TIM-1 is a physiological P-selectin ligand that mediates T-cell trafficking during inflammation

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Abstract

Leucocyte trafficking is an important mechanism of immune surveillance that enables immune cells to migrate to and from peripheral tissues, providing primary and secondary immune responses as requested. The interaction between leukocytes and the inflamed endothelium are mediated by selectins, integrins, and immunoglobulin (Ig) gene super family proteins. Moreover, other important glycoproteins involved in this process are the mucins, which serve as glycoprotein ligands for selectins. Selectins play a central role in leukocyte trafficking by mediating the first phases of tethering and rolling on vascular surfaces. Tims proteins are a class of mucin able to bind a diverse set of ligands. The structure of Tim proteins, in particular the one of Tim-1 is similar to those of the mucin mucosal addressin cell adhesion molecule (MadCAM)-1, a classical adhesion receptor involved in leukocyte trafficking in the immune system able to bind both selectins and integrins. The mucin domain of Tims protein exhibit several sites of O- and N-glycosilation similar to those observed on P-selectin glycoprotein ligand (PSGL)-1; the most charachterized ligand of selectins. Moreover, it has been shown that the IgV domain of Tim-1, exhibits characteristics of the C-type lectins, as its non-species-specific binding to carbohydrate moieties of several cell types is calcium sensitive and is reduced in cells with defective O- and N-linked carbohydrate synthesis. All these structural observations led us to investigate a potential role for Tim-1 in leukocyte trafficking in inflamed tissues as highly glycosilated molecules like C-type lectins, mucins, integrins and Ig-superfamily members are involved in this process. For this reasons we initially tested the ability of Tim-1 to bind selectin, that are known to interact with highly glycosilated mucin like Tim-1.

Here we report that T cell immunoglobulin and mucin domain 1 (TIM-1) is a novel P-selectin ligand. We first reported the ability of both human and murine Tim-1 to bind P-selectin in vitro and under shear stress conditions in a cell free system. We then demonstrated the importance of TIM-1 in mediating tethering and rolling of Th1 and Th17 cells on P-selectin in underflow rolling assays. Cells lacking the mucin domain of Tim-1 displayed a strong reduced ability to interact with P-selectin underflow in vitro. To evaluate the importance of Tim-1 –P-selectin binding in vivo we performed intravital microscopy in thrombin-activated mesenteric venules
displaying that Th1 and Th17 cells lacking the TIM-1 mucin domain showed reduced rolling ability in vivo in a P-selectin dependent model of inflammation. Uniquely, the TIM-1 IgV domain was also required for P-selectin binding. To evaluate a potential physiologic role for Tim-1/P-selectin interaction in mediating leukocyte trafficking in vivo during inflammatory responses, we demonstrated that inhibition of TIM-1 reduced T cell recruitment in a contact hypersensitivity model (CHS) of inflammation. We then demonstrated the importance of Tim-1 in mediating T cell recruitment in the inflamed brain microcirculation adopting intravital microscopy in brain pial venules. Also in this model we have shown that the lacking of Tim-1 mucin domain resulted in a strong reduced ability of Th1 and Th17 cells to interact with the inflamed endothelium. Finally as brain pial venules are a key entry point for T cells in the early phases of development of EAE (experimental autoimmune encephalomyelitis) we checked the involvement of Tim-1 in this model. We discovered that lack of Tim-1 mucin domain resulted in a less severe development of the pathology correlating with a lower T cell accumulation in the CNS. Collectively our data demonstrate that TIM-1 is a major P-selectin ligand with a specialized role in T cell trafficking during inflammatory responses and the induction of autoimmune disease.
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1. Introduction
1.1 Leukocytes trafficking

Leukocyte trafficking is an essential meccanism of immune surveillance. The ability of immune cells to migrate to and from peripheral tissues enables them to patrol the entire body, providing primary and secondary immune responses as needed (Notrangelo and Badolato; J Leukoc Biol 2008). At the site of inflammation, infection, vascular injury, local pro-inflammatory or pathogen-derived stimuli render the luminal vascular endothelial surface attractive for leukocytes (Langer et al, J Cell Mol Med. 2009; Rossi et al., J Leukoc Biol 2011), which extravasate from blood to the site of inflammation. In this process, substitution of interchangeable receptor–ligand pairs at each step provide a combinatorial mechanism for generating specificity and diversity in leukocyte–endothelial cell recognition and hence recruitment (Ley et al., Nat Rev Immunol 2007). Molecular specificity in the targeting of leukocytes at sites of inflammation is mediated by selectins, integrins, and immunoglobulin (Ig) gene super family proteins (Butcher, Cell 1991; Springer, Cell 1994). Moreover, other important glycoproteins are the mucins, which serve as glycoprotein ligands for the selectins (Ley and Kansas, Nat Rev Immunol 2004). The importance of leukocyte recruitment in the regulation of the immune system is exemplified by the leukocyte adhesion deficiency (LAD) diseases, in which multiple genetic defects in molecular mechanisms controlling the function of leukocyte adhesion molecules lead to impairment of the immune reaction (Etzioni et al., Curr Opin Immunol 2009).

Based on in vitro and in vivo observations, leukocyte recruitment and homing may be described as sequential multi-step processes. (Ley et al., Nat Rev Immunol 2007). The “classical” four steps of leukocyte migration through the endothelium are: 1) capture (or tethering) and rolling, which are mediated by interactions between selectins-mucins and integrins-members of the Ig-superfamily; 2) activation, which is characterized by chemoattractant-induced G protein–dependent intracellular signaling, leading to integrin activation; 3) arrest, which is mediated by integrins and their endothelial counter-ligands belonging to the Ig-superfamily; 4) diapedesis/transmigration. Many progress has been made in the last years and additional steps have been defined: slow rolling, adhesion strengthening and spreading, intravascular crawling, and transcellular, along with paracellular, transmigration (Ley et al., Nat Rev Immunol 2007)(Fig 1).
The first phases of capture and rolling consists of the initial transient adhesion contacts of flowing leukocytes with the vascular endothelium, with an already adhered leukocyte or with leukocyte fragment or platelet. Selectins and their counter-ligands from the mucin family are the main adhesion molecules involved in this process during migration into lymphoid or non-lymphoid organs under physiological and pathological conditions. Leukocytes are initially captured from the blood stream by tethering via constitutively expressed leukocyte selectin (L-selectin; CD62L), which recognize glycoprotein ligands, like the peripheral node addressins (PNAd) or the mucin MAdCAM-1, constitutively expressed by lymphoid organs high endothelial venules (HEVs) or up-regulated on cytokine-activated vascular endothelium, mainly during chronic inflammation (Ley and Kansas, Nat Rev Immunol 2004; Rivera-Neves et al., J Immunol 2005). However, tethering process during inflammatory responses is mainly mediated by platelet/endothelial selectin (P-selectin; CD62P) and endothelial selectin (E-selectin; CD62E), which are expressed by activated vascular endothelium, through the interaction with the homodimeric sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) expressed on granulocytes, monocytes, and appropriately activated T cells (Carlow et al., Immunol Rev 2009; Yang et al., J Exp Med 1999; Xia et al., J Clin Invest 2002). After capture from the blood stream, leukocytes started to roll on the vascular bed. During the rolling step, selectin-ligand bonds are formed at the leading edge of the rolling cell and broken at the trailing edge, as these interactions have high mechanical strength, allowing initial tethering to the vessel wall, and have fast on and off rates, permitting rolling in response to hydrodynamic drag (Alon et al., Nature 1995). P-Selectin is the predominant leukocyte rolling receptor on acutely inflamed endothelial cells in vivo, and in resting conditions is stored in vascular endothelial cells Weibel-Palade bodies and in α-granules of platelets. Upon thrombogenic and inflammatory challenges, it is rapidly expressed, by exocytosis, on the cell surfaces of activated platelets and stimulated endothelial cells (Sperandio and Ley, Mod Asp Immunobiol 2005). Leukocyte rolling is also supported by E-selectin, which, except for skin microvessels, is not constitutively expressed on resting vascular endothelium. Expression has to be stimulated with TNF-α, lipopolysaccharide (LPS), interleukin-1, or other pro-inflammatory mediators involving transcriptional mechanisms (Bevilacqua et al., Science 1989). P-selectin is thought to be responsible for slow rolling interactions and possibly co-operates with the chemokine receptors in...

*In vivo* studies using mice deficient in PSGL-1 have shown that this mucin is the predominant, if not the only, P-selectin ligand during inflammation (Yang et al. *J Exp Med* 1999; Xia et al., *J Clin Invest* 2002). PSGL-1 also binds E-selectin, but slow rolling on E-selectin seems to be mediated by different ligands (Xia et al., *J Clin Invest* 2002). Other E-selectin ligands with a role in mediating rolling *in vivo* in inflammatory conditions are CD44 (Katayama et al., *J Exp Med* 2005), CD43 (Matsumoto et al., *J Immunol* 2007) and E-selectin ligand-1 (Levinovitz et al., *J Cell Biol* 1993).

In addition to selectin-mediated rolling, integrin-mediated rolling may take place during leukocyte-endothelium interactions. Integrins are heterodimeric transmembrane glycoproteins ubiquitous expressed, consisting of two covalently bound subunits called α (120-170 kDa) and β subunits (90-100 kDa). 8β subunits and 18α subunits are known; which are differently combined to generate heterodimers characterized by a cell-specific distribution and distinct ligand specificity (Constantin and Laudanna, in *Leukocyte Trafficking* A. E. Hamann, ed). Among integrin proteins, the integrin very late antigen (VLA)-4 (α<sub>4</sub>β<sub>1</sub> integrin; CD49d/CD29) can mediate rolling on vascular cell adhesion molecule (VCAM)-1 (CD106), a cell adhesion molecule belonging to the Ig superfamily expressed on endothelial cells and implicated in leukocytes rolling and firm arrest in inflamed vessels. Moreover, integrin lymphocyte function-associated antigen (LFA)-1 (α<sub>1</sub>β<sub>2</sub> integrin; CD11a/CD18) can mediate rolling on its vascular ligand intercellular adhesion molecule (ICAM)-1 (CD54). This rolling interaction seems to be important to induce slow rolling and leukocyte arrest (Rossi et al., *J Leukoc Biol* 2011; Ley et al., *Nat Rev Immunol* 2007).

Rolling along the endothelium is thought to allow sufficient time for eliciting the activation and the clustering of leukocyte integrins, which then bind to their counter receptors belonging to the Ig-superfamily, resulting in a shear-resistant firm adhesion (Ley et al., *Nat Rev Immunol* 2007). Integrin activation on rolling leukocytes is triggered by the interactions between chemo-attractive proteins, such as chemokines and lipid chemoattractants, exposed on the surface of activated endothelium, and their receptors expressed by leukocytes (Alon and Ley, *Curr Opin Immunol* 2008; Ley et al., *Nat Rev Immunol* 2007; Laudanna, *Nat Immunol* 2005).
Chemokines, the most important chemoattractants exposed by inflamed endothelium, binds with high affinity to specific G-protein-coupled receptors (GPCRs) expressed by rolling leukocytes, and induce the so-called “inside-out” signaling, a complex signaling pathway leading to integrin activation on the surface of rolling leukocytes that induce increase of both integrins affinity and valency for their endothelial ligands (Alon and Shulman, *Exp Cell Res* 2011). This process is crucial for adhesion, stabilization and cell motility on the vascular bed. The most prominent member of the \( \beta_2 \) integrin family is LFA-1, which participates in rolling interactions but predominantly mediates the firm adhesion/arrest of leukocytes in the blood vessels of lymphoid organs or at sites of inflammation, by binding the Ig-superfamily ligands ICAM-1 and ICAM-2 (CD102), expressed by the vascular endothelium (Luster et al., *Nat Immunol* 2005). Another important member of the \( \beta_2 \) integrin family is the macrophage-1 antigen (Mac-1; \( \alpha_M \beta_2 \) integrin; CD11b/CD18), which mediates interactions with vascular ICAM-1 (Luster et al., *Nat Immunol* 2005). Interestingly, LFA-1 and Mac-1 have recently been shown to be activated by intracellular signaling generated when PSGL-1 is cross-linked by P-selectin during leukocyte rolling, suggesting that the PSGL-1 signaling pathway is a key regulator of integrin-mediated firm adhesion in the control of leukocyte recruitment (Wang et al., *Nat Immunol* 2007). The most important \( \beta_1 \) integrin expressed on leukocytes is VLA-4, which binds to VCAM-1 expressed on endothelial cells and is implicated in the control of leukocyte rolling and firm arrest in inflamed vessels (Rossi et al., *J Leukoc Biol* 2011). Finally, the integrin \( \alpha_4 \beta_7 \) and its vascular ligand MAdCAM-1 play a specific role in lymphocyte homing by acting as a brake during naïve lymphocyte interactions in the HEVs (high endothelial venules) of Peyer’s patches (Springer, *Cell* 1994).

Once firmly adhered, leukocytes can transmigrate through the endothelium in the inflamed tissue; this process is in part mediated by the same integrins involved in the adhesion cascade, with the help of such chemo-attractive signal from the tissue. Vascular endothelium forms a nonthrombotic, nonadhesive barrier between the blood and tissue that is impermeable to macromolecules. Inflamed endothelial beds undergo diverse and heterogenous changes in permeability to blood constituents and adhesiveness to leukocytes and platelets (Vestweber, *Curr Opin Cell Biol* 2002). Two routes of leukocyte diapedesis have been noted so far both *in vivo* and *in vitro*: a paracellular route that dominates most extravasation processes, and a transcellular route reported *in vivo* for neutrophils and subsets of activated effector T cells (Ley et
al., Nat Rev Immunol 2007). Both routes demonstrate a proactive function for both apical and junctional endothelial ICAM-1 and VCAM-1, whose expression is differentially increased at specific sites of inflammation (Luster et al., Nat Immunol 2005).

Once migrated in the tissue, chemokine gradients guide activated leukocytes to the site of injury/infection, with migrating cells passing through the tissue extracellular matrix (ECM) (Luster et al., Nat Immunol 2005). During the transmigration through the endothelium, ligation of PECAM1 (platelet endothelial adhesion molecule 1) on leukocytes can lead to activation of members of the β1, β2 and β3-integrin families (Ley et al., Nat Rev Immunol 2007), which are the main receptors for extracellular matrix proteins such as laminin and fibronectin. Further, cell-surface-expressed leukocyte proteases facilitate leukocyte chemotaxis, by exposing binding sites within matrixprotein constituents or generating chemotactic fragments by selective cleavage of basement-membrane constituents (Ley et al., Nat Rev Immunol 2007).
**Figure 1**

**Fig.1 The leukocyte adhesion cascade.** The original steps are shown in bold: rolling, which is mediated by selectins; activation, which is mediated by chemokine and arrest, which is mediated by integrins. Progress has been made in defining additional steps: capture (or tethering), slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration. Key molecules involved in each step are indicated in boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α4β1-integrin).[Picture adapted from Ley et al, *Nature Rev Immunol*, 2007]
1.2 Selectin-dependent leukocyte trafficking into inflamed tissues: evidences and experimental models.

Selectins are a family of three transmembrane glycoproteins (P-, E- and L-selectin) expressed by bone marrow-derived cells and endothelial cells. Selectin structure is characterized by an amino-terminal domain related to those of Ca\(^{2+}\)-dependent (C-type) lectins, followed by an epidermal growth factors-like domain, a variable number of short consensus repeats similar to those found in complement-regulatory proteins, a transmembrane spanning segment and a short cytoplasmatic segment (Crockett-Torabi, *J Leuk Bio* 1998)(Fig 2). L-selectin (CD62L), is constitutively expressed by most leukocytes, E-selectin (CD62E), which is upregulated on endothelial cells following cytokine stimulation, and P-selectin (CD62P), which is stored in endothelial Weibel-Palade bodies and platelet \(\alpha\)-granules and is rapidly expressed by activated endothelium and platelets and by platelet-derived microparticles (Ley, & Kansas, *Nat Rev Immuno* 2004) (McEver, & Zhu, *Annu Rev Cell Dev Biol* 2010). The main physiological function of all selectins is to mediate leukocyte adhesion under flow, but both selectins and their ligands also have signaling functions (Ley, *Trends Mol Med* 2003). Selectins mediate cell-cell adhesion through the carbohydrate recognition domain (CRD) present on lectin-like portion, which mediates cell-cell contact through a Ca\(^{2+}\)-dependent interaction with cell-surface carbohydrates. This motif binds carbohydrates such as sialic acid, fucose, galactose, mannose and an anionic sulfate or phosphate ester moiety (Crockett-Torabi, *J Leuk Bio* 1998). Like other mammalian lectins, the selectins bind selectively, but with low affinity, to particular oligosaccharides. All selectins bind to the tetrasaccharide sialyl Lewis X (sLe\(^X\); NeuAc2,3Gal1,4[Fuc1,3]GlcNAc) and its isomer sialyl Lewis A (sLe\(^A\); NeuAc2,3Gal1,3 [Fuc1,4]GlcNAc) (McEver, *Curr Opin Immunol* 1994). These epitopes are normally not present on leukocyte surface, and all the selectin ligands expressed by leukocytes require post-transcriptional modifications in order to bind the selectin CRD. Several glycosyltransferases such as a1,3-fucosyltransferases,a2,3-sialyltransferases,core-2-acetylgalactosaminyltransferases, b1,4-galactosyltransferases, and polypeptide N-acetylgalactosaminytransferases have been implicated in the generation of functional selectin ligands that mediate leukocyte rolling via binding to
selectins (Sperandio et al., *Immunol Rev* 2009).

Studies using selectin deficient mice or selectin blocking antibodies unveiled the crucial role for selectins in leukocyte trafficking during inflammation (Hartwell and Wagner, *Thromb Haemost* 1999; Barthel et al., *Expert Opin Ther Targets* 2007). The importance of selectins and selectin ligands in mediating leukocytes recruitment in inflamed tissues has been demonstrated in several experimental models of inflammatory pathologies (Ley, *Trends Mol Med* 2003; Rossi and Constantin, *Inflamm Allergy Drug Targets* 2008). It was initially observed that mice genetically lacking endothelial selectins displayed a dramatic reduction in leukocyte trafficking in the inflamed tissues (Hartwell and Wagner, *Thromb Haemost* 1999) and are more susceptible to systemic infections (Munoz et al., *J Clin Invest* 1997). Mice with targeted deletions in E-selectin, P-selectin, or both selectin genes showed significant reductions in AHR (airway hyperresponsiveness), peri-bronchial inflammation, and eosinophil accumulation in a cockroach allergen-induced AHR (Lukacs et al., *J Immunol* 2002). Moreover, in an OVA (ovalbumin)-induced acute lung injury model, mice deficient in all the 3 selectins acute fail to develop an asthma phenotype, due, at least in part, to failure of inflammatory cells migration in the lung (Banerjee E. R, *The Journal of inflammation*, 2011). In ischemia-reperfusion injury (IRI) models, P- and E-selectin blocking with blocking antibodies ameliorated the clinical injury by reducing leukocyte infiltration into the ischemic tissues, such as kidney (Singbartl and Ley, *Crit Care Med* 2000; Singbartl et l., *FASEB J* 2000) and heart (Lefer et al., *Am J Physiol* 1996). Several reports have shown that E- and P-selectin are important for T cell recruitment to the inflamed lung (Ainslie et al., *Thorax* 2002; Wolber et al., *J Immunol* 1998; Curtis et al., *J Immunol* 2002), and L-selectin-deficient mice showed a drastic increased survival in a lipopolysaccharide (LPS)-induced toxic shock model (Tedder et al., *J Exp Med* 1995), in accordance with data indicating a role for L-selectin in mediating leukocyte recruitment in the site of chronic inflammation (Ley and Kansas, *Nat Rev Immunol* 2004). In proteoglycan-induced arthritis models, L-selectin expression in the cells of the innate immune system (granulocytes) seems to be important for their efficient influx into the joints (Sarraj B et al, *The Journal of Immunology*, 2006), and P-selectin appears to be a key adhesion receptor mediating leukocyte recruitment into atherosclerotic arterial lesions (Dong et al., *Circulation* 2000; Manka et al., *Circulation* 2001). Interestingly, in a murine model of epilepsy, the lacking expression or functionality of the most important ligand for P- and E-
selectin which is PSGL-1 led to a drastic seizures reduction by limiting leukocyte recruitment in the brain (Fabene et al., Nat Med 2008). PSGL-1, has a crucial role in leukocyte recruitment into the target organ in experimental models of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Crohn’s disease, type I diabetes, psoriasis, hepatitis, lupus and experimental autoimmune encephalomyelitis (Luster et al., Nat Immunol 2005), but the beneficial role of anti-selectin therapy to block chronic inflammation in autoimmune diseases is not clear (Rossi and Constantin, Inflamm Allergy Drug Targets 2008).

Even if the majority of research on selectin ligands has focused until now on myeloid cells still PSGL-1-independent rolling on P-selectin has been observed for T lymphocytes; suggesting that the repertoire of physiological ligands that interact with endothelial selectins is still incompletely understood (Ley, & Kansas, Nat. Rev. Immunol. 2004).
**Fig. 2 Selectin mediated leukocyte recruitment** A) Structural diagrams representing P-, E- and L-selectin. The selectins are rigid, asymmetric molecules that share structural similarities including the presence of the C-type lectin domain, followed by an epidermal-growth-factor-like (EGF) motif, a variable series of short consensus repeats (nine, six and two for P-, E- and L-selectins, respectively), a transmembrane domain (TM) and a cytoplasmic tail (cyto). (B) Selectin-mediated leukocyte recruitment. PSGL-1 on free-flowing leukocytes tethers to E- and/or P-selectin on activated endothelial cells, allowing leukocytes to roll to sites of infection or inflammation. E-Selectin might also bind to an as-yet-unidentified leukocyte glycosphingolipid. PSGL-1 on adherent leukocytes participates in secondary tethering by interacting with L-selectin on free-flowing leukocytes. (Picture adapted from Hanley et al., *Journal of cell Science*, 2004)
1.3 The Tim gene family

The T-cell Immunoglobulin and mucin domain containing (TIM) gene family encodes for a recently discovered family of glycoproteins involved in a variety of immune processes and in several pathologies, like atopic and autoimmune diseases, both in human and mice (Rodriguez-Manzanet et al., *Immunol Rev* 2009). The TIM family was firstly cloned from the Tarp (T-cell and airway phenotype regulator) locus on mouse chromosome 11B1.1 as a novel allergy and asthma susceptibility gene (McIntire et al., *Nat Immunol* 2001). In mice, four proteins (Tim-1 to 4) from the Tim family have been experimentally studied, while other four Tims (Tim-5 to -8) putative genes have been found in the same chromosomal region. In humans, three members of the TIM family are conserved (TIM-1, TIM-3 and TIM-4), and are locate in a chromosomal region (chromosome 5q33.2) repeatedly linked with asthma, allergy and autoimmunity (McIntire et al., *Springer Semin Immunopathol* 2004). Expression, function, and structural studies confirm that mouse Tim-1, Tim-3, and Tim-4 are the orthologues of human TIM-1, TIM-3, and TIM-4, respectively.

The Tim proteins are type I cell-surface glycoproteins with an immunoglobulin V (IgV)-like N-terminal Cys-rich region, followed by a mucin-like domain at the extra-cellular portion, a single trans-membrane region, and a cytoplasmic tail with tyrosine phosphorylation motifs involved in trans-membrane signalling, apart from TIM-4 (Meyers et al., *Trends Mol Med* 2005). 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 between members, with the number of the predicted O-linked glycosylation sites ranging from 60 in mouse Tim-1 to 3 in mouse Tim-3. (Fig. 2A)

The TIM gene family was initially associated with T-cell functions, but in the last years other cell types have been described to express this class of molecules. There is a clear distinction in the expression and functions among the TIM proteins, and differences in their ability to control T cell responses in the immune system. In mice, Tim-4 is the only protein of the Tim family not expressed by T cells subsets; it is expressed by professional antigen presenting cells (APCs), like macrophages and dendritic cells (DCs); it has bimodal effects on T-cell functions, inhibiting naïve T
cells activation and promoting the survival and the proliferation of already activated T cells (Meyers et al., *Nat Immunol* 2005; Rodriguez-Manzanet et al., *J Immunol* 2008; Mizui et al., *Int Immunol* 2008). The receptor for Tim-4 on naïve T cells is still unknown, while on activated T cells Tim-4 binds to Tim-1 to mediate co-stimulatory signals (Meyers et al., *Nat Immunol* 2005). Also Tim-2 has peculiar characteristics, among the Tim family, as it is not conserved in humans. It is up-regulated by activated T cells, but its expression is exclusively maintained by Th2 cells (Chakravarti et al., *J Exp Med* 2005); moreover, it is also expressed by B cells (Chen et al., *J Exp Med* 2005). Tim-2 binds to the class IV semaphorin Sema 4A expressed by activated APCs, and acts as a feedback loop to down-regulate established and ongoing Th2 responses (Kumanogoh et al., *Nature* 2002; Chakravarti et al., *J Exp Med* 2005; Rennert et al., *J Immunol* 2006).

The other main Tim proteins, Tim-1 and Tim-3, are the most studied, due to their broad impact on immune functions. Tim-3 was initially identified as a Th1-specific molecule, not expressed on Th2 cells (Monney et al., *Nature* 2002); however, this molecule is broadly expressed by other cells types, like human natural killer cells, monocytes and dendritic cells and mouse macrophages and DCs (Rodriguez-Manzanet et al., *Immunol Rev* 2009). Initially, Tim-3 was found to negatively regulate T-cell responses by inducing deletion of Th1 cells and by inducing tolerance (Sabatos et al., *Nat Immunol* 2003; Sanchez-Fueyo et al., *Nat Immunol* 2003). However, emerging data suggest that Tim-3 have opposite roles in innate and adaptive immunity. During the initiation of an immune response, Tim-3 is expressed by DCs, and promotes inflammation by synergizing with Toll-like receptors signals and leading to the production of pro-inflammatory cytokines by DCs (Anderson et al., *Science* 2007). Once an effector Th1 cells response is generated, Tim-3 expressed by terminally differentiated Th1 cells binds to galectin-9 expressed by several cell types (naïve T cells, APCs, CD4+CD25+ regulatory T cells) and inhibits the Th1-cell response by triggering cell death (Zhu et al., *Nat Immunol* 2005).

In contrast to Tim-3 functions, which are mainly associated with Th1 responses, several data indicate an important role for Tim-1 in regulating Th1, Th17, Th2 and regulatory T cells (Tregs)- mediated responses in vivo. In humans, TIM-1 was initially identified as the hepatitis A virus cellular receptor 1 (HAVCR-1); (Kaplan et al., *EMBO J* 1996) and as the kidney injury molecule 1 (KIM-1), a renal epithelial cells protein up-regulated and shedded following kidney injury (Ichimura et
al., *J Biol Chem* 1998). More recently, in mouse, Tim-1 expression was observed on activated T cells; in particular, after polarization, its expression remains high on Th2 cells, while Th1 and Th17 cells express little Tim-1 (Umetsu et al., *Nat Immunol* 2005; Meyers et al., *Nat Immunol* 2005; Nakae et al., *J Leukoc Biol* 2007). Tim-4 is the main ligand for Tim-1, which also binds itself (dimerization), Tim-3, leukocyte mono-immunoglobulin (Ig)-like receptor 5 (LMIR5)/CD300b, oxidized low-density lipoprotein, and immunoglobulin α heavy chain (Rennert, *Immunol Lett* 2011). It has been demonstrated by using an agonist antibody that Tim-1 cross-linking provided a strong co-stimulatory signal for T-cell activation, increasing naïve T-cell proliferation and Th2 cells proliferation and activation, both in vitro and in vivo during antigen challenge (Umetsu et al., *Nat Immunol* 2005). Notably, a role for Tim-1 has been found also in regulating pro-inflammatory responses by Th1 and Th17 cells; administration of a Tim-1 agonist during the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of central nervous system (CNS) inflammation, enhanced pathogenic Th1 and Th17 responses and increased the severity of the disease (Xiao et al., *J Exp Med* 2007), suggesting that also the low levels of Tim-1 expression observed on Th1 and Th17 cells might be important for their functions.

Recently, a new function for Tim proteins has been described. It has been shown that Tim-1, -3, and -4 are receptors for phosphatidylserine (PtdSer) on both human and mouse cells (Freeman et al., *Immunol Rev* 2010). PtdSer are normally localized to the inner leaflet of the plasma membrane, but it is re-distributed or exposed on the outer membrane during apoptosis, cell injury, cell activation and malignant transformation (Balasubramanian et al., *Annu Rev Physiol* 2003); PtdSer recognition provides a key signal to phagocytes that trigger engulfment of the apoptotic cells (Schlegel et al., *Cell Death Diff* 2001). In Tim-4-deficient mice, peritoneal macrophages and B-1 cells do not efficiently engulf and clear apoptotic bodies in vivo, leading to dysregulated lymphocyte activation and signs of systemic autoimmunity such as hyperactive T and B cells, elevated levels of serum Ig and development of antibodies to double-stranded DNA (Rodriguez-Manzanet et al., *PNAS* 2010). Anti-Tim-3 in vivo administration induced blocking of phagocytic activity by CD8+ DCs, again leading to disregulated lymphocyte activation and signs of systemic autoimmunity (Nakayama et al., *Blood* 2009). Finally, Tim-1 expressed on proximal tubular epithelial cells after renal injury coordinates engulfment of
apoptotic cells within the tubular lumen, and this function seems central in the recovery of renal function after acute kidney injury (Ichimura et al., *J Clin Invest* 2008). All these data suggest a physiological role for Tim proteins in regulating apoptotic bodies’ clearance by several populations of professional phagocytes.

Another putative important role for Tim proteins-PtdSer binding is represented by exosome uptake. Exosomes are a particular type of nanovesicles secreted by a wide range of mammalian cells that expose PtdSer 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 (Thery et al., *Nat Rev Immunol* 2009). It has been shown that Tim-1- or Tim-4-induced expression increase exosomes binding via PtdSer in transfected cells, and exosomes stimulated the interaction between Tim-1 and Tim-4 (Miyanishi et al., *Nature* 2007), indicating that Tim proteins may represent a new class of exosomes receptors and may be involved in intercellular signalling in which exosomes are involved.

Collectively, all these data clearly demonstrate a crucial role for the Tim proteins in regulating immune processes.
Fig. 3: Schematic representation of TIM protein structures. (A) The TIM proteins are type I cell-surface glycoproteins with an IgV-like N-terminal Cys-rich region, a mucin-like domain, a single trans-membrane region, and a cytoplasmic tail with tyrosine phosphorylation motifs, except in Tim-4, involved in trans-membrane signalling. The mucin domain is rich in threonine-, serine-, and proline, with a predicted extended conformation and a heavily O-glycosylated pattern. (Picture adapted from Freeman J et al, Immunological reviews, 2010) (B) Comparison between Tim-1 and MadCAM-1 structure. Note the similarities between the two molecules, both containing an highly glycosylated mucin domain followed by 1 (Tim-1) or 2 (MadCAM-1) Ig variable-like domains.
1.3.1 Tim-1 in inflammatory and autoimmune pathologies

Among Tim proteins, Tim-1 and Tim-3 are the most studied members, for their involvement in a variety of immune processes associated with the development of immune-mediated pathologies. In particular, all the observations obtained from in vitro and in vivo studies in mouse models suggest an involvement of Tim-1 also in human pathologies associated with T cell responses.

1.3.2 Tim-1 expression and functions in immune cells

As mentioned above, Tim-1 is expressed by activated T cells and, after polarization, its expression remains high on Th2 cells, while Th1 and Th17 cells express little or no Tim-1 (Rodriguez-Manzanet, Immunol Rev 2009). Cross-linking of Tim-1 on CD4+ T cells with an agonistic monoclonal antibody (mAb; 3B3 clone) provided a potent costimulatory signal for T-cell activation that increased naïve T-cell proliferation and interleukin (IL)-4 production by differentiated Th2 cells. Moreover, in vivo administration of 3B3 mAb along with antigen also greatly increased antigen-specific T-cell proliferation and production of IL-4 and interferon (IFN)-γ and blocked the development of respiratory tolerance (Umetsu et al., Nat Immunol 2005), consistent with the idea that Tim-1 costimulation activates T cells. A recent study showed that in vivo Tim-1 costimulation by 3B3 prevents allogenic transplant tolerance by reducing forkhead box p3 (Foxp3) expression and thereby preventing regulatory T cell (Treg) development. Furthermore, in vitro, 3B3 signalling “de-programmed” Foxp3-expressing Tregs into IL-17-producing pro-inflammatory cells (Degauque et al., J Clin Invest 2008), suggesting that Tim-1 costimulation may prevent tolerance induction both by enhancing T helper cell development and hindering Treg cell development. TIM-1 has been shown to co-localize on human T-cells surface with CD3, and to be recruited to the T-cell receptor (TCR)-signaling complex (Binné et al., J Immunol 2007); moreover, a recent work demonstrated that Tim-1 cross-linking is able to generate a costimulatory signal also in naïve T cell,
independently from the TCR (Mariat et al., *J Immunol* 2009). Over-expression of Tim-1 leads to NFAT/AP-1 transcriptional activation, dependent on tyrosine Y276 in the cytoplasmic tail, with increased IL-4 production (de Souza et al., *PNAS* 2005). In addition, agonistic anti-Tim-1 antibodies led to phosphorylation of Zap-70 and IL-2-inducible T cell kinase (ITK), and recruitment of an ITK and phosphoinositide 3-kinase (PI3K) complex to the TCR signaling complex (Binnè et al., *J Immunol* 2007; de Souza et al., *J Immunol* 2008). The p85 subunit of PI3K is recruited directly to tyrosine 276 in Tim-1 after lymphocyte-specific protein tyrosine kinase (Lck)-dependent phosphorylation of the cytoplasmic tail (de Souza et al., *J Immunol*, 2008).

Finally, Tim-1 promotes T-cell viability through activation of PI3K/AKT pathway and induction of the anti-apoptotic gene bcl-2 (Rodriguez-Manzanet et al., *J Immunol* 2008). These recent results started to unravel the complex signalling pathway downstream of Tim-1 engagement, which seems to be clearly correlated with T cell activation.

An important role for Tim-1 also in other cell subsets of the immune system is now emerging. Tim-1 is expressed by myeloid-derived DCs and Tim-1-mediated signalling induce DCs activation and pro-inflammatory responses *in vivo* (Xiao et al., *Eur J Immunol* 2011). Also murine mast cells constitutively express Tim-1, and Tim-1 cross-linking with recombinant Tim-4 induce cytokine production by mast cells (Nakae et al., *Blood* 2007). Mast cells have been shown to contribute to the development of autoimmune and allergic diseases. (Galli et al., *Nat Immunol* 2005), and Tim-1 expression on these cells could influence these pathologies. Furthermore, invariant natural killer T (iNKT) cells constitutively express Tim-1, (Kim et al., *J Immunol* 2010; Lee et al., *J Immunol* 2010), and Tim-1 recognition of PtdSer induced iNKT cell activation, proliferation, and cytokine production. Induction of apoptosis in airway epithelial cells activated pulmonary NKT cells and resulted in airway hyper-reactivity (Lee et al., *J Immunol* 2010), a classical feature of asthma, in an NKT cell-dependent and Tim-1–dependent fashion, suggesting that Tim-1 may act as a pattern recognition receptor on NKT cells and may regulate asthma-associated pathologies independently from T cells. Finally, consistent Tim-1 expression has been found on B lymphocytes; naïve B cells express basal low Tim-1 levels, that strongly increase after B-cell receptor (BCR) stimulation (Wong et al., *Immunology* 2010; Barlow et al., *Clin Exp Allergy* 2011; Ma et al., *Biochem Biophys Res Commun* 2011). Tim-1 is induced on B cells in a PI3K and nuclear factor-kB (NF-kB) dependent manner, and *in vivo* is
predominantly expressed on germinal centre B cells (Wong et al., *Immunology* 2010). The precise role of Tim-1 on B cells is still elusive. Tim-1 expression on B cells has been correlated with regulation of antibody production (Ma et al., *Biochem Biophys Res Commun* 2011), but no defects have been found in B cells responses in Tim-1 deficient mice (Wong et al., *Immunology* 2010). (Ding et al., *J Clin Invest* 2011) shows that Tim-1 is preferentially expressed by IL-10/IL-4-producing regulatory B cells (Bregs), which promote Th2 responses and can directly transfer allograft tolerance, that are also known to promote tolerance in a number of autoimmune models such as EAE, inflammatory bowel disease, collagen-induced arthritis, allergic airway disease, and diabetes mellitus (Ding et al., *J Clin Invest* 2011).

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.

1.3.3 Tim-1 in experimental models of inflammatory and autoimmune diseases

The importance of Tim-1 in the induction and development of several pathologies have been extensively studied, mainly by using different anti-Tim-1 mAbs. Initially, the agonist high-affinity Tim-1 activating 3B3 antibody and the antagonist low-affinity Tim-1 blocking RMT1-10 antibody have been described (Umetsu et al., *Nat Immunol* 2005; Xiao et al., *J Exp Med* 2007). While both antibodies bind to the Tim-1 IgV domain and induce CD3 capping, mAb 3B3 has a greater affinity and induce cytoskeletal reorganization (Xiao et al., *J Exp Med* 2007). They were initially tested in an EAE (experimental autoimmune encephalomyelitis) model, an autoimmune inflammatory pathology of the CNS, widely accepted as the mouse model of human Multiple Sclerosis. Administration of the high-affinity 3B3 mAb during the induction of autoimmunity enhanced pathogenic Th1 and Th17 responses and increased the severity of EAE, whereas the low-affinity RMT1-10 mAb increased Th2 responses and inhibited the development of EAE (Xiao et al., *J Exp Med* 2007). In accordance, in transplant model studies, *in vivo* administration of agonist 3B3 mAb overcame the protective effects of anti-CD154 mAb and resulted in
allograft rejection, by reducing Foxp3 expression and inhibiting Treg cell development, while enhancing development of Th1 and Th17 pathogenic responses (Degauque et al., *J Clin Invest* 2008). On the contrary, it has been shown that treatment with blocking RMT1-10 mAb inhibited rejection of fully MHC-mismatched mouse cardiac allografts. Prolongation of graft survival was associated with inhibition of alloreactive Th1 responses, enhancement of Th2-type responses, and preservation of Tregs (Ueno et al., *J Clin Invest* 2008). Moreover, administration of RMT1-10 specifically inhibited IL-17-producing CD8+ T cells that mediated resistance to tolerance induction (Yuan et al., *PNAS* 2009). To note, as previously mentioned, TIM-1 expression on Bregs could play a role in the allograft tolerance induced by RMT1-10 treatment (Ding et al., *J Clin Invest* 2011). In a mouse model of liver ischemia-reperfusion injury (IRI), preventive treatment with RMT1-10 antibody ameliorated the hepatocellular damage and improved liver function, with reduced local neutrophil, T lymphocytes and macrophages infiltration, reduced pro-inflammatory cytokines and chemokines production in the liver and reduced liver cell apoptosis (Uchida et al., *Hepathology* 2009). Similar results were obtained in a model of renal IRI (Rong et al., *J Am Soc Nephrol* 2011). In a mouse model of allergic asthma, preventive treatment with anti-Tim-1 antibody (clone 222414) before ovalbumin (OVA) challenge reduced lung inflammation, with lower inflammatory cells infiltrates, reduced mucus deposition and lower Th2-associated cytokines production by OVA-specific T cells (Encinas et al., *J Allergy Clin Immunol* 2006). Surprisingly, in Tim-1 deficient mice the airway hyperactivity (AHR) reaction was normally induced, although Tim1-deficient mice did show a small but significant decrease in cell infiltration (Barlow et al., *Clin Exp Allergy* 2011), 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, mainly by suppression of Th2 cell proliferation and cytokine production (Sonar et al., *J Clin Invest* 2010), again supporting a role for Tim-1 in the generation of AHR pathology.
1.3.4 Tim-1 in human pathologies

The role of Tim-1 is now clearly emerging also in the development of human diseases. TIM-1 gene (HAVCRI) is the most polymorphic gene in both mice and human TIMs gene family, with single nucleotide polymorphisms as well as insertion/deletion variants primarily in the mucin domain in both humans and mice (Nakajima et al., *Gene Immunol* 2005). Tim-1 was initially described as the cellular receptor for HAV (Kaplan et al., *EMBO J* 1996; Feigelstock et al., *J Virol* 1998), and polymorphic forms of TIM-1 have been associated with protection against atopy, especially in individuals who had prior infections with HAV (McIntire et al., *Nature* 2003), suggesting an important interaction between genotype (TIM-1 polymorphism) and environment (HAV infection) in regulating the development of the atopic phenotype (Freeman et al., *Immunol Rev* 2010). Recently, a specific polymorphism in TIM-1 gene has been associated with severe HAV infections (Kim et al., *J Clin Invest* 2011), directly correlating disease clinical course with TIM-1 sequence. An increase in TIM-1 mRNA level has been observed in patients with Systemic Lupus Erythematosus, associated with increase production of IL-10 (Wang et al., *Scand J Immunol* 2007). In multiple sclerosis (MS), both TIM-1 and TIM-3 have been associated with T cells functions: T cell clones isolated from the cerebrospinal fluid (CSF) of MS patients express lower TIM-3 and secrete higher IFN-γ when compared to T cells clones from healthy donors (Koguchi et al., *J Exp Med* 2006); Tim-1 expression is increased in the CSF mononuclear cells of MS patients in remission, suggesting a possible involvement of Tim-1 in this phase of the disease (Khademi et al., *J Immunol* 2004). Furthermore, polymorphisms in HAVCRI gene (exon 4 encoding for the mucin domain) have been associated with susceptibility to rheumatoid arthritis (Chae et al., *Immunogenetics* 2005; Chae et al., *Biochem Biophys Res Commun* 2004), and C-reactive protein or rheumatoid factor levels in patients with rheumatoid arthritis are associated with polymorphisms in the promoter region of TIM-1. (Chae et al., *Immunogenetics* 2005). Finally, very recently, Tim-1 was shown to function as cellular receptor for Zaire Ebola virus and Lake Victoria Marburg Virus (Kondratowicz et al., *PNAS* 2011). All these data suggest a complex pattern of in vivo regulation of T-cell associated functions by Tim-1 and indicate its potential involvement in the development of several inflammatory and autoimmune diseases.
1.4 Experimental hypothesis: a role for Tim-1 in T cell trafficking

In our project, we investigated a possible role for Tim-1 in mediating leukocyte trafficking during inflammatory responses. For their complex structure, Tim proteins are able to bind a diverse set of ligands, belonging to different gene families (Rodriguez-Manzanet et al., *Immunol Rev* 2009). As previously mentioned the structure of Tim proteins, in particular the ones of Tim-1 and Tim-4, are similar to those of the mucin mucosal addressin cell adhesion molecule (MadCAM)-1, a classical adhesion receptor involved in leukocyte trafficking in the immune system (Ley et al., *Nat Rev Immunol* 2007, Fig. 2B). Notably MadCAM-1 is able to bind both selectins and integrins (Berg et al., *Nature* 1993)(Berlin et al., *Cell* 1993). Moreover, it has been shown that the IgV domain of Tim-1, Tim-3 and Tim-4 exhibits characteristics of the C-type lectins, as its non-species-specific binding to carbohydrate moieties of several cell types is calcium sensitive and is reduced in cells with defective O- and N-linked carbohydrate synthesis (Wilker et al., *Int Immunol* 2007). All these structural observations led us to investigate a potential role for Tim-1 in leukocyte trafficking in inflamed tissues, as we and other have previously demonstrated that molecules like C-type lectins, mucins, integrins and Ig-superfamily members are involved in this process (Piccio et al., *J Immunol.* 2002; Battistini et al., *Blood* 2003; Ley et al., *Nat Rev Immunol* 2007; Fabene et al., *Nat Med* 2008; Rossi et al., *J Leuk Biol* 2011).

However Tim-1 does not present in the IgV domain the typical RGD peptide motif, which is found on many integrin ligands and also on Tim-4 (Kuchroo et al., *Nat Rev Immunol* 2003). For this reason, we focused our attention on the possible binding between Tim-1 and selectins, possibly via the Tim-1 highly glycosylated mucin domain trying to elucidate the involvement of Tim-1 in mediating selectin dependent leukocyte trafficking during inflammatory responses.
2. Materials and methods
2.1 Microtiter plate binding assay

The assay was performed as previously described (Deban et al., Nat Immunol, 2010). Briefly, microtiter plates (NUNC) were coated with 5 µg/ml of murine or human P-selectin Fc chimera, E-selectin Fc chimera, L-selectin Fc chimera, ICAM-1 Fc chimera (negative control) (R&D Systems) or TIM-4 (positive control; Creative Biomart). We added 5 µg/ml of mouse TIM-1 Fc-chimera (Sizing et al., J Immunol 2007) or human TIM-1 Fc-chimera (Sino Biological Inc.) to each well for 1 h at 25°C. In some experiment, 10 mM EDTA was added to chelate divalent cations. In some experiments, murine TIM-1 proteins were treated overnight at 37°C with α-(3,4) fucosyltransferase (Sigma-Aldrich), tyrosin-sulfatase (Sigma Aldrich), O-sialoglycoprotein endopeptidase (OSGE; Cedarlane), neuraminidase or PNGase F (New England Biolabs) following manufacturer’s instructions. The corresponding control protein was incubated overnight at 37°C with the same buffer used to reconstitute the glycosidase. In separate experiments, selectin-TIM-1 binding was analyzed by using entire or truncated TIM-1 Fc-chimeras produced in 293T cells as previously described (Santiago et al., Immunity 2007)

2.2 Mice

Selpg-/- mice and C57BL/6J black mice were used as WT age-matched controls. Tim-1Δmucin mice were a kind gift from Prof. Vijay K. Kuchroo (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA) and were obtained replacing the exon 3 of Tim-1 gene with a neo cassette. (described in supplementary). All mice were housed and used in according to the current European Community rules.
2.3 Mice genotyping and DNA extraction

The tip of mouse tail was digested in Tail Lysis Buffer (NaCl 50 mM, Tris pH 7.5 50 mM, EDTA 30 mM and SDS 1%), supplemented with proteinase K (0.5 mg/ml) for 3-4 hours at 55°C. 500 µl of phenol:chloroform:isoamyl alcohol 25:25:1 was added to the sample to separate DNA for proteins. Next, we added in the sample 25 µl of NaCl 5M and 500 µl of isopropanol to precipitate DNA. DNA pellet was then washed with 500 µl of EtOH 70%, re-pelleted and dried at room temperature. Finally, DNA pellet was re-suspend in 40 µl of TE buffer pH 7.5 (10 mM tris pH 7.5; 1 mM EDTA pH 8.0) and quantified with Nanodrop 2000C (Thermo Scientific).

2.4 PCR amplification

The DNA was amplified by polymerase chain reaction (PCR). The reaction mix contained: buffer Taq 5 X dNTPs 10 mM MgCl₂ 25 mM Taq 5 U/ml primer forward (0.2 µM) primer reverse (0.2 µM) H₂O. The reaction mix was heated at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, followed by a final extension of 1 minutes at 72°C. The following primers were used:

TIM1-Forward:        AACTGGGTGAAGTCAGTCACCACT
TIM1-Reverse:         TGTAGCTGTGGGCCTTGTAG
TIM1-Neo Reverse:  AAGGAACAAAGCTGCTATTGGCCG

The forward-reverse primers couple amplified the WT C57Bl/6J mice Tim-1 allele (amplicon length: 385 bp), while the forward-neo reverse amplified the Tim-1Δmucin
allele generated by exon 3 deletion (amplicon length: 581 bp)(described in supplementary). The PCR products were loaded onto agarose gel 1.0% in TAE buffer (Sigma-Aldrich), run by gel electrophoresis and analyzed in an UV lightbox (LAS 4000; GE Healthcare).

2.5 Mouse primary cells culture and CD4+ T cells isolation

Peripheral lymph nodes were harvested from 8- to 10-weeks-old C57Bl/6J WT or Tim-1Δmucin mice, mechanically dissociated and washed twice after treatment with antibiotic solution. CD4+ cells were separated by negative selection with magnetic cell sorting, according to manufacturers instructions (all reagents from Miltenyi Biotec). Briefly, CD4+ cells were enriched by depletion of non-CD4+ T cells by labeling with biotin-antibody cocktail, followed by incubation with anti-biotin MicroBeads. The cell suspension was then loaded onto a column placed in a magnetic field, and the magnetically labeled non-CD4+ cells were retained in the column, whereas the unlabeled CD4+ cells that run through were collected. The purity of the obtained cell population (usually >95%) was determined by flow cytometry analysis with a rat anti-mouse CD4 antibody (Miltenyi Biotec), using the MACSQuant flow cytometer (Miltenyi Biotec).
2.6 CD4+ T cells stimulation and Th1, Th17 cells polarization

2.5x 10^6 CD4+ T cells were stimulated for 2 days at 37°C in a 48 well plate in TK1 medium (RPMI, 10% Fetal Bovine Serum [FBS], ultraglutamine 4 mM, Na pyruvate 1:100, penicillin/streptomycin 100U/ml; all reagents from Lonza) containing concanavalin A (ConA; Sigma-Aldrich) 5µg/ml. For Th1 polarization, CD4+ T cells were cultured with irradiated splenocytes, ConA 5 µg/ml, anti-IL-4 antibody (clone 11B11; 10 µg/ml) and IL-12 (0,1 ng/ml, R&D Systems). After 3 days, IL-2 (Miltenyi Biotec; 10 U/ml) was added to the culture for 2 days. For Th17 polarization, cells were cultured with irradiated splenocytes, ConA 5 µg/ml, anti-IL-4 antibody (20 µg/ml), IL-6 (R&D Systems; 30 ng/ml), transforming growth factor β (TGF-β, R&D Systems; 3 ng/ml), and anti-IFN-γ antibody (clone HB170; 20 µg/ml). After 3 days, IL-7 (R&D Systems; 10 ng/ml) was added for 2 days.

2.7 Generation of MOG35-55-specific Th1 cells

MOG35-55-specific Th1 cells were generated from wild-type or TIM-1Δmucin mice immunized with 100 µg MOG35-55 peptide (GenScript Corporation) in complete Freund adjuvant (CFA; Difco Laboratories). After 14 days, spleens were collected and CD4+ T cells isolated using magnetic beads. Then, 1 x 10^6 CD4+ T cells were re-stimulated with 10 µg /ml MOG35-55 peptide, 10 U/ml IL-2, 10 µg/ml anti-IL-4 antibody and 10 U/ml IL-12, in the presence of APCs (irradiated splenocytes). After two weeks, cells were re-stimulated for 4 days before transfer in mice.
2.8 PMN isolation from mouse bone marrow

Immature neutrophil were isolated from the bone marrow of the femurs and tibias of C57BL/6J WT or Tim-1 mutant mice. The femur and the tibia from both hind legs were removed and the tip of each extremity was cut. Bone marrow was flushed out form the bone with a 25G needle-syringe containing Hanks’ balanced saline solution (HBSS; GIBCO) + 0.1 % Bovine serum albumin (BSA; Sigma-Aldrich). Cell suspension was disaggregated with an 18G needle-syringe and filtered through a 70 mm cell strainer. Cell suspension was centrifuged and erythrocytes cells were lysed with a hypotonic NaCl 0.2% solution. Cells were then suspended into 45% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) in HBSS/BSA, loaded on the top of a Percoll discontinuous density gradient with 81%, 62 %, 50% and 55 % Percoll in HBSS/BSA phases and centrifuged at 2700 rpm for 30 min without brake. Neutrophils were collected from the 81%/62% interface, washed twice in HBSS + 0.1% BSA and resuspended in the appropriate buffer. Cell purity (usually >90%) was assessed by flow cytometry analysis with a PE-conjugated rat anti-mouse Gr-1 antibody (Miltenyi Biotech).

2.9 Under flow assays in capillary tube

Microcap glass capillary tubes (100 µl; Sigma-Aldrich) were coated with different concentrations of mouse P-selectin or E-selectin Fc-chimeras. For the experiments with TIM-1 covered microspheres, 9.7 µm protein A-covered non-deformable polystyrene microspheres (Bangs Laboratories) were washed and incubated for 45 min at room temperature with 10 µg/ml human PSGL-1 Fc chimera (AbD Serotec) or
10 μg/ml murine TIM-1 Fc-chimera (Sizing et al., *Journal of Immunol* 2007), following the manufacturer’s instructions. Beads covered with mouse IgG Fc-chimera (R&D Systems) were used as a negative control. In separate experiments, T-cell populations were fluxed into the tubes at a density of 10⁶ cells/ml. T cells or beads were fluxed at a shear stress of 2 dyne/cm² in a buffer containing 1 mM Ca²⁺/Mg²⁺, 10% vol/vol FBS in PBS. Adhesion interactions were recorded and analyzed frame by frame by computer-assisted digital analysis as described (Deban et al., *Nat Immunol* 2010).

### 2.10 Intravital microscopy in mesenteric vessels

Intravital microscopy experiments were performed as described (Deban et al., *Nat Immunol* 2010). Briefly, 6- to 8-week-old C57Bl/6J mice were anesthetized by intraperitoneal injection of PBS containing ketamine (5 mg/ml) and xylazine (1 mg/ml). A polyethylene catheter was inserted in the lateral tail vein. A segment of the terminal ileum with its accompanying mesentery was exteriorized and bathed with PBS containing bovine thrombin (0.5 U/ml; Sigma). The recipient was maintained at 37°C and placed on a BX50WI microscope (Olympus). After 30 min of stimulation with thrombin, Th1, Th17 cells or PMNs from wild-type or TIM-1 mutant mice were slowly injected through the catheter. Cells were previously labeled with 5-chloromethylfluoresceindiacetate (CMFDA) (Invitrogen). Images were visualized with a silicon- intensified target video camera (VE-1000 SIT; Dage-MTI) and a Sony SSM-125CE monitor. Digitalized video images were analyzed frame by frame using ImageJ software. Interacting cell fractions were defined as the percentage of cells that interacted within a given venule among the total number of cells that entered that
venule during the same period.

2.11 Cutaneous hypersensitivity (CHS)

Mice were sensitized by applying to the shaved abdomen 30 µl of acetone/olive oil (4:1; Sigma-Aldrich) plus 0.5% (v/v) 1-fluoro-2,4-dinitrobenzene (DNFB; Sigma-Aldrich). Five days later, sensitized mice were challenged with 7 µl of acetone/olive oil (4:1) plus 0.3% DNFB on each side of the right ear pinnae. For the control condition the left ear was painted with an identical amount of vehicle. The ear thickness was measured at times 0 and 24 and 48 h after challenge with a dial thickness gauge (Swiss Precision Instruments). In Th1 transfer experiments, 10 x 10⁶ Th1 cells labeled with green carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) were injected intravenously 24 h after ear pinnae painting, and 24 h later, ears were processed as previously described to isolate migrated leukocytes (DeKrey et al., J Immunol Methods 1999).

2.12 Intravital microscopy in brain vessels

Intravital microscopy experiments were performed in inflamed brain microcirculation in an experimental model mimicking early inflammation during EAE as previously described (Piccio et al., J Immunol 2002). To induce the expression of adhesion molecules on CNS vessels mimicking early EAE vascular inflammation, wild-type C57Bl/6 mice were injected intraperitoneally with 12 mg lipopolysaccharide (LPS) 5-6 h before starting the experiment. Animals were anesthetized and a heparinized PE-10 catheter was inserted into the right common carotid artery toward the brain. Blood
vessels were visualized through the bone using fluorescent dextran as described (Piccio et al., *J Immunol* 2002). We labeled 2-3 x 10⁶ wild-type or TIM-1Δmucin Th1 or Th17 cells with (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR) and injected them into the carotid artery by a digital pump. Hemodynamic parameters were determined as described (Piccio et al., *J Immunol* 2002). Lymphocytes that remained stationary on the venular wall for at least 30s were considered adherent. At least 150 consecutive cells per venule were examined. Rolling and firm arrest fractions were determined as the percentage of cells that rolled or firmly arrested within a given venule on the total number of cells entering the venule.

### 2.13 Active and transfer EAE induction

For active EAE induction, 6–8-week-old C57Bl/6J wild-type and TIM-1Δmucin mice were immunized subcutaneously in the flanks and at tail base with 150 mg of MOG35-55 peptide in 200 ml emulsion consisting of equal volumes of PBS and CFA supplemented with 0.8 mg/mouse *Mycobacterium Tuberculosis* (strain H37Ra; Becton-Dickinson). Mice received 20 ng of pertussis toxin (PTX; List Biological Laboratories) intravenously at the time of immunization and 48 h later. For passive transfer EAE induction, C57Bl/6J wild-type mice were injected with 20 ng PTX 4 days and 1 day before cell transfer. The day after the second PTX injection, 5 x 10⁶ wild-type or TIM-1Δmucin MOG₃⁵₋₅₅-specific Th1 cells were injected intravenously. The EAE clinical course was recorded daily as previously described (Piccio et al., *J Immunol* 2002).
2.14 Proliferation assays from EAE mice

CD4+ T cells were isolated from wild-type and TIM-1Δmucin EAE mice 7 days post-immunization with MOG35-55 peptide. Cells were re-stimulated in vitro in the presence of antigen and pulsed for 18 h with 1 µCi/well of [3H]-thymidine. Cells were then collected and radioactivity was detected using a b-counter (Perkin-Elmer).

2.15 Neuropathology

Mice were sacrificed at the disease peak, the spinal cords were collected and frozen, and 10-mm sections were analyzed using hematoxylin and eosin staining for the detection of inflammatory infiltrates and Spielmeyer coloration for myelin.

2.16 Migration of Th1 cells in the brain

C57Bl/6J wild-type mice were treated intravenously with 30 ng PTX as previously described (Kerfoot et al., J Immunol 2004). After 5 h, 5 x 10⁶ wild-type or TIM-1Δmucin MOG 35-55-specific CFSE-labeled Th1 cells were transferred into mice. CFSE+ cells were detected after 60 h from brain homogenates by FACS analysis.

2.17 Migration of MOG35-55-specific Th1 cells

C57Bl/6J wild-type mice were treated intravenously with 30 ng PTX as previously described (Kerfoot et al., J Immunol 2004). After 5 h, 5 x 10⁶ wild-type or TIM-1Δmucin MOG35-55- specific TH1 cells labeled with green carboxyfluorescein
succinimidyl ester (CFSE; Invitrogen) were transferred into mice. After 60 h, mice were sacrificed and brains and spinal cords were collected and treated as previously described to isolate CNS leukocytes (Korn et al., Nat Med 2007). CFSE+ cells were detected by FACS analysis.

2.18 Immunofluorescence staining for confocal microscopy

For surface staining, wild-type or TIM-1Δmucin TH1 cells were incubated with Alexafluor488-conjugated hamster anti-mouse CD3e (Biolegend) and 5F12 anti-TIM-1 (Xiao et al., PNAS 2012) antibody, followed by 7.5 µg/ml rabbit anti-rat biotinylated secondary antibody (VectorLab) and 25 µg/ml Texas Red-conjugated avidin (VectorLab). For intracellular staining, cells were incubated with Alexafluor488-anti-CD3e and permeabilized. Cells were then incubated with 5F12 anti-TIM-1 antibody, followed by biotinylated secondary antibody and Texas Red-avidin. Cells were visualized using a Tandem Confocal Scanning SP5 microscope (Leica, Germany). Collected images were processed and elaborated with Imaris software (Bitplane).

2.19 Antibodies and fluorescence-activated cell sorting (FACS) analysis

The following rat-anti mouse monoclonal antibodies were used: anti-PSGL-1 (clone 4RA10; BD Bioscience), PE- conjugated anti-CD25 (eBioscience), purified anti-α4 integrin (clone PS-2), anti-LFA-1 (clone TIB-213), anti-L-selectin (clone Mel-14) and anti-CD44 (clone MJ64). Rat-anti mouse TIM-1 RMT1-10, 4A2.2 and 5F12
clones (Xiao et al., J Exp Med 2007; Sizing et al., Journal of Immunol 2007; Xiao et al., PNAS 2012). Samples were collected with the MACSQuant Analyzer (Miltenyi Biotec) and analyzed with the FlowJo software (Tree Star Inc.).

2.20 Real time PCR for TIM-1 expression

Total RNA was extracted from T cells using the RNAeasy mini kit (Qiagen, Crawley, UK) and used as a template for the retro-transcription reaction and random hexamers and SuperScript II RT (Invitrogen, Carlsbad, CA, USA). Triplicate RT-PCR reactions for each sample were performed in 20 µl containing 5 ng cDNA, Fast SYBR Green Master Mix (Invitrogen), and primers (200 nM). The PCR reactions were performed in 96-well plates using the DNA Engine Opticon 2 system (MJ Research, Waltham, MA, USA). Amplification plots were analyzed using Opticon Monitor Software, Version 2.02 (MJ Research). Data were calculated with Q-Gene software (www.BioTechniques.com) and expressed as MNE units after b-actin normalization (Muller et al., 2002). Oligonucleotide primers (Invitrogen) for TIM-1 and for b-actin (housekeeping gene) were the following:

TIM-1 fwd: 5’-GCGCTGTGGATTCTTATGTG-3’;
TIM-1 rev: 5’-CTCAACAGAGTTCTCTATCG-3’.
b-actin fwd: 5’-TCTTTGCAGCTCCTCGTTG-3’;
b-actin rev: 5’-CAGGATACCTCTCTGCTCTCG-3’.
2.21 Statistics

Quantitative data are shown as mean values ± SD or 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 followed by Dunnett’s test for multiple comparisons. A $P$-value $<0.05$ was considered statistically significant.
3. Results
3.1 Human and murine TIM-1 bind selectins in vitro in a cell-free system

We hypothesized that TIM-1 may present carbohydrate moieties to selectins as highly glycosylated molecules are the classical ligand for selectins and other trafficking receptors (Sperandio et al., *Immunol Rev* 2009). We therefore carried out microtiter plate binding assays to check for direct binding between both human and murine TIM-1 and the three known selectins. We used TIM-4 as a positive control for TIM-1 ligand binding (Meyers JH et al., *Nat Immunol* 2005) and ICAM-1, a molecule that display no binding with mucins, as negative control (Santiago et al., *Immunity* 2007). We found that both mouse and human TIM-1 were able to bind all three selectins in cell-free assays, with P-selectin showing a higher binding capacity than E-selectin or L-selectin (Fig. 4a, b). As selectin binding to their ligands is absolutely dependent on the presence of divalent cations (Crockett-Torabi, *J Leukoc Biol* 1998), we tested the ability of TIM-1 to bind selectins in the presence of EDTA, which is known to chelate divalent cations. Interestingly, the presence of EDTA in the assay completely blocked the binding of Tim-1 to all the selectin, and, as previously shown, also to Tim-4 (Wilker et al., *Int Immunol* 2007) (Fig. 4 B), suggesting an involvement of the carbohydrates in Tim-1 mucin domain in the binding. Collectively, these results indicate that Tim-1 is able to bind all the selectins, with higher affinity to P-selectin, and considering the Ca$^{2+}$-dependency revealed by EDTA treatment, the binding is probably mediated by the highly glycosylated mucin domain in the Tim-1 structure.

We tested other members of the TIM family and found that murine TIM-2 and TIM-3 were unable to bind selectins, suggesting that TIM-1 may be the sole candidate selectin receptor (Supplementary Fig. 1).
Figure 4

Figure 4: TIM-1 interacts with selectins *in vitro* in a cell-free system. Microtiter plates were coated with 5 µg/ml murine or human P-selectin, E-selectin or L-selectin, TIM-4 (positive control) or ICAM-1 (negative control), and tested for their ability to bind recombinant mouse or human TIM-1 respectively. In some experiments, 10 mM EDTA was added to chelate divalent cations. Both murine (a) and human (b) TIM-1 bound to all three selectins, and binding was dependent on the presence of divalent cations (*P < 0.0001 compared to ICAM-1 binding). Data represent means ± standard error of the mean (SEM) of at least three independent experiments performed in triplicate for each condition.
3.2 TIM-1 requires α1-(3,4)-fucosylation and tyrosine sulfation for efficient binding to P- and E- selectin

The Ca^{2+}-dependency revealed by EDTA treatment suggested us the possible involvement of the carbohydrates in Tim-1 mucin domain in mediating the binding to selectins. Then we next sought to identify the carbohydrate moieties required for high-affinity binding between TIM-1 and endothelial selectins.

By treating recombinant mouse TIM-1 proteins derived from Chinese hamster ovary (CHO) or human embryonic kidney (HEK) 293T cells with different glycosidases, we found that α1-(3,4)-fucosylation and tyrosine sulfation were the post-translational modifications necessary for binding between TIM-1 and selectins (Figure 5 a, b). Treatment with Peptide: N-glycosidase F (PNGaseF) did not inhibit TIM-1 binding, suggesting that N-linked glycans have no role in the interaction with selectins (Figure 5 a,b). Surprisingly, treating TIM-1 with neuraminidase or O-sialoglyco-endopeptidase did not inhibit TIM-1 binding either (Figure 5 a,b), suggesting that sialic acid moieties are dispensable for the interaction with selectins. These data suggest that TIM-1 presents a specific glycosylation profile necessary for selectin binding, but that the profile differs from that of PSGL-1, particularly in the requirement for sialylated carbohydrates (Zarbock et al., Blood 2011).
**Figure 5:** TIM-1 requires a1-(3,4)-fucosylation and tyrosine sulfation for efficient binding to P- and E-selectin Microtiter plate assays showing the binding of recombinant mouse TIM-1 protein from CHO (shown in a) and 293T cells (shown in b) to P- and E-selectin after overnight treatment with a1,(3,4)-fucosidase, tyrosine sulfatase, PNGase, OSGE and neuraminidase treatment (*P<0.001). Fucosylation and tyrosine sulfation are the post-translational modifications necessary for binding between TIM-1 and selectins.
3.3 TIM-1 mediates capture and rolling on P-selectin and E-selectin under physiological flow conditions in vitro

The capture and rolling of leukocytes on vascular endothelium mediated by L-selectin and P-selectin requires shear stress conditions (Zarbock et al., *Blood* 2011). Therefore we carried out additional TIM-1-selectin binding assays under physiological flow conditions in glass capillary tubes using 10-µm protein A polystyrene microspheres covered with a murine TIM-1 Fc-chimera (Sizing et al., *J Immunol* 2007). The TIM-1-covered beads were infused into capillary tubes pre-coated with each of the three murine selectins under physiological shear stress conditions, i.e. 2 dyne/cm² (Deban et al., *Nat Immunol* 2010). The TIM-1-covered microspheres were efficiently captured by P-selectin and E-selectin (Fig. 6a) under these conditions. Notably, TIM-1-mediated tethering and rolling was more efficient on P-selectin compared to E-selectin, suggesting that TIM-1 may preferentially bind P-selectin under physiological flow conditions. TIM-1 also mediated the transition from capture to rolling interactions, and the rolling velocity was dependent on the quantity of each selectin used to coat the capillary tubes (Fig. 6b). The rolling of beads on E-selectin was slow (Ley et al., *Nat Rev Immunol* 2007), and slowed further as the quantity of E-selectin in the coating increased (Fig. 6b). In contrast, TIM-1 did not bind L-selectin under these flow conditions, suggesting that it may interact preferentially with selectins expressed on endothelial cells. These results demonstrated that TIM-1 can mediate capture and rolling on P-selectin and E-selectin under physiological flow conditions and may represent a novel adhesion mechanism that facilitates efficient lymphocyte-endothelium interactions *in vivo*. 
Figure 6: TIM-1 mediates tethering and rolling on endothelial selectins under physiological flow conditions. Protein A-covered microspheres were coated with a murine TIM-1 Fc-chimera or a control mouse IgG Fc-chimera (control beads) and were infused into glass capillary tubes pre-coated with P-selectin, E-selectin or L-selectin, under physiological shear stress conditions (2 dyne/cm²). (a) TIM-1 mediates capture and rolling on P-selectin and E-selectin, but not L-selectin, with a higher number of interacting beads detected in P-selectin-coated tubes (*P < 0.002, **P < 0.0001). (b) The rolling velocity of TIM-1-covered beads declines as the concentration of P-selectin and E-selectin increases under physiological flow conditions. Data represent the means ± SEM of three independent experiments.
3.4 The TIM-1 mucin domain is required for T-cell rolling on P-selectin in vitro

The complete inhibition of TIM-1-selectin binding by the chelation of divalent cations (Figure 4 b) suggested that the TIM-1 mucin domain may be responsible for primary adhesion. We therefore tested the behavior of T cells derived from TIM-1Δmucin mice, which selectively lack the O-glycosylated TIM-1 mucin domain (Xiao et al., PNAS 2012). We used activated T cells, which express TIM-1 on the cell surface (Meyers et al., Trends in Mol Med 2005) and just beneath the plasma membrane, compared to naive T cells, in which TIM-1 is exclusively intracellular (Supplementary Fig. 2). As previously described (Xiao et al., PNAS 2012), we found no differences in the expression of TIM-1 between wild-type and TIM-1Δmucin T-cells (data not shown). Furthermore, activated T helper cells from wild-type and TIM-1Δmucin mice expressed comparable levels of several adhesion molecules and activation markers (Supplementary Fig. 4), suggesting that there were no potential adhesion defects associated with the TIM-1Δmucin mutation. We carried out further in vitro assays with capillary tubes pre-coated with recombinant P-selectin or E-selectin and found that TIM-1Δmucin T-cells activated with concanavalin A (ConA blasts) were consistently deficient (45% reduction) in their ability to tether and roll on P-selectin under physiological flow conditions (Fig. 7a), providing more evidence that the TIM-1 mucin domain is necessary for interactions with P-selectin. Furthermore, Th1 and Th17 cells lacking the mucin domain also showed a significant reduction in P-selectin-mediated tethering and rolling (Fig. 7 a,b). Th1 cells showed a 45% reduction and Th17 cells showed a 50% reduction, suggesting that despite the presence of PSGL-1 on these cells, TIM-1 is nevertheless required for effective P-selectin-dependent tethering and rolling. In contrast, TIM-1Δmucin bone marrow-derived polymorphonuclear neutrophils (PMNs), which do not express TIM-1, displayed normal rolling activity (Fig. 7a), suggesting that the adhesion defect in TIM-1Δmucin mice is specific to T cells. No differences in rolling velocity were observed between wild- type and TIM-1Δmucin T cells (Fig. 7c), suggesting that TIM-1 does not influence the quality of rolling interactions in the presence of high levels of functional PSGL-1. Because TIM-1 also mediated rolling on E-selectin in vitro, we investigated whether
the TIM-1 mucin domain is also required for E-selectin-dependent rolling. In contrast to the P-selectin experiments, we found that TIM-1Δmucin ConA blasts, Th1 and Th17 cells showed no defects in their interactions with E-selectin in vitro under physiological flow conditions when compared to wild-type cells (Fig. 7d). There was also no change in rolling velocity when TIM-1Δmucin cells were tested on E-selectin (Fig. 7e), suggesting that the TIM-1 mucin domain plays a specific role in P-selectin-dependent rolling.
**Figure 7:** The TIM-1 mucin domain is required for the capture and rolling of activated T cells on P-selectin under physiological flow conditions *in vitro*. CD4+ T cells were stimulated with concanavalin A (ConA) or polarized toward Th1 or Th17 phenotypes, and then infused into capillary tubes pre-coated with 5 µg/ml P-selectin or E-selectin. Bone marrow-derived PMNs, which do not express TIM-1, were used as negative controls. (a) TIM-1Δmucin ConA blasts, Th1 and Th17 cells display a significantly reduced capture and rolling ability on P-selectin under physiological shear stress conditions (2 dyne/cm2), whereas PMNs from TIM-1Δmucin mice are unaffected (*P < 0.002; **P < 0.0003; ***P < 0.0001;). (b) Representative images of wild-type and TIM-1Δmucin Th1 cells rolling on P-selectin, showing a consistently lower number of rolling TIM-1Δmucin Th1 cells. (c) Rolling velocities of wild-type and TIM-1Δmucin ConA blasts, Th1 and Th17 cells on P-selectin. No significant differences were found between wild-type and TIM-1Δmucin T cells. (d) TIM-1Δmucin activated T cells show no tethering and rolling defects on E-selectin under physiological flow.
conditions. (e) Rolling velocities of wild-type and TIM-1Δmucin ConA blasts, Th1 and Th17 on E-selectin under physiological flow conditions. No significant differences were found between wild-type and TIM-1Δmucin T cells. Data represent means ± SEM of four independent experiments. For rolling velocities, data represent means ± SEM of at least 100 cells per condition.
3.5 TIM-1 mediates T-cell capture on P-selectin in vivo

To establish the physiological relevance of TIM-1 in leukocyte adhesion interactions, considering the importance of the TIM-1 mucin domain for P-selectin binding in vitro and the higher rolling capacity on P-selectin shown above, we next focused on the interactions between TIM-1 and P-selectin. We carried out intravital microscopy experiments in exposed mesenteric venules pre-treated with thrombin, which rapidly induces the expression of endothelial P-selectin (Deban et al., Nat Immunol 2010). Th1 and Th17 cells from TIM-1Δmucin mice showed a significant reduction in the ability to roll on P-selectin compared to wild-type cells (Fig. 8a, b). Th1 showed a 52% reduction and Th17 cells showed a 48% reduction, suggesting that TIM-1 mediates primary adhesion in vivo. Importantly, the number of total tethers was reduced by 42% for Th1 cells and 50% for Th17 cells (Fig. 8c), suggesting that TIM-1 can also mediate T-cell tethering in vivo. As expected, TIM-1Δmucin PMNs displayed no defects in their ability to interact with P-selectin in vivo (Fig. 8a, c), indicating that the defect in rolling is lymphocyte-specific. As shown in vitro, we found no differences in rolling velocities or the distribution of rolling velocities between wild-type and TIM-1Δmucin cells in mesenteric venules, suggesting that, in the presence of functional PSGL-1, TIM-1 does not influence the quality of activated T-cell rolling on P-selectin in vivo (Fig. 8d, e).
Figure 8: TIM-1 mediates T-cell capture on P-selectin in vivo

Th1 and Th17 cells were generated from murine wild-type and TIM-1Δmucin CD4+ cells. Bone marrow-derived PMNs were used as negative controls. Cells were injected intravenously into the exposed mesenteric vessels of mice pre-treated with bovine thrombin to upregulate P-selectin on the vascular endothelium. (a) The analysis of interactions between cells and the vessel wall showed that activated TIM-1Δmucin T cells have a significantly reduced ability to roll on P-selectin in vivo (*P < 0.002 compared to wild-type cells). Bone marrow-derived PMNs from TIM-1Δmucin mice showed no defects in rolling interactions with mesenteric venules. (b) The number of tethers (each new interaction with the vessel wall) showed that TIM-1Δmucin activated T cells
have fewer total interactions with the venules, whereas the capture of PMNs was not affected by the mutation (*$P < 0.03$ and **$P < 0.004$ compared to the corresponding wild-type cells). Data in (a) and (b) represent means ± SEM of 13-15 independent experiments for a total of 15-20 total venules/condition. (c) Representative images of Th1 cells rolling in mesenteric vessels. Cells are the white spots inside the venules (arrow tips). Note the reduced number of TIM-1$^{\Delta\text{mucin}}$ cells interacting with the vessel endothelium, compared to wild-type cells. (d-e) TIM-1$^{\Delta\text{mucin}}$ Th1 (d) and Th17 (e) cells display no defects in their rolling velocity in mesenteric venules, compared to wild-type cells. Rolling velocities represent the mean ± SEM of at least 100 cells per condition (left panel). The distribution of leukocyte rolling velocities is also shown (right panel).
3.6 The TIM-1 IgV domain is also required for T-cell rolling on P-selectin

We next investigated the potential role of the TIM-1 IgV domain in P-selectin binding by testing cells in the presence of two antibodies that specifically block this domain: RMT1-10 and 4A2.2 (Xiao et al., *J Exp med* 2007)(Sizing et al., *J Immunol* 2007). We carried out intravital microscopy experiments in exposed mesenteric venules pretreated with thrombin. Surprisingly, we found that inhibiting the IgV domain with antibody RMT1-10 in Th1 cells led to a 54% reduction in the ability of the cells to roll on P-selectin *in vivo*, compared to cells treated with a control IgG antibody (Fig. 9a). This suggested that the TIM-1 IgV domain is required for interactions with P-selectin. In addition, the total number of tethers was reduced to 60% when the IgV domain was blocked with antibody RMT1-10, suggesting that the TIM-1 IgV domain is required for tether formation under physiological flow conditions (Fig. 9b). As shown for the mucin domain, there was no difference in rolling velocity between cells treated with antibody RMT1-10 and the control antibody (Fig. 9c), providing further evidence that TIM-1 does not affect the quality of rolling interactions in the presence of functional PSGL-1. The other blocking antibody (4A2.2) had a similar effect on tethering and rolling interactions, reinforcing the physiological relevance of TIM-1 IgV domain *in vivo* (Fig. 9a, b, d). The IgV domain has not previously been shown to play a role in tethering or rolling of other selectin ligands and our results suggest that TIM-1 therefore uses a regulatory mechanism that is unique within the family of selectin receptors.
Figure 9: The TIM-1 IgV domain is required for P-selectin-dependent rolling in vivo. Wild-type Th1 cells were treated with control rat IgG or the blocking anti-TIM-1 antibodies RMT1-10 or 4A2.2, which recognize epitopes in the TIM-1 IgV domain (Xiao et al., J Exp Med 2007)(Sizing et al., J Immunol 2007). Immediately after antibody treatment, cells were tested for P-selectin-dependent rolling in thrombin-treated mesenteric venules. (a) Cells treated with RMT1-10 and 4A2.2 showed a strongly reduced ability to roll on P-selectin in vivo (*P < 0.005 and **P < 0.04 compared to cells treated with rat IgG). (b) RMT1-10 and 4A2.2 treatments also reduced the number of total tethers (*P < 0.008 and **P < 0.03 compared to control cells). Data in (a) and (b) represent means ± SEM of 3-4 independent experiments for a total of 6-8 total venules per condition. (c-d) Cells treated with both RMT1-10 (c) and 4A2.2 (d) showed no defects in their rolling velocity, compared to cells treated with rat IgG. Rolling velocities represent the mean ± SEM of at least 50 cells per condition. Distribution of rolling velocities is also shown (right panels).
3.7 TIM-1 cooperates with PSGL-1 to mediate tethering and rolling in vitro and in vivo

PSGL-1 is currently regarded as the sole physiological P-selectin ligand expressed on activated T cells (Ley and Kansas, Nat Rev Immunol 2004)(Zarbock et al., Blood 2011)(Borges et al., J Exp Med 1997). PSGL-1-deficient leukocytes showed a reduced ability to interact with P-selectin under physiological flow conditions, but residual interactions with P-selectin still occur in PSGL-1-deficient T cells in vitro and in vivo (Supplementary Fig. 5)(Borges et al., J Exp Med 1997). We therefore investigated whether the residual rolling observed in Selplg−/−Th1 cells is mediated by TIM-1-dependent interactions. We crossed the two mutant lines to generate Selplg−/−/TIM-1Δmucin mice and analyzed the interaction between Th1 cells from these mice with P-selectin and E-selectin under physiological flow conditions in coated capillary tubes. We found that the absence of the TIM-1 mucin domain in Selplg−/−/TIM-1 Th1 cells inhibited PSGL-1-independent residual rolling in capillary tubes coated with P-selectin by 42%, whereas rolling on E-selectin was not affected (Fig. 10a).

We next investigated the importance of TIM-1 in PSGL-1-independent rolling in vivo, in thrombin-treated mesenteric venules. In agreement with previous reports, we found that Selplg−/−Th1 cells show a substantial reduction of rolling interactions in vitro compared to wild-type cells (Supplementary Fig. 5). In addition, Selplg−/−Th1 cells showed a reduction of rolling interactions and total tethers and an increase in rolling velocity in vivo (Supplementary Fig. 5). However, in agreement with our in vitro data, we found that Selplg−/−/TIM-1Δmucin Th1 cells showed a 43% reduction in their ability to roll on P-selectin compared to Selplg−/− cells (Fig. 10b), suggesting that TIM-1 mediates a significant proportion of the residual rolling interactions observed in Selplg−/− cells. Notably, the number of total tethers was reduced by 40% in the TIM-1Δmucin cells (Fig. 10b), confirming that the reduction in T-cell capturing is associated with a functional defect in the TIM-1 molecule. Furthermore, the IgV domain-blocking antibody RMT1-10 inhibited both residual tethering and rolling interactions by 44% in Selplg−/− cells (Fig. 10c). Taken together, our data clearly indicate that TIM-1 mediates tethering and rolling in the absence of PSGL-1 and cooperates with PSGL-1 in supporting activated T-cell rolling on P-selectin in vivo.
Figure 10: The mutation or functional blocking of TIM-1 inhibits residual PSGL-1- independent rolling on P-selectin. (a) Selplg−/− and Selplg−/−/TIM-1Δmucin
Th1 cells were infused into capillary tubes pre-coated with P-selectin or E-selectin, under physiological shear stress conditions (2 dyne/cm²). Selplg−/− cells showed a significant residual ability to interact with P-selectin under physiological flow conditions, whereas the Selplg−/−/TIM-1Δmucin double mutant Th1 cells showed a strongly reduced ability to interact with P-selectin (but not E-selectin) compared to Selplg−/− cells, suggesting that PSGL-1 and TIM-1 co-operate to support Th1 cell binding to P-selectin under physiological flow conditions (*P < 0.0001; reduction = 42%). Data represent the mean ± SEM of four independent experiments. (b) Selplg−/−
and Selplg−/−TIM-1Δmucin Th1 cells were tested for in vivo P-selectin-dependent rolling in the mesenteric venules of thrombin-treated mice. As for the in vitro results, the Selplg−/−TIM-1Δmucin double mutant Th1 cells (left panel) showed a reduced ability to roll on mesenteric venules expressing P-selectin, compared to Selplg−/− cells (∗∗P < 0.004; reduction = 43%). The absence of the TIM-1 mucin domain also reduced the number of total tethers formed in mesenteric venules compared to Selplg−/− cells expressing functional TIM-1 (∗P < 0.02; reduction = 42%). Data represent the mean ± SEM of 10 independent experiments for a total of 16-17 total venules per condition.

(c) Selplg−/− Th1 cells were treated with antibody RMT1-10 or a control rat IgG and were tested for P-selectin-dependent rolling in thrombin-treated mesenteric venules. Selplg−/− cells treated with RMT1-10 showed a reduced ability to roll on P-selectin in vivo, compared to cells treated with the control rat IgG (left panel; ∗P < 0.05). Fewer tethers were also formed compared to control cells following treatment with RMT1-10 (right panel; ∗∗P<0.002). Data represent the mean ± SEM of three independent experiments for a total of seven venules per condition.
3.8 TIM-1 mediates T-cell recruitment in inflamed skin

We next evaluated the potential pathophysiological role of the interaction between TIM-1 and P-selectin in leukocyte trafficking *in vivo* during inflammation, using a contact hypersensitivity model (CHS) in which P-selectin on the skin endothelium is necessary for activated T-cell recruitment (Austrup et al., *Nature* 1997)(Catalina et al., *Blood* 1999). To evaluate the ability of wild-type and TIM-1\[^{Δmucin}\] Th1 cells to migrate in the inflamed ear pinnae, wild-type mice were sensitized with DNFB (1-fluoro-2,4-dinitrobenzene ) and challenged 5 days later on the right ear. After 24 h, Th1 cells were labeled with CFSE and injected into the CHS mice. We found that the TIM-1\[^{Δmucin}\] Th1 cells showed a 48% reduction in their ability to migrate in the inflamed skin compared to wild-type cells (Fig. 11a), suggesting that TIM-1 plays a role in activated T-cell migration in the inflamed skin. In addition, the TIM-1\[^{Δmucin}\] Th1 cells failed to amplify the inflammatory response in the challenged ear by increasing ear thickness compared to wild-type Th1 cells (Fig. 11b), clearly supporting a pathological role for TIM-1-dependent T-cell trafficking.
Figure 11: TIM-1 mediates T-cell recruitment in the inflamed skin. Mice were sensitized with 1-fluoro-2,4-dinitrobenzene (DNFB) to induce cutaneous hypersensitivity (CHS) and were challenged again 5 days later on each side of the right ear pinnae with DNFB. The ear thickness was measured 0, 24, 48 and 72 h after challenge with a dial thickness gauge. (a) $10 \times 10^6$ CFSE-labeled Th1 cells from wild-type and TIM-1$^{\Delta$mucin}$ mice were transferred to CHS mice 24 h after the ear pinnae were painted, and Th1 accumulation was evaluated 24 h later by FACS. Th1 cells from the TIM-1$^{\Delta$mucin}$ mice showed a reduced ability to migrate in the inflamed skin compared to wild-type cells ($*P < 0.02$). (b) The injection of wild-type but not TIM-1$^{\Delta$mucin}$ Th1 cells increased the inflammation response in the challenged ear ($*P<0.01$). In both a and b, results represent the mean ± SEM of 9-11 mice per condition.
3.9 TIM-1 controls activated T-cell interactions with inflamed pial vessels

We investigated the impact of interactions between TIM-1 and P-selectin during inflammatory responses in more detail by studying the role of TIM-1 in T-cell adhesion within the inflamed venules of the central nervous system (CNS). We carried out intravital microscopy experiments in the inflamed brain pial vessels, a key point for T-cell entry into the CNS during experimental autoimmune encephalomyelitis (EAE). We therefore used an experimental model mimicking early CNS vascular inflammation during EAE, in which we and others have previously shown that the inhibition of P-selectin almost completely abolishes T-cell tethering and rolling (Piccio et al., *J Immunol* 2002; Kerfoot and Kubes, *J Immunol* 2002). Our results showed that the TIM-1\(\Delta\)mucin mutation caused a significant reduction in the ability of Th1 and Th17 cells to roll (50% reduction for Th1 cells and 40% for Th17 cells), and their capacity to maintain firm adhesion (43% reduction for Th1 cells and 62% for Th17 cells) in the inflamed brain pial venules, compared to wild-type cells (Fig. 12a,b). These results showed that TIM-1 is required on T cells for efficient adhesion to the inflamed CNS endothelium in addition to the previously described requirement for PSGL-1, VLA-4 and LFA-1 (Piccio et al., *J Immunol* 2002). Thus, as shown in mesenteric venules, the TIM-1 mucin domain is also necessary for the ability of TIM-1 to control adhesion interactions in the inflamed brain microcirculation.
Figure 12

(a) TIM-1 controls T-cell trafficking in the inflamed CNS and the induction of EAE. For a and b, wild-type and TIM-1Δmucin Th1 and Th17 cells were injected into the right carotid artery of LPS-treated mice, and the adhesive interactions between T-cells and inflamed vascular endothelium of brain pial vessels were investigated in intravital microscopy experiments. (a) TIM-1Δmucin Th1 and Th17 cells showed a reduced ability to roll (and thus adhere firmly) in the inflamed CNS vessels, compared to wild-type cells (***P < 0.008; *P < 0.01; ***P < 0.007). For both the Th1 and Th17 cells, data represent the mean ± SEM of three independent experiments for a total of 8-9 venules per condition. (b) Representative images of brain pial venules showing adhered Th1 cells (wild-type and TIM-1Δmucin) as white spots inside the vessels (arrow tips). There are fewer TIM-1Δmucin Th1 cells than wild-type cells adhering to the brain pial vessels. Scale bar = 100 µm.
3.10 TIM-1 controls T-cell accumulation in the inflamed CNS and the induction of autoimmune disease

The pathological role of TIM-1 in T-cell trafficking was investigated by studying the ability of TIM-1 to control T-cell recruitment in the CNS during EAE. We therefore generated *in vitro* MOG35-55-specific Th1 cells from wild-type and TIM-1Δmucin mice (Lees et al., *J Immunol* 2008) and evaluated their migration in the CNS using a model of EAE (Kerfoot et al., *J Immunol* 2004). We first determined the phenotype of wild-type and TIM-1Δmucin MOG35-55-specific Th1 cells and found no differences in IFNγ production and adhesion molecule expression among the two cell populations (Supplementary Fig. 6). However, when we investigated the recruitment of myelin-specific T cells in the CNS, we found that TIM-1Δmucin Th1 cells showed a significantly reduction in the ability to migrate compared to wild-type cells (Fig. 13a), suggesting that TIM-1 plays a role in activated T-cell migration in the inflamed CNS. Notably, we also found that MOG35-55-specific Th1 cells obtained from TIM-1Δmucin mice induced a significantly less severe form of EAE compared to wild-type cells (Fig. 13b), with reduced demyelination and inflammatory cell infiltration in the CNS parenchyma (Fig. 13c). This clearly demonstrates that TIM-1 expression plays a pivotal role in the trafficking of T cells into the CNS and consequently in the induction of EAE.

We also investigated the induction of EAE in C57Bl/6J wild-type and TIM-1Δmucin mice by immunization with the MOG35-55 peptide. The disease was significantly less severe in the TIM-1Δmucin mice (Supplementary Fig. 6a), indicating a key role for TIM-1 in EAE pathogenesis. Notably, CD4+ T-cells isolated from draining lymph nodes of the wild-type and TIM-1Δmucin EAE mice 7 days post-immunization had a similar capacity to proliferate (Supplementary Fig. 6b), suggesting that the inhibition of EAE in TIM-1Δmucin mice reflects a defect in T-cell trafficking, rather than antigen-dependent T-cell activation.
Figure 13: TIM-1 controls T-cell trafficking in the inflamed CNS and the induction of EAE. (a) 5 x 10^6 wild-type or TIM-1Δmucin Th1 cells were labeled with green CFSE and injected into C57BL/6J wild-type recipient mice treated 5h previously with 20 ng pertussis toxin (PTX). The brains and spinal cords were removed 60 h after cell transfer. The accumulation of CSFE+ cells in the CNS was evaluated by FACS. TIM-1Δmucin Th1 cells showed a reduced ability to migrate in the CNS of PTX-treated mice, compared to wild-type cells (*P < 0.006). Data represent means ± SEM of 12 mice per condition from two independent experiments involving six mice per condition. (b) Recipient C57BL/6J wild-type mice were injected with 20 ng PTX 4 days and 1 day before the transfer of 5 x 10^6 MOG_{35-55}-specific wild-type or TIM-1Δmucin Th1 cells. The mice receiving TIM-1Δmucin Th1 cells developed significantly less-severe EAE symptoms compared to mice receiving wild-type Th1.
cells. Data represent the mean ± SEM of 10 mice per condition from two independent experiments with five mice per condition (*$P < 0.05$). (c) Neuropathological analysis of lumbar spinal cord tissues from mice receiving wild-type or TIM-1$^{\Delta_{\text{mucin}}}$ Th1 cells and then killed at the disease peak. Hematoxylin/eosin and Spielmeyer staining revealed a significant reduction of inflammatory cell infiltrates and demyelination in TIM-1$^{\Delta_{\text{mucin}}}$ compared to wild-type mice.
4. Discussion
Recruitment of blood flowing neutrophils, monocytes and activated lymphocytes to the site of acute or chronic inflammation is a crucial process during the development of an immune response. Genetic abnormalities leading to defects in leukocyte trafficking have been correlated in humans with the development of the leukocyte adhesion deficiency (LAD) syndromes, in which most of the patients die early in life due to severe impairment in host defense mechanisms (Etzioni, *Current Opinion Immunol* 2009). Leukocyte recruitment to the inflamed tissue and homing to secondary lymphoid organs is a multi-step highly regulated process in which several protein families of cellular and vascular adhesion molecules are involved (Kondratowicz et al., *PNAS* 2011). The purpose of this project was to investigate a role for the mucin Tim-1 as a novel adhesion molecule controlling the recruitment of activated T lymphocytes in inflamed tissues. Tim-1 is a transmembrane glycoprotein belonging to the TIM gene family and expressed by CD4+ T cells upon activation (Rennert, *Immunol Lett* 2011). It is a costimulatory molecule that induces activation of naïve T cells and sustains activated T cells functions (Rodriguez-Manzanet et al., *Immunol Rev* 2009). The Tim proteins present, in the external region, an IgV-like domain, followed by a highly glycosylated mucin domain, with a predicted extended conformation and a heavily O-glycosylated pattern (Kuchroo et al., *Nat Rev Immunol* 2003). This peculiar structure is similar to those of the mucin MadCAM-1, a trafficking receptor able to bind selectins, which is a classical family of adhesion molecules that control leukocyte trafficking in the secondary lymphoid organs and chronically inflamed tissues (Ley and Kansas, *Nat Rev Immunol* 2004; Ley et al., *Nat Rev Immunol* 2007).

Moreover, it has been recently shown that that the IgV domain of human TIM-1 exhibits characteristics of the C-type lectins such as for instance calcium-dependent interactions (Wilker et al., *Int Immunol* 2007). The interaction of C-type lectins with carbohydrate ligands is mediated by a conserved carbohydrate recognition domain (CRD). The TIM family Ig domains appear unrelated to the CRD of C-type lectins based on amino acid sequence comparisons, but are related to type-I lectins, called siglecs, each containing an extracellular Ig domain (Crocker; *Curr Opin. Struct Biol* 2002). Each siglec family member contains a V-set Ig domain that binds specific types of sialic acid attached to the terminal sugars of oligosaccharide chains by specific glycosidic linkages. Thus, siglec family Ig domains provide a clear precedent for carbohydrate recognition by TIM family Ig domains. In support of our
hypothesis of a role of Tim-1 in leukocyte adhesion processes, the O-glycosylation profile predicted for Tim-1 is comparable to the one found on the mucin PSGL-1 (Ley, *Trends Mol Med* 2003), another well characterized mucin adhesion molecule expresses by leukocytes able to bind all known three selectins. Taken together, all these structural informations prompted us to investigate a possible role for Tim-1 in mediating activated T cells trafficking in the inflamed tissues. In particular, due to the presence of a highly glycosylated mucin domain in the Tim-1 structure, we focus our attention in the present study on the interactions between mucin Tim-1 and selectins.

We have provided direct evidence that TIM-1 is a novel ligand for endothelial selectins and that it controls the tethering and rolling of activated T cells in the inflamed microcirculation and the accumulation of T cells at inflammation sites. More specifically we have demonstrated that TIM-1 is a major P-selectin ligand; our results fulfill all criteria for the definitive assignment of TIM-1 as a selectin ligand. In facts we have shown that: (i) Tim-1 is able to bind selectins in a cell free system in a divalent cation dependent manner; (ii) microspheres covered with TIM-1 can support T-cell rolling on selectins under physiological flow conditions; (iii) monoclonal antibodies against TIM-1 block selectin-dependent T-cell rolling *in vitro* and *in vivo*; and (iii) the mutation of the TIM-1 gene impairs selectin-mediated T-cell functions on intact cells *in vitro* and in experimental models *in vivo* (Zarbock et al., *Blood* 2011).

As previously shown for PSGL-1, our data demonstrate that TIM-1 binds all three selectins *in vitro*, but under physiological shear stress conditions TIM-1-covered microspheres tether and roll only on P-selectin and E-selectin, suggesting that interactions between TIM-1 and L-selectin are less relevant *in vivo*. Furthermore inhibiting the mucin domain of TIM-1 in activated T cells causes rolling defects on P-selectin but not E-selectin, suggesting that the interaction between TIM-1 and P-selectin has a more significant role in T-cell trafficking and/or that the interaction between TIM-1 and E-selectin is mediated by other TIM-1 domains. Is well known that activated T cells express high levels of E-selectin ligands such as PSGL-1, CD44 and CD43, thus any E-selectin-dependent rolling mediated by TIM-1 could be masked by the functional redundancy of other selectin ligands. Indeed, the functional redundancy of E-selectin ligands in mice has required the simultaneous deletion of more than one ligand to decipher their individual functions and further studies are needed to clarify the role of TIM-1/E-selectin interactions *in vivo* (Zarbock et
The existence of P-selectin ligands other than PSGL-1 (and their ability to mediate rolling interactions) has been predicted, but the identity and precise role of these ligands in leukocyte trafficking has been unclear (Ley and Kansas, Nat Rev Immunol 2004). Although PSGL-1 has been shown to mediate the rolling of neutrophils, monocytes and T cells, our data show that TIM-1 plays a more specialized role in the trafficking of activated T cells. Furthermore, whereas PSGL-1 is involved in naïve T cell homing to lymphoid organs as well as leukocyte trafficking during inflammation, TIM-1 has a more specialized role in activated T cell recruitment to the sites of inflammation suggesting a new level of diversity between PSGL-1 and TIM-1.

The significant reduction in the ability of TIM-1Δmucin Th1 and Th17 cells to roll on P-selectin is surprising, because these cells have high levels of functional PSGL-1 on their surface. Indeed, our studies showed that the inhibition of TIM-1 in Th1 and Th17 cells strongly reduced rolling on P-selectin in vitro and in vivo, and PSGL-1-independent residual rolling was inhibited by TIM-1 inactivation, clearly indicating that TIM-1 is a major P-selectin ligand on T cells. Our data show that both TIM-1-P-selectin and PSGL-P-selectin pro-adhesive mechanisms are both necessary to achieve “fully efficient” recruitment and reveal that the novel TIM-1-P-selectin molecular pathway is concurrently involved in T cell trafficking at the regulatory level of cell tethering and rolling (D’Ambrosio et al., Life Sci 2004).

The PSGL-1 mucin domain requires dense O-glycosylation in order to bind selectins (Carolw et al., Immunol Rev 2009). Similarly, the TIM-1 mucin domain is rich in threonine, serine and proline residues, and is predicted to undergo substantial O-glycosylation although the glycans have not been analyzed in detail (Kuchroo et al., Nat Rev Immunol 2003). The expression of selectin ligands is inducible in T cells and several glycosyltransferases facilitate the biosynthesis of selectin ligands including α1,3-fucosyltransferases, core 2 β1,6- glucosaminyltransferase-I (C2GlcNacT-I), β1,4-galactosyltransferase-I, sialyltransferases and tyrosine sulfotransferases. The TCR-dependent stimulation of T cells induces the expression of enzymes that are fundamental to P-selectin binding, which may facilitate the correct glycosylation of both PSGL-1 and TIM-1. However, the recombinant murine TIM-1 used in our experiments was produced in Chinese hamster ovary (CHO) cells lacking the enzymes α1,3-fucosyltransferase (Li et al., J Biol Chem 1996) and C2GlcNacT-I,
which is considered the limiting enzyme for P-selectin ligand formation in T cells (Ley and Kansas, Nat Rev Immunol 2004). Other carbohydrates in the TIM-1 mucin domain may control the interaction, e.g. there are two and three predicted sites for N-glycosylation in the murine and human TIM-1 proteins, respectively (Kuchroo et al., Nat Rev Immunol 2003). Indeed, our results show that TIM-1 requires post-translational fucosylation and tyrosine sulfation for efficient binding to endothelial selectins, but does not require sialylated glycans, which are instead critical for the binding of other receptors such as PSGL-1 and CD44 suggesting that TIM-1 presents a specific glycosylation profile necessary for selectin binding, with clear differences to PSGL-1 and other ligands, particularly in the requirement of sialylated carbohydrates (Zarbock et al., Blood 2011)

Our intravital microscopy studies showed that the inhibition of TIM-1 in cells expressing high levels of PSGL-1 strongly reduces rolling on P-selectin in vitro and in vivo, clearly indicating that TIM-1 is a major P-selectin ligand on T cells. PSGL-1 and TIM-1 cooperate to control activated T-cell rolling on P-selectin, as suggested by the inhibition of PSGL-1-independent residual rolling when TIM-1 is inactivated or blocked. However, residual rolling on cells lacking PSGL-1 and functional TIM-1 was not completely abolished, suggesting that T cells may have an additional P-selectin receptor that mediates rolling. TIM-1Δmucin cells show normal rolling velocities on P-selectin, confirming that TIM-1 does not influence the quality of rolling interactions in the presence of functional PSGL-1. During the course of T-cell activation, T cells express P-selectin ligands earlier than E-selectin ligands, suggesting that P-selectin has a more important role in early inflammation (Ley and Kansas, Nat Rev Immunol 2004). Furthermore, TIM-1 is upregulated immediately after TCR activation or generic stimulation with ConA, suggesting that it may mediate the trafficking of less-polarized T cells during early inflammatory responses. Our results show that TIM-1 also controls the rolling adhesion of Th1 and Th17 cells, suggesting a pivotal role for TIM-1 in the trafficking of polarized T-cell populations to inflammation sites. Th2 cells express high levels of TIM-1 and this may be relevant in the control of Th2 cell rolling on P-selectin and trafficking during allergic inflammatory reactions (Bonder et al., 2008). TIM-1 is also expressed on activated B cells (Rennert, Immunol Lett 2011), which have a low capacity to roll on endothelial selectins (data not shown), suggesting a selective role for TIM-1 in T-cell trafficking.

We also sought to determine the role of individual TIM-1 domains in selectin binding.
We showed that the mucin domain is not required for T-cell activation and proliferation in the presence of an antigen (supplementary result 6,7), but it is selectively involved in the interaction with P-selectin in vitro and in vivo, helping to control T-cell trafficking to inflammation sites. TIM-1Δmucin Th1 and Th17 cells display a significant reduction in their ability to roll in the inflamed microcirculation, suggesting that functional inhibition of the mucin domain has anti-inflammatory effects. Unexpectedly, our results revealed that IgV domain-blocking antibodies also inhibit rolling on P-selectin in vivo. The IgV domain has never previously been shown to be required for selectin binding. The structure of TIM-1 is similar to the mucosal addressin cell adhesion molecule 1 (MAdCAM-1), a homing receptor containing two extracellular IgV domains that bind integrin αβ7, and one mucin domain that mediates rolling on L-selectin (Ley et al., Nat Rev Immunol 2007). However, the MAdCAM-1 IgV domains are not involved in rolling, suggesting that the interaction between the TIM-1 IgV domain and P-selectin is a unique feature of TIM-1. Notably, the IgV domain of TIM proteins was shown to share functional characteristics with Ca2+- dependent (C-type) lectins and SIGLEC sialic acid-binding proteins, which also play a role in leukocyte trafficking (Ledbetter et al., PNAS 1987), further supporting a role for the TIM-1 IgV domain in T-cell adhesion.

TIM-1 takes part in diverse homotypic and heterotypic interactions, with ligands such as CD300b, HAV, filoviruses, phosphatidylserine on apoptotic cells and the intracellular nuclear receptor NUR77. In addition to its presence on the cell surface, TIM-1 was previously found in large intracellular pools in early endosomes, the Golgi apparatus and lysosomal compartments (Santiago et al., Immunity 2007). Cell-surface TIM-1 undergoes continuous retrograde trafficking via constitutive clathrin-dependent endocytosis (Balasubramanian et al., Ann Rev of Physiol 2012). In naïve T cells, we found substantial intracellular pools of endogenous TIM-1 but none on the surface. In contrast, TIM-1 expression was upregulated on the surface of in vitro activated T cells, but was found predominantly just beneath the plasma membrane (supplementary results figure 2). These results suggest that TIM-1 is poised to be exposed on the cell surface after activation under specific conditions and that TIM-1 functions in lymphocyte extravasation as an activation-dependent primary adhesion molecule. Indeed, recent data suggest that cells transfected with TIM-1 expose the mucin domain on the cell surface following intracellular calcium release (Santiago et al., Immunity 2007). A flip-flop model was proposed in which the TIM-1 extracellular
domain resides on the cytosolic side of the membrane, with the metal ion-dependent site (MILIBS) of the IgV domain interacting with phosphatidylserine (Santiago et al., *Immunity* 2007). An increase in the concentration of intracellular calcium could therefore enhance the exposure of TIM-1 to the outer leaflet of the membrane, with subsequent release of the IgV domain and presentation of the whole molecule. This model, together with our data, suggest that activating stimuli including TCR engagement (Ledbetter, *PNAS* 1987) induce TIM-1 presentation on the cell surface and thus enhance T-cell rolling capacity and trafficking to inflammation sites.

Physiological selectin ligands such as PSGL-1, CD44 and ESL-1 mediate not only leukocyte rolling but also partial and transient integrin activation in rolling cells, a process that promotes their subsequent stable arrest on the endothelium (Zarbock et al., *Blood* 2011). Signaling through PSGL-1 depends on a constitutive association between the PSGL-1 cytoplasmic tail and Nef-associated factor 1 (Naf1). The binding of P-selectin to PSGL-1 leads to the phosphorylation of Naf1 by Src kinases, and subsequent recruitment of the phosphoinositide-3-OH kinase (PI(3)K) p85-p110δ heterodimer, which triggers β2 leukocyte integrins to adopt an intermediate affinity state mediating slow rolling (Wang et al., *Nat Immunol* 2007). TIM-1 is a co-stimulatory molecule with at least one tyrosine phosphorylation site in its cytoplasmic tail (Kuchroo et al., *Nat Rev Immunol* 2003). TIM-1 crosslinking induces the phosphorylation of its cytoplasmic tail as well as phosphorylation of Zap-70 and IL-2-inducible T-cell kinase (ITK). Interestingly, the p85 subunit of PI3K is recruited directly to the tyrosine-276 residue of TIM-1 after lymphocyte-specific protein tyrosine kinase (Lck)-dependent phosphorylation of the TIM-1 cytoplasmic tail (De Souza et al., *J of Immunol* 2008). We have previously shown that PI3K plays a key role in integrin-mediated adhesion by specifically controlling integrin valency (Constantin et al., *Immunity* 2000) we speculate that the interaction between TIM-1 and selectins may transactivate integrins contributing to T-cell arrest. The high concentrations P-selectin in the blood during autoimmune and inflammatory diseases suggest that P-selectin may crosslink lymphocyte TIM-1, favoring the ingress of activated T-cells at inflammation sites (Wang et al., *Nat Immunol* 2007).

Finally our findings show that TIM-1 mediates T-cell trafficking in three models of inflammatory conditions: thrombin-activated mesenteric vessels, the inflamed brain endothelium during EAE, and CHS in the skin. These results provide compelling
evidence that TIM-1 plays a major role in T-cell trafficking as a rolling receptor for P-selectin as P-selectin play a pivotal role in the development of these inflammatory models.

Moreover we showed that Tim-1 plays a prevalent role in Th1 cell accumulation in CNS when compared to inflamed skin, suggesting that Tim-1 is important to achieve tissue specificity in leukocyte trafficking. We also show that TIM-1 is required for the recruitment of Th1 and Th17 cells, which are potent inducers of inflammation and autoimmunity, suggesting that interference with TIM-1 activity may provide a novel therapeutic approach in T-cell-mediated diseases. Considering that Th1 and Th17 cells facilitate pathogen clearance and promote anti-tumor immunity, we hypothesize that the TIM-1-P-selectin interaction may also play a role during infection and cancer (Ruffell et al., *Cytokine Growth Factor Rev* 2010; Zhu et al., 2010). As well as interacting with P-selectin on endothelial cells, TIM-1 on the surface of T cells may also interact with P-selectin presented by adhered platelets or their microparticle fragments, further contributing to the efficacy of T-cell trafficking.

In conclusion, our findings collectively indicate that TIM-1 is a major P-selectin ligand and a pivotal trafficking mechanism for T cells during inflammation. Our results refine the paradigm of the leukocyte adhesion cascade and show that the primary adhesion of T cells to P-selectin *in vivo* is no longer exclusively dependent on PSGL-1, but also requires TIM-1, thereby providing a physiological role for the interaction between these two critical components of the immune system.
5. Supplementary
Supplementary figure 1: Binding of P-selectin, E-selectin and L-selectin to TIM proteins \textit{in vitro} Microtiter plates were coated with murine P-selectin, E-selectin or L-selectin, TIM-4 (positive control) or ICAM-1 (negative control) and tested for the ability to bind recombinant murine TIM-1, TIM-2 and TIM-3. Although TIM-1 was able to bind all three selectins, TIM-2 and TIM-3 did not. Data represent the mean ± SEM of two independent experiments performed in triplicate for each condition.
Supplementary figure 2: The cellular localization of TIM-1 in naïve and activated T cells. Naïve T cells (a) and Th1 cells (b) were labeled with FITC-conjugated anti-CD3 and the anti-TIM-1 antibody 5F12 as described in the methods section. Labeled cells were spotted onto a glass slide, and images were acquired by confocal microscopy. The figure shows 3-4 superimposed central stacks of the 3D image. Both naïve and activated T cells expressed TIM-1, which is predominantly localized in the cytosol. However, whereas the TIM-1 in naïve T cells (a) is exclusively located immediately beneath the cell surface, a significant amount of the TIM-1 in Th1 cells (b) is also found on the cell surface, suggesting activation-dependent trafficking of TIM-1 on the surface of T cells. (c) TIM-1 mRNA levels in naïve, Th1 and Th17 cells evaluated by Real Time PCR. All T cell populations have very low levels of TIM-1 transcript, compared to β–actin housekeeping gene, with mRNA levels almost undetectable in terminally polarized Th1 and Th17 cells. This suggests that TIM-1 protein may be principally synthesized during T cell development, and stored in intracellular compartment of fully differentiated CD4+ naïve T cells.
Mouse generation and genotyping

The Tim-1 mutant mice we used in this project were generated in the laboratory of Prof. Vijay K. Kuchroo (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA) by 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 caused the ablation of the extracellular mucin domain from the protein structure (Suppl. Fig. 3a). These mutant mice were particularly useful for our purpose to investigate a role for Tim-1 in leukocyte trafficking, as highly glycosylated mucin domain are typical moieties involved in this process (Sperandio et al., *Immunol Rev* 2009). We initially backcrossed WT/Tim-1Δmucin heterozygous to obtain Tim-1Δmucin/Tim-1Δmucin homozygous animals. The presence of WT or Tim-1Δmucin alleles on newborn mice was checked by PCR genotyping, using specific primers for both alleles. Examples of PCR results from mouse genotyping were reported in supplementary Fig. 3b.

The Tim-1Δmucin mice completely lack the Tim-1 highly glycosylated mucin domain, while the rest of the protein is still expressed on the cell surface. To confirm Tim-1 expression on mutant cells, we evaluated Tim-1 expression on concanavalin A-activated T cells (ConA blasts) and anti-IgM-activated B cells, which express consistent Tim-1 levels on their surface (Meyer JH et al., *Nat Immunol* 2005; Ma et al., *Biochem Biophys Res Commun* 2011). By flow cytometry analysis, we detected Tim-1 expression on both WT and Tim-1Δmucin ConA blasts and activated B cells; moreover, Tim-1 expression levels were comparable between WT and Tim-1 mutant cells (Fig. 3C), confirming that cells from Tim-1 mutant mice still express Tim-1 at normal levels.
Supplementary figure 3: Mouse generation and genotyping: (a) Tim-1 mutant mice were generated by inserting a neo-cassette in the exon 3 of Tim-1 gene. The neo-cassette is indicated in red in the gene sequence. This insertion caused the ablation of the extracellular mucin domain from the protein structure. (b) We backcrossed WT/Tim-1^Δmucin heterozygous to obtain Tim-1^Δmucin/Tim-1^Δmucin homozygous animals. Animals were checked by PCR using one forward primer and two different reverse primers. The forward-reverse primers couple amplified the WT C57Bl/6J mice Tim-1 gene (amplicon length: 385, b right image), while the forward-neo-reverse couple amplified the Tim-1^Δmucin gene generated by exon 3 deletion (amplicon length: 581 bp, b left image). In all the images M indicates marker (C) The expression of Tim-1 was evaluated by flow cytometry on concanavalin A-activated T cells (ConA blasts) and anti-IgM-activated B cells. The expression of Tim-1 was comparable between WT and Tim-1 mutant cells, confirming that Tim-1^Δmucin cells express Tim-1 at normal levels.
Supplementary figure 4: Phenotypes of wild-type and TIM-1Δmucin activated T cells. Th1 and Th17 cells were generated in vitro from wild-type and TIM-1Δmucin CD4+ T cells. The expression of several adhesion molecules and activation markers was analyzed by FACS. No differences in the expression of L-selectin, integrins αLβ2 (LFA-1) and α4, PSGL-1, CD44 and CD25 were found between the wild-type and TIM-1Δmucin Th1 cells (a) and Th17 cells (b). Colors: red line = isotype control; blue line = wild-type cells; green line = TIM-1Δmucin cells. Data reflect one representative experiment among of four carried out in total.
Supplementary figure 5: Comparison of wild-type and Selplg−/− Th1 cell interactions with endothelial selectins. (a) Wild-type and Selplg−/− Th1 cells were infused into capillary tubes pre-coated with P-selectin or E-selectin, under physiological shear stress conditions (2 dyne/cm²). The Selplg−/− Th1 cells showed strongly-reduced interactions with both selectins under physiological flow conditions compared to wild-type cells (*P < 0.0001). Data represent the mean ± SEM of four independent experiments. (b) The rolling velocities of Th1 cells in capillary tubes. Selplg−/− Th1 cells showed a significantly higher rolling velocity on both selectins compared to wild-type cells (*P < 0.0001). Data represent the mean ± SEM of at least 100 cells per condition. (c) Evaluation of interactions between wild-type or Selplg−/− Th1 cells and thrombin-treated mesenteric venules. As shown in vitro, Selplg−/− Th1
cells showed a strongly reduced ability to interact with P-selectin-expressing mesenteric venules, compared to wild-type cells, in terms of rolling and the total number of tethers (*$P < 0.0001$). Data represent the mean ± SEM of 10 independent experiments for a total of 14–15 total venules per condition. (d) The rolling velocities of Th1 cells in thrombin-treated mesenteric venules. Selplg−/− Th1 cells showed a significantly higher rolling velocity in mesenteric venules, compared to wild-type cells (*$P < 0.04$). Data represent the mean ± SEM of at least 100 cells per condition (left panel). The distribution of leukocyte rolling velocities is also shown (right panel).
**Supplementary figure 6**

**a**

The expression of several adhesion molecules was analyzed by FACS. No differences in the expression of L-selectin, integrins LFA-1 and α4, PSGL-1 or CD44 were found between wild-type and TIM-1Δmucin cells. Colors: red line = isotype control; blue line = wild-type cells; green line = TIM-1Δmucin cells.

**b**

Wild-type and TIM-1Δmucin cells produce comparable amounts of IFN-γ after *in vitro* re-stimulation for intracellular staining. Both populations produce negligible amounts of IL-17 and IL-4 (data not shown).

**Supplementary figure 6: The phenotype of wild-type and TIM-1Δmucin MOG\textsubscript{35-55} specific Th1 cells.** MOG \textsubscript{35-55}-specific Th1 cells were generated from wild-type and TIM-1Δmucin CD4+ T cells isolated from MOG\textsubscript{35-55} immunized mice. (a) The expression of several adhesion molecules was analyzed by FACS. No differences in the expression of L-selectin, integrins LFA-1 and α4, PSGL-1 or CD44 were found between wild-type and TIM-1Δmucin cells. Colors: red line = isotype control; blue line = wild-type cells; green line = TIM-1Δmucin cells. (b) Wild-type and TIM-1Δmucin cells produce comparable amounts of IFN-γ after *in vitro* re-stimulation for intracellular staining. Both populations produce negligible amounts of IL-17 and IL-4 (data not shown).
Supplementary figure 7

Supplementary Figure 7: Absence of the TIM-1 mucin domain influences the development of active EAE. (a) EAE was actively induced in wild-type and TIM-1^{Δmucin} mice by immunization with the MOG_{35-55} peptide. TIM-1^{Δmucin} mice developed a less-severe EAE compared to wild-type mice. Data represent the mean ± SEM of 10 mice per condition from a representative experiment from a series of two independent experiments with similar results (*P < 0.05). (b) CD4+ T cells were isolated from the draining lymph nodes of wild-type and TIM-1^{Δmucin} mice 7 days post-immunization with the MOG_{35-55} peptide. The proliferative response to increasing concentrations of MOG_{35-55} peptide was determined, and no differences were found in the antigen-specific proliferation of wild-type and TIM-1^{Δmucin} cells, suggesting that the TIM-1 mutation does not affect T-cell priming following immunization. Data are shown as counts per minute (CPM) of [3H]-thymidine radioactivity, and represent the mean ± SEM of four mice per condition.
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