Receptors, cells & mechanisms responsible for antibody-induced tumor therapy

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PREAMBLE

Monoclonal antibodies (mAbs) targeting antigens expressed on tumor cells are used in the treatment of tumors and aim at inducing a reduction in the tumor burden[1,2]. Due to their functional properties these molecules can be considered a double-edged sword. On the one hand, a part of the therapeutic effect of mAbs relies on their Fab portion that can target surface proteins whose signaling is involved in tumor growth and/or survival[3]. On the other hand several studies conducted in animal models, as well as clinical correlations, suggest a crucial role for activating receptors for the Fc portion of IgG (FcγRs) in the anti-tumor activity of mAbs[4]. A role for FcγRs implies that effector cell populations bearing these receptors are contributing to anti-tumor mAb therapy. (Albanesi M. and Daeron M. Imm. letters 2012 *annex review*)

Several FcγRs exist that differ from each other for both their functional activity and their expression pattern among hematopoietic lineages. Moreover, the analysis of the FcγRs expressed in different species reveals that numerous differences exist between mouse and human FcγRs. Thus, whereas some studies addressed the role of FcγRs in anti-tumor mAb therapy, the responsible FcγRs remain elusive. In addition, neither the cell population nor the mechanism responsible for tumor killing has been formally identified.

Using different mouse models of anti-tumor mAb therapy, I have identified during my PhD the mouse FcγRs responsible for mAb-dependent tumor killing in one model (Albanesi M *et al*., J Albanesi M *et al.,* J. Immunol, 2012, in press), and analyzed the involvement of two of their human homologues (Mancardi DA, Albanesi M *et al*., 2nd revision at Blood). Furthermore, I have identified an unexpected role for an innate cell population during mAb therapy and propose a mechanism by which tumor cells are killed in the presence of anti-tumor mAbs in mouse therapy models of solid tumors (Albanesi M, *et al.,* submitted).

INTRODUCTION

INTRODUCTION

Several therapeutic approaches are already available for the treatment of solid tumors and new ones are constantly developed. In this first chapter, I will describe the main treatments of solid tumors with a particular focus on cancer immunotherapies.

TREATMENT OF SOLID TUMORS

The treatment of a solid tumor starts when a neoplasm is clinically visible and diagnosed. At that moment a solid tumor can already be considered a "mature" malignant structure.

1-Rate of growth

One of the main features of cancer cells is that they grow uncontrollably. As a rule, the growth rate of cancer cells is inversely correlated with their level of differentiation. Thus undifferentiated malignant tumors have a higher proliferation rate than differentiated malignant tumors.

In a clinically visible tumor mass the neoplastic proliferating cells (the main core of the tumor) are surrounded by collagen fibers (*tumor parenchyma*). The mass is irrigated by blood vessels (*tumor angiogenesis*) that will ensure the energetic supply. Depending on the stage of the tumor growth, the primary tumor mass can be confined (*non invasive tumor*) or not (*invasive tumor*) to the wall of the organ in which it develops. Furthermore, due to their metastatic potential, tumors are often accompanied by the outgrowth of several, local or distant, metastases. A proper evaluation of the tumor and of its pathological features (described in inserts **1-4**) at the time of diagnosis enables to choose the most adapted, and therefore efficient, treatment.

Solid tumors are firstly treated with surgery, considered as the gold standard. This treatment enables to remove the primary tumor and the detectable metastases. In the case of an invasive tumor, a part of the surrounding sane tissue and the draining lymph nodes are also removed. The lymphatic system is, indeed, considered the main route by which tumors can metastasize.

2- Differentiation

Neoplastic proliferating cells represent with various degrees of fidelity their precursors; benign tumors are generally composed of well differentiated cells (*e.g.* lipomas) whereas malignant tumors are composed of a range of differentiated to undifferentiated cells (*e.g.* adenocarcinomas). Lack of differentiation among tumor cells is called aplasia and is considered a hallmark of malignancy. This particular state can be unraveled by histological analyses of nuclei hypercromacy or pleiomorphisms, i.e. by the identification cancer cells alterations.

Complementary therapeutic approaches (*adjuvant treatments*) that aim at preventing the outgrowth of tumors from residual tumor cells, left behind after the surgical intervention, have been developed to improve the efficacy of surgery. The main adjuvant treatments are chemotherapy and radiation therapy. Both these treatments aim at reducing the proliferation of neoplastic cells. The decision of employing one or

the other approach is based on the type and localization of the tumors, the medical history of the patient and international guidelines.

- *Chemotherapy (CT)* consists of administrating drugs that interfere with the cell cycle. Chemotherapeutic agents can be divided in: *alkylating agents* that via alkylation of the DNA cause cell death (*e.g.* Cycloposphamide); *antimetabolites* that prevent the incorporation of purine and pyrimidine into DNA and RNA (*e.g.* Azathioprine); *antracyclins* that inhibit the DNA and RNA synthesis (*e.g.* Mitoxantrone); *plants alkaloids* (*e.g.* Vincristine) that prevent the microtubule formation; and *topoisomerase inhibitors* that interfere with the enzymatic activity of topoisomerases (*e.g.* Etoposide). As an example, the administration of a anthracycline-based polychemotherapy treatment for 6 months, to patients that underwent surgical removal of breast cancer, reduced the death rate by 38% for woman younger than 50 years old and by 20% for those of age 50-69. [5]

3-Invasiveness

The growth of a malignant neoplasm is accompanied by infiltration, invasion and destruction of the surrounding tissue. Due to invasion processes, most invasive malignant tumors recognize no normal anatomic boundaries, and can be expected to penetrate the wall of the organ in which they develop. Invasiveness is considered the most reliable feature to differentiate anatomically malignant from benign tumors

- *Radiation Therapy (RT)* consists of local irradiation using different types of ionizing radiations (X-rays, and electrons). The ionizing radiation can indeed produce a variety of lesions in the DNA including single and double-strand breaks, oxidation and degradation of bases, cross-linking of the DNA strands and even DNA-protein cross-links. This damage may be produced directly by the radiations themselves or by oxygen-free radicals generated by the interaction of ionizing radiation with H_2O molecules present in the tissues. Cells that have been lethally damaged (*e.g. double strand DNA breaks*) will rapidly die by apoptosis, whereas cells that have been sub-lethally damaged (*e.g. single-strand DNA breaks*) may die while trying to divide and/or have their replication rate reduced. RT can be used in adjuvant settings as treatment of several tumors; the most promising results have been obtained in the treatment of seminoma, a germ cell tumor of testis. In the early stage of disease, an adjuvant RT treatment is associated with an improved survival[6].

RT and CT can also be performed as *neo-adjuvant* therapy in order to reduce the tumor size prior to surgery. Large and/or overgrown tumors, indeed, deeply infiltrate the tissue that either require a massive surgical intervention or may preclude surgery. Furthermore, many types of

tumors generate metastases seeding either locally or, depending on the tumor type, in various tissues/organs and more particularly in the liver, lungs, bones and brain. CT having a systemic diffusion, it may also affect the development of metastases. When metastases are localized in the irradiated area during RT, this therapy may also be beneficial on these metastases. If metastases are localized outside of this area, they will, however, not be affected by the radiation or oxygen radicals induced by these radiations. Neo-adjuvant CT and/or RT led to improve the outcome of some cancer treatments: a neo-adjuvant CT treatment of patients with large breast cancers led to a reduction of tumor size in 80% of the cases that rendered lumpectomy feasible (*National Surgical Adjuvant Breast and Bowel Project B-18 trial*), and a coupled CT and RT neoajuvant treatment of rectal cancers lead to a reduction in the local relapses.[5,7]

4- Metastatic potential

As result of the invasion process, cancer cells can have access to blood vessels, lymphatics and body cavities. This enables cancer cells to spread and to give rise to tumor implants at distant anatomical localizations, *i.e.* discontinuous with the primary tumor. These distant tumor implants that nearly all malignant tumors can generate are called metastases.

HOW CAN CANCER CELLS BE TARGETED?

Despite the positive results in the treatment of solid tumors, the effects CT and RT are not confined to the cancer cells by definition. Normal cells and tissues, indeed, may also suffer from these treatments. CT, due to its systemic diffusion, may induce a severe neutropenia that exposes the patients to opportunistic infections; RT may lead to fibrosis in the site of irradiation causing organ dysfunction. Because both of these treatments are not specific, several other therapeutic approaches have been developed aiming at affecting tumors more specifically and in particular *targeted therapies by interfering compounds* and *targeted immunotherapies.*[8]

Targeted therapies by interfering compounds:

The growing understanding of the molecular events underlying carcinogenesis and cell growth led to the development of cancer-*targeted therapies by interfering compounds.* These therapies consist of providing drugs that interfere specifically with key molecules involved in cancer cell growth and death. The most intensively studied targets are protein kinases, key enzymes involved in the signaling cascade of several receptors required for cancer cell growth and angiogenesis. As an example, the activity of Epidermal Growth Factor Receptors (EGFRs) depends on their intrinsic tyrosine kinase activity. Because EGFRs are found to be mutated and/or over-expressed in different tumors, tyrosine kinases have been considered an important therapeutic target[9]. The first kinase inhibitors were described nearly 20 years ago, and nowadays the effects of more than 30 agents are under study in clinical trials. The best known small molecule inhibitor for the treatment of solid tumors is *Geftinib*. Geftinib is a selective EGFR tyrosine kinase inhibitor. It disrupts the intrinsic kinase activity of EGFR by binding the ATP pocket within the catalytic domain in the intracellular domain of the receptor[10,11]. Geftinib was approved in 2003 by the FDA for the treatment of non-small cells lung cancer (NSCLC); it induces tumor regression in 10-30% of the patients[12-14]. Other approved targeted treatments are: *Lapatinib,* an inhibitor of the HER2 receptor tyrosine kinase that, in combination therapy with the chemotherapeutic agent *capecetabine,* leads to 51% reduction in the disease progression in patients with breast cancer[15]; *Imatinib*, an inhibitor of the Bcr-Abl tyrosine kinase, for the treatment of chronic myelogenous leukemia and gastro intestinal stromal tumors (GISTs)[16]; *Sunitinib*, an inhibitor of the VEGF Receptor tyrosine kinase that, in the treatment of GISTs, reduces the risk of disease progression and death by 67% and 51%, respectively.[17,18]

Targeted immunotherapies

The final outgrowth of a tumor depends on the balance between the rate of tumor cell proliferation and the efficiency of the endogenous immune response that controls the tumor burden. *Targeted immunotherapies* comprise different therapeutic approaches that try to shift this balance in favor of the anti-tumor immune response. In fact, these treatments target different steps of the anti-tumor immune response. To properly describe the basic principles of the targeted immunotherapies in the next paragraphs an overview of an "ideal" anti-cancer immune response has to be given. This overview will be then followed by a description of selected targeted immunotherapies.

Anti-tumor immune response.

- *First step: antigen presentation*. The immune response against a tumor usually starts when the rate of tumor growth and the vascularization of the tumor are misbalanced causing an internal zone of hypoxia that leads to tumor cell death. The dead cell bodies and tumor antigens are picked up by *Dendritic Cells* (DCs) in the tumor environment. Upon antigen uptake, DCs migrate into secondary lymphoid organs, particularly in the tumor-draining lymph nodes. The tumor antigens are processed by DCs into proteolytic peptides that are loaded onto MHC class II molecules ***(n.b.** antigens can also be loaded onto MHC I molecules in the case of cross-presentation)*. This process of antigen uptake, degradation and loading, is called *antigen presentation.* DCs are professional *Antigen Presenting Cells* (*APC*). Other cell subtypes can function as APCs, such as monocytes, macrophages and B lymphocytes. Once the DCs have migrated into the tumor draining lymph nodes, they undergo a process of maturation that enhances the expression of several adhesion and co-stimulatory molecules on their surface. This phenomenon is associated with their rapid re-localization inside the lymph node to the T cell zone.

- *Second step: generation of effectors cells*. In the T cell zone, peptides loaded onto MHC molecules can be recognized by T cells *via* their *T-Cell Receptor* (TCR). The recognition of a peptide-MHC complex by the TCR together with the mandatory interaction of co-stimulatory molecules CD80 and CD86 (on DCs) with the CD28 receptor (on T cells), drives T-cell activation. This activation leads to the generation of tumor-specific $CD8^+$ and $CD4^+$ helper T cells. This process of T cell activation via the DCs is called *T cell priming*.

On the one hand primed $CD8^+$ T cells, leave the lymph node and can migrate into the tumor site. There, the recognition by the TCR of tumor antigen peptides displayed on the tumor cell surface on MHC I molecules triggers $CD8^+$ T cell activation. Upon activation, several cytotoxic molecules such as *granzyme* and *perforin* are released by $CD8^+$ T cells that may result in killing the most proximal tumor cells. Furthermore, molecules expressed by $CD8^+$ T cells can also engage interactions with particular molecules expressed on the target cell surface, globally termed *Death Receptors*, that can trigger pathways leading to cell death by apoptosis *(**n.b**. the biological activity of granzymes, perforin and death receptors are further discussed in chapter 3).*

On the other hand, T cell priming can also lead to the generation of $CD4^+$ helper T cells. These cells do not acquire a cytotoxic phenotype and participate rather to the third step of the immune response.

- *Third step: production immune effector molecules*. Tumor antigens are recognized by B cells through the B-Cell Receptor (BCR). Whereas TCRs recognize peptides processed from antigens on MHC molecules, BCRs recognize antigen in their native form. Once having recognized the antigen, B cells are trapped in the lymph node at the border of the T-cell and B-cell zone. In this zone, primed $CD4^+$ helper T cells interact with B cells that are specific for the same antigen. This process leads to the expression of B cell-stimulatory molecules on $CD4^+$ helper T cells, such as CD40 ligand, and to the secretion of B cell-stimulatory cytokines, such as Interleukin 4 (IL4) and Interleukin 6 (IL6). Subsequently, B cells proliferate and form a secondary structure called *germinal center.* In this structure, B cells undergo a process called *Somatic Hypermutation:* an enzyme called *Activation-Induced (cytidine) Deaminase (AID)* induces DNA brakes that may result in point mutations in the immunoglobulin genes. Hot spots of mutation are present in the variable regions of these genes, thus generating a pool of B cells that may differ in their specificity and/or affinity for

5-Structure of Immunoglobulin molecules

An immunoglobulin is a Y-shaped molecule with a molecular weigh of approximately 150kDa and composed of two different kinds of polypeptide chains: two heavy chains (H-chains) and two light chains (L-chains). Disulfide bonds link the two H-chains to each other and each H-chain is linked to a L-chain by disulfide bonds (Fig. 1). Each L-chain has one constant domain (C_L) and one variable domain (V_L). Two genes encode two types of light chains (lamba-λ; kappa-κ) with no described functional differences. By contrast, five heavy chain classes exist, defining five different antibody subclasses with different functional activities: Immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin E (IgE). IgM, IgD, IgG and IgA H-chains consist of two constant domains (C_H) and two variable domains (V_H), whereas IgE H-chains consist of three C_H and two V_H.

An antibody, i.e. an immunoglobulin with a defined specificity, can be functionally shared into two different portions: the antigen binding site (Fab portion) and the fragment cristallizable (Fc portion). The specificity of an antibody is determined by the combination of the two Complementary Determining Regions (CDRs) that are included one in the V_H and one in the V_L.

the tumor antigen. Subsequently, B cells with the highest affinity for that particular tumor antigen are selected to survive and proliferate though a process called *Clonal Selection*. These proliferating antigen-specific B cells can differentiate into *memory B cells* or *antibody-producing B cells*. Antibody-producing B cells are also called plasma cells and are responsible for production of the main immune effectors molecules of the B cells, the ***antibodies (Abs-fig-1 and insert 5)***. During an immune response, a first population of plasma cells is generated. These cells produce antibodies belonging to the IgM class that may provide a first line of protection. In the meanwhile, proliferating antigen-specific B cells can undergo a process called *isotype switch* that generates plasma cells producing antibodies of a different class such as IgG, IgE and IgA, and among them of different subclasses/isotypes. Importantly, these switch variants maintain their antigenic specificity as the isotype switch does not affect the sequences encoding the variable regions of a given antibody, and in particular not the *Complementary Determining Regions (CDRs)* present in these variable regions (refer to insert 6).

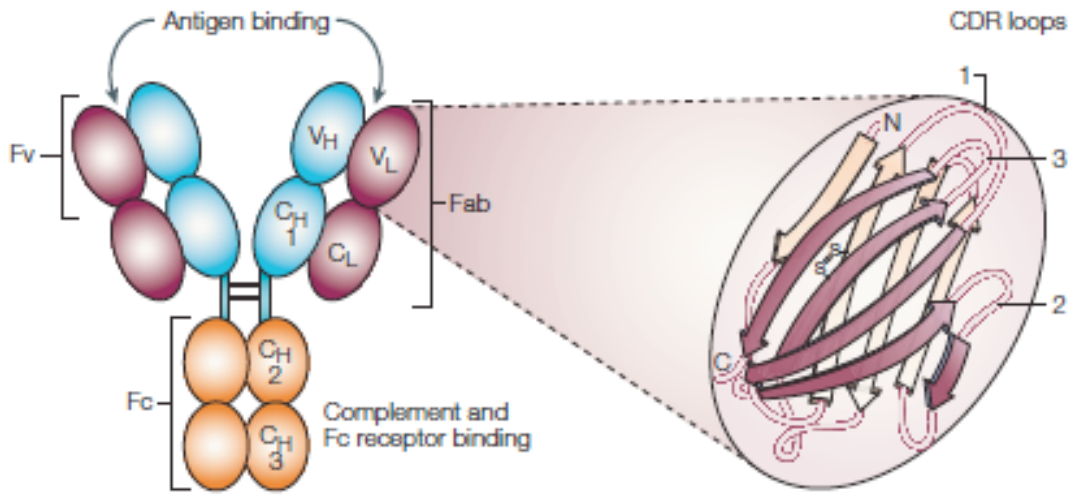

Fig.1 *Schematic representation of an Immunoglobulin molecule (Adapted from. Brekke O.H. Nature reviews, 2012)*

The basic principles of an anti-tumor immune response being described in the previous paragraphs, I will now describe the main targeted immunotherapies. These treatments can be divided into different groups based on the steps of the immune response they target.

Targeting the "antigen presentation" step: Immunotherapies as a mean to induce an immune response

Despite the "theoretical" capability of the immune response to inhibit or block the growth of a given tumor, tumors are able to escape from this response by altering antigen presentation trough a variety of mechanisms[19]. Consequently, the anti-tumor immune response can be inhibited or abolished by the tumor itself. Different therapeutic approaches have been developed aiming at inducing tumor antigen presentation in order to trigger an anti-tumor immune response. These approaches are named *cancer vaccination*. The main treatments that have been developed are: *cellular vaccines*, *peptide vaccines* and *DC-based therapies.*

- *Cellular vaccines* are based on the administration of dead tumor cells or their lysate, mimicking the very fist step of the antigen presentation in which dead cell bodies are picked up by DCs, tumor antigens processed and presented[20]. Although this procedure induces an anti-tumor immune response towards a wide array of tumor antigens, the clinical efficacy of cellular vaccines is still uncertain. Among unsuccessful examples: *Canavaxin*, a cellular vaccine proposed as an adjuvant treatment for metastatic melanoma, failed to provide significant survival benefit for patients[21].

- *Peptide vaccines:* The basic principle of peptide vaccines is the same as that of cellular vaccines. The difference lies in the fact that in this treatment, tumor antigens (*e.g. gp100, melan/A-MART 1* used in the case of a malignant melanoma), and not whole cells, are administered. This administration includes an adjuvant in order to promote the presentation of these tumor antigens by APCs. This vaccination approach has several advantages compared to cellular vaccines: peptides are easily produced; the corresponding antigen is well defined. This allows monitoring the antigen-specific (and thus tumor-specific) immune response over time. Although peptide vaccines induce immune responses in almost 80% of the patients treated, for unknown reasons only 10-20% of these show clinical improvements. [22]

- <u>*DC-based therapy:*</u> This therapy aims at generating DCs *ex vivo* using cells from the patient, load these DCs with a given tumor antigen, and re-inject these DCs into the patient[23]DCs can be obtained *ex vivo* by culturing $CD34^+$ cells or peripheral blood monocytes in the presence of various cytokine cocktails. These DCs can be transfected with cDNA or mRNA coding for tumor antigens, or bulk RNA prepared from the tumor. DCs are then matured in the presence of cytokines and growth factors, and finally re-infused into the patient using intradermal, subcutaneous or intra-nodal routes. [24]. It is commonly believed that once infused into the patients, these DCs may induce the priming of $CD8^+$ T cells and $CD4^+$ helper T cells, thus triggering an anti-tumor immune response. This approach has been used to develop *Sipuleucel*-T, a cellular product based on DCs loaded with prostatic acid phosphatase (PAP), an antigen commonly found expressed by prostate tumors. Sipuleucel-T has, in fact, been approved for the treatment of prostate cancer[25-27]. More than 10 clinical trials have already evaluated the efficacy of DC-based therapy with encouraging results in terms of generation of an immune response and tumor regression. [19]

Targeting the "generation of effector cells" step: Immunotherapies as a mean to reinforce an existing immune response

$CD8^+$ T cells are the main effector cells induced during an anti-tumor immune response. Due to their high killing potential, these cells play a pivotal role in anti-tumor immunity and their detection in the tumor microenvironment is associated with a favorable clinical outcome[28,29]. However, there is growing body of work suggesting that the tumor killing activity of $CD8^+$ T cells is hampered *in vivo* because of their chronic activation state, and because tumors can produce immunosuppressive factors. Different immunotherapies aim at *optimizing $CD8^+$ T cell activation* or to *amplify their number* in order to ameliorate tumor killing:

- <u>*Optimizing the $CD8^+$ T cell activation*</u>: $CD8^+$ T cell activation requires, aside from MHC/TCR interaction, a second signal mediated by CD80 and CD86 expressed on the DCs. These molecules interact with two receptors on the T cell: *CD28* and C*ytotoxic T-lymphocyte antigen*-4 (CTLA-4). The engagement of CD28 amplifies TCR-induced T cell activation. The engagement of CTLA-4, by contrast, negatively regulates TCR-induced cell activation. Therefore during an immune response, the interaction of CD80/CD86 with CTLA-4 may limit the activation of $CD8^+$ T cells. In order to reinforce the activation of $CD8^+$ T cells, it possible to bypass the CTLA-4-mediated inhibition using specific anti-CTLA-4 blocking antibodies, as assessed in the clinic

with promising results[30]. In fact, the treatment of metastatic melanoma with an anti-CTLA-4 mAb called *Ipilimumab*, resulted in 47% and 32,8% survival rate over one and two years, respectively. [31]

- *Amplifying CD8$^+$ T cell numbers:* An Autologous Cell Transfer (ACT) allows boosting the tumor-specific CD8$^+$ T cell response, by amplifying a pool of CD8$^+$ T cells *ex vivo* and re-injecting them *in vivo*[32]. In this treatment scenario, the tumor masses are surgically resected and fragmented to obtain single cell suspensions. These cells are plated in the presence of Interleukin-2 (IL2), a potent T cell growth factor inducing T cell proliferation. From this cell population, CD8$^+$ T cells are isolated, and the ones expressing a TCR specific for a tumor antigen of interest are selected and further expanded in adapted cell media. It is also possible to genetically engineer isolated CD8$^+$ T cells to confer them the specificity for a desired tumor antigen (*e.g.* CD8$^+$ T cells are transfected with DNA vectors encoding a TCR specific for a tumor antigen)[32]. A lympho-depletion of the patient prior to the transfer of CD8$^+$ T cells, as well as a systemic IL2 treatment, are required to enhance the survival of the transferred T cells. ACT appears nowadays to be one of the most efficient treatment for metastatic melanoma, as a recent clinical trial gave an objective clinical response *(n.b. assessed using response evaluation criteria in solid tumors -RECIST)* that ranged from 49 to 72 % [33] [34] [35]. Nevertheless, not every patient can benefit from this treatment since a good performance status is required to undergo the lympho-depletion and IL2 treatment.

Targeting the "production of effector molecules" step: immunotherapies as a mean to induce effector mechanisms.

Using immunotherapies, it is also possible to by-pass the first two steps of the immune response ***(n.b.** antigen presentation and generation of effector cells*), by directly supplying immune effectors molecules, and among those antibodies. This particular therapy is called *antibody-based cancer immunotherapy*.

Antibody-based cancer immunotherapy. After immunization of animals with antigens, specific antibody-producing B cells can be isolated and fused with myeloma cells to obtain immortalized antibody-producing B cells called *Hybridomas*. An hybridoma is a clone derived from a single B cell, thereby all the antibody molecules it produces are identical in structure, including their antigen binding site and their antibody isotype. Such antibodies are called *monoclonal antibodies* (mAbs)[36].

Since the early 1980s, mAbs have been used in clinical practice to different aims, and several of them in the treatment of cancer. Even though mAbs originating from animals or humans have a similar structure, they are antigenically different. Thus, once injected in humans, mAbs originating from animals may induce an anti-mAbs immune response that may lead to shortened half-life of these therapeutic mAbs and reduce their effector function. A first example came from *moromomab* (OKT3) a mouse anti-CD3 mAb approved in 1986 for the treatment of transplant rejection. The patients that underwent OKT3 treatment had, indeed, a strong Human Anti-Mouse Antibody response (HAMA) that diminished the therapeutic efficacy of OKT3 [37]. To overcome this problem, genetic engineering was employed to generate first chimeric, then humanized, mAbs that resemble more human endogenous antibodies than mouse antibodies do. A chimeric mAb contains the variable regions of animal origin (mouse, rat,…) and the constant regions of a human antibody. In a humanized mAb, only the CDRs are of animal origin, and they are grafted into a human antibody framework. The first chimeric and humanized mAbs produced were of the human IgG_1 subclass. This choice was most probably due to the fact that among the IgG molecules in human serum, IgG_1 are the most abundant *(**n.b**. IgG concentration in human serum: $IgG_1>IgG_2>IgG_3=IgG_4$)* and have among the longest half-lives (21 days). More recently, the possibility of cloning of human antibody-producing B cells directly from human blood enabled the generation of fully human mAbs (***fig.***2).[38-40]

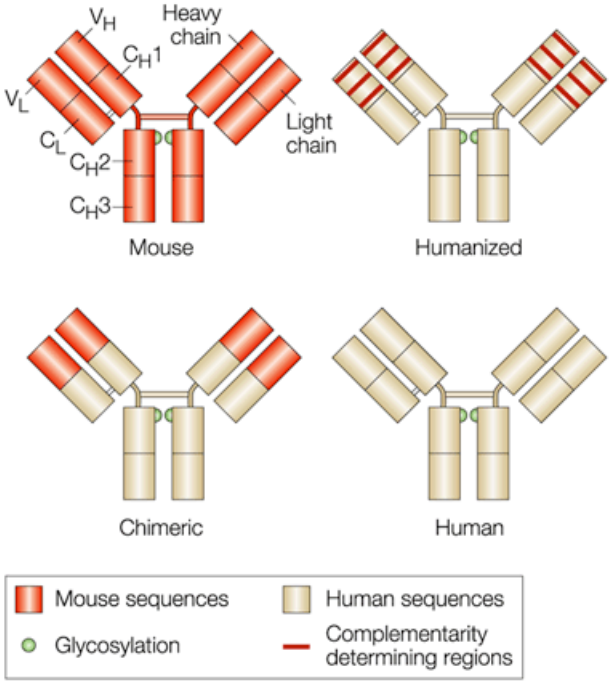

Fig. 2: *Schematic representation of mouse, chimeric, humanized and fully human mAbs Adapted from Carter et al.*[2].

Fig. 2: *Schematic representation of mouse, chimeric, humanized and fully human mAbs Adapted from Carter et al.*[2].

The use of mAbs for cancer therapy has achieved considerable success in the recent years. The best example is *Trastuzumab*, an anti-HER2 humanized IgG_1 mAb. HER2 (*n.b*. described in details later in this thesis) is the second member of the Epidermal Growth *Factor* (EGF) receptor family, and is over-expressed in presenting a breast (30%)[41], ovarian[42] or gastric cancer[43]. Trastuzumab treatment has proven to reduce the risk of relapse in breast cancer patients by 50% when given in an adjuvant setting for one year[44]. More than 30 new mAbs are under study in clinical trials (phase II and phase III), and several mAbs have been approved by the FDA for the treatment of different cancers. As examples, Rituximab (an anti-CD20 mAb) targets CD20 expressed on transformed B cells; Bevacizumab (an anti-VEGF mAb), targets this pro-angiogenic factor produced by tumor cells; Cetuximab/Panitumumab (an anti-EGFR mAb), targets EGFR expressed on tumor cells.[1]

OPEN QUESTIONS IN ANTIBODY-BASED CANCER IMMUNOTHERAPY

Among the different immunotherapies that have been described so far, *antibody-based cancer immunotherapy* gave the best clinical results in cancer treatment. Nevertheless, one of the major challenges since the first approval of these therapeutic mAbs has been to understand by which mechanisms mAbs induce tumor regression. An antibody can, indeed, be considered to be composed of two functionally separated portions: the antigen binding site (*Fab portion*) and the Fragment crystalizable (*Fc portion*) that have very different biological functions and properties (described in insert 6).

6- Fab and Fc portion

The **Fab portion** is composed of one constant and one variable domain of the H-chain and its linked L-chain. The variable domains and more specifically the three variable loops in their V strands (CDRs) are responsible for antigen binding.

The **Fc portion** represents the "tail" of the antibody molecule, composed of the constant domain of the two H-chains. The Fc portion is able to trigger various effector mechanisms via either the complement cascade activation or the interaction with Fc Receptors expressed by immune cells.

Fab- dependent biological effects

Every therapeutic mAb has originally been generated by taking into account only the biological property of its Fab portion. In fact, biological effects can be exerted by the Fab portion itself upon the binding of its target. Depending on the nature of the target molecule different biological effects can arise:

- *mAbs against soluble molecules produced by tumor cells:* mAbs that bind to growth factors and cytokines can block their interaction with their growth factor or cytokine receptor, respectively, and thereby neutralizing their pro-tumoral activity. Importantly, several cytokines and growth factors can be secreted by the tumor cells themselves, including Transforming Growth Factor-β (TGF-β) and Vascular Endothelial Growth Factor-A (VEGF-A). VEGF-A is a growth factor that upon binding on the VEGF Receptor, mostly expressed on endothelial cells, is able to induce vascular permeability and angiogenesis. Tumors that produce VEGF-A can therefore induce their own vascular support. *Bevacizumab* is an anti-VEGF-A humanized IgG_1 mAb that neutralizes the activity of VEGF-A[45]. In a clinical phase III trial, the treatment of lung cancers with a combination of Bevacizumab and chemotherapeutic agents resulted in a significant improvement of progression-free survival. Bevacizumab is since approved for the treatment of lung, colorectal, and renal cancer. [46].

- *mAbs against molecules expressed by tumor cells:* Tumor cells can express surface receptors for growth factors that are involved in tumor growth, such as EGFRs. The activity of these receptors can be blocked by mAbs that prevent either receptor dimerization (*Trastuzumab* humanized IgG_1 anti- HER2)[47], or the binding of their ligand (*Cetuximab:* humanized IgG_1 anti-EGFR mAb)[48]. Other mAbs, via their $F(ab')_2$ portion, can aggregate membrane molecules expressed by tumor cells and trigger biological effects, such as cell death. Thus, anti-CD20 antibodies (*Rituximab:* humanized IgG_1 anti-CD20) and anti-CD52 (*Alemtuzumab:* humanized IgG_1 anti-CD52) deliver pro-apoptotic signals into lymphoma B cells and chronic lymphoid leukemia cells, respectively. [49-51]Likewise, anti-CD40 antibodies can induce apoptosis in CD40-expressing tumor cells.

- *Antibodies against membrane molecules expressed by immune cells*. As mentioned before, mAbs can be used to target immune cells in order to optimize the immune response against tumors. Thus, anti-CTLA-4 antibodies block the binding of CD80 and CD86 to this inhibitory molecule expressed by $CD8^+$ T cells, resulting in an enhanced anti-tumor activity of $CD8^+$ T cells[52]. Likewise, anti-CD40 mAbs are used to up-regulate the expression of co-stimulatory molecules on DCs, thus enhancing the presentation of tumor antigens to T cells, and consequently T cell mediated anti-tumor immune responses[53].

Although the binding of the Fab portion to the antigen can lead to a therapeutic effect by itself, this phenomenon is not sufficient to fully explain the clinical (and experimental) efficacy of anti-tumor mAbs. In fact, with the exception of mAbs directed against soluble molecules, mAbs can mediate many biological activities that are dependent of their Fc portion. These activities can involve soluble plasma molecules such as the complement component C1q, or receptors for the Fc portion of antibodies (Fc receptors, FcRs) that are mostly expressed on immune cells. On the one hand, the activation of the complement cascade on the opsonized tumor cells results in the generation of several effectors molecules that can potentially lead to the target cell death and to the local recruitment of inflammatory cells. On the other hand, the interaction of the mAbs with FcR-expressing cells of the immune system can result in a wide array of responses that range from cell degranulation and phagocytosis, to the delivery of immune-modulatory signals.[3]

The relative contribution of the Fab and of the Fc portion to the anti-tumor effect of therapeutic and endogenous antibodies is still under discussion *(**n.b.** this point is further discussed*

in the review placed in annex, Albanesi M. & Daeron M.). Moreover, it is still unclear which among the several Fc-dependent mechanisms are responsible for target cell killing. In the following chapter, I will describe the Fc-dependent effectors mechanisms that can potentially be involved in the mAb anti- tumor activity.

FC-DEPENDENT MECHANISM OF TUMOR KILLING

The complement system

The complement system is based on proteins synthesized by the liver, which normally circulate as inactive pro-protein precursors. These complement components are identified by the letter C followed by a number ranging from 1 to 9 (C1- C9), and termed the complement cascade. Three different pathways can lead to the activation of the complement cascade triggered either by targets opsonized by antibodies (classical pathway, CP), by microbial repetitive polysaccharide structures (lectin pathway, LP), or by the recognition of other "foreign" surface structures (alternative pathway, AP).

C1, C4, C2 and C3, in this order, represent the activation cascade of the classical pathway. C1 consists of one C1q molecule, formed by six sub-units, bound non-covalently to two C1r and two C1s molecules. The first step of this cascade consists of the binding of C1q to antibodies opsonizing an antigen, cell or surface. This binding induces a conformational change in C1q that triggers a series of protein cleavages, resulting in the activation of C4 and C2 components. Importantly, C1q possesses very different binding abilities towards the different human immunoglobulins classes and isotypes: C1q binds IgM, IgG_1 and IgG_3 very effectively, IgG_2 poorly, and does not bind IgG4, IgA, IgE and IgD. These latter antibodies can therefore non activate the complement cascade. The activation of C4 and C2 leads to the formation of the C4b2a complex, also called C3 convertase. This complex mediates the cleavage of C3 and the subsequent formation of C3a, an inflammatory peptide, and C3b, the main effector molecule of the complement system. C3b can, indeed, induce the destruction of a target cell by phagocytic cells. The association of C3b with the C3 convertase forms the C5 convertase complex (C4b2a3b). The C5 convertase initiates the late events of the complement activation that comprise a sequence of polymerization reaction in which the terminal complement components (C5,C6, C7, C8, C9) interact to form the membrane-attack complex that creates a pore in the target cell and may lead to cell death.

Evidence that the complement cascade participates to the anti-tumor activity of mAbs was obtained from experiments using Rituximab. This humanized anti-CD20 antibody approved for non solid tumor treatment, and in particular B cell malignancies (non-Hodgkin lymphoma/leukemia), can indeed kill the target cells *in vitro* in the presence of the complement components[54]. Moreover, in mouse models, depletion of components of the complement cascade by cobra venom factor

decreased the therapeutic effect of this mAb[55]. Clinical observations showed that patients undergoing Rituximab treatment demonstrated a reduction in complement components in their serum[56]. Finally, an improved therapeutic response in chronic lymphoid leukemia patients could be obtained a modified anti-CD20 mAb that demonstrates an improved affinity for C1q (*Ofantumumab*, a fully human anti-CD20 mAb) [57].

Fc Receptors (FcRs)

Fc receptors (FcRs) are surface molecules that are capable to interact with the Fc portion of immunoglobulin molecules. Studies on IgG, IgM, IgA and IgE demonstrated the existence of distinct receptors for those isotypes on various immune cells. In terms of nomenclature, FcRs are named after their ligands: these are abbreviated by "Fc" followed by the Greek letter corresponding to the heavy chain characteristic of each class of immunoglobulins. Thus, FcαR bind IgA, FcεR bind IgE, FcμR bind IgM and FcγR bind IgG. pIgR, the polymeric Ig receptor that binds IgA and IgM, and FcRn, the neonatal FcR that binds IgG at acidic pH, are exceptions to this nomenclature. [58] Since most of the approved therapeutic anti-tumor mAbs are belonging to the IgG class, in the next paragraphs I will focus on FcγRs, describing their structure, function and expression pattern on immune cells, focusing on those that are potentially involved in tumor killing (***fig.***3-4).

FcγR structure

All the FcγRs are trans-membrane molecules belonging to the immunoglobulin superfamily[59]. FcγRs have two or three extracellular immunoglobulin-like domains that enable the binding of the Fc portion of IgG. If this IgG-binding molecule is associated to other FcR-subunits, the resulting FcγR is termed a *multi-chain receptor*; if not it is termed a *single-chain receptor*[58] ***(fig.****3-4).*

- *Multi-chain receptors* are composed of an IgG-binding α sub-unit (FcRα) associated with the FcRγ sub-unit. The FcRα sub-unit consists of two or three extracellular domains, a transmembrane domain and a short intracytoplasmic domain. The binding site for the IgG Fc portion is situated at the interface between the two extra-cellular domains closest to the membrane. The FcRγ subunit that is associated with the α subunit is a homodimeric protein

in which each chain consists of a short extracellular domain, a transmembrane domain and a 42 aminoacid intracellular domain[60]. This intracellular domain contains an activation motif called *Immunoreceptor Tyrosine-based Activation Motif* (ITAM). The FcRγ subunit is required for association and expression of most FcRα-chains at the cell surface. The FcRγ ITAM motif is responsible for the signal transduction that arises following FcRα-chain aggregation by multimeric ligands[61]. These aggregations may result in cell activation that requires non-FcR-related tyrosine kinase-dependent pathways (*N.B.*: the FcRγ subunit is also shared by other receptors such as some TCRs, OSCAR, Mincle and some integrins) [62,63]

- *Single-chain FcγRs* consist of the FcRα chain. This chain possesses in its intracytoplasmic domain either an activation motif (ITAM) resembling the FcRγ-subunit ITAM or an inhibitory motif capable of inhibiting cell activation induced by FcRs, the BCR, the TCR and other activating receptors. This inhibitory motif is called *Immunoreceptor Tyrosine-based Inhibitory Motif* (ITIM)[64].

Upon aggregation of FcγRs on the cell surface, different and sometimes opposite biological responses may be triggered, ranging from cell activation to cell inhibition or anergy. The nature of the Fc portion and the affinity of the FcγR for this Fc portion does not, *per se*, determine the type of biological response triggered (*described in the following paragraphs*). It depends, however, on the presence of an ITAM or an ITIM in the FcRα-chain of the FcRs engaged by the opsonized antigen, cell or surface. FcγRs possess either an ITAM or an ITIM, distinguishing them as either *activating FcγRs* or *inhibitory FcγRs*, respectively. The human FcγRIIIB is an exception, as it is a GPI-linked molecule that possesses neither an ITAM nor an ITIM.[65]

Activating FcγRs

Human activating FcγRs comprise FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA. Only 20% of the population expresses FcγRIIC due to a polymorphism that aborts transcription (stop codon: *Fcgr2c*-STOP) or not (open reading frame: *Fcgr2c-ORF*). In mice, activating FcγRs comprise FcγRI, FcγRIII and FcγRIV. As described before, most activating FcγRs associate to the ITAM-containing FcRg-subunit, except human FcγRIIA and human FcγRIIC. These latter receptors are single chain activating FcγRs that possess their own ITAM in the intracellular domain of their FcRα-chain. FcγRIIA and FcγRIIC do not associate with the FcRγ-subunit.[66]

The binding of the Fc portion of an IgG to an FcγR is not sufficient to induce cell activation. In fact, in order to deliver signals to the cells, FcγRs require to be aggregated by IgG antibodies bound to multivalent antigens, aggregated antibodies or antibodies bound to cells (opsonized cells). The cross-linking of the FcRα-chain (ligand-binding extracellular domain) results in the phosphorylation of the ITAM it possesses, or the ITAMs it is associated to, by members of the *Src* family kinases (*i.e.* Lyn and Fyn) that are anchored into the cell membrane. These phosphorylation events lead to the subsequent recruitment and binding of SH2-containing signaling molecules to the phosphorylated ITAM, and among them the Syk family kinases. The phosphorylation and activation of Syk family kinases (*i.e.* Syk or ZAP70) leads to the recruitment and activation of a variety of intracellular enzymes, including the Phospho-Inositide-3 Kinase (PI3K) and the PhosphoLipase C-γ (PLCγ). A crucial step in the activation occurs with the activation of the PI3K that phosphorylates membrane inositols, generating $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ can, indeed, propagate throughout the cell, enabling cell activation. Two main pathways are representative of the cell activation induced by activating FcγRs: calcium influx and activation of MAP kinases. The calcium influx leads to the activation of various calcium-dependent processes such as degranulation, phagocytosis and cytokine release; the activation of MAP kinases is mainly responsible for cell proliferation.

Inhibitory FcγRs

In both mice and humans, only one inhibitory receptor exists: FcγRIIB. This receptor is a single chain molecule containing the distinctive inhibitory ITIM sequence in its cytoplasmic domain. FcγRIIB is capable of inhibiting cell activation induced by a variety of activating receptors and, in particular, cell activation induced by activating FcγRs[64]. The ITIM domain is mandatory for the inhibitory activity of FcγRIIB. To exert inhibitory functions, FcγRIIB needs however to be co-aggregated with activating FcγRs by common ligands, such as IgG immune complexes or opsonized cells. This phenomenon enables the Lyn kinase, involved in the activation complex, to phosphorylate the tyrosine of the ITIM of FcγRIIB. The phosphorylation of the ITIM leads to the recruitment and activation of the SH2-containing Inositol Phosphatase SHIP-1. Thus, SHIP-1 can abrogate the ITAM signaling cascade by hydrolyzing $PI(3,4,5)P_3$ into $PI(4,5)P_2$ but also by recruiting the Ras-GAP exchange factor Dok1 that catalyzes Ras-GTP into Ras-GDP[67]. These activities result in inhibition of the Calcium response and inhibition of the MAP kinase pathway, respectively[65]. It has been well established that SHIP-1 is the main effector of the negative

regulation exerted by FcγRIIB, primarily because in SHIP-1$^{-/-}$ mice the inhibitory activity of FcγRIIB is lost.[68]

High Affinity/ low affinity FcγRs

The affinity for the ligand of a given receptor can be quantified using the affinity constant (K_A), defined by the ratio between dissociation (K_{off}) constant and the association constant (K_{on}) of ligand-receptor interactions. Based on the affinity for the Fc portion two different types of FcγRs have been distinguished: *high-affinity* and *low-affinity* FcγRs. [69]

- *High-affinity FcγRs* are defined by their ability to bind monomeric IgG and have an affinity constant for the IgG higher than 10^7 M^{-1}. The high-affinity FcγRs in mice comprise FcγRI and FcγRIV. The only high affinity FcγR in human is FcγRI.

- *Low-affinity FcγRs* are defined by their inability to bind IgG in monomeric form and their ability to bind IgG in a multimeric form (when IgGs are present in an immune complex or when IgG are opsonizing cells). These low-affinity receptors have an affinity constant lower than 10^7 M^{-1}, usually comprised between 10^5 and 10^7. Low-affinity FcγRs in mice comprise FcγRIIB and FcγRIII. Low-affinity FcγRs in humans comprise FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA and FcγRIIIB[66].

In humans, polymorphisms in the ligand-binding domains of FcγRIIA, FcγRIIIA and FcγRIIIB exist that may affect the affinity for their ligands. The main FcγRIIA polymorphism is a point mutation affecting aminoacid position 131, encoding an arginine (R_{131}) to histidine (H_{131}) mutation. These alleles are co-dominantly expressed; an individual might therefore phenotypically be R/R, H/H or H/R. The main FcγRIIIA polymorphism is a point mutation affecting aminoacid position 158, encoding a valine (V_{158}) to phenylalanine (F_{158}) mutation. As for the FcγRIIA alleles, the FcγRIIIA alleles are co-dominantly expressed; an individual may therefore phenotypically be V/V, V/F or F/F. The FcγRIIIB polymorphisms result in the expression of three isoforms of FcγRIIIB with several mutation among which a mutation affecting the glycosylation close to the Fc-binding site. These variants are named FcγRIIIB-*NA1*, FcγRIIIB-*NA2* and FcγRIIIB-*SH*. The polymorphisms at positions 131 in FcγRIIA and at position 158 in FcγRIIIA, but not the FcγRIIIB polymorphisms affect IgG binding when measured in vitro [66]. An example of the biological consequences of these FcγR polymorphisms is that phagocytes obtained from homozygous

FcγRIIA-H/H_{131} individuals ingest IgG_2-coated erythrocytes more efficiently than phagocytes obtained from homozygous FcγRIIA- R/R_{131} individuals[70]. *Moreover, the* FcγRIIIA polymorphisms has been linked to the clinical outcome of patients with breast cancer treated with Trastuzumab [71] *(**n.b**.: this point is further detailed in the chapter describing NK cells, see below).*

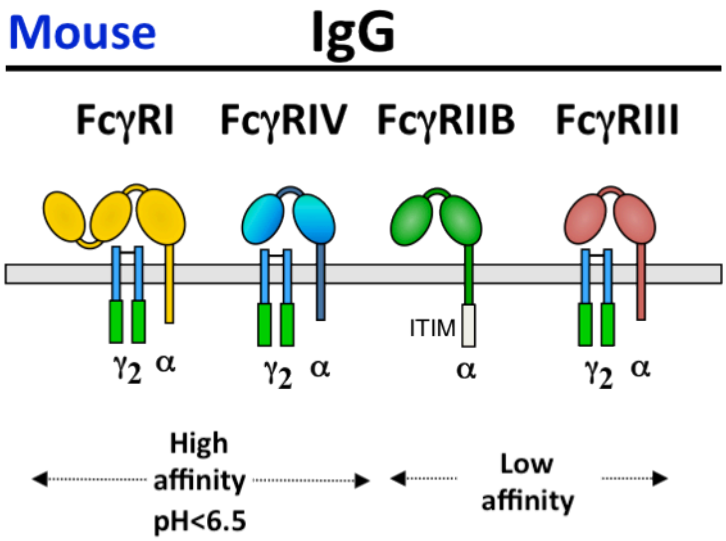

Fig.3: *Schematic representation of mouse IgG receptors at the cell membrane (grey bar) and their association or not to the FcRγ-chain dimmer (black). ITAMs are represented by green boxes, ITIMs by a white box.*

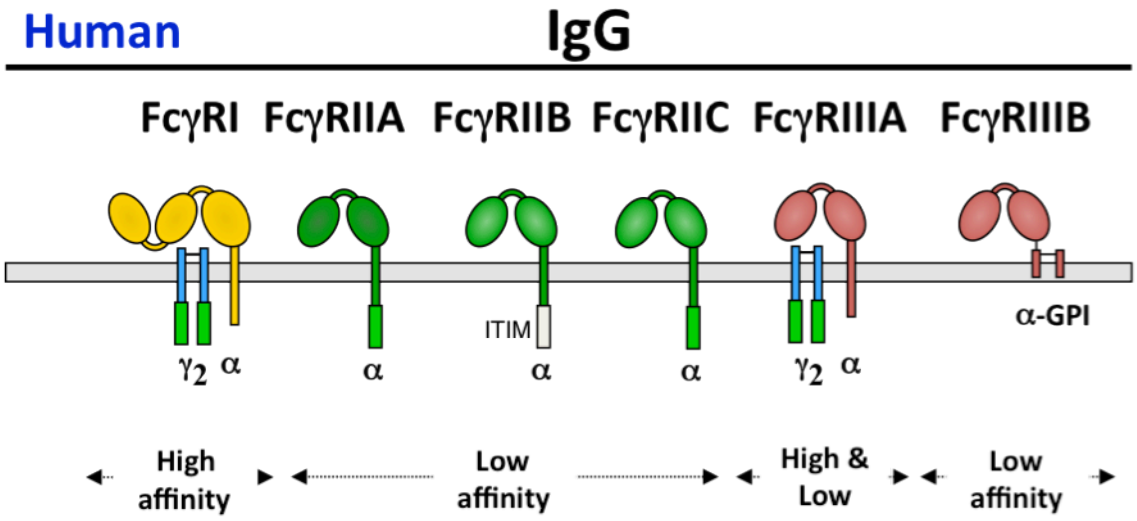

Fig.4: *Schematic representation of human IgG receptors at the cell membrane (grey bar) and their association or not to the FcRγ-chain dimmer (black). ITAMs are represented by green boxes, ITIMs by a white box.*

FcγR-EXPRESSING CELLS

During mAb-based cancer immunotherapy therapeutic mAbs can opsonize cancer cells allowing the aggregation of FcγRs on immune cells in the tumor environment. Different biological responses may be triggered, depending on the cell subtype. FcγRs are, indeed, differentially expressed on hematopoietic cells and are mainly present on innate immune cells. Importantly, FcγRs that exist in humans and in mice do not have the same expression pattern in both species[72] ***(fig.**5)*.

- *In mice:* FcγRI is expressed only on monocyte-derived dendritic cells in tissues [73,74], FcγRIIB on neutrophils, eosinophils, basophils, mast cells, monocyte/macrophages and B cells; FcγRIII on neutrophils, eosinophils, basophils, mast cells, monocyte/macrophages, dendritic cells and NK cells; FcγRIV on neutrophils and monocyte/macrophages.

- *In humans:* FcγRI is expressed on monocyte/macrophages and, inducibly, on neutrophils[75]; FcγRIIA on neutrophils, eosinophils, basophils, platelets, mast cells, monocyte/macrophages and dendritic cells; FcγRIIB on B cells and basophils, and on subpopulations of neutrophils, monocytes and dendritic cells; FcγRIIC on NK cells, neutrophils and monocyte/macrophages when an individual has a *Fcgr2c*-ORF allele; FcγRIIIA on NK cells and monocyte/macrophages; FcγRIIIB on neutrophils and on subpopulations of basophils.

Because *NK cells* and *macrophages*, but also *neutrophils*, have been shown to infiltrate tumors and are thought to participate to the tumor growth and tumor rejection processes, respectively, these cells may potentially contribute to mAb-mediated tumor therapy. In particular, they might be responsible for mAb-mediated tumor killing *in vivo*. The next paragraphs are dedicated to the description of these cell populations in light of their potential involvement in mAb therapy.

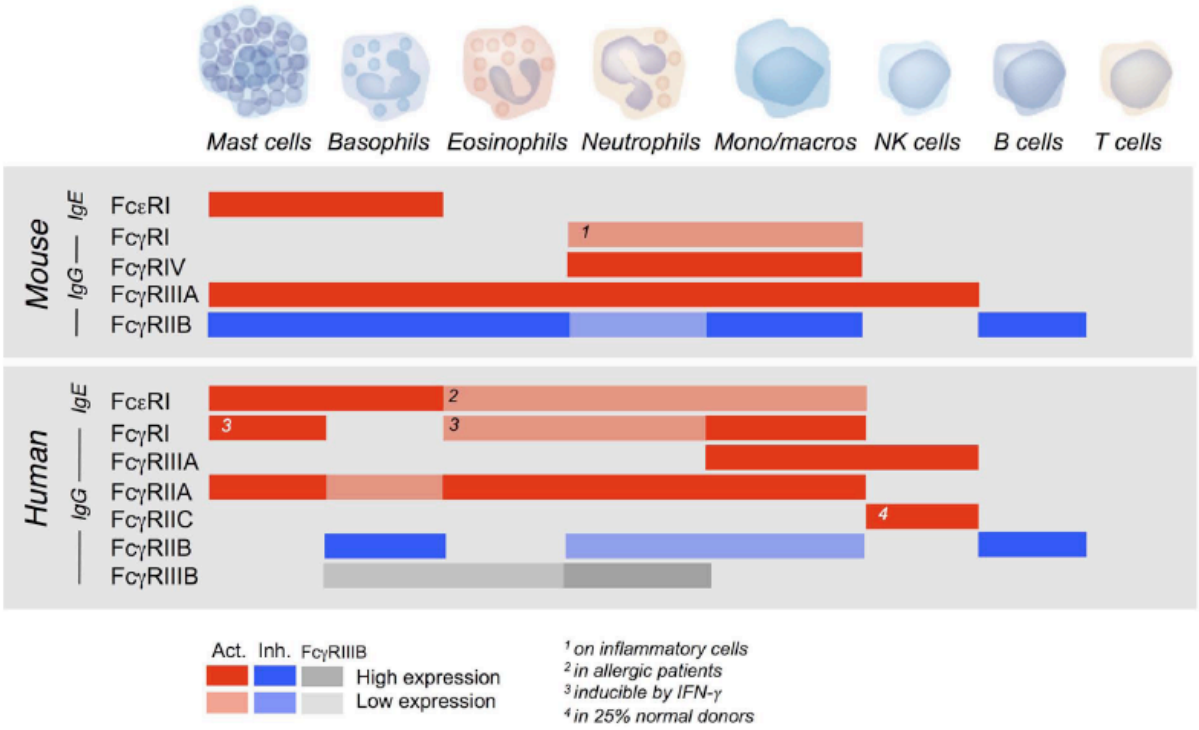

Fig.5: *Tissue distribution of mouse and human FcR for IgE and IgG (From Jonssonn et al.[72])*

NK Cells

Natural Killer cells (NK cells) have been initially identified as a lymphoid population representing 10-20% of the Peripheral Blood Mononuclear Cells (PBMCs). The majority of NK cells is localized in the peripheral blood, lymph nodes, spleen and bone marrow. Morphologically, NK cells are typically large lymphocytes containing granules. However, the large granular lymphocyte morphology is not a hall-mark of NK cells, as it is also associated with other cell subtypes, such as $CD8^+$ T cells. The development of NK cells occurs in the bone marrow from a common lymphoid progenitor that also gives rise to T and B cells, but not to myeloid cells. This process is dependent on transcription factors and cytokines, particularly on IL2, Interleukin-7 (IL7), Interleukin-15 (IL15) and Interleukin-21 (IL21). These cytokines exert their biological effect through the binding to their corresponding interleukin receptor that signals through a common-gamma chain (γ_c) associated with these receptors[76]. Mice deficient for γ_c ($\gamma_c^{-/-}$ mice) therefore lack functional receptors for IL2, IL7, IL15 and IL21. One of the consequences of this absence of cytokine signaling is the lack of NK cells (n.b.: $\gamma_c^{-/-}$ mice also present a reduced production of B and T cells). $\gamma_c^{-/-}$ mice represent therefore a model of NK cell deficiency that has been extensively used to study NK-dependent functions[77].

NK cells express a lytic machinery able to kill target cells. NK cells, in contrast to $CD8^+$ T cells, do not require priming in order to exert their biological function. In fact, NK cells possess activating and inhibitory receptors able to sense the alteration of MHC-I molecules on the target cell surface. The lack of a single MHC-I allele, a frequent event in transformed neoplastic cells, is sufficient to trigger NK cell activation, and eventually to kill that transformed cell[78].

Mature NK cells can be identified in the blood by selective NK cell surface markers. Mouse NK cells, indeed, express CD122 and, in the C57BL/6 mouse strain NK1.1 and NK1.2. Human NK cells can be identified by their CD56 expression.

NK cells express FcγRs: mouse NK cells express FcγRIII, human NK cells express FcγRIIIA and, if that individual has an *FcgR2c-ORF* allele, also FcγRIIC. The aggregation of FcγRs on NK cells can lead to NK cell activation that results in two major biological effects: *release of cytotoxic granules* and *cytokine production.*

- <u>*Release of cytotoxic granules*</u>. NK cell cytotoxic granules contain enzymes like *perforin* and *granzymes*. Perforin, also known as cytolysin, is a 65 kDa protein belonging to the MACPF superfamily (Membrane Attack Complex proteins of Complements and Perforin). This protein is stocked in its inactive form in the granules and, upon exocytosis, released in the

intermembrane space formed between the target cell and the NK cell. In this space, perforin polymerises and forms a pore in the target cell membrane, causing cell damage. The formation of the pore enables the entry of granzymes into the target cell. Granzymes are a family of serine proteases that activate the caspase cascade (particularly through the cleavage of Caspase 3) and DNA fragmentation, which are exocytosed with perforin in the intermembrane space. The release of cytotoxic granules by NK cells may therefore result in target cell death.

- *Cytokine production*. Upon activation NK cells produce several cytokines such as IFNγ, TNFα and GM-CSF, which participate in leucocytes recruitment and in the amplification of the inflammatory process.

When NK cells and antibody-opsonized cancer cells are in close contact thanks to the binding of antibodies to FcγRs on the surface of NK cells, these cells may also kill the target cell by a third mechanism: the *death receptor system*. Death receptors are receptors expressed on the cell surface that upon ligand binding transmit apoptotic signals into the cell via the caspase cascade, which may ultimately lead to cell death. Normal cells and most tumor cells express death receptors (*e.g.* mainly *Fas* and *Death Receptor 4)*. NK cells can induce the death of an opsonized target through the expression of death receptors ligands such as Fas-ligand.

Numerous reports have shown that NK cells can infiltrate tumors of different origins, such as melanoma, non-small cell lung cancer (NSCLC), gastro intestinal stromal tumors (GIST), renal cell carcinoma (RCC), prostate cancer and colorectal carcinoma (CRC)[79-82]. For several tumors, the infiltration of NK cells has been associated with the clinical outcome: for prostate cancer an elevated count of infiltrating NK cells is associated with a lower risk of progression[83]. Correlations between the number of intra-tumoral NK cells and the control of tumor growth by the patient were also reported for squamous cell lung cancer[84]. Moreover, a recent study showed a positive association between the number of tumor-infiltrating NK cells and regression of melanocytic lesions[85].

Clinical results suggest a potential involvement of NK cells in mAb-based cancer immunotherapy, *e.g.* Trastuzumab treatment of patients carrying metastatic breast cancer: 1) Responder patients have indeed a higher number of circulating NK cells, and increased NK cell activity compared to non-responder patients[86]*)*; 2) Polymorphisms affecting FcγRIIIA (expressed by human NK cells) correlated with the clinical efficacy of Trastuzumab. In fact, patients having a FcγRIIIA 158V/V polymorphism have a higher response rate to Trastuzumab compared to the

patients having a FcγRIIIA 158V/F or a 158F/F polymorphism[71]; 3) *In vitro* experiments demonstrated that human NK cells carrying a FcγRIIIA158V/V polymorphism have a higher cytotoxic potential against Trastuzumab-opsonized tumor cells compared to FcγRIIIA 158V/F or 158F/F NK cells [70]. If NK cells are, indeed, the effector cell population during anti-tumor mAb therapy has not been demonstrated formally. Other cells, like monocytes/macrophages that also express human FcγRIIIA may be contributing or even responsible.

Monocytes/Macrophages

Monocytes represent approximately 8% of the cells circulating in the blood, bone marrow and spleen. They originate in the BM from a specific progenitor called myeloblast. Monocytes do not proliferate in a steady state and migrate into the tissues where they differentiate into macrophages. Depending on the tissue, we can identify different types of tissue macrophages: red/white pulp and marginal macrophages in the spleen, lamina propria macrophages in the epithelia, Kupfer cells in the liver, osteoclast in the bones, microglia cells in the central nervous system, and alveolar macrophages in the bronchi. The Macrophage-Colony Stimulating Factor (M-CSF) is the main cytokine regulating macrophage development, proliferation and differentiation. Compared to wt mice, mice carrying a mutation in the M-CSF gene (*op/op* mice) have indeed about 60% fewer macrophages in different compartments[87,88]. In a steady state, macrophages are quiescent cells and their life span can be of several months. They are involved in tissue homeostasis via the clearance of apoptotic cells and the production of growth factors, as well as in multiple inflammatory processes.

Mouse macrophages express CD11b, CD14, CD68 and F4/80 markers ***(n.b.** the expression of F4/80 varies between the different tissue macrophages subpopulation)*. Human macrophages express CD68, CD11b, CD14 and EMR1.

Macrophages express several FcγRs. Mouse macrophages express FcγRI, FcγRIIB, FcγRIII, and FcγRIV. Human macrophages express FcγRI, FcγRIIA, FcγRIIIA and low levels of FcγRIIB. The aggregation of FcγRs on macrophages may lead to their activation that results in various biological effects: *Fc-dependent phagocytosis*, *respiratory oxidative burst*, *reactive nitrogen intermediates production* and *cytokine production:*

. <u>*Fc-dependent phagocytosis*</u> is a process by which macrophages engulf and destroy immune complexes, or antibody-opsonized cells. This process includes different steps: Fc receptor

ligation initiates the ITAM-dependent signaling cascade that results in actin polymerization and extension of the plasma membrane, called pseudopodia, that surround the target cell. Once surrounded, the fusion of the pseudopodia leads to the engulfment of the target and the formation of a phagosome. The mechanism by which FcγRs stimulate the polymerization of actin and induce the formation of phagosomes is not known. Whereas macrophages from *Syk*$^{-/-}$ mice are still capable of polymerizing actin, they are unable of internalization [89], suggesting a role for Syk and Syk-dependent events in this process. Once a phagosome is formed, it is fused with an early endosome, resulting in a slight reduction of the pH. This phenomenon enables the uncoupling of the interaction between FcγRs and IgG. It allows also the recycling of FcγRs to the cell membrane. The subsequent fusion of this phagosome with a late endosome and a lysosome results in the formation of a phagolysosome, resulting in a further reduction of the pH. The lysosome is an highly oxidative compartment that contains several enzymes, proteins and peptides such as *lysozyme*, *defensins*, *chitinases* and *lactoferrin*. The release of the lysosome content into the phagosome results in the digestion and elimination of the engulfed target[90]. *(**n.b**. also neutrophils are capable of phagocytosis, see "Neutrophil" section)*

- *Respiratory oxidative burst.* Macrophages are also equipped with enzymatic machineries that generate highly toxic reactive oxygen compounds. The induction of *Reactive Oxygen Species* (ROS) is dependent on the phagocyte NADPH oxydase system. This system is a multi-component enzymatic complex that consist of three cytosolic subunits (p40phox, p47phox and p67phox) and a membrane-associated flavocytochrome complex (p22phox and p91phox). The activation of macrophage induces the assembly of the different subunits into a functional NADPH complex. Once the complex is assembled, it produces superoxide anions and hydrogen peroxide. These molecules can either be released into phagocytic vacuoles or secreted outside the cell where they exert cytotoxic and anti-microbial effects. *(**n.b.** also neutrophils produce ROS see "Neutrophil" section)*

- *Reactive nitrogen intermediates production.* Upon activation, macrophages can produce a large amount of Nitric Oxyde (NO). The enzyme in macrophages that leads to the production of NO is called inducible Nitric Oxyde synthase (iNOs). Whereas NO has been shown to be toxic for cells and bacteria, the mechanism by which it does is not known. *(**n.b.** also neutrophils produce NO, see "Neutrophil" section)*

- *Cytokine production*. Upon activation, macrophages can produce several cytokines such as Interferon α/β (IFN−α/β), Tumor Necrosis Factor α (TNF−α), IL10, IL12, IL18, IL10 and IL16 that participate to the inflammatory reaction.

It is now appreciated that most solid tumors are abundantly populated with *tumor-associated macrophages (TAMs)* and that these cells can alter the clinical outcome [91] . During the tumor development, monocytes are recruited to the tumor site by growth factors and cytokines produced by the tumor, and in particular M-CSF. M-CSF is widely produced by tumors of the reproductive system including ovarian, uterine, breast and prostate tumors [92].

Once in the tumor, monocytes differentiate into TAMs. It is believed that TAMs are initially recruited to the tumor site in order to reject the tumor. However, the tumor micro-environment is often immunosuppressive and the TAMs, instead of rejecting the tumor, may help tumor growth, invasion and metastases development (*tumorigenic role*). TAMs can indeed produce: Fibroblast-Growth Factor (FGF) Epidermal-Growth Factor (EGF) that may stimulate the tumor growth; Vascular Endothelial-Growth Factor (VEGF) and angiopoietins that promote tumor angiogenesis; metalloproteinases (MMP), and in particular MMP-9 and MMP7, that through the digestion of collagen structures of the surrounding tissue, promote tumor infiltration[91]. Taking multiple reports into account, more than 80% show a significant correlation between high TAM density and poor prognosis, whereas less than 10% associate the presence of TAMs with good prognosis [93].

Some experimental studies suggested a role for macrophages in mAb-based cancer immunotherapy. In the B16 melanoma model of mAb-based anti-tumor therapy (described in details in the following chapters), Mac-1 positive cells have been demonstrated to be involved in mAb anti-tumor activity. Mac-1 is a receptor for complement component 3 that is expressed on macrophages, neutrophils and NK cells[94]. Furthermore, the depletion of circulating monocytes partially inhibited the protective effect of mAb anti-tumor on B16 melanoma metastasis in the liver[95]. These reports suggest a role for monocytes/macrophages in mAb-anti tumor activity that has, however, not been demonstrated formally.

Neutrophils

Neutrophils (PMNs) represent the predominant circulating leukocyte population in humans, and account for 50-70% of circulating leukocytes. They are characterized by their large bi-lobate nucleus and the presence in the cytoplasm of numerous granules that contain toxic mediators[96]. Neutrophils develop in the bone marrow from a common progenitor cell, shared by neutrophils and monocytes/macrophages, called Granulocyte-Monocyte-Progenitor (GMP). The morphologic maturation stages from the GMP to mature neutrophils include: *myeloblast, promyelocyte, myelocyte* and *metamyelocyte*. ***(fig.** 6)*

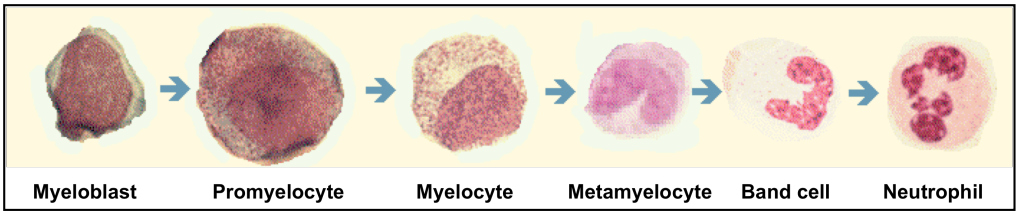

Fig.6: *Schematic representation of neutrophil development.*

The maturation process of PMNs is regulated by a number of gene transcription factors and repressors. In particular, the gene repressor *Growth factor independence-1* (*Gfi-1*) has a pivotal role in the development of PMNs and monocytes/macrophages. *Gfi-1* is indeed a zinc-finger transcription repressor that represses the differentiation axis towards monocytes/macrophages and therefore favors the transition from promyelocytes to myelocytes[97,98]. Its absence in Gfi-1$^{-/-}$ mice therefore leads to a lack of mature neutrophils. In these mice, immature neutrophils accumulate and form an "atypical" myeloid cell population that shares characteristics of neutrophils and macrophages. These atypical myeloid cells can mature only into macrophages, but not into mature neutrophils even in the presence of Granulocyte-Stimulating Factor (G-CSF)[99]. Gfi-1$^{-/-}$ mice therefore constitutes a model of neutropenia.

Once matured, neutrophils are exported from the bone marrow to the blood stream where they circulate approximately six hours before dying. In healthy individuals, circulating neutrophils are cleared from the blood without participating in any inflammatory response. Upon inflammation however, neutrophils are recruited to the inflammatory site by the local production of chemokine IL8 binding to its receptor CXCR2 on neutrophils. (n.b. CXCL-1/KC is the mouse homologue of human IL8).

Mouse neutrophils express high levels of Granulocyte-1 (GR1) and Mac-1 antigens. Human neutrophils express CD66.

Neutrophils express several FcγRs. Mouse neutrophils express FcγRIIB, FcγRIII and FcγRIV. Human neutrophils express FcγRIIA and FcγRIIIB, inducibly FcγRI, and low levels of FcγRIIB. The aggregation of FcγRs on neutrophils may lead to cell activation that results into different biological effects: *Fc-dependent phagocytosis, release of the neutrophilic granules*, *Respiratory Oxidative Burst*, *NET formation* and *cytokine production*

- *Fc dependent phagocytosis.* PMNs, as described for the macrophages, can phagocyte antibody-opsonized targets upon engagement of FcγRs. Although the first phases of phagocytosis (Pseudopodia formation / engulfment/ phagosome formation) are shared by both macrophages and PMNs, there are essential differences between macrophage- and PMN phagocytosis. In PMNs, phagosome maturation results from the fusion of a phagosome with neutrophilic granules, whereas in macrophages it results from the fusion of a phagosome with lysosomes. The phagosomal pH is differently regulated in PMNs compared to macrophages. In fact, while in macrophages the phagosome gradually acidifies; in PMNs phagosomal pH is initially alkaline and remains neutral for prolonged periods. The maintenance of this alkaline pH is indeed absolutely required for the activation of the major neutrophil serine proteases *Neutrophil Elastase (NE)* and *Cathepsin G* (described below).

- *Release of the neutrophils granules.* Neutrophils possess three fundamental types of granules. Primary granules contain *Myeloperoxidase (MPO)*, an enzyme critical for the oxidative burst (see paragraph below), *defensins*, *BPI (Bactericidal/Permeability-Increasing protein*) and a number of serine proteases: NE, *proteinase 3* (PR3) and CG. Secondary granules contain a wide range of antimicrobial compounds including *NGAL*, *hCAP-18* and *lysozyme*. Tertiary granules serve as storage location for a number of metalloproteases (MMP) such as *gelatinase* and *leukolysin*. Upon cell activation, the granules can be released into the phagosome or secreted outside the cell. The content of the granules have mainly anti-microbial and cytotoxic activities. Additional functions can, however, be exerted such as chemotaxis. PR3 can indeed induce monocyte recruitment, thereby amplifying inflammation processes. Moreover, granular proteins in PMNs can also increase the activity of macrophages by enhancing their phagocytic activity[100].

- *Respiratory Oxidative Burst*. Upon activation in PMNs, as described for macrophages, o the NADPH oxydase complex is formed and generates ROS. Neutrophil MPO mediates a further step of ROS production: using the products of the NADPH oxydase pathway

(hydrogen peroxide) as a substrate, MPO generates hypoclorous acid and chloramines that have a potent antimicrobial and cytotoxic activity.

- *Reactive Nitrogen intermediates production.* Neutrophils are capable upon activation of generating Reactive Nitrogen production with the same mechanism described for macrophages. As mentioned before NO has been shown to be toxic for bacteria and cells.

- *NET formation.* Upon stimulation, PMNs can undergo an active form of cell death called NETosis[101]. During this process, PMNs release fibrous structures containing histones, cytoplasmic proteins and granular proteins. These structures are called *Neutrophil Extracellular Traps* (NETs). The mechanism of NET formation is not completely understood, but different neutrophilic enzymes have been shown to be necessary for NET formation, such as MPO and NE. In $MPO^{-/-}$ mice or $NE^{-/-}$ mice, NET formation is, indeed, abolished[102,103]. NETs are thought to kill microbes by exposing them to an high local concentration of antimicrobial neutrophilic enzymes. Several evidences unraveled a role for NET also in clog formation and in propagation of an inflammatory response[104].

- *Cytokine production*. Upon activation neutrophils produce several pro-inflammatory cytokines such as IL-1β, IL6, Tumor Necrosis Factor-α (TNF–α) and IL8 that help recruiting other neutrophils and leucocytes.

Neutrophils make up for a significant proportion of the inflammatory cell infiltrate. It has been shown that tumor cells themselves mediate neutrophil recruitment to the tumor via the secretion of cytokines, and in particular IL8. When neutrophils traffic inside tumors, they are referred as *Tumor Associated Neutrophils (TANs)*[105]. Interestingly, using a mouse fibrosarcoma model, it has been shown that the depletion of neutrophils inhibits tumor growth[106]. In a mouse melanoma model, it has been shown that neutrophils facilitate the seeding of metastases[107]. Moreover, the depletion of neutrophils in a mouse model of pancreatic cancer reduced the amount of tumor angiogenesis [108]. These observations suggest that, as described for TAMs, neutrophils - and in particular TANs - may have tumorigenic potential. Different proteins and enzymes produced by neutrophils may account for this phenomenon. Neutrophil metallo-proteinase-9 (MMP-9) has been shown to inactivate by cleavage anti-angiogenic molecules and to promote the release of VEGF that results in increased tumor angiogenesis[109]. The release of enzymes such as heparanase, collagenase IV and NE mediate the degradation of the extracellular matrix thus favoring tumor invasion[110]. Moreover, the secretion

of arginase-1 by TANs has been reported to suppress T cell proliferation through the cleavage of arginin, a factor required for efficient T cell activation[111]. These experimental studies have also been confirmed by clinical observations: neutrophil infiltration is associated with a worse prognosis in bronchoalveolar and renal carcinoma[112,113].

Despite this tumorigenic effect, some recent findings unraveled also a protective role of neutrophils in tumor development and particularly in the seeding of metastases. After their first contact with the tumor, TANs can indeed migrate out of the tumor into secondary organs, like the lungs. These emigrating neutrophils are called *Tumor Entrained Neutrophils* (TENs). Once in the secondary organs, TENs have been described to exert cytotoxic functions able to eliminate disseminated cancer cells, thus preventing the seeding of metastases[114]. Even though neutrophils have been shown to be able to kill *in vitro* antibody-opsonized cells[115], no evidence of a role for neutrophils in mAb-based cancer immunotherapy has been reported so far.

THESIS OBJECTIVES

mAbs are currently used in clinics for the treatment of solid tumor with impressive results in terms of tumor reduction. However, the relative contribution of the Fab and of the Fc portion to the anti-tumor effect of mAbs is still under discussion. In particular, it remains still elusive which one of the Fc-dependent mechanisms is responsible for tumor killing. During the last decade, however, some preliminary evidences came from both *experimental studies* and *clinical studies*.

- *Experimental studies.* Mouse models of mAb-based cancer immunotherapy showed that that the anti-tumor activity of mAbs depends partially, and in some cases totally, on the presence of activating FcγRs. Nevertheless, which receptors among the three mouse activating FcγRs is/are responsible for tumor killing remains unknown. On this issue, contradictory if not opposite data have been reported in the literature[116,117] ***(n.b.** this issue is further discussed in the article 1).* Because activating FcγRs have different expression patterns among immune cells, mAbs bound to cancer cells may trigger the activation of various cell subtypes. This implies that different effector mechanisms, such as phagocytosis or release of cytotoxic mediators, may lead to tumor killing. Nevertheless, the cell population mandatory for mAbs-induced tumor killing *in vivo* remains unknown.

- *Clinical studies.* Clinical reports have pointed out correlations between human FcγR polymorphisms and the anti-tumor activity of therapeutic mAbs. The majority of these studies focused on hFcγRIIIA (CD16A) those polymorphisms, indeed, correlated with the efficacy of some therapeutic mAbs[71]. Since NK cells have been reported to infiltrate tumors, express hFcγRIIIA and possess cytotoxic potential, they have been considered the main cell population that accounts for mAb-anti tumor activity. However, these observations remain correlative and have never been confirmed in an *in vivo* experimental setting. Furthermore, the possible involvement of the other three human activating FcγRs to mAb-therapy has not been analyzed so far.

During my PhD I have addressed some of these unsolved issues on mAb anti-tumor activity. The main questions of my project have been:

1. Which is/are the mouse and human FcγRs involved in mAb-based cancer immunotherapy?

2. Which is the cell population responsible for mAb-mediated tumor killing?

3. What is the effector mechanism involved during mAb-mediated tumor killing?

To tackle these questions, I have used two different models of mAb-based cancer immunotherapy.

- First, I used the B16F10 mouse melanoma model. B16F10 cells express the glycoprotein gp75, the target of a mAb called *TA99* (mouse IgG2a). In a syngeneic mouse background ***(e.g.*** *C57BL/6*), an intravenous injection of B16F10 cells leads to lung metastases, whereas a subcutaneous injection leads to a solid tumor. A treatment with TA99 mAb results in a reduction of the tumor load.
- Second, I used the BT474 human breast cancer model. BT474 cells over-express HER-2, the target of the monoclonal antibody *Trastuzumab* (a humanized IgG_1). In immunodeficient mice, ***(e.g.*** *Nude foxp1$^{nu/nu}$)* the subcutaneous engraftment of BT474 cells leads to the development of a solid tumor. Recurrent injections of Trasuzumab results in a reduction of the tumor load.
- For both tumor models, I have established luciferase-expressing variants, termed B16Luc2+ and BT474Luc2+, in order to quantify the tumor growth non-invasively using bioluminescence.

In my *fist work (article 1)* I have studied the involvement of the different mouse activating FcγRs during mAb-therapy. I have addressed this question using the B16Luc2+ melanoma model of lung metastases. To study the role of a given FcγR it is possible to use different strategies: **1)** mouse lacking a particular FcγR, **2)** mice lacking all FcγRs except the one of interest, **3)** specific antibodies that block *in vivo* the activity of a particular FcγR. Using these approaches it was possible to show that, among the different activating FcγRs, FcγRI and FcγRIII are both responsible for mAb antitumor activity in the B16 model, whereas FcγRIV is not.

In my *second work (article 2)*, part of a collaborative project with a post-doctoral fellow in the laboratory, I have been in charge of studying the involvement of the human receptor FcγRI (hFcγRI) in mAb-anti tumor therapy. Like in the work performed for *Article 1* I have chosen the B16Luc2+ melanoma model of lung metastases. To study the role of hFcγRI mice that lack multiple FcγRs and express hFcγRI have been generated. My work showed that hFcγRI can mediate mAb TA99 anti-tumor activity *in vivo* in transgenic mice. Importantly, hFcγRI can mediate also the activity of a humanized (CTA99 - hIgG1) or of a fully human (20D7S) anti-gp75 mAb in the same model.

My *third work (article 3)* has been my major project and is dedicated to identifying the cell population and the mechanism responsible for *in vivo* tumor killing following mAb therapy. I have addressed these issues using both the models of B16Luc2+ melanoma and BT474Luc2+ breast cancer. To study the involvement of the different immune cells, it is possible to use either depletion of particular cell population *in vivo*, mice deficient for specific cell populations or *in vivo* reconstitution of a particular cell population by cell transferring. Once the cell population identified, the mechanism by which it kills tumors can be identified using mice rendered unable to mediate particular cytotoxic or phagocytic processes. This can be achieved either by using mice deficient for mediators expected to be involved in these processes, or by using wt mice treated with interfering compounds or inhibitors of biological pathways. Using these different approaches, it was possible to show that the anti-tumor activity of TA99 and Trastuzumab depends on hematopoietic cells. Among these, I unexpectedly identified neutrophils as necessary and sufficient to mediate mAb-induced tumor killing, most probably *via* FcγR–dependent phagocytosis.

ARTICLE 1

ARTICLE 1

"Antibody therapy to metastases requires FcγRIII and FcγRI"

INTRODUCTION

The B16F10 melanoma is a syngeneic mouse tumor that can be used to model a mAb-based cancer immunotherapy. B16F10 cells, indeed, express the glycoprotein gp75, a 75 kDa glycoprotein present on normal and over-expressed in transformed melanocytes (melanomas)[118]. This protein is the target of a mouse IgG2a mAb called TA99[119]. An intravenous injection of B16F10 melanoma cells in a syngeneic mouse background ***(e.g.*** *C57BL/6*) leads to the development of lung metastases. In this compartment, the number and/or the size of metastases present on the lung surface can quantify the tumor load. Recurrent injections of mAb anti-gp75 TA99 lead to a drastic reduction in the tumor load. Interestingly, the anti-tumor activity of TA99 is completely lost in mice lacking the FcRγ subunit, required for the expression of all mouse activating FcγRs (FcRγ$^{-/-}$ mice)[120]. This result has been further confirmed in mice bearing a mutation in the ITAM motif of the FcγRs subunit that abolished the signaling by FcRs. Indeed, the protective effect of mAb TA99 effect is lost in these mice[121]. Thus the therapeutic effect of the mAb depends on the activating FcγRs. However, which one(s) of the mouse FcγRs is/are responsible for TA99-anti tumor activity remains unclear. In the literature, indeed, contradictory results have been reported. Whereas *Nimmerijahn et al.* reported a contribution of FcγRIV, but neither of FcγRI nor of FcγRIII, *Bevaart et al*. reported a mandatory role for FcγRI but not detectable contribution of FcγRIII or FcγRIV [116,117].

The first aim of my PhD has been to solve this contradiction and identify the FcγR(s) involved in TA99 anti-tumor activity. To this aim strategies needed to be chose to assess the *tumor quantification* and to *identify the responsible FcγR(s)* by more than one method.

- <u>*Tumor quantification.*</u> The lung tumor load in B16 metastases model is usually assessed by counting the number of metastases (**n.b.** regardless their size) and, eventually, scoring those by groups of size on the lung surface. These quantifications are rather approximative and neglect not-eye visible metastases and those present inside the lung parenchyma. I chose to use a luciferase-expressing variant of B16F10 cells (B16Luc2+) that may enable an accurate quantification of the tumor load both *in vivo* and *ex-vivo*.

- *Strategies used to identify the responsible FcγR(s)*: previous reports on the involvement of FcγRs to mAb TA99 anti-tumor activity have used either mice lacking one or two FcγRs or a specific anti FcγRIV mAb that blocks *in vivo* the activity of mouse FcγRIV. Neither of these studies, however, confirmed a result obtained with a knock out mouse by using blocking mAb in wt mice, or the reverse. Because these strategies have inherent limitations (see discussion of the article), we chose to combine these strategies and coupled two or more approaches to address the role of a particular FcγR.

Using the above-mentioned tools, I have re-analyzed the contribution of the three mouse activating FcγRs to the protective effect of anti-gp75 mAb TA99. I have confirmed the contribution of FcγRI, identified an unexpected contribution of FcγRIII and excluded any contribution of FcγRIV. FcγRI and FcγRIII are, therefore, responsible together for mAb TA99 anti-tumor activity.

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: FcγRIII (CD16) and FcγRI (CD64) Are Responsible for Anti-Glycoprotein 75 Monoclonal Antibody TA99 Therapy to Experimental Metastatic B16 Melanoma

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mAb therapy for experimental metastatic melanoma relies on activating receptors for the Fc portion of IgG (FcγR). Opposing results on the respective contribution of mouse FcγRI, FcγRIII, and FcγRIV have been reported using the gp75-expressing B16 melanoma and the protective anti-gp75 mAb TA99. We analyzed the contribution of FcγRs to this therapy model using bioluminescent measurement of lung metastases loads, novel mouse strains, and anti-FcγR blocking mAbs. We found that the TA99 mAb-mediated effects in a combination therapy using cyclophosphamide relied on activating FcγRs. The combination therapy, however, was not more efficient than mAb therapy alone. We demonstrate that FcγRI and, unexpectedly, FcγRIII contributed to TA99 mAb therapeutic effects, whereas FcγRIV did not. Therefore, FcγRIII and FcγRI are, together, responsible for anti-gp75 mAb therapy of B16 lung metastases. Our finding that mouse FcγRIII contributes to Ab-induced tumor reduction correlates with clinical data on its human functional equivalent human FcγRIIIA (CD16A). *The Journal of Immunology*, **2012, 189: 000–000.**

Tumor cells can be specifically targeted using mAbs that bind to specific or overexpressed Ags on the tumor cell surface. Some of these mAbs can directly affect tumor growth or survival when their Fab portions are bound to their target, for example, trastuzumab targeting HER2/Neu. Some mAbs, however, target molecules that are not involved in tumor growth or survival. In most situations, however, mAbs bound to tumor cells enable the recruitment of phagocytic and cytotoxic immune cells bearing receptors for the Fc portion of IgG (FcγR). Activating FcγRs are indeed necessary for the protective effect of the mouse anti-gp75 mAb TA99 on the development of lung metastases of gp75-expressing B16F10 mouse melanoma cells (1). The contribution of FcγRs in the context of a therapy using a combination of chemotherapy and anti-gp75 mAb treatment has, however, not been investigated.

Activating FcγRs in humans comprise FcγRI (CD64), FcγRIIA (CD32A), FcγRIIC (CD32C), and FcγRIIIA (CD16A), whereas in mice, FcγRI, FcγRIII, and FcγRIV exist. All mouse activating FcγRs require the association of the FcRγ subunit to be expressed and functional at the cell surface. The generation of FcRγ$^{-/-}$ mice allowed for the discovery that activating FcγRs contribute primarily to the protective effect of mAb TA99 on B16 lung metastases (1). Since then, FcγRI$^{-/-}$ mice (2, 3), FcγRIII$^{-/-}$ mice (4), and FcγRIV$^{-/-}$ mice (5) have been reported; however, studies that have used these mice to identify the activating FcγR(s) responsible for the protective effect of mAb TA99 on B16 lung metastases report contradictory results. Whereas Nimmerjahn et al. (6) described a contribution of FcγRIV, but neither FcγRI nor FcγRIII, Bevaart et al. (7) described a mandatory role for FcγRI, but no detectable contribution of FcγRIII or, by deduction, of FcγRIV. These opposing results may be attributable to differences in techniques used to measure tumor load: Nimmerjahn et al. (6) counted the total number of lung surface metastases, whereas Bevaart et al. (7) scored lung surface metastases based on their number and size.

Using a luciferase-expressing variant of B16F10, allowing bioluminescent quantification of the metastases load, combined with single or multiple FcγR-deficient mice and FcγR-blocking mAbs, we reassessed the contribution of FcγRs to a therapy model of B16 lung metastases. Activating IgG receptors were necessary for the protective effect of mAb TA99 not only in single mAb therapy, but also in combina-

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Abbreviations used in this article: CTX, cyclophosphamide; wt, wild-type.

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tion therapy with cyclophosphamide (CTX). Moreover, we identified a significant and unexpected contribution of FcγRIII, confirmed the contribution of FcγRI, and excluded any contribution of FcγRIV to TA99 mAb therapy.

Materials and Methods

Reagents and flow cytometry

B16F10 cells and hybridoma TA99 (anti-gp75) were from American Type Culture Collection, B16F10-Luc2$^+$ from Caliper-Life Sciences, hybridoma 9E9 (anti-FcγRIV) from J.V. Ravetch, anti-FcγRIII mAb 275003 (8) from R&D Systems, and CTX (Endoxan) from Baxter SAS. Staining by mAb TA99 (30 μg/ml) for 30 min at 0°C was revealed using FITC-labeled anti-mouse IgG.

Mice

C57BL/6J FcγRI$^{-/-}$ mice (3), FcγRIII$^{-/-}$ mice, and FcRγ$^{-/-}$ mice (Jackson Laboratories), FcγRIV$^{-/-}$ mice (5), and FcγRI/IIB/III FcεRI/II (5KO, also known as "FcγRIV-only") mice (9) have been reported previously. Mice deficient in FcγRIIB, FcγRIII, and FcγRIV ("FcγRI-only") were generated at Regeneron Pharmaceuticals by deletion of the entire mouse low-affinity FcγR locus (manuscript in preparation) using Velocigene technology (10). All mice were used at 6–10 wk of age. A total of 1×10^6 B16 cells were injected i.v. on day 0. Where indicated, mice were injected with mAb TA99 (200 μg) or isotype control i.p. on days 0, 1, 2, 4, 7, and 9; CTX (100 mg/kg) i.p. on day 0; anti-FcγRIV (mAb 9E9, hamster IgG, 200 μg), anti-FcγRIII (mAb 275003, rat IgG2a, 100 μg), or respective isotype controls i.v. on days 0, 2, 4, and 6. Mice were shaved, anesthetized, and injected i.p. with 3 mg luciferin to acquire bioluminescence on the whole mouse. Alternatively, explanted lungs were exposed to luciferin (50 μl at 15 mg/ml) 2 min before bioluminescence acquisition (IVIS 100; Caliper Life Sciences), with settings of 2 min exposure time and medium binning. Total photon flux (photons/seconds) was calculated using Living Image software. Mouse protocols were approved by the Animal Care and Use Committees of Paris, France.

In vitro assays

For bioluminescence detection, B16-Luc2$^+$ cells (5000/well) were added to a black 96-well plate, incubated with D-luciferin (150 μg/ml), and bioluminescence acquired.

Statistical analyses

Data were analyzed using Student *t* test (NS: $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars correspond to SEM. In vivo data were analyzed using two-way ANOVA with Bonferroni posttest.

Results and Discussion

An i.v. injection of B16-F10 cells leads to lung metastases of very different sizes (Fig. 1A, *left panel*). Notably, metastases developed also inside the lung parenchyma (Fig. 1A, *right panel*), a parameter that was not considered in previous studies (6, 7). To analyze the contribution of FcγR to the B16-TA99 metastasis immunotherapy model with an accurate measure of tumor load, we took advantage of a B16-F10 variant expressing the enhanced firefly luciferase (luc2) that allows

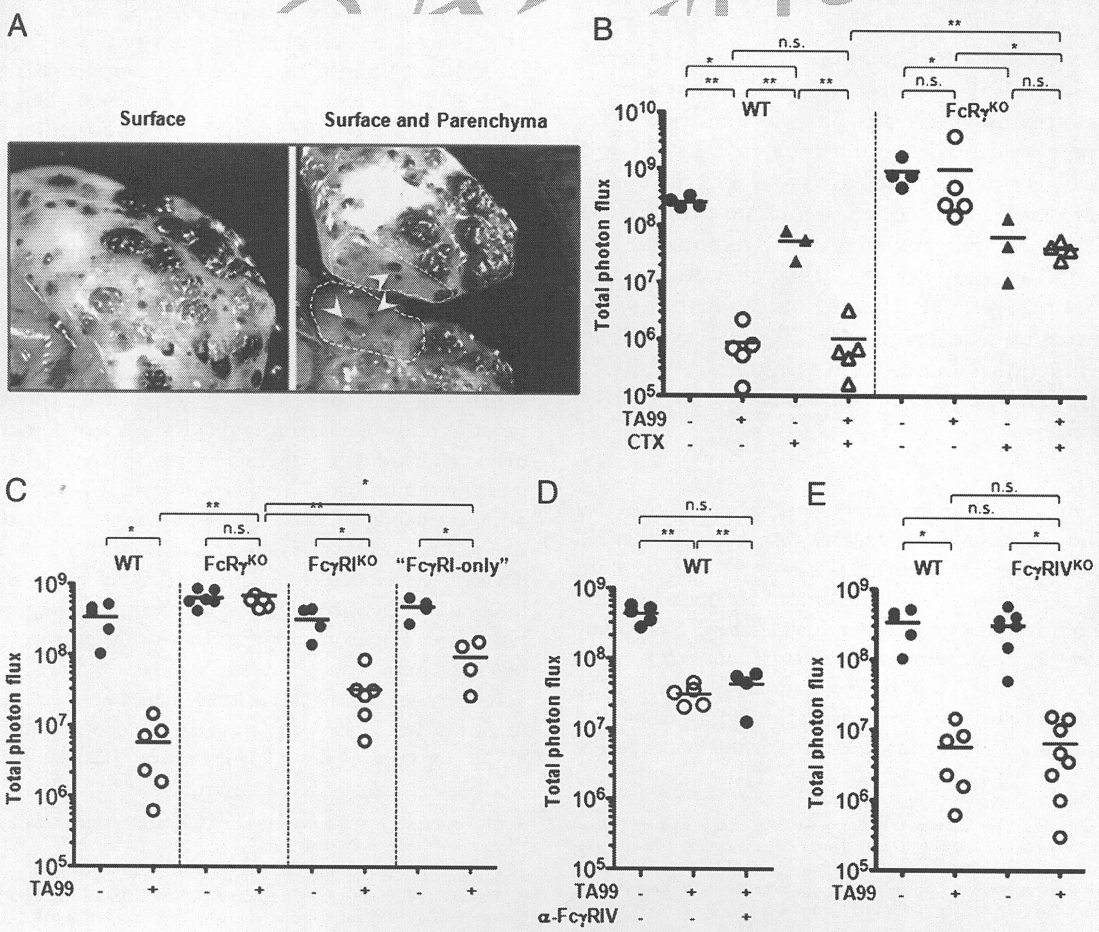

FIGURE 1. FcγRI, but not FcγRIV, contributes to mAb therapy of metastatic melanoma. (**A**) Representative picture of metastases on the lung surface (*left panel*) or on the surface and in the lung parenchyma (*right panel*) of wt mice at day 11 postinjection of B16F10 cells. Arrowheads indicate metastases in the parenchyma. (**B–E**) Mice were injected with B16-luc2$^+$ cells and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs in (B) wt and FcRγ$^{-/-}$ mice that were additionally injected with CTX when indicated ($n \geq 3$); (C) wt, FcRγ$^{-/-}$, FcγRI$^{-/-}$, or "FcγRI-only" mice ($n \geq 4$); (D) wt mice injected with anti-FcγRIV mAb or not ($n \geq 4$); (E) wt and FcγRIV$^{-/-}$ mice ($n \geq 5$, compilation of two identical experiments). Data are representative of two to four independent experiments. Not significant (n.s.): $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$.

assessment of tumor growth by bioluminescence. The expression of luc2 in B16-F10 cells led to photon release in the presence of luciferin but did not alter the expression of gp75 (Supplemental Fig. 1A). In vitro B16-luc2$^+$ cell growth was not affected by anti-gp75 mAb TA99 whether bound or aggregated at the cell membrane (Supplemental Fig. 1B), as expected (11). When injected i.v., B16-luc2$^+$ cells formed metastases specifically in the lung that could be noninvasively monitored in vivo and quantified over time by bioluminescence. Performing the same analysis on explanted lungs from the same mice, however, increased the sensitivity 20-fold (Supplemental Fig. 1C). Subsequent analyses were therefore performed on explanted lungs at day 11 postinjection of B16-luc2$^+$ cells.

B16-luc2$^+$ metastases formed to a greater extent in FcRγ$^{-/-}$ mice than in wild-type (wt) mice (Fig. 1B), as previously reported for wt B16F10 metastases (12). This difference was not due to different homing of tumor cells to the lungs after B16-luc2$^+$ cell injection (Supplemental Fig. 1D, insert). mAb TA99, but not an isotype control mAb (Supplemental Fig. 1E), reduced the tumor load in wt mice, but not in FcRγ$^{-/-}$ mice (Fig. 1B), an effect reliant on the FcRγ ITAMs, as described previously (13). Notably, the quantification of B16-luc2$^+$ metastases using bioluminescence therefore reproduces data from previous studies using metastasis counts by eye (1, 6, 7), and may thus be applied to analyze the contribution of specific FcγRs to the mAb therapy of metastatic melanoma. CTX administration reduced the tumor load to a similar extent in both wt and FcRγ$^{-/-}$ mice (Fig. 1B). Importantly, the combination treatment (CTX+mAb TA99) was more efficient than the CTX treatment alone in wt mice, but not in FcRγ$^{-/-}$ mice. The combination treatment, however, was not more efficient in wt mice than the administration of TA99 mAb alone. These results indicate that FcγRs contribute to both single mAb therapy and CTX+mAb combination therapy in this model.

First, we examined the contribution of FcγRI to mAb TA99 therapy of B16-luc2$^+$ metastases. Injection of B16-luc2$^+$ cells led to similar tumor loads in wt and FcγRI$^{-/-}$ mice that were significantly reduced after mAb TA99 injections in both mouse strains (Fig. 1C). The TA99-mediated reduction in tumor load was, however, less profound in FcγRI$^{-/-}$ mice compared with wt mice. This result indicates that FcγRI contributes to TA99-mediated reduction of B16 lung metastases, in accordance with the report by Bevaart et al. (7). Although no contribution of FcγRI was identified by Nimmerjahn et al. (6), our method of measuring tumor load may show in this study subtle differences that could not be revealed previously. To confirm our finding, we also investigated the contribution of FcγRI to this mAb therapy model in novel mice deficient for all IgG receptors except FcγRI (FcγRIIB/III/IV deficient). These "FcγRI-only" mice had partial reduction of tumor load after mAb TA99 injections (Fig. 1C). Whereas the kinetics of tumor growth was similar in both types of mice, TA99-mediated reduction in tumor load tended to be less efficient in "FcγRI-only" mice than in wt mice (Supplemental Fig. 2A). FcγRI therefore partially mediates the protective effect of mAb TA99.

We then reassessed the contribution of FcγRIV to this model. The injection of anti-FcγRIV mAbs had no significant effect on TA99-mediated tumor reduction in wt mice (Fig. 1D), similar to its isotype control (Supplemental Fig. 2B). Various FcγRIV-dependent disease models were abolished using the same dose of the same batch of anti-FcγRIV mAbs (data not shown and Refs. 8, 14), indicating that FcγRIV was efficiently and systemically blocked in vivo. Notably, this mAb does not, by itself, induce FcγRIV-mediated cell activation in vitro (9). Our in vivo result is unexpected because injections of the same dose of this blocking mAb were reported to partially impair the protective effect of TA99 (6). To ascertain the lack of contribution of FcγRIV to this Ab therapy, we compared wt and FcγRIV$^{-/-}$ mice. No difference in tumor rejection efficacy between wt and FcγRIV$^{-/-}$ mice could be detected after mAb TA99 injections (Fig. 1E).

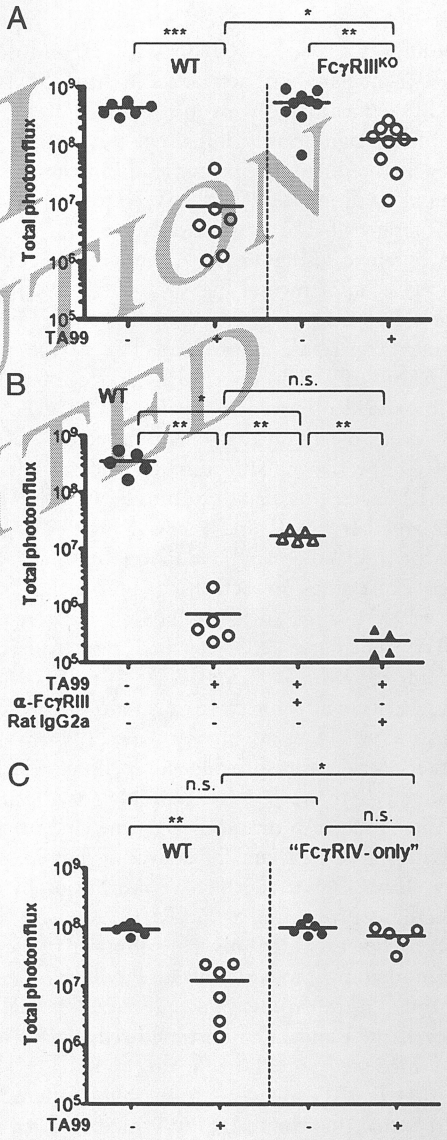

FIGURE 2. FcγRIII and FcγRI are responsible for mAb therapy to metastatic melanoma. Indicated mice were injected with B16-luc2$^+$ cells on day 0 and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs at day 11 in (**A**) wt ($n \geq 7$) and FcγRIII$^{-/-}$ mice ($n = 9$), two identical experiments were compiled; (**B**) wt mice injected or not with anti-FcγRIII mAb ($n = 5$) or isotype control ($n = 4$); (**C**) wt ($n = 6$) and "FcγRIV-only" mice ($n \geq 5$). Data are representative of two (A, C) or four (B) independent experiments. Not significant (n.s.): $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

These results demonstrate that FcγRIV does not contribute to this model of Ab therapy, in agreement with results reported by Bevaart et al. (7), but in contradiction to Nimmerjahn et al. (6).

Considering that FcγRI contributes only partially to the reduction of tumor load (Fig. 1C) and because FcγRIV does not contribute (Fig. 1D, 1E), we investigated whether the last mouse activating IgG receptor FcγRIII may contribute to TA99-mediated reduction of B16 lung metastases. mAb TA99 injections reduced tumor loads in both wt and FcγRIII$^{-/-}$ mice, but this effect was significantly impaired in FcγRIII$^{-/-}$ mice (Fig. 2A). This result is unexpected because it has been reported that wt and FcγRIII$^{-/-}$ mice display similar numbers/scores of B16 metastases after mAb TA99 injections (6, 7). To validate our finding, we took advantage of anti-FcγRIII mAbs that we have described previously, which specifically block FcγRIII in vivo (8), and do not induce FcγRIII-mediated cell activation in vitro (Supplemental Fig. 2C, 2D). Treatment with anti-FcγRIII mAb, but not isotype control, significantly impaired, but did not abolish, TA99-mediated reduction of tumor load in wt mice (Fig. 2B). These results indicate that FcγRIII contributes to TA99-mediated therapy of B16 lung metastases.

Results obtained using FcγRI$^{-/-}$ mice or "FcγRI-only" mice (Fig. 1C, Supplemental Fig. 2A), and FcγRIII$^{-/-}$ mice or anti-FcγRIII mAbs (Fig. 2A, 2B) suggest that FcγRI and FcγRIII may contribute nonredundantly to the protective effect of mAb TA99. The intermediate effect of mAb TA99 on the tumor load observed in FcγRI$^{-/-}$ or in FcγRIII$^{-/-}$ can be deduced to rely on the remaining activity of FcγRIII and FcγRI, respectively. Unfortunately, no mAb exists that blocks FcγRI to test this assumption in FcγRIII$^{-/-}$ mice. To investigate whether FcγRI and FcγRIII may be together responsible for TA99-mediated reduction in tumor load, we used mice expressing no activating IgG receptors except FcγRIV, "FcγRIV-only" mice (9). These mice were unable to mediate TA99-mediated reduction in tumor load (Fig. 2C). Furthermore, FcγRI$^{-/-}$ FcγRIIB$^{-/-}$ FcγRIII$^{-/-}$ triple-deficient mice were also unable to mediate the protective effect of mAb TA99 (data not shown).

Altogether, our results provide compelling evidence that FcγRI and FcγRIII are both responsible for the protective effect of anti-gp75 mAb on mouse B16 melanoma lung metastases. Using mice deficient for activating FcγRs, or blocking anti-FcγR mAbs, we identified a contribution by FcγRIII, reaffirmed the contribution of FcγRI (7), and by three different approaches excluded any contribution of FcγRIV to this immunotherapy model. Indeed, FcγRIII$^{-/-}$ mice and "FcγRIV-only" mice express higher levels of FcγRIV (5, 8, 15); however, these mice demonstrated reduced or undetectable TA99-mediated reduction in lung metastases load, also implying that FcγRIV does not contribute. Interestingly, it has been reported that the mAb TA99-mediated reduction of B16-F10 liver metastases relies on redundant functions of FcγRI and FcγRIV (16), whereas that of B16-F10 solid s.c. tumors relies only on FcγRIV (5). In particular, the mAb TA99-mediated effect was unaffected in both reports in mice deficient for both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$. It remains to be determined why the s.c. solid tumor model relies on FcγRIV (5), the liver metastases model relies on either FcγRI or FcγRIV (16), and the lung metastases model relies on additive roles of FcγRI and FcγRIII (this report), but these discrepancies may be explained by different effector populations mediating tumor clearance in skin, liver, and lung, respectively, and therefore on the IgG receptors they express.

The contribution of FcγRI (CD64) to lung metastases clearance has been reported earlier (7) and is confirmed in this study using FcγRI$^{-/-}$ mice. Importantly, we demonstrate in this article for the first time, to our knowledge, that "FcγRI-only" mice retain the ability to reduce tumor load in this mAb therapy model. A role for FcγRIII (CD16) in contributing to metastases clearance was, however, unexpected based on previous reports (6, 7) but is supported by a report on the contribution of FcγRIII to the protective effect of anti-CD20 mAbs on the growth of CD20-expressing lymphoma (17). In concordance with these results obtained in the mouse, polymorphisms in the gene encoding human FcγRIIIA (CD16A), the human functional homolog of mouse FcγRIII, indeed correlate with the therapeutic efficacy of rituximab (a chimeric anti-CD20 mAb) on non-Hodgkin's lymphoma (18), and of trastuzumab (a humanized anti–HER-2 mAb) on HER-2^{+} metastatic breast cancer (19). Two of our findings have important repercussions for the validity of the mouse as an animal model to study Ab-mediated cancer immunotherapy: 1) that FcγRs contribute to both single mAb therapy and combination (CTX+mAb) therapy; and 2) that mouse FcγRIII, like human FcγRIIIA, contributes to tumor clearance. Furthermore, our result supports the current trend in therapeutic Ab engineering of aiming to improve specific binding to human FcγRIIIA (CD16A) to increase Ab antitumor efficacy.

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Disclosures

L.E.M. and A.J.M. are employees of Regeneron Pharmaceuticals, Inc. The other authors have no conflicts of interest.

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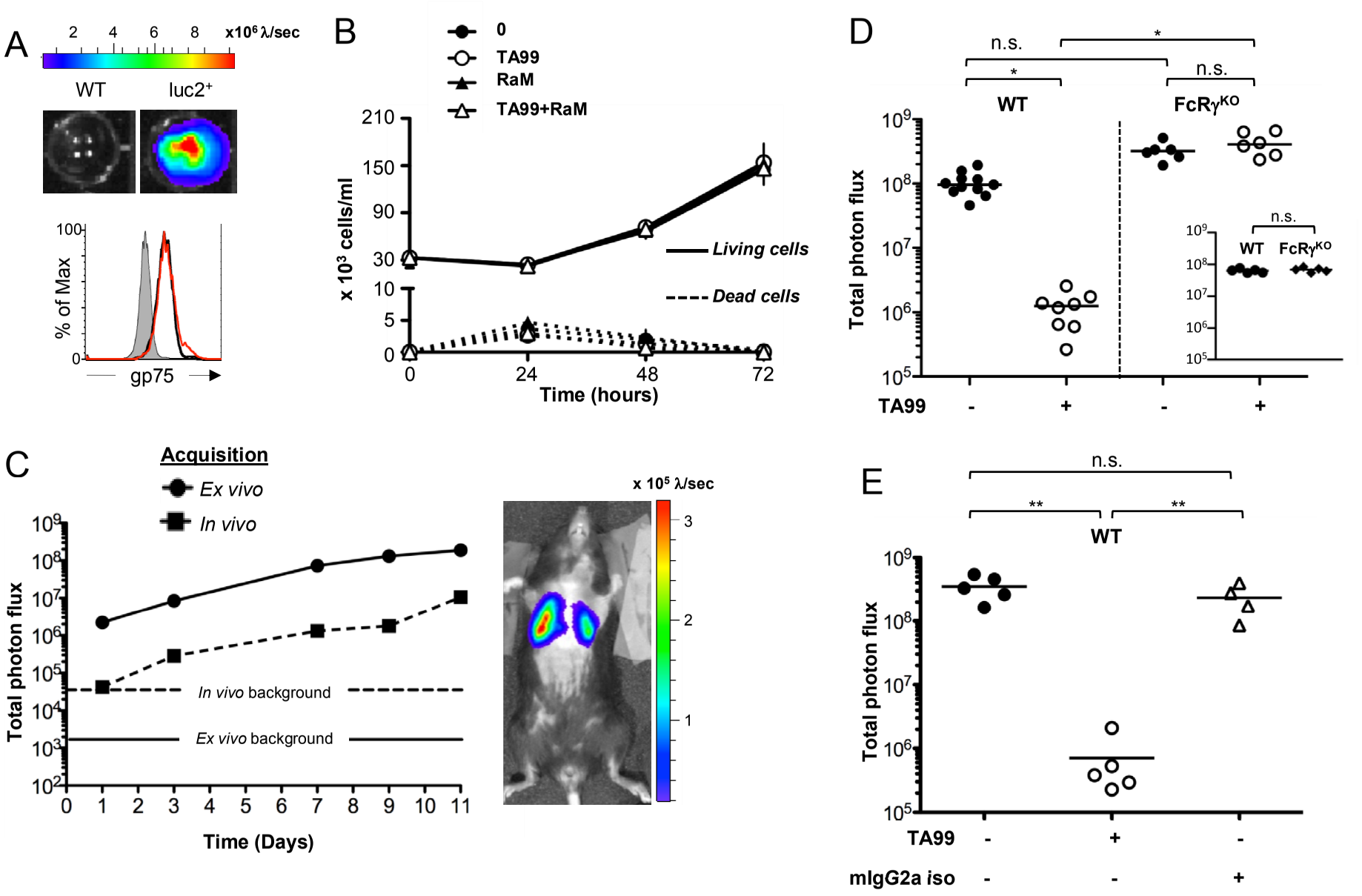
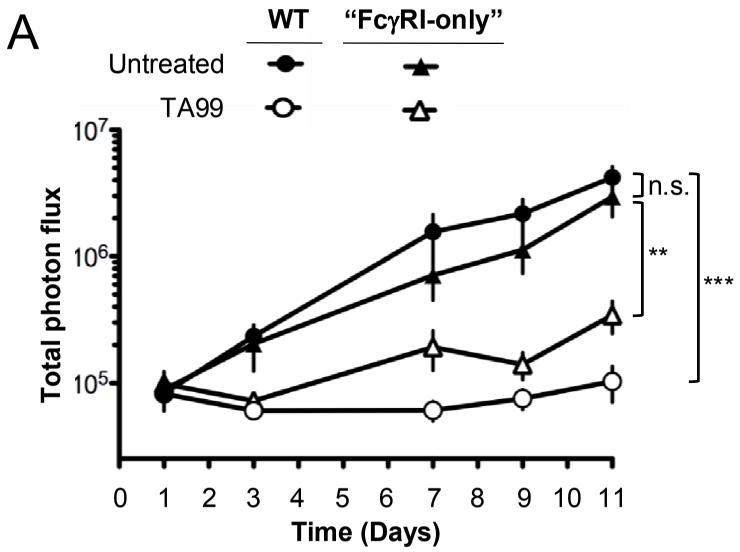
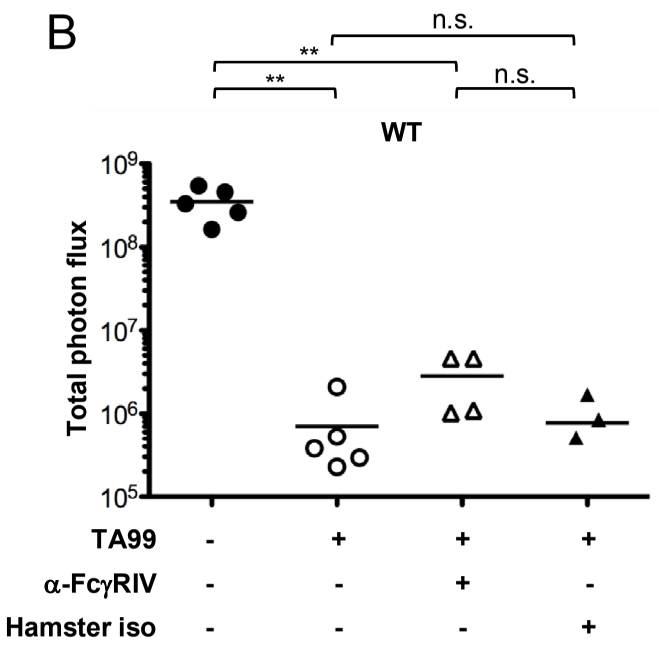
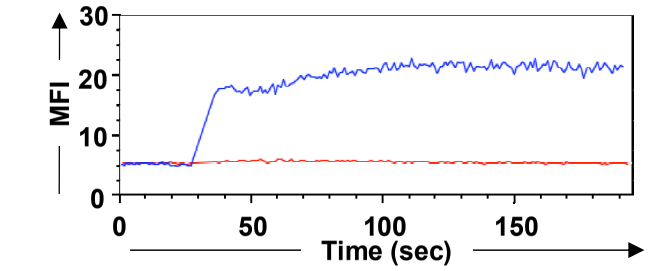
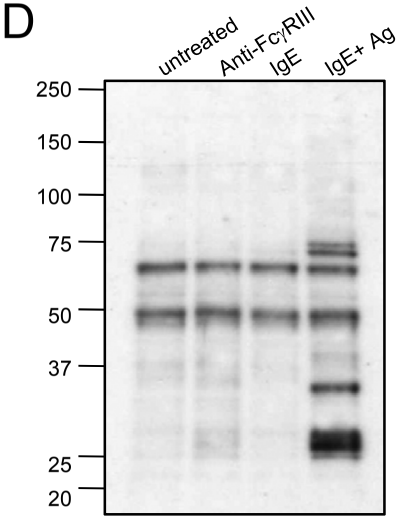


Supplemental Figure 1









Supplemental Figure 2

DISCUSSION

The results I have obtained in this work provide compelling evidence of a non-redundant role of different mouse activating FcγRs to the anti-tumor activity of the anti gp75 mAb TA99, and solved the contradiction found in the literature. Indeed, I could identify a contribution of FcγRIII, demonstrate the contribution of FcγRI in agreement with previous reports, and exclude a contribution of FcγRIV to mAb TA99 anti-tumor activity.

Tumor quantification & unexpected results:

The first difference between my work and the previous studies relies in the methodology used for metastases quantification. In previous reports, indeed, the quantification of the tumor load took into account only the size and/or the number of metastases present on the lung surface. However once injected intravenously, cancer cells enter through the inferior vena cava into the pulmonary circulation that deeply irrigates the lung parenchyma. Therefore the seeding of melanoma cells and the development of metastases foci can occur both into the lung parenchyma and on the lung surface. As a consequence, quantification that takes into account only the size or number of metastases on the lung surface may neglect metastases developing inside the lung parenchyma and not eye-visible metastases. B16 cells that express the enhanced firefly luciferase *Luc2* (*B16 Luc2+*) enabled me to overcome this issue and to quantify the tumor load in accurate and experimentator-independent manner. In this work, eleven days after the intravenous injection of B16Luc2+ cells in mice the lungs have been explanted. The photon emission obtained upon contact with the luciferin *ex vivo* correlates with the amount of lung metastases. This approach leads to a measurement over three logs and therefore enabled to identify contributions of FcγRs that have never been detected before. As an example, none of the previous reports identified a contribution of FcγRIII to this model of immunotherapy. This may also be due to the fact that both FcγRIII$^{-/-}$ mice and wt mice treated with anti-FcγRIII blocking mAbs, after injections of mAb TA99, developed less *eye-visible metastases* on the lung surface than wt mice. In addition these mice had tumor foci inside the lung parenchyma, as revealed by the bioluminescence, indicating that FcγRIII participate to the mAb TA99 anti-tumor activity. The contribution of FcγRIII that I found supports the correlations that have been pointed out from clinical studies. In fact, the polymorphisms in the gene encoding for FcγRIIIA, the human functional homolog of mouse FcγRIII, correlate with the therapeutic efficacy

of different therapeutic mAbs such as Rituximab (anti CD20 mAb) on non-Hodgkin's lymphoma and of Trastuzumab (anti-HER2) on $HER2^+$ metastatic breast cancer[71].

The bioluminescence approach enabled me to make another important observation on the possible involvement of the inhibitory receptor FcγRIIB (data not published). In vitro experiment involving heat-aggregated mAb TA99 molecules *(**n.b.** condition that mimics an immune complex or a cancer cell opsonized with mAb TA99 molecules)* demonstrated binding on to mouse FcγRs, including the inhibitory receptor FcγRIIB. FcγRIIB, may therefore limit mAb TA99 therapeutic effect when present on cells that express FcγRI and /or FcγRIII. Previous reports[4] have indeed proposed that the absence of FcγRIIB in $FcγRIIB^{-/-}$ mice results in an enhanced mAb TA99 antitumor activity. This observation was based on a macropscopic analysis and lung metastases count. However, when I re-analyzed, using bioluminescence, the involvement of FcγRIIB using $FcγRIIB^{-/-}$ mice I could not confirm this result. No differences in the tumor load appeared compared to wt mice following mAb TA99 treatment, indicating that FcγRIIB is not involved in this model of immunotherapy (***fig.***7).

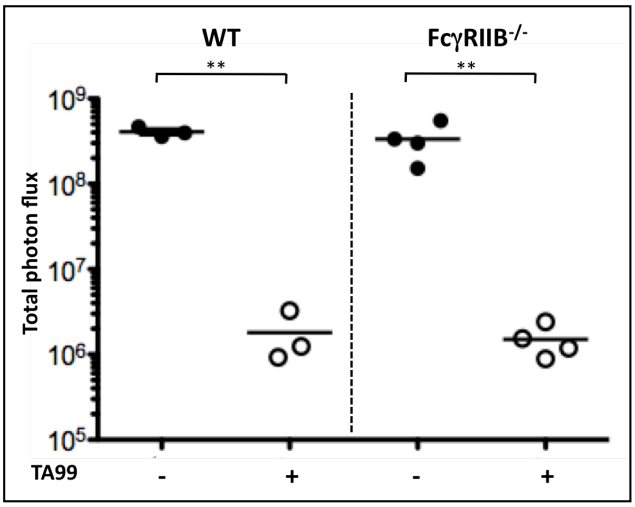


***Fig.** 7. Indicated mice were injected with B16 Luc2+ cells on day 0 and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs at day 11*

This difference could, again, be attributable to the different techniques to quantify tumour load. An alternative possibility is that intrinsic to the $FcγRIIB^{-/-}$ genotype: the inhibitory receptor FcγRIIB is involved in the control of immune cell proliferation and B cell homeostasis, and consequently $FcγRIIB^{-/-}$ mice are prone to spontaneous development of autoimmune diseases, such as Systemic Lupus Erythematosus and myeloproliferation[122]. Importantly, these conditions may impair the formation of an anti-tumor immune response and therefore the efficacy of therapeutic tumor-specific mAbs. Furthermore, it may be considered that different $FcγRIIB^{-/-}$ murine strains bred in different animal facilities may exhibit a differential age of onset of autoimmune diseases; and this may account for different results on the role of FcγRIIB in TA99-induced tumor therapy. The use of FcγRIIB blocking mAbs in wt mice could, therefore, clarify the function of this receptor in this model.

A multi-approach strategy to identify the activating FcγRs responsible:

Different approaches can be used to study the biological activities of FcγRs *in vivo* that, all have their own limitations. The most common approach is to use mice that lack one FcγR. However, it is important remind that, in mice, all activating FcγRs are coupled with the FcRγ subunit required for their signal transduction and expression on the cell surface. Moreover, this subunit represents the limiting factor for FcγR expression. Thereby, in mice lacking one (or more) FcγR(s) more FcRγ subunit is available for the association with the remaining FcγRs. This phenomenon may enhance the expression of one or several remaining FcγRs (***e.g***. *FcγRIII*$^{-/-}$ mice have an augmented expression of FcγRIV^{123}) and may bias the experimental result obtained using these mice. Another approach to study the involvement of FcγRs in mAb-therapy is an *in vivo* injection of blocking mAbs against FcγRs. These different blocking mAbs may, however, have different mechanisms of action *in vivo*. Some mAbs may block the activity of the target FcγR via the Fab portion and have no additional through their Fc portion. Others, upon binding of the Fab portion to their target FcγR, block the activity of other FcγRs that are in close proximity via their Fc portion (**e.g.** occupying the Fc binding pocket of adjacent FcγRs).

Because of the limitations in these approaches I have used a *multi-approach strategy* that consists in using two or more approaches to analyze the role of a particular FcγR. An example can be given by the analysis made for the involvement of FcγRIV in this model of immunotherapy. In fact, the same results could be obtained in wt mice injected with anti FcγRIV mAb and in FcγRIV$^{-/-}$ mice, each approach validating the other. Moreover, "FcγRIV-only" mice, in which the only activating FcγR expressed is FcγRIV, were not protected by the injection of the therapeutic mAb TA99. These results together supported, for the first time, the hypothesis that FcγRIV is not involved in mAb TA99 –mediated tumor killing.

n.b-1. *The differential involvement of the mouse FcγRs during mAb therapy is further discussed in the general discussion –Part I*

n.b.-2 *The identification of the FcγRs involved in mAb anti-tumor activity can help predict, based on the FcgRs expression pattern, the cell population responsible for tumor killing. This particular issue is discussed in the general discussion, Part I*

ARTICLE 2

ARTICLE 2

"Unexpected properties for human FcγRI"

INTRODUCTION

Human FcγRs comprise FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA and FcγRIIIB. Among these receptors the only high affinity receptor is FcγRI (hFcγRI)[66]. Differing from its mouse homolog *mouse FcγRI* (mFcγRI), the pattern of expression of hFcγRI is not restricted to monocyte-derived DCs, and extends to blood monocytes and tissue macrophages. Moreover under certain conditions such as CT, infections and rheumatoid arthritis hFcγRI is also expressed on neutrophils. Since the generation in 1996 of a hFcγRI-transgenic mouse strain, one study has been performed to assess the involvement of hFcγRI on antigen presentation following targeting of antigens to this receptor, and one to study its role in protection from malaria by Abs[124,125]. However, the involvement of this receptor in other antibody- based reactions has not been investigated. In project of a post-Doctoral fellow in the laboratory to which I collaborated, the role of hFcγRI in different Ab-mediated models of disease and therapy has been investigated. *hFcγRI^{Tg} mice* deficient for multiple endogenous FcRs were, indeed, generated to study the role of this human FcγR without the contribution of endogenous FcγRs. We found that hFcγRI was sufficient to trigger autoimmune arthritis and thrombocytopenia, immune complex-induced airway inflammation, active and passive systemic anaphylaxis and mAbs-mediated cancer immunotherapy.

In this work, I have been in charge of analyzing the involvement of hFcγRI in mAb-based cancer immunotherapy. The idea of looking at the involvement of hFcγRI in the mAbs anti- tumor activity came from two observations:

1. The results presented in article 1 demonstrated that, among the different mouse activating FcγRs, FcγRI and FcγRIII are together responsible for mAb TA99 anti-tumor activity. This result may thus give indications about the involvement of their human homologues ***(n.b.** this particular issue is further discussed in the general discussion- Part I)*. As mentioned before, several clinical studies based on differential responses to the treatment that have been linked to human Fcgr3a gene polymorphisms have already suggested the involvement of human FcγRIIIA (*hFcγRIIIA*) in mAb-based cancer immunotherapy. By contrast the involvement of hFcγRI has never been investigated[71].

2. In this article we describe that intravenous injection of anti-platelet IgG2a mAb (6A6) to hFcγRI^{Tg} mice results in a drop of 90-95% of platelet count, a model of platelets Immune Thrombocytopenic Purpura (ITP). hFcγRI can therefore mediate platelet destruction mediated by anti-platelet IgG2a mAb. From this observation we wondered whether hFcγRI could also mediate cancer cell destruction following anti-cancer IgG2a mAb injections.

I therefore used the same B16Luc2^{+} melanoma model of lung metastases that has been described article 1, and the protective IgG2a anti-gp75 TA99. Importantly, I used not only the original murine mAb, but also a humanized, chimeric version of TA99 called CTA99 containing an IgG_1 framework, as well as a fully human IgG_1 anti-gp75 mAb called 20D7S (hIgG1). Using the above-mentioned tools I was able to describe, for the first time, an involvement of hFcγRI in mAb therapy.

The high-affinity human IgG receptor FcγRI (CD64) promotes IgG-mediated inflammation, anaphylaxis and anti-tumor immunotherapy

***Running title*: Unexpected properties for human FcγRI**

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ABSTRACT

IgG receptors (FcγRs) are mandatory for the induction of various IgG-dependent models of autoimmunity, inflammation, anaphylaxis and cancer immunotherapy. Among the three mouse activating FcγRs, the contribution of high-affinity mFcγRI to these models has not been thoroughly investigated. The main reasons reside in its extremely restricted tissue distribution and in the belief that this receptor is occupied by circulating IgG *in vivo*. Its human homolog hFcγRI (CD64) is also believed to be occupied by IgG *in vivo* but its expression extends to monocytes, macrophages and neutrophils; cells identified as mandatory effector populations in most models of inflammation. We therefore investigated the role of hFcγRI in antibody-mediated models of disease and therapy by generating hFcγRI-transgenic mice deficient for multiple endogenous FcRs. We found that hFcγRI was sufficient to trigger autoimmune arthritis and thrombocytopenia, immune complex-induced airway inflammation, active and passive systemic anaphylaxis. We identified monocyte/macrophages to be responsible for thrombocytopenia, neutrophils to be responsible for systemic anaphylaxis, and both cell types to be responsible for arthritis induction. Finally, hFcγRI was capable of sustaining antibody-mediated immunotherapy of mouse metastatic melanoma. Altogether, our results unravel unexpected capabilities of human FcγRI that change our understanding of the function of high-affinity IgG receptors *in vivo*.

INTRODUCTION

Receptors for the Fc portion of IgG (FcγR) are expressed in humans and mice and mediate most biological activities of IgG antibodies. FcγRI (CD64), FcγRIIB (CD32B) and FcγRIIIA (CD16A) exist in both species. FcγRIIA (CD32A), FcγRIIC (CD32C) and FcγRIIIB (CD16B) are specific to humans, whereas FcγRIV is specific to mice. This nomenclature is based on amino acid sequence homology but does not systematically reflect functional homologies or similar expression pattern between FcγRs in both species[1]. Therefore, the role of human FcγRs may not be predicted from the role of their homologs studied in mice. Transgenic mice expressing human FcγRs (hFcγRs) have been generated to enable their analysis in disease and therapy models *in vivo*. Whereas hFcγRIIA has been extensively studied using transgenic mice[2-5], some hFcγRs like hFcγRI have been intriguingly understudied *in vivo*.

Human FcγRI is the only high-affinity IgG receptor in humans. hFcγRI binds human IgG1, IgG3 and IgG4 with a high affinity and has no affinity for IgG2[6]. High-affinity FcγRs ($K_A \approx 10^7$–10^8 M^{-1} for IgG), but not low-affinity FcγRs ($K_A \approx 10^5$–10^6 M^{-1} for IgG), are defined by their ability to bind IgG as monomers. Both types of FcRs however bind IgG when present in immune complexes (ICs) or when opsonizing cells or surfaces. Thus, high-affinity FcγRs are thought to be occupied/saturated by IgG *in vivo*, leading to the belief that pre-bound IgG prevents participation of high-affinity receptors to IC-mediated reactions. Inversely, low-affinity FcγRs are believed to remain free and thus to be responsible for IC-mediated reactions. ICs, however, have been reported to displace monomeric IgG from high-affinity FcγRs within minutes[7]. Furthermore, even when in the presence of elevated IgG levels *in vitro*, high-affinity FcγRs have been reported to retain their ability to bind opsonized red blood cells[8]. It could thus be demonstrated that the

mouse high-affinity IgG receptor mFcγRI, those expression pattern in steady state is restricted to monocyte-derived dendritic cells[1,9,10], contributes to inflammation severity in multiple models of disease[11-16]. The contribution of mFcγRI to these disease models may rely solely on these monocyte-derived $CD11b^+$ $CD11c^+$ $MHCII^+$ $Ly6C^+$ "dendritic cells"[10] as neither circulating monocytes or neutrophils express this receptor[9,17]. Intriguingly, whereas several reports described mFcγRI expression on bone marrow-derived macrophages, contradictory results are reported on its expression on thioglycolate-elicited macrophages[13,17]. No role for mouse FcγRI could however be identified in the passive model of antibody-induced inflammatory arthritis (K/BxN)[18], antibody-induced immune thrombocytopenia or antibody-induced protection from lung metastases[19]. It could nevertheless be demonstrated that the other mouse high-affinity IgG receptor mFcγRIV that is expressed on monocyte/macrophages and neutrophils contributes to several of these models of autoimmunity, inflammation and anaphylaxis[17,20,21].

Differing from its mouse homolog mFcγRI, the expression pattern of hFcγRI is not restricted to monocyte-derived "dendritic cells", and extends to blood monocytes and tissue macrophages[22,23]. Under many circumstances including chemotherapy, multiple myeloma[24], rheumatoid arthritis[22], bacterial infection, sepsis, inflammatory bowel disease or treatment with recombinant G-CSF, hFcγRI is also expressed by neutrophils. Thus, the expression pattern of human and mouse FcγRI appear very different and suggest that their roles in pathology and therapy may also be very different. Whereas a role for hFcγRI on dendritic cells has been reported in enhancement of antigen presentation and cross-presentation[23], its role(s) on monocytes, macrophages and neutrophils, has, however, not been addressed. Monocytes/Macrophages have been involved in IC-induced airway inflammation[25], in antibody-dependent cellular cytotoxicity (ADCC) of opsonized platelets leading to thrombocytopenia[26] or

of opsonized tumor cells in mouse models of metastatic cancer[14]. Monocytes/Macrophages also induce neutrophil recruitment into the inflamed tissue, *i.e.* joints or pulmonary tissues in models of inflammatory arthritis[27] or airway inflammation[28], respectively. Neutrophils have been reported to be mandatory for the induction of inflammatory arthritis[29], IC-dependent airway inflammation and, recently, to contribute to models of systemic anaphylaxis[2,20].

Mice transgenic for the *Fcgr1a* gene have been generated that recapitulate the expression of the high-affinity receptor hFcγRI in humans[1,30]. Based on its expression pattern, we hypothesized that hFcγRI may be capable of inducing antibody-dependent autoimmunity, anaphylaxis and tumor immunotherapy models to which monocytes/macrophages and/or neutrophils have been reported to contribute. To this aim, we crossed hFcγRI^{tg} mice with mice deficient for multiple endogenous FcRs. We found that hFcγRI bound several mouse IgG subclasses as monomers, thereby conserving its properties as a high affinity receptor *in vivo* in these mice. In this context, we demonstrate that hFcγRI was sufficient to induce not only autoimmune arthritis, thrombocytopenia, airway inflammation and fatal systemic anaphylaxis, but could mediate the therapeutic efficacy of clinically-adapted humanized anti-tumor antibodies on metastatic melanoma. Thus human high-affinity IgG receptor hFcγRI might be a pro-inflammatory and pro-anaphylactic IgG receptor in humans that can mediate IgG-based anti-tumor immunotherapies.

RESULTS

hFcγRI can trigger passive inflammatory arthritis.

To investigate the pro-inflammatory potential of hFcγRI *in vivo*, we crossed mice transgenic for hFcγRI ($hFc\gamma RI^{tg}$)[30] to mice deficient for five endogenous FcRs ($Fc\gamma RI/IIB/III^{-/-}$ $Fc\varepsilon RI/II^{-/-}$ mice, aka 5KO mice)[7]. These mice still express the FcRγ-chain that is mandatory for hFcγRI expression and endogenous FcγRIV. In $hFc\gamma RI^{tg}$ 5KO mice, hFcγRI was expressed in the blood specifically on $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes, on neutrophils, and on peritoneal, liver, lung and alveolar macrophages, but not on peritoneal mast cells (Fig.1A), in agreement with a previous report[30]. The expression pattern of hFcγRI in $hFc\gamma RI^{tg}$ 5KO mice therefore mimics its expression pattern in humans in which hFcγRI is constitutively expressed on monocytes and inducible on neutrophils. Noticeably, whereas the expression level of hFcγRI was higher on neutrophils from these mice compared to human neutrophils from two different normal donors, it was similar on mouse monocytes compared to monocytes from normal donors (Fig.1B and Supplemental Fig.S1A). Importantly, hFcγRI bound mouse IgG2a, IgG2b and IgG3, but not mouse IgG1, either as monomers (Fig.1C) or as immune complexes (Fig.1D). Moreover, the analysis of the interaction of hFcγRI with mouse IgG2a or with human IgG1 resulted in similar association (k_{on}) and dissociation (k_{off}) constants, and therefore in a very similar calculated affinity constant ($K_D \approx 40nM$, *i.e.* $K_A \approx 2.5x10^7 M^{-1}$) (Fig.1E-F, Supplemental Fig.S1B). hFcγRI retains therefore its properties as a high-affinity receptor for IgG when expressed in transgenic mice.

Because hFcγRI has been reported to be expressed in the articular synovium of arthritis patients but not in healthy controls[31], we investigated whether hFcγRI could induce arthritic inflammation using hFcγRI^{tg} 5KO mice and K/BxN serum. The serum of spontaneously arthritic K/BxN mice (F1 offsprings from KRN^{tg} mice crossed with NOD mice) indeed contains pathogenic IgG1 and IgG2 anti-Glucose-6-Phosphate Isomerase (GPI) antibodies[17] able to form immune complexes with GPI deposited on the articular cartilage. These immune complexes induce inflammatory arthritis that requires activating FcγRs[18]. Both 5KO and hFcγRI^{tg} 5KO mice developed arthritis (Fig.2A) following K/BxN serum injection (K/BxN PA). Blocking FcγRIV using blocking anti-FcγRIV mAbs abolished arthritis in 5KO, but not in hFcγRI^{tg} 5KO mice. Blocking FcγRIV using anti-FcγRIV mAbs and hFcγRI using blocking anti-hFcγRI.1 mAbs (Supplemental Fig.S1C) was necessary to abolish K/BxN PA in hFcγRI^{tg} 5KO mice (Fig.2A). Blocking hFcγRI significantly reduced arthritis symptoms in hFcγRI^{tg} 5KO mice (Fig.2B). hFcγRI-dependent arthritis (arthritis developing in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice) was milder than arthritis developing in untreated hFcγRI^{tg} 5KO mice. Occupancy of a proportion of this human high-affinity receptor by endogenous mouse IgG may be responsible for these mild arthritic symptoms. hFcγRI-dependent arthritis did not, however, increase in severity when induced in RAG-deficient hFcγRI^{tg} 5KO mice that lack endogenous IgG (Fig.2C). Similar results were obtained for FcγRIV-dependent arthritis (Fig.2C, insert). If occurring *in vivo*, partial occupancy or saturation of hFcγRI (or FcγRIV) by IgG does therefore not affect K/BxN arthritis induction and development. As expected, IgG2 antibodies purified from K/BxN serum induced hFcγRI-dependent arthritis, whereas IgG1 antibodies purified from K/BxN serum induced only very modest pathological symptoms (Fig.2D). Finally, hFcγRI-dependent arthritis was abolished when monocytes/macrophages or neutrophils were depleted (Fig.2E). Altogether, these results

demonstrate that hFcγRI is sufficient to induce K/BxN passive arthritis, mediated by mouse IgG2 autoantibodies, that required both monocytes/macrophages and neutrophils.

hFcγRI can trigger antibody-dependent airway inflammation

We next investigated if hFcγRI could induce lung inflammation in a model of immune complex-mediated airway inflammation[28] as hFcγRI is expressed on lung and alveolar macrophages from hFcγRI^{tg} 5KO mice (Fig. 1A). This disease model of a reverse Arthus reaction consist of an intravenous injection of antigen (OVA) and of intranasal instillation of anti-OVA antibodies that was shown to depend on the expression of activating FcRs on alveolar macrophages[25]. Intravenous injection of OVA followed by intranasal instillation of rabbit anti-OVA serum (hFcγRI binds rabbit IgG, Supplemental Fig.S1D) lead to a massive infiltration of neutrophils in the airways within 18 hours, as determined in broncho-alveolar lavages (BAL). Whereas blocking either hFcγRI or mFcγRIV significantly inhibited neutrophil infiltration, blocking both hFcγRI and FcγRIV was necessary to abolish neutrophil infiltration (Fig.3A,B). No major variation in alveolar macrophage numbers under these different conditions was observed (Fig.3C), as expected[28]. When occurring however, neutrophil infiltration drastically modified the alveolar macrophage/neutrophil ratio in BAL (Fig.3D vs Fig.3B). Similarly whereas myeloperoxidase production in the BAL (Fig.3E), resulting from neutrophil and/or macrophage activation, and hemorrhage (Fig.3F), resulting from tissue damage, had a trend to be reduced following hFcγRI blockade and was significantly reduced following mFcγRIV blockade, both symptoms were abolished following blockage of both receptors. Altogether, these results demonstrate that hFcγRI is sufficient to induce airway inflammation.

hFcγRI can trigger passive systemic anaphylaxis.

We recently reported that FcγRIV was responsible for IgG2b-induced passive systemic anaphylaxis (PSA)[20] that arises following intravenous injection of preformed immune complexes made of mouse IgG2b (anti-DNP) and antigen (DNP-BSA). We therefore investigated the potential of hFcγRI, which has the same expression pattern and ligands as FcγRIV in transgenic mice, to induce PSA in hFcγRI^{tg} 5KO mice using divalent (anti-hFcγRI mAbs) or multivalent (IgG-immune complexes) ligands. An i.v. injection of the non-blocking anti-hFcγRI.2 mAb, but not of the blocking anti-hFcγRI.1 mAb (Supplemental Fig.S1C), induced a significant temperature drop in hFcγRI^{tg} 5KO mice, but not in 5KO mice (Fig.4A). The effect of non-blocking anti-hFcγRI.2 mAb injections on the central temperature of hFcγRI^{tg} 5KO mice was dose-dependent (Fig.4B) and resulted in fatal anaphylactic shocks at higher doses (data not shown). Therefore, whereas anti-hFcγRI.1 mAb is an antagonistic blocking antibody, anti-hFcγRI.2 mAb is an agonistic non-blocking antibody capable of inducing hFcγRI-dependent anaphylaxis. *N.B.* In all further experiments *in vivo* hFcγRI blockade will be achieved by anti-hFcγRI.1 mAb injections. An i.v. injection of mouse IgG2b-immune complexes induced a temperature drop in 5KO and hFcγRI^{tg} 5KO mice that was abolished by FcγRIV blockade in 5KO, as expected[20], but not in hFcγRI^{tg} 5KO mice (Fig.4C). Confirming the anaphylactogenic potential of hFcγRI, blocking hFcγRI reduced the temperature drop in hFcγRI^{tg} 5KO mice, and hFcγRI-dependent PSA (anaphylaxis developing in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice) was abrogated by hFcγRI blockade (Fig.4D). Altogether, these results demonstrate that hFcγRI is sufficient to trigger PSA in transgenic mice.

Neutrophils and PAF mediate hFcγRI-dependent active systemic anaphylaxis.

Because hFcγRI was sufficient to trigger PSA, we investigated if hFcγRI may also trigger active systemic anaphylaxis (ASA). ASA was induced by an i.v. antigen (BSA) challenge in mice repeatedly immunized with the same antigen in Freund's adjuvant (first immunization in complete, second and third immunization in incomplete Freund's adjuvant). This protocol induced a strong body temperature decrease in hFcγRI^{tg} 5KO mice, but not in 5KO mice, when pre-treated with anti-FcγRIV mAbs (Fig.5A), that we termed hFcγRI-dependent ASA. Supporting this result, hFcγRI blockade significantly inhibited ASA-induced temperature drop (Fig.5B) and abolished ASA-induced mortality (Supplemental Fig.S2A) in hFcγRI^{tg} 5KO mice. Blocking both hFcγRI and FcγRIV further inhibited ASA-induced temperature drop in these mice (Fig.5B). hFcγRI is therefore sufficient to trigger active systemic anaphylaxis in transgenic mice.

Both effector cell types that express hFcγRI, *i.e.* monocytes/macrophages[32] and neutrophils[20], can potentially contribute to ASA. hFcγRI-dependent ASA was strongly inhibited by neutrophil depletion following injection of anti-Gr1 mAbs (Fig.5C). Because this rat IgG2b anti-Gr1 mAb injection may lead to activation and depletion of complement components due to *in vivo* immune complex formation as suggested previously[33], we investigated if the inhibition of hFcγRI-mediated active anaphylaxis following anti-Gr1 mAb treatment relied on complement. A dose of cobra venom factor (CVF) that inactivates both C3 and C5 components of the complement[34] did neither prevent hFcγRI-mediated active anaphylaxis nor its inhibition following anti-Gr1 mAb injections (Supplemental Fig.S2B). Therefore, the inhibition of anaphylaxis following anti-Gr1 mAb injection is dependent on neutrophil depletion *per se*, and not on complement. Surprisingly, neither monocyte/macrophage depletion following toxic liposomes injection (Fig.5D), nor inhibition of monocyte/macrophage function following gadolinium injection (Fig.5E) reduced hFcγRI-dependent ASA. Unexpectedly, the injection of

toxic liposomes or of gadolinium rather increased hFcγRI-induced hypothermia. The depletion or inhibition of monocytes/macrophages, when combined with the depletion of neutrophils had, however, a tendency to increase the protection from hFcγRI-dependent ASA (Fig.5D-E). Neutrophils and, possibly to a minor extent, monocytes/macrophages therefore contribute to hFcγRI-dependent ASA. Mediators released and/or secreted by these activated cell types should therefore be responsible for the anaphylactic shock observed. Among them, PAF was shown to be responsible for neutrophil-dependent ASA[20] and for macrophage-dependent ASA[32], whereas histamine was shown to be responsible for mast cell-dependent anaphylaxis[35]. The PAF-R antagonist ABT-491, but not the histamine and serotonin receptor antagonist cyproheptadine, markedly reduced hFcγRI-dependent temperature drop (Fig.5F) and mortality (Supplemental Fig.S2C). PAF therefore accounts for hFcγRI-dependent ASA. The conjunction of both antagonists, however, further reduced hFcγRI-dependent ASA (Fig.5F). Noticeably, in addition to mast cells and basophils, neutrophils have been reported to be able to release histamine[36] but not serotonin, suggesting that histamine released by neutrophils might, to a minor extent, contribute to hFcγRI-dependent ASA.

Monocytes/macrophages mediate hFcγRI-dependent thrombocytopenia

We next investigated if, in addition to exerting pro-inflammatory and pro-anaphylactic properties, hFcγRI may also exert phagocytic properties *in vivo* using a murine model of thrombocytopenia. Immune Thrombocytopenic Purpura (ITP) can be induced by injecting intravenously anti-platelet antibodies (reminiscent of autoantibodies found in ITP patients) and by following circulating platelet consumption. ITP could be induced following injection of mouse IgG2a anti-platelet mAbs both in hFcγRI^{tg} 5KO mice and in 5KO mice. FcγRIV blockade

prevented ITP in 5KO mice (as expected[20,21]), but reduced platelet consumption by less than 50% in hFcγRI^{tg} 5KO mice (Fig.6A,B). The remaining platelet consumption was hFcγRI-dependent, as it was prevented by a further hFcγRI blockade (Fig.6B). hFcγRI-dependent ITP was not affected by neutrophil depletion (Fig.6C), but was significantly inhibited by monocyte/macrophage depletion (Fig.6D). Noticeably, splenectomy had no significant effect on hFcγRI-dependent ITP (Fig.6E), suggesting that other hFcγRI-expressing macrophages than splenic macrophages contribute to platelet clearance in this model. Liver macrophages, *i.e.* Kupffer cells, which belong to the mononuclear phagocyte system express hFcγRI in hFcγRI^{tg} 5KO mice (Fig.1A), could be responsible for platelet consumption in this model.

hFcγRI can mediate Ab-induced anti-tumor immunotherapy

Because hFcγRI can mediate Ab-induced platelet clearance, we wondered whether hFcγRI may also mediate tumor reduction/destruction following anti-tumor Ab injection. To this aim, we used the B16 melanoma (expressing gp75 aka TYRP-1) tumor immunotherapy model that relies on injections of anti-TYRP-1 mouse IgG2a TA99 mAb and that was reported to depend on mouse FcγRs[37]. To allow accurate quantification of lung metastases (*i.e.* tumor load) following i.v. injection of the tumor, we used a luciferase-expressing variant of B16 (B16 luc2^{+}) that expresses similar amounts of TYRP-1 as wt B16 cells (Supplementary Fig.S3A). I.v. injections of B16 wt or B16 luc2^{+} cells in wt C57BL/6J mice lead to metastatic melanoma in the lung that could be quantified by bioluminescence imaging on explanted lungs *ex vivo* in the case of B16 luc2^{+}-injected mice (Supplementary Fig.S3B). Repeated TA99 injections lead to a drastic reduction in tumor load in wt C57BL/6J mice, but neither in FcRγ$^{-/-}$ mice that lack all activating FcRs, as expected[37] (Fig.7A), nor in 5KO mice (Fig.7B). TA99 injections, however, lead to a

significant reduction in tumor load in hFcγRI^{tg} 5KO mice (Fig.7B). hFcγRI can therefore mediate metastatic melanoma reduction following mouse IgG2a anti-TYRP-1 mAb injections.

A chimeric version of TA99 with a human IgG1 heavy chain (CTA99; developed by Imclone, Patent US 2009/0232823 A1) has been constructed to test the therapeutic efficacy of this mAb in clinical trials. Heat-aggregates of CTA99 (or human polyclonal IgG1), mimicking immune complexes, readily bound hFcγRI *in vitro* (Supplementary Fig.S3C). CTA99 injections lead to a significant reduction in tumor load in hFcγRI^{tg} 5KO mice, pre-treated with anti-FcγRIV mAbs, that was abolished by hFcγRI blockade (Fig.7C). A significant reduction in tumor load following CTA99 injection and an abolition of this effect following hFcγRI blockade were also obtained in anti-FcγRIV mAbs pre-treated RAG-deficient hFcγRI^{tg} 5KO mice that cannot produce endogenous antibodies (Fig.7D). Furthermore, injections of a fully human IgG1 mAb anti-TYRP-1[38] had a trend to reduce tumor loads in hFcγRI^{tg} 5KO mice pre-treated with anti-FcγRIV mAbs (Fig.7E). hFcγRI therefore mediates Ab-induced reduction of tumor load in transgenic mice following injection of humanized anti-TYRP-1 mAbs.

DISCUSSION

Our work suggests that although hFcγRI is characterized as a high-affinity receptor for IgG, hFcγRI is readily available *in vivo* to bind IgG-immune complexes or IgG-opsonized targets. Despite its potential saturation by IgG *in vivo*, hFcγRI was indeed sufficient to mediate pro-inflammatory, pro-anaphylactic as well as anti-tumor functions, leading to autoimmune, allergic and therapeutic reactions, respectively, in transgenic mice. Neutrophils contributed predominantly to hFcγRI-induced anaphylaxis, whereas monocytes/macrophages contributed predominantly to hFcγRI-induced autoimmune thrombocytopenia. Both neutrophils and monocytes/macrophages were, however, required for hFcγRI-induced autoimmune arthritis demonstrating their non-redundant roles in this arthritis model. hFcγRI-expressing resident macrophages may attract circulating hFcγRI-expressing neutrophils that are responsible for inflammation and cartilage destruction in this arthritis model, as suggested from studies using wt mice[27,29].

To investigate the role of human FcγRI *in vivo*, we used transgenic mice for this receptor[30] that display an expression pattern of hFcγRI comparable to that found in humans. Monocytes, macrophages and dendritic cells in humans and in these transgenic mice indeed express hFcγRI. Noticeably, however, hFcγRI was reported to be inducible on human neutrophils whereas neutrophils from hFcγRI^{tg} mice constitutively express hFcγRI. Nevertheless, hFcγRI was reported to be expressed on human neutrophils under multiple circumstances including, in particular rheumatoid arthritis[22] and multiple myeloma[24]. One can therefore consider that human

neutrophils may express hFcγRI in most inflammatory contexts. To avoid a possible *in vivo* competition or contribution of endogenous FcγRs to reactions mediated by hFcγRI, we crossed hFcγRI-transgenic mice with 5KO mice that lack FcγRI, FcγRIIB, FcγRIII, FcεRI and FcεRII[7]. The resulting hFcγRI^{tg} 5KO mice express only two activating FcRs, transgenic hFcγRI and endogenous FcγRIV that could be efficiently blocked *in vivo* to study the specific contribution of hFcγRI to a particular disease or therapy model. The expression of the transgene in this background lead to an increased expression level of hFcγRI on neutrophils in transgenic mice compared to humans, but a very similar expression on monocytes. Testing anti-hFcγRI specific mAbs *in vivo* in these mice revealed an agonist/non-blocking activity (anti-hFcγRI.2 mAb) or an antagonist/blocking activity (anti-hFcγRI.1 mAb). hFcγRI bound not only human IgG1/3/4 subclasses[6] but also mouse IgG2a/2b subclasses as monomers. Importantly, the affinity of hFcγRI for mIgG2a was very similar to its affinity for hIgG1 (K_D≈38nM and 40nM, respectively), in the range of the high-affinity mIgG2a-mFcγRIV interaction (K_D≈34nM)[21]. hFcγRI thus functions as a high-affinity IgG receptor not only in humans but also in hFcγRI^{tg} mice. The fact that hFcγRI conserved its high-affinity properties also for mouse IgG validates hFcγRI^{tg} mice as a model to study the contribution of hFcγRI to disease and therapy.

In hFcγRI^{tg} mice, we found that the engagement of hFcγRI alone or of FcγRIV alone resulted in reactions with a lower intensity than following the engagement of both receptors. Because hFcγRI and FcγRIV associate with the same FcRγ-subunit to mediate signal transduction, their aggregation by ICs should not lead to qualitatively different responses. Insufficient expression levels or occupancy of a proportion of these high-affinity receptors by endogenous (monomeric) IgG2 may, however, explain this phenomenon. The latter possibility,

evoked previously[39], certainly dissuaded many to investigate the role of hFcγRI in IgG-mediated effector reactions *in vivo*. Importantly, we demonstrate here that hFcγRI can readily induce inflammatory reactions following passive administration of pathogenic IgG in spite of its ability to be bound/saturated by endogenous monomeric IgG. In addition, the intensity and kinetic of the responses triggered by hFcγRI were comparable to those reported when triggered by low-affinity FcRs. Supporting our observations, mouse high-affinity FcRs, FcγRI and FcγRIV, were reported to play similar roles as mouse low-affinity FcγRIII in models of inflammation[18,20,28]. Finally, we observed no difference in the kinetic of appearance of hFcγRI-dependent arthritic symptoms, nor in their severity, between IgG-sufficient (hFcγRI^{tg} 5KO) and IgG-deficient (RAG-deficient hFcγRI^{tg} 5KO) mice.

Altogether these reports, including ours, support the notion that being of high or of low affinity for IgG, FcγRs engaged by a given multivalent ligand and expressed by a given cell will induce with comparable kinetics the activation of that cell and consequently *in vivo* responses. It follows that the ability of high-affinity FcγRs to bind monomeric IgG has no detectable consequence *in vivo*. One could therefore consider that high-affinity FcγRs remain as unoccupied as low-affinity FcγRs *in vivo*. Nevertheless, the high concentration of circulating IgG favors the hypothesis that at any given time a proportion of high-affinity, but also of low-affinity, FcγRs are interacting with IgG. Low-affinity and high-affinity FcγRs were indeed reported to bind monomeric IgG with a half-life of the interaction varying from less than 1 minute to more than 10 minutes[7,19,21,40], respectively. In line with these previous results, we report here a ≈4 minute half-life for the interaction of hFcγRI with hIgG1 or with mIgG2a. Results obtained *in vivo* nevertheless suggest that these half-lives are sufficiently short to allow low- and high-affinity FcγRs to rapidly bind IgG-immune complexes and to induce cell activation.

We unexpectedly found that hFcγRI can induce several allergy-related reactions in hFcγRI^{tg} mice. In the model of airway inflammation, hFcγRI triggered neutrophil infiltration, hemorrhage and MPO production in the alveolar space, symptoms that are reminiscent with those found in patients. Whereas this model has been reported to be macrophage-dependent, we could not formally demonstrate the contribution of these cells to hFcγRI-induced airway inflammation due to inefficient depletion of alveolar macrophages. Nevertheless, the fact that alveolar macrophages represent more than 90-95% of the cells in the BAL of unchallenged mice and that they express hFcγRI supports a role for alveolar macrophages in this reaction. hFcγRI was also able to induce passive systemic anaphylaxis when triggered by divalent or multivalent ligands, as well as ASA. Similarly as ASA in wt mice[20], hFcγRI-induced ASA relied predominantly on neutrophils and PAF. Surprisingly, whereas monocytes/macrophages were reported to contribute predominantly to human FcγRIIA-induced systemic anaphylaxis[2] and to particular models of passive and active anaphylaxis[32], monocytes/macrophages did not significantly contribute to anaphylaxis in hFcγRI^{tg} mice. Whereas it has been reported that hFcγRI is expressed on *in vitro*-stimulated human cord blood-derived mast cells[41], it has not been reported on human skin mast cells[42] or mast cells from hFcγRI^{tg} mice (this report). Whatever the relative contribution of these cell subsets to allergic and anaphylactic reactions in humans, our results suggest that hFcγRI may be a key player in allergic and anaphylactic reactions in humans when allergen-specific IgG are present.

hFcγRI has been reported to allow antigen targeting to dendritic cells to enhance antigen presentation[23] and we report here that hFcγRI contributes to the induction of several

inflammatory models in hFcγRI^{tg} mice. The mouse homolog of FcγRI, mFcγRI, is also expressed on subsets of dendritic cells[9,10] and has been reported to play similar roles than hFcγRI in enhancing antigen presentation of IgG-bound antigen[13]. We and others[9,17] could not, however, detect mFcγRI on circulating monocytes, macrophage subsets nor on neutrophils neither in steady state nor during inflammatory arthritis or in tumor-bearing mice (data not shown). The absence of mFcγRI on these effector cells suggest that its main activity may be to favor antigen presentation by and activation of dendritic cells, in agreement with its contributions reported following active immunization protocols[12,13]. Passive models of disease using mFcγRI$^{-/-}$ mice nevertheless reported an effect of mFcγRI deficiency in immune complex-induced Arthus reactions in the footpad[13] and in Ab-induced autoimmune hemolytic anemia[12,15]. mFcγRI may therefore be a functional homolog of hFcγRI when considering dendritic cells only. When considering circulating monocytes, macrophages and neutrophils, however, mFcγRIV that does not exist in humans may be a functional homolog of hFcγRI. Like hFcγRI (this report), mFcγRIV is indeed expressed on these cell subsets[7,21] and was reported to contribute to anaphylaxis[20], arthritis[17], airway inflammation[25] and thrombocytopenia[19,20]. We therefore propose that hFcγRI may recapitulate in humans the roles played in mice by mFcγRI on dendritic cells to favor antigen presentation and cell activation, and by mFcγRIV on monocytes/macrophages and neutrophils to trigger effector (pro-inflammatory) reactions.

The model of B16 metastatic melanoma has been extensively used to study the contribution of FcRs to experimental antibody-based immunotherapy. Using a bioluminescent variant of B16 and either the mouse IgG2a anti-TYRP-1 mAb TA99 or its humanized version CTA99 bearing the constant regions of a human IgG1, we report here that hFcγRI can mediate

antibody-based immunotherapy. hFcγRI may thus contribute to (or be responsible for) the reduction of B16 metastatic melanoma recently observed in mice expressing multiple hFcγRs injected with a humanized anti-TYRP-1 mAb TA99[43]. Furthermore, we demonstrated that hFcγRI could mediate the protective effect of a fully human IgG1 anti-TYRP-1 mAb, currently evaluated in a Phase I trial involving patients suffering from malignant melanoma. CTA99 and fully human anti-TYRP-1 were, however, less efficient than TA99 in the mouse model of metastatic melanoma. In mice and humans, the neonatal IgG receptor FcRn is responsible for the protection of IgG degradation and contributes to IgG distribution into tissues[44]. Noticeably, the binding of human IgG1 to mouse FcRn is almost 3-times lower than the binding of mouse IgG2a to mouse FcRn[45]. Consequently, the half-life and/or bio-distribution of human IgG1 may be reduced compared to that of mouse IgG2a when injected in mice, thus suggesting a reduced opsonization and elimination of tumor cells. The mechanism by which hFcγRI mediates the protective effect of anti-TYRP-1 mouse mAb TA99, humanized IgG1 mAb CTA99 and fully human IgG1 mAb on metastatic melanoma remains to be identified, but should not require NK cells as these cells do not express hFcγRI. Myeloid cells however, and among them macrophages in particular, might be responsible for metastasis reduction in this model. Intriguingly, in the absence of all other FcγR, mouse FcγRIV was not sufficient to mediate TA99-based tumor immunotherapy whereas its absence (FcγRIV$^{-/-}$ mice) or *in vivo* blockade in wt mice (anti-FcγRIV mAbs) have been reported to reduce the efficiency of TA99 in this model[19,46]. The expression of hFcγRI was, however, sufficient to restore antibody-based tumor immunotherapy in mice that could not mediate this property anymore. This property of hFcγRI is reminiscent of that found for its mouse homolog mFcγRI to mediate the protective effects of anti-TYRP-1 mAb TA99 on B16 liver metastases[47].

Most current preclinical studies based on hFcγRI only exploit its functions in favoring antigen presentation[48-50]. In addition to these properties, we report here that hFcγRI can also mediate the protective effects of anti-tumor antibodies on melanoma metastases, and therefore potentially also on solid tumors. Supporting this assumption, bi-specific antibodies directed against hFcγRI and c-erbB-2, a transmembrane receptor highly expressed in several human malignancies, indeed trigger hFcγRI-dependent antibody-dependent cell cytotoxicity *in vitro*[48-50]. We also report here that hFcγRI can induce several mouse models of autoimmune and allergic reactions, and can therefore be considered as a potential pro-inflammatory and pro-anaphylactic activating IgG receptor in humans. Anti-hFcγRI blocking mAbs prevented hFcγRI-dependent models of autoimmunity and allergy, and may thus be assessed for their efficiency in human pathologies. Finally our results indicate that hFcγRI, and potentially other high-affinity FcRs, are either not occupied/saturated by IgG *in vivo* or if they are, this comes without functional consequence on their ability to mediate anti-tumor activities and pro-inflammatory and pro-anaphylactic properties.

METHODS

Flow cytometry analysis

Blood cells populations were defined as follows: Mouse B cells ($CD19^+$), T cells ($CD3^+$), monocytes/macrophages (blood/peritoneum: $CD11b^+/Gr1^-$; BAL: $CD11c^+/Gr1^-$), neutrophils ($Gr1^+/SiglecF^-$), basophils ($IgE^+/DX5^+$), eosinophils ($Gr1^{int}/SiglecF^+$), mast cells ($IgE^+/CD117^+$), platelets ($DX5^+/CD61^+$) and NK cells ($NK1.1^+/DX5^+$); Human B cells ($CD19^+$), T cells ($CD3^+$), NK cells ($CD56^+$), monocytes ($CD14^+$) neutrophils ($CD24^+$), basophils ($CD123^+/CD203c^+$), eosinophils ($CD24^+/CD193^+$). Expression of different Flag-tagged FcRs in CHO-K1 cells was compared using anti-FLAG antibody.

Immune complex binding: CHO-K1 cells were incubated with preformed ICs made of 10µg/ml TNP_5-BSA-biotin and 15µg/ml anti-TNP mAbs, for 1h at 4°C. Bound ICs were detected using PE-conjugated neutravidin at 2µg/ml, for 30min at 4°C. *Monomeric Ig binding assays:* CHO-K1 cells were incubated with 10µg/ml monomeric mIgG or rabbit IgG for 1h at 4°C. Cell-bound Ig was detected using 5µg/ml PE-labeled $F(ab')_2$ fragments of anti-mouse $F(ab')_2$-specific or 15µg/ml FITC-conjugated $F(ab')_2$ anti-rabbit Ig, respectively, for 30min at 4°C.

Airway inflammation

Mice were injected intranasally with 20µl of rabbit anti-OVA antiserum and i.v. with 500µg OVA. After 18h, mice were lethally anesthetized and four broncho-alveolar lavages of respectively 0.5, 1, 1 and 1ml PBS were performed. The supernatant of the first lavage was used to quantify MPO content. The cells from all lavages were pooled for cell count analysis.

Hemorrhage was determined in the cell-free supernatant of pooled lavages after RBC lysis by optical density measurement (570nm).

Anaphylaxis

PSA: Immune complexes made of 80µg GPI and 200µl anti-GPI containing serum (K/BxN serum) in 300µl physiological solution were preformed at 37°C and injected i.v. Alternatively, 10 to 200µg of antagonistic blocking anti-hFcγRI.1 or agonistic non-blocking anti-hFcγRI.2 mAbs was injected i.v. Central body temperature was recorded using a digital thermometer (YSI).

ASA: Mice were injected i.p. on day 0 with 200µg BSA in CFA and boosted i.p. on day 14 and day 28 with 200µg BSA in IFA. BSA-specific IgG1, IgG2a/b/c and IgE antibodies in serum were titered by ELISA on day 30 as described[20]. Mice with comparable antibody titers were challenged i.v. with 500µg BSA, 8 days after the last immunization. Central temperature was monitored.

Lung metastases model

$1x10^6$ B16-Luc2$^+$ cells were injected i.v. on day 0, and anti-TYRP-1 mAbs TA99 (200µg), CTA99 (500µg) or human IgG1 anti-TYRP-1 (500µg) i.p. on day 0, 1, 2, 4, 7 and 9. Shaved and anesthetized mice were injected i.p. with 3mg luciferin 5min before, or explanted lungs were exposed to 50µL at 15mg/mL luciferin 2min before bioluminescence acquisition on an IVIS 100 (Caliper LifeSciences), using 5min exposure times with medium binning. Total photon flux (photons/seconds) of the entire lung was calculated using Living Image software.

Please refer to supplemental Methods for information on mice; reagents; *in vivo* blocking and depletion; K/BxN serum-induced passive arthritis (K/BxN PA); Experimental

thrombocytopenia (ITP); Surface Plasmon Resonance analysis; Statistical analyses.

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AUTHORSHIP AND CONFLICT OF INTEREST STATEMENTS

D.A.M. performed all experiments except tumor experiments that were performed by M.A.; F.J. contributed to several experiments; B.I. genotyped mice and produced reagents; N.v.R. an X.K. provided reagents; P.E. designed and analyzed Surface Plasmon Resonance experiments; P.B., D.A.M. and M.A. analyzed and discussed results; P.B. and D.A.M. wrote the manuscript; P.B. designed and supervised the research.

All authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. hFcγRI conserves its properties as a high-affinity IgG receptor in transgenic mice. (A-B) Representative histogram plots of hFcγRI expression on indicated cell populations from (A) blood or tissues of hFcγRI^{tg} 5KO mice or (B) blood of normal human donors (two representative histogram plots from two different donors (#1 and #2) are represented for hFcγRI expression on neutrophils). (C) Histograms show the expression of the respective FcγRs (FLAG), or the binding of indicated mouse monomeric IgG to FLAG-tagged FcγR^{+} CHO transfectants. Solid gray histograms represent the binding of secondary Abs alone. (D) Histograms show the expression of the respective FcγRs (FLAG), or the binding of indicated IgG ICs (black line) or Ag alone (solid gray histograms) to FcγR^{+} CHO transfectants, as revealed by neutravidin staining. *Note: the use of different secondary reagents to detect monomeric IgG- (C) or IC-binding (D) prevents comparing fluorescence intensities between histograms in C and D.* (E,F) Real-time SPR sensorgrams and affinity constants were determined from SPR analysis. (E) Data correspond to the injection of 125nM of hIgG1 (black) or of mIgG2a (grey) onto immobilized hFcγRI. (F) Kinetic parameters determined from experiments presented in Fig.1E and in Supplemental Fig.S1B. (A-F) Data are representative of at least two independent experiments.

Figure 2. hFcγRI can trigger inflammatory Arthritis in transgenic mice. (A-C) K/BxN PA in indicated mice injected with indicated mAbs (A,B, n=3; C, n=4). (D) Arthritis induced in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice by K/BxN serum (n=4) or 80μg of purified K/BxN IgG1 (n=3) or of purified K/BxN IgG2 (n=4). (E) K/BxN PA in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice injected with indicated liposomes (n=3) or mAbs (n=4). (A-E) Data are representative from

at least two independent experiments and are represented as mean ± SEM. Statistical differences (B-D) between curves or (A,E) for each time point are indicated (A, between [anti-FcγRIV] and [anti-FcγRIV+anti-hFcγRI.1]-treated groups).

Figure 3. hFcγRI can trigger IC-induced airway inflammation in transgenic mice. (A,B) Neutrophil (A) count and (B) percentage among leukocytes, (C,D) alveolar macrophage (C) count and (D) percentage among leukocytes, (E) MPO level and (F) hemorrhage score, in BAL from hFcγRI^{tg} 5KO mice following injection of indicated reagents. IC stands for OVA injected i.v. followed by anti-OVA antiserum injected i.n. (n=4 in all groups). (A-F) Data are representative from at least two independent experiments and represented as mean ± SEM.

Figure 4. *In vivo* aggregation of hFcγRI induces passive systemic anaphylaxis. (A,B) Indicated mice were injected with (A) 200µg of anti-hFcγRI.1 blocking mAb or anti-hFcγRI.2 non-blocking mAb, or (B) with indicated amounts of anti-hFcγRI.2 non-blocking mAb, and central temperatures were monitored (n≥3). The same curve corresponding to 200µg anti-hFcγRI.2 non-blocking mAb injected in hFcγRI^{tg} 5KO mice is represented in experiments A and B that were performed together. *Note:* anti-hFcγRI.1 mAb is an antagonistic blocking antibody and anti-hFcγRI.2 mAb an agonistic non-blocking antibody. (C,D) 5KO and/or hFcγRI^{tg} 5KO mice were pretreated with indicated reagents and injected with preformed mouse IC made of mouse polyclonal anti-GPI serum and GPI, and central temperatures were monitored (C, n≥4; D, n≥3). (A-D) Data are representative from at least two independent experiments and represented as mean ± SEM (D: between [unteated] and [anti-FcγRIV+anti-hFcγRI.1]-treated groups).

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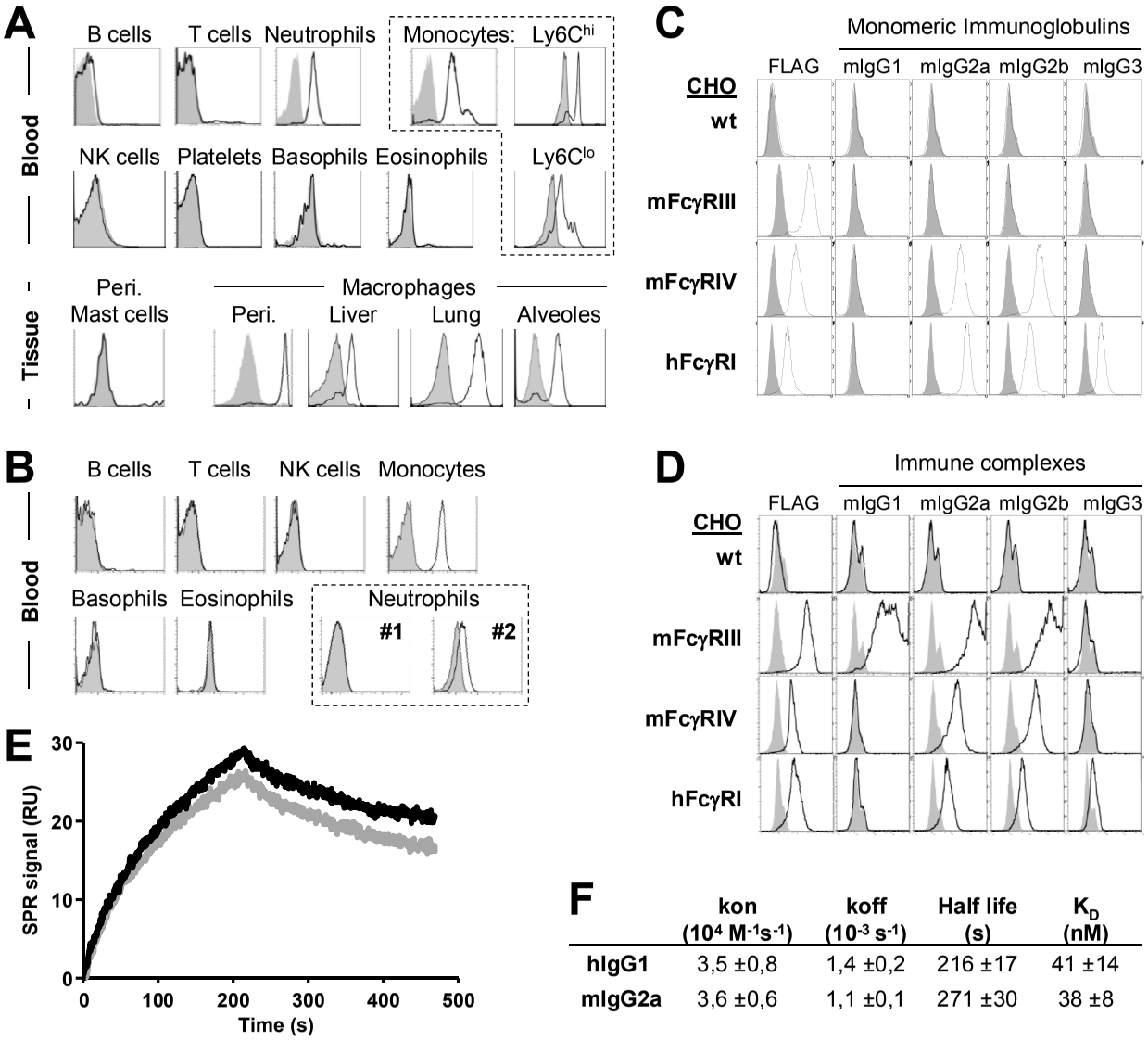
Figure 5. Neutrophils are necessary for hFcγRI-dependent active systemic anaphylaxis. Indicated mice were immunized with BSA in Freund's adjuvant, challenged with BSA and central temperatures and survival rates were monitored. (A,B) ASA in hFcγRI^{tg} 5KO and/or 5KO mice injected with indicated reagents (n=5). (C-F) ASA in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice injected with indicated reagents (C, n≥4; D, n=5; E, n=5; F, n≥3). (A-F) Data are representative from at least two independent experiments and represented as mean ± SEM. Abbreviations: toxic liposomes (Cld2 lipo.); gadolinium chloride (GdCl3); cyproheptadine (Cypro.)

Figure 6. Macrophages are necessary for hFcγRI-dependent thrombocytopenia. (A) hFcγRI^{tg} 5KO (black) or 5KO (gray) mice were pretreated with indicated reagents before being injected i.v. with anti-platelet mAb (α-PLA). Platelet counts were acquired in blood at (left) indicated times presented as curves or (right) at t=4 hours presented as histograms, following α-PLA injection (n=3). (B) hFcγRI^{tg} 5KO mice were pretreated with indicated reagents and platelet counts acquired in blood at t=4 hours following α-PLA injection (n=3). (C-E) 5KO mice (small histograms in inserts) or anti-FcγRIV-treated hFcγRI^{tg} 5KO mice (large histograms, left in each panel) were pretreated with indicated reagents or splenectomized when indicated, and platelet counts acquired in blood at t=4 hours following α-PLA injection (C, D: n=3; E: n≥3). (A-E) Data are representative from at least two independent experiments and represented as mean ± SEM.

Figure 7. hFcγRI can mediate antibody-dependent protection from metastatic melanoma in transgenic mice. Indicated mice were injected i.v. with B16 luc2^{+} cells and injected with anti-

TYRP-1 mAbs (TA99 or CTA99 or human anti-TYRP-1) when indicated, and (C-E) also pretreated with anti-FcγRIV mAbs. (A-E) Quantification of tumor load was performed on *ex vivo* explanted lungs on day 11 after injection of B16 luc2^{+} cells (A, n≥5; B, n≥5; C, n≥4; D, n≥4; E, n≥4). (A-E) Data are representative from at least two independent experiments and represented as mean ± SEM.



F

	kon (10^4 $M^{-1}s^{-1}$)	koff (10^{-3} s^{-1})	Half life (s)	K_D (nM)
hIgG1	3,5 ±0,8	1,4 ±0,2	216 ±17	41 ±14
mIgG2a	3,6 ±0,6	1,1 ±0,1	271 ±30	38 ±8

Figure 1

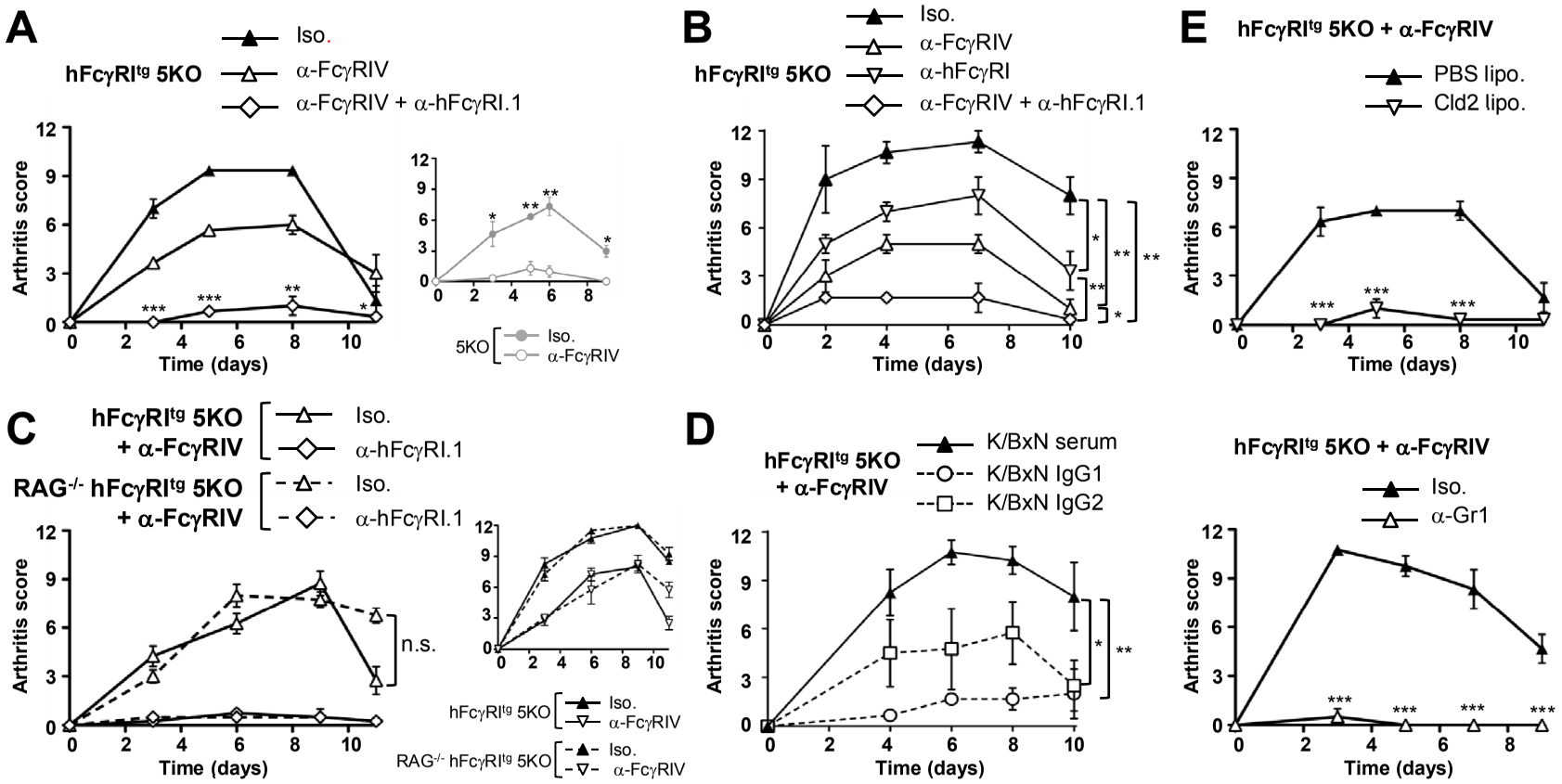

A
hFcγRI^tg 5KO
Iso.
α-FcγRIV
α-FcγRIV + α-hFcγRI.1
Arthritis score
Time (days)
5KO
Iso.
α-FcγRIV
B
hFcγRI^tg 5KO
Iso.
α-FcγRIV
α-hFcγRI
α-FcγRIV + α-hFcγRI.1
Arthritis score
Time (days)
E
hFcγRI^tg 5KO + α-FcγRIV
PBS lipo.
Cld2 lipo.
Arthritis score
Time (days)
C
hFcγRI^tg 5KO + α-FcγRIV
Iso.
α-hFcγRI.1
RAG^-/- hFcγRI^tg 5KO + α-FcγRIV
Iso.
α-hFcγRI.1
n.s.
Arthritis score
Time (days)
hFcγRI^tg 5KO
Iso.
α-FcγRIV
RAG^-/- hFcγRI^tg 5KO
Iso.
α-FcγRIV
D
hFcγRI^tg 5KO + α-FcγRIV
K/BxN serum
K/BxN IgG1
K/BxN IgG2
Arthritis score
Time (days)
hFcγRI^tg 5KO + α-FcγRIV
Iso.
α-Gr1
Arthritis score
Time (days)

Figure 2

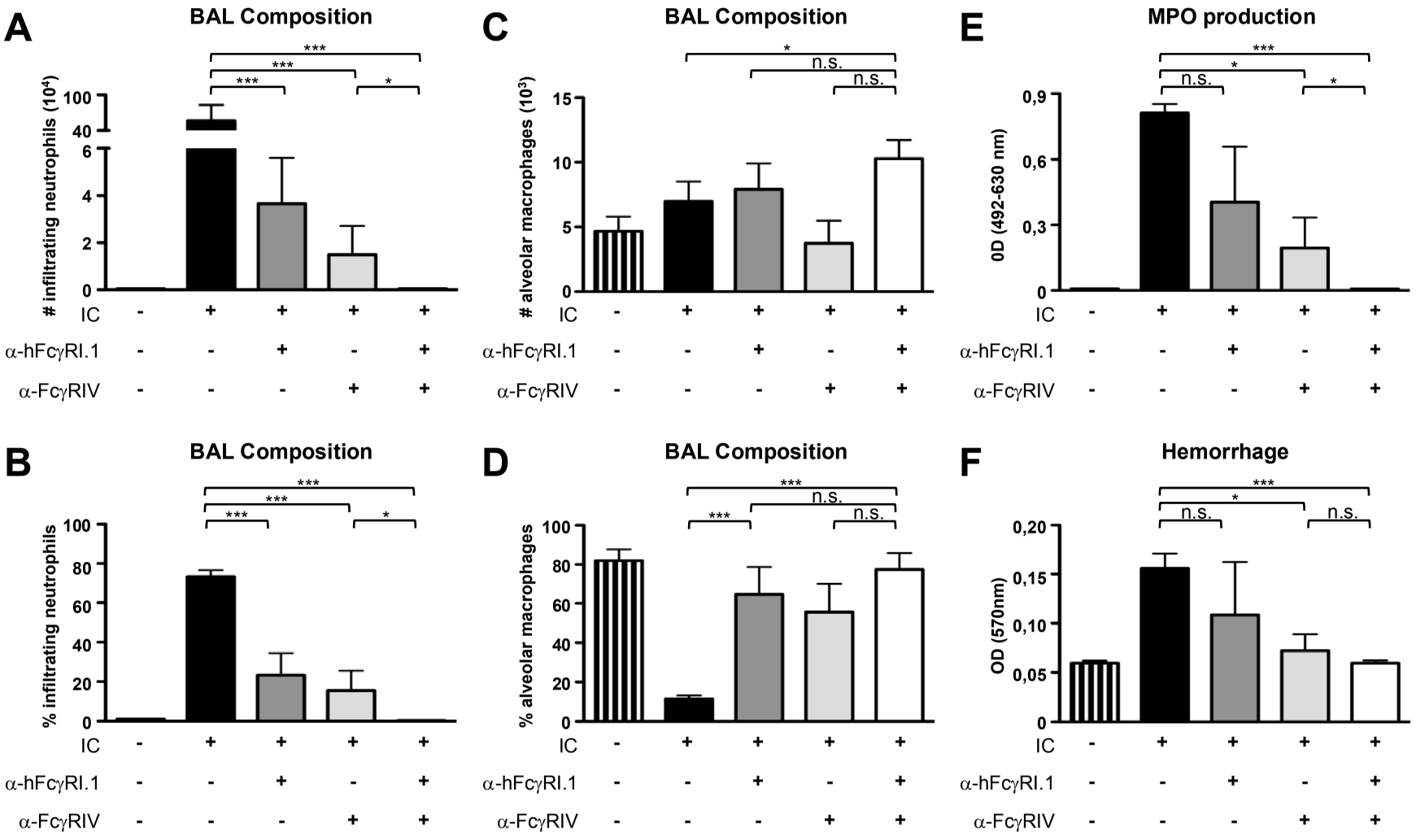
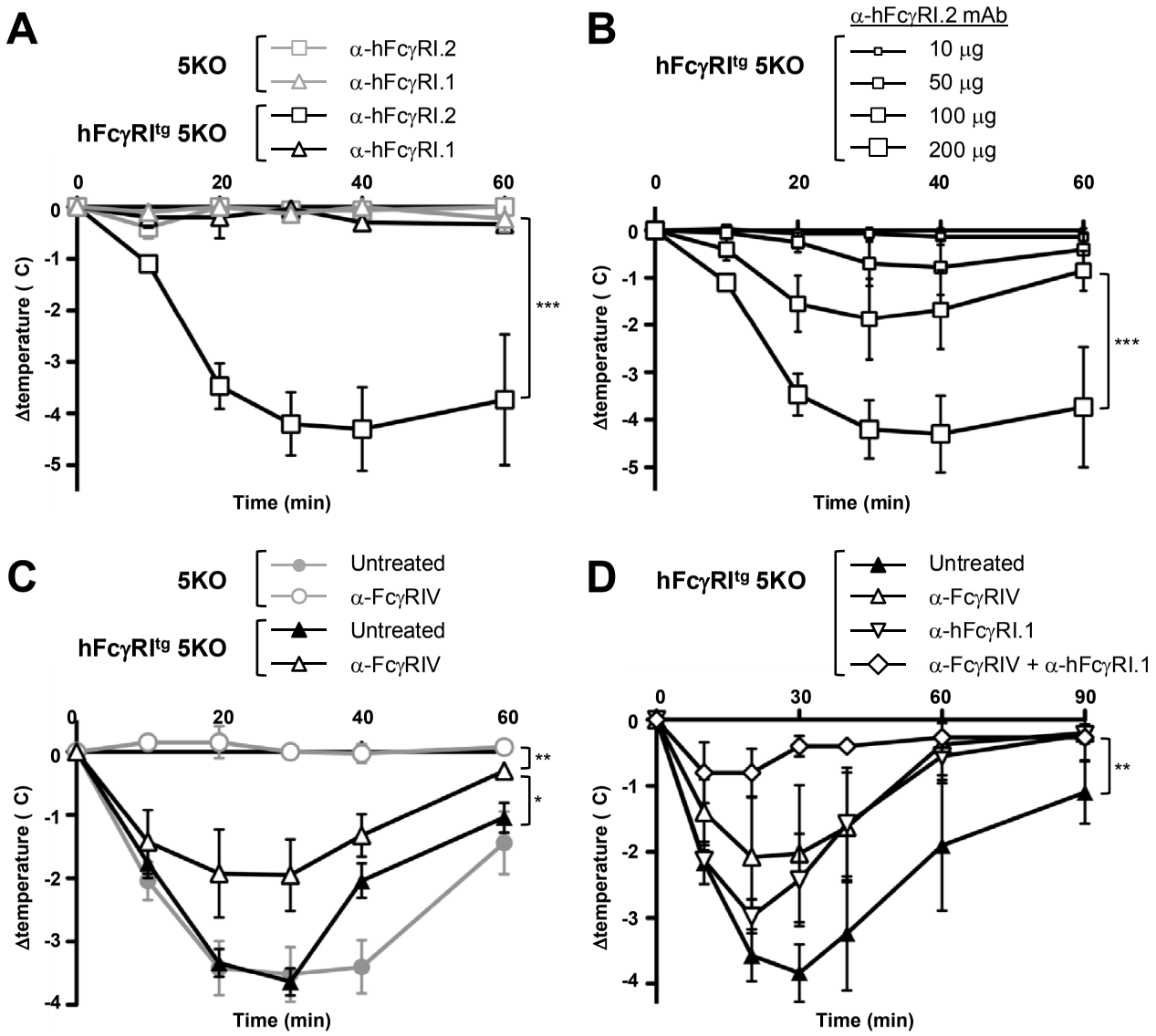

A
BAL Composition
infiltrating neutrophils (10⁴)
100
40
6
4
2
0
***
***
***
*
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +
B
BAL Composition
% infiltrating neutrophils
100
80
60
40
20
0
***
***
***
*
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +
C
BAL Composition
alveolar macrophages (10³)
15
10
5
0
*
n.s.
n.s.
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +
D
BAL Composition
% alveolar macrophages
100
80
60
40
20
0
***
***
n.s.
n.s.
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +
E
MPO production
0D (492-630 nm)
0,9
0,6
0,3
0
***
*
n.s.
*
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +
F
Hemorrhage
OD (570nm)
0,20
0,15
0,10
0,05
0
***
*
n.s.
n.s.
IC - + + + +
α-hFcγRI.1 - - + - +
α-FcγRIV - - - + +

Figure 3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5KO
α-hFcγRI.2
α-hFcγRI.1
hFcγRItg 5KO
α-hFcγRI.2
α-hFcγRI.1
Δtemperature (C)
Time (min)
***
B
α-hFcγRI.2 mAb
hFcγRItg 5KO
10 μg
50 μg
100 μg
200 μg
Δtemperature (C)
Time (min)
***
C
5KO
Untreated
α-FcγRIV
hFcγRItg 5KO
Untreated
α-FcγRIV
Δtemperature (C)
Time (min)
**
*
D
hFcγRItg 5KO
Untreated
α-FcγRIV
α-hFcγRI.1
α-FcγRIV + α-hFcγRI.1
Δtemperature (C)
Time (min)
**

Figure 4

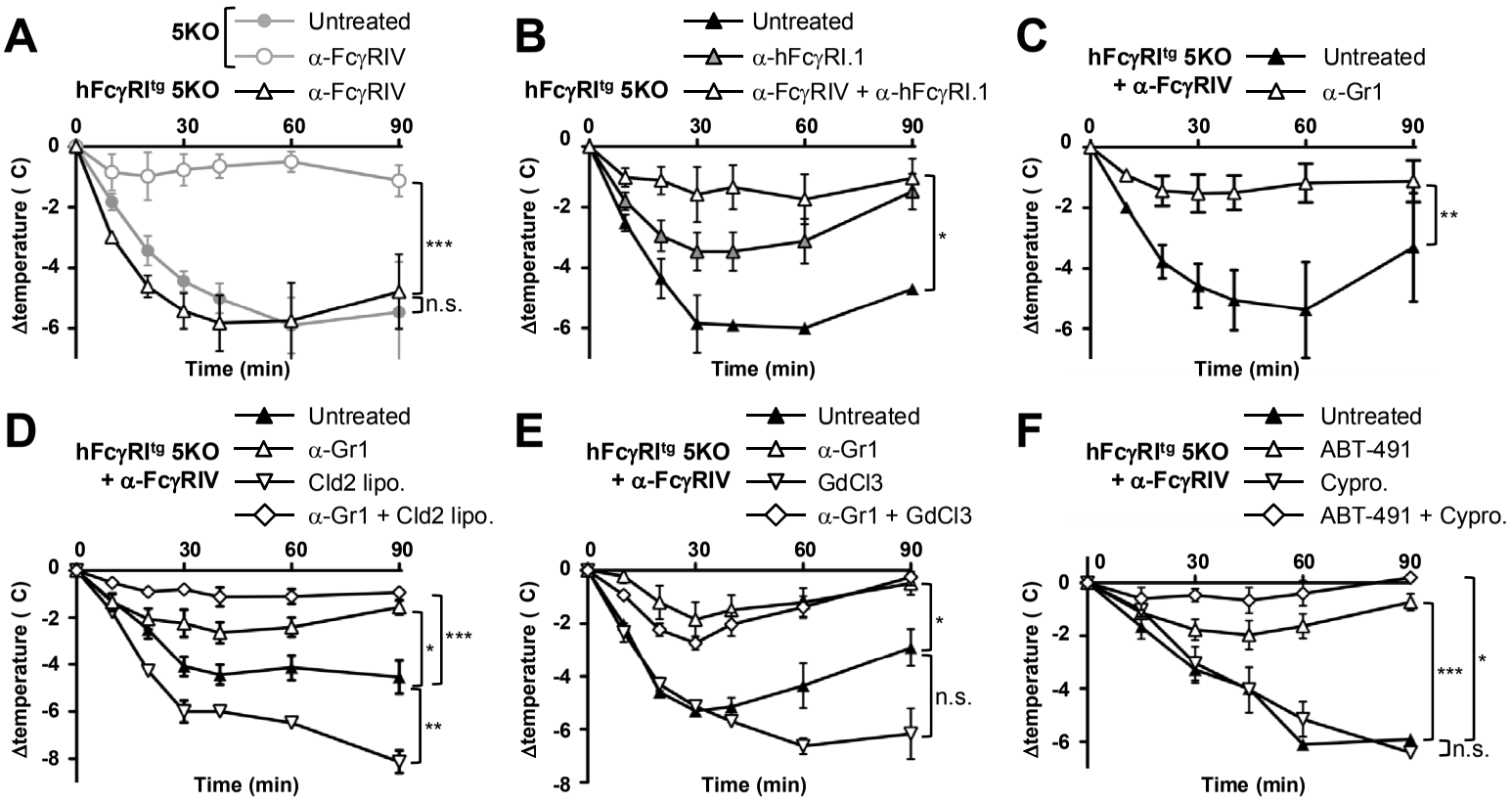

A
5KO
Untreated
α-FcγRIV
hFcγRI^tg 5KO
α-FcγRIV
Δtemperature (C)
Time (min)
***
n.s.
B
Untreated
α-hFcγRI.1
hFcγRI^tg 5KO
α-FcγRIV + α-hFcγRI.1
*
C
hFcγRI^tg 5KO
+ α-FcγRIV
Untreated
α-Gr1
**
D
Untreated
hFcγRI^tg 5KO
+ α-FcγRIV
α-Gr1
Cld2 lipo.
α-Gr1 + Cld2 lipo.
*
***
**
E
Untreated
hFcγRI^tg 5KO
+ α-FcγRIV
α-Gr1
GdCl3
α-Gr1 + GdCl3
*
n.s.
F
Untreated
hFcγRI^tg 5KO
+ α-FcγRIV
ABT-491
Cypro.
ABT-491 + Cypro.
***
*
n.s.

Figure 5

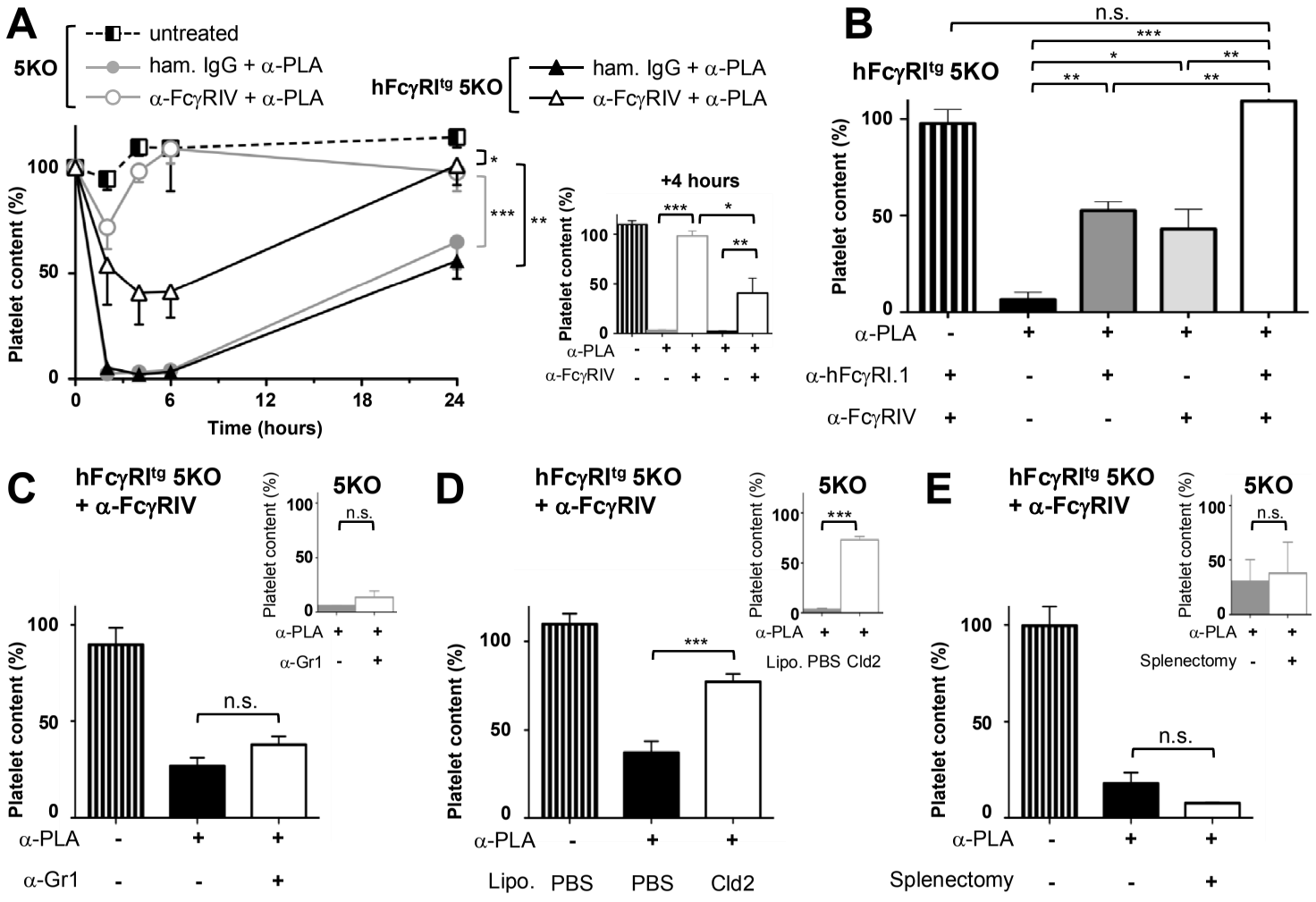

A
5KO
untreated
ham. IgG + α-PLA
α-FcγRIV + α-PLA
hFcγRItg 5KO
ham. IgG + α-PLA
α-FcγRIV + α-PLA
Platelet content (%)
Time (hours)
+4 hours
α-PLA
α-FcγRIV
B
hFcγRItg 5KO
n.s.
α-PLA
α-hFcγRI.1
α-FcγRIV
C
hFcγRItg 5KO
+ α-FcγRIV
5KO
n.s.
α-PLA
α-Gr1
D
hFcγRItg 5KO
+ α-FcγRIV
5KO
Lipo. PBS Cld2
Lipo. PBS PBS Cld2
E
hFcγRItg 5KO
+ α-FcγRIV
5KO
Splenectomy

Figure 6

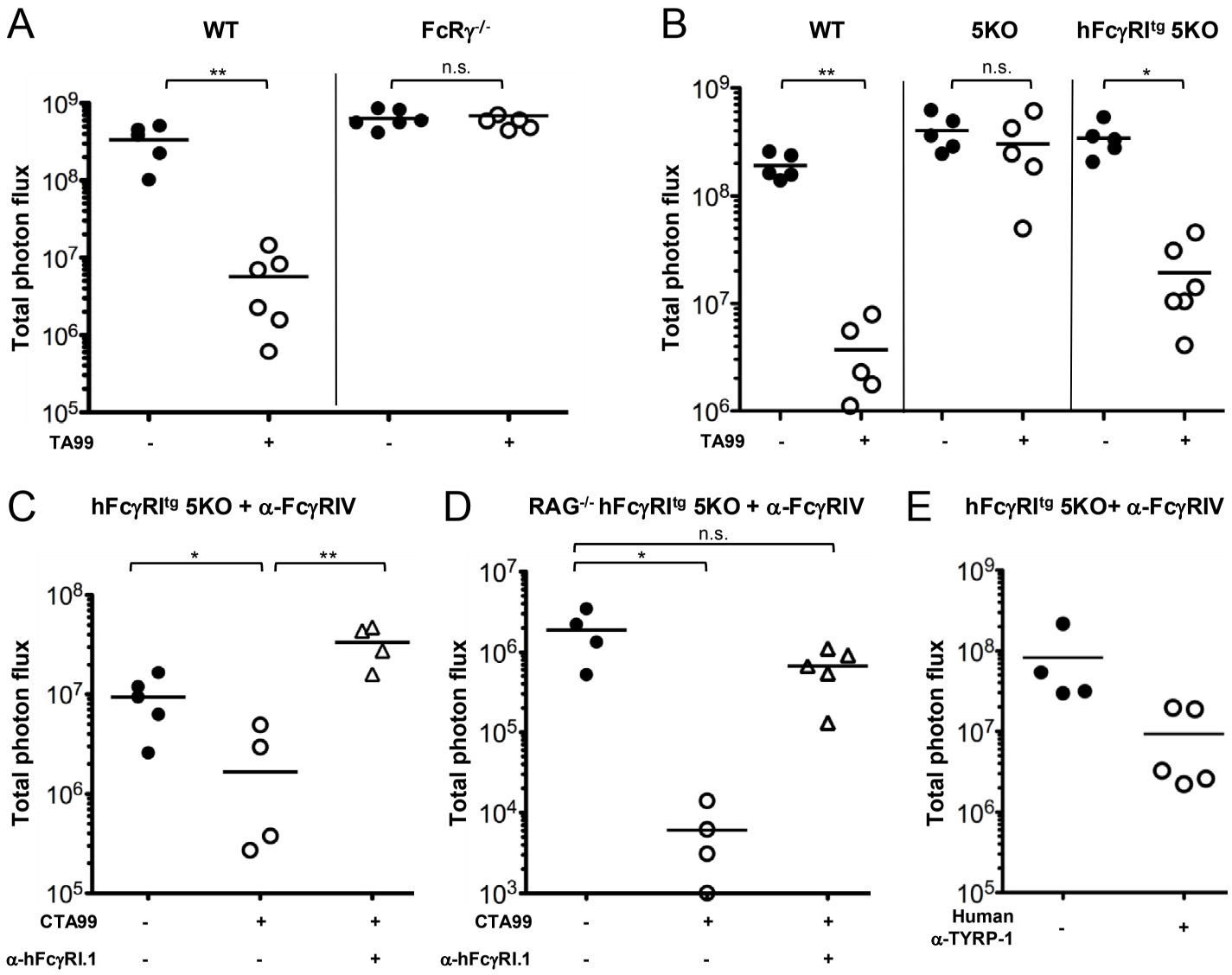

A
WT
FcRγ-/-
**
n.s.
Total photon flux
TA99
B
WT
5KO
hFcγRItg 5KO
**
n.s.
*
Total photon flux
TA99
C
hFcγRItg 5KO + α-FcγRIV
*
**
Total photon flux
CTA99
α-hFcγRI.1
D
RAG-/- hFcγRItg 5KO + α-FcγRIV
n.s.
*
Total photon flux
CTA99
α-hFcγRI.1
E
hFcγRItg 5KO+ α-FcγRIV
Total photon flux
Human α-TYRP-1

Figure 7

SUPPLEMENTAL METHODS

Mice

FcγRI/IIB/IIIA$^{-/-}$ FcεRI/II$^{-/-}$ (5KO) mice have been described[1]. hFcγRI^{tg} mice were obtained from J.G.J. van de Winkel (UMCU, Utrecht, The Netherlands), crossed to 5KO mice to obtain hFcγRI^{tg} 5KO mice. These mice were further crossed to RAG$^{-/-}$ mice to generate RAG$^{-/-}$ hFcγRI^{tg} 5KO mice. All mice carrying the hFcγRI transgene were used as heterozygous animals and non-transgenic littermates served as controls. KRN^{tg} mice were provided by D. Mathis, C. Benoist (HMS, Boston, MA, USA), and IGBMC (Strasbourg, France). Mice used in experiments were on C57BL/6J background (6th-12th generation backcross). Wt mice were purchased from Charles River. All mouse protocols were approved by the Animal Care and Use Committees of Paris, Ile de France, France.

Reagents

Anti-mouse CD11b, CD11c, CD3, CD19, Gr1, SiglecF, CD117, DX5, CD61, NK1.1, IgE and labeled anti-hFcγRI were from BD Biosciences; mouse IgG3 anti-DNP from Serotec; recombinant soluble C-terminal polyhistidine-tagged hFcγRI ectodomains from R&D Systems; human IgG1 from The Binding Site; HRP-coupled anti-mouse IgG subclasses from Southern Biotechnology; anti-FLAG mAbs, OVA, BSA, rabbit GPI, rabbit anti-ova antiserum, gadolinium-(III)-chloride, Freund's adjuvant, ABT-491, cyproheptadine from Sigma-Aldrich; MPO ELISA kit from HyCult Biotech; Cobra Venom Factor from Quidel Corporation. IgG were purified by Protein G-affinity purification from supernatants of hybridomas producing anti-hFcγRI.1 mAb, anti-TYRP-1 (gp75) mAb (clone TA99), anti-mFcγRIV mAb (clone 9E9)

provided by J.V. Ravetch (Rockefeller University, New York, NY, USA), anti-Gr1 mAb (clone RB6-8C5) provided by R. Coffman (DNAX, Palo Alto, CA, USA), anti-DNP mIgG1, mIgG2a and mIgG2b provided by B. Heyman (Uppsala Universitet, Uppsala, Sweden) and mIgG2a anti–platelet mAb (clone 6A6) provided by Dr R. Good (USFCM, Tampa, FL, USA). Purified anti-hFcγRI.2 mAb (clone 10.1) was provided by N. Hogg (CRUK, London, UK), purified humanized anti-TYRP-1 mAb (clone CTA99; humanized IgG1 Fc variant of the mouse mAb TA99) and purified fully human IgG1 anti-TYRP-1 mAb by Imclone (New York, NY, USA. PBS-liposomes and Clodronate-liposomes were prepared as published[2]. CHO K1 cells stably transfected with FLAG-tagged mouse FcγRs[1] or human FLAG-tagged FcγRs[3] were cultured as described. The wt B16F10 mouse melanoma cell line was obtained from the National Cancer Institute (Frederick, MD) and the luciferase-expressing variant (B16-luc2^{+}) from Caliper Lifesciences.

Anti-GPI IgG were purified from K/BxN serum using Protein G, polyclonal mIgG1 and mIgG2 fractions using anti-mIgG1 or anti-mIgG2 sepharose beads (Nordic Immunology). Mouse IgG subclasses were determined by ELISA; IgG1, IgG2a and IgG2b anti-GPI mAbs obtained in collaboration with the Antibody Production Platform (Institut Pasteur, Paris, France) were used as standards.

***In vivo* blocking and depletion**

200μg/mouse of anti-FcγRIV or anti-hFcγRI.1 blocking mAbs were injected i.v. once 30 min before the beginning of the experiment, except for arthritis and B16 melanoma assay, were blocking antibodies were injected every second day.

500μg/mouse anti-Gr1 mAbs, 300μl/mouse PBS- or clodronate-liposomes (at 2,1mg/ mouse), 1mg/mouse $GdCl_3$, 3μg/mouse Cobra Venom Factor (CVF) were injected i.v. 24 hours before the

beginning of the experiment, except for arthritis were anti-Gr1 mAbs and liposomes were injected every second day. Depletion of specific populations was ascertained using flow cytometry on blood samples taken during or after the experiment (data not shown).

ABT-49 (25μg/mouse) or cyproheptadine (50μg/mouse) were injected i.v. 20 or i.p. 30 min before challenge, respectively.

K/BxN serum-induced passive arthritis (K/BxN PA)

K/BxN serum was generated. Arthritis was induced by an intravenous injection of 150μL of K/BxN serum and arthritis was scored as described[4].

Experimental thrombocytopenia (ITP)

Blood samples were taken retro-orbitally before, and at indicated time points after the i.v. injection of 5μg of anti-platelet mAb 6A6. Platelet counts were determined using an ABC Vet automatic blood analyzer (Horiba ABX).

Surface Plasmon Resonance analysis

A BIAcore 2000 SPR biosensor (GE Healthcare), equilibrated at 25°C in PBS buffer, was used to assay the interaction of immobilized His-tagged ectodomains of hFcγRI (His-hFcγRI) with hIgG1 and mIgG2a anti-DNP in solution. His-hFcγRI were covalently bound to a NTA sensorchip surface at two different densities (400 or 1200 Resonance Units; 1 RU ≈ 1 pg/mm^2 as described[5]. A range of Ig concentrations was injected into flow cells at a flow rate of 50μl/min, with a contact and dissociation time of 210 and 240 seconds, respectively. Regeneration was performed using a 20 seconds injection of 10mM NaOH. The SPR response was recorded continuously,

background binding measured on an empty surface was subtracted, and association (k_{on}) and dissociation (k_{off}) rate constants were determined using BIAevaluation v4.1 software. A 1:1 Langmuir binding model closely fitted the observed sensorgram data and was used in all experiments. Equilibrium dissociation constant (K_D) were calculated as the k_{off}/k_{on} ratio. *N.B.* Varying the densities of immobilized hFcγRI did not significantly affect steady-state affinities.

Statistical analyses

Data was analyzed using one-way ANOVA with Bonferroni post-test (Figs. 2, 3, 4A-C, 6A, 7), two-way ANOVA with Bonferroni post-test (Fig. 6B-E), Mantel Cox test for all Survival curves or Student's t-test (all other data). Statistical significance is indicated (ns: $p>0.05$; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). The n given in the Figure Legends corresponds to the number of mice per group in individual experiments.

Supplemental Method's References

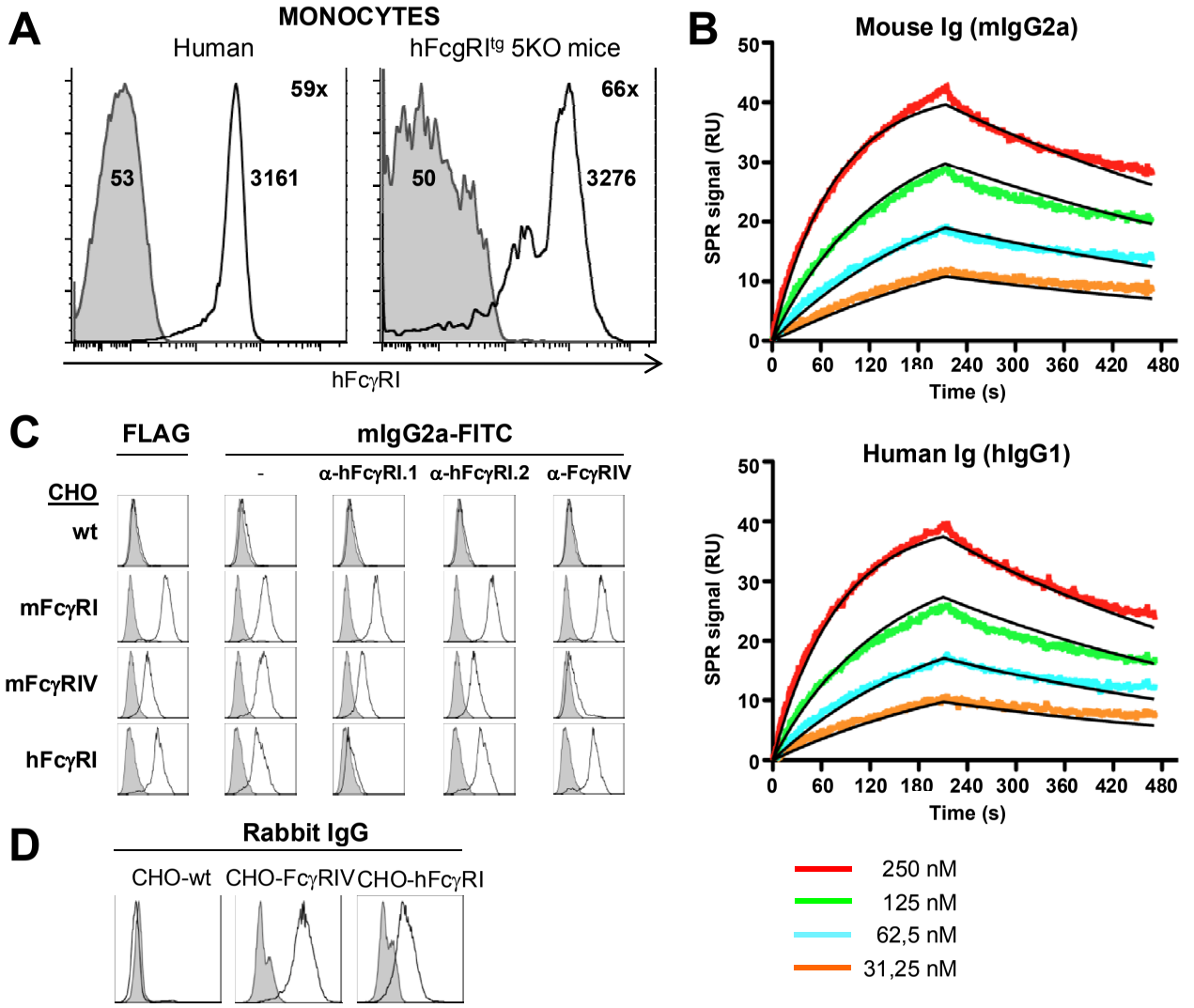
1. Mancardi DA, Iannascoli B, Hoos S, England P, Daeron M, Bruhns P. FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation. *J Clin Invest*. 2008;118(11):3738-3750.
2. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174(1-2):83-93.
3. Bruhns P, Iannascoli B, England P, et al. Specificity and affinity of human Fc{gamma} receptors and their polymorphic variants for human IgG subclasses. *Blood*. 2009;1133716-3725.
4. Bruhns P, Samuelsson A, Pollard JW, Ravetch JV. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity*. 2003;18(4):573-581.
5. Jomain JB, Tallet E, Broutin I, et al. Structural and thermodynamic bases for the design of pure prolactin receptor antagonists: X-ray structure of Del1-9-G129R-hPRL. *J Biol Chem*. 2007;282(45):33118-33131.

SUPPLEMENTAL FIGURE LEGENDS

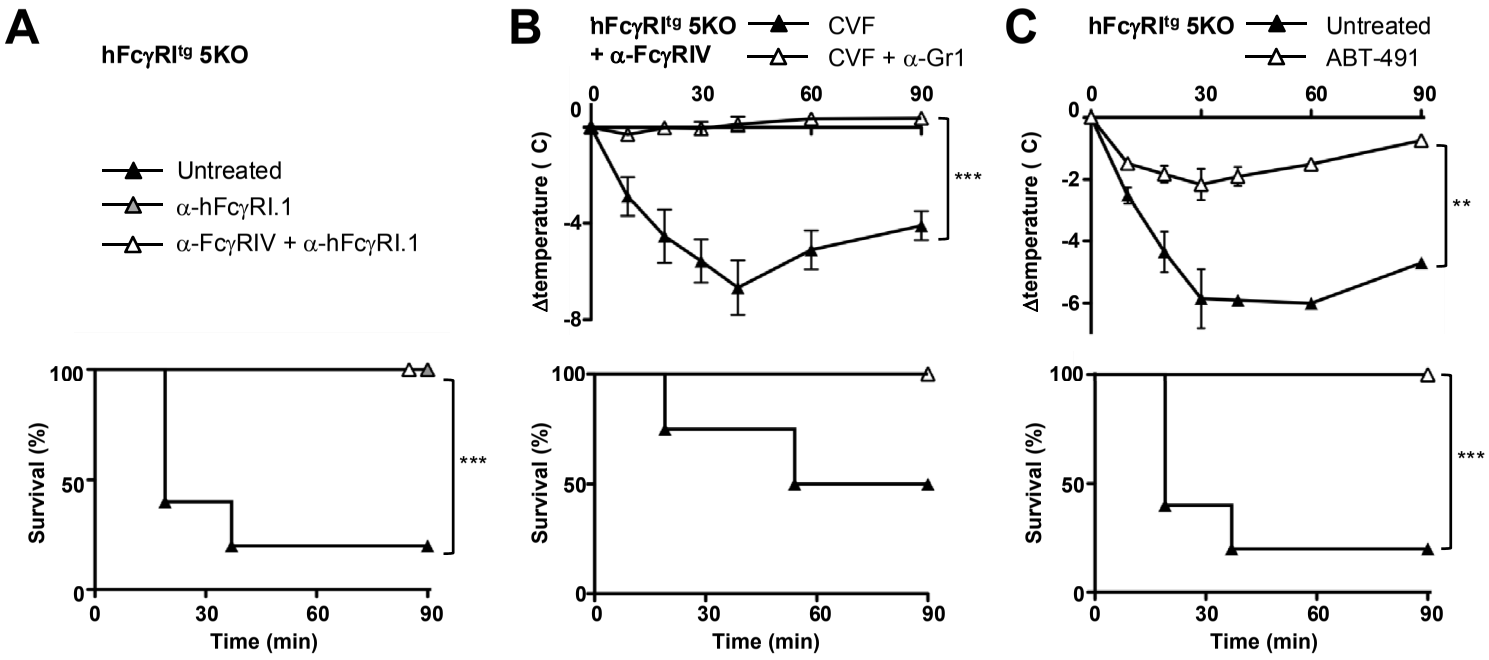
Supplemental Figure 1. (A) Representative histogram plots of hFcγRI expression (black line) on monocytes from human blood (left) or hFcγRI^{tg} 5KO mouse blood. Solid gray histograms represent Isotype control. MFI values for each histogram are indicated, as well as the [specific antibody MFI/isotype control MFI] ratio (x). (B) Real-time SPR sensorgrams were generated by injecting the indicated concentrations of soluble mIgG2a (upper panel) or hIgG1 (lower panel) onto immobilized recombinant hFcγRI. Fitting curved according to a 1:1 Langmuir model are overlaid. (C) Histograms show the binding of (left column) anti-FLAG mAb or (all other columns) FITC-conjugated mIgG2a to indicated FcγR^{+} CHO transfectants pre-incubated or not with indicated mAbs. Solid gray histograms represent background fluorescence. (D) Histograms show the binding of rabbit IgG to FcγR^{+} CHO transfectants. Solid gray histograms represent the binding of FITC-conjugated anti-rabbit Ig alone. (A-D) Data are representative from at least two independent experiments.

Supplemental Figure 2. (A-B) Indicated mice were immunized with BSA in Freund's adjuvant, challenged with BSA, and central temperatures and/or survival rates were monitored. (A) Survival rates during ASA in hFcγRI^{tg} 5KO mice injected or not with anti-hFcγRI or [anti-FcγRIV and anti-hFcγRI] 30 minutes before BSA-challenge (n=5). (B) ASA in anti-FcγRIV-treated hFcγRI^{tg} 5KO mice injected with Cobra Venom Factor (CVF) alone, followed or not by an anti-Gr1 mAb injection (n=4) (C) ASA in hFcγRI^{tg} 5KO mice injected with PAF antagonist ABT-491 or left untreated (n=5). (A,C) Data are representative from at least two independent experiments and (B,C) represented as mean ± SEM.

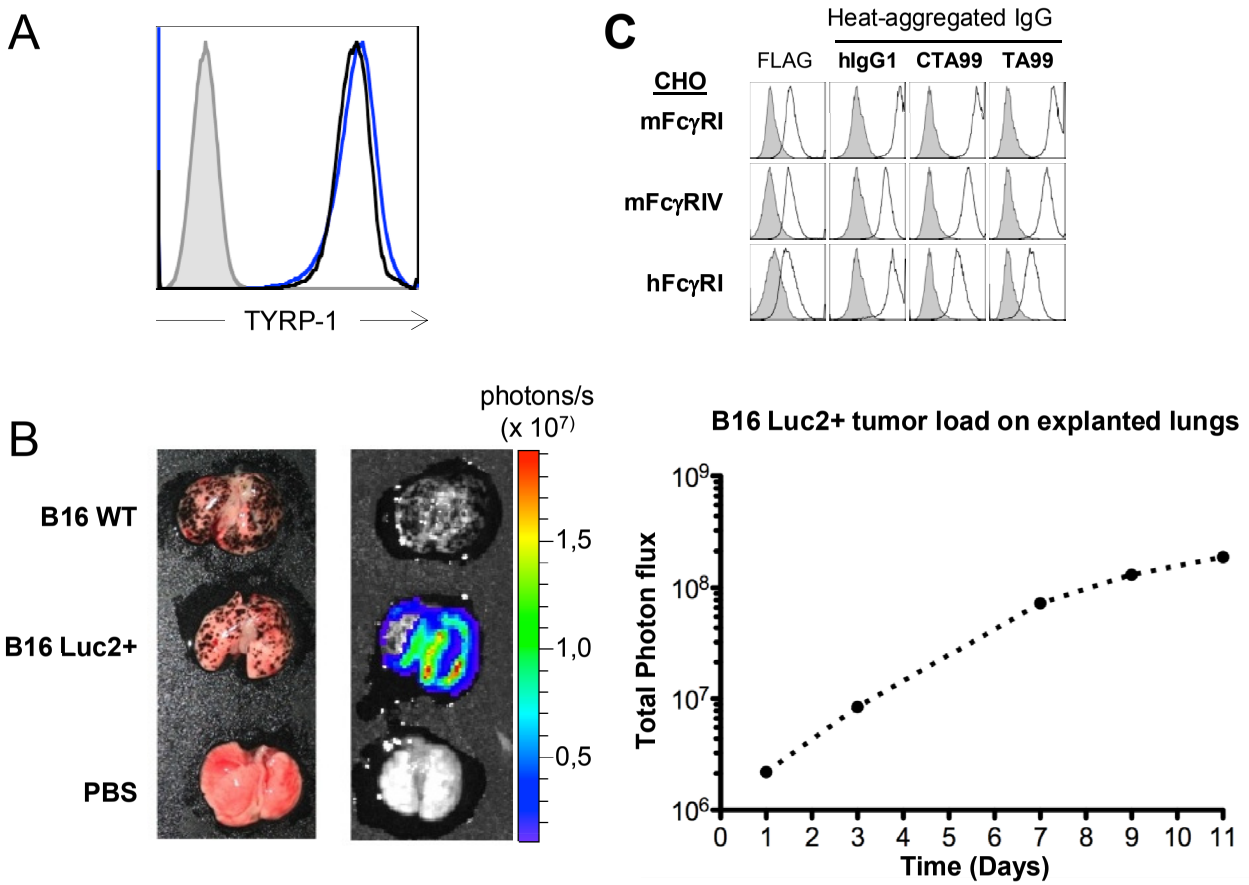
Supplemental Figure 3. (A) Representative expression of TYRP-1 on wt (black line) or luc2$^+$ (blue line) B16F10 cells. Solid gray histograms represent the binding of the secondary Ab alone. (B) Left, representative pictures of B16 metastasis on the lung surface and map of the photon flux superimposed on a black and white photograph of the lung at day 11 post-injection. Right, bioluminescence was acquired on the explanted lungs at indicated days from wt mice injected with B16 luc2$^+$ at day 0 (n=2). (C) Histograms show the binding of indicated heat-aggregated IgG or mAb to FcγR$^+$ CHO transfectants. Solid gray histograms show the binding of heat-aggregated IgG to untransfected CHO cells. (A-C) Data are representative from at least two independent experiments.



Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

DISCUSSION

Our results provide evidence that hFcγRI, the only human high-affinity FcγR, can mediate different pro-inflammatory, pro-anaphylactic as well as anti-tumor functions, leading to autoimmune, allergic and therapeutic reactions, respectively, in hFcγRI^{Tg} mice. In particular, using the B16 melanoma model of lung metastases, we demonstrated, for the first time, that hFcgRI is able to mediate the antitumor activity of the mouse IgG2a anti-gp75 therapeutic mAb (TA99).

Moreover we showed the involvement of hFcγRI in the anti-tumor activity of both a humanized (cTA99) and a fully human (20D7S) anti-gp75 therapeutic mAbs[126].

n.b. *The observation that hFcγRI is involved in mAbs anti-tumor activity has important consequences from clinical point of view. This issue is further analyzed in the part I of the general discussion.*

hFcγRI is the only human high affinity FcγR. hFcγRI binds human IgG_1, IgG_3 and IgG_4 with a high affinity and has no affinity for IgG_2. As mentioned in the introduction, the *high affinity* FcγRs, but not l*ow affinity* FcγRs, are defined by their ability to bind IgGs as monomers. Both types of FcγRs however can bind IgG when present in immune-complexes (ICs) or when opsonizing cells or surfaces. For this reason it is commonly believed that the high affinity hFcγRI is occupied/saturated by IgG molecules *in vivo*, leading to the belief that pre-bound IgG prevents the participation of hFcγRI to IC-mediated reactions.

To understand the biological consequences of the occupation/saturation of hFcγRI *in vivo* RAG2$^{-/-}$/FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} mice were generated. Due to the mutation in the Recombination Activating Gene-2 (RAG2$^{-/-}$) these mice lack mature B cells and T cells preventing endogenous Abs to be produced that could potentially occupy/saturate hFcγRI. In the B16Luc2+ melanoma one may notice that RAG2$^{-/-}$/FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} mice demonstrate enhanced mAb anti-tumor activity compared to FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} mice following mAbs TA99 injections. This could suggest that hFcγRI is occupied/saturated *in vivo* by endogenous IgG, a condition that may limit the efficacy of the injected therapeutic mAbs. However, it has to be noticed that RAG2$^{-/-}$/FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} mice demonstrated a lower tumor growth compared to immunocompetent FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg}, that might account for the difference observed after the treatment with mAbs: a lower tumor load being more efficiently affected than a higher tumor load by protective anti-tumor mAbs.

By contrast, when RAG2$^{-/-}$/FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} in the inflammatory model of autoimmune

arthritis were compared to immunocompetent FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} mice, no difference was observed in the arthritis onset or severity between the two strains of mice. This result suggests that hFcγRI is not occupied/saturated in vivo or that its occupancy has no biological consequence.

This discrepancy between these two models might rely on anatomical differences. In fact the lungs parenchyma is a well-irrigated compartment by the blood stream, compared to the joints that are normally non-vascularized. As a consequence, endogenous Abs might reach more easily resident cells involved in biological processes in the lungs than in the joints thus occupying hFcγRI more easily. A feasible approach to address this question would be to supply Abs to RAG2$^{-/-}$ /FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} in order to mimic the endogenous Abs production and their occupation/saturation of hFcγRI *in vivo* and compare this group with a FcγRIV$^{\text{“only”}}$/hFcγRI^{Tg} group.

ARTICLE 3

ARTICLE 3

"Neutrophils are responsible for specific antibody-induced therapy of tumors"

This article represents the main work of my PhD. It will be submitted before the thesis defense. The version integrated in this thesis is a close-to-final version lacking a few ongoing experiments among which 2-photon data.

INTRODUCTION

Note: The observation that hFcγRI can be involved in mAb anti-tumor activity not only suggests a novel role for this receptor in mAb therapy, but can also help predict the cell population responsible for tumor killing. In the work presented in article 2, indeed, we demonstrated the involvement of hFcγRI to a model of anti-tumor therapy using hFcγRI^{Tg} mice. Noticeably, the expression pattern of hFcγRI in these mice is restricted to monocyte/macrophages, neutrophils and DCs. According to this observation, one might propose that myeloid cells are sufficient (in the absence of a contribution of NK cells) to mediate mAb anti-tumor activities.

The work presented in the following article has been dedicated to the identification **1)** of the cell population involved in anti-tumor mAb therapy, and **2)** of the mechanisms involved in tumor killing.

- *Identification of the cell population*

To investigate the cell population responsible for mAb therapy, different strategies can be envisioned including *in vivo* depletion of cell populations, reconstitution of particular populations *in vivo* by transfer of purified cells, or mice deficient for specific cell populations.

Unfortunately, the majority of these strategies are difficult to realize in the B16 model of lung metastases. In fact, even though I tried several depleting agents, cell populations in the lung compartment appear more difficult to target than equivalent cell populations in other tissues, resulting in inefficient depletions. Moreover, lung resident cells such as alveolar macrophages are insensitive to depletion protocols that do not involve surgery. As a consequence reproducible and effective depletion of cell populations in the lung compartment appear difficult to obtain. The same issue applies for the transfer of specific cell population. In fact, even though specific cell population

can be easily purified with different strategies, once these cells injected into recipient mice, the reconstitution efficacy in the lung compartment is rather poor.

To avoid these issues, I proposed to use subcutaneous tumor models of mAb therapy. In fact, by injecting cancer cells subcutaneously no major limitations apply in the usage of the above-mentioned strategies to investigate cell populations. First I have established subcutaneous injections of B16 cells that lead to the formation of a solid tumor those and tumor load could be drastically reduced following injections of anti-gp75 mAb TA99. Second, I used the previously described model of HER2-expressing BT474 human breast cancer model. In immunodeficient mice, *i.e.* Nude foxp1$^{nu/nu}$, a subcutaneous engraftment of BT474 cells in matrigel lead to the development of a solid tumor. Recurrent injections of Trasuzumab, a humanized IgG_1 anti-HER2 mAb, result in an effective reduction of the tumor load. For both tumor models, I have established luciferase-expressing variants, termed B16Luc2+ and BT474Luc2+, in order to quantify the tumor growth non-invasively using bioluminescence.

- *Identification of the mechanism involved in tumor killing*

The mechanism involved in tumor killing has been analyzed in the following article using different strategies. In fact the above-mentioned tumor models can be carried out in mice rendered unable to mediate particular cytotoxic or phagocytic processes. This can be achieved either by using mice deficient for mediators expected to be involved in these processes, or by using wt mice treated with interfering compounds or inhibitors of biological pathways.

Using the above mentioned tools I have identified the cell population involved in the anti-tumor activity of the murine IgG2a anti-gp75 mAb TA99 and of the humanized IgG1 anti-HER2 mAb Trastuzumab. The unexpected results we obtained show for the first time that neutrophils are necessary and sufficient to mediate mAb anti-tumor activity in both a syngeneic and a xenografts tumor model, most likely through an FcγR-dependent phagocytosis mechanism. They may contribute to change the way anti-tumor mAb therapy is conceived and designed.

Neutrophils are responsible for specific antibody-induced tumor therapy

Marcello Albanesi[1,2], … **to be defined** …… and Pierre Bruhns[1,2]

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SUMMARY

Monoclonal antibody (mAb) therapy targeting antigens present on the tumor cell surface aim at eliminating leukemia and metastases, at reducing solid tumor masses before and preventing tumor reappearance after removal surgery[1-3]. Whether mAbs target cell surface proteins whose signaling is crucial to intrinsic tumorigenicity (*e.g.* Trastuzumab targeting HER-2[4]) or not (*e.g.* Rituximab targeting CD20[5]), animal models have demonstrated a major contribution of receptors for the Fc portion of IgG (FcγRs) to the efficacy of therapy[6,7], which has been correlated with polymorphisms in FcγR-encoding genes in patients[8]. However, the FcγR-expressing cell populations responsible for antibody-induced tumor therapy remain elusive[3]. Here we show that neutrophils are responsible for mAb-induced therapy of both subcutaneous syngeneic melanoma in immunocompetent mice and human breast cancer xenografts in immunodeficient mice. We found that depletion of neutrophils or use of genetically-altered neutropenic mice abolished mAb-induced tumor reduction, whereas depletion of other myeloid populations or of NK cells did not. Transfer of purified neutrophils into mice incapable to respond to therapy restored mAb-induced tumor reduction. Histological analyses confirmed specific neutrophil foci formation in the tumor rim following mAb treatment. Finally, conditional knock-out mice unable to perform FcγR-mediated activation and phagocytosis specifically in neutrophils were resistant to mAb-induced therapy. Our results demonstrate how neutrophils are likely to be necessary and sufficient for mAb-induced therapy of subcutaneous tumors in mice, being recruited locally to the tumor following mAb injection to exert their anti-tumor functions. We anticipate our work to profoundly modify the way mAb-induced specific cancer immunotherapy is perceived[9] and exploited from mouse models to define ameliorated[5] or novel anti-tumor therapeutic mAbs for human cancer treatment.

TEXT

Protocols leading to tumors developing in mice are the main preclinical models to screen and optimize mAbs for potential anti-tumor mAb-mediated therapy in the clinic. These models consist mainly of implanting syngeneic mouse cancer cells into immunocompetent mice or xenogeneic human cancer cells into immunodeficient mice, followed by intravenous injections of potential therapeutic mAbs. Most anti-tumor therapeutic mAbs target an antigen expressed by the tumor and were designed and expected to limit tumor growth by inducing cellular apoptosis or growth arrest[3]. Several reports, however, indicate that the immune effector response is highly relevant to the efficacy of therapeutic mAbs *in vivo* in mouse models[2]. Indeed, whether these mAbs are of mouse origin, chimeric mouse/human or of human origin, their Fc portion can interact with endogenous mouse FcγRs[10,11] once injected mAbs are opsonizing tumor cells. Importantly, mice deficient for all activating FcγRs (FcRγ$^{-/-}$ mice) are neither protected from human CD20-expressing syngeneic lymphoma, from gp75-expressing syngeneic melanoma nor from HER2-expressing breast cancer xenografts development following Rituximab, anti-gp75 mAb or Trastuzumab treatment, respectively[6,7,12]. Whereas polymorphism in human FcγR-encoding genes have been correlated to the efficacy of mAb therapy[8], the FcγR-expressing cell populations responsible for the mAb-induced therapeutical activities on solid tumor have not been formally identified, among FcγR^{+} NK cells and various FcγR^{+} myeloid cells, *i.e.* macrophages, monocytes, neutrophils, eosinophils, basophils, mast cells that can all kill opsonized tumor cells *in vitro*[13] (other REFs?)

To investigate the contribution of FcγR^{+} cell populations to anti-tumor mAb immunotherapy, we used tumor cell lines expressing the enhanced firefly luciferase (luc2) that allows non-invasive and accurate tumor load assessments over time using bioluminescence

acquisition[14]. A subcutaneous injection of luc2-expressing syngeneic gp75^{+} B16-F10 (B16-luc2) melanoma into wild-type mice lead to a localized tumor development those tumor cell burden could be assessed by bioluminescence immediately following injection, visually detected starting day 6-7 and physically measured starting day 10 (Fig. 1a). Recurrent injections of anti-gp75 mAb TA99 reduced bioluminescence to background level as early as 24-48h following the first injection and prevented detectable tumors to appear in wilt-type mice but not in FcR$\gamma^{-/-}$ mice (Supplementary Fig. 1a), as reported[6]. Anti-gp75 mAb injections starting on day 0 or day 2, but not on day 7, post-tumor engraftment efficiently reduced the tumor load (Supplementary Fig. 1b), mimicking the clinical efficacy of anti-tumor mAbs on small or residual tumors and their relative inefficiency on larger tumors (REFs). NK cells did not detectably contribute to anti-gp75 mAb-induced reduction in tumor load, as demonstrated by NK cell depletion (Supplementary Fig.1c) or NK cell deficiency (Fig.1b). Monocytes/macrophages did not detectably contribute either, as demonstrated by monocyte/macrophage depletion (Fig.1c) or by inhibition of monocyte/macrophage activation by gadolinium (data not shown). Absence of mast cells or depletion of basophils or eosinophils did not affect mAb-induced reduction in tumor load either (Supplementary Fig. 1d-f).

Although bone marrow cell transfers from wt mice into FcR$\gamma^{-/-}$ RAG$^{-/-}$ mice restored the protective effect of anti-gp75 mAbs (Fig. 1d), daily transfers[15] were required (Supplementary Fig. 1g), suggesting that a short-lived bone marrow cell population mediated the protection. Among those, neutrophils have been reported to have a lifespan of 12.5 hours in mice[16,17]. Importantly, antibody-induced depletion of neutrophils abolished mAb-induced reduction in tumor load (Fig. 1e). Because antibody-induced cell depletion might also affect other cell populations in this setting, we used a mouse model of neutropenia, relevant to severe congenital neutropenia in

humans[18], induced by the absence of transcriptional repressor growth factor independence-1 (Gfi1)[19]. Whereas tumor growth was identical in Gfi1-deficient and Gfi1-sufficent ($Gfi1^{+/-}$) mice, mAb-induced reduction in tumor load was abolished in Gfi1-deficient mice (Fig. 1f). Thus, our results indicate that neutrophils are mandatory for anti-tumor mAb therapy in this model whereas surprisingly neither NK cells, monocytes/macrophages nor other myeloid cells are required.

We next wondered if these conclusions might be dependent on this particular model of anti-syngeneic tumor immunotherapy. We therefore generated a luc2-expressing variant of the human breast cancer cell line BT474-M1 overexpressing HER2/neu (BT474-luc2; Supplementary Fig. 2a-b). A subcutaneous injection of BT474-luc2 in matrigel leads to a localized bioluminescent tumor mass in immunodeficient nude mice (Fig. 2a). Trastuzumab injections reduced bioluminescence to background level in 7 days and prevented detectable tumors to appear in nude mice but not in $FcR\gamma^{-/-}$ nude mice (Fig. 2a), in agreement with earlier findings[7]. In this model also, depletion of neutrophils abolished (Fig. 2b), whereas reduction of neutrophil numbers only partially reduced mAb-induced reduction in tumor load (Supplementary Fig.2c). Strengthening these results, Gfi1-deficient nude mice were resistant to Trastuzumab treatment (Fig. 2c). Thus, our results indicate that neutrophils are also mandatory for the anti-tumor effect of Trastuzumab on HER2-expressing breast cancer xenografts. Further supporting a role for neutrophils, but not for NK cells in this model also, the transgenic expression of human FcγRIIA (CD32A)[20] restored Trastuzumab efficacy in $FcR\gamma^{-/-}$ nude mice (Fig. 2d); indeed human FcγRIIA is expressed on neutrophils among other myeloid cells, but not on NK cells[10].

We next investigated if neutrophils, in addition of being mandatory for mAb-induced reduction in tumor load, may also be sufficient in an environment resistant to therapy. Daily transfers of purified neutrophils from wt mice, but not from $FcR\gamma^{-/-}$ mice, into recipient $FcR\gamma^{-/-}$

mice restored anti-gp75 mAb-induced reduction in tumor load (Fig. 3a) and undetectable tumor masses (data not shown). Therefore activating IgG receptors are required on neutrophils, and only neutrophils are required to express activating IgG receptors to restore mAb-mediated therapy. This consideration rules out that another cell population is required to interact through its activating IgG receptors with opsonized tumor cells to enable tumor reduction. Neutrophils may thus be responsible by themselves for mAb-induced tumor reduction by cytotoxic and/or phagocytic mechanisms. Human blood neutrophils could, indeed, induce the killing of human $HER2^+$ BT474-luc2 cells *in vitro* only in the presence of Trastuzumab (Fig. 3b) suggesting a requirement for contact between neutrophils and opsonized target cells *in vivo*[13]. Histological analysis revealed foci of $Gr1^+$ cells with a neutrophil morphology in the tumor outer rim only after anti-gp75 mAb injection (Fig. 3c), whereas similar numbers of $CD4^+$, $B220^+$ or $F4/80^+$ cells were present in the presence or absence of therapy (Supplemental Fig. 3). Similar analyses performed comparing wt, neutropenic $Gfi1^{-/-}$ and $FcR\gamma^{-/-}$ mice indicated these $Gr1^+$ cells to be neutrophils that were (NOT?) recruited to the tumor (also?) in the absence of activating IgG receptors (Fig.3d).

Finally, we investigated by which mechanism neutrophils contribute to these models of anti-cancer immunotherapy. Neither a deficiency in cytokines (Tumor Necrosis Factor-α or Interferon-γ), in proteases (elastase or myeloperoxidase), in phospholipase-A2-dependent mediators, nor in reactive oxygen species (superoxide or $gp91^{phox}$-NADPH oxidase complex) affected mAb-induced reduction in tumor load, nor did inhibition of metalloproteases or blocking neutrophil-chemoattractant chemokine CXCL1 (Supplementary Fig. 4). To investigate if neutrophils may contribute to tumor reduction by phagocytosing opsonized tumor cells *in vivo*, we used mice deficient for the kinase syk specifically in neutrophils, *i.e.* $syk^{fl/fl}$ MPR8-cre^+

mice[21]. Syk has indeed been reported to be necessary for antibody-dependent phagocytosis[22] without impairing neutrophil migration to sites of antibody-induced inflammation[23]. Importantly, $syk^{fl/fl}$ MPR8-cre^{+} were resistant to mAb-induced reduction of tumor load (Fig. 3e), demonstrating an essential role for syk-dependent FcγR-induced neutrophil anti-tumor activity.

The studies described here provide a mechanistic basis for the observed reduction in tumor load following anti-tumor mAbs injection in both syngeneic and xenograft models of cancer immunotherapy. The selective requirement and the sufficiency of neutrophils to mediate mAb-induced anti-tumor activities in these models are reminiscent of their ability to control seeding of metastases in the absence of mAb therapy[24]. Although significant differences between mouse and human neutrophils as well as the activating IgG receptors they express have been reported[17,25], the principles that have emerged from these mouse studies are likely to apply to certain human immunotherapy protocols. Such considerations may prove important in the clinic for the combination of anti-tumor mAbs, potentially relying on neutrophil recruitment and activation, with neutropenia-inducing chemotherapy.

METHODS

Mice. RAG $\gamma c^{-/-}$ mice[26], $Gfi1^{-/-}$ mice[27], $Syk^{fl/fl}$ MPR8-cre[21], $FcγRIIA^{tg}$ $FcRγ^{-/-}$ mice[10] and $PLA2^{-/-}$ mice [28] have been described (all C57BL/6J). $FcRγ^{-/-}$, W^{sh}, $Elastase^{-/-}$, $MPO^{-/-}$, $Ncfl^{m1J}$ ($Superoxide^{-/-}$), INF-$γ^{-/-}$, TNF-$α^{-/-}$, $gp91^{phox-/-}$, $RAG2^{-/-}$ and Swiss nude mice were from Jackson Laboratories. All mice were used at age 8-16 weeks of age. Bioluminescence was acquired on a IVIS-100 on shaved anesthetized mice injected i.p. with 3 mg D-luciferin (R&D Systems) (5 minutes exposure time, medium binning) and total photon flux calculated using Living-Image software. Mouse protocols were approved by the Animal Care and Use Committees of Paris, France.

Reagents. B16-luc2 cells were from Caliper-Lifesciences; BT474-M1-luc2 (deposited at CNCM I-4468) cells from B. Hann; Trastuzumab from Roche, Rituximab from Genentech, mAb anti-KC from R&D-Systems; mAb TA99 and mAb Nk1.1 from ATCC; mAb anti-Gr1 (RB6-8C5) from R. Coffman; mAb anti-CD200R3 (Ba103) from H. Karasuyama; mAb anti-CCR3 from J.J. Lee. The following antibodies were used for flow cytometry: anti-mouse CD11b, Gr1, SiglecF, DX5 and IgE from BD Biosciences; anti-hFcγRIIA mAb from StemCell Technologies; anti-human CD15 from Miltenyii Biotech. Mouse neutrophils were purified to >70% purity from bone marrow suspensions using anti-Ly6G microbead kits (Miltenyii Biotec).

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Author Contributions M.A. performed the experiments. ***TO BE COMPLETED***. P.B. conceived and funded the study and wrote the manuscript.

Author Information The authors declare no competing financial interests. Correspondence should be addressed to P.B. (bruhns@pasteur.fr).

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FIGURE LEGENDS

Fig. 1. Neutrophils are required for anti-gp75 mAb therapy of melanoma. (a-f) Indicated mice were injected subcutaneously with $5x10^4$ B16-luc2 cells at day 0, intravenously with 200 μg mAb TA99 on days 0, 1 and 2, and intraperitoneally with D-luciferin immediately before total photon flux acquisition (photon/s). When indicated mice were also injected on days -1, 1, 3, 5 and 7 with (c) clodronate-containing liposomes or (e) anti-Gr1 mAbs as described[29], or on days 0, 1, 2 with $2x10^6$ wt bone marrow cells. (a-f) Data are representative from at least two independent experiments ($n \geq 4$).

Fig. 2. Trastuzumab efficacy on $HER2^+$ xenografts depends on neutrophils. (a-d) Indicated mice were injected subcutaneously with $5x10^6$ BT474-luc2 cells in matrigel at day 0, intravenously with 100 μg Trastuzumab weekly starting day 1, and total photon flux was acquired (photon/s). Mice were also injected (b) on days -1, 1, 3, 5 and 7 with anti-Gr1 mAbs. Data are (a,b,d) representative from at least two experiments with $n \geq 4$ in each group, or (c) compiled from two identical experiments. *N.B*. $Gfi1^{-/-}$ nude and $Gfi1^{+/-}$ nude littermates were kept under sulfamethoxypyridazin plus trimethoprim.

Fig. 3. Neutrophils are sufficient to mediate FcγR-dependent anti-tumor mAb activity *in vivo*. (a) $FcR\gamma^{-/-}$ mice ($n \geq 4$) were injected with B16-luc2 cells and mAb TA99 as in Fig. 1, daily with $2x10^6$ neutrophils purified from indicated mice. (b) *Ex vivo* cytotoxic potential of human neutrophils on opsonized-BT474-luc2 cells at an 50:1 effector:target ratio were assayed as described (REF). (c) Hematoxilin-Eosin (H&E) or anti-Gr1 staining on sections of paraffin-embedded 7-day-old B16luc2 tumors 24 hours after mAb TA99 injection. (d) Mice ($n \geq 4$) were

injected with B16-luc2 cells, mAb TA99 and analyzed as in Fig. 1. (a-d) Data are representative from at least two independent experiments.

Supplemental Fig. 1. FcγR- and cell population-dependency of anti-gp75 mAb therapy of melanoma. (a-f) Mice were injected subcutaneously with $5x10^4$ B16-luc2 cells at day 0, intravenously with 200 μg mAb TA99 on (a, c-f) days 0, 1 and 2, or (b) on days 0, 1 and 2 (0/1/2), days 2, 3 and 4 (2/3/4) or on days 7, 8 and 9 (7/8/9). Total photon flux was acquired (photon/s). Mice were additionally injected on days -1 and 3 with (c) 50 μg NK cell-depleting anti-NK1.1 mAb, (e) 100 μg basophil-depleting anti-CD200R3 mAb Ba103 or (e) 300 μg eosinophil-depleting anti-CCR3 mAb. (g) $FcR\gamma^{-/-}$ mice were injected subcutaneously with $5x10^4$ B16-luc2 cells at day 0, intravenously with 200 μg mAb TA99 on days 0, 1 and 2, and on days 0 and 1 with $2x10^6$ wt bone marrow cells. Total photon flux was acquired (photon/s). (a-g) Data are representative from at least two independent experiments ($n \geq 4$).

Supplemental Fig. 2. (a-b) Characteristics of BT474-luc2 cells. (a) Images shows color-coded photon flux from indicated BT474-M1 cells in the presence of luciferin. (b) Representative expression of HER2/neu on wt BT474-M1 (top) and enhanced luciferase-expressing BT474-luc2 (bottom) cells detected by Trastuzumab (black line). (c) Nude mice ($n \geq 4$) were injected subcutaneously with $5x10^6$ BT474-luc2 cells in matrigel at day 0, intravenously with 100 μg Trastuzumab weekly starting day 1, and when indicted on days -1, 1, 3, 5 and 7 with suboptimal (100 μg/mouse) anti-Gr1 mAbs. Total photon flux was acquired (photon/s). Data are representative from at least two independent experiments.

Supplemental Fig. 3. (????) HISTOLOGY

Supplemental Fig. 4. Enzymes, pathways and cytokines/chemokines involved in anti-gp75 mAb therapy of melanoma. Mice (n≥4) were injected subcutaneously with $5x10^4$ B16-luc2 cells at day 0, intravenously with 200 μg mAb TA99 on days 0, 1 and 2. Mice were additionally injected on days -1 and 3 with (h) 100 μg NK cell-depleting anti-CXCL1 mAb or (i) 100 μg of metalloproteases inhibitor XXX i.p. Total photon flux was acquired (photon/s). (a-i) Data are representative from at least two independent experiments.

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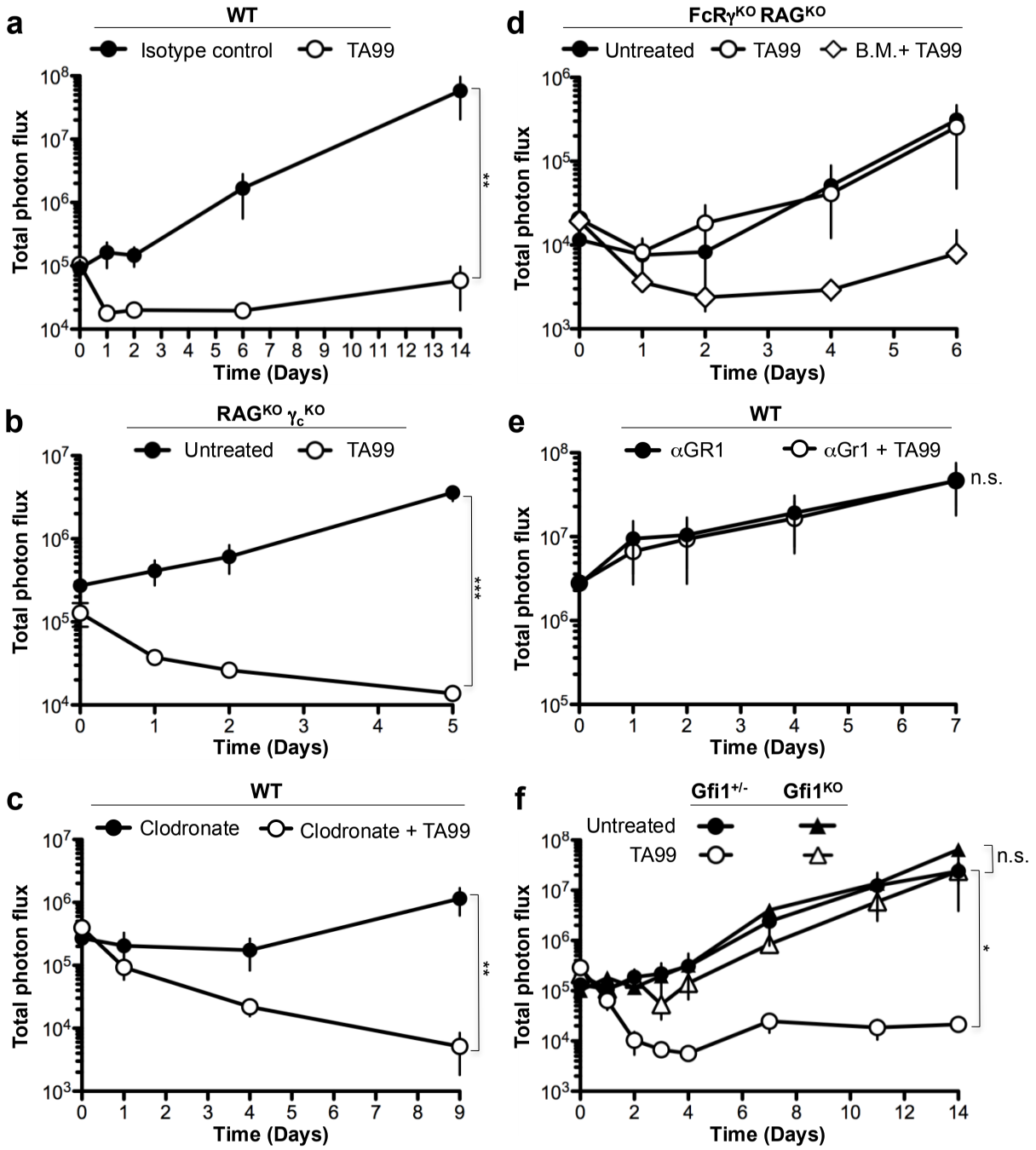
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WT
Isotype control
TA99
Total photon flux
Time (Days)
**
b
RAG^KO γc^KO
Untreated
TA99
Total photon flux
Time (Days)
***
c
WT
Clodronate
Clodronate + TA99
Total photon flux
Time (Days)
**
d
FcRγ^KO RAG^KO
Untreated
TA99
B.M.+ TA99
Total photon flux
Time (Days)
e
WT
αGR1
αGr1 + TA99
n.s.
Total photon flux
Time (Days)
f
Gfi1+/-
Gfi1KO
Untreated
TA99
n.s.
*
Total photon flux
Time (Days)

Fig. 1

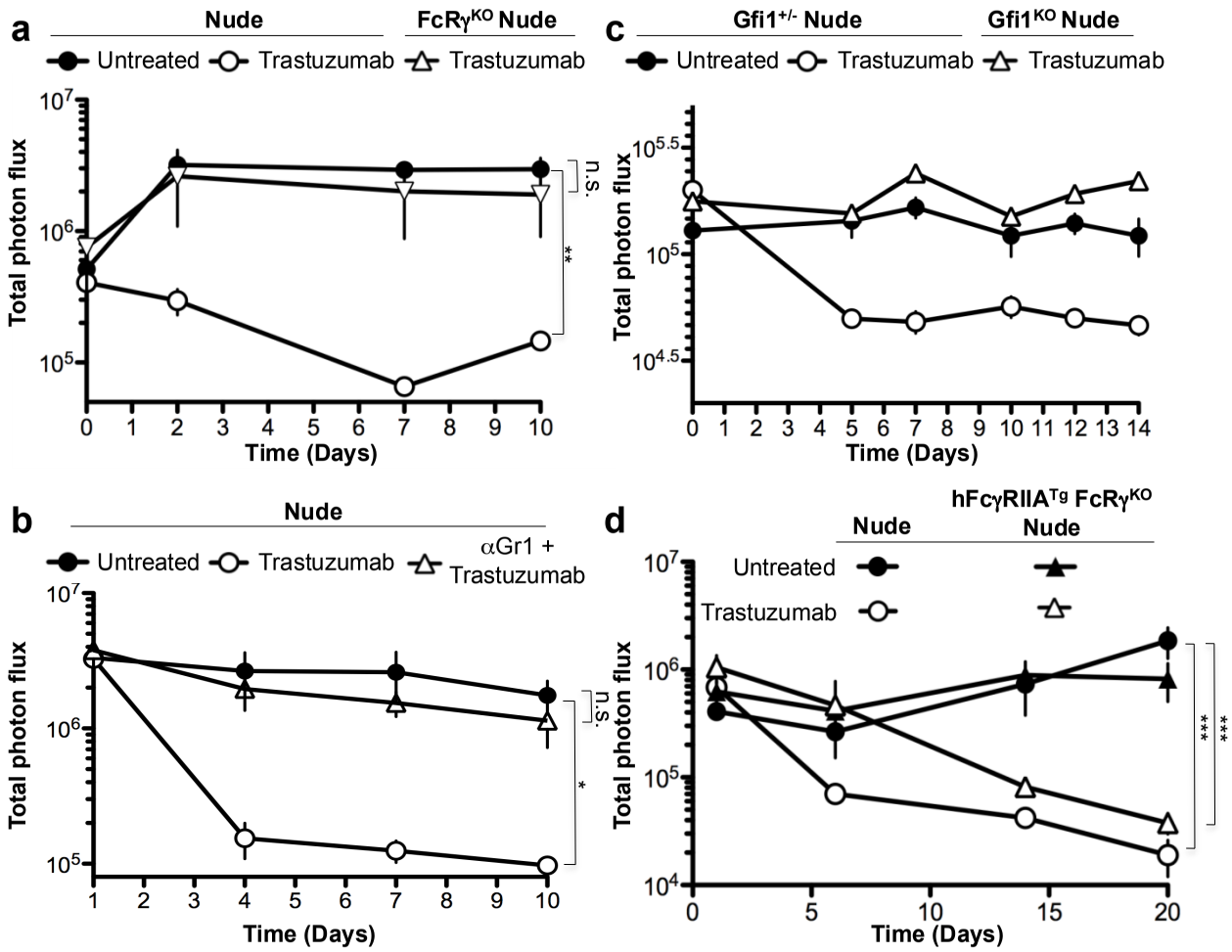
a
Nude
FcRγKO Nude
Untreated
Trastuzumab
Trastuzumab
Total photon flux
Time (Days)
n.s.
**
b
Nude
Untreated
Trastuzumab
αGr1 +
Trastuzumab
Total photon flux
Time (Days)
n.s.
*
c
Gfi1+/- Nude
Gfi1KO Nude
Untreated
Trastuzumab
Trastuzumab
Total photon flux
Time (Days)
d
hFcγRIIATg FcRγKO
Nude
Nude
Untreated
Trastuzumab
Total photon flux
Time (Days)
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Fig. 2

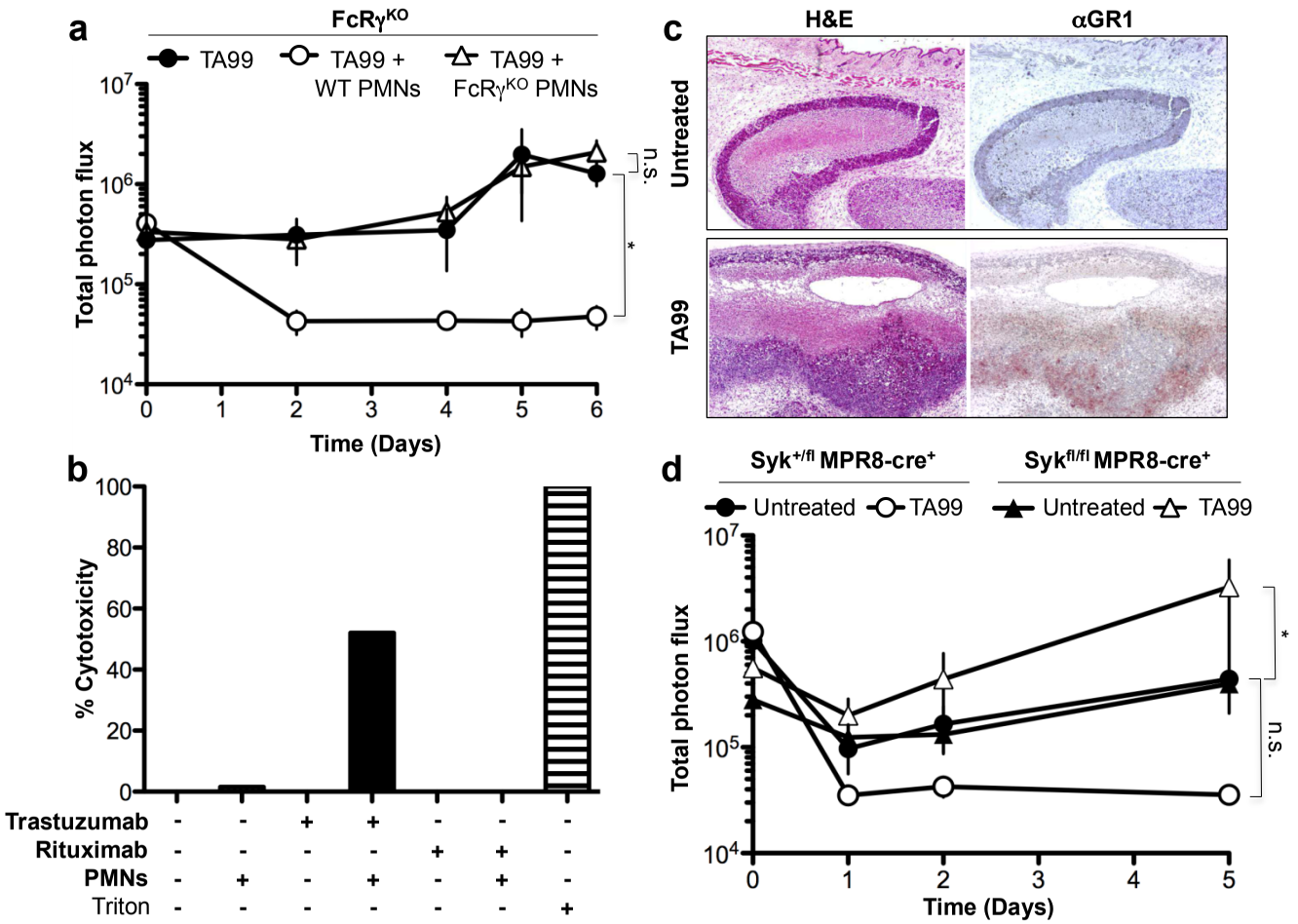
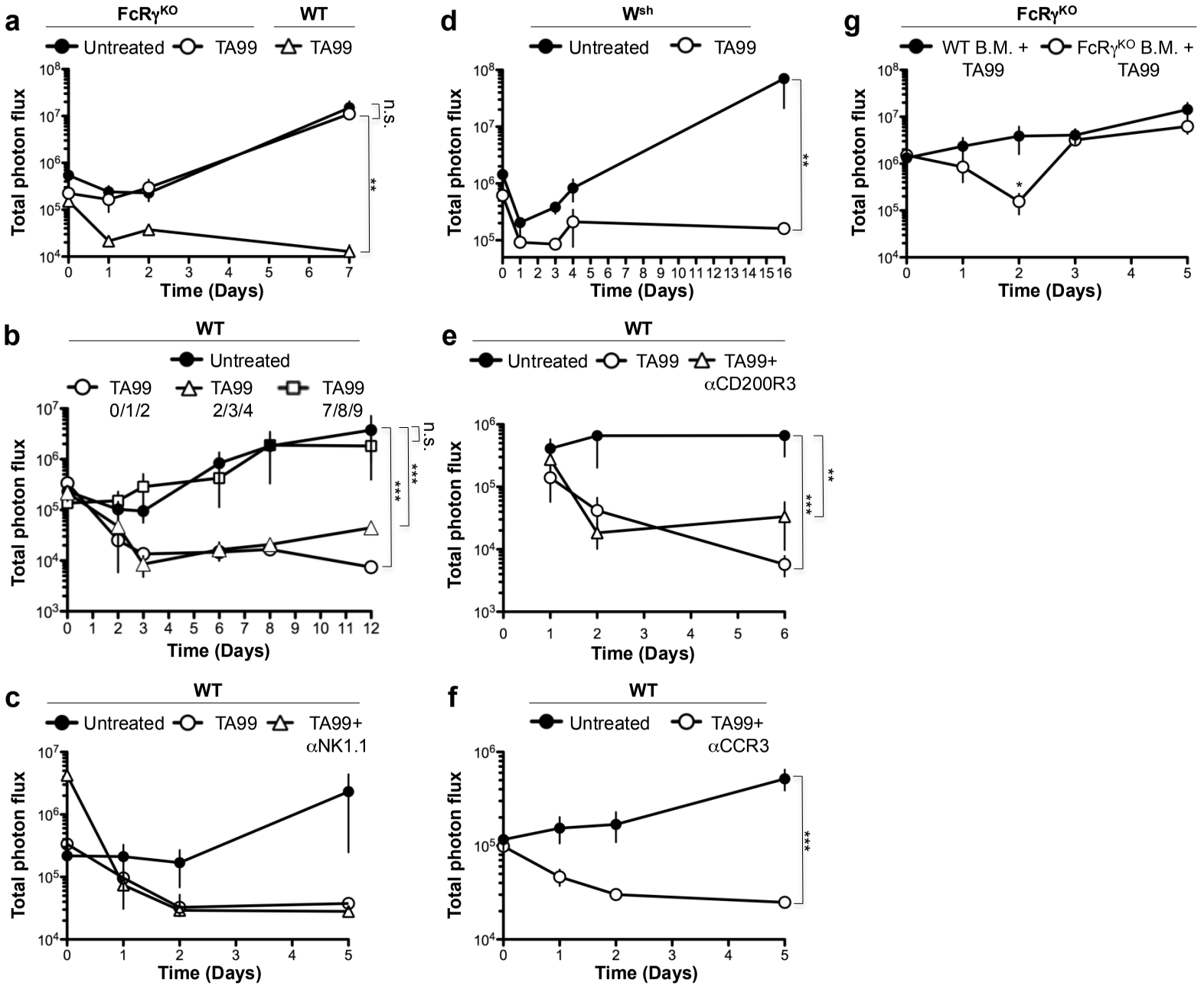
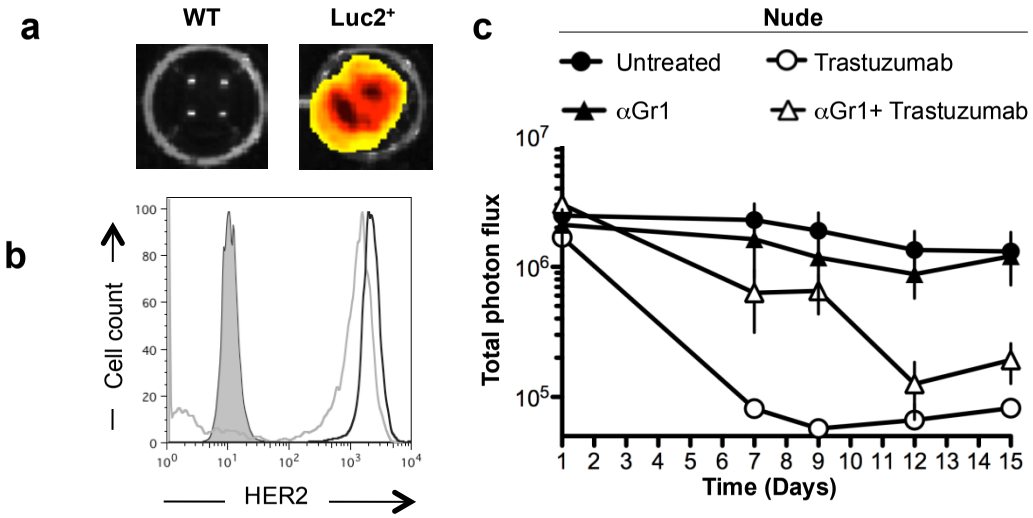

a
FcRγKO
TA99
TA99 + WT PMNs
TA99 + FcRγKO PMNs
Total photon flux
Time (Days)
n.s.
*
b
% Cytotoxicity
Trastuzumab
Rituximab
PMNs
Triton
c
H&E
αGR1
Untreated
TA99
d
Syk+/fl MPR8-cre+
Sykfl/fl MPR8-cre+
Untreated
TA99
Total photon flux
Time (Days)
*
n.s.

Fig. 3



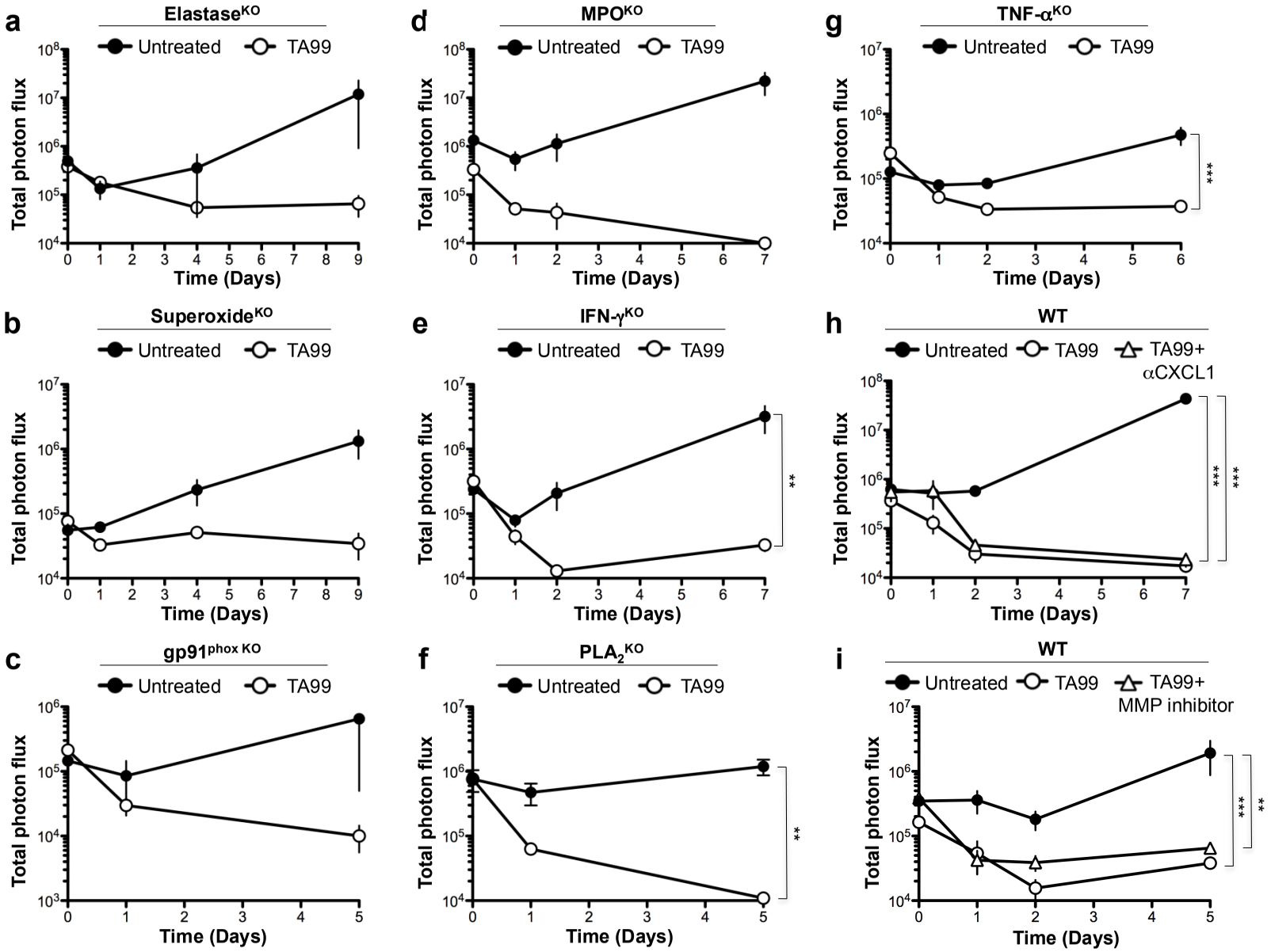
Supplemental Fig. 1



Supplemental Fig. 2

Histology ongoing

Supplemental Fig. 3



Supplemental Fig. 4

GENERAL DISCUSSION

GENERAL DISCUSSION

mAbs targeting antigens expressed on tumor cells can be used in treatment of tumors to achieve a reduction of the tumor burden. Mouse models of mAb therapy have been extensively used aiming at understanding the underlying mechanism of action of mAbs. Different studies have shown that the mAb anti-tumor activity depends partially (or totally) on receptor for the IgG portion (FcγRs). This dependency implies that one or more FcγRs, and therefore one or more FcγR-expressing cells, contribute to the therapeutic activity of mAbs. Addressing which among the different mouse and human FcγRs, which among the multiple FcγR-expressing cell populations and which among the various FcγR-dependent mechanisms are responsible for mAb anti-tumor activity represented the main goals of my PhD.

Using different mouse models of mAb therapy I have analyzed the mouse FcγRs responsible for mAb therapy (Albanesi M *et al.,* J. Immunol, 2012, in press) and extended this analysis to two of their human homologues (Mancardi DA, Albanesi M *et al*., 2nd revision at Blood). Moreover I have identified a primordial role for an innate immune cell population and a mechanism by which it can kill cancer cells during mAb anti-tumor therapy (Albanesi M *et al.,* submitted).

Part I: Lung metastases, FcγRs & mAb-Therapy

The lung is one of the most vascularized organs, irrigated by 2,5 liters of blood flow each minute in humans. For this reason, the lung parenchyma is one of the main compartments where cancer cells, detached from a primary tumor, can extravasate, seed and develop into multiple secondary tumor foci. The development of lung metastases represents a major problem in terms of treatment. In fact only a limited percentage of the lung compartment can be excised with surgery without detrimental effects on the patient.

As already mentioned in the introduction, therapeutic mAbs enable to specifically target cancer cells. For this reason, over last decades several studies have evaluated the efficacy of mAb therapy on lung metastases as well as their mechanism of action. An extensively used mouse model of lung metastases is the B16 melanoma model in which the intravenous injection of mouse B16 melanoma cells into a wt mouse leads to the development of lung metastases. B16 melanoma cells express the antigen gp75. Recurrent injections of mAb anti-gp75 (TA99) lead to a drastic reduction in the tumor load, indicating that mAbs are effective on metastases developing in the lung compartment in this model, and therefore potentially in the clinic[120]. After this pioneer observation, further studies showed that mAb TA99 anti-tumor activity depends on the activating FcγRs. Activating FcγRs in mice comprise FcγRI, FcγRIII and FcγRIV. Contradictory data on the involvement of these three receptors have been reported in the literature[116,117]. The results I obtained applying bioluminescence analyses of the B16 melanoma model of lung metastases (refer to Article 1: Albanesi M *et al*, J Immunol, in press) enabled to identify the mouse receptors FcγRI and FcγRIII as responsible for the mAb TA99 anti-tumor activity. These novel data support some data and contradict other data from the literature, but solve in my view the contradiction found in the literature that was the basis of this work.

Differential involvement of mouse FcγRs

The observation that FcγRI is involved in mAb TA99 anti-tumor activity does not, by itself, represent a novelty in the field but rather a confirmation of what has been reported before in one study. By contrast, the involvement of FcγRIII to TA99 anti-tumor activity had never been described before. From primary articles and reviews correlations and indirect evidences can, however, be found supporting a role for FcγRIII during anti-tumor mAb therapy. The absence of reports on its involvement is therefore surprising. First, FcγRIII is a low-affinity FcγR that does not

bind monomeric IgG but binds ICs or cells opsonized by Abs, as is the case for a cancer cell bound/covered by therapeutic mAbs. Second, FcγRIII has a large expression pattern comprising NK cells, neutrophils, monocytes/macrophages, mast cells, eosinophils and basophils[66,72]. Importantly, the majority of these cells have been shown to be able to kill cancer cells *in vitro* in the presence of mAbs. Third, FcγRIII has been shown to be involved in most Ab-mediated models of disease and inflammation including Ab-induced thrombocytopenia and hemolytic anemia[127]. A particular attention must be given to these latter models in which the injection of mAbs anti-platelets or anti-Red Blood Cells (RBC), respectively, leads to opsonization of platelets or RBCs and to their destruction by FcγR-bearing cells. Therefore, because FcγRIII is involved in Ab-mediated reactions that lead to cell destruction, FcγRIII should be able to mediate also the clearance of the B16 cells once they are opsonized by mAb TA99. For all the above-mentioned reasons, finding an involvement of FcγRIII in mAb TA99 anti-tumor activity it not really surprising and rather confirms its pivotal role in most Ab-mediated reactions in the mouse. As I have already mentioned in the discussion of the article 1, I was probably able to identify an involvement of FcγRIII because of the technical approach chosen for the quantification of the tumor load *(bioluminescence, see discussion of article 1)*.

It is important to notice that FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice are protected by mAb TA99 injections. However, even if the protective activity of mAb TA99 is still present in both the strains of mice, it is reduced compared to the effect observed in wt mice. This difference seems not to be related to the genetic modification leading to FcγRIII deficiency, as the same reduced protection is observed in wt mice following FcγRIII blockade by anti-FcγRIII mAbs. This suggests that the absence of either FcγRI or FcγRIII cannot be fully compensated by the activity of the remaining FcγRs. Because in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice the inhibitory receptor FcγRIIB is still expressed, one may argue that the reduction in the mAb TA99 anti-tumor activity might rely on the inhibition exerted by FcγRIIB upon cross-linking to the remaining FcγR induced by mAb TA99-opsonized tumor cells. No experiment has been conducted to address directly this issue. Previous experiments, however, indirectly suggest that inhibition by FcγRIIB is probably not responsible for the reduced protective effect of mAb TA99 in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice compared to wt mice. Experiments performed using FcγRIIB$^{-/-}$ mice, did not suggest any contribution (or inhibitory activity) of FcγRIIB to this model *(see discussion of article 1)*, in contradiction with a previous report [4]. Moreover, in "FcγRI-only" mice that do not express FcγRIIB, the mAb TA99 anti-tumor activity is also reduced compared to wt mice. Thus, the reduction of the mAb TA99 therapeutic effect in absence of either FcγRI or FcγRIII, might not be due to the presence of the inhibitory receptor

FcγRIIB, but rather to the inherent inability of either receptor to compensate for the absence of the other. Altogether, these considerations are in favor of an additive role of FcγRI and FcγRIII during mAb TA99 anti-tumor activity.

Different explanation may account for this additive role. In fact, FcγRI and FcγRIII might be 1) expressed together on just one cell subtype responsible for tumor killing and the engagement of both these receptor at the same time may result in an improved activation and tumor killing; or 2) expressed on different cell subtypes that collaborate together to increase the efficacy of tumor destruction (please refer to *one cell* and *two cell* hypotheses described later in this discussion).

If FcγRI and FcγRIII are both responsible for the mAb TA99 anti-tumor activity, by contrast, no role could be attributed to FcγRIV in this work. It is interesting to notice that FcγRI and FcγRIV share some similarities: FcγRI and FcγRIV are both high-affinity receptors for IgG2a, and they are both associated with the same FcRγ-subunit that contains the ITAM motif required for the signal transduction upon FcγR aggregation. In the model of mAb TA99 (a mouse IgG2a)-mediated protection from B16 lung metastases, FcγRI and FcγRIV should thus be engaged in a similar manner by TA99-opsonized B16 cells and their aggregation should lead to very similar, if not identical, activation cascades. Despite these considerations, however, FcγRI is involved in mAb TA99 anti-tumor activity whereas FcγRIV is not. *What could be the causes of this difference?*

In both primary articles and reviews, no information on the relative expression of the different activating FcγRs on the effector cells can be found, even though this parameter may influence their contribution to pathology and therapy models and to mAb TA99 anti-tumor activity in particular. In a first hypothesis, FcγRIV may have a lower expression level compared to FcγRI and FcγRIII. Because all activating FcγRs compete for the same ligand during mAb TA99 therapy, this condition may limit the possibility of FcγRIV to interact with the Fc portion of mAb TA99, and therefore its contribution to therapy. It might be possible that FcγRs need to have a minimum level of expression on the cell surface in order to be functional*, i.e.* activate the cells they are expressed on. Thereby one can imagine that, regardless of the relative expression of FcγRI and FcγRIII compared to FcγRIV, FcγRIV have an insufficient expression level on the effector cell populations responsible for mAb TA99 anti-tumor activity. In my work the involvement of FcγRIV in mAb TA99 anti-tumor activity has been analyzed using different approaches including "FcγRIV-only" mice. In these mice, FcγRIV is the only activating FcγR present and its expression is enhanced compared to wt mice [123]. Importantly, "FcγRIV-only" mice were not protected by the mAb TA99 injections whereas they have been reported to be able to sustain antibody-induced arthritis,

anaphylactic reactions and thrombocytopenia. Altogether these considerations suggest that neither the competition potentially exerted by FcγRI and FcγRIII, nor the relative expression levels of these receptors, account for the different biological behavior of FcγRIV compared to FcγRI and FcγRIII in mAb TA99 therapy.

Aside the expression level, another possible explanation could be that FcγRIV is functionally different from FcγRI. In particular, FcγRIV might be unable to trigger the biological functions that are involved in cancer cell clearance, *e.g.* phagocytosis. Interestingly, Ioan-Facsinay *et al.* described that FcγRIII$^{-/-}$ thioglycolate-elicited macrophages that express only FcγRI and FcγRIV as activating FcγRs are able to phagocyte IgG2a-IC. By contrast, FcγRI/III$^{-/-}$ thioglycolate-elicited macrophages that express only FcγRIV as an activating FcγR are unable to perform IgG2a-IC phagocytosis. This result suggested that FcγRI, but not FcγRIV, possess phagocytosis-inducing abilities, and that these two receptors might therefore be functionally different[128].

Different factors can potentially account for this difference. First, even though FcγRIV and FcγRI share the same ITAM motif located in the FcRγ-subunit these receptors are associated with, the analysis of the aminoacid sequence of the alpha chain of FcγRI or FcγRIV shows that these two receptors are profoundly different. In particular, the intracellular domains of their respective alpha chains share only 10% homology (data not shown), and the intracellular domain of FcγRIV, but not of FcγRI, contains a tyrosine residue. Because tyrosine residues are the main targets of kinases activated upon FcγR engagement, the tyrosine residue located in the intracellular domain of FcγRIV may be involved in the signaling cascade following FcγRIV triggering. Further investigations are necessary to address the importance of this tyrosine residue. Second, it is important point out that the activity of the FcγRs can be modulated by other non-FcR related molecules. As an example, Milella *et al* [129] showed that the activity of the human FcγRIIIA can be down-modulated by $\alpha_4\beta_1$ and $\alpha_5\beta_1$ fibronectin receptors. Molecules unrelated to FcγRs might therefore differentially regulate the activity of FcγRI, FcγRIII and FcγRIV during mAb TA99 therapy of lung metastases. Finally, cytokines are also able to modulate the activity of FcγRs, and in particular their ligand-binding capacity, through a process called "inside-out signaling". As an example, the cytokine Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) has been reported to induce an enhanced ligand-binding capacity of human FcγRII[130]; likewise, the cytokine IL3 has been reported to enhance the ligand binding capacity of the human IgA receptor FcαRI[131]. According to these observations, one can hypothesize that cytokines could also modulate the activity of mouse FcγRI, FcγRIII and FcγRIV during mAb therapy. In particular, the aggregation of FcγRs on the effector cells, might lead to cell activation resulting in the production of cytokines that

might, in turn, differentially modulate the activity of FcγRI, FcγRIII and FcγRIV.

Taking together all the above-mentioned considerations into account, that FcγRIV is functionally different from FcγRI and FcγRIII appears the most probable explanation for its non–involvement in mAb TA99 anti-tumor activity. This hypothesis opens the door to the idea that, *in vivo*, aside from binding the Fc portion of IgG and their association to the ITAM motif-bearing FcRγ-subunit, other factors might influence the biological properties, not only of FcγRIV, but also of all FcγRs.

The involvement of human FcγRs

The contribution of human FcγRs to this anti-gp75 mAb therapy model has not been thoroughly analyzed. Their potential to mediate protection against lung metastases, when all human FcγRs are expressed as transgenes together in the same mouse, has been reported recently [132], but not the relative contribution of each of them. Because mouse FcγRs differ from their human homologues (when existing) in several aspects including expression pattern and IgG binding abilities, and therefore in *in vivo* function, I also analyzed during my thesis the contribution of the human receptors FcγRI (CD64) and FcγRIIA (CD32A) to this model of mAb-based therapy.

Even though mouse and human FcγRs are different, based on their function, structure and expression pattern some of the human FcγRs can be considered as the human homologues of mouse FcγRs. The human homologue of FcγRI is the human activating receptor FcγRI (hFcγRI, CD64). hFcγRI is a high-affinity FcγR that shares with mouse FcγRI about 70% homology in the aminoacid sequence (data not shown). Whereas mouse FcγRI is expressed only on particular subset of monocyte-derived dendritic cells in certain tissues [74], lung and skin (*B. Malissen, personal communication*), hFcγRI is expressed on dendritic cells, monocyte/macrophages and, upon induction, on neutrophils[75]. Whereas hFcγRI has been previously described to be involved in antigen presentation, none investigated a possible role of this receptor in mAb anti-tumor activity. To tackle this question in the laboratory, "FcγRIV-only"/ hFcγRI^{tg} mice have been generated. In these mice mFcγRIV and hFcγRI are the only FcγRs expressed. As discussed before, "FcγRIV-only" mice are not protected from B16 lung metastases by mAb TA99 injections. However, the expression of the human receptor hFcγRI was able to restore the mAb TA99 anti-tumor activity. Importantly, the same result can be obtained also using a humanized variant of TA99 (CTA99) and using a fully human (20D7S) anti-gp75 mAb. These results show for the first time an involvement of hFcγRI in an Ab-dependent anti–tumor reaction.

It is important to point out the potential implication of these findings from a clinical point of view. In humans, hFcγRI is constitutively expressed on dendritic cells and monocytes/macrophages. The expression of hFcγRI can be enhanced on these cells or induced on neutrophils by cytokines such as IFNγ and Granulocyte-Colony Stimulating Factor (G-CSF). In line with this observation, previous reports showed that, in patients with non-Hodgkin's lymphoma undergoing CT, a daily treatment with a G-CSF analog *(Filgrastim)* resulted in an enhanced hFcγRI expression on monocytes and neutrophils[133]. If we assume that hFcγRI can participate to anti-tumor mAb therapy, a treatment that enhances the expression of this receptor on effector cells should increase the efficacy of mAb treatment. Thus, as therapeutic mAbs are often used in combination therapies with CT one may suggest to propose a combined treatment that comprises: 1) CT that broadly attacks the tumor cells, 2) therapeutic mAbs that specifically target the tumor cells, and 3) G-CSF to induce the expression of hFcγRI.

When considering the mouse receptor mFcγRIII, one notices that the identification of its human homologue is not straightforward. Historically human FcγRIIIA (hFcγRIIIA) has been described as being the human homologue of mouse FcγRIII. hFcγRIIIA is a low-affinity receptor that shares with mFcγRIII about 70 % of homology in aminoacid sequence (data not shown). However, whereas mFcγRIII is expressed on all myeloid cells and NK cells, hFcγRIIIA is expressed only on subsets of monocyte/macrophages (among myeloid cells) and on NK cells. Because the biological effect triggered by FcγRs depends finally on the cell subtype that is engaged on, these two receptors might have a different involvement in Ab-dependent reactions *in vivo*. This consideration suggest that hFcγRIIIA might not be the (only) human homologue of mFcγRIII and that the actual nomenclature might be somehow misleading. Interestingly, the human receptor FcγRIIA (hFcγRIIA) is a low-affinity receptor that 1) shares with mFcγRIII about 80% of homology in aminoacid sequence (data not shown) and 2) is expressed with the same pattern as mFcγRIII, except for NK cells. Similarly to mFcγRIII, hFcγRIIA has been reported to be involved in different Ab-mediated reactions such as autoimmune arthritis and IgG-dependent anaphylaxis. Moreover, genetic studies based on comparison and evolution of the IgG receptor locus in mice, non-human primates and humans support the hypothesis that mFcγRIII is not the homolog of hFcγRIIIA. Indeed, this work proposes that mFcγRIII is the ancestor of hFcγRIIA, whereas mFcγRIV is the ancestor of both hFcγRIIIA and hFcγRIIIB *(Lejeune J & Watier H, submitted)*. Altogether, genetic data, documented *in vivo* properties, similar expression pattern, converge to propose that hFcγRIIA (CD32A), rather than hFcγRIIIA (CD16A), is the human homologue of mFcγRIII (CD16).

A possible role for hFcγRIIA in models of Ab-mediated cancer immunotherapy has not been investigated in previous reports. To this aim FcRγ$^{-/-}$ / hFcγRIIA^{Tg} mice have been generated in our laboratory. These mice lack all the endogenous activating FcγRs and express hFcγRIIA under the control of its own promoter, reproducing the expression pattern of hFcγRIIA found in humans[134,135]. As discussed before, the lack of all-endogenous activating FcγRs leads to loss of mAb TA99 anti-tumor activity in the B16 melanoma model of lung metastases [120]. This activity could not be restored by the expression of hFcγRIIA, conversely to what we had observed with hFcγRI expression in FcγRIV$^{"only"}$ mice *(**Fig.** 8)*.

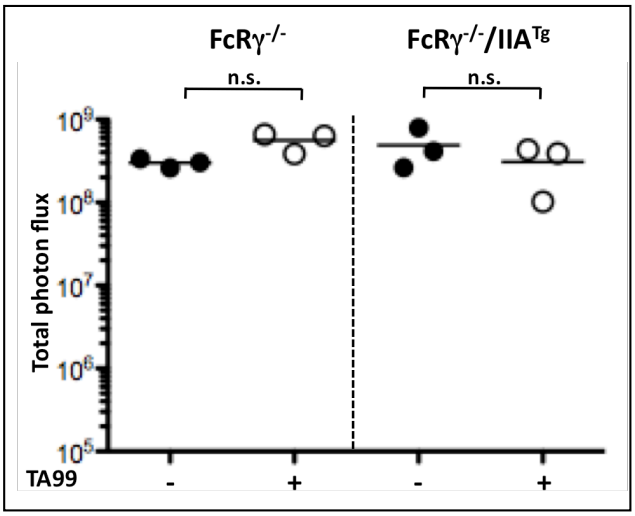


Fig. 8. *Indicated mice were injected with B16 Luc2+ cells on day 0 and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs at day 11*

This result suggests that hFcγRIIA is unable, by itself, to mediate the anti-tumor activity of mAb TA99. It has to be reminded that hFcγRIIA is a single-chain FcγR, which possesses its own ITAM. Importantly, the hFcγRIIA ITAM is atypical and previous reports proposed that it is less potent to induce cell activation *in vitro* [136,137]. According to this hypothesis one may propose that hFcγRIIA is not able to trigger the biological responses that lead to cancer cell clearance. However, this hypothesis is not supported by the existing literature and by our recent findings. In our laboratory, indeed, Jönsson *et al* described not only that hFcγRIIA is able to induce *in vitro* cell activation, but also that this receptor can mediate various Ab-dependent reactions *in vivo,* such as lung inflammation, passive or active systemic anaphylaxis[135] and inflammatory arthritis (*Hogarth, & F. Jönsson, unpublished).* Furthermore, previous reports showed that hFcγRIIA expressed in transgenic mice can mediated Ab-induced thrombocytopenia[134] and that polymorphisms of hFcγRIIA are associated with severity of The Guillain-Barré syndrome, an Ab-mediated acute polineuropathy [138]. Finally, these hFcγRIIA polymorphisms have also been associated with the clinical response to mAb therapy of neuroblastoma [139]. Taking all these considerations into account,

it seems unlikely that the incapacity of hFcγRIIA to mediate mAb TA99 protective effects in transgenic mice is due to an inherent inability to induce cell activation/function. As a consequence its ITAM, even though it has been described as "atypical", should enable this receptor to induce effector cells to phagocytose/kill opsonized tumor cells. This incapacity might, however, be explained by the presence of the inhibitory receptor FcγRIIB in hFcγRIIA^{tg} FcRγ$^{-/-}$ mice. Indeed, as I mentioned earlier e the endogenous inhibitory receptor FcγRIIB is still expressed in the FcγR$^{-/-}$ mice. mAb TA99 is a mouse IgG2a that can bind to both mFcγRIIB [117] and hFcγRIIA (data not shown).

In hFcγRIIA^{tg} FcRγ$^{-/-}$, the co-expression of FcγRIIB may therefore lead to an engagement of mFcγRIIB with hFcγRIIA on the same cells, thus engaging the inhibitory machinery of mFcγRIIB. This inhibitory machinery may also inhibit the signaling pathways used by hFcγRIIA to induce cell activation, as reported before[64]. To address this issue, one may assay mAb TA99 protective effects on B16 melanoma in hFcγRIIA^{tg} / FcγRIV $^{"only"}$ mice that do not express inhibitory FcγRIIB, as those background is refractory to this mAb-induced effect (refer to Article 1 and 2).

The question of the cell population involved.

One of the intriguing issues that still remain unsolved in the B16 melanoma model of lung metastases is the identity of the cell population responsible for mAb TA99 anti-tumor activity. As FcγRs are differentially expressed among immune cells, the expression pattern of the FcγRs responsible for this activity, *i.e.* FcγRI and FcγRIII, may help predict the cell subtype(s) responsible for tumor killing. Human FcγRI also mediates mAb TA99 anti-tumor activity, and its expression pattern is restricted to monocytes, macrophages and neutrophils, which are therefore potential candidates to consider. Because FcγRIII as a broad expression pattern in mice, being expressed on all myeloid cells and NK cells, one cannot exclude a subset among the probable candidates from its expression pattern. The expression pattern of FcγRI, however, appears extremely restricted. In our laboratory, we could not find any expression of this receptor on freshly isolated cells from blood, spleen, liver, broncho-alveolar lavages or bone marrow, in agreement with another group (Tan et al.). Importantly, a report from *Langlet et al.* described FcγRI expression on a particular subset of CD11b^{+} monocyte-derived cells in the muscle tissue[74]. This subset of monocytes shares with DCs the CD11c and MHCII markers, and is therefore considered as a monocyte-derived DCs subpopulation. This particular cell subtype might also be present in the lung compartment (B. Malissen, personal communication), which can thus be considered as the only mouse FcγRI-

expressing cell in the steady state lung. Because mFcγRIII has been reported to be expressed on all myeloid-derived cells, and in particular on the monocytic lineages, this $CD11b^+$ $CD11c^+$ $MHCII^+$ monocyte-derived should theoretically co-express mFcγRI and mFcγRIII. Taking all these considerations into account two possible hypotheses can be formulated:

- *One cell hypothesis*: in this hypothesis only one cell population is responsible for the mAb TA99 anti-metastatic melanoma activity in the lung. This particular cell population should: 1) express both FcγRI and FcγRIII (and hFcγRI when considering the transgenic mouse); 2) be either resident or recruited into the lung compartment; 3) have cytotoxic potential, upon FcγR engagement. An obvious cell population that fits all these criteria is monocytes. Indeed, monocyte-derived cells expressing FcγRI *(B. Malissen, personal communication)* and theoretically FcγRIII (and hFcγRI) are present in the lung. Furthermore, monocytes have been extensively reported to possess cytotoxic potential against opsonized tumor targets *in vitro*, and against platelets in Ab-mediated thrombocytopenia[140].

- *Two cells hypothesis:* In this hypothesis two cell populations are responsible for the TA99 mAb anti-metastatic melanoma activity in the lung. Alveolar macrophages are immune resident cells in the lung compartment, *i.e*. the compartment in which B16 metastases seed and develop. These cells express in steady state the F4/80 and CD11c antigens, FcγRIII and FcγRIV, but not FcγRI. The aggregation of these FcγRs on alveolar macrophages by opsonized tumor cells should lead to cell activation. Thereby, it can be hypothesized that during mAb TA99 treatment the first cells to become activated are alveolar macrophages that are in the vicinity of the developing metastases. This activation may lead on the one hand to target cell killing and, on the other hand, to the production of pro-inflammatory cytokines such as IFNγ that will trigger the recruitment of monocytes. These monocytes may be FcγRI-expressing monocytes or not, but should, once recruited express FcγRI. These $FcγRI^+$ $FcγRIII^+$ monocytes would participate with alveolar macrophages to tumor killing, or be sufficient by themselves to ensure the mAb TA99 anti-tumor activity.

In the literature, there is no report analyzing the involvement of alveolar macrophages in mAb TA99 anti-tumor activity on lung metastases. A well-accepted mouse model of macrophages deficiency is the op/op mouse *(please refer to the "macrophage" section of the introduction)* that unfortunately cannot be used to study alveolar macrophages. Indeed in op/op mice, the deficiency in alveolar macrophages is spontaneously corrected with age [141]. Alternatively, different depleting

strategies such as toxic liposomes (chlodronate-loaded liposomes) or depleting mAbs (anti-F4/80 mAbs) could be envisioned to deplete alveolar macrophages. However, the lung compartment appears more difficult to reach than other tissues and alveolar macrophages are not very sensitive to these protocols (unpublished results).

As far as monocytes are concerned, few reports support the possible involvement of these cells in mAb-dependent tumor killing. Van Spriel *et al* showed that the lack of the CD11b antigen, which which has been reported to cause reduced leucocyte extravasation leads to an impaired mAb TA99 anti-tumor activity in the B16 melanoma model of lung metastases[94]. Intriguingly, CD11b is expressed on circulating neutrophils, monocytes and NK cells but not on alveolar macrophages suggesting that the mAb anti-tumor activity might not depend on this latter cell population. Of note, Otten *et al* reported that B16 melanoma cells could also give rise to liver metastases upon injection into the portal vein. Similarly to the lung metastases model, recurrent injections of mAb TA99 were able to induce a reduction in the number of metastases in this compartment. Finally this work showed that intravenous injections of toxic liposomes reduced the efficacy of mAb TA99 treatment, correlated with the depletion of circulating monocytes and resident liver Kupfer cells[95]. This result indicated for the first time that monocyte and/or Kupffer cells could be contributing to anti-tumor activity. If it is intrinsic to this model of metastases developing in the liver is not known.

Taking all these considerations into account the most likely scenario can be as follows: B16 melanoma metastases are rapidly opsonized upon intra peritoneal injection of mAb TA99. Once bound to the cancer cells, mAb TA99 through the interaction of its Fc portion with FcγRs expressed by $Fc\gamma RI^{+}$ $Fc\gamma RIII^{+}$ monocyte-related cells in the local environment triggers their activation and subsequent tumor killing (one cell hypothesis). Importantly, these two FcγRs participate in an additive manner to the activation of these monocyte-related upon engagement by the Fc portion of mAb TA99 bound to the tumor target.

Part II: Neutrophils & mAb-Therapy

Note: As mentioned in part I of the discussion, the majority of the strategies to analyze the cell population involved in mAb therapy are difficult to realize in the B16 model of lung metastases. In particular, reproducible and effective depletion of cell populations as well as specific cell transfer are difficult to obtain. Therefore, to identify the cell population responsible for mAb anti-tumor activity I decided to use subcutaneous models of mAb therapy that do not present major limitations in the usage of the strategies to investigate cell population.

Neutrophils represent a high percentage of the cells infiltrating a solid tumor, however, their role in tumor biology is still under discussion. In 1995, Pekarek et al showed using a mouse model of fibrosarcoma that the depletion of neutrophils with specific mAbs resulted in reduced tumor growth[106]. This observation indicated for the first time that neutrophils can support the tumor growth possessing therefore a pro-tumorigenic role. Since then, several reports have confirmed these results and have shown that neutrophils can, indeed, participate to different steps of the tumor growth: carcinogenesis through the secretion of ROS and MMP9; angiogenesis through the secretion of cytokines such as IFN-β and growth factors such as V-EGF; extravasation and metastases through the secretion of matrix-degrading proteases[96]. Interestingly, these findings found confirmation also in clinical settings. In fact, clinical studies indicated a correlation between neutrophil infiltration and poorer tumor prognosis[142] [112], and with enhanced metastases development.

Aside this pro-tumorigenic role, a growing body of reports indicates that neutrophils can also acquire a "tumor-cytotoxic" phenotype which consist in the ability of killing cancer cells, thus resulting in lower tumor growth. As an example in 2009 Friendler *et al.* showed in a mouse model of mesothelioma (cancer that develops from transformed cells originating from the mesothelium, the protecting lining that covers most of the organs) that depletion of neutrophils, conversely to results from other reports, lead to enhanced tumor growth[143,144]. These results suggest therefore an anti-tumorigenic role of neutrophils during tumor development. Importantly, this anti-tumorigenic role of neutrophils is not confined to the primary tumor but it may also affect metastases. In fact, a recent report by Granot *et al* showed that neutrophils that migrate from the primary tumor into secondary organs, termed Tumor Entrained Neutrophils (TEN), can inhibit metastase seeding[114]. According to recent reports it seems likely that the acquisition of either an anti-tumorigenic or a pro-tumorigenic phenotype for neutrophils depends on the tumor microenvironment and in particular on the presence of cytokines TGF-β and IFN-β. In fact, it has been proposed that TGF-β

would be responsible for the acquisition of a pro-tumorigenic phenotype, whereas IFN-β would be responsible for the acquisition of an anti-tumorigenic phenotype.[109,144]

Taking these consideration into account it is becoming increasingly clear that neutrophils, as far as tumor biology is concerned, are an ambivalent cell population, and that their "naive/non-primed" state is influenced by the tumor microenvironment to induce either pro-tumor or anti-tumor responses from this cell population.

Despite these important observations on tumorigenesis, the role of neutrophils during mAb therapy of cancer has been poorly investigated. Evidence exist that both mouse and human neutrophils in the presence of anti-tumor mAbs are capable to kill cancer cells *in vitro*[115]. This capability had, however, never been confirmed *in vivo*.

The reason behind neglecting the involvement of neutrophils in mAb-therapy can be attributed to a misconception present in the scientific community concerning the mechanism of action of therapeutic mAbs. In 2000, Clynes *et al.* described in a subcutaneous breast cancer model that the antitumor activity of therapeutic mAbs required the presence of activating FcγRs and, therefore, an FcγR-expressing effector cell population[4]. Based on three observations, it has since then been considered that NK cells are the main effector cells responsible for mAbs anti-tumor activity: 1) NK cells were originally described for their capability to kill cancer cells *in vitro* because of their ability to sense alterations of MHC-I molecule expression on the cancer cell surface; 2) NK cells express FcγRs and are able to kill cancer cells in the presence of anti-tumor mAbs *in vitro*; 3) polymorphisms of the FcγR expressed on human NK cells, FcγRIIIA, have been correlated with the clinical efficacy of therapeutic mAbs such as Trastuzumab and Rituximab [71,76,78,86]. Nevertheless, all these observations are only indirect evidences for a role of NK cells in mAb therapy and, as for neutrophils, there are actually no reports demonstrating the requirement of NK cells for anti-tumor mAb therapy *in vivo*.

The results I obtained applying bioluminescence analyses of the subcutaneous B16 melanoma model and the subcutaneous BT474 breast cancer model *(refer to Article 3: Albanesi M et al, submitted)* enabled to describe a primordial role for neutrophils in mAb anti-tumor activity and exclude a significant role for NK cells. Importantly, neutrophils were not only necessary but also sufficient to mediate the therapeutic effect of anti-tumor mAbs. These data represent in our opinion a major breakthrough in the understanding of the mechanism of action of anti-tumor mAbs,

contradicting also the dogma that NK cell are the cells responsible for tumor killing during mAb therapy.

A critical analysis of the results presented in article 3 and their comparison with existing results from the literature raises several important questions:

- *How do neutrophils reach cancer cells during mAb therapy?*
- *How do neutrophils kill cancer cells during mAb therapy?*
- *Are neutrophils a homogenous cell population?*

How do the neutrophils reach the site of the tumor?

The recruitment of neutrophils during inflammation is normally considered to be cytokine-dependent process. According to the literature, in the mouse two main cytokines are responsible for neutrophil recruitment: 1) Chemokine (CXC motif) ligand-1 (CXCL-1) (n.b. the murine homologue of the human cytokine IL-8), a small cytokine that belongs to the CXC cytokine family produced by macrophages, neutrophils and epithelial cells. This cytokine elicits its biological effect upon binding with the Chemokine Receptor 2 (CXCR2) that is mainly expressed on neutrophils[145]; 2) Interferon-γ (IFN-γ), the only member of the type II class of interferons, is produced by the majority of immune cells. This cytokine elicits its biological effect upon binding with Interferon Receptors (IFN-Rs) that are widely expressed among hematopoietic and non-hematopoietic cells[146].

In the work presented in article 3, the involvement of these two latter cytokines in the recruitment of neutrophils has been investigated using the subcutaneous B16 melanoma model of mAb therapy. To assess the involvement of the cytokine CXCL1, a specific anti-CXCL1 blocking mAb has been used, whereas the role of IFN-γ has been assessed using IFN-γ-deficient mice. In both cases the mAb TA99 therapeutic effect was not altered, suggesting that these two cytokines might not be major contributors in the recruitment process of effector neutrophils during mAb therapy., Different observations, however, need to be pointed out concerning these latter experiments. First, the strategy used to investigate the role of the cytokine CXCL1 has been using a specific blocking mAb that might not be 100% effective *in vivo*, enabling unblocked CXCL1 to still exert its biological function, and therefore mediate the recruitment of neutrophils. As CXCL1 elicits its biological function through the binding of CXCR2 on neutrophils surface, the best way to confirm the result obtained on the involvement of this cytokine would be to envision using mice deficient for CXCR2 ($CXCR2^{-/-}$ mice). Second, according to the results obtained one can also

hypothesize that CXCL1 and IFN-γ might have a redundant role in neutrophils recruitment during mAb therapy. A possible way to address this issue would be to use mice doubly deficient for both IFN-γ and CXCR2, or to block CXCL1 in INF-$\gamma^{-/-}$ mice.

Importantly, we were also able to indirectly exclude a possible involvement of the cytokines IL2, IL7, IL15, and IL21 in the recruitment (and anti-tumor function) of neutrophils during mAb therapy. In fact the $RAG^{-/-}$ $\gamma_c^{-/-}$ mice are still protected by mAb therapy and, as mentioned in the introduction, $RAG^{-/-}$ $\gamma_c^{-/-}$ lack functional receptors for the above-mentioned cytokines.

Even though a cytokine-dependent recruitment of neutrophils is the most accepted mechanism by which neutrophils are recruited to an inflammatory site, in the case of mAbs opsonizing cancer cells, neutrophils might also be recruited to the site of the tumor through a *complement-dependent mechanism*. In fact, as mentioned in the introduction, the binding of mAbs on the cancer cell surface is able to lead to the activation of the complement cascade through the classical pathway. This activation leads to the generation of several products of the complement cascade with diverse biological functions: in particular, the activity of the C5-convertase, generated upon the proteolytic cleavage of the C3 peptide, has a dual role. On the one hand, it initiates the late events of the complement activation that lead to the formation of the membrane-attack complex. On the other hand, it catalyses the cleavage of the C5 factor into the C5a peptide. C5a is a potent chemoattractant that exerts its biological activity through binding to the C5a Receptor (C5aR). C5a is responsible for generating a number of diverse functions on neutrophils. These functions include stimulation of chemotaxis, calcium influx, aggregation and production of superoxide anions [147]. Therefore, one may hypothesize that once cancer cell are opsonized by mAbs, the complement cascade is activated via the classical pathway, and mediates through the C5a peptide the recruitment of neutrophils.

This latter hypothesis is in agreement with results presented in article 3 and by reports from the literature on the involvement of the complement system in mAb therapy. In fact, the histological analysis of the immune cells infiltrating a subcutaneous B16 melanoma showed a differential immune infiltration in the presence or not of mAb TA99. In particular, the tumor explanted from untreated mice showed an infiltration of macrophages, T cells and B cells but not of neutrophils. By contrast, tumor explanted from mice treated with mAb TA99 showed, aside the infiltration of macrophages, T cells and B cells also a significant infiltration of neutrophils. This result indicates that the infiltration of the tumor by neutrophils is linked to the injection of the therapeutic mAb. Interestingly, supporting the hypothesis that the complement can trigger the recruitment of

neutrophils, Allendorf *et al.* demonstrated an essential role of C5a in neutrophil chemotaxis during mAb therapy in a mouse model of mammary adenocarcinoma [148].

According to these observations, it follows that during mAb therapy neutrophils may be recruited via a complement-dependent mechanism. A likely scenario may be that, upon injection, mAbs bind to the cancer cells and trigger the complement cascade activation through the classical pathway of complement. This activation leads to the production of C5a that triggers a *"first wave*" of neutrophil infiltration. Upon infiltration, the interaction between the mAbs opsonizing the cancer cells and the FcγRs expressed by neutrophils, triggers neutrophil activation (and anti-tumor effects). Neutrophil activation, in turn, will lead to the production of CXCL1 and IFN-γ that will cause a *"second wave"* of neutrophil infiltration into the tumor site, starting thus an self-sustaining loop of neutrophil recruitment.

How do neutrophils kill cancer cells?

The results obtained in article 3 indicate that neutrophils are necessary and sufficient to mediate mAb anti-tumor activity. However, there are several mechanisms by which neutrophils can kill a target cell opsonized by mAbs. Some of these mechanisms rely on the exocytosis of cytotoxic mediators such as enzymes and/or cytokines. By contrast, other mechanisms such as phagocytosis or death receptor–induced cell death are also possible and could be been investigated to try identify the responsible mechanism.

Phagocytic mechanisms?

Cancer cells opsonized by mAbs can be eliminated by neutrophils through phagocytosis, a process that require an interaction between the target cell and the effector cell. Two distinct mechanisms of phagocytosis can be potentially triggered in this scenario: 1) the interaction of neutrophil FcγRs with the Fc portion of mAbs opsonizing cancer cells can trigger an Fc-dependent phagocytosis (please refer to the introduction neutrophils section); 2) the binding of the mAbs to the cancer cell surface can activate the complement cascade through the classical pathway leading to complement product C3b deposited onto the target cell and recognition by complement receptors expressed on neutrophils that will trigger phagocytosis. Neutrophils, indeed, express several complement receptors among which CR1 and CR3. Even though the complement system and FcγRs are both able induce phagocytosis of the target, these two processes follow different molecular

mechanisms [149]. In fact, it is possible to distinguish the complement-dependent phagocytosis from the FcR–dependent phagocytosis based on the involvement of the Src-family kinases and, in particular Syk. In 1997 Crowley et al described, indeed, that macrophages from $Syk^{-/-}$ mice are defective in phagocytosis of antibody-coated particles bound by FcγRs, demonstrating a pivotal role for this kinase in the Fc-dependent phagocytosis. By contrast, no role has been reported for Syk during complement-dependent phagocytosis[89,150]. We propose that in the presence of mAbs, neutrophils kill the target cells through an Fc-dependent rather than a complement-dependent phagocytosis, based on our results on the involvement of the kinase Syk in neutrophils during mAb TA99 anti-tumor activity. Mice deficient for the Syk kinase specifically in neutrophils (*$Syk^{fl/fl}$ MPR8-cre^{+} mice*), indeed, were resistant to mAb therapy. FcγR-mediated rather than complement-mediated phagocytosis appears to be essential for anti-tumor mAb activity. Moreover in the subcutaneous B16 melanoma model the transfer of neutrophils purified from wt mice, but not from $FcR\gamma^{-/-}$ mice, into therapy-resistant $FcR\gamma^{-/-}$ mice was able to restore mAb TA99 anti-tumor activity. Neutrophil-dependent anti-tumor activity therefore requires FcγRs to be expressed on neutrophils that cannot be replaced by a complement-dependent activity. Taken together these results strongly suggest that neutrophils eliminate opsonized-cancer cell through an FcγR-dependent rather than a complement-dependent phagocytosis mechanism.

Interestingly, the results obtained in the $Syk^{fl/fl}$ MPR8-cre^{+} mice enabled us to exclude also another killing mechanism that requires contact between the target cell and the effector cells: *death receptor-induced cell death.* Neutrophils, like NK cells, indeed express death receptors ligands. One can imagine that during mAb therapy, because neutrophils and opsonized tumor cells are engaged through Fc-FcγR interactions it will favor the interaction between the death receptors expressed by tumor cells and their ligands on neutrophils. This interaction could potentially induce the death of the cancer cells. However, the absolute requirement for the kinase Syk in neutrophils that has not been reported to neither affects Fc-FcγR interactions nor expression of death receptor ligands strongly suggest that a death receptor-induced cell death does not account for the therapeutic effect of this mAb therapy, *e.g.* mAb-induced tumor killing.

Fc-dependent phagocytosis, however, might not be the only mechanism that accounts for the mAb anti-tumor activity we observed in our models. In fact, the Fc-FcγR interaction should trigger neutrophil activation leading to several mediators being secreted into the extracellular compartment that can potentially participate to tumor killing. Noticeably, Syk is involved in Reactive Oxigen Species (ROS) generation, cytokine production, exocytosis of granules by neutrophils and integrin signaling. In fact, neutrophils from *$Syk^{fl/fl}$ MPR8-cre^{+}* have been described to produce less ROS,

cytokines and to present an impaired degranulation potential. Therefore, one cannot exclude that these contact-induced, phagoctytosis-independent, events also concur to tumor killing[151,152].

Non-phagocytic mechanisms?

It is well known that the activation of neutrophils leads to the production of ROS that have anti-microbial and cytotoxic activities. Upon neutrophil activation, the production of ROS requires the assembly of the NADPH complex at the cell membrane that leads to the production of superoxide anion. Superoxide anion is the substrate of the superoxide dismutase enzyme that generates the highly toxic hydrogen peroxide. The enzyme myeloperoxidase (MPO) mediates a further step of ROS production by generating hypoclorus acid and chloramines from the products of the NADPH oxydase pathway. Once produced, all these ROS can be secreted in the extracellular milieu where they can exert their cytotoxic activities[153]. Importantly, circulating neutrophils from mice bearing intramuscular B16 melanoma were able to exert cytotoxic activities on B16 melanoma cells *ex vivo* that depended on ROS production[154]. For this reason we tested the possible involvement of ROS during mAb TA99-mediated tumor killing of subcutaneous B16 melanoma using mice rendered unable to produce ROS ($gp91^{phox-/-}$, $superoxide^{-/-}$ or $MPO^{-/-}$ mice). No difference between wt and any of these strains of mice could, however, be detected, suggesting that ROS might not be major contributors for this mAb anti-tumor activity.

The activation of neutrophils can also lead to the exocytosis of enzymes contained in the neutrophil granules. Neutrophil Elastase (NE) is a serine-protease stocked in the secondary granules of neutrophils that upon secretion has been reported to exert mainly anti-microbial activities (Belaaovaj a. Nat Med 1998). Importantly, however, Mittendorf et al showed using a mouse model of breast cancer that NE can participate to the process of tumor killing[155]. In our models, the contribution of the NE to the mAb TA99 anti-tumor activity using $Elastase^{-/-}$ mice could not be detected. Neither could we detect a contribution of metallo-proteinases (MMP) or of Phospholipase A2 (PLA2) responsible for the production of multiple lipid mediators.

It is important to point out that the results obtained in $Elastase^{-/-}$ mice and in $MPO^{-/-}$ mice can also give us indications concerning the involvement of Neutrophils Extracellular Traps (NETs) in mAb therapy (for a definition of NETs, please refer to the neutrophil section in the introduction). NETs that are formed upon neutrophil activation have been shown to be involved in several biological phenomenon such as the anti-microbial activity of neutrophils[101], clog formation and cancer-associated thrombosis[104,156]. Therefore one can hypothesize the FcγR aggregation on neutrophils following encounter with opsonized tumor cells may lead to the formation of NETs that

could contribute to mAb anti–tumor activity. Not supporting this view, Papayannopoulos *et al.* showed that NE and MPO are responsible for NET formation and, importantly, that the formation of NETs is abolished in either MPO$^{-/-}$ or Elastase $^{-/-}$ mice. A major role for NETs in anti-tumor mAb therapy can thus be excluded[102,103].

Altogether these considerations suggest that contact–dependent phagocytosis-independent mechanisms are not major contributors for mAb anti-tumor activity. However, it has to be pointed out that the different mediators have been studied using either interfering compounds to block their biological activity or mice deficient for a given mediator/enzyme. Therefore one cannot exclude that the above-mentioned secreted mediators might have a redundant role in tumor killing. Thus, , in the absence of one mediator another (or several other) mediator(s) could still mediate tumor killing. Mice genetically-deficient for several of these mediators may be used but a more feasible approach to tackle this question would be to combine different interfering compounds, to block multiple mediators at the same time in wt mice.

Finally, taking all these consideration into account, it is not yet possible in my opinion to identify a unique mechanism by which neutrophils kill cancer cells in the presence of mAbs. In fact, it seems likely that neutrophils once in the close vicinity of opsonized tumor can mediate tumor killing via both Fc-FcγR-induced phagocytosis and activation-dependent degranulation/secretion of mediators. The cytotoxic potential of these mediators may also affect non-tumor cells in the vicinity of activated neutrophils (bystander effect).

Can neutrophils be considered a homogeneous cell population?

This particular point of discussion is raised by the results obtained on genetically altered neutropenic mice that have been used to study the involvement of neutrophils during anti-tumor mAb therapy. As already mentioned in the first part of the discussion, the usage of mice deficient for a particular cell population is one of the main strategies by which one can investigate the involvement of a particular cell population in a given biological phenomenon. There are two available mouse models of neutropenia to study neutrophils-dependent functions: Gfi-1$^{-/-}$ mice [98,99,157] and Genista mice (described below). The genetic target of both these mouse models is the gene encoding for Gfi-1 transcription factor. Gfi-1 is a zinc-finger transcription repressor that represses the differentiation axis towards monocytes/macrophages and therefore favors the transition from promyelocytes to myelocytes. *(**n.b**. for explanations on the developmental stages of neutrophils please refer to the introduction of this thesis, i.e. in the "neutrophil" section).*

The absence of the Gfi-1 repressor in Gfi-1$^{-/-}$ mice (or Gfi-1-GFP$^{KI/KI}$ mice) results in an altered neutrophil differentiation pathway and an accumulation in the promyelocytic stage of development. As a consequence these mice lack mature neutrophils and cells resembling immature neutrophils accumulate, forming an "atypical" myeloid cell population that shares characteristics of neutrophils and macrophages. The defect in Gfi-1 repressor does not affect only myeloid cells, but it affects also lymphocytes. In particular, a partial block in T cell-development has been reported that results in a reduced number of mature CD4^{+} and CD8^{+} T cells. The Gfi-1-deficient mouse (*e.g.* Gfi-1$^{-/-}$ mouse) is the model of neutropenia that I have used to address the role of neutrophils during mAb-therapy. Importantly, in the subcutaneous syngeneic B16 melanoma model I could observe that Gfi-1$^{-/-}$ mice are not protected by the anti-tumor mAb injections. Similarly, in the BT474 human breast cancer xenograft model, the anti-tumor activity of the anti-tumor mAbs was lost in Gfi-1$^{-/-}$ Nude$^{nu/nu}$ mice. These results represent the first clear evidence for the requirement of neutrophils in anti-tumor mAb therapy.

Unlike Gfi$^{-/-}$ mice, in Genista mice the product of the gene encoding for the Gfi-1 repressor is not absent but mutated. Genista mice indeed result from an N-ethyl-N-nitrosourea (ENU)-mutagenesis screen and carry a point mutation in the third zinc finger domain of the Gfi-1 gene (Genista mutation) [97]. This mutation has no impact on the expression level of the Gfi-1 gene transcript but it leads to a partial loss of function. Subsequently, these mice also lack mature neutrophils. However, conversely to Gfi-1$^{-/-}$ mice, myelocytes and metamyelocytes are present, suggesting that the block in the generation of mature neutrophils occurs after the metamyelocyte stage ***(fig.9)***. Moreover, the Genista mutation has been reported to only mildly affect T cell development, as indicated by the normal distribution of CD4^{+} and CD8^{+} T cells in secondary lymphoid organs.

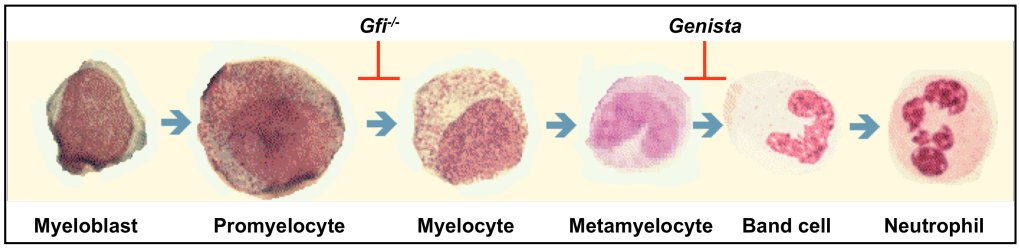


Fig.9: *Schematic representation of neutrophil development. Red bars indicate the block to neutrophils evelopment in Gfi$^{-/-}$ and Genista mice*

It is interesting to notice that the mAb TA99 anti-tumor activity is conserved in neutropenic

Genista mice, whereas it is lost in Gfi-1$^{-/-}$ mice (unpublished data). In fact, no difference could be detected in the mAb TA99 anti-tumor activity when comparing Genista mice and wt mice ***(fig. 10)***

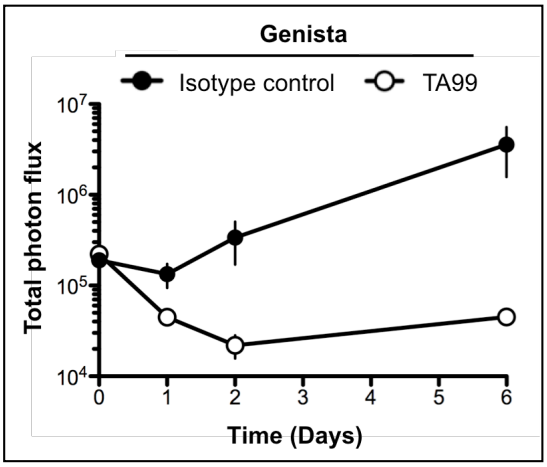


Fig. 10. *Mice were injected sub-cutaneously with 5x104 B16Luc2+ cells at day 0,intravenously with 200mg mAb TA99 on days 0, 1 and 2.*

Even though surprising, this result is in line with previously reported differences on neutrophil-dependent disease models between these two neutropenic mice strains: Ordonez-Rueda *et al* reported that Ab-dependent autoimmune arthritis and IC-mediated alveolitis were reduced in Genista mice whereas they were abolished in Gfi-1$^{-/-}$ mice. Even though both Gfi-1-deficient mice (*i.e.* Gfi-1$^{-/-}$ mice) and Genista mice are genetically-altered "neutropenic" mice, the absence and the point mutation of the gene repressor Gfi-1 differentially affects neutrophil development, respectively. Genista mice, but not Gfi-1-GFP$^{KI/KI}$ mice, indeed present a significant "abnormal" circulating myeloid cell population that can presumably rapidly become an effector neutrophil-like population during inflammatory processes. This "abnormal" population may therefore replace mature neutrophils in Genista mice to mediate anti-tumor activities induced by anti-tumor mAb therapy. It is widely accepted that neutrophils are identified by the expression of the CD11b and Ly6G antigens. Within the neutrophils two different cell populations can be distinguished based on the level of expression of Ly6G antigen: Ly6G intermediate (Ly6G^{int}) and Ly6G high (Ly6G^{hi}). Interestingly, the comparison of the bone marrow cells from Gfi-1$^{-/-}$ and Genista mice revealed a difference in these two latter cell populations. In fact, the Ly6G^{hi} cell population is missing in both strains of mice but, by contrast, Ly6G^{int} cell population is also lacking in Gfi-1$^{-/-}$ mice whereas it is still present in Genista mice. It is important to point out that Ly6G^{int} cells can be mobilized from the bone marrow to the tissue during inflammatory condition[97]. According to these observations, one may propose that in wt mice and Genista mice the Ly6G^{int} cell population might, upon mobilization from the bone marrow, are responsible for mAb anti-tumor activity. According to this hypothesis, this latter cell population might also be responsible for the Ab-dependent arthritis and alveolitis observed in Genista mice. Nevertheless, it is important to point out that from the results obtained in

article 3 also the Ly6G^{hi} cell population appears to be able to mediate mAb anti-tumor activity: the transfer of Ly6G^{hi} cell populations purified from wt mice into FcRγ$^{-/-}$ mice that are resistant to mAb therapy indeed restored anti-tumor mAb therapy.

It is also interesting to notice that a similar phenotypical distinction has already been proposed for different cell subtypes and, in particular, monocytes. Mouse monocytes are normally identified by the expression CD11b, GR1 and lack of B, T and NK cell markers. Additionally monocytes can also express the chemokines receptors CX_3CR1 and CCR2. According to the expression level of GR1, CX_3CR1 and CCR2, two different monocytes subsets could be distinguished: CX_3CR1^{low}/CCR2^{+}/GR1^{+} and CX_3CR1^{high}/CCR2^{-}/GR1^{-}. Importantly these two distinct monocytes subsets have both the potential to differentiate into DCs *in vivo* but they are differentially recruited to inflamed or non-inflamed tissues, suggesting therefore that these cells might be involved in different biological processes.[158,159].

One may therefore consider that neutrophils, similarly to monocytes, does not represent an homogeneous, but rather an heterogeneous, cell population that comprises at least two distinct cell subsets: Ly6G^{hi} and Ly6G^{int} neutrophils. These two distinct cell subsets seem nevertheless to possess the potential to kill cancer cells opsonized by mAbs. A possible experimental setup to confirm this hypothesis would be to transfer purified Ly6G^{hi} or purified Ly6G^{int} neutrophils into mAb therapy-resistant FcRγ$^{-/-}$ mice and assess whether these cell populations can both restore the mAb anti-tumor activity. The Ly6G^{hi} and Ly6G^{int} neutrophils may be considered as two different terminally differentiated neutrophil subpopulations, but also as two different stages of neutrophil development. This latter hypothesis is supported by the observation that Genista mice develop less severe forms of Ab-dependent autoimmune arthritis and IC-mediated alveolitis compared to wt mice[97]. Thus, one can hypothesize that Ly6G^{int} neutrophils that are thought to induce these reactions in Genista mice, might be precursors of Ly6G^{high} neutrophils that possess more efficient biological properties to induce inflammation.

FINAL CONSIDERATIONS / PERSPECTIVES

A new model to explain the Fc-dependent anti-tumor activities of anti-tumor mAbs

My PhD work allows proposing a detailed mechanism engaged by therapeutic mAbs that relies on neutrophils and that may be generalized for most/all models of mAb-induced subcutaneous tumor therapy models. Extension to other tumor models, in particular those based on mice naturally developing tumors, or to human solid tumors, will require more work but one can already propose that:

Once mAbs are bound to cancer cells, the Fc portion of those mAbs induce the activation of the complement cascade through the classical pathway, thus to the generation of chemotactic products that trigger neutrophil recruitment to the tumor site. Once neutrophils reached the opsonized cancer cells they will interact through their FcγRs with the Fc portion of the opsonizing mAbs that results in FcγR-dependent phagocytosis and cell activation (degranulation, secretion of cytotoxic mediators and cytokines/chemokines). In turn, the secreted cytokines/chemokines such as IL8 and CXCL1 will trigger an autocrine loop of neutrophils recruitment and infiltration into the tumor site to further reduce the tumor burden.

The finding that neutrophils are responsible for the anti-tumor activity of some therapeutic mAbs profoundly modifies, in my opinion, the way mAb therapy is conceptually perceived and may have important repercussions in a clinical point of view:

First of all, as mentioned in the introduction, the majority of clinically used therapeutic mAbs are humanized antibodies based on a human IgG1 framework. Bruhns *et al.* described in 2009 that different human FcγRs differentially bind different human IgG isotypes, but that all bind human IgG1[69]. In particular, hFcγRI bind IgG1 molecules with a high affinity ($K_A \approx 6.5.x10^7\ M^{-1}$), whereas the other hFcγRs bind IgG1 with a low affinity ($10^5 < K_A < 10^7\ M^{-1}$). Neutrophils express hFcγRIIA, hFcγRIIIB and, inducibly, hFcγRI. According to the model I propose, generating therapeutic mAb variants possessing an enhanced binding affinity towards the hFcγRs expressed on neutrophils should be a priortity in order to ameliorate the anti-tumor activity of therapeutic mAbs. A potential target for this type of development would be mutations increasing the binding to hFcγRIIA. This receptor can indeed mediate the anti-tumor activity of Trastuzumab in a human breast cancer xenograft model (refer to article 3). The contribution of hFcγRI is still under investigation in this xenograft model, but the fact that hFcγRI proved able to mediate the anti-tumor

effect of anti-gp75 mAbs in the syngeneic B16 lung metastases model (refer to article 2), strongly suggests its potential to do so in the xenograft model.

Therapeutic mAbs are, in general, used in combined treatment with CT. As already mentioned in the introduction, the effects of CT are not confined to the tumor cells but affect also other cell populations. Neutrophils are particularly susceptible to the detrimental effects of CT, as demonstrated by the fact that during CT regimen, a drop in the neutrophil count or a state iof neutropenia is generally observed. Therefore, association between CT and anti-tumor mAb therapy might drastically limit the therapeutic efficacy of these mAbs by depleting the cell population responsible for their Fc-dependent anti-tumor activities. This consideration applies especially to the case of CT and mAbs used together after resection surgery, thus as an adjuvant regimen. Among immune cells, neutrophils are indeed the first cells recruited to the site of tissue injury, i.e. a surgical intervention aiming at removing the tumor. During a combined regimen, CT may therefore reduce cancer cell proliferation and increase antigen uptake from dying cancer cells and therefore anti-tumor immune responses, but CT also reduces neutrophil numbers, i.e. effector cell numbers. One could propose therefore 1) an “uncoupled neoadjuvant treatment” where CT and anti-tumor mAbs are not administered at the same time in order to recover first from the CT-induced neutropenia to enhance the efficacy of the upcoming mAb therapy; or 2) a treatment based on the administration of CT, mAbs and a G-CSF analog (*e.g.* Filgastrim®) in order to compensate the deleterious effect of CT on neutrophil by increasing their generation from the bone marrow.

ANNEX REVIEW

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The interactions of therapeutic antibodies with Fc receptors

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ABSTRACT

During the last two decades, a rapidly increasing number of therapeutic antibodies have been generated and used in a variety of diseases. The rationale of passive immunotherapy is that, due to their unique specificity, antibodies can selectively target and act on molecules associated with pathological processes. However, how therapeutic antibodies actually act on target molecules and cells under specific conditions is poorly known. As a consequence, whether the efficacy of available antibodies could be increased and whether new antibodies could be tailored for specific purposes have not been thoroughly investigated. We discuss in this review how therapeutic antibodies interact with Fc receptors, what are the cellular responses induced by these interactions and how a better knowledge of these interactions and biological responses could improve antibody-based passive immunotherapy.

1. Introduction

The first *Nobel Prize in Physiology or Medicine* was awarded to Emil von Behring in 1901 for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths. Seven years later, Paul Ehrlich shared the same prize with Elie Metchnikoff in recognition of their work on immunity. Von Behring and Ehrlich had worked together in Berlin on humoral immunity. They were both medical doctors, but Ehrlich was also a chemist and a talented histologist. He imagined that, like tissues, blood cells or microbes can be specifically stained by dyes, pathogens could be selectively targeted by chemicals. He screened chemical compounds to find substances having a selective affinity for pathogenic organisms, and which, like "magic bullets", could go straight to the organisms at which they were aimed as antitoxins go to the toxins, and destroy them [1]. The bases of antibody-based immunotherapy were laid. It was more than one century ago.

During the first decade of the 21st century, a rapidly increasing number of therapeutic antibodies have been generated and used in a variety of diseases. The rationale of passive immunotherapy is that, due to their unique specificity, antibodies can selectively target and act on molecules associated with pathological processes. Depending on their affinity, antibodies bind indeed more or less tightly to specific antigens *via* their Fab portions. If some effects are the direct consequences of binding only, other effects are indirect consequences that depend on the Fc portion of antibodies. *Via* their Fc portion, antibodies indeed activate effector systems that can, in turn, act on targets. Antibodies can thus establish complex and multiple interactions with cells that express receptors for the Fc portion of immunoglobulins (FcR) and that can exert a variety of biological activities.

However, how therapeutic antibodies actually *act* on target molecules and cells under specific conditions is poorly known. As a consequence, whether the efficacy of available antibodies could be increased and whether new antibodies could be tailored for specific purposes have not been thoroughly investigated. We discuss in this review how therapeutic antibodies interact with FcR, what are the cellular responses induced by these interactions and how a better knowledge of these interactions and biological responses could improve antibody-based passive immunotherapy. In the accompanying article, by Malbec and Daëron, we provide an example illustrating new possible therapeutic uses of antibodies in cancer.

2. Fab only-dependent effects of therapeutic antibodies

Two main types of therapeutic antibodies have been generated and used in various pathological conditions: antibodies against soluble molecules and antibodies against membrane molecules. Antibodies against soluble molecules have been used in allergy, autoimmunity and cancer. Antibodies against membrane molecules have been essentially used in cancer. They include antibodies against molecules expressed by cancer cells and antibodies against molecules expressed by immune cells (Table 1).

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Table 1
Fab- and Fc-dependent effects of monoclonal antibodies approved for therapeutic use. CDC: Complement-Dependent Cytotoxicity, ADCC: Antibody-Dependent Cell-mediated Cytotoxicity.

	Antibody name	Target molecule	Fab-dependent effects	Fc-dependent effects		
				C-mediated	Cell-mediated (FcR)	
Cell surface molecules	Rituximab	CD20	↑ Apoptosis[a]	CDC[a,b,c]	ADCC[a,b]	(FcγRIIIA)[c]
	Ofantumumab[d]	CD20	↑ Apoptosis[a]	CDC[a,b,c]	?	(?)
	Trastuzumab	HER2	↓ Cell proliferation	?	ADCC[a,b]	(FcγRIIIA)[c]
	Alemtuzumab	CD52	↑ Apoptosis[a]	?	ADCC[b,c]	(?)
	Daclizumab	CD25	↓ T cell activation[a]	?	ADCC[b,c]	(?)
Soluble molecules	Infliximab	TNFα	↓ TNFα[c]	Not investigated	Not investigated	
	Omalizumab	IgE	↓ IgE[c]	Not investigated	Not investigated	
	Bevacizumab	VEGF	↓ VEGF[c]	Not investigated	Not investigated	

[a] *In vitro* models.
[b] *In vivo* models.
[c] Clinical evidence.
[d] Ofantumumab is a fully humanized anti CD20 Ab.

Like any other molecule, antibodies need to bind in order to exert an effect. Because they are generated after a specific B-cell receptor made of heavy and light chains that have the same variable domains, antibodies bind specifically to the antigens that have stimulated B cells, during the induction phase of the adaptive immune response. In some cases, binding is sufficient for antibodies to act on antigen-bearing molecules and cells. Binding is indeed enough for antibodies to block ligand–receptor interactions or to mimic agonists. Antibodies against soluble molecules such as IgE (Omalizumab) [2], TNF-α (Infliximab) [3] or VEGF (Bevacizumab), can block the binding of these molecules to corresponding receptors. Anti-VEGF antibodies thus prevent VEGF receptors from triggering pro-angiogenic signals that are necessary for tumors to be vascularized and grow [4]. Anti-IgE antibodies prevent IgE from binding to IgE receptors, which decreases the sensitization of mast cells and basophils and, ultimately, enhances the degradation of empty receptors [5].

Antibodies against membrane molecules expressed by tumor cells can also block receptor signaling. Anti-Her2-Neu/ErbB2 antibodies (Trastuzumab) thus prevent receptor dimerization on breast cancer cells and other cells such as ovary cancer cells and non-small cell lung cancer cells that over-express this receptor [6]. Anti-EGFR antibodies (Cetuximab) prevent the binding of EGF to receptors on non-Hodgkin lymphoma cells [7]. Other antibodies against membrane molecules expressed by tumor cells can mimic natural ligands and act as agonists. Thus, anti-CD20 antibodies (Rituximab) and anti-CD52 (Alemtuzumab) deliver pro-apoptotic signals in lymphoma B cells [8] and chronic lymphoid leukemia cells [9], respectively. Likewise, anti-CD40 antibodies can induce apoptosis in CD40-expressing tumor cells [10].

Antibodies against membrane molecules expressed by immune cells can also either block or induce receptor signaling. Anti-CTLA4 antibodies (Ipilumumab) block the binding of B7.1 (CD80) and B7.2 (CD86) to this inhibitory molecule expressed by T cells [11]. As a result, they stimulate effector T cell functions. Inversely, anti-CD40 antibodies upregulate the expression of co-stimulatory molecules on dendritic cells, thus enhancing the presentation of tumor antigens to T cells [12].

3. Fc-dependent effects of therapeutic antibodies

In most cases, however, binding to antigen is necessary but not sufficient for antibodies to exert an effect. Therapeutic antibodies against soluble molecules form immune complexes that are rapidly cleared. Antibodies against membrane-bound molecules can lead to the destruction of target cells by various mechanisms. These two effects require more than the mere binding of antibodies to antigen. Indeed, when binding to antigens through their Fab portions, antibodies also interact through their Fc portion with potent effector systems. These are of two kinds: soluble molecules and cells.

Soluble molecules include complement components some of which have an affinity for the Fc portion of several classes of antibodies. These molecules launch an enzymatic cascade that eventually leads to target cell damage. Evidence that therapeutic antibodies induce complement-dependent cytotoxicity (CDC) was obtained for Rituximab, a humanized anti-CD20 antibody approved for B cells malignancies (non-Hodgkin lymphoma/leukemia and chronic lymphoid leukemia) and Rheumatoid Arthritis (RA). Anti-CD20 antibodies could indeed kill CD20-expressing transformed B cells in the presence of complement *in vitro* [13], and complement depletion by cobra venom factor decreased their therapeutic effects in *in vivo* murine models [14]. Indirect arguments also support that complement may contribute to the clinical efficacy of Rituximab. Patients under Rituximab treatment show a reduction of complement components in serum, suggesting that anti-CD20 antibodies may consume complement [15]. Also, better therapeutic responses were induced by Ofantumumab, a second generation of fully human anti-CD20 antibody with an enhanced CDC activity, than by Rituximab, in chronic lymphoid leukemia patients [16].

Cells include the many cells, mostly of hematopoietic origin, which express FcR on their plasma membrane. FcR-expressing cells include myeloid cells of all types and some lymphoid cells such as B cells, NK cells and NKT cells. A few non-hematopoietic cells, such as some endothelial cells and some tumor cells, also express FcR. Antibodies can thus induce a variety of cellular responses, depending on the cell type. When engaged by antibodies and antigen, FcR generate intracellular signals that modulate, positively and negatively, cell responses. Some cells can internalize soluble immune complexes by endocytosis or cell–antibody complexes by phagocytosis, while some effector cells can kill target cells by antibody-dependent cell-mediated cytotoxicity (ADCC). Evidence that therapeutic antibodies engage FcR is as follows. A variety of anti-tumor antibodies, including therapeutic antibodies have been used for NK cell-dependent ADCC that kills target cells *in vitro* [17]. Strong evidence that therapeutic antibodies engage FcR *in vivo* was obtained using mice lacking FcR and transplanted with various murine or human tumor cells. The antitumor effects of Rituximab or Trastuzumab [18] were indeed lost or impaired in mice lacking activating FcR and in mice whose activating receptors were unable to signal [19]. That ADCC is responsible of these *in vivo* effects has been suggested [20] but not firmly established. The nature of effector cells is also unclear. Cell-depletion experiments suggested a role for monocytes/macrophages [21] and possibly other myeloid cells, besides NK cells. Evidence that FcR are involved in anticancer antibody-based immunotherapy is strongly supported by the correlations found between the clinical efficacy of Rituximab

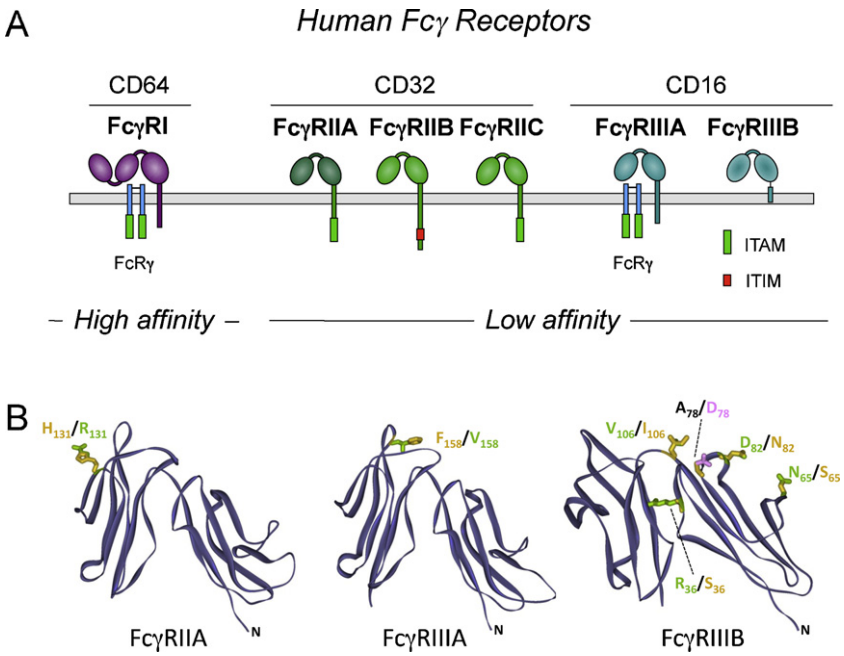

Fig. 1. Human FcγR. (A) High- and low-affinity human IgG receptors (FcγR) and corresponding clusters of differentiations (CD64, CD32 and CD16). (B) The polymorphic variants of FcγRIIA ($H_{131}R$), FcγRIIIA ($F_{158}V$) and FcγRIIIB ($N_{65}S$, $A_{78}D$, $D_{82}N$ and $V_{106}I$) are due to specific substitutions of amino acids in extracellular domains.

in follicular lymphoma patients, and polymorphic variations that affect the affinity of activating FcR for IgG expressed primarily by NK cells, but also by monocyte/macrophages [22]. This correlation was however not found in chronic lymphoid leukemia patients [23].

Although suggestive, these observations altogether underscore how poorly known the mechanisms that account for the clinical efficacy of therapeutic antibodies in specific pathological conditions are. Several mechanisms are likely to concur, but neither their relative contribution nor their possible interactions is well known. In most cases, one mechanism only is considered, and in any case, what is known accounts only partially for a complex situation. One given antibody can indeed trigger a variety of effects by engaging different FcR expressed by different cell types whose biological responses are different. Taking this complexity into account may make the problem more difficult. It may also enable to tailor therapeutic antibodies for specific purposes, open not previously envisioned therapeutic approaches and, in any case, enhance the efficacy of already available antibodies.

4. Human FcR engaged by therapeutic antibodies

Antibodies bind to FcR *via* their Fc portion with a variable affinity. A proportion of high-affinity FcR, which can bind monomeric immunoglobulins in the absence of antigen, are occupied *in vivo* whereas low-affinity FcR, which can bind antibodies as multivalent immune complexes only, are not, in spite of the high concentration of circulating immunoglobulins. Human high-affinity IgG receptors are referred to as FcγRI; low-affinity IgG receptors as FcγRII and III. There are three FcγRII in humans, FcγRIIA, B and C, encoded by distinct genes. There are two FcγRIII in humans, FcγRIIIA and B, encoded by distinct genes [24] (Fig. 1A). The diversity of human FcγRII and III is further increased by polymorphisms of selected

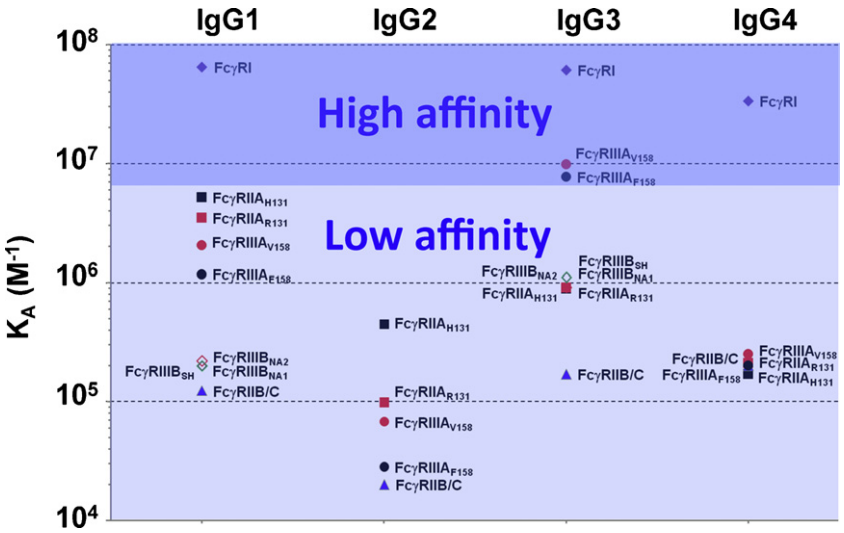

Fig. 2. Affinity of human FcγR for human IgG subclasses. (A) Binding affinity of human FcγR and their polymorphic variants for the four human IgG subclasses. (Adapted from Bruhns et al. [28]).

residues in their extracellular domains. These are $H_{131}R$ in FcγRIIA [25], $F_{158}V$ in FcγRIIIA [26], $N_{65}S$, $A_{78}D$, $D_{82}N$ and $V_{106}I$ in FcγRIIIB [27] (Fig. 1B). Altogether, 10 FcγR were described in humans.

The affinity of the 10 human FcγR for the four subclasses of human IgG was recently re-examined under conditions that enabled an accurate comparison [28]. The affinity constants (K_a) of human FcγR span over 4 Log_{10} (Fig. 2). Human IgG also bind to another high-affinity receptor named FcRn. This receptor is a unique MHC class I-like molecule that binds the Fc portion of IgG instead of peptides [29]. It plays a major role by protecting IgG from degradation [30]. Therapeutic antibodies benefit from this protection, and their half life can be augmented by introducing mutations that enhance the affinity of their Fc portion for FcRn [31]. This increases the blood concentration by modifying the pharmacokinetics of therapeutic antibodies [32].

The affinity with which IgG bind to FcγR further depends on the glycosylation of their Fc portion [33]. Each heavy chain contains a single covalently attached biantennary N-glycan at the highly conserved N_{297} residue in its CH_2 domain. Point mutations of this glycosylation site abrogate the ability of IgG antibodies to bind to FcγR. If engineered with such a mutation (*e.g.* $N_{297}Q$), aglycosylated antibodies therefore no longer engage FcγR [34] and they can be used as blocking-only molecules. Aglycosylated antibodies against specific receptors or ligands can prevent receptor–ligand interactions without engaging FcγR. Noticeably, N_{297} mutations do not affect the binding of IgG to FcRn. Aglycosylated antibodies are therefore similarly protected from degradation as glycosylated antibodies. Other mutations that remove fucose residues from the glycan chain were found to enhance the binding of modified antibodies to FcγRIIIA, irrespectively of the polymorphism that affects this receptor [35,36].

Most therapeutic antibodies derive from mouse mAb that have been genetically engineered so that their V_L and V_H domains, which determine the binding specificity and affinity of these antibodies, have been hooked to the Cκ and Cγ1 domains of human light and heavy chains, respectively. Important biological effects of therapeutic antibodies are therefore determined by the biological properties of the Fc portion of human IgG1. IgG1 bind to all human FcγR with an affinity than spans over 3 Log_{10}. Indeed, FcγRIIB and FcγRIIIB bind human IgG1 with a K_a of $10^{5-6}\,M^{-1}$, FcγRIIA and FcγRIIIA with a K_a of $10^{6-7}\,M^{-1}$, and FcγRI with a K_a of $10^{7-8}\,M^{-1}$ [28]. Interestingly, follicular lymphoma and breast cancer patients with the FcγRIIIA V_{158} polymorphism had better therapeutic responses to Rituximab [37] and Trastuzumab [38] respectively. These two IgG1 antibodies bind with a higher affinity to FcγRIIIA V_{158} than to FcγRIIIA F_{158}. Some therapeutic antibodies such as the anti-EGF Receptor antibody (Panitumumab) were engineered on a human IgG2 backbone. IgG2 bind only to human FcγRIIA, FcγRIIIA and FcγRIIB/C, and with a much lower affinity than IgG1. FcγRIIB/C and FcγRIIIA F_{158} bind IgG2 with a K_a of $\pm 2 \times 10^4\,M^{-1}$, FcγRIIA R_{131} and FcγRIIIA V_{158} with a K_a of $\pm 1 \times 10^5\,M^{-1}$ and FcγRIIA H_{131} with a K_a of $\pm 5 \times 10^5\,M^{-1}$ (Fig. 2). One notices that FcγRIIB/C have a lower affinity than other human FcγR for both IgG1 and IgG2 [28].

5. Biological responses triggered by FcγR

Like other membrane receptors, FcR can both bind specific ligands by their extracellular domains and trigger intracellular signals by their intracytoplasmic domains. FcR trigger no signal when binding immunoglobulins. They signal when aggregated on the cell membrane by antibodies and plurivalent antigens. FcγR can trigger activation signals and/or inhibition signals. The nature of signals depends on molecular motifs contained in the intracytoplasmic domains of FcγR or of receptor subunits with which FcγR associate [39].

Activating human IgG receptors are FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA. FcγRIIA and FcγRIIC are single-chain receptors the intracytoplasmic domains of which are identical and contain a single *Immunoreceptor Tyrosine-based Activation Motif* (ITAM) [40]. FcγRI and FcγRIIIA are associated with the common FcR subunit FcRγ. FcRγ is a homodimer which contains two ITAMs [41]. Upon aggregation, the activating FcγR ITAMs are phosphorylated by src family tyrosine kinases, which initiates the constitution of intracellular signalosomes in which activation signals are dominant over inhibition signals.

Inhibitory receptors are FcγRIIB. FcγRIIB are single-chain receptors the intracytoplasmic domain of which contains one *Immunoreceptor Tyrosine-based Inhibition Motif* (ITIM) [42]. Unlike activating receptors, FcγRIIB trigger no intracellular signal upon aggregation. They trigger negative signals when they are coaggregated with activating receptors by immune complexes [43]. Under these conditions, the ITIM of FcγRIIB is phosphorylated by the same src-family tyrosine kinase that phosphorylates ITAM in activating receptors [44]. Phosphorylated FcγRIIB recruit inhibitory molecules that are brought into signalosomes. This renders inhibition signals dominant over activation signals [45]. The signaling properties of the glycosylphosphatidylinositol-anchored FcγRIIIB, which have no intracellular domain and no known associated subunit, remain unclear.

The nature of biological responses triggered by IgG antibodies primarily depends on the cell type. Activating FcγR can indeed trigger a variety of cellular responses, including endocytosis of soluble immune complexes, phagocytosis of particulate complexes, exocytosis of preformed granular mediators, including vasoactive amines, proteolytic enzymes, and/or cytotoxic molecules, the production of newly-formed lipid-derived proinflammatory mediators or the secretion of newly-transcribed cytokines, chemokines and growth factors. The same set of biological responses can be triggered by different FcRγ-associated FcγR in a given cell type. FcγRIIA/C, however, may not trigger all responses triggered by FcγRI or FcγRIIIA [46]. One likely reason is that the FcγRIIA/C ITAM is different from the FcRγ ITAM. In any case, however, biological responses are determined by the functional repertoire of the cell. Biological responses triggered by therapeutic antibodies therefore depend on the tissue distribution of FcγR.

Most cells express more than one FcγR (Fig. 3). Except NK cells, which express FcγRIIIA only and B cells, which express FcγRIIB only, other FcR-expressing cells co-express one or several activating and inhibitory FcγR. Noticeably, most human cells of hematopoietic origin express no or low-levels of FcγRIIB, except B cells and basophils. Differing from human mast cells, which express little FcγRIIB if any [47], human basophils indeed express more FcγRIIB than any other blood leukocytes (our unpublished results). Human antibodies of every IgG subclass bind to more than one FcγR. It follows that antibodies such as human IgG1, and therefore therapeutic antibodies, engage a complex of several FcγR, the composition of which depends on the cell type. Antibodies thus trigger a mixture of activation and inhibition signals the integration of which determines the intensity and the quality of the responses of effector cells.

6. Interactions of therapeutic antibodies with FcR-expressing cells

If one understands that, except aglycosylated antibodies that can be used as blocking reagents or as agonists, therapeutic antibodies against cell surface molecules are expected to engage FcγR on various cell types, one may consider two types of interactions, depending on whether FcγR and target molecules are on distinct cells or on the same cells.

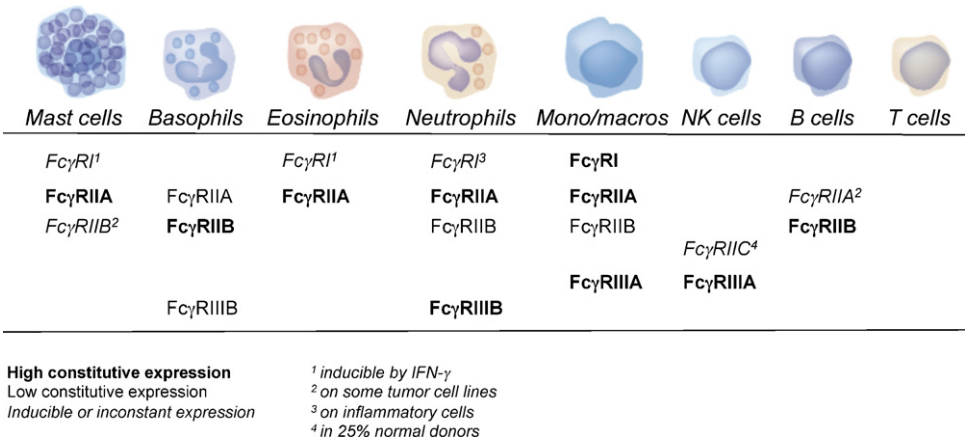

Fig. 3. Tissue distribution of human FcγR. FcγR expression pattern on human myeloid and lymphoid cells.

Antibodies interact in *trans* when the target molecule and FcγR are on different cells. This situation applies for target cells that do not express FcγR, *i.e.* mostly for non-hematopoietic cells. Under this condition, one primarily expects that effector cells are activated and that the consequences of this activation affect target cells. Biological consequences are, for instance, phagocytosis or ADCC of cells expressing target molecules by cells that express ITAM-containing receptors. Typically, therapeutic antibodies are envisioned to act in *trans*. Effector cells can express activating FcγR only (Fig. 4A), such as NK cells, or co-express activating and inhibitory FcγR (Fig. 4B), such as monocyte/macrophages. Both types of cells can act on the same target cell (Fig. 4C).

Antibodies interact in *cis* when the target molecule and FcγR are on the same cell (Fig. 4D). This situation applies for target cells that expresses FcγR, *i.e.* mostly for hematopoietic cells. An important consequence is that the binding of Fab portions to a specific membrane antigen markedly increases the probability that the Fc portion of the same antibody binds to FcγR on the same membrane. As a result, antibodies bind with a high avidity, even to FcR that have an affinity as low as that of FcγRIIB. Under this condition, target/effector cells can either be activated or inhibited, depending on the FcγR. Cell activation resulting from a *cis*-interaction of IgG antibodies was first described in mast cells which could be induced to degranulate vigorously by anti-MHC class I molecule antibodies that bound simultaneously to these molecules by their Fab portions and to FcγR by their Fc portion on the same cell [48,49]. Cell inhibition resulting from a *cis*-interaction of IgG antibodies was first described in B cells which could be activated by F(ab′)2 fragments of anti-immunoglobulin antibodies that could aggregate BCR, but not by intact anti-immunoglobulin antibodies that bound simultaneously to BCR by their Fab portions and to FcγRIIB by their Fc portion on the same cell [50]. Either one outcome or the other can be aimed at, depending on the pathological state. An example of such an approach is provided in the accompanying article (by Malbec and Daëron). Finally, both *cis* and *trans* interactions can conceivably involve a single target cell (Fig. 4E).

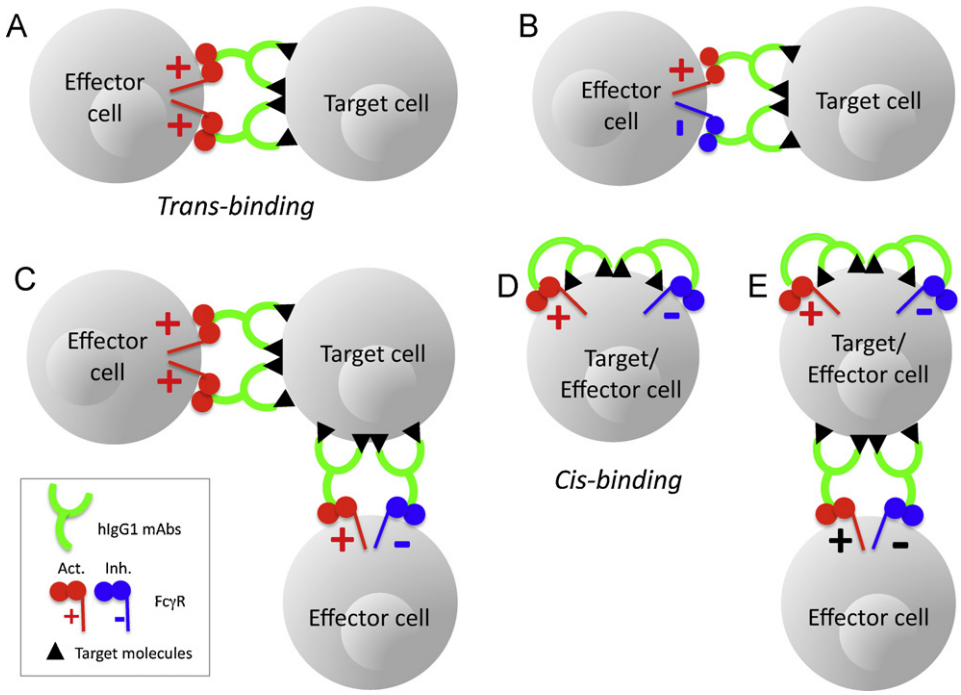

Fig. 4. Different interactions of therapeutic antibodies with FcγR-expressing cells (A–C) *Trans* binding: target molecules and FcγR are on different cells. (D) *Cis* binding: target molecules and FcγRs are on the same cell. (E) *Cis* and *Trans* binding can occur at the same time.

Target molecules recognized by the Fab portions of therapeutic antibodies can, themselves, have signaling properties or not. Therapeutic antibodies against non-signaling molecules can act in *cis* or in *trans*, depending on whether the target cell expresses FcγR or not. For instance, B cells co-express CD20 and FcγRIIB, and one cannot envision that, when binding to CD20, Rituximab does not co-engage FcγRIIB on the same B cell. A similar effect can be anticipated for other cancer cells that express FcγRIIB such as some melanoma cells [51].

Therapeutic antibodies against signaling molecules can be specific for activating receptors. Conceivably, these can be receptors that control cell activation such as FcR themselves, receptors that control cell proliferation such as growth factor receptors (*e.g.* RTK) and cytokine receptors, or receptors that control both such as BCR and TCR. As discussed above, when acting on ErbB2-expressing breast cancer cells, Trastuzumab does not only interfere with RTK signaling [6]. It also engages FcγR, and FcγR signaling contributes to the therapeutic effect [18]. Therapeutic antibodies against activating receptors can act in *cis* or in *trans*, depending on whether the target cell expresses FcγR or not. The outcome can be expected not to be the same.

Therapeutic antibodies against signaling molecules can be specific for inhibitory receptors. These can be CTLA4, on T cells [52], KIRL on NK cells [53], or other inhibitory receptors that use the SH2 domain-containing protein tyrosine phosphatases SHP-1/2 as the intracellular effectors of inhibition [54]. They can also be FcγRIIB that use the SH2 domain-containing inositol phosphatases SHIP1/2. One example is an anti-KIRL mAb developed by Innate Pharma [53]. Therapeutic antibodies against inhibitory receptors can also act in *cis* or in *trans*, depending on whether the target cell expresses FcγR or not, and this should affect the outcome. Thus, anti-KIRL antibodies can be expected to co-engage the inhibitory receptors with activating FcγRIIIA on NK cells. Likewise, anti-FcγRIIB antibodies can be used as a therapeutic tool to increase the efficacy of immune responses. An anti-human FcγRIIB antibody was indeed generated, with a $N_{297}Q$ mutation [34]. This antibody, could be used to prevent the co-engagement of FcγRIIB and therefore FcγRIIB-dependent inhibition. Indeed, when co-engaged in *cis* with CD20 by Rituximab on B cells, FcγRIIB is phosphorylated, enhances the internalization of Rituximab and reduces its clinical efficacy [55]. An aglycosylated anti-FcγRIIB antibody could therefore be proposed as an adjuvant to enhance the effects of Rituximab.

On the basis of the above points, therapeutic antibodies can exert a variety of biological effects that will depend essentially on the target molecule, hence on the target cell that expresses this molecule, and on FcγR expressed by effector cells, hence on the effector cells involved. Four situations can be considered, depending on whether the target molecules recognized by antibodies are (1) non-signaling molecules, (2) activating receptors, (3) inhibitory receptors or (4) a mixture of activating and inhibitory receptors. These four types of antibodies can engage activating FcγR only, inhibitory FcγR only or a mixture of activating and inhibitory FcγR. The biological effects will obviously not be the same in the different combinations.

7. FcR-dependent mechanisms induced by therapeutic antibodies

These complex interactions with target and/or effector cells provide the bases of the mechanisms that account for several of the biological effects of therapeutic antibodies. Below are a few examples of such mechanisms. These mechanisms and others could be further exploited for other purposes.

7.1. Deleting pathological cells or cells associated with pathological processes

Targeting tumor cells with therapeutic antibodies has been an obvious aim of passive immunotherapy of cancer. In this therapeutic approach, antibodies are used as a mean to eradicate malignant cells. They can indeed promote phagocytosis and ADCC and, by this mechanism, somehow reduce the tumor load. This, however, can be nothing but an adjuvant treatment, following surgery and/or associated with chemotherapy. Non-tumor cells can also be targeted by therapeutic antibodies. Anti-CD3 antibodies (OKT3/Muromonab) were among the first approved therapeutic antibodies. They were first used to induce a state of immunodeficiency by deleting T cells and to prevent acute graft rejection following kidney, heart or liver transplantation [56]. They were subsequently used in autoimmune diseases such as psoriasis [57] and type 1 diabetes. In the latter case, a mild immunodeficiency can be induced by aglycosylated anti-CD3 antibodies (Teplizumab) [58] that block CD3 on T cells instead of deleting $CD3^+$ cells. Likewise, anti-CD25 antibodies (Daclizumab) have been used to decrease regulatory T cell numbers [59], anti-CD20 antibodies (Rituximab) and anti-CD22 antibodies (Epratuzumab) to deplete auto reactive B cells in Rheumatoid Arthritis [60] and Systemic Lupus Erythematosus, respectively [61]. According to this reasoning, one can also think of using antibodies to deleted effector cells involved in pathological inflammation such as allergies.

7.2. Enhancing antigen presentation by targeting tumor antigens

Increasing evidence accumulated in the recent years, supporting the idea that an efficient antitumor treatment may not primarily depend on the eradication of tumor cells. Chemotherapy has indeed been recognized as having more complicated effects, involving apoptosis induction and enhanced classical antigen presentation or cross-presentation of dead or altered tumor cells to T cells [62]. FcγR engagement by anti-tumor antibodies affects antigen presentation by dendritic cells. Depending on the type of receptor *i.e.* activating *vs* inhibitory FcγR, antibodies can either enhance [63] or decrease tumor antigen presentation [64]. Both types of receptors are expressed by DC.

7.3. Enhancing antigen presentation by targeting DC molecules

An alternative approach to enhance antitumor immune responses with antibodies consists of targeting antigen-presenting cells. Anti-CD40 antibodies are one example. They have been used as immunostimulatory molecules. By increasing the expression of accessory molecules such as CD80 and CD86 on dendritic cells, CD40 ligation indeed enhances antigen presentation to T cells and, consequently, T cell activation. It was recently found in a murine model that the coengagement of CD40 and FcγRIIB by anti-CD40 antibodies enhanced T cell-dependent cytotoxicity against tumor cells [65]. This immunostimulatory effect was shown to depend on the coengagement of the two molecules *in trans* on two individual cells and not to depend on FcγRIIB-dependent signaling. Noticeably, a similar effect could be induced when coengaging FcR other than FcγRIIB *in vitro*, but not *in vivo*, and it depended on the density of FcγR. Thus, the apparently paradoxical enhancing effect of FcγRIIB could be explained by a "passive" effect of FcγR that enhances CD40 ligation on neighboring cells, and the apparent specificity for FcγRIIB by its tissue distribution and availability on cells present in the environment [66].

7.4. Controlling effector cells in tumor microenvironment

The role of inflammatory processes in tumor microenvironment has been increasingly recognized as being critical both in the initiation and in the outcome of cancer. One could therefore aim either at dampening or, perhaps, at promoting local inflammation. Antibodies that can engage inhibitory FcR could be used to decrease the production of pro-inflammatory mediators of inhibit their biological effects in the early phase of cancer. On the contrary, antibodies that can engage activating FcR could be used to induce the release and/or the secretion of biologically active mediators, including chemokines that could attract inflammatory/effector cells in tumors, and cytokines that would activate them. The presence of immune effector cells, such as CD8T cells and other cytotoxic cells in the tumor microenvironment was found to critically determine the prognosis of colorectal cancer [67].

7.5. Altering the balance between activating and inhibitory FcR

Antibody-induced cell activation depends on the integration of positive and negative signals generated when activating and inhibitory receptors are co-aggregated. One can envision the balance between activating and inhibitory FcR as a therapeutic target for immune intervention at the effector phase of immune responses. One mean to act on this balance is to block one of the receptors using anti-FcR mAbs. FcγRIIA is widely expressed on human myeloid cells, and most FcγRIIB-expressing cells co-express FcγRIIA. Blocking FcγRIIA can therefore be expected to prevent FcγRIIA-dependent cell activation and to favor FcγRIIB-dependent negative regulation. This should be beneficial in antibody-dependent inflammatory diseases such as allergies or some autoimmune diseases. Inversely, blocking FcγRIIB can be expected to inhibit FcγRIIB-dependent negative regulation and to favor FcγRIIA-dependent cell activation. This should be beneficial for anticancer immunotherapy, such as Rituximab treatment. Another mean to alter the balance between activation/inhibition signals is to generate therapeutic antibodies the Fc portion of which have been genetically engineered so that they bind with a higher affinity to activating or to inhibitory receptors. Thus, an anti-human CD19 antibody was produced, the Fc portion of which has a markedly enhanced affinity for human FcγRIIB. By co-engaging FcγRIIB with the BCR complex, this antibody profoundly inhibited the *in vitro* activation and proliferation of B cells from healthy donors or from SLE patients, decreased serum immunoglobulins in mice engrafted with PBMC from a SLE patient and increased survival in these mice [68]. Inversely, an anti-CD20 antibody was produced, the Fc portion of which has a markedly higher affinity for activating FcγR, resulting in an enhanced ADCC [69].

8. Conclusion

Antibody-based passive immunotherapy has now proved to be feasible and clinically useful. It has, however, been used in a limited number of pathological conditions only. Its efficacy could be significantly enhanced, and its indications could be markedly broadened. For this aim to be reached, one needs to take into consideration a complexity that has not been considered before.

Even though the binding of therapeutic antibodies to target molecules can be enough to generate therapeutic effects, one cannot consider therapeutic antibodies as antigen-binding molecules only. Most of the biological effects exerted by antibodies depend on their ability to engage FcR. By being differentially expressed on a variety of lymphoid and myeloid cells, FcγR can trigger a wide array of biological responses. By generating antagonistic signals in a single cell in response to their engagement by a single type of antibody, activating and inhibitory FcγR can finely tune cellular responses as a function of stimuli present in the local environment. By engaging target molecules and FcγR not only *in trans* on different cells, but also *in cis* on the same cell, therapeutic antibodies further enhance the functional repertoire of cellular responses. Therapeutic antibodies can therefore have a multiplicity of effects, some of which may be beneficial, while others can be detrimental.

Favoring the conditions that will enhance the beneficial effects and reduce the detrimental effects is an attractive aim to be reached. This requires that the complex interactions of therapeutic antibodies with target molecules and cellular receptors are better understood. One expects this knowledge to enable therapeutic antibodies to be engineered so that they engage the right combination of FcγR on the right cells. Besides enabling the clinical efficacy of available therapeutic antibodies to be improved, this knowledge may also enable new antibodies to be tailored for new therapeutic indications, as exemplified in the accompanying article. The challenge is to master the *in vivo* combinatorial complexity of interactions between cells and molecules.

Conflict of interest

The authors declare to have no financial conflict of interest.

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