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ROLE OF FUCOSYLTRANSFERASE-VII AND P-SELECTIN GLYCOPROTEIN LIGAND-1 IN THE SUPPRESSOR ACTIVITY OF CD25$^+$CD4$^+$ REGULATORY T CELLS

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Riassunto

Un momento critico nella patogenesi della sclerosi multipla e del suo modello animale, l'encefalomielite sperimentale autoimmune (EAE), è rappresentato dall'entrata dei linfociti dai vasi sanguigni nel parenchima del sistema nervoso centrale (CNS). L'attività' di ricerca durante il progetto si è focalizzata sulla caratterizzazione dei meccanismi molecolari che controllano la migrazione e la capacità soppressoria dei linfociti $CD4^+CD25^+$ (cellule T regolatorie) durante l'EAE, in quanto è noto che i linfociti T $CD4^+CD25^+$ hanno azione soppressoria sull'induzione delle malattie autoimmuni. Nel progetto qui presentato, è stato analizzato il ruolo della mucina P-selectin glycoprotein ligand (PSGL)-1 e di fucosiltrasferasi (FucTs) leucocitarie, molecole che controllano la funzionalità del PSGL-1, nell'azione soppressoria svolta dalle cellule T regolatorie nel contesto delle malattie autoimmuni del CNS.

Sono stati inizialmente eseguiti esperimenti *in vivo* di induzione di EAE su topi WT o che mancano dei geni per le molecole studiate; analizzando l'effetto della mancanza di PSGL-1 e FucTs sull'induzione dell'EAE mediante immunizzazione sottocutanea con l'antigene, abbiamo osservato che topi PSGL-$1^{-/-}$ sviluppano una malattia più grave rispetto ai WT. Inoltre, cellule T encefalitogeniche ottenute da topi FucT-$VII^{-/-}$ e PSGL-$1^{-/-}$ (induzione passiva), inducono una malattia molto più severa rispetto a cellule ottenute da topi WT. Allo scopo di spiegare questi risultati, è stata studiata la proliferazione, la produzione di citochine e la capacità adesiva di cellule T encefalitogeniche provenienti da topi WT o FucT-$VII^{-/-}$ e PSGL-$1^{-/-}$. I risultati ottenuti dimostrano che non vi sono differenze significative nell'espressione di molecole d'adesione e nella produzione di citochine da parte di cellule T autoreattive ottenute da topi PSGL-$1^{-/-}$ e FucT-$VII^{-/-}$, rispetto a cellule WT. Cellule autoreattive isolate da topi PSGL-$1^{-/-}$ e FucT-$VII^{-/-}$ non mostrano inoltre difetti nella capacità proliferativa dopo stimolo antigenico.

La nostra attenzione si è spostata sulla possibilità di un coinvolgimento delle molecole sopra citate nell'azione soppressoria delle cellule T regolatore $CD4^+CD25^+$.

Mediante saggi di inibizione della proliferazione è stata valutata la capacità soppressoria *in vitro* di cellule T regolatorie WT e provenienti da topi PSGL-1$^{-/-}$, FucT-VII$^{-/-}$ e FucT-IV$^{-/-}$. Co-culture effettuate con cellule T regolatorie CD4^{+}CD25^{+} e cellule effettrici ("responder") CD4^{+}CD25^{-} hanno indicato che la mancanza di espressione di PSGL-1 e FucT-VII porta ad una drastica diminuzione della capacità soppressoria sulla proliferazione da parte delle T regolatorie. Nessun effetto significativo è stato osservato in presenza di cellule provenienti da topi FucT-IV$^{-/-}$. Inoltre, la stimolazione delle Tregs con anti-CD3 e IL-2 induce un aumento della capacità di legame per chimere di P- e E-selectin, recettori endoteliali del PSGL-1; questa aumentata espressione di PSGL-1 funzionale è accompagnata da una capacità soppressoria significativamente maggiore sulla proliferazione delle cellule effettrici. È stata infine valutato l'effetto della mancanza dei geni del PSGL-1 e FucTs sulla produzione di citochine in saggi Multiplex. I dati dimostrano che la mancanza di espressione di PSGL-1 e FucT-VII porta ad una netta diminuzione della capacità soppressoria sulla produzione di citochine pro-infiammatorie da parte delle cellule T regolatorie. Questi risultati dimostrano quindi un ruolo critico per PSGL-1 e FucT-VII nell'azione soppressoria svolta dalle cellule T regolatore in vitro.

L'importanza di PSGL-1 e FucT-VII è stata ulteriormente dimostrata anche *in vivo*, in quanto cellule T regolatorie isolate da topi PSGL-1$^{-/-}$ e FucT-VII$^{-/-}$ mostrano una completa incapacità nel migliorare il decorso clinico della malattia; un significativo blocco della malattie si è verificato invece utilizzando cellule Treg WT.

Sono state successivamente studiate le caratteristiche migratorie delle cellule T regulatorie; allo scopo, sono stati eseguiti esperimenti di microscopia intravitale, citofluorimetria a flusso e saggi di migrazione in vivo utilizzando linfociti marcati con glicerolo radioattivo. È stata dimostrata una ridotta abilità migratoria delle cellule CD4^{+}CD25^{+} derivanti da topi PSGL-1$^{-/-}$ e FucT-VII$^{-/-}$ rispetto a cellule regolatorie WT nel CNS infiammato in corso di EAE. I risultati ottenuti dimostrano inoltre che le cellule T regolatore presentano una drastica riduzione della capacità di effettuare "rolling" e adesione salda all'endotelio del microcircolo cerebrale infiammato.

Nell'insieme i dati ottenuti dimostrano un ruolo centrale del PSGL-1 e della FucT-VII nell'azione soppressoria svolta dalle cellule T regolatorie. PSGL-1 e FucT-VII hanno un'importante ruolo sia nel controllo del contatto cellula-cellula necessario per un'efficace soppressione delle funzioni delle cellule effettrici, che nel controllo del reclutamento delle Tregs nei vasi cerebrali infiammati in corso di EAE.

Abstract

CD4+CD25+ regulatory cells (Tregs) represent a lymphocyte population with the capability to downmodulate immune responses. Here we studied the role of P-selectin glycoprotein ligand-1 (PSGL-1) and α(1,3) fucosyltransferase-VII (FucT-VII), a molecule involved in the glycosylation of PSGL-1, in the suppression of EAE by Tregs. We first observed that PSGL-$1^{-/-}$ mice developed a more severe EAE, while encephalitogenic T cells obtained from PSGL-$1^{-/-}$ and FucT-$VII^{-/-}$ mice transferred a more severe disease than WT cells. However, we observed no differences in the expression of adhesion molecules, cytokine production and proliferation of PSGL-1/FucT-VII deficient T cells versus WT cells. We then hypothesized a defect in the suppressor functions of Tregs. Our results showed that PSGL-1 and FucT-VII deficiency led to a marked decrease of the suppression capacity of Tregs on the proliferation and production of proinflammatory cytokines. Activation of Tregs increased the suppression capacity in vitro that correlated with an increased PSGL-1 functionality. Moreover, PSGL-1 deficient Tregs were completely unable to ameliorate EAE when compared to WT Tregs. Finally, PSGL-1 and FucT-VII deficient Tregs lost the ability to migrate into the inflamed CNS. In conclusion our results demonstrate a key role for PSGL-1 and FucT-VII in the suppression of EAE exerted by Tregs.

1. Introduction

1.1 Multiple sclerosis - general concepts

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system, characterized by perivascular evasions of mononuclear cells that include both lymphocytes and macrophages. This infiltration leads to damage of the myelin sheath and the underlying axon. Activation of microglia and astrocytes occurs in MS, but it is secondary to infiltrating lymphocytes. There are two major forms of MS: the Relapsing-Remitting (RR)-MS is the most common and affects women about twice as often as men; most RR-MS patients later develop Secondary Progressive (SP)-MS. The other form is the Primary-Progressive (PP)-MS. In RR-MS, symptoms appear for several days to weeks, after which they usually resolve spontaneously. After tissue damage accumulates over many years, patients usually enter the SP-MS, where preexisting neurological deficits gradually worsen over time; relapses can be seen during the early stages of SP-MS, but become uncommon as the disease progresses. 10-15% of the patients present a chronic course from the beginning of the disease (PP-MS form), with gradually worsening manifestations [1; fig.1].

The episodic inflammation that is classic of MS is clearly visualized by magnetic resonance imaging (MRI) scans of the brain, after administration of contrast materials such as gadolinium. The duration of enhanced inflammation in individuals receiving weekly MRI scans is 4-8 weeks, and virtually all lesions enhance in their earliest phases. When the acute inflammation resolves, it leaves a scar and tissue damage. The new inflammatory focus can be seen appearing adjacent to the ventricle and then beginning to resolve. The inflammatory process of MS is associated with a complex cascade of inflammatory molecules and mediators, including chemokines, adhesion molecules associated with activated endothelial cell walls and matrix metalloproteases [2, 3]. It's not clear which factors are responsible for the different courses, and there is also heterogeneity in morphological alteration of the brain found by MRI or histopathological evaluation, e.g., which CNS areas are primarily affected

and whether a patient responds to treatment. The factors underlying this heterogeneity are not completely understood, but include a complex genetic trait that translates into different immune abnormalities and/or increased vulnerability of CNS tissue to inflammatory insult or reduced ability to repair damage. Also non-genetic factors seem to be important, such as environmental elements, infectious agents, behavioral or lifestyle influences and hormonal variables.

The cause of the recurrent inflammation in MS is now generally accepted to be autoimmune in nature, as a cell-mediated autoimmune attack against the white matter sheath. In particular, MS is considered a $CD4^+$ T_H1-mediated autoimmune disease. This view is based on the cellular composition of brain and cerebrospinal fluid-infiltrating cells and data from experimental allergic encephalomyelitis (EAE), the main mouse model of the disease. In EAE model, injection of myelin components into susceptible animals leads to a $CD4^+$-mediated autoimmune disease that shares similarities with MS, and can be adoptively transferred by encephalitogenic $CD4^+$ T cells into a naïve animal [4, 5]. EAE cannot be transferred by antibodies, and only in two instance it has been transferred by $CD8^+$ T cells [6], emphasizing the importance of $CD4^+$ T cells.

1.2 Multiple sclerosis immunology

The adaptive immune system can be classified broadly into cellular and humoral (antibody)-type responses. Among cellular responses, different types or classes of cellular immune responses have been identified that are essential for understanding the mechanisms of the inflammatory process in MS and to devising strategies to control it. Cellular immune responses can be classified as T_H1-type or T_H2-type responses, depending on how they differentiate from T_H0 precursors [7]. T_H1 (or pro-inflammatory) cells generated when T_H0 cells differentiate in the presence of interleukin 12 (IL-12); they are characterized by the secretion of interferon-γ (IFN-γ) and inflammatory mediators, such as tumor-necrosis factor-α (TNF-α). MS seems to be a cell-mediated autoimmune disease of a T_H1 type [Fig.2].

Anti-inflammatory T-cells include both T_H2 cells and T cells that have been classified as "regulatory cells". T_H2 responses are induced when T cells differentiate in the presence of IL-4, and T_H2-type cells secrete anti-inflammatory cytokines such as IL-4 and IL-10. T_H2-type responses are important in fighting parasitic infections, and T_H1 and T_H2 responses may cross-regulate each other. Another class of T cell is represented by regulatory cells, which can down regulate T_H1-type inflammatory processes. Different types of regulatory cells have been described [8]: T_H3 cells act primarily through the secretion of transforming growth factor-β (TGF-β) and are preferentially induced at mucosal surfaces [9]; T_R1 cells (T-regulatory cell 1) act primarily through the secretion of IL-10 [10]; $CD4^+CD25^+$ regulatory cells are T cells that basally express CD25 (the α-chain of the IL-2 receptor) and exert potent regulatory function through cell contact and also through secretion of cytokines such as IL-10 and TGF-β [10]. If MS is a T_H1-type cell-mediated autoimmune disease, it might be possible to regulate the T_H1 responses by the induction of regulatory cells populations.

The induction of T_H1-type myelin-reactive cells and their migration into the nervous system is shown in Fig. 2. It's postulated that T_HP (T-cell precursors) are induced to differentiate into myelin-reactive T_H1 cells when an antigen cross-reacting with a myelin antigen is presented to a T cell by an antigen-presenting cell in the context of IL-12 and co-stimulatory molecules (molecular mimicry). It is generally thought that viruses with structures that cross-react with myelin antigens act as cross-reactive antigens. T_H1 T cells that react with myelin antigens, such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), cross the blood-brain barrier, where the myelin antigens are presented to the T-cells by antigen-presenting cells in the brain (microglia cells), and an inflammatory cascade is triggered, with the release of inflammatory mediators that cause damage to the myelin sheath and ultimately the underlying axon.

The MBP isoforms (isoform 4-isoform 14) are, with PLP, the most abundant protein components of the myelin membrane in the CNS. They have a role in both its formation and stabilization. The smaller isoforms might have an important role in re-

myelination of denuded axons in multiple sclerosis. The non-classic group of MBP isoforms may preferentially have a role in the early developing brain long before myelination, maybe as components of transcriptional complexes, and may also be involved in signaling pathways in T-cells and neural cells. Differential splicing events combined to optional post-translational modifications give a wide spectrum of isomers, each of them having maybe a specialized function. PLP is the most represented myeloid protein in the CNS; is 276 aminoacids long and has four highly hydrofobic regions, which form the transmembrane domain. Another isoform, called DM20, is generated after alternative splicing of its RNA, and lacks of 35 aminoacids in a region representing and intracellular loop. In the CNS, PLP acts as a scaffold, because its extracellular loop define the right membrane-membrane spacing in the compact myelin. MOG is a minor component of the myelin sheath (about 0.05%); it's a tipe I integral membrane protein, with one variable extracellular domain, Ig-like. The aminoacidic sequence is very conserved between animal species, indicating a functional biological importance. It seems that MOG is important for the formation and the compacting of the myelin sheat, and also in the adesiveness in the developed CNS.

The hypothesis of MS as an inflammatory T_H1-type disease is supported by several observations. First, it has been shown directly by the effects of IFN-γ, the prototypic T_H1 cytokine, which when administered to individuals with MS caused clinical exacerbations [11]. Second, individuals affected with MS have a T_H1 bias, as indicated by increased concentrations of IL-12 and IL-18, both of which induce IFN-γ and increase T_H1-type chemokine receptors expression [12]. Last, IL-12-secreting cells in the peripheral blood are linked to inflammation in the CNS: increased numbers of IL-12-secreting cells in the blood are associated with gadolinium enhancement on MRI imaging, and cyclophosphamide decreases the number of IL-12-secreting cells, which is linked to clinical response. In addition to IL-12, it has been shown recently that osteopontin is important in T_H1 differentiation in autoimmune demyelization diseases. The most widely used immuno-modulatory drug in MS, IFN-β, seems to have two broad mechanisms of action: it decreases IFN-α

secretion by cells in the peripheral blood and blocks the migration of T cells across the blood-brain barrier [13].

One of the primary animal models for MS, experimental autoimmune encephalomyelitis (EAE), is induced by immunizing different mouse or rat strains with a myelin auto antigen (such as MBP, PLP or MOG) given in complete Freund's adjuvant, which induces a T_H1-type cell-mediated response against the myelin antigen. In EAE, myelin-reactive T_H1-type $CD4^+$ T cells migrate from the periphery into the CNS, where they also initiate a cascade of immune-mediated damage [Fig.2]. In animals, EAE can also be induced by the adoptive transfer of T_H1-type $CD4^+$ cells specific for one of the myelin proteins.

1.3 Lymphocyte recruitment in inflamed tissues

Lymphocyte migration into brain and spinal cord represents a critical event in the pathogenesis of MS and its animal model, EAE. However, the mechanisms controlling the recruitment of lymphocytes to the central nervous system via inflamed brain venules are poorly understood, and therapeutic approaches to inhibit this process are consequently few. In 1987–1990, a number of discoveries came together and led us to propose a quite speculative general model [14] to explain the targeting of lymphocytes as well as other leukocytes from the blood. The key elements were a common process involving three or more steps – rolling, chemo-attractant activation of integrin or other inducible adhesion molecules, and firm adhesion (arrest or sticking) – in which substitution of interchangeable receptor-ligand pairs at each step provided a combinatorial mechanism for generating specificity and diversity in leukocyte endothelial cell recognition and hence recruitment [Fig.3].

Molecular specificity in the targeting of leukocytes at sites of inflammation is mediated by selectins, integrins, and immunoglobulin gene super family [15]. A fourth class of glycoproteins are the mucins, that serve as glycoprotein ligands for the selectins [16]. Based on in vitro and in vivo observations, leukocyte recruitment may be described as a sequential process having at least three distinct adhesive events.

Leukocytes are captured by the inflamed endothelium from the blood stream, by tethering via constitutively expressed leukocyte selectin denoted as L-selectin (CD62L), which recognize glycoprotein ligands, like the Peripheral lymph Node Adressin (PNAd) [17], up-regulated on cytokine-activated endothelium. The capturing is also supported by leukocyte PSGL-1 (P-selectine glycoprotein ligand-1), which binds to the P-selectin (CD62P) expressed on the inflamed endothelium [18]. P-selectin on endothelial cells is the primary adhesion molecule for capture and the initiation of the second step, the rolling [19]. The selectins mediates in fact also the rolling process, where P-selectin is the most important molecule involved. P-selectin can support both capture and rolling in the absence of L-selectin. Upon pro-inflammatory stimulation, P-selectin is rapidly surface-expressed on the venular endothelium, and it makes the endothelium "sticky" to leukocytes. PSGL-1 is constitutively expressed on monocytes, eosinophils, and neutrophils; it has a glycosylation pattern allowing it to bind to endothelial P-selectin. As a result, the leukocyte rolls along the endothelium. During rolling, bonds are formed at the leading edge of the rolling cell and broken at the trailing edge. Leukocyte integrins initially remain in their resting state, and endothelial immunoglobulins remain at control levels. Leukocyte rolling is also supported by the endothelial selectin E-selectin (CD62E), which is thought to be responsible for slow rolling interactions below 5mm/s and possibly the initiation of firm adhesion [20]. E- and P-selectin are up-regulated on the plasma membrane in response to cytokine or thrombin and histamine stimulation, respectively. These two selectins can bind PSGL-1 and L-selectin expressed by leukocytes. The carbohydrate and epitope specificity of selectin recognition has been determined by blocking adhesion with mAbs to each selectin, recombinant soluble selectins, and target polysaccharides. These interventions are effective in blocking tethering and rolling in vitro, and they can inhibit the accumulation of PMNs at sites of inflammation in animal models of acute inflammation [16]. The requirement for selectins in primary cell capture and rolling has also been confirmed in transgenic mice deficient in L-selectin, E-selectin, P-selectin or PSGL-1 [21, 22]. In addition to selectin-mediated rolling, Very Late Antigen-4 (VLA-4 or $\alpha_4\beta_1$ integrin), an adhesion receptor of the integrin family that

binds to the Vascular Cell Adhesion Molecule-1 (VCAM-1), has also been found to mediate rolling of certain leukocyte subsets [23].

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, resulting in a shear-resistant firm adhesion. In this context, all three selectins exhibit the capacity to become clustered following ligand binding, which correlates with transmembrane signaling of a diverse set of pro-inflammatory functions, including membrane upregulation of integrins into high-affinity clusters on leukocytes and clustering of E- and P-selectin on rigidified membrane domains of the endothelium [24].

The last step, the firm adhesion, is initiated by the stimulatory effects exerted by growing number of chemokines and other mediators: after rolling, leukocytes become activated and attach to the endothelium through adhesion of β_2-integrins, including the Lymphocyte Function-associated Antigen-1 (LFA-1 or $\alpha_L\beta_2$ integrin) and the Macrophage Antigen 1 (Mac-1 or $\alpha_M\beta_2$ integrin), to immunoglobulin superfamily members such as Intercellular Adhesion Molecule-1 (ICAM-1) [25]. In addition, β_1-integrins and their ligands, such as the VLA-4/VCAM-1 pair, are involved in leukocyte-endothelial cell binding. Pro-inflammatory cytokines, including IFN-γ, TNF-α and IL-1, can increase T cell localization to inflammatory sites through induction of ICAM-1 and VCAM-1 [26]. Once firmly adhered, leukocytes can transmigrate through the endothelium in the inflamed tissue; this process is probably mediated by the same integrins involved in the adhesion cascade, with the help of such chemo-attractive signal from the tissue.

1.4 Selectins structure: importance of glycosylating enzymes

Selectins are a family of three C-type lectins (glycoproteins) expressed exclusively by bone-marrow-derived cells and endothelial cells. Their structure is modular, with an amino-terminal lectin domain, followed by an EGF-like domain, several consensus repeats with homology to complement regulatory proteins, a single

transmembrane domain, and a short carboxy-terminal cytoplasmic tail [27]. The main physiological function of all selectins is in mediating leukocyte adhesion under flow, but both selectins and their ligands also have signaling functions [28]. Selectins mediate cell-cell adhesion through interactions of the lectin domain with specific glyco-conjugate ligands. 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). L- and P-selectins, but not E-selectin, also bind to particular sulfated carbohydrates, such as heparan sulfate, which lack sialic acid and fucose. However, selectins bind with higher affinity or avidity to only a little number of glycoproteins. Most of these are mucins, glycoproteins with multiple Ser/Thr-linked oligosaccharides (O-glycans) and repeated peptide motifs. A key issue is whether any of these molecules mediates biologically relevant interactions with selectins. These perspectives focused the attention on PSGL-1, a sialomucin with the most clearly defined function as selectin ligand [29].

A cDNA encoding PSGL-1 was subsequently isolated from a human HL-60 cell library by expression cloning, using COS cells panned on immobilized P-selectin. Functional expression of PSGL-1 in COS cells required co-transfection with a 1,3 fucosyltransferase, confirming earlier observations that 1,3 fucosylation of surface glycoproteins is required for binding to P- and E-selectin [30]. PSGL-1 is constitutively expressed on T cells, but in a non-functional form; it must be modified with a 2,3-linked sialic acid and a 1,3-linked fucose in order to bind P- and E-selectin. Two α(1,3) fucosyltransferases (FucT), FucT-VII and FucT-IV, are expressed in leukocytes and endothelial cells and catalyze the final reaction in selectin ligand biosynthesis, the addition of fucose to sialylated precursors [31]. PSGL-1 is fucosylated by both FucT-VII and FucT-IV, but FucT-VII is principally responsible for efficient PSGL-1 interactions with endothelial selectins. Expression of Cutaneous Lymphocyte Antigen (CLA), a FucT-VII-dependent carbohydrate modification of PSGL-1, is closely correlated with interactions between PSGL-1 and E-selectin. FucT-VII is essential for the synthesis of all selectins ligands in T cells; its expression absence by naïve T cells explains the lack of selectins ligands on them. T_H1 cells

have high levels of expression of FucT-VII and selectins ligands, whereas the low levels of FucT-VII in T_H2 cells correlate with low levels of selectins on these cells. For this reason, T_H1 cells, but not T_H2 cells, are able to bind to P-selectin and E-selectin [32].

1.5 $CD4^+CD25^+$ regulatory T cells: migration patterns

$CD4^+CD25^+$ regulatory T cells (Tregs) are a subset of suppressor T cells that function regulating the immune responses. A large body of data demonstrates that they are important in the maintenance of immunological homeostasis and self-tolerance. In several animal models it has been convincingly demonstrated the suppressive activity of Tregs in autoimmune and allergic diseases, and in allograft rejection. Indeed, it has been clearly shown that Tregs are able to suppress EAE in various experimental models, including MOG_{35-55}-induced EAE in C57Bl/6 mice. Moreover, it has been shown a significant decrease in the effector function of $CD4^+CD25^{high}$ regulatory T cells from the peripheral blood of MS patients as compared with healthy donors [33].

Tregs appear to be generated as a unique lineage of T cells within the thymus, suggesting that self-reactive Tregs are there generated during normal cell development. Also peripheral induction of suppressive $CD4^+$ T cells from naïve T cells has been reported. These two populations are very similar; their development is mainly controlled and programmed by the transcription factor Foxp3, which is the universal master switch for the generation and the activity of these cells [34]. Thymus-derived and peripheral-induced Tregs are functionally indistinguishable, and are able to inhibit the proliferation and the activation of naïve T cells. For all these evidences, the enhancement of the number and activity of peripheral Tregs seems to represent an obvious goal for the treatment of autoimmune diseases.

Where Tregs act and how they migrate in vivo has hardly been studied so far. L-selectin, which is required for entry into lymph nodes, has been found on the majority of $CD4^+CD25^+$ Tregs. Other papers report the presence of receptors for

inflammatory chemokines and increased levels of adhesion molecules on regulatory subsets, suggesting that these cells are able to enter inflamed tissues and might act directly at sites of inflammation. However, a comprehensive analysis on trafficking properties of Tregs in vivo is still lacking. It has been recently shown that the integrin $\alpha_E\beta_7$ subdivides the Treg compartment into α_E^- and α_E^+ Tregs, which largely differ in their *in vivo* suppressive activity and in the expression of adhesion molecules and chemokine receptors. α_E^- Tregs displayed a naïve-like phenotype and efficiently migrated into lymph nodes, fitting to their high L-selectin expression and their high responsiveness towards CCR7 ligands. They lacked suppressive capacity under acute inflammatory conditions, but turned out to be most potent at suppressing naïve $CD4^+$ T-cell proliferation within the lymph node. On the other hand, α_E^+ Tregs showed increased frequencies of E/P-selectin ligands expression combined with a strong down-regulation of L-selectin and high responsiveness towards several inflammatory chemokines. These characteristics are associated with a high suppressive potential in varius inflammation models, thanks to their efficient entering into inflamed tissues, and a poor capability to migrate into lymph nodes. For these reasons, the α_E^+, but not α_E^-, subsets of Tregs are also able to bind P- and E-selectin, and, when transferred *in vivo*, preferentially migrate in inflamed joints and block antigen-induced arthritis in C57Bl/6 mice. Moreover, it has been recently shown that also regulatory IL-10-producing $CD4^+$ T cells bind E- and P-selectin with higher frequency when compared to T_H1 and T_H2 cells and are able to migrate in inflamed lung, suggesting that functional PSGL-1 might be used by various Treg subpopulations to migrate in sites of inflammation. Supporting this hypothesis, α_E^+ Tregs from FucT-VII deficient mouse, which lack E/P-selectin ligands, fail to migrate into inflamed sites and cannot suppress the skin inflammation; this demonstrates that immigration into inflamed sites is a prerequisite for the resolution of inflammatory reactions *in vivo*, because these selectin ligands merely regulate entry into inflamed tissues [35].

These data demonstrate that different populations of Tregs have the capability to enter sites of inflammation and exert their suppressive activity directly into the site of ongoing immune reaction [Fig.4]. In this project, will be studied the importance of

FucT-VII and PSGL-1 in suppressive activity of Tregs toward antigen-specific T_H1 cells; moreover, it will be analyzed the migration pattern of Tregs lacking these two molecules.

2. Materials and Methods

Mice

6- to 8-weeks-old C57BL/6J female mice were used as WT controls. FucT-VII$^{-/-}$, FucT-VII$^{-/-}$, FucT-IV$^{-/-}$/FucT-VII$^{-/-}$ and PSGL-1$^{-/-}$ mice were obtained from J.B. Lowe (FucTs$^{-/-}$; Case Western University, School of Medicine, Cleveland) and R.P. McEver (PSGL-1$^{-/-}$; University of Oklahoma, Health Science Center). Deficient mice were on a C57BL/6 genetic background (backcrossed for at least 9 generations). Mice were housed and used according to current European Community rules.

Active EAE induction

Mice were immunized sub-cutaneously in the flanks with 200 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide in 100 μl emulsion consisting of equal volumes of PBS and complete Freund adiuvant, supplemented with 8 mg/ml of *Mycobacterium tuberculosis* (strain H37Ra; Becton-Dickinson). Mice received 20 ng of pertussis toxin (Alexis biochemicals) intra-venously at the time of immunization and 48 h later. Clinical scores were recorded daily according to the following scale: 0= no disease; 1= tail weakness; 2= posterior legs weakness; 3= one posterior leg paraplegia; 4= complete posterior paraplegia; 5=paraplegia with forelimb weakness or paralysis; 6= moribund or dead animals.

Production of MOG_{35-55} T cell lines and induction of passive EAE

C57Bl/6J 8- to 10-weeks-old female mice were immunized with 100 μl of emulsion/mouse containing 300 μg MOG_{35-55} peptide in CFA supplemented with 0.8 mg of Mycobacterium tuberculosis. After 10-12 days, mice were sacrificed and MOG specific T cell lines were produced from spleens and draining lymph nodes. Cells were then cultured in tissue culture medium in the presence of 20 μg/ml MOG_{35-55} peptide and 1 ng/ml murine IL-12. After three days, cells were washed three times in D-PBS 1x and injected intra-venously in naïve recipients, in a volume of 0.5 ml/mouse containing 3 - 4 x 10^6 blast cells. Pertussis toxin (150 ng) was administered intra-venously in the same day of cell transfer and two days later.

ELISA assay

The following cytokines were tested on proliferation assay supernatants: mouse TNF-α, mouse IFN-γ and mouse IL-4. NUNC Maxi Sorp plate was coated with anti-mouse cytokine antibody and incubate overnight at 4° C. Plate was washed once with wash buffer (PBS/0.1 % tween 20) by using the automated ELISA plate washer and blocking buffer (PBS 10% FC) was added to each well and incubated for 1 h. at 37° C. Samples were adequately diluted in assay buffer (PBS 5% FC), 100 µl/well and incubated all together with standard ON at 4° C. Plate was washed six times with wash buffer. Anti-mouse cytokine biotinylated antibody was incubated for 1 h at room temperature. Plate was washed six times with wash buffer. Streptavidin-HRP was added and incubated for 45 minutes at room temperature. Developing reaction was made with TMB and stopped with 1M H_2SO_4. The optical density of each well was determined immediately. Standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit.

Flow cytometry for surface staining

The following antibodies and reagents were used for cell surface staining: rat anti-mouse IgG hybridoma culture supernatants PS/2 (anti-VLA-4), MJ64 (anti-CD44), Mel-14 (anti-L-selectin), Tib-213 (anti-LFA-1), 4RA10 (anti-PSGL-1); rat IgG isotype; Goat anti-rat IgG PE; anti-CD4 PE, anti-CD8 PE, anti-CD19 FITC, anti-CD3 FITC; rat IgG isotype FITC; rat IgG isotype PE; hamster IgG isotype. Flow-cytometric analysis was performed on a BD FACSCan using CellQuest 3.3 software.

E- and P-selectin chimeras binding assays

The extracellular domain of mouse E- and P-selectin was fused to the carboxy-terminal 6X histidine tagged Fc region of human IgM via a polypeptide linker. The chimeric protein was expressed in a mouse myeloma cell line, NS0.

Cells were stained by combining $5x10^5$ cells with selectin chimeras in a total volume of 100 µl for 30 minutes at 4° C. Following incubation, the cells were washed

once in 400 μl of DMEM, resuspended in 100 μl of PE-labeled goat anti-human IgM secondary antibody and incubated for 20 minutes at 4° C. The cells were pelleted and resuspended in fresh DMEM immediately before FACS analysis. Nonspecific staining was established by addition of EDTA (10mM) to staining reactions condicted with the selectin chimeras.

Production of $CD4^+CD25^+$ Tregs

Peripheral lymph nodes and spleens were harvested from 8- to 10-weeks-old C57Bl/6J mice, dissociated mechanically and washed twice after treatment with lysis buffer and antibiotic solution. Cells were separated by magnetic cell sorting in a two-step procedure (all reagents from Miltenyi Biotec). Briefly, $CD4^+$ cells were first enriched by depletion of non-$CD4^+$ T cells by indirect labeling with Biotin-Antibody cocktail and Anti-Biotin MicroBeads. In parallel, cells were labeled with anti-CD25-PE antibody. The cell suspension was then loaded onto a column placed in a magnetic field, and the magnetically labeled non-$CD4^+$ cells are retained in th column, whereas the unlabeled $CD4^+$ cells that run through will be collected and labeled in a second step with anti-PE MicroBeads. Subsequently, $CD4^+CD25^+$ cells were isolated by loading the cell suspension onto a second column and collecting the cell fraction, which is retained in the column, by immediate flushing out. The purity of the obtained cell population (usually >85%) was determined by FACS analysis of the PE-labeled fraction.

T regulatory inhibition of proliferation assays

$CD4^+CD25^+$ Tregs were co-cultured with $CD4^+CD25^-$ responder cells using the following ratios $CD4^+CD25^-/CD4^+CD25^+$ T cells: 1/0.06; 1/0.12; 1/0.25; 1/0.5. A fixed number of $CD4^+CD25^-$ cells ($1x10^5$/well) were cultured in U bottom 96-well microtiter plates in the presence of 1 μg/ml anti-CD3 mAb and $2x10^5$ APCs (irradiated splenocytes) for 4 days. Proliferation control was performed using, in the same well, $1x10^5$ of $CD4^+CD25^-$ T cells in the presence of APCs and in absence of Tregs. [^{3}H]-thymidine (1 μCi/well) was added in each well 18 h before the cultures

were terminated. [^{3}H]-thymidine uptake was determined by using a Microplate Scintillation Counter (Perkin-Elmer).

For stimulated Tregs, $CD4^+CD25^+$ cells were coltured in a 48 wells plate with $2x10^6$ Tregs/well in the presence of anti-CD3 5 μg/ml and IL-2 100 U/ml, in 1 ml of total volume, for 3 days. Cells are then collected and the proliferation assay performed as described before.

Bio-plex analysis of cytokine production

Supernatants from inhibition of proliferation assays were collected before the addiction of [^{3}H]-thymidine, and stored at –0° C for short times. At the time of analysis, samples were defrosted and treated with the Bio-Plex cytokine assay (Bio-Rad), which uses the multiplexing technology of Luminex Corp. In this system, every sample's cytokine was firstly bound (reaction in a microplate well) with polystyrene beads internally dyed with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity, thereby creating a family of 100 spectrally addressed bead sets. The binding is possible thanks to mAbs conjugated with the beads; any bead is conjugated with a mAb specific for only one target cytokine. After that, the samples was added with monoclonal- biotinylated antibodies, specific for the several cytokines, in order to form a capture sandwich immunoassay. Finally, streptavidin-PE antibody was added.

Samples so prepared were analyzed with Bio-plex system (Bio-Rad; powered by Luminex xMAP^{TM} technology). In this instrument, samples are illuminated contemporaneously with a red diode "classification" laser (635 nm) and a green "reporter" laser (532 nm). Beads are sorted thanks to the Bio-Plex Manager software, which correlates each bead set (his dye) to the assay reagent that has been coupled to it. On the other hand, the amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allows quantification of each analyte in the sample. With this system we evaluated the concentration of TNF-α, IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF and IFN-γ in the supernatants collected in the inhibition of proliferation experiments.

Migration assay with [^{3}H]-glycerol

Tregs isolated as described before were cultured in a 48 well plate, in 1 ml of total volume (x TK-1 medium) containing 2x10^6 Tregs + 25 μCi/ml of [^{3}H]-glycerol (GE Healthcare). Cells were collected after 18 h, washed 3 times with D-PBS 1x, re-suspended in 500 μl of D-PBS 1x and injected in mice previously immunized. Mice were sacrificed after 24 h and perfused with D-PBS 1x + heparin 5 U/ml; brain, spinal cord, liver, spleen, lymph nodes, thymus and blood (100 μl) were collected. Organs were weighted, put in 700 μl of water and sonicated with Sonoplus (BANDELIN electronic). Blood was mixed with 500 μl of PBS + heparin 10 U/ml + dextran 1%, in order to precipitate red cells. After 30', supernatant was collected and centrifuged 5' x 400g. The pellet obtained was re-suspended in 500 μl of water. All the samples so obtained were added with 17 ml of scintillator liquid (Ultima Gold from Perkin-Elmer); the radioactivity was red with a β-counter (Perkin-Elmer).

Intravital microscopy

Tregs were isolated from WT, FucT-VII$^{-/-}$ orPSGL-1$^{-/-}$ mice as previously described, and stimulated for 3 days in a 48 wells plate: 1 ml/well with 2x10^6 Tregs + anti-CD3 5 μg/ml and IL-2 100 U/ml. At the time of the experiment, they were collected, washed 2-3 times with D-PBS and re-suspended in 1 ml of DMEM medium + HEPES 20 mM + FCS 5% (pH 7.05). Cells were labeled with red CMTMR (5-(and-6)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine) or green CMFDA (5-chloromethylfluorescein diacetate) (Molecular probes).

Wild type C57Bl/6 mice were injected intraperitoneally with 12 μg LPS 5-6 hours before starting the intravital experiment. After this time, animals were anesthetized and a heparinized PE-10 catheter was inserted into the right common carotid artery toward the brain. In order to exclude non-cerebral vessels from the analysis, the right external carotid artery and pterygopalatine artery, a branch from the internal carotid, were ligated [Fig.5A]. Then, the scalp will be reflected and a 24 mm x 24 mm coverslip will be applied and fixed with silicon grease. A round camera with 11 mm internal diameter will be attached on the coverslip and filled with water

as previously described in our group [Fig.5B] (Constantin et al., Immunity 2000; Piccio et al., 2002; Pluchino et al., Nature 2005).

The preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (focal distance 3.3 mm, NA 0.5 ∞) was used. Blood vessels were visualized through the bone by using fluorescent dextran [Fig.5C]. 2-3x10^6 fluorescently labeled cells/condition, resuspended in PBS 1x, were slowly injected into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target videocamera (VE-1000 SIT) and a Sony SSM-125CE monitor, and recorded employing a digital VCR [Fig.5D] (Constantin et al., Immunity 2000).

Image analysis

Video analysis was performed by playback of digital videotapes. Vessel diameter (D), haemodynamic parameters and the velocities of rolling were determined by using a PC based system. The velocities of ≥ 20 consecutive freely flowing cells/venule were calculated, and from the velocity of the fastest cell in each venule (V_{fast}), we calculated the mean blood flow velocities (V_m): $V_m=V_{fast}/(2-\varepsilon^2)$. The wall shear rate (WSR) was calculated from $WSR=8xV_m/D$ (s^{-1}), and the wall shear stress (WSS) acting on rolling cells was approximated by WSR x 0.025 (dyn/cm^2), assuming a blood viscosity of 0.025 Poise. Lymphocytes that remained stationary on venular wall for ≥ 30 s were considered adherent. At least 140 consecutive cells/venule were examined. Rolling and firm arrest fractions were determined as the percentage of cells that rolled or firmly arrested within a given venule in the total number of cells that entered that venule during the same period.

Statistics

Quantitative data are given as mean values ± S.D. or S.E.M. A two-tailed Student's *t* test was employed for statistical comparison of two samples. Multiple comparisons were performed employing Kruskall-Wallis test with the Bonferroni

correction of *P*. Velocity histograms were compared using Mann-Whitney U-test and Kolmogorov-Smirnov test.

3. Results

3.1 Autoreactive T cells from FucT-VII$^{-/-}$ and PSGL-1$^{-/-}$ mice induce severe EAE

As previously reported by our group, α(1, 3) fucosyltransferase (FucT)-VII and PSGL-1 are critical for lymphocyte recruitment in inflamed tissues (Piccio et al., J. Immunol. 2005). Thus we expected to observe a reduced EAE severity by transferring autoreactive T cells obtained from mice deficient of these molecules or by inducing active EAE in mice deficient mice. In contrast with our expectations, we observed that PSGL-1 deficient mice develop a more severe EAE comparing to WT mice (Fig. 6). In addition, mice transferred with cells from PSGL-1$^{-/-}$ mice [8 mice/condition; Fig. 7] developed a more severe disease. Similar results were obtained with FucT-VII$^{-/-}$ MOG-specific donor cells [8 mice/condition; Fig. 8], when compared with mice receiving WT cells. Encefalitogenic T cells obtained from FucT-IV$^{-/-}$ animals induced a disease comparable to WT cells.(the data were not statistically significant and were variable in the several experiments [*P*=0.43; Fig. 8].

3.2 FucTs and PSGL-1 deficiency have no effect on the proliferation, adhesion molecule expression and pro-inflammatory cytokine production of MOG$_{35-55}$ T cells

In order to explain the increase of the disease severity due to FucT-VII or PSGL-1 deficiency, we asked whether the proliferative response after antigen stimulation is modified in FucTs or PSGL-1 deficient MOG$_{35-55}$ T cells. No statistically significant differences were observed between WT and PSGL-1 or FucTs deficient cells [Fig. 9]. We further analyzed the cytokine production of MOG$_{35-55}$ autoreactive cells in FucTs or PSGL-1 deficient autoreactive T cells. For this purpose we performed ELISA on cell supernatants obtained from proliferation assay experiments. No statistically significant differences were observed in the production of IFN-γ and IL-4 between WT and FucTs/PSGL-1 deficient cells [Fig. 10]. As expected, the production of IL-4 was low consistent with the T_H1 phenotype of

MOG-specific encephalitogenic T cells. Moreover, no significant differences were obtained when we measured TNF-α production (data not shown).

We also analyzed the expression of adhesion molecules on WT and PSGL-$1^{-/-}$, FucT-$VII^{-/-}$ and FucT-$IV^{-/-}$ MOG_{35-55} T cells; as shown for the proliferation and cytokine production, no significant differences in the expression of LFA-1, VLA-4, L-selectin, and CD44 were observed (data not shown).

3.3. Stimulation of regulatory T cells with anti-CD3 mAb and IL-2 increases adhesive and suppressor capacities of CD4+CD25+ T cells in vitro

The results presented above gave no apparent explanation on why T cells lacking PSGL-1 or FucT activity induce a more severe disease or on the increased severity in mice deficient in PSGL-1. Proliferation, adhesion molecule expression and the production of cytokines by autoreactive lymphocytes are similar in WT and PSGL-1/FucTs deficient cells. We next hypothesized some functional defects in the suppressor activity of $CD4^+CD25^+$ regulatory T cells.

It is widely accepted that Tregs require cell-cell contact in order to exert inhibitory effects on activated T cells. We have first set up a proliferation assays in order to test the suppressive effect of $CD4^+CD25^+$ T cells on the proliferation of $CD4^+CD25^-$ cells. Recently, it has been shown that pre-culture of $CD4^+CD25^+$ T cells for 1-3 days in the presence of plate-bond anti-CD3 (5 μg/ml) mAb and high doses of IL-2 or IL-4 induces the production of more potent suppressor cells in vitro. Thus, we compared the activity of naïve versus stimulated Tregs.

We initially asked if activation of $CD4^+CD25^+$ T cells modifies their adhesive capability. To this purpose, we compared the expression of adhesion molecules and the ability to bind E- and P-selectin chimeras in functional assays by using flow cytometry. The binding of selectin chimeras measures the functionality of PSGL-1. Our results indicate that after 3 days of activation, no significant differences were observed in the expression of α_4 integrins, LFA-1, PSGL-1 on activated Tregs when compared with naïve Tregs, whereas L-selectin increased after stimulation.

Interestingly, the capacity to bind P- and E-selectin increased significantly after activation, suggesting that PSGL-1 became functional and able to bind its counter-ligands after Treg activation [Tab. I]. These data also suggest that activated $CD4^+CD25^+$ T cells may have increased ability to migrate into sites of inflammation in which endothelium expresses endothelial selectins.

We next studied whether activation of $CD4^+CD25^+$ T cells modifies also their suppressor capacity. To this purpose, we performed proliferation assays using co-cultures naïve $CD4^+CD25^+$ cells or activated $CD4^+CD25^+$ cells plus $CD4^+CD25^-$ cells (responder/effector cells). Different ratios between Tregs/effector cells were used (see Figure legend 11). Our results showed that after 3 days of activation, Tregs increased their inhibitory capability on $CD4^+CD25^-$ proliferation when compared with naïve Tregs inhibition [Fig. 11]. These results reveal that the increased functionality of PSGL-1 is associated with an increase of the immunosupression ability of Treg.

3.4 Anti-L-selectin and anti-PSGL-1 blocking antibodies revert immunosupressive capability of Tregs

In order to confirm that PSGL-1 and FucTs participate to mechanisms involved in the suppression of autoimmune attack against brain antigens and in order to understand which are the adhesion molecules involved in the cell-cell contact, we performed proliferation assays in the presence of monoclonal blocking mAbs. The following mAbs were used: rat anti-mouse IgG hybridoma culture supernatants Mel-14 (anti-L-selectin), 4RA10 (anti-PSGL-1) and PS/2 (anti-VLA-4) as a negative control. The results indicate that both L-selectin that PSGL-1 are involved in the cell-cell contact necessary for the suppressor activity exerted by Tregs [Fig. 12].

3.5 FucTs and PSGL-1 deficient Tregs show a defect in the suppressor activity

We next studied the effect of PSGL-1 and FucTs deficiencies on the suppression of proliferation by CD4CD25+ T cells. We performed co-cultures using different ratios of Tregs/effector cells as described above. We first controlled the

expression of Foxp3 in all Treg populations. We observed a similar expression of Foxp3 in WT and PSGL-1/FucT deficient Tregs suggesting that the potential reduction in the suppressor activity is not due to a decrease in Foxp3 expression (results not shown). Data obtained using naïve Tregs showed that $CD4^+CD25^+$ cells from FucT-VII deficient mice exerted significantly less inhibitory effect on the proliferation of wild type $CD4^+CD25^-$ responder cells [Fig.13A]. Interestingly, when co-cultures were made with both Tregs and responder cells deficient of FucT-VII, we observed that inhibition of proliferation by Tregs was dramatically reverted [Fig. 13C]. No significant role was observed for FucT-IV, when deficiency either in Tregs or in $CD4^+CD25^-$ responder cells or both [Fig.13B]. PSGL-1 deficiency on responder cells, but not on Tregs, partially reverted the inhibitory effect of Tregs on the proliferation [Fig. 13D]. Taken together, these results suggest that glycans fucosylated on both Tregs and responder cells are involved in the suppression of proliferation. Notably, these data were confirmed in proliferation assays performed with antiCD3/IL-2 stimulated WT and PSGL-1/FucT deficient Tregs (data not shown).

3.6 FucT-VII activity has an important role in Treg-mediated suppression of cytokine production

We next asked whether Tregs are able to suppress the production of cytokines by $CD4^+CD25^-$ responder cells. We focused our attention on two different ratios Teff/Tregs, 1/0.12 and 1/0.06, and evaluated the release of IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, TNF-α and IL-1β in the supernatans samples using the Multiplex system from BioRad (see "Material and methods"). FucT-VII deficiency in Tregs and effector T cells led to a reduction of the inhibitory effect on the production of pro-inflammatory cytokines such as GM-CSF, IFN-γ and IL-1 [$P<0.01$. Fig. 14A]. Interestingly, we observed no reduction of the inhibitory effect on the production of T_H2 and anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 [Fig. 14B]. These results support the data obtained in proliferation assays and show an important role

for FucT-VII expression on both Tregs and Teff cells [Fig. 14A and B]. IL-1β and TNF-α were produced in very low amounts in these experiments, and no significant differences were found between KO and WT cells (Tregs or Teff).

However preliminary data obtained from experiments using PSGL-1 deficient cells were less clear. Effector T cells deficient of PSGL-1 produced higher amounts of pro-inflammatory cytokines (GM-CSF, IFN-γ and TNF-α), explaining at least partially the *in vivo* exacerbation of disease in PSGL-1 deficient mice (data not shown). No significant differences were found between WT and PSGL-1 deficient cells (both Tregs and Teff) for other cytokines. These results suggest that more analysis is required for a better understanding of data obtained with PSGL-1 deficient cells.

3.8 PSGL-1$^{-/-}$ and FucT$^{-/-}$ Tregs do not exert suppressor activity in mice with EAE

We next wanted to confirm the in vitro studies also in vivo in the context of brain autoimmune diseases and we analyzed the effect of FucT-VII and PSGL-1 deficiencies on the induction of EAE. We first analyzed the ability of WT and PSGL-1/FucT-VII deficient Tregs to ameliorate the disease course in C57BL/6J mice immunized with the MOG_{35-55} peptide (active EAE). For this purpose, we isolated $CD4^+CD25^+$ cells from WT or PSGL-1 deficient mice and transferred $2{,}5x10^6$ cells in immunized mice at day +7 post-immunization. Data obtained show that naïve PSGL-$1^{-/-}$ Tregs were completely unable to prevent EAE, whereas naïve WT Tregs ameliorated the disease course [Figure 15]. We have also obtained preliminary results suggesting that also FucT-VII deficiency leads to a lack of inhibition of EAE (data not shown). We obtained similar data when we used anti-CD3/IL-2 stimulated Tregs [Fig. 16]. Taken together these in vivo data support the in vitro results and show a key role for PSGL-1 and FucT-VII in the suppressor activity on EAE. Importantly, recent preliminary results show that activated Tregs block EAE more efficiently when compared to naive Tregs, suggesting that in vitro manipulation of Tregs might increase their suppressor effect in vivo.

3.9 FucT-VII and PSGL-1 are involved in the recruitment of Tregs in inflamed central nervous system (CNS)

A. Measurement of the accumulation of 3H-glycerol labeled Tregs in vivo.

We have previously shown that PSGL-1 and FucT-VII are critical molecules for the recruitment of lymphocytes into the inflamed brain (Piccio et al., J. Immunol. 2002; Battistini et al., Blood 2003; Piccio et al., J. Immunol. 2005). We next studied the migration in various organs of WT and PSGL-1/FucT VII deficient Tregs in mice with EAE. We first analyzed the migration of [^{3}H]-glycerol-labeled naïve Tregs, injected at day +7 (pre-clinical phase) or +14 (disease onset) after immunization of mice with the MOG_{35-55} peptide. We collected brain, spinal cords, draining lymph nodes, thymus, liver, spleen and blood. Spinal cord was considered the most important organ in our analysis, as it represents the principal site of the inflammatory autoimmune reactions against CNS parenchyma in MOG35-55 immunized mice. The results obtained showed that naïve Tregs preferentially migrate in the preclinical phase of disease when compared with the migration at disease onset (naïve Tregs migrate two times more at day 7 compared to day 14) [Figure 17A]. In addition we observed that also activated Tregs display a higher migration into the inflamed CNS in the pre-clinical phase of disease [P<0.01. Fig. 17B]. These results are consistent with: i) our previous data showing increased expression of endothelial selectins in the preclinical phase of EAE when compared with disease onset (Piccio et al., 2002) and with ii) flow cytometry results from Table I showing increased binding of E- and P-selectin chimeras (consistent with an increased PSGL-1 functionality). When we compared the migration of naïve versus activated Tregs we observed that activated T cells are preferentially recruited in inflamed spinal cord both at day 7 and day14 post immunization [Fig. 17C and D]. These results suggest that in vitro activation of Tregs increase their delivery into the inflamed CNS. We next studied the role of PSGL-1 and FucT-VII in the migration of Tregs in vivo in mice with EAE. The results obtained showed a drastic reduction of the migration of FucT-VII deficient Tregs, while cells that lack PSGL-1 lost the ability to penetrate into the inflamed CNS [P<0.0001. Fig. 17E]. Interestingly, we observed that at disease onset there is almost

no inhibition of the migration using PSGL-1 and FucT-VII deficient cells [$P<0.05$. Fig. 17E and F]. These results are consistent with the data described above showing a preferential migration of Tregs in the preclinical phase of disease and with our previous results showing that endothelial selectins are basically undetectable at disease onset by in vivo staining (Piccio et al., 2002).

B. Intravital microscopy analysis of Treg adhesion in inflamed brain microcirculation

The formidable technical challenges associated with efforts to visualize brain microcirculation in vivo have represented major obstacles to understanding how leukocytes gain access into the brain during inflammation. Our initial efforts in this area, using intravital microscopy, have implicated P-selectin glycoprotein ligand-1 (PSGL-1) as a concomitant of recruitment of phenotypically heterogeneous autoreactive lymphocytes in inflamed brain venules (Piccio et al. J. Immunol. 2002; Battistini et al., Blood 2003). In addition, it has been shown by our laboratory, as well as by others, that endothelial E- and P-selectin, both PSGL-1 ligands, might be involved in the recruitment of leukocytes in murine and human brains during inflammatory diseases including EAE and MS.

We completed our study on the capacity of Tregs to migrate into the inflamed brain by visualizing and analyzing the adhesive events in inflamed brain microcirculation with intravital microscopy. We have previously shown that mice treatment with TNF-α or LPS induces expression of P- and E-selectin, ICAM-1, and VCAM-1 on brain endothelium (Piccio et al., 2002). This experimental model resembles early inflammation during EAE, as we and others brought evidence that brain endothelium expresses E- and P-selectin, ICAM-1 and VCAM-1 during early inflammation in preclinical phase of autoimmune disease (Cannella et al., Lab Investig. 1991; Piccio et al., J. Immunol. 2002; Kerfoot & Kubes. J. Immunol. 2002). We studied the effect of PSGL-1 and FucT-VII deficiency on the behavior of Tregs in inflamed brain venules. In agreement with our previous results obtained with T_H1 and encephalitogenic T cells, we observed a dramatic reduction of all adhesive events in

cells deficient of PSGL-1 and FucT-VII [$P<0.0001$. Fig. 18 and 19] (Piccio et al., 2005). These results confirm the data obtained at point A and clearly demonstrate that PSGL-1 and FucT-VII are key molecules in the recruitment of Tregs into the inflamed brain.

4. Discussion

CD4+CD25+ Tregs are potent immunoregulatory cells that function controlling immune responses. In fact, multiple animal models have convincingly demonstrated the suppressive activity of Tregs in autoimmune, allergic diseases and allograft rejection. The use of Tregs in the treatment of autoimmune pathologies such as colitis, rheumatoid arthritis or multiple sclerosis is appealing and have the potential to represent a major advance in the treatment of such diseases. It has been clearly shown that Tregs are able to suppress EAE in various experimental models, including MOG_{35-55}-induced EAE in C57Bl/6 mice. Moreover, Viglietta et al. have recently shown a significant decrease in the effector function of $CD4^{+}CD25^{high}$ regulatory T cells from the peripheral blood of MS patients as compared with healthy donors. Thus, the enhancement of the number and activity of Tregs seems to represent an obvious goal for the treatment of autoimmune diseases.

Recent data by Huehn et al. demonstrate that a subpopulation of $CD4^{+}CD25^{+}$ Tregs expressing α_E integrin and able to bind E-and P-selectin (both PSGL-1 ligands) preferentially migrates in sites of inflammation and blocks the development of autoimmune arthritis. Data obtained in the present study suggested that PSGL-1 might be involved in the suppressor activity mediated by $CD4^{+}CD25^{+}$ cells during MOG-induced EAE.

The results presented here started from the observation that transfer of T cells lacking FucT-VII activity or PSGL-1 lead to an increase of disease severity suggesting that FucT-VII and PSGL-1 are critical to protective mechanisms during EAE. We thus wanted to dissect the potential role of PSGL-1 and FucTs in the suppressor activity mediated by CD4+CD25+ Foxp3+ regulatory T cells in vitro and in vivo.

We studied the role of PSGL-1 and FucTs in *in vitro* the suppression of effector T cell functions, which requires cell-cell contact between Tregs and effettor/responder T cells. FucT-VII Tregs showed a drastic decrease in the suppressor activity exerted on effector T cells, suggesting that glycans fucosylated on

Tregs are involved in the suppression of proliferation. Moreover, proliferation of effector cells (CD4+CD25-) deficient of FucT-VII in the presence of wt CD4+CD25+ T cells was 2-3 times less inhibited (depending on the responder/Treg ratio) when compared with wt effector cells. Thus the lack of FucT-VII also on effector cells significantly reverted the inhibitory effect on proliferation by Tregs. In contrast, we observed no effect on the inhibition of proliferation when we used responder cells or Tregs deficient in FucT-IV. Moreover, analysis of cytokine production in supernatants collected after proliferation assays demonstrated that FucT-VII deficiency either on Tregs or on effector cells strongly reverted the inhibitory effect on the production of pro-inflammatory cytokines, but not on Th2 and anti-inflammatory cytokines. These results show that FucT-VII has an important role in the establishment of cell-cell contacts responsible for the suppressive activity of Tregs. Our results indicate that FucT-VII activity is required on both sides: effector and regulatory cells, suggesting that similar carbohydrate structures might be involved in these cell-cell interactions. These results might also suggest a mechanism of control of T_H1 cells by Tregs in vivo, as T_H1 cells, but not T_H2 cells, up-regulate expression of FucT-VII and express functional PSGL-1. However, recent results by Xu et al. suggest that $CD4^+CD25^+$ T cells are able to suppress both T_H1 and T_H2 cells in vivo and in vitro. Tregs cells are heterogeneous cells and different mechanisms of suppression may be involved in the inhibition of T_H1 versus T_H2 mediated responses. Moreover, our results show that Tregs activation increase the inhibitory capability to inhibit the proliferation of $CD4^+CD25^-$. These results correlate the increased adhesive functionality of PSGL-1 (see Table I) to the increase of the suppression ability of Tregs.

Proliferation of responder cells in the presence of CD4+CD25+ T cells deficient in PSGL-1 was similar to wt Tregs, thus the lack of PSGL-1 had no effect on the inhibition of proliferation by Tregs. However, the proliferation of effector cells (CD4+CD25-) deficient in PSGL-1 in the presence of wt CD4+CD25+ T cells was significantly reverted when compared with wt effector cells. Taken together these results show that PSGL-1 has a role on effector T cells, but not on Tregs. The discrepancy between the results obtained with cells deficient of FucTs versus cells

deficient of PSGL-1 suggest that a not yet identified fucosylated molecule (not PSGL-1) is involved in the cell-cell contacts responsible for suppressor activity mediated by Tregs.

In addition, we observed that anti-L-selectin and anti-PSGL-1 blocking antibodies revert immunosuppression by Tregs, suggesting that selectins and mucins control, at least in part, cell-cell contact between Tregs and effector T cells. Taken together, these data, together with EAE data described above, show that glycans are critical for suppressive mechanisms regulating the immune responses. Recent data obtained with the two-foton intravital microscopy technology show that Tregs exert suppression in secondary lymphoid organs by blocking the interactions between effector T cells and APCs (Tadokoro et al., JEM 2006; Tang et al., Nature Immunol. 2006). In fact, in order to validate our results obtained in vitro on the role of PSGL-1 and FucT-VII in cell-cell contact between Treg and responder cell, we are presently performing in vivo two photon laser scanning microscopy in collaboration with Dr. Mark Miller from Washington University (St. Louis).

Where Tregs act and how they migrate in vivo has hardly been studied so far and a comprehensive analysis on trafficking properties of Tregs in vivo is still lacking. [35]. In fact, the mechanisms involved in the migration of Tregs into the brain are completely unknown. The main limitation in the identification of the mechanisms controlling leukocyte recruitment in brain venules was the lack of methods to directly visualize and analyze the interactions between leukocytes and endothelium in microcirculation in live animal. To overcome this limitation our group had previously set up a new intravital microscopy method that allows the visualization of brain microcirculation directly through the skull and analyze the behavior of blood cells in mouse brain microvessels (Piccio et al., J. Immunol. 2002; Battistini et al., Blood 2003; Pluchino et al., Nature 2005). Our results indicated that after activation, the capacity of Tregs to bind P- and E-selectin increases significantly, suggesting that PSGL-1 become functional and able to bind its counter-ligands. This data also suggest that activated $CD4^+CD25^+$ T cells may have increased ability to migrate into sites of inflammation. In fact, confirming the data obtained *in vitro*, our in vivo experiments using [^{3}H]-glycerol labeled Tregs show that activated

Tregs are able to better migrate during the pre-clinical phase and at disease onset in mice immunized with MOG35-55 peptide. Moreover, our results indicate that Tregs are preferentially recruited during pre-clinical phase of disease (a time point when we and others have shown that endothelial selectin are expressed on brain endothelium at a higher level). Thus our results give important indication on the phenotype and the kinetics of the migration of Tregs in mice with EAE.

The mucin PSGL-1 expressed by the leukocytes is classically responsible for efficient tethering and rolling in vivo on either P- or E-selectin (Norman et al., 2000). We also quantified the accumulation of Tregs obtained from PSGL-1 and FucT deficient mice into the organs of animals in which we induced EAE with MOG35-55 peptide. CD4+CD25+ T cells deficient in FucTs or PSGL-1 accumulated efficiently into the lymphoid organs at all time points. However, the accumulation of CD4+CD25+ cells deficient in PSGL-1 or FucT-VII was significantly decreased in the spinal cord of EAE animals, suggesting that PSGL-1 and FucT-VII activity are involved in the recruitment of CD4+CD25+ T cells into the inflamed brain. Moreover, data obtained in intravital microscopy experiments performed in an experimental model that mimics endothelial inflammation during pre-clinical phase of EAE showed that cells from PSGL-1 and FucT-VII deficient mice have a dramatic decrease of the adhesive interactions (tethering and rolling and arrest) in inflamed brain microcirculation when compared with WT cells. These in vivo results clearly demonstrate that PSGL-1 and FucT-VII activity have a key role in the recruitment of Tregs in the inflamed CNS.

Taken together, our results allow us to demonstrate two different mechanisms involving FucT-VII and PSGL-1 in the suppressor activity of Tregs. The first mechanism is the recruitment of Tregs into the inflamed brain and it is based on our evidence showing that both PSGL-1 and FucT-VII are important for the migration capability of CD4+CD25+ cells. PSGL-1 and FucT-VII deficiencies dramatically decrease the ability of Tregs to efficiently interact with inflamed endothelium expressing E- and P-selectin and to migrate to inflamed tissues. The second mechanism is cell-cell contact necessary for the suppressor activity exerted by Tregs on effector cells. While FucT-VII is critical and is required on both Tregs and effector T cells, PSGL-1 has a role only when expressed on effector T cells. This discrepancy

suggest us that probably other fucosylated molecules are expressed on Tregs. In fact, . in the last few years it has been shown that new classes of highly glycosylated molecules such as T Cell Ig- and mucin-domain-containing molecules (TIMs) and galectins are important for T cells activity, and we are presently exploring the potential role of these molecules in cell-cell contact and migration required for efficient suppressor activity by Tregs.

In conclusion the multiparametric approach used in this study allowed us to analyze the role of molecules containing many carbohydrate branches (glycans) in the control of suppressive action exerted by CD4+CD25+ cells during EAE, the animal model of multiple sclerosis. The data obtained strongly indicate that PSGL-1 and the fucosylation of its glycans by fucosultransferases (enzymes that add the carbohydrate molecule fucose) are involved in the suppressor activity mediated by CD4+CD25+ cells during EAE. The project also used a sophisticated video-microscopy technique that enabled direct visualization of the Treg cells as they circulate in blood vessels; this technique helped us to clarify the role of PSGL-1 and FucTs in the selective recruitment of Tregs cells into the inflamed brain. The knowledge of the molecular mechanisms controlling the recruitment of Treg into the brain is of critical importance, as potentially new therapies targeting migratory capabilities of T cells should not interfere with the extravasation of Tregs into the brain. The project also helped us to unveil the fact that activated Treg have a higher ability to migrate into the inflamed microcirculation of the brain. Our results suggest that in vitro activated Tregs could have a stronger therapeutic potential in demyelination diseases. Further studies are needed to clarify whether in vitro Treg activation and manipulation have a beneficial effect also on disease course. The knowledge of how to manipulate $CD4^{+}CD25^{+}$ Tregs, in order to obtain cells with enhanced suppressive and migratory capacity, may help to increase the inhibitory action exerted by regulatory T cells on the autoimmune process.

5. Figures and legends

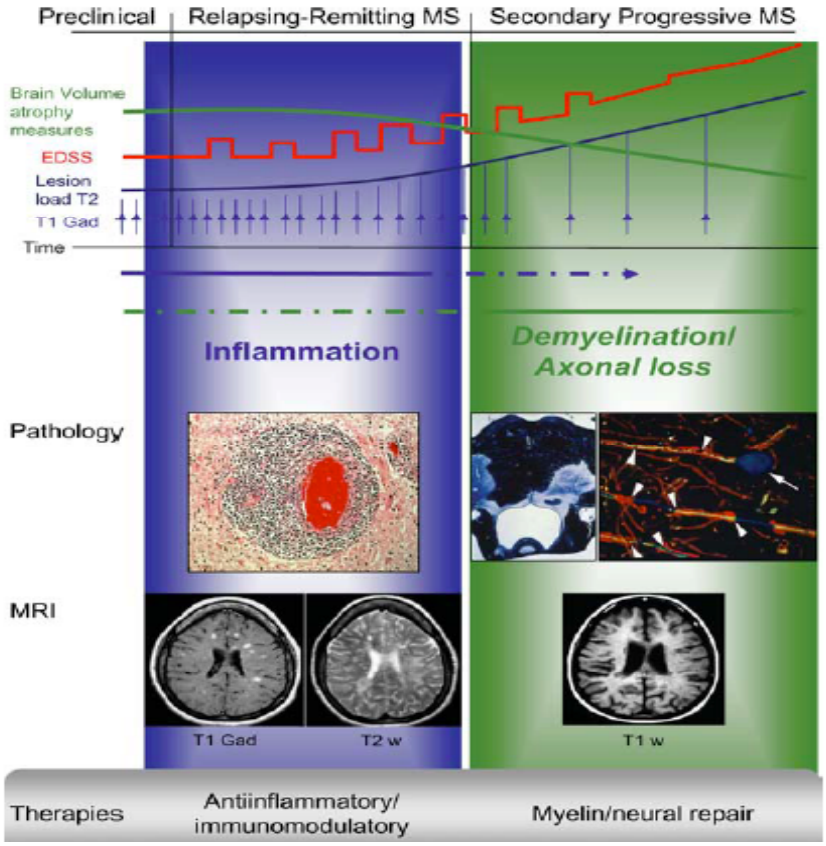


Fig. 1: ***Top:*** Schematic depiction of the clinical evolution of MS by a clinical scale (*red line*). The frequency of inflammatory events when studied by MRI (T1 lesions with contrast showing blood-brain barrier opening, *blue arrows*); T2 lesion load documenting all tissue damage (*blue line*); brain atrophy (*green line*). ***Pathology****:* main pathological characteristics of MS. On the left, perivascular inflammation with mononuclear cells and open blood-brain barrier; on the right, demyelinated areas shown in light blue and white, and, on the far right, axonal transactions (blue onion bulb-like structure) and segmental demyelination. ***MRI****:* Typical MRI characteristics. On the left, T1-weighted image with Gadolinium contrast enhancement. White lesions indicate areas of fresh inflammation and open blood-brain barrier. T2-weighted image shows the CSF-filled ventricles in white and MS lesions in the brain parenchyma. On the right, brain atrophy with widened lateral ventricles and cortical sulci. [Figure adapted from Sospedra M. and Martin R., *Annu. Rev. Immunol*. (2005)].

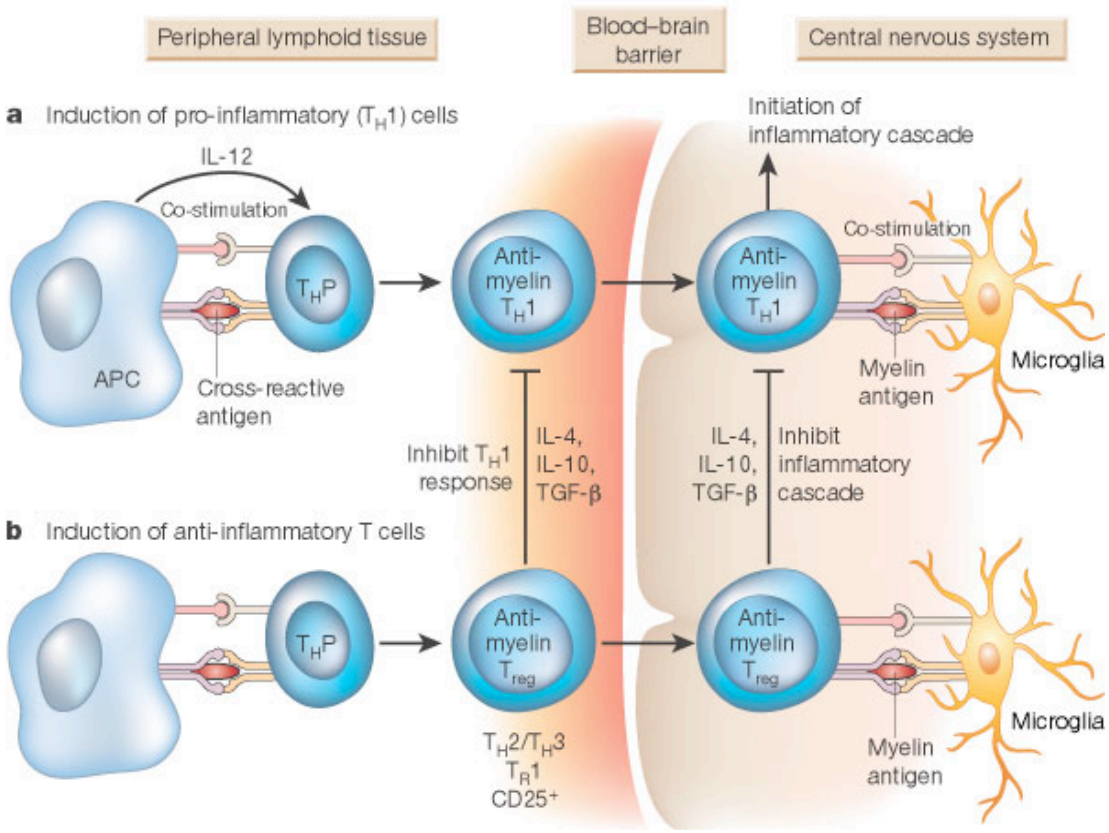


Fig. 2: Inflammation and immune mechanisms in multiple slerosis. a. Multiple sclerosis is probably induced by the generation of T_H1-type myelin-reactive cells from precursor cells (T_HP), which are presumably triggered by cross-reactive antigens, such as viruses, in the context of co-stimulatory molecules and IL-12. T_H1-type cells directed against myelin migrate into the nervous system where they re-encounter myelin antigens presented by microglia and are re-stimulated to initiate a destructive inflammatory cascade. **b**. Immune therapy involves the induction of anti-inflammatory regulatory T cells (T_H2, T_H3, T_R1, $CD25^+$ cells) that secrete anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β. Regulatory cells, that act by cell/cell contact ($CD25^+$ cells), inhibit T_H1 responses in the periphery and/or migrate to the CNS, where they are re-stimulated by local microglia cells and inhibit or suppress the local inflammatory cascade in the CNS.

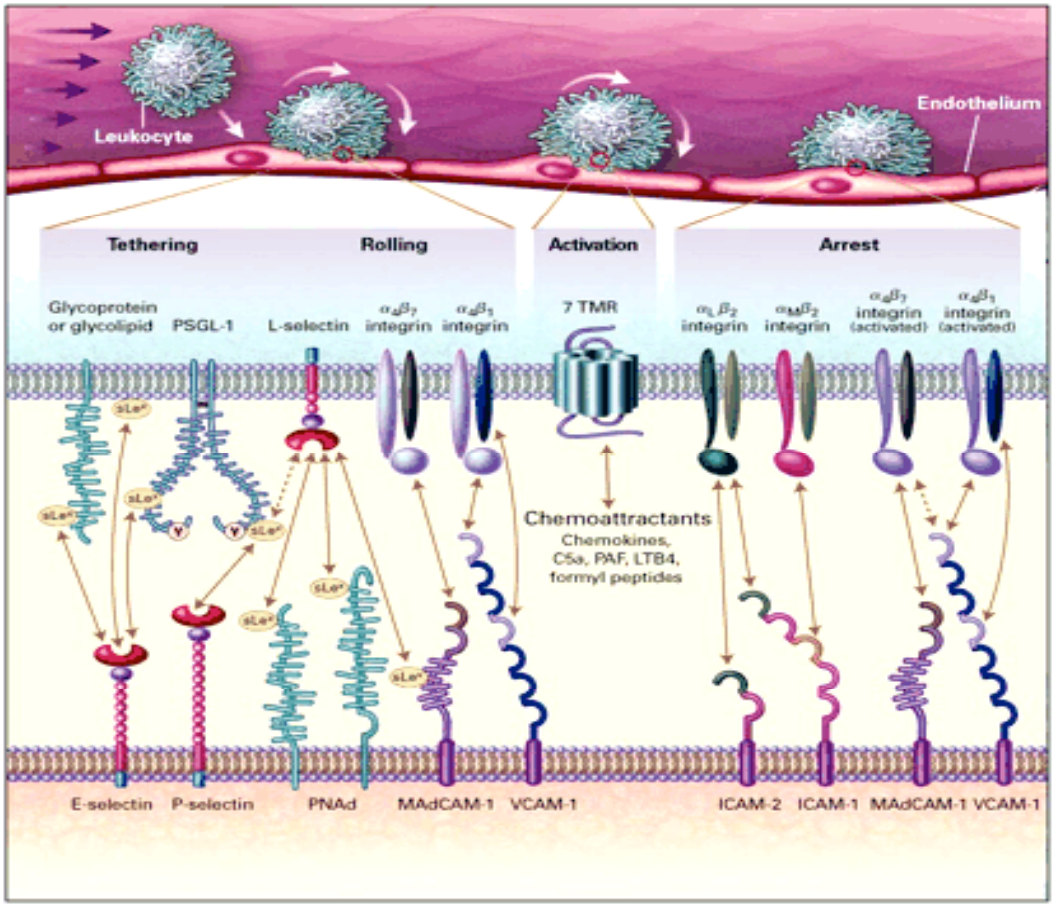


Fig. 3: The multistep model of leukocyte migration through the endothelium. The process known as "capture" or "tethering" represents the first contact of a leukocyte with the activated endothelium. Subsequent rolling consists of reversible adhesive interactions and is mediated by selectins, integrins and their counterligands: mucins and immunoglobulins. During an early inflammatory response, P- and E-selectin on endothelial cells are primary molecules for capture and initiation of slow, inflammatory rolling. During the rolling, the leukocyte is activated by chemokines released from the endothelium; via G-protein pathways, the production of integrins on the leukocyte is up-regulated. Integrins interact with their counter-ligands on endothelial cells, permitting the arrest of the cells. The last step will be the migration of the leukocyte through the endothelium in the tissue. [Figure adapted from Luster A. D. *et al.*, Nat. Immunol. (2005)].

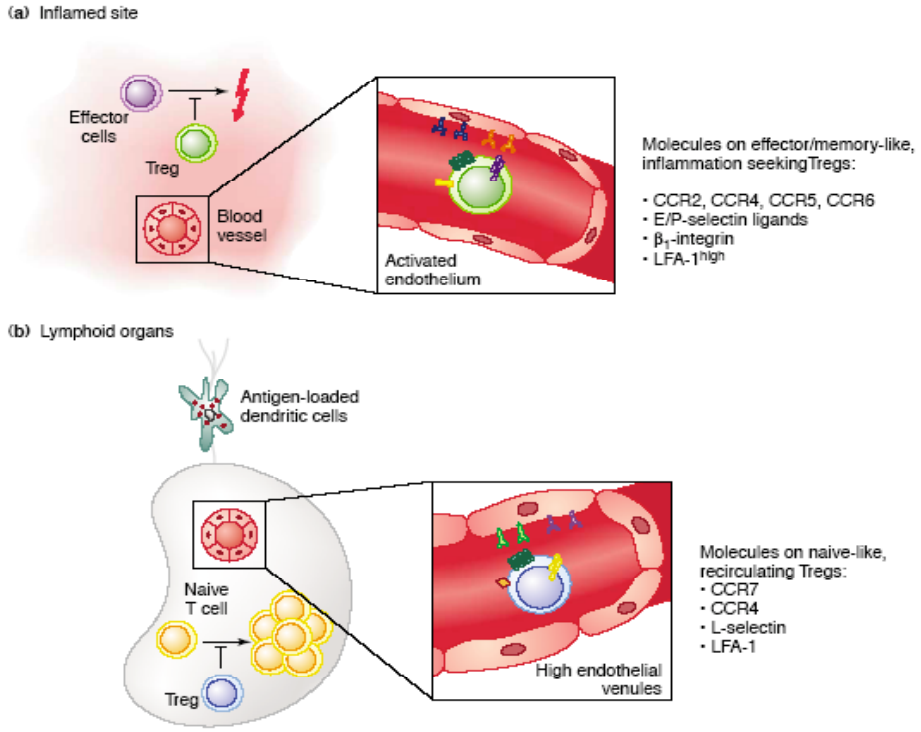


Fig. 4. Treg compartmentalization determines suppressive activity in vivo. Peripheral antigen, transported from the inflamed site to the local draining lymph node (**b**) by professional antigen-presenting cells (APCs), leads to expansion and differentiation of naive T cells. This priming step is under the control of naive-like Tregs, which express high levels of L-selectin (CD62L) and CCR7. This enables efficient migration of these recirculating Tregs into lymphoid organs via the high endothelial venules expressing ligands for L-selectin and presenting CCR7 ligands on their surface. The chemokine receptor CCR4 might have a role in the interaction between naive-like Tregs and APCs within lymph tissues. (**a**) By contrast, effector/memory-like Tregs display an inflammation-seeking phenotype by the expression of diverse CCRs, E/P-selectin ligands, β_1-integrins and high levels of LFA-1. This enables an efficient entry into inflamed sites because it is expected that activated endothelia will express high levels of E-selectin, P-selectin, ICAM-1 and VCAM-1, and will present inflammatory chemokines on their surface. Thereby, these inflammation-seeking Tregs are predestinated to control the inflammatory action of effector cells at peripheral sites. [Figure adapted from Huehn J. and Hamann A., *Trends Immunol.* (2005)].

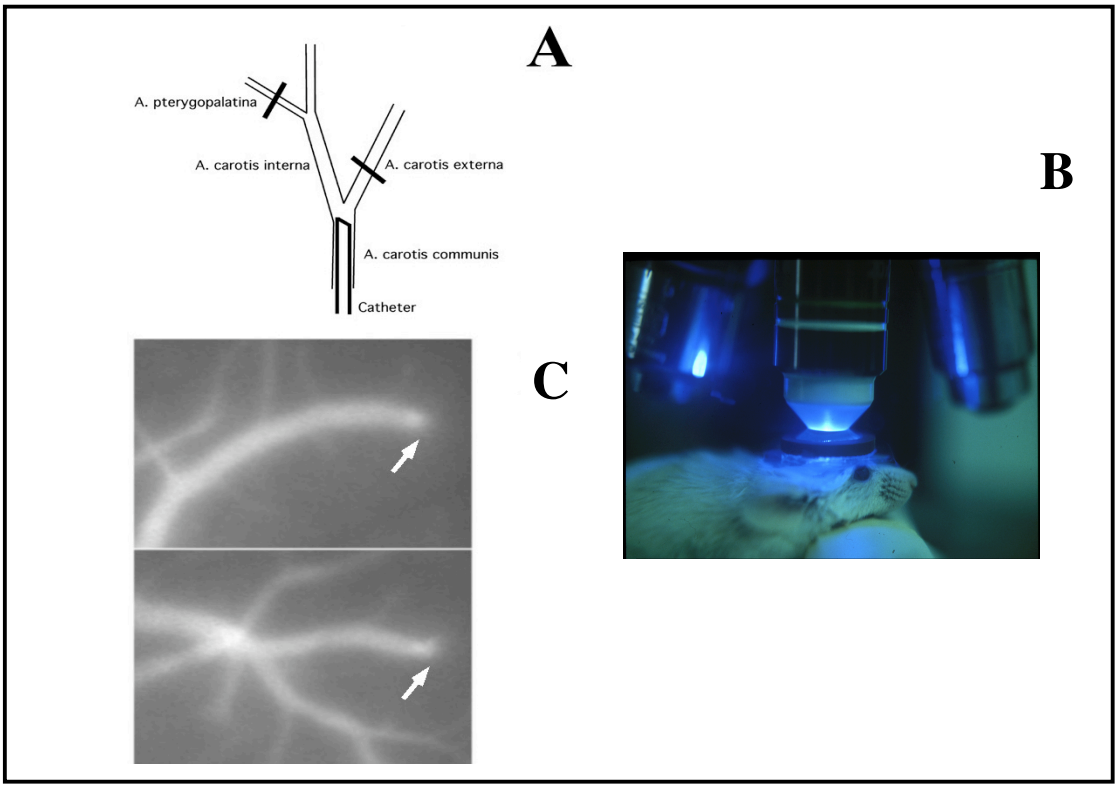


Fig. 5: Intravital microscopy in mouse brain microcirculation. A. Ligations performed to exclude the arrival of fluorescent cells into the non-cerebral vessels. **B.** Mouse scalp is reflected and a coverslip will be applied and fixed with silicon grease. A round camera is attached on the coverslip and filled with water. **C.** The characteristic convex origin of superficial cerebral venules (arrows) is due to the emergence of the vessel on the brain surface from the more profound layers. Cerebral vessels are labeled with fluorescent dextran. **D.** Intravital microscopy equipment (see below).

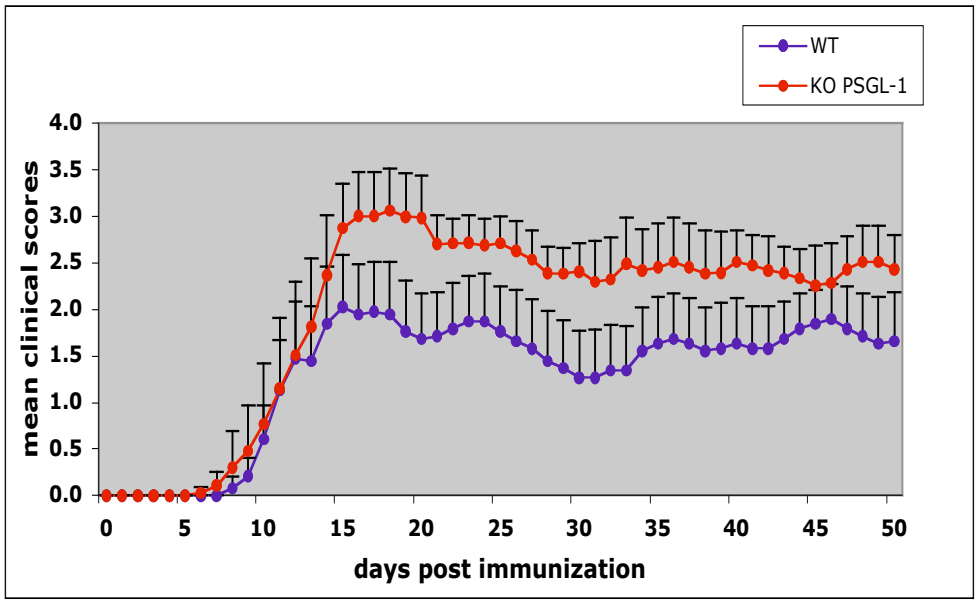


Fig. 6: PSGL-1 deficiency increases disease severity in actively-induced EAE. The figure represents the mean results obtained from 4 different experiments of actively-induced EAE with MOG_{35-55} peptide. ($P<0.01$).

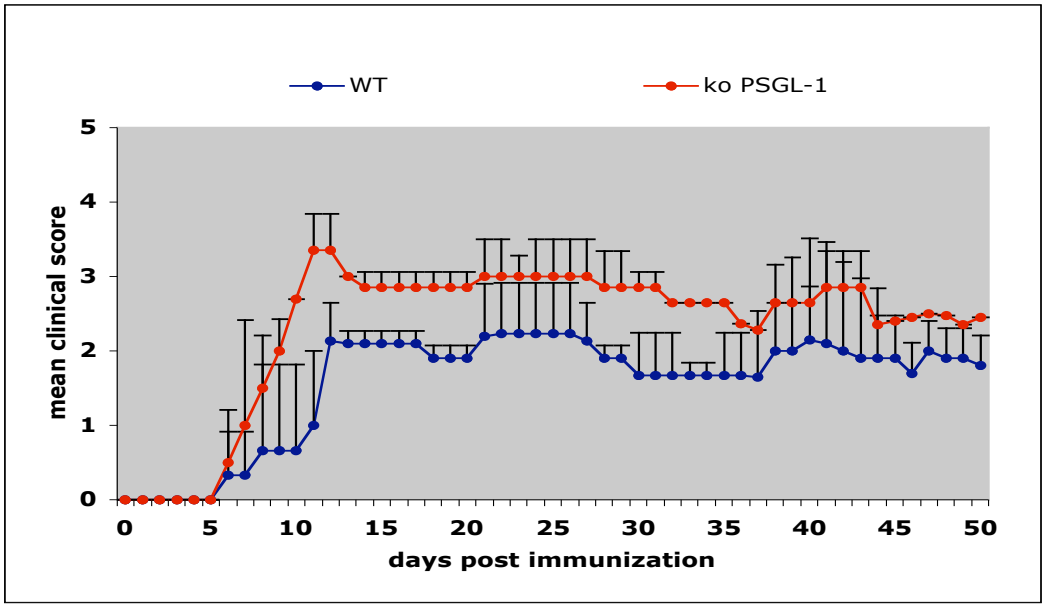


Fig. 7: PSGL-1 deficiency induces increased severity in passively-induced EAE. MOG specific T cell lines, produced from spleen and draining lymph nodes of WT and PSGL-$1^{-/-}$ animals immunized with MOG_{35-55} peptide were transferred in WT recipients. PSGL-1 deficient T cells were able to transfer a more severe disease when compared to wt cells.

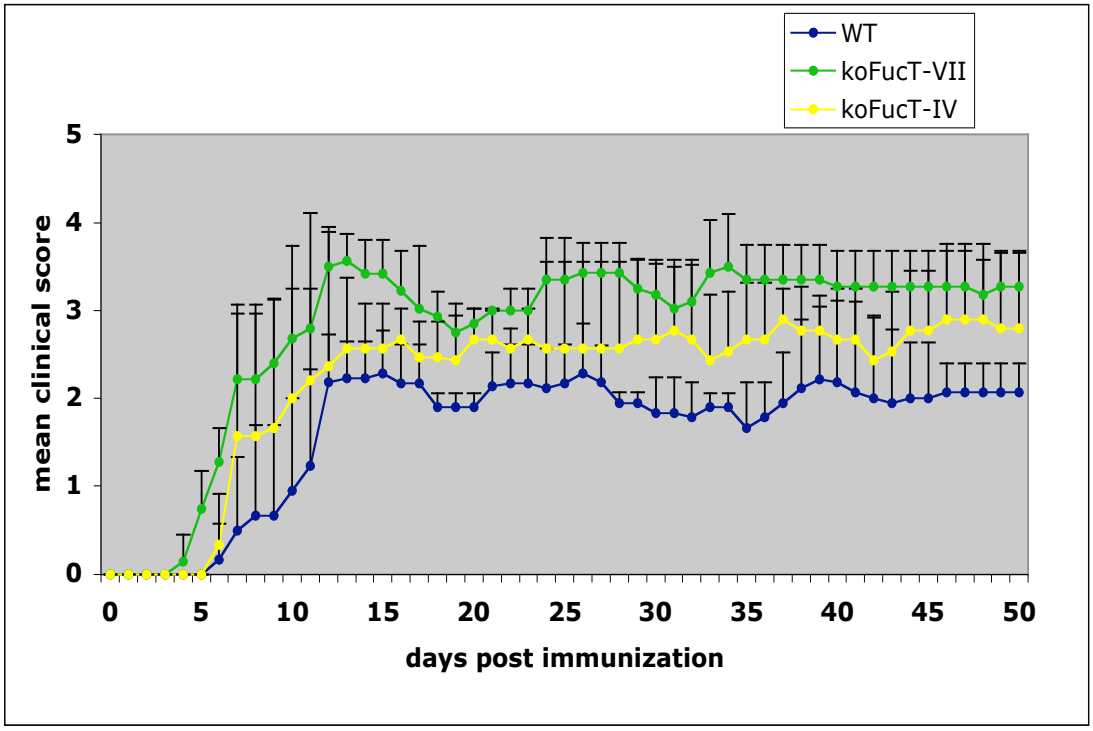


Fig. 8: FucT-VII deficiency increased disease severity in passively-induced EAE. MOG specific T cell lines were produced from spleen and draining lymph nodes of WT and FucT$^{-/-}$ animals immunized with MOG_{35-55} peptide, and then transferred in WT recipient mice. Note the increased disease severity observed when we transferred T cells deficient of FucT-VII, while results obtained with FucT-IV$^{-/-}$ cells were not statistically significant.

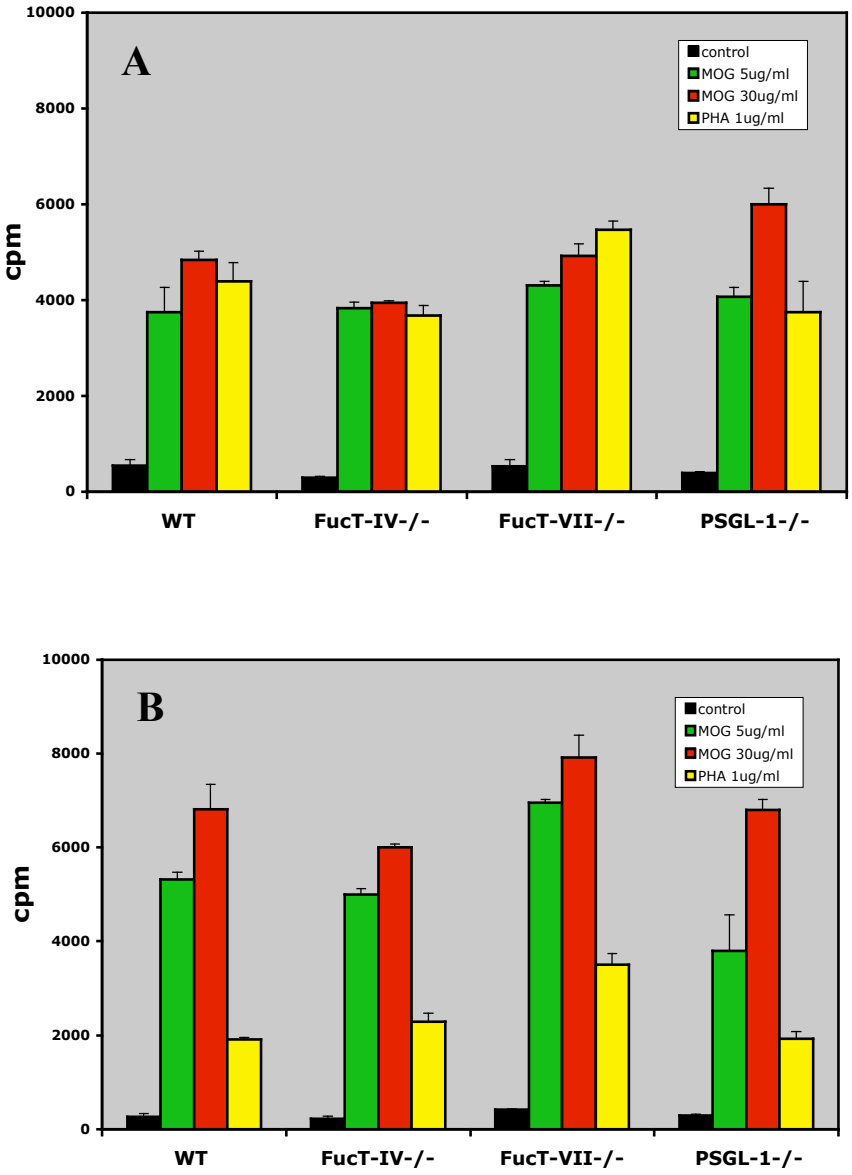


Fig. 9: Proliferative response to MOG_{35-55} peptide in lymph node cells from FucT-IV$^{-/-}$, FucT-VII$^{-/-}$ and PSGL-1$^{-/-}$ mice. MOG_{35-55} specific T cells were produced as described at "Materials and Methods". $5x10^5$ $CD4^+$ T cells isolated from spleens (A) or from peripheral lymph nodes (B) were cultured in a volume of 200 µl/well with MOG 0 (control) - 5 - 30 µg/ml or PHA 1 µg/ml. 3 wells/condition were used. No significant differences were observed between the T cell populations isolated from wt mice or mice deficient in PSGL-1 and FucTs.

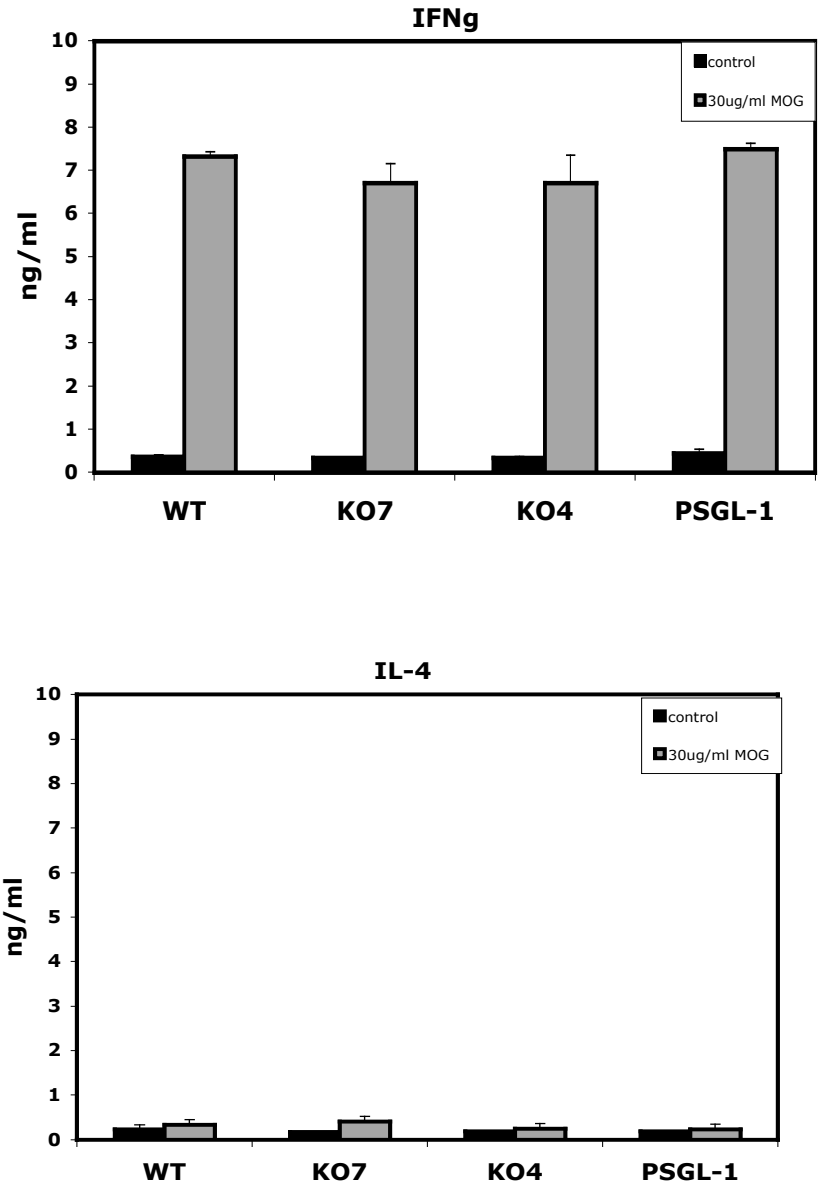


Fig.10: Analysis of cytokine production by MOG_{35-55} auto-reactive T cells from WT and PSGL-1/FucTs deficient mice. $5x10^5$ CD4+ T cells isolated from peripheral lymph nodes and spleens were cultured in a volume of 200 μl/well with MOG_{35-55} peptide 30 μg/ml. The results are expressed as mean cytokine production of 3 wells/condition. As expected, the production of IL-4 was very low consistent with a Th1 phenotype of the encephalitogenic T cells. No significant differences were observed between the auto-reactive T cell populations.

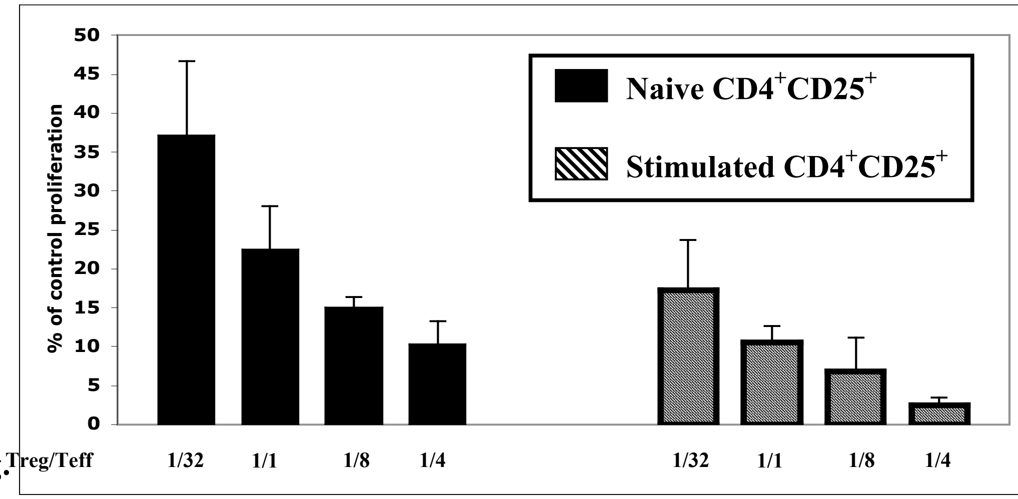


Fig. ... **on between stimulated and naïve Tregs.** Tregs stimulated with anti-CD3 (5 μg/ml) and IL-2 (100 U/ml) had increased suppressor activity in inhibiting the proliferation of $CD4^+CD25^-$ responder cells. Co-cultures were performed using different Treg/Teff cell ratios.

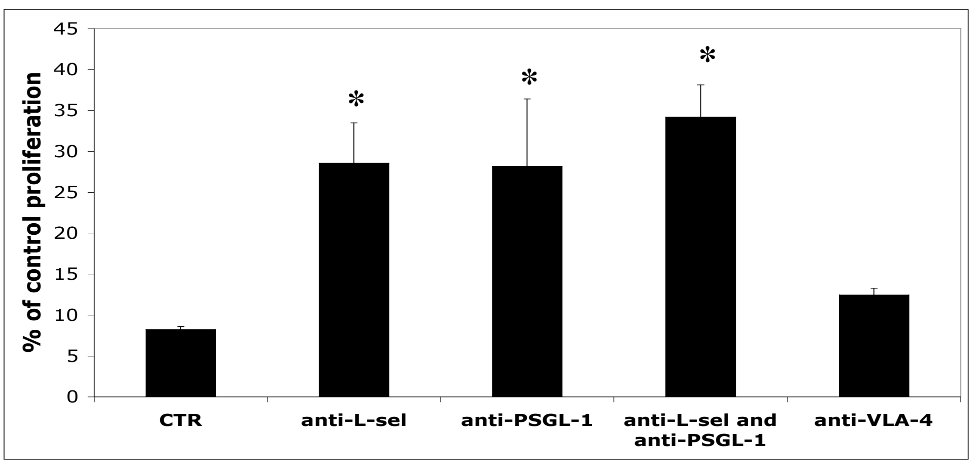
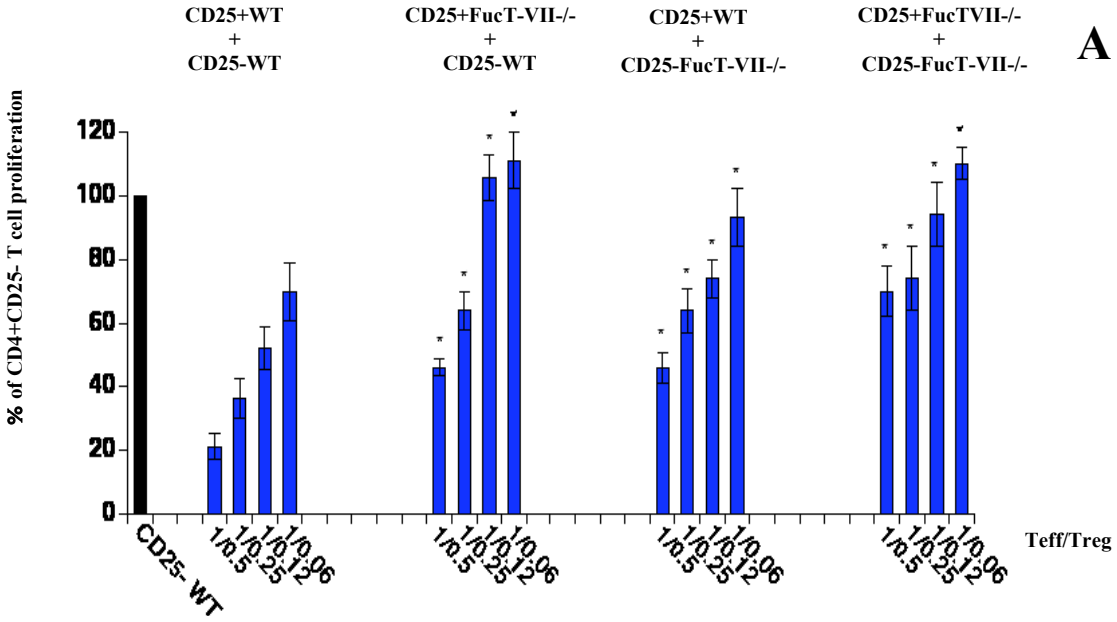
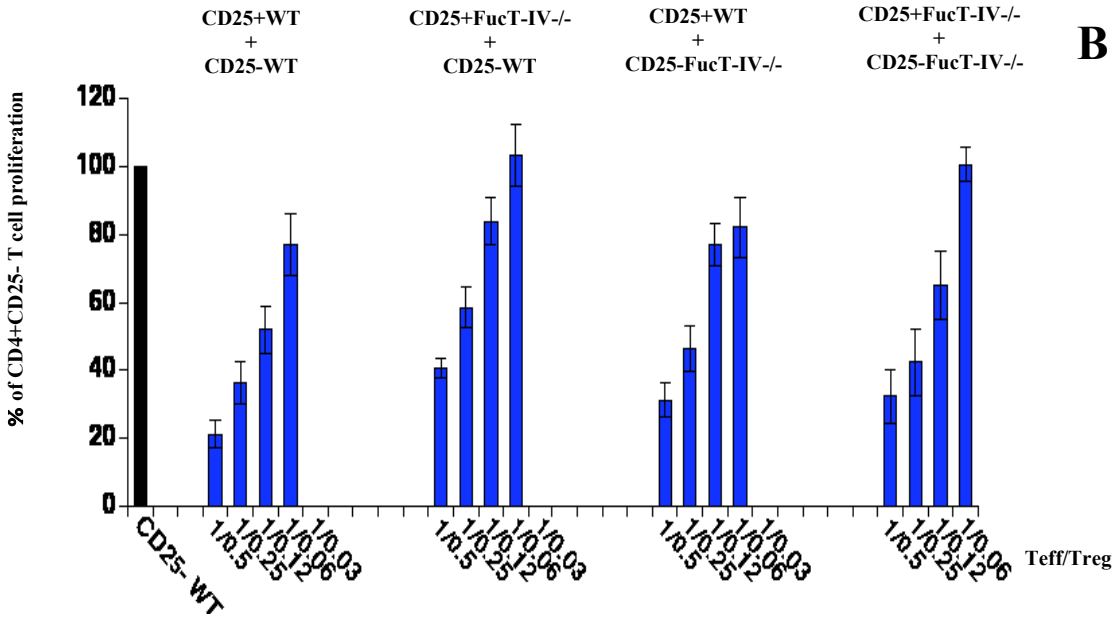


Fig. 12: The effect of adhesion blocking mAbs on the suppressor activity of stimulated Tregs. Co-cultures were made with CD4+CD25+Tregs and $CD4^+CD25^-$ T cells (responder cells) for 72h in the presence of APCs and with 1μg/ml anti-CD3. The results are expressed as % of $CD4^+CD25^-$ T cells proliferation. $CD4^+CD25^-$ / $CD4^+CD25^+$ T cells ratio was 1/1. The following antibodies were used: anti-L-selectin (Mel-14), anti-α_4 integrins (PS/2), anti-PSGL-1 (4RA10) and a control anti-rat isotype mAb. The results show that L-selectin and PSGL-1 are involved in the suppression exerted by Tregs on responder cell proliferation. (*: *P*<0.001).


A
CD25+WT + CD25-WT
CD25+FucT-VII-/- + CD25-WT
CD25+WT + CD25-FucT-VII-/-
CD25+FucTVII-/- + CD25-FucT-VII-/-
% of CD4+CD25- T cell proliferation
120
100
80
60
40
20
0
CD25- WT
1/0.5
1/0.25
1/0.12
1/0.06
Teff/Treg


B
CD25+WT + CD25-WT
CD25+FucT-IV-/- + CD25-WT
CD25+WT + CD25-FucT-IV-/-
CD25+FucT-IV-/- + CD25-FucT-IV-/-
% of CD4+CD25- T cell proliferation
120
100
80
60
40
20
0
CD25- WT
1/0.5
1/0.25
1/0.12
1/0.06
1/0.03
Teff/Treg

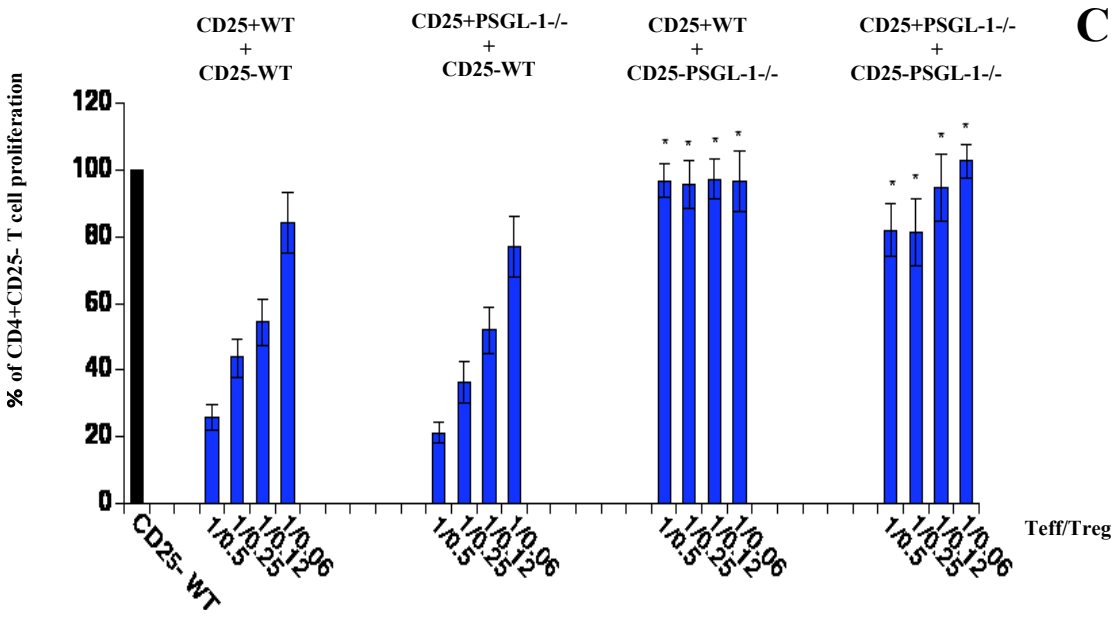
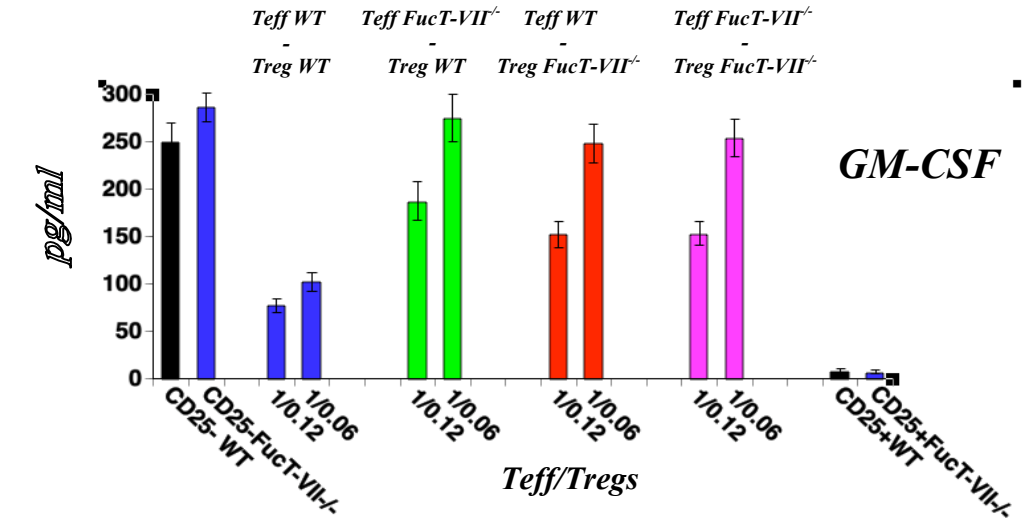
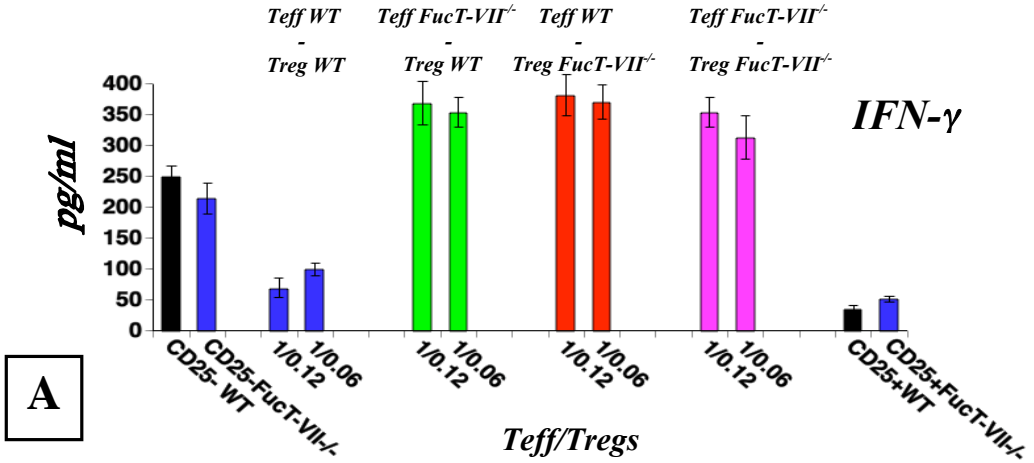
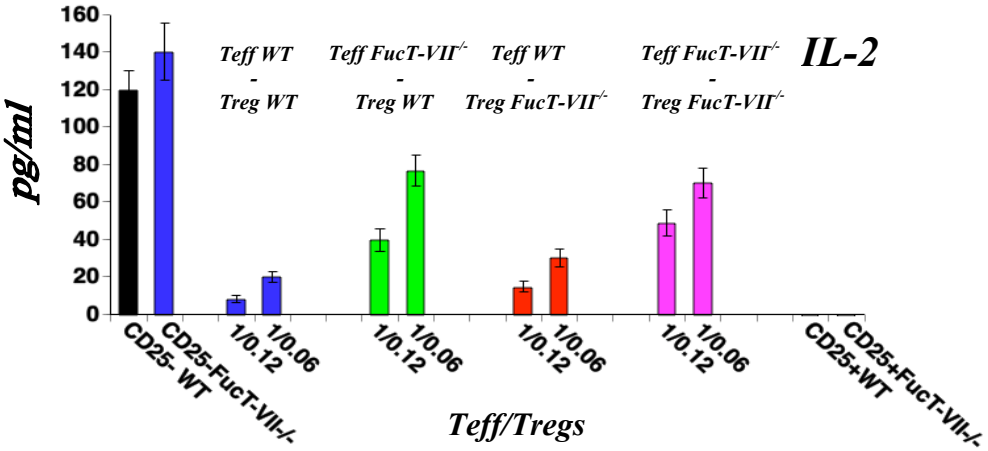


Fig. 13 (pag. 48 and 49): The effect of FucTs and PSGL-1 deficiencies on the suppressor activity of $CD4^+CD25^+$ regulatory T cells. The experiments were performed with naïve Tregs. Different ratio Tregs/Teff ratios were used. Data are shown as percentage of proliferation compared with a control (100%) represented by $CD4^+CD25^-$ from the recipient mice. To note, FucT-VII deficiency leads to a reduction of suppressor activity when present on Tregs or responder cells or both populations (A). PSGL-1 expression is requires only on effector cells for efficient cell-cell contact (C) (*P<0.01). No significant effects are associated with FucT-IV deficiency (B).


Teff WT - Treg WT
Teff FucT-VII-/- - Treg WT
Teff WT - Treg FucT-VII-/-
Teff FucT-VII-/- - Treg FucT-VII-/-
GM-CSF
pg/ml
300
250
200
150
100
50
0
CD25- WT
CD25-FucT-VII-/-
1/0.12
1/0.06
CD25+WT
CD25+FucT-VII-/-
Teff/Tregs


Teff WT - Treg WT
Teff FucT-VII-/- - Treg WT
Teff WT - Treg FucT-VII-/-
Teff FucT-VII-/- - Treg FucT-VII-/-
IFN-γ
pg/ml
400
350
300
250
200
150
100
50
0
CD25- WT
CD25-FucT-VII-/-
1/0.12
1/0.06
CD25+WT
CD25+FucT-VII-/-
Teff/Tregs

A


Teff WT - Treg WT
Teff FucT-VII-/- - Treg WT
Teff WT - Treg FucT-VII-/-
Teff FucT-VII-/- - Treg FucT-VII-/-
IL-2
pg/ml
160
140
120
100
80
60
40
20
0
CD25- WT
CD25-FucT-VII-/-
1/0.12
1/0.06
CD25+WT
CD25+FucT-VII-/-
Teff/Tregs

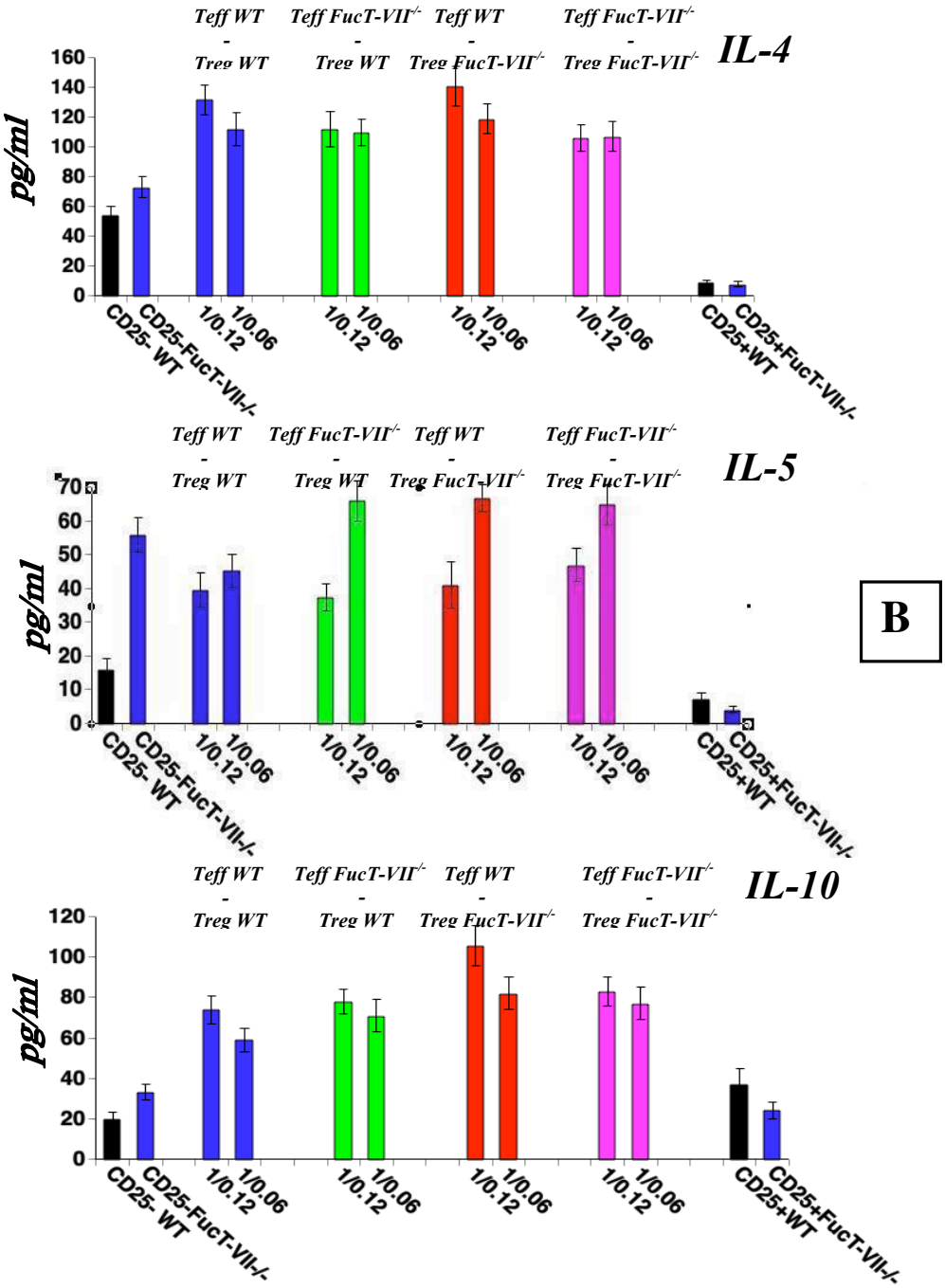


Fig. 14 (pag. 50 and 51). The effect of FucT-VII deficiency on the regulation of cytokine production by Tregs. Data were obtained with in Multiplex assays (BioRad) on supernatants collected from co-cultures performed as described for Figure 13 (proliferation assays). Effects of FucT-VII deficiency on GM-CSF, IFN-γ, and IL-2 secretion are shown in A (P<0.01). The effect of FucT-VII KO on IL-4, IL-5, and IL-10 secretion is shown in B.

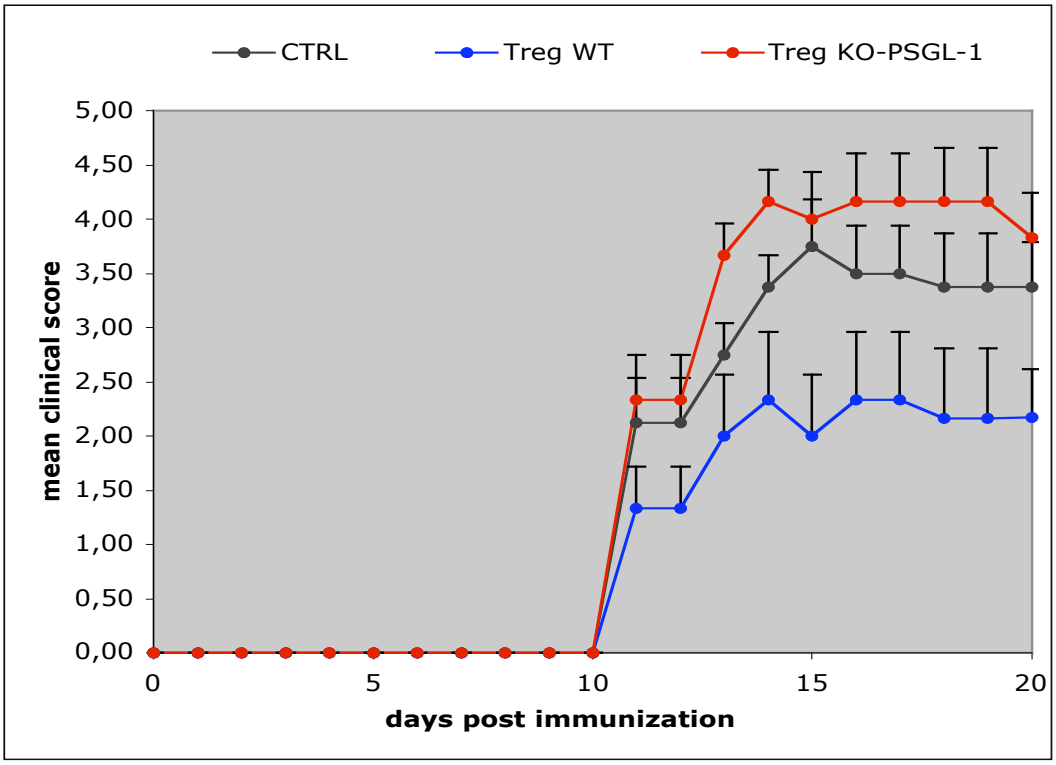


Fig. 15: Effect of PSGL-1 deficiency on naïve Tregs suppression of EAE. C57BL/6J mice immunized with MOG_{35-55} peptide (active EAE) were injected i.v. at day +7 post-immunization with $2,5x10^6$ Tregs from WT or PSGL-1 deficient mice. Data showed are from one representative experiment with 6 mice per condition from a series of three with similar results. We observed a complete lack of suppression by PSGL-1 deficient Tregs when compared with WT Tregs ($P<0.02$ when compared with WT Tregs-transplanted group). Interestingly, we observed a higher disease score in animals injected with Tregs from PSGL-$1^{-/-}$ mice after day +25 post-immunization.

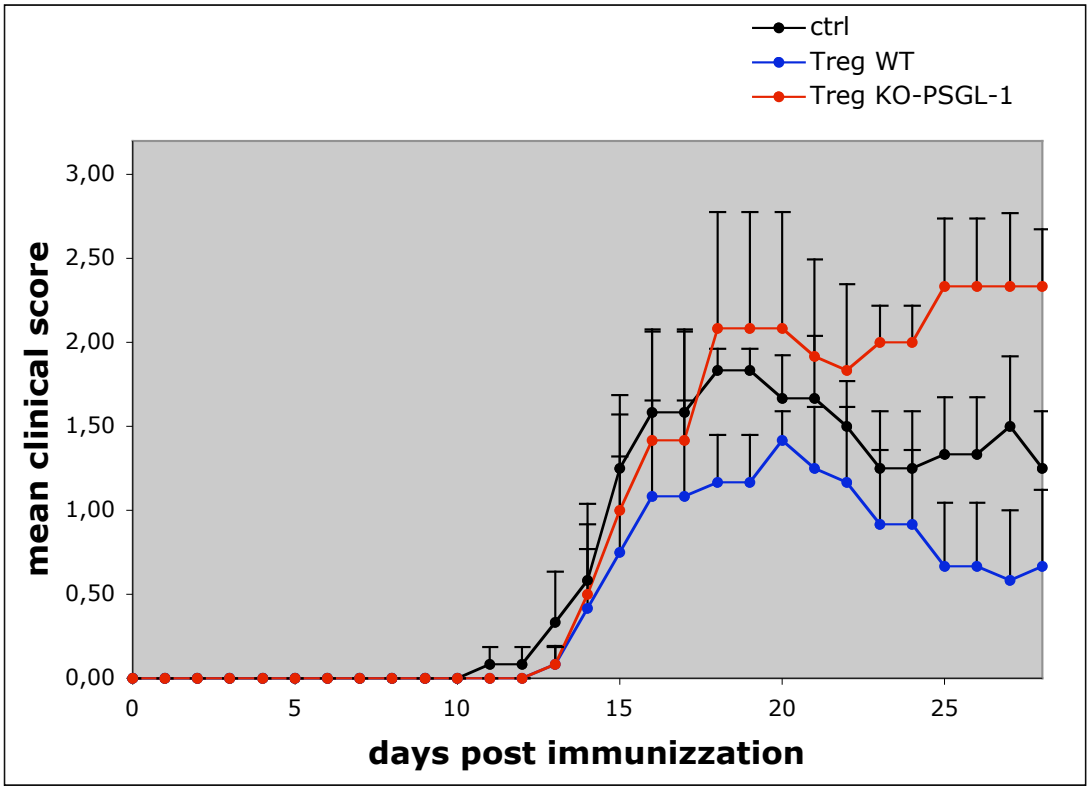
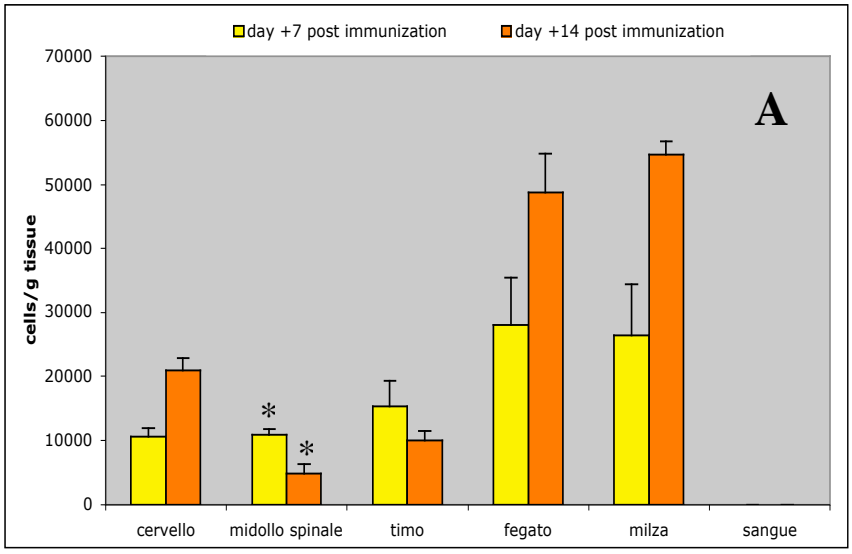


Fig. 16: The effect of stimulated PSGL-1 deficient Tregs on EAE. C57BL/6J mice immunized with MOG_{35-55} protocol (active EAE) were injected i.v. at day +7 post-immunization with $2{,}5x10^6$ anti-CD3/IL-2-stimulated Tregs from WT or PSGL-1 deficient mice. Data showed are from one experiment with 6 mice per condition. As shown for naïve Tregs, animals treated with PSGL-1 deficient Tregs displayed a higher mean disease clinical score than controls and mice transplanted with WT Tregs ($P<0.01$).


day +7 post immunization
day +14 post immunization
A
cells/g tissue
70000
60000
50000
40000
30000
20000
10000
0
cervello
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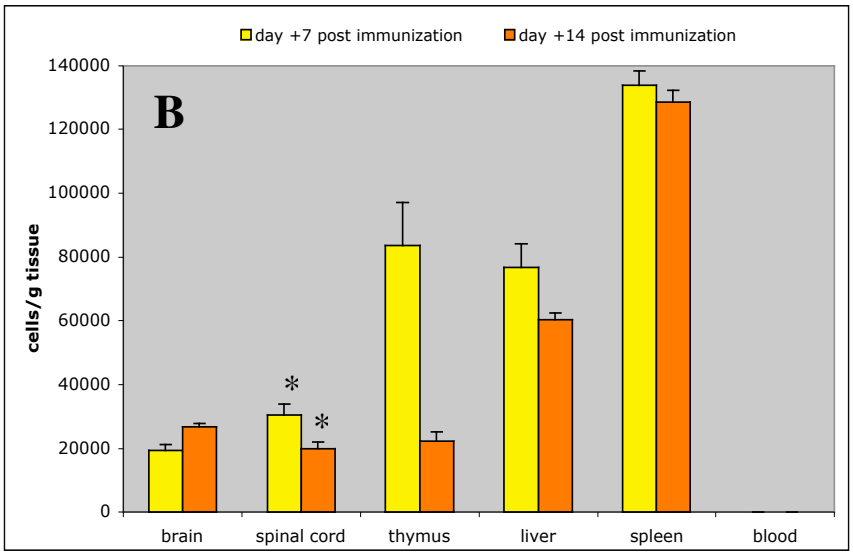
timo
fegato
milza
sangue


day +7 post immunization
day +14 post immunization
B
cells/g tissue
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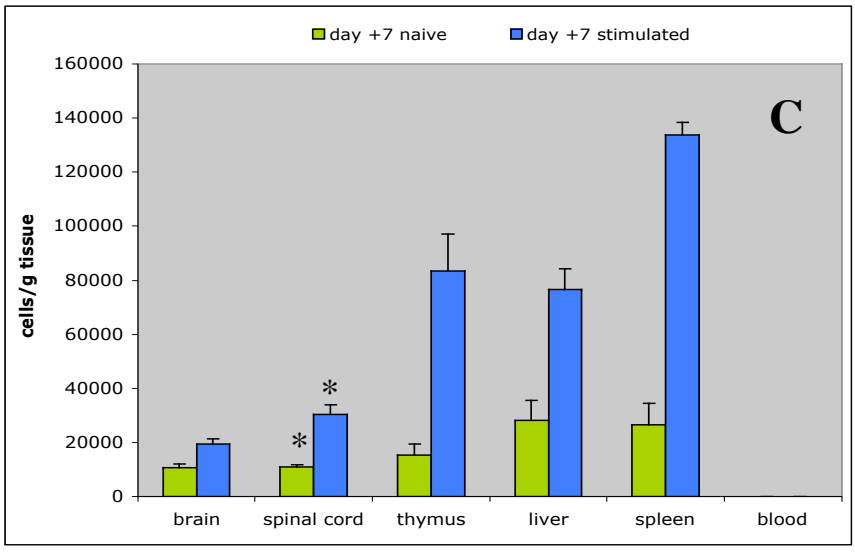
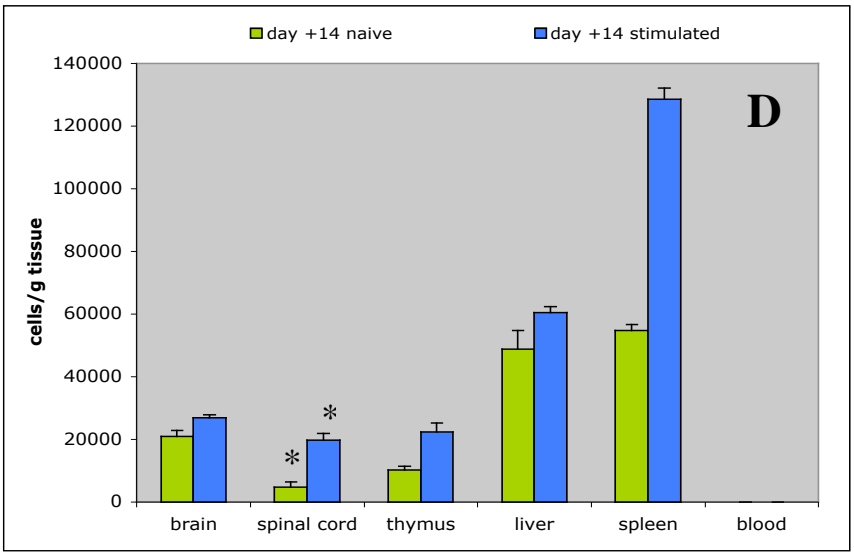
120000
100000
80000
60000
40000
20000
0
brain
spinal cord
thymus
liver
spleen
blood


day +7 naive
day +7 stimulated
C
cells/g tissue
160000
140000
120000
100000
80000
60000
40000
20000
0
brain
spinal cord
thymus
liver
spleen
blood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day +14 stimulated
D
cells/g tissue
140000
120000
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80000
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40000
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brain
spinal cord
thymus
liver
spleen
blood
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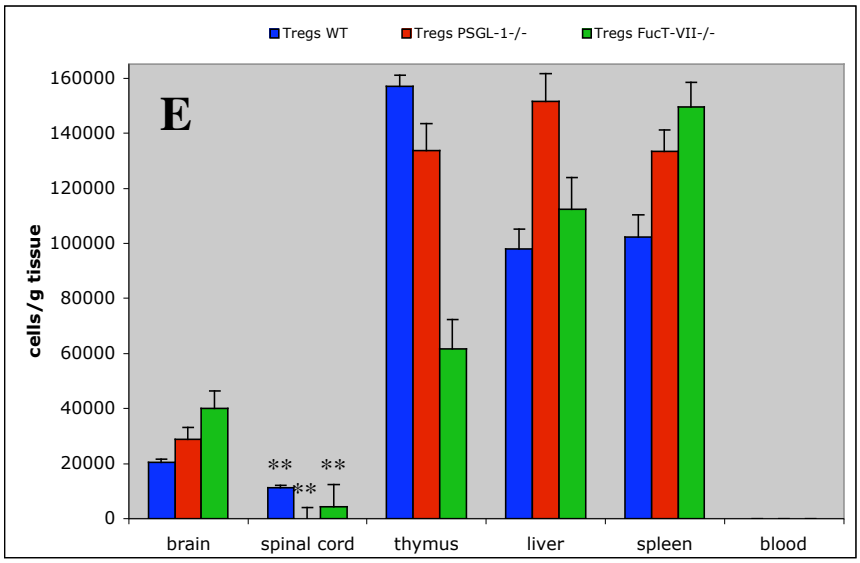
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Tregs PSGL-1-/-
Tregs FucT-VII-/-
E
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160000
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brain
spinal cord
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liver
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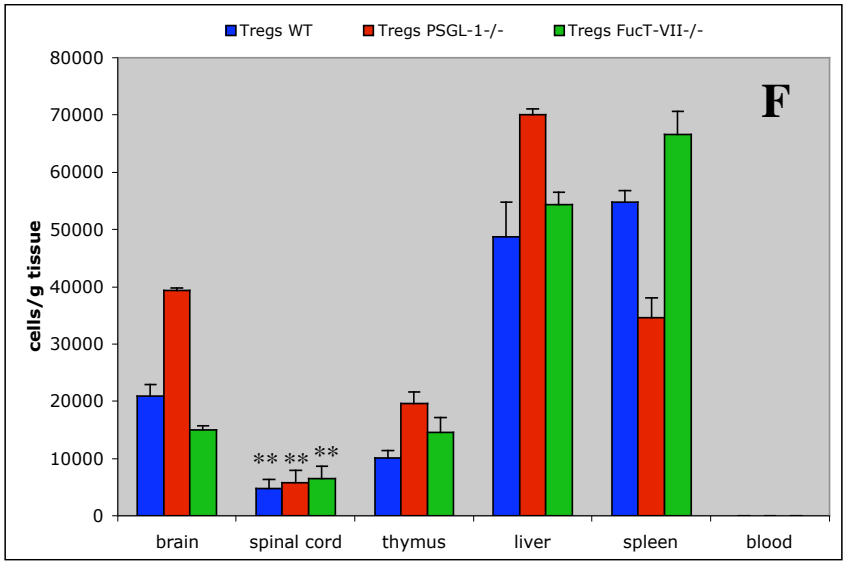
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Tregs PSGL-1-/-
Tregs FucT-VII-/-
F
cells/g tissue
80000
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60000
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brain
spinal cord
thymus
liver
spleen
blood
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Fig. 17 (pag. 54 and 55): migration ability of Tregs in vivo. WT C57BL/6J mice were immunized with the MOG_{35-55} peptide. Tregs from WT, FucT-VII-/- or PSGL-1-/- donor mice were used. $2{,}5x10^6$ [^{3}H]-glycerol-labeled naïve WT Tregs (A) or activated Tregs (B) were injected at day 7 and day 14 post immunization. The migration of naïve versus stimulated Tregs was compared at day 7 (C) and at day 14 (D) post immunization. The effect of PSGL-1 and FucT-VII deficiency was studied with naïve Tregs at day 7 (E) and day 14 (F) post immunization. In all experiments mice were sacrificed at 24h after Treg administration and then were perfused with PBS through the left ventricle. Organs were collected, sonicated and added in scintillation liquid; the radioactivity was evaluated with a β-counter. The results show that naïve and activated Tregs better migrate in the pre-clinical phase of disease (A and B) (*P<0.01), while activated T cells have increased migration capacity when compared to naïve Tregs (C, D) (*P<0.01). A clear role for PSGL-1 and FucT-VII is seen in the inflamed spinal cord in the pre-clinical phase of disease (E) (**P<0.0001 in E and **P<0.05 in F). For each point, data are from one representative experiment with 3-4 mice/condition from a series of two.

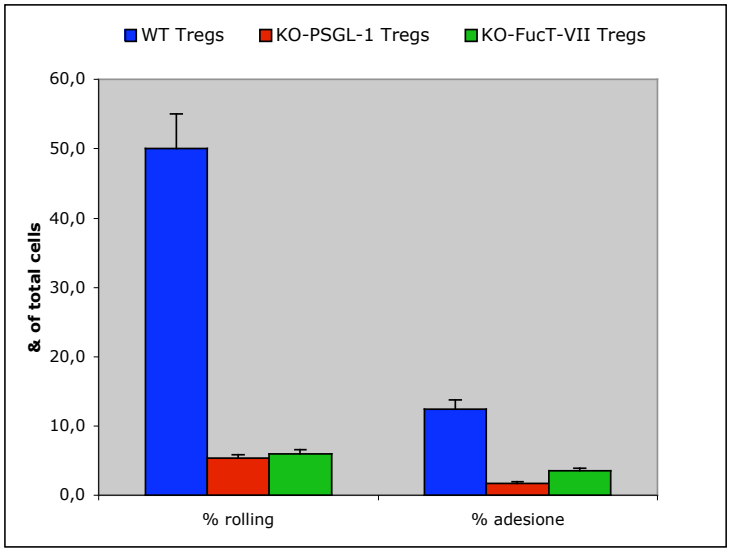

Fig. 18: Analysis of Tregs adhesion ability with intravital microscopy. Tregs from WT, PSGL-1$^{-/-}$ and FucT-VII$^{-/-}$ mice were stimulated with the anti-CD3/IL-2 protocol. After 3 days, 2,5x10^{6} cells were labeled with CMTMR or CMFDA (see Materials and methods) and injected via-catheter in a 7 weeks-old SJL mice, previously treated with 12 μg of LPS in order to subacutely inflame brain endothelium (6h before the intravital microscopy experiment). The model of endothelium activation used here mimics the inflammatory conditions shown during pre-clinical phase of EAE (Piccio et al., 2002). Data are expressed as percentage of total cells observed. The results are the average of 2 experiments, analyzing 2-3 venules per brain. Tregs from PSGL-1 and FucT-VII deficient mice presented a dramatic reduction in the ability to adhere to endothelium in inflamed brain microcirculation (P<0.0001).

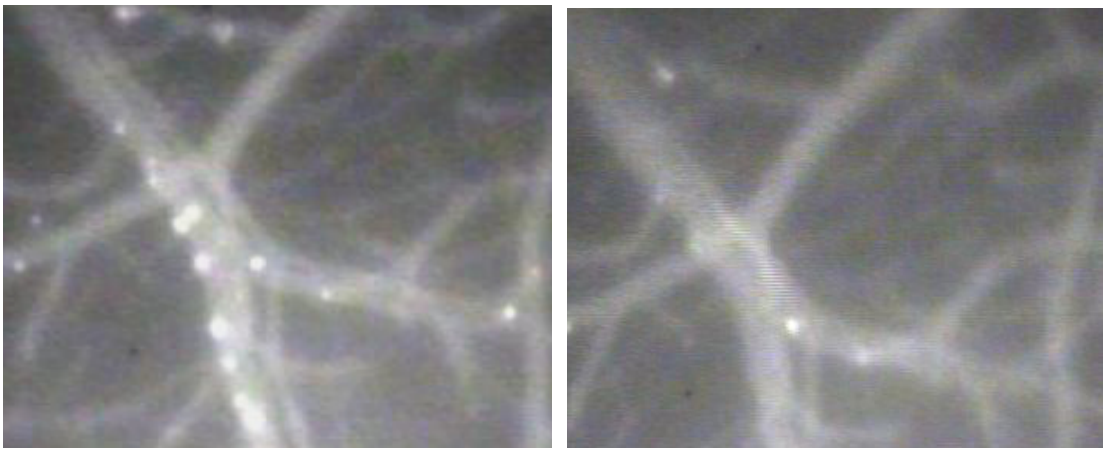

Fig. 19: Intravital microscopy imaging. To note, CMFDA-labeled WT Tregs highly adhere on inflamed brain endothelium (left), while CMTMR-labeled PSGL-1 deficient Tregs (right) display an almost complete inhibition of the adhesion. Cells are the white spots inside blood vessels labeled with fluorescent dextrans.

6. Table and legend

	α_4 integrins	LFA-1	PSGL-1	L-selectin	CD44	P-sel. Chimera	E-sel. Chimera
naive Tregs	53%	99%	96%	51%	88%	4%	0,70%
% (MFI)	(21.14)	(54.2)	(92.27)	(33.64)	(63.31)	(273.97)	(77.87)
stimulated Tregs	42%	99%	98%	75%	93%	23.5%	2,40%
% (MFI)	(14.93)	(59.86)	(116.47)	(52.79)	(55.64)	(511.41)	(308.86)

Table I. Tregs display higher binding activity to E- and P-selectin after activation with anti-CD3 mAb and IL-2.

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