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# Design and Optimization of Recombinant Antibodies Directed Against Platelet Glycoprotein VI with Therapeutic and Diagnostic Potentials

Muhammad Zahid

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# UNIVERSITÉ PARIS-SUD 11

## ECOLE DOCTORALE :

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*PÔLE : PHYSIOPATHOLOGIE MOLECULAIRE ET CELLULAIRE*

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par

**Muhammad ZAHID**

### **Design and Optimization of Recombinant Antibodies Directed Against Platelet Glycoprotein VI with Therapeutic and Diagnostic Potentials**

**Directeur de thèse :** Professeur Philippe BILLIALD, EA4530, Université Paris-Sud 11

**Co-directeur de thèse :** Docteur Martine JANDROT-PERRUS INSERM U698

#### **Composition du jury :**

*Président du jury :* Professeur Dominique PORQUET, Doyen de la Faculté de Pharmacie, Université Paris-Sud 11

*Rapporteurs :* Docteur Richard LE NAOUR, EA 4303, Université de Reims, Champagne-Ardenne

Professeur Philippe NGUYEN, EA 3801, Université de Reims, Champagne-Ardenne

*Examineurs :* Professeur Philippe BILLIALD, Faculté de Pharmacie, Université Paris-Sud 11

Professeur Delphine BORGEL, EA4531, Faculté de Pharmacie, Université Paris-Sud 11

Docteur Martine JANDROT-PERRUS, Inserm U698, Hôpital Bichat, Paris



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Annexe 1:

### **Scorpion antivenoms: Progresses and challenges.**

**M. ZAHID**, S. ADI-BESSALEM, J. MUZARD, M. JUSTE, M.F. MARTIN-EAUCLAIRE, N. AUBREY, F. LARABA-DJEBARI, P. BILLIALD.

In J. Barbier, E. Benoit, P. Marchot, C. Mattei, D. Servent (Eds), *E-Book Rencontres en Toxinologie 18 « Advances and new technologies in Toxinology »*. Société française pour l'Etude des Toxines. 2010, pp 243-252.

Annexe 2:

### **Design and humanization of a murine scFv that blocks human platelet glycoprotein VI *in vitro*.**

J. MUZARD, M. BOUABDELI, **M. ZAHID**, V. OLLIVIER, J.J. LACAPERE, M. JANDROT-PERRUS, P. BILLIALD.

**FEBS Journal**. 2009, 276, 4207-4222.

## **Part A**

### **State of the Art**

# 1 Historical Development of Therapeutic Antibodies

Among the marvels of scientific research which have distinguished our century, no achievements are more remarkable, nor of greater moment to the welfare of mankind, than those pertaining to the field of biology, pathology and therapeutic investigation.

A glance at the history of therapeutic procedure in the prophylactic treatment of infectious diseases shows that the general principle underlying all later discoveries had been, though crudely, divined at a much earlier period than we are wont to suppose. It is known that the ancient Hindus and Persians, as well as the nomad tribes and caravans of farther Asia, practised inoculation of equine virus or horse-pox - the mammary pustule developed during early lactation in the horse, camel, cow and even in woman. The inoculation of human virus is of immemorial origin, probably coeval with the importation of variola from Asia into Africa by the Saracens. It is certain that as early as the tenth century the Arabs and Chinese adopted the custom of variolization (inoculation of small-pox).

It was Jenner (1776), who started the systematic and exhaustive study of the subject destined to prove inestimably beneficial to mankind and then Pasteur who, in 1880, announced to the world the issue of his labors, touching the protective inoculation of animals and thus was the broken thread of pathogenic research taken up a new and the task of solving its mysteries resumed - be it said with more profound acumen and far more complete appliances than ever before.

The French savant demonstrated that cultures of the bacilli of chicken-cholera, when thoroughly dried and long exposed to the air, lost their virulence and that fowls inoculated with the attenuated virus were rendered insensible to the attacks of more energetic micro-organisms. It was, *mutatis mutandis*, a modification or development of the Jennerian principle: "The history of vaccination constitutes the first step in a long series of labours inspired by the admirable discoveries originating in the genius of Pasteur. The principle is always the same - to diminish the strength of the virus and inject it into the animal which we wish to render immune "(Bernheim). Salmon and Smith, in 1886-87, showed conclusively that animals may be rendered immune against certain infectious diseases by inoculating them with filtered cultures containing the toxic products of pathogenic micro-organisms entirely free from the

living bacteria to which they owe their origin. By this process immunity against the bacillus of hog-cholera was attained in pigeons.

Roux (1888), employing similar sterilized cultures, succeeded in protecting susceptible animals against the anthrax bacillus (Roux and Yersin, 1888); and then Behring and Kitasato (1890) have proved that immunity against the action of the tetanus bacillus may be conferred by the use of toxic products in solution free from the presence of active germs ( von Behring and Kitasato, 1890, von Behring and Kitasato 1991b). The significance of this discovery could hardly be over-estimated. By it the entire theory of causal phenomena- the protective force in which the immunizing property was supposed to reside - became modified. If not a living organism, but a chemical substance, proved to be the immunizing agent, then resistance to toxic influences must proceed from some source other than bacterial metabolism - some organic force inherent in the inoculated system. To ascertain the nature and operation of this bactericidal power and determine the rationale of acquired immunity now engaged the earnest attention of savants throughout the world.

Despite use of the antitoxin, death rates from diphtheria were still high in the early 1900s and the need for a vaccine was clear. In 1913, von Behring had produced long lasting immunity in guinea pigs, monkeys and asses using a carefully balanced mixture of toxin and antitoxin ( Von Behring and Kitasato, 1913). This was used in the first vaccination studies on humans. A widespread immunization program followed the development of formalin-inactivated toxin by A. T. Glenny and Barbara Hopkins (Glenny and Hopkins, 1923) and Gaston Ramon in the early 1920s. The antitoxin was first used to treat a seriously ill girl in 1891, who subsequently recovered.

Production of the antitoxin on a large scale was achieved in horses, with both the diphtheria serum and the antiserum being standardised using guinea pigs. Widespread use of the antitoxin followed and studies in rabbits showed that it had to be administered soon after infection to be effective (Zinsser, 1931).

Anti-infectious serotherapy was used for the first time in humans in 1891, followed by the development of antivenom by Phisalix and Calmette (1894). The term antibody (antikörfs) was coined by Pfeiffer in 1898 later 'magic bullet' by Paul Ehrlich (1908).

Thus, active immunity, passive immunity and transfer of immunity were discovered, understood and used in human medicine in a record time. At the same time, supporters of humoral confronted with supporters of cellular immunity: Metchnikoff discovered phagocytosis in 1883. Later, Wright made the concept of opsonization and described opsonins. Antitoxins or antibodies are the body's soldiers in the fight against a disease-causing organism (Bockemuhl, 1994).

It was the beginning of using serum for anti-infective and anti-venomous immunotherapy. As all antibodies preparations are derived from the serum of immunized animals or immune human donors, hence this form of therapy is known as “serum therapy”. Serum therapy was effective, but administration of large amounts of xenogenic proteins was often associated with side effects ranging from hypersensitivity reactions to serum sickness (a form of antibody complex disease). Later on (1930s), improved antibody purification methods reduced the magnitude of toxicity and the serum therapy became effective mainly in infectious diseases. In the 1940s, the discovery of antibiotics has contributed to the decline of anti-infective immunotherapy, due mainly to their greater ease of use (oral administration), their broader spectrum of action, but also soon after in terms of cost. Thus, the use of serum therapy declined due to the use of antimicrobial chemotherapy but antibody-based therapies retained a niche as a treatment for envenomations, intoxications and certain viral infections.

In the second half of the 20th century, the inability to treat certain viral diseases divert the attention to the development of antibodies preparations derived from immunized human donors for the prophylaxis and treatment of hepatitis A and B, rabies, and pneumonia caused by respiratory syncytial virus (RSV) (Casadevall *et al.*, 2004).

In contrast, in the envenomation domain, the xenogenic serum therapy always remained the only specific treatment. Even today, the antibodies of animal origin (from immunized horses) have been used for the treatment of envenomations. The only improvement is the fragmentation of these molecules into Fab and Fab'<sub>2</sub> and an improvement in their purification. Recombinant antibody technology may provide significant advantages in the near future as discussed in our review (Zahid *et al.*; 2010). The polyclonal serum therapy, carried out using sera of animal origin is also used in very limited domains. This is the case of preventive therapy in transplant rejection by



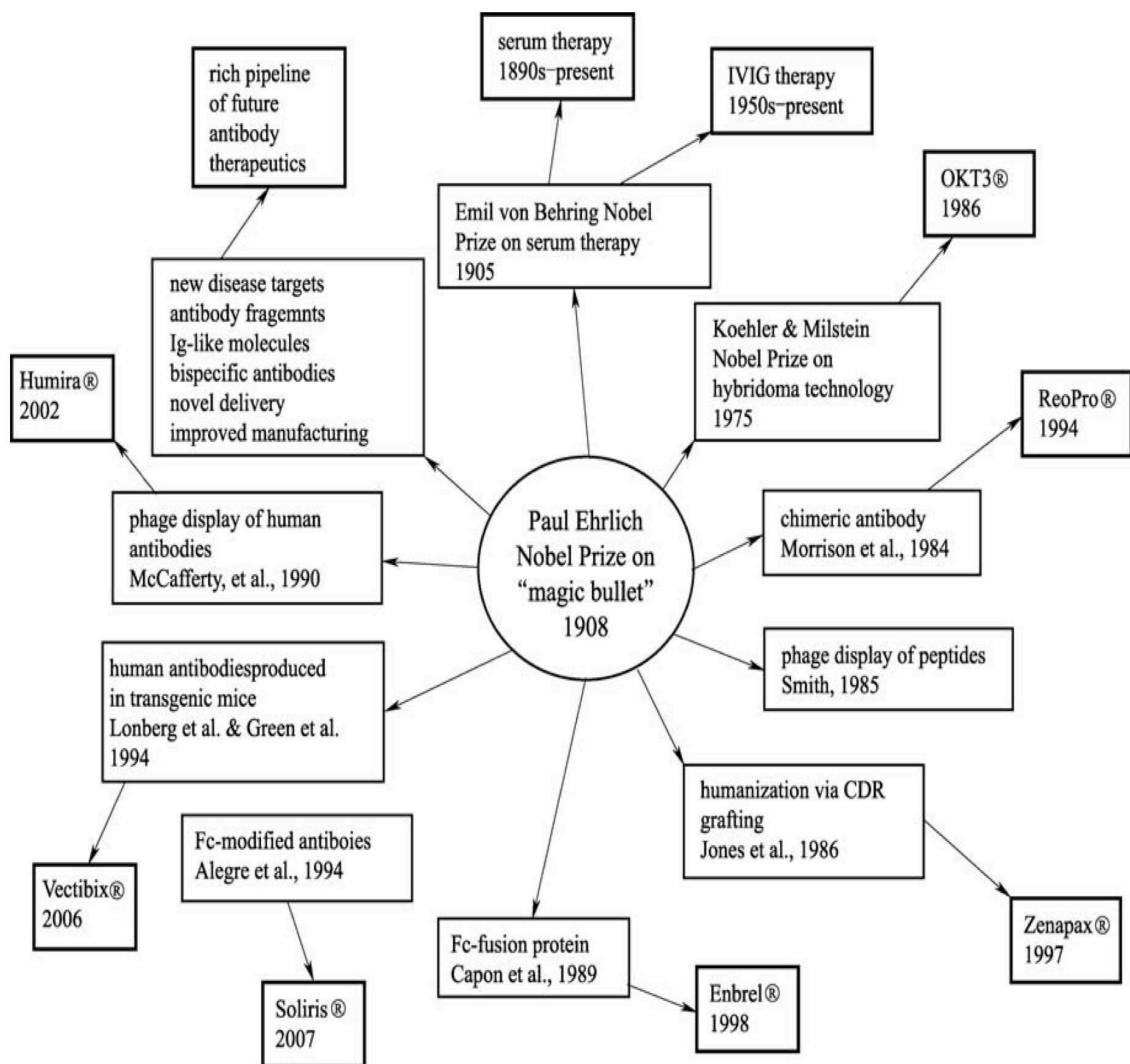
using rabbit antibodies (Thymoglobulin<sup>®</sup>; anti-human thymocytes IgG), or the curative treatment of digitalis intoxications (DigiFab<sup>®</sup>; sheep polyclonal Fab fragments). Polyclonal antibodies, from human donors, vaccinated or not, are better tolerated and administered in some patients as serum therapy in tetanus or immunodeficiencies. Traditional Serotherapy can present a number of drawbacks such as, the low efficiency of the preparations, because only a small fraction of antibodies are directed against the target of interest.

In 1975, the recipients of the Nobel Prize (1980), Köhler and Milstein developed a revolutionary method for the production of highly specific monoclonal antibody. The technology is to merge an antibody producing cell with an immortal cell line (myeloma), resulting in the possibility of isolating a single generation of B lymphocytes producing antibodies with the desired characteristics. They offer the opportunity to keep an unlimited amount of antibody with unique specificity and reproducible affinity and well defined, the two important properties in many experimental or clinical situations (Kohler and Milstein, 1975). Thus, hundreds of hybridomas have been described. The first hybridoma derived murine monoclonal anti-CD3 was introduced into clinical practice in the mid-1980s to prevent organ rejection and this give rise to the hope for the rapid development of many therapeutic applications. However, this hope faced earlier failure. In fact, the administration of heterologous proteins in particular (murine monoclonal antibodies) to humans is generally associated with side effects, mainly immunological (HAMA: human anti-mouse antibody). Thus, modulation of immune responses has been studied by two approaches in order to reduce the anti-mouse antibody (HAMA) responses. The first one is to produce chimeric antibodies less immunogenic, where constant domains of a murine monoclonal antibody are replaced by constant domains of human origin. The second approach is to produce humanized or entire human antibody fragments even less immunogenic. It took a decade for the first chimeric monoclonal antibody, Abciximab<sup>®</sup> for hemostasis, to be approved by FDA in 1994 (Faulds and Sorkin, 1994). While the first humanized monoclonal antibody; Daclizumab (Zenapax<sup>®</sup>) for kidney transplant rejection, was approved for clinical use by Food and Drug Administration (FDA) in 1997 (Vincenti *et al.*, 1998). Humanization alleviated the HAMA response to various degrees, but many other drawbacks became evident. For example, the humanization process is technology demanding and the process may

result in reduced antigen binding affinity and decreased efficacy. Two major approaches were developed in order to avoid the human immune response to murine-derived mAbs and to overcome the technical challenges associated with humanizing murine mAbs. The first one is to express human antibody fragments on bacteriophage surfaces (Vaughan *et al.*, 1996). Adalimumab (Humira®), the first fully human mAb derived from a bacteriophage display antibody library, was approved by FDA in 2002 for the treatment of rheumatoid arthritis (Weinblatt *et al.*, 2003). The second approach was the use of transgenic mice to produce fully human antibodies (Russell *et al.*, 2000). Panitimumab (Vectibix®), an anti-EGFR antibody approved for colorectal cancer therapy in 2006, was the first fully human therapeutic antibody derived from a transgenic mouse system (Chu, 2006).

Thus, to date, twenty five antibodies or antibody fragments, have received authorization for marketing by the FDA for therapeutic use and hundreds more in development (An, 2010). It is likely that this number will significantly increase in coming years. Indeed, antibodies are now the leading source of recombinant proteins being in clinical trials with targets and with varied medical applications: hundreds of molecules are reportedly in clinical trials with potential applications in oncology, transplantation, infection and rheumatology.

In the cardiovascular domain, a chimeric Fab fragment (Abciximab), has proved efficient for the treatment of certain acute phase of coronary syndromes, in combination with other molecules. However, the use of Abciximab is largely associated with adverse bleeding effects, risks of thrombocytopenia and hypersensitivity reactions due to residual immunogenicity. Thus, research and development of safer and more efficient biodrugs is needed (Figure1). For a detailed progress in antivenoms, refer to the following article published on line (<http://www.sfet.asso.fr>) (Annex 1).



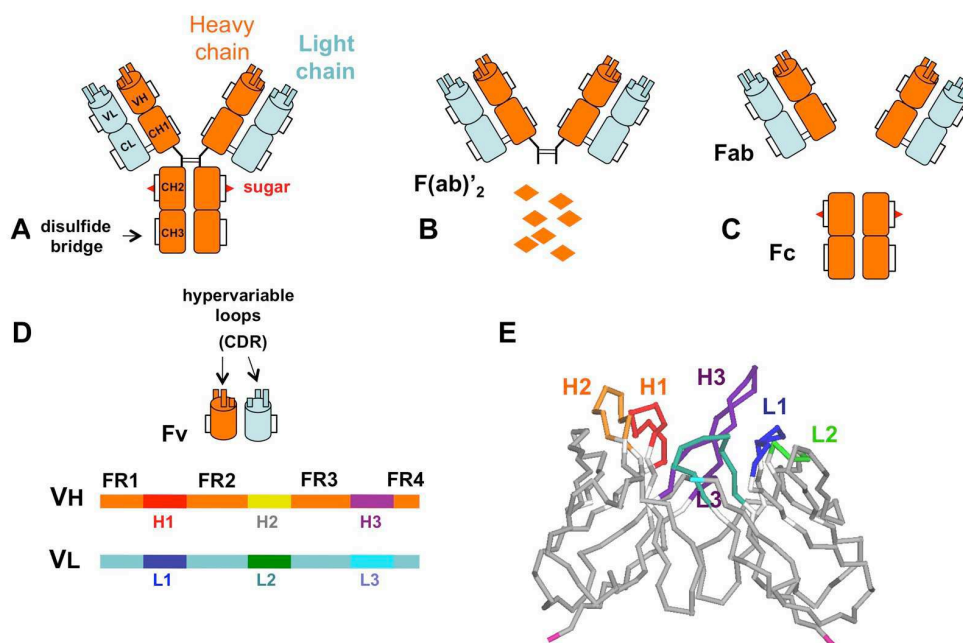
**Figure 1: A schematic representation of the history of antibody therapeutics.**  
Adapted from Higher Education Press and Springer-Verlag Berlin Heidelberg, 2010

## **1.1 Antibodies Engineering**

Antibody belongs to the glycoprotein family of immunoglobulins. It is synthesized during the immune response triggered by the introduction of a foreign agent, to neutralize the antigen and then eliminated. The naive immunocompetent cells of B cell lines (specific immune-mediated humoral response) are selected and activated by the pathogenic agents. They differentiate into clones of plasma cells which are then able to secrete a specific type of antibody capable to recognize and bind to the antigen that has induced it. The binding of the antibody paratope and the epitope of the antigen allows the formation of antigen-antibody complex. It gives the signal to the effector cells of the immune system, telling them that the time has come to destroy the antigen. Antibodies have the advantages of greater specificity, less risk of off-target toxicity and a faster and surer pathway through the clinic. It binds to the target and direct the immune system to attack it through two powerful mechanisms (i) Antibody-dependent cellular cytotoxicity (ADCC) and (ii) complement-dependent cytotoxicity (CDC). Antibodies have physiological properties and activities that have not been duplicated by small molecule drugs and these include toxin neutralization, complement activation, microbial opsonization and antibody-directed cellular cytotoxicity (Casadevall, 1999).

### **1.1.1 Structure of Ig Immunoglobulin**

Antibodies serve a dual purpose, both specifically and stably binding antigens and also attracting effector cells. Antibodies may neutralize a pathogen such as a virus, by simply blocking its surface functions itself (Burton, 2002) or by labeling it for destruction by innate killer cells. These two functions reflect the Y-shaped structure of the antibody, consisting of two homologous arms with specificity-determining function and one opposite arm designed for effector functions. Sequence-wise, this bi-functionality is reflected by one sequentially variable and one sequentially constant domain of the molecule (figure 2). Structurally, the antibody is a globular tetramer, consisting of two longer heavy (H) chains (50 kDa, about 450 residues) and two shorter light (L) chains (25 kDa, about 220 residues), connected together by disulfide bridges and stabilized by noncovalent bonds (Figure 2). Each heavy and light chain has constant domains that contribute to the binding of effector molecules on host cells



**Figure 2: Immunoglobulin G and its functional fragments**

A- Monomeric IgG.

B- Bivalent  $F(ab)_2$  produced after pepsinolysis.

C- Monovalent Fab fragments and Fc fragment obtained after papainolysis.

D- Unstable Fv fragments made of VH and VL domains.

E- Three dimensional model of an Fv. Hypervariable loops L1, L2, L3 and H1, H2, H3 (or CDRs) constitute the paratope which interacts with the target antigen.

and variable domains that recognizes the target antigen. The enzymatic cleavage can isolate different fragments: The Fab portion, which participates in antigen binding and the Fc portion which is the support of the biological properties of immunoglobulin. There are five functionally different major subclasses of heavy chain fragments (IgA, IgD, IgE, IgG, IgM), but only two variants of the light chain subclasses (kappa and lambda) without any known functional differences (Bengtén *et al.*, 2000). The majority of antibodies found in the serum belong to the IgG class and most structural information has been derived from this class of antibody. Being heavily stabilized by cysteine bonds, antibodies subjected to cysteine protease papain digestion, form three characteristic fragments frequently referred to; two identical antigen binding fragments (Fab) and one crystallizable (Fc) fragment. The binding site is formed by the convergence of six hypervariable peptide loops known as complementarity determining regions (CDRs). It is primarily the variation in amino acids sequence in these regions that produces antibodies of different specificities. Three CDRs are provided by each heavy (H)- and light (L)-chain protein (Kabat and Wu, 1971, Wu and Kabat., 1970). *In vivo*, diversity in antibody combining sites is produced by multiple variable (V), diversity (D) and joining (J) gene segments and somatic mutation. CDRs 1 and 2 of H and L chains are encoded by V regions. Light-chain CDR 3(LCDR3) is produced by the combination of V and J regions, where as heavy-chain CDR3 (HCDR3) is formed by the combination of V, D and J regions. The HCDR3 have the potential to generate more than  $10^{14}$  peptides in this region, which differ both in length and sequence (Sanz, 1991).

### **1.1.2 Antibodies in Bodies**

The ability of antibody to recognize antigen is due to an advanced combinatorial process involving fusion of different sets of gene segments (V, D and J) each existing in multiple different copies. These gene segments, separated by thousands of base pairs in the genome, are brought together by a stepwise recombination process and fused into functional heavy and light chain genes during the differentiation from a hematopoietic stem cell to a mature naïve B cell (Tonegawa, 1983). In addition to the great number of different gene segment copies, the actual variability is further increased by imperfections in the recombination and segment joining process. As mature antibody presenting B cells circulating the body, the antibody affinity is relatively low ( $10^{-5} - 10^{-6}$  M) (Foote and Eisen, 1995), so upon antigen encounter the

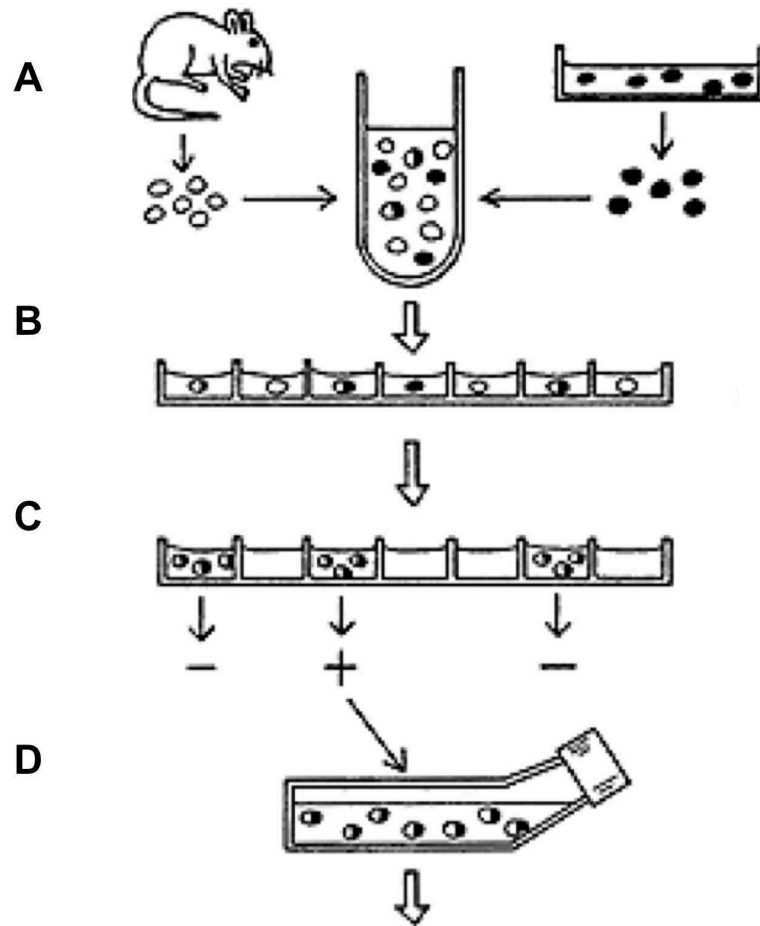
B-cell migrates to secondary lymphoid organs (e.g. lymph nodes) for further binding improvements of the antibody. In broad terms two major changes appear. Firstly class switching, which leads to a change in constant region of the expressed antibody enabling a choice of innate effector functions while still retaining the same antigen specificity (Harriman *et al.*, 1993). The second change is a somatic hypermutation, which greatly contributes to diversifying the information encoded by the germline gene segments. This process, typically involving a change between zero and 25 amino acids, is mainly achieved by mutations all over the variable region, especially in mutational hotspots that are more susceptible to replacement mutations (Neuberger and Milstein, 1995, Wagner *et al.*, 1995). B-cells with improved binding affinity will proliferate and outcompete other cells in the fight for limited amount of antigen displayed on the follicular dendritic cells and they will thus get the T-cell signal necessary for survival (Kosco-Vilbois, 2003). In a study the affinities found in matured antibody populations after immunization with tetanus toxoid varied substantially ranging from micromolar to picomolar values (Poulsen *et al.*, 2007).

### **1.1.3 Murine Monoclonal Antibodies Production**

Monoclonal antibodies represent a single B lymphocyte generating antibodies to one specific epitope. B-cells can be isolated easily from the spleen and lymph nodes of immunized animals; but, these cells have a limited life span, and can only divide a limited number of times. For an antibody to be useful in research or industry, it must be readily available in large quantities. Due to the limited life span, this would not be possible using B-cells cultured *in vitro* as they would eventually stop dividing and the population would die out.

Consequently, the discovery of hybridoma technology by Kohler and Milstein (1975) aroused great enthusiasm about the use of monoclonal antibodies in the field of immuno-detection, as well as in human immunotherapy. This technology is based on fusion of lymphoid cells, secreting a single population of antibodies (monoclonal) of known specificity, with myeloma cells, thus conferring immortality to hybrid cells known as hybridomas (Kohler and Milstein, 1975) (Figure 3).

To generate hybridomas, B cells, usually from the spleen of mice immunized against an antigen (polyclonal response), are fused with a non-secretory myeloma cell line and deficient in HGPRT (hypoxanthine guanine phosphoribosyl transferase), an



**Figure 3: Schematic representation of hybridoma technology**

- A- Immunized mouse spleen cells are fused with myeloma cells.
- B- Sparse seeding of cells in a medium allowing selection of hybrid cells.
- C- Expansion of clones with a predetermined antibody specificity.
- D- Collection of antibodies from the tissue culture medium.



enzyme of the purine *salvage* pathway. The technology usually allows a small proportion of the cells to fuse. The mixture is cultured in a medium containing hypoxanthine, aminopterin which blocks *de novo* synthesis of purines and thymidine (HAT medium) which provides the substrate for salvage path way. Both non-fused myeloma and the B cell die. Each hybrid cell capable of dividing indefinitely, is then isolated, often by limiting dilution method, and then selected for its ability to synthesize immunoglobulins directed against the antigen of interest. Clones from a single cell that is both immortal and producing a particular antibody (monoclonal) are selected and thus isolated.

The majority of hybridomas have been obtained by fusing the immunized mouse lymphocytes with murine myeloma cells. It poses few problems of genetic instability, the fact remains that the effectiveness of fusion may be low and thus constitute a limiting factor for a rich and varied repertoire of monoclonal antibodies. Clones of interest in the immune repertoire are weakly represented. For example, Bahraoui *et al.* (1988) have obtained two murine monoclonal antibodies directed against scorpion AahII toxin and only one had high affinity and neutralize the toxin. Despite numerous attempts, since no other neutralizing antibody of the toxin could be isolated (Clot-Faybesse *et al.*, 1999) and this irrespective of the immunogen used (toxin, toxin coupled to a tetanus carrier protein).

Although the interest of monoclonal antibodies from murine hybridoma is perfectly demonstrated in diagnosis, their therapeutic use is limited or impossible due to their intrinsic characteristics. The first point is an immune response when administered repeatedly to the human. Indeed, the murine monoclonal antibodies, once injected to humans induce an immune response and production of human anti-mouse immunoglobulin (HAMA for Human Anti-Mouse Antibody) (Bruggemann *et al.*, 1989). Another major drawback is the inability of the Fc fragment of murine antibodies to interact efficiently with the effector cells of the immune system in humans. Fc fragment also contributes to the strong immunogenicity of murine antibodies.

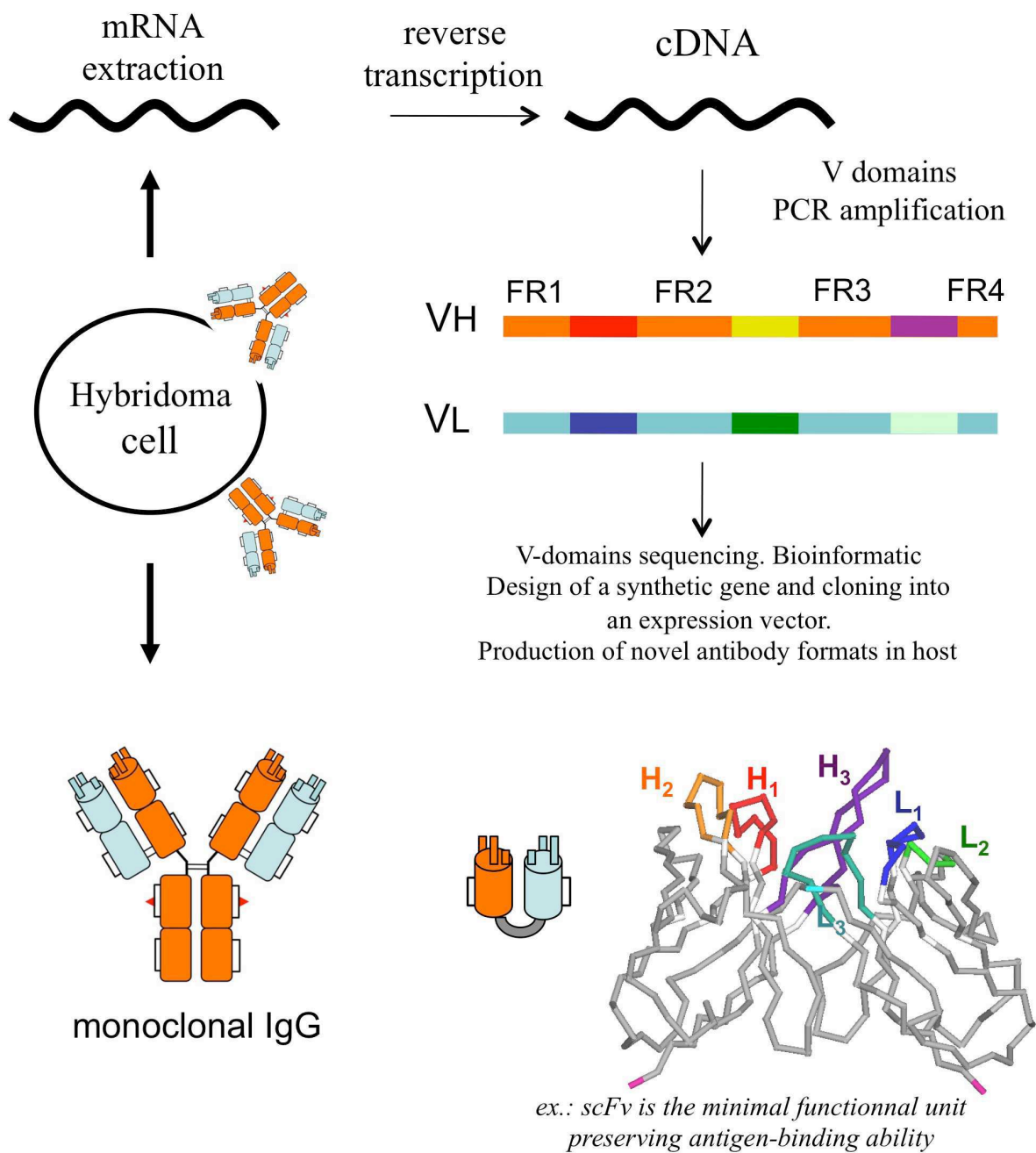
So in 35 years, only three (tositumomab, muromonab-CD3 and ibritumomab tiuxetan) murine monoclonal antibodies have received FDA approval for therapeutic use under strict conditions of supervision which is only 17% of the approved therapeutic antibodies: the first mAb approved was a murine anti-CD3 (Orthoclone<sup>®</sup> OKT3;

Johnson and Johnson) used as a treatment to reverse transplant rejection in immunosuppressed patients. Moreover, two radiolabeled murine anti-CD20 mAbs, ibritumomab (Zevalin<sup>®</sup>; Biogen Idec) and Tositumomab (Bexar<sup>®</sup>; GlaxoSmithKline) are used for the treatment of non-Hodgkin lymphoma. Bexxar<sup>®</sup> is a iodine radiolabeled antibody (Laffly and Sodoyer, 2006) while Zevalin<sup>®</sup> is radiolabelled either with Yttrium-90 or indium-111 (Milenic *et al.*, 2004). Overall, the approval success rate calculated for murine products is only 3%. The concerns associated with murine mAbs have been their: (1) low overall tissue uptake (slow diffusion of the antibodies from the blood into the tissue), (2) slow clearance from blood because of their large size, and (3) their immunogenicity, which shortens their efficiency during therapy and may cause immunity related side effects (Illidge and Brock, 2000). Today different kinds of recombinant antibody constructs have been engineered in order to reduce their immunogenicity, to increase their tissue penetration ratios and to connect them to cytotoxic agents for better therapeutic index (Figure 4)

#### **1.1.4 Chimerization**

The development of chimeric antibodies was found necessary when clinical studies with murine antibodies had failed because of the development of immune responses (HAMA response). An important application of antibody engineering is the possibility to create chimeric antibodies. The binding activity of an immunoglobulin (IgG) is generated by the variable domains. Chimeric antibodies comprise the variable domains (VH and VL) of a murine monoclonal antibody fused to constant regions of heavy and light chains of human immunoglobulin (Figure 5). The framework regions of natural antibody is conserved, which is favourable in maintaining the three-dimensional conformation of the hypervariable regions essential for antigen interaction. Such antibodies are 75% of human origin and less immunogenic than murine antibodies (Hwang *et al.*, 2005), because interspecies epitopes are mainly located at the CH2 and CH3 domains (Bose *et al.*, 2005). In contrast, immunogenic reactions directed against the framework regions of murine variable domains remain possible (Bruggemann *et al.*, 1989) leading to the development of a new generation of therapeutic antibody (Reichert *et al.*, 2005).

The facility with which murine monoclonal antibodies can be produced using hybridoma technology and the serious constraints these antibodies impose upon their use in the clinical setting led to the development of new approaches. Techniques have



**Figure 4: Generation of recombinant antibody fragments from hybridoma cells.**

After extraction of mRNA from hybridoma cell, the cDNA encoding the variable domains of the antibody are amplified by PCR, cloned and sequenced. A gene encoding the scFv made of the variable domains of the antibody fused together via a peptide linker is synthesized and cloned in the expression vector. Recombinant bacteria are used for the production of the scFv.

been developed to modify, at least partly, the readily available rodent monoclonal antibodies into antibodies with predominantly human immunoglobulin chains, preserving those parts of the murine antibody which correspond to the antigen binding sites. Initially, the Fc portion of the antibody molecule, which dictates the functions of the antibody, was chemically exchanged with a human constant portion, giving rise to chimeric monoclonal antibodies (Boulianne *et al.*, 1984). The antigen specificity of the chimeric antibodies is identical to that of the initial mouse monoclonal antibody, whereas the effector functions are determined solely by the human Fc domain. In comparison to the original mouse monoclonal antibodies, the chimeric molecules are less “murine” and they therefore induce a significantly decreased HAMA response in human recipients. However, the remaining immunogenicity renders even these antibodies non-tolerable.

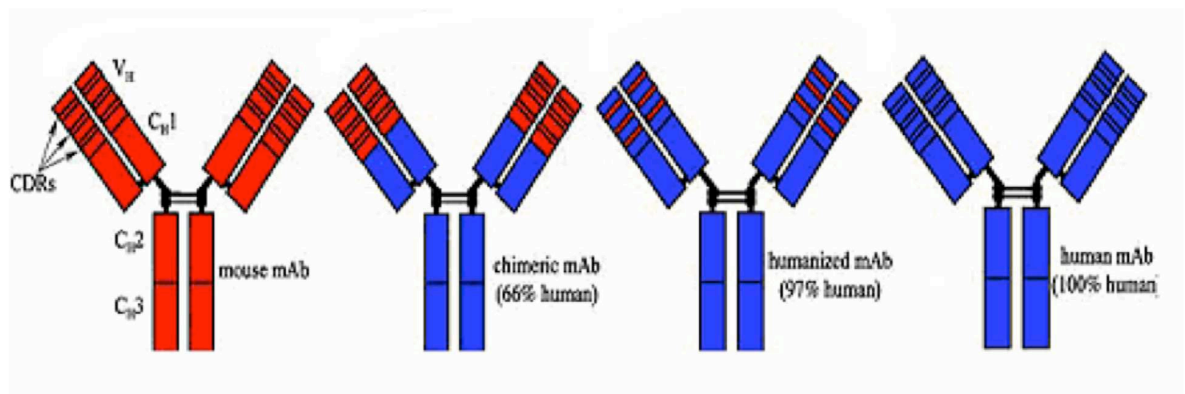
In designing chimeric mAbs, genetic engineering techniques have to be used to replace the murine Fc region with one of the human sequence (Morrison *et al.*, 1984). Surprisingly, the chimeric mAbs have proved to be superior to the early human mAbs because replacement of the murine Fc was sufficient for improving efficacy and reducing HAMA response for at least some products. Following administration of a chimeric antibody to human, the frequency of secretion of human antibodies directed against a chimeric antibody (HACA: Human Anti-Chimeric Antibodies, 30% of recipients) can be halved compared to the frequency of secretion of antibodies directed against parental murine monoclonal antibody (HAMA Human Anti-Mouse Antibodies; 60% recipients) (Khazaeli *et al.*, 1994). In another study, a chimeric Fab (c7E3) can induce a HACA type response in 1% of subjects, compared to 20% for parental murine Fab administered under the same conditions (Knight *et al.*, 1995). Differences observed between these studies could be explained by differences in existing structures between these antibodies (or antibodies fragments) and human antibodies (or antibody fragments). In some cases, when antibodies are detected against a chimeric antibody, their concentration can be on average 10 to 50 times lower than the concentration of antibodies directed against the unmodified parental antibody (Khazaeli *et al.*, 1991). Finally, only four therapeutic antibodies of chimeric nature are currently available. Basiliximab (Simulect<sup>®</sup>; IgG1 anti-CD25) and cetuximab (Ertuximab<sup>®</sup>; IgG anti-EGFR). Abciximab (c7E3 Fab)(Reopro<sup>®</sup>) targeting integrin GPIIbIIIa (Faulds and Sorkin, 1994) is the only chimeric antibody fragment

(Fab) used in therapy (Knight *et al.*, 1995). Infliximab (Remicade<sup>®</sup>; anti-TNF $\alpha$ ) is used to treat autoimmune diseases (Knight *et al.*, 1993). Although chimeric antibodies are less “foreign” than murine monoclonal antibodies and also less immunogenic, but human anti-chimeric antibody responses (HACA) have been observed (Baert *et al.*, 2003).

### 1.1.5 Humanization

It is possible to reduce further the immunogenicity by performing a more complete transformation, resulting in humanized antibodies, by exchanging the hypervariable loops (CDRs) of a human immunoglobulin by those of a murine immunoglobulin-specific to an antigen of interest (Figure 5). The humanized antibody so obtained is 85-90% of human origin (Chames *et al.*, 2009). Verhoeyen *et al.* (1988) have demonstrated the feasibility of this method by conferring specificity of a mouse anti-lysozyme antibody to a human antibody. This method can be very efficient (Gorman and Clark, 1990, Presta *et al.*, 1997, Riechmann *et al.*, 1988). However, it is often necessary to retain the amino acid residues located in the framework regions. These amino acid residues play a key role in the conformation of the hypervariable loops and are thus retained to preserve better affinity of the antibody for the target antigen (Caldas *et al.*, 2003). Residues located at the CDR-FR junction are important in particular, as that of the VH-VL interface (Hurle and Gross, 1994). In some cases, identification of amino acids located in the framework regions, are important, either to maintain CDR conformation, or for the interaction with the epitope, and permitted to restore the activity and the affinity of the antibody 6.7, anti-CD18 (Caldas *et al.*, 2003).

The molecular methods developed and improved in the past two decades and the greater comprehension of the structure and function of the different antibody domains led to novel revolutionary approaches to the production of monoclonal antibodies. While the hybridoma and Epstein Barr virus (EBV) methods facilitate immortalization of specific antibody-secreting B cells, the molecular techniques focus on immortalization of genes corresponding to specific antibodies (see details later). Molecular techniques have been used to further eliminate those portions in the murine immunoglobulin chains that are not involved in the binding of antigen and to replace them with the corresponding human sequences. Complementarity determining regions (CDR's) within the heavy and light chains variable regions, play a prominent role in



**Figure 5: Diagrams of various recombinant monoclonal antibody structures.**

In red fragments of murine origin. In blue fragments of human origin

(Adapted from Higher Education Press and Springer-Verlag Berlin Heidelberg, 2010)

the binding specificity of the antibody. DNA fragments corresponding to the CDR's were grafted into the framework of human immunoglobulin genes (Jones *et al.*, 1986). Further more, replacement of some amino acid residues in the frame work regions of the "recipient" human immunoglobulin with the corresponding amino acids of the mouse "parental" monoclonal antibody proved advantageous (Carter, 2006). Thus, humanized antibodies retain the specificity and binding affinity of the "parental" murine monoclonal antibody, they exhibit reduced immunogenicity in humans and they acquire biological functions of choice.

Different humanization approaches can be considered, depending on one hand on the nature of the human frame work scaffold, the sequence of murine variable domains (donor CDR), and on the other, on human sequences used to support the murine hypervariable loops. The latter may be derived from antibodies whose structure was solved by crystallography (Riechmann *et al.*, 1988), germline sequences (Tan *et al.*, 2002) or even a consensus sequence (Carter *et al.*, 1992). However, CDRs grafting is more technically demanding than a mere fusion, and directed mutagenesis approaches are often needed to restore the parental murine antibody affinity. To this would be added other approaches, such as resurfacing, which are designed to change only the murine amino acids exposed on the surface of the antibody (Padlan, 1991).

The first strategy to produce humanized antibodies is to choose a sequence of human origin as close as possible among those whose three dimensional structure is known in order to preserve the maximum environment for folding of hypervariable loops. Using a crystallographic structure as support to the murine hypervariable loops may allow identification of residues involved in antigen binding (Yazaki *et al.*, 2004).

A second approach is to identify the human germline sequence more close to that of the murine antibody in order to serve as receiver of murine hypervariable loops (CDRs) (Gonzales *et al.*, 2004). The use of germline sequences combined with the construction of combinatorial libraries of antibody was also used. This approach is uncommon and has been used in particular for the humanization of the BR96 Fab, and have allowed to obtain up to five active humanized Fab which retained a high affinity for the antigen expressed on the surface of many carcinomas in humans (Rosok *et al.*, 1996).

"Consensus" strategy on the other hand, utilizes variable light (VL) and variable heavy (VH) domain frameworks derived from the most common amino acid found at each position within a given human sub-group. Whatever the method, CDR grafting may not result in the complete retention of antigen binding properties because some framework residues can interact directly with antigen (Mian *et al.*, 1991), or affect the conformation of CDRs loops (Foote and Winter, 1992). In this case the antibody must be engineered to restore high affinity. On the other hand, humanization of a xenogenic antibody may still have immunogenicity and immune response can be induced against its xenogenic CDRs. Not all residues within the CDRs of an antibody are essential for antigen binding. About 20-30% of the residues are involved in antigen binding (Padlan, 1994). These residues are designated as specificity determining residues (SDRs) (Padlan *et al.*, 1995). Thus, a murine antibody can be humanized through grafting SDRs onto human template and therefore, minimizing the immunogenicity (Gonzales *et al.*, 2004). It overcomes some somatic mutations creating a potentially immunogenic site on the humanized antibody (Presta *et al.*, 1993). This technique has been used successfully for antibody HPC4 directed against human protein C (O'Connor *et al.*, 1998).

Another one is resurfacing strategy, which was proposed for the first time by Padlan (Padlan, 1991). This approach involves the replacement of solvent exposed murine framework residues in the variable regions with human residues (Roguska *et al.*, 1994).

This strategy rests on the assumption that only exposed residues are likely to trigger an immune response in human. The number of residues to substitute are relatively low (Roguska *et al.*, 1994). Whatever the strategy, a humanization does not generally recover the original affinity of the murine antibody, knowing that some structural residues can interact directly with antigen or have an effect on remote CDR conformation (Caldas *et al.*, 2000, Foote and Winter, 1992). Indeed, it is generally necessary to introduce additional mutations in framework regions or even within the CDR to find an affinity similar to the original antibody for its target. Thus, it was possible to restore the affinity of humanized antibodies who had lost their affinity when the CDR of mouse antibody were grafted onto a structure of human origin (Caldas *et al.*, 2003, Tempest *et al.*, 1991). Thus, CAMPATH-1 antibody, directed against CD52 and proposed for the treatment of non-Hodgkin B lymphoma was the



first humanized antibody tested clinically in human. A mutation in the framework regions of the heavy chain and three mutations in framework regions of the light chain were needed to restore a close affinity (2 to 3 times lower) than that of the parental antibody (Vaughan *et al.*, 1998). Although these different approaches can lead to a molecule relatively close to a human antibody, there are still very few therapeutic antibodies from these technologies which concluded the superiority of one approach over another in terms of immunogenicity of the humanized antibody.

Finally, 'Guided selection' is a process that transfers the specificity of a murine monoclonal antibody to novel human monoclonal antibody by creating a hybrid library of the murine heavy chain and random human light chains, then the selection for binding antibodies and repeating the process with the human heavy chains. Adalimumab (Humira<sup>®</sup>) was the first phage display derived human antibody and was generated by "Guided selection" starting from a murine monoclonal antibody (Baca *et al.*, 1997, Jespers *et al.*, 1994).

Although humanization allows virtually to eliminate the murine antibody sequences, some clinical observations indicate that the residual immunogenicity of the antibody can be varied from negligible to intolerable. It is known as Humanized Anti-Human Antibodies type (HAHA) immune responses (Hwang *et al.*, 2005). It is difficult to imagine that a total lack of immunogenicity can be achieved even with antibodies of human origin, considering the contribution of anti-idiotypic and anti-allotypic responses (Clark, 2000, Stephens *et al.*, 1995). However, it appears that the humanized antibodies are less immunogenic than chimeric antibodies, which are themselves much less immunogenic than unmodified murine antibodies (Baert *et al.*, 2003, Bruggemann *et al.*, 1989, Carter, 2006, Hwang *et al.*, 2005). Moreover, other factors besides the number of amino acids of murine origin and the intrinsic properties of the therapeutic antibody can help to induce secondary immune responses, including the patient's immune status, route of administration, the dose used, as well as co-therapy (Chirino *et al.*, 2004, Clark, 2000).

Advancement in genetic engineering technology have made it possible to design human mAbs and have also opened the door to the study of a variety of mAbs fragments. Increased control over the design of these fragments might increase the rate of success in future (Figure 5).

## **1.1.6 Alternatives**

The focus for protein therapeutics is the generation of clinically safe and effective molecules which are tolerated by the human immune system. So in addition to already described methods the following alternative approaches may also be used.

### ***1.1.6.1 Deimmunisation***

As an alternative, deimmunisation has been developed by Biovation (UK, [www.biovation.co.uk](http://www.biovation.co.uk)) which consist of the identification and removal of T helper cell epitopes from the antibody (Mateo *et al.*, 2000, Roque-Navarro *et al.*, 2003). Two products of deimmunisation are actually under clinical trials; J591, a modified antibody binding to prostate specific membrane antigen (PSMA) and the radiolabelled antibody Tromboview. A phase 1 clinical study have shown that J591 is well tolerated, non-immunogenic and can be injected in multiple doses (Bander *et al.*, 2005).

### ***1.1.6.2 Primatization***

Another method for obtaining antibodies that are closely related to human antibodies involves the use of non-human primates. Primate antibodies are more similar in sequence to human antibodies than that of murine antibodies and are less susceptible to be immunogenic in humans. Indeed, the gene segments of macaques are as closely related to human immunoglobulin genes that human genes to each other (Lewis *et al.*, 1993, Newman *et al.*, 1992). The most straightforward approach to isolate human antibodies with high affinities, is the construction and screening of human immune libraries, but humans cannot be immunized with all antigens of interest, for ethical and practical reasons. This difficulty can be circumvented by utilizing non-human primates (NHP) instead of humans. Antibody fragments with high affinities (from 3 nM to 50 pM) and neutralizing properties have been obtained. The framework regions (FR)- the regions most implicated in tolerance - of these NHP antibody fragments were shown to be very similar to FR encoded by human germline genes. The isolation of NHP antibody fragments from immune libraries, followed by the "super-humanization" process, opens a new and highly efficient approach for the production of high-quality recombinant antibodies for therapeutic uses (Pelat and Thullier, 2009).

Three primatized antibodies directed against human antigens are currently in clinical trials (IDEC C9.1, IDEC 114, and IDEC 151) and the preliminary results do not show any anti-primate antibody response (Bugelski *et al.*, 2000). In addition, Biogen Idec is developing Lumiliximab, a primatized anti-CD23 macaca/human chimeric antibody that inhibits the production of IgE antibody, for potential treatment of allergic conditions (Reichert, 2004).

Preclinical data demonstrated an enhanced antitumor effect when lumiliximab, an anti-CD23 monoclonal antibody, is combined with fludarabine or rituximab. Clinical data from a phase 1 trial with lumiliximab demonstrated an acceptable toxicity profile in patients with relapsed or refractory chronic lymphocytic leukemia (CLL) (Byrd *et al.*, 2010).

## **1.2 Fully Human Antibodies**

Monoclonal antibodies of human origin (third generation of antibodies) may be of utmost importance in therapeutics utilization. Therefore, several procedures are in use for the generation of human mAb (Humab) such as selection from human hybridoma, transgenic mice or from *in vitro* libraries. Technologies are in use essential because (i) human hybridomas are not stable and (ii) human immunization with some antigen is not feasible for ethical reasons.

### **1.2.1 Human Monoclonal Antibodies**

Human monoclonal antibodies are virtually indispensable for immunotherapy of infectious diseases, autoimmune diseases, to prevent rejection of organ transplantation and for the treatment of cancer. The hybridoma technique has been proved to be the most and highly producible method for the generation of murine monoclonal antibodies but it does not work well using human B lymphocytes. While murine monoclonal antibodies are immunogenic when administered in human bodies. Grace of biotechnological development, human monoclonal antibodies have been manufactured with higher efficiency (Ohnuma and Morimoto, 2010). Although the original hybridoma technique has proved to be extremely reproducible for murine monoclonal antibodies, new strategies were introduced to improve the production of monoclonal antibodies in general and of human monoclonal antibodies in particular. Alternative techniques have also been developed to create native and even non-native, newly composed antibodies. Because of the enormous clinical potential initially

ascribed to monoclonal antibodies, there have been continuous attempts to construct human therapeutic monoclonal antibodies. The pharmacological industries are intensely involved in these developments. The current approaches for the production of human monoclonal antibodies are still inadequate and continuous attempts have been made to improve the techniques. In addition, the methods offered now a days to test the *in vivo* efficacy and effectiveness of human monoclonal antibodies are unsatisfactory. Finally, the fact that today only very few completely-humanized monoclonal antibodies are used in the clinical setting indicates that the field is in its initial phase (Steinitz, 2009).

It is possible to perform hybridizations of human lymphocyte cells with myeloma of other species (mouse and rat) or human myeloma. However, the lines obtained are unstable, frequently lose human chromosomes and their ability to secrete antibodies (Carson and Freimark, 1986). Cellular technology, still used, consist generally to immortalize B cells from a donor with the Epstein Barr virus (EBV) (Nakamura *et al.*, 1988). The use of this method remains limited due to a lack of efficient immortalization process, but also for ethical reasons. Indeed, it is difficult to consider the immunization of an individual with toxic antigens or pathogens. The only human lymphocyte cells that may to be immortalized are specific vaccine antigens, bacteria or viruses from naturally infected patients.

### **1.2.2 Different Approaches for Human Monoclonal Antibodies Production**

Attempts to obtain human monoclonal antibodies by the conventional methods of cell fusion have so far been mostly confronted with failures. Several approaches have helped to overcome these difficulties.

### **1.2.3 Human B Cell Hybridoma**

It is very difficult to create a human B cell hybridoma for the production of high affinity IgG monoclonal antibody. Encouraging results have been obtained by using a heteromyelomas (Mouse x human hybrid myeloma) as fusioner partners. Problem again in heteromyelomas is that the human lymphocytes are often unstable. Alternative, is the immortalization of human antibody secreting cells by Epstein-Barr Virus (EBV) infection. But, it is difficult to clone EBV infected cells and usually produces a very low amounts of IgG, however, now the addition of a polyclonal B

cell activator (CPG) has ameliorated the situation (Karpas *et al.*, 2001, Traggiai *et al.*, 2004). As the human antibody does not have specificity to “self” and is the major problem in human antibody therapeutics.

#### **1.2.3.1 Transgenic Mice**

Human monoclonal antibodies can also be isolated using transgenic mice which is able to secrete human antibodies in response to immunization (Bruggemann and Neuberger, 1996).

Transgenic ‘humanized’ mice were created by replacing the entire mouse IgG repertoire with a human repertoire (Lonberg, 2008). Upon immunization, these humanized mice produce human IgGs and conventional hybridoma techniques can be used to clone human antibodies with the required properties. This approach has the advantage of yielding *in vivo* matured antibodies, circumventing the need for additional affinity maturation. Moreover, they directly lead to full length IgG, which is often the preferred format for therapy. However, humanized mice cannot be used effectively when the immunogen is toxic or when the targeted antigen shares a high degree of homology with its murine ortholog. The later problem represents a real limitation. The transgenic mouse is ideally suited for the generation of antibodies against multispinning membrane proteins or protein-antigen complexes which are difficult to produce and purify in functional form. Moreover, transgenic mice may facilitate the production of high affinity antibodies which does not need further affinity maturation (Tabrizi *et al.*, 2009). An alternative approach, is the introduction into heavy and light chain gene-deficient knockout mice the human minichromosomes (derived from human chromosomes 2 and 14) containing the complete germline clusters for heavy and k light chains and the mice is known as transchromo mice. Upon vaccination, these knockout/knockin mice produced human antibodies and their spleens were used to make human monoclonal antibodies, applying the conventional hybridoma technique (Ishida *et al.*, 2002a, Ishida *et al.*, 2002b, Tomizuka *et al.*, 2000). At the moment some problems related to the technology remain unresolved. First, the human immunoglobulin genes transferred into the knockout mice are incomplete. Second, the Ig-“humanized” transgenic mice possess murine T cells and therefore their humoral response is not purely human-specific. Third, in this system glycosylation of the human antibodies is mouse-specific. Thus, if the antibodies are intended for immunotherapy they will be

recognized by anti-Gal $\alpha$  1-3Gal antibodies normally present in human serum (Borrebaeck *et al.*, 1993). Fourth, the durability of the foreign human chromosomal material is of major concern. A disturbing drawback is that biological industries are the proprietors of the knockout/knockin mice, which are, therefore, not freely available to the scientific community. Many companies have transgenic rodents platforms such as Medarex, Kirin, Abgenix (Amgen), Alexin, Roche, Boehringer etc. Human monoclonal antibodies can be produced by transplanting a functional human immune system into immunodeficient mouse strains. Several transgenic mice expressing human Ig genes are available (HuMab Mouse, XenoMouse, Taranschromo Mouse etc) which are producing human mAbs (Davis *et al.*, 2004). For example, transplanting a functional human immune system into immunodeficient mouse (SCID), SCID-bg, Trimera. Severe combined immunodeficient (SCID) mice lacks mature T and B cells and are devoid of endogenous serum immunoglobulins, can be successfully reconstituted with human peripheral blood lymphocytes. Such mice reconstituted with a competent human immune system would represent an invaluable tool for producing large amounts of human antibodies, after immunization with antigen. However, the limitations of such mice is their short life spans, spontaneous production of functional lymphocytes with aging and residual immunity leading to variable levels of engraftment. Natural killer (NK) functions in particular would be detrimental to engraftment of human lymphoid cells (Goldman *et al.*, 1998). The Trimera mouse has been developed in three step process. Firstly, normal mice is rendered immuno-incompetent by a lethal split-dose total body irradiation. Secondly, the myeloid and erythroid lineages are reconstituted by transplantation of bone marrow cells from a genetically immune-deficient mouse donor. Thirdly, the resulting preconditioned mouse is transplanted with human cells that can be maintained in the host (Ilan *et al.*, 2002).

### 1.3 Antibodies Selecting Methods

In recent years, the use of display vectors and *in vitro* selection technologies has transformed the way in which antibodies specific for a given target can be generated. Using these new emerging technologies, it is now possible to design repertoires of ligands from scratch and use the power of libraries panning to select those antibody fragments having the desired properties. With phage, ribosome or yeast display,

tailor-made antibodies may be synthesized and selected to acquire the desired affinity of binding and specificity for *in vitro* and *in vivo* diagnosis, or for immunotherapy of human diseases.

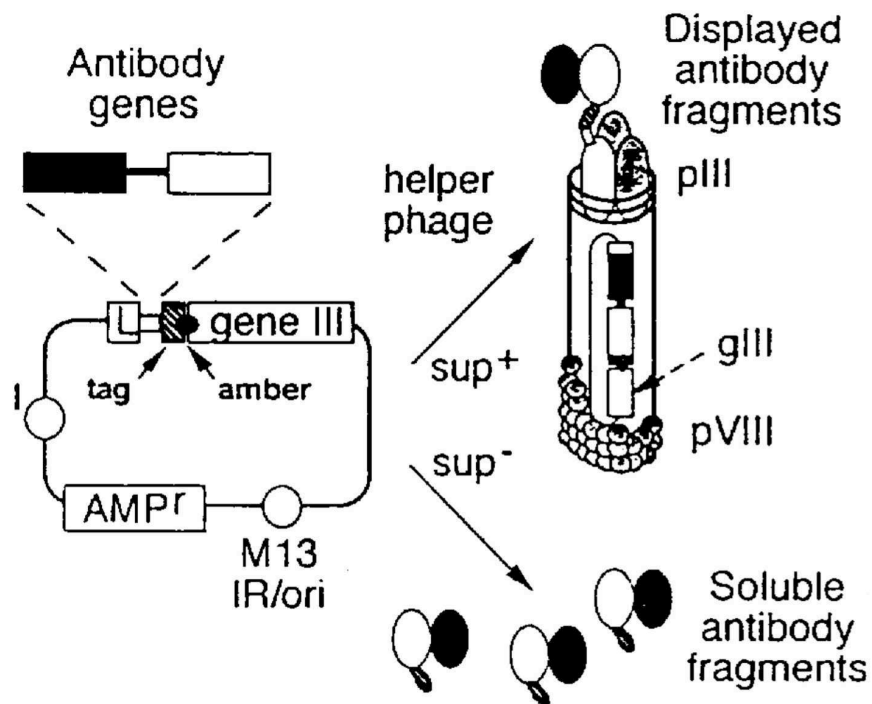
### **1.3.1 Exposure on the Surface of Phage (Phage Display)**

This technique is used for producing antibody fragments (Fabs, scFvs). Gene segments encoding the antibody fragments are fused to the gene encoding one of the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophage (Figure 6).

A collection of recombinant phage, each displaying a different antigen-binding domain on its surface, is known as a phage display library. In a similar way that antibodies specific for a particular antigen can be isolated from a complex mixture by affinity chromatography, phage expressing antigen-binding domains specific for a particular antigen can be isolated by selecting the phage in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody.

The genes encoding the antigen binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining them to parts of immunoglobulin genes that encode the invariant parts of the antibody. When these reconstructed antibody genes are introduced into a suitable host cell line, the transfected cells can secrete antibodies with all the desirable characteristics of monoclonal antibodies of the suitable host cell line.

The antibody fragment gene can be fused to the gene of any of the filamentous phage capsid proteins. Protein III is present in 3 to 5 copies only on the surface of phage. It is generally tolerant for fusion with large protein at its N-terminus, while pVIII is a small protein of 50 amino acid residues, which poorly tolerates the insertion of proteins. However, to maintain proper capsid assembly of the fusions with protein VIII (pVIII) should not represent more than 20% of the pVIII capsid (Adams and Schier, 1999, Holliger and Riechmann, 1997, Kretzschmar *et al.*, 1995). If the



**Figure 6: A schematic representation of Phage display.**

Phagemid allows antibody domains to be displayed on phage after rescue with helper phage from *E. coli* suppressor strain or secreted as (tagged) soluble fragments from non-suppressor strains. AMP= ampicillin resistance gene, L= leader peptide sequence, tag= c-myc peptide sequence. Adapted from Winter *et al.* 1994



molecule is immunopurified by binding to the antigen of interest, its gene is available, allowing sequencing and further multiplication to the specific clone. It made it possible to rapidly and efficiently select fully human antibody fragments against virtually any antigen by using “universal” large non-immunized libraries (Hoogenboom, 2005, Nejatollahi *et al.*, 2002). By repeating rounds of selection / amplification by infecting *E. coli* bacteria, a rare clone may be isolated from many non-specific clones (Winter *et al.*, 1994).

All reported naïve libraries have been constructed in phagemid vectors as fusions to pIII, partially due to the higher transformation efficiency of phagemid vectors compared to phage vectors. The phagemids are plasmids in which the encapsidation signal and the replication origin of phage M13 were incorporated. These vectors have the ability to maintain and replicate in any plasmid in *E. coli*. Both systems, phages or phagemids were used to express antibody fragments fused to pIII (Hoogenboom *et al.*, 1991). In phagemid systems, helper phage is provided *in trans* to supply the other phage genes and gene products for phage particle generation (Marks *et al.*, 1992). As a result, wild-type pIII competes with antibody fragment–pIII fusion for incorporation into phage. The resulting phage population consists of phage bearing between none and five copies of antibody fragment per phage. In fact, the majority of phage bear no antibody fragment, with the next most frequent phage bearing a single copy of antibody fragment. Such monovalent display has the potential advantage of allowing more efficient selection of higher-affinity antibodies compared to multivalent display as occurs with phage vectors. With multivalent display, the presence of multiple antibodies per phage permits avidity and a higher functional affinity when the antigen is multivalent, as occurs with solid-phase immobilization of antigen.

During the screening procedure generally termed as “biopanning” phages that display a relevant antibody will be retained on a surface coated with antigen while non-specific phages will be washed away. Multiple rounds of biopanning makes possible the selection of best binder. Each system has its advantages and disadvantages. The phage system does not require superinfection with helper phage and cultures are simplified. All pIII are fused with the antibody fragment and a greater avidity of the resulting phage particle is expected. It thus promotes the selection of antibodies with low affinity. However, phages are genetically unstable (the inserted fragment is often deleted) and large-scale transformations necessary for the

construction of large banks are weak. Phagemids have, in turn, the characteristics of plasmids for everything concerning the DNA preparations and transformations. Nevertheless, the phage particles obtained are heterogeneous at the genome level (helper phage genome or phagemid incorporated into the capsid) and protein (only 1% of the particles will incorporate a fusion pIII-antibody fragment as the helper phage leads to a pIII wild incorporated more easily). A low prevalence of recombinant phages is an advantage when one wish to select a high-affinity antibodies without being bothered by the effects of avidity (Souriau *et al.*, 2004).

Anti-TNF $\alpha$  antibody, Adalimumab (Humira<sup>®</sup>), derived from phage display is the only human antibody approved by the FDA to treat rheumatoid arthritis.

### **1.3.2 Exposure on the Surface of a Ribosome (Ribosome Display)**

A less common route to generate human antibodies is ribosome display (Hanes and Pluckthun, 1997). The principle is based on the *in vitro* expression of an antibody fragment and stopping of translation, while the protein is still attached to the ribosome (Hanes and Pluckthun, 1997, Irving *et al.*, 2001). In this case, mRNA is transcribed from antibody cDNA libraries and subsequently translated *in vitro* to produce complexes where ribosomes are still connected both to mRNA and nascent polypeptide. The antibody-ribosome-mRNA complex being selected by binding to an immobilized target antigen. Then, isolated messenger RNA serves as template for reverse transcriptase to obtain the cDNA which is then amplified by PCR reaction to generate a population of selected DNA, as a starting point for further rounds of selection. A new *in vitro* transcription/translation can recreate the RNA/protein/ribosome complex and to make further rounds of selection (Levin and Weiss, 2006). This system does not suffer from limitations caused by the cloning step and *transformation in vivo*. Great diversity can also be achieved (Hanes *et al.*, 2000). Although still uncommon, the selection on ribosome surface can form rich banks ( $> 10^{12}$ ), but also to obtain mutants with higher affinities, 30 to 40 times higher affinity than that of the original antibody fragments (Hanes *et al.*, 2000). An affinity maturated ribosomal display antibody has been developed, CAT-354, which is a human anti-IL-3 monoclonal antibody for potential treatment of severe asthma (Blanchard *et al.*, 2005) (FIGURE 7).

### **1.3.3 Exposure on the Yeast Surface (Yeast Display)**

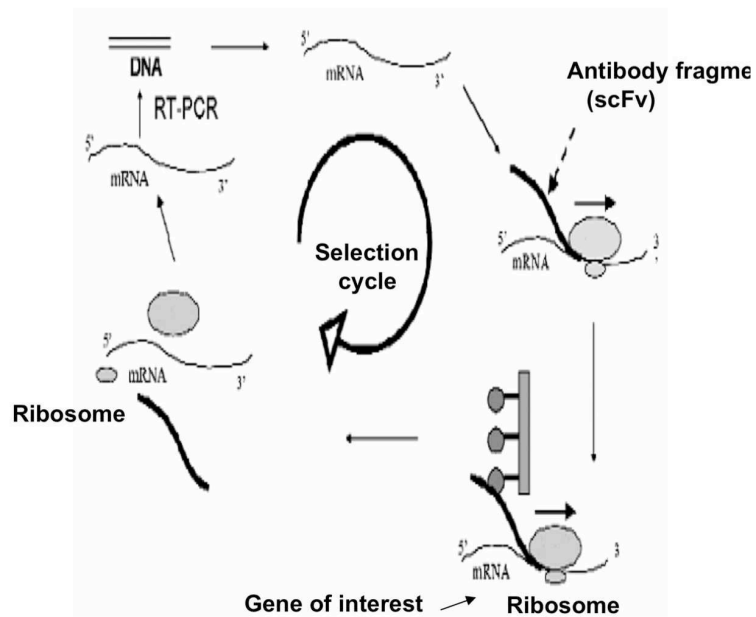
This method allows the expression of antibody fragments fused to a membrane protein on the surface of yeast (Kieckhefer *et al.*, 1997). This method has two advantages. First, the selection of mutants expressed on the surface can be performed using a cell sorter (FACS) (Feldhaus and Siegel, 2004). Then, the eukaryotic expression system promotes protein folding similar to that performed in mammalian cells (Cho *et al.*, 1998). This system could be ideal for expressing antibodies on the surface of a cell (Levin and Weiss, 2006).

Recently a novel adapter-directed yeast display system with modular features was developed. This display system consists of two modules, a display vector and a helper vector, and is capable of displaying proteins of interest on the surface of *Saccharomyces cerevisiae* through the interaction of two small adapters that are expressed from the display and helper vectors. An anti-VEGF scFv antibody gene was cloned into the display vector and introduced alone into yeast *S. cerevisiae* cells. This led to the expression and secretion of a scFv antibody that was fused in-frame with the coiled-coil adapter GR1. For display purposes, a helper vector was constructed to express the second coiled-coil adapter GR2 that was fused with the outer wall protein Cwp2, and this was genetically integrated into the yeast genome. Co-expression of the scFv-GR1 and GR2-Cwp2 fusions in the yeast cells resulted in the functional display of anti-VEGF scFv antibodies on the yeast cell surfaces through pairwise interaction between the GR1 and GR2 adapters. When the adapter-directed phage and yeast display modules are combined, it is possible to expand the adapter-directed display to a novel cross-species display that can shuttle between phage and yeast systems (Wang *et al.*, 2010) (Figure 8).

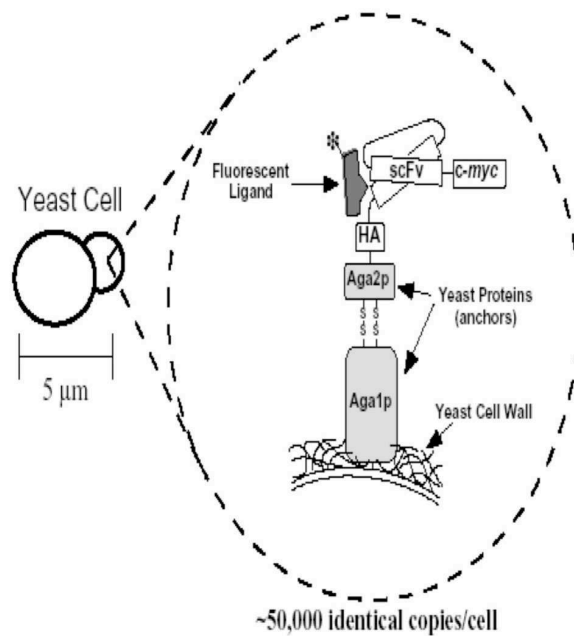
## **1.4 Design of Novel Antibody Formats with Antigen-binding Activity**

### **1.4.1 Monovalent Antibody Formats**

Since the early 1990s, it is possible to create small sized recombinant proteins, retaining the recognition function of an antigen carried by the parental immunoglobulin (Laffly and Sodoyer, 2005). The first fragment created was a monovalent Fv fragment, consisting of non-covalent association of VH and VL



**Figure 7: A Schematic representation of the general principle of the “Ribosome Display” technology.**



**Figure 8 : A Schematic representation of Yeast surface display**

domains of an antibody (Pluckthun and Skerra, 1989). ScFv fragments are much more stable, in which the variable domains of heavy and light chains associated via a peptide bond were then constructed. They usually preserve the functional properties (antigen recognition) of the parent antibody. They can also be used as a basic module to design more complex antibody fragments, multivalent, single-or multispecificity (Aubrey *et al.*, 2001, Holliger and Hudson, 2005, Holliger *et al.*, 1993, Kipriyanov and Little, 1999, Kipriyanov *et al.*, 1996, Pluckthun and Pack, 1997).

#### **1.4.1.1 ScFvs**

Single-chain variable fragments (scFvs) antibodies are the smallest fragments of an antibody molecule (25-30 kDa) which still retain full antigen binding properties (Figure 9). The first scFv molecules were independently developed by Huston *et al.* and Bird *et al.* (1988) (Bird *et al.*, 1988, Huston *et al.*, 1988). Since then many scFvs and scFv-fusion proteins have been constructed. These molecules consist of VH and VL variable domains of an antibody which are held together and stabilized covalently by a peptide linker. They can be arranged in the VL-VH (Bird *et al.*, 1988) or VH-VL orientation (Huston *et al.*, 1988), the C-terminal of one domain is connected to the N-terminal of the other domain. The peptide linker maintains the cohesion of the two VH and VL variable domains without disturbing the inter-domain contacts. It must be flexible, hydrophilic and its length exceeds 12 residues in response to the structural features association of VH and VL. Peptide linker of same length, however, creates even more constraints when the orientation is VL-VH (Huston *et al.*, 1993). Indeed, structural analysis of Fab fragments show that the distance between the C-terminus of VL and the N-terminus of VH is greater than that between the C-terminus of VH and the N-terminus of VL (39-43 Å vs 30-34 Å) (Harris *et al.*, 1998). Huston *et al.* (1988) for the first time used the peptide linker (Gly<sub>4</sub>Ser)<sub>3</sub> to fused the heavy and light variable domains, small Gly residues conferring flexibility and polar Ser residues contributing to greater solubility. This linker has no defined secondary structure and generally not interfering with the variable domains. It still remains the most commonly used, although many others have been studied with a view to further improving the recognition activity and stability of scFv (Huston *et al.*, 1988, Huston *et al.*, 1993). scFv banks in which the peptide bond has a random composition, have also been constructed (Hennecke *et al.*, 1998, Turner *et al.*, 1997). Tang *et al.* (1996) analyzed the sequences of 22 linkers, corresponding to the most active scFv and

produced in soluble form. The analysis reveals the importance of the hydrophilic nature of the linker with an abundance of arginine residues. They noted the presence of a proline residue (forming an elbow) in the peptide bond of many isolated active individual scFv. Moreover, Hennecke *et al.* (1998) have not identified a link that allows improved binding and stability properties of an scFv constructed with the linker (Gly<sub>4</sub>Ser)<sub>3</sub>. Various linker lengths have been designed to provide flexibility and enhance solubility with the most used linkers, a (Gly<sub>4</sub>Ser)<sub>3</sub> peptide (Huston *et al.*, 1991).

scFv molecules, derived from genes isolated from murine hybridoma cell lines, are capable to specifically bind to their target antigens with affinities ranging up to those of their parent mAb (Adams and Schier, 1999). ScFv represent potentially very useful molecules for the targeted delivery of drugs, toxins or radionuclides to a tumor site (Milenic *et al.*, 1991). Due to their small size, these targeting molecules are cleared more rapidly from the blood, penetrate faster and deeper into the tissue, offer enhanced accumulation in target tissues, display reduced immunogenic properties, but they also have a reduced tissue retention time, which makes them more suitable for therapeutic candidate (Yokota *et al.*, 1992).

The scFv can be multimerized mainly into dimers and trimers (Holliger *et al.*, 1993, Kortt *et al.*, 1994). A balance exists between the oligomeric forms, but the monomeric form is thermodynamically the most stable (Aubrey *et al.*, 2004). This balance depends on many factors which are the main force of interaction between the variable domains, high stability of the VH/VL leading to a higher proportion of dimeric and multimeric scFv (Kipriyanov *et al.*, 2003). In solution, 18HD37 scFv directed against the molecule CD19, with a linker of 18 amino acid residues, was in the form of monomer, dimer and multimer while 18OKT3 scFv, anti-CD3 was only isolated as a monomer (90 %) (Kipriyanov *et al.*, 1998).

The construction and production of such recombinant antibody fragments is not as simple as it might seem. The solubility, stability and immunoreactivity are the properties of these fragments which require careful considerations and much work to deal with.

One major problem with the use of scFvs is their tendency to aggregate following purification, particularly if they are derived from hybridoma cells (Kipriyanov *et al.*,

1997a). This is due to circumstances which include the presence of a disulfide bridge in the VH and VL domain, exposed hydrophobic patches at the variable/constant domain interfaces, ionic strength, pH, linker length and at last but not the least, the primary sequence, which is the most causative factor in aggregation tendency (Pluckthun and Pack, 1997, Ramm *et al.*, 1999).

Furthermore, the extent of interconnection between the scFv (fragments) increases the risk for aggregation. The stability of a particular scFv is dependent primarily on the nature and strength of the VH-VL interface interactions and the type of linker used (Huston *et al.*, 1991, Raag and Whitlow, 1995). Several strategies have been developed to overcome the aggregation and instability problem of scFv during expression and purification, including use of different detergents (Arndt *et al.*, 1998), design of inter-domain disulfide bridges (Brinkmann *et al.*, 1997), use of different soluble tag-fusion proteins (LaVallie *et al.*, 1993, Smith and Johnson, 1988) or expression hosts (Baneyx, 1999), removal, addition or substitution of some amino acids in the framework region (Brinkmann *et al.*, 1997). Another obstacle associated with the use of scFvs is their short retention time in the tissue. Due to the rapid clearance of these small 25-30 kDa proteins from the blood pool (with a half-life ranging from less than 15 min and up to hours), the absolute amount of scFv uptake by the tissue is limited (Pavlinkova *et al.*, 1999).

Recombinant antibody fragments can also be used as selective carriers for delivering toxins or cytotoxic agents to the malignant cell population to induce growth arrest, cell death or apoptosis (Maloney *et al.*, 1999). Such fusion proteins, immunotoxins, can selectively bind to cell surface receptors/antigens, block the binding site for growth factors, or kill cells by either catalytic inhibition of protein synthesis or induction of apoptosis depending on the toxin moiety.

The scFv has different pharmacokinetic properties from those of other recombinant antibody formats. The plasma half-life and elimination of B6.2 scFv (anti-tumor) is lower than that of his counterpart Fab. The scFv and Fab have half-lives of 2.4 min to 2.8 h and 14.8 min to 7.5 h respectively (Colcher *et al.*, 1990). This phenomenon seems to be general, and it is accepted that the scFv have a higher diffusivity in the blood and tissues than other antibody formats, but their removal from circulation by the kidney is also much faster (Kipriyanov *et al.*, 1997a, Kipriyanov *et al.*, 1997b). Thus, the half-lives of distribution and elimination are very short, respectively, from

2-12 min and 1.5-4 h (Adams and Schier, 1999). ScFvs are able to penetrate into solid tumors, but their rapid renal elimination limit the accumulation (Colcher *et al.*, 1990).

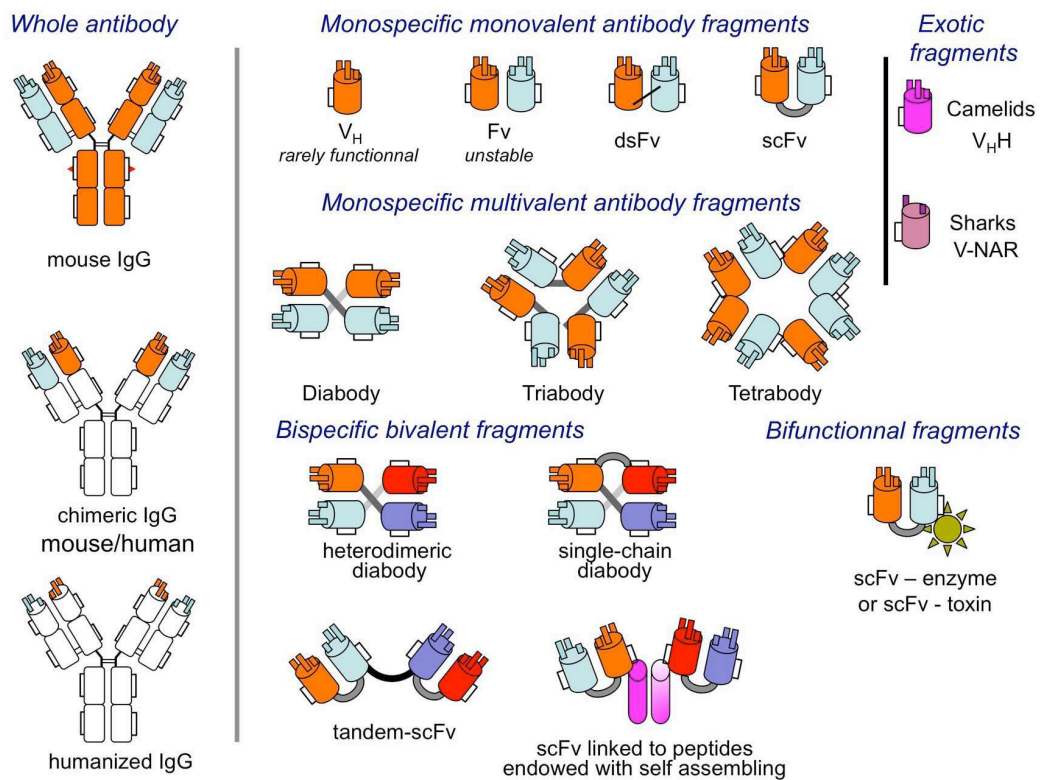
scFv as diagnostic, can be manipulated *in vitro* at the gene level to introduce linker groups for immobilization and coupling to nanoparticles. For example, the introduction of a cysteine residue at the C-terminus of an scFv construction allows covalent attachment to gold-coated sensor interfaces in directed orientation (Backmann *et al.*, 2005). Shen *et al.*, (2005) (Shen *et al.*, 2005a, Shen *et al.*, 2005b) used an additional cysteine or two additional histidine within the linker peptide of the scFv to immobilise the antibody to a gold piezoimmunosenser surface. All scFvs retained antigen-binding activity, and coupled at high surface concentration. These results suggest that the location on the linker sequence, in which the amino acids were incorporated, was well tolerated and did not interfere with the scFv binding activity. The same group further improved this approach by generating a polycationic arginine peptide linker absorbed to anionic charged template surfaces. These immunosensors have the potential applications as highly sensitive detection assays (Hagemeyer *et al.*, 2009, Shen *et al.*, 2008).

scFv as therapeutic agents, Pexelizumoab (Alexion Pharmaceuticals), a recombinant humanized scFv to C5, was the first scFv to enter clinical trial (Evans *et al.*, 1995, Fitch *et al.*, 1999) and is used to inhibit complement-mediated tissue damage associated with reperfusion injury and inflammation that occurs during open heart surgery. It give satisfactory results in clinical trails and can also be used as adjunctive therapy to fibrinolysis and primary percutaneous coronary intervention (Mahaffey *et al.*, 2006) (Figure 9).

#### **1.4.1.2 Fab Fragment**

A recombinant Fab fragment is a heterodimer consisting of the light chain (VL-CL) and the Fd portion of heavy chains (VH-CH1) of the antibodies (Figure 2). The association is made by forming disulfide bridge between CH1 and CL domains. Fab, whether recombinant (rFab) or derived from papain proteolysis (pFab), is the monovalent antibody fragment which, according to numerous studies, have the best affinity for the antigen, often very close to that of the parental antibody. The Fab molecules are more stable than scFv due to the presence of constant domains. Indeed, in a study where different monovalent formats (scFv, Fv, Fab) of





**Figure 9 : Recombinant antibodies: Novel structures for new applications.**

Molecular engineering allows to create chimeric antibodies in which the constant domains of murine origin have been replaced by domains of human antibodies. In humanized antibodies, only the hypervariable loops are of murine origin. Multiple formats consisting only of the variable domains of antibodies can also be invented. Finally, in some animal species (camels and sharks), the site of antigen recognition is made of a single antibody domain.

antiphosphorylcholine MCP603 antibody were produced, the Fab appeared less susceptible to denaturation by urea, than scFv and Fv formats (Arndt *et al.*, 2001).

In contrast, production of recombinant Fab is often difficult and sometimes production yield is very low (Laffly and Sodoyer, 2005). The proportion of active molecule is sometimes also very low. Indeed, it was found that only 6% of rFab derived from 9C2 antibody (anti-toxin AaH1) was active against its antigen (Aubrey *et al.*, 2004). A small proportion of correctly folded Fab (30%) was also obtained in yeast (*P. pastoris*) (Lange *et al.*, 2001). To improve expression of recombinant Fab, Humphreys *et al.* (2002) have modified the nucleotide sequence of signal peptides in order to balance the output of two chains co-expressed in *E. coli*. A unique periplasmic expression yield of 580mg/L of culture in a fermenter has been obtained using the constructed vector (Humphreys *et al.*, 2002). Finally, the recombinant Fab sometimes tend to dimerize and aggregate (Cyster and Williams, 1992).

The pharmacokinetic characteristics of Fab is different from those of whole antibody, particularly well illustrated by the numerous studies carried out on antivenoms (Chippaux and Goyffon, 1998). The plasma half-life and elimination in rats are from 6h to 104h for IgG and from 30 minutes to 23 hours for the Fab format respectively (Weir *et al.*, 2002). The murine Fab have a high degree of immunogenicity and are thus not adapted to therapeutic requirements. The immunogenicity can be reduced by chimerization or humanization (Kipriyanov and Little, 1999). The chimerization which involves replacing the murine constant domains with human constant domains is better tolerated, preserves the affinity of the parental murine Fab and improves the half-life in blood. A humanized Fab can also be obtained through recombinant technology. It contains only the hypervariable loops of murine monoclonal antibodies, and the rest of the structure being of human origin (Carter, 2006) (Figure 9).

#### ***1.4.1.3 Single Domain Antibodies (Specific antibodies) : VHH, VNAR and Human Domain Antibody (dAbs)***

##### ***1.4.1.3.1 Camelids (Camel, llama)***

These antibodies naturally devoid of light chains (Figure 9). These are called heavy chain antibodies (HcAbs) in camelids. These antibodies have a single variable domain (called VHH for camelids), which generates high affinity towards a large spectrum of

antigens. These small domains (13-16kDa) can be easily produced in bacteria or yeast. In addition, these single-chain structures, limited to the heavy chain antibodies, have no CH1 domain, constant region is therefore consists only of CH2 and CH3 domains. The variable domain, or VHH has a common CDR3 very long (up to 24 residues for the camel or llama antibodies). This length may explain the adaptation to the setting of molecular cavities (Koch-Nolte *et al.*, 2007). This characteristic seems to be necessary to provide the antigen contact area sufficient for proper interaction and compensate the absence of the VL domain. Moreover, the primary sequence of the FR regions of these antibodies and that of FR of VH domains of human antibodies is more than 80% homologous. Their three-dimensional structures are very close, and often superposable (Harmsen and De Haard, 2007, Holliger and Hudson, 2005). The VHH are particularly well suited for expression in the *E. coli* or yeast systems. Technologies such as phage or ribosome display, to isolate the highly VHH specific for an antigen of interest from immune, non-immune or semi-synthetic banks, have already been used (Harmsen and De Haard, 2007).

Ablynx oriented for several years on nanobodies technology (nanobodies) derived from llama antibodies. The Nanobody ALX-0081 give positive results in phase I clinical trial. This molecule directed against the von Willebrand factor (vWF) would be able to reduce the risk of thrombosis in patients with acute coronary syndromes without inducing side effects. The VHH represent ideal tools to target other antigens. Thus, a Nanobody specifically recognizing an epitope expressed consistently by different subspecies of the parasite *Trypanosoma brucei*, the causative agent of African trypanosomiasis, was obtained after immunization of camels and selection by phage display. This process allowed obtaining the Nanobodies NbAn33, which then produced as a fusion protein (NbAn33-APOL1), proved capable of durable cure of experimental trypanosomiasis *in vivo* in mice. Finally, the company developed a portfolio of Nanobody other candidates in preclinical trials in a variety of applications, including inflammation, oncology, pulmonology, and Alzheimer's disease (source: ablynx.com).

#### **1.4.1.3.2 IgNAR**

Other species of mammals have antibodies that are structurally particular, as is the case for sharks which also lack light chain and are called IgNAR. Thus, the structure

of an immunoglobulin isolated from serum of shark, have been determined (Ignar, Novel Antigen Receptor) (Figure 9). This is a heavy chain homodimer, stabilized by disulfide bonds, and naturally devoid of light chain. In contrast to camelid antibodies, this molecule is composed of five constant domains and a single variable domain comprising two regions similar to the conventional hypervariable loops (Nuttall *et al.*, 2003).

Antibodies that recognize a parasitic antigen or cholera toxin, have been isolated from naive antibody variable regions libraries of sharks (Liu *et al.*, 2007). In addition, strategies to modulate the characteristics of the IgNAR have already been proposed, particularly for producing bivalent forms of variable domains, and their high avidity for their target (Nuttall *et al.*, 2003). Walsh *et al.* (2011) described a novel reagent, a single variable domain (V(NAR)) of the shark immunoglobulin new antigen receptor (IgNAR) antibodies. The V(NAR) domain displayed biologically useful affinity for recombinant and native hepatitis B e antigen (HbeAg), and recognised a unique conformational epitope (Walsh *et al.*, 2011).

#### ***1.4.1.3.3 Domain Antibody***

A domain antibody (dAb) is either the variable domain of an antibody heavy chain (V<sub>H</sub> domain) (Ward *et al.*, 1989). or the variable domain of an antibody light chain (V<sub>L</sub> domain) (Pereira *et al.*, 1998). Each dAb therefore contains three of the six naturally occurring complementarity determining regions (CDRs) from an antibody, which are the highly diversified loop regions that bind to the target antigen. Although it might seem surprising that three CDR regions are sufficient to confer antigen-binding specificity and high affinity, Darwinian evolution has itself arrived at that very solution in camelids, which produce antibodies comprising only a heavy chain. The antigen-binding site of these antibodies consists of a single unpaired variable domain. dAbs are the smallest functional antigen-binding fragments of an antibody. Their small size and ability to be isolated and affinity-matured by using *in vitro* selection such as phage display means that very specific, high-affinity dAbs can be generated in a matter of weeks from vast ‘single pot’ libraries

In comparison with other antibody formats, domain antibodies (dAbs) technology has several advantages. Firstly, dAbs consist of only a single immunoglobulin variable domain and therefore have a particularly short serum half-life (Holliger and

Hudson, 2005). Secondly, they can also be expressed as soluble protein in bacterial culture with no requirement for mammalian tissue culture expression system and thirdly, it can be reasoned that larger antibody constructs pose a risk for receptor multimerization and systemic activation of platelets. In summary dAbs, with their fully human scaffolds, potentially provide a novel format for the development of anti-platelet therapeutics (Walker *et al.*, 2009).

Gay *et al.*, (2010) reported preclinical data for CEP-37247, the first human framework domain antibody construct to enter the clinic, having high selectivity and affinity for target antigen TNF $\alpha$  (Gay *et al.*, 2010). Thus, the small size of these molecules, their complete solubility and stability could soon be exploited for diagnostic purposes or therapy.

#### ***1.4.1.4 Other Molecules of Interest***

##### ***1.4.1.4.1 Ankyrin***

Totally natural molecules other than antibodies are capable to recognize and neutralize a target. The immune system of lower vertebrates, such as eel, is based on repeated protein motifs (leucine-rich), which constitute a class of proteins endowed with binding activity and abundant in nature. As vertebrates generate immune repertoire from recombinations gene, eels rearrange the gene segments of their repeated proteins to generate a diverse repertoire. These repeated domains proteins play a major role in the immune system in humans, animals, plants, insects, bacteria. The ankyrin molecule is composed of a compact framework regions of variable size, which make it possible to merge peptide loops variability (Binz *et al.*, 2005, Binz *et al.*, 2003). Thus, certain structural repeat motifs (ankyrin repeat) of ankyrin can permit to use this protein to build paratopes banks. The structures, are monomeric and small size (17kDa), fully soluble, and without disulfide bond, which facilitates their production in the form of recombinant protein in prokaryotic organisms (*E. coli*). Their pharmacokinetics properties can be modulated by crosslinking polyethylene glycol (PEG). This strategy is currently used by the Molecular Partner company, which generates the Designed Ankyrin Repeat Protein or DARPin. For example, one of these target molecules with high affinity, the same epitope as trastuzumab, a therapeutic anti-Her2 antibody. Moreover, this DARPin is capable of binding Her2 *in*

*in vivo*. The potential diagnostic imaging (*in vivo*) and treatment of these molecules should now be confirmed in clinical studies (source: molecularpartners.com).

#### **1.4.1.4.2 Anticalin**

The molecules developed by the Pieris-Proteolab company (Anticalins) are based on the use of compact structures, derived lipocalin, a protein family naturally expressed in humans and endowed with binding properties (apolipoprotein D, carrier of retinol). The lipocalin are molecules composed of a single polypeptide chain of 160 to 180 amino acids, which has a combination of four flexible hypervariable regions (Schlehuber and Skerra, 2002). Thus, the lipocalin cloning and the creation of a banks of hypervariable loops allowed to generate Anticalins, whose main characteristics are their stability and solubility. The affinity of these molecules is usually equivalent to that of murine monoclonal antibodies, but their human origin is an advantage with a view to use it in man. The small size of Anticalins (20 kDa) and their stability appear to be essential properties to consider it for targeting an intratissular antigen. In addition, the marking of these molecules with a radioactive agent is usually easy. The fusion of two anticalins molecules has also been developed for obtaining bivalents duocalins recognizing two different targets (Schlehuber and Skerra, 2001). Thus, anticalins and duocalins may be particularly suited for imaging in oncology *in vivo*, but also in therapy. In this area, two anticalins (PR-050, PR-055), anti-VEGF have already been produced. One of them could be a substitute for ranibizumab (Lucentis®) a humanized Fab used in patients with the exudative of age-related macular degeneration (source: Pier-ag.com).

#### **1.4.1.4.3 Microbodies**

Microbodies are novel pharmacophoric entities derived from naturally occurring cysteine-knot microproteins, which provide stable scaffold and can be engineered to high affinity binding proteins (Hu, 2009). The microbodies (microproteins), developed by the Nascacell company. They exhibit various biological functions: inhibition of signal transduction, cellular regulation. In plants, these microproteins have a role in defense against parasites, while in insects, they act as toxins to block a target. These molecules are small, less than 50 amino acids. The structure of the microproteins, perfectly stable, is based on the presence of 3 to 4 disulfide bridges. The microbodies are highly stable and resistant to temperature, pH and proteolytic

degradation. These molecules can be synthesized or produced recombinantly. Their potential applications are varied, the validation of new therapeutic targets, through the stabilization of peptides or inhibitors of target therapeutic potential until administered orally (Source: [nascacell.com](http://nascacell.com)).

#### ***1.4.1.4.4 Aptamers***

RNA aptamers are short nucleic acid sequences that have the capacity to bind a target with high affinity and in a specific manner. Some aptamers are potent inhibitors of protein function. They are conventionally isolated from combinatorial oligonucleotide banks ( $10^{15}$  different sequences) using *in vitro* selection methods. Aptamers have many advantages over monoclonal antibodies. They can be selected quickly by a simple *in vitro* process and effective for many targets, even those that are toxic or weakly immunogenic. They are stable and resistant to nucleases, physical or chemical denaturation, without loss of activity. Finally, the development of chemically stabilized aptamers allowed us to target and inhibit growth factors, receptors, coagulation factors and intracytoplasmic proteins such as thrombin or kinases. The Archemix company has several therapeutic aptamers in advanced clinical phase. Some target proteins or coagulation enzymes, such as Factor (Regado Biosciences, [www.regadobio.com](http://www.regadobio.com)), von Willebrand factor (vWF) or thrombin (NU172, phase II). Other aptamers, target proteins involved in development of certain cancers and chronic autoimmune diseases (Sources: [archemix.com](http://archemix.com), America Heart Association, [www.heart.org](http://www.heart.org))

### **1.4.2 Polyvalent Antibody Fragments**

There are several possibilities for the production of multivalent scFvs. Some of the strategies described exploit the multimerization behavior of scFv when the variable domains of one scFv molecule pair with the complementary domains of another one (Whitlow *et al.*, 1993). The production level of dimers and higher multimers are linker dependent and can be increased under certain conditions (Desplancq *et al.*, 1994, Whitlow *et al.*, 1994). The stabilities of such multivalent Fv, however, differ significantly depending on the specific variable domains from which they are derived/constructed (Whitlow *et al.*, 1994). Polyvalent antibody fragments evolution were aimed to enhance affinity for the antigen, in addition to stability and expression levels (Holliger and Hudson, 2005).

#### ***1.4.2.1 Monospecific Antibody Fragments***

Antibody that can bind to a single antigen epitope are known as monospecific antibodies. The dimeric (diabody), trimeric (triabody) and tetrameric (tetrabody) forms of scFvs can be produced using short peptide linkers to link the two variable domains (Figure 9) (Hudson and Kortt, 1999, Kortt *et al.*, 2001, Kortt *et al.*, 1997). A short peptide linker (11 amino acid residues or less) induces multimerization of fragment because of steric constraints occurrence and prevent the association of variable heavy and light domains of a single subunit between them. Several subunits may then assemble the variable domain of a heavy subunit pairing with the variable light domain of another subunit. The nature and number of amino acid residues of the arm and also the arrangement of the two variable domains, influence valency, stability and the proportions of different oligomers (Kortt *et al.*, 2001). It was observed that a peptide linker of 3 to 12 amino acids residues promotes diabody formation (Kortt *et al.*, 1994). Reducing the linker to less than three residues leads to triabody or tetrabody (Dolezal *et al.*, 2000). However, this is not a general phenomenon (Le Gall *et al.*, 2004).

The presence of several antigenic recognition sites can usually increase the apparent affinity (avidity) of the fragment to its antigen (Kortt *et al.*, 1997, Pluckthun and Skerra, 1989). However the gain of avidity is not always significant and depends on the type of antigen (soluble or membraneous) and the presence or absence of multiple copies of the epitope on the target antigen. Simply changing the size of the peptide linker thus leads to a panel of antibody fragments, 25 to 100 kDa, with different pharmacokinetic characteristics. Le Gall *et al.* (1999) produced the scFv HD37 anti-human CD19, in the VH-VL orientation, as a diabody (10 residues linker), a triabody (no linker) and tetrabody (1 residue linker) (Le Gall *et al.*, 1999). Their results also demonstrated that different formats are not only based on the size of the peptide linker between the variable domains, but also dependent on the nature of amino acid residues located in the vicinity of their proximity.

#### ***1.4.2.2 Bispecific Antibody Fragments (bsAb)***

Antibody that can bind to two different antigen epitopes is known as bispecific. Although bispecific antibodies do not occur naturally, they can be generated through a number of strategies. They can be designed to bind either two adjacent epitopes on a



single antigen, or to bind two different antigens for numerous applications, but particularly for recruitment of cytotoxic T- and natural killer cells (NK) or retargeting of toxins, radionuclides or cytotoxic drugs for cancer treatment. Bispecific antibodies can be produced by fusion of two hybridomas into a single 'quadroma' by chemical cross-linking or genetic fusion of two different Fab or scFv molecules.

Several recombinant fragments have been developed. The most studied recombinant formats include

#### **1.4.2.2.1 Tandem scFv**

Tandem scFv, the bispecific single-chain Fv antibodies comprise four covalently linked immunoglobulin variable domains (VH and VL) of two different specificities. The variable domains of each antibody is fused with the other by a peptide linker of less than 12 amino acid residues and also associated in the form of scFv. The length and composition of the peptide linker used between the two scFv as a general rule have no influence on the two binding activities, however they affect the flexibility of the molecule. The distance between the two paratopes of the molecule varies from 50 Å (5 amino acid residue linker) to 100-120Å (linker of 20 residues) (Kipriyanov *et al.*, 2003). For a good stability a short linker is of importance. Concerning the orientations of variable domains, many different construction are possible (Brandao *et al.*, 2003, Yoshida *et al.*, 2003). Kipriyanov *et al.*, 2003 described tandem anti-CD3 (OKT3) and anti-CD19 (HD37) scFv fragments in which only anti-CD19 activity was observed, regardless of the domains sequence. The dual activity, however, were found in the heterodimeric diabody and single-chain diabody formats (Figure 9).

#### **1.4.2.2.2 Bispecific Diabodies**

Bispecific diabodies, two subunits of 25kDa constitute a heterodimeric diabody. The first subunit constitute the heavy variable domain of an antibody fused to the light variable domain of the second antibody by a short peptide linker. The second subunit have the same structure but the origin of domains is inverted (reversed).

The compact structure of the diabodies give them attractive properties such as good tissue penetration, expression and solubility as well as enhanced stability. Several preclinical studies have shown the efficacy of these molecules but no clinical trials have yet been reported (Fisher, 2001). The disadvantages of the heterodimeric

diabody are (i) need a bicistronic expression vector to express it simultaneously and the same proportion of the two subunits and (ii) the formation of an inactive homodimer is always possible, under a weak interaction between the complementary variable domains. Kipriyanov *et al.*, (2003) elaborated heterodimeric bifunctional diabodies of anti-CD19 and anti-CD3. They observed the formation of aggregates (VHCD3-VLCD19) and soluble inactive homodimers (Figure. 9).

#### **1.4.2.2.3 Single-chain Diabody (scDb)**

Single-chain diabody (scDb), in which the two chains of the fragment are linked via an additional linker, which combines all domains in a single polypeptide. The VH and VL variable domains of the same antibody is arranged in the centre of the molecule and the variable domains of the other antibody is situated at the extremity. The possible arrangement is therefore, VH<sub>a</sub>-VL<sub>b</sub>-VH<sub>b</sub>-VL<sub>a</sub> or VH<sub>b</sub>-VL<sub>a</sub>-VH<sub>a</sub>-VL<sub>b</sub>. The choice of the linker peptide located in the center of the molecule between the two variable domains of one antibody is crucial. It is responsible for the proper folding of the molecule. So it must be long (more than 15 residues) and flexible (Nettelbeck *et al.*, 2001, Nettelbeck *et al.*, 2004). The linkers between the other variable domains are generally identical and shorter in order to prevent the association of neighbours variable domains (Kipriyanov *et al.*, 2003). The presence of the central peptide linker participate in increasing the stability of the recombinant molecule and prevents the formation of inactive homodimers (Kipriyanov *et al.*, 2003).

Design a bifunctional scDb remains still delicate. This requires a fine structural analysis and modeling to determine which arrangement of different variable domains is the most suitable one. It may also be determined which peptide linker, is critical for the proper folding and the stability of the molecule (Volkel *et al.*, 2001). This single chain diabody format has also shown potency in preclinical trials (Muller *et al.*, 2007).

All these bispecific formats rely on the use of flexible peptide linkers and although these linkers have obvious advantages in term of antibody engineering but also have some disadvantages due to their foreign nature which may lead to an unwanted immune response. Moreover, the flexible nature make linkers prone to proteolytic cleavage in serum, potentially leading to production issues, poor antibody stability, aggregation and increased immunogenicity (Fisher and DeLisa, 2009).

Bispecific antibodies have been used in various ways, the most exciting application being the possibility to simultaneously target the effector cells of the immune system and stimulate them through the interaction with an activating receptor in order to achieve an efficient lysis of tumour cells. One of these molecules, MT103 has given very encouraging results in clinical trials, including complete remission, for the treatment of non-Hodgkin's lymphoma. Another bispecific T-cell engager format, MT101 (anti-CD3 x EpCAM) is also being tested at the moment against colorectal cancers (Amann *et al.*, 2009). Catumaxomab (Removab ®; an anti-EpCAM x CD3, has demonstrated efficacy and safety in phase II/III clinical trial against malignant ascites (Shen *et al.*, 2008). Hagemeyer *et al.*, (2004) have developed an scFv-TAP which have both fibrin targeting and anti-FXa activity (Hagemeyer *et al.*, 2004) (Figure 9).

### **1.4.3 Armed Antibodies (Cancer/Immunoconjugates)**

Antibody engineering have made it possible to increase the effector function of monoclonal antibodies by arming them with either radionuclide drugs or potent toxins or by engineering recombinant bispecific antibodies that simultaneously bind the target and activate receptors or immune effector cells such as CD3 and FcR (Kreitman, 1999, Kreitman *et al.*, 2000, Wu and Senter, 2005).

Development of mAb for imaging or for therapeutic application is far from being optimal. Intact antibody (150kDa) penetrates solid tumors very slowly and with slow blood clearance. Therefore, researchers developed smaller antibody fragment with shorter half-life and rapid blood clearance and therefore with reduced background in imaging or alternatively with limited unwanted exposure to normal tissues. Preclinical imaging studies were carried out using antibody fragments targeting CEA and HER2/Neu (Wu and Senter, 2005).

Another approach to increase the effector function of mAb is by antibody-directed enzyme prodrug therapy (ADEPT), which uses mAb to specifically deliver an enzyme that activates a subsequently administered prodrug. Phase 1 trial of ADEPT reported using murine F(ab')<sub>2</sub> anti-CEA fragment linked to carboxypeptidase G2 followed by prodrug bis-iodo-phenol mustard (ZD276P) in patients with advanced colorectal carcinoma, but the main problem with trial was the immunogenicity of the murine F(ab')<sub>2</sub> and the conjugated enzyme (Francis *et al.*, 2002). FDA has approved three

mAb conjugate for cancers, out of which two are murine radiolabeled mAb to treat B cell lymphoma, a CD-20-specific IgG2I<sub>k</sub> radiolabeled with Y90 (Ibritumomab Tiuxetan) (Jacene *et al.*, 2007) and a CD-20 IgG2aI radiolabeled with I131 (Tositumomab) (Borghaei *et al.*, 2004). While the third mAb conjugate is a humanized CD-33 specific IgG4<sub>k</sub> mAb chemically conjugated to a calicheamicin derivative that induces double strand breaks (Gemtuzumab Ozogamicin) for the treatment of leukemia (Linenberger, 2005).

#### **1.4.4 Antibody Fragment Expression System**

Various expression systems can be used for the production of whole antibodies and antibody fragments. These includes bacterial or mammalian cell culture and transgenic animals or plants. The antibodies are the products of highly differentiated cells, which offers difficulty to produce them in a host cell other than B lymphocytes and that does not have an equivalent complexity. The choice of expression system, a host and a vector is based on different criteria: (i) the predicted final use of recombinant antibodies (therapeutic or diagnostic *in vitro* or *in vivo*), (ii) the necessary presence or absence of carbohydrate residues, (iii) the desired production yields, this yield affects the cost of goods, which is compromised principally of two factors: the cost of the upstream process that generates the antibody, and the cost of the down stream process, that is, purification and final fill of the active pharmaceutical ingredient. (iv) the technologies available in each laboratory. The amplified gene of the variable domains of the antibody is inserted into a vector suitable for expression in the system of the selected host. In this manner, the antibody fragments can be produced in prokaryotic systems (Bird *et al.*, 1988, Huston *et al.*, 1988, Skerra and Pluckthun, 1988), in yeast (Davis *et al.*, 1991), in insects cells (Poul *et al.*, 1995), in mammalian cells (Riechmann *et al.*, 1988) and in plant cells (Owen *et al.*, 1992).

##### **1.4.4.1 *E. coli***

The first expression of a complete antibody gene in *E. coli* was reported by Cabilly *et al.* (1984) and Boss *et al.* (1984). They produced the heavy and light chains of the antibody in the form of insoluble inclusion bodies and without specific activity. A solubilization of the aggregates, denaturation, renaturation and then purification were found necessary for the active antibody fragments. The production of antibody

fragment (scFv, Fv and Fab) in soluble and active form were first shown by different researchers (Glockshuber *et al.*, 1992, Skerra and Pluckthun, 1988). *E. coli* have many advantages as a host for the production of antibody fragments such as rapid production, inexpensive, requires very simple materials, the periplasm favours the disulfide bonds formation necessary for antibody fragments activity.

While disadvantages are that there is no glycosylation of proteins in *E. coli*. Level of expression may also be a problem. It is often very difficult to find correct agreement between, (i) a mass production within the bacterial cytoplasm as inclusion bodies, which must be solubilized and purified, (ii) a reduced production in the form of native protein exported into the periplasm or into the culture medium or, finally, (ii) a production in the form of fusion protein directly into the periplasm, on the bacterial surface, or on the surface of bacteriophage envelope.

#### **1.4.4.1.1 Cytoplasmic Expression**

The antibody fragments have also been produced in insoluble form in the cytoplasm of *E. coli* (Bird *et al.*, 1988, Huston *et al.*, 1988). After extraction of the inclusion bodies, denaturation, and renaturation, a small fraction of the recombinant protein were recovered in native active conformation. The *in vitro* refolding of the protein and the arrangement of disulfide bridges can be heterogeneous, even after purification and hence lead to errors in the analysis of functional parameters of the protein, for example, to calculate its affinity (Huston *et al.*, 1991). There are multiple causes of the aggregation of recombinant antibodies in the bacterial cytoplasm. Maturation of the overproduced recombinant protein may be incomplete because of the inability of the cellular machinery to process large amounts of heterologous protein. The chemical environment of the cytoplasm is incapable for the formation of disulfide bonds, the antibody fragments aggregates in the subform of polypeptide leads to their precipitation (Pluckthun, 1994).

The newly synthesized proteins, according to their final location, are supported by different proteins: the chaperones (Ellis, 1990). The chaperone proteins are bacterial stress proteins produced in large amounts when the bacteria are subjected to aggression on the physiological state such as the sudden rise in temperature or abnormal overproduction of an exogenous protein (Ellis, 1990). Early work highlighted the *GroE* complex formation, composed of two proteins *GroEL* and

*GroES*, which promotes the polymerization of ribulose biphosphate carboxylase (Rubiscos) *in vivo* (Goloubinoff *et al.*, 1989). In addition, the *GroE* complex preserves the aggregation of bacterial polypeptides promoting the acquisition of the native structure of the cytoplasmic proteins and maintain proteins in a state held responsible for the translocation to the periplasmic membrane (Martin *et al.*, 1991). Another complex *DnaJ/DnaK* proteins are associated with proteins in synthesis stage and which is still attached to ribosomes, which showed that the two complexes would act at different stages of proteins maturation. Skerra and Pluckthun (1991) discussed the importance of these chaperones in the folding mechanism of newly synthesized proteins.

Both prokaryotes and eukaryotes have reducing cytoplasm while the periplasm of prokaryotes is oxidized which promote disulfide bonding and correct folding of proteins. In the cytoplasm, an enzyme thioredoxin reductase, coded by *trxB* gene destroy the disulfide bond formed. Thus, those proteins which do not require the formation of disulfide bonds for reaching its native structure can be produced in a functional form in the cytoplasm. However, Beckwith and co-workers mutated the thioredoxin reductase by silencing its activity and were able to produce a correctly folded alkaline phosphatase in the bacterial cytoplasm (Derman *et al.*, 1993). The preliminary model indicated that NADPH was the source of reducing potential used by thioredoxin reductase to reduce oxidized thioredoxin but later it was found that there was a second thioredoxin and a glutathione oxidoreductases, all are responsible for the reducing cytoplasmic cysteines (Stewart *et al.*, 1998). It was shown that thioredoxin acts as a reductase only when it remains constantly reduced by the thioredoxin reductase activity. Also glutaredoxin 3 can catalyze disulfide bond formation in the periplasm, but its activity depends on oxidized glutathione availability rather than disulfide bond formation protein B (DsbB) (Eser *et al.*, 2009).

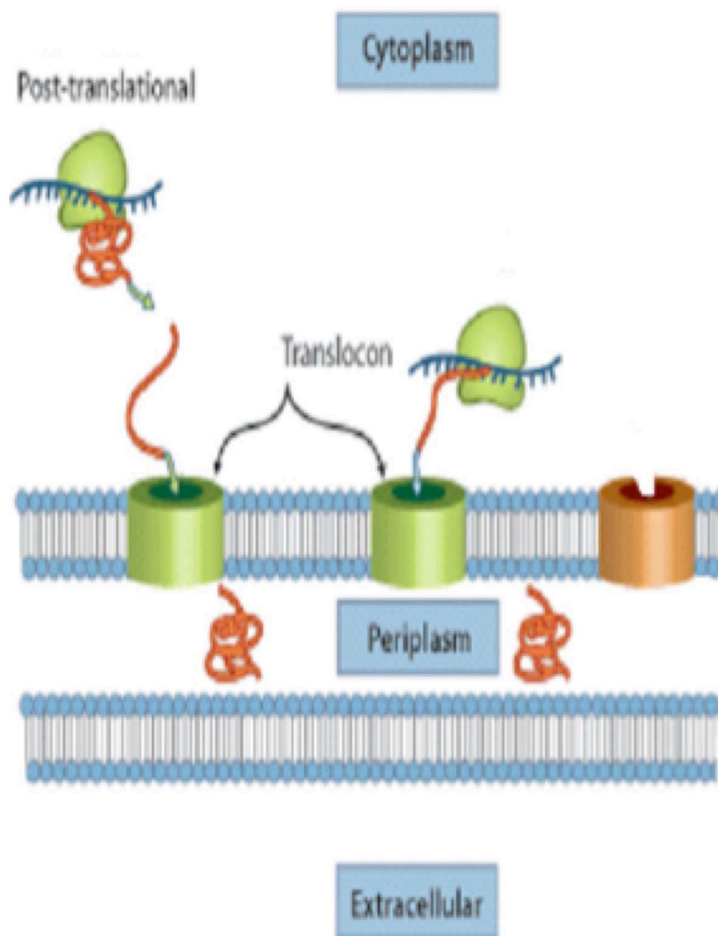
Therefore, it is possible to obtain an oxidizing cytoplasm in a cellular system in which glutaredoxin activity is abrogated by *gor<sup>-</sup>* mutation, and both thioredoxin 1 and 2 are kept oxidized as a consequence of thioredoxin reductase mutations. Reduced glutathione, necessary to preserve cell viability, is produced by disulfide reductase activity of mutated peroxiredoxin *AhpC* (Faulkner *et al.*, 2008, Yamamoto *et al.*, 2008). Interestingly, *AhpC* reductase activity does not significantly influence the oxidizing conditions of the cytoplasm in *trxB<sup>-</sup>*, *gor<sup>-</sup>* cells (Proba *et al.*, 1995). So now

single (*trxB*-) and double (*trxB*-, *gor*-) mutant strains are commercially available with the names of AD494 and Origami (Novagen), respectively for the cytoplasmic expression of recombinant proteins with multiple disulfide bonds in their native structure.

#### **1.4.4.1.2 Periplasmic Expression**

In gram negative bacteria, the periplasm, a compartment between the external and internal membrane, provide a favourable environment for the production of active heterologous proteins (Figure 10). The periplasmic proteins are generally soluble, correctly folded with disulfide bonds because of the presence of periplasmic ‘disulfide binding isomerases (Wulfing and Pluckthun, 1994) and a favourable oxydoreductible environment. Their proteolytic degradation is habitually limited and further purification is facilitated because the recombinant protein represents the majority protein fraction of the bacterial compartment which is equivalent to 30% of the bacterial volume (Van Wielink and Duine, 1990). The strategy generally used for the production of the variable domains of the antibody in the periplasm consist of fusing the protein to the C-terminus of a signal peptide which transport the protein towards the periplasm. The signal peptide is cleaved at the membrane level and liberate the recombinant proteins in the periplasm. The translocation of the new-synthesized protein across the internal membrane requires a signal peptide and a proteinic machinery for the maturation (Hockney, 1994).

Considering the potential danger of having an over crowded cytoplasm, some authors proposed that protein secretion could be effectively modulated at the translational level by modifying the shine-dalgarno sequence (Mavrangelos *et al.*, 2001), while other found that the crucial turning region starts upstream of the shine-dalgarno region and spans approximately twenty nucleotides downstream of the initiation codon (Simmons and Yansura, 1996). The other parameters influencing the expression rate have been evaluated in relationship with the production of disulfide-bond dependent proteins, such as growth medium, plasmid origin of replication, and expression promoters. For instance, the expression features of *tac*, *uspB*, *T7*, *trc*, *lacUV5*, *malk*, *pm/xyIS* have been investigated to achieve high product yields by preventing protein precipitation and cell lysis (Jeong and Lee, 2000, Paal *et al.*, 2009). The choice of a suitable leader peptide sequence also play an important role in protein



**Figure 10. A Schematic diagram for the production of recombinant protein in the periplasm of *E. coli*.**

Adapted from Broedel and Papciak, 2007.



expression level. The signal peptide which consist of about dozens residues, have a very conserved architectures (Von Heijne, 1985). The N-terminal extremity of signal peptide is hydrophilic (2-14 residues) in which three are positively charged, followed by a long hydrophobic region (more than 8 residues) rich in alanine and leucine residues but lacking polar or charged residues, which give it  $\alpha$ -helical secondary structure in the apolar environment. The C-terminus of the signal peptide contain a recognition site for the signal peptidase for cleavage of the precursor proteins (Pugsley, 1993). In the absence of an N-terminal signal peptide for periplasmic secretion, recombinant protein expressed in bacteria accumulate in the cytoplasm. The fusion of suitable leader sequence allows for the translocation of unfolded precursors into the periplasm by either the sec (relatively slow, post-translational translocation) or the SRP (fast, co-translational translocation) system (Luirink and Sinning, 2004). The search for optimal leader peptides to use in the combination with recombinant proteins has been initially undertaken by comparing the efficiency of natural signal sequences identified in the precursors of bacterial periplasmic proteins, including the leader peptide from *spA*, *phoA*, *ribose binding protein*, *pelB*, *ompA*, *ompT*, *dsbA*, *torA*, *torT*, and *tolT*. Moreover , synthetic and phage pIII leader peptide were also used (Mergulhao *et al.*, 2003, Thie *et al.*, 2008). Initially, the approach was not systematic and no clear preference for any among them was apparant, although *ompT* resulted preferable when coupled to overexpression of chaperones involved in the stabilization of intermediates translocated through the sec export machinery (Ignatova *et al.*, 2003). However, there also exist a strong correlation between hydrophobicity of the leader sequence and the export mechanism (Huber *et al.*, 2005). Apparently, cotranslational translocation by SRP needs the presence of highly hydrophobic leader sequences, even though further unknown biophysical features may be critical. The physiological necessity of the SRP pathway as an alternative to the post-translational secretion mediated by the sec route is required to avoid premature folding of the proteins in the cytoplasm. The biotechnological implication of these conclusions is that poor periplasmic accumulation of rapidly folding recombinant proteins may be the consequence of their non-productive cytoplasmic misfolding that prevents efficient translocation and correct periplasmic folding. Therefore, the choice of the leader peptide may make the difference in terms of secretion efficiency, as demonstrated for thermodynamic stable proteins (Steiner *et al.*, 2008).

Another important point is the mechanism of protein oxidation in the periplasm. Spontaneous protein oxidation in periplasm is extremely slow and is incompatible with cell activity. Therefore, it is necessary that the disulfide bond formation is enzymatically catalyzed. Periplasmic protein oxidation is regulated by the five members of the Dsb protein system (DsbA, B, C, D, G) (Messens and Collet, 2006). With the exception of *DsbB*, these proteins belong to the thioredoxin protein superfamily and are involved in both disulfide bond formation and rearrangement.

Co-expression of periplasmic chaperons and foldases are also important for the correct protein folding in the *E. coli* periplasm. These chaperons and foldases belong to several classes and often have partially overlapping functions. There are peptidyl-prolyl isomerases and chaperons such as SurA, FkpA, PpiA, PpiD, and *Skp* and chaperone/proteases such as *DegP* (Dartigalongue and Raina, 1998, Narayanan and Chou, 2008). The prolyl-cis/trans isomerase activity of Ppi apparently catalyzes the rate limiting step of the recombinant antibody folding represented by the isomerization of proline 95 (Kabat numbering) (Jager and Pluckthun, 1997). Its coexpression increased the solubility of scFvs and Fab fragments (Lin *et al.*, 2008). Similarly, fusions to *FkpA* resulted in increased yields of functional recombinant antibodies (Padiolleau-Lefevre *et al.*, 2006, Zhang *et al.*, 2003). Similarly the cytoplasmic chaperons also affects the accumulation of periplasmic proteins.

It was also found that the level of accumulation of a given protein was medium composition-dependent. A simple screening technique was developed to rapidly identify the best available medium for any given recombinant protein. The screen was tested on different recombinant proteins and shown to be an effective means of empirically determining the best medium. It was further observed that the medium composition affected the relative level of soluble protein that accumulated. These results suggested that a medium screen should be employed as a routine part of developing bacterial strains which produce recombinant proteins. The application of such a screen could aid in improving the recovery of recombinant proteins (Baneux, 1999)

Analysis of products approved till 2010 confirm that system based on mammalian cells and *Escherichia coli* remain the workhorses of biopharmaceutical production. Bacterial system are suitable for the expression of scFvs and Fabs, because bacteria lack the cellular machinery to glycosylate proteins, only aglycosyl-antibody

fragments are produced from bacterial fermentation. Recombinant fragments can be localized intracellularly or within the periplasm. The yield of scFvs in bacterial expression system is usually low, primarily as a result of incorrectly folded protein retained either at the inner cell membrane or as an insoluble aggregate in the periplasm. Although the insoluble periplasmic protein can be extracted and refolded, a high periplasmic expression can cause toxicity and cell death (Sanchez *et al.*, 1999).

2 out of 3 Fab fragments approved by the FDA for therapeutic applications are produced in the periplasm of bacteria. Ranibizumab (Lucentis®) for the treatment of Age-related Macular Degeneration (AMD) and certolizumab (Cimzia®) which is a pegylated Fab targeting TNF $\alpha$  using for the treatment of Crohn's disease.

Bacterial expression systems are cost-effective for the production of antibody fragments where no effector function or extended pharmacokinetic activity is required. Because the assembly and glycosylation of full-length antibodies is not possible in bacterial systems, mammalian cell culture or transgenic organisms are better suited for production of these molecules (Figure. 10).

#### ***1.4.4.2 Baculovirus Expression System***

The baculovirus system has been used for the production of functionally active antibodies (Guttieri and Liang, 2004). In addition, cassette of baculovirus vectors with human Fc regions have also been engineered to allow the transfer of specific variable regions selected by phage display and their subsequent expression as a complete antibody (Liang *et al.*, 2001). A disadvantage of the baculovirus expression system is the catalytic properties of infectious baculovirus that make it unsuitable for the optimal antibody production.

#### ***1.4.4.3 Mammalian Cells***

Any human antibody IgG subclass can be generated in mammalian cell culture systems using either Chinese hamster ovary (CHO) cells, or NSO cells or Human embryo kidney (HEK-293) (Grunberg *et al.*, 2003). Mammalian cell culture has emerged as the method of choice for the production of most of the currently commercialized monoclonal antibodies, but the cost of production using this system is very high. In general, productivity in mammalian cell culture continue to increase because of improved recombinant expression vectors, the identification of 'hot integration spots' within the genome and the enhancement of biomass accumulation

which has resulted in cell-culture processes achieving yields of 1-2g/L of unpurified antibody (Zhou *et al.*, 1997). Furthermore, the increase in the productivity of mammalian cell culture could be achieved by enhancing cell growth and the inherent specific productivity of the manufacturing cell line (Chadd and Chamow, 2001). Only one Fab out of the three FDA approved are produced in sp<sub>2</sub>/0 abciximab (Reopro®) used for the treatment of angioplasty.

#### **1.4.4.4 Yeast**

The ability of yeast to grow in chemically defined medium in the absence of animal-derived growth factors and to secrete large amounts of recombinant protein have made it a system widely used to produce proteins that can not be functionally expressed in *E. coli* due to folding problem or absence of glycosylation in *E. coli*. Several antibody fragments have been produced at a high yield in *Pichia pastoris* such as scFv or Fab (Fischer *et al.*, 1999, Ning *et al.*, 2003). The methylotrophic yeast *Pichia pastoris* is as easy as *E. coli* in genetical manipulation and is the eukaryotic protein synthesis pathway (Fischer *et al.*, 1999). Moreover, antibody fragments can be produced in *Pichia* under fully validated conditions, which are essential for the production of therapeutic antibodies. Yeast can express recombinant proteins in a define medium which are secreted into the medium, enabling purification from a starting material that does not contain a great number of contaminants.

#### **1.4.4.5 Transgenic Plants**

Antibody DNA is introduced into the plant using either *Agrobacterium* infection or gene bombardment in the presence of a carrier molecule and gold particles. In the case of corn, antibody is produced in the seed while in tobacco plants, antibody is produced in the leaf. Antibodies extracted from plants (called plant antibodies) have particular advantages in term of production cost, ease of scaling up or down production to meet market demand, and freedom from animal-derived pathogens including prions and viruses (Schillberg *et al.*, 2003, Stoger *et al.*, 2002) The first functional antibody was expressed in plants by Hiatt *et al.*, 1989 and since then plant antibodies have been produced in different transgenic plants such as in tobacco, soybean, alfalfa and other plants (Khoudi *et al.*, 1999, Stoger *et al.*, 2005). Plants have proven to be effective systems for the production of functional therapeutic monoclonal antibodies which are capable to confer passive protection against

bacterial and viral pathogens in different animals (Ma *et al.*, 2005, Stoger *et al.*, 2002). The generation of transgenic plants for preclinical phase 1 studies takes approximately 20 months, although now advancement of inducible promoters make it possible to control the expression only in harvested plant tissue so that antibody is generated in a good manufacturing facilities (GMP) facility and not out in the field (Doran, 2000, Shih and Doran, 2009).

The only disadvantage, is that the carbohydrate structures generated in plants are quite distinct in comparison and structure from human glycoproteins. To overcome this problem, aglycosyl-antibodies are typically produced in transgenic plants. For IgG<sub>1</sub> subclasses requiring effector functions, this type of expression has obvious limitations, but, an aglycosyl mutein of IgG<sub>2</sub> or IgG<sub>4</sub>, both of which lack significant effector function, might be successful. The antibody huNR-LU-10mAb generated in corn has been genetically engineered to knockout the glycosylation site and also another humanized aglycosylated IgG<sub>1</sub> has been produced which treat herpes simplex virus 2, produced in soyabean (Zeitlin *et al.*, 1998). The first anti-HIV antibody P2G12 produced in genetically modified plants has been approved by EU (Home Media Latest news, 20 July, 2011).

#### **1.4.4.6 Transgenic Animals**

Transgenic animals are also used for the large scale production of antibodies. The generation of a transgenic goat and cow herd able to produce antibody for phase 1 clinical trials takes up to two years. Antibody DNA fused to a milk-specific regulatory element is inserted into a single cell embryo by microinjection and then the transgenic animals produces the antibody in the milk (Limonta *et al.*, 1995). The first milk from farm transgenic mammals which contain a human antithrombin III protein, received agreement from EMEA (European Agency for the Evaluation of Medicinal Products) was to put in the market in 2006 (Houdebine, 2009).

Despite limitation by the relatively long interval from birth to first lactation encountered with domestic livestock, the discontinuous nature of the lactation cycle and substantial material investments required to produce transgenic dairy animals (Dyck *et al.*, 2003).

It has been reported that the production of antibodies in the egg white take about 18 months. This expression system has the potential to supply large quantities of material

for clinical trials relatively inexpensively. The commercial cost of chicken eggs (produced under conditions that do not comply with current good manufacturing practice (cGMP)) is currently about 5 cent per egg or about 50 cent per gram (Dollars) (Chadd and Chamow, 2001). Two monoclonal antibodies and human interferon- $\beta$ 1a could be recovered from chicken egg white (Houdebine, 2009).

## 2 Platelets, Thrombosis and Anti-Thrombotics

### 2.1 Hemostasis and Thrombosis

Hemostasis is the physiological process aimed to limit blood loss at sites of injury. It involves complex interactions between the vessel wall, blood cells (mainly platelets) and blood proteins (mainly coagulation factors and inhibitors). The clot plugs the injured vessels and provide large amounts of repairing factors required for tissue healing.

Thrombosis as a pathological entity was recognized in the early 19<sup>th</sup> century. Virchow postulated that thrombosis is initiated by blood stasis, vascular wall damage and blood cell activation (Wu, 1984). Platelets were identified in the late 19<sup>th</sup> century as crucial blood cells that are involved in thrombosis (Bizzozero, 1882) and fibrinogen was identified as a plasma protein involved in clot formation (Hammarsten, 1879). Knowledge of platelet physiology and coagulation cascade advanced through the first half of the 20<sup>th</sup> century and laid the foundation of the discovery for the molecular mechanisms of thrombosis. The second half of the 20<sup>th</sup> century witnessed a rapid increase in knowledge of the molecules that mediate platelet function. Major advances have been made in understanding the mechanisms of platelet activation and the biochemistry of the cascade of coagulation reactions (Born, 1962, Zucker and Nachmias, 1985).

Platelet activation, coagulation, and resulting thrombus formation are crucial to limit blood loss after tissue trauma. However, in diseased arteries, these processes may lead to thrombotic vessel occlusion with obstruction of blood flow and subsequent tissue damage, as in myocardial infarction and ischemic stroke (Ruggeri, 2002). Therefore, progress in understanding the mechanisms of platelet adhesion, activation, and aggregation is essential to identify new therapeutic targets for the treatment of these two leading causes of mortality and severe disability in the developed world (Stegner and Nieswandt, 2010).

Hemostasis is the set of physiological mechanisms aimed at monitoring and maintaining vascular integrity by preventing blood loss. The main objective of this process is, to arrest bleeding but also to prevent thrombosis. Three principal actors

actively participate in hemostasis: the vessel wall, platelets and coagulation factors. Under physiological conditions, endothelial cells have a non-thrombogenic surface that prevents adhesion of blood cells and activation of coagulation. When the endothelium is damaged, the secretion of anti-thrombotic molecules is disturbed and prothrombotic molecules are exposed. The subendothelial matrix components have a high prothrombotic potential, particularly the collagen. The most important substance for platelets binding is collagen. Collagen binds to von Willebrand factor which interacts with the GPIb/IX/V complex. It also directly interacts with the glycoprotein VI and with the integrin  $\alpha 2\beta 1$  (Ruggeri, 2002). Adhesion initiates platelet activation through key processes: shape change of the platelet surface membrane, which results in membrane protrusions to facilitate tethering to other platelets (Jackson, 2007), and the activation of complex intracellular pathways that ultimately results in the release of platelet agonists such as ADP, thromboxane A<sub>2</sub> (Meadows and Bhatt, 2007). Such agonists subsequently activate other platelets. The last step in this process, aggregation results from a modification of the GPIIb/IIIa complex on the surface of activated platelets. This receptor is an integrin that undergo a shape change with activation, allowing it to bind fibrinogen and von Willebrand factor. Since fibrinogen and von Willebrand factor present several motifs for binding to GPIIb/IIIa, they could thereby effecting the cross-linking of platelet to platelet, ultimately leading to the formation of a platelet thrombus (Davi and Patrono, 2007). This allows the clogging of the vascular gap.

Coagulation consolidates the thrombus, forming a network of fibrin and stabilizes the surface pressure. The activation process is triggered by exposure of tissue factor. Thrombin, an enzyme produced at the end of the coagulation cascade converts fibrinogen into solid soluble fibrin clot. The rate of thrombin formation is critical for the effective thrombus formation. It depends on the coordinated action of several factors: zymogens enzymes, or cofactors on an anionic phospholipid surface provided by activated platelets. The formation and activity of thrombin inhibitors are limited acting at different levels of the cascade [antithrombin,  $\alpha 2$ -macroglobulin complex protein S, tissue factor pathway inhibitor (TFPI), heparin co-factor or HC II].

Fibrinolysis allows destruction and limits the extension of clots. This step involves in particular the zymogen plasminogen, which circulates in an inactive form in plasma. Bound to fibrin it is cleaved by tissue type plasminogen activator (tPA) or



urokinase and is transformed into plasmin, a very powerful proteolytic enzyme capable of degrading the fibrin clot but also destroy fibrinogen. Fibrinolysis normally occur at the clot level. The fibrinolytic system is regulated by two types of inhibitors: plasmin inhibitors ( $\alpha$ 2-anti-plasmin  $\alpha$ 2-macroglobulin) and plasminogen activator inhibitor (PAR-1).

### **2.1.1 Major Types of Thrombosis**

Human thrombosis is a common disorder that may occur in diverse blood vessels but coronary arterial, cerebrovascular and deep venous thrombosis are the most important manifestations.

Collectively, arterial and venous thrombosis have the highest morbidity and mortality. The mechanisms leading to arterial and venous thrombosis are different. Arterial thrombosis occurs in conditions of high shear rates and platelets are critical in the initiation of arterial thrombosis. Venous thrombosis occurs in conditions of low shear rates (stasis) and coagulation in critical.

#### ***2.1.1.1 Arterial Thrombosis***

The formation of a thrombus within an artery is known as arterial thrombosis. In most cases, arterial thrombosis follows rupture of atheroma plaques (accumulation and swelling in artery walls), and is therefore referred to as atherothrombosis.

Atherothrombosis could affect different areterial territories: coronary artery thrombosis results in acute coronary syndromes (myocardial infarction) while cerebral arteries thrombosis results in stroke. One of the major clinical problems in the developed countries is arterial thrombosis caused by erosion of an atherosclerotic plaque.

#### ***2.1.1.2 Venous Thrombosis***

Venous thrombosis is the formation of a thrombus within a vein. There are several diseases which can be classified under this category. (i) Deep vein thrombosis (DVT) is the formation of a blood clot within a deep vein. It most commonly affects leg veins (the femoral vein). The triggering mechanism for deep venous thrombosis has not been clearly elucidated. Three important factors in the formation of a blood clot within a deep vein are, the rate of blood flow, the blood thickness and quality of the vessel wall. Classical signs of DVT include swelling, pain and redness of the affected

area. (ii) Portal vein thrombosis is a form of venous thrombosis affecting the hepatic portal vein, which can lead to portal hypertension and reduction of the blood supply to the liver. It usually has a pathological cause such as pancreatitis, cirrhosis, diverticulitis or cholangiocarcinoma. (iii) Renal vein thrombosis is the obstruction of the renal vein by a thrombus which lead to reduced drainage from the kidney. (iv) jugular vein thrombosis is a condition that may occur due to infection, intravenous drug use or malignancy (Webster *et al.*, 2005).

Venous thrombosis is initiated at the junction of valves and the vascular wall. It was proposed that local blood stasis could be triggering event. This proposal was based on a series of *in vitro* experiments, although this proposal has not been proven *in vivo*, but several pieces of evidences strongly support the notion that venous thrombosis is initiated by activation of the cascade of coagulation reactions. (i) Morphological analysis of venous clots reveals the nidus of such a clot to be composed exclusively of fibrin. (ii) Elevation of coagulation factors, mainly factor VIII, increases the risk of deep venous thrombosis (Koster *et al.*, 1995). (iii) Mutation of factor V (Factor V Leiden), which is resistant to digestion by activated protein C, a powerful inhibitor of coagulation, results in increased risk of deep venous thrombosis (Dahlback, 1997). (iv) Finally, deep venous thrombosis responds to anticoagulants but not to platelet inhibitors. Deep venous thrombosis may lead to life-threatening pulmonary embolization and infarction.

## **2.1.2 Different Phases of Thrombus Formation**

Formation of platelet plugs at the sites of vascular damage require a co-ordinated series of events leading to the following phases

### **2.1.2.1 Initiation Phase**

(platelet arrest onto the exposed subendothelium creating a monolayer of activated cells). Upon vascular injury extracellular matrix components are exposed to blood, particularly VWF, collagen, fibronectin, thrombospondin, and laminin. The rheological conditions largely influence the adhesive interaction of platelets. Blood flow has a greater velocity in the centre of the vessel than near the wall, thereby generating shear forces between adjacent layers of fluid that become maximal at the wall. These shear forces are ranging from less than  $500\text{s}^{-1}$  in venules and large veins, upto  $5000\text{s}^{-1}$  in small arterioles and upto  $40000\text{s}^{-1}$  in stenosed arteries (Bluestein *et al.*,

1997). Thus at low shear rate (as in veins and large arteries), platelet adhesion to the vessel wall primarily involves binding to fibrillar collagen, fibronectin and laminin while at conditions of elevated shear stress, such as those encountered in the microvasculature or in stenotic arteries, platelet tethering to the damaged subendothelium is critically dependent on their interaction with subendothelial bound VWF (Jackson *et al.*, 2003, Savage *et al.*, 1998).

#### **2.1.2.2 Extension Phase**

(Recruitment and activation of additional platelets through the local release of major platelet agonists). After deposition of a platelet monolayer over the exposed VWF and collagen, the next step in thrombus formation is the recruitment of additional platelets from the flowing blood, which upon activation acquire the ability to stick to each other in a process commonly referred to as platelet aggregation. This is made possible by the local accumulation of soluble agonists and metabolites that are produced by adherent-activated platelets, including ADP, TXA<sub>2</sub>, serotonin, and epinephrine. The final step is activation of the integrin  $\alpha$ IIb $\beta$ 3 (GPIIb/IIIa), causing a conformational change that enables it to bind fibrinogen and VWF, allowing stable bridges between platelets. The great number of  $\alpha$ IIb $\beta$ 3 copies on the platelet surface, (40000-80000), allows the assembly of large aggregates at the site of vascular injury (Petrich *et al.*, 2007).

#### **2.1.3 Stabilization Phase or Perpetuation**

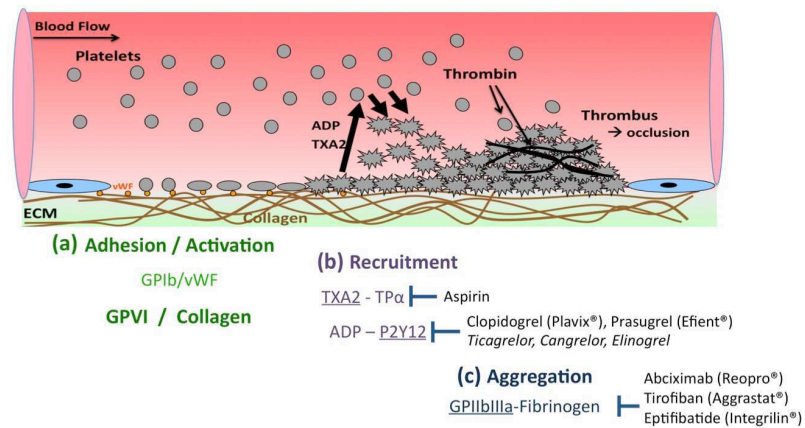
Stabilization of the platelet plug prevents premature disaggregation until wound healing occurs. Currently, the existing static model of thrombus formation has been visualized as a dynamic model of thrombus build-up and stabilization in which continuous signaling is needed to stabilize thrombi and prevent their dissolution (Cosemans *et al.*, 2006).

Arterial thrombosis is schemed in Figures 11 and 12.

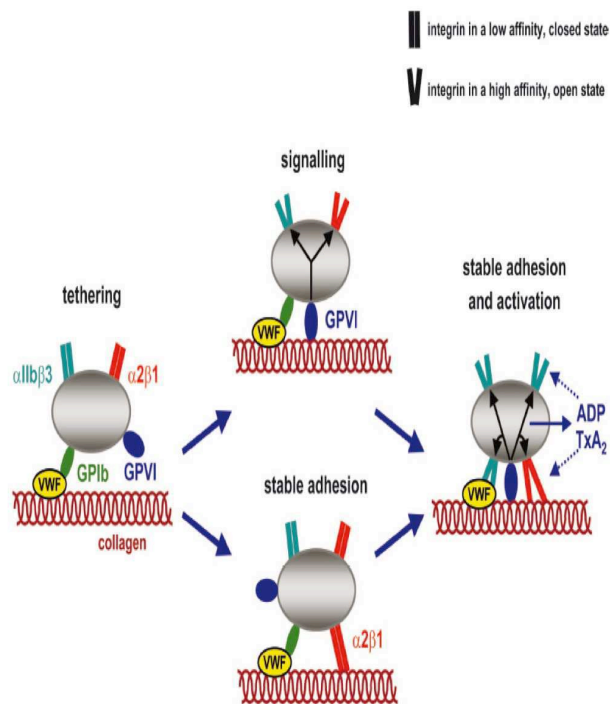
#### **2.1.4 Actors Involved in Thrombus Formation**

##### **2.1.4.1 The Vessel Wall**

Structure of the vessel of the vessel wall:



**Figure 11: Schematic representation of platelet thrombus formation after lesion of the vascular endothelium and common therapeutic targets.**



**Figure 12: Unifying model of platelet adhesion to collagen at arterial shear.**

Two pathways for platelets firm adhesion to collagen at arterial shear are illustrated. In the first pathway (upper), signaling from GPVI first leads to activation of integrins  $\alpha$ 1 and II $\beta$ 3, which then firmly attach the platelet to collagen directly or via collagen-bound VWF (II $\beta$ 3) (right). In the second pathway (lower), platelets first adhere to collagen via integrin  $\alpha$ 1 before GPVI engages collagen and induces activation. These two pathways are likely to reinforce each other and the events of thrombus formation. Release of secondary mediators (ADP and TxA<sub>2</sub>) would further potentiate these events. Adapted from (Auger *et al.* 2005).

The vascular wall consists of three layers:

#### **2.1.4.1.1 Tunica Adventitia**

The strong outer covering of arteries and veins. It consists of a connective tissue, poorly organized, rich in collagen and elastic fibers and containing fibroblasts and adipocytes. These fibers allow the arteries and veins to stretch to prevent overexpansion due to the pressure that is exerted on the walls by blood flow. It forms an envelope that ensures the anchoring of the arteries to surrounding structures. It is irrigated by vasa vasorum which have a nurturing role for adventitia itself and for the outer part of the media. A network of unmyelinated vasomotor nerves joined the smooth muscle of the media. It is sometimes traversed by longitudinal smooth muscle fibers (Figure 13).

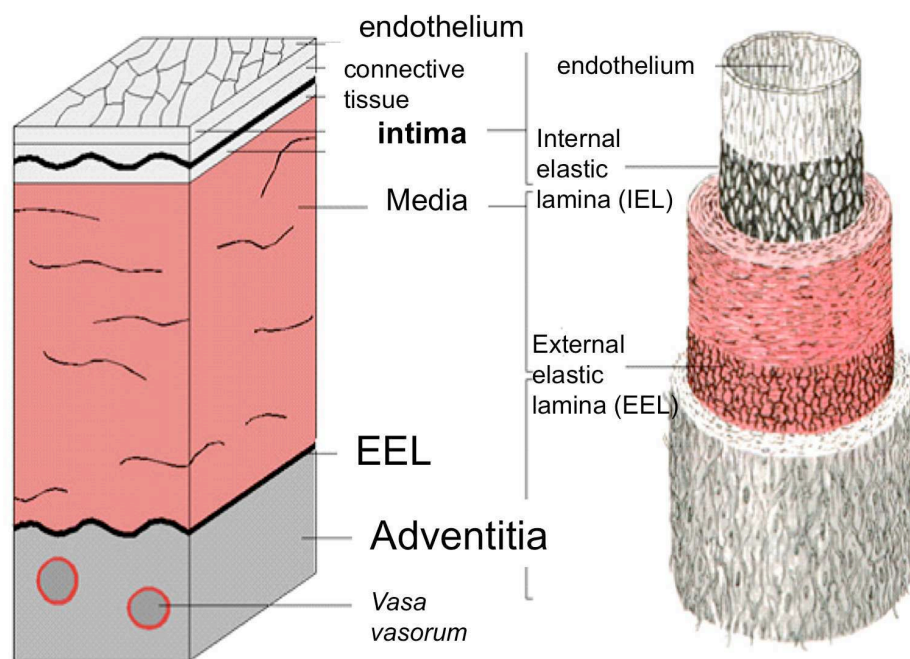
#### **2.1.4.1.2 Tunica Media**

The middle layer of the walls of arteries and veins. This layer is thicker in arteries than in veins.

The media, consisting of smooth muscle cells and elastic fibers, is the thickest and the main constituent of the artery. It consists mainly of smooth muscle cells, stacked concentrically layered called lamellar units. The number of layers depends on the type of artery (a layer for arterioles and several layers in the elastic arteries). Each lamellar unit consists of smooth muscle cells layers surrounded by an extracellular matrix composed of fibrous, elastic proteins (collagen and elastin) and mucopolysaccharides. Except in the thoracic aorta, the media is avascular, and receiving irrigation at its external part from the vasa vasorum of the adventitia. A strip of elastin, the external elastic lamina separates the media from adventitia in the vessels of high caliber (Figure 13).

#### **2.1.4.1.3 Tunica Intima**

The inner layer of arteries and veins. In arteries this layer is composed of an elastic membrane lining and smooth endothelium (special type of epithelial tissue) that is covered by elastic tissues (Figure 13). It is in direct contact with sub-endothelium and blood flow. It consists of a single layer of endothelial cells, joined, forming a blanket seal between blood flow and subendothelium. The endothelium is a full organ with a specific differentiation of organ interacting with its environment. The vascular



**Figure 13 :A Schematic diagram of the vascular wall.**

endothelium has different and important properties in the defense against thrombosis and inflammation and control of the vascular tone. Endothelial cells respond differently depending on the mechanical and biochemical agents, which are applied. Indeed, the pulsatile blood flow generates different types of hemodynamic forces: hydrostatic pressures, cyclic stretches and shear forces or shear stress. Endothelial cells are sensitive to oxidative stress caused by the increased amounts of oxygen derivatives, such as superoxide ions, hydrogen peroxide and the hydrolyse radicals, overflowing the antioxidant system. This stress leads to the acquisition by the cell of a proinflammatory phenotype and a change of interactions between the endothelium, leukocytes and platelets.

Regulating role of the endothelium:

The endothelium, forming the luminal surface of vessels is physiologically non-thrombogenic surface. Natural inhibitors of platelet aggregation are expressed by endothelial cells: prostacyclin (PGI<sub>2</sub>), nitric oxide (NO) increase respectively the intracellular concentration of cAMP and cGMP which are potent inhibitory signals. CD39 is an ecto-ADPase, which degrade ADP and prevent it from activating platelets.

The endothelium also expresses an important anticoagulant activity. Thrombomodulin (TM) is a transmembrane protein expressed constitutively on endothelial surface. TM has a large extracellular domain, which contains binding sites for thrombin and protein C (Sadler, 1997). The conformation of thrombin is altered after binding to TM leading to the loss of its catalytic activity on converting fibrinogen to fibrin. On the other hand, thrombin acquires the capacity to convert protein C bound to the endothelial cell proteins C receptor (EPCR) to activated protein C (APC). APC binds to protein S on the platelet surface where it degrades factor Va and VIIIa, thereby downregulating coagulation reactions (Dahlback, 2004). Furthermore, glycosaminoglycans present at the surface of endothelial cells, provide sites for the binding of coagulation inhibitors, mainly antithrombin and catalyse their activity.

Endothelium also plays a role in the lysis of fibrin clots by a well-coordinated program.

When it is damaged, the endothelium loses its protective role and acquires deleterious properties: it exposes adhesive proteins favouring the recruitment of inflammatory cells and of platelets.

Subendothelial layer of connective tissue is the site where the atherosclerotic plaque forms. Functionally intact endothelial cells are markedly reduced in atherosclerotic lesions. Thus, production of PGI<sub>2</sub>, NO and CD39 is greatly compromised. As a result, platelet aggregation proceeds with little check and balance in the atheromatous plaque.

#### **2.1.4.2 Platelets**

Platelets are the principal effectors of cellular hemostasis in humans and other mammals. They adhere to exposed subendothelial matrix and aggregate in response to prothrombotic signals which contribute to the formation of platelet-fibrin clots that are critical for sealing vascular disruptions and for ultimate vessel and wound repair (White, 2000). In addition, platelets have roles in acute and chronic inflammation, including release of proinflammatory mediators, display of surface molecules that have inflammatory function, and interactions with leukocytes and endothelial cells (McIntyre *et al.*, 2003, Senturk, 2010).

Platelets are in the first line in physiological hemostasis and in arterial thrombosis. Patients presenting severe quantitative or qualitative platelet deficiencies suffer from hemorrhagic syndromes. As a corollary, patients treated with antiplatelet agents which block the main platelet functions have an increased risk of bleeding and there is a constant search for treatments capable to limit pathologic platelet activation while keeping a minimal level of physiologic responses.

The properties of platelets are developed in chapter 3.

#### **2.1.4.3 The Coagulation System**

At the site of tissue injury, the coagulation cascade is triggered by the exposure of tissue factor (TF). TF is a transmembrane glycoprotein that becomes exposed to blood after injury and can also be expressed by macrophages in particular during sepsis (Taubman *et al.*, 1997). Clotting is initiated by the binding of FVII/FVIIa to TF (extrinsic pathway). Free factor VIIa in blood has a weak catalytic activity that is enhanced by 3 to 4 times when it binds to cell surface TF (Losche, 2005). The



TF/FVIIa complex initiates blood coagulation by activating both FX and FIX. Factor X appears to be a preferred substrate and is rapidly converted to factor Xa, which results in the generation of a small amount of thrombin. The FVIIIa/FIXa complex provides an alternative route to activate FX. Factor Xa generated by VIIa/TF, is rapidly inactivated by tissue factor pathway inhibitor (TFPI), a natural anticoagulant produced by vascular endothelial cells (Bajaj *et al.*, 2001, Dahlback, 2000). Tissue factor pathway inhibitor (TFPI) binds tightly to the TF/VIIa/Xa complex, thereby completely blocking this pathway. Thus, the TF/VIIa/Xa pathway has been named the initiation phase of coagulation. While factor IXa generated by VIIa/TF is not inhibited by TFPI and is the predominant pathway for generating a large quantity of thrombin and fibrin. Thus VIIa/TF/IXa pathway is called the propagation phase of coagulation (Wu and Matijevic-Aleksic, 2005).

Coagulation is also triggered by the contact of blood with negatively charged molecules such as polyphosphates released from activated platelets or nucleic acids released by apoptotic or necrotic tissues. FXII is then activated, FXIIa in turns activate FXI, FXIa finally activates FIX. The contact activation is closely linked to the activation of the kininogen/kinin pathway and to inflammation.

The coagulation cascade ends in the activation of prothrombin in thrombin by FXa.

The reactions of the coagulation cascade are catalyzed by electronegative surfaces provided by activated platelets. The plasma membrane of activated platelets, altered during activation, exposes procoagulant anionic phospholipids in particular phosphatidylserine (PS). In the presence of  $Ca^{2+}$ , the exposure of PS by activated platelets provide support to the assembly of the complexes of activation of factors X and II (tensease and prothrombinase).

Thrombin amplify its own production by upregulating loops including the activation of the cofactors FVIII and FV, activation of FX and platelets.

Thrombin selectively cleaves two peptidic bonds on fibrinogen producing a fibrin monomer and fibrinopeptides A and B. Conformational changes in fibrin monomers expose polymerization sites allowing fibrin to non covalently assemble as thicken fibrils. FXIII activated by thrombin stabilizes fibrin by forming covalent bonds. The platelets contained in the clot allow it to retract via their contractile cytoskeleton.

Growth factors, cytokines and chemokines released by the clot allow cell recolonization and contribute to tissue repair.

Inhibitors finally tune the coagulation cascade in order to limit the process in the space and in the time. Inhibitors include TFPI and the serpins antithrombin and heparin cofactor II. Antithrombin is the main inhibitor of thrombin and factor Xa. It behaves as a suicide substrate and forms a covalent complex with its targets. The rate of inactivation is considerably increased in the presence of glycosaminoglycans and in particular of heparin which is as a consequence a very commonly used anticoagulant.

Another regulating system, the protein C pathway is supported by the endothelium: thrombin is captured by TM and activates protein C; Activated Protein C (APC) proteolitically inactivates FVa and FVIIIa, stopping the coagulation cascade.

#### **2.1.4.4 The Fibrinolytic System**

Fibrinolysis weakens the clot and allows its elimination.

Thrombin serves as a bridging molecule to signal the activation of the fibrolytic programme, primarily by inducing the production and release of tissue plasminogen activator (tPA) from endothelial cells (van Hinsbergh, 1988). When tPA and its substrate, plasminogen, bind to fibrin, tPA converts plasminogen to plasmin, which then degrades fibrin step-wise into multiple degradation products (Lucas *et al.*, 1983). Co-localization of tPA and plasminogen to fibrin ensures the targeting of plasmin to fibrin. Plasmin that leaks into circulating blood is rapidly neutralized by a specific inhibitor,  $\alpha$ 2-antiplasmin. Further more, excess of free tPA is bound by plasminogen activator inhibitor-1 (PAI-1) and is removed. It has been speculated that fibrinolysis is impaired in atheromatous plaque, which contributes to mass accumulation of thrombus. Administration of tPA has been shown to dissolve coronary or cerebrovascular thrombi and thus alleviate myocardial infarction or cerebral infarction (Chalela *et al.*, 2004, Nordt and Bode, 2003).

## **2.2 Platelets**

Bizzozero (1881) coined the term “blut plattchen” (blood platelets) to describe tiny blood elements that clumped at the site of vascular puncture (Jackson, 2007). Platelets are small anucleate cell fragments, originating from megakaryocytes in the bone

marrow that circulate in the blood stream for about 7-12 days (Figure 14 and 15). Each mature megakaryocyte produces 1000 to 3000 platelets (Bluteau *et al.*, 2009). But recently, some researchers assumed that platelets are derived from the existing platelets and are of the views that platelets have the capacity for independent replication (Schwartz *et al.*, 2010).

Their numbers in blood varies from  $150$  to  $350 \times 10^9$  /L (George, 2000). This is much more than required to ensure physiologic hemostasis, a bleeding risk appearing for a platelet count lower than  $5 \cdot 10^9$ /L.

Platelets are critical effectors of hemostasis, blood clotting, and wound repair. Platelets monitor the integrity of the vascular system and most platelets never undergo firm adhesion and are finally cleared by macrophages in spleen and liver (Stegner and Nieswandt, 2010).

### 2.2.1 Platelet Structure

Platelets circulate in a discoid form of about  $2 \mu\text{m}$  in diameter, but change shape to become spherical after activation. They have the ability to spread when joining, forming lamellipodia, and produce extensions, filopodia and pseudopodia.

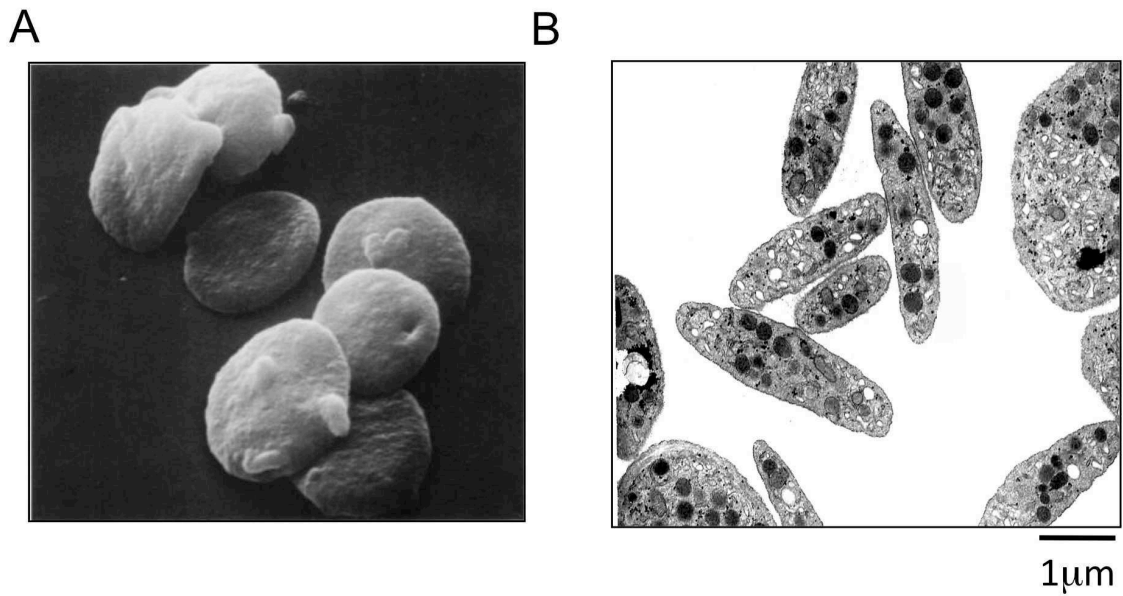
Platelets have a complex ultrastructure:

#### 2.2.1.1 *Peripheral Zone*

It consists of the membrane and its associated structures (Figure 16). The membrane is a phospholipid bilayer in which many glycoproteins are embedded, some of which are receptors involved in platelet activation. Beneath the membrane and bound to it, is the skeleton membrane, which is associated with proteins involved in intracellular signal transduction. Specialized microdomains of the plasma membrane (rafts or lipid rafts) are enriched in proteins anchored to the membrane, playing an important role in platelet signaling.

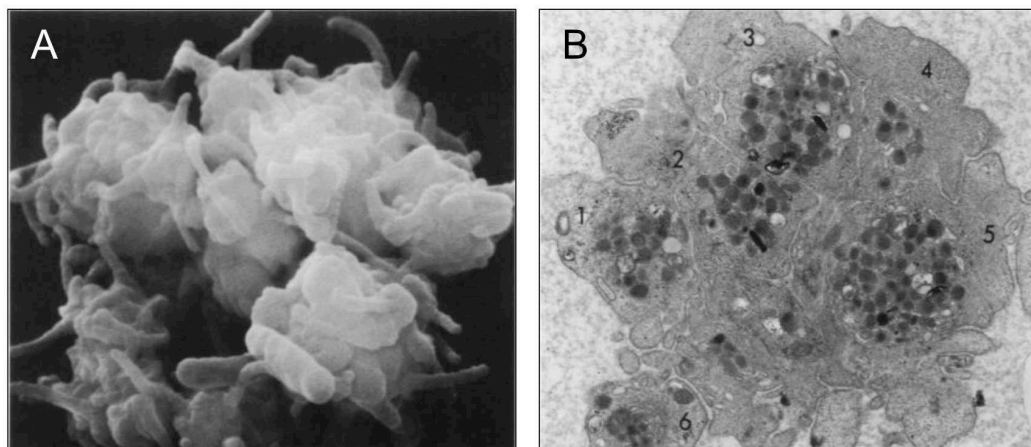
##### 2.2.1.1.1 *The Platelet Membrane*

The Platelet membrane constitutes the exchange surface with the environment; its components are involved in all platelet functions and are therefore main targets of a pharmacological action.



**Figure 14 : View of platelets in resting condition.**

A.Scanning electron microscopy,  
 B.Transmission electron microscopy  
 By courtesy of Dr Paquita Nurden.



**Figure 15: View of ADP-activated platelets.**

A. Scanning electron microscopy,  
 B. Transmission electron microscopy  
 By courtesy of Dr Paquita Nurden.

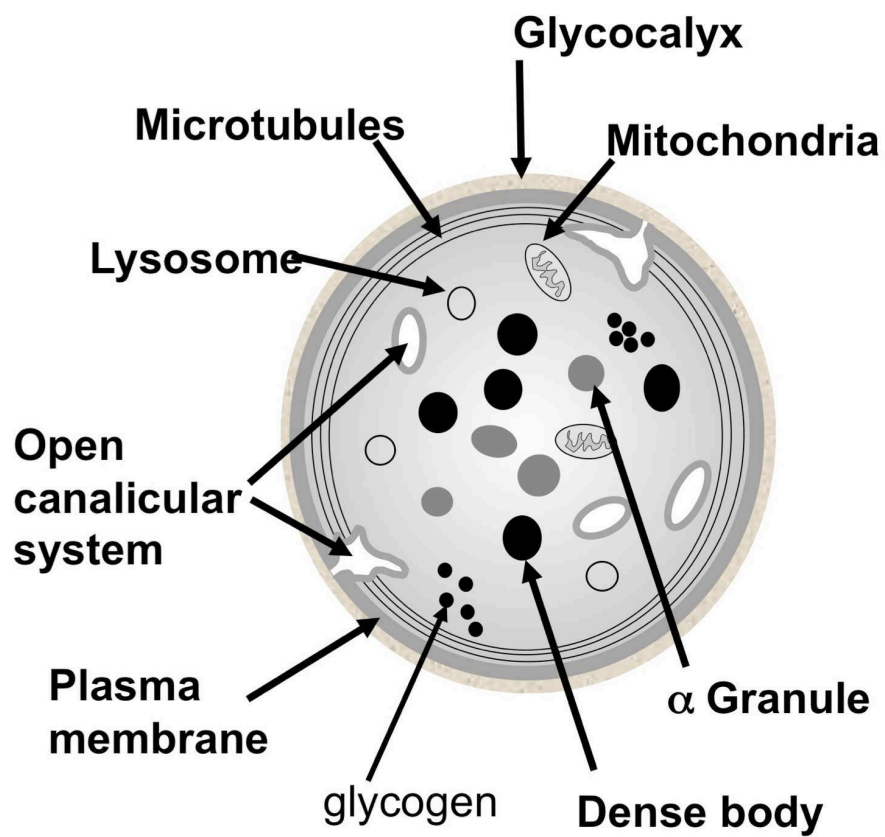


Figure 16: Different organelles of human platelet

#### 2.2.1.1.1.1 Lipids

The membrane is a typical trilaminar structure, with two outer and inner lipid layers of different phospholipids (PL) composition. Phosphatidylcholine and sphingomyelin are mainly located in the outer leaflet (monolayer), while phosphatidylethanolamine, phosphatidylserine (approximately 13%) and phosphatidylinositol (less than 5%) predominate in the inner leaflet. Upon activation, the negatively charged phospholipids migrate to the outer leaflet, an important step that will promote coagulation.

Formation of a pore for platelet granule secretion necessitates the fusion of two lipid membranes. The fusion of opposing lipid bilayers in an aqueous environment requires sufficient energy to overcome electrostatic repulsive and hydration forces between the two membranes (Reed *et al.*, 2000). Relatively little is known about the specific lipids required for platelet membrane fusion. The exact role of PL in potentiating membrane fusion has not been defined. However, altering membrane curvature, serving as protein attachment sites, and signaling are proposed functions for PL in membrane fusion (Jones *et al.*, 1999, Vitale *et al.*, 2001).

Cholesterol is a stabilizer of the membrane. Platelet membrane cholesterol concentration reflects the plasma cholesterol concentration and is higher in hypercholesterolemic individuals (Carvalho *et al.*, 1974, Shattil *et al.*, 1977). Platelets are incapable of synthesizing cholesterol and the contents of the cholesterol in platelet membrane is probably established during their formation from megakaryocytes and may also acquire cholesterol through exchange with plasma lipoprotein (Schick and Schick, 1985). The repitition of choslesterol is not homogenous and membrane microdomains enriched in cholesterol (rafts) have been identified.

The platelet membrane is particularly rich in arachidonic acid released by phospholipase A2 upon platelet activation. A cyclooxygenase will induce the formation of endoperoxides and thromboxane synthetase will lead to the formation of thromboxane A2, which is a platelet activator. This pathway is the target of aspirin, antithrombotic drug most commonly used in primary and secondary prevention of cardiovascular diseases (Patrono and Rocca, 2009).

In platelets, phosphatidylinositol 4, 5-biphosphate (PIP<sub>2</sub>) is synthesized in an activation-dependent manner by both type I and type II phosphatidylinositol

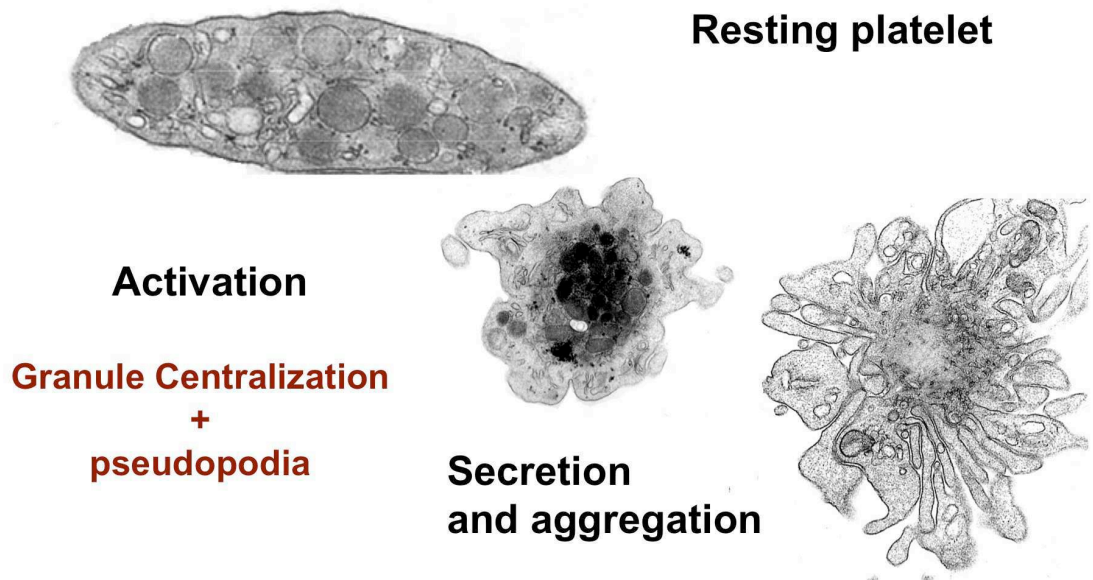
phosphate kinases (PIPKs) (Rozenvayn and Flaumenhaft, 2001, Tolias *et al.*, 2000) [http://atvb.ahajournals.org/cgi/content/full/23/7/1152 - R25-130349](http://atvb.ahajournals.org/cgi/content/full/23/7/1152-R25-130349)). A role for PIP<sub>2</sub> in platelet granule secretion is evidenced by several observations in permeabilized platelets. When phosphatidylinositol-specific phospholipase-C is infused into permeabilized platelets, it cleaves PIP<sub>2</sub> and inhibits  $\alpha$ -granule secretion (Rozenvayn and Flaumenhaft, 2001). Exogenously added PIP<sub>2</sub> also inhibits platelet  $\alpha$ -granule secretion, presumably by competing with endogenous PIP<sub>2</sub> localized in platelet membranes. Antibodies directed at type II PIPKs inhibit PIP<sub>2</sub> synthesis and interfere with  $\alpha$ -granule secretion mediated by Ca<sup>2+</sup>, a thrombin receptor agonist peptide (TRAP), or the PKC agonist PMA (Rozenvayn and Flaumenhaft, 2001, Tolias *et al.*, 2000). Furthermore, recombinant type II $\beta$  PIPK augments TRAP- or PMA-induced  $\alpha$ -granule secretion (Rozenvayn and Flaumenhaft, 2003). These experiments demonstrate that PIP<sub>2</sub> synthesis resulting from the activity of type II PIPK contributes to agonist-induced granule secretion.

At the inner leaflet of the membrane, phosphoinositides metabolism is playing a critical role in the cascade of activating signals. A complex network of lipid kinases and phosphatases tightly regulates the equilibrium between the different metabolites. Briefly, hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>) by phospholipase C constitutes a key step in platelet activation. Its products, diacyl glycerol (DAG) and inositol triphosphate (IP<sub>3</sub>) are responsible of calcium mobilisation and protein kinase C activation. On the other hand, the production of PI<sub>3,4,5</sub>P<sub>3</sub> by PI-3 kinase is also a key point allowing the recruitment of signaling proteins and the building up of signaling complexes (Gratacap *et al.*, 2009).

#### **2.2.1.1.2 Membrane Cytoskeleton**

The discoid shape of resting platelet is achieved by an internal coil formed from microtubules that sits just beneath the plasma membrane. Microtubules are rigid, hollow polymers assembled from subunits. The dominant tubulin isoform expressed in platelets and megakaryocytes is  $\beta$ 1.

A dense membrane cytoskeleton laminates the cytoplasmic side of the plasma membrane. The main components of this network are spectrin, adducin, filamin, and the vWF receptor, GPIb. This membrane skeleton is closely associated to the



**Figure 17: Changes in the platelet structure during different stages of activation.**



scaffolding actin cytosolic skeleton which maintains platelet integrity (Hartwig, 2006).

The cytoskeletal rearrangements that occur following platelet activation begin by the loss of discoid shape: platelets first become spherocytic and then rapidly extend filopodia. Following platelet activation, the membrane cytoskeleton disassembles, the cortical actin filament network is disrupted and new actin filament assembly is initiated from the ends of actin filaments that abut the plasma membrane. When activation occurs on a surface, platelets form lamellae, broad actin-filament rich sheets, and spread (Figure 17). Filopodia, long thin processes with actin filament bundle cores, are prominent in platelets at early times following activation.

#### **2.2.1.1.2.1 Glycoproteins**

The platelet membrane is rich in glycoproteins, which act as sensors for external signals. Most are integral membrane proteins and few are GPI-coupled proteins. Platelet membrane glycoproteins have been classified initially according to their electrophoretic mobility and subsequently according to function. The development of monoclonal antibodies enabled functional analysis and purification of receptors. Analysis of these proteins brought the observation that platelet membrane glycoprotein receptors belong to receptor 'superfamilies' expressed by many cell types and fundamental to biological processes including haemostasis, inflammation, wound healing, foetal maturation and morphogenesis, and cancer growth and metastasis. These include cell surface adhesion receptors, receptors of the immunoglobulin superfamily, seven transmembrane domain G-protein coupled receptors, tetraspanins, leucine-rich glycoproteins. The expression and functionality of cell surface adhesion receptors is dynamic and depends on the cell's environment and state of activation. As example, the partition of these proteins in membrane microdomains may vary during activation with modification of raft composition and tethered sites of adhesive receptors become exposed when platelets are activated. Platelet receptors will be described later on (chapter 4).

#### **2.2.1.2 Cytoplasm**

The cytoplasm is rich in organelles.

#### 2.2.1.2.1 **Tubular Systems**

A tubular system connected to the surface canalicular system (open canalicular system, OCS) forms deep invaginations constituting a large membrane surface in contact with the outside and which facilitates the spreading of platelets (Figure 16). A membrane system not connected to the surface, the dense tubular system (DTS), is similar to smooth endoplasmic reticulum residual of megakaryocyte. It contains enzymes for lipid metabolism, and is a reservoir of  $\text{Ca}^{2+}$ , which plays a critical role in platelet activation and enzymes regulating its intracellular transport.

#### 2.2.1.2.2 **Cytoskeleton**

Platelet integrity is maintained by an elaborate internal actin filament cytoskeleton including actin. Of the actin contained within a resting platelet, 40% is assembled into 2,000 to 5,000 filaments. Actin forms a spacefilling network within the platelet cytoplasm; filaments run from the cell center toward the plasma membrane, where they turn and run parallel to it. Before new actin assembly is used to construct filopodia and lamellae, the actin-based cytoskeleton of the resting platelet is reorganized. Reorganization is mediated by the activation of two proteins, gelsolin and cofilin, which combine to actin filaments fragment and promote disassembly of F-actin net. Calcium is important for actin network reorganization. Two convergent pathways then are used to initiate actin assembly: fragmentation/uncapping of barbed filament ends, and activation of the actin-related protein (Arp) 2/3 complex, which nucleates filaments. Gelsolin inactivation provides actin fragments onto which the actin-related protein (Arp) 2/3 complex binds and nucleates a second filament. The actin organization in filopodia versus lamellipodia is drastically different .

#### 2.2.1.2.3 **Granules**

The cytoplasm is rich in: alpha and dense granules and lysosome (Figure 16). Some mitochondria are present. Each human platelet contains 50 to 80  $\alpha$ -granules, 3 to 8 dense granules, and a few lysosomes

The alpha granules contain a wide variety of proteins (more than 300 releasable proteins identified by proteomic analysis) including adhesion molecules such as fibrinogen, von Willebrand factor (vWF), thrombospondin, chemokines and cytokines (PF4) fibrinolytic regulators, immunologic modulators, and an assortment of

coagulation, complement, growth (PDGF), and proangiogenic and antiangiogenic factors. Some of these proteins are synthesized by megakaryocytes, the precursor of platelets (i.e; thrombomodulin or PF4) whereas others are incorporated by an endocytosis process (i.e. fibrinogen; albumin, immunoglobulins). The membrane of alpha granules also express adhesion receptors such as P-selectin, the GPIb-V-IX and integrin  $\alpha$ IIB $\beta$ 3 (GPIIb/IIIa) that are exposed during exocytosis by fusion of the granule membrane with the plasma membrane.

The dense granules mostly contain small molecules, including calcium, magnesium, polyphosphate, ATP, ADP, GTP, GDP, and serotonin (critical for platelet activation and vasoconstriction).

In the resting state, platelet  $\alpha$ -granules and dense granules are distributed in an apparently random fashion throughout the platelet. On platelet activation, however, platelets undergo a dramatic shape change. The  $\alpha$ -granules coalesce in the center of the platelet and fuse with the open canalicular system (OCS) and also with one another (Ginsberg *et al.*, 1980, Stenberg *et al.*, 1984). Contraction of cyto skeleton causes the release of granules (dense and alpha granules from the platelets) and their contents into the extracellular milieu (Rendu and Brohard-Bohn, 2001). Granule contents are released into the OCS and diffuse out into the extracellular environment ([http://atvb.ahajournals.org/cgi/content/full/23/7/1152 - R64-130349](http://atvb.ahajournals.org/cgi/content/full/23/7/1152-R64-130349)).

Exocytosis via fusion with plasmalemma has also been observed and described (Morgenstern , 1987). The prominent morphologic changes that occur in the platelet after activation result from rapid microtubule reorganization and actin polymerization.

Centralization of granules occurs concurrently with activation-induced shape change and precedes granule release. Thus, it is possible that the morphologic changes induced by cytoskeletal reorganization contribute to granule secretion.

Platelet granule release is important for hemostasis, because patients with inherited granule defects have bleeding problems.  $\alpha$ - granules are absent in the gray platelet and ARC syndromes (Lo, 2005), while deficient dense granules are observed in isolation, in combination with  $\alpha$ -granules deficiency, or as part of a syndrome in the Hermansky-Pudlak, Chediak-Higashi, and Griscelli syndromes (Nurden and Nurden, 2008). The molecular mechanisms involved in platelet granule secretion are complex and incompletely understood (Coppinger *et al.*, 2004, Flaumenhaft, 2003, Reed, 2004,

Ren *et al.*, 2008). Italiano and colleagues have shown that  $\alpha$ -granules can be distinguished based on the pro- or anti-angiogenic factors that they contain (Italiano *et al.*, 2008).

Platelets also release lysosomal enzymes such as cathepsins and hexosaminidase which may play a role in clot remodeling or in further platelet activation (Anitua *et al.*, 2004). Platelets also release fibrinolytic agents such as plasminogen activator inhibitor-1 (PAI-1) and thrombin- activator fibrinolysis inhibitor (TAFI) which are important for clot remodeling. Perhaps the most diverse category is the mitogens such as PDGF, IGF-1, VEGF, and bFGF. These proteins are thought to promote wound healing and angiogenesis through their stimulation of chemotaxis, cell proliferation, and maturation of the cells surrounding the wound site. Finally, platelets release a number of chemokines and cytokines such as RANTES, IL-8, and MIP1 $\alpha$ , which promote activation of passing leukocytes and lead to a range of immune responses. This releasate catalogue suggests that platelet secretion is pivotal in establishing the microenvironment at the wound site.

Therefore, controlling release from the granule stores, either specifically or globally, may prove to be an effective strategy to manipulate this microenvironment (Italiano *et al.*, 2008).

### **2.2.2 Platelet Plug Formation**

Platelet plug formation results from the coordinated action of different agonists, receptors and signaling pathways.

#### **2.2.2.1 Initiation Phase**

The initiation phase is assumed to results from the contact of platelets with the injured vessel wall. Extracellular matrix exposed at the surface of a ruptured vessel is enriched in platelet-activating substances among which collagen is the best characterized one (Bornstein and Sage, 1980). Collagen attracts platelets by several mechanisms. When platelets come into contact with the sub-endothelium, they adhere to matrix proteins, principally von Willebrand factor (VWF) and collagen (Ruggeri and Mendolicchio, 2007, Varga-Szabo *et al.*, 2008). The first interaction is claimed to be formed between vWF and the GPIb-V-IX complex leading to platelet adhesion (tethering) (Pareti *et al.*, 1986). Interaction between vWF and platelet GPIb/IX/V

results in the attachment of a single layer of platelets to the plaque surface (Ruggeri, 2002). Platelet adhesion is essential for the subsequent accumulation of platelets.

Platelets roll and are slowed-down allowing the direct contact between collagen and its receptor GPVI and GPIaIIa (integrin  $\alpha 2\beta 1$ ). It has been reported that collagen binds to these two classes of receptors in a sequential manner (Mangin *et al.*, 2003, Nieswandt and Watson, 2003). Binding of collagen to platelets results in platelet activation.

#### **2.2.2.2 Progression Phase**

Activation signals are emitted leading to secretion of granules content and synthesis of thromboxane A<sub>2</sub> (TXA<sub>2</sub>). This is the progression phase. Secreted ADP and TXA<sub>2</sub> are soluble agonists that allow the recruitment of circulating platelets (Cattaneo and Gachet, 2001, Murugappan *et al.*, 2004). ADP recruits circulating platelets and induces activation of the newly recruited platelets, release of additional ADP, generation of TXA<sub>2</sub> via arachnidonic acid metabolism and conformational changes of GPIIbIIIa. Thus, ADP is an important endogenous mediator of platelet aggregation and plays a key role in the expansion of platelet aggregates induced by collagen. The action of ADP is receptor-mediated. It binds to purinergic receptor isoforms P<sub>2</sub>Y<sub>1</sub> and P<sub>2</sub>Y<sub>12</sub> on platelet surface (Dorsam and Kunapuli, 2004).

Very rapidly, Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) catalyzes the release of arachidonic acid from the sn-2 position of membrane phospholipids, notably phosphatidylcholine (Kudo and Murakami, 2002, Murakami and Kudo, 2003). Cyclooxygenase-1(COX-1) and thromboxane synthase (TXAS) are constitutively expressed in human platelets. COX-1 converts arachidonic acid to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) in turn converted into TXA<sub>2</sub> by TXAS; TXA<sub>2</sub> is released into the extracellular milieu; it has a short half-life and is rapidly degraded to an inactive and stable metabolite, TXB<sub>2</sub>. TXA<sub>2</sub> binds to a specific receptor on the platelet surface and induces platelet release reaction and aggregation (So, 2003). TXA<sub>2</sub> receptor is G-coupled seven-transmembrane domain receptor. It causes contraction of smooth muscle cells and is an important mediator of coronary arterial vasospasm (Calvete, 1999).

#### **2.2.2.3 Aggregation**

The final step is the aggregation of platelets: activated platelets expose at their surface a receptor for fibrinogen that is the activated integrin  $\alpha$ IIb $\beta$ 3 (GPIIbIIIa).

GPIIb/IIIa is a constitutively expressed on the external surface of platelets (Naik and Parise, 1997). It weakly binds to fibrinogen when platelets are at resting state. When platelets are activated by collagen, thrombin, ADP or TXA<sub>2</sub>, the GPIIb/IIIa conformation is altered, and a high affinity binding site for fibrinogen is formed (Fullard, 2004). Fibrinogen possesses several sites for binding to GPIIb/IIIa and forms bridges between adjacent platelets. This stage is the target of GPIIb/IIIa antagonists (Coller and Shattil, 2008).

#### 2.2.2.4 Platelet Procoagulant Activity

Apart from forming platelet plugs, activated platelets have an important procoagulant role. During activation their plasma membrane undergoes remodeling with exposure of PS and emission of procoagulant microparticles that allow catalyzes of thrombin formation. Collagen and thrombin are the most efficient agonists inducing PS exposure (Edouard M. BEVERS, 1982, Lenta, 2003). The major role of PS (negatively charged) is to bind factor VIIa, the zymogens factor X and II and the co-factors IX. These factors are vitamin K dependent that means that they are gamma-carboxylated by a vitamin K dependent carboxylase and thus are negatively charged. Vitamin K dependent factors bind to PS via a Ca<sup>2+</sup> bridge. Release of FV by activated platelets also contributes to thrombin generation. Factor V and VIII also bind to lipids. Thus, PS allows localization of the coagulation cascade and increases the efficiency of the activation of factor X and II.

Thrombin in turn is a very potent platelet agonist, at least based on results from *in vitro* experiments. It binds to specific receptors and amplifies platelet activation. It induces the formation of fibrin which traps the platelet aggregates in a stable clot.

Coagulation factors VIIa (FVIIa) and Xa (FXa) are proteases that act upstream of thrombin in the coagulation cascade and require cofactors to interact with their substrates. These proteases elicit cellular responses. Co-expression of tissue factor (TF), the cellular cofactor for FVIIa, together with PAR1, PAR2, PAR3, or PAR4 conferred TF-dependent FVIIa activation of PAR2 and, to lesser degree, PAR1. The TF/FVIIa complex converts the inactive zymogen Factor X (FX) to FXa. Strikingly, when FX was present, low picomolar concentrations of FVIIa caused robust signaling in cells expressing TF and PAR2 (Camerer, 2000). And it cleaves a peptide from the receptor; the peptide serves as an active ligand to activate platelets. The first thrombin

receptor that has been identified is the prototype of the protease activated receptors (PAR). These receptors belong to the 7 transmembrane domains receptors family but they are characterized by a particular mechanism of action. Cleavage of a peptide bond in their N-terminal extracellular domain unmask a tetrapeptide motif that forms intramolecular interactions leading to receptor activation. PAR-1 and PAR-4 are thrombin receptors on human platelets while PAR-3 and PAR-4 are thrombin receptors on mouse platelet (Camerer, 2000). Thus, thrombin plays a key role in enlarging the platelet aggregate mass.

The platelet clot is also stabilized by numerous interactions involving soluble proteins such as thrombospondin or interactions between surface receptors (Eph-Ephrine) and receptors for unknown ligands. CLEC-2, CD40L, SLAM (signaling lymphocyte activation molecules), Gas6 (growth arrest-specific gene 6) have been proposed to participate to platelet-platelet interactions. Possible candidates for platelet plug stability are JAM-A, JAM-C, CD226 and CD84. Molecules that limit thrombus growth have also been identified: ESAM (endothelial cell specific adhesion molecule) or PECAM-1 (platelet endothelial cell adhesion molecules, CD31). The development of transgenic mice deficient for specific proteins has considerably increased the number of partners suspected to be involved in thrombus formation or stabilization. However the part they play in physiological hemostasis or in thrombosis in human is not yet established (Figure 18).

#### 2.2.2.5 *Platelet Activation Pathways*

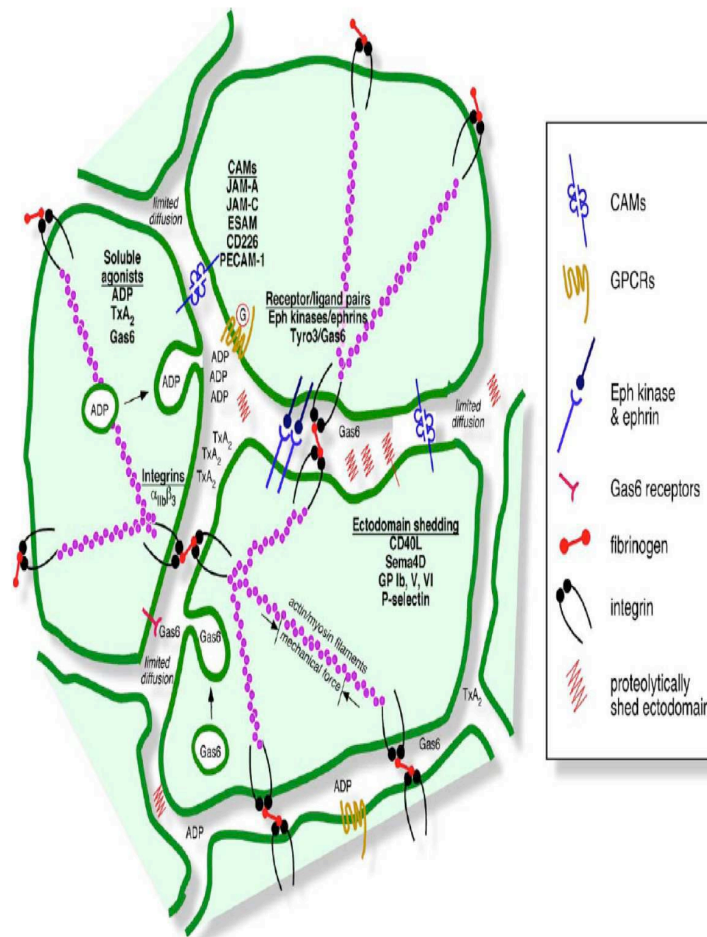
Thus, schematically, platelets are activated by insoluble agonists (proteins of the extracellular matrix such as collagen and vWF) and by soluble agonists such as thrombin, ADP and thromboxane A2 (TxA2).

The receptors of these agonists on the surface of platelets, are of the following different types:

- 7-transmembrane receptors coupled to G proteins (PAR1 and 4: thrombin receptor, P2Y1 and P2Y12: the ADP receptor; TP $\alpha$  : receptor of TxA2).

- glycoproteins of the superfamily of leucine-rich proteins (GPIb, GPV, GPIX)

- integrins ( $\alpha$ 2 $\beta$ 1,  $\alpha$ IIB $\beta$ 3,  $\alpha$ 6 $\beta$ 1)



**Figure 18 : Contact-dependent and contact-facilitated events during thrombus formation.** The onset of aggregation brings platelets into sufficiently close contact for integrins and other cell adhesion molecules to interact and for the activation of Eph receptor kinases by their cell surface ligands known as ephrins. The space between platelets also provides a protected environment in which soluble agonists for G-protein-coupled receptors (ADP, thrombin and TxA<sub>2</sub>) and receptor tyrosine kinases (Gas-6), and the proteolytically shed bioactive exodomains of platelet surface proteins (CD40L and sema4D) can accumulate. The mechanical forces generated by the contraction of actin/myosin filaments helps to compress the space between platelets, improving contacts and increasing the concentration of soluble agonists. Adapted from (Brass *et al.* 2006).



-Immunoglobulin like superfamily receptors (FcγRIIa: Low affinity receptor of the IgG constant domain, GPVI: major collagen receptor)

-calcium channel (P2X1, ATP receptor)

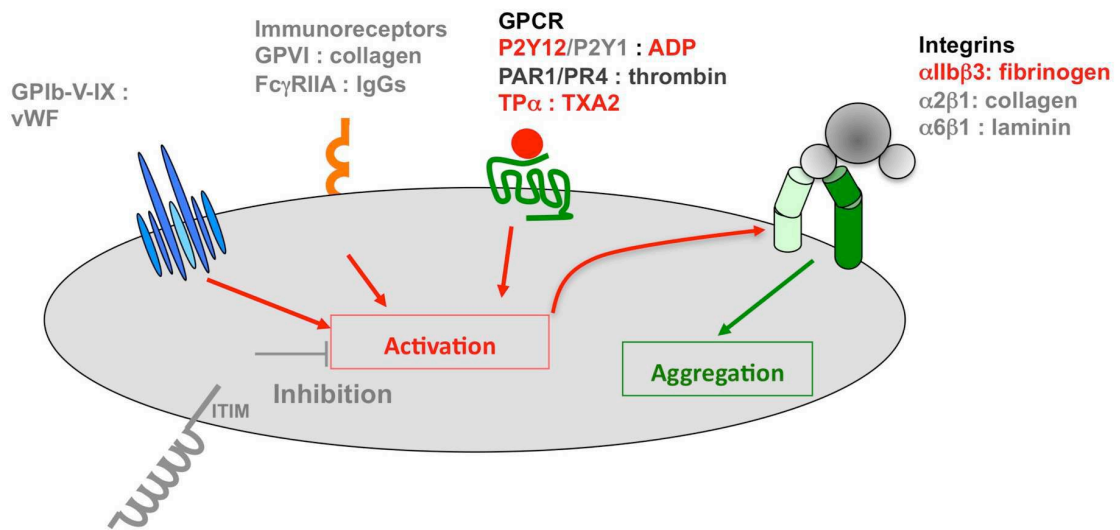
-Tetraspanin (CD63 and CD9, domain 4 transmembrane receptor).

The main platelet receptors and their properties as targets for antiplatelet agents are described in chapter 4.

These receptors are coupled to various signaling pathways. As a consequence, receptor engagement induces the sequential activation of a network of complementary signals ending in mobilization of calcium. A cross-talk between these pathways allows optimal responses. Conversely blockade of the knots of this network impair more or less severely and specifically platelet responses. Soluble agonists (thrombin, ADP, and TxA<sub>2</sub>) stimulate receptors that couple to heterotrimeric G proteins and induce distinct downstream signaling pathways (Offermanns, 2006). Gq proteins activate PLCβ leading to calcium mobilization (via inositol-1,4,5-trisphosphate, IP<sub>3</sub>) and activation of protein kinase (PK) C (via DAG) (Offermanns *et al.*, 1997). G<sub>12</sub>/G<sub>13</sub> proteins regulate multiple pathways, of which the Rho/Rho-kinase pathway, leading to myosine light chain phosphorylation and platelet shape change, has been well explored (Hart *et al.*, 1998). The α-subunit of G<sub>i</sub> family proteins inhibits adenylyl cyclase, while its βγ complexes can regulate several channels and enzymes, most notably PI3Ks (Cantley, 2002) (Figures 19 and 20).

Adhesive proteins activate different pathways. Those coupled to the GPIb-V-IX complex are still a matter of debate.

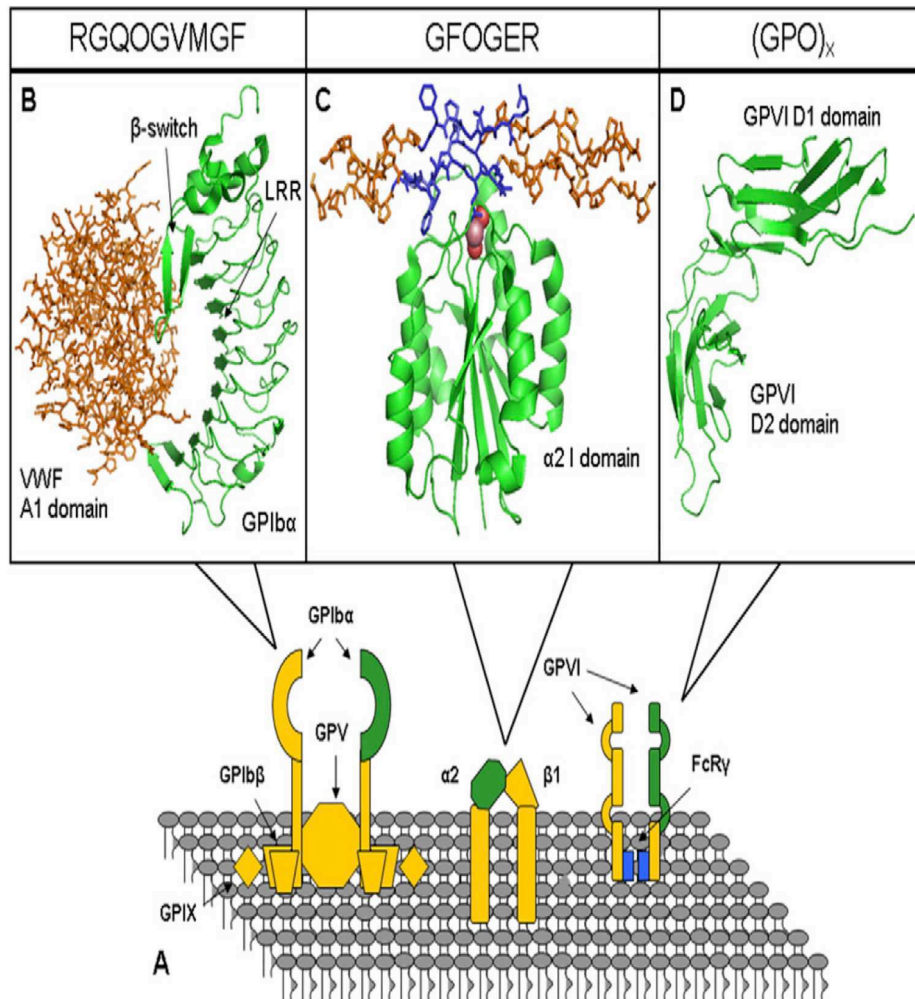
Receptors belonging to the immunoreceptor superfamily could be activating when presenting an immunotyrosine based activation motif (ITAM) or inhibitory when presenting an immunotyrosine based inhibitory motif (ITIM). GPVI, FcγRIIa and C-type lectin receptor 2 (CLEC-2) belong to the first category and are coupled to the activation of tyrosine phosphorylation cascades downstream of the ITAM, or hemITAM, respectively, leading to full platelet activation. PECAM-1 and G6Bb belongs to the second category and are coupled to the activation of protein tyrosine phosphatases downstream of their ITIM.



**Figure 19 : A schematic representation of different receptors on platelet surface and their antagonists that support platelet activation.**

PLC, phospholipase C; PKC, protein kinase C; IP3, inositol-1,4,5-trisphosphate;  $\alpha$ IIb $\beta$ , refers to the platelet integrin;

IP and TP, PGI<sub>2</sub> and TxA<sub>2</sub> receptors.



**Figure. 20 : Overview and crystal structure of the major platelet receptors interacting with the vessel wall.** (A) Schematic representation of the major direct and indirect collagen receptors (yellow), ligand binding domains (green) and FcRγ chain associated with GPVI dimers (blue). (B) Wild-type GPIIbα interacting with VWF-A1 domain. The Leucine-Rich Repeat (LRR) domain and β-switch of GPIIbα are represented as a cartoon in green, the VWF-A1 domain is depicted in an orange stick representation. (C) The α2-I domain (cartoon in green) interacting with triple-helical collagen peptide (orange stick) except GFOGER sequence (blue). Co<sup>2+</sup> ion (sphere). (D) Monomeric GPVI with the immunoglobulin domains D1 and D2 represented as a green cartoon. Adapted from (Benedicte *et al.* 2011)

Integrins are also coupled to an outside –in signaling pathway that involves Tyrosinases as the ITAM-triggered signals.

### **2.2.3 Other Functions of Platelets besides Hemostasis**

Platelets have also other functions that are now recognized in the tissue repair, angiogenesis, inflammation, however, there is a growing appreciation for their role in inflammation. Platelets promote leukocyte recruitment and produce microparticles which are vectors to spread inflammation (Smyth *et al.*, 2009). Platelets release a broad range of inflammatory mediators in chronic inflammatory diseases that support endothelial cells activation, leukocytes adhesion and transmigration, monocyte maturation, and elaboration of cytokines and reactive oxygen species (Davi and Patrono, 2007). Platelet is a storehouse for a host of inflammatory mediators and growth factors that play a role in inflammation (atherothrombosis). These include adhesion proteins (fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, p-selectin, integrin  $\alpha$ IIb $\beta$ 3), growth factors (platelet-derived growth factors, transforming growth factor-beta, epidermal growth factor, basic fibroblast growth factor), chemokines (eg, PF-4, regulated on activation, normal T-cell expressed and secreted, epithelial neutrophil-activating protein 78, macrophage inflammatory protein-1alpha), cytokines and cytokine-like factors (eg, interleukin-1beta, CD40 ligand, thromboglobulin-beta), and coagulation factors (eg, factor V, factor XI, plasminogen activator inhibitor type 1, plasminogen, protein S). All these substances act in concert to mediate a wide range of functions, including cell adhesion, cell activation, chemotaxis, cell survival, proliferation coagulation and proteolysis (Jennings, 2009).

As previously suspected that platelets play a role in rheumatoid arthritis: due to their accumulation in the joints of affected patients; platelet thrombi were observed in synovial vessels of patients with rheumatoid arthritis, and increased numbers of platelets in synovial fluid and of microparticles in blood were associated with the condition (Diaz-Gonzalez, 2010, Yaron *et al.*, 1978).

Boilard *et al.* (2008) did not detect intact platelets in the synovial fluid of the rheumatoid arthritis patient. Then how did the incendiary microparticles gain access to the joints?. A possible explanation is that platelets adhere to activated polymorphonuclear leukocytes and monocytes in the circulation in many

inflammatory conditions (Weyrich and Zimmerman, 2004) including rheumatoid arthritis (Weissmuller *et al.*, 2008). Jerry Ware, a hematologist at the university of Arkansas for Medical sciences in Little Rock concluded that platelets do not instigate rheumatoid arthritis but just worsen it.

Besides all these, platelets are chock-full of biologically influential molecules-not just ingredients for blood clotting but a wealth of growth factors, immune system messengers, enzymes, and other compounds. Researchers have identified more than 1100 kinds of proteins inside platelets or on their surface (Leslie, 2010). Platelets also appear to guard against microbial invasion because their Toll-Like receptors (TLRs) recognizes molecular features of microbes. Semple and colleagues have found that when pathogens trip a platelet's TLRs, there's a surge in TNF- $\alpha$ , a compound that fuels inflammation, one of the body's most potent protections against infection (Aslam *et al.*, 2006).

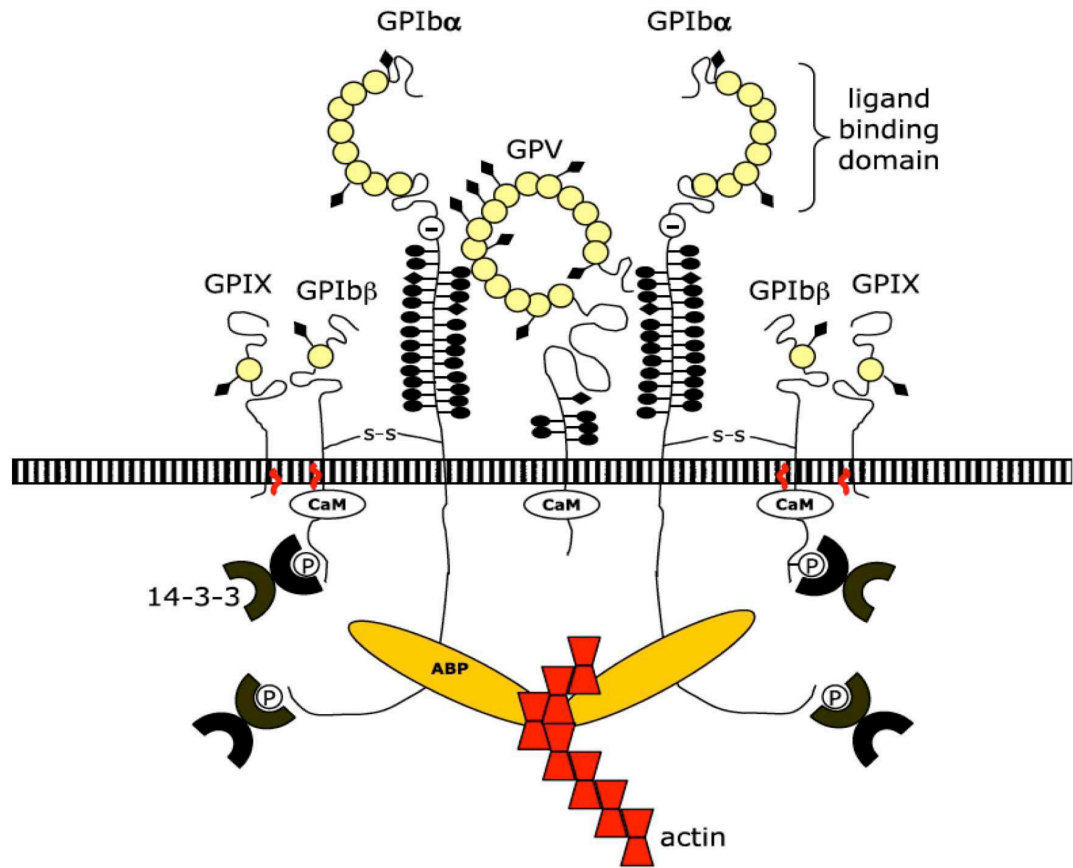
## **2.3 Platelet Receptors: Structure and Function**

### **2.3.1 The GPIb/V/IX Complex and vWF**

VWF permits the interaction of platelets with the sub-endothelial matrix of the vessels under high shear rates. It is responsible for the initial adhesion of platelets to the collagen.

#### **2.3.1.1 GPIb/V/IX Complex**

The GPIb/V/IX complex is a highly abundant (25000 copies per platelet) and structurally unique receptor complex exclusively expressed in platelets and megakaryocytes. Four different genes encodes the receptor complex, namely the alpha and beta subunits of GPIb, GPIX and GPV (Cauwenberghs *et al.*, 2000, George *et al.*, 1984), all of which belong to the leucine-rich repeat protein superfamily and are characterized by the presence of one or more leucine-rich repeats (Berndt *et al.*, 2001). GPIb $\alpha$  (150kDa) and GPIb $\beta$  (27kDa) are associated by a disulfide bridge and are also associated by non-covalent bonds with GPIX (22kDa) and GPV (82 kDa) with a stoichiometry of 2:2:1. GPIX and GPV are considered to serve as a scaffold for GPIb, which harbors binding sites for vWF (Figure 21).



**Figure 21 : Structure of GPIIb-V- IX.**

Platelet GPIIb-V- IX is composed by four different units: GPIIb $\alpha$ , GPIIb $\beta$ , GPIIb $\gamma$ , and GPIIb $\delta$ . GPIIb $\alpha$  is disulphide-linked to GPIIb $\beta$ , which, in turn, is noncovalently associated with GPIIb $\gamma$ . A single GPIIb $\delta$  molecule interacts noncovalently with two adjacent GPIIb $\gamma$  complexes to form the complete receptor. Each member of the complex contains one or multiple leucine-rich repeats (yellow circle) in the extracellular domain. Carbohydrate moieties are represented as black line (diamonds on stalks represent N-linked oligosaccharides, while circles are O-linked oligosaccharides). The short cytoplasmic domains of GPIIb $\beta$  and GPIIb $\gamma$  contain acylated cysteine residues (drawing in red) that represent additional sites for membrane anchorage. Adapted from Canobbio *et al.* (2004)

The binding site for VWF is situated on GPIb $\alpha$ , on the N-terminus in first 282 amino acid residues. This region contains six leucine rich domains, one domain of sulfated tyrosine and loop formed by a disulfide bridge. These three elements participates in interaction with VWF (Berndt *et al.*, 2001, Cauwenberghs *et al.*, 2000).

A thrombin binding site is also identified on this region. A growing number of proteins have been shown to bind to this extracellular domain of GPIb $\alpha$  : this include other coagulation factors such as FXI, FXII and high molecular weight kininogen ; GPIb-V-IX is also recognized as a counter receptor for P-selectin and this could contribute to platelet interactions with inflammed endothelial cells or with leukocytes. The integrin Mac-1 is proposed to contribute to platelet clearance by macrophages via its binding to clustered GPIb-V-IX. GPIb can initiate adhesion by interacting with other ligands. Thrombospondin-1 has also been shown to interact with GPIb under high shear flow conditions *in vitro* (Jurk *et al.*, 2003).

The receptor mediates the initial contact of platelets with the exposed Extracellular matrix (ECM) that function irrespectively of cellular activation and thereby facilitates rapid interaction that resist shear forces acting on the cells (Sakariassen *et al.*, 1986).

At the high shear rates found in small arteries and arterioles, the instantaneous onset of the interaction between GPIb $\alpha$  and the A1 domain of vWF immobilized on collagen or on the surface of the activated platelets is crucial for the initial tethering or capture of flowing platelets. This interaction is, however, characterized by a rapid dissociation rate and is insufficient for stable adhesion. This can be illustrated by the rolling of GPIb-IX-expressing cells or platelets on a vWF monolayer at high shear (Savage *et al.*, 1998). Thus, the principal function of GPIb/V/IX is to recruit platelets to the site of injury and to reduce their velocity to enable the interaction of other receptors with the thrombogenic surface.

The intracellular signal triggered by vWF binding to GPIb-V-IX is complex and not yet well understood: ITAM containing receptors, phospholipase C, phospholipase A2, the cytoskeleton, interactions with the 14.3.3 protein and activation of PI3-kinase have all been reported to be involved (Canobbio *et al.*, 2004). In conditions of high shear flow, vWF/GPIb interaction allows the formation a a single layer of adherent platelets but downstream signal are not sufficient by themselves to permit thrombus growth (Pugh *et al.*).

In human the lack or dysfunction of GPIb/IX/V is associated with the Bernard-Soulier syndrome (BSS), a congenital bleeding disorder characterized by mild thrombocytopenia, giant platelets and inability of the cells to aggregate in response to ristocetin (Lopez *et al.*, 1998).

### **2.3.1.2 Von Willebrand Factor**

The Von willebrand factor (vWF) is a glycoprotein synthesized by endothelial cells (stored in Weible Palade bodies) and megakaryocytes. Stored in  $\alpha$ -granules, it is released upon platelet activation. vWF is a multimeric adhesive glycoprotein and is the central mediator in platelet adhesion. The monomers (about 220kDa) assemble into multimers (up to  $20 \times 10^3$  kDa) in the Golgi complex. vWF is found in the Weibel-palade bodies of endothelial cells, in the alpha granules of platelets and in the plasma at a concentration of about  $10\mu\text{g/mL}$  in human (Berndt *et al.*, 2001). The high molecular weight multimers of vWF are specifically processed by a protease ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif, member 13) repeats causing VWF size reduction. This proteolytic cleavage is essential to reduce the size of ultra large VWF polymers, which, when exposed to high shear stress in the microcirculation, are prone to form platelets clumps.

The biological functions of vonWillebrand factor are exerted through specific domains that interact with extracellular matrix components and cell membrane receptors to promote the initial tethering and adhesion of platelets to sub- endothelial surfaces, as well as platelet aggregation. It contains binding sites for collagen as well as for the two major platelet receptors (GPIba and integrin  $\alpha\text{IIb}\beta\text{3}$ ). Moreover, it binds the procoagulant co-enzyme, factor VIII, contributing to its stability and, indirectly, to its function in the generation of fibrin. Factor VIII, which is a necessary co-factor for the rapid generation of factor Xa at sites of injury (Ruggeri, 2001).

The mature subunit of vWF consists of 2050 amino acids and is composed of four different repeating domains (A-D) (Shelton-Inloes *et al.*, 1986). The three homologous A domains span residues 497 to 1111 and regulate interaction with different receptors and prothrombotic ligands of the subendothelial matrix. The A1 domain exclusively binds collagen type VI, whereas collagen I and III are bound via the A3 domain (Hoylaerts *et al.*, 1997). The C1 domain contains the sequence Arg-Gly-Asp (RGD), which represents a binding motif for both of the platelet Beta3-



integrins, namely  $\alpha\text{IIb}\beta_3$  and  $\alpha_v\beta_3$ . The interaction between vWF and the receptor complex GPIb-V-IX occurs via the A1 domain and is of pivotal importance in the initial adhesion of platelets to the subendothelium under conditions of high shear, such as found in atherosclerotic vessels. Activation of the integrin  $\alpha\text{IIb}\beta_3$  occurs during the transient tethering, mainly through signaling initiated by membrane receptors that bind collagen or other components of exposed thrombogenic surfaces or respond to stimulation by agonists released (e.g. ADP) or generated (e.g.  $\alpha$ -thrombin) locally. The final result is the stable adhesion of recruited platelets to the surface (Ruggeri, 2007).

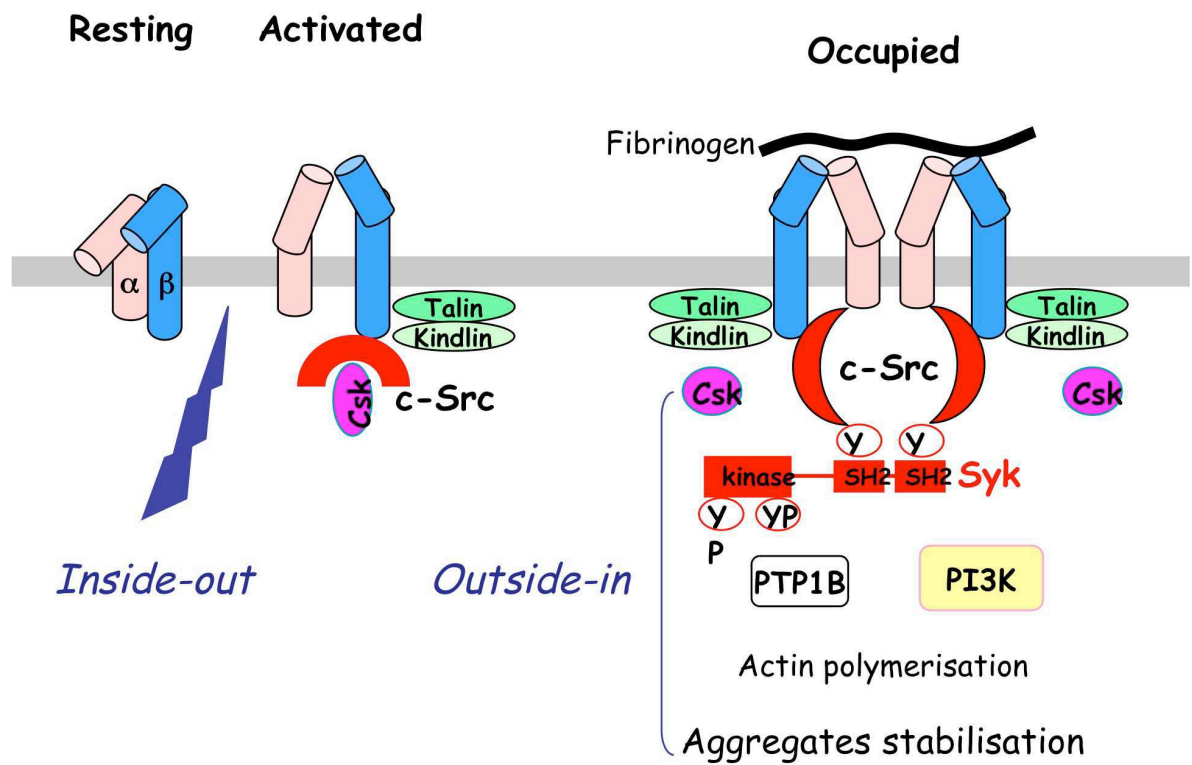
Under normal conditions, soluble vWF does not undergo significant interactions with its platelet receptor, GPIb-V-IX. However, when immobilized on exposed collagen at sites of injury, it becomes a strong adhesive substrate. As a consequence of this binding and under high shear stress, conformational changes occur in VWF, exposing the normally cryptic binding site on VWF-A1 for the GPIb-V-IX complex. *In vitro* studies suggest that conformational changes in the A1 domain of vWF could alter its affinity for interaction partners (Ruggeri, 2003).

## **2.3.2 Integrins**

### **2.3.2.1 $\alpha\text{IIb}\beta_3$ (GPIIb/IIIa)**

$\alpha\text{IIb}\beta_3$  is the most abundant glycoprotein on the platelet surface with 60,000 to 80,000 copies per cell and an additional intracellular pool that is exposed on the surface on activation (Shattil *et al.*, 1998). As a typical integrin  $\alpha\text{IIb}\beta_3$  is a non-covalent complex of an  $\alpha$ -subunit (1008 residues) and a  $\beta$ -subunit (762 residues) which is proteolytically processed into a heavy and light chain. Each subunit spans the membrane once in a type I orientation (Figure 22). Both are glycosylated and each is the product of a single gene localized on chromosome 17 (Quinn *et al.*, 2003).

Like other members of the integrins family  $\alpha\text{IIb}\beta_3$  is in an inaccessible conformation, “low-affinity” or “off” state on resting platelets: a highly bent conformation keeps the binding site for RGD (arginine-glycine-aspartic acid) sequence hidden. On agonist-induced platelet activation “inside-out” signaling events lead to a large conformational rearrangement of the extracellular domains;  $\alpha\text{IIb}\beta_3$  changes into the “highly-affinity” or “on” state, and the RGD binding site becomes unmasked. This process called switchblade-like opening can be initiated also



**Figure 22 : Integrin  $\alpha\text{IIb}\beta\text{3}$**

The inside-out signaling triggered a platelet agonist leads to a conformational change of the integrin which acquires the capacity to bind plasma fibrinogen. Fibrinogen bridges integrins resulting in platelet aggregation. When the integrin is occupied by fibrinogen an outside-in signaling cascade is triggered which is important for thrombus growth and stabilization.

passively by manganese ions (Smith *et al.*, 1994). The “inside-out” activation of  $\alpha$ Ib $\beta$ 3 seems to be regulated through a mechanism that essentially involves the cytoplasmic tails of the two subunits. Studies on mice have established a requirement for talin and talin binding to  $\beta$ 3 in inside-out  $\alpha$ Ib $\beta$ 3 signaling. Another identified partner is kindlin-3.

The main role of  $\alpha$ Ib $\beta$ 3 after activation is binding with fibrinogen in the manner dependent on  $\text{Ca}^{2+}$ . Fibrinogen is an heterodimer ( $\text{A}\alpha$ ,  $\text{B}\beta$ ,  $\gamma$ )<sub>2</sub> and due to its multivalency it supports platelet aggregation. Fibrinogen concentration is 4 $\mu$ g/L and is the most represented  $\alpha$ Ib $\beta$ 3 ligand. Fibrinogen binding to  $\alpha$ Ib $\beta$ 3 involves the RGD motif on  $\alpha$ -chain of fibrinogen and a dodecapeptide at the C-terminus of the fibrinogen  $\beta$ -chain.  $\alpha$ Ib $\beta$ 3 also binds several other ligands each containing the RGD sequence. Although fibrinogen is the predominant ligand in plasma, the role of von Willebrand factor (VWF) in conditions of high shear should be emphasized, while fibronectin, vitronectin and CD40L may also participate (Naik and Parise, 1997, Ruggeri, 2002, Shattil *et al.*, 1998).

Once bound to immobilized ligands,  $\alpha$ Ib $\beta$ 3 induces processes such as spreading. It is also necessary for clot retraction that is due its interaction with the platelet cytoskeleton. Binding of its ligand  $\alpha$ Ib $\beta$ 3 triggers an outside-in signaling pathway that reinforce the level of activation signals in platelets and is required for aggregates stabilization (Shattil *et al.*, 1998). The network of proteins involved in outside-in  $\alpha$ Ib $\beta$ 3 signaling is very large (Coller and Shattil, 2008). Many parallels are apparent between this process and signaling triggered by immunoreceptors, including dependence on Src family kinases (SFKs) and Syk protein tyrosine kinase. The cytoplasmic domain of  $\alpha$ Ib $\beta$ 3 may serve a nucleating function for some of these proteins.

Quantitative and/or qualitative abnormalities of  $\alpha$ Ib $\beta$ 3 (Glanzmann thrombasthenia) is characterized by a major abnormality of platelets aggregation in response to all agonists and the clinical manifestation is an hemorrhagic syndrome which may be very severe (Nurden, 2009, Nurden and Nurden, 2008, Seligsohn, 2002). In man, expression of the  *$\alpha$ Ib* gene (ITGA2B) (and therefore of  $\alpha$ Ib $\beta$ 3 integrin) is restricted to cells of the megakaryocytic cell lineage. Expression of the  $\beta$ 3 gene (ITGB3) is more widespread, with the vitronectin receptor ( $\alpha$ v $\beta$ 3) being expressed in many cell types, including endothelial cells, osteoblasts, smooth muscle

cells, and leukocytes. The inability to bind adhesive proteins when stimulated explains the platelet phenotype in GT.

Due the importance of GPIIb/IIIa in platelet aggregation it has become an attractive pharmacological target for the prevention of cardiovascular events.

### **2.3.2.2 Integrins $\alpha v\beta 3$ , $\alpha 5\beta 1$ (or VLA-5), $\alpha 6\beta 1$ (or VLA-6)**

These integrins are also expressed on platelet surface. These may bind to different proteins of the extracellular matrix such as  $\alpha v\beta 3$  that binds to vitronectin and can also bind to fibronectin and osteopontin, while  $\alpha 5\beta 1$  is the receptor for fibronectin and  $\alpha 6\beta 1$  is a receptor for laminin on platelet surface (Kamata *et al.*, 2005, Sonnenberg *et al.*, 1988). Various *in vitro* and *in vivo* studies have demonstrated that these receptors may modulate platelet responses and that their relative importance to the overall processes of adhesion, activation and spreading is determined by the nature of extracellular matrix, which differ in different parts of an individual's body (Inoue *et al.*, 2006).

### **2.3.3 Collagen Receptors**

In addition to the indirect interaction of platelets with collagen via the vWF/GPIb-V-IX axis, direct interactions are also made with the collagen receptors present on platelet surface (Figure 23). Collagen triggers the initiation of platelet aggregation using two classes of collagen receptors: GPVI and GPIIa/IIb (integrin  $\alpha 2\beta 1$ ). It has been reported that collagen binds to these two classes of receptors in a sequential manner (Mangin *et al.*, 2003, Nieswandt and Watson, 2003).

#### **2.3.3.1 Integrin $\alpha 2\beta 1$**

The direct collagen adhesion receptor integrin  $\alpha 2\beta 1$  (GPIIa/IIb or VLA-2 or CD49b/CD29), is the first collagen receptor that has been identified (Santoro, 1986). It is next to  $\alpha IIb\beta 3$  the second most important platelet integrin with approximately 2000–4000 copies per platelet. The expression of integrin  $\alpha 2\beta 1$  is not restricted to the platelet lineage and it is present on many different cell types. Patients with inherited reduced expression of  $\alpha 2\beta 1$  or acquired deficient activity show a mild bleeding tendency and impaired platelet responses to collagen. This receptor recognizes a GFOGER (O stands hydroxyproline) motif present in the sequence of collagen type I

and IV (Emsley *et al.*, 2000). Integrin  $\alpha 2\beta 1$  can bind to collagen type I monomers and polymers. Both integrin  $\alpha$ - and integrin  $\beta$ -subunits fold into a N-terminal extracellular globular head that creates the ligand binding surface sitting on two membrane-spanning legs. All  $\alpha$ -subunits of integrins contain at the N-terminal of  $\alpha$  seven-bladed  $\beta$ -propeller structure. The  $\alpha 2$  extracellular domain furthermore contains a unique inserted (I) domain, with a conserved cation-binding site known as the metal ion-dependent adhesion site (MIDAS) with a clear preference for  $Mg^{2+}/Mn^{2+}$ . The integrin  $\alpha 2\beta 1$  is assumed to have a primarily adhesive role. An early model of platelet-collagen interactions, the so called “2-step, 2-site” model incorporated the idea that platelets in a first step firmly bind to collagen via high affinity  $\alpha 2\beta 1$  allowing subsequent interaction of a second receptor leading to platelet activation (Santoro *et al.*, 1991). This second, low affinity receptor was later proposed to be GPVI. However, the idea that  $\alpha 2\beta 1$  integrin is active on unstimulated platelets was challenged by Moroi and coworkers who demonstrated that the affinity of the integrin for soluble collagen increases on cellular stimulation (Jung and Moroi, 2000). Upon activation the integrin shifts into a high-affinity state, with the extracellular domains extended upwards. This rapid conformational switch is usually triggered by inside-out signaling. The mechanism of  $\alpha 2\beta 1$  activation is believed to be similar to that of other integrins. Both talin and kindlin-3 that bind to the cytoplasmic tail of the integrin subunits are required and cooperate to regulate integrin activation. Inside-out activation of GPIIb/IIIa can be induced through ligation of GPVI as demonstrated by defective adhesion of GPVI-deficient or –inhibited platelets to the matrix proteins. Human platelet deposition on collagen depends on the concerted interplay of several receptors: GPIb in synergy with integrin  $\alpha 2\beta 1$  mediating primary adhesion. However, even in combination, these 2 axes provide an insufficient stimulus for full thrombus. Engagement of GPVI is required to ensure platelet activation (Lecut *et al.*, 2005, Nieswandt *et al.*, 2001b, Siljander *et al.*, 2004b). The relative contribution of each of these receptors very much depends on the experimental conditions but it is now generally accepted that they act synergistically in the process of platelet adhesion, activation and expression of procoagulant activity on the extracellular matrix. Binding of integrin  $\alpha 2\beta 1$  to collagen contributes to cellular activation indirectly by reinforcing GPVI-collagen interactions and directly by a series of intracellular signaling events summarized as “outside-in” signaling. These are very similar to those induced by GPVI, including Src, Syk, SLP-76 and PLC (Li *et al.*). Thus, although structurally

unrelated, the two major collagen receptors share important signaling molecules and act in a co-operative manner, reinforcing each other's activity.

### **2.3.3.2 GPVI**

GPVI is thus the major receptor for platelet activation by collagen. As the target of this work, it will be described in more details in the next chapter.

Other proposed collagen receptors

-P65, specific receptor for collagen type III and P47, TIIICBP, specific receptors for collagen type III whose nature and role is still not well understood. GPIV is also considered as collagen receptor but GPIV (CD36) deficient patient (5% in Japan) have no abnormal collagen response.

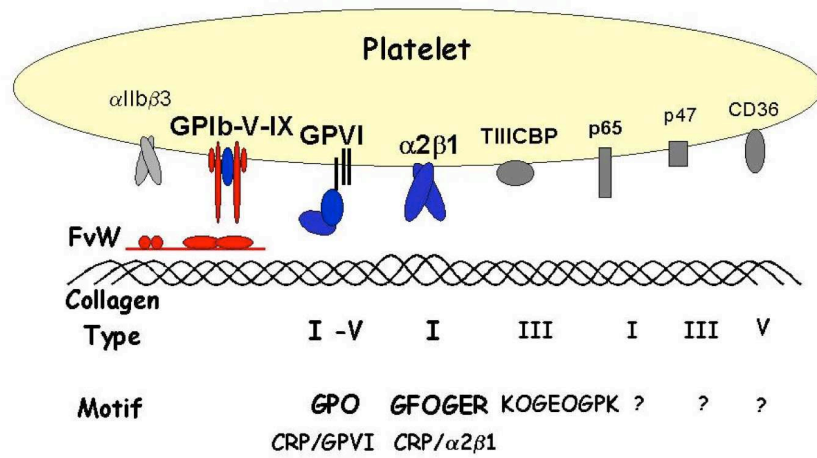
## **2.3.4 G-Protein-Coupled Receptors (GPCRs)**

### **2.3.4.1 ADP Receptors (P2Y1 and P2Y12) and ATP Receptor (P2X1)**

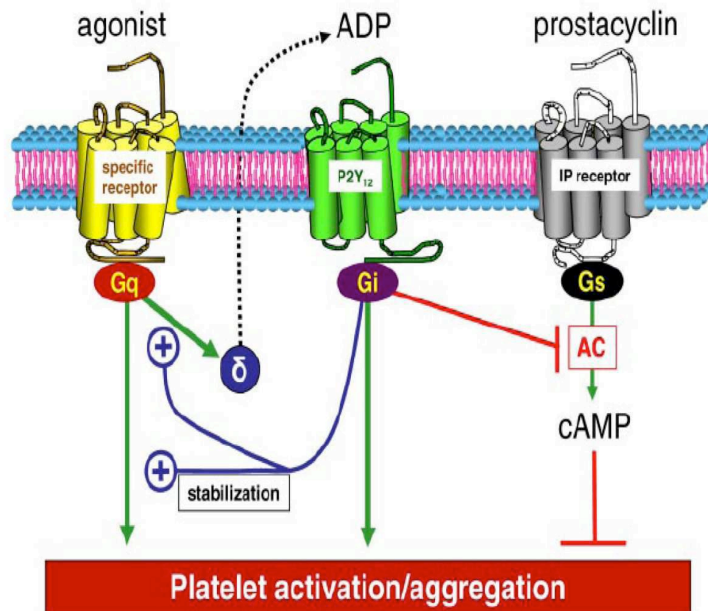
By itself ADP is a weak agonist of platelet aggregation inducing only reversible responses as compared to strong agonists such as thrombin or collagen. However, due to its presence in large amounts in the platelet dense granules and its release upon activation at sites of vascular injury, ADP is an important so-called secondary agonist, which amplifies most of the platelet responses and contributes to the stabilization of the thrombus. The receptors for extracellular nucleotides belong to the P2 family which consists of two classes of membrane receptors: P2X ligand-gated cation channels (P2X1–7) and G protein-coupled P2Y receptors (P2Y1,2,4,6,11,12,13,14) (for a review see (Gachet, 2008).

Platelets express two ADP receptors, P2Y1 and P2Y12 (Figure 24). Co-activation of the P2Y1 and P2Y12 receptors is necessary for normal ADP-induced platelet aggregation since separate inhibition of either of them with selective antagonists results in a dramatic decrease in aggregation. However, the two receptors have different functions since, except in the case of collagen-induced activation, P2Y1 plays a minor role when other agonists induce platelet aggregation whereas P2Y12 supports amplification of these responses.

The P2Y1 receptor is responsible for ADP-induced calcium mobilization required for platelet shape change and aggregation (Hechler *et al.*, 1998, Leon *et al.*, 1999). P2Y1 is expressed at a very low level on the platelet membrane (around 150 sites per



**Figure 23 : A Schematic representation of the platelet receptors for collagen.**



**Figure 24 : Central role of P2Y12 in platelet function.**

Green arrow represents, activation; truncated red line, inhibition; blue line ending with+, amplification; and dotted black line, secretion.  
Adapted from (Cattaneo, 2011).

platelet). P2Y1 is coupled to a Gq protein and activates phospholipase C and, downstream, protein kinase C (PKC) and mobilization of Ca<sup>+2</sup>. Lack of P2Y1 leads to reduced aggregation to all major agonists, resistance collagen/epinephrine and ADP-induced thromboembolism, and moderately increased bleeding times (Leon *et al.*, 1999). Conversely, mice overexpressing P2Y1 in their platelets have shorter tail bleeding times and are susceptible to thromboembolism and thrombosis (Hechler *et al.*, 2003). The P2Y1 receptor should be regarded as an attractive target for antiplatelet compounds. It is worthy of note that inhibition of the P2Y1 receptor results in only moderate prolongation of the bleeding time, which could be advantageous in terms of safety.

P2Y12 is expressed at a higher level (in the order of 1500 copies per platelet). Its tissue distribution is limited although not entirely restricted to platelets. The P2Y12 receptor strongly coupled to Gi2 leading to inhibition of adenylate cyclase and lowering cAMP, thereby enabling amplification of platelet activation via P2Y1 and also by other agonists which would otherwise be hindered (Cattaneo and Gachet, 2001, Cattaneo *et al.*, 2003, Hollopeter *et al.*, 2001, Kahner *et al.*, 2006). It also activates the phosphoinositide 3-kinase (PI3K) pathway. Patients lacking P2Y12 receptor display selective defects of ADP-induced platelet aggregation. Mice lacking P2Y12 form only small and unstable thrombi and show marked protection from arterial occlusion and prolonged bleeding times (Andre *et al.*, 2002). Owing to its central role in the growth and stabilization of a thrombus, the P2Y12 receptor is an established target of antithrombotic drugs

P2Y1 and P2Y12 have been shown to display gene sequence variations, which have been proposed to be associated with variable platelet responsiveness to ADP.

The third component of the platelet P2 receptors is P2X1, a ligand-gated cation channel responsible for a fast calcium entry induced by ATP. Although unable to trigger platelet aggregation by itself, the P2X1 receptor induces transient shape change and participates in collagen- and shear-induced aggregation. One hallmark of this receptor is that it requires high shear conditions to fully play its role in the formation of a thrombus. Since the P2X1 receptor plays an important role in thrombus formation only under high-shear conditions, it might represent the ideal target for an antithrombotic drug. The P2X1 receptor may also be considered as a potential target for antiplatelet strategies, with the interesting feature that P2X1 antagonists should be



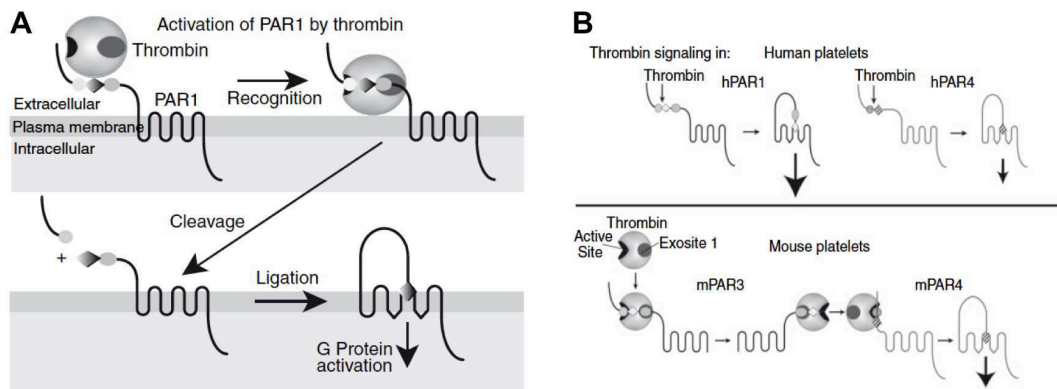
effective only at sites of severe stenosis where shear forces are very high, without having a deleterious effect on normal haemostasis.

#### **2.3.4.2 Thrombin Receptors (PAR1 and PAR4)**

Thrombin is the most potent platelet activator at least *in vitro*. Its receptors called Protease Activated Receptors (PARs) belong to the seven transmembrane domain GPCR superfamily but present a unique mechanism of activation (Varga-Szabo *et al.*, 2008). The activation of a PAR is a 2-step process. First, the cryptic ligand is unmasked by proteolytic cleavage of the receptor N-terminal domain; then, an intramolecular rearrangement allows the ligand and the receptor moieties to interact. On human platelets, thrombin activates PAR1 and PAR4 (Figure 25). PAR1 is a high-affinity receptor for thrombin by virtue of a hirudin (Hir)-like sequence that resides in its N-terminal extracellular domain. The Hir sequence allows PAR1 to compete with the much more plentiful fibrinogen, and, as a result, PAR1 is activated by thrombin at even subnanomolar concentrations. PAR4 has evolved a different strategy for interacting with thrombin. Bereft of a high-affinity Hir-like sequence, PAR4 has instead optimized its interactions with the active site of thrombin and uses a negatively charged cluster of amino acid residues to slow dissociation from the positively charged thrombin molecule. PAR4 is activated at a high thrombin concentrations; it is cleaved and signals more slowly but, despite its slower response, generates the majority of the intracellular calcium flux and does not require additional input from the P2Y<sub>12</sub> ADP receptor to form stable platelet–platelet aggregates (Kahn *et al.*, 1998). Mouse platelets express PAR3 and PAR4 (Nakanishi-Matsui *et al.*, 2000).

In the case of murine platelets, PAR4 binds to the active site in a conformation that leaves exosite I free for interaction with cofactors like PAR3.

PAR1 and PAR4 are coupled to a heterotrimeric G proteins G<sub>q</sub>, which activate the phospholipase C (PLC), protein kinases C (PKCs) and the mobilization of Ca<sup>2+</sup>. These signaling systems are critical in the platelet and bring numerous factors in to action such as Ca<sup>2+</sup> calmodulin dependent kinases, MAP kinases or integrin activation modulators and to the dynamics of the cytoskeleton and the secretion.



**Figure 25 : Activation of PARs by thrombin and thrombin signaling in human platelets.**

A depicts the mechanism of PAR-1 activation by thrombin

B depicts thrombin signalling on human (upper panel) and mouse (lower panel) platelets

PAR1 and PAR4 are also coupled to G12/13, a heterotrimeric G protein can lead to the activation of RhoA-ROCK pathway, a regulator of phosphatase of myosin light chain (Becker *et al.*, 2009, Macaulay *et al.*, 2010).

PAR1 signaling is strengthened by additional inputs from G<sub>i</sub>-coupled signals evoked by stimulation of P2Y<sub>12</sub> by ADP and leading to a decrease in the platelet concentration of cAMP whereas PAR4 signaling does not require additional input (Offermanns, 2006) (Figure 25).

Patients with arterial thrombosis receive in hospital anticoagulant treatments aimed to inhibit thrombin formation and or thrombin activity. However, inhibition of PAR1 could be of interest in the secondary prevention of arterial thrombosis and specific antagonists have been developed.

#### **2.3.4.3 Thromboxane A<sub>2</sub> Receptor (TP $\alpha$ )**

Like ADP, TXA<sub>2</sub> functions as a positive-feedback mediator during platelet activation. The action of TXA<sub>2</sub> is locally restricted because of its short half-life. The TXA<sub>2</sub> receptor identified on platelets (TP $\alpha$ ). It is also activated by the prostaglandin endoperoxides PGG<sub>2</sub> and PGH<sub>2</sub>, couples to G<sub>q</sub> and G12/G13 and to PLC (Brass *et al.*, 1987, Shenker *et al.*, 1991).

The role of TP as the platelet TXA<sub>2</sub> receptor has been demonstrated in studies using platelets from TP deficient mice, which become unresponsive to TXA<sub>2</sub>. TP-deficient mice have prolonged bleeding times and are unable to form stable thrombi (Thomas *et al.*, 1998). A reduced activation of TP-deficient platelets has been suggested to contribute to a reduced injury induced vascular proliferation as well as to a reduced progression of atherosclerosis observed in mice lacking TP. Some studies using the radiolabeled TXA<sub>2</sub> analogues as ligands demonstrated two classes of binding sites in platelets, and indicated that they might represent the receptor subtypes (Dorn, 1989, Hedberg *et al.*, 1988, Morinelli *et al.*, 1987). They further suggested that the two putative subtypes of the receptor may independently mediate shape change and aggregation (Dorn, 1989, Dorn and DeJesus, 1991). This suggestion has been supported by the reports that platelet shape change and aggregation can be differentiated by several TXA<sub>2</sub> analogues. For example, in some species, the TXA<sub>2</sub> agonist U46619 induces platelet shape change and an increase in calcium without aggregation (Nakano *et al.*, 1987). In addition, the TXA<sub>2</sub> analogue S-145 prevents

aggregation and secretion by U46619 but itself induces shape change (Hanasaki and Arita, 1988). One model consistent with these observations is that the TXA<sub>2</sub> receptor mediated signaling consists of two separate receptor-effector systems mediating distinct functional responses; one linked to PLC activation resulting in platelet aggregation and secretion, and the other mediating an increase in cytosolic calcium and platelet shape change (Takahara *et al.*, 1990).

### 2.3.5 Recently Identified Receptors Stabilizing Thrombosis

Many other receptors involved are gradually implicated in the growth of the clot and its stabilization. Their function is mainly known through the study of genetically modified mice, but their role in human diseases and the possibility whether they may be good therapeutic targets is not yet established.

The stabilization of a newly formed thrombus is essential to arrest bleeding at sites of vascular injury. The final thrombus is embedded in a fibrin network to withstand the shear forces generated by the flowing blood. In addition, outside-in signaling through ligand-occupied integrin  $\alpha$ IIb $\beta$ 3 plays a central role in thrombus stabilization and beside CLEC-2, whose involvement in thrombus perpetuation is currently under discussion (see below), other proteins including CD40L (Andre *et al.*, 2002), SLAM (signaling lymphocyte activation molecule), Gas6 (growth arrest-specific gene 6) (Cosemans *et al.*, 2010) and semaphorin 4D (Zhu *et al.*, 2007) have been established to participate in platelet–platelet interaction. It has also been reported that blockade of Eph kinases/ephrins interactions resulted in a decreased thrombus volume *in vitro* under flow conditions, suggesting that this interaction contributes to the stabilization of platelet–platelet contacts (Prevost *et al.*, 2005). It can be anticipated that other platelet receptors will be identified to be involved in promoting platelet plug stability and possible candidates include JAM-A, JAM-C, CD226 and CD84 (Brass *et al.*, 2008). TREM-like transcript-1 has recently been reported to protect against inflammation-mediated hemorrhage and to facilitate platelet aggregation in mice and human (Brass *et al.*, 2008, Stalker *et al.*, 2009, Washington *et al.*, 2009).

However, molecules that limit thrombus growth were also identified. Mice lacking the endothelial cell specific adhesion molecule (ESAM) (Stalker *et al.*, 2009) or platelet endothelial cell adhesion molecule-1 (PECAM-1) (Falati *et al.*, 2006) developed larger thrombi *in vivo* as compared to control mice, indicating that these

molecules serve as negative regulators of thrombus formation, although the effects may be limited.

#### **2.3.5.1 CLEC-2**

CLEC-2 is a C-type lectin-like type II transmembrane receptor identified to mediate the potent platelet activation responses to snake venom toxin, rhodocytin (Suzuki-Inoue *et al.*, 2006). CLEC-2 lack a signal sequence but show a putative hydrophobic transmembrane region. The carbohydrate recognition domain (CRD) conserved six cysteine residues and these cysteine residues generate three interchain disulfide bonds that are typical of C-type lectins (Weis and Drickamer, 1996). CLEC have a tyrosine residue in their predicted cytoplasmic domain and also contain several serine and threonine residues which are potential sites of phosphorylation (Colonna *et al.*, 2000). CLEC-2 uses a similar signaling pathway to GPVI, initiated by tyrosine phosphorylation of a single ITAM motif, termed hem ITAM, within its cytoplasmic tail (Suzuki-Inoue *et al.*, 2006, Watson *et al.*, 2010). The only identified physiological ligand of CLEC-2 is podoplanin (which is a transmembrane protein), an endothelial sialomucin glycoprotein. Podoplanin is expressed in the lymphatic sac, and it was shown that the CLEC-2-podoplanin interaction is essential for the separation of the lymphatic from the blood vascular system during embryonic development (Bertozzi *et al.*, 2010, Uhrin *et al.*, 2010). Mice pretreated with a specific antibody to CLEC-2 exhibit a selective loss of the C-type lectin receptor and impaired platelet activation on collagen at high shear *in vitro* or *in vivo* (May *et al.*, 2009).

CLEC-2 knockout mice displayed reduced thrombus stability under flow *in vitro* and *in vivo* leading to prolonged tail bleeding times and protection from arterial thrombus formation (May *et al.*, 2009, Suzuki-Inoue *et al.*, 2010).

#### **2.3.5.2 FcγRIIA**

Human platelets express on their surface the low affinity receptor for IgG FcγRIIA. This receptor belongs to the immunoreceptor superfamily and shares its signaling mechanism with GPVI. FcγRIIA is involved in heparin-induced thrombocytopenia, the immune complexes triggering platelet activation. The molecules targeting Tyrosinase below the FcγRIIA could be useful in the treatment of Heparin-induced thrombocytopenia (HIT) by inhibiting, on one hand platelet activation and, on the other side, by controlling their elimination by macrophage (Worth *et al.*, 2006).

### **2.3.5.3 Inhibitory Receptors**

Although the inhibitory receptors CD31 (PECAM-1), G6Bb and CAECAM seem to raise the threshold of doses of agonists needed to induce platelet aggregation, it is still too early to assume interest as therapeutic targets.

## **2.4 Antithrombotic Drugs**

There are considerable differences in drug therapy for arterial and venous thrombosis, which reflect different mechanisms underlying these two types of thrombosis. The therapeutic goal is not only to control thrombus formation but also to prevent new plaque rupture events by stabilizing the atheromatous plaque. In contrast, for prevention and treatment of venous thrombosis the major approaches are the control of thrombus formation and removal of environmental risk factors.

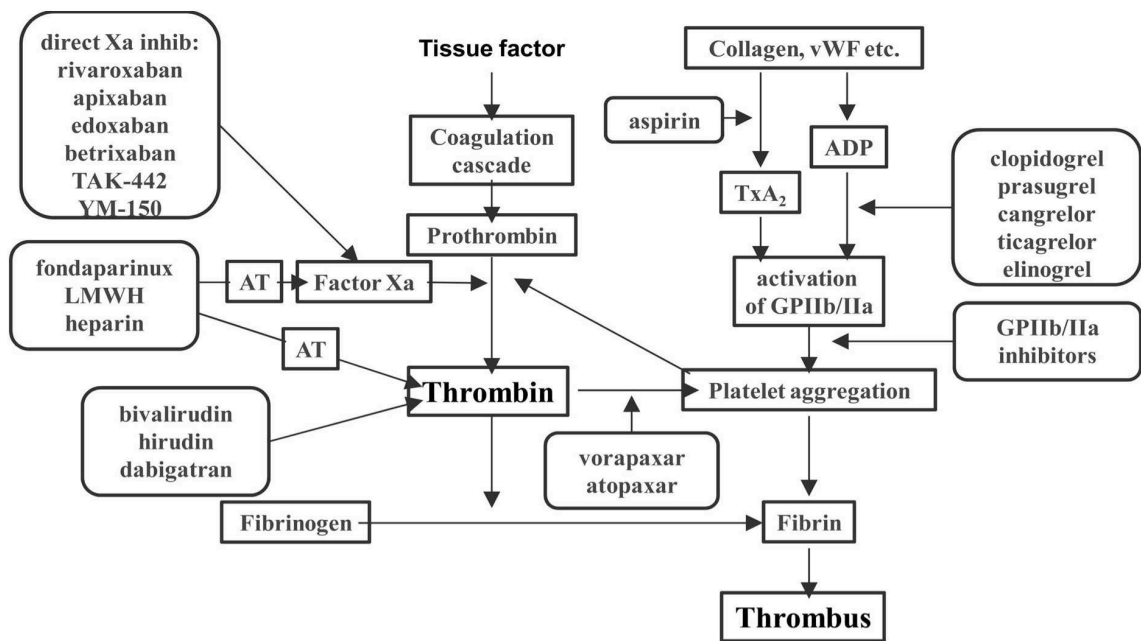
The antithrombotic drugs are classified according to their targeted constituents: antiplatelet agents and anticoagulants (Figure 26).

### **2.4.1 Anticoagulants**

As anticoagulants are not the purpose of this study, they will be shortly mentioned. Anticoagulant molecules are divided into non-specific anticoagulants, such as vitamin K antagonists and heparin, and direct thrombin inhibitors, including hirudin and argatroban.

Vitamine K antagonists block thrombin formation by inhibiting the gamma carboxylation of the VII, IX, X and II factors, preventing their calcium dependent binding to anionic phospholipids. Inhibition of the formation of the tenase and prothrombinase complexes block thrombin formation. Vitamine K antagonists are given orally and treatment needs to be adjusted on biological efficacy (prothrombin ratio and international normalized ration, INR).

Unfractionated heparin is a naturally occurring highly sulfated glycosaminoglycan of molecular weights ranging from 5000 to over 40,000 Daltons. It is inhibiting both thrombin generation and thrombin activity. Heparin binds to the enzyme inhibitor antithrombin (AT) causing a conformational change that results in its activation through an increase in the flexibility of its reactive site loop (Chuang *et al.*, 2001).



**Figure 26: The coagulation cascade, platelet activation pathways, and targets of antithrombotic agents.** inhib indicates inhibition; LMWH, low-molecular weight heparin; AT, antithrombin; vWF, von Willebrand factor; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; and ADP, adenosine diphosphate. Adapted from (Van de Werf, 2011)

The activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably. The rate of inactivation of these proteases by AT can increase by up to 1000-fold due to the binding of heparin. The efficacy of the treatment thus depends on AT availability. Heparin is given parenterally (iv or sc). It has a short half-life and the treatment must be adjusted on the biological efficacy measured by the activated partial thromboplastin time (APTT).

A serious side effect of heparin is heparin-induced thrombocytopenia (HIT). HIT is caused by an immunological reaction that makes platelets a target of immunological response, resulting in the degradation of platelets. This is what causes thrombocytopenia. This condition is usually reversed on discontinuation, and can generally be avoided with the use of synthetic heparins.

The conformational change in AT on heparin-binding mediates its inhibition of factor Xa. For thrombin inhibition, however, thrombin must also bind to the heparin polymer at a site proximal to the pentasaccharide. The highly-negative charge density of heparin contributes to its very strong electrostatic interaction with thrombin. The formation of a ternary complex between AT, thrombin, and heparin results in the inactivation of thrombin. For this reason, heparin's activity against thrombin is size-dependent, the ternary complex requiring at least 18 saccharide units for efficient formation. In contrast, anti-factor Xa activity requires only the pentasaccharide binding site. This size difference has led to the development of low-molecular-weight-heparin (LMWHs) and, more recently, to fondaparinux as pharmaceutical anticoagulants. Low-molecular-weight heparin (LMWH) consists of only short chains of polysaccharide, having an average molecular weight of less than 8000 Da. These are obtained by various methods of fractionation or depolymerisation of polymeric heparin. Low-molecular-weight heparins and fondaparinux target anti-factor Xa activity rather than anti-thrombin activity, with the aim of facilitating a more subtle regulation of coagulation and an improved therapeutic index. Heparin and its low molecular weight derivatives (e.g. enoxaparin, dalteparin, tinzaparin) are effective at preventing deep vein thrombosis and pulmonary emboli in patients at risk (Bergqvist *et al.*, 2002).

Fondaparinux is a synthetic pentasaccharide, whose chemical structure is almost identical to the AT binding pentasaccharide sequence that can be found within polymeric heparin and heparan sulfate (Bauer, 2004). It is now prescribed for both



prophylaxis and treatment of venous thromboembolism (Boehlen *et al.*, 2010). With LMWH and fondaparinux, there is a reduced risk of HIT. Monitoring of the APTT is also not required and does not reflect the anticoagulant effect, as APTT is insensitive to alterations in factor Xa.

Direct thrombin inhibitors (DTI) have been developed. The rationale for the clinical use of direct thrombin inhibitors is their ability to inactivate fibrin bound thrombin and to be independent on the availability of AT. Direct thrombin inhibitors include hirudin and argatroban. Bivalirudin (Angiomax or Angiox, manufactured by The Medicines Company) is a specific and reversible DTI. Chemically, it is a synthetic congener of the naturally occurring drug hirudin (found in the saliva of the medicinal leech *Hirudo medicinalis*). It overcomes many limitations seen with indirect thrombin inhibitors, such as heparin. Bivalirudin is a short, synthetic peptide that is potent, highly specific, and a reversible inhibitor of thrombin. It inhibits both circulating and clot-bound thrombin, while also inhibiting thrombin-mediated platelet activation and aggregation. Bivalirudin has a quick onset of action and a short half-life. It does not bind to plasma proteins (other than thrombin) or to red blood cells. Therefore it has a predictable antithrombotic response. There is no risk for HIT. It does not require a binding cofactor such as antithrombin and does not activate platelets. These characteristics make bivalirudin an ideal alternative to heparin. Bivalirudin clinical studies demonstrated consistent positive outcomes in patients with stable angina and unstable angina and in patients undergoing percutaneous coronary intervention (PCI) (Stone *et al.*, 2008).

Argatroban is a derivative of arginine that structurally mimics the active site residue of thrombin (Fareed *et al.*, 2002). It binds to the active site of thrombin in a reversible manner. It is as effective as heparin and is now used as an alternative of heparin in treating venous thromboembolism (VTE). Bleeding complications are a major adverse effect of argatroban.

#### **2.4.2 Antiplatelet Agents**

The objective of antiplatelet therapy is to reduce the platelet aggregation and inhibit thrombus formation in arterial circulation. Recent years have witnessed important therapeutic advancement of molecules targeting specific platelet surface receptors or regulators of cellular haemostasis.

The field of antiplatelet therapy has experienced very substantial changes during the past 20 years. Attempts to separate improved antithrombotic efficacy from enhanced bleeding liability have met limited success so far. Quantitative assessment of bleeding risk has lagged behind the successful development and validation of tools for assessing the risk of ischemic complications (Subherwal *et al.*, 2009). The interaction between the antiplatelet drug and its molecular target largely depends on the following determinants (i) the availability of the active moiety of the drug at its receptor site, as a function of drug absorption, liver metabolism, and elimination, i.e, pharmacokinetics (PK); (ii) the mechanism of transient or permanent inhibition of the target and the rate of its renewal, i.e, pharmacodynamics (PD); and (iii) genetic variation of the drug target and/or drug-metabolizing enzymes, i.e, pharmacogenetics (Patrono *et al.*, 2008).

- Actual strategies of anti-thrombosis: Many distinguished anti-platelets drugs are actually in use for therapy which are as follows;

#### **2.4.2.1 GPIIb/IIIa (Integrin $\alpha$ IIb $\beta$ 3) Antagonists: (Platelet Anti-aggregatory Antibodies)**

GPIIb/IIIa (integrin  $\alpha$ IIb $\beta$ 3) was among the first pharmacologic targets for the reduction of ischemic events.

Strategies to inhibit its function include antibodies (abciximab), cyclic peptides adapted from a snake venom disintegrin (eptifibatide), and non-peptides analogues of an RGD peptide (tirofiban and lamifiban) that inhibit ligand binding. Although these inhibitors have been beneficial for patients undergoing percutaneous coronary intervention, these do not have widespread clinical use because of their side effect of undesired bleeding (Quinn *et al.*, 2003).

Parenteral GPIIb/IIIa antagonists have been successful in the reduction of ischemic events and mortality in patients undergoing percutaneous coronary intervention but oral analogues have not been proved successful clinically (Jennings and Saucedo, 2008).

##### **2.4.2.1.1 Antibodies/ Abciximab**

Numerous anti-GPIIb/IIIa monoclonal antibodies (mAb) have been produced, some of which abolished platelet aggregation *in vitro*. For a small number of them, *in vivo*

antithrombotic effect in animals and rarely in humans have been reported. One of them was used to develop for a drug.

The mAb obtained by injecting human platelets into mice, AP-2, LJ-CP8 and MA-16NC2, are effective in thrombosis models in baboons but causes thrombocytopenia and/or increasing bleeding time (Hanson, 1988, Krupski, 1993).

The fragments of two mAb, F(ab')<sub>2</sub> CRC64 (FRaMon) and F(ab')<sub>2</sub> YM337 were humanized by CDR grafting, which inhibits platelet aggregation *in vitro* and *ex vivo*. They were only tested on a small series in humans (FRaMon angioplasty with high risk and YM337 in healthy subjects) (Harder, 1999., Mazurov, 2002).

The mAb 7E3 and 10E5 obtained in the early 80s by B.S.Coller inhibit the binding of fibrinogen and vWF to activated GPIIb/IIIa and aggregation of human platelets *in vitro* (Coller *et al.*, 1986) and also in cynomolgus monkeys (Coller *et al.*, 1989). A major protective effect of F(ab')<sub>2</sub> 7E3 was also observed in the thrombolysis in dogs by promoting the dissolution of clots by thrombolytic agents (Gold, 1990.). These studies demonstrate that 7E3 was a suitable candidate to carry on clinical trials in coronary angioplasty during acute coronary syndromes. So, a chimeric Fab (c7E3 Fab; abciximab; ReoPro®) was produced by fusing the variable domains of the mouse monoclonal antibody to the constant regions of human Ig to decrease the immunogenicity of the product and was the first therapeutic antibody administered to human. Plasma half-life of abciximab is short (10 to 30min), but the molecule bound to platelets can be detected up to 10 to 14 days after administration. Its elimination is poorly understood (Coller and Shattil, 2008, Tchong *et al.*, 1994) Abciximab has been approved by the FDA in 1994 and is the only recombinant antibody used in the treatment of thrombosis. Many randomized clinical trials have been conducted involving over 20,000 patients undergoing PCI and/or treatment for unstable angina (De Luca *et al.*, 2005). These studies demonstrated a therapeutic benefit of abciximab in angioplasty treatment of myocardial infarction and acute coronary syndromes with elevation of troponin level and angioplasty (prevention of ischemic complications and mortality). Long-term mortality advantages have also been demonstrated, with most of the benefit paradoxically occurring long after the antiplatelet effects of the drug wore off (Bhatt and Topol, 2000). Abciximab is not considered as beneficial in patients with acute coronary syndrome, which do not need urgent revascularization. It is also associated with secondary effects (hemorrhage, thrombopenia,

hypersensitivity), which may be serious. The increased bleeding observed in some studies may be potentiated by administering the combination of abciximab and unfractionated heparin jointly. The site of catheterization (radial vs femoral) is also important. Overall, the severe bleeding is less than 0.2% in all studies (Kam and Egan, 2002). At the present stage of study, abciximab remains the most widely antagonist of integrin  $\alpha$ Ib $\beta$ 3 used but is not possible to conclude the superiority of one class over another due to advantages and disadvantages of each.

Thus, globally about 80% of patients with acute coronary syndrome do not use it. The doors are opened for other classes of GPIIb/IIIa antagonists.

#### **2.4.2.1.2 Eptifibatide (Integrilin®)**

Based on the observation that small peptides and snake venoms containing the Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequences could inhibit ligand binding to  $\alpha$ Ib $\beta$ 3, a cyclic peptide inhibitor (eptifibatide) and a nonpeptide RGD mimic inhibitor (tirofiban) were developed.

Eptifibatide is a cyclic heptapeptide derived from a protein found in the venom of the south eastern pygmy rattlesnake (*Sistrurus miliarus barbouri*). It belongs to the class of the so-called arginin-glycin-aspartate-mimetics and reversibly binds to platelets. Eptifibatide inhibits platelet aggregation by reversibly binding to the platelet receptor glycoprotein (GP) IIb/IIIa of human platelets, thus preventing the binding of fibrinogen, von Willebrand factor, and other adhesive ligands. Inhibition of platelet aggregation occurs in a dose- and concentration-dependent manner. Half-life is approximately 2.5 hours.

As abciximab, eptifibatide reduces ischaemic complications of percutaneous intervention and the treatment of acute coronary syndrome and increases the bleeding risk with molar bleeding episodes. In the PURSUIT (Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy) trial, which included 10,948 patients with non-ST-elevation acute coronary syndromes, eptifibatide significantly reduced the primary end point of death and non-fatal myocardial infarction at 30 days compared with placebo. In patients with ST-segment elevation myocardial infarction (STEMI), eptifibatide has been studied as adjunct to primary PCI and improved epicardial flow and tissue reperfusion. Studies evaluating eptifibatide in high-risk patients with non-ST elevation acute coronary syndromes

(NSTE-ACS) and a planned early invasive strategy in the EARLY-ACS (Eptifibatide Administration prior to Diagnostic Catherization and Revascularization to Limit Myocardial Necrosis in Acute Coronary Syndrome) trial and in patients with primary PCI for STEMI in comparison to abciximab in the EVA-AMI (Eptifibatide versus Abciximab in Primary PCI for Acute Myocardial Infarction) trial. After the completion of these trials, the value of eptifibatide in patients undergoing PCI in different indications can be determined (Zeymer, 2007).

In order to evaluate the effect of pre-catheterization laboratory (cath lab) administration of eptifibatide on pre-percutaneous coronary intervention (PCI) thrombolysis in myocardial infarction (TIMI) flow and its correlation with ischemia duration, was studied in 438 STEMI patients treated with primary PCI: 310 patients were pretreated with eptifibatide, while 128 patients received either no glycoprotein IIb/IIIa inhibitors or were only given them in the cath lab. All ischemia times (chest pain onset, diagnostic electrocardiogram, eptifibatide administration, cath lab arrival, first balloon inflation) were recorded. At angiography, TIMI grade 2 or 3 flow was observed in 54% of cases in Patients already treated with eptifibatide vs. 34% in already non-treated patients. In already treated patients, there was a strong correlation between pre-PCI TIMI flow and timing of eptifibatide administration. Pretreatment with eptifibatide and symptom duration of  $\leq 90$  minutes resulted in independent predictors of pre-PCI TIMI flow on multivariable analysis. Thirty day mortality was 1.9% in already treated patients and 9.5% in already non-treated patients. Eptifibatide therapy prior to primary PCI achieves a higher rate of pre-PCI TIMI flow with respect to late administration (Aquilina *et al.*, 2009).

#### **2.4.2.1.2.1 Tirofiban (Aggrastat®)**

Tirofiban hydrochloride is a non-peptide, intravenous administered and reversible antagonist of fibrinogen binding to the GPIIb/IIIa receptor and inhibits platelet aggregation. When administered intravenously, it inhibits *ex vivo* platelet aggregation in a dose- and concentration-dependent manner.

When given according to the recommended regimen, >90% inhibition was attained by the end of the 30-minute infusion. Tirofiban has a half-life of approximately 2 hours. It is cleared from the plasma largely by renal excretion, with about 65% of an administered dose appearing in urine and about 25% in feces, both largely as

unchanged tirofiban. Metabolism appears to be limited. Side effects mostly include bleeding problems. A recent study analyzing two large, randomized trials, PROVE IT–TIMI 22 on 8659 patients, which compared lower- versus higher-intensity statin therapy to evaluate whether higher-intensity statin therapy lowered the risk of atrial fibrillation (AF) onset during the 2 years of follow-up. It was concluded that the higher-dose statin therapy did not reduce the short term incidence of AF among patients after acute coronary syndromes when compared with standard dose statin treatment (McLean *et al.*, 2008).

#### **2.4.2.2 ADP/P2Y12 Receptor Antagonists**

Long before its molecular cloning, the pharmacological importance of this receptor in haemostasis and thrombosis was well recognized. This was due to the fact that the potent antithrombotic thienopyridine compounds ticlopidine and clopidogrel, of which an active liver metabolite selectively and irreversibly targets the P2Y12 receptor, were used as molecular tools to characterize platelet responses to ADP and the role of the latter in thrombosis. P2Y12 is the pharmacological target of thienopyridines antiplatelet drugs family (Ticlopidine, clopidogrel and prasugrel) and their derivatives. The thienopyridine compounds are prodrugs, which have to be metabolized by the liver in order to generate active metabolites.

Introduction of platelet ADP receptor inhibitors represented a breakthrough in the modern treatment of acute coronary syndrome (ACS). New agents resolving the bioavailability issues of clopidogrel are expected to minimize treatment failures and improve outcomes whereas it seems reasonable that agents with reversible inhibition of the ADP receptor will result in less bleeding complications.

##### **2.4.2.2.1 Clopidogrel (Plavix®)**

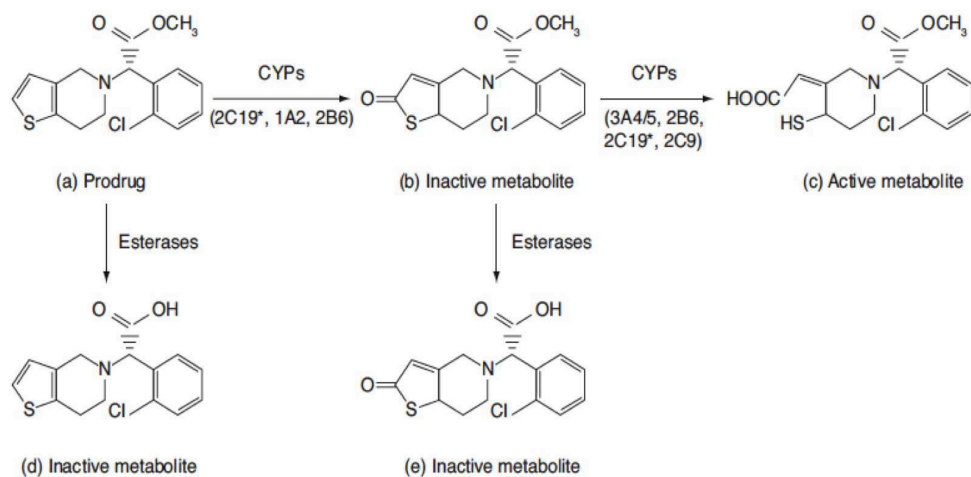
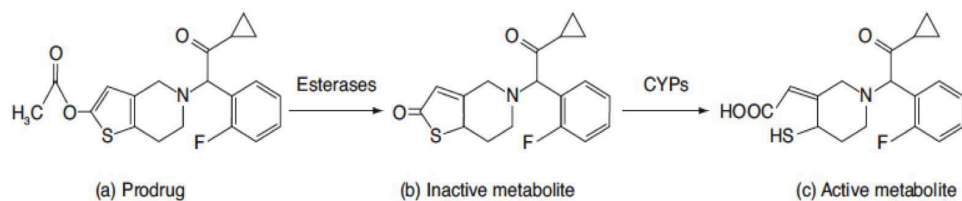
It is an oral, thienopyridine class of ADP receptor antagonist, which blocks ADP-induced platelets activation and aggregation. It is a prodrug that is converted in two steps to an active thiol metabolite by cytochrome P450 (CYP) enzymes and irreversibly inhibits the platelet ADP receptor P2Y12 (Figure 27) (Wiviott *et al.*, 2007). Platelet inhibition can be demonstrated two hours after a single oral dose of clopidogrel, but the onset of action is slow, so that a loading-dose of 300–600 mg is usually administered. CURRENT-OASIS 7 tested it in ACS patients by comparing a 600 mg loading dose and one week of a 150 mg maintenance dose (followed by 75

mg thereafter) to a 300 mg loading dose and 75 mg maintenance dose and observed a significant reduction in stent thrombosis and adverse events (myocardial infarction) in the higher dose group (Mehta *et al.*, 2009). Adverse effects of clopidogrel include hemorrhage, severe neutropenia and thrombotic thrombocytopenic purpura (TTP). The clopidogrel combined with aspirin is the treatment of choice for patients with acute coronary syndrome.

However recently many studies have reported variables biological responses correlated with clopidogrel in clinical efficacy (Cattaneo, 2007, Serebruany, 2010). A major part of Clopidogrel is inactivated by esterases. Additionally, the gene CYP2C19 codes for several alleles of differential efficacy to transform clopidogrel in its active metabolite. This account at least in part for the so-called clopidogrel resistance observed in some patients. The clinical response can be improved by increasing the dose and individual adaptation or the change for prasugrel.

#### ***2.4.2.2 Prasugrel (Efient®)***

Prasugrel is an orally administered thienopyridine pro-drug which is activated in the liver through cytochrome p450 (CYP); it is biologically more effective with less interindividual variability than clopidogrel because it is less degraded by esterases and only one step is needed for the active metabolite to be produced (Figure 27). The active metabolite irreversibly binds platelet ADP receptor (P2Y<sub>12</sub>) to a similar extent as the active metabolite of clopidogrel. However, in the case of prasugrel, *in vivo* availability of the active metabolite is significantly higher compared to clopidogrel. As a result, the recommended loading dose of 60 mg followed by a 10 mg/day maintenance regimen induces a more rapid, potent and consistent inhibition of platelet function compared to the currently used doses of clopidogrel (300 - 600 mg loading, followed by 75 mg/day for maintenance) (Michelson *et al.*, 2009). Prasugrel has already been established as a valuable therapeutic option in clinical practice following the results of the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction (TRITON- TIMI 38), in a Phase III 13,608-patient randomized trial, including moderate to high risk ACS patients undergoing PCI. In this study in which prasugrel (60 mg loading and 10 mg maintenance) in addition to aspirin was immediately compared to clopidogrel (300 mg loading and 75 mg maintenance) plus aspirin,

**A****B**

**Figure 27: Chemical structures and pathways for metabolism of clopidogrel and prasugrel.**

(A) Clopidogrel metabolism from the prodrug form to its active metabolite occurs through two reactions that are mediated by cytochrome P450. Competing pathways for inactivation by esterases are also shown.

(B) Prasugrel metabolism occurs through two steps, one mediated by esterases and the other by cytochrome P450.



prasugrel was associated with a significant reduction of the primary end point (cardiovascular death, non-fatal MI or non-fatal stroke) over a 15-month follow-up period, at the expense of an increase in major bleeding (including fatal bleeding) (Wiviott *et al.*, 2007). The beneficial results of prasugrel were associated with a significant reduction of definite or probable stent thrombosis (1.1 vs 2.4%) while predicting determinants of major bleeding were identified as the history of stroke or transient ischemic attack, age of > 75 years and body weight of < 60 kg. In a prespecified study of patients undergoing primary PCI, prasugrel also proved more effective than clopidogrel in preventing ischemic events, without a significant excess of bleeding complications (Montalescot *et al.*, 2009). Prasugrel has now been approved both in Europe and by the FDA for the prevention of ischemic events in ACS patients undergoing PCI.

Prasugrel is more effective than clopidogrel, but has a higher bleeding risk. The emergence of competitive inhibitors with oral administration (ticagrelor) or intravenous (Cangelor) or both (Elinogrel) led to numerous clinical trials (Cattaneo and Podda, 2010).

#### **2.4.2.2.3 New Antagonists of the P2Y12 Receptor**

##### **2.4.2.2.3.1 Ticagrelor (Brilique®)**

Ticagrelor belongs to a new class of antiplatelet agents, the cyclopentyltriazolopyrimidines. Its mechanism of action is also exerted through P2Y12 platelet receptor inhibition; in contrast to clopidogrel and prasugrel, this inhibition is reversible. It is directly active (no metabolism of a pro-drug is required) with a rapid onset of action and greater degree of platelet inhibition compared to clopidogrel. The efficacy and safety of ticagrelor were evaluated in the Platelet Inhibition and Patient Outcomes (PLATO) trial where 18,624 ACS patients (38% of them with STEMI) were randomly assigned to either ticagrelor (180 mg loading dose followed by 90 mg twice daily) or clopidogrel (300-600 mg loading dose followed by 75 mg/day) for 1 year. All patients were also receiving aspirin. At the end of the follow-up period, patients on ticagrelor presented significantly lower rates of the composite primary end point (cardiovascular death, MI or stroke) compared to clopidogrel (9.8 vs 11.7%) without any significant difference in the rates of major bleeding among the two groups (Wallentin *et al.*, 2009). Despite the encouraging

results while some have serious concerns regarding the effects of a possible poor compliance to medication given the reversible nature and the not yet fully explained side effect of dyspnea (Wijns *et al.*, 2010). Ticagrelor approval by the FDA requires some additional studies.

#### **2.4.2.2.3.2 Cangrelor**

Cangrelor is a direct acting reversible platelet P2Y<sub>12</sub> inhibitor. Cangrelor is administered intravenously with its effect rapidly reversed following end of the infusion. A dose of 4µg/Kg per minute appears to result in 100% platelet inhibition in approximately 70% of patients (Greenbaum *et al.*, 2006, Storey *et al.*, 2001). Similar to prasugrel and ticagrelor, cangrelor is characterized by a rapid onset of action and more effective platelet inhibition compared to clopidogrel, with a favourable safety profile concluded from the initial Phase II trials. Cangrelor underwent two Phase III clinical trials, the ‘Clinical Trial to Demonstrate the Efficacy of Cangrelor (PCI) (Harrington *et al.*, 2009) and the ‘Cangrelor Versus Standard Therapy to Achieve Optimal Management of Platelet Inhibition (Platform), which were discontinued due to insufficient evidence of cangrelor’s clinical effectiveness (Bhatt *et al.*, 2009).

Cangrelor is still being studied as a bridge for patients on clopidogrel who are planned for Coronary Artery Bypass Graft Surgery (CABG) (Topol and Schork, 2011), [Ahttp://www.clinicaltrials.gov/ct2/show/NCT00767507](http://www.clinicaltrials.gov/ct2/show/NCT00767507)].

#### **2.4.2.2.3.3 Elinogrel**

Elinogrel is a novel, direct-acting, reversible P2Y<sub>12</sub> antagonist that can be administered both orally and intravenously resulting in a simplified and effective treatment regimen and covering the full spectrum of care from acute onset to chronic care. A recent pilot trial provided preliminary data about the feasibility and tolerability of escalating doses of intravenous elinogrel as an adjunctive therapy for primary PCI for STEMI (Berger *et al.*, 2009). In a trial 2a study Patients received intravenous Elinogrel before PCI to ERASE-MI trial, evaluated the safety of escalating doses of intravenous elinogrel versus clopidogrel alone before PCI. All 70 patients also received 600 mg of clopidogrel at the time of PCI, followed by 300 mg of clopidogrel four hours after PCI. There was no increase of bleeding rates with doses up to 40 mg (Berger *et al.*, 2009). Another double-blind, randomized, Phase II trial evaluated the safety, tolerability and efficacy of elinogrel in patients undergoing

non-urgent PCI (Leonardi *et al.*, 2010). On the other hand, several new P2Y12 antagonists molecules are arriving or in evaluation stage.

#### ***2.4.2.3 Thromboxane Synthetase and Thromboxane Receptor Inhibitors***

##### ***2.4.2.3.1 Aspirin (ASA) (acetyl salicylic acid)***

It is orally administered drug and is an irreversible inhibitor of COX-1 that blocks platelet activation by reducing the production of TXA2 (Schulman, 2004). Its effect decreases with the production of new platelets (about 10 days). Since TXA2 amplifies platelet activation, aspirin inhibits secondary platelet aggregation. As a long term anticoagulant or preventative measure for heart disease the standard dose is 75-100mg per day. It is thus largely used for secondary prevention in patients with CAD (coronary artery disease). This will vary depending on the medical professionals evaluation of the patient (Baigent *et al.*, 2009).

Blockage of platelet function by aspirin alone is not sufficient for the treatment of acute events so a dual therapy with P2Y12 antagonists is needed for efficient treatment. Variable platelet reactivity so called “resistance to aspirin” has been reported. Insufficient compliance to the treatment appears as a major contributor to aspirin resistance. Competition with non-steroidal anti-inflammatory drugs could also offset the clinical effect of aspirin. Absorption of the active drug and polymorphism of Phospholipase A2 (PLA2) are other factors.

The major risk of aspirin is to increase gastrointestinal bleeding (attack of the gastrointestinal mucosis), other extra-cranial bleeding by about 50% compared with control bleeding and risk of bleeding particularly when therapeutic associations are too efficient.

##### ***2.4.2.3.2 Other Inhibitors of TXA2 Pathway***

A major limitation of aspirin is that it irreversibly inhibits COX-1 of both platelets and endothelium, therefore, reducing the production of beneficial prostacyclin as well. For the inhibition of the TXA2 pathway, three different alternatives would seem feasible: selective inhibition of only platelet COX-1, direct thromboxane-synthase inhibition (which reduce the end product) and thromboxane-receptors blockade as it has been shown that accumulating peroxides can per se activate them, the same way as TXA2 (FitzGerald, 1991).

#### **2.4.2.3.3 Cox-1 Inhibitor**

Triflusal is an antiplatelet agent structurally related to aspirin. Its mechanism of action involves inhibition of TXA<sub>2</sub> production through selective COX-1 inhibition, while at the same time preserving vascular prostacyclin synthesis. Moreover, triflusal is also a phosphodiesterase inhibitor resulting in cyclic AMP increase and, therefore, leading to reversible inhibition of platelet aggregation, vasodilation and inhibition of vascular smooth muscle cell proliferation (Costa *et al.*, 2005). In clinical trials (2275 patients with established AMI ; 2113 patients with transient ischemic attack) there were no significant differences between Triflusal and aspirin in the primary endpoints, but a tendency to lessen major bleeding was noticed for Triflusal.

Keeping in view the importance, to the amplification of platelet activation by TXA<sub>2</sub>, several TP $\alpha$  receptor antagonists have been developed and are under study (Amaro *et al.*, 2009).

#### **2.4.2.3.4 Terutroban (S 18886)**

It is a selective oral antagonist of thromboxane receptor (TP $\alpha$ ), which inhibits thromboxane-induced platelet aggregation and vasoconstriction. Preliminary studies in humans have shown that terutroban induced regression and stabilization of atherothrombotic plaques in magnetic resonance studies and that it successfully inhibited platelet aggregation in peripheral artery disease (PAD) patients (an effect comparable to aspirin) (Viles-Gonzalez *et al.*, 2005). The biological effects of terutroban is not limited to platelet functions, it has inhibiting effects on vasoconstriction of the vessels and a beneficial effect on atherosclerotic plaques has also been described (Sakariassen *et al.*, 2009).

A Phase III clinical study (Prevention of cerebrovascular and cardiovascular events of ischemic origin with terutroban in patients with a history of ischemic stroke or transient ischemic attack, PERFORM) has recently been completed. Recruiting 18,000 patients, this study investigated the efficacy of terutroban in secondary prevention of further cerebrovascular and cardiovascular events following a stroke or a Transient ischemic attack (TIA), compared to aspirin but was not superior to aspirin in preventing a second stroke, and was stopped prematurely (Bousser *et al.*, 2011, Hennerici, 2009).

#### ***2.4.2.4 Phosphodiesterase Inhibitors***

Since elevation of cAMP is a physiologic pathway to prevent activation, agents increasing its intracellular concentration are expected to have antithrombotic effects.

##### ***2.4.2.4.1 Cilostazol (Pletal®)***

Cilostazol (Pletal®) as a phosphodiesterase (PDE3) inhibitor limits cAMP degradation. It inhibits platelet aggregation *in vitro* and is a direct arterial vasodilator.

Cilostazol is indicated in intermittent claudication. It is administered orally and the typical dose is 100 mg twice a day. The effects may take as long as 3 months to be evident and has been shown to improve pain-free walking distance by 50%. Possible side effects of cilostazol use include headache (the most common), diarrhea, abnormal stools, increased heart rate, and palpitations (<http://www.drugs.com/pro/cilostazol.html>, 2008). The interest for Cilostazol is renewed regarding the treatment and secondary prevention of stroke and for patients with diabetes.

### **2.4.3 New Class of Antiplatelet Agents in Clinical Trials**

#### ***2.4.3.1 PAR-1 Receptor Inhibitors***

Thrombin is arguably the most potent activator of platelets. *In vitro* studies suggest that minimal concentrations of thrombin are sufficient to activate PAR-1 leading to platelet shape modification and aggregation. Thus, inhibiting PAR1 would permit platelet activation by the initial traces of thrombin formed at the start of the thrombosis event. It would thus stop thrombosis progression but PAR-1 inhibitors development is a challenging therapeutic option.

##### ***2.4.3.1.1 Vorapaxar (SCH 530348)***

Vorapaxar is an orally administered agent based on the natural product himbacine, that reversibly inhibits platelet PAR-1 inhibiting thrombin-induced platelet aggregation and thrombus formation.

A number of Phase II clinical trials have provided promising results and two Phase III clinical trials are ongoing. Effects of Vorapaxar in Preventing Heart Attack and Stroke in Patients With Acute Coronary Syndrome (TRA.CER) (TRA\*CER, 2009) and Effects of Vorapaxar in Preventing Heart Attack and Stroke in Patients With

Atherosclerosis (Morrow *et al.*, 2009), examining the safety and efficacy of vorapaxar in preventing the composite end point of cardiovascular death, MI, stroke or urgent coronary revascularization in patients with an ACS or atherosclerosis. Results of these studies are still pending however, one arm on 3 was stopped in TRA2P and it was recommended to stop in subjects with a history of ischemic stroke because of an excess intracranial bleeding; in the other study TRACER, the patients (12977 with recent non ST-elevation ACS) received Vorapaxar on top of standard dual antiplatelet therapy (aspirin + clopidogrel/prasugrel) and this study was prematurely close (Van de Werf, 2011).

#### **2.4.3.1.2 Atopaxar (E5555)**

Atopaxar is a reversible protease-activated receptor-1 antagonist that interferes with thrombin-mediated platelet effects.

Another agent of this class, atopaxar, with potential antithrombotic and anti-inflammatory properties has recently completed two Phase II trials (Japanese – Lesson from Antagonizing the Cellular Effect of Thrombin or J-LANCELOT and Lesson from Antagonizing the Cellular Effect of Thrombin in Acute Coronary Syndromes in a Japanese population with either ACS or high risk CAD (<http://www.clinicaltrials.gov/>)). Results from these studies have been demonstrating a satisfactory safety profile in terms of bleeding complications and a potential to reduce major adverse cardiovascular events. There were some concerns regarding the liver function and prolongation of the QTc interval which may be due to the increased atopaxar doses used (Goto *et al.*, 2010).

In the first study, 603 patients with a non-ST-elevation acute coronary syndrome (ACS) treated within 72 hours after onset of symptoms, were given a charge dose of atopaxar per os (orally) followed a daily treatment or matching placebo for 12 weeks. The primary end point, the incidence was similar between the atopaxar and placebo groups although the incidence of major bleeding was numerically higher in the atopaxar groups, including 1 hemorrhagic stroke. A statistically significant reduction in signs of ischemia was found during the first 48 hours after the start of study treatment in the atopaxar groups when compared with placebo. Transient liver function abnormalities and a relative QTc prolongation were observed with the 100-

and 200-mg doses apparently without clinical consequences (O'Donoghue *et al.*, 2011).

In the second study, 720 high-risk stable coronary artery patients were randomized to the same maintenance doses of atropaxar or placebo for 24 weeks. The overall incidence of bleeding complications was low, but more major bleeding complications were seen in the atropaxar groups (difference not significant). With regard to ischemic end points, a numerically lower incidence of major adverse cardiac events was found with each dose of atropaxar when compared with placebo. As in the ACS trial, transient elevations of liver transaminases and a dose-dependent QTc prolongation without apparent clinical correlates were noticed (Wiviott *et al.*, 2011).

The overall results of these two phase-II studies could be considered sufficiently positive to embark on a phase-III program. However, the numerically higher incidence of major bleeding complications, the above-mentioned liver dysfunction and relative QTc prolongation, and the lack of a convincing dose-related trend for bleeding risk and efficacy are somewhat troublesome (Van de Werf, 2011).

In addition, Van de Werf pointed out that it might be difficult to get patients to take two or three antiplatelet agents, on top of other medications such as statins and antihypertensive agents, for long periods of time. In the future, trials will be needed to test the PAR-1 inhibitors with new drugs like dabigatran (Pradaxa, Boehringer Ingelheim) and direct factor Xa inhibitors, such as rivaroxaban (Xarelto, Bayer/Johnson & Johnson) and apixaban (Eliquis, Pfizer/Bristol-Myers Squibb).

## **2.5 Adhesion : Target of the Future ?**

### **2.5.1 Inhibiting Platelet Adhesion**

Under arterial flow conditions and especially in stenosed arteries, platelets are subjected to high shear rates. Under these conditions the initial platelet contact with subendothelium is via the binding of GPIb/V/IX with vWF bound to collagen followed by direct platelet interaction with collagen. Inhibition of platelet adhesion appears to be a promising approach for developing antithrombotic agents which would have the following advantages over existing drugs

(i) adhesion is the first step of platelet thrombus formation, its inhibition would be effective. (ii) restenosis is a serious clinical problem that occurs in 10% patients undergoing angioplasty with stent. Activated platelets release growth factors (PDGF) and cytokines (TGF $\beta$ , IL-1, RANTES) that contribute to the migration and proliferation of smooth muscle cells, leading to the formation of the neointima, supporting an inflammatory condition. By blocking the stable adhesion, the release of these factors would be inhibited and prevent restenosis. Indeed, in animal models, inhibition of platelet deposition has a beneficial effect on the formation of the neointima. Several ligand-receptor pairs can be targeted.

### **2.5.2 GPIb-VWF Axis**

The specific requirement for GPIb-vWF interaction under conditions of high shear, such as found in diseased arteries, makes it a potentially attractive target for the pharmacological inhibition of pathological thrombus formation in the setting of precipitate diseases such as myocardial infarction or stroke. Although anti-GPIb $\alpha$  treatment results in prolonged bleeding times, this does not necessarily translate into an increased bleeding risk.

#### **2.5.2.1 Anti-GPIb/V/IX Antibodies**

Many anti-GPIb mAb inhibiting binding to vWF have been produced and characterized *in vitro*. The problems encountered in their development are the lack of cross-reactivity with GPIb in laboratory animals and in fact induce severe thrombocytopenia. Thus, AP-1 induced severe acute thrombocytopenia within 5 minutes (Cadroy et al, 1994). Other mAb, tested *in vivo* have not been developed (PP4-3C, PG-1). The team of H. Deckmyn produced two mAb anti-GPIb (6B4 and 24G10) which can block the binding of vWF to platelet and vWF- dependent collagen adhesion and recognize the two distinct binding sites of vWF on GPIb (Cauwenberghs *et al.*, 2001).

6B4 has been characterized for its potential antithrombotic properties (Cauwenberghs *et al.*, 2000). *Ex vivo* and *in vivo* studies in baboons indicated that IgG induced severe acute thrombocytopenia while the Fab induce only a minor decrease in the number of platelets. In addition, the Fab blocked human platelets adhesion with an efficiency that increases with shear rate. The Fab 6B4 also did not induce thrombocytopenia nor prolonged bleeding time. The occupancy rate of GPIb reached



a maximum of 80%, remained high for several hours and then decreased in 24 hours. The antibody 6B4 was humanized by re-surfacing (Fontayne *et al.*, 2006). The strategy used was to construct a chimeric Fab in which variable domains of 6B4 were fused to constant domains of human IgG4. The three-dimensional model was realized for the construct after selecting the Ig having a degree of greater than 75% identity in the framework regions (FR) and whose structure had been obtained with a good resolution. A composite model was constructed from the VH and VL of the two different Ig, by replacement of the H3 loop of VH, conflict resolution by steric energy minimization, conformational interactive modifications, *in silico* mutation, and re-surfacing by replacement of exposed mouse residues (immunogens) with human residues were carried out. The coding sequence of the Fab 6B4 was synthesized *de novo*. The affinity of humanized Fab for GPIb was the same as that of the parental IgG ( $0.8 \pm 0.1 \times 10^{-8} \text{ M}^{-1}$  vs  $2.2 \pm 0.6 \times 10^{-8} \text{ M}^{-1}$ ) and the Fab inhibited vWF/GPIb binding with the same efficiency. *In vivo* in baboons, it had no effect on platelet count and bleeding time up to a dose of 1.5mg/kg. On the other hand, it inhibited the GPIb-vWF interaction *ex vivo* in correlation with the occupancy rate of platelet GPIb (Staelens *et al.*, 2006).

Another Fab fragment obtained from the antibody p0p/B, abolished platelet tethering and subsequent thrombus formation at sites of arterial injury in mice (Massberg *et al.*, 2003). The injection of p0p Fab (80 to 600µg/Kg) prevented the deposition of platelets in a Folts cyclic model of thrombosis and arterio-venous shunt occlusion (Wu *et al.*, 2002).

#### **2.5.2.1.1 Anti-VWF Antibodies**

Various antibodies have been described against different domains of VWF.

Antibodies directed against A1 domain of vWF

Although several mAbs (712 and BB3BD5) directed against the A1 domain of vWF have been described to inhibit the binding of vWF to GPIb/V/IX, but having rare anti-thrombotic properties *in vivo*, are largely because of lacking inter-species crossreactivity (712 mAb effective in guinea pigs; BB3 BD5 effective Folts model in baboons) but prolonging bleeding time (Cadroy *et al.*, 1994).

The best-characterized antibody is AJvW2. Its *in vitro* inhibitory effect increases with the shear rate. *In vivo* in the guinea pig, AJvW2 (1.8 mg/kg) inhibits thrombosis without affecting bleeding time (Kageyama *et al.*, 1997). It is more effective in arterial thrombosis than venous thrombosis in hamsters. As compared to abciximab, the Fab AJvW2 proved to be more effective against thrombus formation with a lesser effect on bleeding time. A humanized antibody (AJW200) was obtained by grafting CDRs on a human frame. The administration in cynomolgus monkeys AJW200 *ex vivo* induced sustained inhibition of vWF-GPIb interaction. A wide window between the effective dose and prolongation time of bleeding was noted (Kageyama *et al.*, 2000).

Greater efficiency of AJW200 compared to abciximab was confirmed in the Folts model in dogs. AJW200 was tested in a population of healthy individuals with no side effects, but inhibiting vWF-GPIb interaction *ex vivo* (Kageyama *et al.*, 2002). In the pharmacokinetic/pharmacodynamic study healthy volunteers received placebo or AJW200 (0.01, 0.03 or 0.05 mg·kg<sup>-1</sup>) intravenously. C<sub>max</sub> (205, 586 and 833 ng·mL<sup>-1</sup>), T<sub>max</sub> (1.0, 0.92 and 0.57 h) and T<sub>1/2</sub> (23.5, 24.3, 27.2 h) were dose-dependent. The maximum vWF occupancy by AJW200 was 19, 51 and 62% respectively. AJW200 prolonged closure times in the Platelet Function Analyzer 100, which lasted for 3–6h with the lower and up to 12h for the higher dose. No clinically significant adverse events were recorded and there was no evidence of immunogenicity (Siller-Matula *et al.*, 2010).

The ALX-0081 is a bivalent Nanobody that specifically targets the A1 domain of vWF and blocks its interaction with GPIb. ALX-0081 selectively prevents thrombus formation under high shear stress conditions (Bartunek, 2008). Due to its bivalency, ALX-0081 is able to avidly interact with vWF resulting in an increased potency compared with its monovalent analogue. In Preclinical studies ALX-0081 completely inhibited platelet adhesion in collagen perfusion studies using blood obtained from patients undergoing PCI. In contrast, when aspirin and clopidogrel were used only an incomplete platelet inhibition was observed. In a combined baboon efficacy and safety model measuring acute thrombosis and surgical bleeding, ALX-0081 showed a superior therapeutic window compared with aspirin, clopidogrel and abciximab. The half-life of ALX-0081 was prolonged by vWF binding, enabling predictable drug levels in cynomolgus monkey (Ulrichs *et al.*, 2011). In phase I clinical studies

treatment with ALX-0081 was well tolerated and safe, no signs of bleeding were reported and no immunogenic response was detected after intravenous infusions of ALX-0081 for 1h at doses 0.5–12 mg in 40 male healthy volunteers. ALX-0081 displayed non-linear pharmacokinetic properties, following a two-compartment model. Full inhibition of ristocetin-induced platelet aggregation was observed at ALX-0081 concentrations of 400 ng·mL<sup>-1</sup>. A total dose of ALX-0081 >2 mg caused complete inhibition of ristocetin-induced platelet aggregation 1h post-dosing with a maximal duration of 12 h. Mild and transient laboratory changes in the reduction of factor VIII and vWF plasma levels were observed, all events were fully reversible within 24 h. These results demonstrate that ALX-0081 has a high efficacy with an improved safety profile compared to currently marketed antithrombotics (Ulrichs *et al.*, 2010).

In September 2009, Ablynx commenced a Phase II clinical trial with ALX-0081 in “high risk” patients with ACS undergoing a percutaneous coronary intervention (PCI) procedure. This study compares ALX-0081 head-to-head with ReoPro<sup>®</sup> (abciximab) as adjunctive therapy to a PCI procedure. All patients will receive standard anti-thrombotic therapy (aspirin, heparin and Plavix<sup>®</sup>) and, in addition, are randomly assigned to receive either ALX-0081 or ReoPro<sup>®</sup>. The primary endpoint of the Phase II trial is the number of bleeding events and the goal is to show a significant reduction in these events for ALX-0081 compared to ReoPro<sup>®</sup>. Data from this study are expected by the end of 2011.

#### Antibodies directed against A3 domain of vWF

The importance of the interaction between the A3 domain of vWF and collagen for hemostasis is indicated by the fact that in a family with von Willebrand disease, the lack of binding of vWF to collagen leads to a hemorrhagic syndrome (Ribba *et al.*, 2001). A vWF/collagen binding antagonist could thus block the formation of a thrombus in inappropriate conditions of high shear rates as in the stenosed arteries while maintaining normal hemostasis in healthy vessels.

The group of H. Deckmyn produced a monoclonal antibody (82D6A3) that inhibits the interaction between vWF and fibrillar collagen type I and III (Hoylaerts *et al.*, 1997). The inhibitory effect increases with shear. The antithrombotic effect of 82D6A3 has been demonstrated in the baboon in the Folts model: at the 0.3mg/kg

dose, it abolishes the cyclic flow reduction and had no effect on bleeding time and platelet count (Wu *et al.*, 2002). In *ex vivo* experiments, a 0.3mg/kg dose resulted in complete inhibition of vWF/collagen binding reached for an occupancy rate of 100% of vWF. An overdose of 0.6mg/Kg induces a small prolongation of bleeding time, this compound would have a safety zone wider than abciximab (Wu *et al.*, 2002).

The variable domains 82D6A3 were humanized by re-surfacing method, close to that used for 6B4. Then to increase the half-life and decrease immunogenicity, they were grafted onto the constant domains of human IgG4. The Fab have obtained same *in vitro* activity comparable to that of the parent IgG. Injection of doses of 0.1 to 1.5 mg/kg of h6B4-Fab in baboons showed that both pharmacokinetics and *ex-vivo* bio-activity of the molecule were to a large extent preserved (Staelens *et al.*, 2006).

#### **2.5.2.2 Aptamers to vWF**

ARC1779 is a nuclease resistant aptamer that binds to the vWF A1-domain with high affinity (Kd 2nM). The aptamer inhibits *in vitro* VWF-induced platelet aggregation and platelet adhesion and the formation of occlusive thrombi in cynomolgus monkeys. Because ARC1779 targets and binds to only activated vWF, the anti-platelet effect of ARC1779 should only be present in regions subject to high physical shear forces. These shear forces are only present in the arteries, including those leading into and within the brain. Therefore, ARC1779 could locally suppress platelet function and thrombus formation in the carotid arteries, while not disrupting normal platelet function and blood clotting in the remainder of the body.

A Phase 1 clinical trial of ARC1779 has been completed in 47 healthy volunteers. The primary objectives of the Phase 1 trial were to assess the safety and tolerability of ARC1779 and to establish proof of mechanism by determining the relationship between the administered doses of ARC1779 and the inhibition of plasma vWF activity and platelet function. The study evaluated ascending doses of ARC1779. Both vWF activity and platelet function were inhibited. No serious adverse events were observed to the exception of an allergic-like reaction in one subject.

Two phase 2a have been initiated:

One randomized, double-blind, placebo controlled single dose study will evaluate the safety and efficacy of ARC1779 in up to 100 patients undergoing selective carotid endarterectomy. The primary objectives are: (i) reduction of the number of small

blood clots which form immediately following the operation: the time to the first embolic signals is significantly prolonged by ARC1779 and the counts was decreased, (ii) perioperative bleeding was increased (Markus *et al.*, 2011).

The second, in patients with thrombotic thrombocytopenic purpura which results from a deficiency in ADAMTS13 leading to the consumption in ultra large, hyper-functional VWF polymers. ARC1779 could bind to and inhibit the activity of ultra-large vWF molecules, thereby potentially reducing the formation of blood clots in patients experiencing acute episodes of thrombotic thrombocytopenia purpura (TTP). Patients with a VWF disease of type 2B who also have an excessive unregulated VWF binding to platelets are also included. One serious adverse allergic-like reaction was observed and slow iv administration is required. The tested doses did not correct all clinical and laboratory features and more studies are necessary (Jilma-Stohlawetz *et al.*, 2011).

### **2.5.3 Integrin $\alpha 2\beta 1$ and GPVI**

#### **2.5.3.1 Integrin $\alpha 2\beta 1$**

It is now generally accepted that GPIb, integrin  $\alpha 2\beta 1$  and GPVI act synergistically in the process of platelet adhesion, activation and expression of procoagulant activity on the extracellular matrix.

However the therapeutic potential of targeting integrin  $\alpha 2\beta 1$  is questionable for several reasons contradictory results have been obtained in different experiments (Vanhoorelbeke *et al.*, 2003). The absence of integrin  $\alpha 2\beta 1$  has been shown to have no or only minor protective effect in arterial thrombosis (Gruner *et al.*, 2003, He *et al.*, 2003). Furthermore, the expression of the integrin  $\alpha 2\beta 1$  is not limited to platelets and it has other functions in addition to its role in platelet adhesion. This implies that antagonists to integrin  $\alpha 2\beta 1$  would not be specific.

#### **2.5.3.2 Glycoprotein VI (GPVI)**

GPVI is required for platelet activation and thrombus growth on collagen; GPVI is now considered as a very promising target for the development of a new class of antiplatelet agents.

## 2.6 GPVI, a Major Receptor of Collagen

GPVI was named during a membrane labelling study performed in 1970s which also identified other platelet glycoproteins, including GPIIb-IIIa (integrin  $\alpha$ IIb $\beta$ 3) and the GPIb. The function of GPVI as a major receptor to collagen has been identified before the receptor itself.

It was in 1980s that GPVI emerged as a candidate receptor for collagen through identification of a patient with an auto-immune thrombocytopenia whose platelets presented a GPVI deficiency and absent responses to collagen (Sugiyama *et al.*, 1987). The antibody from the Japanese patient was for 10 years, the only GPVI-sepecific tool available but it permitted to determine that GPVI was coupled to the common  $\gamma$ -chain of Fc receptors (Watson and Gibbins, 1998) and belonged to the immunoreceptor superfamily. In the late 1990's identification of the snake venom convulxin as a GPVI specific ligand (Jandrot-Perrus *et al.*, 1997, Polgar *et al.*, 1997) offered new possibilities for characterizing GPVI. Finally human and mouse GPVI genes were cloned simultaneously in 1999 by different approaches: peptidic sequencing obtained by protein purification (Clemetson *et al.*, 1999) and screening of megakaryocytes cDNA libraries for a protein with expected properties (Jandrot-Perrus *et al.*, 2000).

Since then, considerable work have been performed with up to 500 manuscripts published (PubMed). This allowed to characterize GPVI in physiology and pathology and to show that it presents the characteristics of a promising target for the development of a new class of anti-platelet agents.

It was proposed in early 1990s that collagen induced-platelet activation was mediated through the combination of two receptors, with integrin  $\alpha$ 2 $\beta$ 1 mediating adhesion and a second, unknown receptor inducing activation. It was termed as two site, two-step model of platelet activation by collagen (Morton *et al.*, 1989, Santoro *et al.*, 1991).

The identification of GPVI as the activation receptor led to refinement of the model with binding to GPVI being the initiating event, leading to inside-out activation of

integrin  $\alpha 2\beta 1$  and further activation of GPVI as a result of enhanced adhesion to collagen (Nieswandt *et al.*, 2001b, Watson *et al.*, 2000).

More recently, the observation that platelets have a low level of constitutively active integrin  $\alpha 2\beta 1$  and that collagen can bind to the integrin in its low affinity state (Siljander *et al.*, 2004a) has led to recognition that both receptors support the initial interaction with collagen, but the role of GPVI is predominant (Auger *et al.*, 2005). Meanwhile, GPVI had been confirmed as the major signaling receptor on platelets for collagen through generation of mice deficient in the glycoprotein receptor (Kato *et al.*, 2003).

### **2.6.1 Nucleotide Sequence, Genomic Structure of GPVI and Polymorphisms**

Expression of GPVI is restricted to megakaryocytes and platelets (Jandrot-Perrus *et al.*, 2000).

The GPVI gene was mapped on chromosome 19 (q13.4) of the human genome within the leukocyte receptor cluster (LRC). The nucleotide sequence of human GPVI is composed of 8 exons, spanning 23kb (Figure 28). Exon 1 and 2 code for the signal peptide of the protein, exons 3 and 4 for two IgC2 domains, exons 5 to 7 for the juxta-membraneous residues and finally exon 8 code for the transmembrane and cytoplasmic domains of GPVI. The last exon contains more of the regulatory region of the 3' non translated end of the gene and also a polyadenylation signal (Ezumi *et al.*, 2000).

Two variants of human GPVI have been described, from mRNA of megakaryocytic lineage, by the group of Ezumi and colleagues. The GPVI-2 isoform, results of a deletion of exon 5 and is characterized by a juxta-membraneous region truncation of 18 amino acids residues. The nucleotide sequence of GPVI-3 variant possesses an insertion 4bp at the extremity of exon 7, resulting a reading frame decalage, which is translated, by the absence of transmembrane domain and by an extension of the sequence of 361 residues. The 3-isoforms were co-transfected with FcR $\gamma$  into COS-7 cells, and studies during their expression, their association with gamma chain and their binding properties: the GPVI-1 and 2 isoforms were highly expressed on cell surface, associated with FcR $\gamma$  and binds to CRP, in contrary, the isoform-3, lacking

the transmembrane domain, is not associated with FcR $\gamma$  and not expressed at the surface (Ezumi *et al.*, 2000).

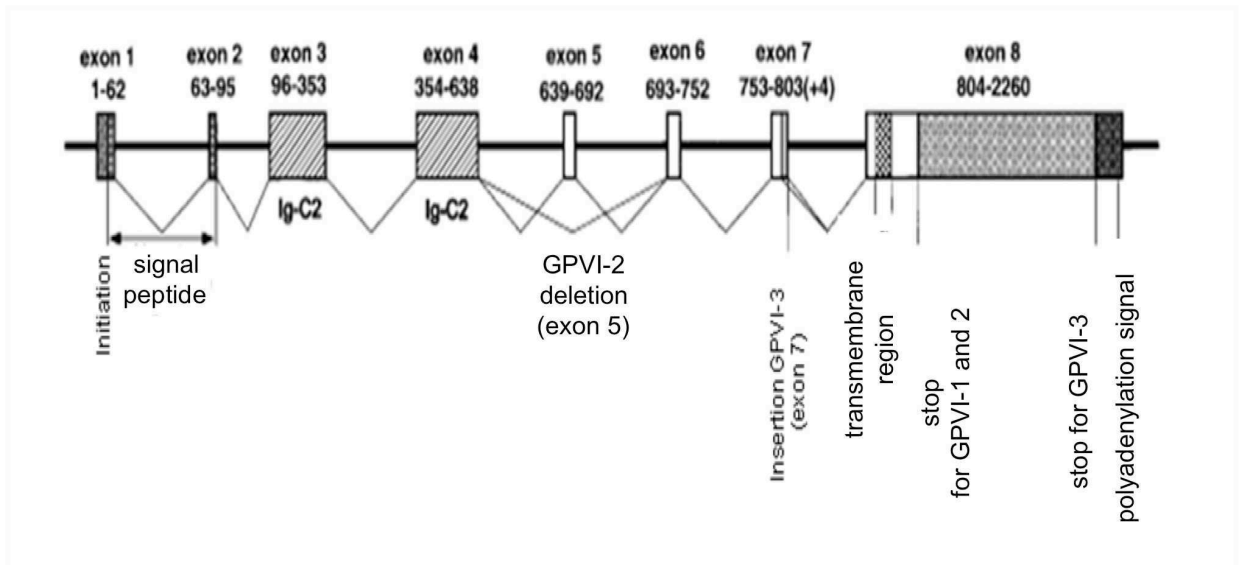
The promoter of GPVI gene is localized at -199 to -39 position in the regulatory region at 5' end. Like other specific genes of megakaryocytic lineage, this promoter neither possesses CAAT nor TATA sequence but possesses many binding sites for transcription factors. In particular, GATA motif at position -176 as well as a binding site for proteins of Ets family, situated at -45 position, are important for transcriptional activities of GPVI gene (Furihata and Kunicki, 2002).

At least ten polymorphisms in the GPVI sequence of healthy individuals have been identified (Cole *et al.*, 2003, Takagi *et al.*, 2002). Of these, seven are commonly inherited together as a core haplotype, with five of the seven resulting in amino acid substitutions: Ser219Pro, Lys237Glu, Thr249Ala, Gln317Leu and His322Asn. The two common alleles of GP6, 'a' and 'b', differ by these five amino acid replacements, clustered within the mucin or cytoplasmic domains and have been observed at population frequencies of 65% and 2% homozygotes respectively (Joutsu-Korhonen *et al.*, 2003). The high frequency GP6 'a' encodes amino acids Ser219, Lys237, Thr249, Gln317 and His322, whereas the low frequency GP6 'b' encodes Pro219, Glu237, Ala249, Leu317 and Asn322.

Platelets from subjects homozygous for the low frequency allele 'bb' had lower expression and apparently reduced glycosylation of GPVI relative to the platelets from 'aa' subjects (Joutsu-Korhonen *et al.*, 2003). Similarly, lower GPVI densities (number of copies on platelet) have been detected in individuals carrying the Pro219 mutation relative to individuals homozygous for common Ser219 (Best *et al.*, 2003). Functionally, 'bb' platelets had reduced thrombogenicity on collagen and lacked the CRP-induced early wave of tyrosine phosphorylation observed in 'aa' platelets (Joutsu-Korhonen *et al.*, 2003). The amino acid substitutions at Ser219Pro and Thr249Ala in the extracellular domain of GPVI alter potential O-linked glycosylation sites substitution at Lys237Glu, which results in the exchange of a positive charge for a negative

The Gln317Leu and His322Asn mutation in the cytoplasmic domain potentially alter the interaction of GPVI with its intracellular partners. It has been reported that N322 attenuates binding of Fyn/Lyn to GPVI and, consistent with the latter finding,





**Figure 28 : Genomic structure of the GPVI**

The figures indicate the number of amino acids residues with and (without) the signal peptide. Taken and adapted from (Ezumi, 2000).

convulxin-induced Syk phosphorylation was significantly attenuated in Dami cells stably transfected with GPVIb, relative to GPVIa. These allelic differences in GP6a and GP6b explain functional differences in the respective isoforms, but not the variability in the levels of GPVI on human platelets (Trifiro *et al.*, 2009).

## **2.6.2 GPVI Ligands**

### **2.6.2.1 Collagens**

Collagens are among the most abundant proteins in the human body, with more than 29 varieties known so far of which seven (types I–III, V, XI, XXIV, and XXVII) are fibrillar, able to assemble as stable triple helices, which then form a complex higher order three-dimensional fibrous superstructure. They are found in basement membranes of epithelium, connective tissue, tendons and cartilage. Nine types are detected in the vascular wall and surrounding tissue: collagens I, III, IV, V, VI, VIII, XV, XVIII and XIX, types I and III being the most abundant. Fibrillar collagens type I, III and V mostly participate in strength and flexibility of the vessel wall. Collagen type I constitutes approximately 50-75% of collagen present in the arterial wall (Farndale *et al.*, 2004).

At the simplest level, the collagen polypeptide sequence is composed of Gxx' triplets containing glycine followed by variable residues that often include proline at position x and hydroxyproline at position x'. The composition of this triplet of residues generates a bend in the polypeptide chain ( $\alpha$  chain) and gives a left helical structure to the procollagen molecule. Three procollagen molecules assemble to form a right super-helix: tropocollagen (Gelse *et al.*, 2003). The pattern of Gxx' and triple helix structure that results is called CoI domain.

The three polypeptide chains that assemble to form a tropocollagen triple helix can be identical gene products, as for collagen III (three  $\alpha$ 1 chains), or may differ, as for collagen I (two  $\alpha$ 1 chains and one  $\alpha$ 2 chain). The tropocollagen triple helices assemble to form a supermolecular fiber. These fibrillar collagens also possess short telopeptide extensions at their extremities and a second N-terminal CoI domain, which has an important role in the assembly of triple helix. The second domain CoI is cleaved upon secretion of collagen by cell. Once secreted, the monomers of fibrillar collagens (that is to say the tropocollagen molecules) are assembled to form fibrils,

which in turn fit together to form structure of vessels (Baum and Brodsky, 1999, Farndale *et al.*, 2007).

GPVI binds directly to glycine-proline-hydroxyproline (GPO) motifs within types-I and III collagen fibres (Jarvis *et al.*, 2008). Importantly, GPVI only binds to collagen fibres but not to monomeric collagen and this is in contrast to the integrin  $\alpha_2\beta_1$ .

#### **2.6.2.2 Collagen Derived Peptides**

Due to the complexity of the intact collagen fiber, most of our insights into the nature of platelet receptor-collagen interactions are based on modelled collagen-related peptides (CRP). Early studies identified a high-affinity site within collagen type I for the I-domain of integrin  $\alpha_2\beta_1$  (GFOGER, where O is hydroxyproline) and a low-affinity site in collagen type III for GPVI composed of repeating GPO triplets.

GPVI specific CRP are synthetic triple-helical collagen related peptide (CRP), which contains 10 GPO triplets and acts as a specific GPVI agonist (Knight *et al.*, 1999, Morton *et al.*, 1995). They consist of a triplet repeat of the GPO motif (> 5) spaced by inert GPP motifs and spontaneously form stable helical structures very similar to those of procollagen. Covalent bond formation by cross-linking between monomers of CRP causes their polymerization into a structure similar to the quaternary structure of collagen fibrillar. GPVI binds directly to glycine-proline-hydroxyproline (GPO) motifs within the collagen. The polymers of CRP (CRP-XL) are potent platelet GPVI specific agonists (Kehrel *et al.*, 1998, Knight *et al.*, 1999, Morton *et al.*, 1995). GPO-containing triple helical peptides bind GPVI with affinity that increases with the number of contiguous GPO triplets. However, such tracts of GPO triplets are scarce in collagen. In collagen III, GPOGPOGPO occurs at positions -8 to 0, not present in collagen I, and this region binds GPVI quite well. The best GPVI-binding motif in collagen III is a split GPO ... GPOGPO motif in the middle of the molecule (D3) that may bind two or more GPVI molecules side-by-side. How collagen I is recognized by GPVI remains to be established.

#### **2.6.2.3 Convulxin**

Convulxin (Cvx), a venom protein from the tropical rattlesnake *Crotalus durissus terrificus* and *Crotalus durissus cascavella*, is also an agonist of GPVI (Jandrot-Perrus *et al.*, 1997, Polgar *et al.*, 1997, Prado-Franceschi and Brazil, 1981).

Convulxin is a C-type lectin composed of two subunits,  $\alpha$  and  $\beta$ . These units are cross-linked by disulfide bonds to form a heterotetrameric structure ( $\alpha_4\beta_4$ ) (Batuwangala *et al.*, 2004, Murakami *et al.*, 2003).

Convulxin is a potent platelet agonist: a very low concentration (50-200pM) is sufficient for platelet aggregation in normal human. It binds with a very high affinity to the rabbit platelets (Francischetti *et al.*, 1997) and to human platelets with a very weak dissociation.

Convulxin may be a choice to study the role of GPVI in platelet-activation and has been used as a specific GPVI agonist.

One study has suggested that convulxin may bind to native human GPIb $\alpha$ : convulxin binds to mice cells (CHO) expressing recombinant human GPIb $\alpha$  and the binding was inhibited by an anti- GPIb $\alpha$  antibody which block its interaction with VWF factor but also by a soluble recombinant human GPVI (Kanaji *et al.*, 2003) but the relevance of this observation on platelets is not established.

Convulxin exists in solution as a dimer of  $\alpha_4\beta_4$  rings, yielding eight potential binding sites for GPVI. Binding studies confirm that all eight sites are able to bind GPVI tightly, each with high picomolar or low nanomolar affinity. The dimeric nature of CVX and its ability to bind eight GPVI molecules suggest that it might be capable of binding to GPVI expressed on two opposing surfaces. Thus, in addition to clustering up to eight GPVI receptors, CVX may facilitate platelet activation by bridging platelets directly (Horii *et al.*, 2009).

Other C-type snake venom which are GPVI agonists are alborhagin and ophioluxin (Andrews *et al.*, 2001, Du *et al.*, 2002).

Two endogenous ligands for GPVI have also been identified which are globular adiponectin (hormone) and laminin (Inoue *et al.*, 2006, Riba *et al.*, 2008). CD147 (EMMPRIN) has also recently been reported to bind to GPVI (Seizer *et al.*, 2009).

### **2.6.3 Structure of GPVI**

GPVI is a 63kDa (reduced condition) or 58 kDa (non-reduced condition) type-1 transmembrane protein, whose expression is restricted to platelets and megakaryocytes (Clemetson, 1999a, Jandrot-Perrus *et al.*, 2000). The peptide sequence is composed of 319 amino acids with a signal sequence of 20 amino acids

residues. The molecular weight of the peptidic core is about 34836 Da while the apparent molecular mass deduced from SDS gel electrophoresis is 62 kDa (reducing conditions), so about 45% of the molecular weight of GPVI is due to carbohydrate residues conjugated to the polypeptide chain.

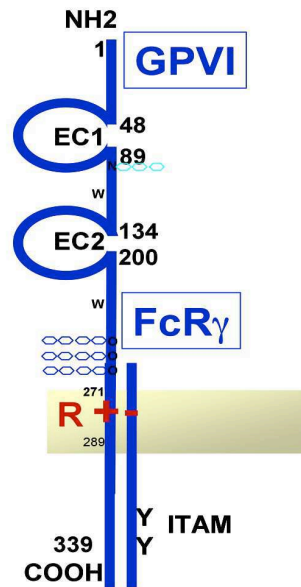
The GPVI chain is organized in three main domains from N to C-terminus: the N-terminal extracellular domain, a transmembrane domain and a cytoplasmic domain (Figures 29 and 30).

### **2.6.3.1 GPVI Extracellular Domain**

The extracellular domain contains 249 amino acids and is extensively glycosylated. At the the N-terminus, two immunoglobulin C2-like folds (D1 and D2) are assembled by two disulfide bridges linking Cysteines at position 48 and 88 for the D1-loop and 134 and 180 for the D2-loop, indicating that GPVI is a member of the paired immunoglobulin-like receptor family (Figure 29) (Clemetson *et al.*, 1999, Jandrot-Perrus *et al.*, 2000, Miura *et al.*, 2000). The most closely related molecules in the Swiss Prot database are the Fc $\alpha$  receptor and natural killer receptor class. As an example, the immunoglobulin-like domain of GPVI showed a 40% sequence homology with that of the killer cell inhibitory receptor cL-42.

A putative glycosylation site for the N-linked carbohydrate chain is identified at Asn72, while many O-linked carbohydrate chains are conjugated to a Ser/Thr-rich region proximal to the membrane. This heavily O-glycosylated mucin-like rigid stalk is predicted to push the collagen binding domain away from the membrane (Moroi and Jung, 2004) and accounts for the difference between the theoretical molecular weight (37 kDa) determined by the primary sequence and the apparent mass (60 kDa).

Homologies with NK receptors suggested that D loops contained the collagen-binding domain. Several approaches have been used to identify ligand binding sites including point mutations and domain exchanges. These studies have been performed on soluble recombinant forms of GPVI. Smethurst *et al.* (2004) observed that human GPVI (hGPVI) bound more strongly to CRP than mouse GPVI (mGPVI). They observed key differences by sequence comparison and molecular modeling and produced corresponding mutants. They identified Lys59 within the D1 domain, as a key residue for binding of hGPVI to CRP (Smethurst *et al.*, 2004). Lecut *et al* used a monoclonal antibody that inhibits GPVI binding to collagen to screen a peptide



**Figure 29 : Schematic of the transmembrane structure of platelet GPVI.** The extracellular domain contains one N-glycosylation site and several sites of O-glycosylations. W: wsx motif. A: transmembrane arginine is essential for binding to the gamma chain (salt bridge), Ig-like D1 and D2 extracellular loops.

```

1         10        20         30
MSPSPALFC LGLCLGRVPA QSGPLPKPSL QALPSSLVPL EKPVTLCRQG

40        50        60        70        80
PPGVDLYRLE KLSSSRVQDQ AVLFIPAMKR SLAGRYRCSY QNGSLWSLPS
          Domaine IgC2-1 (EC1)

90        100       110       120       130
DQLELVATGV FAKPSLSAQP GPAVSSGGDV TLQ CQTRYGF DQFALYKEGD

140       150       160       170       180
PAPYKNPERW YRASFPITV TAAHSGTYRC YSFSSRDPYL WSAPSDPLEL
          Domaine IgC2-2 (EC2)

190       200       210       220       230
VVTGTSVTPS RLPTEPPSSV AEFSEATAEL TVSFTNKVFT TETSRSTITTS

240       250       260       270       280
PKESDSPAGP ARQYYTKGNL V RICLGAVIL IILAGFLAED WHSRRKRLRH
          Domaine transmembranaire

290       300       310       319
RGRAVQRPLP PLPLPQTRK SHGGQDGGRQ DVHSRGLCS

```

**Figure 30 : Peptide sequence of human GPVI.**

Bold: the two cysteines flanking Ig-like extracellular domains and transmembrane arginine. The residue numbering corresponds to that of (Horii *et al*, 2006).

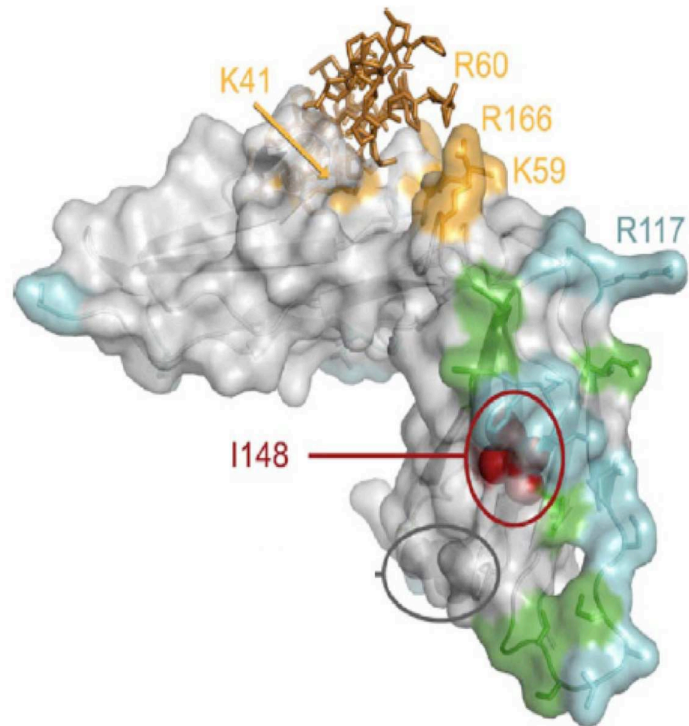
library. They identified Val34 and Leu 36 within D1 as residues important for binding to collagen and CRP (Lecut *et al.*, 2004a). Later, mutations of Arg 60 within D1 and Arg 166 within D2 were found to reduce GPVI binding to collagen but not to CRP (O'Connor *et al.*; 2006). N-linked glycosylation of Asn92 was proposed to contribute to GPVI-dependent cell adhesion to collagen (Kunicki *et al.*, 2005). Dumont *et al.* (2006) constructed chimeric proteins in which the Ig loops were successively or simultaneously exchanged by their Fc $\gamma$ RI homologues. They confirmed the importance of the D1 loop for GPVI binding to collagen and they unexpectedly observed that the D2 loop also contributes to optimal binding to collagen. Regarding GPVI binding to its different ligands, the binding sites of collagen, CRP and convulxin are partially overlapping. D1 and D2 domains contribute to collagen binding, whereas CRP binding is increasingly dependent on D1 domain. The D1 and D2 hinge region is particularly important for binding to convulxin (Dumont *et al.*, 2006).

#### 2.6.3.1.1 **Three-dimensional Structure of the GPVI Ectodomain**

Three dimensional structures of extracellular domain of GPVI have been modeled on the crystallographic structure of KIR as it was done for Fc $\alpha$ RI (Figure 31) (Smethurst *et al.*, 2004, Wines *et al.*, 2001). It was estimated that the two IgC2 domains were organized in  $\beta$ -sheeth, forming an angle of 60° to 90° to one another.

Horii and colleagues (Horii *et al.*, 2009) have crystalized the extra-cellular domain of GPVI and its interaction with CRP has been modeled. The GPVI crystalization results confirmed that the two IgC2 domains form an angle of 90°-92°. The D1 domain is composed of two  $\beta$ -sheeths, a short helix  $3_{10}$  and two polyproline helix. The D2 domain is composed of two  $\beta$ -sheeths. In this crystal, GPVI forms a dimer having a particular configuration: the two D2 domains of GPVI are positioned “back-to-back” and interact with each other to form a  $\beta$ -sheeth along the interface. This zone of contact between two D2, is stabilized by hydrophobic interactions and hydrogen bonds. This dimerization mode is similar to that observed for heterodimeric receptor CD3 $\epsilon\gamma$  and CD3 $\epsilon\delta$  complex of T-cells.

The GPVI structural data provided a framework for understanding the interaction between GPVI and collagen or CRP by allowing accurate mapping of mutagenesis results onto the surface of the GPVI dimer. The residues implicated in collagen or



**Figure 31 : Three dimensional structure of the GPVI extracellular domain.**

On the top left the D1 domain with a computational model of the triple helix of CRP docked in the proposed collagen binding site; on the lower right part, the D2 domain with in red a potential site for homotypic interactions. Taken from {O'Connor, 2006}.



CRP binding fall into two clusters: the primary region includes basic residues on the surface of D1 including K41, K59, R60, and R166. A second cluster of residues implicated in collagen or CRP binding is found at the distal end of D1; these residues include L36, implicated in collagen binding, and both V34 and the N-glycan attached to N72, both of which are involved in collagen and CRP binding (Horii *et al.*, 2009). In the GPVI dimer the two collagen binding sites are nearly parallel and at a distance of 55Å°, which may be compatible with the distance between GPO motif determined after dimension and configuration of a collagen fibre. Interestingly, the use of soluble recombinant GPVI either dimeric (GPVI-Fc) or monomeric (GPVI-His) has evidenced that only GPVI dimers bind to GPVI with a good affinity (Miura *et al.*, 2002). It was thus very important to determine whether GPVI is expressed as a monomer or as a dimer on resting platelets since the second form will confer a greater reactivity towards collagen than the first. Studies conducted in the laboratory are in favour of a mechanism maintaining GPVI in a monomeric little active form (Loyau and Jandrot-Perrus personal communication).

### **2.6.3.2 Transmembrane Domain and Complex with FcR $\gamma$**

The transmembrane domain of GPVI is composed of 19 amino acids (Clemetson, 1999b, Jandrot-Perrus *et al.*, 2000). It is characterized by the presence of a charged residue Arg 272, which allows it to undergo a non-covalent association with the Fc receptor-gamma (FcR $\gamma$ ) chain (Tsuji *et al.*, 1997, Watson and Gibbins, 1998). Arg272 forms a salt bridge with the aspartic acid (11) of the FcR $\gamma$ -chain transmembrane domain. FcR  $\gamma$ -chain is a covalently linked 20 kDa homodimer with each chain containing one copy of an ITAM defined by the presence of two YxxL sequences separated by seven amino acids (Feng *et al.*, 2005).

Thus, GPVI is constitutively expressed on the platelet surface as noncovalent complex with FcR $\gamma$  and this interaction plays an important role in GPVI expression and function. The association of GPVI with FcR $\gamma$ -chain has a functional prerequisite role in signaling (Berlanga *et al.*, 2002, Zheng *et al.*, 2001).

In mice, the expression of FcR $\gamma$  determines the expression of GPVI at the membrane: the platelets of mice deficient in FcR $\gamma$  does not express GPVI on their surface (Nieswandt *et al.*, 2000, Poole *et al.*, 1997). The association GPVI-FcR $\gamma$  appears therefore critical for the expression of platelet complex. Kato and colleagues

have demonstrated that the converse was not true, since mice deficient in GPVI expressed FcR $\gamma$  on the surface of their platelets (Kato *et al.*, 2003).

FcR $\gamma$  is expressed in early hematopoiesis, from the progenitor stage, and prior to GPVI (Lagrue-Lak-Hal *et al.*, 2001). FcR $\gamma$  have other partners to which it may associate in the platelets like GPIb-V-IX complex (Falati *et al.*, 1999) and Fc $\epsilon$ RI (Joseph *et al.*, 1997, Wines *et al.*, 2006). These observations are in favour of an independent expression of FcR $\gamma$ .

No FcR $\gamma$  deficiency has been so far reported in human beings but several cases of GPVI deficiency have been reported with in some cases an apparent decreased expression of FcR $\gamma$  (Tsuji *et al.*, 1997). However, this observation is not systematic and decreased FcR $\gamma$  expression could be related to the processing of the receptor triggered by antibody-induced internalisation. Interestingly, the expression of GPVI on the surface of CHO cells that do not express FcR $\gamma$  was observed after transfection of cells with vectors coding for human GPVI (Jandrot-Perrus *et al.*, 2000). Indeed, recombinant GPVI can be expressed on the surface of hematopoietic cells constitutively expressing FcR $\gamma$  (Jandrot-Perrus *et al.*, 2000, Jandrot-Perrus *et al.*, 1999, Lecut *et al.*, 2003) and, mutants of GPVI deficient for the capacity to associate with FcR $\gamma$  were also found to be expressed at the cell surface (Zheng *et al.*, 2001). These observations suggest that this is not the association of both proteins in the cytoplasm that directs expression of the complex.

### **2.6.3.3 Cytoplasmic Domain**

The cytoplasmic region/tail of human GPVI contains 51 amino acids having no apparent homology with other receptor proteins and contains no Tyr residues. The cytoplasmic region of mouse GPVI contains only 27 residues and the region corresponding to 24 residues from the C-terminal sequence of human GPVI is absent (Figure 32) (Jandrot-Perrus *et al.*, 2000). Since GPVI triggered responses are globally similar in human and mouse platelets, the C-terminal region is not critical for GPVI function but it could drive differences in the efficiency of signaling in mice versus human platelets. The remaining portion of the cytoplasmic region shows a strong similarity between human and mouse GPVI. As compared to other members of the immunoreceptors family, the cytoplasmic region of GPVI has two unique functional

motifs. One sequence is a basic amino acid-rich region close to the transmembrane domain, which reinforces the interaction with FcR $\gamma$  and also presents a calmodulin binding domain (Andrews *et al.*, 2002). The interaction with calmodulin is assumed to protect GPVI from the cleavage of its ectodomain by metalloproteases. The calmodulin inhibitor W7 blocks the interaction of calmodulin with the peptidic motif of GPVI and triggers the proteolytic loss of GPVI from the platelet surface. These findings suggest that disruption of calmodulin binding to GPVI cytoplasmic tail by agonist binding to the receptor triggers metalloproteinase-mediated loss of GPVI from the platelet surface (Gardiner *et al.*, 2004).

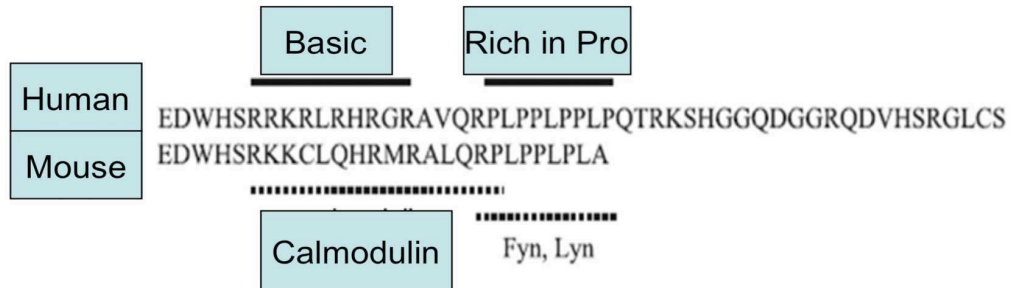
GPVI is unique relatively to other ITAM receptors in possessing a conserved proline rich domain (PxxP, PRD) within the cytoplasmic region. This motif is a docking site for the Src homology 3 (SH3) domain of the Src family tyrosine kinases Fyn and Lyn (Suzuki-Inoue *et al.*, 2002). This interaction confers to the receptor a 'ready-to-go' state. In platelets, Lyn has been shown to bind selectively and in an active conformation to the proline rich domain of GPVI and is needed for rapid cell adhesion to collagen at high shear rate but not in the absence of shear (Feng *et al.*, 2005, Schmaier *et al.*, 2009).

An unpaired Cys residue present in the cytoplasmic tail of mouse GPVI is assumed to contribute to the rapid formation of covalent GPVI dimers formed after platelet treatment with GPVI agonists (Arthur *et al.*, 2007b). However, no unpaired Cys residue is present in the human cytoplasmic tail. It is thus possible that the mechanism leading to the formation of covalent dimers of GPVI on the platelet surface, is different in human and mice

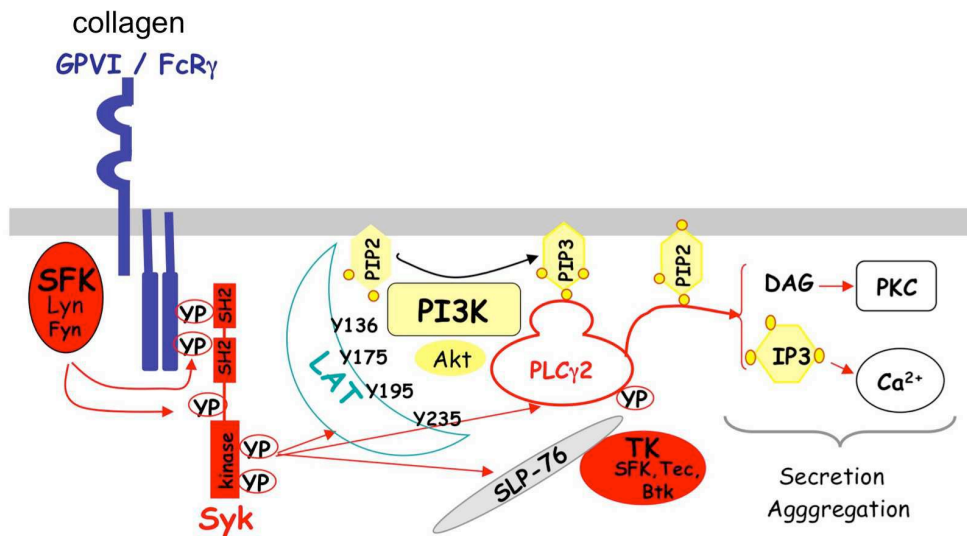
#### **2.6.4 GPVI Coupled Signaling Pathway**

The signaling pathway coupled to the GPVI-FcR $\gamma$  complex is globally similar to that of other immunoreceptors (for a review see (Watson *et al.*, 2001) and consists of a cascade of tyrosine phosphorylation (Figure 33).

In resting platelets the C-terminus of the  $\gamma$  chain dimer is assumed to be tethered within the inner layer of the membrane and its ITAM domains are protected from phosphorylation by Lyn/Fyn constitutively bound to the Pro rich domain of GPVI and already in a preactivated conformation (Quek *et al.*, 2000, Schmaier *et al.*, 2009).



**Figure 32: Cytoplasmic domain of human and murine GPVI.**  
 Basic domains and rich in Proline as well as the binding sites of calmodulin and tyrosine kinases  
 Fyn and Lyn are shown. Adapted from Moroi, 2004



**Figure 33 : A schematic representation for the signaling pathway of human GPVI.**

The bridging of several GPVI-FcR $\gamma$  complexes by the GPVI multivalent ligands is thought to unmask the C-terminus of the  $\gamma$  chain, to trigger interaction between cytoplasmic domains (Sigalov, 2008). This causes ITAM phosphorylation mediated by Src family kinases, Lyn (and Fyn) that occurs in cholesterol-rich membrane domains known as lipid rafts. GPVI is partially or completely excluded from lipid rafts in resting platelets, but translocates to these domains upon ligand engagement see Watson review (Watson, 1999).

Then, the tyrosine kinase Syk (spleen tyrosine kinase) is recruited via its SH2 domain to the phosphorylated ITAMs and is activated by tyrosine phosphorylation. Syk initiates a cascade of tyrosine phosphorylation resulting in the assembly of a large signaling complex consisting of adapter proteins including LAT (Linker for Activation T-cells), Gads and SLP-76 (SH2-domain containing of Leukocyte-specific Phosphoprotein 76 kDa), tyrosine kinases of the Src family (Fyn and Lyn) and Bruton Family (Bruton's kinase or Btk and Tec), the small G protein Rac1, the GTP exchange factors Vav1 and Vav3, the ubiquitinating protein, c-Cbl, and two metabolic enzymes of phosphoinositide (PI): PI3-kinase a and b isoform (PI3K) and phospholipase C (PLC $\gamma$ 2) (Moroi and Jung, 2004, Nieswandt and Watson, 2003, Watson *et al.*, 2010).

Once phosphorylated by Syk, LAT is associated with (i) PI3K, which catalyzes the formation of PIP3 by phosphorylation of PIP, and (ii) the PLC $\gamma$ 2 allowing its recruitment to the membrane via the interaction of the PH domain of the enzyme with PIP3. Activation of PLC $\gamma$ 2 is regulated by tyrosine phosphorylation by kinases Lyn and Btk, the latter also being recruited to the membrane via PIP3. Associated with LAT, SLP-76 plays an important role in the activation of PLC $\gamma$ 2. These numerous proteins play qualitatively and quantitatively distinct roles in activation of PLC $\gamma$ 2. Studies in mouse platelets have shown that Syk and SLP-76 are essential for activation of the phospholipase and that functional redundancy exists between several of the other proteins, including Btk and Tec, Vav1 and Vav3, and PI 3-kinase a and b.

Activation of PLC $\gamma$ 2 leads to the cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP2) to inositol-(1,4,5) trisphosphate (IP3) and diacylglycerol (DAG). IP3 binding to its receptor on sarcoplasmic membrane, causes the release of Ca<sup>2+</sup> from cytoplasmic storage pools. The activation of protein kinase C (PKC) by DAG and the elevation of intracellular Ca<sup>2+</sup> causes platelet cytoskeletal reorganization: and shape change of platelets, formation of pseudopodia and filopodia, and the release

contents of secretory granules. CalDAG-GEFI has recently been shown to play a central role in Ca<sup>2+</sup>-dependent integrin activation, TxA<sub>2</sub> generation and granule release and to be preferentially activated over PKC downstream of the GPVI (Stefanini and Bergmeier, 2010). Finally, these signals also lead to activation of integrin  $\alpha 2\beta 3$  and platelet aggregation (Moroi and Jung, 2004).

## 2.6.5 GPVI Down-regulation

### 2.6.5.1 *Mechanisms Leading to GPVI Down-regulation*

The level of GPVI expression at the platelet surface is also regulated by external events. Down-regulation of GPVI from the platelet surface can occur through internalization or metalloproteinase-dependent ectodomain shedding (Figure 34). Signaling induced by activation of GPVI by collagen, CRP or convulxin, leads to the proteolytic activation process and cleavage of the extracellular domain of GPVI. The extracellular domain of GPVI is cleaved by metalloproteinases (MMP). A soluble form of GPVI (55 kDa) and a 10 kDa remnant are produced (Gardiner *et al.*, 2004, Stephens *et al.*, 2005). *In vitro*, GPVI shedding is induced by ligands of GPVI including anti-GPVI antibodies, during platelets mitochondrial alterations associated with aging or in the presence of the calmodulin inhibitor W7, which blocks the association between calmodulin and GPVI. The shedding of GPVI is more effective when platelets are activated by GPVI agonists compared to other agonists such as thrombin. The cleavage is not a specific process of GPVI and allows the regulation of various platelet receptors, including GPIIb $\alpha$  whose shedding is mediated by ADAM 17 (Berndt *et al.*, 2007).

The process of cleavage or "shedding" is metalloproteinase dependent (Gardiner *et al.*, 2007, Rabie *et al.*, 2007). Platelets abundantly express sheddases of the a disintegrin and metalloproteinase (ADAM) family, most notably ADAM17 (also referred to as the tumor necrosis factor– converting enzyme, TACE) and ADAM10. Studies on GPVI-based synthetic peptides indicated a role for ADAM10 in GPVI cleavage, but direct evidence for this has been lacking. The analysis of mice lacking functional ADAM17 revealed that this enzyme mediates the constitutive and agonist-induced shedding of GPIIb and is required for the agonist-induced shedding of GPVI. Studies conducted in mice lacking both ADAM10/ADAM17 in their platelets demonstrate that GPVI cleavage *in vitro* can occur independently through either

ADAM10 or ADAM17 in response to distinct stimuli, however, of a third GPVI cleaving platelet enzyme is proposed to also trigger GPVI shedding *in vivo* (Bender et al., 2010).

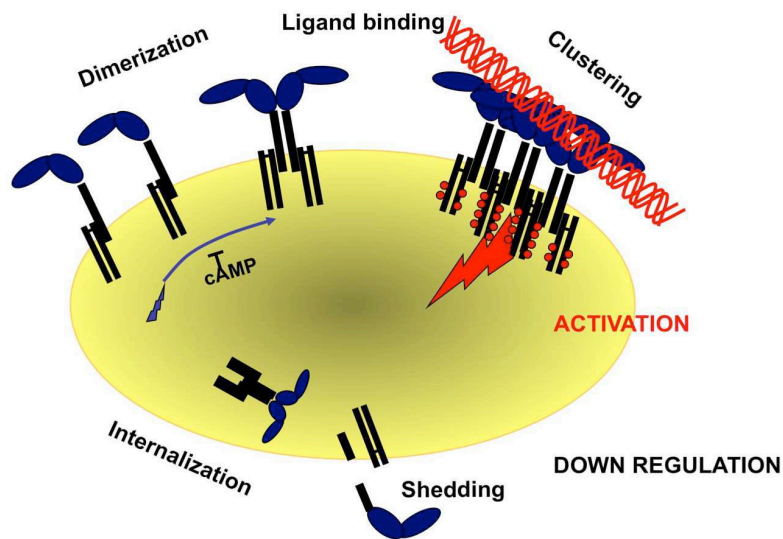
The shedding requires prior activation of the signaling pathway of GPVI and can be blocked by inhibitors directed against signaling proteins, such as antagonists of Src family kinases (PP1 and PP2), of PI3K (wortmannin) or of Syk (piceatannol).

#### 2.6.5.2 **Antibody-induced GPVI Down-regulation**

Antibody binding to GPVI is capable of inducing the internalization and/or cleavage (shedding) of GPVI (Figure 35). The injection of the rat anti-mouse GPVI monoclonal antibodies induces a transient thrombocytopenia and a prolonged GPVI depletion, the most documented being JAQ-1. But, JAQ1 does not modify GPVI expression *in vitro* (Nieswandt *et al.*, 2001a, Schulte *et al.*, 2006). In contrast, some monoclonal antibodies to human GPVI are capable to trigger the cleavage of the GPVI ectodomain and this is the case of the 9O12.2 antibody (Stephens *et al.*, 2005, Takayama *et al.*, 2008). The injection of a monoclonal antibody to human GPVI into mice immunodepleted for their platelets and reconstituted with human platelets also resulted in the shedding of GPVI (Boylan *et al.*, 2006). Internalization and cleavage of GPVI require both the functionality of signaling pathways. Indeed, a single mutation of tyrosine within ITAM domain of FcR $\gamma$  abolished the internalization induced *in vivo* by antibodies and shedding of GPVI. The deficiency of LAT and PLC $\gamma$  in mice can inhibit the shedding of GPVI which are internalized irreversibly (Rabie *et al.*, 2007).

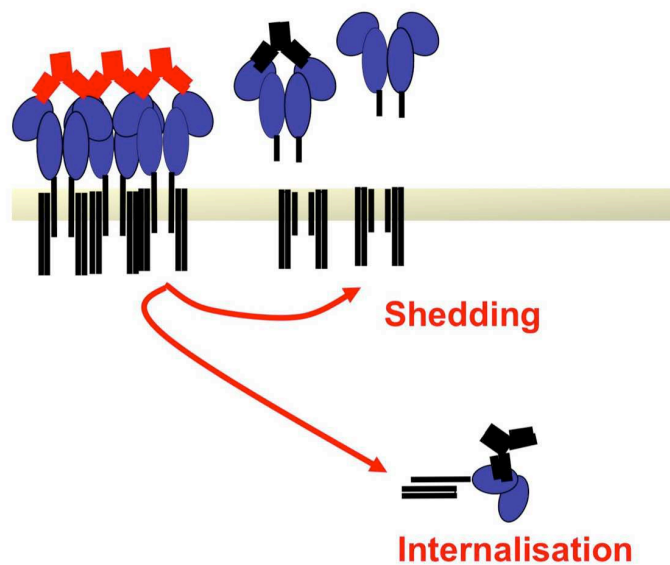
Antibody induced GPVI depletion is assumed to be responsible for the GPVI deficiency observed in some patients with immune thrombocytopenia (Boylan *et al.*, 2004, Moroi *et al.*, 1989). Furthermore, isolated patient's IgG are in some cases activating antibodies and keep the capacity to trigger the cleavage of the ectodomain *in vitro* (Nurden *et al.*, 1999) and to finally, a down-regulation of GPVI can also be observed after administration of either anti-GPVI Fab in mice (Schulte *et al.*, 2003) or human platelets treated with Fab fragments of immunodeficient mice (Boylan *et al.*, 2006).

Interestingly, the endocytosis of GPVI-antibody complex by a cAMP dependent mechanism has been shown for non-activator monoclonal mF1232 antibody, whose



**Figure 34: GPVI signaling leading to its activation and downregulation**

**Activation-dependent immunodepletion**



**Figure. 35 : Activation-dependent immunodepletion of GPVI.**  
 The mechanism of GPVI loss involves the cleavage of its extracellular portion by a metalloprotease and/or internalization of the immune complex.



epitope is situated on D2 domain of GPVI (Takayama *et al.*, 2008). The injection of chimeric h/m F1232 antibody (cF1232) to monkeys showed depletion of GPVI on the surface without augmentation of soluble GPVI in the plasma, which indicates the internalization phenomenon of the antibody receptor complex. In contrast, in the same study, another antibody directed to the D1 domain lead to shedding and internalization of the antibody-receptor complex. It was thus hypothesized that the shedding of the receptor is carried out by anti-GPVI antibody whose epitope is situated on D1 domain, while internalization is common for the antibody against D1 or D2 domains.

The precise mechanisms of internalization/shedding of GPVI remain to be characterized as a function of the antibody in question, its concentration, the active capacity or not.

### **2.6.6 GPVI Deficiencies**

About 11 different cases has been reported having GPVI-related defects (Arthur *et al.*, 2007a).

GPVI-related defects are of the following types:

#### ***2.6.6.1 Acquired Deficiency***

In this case the patients are GPVI-deficient due to anti-GPVI autoantibodies. In human antibody induced GPVI deficiency and the first case of GPVI deficiency was reported in a japani patient since then several cases have been registered/reported. All the reported cases with GPVI deficiencies induced by autoantibodies have mild bleeding tendency and platelet have severly impaired or no response to collagen. (Boylan *et al.*, 2004, Moroi *et al.*, 1989, Sugiyama *et al.*, 1993, Sugiyama *et al.*, 1987, Takahashi and Moroi, 2001, Tsuji *et al.*, 1997) or other causes (Bellucci *et al.*, 2005). These antibodies causes GPVI deficiencies either by activation-dependent or activation-independent immunodepletion (Gardiner *et al.*, 2008, Nurden, 2009, Takayama *et al.*, 2008). For some the antibody could have been purified and showed to induce GPVI cleavage (Nurden, 2009). Whether the occurance of anti-GPVI antibodies could be related to GPVI polymorphism is not known, generally not associated with bleeding.

#### **2.6.6.2 Congenital Deficiencies**

Only recently has the absence of collagen-induced platelet activation been linked to mutations in the GP6 gene. In France, a young girl with a lifelong mild bleeding syndrome associates an Arg38Cys mutation in exon 3 of one allele with an insertion of 5 nucleotides in exon 4 of the second allele (leading to a premature stop codon and mRNA instability); the result was low expression of nonfunctional GPVI. In Belgium, a patient with strongly reduced platelet expression of non-functional GPVI has a combination of an out-of-frame 16-bp deletion and a Ser175Asn missense mutation. (Dumont *et al.*, 2009, Hermans *et al.*, 2009).

#### **2.6.6.3 Signaling Deficiencies**

Analysis of the GPVI patient profiles reveals a link with immune dysfunction. In addition to patients diagnosed with immune thrombocytopenic purpura and anti-GPVI IgG, in some cases the selective GPVI defect occurred in the presence of normal GPVI levels and anti-GPVI antibodies were absent. Defective GPVI function due to signaling abnormality have been identified in patients diagnosed with myelodysplasia, chronic lymphocytic leukemia (Bellucci *et al.*, 2005), and appeared in these cases as acquired defects. In one patient presenting prominent immune (Sjogren's syndrome) a congenital GPVI-related signaling defect has been reported (Dunkley *et al.*, 2007).

Two general observations can be made on the basis of different types of GPVI defects and co-associated diseases in the reported cases first, the characteristics of bleeding disorders (including epistaxis, recurrent purpura, subcutaneous and gingival bleeding, menorrhagia, bleeding postpartum or post-surgery/trauma and how these conditions vary from individual to individual through out life. Second, while the specific effects of GPVI defects on platelet function may in part be masked on a clinical background of other pathology, what is very striking is how GPVI defects occur predominantly in females (90%) and how GPVI defects are commonly associated with other immunological disorders (Arthur *et al.*, 2007a).

#### **2.6.6.4 Mouse Models of GPVI Deficiencies**

The first description of a GPVI deficiency in mice was obtained by gene invalidation of FcR $\gamma$  since GPVI is not expressed on platelets in the absence of the gamma chain. However one limitation to the interpretation of the data in these mice comes from the fact the FcR $\gamma$  deficiency could have GPVI-independent functional

consequences impairing the interpretation of the data obtained *in vivo* in some models (Berlanga *et al.*, 2002).

Gene invalidation of GPVI allowed obtaining a pure GPVI deficiency (Kato *et al.*, 2003) without abnormality in platelet count and size.

Alternatively, immunological depletion of GPVI is induced by one bolus injection of the monoclonal antibody JAQ1 by an internalization/shedding process. The depletion is long lasting and accompanied by a transient thrombocytopenia (Nieswandt *et al.*, 2001b). Although it is largely used this model has the inconvenient to require an exogenous IgG.

In all cases, GPVI deficiency is accompanied by a major defect of platelet response to collagen *in vitro* (no aggregation in response to collagen and CRP, the prevalence of primary adhesion but no thrombus formation in whole blood and flow condition).

#### **2.6.6.5 GPVI Expression in Cardiovascular Diseases**

Given the important role of GPVI in platelet thrombus formation, several studies have been focused on whether variations of GP6 might be associated with an increased risk of thrombotic disease, such as myocardial infarction (MI). Considering the common GPVI polymorphisms (SKTQH/PEALN) a low GPVI density has been reported on the platelets of subjects homozygous for the low frequency b allele (Best *et al.*, 2003). Functionally, 'bb' platelets had reduced thrombogenicity on collagen (Joutsu-Korhonen *et al.*, 2003). Based on these results, individuals homozygous for the low-frequency allele could be more susceptible to bleeding than thrombotic events. On the other hand, one group reported that platelet activation increases surface exposure of GPVI as indicated by the increased in the mean fluorescence of platelets in flow cytometry using an anti-GPVI antibody. The authors proposed that the level of GPVI expression could be related to the risk of thrombosis. The same group reported that patients with ACS showed a significantly enhanced GPVI expression compared with patients with stable angina and healthy controls. The expression of GPVI correlated well with CD62P. Elevated platelet GPVI expression was associated with ACS independent of markers of myocardial necrosis (Bigalke *et al.*, 2007). Fc receptors expression correlated with GPVI expression was also found significantly increased in type 2 diabetics patients (Cabeza *et al.*, 2004). Platelet GPVI surface expression was also found significantly enhanced in patients with transient ischemic

attack and stroke compared to patients with MI events (Bigalke *et al.*, 2010).

Therefore, the determination of platelet surface GPVI expression could be of value to identify patients with an abnormal GPVI expression, either low with an increased risk of bleeding, or high with a risk of arterial thrombosis. GPVI expression at the platelet surface is most often analysed by flow cytometry but there are no commercially available antibody. In another approach, methods to measure soluble GPVI in plasma have been developed in different laboratories but their usefulness in diagnosis is not completely established.

### **2.6.7 GPVI in Haemostasis and Thrombosis**

GPVI deficiency in humans is associated from a low to mild bleeding tendency. This is in contrast with the severe bleeding episodes in patients with Von Willebrand disease or with Glanzmann thrombasthenia (deficiency in  $\alpha\text{IIb}\beta\text{3}$ ). Similarly, the absence of GPVI has no serious hemorrhagic impact in mice and longer bleeding time is observed sporadically. The genetic background is probably important and may enhance the bleeding tendency. GPVI thus appears not to be essential for physiological haemostasis, the initial adhesion phase facilitated by vasoconstriction being ensured by the vWF/GPIb axis (Konstantinides *et al.*, 2006). However, when associated to a treatment by antiplatelet agents, a GPVI deficiency could increase the risk of bleeding as it was observed in GPVI-immunodepleted mice treated by aspirin (Gruner *et al.*, 2004).

Most of the informations concerning the importance of GPVI in thrombosis come from animal models. The first evidence was provided by Nieswandt and colleagues who observed that after one injection of the antibody JAQ1, platelets were depleted in GPVI and mice were protected in a severe model of thromboembolism (Nieswandt *et al.*, 2001a). Still using JAQ1-induced GPVI depletion, the same group demonstrated by intravital fluorescence microscopy of the mouse carotid artery that inhibition or absence of the major platelet collagen receptor, GPVI, abolishes platelet–vessel wall interactions after endothelial denudation. These findings were found in a carotid ligation arterial model, in ferric chloride induced carotid artery thrombosis model and in wire –induced endothelial denudation of the carotid (Massberg *et al.*, 2003). The injection of soluble recombinant GPVI (GPVI-Fc) to mice was further reported to

inhibit thrombosis in a carotid artery ligation model (Massberg *et al.*, 2004) but this was not confirmed by others (Gruner *et al.*, 2005). Furthermore, in a model of coronary ischemia using wild type mice and FcR $\gamma$ <sup>-/-</sup> mice that lack GPVI, the infarct size was significantly smaller in FcR $\gamma$ <sup>-/-</sup> mice subjected to occlusion and reperfusion of the coronary artery than in control FcR $\gamma$ <sup>+/+</sup> mice (Takaya *et al.*, 2005).

Then emerged a controversy on the real role of GPVI in thrombosis. This was related to the appearance of new models of arterial thrombosis and in particular laser induced thrombosis in platelet activation by thrombin appeared more important than platelet activation by GPVI-collagen (Dubois *et al.*, 2006, Mangin *et al.*, 2006). The relevance of the models used has confirmed the complementary roles of GPVI-collagen and thrombin in models of arterial thrombosis on healthy vessels (Hechler *et al.*, 2010) and most recently in a forceps induced model GPVI still appeared to be important (Bender *et al.*, 2011). A common weakness of all these models is that they consider thrombosis in healthy vessels that is indeed completely different from arterial thrombosis in humans occurring in diseased atherosclerotic arteries.

Importantly, *ex vivo* thrombosis models using flow chambers have brought new evidences in favour of an important role of GPVI in atherothrombosis. When anticoagulated whole blood is perfused at an arterial shear rate over a surface of collagen, GPVI deficiency or inhibition blocks thrombus growth. Similarly, when extracts from atherosclerotic plaques were used in these devices in place of collagen, blocking GPVI consistently limited thrombus development (Cosemans *et al.*, 2005, Penz *et al.*, 2005). Type I and III collagens accumulated within the atherosclerotic plaque revealed to be highly thrombogenic. Furthermore, it is well established that collagen-GPVI is the most potent activator of platelet procoagulant activity and blocking GPVI very efficiently delays and limits thrombin generation (Lecut *et al.*, 2004a, Lecut *et al.*, 2003, Lecut *et al.*, 2004b).

GPVI deficiency was further shown to prevent thrombus formation *in vivo* on injured plaques in ApoE<sup>-/-</sup> mice (Hechler and Gachet, 2011). *In vivo*, soluble recombinant GPVI was shown to accumulate within atherosclerotic plaques in rabbit and to protect ApoE<sup>-/-</sup> mice from arterial remodelling after mechanical injury (Bultmann *et al.*, 2010). Together, these observations bring solid evidence, that GPVI plays an important role in atherothrombosis.

### **2.6.8 GPVI and Inflammation**

The capacity of GPVI-collagen activated platelets to recruit leukocytes was first reported by Konishi and colleagues (Konishi *et al.*, 2002). In a model of wire induced injury of the femoral artery, these authors observed marked decreases in platelet adhesion and neutrophil attachment to the vascular wall surface in FcR $\gamma$ -knockout mice compared with wild-type mice. On the 7th day after injury, fewer neutrophils were detected and at 28 days after injury, the neointima was significantly smaller in FcR $\gamma$ -knockout mice than in wild-type mice. These data suggested that GPVI-collagen interaction triggers efficiently the recruitment of inflammatory cells by platelets.

The immunoglobulin-like glycoprotein extracellular matrix metalloproteinase induced EMMPRIN (CD147) is upregulated on monocytes of patients with acute myocardial infarction. Interestingly, EMMPRIN has recently been recognized as counter receptor for EMMPRIN and the interaction of EMMPRIN with GPVI supports platelet monocyte interaction and promotes monocyte recruitment to the arterial wall (Schulz *et al.*, 2011).

GPVI also appears to contribute to platelet-dependent amplification of inflammation outside of the thrombosis field. Using pharmacological and genetical approaches, Boilard and colleagues very recently identified that GPVI is involved in the progression of inflammatory arthritis (Boilard *et al.*, 2010). Collagen induced-platelet activation leads to the production of microparticles in inflammatory joint diseases and GPVI deficient mice exhibited a marked reduction in arthritis.

In another model of inflammation, antibody induced glomerulonephritis in mice, Devi and colleagues also observed very rapid platelet recruitment to the glomeruli that was prevented by the absence of GPVI and triggered P-selectin mediated neutrophils recruitment (Devi *et al.*, 2010).

### **2.6.9 Strategies for Therapeutic Targeting of GPVI**

The current antiplatelets have several drawbacks: such as they intervene on a late phase of the platelet aggregation process when thrombus formation is already well initiated; they may induce serious secondary effects and they may not be as effective

as suited leading to their association with increased risks. There is not yet commercialized anti-thrombotic drug in market which can interrupt the initial phase of thrombosis, i.e the adhesion leading to activation of platelets

GPVI is generally accepted as a promising target for the treatment of arterial thrombosis. Targeting GPVI would have the following advantages: (i) GPVI expression is restricted to megakaryocytes and platelets (Lagrue-Lak-Hal *et al.*, 2001) so GPVI antagonists may have limited side effects (ii) blocking GPVI permits the coverage of the lesion by a single layer of platelets but prevents activation of these platelets, release of soluble mediators and thrombus growth; (iii) GPVI plays a central role in collagen-induced exposure of procoagulant phospholipids at the platelet surface and catalyzes thrombin generation (Heemskerk *et al.*, 1999). So blockage of thrombin generation through anti-GPVI antagonist would further limit thrombus growth; (iv) GPVI deficiency usually results in a mild bleeding phenotype (Arthur *et al.*, 2007a), thus blocking GPVI would respect normal haemostatic processes.

Several groups are on the way of conducting preliminary research targeting GPVI with different strategies (FigureS 36 and 37).

#### **2.6.9.1 GPVI Mimics**

An agent that interferes with GPVI/collagen interactions might be soluble GPVI acting as a competitive inhibitor of collagen-platelet interactions at the sites of injury. In this approach a soluble form of recombinant GPVI could be used to compete with blood platelet GPVI for collagen binding. Jandrot-Perrus (2000) laid down the foundation stone for this strategy by constructing recombinant GPVI in the form of a fusion protein, coupling the extracellular domain of human GPVI with the Fc domain of a human immunoglobulin, GPVI-Fc (Figure 38) (Jandrot-Perrus *et al.*, 2000). This protein (immunoadhesin) being dimeric, it has a high affinity for collagen. When preincubated with collagen, it prevented platelet activation but its efficacy was low in absence of preincubation.

Massberg *et al.* (2004) reported that GPVI-Fc reduced platelet adhesion and aggregation at the injured carotid artery of mice, indicating that soluble GPVI-dimer might confer similar antithrombotic protection as direct anti-GPVI treatment (Massberg *et al.*, 2004). In contrast the team of Nieswandt observed little effect of GPVI-Fc on platelet adhesion and thrombus formation at the injured arterial wall

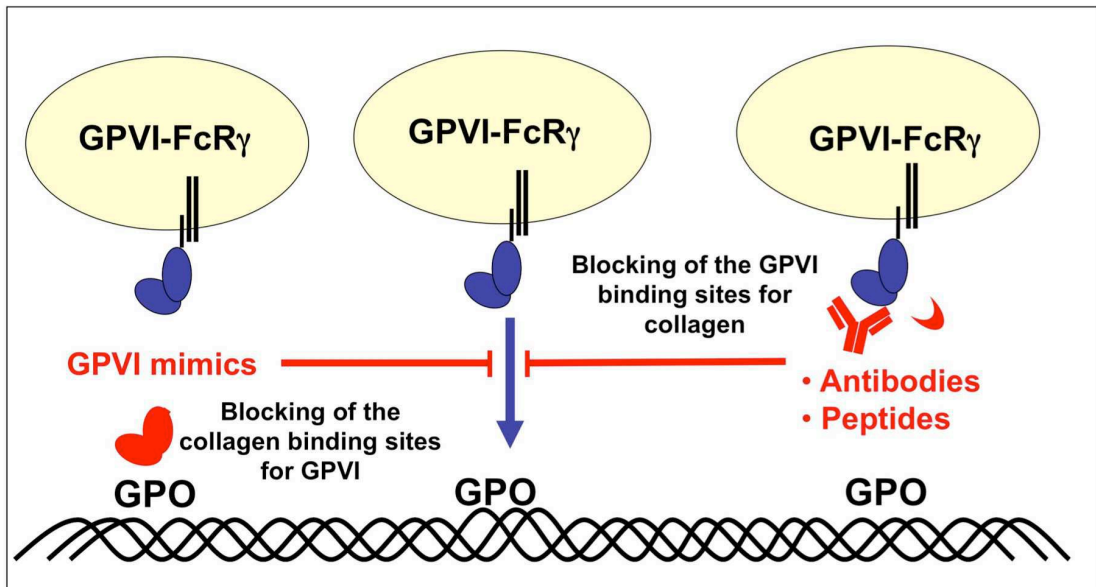


Figure 36: Different approaches to block GPVI binding to collagen by using different antagonists.

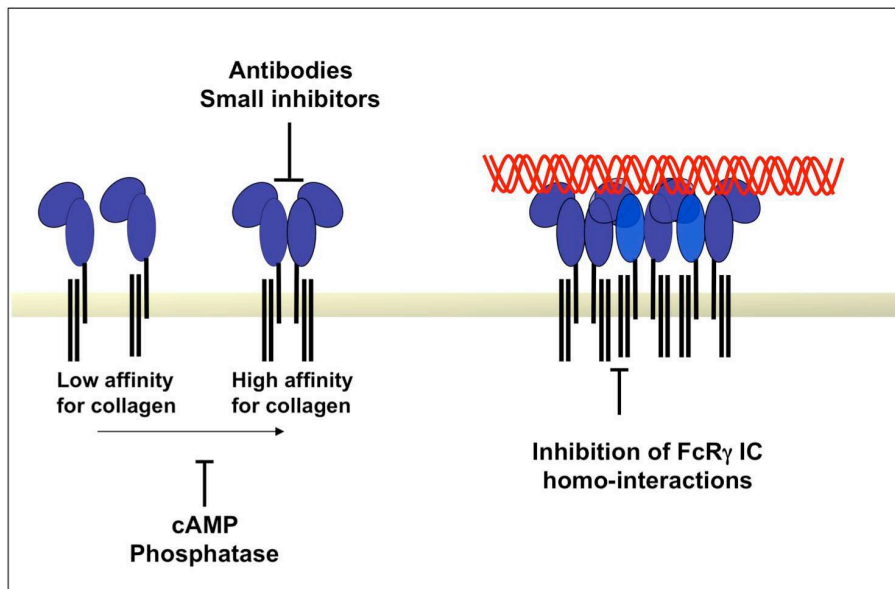
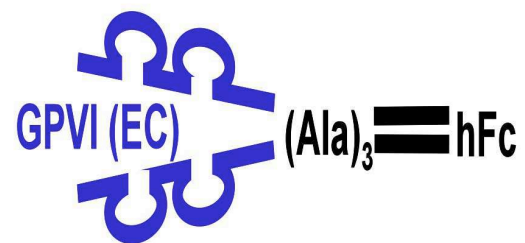


Figure 37: Blocking of GPVI dimerization (left) or oligomerization (right) either by increasing intracellular cAMP or inhibiting phosphatase or FcR $\gamma$  homo-interaction.





**Figure 38: Schematic representation of the fusion protein combining the extracellular domain of GPVI and the Fc domain of human Ig (Fc-GPVI) via a Alanine bridge.**

whereas anti-GPVI antibodies profoundly inhibited these processes (Gruner *et al.*, 2005).

Recently, an injectable form of GPVI-Fc (Revacept) was shown to inhibit lesion-directed thrombus formation with out any risk of bleeding complications in animals and a clinical phase 1 study demonstrated promising results regarding safety, tolerability, pharmacokinetics and pharmacodynamics aspects (Ungerer *et al.*, 2011).

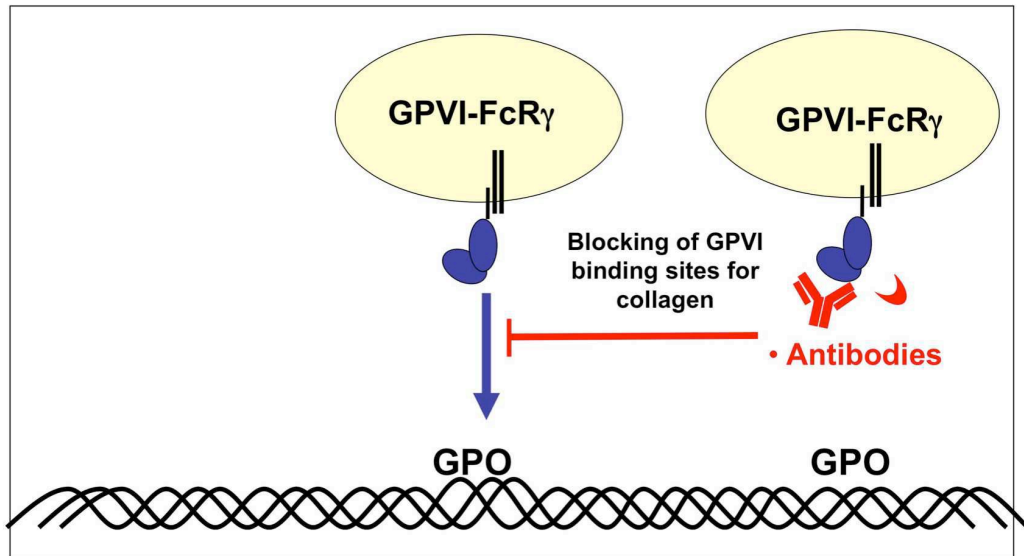
This approach has major drawbacks due to structural aspect (high molecular weight, sensitivity to proteases), functional (risk of non-specific effects related to the presence of Fc domain) and pharmacological (difficulty of estimating the dose necessary to saturate the collagen exposed by vascular injury). In addition, it is not excluded that the fusion proteins presents new epitopes that could induce an immune response.

Another approach would be to develop low molecular weight GPVI mimics. At present one peptidomimetic of GPVI has been reported. It binds to collagen but its affinity is too low to allow a competition with GPVI. However it is being developed as an imaging tool of collagen (Muzard *et al.*, 2009).

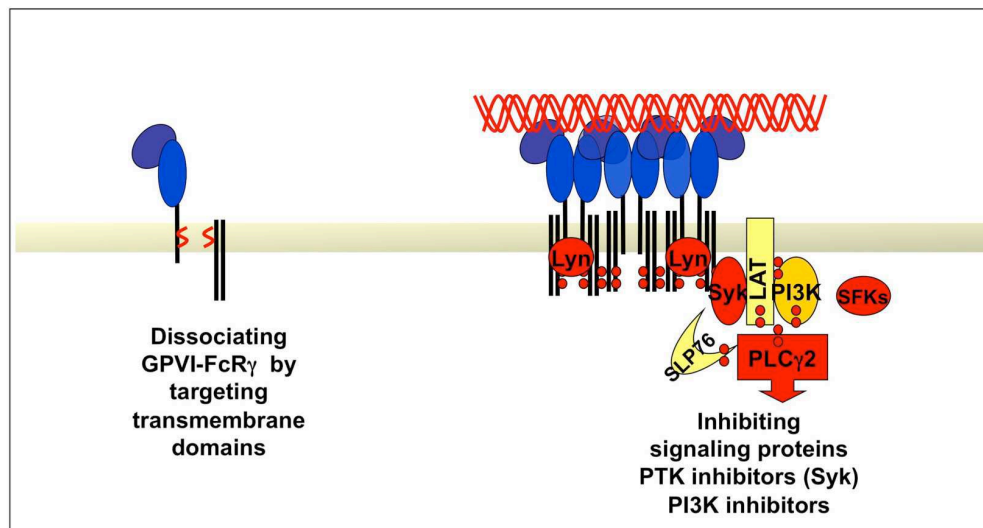
#### **2.6.9.2 Anti-GPVI Antibodies**

Another very attractive, promising and suitable strategy is the design and production of GPVI specific antibodies which would bind to platelet GPVI and inhibit its binding to exposed collagen at the site of vascular injury (Figure 39). Here, the main hindrance is the use of a whole IgG that would bridge platelet membrane GPVI and possibly the low affinity IgG receptor FcγRIIA, leading to platelet activation. The whole IgG may also induce GPVI deficiency because of internalization and shedding. It has been proposed that inducing GPVI depletion could be a therapeutic strategy.

Indeed, the first anti-GPVI antibody was identified in a patient with thrombocytopenia having impaired collagen-induced platelet aggregation (Moroi *et al.*, 1989, Sugiyama *et al.*, 1987). This initial observation led to the identification of GPVI as the major collagen receptor. IgG isolated from plasma of patients activate platelets from healthy donors while their Fab fragments specifically inhibit aggregation induced by collagen. Interestingly, the platelets of this patient were deficient in GPVI and FcRγ. Since then several other patients have been identified with autoantibodies. None of these antibodies have been cloned or further processed to obtain therapeutic antibody fragments. However, mouse monoclonal antibodies



**Figure 39: The inhibition of GPVI/collagen interaction by using GPVI antagonists.**



**Figure 40: Blocking of GPVI signaling pathway:** Disruption of the intra-receptor GPVI-FcR $\gamma$  trans-membrane interaction or inhibition of signaling kinases (Syk and PI3kinase).

(mF1201 and mF1232) mimicking the properties of the patient's IgGs have been obtained and chimeric Fab produced. *In vitro* study showed that mF1201 but not mF1232 caused human platelet activation and GPVI shedding while mF1232 inhibited collagen-induced platelet aggregation. In monkey, mF1201 and mF1232 caused GPVI immunodepletion with and without significant thrombocytopenia and GPVI shedding respectively. The chimeric cF1232 have the ability to bind to platelet surface expressed GPVI and induced GPVI endocytosis *in vivo* by a cAMP dependent mechanism (Takayama *et al.*, 2008). Some drawbacks to this strategy could be identified (i) antibodies capable to down regulate GPVI are coupled to intraplatelet signaling and platelets could thus be “primed”; (ii) shedding could affect other receptors important to ensure haemostasis; (iii) GPVI depletion is long lasting (as long as platelets survive) and there is no antidote. This approach thus needs to be further explored.

Human single chain antibodies obtained by phage display have been obtained but do not appear to have a sufficiently high affinity for therapeutic applications. The scFv 10B12 is an example (Qian *et al.*, 2002, Smethurst *et al.*, 2004). The scFv10B12 binds in the apical surface of GPVI, i.e is at the interface common to both Ig-like D1 and D2 loops close to hinge sequence, an area known for the interaction of certain GPVI ligands (O'Connor *et al.*; 2006). Another humanized scFv, 1C3, binds to an area involved in the dimerization of GPVI and including isoleucine 148 of D2. Another group isolated from phage display libraries a human domain antibody (dAbs) BLO8-1 that binds human GPVI and inhibits its function. BLO8-1 specifically inhibits the binding of recombinant human GPVI to cross-linked collagen related peptide (CRP-XL) *in vitro*. BLO8-1 binds to the platelet cell surface and prevents CRP-XL induced platelet aggregation in platelet-rich plasma, as well as inhibiting thrombus formation in whole blood under arterial shear conditions. BLO8-1 recognizes an epitope within the collagen binding domain of GPVI (Walker *et al.*, 2009).

Another possibility is to use blocking monoclonal antibodies. Several teams have produced hybridoma secreting monoclonal antibodies directed against human GPVI, but very few of them efficiently block the collagen-induced platelet activation. In fact, some of these monoclonal IgG activate platelets, while others do not. The activation results from bridging of GPVI and can be reproduced by the F(ab')<sub>2</sub> and/or cross-linking of GPVI with the low affinity receptor Fc (FcγRIIA). Of note, only human

platelets are activated by the latter mechanism, platelets of mice were deprived of Fc $\gamma$ R1IA.

Nevertheless, some monoclonal antibodies have interesting characteristics. One group obtained monoclonal anti-human GPVI after immunization of mice GPVI-deficient. One of them has a good affinity for GPVI and its injection to Cynomolgous monkey inhibits platelet activation by collagen *ex vivo* (Matsumoto *et al.*, 2006). A bolus injection of OM2 Fab at a dose of 0.4 mg / kg leads to inhibition of platelet aggregation induced by collagen up to 6 hours after administration of the antibody to the animal, with a half-life time longer than abciximab. Interestingly, the administration of OM2 Fab did not induce thrombocytopenia or shedding of GPVI in monkeys (Matsumoto *et al.*, 2006). Moreover, the Fab fragment of antibody OM4 (anti-rat GPVI) inhibits platelet aggregation induced by collagen *in vitro*, *ex vivo* and *in vivo* thrombosis model in rats without increasing bleeding time, unlike what was observed with whole IgG (Li *et al.*, 2007, Matsumoto *et al.*, 2006).

Recently, Jung *et al.* (2009) described six recombinant Fab fragments that specifically binds to recombinant GPVI dimer but not to monomeric GPVI. It was observed that all the bivalent form of Fabs induced platelet activation while only the monovalent form inhibited collagen-induced platelet aggregation (Jung *et al.*, 2009).

The monoclonal Ig 9O12.2 obtained through gene-gun immunization has been extensively characterized and presents the prerequisites to develop an antithrombotic antibody (Lecut *et al.*, 2004a, Lecut *et al.*, 2003, Ohlmann *et al.*, 2008, Smethurst *et al.*, 2004).

### **2.6.9.3 Blocking GPVI Dimerization/Oligomerization and Signaling**

Other strategies may include the blocking of GPVI dimerization or oligomerization and signaling transmission (Figure 40) (for a review see Sigalov, 2008).

Dimerization of GPVI favours optimal collagen interaction, which may be controlled by using cyclic AMP and phosphatases. Dimerization could also be blocked by using extracellular agents such as antibodies or small inhibitors that could block D2 homotypic interactions.

Disruption of intra-receptor GPVI-FcR $\gamma$  transmembrane interactions could theoretically be another approach. It will lead to disconnection of subunits and thus will block downstream activation.

Homotypic-interactions between the cytoplasmic domains of FcR $\gamma$  subunits contribute to GPVI oligomerization and subsequent activation of the signaling cascade. These interactions may be disrupted by small membrane permeable molecules. One potentially major disadvantage of this approach is the lack of specificity, because gamma chain is also the signaling subunit of several other immunoglobulin receptors.

Blocking GPVI signaling might also be achieved by signaling cascade: kinase inhibitors such as those developed for cancer therapy represent here the target. As an example, the Syk inhibitors and PI3kinase inhibitors may be useful but with the major drawback of being non specific (Gratacap *et al.*, 2009).

## **Part B**

# **Experimental Approaches**

### **3 Aim of the study**

Cardiovascular diseases and stroke are the predominant causes of death in developed countries. Rupture of atherosclerotic plaque in an artery wall and the ensuing thrombotic events are the triggering events for acute ischemic injury in cardiovascular diseases. Platelet activation and aggregation play key roles in atherothrombosis process. GPVI constitutively expressed collagen receptor is platelet-specific and seems to be a useful biomarker tool for the early detection of atherosclerotic diseases, in particular acute coronary syndrome and ischemic stroke. In addition, GPVI is also the target of therapeutic drugs. The blockade of GPVI/collagen interaction would be a promising strategy for antithrombotic and antiatherosclerotic therapy in future.

Antibodies are the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines. Currently, several antibodies are approved for therapeutic use in diverse, clinical settings, including oncology, chronic inflammatory diseases, transplantation, infectious diseases and cardiovascular medicine. Recombinant antibody fragments are becoming popular therapeutic alternatives to full length monoclonal antibodies since they are smaller, possess different properties that are advantageous in certain medical applications, can be produced more economically and are easily amendable to genetic manipulation. Single-chain variable fragment (scFv) antibodies are one of the most popular recombinant antibody format as they have been engineered into larger, multivalent, bi-specific and conjugated forms for many clinical applications. scFv show tremendous versatility and provide the basic antigen binding unit for a multitude of engineered antibodies for use as human therapeutics and diagnosis.

In the present study two anti-GPVI monoclonal antibodies were used for the design and production of recombinant antibody fragments with therapeutic and/or diagnostic potentials. One murine monoclonal antibody, 3J24, has diagnostic potential as it recognizes both monomeric and dimeric form of GPVI. The other murine monoclonal antibody, 9O12, has a therapeutic potential because it blocks the binding of GPVI to collagen.

The aim of the present work was the design, production, optimization and characterization of anti-GPVI antibody fragments with diagnostic and or therapeutic



potentials. We designed and reshaped a single-chain antibody fragment (scFv) based on 3J24 variable domains for the quantification of platelet surface GPVI with diagnostic potential. In addition, a single point mutation in V-kappa FR1 conferred to the scFv (3J24-P8) a Protein L (PpL) recognition site, which provides a way for rapid single-step purification or detection using PpL conjugates. The point mutated scFv 3J24-P8 retained the functional properties of the parental antibody for binding to GPVI. Moreover, scFv 3J24-P8 binding to platelets induces neither platelet activation nor aggregation. Therefore, the scFv preserves all the functional properties required in monitoring platelets and assays of GPVI quantification (Zahid *et al.*, 2011).

We were also involved in the design, production and functional evaluation of humanized anti-GPVI recombinant antibody fragments (scFvs and Fabs) with therapeutic properties. Concerning the therapeutic aspects, a murine scFv 9O12 (m<sub>scFv</sub> 9O12) was designed and produced in functional form which was then humanized using recombinant antibody engineering techniques. The humanized version of the scFv 9O12 (h<sub>scFv</sub> 9O12) was produced in functional form (Muzard *et al.*, 2009) but the production yield was very low and insufficient for performing all functional analysis. Different approaches were carried out for the better production of the h<sub>scFv</sub> 9O12. Finally, we succeeded to produce a reshaped scFv (L<sub>h</sub>scFv 9O12) in enough quantity, sufficient for all the functional evaluation. Also, a PpL recognition site was grafted on to it, in order, to make easy its detection and purification, without requiring any additional tag.

Monovalent Fab antibody fragment may be more suitable and valuable recombinant antibody format than scFv for therapeutic purposes due to their pharmacokinetic properties and other specific characterization. Here, scFv 9O12 was used as a building block for the construction of recombinant antibody Fab 9O12 fragments. Several constructs were designed including chimeric, hemi-humanized and humanized Fabs. All the three constructs were expressed in bacterial periplasm in functional forms. The preliminary results of the recombinant Fabs are very encouraging which is a motivation for their further elaborated evaluation. These recombinant Fab fragments especially humanized version may become pilot molecules to design valuable antithrombotic therapeutic drugs in near future.

### **3.1 Design of Anti-GPVI scFv 9O12 with Diagnostic Potential**

Although GPVI is constitutively surface-expressed, activation of platelets lead to further release and enhanced plasma membrane expression of GPVI. Preliminary data indicate that individual with low GPVI expression have low-cardiovascular risk, where as enhance GPVI surface expression has been associated with myocardial infarction and acute coronary syndrome (Bigalke *et al.*, 2007, Samaha *et al.*, 2005). Thus, an altered GPVI expression level on circulating platelets may indicate a prothrombotic disease state such as imminent MI. Therefore, determination of GPVI levels may be an early marker of acute coronary syndrome (ACS) before myocardial ischemia is evident.

Different GPVI defects can be distinguished by determining the levels of GPVI by flow cytometry (surface expression) or by western blot (total protein) using different antibodies, but whole antibody can cause bridging surface GPVI leading to internalization or shedding of GPVI making impossible the precise GPVI quantification. Therefore, the need of a monovalent anti-GPVI antibody fragment is evident, in order, to quantify GPVI without any loss to GPVI. That is why, we constructed various anti-GPVI recombinant antibody fragments either with diagnostic and/or therapeutic potentials.

The first part of our work was the construction, production and characterization of recombinant anti-GPVI antibody fragments with diagnostic potential. For this purpose, we used the already described monoclonal antibody 3J24 directed to ectodomain of human platelets GPVI. The 3J24 was produced by immunizing Balb/c mice with the DNA encoding a fusion protein corresponding to the extracellular domain of GPVI fused at its C-terminus via a 3 Ala linker to the human IgG1 Fc sequence using the Rapid Immunization Gene Gun delivery (Jandrot-Perrus *et al.*, 2000). The interest of such an antibody was its ability to recognize both monomeric and dimeric form of recombinant GPVI which would have diagnostic potential.

The variable domains (VH and VL) of the antibody responsible for the specific antigen binding activity were cloned and assembled into a synthetic gene encoding a monovalent scFv capable to bind GPVI. Guided by computer modelling and sequence analysis, a variant, designated as scFv 3J24-P8, was constructed. A single point

mutation in V-Kappa FR1 conferred to the scFv a Protein L (PpL) recognition site, which provides a way for rapid single-step purification or detection using PpL conjugates. The point-mutated scFv 3J24-P8 is fully functional. It retains the ability of the parental antibody to bind monomeric and dimeric recombinant GPVI. Binding of scFv 3J24-P8 to platelets neither induces platelets activation nor aggregation. Therefore scFv 3J24-P8 preserves all the functional properties required in monitoring platelets and assays of GPVI quantification.



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## Design and reshaping of an scFv directed against human platelet glycoprotein VI with diagnostic potential

Muhammad Zahid<sup>a,b</sup>, Stéphane Loyau<sup>b</sup>, Maxime Bouabdelli<sup>b</sup>, Nicolas Aubrey<sup>c</sup>, Martine Jandrot-Perrus<sup>b,d,\*</sup>, Philippe Billiald<sup>a,e,\*</sup>

<sup>a</sup> Université Paris-Sud 11, IFR 141, Faculté de Pharmacie, 92260 Châtenay-Malabry, France

<sup>b</sup> INSERM, U698, Hôpital Bichat, F-75018 Paris, France

<sup>c</sup> Faculté de Pharmacie, UMR Université-INRA 483, 37200 Tours, France

<sup>d</sup> Université Paris 7, UMR698, F-75018 Paris, France

<sup>e</sup> Muséum National d'Histoire Naturelle, USM 505, F-75005 Paris, France

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### ABSTRACT

Blood platelets play a key role in physiological hemostasis and in thrombosis. As a consequence, platelet functional analysis is widely used in the diagnosis of hemorrhagic disorders as well as in the evaluation of thrombosis risks and of the efficacy of antithrombotics. Glycoprotein (GP) VI is a platelet-specific collagen-signaling receptor. Clinical studies suggest that increased GPVI expression is associated with a risk of arterial thrombosis. Conversely, GPVI deficiencies have been identified in patients with defective platelet responses to collagen. Currently, there is no standard test available for measuring GPVI expression, essentially because antibodies usually cross-link GPVI upon binding, leading to platelet activation and consecutive changes in GPVI expression. Here, we designed a recombinant monovalent antibody fragment (scFv) derived from an anti-GPVI monoclonal IgG, 3J24, with the characteristics required to analyze GPVI expression. Guided by *in silico* modeling and V-KAPPA chain analysis, a Protein L (PpL) recognition pattern was engineered in the scFv, making possible its purification and detection using PpL conjugates. The PpL affinity-purified scFv is functional. It retains GPVI-binding specificity and allows detection of platelet surface-expressed GPVI without inducing platelet activation. In conclusion, the reshaped scFv may be very useful in the development of diagnostic approaches.

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The physiological role of blood platelets is to limit bleeding by forming a clot filling vascular gaps and facilitating healing. On the other hand, inappropriate activation of platelets could lead to arterial thrombosis and ischemic damage of downstream tissues. Several analytical methods are in use to explore platelets. Among them, flow cytometry is a powerful and versatile tool that provides definitive quantitative information regarding the phenotypic status and antigenic properties of platelets (e.g., surface expression of receptors, bound ligands, granules components, or platelet–platelet interactions, interactions with other blood cells or components of the plasma coagulation system), thereby facilitating the diagnosis of inherited or acquired platelet disorders (e.g., Bernard–Soulier syndrome, Glanzmann thrombasthenia, storage pool disease), the pathological activation of platelets (e.g., in the setting of acute coronary syndromes, cerebrovascular ischemia,

peripheral vascular disease, cardiopulmonary bypass), and changes in the ability of platelets to activate in response to specific stimuli (e.g., efficacy of antiplatelet therapies). Reliable methods have been developed to characterize the expression of the main platelet receptors such as glycoprotein (GP)Ib<sup>1</sup> (von Willebrand factor receptor) and integrins  $\alpha 2\beta 1$  and  $\alpha IIb\beta 3$  (fibrinogen receptor). Flow cytometry is also used to measure the exposure of platelet activation markers such as CD62P (P-selectin).

One of the most important receptor expressed at the surface of platelets is GPVI, which plays a key role in collagen-induced platelet activation [1]. GPVI is a glycosylated protein of 339 amino acid residues with an apparent  $M_r$  of 62 kDa, only expressed on megakaryocytes and platelets as a noncovalent complex with the signaling  $\gamma$  chain common to the Ig receptors (FcR $\gamma$ ) [2,3]. Binding of GPVI-FcR $\gamma$  to collagen exposed at the site of vascular injury induces clustering of GPVI molecules and initiates a signaling

\* Corresponding authors. Address: INSERM, U698, Hôpital Bichat, F-75018 Paris, France (M. Jandrot-Perrus), Université Paris-Sud 11, IFR 141, Faculté de Pharmacie, 92260 Châtenay-Malabry, France (P. Billiald). Fax: +33 1 40 793 594.

E-mail addresses: [martine.jandrot-perrus@inserm.fr](mailto:martine.jandrot-perrus@inserm.fr) (M. Jandrot-Perrus), [philippe.billiald@u-psud.fr](mailto:philippe.billiald@u-psud.fr) (P. Billiald).

<sup>1</sup> Abbreviations used: GP, glycoprotein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PE, phycoerythrin; PpL, Protein L; PRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

pathway leading to platelet degranulation, activation, aggregation, and procoagulant activity.

Some preclinical studies have provided evidences that enhanced GPVI expression at the platelet surface may be a marker for acute coronary syndrome [4]. In contrast, deficiency or defects of GPVI can lead to profoundly altered platelet responses to collagen. Most often, GPVI deficiencies are revealed by mild bleeding disorders and are acquired in the context of immune dysfunction [5,6]. In rare cases, functional GPVI defects are associated with genetic protein deficiency and dysfunction [7,8]. Furthermore, GPVI has recently been shown to be down-regulated by proteolysis by the coagulation factor Xa which is the target of newly developed anticoagulant drugs [9]. For all these reasons, monitoring of GPVI function and count may be useful in medical practice to understand GPVI-related defects and thrombotic disorders [5,10,11].

Several anti-GPVI monoclonal antibodies have been produced and some of them have great potential for therapeutic antithrombotic applications [12–14]. Others have been used in cytometry for the quantification of surface expressed GPVI or in Western blot to analyze the total cell protein. However, no standardized assays are yet available. Studies conducted in different research laboratories using these various antibodies have contributed to the understanding of GPVI structure, function, and regulation. They allowed the evaluation of normal expression and pathological variations of GPVI: congenital or acquired deficiencies [7,8] or increased expression of GPVI proposed to be associated with a major risk of arterial thrombosis [1]. However, data indicated that the level of GPVI expression varies among healthy individuals. The degree of these variations is a matter of debate, either tightly regulated, varying by only 1.5 or up to 5-fold [10,15,16]. These discrepancies may be due to the use of whole bivalent antibodies that cross-link surface-exposed GPVI and thus may lead to activation signals and modifications in GPVI expression. New sites could be exposed or conversely antibody binding could induce GPVI shedding or internalization [17–19]. This highlights the difficulties in quantifying GPVI expression and the requirement of specific characteristics of antibodies to make them valuable reagents for accurate quantification.

In this study we used the monoclonal antibody 3J24 directed to the ectodomain of human platelets GPVI [20]. The V-domains of this antibody responsible for the specific antigen-binding activity were cloned and assembled into a synthetic gene encoding a monovalent scFv capable of binding GPVI. Guided by computer modeling and sequence analysis, a variant, here designated as scFv 3J24-P8, was constructed. A single point mutation in V-Kappa FR1 (T 8>P) conferred to the scFv a Protein L (PpL) recognition site, which provides a way for rapid single-step purification or detection using PpL conjugates. The point-mutated scFv 3J24-P8 is fully functional. It retains the ability of the parental antibody to bind monomeric and dimeric shGPVI. Binding of scFv 3J24-P8 to platelets induces neither platelet activation nor aggregation. Therefore scFv 3J24-P8 preserves all the functional properties required in monitoring platelets and assays of GPVI quantification.

## Materials and methods

### Materials

The anti-GPVI monoclonal antibodies were produced by immunizing Balb/c mice with the DNA encoding a fusion protein corresponding to the extracellular domain of GPVI (residues 1–269) fused at its C terminus via a 3 Ala linker to the human IgG<sub>1</sub> Fc sequence (shGPVI-Fc) using the Rapid Immunization Gene Gun delivery followed by one intravenous injection of 100 µg shGPVI-Fc, 4 days prior to fusion [2]. Hybridomas were screened for secretion of GPVI-specific antibodies by enzyme-linked immunoassay (ELISA) using plate-bound shGPVI-Fc. Selected cell lines were cloned

using ClonalCell™-HY medium D (Stem Cell Technology, Vancouver, British Columbia, Canada). Ascitic fluids were produced, and antibodies were purified by chromatography on Protein A–Sepharose (GE Healthcare, Europe). 3J24 and 9012 were isotyped as IgG<sub>1</sub> and previously characterized [20,21].

The murine scFv 9012 directed to the GPVI of human platelets and scFv 9C2 directed against scorpion toxins irrelevant to GPVI have been described elsewhere [13,22].

PpL immobilized on crosslinked beaded agarose resin, peroxidase-conjugated PpL, and biotinylated PpL were from Pierce Biotechnology (Rockford, USA).

The antibodies anti-mouse IgGs and anti-cMyc (9E10) conjugated to horseradish peroxidase (HRP) (Sigma–Aldrich, St. Louis, MO, USA) or FITC (Invitrogen, Cergy Pontoise, France), anti-P-selectin–FITC or phycoerythrin (PE), anti-mouse IgG<sub>1</sub>–FITC, and anti-CD41–FITC (Beckman Coulter, Marseille, France) were used according to the manufacturer's instructions.

ScFv 3J24-P8 was coupled to FITC as previously reported and free FITC was removed by extensive dialysis against PBS, pH 7.4 [2].

Dimeric shGPVI-Fc was produced and purified as described [23]. Monomeric recombinant shGPVI-His consisting of GPVI ectodomain fused to a hexahistidine tag at its C-terminal extremity was produced in transfected HEK293 cells and purified using the ProBond purification system (Invitrogen).

Blood was collected from healthy volunteers. Platelet-rich plasma (PRP) and washed platelets were obtained according to the previously described procedure [2]. Whole platelet lysates were obtained by solubilization of washed human platelets ( $10^9$  platelets mL<sup>-1</sup>) in Tris–HCl 20 mM, pH 7.4, containing NaCl 150 mM, EDTA 3 mM, and SDS 2%, according to a described procedure [2]. All chemicals were of standard grade from Sigma–Aldrich or equivalent.

### Methods

#### Construction of single-chain antibody fragment genes

Total RNA was isolated from freshly subcloned hybridoma 3J24. cDNAs encoding the antibody variable domains (IGHV and V-KAPPA) were cloned after RT-PCR using degenerated primers IGH-For (5'-CGG GAT CCT CTA GAC AGT GGA TAR ACM GAT GG-3') and IGH-Rev (5'-CGG GAT CCT CTA GAG GTS MAR CTG CAG SAG TCW GG-3') for 3J24 VH amplification or Vκ-For (5'-GGA TAC AGT TGG TGC AGC ATC-3') and Vκ-Rev (5'-GAY ATT GTG MTS ACM CAR WCT MCA-3') for 3J24 V-KAPPA and sequenced as previously reported [22,24]. Then, a synthetic gene encoding 3J24 IGHV fused to 3J24 V-KAPPA via a (G<sub>4</sub>S)<sub>3</sub> peptide linker was designed. Codon usage was adapted to the bias of *Escherichia coli* resulting in a codon adaptation index value of 0.98 (GeneArt, Regensburg, Germany). The scFv gene was cloned into the prokaryotic expression vector pSW1, in frame with the pelB leader sequence at its 5'-end and, downstream, a sequence encoding the c-Myc tag [25].

Plasmid pSW-3J24-P8 encoding the mutated scFv which contains the V-KAPPA point mutation T 8>P was prepared using the Quick Change Kit (Agilent Technologies, Massy, France) and primers 3J24P8For (5'-GCG CTC AGG CTA CTG GGG GTC TGG GTC AGA AC-3') and 3J24P8Rev (5'-GTT CTG ACC CAG ACC CCC AGT AGC CTG AGC GC-3').

The constructed vectors pSW-3J24 and pSW-3J24-P8 were sequenced to ensure accuracy before being cloned into *E. coli* TOPP I for expression.

All standard molecular biological procedures were carried out as reported earlier [26].

#### Bioinformatics

*Sequence analysis and data banks search.* In order to easily compare V-REGION sequences of IgG 3J24 we used the IMGT unique

numbering and standards that have been approved by the WHO-IUIS [27]. Antibody V-KAPPA FR1 sequences were also analyzed using the IMGT tools (IMGT/V-QUEST) and databases (IMGT/LIGM-DB, IMDT/3D structure-DB).

*In silico modeling and visualization of complexes.* Since no structural analysis of antibody 3J24 had ever been performed, a three-dimensional structural model of its Fv domains was built using the online Web Antibody Modeling facility (<http://antibody.bath.ac.uk>). Framework regions were modeled using homologous antibody templates. Visualization, superimposition of the structures, modeling of the mutants, and graphic images were done using PyMol [28].

#### Periplasmic expression of scFvs and purification

Bacteria were grown under rotative agitation (125 rpm, 37 °C) in 500 mL 2xTY broth (DIFCO, Le Pont de Claix, France) containing ampicillin (100 µg mL<sup>-1</sup>) until  $A_{600nm}$  reached 0.8. Then, 0.8 mM isopropyl-thio-β-D-galactoside was added to the medium and incubation was continued for 16 h at 16 °C. The cells were pelleted by centrifugation (3600g, 20 min, 4 °C). Periplasmic proteins were extracted according to the osmotic shock method previously reported [13].

To purify the scFv, the periplasmic extract (30 mL) was incubated with 500 µL of PpL coupled to agarose beads for 90 min at room temperature. The mixture was loaded onto a microcolumn. After washing with PBS, pH 7.4, bound proteins were eluted by glycine-HCl 0.1 M, pH 2.0, in 0.5-mL fractions, and immediately neutralized with 40 µL Tris 1 M on ice. Fractions with  $A_{280nm}$  higher than 0.1 were pooled and extensively dialyzed against PBS, pH 7.4. The concentration of the purified scFv was evaluated after measuring absorbance at 280 nm and using the Swiss Institute of Bioinformatics software (ProtParam tool) to determine its theoretical  $M_r$  and extinction coefficient [29].

Profiling of affinity PpL-purified scFv 3J24-P8 dialyzed in PBS was performed using hydrophilic NP20 ProteinChip Arrays (Bio-Rad, Marnes-La-Coquette, France). Briefly, 1 µg of purified scFv3J24P8 was applied to the NP20 chip and air-dried. Sinapinic acid was used as the ionization matrix and ProteinChip Arrays were determined from time-of-flight measurements using a Protein-Chip Reader (PCS 4000; Bio-Rad). Data were collected by averaging 795 laser shots and after intensity optimization.

#### Surface plasmon resonance (SPR)

A BIAcore T100 instrument and all the reagents for analysis were obtained from GE Healthcare, Europe. Biotinylated PpL (50 µg mL<sup>-1</sup>) was captured on a SA sensorchip (approximately 250 RU). Samples were then passed over the immobilized PpL in PBS, pH 7.4, at a flow rate of 20 µL min<sup>-1</sup> at 25 °C. Glycine-HCl 10 mM, pH 2.0, was injected for 1 min at 20 µL min<sup>-1</sup> to regenerate the sensorchip between successive samples. Kinetic constants ( $k_{on}$ ,  $k_{off}$ ) were deduced from the analysis of association and dissociation rates at five different antibody fragment concentrations ranging from 4 to 50 µg mL<sup>-1</sup>. The dissociation constant  $K_D$  was calculated from  $K_D = k_{off}/k_{on}$ . Sensorgrams were analyzed using the BIAevaluation version 1.1.1 software. All experiments were carried out in duplicate at the IFR 141-IPSIT platform (Châtenay-Malabry, France).

#### Electrophoresis and immunoblotting

Protein samples were separated by SDS-PAGE using either a 10% polyacrylamide gel or a 8–16% Tris-Hepes-SDS gradient gel (Thermo Fisher Scientific, Rockford, IL, USA) followed by staining with Coomassie brilliant blue, or Western blotting after transfer onto nitrocellulose membranes and blocking with 5% nonfat milk in PBS, pH 7.4, containing 0.1% Tween 20 for 90 min. Probing of transferred scFvs was carried out using either HRP-conjugated

anti-cMyc monoclonal antibody 9E10 or PpL-peroxidase. Western blots of platelet lysates and shGPVI were carried out after incubating the membrane with either IgG 3J24 or periplasmic extracts containing scFv 3J24-P8. Bound IgGs were detected using HRP-conjugated anti-mouse IgG antibody. The detection of the bound scFv was carried out using PpL-peroxidase. Immunocomplexes were stained with appropriate substrate (diaminobenzidine or chemiluminescent (Thermo Fisher Scientific, Rockford, IL, USA)). All incubations were carried out for 1 h at room temperature, and the membranes were washed five times with PBS, pH 7.4, containing 0.1% Tween 20 between the intermediate steps.

#### Immunoprecipitation

Purified scFv 3J24-P8 was incubated with the shGPVI-agarose at 4 °C for 16 h under agitation. The mixture was centrifuged at 112g for 4 min at room temperature. Pellets and supernatants were collected and analyzed by Western blot using either anti-cMyc antibody or PpL conjugated to peroxidase.

#### ELISA

Immulon 2HB 96-well plates (Thermo Fisher Scientific, Rockford, IL, USA) were coated with either dimeric shGPVI-Fc or monomeric shGPVI-His (100 µL, 2 µg mL<sup>-1</sup>) by incubating for 16 h at 4 °C, and then saturated with 1% bovine serum albumin for 90 min at 37 °C. The plates were then incubated with increasing concentrations of the scFv preparations (0–5 µg mL<sup>-1</sup>, 100 µL) for 90 min. They were then incubated for 1 h with 100 µL of peroxidase-coupled PpL or peroxidase-coupled anti-c-Myc monoclonal antibody 9E10. Substrate solution (100 µL) (*ortho*-phenylenediamine) was then added to the wells for 5 min. The reaction was stopped by sulfuric acid 3 M and the absorbance was read at 485 nm in an ELISA plate reader. The wells were washed five times with PBS, pH 7.4, containing 0.1% Tween 20 and 0.1 mg mL<sup>-1</sup> bovine serum albumin, between the intermediate steps. Controls were performed either omitting coating with shGPVI or using periplasmic extracts containing irrelevant scFvs (anti-scorpion toxin scFv-9C2 having reactivity with PpL or the anti-GPVI scFv 9O12 which does not interact with PpL). All assays were conducted in triplicate. Data were analyzed and graphs were produced using PRISM GraphPad (San Diego, CA, USA). Specific binding ( $Y$ ) was determined using formula  $Y = B_{max} \cdot X / (K_D + X)$ , where  $B_{max}$  is maximum binding,  $X$  is the concentration of the ligand, and  $K_D$  is the apparent dissociation constant.

In competitive ELISA scFv preparation was mixed with increasing concentrations of IgG 3J24 (0–50 µg mL<sup>-1</sup>) before being delivered into shGPVI-coated wells.

#### Flow cytometry

PRP ( $3 \times 10^8$  platelets mL<sup>-1</sup>) was incubated with purified scFv 3J24-P8 labeled with 5 µL FITC-coupled anti-cMyc antibody. The samples were analyzed on a LSRII flow cytometer (BD Biosciences, Le Pont de Claix, France) for measuring the cell fluorescence. Platelets were gated on forward and side scatter and more than 95% of events were positive for CD41. A mouse IgG<sub>1</sub> (isotypic control) was used to define negative events in the population of gated platelets. Alternatively, PRP was incubated with either the FITC-coupled scFv 3J24-P8 or the IgG 3J24 and binding to platelet was directly estimated.

In competition test, PRP ( $3 \times 10^8$  platelets mL<sup>-1</sup>) was incubated with a mixture of shGPVI-Fc (2.5 µg mL<sup>-1</sup>) and scFv 3J24-P8 for 20 min at room temperature, followed by centrifugation (140g, 5 min). The pellet were resuspended in 5 µL FITC-coupled anti-cMyc antibody and incubated for 20 min at room temperature for fluorescence analysis.

In order to analyze P-selectin (CD62P) exposure at the platelet surface, varying amounts of FITC-coupled scFv 3J24-P8 were added



to washed platelets ( $3 \times 10^8$  platelets  $\text{mL}^{-1}$ ) in the presence of 5  $\mu\text{L}$  of PE-conjugated anti-P-selectin IgG.

All incubations were carried out for 20 min in the dark. Binding was estimated using either irrelevant scFv or PBS, pH 7.4, and all experiments were carried out at least in triplicate.

#### Platelet aggregation

PRP ( $3 \times 10^8$  platelets  $\text{mL}^{-1}$ ) was incubated with IgG 3J24 ( $1\text{--}2.5 \mu\text{g mL}^{-1}$ ) or PpL affinity-purified scFv 3J24-P8 ( $1 \mu\text{g mL}^{-1}$ ) under stirring conditions at 37 °C for 20 min. Platelet aggregation was continuously recorded as changes in light transmission using a Chronolog Aggregometer (Chrono Log Corps, Harveston, PA, USA).

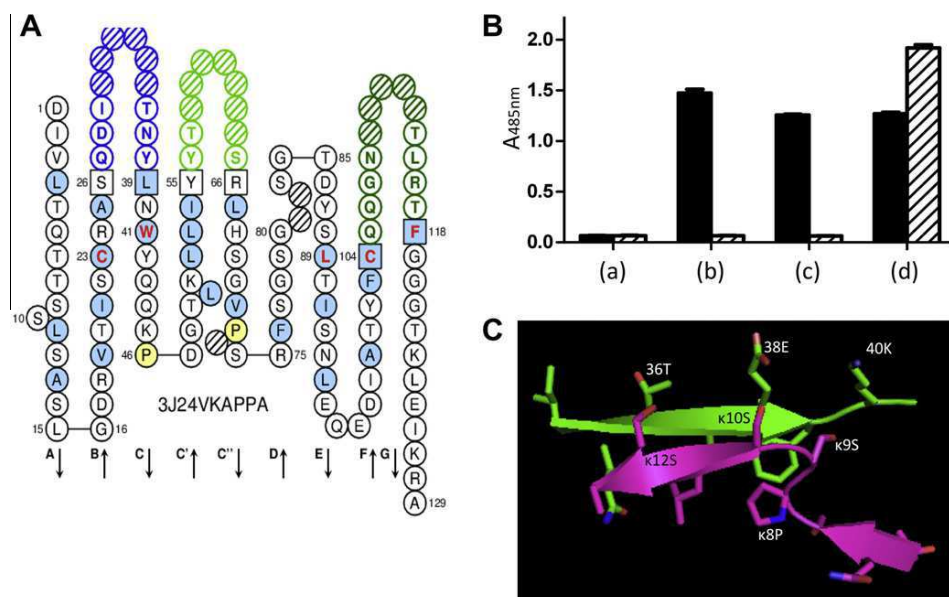
## Results

### Design and bacterial expression of scFvs

Total RNA was isolated from freshly subcloned hybridoma 3J24 cells for RT-PCR amplification of both VH and V-KAPPA cDNAs. VH and V-KAPPA PCR products were acquired from two distinct batches of RNA to ensure accuracy. Sequencing of VH led to a single sequence. Conversely, sequencing of V-KAPPA was scrambled because of the amplification of the aberrant MOPC21-derived V- $\kappa$  gene. The V-KAPPA PCR product was treated with *Bci*VI, which cut a unique restriction site contained within the aberrant gene that is missing from most (95.81%) functional  $\kappa$  chain variable sequences [24]. The treatment enabled us to clone the 3J24 V-KAPPA gene. VH and V-KAPPA primary structures were deduced from the cDNA sequences and analyzed using the IMGT facilities (Fig. 1A). The 3J24 VH domain belongs to the IGHV5 subfamily while the V-KAPPA chain sequence exhibits 96.77% identity with IGKV10-96\*01 which belongs to the murine IGKV10 subgroup. A gene encoding scFv 3J24 in which VH and V-KAPPA were joined together via a short flexible peptide ( $\text{Gly}_4\text{Ser}$ )<sub>3</sub> was chemically synthesized after optimizing the codon sequence for expression in *E. coli*. The

gene was inserted into the periplasmic expression vector pSW1 in frame with the pelB signal sequence, upstream of the c-Myc tag (pSW-3J24). pSW-3J24 was then cloned into *E. coli* and the recombinant protein was produced. Periplasmic extracts were assayed for shGPVI-Fc binding in direct ELISA. scFv 3J24 bound to immobilized shGPVI-Fc and the immunocomplexes were easily detected using the anti-cMyc antibody (9E10) as a secondary reagent (Fig. 1B).

However, the use of PpL-peroxidase conjugate as a secondary reagent did not allow the detection of immunocomplexes. PpL is a cell wall protein isolated from *Peptostreptococcus magnus* which has been reported to interact with most of the V-KAPPA chain and is used for the detection of antibody fragments in immunoassays as well as for affinity purification [30]. Recently, we highlighted the critical function of V-KAPPA FR1 in the interaction between PpL and antibody fragments [31]. Crystal structural analysis of antibody fragments in complex with PpL has shown that the integrity of the backbone conformation and tertiary structure of strand A from the V-KAPPA FR1 is essential for the interaction with the PpL  $\beta$ 2 sheet [32]. Here, we constructed an *in silico* model of 3J24 V-KAPPA and focused on strand A. We noted that it preserved all the key residues of the human antibody 2A2, which have been reported to be involved in hydrogen bonds (Ser9, Ser10, Ser12, Thr20) and salt bridges with PpL (Ser10, Ser12, Arg18, Arg2) [32]. We also concentrated our attention on the main-chain conformation of segment 5–12 which is involved in a  $\beta$  zipper interaction with the PpL  $\beta$ 2-strand. We observed that the residues of this segment which are well conserved among PpL-recognized V $\kappa$  regions were well preserved in 3J24 V-KAPPA with the exception of two (Thr7 and Thr8). We assumed that the nonconservative substitution of the residue Thr8 by the hydrophobic residue Pro which in addition has a particular ring structure may help to confer to V-KAPPA strand A the conformation required for the  $\beta$  zipper interaction with PpL while the substitution S 7 > T would have no effects since both residues have similar properties (hydrophilic

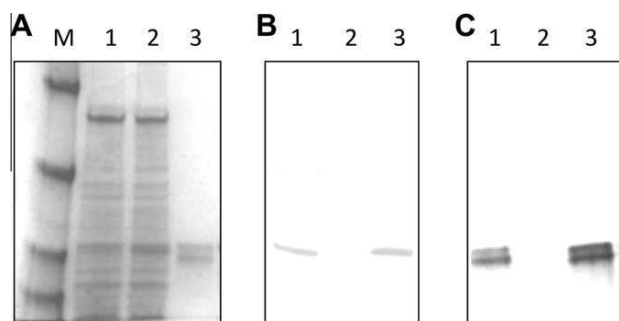


**Fig. 1.** Structural analysis of antibody 3J24 V-KAPPA domain and grafting Protein L-binding activity. (A) IMGT "collier de perles" representation of 3J24 V-KAPPA displayed according to IMGT unique numbering [27]. Hydrophobic residues are shown in blue and all proline residues in yellow. The CDR-IMGT sequences are limited by residues shown in squares. Hatched circles correspond to missing positions according to the IMGT unique numbering. Arrows indicate the direction of the  $\beta$ -strand (from IMGT Repertoire, <http://imgt.org>). CDR1-IMGT is shown in blue, CDR2-IMGT in light green, and CDR3-IMGT in dark green. (B) Analysis of the shGPVI-Fc binding activity of periplasmic extracts containing recombinant scFvs 9C2 (a), 9O12 (b), 3J24 (c), 3J24-P8 (d) using either the anti-c-Myc antibody (black) or PpL-HRP conjugate (hatched). (C) Enlargement of a model showing the  $\beta$  zipper interaction surface between PpL  $\beta$ 2 sheet (green) and 3J24-P8V-KAPPA segment L5–L12 (pink). P8 and residues expected to be involved in hydrogen bonds are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

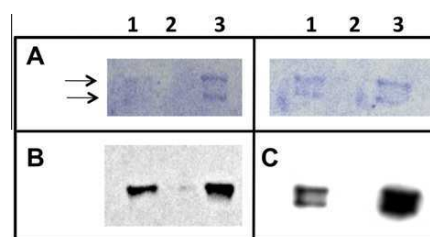
with uncharged side chains) (Fig. 1C). Thus, the mutation T 8>P was introduced in the 3J24 V-KAPPA FR1 sequence and the mutated scFv (here designated scFv 3J24-P8) was produced. The shGPVI-Fc-binding activity of scFv 3J24-P8 was investigated in ELISA (Fig. 1B). Detection of immunocomplexes using the anti-cMyc antibody allowed us to demonstrate that scFv 3J24-P8 preserved antigen-binding activity. We also observed that mutation T 8>P conferred PpL-binding activity, allowing detection of immunocomplexes in a rapid single-step immunoassay using PpL-peroxidase.

#### Affinity purification of functional scFv using PpL-agarose beads

scFv 3J24-P8 was purified from periplasmic extracts by PpL affinity chromatography. This procedure made it possible to recover scFv with a yield of  $300 \mu\text{g L}^{-1}$  from bacterial culture. The preparation appeared to be homogeneous when analyzed by Western blot using the antibody against c-Myc with a single band (Fig. 2). However, Coomassie blue staining and Western blot carried out with PpL-peroxidase indicated that the scFv preparation migrated as a doublet with an apparent  $M_r$  close to 28 kDa when compared to prestained molecular weight markers. We postulated that the component with the lower apparent  $M_r$  was a truncated form of scFv 3J24-P8 lacking the c-Myc tag. Indeed, MS analysis confirmed the heterogeneity of the preparation and allowed us to identify the two components: one with an experimental relative molecular mass ( $M+H^+$ ) of 28 199 Da, close to the theoretical molecular mass of 28,197 Da calculated from the amino acid sequence of scFv 3J24-P8, the other one being slightly lower (26,822 Da) likely being devoid of the Myc tag. To further investigate whether both proteins preserved antigen-binding activity, the PpL affinity-purified preparation was incubated with shGPVI-Fc agarose beads and then centrifuged. Supernatant and pellet were analyzed in Western blot for their content in scFv 3J24-P8 (Fig. 3). Red Ponceau staining allowed us to ensure correct transfer of proteins onto the nitrocellulose membrane. When analyzed with the anti-c-Myc antibody we observed a single band in the pellet corresponding to full size functional scFv 3J24-P8. Detection using PpL-peroxidase allowed us to distinguish two bands in the pellet corresponding to the full size scFv and the truncated form. No signal was seen in the supernatant whatever the detection system was (anti-c-Myc or PpL), demonstrating that most if not all scFv contents were trapped by the shGPVI-Fc agarose beads. All together these results demonstrated that both components of the PpL affinity-purified scFv preparation preserved antigen-binding activity and that the truncated form was functional but not detected using the anti-c-Myc antibody.



**Fig. 2.** Expression of scFv-3J24-P8 and analysis of the PpL affinity-purified scFv preparation. (A–C) Purification of scFv 3J24-P8 using PpL-agarose and analysis after gradient SDS–PAGE and staining with Coomassie blue (A) or Western blotting on nitrocellulose membrane (B and C) followed by staining using antibody 9E10 against c-Myc (B) or PpL (C). Lane 1: periplasmic extract containing scFv 3J24-P8. Lane 2: Flowthrough fraction. Lane 3: retained fraction eluted from the column at pH 2.0. Lane M: prestained molecular mass standards (97, 50, 36, 28, 19 kDa).



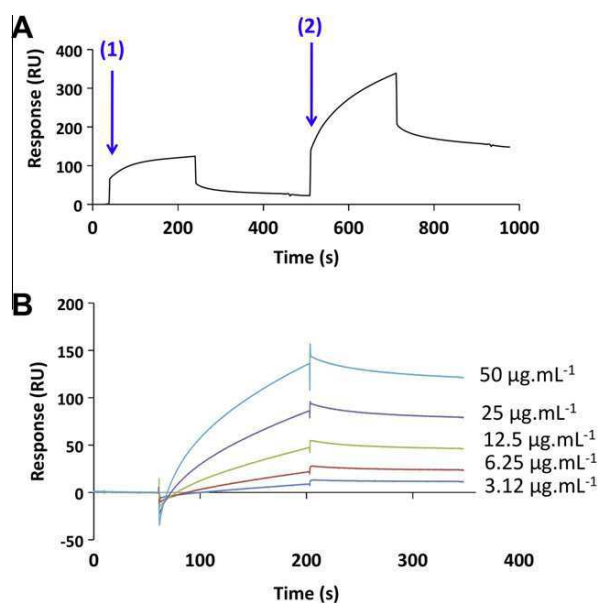
**Fig. 3.** Immunoreactivity of Protein L affinity-purified scFv 3J24-P8 with shGPVI-Fc. PpL affinity-purified scFv 3J24-P8 was incubated with shGPVI-Fc agarose beads. Samples were then centrifuged. Supernatant and pellet protein contents were analyzed after gradient SDS–PAGE and transferred onto nitrocellulose membrane. (A) Staining of the membrane using Red Ponceau. (B) Immunoblotting using anti c-Myc 9E10 antibody. (C) Blotting using PpL. Lane 1: PpL affinity-purified scFv 3J24-P8 alone. Lane 2: Supernatant. Lane 3: Pellet content.

scFv–PpL interaction was also investigated in real time using SPR technology (Fig. 4). Analysis of crude periplasmic extracts confirmed the capacity of the scFv 3J24-P8 mutant to interact with PpL while no PpL-binding activity was detected with scFv 3J24 even though these two scFv periplasmic extracts had similar antigen-binding affinity when tested against shGPVI-Fc in ELISA (Fig. 1B). Injection of increasing amounts of purified scFv 3J24-P8 allowed us to determine the kinetic parameters of the interaction between PpL and scFv 3J24-P8:  $k_{on} = 252.7 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{off} = 3.3 \times 10^{-6} \text{ s}^{-1}$ , and the dissociation constant  $K_D = 1.3 \times 10^{-8} \text{ M}$ . No PpL interaction was detected when we investigated the ability of the purified parental IgG 3J24 to bind to immobilized PpL under similar experimental conditions (data not shown).

#### Functional evaluation of scFv 3J24-P8

##### Reactivity in ELISA

The antigen-binding activity of scFv 3J24-P8 was first investigated in ELISA. Increasing quantities of PpL affinity-purified scFv were incubated with immobilized monomeric shGPVI-His or



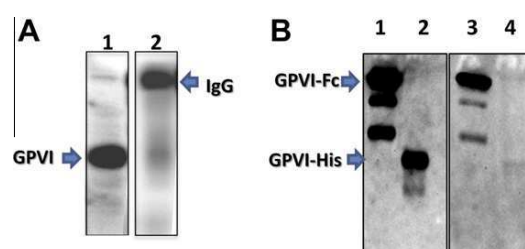
**Fig. 4.** Real-time analysis of the interaction of antibody fragments with biotinylated Protein L captured on a SA sensorchip. (A) Manual run in which periplasmic extracts containing scFv 3J24 (1) and scFv 3J24-P8 (2) were injected. (B) Injection of increasing amounts of purified scFv 3J24-P8.



dimeric shGPVI-Fc and immunocomplexes were detected using PpL-peroxidase conjugate (Fig. 5A). This assay demonstrated the ability of scFv 3J24-P8 to bind to each form of shGPVI as is the case for IgG 3J24. The  $K_{Dapp}$  was  $5.88 \pm 0.76$  nM for shGPVI-Fc and  $2.17 \pm 0.74$  nM for shGPVI-His. Thus, scFv 3J24-P8 retained a high affinity for both types of shGPVI. Competitive ELISA was also carried out using increasing amounts of IgG 3J24 as a competitor. Detection was made using PpL-peroxidase which interacts with scFv 3J24-P8 but not IgG 3J24. IgG 3J24 inhibited the binding of scFv 3J24-P8 to shGPVI-Fc (Fig. 5B) or shGPVI-His (Fig. 5C) and this inhibition was dose dependent. This assay confirmed that scFv 3J24-P8 preserved the antigen-binding specificity of the parental monoclonal antibody.

#### Reactivity in immunoblotting

Monoclonal antibody 3J24 is commonly used in Western blot to investigate GPVI defects [6]. Here, SDS-soluble extracts of human platelets were submitted to a Western blot and probed for GPVI using either IgG 3J24 or scFv 3J24-P8 (Fig. 6) detected with peroxidase-conjugated goat anti-mouse IgG and PpL-peroxidase conjugate, respectively. When a whole platelet lysate (20  $\mu$ g/well) was analyzed, a 58-kDa band corresponding to full-length GPVI was observed whatever the method of probing (scFv 3J24-P8 or IgG 3J24). An additional band with high apparent  $M_r$  was observed when using scFv 3J24-P8 and PpL-peroxidase conjugate. This band corresponds to direct binding of PpL to IgGs stored in platelet alpha granules as usually observed with Protein A conjugates. The Western blot confirmed the ability of scFv 3J24-P8 to bind to shGPVI-Fc (0.5  $\mu$ g/well) and its truncated forms as is the case for IgG 3J24. However, only a weak signal was observed when shGPVI-His (0.5  $\mu$ g/well) was probed with scFv 3J24-P8.

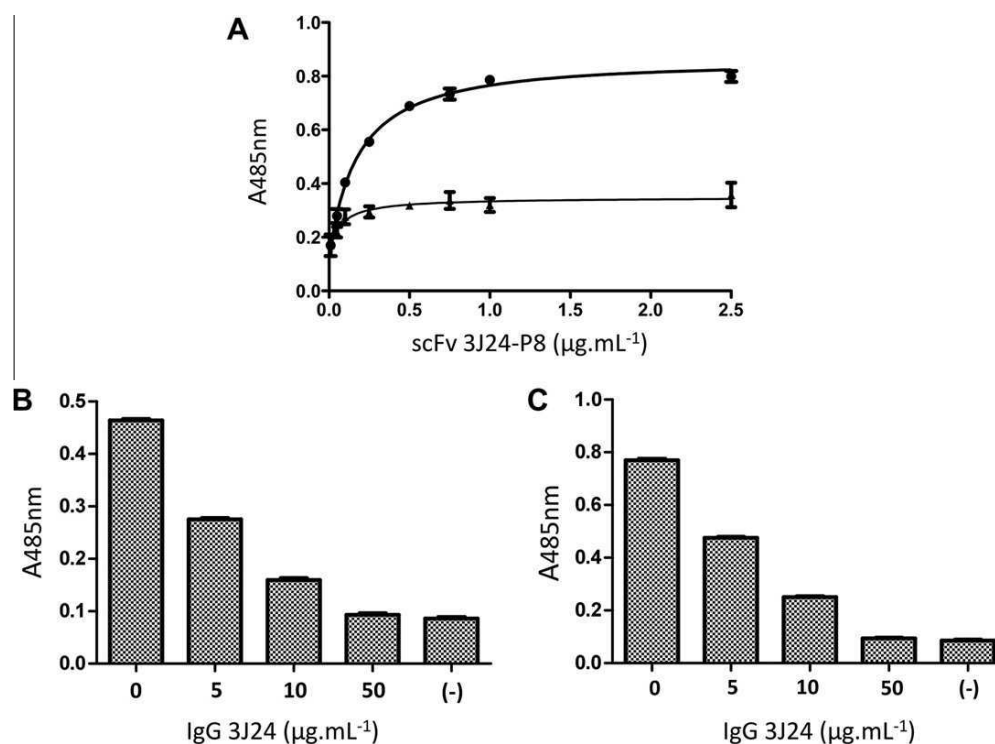


**Fig. 6.** Western blotting of SDS-soluble extracts of platelet lysate. (A) Whole platelet lysates: Incubation with IgG 3J24 (1) or periplasmic extract containing scFv 3J24-P8 (2). (B) shGPVI-Fc (1, 3) or shGPVI-His (2, 4): incubation with IgG 3J24 (1, 2) or periplasmic extract containing scFv 3J24-P8 (3, 4).

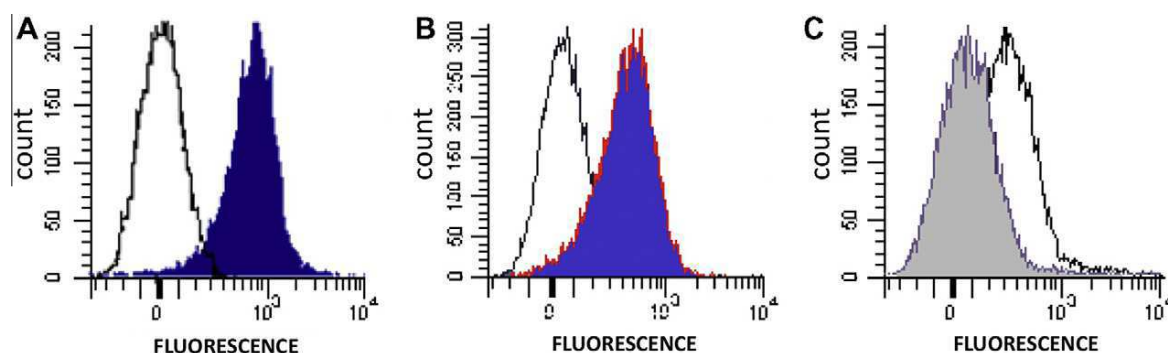
#### Reactivity in flow cytometry and aggregometry

The ability of scFv 3J24-P8 to bind native GPVI expressed at the surface of platelets was investigated in flow cytometry (Fig. 7). First, scFv 3J24-P8 binding onto platelets was indicated by a shift of the fluorescence to the right when using FITC-coupled anti-c-Myc antibody as a secondary reagent (Fig. 7A). scFv 3J24-P8 directly conjugated to FITC also preserved the capacity to label platelets and made it possible to perform the assay without requiring any secondary reagent (Fig. 7B). Finally, the binding of FITC-coupled scFv to platelets was inhibited in the presence of shGPVI-Fc, indicating that it is GPVI specific (Fig. 7C).

We also investigated whether binding of scFv 3J24-P8 to platelets induces their activation or aggregation (Fig. 8). First, we observed that incubating washed platelets with increasing amounts of FITC-coupled scFv 3J24-P8 increased platelet labeling but without inducing P-selectin exposure, indicating that the scFv 3J24-P8



**Fig. 5.** Analysis of PpL affinity-purified scFv 3J24-P8 binding to shGPVI. (A) shGPVI-Fc (circles) or shGPVI-His (triangles) was immobilized on a microtitration plate and incubated with increasing amounts of scFv 3J24-P8. (B) Immobilized shGPVI-Fc was incubated with scFv 3J24-P8 (1  $\mu$ g mL<sup>-1</sup>) in competition with increasing amounts of IgG 3J24 (0 to 50  $\mu$ g mL<sup>-1</sup>). (C) Immobilized shGPVI-His was incubated with scFv 3J24-P8 (0.2  $\mu$ g mL<sup>-1</sup>) in competition with increasing amounts of IgG 3J24 (0–50  $\mu$ g mL<sup>-1</sup>) (–) Incubation was carried out with IgG3J24 (50  $\mu$ g) but no scFv 3J24-P8. In all assays immunocomplexes were detected using PpL-peroxidase conjugate.



**Fig. 7.** Flow cytometry analysis of scFv 3J24-P8 binding to platelets. (A) Platelets were incubated with irrelevant FITC-conjugated mouse IgG<sub>1</sub> (white) or FITC-conjugated IgG 3J24 (blue) ( $4 \mu\text{g mL}^{-1}$ ). (B) Platelets were incubated with PpL-purified scFv 9C2 (white) or 3J24-P8 (blue) ( $7 \mu\text{g mL}^{-1}$ ) revealed by an FITC-coupled anti c-Myc Ig. (C) Platelets were incubated with FITC-conjugated scFv 3J24-P8 ( $7 \mu\text{g mL}^{-1}$ ) in competition with shGPVI-Fc ( $2.5 \mu\text{g mL}^{-1}$ ) (gray) or alone (white). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

up to  $7 \mu\text{g mL}^{-1}$  did not activate platelets (Fig. 8A). In addition, we observed that the purified scFv 3J24-P8 did not induce platelet aggregation at least over a 20-min period while platelets aggregation was induced by the parental IgG even in a molar concentration ( $6.7 \text{ nM}$ ) much lower than that of the scFv ( $40 \text{ nM}$ ) (Fig. 8B).

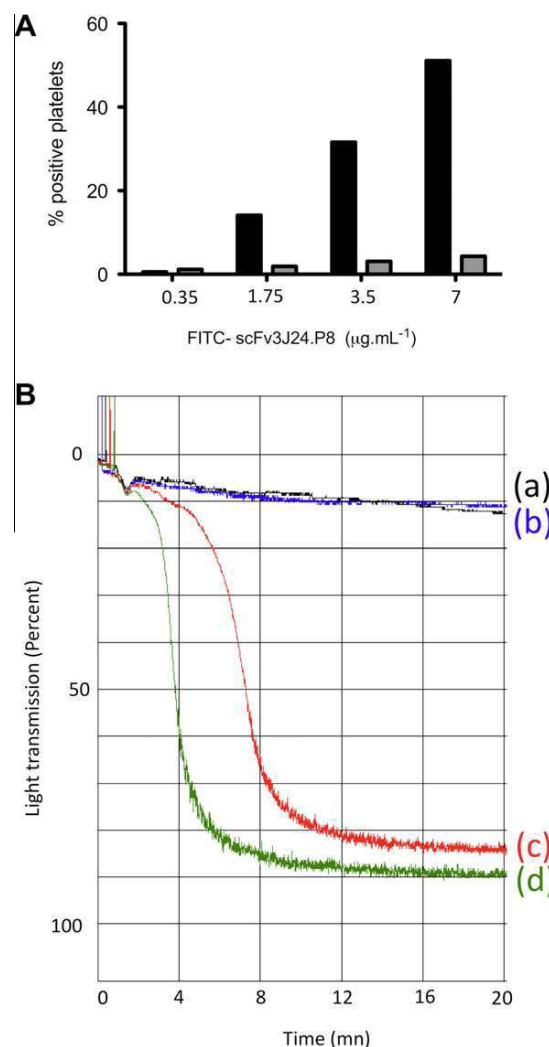
## Discussion

Quantification of platelet surface-expressed GPVI is of utmost importance for the diagnosis of inherited or acquired platelet disorders. Several anti-GPVI monoclonal antibodies have been used for this purpose, but the whole antibody usually activates platelets by favoring dimerization and/or cross-linking GPVI with the Fc $\gamma$ RIIA. As a consequence, the exact number of GPVI copies is altered due to externalization of internal membrane pools, internalization, or ectodomain shedding of the receptor [17,19,33,34]. Therefore, whole antibodies are not fully appropriate for GPVI quantification by cytometry.

The design of a monovalent antibody fragment retaining the parental antibody specificity but with no GPVI-clustering activity appears to be a promising approach for the development of GPVI quantification tools. For this purpose, we selected the construction of an scFv, which represents the minimal size unit that preserves the antigen-binding activity of an antibody. scFvs are only produced by recombinant technologies but they do not require glycosylation to be functional and therefore can be expressed quickly and at low cost in a prokaryotic system. Usually, they are easily produced even if, in some rare cases, difficulties in their proper folding, depending on primary sequences, have been reported [35,36]. Thus engineering of an scFv is an attractive approach as compared to limited papainolysis of whole antibodies into Fab fragments, which is laborious, time-consuming, not always reproducible, and sometimes leading to unfunctional fragments [37]. In addition, recombinant technologies have several advantages because they allow modulating affinity and specificity, to tailor functional properties by guided maturation and to graft novel desired properties [38,39].

The scFv, we designed here, has dual characteristics. It preserves the parental antibody-specific GPVI binding activity and acquires the advantage of not activating platelets. Furthermore, a PpL-binding activity was conferred to the scFv in order to make it feasible for rapid purification and one-step detection without deleterious effects on its natural antigen-binding specificity.

In this context, the first technological challenge was the cloning of an appropriate functional V-domain. As is the case for several hybridomas in which the myeloma fusion partner is Sp2/0-Ag14, the amplicon we got after RT-PCR using primers specific for Vk chain cDNA was heterogeneous. It contained the cDNA correspond-



**Fig. 8.** Analysis of platelet activation and aggregation upon antibody binding. (A) Flow cytometry of washed platelets incubated with increasing amounts of FITC-conjugated scFv 3J24-P8 and PE-conjugated anti-P-selectin antibody. Cells labeled with scFv 3J24-P8 (black) or anti-P-selectin antibody (gray) were counted. (B) Platelet-rich plasma ( $3 \times 10^8$  platelets  $\text{mL}^{-1}$ ) was incubated with (a) PBS, pH 7.4, (b) purified scFv 3J24-P8 ( $1 \mu\text{g mL}^{-1}$ ), (c) purified IgG 3J24 ( $1 \mu\text{g mL}^{-1}$ ), (d) purified IgG 3J24 ( $2.5 \mu\text{g mL}^{-1}$ ).

ing to the aberrant transcript (GenBank Accession No. M35669) that is often produced to a greater extent than the functional one. Here again, the procedure we reported earlier was efficient and allowed us to clone the 3J24 V-KAPPA gene [24].

Another major challenge in scFv generation is its purification and detection. Usually one can take advantage by fusing an additional tag but sometimes it is unsatisfactory. Sometimes tags are not surface exposed or may interfere with the correct folding of the recombinant protein, promote aggregation, and even be cleaved by host proteases or not synthesized. Natural antibody binders such as Protein A, Protein G, or Protein H are widely used for purification of whole antibodies or Fab fragments but cannot be used to purify scFvs because they only interact with antibody constant domains. PpL is the only natural binder of antibody V-domains. PpL has great potential for purification of label free scFvs as well as a probe reagent to detect antigen–scFv complexes, but only about 60% of mammalian immunoglobulin light chains interact with it. Human IGKV subgroups I, III, and IV are considered to be PpL binders as well as murine IGKV subgroup I and some rat, hamster, and pig  $\kappa$  chains but the pattern is ambiguous [30]. Here, we clearly demonstrated that the conservation of all the residues previously identified to be involved in hydrogen bonds and ionic interactions with PpL is not sufficient to preserve strong interaction. These residues also must be properly located in spatial arrangement. We focused on V-KAPPA 5–12 segment, which is involved in  $\beta$  zipper interaction with PpL strand  $\beta$ 2 and we postulated that a specific backbone conformation is required to allow appropriate weak interactions (hydrogen bonds and salt bridges). This was supported by previous structural analysis of antibody fragments in complex with PpL and also by the observation that no V $\lambda$  chain reacts with PpL, probably because their 5–12 segment is one residue shorter [32]. We also observed that all residues of 3J24 V-KAPPA segment 5–12 were identical or similar to those reported in PpL reacting V-KAPPA chain with the exception of residue 8 [31]. After consulting different data banks we noted that Pro is mostly reported at position 8 with some exceptions including all the V-KAPPA belonging to the mouse IGKV10 subgroup (Thr8) and some others having Ser, Gln, His, or Ala at position 8 (results from IMGT/V-QUEST). Undoubtedly, the ring structure of Pro 8 plays a critical role and introduces major steric constraints that favor the correct main-chain conformation of segment 5–12 on which the spatial arrangement of residues involved in interactions with PpL is highly dependent.

The single point mutated scFv 3J24-P8 acquired the capacity to bind PpL with sufficient affinity. The engineered PpL-binding site fulfilled all the criteria for a rapid single step purification and allowed us to capture all the functional forms of the scFv from the periplasmic protein extract. scFv 3J24-P8 was produced in sufficient amounts for all functional characteristics analysis.

Competitive immunoassays confirmed that scFv 3J24-P8 preserved the specificity of the parental antibody. However, when we analyzed scFv 3J24-P8 binding to recombinant GPVI molecules (monomeric shGPVI-His and dimeric shGPVI-Fc), the signal observed with shGPVI-His was lower than that of shGPVI-Fc, whatever the method used (ELISA or Western blot). This difference in reactivity may be related to degree of interactions between the antigen and the immobilizing surface. There may be several plausible explanations. shGPVI-His may interact randomly with the solid surface and there may be masking of the paratope or steric hindrances leading to early saturation of exposed scFv binding sites. Conversely, the Fc fragment of shGPVI-Fc may play the role of a spacer, facilitating the access of the scFv to the paratope and maximizing the signal.

We also observed some discrepancies when scFv 3J24-P8 was investigated for platelet lysate GPVI contents in Western blot. The signal observed in Western blot allowed the detection of

dimeric shGPVI-Fc and platelet lysate GPVI. However it was not sensitive enough for the detection of monomeric shGPVI-His even if apparent affinities of scFv 3J24-P8 for both forms of shGPVI are nearly similar. This lack of sensitivity may underline some difficulties in the use of minimal size antigen-binding molecules such as scFvs to probe particular antigens embedded in nitrocellulose membranes. One must also consider that molecular binding properties are influenced by environmental conditions (ionic strength, pH, interactions with the solid phase), which may differ in each method. Several examples of antibodies efficient for probing in ELISA but not in Western blot and vice versa have also been reported [40].

Finally cytometry experiments allowed us to demonstrate that scFv 3J24-P8 is efficient in specifically recognizing GPVI expressed at the surface of human platelets. Direct coupling of scFv 3J24-P8 with FITC does not interfere with the GPVI-binding activity nor specificity. In addition, no significant activation of platelets was observed as compared to the parental IgG 3J24.

In conclusion, we have demonstrated here that a single point mutation based on sequence analysis of the V-KAPPA chain can confer a PpL recognition site to a recombinant scFv, making feasible its rapid purification and detection without requiring additional tags and also without deleterious effects. In addition, the scFv 3J24-P8 scaffold obtained in this work represents an ideal candidate to design quantitative flow cytometry application kits useful for the diagnosis of platelet disorders related to GPVI expression which are not yet in current practice.

## Acknowledgments

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### **3.2 Design of Anti-GPVI Recombinant Antibody Fragments with Therapeutic Potential**

Collagen binding to GPVI mediates the initial adhesion of the platelet to the vascular wound. Signaling by GPVI leads to the onset of the platelet activation cascade that is finally crowned by a firm and shear-resistant integrin-based adhesive clot. Blockade of GPVI/collagen interaction would prevent initial adhesion and further activation of the platelet and this would have an enormous impact in antithrombotic therapy. Moreover, recent experimental and clinical evidences indicate that GPVI-deficient patients or mice are protected against thrombosis and also not associated with any severe bleeding tendency. This suggests that a GPVI inhibitor would be able to inhibit thrombus formation without causing any significant bleeding tendency. Thus GPVI/collagen interaction inhibitor would show promise as an anti-thrombotic agent for clinical use. Keeping all these in mind, we also converged attention on the therapeutic side.

Therefore, the second part of our present work was the design, production and characterization of various formats of anti-GPVI humanized antibody fragments with therapeutic potential. Previous studies conducted in our laboratory led to the characterization of 9O12.2 monoclonal antibody directed against the glycoprotein VI (GPVI) expressed on the surface of human platelets. This antibody was also obtained by the same gene gun immunization method as for 3J24. The reason for further study of this antibody, was due the fact that the Fab fragment derived from proteolysis of the IgG 9O12.2 was an inhibitor of GPVI functions *in vitro*. Indeed, in whole blood and in flow condition, the Fab 9O12.2 has no effect on primary adhesion of platelets to collagen, but inhibited thrombus formation. More over, it effectively blocks thrombin generation induced by platelets stimulated by collagen. However, the demonstration of its antithrombotic effect *in vivo* requires studies in non-human primates because of the lack of cross-reactivity with a GPVI different from that of human. The mechanism of action of the antibody 9O12.2 is the blockage of GPVI/collagen interaction has been previously demonstrated (Lecut *et al.*, 2004a, Lecut *et al.*, 2003, Lecut *et al.*, 2005).

The Fab fragment of 9O12.2 thus has the expected characteristics of an effective antiplatelet molecule. However, the fully murine origin of the antibody can be the source of serious side effects if administered to humans, including HAMA type immune reactions. Thus, the development of therapeutic potential for therapy is limited. To make it more suitable as a therapeutic tool, we worked on the building blocks (scFvs) for stable monovalent antibody fragments (Fabs)

Thus, first of all the variable domains (VH and VL) of the antibody 9O12.2 responsible for the antigen recognition specificity were identified. They were then produced as a murine scFv, the smallest entity capable of preserving the antigen recognition function. The functional properties of scFv were evaluated. It was observed that the murine scFv preserved all the functional properties of the parental antibody (Annex 2) but murine scFv is unsuitable for therapeutic use so to make it suitable for therapeutic purposes, it must be less immunogenic. Therefore, we decided to carry out the design of scFv humanization, less immunogenic and then further its use as a building block for the construction and design of chimerized and humanized Fabs with more suitable therapeutic potentials.

For this purpose a humanized form of scFv was designed. The final molecule retained the antigen recognition specificity and high affinity for GPVI, both properties that may be altered during humanization of an antibody. Our contributed preliminary results were published in 2009 (Annex 2). The only problem with the construct was its low production yield. We carried out various attempts to enhance the production rate using different possible approaches. For this purpose we first constructed the scFv in the VL-VH orientation. Later on different expression vectors with strong promoters, *E. coli* strains, and media were tested. Moreover, a PpL recognition site was also conferred on it through point mutation for easy detection and purification. All these enable us to produce a humanized scFv construct in high quantity sufficient enough for a detailed functional evaluation.

Our main goal, here was the optimization of the humanized scFv production, evaluation of its functional properties and then its use as a building block in the construction of recombinant Fab fragments.



# **Redesigning of a humanized single chain Fv directed against human platelets GPVI**

**Muhammad ZAHID<sup>a,b</sup>, Maxime BOUABDELLI<sup>b</sup>, Stéphane LOYAU<sup>b</sup>, Philippe BILLIALD<sup>a,c</sup>, Martine JANDROT-PERRUS<sup>b,d</sup>**

<sup>a</sup> Université Paris-Sud 11, IFR 141, Faculté de Pharmacie, 92260 Châtenay-Malabry, France

<sup>b</sup> INSERM, U698, Hôpital Bichat, F-75018 Paris, France

<sup>c</sup> Muséum National d'Histoire Naturelle, USM 505, F-75005 Paris, France

<sup>d</sup> Université Paris 7, UMR698, F-75018 Paris, France

## **1- INTRODUCTION**

Atherothrombotic diseases are one of the leading causes of mortality and morbidity in both men and women in the developed countries and, therefore, has a major burden in public health. Thrombus formation is a major cause of acute arterial ischemia and their clinical manifestations, acute coronary syndrome and stroke. At sites of vascular lesions, blood platelets undergo a rapid transition from a circulating state to an adherent state, followed by their activation and aggregation. The platelet plug actively sustains coagulation and fibrin formation.

The treatment of acute coronary syndromes has been considerably improved in recent years with the introduction of highly efficient antiplatelet drugs, the current standard treatment being based on dual antiplatelet therapy with aspirin and a thienopyridine. However, this strategy still has significant limitations: the recurrence of adverse vascular events remains a problem and the improvement in efficacy is counterbalanced by an increased risk of bleeding. The search for better antiplatelet drugs which efficiently prevent platelet thrombus formation while having a minimal effect on general hemostasis remains a competitive challenge (Jackson *et al.*, 2003).

GPVI may be considered as an attractive target for the development of new molecules with potential antithrombotic activity (Bigalke *et al.*, 2010). GPVI is of central importance for the

activation of platelets by fibrillar collagen of types I and III, the most thrombogenic compounds of the subendothelial matrix and abundantly present in atherosclerotic plaques.

Despite the accumulating data in favour of GPVI as a major target, controversies subsist when considering the different mouse models of thrombosis. Depending on the model used to induce thrombosis, the respective parts played by GPVI and thrombin in mediating platelet activation and aggregation vary. GPVI appears to be important in the carotid ligation model [Jandrot-Perrus *et al.* 2000, Suguyama *et al.* 1987], but dispensable or less critical in severe injuries induced by FeCl<sub>3</sub> or a laser, as compared to the increasing involvement of thrombin in these models (Dubois *et al.*, 2006). A major limitation of all these *in vivo* models is that thrombosis is triggered on healthy vessels. The mechanisms triggered in these conditions are expected to resemble more those insuring physiological hemostasis than those involved in pathological thrombosis. There are several lines of evidence that GPVI is of critical importance in atherothrombosis. *In vitro* studies of platelet thrombus formation under flow on immobilized plaque extracts indicated that collagen-GPVI interaction triggers platelet accumulation on atherosclerotic lesions. Moreover, GPVI deficiency prevented thrombus formation on injured plaques in ApoE<sup>-/-</sup> mice (Hechler and Gachet, 2011). *In vivo*, recombinant soluble GPVI was shown to accumulate within atherosclerotic plaques in the rabbit and to protect ApoE<sup>-/-</sup> mice from arterial remodeling after mechanical injury .

Interestingly, targeting GPVI appears to carry a low bleeding risk. One clear cut result yet obtained in mice is the absence of bleeding when GPVI is absent or blocked. Moreover, patients presenting a congenital or autoimmune GPVI deficiency display a mild bleeding. Furthermore, GPVI expression being restricted to platelets and megakaryocytes, the side effects of GPVI antagonists should be limited.

Several strategies may be employed to inhibit GPVI-triggered platelet activation. Use of the soluble immunoadhesin GPVI-Fc to compete for collagen binding has been reported to reduce thrombosis in different animal models, limit plaque development in hypercholesterolemic rabbits and inhibit neointima formation after plaque denudation in ApoE<sup>-/-</sup> mice. Very recently, a phase I study demonstrated that GPVI-Fc efficiently inhibited collagen-induced platelet aggregation with no alteration of primary hemostasis. A second possibility is the use of antibodies directed against GPVI because some of them have the capacity to block the interaction of GPVI with collagen, while others induce platelet GPVI depletion.

Our group has also developed and characterized one murine monoclonal antibody 9O12.2 of high affinity for human GPVI. Purified IgGs block GPVI binding to collagen but they activate



platelets by a mechanism including homotypic crosslinking of GPVI and heterotypic crosslinking with Fc $\gamma$ RIIA at the platelet surface (Lecut *et al.*, 2003). In contrast, the monovalent Fab 9O12 inhibits collagen-induced platelet aggregation as well as the procoagulant activity of collagen-stimulated platelets and prevents thrombus formation under static and flow conditions. One bolus injection of the Fab 9O12.2 rapidly and reversibly inhibited collagen induced platelet aggregation and procoagulant responses *ex vivo* in non-human primates (Ohlmann *et al.*, 2008). Furthermore, the proof of concept that the Fab 9O12 inhibits thrombosis *in vivo* has recently been obtained in transgenic mice expressing human GPVI (Mangin *et al.*, submitted for publication). However, the murine origin of the Fab 9O12 is the major hindrance for its clinical development. A murine scFv has been constructed and retained *in vitro* the affinity and inhibitory properties of the Fab 9O12.2 (Muzard *et al.*, 2009b). A first humanized version of the scFv was functional. However, the level of production of the soluble recombinant antibody fragment in *E. coli* was extremely low, preventing any preclinical trial.

So here, we are reporting the cloning, engineering and an elaborated evaluation of the derived VL-VH orientated humanized scFv produced in recombinant bacteria using different media. It has the ability to retain all the functional activities of the parental antibody and in addition presents the recognition motif for protein L (PpL), an important feature which render to recombinant protein the characteristics of easy purification without requiring any external tag. Moreover, the new constructed humanized scFv 9O12 was produced in enough high quantity which allowed us to carry out all the functional tests and its antithrombotic potentials.

## **2- MATERIALS and METHODS**

### **2-1 Materials**

The recombinant soluble human GPVI (shGPVI-Fc) consisting of two extracellular domains of the receptor (GPVI), coupled to the Fc fragment of human IgG1 was produced as already reported (Jandrot-Perrus *et al.*, 2000).

Monoclonal antibody 9O12.2 was obtained by immunizing Balb/C mice with the cDNA encoding the recombinant GPVI-Fc fusion protein using GeneGun technique as already reported (Lagrue-Lak-Hal *et al.*, 2001).

The murine scFv 9O12 ( $_m$ scFv9O12) directed to the GPVI of human platelets and scFv 9C2 directed against scorpion toxins irrelevant to GPVI have been reported elsewhere (Devaux *et al.*, 2001, Muzard *et al.*, 2009a, Muzard *et al.*, 2009b). GPVI-Fc was coupled to cyanogen

bromide-activated Sepharose according to the manufacturer's instructions (Amersham-Pharmacia, Les Ulis, France). Protein L (PpL) immobilized on agarose or peroxidase-conjugated were from Pierce Biotechnology (Rockford, USA). Anti-mouse IgG, anti-human IgG (Fc specific) from Jackson Immuno-Research Labs Inc (West Grove, PA, USA), and anti-cMyc (9E10) conjugated to horseradish peroxidase (HRP) (Sigma Aldrich, Saint Louis, USA), anti-P-selectin-FITC, anti-mouse IgG1-FITC and anti-CD41-FITC (Beckman Coulter, Marseille, France) were used according to manufacturer instructions.

Washed human platelets were obtained according to a previously described procedure (Jandrot-Perrus *et al.*, 2000). All chemicals were of standard grade from Sigma-Aldrich or equivalent.

### **Vector**

The bicistronic pCOMB3HSS which is originally designed for the expression of Fab fragments as previously reported (Barbas *et al.*, 1991) was used for the construction of a universal vector. Gene III, *pelB* leader sequence and human heavy chain stuffer were removed in order to make it monocistronic for the expression of scFv. A cDNA encoding 9C2 V-kappa FR1 was then cloned in the monocistronic vector for conferring PpL-recognition ability. This universal constructed vector (pPpLK7) can be used for the periplasmic expression of any VL-VH oriented scFv and can also confer PpL-recognition motif to the expressed scFv.

A synthetic gene was designed encoding VL-linker-VH oriented humanized scFv 9O12 (L<sub>h</sub>scFv 9O12) which was synthesized by GenArt without any optimization (GenArt, Regensburg, Germany). In the synthetic gene natural codons of amino acid residues non-concerned with humanization were preserved. All molecular biological techniques were used as previously described (Sambrook, 2001). All mutations in the sequence were carried out using Quik Change mutation Kit (Agilent Technologies, Massy, F).

## **2-2 Methods**

### **Protein expression**

100µL of an overnight preculture of *E. coli* strain TG1 was inoculated into five different media (100mL each) including 2xTY broth (MP Biomedicals, LLC Fountain, France), Turbo broth<sup>TM</sup>, Power broth<sup>TM</sup>, Superior broth<sup>TM</sup> and Hyper broth<sup>TM</sup>, (AthenaES, Baltimore, MD) containing 100µg.mL<sup>-1</sup> ampicillin and grown at 37°C at 125 g until A<sub>600</sub> reached 0.9. The bacterial cultures were induced for expression of recombinant protein by the addition of Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.8mM) followed by incubation at 16°C at 110 g for 16h. The cells were then harvested by centrifugation at 15000 rpm for 20 min in ice. The

cells were resuspended in TES buffer (0.2 M Tris/HCl, PH 8.0, containing 0.5 mM EDTA and 0.5 M sucrose), and incubating for 30 min. Cells were then subjected to osmotic shock by adding TES buffer diluted 1:4 for 30 min on ice, and then centrifuged at 15000 g for 30 min at 4°C to remove insoluble materials.

#### **SDS-PAGE and Western blot analysis**

The expressed recombinant protein was analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (Laemmli, 1970). Soluble protein fractions were mixed with 2xSDS sample loading buffer and boiled for 5 min. The samples were run on 12% SDS-PAGE gels at 150 V for 90 min using Bio-Rad mini protein system (Bio-Rad Laboratories). The resolved proteins samples were visualized by staining with Coomassie brilliant blue.

The presence of the recombinant L<sub>h</sub>scFv 9O12 in the periplasmic extracts and the affinity purified fractions was analyzed by Western blot analysis. The recombinant proteins resolved on a 12% SDS-PAGE gel were transferred to nitrocellulose membrane for 2h at 150mA in transfer buffer using Bio-Rad mini trans blot apparatus. After blocking with 5% skimmed milk in PBS, pH 7.4 containing 0.1% Tween 20, membranes were incubated with the anti-cMyc antibody 9E10 overnight at 4°C and then with anti-mouse IgG (Fc specific) conjugated with alkaline phosphatase for 2h at room temperature. Membranes were washed five times with PBS, pH 7.4 containing 0.1% Tween 20 between each steps The positive reactivity was visualized using 5-bromo-4-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) as a substrate (SIGMA-ALDRICH, St. Louis, USA).

#### **Purification of recombinant antibody fragments**

The periplasmic extracts (30mL) were incubated with GPVI-Sepharose gel (500µL) overnight at 4°C and then for 2h at room temperature under slow rotation. The mixture was loaded on a microcolumn and washed with PBS, pH 7.4. The bound recombinant proteins were then eluted by glycine-HCl 0.1M, PH 2.5, in 0.5 mL fractions, and immediately neutralized with 40µL Tris 1M on ice. Fractions with A<sub>280</sub> equal and higher than 0.1, were pooled and extensively dialyzed against PBS, pH 7.4 at 4°C. Alternatively, purification was carried out using PpL-Agarose beads (Pierce Biotechnology, Rockford, USA) and elution was performed as for GPVI-Sepharose gel.

The concentration of the purified recombinant protein was evaluated after measuring absorbance at 280 nm and using the Swiss Institute of Bioinformatics software (ProtParam tool) to determine its theoretical Mr and extinction coefficient (Gasteiger *et al.*, 2003).

#### **ELISA**

Human soluble GPVI-Fc (100  $\mu\text{L}$ , 2  $\mu\text{g.mL}^{-1}$  in PBS, pH 7.4) was immobilized on Immulon 2HB 96-well plates (Thermo Fisher Scientific, Rockford, IL, USA) by incubating for 16 h at 4°C. Wells were next saturated with 1% bovine serum albumin. The plates were then incubated with the scFv (20  $\mu\text{g.mL}^{-1}$ , 100  $\mu\text{L}$ ), followed by incubation with 100  $\mu\text{L}$  of HRP-coupled PpL or HRP-coupled anti-cMyc monoclonal antibody 9E10. The substrate OPD (ortho phenylene diamine/ $\text{H}_2\text{O}_2$ , 100  $\mu\text{L}$ ) was then added to the wells for 5 min. The reaction was stopped by sulfuric acid 3M and the absorbance was read at 485 nm. The wells were washed five times with PBS, pH 7.4 containing 0.1% Tween 20 and 0.1  $\text{mg.mL}^{-1}$  bovine serum albumin, between each steps. Controls were performed either omitting coating with shGPVI-Fc or using periplasmic extracts containing irrelevant scFvs (anti-scorpion toxin scFv-9C2 having reactivity with PpL or the anti-GPVI<sub>m</sub>scFv 9O12 which does not interact with PpL).

### **GPVI binding to collagen**

To analyze the blocking effect of the antibody fragments on the GPVI-collagen interaction, microtitration plates were coated with fibrillar type 1 collagen (20  $\mu\text{g.mL}^{-1}$ , 100  $\mu\text{L}$ , equine tendon; Horm, Nycomed, Munich) and saturated as previously described. Then, 100  $\mu\text{L}$  of shGPVI (20  $\mu\text{g.mL}^{-1}$ ) that was preincubated for 30 min at room temperature with the affinity purified antibody fragments (20  $\mu\text{g.mL}^{-1}$ ) was added to each well. After incubation for 90 min, the bound GPVI was detected using a HRP-coupled anti-human Fc and OPD. All incubations were performed at room temperature, and five washing with 200  $\mu\text{L}$  of PBS, pH 7.4 containing 0.1  $\text{mg.mL}^{-1}$  BSA and 0.1% Tween 20 were carried out between each intermediate step. All assays were conducted in triplicate. Data were analyzed and graphs were produced using PRISM GraphPad (San Diego, CA, USA)

### **Flow cytometry**

Washed human platelets ( $3 \times 10^8$  platelets. $\text{mL}^{-1}$ ) obtained as previously reported (Jandrot-Perrus *et al.*, 2000), were preincubated with purified L<sub>h</sub>scFv 9O12 or control antibody fragments (m<sub>h</sub>scFv 9O12 or irrelevant scFv 9C2), and then incubated with 5 $\mu\text{L}$  FITC-coupled anti-cMyc antibody. Samples were analyzed for cell fluorescence on a LSRII flow cytometer (BD Biosciences, Le Pont de Claix, France). Platelets were gated on forward and side scatter and more than 95% of events were positive for CD41. A mouse IgG<sub>1</sub> (isotypic control) was used to define negative events in the population of gated platelets. Binding was estimated using either irrelevant scFv 9C2 or PBS, pH 7.4.

In order to analyze the inhibition of collagen-induced platelet activation, platelets were incubated with L<sub>h</sub>scFv 9O12 (10-40 $\mu\text{g. mL}^{-1}$ ). Then platelets were activated with collagen for

15 min at room temperature. 5 $\mu$ L anti-P-selectin IgG conjugated to FITC (Beckman Coulter, Villepinte, France) was added to the cells for 30 min before analysis of the cell suspension by flow cytometry. All incubations were carried out for 30 min in the dark at room temperature. In control experiments L<sub>h</sub>scFv 9O12 preparations were replaced by murine Fab 9O12 or irrelevant scFv 9C2 or PBS, pH 7.4. All experiments were performed at least in triplicate.

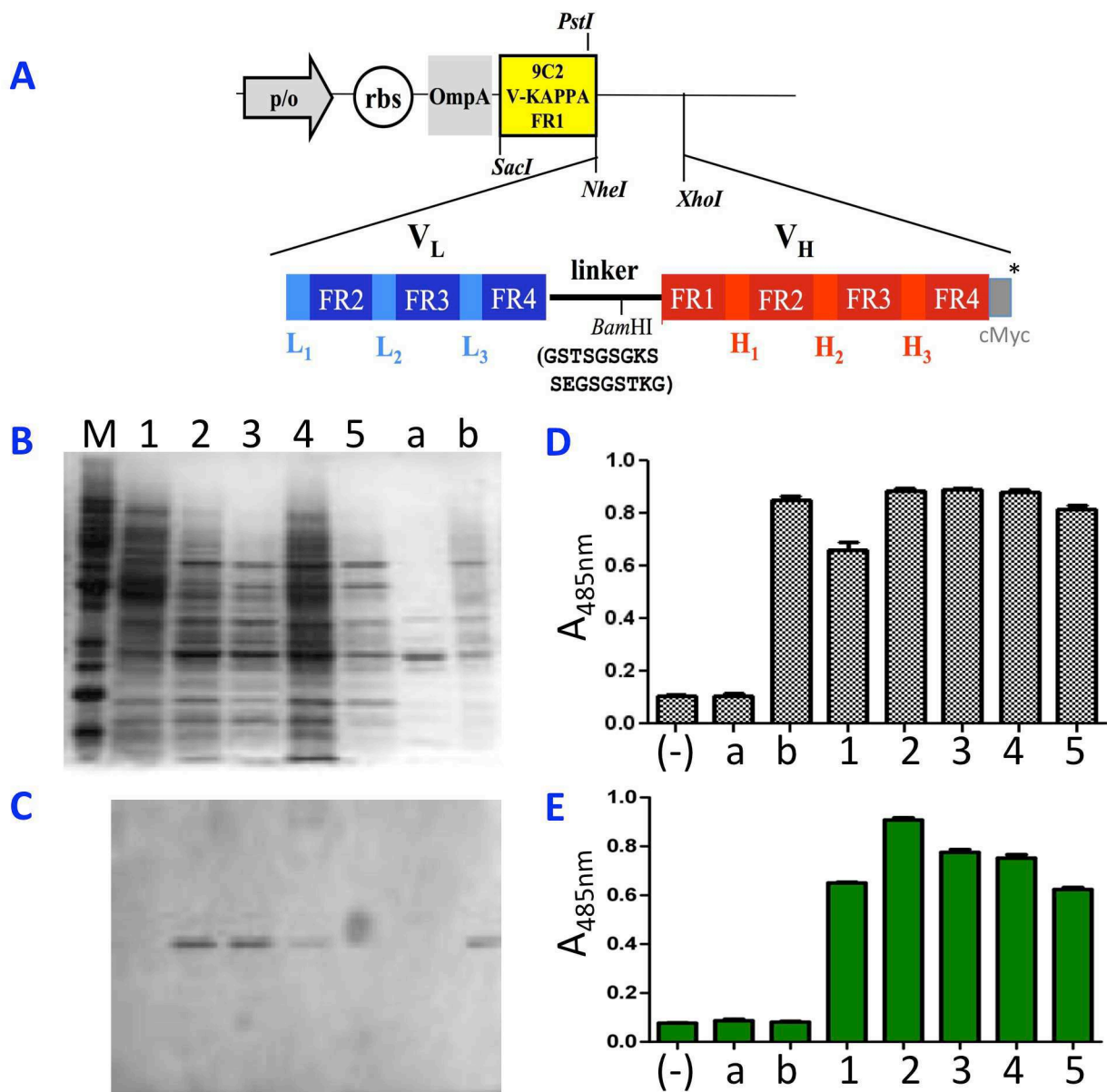
### **Platelet aggregation**

Washed human platelets (3 $\times$ 10<sup>8</sup> platelets.mL<sup>-1</sup>) were incubated with murine Fab 9O12 (2.5  $\mu$ g.mL<sup>-1</sup>) or affinity purified L<sub>h</sub>scFv 9O12 (25  $\mu$ g.mL<sup>-1</sup>) or irrelevant scFv 9C2 in PBS, PH 7.4 without stirring for 5 min at 37°C. Platelet aggregation was then initiated by adding collagen type 1 to final concentration of 1 $\mu$ g.mL<sup>-1</sup> under stirring conditions at 37°C for 20 min. Platelets aggregation induced changes in light transmission that were continuously recorded using a Chronolog Aggregometer (Chrono Log Corp; Harveston, PA, USA).

## **3- RESULTS**

### **3-1 Design and bacterial expression of scFv**

A universal vector was constructed with dual characteristics starting from the bicistronic phagemid vector pCOMB3HSS which was originally designed for the expression of Fab fragments. On one hand this constructed vector designated as pPpLK7 will be used for the expression of any recombinant scFvs in VL-VH orientation and on the other hand it would directly confer Protein L recognition motif to the expressed recombinant scFv which will make its purification and detection easier. For the construction of this universal vector, the pCOMB3HSS was digested with *NheI* and *SpeI* and then self ligated in order to remove gene III and distortion of the said restriction sites. Again self-ligated vector was double digested with *SacI* and *XhoI*, removing *pelB* leader sequence and human heavy chain stuffer, which makes it monocistronic and suitable for the expression of scFvs. Then, a cDNA encoding 9C2 V-kappa FR1 followed by a spacer having the residues (ELDVQMTQSPASLSVSVGETVTITCRASQSLE) recognizing PpL was cloned in the vector down stream to *OmpA* leader sequence with *SacI* at its 5' end and *XhoI* at the 3' end (AGCTCGACGTCCAGATGACTCAGTCTCCAGCCTCCCTATCTGTATCTGTGGGAGAACTGTCACCATCA CCTGCAGAGCTAGCCAAAGCCTCGAG) (Fig. 1A). Finally, a synthetic gene encoding humanized scFv 9O12 (hscFv 9O12) with removed FR1 sequence was inserted in the constructed vector in between *NheI* (GCTAGC) /*XhoI* (CTCGAG) restriction sites. This synthetic gene encodes the humanized VL 9O12 (hVL 9O12) devoid of its FR1 fused with the humanized VH 9O12 (hVH 9O12) via a flexible linker (GSTSGSGKSSEGSSTKG) and a c-Myc tag at its C-



**Figure 1: Construction of vector pPpLK7 and evaluation of the  $L_h$ scFv 9012 protein from periplasmic extracts of recombinant *E. coli* TG1.**

**A-** Schematic representation of vector pPpLK7.

**B-** SDS-PAGE stained with Coomassie brilliant blue.

**C-** Western blot carried out using the anti-cMyc antibody 9E10.

**D-E-** ELISA of periplasmic extracts containing scFv using immobilized  $_{sh}$ GPVI-Fc. Detection was carried out using either the anti-cMyc antibody 9E10 (**D**) or PpL-HRP conjugate (**E**).

M: molecular mass standards (19, 20, 25, 30, 40, 50, 60, 70, 80). Bacteria expressing the  $L_h$ scFv 9012 were grown in 2xTY (1), Turbo broth™(2), Power broth™(3), Superior broth™(4) or Hyper broth™(5). Periplasmic extracts of bacteria expressing the irrelevant scFv 9C2 (a) or  $_{m}$ scFv 9012 (b) were used as controls. (-) ELISA plate coated with BSA.

terminus. As restriction site *NheI* rarely occur in the scFv sequence so to inculcate this site a mutation of S25>A was carried out in the CDR1 of VL. Another point mutation (T90>K) of VL was also introduced in the <sub>h</sub>VL, since previous observations have shown that such a basic residue is required at position 90 in the VL chain for PpL binding (Zahid *et al.*, 2011).

The production of L<sub>h</sub>scFv 9O12 was evaluated in periplasmic extracts by electrophoresis, immunoblot and ELSA. The scFv was not well expressed using conventional culture media (LB and 2XTY) even under various other conditions (temperature, IPTG concentrations, phase and duration of induction and using different *E. coli* strains).

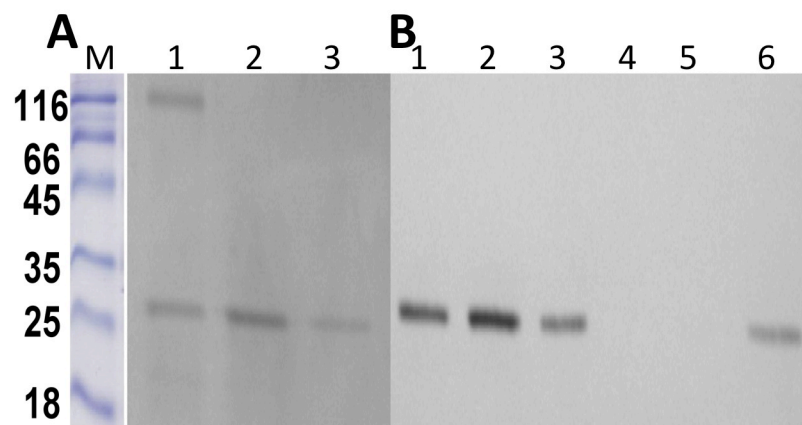
However, improved expression of the L<sub>h</sub>scFv 9O12 was observed when testing other culture media under similar induction conditions. Fig 1B shows the expression of the scFv (28kDa) in different culture media: while using conventional medium (2XTY) the band corresponding to the scFv is very faint, its intensity greatly increased when bacteria were cultured in Turbo and Power broth media. The scFv was then produced almost as the murine scFv 9O12 (<sub>m</sub>scFv 9O12), allowing to estimate the yield at approximately 500 µg.mL<sup>-1</sup> (Fig. 1B, C).

ELISA showed that bound to its ligand GPVI indicating that the expressed recombinant antibody fragment was functional. Furthermore, the L<sub>h</sub>scFv 9O12 and <sub>m</sub>scFv 9O12 were both detected by anti-cMyc indicating that the scFv has conserved c-Myc tag (Fig. 1D); L<sub>h</sub>scFv 9O12 was detected using anti-PpL indicating that the mutations and expression in pPpLK7-L<sub>h</sub> 9O12 has been successful for introducing the PpL motif (Fig. 1E). Thus, the scFv production was enhanced with the advantage of conferring it the PpL recognition motif which should make its purification and detection easier without disturbing the functional properties.

### 3-2 Affinity purification of functional scFv

L<sub>h</sub>scFv 9O12 was purified from periplasmic extracts by GPVI-Sepharose gel. The GPVI-Sepharose purification procedure made it possible to recover the recombinant scFv with a high yield similar to that of murine version of the scFv 9O12. The preparation was homogeneous when analyzed by Coomassie blue staining and Western blot using the antibody against c-Myc with a single band of an apparent Mr close to 28kDa when compared to pre-stained molecular weight markers (Fig. 2A, B), close to the theoretical molecular mass of 28586 Da calculated from the amino acid sequence of L<sub>h</sub>scFv 9O12. Preliminary assays also show the feasibility of purification using PpL-affinity chromatography. Large batches are now under purification phase after the production in optimized medium.

### 3-3 Functional evaluation of L<sub>h</sub>scFv 9O12



**Figure 2: Analysis of the  $_{sh}GPVI-Fc$  affinity purified  $L_hscFv$  9O12 preparation**

**A-** SDS-PAGE stained with Coomassie brilliant blue.

**B-** Western blot carried out using the anti-cMyc antibody 9E10.

M: molecular mass standards.

Lanes 1 to 3: Retained fractions eluted from the column at pH 2.5.

Lanes 4 and 5: Flowthrough fractions

Lane 6:  $_{m}scFv$  9O12



### **Reactivity in ELISA**

The antigen-binding activity of the affinity purified L<sub>h</sub>scFv 9O12 was first investigated in ELISA (Fig. 3A, B). This assay demonstrated the ability of L<sub>h</sub>scFv 9O12 to bind to shGPVI-Fc as is the case for mscFv 9O12. Binding was detected with both the anti-cMyc antibody and PpL-conjugate. These data indicate that the purification did not alter the functional property of the L<sub>h</sub>scFv 9O12 (Fig. 3C).

We also investigated whether the affinity purified L<sub>h</sub>scFv 9O12 interaction with GPVI could inhibit the binding of shGPVI-Fc to immobilized collagen by preincubating GPVI with L<sub>h</sub>scFv 9O12. As demonstrated in Fig. 3C, L<sub>h</sub>scFv 9O12 inhibited shGPVI-Fc (20 µg.mL<sup>-1</sup>) binding to collagen similar as that of mscFv 9O12. In contrast, the irrelevant anti scorpion toxin scFv 9C2 had no effect on the binding of GPVI to collagen.

### **Binding to platelet GPVI and inhibition of platelet activation**

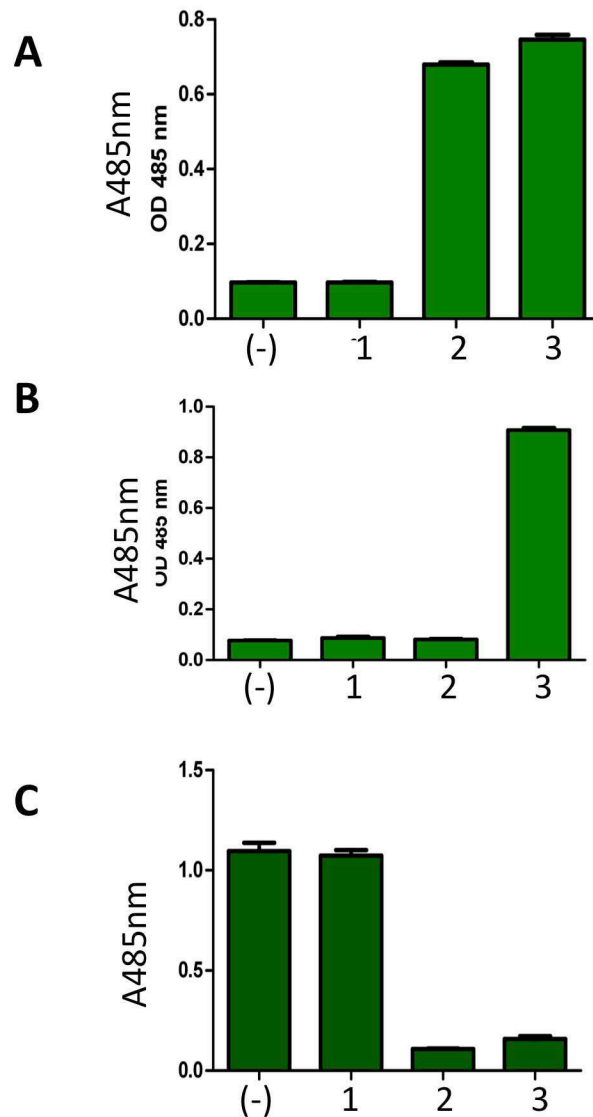
The ability of L<sub>h</sub>scFv 9O12 to bind native GPVI expressed at the surface of platelets was investigated in flow-cytometry (Fig. 4). L<sub>h</sub>scFv 9O12 retains the ability for binding to platelets GPVI similar to the mscFv 9O12 when using FITC-coupled anti-cMyc antibody as a secondary reagent for detection (Fig. 4A).

The effect of L<sub>h</sub>scFv 9O12 on platelet activation was analyzed by measuring the exposure of P-selectin. Incubation of platelets with L<sub>h</sub>scFv 9O12 did not induce exposure of P-selectin on platelets as compared to resting conditions indicating that L<sub>h</sub>scFv 9O12 does not activate platelets. In addition, preincubation of platelets with L<sub>h</sub>scFv 9O12 binding inhibited the exposure of P-selectin in response to collagen (Fig. 4B). Collagen-induced platelet activation was not inhibited by the irrelevant scFv 9C2 indicating that the effect of L<sub>h</sub>scFv 9O12 is specific.

The inhibitory effect of affinity-purified L<sub>h</sub>scFv 9O12 was further analyzed on collagen-induced platelet aggregation. The L<sub>h</sub>scFv 9O12 (25µg.mL<sup>-1</sup>) fully inhibited collagen induced aggregation as does the murine Fab 9O12 (Fig. 5). In addition, we also observed that the antibody fragments do not inhibit aggregation of platelets induced by thrombin or thrombin receptor agonist peptide (TRAP) (results not shown).

## **4-DISCUSSION**

We have already reported the construction and design of the murine scFv 9O12 and then its humanization (Annex 1) but the production level of the humanized version was very low and insufficient for detailed functional evaluation.



**Figure 3: Analysis of affinity purified L<sub>h</sub>scFv 9O12 binding to <sub>sh</sub>GPVI-Fc**

**A-B** <sub>sh</sub>GPVI-Fc was immobilized on a microtitration plate and incubated with preparation of scFvs. Immunocomplexes were detected using either anti-cMyc antibody conjugated to peroxydase (**A**) or PpL-peroxydase (**B**).

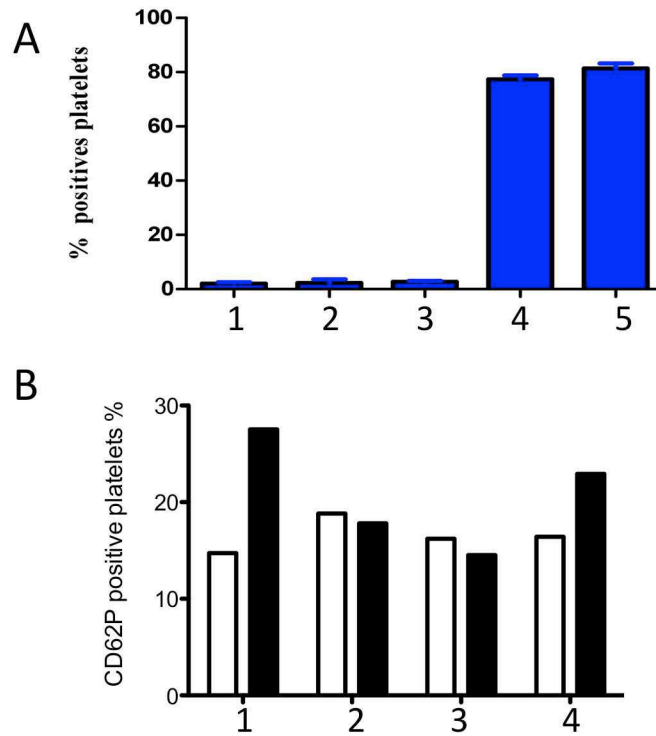
**C**- Collagen was immobilized on a microtitration plate and <sub>sh</sub>GPVI-Fc binding to collagen was investigated in the presence of scFv preparation.

(-) BSA was used in place of <sub>sh</sub>GPVI-Fc

(1) irrelevant scFv 9C2

(2) <sub>m</sub>scFv 9O12

(3) L<sub>h</sub>scF v9O12



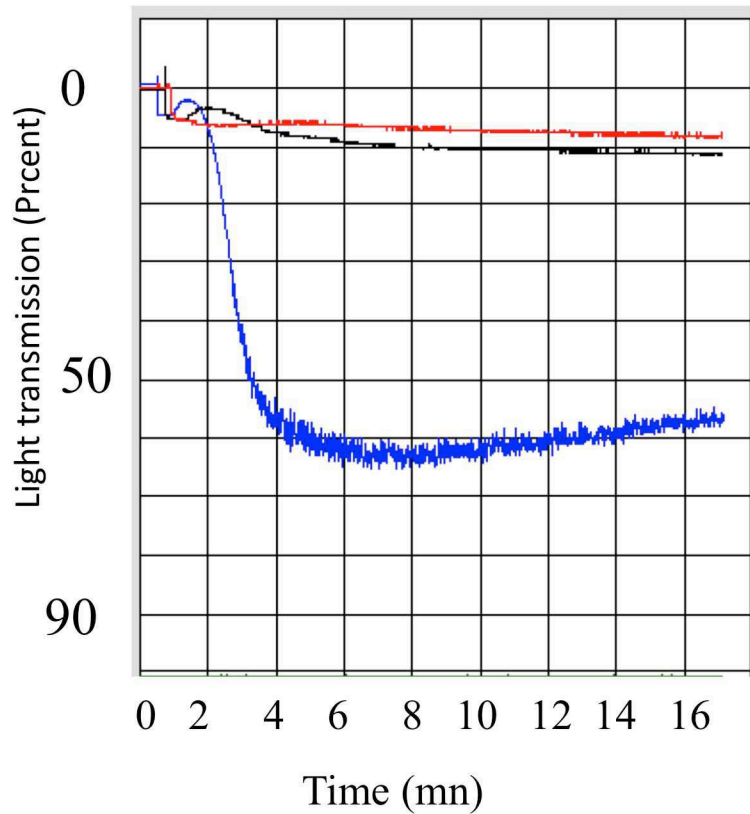
**Figure 4: Flow cytometry analysis of  $L_h$ scFv 9O12 binding to platelets**

**A-** Platelets were incubated with an scFv preparation and revealed by an FITC-coupled anti-cMyc Ig.

- (1) direct incubation of platelets with an irrelevant FITC-conjugated IgG.
- (2) direct incubation of platelets with the FITC conjugated anti-cMyc antibody.
- (3) irrelevant scFv 9C2.
- (4)  $m$ scFv 9O12.
- (5)  $L_h$ scF v9O12.

**B-** Platelets were incubated with an antibody preparation (scFv or Fab) and then activated with collagen (black) or not (white). P-selectin exposure was detected using FITC-conjugated antibody against P-selectin.

- (1) Incubation with an antibody fragment was omitted
- (2)  $L_h$ scFv 9O12
- (3) murine Fab 9O12
- (4) Irrelevant murine scFv 9C2



**Figure 5: Effects of L<sub>h</sub>scFv 9O12 on platelet aggregation induced by collagen**  
Washed human platelets were incubated with the antibody fragment and then, collagen was added. Aggregation was analyzed at 37°C while stirring, and the change in light transmission was recorded  
in red: murine Fab 9O12  
in black: L<sub>h</sub>scFv 9O12  
in blue: irrelevant scFv 9C2

The objective of this study was, therefore, the production of the humanized scFv 9O12 (L<sub>h</sub>scFv 9O12) in high enough quantity which would be sufficient for all functional evaluation. In order to increase the production yield of the recombinant proteins various aspects and factors were considered. Here, we succeeded to improve the production level sufficient enough for a detailed functional analysis by using VL-VH oriented scFv and its expression in *E. coli* grown in optimized medium.

A combination of several factors influence productivity such as efficiency of transcription, mRNA stability, mRNA folding, codon usage, solubility and protein folding (Jana and Deb, 2005). Redesigning a heterologous gene for optimal expression requires both adjusting the codon and codon pair bias as well as eliminating unfavourable mRNA structure (Gustafsson *et al.*, 2004). We investigated new constructs, with inversion of the orientation (VL-VH) using either codons optimized for prokaryotic expression or codons identical with those of the natural mscFv with the exception of codons encoding mutated residus and not concerned with humanization. But in both cases, no improvement in production was observed using conventional medium.

Other parameters which may also influence the yield of production of recombinant protein are the choice of culture medium, mode of cultivation, promoter, strain improvement, expression system control, arrangements of the sequence (VH-VL or VL-VH orientation), culturing temperature, duration and temperature of induction. As much of the effort aimed at increasing recombinant protein production in bacterial strains has been directed at maximizing the biomass production and little is known about the effects of media composition on the expression of recombinant proteins. Therefore, we focused on the use of optimized media. The accumulation of recombinant proteins as insoluble products is a major shortcoming to using *E. coli*-based expression systems and different approaches reported to increase the relative accumulation of soluble protein included lowering the temperature during induction (Cabilly, 1989, Piatak *et al.*, 1988), reducing the concentration of inducer, using mutant strains which affect protein folding, fusing the target protein to a bacterial protein (Forrer and Jaussi, 1998, Kapust and Waugh, 1999, Sachdev and Chirgwin, 1998). It now seems that medium composition may also affect protein folding and most likely the effect is through modulation of expression of the accessory proteins involved in protein folding. Therefore, the best medium must be determined empirically (Baneyx, 1999). Here, a simple screening technique was developed to rapidly identify the best available medium for the production of a recombinant scFv. The screen was performed on five different media and shown to be an

effective means of empirically determining the best medium for the production of recombinant scFv. It was observed that the medium composition affected the relative level of soluble protein that accumulated. It was further observed that functional recombinant antibody fragments production was independent of biomass accumulation. During expression of the recombinant antibody fragments, we noted a lower biomass for the Turbo broth than in Hyper, 2xTY and Power broths but a higher amount of functional recombinant antibody fragments production for Turbo broth.

The general aim of recombinant protein expression is a high productivity and fusion tags are commonly used for their purification because Protein A, G can not be used for scFvs purification as these proteins recognizes only constant domains. But the external tag may be immunogenic in nature, sometimes it can interfere with proper folding and, therefore, with the functional properties of the recombinant proteins. In addition, sometimes, it promotes aggregation as it has been observed with (His)<sub>6</sub> tags. An internal sequence, such as protein L recognition motif, which can make easy the detection and purification of recombinant protein, without being immunogenic, would be a better option. Protein L binds to some but not all kappa light chain of the antibodies. Here, based on previous structural analysis (Muzard *et al.*, 2009a, Zahid *et al.*, 2011), we constructed the universal pPpLK7 vector which introduces a specific FR1 which has the PpL-recognition motif. As already demonstrated that this FR1 does not interfere with the antigen binding ability. This vector would have a great potential for the expression of any scFv without requiring an external tag as it will confer PpL-recognition ability to the expressed recombinant antibody fragments (scFvs).

The need for the better, specific and efficient antithrombotic drugs, and approval of many antibody-derived molecules for the treatment of various diseases, stimulated the urge and search for potent antithrombotic antibodies or recombinant antibody fragments. The first fruit of this search was the approval of a chimeric mouse-human Fab abciximab (Reopro<sup>®</sup>), which has the ability to block platelet GPIIb-IIIa receptor, and is used for the treatment of the patients with acute coronary syndrome undergoing percutaneous coronary surgery (De Luca *et al.*, 2005, Kastrati *et al.*, 2006). However, abciximab can induce the formation of human anti-chimeric antibodies (HACA) and is not used by most of the patients.

Very few anti-GPVI antibodies which can efficiently block the collagen-induced platelet activation have been reported (Lecut *et al.*, 2003, Matsumoto *et al.*, 2006). It was observed that Fab 9O12 obtained after enzymatic treatment of murine 9O12 antibody have antithrombotic effects by blocking GPVI-collagen interaction in non-human primates without

platelet GPVI depletion (Ohlmann *et al.*, 2008). These characteristics make 9O12 as an excellent template for the design of antithrombotic molecules with potential clinical applications. But the murine nature of 9O12 make it highly immunogenic and unsuitable for therapeutic use and moreover, whole IgG can cause GPVI bridging leading to platelet activation, so may not be a suitable antithrombotic candidate. Thus, scFv which is the minimal size functional unit of the antibody and produced using *E. coli*, was selected. As the development of recombinant antibodies in single chain Fv (scFv) format is a good alternative to obtain high affinity antibodies against any target (Liu *et al.*, 2002). The affinity of scFvs for the targets is comparable or even higher than the parental antibody (Hoet *et al.*, 2005). As general rules, scFvs possess several advantages in comparison to IgG or Fab such as higher tissue penetrance and more rapid clarification (Adams *et al.*, 2001, Jain, 1996). The major drawback may be its short half life, which can be counterbalanced by using different approaches of chemical modifications such as pegylation (Certolizumab pegol, a pegylated Fab', anti-TNF $\alpha$  used for the treatment of Crohn's disease) or genetic fusion to a peptide which prolong the plasma half life (developped by XL-Protein) and/or the use of the scFv as building blocks for the construction of Fab, a better format therapeutic agent. Another problem may be the tendency to dimerize or to make aggregates particularly when mutations are introduced as is the case in humanization. It is often observed that scFv exhibited several multimeric forms which are also functional but less stable and are reverted back to dimeric or monomeric forms (Aubrey *et al.*, 2003, Devaux *et al.*, 2001).

Here all the mutated residues for humanizations were retained as the same group as that of original, acidic residues were mutated for acidic, basic for basic and hydrophobic for hydrophobic. But, it can not be ensured that these mutations would not promote aggregation and this should be a major drawback. Thus, this means that other monovalent format may be more suitable. Fabs are stabilized by the association of constant domains, CL and CH1 which favours correct orientation of the V-domains and hinder the hydrophobic part which is in contact with the constant domains but exposed to the solvent in scFvs, favouring association with another scFvs as it is well described (Aubrey *et al.*, 2003, Rothlisberger *et al.*, 2005).

As described, our group have already designed a murine scFv 9O12 and then carried out the humanization, retaining the functional characteristics but with low production yield (Muzard *et al.*, 2009b). Here, a high production yield was obtained of the L<sub>h</sub>scFv 9O12 for all the functional *in vitro* tests. In addition, this scFv now also has the PpL-recognition motif. The affinity purified L<sub>h</sub>scFv 9O12 prevented all the collagen-induced platelet responses tested *in vitro*, including collagen-induced platelet activation and aggregation which make it a very

promising antithrombotic tool in the future. Further, evaluation for the inhibition of aggregation in flux, *ex vivo* and *in vivo*.

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### 3.2.2 Recombinant Antibody Fab Fragments

Here, we report the preliminary results concerning the engineering of the recombinant Fab fragments with 9O12 antigen-binding specificity in order to evaluate their antithrombotic activity and therapeutic potential.

We constructed recombinant chimeric and humanized Fabs. Chimeric Fab is not ideal because one can anticipate residual immunogenicity as reported with chimeric antibodies in the market (ex: Reopro®) but the advantage here is that binding properties are well-preserved since no modifications in the CDRs and Frameworks had occurred. Humanized Fab fragments are less immunogenic but sometimes functional properties are altered. Here, we can anticipate that it will not be the case as demonstrated by the results got with the  $_{\text{hscFv}}9\text{O}12$

The recombinant monovalent antibody fragment scFv 9O12 was humanized without deleterious effects which make it a powerful building block for the generation of new compounds with more suitable therapeutic properties. We attained an efficient process for the production of the humanized version of scFv 9O12 ( $_{\text{hscFv}}9\text{O}12$ ). Moreover,  $_{\text{hscFv}}9\text{O}12$  could be easily purified from the periplasmic extracts in functionally active form.

However, it is well established that pharmacokinetic properties of scFvs are unsuitable for therapeutic applications because of their short plasma half-life. Therefore, monovalent Fab remains the most suitable and valuable recombinant antibody format for therapeutic purposes.

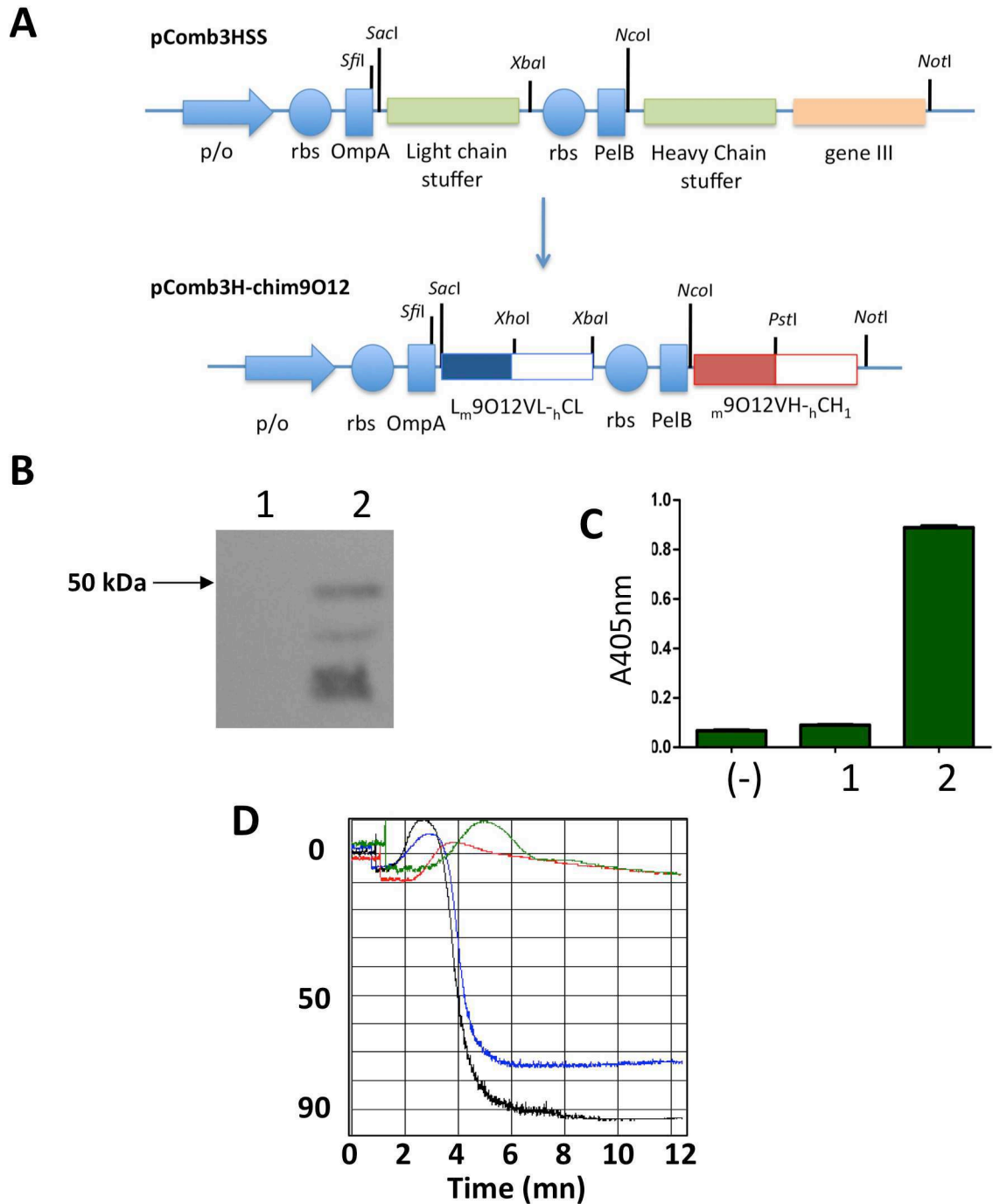
Here, we are involved in the process of designing recombinant Fabs derived with 9O12 specific binding characteristics. The  $_{\text{hscFv}}9\text{O}12$  was reformatted into recombinant Fab fragments with higher molecular weight (50 kDa), more appropriate in terms of pharmacokinetic properties. We constructed two different recombinant 9O12 Fabs, chimeric and humanized. Both Fab fragments retained the properties of binding to GPVI, with the exception that their production yield was very low as is the case with many Fabs. We are now using different strategies for the better production of the recombinant Fabs including using different media and have recently got some very encouraging results.

### 3.2.2.1 *Vector construction for recombinant chimeric Fab 9O12*

We used the bi-cistronic vector pCOMB3HSS a variant of pCOMB3H (Barbas *et al.*, 1991) for the expression of the recombinant Fabs 9O12. Phage III sequence was removed from pCOMB3HSS by double digestion (*SpeI/NheI*) for soluble expression of the recombinant Fabs 9O12 in the *E. coli*. A synthetic cDNA (GeneArt, Regensburg, Germany) encoding the chimeric Fd (VH-CH1) having murine 9O12 VH with CH1 of human IgG1, was digested with *NcoI* and *NotI* and cloned into the vector down stream to the *pelB* leader sequence. The resulting construct (pComb3H-Fd-chim) was transformed into chemically competent *E. coli* XL1-Blue cells by standard chemical method (Sambrook, 2001). The recombinant plasmid pComb3H-Fd-chim was isolated and digested with *SacI* and *XbaI*. A synthetic cDNA encoding murine variable light chain and human Ck of IgG1 (GeneArt, Regensburg, Germany), digested with the same enzymes (*SacI/XbaI*) was cloned into the pComb3H-Fd-chim down stream to *ompA* leader sequence, to generate the construct pComb3H-chim9O12 as indicated in Figure 1A. This construct was transformed into chemically competent XL1-Blue cells by standard chemical transformation method and after overnight incubation, recombinant clones were checked for the presence of inserts by restriction digestion and sequencing to ensure that no deletion or mutation had occurred (GATC- Biotech, Konstanz, Germany).

### 3.2.2.2 *Construction of recombinant humanized Fab 9O12*

We constructed an intermediate vector encoding hemi-humanized Fab having murine variable light domain (mVL) and humanized heavy chain using pComb3H-chim9O12. pComb3H-chim9O12 was digested with *NcoI* and *PstI* and the cDNA encoding the humanized variable heavy chain (hVH) with the same restriction sites (*NcoI/PstI*) was cloned in it. The resulting construct pComb3H-hem9O12 was then cloned and isolated. This plasmid was then digested with *SfiI* and *XhoI* and a synthetic gene encoding the humanized VL sequence of 9O12 was cloned (Fig. 2A). Sequencing allowed to check that the sequence was correctly inserted without any mutation (GATC- Biotech, Konstanz, Germany). The methods which are not discussed here were carried out similar to that described in section “Redesigning of a humanized single chain Fv directed against human platelets GPVI”.



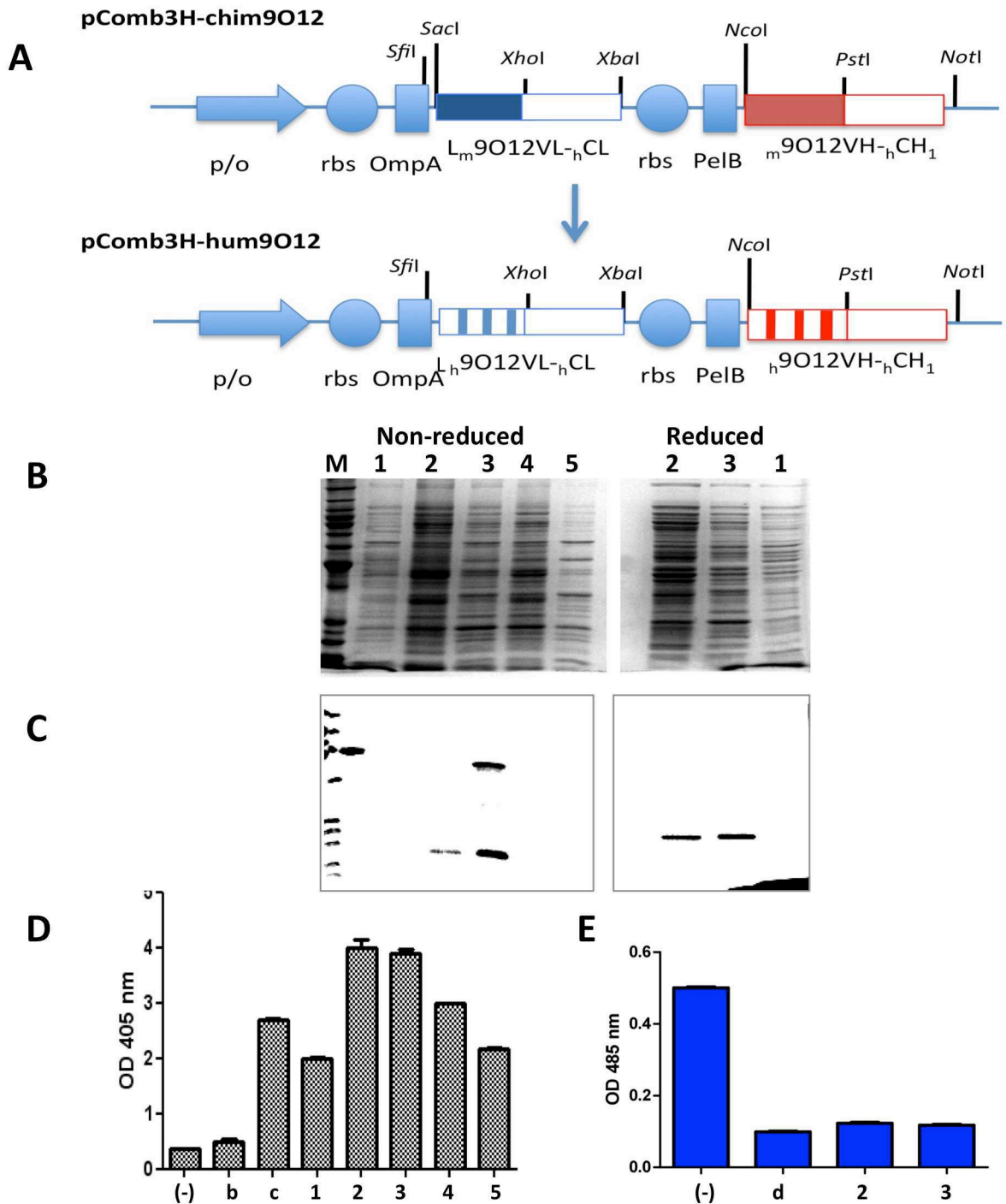
**Figure 1: Construction, expression and functional characterization of the chimeric Fab 9012**

**A-** Construction of the expression vector pComb3H-chim9012: p/o, region containing the lac Z promoter, operon; rbs: ribosome binding site. White boxes: sequence encoding antibody domains from human origin.

**B-** Western blot analysis of periplasmic extracts from induced XL1-Blue bacteria transformed with empty vector (1) or pComb3H-chim9012 (2).

**C-** Reactivity with  $_{sh}$ GPVI-Fc in ELISA. Irrelevant Fab 9C2 (1); chimeric Fab9012 (2); BSA was coated in place of with  $_{sh}$ GPVI-Fc (-)

**D-** Effects on platelets aggregation induced by collagen. Periplasmic extracts containing Chimeric Fab9012 (green), mscFv9012 (red), irrelevant scFv 4C1 (black) or PBS (blue).



**Figure 2: Construction, expression and functional characterization of the humanized Fab 9O12**  
**A-** Construction of the expression vector pComb3H-chim9O12: p/o, region containing the lac Z promoter, operon; rbs: ribosome binding site. White boxes: sequence encoding antibody domains from human origin. Coloured bars: CDRs from murine origin  
**B-C-** SDS-PAGE (**B**) and Western blot (**C**) analysis of periplasmic extracts of transformed bacteria induced in 2xTY (1); Turbo™ broth (2), Power™ broth (3); Superior™ broth (4) and Hyper™ broth (5).  
**D-** Reactivity with  $_{sh}$ GPVI-Fc in ELISA. Irrelevant anti-tetanus Fab (b); chimeric Fab9O12 (c); humanized Fab 9O12 from bacteria induced in different medium as above (1 to 5).  
**E-** Inhibition of GPVI/collagen interaction by murine Fab 9O12 (d) or periplasmic extracts from bacteria induced in Turbo™ broth (2) or Power™ broth (3).  
 (-) BSA was coated in place of with  $_{sh}$ GPVI-Fc.

### ***3.2.2.3 Production and Functional Evaluation***

The two expression vectors, pComb3H-chim9O12 and pComb3H-hum9O12, encoding the chimeric Fab 9O12 and the humanized Fab 9O12 respectively were transformed into *E. coli* strain TG1 for expression. The chimeric clone was cultivated in 2XTY while humanized clone in five different culturing media (2XTY, Turbo, Power, Superior and Hyper broth) containing ampicillin ( $100 \mu\text{g. mL}^{-1}$ ) at  $37^\circ\text{C}$  until  $A_{600\text{nm}}$  reached 0.9. IPTG ( $0.8 \mu\text{g. mL}^{-1}$ ) was added and the cultures were grown overnight at  $16^\circ\text{C}$  at 110 rpm. The leader sequences present upstream to the genes (Fd and VL) drag both the chains to the bacterial periplasm, where they form inter- and intra-chain disulfide bonds. Cells were pelleted by centrifugation and soluble Fabs were recovered from periplasm by osmotic shock. The periplasmic fractions were cleared by centrifugation ( $12000g$ ,  $4^\circ\text{C}$  for 25min). The periplasmic extracts were used for various functional tests.

### ***3.2.2.4 Expression and Functional Characterization of Chimeric Fab 9O12***

#### ***3.2.2.4.1 Immunoblot Analysis***

Periplasmic extracts containing recombinant chimeric Fab fragments was analyzed on a 12% SDS-PAGE gel under non-reducing conditions, then the resolved recombinant proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS, and then incubated with anti-human IgG (Fab specific) conjugated to alkaline phosphatase followed by visualization with NBT/BCIP. Fig 1B shows the expression of chimeric Fab, with an apparent 50kDa band for whole Fab and a non-covalently bonded band of about 25kDa for Fd and/or light chains.

#### ***3.2.2.4.2 Binding and Inhibitory Properties of the Recombinant Chimerized Fab 9O12***

The binding ability of the recombinant chimeric Fab 9O12 was investigated to the immobilized GPVI-Fc on microtitration wells. The plates were then incubated with the periplasmic extracts containing recombinant Fab 9O12 and then with anti-human IgG (Fab specific) conjugated to alkaline phosphatase followed by immunocomplex detection. It was observed that the chimeric Fab 9O12 retained the parental antibody ability for binding to recombinant GPVI (Fig. 1C).

#### **3.2.2.4.3 Inhibition of Collagen-induced Platelet Aggregation**

The ability of the chimeric Fab 9O12 to inhibit collagen-induced platelet aggregation was investigated by light transmission aggregometry. Human washed platelets were incubated with the recombinant chimeric Fab 9O12 and then aggregation was induced by collagen. It was observed that the periplasmic extracts containing chimeric Fab 9O12 completely inhibit collagen-induced platelets aggregation (Fig. 1D).

#### **3.2.2.5 Expression and Functional Characterization of Recombinant Humanized Fab 9O12**

##### **3.2.2.5.1 Immunoblot Analysis**

To check the presence of the humanized recombinant Fab 9O12, the periplasmic extracts containing humanized recombinant 9O12 were resolved by 12% SDS-PAGE in non-reducing as well as reducing conditions. The resolved proteins samples were visualized by staining with Coomassie brilliant blue (Fig. 2B).

The presence of recombinant humanized Fab 9O12 in the periplasmic extracts was further confirmed by Western blot analysis (same as described for chimeric Fab). The recombinant Fab resolved on 12% SDS-PAGE gel under both reduced and non-reduced conditions, was transferred to nitrocellulose membrane. The recombinant Fab expressed as heterodimer (~ 50kDa) of the Fd and light chain (Fig. 2C). In reducing conditions, both the chains were detected in monomeric form (~ 25kDa) (Fig. 2C). It was observed that the production was better when bacteria were cultured in Power broth. We also observed a band with an apparent Mr of 25kDa indicating that all the chains did not associate in Fab. This may also be an indication that one of the chain light or Fd is synthesized in excess compared to the other.

##### **3.2.2.5.2 Binding and Inhibitory Properties of the Recombinant Humanized Fab 9O12**

The binding ability of the recombinant humanized Fab 9O12 was investigated to the immobilized GPVI-Fc on microtitration wells. We observed that the recombinant humanized Fab 9O12 retained the parental antibody ability for binding to recombinant GPVI (Fig. 2D).

An inhibitory ELISA was also performed to confirm that the recombinant humanized Fab 9O12 has also retained the epitope specificity. The humanized

recombinant Fab 9O12 inhibited the interaction of GPVI to collagen immobilized on microtitration plates, as similar as that of murine Fab 9O12 (Fig. 2E). In contrast, as irrelevant Fab anti-tetanus has no effect on the binding of GPVI to collagen.



## **Part C**

# **General Discussion and Perspectives**

## 4 General Discussion and Perspectives

GPVI has revealed to be a platelet receptor of major importance in the occurrence of arterial thrombosis. It is, thus, the subject of major interest in diagnosis and therapeutic fields. Antibodies, in particular monoclonal antibodies, are powerful molecules largely used in both fields. Indeed, these molecules have applications in almost every field of biomedical sciences. Their advantages are their restricted specificity, their good affinity for their target. Moreover, the antibodies are more advantageous than chemical drugs, as their use is safer due to their human origin and easily demonstratable in clinical trials. In addition, the sufficient knowledge of their structure and the possibility to engineer them allow to make them adaptable for better therapeutic use.

The therapeutic use of antibodies has now more than a century, and still raises great interest. By the mid-1990s, especially with the emergence of molecular engineering technology, recombinant antibodies have emerged as a new class of therapeutic proteins. By using various technologies, antibodies derived directly from the immune system or synthetic libraries and/or the derived fragments, it now becomes possible to design specific molecules of high affinity for a wide variety of targets.

Immunoglobulins are bivalent molecules, of high molecular weight, their assembly in multi-domain form is complex, particularly as it requires the formation of disulfide bonds and specific glycosylation patterns, which are the two essential elements for recognition and effector functions of the antibody. Therefore, the production of functional recombinant antibodies is often difficult, which can lead to high production costs.

A score of molecules have been derived from murine monoclonal hybridomas are now in the market and in daily use for the treatment of various disorders such as oncology, the treatment of chronic inflammatory or rejection transplantation, infectious diseases and cardiovascular diseases. These therapeutic antibodies are either full IgG or immunoconjugated with a radioisotope or toxin, or active Fab fragment of the antibody (Abciximab®). These now account for more than a quarter of proteins for therapeutic use. Many antibodies are in clinical trials phase, majority in phase II and III. The development of these molecules is immense and is likely to

increase over the coming years, with a range of diagnostic and therapeutic applications and in an even larger arsenal consists the generations of new effective antibodies.

It is astonishing that some cardiovascular diseases, which are the leading cause of death in industrialized countries have not yet benefited from molecular engineering technologies which allow to generate antibodies for the diseases. In such a field of application, although it is usually very complex process, such as thrombosis, only one chimeric Fab is used in combination with other antithrombotic molecules (aspirin, thienopyridines) whose effectiveness is disputed.

In this context, the laboratory has produced several murine monoclonal antibodies to human GPVI by gene gun immunization against the immunoadhesin hGPVI-Fc. These antibodies differ by their epitope, affinity and functional properties (Lecut *et al.*, 2003). One of them, 3J24 is used to detect GPVI (Lagrue-Lak-Hal *et al.*, 2001). Another, 9O12.2 is an inhibitory antibody which blocks the binding of GPVI to collagen. Its Fab fragment has been extensively characterized *in vitro*, *ex vivo* and *in vivo* for its antithrombotic properties.

Our objectives were;

- first to produce a useful tool for GPVI quantification at the platelet surface; for this purpose, we produced a recombinant scFv fragment derived from 3J24 IgG
- second to produce an antibody fragment that could be developed as an antithrombotic drug in humans; for the purpose, we engineered a mouse scFv fragment, a humanized scFv fragment and a humanized Fab.

Although many stage remain to be validated, this study however, creating hope for the development of strategies for the upcoming effective antithrombotic recombinant antibody molecules.

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# **Annexes**

# **Annexe 1**

## **Scorpion antivenoms: Progresses and Challenges**

**M. ZAHID, S. ADI-BESSALEM, J. MUZARD, M. JUSTE, M.F. MARTIN-EAUCLAIRE, N. AUBREY, F. LARABADJEBARI, P. BILLIALD.**

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## **Scorpion antivenoms : progresses and challenges**

Muhammad ZAHID<sup>1</sup>, Sonia ADI-BESSALEM<sup>2</sup>, Julien MUZARD<sup>1</sup>, Matthieu JUSTE<sup>3</sup>,  
Marie-France MARTIN-EAUCLAIRE<sup>4</sup>, Nicolas AUBREY<sup>3</sup>, Fatima LARABA-DJEBARI<sup>2</sup>,  
Philippe BILLIALD<sup>1\*</sup>

<sup>1</sup> Université Paris-Sud, Faculté de Pharmacie & Muséum national d'Histoire naturelle, F-75005 Paris, France ;

<sup>2</sup> Université des Sciences et de la Technologie Houari Boumedienne & Institut Pasteur d'Alger, Algérie ;

<sup>3</sup> Université de Tours, Faculté de Pharmacie & INRA, F-37200 Tours, France ; <sup>4</sup> ToxCiM, Centre de Recherche en Neurobiologie-Neurophysiologie de Marseille (CRN2M), CNRS/Universités d'Aix-Marseille II-III, Faculté de Médecine - Secteur Nord, F-13344 Marseille, France

\* Corresponding author ; Tel : + 33 (0) 140793155 ; Fax : +33 (0) 140798857 ;

E-mail : [philippe.billiald@u-psud.fr](mailto:philippe.billiald@u-psud.fr)

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### **Abstract**

*Serum therapy is the only specific treatment against scorpion envenoming, and antivenoms are still prepared by fragmentation of polyclonal antibodies isolated from hyperimmunized horse serum to form Fabs or F(ab)<sub>2</sub>. Most of these antivenoms are efficient, but there is still considerable need for improvement in their production and safety profile. Recombinant antibodies and smaller functional recombinant antibody units are now emerging as credible alternatives, and constitute a source of still unexploited biosubstances capable of neutralizing the effects of venoms. Recent advances suggest the possibility of important innovations in the development of specific recombinant antibody fragments that have better properties than conventional antivenoms in terms of homogeneity, specific activity and possibly safety.*

### **La sérothérapie anti-scorpionique : anticorps thérapeutiques d'aujourd'hui et de demain**

*La sérothérapie demeure le seul traitement spécifique des envenimations scorpioniques et aujourd'hui encore, les sérums antivenimeux sont préparés à partir d'immun-sérums de chevaux dont les anticorps polyclonaux ont été fragmentés en Fab ou F(ab)<sub>2</sub> par protéolyse ménagée. Même si ces sérums antivenimeux sont habituellement efficaces, leur emploi présente des inconvénients, principalement en raison de leur hétérogénéité. Aujourd'hui, les anticorps recombinants dirigés contre des toxines ainsi que leurs fragments actifs issus des biotechnologies constituent une source encore inexploitée de biomolécules capables de neutraliser les effets d'un venin. De récents travaux laissent entrevoir des innovations importantes pour le développement d'antidotes spécifiques aux propriétés améliorées avec une homogénéité, une activité spécifique et une sécurité d'emploi accrues par rapport à celles des sérums antivenimeux conventionnels.*

**Keywords :** Androctonus, antivenom, scorpion, single-chain antibody, toxin.

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### **Introduction**

Scorpion envenoming is an important public health issue in many tropical and subtropical countries (Theakston *et al.*, 2003). Species capable of inflicting fatal stings are mainly found in North Africa and the Middle East (genera *Androctonus*, *Buthus* and *Leiurus*), in Central and Southern America (*Centruroides* and *Tityus*), in India (*Mesobuthus*) and in South Africa (*Parabuthus*). Scorpion venoms contain relatively low quantities of toxins compared to snake venoms, but their neurotoxins are very potent (Goyffon and Billiald, 2007). These toxins target the sodium, potassium, calcium and chloride channels. They induce direct effects and trigger the release of neurotransmitters (Gazarian *et al.*, 2005). The toxic effects of scorpion venoms are essentially due to the so-called long toxins consisting of 60-70 residues, which have a high affinity for the sodium channels of excitable cells. Following the binding of these toxins, a massive release of acetylcholine and catecholamines is induced, which in turn leads to signs of overactivity of the autonomic system or an "autonomic storm". Intense local pain is the first effect, even when the venom has no life-threatening potential. Systemic clinical signs (fever, sweating, hypertension, vomiting, and priapism) are observed 2-4 h after the sting. The symptoms evolve rapidly, and several stages of increasing severity can be distinguished (Goyffon and Billiald, 2007). Stage 3 corresponds to very severe envenoming, and is potentially lethal. Here, the greatest risk is that of cardiovascular collapse associated with major respiratory complications. Patients at this stage are usually kept under close surveillance in an intensive care unit. Apart from symptomatic treatment, which involves the use of local anesthetics and anti-histamine drugs, serum therapy is the only specific treatment. It is widely used in serious cases of envenoming,

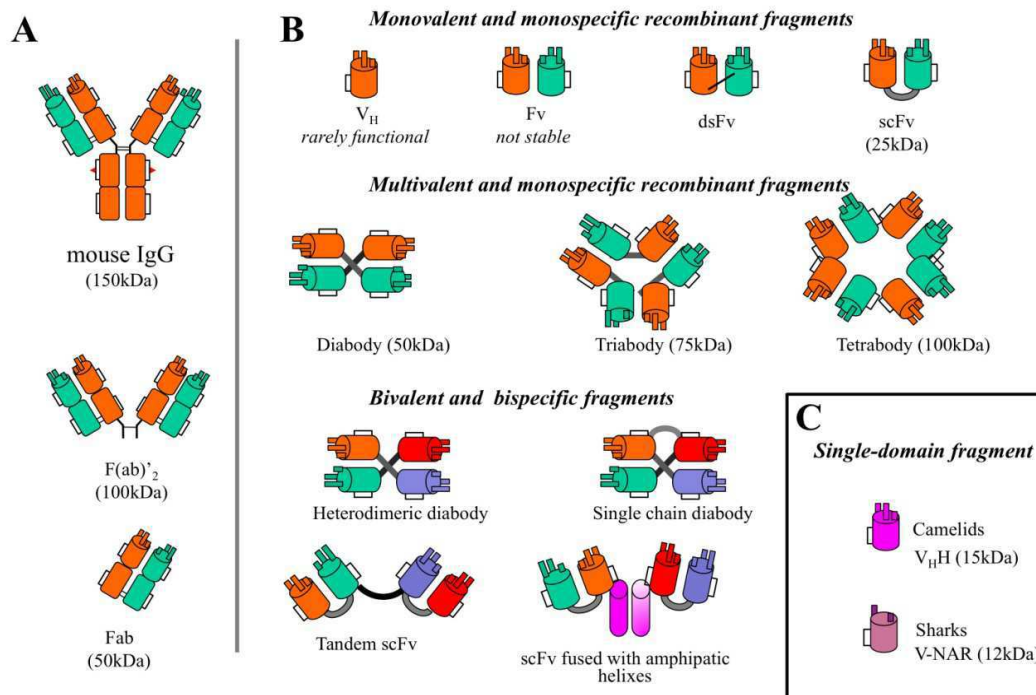


and has contributed to a significant decrease in mortality (Boyer *et al.*, 2009). This is particularly well exemplified in Mexico, where the mortality has significantly fallen from 700-800 to less than 100 within twelve years. However, the interest of using antivenoms remains controversial in several countries (Abroug *et al.*, 1999). Such discrepancies could be related to the lack of standardization in the use of antivenoms and in their preparation. Scorpion neurotoxins diffuse into tissues very quickly, and bind to their target almost irreversibly. Some clinicians are of the opinion that the time between the scorpion sting and the administration of the antivenom is often too long to allow the antibodies to trap the unbound toxins. Conventional therapeutic antibody fragments (100 kDa in the case of  $F(ab)_2$ ) are much larger than toxins (7 kDa), a feature which limits their ability to diffuse from the vascular compartment into the tissues. In addition, antivenoms are products of biological origin. These are still derived from equine polydonal antibodies raised against the entire venom. They have a low specific activity, which may be the potential cause of ineffectiveness. This low specific activity also means that patients are injected with large quantities of heterologous immunoglobulins, thus increasing the risk of major immune adverse reactions such as serum sickness. In a few cases, hypersensitivity reactions, which can reach the severity of anaphylactic shock, have also been reported.

What is required is a standardized source of high-titer antibodies targeting the most potent toxins that are responsible for the lethality, and which represent just a few components of the venom. Monoclonal antibodies derived from the hybridoma technology could meet these criteria, but they have never been approved for therapeutic use due to their murine origin (Nissim and Chernajovsky, 2008). Today, molecular engineering has made it possible to create recombinant antibody fragments specific for any antigen, sometimes without requiring the immunization of animals and the fragmentation process. The various features of natural antibodies can be tailored by a variety of methods to fit the requirements of a particular treatment. These include size, tissue penetration and distribution, half-life, stability, affinity and immunogenicity (Laffly and Sodoyer, 2005). Several research projects are currently investigating the therapeutic potential of recombinant antibody fragments as substitutes for conventional polyclonal  $F(ab)_2$  derived from immune sera.

### Fragments of recombinant antibodies : an unexploited diversity

Many hybridomas that secrete well-characterized monoclonal antibodies are available. Access to their genes has made it possible firstly to clone the  $V_H$  and  $V_L$  domains responsible for the specificity of antigen recognition, and then subsequent generation in the form of recombinant proteins (Figure 1).



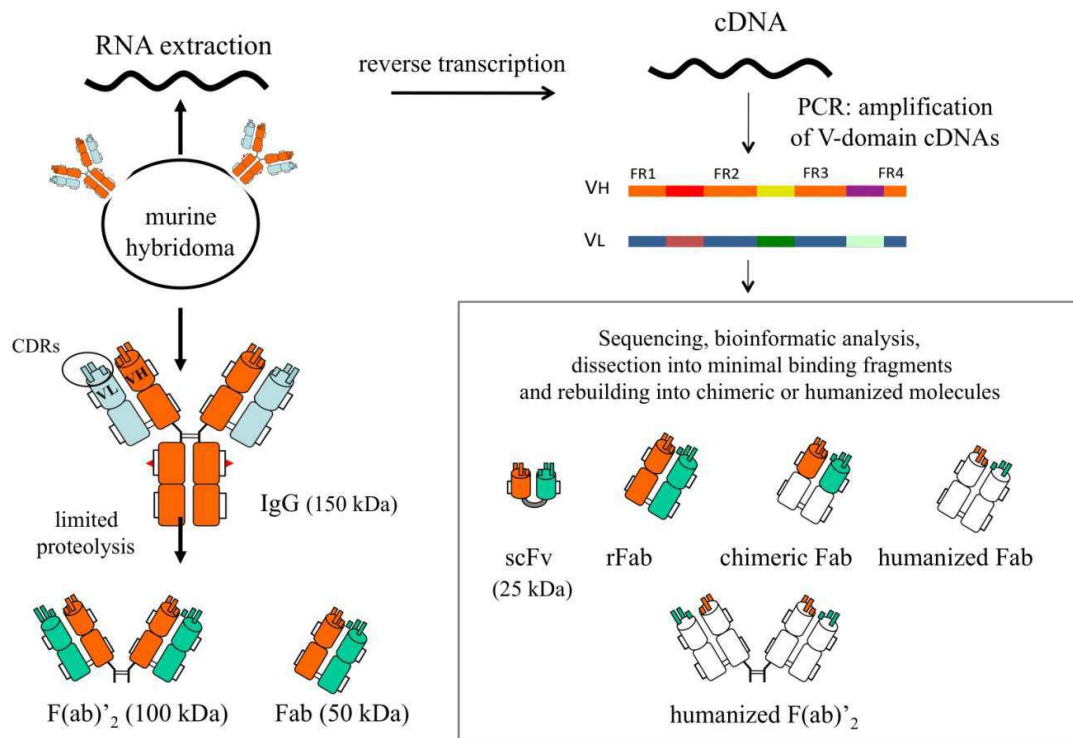
**Figure 1.** Dissection of immunoglobulins into minimal binding fragments and reformatting into multivalent recombinant reagents. **(A)** Monoclonal IgG and its functional fragments prepared by limited proteolysis. **(B)** A selection of recombinant antibody fragments derived from the antibody variable domains. **(C)** The minimal functional units, simply made of one antibody domain, that are extracted from exotic animal species.

**Figure 1.** Immunoglobulines et fragments d'anticorps recombinants. **(A)** IgG monoclonale et ses fragments actifs issus de la protéolyse ménagée. **(B)** Sélection de fragments d'anticorps recombinants constitués des domaines variables d'anticorps. **(C)** Unités fonctionnelles minimales, constituées d'un seul domaine d'anticorps, qui sont isolées d'espèces animales "exotiques".

Very few examples of isolated VHs or of Fvs (VH and VL associated in a non-covalent manner) were initially described, then new arrangements were invented, which made it possible to modify their size and valence, and to create a wide range of diverse active antibody fragments (Holliger and Hudson, 2005). These new formats can be used, for instance, to improve the tissue diffusibility and the bioavailability of the antibody, and also to make them less immunogenic, more "human" or even fully human.

In a fragment of recombinant monovalent antibody, consisting solely of variable domains, the ability to recognize antigens is maintained properly only if the association of VH and VL is stabilized by a disulfide bridge (dsFv) inserted by molecular engineering, or a peptide bond (scFv), which provides the junction between the C- and N-terminals of the two domains. 25-kDa single-chain variable fragments (scFv) are the molecules most often described. Various linkers have been used, depending on whether the association takes place in VH/VL direction or VL/VH direction, and the size of these bonds is also important. Several linkers of about fifteen residues have been reported to induce the formation of monovalent, monomeric molecules. Shorter linkers (5 residues) create a constraint between the variable domains of the same molecule, which favors the formation of a dimer also called diabody, which has the same size as a Fab fragment (50 kDa) but is bivalent. An even shorter linker (1 residue or even no residue) results in trimeric (triabody) or even tetrameric (tetrabody) multivalent assemblies.

Various molecular engineering strategies have been developed to create the basic module: scFv. A widely used one consists of cloning antibody domains from a murine hybridoma, which can subsequently be chimerized or humanized (Figure 2). Humanization consists of grafting the hypervariable regions (CDRs) responsible for the antigen recognition specificity onto the scaffold of human immunoglobulins. This is a challenge which has been made much easier by crystallographic analyses that provide details of the interface between the antigen complexes/antibodies at the atomic scale. Access to data banks and softwares that list, analyze, and compare all available antibody sequences also provides useful information (Lefranc *et al.*, 2009).



**Figure 2.** Hybridoma as a source of recombinant antibodies. After extraction of the hybridoma mRNA and reverse transcription, the cDNAs encoding the antibody variable domains are PCR amplified, sequenced and cloned. Recombinant antibody fragments with low immunogenicity can be designed. In chimeric antibody fragments all the murine constant domains are substituted by constant domains of human origin. In humanized antibody fragments the murine antibody CDRs are grafted onto human V-domain frameworks

**Figure 2.** Les hybridomes, sources d'anticorps recombinants. Après extraction de l'ARNm et transcription inverse, l'ADNc codant les domaines variables d'anticorps est amplifié par PCR, séquencé et cloné. Des fragments d'anticorps recombinants de faible immunogénicité peuvent être conçus. Dans les fragments d'anticorps chimériques, tous les domaines constants d'origine murine sont remplacés par des domaines constants d'origine humaine. Dans les fragments humanisés, les boucles hypervariables (CDR) murines sont greffées sur des molécules d'immunoglobulines humaines.

Another very attractive strategy is to construct antibody V-domains gene repertoires expressed *in vivo* on the surface of bacteriophages (phage display) or yeast (yeast display) or *in vitro* (ribosome display). Then,

these libraries are screened against the antigen of interest (Bradbury and Marks, 2004). Such repertoires can be constructed from an inventory of human antibodies, which is an advantage if therapeutic applications are expected. However, the scFvs isolated from these repertoires are often of mediocre quality in terms of their affinity for the target antigen, and affinity maturation processes have to be carried out. An alternative consists of using an immune repertoire. For obvious ethical reasons, such libraries must only be constructed against antigens to which Man is naturally exposed. For the other antigens, such as toxins, an alternative approach has been suggested. It consists of creating an immune repertory of scFv or Fab of antibodies of primates (macaques), which are closely related to human beings. Fragments of antibodies that target plant (ricin) or bacterial (anthrax) toxins have been isolated from such libraries (Pelat *et al.*, 2009).

### Towards a new generation of anti-venoms

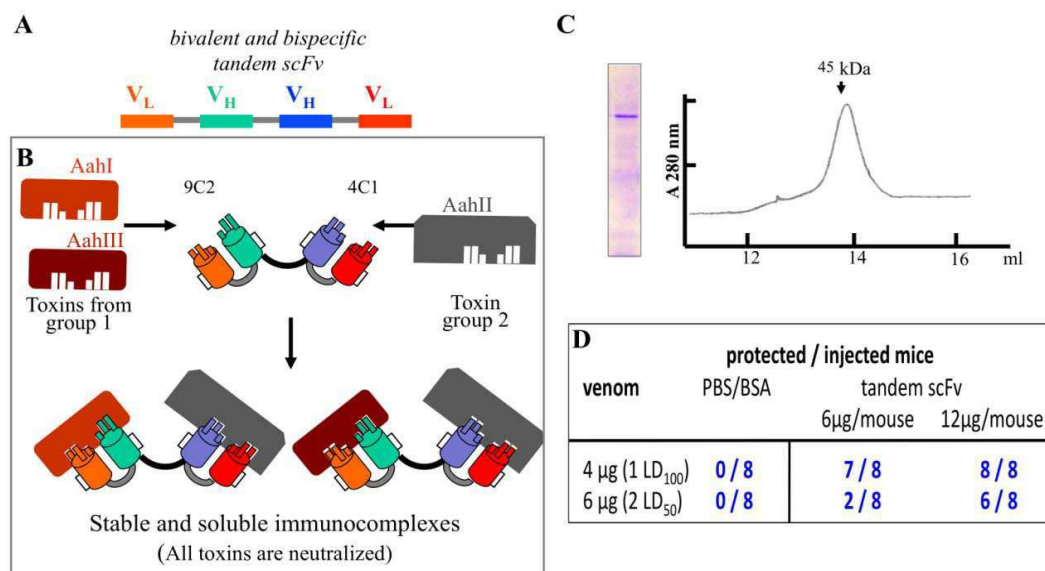
Conventional antivenoms still consist of a mixture of polyclonal antibody fragments, only a small fraction of which targets the dangerous toxins, and a still smaller proportion consists of neutralizing antibodies. The recombinant antibody technology offers a way of isolating an antibody of interest from such a heterogeneous population. Then, this antibody can be "rebuilt" in the form of a single small molecule well suited for particular applications. Thus, several groups have tried to assess the potential of recombinant antibody fragments to neutralize the action of toxins, or even provide protection against envenoming. At present these studies essentially concern scorpion venoms, which are less complex than snake venoms, and the toxic effects in human beings are due to the effects of a limited number of neurotoxins that act on the membrane sodium channels activated by the voltage of the so-called "excitable" cells.

In central and north America, the scorpions of the genus *Centruroides* are amongst the most dangerous for Man. The toxicity of their venom is essentially due to structurally similar neurotoxins, the action of which can be neutralized experimentally by the monoclonal murine antibody BCF2. A scFv fragment derived from this antibody, and variants that have undergone *in vitro* "maturation", have been created and assessed for their ability to neutralize the action of the venom (Juarez-Gonzalez *et al.*, 2005). The authors have already shown that the action of the venom incubated with these scFvs before being injected into mice was neutralized. Other scFvs have also been selected by the same group by screening a repertoire of human phages-antibodies, with the potential advantage of improved acceptability (Riano-Umbarila *et al.* 2005). However, the protective potential of these substances has never been assessed under experimental conditions that mimic those of natural envenoming in which the antidote is injected after the venom, and via a different route.

Other studies also show that scFvs diffuse rapidly into tissues, thus facilitating the uptake and neutralization of any free toxins not yet bound to a receptor. However, their plasma half-life is extremely short (a few minutes) and insufficient to provide effective protection *in vivo*. Cross-linking scFvs with polyethyleneglycol (PEG) could improve their stability and solubility, prolonge their circulation time, slower their clearance and reduce their immunogenicity. An alternative strategy consists in creating multimeric scFv molecules, which still have low immunogenicity, because they lack the constant domains of murine antibodies, but which have modified pharmacokinetic characteristics and a longer half-life. Thus, diabodies, which are of the same size as Fab but with two antigen recognition sites, have greater efficacy (Aubrey *et al.*, 2003). It has been shown that a diabody that targets the *AahII* neurotoxin of the venom of the north-African scorpion *Androctonus australis* could be adapted to protect experimentally envenomed mice, with greater efficacy than the conventional antivenoms.

For the dangerous scorpions of the Old World, such as *Androctonus australis*, the toxins that are active at the sodium channels are characterized by having greater structural and antigenic polymorphism. Thus, it has been estimated that 90% of the lethal activity of the venom is attributed to 3 toxins (*AahI*, *AahII* and *AahIII*) belonging to two distinct immunological groups, so that the action of the venom can be neutralized only by using at least two antibodies with different specificities. Molecular engineering has made it possible to develop a single recombinant antibody fragment, which is the same size as a Fab, but bivalent and bispecific, that can simultaneously recognize the toxins of both groups (Juste *et al.*, 2007). Various molecular constructions can be envisaged (Figure 1). The tandem scFv format has been constructed using the variable domains of the 9C2 and 4C1 antibodies, which target the toxins of groups I (*AahI* and *AahIII*) and II (*AahII*), respectively (Figure 3). The recombinant scFv protein produced in bacteria is fully functional, bivalent and bispecific. It has high affinity for both groups of toxins, and its protective capacity has been shown to be 150 DL<sub>50</sub>/mg in the experimentally envenomed mouse, a value which is 300-fold greater than that usually found for polyclonal antivenoms.

Other avenues have also been explored. Unique antibodies lacking the light chain have been reported in sharks (Ig-NAR) or in Camelids (HCAb). A V-NAR single domain (12 kDa) directed to a scorpion toxin has been isolated from a phage display library immunized with scorpion toxin Cn2 (GenBank AAX10141.1). However its functional properties remain to be explored. In HCAbs, the antigen-binding site is a single protein domain (V<sub>H</sub>H) of 15 kDa (Harmsen and Haard, 2007). The V<sub>H</sub>H is extremely stable and easily produced in bacteria or yeast. V<sub>H</sub>Hs that neutralize *Androctonus australis* toxins have recently been identified by screening an immune library. Two of these antibody fragments have been reformatted into a bispecific molecule that fully neutralizes the whole venom (Hmila *et al.*, 2010). Although these innovative molecules (scFv, V-NAR and V<sub>H</sub>H) are very attractive, their therapeutic potential has now to be compared in a reproducible and rigorous manner by using well-standardized assays in which the LD<sub>50</sub> of the venom is experimentally determined. Such assays will have to take into account the time interval between the sting and the treatment, the administration routes of the venom and the antidote. Assays such as the intra-cerebroventricular injection of venoms pre-incubated with antidotes have no longer to be used to predict the therapeutic potential of antibodies fragment even if they provide interesting information.



**Figure 3.** A bispecific tandem scFv that protects against experimental envenomation. **(A)** Schematic representation of the cDNA encoding the tandem scFv. **(B)** Schematic representation of the neutralization of *Androctonus australis* toxins AahI, AahII, AahIII using the tandem scFv. **(C)** Electrophoretic and gel filtration analysis of the purified tandem scFv. **(D)** The intraperitoneal injection of tandem scFv to mice experimentally envenomed by a subcutaneous administration of the venom.

**Figure 3.** Un fragment d'anticorps scFv en tandem protège contre une envenimement expérimentale. **(A)** Représentation schématique de l'ADNc codant un scFv en tandem. **(B)** Représentation schématique de la neutralisation des toxines AahI, AahII, AahIII d'*Androctonus australis* par le scFv en tandem. **(C)** Analyse des préparations de scFv en tandem par électrophorèse SDS et chromatographie d'exclusion stérique. **(D)** Analyse des effets de l'injection intrapéritonéale du scFv en tandem à des souris expérimentalement envenimées par voie sous-cutanée.

### Polyvalent recombinant antivenoms?

Several venomous species often cohabit, which makes it necessary to produce polyvalent antivenoms with several targets. In Tunisia, for example, the scorpion *Buthus occitanus* shares the same territory as *Androctonus australis*, but the tandem scFv that targets *A. australis* is unable to interact with the most potent toxin of *B. occitanus* (BotIII), even though it differs from the AahII toxin by three amino acid residues only. A crystallographic analysis of the AahII/Fab 4C1 toxin complex has shown that the nature of the C-terminal residue (H64>N), which is located in the interaction zone, is probably responsible for this (Fabrichny *et al.*, 2007). The construction of a library of 4C1-derivative phage-antibodies, mutated in a random fashion at the CDR residues in contact with His64, and its dual screening versus the BotIII and AahII toxins, could lead to the selection of mutants with crossed reactivity for both toxins. Powerful methods of affinity maturation and selectivity have already been developed to optimize the specificity of antibodies, in particular of anti-steroid antibodies (Dubreuil *et al.*, 2005). Replacing the variable domains derived from 4C1 in the tandem scFvs by the variable domains of the selected mutants would then lead to a polyvalent antivenom, consisting of a single molecule able to neutralize the major toxins of the venoms of *Androctonus australis* and of *Buthus occitanus*.

### What are the prospects for the future?

With the rise of molecular engineering, the diversity of active antibody fragments has increased considerably, and they are no longer limited to Fab or F(ab')<sub>2</sub> fragments, which were the only formats accessible for many years, due to the limits imposed by limited proteolysis. Many formats of recombinant antibody fragments have already been designed, and there is no doubt that many others remain to be invented.

It is clear that the therapeutic management of serious envenoming could one day benefit from this progress. Results obtained recently by various groups in Europe, in Africa and in central and south America clearly demonstrate the therapeutic potential of recombinant antibody fragments, and the advantages that they could offer compared to preparations derived from animal immunosera, which are always broad mixtures and produced in a manner that is difficult to reproduce.

Advances in recombinant antibody technology have made it possible to renew the interest in therapeutic antibodies (Nelson *et al.*, 2010). Today, more than twenty-four molecules have been approved by the FDA with a wide range of applications and several hundred are in preclinical trials or under FDA review (Nelson *et al.*, 2010).

The manufacturers of antivenoms are interested in these studies, but the socio-economic constraints faced by the countries exposed to scorpionism are still a major obstacle to the development and implementation of innovative therapies.

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## **Annexe 2**

**Design and humanization of a murine scFv that blocks human platelet glycoprotein VI *in vitro*.**

J. MUZARD, M. BOUABDELI, **M. ZAHID**, V. OLLIVIER, J.J. LACAPERE, M. JANDROT-PERRUS, P. BILLIALD.

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## Design and humanization of a murine scFv that blocks human platelet glycoprotein VI *in vitro*

Julien Muzard<sup>1,2</sup>, Maxime Bouabdelli<sup>2</sup>, Muhammad Zahid<sup>3</sup>, Véronique Ollivier<sup>2</sup>, Jean Jacques Lacapère<sup>4</sup>, Martine Jandrot-Perrus<sup>2</sup> and Philippe Billiald<sup>1,3</sup>

<sup>1</sup> Muséum national d'Histoire naturelle, CNRS FRE 3206, CP39, Paris, France

<sup>2</sup> Inserm U698, Hôpital Bichat, Université Paris 7, France

<sup>3</sup> Université Paris-Sud 11, JE 2493, Faculté de Pharmacie, Châtenay-Malabry, France

<sup>4</sup> Inserm U733, CRB3 Faculté Xavier Bichat, Université Paris 7, France

### Keywords

antibody humanization; arterial thrombosis; collagen; platelet

### Correspondence

P. Billiald, Muséum national d'Histoire naturelle, CNRS FRE 3206, CP39, 12, rue Buffon, 75231 Paris cedex 05, France

Fax: +33 140 793 594

Tel: +33 140 793 155

E-mail: billiald@mnhn.fr

M. Jandrot-Perrus, Inserm U698, Hôpital Bichat, 46 rue Henri Huchard; University Paris 7, Paris, France

Fax: +33 140 258 602

Tel: +33 140 256 531

E-mail: martine.jandrot-perrus@inserm.fr

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Platelet adhesion and aggregation at the site of vascular injury is essential for hemostasis, but can also lead to arterial occlusion in thrombotic disorders. Glycoprotein (GP) VI is the major platelet membrane receptor that interacts directly with collagen, the most thrombogenic compound in the blood vessels. GPVI could therefore be a major therapeutic target. Fab fragments of the anti-GPVI murine monoclonal IgG 9O12 have previously been shown to completely block collagen-induced platelet aggregation, to inhibit the procoagulant activity of collagen-stimulated platelets, and to prevent thrombus formation under arterial flow conditions without significantly prolonging the bleeding time. Here, we engineered recombinant scFvs that preserve the functional properties of 9O12, and could constitute building blocks for designing new compounds with potentially therapeutic antithrombotic properties. First, the 9O12 variable domains were cloned, sequenced, and expressed as a recombinant murine scFv, which was fully characterized. This scFv preserved all the characteristics that make 9O12 Fab potentially useful for therapeutic applications, including its high affinity for GPVI, ability to inhibit platelet adhesion, and aggregation with collagen under arterial flow conditions. A humanized version of this scFv was also designed after complementarity-determining region grafting and structural refinements using homology-based modeling. The final product was produced in recombinant bacteria. It retained GPVI-binding specificity and high affinity, which are the main parameters usually impaired by humanization procedures. This is a simple, efficient and straightforward method that could also be used for humanizing other antibodies.

## Introduction

Platelets play a crucial role in thrombus formation and limiting blood loss from sites of vascular injury. This is a complex process and entails the following series of steps: adhesion of platelets to the damaged vascular subendothelium, activation of the platelets, their

recruitment and, finally, aggregation. In some circumstances, such as disruption of the atherosclerotic plaques, platelet activation can lead to arterial occlusion and irreversible tissue damage. Acute coronary and cerebrovascular accidents usually result from this

### Abbreviations

CDR, complementarity-determining region; FITC, fluorescein isothiocyanate; FR, framework region; GP, glycoprotein; PRP, platelet-rich plasma; SPR, surface plasmon resonance.



phenomenon, and they are currently the leading causes of death in the world. As a consequence, antiplatelet drugs constitute a major class of therapeutics intended to prevent and/or reverse platelet aggregation in arterial thrombosis [1]. Drugs that act on the platelet recruitment step have been used for a long time, and include inhibitors of cyclooxygenase (aspirin), phosphodiesterase inhibitors (dipyridamole) and, more recently, ADP receptor antagonists (clopidogrel). A chimeric antibody fragment (Fab) (abciximab) directed to glycoprotein (GP) IIb-GPIIIa that acts on the final step of the aggregation has also been approved for specific subsets of patients with acute coronary disease. However, all of the drugs currently utilized to counteract platelet function suffer from a lack of potency and/or specificity, and major risks of hemorrhage and thrombocytopenia are associated with their use [2]. There is therefore a continuing need for new and improved drugs that will inhibit platelet function in diseases characterized by thrombosis, and in particular for drugs with limited hemorrhagic effects, this point being crucial for safe antithrombotic therapy.

An attractive antithrombotic strategy would consist of targeting the initial interaction of platelets with the vessel wall rather than the later recruitment and aggregation phases. GPVI is the major receptor that interacts directly with collagen, which is the most thrombogenic compound in the vessel wall. GPVI mediates platelet aggregation and secretion of the secondary agonists responsible for thrombus growth. In addition, the release of growth factors and cytokines contributes to the recruitment of inflammatory cells and to the proliferation of smooth muscle cells, leading to stenosis [3,4]. Finally, GPVI expression is restricted to the platelet lineage [5]. There is also direct experimental and clinical evidence that platelets deficient in GPVI cannot be activated by collagen, and that a deficiency in GPVI expression does not produce major bleeding [6,7]. For all of these reasons, GPVI antagonists can be expected to be good candidates for preventing secondary thrombosis in an efficient and specific manner and also to be associated with a low risk of hemorrhage.

GPVI antagonists can be designed in several ways. One approach consists of generating soluble GPVI recombinant proteins or GPVI mimetics that will compete with platelets to bind to collagen. Encouraging results have been reported in a murine thrombosis model using the extracellular domain of murine GPVI produced in fusion with the human immunoglobulin Fc domain [8]. However, these results have not been confirmed, and the use of competitive ligands such as soluble recombinant GPVI may be associated with

considerable pharmacological and functional drawbacks [9]. A more effective way to block platelet GPVI–collagen interactions consists of directly targeting GPVI with molecules such as specific antibodies. Previous studies have shown that targeting murine GPVI with the rat monoclonal antibody JAQ1 abolishes collagen responses in circulating platelets, leading to the depletion of the receptor and to long-term anti-thrombotic protection in mice [10–12]. However, this antibody does not crossreact with human GPVI. Recombinant scFvs directed against human GPVI have also been reported over the last few years [13–15]. These antibody fragments have been isolated from combinatorial phage display libraries expressing human scFvs, and some of them specifically block GPVI binding to collagen under experimental conditions. However, they all exhibit low affinity for their target ( $K_D$  in the range of  $10^{-7}$  M), making them unsuitable for therapeutic applications. Recently, monoclonal Fab fragments derived from four distinct murine hybridomas (OM 1–4) were found to inhibit the binding of GPVI to collagen, collagen-induced secretion and thromboxane  $A_2$  formation *in vitro*, as well as *ex vivo* collagen-induced platelet aggregation after intravenous injection in cynomolgus monkeys [16,17]. Fab OM 4 also inhibited thrombus formation *in vivo* in rats without prolonging the bleeding time, thus confirming the therapeutic potential of antibody fragments directed to GPVI [18]. A human–mouse chimeric monoclonal antibody (cF1232) has also been reported to cause GPVI immunodepletion with a long-term *in vivo* antiplatelet effect in monkeys. However, such a process is not reversible and implies GPVI depletion on megakaryocytes, the consequence of which has not been addressed [19].

Another murine monoclonal antibody-derived Fab (9O12) specific for human GPVI has been developed and characterized by our group [20]. This high-affinity inhibitory monovalent Fab not only completely blocks collagen-induced platelet aggregation, but also inhibits the procoagulant activity of collagen-stimulated platelets, and prevents thrombus formation under both static and flow conditions. However, the immunogenicity of murine antibody fragments is a major obstacle to their clinical development, so it is necessary to reduce this immunogenicity by a humanization procedure.

Here, we report the cloning and sequencing of 9O12 variable domains, and the engineering and detailed evaluation of the derived scFv produced in recombinant bacteria. In view of its high potential, the murine scFv ( $_m$ scFv 9O12) was then humanized. At this stage, the 9O12 complementarity-determining regions (CDRs) were grafted onto closely related human antibody



variable domains, and refinements were carried out after *in silico* modeling and inspection of the paratope. The cDNA encoding the humanized construct ( $_{\text{hscFv}}$  9O12) was chemically synthesized, cloned, and expressed in recombinant bacteria. We were able to purify this molecule and to test it on human platelets by flow cytometry analysis. The final product did not suffer loss of affinity and specificity, which are the parameters usually affected by humanization procedures.

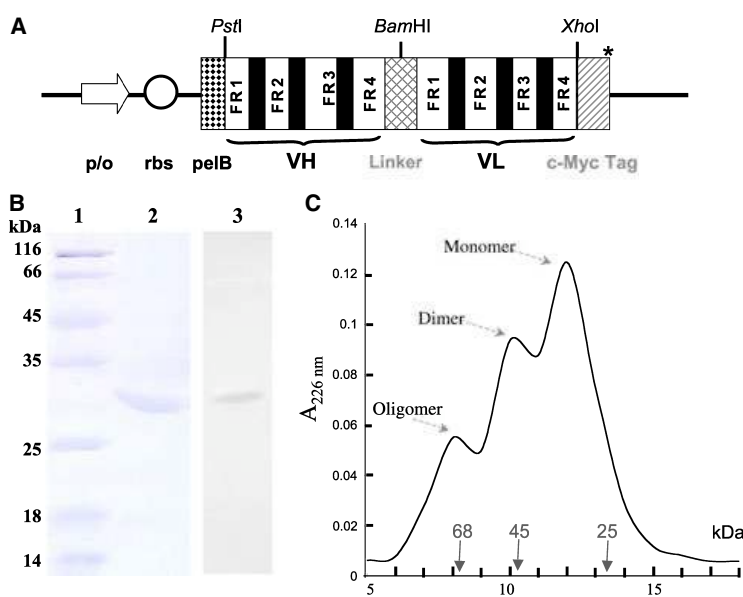
## Results

### Design, bacterial expression and purification of $_{\text{mscFv}}$ 9O12

cDNAs encoding the VH and VL domains of 9O12 were derived from hybridoma cell mRNA in an RT-PCR using consensus primers. Sequencing of the PCR product corresponding to the VH domain led to a single sequence, whereas the sequence of the PCR product corresponding to the VL domain was scrambled because of the amplification of the aberrant MOPC21-derived Vk gene. Digestion of the VL PCR product with *Bci*VI, and treatment as previously reported, made it possible to bypass the aberrant MOPC21-derived transcript, and to clone the 9O12 kappa light chain V-gene [21]. To ensure accuracy, VH and VL cDNAs were independently amplified from two distinct batches of mRNA. Sequencing confirmed that no mutation had occurred during the PCR reac-

tion. The 9O12 VH and VL cDNA sequences are now registered in the EMBL data bank (AM 887763 and AM 887764, respectively). The scFv-encoding gene derived from the variable regions of 9O12 [VH and VL linked together via a short linker ( $\text{G}_4\text{S}$ )<sub>3</sub>] was then constructed, and inserted in frame with the pelB signal sequence, upstream of the c-Myc tag, into the pSW1 expression vector (pSW- $_{\text{mscFv}}$  9O12). The construct is shown in Fig. 1A. Plasmid pSW- $_{\text{mscFv}}$  9O12 was cloned into the TOPPI *Escherichia coli* strain, and the recombinant protein was produced and purified by affinity chromatography using GPVI-Sepharose gel. This procedure made it possible to recover fully functional scFv with a yield of  $200 \mu\text{g}\cdot\text{L}^{-1}$  of culture. The preparation was homogeneous, as shown by SDS/PAGE under reducing conditions, western blot using the antibody against c-Myc, and MS analysis, which indicated an experimental relative molecular mass ( $M + \text{H}^+$ ) of 28 402.3 Da, close to the theoretical molecular mass of 28 394.5 Da calculated from the amino acid sequence (Fig. 1B). The purified scFv was further analyzed by size exclusion chromatography (Fig. 1C). This revealed the presence of a main peak (62%) eluting at 12.5 mL with an apparent molecular mass of about 27 kDa, corresponding to the monomeric  $_{\text{mscFv}}$  9O12. Two other minor peaks were also observed: one eluted at 11 mL (25%), and the other at 8 mL (13%). These minor peaks could correspond to the dimeric and multimeric forms of  $_{\text{mscFv}}$ , as previously reported for other scFvs [22]. After storage at 4 °C, the isolated dimeric and multimeric fractions

**Fig. 1.** Construction and structural characterization of  $_{\text{mscFv}}$  9O12. (A) Diagram of the expression vector pSW- $_{\text{mscFv}}$ 9O12. The gene encoding  $_{\text{mscFv}}$  9O12 is cloned between the *Pst*I and *Xho*I sites in-frame with the pelB leader sequence and the c-Myc tag sequence followed by a stop codon (\*). p/o, operon; rbs, ribosome-binding site; pelB, signal sequence. (B) Affinity-purified  $_{\text{mscFv}}$  9O12 analyzed after SDS/PAGE and staining with Coomassie brilliant blue (lane 2) or western blotting on nitrocellulose membrane and immunostaining using a monoclonal antibody against c-Myc (lane 3). Lane 1: molecular mass standards. (C) Size exclusion chromatography of affinity-purified  $_{\text{mscFv}}$  9O12 performed on a Superdex 75 HR 10/30 column calibrated versus standards of known molecular mass.



were subjected to another gel filtration step. This resulted in a two-peak elution profile, one peak corresponding to the dimer, and the other to the monomer. The proportion of monomer increased when the dimer fraction was left for 3 days before being reapplied, demonstrating the instability of dimers and higher-order oligomers (data not shown). Monomeric scFv remained stable after prolonged storage at 4 °C. Affinity-purified scFv 9O12, but not the irrelevant scFv 9C2, was able to bind to GPVI in a dose-dependent manner in a direct ELISA, and competed with 9O12 (data not shown).

### Functional properties of $m_{scFv}$ 9O12

Analysis of the scFv–GPVI interaction in real time using surface plasmon resonance (SPR) technology allowed us to determine the kinetic parameters of  $m_{scFv}$  9O12 using the BIAcore 2000 system and BIA-EVALUATION version 3.1 software (BIAcore, Uppsala, Sweden) (Fig. 2A). This gave the following kinetic parameters:  $k_{on} = 6.5 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ ,  $k_{off} = 1.7 \times 10^{-4}\cdot\text{s}^{-1}$ , and the dissociation constant  $K_D = 2.6 \text{ nM}$ . These parameters were similar to those calculated under the same conditions for the 9O12 proteolytic

Fab fragment ( $K_D = 2.3 \text{ nM}$ ) and parental IgG ( $K_D = 4.0 \text{ nM}$ ). We investigated the ability of purified  $m_{scFv}$  9O12 to bind to immobilized GPVI after prolonged storage at 4 and 20 °C (for 3 days), and no significant decrease in antigen-binding ability was observed.

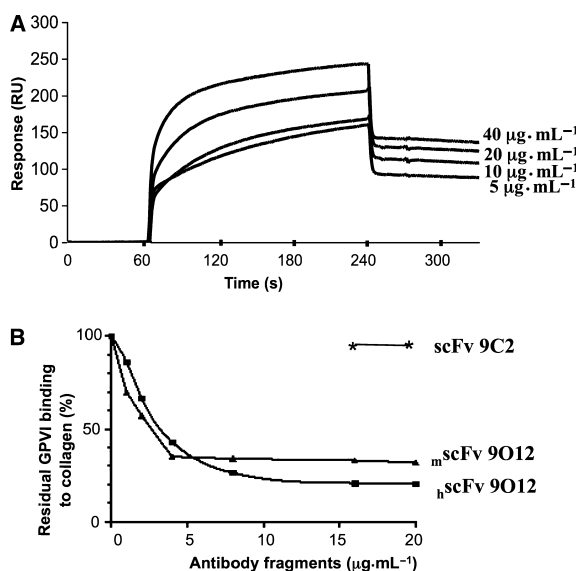
We also investigated whether the interaction of  $m_{scFv}$  9O12 with GPVI could inhibit the binding of GPVI to collagen immobilized on microtitration plates. GPVI preincubated with increasing amounts of antibody fragments was added to the wells. As shown in Fig. 2B,  $m_{scFv}$  9O12 inhibited GPVI ( $20 \mu\text{g}\cdot\text{mL}^{-1}$ ) binding to collagen with an  $\text{IC}_{50}$  of approximately  $1.17 \mu\text{g}\cdot\text{mL}^{-1}$ , 80% inhibition being reached at a concentration of  $5\text{--}10 \mu\text{g}\cdot\text{mL}^{-1}$  of  $m_{scFv}$  9O12. This inhibitory capacity was comparable to that observed for 9O12 Fab prepared after papain digestion of the parental IgG ( $2.1 \mu\text{g}\cdot\text{mL}^{-1}$ ). Similar results were observed after incubation of GPVI ( $10$  or  $40 \mu\text{g}\cdot\text{mL}^{-1}$ ) with the antibody fragments. In contrast, the irrelevant scFv 9C2 had no effect on the binding of GPVI to collagen.

### Effects induced by $m_{scFv}$ 9O12 binding to GPVI

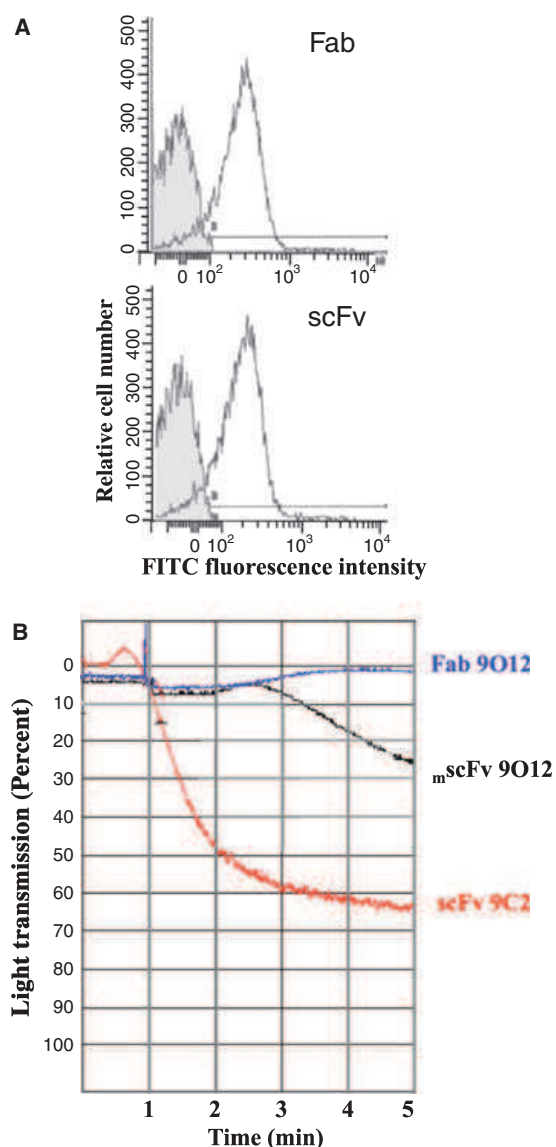
The binding of  $m_{scFv}$  9O12 to native GPVI expressed at the platelet surface was further observed by flow cytometry, as indicated by the shift of the fluorescence peak to the right (Fig. 3A). Its ability to inhibit collagen-induced platelet aggregation was tested by light transmission aggregometry. The affinity-purified  $m_{scFv}$  9O12 ( $25 \mu\text{g}\cdot\text{mL}^{-1}$ ) delayed the aggregation and reduced its extent from 65% to 25%, whereas the Fab was fully inhibitory (Fig. 3B). We further tested the monomeric form of  $m_{scFv}$  9O12 purified after size exclusion gel chromatography (Superdex 75 column) at the same concentration, and observed total inhibition of platelet aggregation, as observed with the Fab (not shown).

In addition, the effects of  $m_{scFv}$  9O12 on platelet adhesion and aggregation to collagen were investigated under arterial flow conditions, and compared with those of 9O12 Fab and an irrelevant scFv (Fig. 4). Once again, platelet aggregation induced by collagen was inhibited. In the presence of 9O12 fragments (scFv or Fab), only isolated platelets attached to the collagen fibers were observed, in agreement with previous results [20,23]. In contrast to control conditions, no large platelet aggregates were observed over a 5 min period.

As 9O12 Fab is known to inhibit thrombin generation at the surface of collagen-stimulated platelets, the effect of the purified  $m_{scFv}$  9O12 was tested using the thrombogram method (Fig. 5).  $m_{scFv}$  9O12 and 9O12



**Fig. 2.** Analysis of  $m_{scFv}$  9O12 binding to GPVI. (A) GPVI was immobilized on a CM5 sensor chip, and the binding of purified scFv 9O12 was analyzed by SPR after injection of increasing amounts. Nonspecific binding analyzed using scFv 9C2 has been subtracted. (B) Collagen was immobilized on a microtitration plate, and GPVI binding to collagen was investigated in the presence of increasing amounts of antibody: triangle,  $m_{scFv}$  9O12; square, 9O12 Fab; star, irrelevant scFv 9C2.



**Fig. 3.** Analysis of  $m_{scFv}$  9O12 binding to platelets. (A) Flow cytometry analysis: washed platelets were incubated for 30 min with antibody fragments ( $40 \mu\text{g}\cdot\text{mL}^{-1}$ ): Fab (upper panel) and scFv (lower panel) revealed by an FITC-coupled anti-mouse Ig and by an FITC-coupled anti-c-Myc Ig, respectively. Dark line: 9O12 Ig fragments. Gray histogram: control 9C2 antibody fragments. (B) Effects of  $m_{scFv}$  9O12 on platelet aggregation induced by collagen. Washed human platelets were incubated with control scFv 9C2, 9O12 Fab or affinity-purified scFv 9O12 for 5 min at  $37^\circ\text{C}$ . Then, collagen was added. Aggregation was analyzed at  $37^\circ\text{C}$  while stirring, and the change in light transmission was recorded.

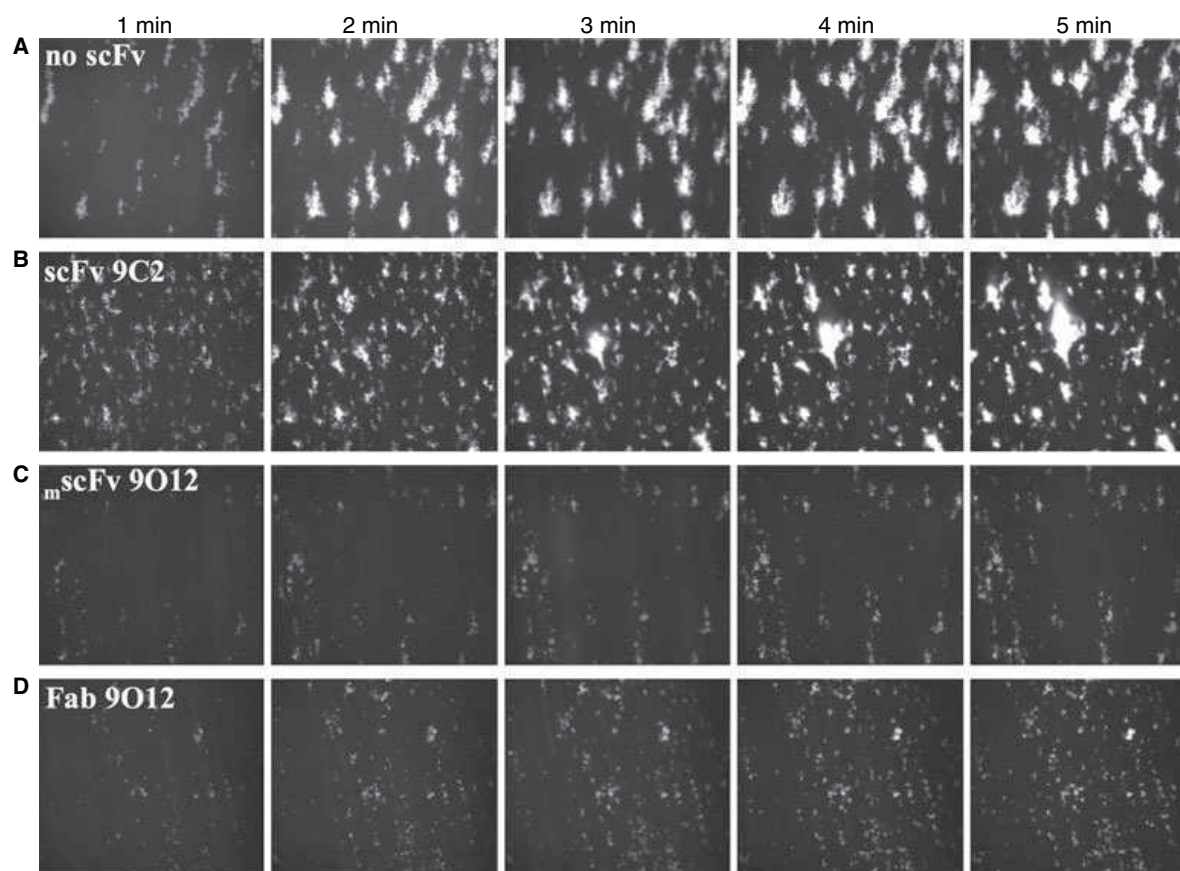
Fab reduced the thrombin peak to similar extents, and increased the lag preceding thrombin generation, indicating that  $m_{scFv}$  9O12 is as efficient as 9O12 Fab

in inhibiting collagen-induced platelet procoagulant activity.

### Humanization of $m_{scFv}$ 9O12 and functional evaluation

Murine antibodies would trigger an immune reaction if injected into human beings, and so humanization is required before any clinical investigation can be undertaken. To do this, we first constructed a 3D structural model of  $m_{scFv}$  9O12 *in silico* after identifying the crystal structures with sequences very similar to the 9O12 variable domains. All of these sequences were of murine origin. The top four scoring structures of murine origin were used for modeling. For the VH gene, we used antibodies designated 1PLG, 1MNU, 1A5F and 1IGI in the Protein Data Bank, which have 66–78% sequence identity (79–85% similarity) with 9O12. For the VL gene, we used antibodies 1PLG, 1IGI, 1MNU, and 1AXT, which have 87–90% sequence identity (94–95% similarity). The 3D structures of all these sequences were solved with a resolution higher than  $2.8 \text{ \AA}$ . Twenty models were generated for each domain, using MODELER 3.0 software, and the best one was selected on the basis of the rmsd value ( $0.13 \text{ \AA}$  for VH and  $0.703 \text{ \AA}$  for VL) and detailed inspection. The model is shown in Fig. 6A.

We then proceeded to the humanization of 9O12 variable domains. To do this, FASTA searches were performed to independently align VH and VL amino acid sequences against a repertoire of human antibody sequences registered in the Protein Data Bank. Among the human variable domains that matched 9O12, we first selected a VH domain and a VL domain from the same antibody molecule in order to preserve the inter-domain contacts that occur in a natural antibody. The human antibody 1VGE was selected because it had the best identity score with 9O12 when the entire variable domain sequences were spanned, and was found to exhibit 62% and 55% identity with the VH domain and VL domain, respectively. When calculated over framework region (FR) sequences alone, the identity was even slightly better, at 69.5% and 65.4%, respectively. In addition, the crystallographic structure of 1VGE was solved at high resolution ( $2 \text{ \AA}$  and  $R = 0.18$ ). We therefore decided to graft 9O12 CDRs onto the 1VGE template *in silico*. A gene encoding this construct was chemically synthesized, and inserted into pSWI exactly as had been done for  $m_{scFv}$  9O12. TOP-PI cells transformed with this vector were induced to express the recombinant protein. However, the recombinant protein was never detected in the periplasm of induced bacteria, and so some refinements of the



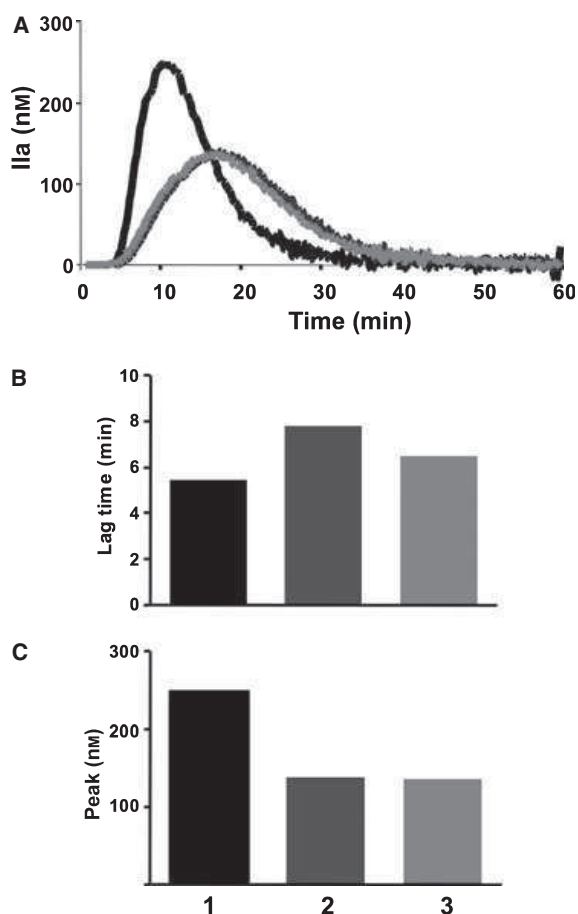
**Fig. 4.** Effect of  $m_{scFv}$  9O12 on platelet aggregation induced by collagen under arterial flow conditions. Whole blood cells labeled with fluorochrome DIOC-6 were incubated with NaCl/P<sub>i</sub> (A) or antibody fragments (B–D), and then perfused onto collagen-coated coverslips in a flow chamber at 1500 s<sup>-1</sup>. The formation of platelet aggregates bound to the collagen matrix was recorded with a fluorescence microscope at various time intervals. (B) Control scFv 9C2. (C)  $m_{scFv}$  9O12. (D) 9O12 Fab.

construct were called for. First, we observed that 9O12 V-kappa CDR1 is five residues longer than that of the template (1VGE), and that 9O12 and 1VGE V-kappa FR1 and FR2 have low identity scores (48% and 73%, respectively) (Fig. 6C). Extra FASTA searches were then performed using 9O12 V-kappa FR1 and FR2. An excellent match was found with V-kappa FR1 and FR2 from human antibody 1X9Q (95.6% and 86.6% identity scores, respectively, and 100% similarity in both cases). In addition, V-kappa CDR1 of the selected antibody 1X9Q was similar in length to that of 9O12. We therefore decided to preserve the original 9O12 V-kappa FR1 and FR2 in the novel humanized scFv construction. Other refinements were carried out on the basis of close inspection of the model, and the final construct is shown in Fig. 6B. All of the humanized 9O12 frameworks exhibit 100% similarity with human frameworks, apart from the IGHV

and V-kappa FR3 (90.62% and 93.75%, respectively) (Table 1). The 11 N-terminal residues from the murine IGHV FR3 were preserved in the final construct, because they are clearly located close to the flat part of the pocket in which the antigen is expected, and so could interact with it. Nevertheless, IGHV FR3 exhibits 25/32 residue identity with 1VGE. Only three residues of this framework (Ala71, Lys73, and Arg76; Kabat numbering) had no similarity with 1VGE. The 9O12 V-kappa FR3 was substituted for its 1VGE counterpart, with the exception of two residues (L59P and D60S), essentially because Leu is not frequently encountered at this position, and Asp is an acidic residue.

The gene encoding the scFv in which humanized 9O12 variable domains are fused together via the short flexible linker (G<sub>4</sub>S)<sub>3</sub> was synthesized and cloned into the pSW1 vector. The final humanized scFv 9O12





**Fig. 5.** Effect of  $m_{scFv}$  9O12 on thrombin generation induced by collagen in PRP. (A) PRP was preincubated with vehicle (black curve), 9O12 Fab (dark gray) or  $m_{scFv}$  9O12 (light gray) before addition of collagen. Thrombin generation was initiated by adding tissue factor and  $CaCl_2$ . The thrombin concentration was determined using a fluorescent substrate and was calculated relative to a calibrator. (B, C) The traces are from one representative experiment. Bar graphs represent mean  $\pm$  standard deviation of the lag phase (B) and the peak values ( $n = 3$ ) (C). The bars corresponding to the standard deviation are too small to be visible. 1, collagen; 2, 9O12 Fab + collagen; 3,  $m_{scFv}$  9O12 + collagen.

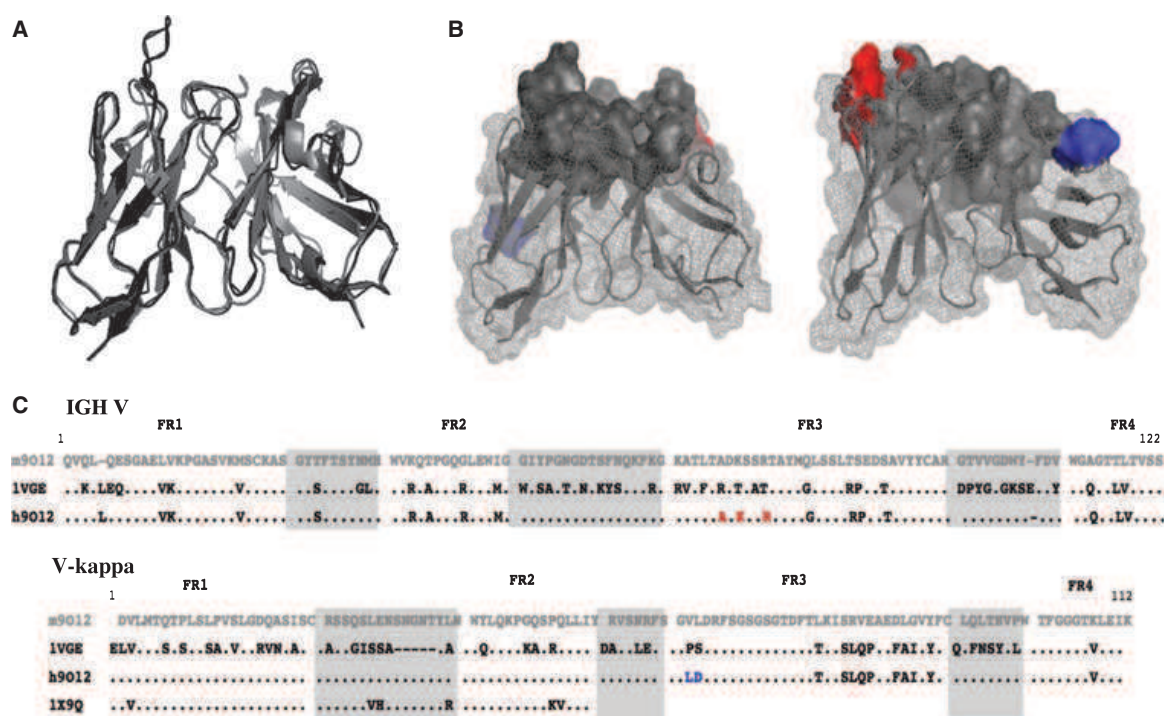
protein ( $h_{scFv}$  9O12) was produced in the periplasm of recombinant ToppI *E. coli* cells and purified to homogeneity by affinity chromatography using GPVI–Sepharose bead columns. A single band with the size expected (28 kDa) was observed after SDS/PAGE. However, the production yield of  $h_{scFv}$  9O12 was very low. We observed a slightly better level of expression when using BL21DE3 *E. coli* transformed with pET-22- $h_{scFv}$  9O12 (60  $\mu g \cdot L^{-1}$  of culture), and we selected this expression system to produce  $h_{scFv}$  9O12 for further characterization (Fig. 7A). The purified

$h_{scFv}$  9O12 conserved high affinity for its target, as demonstrated by SPR analysis against immobilized GPVI ( $k_{on} = 5.8 \times 10^4 M^{-1} \cdot s^{-1}$ ,  $k_{off} = 1.86 \times 10^{-4} s^{-1}$ , and dissociation constant  $K_D = 3.2$  nM) (Fig. 7B). It was also able to bind to freshly prepared human platelets in flow cytometry, and the shift to the right of the fluorescence peak was similar to that of cells labeled with  $m_{scFv}$  9O12 under similar experimental conditions (Fig. 7C). Finally, nearly total inhibition of  $h_{scFv}$  9O12 binding was observed when platelets were premixed with an excess of 9O12 Fab. In addition,  $h_{scFv}$  9O12 binding to platelets inhibited platelet activation induced by convulxin which is a specific GPVI agonist [24]. Indeed, P-selectin exposure was not observed at the surface of platelets preincubated with  $h_{scFv}$  9O12 and then activated by convulxin (Fig. 8A). In addition, we observed that  $h_{scFv}$  9O12 inhibited the aggregation of platelets induced by collagen but not by thrombin or the thrombin receptor agonist peptide (TRAP), which demonstrates that  $h_{scFv}$  9O12 inhibition is GPVI-dependent (Fig. 8B).

## Discussion

Platelets play a crucial role in arterial thrombosis and represent a major therapeutic target. All the drugs currently available act on late phases of thrombus formation, and their use is often associated with major drawbacks, such as prolongation of the bleeding time and/or induction of thrombocytopenia. Acting at an earlier stage of the platelet aggregation process could offer several advantages. GPVI and GPIa–IIa are the main platelet receptors, interacting directly with the collagen exposed by the subendothelial matrix as a result of vascular injury, but GPVI is the only one specifically expressed at the surface of platelets. Early GPVI interaction with collagen is a major event leading to platelet activation, the release of secondary agonists, and thrombus formation. Thus, inhibition of GPVI–collagen interaction could have potent antithrombotic effects. This has been demonstrated both *in vitro* and under physiological conditions *in vivo*. Deficiency in GPVI expression does not lead to impaired hemostasis in mice or humans [6,10]. Furthermore, anti-GPVI molecules inhibit thrombus growth without producing any significant side effects [8,10].

In this context, the need for better antithrombotic drugs, and the emergence of numerous antibody-derived molecules approved for the treatment of a wide range of disorders, have stimulated the search for potent antithrombotic antibodies. This is exemplified by the chimeric mouse–human Fab abciximab



**Fig. 6.** Humanization of the 9O12 antibody variable domains. (A) Superimposed variable domain ribbon diagram of murine 9O12 (gray) and human 1VGE (black) antibodies in lateral view. (B) Models of the humanized 9O12 variable domains in both lateral views. CDRs are shown in surface representation, and frameworks in ribbon representation. Framework residues having no similarity with their counterparts in human antibody sequences are shown in blue (V-kappa FR3) or in red (IGHV FR3). (C) Sequence analysis of antibody IGHV and kappa-V domains: murine 9O12 (m9O12); 1VGE; humanized 9O12 (h9O12); and 1X9Q. (.) indicates residues identical to murine 9O12. (–) indicates a gap. Residues of the humanized variable domains having no similarity with murine 9O12 are shown in red and blue (residues A71, K73 and R76 for IGHV, and L59 and D60 for kappa-V; numbering according to the Kabat nomenclature). CDRs are highlighted in gray.

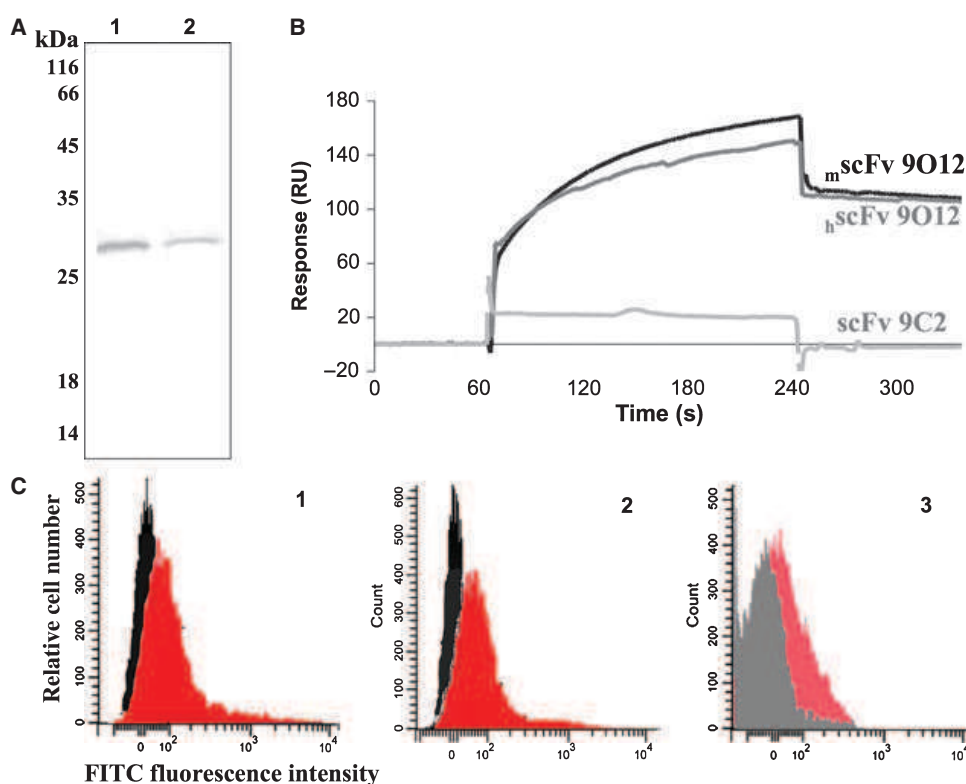
**Table 1.** Identity and similarity scores of the 9O12 humanized variable domain frameworks, with human antibody frameworks used as template; x is the number of residues in the humanized FR that are identical to residues from the human FR; y is the total number of residues in the FR.

Domain	Identity (x/y)	Identity (%)	Similarity (%)
IGHV domain			
FR1	23/26	88.46	100
FR2	14/14	100	100
FR3	25/32	78.12	90.62
FR4	11/11	100	100
V-kappa domain			
FR1	22/23	95.65	100
FR2	13/15	86.60	100
FR3	30/32	93.75	93.75
FR4	10/10	100	100

(Reopro<sup>®</sup>), which blocks the platelet GPIIb–IIIa receptor, and is now used for the treatment of patients with acute coronary syndrome undergoing percutaneous coronary surgery [25,26].

Few antibody molecules directed against GPVI have been prepared, because GPVI cDNA has only recently been cloned and expressed as a recombinant soluble protein [5,27]. Some anti-GPVI scFvs have been selected after panning phage libraries expressing non-immune human repertoires [13–15]. However, this approach has not yet been demonstrated to be fully effective. Indeed, although some of the anti-GPVI scFvs isolated do lead to dose-dependent inhibition of the GPVI–collagen interaction *in vitro*, they have a weak affinity for GPVI, making them unsuitable for clinical investigations. Such difficulties may be related to technical problems, including difficulties in constructing large libraries of scFvs, maintaining the repertoire over time, and slowing its inevitable drift [28].

All of the high-affinity antibodies against GPVI isolated so far have been prepared using the more conventional hybridoma technology after immunization of Balb/C mice with 3T3 fibroblasts expressing human GPVI or with the cDNA encoding the human GPVI protein, as is the case for 9O12, or after inoculation of



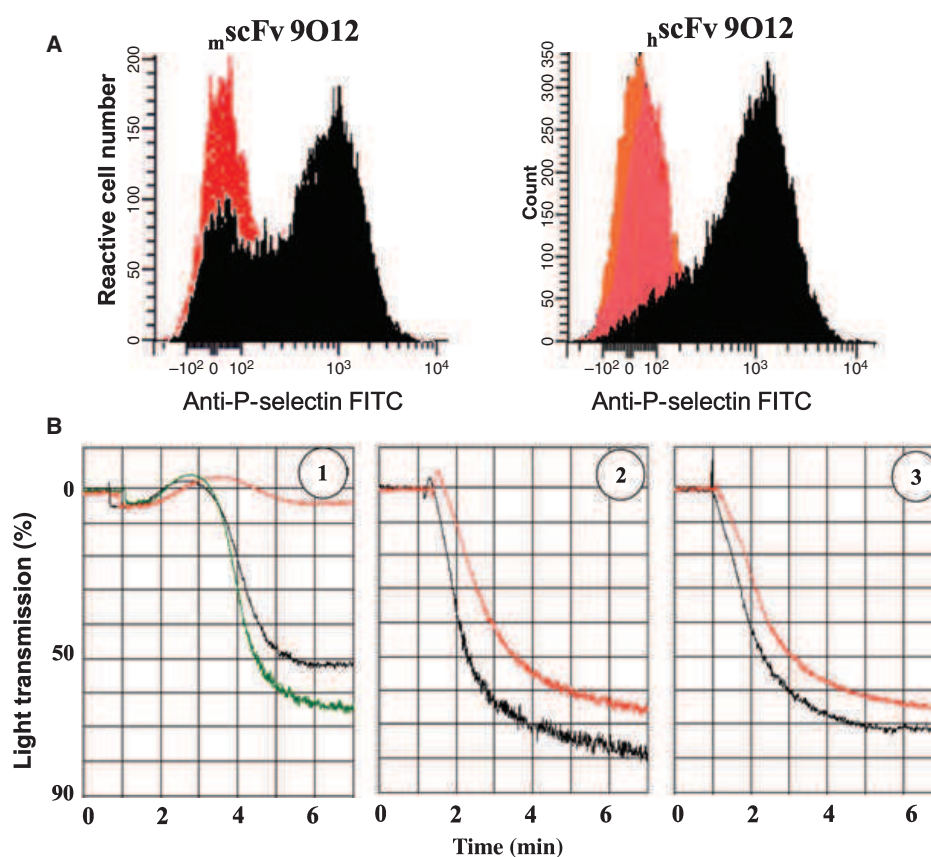
**Fig. 7.** Characterization of  $hscFv$  9O12. (A) Western blot detection of recombinant scFvs using antibody against c-Myc. 1,  $mscFv$  9O12; 2,  $hscFv$  9O12. (B) SPR analysis. GPVI was immobilized on a CM5 sensor chip, and the binding of purified scFv ( $10 \mu\text{g}\cdot\text{mL}^{-1}$ ) was analyzed. 1,  $mscFv$  9O12; 2,  $hscFv$  9O12; 3, control scFv 9C2. (C) Flow cytometry analysis. Washed human platelets were incubated with antibody fragments (red histogram), and bound antibody was detected using an FITC-coupled antibody against c-Myc. Panels 1 and 2:  $mscFv$  9O12 (1) and  $hscFv$  9O12 (2) are shown in red, and the control scFv 9C2 in black. Panel 3: platelets preincubated with 9O12 Fab (gray histogram) or not (red histogram) were then incubated with  $hscFv$  9O12.

GPVI-knockout mice with transfected Chinese hamster ovary cells expressing human GPVI and FcR $\gamma$ , as has recently been reported [17,18,20,29]. Some of these antibodies are devoid of blocking activity, such as HY101, 6B12, and 3J24.2 [29–31]. Others have good potential in terms of their biological and pharmacological properties, displaying direct blocking activity against the GPVI–collagen interaction when tested as monovalent Fab fragments. In addition, the *ex vivo* antithrombotic effects of anti-GPVI Fabs obtained by papainolysis of murine IgGs OM4 and 9O12 have been observed in nonhuman primates without the induction of platelet GPVI depletion [18,32]. For all of these reasons, 9O12 is an excellent template to use when designing antithrombotic molecules with potential human clinical applications.

Murine antibodies are highly immunogenic when injected into humans, and thus they have to be chimerized or humanized for therapeutic applications [33]. Chimerization is achieved by transferring variable

domains to human constant antibody domains. The chimeric Fab abciximab, which binds to the platelet fibrinogen receptor GPIIb–IIIa, inhibits thrombus growth, but it still induces adverse immune responses against mouse epitopes in small groups of patients [34]. A humanized antibody, ‘eculizumab’, has recently been approved by the US Food and Drug Administration for the chronic treatment of paroxysmal nocturnal hemoglobinuria. It is nonimmunogenic, safe, and well tolerated [35]. Several possible methods of humanizing antibody variable domains have been suggested [36–38]. None of these methods is simple, and all often result in impaired specificity and/or affinity [39–42].

In view of the functional properties of the murine 9O12 Fab fragment, we designed and evaluated the activity of derived scFvs that can be viewed as possible building blocks for future drug development. First, murine scFv 9O12 was engineered with the intention of determining the 9O12 variable domain sequences and checking their functionality when produced as a



**Fig. 8.** Effects induced by  $h$ scFv 9012 binding on platelet activation and aggregation. (A) P-selectin exposure: washed human platelets were preincubated with NaCl/ $P_1$  (black histograms) or the antibody fragments (red histograms) for 30 min, and then activated by convulxin. P-selectin exposure was detected using FITC-conjugated antibody against P-selectin. (B) Effects of  $h$ scFv 9012 on platelet aggregation: washed human platelets were incubated alone (dark line), with affinity-purified  $h$ scFv 9012 (red line), or with the flow-through fraction collected upon affinity purification of  $h$ scFv 9012 (green line). Aggregation was induced with collagen (1), thrombin (2), or TRAP (3).

recombinant molecule in a heterologous expression system. Affinity-purified scFv preserved high functional affinity for GPVI, which allowed it to block collagen-induced platelet aggregation, and inhibit thrombus formation under flow conditions as well as preventing thrombin generation. To reduce the immunogenicity of  $m$ scFv, we then proceeded to humanize it by a procedure essentially based on CDR grafting combined with refinements based on *in silico* modeling. The final product reported here exhibits 100% similarity with human antibody frameworks, with the exception of VH FR3 (90.62%) and VL FR3 (93.75%), and its framework sequences differ by 25 residues from that of  $m$ scFv 9012. The strategy consisted of grafting murine CDRs onto the frameworks of human antibody 1VGE in order to preserve interdomain contacts. This strategy was effective, but required further refinement. Indeed, during this first attempt, the recombinant scFv resulting from the grafting of 9012 CDRs onto 1VGE

frameworks was not produced efficiently. We postulated unforeseen structural incompatibilities between the original murine CDRs and the human acceptor frameworks that could have led to misfolding of the variable domains. To circumvent these difficulties, some minor refinements were made. First, we observed that 9012 V-kappa CDR1 is particularly long and has five more residues than 1VGE, suggesting that 1VGE V-kappa FR1 and FR2 are not suitable for the correct folding of CDR1. Back-mutation to 9012 wild-type V-kappa FR1 and FR2 was therefore required, and this was encouraged by the observation that the sequences of these murine frameworks fit well with another human antibody framework (1X9Q). V-kappa CDR1 of antibody 1X9Q is exactly the same size as that of 9012 (Fig. 6). We also retained in the final construct a very limited number of residues that could influence the ability of CDR loops to adopt their conformation.



Two critical areas were preserved. The first one was the dipeptide Leu-Asp at position 59–60 in the V-kappa domain. Leu59 (Pro in the human template) was considered to be potentially significant, as it is in the close vicinity of the residues of V-kappa CDR2. Although Leu and Pro are both hydrophobic, Pro has a cyclical side chain and is known to have specific effects on the protein backbone structure [43]. In addition, we noticed that Leu occurs much less often than Pro at this position (2%), and this may be indicative of a specific role [44]. The other unmutated murine residues with no similarities to the human template (1VGE) were located in IGHV FR3 (Ala71, Lys73, and Arg76; Kabat numbering). *In silico* observations and previous analysis have shown that Ala71 has an important role in the conformation of IGHV CDR2 [45]. Finally, only five murine residues in V-kappa and 10 in IGHV were maintained in the human frameworks selected for humanization. Other humanized scFvs reported in the literature have almost always required the insertion of numerous murine residues into the reshaped molecule in order to preserve strong binding activity and specificity [46]. FASTA searches of the final humanized IGHV and V-kappa sequences (with the exclusion of the CDRs) against the UniProt data bank showed that the five best-fitting sequences were all derived from human antibodies, whereas the same search performed for murine 9O12 variable domains led exclusively to murine antibody sequences.

The main parameters usually affected by humanization were well preserved in our final product. Affinity-purified  $_{\text{h}}$ scFv 9O12 was fully functional, with high affinity for GPVI, a major point for biological applications. Fluorescence-activated cell sorting analysis also indicated that  $_{\text{h}}$ scFv 9O12 recognizes the same epitope on human platelets as mouse 9O12 Fab, because its binding was specifically blocked in the presence of a molar excess of 9O12 Fab. However, despite the use of codons optimized for prokaryotic expression in the synthetic cDNA encoding  $_{\text{h}}$ scFv 9O12, the production yield remained low, and other expression systems will have to be tested in the future [47,48]. For this reason, the ability of  $_{\text{h}}$ scFv to inhibit aggregation in flow conditions was not investigated here. However, we clearly demonstrate that binding of  $_{\text{h}}$ scFv 9O12 inhibits GPVI-dependent platelet activation and aggregation.

In conclusion, this study provides the first experimental evidence that murine anti-human GPVI platelets can be humanized without deleterious effects, making  $_{\text{h}}$ scFv 9O12 a powerful building block for the generation and evaluation of new compounds with potentially therapeutic antithrombotic properties.

## Experimental procedures

### Materials

Hybridoma cell line 9O12 secreting a monoclonal IgG<sub>1,k</sub> directed against the GPVI of human platelets, and IgG and Fab fragment preparation and purification, have been reported previously [20]. The scFv 9C2 directed against a scorpion toxin, irrelevant to GPVI, was prepared as in [49]. The antibody against flag (9E10) was used either free or conjugated to horseradish peroxidase (Sigma-Aldrich, Saint Quentin Fallavier, France) or to fluorescein isothiocyanate (FITC) (Invitrogen, Cergy Pontoise, France). FITC-coupled goat anti-mouse IgGs were from Sigma-Aldrich.

The recombinant soluble human GPVI protein, consisting of two extracellular domains of the receptor, coupled to the Fc fragment of human IgG<sub>1</sub> was produced and purified as in [20]. GPVI was coupled to cyanogen bromide-activated Sepharose according to the manufacturer's instructions (Amersham-Pharmacia, Les Ulis, France). The gel was stored at 4 °C in NaCl/P<sub>i</sub> containing sodium azide (1%).

Blood was collected from healthy volunteers. Washed human platelets were obtained according to a previously described procedure [24].

All chemicals were of standard grade from Sigma-Aldrich or equivalent.

### Construction of the single chain antibody genes

mRNA was isolated from freshly subcloned hybridoma 9O12. cDNAs encoding the antibody variable domains (V-kappa and IGHV) were cloned after RT-PCR, essentially as previously reported [21,22]. Murine scFv 9O12 was created by PCR splicing with overlap extensions using oligonucleotides that encode the (G<sub>4</sub>S)<sub>3</sub> peptide linker between the C-terminus of IGHV and the N-terminus of V-kappa. First, IGHV and V-kappa genes were modified by PCR amplification with primers VHR<sub>rev</sub> and VHlinkFor or VLlinkRev and VLFor, respectively. VHR<sub>rev</sub> (5'-CAG GTG CAG CTG CAG GAG TCA GG-3') encoded the N-terminal sequence of IGHV containing a *Pst*I site, and VHlinkFor (5'-ACC ACC GGA TCC GCC TCC GCC TGA GGA GAC GGT GAC CGT-3') encoded the C-terminus of IGHV and part of the linker. VLlinkRev (5'-GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAT GTT TTG ATG ACC CAA ACT CCA CT-3') and VLFor (5'-GAC CCT CGA GCG TTT GAT CTC CAG CTT GGT-3'), which contains a *Xho*I site, were used to amplify and modify the V-kappa domain. Both genes were assembled by 'splicing by overlap extension' with primers VHR<sub>rev</sub> and VLFor. The gene fragment of the appropriate size was purified, cleaved with *Pst*I and *Xho*I, and cloned into the expression vector pSWI restricted in the same manner before being sequenced. This made it possible to clone the scFv gene in-frame with the

pelB leader sequence at its 5'-end and, downstream, a sequence encoding the c-Myc tag. The constructed vector pSW<sub>-h</sub>scFv9O12 was cloned into the Toppl *E. coli* strain (Stratagene, La Jolla, USA).

### Computational analysis and synthetic gene design

Frameworks and the hypervariable loops (CDRs) of antibody variable domains were identified using the Kabat nomenclature [50]. An *in silico* 3D model of 9O12 Fv was constructed by homology modeling based on V-kappa and IGHV domains with maximum sequence identity and a known tertiary structure. First, the protein structure sequence Protein Data Bank was searched for antibody sequence similarities with each individual 9O12 variable domain, using the FASTA 3 program at the European Bioinformatics Institute (EMBL-EBI, Cambridge, UK). Then, MODELER 3.0 software (Accelrys, San Diego, CA, USA) was used to generate up to 20 models for each variable domain, the best one being selected on the basis of the rmsd value.

To design a humanized scFv (<sub>h</sub>scFv), the 9O12 variable domain sequences were independently subjected to a FASTA search against the Protein Data Bank, and the closest human antibody variable domains were selected. The same search was also performed after excluding the CDR regions. The scaffolds of variable domains belonging to the same antibody molecule were chosen in order to minimize the risk of lowering the stability of the interaction between variable domains in the recombinant scFvs while attempting to preserve the scaffold required for the integrity of the antigen-binding site. In the first step, the human antibody 1VGE variable domains were selected as templates, and the murine 9O12 CDRs were grafted onto them *in silico*. The framework sequences were inspected to see whether any buried residues had been conserved. The packing of the grafted loops was also evaluated by visual inspection, and FR refinements were performed by *in silico* substitution.

The changes in the amino acids required for the humanization were obtained by *de novo* DNA synthesis. Optimization of the DNA design was performed using the codon usage table for expression in *E. coli* cells [51]. Restriction sites were also inserted at the extremities of the CDRs to make it possible to carry out the cDNA sequence adjustments that would be required to optimize the structural or functional characteristics of the recombinant protein. The synthetic gene encoding <sub>h</sub>scFv 9O12 was cloned into the expression vector pSW1 restricted with *Pst*I and *Xho*I, leading to a vector designated pSW<sub>-h</sub>scFv 9O12, which allows periplasmic expression of <sub>h</sub>scFv 9O12 fused to the c-Myc tag. The gene encoding <sub>h</sub>scFv 9O12 fused to the c-Myc tag was also cloned into the pET-22b(+) vector (Merck Chemicals Ltd., Nottingham, UK), which carries a T7

promoter. To do this, the sequence encoding <sub>h</sub>scFv 9O12 fused to the c-Myc tag was first reamplified from pSW<sub>-h</sub>scFv 9O12 by PCR using primers VHpEtRev (5'-TG GCC ATG GCC CAG GTG CAG CTG CAG G-3') and 9O12pEtFor (5'-TG GTG CGG CCG CTT AT-T AAT TCA GAT CCT CTT CTG A-3'). The amplified cDNA was then digested with *Nco*I and *Not*I, leading to a 820 bp sequence that was cloned in-frame with the pelB leader sequence into pET-22b(+) restricted in the same manner. The constructed vector, designated pET-22<sub>-h</sub>scFv 9O12, was cloned into the BL21DE3 *E. coli* strain (Merck Chemicals Ltd.).

### Production and purification of recombinant antibody fragments

For expression of the recombinant scFvs, bacteria containing the desired plasmids were grown in 500 mL of 2× TY medium (Difco, Le Pont de Claix, France) containing ampicillin (100 µg·mL<sup>-1</sup>) at 37 °C under rotative agitation (125 r.p.m.), until *A*<sub>600 nm</sub> reached 0.6 (BL21DE3 *E. coli*) or 1.5 (Toppl *E. coli*). Then, 0.8 mM isopropyl-thio-β-D-galactoside was added to the medium, and incubation was continued for 16 h at 16 °C under rotative agitation (75 r.p.m.) to induce scFv production. Bacteria were then collected by centrifugation (3600 g, 20 min, 4 °C). Periplasmic extracts containing scFv were prepared by resuspending the pellet in 10 mL of ice-cold TES buffer (0.2 M Tris/HCl, pH 8.0, containing 0.5 mM EDTA and 0.5 M sucrose), and incubating for 30 min. Cells were subjected to osmotic shock by adding TES buffer diluted 1 : 4 for 30 min on ice, and then centrifuging at 15 000 g for 30 min at 4 °C to remove insoluble material. Deoxyribonuclease A (50 U) and aprotinin (2 µg·mL<sup>-1</sup>) were added to the supernatant before extensive dialysis against NaCl/P<sub>i</sub> at 4 °C.

To purify scFv, the periplasmic extract (30 mL) was incubated with 500 µL of GPVI coupled to Sepharose beads for 12 h at 4 °C and for 4 h at room temperature. The mixture was loaded onto a microcolumn. After washing with NaCl/P<sub>i</sub> at pH 7.4, bound proteins were eluted with 0.1 M glycine-HCl (pH 3.0) in 0.4 mL fractions, and immediately neutralized with 5 µL of 3 M Tris on ice. Fractions with *A*<sub>280 nm</sub> higher than 0.2 were pooled and extensively dialyzed against NaCl/P<sub>i</sub>.

The purity was checked by SDS/PAGE using a 15% gel followed by staining with Coomassie brilliant blue, or by western blotting and immunostaining with horseradish peroxidase-coupled antibody against c-Myc, essentially as in [22]. The integrity of the purified recombinant protein was also investigated by MALDI-TOF MS on a 4700 Proteomics Analyzer MALDI-time of flight (TOF)/TOF apparatus (Applied Biosystems, Foster City, CA, USA).

Finally, the affinity-purified scFv (200 µL, 100 µg·mL<sup>-1</sup>) was analyzed by gel filtration using a Superdex 75 column

(Amersham Bioscience, Les Ulis, France) calibrated using standards from Boehringer Mannheim (Meylan, France). Proteins were eluted with NaCl/P<sub>i</sub> at a flow rate of 0.5 mL·min<sup>-1</sup>, and detected with a UV recorder at 280 nm.

The concentration of the purified scFvs was evaluated after measuring their absorbance at 280 nm, and using the Swiss Institute of Bioinformatics software (PROTPARAM tool) to determine the theoretical molecular mass of the recombinant scFvs and their extinction coefficient [52]. Affinity-purified hscFv 9O12 was aliquoted and stored at -20 °C in NaCl/P<sub>i</sub> containing 0.1% BSA until further use.

### Immunochemical characterization of the scFv fragments

#### ELISA

Microtitration plates were coated with GPVI in NaCl/P<sub>i</sub> (10 µg·mL<sup>-1</sup>, 100 µL per well) overnight at 4 °C. Nonspecific binding sites were saturated with 100 µL of 1% BSA in NaCl/P<sub>i</sub> for 90 min. The plates were then incubated with increasing concentrations of the scFv preparation (0–20 µg·mL<sup>-1</sup>; 100 µL) for 90 min. They were incubated for an additional 90 min with peroxidase-coupled antibody against c-Myc (100 µL, 1 : 750 in NaCl/P<sub>i</sub>). All incubations were carried out at room temperature. Finally, 100 µL of the substrate solution (orthophenylene diamine; Sigma-Aldrich) was added to each well for 5 min, and the absorbance was read at 492 nm. Two controls were performed: the first one used the irrelevant scFv 9C2 instead of scFv 9O12, and for the second, coating with GPVI was omitted. Five washes with NaCl/P<sub>i</sub> containing 0.05% Tween and 0.1 mg·mL<sup>-1</sup> BSA were performed between each of the intermediate steps. In competitive assays, scFv 9O12 (100 µg·mL<sup>-1</sup>) was mixed with increasing concentrations of 9O12 (0–100 µg·mL<sup>-1</sup>), before being delivered into GPVI-coated microtitration wells. Bound scFv was detected as described above.

#### SPR

The BIAcore 2000 instrument and all the reagents for analysis were obtained from BIAcore. GPVI was immobilized (approximately 600 RU) on a carboxymethyl dextran CM5 sensor chip activated with a 1 : 1 mix of *N*-hydroxysuccinimide (50 mM) and *N*-ethyl-*N'*-(dimethylaminopropyl)-carbo-di-imide (200 mM) by a 7 min pulse. Affinity-purified antibody fragments were then passed over the immobilized GPVI in HBS-EP buffer [0.01 M HEPES (pH 7.4), 0.15 M NaCl, 0.005% polysorbate 20 (v/v)] at a flow rate of 20 µL·min<sup>-1</sup> at 25 °C. Glycine-HCl (10 mM, pH 2.5) was injected for 30 s at 20 µL·min<sup>-1</sup> to regenerate the sensor chip between successive samples. Kinetic constants ( $k_{on}$ ,  $k_{off}$ ) were deduced from the analysis of association and dissociation rates at four different antibody fragment

concentrations, ranging from 5 to 40 µg·mL<sup>-1</sup>. The dissociation constant  $K_D$  was calculated from  $K_D = k_{off}/k_{on}$ . Sensorgrams were analyzed using BIAEVALUATION version 3.1 software. All experiments were carried out in quadruplicate at the Institute Jacques Monod platform (Paris, France).

### Flow cytometry

#### scFv binding

Washed human platelets ( $2 \times 10^7$  mL<sup>-1</sup>) from several healthy volunteers were incubated for 30 min at room temperature with 10–40 µg·mL<sup>-1</sup> purified scFv, and then incubated again for 30 min at room temperature with 5 µL of FITC-coupled anti-c-Myc IgG (dilution 1 : 60). Cell fluorescence was measured using a flow cytometer (Epics XL, Beckman Coulter, Villepinte, France). Background was determined by using the irrelevant scFv 9C2 instead of the scFv 9O12 variants. All incubations were carried out in the dark. When 9O12 Fab (40 µg·mL<sup>-1</sup>) binding to platelets was investigated, FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) (1 : 100) was used instead of the FITC-coupled anti-c-Myc IgG.

#### scFv binding inhibition

Cells were incubated with blocking Fab 9O12 (10 µg·mL<sup>-1</sup>) for 10 min. They were then mixed with purified hscFv 9O12 (40 µg·mL<sup>-1</sup>), and incubated for 30 min. Finally, 5 µL of FITC-coupled anti-c-Myc IgG (diluted 1 : 60) was added to the cells for a further 30 min before analysis of the cell suspension by flow cytometry.

#### Inhibition of platelet activation

Platelets were incubated for 30 min. with scFv (10–40 µg·mL<sup>-1</sup>) or NaCl/P<sub>i</sub>. Then, platelets were activated with convulxin (0.3 nM) for 15 min at 20 °C. Five microliters of an anti-P-selectin IgG conjugated to FITC (Beckman Coulter, Villepinte, France) was added to the cells for 30 min before analysis of the cell suspension by flow cytometry. All experiments were carried out at least in triplicate.

#### Ability of the antibody fragments to block GPVI binding to collagen

Wells of a microtitration plate (Immulon 2; Dynex, VWF, France) were coated overnight with 100 µL of collagen type I (equine tendon; Horm, Nycomed, Munich) (20 µg·mL<sup>-1</sup>) and blocked with BSA (0.2% in NaCl/P<sub>i</sub>) for 2 h. Then, 100 µL of GPVI (10, 20 or 40 µg·mL<sup>-1</sup>) that had been preincubated for 30 min with increasing amounts of antibody fragments (0–20 µg·mL<sup>-1</sup>) was added to each well. After incubation for 2 h, the bound GPVI was detected

using a peroxidase-coupled anti-human Fc (Jackson Immuno-Research Labs Inc., West Grove, PA, USA) and orthophenylene dimine. All incubations were performed at room temperature, and five washes with 300  $\mu\text{L}$  of NaCl/P<sub>i</sub> containing 0.1% Tween-20 and 1% BSA were carried out between each step. All assays were conducted in triplicate. The percentage of residual GPVI binding to collagen was determined using mean values.

### Platelet aggregation assays

#### Platelet aggregation

Washed human platelets ( $3 \times 10^8 \text{ mL}^{-1}$ ) were preincubated for 5 min at 37 °C with antibody fragments in NaCl/P<sub>i</sub> (25  $\mu\text{g mL}^{-1}$ ), without stirring. Platelet aggregation was then initiated by adding type I collagen to a final concentration of 1  $\mu\text{g mL}^{-1}$ , or thrombin (1 nM) or the thrombin receptor agonist peptide TRAP (10  $\mu\text{M}$ ) [20]. Platelet aggregation induced changes in light transmission that were continuously recorded (Chronolog Aggregometer Chrono Log Corp., Harveston, PA, USA).

#### Platelet aggregation under flow conditions

Platelet adhesion to collagen under flow conditions was measured essentially as described previously [20]. Glass coverslips were coated with fibrillar type I collagen (50  $\mu\text{g mL}^{-1}$ ). Blood from healthy volunteers was collected on 40  $\mu\text{M}$  PPACK, and labeled with DIOC-6 (1  $\mu\text{M}$ ). Blood aliquots were incubated for 15 min at room temperature with buffer or purified antibody fragment (9O12 Fab, mscFv 9O12, scFv 9C2) at a final concentration of 50  $\mu\text{g mL}^{-1}$ . The mixture was then perfused over the collagen-coated coverslips inserted in a flow chamber at 1500  $\text{s}^{-1}$  for 5 min. Transmission and fluorescent images were recorded in real time using a fluorescence microscope. Fluorescent images were obtained from at least 10 different collagen-containing microscopic fields, which were arbitrarily chosen at the end of perfusion. The area coverage of fluorescent images was analyzed off-line using HISTOLAB software (Microvision, Evry, France). Assays were performed using blood from two healthy volunteers and two distinct preparations of affinity-purified scFv.

#### Thrombin generation

Thrombin generation was continuously measured in platelet-rich plasma (PRP) using the thrombogram method as previously described [53]. Briefly, citrated PRP ( $1.5 \times 10^8$  platelets  $\text{mL}^{-1}$ ) was incubated with the antibody fragments (50  $\mu\text{g mL}^{-1}$ ) for 10 min at 37 °C before addition of the collagen (5  $\mu\text{g mL}^{-1}$ ). Ten minutes later, thrombin generation was initiated by transferring the samples into the wells of a microtitration plate containing tissue factor

(0.5  $\mu\text{M}$ ). After 5 min at 37 °C, the reaction was initiated by addition of buffer containing CaCl<sub>2</sub> (16.6 mM) and the fluorescent thrombin substrate Z-GGR-AMC (Stago, Asnières, France). Fluorescence accumulation of the cleaved substrate was continuously measured at excitation and emission wavelengths of 390 and 460 nm, respectively. First derivative curves of fluorescence accumulation were converted into thrombin concentration curves using a thrombin calibrator [54]. The peak height is an indicator of the maximum rate of thrombin formation, and is sensitive to platelet activation.

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Abstract:

Human platelets glycoprotein VI (GPVI) is evidenced to be a platelet receptor of major importance in the occurrence of arterial thrombosis. Thus, it can be considered to be of great interest in diagnosis and therapeutic of atherosclerotic diseases. Antibodies are powerful molecules which can be used in both diagnostic as well as for therapeutic purposes due to their unique characteristics. Monoclonal and recombinant antibodies have antigen restricted specificity, high affinity and can be used in various assays. Moreover, the good knowledge of their structure and molecular engineering facilities now allows the antibody modulation according to desired properties.

Our group has already produced several monoclonal antibodies to human GPVI by gene gun immunization against the immunoadhesin hGPVI-Fc, which differ in fine epitope specificity, affinity and other functional properties (Lecut *et al.* 2003). One, 3J24, with diagnostic potential while the other, 9O12, has a therapeutic potential because it blocks the binding of GPVI to collagen. Its Fab fragment has been extensively characterized *in vitro*, *ex vivo* and *in vivo* for its antithrombotic properties.

Here, we designed and reshaped a single-chain antibody fragment (scFv) based on 3J24 variable domains for the quantification of GPVI with diagnostic potential. We were also involved in the design, production and functional evaluation of humanized anti-GPVI recombinant antibody fragments (scFvs and Fabs) with therapeutic properties.

Key words: recombinant antibody fragments, scFv, arterial thrombosis, platelets, glycoprotein VI