Metabolic adaptation of inflammatory neutrophils in human diseases revealed by retroviral envelope-derived ligands: focus on cystic fibrosis

Julie Laval

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Metabolic adaptation of inflammatory neutrophils in human diseases revealed by retroviral envelope-derived ligands: focus on cystic fibrosis

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À ma plus grande force,
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ABBREVIATIONS

4E-BP	eIF4E -binding protein
AA	amino acids
AMP	adenosine mono-phosphate
APC	antigen-presenting cells
APE	acute pulmonary exacerbation
BM	bone marrow
CF	cystic fibrosis
CFRD	cystic fibrosis related-diabetes
CFTR	cystic fibrosis transmembrane conductance regulator
DAMP	damage-associated molecular pattern
DC	dendritic cells
DC	disease control
DNA	deoxyribonucleic acid
eIF4E	eukaryotic initiation factor 4E
Env	retrovirus envelope-glycoprotein
FAO	fatty acid oxidation
HC	healthy control
HIF-1α	hypoxia inducible factor-1α
MAMP	microbe-associated molecular pattern
MPO	myeloperoxidase
mTOR	mammalian Target of Rapamycin
NE	neutrophil elastase
NET	neutrophil extracellular traps

OxPhos oxidative phosphorylation

PAMP pathogen-associated molecular pattern
PI3K phosphatidylinositol 3-kinase
PPP pentose phosphate pathway

RA rheumatoid arthritis
RBC red blood cell
RBD receptor binding domain
RNA ribonucleic acid
RNS reactive nitrogen species
ROS reactive oxygen species

S6K ribosomal protein S6 kinase b1
SLC solute carrier
SS steady-state

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INTRODUCTION

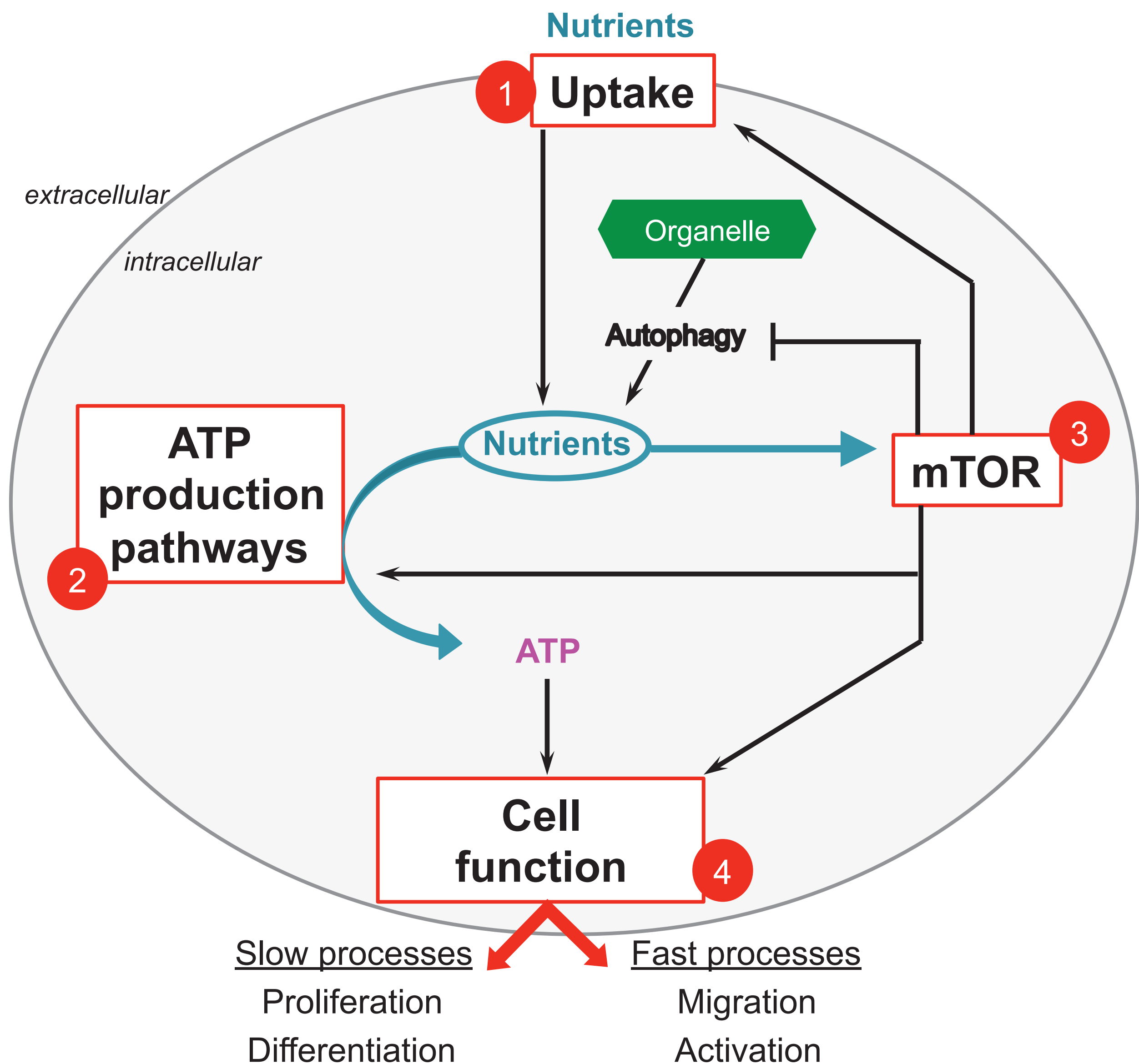


Fig. 1 Impact of metabolism on immune cell function. Schematic representation of immune cell functions that can be modulated by metabolism, notably nutrient uptake and utilization and mTOR pathway activation.

CHAPTER I: IMMUNOMETABOLISM

The interconnections and interdependence between immunity and metabolism are now largely documented and integrated models that articulate both systems are now emerging *(Odegaard and Chawla 2013)*, which has led some investigators to coin the term "immunometabolism". In the overall organism, the main outcome of these two system interactions is the control of basic physiological processes responsible for appetite / food intake, growth, development and reproduction. At levels of cells and tissues, immunometabolism governs nutrient availability, nutrient consumption and subsequent energy production (generally as ATP), thus allowing an adequate immune response to unfold.

In this introductory chapter, we are focusing on pathways that preside over cell metabolism and drive immune cell activity (Fig. 1), most notably pathways that involves the anabolic mammalian target of rapamycin (mTOR), which plays a central role in immunometabolism. This pathway is activated in response to environmental cues including nutrients, and is thus driven and modulated by nutrient uptake (see section I-A.1). Once nutrients such as glucose, amino acids (AA) or fatty acids are taken up, they can be used by immune cells to produce ATP, through either fast or slow catabolic processes, which condition their activation level and the intensity and duration of the immune response (see section I-A.2). Therefore, mTOR is involved in the on-switch of cellular anabolism, which in immune cells is linked to the modulation of specific defense functions (see section I-B).

I-A. Overview of cell metabolism

I-A.1. Nutrients and uptake mechanisms

In order to engage in metabolic activity and produce energy, cells need to take up nutrients from the external environment, which is accomplished at the cell membrane by means of various absorption processes including endocytosis and transmembrane transporters (Table 1).

I-A.1.a. Endocytosis

Endocytosis is the general term use to define the engulfment of extracellular material by cells, along with plasma membrane proteins and lipids *(Grant and Donaldson 2009)*. In addition to aiding in nutrient uptake, as performed by all cell types, this process has been involved in regulating cell polarity, signal transduction, membrane recycling, immune cell adhesion and migration and other functions such as virus entry *(Turley, Inaba et al. 2000, Fabbri, Di Meglio et al. 2005, Masilamani, Narayanan et al. 2008, Grant and Donaldson 2009, Knorr, Karacsonyi et al. 2009, Capuano, Paolini et al. 2012)*.

Endocytosis occurs via two main processes, named clathrin-dependent and independent *(Sorkin and von Zastrow 2009)*. Clathrin is a protein found at sites of endocytosis on the cytoplasmic side of the plasma membrane and arranged in specialized "pits" (clathrin-coated pits) that facilitate surface receptor endocytosis via small vesicle formation. Another type of endocytic structures, caveolae, involves vesicle formation by membrane invagination and depends on a multipass integral membrane protein named caveolin *(Alberts 2002)*. Clathrin-coated pits and caveolae are associated with receptor mediated-endocytosis (Table 1) and are responsible in part for the ingestion of small molecules.

The non-receptor-mediated form of endocytosis is known as pinocytosis (a greek term meaning “cell drinking”). Pinocytosis is associated with the uptake of large particles, including microorganisms or dead cells, for which the formation of big vesicles is essential *(Alberts 2002)*.

Types	Specific	Active	Examples of metabolites and small molecules
Endocytosis			
- Receptor mediated	Yes	Yes	Fatty acids, Vitamins
- Non-receptor mediated	No	Yes	Glucose, AA, Pi
Transmembrane transport			
- Diffusion	Yes	No	O_2, H_2O
- Pores / Channels	Yes	No	H_2O
- Transporters / Channels	Yes	Yes	Glucose, AA, Pi, Vitamins, Nucleotides

Table 1 **Metabolite and small molecule uptake mechanisms in cells.** Nutrient uptake is mediated by endocytosis and transmembrane transport. These processes can be specific for a particular metabolite or metabolite family. They can use the electrochemical gradient (passive) or go against the solute concentration (active). Pi= inorganic phosphate.

Pinocytosis has been associated with "shaping cups", characteristic membrane extensions shaped as a pocket, which then fuse to form the macropinosome or phagosome *(Swanson 2008)*. Macropinocytosis is an actin-dependent process of membrane invagination, where molecules and a significant amount of extracellular fluid are engulfed into the vesicle *(Grant and Donaldson 2009)*. While macropinocytosis has been described in various cell types, phagocytosis (a greek term meaning "cell eating") is only observed in macrophages, dendritic cells and neutrophils. Indeed, these professional phagocytes extend and fuse their pseudopods around the external material to form a specific endocytic vesicle that contains microorganisms or dead cells *(Swanson 2008, Grant and Donaldson 2009)* (Table 1).

Once the cargo has been captured, the endocytic vesicle fuses with the early endosome. Then, the endosomal trafficking can either follow a recycling pathway (e.g., integrin turnover during neutrophil chemotaxis *(Fabbri, Di Meglio et al. 2005)*) or shuttle the internalized molecule to late endosomal and lysosomal compartments for full degradation *(Stoorvogel, Strous et al. 1991, Luzio, Rous et al. 2000, Grant and Donaldson 2009, Sorkin and von Zastrow 2009)*.

I-A.1.b. Transmembrane transport

Gases such as O_2, CO_2, N_2, and other *hydrophobic* molecules readily dissolve in the lipids of the plasma membrane and other intracellular compartments and diffuse through. Just as well, but usually with slower kinetics, small uncharged molecules such as H_2O and urea also diffuse freely across membranes *(Alberts 2002)*, although, in addition to passive diffusion, H_2O can also flow via facilitative channels, the aquaporins. Transmembrane transport of *hydrophilic* molecules (ions, nutrients, etc.) is more complex. The lipid bilayer of the plasma membrane (and that of intracellular organelles, too) contains a plethora of specific transmembrane transport structures that allow exchanges of hydrophilic molecules (ions, nutrients, etc.) between extra- and intra-cellular compartments. The size, polarity and relative extra- vs. intra-cellular concentrations of transported molecules cause transport to occur through two different systems, i.e., channels and carrier proteins / transporters.

Channels use the electrochemical and / or concentration gradient(s), and thus qualify as *passive* or facilitative transport. They are multitransmembrane proteins with specific pore properties (size, selectivity and specificity) that can fluctuate between opened or closed states. Their activity is controlled by the membrane potential, extra- or intra-cellular ligand binding or mechanical stress *(Alberts 2002)*.

By contrast, *active* transport through carrier proteins / transporters goes against electrochemical and / or concentration gradient(s) and requires energy, often in the form of ATP to allow the transport across the membrane to occur *(Alberts 2002, Ren and Paulsen 2005)* (Table 1). Carrier proteins / transporters are specialized membrane transporters that promote transmembrane transport of ions, peptides, lipids and others molecules such as sugars *(Hediger, Clemencon et al. 2013)*. In eukaryotes, there are two main types of transporters:

- Primary transporters, for example, the ATP-Binding Cassette (ABC) family of transporters. ABC transporters comprise two intracytoplasmic sites for ATP binding and hydrolysis that regulate structural and conformational changes that are necessary for transmembrane transport to occur. Another example of primary transporters is the ATPase-coupled family of transporters, which also hydrolyze ATP to allow transmembrane solute transport.
- Secondary or co-transporters involving the transport of a second solute in the same (symport) or opposite (antiport) direction.

An important group of membrane-spanning transporters in mammals is that of the solute carriers, or SLC, which include facilitative and secondary active transporters. This group is divided in distinct families (43 in 2004, 52 as in 2013), relative to the solute(s) they transport. For example, solute carriers transporting neutral AA, glucose or phosphate belong to the SLC1, SLC2 and SLC20 families, respectively. It has been shown that about more than 2000 genes in human are transporter-related, of which ~15% are identified as SLC.

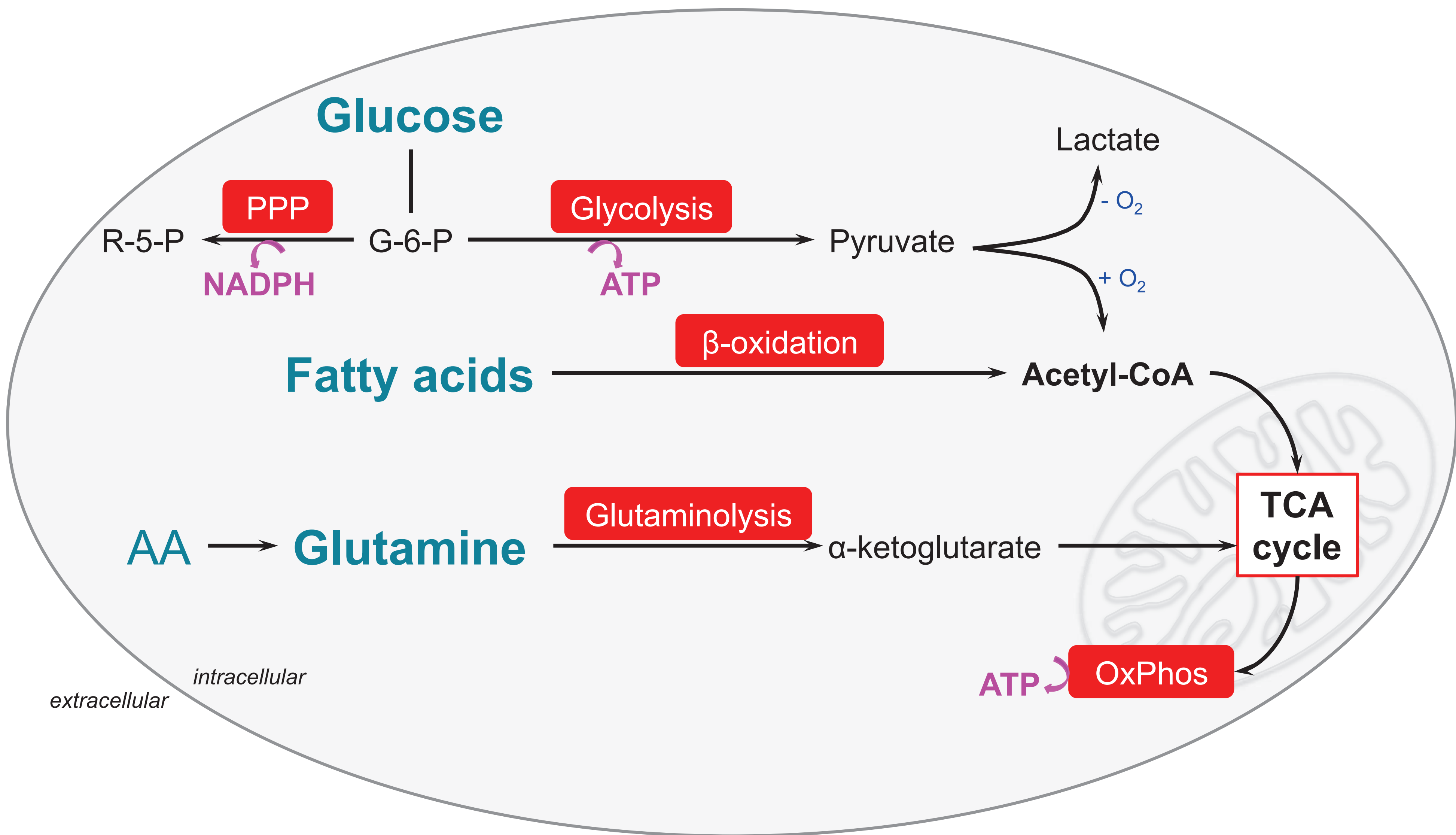


Fig. 2 Metabolic pathways and energy production. Cells are able to produce ATP (as well as NADPH) through metabolic conversion of glucose, fatty acids and AA (notably glutamine). AA= Amino acids; Acetyl-CoA= Acetyl-coenzyme A; G-6-P= Glucose-6-phosphate; OxPhos= Phosphorylation oxidative; PPP= Pentose phosphate pathway; R-5-P= Ribose-5-phosphate; TCA= Tricarboxylic acid.

Mutations in specific SLC genes have been involved in human diseases linked to metabolite absorption deficiencies, hypertension, and neurological diseases *(Wang, Li et al. 2012, Hediger, Clemencon et al. 2013, Pearson, Akman et al. 2013)*. Activity of SLC transporters has been involved with cell activation, shown for instance with Glut1/SLC2A1, the main glucose transporter *(Swainson, Kinet et al. 2005, Kinet, Swainson et al. 2007, Lavanya, Kinet et al. 2008, Loisel-Meyer, Swainson et al. 2012)*, the inorganic phosphate transporter PiT1/SLC20A1 *(Beck, Leroy et al. 2009)*, and notably with ASCT2/SLC1A5, through the modulation of mTOR-dependent induction of anabolic responses *(Wullschleger, Loewith et al. 2006, Nicklin, Bergman et al. 2009)*. Moreover, a key role of SLC regulation is expected in immune cells, since both the Glut1-AKT and ASCT2-mTOR pathways are tightly associated with the unfolding of immune responses (see section I-B.2 for the latter).

I-A.2. Metabolic pathways and energy production

Recruitment to a peripheral area of quiescent immune cells followed by cytoskeletal rearrangements, *de novo* gene transcription and protein production, and potentially, proliferation, require high amount of energy, generally in the form of ATP. Within a given organism, each cell metabolizes nutrients into energy for its own use and the level of activation and corresponding energy availability will condition the capability of cells to enter into fast processes of energy production (generally linked to cytosolic enzymes and acute activation) or slow processes of energy production (generally linked to mitochondrial activity and long-term differentiation and survival pathways) (Fig. 2).

I-A.2.a. Fast processes of energy production

Glycolysis is a well-described metabolic process, by which the transformation of glucose in the cytoplasm forms ATP, with pyruvate as a by-product. The downstream fate of pyruvate then distinguishes between different pathways of cell metabolism and activity. In presence of O_2, pyruvate is generally taken into mitochondria and transformed in acetyl-CoA to enter

the tricarboxylic acid (TCA) cycle (see section I-A.2.b). In humans, this metabolic pathway is active in most quiescent cells and differentiated tissues. However, during inflammatory or stress responses (e.g., lymphocyte proliferation or tumor growth), pyruvate undergoes quick cytoplasmic fermentation to lactate. This pathway, known as aerobic glycolysis or Warburg effect has been described as the main pathway for ATP production in immune cell activation *(Vander Heiden, Cantley et al. 2009, Pearce and Pearce 2013)* (Fig. 2 and Table 2).

Aerobic glycolysis takes on a different importance in innate and adaptive immune subsets:

- Among *innate* subsets, activated pro-inflammatory M1 macrophages, myeloid-derived dendritic cells and neutrophils have been described to increase energy output from aerobic glycolysis, along with an active pentose phosphate pathway (PPP) *(Haschemi, Kosma et al. 2012, Pearce and Pearce 2013)*. The PPP is an important ancillary glucose catabolic process for nicotinamide adenine dinucleotide phosphate (NADPH) synthesis, thus providing the NADPH oxidase enzyme its co-factor PPP reaction also provides pentose sugars that can be used for nucleotide synthesis *(Grant 2008)*. In activated neutrophils, increased O_2 consumption, combined with high levels of NADPH, supports significant NADPH oxidase activity and reactive oxygen species (ROS) production. In M1 macrophages, NADPH is used in conjunction with L-arginine to promote NO synthesis and the production of downstream reactive nitrogen species (RNS), and also contributes to the cell redox balance *(Aktan 2004)* (Fig. 2). Thus, aerobic glycolysis in the context of neutrophil, M1 macrophage, or myeloid-derived dendritic cell activation is mainly associated with lactate and ROS/RNS production *(Pearce and Pearce 2013)*.
- Among *adaptive* subsets, aerobic glycolysis has also been described in highly proliferative cells, such as clonally expanding T lymphocytes, which undergo an acute switch from their normal, mitochondria-based metabolism, to this faster process.

Studies of long-lived *adaptive* immune cells however reveal persistent energy production through mitochondria *(van der Windt, Everts et al. 2012)* (Table 2).

I-A.2.b. Slow processes

As introduced above, mitochondria are able to produce energy in the form of ATP through the process of oxidative phosphorylation (OxPhos), using the electron transport chain in their inner membrane. Indeed, the TCA cycle that is centered on the transformation of acetyl-CoA generates by-products (NADH and FADH2) which donate electrons that start the electron transport chain. In addition to glucose-derived pyruvate, acetyl-CoA can originate from glutamine or fatty acids. Glutamine is an AA and an end-product of degradation of other complex AA, which can be further degraded within mitochondria (a process termed glutaminolysis), leading to the formation of α-ketoglutarate that then directly feeds into the TCA cycle *(Duran and Hall 2012)*. Fatty acids are degraded by β-oxidation, leading to the formation of acetyl-CoA, through successive enzymatic steps occurring in large part within the mitochondrial matrix. Thus, glycolysis (for glucose), glutaminolysis (for glutamine) and β-oxidation (for fatty acids) are three major mechanisms that yield metabolic precursors, or "fuel", for OxPhos *(Carracedo, Cantley et al. 2013)* (Fig. 2).

OxPhos has been described in all differentiated tissues. However, in the immune system, only *adaptive* subsets and long-lived *innate* subsets seem to use it. Resting T lymphocytes have been shown to engage in OxPhos using glutamine (naïve T cells) or fatty acids (memory and regulatory T cells) as fuel *(Michalek and Rathmell 2010, Michalek, Gerriets et al. 2011)*. Long-lived anti-inflammatory M2 macrophages and resting myeloid-derived dendritic cells oxidize fatty acids and glucose, respectively, to produce ATP by OxPhos *(Vats, Mukundan et al. 2006, Pearce and Pearce 2013)* (Table 2). By contrast, in neutrophils, energy production has never been associated with OxPhos. In these cells, mitochondrial activity is linked to anti-microbial and survival pathways *(Pearce and Pearce 2013)*.

Metabolic pathway	Cell type	Condition
Glycolysis	Neutrophils, Basophils, Eosinophils, DC, Macrophages (M1)	Resting / Activated
	Lymphocytes T and B	Proliferating
PPP	Neutrophils, Macrophages (M1)	Activated
FAO	Lymphocytes (Memory / Regulatory), Macrophages (M1 / M2)	Activated
Glutaminolysis	Lymphocytes (Naïve)	Resting
OxPhos	Lymphocytes, DC	Resting
	Macrophages (M2)	Activated

Table 2 **Energy production in immune cells.** Glycolysis, Pentose Phosphate Pathway (PPP), Fatty Acid Oxidation (FAO), Glutaminolysis and Oxidative phosphorylation (OxPhos) are metabolic pathways producing ATP. DC= dendritic cells; M1 / M2 designate two types of monocytes / macrophages.

I-B. mTOR pathway: a central player in immunometabolism

Cues from the environment and neighboring cells can modulate cell function via activation / inhibition of the mTOR signaling pathway. Owing to its versatile responses to the cell environment, this pathway has been involved, not only in development and tumorigenesis, but also during ATP production, activation and deployment of defense mechanisms by innate and adaptive immune cells.

I-B.1. mTOR: upstream and downstream

The mammalian *tor* gene, conserved throughout evolution, codes for a serine-threonine kinase forming two protein complexes, mTORC1 and mTORC2, which perform non-overlapping functions within cells *(Russell, Fang et al. 2011)*, and refer to the assembly of different subunits around the mTOR protein. The most studied complex, mTORC1, has been involved in various cell functions during proliferation, while mTORC2 has been mostly associated with cytoskeletal organization. Henceforth, we refer to "mTOR" as the entity that encompasses both mTORC1 and mTORC2, unless otherwise stated. The drug rapamycin is a specific inhibitor of mTOR activation, hence its appellation. Rapamycin was described as directly binding mTOR, disrupting the interaction with its binding partners, thus regulating complex formation.

I-B.1.a. Upstream modulators of the mTOR pathway

The mTOR pathway is activated when hormones, growth factors and nutrients such as AA are taken up via cognate receptors or by pinocytosis *(Hara, Yonezawa et al. 1998, Russell, Fang et al. 2011)*. Tonic inhibition of mTOR signaling is mostly performed by tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2), which exert negative control over the small GTPase Rheb. Inhibition of TSC1/TSC2 by phosphorylation leads to stabilization of Rheb in its GTP-bound, active form, which. in turn, increases mTOR activity *(Inoki, Li et al. 2003)*. Consequently, upstream modulators of TSC1/2 phosphorylation impact mTOR signaling *(Russell, Fang et al. 2011)* (Fig. 3).

The predominant signaling pathway activated in response to nutrients such as AA involves phosphatidylinositol 3-kinase (PI3K) and Akt (also known as protein kinase B or PKB). After binding to their cell surface receptors, growth factors such as insulin and insulin growth factors (IGF) lead to the phosphorylation of their receptor substrate (insulin receptor substrate-1, or IRS-1, in the case of insulin and IGF) and recruitment of PI3K. This intracellular activation of PI3K induces the conversion of phosphatidylinositol-4,5-phosphate (PIP2) into phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 formation activates the phosphoinositide-dependent protein kinase 1 (PDK1) that induces Akt activity and inhibitory phosphorylation of TSC1/TSC2, leading to mTORC1 activation *(Wullschleger, Loewith et al. 2006, Sengupta, Peterson et al. 2010, Russell, Fang et al. 2011)*. In addition to their positive regulation on PI3K activity, AA can activate mTOR via the recruitment of the mTORC1 complex to lysosome membrane-coupled Rheb. This effect is mediated by some of the Rag GTPases present in mTORC1 *(Kim, Goraksha-Hicks et al. 2008, Sancak, Peterson et al. 2008, Sancak, Bar-Peled et al. 2010)*.

The mTOR protein is also sensitive to energy levels, oxygen and stress. In the case of hypoxia or metabolic stress, nutrient uptake and energy production are slowed down, inducing the downregulation of intracellular ATP and an increase in AMP level. The ATP:AMP ratio modulates the AMP-activated protein kinase (AMPK) activity, which, in turn, ibits mTORC1. This negative regulation is mediated by AMPK phosphorylation, directly on mTOR subunit or indirectly by active phosphorylation of TSC1/TSC2 *(Inoki, Zhu et al. 2003, Gwinn, Shackelford et al. 2008)* (Fig. 3).

I-B.1.b. Downstream modulations of the mTOR pathway

Activation of PI3K, Akt or AMPK (and other signaling components upstream of mTOR) that follows various situations, significantly impact cell metabolism, growth, activation and proliferation. These key cell functions are regulated by mTOR phosphorylation of specific effectors that in turn exert strong control over mRNA transcription, protein translation, post-translational modification and autophagy.

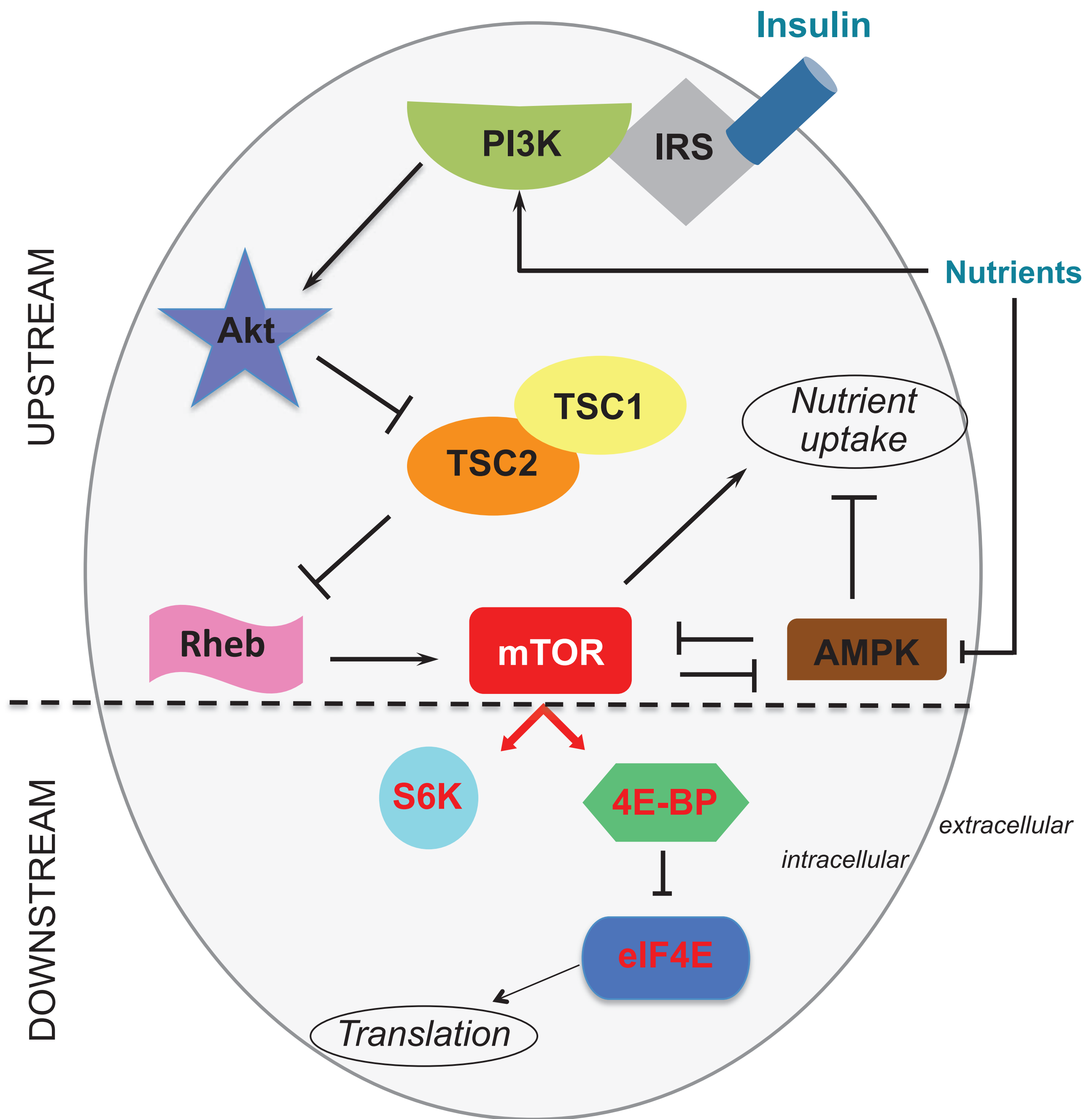


Fig. 3 Upstream and downstream components of the mTOR pathway. Insulin and nutrients, via the mTOR kinase, can induce translation initiation and regulate various cell processes including proliferation, differentiation and nutrient uptake. Akt or PKB= Protein kinase B; AMPK= AMP-activated protein kinase; IRS= Insulin receptor substrate; mTOR= mammalian target of rapamycin; PI3K=Phosphatidylinositol 3-kinase; S6K= ribosomal protein S6 kinase b1 ; TSC1/2= Tuberous sclerosis complex 1 or 2; 4E-BP= eukaryotic initiation factor 4E (eIF4E)-binding protein.

mTOR regulates the activity of the translational machinery, specifically towards mRNA that are thought to promote cell growth and proliferation *(Sengupta, Peterson et al. 2010)*. Its main targets are the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) and ribosomal protein S6 kinase b1 (S6K), which widely promote mRNA translation. Indeed, phosphorylated 4E-BP induces the separation of the complex 4E-BP/eIF4E. Once free, the mRNA cap-binding protein eIF4E recruits the translation initiation complex to the 5' mRNA cap structure *(Pause, Methot et al. 1994)*. In addition, the phosphorylation of S6K activates the ribosomal protein S6, as well as substrates involved in translation initiation by ribosome-mRNA association *(Ma and Blenis 2009)* (Fig. 3).

Many among newly translated proteins are transcription factors or subunits thereof, which, in turn, promote DNA transcription. Notably, mTOR modulation influences mRNA levels of metabolic components regulated by the hypoxia-inducible factor-1α (HIF-1α) and sterol regulatory element binding protein (SREBP) *(Sengupta, Peterson et al. 2010)*. In addition, mTOR-mediated transcriptional activation promotes expression of enzymes involved in glycolysis, PPP and the synthesis of lipids and nucleotides *(Peng, Golub et al. 2002, Duvel, Yecies et al. 2010)*. On the flipside, inhibition of mTOR has been associated with downregulation of mitochondrial activity. This effect is related to mTOR-dependent transcriptional induction of the peroxisome proliferator-activated receptor gamma (PPARγ) co-activator (PGC1α), which affects mitochondrial activity through mitochondria biogenesis and DNA synthesis *(Cunningham, Rodgers et al. 2007)*.

In addition to multiple anabolic functions that are regulated by mTOR, the mTOR phosphorylation pathway is also interdependent with catabolism. Indeed, as nutrient intake positively affects mTOR activity, deprivation of glucose, AA or growth factors releases the inhibitory effect of mTOR on autophagy *(Noda and Ohsumi 1998)*. Thus, starvation and low energy levels activate the AMPK pathway, with concomitant downregulation of mTOR responses.

As a result, protein synthesis stops and autophagolysosome formation is triggered, leading to the breakdown of cytoplasmic components and the subsequent increase of nutrient (notably AA) availability while maintaining essential cellular activities *(Sengupta, Peterson et al. 2010)* (Fig. 3). Interestingly, this mechanism is auto-regulated as AA derived from autophagy can directly activate mTOR and hence, dowregulate autophagy *(Yu, McPhee et al 2010)*. Finally, downregulation of mTOR can also stem from negative feedback loops. This has been demonstrated in the case of IRS-1 and other growth factor receptor substrates, the activity of which is reduced upon phosphorylation by S6K (a mTOR target), or by mTOR itself *(Harrington, Findlay et al. 2004, Shah and Hunter 2006)*.

I-B.2. Impact of mTOR on immune regulation

It is now firmly established that immune cell survival and functions are linked to cell metabolism *(Afacan, Fjell et al. 2012)*. Immune cell physiology is rapidly modulated by changes in their environment, which impact on nutrient uptake, allowing the development of reactions to stress and infection. Not surprisingly, the mTOR pathway has been implicated in the regulation of fast- and slow-onset immune responses *(Weichhart, Costantino et al. 2008, Powell and Delgoffe 2010)* (Table 3) and rapamycin significantly modulates innate and adaptive immunity, with general immunosuppressive properties.

I-B.2.a. Fast-onset immune responses (occur within minutes to hours)

An important way by which the mTOR pathway influences innate immunity is via induction of the transcription factor HIF-1α. Indeed, several studies have described HIF-1α as a key contributor to neutrophil survival and activation *(Walmsley, Print et al. 2005)*. In particular, HIF-1α regulates NFκB activation and glycolysis, both mechanisms critical to neutrophil motility, delay of apoptosis and bacterial killing *(Cramer, Yamanishi et al. 2003)*. In addition, mTOR activation in neutrophils can induce extracellular trap formation (see section II.A.2b), also via HIF-1α *(McInturff, Cody et al. 2012)*. However, another study demonstrated that extracellular traps can also be formed upon mTOR

Type of process	Immune subset	Associated response	References
Fast	Neutrophils	Chemotaxis	*Gomez-Cambronero 2003, Lehman 2003, Liu 2010*
		Survival, Phagocytosis	*Walmsley 2005, Cramer 2003*
		NETosis	*McInturff 2012, Itakura 2013*
	Macrophages	Cytokine production	*Tannahill 2013*
	Dendritic cells	Maturation	*Hackstein 2003, Pan 2013*
Slow	T cells	Survival, Effector generation	*Delgoffe 2009, Swainson 2007, Powell 2010*
		Treg differentiation	*Kang 2008*
		Migration	*Finlay 2010*
	B cells, NK cells	Cell cycle progression	*Li 1999, Donahue 2003 and 2007, Wai 2008*

Table 3 Role of mTOR in immune cell regulation. Presented here are examples of fast and slow immune responses modulated by mTOR.

inhibition, correlating this time with autophagosome formation rather than HIF-1α activation *(Itakura and McCarty 2013)*.

Activation of the mTOR pathway has also been implicated in neutrophil chemotaxis. First, rapamycin was able to suppress the S6K activity and its positive regulation of GM-CSF- and IL-8-induced neutrophil recruitment *(Gomez-Cambronero 2003, Lehman, Calvo et al. 2003)*. Second, mTORC2 was identified as a contributor to cytoskeletal regulation and its inhibition in mice induces a reduction of cAMP, leading to subsequent impairment in neutrophil polarization and migration *(Charest and Firtel 2010, Liu, Das et al. 2010)*. Thus, both mTORC1 and mTORC2 are able to impact on neutrophil functions and their role in innate immunity.

With regards to inflammatory signaling, mTOR induction has been shown to reduce T-cell priming by macrophages and monocytes through their decreased secretion of IFN-γ and IL-17 and enhanced release of the anti-inflammatory cytokine IL-10 *(Weichhart, Costantino et al. 2008)*. Consistently, inhibition of mTOR by rapamycin enhances pro-inflammatory signaling. However, this is not a generalizable notion, since rapamycin treatment of activated neutrophils decreases pro-inflammatory signaling, notably by slowing down TNF-α and IL-6 production, as well as NFκB activity. These effects of rapamycin are linked to AMPK activation and were responsible for an attenuation of tissue damage in mouse models of neutrophil-induced lung injury *(Zhao, Zmijewski et al. 2008, Lorne, Zhao et al. 2009)*.

I-B.2.b. Slow-onset immune processes (occur in hours to days).

T-cell activation, differentiation and function are closely dependent on their process of energy production, OxPhos (quiescent cells) or others (see section I-A.2). Activation of metabolic pathways during T-cell responses, as well as the regulation of glucose transport, protein and lipid synthesis have been shown to be dependent upon mTOR modulation. This regulation is essential to effector cell differentiation, trafficking and proliferation *(Delgoffe and Powell 2009, Powell and Delgoffe 2010, Powell, Pollizzi et al. 2012)*. Indeed, TCR engagement combined with CD28 activation requires concomitant PI3K

stimulation that prevents T-cell anergy and promotes full activation *(Delgoffe and Powell 2009)*. This mechanism can be dependent or independent of IL-2 and IL-2 receptor signaling and is sensitive to rapamycin *(Powell, Lerner et al. 1999, Appleman, Berezovskaya et al. 2000, Colombetti, Basso et al. 2006)*. T-cell apoptosis is also prevented by cytokine-induced mTOR stimulation. For example, exogenous IL-7 has been shown to induce survival and proliferation of naïve T cells via PI3K through induction of glucose transporter 1 (Glut1) expression *(Rathmell, Farkash et al. 2001, Swainson, Kinet et al. 2007)*.

Interestingly, inhibition of PI3K-Akt signaling leads to enhanced expression of the transcription factor Foxp3, which is critical for the differentiation of regulatory T cells (Treg) *(Haxhinasto, Mathis et al. 2008, Sauer, Bruno et al. 2008)*. Rapamycin treatment in itself can promote Treg development *(Kang, Huddleston et al. 2008)*. By contrast, mTOR pathway activation, following treatment with inducing cytokines, is necessary for CD4 effector T-cell generation *(Stephenson, Park et al. 2005, Powell and Delgoffe 2010)*. Also, mTOR inhibition by rapamycin or AMPK activation by metformin showed immunostimulatory effects on CD8 effector T-cell generation, via induction of catabolism and memory cell initiation *(Araki, Turner et al. 2009, Pearce, Walsh et al. 2009, Rao, Li et al. 2010)*. Finally, mTOR pathway activation has been involved in T-cell motility via the regulation of L-selectin and C-C chemokine receptor-7 (CCR7) expression, allowing T-cell trafficking between lymphoid organs. Thus, after antigen presentation, mTOR induces downregulation of these proteins, promoting T-cell migration into inflammation sites *(Finlay and Cantrell 2010, Powell and Delgoffe 2010)*.

While the impact of mTOR modulation has been primarily studied in association with specific T-cell developmental and functional processes, other leukocyte subsets are also affected by this pathway. For example, in NK cells and B lymphocytes, rapamycin can block cell cycle progression, demonstrating an important role of mTOR in their proliferation *(Gourlay, Chambers et al. 1998, Li, Davis et al. 1999, Donahue and Fruman 2003, Donahue and Fruman 2007, Wai, Fujiki et al. 2008)*. In antigen-presenting cells (APC), the mTOR pathway plays a dual role in cell differentiation and function. First,

mTOR is necessary for the maturation and mobilization of APC *(Hackstein, Taner et al. 2003, Thomson, Turnquist et al. 2009)*. Once APC have matured, downregulation of mTORC1 is required for efficient class II major histocompatibility complex expression and subsequent antigen presentation to CD4 T cells, revealing an unexpected role for mTOR downregulation in APC function *(Pan, O'Brien et al. 2013)*.

CHAPTER II: NEUTROPHILS

II-A. Neutrophil biology

II-A.1. Generalities

Neutrophils were identified for the first time in 1865 by Max Schultze (1825-1874). He described four different leukocyte types, with among them, a specific one with the nucleus shape divided into several lobes, the presence of “refracted” granules and an ability to move, although he never formally studied their function *(Brewer 1994, Cavaillon 2011)*.

In 1883, Elie Metchnikoff (1845-1916) published his discovery about “intracellular digestion”. He described the ability of cells to take up foreign material as well as nutrients. He observed this phenomenon for the first time when he hammered rose thorns in starfish larvae, which led to the surrounding of the foreign object by migratory cells. His concept later baptized “phagocytosis” by Carl Claus (1835-1899) indicates the process driven by specific “phagocytes” (derived from the Greek: “devoring cells”) *(Cavaillon 2011)*. As a zoologist, Metchnikoff studied phagocytosis among several kinds of organisms, from marine species to vertebrates, and upon various experimental conditions, including physical injuries, infection and differentiation. In addition to their central role in host defense during pathogen invasion, he observed that phagocytes could also engulf dying and dead cells. In 1887, he categorized two types of phagocytes: macrophages and microphages and functionally described them as scavengers *(Kaufmann 2008)*.

Metchnikoff characterized neutrophils (or microphages) based on their ability to phagocyte microorganisms during infection. However, we owe to Paul Ehrlich (1854-1915) the phenotypic distinction between white blood cell subpopulations. Indeed, he distinguished leukocytes based on their chemical properties using specific dyes. Polymorphonuclear leukocytes showed a tendency to retain neutral dye, thus their given name “neutrophils” *(Kaufmann 2008, Amulic, Cazalet et al. 2012)*. Taken together, research by Schultze,

Metchnikoff and Ehrlich highlighted important characteristics and functions of neutrophils in host defense.

II-A.1.a. Diversity and evolution of neutrophils and phagocytes

Since Metchnikoff placed phagocytes as central actors in defense mechanisms, these cells have been studied in various organisms. Through evolution, phagocytes are defined by a conserved, cardinal feature of engulfing and (when possible) destroying foreign bodies. Their characterization from protozoans to multicellular organisms suggest that these cells and their ability to perform phagocytosis is derived from a common, ancestral cell type. In mammals, the formation of effector granules and the presence of a polylobulated nucleus defines the neutrophil subset.

Dictyostelium discoideum, a protozoan with free-standing and communal life forms that commonly performs phagocytosis to feed on bacteria, is known to induce the differentiation of specific cells with immune function during its multicellular aggregation. Indeed, formation of the “social” amoeba has been associated with the development of “sentinel” cells, which provide enhanced phagocytosis-based protection during infection *(Chen, Zhuchenko et al. 2007)*.

Defense against pathogens in insects is partly controlled by circulating immune cells, the haemocytes. While there is significant variability between insect species, the most common haemocyte subsets are described by plasmatocytes and granular haemocytes. They both have a round nucleus, but the latter contain different granules types and are able to release them when entering in contact with foreign bodies, while the earlier function as more classical phagocytes *(Ribeiro and Brehelin 2006)*.

In zebrafish (*Danio rerio*), the cells with segmented nuclei and myeloperoxidase (MPO) positive granules analogous to neutrophils are named heterophils. Because of the strong resemblance and functional similarities between heterophils and neutrophils, the zebrafish model has

been used to mimic various immune disorders, notably neutrophilic airway disease *(Bennett, Kanki et al. 2001, Martin and Renshaw 2009)*.

Neutrophils, macrophages and dendritic cells (DC) constitute the phagocyte family in humans and other mammals. Whereas neutrophil morphology and phagocyte function are very similar between species, they vary in their frequencies. Among total blood leukocytes, 10-25% in mice and around 50% in chimpanzees are neutrophils, versus 50-70% in humans *(Hawkey 1985, Doeing, Borowicz et al. 2003, Mestas and Hughes 2004)*. In humans, neutrophils are the most abundant circulating leukocyte subset and generally represent the first cells to be recruited to sites of injury, where they appear essential to pathogen elimination.

II-A.1.b. Human neutrophil biogenesis

In adult humans, neutrophils are produced and released from the bone marrow (BM) at an estimated rate of ~10^9 cells/kg/day, and thus represent the most abundant immune cells of the human body *(Bugl, Wirths et al. 2012)*. Neutrophils originate from hematopoietic stem cells (HSC), and downstream, multipotent common myeloid progenitors (CMP). CMP give rise to red blood cell (RBC) progenitors, platelet progenitors, mast cell progenitors or granulocyte-macrophage progenitors (GMP). GMP can differentiate into monoblasts (monocytic and myeloid dendritic cell lineage) or myeloblasts. During granulopoiesis, myeloblasts differentiate into specific promyelocytes to become distinct granulocytes: basophils, eosinophils and neutrophils. From the myeloblast stage on, neutrophil differentiation proceeds in defined, orderly steps, morphologically and transcriptionally, leading to nuclear shape changes and in the formation of diverse granule compartments *(Theilgaard-Monch, Jacobsen et al. 2005)*. Figure 4 illustrates steps in human myelopoiesis and granulopoiesis (Fig. 4).

Terminally differentiated neutrophils comprise three distinct granule types in addition to secretory vesicles, all of which are formed by carefully timed waves of protein biosynthesis and packaging during differentiation

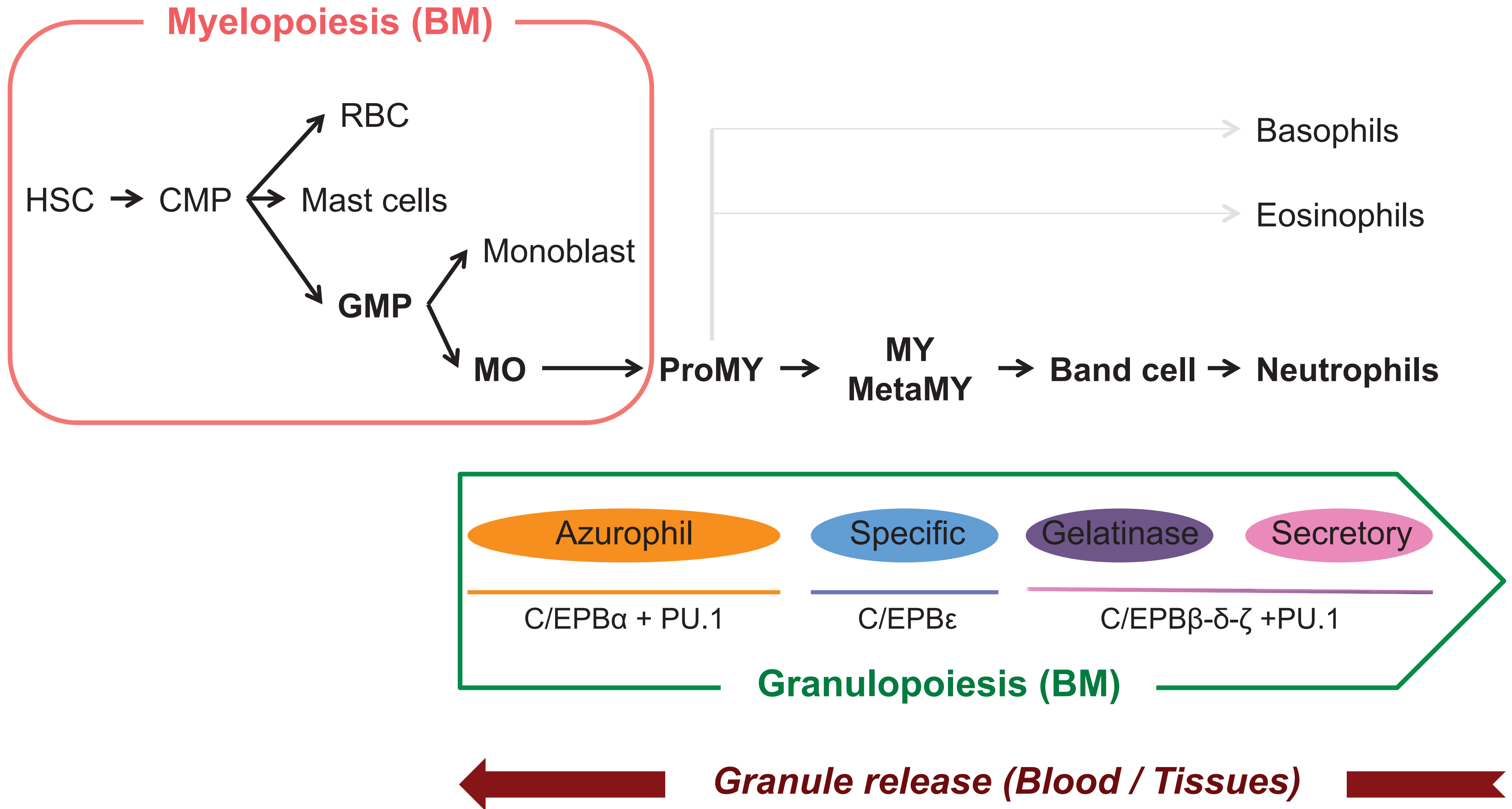


Fig. 4 Myelopoiesis and granulopoiesis. Schematic representation of neutrophil biogenesis. Myelopoiesis and granulopoiesis both occur in the bone marrow (BM). Granulopoiesis includes successive formation of different granule sub-types, controlled by specific transcription factors. During recruitment to inflammatory sites, neutrophils release secretory vesicles and then gelatinase, specific, and rarely azurophil granules. The order of granule release is generally inverse to that of granule biogenesis. C/EPB= CCAAT/enhancer binding protein; CMP or GMP= Common / Granulocyte myeloid progenitors; HSC= Hematopoietic stem cells; MO= Myeloblasts; (Pro/Meta)MY= (Pro/Meta)Myelocytes; RBC= Red blood cells.

(Borregaard, Sehested et al. 1995). Indeed, a stepwise transcriptional program has been shown to regulate the successive generation of azurophil (primary), specific (secondary) and gelatinase (tertiary) granule proteins *(Theilgaard-Monch, Jacobsen et al. 2005)*. Granule formation starts at the promyelocyte stage with the synthesis of azurophil granule proteins, regulated by the CCAAT/enhancer binding protein α (C/EBPα) and PU.1 transcription factors. After these are downregulated, the formation of specific granules is triggered and controlled by the transcription factor C/EBPε. Then, expression of the transcription factors C/EBPβ-δ- ζ and PU.1 regulates gelatinase granule and the secretory vesicle membrane protein synthesis, the content of the latter being acquired by endocytosis of plasma proteins *(Borregaard 2010)*. This wave-like transcriptional program regulates consecutive steps of granule formation and comes to an end when neutrophils are segmented and ready to leave the BM *(Borregaard, Sorensen et al. 2007)* (Fig. 4).

Neutrophil granules function as stores of intragranular effector proteins that can kill microbes and digest tissues when released into the phagosome or the extracellular fluid. However, the membrane of neutrophil granules also serves as a reservoir of membrane proteins that incorporate to the plasma membrane of neutrophils and play a role in signaling when these granules fuse with the plasma membrane to exocytose their intragranular content *(Borregaard and Cowland 1997, Lominadze, Powell et al. 2005)*. Upon priming and activation, neutrophil degranulation is initiated. This process starts with secretory vesicles, followed by release of gelatinase, specific and azurophil granules (the latter is not as easily mobilized to the surface). Through this tightly controlled mechanism neutrophils specifically express surface molecules required for recruitment to the site of infection, microorganism killing processes, and the resolution of the inflammatory reaction (see section II-A.2).

II-A.1.c. Release and turnover

Neutrophil homeostasis is dependent on their production within the BM (a 7-10 day long process), egress from the BM, circulation in blood,extravasation and clearance.

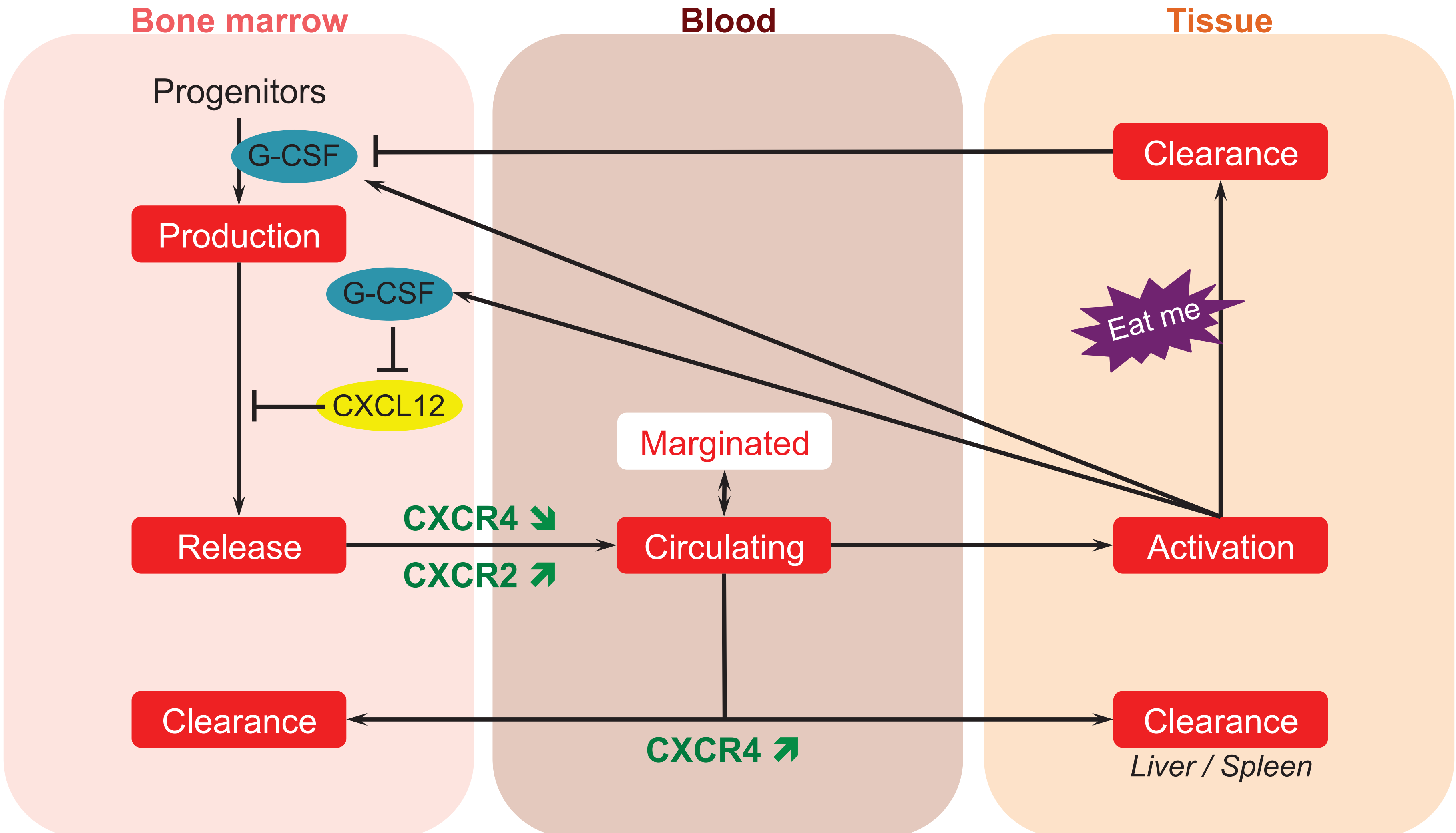


Fig. 5 Neutrophil release and turnover. Schematic representation of neutrophil release and turnover between bone marrow, blood and tissue. CXCL12= Chemokine (C-X-C motif) ligand 12; CXCR2 and CXCR4=Chemokine (C-X-C motif) receptor 2 / 4 ; G-CSF= Granulocyte-colony stimulating factor; SS= Steady-state.

These processes are regulated by local cytokine and growth factors, notably granulocyte colony-stimulating factor (G-CSF).As illustrated in Figure 5, G-CSF produced and secreted by stromal cells in BM modulates progenitor mobilization and differentiation, as well as mature neutrophil release *(Demetri and Griffin 1991, Lieschke, Grail et al. 1994, Heissig, Hattori et al. 2002, Richards, Liu et al. 2003)* (Fig. 5). G-CSF regulates neutrophil egress from the BM by inducing a down-regulation of the CXCL12-CXCR4 interaction, which keeps developing neutrophils tethered to the stroma *(Semerad, Liu et al. 2002, Summers, Rankin et al. 2010)*. Moreover, CXCR4 downregulation in mature neutrophils is concomitant with the upregulation of surface CXCR2 (IL-8 receptor) expression, which makes mature neutrophils more sensitive to peripheral IL-8 upon release from the BM *(Eash, Greenbaum et al. 2010)*. While high CXCR4 expression is associated with retention of developing neutrophils in the BM and with the sequestration of senescent neutrophils in either BM, liver or spleen *(Kolaczkowska and Kubes 2013)*, CXCR4 upregulation has also been documented in activated neutrophils homing to peripheral sites of inflammation. Clearance of recruited neutrophils in sites of inflammation is performed by resident macrophages and DC, and leads to the downregulation of G-CSF secretion and neutrophil release *(Stark, Huo et al. 2005)*.

In absence of inflammation, newly released neutrophils from the BM have a short lifespan estimated to last less than 24 hours in humans, with a half-life estimated around 8 hours *(Dancey, Deubelbeiss et al. 1976)*. However, this question is still controversial since a recent study proposed that human neutrophil half-life is around 3.8 days *(Pillay, den Braber et al. 2010, Tofts, Chevassut et al. 2011)*. Irrespective of what occurs during normal homeostasis, inflammation strongly impacts neutrophil lifespan, production and release. Commensal microbiota modulate “steady-state” neutrophil production due to bacterial products that can stimulate neutrophil maturation and maintain an appropriately sized circulating pool *(Clarke, Davis et al. 2010; Bugl, Wirths et al. 2012).* In contrast to this “steady-state” regulation, “emergency” granulopoiesis occurs when there is a sudden increase in systemic cytokine levels, inducing BM egress of mature neutrophils and accelerating production of new cells from precursors through G-CSF upregulation, thus leading to BM and blood

neutrophilia. The increase in circulating neutrophils can stem from *de novo* neutrophil production from BM progenitors, but also from the release of neutrophils from the marginated pools (mature cells tethered to the inside wall of bone marrow sinusoids and other vascular beds) *(Terashima, English et al. 1998, Cain, Snowden et al. 2011)*.

II-A.2. Function

Since their first description in 1887 by Elie Metchnikoff, neutrophils have been widely recognized as the first barrier to microbial infections and critical effector cells of the immune system. As introduced above, local and systemic stimuli regulate neutrophil fate and notably their release and turnover. Here, we review neutrophil functions during activation, as summarized in Table 4. Endogenous and exogenous inflammatory signals can activate signaling pathways and prime neutrophils to carry out functional responses. Signal transduction is mediated by a plethora of cellular receptors and adaptor proteins (surface and intracellular) and directs neutrophils through the various steps of:

(i) peripheral recruitment (chemotaxis and diapedesis)
(ii) unfolding of killing mechanisms, and
(iii) regulatory functions that lead to the resolution of inflammation *(Mantovani, Cassatella et al. 2011)* (Table 4).

II-A.2.a. Chemotaxis and diapedesis

Upon priming and activation, neutrophils adhere to the endothelium, migrate through and reach the site of infection and/or inflammation. Therein, pathogen-associated or microbe-associated molecular pattern (MAMP or PAMP, e.g., lipopolysaccharide, mannose) and damage-associated molecular pattern (DAMP, e.g., high-mobility group box protein-1) molecules as well as chemoattractants and cytokines (e.g., IL-1β, IL-6, IL-6 or LTB_4) induce P- and E-selectin expression on endothelial cells *(Borregaard 2010, Amulic, Cazalet et al. 2012)*. While neutrophils are circulating in the blood stream, they migrate to the MAMP / DAMP / PAMP / chemokine / cytokine gradient until they come in

Stimulus >>>	Receptor >>>	Signaling >>>	Priming >>>	Activation
Endogenous / Exogenous molecules	**Surface / Intracellular receptors**	**Transduction pathways**	**Exocytosis (SVs, 3^{ry} granules), NOX assembly**	**Exocytosis (2ry and 1ry granules), Motility**
Complement Cytokines / Chemokines PAMPs / DAMPs Integrins Immunoglobulins	Fc receptors GPCRs Integrins PRR (NLR, TLR)	ERK Inflammasome MAPK NF-κB PKA / Pi3K / PKC	Surface changes (new receptors) ROS / RNS production	Chemotaxis Diapedesis Immune cell crosstalk Killing

Table 4 Neutrophils: from stimulus to activation. Examples of stimuli, receptors, signaling pathways, priming and activation processes are indicated. DAMP= Damage-associated molecular patterns; ERK= Extracellular signal-regulated kinase; GPCR= G-protein coupled receptors; MAPK= Mitogen-activated protein kinase; NF-κB= Nuclear factor-kappa B; NLR= NOD-like receptors; PAMP= Pathogen-associated molecular patterns; PK= Protein kinase; PRR= Pattern-recognition receptors; RNS= Reactive nitrogen species; ROS= Reactive oxygen species; SV= secretory vesicles; TLR= Toll-like receptors.

contact with endothelial selectins. Neutrophils constitutively express P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin. The recognition and binding of neutrophil and endothelial selectins trigger the "tethering" and lead to neutrophil "rolling" along the inflamed endothelium. After neutrophils reach firm adhesion, dependent on neutrophil β_2-integrin (CD18) and their endothelial ligands (intercellular adhesion molecule-1, ICAM-1), they begin their transendothelial migration *(Kolaczkowska and Kubes 2013)*.

Neutrophil transmigration through the endothelial barrier has been shown to be either dependent or independent of β_2-integrins *(Doerschuk, Winn et al. 1990)*. Integrin-independent migration occurs most particularly in the specialized capillary beds of tissues such as lung, spleen, liver and BM. These capillaries' diameter are small enough to stop circulating neutrophils, which actively deform the endothelium and make their way into the tissue *(Doerschuk 2000)*. Once neutrophils have traversed the endothelial barrier, they navigate through the basement membrane made of fibroblasts and collagen. Then, following the chemotactic gradient, they crawl until they reach the site of inflammation. To do so, neutrophils may have to migrate through another layer of tissue (e.g., synoviocytes in the joint, or epithelium in the lung).

Chemotaxis and diapedesis are generally concomitant with priming and activation of neutrophils. While neutrophils physically reach a site of inflammation, the first signs of priming are the exocytosis of secretory vesicles and tertiary granules and enhanced intracellular signaling, leading to the assembly of the NADPH oxidase enzyme from multiple cytosolic and granule subunits. Further stimulation leads to full-blown activation, with accelerated NADPH oxidase assembly at the plasma membrane and at the phagosomal membrane and induction of the oxidative burst, leading to ROS and RNS release *(El-Benna, Dang et al. 2008)*. Secondary and primary granule exocytosis and fusion with the phagosome are also important steps in neutrophil activation.

Killing mechanism	Effector method		Effector molecules	Location
Phagocytosis	Capture	Phagocytic vesicle	Cathepsin G MPO NE Proteinase 3 ROS/RNS	Intracellular
NETosis	Capture	DNA mesh	Histones Lactoferrin NE MMP-9 MPO Pentraxin 3	Extracellular
Anti-microbial proteins	Secretion / Degranulation	Diffusible factors	BPI Calprotectin (heteromer of S100A8/S100A9) Defensins Lactoferrin Lysozyme NE MPO ROS/RNS	Extracellular

Table 5 **Neutrophil killing mechanisms.** Neutrophils target and kill microbes by way of phagocytosis, NETosis and anti-microbial proteins. BPI= Bactericidal/permeability-increasing protein; MMP-9= Matrix metalloproteinase-9; MPO= Myeloperoxidase; NE= Neutrophil elastase; RNS= Reactive nitrogen species and ROS= Reactive oxygen species.

II-A.2.b. Killing mechanisms

Neutrophils play an important role in the elimination of pathogens such as bacteria and fungi. Once recruited to the site of infection, neutrophils deploy a wide range of killing mechanisms and toxic molecules that together promote pathogen killing. These mechanisms, presented in Table 5, comprise phagocytosis, antimicrobial protein secretion, degranulation and extracellular trap formation (a.k.a., NETosis) (Table 5). In response to this plethora of antimicrobial weapons, some bacteria and fungi have evolved resistance mechanisms that allow them to escape neutrophil killing, including disruption of phagosomes or prevention of granule-phagosome fusion.

Phagocytosis is the major mechanism for pathogen uptake and removal *(Amulic, Cazalet et al. 2012)*. It consists in the engulfment of microbes tagged by antibodies or complement through Fc- or complement receptors, respectively *(Underhill and Ozinsky 2002)*. In the absence of complement or antibody "tagging", microbes can also be recognized through PAMP or pattern-recognition receptors (PRR), such as the Toll family, and others. Once the phagosome is formed, it matures by intracellular fusion with neutrophil secondary and primary (lysosomal) granules and the delivery of their antimicrobial molecules therein. From this point on, the phagosome becomes a phagolysosome. NADPH oxidase subunits get assembled at the phagolysosomal membrane and ROS/RNS production is triggered, maintaining pH levels that enable full activity of proteolytic enzymes (e.g., neutrophil elastase from primary granules -NE-) *(Jankowski, Scott et al. 2002)*. Myeloperoxidase, also from primary granules, transforms the hydrogen peroxide produced by NADPH oxidase into the potent actimicrobial hypochlorous acid (HOCl, the active component of bleach) *(Winterbourn, Hampton et al. 2006)*.

As mentioned above (see section II-A.1.b), neutrophils granules can fuse with the phagosome during phagocytosis, but also with the plasma membrane, thereby releasing their content in the extracellular milieu, a process named exocytosis or degranulation. Numerous antimicrobial molecules and proteases can thus be released, especially those contained in secondary and primary granules (e.g., defensins, lysozyme, lactoferrin, NE,

MPO) *(Nathan 2006)*. By the same token, NADPH oxidase can also assemble at the neutrophil surface, which enables ROS/RNS production and release into the extracellular milieu *(El-Benna, Dang et al. 2008)*.

In the past decade, another neutrophil-killing mechanism has been described, which consists in the formation of an extracellular DNA mesh that traps and kills microorganisms by sequestering them in contact with neutrophil effectors *(Brinkmann, Reichard et al. 2004)*. This process of extracellular trap formation, or NETosis, can be accomplished with DNA from the nucleus (associated with cell death) or from the mitochondria *(Fuchs, Abed et al. 2007, Yousefi, Mihalache et al. 2009)*, leading to the extracellular localization of histones, secondary and primary granule effectors (all cationic) with anionic DNA *(Wang, Li et al. 2009, Papayannopoulos, Metzler et al. 2010)*. This mechanism is increasingly recognized as a common killing modality used by neutrophils in acute and chronic inflammation.

II-A.2.c. Regulatory functions

Neutrophils are generally among the first immune cells present at a site of inflammation (besides resident immune cells), and thus play a critical role in the establishment of the large network of signals that presides over the various stages of an inflammatory response. We present in Figure 6 that neutrophils reaching the inflammatory milieu exchange signals with the tissue, stimulating the recruitment of further leukocytes in the early phase of inflammation. Along with the local endothelium, fibroblasts (and when relevant, epithelial cells), neutrophils engage in cytokine secretion, by-product release and cell-cell contacts that will shape the immune response. At a later stage, neutrophils can participate in the resolution of the inflammatory response *(Zemans, Briones et al. 2011)*, or initiate a chronic inflammatory syndrome. In addition to their ability to interface with other cells, neutrophils also influence one another, particularly when a first wave of neutrophils recruited to an inflammatory tissue submits the circulating neutrophils to a chemoattractant gradient, which essentially forms a signal relay between sequential waves of neutrophils *(Afonso, Janka-Junttila et al. 2012)*.

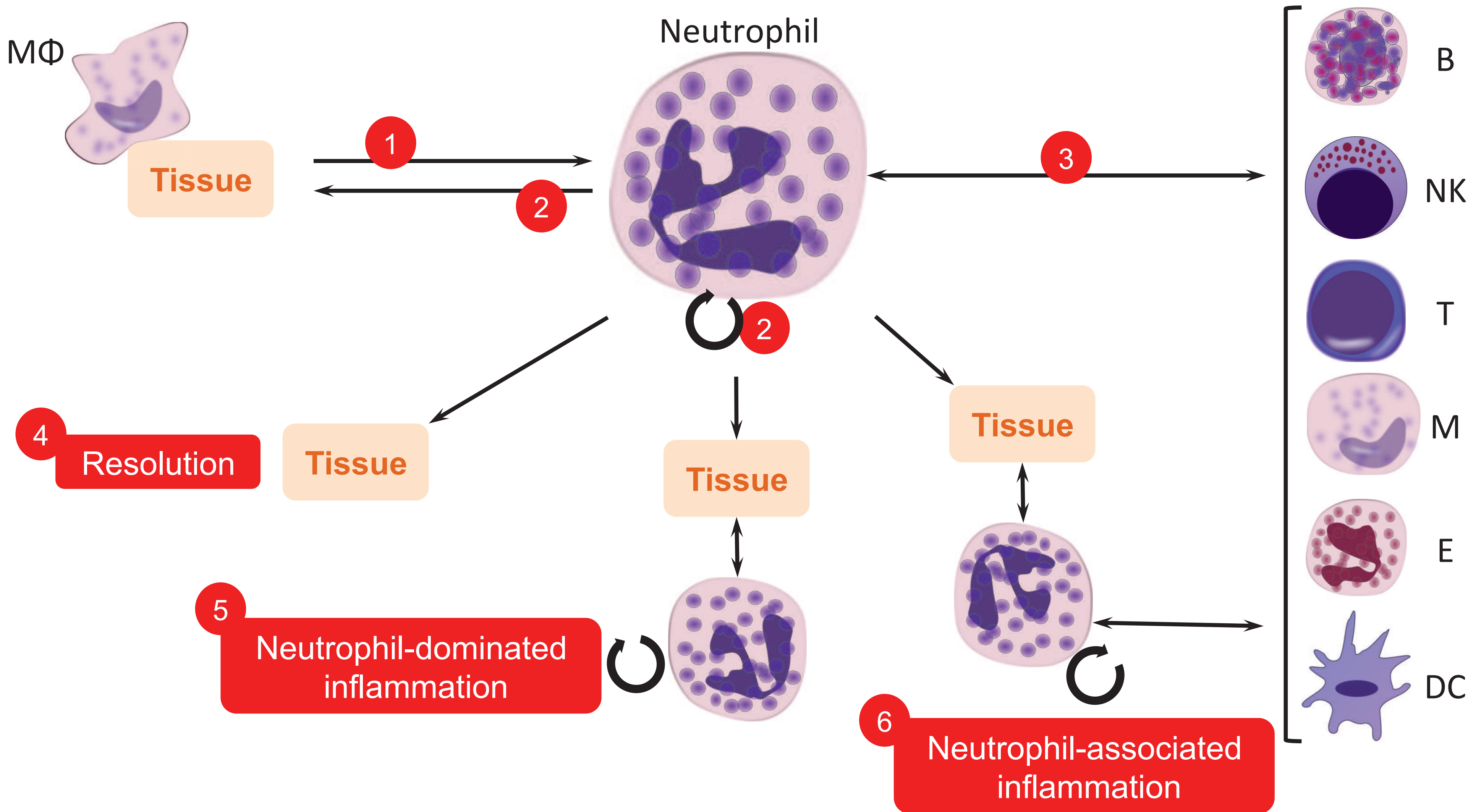


Fig. 6 Neutrophils: actors and regulators of inflammation. Schematic representation of neutrophil impact on inflammation . Cells present in tissues, including resident macrophages, exchange signals with neutrophils (1 and 2), stimulating their recruitment, as well as that of other leukocytes (3). Neutrophil actions can lead to the resolution of inflammation (4) or chronic disease in the form of neutrophil-dominated (5) or neutrophil-associated (6) inflammation. B= Basophils; DC= Dendritic cells; E= Eosinophils; M= Monocytes; MΦ= Macrophages; NK= Natural killer cells; T= T Lymphocytes. *Cliparts from www.clker.com.*

The impact of neutrophils on other immune subsets is also very significant, as they can: (i) induce monocyte/macrophage recruitment, differentiation and activity *(Scapini, Laudanna et al. 2001, Soehnlein, Kenne et al. 2008)*; (ii) interact directly or indirectly with NK cells, which are major sources of interferon gamma (IFN-γ) and thereby can support further neutrophil activation *(Costantini, Calzetti et al. 2011, Costantini and Cassatella 2011)*; (iii) influence B-cell maturation *(Scapini, Bazzoni et al. 2008)*; (iv) modulate DC recruitment and maturation, and the downstream T-cell response *(Yang, Strong et al. 2010, Blomgran and Ernst 2011)*; and (v) regulate T-cell chemotaxis, proliferation and activity (notably by L-arginine depletion) *(Munder, Schneider et al. 2006, Beauvillain, Delneste et al. 2007)*. In addition, neutrophils can upregulate expression of MHC and T-cell costimulatory molecules *(Tirouvanziam, Gernez et al. 2008)*, which suggests that they may play a direct antigen-presenting role in peripheral inflammatory sites and thereby modulate adaptive immune responses in more profound ways than previously thought *(Mantovani, Cassatella et al. 2011, Amulic, Cazalet et al. 2012)* (Fig. 6).

In addition to their ability to drive immune responses while they are alive and active at an inflammatory site, the end-stage and ultimate fate of neutrophils is also crucial to the proper resolution of an inflammatory response. For example, an orderly death of neutrophils through apoptosis / programmed cell death is associated with clearance by resident macrophages *(Stark, Huo et al. 2005)*. Phlogistic removal of inflammatory neutrophils also modulates neutrophil production and release from BM and induces anti-inflammatory cytokine secretion as TGF-β and IL-10 *(Kennedy and DeLeo 2009)*. Another important step in the resolution of inflammation is the switching of neutrophil production of pro-inflammatory (e.g., prostaglandin and leukotrienes as LTB_4) to anti-inflammatory lipids (e.g., lipoxins, resolvins, protectins) *(Godson, Mitchell et al. 2000, Serhan 2007)*. Pathologically, optimal neutrophil clearance can become as important as an optimal killing function, since defects in neutrophil apoptosis and/or clearance can lead to chronic inflammation (see section II-B).

II-B. Neutrophil-related diseases

Neutrophil disorders have been associated with: (i) mutations in genes coding for proteins essential for neutrophil development and function (e.g., transcription factors, or effectors, such as NADPH oxidase subunits), leading to primary immunodeficiencies; (ii) hijacking of neutrophil signaling by tumor cells to promote angiogenesis and induce T-cell tolerance towards the tumor; (iii) systemic and tissue pathologies *associated* with neutrophils; and (iv) autoimmune and mucosal pathologies *dominated* by neutrophils. The primary immunodeficiencies impacting directly neutrophil development and function and the role of neutrophil during tumor development will not be presented in detail here, but we would like to direct the reader to the following references, for further development *(Dinauer, Lekstrom-Himes et al. 2000, Hager, Cowland et al. 2010, Gregory and Houghton 2011)*. In the next sections, we will explore examples of specific inflammatory diseases or associated disorders in which neutrophils play a significant pathological role.

II-B.1. Disease associated with neutrophil dysfunction

II-B.1.a. Systemic pathologies

Whereas most past research on anaphylaxis and allergic sensitization has focused on other immune subsets, recent evidence has emerged that neutrophils can play a significant role in these systemic syndromes. Indeed, murine and human neutrophils can induce an IgG-triggered systemic anaphylaxis, in a similar way that mast cells and basophils can be triggered by IgE crosslinking to induce classical anaphylaxis *(Jonsson, Mancardi et al. 2011)*. In addition, neutrophils are necessary to allergen sensitization and the following T-cell recruitment occurring during skin allergic reactions *(Mocsai 2013)*.

In addition, neutrophil dysfunction has been linked to autoimmune disorders caused by the presence of systemic autoantibodies. These antibodies are notably found in blood of patients with rheumatoid arthritis (RA, see section II-B.2.a), Wegener's granulomatosis and systemic lupus

erythematosus (SLE) *(Brouwer, Huitema et al. 1994, Kessenbrock, Krumbholz et al. 2009)*. Common targets for such autoantibodies are MPO and proteinase-3 (yielding anti-neutrophil cytoplasmic antibodies or ANCA), and chromatin associated to NET formation (only SLE).

Recently, a role for neutrophil during metabolic syndromes such as type II diabetes has also been revealed. While the presence of neutrophils in adipose tissue and liver has been long noted in obese mice and humans, NE was recently attributed a specific role in cleaving the IRS-1 signaling intermediate in cells, causing impaired glucose tolerance and insulin resistance *(Talukdar, Oh da et al. 2012)*. Additionally, neutrophils have been described as important inflammatory mediators during the development of autoimmune type I diabetes *(Diana, Simoni et al. 2013)*.

In addition to non-infectious systemic disorders, the role of neutrophils in sepsis is long known. In sepsis, systemic inflammation featuring massive and sustained levels of pro-inflammatory signals can alter neutrophil chemotaxis to the primary site of infection through modulation of receptor and adhesion molecule surface expression, and thereby perpetuate infection and inflammatory signaling *(Phillipson and Kubes 2011)*.

II-B.1.b. Mucosal and tissue pathologies

Asthma is a reversible airway hyperresponsiveness and obstructive syndrome involving various immune cell types. Typically, asthma is associated with eosinophil recruitment and with the chronic production of inflammatory mediators from eosinophils and Th2 T-cells. In severe asthma, it is common to observe high neutrophil numbers and high IL-8 levels in sputum samples *(Nakagome, Matsushita et al. 2012)*. In humans (as opposed to mice), coexistence of neutrophils with eosinophils in asthma tissue is the rule rather than the exception, and some investigators have even proposed that recruited neutrophils are driving secondary eosinophil migration and accumulation through ROS production *(Nagata, Yamamoto et al. 2000)*.

Another important role for neutrophils lies in the regulation of intracellular pathogens, and of their dissemination in the organism. For example, neutrophils have been identified as vectors for *Mycobacterium tuberculosis* in the blood and lymphatic circulation, and may help bacteria that escape phagocytic killing infect distant organs *(Abadie, Badell et al. 2005)*. This mechanism observed for mycobacteria is analogous to the propagation of *Leishmania* from neutrophils to macrophages during clearance of the former by the latter, in a process called efferocytosis *(Mocsai 2013)*. In addition, a study in mice demonstrated that in addition to their effective role in mycobacterial clearance during chronic infection, neutrophils downregulate the acute inflammation by producing IL-10, which promotes resolution of inflammation *(Zhang, Majlessi et al. 2009)*.

II-B.2. Diseases dominated by neutrophil dysfunction

II-B.2.a. Autoimmune diseases

RA is a chronic autoimmune disease leading to local inflammation in joints and characterized by synovial hyperplasia and joint destruction *(Cascao, Rosario et al. 2010)*. RA can affect up to 1% of the adult population in developed countries and genetic factors account for 50% of the risk for development of RA.

Clinical diagnostic and/or evaluation of the severity of inflammation in RA follow the level of serum autoantibodies *(Schaeverbeke, Truchetet et al. 2012)*. The first type of autoantibody associated with RA pathology is rheumatoid factor (RF), which is an antibody directed against the Fc portion of host IgG. Anti-citrullinated peptide antibodies (ACPA) are also characteristic of RA and can often be detected before the onset of symptoms. These anti-bodies are specific for citrullinated peptides produced within the synovial membrane *(Masson-Bessiere, Sebbag et al. 2000)*. In addition to significant RF / ACPA levels, other inflammatory markers are found in the blood of RA patients, including C-reactive protein, S100 proteins (specifically S100A8, S100A9 and S100A12), and cytokines (IL-1, IL-6, IL-10, IL-12, IL-15, TNF-α, GM-CSF, as well as Th17-derived cytokines) *(Chen, Yan et al. 2009, Cascao,*

Moura et al. 2010). These mediators trigger systemic priming and activation of blood neutrophils in RA, leading to abnormal ROS production *(Cedergren, Forslund et al. 2007)*. In addition to neutrophil priming and activation, the endothelium surrounding the joints has been shown to increase E- and P-selectin expression in RA patients, thus promoting neutrophil and T-cell migration to the site of inflammation *(Oppenheimer-Marks and Lipsky 1998)*.

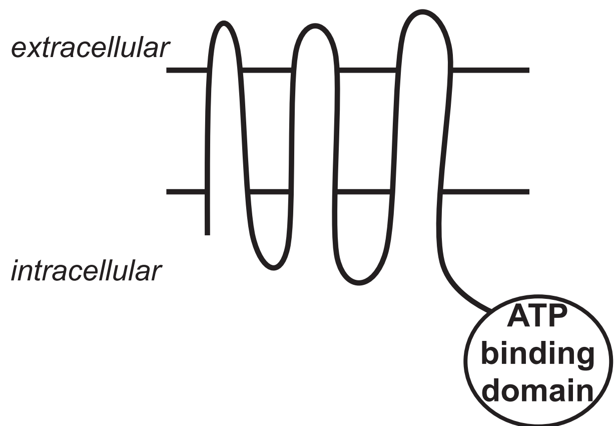
Hyperplasia and a high amount of pro-inflammatory cytokines characterize the inflamed joint in RA. Besides lymphocytes and monocyte-macrophages, neutrophils are frequently recruited to both synovial fluid (SF) and synovial membranes *(Wittkowski, Foell et al. 2007)*. The high concentration of RF in RA joints leads to neutrophil crosslinking and activation after recruitment, and to subsequent granule and ROS release. Studies of neutrophils recruited to inflamed RA joints revealed specific features consistent with a significant inflammatory role. First, upon activation and recruitment to the RA joint, neutrophils present a delayed apoptosis, which leads to increased destructive potential towards the local tissue *(Raza, Scheel-Toellner et al. 2006)*. Second, neutrophils isolated from the synovial fluid of RA patients are able to present antigens via class II MHC, CD80 and CD86 co-stimulatory molecules and can induce T-cell proliferation *in vitro*, which suggests a role in sustaining adaptive immune activation in RA *(Cross, Bucknall et al. 2003)*. Third, activated RA neutrophils secrete chemoattractants that recruit blood neutrophils and other immune cells to RA joints, including IL-8, IL-1β, IL-6, TNF-α and LTB_4 *(Wright, Moots et al. 2010)*. Finally, metalloproteinases, elastase, and collagenase contained in neutrophil granules are found in synovial fluid and are responsible for the destruction of collagen matrix and joint tissues. Thus, neutrophil-associated mediators and effectors contribute to the course of the disease *(Pillinger and Abramson 1995)*. Consistently, drugs used for therapy in RA have been shown to decrease neutrophil migration and activation *(Wright, Moots et al. 2010)*.

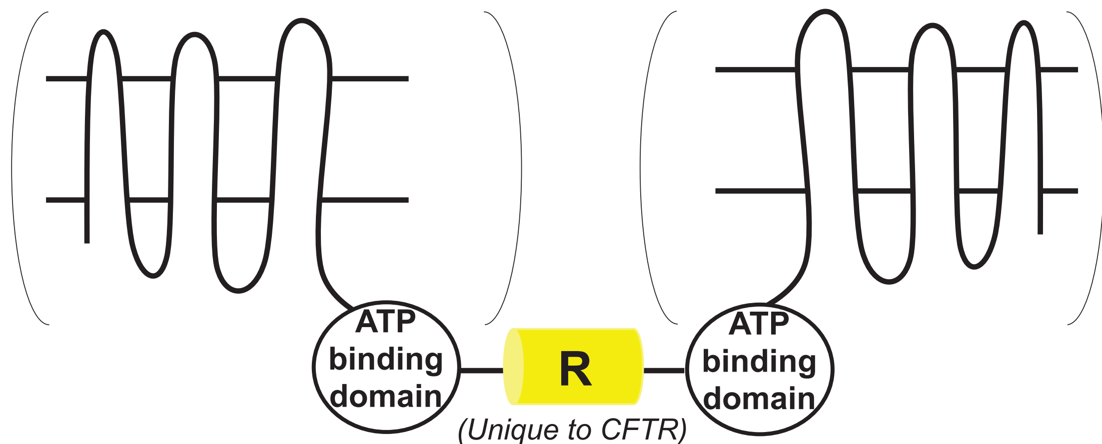
II-B.2.b. Mucosal pathogen-related pathologies

Inflammatory bowel diseases (IBD) are characterized by chronic active intestinal inflammation and massive recruitment of neutrophils due to mucosal injury, increased epithelial permeability and bacterial infiltration into the lamina propria. The main studied IBD are ulcerative colitis (UC) and Crohn's disease (CD) *(Fournier and Parkos 2012)*. The main function of neutrophils in the gut is to kill invasive microbes that cross the epithelium, as may occur when the normal commensal microbiota is perturbed. In the case of IBD, high concentrations of IL-8, IL-17 and LTB_4 from neutrophils, injured epithelial cells and resident macrophages, as well as bacterial PAMP, are detected in the luminal fluid *(Anton, Targan et al. 1989, Ina, Kusugami et al. 1997, Fujino, Andoh et al. 2003, Jupp, Hillier et al. 2007)*. These mediators are thought to contribute to the massive neutrophilic infiltration and local inflammation. Moreover, effector molecules released from neutrophil recruited to the injury sites, e.g., ROS and granule anti-microbial proteins pentraxin 3 and NE, have been identified as significant contributors to IBD pathology *(Adeyemi and Hodgson 1992, Bao, Carr et al. 2011, Savchenko, Inoue et al. 2011)*. Taken together, these data support a significant role for neutrophils in the onset and chronic phase of IBD.

In the respiratory mucosa, neutrophilic inflammation occurs in the contexts of various airway diseases, notably bronchiectasis and chronic obstructive pulmonary disease (COPD). These airway diseases, while distinct, share a central feature of chronic obstruction associated with infection and concomitant inflammation leading to progressive destruction of the lung tissue. Bronchiectasis is commonly associated with idiopathic disease or with cystic fibrosis (CF) airway disease (see section III-B). Bronchiectasis is generally characterized by failure of the immune system to clear lower airway infections, which sustains a vicious cycle of chronic infection and neutrophilic inflammation leading to airway destruction *(Chalmers and Hill 2013)*. COPD is often characterized by chronic bronchitis with prominent airway neutrophilia, leading to decreased lung function and hypoxia, and is generally associated with a history of smoking. In COPD, acute phases are caused by viral or bacterial infections, with high secretion of pro-inflammatory cytokines that enhance the preexisting neutrophilic inflammation *(Hoenderdos and Condliffe 2013)*. Both bronchiectasis and COPD also display an impairment of

macrophage phagocytosis and clearance of apoptotic airway neutrophils (efferocytosis). Neutrophil overactivation and dysfunctional efferocytosis are thought to lead to neutrophil necrosis, and to the passive release of proteolytic (NE, collagenase) and oxidative (MPO) molecules into the extracellular fluid. Therefore, lung damage associated with these disorders is clearly related to neutrophils and their by-products, including, but not limited to, proteases and oxidases contained in primary granules.





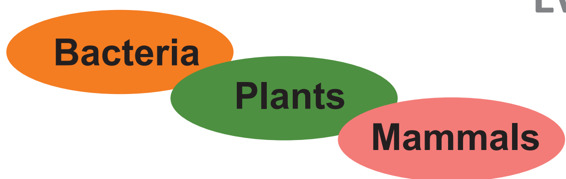


Fig. 7 Structure and evolution of CFTR. Structure and evolutionary history of ABC transporter family members, in general (minimum requirement) and of the CFTR protein in particular. While CFTR first arose in cartilaginous fish, only mouse, ferret and pig are used as models of CF (cloned animals with engineered mutations). Indeed, only humans have spontaneously occurring *cftr* mutations. ABC= ATP-binding cassette; TM= Transmembrane.

CHAPTER III: CYSTIC FIBROSIS: FROM THE GENE TO FATAL AIRWAY INFLAMMATION

III-A. CFTR disorders

Cystic fibrosis (CF) or mucoviscidosis *(Littlewood 2012)* has been described for the first time as a specific infant disorder of the pancreas in 1938 by Dorothy Andersen *(Andersen 1938)*. In 1944, Philip Howard traced familial occurrences of fibrocystic disease of the pancreas and suggested that CF was an inherited disorder. This conjecture was supported by a study of Andersen in 1946, which indicated the recessive mode of transmission of the disease *(Howard 1944, Andersen and Hodges 1946)*. Later, during the fifties, the deregulation in sweat electrolytes led to a possible diagnosis of CF, until then performed by measuring tryptic activity from duodenal intubation samples *(Disantagnese, Darling et al. 1953)*. In addition to the sweat diagnostic test, improvement was made with the therapeutic management of the disease, notably with the advent of pancreatic enzyme replacement therapy *(Harris, Norman et al. 1955, Gibson and Cooke 1959)*. Also, physicians from all over the world linked pulmonary infections and lung function decline to CF and started patients on antibiotic treatments, as well as physical therapy *(West, Levin et al. 1954, Shwachman and Kulczycki 1958, Doyle 1959, Wright and Mc 1959)*.

In 1989, after several years of painstaking genetic research in pursuit of the CF gene, Lap-Chee Tsui, Francis Collins and Jack Riordan's groups identified the cystic fibrosis transmembrane conductance regulator gene (*cftr*), on chromosome 7, as responsible for the disease *(Kerem, Rommens et al. 1989, Riordan, Rommens et al. 1989, Rommens, Iannuzzi et al. 1989)*. Immediately after the discovery of the CF gene, much effort was dedicated to the exploration of potential gene therapy strategies and in the development of animal models for the disease *(Dorin, Dickinson et al. 1992, Snouwaert, Brigman et al. 1992, Ratcliff, Evans et al. 1993, Zabner, Couture et al. 1993, Keiser and Engelhardt 2011)*.

CF is the most common genetic disease in Caucasians, affecting more than 70,000 individuals worldwide. Approximately, 1 in 2,000-3,500 newborns is affected in Europe and the United States of America (USA), according to the World Health Organization. While the incidence of CF is elevated in Caucasians (3-4% are carriers of one mutated allele), CF has also been described in other ethnicities.

III-A.1. Molecular and cellular pathology

III-A.1.a. CFTR: a member of the ABC family of proteins

The *cftr* gene encodes a 1,480 AA transmembrane protein, which main site of expression is the apical surface of exocrine epithelial cells. CFTR belongs to the ABC family of transporters and is formed by a duplication of the minimal ABC family structural module which comprises 6 transmembrane α-helices (the CFTR protein has twelve), and one ATP nucleotide-binding domain -NBD- (the CFTR protein has two, NBD1 and NBD2). In addition to its twelve transmembrane helices and two NBD domains, CFTR comprises a unique regulatory domain (R) that can be phosphorylated by protein kinase A or C (PKA/PKC) in cAMP-dependent fashion. Figure 7 illustrates the putative structure for CFTR (no crystal structure is available of the protein as of today) (Fig. 7). While all other ABC family members are transporters, the CFTR is unique among the family in that it functions primarily as a channel, conducting anions across the membrane along the electrochemical gradient (see below). Evolutionarily, CFTR is a recent addition to the ABC family as it is present only in cartilaginous fish (sharks) and onward, while other mammalian ABC family members have homologs in bacteria *(Sebastian, Rishishwar et al. 2013)*.

III-A.1.b. CFTR functions and mutations

As shown in Table 6, CFTR has been involved in transport of the chloride anion but also of other small anions such as glutathione, bicarbonate and thiocyanate.

Function	Associated molecules	Impact on cell/tissue function	References
Anion channel	Cl^-	Osmolarity	*Schwiebert 1998*
	HCO_3^-	pH (notably of intracellular compartments)	*Welsh 1987*
	GSH	Redox balance	*Linsdell 1998*
	S1P	Lipid signaling	*Meissner 2012,*
Regulator of other channels	ENac	Sodium transport, Osmolarity	*Hwang 1996*
	CaCC, CLC, ORCC	Chloride transport, Osmolarity	*Jentsch 1994*
			Schwiebert 1995
			Stutts 1995
Regulators of other functions	Channels and receptors subject to recycling	Fusion, Trafficking, Endo/Exocytosis	*Barasch 1991*
		Membrane protein turnover	*Bradbury 1992*

Table 6 Cellular functions assumed by the CFTR protein. CaCC= Calcium-activated Cl^- channels; CLC= Voltage sensitive Cl^- channels; ENac= Epithelium sodium channel; GSH= Glutathione; ORCC= Outwardly rectifying Cl^- channels; S1P= sphingosine-1-phosphate.

In some tissues, like airway epithelia, CFTR also regulates Cl^- and Na^+ transport and ATP release through its interaction with other channels. CFTR has also been shown to interact with other membrane receptors such as G-coupled proteins directly through its cytoplasmic domains or indirectly via cytoskeletal linkage. Finally, CFTR is believed to play an important role in vesicle trafficking, endocytosis and exocytosis and therefore in membrane protein turnover *(Schwiebert, Benos et al. 1998, Rowe, Miller et al. 2005)* (Table 6).

Since the identification of *cftr* as the gene responsible for CF, 1,940 mutations have been listed in the "CF mutation database". The most frequent mutations of this 215 kb gene fall into these categories: missense (40.2%), frameshift (15.9%), splicing (11.7%) and nonsense (8.3%), the remainder being deletions and promoter alterations *(Lommatzsch and Aris 2009)*. Mutations can also be classified in classes (I to VI) reflecting their level of residual CFTR function, which depends on the efficiency with which a given mutant *cftr* allele undergoes transcription, protein translation, post-translational maturation, membrane integration and channel function *(Welsh and Smith 1993, Zielenski and Tsui 1995)*. We present in Table 7 the most common mutations in the five countries with the most CF patients, namely, USA, France, United Kingdom (UK), Germany and Australia (Table 7).

Various studies have attempted to link the *cftr* genotype and mutation class to CF disease phenotype. As an example, the ΔF508 and G551D mutations have been correlated with severe respiratory disease, pancreatic insufficiency and high sweat Cl^- concentration (see section III-A.1.c) *(Zielenski 2000)*. However, as shown in Figure 8, the relation between genotype and phenotype is not simple and might not be related directly to a specific mutation or its class, but rather due to their different effects on CFTR functions in a given cell and tissue, under particular homeostatic or stress conditions. Beyond mutations and function of the CFTR itself, the role of other genetic loci (a.k.a., modifier genes) in influencing CF phenotype has been suggested, along with that of environmental factors *(Moskowitz, Gibson et al. 2005)* (Fig. 8).

Country	Mutations	Frequency	Class
Australia	ΔF508	76.9%	II
	G551D	2.8%	III
	G542X	4.5%	I
	R553X	1.3%	III
	621+1G➔T	1.1%	I
France	ΔF508	67.7%	II
	G542X	2.9%	I
	N1303K	1.8%	II
	1717-1G➔A	1.4%	I
	W1282X	0.9%	I
Germany	ΔF508	71.8%	II
	R553X	2%	III
	N1303K	1.8%	II
	G542X	1.2%	I
	R347P	1.2%	IV
United Kingdom	ΔF508	75.3%	II
	G551D	3.1%	III
	G542X	1.7%	I
	621+1G➔T	0.9%	I
	1717-1G➔A	0.6%	I
United States of America	ΔF508	68.6%	II
	G542X	2.4%	I
	G551D	2.1%	III
	W1282X	1.4%	I
	N1303K	1.3%	II

Table 7 Most common *cftr* mutations in Australia, Europe (France, UK and Germany) and the USA. Mutation classes are associated with defects in: I= Protein synthesis; II= Protein maturation; III= Channel regulation; IV= Channel conductance; V= mRNA stability and VI= Protein stability. *Bobadilla 2002 and Green 2010*

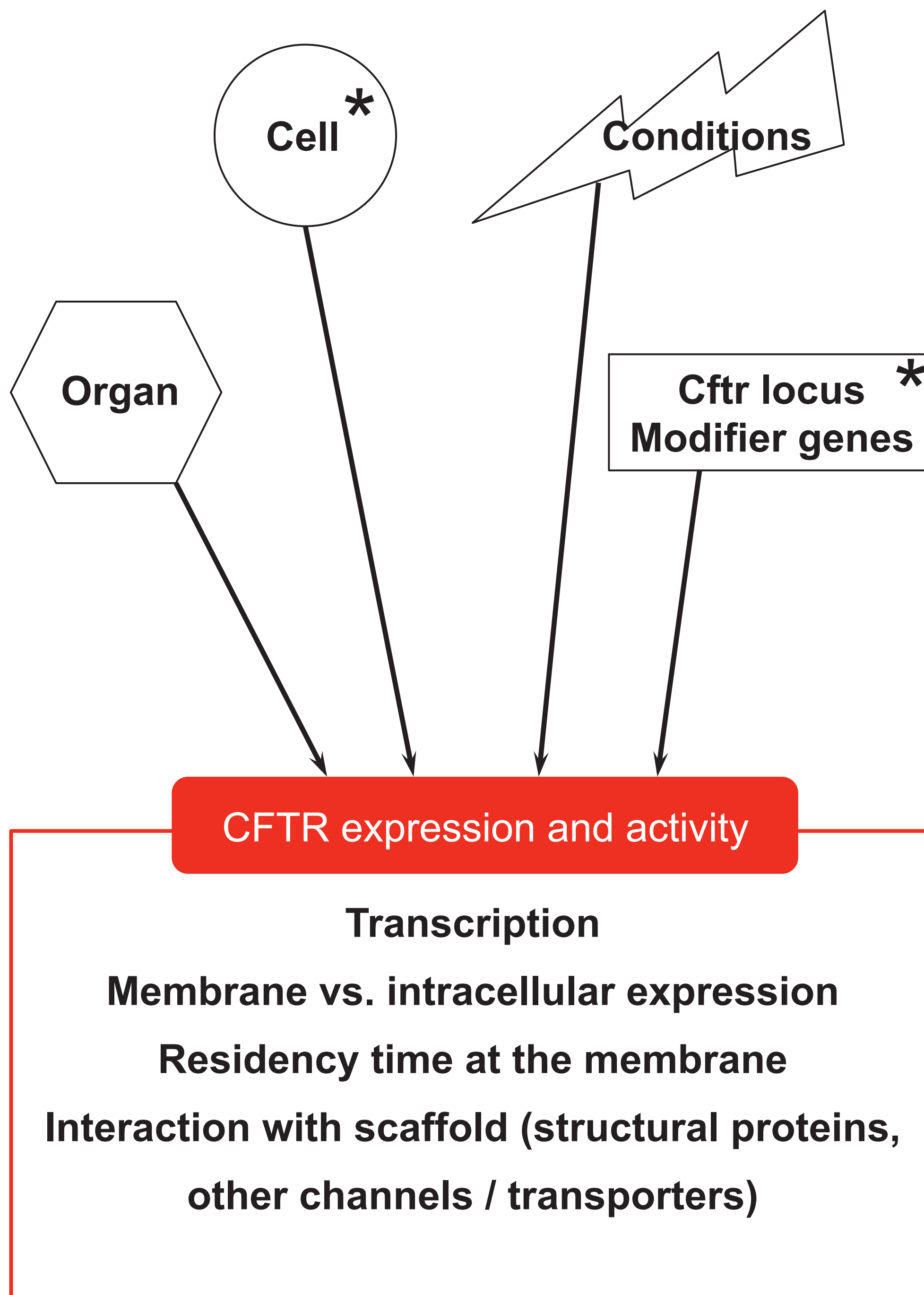


Fig. 8 Multiple levels of control for CFTR expression and activity. Mutations in the *cftr* gene and in modifier genes influence CFTR expression. The organ of interest, cell type within this organ, and physiological conditions (steady-state, inflammation) also modulate CFTR expression and activity. * marks species-specific differences that may explain the phenotypic discrepancies between human CF and cloned mouse, ferret and pig models of CF.

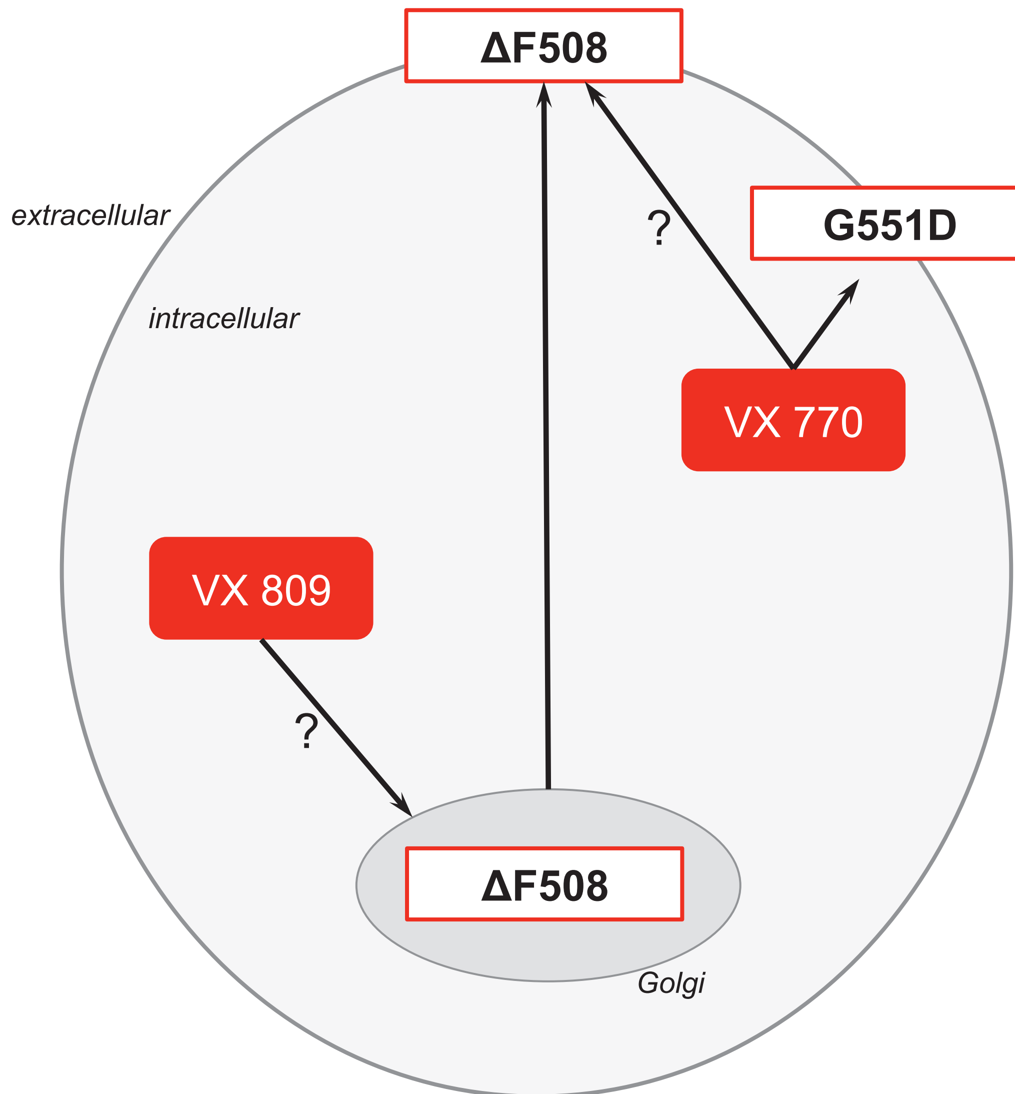


Fig. 9 **ΔF508 and G551D**. Associated with severe lung disease in CF, these particular mutations of the *cftr* gene prevent protein folding in the Golgi (ΔF508) and proper channel conductance (ΔF508 and G551D). Biosynthetic “corrector” VX 809 and “potentiator” VX 770 have been tested for ΔF508 and G551D, respectively. The effects of VX 809 on protein folding as well as VX 770 on conductance of the ΔF508 mutation are still under investigation.

III-A.1.c. ΔF508 and G551D: two particular mutations of the *cftr* gene.

The most frequent mutation in CF, found in approximately 66% of all CF alleles in Caucasians is ΔF508 *(Bobadilla, Macek et al. 2002)*. This class II mutation leads to a phenylalanine deletion at position 508 in the CFTR protein (NBD1), which prevents proper post-translational folding in the trans-Golgi network and leading to its degradation and low expression at the membrane. In addition, any residual ΔF508 CFTR protein that makes it at the membrane has lower open probability than wild-type CFTR such that this mutant is also defective in terms of conductance. Another common mutation, the class III mutation G551D, alters CFTR channel function and its regulation of outwardly rectifying chloride channels (ORCC) and epithelium sodium channels (ENaC). This mutation is also located in the NBD1 and alters ATP binding, CFTR conductance and regulatory functions.

These two mutations, both associated with severe lung disease, are located in a sensitive regulatory domain of CFTR *(Cutting, Kasch et al. 1990, Schwiebert, Benos et al. 1998)*, which led itself to the development of pharmacological therapies, as presented in Figure 9. Biosynthetic compounds known as “correctors” and “potentiators” may help mutant CFTR proteins translocate to the plasma membrane and improve its function once there *(Sloane and Rowe 2010)*. VX-809 and VX-770 (Vertex Pharmaceuticals, Inc.; Cambridge, Massachusetts), respectively a ΔF508 corrector and G551D or class III/IV potentiator, have been tested recently with some success (Fig. 9). Results of *in vitro* and *in vivo* studies for each component are promising since increases of mature mutated CFTR at the membrane, Cl^- secretion and transepithelial current were observed along with improvement in lung function and lower sweat chloride concentration during human clinical trials *(Van Goor, Hadida et al. 2009, Van Goor, Hadida et al. 2009, Accurso, Rowe et al. 2010, Clancy 2010, Rogan, Stoltz et al. 2011)*.

III-A.2. CFTR dysfunction and organ pathophysiology

CFTR is mostly expressed at the membrane of exocrine epithelia, as well as exocrine glands. Therefore, defects in CFTR function caused by *cftr*

mutations affect several exocrine organs throughout the body. Morbidity and mortality in CF is primarily associated with airway disease, characterized by infection, inflammation and high mucus secretion leading to obstruction (see section III-B). In addition to airway pathology, primary disorders in CF relate to high salt concentration in sweat, pancreatic, intestinal and genital tract dysfunction as well as bone disease. Relationships between genotype and phenotype are strongest for genital, sweat gland, and pancreatic symptoms of CF, and lowest for airway disease *(Schwiebert, Benos et al. 1998, Rowe, Miller et al. 2005, Lommatzsch and Aris 2009)*.

III-A.2.a. CF-associated gastrointestinal disease

The pancreatic disease in CF is characterized by an abnormal hydration of pancreatic secretions, leading to a blockade of pancreatic ducts and an early destruction of the exocrine pancreas, with a deficit in the secretion of digestive pancreatic enzymes (notably lipases). This leads to the formation of pancreatic cysts, hence the term "cystic fibrosis". While pancreatic insufficiency can be treated by enzyme supplementation, pancreatic disease phenotype correlates well with severe *cftr* mutations *(Cohn, Friedman et al. 1998, Sharer, Schwarz et al. 1998)*. Other primary symptoms in the gastrointestinal tract in CF center around duct obstruction, leading to organ damages. Duct obstruction is apparent in the liver, the gallbladder and the colon, leading respectively to an obstruction of the bile canaliculi and cirrhosis, gallstone formation, and newborn meconium ileus and distal intestinal obstruction syndrome. No direct links between non-pancreatic GI phenotype and *cftr* mutations has been observed *(Davis, Drumm et al. 1996, Wilschanski, Rivlin et al. 1999, Lommatzsch and Aris 2009)*.

III-A.2.b. CF-associated genital tract disease

Mutations in the CFTR protein are highly associated with infertility in men, due to azoospermia. Indeed, congenital bilateral absence of vas deferens is a syndrome affecting 95% of CF males. Moreover, CBAVD in non-CF men has been related to mutation in the CF gene with compound heterozygosity *(Taulan, Girardet et al. 2007, Lommatzsch and Aris 2009)*. In

women, although congenital absence of uterus and vagina (CAUV) is usually associated to sporadic mutations, *cftr* mutations double its incidence *(Timmreck, Gray et al. 2003, Radpour, Gourabi et al. 2008)*.

III-A.2.c. Secondary CF-associated disorders

Reduced bone density is secondary to CF disorders and is linked to poor nutrition status, vitamin deficiency and bone resorption *(King, Topliss et al. 2005)*. Moreover, pancreatic damages due to impairment of the exocrine pancreas can alter islet cells in the endocrine pancreas. This complication of CF leads to the development of CF-related diabetes (CFRD) and associated diabetic disorders characterized by increased insulin resistance, poor glucose control and worsened lung function *(Kelly and Moran 2013)*.

III-B. CF airway disease

III-B.1. CF airway triad: epithelial dysfunction, infection and inflammation

Infants with CF display normal lung structure at birth, with obstructions in the small airways, as evidenced by air trapping, that seem to be the earliest manifestations of airway disease. However, recent studies in cloned CF pigs and young CF children showed early defects of the trachea lumen associated with CFTR dysfunction *(Meyerholz, Stoltz et al. 2010, Ratjen 2012)*. In general, CF airway disease is characterized by an obstruction of airway ducts. In the upper airways, this is reflected by nasal polyposis and chronic sinusitis while in the lung obstruction is associated with mucus plugging, chronic pathogen infection and inflammation *(Davis, Drumm et al. 1996)*.

CF airway disease encompasses the absence of functional CFTR in epithelial cells, the chronic presence of bacteria, fungi and viruses and massive and sustained neutrophil recruitment to the lungs. This triad is responsible for chronic bronchiectasis leading to a progressive decline in respiratory function and destruction of the lung tissue. Over time, tissue

damage may lead to respiratory failure, with lung transplantation as the only therapeutic option.

III-B.1.a. Primary defects in the CF epithelium

Based on a recent study, wild-type CFTR is differentially expressed according to the airway region and is highly detected at the plasma membrane of ciliated cells from upper, large and small airways. CFTR expression is weaker in gland acini from all regions and in superficial epithelia from respiratory bronchioles and alveoli *(Kreda, Mall et al. 2005)*. In CF, the absence of functional CFTR causes changes in Cl^- and Na^+ conductance leading to airway surface liquid (ASL) dehydration, increased thickness of the mucus layer and impairment of mucociliary clearance of secretions *(Matsui, Grubb et al. 1998, Boucher 2003)*. These primary dysfunctions have been associated with increased bacterial attachment to the epithelium and the development of hypoxia, favoring the growth of anaerobic pathogens and inflammation of the airways *(Worlitzsch, Tarran et al. 2002, Moskowitz, Gibson et al. 2005)*.

III-B.1.b. Infection

CF airway pathophysiology is characterized by the presence and persistence of pathogens, causing chronic airway infections. Upon birth, CF and non-CF infants can be affected by spontaneous respiratory viral infections, which in CF have been associated with a higher decrease in lung function and higher probability of hospitalization *(Hiatt, Grace et al. 1999, van Ewijk, van der Zalm et al. 2005)*. Primary infection with bacteria may also be observed in CF infants and largely caused by opportunistic bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Haemophilus influenzae* (*H. influenzae*). This picture evolves with time, with more than 80% of CF teenagers showing chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) infection. Infections with other strains like *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cepacia* are less frequent *(Davis, Drumm et al. 1996, Moskowitz, Gibson et al. 2005, Hartl, Gaggar et al. 2012)*. In addition to bacteria, CF patients can be colonized by mycobacteria, yeast and fungi such as *Aspergillus fumigatus*, the latter causing in some patients a

complex allergic syndrome termed allergic bronchopulmonary aspergillosis (ABPA) *(Olivier, Weber et al. 2003a, Olivier, Weber et al. 2003b, Stevens, Moss et al. 2003, Chotirmall, O'Donoghue et al. 2010, Moss 2010)*.

Adaptation of bacteria to CF airways is achieved through production and secretion of exopolysaccharides, which are essential to biofilm formation. For example, alginate is secreted by *P. aeruginosa* when the bacteria acquires a mucoid resistant phenotype, associated with the loss of virulence factors and motility *(Costerton, Stewart et al. 1999, Singh, Schaefer et al. 2000)*. Biofilms have been associated with increased resistance to antibiotic treatment, protection from phagocytosis and other anti-bacterial responses mounted by recruited neutrophils *(Meluleni, Grout et al. 1995, Jesaitis, Franklin et al. 2003)*, which makes mucoid *P. aeruginosa* difficult, if not impossible, to eradicate. *S. aureus* strains can also become resistant to antibiotics and formed methicillin-resistant *S. aureus* (MRSA) colonies, which are also difficult to eradicate. Several groups have attempted to correlate bacterial phenotype, density and proliferation rate with CF airway disease severity, notably with regards to the frequency of acute pulmonary exacerbations (APE) *(Moskowitz, Gibson et al. 2005)*. However, so far, no such correlation has been identified and the definition of APEs is mainly based on a combination of parameters including, but not restricted to, decreased nutritional status, increased sputum production and worsened lung function *(Rosenfeld, Emerson et al. 2001, Stenbit and Flume 2011)*.

III-B.1.c. Inflammation

As illustrated in Table 8, epithelia and resident immune cells such as tissue macrophages secrete cytokines and chemokines in response to pathogen infection, which induces innate immune activation and neutrophil recruitment. The recognition of PAMP and DAMP by CF airway epithelial cells induces an inflammatory cascade with production and secretion of pro-inflammatory cytokines including but not limited to IL-1β, TNF-α, IL-6 and IL-8 and LTB_4, which are all potent neutrophil chemoattractants (Table 8). In the lungs, neutrophils have been described to effectuate diapedesis via the alveolar capillary bed and post capillary venules in small airways *(Downey, Bell et al. 2009)*. This mechanism is thought to be deficient in CF, but no direct link

Cells		Associated functions and molecules
Microbial cells	Bacteria Fungi Viruses	Biofilm formation (exopolysaccharide, PAMP), Growth (metabolites), Virulence factors
Host cells	Epithelium Macrophages Neutrophils	Cytokine production (GM-CSF, IL-1β, IL-6, IL-8, LTB_4, TNF-α), Exocytosis (MMP-9, MPO, NE), NETosis, Oxidative burst, Phagocytosis, Sensing (Complement receptors, Fc receptors, NLR, TLR)

Table 8 Microbial and host cell contributions to the CF airway inflammatory environment. GM-CSF= Granulocyte macrophage colony-stimulating factor; IL= interleukin; LTB_4= Leukotriene B_4; MMP-9= Matrix metalloproteinase-9; MPO= Myeloperoxydase; NE= neutrophil elastase, NLR= NOD-like receptors; PAMP= Pathogen-associated molecular patterns; TLR= Toll-like receptors.

has been established and neutrophil transmigration through CF epithelia seems not to be affected by CFTR dysfunction *(Pizurki, Morris et al. 2000)*. CF airway disease is characterized by chronic inflammation, linked to persistent infection- and neutrophil-driven tissue damage. Inflammation is observed early in the course of the disease and several studies showed higher IL-8 levels and significant neutrophil recruitment prior to overt infection in both CF infants and human CF fetal xenografts *(Tirouvanziam, de Bentzmann et al. 2000, Rosenfeld, Gibson et al. 2001, Tirouvanziam, Khazaal et al. 2002, Armstrong, Hook et al. 2005)*. Nevertheless, the presence of significant inflammation before infection in CF airways remains a controversial notion.

III-B.2. The impact of neutrophils on CF airway disease

III-B.2.a. Old paradigm

An early, massive and sustained recruitment of blood neutrophils into airways is a hallmark of CF airway disease. The prevailing view is that upcoming CF airway neutrophils: (i) are unable to kill bacteria and fungi upon recruitment; (ii) undergo rapid necrosis; (iii) release passively their toxic granule contents (NE, and MPO, notably) and other toxic material such as oxidants, actin and DNA. Neutrophil by-products such as proteases and oxidants are largely to blame for increasing mucus viscosity and perpetuating inflammation and tissue destruction, which has been strongly correlated with decreased lung function among CF patients *(Sagel, Sontag et al. 2002)*. Another common view of airway neutrophils in CF is that phagocytosis is reduced, maybe due to the degradation of opsonins, the inability of neutrophils to handle mucoid strains, and a higher level of neutrophil necrosis *(Coakley, Taggart et al. 2002, Jesaitis, Franklin et al. 2003, Morris, Doull et al. 2005, Downey, Bell et al. 2009)*. Taken together, neutrophil-associated factors found in the CF airway fluid suggest that neutrophils are very significant, yet somewhat passive, actors in the disease process.

III-B.2.b. New paradigm

In a string of recent studies looking at CF blood and airway samples collected from patients *in vivo*, our group has shown that CF neutrophils maintain an oxidative burst activity and ROS production and release, leading to oxidative stress in both systemic and airways compartments *(Tirouvanziam, Conrad et al. 2006)*. Then, we demonstrated that a large amount of the neutrophils found within CF patients' sputum are alive and have undergone marked mobilization of secretory vesicles, tertiary granules and secondary granules (based on surface CD11b and CD66b expression) when compared to their blood counterparts, a phenomenon also seen in healthy controls. However, a cardinal feature of CF is that live airway neutrophils also express very high levels of CD63 at their surface, a marker indicating highly active exocytosis of primary granules by these cells and the concomitant active release of their toxic content, including NE and MPO, in the extracellular milieu. This unexpected neutrophil activation profile was concomitant with a decrease of phagocytosis-associated receptors CD14 and CD16, both of which are targets for cleavage by extracellular NE. Moreover, live CF airway neutrophils showed increased levels of unconventional neutrophil receptors, generally associated with antigen-presenting cells lineage (MHCII, CD80) and T-cell modulation (CD294) *(Tirouvanziam, Gernez et al. 2008)*. CF airway neutrophils also displayed increased surface expression of the G-CSF receptor (CD114), the receptor for advanced glycation endproducts (RAGE, a component of oxidant signaling) and the ecto-nucleoside triphosphate diphosphohydrolase (CD39, an enzyme hydrolysing extracellular ATP), suggesting that these cells not only remain alive, but also mount an active response to the stress conditions (high extracellular oxidant and ATP levels) reigning in CF airways *(Makam, Diaz et al. 2009)*.

We further investigated CF airway neutrophil physiology and discovered that these cells activate critical signaling pathways. As compared to blood, live CF airway neutrophils showed higher level of phosphorylated cAMP response element binding protein (CREB), another indicator of an active stress response. Most interestingly, CF airway neutrophils also presented increased phosphorylation of the eIF4E, 4E-BP and S6 ribosomal

protein, three canonical targets of the mTOR pathway *(Makam, Diaz et al. 2009)*. As presented earlier (see section I-B.1), the mTOR pathway can be activated by glucose and AA, and higher concentrations of those metabolites are also found in CF airways *(Barth and Pitt 1996, Baker, Clark et al. 2007, Garnett, Nguyen et al. 2012)*.

Taken together, the activation of the stress-responsive CREB pathway and anabolic mTOR pathway, combined with the high level of extracellular metabolites and changes in surface receptors suggest that neutrophils homing to CF lungs undergo a concerted set of reprogramming processes, as argued in our seminal publication in the Proceedings of the National Academy of Sciences of the USA in 2009 (Article 1) *(Makam, Diaz et al. 2009)*. Based on this recent data, we propose a new paradigm of CF airway disease (illustrated in Figure 10) in which CF neutrophils are not rapidly necrosing, but rather stay viable, playing an active, rather than passive, role in the course of the disease (Fig. 10).

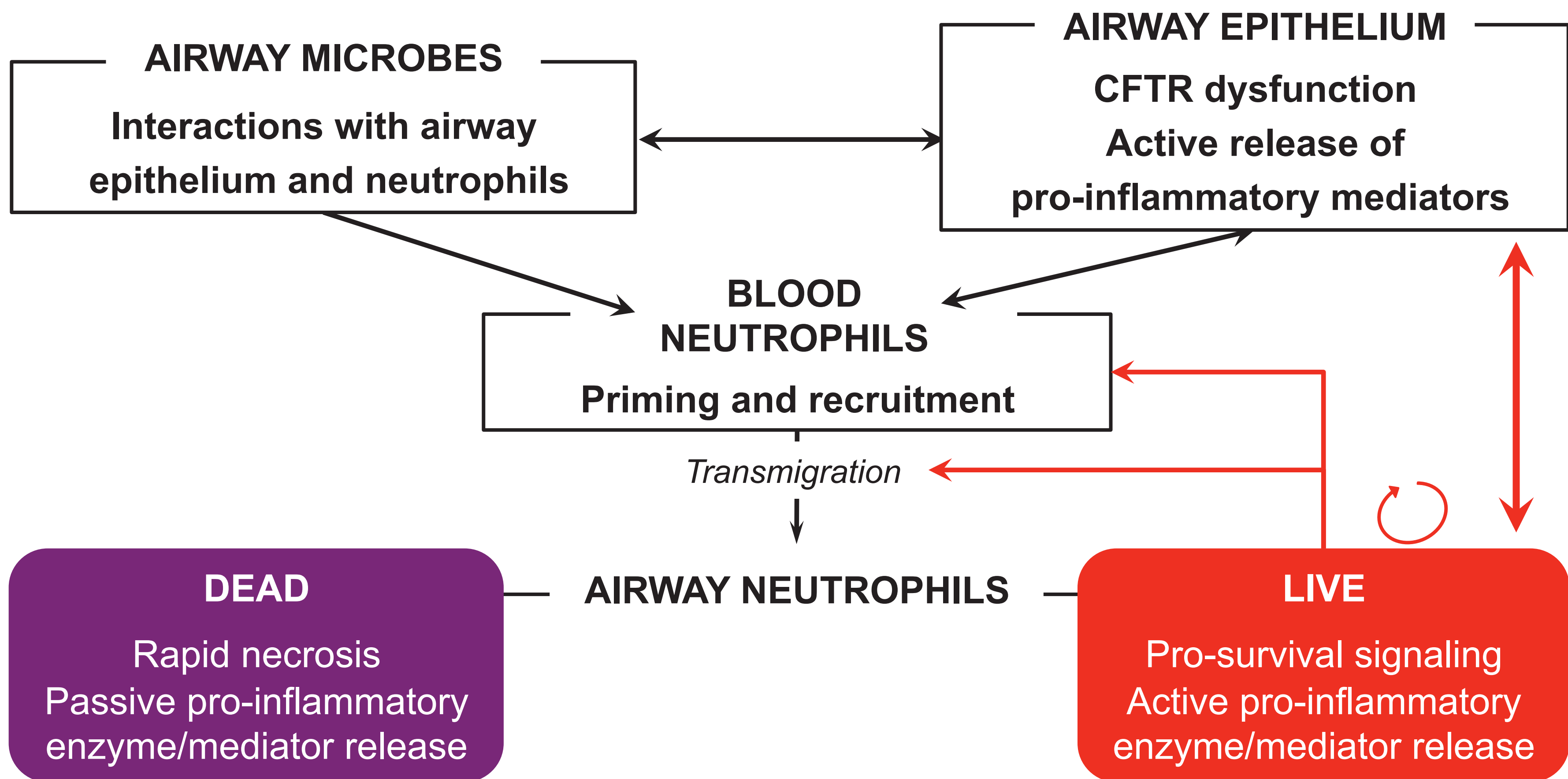


Fig. 10 Paradigms of CF airway disease. Airway microbes and epithelium stimulate blood neutrophil priming and recruitment. Once neutrophils transmigrate into CF lung, the conventional paradigm holds that they die rapidly and passively release toxic mediators. Based on *in vitro* data, we propose a new disease paradigm, according to which neutrophils stay alive in CF airway and actively release pro-inflammatory components.

Activation of critical, host-induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis lungs

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Cystic fibrosis (CF) patients undergo progressive airway destruction caused in part by chronic neutrophilic inflammation. While opportunistic pathogens infecting CF airways can cause inflammation, we hypothesized that host-derived metabolic and stress signals would also play a role in this process. We show that neutrophils that have entered CF airways have increased phosphorylation of the eukaryotic initiation factor 4E and its partner the 4E-binding protein 1; 2 key effectors in the growth factor- and amino acid-regulated mammalian target of rapamycin (mTOR) pathway. Furthermore CF airway neutrophils display increased phosphorylation of the cAMP response element binding protein (CREB), a major transcriptional coactivator in stress signaling cascades. These active intracellular pathways are associated with increased surface expression of critical adaptor molecules, including the growth factor receptor CD114 and the receptor for advanced glycation end-products (RAGE), a CREB inducer and sensor for host-derived damage-associated molecular patterns (DAMPs). Most CF airway fluids lack any detectable soluble RAGE, an inhibitory decoy receptor for DAMPs. Concomitantly, CF airway fluids displayed high and consequently unopposed levels of S100A12; a potent mucosa- and neutrophil-derived DAMP. CF airway neutrophils also show increased surface levels of 2 critical CREB targets, the purine-recycling enzyme CD39 and the multifunctional, mTOR-inducing CXCR4 receptor. This coordinated set of events occurs in all patients, even in the context of minimal airway inflammation and well-preserved lung function. Taken together, our data demonstrate an early and sustained activation of host-responsive metabolic and stress pathways upon neutrophil entry into CF airways, suggesting potential targets for therapeutic modulation.

CFTR | EN-RAGE | flow cytometry | S6 ribosomal protein | stromal derived factor-1

Airway disease is the most common cause of morbidity and mortality in cystic fibrosis (CF), a recessive disease affecting more than 70,000 individuals worldwide (1). Early, massive, and sustained inflammation by neutrophils is a hallmark of CF airway disease and neutrophil by-products drive disease progression (2). In particular, human neutrophil elastase (HNE) destroys the elastic fabric of airways and its activity is the best known predictor of CF lung function (3). Also, neutrophil-derived DNA and actin strings spill into mucous secretions and increase their viscosity, a characteristic of CF airway disease that underlies its other common name, mucoviscidosis.

Neutrophils are first-line immune sentinels, endowed with a plethora of receptors and weapons to sense and eliminate pathogens (4). In CF patients, neutrophils function properly throughout the body, except for the airway lumen where their presence seems to favor, rather than antagonize, chronic infections. Hence, CF airways must provide specific conditions that promote early neutrophil dysfunction (5). In a xenograft model of human airway development, we observed that before any infection, neutrophils from the mouse host would migrate into and destroy CF, but not non-CF, xenografts (6); this evidence further supports a role for host-derived cues in the conditioning of airway neutrophils.

The common view of peripheral neutrophils is that of a terminally differentiated population, with little if any ability to become anabolic and escape the default apoptotic program that they embark on as they leave the bone marrow. However, recent studies have outlined the ability of human neutrophils to modify their transcriptional profile upon migration to organs, likely under the influence of local conditioning by host-derived cues (7). Indeed, neutrophils are also endowed with the ability to recognize host-derived metabolites and stress signals (8). Some of these signals, collectively referred to as damage-associated molecular patterns (DAMPs), are emerging as important biomarkers in inflammatory diseases and represent a heterogeneous class of self-proteins that have undergone chemical transition (e.g., oxidation), relocation (e.g., nuclear protein in the extracellular milieu), or other stress-induced modifications (9).

For a long time, neutrophil dysfunction in CF airways has been equated with necrosis and passive release of elastase, DNA, and actin. However, we established recently by direct ex vivo analysis of airway neutrophils from CF patients that a large fraction of these cells are viable and appear to actively release HNE-containing granules (10). We also provided evidence that neutrophils undergo a highly unusual set of surface and intracellular changes suggesting significant functional reprogramming upon their migration to CF airways. Here, we hypothesized that viable CF airway neutrophils would bear the signs of pathophysiological conditioning by endogenous cues such as DAMPs, including significant activation of key metabolic and stress signaling pathways.

To test this hypothesis, we undertook direct ex vivo analyses of CF airway neutrophils focusing on critical intracellular phosphoepitopes and surface molecules associated with metabolic and stress pathways. Our data demonstrate an early and sustained activation of the anabolic mammalian target of rapamycin (mTOR) pathway and the DAMP-/stress-responsive cAMP-response element binding protein (CREB) pathway upon neutrophil entry into CF lungs. These results shed new light onto neutrophil regulatory pathways and emphasize the importance of host-derived signaling cues in CF pathobiology.

Results

Direct Profiling of Intracellular Phosphoepitopes in CF Airway Neutrophils. We demonstrated previously (10) that viable CF airway neutrophils, as compared to their blood counterparts, main-

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The authors declare no conflict of interest.

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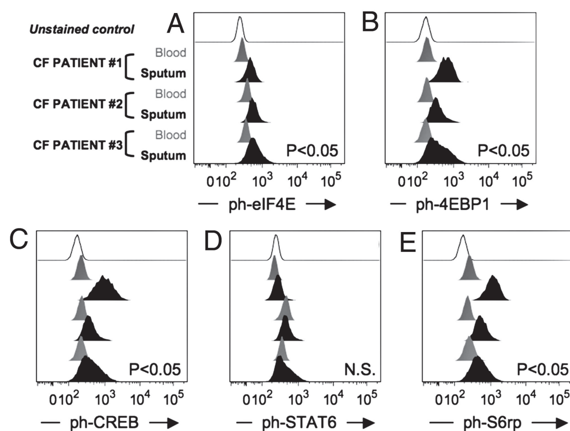

Fig. 1. Activation of mTOR and CREB pathways in CF airway neutrophils. (*A–E*) Viable neutrophils from induced sputum (black histograms) are compared to their blood counterparts (gray histograms) for expression of phospho-eIF4E, 4E-BP1, CREB, Stat6, and S6rp, respectively. A fluorescence control (left unstained in the relevant channel) is also shown (open histogram, first line of each panel). Data are from 3 representative patients with low, medium, and high airway neutrophil count, respectively (from *Top* to *Bottom* in each panel). Differences in median fluorescence intensities between airway and blood neutrophils were similar across all patients in the study (N.S.: not significant; see Table S1 for detailed statistics).

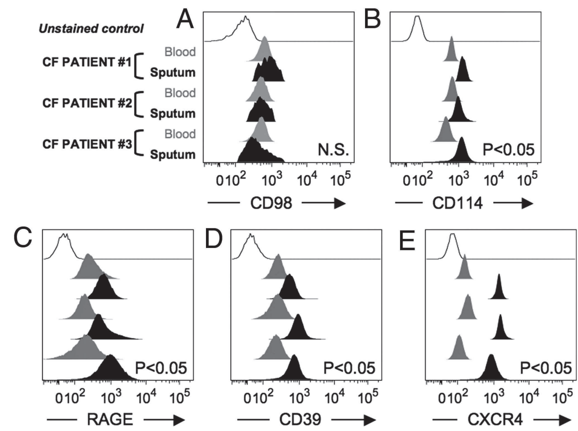

Fig. 2. Modulation of mTOR and CREB-associated surface adaptor molecules in CF airway neutrophils. (*A–E*) Viable neutrophils from induced sputum (black histograms) are compared to their blood counterparts (gray histograms) for expression of CD98, CD114, RAGE, CD39, and CXCR4, respectively. A fluorescence control (left unstained in the relevant channel) is also shown (open histogram, first line of each panel). Data are from 3 representative patients with low, medium, and high airway neutrophil count, respectively (from *Top* to *Bottom* in each panel). Differences in median fluorescence intensities between airway and blood neutrophils were similar across all patients (N.S.: not significant; see Table S1 for detailed statistics).

tained similar levels of phosphorylated intermediate kinases (Akt, p38 mitogen-activated kinase, p44/42 and cJun N-terminal kinase) and significantly increased their levels of phosphorylated S6 ribosomal protein (S6rp). A final effector in the anabolic mTOR pathway, S6rp can also be activated in mTOR-independent fashion (11). Here, we assembled an independent cohort of CF patients with variable ongoing inflammation, lung function, age, gender, and genotype ($n = 20$, see supporting information (SI) Table S1 for demographic details) and screened their airway neutrophils, at baseline, for the above epitopes and 4 new ones relevant to the question of stress signaling (see SI Methods). Analytical gating of viable blood and neutrophils was as described previously (10) and illustrated in Fig. S1.

Activation of mTOR and CREB in CF Airway Neutrophils. We observed increased phosphorylation within CF airway neutrophils of the eukaryotic initiation factor 4E (eIF4E), a translation apparatus-associated effector and anabolic switch (12) in the mTOR pathway (Fig. 1*A*; median change from blood neutrophils, +24%, see Table S2 for detailed statistics). We also observed increased phosphorylation within CF airway neutrophils of the 4E-binding protein 1 (4E-BP1, Fig. 1*B*; median change from blood neutrophils, +68%), an inhibitory binding partner of eIF4E that is itself inhibited by phosphorylation (11). Phosphorylation of the host stress-responsive CREB transcriptional co-activator (13) was also increased in these cells (Fig. 1*C*; median change from blood neutrophils, +67%). Meanwhile, the cytokine-regulated signal transducer and activator of transcription (STAT) 1 was not detectable in CF airway neutrophils (not shown) while STAT6 was detectable but not significantly modulated compared to blood neutrophils (Fig. 1*D*). Data for this new cohort confirmed previous data (10) on Akt, p38 mitogen-activated kinase, p44/42, cJun N-terminal kinase (stable expression, not shown), and S6rp (increased expression, Fig. 1*E*; median change from blood neutrophils, +69%).

Modulation of the mTOR-Associated Surface Molecules CD98 and CD114 in CF Airway Neutrophils. The mTOR pathway is the main orchestrator of cell anabolism; responding to nutrient (notably amino acids) availability and growth factor exposure (12). Extracellular amino acids are in large excess in CF lungs, driving opportunistic bacteria toward auxotrophy (14). Thus, we tested whether high amino acid levels would drive up expression of CD98, the common heavy chain subunit in heterodimeric amino acid transporters (15). CD98 expression (Fig. 2*A*) was neither up- nor down-regulated on CF airway neutrophils compared to blood neutrophils, unlike previous data in activated macrophages (16). Next, we assessed modulation on CF airway neutrophils of CD114, receptor for the granulocyte-colony stimulating factor (G-CSF), and a major neutrophil growth factor (17). CF neutrophils showed strongly up-regulated CD114 expression in airways compared to blood (Fig. 2*B*; median increase from blood to airways, +46%), although G-CSF was detectable in some, but not all, CF airway fluids (Table 1).

Modulation of the Receptor for Advanced Glycation End-Products (RAGE), a Potent CREB Inducer, in CF Airway Neutrophils. The CREB pathway mediates the response to various host-derived stress signals, including DAMPs. A versatile DAMP sensor and CREB inducer (18) is the receptor for advanced glycation end-products (RAGE). We show that RAGE expression is up-regulated on CF airway neutrophils compared to their blood counterparts (Fig. 2*C*; median increase from blood to airways, +291%). We also observed that the soluble form of RAGE (sRAGE), an inhibitory decoy receptor up-regulated in pulmonary injury settings (19) was not detectable in 18 out of 20 CF airway fluids tested, although it was present in high amounts in plasma (Table 1). Furthermore, the major RAGE inducer S100A12 (5, 20), also called EN-RAGE, was induced by about 100-fold in CF airway fluids compared to plasma (Table 1). Another RAGE ligand, S100A4 (8), was detected in some but not all CF fluids (Table 1).

Modulation of Inflammation-Associated CREB Targets, CD39 and CXCR4, in CF Airway Neutrophils. Several neutrophil-associated surface effector molecules have been previously shown to crit-

Table 1. Measures of metabolic and stress signaling in CF patient fluids

Analyte	Detectability	Median [IR]	Correlations	Difference
CXCL12 (pg/ml)	Plasma: 8/20	142 [75.6; 287]	None	None
	Sputum: 11/20	377 [83.8; 617]	None	
G-CSF (pg/ml)	Plasma: 8/20	20.8 [5.1; 27.2]	None	Plasma < Sputum ($P = 0.02$)
	Sputum: 9/20	1197 [75.1; 1415]	None	
S100A4 (ng/ml)	Plasma: 20/20	10.8 [6.8; 27.2]	None	Plasma > Sputum ($P = 0.02$)
	Sputum: 12/20	3.4 [3.1; 6.2]	None	
S100A12 (pg/ml)	Plasma: 20/20	41.3 [27.6; 91.8]	None	Plasma << Sputum ($P < 0.01$)
	Sputum: 20/20	6762 [3948; 7629]	Neutrophil sputum count ($P = 0003$)	
sRAGE (pg/ml)	Plasma: 20/20	1354 [1119; 1520]	None	Plasma >> Sputum ($P < 0.01$)
	Sputum: 2/20	2 datapoints 1091; 3058	None	

IR, interquartile range defined by encompassing 25th and 75th percentile. Correlations were sought between the fluid measures above and disease predictors. These included age, gender, genotype (homozygous for DF508 mutations, compound heterozygotes harboring one DF508 and one other mutations and patients with other mutations), infection with opportunistic pathogens, ongoing Pulmozyme, TOBI, Zithromax, inhaled steroid treatments, functional expiratory volume in 1 sec (a measure of lung function), neutrophil blood and sputum counts. Differences were calculated between fluid measures in blood and sputum.

ically depend on CREB activation for expression. CD39 is up-regulated in activated myeloid cells via the CREB pathway (21) and is the rate-limiting enzyme in neutrophil autocrine/paracrine feedback regulatory loops involving purines (22). Here, we show a strong up-regulation of surface CD39 on CF airway neutrophils compared to their blood counterparts (Fig. 2*D*; median increase from blood to airways, +162%). CXCR4, a CREB target (23) with high expression in both immature young and mature senescent neutrophils that causes their sequestration in the bone marrow (24, 25), was also highly up-regulated on mature, nonsenescent CF airway neutrophils (Fig. 2*E*). The median increase in CXCR4 expression as neutrophils migrate from blood to airways (+634%) exceeded that measured for conventional granule activation markers such as CD11b or CD66b (+233% and +545%, respectively; see Table S1 for detailed statistics). Interestingly, CXCL12 (also called stromal-derived factor 1), the only known ligand for CXCR4, was detected in some but not all CF airway fluids, with median levels comparable to those in plasma (Table 1).

Metabolic and Stress Signaling in CF Airway Neutrophils Occurs Early and Independently of Conventional Disease Predictors. The coordinated set of events described above, featuring increased eIF4E and CREB phosphorylation in CF airway neutrophils and up-regulated surface CD114, RAGE, CD39, and CXCR4 expressions, occurred in all CF patients tested and did not correlate with age, gender, genotype, airway and blood neutrophil count, infection status, and lung function ($P > 0.1$ for all) (Table 1). In particular, the presence or *P. aeruginosa* bacteria in the airways of patients did not influence this phenomenon. We did not find any indication that chronic therapies, some of which have been claimed to modulate neutrophilic inflammation (e.g., Pulmozyme, TOBI, Zithromax, or inhaled steroids as listed in Table S1), had any influence on the induction of mTOR and CREB in CF airway neutrophils. Additional data are required however to study potential correlations within complex combined classes of patients (e.g., with or without *P. aeruginosa*, combined with specific therapies). Finally, and of significant importance for our understanding of CF airway inflammation, we found that functional CF airway neutrophil subsets defined by high and low CD16 expression (an indication of active HNE release; see Fig. 3 and ref. 10) did not differ in the expression of the above metabolic/stress markers (Fig. S1). Thus, metabolic and stress signaling occurs right upon entry of neutrophils into CF airways, earlier than HNE release, and is sustained throughout their lifespan.

Discussion

Research on CF airway disease has often focused on mechanisms of epithelial dysfunction and opportunistic infections, relegating the

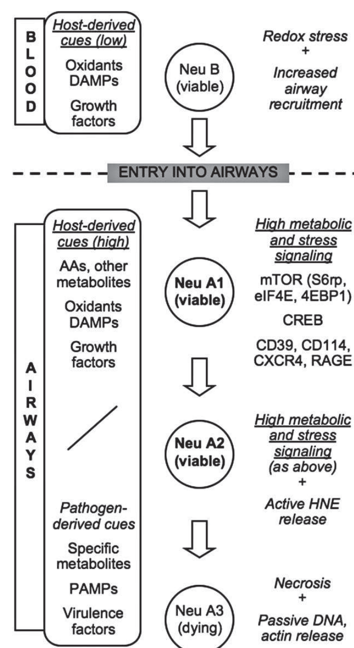

Fig. 3. Putative model of CF airway inflammation highlighting the role of host-derived metabolic and stress signals. Results presented here and previously (refs. 6, 10, 28), support the existence of 4 discrete states of neutrophils, 1 in CF blood (Neu B) and 3 in the airways (Neu A1–3). We propose that host-derived cues such as oxidants, DAMPs, growth factors, and, in the airways, amino acids (AAs) and other metabolites, drive the transition between these discrete states. Pathogen-derived cues mirroring the categories defined for host-derived cues (i.e., pathogen-associated molecular patterns or PAMPs, pathogen-derived metabolites and virulence factors) also impact the biology of Neu A1, A2, and A3 subsets. Early and sustained activation of mTOR and CREB pathways and associated surface molecules is readily detectable in the Neu A1 subset, while active HNE release defines the Neu A2 subset. Both Neu A1 and A2 subsets are viable and thus may be therapeutically targeted to prevent their transition into the dying Neu A3 subset, which passively releases undigested DNA and actin fibers in the lumen.

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third hallmark of the disease, neutrophilic inflammation, to a secondary role. Here, we demonstrate that the mTOR and CREB pathways, 2 intracellular cascades responsive to host-derived metabolic and stress signals, are activated immediately upon migration of neutrophils to CF airways. Importantly, these events were readily detectable in patients with low airway neutrophil count and normal lung function. Taken together, our results support a pathological model (Fig. 3) that emphasizes the role of host-derived cues in a stepwise, abnormal conditioning of neutrophils entering into CF airways.

The extreme sensitivity of human neutrophils to experimental artifacts make it essential for signaling studies focusing on inflammatory pathologies to rely on cells collected from affected organs and on methods for their direct analysis, as used here. In vivo, neutrophils are exposed to various signals and integrate them into concerted responses, unless signals induce their rapid necrosis. This was believed to occur in CF, until we showed that neutrophils homing to CF airways do, in fact, remain viable, albeit with a highly unusual set of phenotypic changes (10). These included an active, rather than passive, release of HNE and an increased phosphorylation of the S6rp. S6rp is a conventional effector in the anabolic cascade downstream of mTOR activation, yet it can also be phosphorylated in mTOR-independent fashion (11). Our evidence that CF airway neutrophils activate eIF4E and 4E-BP1, in addition to S6rp, demonstrates an activation of the canonical mTOR pathway in these cells.

Our results are the first demonstration of selective mTOR activation in neutrophils in human disease. The mTOR pathway is an evolutionary conserved anabolic cascade generally associated with long-lived cells and triggered by a range of signals such as amino acids and selected growth factors (12). In CF airway fluid, free amino acid levels are high due to: (*i*) high extracellular amino acid production caused by neutrophil- and bacteria-derived proteases, the activity of which is potentiated by redox stress (26); and (*ii*) defective amino acid reuptake, caused by dysfunction of the CF ion channel and downstream transepithelial charge gradient anomalies (27). Neutrophils are equipped with several heterodimeric amino acid transporters involving the heavy CD98 subunit. Our results demonstrate that CD98 expression, while not up-regulated in CF airway compared to blood neutrophils, still remained detectable, consistent with a role in amino acid uptake (15). Comprehensive metabolite profiling experiments will be necessary to assess whether extracellular amino acids are indeed the main/sole inducers of mTOR pathway activation in CF airway neutrophils. Alternatively, mTOR pathway activation in CF airway neutrophils may proceed via receptors to anabolic growth factors such as CD114, which indeed was up-regulated in these cells.

The mTOR pathway may not operate in isolation, but rather in concert with other pathways, to deliver prosurvival and anabolic cues to CF airway neutrophils. Since CF features chronic metabolic and redox stresses (28, 29), we hypothesized that airway neutrophils would display significant activation of the RAGE pathway, a sensor for host-derived DAMPs generated under such stresses. Indeed, CF airway neutrophils demonstrated highly increased RAGE expression, concomitant with decreased levels of the inhibitory decoy receptor sRAGE, as well as high and consequently unopposed levels in the airway fluid of the RAGE ligand S100A12 (>100-fold up-regulation compared to plasma). S100A12 is believed to derive chiefly from neutrophils (perhaps actively, like HNE; see ref. 10) and we found a strong correlation between sputum S100A12 levels and neutrophil count. Airway epithelial cells themselves may release this DAMP into the lumen, as demonstrated in other inflamed mucosae (30). Other RAGE ligands, such as nuclear high mobility group box protein 1 (31) from dead epithelial cells and neutrophils, may provide additional host-dependent activation of RAGE in CF airway neutrophils.

RAGE is a potent inducer of the stress-responsive CREB pathway (18) and our data demonstrate a specific up-regulation of phosphorylated CREB in these cells. CREB induces several adaptor molecules involved in neutrophil regulatory loops, and we identified 2 such CREB targets, CD39 and CXCR4, as being significantly up-regulated in CF airway neutrophils. CD39 is a critical enzyme that metabolizes ATP into AMP and primes purine-dependent regulatory cascades during various neutrophil effector responses (32). CD39 up-regulation in CF airway neutrophils is further evidence for an active, rather than passive, role for these cells. This result is also consistent with the recent evidence that purine levels track neutrophil count and CF airway disease (33).

With regards to CXCR4, the level of up-regulation observed on CF airway neutrophils compared to their blood counterparts is the highest of any of the molecules we have identified so far, including markers of granule mobilization such as CD11b and CD66b (ref. 10 and Table S2). CXCR4 is responsible for neutrophil retention in the bone marrow during maturation and upon senescence, and in that context, signaling via the G-CSF receptor CD114 provides the main driving force for CXCR4 down-regulation as needed for mature neutrophils to avoid bone marrow retention (24, 25). The concomitant up-regulations of CXCR4 and CD114 surface receptors in CF airway neutrophils thus constitute a rather intriguing coexpression pattern. Another potentially important functional interplay is that of CXCR4 with mTOR, the former having been shown to induce activation of the latter in cancer cells (34).

Interestingly, the only known ligand for CXCR4, CXCL12, was detectable in some but not all CF airway fluids tested. CXCL12 was previously measured in other lung diseases (35), and its absence in some CF fluids may be linked to high activity of HNE, which can cleave CXCL12 (36). Whether cleavage products of CXCL12 may retain some bioactivity toward CXCR4 is currently unclear. The absence of full length G-CSF in a large portion of CF airway fluids tested in this study, despite increased CD114 expression on CF airway neutrophils, may follow the same line of explanation since G-CSF can be cleaved by HNE (37) and yet may not be construed as a failure to activate the receptor since cleavage products may retain such bioactivity. Overall, an emerging set of regulatory mechanisms at play in CF airways rely on selective, HNE-dependent cleavage of conventional receptor/ligand pairs endowed with neutrophil-tropic effects (10, 38). Functional mismatches and the emergence of novel functional/signaling modules may ensue, regulating the balance of chronic/acute neutrophilic inflammation and hampering the normal resolution of infections (Fig. 3).

In this context, it is particularly important to consider that active HNE release by CF airway neutrophils occurs at all stages of the disease and that, as shown here, activation of mTOR and CREB pathways occur in these cells even before HNE release. While only associative at this point, the relation between these active metabolic and stress pathways and HNE release are worth exploring, since extracellular HNE is a key contributor to CF airway disease. Due to the sensitivity of neutrophils to exogenous manipulation, accurate mechanistic studies in vitro are difficult to conduct, but are necessary to confirm the relation between these 2 sets of events.

In summary, we show that the mTOR and CREB pathways, 2 key pathways involved in cellular responses to host-derived metabolic and stress signals, are activated upon migration of neutrophils to CF airways, early on in the disease. Within the context of CF, our results suggest that airway neutrophils are active disease contributors and bona fide therapeutic targets. CF airway neutrophils also emerge as a potential source of dynamic cellular biomarkers that could be interrogated to assess the disease course and, possibly, the effect of new targeted therapies. Such biomarkers, identified in baseline conditions on a limited set of patients, require additional data on large cross-sectional (infants to transplantees) and longitudinal (yearly baseline vs. disease exacerbations) cohorts to ascertain their usefulness as potential tools in clinical settings. More broadly, it is interesting to consider that the reliance on host-derived cues to drive innate immunity has been observed and thoroughly described

before in the plant kingdom (39). In humans, such cues may represent a primary evolutionary layer responsible for the robustness of neutrophilic immunity, notably in contexts where pathogen-sensing pathways are genetically or functionally debilitated (40).

Materials and Methods

Human Subjects. This study received the approval of the Stanford Administrative Panel on Human Subjects in Medical Research. All subjects signed informed consent forms before undergoing study procedures. Diagnosis of CF was by documented sweat chloride >60 mEq/L by quantitative iontophoresis test and/or one or more clinical features consistent with CF and/or preexisting documentation of 2 identifiable mutations. The presence in the patients' lungs of common opportunistic pathogens (as indicated in Table S1) was tested by routine sputum culture. Lung function was tested by spirometry using American Thoracic Society criteria.

Collection and Processing of Samples. Blood and airway fluid were simultaneously collected from subjects by venipuncture and sputum induction, respectively. Sputum induction is a minimally invasive, standardized, procedure that was shown by several independent groups to faithfully reflect CF inflammatory lung disease. Since our study focused on characterizing baseline functional and signaling profiles of CF airway neutrophils, we used a protocol for sputum processing that avoids DTT treatment and associated artifacts, as described earlier (10).

Multiparametric Flow Cytometry. Screening of viable CF airway neutrophils for intracellular and surface adaptor molecules of interest was performed as indicated in details in ref. 10 and in the *SI Methods*. In brief, cells were stained with the Live/Dead near infrared probe from Invitrogen to assess viability, as well as with fluorescent antibodies from Invitrogen, BD Biosciences, eBioscience and Cell Signaling Technologies, as listed in the *SI Methods*, online. Cells were acquired on a 4-laser LSRII digital FACS (BD Biosciences).

Fluid Assays. Fluid mediators were measured by specific commercial ELISA kits, including kits against CXCL12, G-CSF, and sRAGE (from R&D Systems) as well as S100A4 and S100A12 (from MBL International), as per the manufacturer's guidelines. Detection limits were 18 pg/ml, 20 pg/ml, 4 pg/ml, 0.25 ng/ml and 50 pg/ml, respectively. Absorbance data were acquired at 450 nm on a Modulus microplate reader (Turner Biosystems).

Analysis and Presentation of Data. Flow cytometry data were exported to the FlowJo software (Treestar) and compensated using single-stained beads or cells, as detailed elsewhere. Median fluorescence values were calculated and compared to the appropriate background controls. Statistical analysis of flow cytometry and ELISA datasets was performed using the JMP6 software (SAS Institute). Distributions were compared between blood and airway neutrophils using the paired Wilcoxon signed-rank test. Correlations between continuous variables were tested using the Spearman Rho test. Differences in outcome measures following nominal categories (e.g., gender, genotype) were assessed using the unpaired Wilcoxon rank-sum test. Differences or correlations were considered significant with $P < 0.05$.

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Supporting Information

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SI Methods

Foreword. Microbiological assessment of patients as well as methods for blood and sputum sample processing, surface and intracellular staining, data acquisition on the flow cytometer and multistep analytical gating strategy of viable neutrophils were exactly as described in extensive details in the supporting information *(SI) Text* [Tirouvanziam R, et al. (2008) Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci USA* 105:4335–4339.]. Core technical details are given below, as well as additional information regarding antibodies against intracellular phosphoepitopes and surface markers that are specific to this study.

Processing of Samples. To limit artifactual activation of blood and lung neutrophils as much as possible, samples were kept on melting ice immediately upon collection and throughout all experimental procedures. Blood samples were centrifuged at 400G for 10 min to remove platelet-rich plasma and resuspended to their original volume in sterile PBS with EDTA at 5 mM (PBS-EDTA). Sputum samples were weighed, their volume measured and 1–2 volumes of ice-cold PBS-EDTA were added to sputum samples to loosen adherences and inhibit extracellular proteases. Cells were collected after gentle mechanical dissociation by repeated slow passage through a sterile 18“G needle, followed by filtration through a sterile nylon 70 μm sieve (BD Biosciences) and low-speed centrifugation at 400G for 10 min to remove the sputum supernatant. Both platelet-rich plasma and sputum fluid were spun at 3000G for 10 min to pellet down platelet and debris, respectively, and then aliquoted for use in further fluid assays, as described in the *Materials and Methods* section.

Surface Marker Profiling. Whole blood (50 μl) and sputum cells (50 μl, after resuspension at 5×10^6 leukocytes per ml, similar to whole blood) were stained in PBS-EDTA for 20 min on ice, in the dark, with the Live/Dead near InfraRed viability probe (Invitrogen) and several antibodies against surface determinants. Previously tested antibodies (1) that were maintained for the analysis of the present cohort of patients were directed against CD11b, CD16, CD45, CD63, and CD66b (all from BD Biosciences) and CD14 (from Invitrogen). We also included novel antibodies against CD39, from eBioScience, CXCR4 (CD184) from BD Biosciences, and RAGE, from Santa Cruz Biotechnologies. After staining, cells were washed with excess PBS-EDTA, centrifuged, and the supernatant was removed. Upon resuspension in 100 μl of PBS-EDTA, cells were fixed with 2 ml of 1X Lyze/Fix PhosFlow (BD Biosciences). To ascertain the efficiency of individual stains, we used fluorescence-minus-one controls, consisting of all stains but the stain of interest. For quantitative assessment of antibody binding to key markers (CD39, CD98, CD114, CXCR4, RAGE), we used fluorescence channels with minimal overlap with other channels, as previously discussed (2). For standardization of cell staining, antibodies were purchased in large batches and titrated for epitope saturation. Saturating concentrations were used throughout the study.

Intracellular Phosphoepitope Profiling. Whole blood (50 μl) and sputum cells (50 μl, after resuspension at 5×10^6 leukocytes per ml, similar to whole blood) were stained in PBS-EDTA for 20 min on ice, in the dark, with the Live/Dead near InfraRed viability probe (Invitrogen). Cells were then washed with excess PBS-EDTA, centrifuged, and the supernatant was removed. Upon resuspension in 100 μl of PBS-EDTA, cells were fixed with 2 ml of 1X Lyze/Fix PhosFlow (BD Biosciences). After one wash cycle with excess PBS-EDTA, the supernatant was removed. Cells were resuspended in 100 μl of PBS-EDTA and permeabilized by drop-by-drop addition of 400 μl ice-cold Perm III buffer (BD Biosciences) while vortexing. Upon 30 min incubation on ice, the Perm III solution was washed twice with excess PBS-EDTA and the supernatant was removed. Cells were then resuspended in 50 μl PBS-EDTA and stained with antibodies against phosphoepitopes of choice, for 20 min, in the dark, followed by a wash in excess PBS-EDTA. Previously tested anti-phosphoepitope antibodies (2) that were maintained for the analysis of the present cohort of patients were directed against: Akt (phospho-S473), JNK (phospho-T183/phospho-Y185), NFkB p65 (phospho-S536), p38 MAP kinase (phospho-T180/Y182), p44/42 (phospho-T202/phospho-Y204), S6rp (phospho-S235/236), all from Cell Signaling Technologies and STAT5 (phospho-Y694) from BD Biosciences. Novel antibodies introduced in this study were against: CREB (phospho-S133) and 4E-BP1 (phospho-T37/46) from Cell Signaling Technologies; eIF4E (phospho-Y285), Stat1 (phospho-Y701) and Stat6 (phospho-Y694) from BD Biosciences.

Acquisition of Flow Cytometry Data. Data were acquired on a FACS LSRII digital flow cytometer (BD Biosciences) equipped with 4 lasers (407, 488, and 633 nm), 2 light scatter detectors (yielding forward and side scatter data) and 18 fluorescent detectors. Acquisition was controlled using the DiVa software (BD Biosciences). Threshold for data acquisition was set in the forward scatter channel, excluding dead cells and debris with very low size. To standardize data acquisition, several steps were taken. First, to limit day-to-day variability, all antibodies were purchased in large batches and used at saturating concentrations throughout the study. Second, to provide fluorescence background quantification in measurement channels, one aliquot of each sample was stained with the fixable viability dye, but left unstained in other channels (background control aliquots). Third, to standardize signal output by the flow cytometer, we ran before each session a thorough calibration procedure using a standard set of multicolor fluorescence beads.

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Table S1. Demographics of patients included in the study (sorted by FEV1, percent predicted)

Gender	Age	Genotype	Neutrophil sputum count (10^6 cells)	Neutrophil blood count (10^6 cells)	FEV1 (%Pred.)	Opportunistic infections	Inhaled steroids	Pulmozyme	TOBI	Zithromax
F	10	HO	10.2	4.1	119	N	N	Y	N	N
M	15	OT	28.3	4.6	106	N	Y	Y	N	N
M	12	OT	7.5	2.3	96	N	Y	Y	N	N
F	18	HO	2.46	6.8	95	Y: PA	Y	Y	Y	Y
F	11	HO	0.48	3.6	91	Y: SA	N	Y	N	N
F	44	OT	15.3	3.5	90	Y: PA, SA, AF, CA	Y	Y	Y	N
M	29	CH	45.7	5.6	89	Y: PA	Y	Y	N	N
F	37	CH	10.2	3.3	85	Y: PA	Y	Y	Y	N
M	11	CH	1.63	7.5	82	Y: PA	Y	Y	N	Y
F	48	HO	0.62	6.2	68	Y: PA	Y	N	N	Y
M	49	HO	60	7.8	67	Y: PA	N	Y	Y	Y
M	22	HO	38.2	5.7	66	Y: PA, CA	N	N	N	N
M	12	HO	0.75	3.4	66	Y: PA, HP, CA	N	Y	Y	Y
M	11	CH	4.5	10.4	65	Y: PA, SA, CA	Y	Y	Y	Y
F	50	CH	31.2	8.7	65	Y: PA, CA	Y	Y	N	Y
M	43	CH	73.5	5.3	65	Y: PA	Y	Y	Y	N
F	44	CH	127	6.4	62	Y: SA	Y	Y	N	N
M	25	HO	96	8.3	59	Y: PA	N	N	N	N
M	33	CH	16.2	4.1	48	Y: PA	Y	Y	Y	Y
F	26	CH	21	3.8	48	Y: PA	N	N	Y	N
M = 11 F = 9	27.5 ± 3.27 (Mean ± SE)	CH = 9 HO = 8 OT = 3	15.8 [2.97; 43.8] (Median [IR])	5.57 ± 0.48 (Mean ± SE)	76.6 ± 4.28 (Mean ± SE)	Y = 17 *N* = 3	Y = 7 *N* = 13	Y = 16 *N* = 4	Y = 11 *N* = 9	Y = 8 *N* = 12

Neutrophil counts are expressed as 10^6 cells for sputum (total count) and as 10^6 cells/ml for blood. Opportunistic infections include *P. aeruginosa* (PA), *S. aureus* (SA), *H. parainfluenzae* (HP), *A. fumigatus* (AF), *C. albicans* (CA). CH = compound heterozygotes, HO = DF508 homozygotes, OT = other mutations, Y = Yes, N = No.

Table S2. Detailed statistics for metabolic and stress markers induced in sputum vs. blood neutrophils (alphabetical order)

Analyte	Type	Percent change: Median [IR]	P
4EBP1	Phosphoepitope	+68.2 [+37.4; +96.8]	0.002
CD114	Surface marker	+46.4 [+41.3; +136]	0.04
CD11b	Surface marker	+233 [+133; +366]	0.03
CD39	Surface marker	+162 [+96.5; +268]	0.03
CD66b	Surface marker	+545 [+230, +1438]	0.03
CREB	Phosphoepitope	+67.5 [+40.0; +117]	$<10^{-3}$
CXCR4	Surface marker	+634 [+335, +722]	0.03
eIF4E	Phosphoepitope	+23.6 [+19.4; +55.4]	0.004
RAGE	Surface marker	+291 [+146; +572]	0.016
S6rp	Phosphoepitope	+69.4 [+53.7; +154]	$<10^{-3}$

All above data relate to new phosphoepitopes and surface markers, as detailed in text, except S6rp, CD11b, and CD66b (initially described in ref. 2), which data was also collected on patients listed in Table S1 and is provided here for comparison. Numerical data presented as percent change [median, with interquartile range (IR) defined by 25th and 75th percentiles]. P values were calculated by the Wilcoxon signed-rank test for paired data.

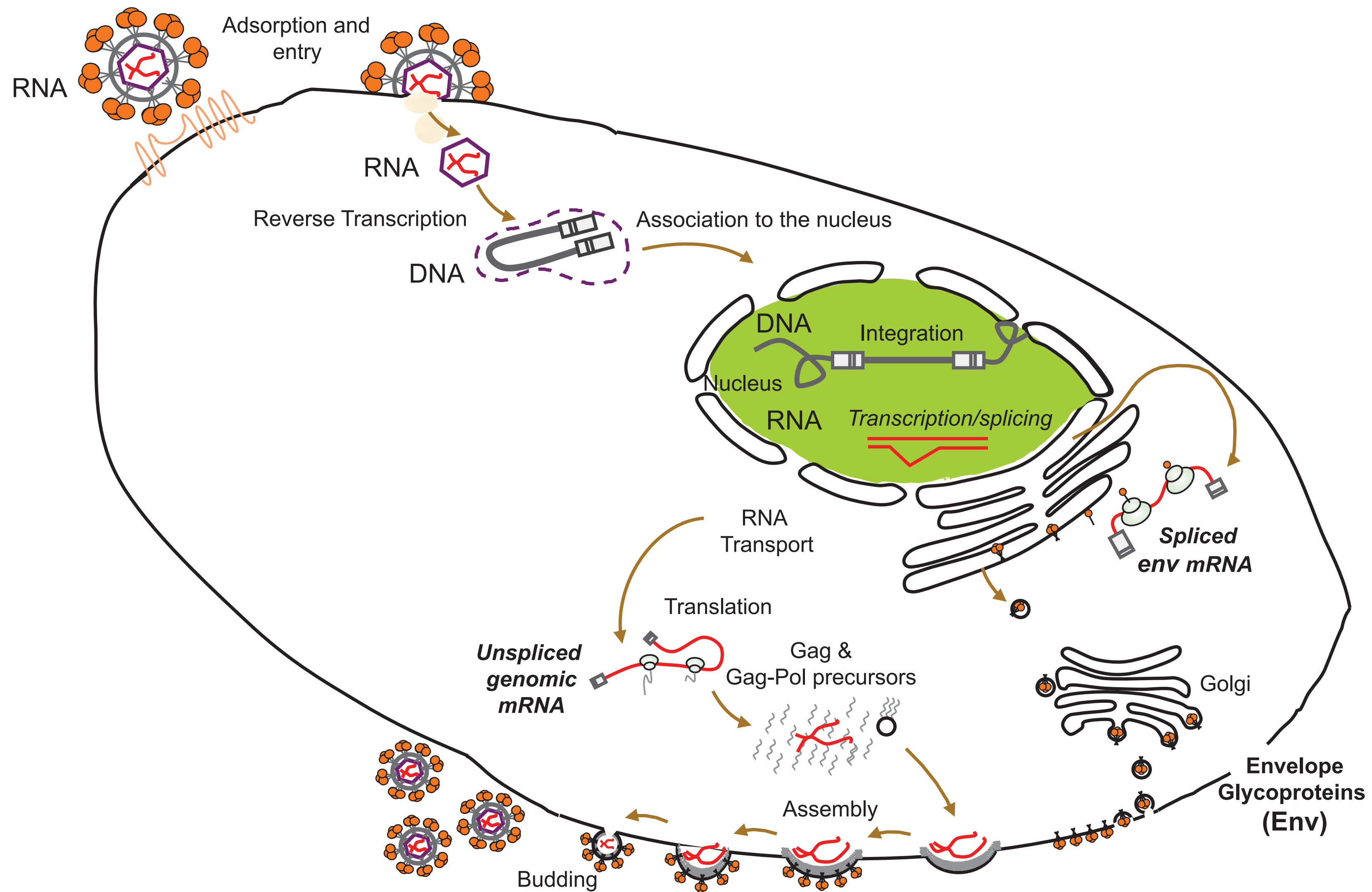


Fig. 11 Retrovirus infection cycle. Infectious retroviruses enter host cells after fusion of their envelope either directly at the cell plasma membrane or after endocytosis (Adsorption and entry). The rest of the cycle encompasses reverse transcription of the retroviral RNA into a double-stranded DNA copy, integration of the new-formed DNA into the host genome, leading to the so-called proviral DNA, transcription of the provirus, mRNA direct export or splicing and mRNA translation in two Gag and Env polyprotein precursors (depending on the retrovirus genus, alternative multisplicing can take place). The cycle ends by maturation of the polyproteins, assembly and the budding of virion progeny (assembly and budding).

CHAPTER IV: RETROVIRAL-DERIVED METABOLIC MARKERS

As developed in the chapters above, documenting changes in cell metabolic status has a major interest in the assessment of inflammatory processes that involve neutrophils. A large part of the present work has taken advantage of a new set of ligands derived from retrovirus envelope glycoproteins (Env) that bind exofacial determinants of nutrient and metabolite transporters. These ligands have been derived in our laboratory on the basis of receptor-binding properties of Env from specific generae of retroviruses.

In this Chapter, I will present a brief background on retroviral genes and on the origin and the function of these retrovirus-derived ligands that filled in for the absence of valid antibodies directed against cell surface determinants of nutrient transporters *(Kinet, Swainson et al. 2007, Yonezawa, Masuda et al. 2008)*.

IV-A. Biology of vertebrate retroviruses and receptors

IV-A.1. Vertebrate retroviruses and endogenous retroviral sequences

IV-A.1.a. Structure

Retroviruses are enveloped viruses whose genome is a dimer of a single-strand RNA(+) structured as an mRNA *(Coffin 1990)*. The name Retrovirus has been coined after their polymerase, the reverse transcriptase (RT), whose discovery by Howard Temin and David Baltimore led to a revolution in molecular biology *(Baltimore 1970, Temin and Mizutani 1970)*. As shown in figure 11, RT copies the viral RNA into a double-stranded DNA that can associate with the chromatin and integrate into the host cell genome to consitute bona fide gene transcriptional units (Fig.11).

Genus	Examples
Alpharetrovirus	*ALV, RSV*
Betaretrovirus	*MMTV, MPMV, sheep Jaagsiete retrovirus*
Gammaretrovirus	*MLV, FeLV, GaLV, KoRV*
Deltaretrovirus	*HTLV, STLV, BLV*
Epsilonretrovirus	*WDSV*
Lentivirus	*HIV / SIV, ongulate Visna, CAE, FIV*
Spumavirus	*Foamy viruses, primate, feline, bovine*

Table 9 The Retroviridae family. The Retroviridae family. Vertebrate retroviruses comprise 7 generae: Alpharetroviruses, Betaretroviruses, Gammaretroviruses, Deltaretroviruses, Epsilonretroviruses, Lentiviruses and Spumaviruses. ALV= Avian leukemia virus; BLV= Bovine leukemia virus; CAEV= Caprine arthritis and encephalitis virus; FeLV= Feline leukemia virus; FIV= Feline immunodeficiency virus; GaLV= Gibbon ape leukemia virus; HIV= Human immunodeficiency virus; HTLV= Human T cell leukemia virus; KoRV= Koala endogenous retrovirus; MLV= Murine leukemia virus; MMTV= Mouse mammary tumor virus; MPMV= Mason Pfizer Monkey virus; RSV= Rous sarcoma virus (avian); SIV= Simian immunodeficiency virus; STLV= Simian T cell leukemia virus; WDSV= Walleye dermosarcoma virus (fish).

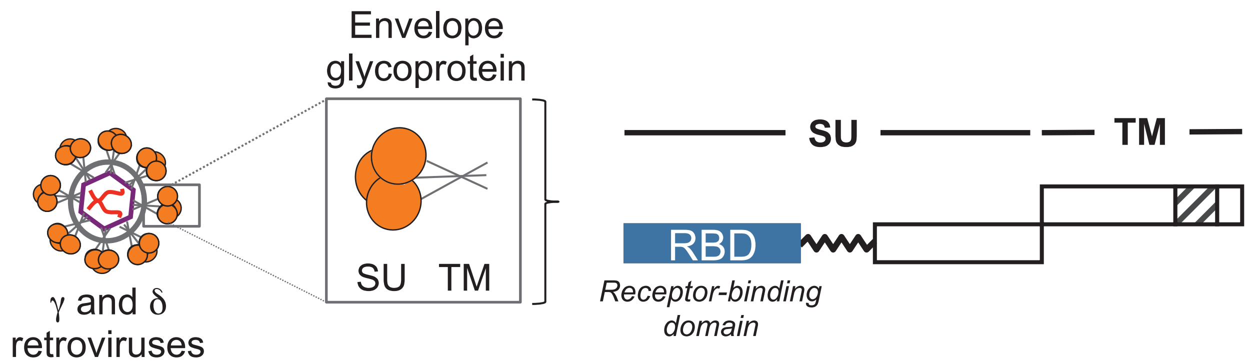
Vertebrate retroviruses comprise 7 generae (Table 9) that are classified according to their viral genome organization and the capsid sequences. All generae include the *LTR-gag-pol-env-LTR* framework, wherein LTR, for long-terminal repeat, comprises the transcription regulatory sequences such as the promoter, enhancer, transcription start and poly(A) signal sequences. The full-length transcribed RNA(+) has mRNA general features and can be exported to the cytoplasm to either:

- (i) be packaged into virions as a dimer to form the retrovirus genome;
- (ii) be translated to yield the *gag*-encoded viral core proteins, the reverse transcriptase and the other enzymes necessary to accomplish the viral cycle;
- (iii) be single-spliced and translated to yield the *env*-encoded Env glycoprotein. The full length RNA of several generae also includes multi-splicing steps that yield a complex array of regulatory proteins (Fig.11).

IV-A.1.b. Endogeneous sequences

Integration of proviral DNA into host genomes has constituted a powerful evolutionary engine as metazoan genomes have evolved with the recurrent accidental integration of retroviral sequences in the germline. Thus, it is admitted that nearly 8% of the human genome is composed of bona fide retroviral sequences that have lost their replicative abilities *(Bannert and Kurth 2004)*. Retroviruses and retroviral sequences can therefore be distinguished as exogenous viral agents (actual infectious retroviruses), which infect somatic cells and most rarely germ cells, and endogenous retroviral sequences (ERV) that are present in the germ line. Human ERV (HERV) are defective for replication although entire open reading frames are conserved. In contrast, several other mammalian species harbor replication-competent infectious ERV, including murine, porcine or simian species *(Martin, Kabat et al. 2003)*.

A



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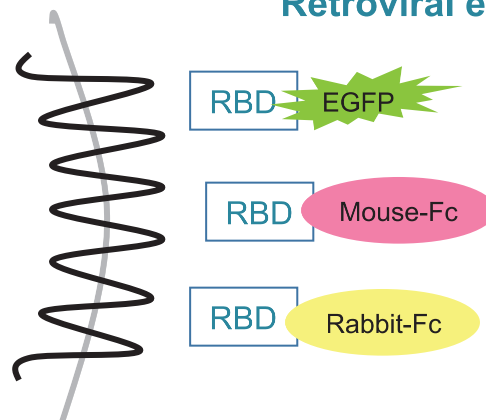

Fig. 12 Construction of retroviral envelope-derived ligands. (A) Schematic representation of γ and δ retroviral Env and RBD. Surface (SU) and the transmembrane (TM) subunits are indicated. **(B)** Retroviral envelope-derived ligands are obtained by fusing specific Env RBD to enhanced green fluorescent protein (EGFP), a Mouse-Fc (or mFc) or Rabbit-Fc (or rFc) tag. These "tagged RBD" are then used to detect surface metabolite transporters on cells by flow cytometry.

IV-A.2. The mammalian retroviral envelope glycoproteins and their cellular receptors

IV-A.2.a. Env, the receptor-binding and membrane fusion entity

Env is the retroviral fusion protein that allows retroviruses to enter target cells. All vertebrate retroviral Env present the same general structure and maturation process: an Env polyprotein precursor is formed from the translation of the single-spliced *env* mRNA. This precursor is translocated into endoplasmic reticulum (ER) as driven by the aminoterminal signal peptide. The cleavage of the signal peptide in the ER releases the Env aminoterminus in the lumen, which gives the Env protein orientation (idem for all Env), i.e. an extracellular aminoterminus and a cytoplasmic carboxyterminus. Glycosylation occurs in the ER followed by maturation in the Golgi where the Env precursor is further cleaved by a cellular protease at a specific site that splits Env into what will become an entirely extracellular surface component (SU) and an integral and membrane-anchoring transmembrane component (TM) (Fig.12-A).

The general accepted scheme of successful viral entry includes the binding of a specific cellular receptor by the SU aminoterminus, ensuing conformational changes that expose a fusion peptide located at the extracellular aminoterminal end of TM, and membrane fusion either directly at the cell surface or after endocytosis, depending on the Env and the target cell type *(Hunter and Swanstrom 1990)* (Fig.13 and 12-A).

IV-A.2.b. Receptors of gamma- and deltaretroviruses: a distinct family of receptors directly linked to metabolism

The first human retrovirus identified is the human T-cell leukemia/lymphoma virus (HTLV-1), a deltaretrovirus isolated in the early ‘80s *(Poiesz, Ruscetti et al. 1981)*, a couple of years before HIV-1, the lentivirus responsible for the AIDS epidemics, was discovered *(Barre-Sinoussi, Chermann et al. 1983).* However, the first retrovirus receptor identified is that of HIV-1, known to be a complex formed by CD4, an integral glycoprotein, which is part

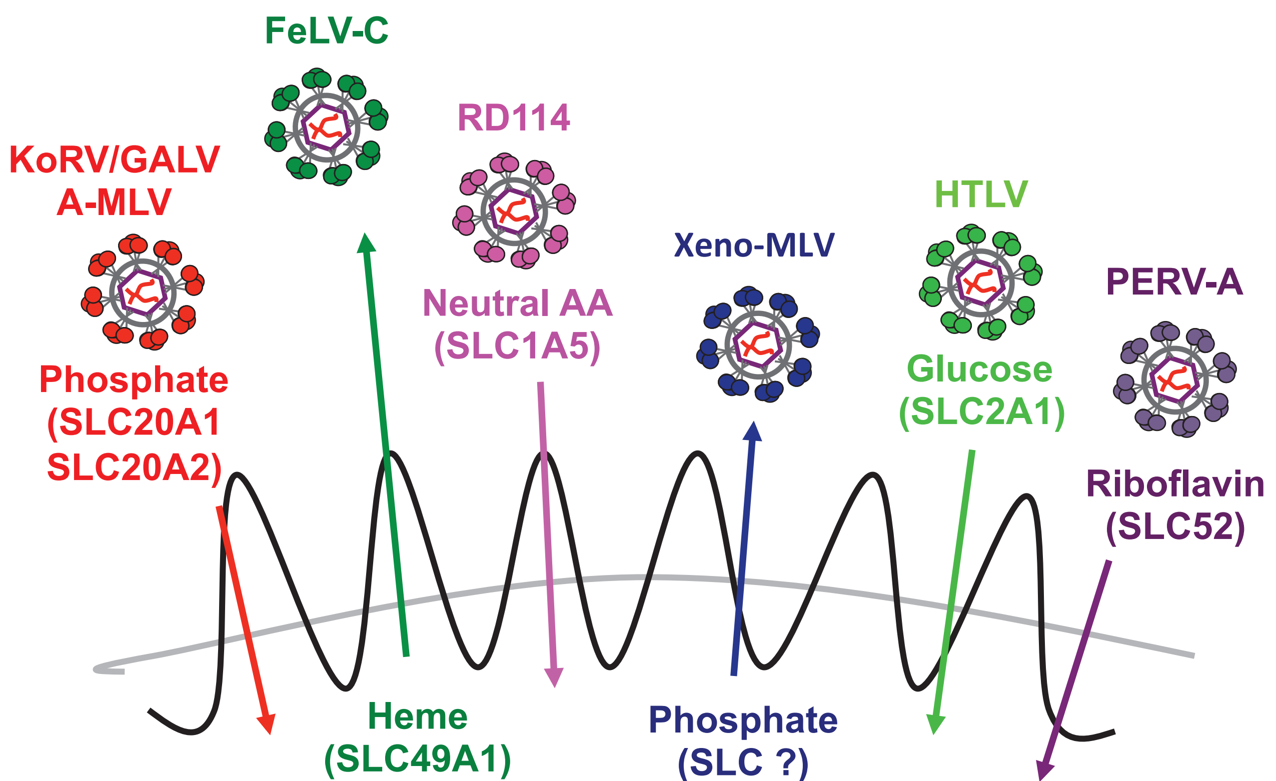

Fig. 13 **Metabolism-associated retrovirus receptors on host cells**. All mammalian receptors for γ and δ (HTLV) retroviruses that are characterized so far are multimembrane-spanning nutrient transporters. Examples of some retroviruses and their cognate receptor (solute carrier or SLC) are shown.

of the T-cell receptor complex *(Klatzmann, Champagne et al. 1984)*, and multitransmembrane chemokine receptors, CXCR4 or CCR5, depending on the target cell types *(Moore, Kitchen et al. 2004)*. On the other hand, it was only over 20 years after the discovery of HTLV, that the HTLV receptor has been identified, by our group, as Glut1/SLC2A1, the main glucose transporter *(Kim, Seiliez et al. 2000, Manel, Kim et al. 2003, Manel, Battini et al. 2005, Kinet, Swainson et al. 2007)*.

Between these two major discoveries of human retrovirus receptors, a series of receptors started to be identified for retroviruses that belonged to the gammaretrovirus generae. Interestingly, all receptors identified for these retroviruses were multipass proteins and those for which a function has been assigned were SLC of nutrients or of metabolic-related substrates. The substrates identified include diverse types of amino acids (neutral AA with ASCT1 and 2/SLC1A4 and 5 or cationic AA for CAT1/SLC7A1), inorganic phosphate, heme exporter, etc. (Fig.13 and Table 10).

It became clear that a tight co-evolution process has driven the selection of SLC as entry receptors for gammaretroviruses *(Overbaugh, Miller et al. 2001, Manel, Battini et al. 2005)*. This feature and the observation that HTLV Env sequence was close to those found in gammaretroviruses oriented our group towards the identification of Glut1/ SLC2A1 as the receptor of HTLV Env. Moreover, the same frame has served to our most recent identifcation of the function of XPR1, the receptor for xenotropic and polytropic murine leukemia viruses (Xeno- and Poly-MLV). Thus, XPR1 is the first inorganic phosphate exporter identifed in metazoans *(Giovannini, Touhami et al. 2013)*, a discovery that led to the classification of XPR1 as SLC53.

Another consequence of this exclusive interaction of gamma- and deltaretroviral Env with SLC, has led to the assumption that all gamma- and deltaretroviral Env for which no receptor has been identified, or when identified, for which no function has been yet assigned, are transporters directly associated with metabolism. This assumption is at the origin of using

RBD ligand	Receptor / Transporter	Substrates	Remarks	References
HTLV-2	**Glut1**	Glucose DHA	Glut1 is mostly a DHA transporter in mature human erythroid cells	*Manel 2003* *Montel-Hagen 2008*
KoRV-A GaLV	**PiT1**	Pi	Importer	*Hanger 2000* *O'Hara 1990*
Ampho-MLV	**PiT2**	Pi	Importer	*Van Zeijl 1994*
RD114	**ASCT2**	Gln Neutral AA	Importer	*Rasko 1999*
FeLV-C	**FLVCR**	Heme	Exporter	*Tailor 1999b*
PERV-A	**hRFT1 / 3**	Riboflavin	Importer	*Ericsson 2003* *Yao 2010*
Xeno-MLV	**XPR1**	Pi	Exporter	*Battini 1999* *Giovannini 2013*
BLV	**BLV-R***	*Not identified*	B and T lymphocyte activation marker	*Lavanya 2008*
PERV-B	**PERVB-R***	*Not identified*	-	*Takeushi 1998*

Table 10 List of RBD ligands and their cognate receptors. RBD-derived ligands from γ and δ (HTLV and BLV) retroviruses generated in our laboratory. R*= Unidentified specific retroviral receptor / transporter. Ampho-MLV= amphotropic murine leukemia virus; BLV= Bovine leukemia virus; DHA= Dihydroascorbate; FeLV-C= Feline leukemia virus type C; GaLV= Gibbon ape leukemia virus; HTLV= Human T cell leukemia virus; KoRV-A= Koala endogeneous retrovirus type A (a KoRV type B/J that binds different receptor has very recently been described *(Shojima, Yoshikawa et al. 2013, Xu, Stadler et al. 2013)*) ; PERV-A and B= Porcine endogenous retrovirus type A and B; Pi= inorganic phosphate; RD114= feline RD114 endogenous retrovirus; Xeno-MLV= Xenotrope murine leukemia virus.

these Env and their receptor binding domain (RBD) as new and unique exofacial ligands of metabolic transporters.

IV-B. RBD, the Env-derived soluble ligands of nutrient / metabolite transporters.

IV-B.1. Gamma- and deltaretrovirus RBD as soluble ligands of nutrient transporters

As shown on Figure 12, a modular organization that distinguishes an amino terminal RBD has been described about two decades ago *(Heard and Danos 1991, Battini, Heard et al. 1992)*. This provided the framework to isolate RBD from different Env of interest, to fuse them to a tag and produce a potential soluble ligand that binds the corresponding receptor/transporter when present at the cell surface (Fig 12). Under this principle, a panel of new metabolic ligands has been constituted and used in different physiological models to monitor metabolic alterations (Table 10).

IV-B.2. Application of the use of RBD as metabolic markers of physiological processes

The first applications of the RBD as new ligands have been the use of a HTLV Env-derived RBD to track Glut1 at the cell surface of T lymphocytes in different models, notably T-cell activation *(Manel, Kim et al. 2003, Kinet, Swainson et al. 2007)*. Furthermore, the same groups have unveiled the existence of a sub-population of thymic T cells with a remarkably high metabolism *(Swainson, Kinet et al. 2005)* and that HIV-1 infection of primary T lymphocytes remained dependent on the expression of Glut1, although this transporter is not used as a receptor by HIV *(Loisel-Meyer, Swainson et al. 2012)*. In both cases, the authors have shown that overexpression of Glut1 was accompanied by higher glucose consumption of the corresponding T cells.

By contrast, the same teams have shown that considerable overexpression of Glut1 accompanied human erythroid cell differentiation although no increase in glucose consumption was observed. Indeed, in the differentiated context of human erythroid cells, Glut1 served mainly as transporter of oxidized vitamin C (dehydroascobic acid), a trait that seems to be conserved in mammals that are unable to synthesize their own vitamin C *(Liang, Johnson et al. 2001, Montel-Hagen, Kinet et al. 2008, Montel-Hagen, Sitbon et al. 2009).*

Most recently, the use of a panel of RBD has been successfully applied to distinguish human solid tumors xenografted into mouse recipients *(Petit, Massonet et al. 2013).* The combinatorial use of several RBD allowed identifying xenografts that were derived from the same patients.

RESULTS
&
DISCUSSION

CHAPTER I:
RETROVIRAL ENVELOPE-DERIVED LIGANDS: NEW TOOLS TO STUDY METABOLISM DURING INFLAMMATORY RESPONSES

I-A. Foreword

Leukocytes play a major role in the body's response to stress. Infection by pathogens lead to rapid changes in cells and tissues, at the local level (e.g., mucosal epithelial cells, fibroblasts, circulating hematopoietic cells, etc.). These changes translate in further responses locally, but also at a systemic level and in specialized immune organs. The ensuing recruitment of inflammatory cells leads to an immune response involving subsets of innate (granulocytes, macrophages) and adaptive (lymphocytes) circulating cells. These events occur with acute reprogramming of quiescent, circulating leukocytes into activated leukocytes that usually relocate at the injury site. This adaptive response is accompanied by active transcription and protein synthesis and is energy-dependent. Energy is produced in part from the active absorption of metabolites (glucose, amino acids, phosphate, and others), which is reflected by an increase in expression of the cognate metabolite transporters at the surface of inflammatory cells. The monitoring of nutrient and metabolite transporters directly at the cell surface would thus be warranted. However, for reasons that are not completely clear and that in part include poor immunogenicity and high levels of sequence conservation, no reliable antibodies directed against cell surface determinants of nutrient transporters are available (see for instance *(Kinet, Swainson et al. 2007, Yonezawa, Masuda et al. 2008)*). This context enticed us to explore the use of Env RBD as metabolic markers of inflammation and immune responses (see Introduction Chapter IV).

Retroviruses are enveloped viruses that bind to host cells by direct interaction between the receptor-binding domain (RBD) on their envelope

glycoprotein (Env) and specific cell surface proteins. Gamma and deltaretroviruses, in particular, have been shown to interact with the host cell surface through active receptors that belong to the multimembrane protein family. Those receptors for which a function has been identified are directly involved in cellular metabolism *(Battini, Rodrigues et al. 1996, Overbaugh, Miller et al. 2001, Manel, Kim et al. 2003)*. Harnessing this evolutionary trait of gamma and deltaretroviruses, we developed a set of retroviral envelope-derived ligands (using the RBD of respective retroviral Env) as probes for high-affinity tagging of metabolite transporters on human cells *(Kim, Battini et al. 2004)*. These transporters, so far all identified as members of the SLC superfamily, carry a wide variety of metabolites, including, but not limited to: neutral amino acids (AA), cationic AA, glucose, phosphate, heme and vitamins.

Airway disease in cystic fibrosis (CF) is due to the massive and sustained recruitment of blood neutrophils into the lungs. Recent discoveries have helped to understand how neutrophilic inflammation is made chronic in CF lungs. In particular, our group has demonstrated metabolic reprogramming of CF airway neutrophils by intracellular staining of specific phosphoproteins, which led us to show strong activation of the mTOR pathway, the main anabolic switch, in these cells *(Tirouvanziam, Gernez et al. 2008, Makam, Diaz et al. 2009)*. To bring additional perspective onto this discovery, we aimed to characterize metabolite transporter expression by mTOR-positive CF airway neutrophils using flow cytometry. To accomplish this task, we needed reliable probes with which to identify surface metabolite transporters receptors (generally, SLC superfamily members, see Introduction section I-A.1.b) with high affinity and specificity. Our aim was thus to develop and validate RBD-derived probes in the context of inflammatory responses.

I-B. Original patent summary

We isolated soluble RBD from retrovirus Env, in combination with diverse tags, thus forming retroviral Env-derived ligands that allowed us to measure surface expression of their cognate receptors by flow cytometry. We used RBD generated from Env of the Human T-cell Leukemia Virus-2 (HTLV-

2) deltaretrovirus and the following gammaretroviruses: Feline endogenous Retrovirus (RD114), Koala endogenous Retrovirus (KoRV-A) and Amphotropic Murine Leukemia Virus (AMLV). The corresponding receptors bound by these RBD are Glut1 (SLC2A1, glucose transporter), ASCT2 (SLC1A5, neutral AA transporter), PiT1 and PiT2 (SLC20A1 and 2, both inorganic phosphate importers), respectively *(van Zeijl, Johann et al. 1994, Rasko, Battini et al. 1999, Hanger, Bromham et al. 2000, Manel, Kim et al. 2003)* (Fig. 13).

These probes, combined with our established protocols for flow cytometric analysis of blood and airway leukocytes, enabled us to gate singlets and live cells, discriminate leukocyte subsets and evaluate their respective levels of metabolite transporter expression. Thus, we compared expression of Glut1, ASCT2, PiT1 and PiT2 on the surface of blood leukocytes (granulocytes, monocytes, lymphocytes) and airway neutrophils in 16 patient samples. All leukocyte subsets expressed each of the four transporters tested, albeit at different levels. Interestingly, blood and airway neutrophils differed significantly in their expression levels. The use of these four RBD thus demonstrates alterations in metabolite transporter expression on neutrophils recruited to CF airways, which bring further evidence for pathological reprogramming of these cells being a part of the pathological process (International patent application)
(Tirouvanziam, Laval et al. 2010).

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(54) Title (EN): METHOD FOR THE DIAGNOSIS AND/OR PROGNOSIS OF INFLAMMATORY STATES

(54) Title (FR): PROCÉDÉ POUR LE DIAGNOSTIC ET/OU LE PRONOSTIC D'ÉTATS INFLAMMATOIRES

(57) Abstract:
(EN): The invention relates to a method for the diagnosis and/or prognosis of inflammatory states. We describe the use of at least one soluble receptor-binding (RBD), for the identification and quantication of the expression of membrane receptors present on the surface of target granulocytes, said identification and quantification taking place at a given time or during a given time interval, and allowing the diagnosis and/or prognosis of inflammatory states in a mammal.

(FR): L'invention porte sur un procédé pour le diagnostic et/ou le pronostic d'états inflammatoires. On décrit l'utilisation d'au moins un domaine de liaison au récepteur soluble (RBD) pour l'identification et la quantification de l'expression de récepteurs membranaires présents sur la surface de granulocytes cibles, ladite identification et ladite quantification ayant lieu à un temps donné ou durant un intervalle de temps donné, et permettant le diagnostic et/ou le pronostic d'états inflammatoires chez un mammifère.

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European Patent Office (EPO) : AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR
African Intellectual Property Organization (OAPI) : BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
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Eurasian Patent Organization (EAPO) : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

I-C. Discussion and contributions

Retroviral Env-derived ligands are unique tools to identify cell surface metabolite transporters, reflecting an important aspect of the cell physiological status. We used these markers to investigate changes occurring during chronic inflammation, in CF patients, a hallmark of which is the massive migration of neutrophils from blood into airways. We developed a unique protocol, allowing us to quantify transporter expression with these new probes and established a metabolic profile of cells from two different cellular compartments. Thus, we were able to distinguish blood leukocyte subsets (neutrophils, eosinophils, monocytes, and lymphocytes) and airway (sputum) neutrophils.

This invention illustrates the use of the new RBD set of probes to identify and characterize metabolic aspects of inflammation in leukocytes of different compartments. We demonstrated that our RBD can be used as biomarkers for activated inflammatory cells, such as airway neutrophils in CF and lead to a better characterization of disease processes. More extensive data using these RBD to characterize other stages of CF might support their use as disease-monitoring tools. Thus, we plan to extend this study to a larger set of CF patients, as well as healthy and disease controls. We also plan to test RBD derived from other Env that use additional metabolite transporters as receptors (Table 10). Owing to the physiological importance of metabolite tranporters and the wide availability of flow cytometers, the potential field of application for this approach is very large.

CHAPTER II:
METABOLITE TRANSPORTER EXPRESSION ON BLOOD NEUTROPHILS: COMPARISON BETWEEN INFLAMMATORY STATES

II-A. Foreword

Inflammation is a highly regulated process, characterized by the recruitment of blood leukocytes into peripheral sites of injury. Inflammation involves the release of immune mediators such as pro-inflammatory cytokines into the bloodstream, initiating systemic priming and activation of leukocytes. Inflammation can be classified as "chronic" or "acute", the former state being characterized by an extended stimulation of the immune system. Different inflammatory states impact the immune cells in various ways and can for example be associated with specific neutrophil phenotypes, while these cells are still in the circulation.

During the autoimmune disease process associated with rheumatoid arthritis (RA), systemic inflammation is linked to synovial membrane alterations and chronically inflamed joints. Neutrophils in RA patients have been shown to be readily primed in blood, which suggests that these cells play a role in the systemic course of RA *(Cedergren, Forslund et al. 2007, Cascao, Moura et al. 2010, Cascao, Rosario et al. 2010)*. Moreover, a recent study demonstrated specific alterations in blood metabolites, such as AA and lipids, distinguishing RA from other inflammatory arthritides *(Young, Kapoor et al. 2013)*. The emergence of a blood metabolite fingerprint for RA suggests that systemic metabolic imbalance may be part of RA pathogenesis, which may in turn affect blood neutrophils.

In CF, airway inflammation is linked to the absence of functional CFTR in the epithelium, the presence of bacteria and fungi in the lumen and a continuous and massive influx of neutrophils from blood *(Elizur, Cannon et al*

2008). While CF airway inflammation is in essence chronic, patients often undergo acute exacerbations characterized by rapid and severe decline in lung function, often requiring hospitalization and intravenous antibiotic treatment *(Stenbit and Flume 2011)*. These so-called acute pulmonary exacerbations (APE) are believed to stem from an alteration in the pathological ecosystem of CF airways, involving changes in the microbiota, inflammatory neutrophils, and components of the fluid, including inflammatory mediators and metabolites *(Carpagnano, Resta et al. 2002, Wolak, Esther et al. 2009, Liou, Adler et al. 2012, Montuschi, Paris et al. 2012)*. As they grow in age, CF patients can also develop glucose intolerance and insulin resistance, which typify CF-related diabetes, or CFRD *(Kelly and Moran 2013)*. This complication of CF is associated with abnormal systemic metabolism and poor nutritional status of patients, which could impact metabolic signaling and function within inflammatory cells.

Since changes in systemic inflammatory states as described above can alter leukocyte activation and functional profiles, we characterized the expression of metabolite transporters on blood leukocytes (particularly neutrophils) from patients experiencing different chronic inflammatory states. We assessed subjects from four distinct groups: (i) healthy controls (HC); (ii) RA patients; (iii) non-inflammatory arthritis patients (a.k.a., disease controls -DC-); and (iv) CF patients. We screened for changes in cellular expression of surface metabolite transporters in blood RA and CF neutrophils as compared to those of control subjects, and also compared between various CF inflammatory states (steady-state vs. APE). In addition to RBD-derived probes for Glut1, ASCT2, PiT1 and PiT2, as described above (see Results section I), we used XPR1-RBD (phosphate export), FLVCR-RBD (heme export), and hRFT1/3-RBD (riboflavin transport), as well as BLV-RBD and PERVB-RBD, for which no function has been yet assigned *(Battini, Rodrigues et al. 1996, Tailor, Willett et al. 1999, Ericsson, Takeuchi et al. 2003, Yao, Yonezawa et al. 2010, Giovannini, Touhami et al. 2013).*

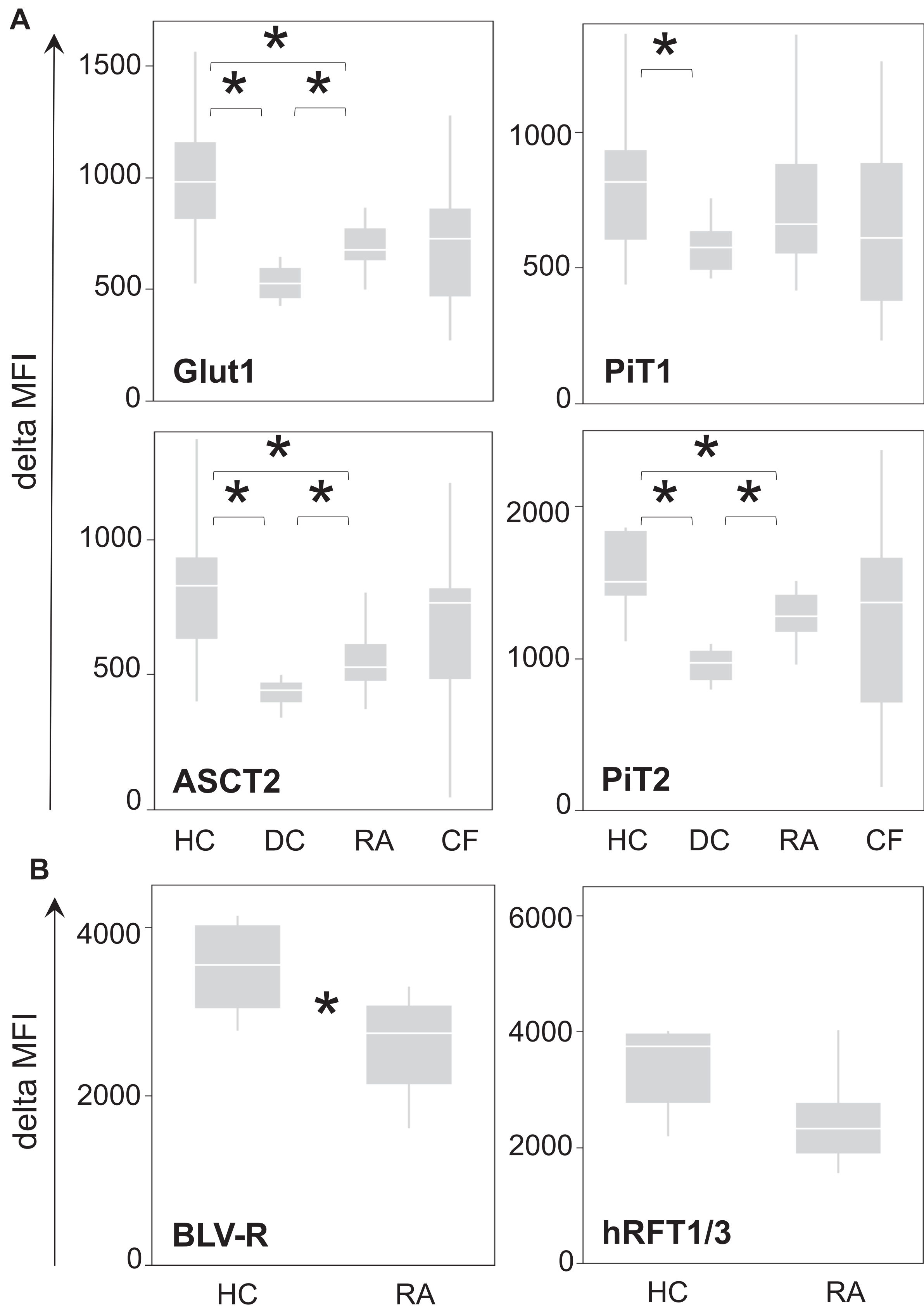

Fig. 14 Metabolite transporter expression on HC, DC, RA and CF blood neutrophils. Expression levels (delta MFI) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). (**A**) Glut1, ASCT2, PiT1 and PiT2 expression (*n*: HC = 11; DC = 9; RA and CF = 14 /group). (**B**) BLV-RBD cognate receptor and hRFT1/3 expression (*n*: HC = 8 and RA = 9). *$p < 0.025$

II-B. Results

II-B.1. Metabolite transporter expression on blood neutrophils in various inflammatory conditions

Blood neutrophils from RA patients displayed distinct surface expression levels of metabolite transporters when compared to healthy and disease control subjects. Indeed, we observed significantly lower expression of Glut1, ASCT2, PiT2 and BLV-RBD-tagged transporters on blood neutrophils from RA patients when compared to healthy controls (0.69-, 0.64-, 0.85- and 0.77-fold, respectively). In contrast, RA blood neutrophils displayed higher levels of surface Glut1, ASCT2 and PiT2 metabolite transporter expression when compared to blood neutrophils from disease control subjects (1.47-, 1.20- and 1.31-fold, respectively). No changes were observed in surface PiT1 and hRFT1/3 expression between blood neutrophils from RA and healthy or disease control subjects. Also, we found significantly higher surface PiT1, Glut1, PiT2 and ASCT2 on blood neutrophils from healthy than diseased control subjects (1.42-, 1.87-, 1.55- and 1.88-fold, respectively) (Fig. 14).

There was a tendency for CF blood neutrophils to exhibit lower transporter expression than in healthy subjects, and higher transporter expression than in disease control subjects, as seen in RA (Fig. 14). However, unlike RA, these differences did not reach significance for CF. This may be explained by the fact that in CF, blood is not exposed to high inflammatory signaling like in RA, such that metabolic priming of blood neutrophils is more pronounced in the latter than in the former.

II-B.2. Metabolite transporter expression on blood neutrophils in various inflammatory states in CF

We investigated whether the intensity of inflammation during CF disease affects metabolite transporter expression in blood neutrophils. The comparison of blood neutrophils from CF patients during acute pulmonary

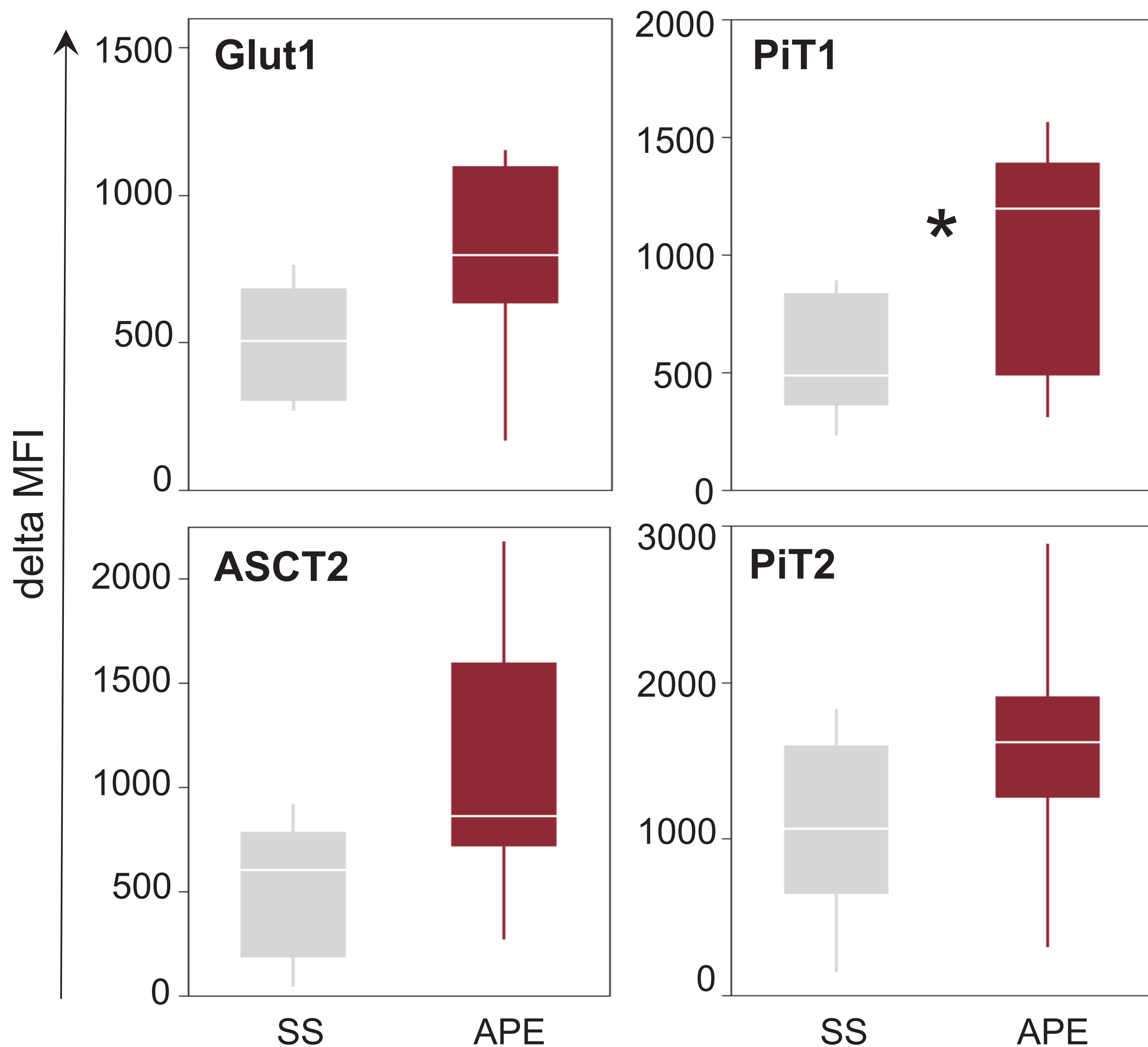

Fig. 15 Glut1, ASCT2, PiT1 and PiT2 expression on CF blood neutrophils (SS and APE). SS and APE shown in grey and red, respectively (*n* = 8/group, matched samples). Expression levels (delta MFI) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.025$

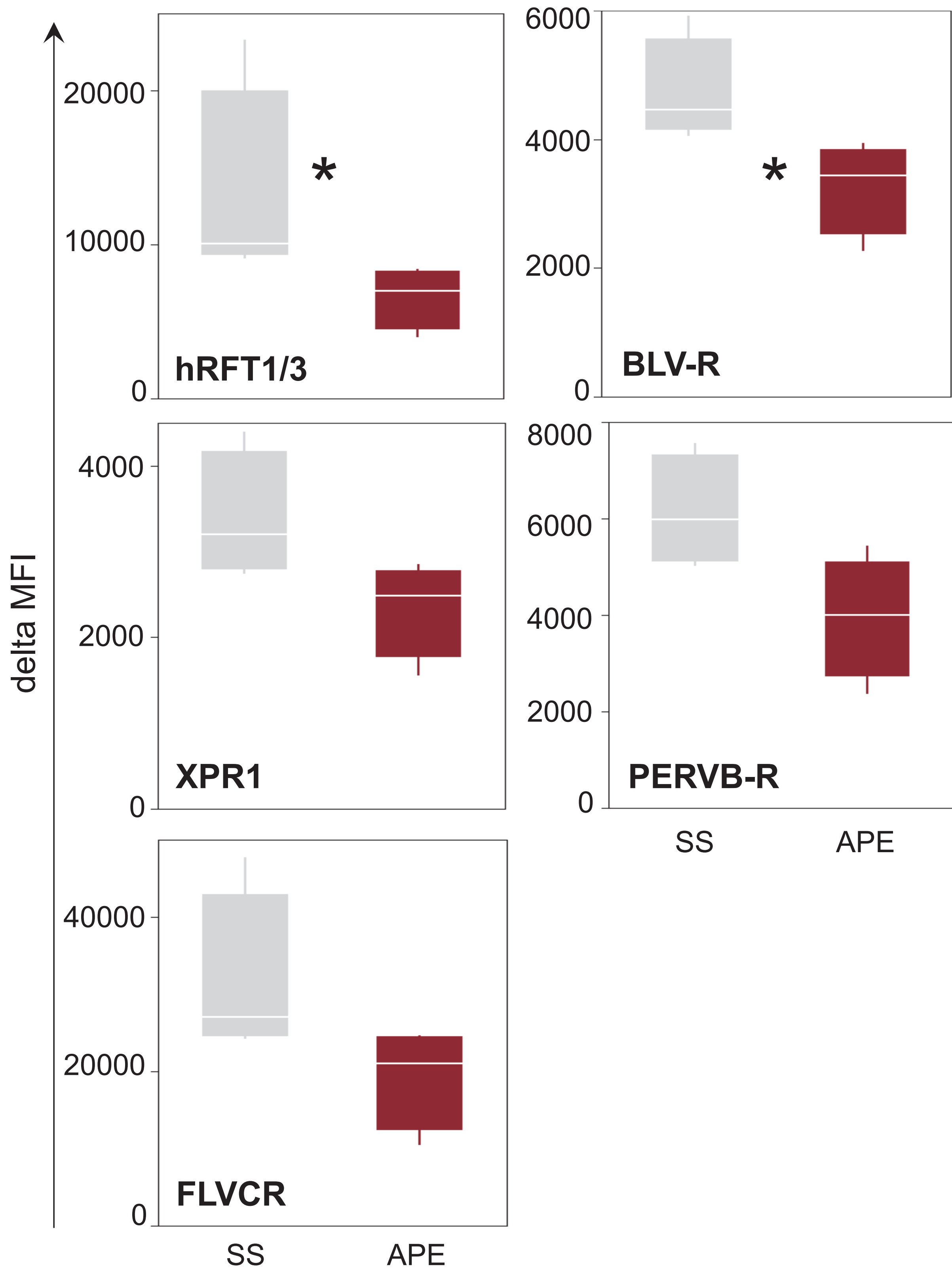

Fig. 16 Expression of hRFT1/3, XPR1, FLVCR and BLV-RBD and PERVB-RBD cognate receptors on CF blood neutrophils (SS and APE). SS and APE shown in grey and red, respectively (*n* = 4/group, unmatched samples). Expression levels (delta MFI) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.025$

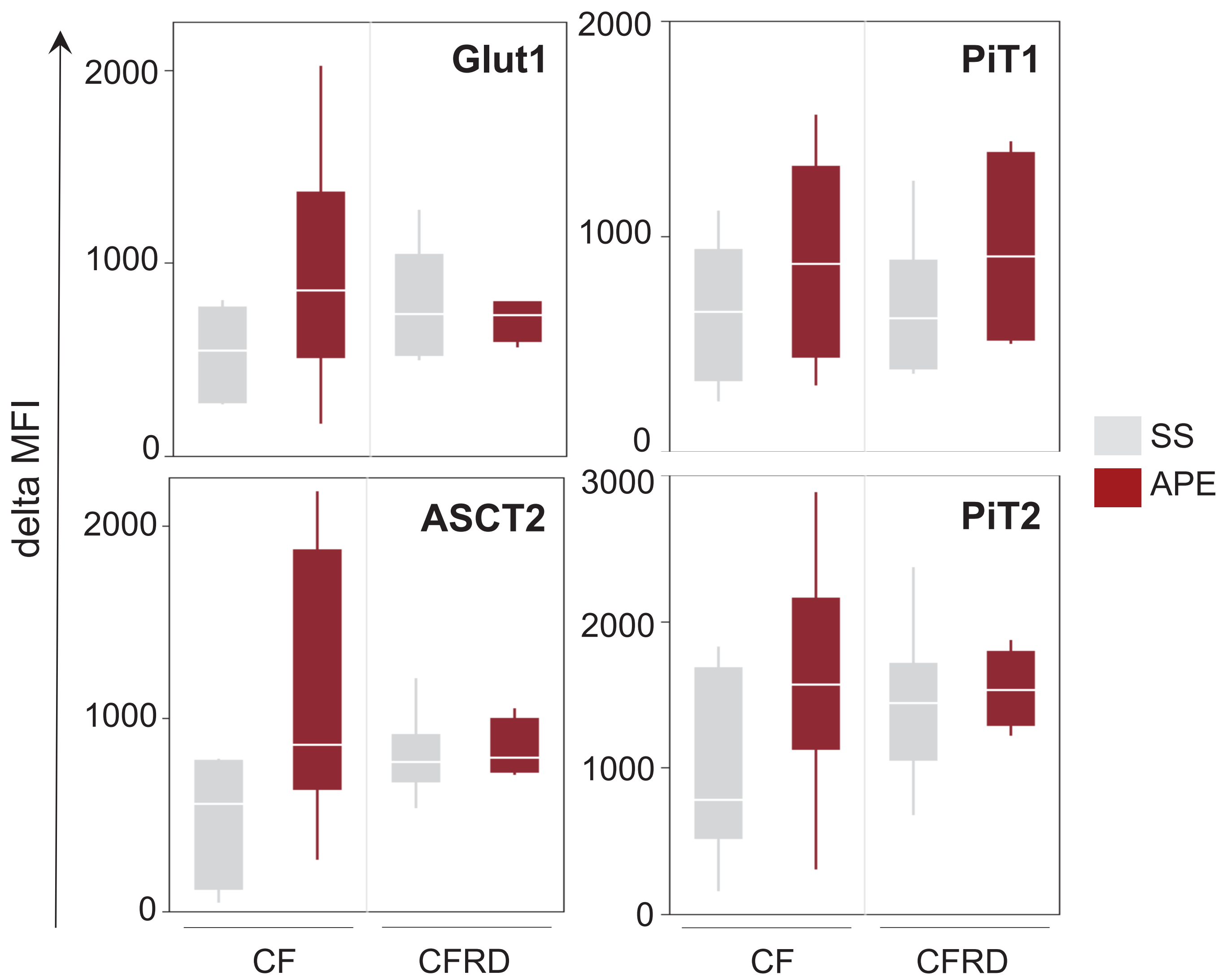

Fig. 17 Glut1, ASCT2, PiT1 and PiT2 expression on CF and CFRD blood neutrophils (SS and APE). SS and APE shown in grey and red (*n* = 7 for both groups at SS, *n* = 6 for CF/APE and *n* = 4 for CFRD/APE, unmatched samples). Expression levels (delta MFI) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.025$

exacerbation (APE) revealed an increase in surface PiT1 expression when compared to neutrophils collected at steady-state (SS) from the same patients (2.44-fold). Expression of Glut1, PiT2 and ASCT2 did not differ significantly in matched APE and SS blood samples (Fig. 15). Next, we compared expression of five other metabolite transporters on CF blood neutrophils during APE and SS (non-paired samples). We found that surface hRFT1/3 and BLV-RBD-tagged transporters were downregulated on neutrophils collected during an APE vs. SS (0.70- and 0.77-fold, respectively). Expression of XPR1, FLVCR and PERVB-RBD-tagged transporter did not differ significantly between blood neutrophils collected during APE vs. SS (Fig. 16).

Next, we assessed surface metabolite transporter expression on neutrophils from CF patients diagnosed with impaired glucose tolerance, comprising pre-diabetic and good- or poor-glucose control diabetic subjects (all together forming the CFRD group). Despite some variations, we observed no significant modulation of surface Glut1, ASCT2, PiT1 and PiT2 expression levels between CF and CFRD blood neutrophils, at both SS and APE visits (unpaired samples) (Fig. 17).

II-C. Discussion

In this chapter, we presented the application of retroviral envelope-derived ligands to the study of metabolite transporter expression in blood cells from patients in various inflammatory states. In addition to analyses of surface Glut1, ASCT2, PiT1 and PiT2 expression, as validated in the patent application with the corresponding RBD, we tested retroviral Env-derived RBD from other Env. From a technological standpoint, these data further demonstrate the validity of RBD ligands for investigating metabolic changes occurring during inflammation, although this work is still in progress and needs further demonstration.

We described specific profiles of blood neutrophils from RA patients, with surface metabolite transporter downregulation and upregulation when compared to healthy subjects and disease controls, respectively . Similar

trends, albeit not reaching significance, were observed in blood neutrophils from CF patients. These results suggest that systemic inflammation can modulate metabolite transporter expression on blood neutrophils.

In addition, we investigated metabolite transporter expression on blood neutrophils in distinct inflammatory states of CF disease (acute pulmonary exacerbation, APE, vs. steady-state, SS). Among all metabolite transporters tested, only three differed significantly between APE and SS conditions. PiT1 was upregulated, while hRFT1/3 and BLV-RBD-recognized transporter were downregulated on APE blood neutrophils, suggesting systemic modulation of some, but not all, metabolite transporters in blood neutrophils during acute inflammation.

By contrast, despite the fact that CFRD is linked to systemic metabolic changes in CF, we did not detect any significant modulation of metabolite transporter expression on blood neutrophils in this context. It should be clearly stated that these data represent preliminary results obtained from a limited cohort of CF patients, which definitely warrants further investigation. It would be of importance to test whether the trends for altered metabolite transporter expression when comparing CF neutrophils in APE vs. SS visits, and also in CFRD vs. CF, reach statistical significance with a larger number of subjects.

CHAPTER III:
METABOLIC ADAPTATION OF NEUTROPHILS RECRUITED FROM BLOOD INTO THE AIRWAYS OF CF PATIENTS

III-A. Foreword

Airway disease in CF is a progressive and destructive process rooted in a vicious cycle of obstruction by mucus, infection and inflammation. The continuous and massive recruitment of blood neutrophils into lungs is characteristic of CF airway inflammation. Previously, we showed that changes undergone by neutrophils upon their recruitment into CF airway included the activation of anabolic signaling *(Makam, Diaz et al. 2009)*. In addition, we have shown here that the use of specific new tools, the RBD-derived ligands, could reveal surface metabolite transporter expression in cells, adding a critical piece to our toolkit for the study of inflammatory cell metabolism (see Results sections I and II). In preliminary experiments, RBD revealed significant alterations in neutrophils from CF airways compared to their blood counterparts. This is consistent with the observation, made by our group and others, that metabolites such as AA and glucose accumulate to reach very high extracellular levels in CF *(Barth and Pitt 1996, Baker, Clark et al. 2007, Garnett, Nguyen et al. 2012)* (Fig. 18).

The metabolite-rich environment provided by the CF airway fluid may be particularly important in inducing neutrophil anabolic signaling. In this chapter, we present our published work (*Journal of Immunology*, June 2013) describing the in-depth study of metabolite transporter expression on CF neutrophils comparing blood and airway compartments. In addition of the use of RBD ligands, we investigated other functional features of neutrophils, including granule exocytosis and glucose transport. Our data bring strong support to the notion that neutrophils undergo significant metabolic adaptation within the CF airway environment. Our data also highlight the complexity of

within the CF airway environment. Our data also highlight the complexity of pathological processes at play in CF airway neutrophilic inflammation, which clearly go beyond rapid neutrophil necrosis upon recruitment, as previously thought.

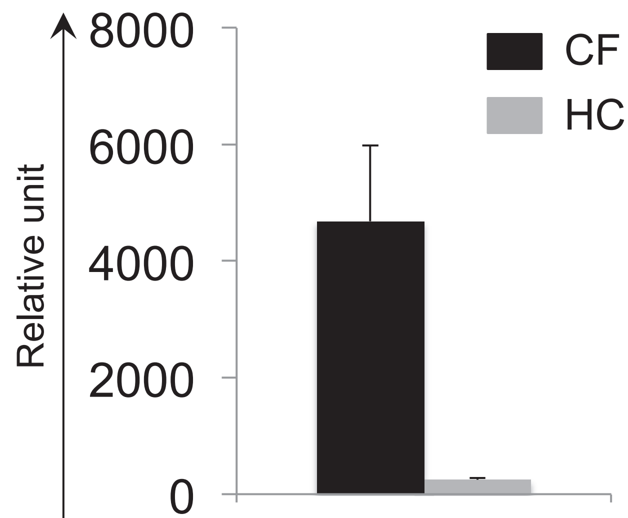

Fig. 18 AA accumulation in CF airway fluid. Total AA levels are highly increased in airway fluid from CF patients (black, *n* = 14) when compared to HC subjects (grey, *n* = 3).

III-B. Original manuscript

(Article 2)

(Laval, Touhami et al. 2013)

Metabolic Adaptation of Neutrophils in Cystic Fibrosis Airways Involves Distinct Shifts in Nutrient Transporter Expression

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Inflammatory conditions can profoundly alter human neutrophils, a leukocyte subset generally viewed as terminally differentiated and catabolic. In cystic fibrosis (CF) patients, neutrophils recruited to CF airways show active exocytosis and sustained phosphorylation of prosurvival, metabolic pathways. Because the CF airway lumen is also characterized by high levels of free glucose and amino acids, we compared surface expression of Glut1 (glucose) and ASCT2 (neutral amino acids) transporters, as well as that of PiT1 and PiT2 (inorganic phosphate transporters), in blood and airway neutrophils, using specific retroviral envelope-derived ligands. Neither nutrient transporter expression nor glucose uptake was altered on blood neutrophils from CF patients compared with healthy controls. Notably, however, airway neutrophils of CF patients had higher levels of PiT1 and Glut1 and increased glucose uptake compared with their blood counterparts. Based on primary granule exocytosis and scatter profiles, CF airway neutrophils could be divided into two subsets, with one of the subsets characterized by more salient increases in Glut1, ASCT2, PiT1, and PiT2 expression. Moreover, in vitro exocytosis assays of blood neutrophils suggest that surface nutrient transporter expression is not directly associated with primary (or secondary) granule exocytosis. Although expression of nutrient transporters on CF blood or airway neutrophils was not altered by genotype, age, gender, or *Pseudomonas aeruginosa* infection, oral steroid treatment decreased Glut1 and PiT2 levels in blood neutrophils. Thus, neutrophils recruited from blood into the CF airway lumen display augmented cell surface nutrient transporter expression and glucose uptake, consistent with metabolic adaptation. ***The Journal of Immunology*, 2013, 190: 6043–6050.**

Neutrophils provide a critical defense against infection (1) by phagocytosis of microorganisms or extracellular killing via exocytosis of toxic granule effectors, sometimes in combination with the formation of extracellular traps (2). Blood neutrophils are commonly viewed as terminally differentiated effector cells with very limited capacity to stray away from a default program of activation/apoptosis, which they generally follow upon leaving the bone marrow. However, recent evidence suggests that profound changes in neutrophil survival and function can occur upon their recruitment from blood into diseased organs, as a result of permissive/stimulatory environmental conditions therein (3).

In the fatal disease cystic fibrosis (CF), we previously demonstrated that neutrophils recruited from blood into the diseased airway environment undergo marked functional changes (4). In particular, CF airway neutrophils undergo active exocytosis of primary granules, leading to massive release of enzymes (e.g., elastase, myeloperoxidase) that damage the airway tissue and perpetuate inflammation. More intriguingly, we also observed that CF airway neutrophils express high levels of markers conventionally found on long-lived APCs, including class II MHC, the costimulatory molecule CD80, and the chemoattractant receptor of Th2 cells (CD294), all of which suggest profound reprogramming (4).

CF airway neutrophils also undergo strong activation of CREB and mTOR prosurvival pathways (5). The CREB pathway is commonly induced in stress responses, as expected in the inflammatory environment of CF airways (6). However, the mTOR pathway is the main anabolic switch in eukaryotes and is generally associated with cell survival and growth (7). Therefore, its activation in catabolic neutrophils is rather perplexing. The mTOR pathway is responsive to extracellular insulin, as well as to extracellular nutrients, notably glucose (7) and amino acids (8). Both glucose (9) and amino acids (10) tend to accumulate in CF airway fluid as the result of an apparent failure of the epithelium, lacking functional CFTR protein, to transport these anabolic nutrients (11–13). Additionally, CF airway fluid is enriched in breakdown products of phosphonucleotides (14), which fuel cellular energy production.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AMLV, amphotrophic murine leukemia virus; CF, cystic fibrosis; delta MFI, differential median fluorescence intensity; EGFP, enhanced GFP; Env, envelope glycoprotein; HC, healthy control; HTLV, human T cell leukemia virus type 2; KoRV, Koala endogenous retrovirus; LB, latrunculin B; mFc, mouse Ig-Fc tag; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose; OS, oral steroid; RBD, receptor-binding domain; RD114, feline endogenous retrovirus; rFc, rabbit Ig-Fc tag.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201755

To investigate the process of metabolic adaptation by neutrophils recruited to CF airways, we used novel retroviral envelope glycoprotein (Env)-derived ligands (15, 16) combined with multicolor flow cytometry to characterize the expression of glucose/dehydroascorbic acid (Glut1), neutral amino acid (ASCT2), and inorganic phosphate (PiT1 and PiT2) transporters on CF blood and airway neutrophils. Using imaging flow cytometry (ImageStreamX platform; Amnis), we also characterized the expression and cytoplasmic localization of the conserved RNA-binding protein vigilin in blood and airway neutrophils. Vigilin has been associated with anabolic activity as a positive regulator of mRNA translation in multiple eukaryotic organisms, including humans (17–19). Simultaneously to vigilin, we characterized the expression of the LC3 protein, previously linked to multiple catabolic activities, including autophagy, phagocytosis, and the exocytosis of proteolytic enzymes (20–23). Our results demonstrate significant, distinctive metabolic changes among CF airway neutrophil subsets and suggest an important role for nutrient transporters in the inflammatory process observed in CF airway disease.

Materials and Methods

Human samples

This study received the approval of the Institutional Review Boards at Stanford University and Emory University. All subjects provided written informed consent before undergoing study procedures. Healthy control (HC) subjects ($n = 13$) were >18 years of age, with no restriction based on race or gender, excluding pregnant and breast-feeding individuals. CF was diagnosed by sweat chloride (>60 mEq/l), using a quantitative iontophoresis test and/or pre-existing documentation of two identifiable *cftr* mutations (see demographic data for CF subjects in Supplemental Table I, $n = 24$). The presence of common opportunistic pathogens in patient lungs was tested by routine sputum culture. Lung function was tested by spirometry, as per American Thoracic Society criteria. Blood (CF and HC subjects) and airway fluid (CF subjects only) were collected by venipuncture and sputum induction or spontaneous expectoration, respectively, and immediately placed on ice. Airway fluid was dissociated by addition of PBS-EDTA and repeated pipetting, a procedure that minimizes activation (4). Blood and airway fluid were centrifuged at $400 \times g$ and $1500 \times g$ for 10 min, respectively. The supernatant was removed, and the cell pellet was washed, resuspended in PBS-EDTA, and used immediately for glucose uptake, exocytosis, and cell sorting/quantitative RT-PCR assays. For analyses of nutrient transporter expression and vigilin and LC3 expression, blood and airway cells were fixed in Phosflow Lyse/Fix Buffer (BD Biosciences) to preserve leukocytes in their native state and stored at −80°C until use.

Cell surface assessment of Glut1, ASCT2, PiT1, and PiT2 nutrient transporters

Receptor-binding domains (RBDs) of gamma- and deltaretroviruses Env specifically interact with their cell surface receptor, all of which were shown to be metabolite transporters (24–27). In this study, we derived RBD ligands, as previously described (15), comprising the sequence encoding the 18–35 aa of the signal peptide with the corresponding RBD of the (i) 178 N-terminal amino acids of the human T cell leukemia virus type 2 (HTLV) Env, which is a ligand of Glut1 (glucose/dehydroascorbic acid transporter); (ii) 222 N-terminal amino acids of the RD114 (feline endogenous retrovirus) Env, which is a ligand of ASCT2 (neutral amino acid transporter); (iii) 253 aminoterminal amino acids of the koala endogenous retrovirus (KoRV) Env, which is a ligand for PiT1 (inorganic phosphate transporter); and (iv) 245 aminoterminal amino acids of the amphotropic murine leukemia virus (AMLV) Env, which is a ligand for PiT2 (another inorganic phosphate transporter). HTLV RBD was fused to the enhanced GFP (EGFP) at the carboxyl-terminal end, and all of the other RBDs were fused to either a specific mouse Ig-Fc tag (mFc) or rabbit Ig-Fc tag (rFc). The specificity of HTLV-RBD EGFP for Glut1 was established previously (15, 27–29). To assess the specificity of recognition of RD114-RBD mFc, KoRV-RBD mFc, and AMLV-RBD rFc for ASCT2, PiT1, and PiT2, respectively, we transduced CHO cells with the pLXSN vector containing an empty cassette or a cassette with the DNA sequence coding for human HA-tagged ASCT2, PiT1, or PiT2 (National Center for Biotechnology Information Reference Sequences: NM_001145144.1, NG_028281.1, and NC_000008.10, respectively). Following selection (G418 treatment for 15 d), staining of the parental and transduced CHO cells with the RBD ligands was assessed by flow cytometry (Fig. 1).

Flow cytometric analysis of nutrient transporter expression

Fixed frozen blood and airway cell samples were thawed, washed with PBS-EDTA, and surface stained at 37°C with two combinations of two RBD ligands: HTLV-RBD EGFP (Glut1)/KoRV-RBD mFc (PiT1) or RD114-RBD mFc (ASCT2)/AMLV-RBD rFc (PiT2). Although the HTLV-RBD EGFP ligand is inherently fluorescent, the other RBD ligands were used in a combination with fluorescently labeled Abs against mouse or rabbit IgG Fc (Life Sciences). Samples were also stained with cholera toxin B (Life Sciences); after a wash in PBS-EDTA and permeabilization with Perm I buffer (BD Biosciences), they were stained with the DNA-binding probe DRAQ5 (Life Sciences). The combination of cholera toxin B and DRAQ5 enables robust gating of live neutrophils from blood and airway samples (30), as illustrated in Supplemental Fig. 1A. Control stainings combined anti-mFc or -rFc with cholera toxin B and DRAQ5, in the absence of RBD ligands. After a final wash in Perm I buffer at room temperature, samples were analyzed on a LSR II flow cytometer (BD Biosciences), and expression of nutrient transporters was determined after fluorescence compensation (FlowJo software; TreeStar) (30). Data are reported as differential median fluorescence intensity (delta MFI) between the specific RBD staining and the corresponding background control for each individual sample.

Glucose-uptake assays

Glucose uptake was analyzed using the fluorescent glucose analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose (2-NBDG; Cayman Chemical). Blood was treated with ammonium chloride to lyse RBCs, after which blood leukocytes were spun down and washed with PBS-EDTA. Leukocytes from blood and airway were prestained at 4°C with Live/Dead (Life Sciences) and anti-CD16 and -CD63 (both from BioLegend) to enable gating of live neutrophils (4). After a wash with PBS-EDTA, leukocytes were equilibrated in serum-free RPMI 1640 (Cellgro) at 4°C for ≥45 min. Leukocytes were then washed and resuspended in PBS, and 2.5×10^5 leukocytes were incubated for 1 min at 37°C with 2-NBDG at 100 μM (31). After a wash in PBS-EDTA, samples were analyzed on a LSR II flow cytometer, as above, and uptake was determined as delta MFI between 2-NBDG–stained and unstained conditions for each sample.

Cell sorting and quantitative RT-PCR to assess nutrient transporter transcript levels

Blood was treated with ammonium chloride to lyse RBCs and then spun down and washed with PBS-EDTA. Blood and airway leukocytes (1×10^6) were stained at 4°C with Live/Dead, anti-CD15 (BioLegend), anti-CD16 (BioLegend), and anti-CD63 (BD Biosciences) Abs to enable gating of live blood neutrophils (B: $CD15^{hi}CD16^{hi}$) and live airway neutrophil subsets (A1 subset: $CD15^{hi}CD16^{hi}CD63^{lo}$; A2 subset: $CD15^{hi}CD16^{lo}CD63^{hi}$), as described previously (4, 5). After a wash with PBS-EDTA, blood and airway neutrophils were sorted with a FACS Jazz Cell Sorter using BD FACS Software (BD Biosciences). Nucleic acids from sorted blood and airway neutrophils were extracted using the QIAshredder kit (QIAGEN), and extracts were stored at −80°C until use. RNA isolation and genomic DNA removal were performed using RNeasy plus columns (QIAGEN), and the quality/quantity of RNA were determined using the ND-1000 spectrophotometer (NanoDrop Technologies). First-strand cDNA synthesis was carried out using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Specific paired primers (forward; reverse) were used to determine the levels of the following transcripts—Glut1 (5′-TGCTGATGATGAACCTGCTG-3′; 5′-GATGAGGATGCCGACGAC-3′), PiT1 (5′-CAGCGTGGACTTGAAAGAGG-3′; 5′-TGACGGCTTGACTGAACTGG-3′), and PiT2 (5′-TCTCATGGCTGGGGAAGTTAGT-3′; 5′-TTGCGACCAGTGAGAATCCTAT-3′)—and β-actin (ACTB) as the reference gene (32). Transcripts were amplified and measured using the ABI 7500 thermocycler with SYBR Select Master Mix (Life Sciences), according to the manufacturer's instructions. Transcript levels were quantified relative to ACTB in each sample. Efficiency of RNA recovery per number of cells and number of cycles required for ACTB RNA amplification were equivalent for all sample categories (B, A1 and A2 sorted neutrophils), indicative of an absence of particular RNA degradation for the different subsets of neutrophils.

Exocytosis and nutrient transporter surface expression assays

Exocytosis (secondary and primary granules) and nutrient transporter surface expression were analyzed on blood neutrophils upon incubation

with latrunculin B (LB) combined with the bacterial formyl peptide fMLF; LB and fMLF (both from Sigma-Aldrich) were prepared as 1000× frozen stocks in DMSO and diluted immediately prior to use. Blood was washed and resuspended in medium (serum-free RPMI 1640) at 5×10^6/ml and incubated with LB (1.25 μM) for 5 min at 37°C, followed by fMLF (5 μM) for 10 min at 37°C. In these conditions, primary and secondary neutrophil granules (and tertiary granules) are exocytosed to a large extent (33). Incubations were stopped by washing the cells with ice-cold PBS-EDTA and transferring tubes on ice. Samples were then stained at 4°C with Live/Dead and cholera toxin B to enable live neutrophil gating, as well as anti-CD66b (BioLegend) and anti-CD63 (BD Biosciences) to measure secondary and primary granule release, respectively (4). Samples were then fixed in Phosflow Lyse/Fix Buffer to preserve stained leukocytes, stored at −80°C, and thawed for batch staining with RBD probes and analysis of nutrient transporter expression, as described above.

Multispectral imaging flow cytometric analysis of vigilin and LC3

Fixed and frozen blood and airway cell samples were thawed, washed with PBS-EDTA, permeabilized with Perm I buffer (BD Biosciences), and stained with DRAQ5 and Abs directed against the nucleic acid–binding protein vigilin (Santa Cruz Biotechnology) and the autophagolysosome protein LC3 (MBL). The combination of LC3 and DRAQ5 also enables a robust gating of live neutrophils from blood and airway samples, as illustrated in Supplemental Fig. 1B. After a final wash in Perm I buffer, samples were analyzed on an ImageStreamX system (Amnis), and expression and localization (nuclear versus cytoplasmic) of vigilin and LC3 were quantified (IDEAS software) after fluorescence compensation (30, 34).

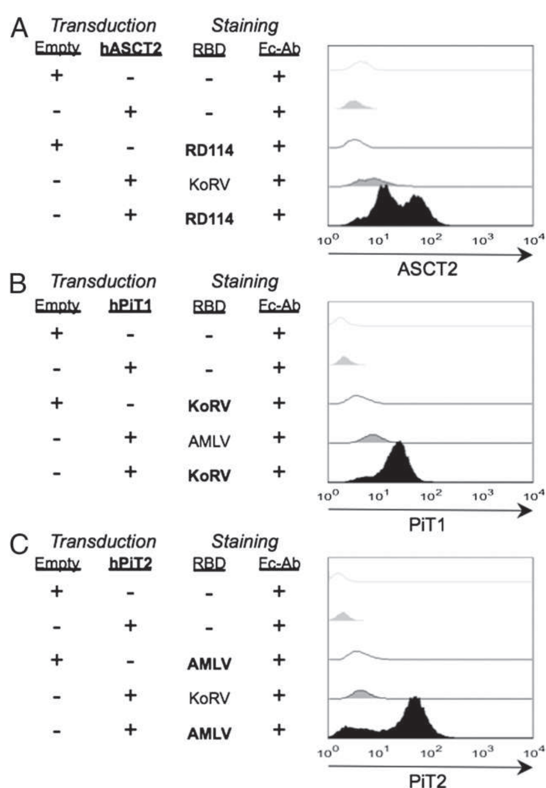

FIGURE 1. Detection of nutrient transporter expression by RBD-derived ligands. CHO cells were transduced with either an empty LXSN vector or an LXSN vector coding for the human ASCT2 (**A**), PiT1 (**B**), or PiT2 (**C**) transporter. Control- and transporter-transduced cells were then stained with cognate RD114-, KoRV-, and AMLV-RBD ligands. Data are presented for negative controls (open gray graphs) and background controls (transporter-transduced cells stained with secondary Ab alone, filled gray graphs), as well as for control- and transporter-transduced cells stained with the specific RBD (open and filled black graphs).

Statistical analysis

Statistical analyses of flow and image cytometry datasets were performed using the JMP9 software (SAS Institute). Nonparametric statistics were used throughout the study, because flow and image cytometry data are generally not distributed normally. Differences were assessed using paired statistics (Wilcoxon signed-rank test) when comparing distributions between blood and airway neutrophils and unpaired statistics (Wilcoxon rank-sum test) when comparing data between groups (e.g., CF versus HC, treatment with given medications, male versus female, presence or absence of given bacterial infections, and genotype). Correlations between flow and image cytometry data and continuous demographic data (age) were assessed by the nonparametric Spearman test. Differences were considered significant at $p < 0.05$. When appropriate, Bonferroni corrections were applied according to the number of simultaneous outcome measures for each set of experiments.

Results

Novel retroviral Env ligands specifically bind to the ASCT2, PiT1, and PiT2 cell surface nutrient transporters

We demonstrated previously that the RBD from HTLV was a specific ligand for the glucose/dehydroascorbic acid transporter Glut1 (15, 28, 29, 35). Based on previously established binding specificities for the Env of RD114 (36), KoRV (37), and AMLV (24) for the human neutral amino acid transporter ASCT2 and inorganic phosphate transporters PiT1 and PiT2, respectively, we developed specific RBD ligands for each of these three human nutrient transporters. Env-derived RBD ligands for ASCT2 (Fig. 1A), PiT1 (Fig. 1B), and PiT2 (Fig. 1C) specifically stained CHO cells transduced with the corresponding human nutrient transporter. In contrast, no significant RBD ligand labeling was observed when CHO cells were transduced with an empty vector (negative control), confirming that none of the three corresponding Envs recognizes the endogenous hamster nutrient transporters (27). Additionally, there was no significant RBD ligand staining of CHO cells transduced with a noncorresponding human transporter, confirming the ligand-to-transporter specificity.

Neutrophils recruited to CF airways modulate nutrient transporter expression and glucose uptake

A hallmark of airway neutrophils in CF patients is a robust activation of the anabolic mTOR pathway (5), which responds to ex-

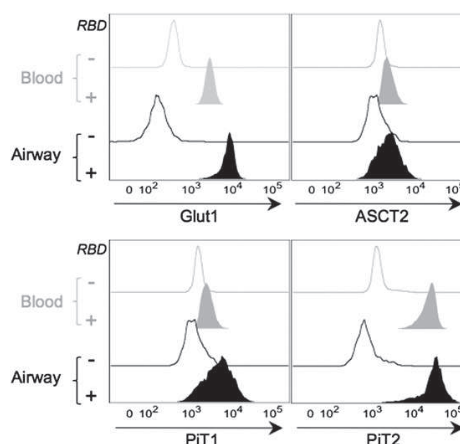

FIGURE 2. Expression of Glut1, ASCT2, PiT1, and PiT2 nutrient transporters on CF neutrophils. Blood and airway neutrophils from CF patients were stained with ligands for Glut1, ASCT2, PiT1, and PiT2. Representative graphs showing background (open) and specific RBD staining (filled) on blood (gray) and airway (black) neutrophils from a representative CF patient.

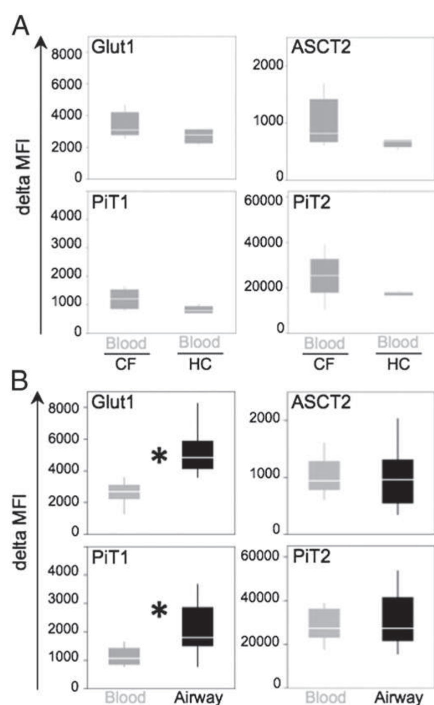

FIGURE 3. Differences in nutrient transporter expression in CF and HC blood and airway neutrophils. Glut1, ASCT2, PiT1, and PiT2 expression levels (delta MFI) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). (**A**) Blood neutrophils from CF and HC subjects (gray, $n = 6$/group). (**B**) Blood (gray) and airway (black) neutrophils from CF patients ($n = 12$). *$p < 0.05$.

tracellular metabolites, such as glucose and amino acids, and induces ATP production (7). Hence, we investigated the expression of glucose, amino acid, and phosphate transporters on blood and airway neutrophils from CF patients. Staining with RBD ligands revealed significant expression of Glut1, ASCT2, PiT1, and PiT2 on neutrophils from both compartments (Fig. 2). Comparison of blood neutrophils from HC ($n = 6$) and CF ($n = 6$) subjects showed no significant difference in nutrient transporter expression (Fig. 3A). However, comparison of paired blood/airway samples from CF patients ($n = 12$) showed a marked upregulation of Glut1 and PiT1 on airway neutrophils compared with blood neutrophils (1.81- and 1.77-fold, respectively, Fig. 3B, Table I), whereas ASCT2 and PiT2 expression profiles appeared unchanged. We then assessed glucose uptake by blood neutrophils and airway neutrophils using the fluorescent D-glucose analog 2-NBDG (Fig. 4). Although glucose uptake was similar in HC and CF blood neutrophils, it was markedly increased in CF airway neutrophils. Thus, differences in baseline surface Glut1 levels between CF blood and airway neutrophils (Fig. 3B) translated into differences in glucose uptake (Fig. 4).

CF airway neutrophil subsets display distinct nutrient transporter surface expression profiles

We showed previously that CF airway neutrophils can be split into two distinct subsets, designated as A1 and A2, based on low and high levels of exocytosis of toxic primary granules (and reflected by scatter properties), respectively (4). We found that these subsets also differed in their expression of nutrient transporters (Fig. 5). The A2 subset, which presented with higher levels of exocytic toxic granules according to scatter profiles (Supplemental Fig. 1), showed higher levels of Glut1 (1.29-fold, Table I) and more markedly increased levels of PiT1 (4.15-fold), PiT2 (3.59-fold), and ASCT2 (2.45-fold) compared with the A1 subset (Fig. 5, Table I). Although Glut1 was uniformly higher on airway neutrophils compared with blood neutrophils, regardless of the subset considered (A, A1, or A2), other nutrient transporters showed more complex patterns of modulation (Table I). Compared with blood neutrophils, ASCT2 expression was significantly lower on the A1 subset but was higher on A2 neutrophils (0.46- and 1.28-fold, respectively). This pattern was similar to that observed for PiT2 on airway neutrophils compared with blood neutrophils, with lower and higher expression on A1 and A2 neutrophils, respectively (0.38- and 1.30-fold, respectively). Thus, cell surface nutrient transporters in neutrophils that migrated from blood to the airways have patterns that distinguished the A1 and A2 neutrophil subsets. When assessing levels of Glut1, PiT1, and PiT2 transcripts in sorted blood (Supplemental Fig. 2), A1, and A2 neutrophil subsets, we found equivalent RNA recovery and actin RNA amplification, but no discernable increase in the transporter RNAs, despite increased surface expression. Rather, we noted a tendency for lower nutrient transporter transcript levels in airway versus blood neutrophils. Therefore, differences in transporter surface level expression are not reflected by synchronous modulation of the cognate transcripts.

Table I. Comparative expression of nutrient transporters, vigilin (% cytoplasmic), and LC3 in CF blood and airway neutrophils

Marker	A Versus B	A1 Versus B	A2 Versus B	A2 Versus A1
Glut1	1.81	1.37	1.80	1.29
	$<10^{-3}$	0.032	$<10^{-3}$	0.002
ASCT2	1.09	0.46	1.28	2.45
	0.584	0.011	0.17	$<10^{-3}$
PiT1	1.77	0.64	2.24	4.15
	0.002	0.190	$<10^{-3}$	$<10^{-3}$
PiT2	1.08	0.38	1.30	3.59
	0.396	0.011	0.038	$<10^{-3}$
Vigilin (% cytoplasmic)	5.68	7.58	4.89	0.93
	0.001	0.001	0.001	0.097
LC3	1.82	0.92	2.18	2.10
	$<10^{-3}$	0.335	$<10^{-3}$	$<10^{-4}$

Fold differences in the expression of the indicated marker between total (A) or the A1 and A2 subsets of airway neutrophils over blood neutrophils (B) and A2 subset over A1 subset of airway neutrophils.

The p values provided are for paired analyses.

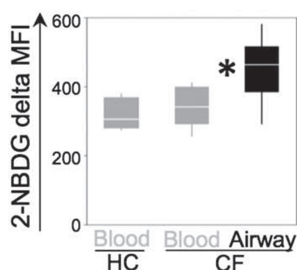

FIGURE 4. Glucose uptake by blood and airway neutrophils. Glucose uptake in blood neutrophils from HC and CF subjects (gray) and CF airway neutrophils (black) was determined upon 1 min of incubation with the fluorescent D-glucose analog 2-NBDG. Box plots show the delta MFI (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars) (n = 6/group). $*p < 0.05$.

Oral steroids modulate Glut1 and PiT2 expression in CF blood neutrophils

Oral steroids (OSs) are powerful inhibitors of inflammation, often used to curb acute symptoms of inflammatory airway disease in CF (6), and inhibition of Glut1 expression and its membrane localization are among the multiple parameters altered by OS treatment (38). Consistent with this mode of action, we observed that OS treatment significantly decreased Glut1, as well as PiT2, expression on CF blood neutrophils (Fig. 6). OS treatment did not significantly affect the upregulation of Glut1 expression on airway neutrophils. However, OS treatment significantly increased the proportion of the A2 subset, which expressed higher levels of PiT2 transporter than did the A1 subset (3.59-fold, Table I), thus increasing PiT2 expression among total CF airway neutrophils (A subset) in OS-treated versus untreated patients. No significant change was observed in either ASCT2 or PiT1 surface expression on blood and airway neutrophils upon OS treatment. Unlike OS treatment, other common modifiers of CF airway disease (genotype, age, gender, or *Pseudomonas aeruginosa* infection) did not affect nutrient transporter expression (data not shown).

Increased surface expression of nutrient transporters is not due to exocytosis of secondary or primary granules

Compared with blood neutrophils, the A1 subset of CF airway neutrophils is characterized by a marked exocytosis of secondary granules (high CD66b with low CD63 surface expression), whereas

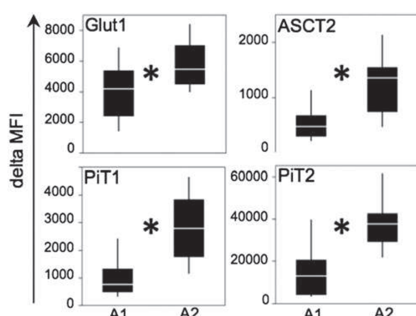

FIGURE 5. Modulation of nutrient transporter expression in the A1 and A2 subsets of CF airway neutrophils. Glut1, ASCT2, PiT1, and PiT2 expression was assessed in the A1 and A2 airway neutrophil subsets from CF patients (n = 12). Box plots show the delta MFI (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). $*p < 0.05$.

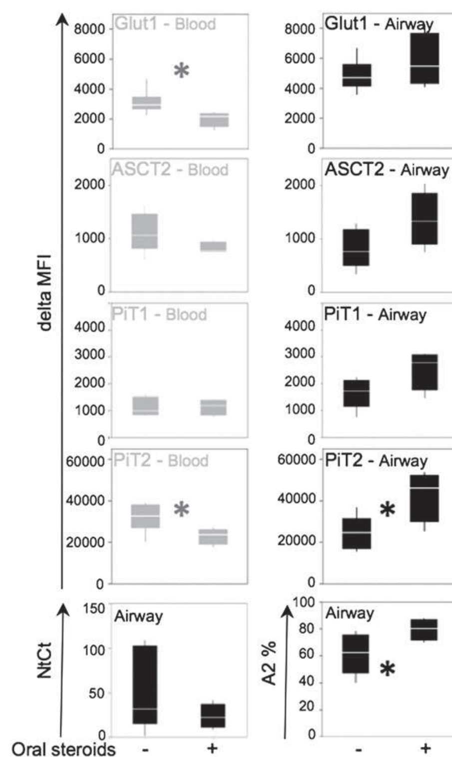

FIGURE 6. Modulation of nutrient transporter expression by OS treatment. Glut1, ASCT2, PiT1, and PiT2 expression in blood (gray) and airway (black) neutrophils was monitored as a function of OS treatment. Total airway neutrophil counts (NtCt, expressed as 10^6 cells/sample) and the percentage of A2 neutrophils present in the total airway neutrophil population were determined in CF patients undergoing OS treatment (+) versus untreated CF patients (-). Box plots show delta MFI (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars) (OS treatment, n = 4; no treatment, n = 8). $*p < 0.05$.

the A2 subset is characterized by a marked exocytosis of both secondary and primary granules (high CD66b and CD63 surface expression) (4, 5). Because both subsets showed significant changes in nutrient transporter expression, we tested whether in vitro neutrophil activation and induction of granule exocytosis could alter surface expression of nutrient transporters. As previously reported (33), efficient secondary and primary granule exocytosis was triggered by the combined treatment with LB and fMLF and evidenced by marked increases in the CD66b and CD63 surface markers (5.66–6.87-fold and 23.74–29.4-fold, respectively, Supplemental Table II). In contrast, upon LB+fMLF treatment, nutrient transporter levels in HC and CF neutrophils remained within a range (0.61–1.67-fold) of those observed in control conditions. This range was markedly lower than that observed in vivo for the A2 subset, in which nutrient transporter expression ranged from 1.29- to 4.15-fold higher than in blood neutrophils (Table I). Of note, short-term incubation (10 min) of CF and HC blood neutrophils in CF airway fluid (cell- and bacteria-free) promoted changes in nutrient transporter expression that ranged from 0.96- to 1.5-fold (data not shown) lower that those observed for A2 cells in vivo. Thus Glut1, ASCT2, PiT1, and PiT2 are not components of neutrophil secondary and primary granules, and changes in their cell surface expression cannot be recapitulated by exocytosis of these granules or exposure to CF airway fluid in vitro.

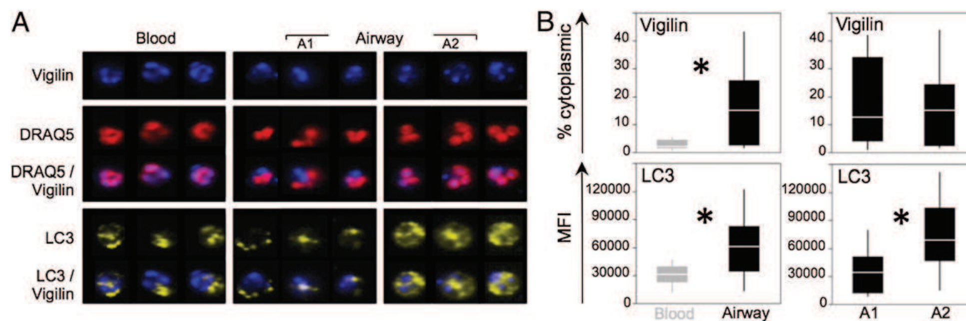

FIGURE 7. Expression and compartmentalization of vigilin and LC3 in CF blood and airway neutrophils. (**A**) Neutrophils from blood and airways (A1 and A2 subsets) were stained with Abs directed against the nucleic acid–binding protein vigilin and the autophagolysosome protein LC3, and with the DNA-binding DRAQ5 reagent. Colocalization was assessed on the ImageStreamX imaging flow cytometer (data acquired at original magnification ×40). Pseudocolor images show vigilin (*top panels*), DRAQ5 (*middle panels*), and LC3 (*bottom panels*) localization in a representative CF patient. (**B**) The percentage of cytoplasmic vigilin and total LC3 expression was compared between blood (gray) and airway (black) neutrophils (*left panels*), as well as between the A1 and A2 airway subsets (*right panels*) of CF patients (n = 14). *$p < 0.05$.

Recruitment of neutrophils to CF airways is associated with altered intracellular expression of the nucleic acid–binding protein vigilin and the autophagolysosome marker LC3

Vigilin is an RNA-binding protein with nuclear and cytoplasmic localizations, the latter being linked to anabolic activity in the form of increased RNA translation (17–19). Recent evidence from our group (J. Laval, M. Makam, D. Diaz, M. Preininger, M. Miglianico, L.A. Herzenberg, and R. Tirouvanziam, manuscript in preparation) and other investigators (39) suggests that CF airway neutrophils are transcriptionally and translationally active. In this study, we used image cytometry to monitor vigilin compartmentalization and observed a nuclear localization in CF blood neutrophils, although with a significant cytoplasmic translocation in airway neutrophils (Fig. 7). This translocation occurred in both the A1 and A2 subsets of airway neutrophils (7.58- and 4.89-fold compared with blood, Table I). Remarkably, expression of the catabolic protein LC3 (20–23) was increased markedly in the A2 subset of airway neutrophils (Fig. 7), but it was essentially unchanged in the A1 subset (2.1-fold compared with A1, Table I). Although vigilin and LC3 proteins could both be detected in the cytoplasm of CF airway neutrophils, they did not appear to colocalize (Fig. 7A). Taken together, our results suggest a complex process of neutrophil adaptation to the CF airway environment (Fig. 8).

Discussion

In this study, we demonstrate marked changes in glucose, amino acid, and phosphate transporters among subsets of CF airway neutrophils. Using imaging flow cytometry, we further show that CF airway neutrophils express higher cytoplasmic levels of vigilin and LC3 compared with their blood counterparts, with significant differences among subsets for the latter. Together with our previous observation of sustained mTOR and CREB pathway activation in CF airway neutrophils (4, 5), these results suggest that neutrophils undergo significant metabolic adaptation as they are recruited to CF airways.

Under the nutrient-rich conditions provided by the CF airway milieu (9, 11, 12, 14), environmental strains of autotrophic bacteria acquired by patients overcome the many negative aspects of this hypoxic and antimicrobial-, protease-, and oxidant-laden environment to thrive and undergo auxotrophic adaptation over time (10). Hence, the CF airway milieu exerts significant selective pressure on incoming bacteria, in large part through metabolic routes. Our results support the idea that inflammatory neutrophils recruited to the metabolically permissive CF airway milieu also undergo a metabolic adaptation.

The significant increase in Glut1 surface expression and glucose uptake detected in airway neutrophils compared with blood neutrophils supports the notion that these cells are able to use the high amount of glucose in the CF extracellular milieu (9). This is consistent with our previous data on the immediate activation of the glucose-sensitive mTOR pathway in neutrophils recruited to CF airways (5). Studies by other groups clearly established that neutrophils are avid consumers of glucose (40) and that the upregulation of surface Glut1 expression is associated with neutrophil activation upon exposure to inflammatory conditions (41). A similar increase in glucose-dependent metabolism is seen in other activated leukocyte subsets, notably T and B cells (29, 42–45). In contrast with Glut1, expression of the amino acid transporter ASCT2 is not uniformly increased on CF airway neutrophils (decreased on A1 and increased on A2 neutrophils compared with blood neutrophils, respectively). Because ASCT2 is required for sustained branched amino acid–dependent mTOR activation in anabolic cells (8), our findings suggest that, among CF airway neutrophils, glucose-driven and amino acid–driven anabolic activities may be regulated independently. The absence of a marked difference in nutrient transporter expression between CF and HC neutrophils in blood suggests that, although chronic inflammation

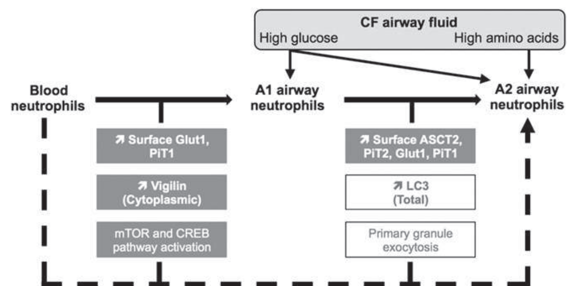

FIGURE 8. Schematic representation of neutrophil adaptation recruited from blood to CF airways. CF airways are rich in glucose and amino acids. Neutrophils recruited from blood into this pathological environment undergo complex adaptation, with changes in nutrient transporter expression and levels of LC3 and cytoplasmic vigilin (this study), as well as granule exocytosis (4) and mTOR and CREB pathway activation (5). Two models of cell differentiation from blood neutrophil phenotype to airway neutrophil phenotypes are represented: a model with sequential changes from A1 to A2 neutrophil subsets (solid arrows) and a model with a direct transition to either A1 or A2 subsets (dashed arrows).

in CF patients may impact on the metabolism of circulating neutrophils, active recruitment to the airways seems to be required to fully promote anabolic adaptation.

The RNA-binding protein vigilin undergoes a major relocalization from the nucleus to the cytoplasm upon neutrophil recruitment from blood into CF airways. Cytoplasmic vigilin plays a key role in mRNA stabilization and positively regulates translation, notably in response to stress (17–19, 46). Interestingly, we observed that CF airway neutrophils undergo marked changes in their transcriptional profile compared with CF blood neutrophils (Laval et al., manuscript in preparation), consistent with reprogramming (3, 39). Thus, the cytoplasmic relocalization of vigilin detected in all airway neutrophils (A1 and A2 subsets) is consistent with the notion that neutrophils are primed for anabolic activation following their entry into CF airways. In contrast, the increase in LC3 expression occurs only in A2 cells compared with blood neutrophils. This result is consistent with our observations of a major shift in primary granule exocytosis in the A2 subset (4) and with the recently proposed role for the LC3 protein in granulocyte exocytosis (21). Alternatively, the LC3 protein may be involved in other catabolic functions in these cells, including LC3-associated phagocytosis or even conventional autophagic activity (20, 22, 23).

Interestingly, conditions inducing primary and secondary granule exocytosis, as produced by LB+fMLF treatment, did not induce nutrient transporter surface expression in the range observed in vivo. The lack of an obvious association between surface CD66b and CD63 and Glut1, ASCT2, PiT1, and PiT2 expression upon in vitro exocytosis is consistent with proteomic data, which did not identify nutrient transporters, such as Glut1, ASCT2, PiT1, or PiT2, in secondary and primary granules (47). Thus, these four nutrient transporters likely originate from other neutrophil compartments that are also subject to membrane mobilization. Rather than exocytosis processes per se, it is more likely that transepithelial migration, a process that induces strong signaling loops in migratory neutrophils and the surrounding tissue (48–50) and that was not reproduced in our in vitro activation assay, played a role in the induction of nutrient transporter surface expression. Consistently, in vitro incubation of blood neutrophils with CF airway fluid does not fully recapitulate this process. Additionally, changes in surface expression of these four nutrient transporters, as observed in vivo, did not appear to be due to changes in intracellular levels of the cognate mRNAs, consistent with the established notion that RNA transcription and protein expression are often asynchronous in neutrophils (3).

Taken together, our data are consistent with a sequential model of neutrophil adaptation to the glucose- and amino acid–rich CF airway milieu. In the first step (blood → A1), neutrophils upregulate surface Glut1 and cytoplasmic vigilin expression, together with mTOR and CREB pathway activation (5). In the second step (A1 → A2), neutrophils further upregulate surface ASCT2, PiT1, and PiT2 and cytoplasmic LC3 expression, along with primary granule exocytosis (4). An alternative model, also compatible with our results, would consider A1 and A2 subsets to be mutually exclusive, rather than sequential adaptive states of CF airway neutrophils (blood → A1 and blood → A2). Both models are presented in Fig. 8.

Further studies are needed to delineate the precise developmental relationships between these distinct neutrophil subsets. Longitudinal studies are also required to determine the potential associations between nutrient transporter expression and the severity of chronic and acute CF airway disease, as well as the effect of treatments. For instance, previous studies suggested that OSs significantly modulate Glut1 expression (38). In the course of the current study, we observed significant alterations in both Glut1 and PiT2 levels following OS treatment. Beyond CF, our findings indicate that the RBD ligands against surface-exposed Glut1, ASCT2, PiT1, and PiT2, and similar ligands derived from the Env of related retroviruses with high affinity for nutrient transporters, are likely to prove useful in investigating mechanisms of metabolic adaptation in response to inflammation and stress.

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Disclosures

J.L., J.-L.B., M.S., and R.T. are inventors on a provisional patent describing the use of RBD ligands for cell analysis in human inflammatory diseases (PCT/FR2010/051945 and PCT/EP2011/066231). The other authors have no financial conflicts of interest.

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SUPPLEMENTAL TABLE I. Demographic characteristics of the CF patients. Pulmonary function is represented as functional expiratory volume in 1 second (FeV1, % predicted). Ab=Absent, CH=Compound heterozygotes (comprising Delta F508 and a second mutation of the CF gene), F=Female, HO=Delta F508 homozygotes, M=Male, Mu=Mucoid, N/A=Not

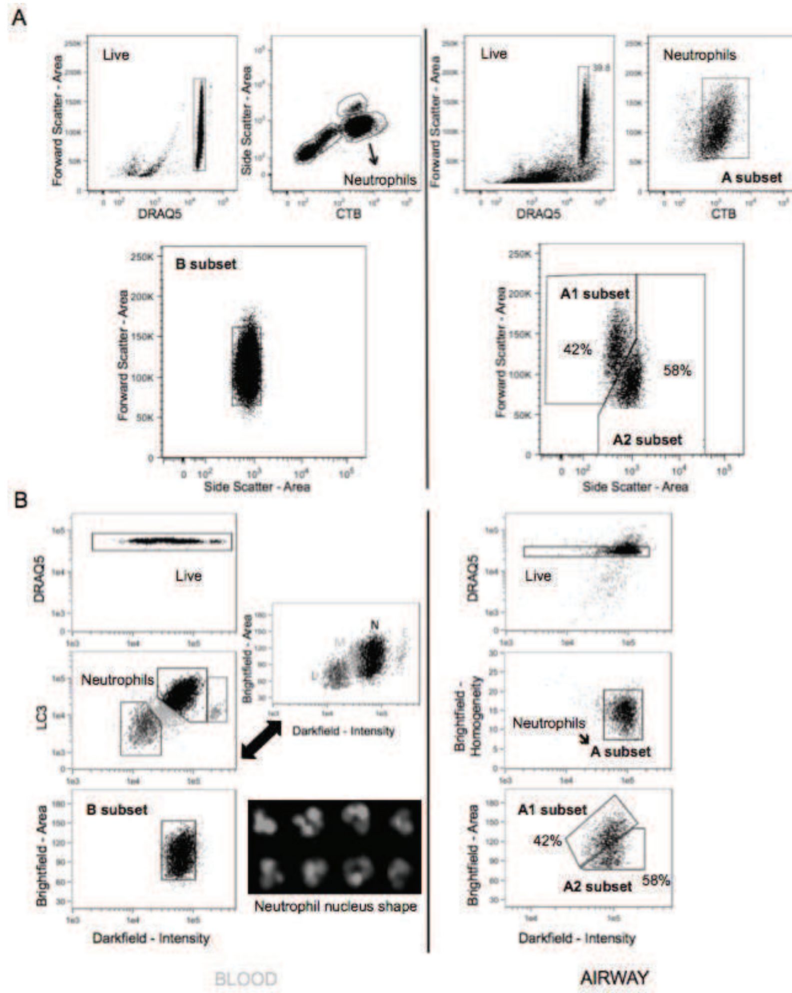
Gender	Age (years)	CFTR mutations	*P. aeruginosa* infection	FeV1 (% predicted)	Oral steroids
M	22	HO	Mu	68	N
M	49	HO	Mu	52	N
F	26	CH	Mu	50	N
M	11	CH	Mu	56	N
M	25	HO	Mu	53	Y
F	50	CH	Ab	67	Y
F	26	HO	Mu	53	N
F	50	HO	Mu	34	Y
F	32	CH	Ab	46	N
F	45	CH	Ab	57	N
M	33	OT	Pl	63	N
F	39	CH	Mu	58	N
M	32	HO	Mu	81	Y
F	44	OT	Ab	75	N
F	12	HO	Ab	56	N
M	50	HO	Ab	35	N
M	22	OT	Mu	41	N
F	22	CH	Mu	N/A	N/A
M	22	CH	Mu	49	N
F	22	OT	Mu	35	N
M	30	HO	N/A	75	N/A
F	23	OT	Ab	67	N/A
F	22	CH	Ab	62	N/A
F	39	CH	N/A	N/A	N/A
F=14, M=10	31.2 ± 2.4 (mean ± SE)	CH=10, HO=9, OT=5	Mu=13, Pl=1, Ab=8, N/A=2	56 ± 2.8 (mean ± SE)	Y=3, N=16, N/A=5

available, N=No, OT=Other mutations, Pl=Planktonic, Y=Yes.

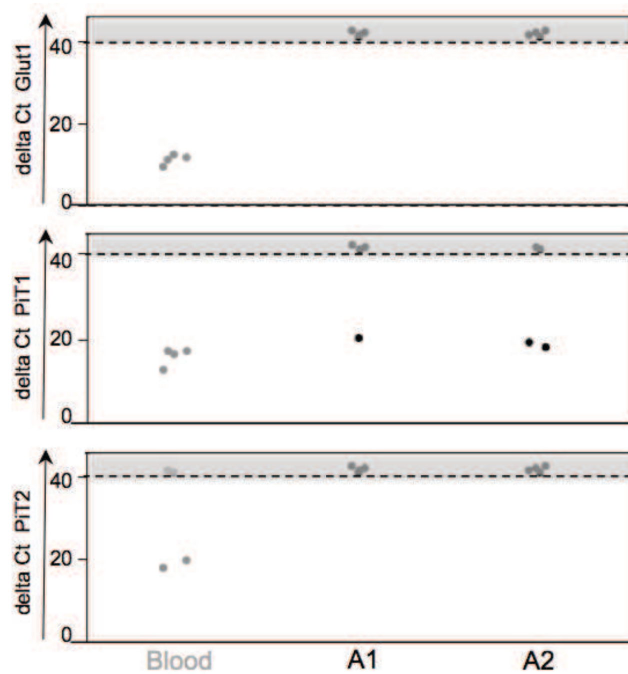
SUPPLEMENTAL TABLE II. Granule exocytosis and nutrient transporter surface expression on *in vitro* stimulated blood neutrophils. Cell surface expression of CD63 and CD66b, markers of primary and secondary granule exocytosis, respectively, as well as that of Glut1, ASCT2, PiT1 and PiT2 were assessed on blood neutrophils from healthy control (HC) and CF subjects (N=3 for each group) following 10 minute incubation with LB+fMLF. The median fold change in median fluorescence intensity as compared to unstimulated conditions for each cohort are shown.

	HC	CF
CD63	29.40	23.74
CD66b	6.87	5.66
Glut1	0.79	0.75
ASCT2	0.83	1.01
PiT1	1.67	1.41
PiT2	0.61	0.69

SUPPLEMENTAL FIGURE 1. Flow and image cytometry gating strategies for the analysis of blood and airway neutrophils. (A**)** Sequential gating based on forward scatter (FSC) and DRAQ5 staining yields live singlets. Leukocytes and neutrophils can be distinguished by their combined side scatter (SSC) and cholera toxin B (CTB) profiles. Flow cytometry analyses of blood and airway neutrophils are shown on the left and right hand panels, respectively. Airway neutrophils can be further subdivided into A1 and A2 subsets (as detailed in references 4, 5). **(B)** Image cytometry analysis allows separation of live cells as a function of DRAQ5/darkfield profiles. LC3 staining and darkfield intensity facilitates separation of neutrophils (N) from lymphocyte (L), monocyte (M), and eosinophil (E) subsets. Neutrophils are characterized by their scatter properties (brightfield area vs. darkfield intensity plot, assimilated to forward and side scatter, respectively) and lobulated nuclei (DRAQ5 image obtained on ImageStreamX).


A
Live
Forward Scatter - Area
DRAQ5
Side Scatter - Area
CTB
Neutrophils
Live
Neutrophils
A subset
B subset
Side Scatter - Area
A1 subset
42%
58%
A2 subset
B
DRAQ5
Live
LC3
Neutrophils
Brightfield - Area
Darkfield - Intensity
N
B subset
Neutrophil nucleus shape
Brightfield - Homogeneity
A subset
A1 subset
A2 subset
BLOOD
AIRWAY

SUPPLEMENTAL FIGURE 2. Nutrient transporter mRNA levels in sorted CF blood and airway neutrophils. The level of Glut1, PiT1 and PiT2 transcripts in sorted blood ($CD15^{hi}CD16^{hi}$) and airway neutrophil subsets (A1: $CD15^{hi}CD16^{hi}CD63^{lo}$; A2 $CD15^{hi}CD16^{lo}CD63^{hi}$, N=4, paired with blood samples) from CF patients were assessed by qRT-PCR. Levels were quantified relative to beta-actin (ACTB) and each point represents the calculated delta Ct (Ct_{Target} - Ct_{ACTB}), with Ct representing the number of cycles required to obtain a signal above background. Undetected transcripts are displayed with a delta Ct above 40 cycles.



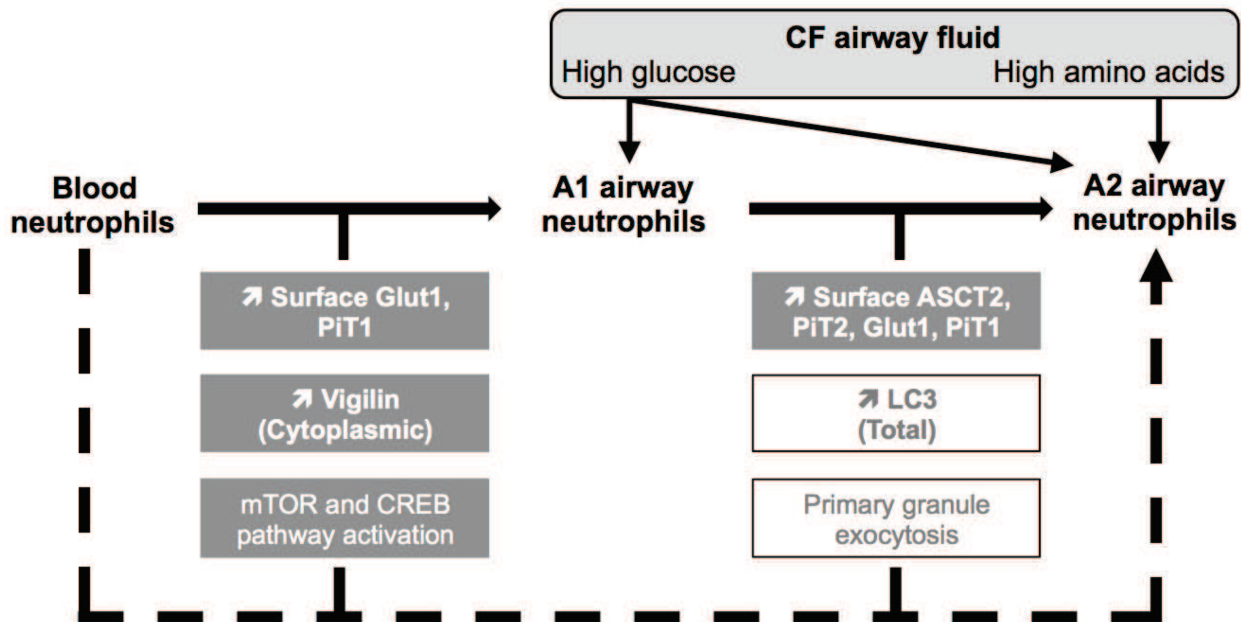


Fig. 19 Adaptation of neutrophils recruited to CF airways. Schematic representation of neutrophil adaptation upon recrutement from blood to CF airways. CF airways are rich in glucose and amino acids. Neutrophils recruited from blood into this pathological environment undergo complex adaptation, with changes in nutrient transporter expression and levels of LC3 and cytoplasmic vigilin (this study), as well as granule exocytosis and mTOR and CREB pathway activation. Two models of cell differentiation from blood neutrophil phenotype to airway neutrophil phenotypes are represented: a model with sequential changes from A1 to A2 neutrophil subsets (solid arrows) and a model with a direct transition to either A1 or A2 subsets (dashed arrows). *Figure from Laval et al., JI, 2013*

III-C. Discussion

In this chapter, we describe the modulation of Glut1, PiT1, PiT2 and ASCT2 expression comparing blood and airway neutrophils from CF patients. We also describe further modulation of metabolite transporter expression among discrete airway neutrophil subsets (A1 and A2). These results indicate that CF airway neutrophils adapt to the metabolically permissive environment in which they migrate, using resources available in the extracellular milieu. Furthermore, we show that CF airway neutrophils are able to maintain both catabolic (LC3-positive compartments) and anabolic (vigilin translocation from nucleus to cytosol) activities. This is rather remarkable, since catabolic and anabolic activities are generally thought to be mutually exclusive within a cell, and anabolic activities are not commonly associated with mature neutrophils.

Our data are consistent with a model in which blood neutrophils recruited into CF airways adopt the A1 phenotype, with activation of the CREB and mTOR pathways and increased Glut1 expression and glucose uptake (Fig. 19). A subset of neutrophils adopts the A2 phenotype, with certains A1 characteristics while enhancing rates of primary granule exocytosis and further enhancing metabolite transporter expression. The A2 subsets may either transition from A1 neutrophils or may derive directly from neutrophils recruited to CF airways. Further functional investigation of A1 and A2 subsets is required to determine the role of these metabolic and functional adaptations in the disease pathogenesis.

CHAPTER IV:
IMMUNOCOMPETENCE AND FUNCTIONAL PLASTICITY OF CF AIRWAY NEUTROPHILS

IV-A. Foreword

We previously described that neutrophils entering CF airways undergo anabolic reprogramming *(Makam, Diaz et al. 2009)* and metabolic adaptation *(Laval, Touhami et al. 2013)* to their new nutrient-rich and pro-inflammatory environment. Moreover, we observed functional alterations in CF airway neutrophils featuring the expression of novel surface molecules, usually associated with other immune lineages such as antigen-presenting cells. The metabolic reprogramming observed in CF airway neutrophils suggests that these cells may be able to assume functions not commonly ascribed to activated neutrophils. To address this issue, we investigated functions that are classically known to be fulfilled by neutrophils recruited to peripheral tissues, *(Davis, Drumm et al. 1996, Amulic, Cazalet et al. 2012)*. Then, we assessed CF neutrophils for the presence of significant transcriptional activity, generally associated with anabolic signaling in other cells *(Thomson, Turnquist et al. 2009, Powell, Pollizzi et al. 2012)*.

To assess CF neutrophil functions, we used flow cytometry, image cytometry, ELISA and microarray assays. We compared CF blood and airway neutrophils, as well as A1 and A2 subsets and different inflammatory states (when possible) for the following features:

- Cell priming, based on CD62L (L-selectin) shedding *(Waddell, Fialkow et al. 1994)*;
- Activation of the inflammasome pathway, based on intracellular caspase-1 activity (FAM FLICA) and extracellular IL-1β levels *(Ayala, Yamin et al. 1994)*;
- ROS production (CellRox probe) *(Cheng, Lee et al. 2013)*;

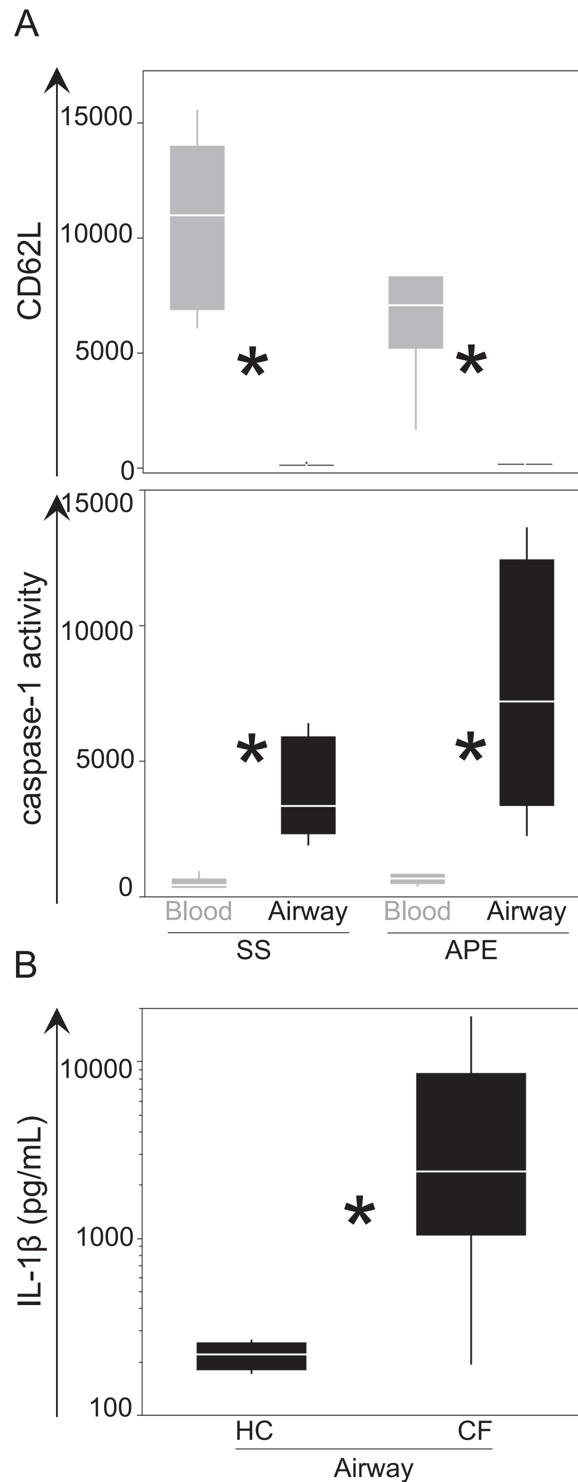


Fig. 20 Surface CD62L and intracellular caspase-1 in CF neutrophils and IL-1β levels in CF airway fluid. (**A**) Levels (MFI) of surface CD62L (*n*: SS = 8; APE = 7) and Caspase-1 activity (*n* = 8 for each group) in blood (grey) and airway (black) neutrophils. (**B**) IL-1β levels in airway fluid (*n*: HC = 4 and CF = 18). Results are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.05$

- Bacterial uptake and phagosome acidification (pHrodo probe) *(Simons 2010)*;
- Chromatin modifications, including: (i) quantification of cellular DNA and Ki67 expression *(Scholzen and Gerdes 2000)*; (ii) histone 4 citrulline 3 (H4Cit3) levels *(Wang, Li et al. 2009)* and (iii) HIF-1α expression and nuclear translocation index *(Duvel, Yecies et al. 2010)*;
- mRNA transcription (Affymetrix microarray chip) and pathway analysis by gene ontology (JMP Genomics).

IV-B. Results

IV-B.1. CF airway neutrophils are immunocompetent

IV-B.1.a. Priming and inflammasome activation

Surface CD62L expression is high in neutrophils that leave the bone marrow and circulate in blood before it is typically shed upon priming and activation. Consistent with the notion that neutrophils undergo a strong activation process when they migrate into CF airways, we observed significantly lower CD62L expression on CF airway vs. blood neutrophils (>90% decrease), at both SS and APE visits (Fig. 20-A). Caspase-1 activity, which is essential for production of the pro-inflammatory cytokine IL-1β by activated neutrophils, was also significantly higher in CF airway vs. blood neutrophils, at both SS and APE visits (increased by 7.9- and 11-fold, respectively) (Fig. 20-A). Consistently, IL-1β levels in airway fluid from CF patients was significantly higher (10.9-fold) than in healthy controls (Fig. 20-B). No difference was observed when comparing CD62L expression or caspase-1 activity in blood or airway neutrophils between SS and APE.

IV-B.1.b. ROS production and bacterial uptake

Two cardinal functions of activated neutrophils are ROS production and bacterial uptake. We used flow cytometry methods to assess these functions in CF neutrophils (Fig. 21).

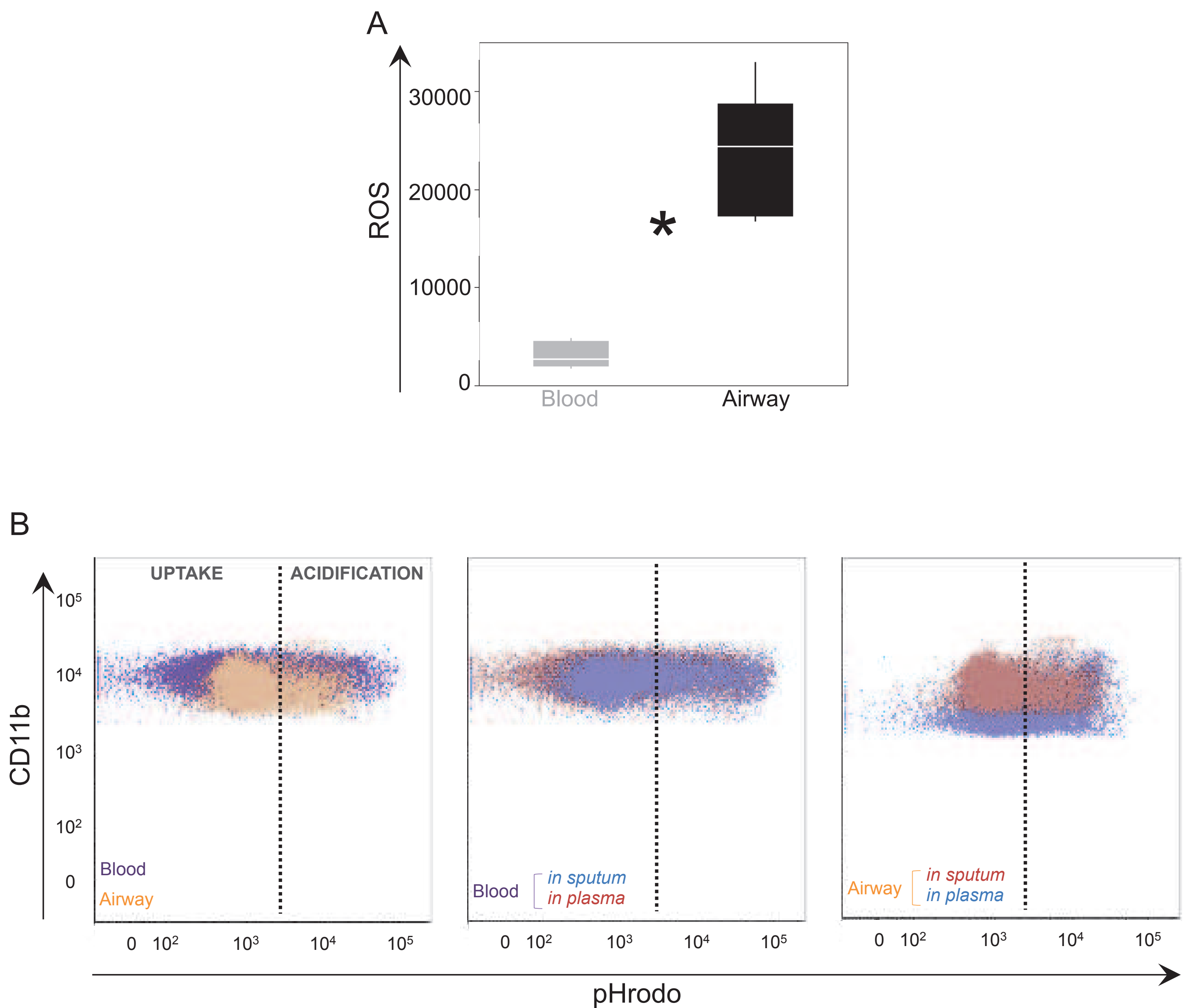


Fig. 21 ROS production and bacterial uptake by CF neutrophils. (**A**) Levels (deltaMFI) of ROS production (*n*: CF = 5) in blood (grey) and airway (black) neutrophils measured with the cell-permeable CellRox probe, are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). (**B**) pHrodo probe enabling quantification of bacterial uptake and acidification (shown is one representative plot, *n*: CF = 6). *p < 0.05

Both CF blood and airway neutrophils were able to trigger these two functional responses. Airway neutrophils showed higher (8.68-fold) levels of intracellular ROS when compared to blood (Fig. 21-A). Bacteria uptake and acidification was similar in airway and blood neutrophils and did not depend on the extracellular fluid in which neutrophils bathed. Indeed, plasma and CF airway fluid worked equally well in enabling bacterial uptake and acidification by blood and airway neutrophils (Fig. 21-B).

IV-B.2. CF airway neutrophils are transcriptionally active

IV-B.2.a. Chromatin modificiations

mTOR activation is generally linked to *de novo* transcription, anabolic activity and, potentially, cell proliferation. To investigate whether CF airway neutrophils showed signs of proliferation, we quantified total cellular DNA (using the DNA-intercalating dye DRAQ5) and assessed expression of the proliferation-associated antigen Ki67 in CF blood and airway neutrophils. No significant changes were observed in DRAQ5 and Ki67 levels between CF blood and airway neutrophils, thus excluding any proliferative activity (Fig. 22). Next, we assessed whether the chromatin in CF airway neutrophils showed any sign of remodelling, as evidenced by the presence of hypercitrullinated histones (anti-H4 citrulline 3 antibody) (Fig. 22). We found that H4Cit3 expression was decreased in CF airway neutrophils when compared to blood (0.77), indicating that chromatin was less condensed in blood compared to the whole airway population of neutrophils. However, we observed a modulation of H4Cit3 expression between previously described CF airway neutrophil subsets *(Makam, Diaz et al. 2009, Laval, Touhami et al. 2013)*, with A2 neutrophils showing an increase in H4Cit3 (1.21-fold) levels as compared to A1 neutrophils. These data suggest that live neutrophils undergo subtle chromatin changes in CF airway, which are not likely related to either proliferation (net DNA gain) or NET formation (net DNA loss), since cellular DNA was not quantitatively altered.

Airway neutrophils in CF are exposed to a hypoxic environment and thus are likely to undergo transcriptional adaptation along the HIF-1α

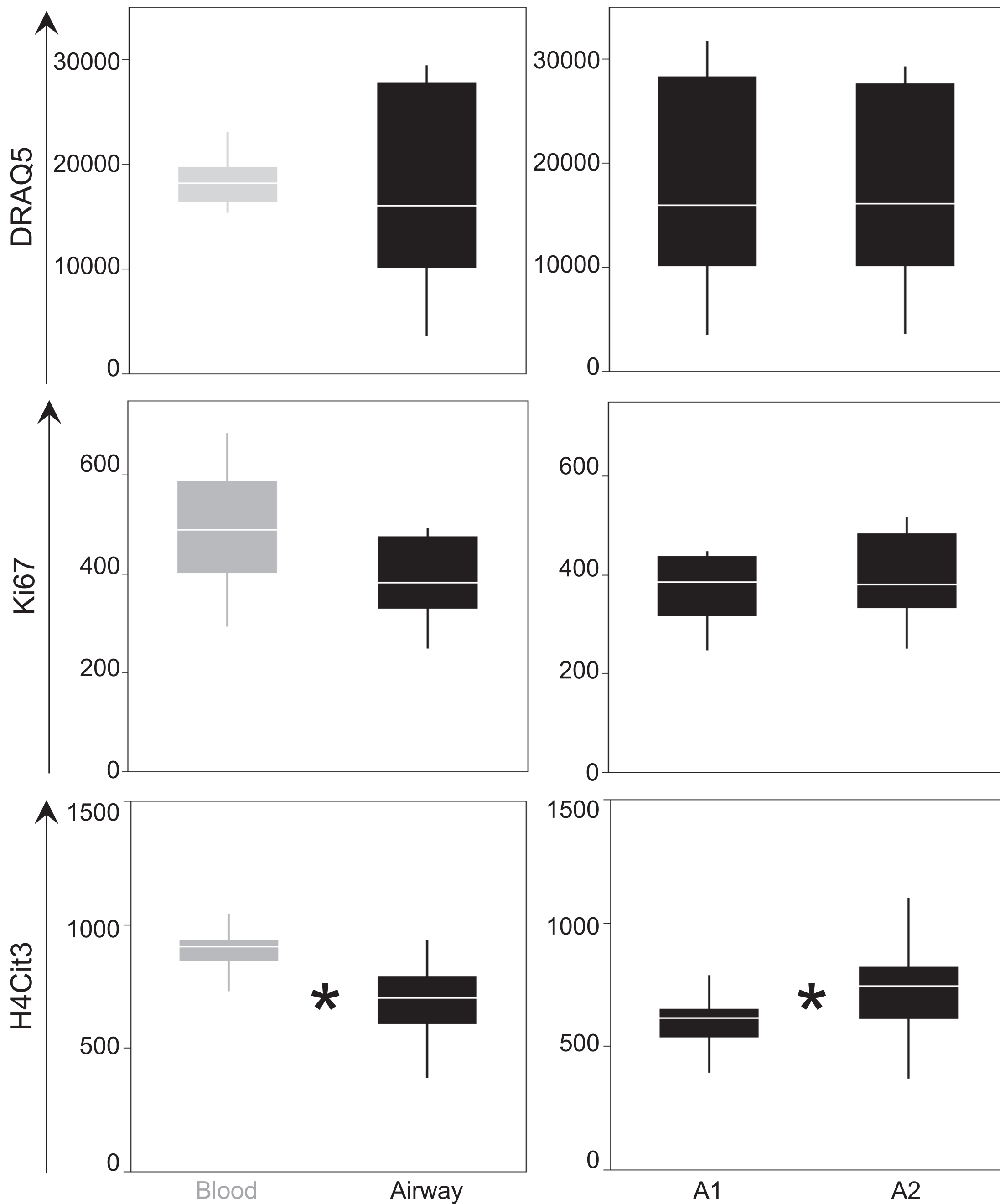


Fig. 22 Total cellular DNA and chromatin-associated proteins in CF neutrophils. Levels (MFI) of DNA (DRAQ5), Ki67 and H4Cit3 expression (*n*: CF = 12) in blood (grey), airway (black) and airway subsets (A1 and A2) neutrophils are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.001$

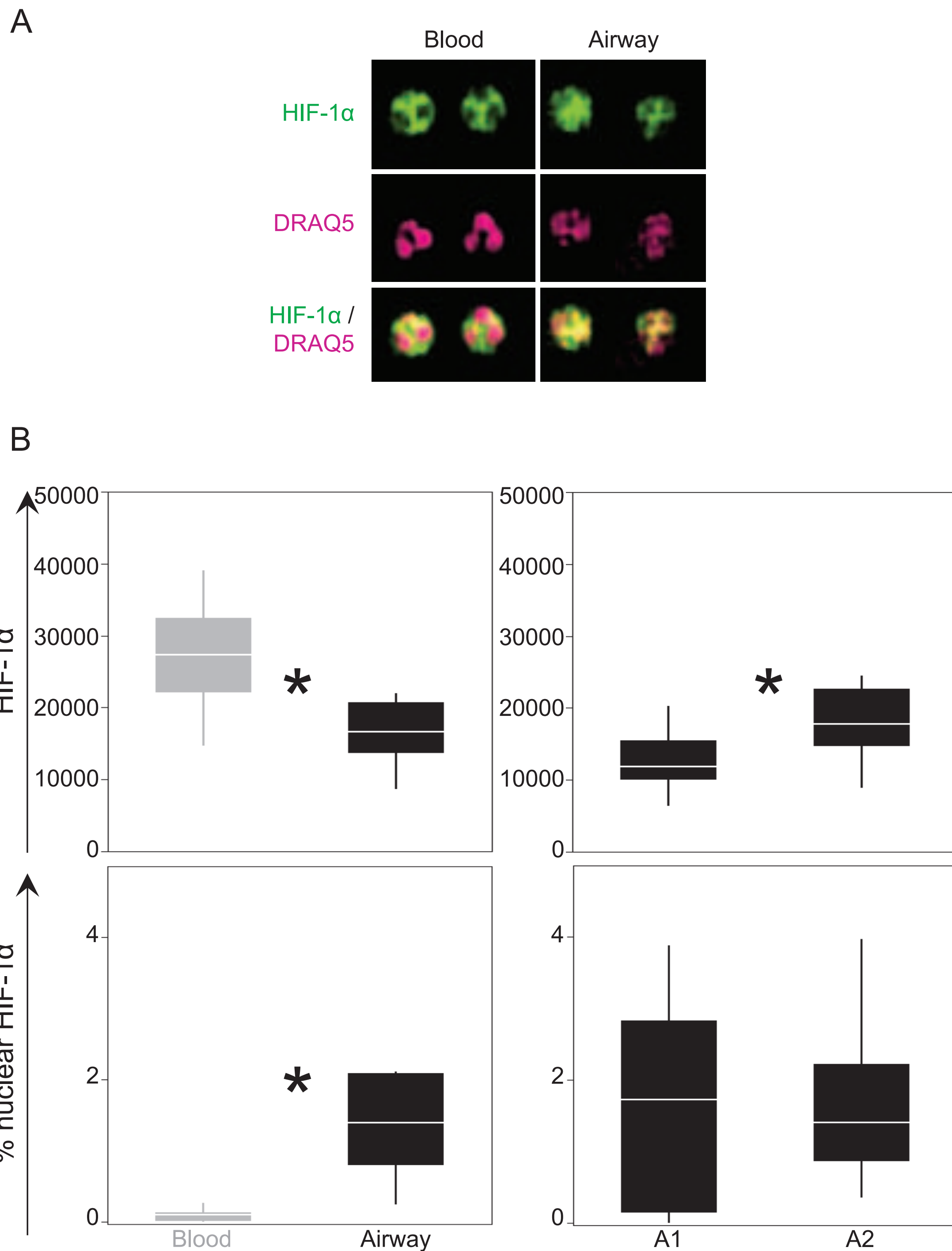


Fig. 23 HIF-1α expression and nuclear translocation in CF neutrophils. (**A**) HIF-1α (green) and nucleus (DRAQ5, pink) expression and colocalization in blood and airway neutrophils. Images are representative of 12 patients. (**B**) Levels of HIF-1α expression (MFI) and its nuclear translocation (% of positive cells) in blood (grey), airway (black) and airway subsets (A1 and A2) neutrophils from CF subjects (n = 12) are presented as box plots (delimited by 25th and 75th percentiles, with median line, and 10th and 90th percentile bars). *$p < 0.05$

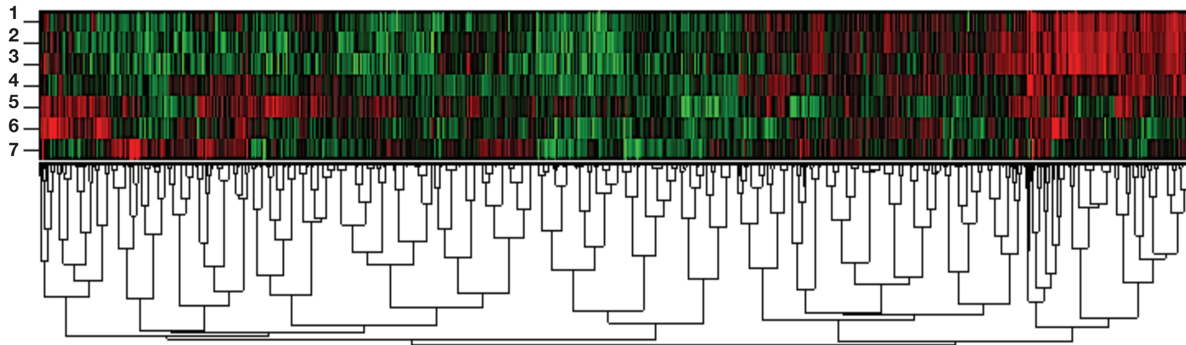


Fig. 24 Microarray analysis of transcript expression in CF airway and blood neutrophils. Competitive hybridization of transcripts from airway and blood neutrophils (*n*: CF = 7, paired samples) shows multiple transcripts that are upregulated (green) and downregulated (red), in the former vs. the latter, by a minimum of 2-fold.

Change	GO #	Function	P value
	0006415	Translational termination	6.6 E-98
	0006414	Translational elongation	1.5 E-95
B<A	*0003735*	Structural constituent of ribosome	6.8 E-84
	0071822	Protein complex organization	2.3 E-40
	0071843	Cellular component biogenesis	1.0 E-11
	0002376	Immune system process	5.8 E-20
	0009611	Response to wounding	1.0 E-15
B>A	*0043067*	Positive regulation of PCD	1.0 E-9
	0042981	Positive regulation of apoptosis	3.9 E-7
	0004721	Protein phosphatase activity	2.4 E-3

Table 11 Most changed transcript families in CF airway neutrophils. Gene ontology analysis identifies the five most upregulated (green) and downregulated (red) transcript families (of Fig. 23) in airway neutrophils compared to blood as consistent with an increased lifespan and activation in the former.

pathway, which regulates hypoxic responses in cells *(Worlitzsch, Tarran et al. 2002)*. In addition, the HIF-1α pathway is strongly influenced by mTOR activation (see Introduction section I-B.1.b). When comparing intracellular HIF-1α expression between CF blood and airway neutrophils, we observed a decrease in total HIF-1α protein level in the latter (0.61-fold). However, CF airway neutrophils presented a significant increase (13.9-fold) in the nuclear translocation of HIF-1α, suggesting a strong transcriptional activity, when compared to blood (Fig. 23). We also compared these parameters between the previously described A1 and A2 airway neutrophil subsets. The A2 subset displayed higher (1.5-fold) HIF-1α expression, as compared to A1 (Fig. 23-B). This change in expression did not correlate with a significant change in nuclear translocation between A1 and A2 subsets, however. Taken together, these data suggest that HIF-1α is expressed and potentially active as a nuclear transcription factor in CF airway neutrophils.

IV-B.2.b. mRNA transcript modulation

Next, we compared transcript levels between pure FACS-sorted fractions of live CF blood and airway neutrophils. We purified the corresponding mRNA and made cDNA from pairs of blood and airway samples (originating from 7 patients) and performed a two-color competitive hybridization (airway vs. blood) on microarray slides. Out of 10,877 genes interrogated, 750 were upregulated and 1,088 were downregulated in CF airway vs. blood neutrophils (Fig. 24). The gene ontology analysis of the upregulated genes identified biological functions such as translational elongation and termination, ribosomal constituent formation and biogenesis as being significantly enriched. In contrast, downregulated genes related to positive regulation of apoptosis and phosphatase activity. Thus, our transcriptional data suggest increase biosynthetic activity, decreased apoptosis and higher activation for airway neutrophils, which is consistent with our previous data (Table 11). Of note, whole-genome microarray analysis did not show significant differences in gene expression between A1 and A2 subsets (only 3 pairs analyzed so far, data not shown)

IV-C. Discussion

In this chapter, we brought evidence that neutrophil recruitment to CF airways is accompanied by L-selectin shedding and inflammasome activation. In addition, these cells produce high amounts of ROS and are able to take up bacteria and acidify formed vesicles. Therefore, the unconventional reprogramming that we observed in CF airway neutrophils (altered surface receptor expression, mTOR pathway activation, increased metabolite transporter expression) does not seem to impact their ability to carry out cardinal functions expressed by activated neutrophils.

H4Cit3 expression was higher in blood neutrophils, suggesting early chromatin decondensation, possibly linked to priming. Recruitment of neutrophils to CF airways was accompanied by nuclear translocation of HIF-1α expression and marked changes in their transcriptional profile, supporting the notion that these cells undergo profound adaptation therein. Highly modulated transcript families in CF airway neutrophils suggested an increase in biosynthetic activity and decreased the cell death, which together suggest increased lifespan and activity in these cells. While consistent with our previous data, these results remain preliminary and need to be reproduced in a second, independent cohort.

Overall, our data demonstrate that CF airway neutrophils, in addition to being metabolically reprogrammed, undergo transcriptional alterations, yet remaining functionally competent. Our identification of two CF airway neutrophil subsets (A1 and A2) may be followed by that of further discrete subsets of neutrophils, since our data suggest that metabolic, functional (e.g., degranulation) and transcriptional adaptations are occurring in asynchronous fashion among neutrophils exposed to the pathological CF airway fluid environment.

CONCLUSIONS
&
PERSPECTIVES

We developed and validated the use of the RBD-derived probes in the monitoring of inflammatory cell metabolism. We showed that these probes, which are derived from the retroviral-binding domains of retroviral envelope glycoproteins, enable the investigation of adaptive metabolic changes occurring in leukocytes during an immune response. Our methods, primarily validated for the flow cytometry analysis of blood and airway neutrophils from CF patients, is applicable to all leukocytes and cells isolated from other compartments or organs (e.g., synovium) and to other inflammatory disease conditions. During this study, we used this novel set of tools to:

- (i) Characterize specific metabolic profile of blood neutrophils from patients undergoing inflammatory disease (CF and RA) and controls subjects (healthy or suffering of a non-inflammatory disease),
- (ii) Identify possible metabolite transporter expression fluctuations that could occur during distinct inflammatory states observed during CF disease (APE vs. SS and CFRD),
- (iii) Distinguish between activated neutrophil subsets that arise upon recruitment to the inflammatory site (e.g., CF airways).

Although our pilot studies showed significant modulations of metabolite transporter expression in inflammatory cell populations, this work needs to be expanded to larger cohorts. In particular, potential correlations between metabolite transporter expression and conventional inflammatory markers (e.g., CRP) should be addressed in further work. This will help establish RBD-derived probes as valid biomarkers for inflammatory disease diagnosis and monitoring of associated pathophysiological states.

Previously, RBD-derived probes have been used to characterize lymphocyte activation and red blood cell maturation *(Manel, Battini et al. 2005, Swainson, Kinet et al. 2005, Kinet, Swainson et al. 2007, Lavanya, Kinet et al. 2008, Montel-Hagen, Kinet et al. 2008)*. More recently, the use of the specific RBD-derived ligand for Glut1 detection identified a specific lymphocyte subset susceptible to HIV infection *(Loisel-Meyer, Swainson et al. 2012)*. Here, we extended the application of these RBD-derived probes to the study of metabolite transporter expression by neutrophils from patients suffering from various inflammatory states. RBD-derived probes for Glut1, ASCT2, PiT1 and

PiT2 principally, but also probes derived from other Env, distinguished between inflammatory states, notably in systemic inflammation occurring in RA disease. Consistent with the recent discovery of a significant modulation of blood metabolites in RA patients and with prior experimental evidence that blood neutrophils in RA are primed *(Cedergren, Forslund et al. 2007, Cascao, Moura et al. 2010, Cascao, Rosario et al. 2010, Young, Kapoor et al. 2013)*, we described that blood neutrophils in RA display a specific metabolite transporter expression profile when compared to those from HC or DC. By contrast, in CF disease, we did not observe significant changes in blood neutrophil metabolite transporter expression as compared to control groups. However, CF blood neutrophils collected during an APE displayed different levels of transporter expression than CF blood neutrophils at SS, suggesting a metabolic adjustment during acute phases, which may be associated with the intensification of neutrophilic inflammation occurring in CF APE *(Stenbit and Flume 2011)*. Unlike the observations made on CF blood neutrophils in APE, CFRD status was not associated with changes in metabolite transporters at the neutrophil surface, even though this complication of CF is metabolic in nature. Taken together, these results attest to the metabolic plasticity of blood neutrophils in chronic and acute inflammation.

In recent *in vivo* studies, our group has demonstrated that a large fraction of neutrophils remains viable and active upon recruitment to CF airways, although they were generally thought to undergo rapid necrosis and passive release of toxic mediators when exposed to this pathological environment *(Tirouvanziam, Gernez et al. 2008)*. These results were buoyed by our later demonstration that neutrophils recruited to CF airways undergo anabolic reprogramming *(Makam, Diaz et al. 2009)*. Consistent with the observed accumulation of anabolic nutrients in CF airway fluid (notably AA and glucose) *(Barth and Pitt 1996, Baker, Clark et al. 2007, Garnett, Nguyen et al. 2012)*, we show a strong modulation of Glut1 and PiT1 expression on neutrophils recruited to this compartment. Moreover, we demonstrate that these neutrophils cluster into two distinct subsets, which differ regarding their metabolite transporter profiles as well as their anabolic and catabolic activities, suggesting a complex process of metabolic adaptation of

neutrophils to the permissive CF airway milieu *(Laval, Touhami et al. 2013)*. Interestingly, our additional results presented here show that metabolically reprogrammed CF airway neutrophils remain largely immuno*competent* since they are able to implement conventional functions of activated neutrophils (e.g., L-selectin shedding, inflammasome activation, bacterial uptake). Of note, CF airway neutrophils also display chromatin rearrangements and altered transcriptional activity. The identification of live neutrophils as active contributors to CF airway disease places them as an attractive target for anti-inflammatory therapy. Further studies in this direction will need to address key questions, raised by our recent discoveries. These questions include: What is the impact of neutrophil metabolic reprogramming in the course of the disease? How does this large fraction of viable and functional CF airway neutrophils tolerate chronic airway infection by selected organisms? And finally, how does the genetic defect in *cftr* (presumably acting in epithelial cells, primarily) modulate neutrophil fate, once these cells are recruited to CF airways?

From a broader theoretical standpoint, our studies of the metabolic profile and functions of neutrophils in RA and CF allowed us to discover unconventional activities not predicted by immunological theory. Indeed, neutrophils have been described as being short-lived, catabolic cells, pre-programmed to undergo apoptosis after quick activation processes. In the past few years, neutrophil research highlighted the plasticity of these cells, notably emphasizing their ability to undergo sweeping transcriptional changes during priming and recruitment *(Borregaard, Sorensen et al. 2007)*. Here, in addition to transcriptional plasticity, we demonstrated that neutrophils undergo physiological reprogramming driven by metabolic adaptation, associated with modulations in their surface metabolite transporter expression profile, nutrient uptake and anabolic functions. These new characteristics (observed in CF airway neutrophils) suggest a new paradigm for neutrophil regulation in human inflammatory diseases.

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RÉSUMÉ SUBSTANTIEL (Français)

CHAPITRE I :
LIGANDS DÉRIVÉS D'ENVELOPPES RÉTROVIRALES: NOUVEAUX OUTILS POUR ÉTUDIER LE MÉTABOLISME AU COURS DE LA RÉPONSE INFLAMMATOIRE

I-A- Avant-propos

Les rétrovirus sont des virus enveloppés qui se lient à leurs cellules-hôtes par interaction directe entre le domaine de liaison au récepteur (*Receptor Binding Domain* ou RBD) de la glycoprotéine d'enveloppe (Env) et des récepteurs spécifiques à la membrane plasmique (Fig. 12). Dans le cas des gamma- et deltarétrovirus, les récepteurs en question appartiennent à la famille des "multipass transmembrane proteins" (protéines à multiples domaines transmembranaires). Les récepteurs de ce type dont la fonction a été identifiée sont des transporteurs, généralement de la famille SLC (pour « *solute carriers* »). Ces transporteurs assurent le passage, généralement unidirectionnel, transmembranaire de multiple métabolites, dont les acides aminés (AA) neutres, les AA cationiques, le glucose, le phosphate, l'hème et certaines vitamines (Fig. 13). Afin de caractériser ces acteurs-clés du métabolisme cellulaire, mon laboratoire d'accueil à Montpellier a développé un panel de ligands dérivés des RBD spécifiques de différents transporteurs. Ces RBD peuvent être utilisés comme marqueurs des transporteurs métaboliques des cellules humaines, notamment les leucocytes (Fig. 12).

Les leucocytes jouent un rôle majeur dans la réponse au stress. L'infection par des agents pathogènes induit des changements rapides dans les cellules et tissus périphériques (par exemple, l'épithélium et la sous-muqueuse du tractus respiratoire ou gastro-intestinal) qui se traduisent aussi au niveau systémique et dans les tissus lymphoïdes (ganglions lymphatiques, moelle osseuse), aboutissant au recrutement de leucocytes de lignées innées (granulocytes, macrophages) et acquises (lymphocytes T et B). La réponse immuno-inflammatoire ainsi engagée s'accompagne de migration cellulaire,

de dégranulation et d'endocytose, ainsi que de transcription de nouveaux gènes et de néo-synthèse de protéines, lesquels requièrent de l'énergie. Cette énergie nécessaire à la réponse immuno-inflammatoire dépend de l'absorption active de métabolites (glucose, AA, phosphate, notamment), elle-même modulée par l'expression des transporteurs métaboliques en question à la surface des leucocytes. Malheureusement, il est reconnu qu'aucun anticorps fiable contre les domaines extracellulaires de ces transporteurs n'est disponible à des fins d'analyse phénotypique de surface. Ceci est en partie dû à la très forte conservation et très faible immunogénicité des boucles extracellulaires des SLC. Afin de pallier ce manque, nous avons développé l'utilisation de ligands RBD comme marqueurs métaboliques reflétant les changements phénotypiques des leucocytes lors de l'inflammation et des réponses immunitaires.

La maladie pulmonaire liée à la mucoviscidose (*cystic fibrosis* ou CF en anglais) est caractérisée par un recrutement massif et chronique de neutrophiles, un type de leucocytes appartenant à l'immunité innée, du sang vers les poumons. Des études récentes menées par mon groupe d'accueil américain ont démontré que, contrairement au modèle établi, les neutrophiles recrutés dans le poumon CF ne meurent pas de nécrose rapide, mais subissent une reprogrammation métabolique liée à une survie prolongée (Fig. 10). Par quantification de phosphoprotéines à l'échelle unicellulaire, nous avons ainsi montré que ces cellules enclenchent la voie anabolique mTOR, laquelle dépend de l'absorption de métabolites (Fig. 3). Afin d'étendre ces résultats, nous avons quantifié l'expression de transporteurs métaboliques-clés à la surface des neutrophiles de patients CF. À cette fin, nous avons utilisé les ligands RBD, permettant d'identifier ces transporteurs métaboliques avec hautes affinité et spécificité.

Ce faisant, nous avons validé l'utilisation des ligands RBD pour l'étude phénotypique des neutrophiles par cytométrie de flux, dans le cadre de la pathologie pulmonaire liée à la mucoviscidose, et plus largement, dans le cadre d'autres pathologies immuno-inflammatoires humaines.

I-B- Résultats et discussion

Nous avons isolé les RBD solubles des Env rétrovirales, que nous avons fusionné avec diverses étiquettes, pour mesurer par cytométrie de flux l'expression de surface des récepteurs correspondants (Fig. 12). Les ligands RBD utilisés ont été générés a partir des Env du deltarétrovirus de la leucémie humaine à cellules T de type 2 (HTLV-2) et des gammarétrovirus suivants : les rétrovirus endogène du félin (RD114), rétrovirus endogène du Koala (KoRV-A) et virus amphotrope de la leucémie murine (A-MLV) dont les récepteurs correspondants de ces ligands sont respectivement Glut1 (SLC2A1, transporteur de glucose), ASCT2 (SLC1A5, transporteur d'AA neutres), PiT1 et PiT2 (SLC20A1 et 2, tous deux importateurs de phosphate inorganique).

Combinées avec nos protocoles d'analyse de cytométrie en flux pour les leucocytes sanguins et pulmonaires, les sondes RBD nous ont permis de sélectionner les cellules vivantes et de discriminer les sous-populations leucocytaires dont nous avons évalué les niveaux respectifs d'expression en surface des transporteurs métaboliques. Ainsi, nous avons comparé l'expression de Glut1, ASCT2, PiT1 et PiT2 à la surface des leucocytes sanguins (granulocytes, monocytes, lymphocytes) et des neutrophiles des voies respiratoires dans 16 paires d'échantillons de patients. Toutes les sous-populations leucocytaires expriment les quatre transporteurs testés, mais à des niveaux différents. Nous avons remarqué que les neutrophiles du sang et des voies respiratoires des patients atteints de mucoviscidose diffèrent significativement dans leurs niveaux d'expression des transporteurs analysés grâce aux ligands RBD. La modulation que nous avons observé de l'expression de ces transporteurs métaboliques å la surface des neutrophiles recrutés vers les voies respiratoires mucoviscidosiques, appuie ainsi l'hypothèse d'une reprogrammation métabolique de ces cellules au cours de la maladie pulmonaire de la mucoviscidose (Demande de brevet en cours).

La demande d'invention déposée démontre que l'utilisation des ligands dérivés des RBD est unique pour identifier les transporteurs métaboliques de surface, associés à un état inflammatoire des cellules examinées. Nous

avons utilisé ces marqueurs pour documenter les changements survenus au cours de l'inflammation pulmonaire chronique chez les patients atteints de mucoviscidose, caractérisée par une migration massive des neutrophiles du sang vers les voies respiratoires. Nous avons développé un protocole unique, qui nous permet de quantifier l'expression des transporteurs par l’utilisation des ces nouvelles sondes et établi un profil métabolique des cellules de chacun des deux compartiments cellulaires étudiés (sang et expectorât pulmonaire) permettant une meilleure compréhension du processus pathologique. Des études supplémentaires, utilisant ces ligands dans certaines conditions pathologiques spécifiques de la mucoviscidose pourraient permettre leur utilisation comme outils de suivi de ces différents états. Ainsi, nous prévoyons d'étendre cette étude à un ensemble plus large de patients atteints de mucoviscidose, ainsi que des contrôles sains et d’autres pathologies inflammatoires. Il serait également intéressant de tester d’autres ligands spécifiques pour l’identification de différents transporteurs métaboliques (Tableau 10). En raison de l'importance physiologique des transporteurs métaboliques et de l’accessibilité à la cytométrie en flux, le champ d'application de cette approche est potentiellement très large.

CHAPITRE II :
EXPRESSION DES TRANSPORTEURS MÉTABOLIQUES SUR LES NEUTROPHILES DU SANG : COMPARAISON ENTRE DIFFÉRENTS ÉTATS INFLAMMATOIRES

II-A- Avant-propos

L'inflammation est un processus très contrôlé, caractérisé par le recrutement de leucocytes du sang vers les sites périphériques. La réaction inflammatoire implique la libération de médiateurs immunitaires tels que les cytokines pro-inflammatoires dans la circulation sanguine, initiant l'amorçage et l'activation systémique des leucocytes. L'inflammation peut être catégorisée comme «chronique» ou «aiguë», le premier état étant caractérisé par une stimulation prolongée du système immunitaire. Différents états inflammatoires impactent les cellules immunitaires de diverses façons et peuvent par exemple être associés à des phénotypes spécifiques des neutrophiles, alors que ces cellules sont toujours circulantes.

Au cours de la maladie autoimmune associée à la polyarthrite rhumatoïde (*rheumatoid arthritis* en anglais ou RA), l'inflammation systémique est liée à des altérations de la membrane synoviale et une inflammation chronique des articulations. Les neutrophiles des patients atteints de polyarthrite rhumatoïde ont été montrés comme étant « pré-activés » dans le sang, ce qui suggère que ces cellules jouent un rôle dans l'inflammation systémique de cette maladie. Par ailleurs, une étude récente a montré des modulations des métabolites sanguins, tels que AA et lipides, en distinguant la polyarthrite rhumatoïde d'autres arthrites non-inflammatoires. L'émergence de caractéristiques métaboliques dans le sang des patients atteints de polyarthrite rhumatoïde suggère que ce déséquilibre métabolique systémique pourrait participer à la pathologie, ce qui affecterait à son tour les neutrophiles sanguins.

L'inflammation pulmonaire de la mucoviscidose est liée à l'absence de

la protéine CFTR fonctionnelle à la surface de l'épithélium, la présence de bactéries et de champignons et un afflux continu et massif de neutrophiles du sang. Alors que cette inflammation des voies aériennes est en général chronique, les patients peuvent souffrir d'exacerbations aiguës caractérisées par un déclin rapide et sévère de la fonction pulmonaire, ce qui nécessite souvent une hospitalisation et un traitement antibiotique par voie intraveineuse. Ces exacerbations pulmonaires aiguës (*acute pulmonary exacerbation* en anglais ou APE) proviennent d'une altération de l'écosystème pathologique des voies respiratoires, impliquant des changements dans le microbiotope, les neutrophiles inflammatoires, et les composants du fluide, y compris les médiateurs de l'inflammation et les métabolites. De plus, le développement de la mucoviscidose se traduit chez certains patients par le développement d'une intolérance au glucose et de résistance à l'insuline, caractérisant le diabète associé à la mucoviscidose (*cystic fibrosis related-diabetes* en anglais ou CFRD). Cette complication est associée à une dérégulation du métabolisme systémique et un mauvais état nutritionnel des patients pouvant affecter l'activation et les fonctions métaboliques des cellules inflammatoires.

Puisque des changements d'états inflammatoires systémiques tels que décrits ci-dessus peuvent modifier l'activation des leucocytes et leur(s) fonction(s), nous avons tenté de caractériser l'expression des transporteurs métaboliques à la surface des leucocytes sanguins (en particulier les neutrophiles) de patients souffrant de différents états inflammatoires chroniques. Nous avons étudié le métabolisme des neutrophiles de sujets issus de quatre groupes distincts: (i) donneurs sains (HC, pour *healthy controls*), (ii) patients atteints de polyarthrite rhumatoïde (RA), (iii) patients atteints d'arthrite non-inflammatoire (maladies témoins, ou DC pour *disease controls*), et (iv) patients atteints de mucoviscidose (CF). Nous avons aussi comparé différents états inflammatoires observés au cours de la mucoviscidose (exemple : état d'équilibre et APE). En plus des RBD spécifiques pour Glut1, ASCT2, PiT1 et PiT2, comme décrits dans la première partie, nous avons utilisé XPR1-RBD (exportateur de phosphate), FLVCR-RBD (exportateur de l'hème), et hRFT1/3-RBD (importateur de riboflavine),

ainsi que BLV-RBD et PERVB-RBD, pour lesquels la nature des transporteurs/récepteurs n’est pas encore connue.

II-B. Résultats et discussion

Dans ce chapitre, nous avons présenté l'application de ligands RBD à l'étude de l'expression des transporteurs métaboliques dans les cellules du sang de patients souffrant de différents états inflammatoires. En plus de l’analyse de Glut1, ASCT2, PiT1 et PiT2, telle que validée dans la demande de brevet par l’utilisation des ligands correspondants, nous avons testé des ligands supplémentaires. D'un point de vue technologique, ces données démontrent en outre la validité des ligands pour étudier les changements métaboliques qui se produisent au cours de l'inflammation, bien que ce travail soit toujours poursuivi.

Nous avons décrit des profils spécifiques des neutrophiles du sang des patients atteints de polyarthrite rhumatoïde, avec une augmentation et une diminution de l’expression des transporteurs métaboliques à la surface cellulaire quand comparés respectivement aux neutrophiles des sujets sains et des témoins (Glut1, ASCT2, PiT1 et PiT2). Des tendances similaires, mais pas statistiquement significatives, ont été observées dans les neutrophiles sanguins des patients atteints de mucoviscidose (Fig. 14). Ces résultats suggèrent que l'inflammation systémique peut moduler l'expression des transporteurs métaboliques à la surface des neutrophiles sanguins.

De plus, nous avons étudié l'expression des transporteurs métaboliques à la surface des neutrophiles sanguins dans des états inflammatoires distincts de la mucoviscidose (exacerbation pulmonaire aiguë, APE, par rapport à l'état d'équilibre ou *steady-state* en anglais ou SS). Parmi tous les transporteurs de métabolites testés, seuls trois différaient significativement entre les conditions SS versus APE. L’expression de PiT1 augmentait, tandis que hRFT1/3 et le transporteur reconnu par BLV-RBD étaient diminués à la surface des neutrophiles sanguins APE, suggérant une modulation systémique spécifique de certains transporteurs à la membrane

des neutrophiles sanguins lors de l'inflammation aiguë associée à la mucoviscidose (Fig. 15 et 16).

Curieusement et bien que le diabète, et notamment CFRD, soit lié à des modulations métaboliques systémiques chez les patients atteints de mucoviscidose, nous n'avons détecté aucune modulation significative de l'expression des transporteurs métaboliques à la surface des neutrophiles sanguins (Fig. 17). Il est important d'indiquer que ces données sont des résultats préliminaires obtenus à partir d'une cohorte limitée de patients, et que cette étude doit être approfondie. Il serait important, notamment, de vérifier si les tendances observées dans l'expression des transporteurs métaboliques (APE vs. SS, et aussi dans CFRD versus CF), pourraient devenir significatives avec un plus grand nombre de sujets.

CHAPITRE III :
ADAPTATION MÉTABOLIQUE DES NEUTROPHILES RECRUTÉS DU SANG VERS LES VOIES RESPIRATOIRES CHEZ LES PATIENTS ATTEINTS DE MUCOVISCIDOSE

III-A. Avant-propos

La maladie pulmonaire de la mucoviscidose est un processus progressif et destructeur entrainé par une obstruction continue générée par le mucus, l'infection et l'inflammation. Le recrutement continu et massif des neutrophiles sanguins vers les poumons est caractéristique de la pathologie pulmonaire inflammatoire de la mucoviscidose. Nous avons montré auparavant que les changements subis par les neutrophiles lors de leur recrutement dans les voies respiratoires mucoviscidosiques incluent l'activation des voies anaboliques (Article 1). De plus, nous avons montré ici que l'utilisation de nouveaux outils spécifiques, les ligands RBD, pourrait révéler l'expression des transporteurs métaboliques à la surface des cellules, élément essentiel à notre boîte à outils pour l'étude du métabolisme des cellules inflammatoires. Dans des expériences préliminaires, nous avons observé des altérations significatives des transporteurs sur les neutrophiles des voies respiratoires mucoviscidosiques par rapport à leurs homologues du sang. Ceci est cohérent avec l'observation, faite par mon groupe d'accueil américain et d'autres, que les métabolites tels que AA et glucose s'accumulent pour atteindre des niveaux extracellulaires très élevés dans les poumons des patients atteints de mucoviscidose (Fig. 18).

L'environnement riche en métabolites fourni par le fluide des voies respiratoires mucoviscidosiques peut être particulièrement important dans la régulation des voies anaboliques des neutrophiles. Dans ce chapitre, nous présentons notre travail publié (*Journal of Immunology*, Juin 2013) (Article 2) décrivant l'étude en profondeur de l'expression des transporteurs métaboliques sur les neutrophiles des patients atteints de mucoviscidose, en comparant les compartiments du sang et des voies respiratoires. En plus de l'utilisation de ligands RBD, nous avons étudié d'autres caractéristiques

fonctionnelles des neutrophiles, dont l'exocytose des granules et le transport du glucose. Nos données apportent un appui solide à la notion que les neutrophiles subissent une adaptation métabolique importante dans l'environnement pulmonaire spécifique à la maladie. Nos résultats mettent également en évidence la complexité des processus pathologiques en jeu dans l'inflammation à neutrophiles des voies respiratoires mucoviscidosiques qui vont au-delà de la nécrose rapide des neutrophiles lors du recrutement, comme décrits jusque ici.

III-B. Résultats et discussion

Dans ce chapitre, nous décrivons la modulation de l'expression de Glut1, PiT1, PiT2 et ASCT2 en comparant les neutrophiles du sang et des voies respiratoires de patients atteints de mucoviscidose. Nous décrivons également davantage la modulation de leur expression parmi des sous-populations discrètes de neutrophiles extraits d'expectorâts pulmonaires (A1 et A2). Ces résultats indiquent que les neutrophiles recrutés vers les voies respiratoires des patients s'adaptent à l'environnement métaboliquement permissif dans lequel ils migrent, en utilisant les ressources disponibles dans le milieu extracellulaire. De plus, nous montrons que ces mêmes neutrophiles sont en mesure de maintenir à la fois une activité catabolique (compartiments LC3-positif) et anabolique (translocation de la vigiline depuis le noyau vers le cytosol). Ces résultats sont surprenants puisque les activités cataboliques et anaboliques sont généralement considérées comme mutuellement exclusives dans une cellule. De plus, les activités anaboliques sont rarement associées aux neutrophiles matures (Article 2).

Nos résultats sont compatibles avec la description d'un nouveau modèle dans lequel les neutrophiles sanguins recrutés vers les voies respiratoires mucoviscidosiques adoptent le phénotype A1, avec activation des voies CREB et mTOR et augmentent l'expression de Glut1 et la consommation de glucose. Une deuxième sous-population de neutrophiles adopte le phénotype A2, avec en plus de certaines caractéristiques de A1, l'exocytose des granules primaires et une amplification de l'expression des

transporteurs métaboliques. La sous-population A2 peut résulter d'une transition a partir de A1 ou directement découler des neutrophiles recrutés dans les voies respiratoires (Fig. 19). Une étude plus approfondie fonctionnelle de A1 et A2 est nécessaire pour déterminer le rôle de ces adaptations métaboliques et fonctionnelles dans la pathogenèse de la maladie.

CHAPITRE IV :
IMMUNOCOMPÉTENCE ET PLASTICITÉ FONCTIONNELLE DES NEUTROPHILES PULMONAIRES DES PATIENTS ATTEINTS DE MUCOVISCIDOSE

IV-A. Avant-propos

Nous avons précédemment décrit que les neutrophiles entrant dans les poumons mucoviscidosiques sont soumis à une reprogrammation anabolique et à une adaptation métabolique dues à leur nouvel environnement, riche en nutriments et médiateurs pro-inflammatoires. De plus, nous avons observé des modifications fonctionnelles des neutrophiles issus des voies respiratoires des patients atteints de mucoviscidose, notamment par l'expression de nouvelles molécules de surface, généralement associées à d'autres lignées immunitaires comme les cellules présentatrices d'antigène. La reprogrammation métabolique observée dans les neutrophiles des voies respiratoires mucoviscidosiques suggère que ces cellules peuvent être en mesure d'assurer des fonctions non communément attribuées aux neutrophiles activés. Pour tenter de mieux comprendre ces phénomènes, nous avons étudié les fonctions classiquement observées dans les neutrophiles recrutés au sein des tissus périphériques. Ensuite, nous avons exploré l'activité transcriptionnelle de ces cellules, généralement associé à l'anabolisme.

Pour évaluer les différentes fonctions des neutrophiles des patients atteints de mucoviscidose, nous avons utilisé la cytométrie en flux, la cytométrie en image, la méthode ELISA et l'hybridation des ARNm sur puce ADN. Nous avons comparé les neutrophiles sanguins à ceux des voies respiratoires, ainsi que des sous-populations A1 et A2 et différents états inflammatoires (quand les échantillons le permettaient) pour les caractéristiques suivantes:

- Activation cellulaire, basée sur le marqueur CD62L (L-sélectine);
- Activation de l'inflammasome, basée sur l'activité intracellulaire de la caspase-1 (FAM FLICA) et extracellulaire via les niveaux de IL-1β;

- Production de ROS (dérivés réactifs de l'oxygène, sonde CellRox);
- Absorption bactérienne et l'acidification du phagosome (sonde pHrodo);
- Modifications de la chromatine, y compris: (i) quantification de l'ADN cellulaire et prolifération (respectivement par la mesure de DRAQ5 et l'expression de Ki67), (ii) expression des histones 4 citrulline 3 (H4Cit3) et (iii) expression de HIF-1α et l'indice de translocation nucléaire
- Transcription de l'ARNm (Affymetrix *microarraychip*) et l'analyse des voies par *Gene Ontology* (JMP Genomics).

IV-B. Résultats et discussion

Dans ce chapitre, nous avons apporté la preuve que le recrutement des neutrophiles vers les voies aériennes mucoviscidosiques est accompagné du clivage de la protéine L-sélectine et de l'activation de l'inflammasome (Fig. 20). En outre, ces cellules produisent de grandes quantités de ROS et sont capables d'ingérer des bactéries et d'acidifier les vésicules d'absorption (Fig. 21). Par conséquent, la reprogrammation non conventionnelle que nous avons observée dans ces neutrophiles (altération de l'expression des récepteurs de surface, activation de la voie mTOR, augmentation de l'expression de surface des transporteurs de métaboliques) ne semble pas influer sur leur capacité à exercer des fonctions cardinales effectuées par les neutrophiles activés.

L'expression de H4Cit3 était plus élevée dans les neutrophiles sanguins, ce qui suggère une décondensation précoce de la chromatine, peut-être liée à la « pré-activation » (Fig. 22). Le recrutement des neutrophiles pulmonaires des patients atteints par la mucoviscidose est accompagné par la translocation nucléaire de HIF-1α marquant l'évolution de leur profil transcriptionnel, et soutenant l'idée que ces cellules subissent une adaptation profonde (Fig. 23). Les familles de transcrits fortement modulés dans les neutrophiles pulmonaires mucoviscidosiques suggèrent une augmentation de l'activité de biosynthèse et une diminution de la mort cellulaire (Fig. 24 et Tableau 11). L'ensemble de ces résultats suscite une durée de vie et une

activité accrues de ces cellules. Bien que compatibles avec nos données précédentes, ces résultats restent préliminaires et doivent être reproduits à partir d'une seconde cohorte indépendante.

Dans l'ensemble, nos résultats montrent que les neutrophiles des patients atteints de mucoviscidose, en plus d'être métaboliquement reprogrammés, subissent des modifications transcriptionnelles, tout en restant fonctionnellement compétents lorsqu'ils sont recrutés vers les voies aériennes. L'identification des sous-populations (A1 et A2) peut être suivie par celle de nouvelles sous-populations de neutrophiles, distinctes, puisque nos données suggèrent que les adaptations métaboliques, fonctionnelles (dégranulation, par exemple) et transcriptionnelle se déroulent en mode asynchrone entre les neutrophiles exposés à l'environnement spécifique de l'inflammation pulmonaire de la mucoviscidose.

Adaptation métabolique des neutrophiles inflammatoires dans les maladies humaines révélée par des ligands dérivés d'enveloppes rétrovirales : étude dans la mucoviscidose

Résumé : Notre étude est consacrée à la caractérisation des mécanismes d'adaptation métabolique des neutrophiles au cours de l'inflammation et plus particulièrement au cours de leur recrutement dans les voies aériennes des patients atteints de mucoviscidose (*cystic fibrosis* ou CF en anglais). Dans la mucoviscidose, nous avons précédemment décrit que les neutrophiles présents dans la lumière du poumon sont viables et soumis à une reprogrammation, notamment via l'activation de la voie anabolique mTOR. Ce travail est fondé sur les propriétés spécifiques de ligands solubles dérivés des domaines liant le récepteur (RBD, pour *Receptor-Binding Domain*) des glycoprotéines d'enveloppes rétrovirales permettant leur utilisation pour la détection de transporteurs métaboliques à la surface des cellules. Dans un premier temps, nous avons validé l'utilisation de ce nouveau groupe de marqueurs pour identifier et caractériser le phénotype métabolique des leucocytes CF obtenus à partir d'échantillons de différents compartiments (sang et expectorât pulmonaire). Dans un deuxième temps, l'étude de l'expression de ces transporteurs métaboliques sur les neutrophiles sanguins de patients atteints de CF ou de polyarthrite rhumatoïde et de sujets contrôles, a permis la distinction de phénotypes métaboliques au niveau systémique selon différents états inflammatoires. Ensuite, nous avons comparé l'expression des transporteurs métaboliques entre les neutrophiles sanguins et pulmonaires de patients CF et révélé une adaptation métabolique de ces cellules lors de leur recrutement vers les poumons. Enfin, les neutrophiles présents dans les voies aériennes des patients CF présentent une modulation de leur activité transcriptionnelle. De façon surprenante, nos travaux démontrent que malgré leur reprogrammation, les neutrophiles recrutés vers les poumons CF sont fonctionnellement compétents, ajoutant ainsi un nouvel angle d'approche dans l'étude des neutrophiles au cours de l'inflammation, notamment dans le cas de la pathologie pulmonaire liée à la mucoviscidose.

Mots-clés : neutrophiles, enveloppes rétrovirales, mucoviscidose, métabolisme, inflammation, transporteurs de nutriments.

Metabolic adaptation of inflammatory neutrophils in human diseases revealed by retroviral envelope-derived ligands: focus on cystic fibrosis

Summary: The present study focuses on adaptive metabolic steps adopted by neutrophils during inflammation, particularly during their recruitment into the cystic fibrosis (CF) airways. In CF, we previously described that airway neutrophils are alive and undergo reprogramming, featuring notably the activation of the anabolic mTOR pathway. The present work is based on specific properties of soluble ligands derived from the receptor-binding domains (RBD) of retroviral glycoprotein envelopes, which can be used for the detection of metabolite transporters at the cell surface. First, we validated the use of this new set of markers for the identification and characterization of the metabolic phenotype of CF leukocytes obtained from distinct compartments (blood and sputum). Second, by studying the metabolite transporter expression on blood neutrophils from CF or rheumatoid arthritis patients and control subjects, we distinguished metabolic phenotypes characteristic of specific inflammatory states. Then, we compared metabolite transporter expression between CF blood and airway neutrophils and showed that neutrophils undergo significant metabolic adaptation upon recruitment into the lungs. Finally, we demonstrated that CF airway neutrophils display significant transcriptional modulation and that despite their metabolic reprogramming, they remain functionally competent, thus adding an additional angle of approach to neutrophil studies with regard to inflammation, notably during CF airway disease.

Key words: neutrophils, retroviral envelope, cystic fibrosis, metabolism, inflammation, nutrient transporters.