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TIM-1 glycoprotein mediates neutrophil peripheral recruitment during inflammation

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ABSTRACT

In the last decade, the T cell immunoglobulin and mucin domain 1 (TIM-1) has emerged as a key regulator of innate and adaptive immune responses, representing a promising therapeutic target to be investigated. TIM-1 is widely distributed across immune cells, such as T, B and dendritic cells, where it exerts multiple functions. Despite being well investigated in cancer, transplantation, atopic and autoimmune diseases, several functional aspects of TIM-1 are still unclear. Recently, we have shown a novel function for TIM-1 as adhesion receptor for T helper (Th) 1 and Th17 cells, controlling their recruitment during inflammatory conditions. Neutrophils represent the first leukocyte population recruited to the injured site and several adhesion molecules are implicated in this process. Although neutrophils are classically associated with acute inflammatory processes, they are now emerging as crucial players also in chronic inflammatory diseases, including Alzheimer's disease (AD). However, the potential involvement of TIM-1 in neutrophil biology has never been investigated.

The main goal of this project was to investigate neutrophil TIM-1 expression and its role in neutrophil recruitment during inflammation.

By using flow cytometry and immunofluorescence staining, we demonstrated for the first time, that TIM-1 was expressed in mouse and human neutrophils, mainly stored in the cytoplasm under resting conditions. However, neutrophil activation after exposure to different inflammatory stimuli, including chemokines, fMLP, C5a, PMA and toll-like receptor (TLR) ligands rapidly triggered TIM-1 translocation to the cell surface. In the light of previous studies describing TIM-1 as an adhesion molecule and our results showing rapid translocation on neutrophil plasma membrane in response to chemotactic signals, we next tested the involvement of TIM-1 in neutrophil adhesion during inflammatory responses. By using a model of sterile thioglycolate-induced peritonitis, we demonstrated that the systemic blockade of TIM-1 or injection of exogenous neutrophils lacking a TIM-1 functional domain, strongly reduced neutrophil accumulation in the inflamed peritoneum, indicating that TIM-1 had a role in neutrophil recruitment during acute inflammation.

We also found that the oligomeric form of amyloid β 1-42 ($A\beta_{1-42}$) peptide, a potent inflammatory mediator and pivotal key player in the development and progression of AD, strongly upregulated TIM-1 expression on neutrophil surface, suggesting a potential TIM-1 contribution in neutrophil responses during neuroinflammation. By flow cytometry, we demonstrated that TIM-1 expression increases on circulating neutrophils of 3xTg-AD mice, an animal model of AD, compared to sex- and age-matched WT controls. Moreover, in 3XTg-AD mice, TIM-1-positive neutrophils accumulated in the meninges and in the choroid plexus, which are main access sites of leukocytes to the inflamed brain. Since a soluble form of TIM-1 (sTIM-1) was detected in several pathological diseases, we also measured sTIM-1 levels in the serum of 3xTg-AD mice. Interestingly, sTIM-1 significantly accumulated in the serum during disease progression, while it remained stable in the controls during aging, indicating TIM-1 as a new potential biomarker for AD. We also investigated the impact of TIM-1 on memory decline and neuropathological changes in 3xTg-AD mice crossed with TIM-$1^{\Delta mucin}$ mice lacking functional TIM-1. By using different behavioral tests we observed a significant restoration of spatial and associative memory in 3xTg-AD/Tim-$1^{\Delta mucin}$ mice when compared to 3xTg-AD control animals. Moreover, TIM-1 deficiency led to a drastic reduction of neutrophil accumulation in the brain during early disease and reduced neuropathological features, such as amyloid deposition, microglial activation and tau hyperphosphorylation, suggesting a role for TIM-1 in the induction of brain inflammation and neuropathological changes in an animal model of AD.

Collectively, our findings shed a new light on the role of TIM-1 as a novel trafficking receptor for neutrophils suggesting that TIM-1 blockade may have a beneficial effect on the development of acute and chronic inflammatory responses.

ABBREVIATIONS

AD *Alzheimer's disease*

ADAM *A disintegrin metalloproteinase*

AICD *Amyloid precursor protein intracellular domain*

ALS *Amyotrophic lateral sclerosis*

APC *Antigen-presenting cell*

APP *Amyloid precursor protein*

APRIL *A proliferation-inducing ligand*

Aβ *Amyloid β*

BACE1 *β-Site APP cleaving enzyme 1*

BBB *Blood brain barrier*

BLyS *B lymphocyte stimulator*

BSA *Bovine Serum Albumin*

C5a *Complement 5a*

CDK-5 *Cyclin-dependent kinase-5*

CF *Cystic fibrosis*

CNS *Central Nervous System*

CSF *Cerebro- spinal fluid*

CXCL *CXC ligand*

CXCR *C-X-C-motif chemokine receptor*

DAMP *Damage associated molecular patterns*

DCs *Dentritic cells*

EAE *Experimental autoimmune encephalomyelitis*

ECM *Extracellular matrix*

FBS *Fetal bovine serum*

FcR *Fc receptor*

fMLP *N-formyl-methionyl-leucyl-phenylalanine*

GD *Gamma Delta T cells*

GMP *Granulocyte-macrophage progenitors*

GPCRs *G-protein-coupled receptors*

HAV *Hepatitis A Virus*

HBSS *Hank's Balanced Salt Solution*

HEV *High endothelial venules*

IP *Intraperitoneal*

IV *Intravenously*

ICAM-1 *Intercellular adhesion molecule-1*

IgV *Immunoglobulin variable like-domain*

IL *Interleukin*

IRI *Ischemia-reperfusion injury*

KIM *Kidney injury molecule*

LAD *Leukocyte adhesion deficiency*

LFA-1 *Lymphocyte function-associated antigen-1*

LPS *Lipopolysaccharide*

LTB4 *Leukotriene B4*

MAdCAM-1 *Mucosal addressin cell adhesion molecule-1*

MAPK *Mitogen-activated protein kinase*

MAPs *Microtubule-associated proteins family*

MCI *Mild cognitive impairment*

MHC *Major histocompatibility complex*

MPO *Myeloperoxidase*

MS *Multiple sclerosis*

NET *Neutrophil extracellular traps*

NFTs *Neurofibrillary tangles*

NK *Natural killer*

NLR *Neutrophil-lymphocyte ratio*

PAF *Platelet-activating factor*

PAMP *Pathogen associated molecular patterns*

PBS *Phosphate buffered saline*

PCNSL *Primary CNS lymphoma*

PD *Parkinson's disease*

PFA *Paraformaldehyde*

PHF *Paired helical filament*

PMA *Phorbol 12-myristate 13-acetate*

PMN *Polymorphonuclear cell*

PRRs *Patterns recognition receptors*

PS *Phosphatidylserine*

PSEN *Presenilin*

PSGL-1 *P-selectin glycoprotein ligand-1*

RAGE *Receptor for advanced glycation end products*

ROS *Reactive oxigen species*

SLE *Systemic lupus erythematosus*

sTIM-1 *Soluble TIM-1*

Th *T helper cell*

TIM *T cell immunoglobulin and mucin domain*

TIM-1 *T cell immunoglobulin and mucin domain 1*

TLR *Toll-like receptor*

TNF-α *Tumor necrotic factor α*

Treg *T regulatory cells*

VCAM-1 *Vascular cell adhesion molecule-1*

VLA-4 *Very-late antigen-4*

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INTRODUCTION

1. TIM-1

1.1. THE TIM FAMILY

The T cell immunoglobulin and mucin domain (TIM) family of genes encode for a series of type I cell-surface glycoproteins involved in a variety of immune processes, including transplant tolerance, autoimmunity, allergy and asthma, viral infections and anti-cancer immunity[1]. The TIM family was first discovered in 2001 and since then received much attention due to its location on mouse chromosome 11B1.1, a genetic region associated with susceptibility to asthma, allergy and autoimmunity[2].

In mice, there are four expressed *Tim* genes, *Havcr1* (hepatitis A virus cellular receptor 1, encoding for TIM-1), *Timd2* (which encodes TIM-2), *Havcr2* (encoding for TIM-3) and *Timd4* (which encodes TIM-4), and four predicted genes have been found in the same chromosomal region: *Tim5*, *Tim6*, *Tim7* and *Tim8.* In humans, three members of the TIM family are conserved (TIM-1, TIM-3 and TIM-4) and the locus is located on human chromosome 5q33.2, again linked with atopy, asthma and autoimmune pathologies[3]. The three human TIMs are considered the orthologous of mouse TIM-1, TIM-3, and TIM-4, respectively.

The proteins of the TIM family share a common structure: an extracellular portion with an immunoglobulin variable (IgV)-like N-terminal Cys-rich region, followed by a mucin-like domain, a single transmembrane region and a cytoplasmic tail with tyrosine phosphorylation motifs involved in intracellular signaling, with the unique exception of TIM-4 (Figure 1)[4]. The mucin domain is rich in threonine, serine and proline, with a predicted extended conformation and a heavily O-glycosylated pattern. However, the length of the mucin domain is variable among the TIM members, in fact the number of O-linked glycosylation sites range from 60 in TIM-1 protein to 3 in TIM-3 in mice (Figure 1). Particularly TIM-1 is formed by signal sequence motif of 21 residues, an Immunoglobulin V-set (IgV) domain of 100 residues, a mucin domain containing sixty glycosylation sites, the majority of which are O-linked glycosylation sites, a transmembrane domain of 20 residues, and a

intracytoplasmic tail 47 residues long[2]. Close to the C-terminal domain there is a tyrosine phosphorylation site, suggesting that TIM-1 may be a substrate for Src family kinases[5].

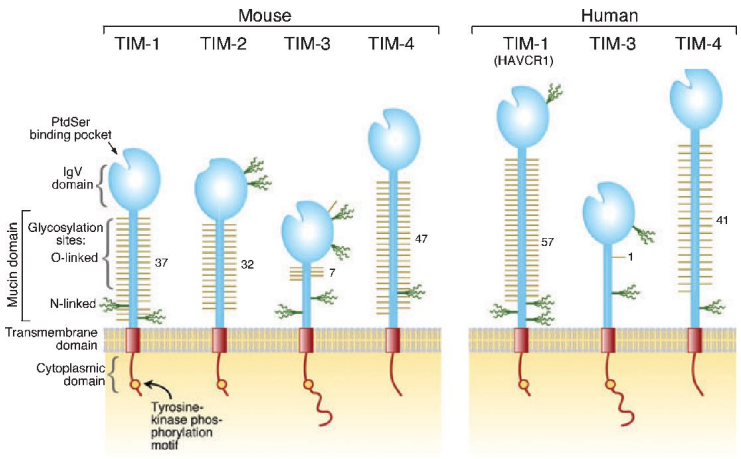


Figure 1. Schematic representation of TIM protein structures. The TIM proteins are type I cell surface glycoproteins expressed in mice and humans. Their structure consists of an IgV-like N-terminal Cys-rich region, a mucin-like domain, a single transmembrane region and a cytoplasmic tail with tyrosine phosphorylation motifs, except for TIM-4. The mucin domain is rich in threonine, serine and proline, with a predicted extended conformation and a heavily O-glycosylated pattern[6].

TIMs were initially thought to be specific molecules expressed on T lymphocytes and to exclusively regulate T cell functions such as proliferation, activation, transmigration, cytokines production, phagocytosis and apoptosis. However, in the last years other cell types have been described to express this class of molecules and it is now clear that TIM glycoproteins are distinctly expressed among immune cells.

TIM-1 and TIM-3 are the most studied TIM proteins in literature, due to their broad impact on immune functions. TIM-1 is mainly expressed on T-helper 2 (Th2) cells, acting as a strong costimulatory molecule for T cell activation, while it is less present on Th1 and Th17 subsets[7]. TIM-1 is also expressed on activated B cells leading to antibody formation and it is involved in the maturation of B cells in plasma cells[8]. TIM-3 was initially identified as a Th1 specific molecule[9]; however, some authors recently observed TIM-3 expression also in natural killer (NK) cells, monocytes and dendritic cells (DC)[1], where it drives the phagocytosis of apoptotic cells and the cross-presentation of antigens[6]. Emerging data suggest that TIM-3 has opposite functions in innate and adaptive immunity. During the initiation of the

immune response, TIM-3 is primarily expressed by DCs and it promotes inflammatory responses triggering the production of proinflammatory cytokines and the activation of Toll-like receptors (TLR) signals[10]. In mice, TIM-4 is only expressed by professional antigen presenting cells (APCs), like macrophages and DCs. Although it is not expressed by T cell subsets, TIM-4 is described to contribute to T cell activation, exerting a bimodal effect: it inhibits the activation of naïve T cells and it promotes the survival and proliferation of already activated cells[11, 12]. The latter function is due to the interaction of TIM-4 with TIM-1[12, 13].

Recently, a new function for TIM proteins has been described. It has been shown that TIM-1, -3, and -4 are receptors for phosphatidylserine (PS) on human and mouse cells[6]. PS is normally localized to the inner leaflet of the plasma membrane in healthy cells and its upregulation or exposure to the outer membrane is a sign of apoptosis, cell injury and malignant transformation[14]. PS recognition provides a key signal to phagocytes that triggers engulfment of apoptotic cells[15]. The interaction between TIMs and PS suggests a physiological role for TIM proteins in the regulation of tissue homeostasis and immune responses. Thus, depending on which TIM molecule is involved and on which cell type it is expressed, the TIM family establishes whether apoptotic cell recognition drives immune activation or tolerance[6]. Another putative important role for TIM proteins-PS binding is represented by exosome uptake. Exosomes are types of nanovesicles secreted by a wide range of mammalian cells that expose PS at their outer leaflet and contain various cellular proteins. There is growing evidence that exosomes participate in cell communications and immune responses, and exosomes also appear to play an important role in tumor growth and host-tumor relationships[16]. It has been shown that TIM-1- or TIM-4-induced expression increase exosomes binding via interaction with PS in transfected cells, and exosomes stimulated the interaction between TIM-1 and TIM-4[17], indicating that TIM proteins may represent a new class of exosomes receptors and may be involved in intercellular signaling in which exosomes are involved.

1.2 TIM-1 EXPRESSION AND FUNCTIONS IN IMMUNE CELLS

As mentioned before, TIM-1 is expressed by activated T cells and, after polarization, its expression remains high on Th2 cells, while Th1 and Th17 cells

express lower levels of TIM-1 on their surface[1]. TIM-1 is absent on naïve T cells. A proposed model for TIM-1 expression was suggested in T cells (Figure 2). In naïve lymphocytes, TIM-1 is largely stored in intracellular pools such as early endosomes, the Golgi apparatus and lysosomes, and it is present at only low levels on the plasma membrane[18, 19, 20]. Upon activation of T cells TIM-1 expression strongly increases on the cell surface but still predominantly accumulates within intracytoplasmic vesicles and beneath the plasma membrane. Besides, a continuous recycling of TIM-1 from the membrane to the cytoplasm was also described[20] and TIM-1 surface translocation can be rapidly induced by the release of intracellular calcium following in situ activation with specific stimuli, for instance TCR ligation or chemokine stimulation[19]. A flip–flop model has been proposed in which the TIM-1 extracellular domain resides in a bent conformation on the cytosolic side of the membrane, anchored to PS with a PS-binding pocket exposed in the N-terminal IgV domain[19]. An increased concentration of intracellular calcium could, therefore, induce the exposure of PS (and consequently of TIM-1) to the outer sheet of the membrane, promoting the release of the IgV domain and the presentation of the whole molecule (Figure 2).

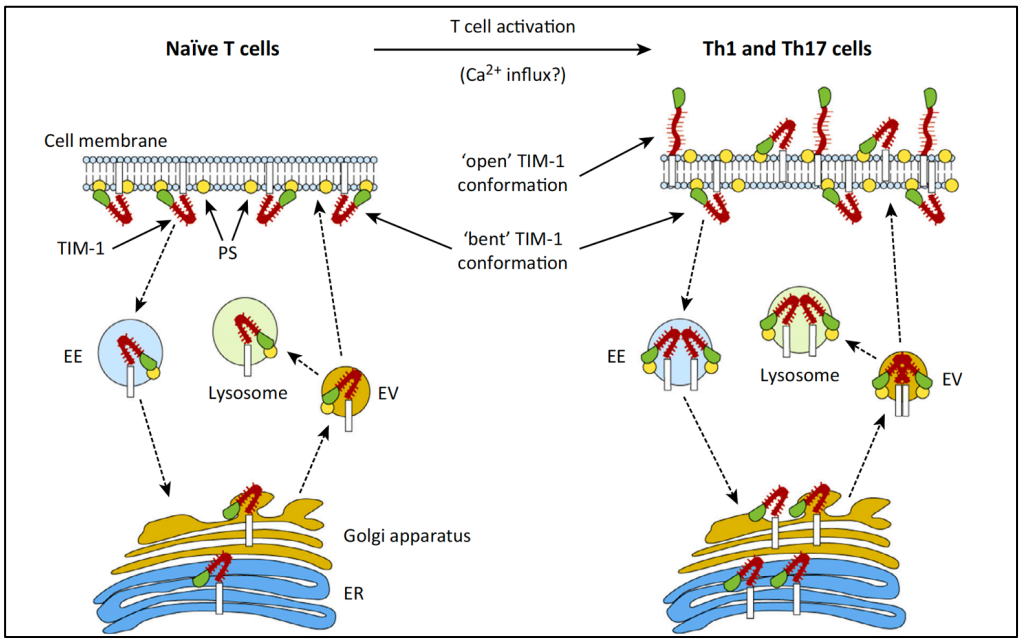


Figure 2. Cellular localization of TIM-1 in naïve and activated T cells. In naïve T cells, TIM-1 is mainly stored in intracellular pools in the Golgi apparatus, endoplasmic reticulum (RE), in lysosomes and early endosomes (EE); however, it is also present in the inner leaflet of the cell membrane in a bent conformation. Upon T cell activation to Th1/Th17 phenotypes,

TIM-1 levels increase intracellularly and on the cell surface, here also in a functional open structure exposing the IgV and mucin domains[21].

Regarding the function of T cells, TIM-1 mainly acts as costimulatory signal for T cell activation. Indeed, the cross-linking of TIM-1 on CD4+ T cells with an agonistic monoclonal antibody (mAb) (3B3 clone) was shown to trigger T cell activation also increasing cell proliferation and cytokines production, especially interleukin (IL)-4 produced by mature Th2 cells[22]. Moreover, the administration of 3B3 mAb *in vivo* along with antigen increases antigen-specific T cell proliferation and production of interferon (IFN)-γ leading to the blockade of the development of respiratory tolerance[23].

TIM-1 has been shown to co-localize on human T cell surface with CD3, and to be recruited to the T cell receptor (TCR) complex[24]. Moreover, a recent work demonstrated that one of the TIM-1 main functions is a cross-linking ability to generate a costimulatory signal also in naïve T cell, independently from the TCR[25]. Agonistic anti-TIM-1 antibodies, in fact, are capable of inducing T cell activation without additional stimuli. The biochemical mechanisms underneath are still under review, but it is clear that the phosphoinositide 3-kinase (PI3K) pathway is required for TIM-1 signaling: the p85 subunit of PI3K is directly recruited to TIM-1 tyrosine Y276 after the phosphorylation of the cytoplasmic tail by the lymphocyte-specific protein tyrosine kinase (Lck)[24]. Finally, through the PI3K/AKT pathway TIM-1 promotes T cell viability and induction of the antiapoptotic gene *Bcl*-2[12]. Overall, these recent results unravel a complex signaling pathway downstream TIM-1 engagement, which is correlated with T cell activation. If over-expressed, TIM-1 also leads to NFAT/AP-1 transcriptional activation, depending on tyrosine Y276 in the cytoplasmic tail and consequent increase in IL-4 production[26].

Consistent TIM-1 expression has been found on B lymphocytes. Particularly, naïve B cells express basal low levels of TIM-1, that strongly increase after B cell receptor stimulation in a PI3K and nuclear factor-kB (NF-kB) dependent manner[8, 27]. TIM-1 expression on B cells has been correlated with the regulation of antibody production[8], but no defects have been found in B-cell responses in TIM-1 deficient mice[27] thus, the precise function of TIM-1 on B cells needs further investigations. A recent study also proposed TIM-1 as an inclusive marker for a subset of B lymphocytes, named regulatory B cells (Bregs). In fact, TIM-1 is preferentially expressed by IL-10/IL-4-producing Bregs in all major B cell subpopulations,

including transitional, marginal zone, and follicular B cells[28]. TIM-1-expressing Bregs can promote Th2 responses and maintain tolerance in several models of inflammatory bowel disease, collagen-induced arthritis, allergic airway disease, and diabetes mellitus[28].

In the last years, other cell types have been shown to express TIM-1, revealing a more complex regulation of the immune functions. TIM-1 is constitutively expressed by myeloid-derived DCs and its expression further increases after DC maturation and activation. TIM-1 signaling (partly linked to NF-kB activity) into DCs, upregulates the expression of costimulatory molecules and the production of pro-inflammatory cytokines. Specifically, due to the production of cytokines IL-6, IL-23 and IL-1, TIM-1-activated DCs enhance Th17 responses and inhibit Foxp3+ T regulatory (Treg) cell generation[29].

In addition, Nakae and colleagues found that TIM-1 is constitutively expressed on mouse peritoneal mastocytes and TIM-1 cross-linking with recombinant TIM-4 induces cytokine production by these cells[30]. Mast cells have been shown to contribute to the development of autoimmune diseases[31], and TIM-1 glycoprotein present on mast cells could influence these pathologies.

Furthermore, invariant NKT (iNKT) cells constitutively express TIM-1, and TIM-1/PS recognition induces iNKT cell activation, proliferation and cytokine production. Induction of apoptosis in lung epithelial cells activate pulmonary NKT cells resulting in airway hyperreactivity, a classical feature of asthma[32]. In this context, TIM-1 may act as a pattern recognition receptor (PRR) on NKT cells and may regulate asthma-associated pathologies independently from T cells[33].

Overall, the broad and complex pattern of TIM-1 expression in the immune system and its immuno-modulatory properties strongly suggest a central role for this receptor in the regulation of immune responses.

Moreover, TIM-1 glycoprotein has been found not only on immune cells, but also in other cellular types both in rodents and humans. Ichimura et al. were the first to report the presence of TIM-1 protein and mRNA levels in proximal tubule renal epithelial cells. Indeed, they found that TIM-1 is present at low levels in normal tissue, but its expression is dramatically upregulated more than any other protein in the post-ischemic kidney[34, 35]. Moreover, they proved in vivo that apoptotic and necrotic cells of the injured kidney were phagocytosed by surviving epithelial cells that express TIM-1. Thus, TIM-1 confers epithelial cells the property

to recognize and engulf apoptotic cells by binding the phosphatidylserines and oxidized lipid epitopes exposed on their cell surface[35, 36].

Moreover, many authors have reported increased TIM-1 expression in the lung[37], liver[38], prostate[39], gastric[40] and colorectal tissues[41] under inflammatory conditions, including cancer. Besides, some data also demonstrate that TIM-1 is expressed on human endothelial cells, and that it may have a novel function as part of the regulatory apparatus for tight junction of endothelial cells[42]. Finally, a very recent study showed the presence of TIM-1 glycoprotein on primary human oligodendrocytes of the temporal lobe isolated from surgical resection of patients with epilepsy[43]. In this study, the authors demonstrated a novel neuro-immune axis between TIM-1, semaphorin 4A (SEMA4A) and H-ferritin.

1.3 TIM-1 IS A NOVEL TRAFFICKING RECEPTOR ON T CELLS

Recruitment of circulating leukocytes to sites of inflammation is a crucial process of an immune response. Genetic abnormalities leading to defects in leukocyte trafficking have been correlated in humans with the development of leukocyte adhesion deficiency (LAD) syndromes, in which most of the patients die early in life due to severe impairment in host defense mechanisms[44]. Leukocyte trafficking to the inflamed tissue and homing to secondary lymphoid organs is a highly regulated sequential multistep process including tethering, rolling, integrin activation, arrest, crawling and transmigration[45], in which chemoattractants and cellular and vascular adhesion molecules are tightly involved[46].

In the last years, TIM-1 glycoprotein has emerged as a novel adhesion molecule controlling the recruitment of activated T lymphocytes during inflammation (Figure 3)[18]. TIM-1 has three main structural characteristics in common with other adhesion receptors. First, the TIM-1 mucin domain is highly glycosylated, and shows a specific predicted pattern of O-linked glycosylation sites that is comparable to that of P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is able to bind E-, L- and P-selectins, but it is currently regarded as the major P-selectin ligand, and it is expressed on myeloid and activated T cells[47, 48]. PSGL-1-deficient leukocytes showed a reduced ability to interact with P-selectin under physiological flow conditions *in vitro*[49]. Second, the whole extracellular domain of TIM-1 has a great similarity with mucosal addressin cell adhesion molecule-1 (MAdCAM-1), that

normally binds α4β7 integrin via its IgV-like domain, and L-selectin via the mucin domain during lymphocytes homing to mucosal tissues[21, 50]. The third feature of TIM-1 is the IgV-like domain that shares functional characteristics with C-type lectins[51].

The potential involvement of TIM-1 in leukocyte extravasation was previously suggested by the fact that TIM-1 blockade reduced leukocyte accumulation in target tissues in models of allergic asthma[52] and liver ischemia reperfusion[38]. Recent data have clearly shown that TIM-1 expressed on Th1 and Th17 cells controls T cell tethering and rolling, the first steps of the leukocyte adhesion cascade to the vascular endothelium, by binding to endothelial P-selectin in acute and chronic inflammatory conditions. Moreover, the TIM-1 mucin domain is the functional one involved in the interaction with P-selectin *in vitro* and *in vivo*. Of note, TIM-1 can also bind E- and L-selectins *in vitro*, but with lower affinity[18]. Therefore, these evidences suggest that PSGL-1 is not the sole ligand for selectins during inflammatory responses, that TIM-1 is a P-selectin ligand and T cell trafficking receptor, and TIM-1 acts concurrently with PSGL-1 to achieve efficient tethering and rolling interactions on the vascular wall during inflammatory responses *in vivo* (Figure 3).

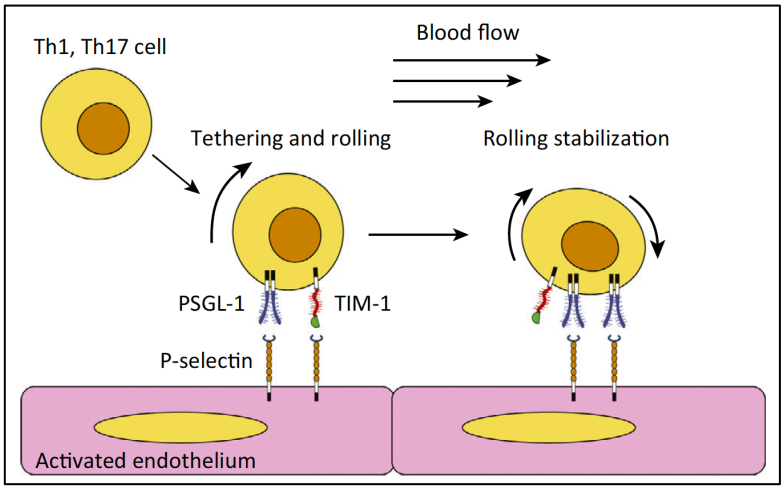


Figure 3. Schematic representation of activated T cell interaction with the inflamed vessel wall. P-selectin, expressed on the activated endothelium, captures Th1 and Th17 cells on the vessel wall through binding to PSGL-1 and TIM-1. These molecules concurrently control T cell attachment and initial rolling on the vascular endothelium; subsequent rolling stabilization appears to require only PSGL-1[21].

1.4 ROLE OF TIM-1 IN EXPERIMENTAL MODELS OF INFLAMMATORY DISEASES

The importance of TIM-1 in the induction and development of several inflammatory and autoimmune diseases have been extensively investigated. Indeed, two different TIM-1 specific monoclonal antibodies have been described: the agonist 3B3 antibody, that binds TIM-1 with high affinity and the blocking RMT1-10 antibody that binds TIM-1 with lower affinity[7, 23]. Depending on the TIM-1 mAb, which both target the TIM-1 IgV domain, there are opposing effects on T cell activation and tolerance in vivo, probably due to a different signaling pathway activated downstream of TIM-1. Administration of the higher affinity 3B3 mAb during the induction of experimental autoimmune encephalomyelitis (EAE), an autoimmune inflammatory pathology of the central nervous system (CNS) well-known as the model of human Multiple Sclerosis (MS), strongly enhances pathogenic Th1 and Th17 responses and increases the severity of EAE. By contrast, the low-affinity RMT1-10 mAb increases Th2 responses and inhibits antigen specific T cell proliferation, the production of Th1/Th17 type cytokines and the development of EAE[7]. Similarly, in transplant model studies in vivo administration of agonist 3B3 mAb overcomes the protective effects of anti-CD154 mAb (commonly used for long-term survival of allogeneic transplant) and causes allograft rejection, by reducing Foxp3+ Treg cell development and enhancing Th1/Th17 pathogenic responses[22]. Moreover, administration of RMT1-10 specifically inhibits IL-17-producing CD8+ cytotoxic T cells that mediate resistance to tolerance induction[53]. In an experimental model of liver ischemia-reperfusion injury (IRI), preventive treatment with RMT1-10 antibody has been shown to ameliorate the hepatocellular damage and to rescue liver function by reducing the infiltration of neutrophil, T lymphocytes and macrophages in the liver and the production of proinflammatory signals, and decreasing hepatocyte apoptosis[38]. Similar results were also described in a model of renal IRI[54].

As previously reported, *Tim-1* gene is associated with several atopic diseases, as the locus co-segregates with those genes encoding for Th2-type cytokines, such as IL-4 and IL-13. Notably, one hallmark of asthma is lung inflammation and is partially caused by dysregulated Th2 cytokine expression. Interestingly, it was reported that the preventive treatment with anti-TIM-1 antibody in a model of

ovalbumin (OVA)-induced lung inflammation had a beneficial effect, diminishing the infiltration of inflammatory leukocytes and mucus deposition, and strongly decreasing the production of Th2-type cytokines by OVA-specific T cells[52, 55]. Surprisingly, TIM-1 deficient mice normally developed an airway hyperactivity (AHR) reaction, although they did show a small but significant decrease in cell infiltration[56], raising some controversies about TIM-1 role in the development of allergic asthma. However, in a humanized mouse model of experimental asthma, therapeutic treatment with a newly generated anti-human TIM-1 antibody ameliorated inflammation and airway hyperresponsiveness, mediated again by suppression of Th2 cell proliferation and cytokine production[57].

1.5 TIM-1 IN HUMAN INFLAMMATORY PATHOLOGIES

The contribution of TIM-1 in acute and chronic inflammatory diseases is now clearly emerging also in humans. *Havcr1* is highly polymorphic and displays single nucleotide or insertion/deletion variations that primarily affect the mucin domain[58]. Association analysis of TIM-1 in patients suffering from atopic diseases proved that some allelic polymorphisms are protective against asthma and allergy in those patients previously infected with the Hepatitis A Virus (HAV)[59]. However, some other polymorphisms in the TIM-1 gene are associated with a higher severity of HAV-induced hepatitis[60], directly correlating the disease clinical course and TIM-1 sequence. In fact, patients suffering from severe HAV-induced hepatitis exposed on their hepatic cells the longest polymorphism of TIM-1[60]. A longer TIM-1 protein gives hepatocytes the capacity to bind pathogens more efficiently, but renders NKT cells more cytotoxic towards HAV-infected liver increasing the disease severity.

In addition to binding to HAV virus, TIM-1 was also described to function as a cellular receptor for several viruses, such as Zaire Ebolavirus and Lake Victoria Marburgvirus[46], and Dengue virus[61], promoting their endocytosis.

It has been also reported that TIM family is related to organ-specific inflammatory and autoimmune diseases[6]. Particularly, in patients with systemic lupus erythematosus (SLE), a heterogeneous autoimmune disease, increased TIM-1 mRNA level has been observed in peripheral blood mononuclear cells, and this also correlated with a higher production of IL-10[62], indicating a role of TIM-1 in the

development of SLE. Furthermore, polymorphisms in the exon 4 encoding for the TIM-1 mucin domain have also been associated with susceptibility to rheumatoid arthritis[63, 64].

Interestingly, TIM-1 expression was also described in MS. For the first time, Khademi and colleagues demonstrated that TIM-1 mRNA is upregulated in cerebrospinal fluid (CSF) mononuclear cells of patients with MS in clinical remission, suggesting a possible involvement of TIM-1 in this phase of the disease[65]. Moreover, TIM-1 was found expressed in several leukocyte populations, including CD4+ Th2, CD8+, NK and NKT cells in MS[65].

Of note, TIM-1 was detected in most patients with primary CNS lymphoma (PCNSL), an uncommon form of non-Hodgkin's lymphoma arising within the CNS. Interestingly, a soluble form of TIM-1 (sTIM-1) was found in the CSF of PCNSL patients in the active phase of the disease, suggesting that sTIM-1 in the CSF may be useful for the diagnosis or the evaluation of disease course of PCNSL[66]. The sTIM-1 was also measured in urine and it is currently used as a new diagnostic marker for kidney injury[67, 68]. In fact, under normal conditions, TIM-1 shows very restricted expression in healthy tissue, while it is significantly upregulated on the surface of renal epithelial cells and shed after tissue damage or in patients with renal cell carcinoma. The release of the TIM-1 ectodomain is mediated by members of a disintegrin and metalloprotease (ADAM) 10 and ADAM17 and accelerated by the activation of p38/MAP kinase signaling pathway[69, 70].

The strong upregulation of TIM-1 glycoprotein was also observed in several human cancers, most notably in renal and ovarian carcinomas, representing a promising target for new biological therapies. Recently, a fully human monoclonal IgG1 antibody (CDX-014) against the extracellular domain of TIM-1 has been developed as a possible therapeutic approach. It is rapidly internalized by target cells and has therefore been conjugated to a potent cytotoxin like monomethyl auristatin E to express antitumor activity. The drug binds intracellular tubulin, inhibiting its polymerization and resulting in G2 phase arrest and cell apoptosis[71]. Anti-TIM-1 antibody-drug showed promising results on mice models against potentially all TIM-1-expressing tumors, which led to clinical trials now in phase I[71].

2. NEUTROPHILS

2.1 NEUTROPHIL BIOLOGY

Neutrophils, also known as polymorphonuclear (PMN) leukocytes, are the first line of innate immune defense playing a crucial role for tissue homeostasis maintenance[72] and for the elimination of pathogens and self-components, such as nucleic acids and products of sterile tissue damage[73].

Neutrophils are the predominant immune cell population in the blood, representing in humans the 50–70% and in mice the 10–25% of blood leukocytes[74]. Neutrophils are relatively short-lived cells displaying a half-life of 8-12 hours in the circulation, and they can survive up to 1-2 days in mouse tissues[75, 76]. However, under homeostatic conditions, human neutrophils can have a more prolonged circulatory life span up to 5 days[76, 77]. Nevertheless, neutrophils that are activated during the inflammatory response increase their longevity in order to carry out their effector activities in the target tissue[78].

Neutrophils are generated in the bone marrow of adult subjects from the hematopoietic stem cell niche[77] within approximately 5 days in a process called granulopoiesis[79]. At the steady-state, neutrophils are generated in the bone marrow at a rate of 5×10^{10}–10×10^{10} cells per day, but it can increase to 10^{12} cells daily during infection[80]. Despite this impressive turnover, the number of neutrophils in circulation remains relatively constant thanks to a fine balance between their production and mobilization, and their elimination[81]. During granulopoiesis bone marrow neutrophil lineage cells can be divided into three compartments, according to their degrees of development and maturation: (i) the stem cell pool, composed of hematopoietic stem cells and pluripotent progenitors; (ii) the mitotic pool consisting of active proliferating, lineage-committed myeloblasts, promyelocytes, and myelocytes, that are in an intermediate differentiated degree of maturation; (iii) the post-mitotic pool composed of metamyelocytes, band cells and fully differentiated and mature neutrophils[82] (Figure 4). During differentiation, the developing neutrophil goes through morphological changes, such as condensation and hyper-segmentation of the nucleus, cytoplasmic granules formation, and, also, the expression of various receptors[82]. All of them are considered morphological markers useful to distinguish mature from immature neutrophils[83]. Several

cytokines and growth factors released by hematopoietic and stromal cells finely regulate the process of granulopoiesis. Granulocyte colony-stimulating factor (G-CSF) is a crucial factor as it regulates neutrophil life cycle at multiple levels, influencing cell proliferation, survival, differentiation and promoting neutrophil trafficking/mobilization out of the bone marrow in the circulation[82]. Moreover, granulocyte macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), IL-3, IL-6, FMS-like tyrosine kinase 3 ligand (FLT3-L) are involved in neutrophil development[84]. Recently, myeloid cell-derived reactive oxygen species (ROS) are also shown to externally regulate and elicit myeloid progenitor proliferation and differentiation during infection and sterile inflammation[85].

Retention or release of mature neutrophils is tightly controlled since only 1-2% of neutrophils circulate in the blood stream under normal homeostatic conditions, and it depends on the expression of two C-X-C-motif chemokine receptors (CXCRs), CXCR2 and CXCR4 (Figure 4). Neutrophil storage is maintained through the interaction between CXCR4 expressed on neutrophils and CXC ligand (CXCL) 12 (also known as stromal cell-derived factor 1 (SDF-1)) produced by hematopoietic stem cells and bone marrow stromal cells[86]. G-CSF induces neutrophils exit from the bone marrow by downregulating the CXCR4 expression and favoring upregulation of CXCR2 on neutrophils and inhibiting the production of CXCL12 on stromal cells[87]. In addition, G-CSF prompts the release of neutrophils by inducing the expression of CXCR2 ligands, such as CXCL1, CXCL2, CXCL5 and CXCL8 (in humans) on megakaryocytes and endothelial cells outside the bone marrow when neutrophils need to be mobilized into the blood[88, 89]. Finally, mature neutrophils leave hematopoietic parenchyma for blood stream preferentially by transcellular migration through tight-fitting pores of sinusoidal endothelial cells[90].

Once released from the bone marrow, neutrophils disseminate in the periphery and passively circulate to patrol the organism and are rapidly recruited in infected or injured tissues in the presence of danger signals such as circulating pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs) and several cytokines and chemokines (Figure 4). Nevertheless, a fraction of circulating neutrophils disappears from the bloodstream slowly transiting in the vascular compartments of the spleen, liver, bone-marrow, lung and some interstitial tissues[80]. This subset of neutrophils constitutes the marginated pools.

Recently, some studies performed in mice and human volunteers demonstrated that radiolabeled circulating neutrophils can be captured and cleared by other compartments besides the bone marrow, that are reticular endothelial macrophages in the spleen and liver[86, 91]. However, in normal conditions, bone marrow remains the major compartment for neutrophil clearance[86]. Senescent pre-apoptotic neutrophils in blood display high expression of CXCR4 and low levels of CXCR2, this phenotype allows them to migrate to the bone marrow in a CXCR4/CXCL12 dependent fashion for final clearance[92]. Interestingly, it was shown that homing of aged neutrophils back to the bone marrow influences the circadian release of hematopoietic progenitors in the circulation during homeostasis[93], particularly by stimulating resident stromal macrophages-dependent release of G-CSF[94].

Neutrophil distribution in the body can be affected by several environmental factors, including drugs, physical exercise, prolonged inflammation and infections modulating their maturation and activation status or extending their lifespan[80]. All these conditions contribute to rapidly mobilize neutrophils from bone marrow increasing their circulating number even 10 times within a matter of hours. Also chemokines that are released locally at sites of inflammation are able to promote neutrophil mobilization and recruitment into tissues. In addition, it was shown that systemic infections and prolonged inflammatory responses induce a process called "emergency" granulopoiesis, consisting of an accelerated production of neutrophils and prematurely release of immature cells from the post-mitotic pool in the blood stream[95]. As they have not properly completed their differentiation, immature circulating neutrophils do not contain an appropriate equipment of granules[96] resulting in a weak ROS production, a lower quantity of antimicrobial peptides and a limited phagocytosis, chemotaxis and tissue migration[95, 97]. However, these immature cells are still able to release cytokines that act back on the bone marrow stimulating the production of neutrophils, generating a loop that maintains a condition of low-grade inflammation.

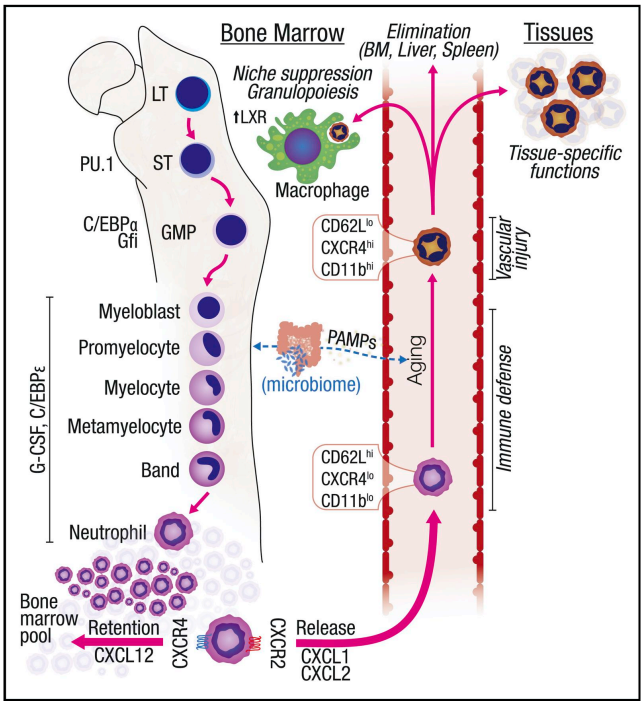


Figure 4. **Neutrophil Life-Cycle**. Neutrophils are produced in the bone marrow through granulopoiesis, starting from the stem cells and granulocyte-macrophage progenitors (GMPs) to committed progenitors that finally generate mature neutrophils. Newly generated neutrophils are released in the circulation through the modulation of CXCR4 and CXCR2. Once in the bloodstream, neutrophils quickly change phenotype and go through a process of aging. After about 12 hours, neutrophils are ready to leave the bloodstream and are targeted to the bone marrow, liver, and spleen for elimination. In the bone marrow and in some tissues, neutrophils are phagocytosed by resident macrophages to maintain homeostasis, alternatively, neutrophils might infiltrate other tissues to execute tissue-specific functions (Adapted from Nicolàs-Avila JA et al., *Immunity*, 2017)[98].

2.2 NEUTROPHIL PHENOTYPES AND HETEROGENEITY

Neutrophils are heterogeneous transcriptionally active cells[99] producing a wide panel of cytokines[100], and are able to modulate the activities of neighboring cells through cell-cell contact or soluble mediators. They show an unexpected plasticity in term of phenotype, trafficking, activity and function thus expanding their field of actions from homeostasis maintenance to the pathogenic inflammation and tissue damage that, if not resolved, could develop to chronic diseases as well as autoimmune reactions[101].

Currently, it is well established that neutrophils are a heterogeneous population of cells that rapidly change their characteristics and behavior when they get activated, age, or enter new environments. This heterogeneity is a fundamental property for

neutrophils as it confers them specific functions and the ability to adapt to environmental changes under homeostatic and disease conditions[102].

Neutrophil subtypes are defined by several characteristics such as expression of surface markers, level of maturation, density, morphology and anatomical site. When mature neutrophils leave the bone marrow and enter the peripheral circulation, they downregulate the surface expression of integrin α4β1 or very late antigen-4 (VLA-4 or CD49d) and CXCR4, while they upregulate CXCR2 and TLR-4[103]. Mature human neutrophils express the neutrophil lineage markers CD15 and CD66b, along with high surface levels of CD16, CD62L (L-selectin) and CD10 (neutral endopeptidase)[104, 105].

Neutrophil activation by cues derived from sites of inflammation results in the exposure of distinct surface molecules from intracellular pools, contributing to change their phenotype. Indeed, activated neutrophils display increased expression of integrin CD11b/CD18 (or Mac-1)[106], complement surface receptors such as CR1 (CD35), which mediates the binding and phagocytosis of C3b-coated particles and immune complexes[107]. Some other surface markers such as CD62L are dramatically downregulated or lost through rapid shedding in activated neutrophils[108].

The mature neutrophils that are normal present in the circulation are defined as Ly6G+ CXCR2+ CD101+, whereas the immature fraction, that is nearly absent in the blood in physiological conditions, are recognized as Ly6G*lo*/+ CXCR2- CD101-[97]. Particularly, aged neutrophils have distinct properties from newly formed neutrophils, that is they upregulate the chemokine receptor CXCR4, which allows them to turn back to the bone marrow for clearance, and they downregulate CD62L and CD47, an inhibitor of phagocytosis[109]. In mice, the neutrophil marker Ly6G is also reduced in senescent cells[110]. In addition, some authors recently reported increased surface expression of CD11c, CD24, CD45 and molecules involved in cell migration and intercellular interactions such as CD11b and ICAM-1 in senescent neutrophils[111]. Functionally, these cells also display higher expression of several activation pathways distinct from activated neutrophils, including signaling via TLRs, NOD like receptors, and the transcription factor NF-κB, greater ROS production, NETs formation[109], suggesting increased efficacy to migrate into sites of inflammation[112].

Regarding the anatomical sites of neutrophil accumulation, the lung was observed to be populated by large numbers of neutrophils in the steady state[113]. These cells

are strategically positioned in the lungs to either supply the circulation or respond to injury, and their retention in the lung is mediated by active signaling through CXCR4. In fact, treatment with CXCR4 antagonists leads to fast release of neutrophils into the blood stream[114]. Additionally, neutrophil entry into lymph nodes relies on receptors that are upregulated in aged neutrophils such as CD11b or CXCR4[115]. Interestingly, it was reported in the context of infection that neutrophils can enter lymph nodes from the peripheral circulation by crossing high endothelial venules (HEVs), in a similar manner like naïve T and B cells[116].

Overall, these data suggest that neutrophils are multifaced heterogeneous cells, playing complex roles, from supporting homeostasis to pathological tissue damage. Several factors including the anatomical site and the inflammatory environment together with the expression of surface markers, determine neutrophil phenotype and functionality.

2.3 NEUTROPHIL TRAFFICKING AND EFFECTOR FUNCTIONS

Peripheral neutrophils reach the target tissue through the process known as leukocyte adhesion cascade, attracted by local chemokines that orchestrate their adhesion, transmigration and chemotaxis[117] (Figure 5). Vascular endothelial cells close to the affected site get activated by pro-inflammatory cues, and they upregulate adhesion receptors such as E- and P-selectins that bind glycoprotein ligands on neutrophils supporting their rolling on the endothelium. Chemokine binding to neutrophil receptors leads to activation of integrins such as lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1); subsequently, interactions of neutrophil integrins to their endothelial counter-ligands such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 cause firm arrest of the neutrophil to the vessel wall. After adhesion, neutrophils start scanning the endothelium by means of their pseudopods looking for an appropriate site of transmigration, that are mainly endothelial cell-cell junctions (paracellular transmigration)[118]. Once extravasated, neutrophils follow gradients of chemoattractans, for instance formyl-methionyl-leucyl-phenylalanine (fMLF) and the anaphylatoxin C5a, they and reach the site of inflammation where local microbes and/or tissue stress cues activate specific effector functions of neutrophils[73, 119].

Of note, circulating neutrophils are quiescent, and their activation is a crucial step to generate the inflammatory response. Moreover, neutrophils are relatively nonresponsive to a single stimulus, but exposure to a first stimulus (e.g., lipopolysaccharide, tumor necrosis factor (TNF), chemokines, growth factors etc.) triggers their cellular response to a second stimulus[83]. This effect, known as neutrophil priming, is a reversible process that permits rapid neutrophil activation and potentiates their effector functions. Thus, during vascular transmigration neutrophils get partially activated (priming state) typically by the presence of cytokines, chemokines or bioactive lipids, then once entered the inflamed site neutrophils become fully activated and explicate their antibacterial effector functions: phagocytosis, release of neutrophil extracellular traps (NETs)[120], production of ROS, degranulation of antimicrobial enzymes (myeloperoxidase, defensins, matrix metalloproteinases, serine proteases) and secretion of pro-inflammatory cytokines and chemotactic factors[82, 121] (Figure 5). Nevertheless, through inflammasome activation by chemokines and DAMPs signals, neutrophil infiltration also occurs during sterile conditions where they mount acute inflammatory reactions[122].

At the site of inflammation, once entered the tissues and carried out their functions, many neutrophils undergo apoptosis and are then cleared by resident macrophages and DCs leading to the resolution of the inflammatory response and the return to tissue homeostasis[92]. Neutrophils can also actively contribute to resolution by local degradation of inflammatory cytokines, DAMPs cleanness and secretion of anti-inflammatory cytokines including IL-1Ra, IL-10, IL-1β, IL-6[123].

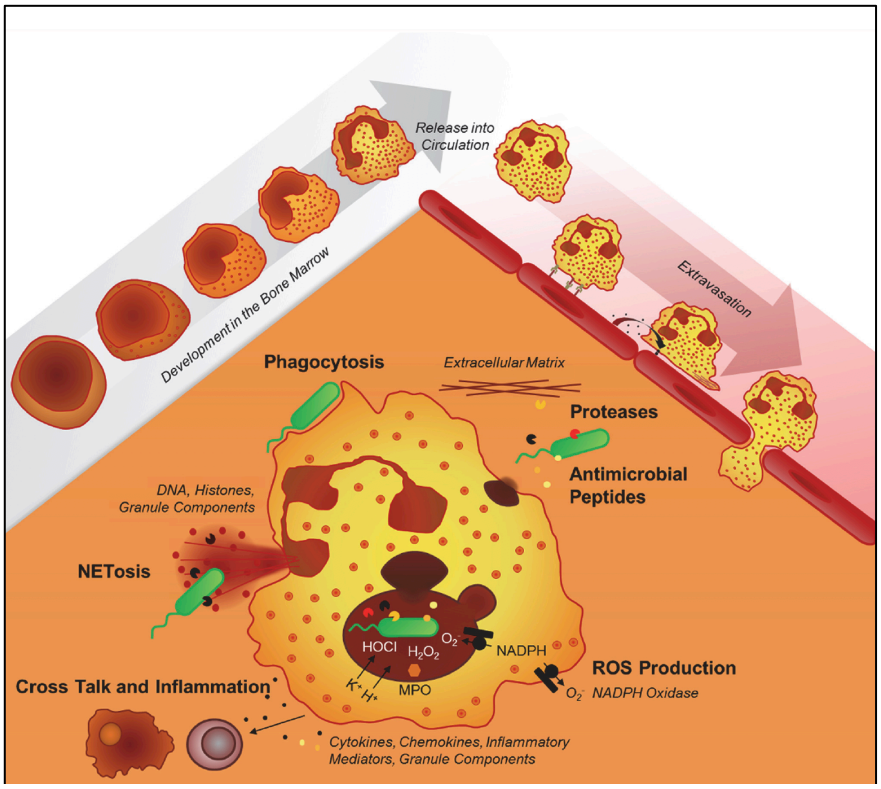


Figure 5. Neutrophil trafficking and effector mechanisms. In response to infection or sterile inflammation, circulating neutrophils display surface molecules that facilitate their interaction with the activated endothelium and their extravasation. Once in the interstitial space, neutrophils follow chemotactic gradients formed by pathogen-derived molecules or inflammatory mediators. Neutrophils display a battery of defense mechanisms, including the internalization of pathogens for intracellular killing, the release of proteases and ROS that generate a hostile environment and contribute to the neutrophil microbicidal function, the release of NETs used to trap bacteria, and the release of various granule components, mainly proteases, oxidants, antimicrobial peptides. During the inflammatory response, neutrophils also release mediators that contribute to shaping the subsequent immune response by modulating adaptive immune cell function[82, 101].

Another layer of complexity has been added to the classical concept of unidirectional neutrophil migration from the blood to the target tissue. Recent studies reported the ability of neutrophil to turn back to the blood stream from peripheral organs. Neutrophils undergoing reverse transmigration exhibit a pro-inflammatory phenotype, characterized by a high ICAM-1 expression, whereas endothelial cells show downregulation of the junctional adhesion molecule C (JAM-C). Interestingly, reverse-transmigrating cells were found to be more resistant to apoptosis, thus their prolonged lifespan could modulate their phenotype and function, contributing to neutrophil heterogeneity[124, 125]. However, the purpose of this process is still unclear. On one hand, it could help to keep neutrophils alive when they are not needed to fight infection; on the other, these cells re-entering the

circulation could disseminate inflammation into other organs, eventually extending tissue damage and/or chronic inflammation[73]. An alternative route that activated neutrophils may use to leave infected tissues is to migrate to secondary lymphoid organs through lymphatic and circulatory systems[126, 127]. Neutrophils that exit from tissues to the blood or emigrate into secondary lymphoid tissues seem to have a more prolonged lifespan and a different phenotype contributing to their heterogeneity.

2.4 NEUTROPHIL CROSS-TALK WITH IMMUNE CELLS

The secretion of cytokines with pro- and anti-inflammatory or immunoregulatory properties confer neutrophils the ability to attract and modulate the activation of other innate and adaptive immune cells such as monocytes, macrophages, DCs, NK cells, T and B subsets[76, 128].

Many products secreted by neutrophils, including lactoferrin, α-defensins and CCL3, are required for rapid DC recruitment to sites of infection and direct interaction of neutrophils with DCs promotes maturation of DCs into more effective antigen-presenting cells and provides DCs access to neutrophil-captured pathogen products[129, 130]. NETs induce IFN-α production from plasmacytoid DCs, which in turn drives formation of self-reactive lymphocytes that recognize chromatin–peptides complexes, leading to production of autoantibodies. IFN-α also, stimulates neutrophils, further promoting NET formation and thereby amplifying the inflammatory response generated by neutrophils and DCs[76].

Neutrophils and T cells modulate each other at several levels. It is supposed that neutrophils may directly function as APCs. In the synovial fluid of rheumatoid arthritis patients, neutrophils were found to express high levels of major histocompatibility complex (MHC)-II and costimulatory molecules such as CD80 and CD89 and they were suggested to stimulate T cell proliferation and Th1/Th17 differentiation[131]. Furthermore, neutrophils can also carry antigens to lymph nodes by migrating through the lymphatic system, where they either directly present the antigen to T cells or deliver it to DCs[132]. The blockade of neutrophils migration to the lymph nodes was indeed shown to limit T cell proliferation suggesting that these cells may act as APCs in the initiation of adaptive immune responses in mouse models[133].

Moreover, neutrophils are major producers of the cytokines B lymphocyte stimulator (BLyS) and A proliferation-inducing ligand (APRIL), which are required for B-cell survival and activation[134]. Interestingly, it was shown that circulating neutrophils can also colonized the spleen white pulp, particularly in the peri-marginal zone at the steady state. These splenic neutrophils defined B cell-helper neutrophils (N_{BH}) are able to activate B cells in the marginal zone promoting immunoglobulin class switching, somatic hypermutation, and production of T cell-independent antibody by release of BLyS, IL-21 and APRIL[135]. In turn, the subset of splenic B cells produces GM-CSF[136] providing differentiation signals for splenic neutrophils[137].

Numerous interactions between neutrophils and NK cells have recently been defined. Although this cross talk remains to be determined in the steady state, it was shown that during infectious disease release of neutrophil-derived IL-18 cytokine directly activates NK production of IFN-γ in mice[138]. Besides, colocalization of neutrophils, NK cells, and DCs has also been observed in inflammatory lesions in Crohn's disease patients[139]. Importantly, many NK cell–derived cytokines, such as IFN-γ and GM-CSF, act priming or prolonging neutrophil survival, thereby enhancing inflammatory responses[76].

Murine and human studies demonstrate the existence of a distinct neutrophil phenotype, who were termed granulocytic myeloid-derived suppressor cells (MDSCs) in analogy to monocyte subsets, in cancer patients, that inhibit T and NK cell proliferation probably involving arginase, ROS, Mac-1, and STAT3[140, 141, 142]. Importantly, in patients with systemic inflammation another subset of neutrophils was found to suppress T cell proliferation through a Mac-1-dependent mechanism[143].

Interactions between neutrophils and macrophages are important in both the initiation and resolution of the inflammatory response. Once recruited at sites of inflammation, neutrophils attract the migration of monocytes by secreting chemokines (e.g. CCL2, CCL3, CCL19, CCL20) and granule proteins (e.g. S100A and various antimicrobial peptides). Neutrophil primary granule proteins also enhance the antimicrobial activity of macrophages by increasing their phagocytosis ability[144]. During the resolution of inflammation, clearance of apoptotic neutrophils by macrophages decreases the production of macrophage IL-23, which results in diminished IL-17 secretion by T cells and hence reduced G-CSF and neutrophil

production. Phagocytosis of apoptotic neutrophils by macrophages also stimulates the macrophages themselves to produce IL-10 and downmodulate IL-12, polarizing towards an M2-like phenotype, to promote tissue repair during resolution of inflammation[145].

3. INFLAMMATION

3.1 THE INFLAMMATORY RESPONSE: AN OVERVIEW

Several mechanisms occur to control the body homeostasis in response to adverse stimuli, ensuring maintenance of an optimal tissue function[146]. In normal conditions, tissue is in a basal state maintained by the availability of nutrients, oxygen and growth factors, a correct osmolarity, temperature and so on. A change in any of these vital parameters induces a stress response, that affords a sort of transient cellular adaptation to the new condition, or a more sustained adaptive change. Once homeostasis is disturbed, and in response to certain stimuli, our body may launch a process that we define as inflammation, characterized by macroscopic signs (i.e., redness, swelling, fever, and pain) and underlined by microvascular, cellular, and molecular events that culminate in leukocyte accumulation in affected tissue areas (from infections, tissue damage, etc.)[147, 148].

The inflammatory process can be divided into three main phases[148]: (i) onset, when pro-inflammatory soluble mediators initiate the inflammatory cascade; (ii) resolution, when another set of mediators dictate events that terminate the inflammatory process; and (iii) the post-resolution phase, when the affected tissue develops adaptive immunity and regains a status of "adapted homeostasis"[149]. If successful, the inflammatory response tends to progress from the onset to the post-resolution phase through a coordinated series of molecular and cellular events that lead to the restoration of tissue structure, organ function, and adapted homeostasis. Failed or impaired resolution may underpin the pathogenesis of various chronic inflammatory diseases.

A typical inflammatory response also consists of four components: inducers, sensors, mediators, and the target tissues[150]. Inducers are the signals that initiate the inflammatory response through their specific recognition by specialized sensors. Inducers can be exogenous, and comprise microbes, PAMPs, virulence factors and

non-microbial agents such as allergens, irritants, toxic compounds, or endogenous, which are signals released by stressed, malfunctioning or damaged tissues (PAMPS) or by disrupted basement membranes, epithelia and vascular endothelium[147]. Another class of endogenous agents relevant to chronic inflammation comprise advanced glycation end products (AGEs) and oxidized lipoproteins, such as high-density and low-density lipoproteins. These molecules are mainly recognized by AGE receptor (RAGE) and TLRs, particularly TLR-4[151]. Sensors activation elicits the production of specific sets of mediators. Many molecules have been described as mediators of inflammation so far, and new players are still being continuously discovered[148]. Cellular mediators can be derived from plasma proteins or produced by specialized leukocytes (tissue-resident macrophages and mast cells) or by tissues themselves. Some mediators are preformed and stored in intracellular granules of mast cells, basophils, platelets and neutrophils, while others circulate as inactive precursors in the plasma. Inflammatory mediators comprise vasoactive agents, fragments of complement components (C3a, C4a, C5a), lipid mediators (platelet-activating factor or PAF), cytokines, chemokines and proteolytic enzymes (cathepsins, matrix metalloproteinases)[147, 148]. Inflammatory mediators, in turn, alter the functionality of many tissues and organs (which are the effectors of inflammation) allowing them to adapt to the conditions indicated by the particular inducer of inflammation.

The response to disturbances of the homeostasis can be managed at three levels by tissue cells, local action of resident immune cells and the systemic intervention of circulating leukocytes[147, 152] (Figure 6). If the change in a parameter is greater than the stress response can handle, the tissue cell undergoes apoptosis or necrosis. When the insults are not strong enough to cause cell death, a tissue autonomous inflammatory response take place, resulting in the upregulation of heat shock proteins, activation of autophagy, and also the production of inflammatory cytokines and chemokines that trigger the intervention of resident immune cells of the tissue, such as resident macrophages, which in turn may release cytokines and growth factors to further promote the repair of the damage and restore the basal homeostasis. The difference between the cell autonomous response and the intervention of local immune cells is that the first type of response promotes the survival of itself, whereas the latter assists in the survival of tissue cells and ensures the integrity of tissue structure and functionalities. Thus, very mild stress

conditions, where the damage is restricted to a limited number of cells, can be handled by tissue-resident cells with minimal disturbance of local or systemic immune cells, resulting in a local tissue inflammatory response[153] (Figure 6). However, if the level of the tissue stress exceeds the reparatory capacity of resident cells, they react by producing and releasing additional pro-inflammatory cytokines and chemokines in the bloodstream to recruit circulating leukocytes, including neutrophils[154, 155]. This reaction leads to a systemic inflammatory response. Moreover, if the abnormal conditions are transient, then a successful acute inflammatory response returns the system to the basal homeostatic condition. By contrast, if they are sustained over time, the ongoing inflammatory state leads the tissue to adapt to the stressful condition. This response is called parainflammation (Figure 6). Finally, a dysregulated prolonged parainflammatory condition might be responsible for chronic inflammatory states that have been associated with many modern human diseases (Figure 6)[148, 153].

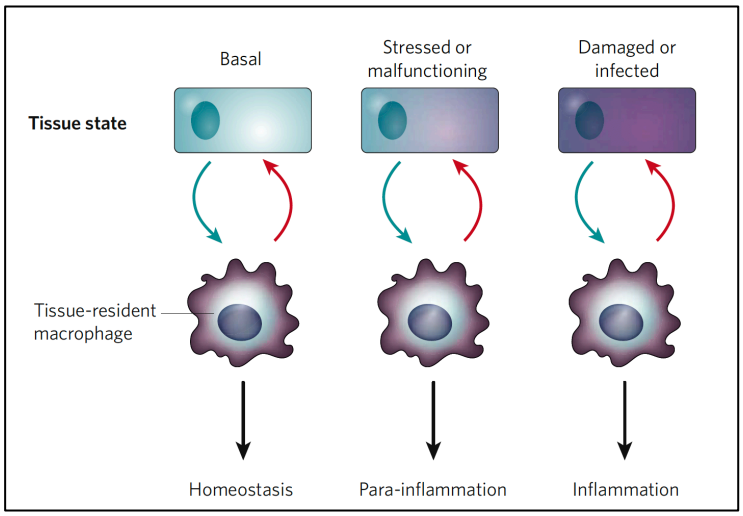


Figure 6. Models of adaptation and maintenance of tissue homeostasis. Tissues can exist in different graded states ranging from basal, stressed/malfunctioning to damaged. The state affects the mode of maintenance of tissue homeostasis or activates adaptive responses engaged by tissue-resident macrophages and, in some tissues, by other types of leukocyte. Under normal conditions, tissue-resident macrophages maintain tissue homeostasis by removing dead cells and other debris and by producing growth factors. At one extreme of the range of responses is inflammation, which follows infection or tissue damage. By contrast, tissue stress or malfunction induces parainflammation, which helps a tissue to adapt to the noxious conditions and restore tissue functionality. Dysregulated para-inflammation might be responsible for the chronic inflammatory states that are associated with many modern human diseases[147].

3.2 PARAINFLAMMATION AND NEUTROPHILS

Parainflammation, also named low-grade chronic inflammation, is an adaptive response of the immune system to low levels of noxious agents or tissue stress conditions, that aims to maintain homeostasis and restore tissue functionality[147, 156, 157]. The induction of a parainflammatory response does not require overt tissue injury, instead, it is switched on by tissue malfunction, which can result from mutations or environmental factors. Several human diseases are characterized by this low-grade chronic inflammatory state, such as obesity, type 2 diabetes, atherosclerosis, asthma and age-related neurodegenerative diseases, including Alzheimer's and Parkinson diseases[158, 159, 160, 161, 162]. Despite their precise role is still poorly understood, neutrophils are reported to contribute to the pathogenesis of these diseases.

Several studies reported a key role of neutrophils in chronic low-grade adipose tissue inflammation, that causes metabolic syndromes. Among them, obesity and the consequent development of insulin resistance is one of the major causes of type 2 diabetes. In this context, neutrophils have been shown to be rapidly recruited to the adipose tissue after the initiation of a high-fat diet in mice[163] and that leukotriene B4 (LTB4) receptor 1 or CXCR2 deficiency, both fundamental for neutrophil recruitment during acute inflammation, reduces phlogosis in adipose tissue and protects against insulin resistance[164]. Moreover, neutrophils were found in human and mouse atherosclerotic lesions at all stages of the disease[165, 166]. Interestingly, while neutrophil counts in humans are predictors of a cardiovascular event, neutrophil counts in mice are shown to correlate with the sizes of initial lesions[167]. Moreover, it is widely accepted that neutrophils, in cooperation with monocytes and platelets, contribute to thrombosis in the context of cardiovascular diseases such as myocardial infarction[168] as revealed by the crawling of blood neutrophils adhering to venous endothelium and the release of NETs[169], that contribute to sustain coagulation[170]. During respiratory chronic diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), inflammation is maintained by the constant influx of neutrophils and persistent NETs release in airways which contribute to the reduction of pulmonary function[171, 172]. Besides, neutrophils recruited from the blood into the lungs of CF patients undergo profound reprogramming, as they express higher levels of MHC II, CD80, the

chemoattractant receptor of Th2 cells (CD294)[173], and they show metabolic changes including marked increases in glucose, amino acid and phosphate transporters, as compared with circulating neutrophils[174].

Moreover, it has been hypothesized that low-grade chronic inflammation is also involved in age-related degenerative diseases, including Parkinson's Disease (PD)[175]. PD is a long-term CNS disorder associated with Lewy bodies and loss of dopaminergic neurons in the substantia nigra of the midbrain. Despite a recent study showed normal peripheral immune responses in term of neutrophils and lymphocyte chemotaxis, phagocytic capacity, as well as NK cytotoxic activity in PD patients[176], myeloperoxidase (MPO), a marker enzyme for neutrophils, was found significantly upregulated in correspondence to damaged areas of the midbrain in both human PD and experimental mouse model[177]. Another example of pathology characterized by systemic low-grade inflammation is amyotrophic lateral sclerosis (ALS), a motor neuron degeneration disorder[178]. Recent studies on ALS blood expression profiling identified low-grade neutrophilia and hypoxia as new biomarkers for the disease[179]. Interestingly, it was demonstrated in a rat model of ALS that mast cells and neutrophils abundantly accumulate around motor axons in the major affected areas by the disease, indicating that leukocyte infiltration extends along the entire peripheral motor pathway[180].

Thus, research advances in neutrophil biology and elucidation of the mechanisms of action of neutrophil in inflammatory diseases will hopefully not only better define how neutrophils contribute to the disease but also identify potential novel therapeutic targets for the treatment of several inflammatory diseases, including autoimmune and neurodegenerative disorders.

4. ALZHEIMER'S DISEASE

4.1. ALZHEIMER'S DISEASE: AN INFLAMMATORY NEURODEGENERATIVE PATHOLOGY

Alzheimer's disease (AD) is the most common neurodegenerative cause of dementia in the elderly, affecting more than 35 million people worldwide[181, 182]. The current prevalence is 7% under 65 years of age and raises up to 40% over 80, with a weak predominance in women, depending on their longer life expectancy. In

Italy, more than a million citizens are affected by dementia, and 600.000 of them are suffering of AD, with an annual outlay of more than 6 billion euros of social sanitary costs[183].

AD is usually sporadic and commonly occurs in aged people, instead the genetic or familial variants occur before 60 years of age. Both the familial and the sporadic forms of AD share a common phenotype converging towards similar neuropsychiatric symptoms, emotional disturbance and the progressive impairment of daily activity, resulting in dependence, disability and mortality[182]. Till now the exact etiology of AD remains unclear and there is currently neither a cure nor adequate clinical treatment available for patients.

In AD three stages of dementia can be detected: mild, moderate and severe (Figure 7). Memory loss is the first visible sign and the main feature of mild cognitive impairment, which is an initial, transitional clinical phase between normal aging brain and AD. It is characterized by a progressive cognitive impairment such as attention deficits, language disturbance and personality/behavior changes[184]. Diagnosis is often made at this stage. The moderate stage is typically the longest one where the clinical features of the disease become more pronounced. Typical of this stage is the struggle with conscious thoughts and the loss of judgment and impulse control, increased memory loss and confusion, and people may have also hallucinations and paranoia. The severe stage of dementia is characterized by the complete loss of identity and sense of self. The median time of survival from clinical onset is at least eight years[185].

From a histological point of view, with the progression of the disease neuronal loss together with ventricles enlargement and macroscopic atrophy occur in the most affected areas, that are the entorhinal cortex, hippocampus, amygdala and associative regions of the neocortex (Figure 7).

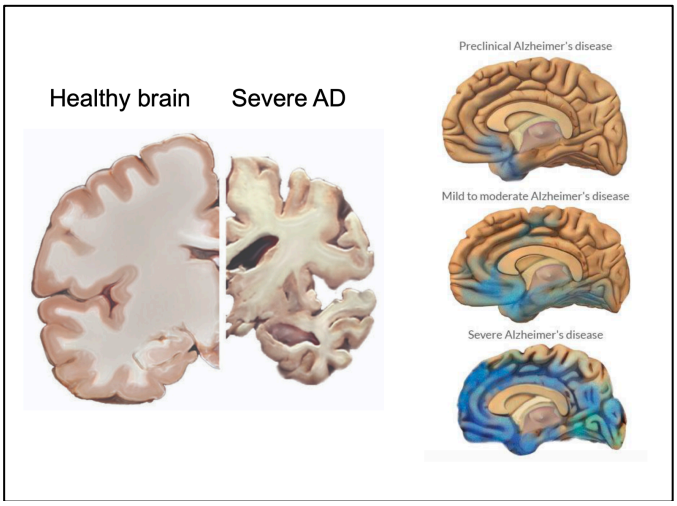


Figure 7. **Differences in healthy and AD brains.** Depiction of the extreme shrinkage in hippocampus and cortex occurring in the brain of severe AD patient, and ventricles enlargements (left). Different stages of AD (right), showing the areas of the brain (blue) that become progressively damaged during AD, from pre-clinical, to mild-moderate, to severe phase of the disease (right). The pathological alterations start decades before any visible sign or symptom of the disease and begin in the entorhinal cortex, spreading later on towards the hippocampus, a fundamental region for short- and long-term memories[186].

The neuropathological hallmarks of AD include extracellular accumulation of plaques containing β-amyloid (Aβ) peptides, intracellular aggregation of hyperphosphorylated tau to form neurofibrillary tangles (NFTs), angiopathy, oxidative stress, neuronal and synaptic loss resulting in brain atrophy, and a cerebral diffused low-grade sterile inflammation[181].

Amyloid plaques result from the extracellular accumulation and deposition of Aβ peptide, characterized by an abnormal β-pleated sheet configuration. Aβ is produced by the sequential proteolytic cleavage of the amyloid precursor protein (APP) by α-, β-, and γ-secretases[187], an enzymatic complex composed by presenilin 1 (PS1) or presenilin 2 (PS2) at its catalytic core. An imbalanced production, clearance and aggregation of peptides causes Aβ accumulation, and this excess may be the initiating factor in AD[181]. Monomers of Aβ are soluble structures that tend to assembly with one another leading to the formation of dimers or trimers[188] (Figure 8 a). Oligomers are normally found at an intracellular level but aggregate to form protofibrils in a process taking place in the extracellular matrix or on cell surfaces. Protofibrils are the precursors of amyloid fibrils. Amyloid fibrils (Figure 8 a) are highly insoluble and are composed by repetitive units of β-sheets; fibrils growth is directly proportioned to the number of Aβ monomers[189]. In the end,

amyloid fibrils can assemble to form extracellular amyloid plaques which deposit in the brain parenchyma during AD progression (Figure 8 b). Aβ oligomers are the most neurotoxic structures. The disease severity correlates with the levels of oligomers deposited in the brain, and not with the total Aβ burden. In addition, the more oligomers are smaller in size, the more they are neurotoxic as they can alter membrane permeability by forming pores[190] and bind to mature synapses on hippocampal and cortical neurons leading to the blockade of synaptic plasticity and consequently neuronal cell death[191].

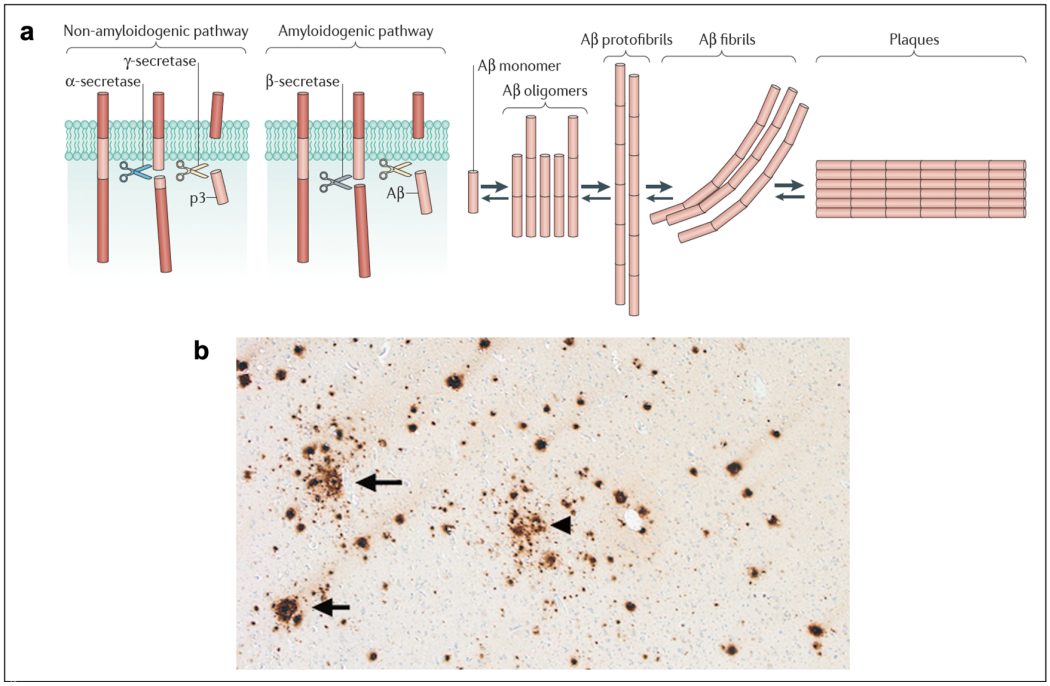


Figure 8. Generation of amyloid- β plaques during AD. (a) Schematic representation of the two pathways for APP processing and Aβ aggregation**.** APP (605-770 aa) can be processed through the amyloidogenic pathway, which depends on β-, and γ-secretases, or through the non-amyloidogenic pathway, that involves α- and γ-secretases. The first pathway leads to the Aβ peptide formation, whereas the other demolishes the production of Aβ protein. In physiological condition the major part of APP (90%) is processed by non-amyloidogenic pathway, while the remaining part (10%) follows the other pathway. **(b)** Microscopy of the left frontal lobe of AD patient showing numerous deposits of diffuse Aβ plaques (arrowheads) and ring-with-core plaques (arrows). Extracellular Aβ plaques are commonly classified in diffuse and dense-core based on their morphology. Diffuse plaques are usually non-neuritic and they are not considered for the pathological diagnosis of AD as present in physiological aging brain. On the other hand, dense-core plaques have neuritic properties and consist of foci of extracellular Aβ peptides associated with axonal and dendritic enlargements that contain degenerated mitochondria, organelles and lysosomes (dystrophic neurites). (Adapted from Heppner F et al., *Nat Rev Neurosci*, 2015)[192].

Neurofibrillary tangles (NFTs) are intraneuronal filamentous inclusions within pyramidal neurons whose main component is a hyperphosphorylated and aggregated form of tau protein[193] (Figure 9). Tau is normally a soluble axonal protein which, interacting with tubulin, promotes assembly and stability of microtubules and vesicles transport[194]. Hyperphosphorylated tau becomes insoluble and loses its affinity for microtubules, thus self-associates into paired helical filament structures (PHF). Like Aβ oligomers, also these intermediates of abnormal tau molecules are cytotoxic. In fact, PHF accumulation into pyramidal neurons leads to the formation of NFTs that destabilize microtubules, interfering with axonal flow, and leads to the destruction of a vital cell transport system causing neuronal degeneration and death. Although tau protein is the main component of NFTs, other proteins such as cholinesterase and ubiquitin have been identified[195]. Moreover, there is a correlation between tau accumulation and Aβ plaques: experimental evidence indicates that Aβ accumulation precedes and drives tau aggregation[181, 196].

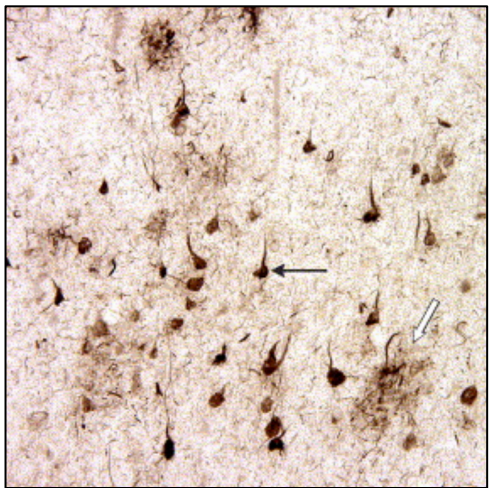


Figure 9. Neurofibrillary tangles. Anti-phospho-tau antibody reveals hyperphosphorylated tau protein accumulation in neuronal cell bodies. NFTs (black arrow) and neuritic plaques (white arrow)[197].

The progressive development of AD is associated with an intense neuronal loss, which determines brain atrophy. This loss of neurons starts in the pre-clinical phase of the disease, before the formation of amyloid plaques and NFTs, and is highly selective for specific brain areas, such as hippocampal field CA1, the dentate fascia, the subiculum and the layer 2 of the entorhinal cortex. Furthermore, during the

pathological process neuronal loss is extended also to the temporal, frontal and parietal cortex[198].

Another hallmark of AD is synaptic loss, which emerges in the earliest phase of AD and occurs mainly in the hippocampal region. Several mechanisms contribute to synaptic dysfunction such as alterations of synaptic proteins, membrane lipids, vesicular function, oxidative stress and loss of plasticity[199]. The initial triggers may be Aβ plaques toxicity or disrupted intracellular transport of aggregated tau[200]. Synaptic loss occurs in the same regions where neuronal loss takes place. Indeed, in these regions the levels of synaptophysin decrease in parallel with AD progression and cognitive dysfunctions. However, nowadays, it is not clear if synaptic loss precedes, follows or is coincident with neuronal loss[201].

Emerging evidences suggest that a low-grade chronic inflammation has a central role in the pathogenesis of AD[161]. Neuroinflammation is mainly driven by microglia, the resident phagocyte of the CNS and astroglia (Figure 10)[202]. Under physiological conditions, microglial cells are in a resting, ramified state monitoring the CNS through their motile processes. Microglial cells have the role of surveillance, microenvironment scanning and maintenance of neuronal plasticity through the release of trophic factors[203, 204]. Microglia is therefore fundamental in morbid conditions, however, if constantly triggered, as in AD, they lead to detrimental effects: excessive release of cytokines, ROS, proteinases and complement proteins, and extension of neuronal damage, establishing a chronic non-resolving inflammation. Microglia can be activated by Aβ oligomers and tau proteins, recognized as PAMPs, via cell-surface receptors. In response to receptor ligation, microglia clear Aβ by phagocytosis or by the release of enzymes that are able to degrade Aβ in the extracellular space[203]. However, in AD the clearance mechanism of Aβ can be compromised, leading to excessive accumulation of Aβ and neuronal debris[203]. Thus, Aβ oligomers could be considered both as the cause and the consequence of neuroinflammation in AD brains.

Tau pathology is closely related to microglia and previous studies have shown that activated microglia induces an increase in tau phosphorylation levels. In fact, during inflammation, microglial cells secrete IL-6 to activate kinases, such as Cyclin-dependent kinase 5 (Cdk5)[205], p38-MAPK and GSK3β producing anomalous tau hyperphosphorylation.

Astrocytes activated by Aβ and NFTs also contribute to the inflammatory process in AD (Figure 10)[206]. Like microglia, astrocytes belong to the glial cell family and have heterogeneous functions within the CNS, such as homeostatic functions, bi-directional communication with neurons, maintenance of the permeability of the blood brain barrier (BBB), and inflammation control[207]. In normal conditions, astrocytes have defensive function in the brain; they trigger a mechanism known as reactive gliosis or astrogliosis, a process in which astrocytes meet morphological changes and increase in number, and they repair and remodel damaged brain tissue[208]. Aβ and NFTs trigger the reactive phenotype of astrocytes, which respond with the release of TNF-α and IL-1β cytokines, nitric oxide and other cytotoxic molecules intensifying the inflammatory response. In addition, reactive astrocytes have an increased expression of β-secretases and PS1, enzymes fundamental for the generation of Aβ fragments, increasing the deposition of insoluble neurotoxic oligomers[209]. This association is also supported by the co-localization of amyloid plaques and activated astrocytes[210].

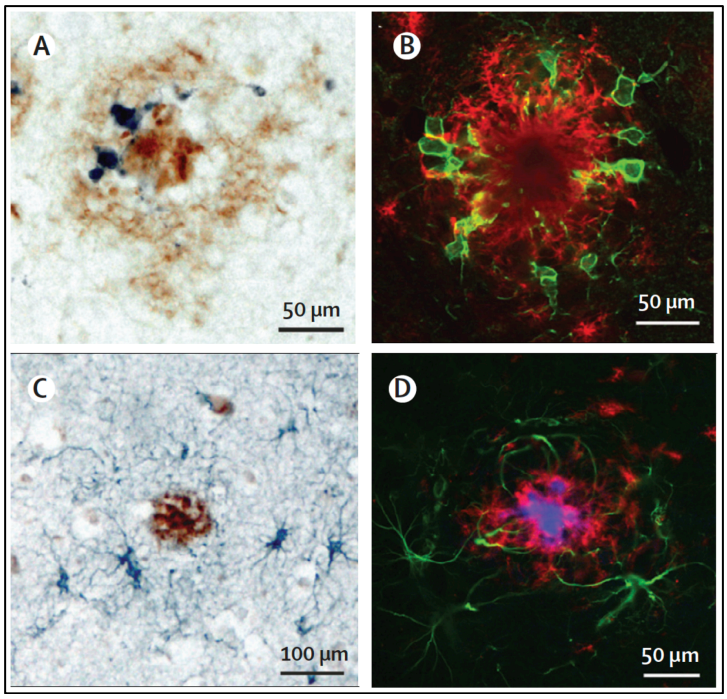


Figure 10. Changes in microglia and astroglia in AD. Microglia and astroglia are key players in the inflammatory response: changes in microglia and astroglia are evident in the post-mortem brains of patients and in AD animal models. (**A-B)** Activated CD11b+ microglia (blue, green) within an amyloid β plaque (brown, red) in the parietal cortex of a brain section from an AD patient. (**C-D**) GFAP+ astrocytes (blue, green) surround the site of Aβ deposit (brown, red) in the cortex of AD patient. (Adapted from Heneka MT et al., *Lancet Neurol*, 2015)[203].

Increasing evidences show that peripheral inflammatory cells also, play a crucial role in the onset and progression of the disease[211, 212].

Lymphocytes can also cross the activated BBB during AD. Both CD4+ and CD8+ T cells were observed adhering to the vascular endothelium or migrating into the parenchyma of AD patients[213, 214]. The number of these cells was higher in AD patients than in healthy, age-matched controls, with the majority of the T cells infiltrating the hippocampus and other limbic structures affected during AD[213]. Furthermore, mild AD patients contained a greater number of activated CD4+ and CD8+ T cells in the CSF, supporting the hypothesis that activated T cells migrate from the blood into the brain during AD[215, 216]. Similarly, T cells were also shown to infiltrate the CNS of AD transgenic models and secrete IFNγ and IL-17 cytokines that could increase glial activation and Aβ deposition[217]. This suggests that peripheral inflammation may favor the entry of circulating activated T cells into the brain, which may in turn exacerbate AD pathology[217].

4.2 THE 3xTg-AD MOUSE MODEL OF AD

The triple transgenic mouse (3xTg-AD) generated by the group of LaFerla is the only AD model currently available which reproduces the main features of human AD pathology[218]. 3xTg-AD model develops an age-related and progressive neuropathological phenotype that includes both Aβ plaques and tau pathology and mimic some cognitive and behavioral alteration reported in AD subjects[219]. Additionally, this model expresses three human mutant genes that were observed in AD familial patients: PS1 (M146V), βAPP (Swedish) and tau (P301L). *PS1* gene encodes for presenilin transmembrane protein involved in the activity of γ-secretase. Several mutations in the *PS1* gene are missense mutations and result in the formation of an abnormal PS1 protein, that causes a defective APP processing due to the failure of γ-secretase cleavage, and an increased production of Aβ species, typical of AD patients. *APP* gene encodes a cell surface receptor and transmembrane APP that is cleaved by β-secretase to form different peptides. APP is produced in the neuronal soma and it is transported on the cell surface by anterograde axonal transport[220]. Mutation of APP gene leads to APP abnormal protein production, which results again in a pathological accumulation of Aβ plaques.

From a pathological point of view, in 3xTg-AD mouse the extracellular Aβ deposits initially accumulate in frontal cortex at 6 months of age and become marked at 12 months of age in cortical regions and hippocampus. By 15 months, Aβ plaques appear in posterior cortical regions such as the occipital and parietal cortices, suggesting a related regional dependence to the Aβ deposits in 3xTg-AD. NFTs are evident at 12-18 months of age in hippocampus, then they progress to the cortex, suggesting that their formation may be influenced by generation of Aβ[218, 221].

The first memory deficits in 3xTg-AD mice are detectable at 4 months of age and correlates with intracellular deposits of Aβ plaques in hippocampus and amygdala. At 6-months of age, these mice start presenting difficulties to retain the information day by day. The continued accumulation of Aβ is likely to account for the continued decline of the cognitive phenotype to include short-term, as well as long-term, memory deficits[221].

Another crucial characteristic to take into account is that 3xTg mouse develops systemic inflammation as observed by the higher expression of adhesion molecules (P-selectin, VCAM-1, ICAM-1) in the vessels of the meninges, cortex, choroid plexus, hippocampus and amygdala of the mutant mice at 6 months of age[222]. This data indicates a general vascular inflammation that may play a continual pathogenic role. Interestingly, among the CD45+ leukocytes migrated into the brain parenchyma of 3xTg-AD mice, there is a massive neutrophils accumulation that peak at 6 months of age, coinciding with the onset of memory loss in cognitive tests (Figure 11)[222]. However, neutrophils also infiltrate the brain before the onset of cognitive deficits and at 6 months of age compared to sex- and age-matched wild type (WT) controls, suggesting that neutrophils play a role in the induction of cognitive decline as well as in disease progression (Figure 11)[222].

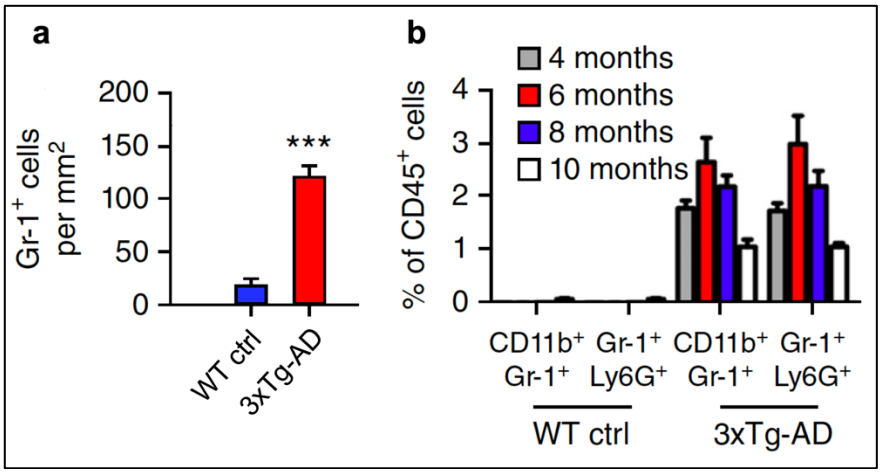


Figure 11. Neutrophil infiltration in the brain in the early stage of disease. (**A**) GR-1+ neutrophils significantly accumulated in the brain of 3xTg-AD mice compared to WT controls (*** $P < 0.0005$). (**B**) Frequency of neutrophils in the brains of 3xTg at different stages of the disease, in comparison with WT healthy controls. (Adapted from Zenaro E et al., *Nat Med*, 2015)[222].

4.3 NEUTROPHILS IN AD

Growing evidences reported by several groups support the idea that also neutrophils could contribute to the pathogenesis of AD (Table 1). Some authors showed a greater hyperactivation of peripheral blood neutrophils in AD subjects in comparison with healthy controls, as indicated by the augmented production of ROS[223], the higher levels of intravascular NETs[222], and a significant shift in the ratio between the harmful senescent (CXCR4*high*/CD62L*low*) and the immunosuppressive (CD16*bright*/CD62L*dim)* neutrophil subsets[224]. These data suggest that the expanded percentage of aged pro-inflammatory circulating neutrophils may play an important role in establishing systemic chronic inflammation, and that their hyperreactive phenotypes correlate with the rate of cognitive decline, thus it was proposed as an innovative and prognostic blood biomarker in AD patients[224]. Moreover, other studies confirmed the hyperactivation phenotype of neutrophils showing an increased expression of several surface molecules including αMCD11b integrin, HLA-DR, COX2 and CD177 in mild AD subjects[225, 226].

Of note, several studies in patients and experimental models reported neutrophil migration into the brain during AD[222, 226, 227]. CD177 is a well-known marker of neutrophil activation[226], which associates with β2 integrins and actively bind CD31 expressed by endothelial cells[228]. The interaction between CD177 and CD31 was

hypothesized to serve for neutrophil recruitment into the brain parenchyma in AD, similarly for ICAM-1 and LFA-1[222]. Recently, the integrin LFA-1 was shown to control the intravascular adhesion of neutrophils in the cerebral microcirculation of transgenic AD mice but also their intraparenchymal motility. The blockade of LFA-1 integrin in mice during the early phases of AD, prevented neutrophil adhesion and extravasation, reducing neuropathological hallmarks and restoring cognitive functions[222]. A similar result was obtained by depleting circulating neutrophils with anti-GR-1 /anti-Ly6G antibodies, suggesting that neutrophils play a key role in AD pathogenesis[222]. Neutrophils typically access sites of tissue damage through the circulation, following chemoattractant cues and responding to local inflammatory mediators. In the AD brain, Aβ represent a crucial chemoattractant for the recruitment of neutrophils, contributing to precise directional movements observed for some extravasated neutrophils in different AD mouse models[119, 229]. In fact, GR-1+ cells infiltrate the brain and migrate towards Aβ plaques in a transgenic AD mouse model[222, 230]. Additionally, two-photon laser-scanning microscopy experiments also revealed neutrophil extravasation inside the cerebral parenchyma preferentially in areas rich in Aβ deposits, suggesting that Aβ might be involved in neutrophil recruitment and movement inside the brain[222].

Interestingly, neutrophils do not necessarily need to accumulate in high numbers in order to induce tissue damage: intravascular adhesion without transmigration is sufficient to induce endothelial injury and the resulting tissue damage during low-grade chronic sterile inflammation[231, 232]. This is supported by a recent report in a mouse model of AD showing that antibody against Ly6G removed capillary stalls resulting from intravascular accumulation of neutrophils, and led to an improvement in spatial and working memory within few hours from the treatment[233]. These data support the detrimental role of neutrophil to the brain without its physical infiltration.

Since the role of neutrophils in AD has been defined only in the last years, the mechanisms controlling neutrophil trafficking in the CNS and interactions with the resident cells need further investigations.

Table I. Summary of experimental evidences of neutrophils involvement in AD pathogenesis (Adapted from Rossi B et al., *Immunobiology*, 2019)[262].

Study design	Main findings	Putative neutrophils involvement	References
Autopsy material from AD patients	Brain infiltrating neutrophils releasing MPO, NE and citrullinated histone H3, essential components of NETs.	Neutrophil infiltration in the brain represents a phenomenon in the inflammatory reactions in AD patients.	Zenaro et al. 2015
AD patients	Brain infiltrating neutrophils express inflammatory mediator CAP37.	Infiltrating neutrophils promote perpetuating recruitment of additional neutrophils and monocytes into the brain by CAP37 contributing to neuronal injury in AD.	Pereira et al. 1996 Brock et al. 2015
AD patients	AD patients are characterized by a high neutrophil-lymphocyte ratio (NLR) in the blood, which tends to increase during disease progression.	Elevated number of peripheral neutrophils contributes to AD neurodegeneration and pathogenesis. NLR is investigated as markers of AD-related peripheral inflammation.	Kuyumcu et al. 2012
AD patients	AD patients have high number of circulating neutrophils with a primed phenotype based on over-expression of CD11b, CD177, HLA-DR, COX-2 and NETs and elevated levels of ROS production. Activation markers in neutrophils positively correlate with disease severity and progression rate of mental decline.	Elevated number of activated peripheral neutrophils increases BBB permeability, brain inflammation contributing to neurodegeneration and clinical progression of AD. Neutrophils activation markers must be investigated as biomarkers of AD-related peripheral inflammation.	Scali et al. 2002 Vitte et al. 2004 Shad et al. 2013 Dong et al. 2018
AD patients	AD patients have high expression of APP in circulating neutrophils.	APP expression level in peripheral blood granulocyte is a potential biomarker for early diagnosis of AD.	Wang et al. 2016
AD patients	In the blood of AD patients harmful aged neutrophils (CXCR4high/CD62Llow) and immunosuppressive neutrophil (CD16bright/CD62Ldim)	Aged related changes in neutrophils phenotype and activity, plays a role in establishing systemic chronic inflammation during AD.	Dong et al. 2018

	ratio increases in the later stage of the disease.		
Mouse models	Neutrophils infiltrate the brain where they produce NETs and IL-17.	Brain infiltrating neutrophils are one of the major sources of inflammatory cells in the early AD-like development.	Zenaro et al. 2015
Mouse models	Neutrophils are attracted from the blood vessels into the brain by chronic Aβ deposition and accumulate preferentially in Aβ-rich areas.	Neutrophil responses to senile plaques influence the progress of AD.	Baik et al. 2014 Zenaro et al. 2015
Mouse models	Brain infiltrating neutrophils reduction (depleting circulating neutrophils or interfering neutrophil CNS recruitment by LFA-1 inhibition) reduce AD–like neuropathology and improve memory.	CNS infiltrating neutrophils have a role in preparing local inflammation contributing to AD-like pathogenesis and cognitive impairment.	Zenaro et al. 2015
Mouse models	Prevent neutrophil adhesion on cortical capillaries (depleting circulating neutrophils) restore cerebral blood flow and improve memory.	Neutrophils indirectly promote cognitive impairment by adhering in brain capillary segments occluding or reducing cerebral blood flow.	Cruz Hernandez et al. 2019

MATERIALS AND METHODS

1. MICE

3xTg-AD mice (MMRRC Stock No: 34830-JAX) and their WT control B6129SF2/J (Stock No: 101045) were purchased from Jackson Laboratories (Sacramento, CA). For our project we also used C57BL/6J mice from Jackson Laboratories and TIM-1$^{\Delta mucin}$ mice were provided by Prof. Vijay K. Kuchroo (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). TIM-1$^{\Delta mucin}$ mouse was obtained introducing a NEO-cassette in the third exon of the TIM-1 gene sequence. This insertion leads to the complete deletion of the exon 3 from the gene sequence and causes the ablation of the extracellular mucin domain from the protein structure. Finally, we used 3xTg-AD/TIM-1$^{\Delta mucin}$ homozygous mice, previously generated in our laboratory in two steps: 1) we crossed 3xTg-AD with TIM-1$^{\Delta mucin}$ to obtain hemizygous 3xTg-AD/TIM-1$^{\Delta mucin}$ mice; 2) we crossed hemizygous 3xTg-AD/TIM-1$^{\Delta mucin}$ each other to obtain homozygous mice for all transgenes. The insertions of APP and PSEN1 genes and the deletion of TIM-1 were confirmed by PCR analysis.

Animals were housed under standardized conditions with a 12-hours (h) photoperiod in climate-controlled facilities and were provided with food and water ad libitum. All animal experiments were supervised by the local Institutional Animal Care Committee (OPBA) of the University of Verona and were approved by the Italian Ministry of Health, Department of Veterinary Public Health, Nutrition and Food Safety, Directorate General of Animal Health and Veterinary Medicine, as required by Italian legislation (D. Lgs 26/2014, application of European Directive 2010/63/EU).

2. ISOLATION OF MURINE NEUTROPHILS

Mouse neutrophils were isolated from the bone marrow of healthy adult C57BL/6J and TIM-1$^{\Delta mucin}$ mice. Mice were sacrificed and tibias and femurs were surgically removed. To prevent unwanted early neutrophil activation, bone marrow cells were rapidly flushed out of the bones with 1X Hank's Balanced Salt Solution (HBSS)

supplemented with 0.1% Bovine Serum Albumin (BSA) and lacking Ca2+ and Mg2+. Cells were then treated for few seconds (sec) with a 0.2% hypotonic NaCl solution to lysate red blood cells, then a 1.2 % isotonic NaCl solution was added to the sample for restoring physiological osmolarity. Meanwhile, 81%, 62% and 55% Percoll solutions were carefully stratified into a falcon tube to obtain a Percoll density gradient. After red blood cell lysis, cells were resuspended in HBSS 1X-BSA 0.1% solution, stratified over the Percoll gradient and centrifuged at 2700 rpm for 30 minutes (min) without brake. Neutrophils were collected from two milky rings the 81-62% Percoll phase, corresponding to more mature and differentiated neutrophils, and the 62-55% one, comprising more immature and less differentiated progenitors and precursors. Cells were washed twice with HBSS 1X-BSA 0.1% solution at 1200 rpm for 10 min, and finally resuspended in HBSS 1X-BSA 0.1% solution and counted. More than 90% of the isolated cells were Ly6G+ pure neutrophils as determined by flow cytometry (data not shown).

Blood samples were collected from the retro-orbital plexus of anesthetized mice by sodium heparinized capillaries in this ratio 50:50 (blood:1% dextran plus sodium heparine 10 U/ml). After 1 h of erythrocytes sedimentation, overlying supernatant plasma-dextran suspension of leukocytes was washed in phosphate-buffered saline (PBS), followed by red cells lysis.

3. ISOLATION OF HUMAN BLOOD NEUTROPHILS

Written informed consent was given to healthy volunteers before sampling execution. Neutrophils isolation was performed from buffy coats. Briefly, 25 ml of sample were stratified over 17,5 ml of Ficoll and centrifuged at 1500 rpm for 30 min without brake. The supernatant was removed and the neutrophil layer collected. Red blood cells were dismissed by sedimentation in a solution of 4% Dextran 500 in PBS, for 20 min at room temperature (RT), followed by hypotonic lysis. The surviving was washed in PBS and centrifuged at 1200 rpm for 5 min. Neutrophils were resuspended in HBSS 1X + 0,1% BSA + 0,5 mM Ca2+/Mg2+ at a density of 5×10^6 /ml and stimulated as described below.

4. Aβ PREPARATION

$A\beta_{1-42}$ peptide was purchased from Bachem AG. Oligomeric Aβ was prepared as previously described [222,234]. Briefly, after the removal of hexafluoroisopro-panol, $A\beta_{1-42}$ was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM and diluted in F12 medium to 100 μM. After incubation for 24 h at 4°C, the Aβ solution was centrifuged at 15,000 *g* for 10 min at 4°C and the supernatant, containing soluble oligomeric Aβ, was collected and quantified using a MicroBCA assay (Pierce). For all the experiments, the reverse peptide $A\beta_{42-1}$was used as control and processed as described above.

5. NEUTROPHIL STIMULATION

For TIM-1 expression studies, murine neutrophils were suspended at a density of 5 × 10^6 cells/ml in HBSS 1X + 0.1% BSA and stimulated with 10 ng/ml recombinant mouse tumor necrosis factor-α (TNF-α), 100 ng/ml lipopolysaccharide (LPS), 1 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP), 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 30 μg/ml PAM3CSK4, 300 nM recombinant complement 5a (C5a) for 1, 5, 10, 15, 30, 60 min while stirring at 37°C. Human neutrophils were primed with 10 ng/ml recombinant human TNF-α for 10 min and then treated with 10 μM oligomeric $A\beta_{1-42}$ or $A\beta_{42-1}$ as control for 15 min at 37 °C. Where indicated, neutrophils were treated with TNF-α, $A\beta_{1-42}$ or $A\beta_{42-1}$ alone as controls. In some experiments, neutrophils were also exposed to 1 μM fMLP for 1, 5, 10 and 15min at 37°C. Untreated samples were considered as resting controls. At the end of the treatment, samples were washed at 1200 rpm for 5 min in PBS + 10% fetal bovine serum (FBS; Sigma, Cat-no: F7524) and analyzed by flow cytometry or by immunofluorescence staining.

6. FLOW CYTOMETRY ANALYSIS

To assess the TIM-1 intracellular expression, resting neutrophils were fixed for 20 min at RT in the dark (fixation buffer; BioLegend), centrifuged and washed in permeabilization buffer according to the manufacture's protocol (BioLegend). In the stimulation protocol and for blood leukocytes, samples were assessed for TIM-1 surface expression without cell fixation and dead cells were excluded by 7-AAD

staining (BioLegend). To exclude non-specific Fc receptor (FcR) binding, murine samples were blocked with purified anti-CD16/CD32 (BioLegend) and mouse IgG (Sigma-Aldrich) in PBS + 10% FBS and human neutrophils with 10% human serum (Sigma) in PBS for 15 min at RT. Cells were then stained for 15 min at 4°C with the following fluorochrome-labeled monoclonal antibodies: panel 1 for mouse) anti-CD45 Vioblue, anti-CD11b APC/Cy7, anti-Ly6G FITC, anti-CD8α PE-Cy7, anti-CD4 APC, anti-TIM-1 PE; panel 2 for human) anti-CD45 APC/Vio770, anti-CD11b APC/Cy7, anti-CD66b APC, anti-CD16 Pacific Blue, anti-TIM-1 PE, anti-CD62L PE/Cy7(BioLegend). Specimens were then washed and acquired by flow cytometry with MACSQuant Analyzer (Miltenyi Biotec). The percentage of TIM-1 expressing neutrophils and the mean fluorescence intensity (MFI) were determined for each experiment using FlowJo software. For the analysis of TIM-1 expression, all values were normalized to the resting control (ctrl) cells set equal to 100 (for MFI) and 1 (for the percentage) as arbitrary units, and represented as the subtraction of the control.

7. IMMUNOFLUORESCENCE STAINING

Resting and stimulated mouse or human neutrophils were labelled for 10 min with 5 µg/ml of CellMask™ Deep Red probe to counterstain the plasma membrane (Invitrogen). Cells were washed in PBS and fixed in paraformaldehyde (PFA) 0.4% for 20 min at 4°C. After 2 x PBS washings, cells were incubated in blocking buffer with 2% normal goat or donkey serum (Vector Labs) for 1 h at RT in the presence or not of 0.2 % saponin permeabilizing agent. The following primary antibodies were added for 1 h at RT: 3.5 µg/ml rat anti-mouse TIM-1 (clone 5F12)[235] and 1 µg/ml rabbit anti-human TIM-1 (Abcam). After washing, 1:500 of Alexa Fluor 488 conjugated goat anti-rat, Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibodies (Molecular Probes, Invitrogen) were added in the blocking solution for 1 h at RT. Nuclei were stained with 1 µg/ml DAPI (Sigma) for 8 min in the dark. Finally, droplets of cell suspension were transferred to glass slides and mounted with Fluoro Gel with DABCO (EMS). Images were acquired with Axio Imager Z2 fluorescence microscope with Apotome system (Zeiss, Germany), and were spatially deconvoluted using linear unmixing algorithm using ZEN software (Carl Zeiss).

8. THIOGLYCOLATE-INDUCED PERITONITIS

Sterile peritonitis was induced as previously reported[236]. Briefly, 10- to 16-week-old male and female WT C57BL/6J mice were injected intraperitoneally (i.p.) with 1 ml of sterile thioglycolate broth 4% (wt/vol) or PBS as control. After 1, 3 and 6 h, mice were anesthetized by i.p. injection with a solution of PBS containing 5 mg/ml ketamine and 1 mg/ml xylazine and peritoneal cavities were washed with 5 ml ice-cold sterile Ca2+/Mg2+-free HBSS 1X containing EDTA 2 mM. Peritoneal exudate cells (PECs) were centrifuged at 1200 rpm for 5 min at 4°C and red cells lysed. PECs were washed, resuspended in PBS 10% FBS, incubated with anti-CD16/CD32 and mIgG FcR blockers and stained with the fluorescently conjugated antibodies of panel 1) (see above). Cell viability was measured by 7-AAD exclusion. Where indicated, blood was collected from healthy and thioglycolate-challenged WT mice before peritoneal lavage, to compare TIM-1 expression on circulating neutrophils to that of neutrophils accumulated to the inflamed peritoneum. Blood was processed and analyzed as described above.

To assess the role of TIM-1 in neutrophil recruitment during inflammation, 8-15 x 10^6 WT or TIM-$1^{\Delta mucin}$ neutrophils isolated from the bone marrow were labelled in HBSS 1X + 0.1% BSA with 7-amino-4-chloromethylcoumarin (CMAC) fluorescent cell tracker (Molecular Probes) for 30 min at 37°C, washed and injected intravenously (i.v.) in WT recipient mice. After 30 min from cell injection, sterile peritonitis was induced, and PECs harvested by peritoneal lavage 1 h after thioglycolate injection. The number and percentage of CMAC+ neutrophils infiltrated to the inflamed peritoneum were counted by flow cytometry. To assess the effect of TIM-1 blockade on neutrophil recruitment, C57BL/6J mice of both sex were treated i.v. with 0.4 mg of anti-TIM-1 blocking antibody (RMT1-10 clone) or anti-RAS control antibody (Y13259 clone) in sterile PBS. After 1 h of treatment, peritonitis was induced with i.p. injection of sterile 4% thioglycolate broth. After 1 and 3 h, PECs were harvested and assessed by flow cytometry as described above.

9. ELISA ASSAY

Whole blood from 3xTg-AD and B6129SF2/J mice was collected from the retro-orbital plexus in sampling tubes. Serum was obtained by spontaneous clotting blood

at RT for 15-30 min, then was centrifuged at 2000 *g* for 10 min and the supernatant stored in tubes at -80°C prior to ELISA assay.

Murine TIM-1 concentration was detected by using Quantikine ELISA immunoassay (R&D) according to the manufacturer's instructions. Briefly, a 96-well plate precoated with immobilized anti-TIM-1 capture antibody was incubated with 50 µl standards, controls and serum specimens for 2 h at RT. After washings, anti-TIM-1 detection antibody conjugated to horseradish peroxidase (HRP) was added for 1 h at 4°C, washed and incubated with substrate solution. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of TIM-1 bound in the initial step. Optical density (OD) was determined for each well using a microplate reader set to 450 nm, with wavelength correction set to 570 nm. The sample concentration was finally determined from the standard curve.

10. ISOLATION OF BRAIN LEUKOCYTES

Mice were anesthetized and perfused through the left cardiac ventricle by injection of cold PBS. The brain was digested with 20 U/ml of DNase I and 1 mg/ml collagenase at 37 °C for 45 min. Cells were isolated by passing the digested tissue through a 70 µm cell strainer, resuspended in 30% Percoll and loaded onto 70% Percoll. Tubes were then centrifuged at 1300 *g* for 20 min at 4°C. Cells were removed from the interphase, washed, labelled with the anti-mouse antibodies of panel 1) and acquired by flow cytometry.

11. INTRACARDIAL PERFUSION

Female 3xTg-AD and B6129SF2/J mice at 6 months of age were anaesthetized by i.p. injection with a solution of PBS containing 5 mg/ml ketamine and 1 mg/ml xylazine. Once sedated, the mouse was placed on a surgical support, with the abdomen facing up, and its four paws were pinned as stretched as possible. Skin was grabbed with forceps at diaphragm level, and the ribs were cut to make the heart easily accessible. Subsequently, the right atrium was incised, and a butterfly needle was inserted into the left ventricle. A peristaltic pump (Minipuls3 GILSON®) with a flow no higher than 0.5 ml/min is used to inject 25 ml of PBS 1X with 1mM Ca2+/Mg2+ and glucose through the mouse systemic circulation in

order to wash away all blood. If the perfusion is successful, tongue color becomes light pink, ears and tail veins cannot be seen any more. Then, the buffer solution is replaced with 25 ml of cold 4% PFA fixative solution.
Once perfusion was finished brains were removed and maintained in ice-cold 4% PFA solution overnight (O/N) at 4°C. The day after, organs were rinsed with PBS for at least 30 min at RT and then transferred in sucrose 30% solution in PBS until they sink. Sucrose was used to cryo-protect and to prevent freeze artefact and loss of tissue architecture.
Organs were included in a cryo-embedding matrix such as OCT (CDK Italia) and stored at -80°C.

12. TISSUE IMMUNOFLUORESCENCE STAINING

Frozen mouse brains were cut into 20-µm coronal sections. Tissue sections were treated with 2% BSA and incubated O/N at 4°C with 5 µg/ml rabbit anti-mouse TIM-1 (Abcam) and 1 µg/ml Alexa Fluor 488 conjugated rat anti-mouse Ly6G (Biolegend). After washings with PBS, slices were incubated in blocking solution with 4 µg/ml of Alexa Fluor 647 conjugated donkey anti-rabbit secondary antibody (Molecular Probes, Invitrogen). After washings with 0.05% Tween-20 in PBS, nuclei were stained with 1 µg/ml DAPI (Sigma) for 8 min in the dark and slides mounted with Fluoro Gel with DABCO (EMS). Images were acquired with Axio Imager Z2 fluorescence microscope with Apotome system (Zeiss, Germany), and were spatially deconvoluted using linear unmixing algorithm using ZEN software (Carl Zeiss).

13. HISTOPATHOLOGICAL ANALYSIS

Frozen mouse brains were cut into 30-µm coronal sections. Brain sections were stained in free-floating and first treated with a blocking solution containing 2% normal goat serum and 0.4% Triton, and then incubated for 18 h at 4°C with the following primary antibodies: anti-mouse Iba-1, anti-human Aβ 6E10, anti-human tau HT7 and anti-human phospho-tau AT180. Aβ and tau staining requires epitope retrieval with 70% formic acid for 20 min and 10 mM sodium citrate buffer (pH 8.5) pre-heated to 85°C in water bath, respectively. After washing with 0.05% Tween-20 in PBS, we added 3% H_2O_2 for 10 min at RT before washing the sections

and incubating them with the biotinylated secondary antibody (goat anti-rabbit and goat anti-mouse antibodies, Sigma). The immunoreactivity was visualized using the VECTASTAIN® ABC kit (Vector) for 30 min and Vector® NovaRED™ (Vector) as the chromogen for 3 min at RT. Finally, brain portions were washed with distilled water, transferred to glass slides and mounted with Eukitt® mounting medium (Sigma). Images were acquired with Axio Imager Z2 (Zeiss) and quantified. The area of Iba-1+ reactive microglia, amyloid deposits, human total tau and phospho-tau positive neurons were determined in coronal sections throughout the cortex and the hippocampus. Sections were taken from the anterior hippocampus through the bregma −2.9 mm at an intersection interval of 500 μm (every fourth section) in order to analyze the whole area of the cortex and the hippocampus. We quantified blindly with ImageJ v1.32j software.

14. BEHAVIOURAL ASSESSMENTS

Learning and memory capacity of 3xTg-AD/TIM- $1^{\Delta mucin}$ and 3xTg-AD controls at 9- and 12-months of age were assessed using Y maze and Contextual Fear Conditioning tests. Mice were selected on the bases of specific inclusion and exclusion criteria. For instance, mouse body weight (20-40 g) was checked and mice with body weight higher than 40 g were excluded from the experiments. Mice with evident physical defects such as loss of the whiskers or dwarfism, and cutaneous defects (i.e. alopecia) were excluded from the experiment. Additionally, mice were previously checked for possible undesirable effects related to general deficits in task performance, such as alterations in vestibular function, which may potentially cause difficulties during behavioral assessment.

All behavioral tests were conducted as described by Imbimbo et al.[237], with approximately 8-12 mice for both tasks, with equal number of males and females. At the end of behavioral tests, mice were sacrificed for pathological analyses. Half brains were cut in sagittal sections, without the hindbrain. The first half of the brain was fast-frozen with dry ice for subsequent isolation of brain leukocytes. Instead, the second half of the brains were fixed and frozen for immunohistochemistry studies.

14.1 Y MAZE

Y Maze Spontaneous Alternation is a behavioral test used to evaluate, without training, reward, or punishment, the willingness of rodents to explore new environments and to assess hippocampus-dependent spatial working memory, which is classified as short-term memory. Testing occurs in a Y-shaped maze with three gray opaque plastic arms at a 120° angle from each other, extending from a central space (Figure 12). Once in the center of the maze, the naïve animal was allowed to freely explore the maze for 8 min. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. Thus, during the session, the mouse should show a lower tendency to enter in the recently visited arm. The sequence and the total number of arm entries were recorded to calculate the percentage of alternation. An entry occurs when all four limbs are within the arm. Each arm of the Y maze was arbitrarily assigned as zone A, B, or C. Alternation was defined as successive entries into the three arms in overlapping triple sets (e.g., ABC or ACB but not ABA). The alternation percentage was calculated as (number of triads containing entries into all three arms / maximum possible alternations) x 100. To diminish odor cues, the maze was cleaned with 70% ethanol solution prior to test each mouse.

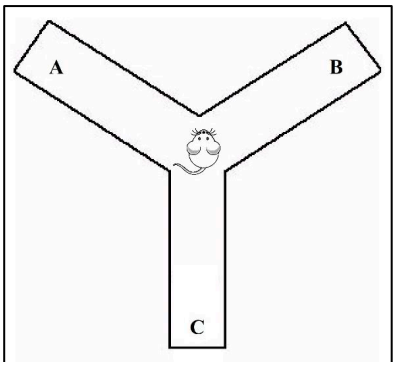


Figure 12. Representation of Y maze.

14.2 CONTEXTUAL FEAR CONDITIONING TEST

Contextual Fear Conditioning (CFC) is an associative learning task in which mice learn to associate a particular neutral conditional stimulus (CS; a tone) with an aversive unconditional stimulus (US; a mild electrical foot shock) and show conditional response (freezing) (Figure 13). After repeated pairings of CS and US,

the animal learns to fear both the tone and training context. CFC test is useful for neurobehavioral, genetic, and pharmacological studies and amygdala and hippocampus are key structures for learning and retrieval of memories in this task. CFC was performed in 30 × 24 × 21 cm operant chambers (Ugo Basile). Each chamber was equipped with a stainless-steel rod floor through which a footshock could be administered, two stimulus lights, one house light, and a solenoid, all controlled by ANY-maze computer software (Stoelting). Mice were trained and tested on 2 consecutive days. Training consisted in placing a subject in a chamber, illuminating stimulus and house lights, and allowing exploration for 2 min. A 15 s tone stimulus (2 Hz) then co-terminated with 2 s foot shock (1.5 mA). The pairing of stimuli was repeated twice with an interval of 2 min. Thirty seconds after the second shock, mice were removed from the chamber. After 20 h, mice were placed back into the same training chamber for the contextual test for 5 min, with no tones or shocks delivered, where the freezing behavior was recorded by the experimenter. Freezing was defined as lack of movement except that required for respiration. At the end of the contextual test, mice were returned to their home cage. Approximately 2 h later, mice were placed in a novel environment for cued fear memory test. The new environment consists in a colored Plexiglas that covers the steel rods of the floor, a black and white striped plastic put on the walls of the chamber, and in the introduction into the testing chamber of a novel odor. After 2 min and 30 s without any stimulus presentation, mice were exposed to the auditory cue for remaining min, and freezing was again scored. The freezing score was expressed as a percentage for each portion of the test. Memory for the context for each subject was obtained by subtracting the percentage of freezing in the novel environment from that in the context. Experiments were blinded with respect to the genotype of the mice and the treatment.

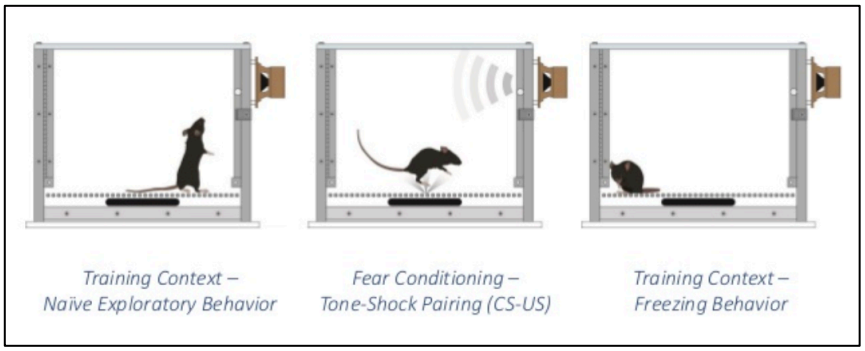


Figure 13. Schematic representation of the CFC test.

15. STATISTICS

Quantitative data are shown as mean values ± standard error (SEM). A two-tailed Student's *t*-test was used for the statistical comparison of two samples. Multiple comparisons were performed by one-way ANOVA.

AIM OF THE PROJECT

TIM-1 is considered a key regulator of immune responses, and recently it was shown to be involved in the trafficking of activated T cells under inflammatory conditions. Despite TIM-1 is expressed on adaptive immune cells, this glycoprotein has never been investigated in relation to neutrophil functions. Importantly, TIM-1 was shown to be crucial in many inflammatory pathologies, representing a promising therapeutic target. All these observations led us to investigate the expression of TIM-1 in neutrophils, which has never been studied before, and to explore a potential role for TIM-1 in mediating neutrophil trafficking during inflammatory responses. By using flow cytometry analysis and immunofluorescence staining we first analyzed TIM-1 glycoprotein expression in mouse and human neutrophils under resting conditions and in the presence of proinflammatory stimuli.

Furthermore, we studied the involvement of TIM-1 in neutrophil recruitment in acute inflammatory responses, adopting a model of sterile thioglycolate-induced peritonitis. To address this point, by flow cytometry we counted the numbers of neutrophils infiltrated into the peritoneal cavity of C57BL/6J mice after either treatment with anti-TIM-1 blocking antibodies or injection of TIM-1$^{\Delta mucin}$ (lacking the mucin functional domain) and WT neutrophils.

We also investigated the implication of neutrophil TIM-1 in chronic inflammatory responses, focusing our attention on AD. All the experiments were performed by using 3xTg-AD mice as animal model of AD, which develop the classical neuropathological features of the disease such as progressive deposition of Aβ plaques, NFTs formation, brain atrophy, microglia activation and enhanced brain inflammation.

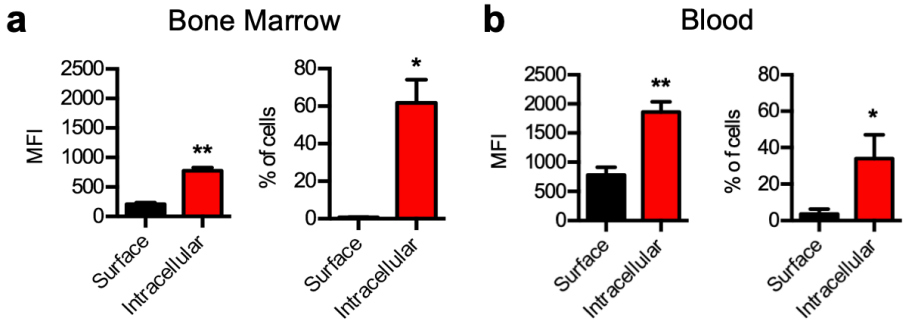
Overall, this study was focused on a new potential role of TIM-1 in controlling neutrophil trafficking during acute and chronic inflammatory responses. Our investigation shed new light on the role of TIM-1 as a novel trafficking receptor for neutrophils migration into impaired tissues and emphasized how TIM-1 deficiency could have a beneficial effect on the development of acute and chronic inflammatory responses in mice. In conclusion, this project highlighted how TIM-1 targeting may represent an attractive therapeutic approach, not only in

neurodegenerative disorders, but also in several other diseases in which inflammation plays a detrimental role.

RESULTS

1. RESTING NEUTROPHILS STORE TIM-1 INTRACELLULARLY

Previous studies have shown that TIM-1 is expressed by activated lymphocytes, but no data are currently available for TIM-1 expression in neutrophils.
Therefore, by using flow cytometry analysis, we firstly checked a potential TIM-1 expression in neutrophils. In particular, we investigated mature neutrophils isolated from bone marrow and blood of male and female 4-month-old C57BL/6J mice. We assessed TIM-1 expression both on permeabilized and non-permeabilized cells. Surprisingly, we found that TIM-1 was mainly stored intracellularly, whereas a very low expression was present on the cell membrane under resting conditions. In particular, the MFI of TIM-1 stored intracellularly and expressed on the cell membrane was respectively 710 ± 55 and 229 ± 29 (mean ± SEM; ** $P < 0.005$) in bone marrow-derived neutrophils (Figure 14 a) and 1860 ± 176 and 780 ± 133 (mean ± SEM; ** $P < 0.005$) in circulating neutrophils (Figure 14 b). These data revealed that under resting conditions in peripheral blood- and bone marrow-derived neutrophils, TIM-1 glycoprotein is mainly stored intracellularly. These data were confirmed by immunofluorescence staining, showing a higher expression of TIM-1 located beneath the cell membrane and widespread at the cytoplasm level, compared to rare dots of fluorescent signal on neutrophil surface (Figure 14 c, d).



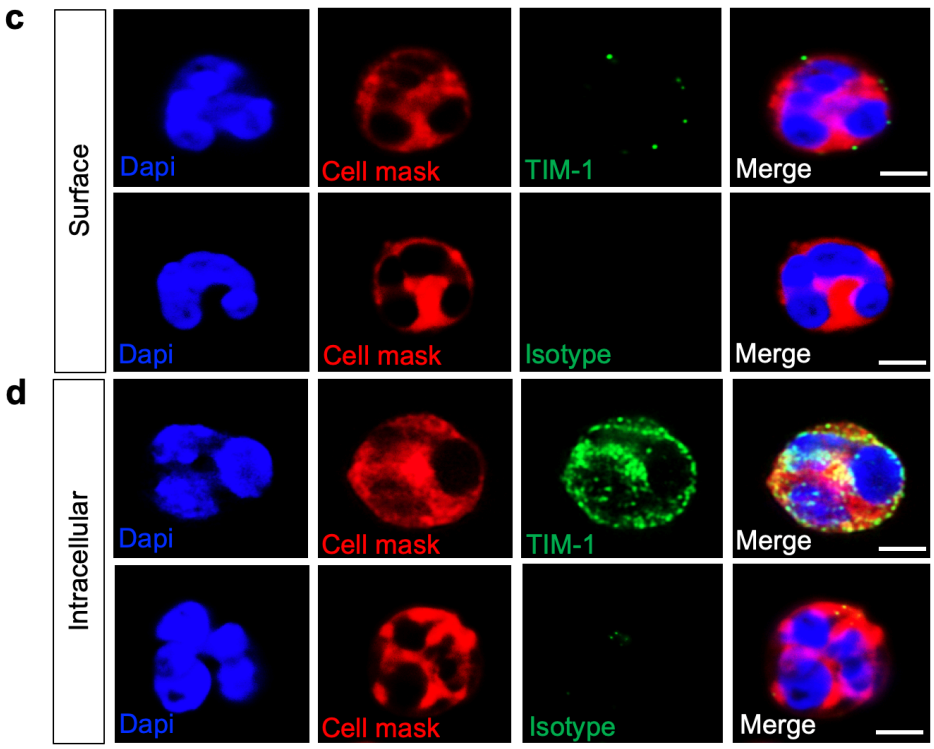


Figure 14. Quantitative and qualitative expression of TIM-1 in resting murine neutrophils. (a-b) Flow cytometry analysis of neutrophils isolated from bone-marrow (**a**) and blood (**b**) of C57Bl/6J mice and assessed for membrane surface or intracellular expression of TIM-1. Values represent mean of fluorescence intensity (MFI) and percentage of TIM-1 expressing neutrophils. Permeabilized cells express higher levels of TIM-1 intracellularly compared to non-permeabilized cells. Data represent the mean ± SEM of 4 different experiments (Student's *t* test, * $P < 0.05$; ** $P < 0.005$). (**c-d**) Representative images of TIM-1 expression (green) in bone marrow-derived neutrophils on the cell membrane (red) (**c**) and intracellularly **(d)**. Nuclei are stained in blue. Scale bars = 5 µm.

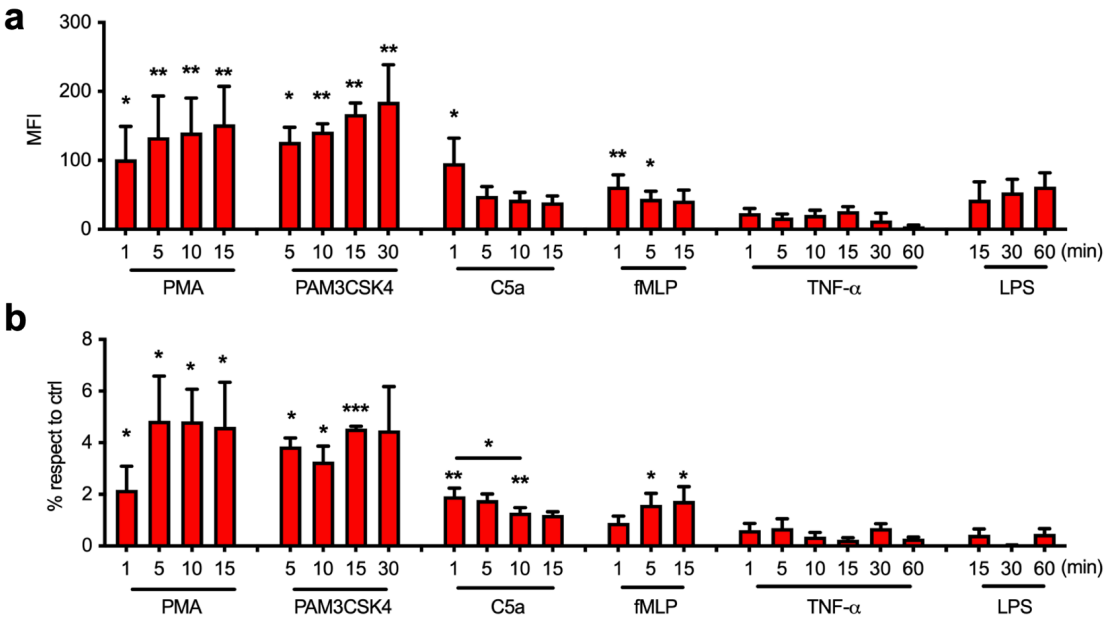
2. PRO-INFLAMMATORY STIMULI INDUCE RAPID TIM-1 MEMBRANE UPREGULATION IN NEUTROPHILS

Next, we investigated whether inflammatory stimuli could influence TIM-1 translocation to the cell membrane, and its potential biological implication.

We treated murine bone marrow-derived mature neutrophils with several pro-inflammatory stimuli including fMLP, PMA, PAM3CSK4, a TLR-1/2 ligand, C5a, TNF-α and LPS and we assessed TIM-1 expression by flow cytometry. For each stimulus we first determined the optimal concentration in order to obtain a good modulation of the expression of TIM-1. Our data indicated that TIM-1 was upregulated on the cell membrane following the exposure to certain pro-inflammatory stimuli. Particularly, we found that PMA and PAM3CSK4 triggered TIM-1 translocation to the neutrophil membrane in terms of MFI and percentage of

TIM-1-positive cells in a time-dependent manner compared to unstimulated control cells (Figure 15 a, b). Interestingly, the percentage of TIM-1-positive cells was also strongly increased already after 1 min of C5a stimulation and then decreased very quickly over time (Figure 15 b). Besides, the MFI of TIM-1 was rapidly increased within 1-5 min of fMLP treatment compared to the control, leading to a doubling in the percentage of TIM-1-expressing neutrophils after 5 min of stimulation (Figure 15 a, b). Similar results were confirmed by immunofluorescence staining (Figure 15 c). Notably, TNF-α and LPS did not significantly modulate TIM-1 surface expression at any time of the performed assay compared to the control (Figure 15 a, b). When treated with TNF-α, neutrophils displayed only a slight, not statistically significant, increase of TIM-1 levels on the surface, that was maintained over time. Notably, LPS did not impact TIM-1 expression at any time after stimulation (Figure 15 a).

These data clearly indicated that when stimulated with TLR-1/2 and G protein coupled receptors ligands, TIM-1 levels was very rapidly translocated on neutrophil surface.



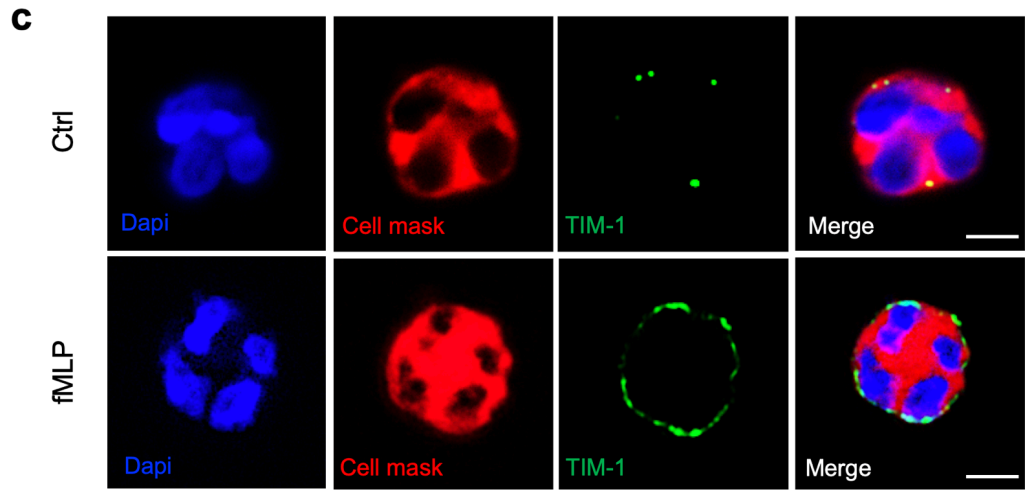


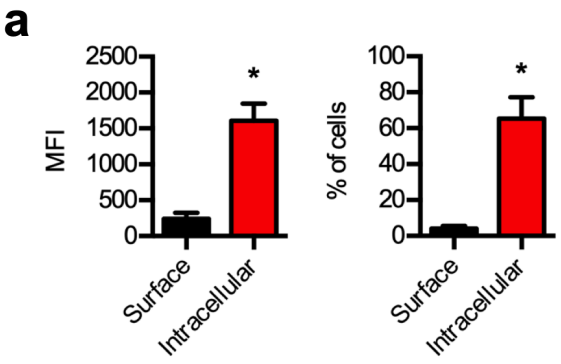
Figure 15. TIM-1 glycoprotein is upregulated on murine neutrophil cell membrane upon stimulation with pro-inflammatory stimuli. (a-b) Flow cytometry analysis of bone marrow derived neutrophils exposed to different stimuli (red bars). Values represent the MFI (**a**) and percentage (**b**) of TIM-1 expressing neutrophils after the exposure to 50 ng/ml PMA, 30 µg/ml PAM3CSK4, 300 nM C5a, 1 µM fMLP, 10 ng/ml TNF-α and 100 ng/ml LPS, normalized to control untreated cells set equal to 100 (**a**) and 1 (**b**), as arbitrary units. Among the stimuli PMA, PAM3CSK4, fMLP and C5a show a higher upregulation of TIM-1 on neutrophil cell membrane compared to ctrl. All represented values are subtracted by the arbitrary unit of the ctrl. Data represent mean ± SEM of 4 different experiments (Student's *t* test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ compared to ctrl). (**c**) Representative images of TIM-1 surface expression in neutrophils stimulated with fMLP for 5 min. TIM-1 expression (green) is higher on the cell membrane (red) upon stimulation with fMLP, compared to untreated ctrl cells. Nuclei are stained in blue. Scale bars = 5 µm.

3. TIM-1 EXPRESSION INCREASES IN HUMAN NEUTROPHILS UPON STIMULATION

Given the promising data obtained on mouse cells, we next investigated the expression of TIM-1 on human neutrophils isolated from the blood of healthy donors.

We confirmed that TIM-1 was retained in the cytoplasm of human neutrophils under resting conditions, as observed by both flow cytometry and immunofluorescence staining (Figure 16 a, b). In particular, the MFI of TIM-1 stored intracellularly and expressed on cell membrane of neutrophils was 1608 ± 238 and 241 ± 60 (mean ± SEM; * $P < 0.05$) and the percentage of TIM-1 positive cells were 65 ± 12 and 4 ± 1 (mean ± SEM; * $P < 0.05$), respectively (Figure 16 a). We further analyzed whether inflammatory stimuli could modulate TIM-1 translocation to the cell membrane similarly to mouse neutrophils. Flow cytometry analysis showed that when stimulated with fMLP, the percentage of TIM-1-

expressing neutrophils rapidly increased, starting already after 1-5 min of stimulation compared to unstimulated cells and dropped to the level of untreated cells over time (fMLP 5 min 2 ± 0.3 *vs* fMLP 15 min 0.5 ± 0.2; mean ± SEM; ** $P < 0.005$) (Figure 16 c). Similar results were obtained by immunofluorescence staining as shown in Figure 16 d. We also investigated the modulation of TIM-1 expression on human neutrophils by treating the cells with another FPR ligand, the oligomeric form of $A\beta_{1-42}$ peptide, which is a potent inflammatory mediator and pivotal key player in the development and progression of AD[238]. We first primed freshly isolated human neutrophils with TNF-α for 10 min, then we stimulated the cells with $A\beta_{1-42}$ or with the reverse peptide $A\beta_{42-1}$ as control for 15 min, and finally we assessed TIM-1 expression by flow cytometry. Surprisingly, we found a significant increase in TIM-1 levels on the cell membrane upon stimulation with oligomeric $A\beta_{1-42}$ compared to cells stimulated with the reverse peptide $A\beta_{42-1}$ or TNF-α alone (Figure 16 e). In particular, the MFI values of TIM-1 expression upon exposure to $A\beta_{1-42}$, $A\beta_{42-1}$ and TNF-α were 110 ± 9, 27 ± 11 and 1 ± 0.4 respectively (mean ± SEM, * $P < 0.05$; ** $P < 0.005$). Indeed, the percentage of TIM-1 expressing neutrophils was 10-fold higher with respect to control cells (Figure 16 e), suggesting that $A\beta_{1-42}$ could trigger TIM-1 expression on human neutrophils during the chronic, inflammatory process occurring during AD pathogenesis.
Overall, these data suggest a potential role of TIM-1 in neutrophil effector functions during the acute and chronic inflammatory responses.



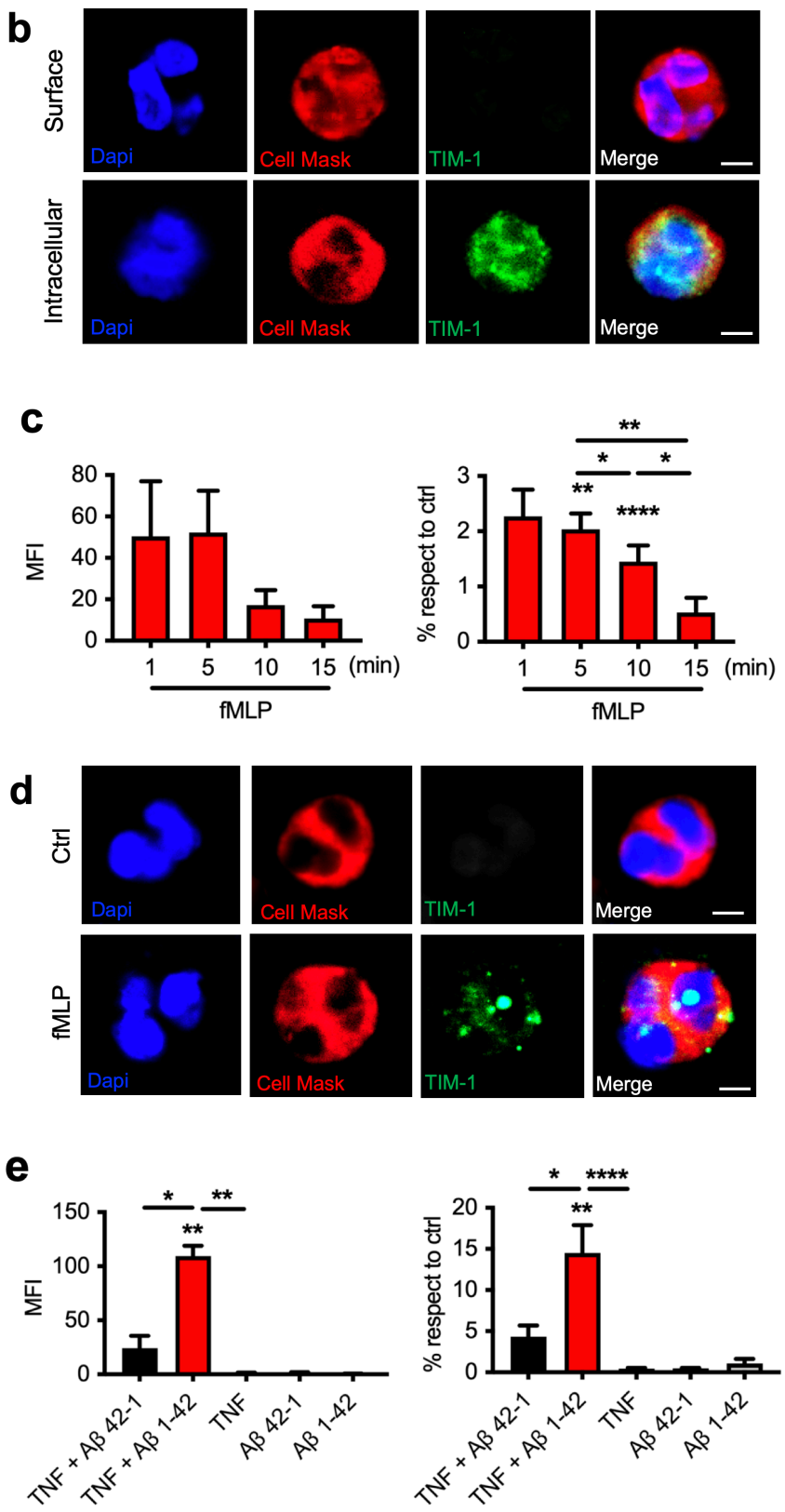


Figure 16. TIM-1 expression is modulated in human neutrophils. (a) Flow cytometry analysis of resting neutrophils isolated from peripheral blood of healthy donors and assessed for membrane surface or intracellular expression of TIM-1. Data represent the mean ± SEM of 3 different experiments (Student's *t* test, * $P < 0.05$ compared to ctrl). (**b**) Representative images of TIM-1 expression (green) in neutrophils on the cell membrane (red) and intracellularly by

immunofluorescence staining. Nuclei are stained in blue. Scale bars = 5 μm. As demonstrated in mouse, human neutrophils retain all TIM-1 glycoprotein intracellularly under normal conditions expressing little level of TIM-1 on the cell surface. (**c-d**) Quantitative and qualitative TIM-1 expression on human neutrophils after stimulation with 1 μM fMLP. (**c**) Flow cytometry analysis of neutrophils stimulated for 1-15 min with fMLP; graphs show TIM-1 expression as MFI and percentage of TIM-1 positive cells. All mean values were normalized to the control resting condition set equal to 100 (MFI) and 1 (percentage) as arbitrary units, and subtracted by the control. Data represent the means ± SEM of 3 different experiments (Student's *t* test, * $P < 0.05$; ** $P < 0.005$; **** $P < 0.0001$ compared to ctrl). (**d**) Representative images of blood-derived neutrophils expressing TIM-1 on the cell membrane under resting condition and upon 5 min of fMLP stimulation. Scale bars = 5 μm. (**e**) Flow cytometry analysis of human neutrophils stimulated with 10 μM of soluble oligomeric $A\beta_{1-42}$ after 10 min of priming with 10 ng/ml of TNF-α. Graphs show the MFI and the percentage of TIM-1+ cells normalized and subtracted from the untreated control. As a comparison we also show the stimulation with the combination of TNF-α and the $A\beta_{42-1}$ reverse peptide, as well as single stimuli. Data represent the means ± SEM of 2 different experiments (Student's *t* test, * $P < 0.05$; ** $P < 0.005$; **** $P < 0.0001$ compared to ctrl).

4. TIM-1 MEDIATES NEUTROPHIL TRAFFICKING DURING STERILE PERITONITIS

As TIM-1 was shown to function as trafficking receptor for activated T lymphocytes[18], we next investigated a potential role for TIM-1 in neutrophil recruitment during acute inflammatory responses using a model of sterile thioglycolate-induced peritonitis.

First, we treated 10-16 weeks-old C57BL/6J mice with 0.4 mg of anti-TIM-1 blocking antibody (RMT1-10 clone) or anti-human RAS (Y13259 clone) control antibody for 3 h, and then we induced peritonitis by i.p. injection of sterile 4% thioglycolate broth. Neutrophils are reported to accumulate in the peritoneal cavity in a time-dependent manner, with a maximal cell influx at 6-10 h after induction[239]. However, in our experimental settings we obtained a significant accumulation of neutrophils in the peritoneum after 1, 3 and 6 h from the thioglycolate administration (data not shown). In order to evaluate an effect by TIM-1 blockade on neutrophil recruitment, we collected peritoneal cell exudates at 1, 3 and 6 h after the induction of the peritonitis. Neutrophils were then assessed by flow cytometry and detected as CD11b+ Ly6G+ cells gated on the CD45+ live cell population (Figure 17 a). TIM-1 blockade significantly reduced neutrophil recruitment to the inflamed peritoneum in comparison with the antibody control treated mice after 3 h from the induction of peritonitis (Figure 17 b), whereas we did not find a statistical significant reduction after 1 and 6 h after thioglycolate administration (Figure 17 b). Of note, the blockade of TIM-1 drastically decreased the absolute number as

well as the percentage of CD11b+ Ly6G+ neutrophils on the total CD45+ infiltrated cells into the peritoneal cavity in comparison with control mice (Figure 17 b).

To further confirm the involvement of TIM-1 as neutrophil trafficking receptor during acute inflammatory responses, we adoptively transferred TIM-1$^{\Delta mucin}$ (lacking the mucin TIM-1 functional domain) or WT neutrophils fluorescently labeled with CMAC cell tracker into C57BL/6J recipient mice. After 30 min from i.v. injection of endogenous cells we induced sterile peritonitis and we assessed the numbers of CMAC+ neutrophils in the peritoneal cavity by flow cytometry (Figure 17 c). From this experimental setting, we obtained a significant difference in the percentage of neutrophils migrating in the inflamed peritoneum 1 h after the induction of peritonitis. In accordance with the data obtained with the anti-TIM-1 blocking antibody, our results showed a 35% reduction in the percentage of neutrophils lacking functional TIM-1 accumulated in the peritoneum in respect to WT cells (Figure 17 c), confirming a role for TIM-1 in neutrophil recruitment during acute inflammation.

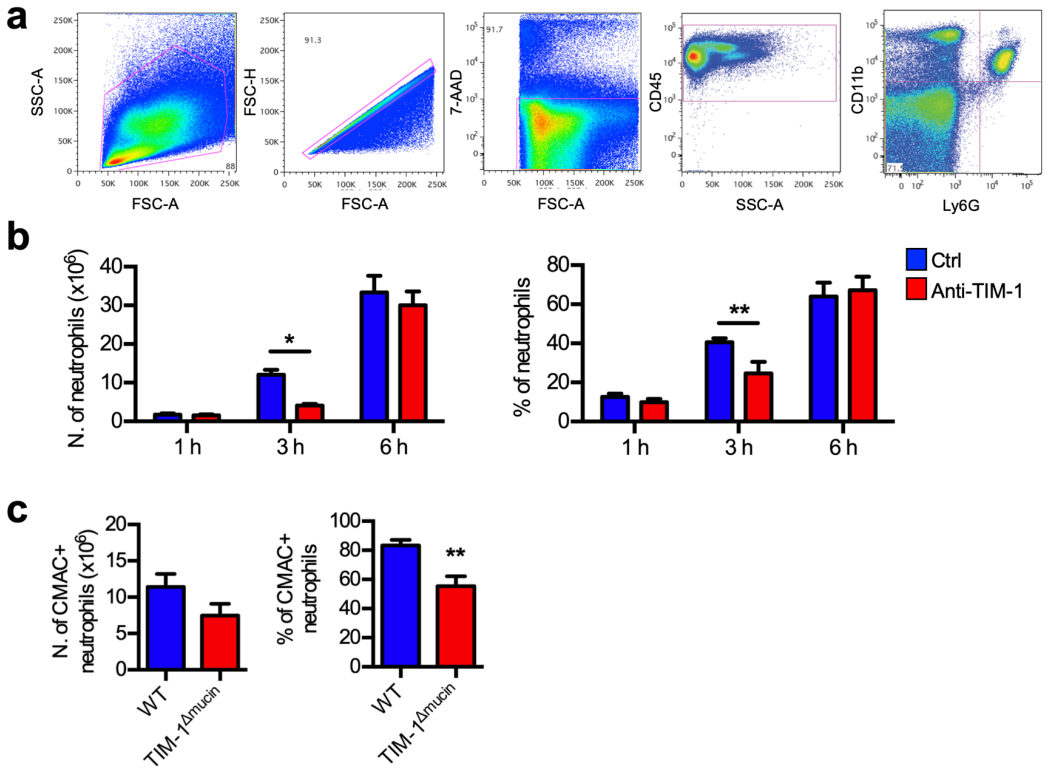


Figure 17. TIM-1 modulates neutrophils migration to the inflamed peritoneum. (**a**) Flow cytometry gating strategy to obtain neutrophils migrated to the inflamed peritoneum. (**b**) Treatment of C57BL/6J mice with 0.4 mg of control antibody or anti-TIM-1 blocking antibody (RMT1-10). After 1 h of treatment, mice were injected with thioglycolate broth to induce peritonitis and peritoneal neutrophils were harvested after 1, 3 and 6 h from the induction of peritonitis. Values are displayed as absolute numbers and percentages of Ly6G+ CD11b+ neutrophils gated on CD45+

leukocytes accumulated into the inflamed peritoneum after 1 h of treatment with anti-TIM-1 antibody. Data represent the mean ± SEM of 6 different experiments for each time point (Student's *t* test, * $P < 0.05$; ** $P < 0.005$ compared to ctrl). (**c**) Quantitative flow cytometry of the absolute number and frequency of CMAC fluorescent WT or TIM-1$^{\Delta mucin}$ neutrophils accumulated in the peritoneum of WT C57BL/6J recipient mice 1 h after peritonitis induction. Data represent the mean ± SEM of 6 different experiments for each condition (Student's *t* test, ** $P < 0.0005$ compared to WT).

5. NEUTROPHILS UPREGULATE TIM-1 EXPRESSION DURING PERITONITIS

Since neutrophils increased TIM-1 protein levels on the cell surface in the presence of inflammatory stimuli *in vitro,* we next checked whether the expression of TIM-1 glycoprotein could be modulated also *in vivo* during inflammatory responses. At this purpose, after 1 h from sterile peritonitis induction in WT C57BL/6J mice, we collected the blood and the peritoneal exudate and assessed the expression of TIM-1 by flow cytometry. As healthy controls, we injected sex- and age-matched mice with PBS.

Our results clearly indicate that under inflammatory conditions, circulating neutrophils displayed higher TIM-1 protein expression on the cell surface in respect of healthy control mice (Figure 18 a, b). Additionally, after 1 h from the induction of peritonitis, where the percentage of migrated neutrophils in the peritoneum generally represents the 30-50% of total CD45+ leukocytes, at least half of infiltrated neutrophils expressed TIM-1 on their cell surface. Besides, the frequencies of neutrophils expressing TIM-1 on their cell surface and accumulated into the inflamed peritoneum was 8-fold higher than the average expression of TIM-1 in blood circulating neutrophils (58 ± 4 and 10 ± 3 respectively; mean ± SEM; **** $P < 0.0001$) (Figure 18 b).

Taken together, these experimental evidences suggest that an acute inflammatory condition could rapidly trigger TIM-1 expression on the cell surface in a specific subject of circulating neutrophils leading their preferential recruitment into the inflamed site.

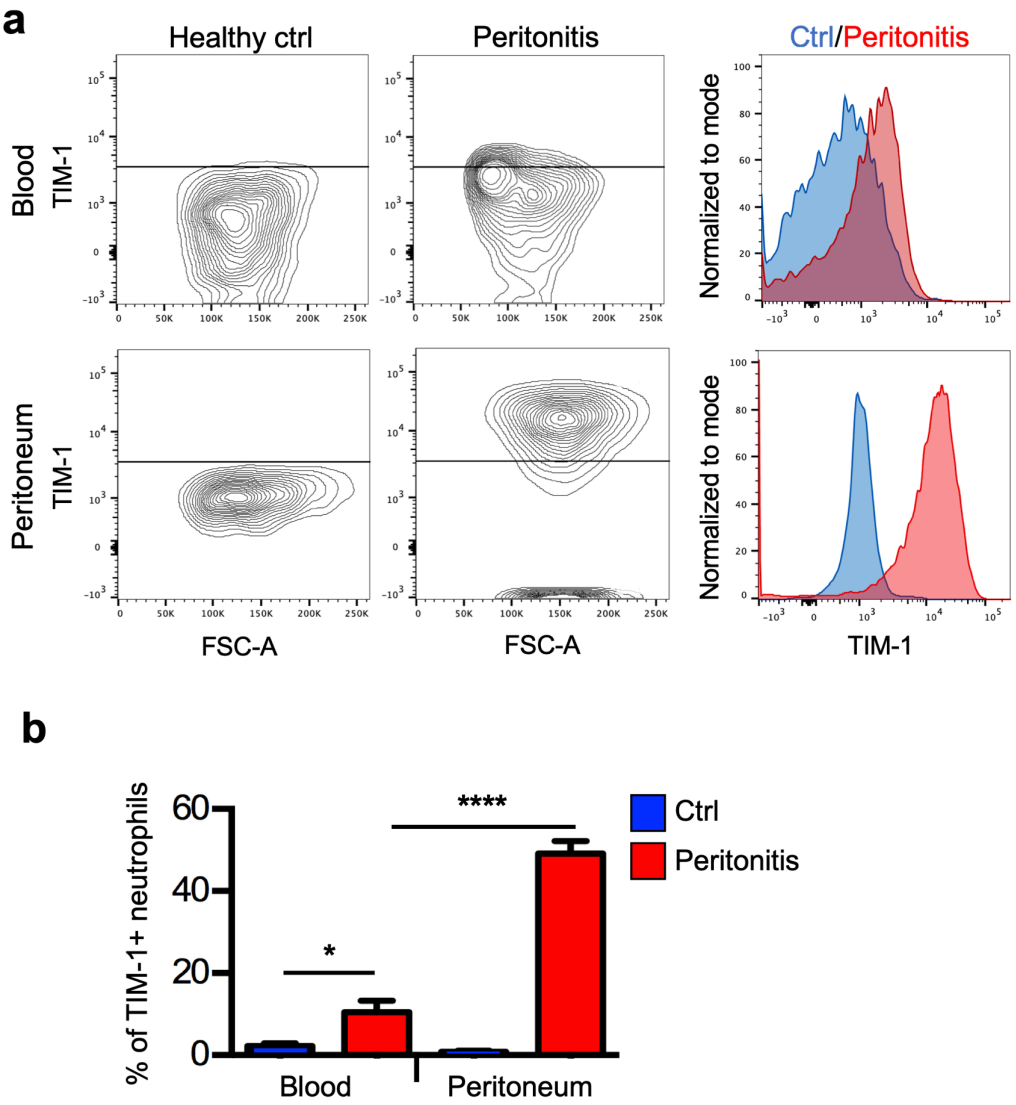


Figure 18. TIM-1 expression is upregulated on neutrophils surface during thioglycolate-induced peritonitis. (**a**) Qualitative and (**b**) quantitative flow cytometry analysis of TIM-1 expression in circulating and peritoneal migrated CD45+ Ly6G+ CD11b+ neutrophils collected after 1 h of thioglycolate-induced peritonitis (red bars) compared to the control (blue bars). TIM-1 levels significantly increase on the cell surface of peripheral blood neutrophil as well as cells isolated from the peritoneal exudate with respect to neutrophils from the healthy ctrl. Data represent the means ± SEM of 6 different experiments (Student's *t* test, * $P < 0.05$; **** $P < 0.0001$ compared to ctrl).

6. CIRCULATING NEUTROPHILS EXPRESS TIM-1 IN 3xTg-AD MICE

Peripheral blood neutrophils upregulated TIM-1 protein expression during acute inflammatory processes, as we observed in the mouse model of thioglycolate-induced peritonitis. We further investigated a potential contribution of TIM-1 in chronic inflammatory conditions focusing our attention on a mouse model of AD.

For this purpose, we used 3xTg-AD mice, which harbor three mutant human transgenes: PS1 (M146V), βAPP (Swedish) and tau (P301L). This mouse model displays two peculiarities: it accumulates extracellular Aβ deposits starting from 6 months of age, and by 9-12 months of age it exhibits tau pathology[218]. Thus, we evaluated by flow cytometry the expression of TIM-1 glycoprotein in peripheral blood neutrophils at different stages of the disease of 3xTg-AD mice and their age- and sex-matched B6129SF2/J controls. Our data showed that the percentage of Ly6G+ CD11b+ neutrophils in the blood was significantly increased in 3xTg-AD mice compared to their age- and sex-matched WT controls at 6, 9 and 12 months of age (Figure 19 a). We also found a propensity in the frequency of circulating neutrophils to augment during the progression of the disease (Figure 19 a).

Surprisingly, at 12 months of age 3xTg-AD mice showed a significant increase in the expression of TIM-1 on the surface of circulating Ly6G+ neutrophils in terms of MFI and percentage of TIM-1 positive cells compared to WT control mice (Figure 19 b). On the other hand, our data in 3xTg-AD mice at 6 and 9 months of age did not show significant difference in the expression of TIM-1 in blood neutrophils compared to WT control (not shown).

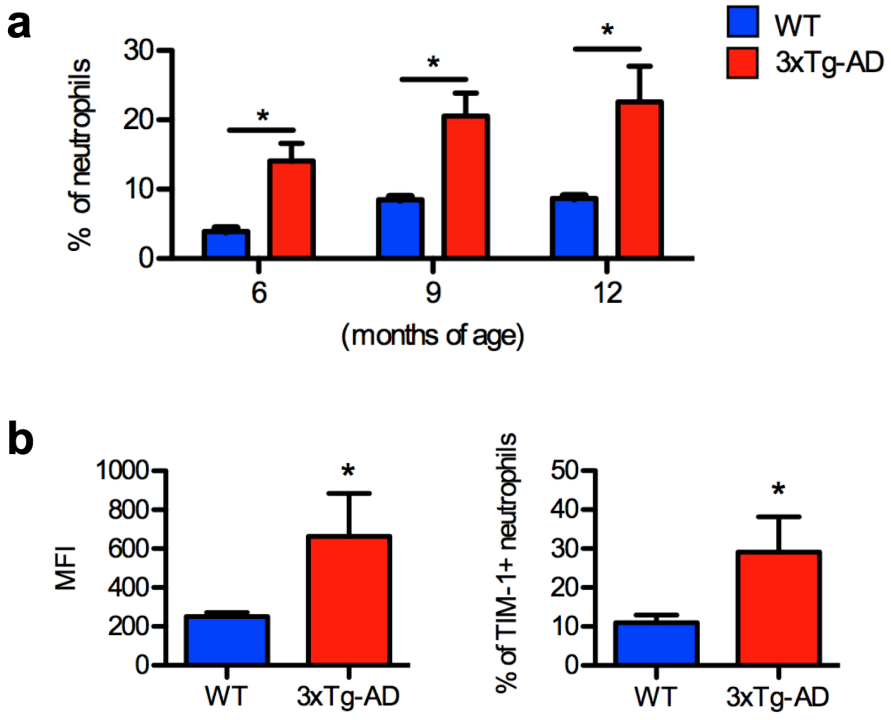


Figure 19. Peripheral blood neutrophils express TIM-1 in 3xTg-AD mice at 12 months of age. (**a**) Flow cytometry quantification of CD45+ Ly6G+ CD11b+ neutrophils isolated from the peripheral blood of 3xTg-AD (red bars) and age- and sex-matched WT control mice (blue bars) at 6, 9 and 12 months of age. (**b**) TIM-1 expression on circulating neutrophils from 3xTg-AD and control mice at 12 months of age. TIM-1 significantly increases on neutrophil surface in 3xTg-AD mice at 12 months with respect to blood cells in WT mice. Data represent the means ± SEM of 3 different experiments at 6 month of age and 4 different experiments at 9 and 12 months of age (Student's *t* test, * $P < 0.05$ compared to WT mice).

7. TIM-1 AS A NEW BIOMARKER IN AD

It is now clear that a soluble form of TIM-1 is released in the urine after kidney injury[240] and tends to accumulate in the CSF of patients with CNS lymphoma[66]. Moreover, the quantification of serum soluble TIM-3 has been recently reported as diagnostic and prognostic marker for osteosarcoma patients[241]. These evidences led us to examine whether the sTIM-1 might be released under the low-grade chronic inflammatory process of AD pathogenesis.

Therefore, we measured TIM-1 protein levels in the serum of 3xTg-AD and relative age- and sex-matched control mice at 6, 9 and 20 months of age by ELISA assay. The sTIM-1 level positively correlated with the progression of the disease. In fact, in the serum of 3xTg-AD at 6 months of age, the pre-clinical stage of the disease, we detected a very low level of sTIM-1 and, importantly, this level was comparable to those found in the serum of age- and sex-matched controls mice (26 ± 3 pg/ml *vs* 31 ± 8 in 3xTg-AD and WT mice respectively; mean ± SEM) (Figure 20). However, sTIM-1 was significantly increased at 9 months of age and more importantly at 20 months of age (82 ± 23 pg/ml and 154 ± 51 pg/ml at 9 and 20 months of age, respectively; mean ± SEM; * $P = 0{,}043$; ** $P = 0{,}0055$ compared to 3xTg-AD mice at 6 months of age) (Figure 20) but it did not change in control mice during aging suggesting that sTIM-1 serum dosage might serve as an early marker for predicting progression of AD. Moreover, the *in vivo* increasing level of sTIM-1 during inflammation strengthens the hypothesis of a role of TIM-1 in leukocyte activation, corroborating the increased TIM-1 expression upon neutrophil stimulation observed by our previous *in vitro* in studies.

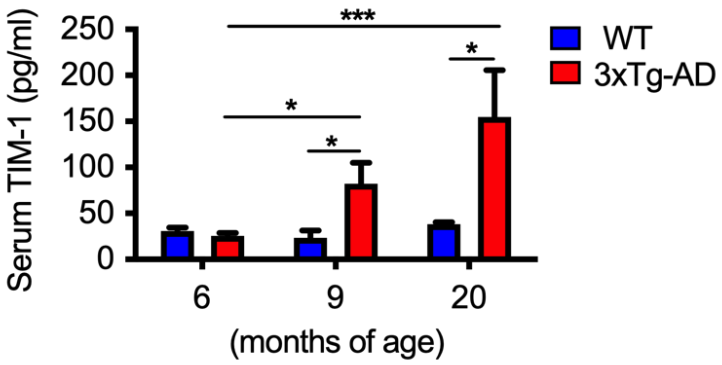


Figure 20. Soluble TIM-1 increases during AD progression in mice. Soluble TIM-1 protein levels were assessed by ELISA in the serum of 3xTg-AD (red bars) and age- and sex-matched control (blue bars) mice at different stages of the disease. Compared to the onset of the disease (at 6 months of age), soluble TIM-1 levels strongly increase at 9 months of age, and more predominantly at 20 months of age, suggesting sTIM-1 as a new marker for AD progression. Data represent the mean ± SEM from one representative experiment obtained from at least 6 mice per condition. (Student's *t* test, * $P < 0.05$; *** $P < 0.0001$).

8. NEUTROPHILS EXPRESS TIM-1 IN THE BRAIN OF 3xTg-AD MICE

The data obtained by a mouse model of peritonitis, clearly showed a stronger increase in TIM-1 expression on the surface of neutrophils accumulated in the peritoneum with respect to circulating cells. Moreover, we demonstrated that circulating neutrophils upregulated TIM-1 protein expression in AD mice compared to WT mice. Based on these results, we further investigated TIM-1 expression in infiltrating neutrophils into the brain of 3xTg-AD mice. As previously reported, in this murine model of AD, neutrophils accumulate preferentially in certain regions of the brain such as meninges and deep cortical layers[222].

By immunofluorescence staining we observed that during the early phases of the disease (7-9 months of age), TIM-1-positive neutrophils mainly localized in the leptomeninges (Figure 21 b) and in the choroid plexus (Figure 21 c), which are privileged sites of entrance of leukocytes in the brain during inflammatory processes[242,243]. However, our preliminary data show that infiltrating neutrophils at the level of brain parenchyma not apparently express TIM-1 on their surface (Figure 21 a); this suggests that TIM-1 could be crucial in driving the entrance of blood neutrophils in the brain during AD pathology, and once entered in the tissue TIM-1 could be shed away. As expected, neutrophils were not detected in the brain of WT control mice.

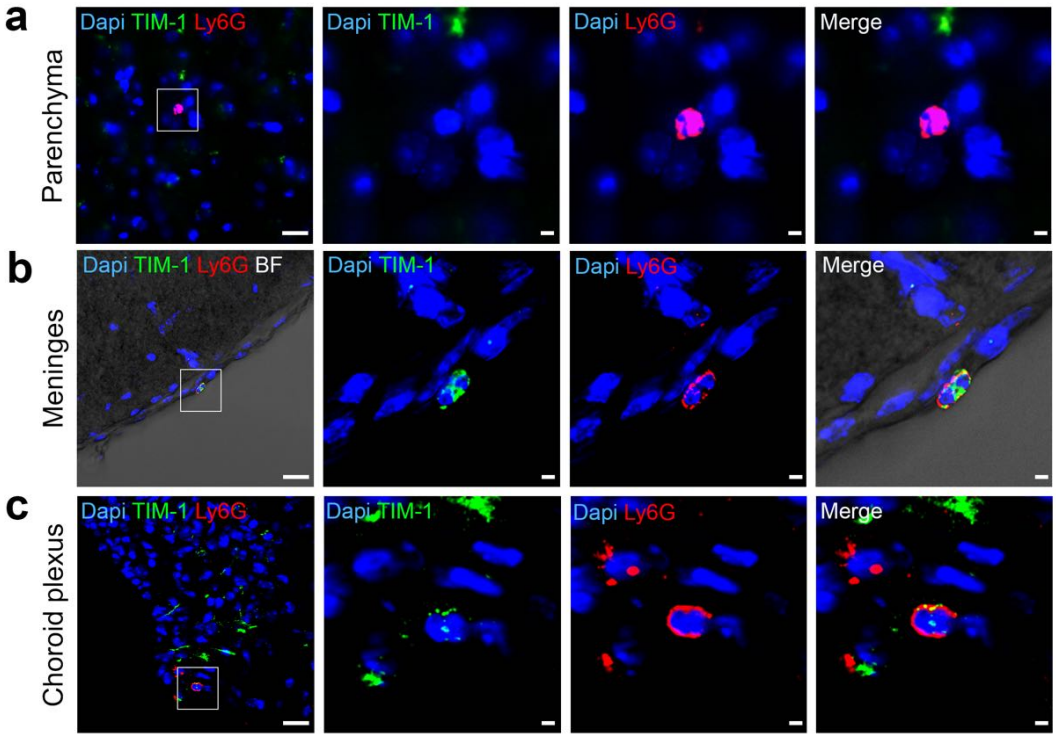


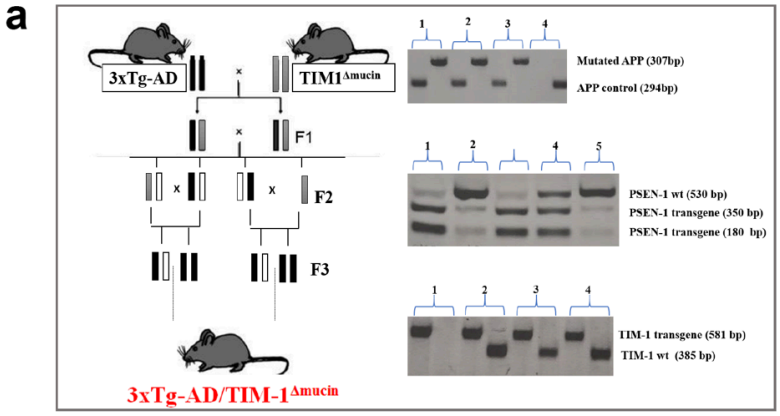
Figure 21. Neutrophil expression of TIM-1 in the brain of 3xTg-AD mice. Representative images of neutrophils labeled with the neutrophil marker Ly6G (red) and TIM-1 glycoprotein (green) inside the brain of AD mice at 7-9 months of age. Neutrophils inside the brain parenchyma are predominantly negative for TIM-1 expression **(a)**, whereas TIM-1-expressing neutrophils are mainly detected in the meninges (**b**) and choroid plexus (**c**), which are main sites of entrance of leukocytes in the CNS during inflammation. Neutrophils are not detected in the brain of age- and sex-matched WT mice. Nuclei are stained with DAPI. First images to the left: scale bars = 10 μm. Magnified images: scale bars = 5 μm.

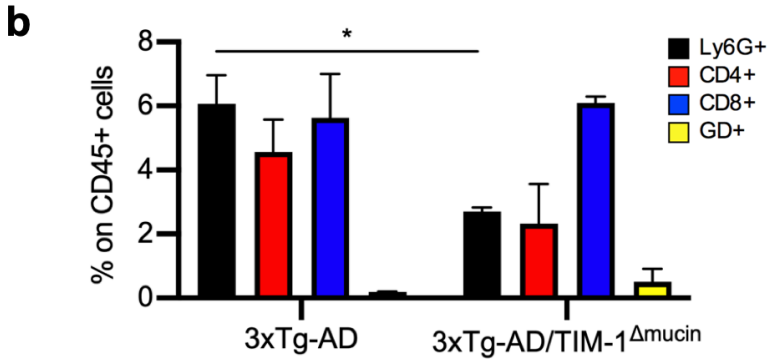
9. 3xTg-AD MICE LACKING FUNCTIONAL TIM-1 SHOW A REDUCED NEUTROPHIL INFILTRATION IN THE BRAIN

To further study the relevance of TIM-1 in neutrophil trafficking during AD, we investigated the effect of TIM-1 genetic deficiency on the development of AD-like disease in 3xTg-AD mice. Therefore, we crossed 3xTg-AD mice with those lacking the functional mucin domain of TIM-1 (TIM-1$^{\Delta mucin}$)[235] to generate a colony of homozygous 3xTg-AD/TIM-1$^{\Delta mucin}$ (Figure 22 a).

Afterwards, we analyzed by flow cytometry, leukocytes accumulation in the brains of 3xTg-AD/TIM-1$^{\Delta mucin}$ and 3xTg-AD control mice at 6 months of age, corresponding to the onset of the disease and the peak of neutrophil infiltration into the brain[218, 222]. Interestingly, the analysis of brain homogenates of 3xTg-AD/TIM-1$^{\Delta mucin}$ showed a 50% reduction in Ly6G+ neutrophils infiltration compared to

3xTg-AD control mice (Figure 22 b). Moreover, the percentage of CD4+ T cell population was also reduced in mice lacking the functional TIM-1, whereas CD8+ and gamma delta (GD) T cell subsets apparently were not affected (Figure 22 b). Additionally, Ly6G+ neutrophils were the most reduced leukocyte subpopulation in mice lacking functional TIM-1 both at 6 and 9 months of age with respect to control mice, indicating that neutrophil infiltration is strongly affected by the functional TIM-1 deficiency (Figure 22 c). Taken together, our data showed a pivotal role of TIM-1 in neutrophils trafficking during the early phases of the disease.





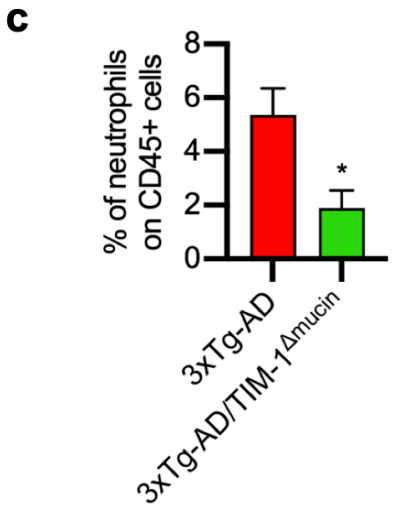


Figure 22. TIM-1 affects neutrophil trafficking into the brain of 3xTg-AD mice. (a) Schematic representation of the generation and genotyping of 3xTg-AD/TIM-1$^{\Delta mucin}$ mice. (**b**) Flow cytometry characterization of leukocyte (LY6G+ CD4+, CD8+, and gamma delta - GD – cell positive) subpopulations accumulated in the brains of 3xTg-AD and 3xTg-AD/TIM-1$^{\Delta mucin}$ mice at 6 months of age. At least 3 animals per group of the same age were analyzed (Student's *t* test; * $P < 0.05$ compared to 3xTg-AD mice group). (**c)** Neutrophil infiltration into the brains of mice lacking functional TIM-1 in comparison to 3xTg-AD mice at 9 months of age. (**b, c**) Data represent the mean ± SEM of at least 3 animals per group of the same age (Student's *t* test; * $P < 0.05$ compared to 3xTg-AD mice).

10. LACK OF FUNCTIONAL TIM-1 IMPROVES COGNITIVE PERFORMANCES IN 3xTg-AD MICE

To further study the implication of TIM-1 glycoprotein in the pathogenesis of AD, we evaluated the effect of TIM-1 deficiency on cognitive functions by performing the following behavioral tests: 1) pre-cognitive tests, used to check correct muscle function and motor coordination and exclude mice with alterations in vestibular function; 2) Y-maze test, which measures the spatial working memory and is dependent on the integrity of the limbic and non-limbic pathways; 3) CFC, which measures associative memory and is highly dependent on the function of the cortex, hippocampus and amygdala.

After excluding any potential motor dysfunctions in 3xTg-AD/TIM-1$^{\Delta mucin}$ mice compared to sex- and age- matched 3xTg-AD control mice, we could proceed with the evaluation of spatial memory by performing the Y-maze. For the test we monitored the entrances of 3xTg-AD/TIM-1$^{\Delta mucin}$, 3xTg-AD and WT mice at 9 and 12 months of age into the 3 arms of the maze to evaluate their spatial working memory. This allowed us to calculate the total arm entries and the alternation score

(percentage of alternation). The total number of arm entries during the session of the test resulted comparable indicating normal motor functions and normal exploratory behavior in all three groups of mice (Figure 23 a, c). As expected, the memory impairment that characterizes 3xTg-AD mice led to a decreased percentage of spontaneous alternation during the Y-maze test in these mice if compared with WT age-matched mice (Figure 23 a, c). Notably, 3xTg-AD/TIM-$1^{\Delta mucin}$ mice showed a significant improvement in cognitive functions with a higher performance of spontaneous alternation when compared to 3xTg-AD mice. In addition, the performance of 3xTg-AD/TIM-$1^{\Delta mucin}$ mice was comparable to the one obtained for healthy age- and sex- matched WT littermates (Figure 23 a, c).

The same group of mice was then tested in the CFC test. In agreement with the results obtained during the Y maze test, mice lacking functional TIM-1 performed better. 3xTg-AD/TIM-$1^{\Delta mucin}$ displayed, indeed, a memory improvement spending more time freezing than sex- and age- matched 3xTg-AD mice group (Figure 23 b, d). Interestingly, 3xTg-AD/TIM-$1^{\Delta mucin}$ showed comparable levels in the freezing response compared to WT control mice, suggesting an almost complete restoration of cognitive impairment (Figure 23 b, d).

Collectively our data indicated that functional TIM-1 deficiency ameliorated cognitive functions in AD mice suggesting a role for TIM-1 glycoprotein in the AD pathogenesis.

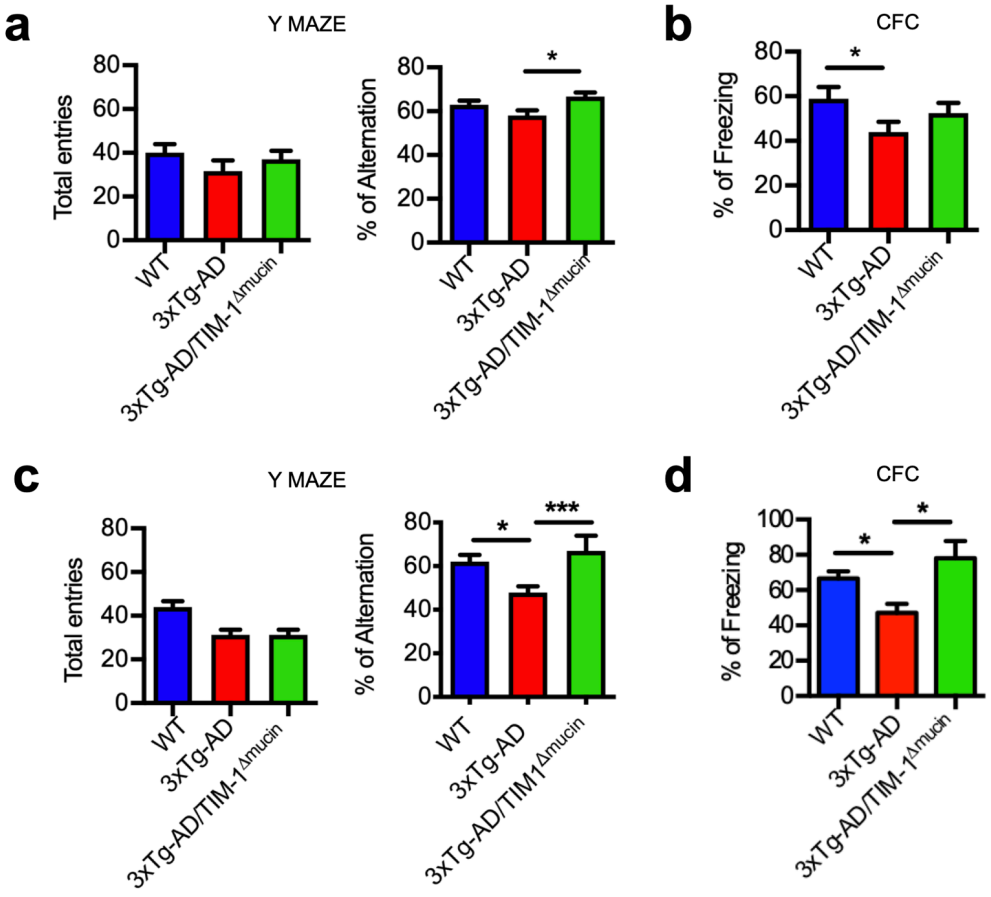


Figure 23. TIM inhibition improves cognition in 3xTg-AD mice at 9 and 12 months of age. 3xTg-AD/TIM-1$^{\Delta mucin}$ mice were tested in the Y-maze and CFC tests at 9 and 12 months of age in comparison with 3xTg-AD mice and, as healthy WT control, B6129SF2/J mice. (**a, c**) The number of total entries in the arms and the percentage of spontaneous alternation performance in the Y-maze test of mice at 9 (**a**) and 12 (**c**) months of age. (**b, d**) The freezing response in the CFC test of mice at 9 (**b**) and 12 (**d**) months of age. Data represent the mean ± SEM of one representative experiment with 12-15 mice per condition. (Student's *t* test; * $P < 0.05$; *** $P < 0.0005$).

11. LACK OF FUNCTIONAL TIM-1 REDUCES NEUROPATHOLOGICAL HALLMARKS OF AD IN 3xTg-AD MICE

Immunohistological analysis of different brain areas allows the identification of subtle pathophysiologic changes in neuronal population that might be associated to behavioral changes.

For this reason, we next investigated the effect of functional TIM-1 deficiency on AD neuropathology by immunohistochemical evaluation of brain samples from 3xTg-AD/ TIM-1$^{\Delta mucin}$ and 3xTg-AD mice, focusing on cortical regions and the CA1 area of the hippocampus, known to be severely affected at earlier stages of the disease in 3xTg-AD mice. Particularly we quantified the Aβ deposition and the

hyperphosphorylated form of tau protein as classical neuropathological hallmarks of AD and microglia activation as hallmark of neuroinflammation.

11.1 FUNCTIONAL TIM-1 DEFICIENCY REDUCES AMYLOID-BETA DEPOSITION IN 3xTg-AD MICE

3xTg-AD and 3xTg-AD/TIM-1$^{\Delta mucin}$ were sacrificed after behavioral assessment at 9 and 12 months of age and we quantified the accumulation of Aβ by immunohistochemistry staining using 6E10 antibody, which recognizes amino acid residues 1-16 of Aβ protein.

As previously reported in literature[244], at these stages of the disease, only intracellular Aβ accumulation was detectable. A significant difference in Aβ levels in the CA1 region of the hippocampus between the two experimental conditions was observed (Figure 24). Particularly, Aβ deposits were drastically reduced in 3xTg-AD/TIM-1$^{\Delta mucin}$ mice compared to sex- and age-matched 3xTg-AD mice at 9 (Figure 24 a) and 12 months of age (Figure 24 c). These qualitative observations were also confirmed by quantitative stereological analysis to determine the total area occupied by Aβ-positive neurons (expressed as pixel^2 / total examined area) (Figure 24 b, d). The values of area occupied by Aβ-positive neurons were significantly decreased with the lack of functional TIM-1 when compared with 3xTg-AD mice, indicating a marked reduction of Aβ deposition in the CA1 region of hippocampus (Figure 24 b). Similar results were confirmed at 12 months of age (Figure 24 d). These data clearly showed a substantial amelioration of Aβ neuropathology following the lack of TIM-1 glycoprotein in 3xTg-AD, suggesting a detrimental role for TIM-1 in the pathology.

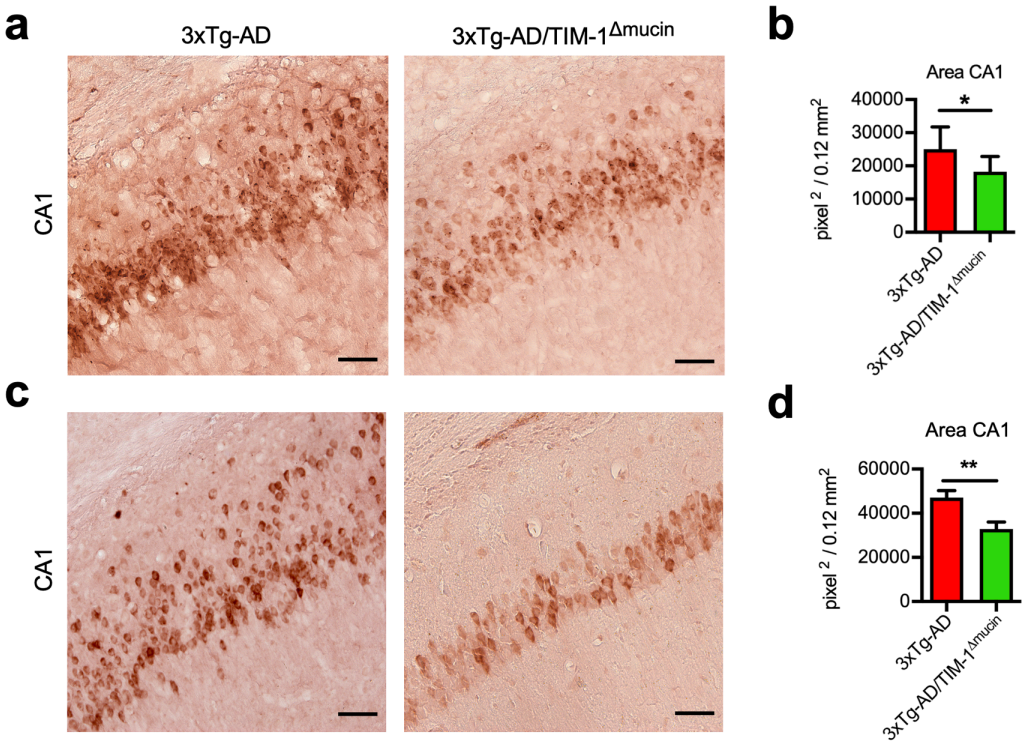


Figure 24. **Lack of functional TIM-1 ameliorates Aβ pathology in AD mice.** (**a-c**) 6E10 monoclonal antibody staining reveal differential Aβ accumulation in 3xTg-AD mice compared to 3xTg-AD/TIM-1$^{\Delta mucin}$ in the CA1 region of hippocampus at 9 (**a**) and 12 (**c**) months of age. 3xTg-AD mice show higher intracellular expression of Aβ in CA1, whereas on the contrary, 3xTg-AD/TIM-1$^{\Delta mucin}$ show a lower intracellular expression of Aβ in the CA1 region compared to the marked accumulation of Aβ in 3xTg-AD control mice. Scale bars, 50 µm. (**b-d**) Quantitative analysis performed in the CA1 region of AD mice at 9 (**b**) and 12 (**d**) months of age. showing the area of Aβ-positive neurons, expressed as pixel^2 / total examined area. Values represent mean ± SEM in each group (Student's *t* test; * $P < 0.05$; ** $P < 0.0075$).

11.2 TIM-1 DEFICIENCY REDUCES MICROGLIA ACTIVATION IN 3xTg-AD MICE

Microglia constitute the main resident immune cell population of the brain and they respond to the presence of Aβ plaques, but the chronic activation of these cells could exacerbate the inflammatory condition and contribute to Aβ plaques deposition[204]. Thus, we further investigated the effect of TIM-1 deficiency on microglia activation, by performing immunohistochemistry staining on 3xTg-AD/TIM-1$^{\Delta mucin}$ mice and 3xTg-AD mice at 12 months of age. To detect microglial cells, we used the antibody against Iba-1, which is a specific marker for these cells. Additionally, we compared the degree of microglia activation between the two experimental conditions, quantifying two parameters: the area occupied by

microglial cells (expressed as pixel^2 / total examined area) and the density of the cells (expressed as number of Iba-1-positive cells / total examined area).

Our data indicated that microgliosis was overall reduced in mice lacking TIM-1 functional domain compared to 3xTg-AD control mice (Figure 25 a). In fact, in 3xTg-AD/TIM-1$^{\Delta mucin}$ microglia changed morphology, appearing with smaller soma and less and thinner ramifications in the examined areas (Figure 25 a, * and #). These qualitative observations were also confirmed by quantitative stereological analysis, showing a significant reduction in the area and density of Iba-1+ microglial cells in the cerebral cortex of TIM-1 deficient mice (Figure 25 b). Besides, we also found a significant reduced microglia activation (in terms of area) in the CA1 region of hippocampus in 3xTg-AD/TIM-1$^{\Delta mucin}$ compared to 3xTg-AD mice at 12 months of age.

In agreement with the data obtained for Aβ accumulation, Iba-1 staining confirmed the inhibitory effect of TIM-1 deficiency on microglia activation, thus contributing to ameliorate the inflammatory milieu in the brain.

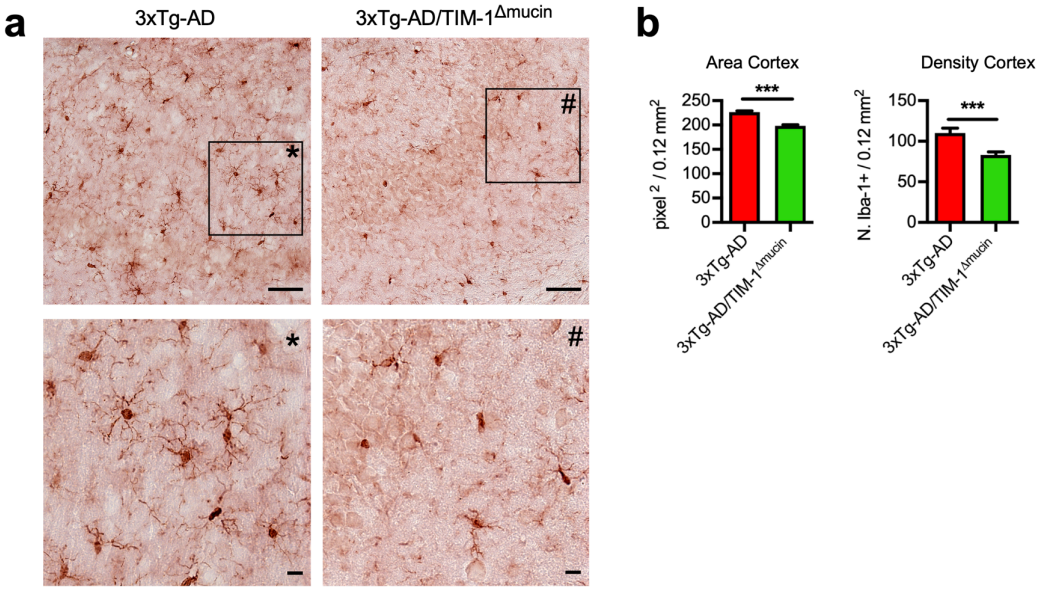
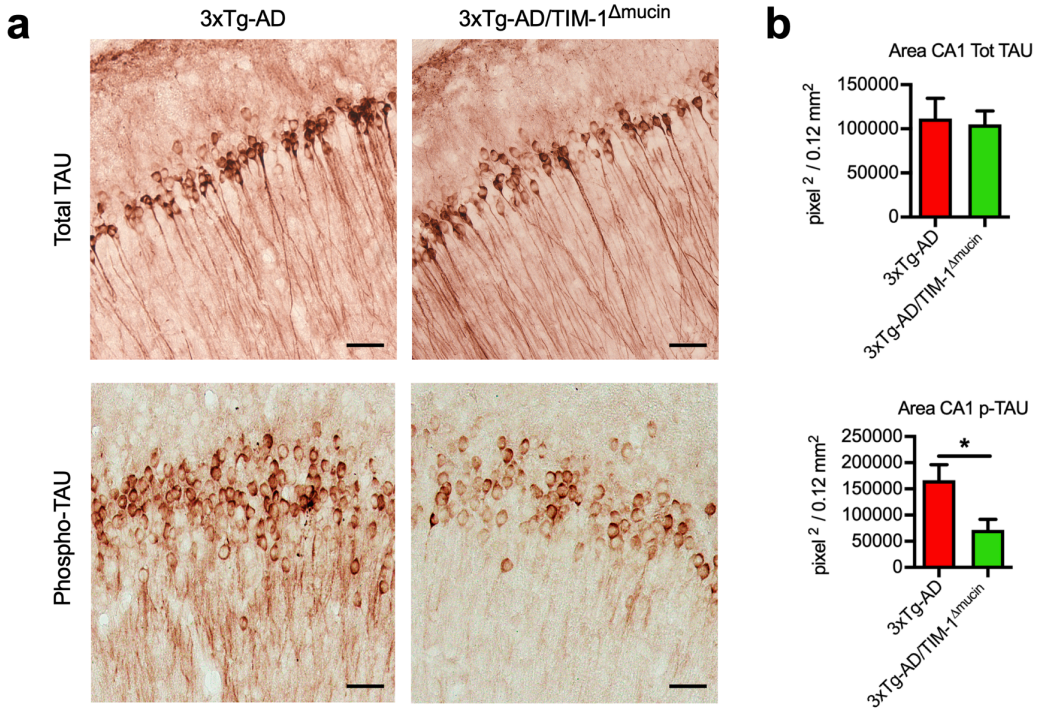


Figure 25. Lack of functional TIM-1 reduces microglia activation in AD mice at 12 months of age. (**a**) Iba-1 monoclonal antibody staining reveals lower microglia activation in 3xTg-AD/TIM-1$^{\Delta mucin}$ compared to 3xTg-AD mice in the cerebral cortex (upper left). Scale bars 50 µm. Lower left are zoomed images of the region of interest (* and #), showing the change in morphology of microglial cells. Scale bars 25 µm. (**b**) Quantitative analysis performed in the cerebral cortex to determine the area and density of microglia cells. Values are mean ± SEM (Student's *t* test, *** $P <$ 0.0001).

11.3 TIM-1 DEFICIENCY REDUCES TAU PHOSPHORILATION IN 3xTg-AD MICE

As abnormalities of tau protein were shown to play a central role in the pathogenesis of AD by leading to a progressive loss of fast axonal transport[244], we finally investigated the effect of the lack of functional TIM-1 protein on tau pathology in 3xTg-AD and 3xTg-AD/TIM-1$^{\Delta mucin}$ mice at 9 and 12 months of age. We used the HT7 antibody to detect total tau expression and AT180 antibody to stain phosphorylated tau at residue Thr231. At 9 months of age AD mice had comparable levels of total tau protein in the CA1 region of the hippocampus, as shown by the qualitative and quantitative immunohistological analysis (Figure 26 a, b). On the contrary, we observed a significant difference in the level of tau phosphorylation in the same brain region. Notably, mice lacking TIM-1 functional domain exhibited a reduction in tau phosphorylation compared to the marked hyperphosphorylation of tau of 3xTg-AD control mice at 9 months of age (Figure 26 a, b). At 12 months of age we did not observe any significant reduction of phosphorylated tau in the CA1 region of the hippocampus between 3xTg-AD/TIM-1$^{\Delta mucin}$ and 3xTg-AD control mice (Figure 26 c), probably due to the lower number of mice used for this analysis. Further experiments are needed to validate an effect of the lack of functional TIM-1 on tau pathology at 12 months of age.



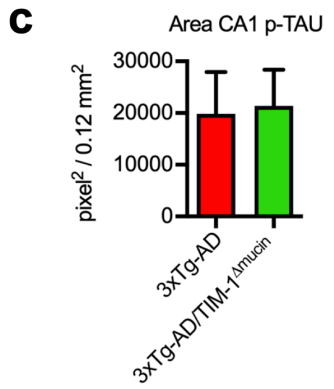


Figure 26. Lack of functional TIM-1 protein ameliorates tau pathology at 9 months of age. (a) Representative images of the CA1 region of the hippocampal area showing lower phosphorylation of tau in 3xTg-AD/TIM-$1^{\Delta mucin}$ compared to 3xTg-AD mice at 9 months of age. Scale bars, 50 μm. (**b**) Quantification of the area occupied by cells expressing total tau (detected with HT7 antibody) and phosphorylated tau (detected with AT180 antibody) in the CA1 hippocampal area at 9 months of age. (**c**) Quantification of the area occupied by cells expressing phosphorylated tau in the CA1 region of the hippocampus at 12 months of age. Values are mean ± SEM (Student's *t* test, * $P < 0.01$).

Taken together our data showed a pivotal role for TIM-1 in a mouse model of AD contributing to reduced neutrophil infiltration in the brain, decreased neuroinflammation in terms of lower microglial activation, Aβ deposits and phosphorylated tau, and it contributed to restore memory. These suggest that TIM-1 may play a role in the development of the disease and that TIM-1 blockade may represent an attractive new therapeutic approach in AD.

DISCUSSION

Previous studies have shown that TIM-1 glycoprotein plays crucial roles in immune responses, including transplant tolerance, autoimmunity, regulation of allergy and asthma, and response to viral infections[67, 70], pointing to TIM-1 as a potential therapeutic target for inflammatory diseases. TIM-1 was initially described as a marker of T cells, and since then, it has aroused great interest as it was found in a variety of immune cells including B cells[8], dendritic cells[29], NK cells[30], NKT[245], macrophages[246] and mast cells[31]. However, no published data are currently available regarding a potential TIM-1 expression in neutrophils. Neutrophils are crucial players in the innate immune response as first responders to tissue damage. Their multiple defense mechanisms, such as the phagocytic capacity, ROS production and ability to degranulate and form NETs, contribute to the effective clearance of pathogens and non-microbial agents responsible for tissue malfunctioning or injury[73]. Till now, there are very few studies reporting indirect association of TIM-1 with neutrophil infiltration. In one study, for instance, the authors showed that in a mouse model of renal IRI, TIM-1, which is upregulated on renal epithelial cells, induced a strong neutrophil accumulation in the inflamed kidney through the binding of leukocyte mono-immunoglobulin (Ig)–like receptor 5 (LMIR5)/CD300b, a neutrophil counter-ligand[240]. Moreover, a study on a model of partial warm liver IRI has revealed that TIM-1 blockade decreased local neutrophil infiltration[38]. However, in these studies TIM-1 expression on neutrophils was not investigated. Interestingly, it was reported that neutrophils can express TIM-3, another member of the TIM protein family, and this molecule is involved in mediating bacterial killing during acute inflammation[248].

Thus, in the light of these observations we were prompted to investigate whether murine neutrophils express TIM-1. By using flow cytometry analysis and immunofluorescence staining, we found that both mouse and human neutrophils expressed TIM-1. Particularly, under resting conditions in human peripheral neutrophils and in mouse blood- and bone marrow-derived neutrophils, TIM-1 glycoprotein is almost absent on the plasma membrane and it is mainly stored intracellularly. Additionally, by immunofluorescence staining we observed that TIM-1 is not only widespread in the cytoplasm, but it is predominantly located just

beneath the cell membrane. This cellular localization in resting neutrophils is similar to that observed in naïve T cells[18, 19, 20] where TIM-1 is largely stored in intracellular pools such as early endosomes, the Golgi apparatus and lysosomes, and it is present at only low levels on the plasma membrane[19, 20]. By flow cytometry analysis and immunofluorescence staining, we further demonstrated that resting neutrophils in response to certain DAMPs and PAMPS stimuli promptly upregulated TIM-1 on their surface. Particularly, when exposed to TLR-1/2 and FPR ligands, TIM-1 levels rapidly increased on neutrophil surface, starting already after 1-5 minutes from stimulation compared to unstimulated cells. However, TNF-α and LPS did not significantly change TIM-1 surface expression at any time of the performed assay compared to untreated controls, suggesting that TIM-1 expression is induced by specific cell stimuli. Given the high rate of glycosylation of the mucin domain and the rapidity of the translocation in response to the stimulation, our data corroborate the assumption that TIM-1 glycoprotein is already present intracellularly, ready to be exposed. Previous reports on T cells[18, 21, 249] and our own data indicate that TIM-1 could be accumulated within cytoplasmic vesicles, also possibly in neutrophil granules, and beneath the cell membrane. In this case, the TIM-1 translocation on the cell surface could be induced by the release of intracellular calcium following neutrophil activation. This occurs in response to fMLP stimulation, in which the binding to the GPCRs activates several signaling pathways, including PLC and PI3K[252]. PLC leads to the downstream release of calcium, contributing in turn to PKC-dependent degranulation[252]; the activation of PI3K in lymphocytes is mainly required for TIM-1 signaling[24]. Additionally, TIM-1 has been proposed to accumulate beneath the cellular membrane, anchored to PS in a bent conformation. An increased concentration of intracellular calcium could, therefore, induce the exposure of PS (and consequently of TIM-1) to the outer sheet of the membrane, promoting the presentation of the whole molecule with a flip-flop mechanism[19]. The prompt TIM-1 translocation to the cell membrane in response to signals that initiate the inflammatory response, suggests that TIM-1 could be necessary to mediate specific neutrophil functions. Notably, the upregulation of TIM-1 on neutrophil surface after fMLP stimulation, which is a potent leukocyte chemotactic factor, could be particularly relevant for neutrophil trafficking[73].

In this PhD thesis, we further tested the potential involvement of TIM-1 as neutrophil adhesion receptor during acute inflammatory responses. By adopting a

model of sterile thioglycolate-induced peritonitis, we demonstrated that the systemic TIM-1 blockade drastically halved the recruitment of CD11b+ Ly6G+ neutrophils into the peritoneal cavity after 3 h from the induction of peritonitis. This reduction was not significant at later time points (6 h from the peritonitis induction), suggesting that TIM-1 is as an early signal for neutrophil migration at the beginning of the inflammatory process and before to the upregulation of others adhesion molecules[253]. These data were confirmed by studies in which we injected exogenous TIM-1$^{\Delta mucin}$ (lacking the mucin TIM-1 functional domain) or WT neutrophils into C57BL/6J mice. In this experimental setting, the lack of functional TIM-1 strongly reduced neutrophil accumulation in the inflamed peritoneum of recipient mice, sustaining our hypothesis that TIM-1 could take part in neutrophil recruitment during acute inflammation. In support of these data, several studies have previously shown that TIM-1 inhibition reduces leukocytes recruitment in models of inflammatory diseases. In experimental model of liver IRI, preventive treatment with the low-affinity RMT1-10 antibody was reported to ameliorate the hepatocellular damage and to rescue liver function by reducing the infiltration of neutrophils and the production of proinflammatory cytokines[38]. Similarly, in a model of cerebral stroke the authors confirmed that blockade of TIM-1 effectively reduced the number of neutrophils and CD3, macrophage functionality and cytokine and chemokine production, thus having a protective effect on the brain tissue[247]. In accordance, our group recently confirmed a role of TIM-1 in neutrophil recruitment in EAE-immunized mice. By using epifluorescence intravital microscopy technique we found that TIM-1 blockade greatly inhibited rolling and firm adhesion of neutrophils in the spinal cord venules during early phases of EAE (unpublished data). Another reason that supports TIM-1 as a cell adhesion receptor is related to its structure. TIM-1 presents in the external region an IgV-like domain, followed by a highly glycosylated mucin domain, enriched in O-glycosylation sites[13]. This peculiar structure is similar to the mucin-like domain of MadCAM-1, an adhesion molecule which classically control leukocyte trafficking into secondary lymphoid organs and chronically inflamed tissues[47, 250]. Moreover, the O-glycosylation profile predicted for TIM-1 is comparable to the one found in PSGL-1[251], another well characterized mucin adhesion molecule expressed by leukocytes and involved in cell trafficking.

Currently, it is well established that neutrophils are heterogeneous plastic cells that rapidly change their characteristics and behavior after activation, aging or migration into peripheral tissues. This heterogeneity is a fundamental property for neutrophils as it confers them specific effector functions and the ability to adapt to environmental changes under homeostatic and inflammatory conditions[102]. Neutrophil activation by cues derived from sites of inflammation results in the exposure of distinct surface molecules from intracellular pools, contributing to change their phenotype. Indeed, activated neutrophils display for instance increased expression of integrin CD11b/CD18 (or Mac-1)[106] and complement surface receptors such as CR1 (CD35)[107], or downregulation of surface markers such as CD62L[108]. Our data show that under inflammatory conditions circulating neutrophils display higher TIM-1 protein expression on the cell surface compared to healthy control mice, suggesting TIM-1 may represent a novel marker of neutrophil activation under inflammatory conditions. Moreover, after 1 h from the induction of peritonitis, when the percentage of migrated neutrophils in the peritoneum generally represents the 30-50% of total CD45+ leukocytes, at least half of infiltrating neutrophils expressed TIM-1 on their cell surface. Besides, the frequencies of extravasated neutrophils expressing TIM-1 on their cell surface was 8-fold higher than the average expression of TIM-1 in blood circulating neutrophils. We observed similar results during the low-grade chronic inflammation in 3xTg-AD mice, the animal model of AD. In this pathological condition, the frequency of circulating neutrophils is generally increased in 3xTg-AD mice compared to their age- and sex-matched WT controls at 6, 9 and 12 months of age, and they tend to augment during disease progression. Surprisingly, at 12 months of age 3xTg-AD mice showed a significant increase in the expression of TIM-1 on the surface of circulating neutrophils compared to WT control mice. Overall, our data clearly demonstrated that acute and chronic inflammatory conditions could rapidly trigger TIM-1 expression on the cell surface of neutrophils *in vivo*, probably in a specific subject of circulating neutrophils, leading to their preferential recruitment into the inflamed site.

In this PhD thesis we also investigated whether the soluble form of TIM-1 might be released under the low-grade chronic inflammatory process of AD. In fact, numerous studies revealed that cell membrane molecules of diverse functional groups are subjected to proteolytic cleavage, and the released soluble form of

proteins may modulate various cellular processes and contribute to disease pathologies[254]. Therefore, in addition to the upregulated expression of surface proteins, the shedding of membrane proteins may comprise an additional resource of noninvasive and accessible biomarkers. Interestingly, sTIM-1 significantly accumulated in the serum of 3xTg-AD mice at 9 months of age and more importantly at 20 months of age, but did not change in control mice during aging. Conversely, in the serum of 3xTg-AD at 6 months of age, which corresponds to the pre-clinical stage of the disease, we detected a very low level of sTIM-1 that was comparable to that found in the serum of age- and sex-matched control mice. Our data suggest that sTIM-1 in the serum might be useful as a marker for AD progression. In this regard, sTIM-1 is currently used as diagnostic marker for acute kidney injury and renal carcinomas in the clinics, as it is released in urine after tissue injury[67, 68]. Interestingly, sTIM-1 is recently found in the CSF of patients with CNS lymphoma in the active phase of the disease[66]. Thus, it would be interesting to test the levels of sTIM-1 not only in the serum, but also in the CSF of AD patients. Another potent inflammatory mediator and pivotal key player in the development and progression of AD is the oligomeric form of $A\beta_{1-42}$ peptide[238, 255]. Particularly, $A\beta_{1-42}$ peptide was recently described as a strong chemoattractant for neutrophils during AD. Most of MPO+ neutrophils migrated in the brain parenchyma were indeed found in close proximity to Aβ deposits, in a non-random distribution[222, 230]. Moreover, vascular deposition of Aβ in the CNS was shown to induce the expression of endothelial adhesion molecules, promoting the transmigration of circulating neutrophils[255]. The increasing level of serum sTIM-1 during inflammation strengthens the hypothesis of a role of TIM-1 in leukocyte activation, corroborating the increased TIM-1 expression upon neutrophil stimulation, as observed by our previous *in vitro* studies. Thus, we evaluated the *in vitro* effect of $A\beta_{1-42}$ peptide on TIM-1 expression in human neutrophils. Our data have confirmed that Aβ peptide stimulation strongly upregulated TIM-1 levels on neutrophil surface.

In the light of these results, by using a murine model of AD, we further explored the contribution of TIM-1 in neutrophil responses during neuroinflammation. Our results sustain the hypothesis that among all the potential biological functions that TIM-1 could exert in neutrophils, it can potentially have a role as a trafficking receptor. Although leukocyte trafficking during AD has not

been investigated in great detail, several lines of evidence now support the hypothesis that both innate and acquired immune cells migrate into the brains of AD patients and relative animal models[230, 256, 257]. Particularly, Zenaro et al. reported that neutrophils accumulate preferentially in certain regions of the brain in the 3xTg-AD mouse, such as meninges and deep cortical layers, and inside the cortex and hippocampus of individuals with AD, contributing to tissue inflammation and neuronal damage[222]. Thus, we further asked whether TIM-1+ neutrophils could infiltrate the brain, and in which brain regions of 3xTg-AD mice were mainly accumulated. By immunofluorescence staining we observed that during early phases of the disease (7-9 months of age), TIM-1-positive neutrophils mainly localized in the leptomeninges and in close proximity of the choroid plexus, which are privileged sites of entrance of leukocytes in the brain during inflammation[242, 243]. However, we our preliminary data apparently did not show TIM-1 expression on the surface of infiltrating neutrophils in the parenchyma. These data, together with the higher expression of the molecule in circulating neutrophils, suggest that TIM-1 could be crucial in driving the entrance of blood neutrophils in the brain during AD pathology, and once entered in the tissue TIM-1 could be shed away.

We further studied the relevance of TIM-1 during the inflammatory process of AD, by investigating the effect of TIM-1 genetic deficiency on the development of AD-like disease in 3xTg-AD mice crossed with those lacking the functional mucin domain of TIM-1 (TIM-1$^{\Delta mucin}$)[235]. We analyzed the levels of leukocyte accumulation in the brains of 3xTg-AD/TIM-1$^{\Delta mucin}$ and 3xTg-AD control mice at 6-9 months of age, the time point corresponding to the onset of the disease and the peak of neutrophil infiltration into the brain in 3xTg-AD mice[218, 222]. Even though 3xTg-AD/TIM-1$^{\Delta mucin}$ showed also a reduced infiltration of CD4+ T cells compared to control mice, Ly6G+ neutrophils were the most reduced leukocyte sub-population in mice lacking functional TIM-1 both at 6 and 9 months of age. These data indicate that TIM-1 could play a pivotal role in neutrophil trafficking during the early phases of the disease.

In order to evaluate the contribution of TIM-1 on the cognitive aspect of the pathology, we also carried out Y-maze and CFC behavioral tests, which assess respectively the spatial working memory dependent on the hippocampus, and the associative memory dependent on the cortex, hippocampus and amygdala[218, 258, 259].

We used two behavioral tests in order to obtain robust and reproducible results and to better assess the effect of functional TIM-1 deficiency in different cognitive domains. In addition, we analyzed two time points of the disease to better appreciate a different effect of functional TIM-1 deficiency at earlier and later time points of the disease. Additionally, from a technical point of view, at 9 months of age mice did not show any apathic behavior yet, as a result of the progression of AD, but they were easily stimulated to explore the environment, differently from later time points of the disease[218, 259]. In both Y-maze and CFC tests, functional TIM-1 deficiency led to a significant restoration of memory impairment at 9 months of age compared to 3xTg-AD age-matched control animals, but this effect appeared more marked in 12 months old mice. These data suggest that inhibition of TIM-1 has therapeutic effect on disease in a transgenic mouse model of AD.

Brain immunohistological analysis allows the identification of subtle pathophysiologic changes in neuronal population such as Aβ plaques deposition, tau phosphorylation and microglia activation, that might be associated to behavioral changes. Notably, the first memory impairment in 3xTg-AD mouse model, is detectable at 4 months of age and correlates with intracellular deposits of Aβ plaques in hippocampus and amygdala. At 6-months of age, these mice start presenting difficulties to retain information day by day. The continued accumulation of Aβ is likely to account for the continued decline of the cognitive function[221]. In accordance with the behavioral assessments, we found a significant reduction of intraneuronal Aβ deposits in 3xTg-AD/TIM-1$^{\Delta mucin}$ mice compared to age-matched control 3xTg-AD in the CA1 hippocampal region at 9 and 12 months of age.

The distribution of activated microglia is different in the brain regions of 3xTg-AD mice. In fact, activated microglia is evenly distributed throughout the hippocampus from 3 to 15 months of age in 3xTg-AD mice, while they get progressively activated in the cortical region during the disease[244, 260]. Thus, we performed the immunohistochemical analysis in the cortex in order to see a more significant modulation of the cell activation. In addition, it is noticeable that the cognitive functions assessed through the behavioral tests seem to go hand in hand with the activation of microglia in the cortex; when mice show more evident cognitive dysfunctions (as we observed at 12 months of age) the microglia is more activated, and *vice versa*[244]. For these reasons we further assessed the effect of functional

TIM-1 deficiency on microglia activation in the cerebral cortex where, particularly at 12 months of age, we observed a marked reduction of microglia activation in mice lacking functional TIM-1. The presence of the activated microglia in the cortex could be considered as an index of disease progression[244].

Together with Aβ deposition and microglia activation, we also assessed another common pathological feature of AD, that is the phosphorylation of tau. Our preliminary data indicated that TIM-1 deficiency has also a beneficial effect on tau pathology, by reducing the levels of phosphorylated tau in the CA1 region at 9 months of age. However, at 12 months of age we did not see any differences in terms of phosphorylated tau between 3xTg-AD/TIM-1$^{\Delta mucin}$ and 3xTg-AD control mice. Our findings are apparently in conflict with some reports, showing that 3xTg-AD mice exhibit age-dependent tau pathology in the same hippocampal region[218, 261]. However, given the lower number of mice used for this analysis further experiments are needed to better understand the effect of functional TIM-1 deficiency on tau pathology at 12 months of age. Moreover, the phosphorylation of tau is only detectable in the CA1 region of the hippocampus of 3xTg-AD mice, therefore we could not perform any evaluation of tau phosphorylation in cortical regions. These observations are in agreement with what was reported by others showing that AT180-positive cells were not detectable until later time points of the disease (approximately 12-20 months of age) in cortical regions of 3xTg-AD mice[244].

Since PSGL-1 has long been indicated as the main P-selectin ligand expressed by leukocytes[48], by using different animal models of inflammation, recent data demonstrated that P-selectin expressed by inflamed blood vessels binds also TIM-1 expressed on the surface of Th1 and Th17 cells, promoting their adhesion and migration into peripheral tissues such as skin and CNS[18]. At the same time, it is now well established that mouse and human P-selectin contribute significantly to neutrophil recruitment to the inflamed tissue[263, 264], leading P-selectin antagonists to be proposed as inflammatory modulators in several pathologies[265]. Moreover, some authors, by using the same model of thioglycolate-induced peritonitis we used in this study, reported that P-selectin blockade or P-selectin deficiency have anti-inflammatory effects compromising neutrophil accumulation into the inflamed peritoneum[263, 264, 266, 267]. In addition, in the 3xTg-AD mouse model the expression of P-selectin is significantly augmented in cerebral

vessels compared to the brain of healthy animals, indicating the presence of activated endothelium that may drive leukocytes migration in the inflamed CNS[222]. Besides, P-selectin was also found increased in the plasma of AD patients with faster cognitive decline[268, 269]. Furthermore, to sustain the amelioration of AD pathology obtained from targeting TIM-1, we also treated 3xTg-AD mice at 9 months of age with an anti-P-selectin blocking antibody (RB-40 clone) and assessed cognitive functions. Our preliminary results indicated that the blockade of P-selectin in 3xTg-AD mice ameliorated cognitive functions in Y-maze and CFC tests compared to mice treated with the control antibody.

On these bases, we hypothesized that endothelial P-selectin could be one of the TIM-1 counter-ligands, that could potentially drive the neutrophil recruitment to sites of inflammation. A very preliminary result showed that when neutrophils isolated from 3xTg-AD, which express higher TIM-1 levels on their surface, are pre-treated with anti-PSGL-1 blocking antibody (4RA10 clone) and then fluxed in capillary tubes coated with P-selectin, the number of rolling neutrophils is significantly higher if compared to control WT cells. However, it remains to be investigated the impact of TIM-1 inhibition on neutrophil rolling under flow adhesion assays. Moreover, the role of TIM-1 in T cell trafficking was also previously demonstrated in the presence of PSGL-1 indicating a concurrent cooperation between these two trafficking molecules in the control of P-selectin-dependent rolling[18, 48, 250].

In conclusion, our findings indicate that TIM-1 is a novel trafficking receptor required for the recruitment of neutrophils during acute and low-grade chronic inflammatory responses, and may represent a novel biomarker of disease progression as well as an attractive therapeutic target in neutrophil-mediated inflammatory diseases.

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