Chiara Uboldi

SJL/L-selectin-deficient mice

develop experimental autoimmune encephalomyelitis

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vorgelegt von

Chiara Uboldi

aus Cittiglio (Italien)

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Dekan:	Herrn Prof. Dr. Alexander Steinbüchel
Erste Gutachter:	Herrn Prof. Dr. Dietmar Vestweber
Zweiter Gutachter:	Herrn Prof. Dr. Volker Gerke
Prüfer:	Herrn Prof. Dr. Andreas Puschel

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I. INTRODUCTION

I.1. Lymphocyte homing receptor: L-selectin

In the 1950s and 1960s Gowans and collaborators noticed that lymphocytes continuously recirculate from the blood into the lymph nodes and then back into the blood (Gowans and Knight, 1964; Marchesi and Gowans, 1964). This migration or homing of lymphocytes into the peripheral lymph nodes (PLN) is necessary to expose lymphocytes to antigens. As demonstrated by histological studies, circulating lymphocytes migrate into the tissues through the postcapillary venules of lymph nodes, which are characterized by cuboidal endothelial cells (Ford et al., 1969; Ford, 1975). In 1976 Stamper and Woodruff were able to demonstrate, *in vitro*, that lymphocytes bind to the high endothelial venules (HEV). They added fresh lymphocytes to frozen sections of lymph nodes and observed their binding. Later, antibodies were developed to block lymphocyte binding to HEV and to identify the adhesion molecules involved in lymphocyte homing to PLN and to sites of inflammation. A great number of different adhesion molecules were identified, including three proteins which belong to the family of selectin proteins: E-, P- and L-selectin. Selectins (Figure I.1.1) are C-type lectin calcium-dependent proteins expressed by bone marrow-derived and by endothelial cells.

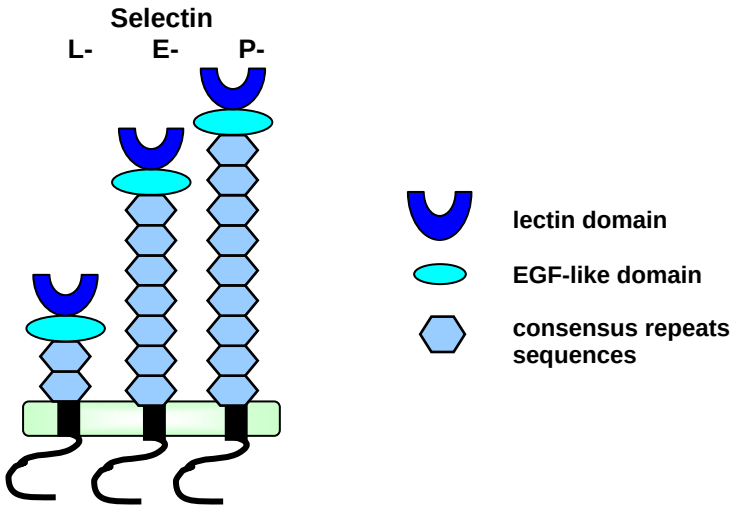

Figure I.1.1. Selectin family proteins. Schematic representation of L-, E- and P-selectin proteins.

Due to their involvement in the migration of leukocytes into PLN and inflamed tissue (McEver et al., 1991), these molecules have received great attention and have been studied in detail. *In vitro*, selectins were demonstrated to be involved in neutrophil adhesion to cytokine-stimulated endothelial cells (Smith et al., 1991; Bargatze et al., 1994) and in neutrophil rolling under shear stress (Lawrence et al., 1993; Abbassi et al., 1993; Fuhlbrigge et al., 1996). I*n vivo*, all three selectin proteins were shown, independently, to take part in leukocyte rolling on the endothelium (Doré et al., 1993; Ley et al., 1993; Ley et al., 1995; Finger et al., 1996).

L-selectin is the homing receptor for lymphocytes at high endothelial venules (HEV) within peripheral lymph nodes (PLN). It is localized on the microvilli of naïve T cells, myeloid cells (Kansas, 1996) and central memory T cells (Sallusto et al., 1999). I*n vivo*, L-selectin mediates rolling of circulating lymphocytes on HEV (Von Andrian et al., 1995; Warnock et al. 1998; Stein et al., 1999). The monoclonal antibody MEL-14, *in vivo,* completely blocked the migration of lymphocytes from the blood into PLN, but not into Peyer's patches (Gallatin et al., 1983; Geoffroy and Rosen, 1989), by binding the lectin domain of L-selectin (Bowen et al., 1990). While E- and P-selectins were demonstrated not to be involved in lymphocyte migration into the central nervous system during inflammation (Engelhardt et al., 1997), L-selectin was demonstrated to be involved in lymphocyte binding to myelinated regions of the CNS. Rosen and his co-workers demonstrated that L-selectin binds, on brain and spinal cord frozen sections, to the white matter regions and this binding can be impaired by adding the monoclonal antibody MEL-14 (Huang et al., 1991). L-selectin (CD62L) used to be called LECAM-1 (leukocyte-endothelial cell adhesion molecule-1), Ly-22 (lymphocyte antigen-22), Leu8 (leukocyte surface antigen-8), TQ1, DREG-56, and LAM-1 (leukocyte adhesion molecule-1), but these terms are no longer in use. In fact, after cloning and sequencing (Tedder et al., 1989; Siegelman et al., 1989; Lasky et al., 1989), L-selectin was identified as a type-I membrane protein composed by different domains which are all involved in the regulation of L-selectin function. The cytoplasmic tail is important for the anchorage of L-selectin to the cytoskeleton and modulates the ability of L-selectin to mediate tethering and rolling of lymphocytes along HEV (Kansas et al., 1993). In fact, tail-truncated L-selectin molecules were created and it was demonstrated that the 6 amino acids in close proximity to the cell membrane are important for the localization of L-selectin on microvilli. Instead, the last 11 amino acids at the C-terminus are important for the anchoring of L-selectin to the cytoskeleton (Dwir et al., 2001), which is mediated by

α-actinin (Pavalko et al., 1995) and by the ezrin-radixin-moesin (ERM) proteins (Ivetic et al., 2002; Ivetic et al., 2004; Ivetic and Ridley, 2004). A correct linkage of L-selectin to the cytoskeleton was shown to facilitate ligand binding, to stabilize tethering and to switch tethering to rolling (Schwarz et al., 2004). The binding of L-selectin to its carbohydrate ligands is mediated by calcium-dependent lectin domain at the N-terminus. The first suggestion that carbohydrates play a role in lymphocyte homing was made by Gesner and Ginsburg in 1964. Their hypothesis was then confirmed by Stoolman and Rosen: they demonstrated that cell surface carbohydrates on the endothelium and carbohydrate-binding molecules on lymphocytes contribute to lymphocyte homing at HEV (Stoolman et al., 1983). Lymphocytes are able to recognize HEV (Jalkanen et al., 1986; Berg et al., 1989) because HEV present specific carbohydrates on their luminal site (Streeter et al., 1988; Berg et al., 1989). The presence of these HEV specific antigens was defined by MECA-79. MECA-79 is a monoclonal antibody binding to specific sulphate- and fucose-dependent carbohydrate epitopes on HEV and is able to block lymphocyte extravasation into PLN. *In vitro*, MECA-79 binds to epitopes on the surface of high endothelial venules of peripheral lymph nodes and not to the HEV of the mucosa-associated lymphoid organs like Peyer's patches (Streeter et al., 1988). L-selectin and MECA-79 precipitated the same proteins (Imai et al., 1991), thus the protein complexes recognized by MECA-79 were thought to include also L-selectin ligands. Both L-selectin and MECA-79 bind to O-glycans attached to serine and threonine residues on glycoproteins which are called mucins or peripheral node addressin (PNAd) (Figure I.1.2). The O-glycans contain tetrasaccharides rich in fucose moieties and sialic acid called sialyl Lewis X (sLeX). The structural analysis of sialomucins confirmed that L-selectin and MECA-79 require sulfation of sLeX (6-sulfo sLeX) to bind their fucosylated ligands (Moore et al., 1991; Hemmerich et al., 1994; Toppila et al., 1997; Renkonen et al., 1997). In fact, recently two independent studies focused on GlcNAc6ST-1 and -2 (N-acetylglucosamine-6-O-sulfotransferase-1 and -2), which are the enzymes responsible for the correct biosynthesis of 6-sulfo sLeX, and confirmed the biological relevance of sulfation for lymphocyte homing *in vivo*. Mice lacking both sulfotransferases were shown to have an impaired recruitment of lymphocytes into PLN and the 6-sulfo sLeX and the MECA-79 epitope are missing on HEV (Kawashima et al., 2005). Using intravital microscopy, in GlcNAc6ST-1 and -2 knock-out mice, only few lymphocytes were seen to roll on HEV and their rolling velocity was higher compared to wild type mice (Uchimura et al., 2005). In both studies, could be still detected a minor group of

lymphocytes homing to PLN, suggesting that L-selectin can bind also ligands which are not recognized by MECA-79, such as PSGL-1. In addition, it was demonstrated that the absence of fucosylation provokes absence of L-selectin ligands on HEV and therefore a reduction in the number of lymphocytes in the PLN (Maly et al., 1996; Homeister et al., 2001; Piccio et al., 2005).

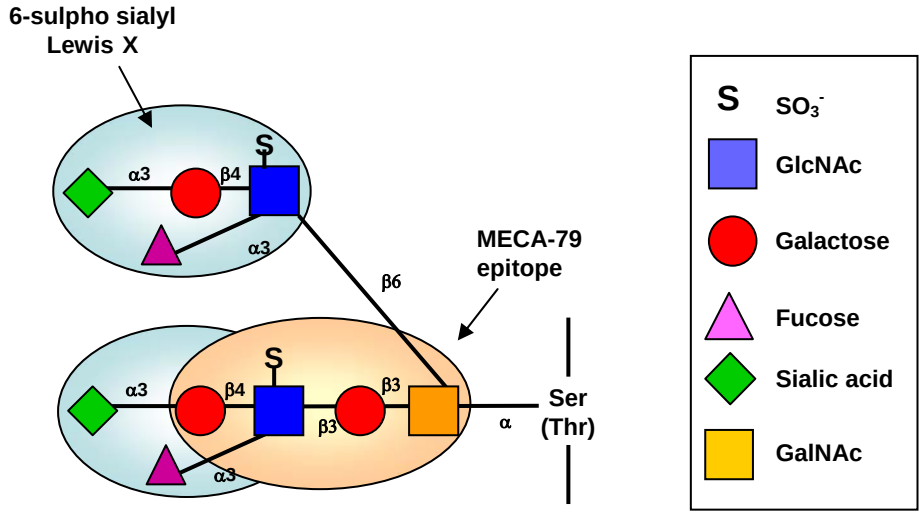

Figure I.1.2. Peripheral node addressin and MECA-79 antibody. (Modified from R.P. McEver, 2005. *Nature Immunology 11(6): 1068*). The L-selectin binding determinant 6-sulpho sialyl Lewis X is created by two Golgi enzymes (GlcNAc6ST-1 and -2) which attach sulphate esters to GlcNAc residues on the sLeX group. MECA-79 recognizes mucins on HEV.

PSGL-1 (P-selectin glycoprotein ligand-1) (Figure I.1.3) is a disulfide-bonded homodimeric mucin-like glycoprotein of 120kDa which is expressed, on leukocytes, on the top of microvilli (Moore et al., 1995). During inflammation, PSGL-1 can bind P-selectin and mediates leukocyte-endothelial and leukocyte-platelet adhesion, but PSGL-1 can also mediate leukocyte-leukocyte interaction by binding to L-selectin (Walcheck et al., 1996; Moore, 1998). In neutrophils, L-selectin and PSGL-1 interact and mediate leukocyte recruitment to inflamed tissues (Spertini et al., 1996). L-selectin binding to PSGL-1 requires that the ligand has an appropriate glycosylation (Cummings et al., 1999) and can be blocked by antibodies (Guyer et al., 1996; Snapp et al., 1998). Furthermore, the binding is calcium-dependent and is regulated by the lectin domain with the EGF domain and the two consensus repeats sequences of L-selectin (Tu et al., 1996).

The glycosylated ligands for L-selectin on HEV, GlyCAM-1, CD34 and the novel ligand endomucin (Figure I.1.3), were identified using the MECA-79 antibody.

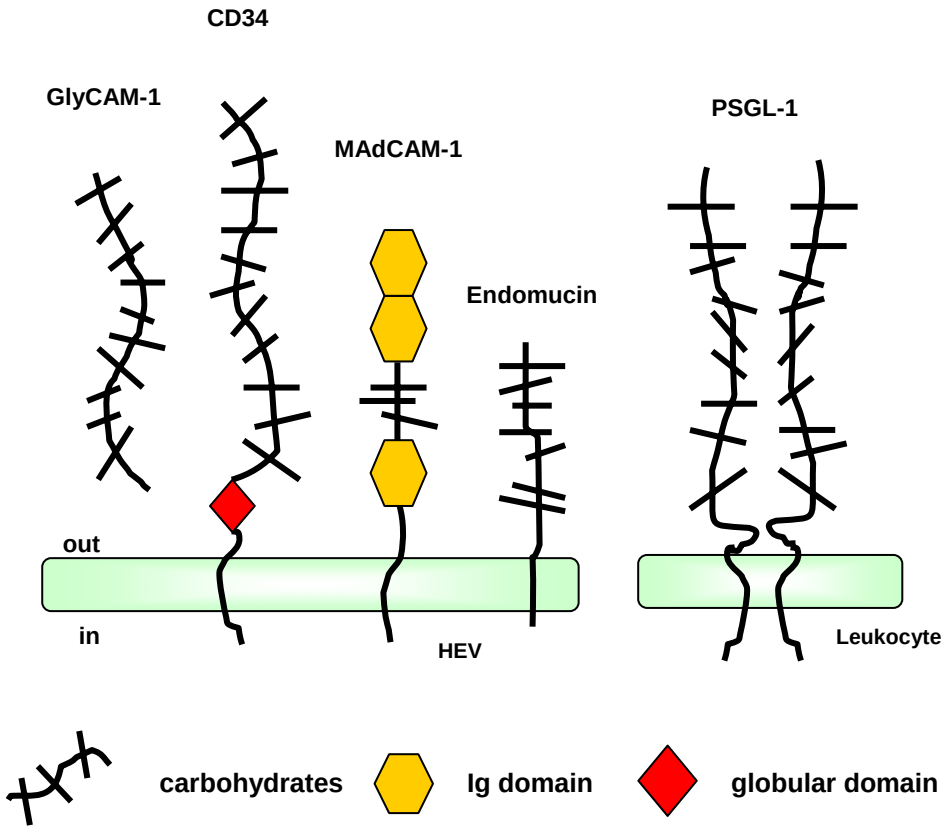

Figure I.1.3. L-selectin ligands GlyCAM-1, CD34, PSGL-1, MAdCAM-1 and endomucin. Schematic representation of glycosylated L-selectin ligands expressed on HEV or leukocytes.

GlyCAM-1 (glycosylation-dependent cell adhesion molecule-1) was isolated from culture media of mouse lymph node (Imai et al., 1991). It was first named sulfated glycoprotein Sgp50 because of its molecular weight of 50kDa and only after sequencing was it recognized as a mucin-like adhesion molecule (Brustein et al., 1992; Lasky et al., 1992). GlyCAM-1 is detectable, by immunocytochemistry, in the intracellular vesicles of high endothelial cells and not on the surface of HEV, suggesting that GlyCAM-1 is secreted, upon inflammatory stimuli, in a sialylated form into the blood (Imai et al., 1993; Kikuta et al., 1994), where it binds soluble L-selectin (Hemmerich et al., 1994; Suguri et al., 1996).

The second L-selectin ligand identified using MECA-79 was CD34 (Baumhueter et al., 1993; Baumhueter et al., 1994), which was first named Sgp90 because of its molecular

weight (Imai et al., 1991). The sialomucin CD34 is abundant in serine, threonine and carbohydrates and can support lymphocyte adhesion under shear flow *in vitro* (Puri et al., 1995). CD34 is a transmembrane glycoprotein expressed on HEV (Lin et al., 1995), where it is sulfated to bind L-selectin. CD34 is also expressed in a non-sulfated form on hematopoietic precursor cells, where is not an L-selectin ligand. Electron microscopy revealed that CD34 is expressed on the luminal site of HEV and is localized at cell junctions (Fina et al., 1990).

Endomucin is a novel ligand for L-selectin. It was previously identified as an endothelial sialomucin (Morgan et al., 1999), but more recent investigations have shown that endomucin is also expressed on the luminal site of HEV in lymphoid organs and carries the MECA-79 epitope. Because of its expression on HEV and the presence of the MECA-79 epitope, endomucin is considered to be a ligand for L-selectin (Samulowitz et al., 2002) and supports L-selectin-mediated rolling of lymphocytes on HEV (Kanda et al., 2004).

Lymph node high endothelial venules present Sgp200, which is another sulfated glycoprotein, not yet characterized, that binds soluble L-selectin via the carbohydrate groups (Hemmerich et al., 1994).

L-selectin also binds to another ligand which is expressed on HEV in mucosa-associated lymphoid tissue (MALT) and gut-associated lymphoid tissue (GALT) (Berlin et al., 1995). This ligand (Figure I.1.3), the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), was recognized by the monoclonal antibody MECA-367 and is the ligand for $\alpha_4\beta_7$ integrin on Peyer's patches and mesenteric lymph nodes (Streeter et al., 1988; Berlin et al., 1993; Briskin et a., 1993; Bradley et a., 1997). MAdCAM-1 is a 60kDa protein which is recognized by the lectin domain of L-selectin. In fact, L-selectin recognizes and binds to a mucin-like structure of MAdCAM-1 (Berg et al., 1993). MAdCAM-1 expression can be increased *in vitro* and *in vivo* upon inflammation (Sikorski et al., 1993).

The generation of L-selectin-deficient mice allowed an improved investigation of the role of L-selectin in lymphocyte homing *in vivo*. The L-selectin-deficient mice were generated in the early 1990s by gene targeting, and their characterization reported an unique phenotype. Lymphocytes from those mice do not bind to HEV and, compared to the wild type littermates, the L-selectin knock-out mice had a strong reduction in lymphocyte recruitment into PLN (Arbones et al., 1994; Catalina et al., 1996) and an increased splenic cellularity (Steeber et al., 1996; Steeber et al., 1996). The role of L-

selectin in inflammation is controversial. In fact, L-selectin-deficient mice have shown an impaired migration of neutrophils, lymphocytes and monocytes into the inflamed peritoneum, indicating that L-selectin also plays a role in cell migration into non-lymphoid tissues (Tedder et al., 1995). Indeed, no significant difference was reported in lymphocyte homing to inflamed skin in L-selectin-deficient mice (Catalina et al., 1996).
Many investigations focused on the involvement of L-selectin in leukocyte-leukocyte interaction during inflammation (Tedder et al., 1995; Ley et al., 1995) and on the role of L-selectin in driving leukocytes to sites of inflammation and infection (Michie et al., 1993). It is well known that cells are able to cross the endothelium and enter sites of inflammation, and those infiltrating cells display a particular phenotype. The CD4-positive T cells infiltrating the inflamed central nervous system (CNS) express high levels of the activation marker CD44, high levels of LFA-1 and are also positive for ICAM-1. In addition, CNS infiltrating lymphocytes are negative for L-selectin (Engelhardt et al., 1995; Engelhardt et al., 1998). In fact, once lymphocytes are activated, L-selectin is down regulated from the cell surface by shedding. L-selectin shedding occurs within minutes after lymphocytes activation (Jung et al., 1988) and this is due to the cleavage of the extracellular domain of L-selectin proximal to the cell membrane (ectodomain) (Kahn et al., 1994; Migaki et al., 1995; Preece et al., 1996; Stoddart et al., 1996). Several studies focused on L-selectin shedding in order to elucidate the mechanisms and the molecules involved. In 1997 a member of the A Disintegrin and Metalloprotease family, ADAM17 or TACE (TNF-α converting enzyme) was identified as responsible for L-selectin shedding in mouse thymocytes upon PMA (Phorbol Myristate Acetate) stimulation (Peschon et al., 1998). *In vitro*, a role for calmodulin in L-selectin shedding was demonstrated. Calmodulin binds the cytoplasmic tail of CD62L and is released when the intracellular Ca^{2+} levels increase after cell activation. Loss of calmodulin determines a conformational change in L-selectin and allows the cleavage molecules to access the ectodomain (Kahn et al., 1998; Fors et al., 2001; Matala et al., 2001). The function of L-selectin shedding is controversial. In fact it was shown that inhibition of shedding has no effect in blocking neutrophil adhesion and transmigration under flow (Allport et al., 1997), but another study revealed that blocking L-selectin cleavage on lymphocytes results in an increased integrin activation and in a slower rolling velocity (Hafezi-Moghadam et al., 2001).

I.2. The multi-step cascade for lymphocyte extravasation

Lymphocytes selectively recognize and adhere to the endothelium in different part of the body (Picker et al., 1993). It was shown that HEV are responsible for lymphocyte recruitment and extravasation from the blood into peripheral lymph nodes under physiological conditions (Streeter et al., 1988; Weisz-Carrington et al., 1991). The discovery of adhesion molecules and receptors lead to the conclusion, in the 1980s, that there are tissue-specific molecules on the cell surface and lymphocyte migrate in a distinct manner in different tissues (Butcher, 1986). Further investigations clarified that lymphocyte extravasation is a controlled multistep process, in which adhesion molecules and their receptors actively participate (Butcher, 1991; Shimizu et al., 1996; Springer, 1994). The multi-step cascade for lymphocyte migration into lymph nodes consists of four different steps (Figure I.2.1):

- *Tethering and rolling* of the lymphocytes along HEV
- *Activation* of lymphocyte integrins upon chemokine interaction with their G-protein coupled receptors
- *Firm adhesion* mediated by integrins
- *Transmigration* of activated lymphocytes through the endothelium

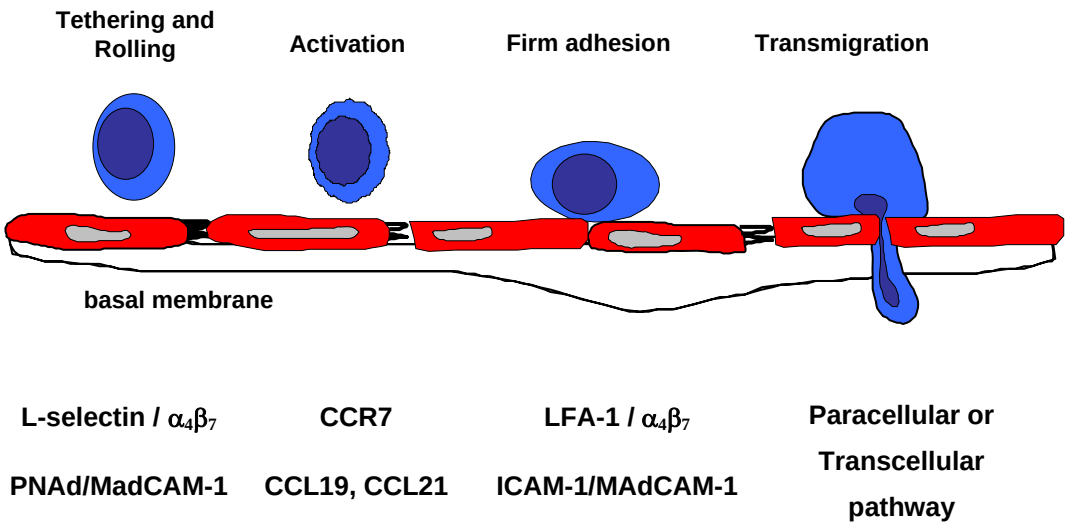

Figure I.2.1. The multi-step cascade at peripheral lymph nodes. Circulating lymphocytes bind via L-selectin or $\alpha_4\beta_7$ integrin their receptors on HEV and reduce their velocity (tethering and rolling). Integrins activation is followed by firm adhesion of lymphocytes to the endothelium and subsequent transmigration across the endothelium.

Tethering and rolling. Tethering and rolling are transient and reversible adhesive contacts between lymphocytes and the endothelium. In PLN tethering and rolling is mediated by the selectin protein L-selectin and PSGL-1, in Payer's patches by $\alpha_4\beta_7$ integrin and MAdCAM-1 (see section I.1).

Activation. Once the cells decrease their velocity and are rolling on HEV, the chemokines CCL21 on the high endothelial cells binds its serpentine receptor CCR7 expressed on lymphocytes. This interaction starts an inside-out signalling and activates integrins on the lymphocyte surface. Integrins are transmembrane glycoproteins responsible for firm attachment to the endothelium. Structurally, integrins are heterodimers composed of an α-chain of about 120-170kDa and a β-chain of 90-100kDa. The following integrins are directly involved in lymphocyte extravasation:

- $\alpha_4\beta_1$ integrin (VLA-4), which is expressed on lymphocytes, eosinophils and monocytes;
- $\alpha_4\beta_7$, which is expressed on lymphocytes and mediates, via MAdCAM-1 interaction, rolling and adhesion of leukocytes to the mucosal tissues;
- LFA-1 (lymphocyte function-associated antigen-1) which is expressed on lymphocytes and monocytes;
- Mac1 (integrin alpha-M), which is exclusively on granulocytes and monocytes.

The adhesion of lymphocytes to the endothelium is mediated by integrins. Upon activation integrins need to change their conformation and form clusters to increase affinity and avidity to their specific ligands (Takagi et al., 2002; Hogg et al., 2003; Hogg et al., 2004).

Firm adhesion. LFA-1 mediates firm adhesion of lymphocytes to HEV by binding to ICAM-1 (Dustin et al., 1998; Von Andrian et al., 1998). I*n vitro* and *in vivo*, it was demonstrated that blocking LFA-1 with antibodies leads to an impairment in lymphocyte homing to secondary lymph nodes (PLN and Peyer's patches). The importance of LFA-1 in lymphocyte homing to PLN was also demonstrated using LFA-1-deficient mice, where in the absence of LFA-1, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins mediate, via VCAM-1 interaction, the lymphocyte migration through HEV (Berlin-Rufenach et al., 1999).

Transmigration. After firm adhesion, lymphocytes are ready to migrate across the endothelium and cells are driven by chemotactic agents into the lymph nodes. The

molecules involved in lymphocyte transmigration at HEV are LFA-1 and ICAM-1. The transmigrating lymphocytes cross the endothelium via a paracellular pathway, by which cells migrate through the endothelial junctions, or via a transcellular pathway, by which cells migrate directly through the endothelium leaving the cell junctions intact.

I.3. The blood-brain barrier

The blood brain barrier (BBB) is a highly specialized network of tightly connected endothelial cells that regulates the flow of cells, nutrients and fluids between the blood and the central nervous system. The concept of the BBB was postulated during the 20th century. Paul Ehrlich injected some water-soluble dyes intravenously and he detected them in all organs but not in the central nervous system (CNS). He did not consider that the brain endothelium could be a selective barrier and he concluded that the CNS had no affinity for the dyes (Ehrlich, 1904). After a few years, his associate Edwin Goldmann repeated the same experiments, but this time the dyes were injected directly into the CNS. The dyes stained the CNS and not the other organs, and Goldmann concluded that there is a structure within the CNS that is both blood-brain and brain-blood barrier (Goldmann, 1913). Only in 1967 Reese and Karnovsky and in 1969 Brightman and Reese were able to describe the components of the BBB. A horseradish peroxidase tracer was injected into the CNS, or into the blood circulation, and the dye was seen to be blocked at the endothelial tight junctions (TJ) of the BBB. The tight junctions consist of transmembrane proteins that link together neighbouring endothelial and epithelial cells. The tight junctions are responsible for some of the unique characteristics of the barrier such as the high electrical resistance of the barrier (1500-2000$\Omega \cdot cm^2$) (Crone and Olesen, 1982). The first protein of the tight junctions identified was occludin (Furuse et al., 1993). Occludin is an important, but not essential, component of the endothelial tight junctions as demonstrated by occludin-deficient mice, which were able to develop fully functional tight junctions (Saitou et al., 2000). Essential for the BBB development are claudins (Tsukita and Furuse, 1999). The claudin family consists of more than 20 members. Claudin-5, claudin-12 and claudin-3, which is lost during inflammation (Nitta et al., 2003; Wolburg et al., 2003), were shown to be expressed at the tight junction level on endothelial cells in the CNS (Wolburg and Lippoldt, 2002; Wolburg et al., 2003). In

addition, the junctional adhesion molecules JAM (Martin-Padura, 1998) and the endothelial cell-selective adhesion molecule ESAM (Nasdala et al., 2002) were shown to be localized at TJ. At the endothelial cell contacts CD99 was detected, a new molecule that participates in the migration of lymphocytes to the site of inflammation *in vivo* (Bixel et al., 2004) and of monocytes *in vitro* (Schenkel et al., 2002). PECAM-1 (platelet endothelial cell adhesion molecule-1) is essential for the leukocyte extravasation *in vivo* (Graesser et al., 2002). The endothelial cells are the major cell population at the BBB, but the regulation and the functionality of the barrier depends on its interaction with astrocytes and pericytes. Astrocytes are cells interacting with the endothelium during development and maturation of the BBB. Pericytes are the cells that surround the brain capillaries and play an active role in immunity in the brain (Thomas, 1999). Both cells types were used during the last years to develop *in vitro* BBB models, which are useful instruments to study molecules and cell types playing a role in lymphocyte trafficking across the endothelium. These models were able to reproduce only some of the *in vivo* BBB properties. Recently, a model that retains most of the BBB characteristics has been established, showing high electrical resistance, permeability, absence of MECA-32 expression and up-regulation of cell adhesion molecule ICAM-1 and VCAM-1 upon inflammatory stimuli (Coisne et al., 2005).

I.4. Multiple sclerosis and experimental autoimmune encephalomyelitis

The central nervous system has three peculiarities. First, the blood-brain barrier, with its specialized endothelial cells and tight junctions, which clearly separates the CNS parenchyma from the blood and prevents blood components from entering the CNS. Second, the CNS lacks lymphatic vessels. Third, the major histocompatibility complexes (MHC) molecules on parenchymal cells, the antigen presenting cells (APC) and the dendritic cells (DC) are absent. Because of these peculiarities, the CNS was for long time considered to be an immunoprivileged organ where immunosurveillance is completely absent. At present, the CNS is considered to be an immune specialized organ where immune cell migration is tightly controlled and regulated (Engelhardt and Ransohoff, 2005). Sir Peter Medawar was the first person to observe the peculiarity of the CNS. He performed tissue transplantations and noticed that tissues implanted into the CNS were

rejected after a longer time period compared to the tissues transplanted into other organs (Medawar, 1948). Physiologically, the lymphocytes migrating across the BBB into the CNS are low in number, but when the integrity of the BBB is disrupted, a massive number of cells enters the brain and the spinal cord, starts to damage the CNS and induces inflammation. In humans, the disruption of the integrity of the BBB promotes the development of multiple sclerosis (MS). MS is a chronic inflammatory progressive disease of the central nervous system. It is more common in women than in men and the mean onset of disease is between 20-40 years of age. MS is characterized by damage of the myelin, the sheath surrounding the nerve fibres in the CNS. Its aetiology is still unknown, but a malfunction of the immune system seems to trigger the disease, as well as genetic factors and virus infections in childhood (Sibley, 1985; Poser, 1994). MS symptoms are tremor, paralysis and disturbances in coordination, vision and speech. Depending on course of the disease, multiple sclerosis is classified in relapsing remitting, progressive remitting, primary progressive and secondary progressive which is the most severe form of MS. Within the last decades, many investigations have focused on possible therapies to modify the course of the disease, exacerbations and to enhance the quality of life for patients suffering of MS. Based on findings obtained from animal models for MS, several treatments to modify the course of the disease and to reduce the exacerbations, such as corticosteroids and IFN-β, have been proposed. The animal models used to investigate MS, like experimental autoimmune encephalomyelitis (EAE), provide important information for a better understanding of the disease, the cell types and the molecules involved and MS progression. EAE has some characteristics resembling MS, for example paralysis, demyelination, weakness and incontinence. EAE is an autoimmune disease in which inflammatory cells accumulate within the CNS in perivascular spaces and form inflammatory cuffs (Levine, 1974). EAE can be induced only in susceptible species, such as mice and rats, by active immunization (aEAE) or by passive transfer of encephalytogenic T cells (tEAE). The first description of EAE was made by Louis Pasteur. In order to develop vaccines against rabies, Pasteur injected rabies virus particles from rabbit brains. This vaccination induced neuroparalysis, demyelination and death in some of the human recipients who were given the vaccination (Pasteur, 1885). The vaccination was modified by Thomas Rivers, who induced neurological reactions and immune response in monkeys following multiple injection of brain emulsion of rabbits. He concluded that the brain tissue injected, and not the rabies virus, has the capacity to trigger CNS disease, demyelination and immune cell infiltration

into the CNS (Rivers et al., 1933). Later on Kabat improved the protocol established by Thomas Rivers. Kabat in fact was the first scientist to inject an emulsion of brain and complete Freund's adjuvant in monkeys (Kabat et al., 1947). Further studies demonstrated that spinal cord homogenate contains encephalitogenic myelin components like MBP (myelin basic protein), MOG (myelin oligodendrocyte glycoprotein) and PLP (proteolipid protein), which are able to induce active EAE (aEAE) in susceptible recipients (Laatsch et al., 1962; Zamvil et al., 1985; Zamvil et al., 1986; Tuohy et al., 1989). In 1960, it was demonstrated in rats that T cells can invade the CNS and transfer experimental autoimmune encephalomyelitis (tEAE) (Paterson, 1960). Only in 1982, the first stable rat T cell line was established and tEAE was induced in syngenic animals (Ben-Nun et al., 1982; Ben-Nun et al, 1983). Later on, further investigations demonstrated that only freshly activated encephalitogenic T lymphocytes can enter the CNS and invade the parenchyma inducing EAE in recipient animals (Wekerle et al., 1994).

The lymphocytes entering into the inflamed CNS parenchyma follow a four step cascade which resembles the situation at PLN. Encephalitogenic T cells migrating across the BBB do not present L-selectin. On the endothelium, E- and P-selectin were demonstrated to be absent during inflammation *in vivo* (Engelhardt et al., 1997). Nevertheless, *in vitro* it could be shown that, upon the induction of inflammation, brain capillary endothelial cells express E- and P-selectin on their surfaces (Coisne et al., 2006). In addition, circulating lymphocytes were observed to be captured, and not rolling on the endothelium, via the interaction between α_4-integrin and VCAM-1 by intravital microscopy at spinal cord (Vajkoczy et al., 2001). It was also shown that during CNS inflammation, chemokines and G-protein mediated signals are required to promote T cell adhesion on the BBB. In addition, it was demonstrated that the chemokines CCL19 and CCL21 on the endothelium are involved in T cell adhesion to the inflamed BBB. In fact, by means of in situ hybridization, it was demonstrated that CCL19 and CC21 are present on the vessels surrounded by inflammatory cells (Alt et al., 2002). The molecules mainly involved in lymphocyte transmigration are ICAM-1 and VCAM-1. Antibodies against ICAM-1 were able to reduce inflammation in many animal models (Carlos et al., 1994) and the cytoplasmic tail of ICAM-1 was demonstrated to be essential for the lymphocyte transmigration *in vitro* (Greenwood et al., 2003; Lyck et al., 2003). There are two theories regarding the pathway used by the lymphocytes to transmigrate. On the one side there is evidence supporting the paracellular pathway, by which cells migrate through the

endothelial junctions. If cells use this pathway to cross the endothelium, a rearrangement of the junction proteins and their ligands is required. On the other hand, there is also evidence for the transcellular pathway, by which cells migrate through the endothelium leaving the tight junctions intact (Engelhardt and Wolburg., 2004). Electron microscopy studies on murine inflamed CNS sections demonstrated that the endothelial cells extend some protrusions on the luminal site and embrace the migrating cell. The abluminal site of the protrusion is then surrounded with basal lamina and the cell membrane changes its characteristics (Wolburg et al., 2005). At the end of the migration, the cell is released on the abluminal site of the endothelium and can invade the CNS parenchyma.

The molecules involved in the multistep cascade for lymphocyte migration are not yet completely identified. In fact focusing on all the steps here described, there still many investigations are required to identify possible new adhesion molecules involved in the diapedesis of lymphocytes across the endothelium. At the same time, there are still many investigations which focus on already known molecules, to better characterize their role in the transmigration of lymphocytes through the endothelium.

AIM OF THE STUDY

The aim of this study was to investigate the role of L-selectin in the recruitment of inflammatory cells across the blood-brain barrier (BBB) and in the immunopathogenesis of experimental autoimmune encephalomyelitis (EAE). To evaluate the role of L-selectin in EAE in SJL mice, SJL/L-selectin-deficient mice were generated by backcrossing the original L-selectin-deficient strain into the EAE susceptible SJL background and the phenotype of SJL/L-sel$^{-/-}$ mice was investigated. To study the role of L-selectin during the recruitment of inflammatory cells into the CNS, EAE was induced in SJL wt and SJL/L-sel$^{-/-}$ mice by active immunization (aEAE) with a single injection of proteolipid protein (PLP aa139-151) emulsified in complete Freund's adjuvant (CFA). To test the ability of SJL/L-selectin-deficient and SJL wild type PLP-specific T cells to transfer the disease in syngenic recipients, EAE was induced by passive transfer of *in vitro* activated T cell blasts (tEAE). The presence of CD45-positive inflammatory cells within the CNS was detected by immunohistochemistry.

To characterize the phenotype of SJL wild type and SJL/L-sel$^{-/-}$ naïve and effector/memory T cells, the presence of cell surface molecules such as integrins involved in lymphocyte adhesion and transmigration, and PSGL-1, which is involved in lymphocyte rolling, were investigated by flow cytometry. In addition, the presence of cytokine receptors (IL-2 receptor) and the presence of costimulatory molecules (CD28, CD152 and CD154), which are involved in cell proliferation, were investigated and were compared in SJL wt versus SJL/L-selectin-deficient T cells.

Finally, the migration efficiency of SJL/L-selectin-deficient T cells across an endothelium was investigated in a functional *in vitro* assay (Trans-Endothelial Migration) and compared to SJL wild type T cells.

II. MATERIALS AND METHODS

II.1.Devices and materials

Here is a list of devices and materials used.

Devices

β-scintillation counter	Beckman-Coulter; Krefeld, Germany
Cell Harvester	Inotech; Dottikon, Switzerland
CO_2 Incubator IR Autoflow	Nuaire; Fermald, Switzerland
Digital Camera DMX1200F	Nikon; Egg, Switzerland
Gel chamber Sub-Cell GT	Bio-Rad; Munich, Germany
Heating Block Thermomixer	Eppendorf; Hamburg, Germany
Laminar flow cabinet	Nuaire; Fermald, Switzerland
Magnetic stirrer	IKA-WERKE; Staufen, Germany
MasterCycler	Eppendorf; Hamburg, Germany
Microscope Axiovert 25	Zeiss; Feldbach, Switzerland
Microscope Eclipse E600	Nikon; Egg, Switzerland
Microtome HM 550 OM	Microm; Walldorf, Germany
Multiple pipet	Eppendorf; Hamburg, Germany
Nikon microscope and camera	Nikon; Egg, Switzerland
Pipetboy acu	Integra Bioscience; Fernwald, Germany
PCR reaction machine	Eppendorf; Hamburg Germany
Vortex Genie	Scientific Industries; Bohemia, NJ, USA
Water bath	Julabo; Seebach, Germany

Expendable materials

Cell culture vented cap flasks	Greiner bio-one; Nürtingen, Germany
Cell culture Petri dishes	BD Falcon, Heidelberg, Germany
15ml , 50ml polypropylene tubes	BD Falcon; Heidelberg, Germany
0.22µm pore size 45mm diameter filters	Millipore; Volketswil, Switzerland
0.22µm pore size syringe filters	Pall; Dreiech, Germany

Reaction tubes 1.5ml, 2ml	Eppendorf; Hamburg, Germany
Pipette tips	Greiner bio-one; Nürtingen, Germany
Cryotubes 1.5ml	Greiner bio-one; Nürtingen, Germany
10µl, 200µl, 1000µl pipettes	Gilson; Middleton, WI, USA
PCR reaction tubes	Greiner bio-one; Nürtingen, Germany

Serum

Foetal bovine serum	PAN Biotech; Aidenbach, Germany
Foetal bovine serum	BioWest; Nuaillé, France
Newborn calf serum	Gibco; Karlsruhe, Gemany
Calf serum	PAA; Cölbe, Germany
Normal mouse serum	Serotec; Düsseldorf, Germany
Normal rat serum	Serotec; Düsseldorf, Germany
Goat serum	Serotec; Düsseldorf, Germany
Rabbit serum	Serotec; Düsseldorf, Germany

Cell culture media

RPMI 1640, DMEM (Dulbecco's Modified Eagle Medium) 4500g/l glucose, HBSS (Hanks' Balanced Salt Solution) and PBS (phosphate buffer solution) were purchased from Invitrogen (Basel, Switzerland)

Cell culture media supplements

HEPES, L-glutamine, Penicillin/Streptomycin, non-essential amino acids, Na-Pyruvate, Concanavalin A, Trypan blue 0.4% and Trypsin/EDTA solution were purchased from Invitrogen (Basel, Switzerland)

II.2. Tissue culture media

All media, sera and supplements used were filtered using a 0.22µm pore size sterile filter (Millipore AG, Volketswil, Switzerland). Cell lines were maintained at 37°C, 5-10% CO_2 and 100% humidity in sterile conditions in CO_2 incubators (Nuaire, Fermwald, Switzerland). Cells were cultivated in sterile Petri dishes (BD Falcon; Heidelberg, Germany) or vented-cap flasks (Greiner bio-one; Nürtingen, Germany).

Preparation of culture media for T cell lines: IL-2 containing TCGF medium and Restimulation medium

T cells were cultured in T cell growth factor (TCGF) medium, which is composed by RPMI 1640 supplemented with 10% foetal serum (PAN Biotech), 4mM L-Glutamine, 1mM Na-Pyruvate, 100U/ml Penicillin, 100µg/ml Streptomicyn, 1% MEM non-essential amino acids (100x), 0.05mM β-Mercaptoethanol and Concanavalin A supernatant.

T cells were restimulated in RPMI 1640 supplemented with 10% foetal serum, 4mM L-Glutamine, 1mM Na-Pyruvate, 100U/ml Penicillin, 100µg/ml Streptomycin, 1% MEM non-essential amino acids (100x), 0.05mM β-mercaptoethanol (Restimulation medium).

Preparation of endothelioma medium

Endothelioma cells (bEnd.5) were cultured in DMEM supplemented with 10% foetal bovine serum (BioWest), 4mM L-Glutamine, 1mM Na-pyruvate, 100U/ml Penicillin, 100µg/ml Streptomycin, 1% MEM non-essential amino acids (100x), 0.05mM β-Mercaptoethanol.

Preparation of migration assay medium (MAM)

Transmigration and adhesion assay were performed in DMEM supplemented with 5% calf serum, 4mM L-Glutamine and 25mM HEPES

Preparation of washing buffer

Complete washing buffer was prepared with 10% FCS and 25mM HEPES in HBSS. Incomplete buffer, used during density gradient and ConcanavalinA supernatant preparation, was supplemented only with 25mM HEPES

Preparation of Concanavalin- A medium

Splenocytes of C3H mice (Harlan Nederlands; Horst, Netherland) were homogenized, resuspended and cultured overnight in DMEM low-glucose supplemented with 1% normal mouse serum (Serotec), 4mM L-Glutamine, 1mM Na-Pyruvate, 100U/mL Penicillin, 100µg/mL Streptomyicin and 1% MEM non-essential amino acids (100x) and 5g Concanavalin A powder (Sigma Aldrich; Basel, Switzerland).

II.3. T cell lines

Here are the protocols regarding T cell lines: preparation of primary $PLP^{139-151}$ specific CD4-positive T cell lines, isolation of PLP-specific blasts, restimulation of resting cells using their respective antigen, freezing and thawing. Cells were cultured in sterile conditions in Nuaire incubators at 37°C, 7% CO_2, 100% humidity in Petri dishes (PD).

Preparation of primary $PLP^{139-151}$ specific T cell lines

PLP-specific T cell lines were obtained from SJL wild-type (Taconic; Ry, Denmark) and SJL/L-selectin-deficient mice bred in our own animal facility according to the requirements of the local government. Mice were immunized with 50µg/ml of $PLP^{139-151}$ (H_2N-H-C-L-G-K-W-L-G-H-P-D-K-F-COOH) (ThermoHybaid; Ulm, Germany) dissolved in PBS and emulsified in complete Freund's adjuvant (CFA; Santa Cruz Biotechnology; Santa Cruz, CA, USA). CFA was prepared adding to the incomplete Freund's adjuvant 4mg/ml heat-inactivated *Mycobacterium tuberculosis*

micro-organisms. $PLP^{139-151}$ and PBS and CFA were mixed and 100µl of this emulsion were subcutaneously injected in flanks and tail root of anaesthetized mice. At 10-14 days post immunization, mice were sacrificed by cervical dislocation and their spleens were dissected in sterile conditions. Spleens were cut into small pieces, suspended in complete washing buffer and homogenized with a pestle (Wheaton; Millville, NJ, USA). Cell suspension was then filtered through a 100µm pore size nylon mesh and centrifuged (RT, 1200rpm, 10 min). The supernatant was removed, the pellet was filtered once again and the cells were counted. This assay was performed in 60mm PD containing 5ml of Restimulation medium supplemented with 10µg/ml $PLP^{139-151}$ antigen. After three days, 1ml TCGF medium was added to the culture and after 16 hours T cell blasts were isolated by density gradient and cultured in IL-2 containing medium (TCGF).

- Immunization of mice using an emulsion of CFA, PBS and $PLP^{139-151}$
- After 10-14 days, mice are sacrificed and spleens are dissected in sterile conditions
- Spleens are homogenized, filtered and splenocytes are counted
- $5 \cdot 10^7$ splenocytes are plated in 60mm PD in 5ml Restimulation medium containing 10µg/ml $PLP^{139-151}$ antigen
- After 3 days, 1mL TCGF medium is added to the culture
- After 16h, activated blasts are isolated by density gradient

Table II.3.1. Preparation of primary PLP-specific T cell lines.

T cell blasts isolation by density gradient

T cell blasts, highly activated against their specific antigen $PLP^{139-151}$, were isolated by density gradient and separated from debris and unspecific cells. Cells from two 60mm PD were collected, washed with complete washing buffer and centrifuged (RT, 1200rpm, 10 min). Pellet was resuspended in 3ml incomplete washing buffer (HBSS and 25mM HEPES) and this suspension was placed carefully on the top of 5ml NycoPrep 1.077A solution (Axis-Shield; Oslo, Norway), at room temperature, in a 15ml polystyrene tube (BD Falcon; Heidelberg, Germany) and centrifuged (1300rpm, 20min, without brake). At the end of centrifugation, T cell blasts formed a layer between the supernatant and NycoPrep solution. Blasts were collected and

washed 3 times in complete washing buffer, thus counted and plated (2-3·10^6 cells / 100mm PD in 10ml TCGF medium) or used for experiments.

- Cells from two 60mm PD are collected and centrifuged
- Pellet is resuspended in 3ml washing buffer and cells are added on the top of 5ml NycoPrep 1.077A solution at RT in 15ml polypropylene tubes
- After centrifugation, T cell blasts are collected and washed three times with washing buffer
- Cells are then counted and plated in 100mm PD or used for experiments

Table II.3.2. T cell blasts isolation by density gradient.

Restimulation of PLP-specific T cell lines

T cells were cultured in TCGF medium for 5-8 days and checked daily under a microscope to evaluate their status. Freshly activated blasts were large and exhibited a tennis racket shape, resting cells were small and round. When cells reached the resting phase, they had to be restimulated in order to maintain them in culture and to keep them available for further use. To restimulate PLP-specific T cell lines it is necessary to co-culture those cells together with splenocytes and $PLP^{139-151}$ antigen. Splenocytes were used as antigen presenting cells (APC) and came from 8-12 weeks old females of SJL mice. Mice were sacrificed by cervical dislocation, spleens were dissected, homogenized, filtered and centrifuged (RT, 1200rpm, 10 min). After centrifugation, pellet was resuspended in complete washing buffer and cells were irradiated (45Gy at University Hospital Münster, Germany and University Hospital InselSpital, Bern, Switzerland) to obtain a suspension of living but not replicating cells. Therefore, splenocytes were filtered again through a nylon mesh (100µm pore size), centrifuged, counted and plated in a 60mm PD, together with 1.5-2·10^6 resting lymphocytes in 5ml Restimulation medium containing 10µg/ml $PLP^{139-151}$ antigen. After two days, 1ml TCGF medium was added to the culture and after 16 hours activated T cell blasts were separated by density gradient.

- 8-12 weeks old syngenic mice are sacrificed and spleens are dissected under sterile conditions
- Spleens are homogenized, filtered and sublethally irradiated
- Splenocytes are filtered again, centrifuged and counted
- $4 \cdot 10^7$ splenocytes are plated together with $1.5\text{-}2 \cdot 10^6$ resting lymphocytes in 5ml Restimulation medium containing 10µg/ml $PLP^{139\text{-}151}$ antigen
- After 2 days, 1ml TCGF is added to the culture
- After 16h, T cell blasts are separated by density gradient

Table II.3.3. Restimulation of PLP-specific T cell lines.

Antigen specificity assay

To detect the specificity of T cells for their specific antigen $PLP^{139\text{-}151}$, in parallel with restimulation a specificity assay was performed. In each well of a 96 well-plate U shaped bottom (Greiner bio-one), $2 \cdot 10^4$ resting lymphocytes were co-cultured with $5 \cdot 10^5$ irradiated splenocytes. To the cell medium were supplemented the specific antigen $PLP^{139\text{-}151}$ (10µg/ml), the unspecific antigen purified protein derivate (PPD) from *Mycobacterium tuberculosis* (10µg/ml), the mitogenic factor Concanavalin A (2.5µg/ml) as positive control and no antigen as negative control. Samples were plated in triplicate. After two days, 1µCi ^{3}H-thymidin (^{3}H-dT) (Amersham Pharmacia, Otelfingen, Switzerland) was added to the culture and after 16 hours the specificity of T cell lines was determined by measuring the incorporation of the radioactive tracer ^{3}H-thymidine. Cells were aspirated by a cell harvester machine (Inotech; Dottikon, Switzerland), locked in a glass fibre filter (Inotech; Dottikon, Switzerland) and after that lysed with ddH_2O. The radioactivity incorporated in proliferating cells was then measured by a β-scintillation counter (Beckman-Coulter).

- $2 \cdot 10^4$ resting lymphocytes are co-cultured, in triplicates, with $5 \cdot 10^5$ irradiated splenocytes in a 96 well-plate U shaped bottom
- $PLP^{139\text{-}151}$ (10µg/ml), the unspecific antigen purified protein derivate (PPD) (10µg/ml), the mitogenic factor Concanavalin A (2.5µg/ml) as positive control and no antigen are added to the cells in culture
- After 2 days, 1µCi ^{3}H-thymidin is added and 16h after that cells are aspirated by a cell harvester machine and release radioactivity in a glass fibre filter
- Proliferation and specificity are measured by a beta-counter machine

Table II.3.4. Antigen specificity assay

Freezing and thawing of T cells

In order to freeze T cells, they were collected from 2-5 100mm PD, centrifuged (RT, 1200rpm, 10min) and suspended in 1.5ml freezing medium composed of RPMI 1640, 50% FCS and 10% DMSO. The cell suspension was placed in cryotubes (Greiner bio-one) and slowly cooled down to -80°C. After 2-3 days, cells were transferred into a liquid nitrogen tank. To thaw cells, T lymphocytes were rapidly taken at RT, resuspended in complete washing buffer, centrifuged and plated in culture dishes in TCGF medium or used for restimulation assays.

Freezing of T cells	**Thawing of T cells**
• Cells are collected and centrifuged • Pellet is resuspended in 1.5ml freezing medium • Cells are pipetted into cryotubes • Cells are frozen at -80°C • After 2 days, cells are transferred into a liquid nitrogen tank	• Cells are thawed at 37° • Cells are resuspended in washing buffer and centrifuged • Pellet is resuspended in medium

Table II.3.5. Freezing and thawing of T cell lines

IL-2 containing Concanavalin A supernatant

To prepare IL-2 containing Concanavalin A supernatant (ConAS), spleens from 8-10 week old C3H mice (Harlan Nederlands; Horst, Nederland) were dissected and homogenized, then filtered through 100µm nylon mesh and centrifuged (RT, 1200rpm, 10min). Pellet was resuspended in ConcanavalinA medium and 10^7 cells/ml were plated in 10ml in 100mm PD and incubated 24h at 37°C and 7% CO_2. After incubation, supernatant was collected and aliquots of 45ml were stored at -20°C in sterile tubes. To determine the appropriate concentration of ConAS within the TCGF medium, $1 \cdot 10^4$ cells/100µl per well were plated in triplicates in a 96 well-plate U-shaped bottom. The proliferation of those cells in presence of different ConAS dilutions (50%, 25%, 12.5%, 6.25%, 3.125%, no Concanavalin A supernatant) was assessed by ^{3}H-dT incorporation: cells were incubated and after 3 days 1µCi of ^{3}H-thymidine was added. 16h after that, cells were harvested and radioactivity incorporation was measured with a β-scintillation counter.

Preparation of Concanavalin A supernatant (ConAS)

- Spleens were dissected from C3H mice, homogenized, filtered and centrifuged
- Pellet was resuspended in Concanavalin A medium and 10^7cells/ml plated in PD
- Cells were incubated 24h
- Supernatant was collected and 45ml aliquots stored at -20°C

ConAS test

- $1 \cdot 10^4$cells/100µl were plated in triplicates in a 96 well-plate U-shaped bottom
- Dilutions of 50%, 25%, 12.5%, 6.25%, 3.125% and no ConAS were added to the culture
- After 3 days, 1µCi of ^{3}H-thymidine was added
- After 16h, cells were harvested and proliferation was measured to determine which concentration ConAS should be used to prepare TCGF medium

Table II.3.6. IL-2 containing Concanavalin A supernatant

II.4. Animal models

To investigate the role of L-selectin in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), it was necessary to induce the disease in SJL wild-type and SJL/L-selectin-deficient mice. Here are presented the protocols used to induce active (aEAE) or passively transfer (tEAE) experimental autoimmune encephalomyelitis.

Breeding and backcrossing of L-selectin-deficient mice into SJL background

L-selectin-deficient mice were generated (Catalina et al., 1996) and provided by Prof.Dr. Siegelman (Southwestern Medical Centre, University of Texas – USA). Mice were backcrossed into the SJL background because this strain is susceptible to experimental autoimmune encephalomyelitis. The first generation was obtained breeding L-selectin-deficient mice and SJL wild type mice. The generated

heterozygous mice were then backcrossed with wild type SJL animals until generation F8. At this time, heterozygous mice were bred to generate SJL/L-selectin-deficient mice. Mice between backcrossing generation F8 and F18 were used during this study. The genotype of the animals was detected by PCR (see section II.8).

Active experimental autoimmune encephalomyelitis (aEAE)

As has already been described (Engelhardt 1997 and Engelhardt and Laschinger 1998), anaesthetised mice were immunized by injecting subcutaneous in the tail root and flanks - 50µg/ml $PLP^{139-151}$ emulsified in PBS and CFA. At day 1 and 3 post immunization, $3 \cdot 10^9$ *Bordetella pertussis* heat-inactivated organisms were injected intravenously in the tail. Daily, weight and clinical score were controlled in order to monitor signs and ongoing of disease. Disease started after around 10 days and clinical symptoms were scored as:

0	healthy
0.5	limp tail
1	hind leg weakness
2	hind leg paralysis
3	hind leg paraparesis and incontinence
4	death

Figure II.4.1. Healthy SJL mouse, clinical score 0. EAE symptoms are dot detectable.

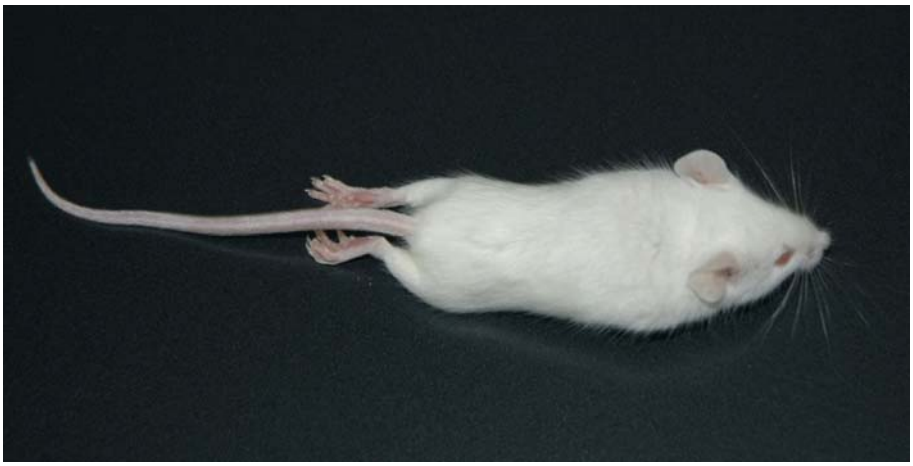

Figure II.4.2. EAE affected SJL mouse, clinical score 2, hind leg paralysis.

Passive transfer of experimental autoimmune encephalomyelitis (tEAE)

Freshly activated PLP-specific T cell blasts were injected in syngenic mice to test their encephalitogenicity. $3 \cdot 10^6$ blasts of established T cell lines or $2 \cdot 10^7$ primary blasts were diluted in PBS and injected intravenously in 8-12 week old mice. The disease started 7-14 days after injection and clinical symptoms were scored as for aEAE.

Animal perfusion and tissue freezing

To evaluate whether injected blasts were encephalitogenic and if CD45 positive cells infiltrated the CNS, tissues from EAE affected mice and healthy control animals were prepared and snap frozen. Mice were first anaesthetised with isoflurane (CuraMed; Karlsruhe, Germany) and then perfused with freshly prepared and cold 1% (w/v) PFA/PBS to remove blood and fix infiltrating cells. At this time brain and spinal cord were dissected, cut in pieces and snap frozen in Tissue-Tek O.C.T. compound (Sakura Finetek; Giessen, Germany) in cryomolds (Miles Inc.; Giessen, Germany). The cryomolds were frozen in a solution of 2Methyl-butan and dry ice and afterwards stored at -80°C. To prepare cryosections of the CNS, tissues were removed from their location at -80°C and placed at -20°C for around 30min before mounting them on a blockholder in a microtome (Microm). 6µm tissue sections were cut and mounted on silanized slides (Vogel; Giessen, Germany). Samples were dried overnight at RT, fixed in acetone at -20°C for 10min and dried again at RT at least 30min. Slides were ready for analysis (see section II.7) or were frozen at -20°C in presence of silica gel (Carl Roth; Karlsruhe, Germany).

II.5. Endothelioma cell line

The brain endothelioma cell line bEnd5 was prepared from brain microvessels of BALB/c mice and immortalized using Polyoma middle-T (pymT) oncogene expression (Kiefer et al., 1994; Wagner and Risau, 1994). Because of their adhesion molecule expression pattern (Reiss et al., 1998), bEnd5 cells are considered to be a wild-type endothelial cell line.
Here are reported the protocols regarding culture and freezing and thawing of the endothelioma cell line bEnd5.

Culture of endothelioma cell line bEnd5

bEnd5 cells were cultured in vented cap flasks (Greiner Bio-One) at 37°C and 10% CO_2 in sterile conditions. Cells were fed twice per week and reached confluency in 4-5 days. Cells were split after 10 days by trypsinization. In order to passage them, cells first have to be washed 5min with PBS/5mM EDTA and then detached by incubating them at 37°C with 0.05% Trypsin/EDTA (Invitrogen). After 5min, cells are washed with washing buffer, collected and centrifuged (RT, 1200rpm, 10min) and then plated again in a 1:4 dilution or used for experiments.

- bEnd5 cells are washed with PBS/EDTA 5mM
- PBS is removed and cells are digested with 0.05% Trypsin/EDTA
- Cells are washed to remove Trypsin, collected and centrifuged
- Cells are plated with endothelioma medium in dilution 1:4 or used for experiments

Table II.5.1. Culture of bEnd5 cells

Freezing and thawing of bEnd.5 cell lines

Confluent cells were trypsinized as described above. Pellet was resuspended in freezing medium (DMEM, 30% FCS, 10% DMSO) and cooled down at -80°C in cryotubes. After 2-3 days cryotubes were moved into a liquid nitrogen tank. Cells were rapidly warmed up to thaw them and washed in washing buffer, centrifuged and plated in endothelioma medium.

Freezing of bEnd5 cells	Thawing of bEnd5 cells
• Confluent cells are washed with PBS/EDTA • Cells are digested with Trypsin/EDTA • Cells are resuspended in washing buffer and centrifuged • Pellet is resuspended in freezing medium • Cells are put in cryotubes and those were cooled down at -80°C • After 2-3 days, cryotubes are moved into a liquid nitrogen tank	• Cryotubes are rapidly warmed up at 37°C • Cells are resuspended in Washi buffer and centrifuged • Pellet is resuspended in endothelioma medium and cells plated in flasks or used for experiments

Table II.5.2. Freezing and thawing of bEnd5 cells

II.6. *In vitro* Trans-Endothelial Migration assay (TEM)

The trans-endothelial migration (TEM) assay is a highly reproducible *in vitro* assay that allows us to study leukocyte migration across an endothelium and molecules involved in this pathway. The protocol presented here has been extensively described in Leukocyte trafficking - molecular mechanisms, therapeutic targets and methods (Chapter 19, Alf Hamann and Britta Engelhardt Editors, Wiley-VCH).

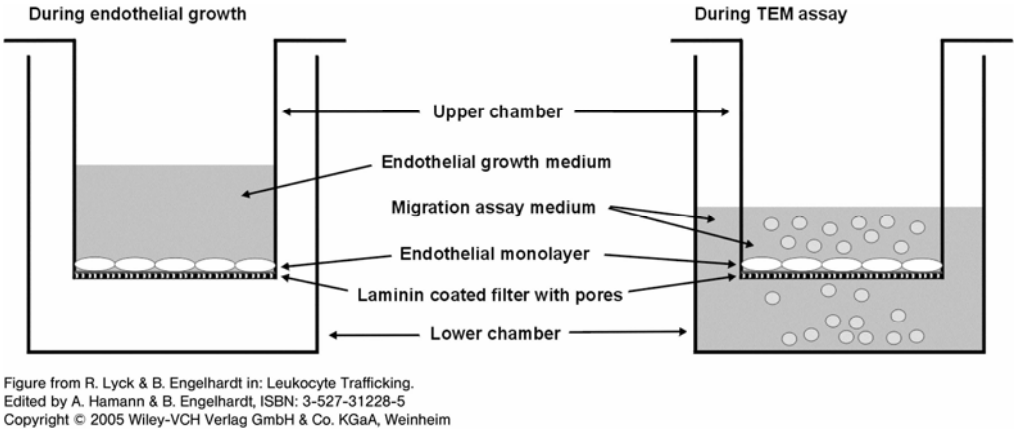

Figure II.6.1. Trans-Endothelial Migration

During the first day, endothelial cells (bEnd5) were plated on laminin coated Transwell® culture inserts of 6.5mm diameter and 5µm pore size (Costar; Cambridge, MA, USA). Filters were coated with 50µg/ml laminin (Roche; Basel, Switzerland) to permit the endothelial cells to form a confluent monolayer. 50µl laminin were incubated for 30min on each insert, then aspirated and dried 1-2h at RT under laminar flow. bEnd5 cells were trypsinized (see *Culture of endothelioma cell line bEnd5* section) and $5 \cdot 10^4$ cells were plated in 200µl of endothelial medium each filter. Medium was added only into the upper chamber to prevent non physiological sprout formation through the pores in the lower chamber. After 48h, bEnd5 cells were treated with 5nM TNF-α, a proinflammatory cytokine inducing breakdown of the monolayer, and incubated for 16h. The day after, filters were washed twice with 200µl migration assay medium -MAM- (DMEM, 5% calf serum, 4mM L-Glutamine and 25mM HEPES) and incubated with antibodies 30min. At the same time, also T cells were incubated at 37°C with antibodies in order to investigate molecules involved in transmigration of leukocytes across the endothelium. After the incubation time expires, cells were washed to remove excess of antibodies and 10^5 T cells in100µl MAM medium were pipetted in the upper chamber. 600µl MAM were added into the lower chamber and the assay was running for 3-4h in a humidified environment at 37°C and 10% CO_2. At the end of this incubation time, 500µl MAM and migrated cells from the lower chamber were added to 9.5m filtered CASY® cell counting buffer into CASY® tubes and analysed with CASY® cell counter (Schärfe System; Reutlingen, Germany). Data, in triplicates, were given as percentage of migrated cells (mean value and standard deviation). To asses the status of the endothelium, filters were washed in PBS, fixed in a formalin saturated chamber for 1-2h and stained with 5% (v/v in tap water) Giemsa's azure eosin methylene blue solution (Merck) for 10min. Dyes in excess was removed by aspiration and filters were dried overnight. The day after, filters were mounted with Entellan (Merck) on glass slides and checked at microscope. Migration values from damaged or not confluent monolayers were excluded from the assay.

Day 1

- Transwell® filters are coated with 50µg/ml laminin for 30min
- Laminin is aspirate and filters let dry at RT 1-2h
- $5 \cdot 10^4$ endothelial cells are plated in 200µl endothelial medium each insert

Day 2

- Endothelial cells are treated with 5nM TNF-α 16h

Day 3

- Endothelial and T cells are incubated 30min with antibodies
- Antibodies are washed away with MAM medium
- 10^5 T cells are pipetted into the upper chamber on the top of the bEnd.5 monolayer
- 600µl MAM are pipetted into the lower chamber
- Assay is started in a humidified incubator at 37°C and 10% CO_2
- After 3-4h, 500µl medium and migrated cells are collected from the lower chamber and pipetted into a CASY® tube containing 9.5ml CASY® cell counting buffer
- Samples are analyzed at CASY® cell counter and given as percentage of migrated cells across the endothelium
- Filters are washed in PBS, fixed with gaseous formalin 1-2h and stained with 5% Giemsa's azure solution 10min
- Filters are dried overnight

Day 4

- Filters are mounted with Entellan on glass slides and analysed at microscope
- Values coming from damaged or not confluent monolayers are excluded from the assay

Table II.6.1. Trans Endothelial Migration assay (TEM)

II.7. Immunofluorescence

Immunofluorescence techniques were applied to living cells or to frozen tissue sections to detect surface molecules expression (Flow cytometry) or to detect the presence of infiltrating cells into the CNS (Immunohistochemistry). The following protocols and antibodies were used.

Tissue culture supernatants

Tissue culture supernatants were prepared from rat anti-mouse hybridoma cells and used directly or as purified monoclonal antibodies. Supernatants were prepared in our laboratory as described in the PhD thesis of Dr.Melanie Laschinger (2002) and the presence of endotoxins was tested at the Fresenius Laboratories in Berlin (Germany).

Antibody	Antigen	Isotype	Source	Reference
9B5	Human CD44, negative control	IgG2a	E.Butcher, Stanford, CA, USA	Jalkanen et al., 1986
Mec 13.3	PECAM-1	IgG2a	ATCC, Rockville, MD, USA	Vecchi et al., 1994
Mel 14	L-selectin	IgG2a	E.Butcher, Stanford, CA, USA	Gallatin et al., 1983
GK 1.5	CD4	IgG2b	ATCC, Rockville, MD, USA	Wilde et al., 1983
M1/9	CD45	IgG	ATCC, Rockville, MD, USA	Springer et al., 1978
PS/2	α4 integrin	IgG2b	P.Kincade, Oklahoma City, OK, USA	Miyake et al., 1991
9EG7	β1 integrin	IgG2a	D.Vestweber, Münster, Germany	Lenter et al., 1993
DATK 32	α4β7 integrin	IgG2a	E.Butcher, Stanford, CA, USA	Andrew et al., 1994
Fib 504	β7 integrin	IgG2a	E.Butcher, Stanford, CA, USA	Andrew et al., 1994
FD441.8	LFA-1	IgG2b	ATCC, Rockville, MD, USA	ATCC, Rockville, MD, USA

25ZC7	ICAM-1	IgG2a	D.Vestweber, Münster, Germany	Steffen et al., 1996
30H12 FITC	Thy1.2	IgG2b	BD Pharmingen, Heidelberg, Germany	Ledbetter et al., 1979
B220 FITC	CD45R	IgG2a	BD Pharmingen, Heidelberg, Germany	Coffman et al., 1982
Gr-1 FITC	Ly6G and Ly6C	IgG2b	BD Pharmingen, Heidelberg, Germany	Malek et al., 1993
M1/70 FITC	CD11b	IgG2b	BD Pharmingen, Heidelberg, Germany	Springer et al., 1983
4RA10	PSGL-1	IgG1	D.Vestweber, Münster, Germany	Frenette et al., 2000
23G2	CD45RB	IgG2a	D.Vestweber, Münster, Germany	Lee et al., 1990
7D4	CD25	IgM	BD Pharmingen, Heidelberg, Germany	Malek et al., 1983
PC61	CD25	IgG1	BD Pharmingen, Heidelberg, Germany	Moreau et al., 1987
3C7	CD25	IgG2b	BD Pharmingen, Heidelberg, Germany	Ortega et al., 1984
TMβ1	CD122	IgG2b	BD Pharmingen, Heidelberg, Germany	Tanaka et al., 1991
5H4	CD122	IgG2a	BD Pharmingen, Heidelberg, Germany	Furse et al., 1993
TUGm2	CD132	IgG2b	BD Pharmingen, Heidelberg, Germany	Guesdon et al., 1979
4G3	CD132	IgG2a	BD Pharmingen, Heidelberg, Germany	Gillis et al., 1978
F4/80 FITC	F4/80	IgG2b	Serotec, Dusseldorf, Germany	Gordon et al., 1981

Table II.7.1. List of antibodies used to detect cell surface molecules

Flow cytometry

Flow cytometry was used to analyse the phenotype of T cells or naïve lymphocytes. Cell surface molecule expression, detected using antibodies (see Table II.7.1), was compared in different cell lines and at different stages of activation, as well as in primary naïve lymphocytes from different organs (spleen, peripheral lymph nodes PLN, mesenteric lymph nodes MLN) and different mouse strains (SJL wild-type, SJL/L-selectin-deficient and L-selectin-deficient mice). Cells in culture were collected, centrifuged and resuspended in FACS buffer (PBS, 2.5% Calf serum, 0.01% NaN_3) to obtain a cell suspension of 10^5 cells/well in a 96 well-plate U-shaped bottom. Naïve lymphocytes were obtained by sacrificing mice and dissecting organs of interest. Those were afterwards homogenized, filtered, centrifuged, counted and diluted. Plates were centrifuged (4°C, 1400rpm, 4min), supernatant was removed and, working on ice, incubated 30min with the first antibody (100µl/well). The first primary antibodies were either hybridoma cell supernatant produced in our laboratory or purified antibodies at the concentration of 10µg/ml in PBS. At the end of incubation time, cells were washed twice using FACS buffer and centrifuged (4°C, 1400rpm, 4min). Working in dark and on ice, cells were incubated with 10µg/ml phycoerythrin (PE)-conjugated goat anti-rat IgG antibody (Jackson Labs; Dianova, Hamburg, Germany), previously neutralized with 10% normal mouse serum to prevent unspecific bindings. After 30min, cells were washed and centrifuged twice, then incubated 10min with 10% rat serum - 10µl/well (Serotec). Cells were then ready to be incubated 30min with the antibody Thy1.2-FITC (fluorescein isotiocyanate) (CD90.2) (Pharmingen; Heidelberg, Germany), specific for lymphocytes detection. Cells were washed and fixed in 1% (w/v) PFA/PBS and analysed at FACScalibur using the CellQuest software (BD Bioscience; Heidelberg, Germany).

- Cells in culture or naïve lymphocytes are collected and diluted to 10^5cells/well
- 100µl of cell suspension are plated in a 96 well-plate U-shaped bottom
- Cells are centrifuged (4°C, 1400rpm, 4min) and working on ice the first antibody is incubated
- After 30min, cells are washed and centrifuged twice
- The second antibody (PE-conjugated goat ant-rat IgG) is incubated (30minutes)
- Cells are washed and centrifuged twice
- 10% normal rat serum (10µl/well, 10min) is incubated
- Thy1.2-FITC coupled antibody is incubated 30min
- Cells are washed and centrifuged and resuspended in 1%PFA/PBS
- Cell surface molecules expression is analysed with FACScalibur and CellQuest software

Table II.7.2. Flow cytometry

Immunohistochemistry

As described in *Animal perfusion and tissue freezing*, 6µm CNS sections were prepared and fixed. Samples were delimited using a PAP pen (DAKO; Hamburg, Germany) to avoid cross contaminations between different tissues and antibodies. After re-hydration of the slides in a wet close dark chamber with PBS for 5min, unspecific bindings were blocked treating samples for 15min with a solution of 90% (v/v) goat serum (Serotec) and 10% (v/v) rabbit serum (Serotec). Blocking solution was then removed and the primary antibody (100µl) , supernatant or purified at the concentration of 10µg/ml, was incubated 30min. Slides were washed 1min with PBS and 5min with PBS/0.1% TWEEN-20 (v/v) (Merck). The secondary biotinylated goat anti-rat antibody was incubated 30min and latterly sections were washed as described above. Afterwards, tissues were incubated with streptavidin-horseradish peroxidase SA/HRP (VectorStain Kit, Vector; Wertheim, Germany) 30min and, after another washing step, AEC kit, consisting of 3-amino-ethyl-carbazol (Vector, Wertheim, Germany), was incubated for 7-10min before slides were stained 1min in Hematoxyline (Merck). At this time slides were washed several times in normal water to remove excess of dyes and later on mounted with Aquatec (Merck). Using bright field microscope (Nikon; Egg, Switzerland), sections pictures were acquired using a digital camera (Nikon; Egg, Switzerland) and an ACT1 software (Nikon; Egg, Switzerland).

- 6µm sections are cut at microtome, mounted on silanized slides and fixed in acetone
- Samples are delimited with PAP pen and re-hydrated in PBS
- Unspecific bindings are prevented incubating slides with goat and rabbit serum 15min
- 30min incubation with primary antibody
- Washing step: PBS and PBS/TWEEN 20
- SA/HRP incubation (30min)
- Washing step: PBS and PBS/TWEEN 20
- AEC kit incubation (7-10min)
- 1min staining in Hematoxyline
- Washing steps in normal water
- Mounting with Aquatec and analysis at microscope

Table II.7.3. Immunohistochemistry

Preparation of silanized slides

Glass slides (Vogel) were silanized to prevent tissue's detachment. They were washed in acetone 5min and coated with 2% (v/v) 3-aminopropyltri-methoxysilane (MESPA) in acetone at RT for 5min. Afterwards slides were washed three times 5min in acetone and air dried 1h before they were used or stored.

- Slides are washed in acetone at RT 5min
- Slides are coated in 2% (v/v) TESPA solution in acetone for 5min
- Slides are washed three times in acetone and then dried 1h

Table II.7.4. Preparation of silanized slides

II.8. Polymerase chain reaction

Here are reported the protocols which were used to extract DNA from mouse tail biopsies and to analyse it by polymerase chain reaction (PCR) to identify the genotype of mice of interest.

DNA extraction from mouse tail biopsies

Tail biopsies of 15-21 day old mice were cut using sterile scissors. Biopsies were then singularly placed in 1.5ml reaction tubes (Eppendorf) and lysed overnight at 55°C in 150µl lysis buffer (50mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.3, 0.45% NP-40, 0.45% Tween 20) and 20mg/ml proteinase K (Merck). The lysates were then heat-inactivated at 95°C for 20min and the samples were stored at -20°C or directly used for PCR analysis.

- Tails of 15-21 day old mice are cut using sterile scissors
- Biopsies are lysed overnight at 55°C in appropriate buffer
- Lysates are heat-inactivated at 95°C for 20min
- Samples are stored at -20°C or directly used for PCR analysis

Table II.8.1. DNA extraction from mouse tail biopsies

L-selectin neo PCR

Two PCR reactions were done to analyse DNA of SJL/L-selectin-deficient mice. The first reaction was to identify the wild type L-selectin allele and required the primers FW1 (5'-TGC CTG GTC CTA GAA CAC TC -3') and Rev1 (5'-GTG GTC CCA CAT CAG CGT AG -3') to generate a band of 480bps. The second reaction was to detect the phosphoglycerate kinase promoter / neomycin resistance gene (PGKneo) on exon 3. This promoter was inserted into the lectin domain of the targeting vector containing exons 2-6 of the L-selectin genomic DNA. This vector was created at the University of Dallas (USA) in the group of Prof.Dr. Siegelman to disrupt the L-selectin locus and generate L-selectin-deficient mice (Catalina et al., 1996). To detect PGKneo were used the primers FW2 (5' -TGT GCC TGG TCC

TAG AAC ACT C -3') and PGK-Rev2 (5' -TGT CAC GTC CTG CAC GAC GC -3'), generating a band of 596bps detectable on 1% agarose gel. As internal controls the primers Tie1-FW3 (5' -CGA AGG GAT GGG AGA GAG AGC -3') and Tie1-Rev2 (5' -TGA CGC TAT GAC GAC GAC GAT G -3' were used as internal controls).

The analysis of DNA of L-selectin mice was done mixing 1µl DNA and 19µl of PCR master mix composed by

wild type allele		*PGKneo allele*	
2µl	10x PCR Buffer	2µl	10x PCR Buffer
1.2µl	25mM $MgCl_2$	1.2µ	25mM $MgCl_2$
0.8µl	5mM dNTPs	0.8µl	5mM dNTPs
14.28µl	ddH_2O	14.54µl	ddH_2O
0.13µl	FW1 primer	0.13µl	FW2 primer
0.13µl	Rev1 primer	0.13µLl	Rev2 primer
0.13µl	Tie1-FW3 primer		
0.13µl	Tie1-Rev2 primer		

The reaction was performed in a PCR reaction machine (Eppendorf; Hamburg, Germany) and were used the following conditions:

Initial denaturation	90°C	4min	
Denaturation	94°C	45sec	35 cycles
Hybridization	60°C	45sec	
Elongation	72°C	1min	
Final elongation	72°C	10min	
	4°C	∞	

PCR reaction results were thus separated by agarose gel-electrophoresis. 1% agarose (Invitrogen) was dissolved in 0.5% TBE buffer (45mM Tris, 45mM boric acid, 1mM EDTA) and 0.5µg/ml ethidium bromide, and solidified at RT. Gel was placed

in a chamber containing 0.5% TBE buffer and probes, labelled with 6x loading buffer, were loaded on the gel and run 30-45min at 10V per centimetre gel length. On the gel was also loaded a size marker (1kb marker). Probes were visualized exciting ethidium bromide with UV light (λ= 312nm) and picture were printed on thermo-paper.

III. RESULTS

III.1 Establishment of SJL-L-selectin-deficient mice

The L-selectin-deficient mice, provided by Mark Siegelman (University of Texas Southwestern Medical Centre in Dallas, Texas, USA), were backcrossed into the EAE susceptible SJL background to generate heterozygous mice (F1), which were then again backcrossed with SJL wild type mice for seven generations (F7). Heterozygous mice at generation F7 were bred together to generate SJL/L-selectin-deficient mice (F8) (Figure III.1.1). All mice used and presented in this study were females between generation F8 and generation F18, and aged 8-10 weeks.

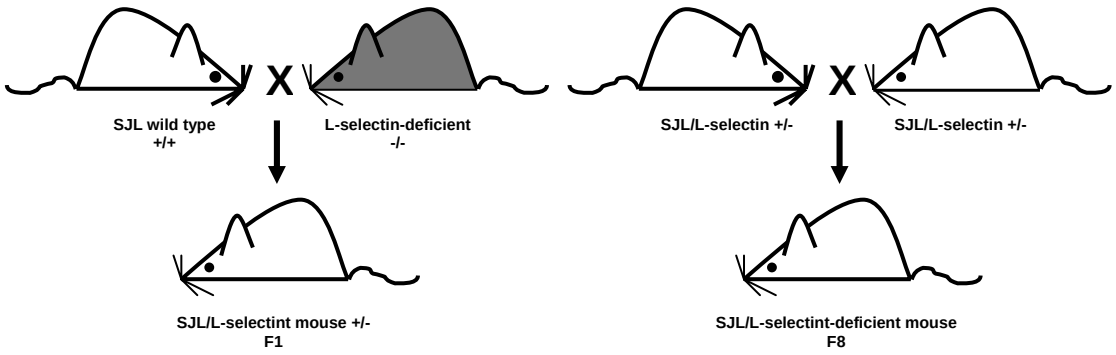

Figure III.1.1. Generation of SJL/L-selectin-deficient mice. Backcrossing the L-selectin-deficient strain into the SJL background resulted in the generation of SJL/L-selectin heterozygous mice (generation F1-F7). Heterozygous F7 mice were bred together to generate SJL/L-selectin-deficient mice F8.

The genotype of the mice was assessed, as described in Material and Methods, by polymerase chain reaction. Tail biopsies were lysed and samples were analyzed by a double PCR to identify the wild type and the PGK neo allele. PCR results were separated by electrophoresis and then visualized in UV light. The genotype of mice tested was determined as follows:

Wild type band	PGK neo band	Genotype
absent	present	-/-
present	absent	+/+
present	present	+/-

Once SJL/L-sel$^{-/-}$ mice were established, their phenotype was analyzed and compared to SJL wild type. In particular, a comparison was made of the lymphocyte populations into lymph nodes, spleen and blood and differences are summarized in Table III.1.1. Females of SJL wild type and SJL/L-sel$^{-/-}$ mice, of the same age, were sacrificed by cervical dislocation. After that, organs were dissected, homogenized and cells per organ counted. Blood lymphocytes, were isolated by density gradient and then counted. No differences in the number of lymphocytes in blood and mesenteric lymph nodes (MLN) were detectable between SJL wild type and SJL/L-sel$^{-/-}$ mice. SJL/L-sel$^{-/-}$ mice showed a dramatic reduction in the number of lymphocytes present in peripheral lymph nodes and showed splenomegaly compared to SJL wt mice. This experiment was repeated five times and data were reproducible. We can conclude that the phenotype of SJL/L-selectin-deficient mice does not differ from the one reported in the literature for L-selectin-deficient mice (Arbones et al., 1994; Catalina et al., 1996).

	Number of lymphocytes per organ	
	SJL/L-sel$^{-/-}$ mice	**SJL wild type mice**
Blood	$2.2 \cdot 10^7$/mL	$2.5 \cdot 10^7$/mL
Mesenteric LN	$6.7 \cdot 10^6$	$6.3 \cdot 10^6$
Popliteal LN	$0.6 \cdot 10^5$	$3.5 \cdot 10^6$
Axillary LN	$2.5 \cdot 10^5$	$6.2 \cdot 10^6$
Aortic LN	$4.2 \cdot 10^5$	$4.2 \cdot 10^6$
Inguinal LN	$1.6 \cdot 10^5$	$6.3 \cdot 10^6$
Spleen	$5.1 \cdot 10^8$	$2.3 \cdot 10^8$

Table III.1.1. SJL/L-selectin-deficient mice phenotype in comparison to SJL wild type littermates. 8-10 week old mice were sacrificed by cervical dislocation. The spleen, PLN and MLN were isolated from each mouse, homogenized separately and cells were counted. Blood lymphocytes were separated by density gradient and then counted. One representative experiment of five.

By means of flow cytometry the phenotype of cells populating spleen, PLN and MLN of SJL wild type, SJL/L-selectin$^{-/-}$ and L-selectin-deficient mice was examined. Results were compared to investigate possible differences in surface molecule expression between different mouse strains and between different organs. Littermates were sacrificed by cervical dislocation and spleen, peripheral lymph nodes and the mesenteric lymph nodes were surgically dissected. After homogenization, cells were counted and

$5 \cdot 10^5$ cells per well plated in 96 well plates. Cells were then stained for 30 minutes with monoclonal rat anti-mouse antibodies directed against cell surface molecules. After two washing steps, cells were stained with a phycoerythrin (PE)-coupled goat anti-rat antibody. After 30 minutes, cells were washed and stained with a fluorescein isothiocyanate (FITC) antibody, Thy 1.2-FITC, which specifically recognizes mature T cells. After fixation with 1% (w/v) paraformaldehyde (PFA) diluted in PBS, cell fluorescence was detected at FACS Calibur (BD, Heidelberg, Germany) and acquired data, here presented as plots of gated living cells, were analyzed using CellQuest software (BD, Heidelberg, Germany). PE-positive cells are identified by FL2 channel and FITC-positive cells are detected by FL1 channel. Several cell surface molecules were investigated: because interleukin (IL)-2 binding to its receptor induces cell proliferation, was investigated the IL-2 receptor (IL-2R). IL-2 receptor is composed of three chains: alpha-chain (CD25), beta-chain (CD122) and by the common gamma-chain (CD132). These chains can bind together to form receptors with different affinity for their ligand IL-2. CD25 is the low affinity receptor, CD122 and CD132 form the intermediate affinity receptor and only when all three chains bind together do they constitute the high affinity receptor (Figure III.1.2). To obtain more accurate information, different monoclonal antibodies (Pharmingen, Heidelberg, Germany) binding to different epitopes were used for each chain. CD25, which is expressed on mature and activated T cells and on regulatory T cells, was stained using the antibodies 7D4, PC61 and 3C7. CD122 is expressed on NK cells and T cells, and on T cells is upregulated upon stimulation. As recommended by the manufacturer, the signal produced by the antibodies TUGm2 and 4G3, which recognize different epitopes on the common gamma-chain, was amplified using biotin and streptavidin, instead of phycoerythrin, and detected at FACS Calibur by the FL2 channel.

Cells were stained for α_4, $\alpha_4\beta_7$, β_7 and β_1 integrins, which are involved in lymphocyte homing and transmigration across endothelia, and lymphocytes were also stained for PSGL-1, which is involved in the rolling of lymphocytes on HEV. Using an antibody directed against CD45RB, it was possible to discriminate between naïve (CD45RB high) and memory/effector (CD45RB low) CD4 positive T cells. CD62L (L-selectin) was used as a control. A representative experiment of the three is shown here.

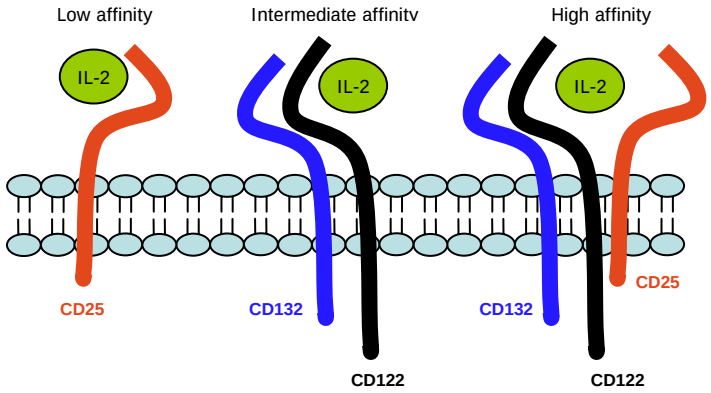

Figure III.1.2. IL-2 receptor chains. There are three chains composing IL-2R: CD25 (alpha-chain), CD122 (beta-chain) and CD132 (gamma-chain or common chain). The chains can arrange themselves together to form receptors which show different affinity for their specific ligand. CD25 is the low affinity receptor, CD122 and CD132 form the intermediate affinity receptor and all three chains together create the high affinity receptor.

As in this experiment fresh naïve primary spleens were used and these are composed of a mixture of cells, analysing splenocytes of SJL wild type (Figure III.1.3), SJL/L-selectin-deficient (Figure III.1.4) and L-selectin-deficient (Figure III.1.5) mice, it is immediately evident that there is a population of cells which is negative for the T cell marker Thy1.2 and a second population which is composed of mature Thy1.2 positive cells. CD25 could be detected on less than 1% of Thy1.2 positive cell surface in all three mouse strains investigated, and any significant difference was noticed between SJL wt and SJL/L-sel$^{-/-}$ and L-selectin-deficient splenocytes. With reference to CD122, in comparison to wild type mice, there is a slight increase in SJL/L-sel$^{-/-}$ and in L-selectin-deficient CD122 positive splenic lymphocytes. CD132 detection is different, as both antibodies used gave different results. On the one hand, the amount of double positive cells detected using the monoclonal antibody TUGm2 is higher in SJL/L-selectin-deficient mice compared to SJL wild type and L-selectin-deficient mice. On the other hand, 31.6% of Thy1.2 positive cells are clearly positive for IL-2R using 4G3 in SJL wt splenocytes, whereas in SJL/L-selectin-deficient mice CD132 positive cells are less (18.6%) and only few IL-2R common gamma-chain positive T cells are detectable in L-selectin-deficient mice using 4G3 (2.1%). α_4 and $\alpha_4\beta_7$ integrin positive T cells are detectable in all splenocytes here investigated and LFA-1 expression on Thy1.2 positive cells is always high, particularly in SJL/L-selectin-deficient mice (53.4%). Integrin β_7 positive T cells were more detectable in SJL wild type and in SJL/L-sel$^{-/-}$ than in L-selectin-deficient splenocytes, whilst β_1 positive splenocytes are detectable only in SJL wt mice. In fact, β_1 integrin

could hardly be detected on the cell surface of SJL/L-sel$^{-/-}$ (0.33%) and L-selectin-deficient (1.6%) splenocytes. CD45RB high cells were present in all populations investigated and, interestingly, also a population of cells which is Thy1.2 positive and CD45RB low is present in SJL wt splencytes. PSGL-1 is expressed not only on T cells; in fact also the Thy1.2 negative cells expressed this molecule on their surface. CD62L was used as a control, and, as expected, L-selectin positive cells were detectable only in SJL wild type mice.

The same protocol was used to stain and analyze lymphocytes populating peripheral lymph nodes. In this case only cells of SJL wild type (Figure III.1.6) and SJL/L-sel$^{-/-}$ (Figure III.1.7) mice were used. In fact, due to the small size of PLN in L-selectin-deficient mice, it was not possible to isolate organs and to investigate them. From the plots presented here, a difference immediately arises compared to splenocytes: in PLN, the main population is composed of Thy1.2 positive cells. With reference to IL-2R alpha-chain, no significant differences were noticed between the two groups analyzed. In fact, only few lymphocytes within the PLN express CD25 on their surface and none of the antibodies used could detect it. CD122 levels detected using TMβ1 and 5H4 were low in SJL wild type and in SJL/L-sel$^{-/-}$ peripheral lymph nodes lymphocytes. In the case of SJL/L-selectin-deficient mice, Thy1.2 positive and CD122 negative cells are subdivided in two subpopulations. A quarter of the Thy1.2 positive T lymphocytes, either SJL wt or SJL/L-sel$^{-/-}$, was positive also for CD132 using the antibody TUGm2. The number of CD132 positive Thy1.2 positive cells was higher using the TUGm2 antibody compared to 4G3. α_4, $\alpha_4\beta_7$ and β_1 integrin were clearly positive on Thy1.2 positive SJL wild type PLN cells and not on SJL/L-sel$^{-/-}$ PLN lymphocytes. On the contrary, in SJL wt and SJL/L-sel$^{-/-}$ PLN, LFA-1 was also detectable on Thy1.2 positive cells. On Thy1.2 positive cells of SJL/L-selectin-deficient PLN, β_7 integrin was detected only on 5.4% of the lymphocytes. The CD45RB fluorescence intensity detected in SJL wild type and SJL/L-sel$^{-/-}$ PLN was extremely high (naïve cells). The percentage of PSGL-1 positive cells was also high: 74% on SJL wt and 58.8% on SJL/L-sel$^{-/-}$ PLN lymphocytes. As expected, in SJL/L-sel$^{-/-}$ PLN lymphocytes were not positive for CD62L, but only a small population of lymphocytes was Thy1.2 positive. In SJL wt mice, PLN cells were CD62L positive, but again only a minor portion was Thy1.2 positive.

Mesenteric lymph nodes in L-selectin-deficient mice were tiny and it was not possible to isolate a sufficient quantity of cells to analyze them. Thus only data relative to SJL wild type (Figure III.1.8) and SJL/L-sel$^{-/-}$ (Figure III.1.9) mice is presented here. Littermates

were sacrificed and MLN dissected as described above. The staining procedure did not differ from the one already described for splenocytes and PLN cells. In MLN the main cell population is composed by Thy1.2 negative cells, as in spleen. Within the Thy1.2 positive cells detectable in MLN, neither SJL wt nor SJL/L-selectin-deficient mice expressed IL-2R alpha- and beta-chain at all. CD132 is, instead, detectable on MLN Thy1.2 high cells, and compared to spleen and PLN, using TUGm2 or 4G3, there is a reduction of lymphocytes expressing IL-2R gamma-chain on their surface. By comparison, Thy1.2 positive mesenteric T cells display α_4, $\alpha_4\beta_7$, β_7 integrins and LFA-1 on their surfaces. It is important to note that β_1 integrin on SJL/L-selectin-deficient mesenteric lymphocytes is not expressed (0.34%) and is only partially detectable on SJL wild type Thy1.2 positive cells (7.7%). On Thy1.2 positive cells, CD45RB was equally present on SJL wt (27%) and SJL/L-sel$^{-/-}$ (28%) mesenteric lymphocytes. There were no differences Thy1.2 high PSGL-1 high comparing SJL wt (35%) and SJL/L-selectin-deficient (30%) MLN lymphocytes.

To summarize, the IL-2R alpha-chain is not detectable with any of the monoclonal antibodies used in spleen, PLN and MLN of SJL wild type, SJL/L-sel$^{-/-}$ and L-selectin-deficient mice. CD122 is partially detectable only on splenocytes of SJL/L-selectin and L-selectin-deficient littermates. In PLN and MLN levels of IL-2R beta-chain could not be detected. Compared to what was seen in the spleen and peripheral lymph nodes, in mesenteric LN there is a reduction in CD132 detection. With regard to integrins, are all present on SJL wild type, SJL/L-sel$^{-/-}$ and L-selectin-deficient splenocytes, PLN and MLN lymphocytes. The only exception is β_1 integrin, which is only detectable on SJL wt Thy1.2 positive cells in spleen and PLN. All mice used for the experiment were naïve mice and, as expected, the CD45RB levels were high. PSGL-1 is always present on the cells analyzed, either Thy1.2 positive or negative. However, on SJL wt and SJL/L-selectin-deficient splenocytes and in MLN, as well on SJL/L-selectin-deficient PLN, there is a cell population which is double negative for Thy1.2 and for PSGL-1. CD62L is only present on cells originating from SJL wild type littermates.

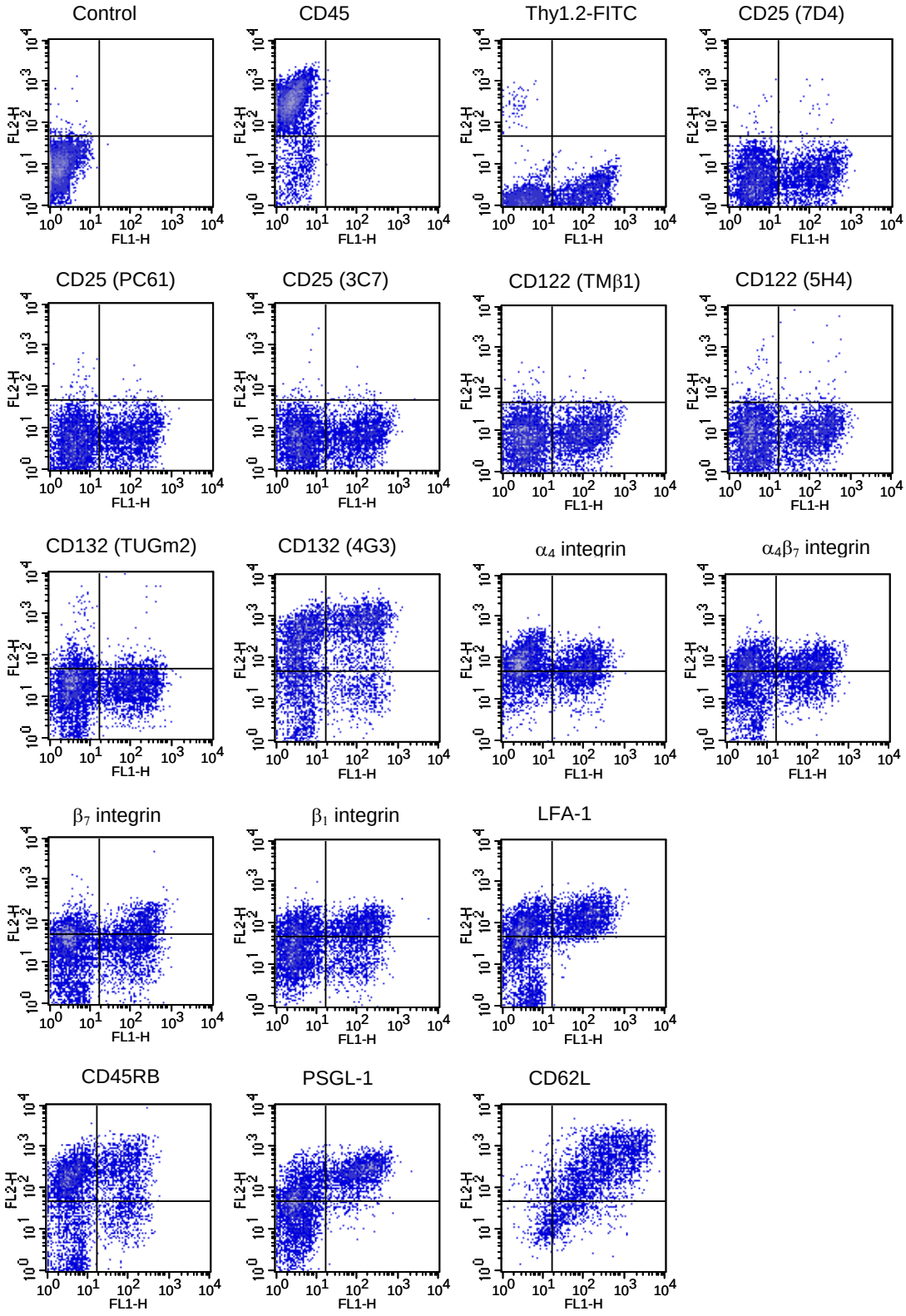

Figure III.1.3. Phenotype of SJL wild type splenocytes. SJL wild type splenocytes were stained to detect cell surface molecules expression. CD25, CD122 and CD132 are not detectable on cells surface. CD132, detected by the monoclonal antibody 4G3, is expressed at high levels on Thy1.2 positive SJL wt splenocytes. All integrins are detectable and CD45RB is highly expressed, as PSGL-1. CD62L is detectable on splenocytes. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

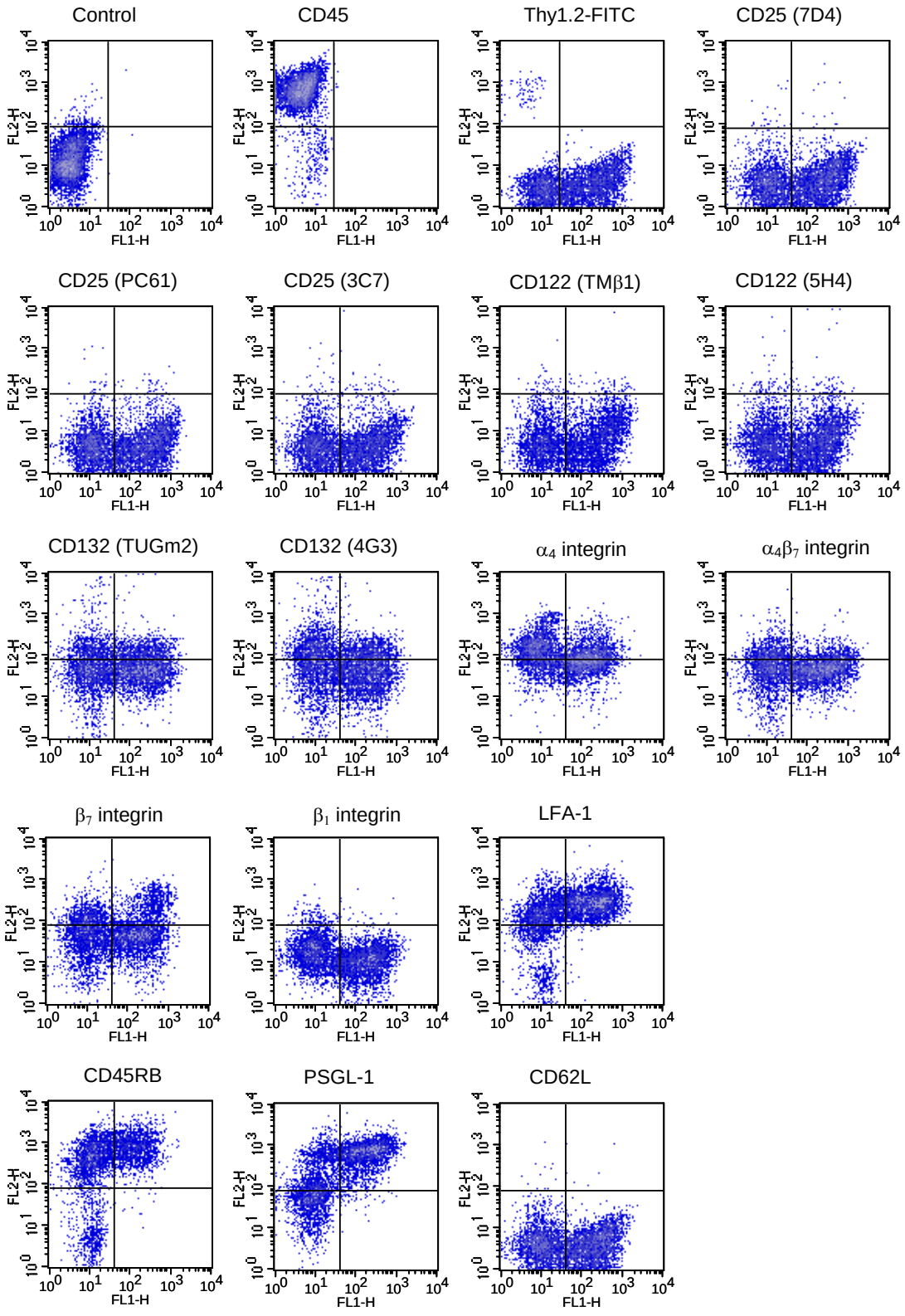

Figure III.1.4. Phenotype of SJL/L-selectin-deficient spleen. CD25 is poorly expressed on splenocytes. CD122 and CD132 (TUGm2) are slightly upregulated on cells surface. There is any impairment in integrin expression, with the exception of β_1 integrin, which is not expressed. CD45RB and PSGL-1 are high. CD62L is not detectable on splenocytes. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

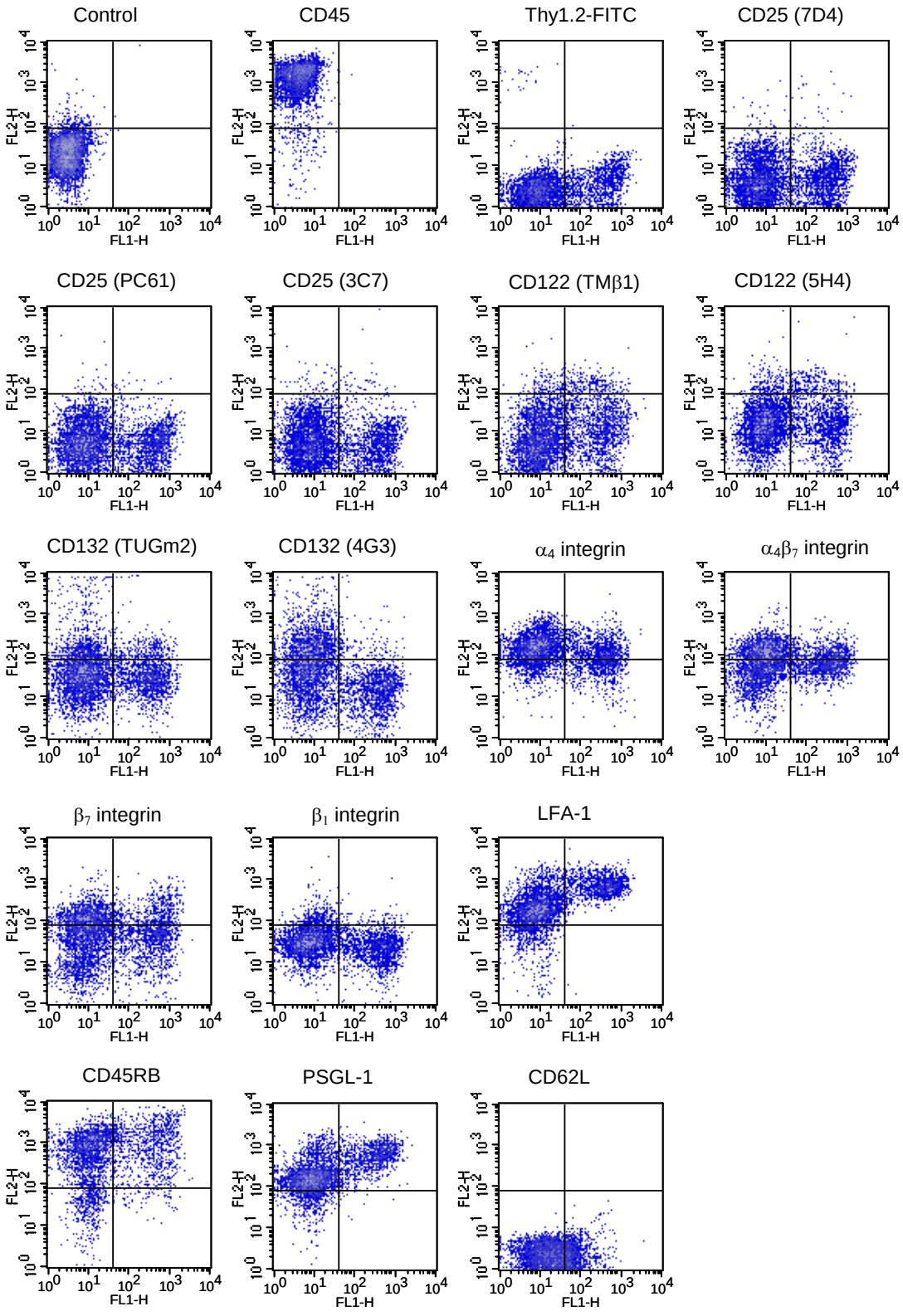

Figure III.1.5. Phenotype of L-selectin-deficient spleen. Low expression of IL-2receptor molecules on cells surface. There is an impairment in β_7 and β_1 expression. CD45RB and PSGL-1 levels are high. CD62L is not detectable on splenocytes. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

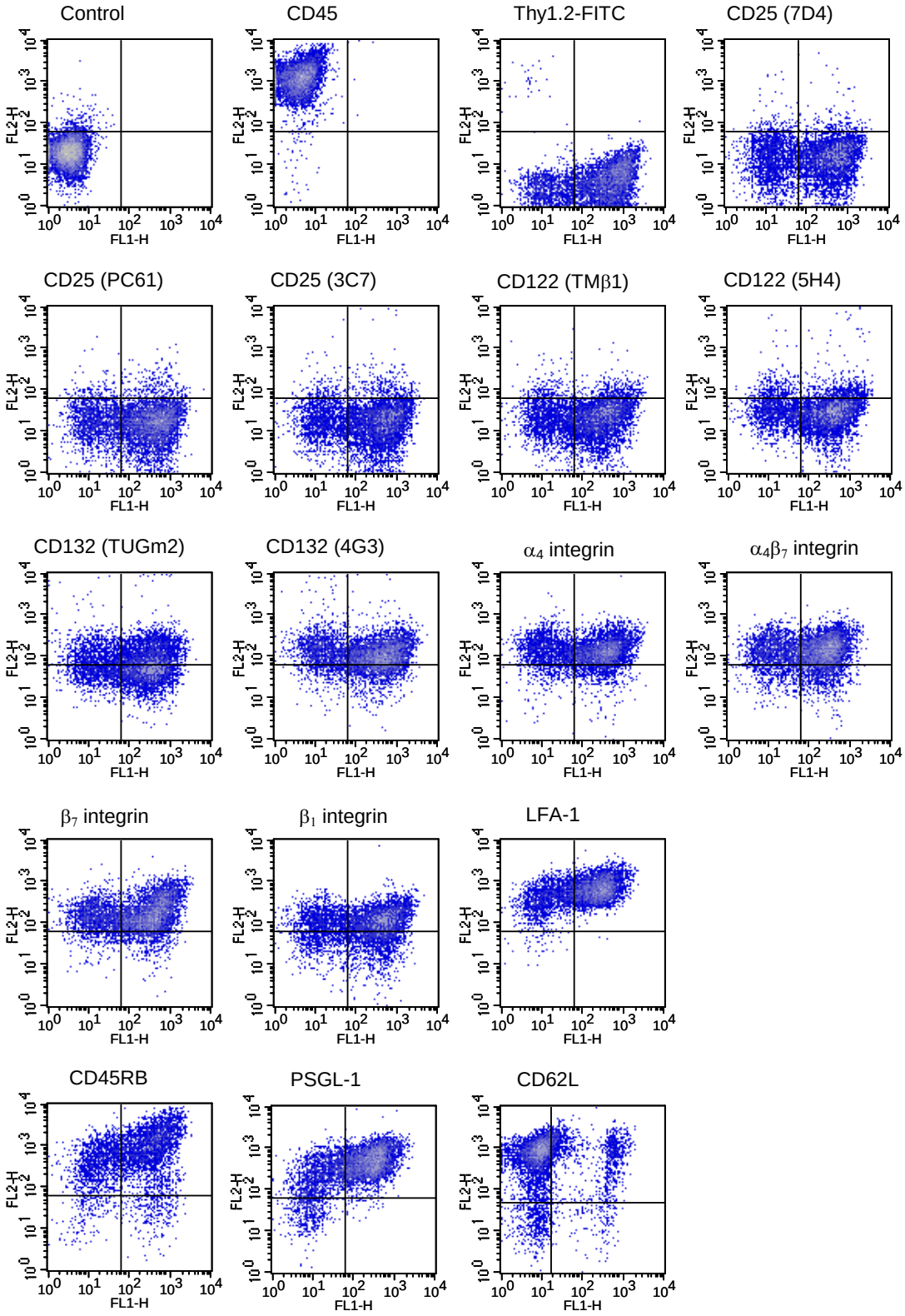

Figure III.1.6. Phenotype of SJL wild type PLN cells. CD25 and CD122 are not detectable on PLN cell surface. CD132 (4G3) is instead expressed on PLN lymphocytes. α_4, $\alpha_4\beta_7$, β_1, β_2 and β_7 integrin levels are high. CD45RB, PSLG-1 and CD62L are detectable on lymphocytes. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

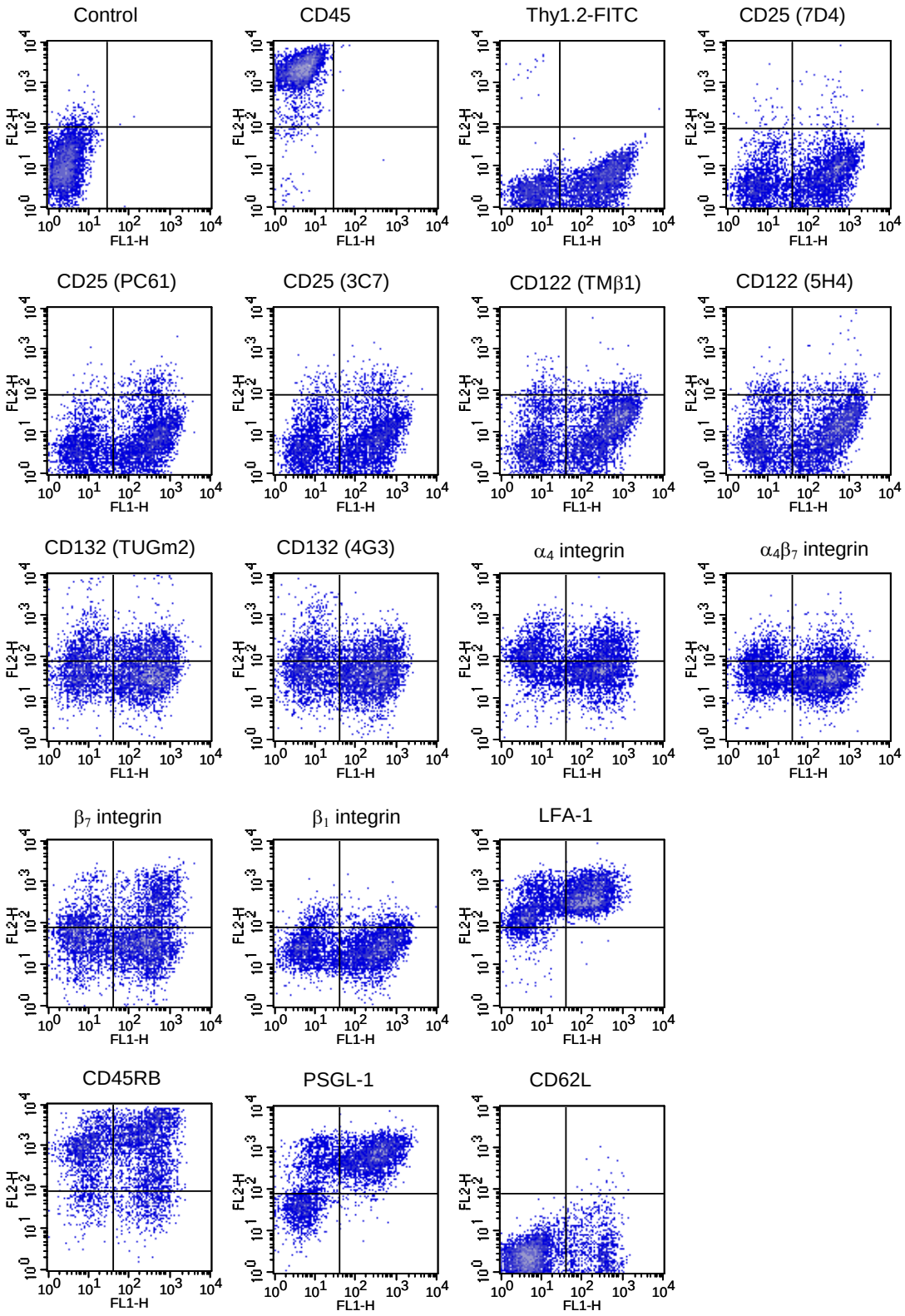

Figure III.1.7. Phenotype of SJL/L-selectin-deficient PLN cells. The only IL-2R molecule expressed is the CD132 (4G3). β_2 and β_7 integrin levels are comparable to SJL wt PLN cells, there is impairment in α_4, $\alpha_4\beta_7$ and β_1 expression on cells surface. Thy1.2 positive CD45RB and PSGL-1 positive cells are detectable, CD62L is not expressed on SJL/L-sel$^{-/-}$ cells. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

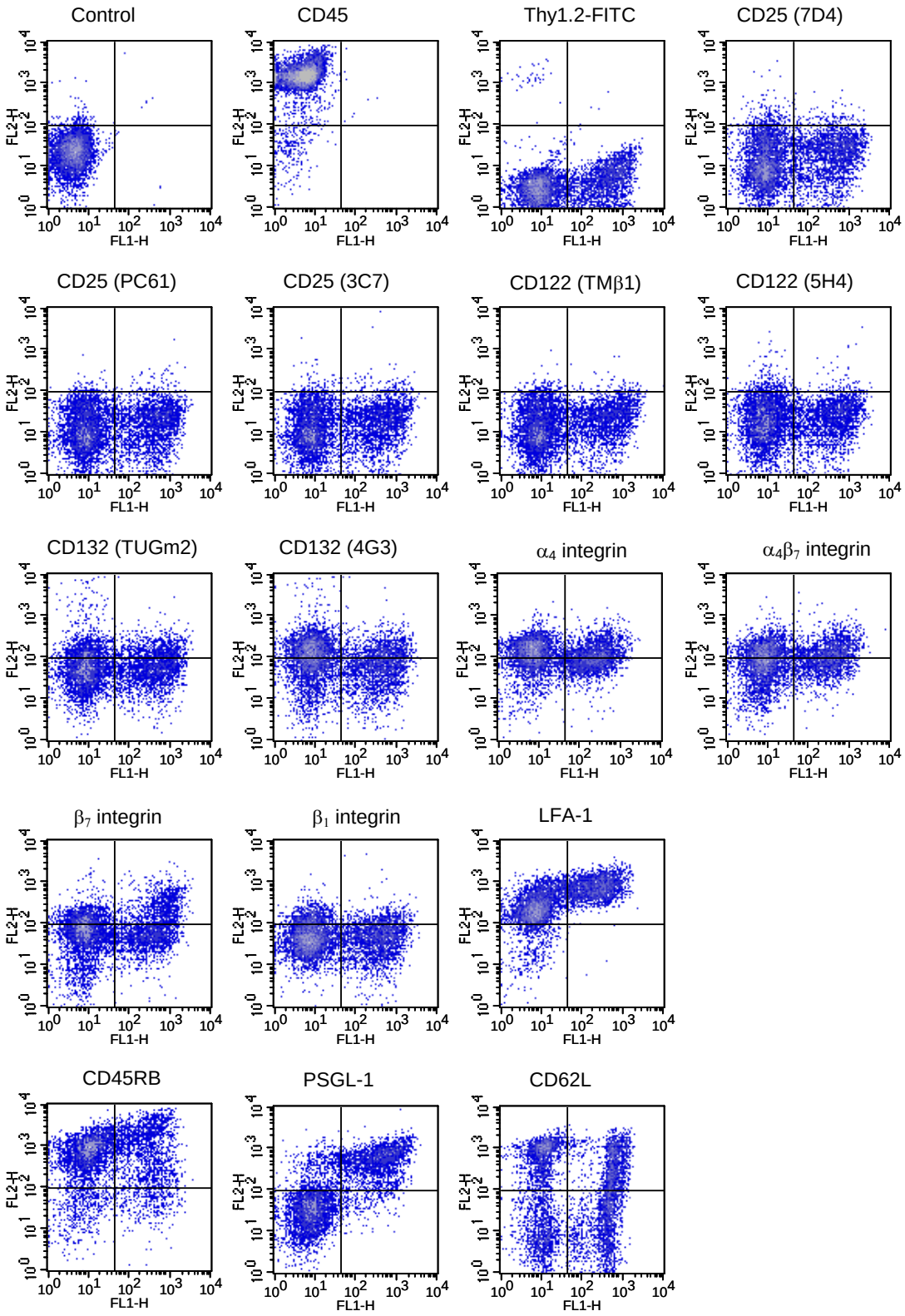

Figure III.1.8. Phenotype of SJL wild type mesenteric lymph node cells. IL-2 receptor molecules and β_1 integrin are not expressed on MLN cell surface. α_4, $\alpha_4\beta_7$, β_7 and β_2 integrins are expressed. CD45RB, PSGL-1 and CD62L positive lymphocytes are present on cells surface. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

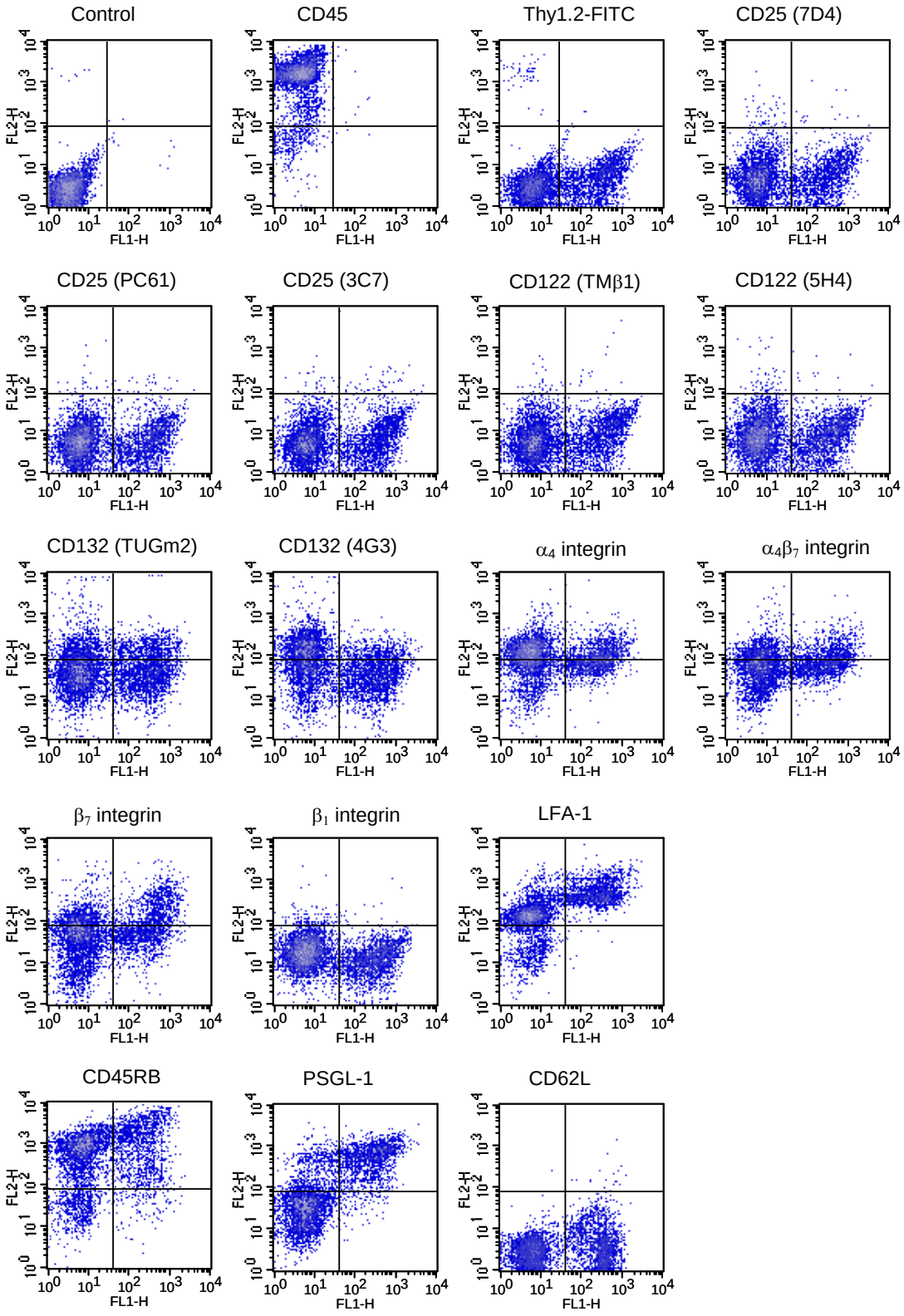

Figure III.1.9. Phenotype of SJL/L-selectin-deficient mesenteric lymph node cells. IL-2 receptor molecules and β_1 integrin are not expressed on MLN cell surface. α_4, $\alpha_4\beta_7$, β_7 and β_2 integrins are expressed. CD45RB and PSGL-1 positive lymphocytes are present on cells surface. CD62L is not detectable on SJL/L-sel$^{-/-}$ MLN lymphocytes. Data is represented as plots of living cells. FITC staining is detected at FACS Calibur by the FL1 channel, PE fluorescence is captured by FL2 channel. One experiment of three.

To draw a conclusion, it was possible to confirm that Thy1.2 positive cells are lacking CD25 and CD122 on splenocytes, PLN and MLN derived cells in SJL wt, SJL/L-sel$^{-/-}$ and L-selectin-deficient mice. Instead, CD132 was detectable using TUGm2 and 4G3 antibody. Intermediate values of α_4 integrin were detectable on SJL wild type and SJL/L-sel$^{-/-}$splenocytes, PLN and MLN lymphocytes. In the experiment described here, we detected integrins on lymphocyte surface, with the exception of β_1 which is only present on SJL wt splenocytes and PLN cells. In contrast, in the other two tests performed, we identified only LFA-1 on cell surface of SJL wild type and SJL/L-selectin-deficient lymphocytes. Data relative to CD45RB and PSGL-1, which were high in all cell types investigated, were reproducible. As expected, CD62L was detectable only on the surface of cells coming from SJL wild type mice.

III.2. active experimental autoimmune encephalomyelitis (aEAE)

After the generation of SJL/L-selectin-deficient mice, the goal was to investigate if L-selectin plays a role *in vivo* in the recruitment of encephalitogenic T cells across the BBB and in the immunopathogenesis of EAE. As described in Material and Methods, aEAE was induced in SJL wild type and SJL/L-sel$^{-/-}$ by active immunization with an emulsion of CFA and PLP peptide, amino acids 139-151 (PLP$^{139-151}$). On day 1 and day 3 post immunization (p.i.) an intravenous injection of heat-inactivated *Bordetella pertussis* micro-organisms was given to SJL wild type and SJL/L-selectin-deficient mice. Clinical symptoms and weight change were scored daily. Figure III.2.1 shows one experiment of four in which aEAE was induced and compared in SJL wild type and SJL/L-sel$^{-/-}$ mice. The experiment presented here was performed with four mice per group. Comparison of the day of disease onset did not show any significant difference between SJL wild type (10.3±1.50) and SJL/L-sel$^{-/-}$ (11±1.0). In addition, both groups analyzed suffered a comparable severity of disease. In fact, mice developed parallel disease and after a peak of severity of disease on day 12 post immunization, which was slightly more severe in SJL wt mice, animals exhibited a mild disease for about two weeks before they fully recovered. Furthermore, the SJL/L-sel$^{-/-}$ mice seemed to recover more gradually between day 22 and day 28 p.i. compared to the SJL wt mice. The weight change graph presented in Figure III.2.1 illustrates a parallel reduction in body weight in SJL wild type and

SJL/L-sel$^{-/-}$ mice until day 12 post immunization, which corresponds to the peak of severity of disease. This reduction in body weight is followed by a continuing reconstitution of the original weight detected prior to immunization. The SJL/L-sel$^{-/-}$ mice, even if completely recovered and not exhibiting any longer detectable clinical symptoms, were not able to fully reconstitute their original body weight within the observation period (33 days).

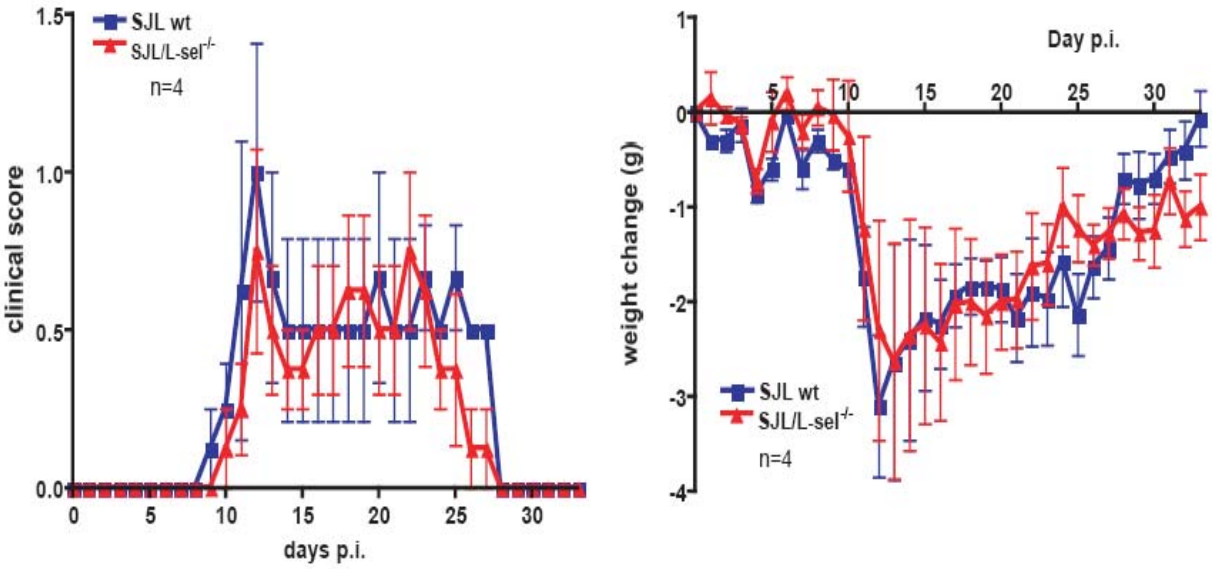

Figure III.2.1. active experimental autoimmune encephalomyelitis (aEAE) comparison in SJL wild type and SJL/L-sel$^{-/-}$ mice. aEAE was induced in 8-10 week old females of SJL wt (blue) and SJL/L-sel$^{-/-}$ (red) mice. Mice develop parallel disease and no significant differences were noticed on the day of onset of disease and severity of disease between the two groups. Within the observation period (33 days) SJL wt mice were able to fully recover their original body weight whereas the SJL/L-sel$^{-/-}$ group did not. Clinical score and weight change are expressed as mean value ± SD. One experiment of four.

At the end of the observation time (33 days p.i.), animals were sacrificed and perfused with a 1% solution (w/v) paraformaldehyde (PFA) in PBS. Surgically the CNS was dissected, cut in pieces and snap frozen in cryomolds, as described in Material and Methods. Frozen brain and spinal cord were cut at microtome (6µm tissue sections) and stained to detect CD45-positive cell infiltrates in SJL wt and SJL/L-selectin-deficient mice. Tissue sections were fixed in acetone at -20°C and air dried for 1 hour. After re-hydration with PBS and washing with a solution of PBS and Tween-20, samples were stained with the first antibody, which in our case were anti-CD45 and anti-PECAM-1: anti-CD45 was used to detect cells infiltrating into the CNS; anti-PECAM-1 was used as

positive control to detect blood vessels. Then sections were stained with the secondary biotinylated antibody which was followed by a streptavidin-horseradish peroxidase and visualized by a hematoxyline counterstaining. At the end of the staining procedure, slides were observed under a microscope. In Figure III.2.2 is shown an example of immunohistochemical analysis of CNS (cerebellum) of EAE affected mice, which were sacrificed on day 33 post injection. CD45 positive cell infiltrates were detected both in SJL wild type and SJL/L-selectin-deficient mice, brain and spinal cord. CD45 positive cells accumulate into the perivascular spaces, as demonstrated by the colocalization with PECAM-1 positive staining, and form inflammatory cuffs.

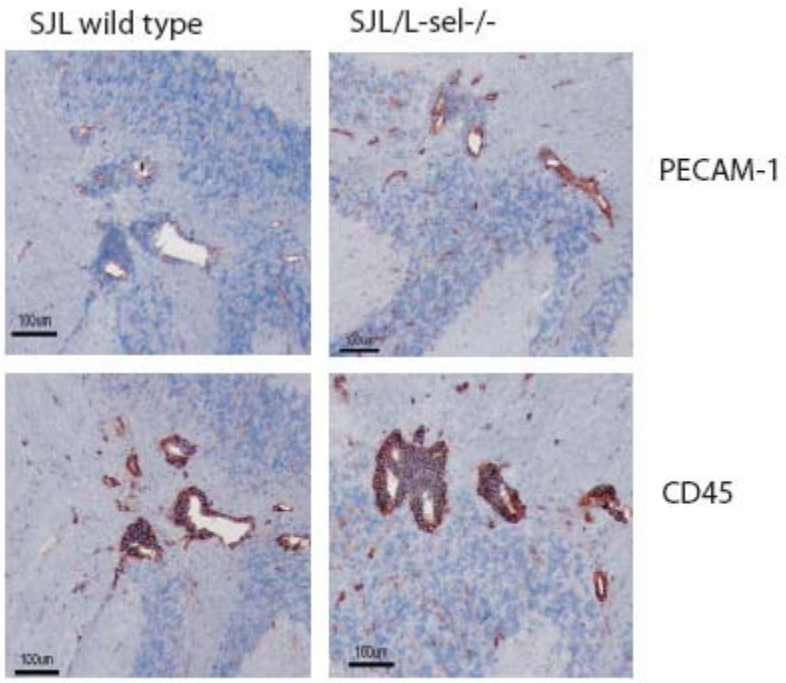

Figure III.2.2. Inflammatory CD45 positive cell infiltrates. CD45 positive cells in SJL wild type and SJL/L-selectin-deficient cerebellum. CD45 positive cells form inflammatory cuffs as shown by colocalization with PECAM-1, which is a marker for blood vessels. Mice were sacrificed on day 33 post immunization. CD45 positive cells were stained using the monoclonal antibody M1/9; PECAM-1 positive cells were stained using the monoclonal antibody Mec13.3. Hematoxyline counterstaining.

This experiment was repeated 3 times using animal of the same age (8-10 weeks old) and from different backcrossing generations (Table III.2.1). Only during the first test, in SJL wild type and in SJL/L-selectin-deficient mice disease was delayed compared to the other experiments, and in this particular experiment both mouse groups developed mild disease. However, during the last three aEAE tests there were no significant differences

concerning the day of the onset of disease and the severity of disease scored in SJL wt and SJL/L-sel$^{-/-}$ mice.

Experiment number	Number of mice per group	Day of onset of disease		Severity of disease	
		SJL wt mice	SJL/L-sel$^{-/-}$ mice	SJL wt mice	SJL/L-sel$^{-/-}$ mice
1	8	19.50±6.39	23.71±4.23	0.88±0.88	0.71±0.27
2	6	12.83±1.83	14.67±2.73	1.25±1.37	1.50±0.55
3	4	13.25±1.50	16.57±2.64	1.87±1.30	1.20±0.90
4	4	10.30±1.50	11.0±1.0	1.33±0.57	0.75±0.40

Table III.2.1. aEAE comparison in SJL wild type and SJL/L-sel$^{-/-}$ mice. Comparison of the day of onset of disease and severity of disease in four different experiments in SJL wild type and SJL/L-sel$^{-/-}$ mice. In one case mice developed a delayed and less severe disease in comparison to the next experiments. Regarding the last three aEAE test performed, both mouse groups had a similar severity of disease and day of onset of disease. Data are expressed as mean value ± SD.

III.3. Passive transfer of experimental autoimmune encephalomyelitis (tEAE) with SJL wild type PLP-specific T cell blasts

From aEAE studies it was evident that L-selectin does not play a role in the recruitment of encephalitogenic across the BBB and in the immunopathogenesis of EAE. Previous investigations demonstrated that, when encephalitogenic T cells are activated *in vitro* and are injected in mice to transfer EAE, inflammatory cells do not enter the lymph nodes to be activated, but rather, encephalitogenic T cells directly circulate in the blood, reach the BBB and induce inflammation (Wekerle et al., 1986; Hickey and Kimura, 1988).Thus, the next goal was to investigate if *in vitro* activated SJL wild type PLP-specific T cell blasts can transfer EAE also in SJL/L-sel$^{-/-}$ mice. Disease was passively transferred (tEAE) by intravenous injection of established PLP-specific SJL wild type T cell lines. The T cell line SJL.PLP4, which was previously established and characterized, was restimulated, as described in Material and Methods, in the presence of the specific antigen PLP$^{139-151}$ at the concentration of 10µg/mL. The proliferation efficiency and the specificity for the specific antigen PLP$^{139-151}$ was assessed, by a ^{3}H-Thymidine incorporation assay, for each T cell line used for tEAE induction. Cells were co-cultured

in 96 well plates, in triplicates, with sublethally irradiated splenocytes in the presence of their specific antigen $PLP^{139-151}$, in the presence of the mitogen Concanavalin A (ConA) and of the unspecific antigen *Mycobacterium tuberculosis* purified protein derivate (PPD). As a control, cells were also plated in the absence of antigens to obtain data relative to baseline proliferation. After three days, cells were labelled with ^{3}H-Thymidine which was incorporated into the DNA. After 16h, cells were harvested and ^{3}H-dT incorporation was measured by a beta-counter as counts per million (cpm). Elevated cpm have been detected in those samples which had high proliferation. As shown in Figure III.3.1, SJL.PLP4 T cells have a high response and an extremely significant specificity for the $PLP^{139-151}$ antigen. ConA was the positive control in this assay and cells moderately proliferated in its presence. Baseline levels are measured in the absence of antigens and in the presence of PPD. The same results were obtained with the T cell lines SJL.PLP36, SJL.PLP37 and SJL.PLP42.

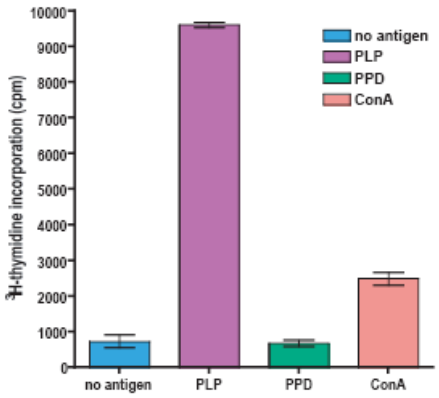

Figure III.3.1. Antigen specificity of the SJL.PLP4 T cell blasts. T cells were co-cultivated with sublethally irradiated splenocytes and labelled with ^{3}H-dT after three days. In the presence of their specific antigen $PLP^{139-151}$ the SJL.PLP4 T cells have an high proliferation. In response to the mitogen ConA, our internal positive control, cells moderately respond. In the presence of the non specific antigen *Mycobacterium tuberculosis* protein purified derivate (PPD) cells do not proliferate as in the absence of antigens in culture (baseline proliferation). Data is represented as mean value of the ^{3}H-dT incorporation (cpm) detected at beta-counter ± SD.

After three days in culture with sublethally irradiated splenocytes, which function as antigen presenting cells, T cell blasts were isolated by density gradient. SJL.PLP4 T cell blasts were diluted in PBS and $3 \cdot 10^{6}$ cells per mouse were injected intravenously in SJL wild type and SJL/L-selectin-deficient mice (n=3). Daily clinical symptoms and weight change were scored and are reported here as mean values ± standard deviation, in Figure III.3.2. The day of the onset of disease in SJL wild type (8.33±0.58) was comparable to

the disease onset in SJL/L-sel$^{-/-}$ mice (8.67±1.15). The disease curves for both mouse groups proceeded in parallel and reached the peak of severity on day 10-17 post injection (p.i.). SJL wild type mice (blue curve) developed a non-significant slightly less severe disease compared to the SJL/L-sel$^{-/-}$ mice (red curve) and had a second peak of disease around day 18 p.i. Within the observation period (30 days) mice were not able to recover and were sacrificed when they still had severe disease. Observing the weight change graph, after injection of SJL.PLP4 blasts, mice increased their body weight and then, as clinical symptoms started to be detectable, they gradually lost body weight and reached a peak corresponding to the peak of severity of disease. Interestingly, SJL wt mice lost more weight compared to SJL/L-selectin-deficient mice. At around day 13 p.i. both mouse groups regularly started to regain body weight, but SJL wt and SJL/L-sel$^{-/-}$ littermates did not fully recover within the observation period. To conclude, injections of SJL wild type PLP-specific SJL.PLP4 T cell blasts in SJL wild type and SJL/L-selectin-deficient recipients induced the development of clinically detectable EAE and induced a loss of body weight. In addition, it is important to mention that, even if SJL wild type mice lost more body weight than SJL/L-selectin-deficient recipients on day 9-12 p.i., this did not correlate with an increased severity of disease. In fact, in the experiment shown here, the SJL/L-sel$^{-/-}$ mice were to develop a slightly more severe tEAE compared to SJL wt mice.

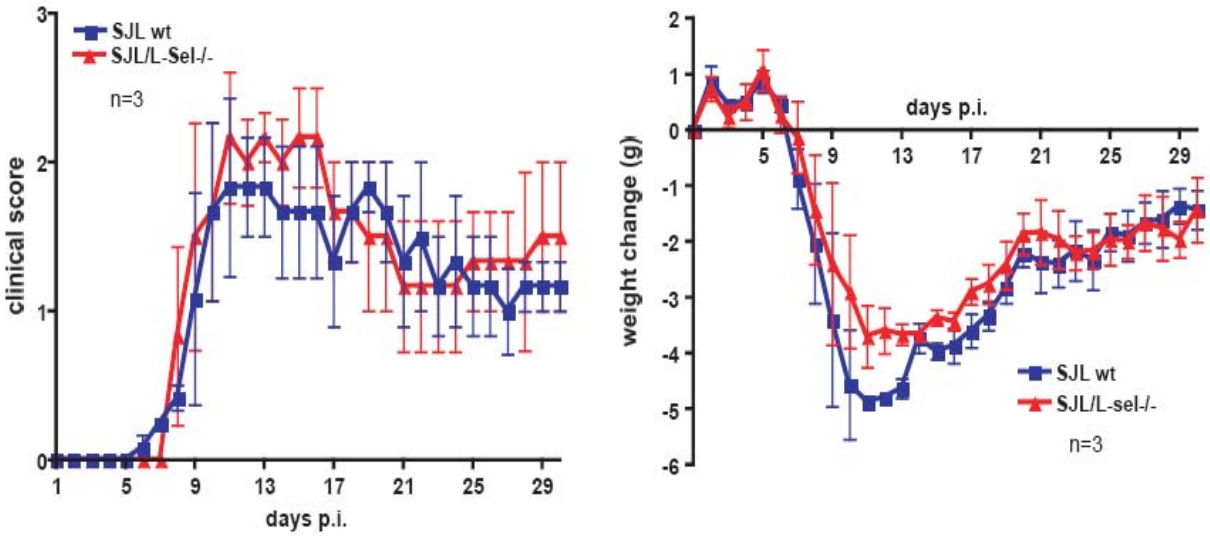

Figure III.3.2. Adoptive transfer of experimental autoimmune encephalomyelitis (tEAE) comparison in SJL wild type and SJL/L-sel$^{-/-}$ littermates. 3·10^6 SJL.PLP4 T cell blasts per mouse were intravenously injected in SJL wt (blue) and SJL/L-sel$^{-/-}$ mice (red). Mice developed parallel disease and there was a significant difference in the severity of disease. In correspondence to the development of disease, mice lose body weight and are not able to completely recover it within the observation period (30 days p.i.). Data are presented as mean values of clinical score and weight change ± SD. One experiment of four.

tEAE was induced in SJL wt and SJL/L-sel$^{-/-}$ mice four times using different SJL wt PLP-specific T cells lines (Table III.3.1). All T cell lines used were restimulated five times before being injected in mice to transfer EAE. SJL wild type and SJL/L-selectin-deficient mice develop disease around day 8-13 p.i. and there are not significant differences in regard to the day of onset of disease. Concerning the severity of disease, in the case of SJL.PLP4 blasts injection SJL/L-sel$^{-/-}$ mice developed a higher severity of disease compared to the other tests performed. In SJL wild type mice, with the exception of disease induced by SJL.PLP42 blasts, which was comparable with the score detected in SJL/L-sel$^{-/-}$ recipients, SJL wild type mice developed a higher severity of disease than their counterparts.

SJL wt PLP-specific T cell lines injected	**Day of onset of disease**		**Severity of disease**	
	SJL wt mice	**SJL/L-sel$^{-/-}$ mice**	**SJL wt mice**	**SJL/L-sel$^{-/-}$ mice**
SJL.PLP4	8.33±0.58	8.67±1.15	2.33±0.58	2.33±1.15
SJL.PLP36	9.50±0.70	10.0±0.0	1.60±1.50	0.83±0.28
SJL.PLP37	8.50±2.12	13.3±4.94	2.503±0.70	0.75±0.35
SJL.PLP42	9.50±0.70	11.5±0.70	0.50±0. 0	0.75±0.35

Table III.3.1. tEAE comparison in SJL wild type and SJL/L-sel$^{-/-}$ mice. Comparison of the day of onset of disease and severity of disease in four different experiments in SJL wild type (blue) and SJL/L-sel$^{-/-}$ mice (red) injected with $3 \cdot 10^6$ SJL wt PLP-specific blasts per mouse. No significant differences are emerging from the analysis of the day of onset of disease between the two groups analyzed and between different experiments. The severity of disease is apparently higher in SJL wt mice, with the exception of the test performed with SJL.PLP4 and SJL.PLP42 T cell blasts. Data are expressed as mean value ± SD.

The phenotype of PLP-specific SJL wild type T cells was investigated by flow cytometry as already published (Laschinger et al., 2000). The freshly isolated T cell blasts of the T cell line SJL.PLP38 (Figure III.3.3) were collected and 10^5 cells per well plated in a 96 well plate and stained with monoclonal antibody. The monoclonal antibodies used were directed against the IL-2 receptor (see Figure III.1.3), to investigate if there was an impairment in cell proliferation. Antibodies directed against cell surface molecules involved in lymphocyte homing and transmigration were also used, such as α_4, $\alpha_4\beta_7$, β_1 and β_7 integrin, and PSGL-1, which is involved in the rolling of lymphocytes on HEV. CD45RB was investigated to distinguish naïve (CD45RB high) and effector/memory T cells (CD45RB low). As positive control CD45 was detected and CD8 as negative control. After 30 minutes, cells were washed and stained with the secondary antibody

PE-coupled. After fixation with 1% (w/v) paraformaldehyde in PBS, cell fluorescence was detected at FACS Calibur (BD, Heidelberg, Germany) and data were analyzed with the CellQuest software (BD, Heidelberg, Germany). Data are here presented as histograms of gated living PE labelled cells (FL2 channel). Freshly isolated T cell blasts are CD45 positive and CD8 negative. The fluorescence intensity detected for LFA-1 and for PSGL-1 was high. Compared to the positive and the negative control, the SJL.PLP38 T cell blasts here analyzed present intermediate levels for CD3, integrins α_4, $\alpha_4\beta_7$, and β_7, and for CD45RB. β_1 integrin was only slightly positive, compared to the isotype control.

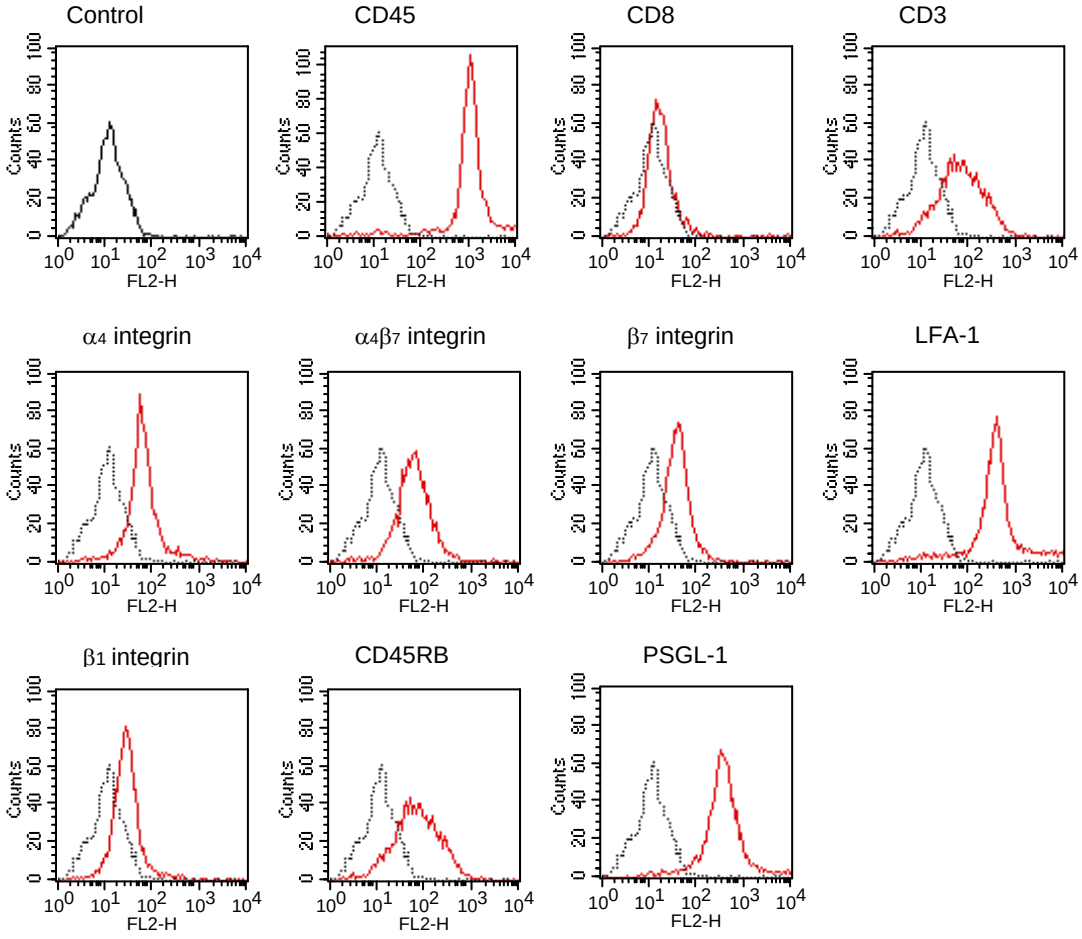

Figure III.3.3. Phenotype of PLP-specific SJL wild type SJL.PLP38 T cell blasts. By flow cytometry was investigated the phenotype of freshly isolated PLP-specific SJL.PLP38 blasts. Blasts are positive for CD45 and PSGL-1, negative for CD8 and only slightly positive for integrins and CD45RB. The isotype control is represented in black. Data is shown as histograms of gated living PE labelled cells and detected at FACS Calibur in FL2 channel.

III.4. Passive transfer of experimental autoimmune encephalomyelitis (tEAE) with SJL/L-selectin-deficient PLP-specific T cells

After the generation of SJL/L-selectin-deficient mice and the induction of aEAE and the transfer of disease with SJL wt T cells blasts, the goal was to transfer EAE using *in vitro* activated PLP-specific SJL/L-sel$^{-/-}$ T cell blasts. For this reason, PLP-specific SJL/L-sel$^{-/-}$ T cell lines were generated. As described in Material and Methods, mice were immunized with an emulsion of CFA and PLP$^{139-151}$. After 10-14 days post immunization, mice were sacrificed by cervical dislocation and spleens were surgically isolated. Organs were homogenized and cells plated in the presence of PLP$^{139-151}$ at the concentration of 10µg/mL. After four days in culture, cells were isolated by density gradient and plated in Petri dishes containing TCGF medium. Under the microscope, cells looked like their counterparts SJL wild type, but it was not possible to maintain them in culture because they all died within 36 hours in the culture. The hypothesis was about an impairment in IL-2 synthesis and IL-2R expression on cell surface. Therefore, freshly isolated SJL/L-selectin-deficient T cells were cultivated in the presence of mouse recombinant (mr) IL-2 and/or IL-15 and compared to cells cultivated in normal TCGF medium. Cells were kept in the culture and, even with difficulties, cells cultivated with mr IL-2 and mr IL-15 were able to survive longer than cells cultivated only with TCGF medium, but did not proliferate. After two days in culture, cells were analyzed by flow cytometry for CD25 and CD122 expression (Figure III.4.1). CD25 was detected by the monoclonal antibody anti-mouse 7D4, PC61 and 3C7; CD122 expression was investigated by the anti-mouse monoclonal antibody TMβ1 and 5H4. IL-2R alpha-chain detection with the antibody 7D4 and PC61 did not show any difference compared to the isotype control. In the presence of mouse recombinant IL-2 and IL-15, using 7D4 and PC61 antibodies could not be detected CD25, suggesting that mouse recombinants did not stimulate cells to synthetize IL-2 receptors. 3C7, which recognizes CD25 on activated T cells, was detectable on all the cells analyzed. In SJL/L-sel$^{-/-}$ PLP7 T cells maintained in a culture medium containing both mr IL-2 and mr IL-15, using 3C7 was detected a double peak: one was negative for 3C7 (naïve T cells), the second one was slightly positive. The presence of mr IL-2 or mr IL-15 in culture media did not increase the amount of CD122 positive detectable.

Because the addition of mr IL-2 and mr IL-15 to cell culture medium did not result in an increase in cell proliferation this approach was dismissed. Thus the strategy was to generate primary PLP-specific T cell lines and to test them *in vitro* and *in vivo*.

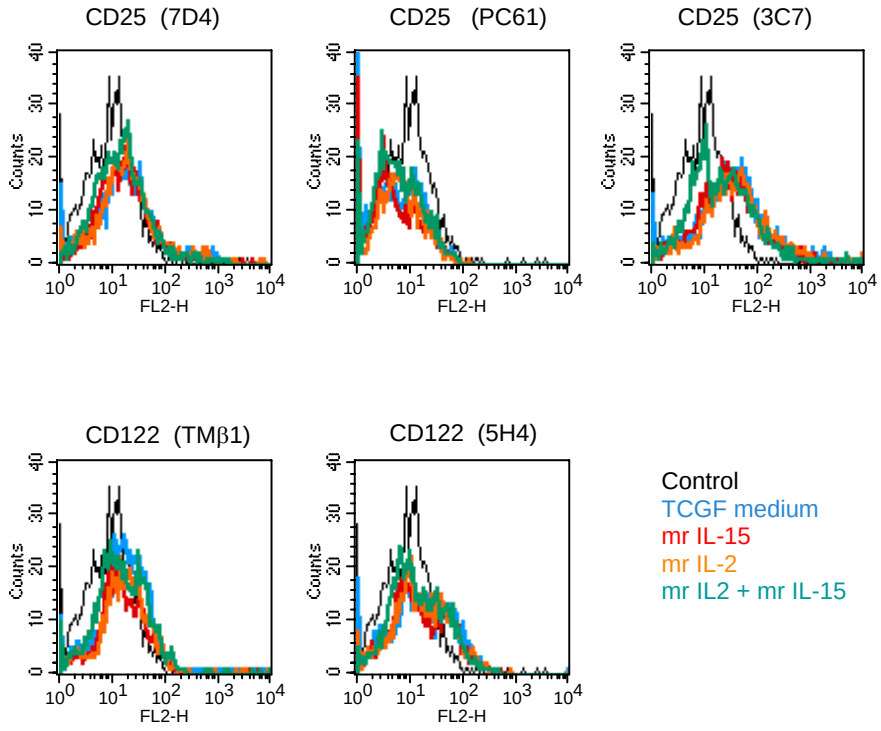

Figure III.4.1. CD25 and CD122 expression of SJL/L-sel.PLP 7 cells cultivated with mouse recombinant IL-2 and IL-15. SJL/L-sel$^{-/-}$.PLP7 T cells were kept in culture in the presence/absence of mr IL-2 and mr IL-15. CD25 is not detectable using the antibody 7D4 and PC61. 3C7, which recognize another epitope of CD25, is slightly positive also in cells cultivated with the normal cell culture medium TCGF. A small portion of CD122 positive cells is present in all cultures. The cells grown in TCGF medium are shown in blue; the cells cultivated in presence of mr IL-15 are shown in red ; the cells grown in presence of mr IL-2 are shown in orange and the cells cultivated with both mr IL-2 and mr IL-15 are represented in green. The isotype control is shown in black.

III.5. Comparison of tEAE induced with primary T cells

In vitro activated primary PLP-specific T cells lines were generated as described in Material and Methods. Briefly, 10-14 days post immunization with CFA and PLP$^{139\text{-}151}$, mice were sacrificed and spleen were isolated, homogenized and splenocytes plated four days in presence of PLP$^{139\text{-}151}$ antigen. After isolation by density gradient, newly generated blasts were cultivated in cell culture. Ten PLP-specific SJL/L-sel$^{-/-}$ primary T cell lines were created in Germany and seven more were generated in Switzerland. All

primary cell lines generated in the laboratory in Münster failed to be maintained in the culture. The seven primary SJL/L-selectin-deficient T cell lines generated in Switzerland could be cultivated in normal TCGF medium. For reasons still unclear, freshly isolated PLP-specific blasts generated in Switzerland could be kept in culture and replicated, but lower than SJL wild type PLP-specific primary T cell blasts. Thus, the phenotype of the primary SJL/L-sel.PLP17 T cells was investigated and compared to the primary SJL.PLP49 T cells. Blasts on day 0 and cells on day 1 and day 3 post gradient were stained to detect CD25, CD122 and CD132, which together form the receptor for IL-2, a cytokine which stimulates cell proliferation. α_4, β_1 and β_7 integrins were also detected, which are involved in lymphocytes homing and diapedesis, and CD45RB to discriminate between naïve (CD45RB high) and effector/memory cells (CD45RB low). PSGL-1, which is involved in lymphocyte rolling on HEV, was investigated using the monoclonal antibody 4RA10. Cells were stained for CD45 as a positive control. The protocol for staining has already been described above. At FACS Calibur (BD, Heidelberg, Germany) the fluorescence intensity of the surface molecules investigated was detected and the data were analyzed using CellQuest software (BD, Heidelberg, Germany). Data are here represented as overlay histograms of gated Thy1.2-FITC positive living cells. Only a few CD25 positive cells are detectable on the surface of SJL/L-sel.PLP17 T cells on day 0 (blue line), day 1 (red line) and day 3 (black line) post gradient (Figure III.5.1). CD122 was not detectable on primary SJL/L-sel$^{-/-}$ PLP-specific blasts and cultivated cells. CD132 expression detected using the monoclonal antibody TUGm2 revealed that on day 0 and on day 1 over half of the SJL/L-sel.PLP17 T cells present IL-2R gamma-chain on their surface. A slight reduction in cell counts and fluorescence intensity was observed on day 3 post gradient. The use of 4G3 to detect CD132 revealed that this antibody recognized only few Thy1.2 positive CD132 positive cells. While β_7 and β_1 integrin were identified only on the surface of few primary SJL/L-sel$^{-/-}$ cells, α_4 integrin recognition was different at each analysis. In fact, on day 0 only few SJL/L-sel.PLP17 T blasts presented α_4 integrin and detection diminished on day 1. On day 3, instead, α_4 integrin was present again on the surface of half of the SJL/L-sel.PLP7 T cells analyzed. SJL/L-sel.PLP17 T cells were effector/memory cells (CD45RB low) but PSGL-1 high.

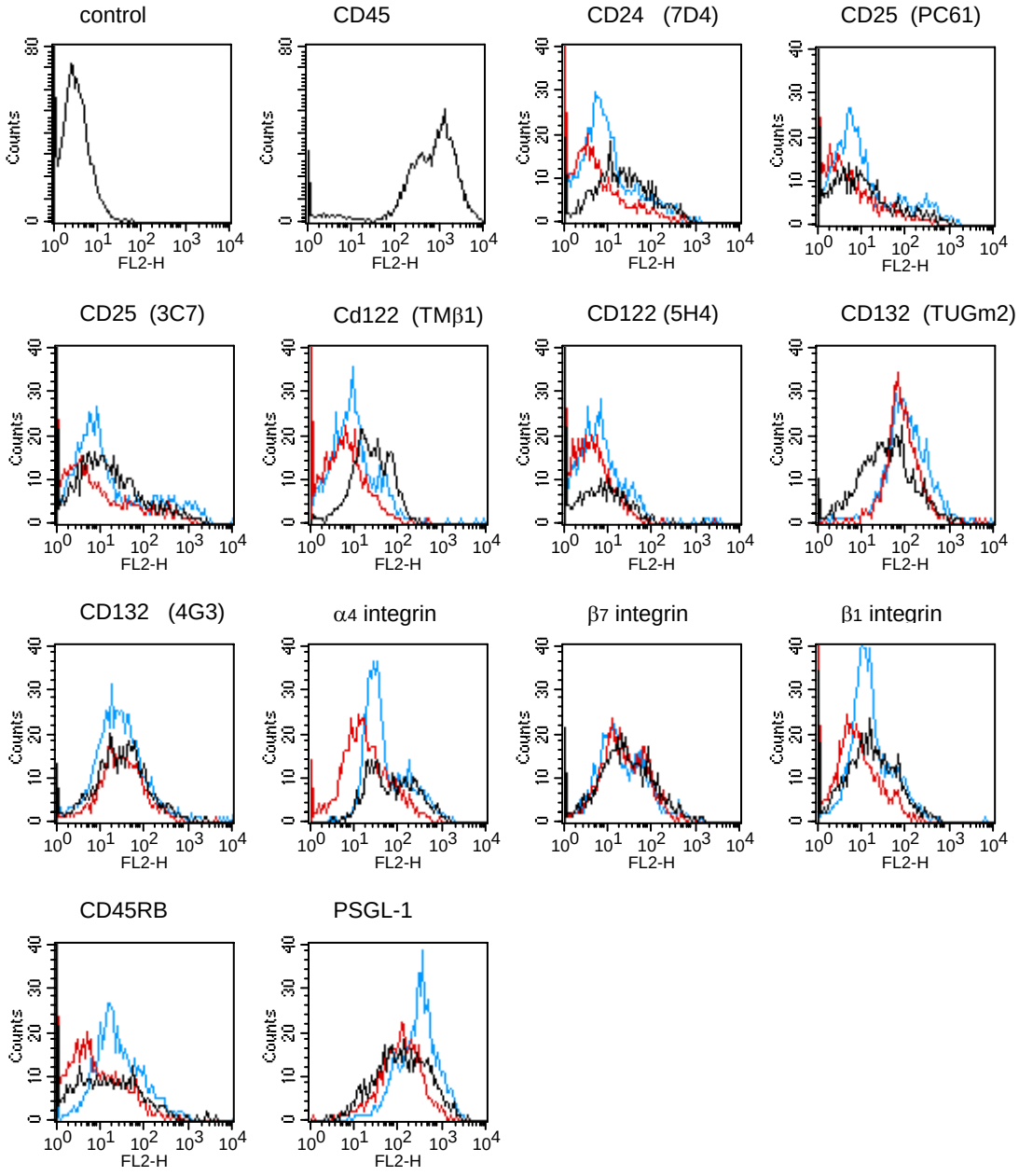

Figure III.5.1. Phenotype of PLP-specific primary SJL/L-sel.PLP17 T cells on day 0, day 1 and day 3 post isolation. Only few SJL/L-sel.PLP17 T cells expressed CD25 and CD122 on their surface. TUGm2 was the only antibody able to recognize the expression of CD132 on SJL/L-sel.PLP17 T cells. Cells were negative for β_7 and β_1 integrins and for CD45RB, and slightly positive for α_4 integrin. PSGL-1 levels on cell surface were high. The analysis on day 0 is represented in blue; the analysis on day 1 is shown in red and the analysis on day 3 post isolation per density gradient is shown in black. Data are shown as histograms overlays of gated Thy1.2-FITC living cells. FL2 indicates the fluorescence intensity of PE positive cells.

In comparison, the phenotype of SJL.PLP49 T cells on day 0 and day 1 and day 3 after isolation was investigated (Figure III.5.2). Only few cells expressed CD25 and CD122 on their surface. An exception was the detection of CD122 by the antibody 5H4. In fact, on

day 1 some CD122 positive cells could be found. Using 4G3 antibody, only few CD132 positive cells were measurable, but using TUGm2 on day 0 and 1, it was possible to identify CD132 on the surface of SJL.PLP49 T cells. α_4 and β_7 integrins were detectable on few SJL.PLP49 primary cells, whilst β_1 levels were slightly positive on day 1. SJL.PLP49 T cells were CD45RB high and PSGL-1 high.

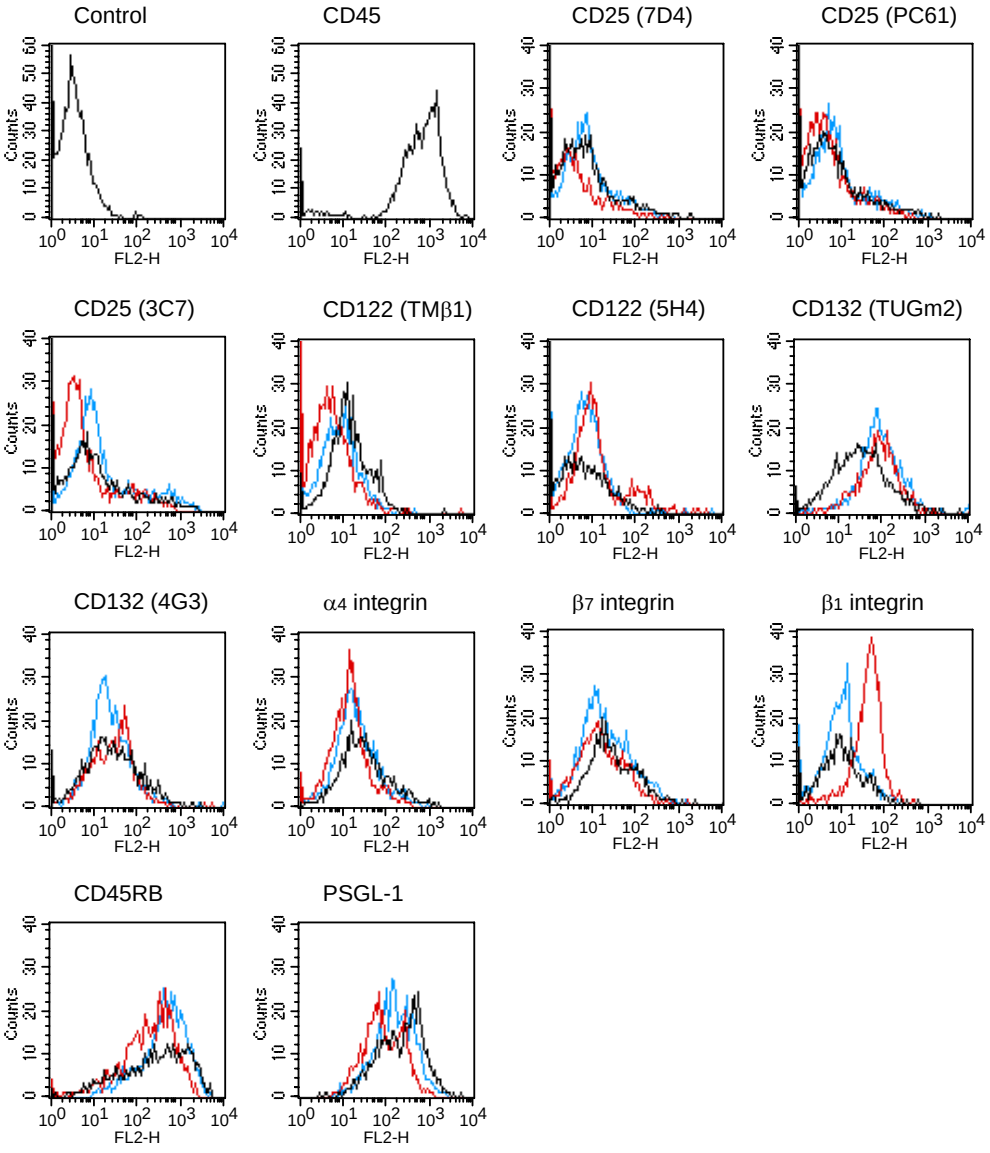

Figure III.5.2. Phenotype of PLP-specific primary SJL.PLP49 T cells on day 0, day 1 and day 3 post isolation. SJL.PLP49 T cells poorly express CD25 and CD122. CD132 is only detectable using the antibody TUGm2. α_4 and β_7 integrins were not identified on SJL.PLP49 T cell surface and β_1 integrin is slightly positive on day 1. Cells were CD45, CD45RB and PSGL-1 positive. Cells analyzed on day 0are represented in blue; in red on day 1 in red and on day 3 post isolation per density gradient in black. Data is shown as histograms overlays of gated Thy1.2-FITC living cells. FL2 indicates the fluorescence intensity of PE positive cells.

To conclude, few SJL/L-sel.PLP17 and SJL.PLP49 primary T cells present CD25 and CD122 on their surface. CD132 was detectable only using the monoclonal antibody TUGm2. α_4 integrin positive cells were detectable on SJL/L-sel$^{-/-}$ primary T cells and on SJL wt primary T cells. SJL.PLP49 cells were slightly positive for α_4 and β_1 integrin and none of the primary cell lines here analyzed presented β_7 integrin on cell surface. PSGL-1 positive cells were detectable on SJL wild type and SJL/L-sel$^{-/-}$ primary T cells on day 0, day 1 and day 3 post gradient. SJL.PLP49 T cells were CD45RB positive, instead SJL/L-sel.PLP17 T cells were effector/memory cells (CD45RB low). Following analysis confirmed the data here shown.

Costimulatory molecules were detected on day 2 post gradient on the surface of SJL.PLP45 (Figure III.5.3) and SJL/L-sel.PLP7 primary T cells (Figure III.5.4). Cells in culture were collected and stained with the primary antibody Thy1.2 followed by a goat anti-rat antibody PE-coupled. Afterwards, cells were stained using hamster anti-mouse CD28, CD152 and CD154 antibody followed by Alexa-Fluor 488, which is detectable at FACS Calibur in FL1 channel. CD28 was investigated because, working with the complex TCR/CD3, promotes T cells proliferation, IL-2 production and cell survival. CD152, known also as CTLA-4, is a negative regulator of T cell activation., also called CD40 Ligand, is present, for a short time upon activation, on the surface of CD4 positive T lymphocytes and belongs to the tumor necrosis factor superfamily. Thy1.2 was used as a positive control and its fluorescence was detected by FL2 channel. On SJL.PLP45 primary T cells at day 2 post gradient, CD28 was detectable on the majority of Thy1.2 positive cells. On SJL/L-sel.PLP7 primary T cells, the amount of CD28 positive Thy1.2 positive cells is reduced compared to SJL.PLP45 primary cells. Low levels of CD152 were detectable on Thy1.2 positive SJL.PLP45 and SJL/L-sel.PLP7 primary T cells, indicating that cells were activated. Intermediate levels of CD40 Ligand were detectable on the surface of Thy1.2 positive SJL.PLP45 and SJL/L-sel.PLP7 T cells. In conclusion, SJL.PLP45 and SJL/L-sel.PLP7 primary T cells, on day 2 post gradient, expressed intermediate levels of CD152 and CD154. On Thy1.2 positive SJL/L-sel.PLP7 primary T cells were detectable intermediate levels of CD28, whilst on Thy1.2 high SJL.PLP45 T cells CD28 was clearly positive, suggesting probably a better proliferation and survival.

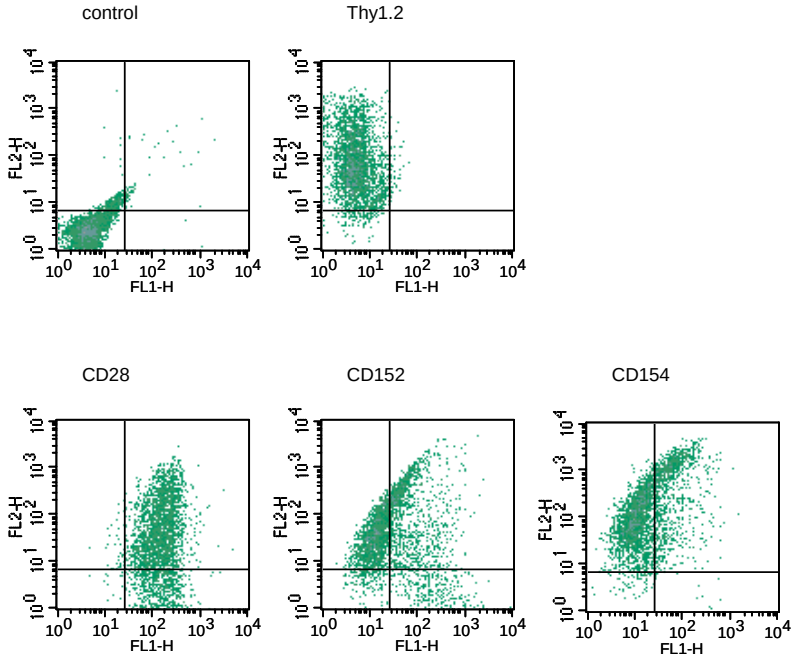

Figure III.5.3. Costimulatory molecules expression on SJL wt PLP-specific T cells. Detection of costimulatory molecules CD28, CD152 and CD154 expression on cells surface of SJL.PLP45 primary T cells on day 2 post gradient. On Alexa-Fluor positive cells are detectable high levels of CD28 and intermediate levels of CD152 and CD154. Data are shown as plots of living gated cells. AlexaFluor 488 positive cells are detected by FL1channel; PE positive cells are detected by FL-2 channel.

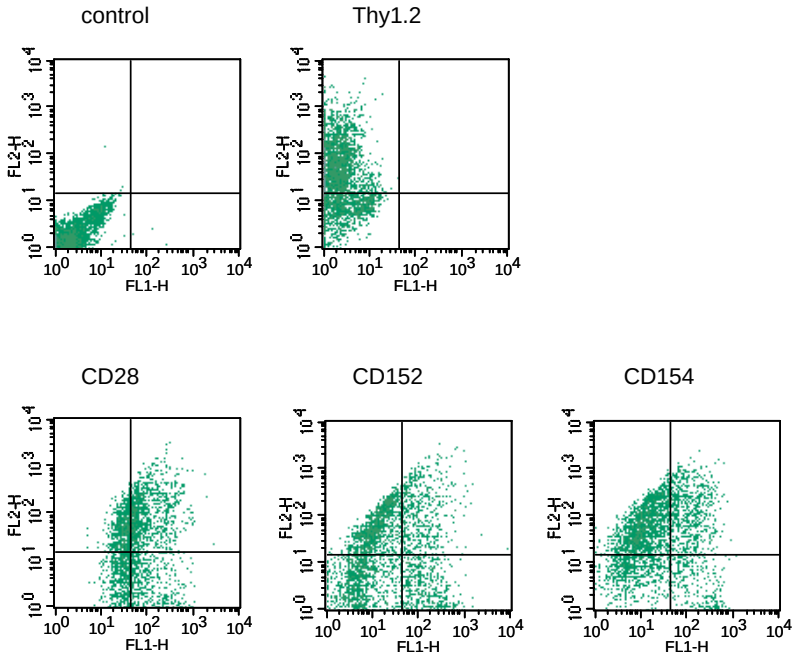

Figure III.5.4. Costimulatory molecules expression on SJL/L-sel.PLP7 T cells. Detection of costimulatory molecules CD28, CD152 and CD154 expression on cells surface of SJL/L-sel.PLP7 primary T cells on day 2 post gradient. On Alexa-Fluor cells are detectable intermediate levels of CD28, CD152 and CD154. Data are shown as plots of living gated cells. AlexaFluor 488 positive cells are detected by FL1channel; PE positive cells are detected by FL-2 channel.

After the characterization of the phenotype of SJL wild type and SJL/L-selectin-deficient PLP-specific primary T cells, the aim was to restimulate these cells co-cultivating them with sublethally irradiated splenocytes in the presence of $PLP^{139-151}$ antigen. First in Germany and then in Switzerland, cells did not proliferate properly in response to their specific antigen. It was then decided to titrate $PLP^{139-151}$ and test if the concentration of 10µg/mL used previously in our assay had to be adapted. The concentration of $PLP^{139-151}$ antigen was titrated using primary SJL wild type and SJ/L-$sel^{-/-}$ T cell lines and also established wild type T cells lines. In Figure III.5.5 a representative experiment of four is shown and it was performed using the primary SJL.PLP57 and SJL/L-sel.PLP20 T cell lines. T cells were cultivated in triplicates in a 96 well plate in the presence of the mitogen Concanavalin A (ConA), of the *M. tuberculosis* protein purified derivate (PPD) and in the presence of $PLP^{139-151}$ at different concentrations. After three days a solution of ^{3}H-Thymidine was added to the cell culture medium and cells were harvested after 16 hours. ^{3}H-dT incorporation and T cell proliferation were measured as counts per million (cpm) using a beta counter. Here data are shown as percentage values and ConA (orange bar) was assumed as positive control having 100% proliferation. Primary cell lines are composed by a mixture of cells specific for different antigens. For this reason, SJL.PLP57 primary T blasts cells have a high proliferation in the presence of PPD (pink bar). Using $PLP^{139-151}$ at the concentration of 100µg/mL (dark blue bar) and 50µg/mL (purple bar) it was not possible to detect any ^{3}H-Thymidine incorporation into the cells. $PLP^{139-151}$ at the concentration of 20µg/mL (blue bar) and 10µg/mL (red bar) induced a proliferation as high as the non specific antigen PPD. 1µg/mL (yellow bar) induced a lower proliferation, probably because the solution did not contain enough antigen to properly stimulate T cell proliferation. Cells were also tested in the absence of antigens (green bar) to give baseline proliferation which was, surprisingly, higher than ConA. In SJL/L-sel.PLP20 primary T cell blasts, surprisingly, cells did not proliferate when stimulated by PPD. In addition, the $PLP^{139-151}$ solutions (100µg/mL, 50µg/mL, 20µg/ml, 10µg/mL and 1µg/mL) induced cells to proliferate significantly more than the baseline values obtained in the absence of antigens, but all values assessed at around 100% and were not significantly different compared to ConA stimulation. Taken together, SJL/L-sel.PLP20 primary T cell blasts presented here, but also in other titration performed with other primary T cell lines, had a less efficient response in the presence of specific and unspecific antigens when compared to SJL wild type primary PLP-specific T cell lines. According to the data acquired, it was assumed that $PLP^{139-151}$ at the concentration of

10µg/mL was satisfactory. In fact, at the concentration of 100µg/mL and 50µg/mL it was not detected proliferation in SJL wild type blasts and the $PLP^{139-151}$ concentration of 20µg/mL did not significantly differ compared to the cell proliferation observed with 10µg/mL.

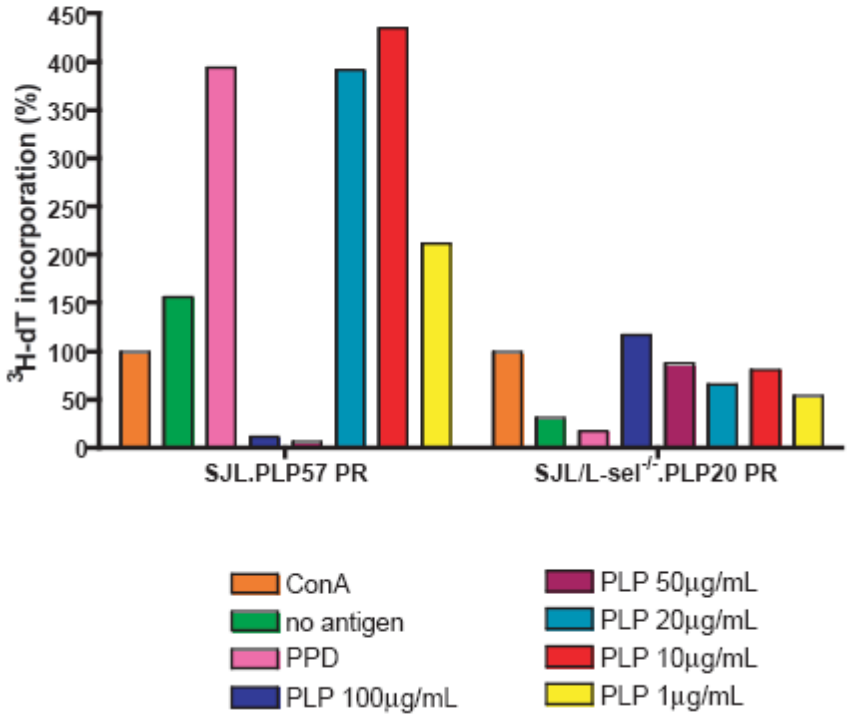

Figure III.5.5. $PLP^{139-151}$ titration in SJL wild type and SJL/L-sel$^{-/-}$ PLP-specific primary T cells. SJL.PLP57 and SJL/L-sel.PLP20 primary T cells were plated in the presence of specific and unspecific antigens. ConA is the positive control within the assay (^{3}H-dT incorporation 100%). SJL.PLP57 primary T cells do not proliferate when stimulated with excessively concentrated solutions of $PLP^{139-151}$ (100µg/mL and 50µg/mL). Cells optimally proliferate in the presence of 20µg/mL and 10µg/mL $PLP^{139-151}$ solutions and at the concentration of 1µg/mL cells have an impaired proliferation. PPD is the non specific antigen which stimulates SJL.PLP57 T cells to proliferate and baseline levels are given by cells plated in the absence of antigens in culture. SJL/L-sel.PLP20 primary T cells blasts have a reduced ^{3}H-dT incorporation compared to their wild type counterparts, but significantly higher than baseline values. Surprisingly SJL/L-sel.PLP20 T cells did not proliferate in the presence of PPD. Data are shown as percentage values of ^{3}H-dT incorporation calculated on the positive control ConA.

However, from the restimulations it was not possible to obtain a sufficient number of T cells to transfer EAE in mice. Thus primary PLP-specific T cell blasts were generated and injected into the SJL wild type and SJL/L-sel$^{-/-}$ recipients. Primary SJL wild type and SJL/L-sel$^{-/-}$ PLP-specific T cell blasts were prepared in parallel and cells isolated were cross-injected in syngenic recipients:

	Injected in
SJL wild type PLP-specific primary blasts	SJL wild type mice SJL/L-sel$^{-/-}$ mice
SJL/L-sel$^{-/-}$ PLP-specific primary blasts	SJL wild type mice SJL/L-sel$^{-/-}$ mice

As an example, the primary SJL.PLP50 T cell blasts are shown here. After the generation of the primary SJL.PLP50 cells, the ^{3}H-Thymidine incorporation and cell proliferation was assessed as described above. SJL.PLP50 primary T cell blasts, as shown in Figure III.5.6, had a high proliferation and ^{3}H-dT incorporation in the presence of the mitogen Concanavalin A. ConA represents the positive control within this assay and induces T cells to proliferate and consequently the measured cpm are elevated. SJL.PLP50 primary T blasts, compared to ConA stimulus, had a significantly reduced proliferation in the presence of the specific antigen PLP$^{139-151}$ at the concentration of 10µg/mL. As explained above, primary cell lines are peculiar: in fact are composed of cells specific for different antigens and cell lines become PLP-specific only after several restimulations performed *in vitro* only in the presence of PLP$^{139-151}$ (i.e. Figure III.3.2). Thus it was unusual to detect less than 1000cpm and was comparable to basal proliferation in the absence of antigens. The data shown here were reproducible at the generation of the other SJL wild type PLP-specific T cell lines.

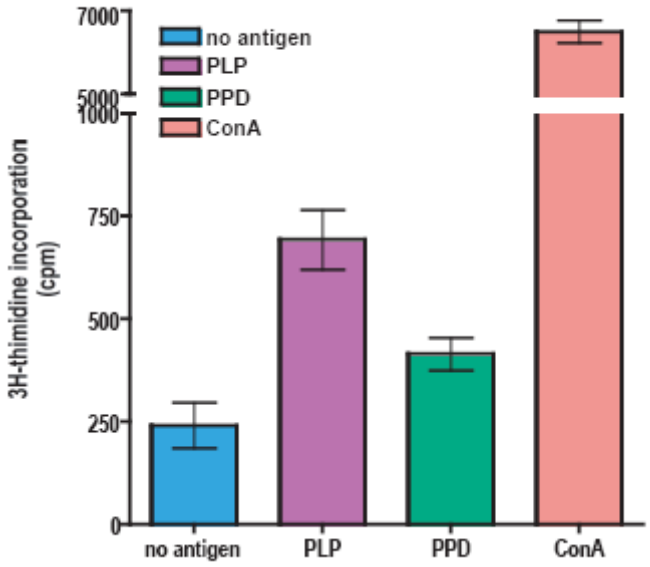

Figure III.5.6. ^{3}H-Thymidine incorporation and proliferation of the primary SJL.PLP50 T cell blasts. SJL.PLP50 T cell blasts massively proliferate when stimulated with the mitogen factor Concanavalin A. PLP$^{139-151}$ induces a proliferation significantly lower than ConA, but higher than the non specific antigen PPD and higher than the basal proliferation obtained in the absence of antigens. Data are presented as mean values of the ^{3}H-dT incorporation measured as count per million (cpm) ± SD.

Because of the specificity for their specific antigen PLP$^{139-151}$, SJL.PLP50 primary T cell blasts were used to transfer EAE in SJL wild type and SJL/L-selectin-deficient mice (Figure III.5.7). After isolation by density gradient, $2 \cdot 10^{7}$ SJL.PLP50 blasts per mouse were intravenously injected into mice. As described in Material and Methods, clinical symptoms and weight change were daily scored. SJL/L-sel$^{-/-}$ mice developed EAE earlier (day 10.33±0.51) than their counterparts SJL wild type mice (day 14.33±0.57). In addition, SJL/L-selectin-deficient mice suffered a significantly more severe disease (2±1) than SJL wt mice (0.58±0.14). SJL/L-sel$^{-/-}$ mice suffered a long disease and they did not recover within the observation period of 23 days. In comparison, SJL wild type littermates not only developed a less severe and delayed disease, but their disease curve was shorter than SJL/L-sel$^{-/-}$ mice and they recovered within 21 days. From the weight change graph it can be observed that the curves relative to SJL wild type and SJL/L-selectin-deficient mice develop parallel. As shown in the weight loss graph, after injection mice gain weight and interestingly SJL wt mice start to loose body weight earlier than SJL/L-sel$^{-/-}$ littermates. Furthermore, SJL wild type mice weight change reached the peak on day 15 and was immediately followed by a gradual, but incomplete reconstitution of the body weight as score at the beginning of the experiment. SJL/L-sel$^{-/-}$ mice, instead, have lost more weight than SJL wt mice and reach their peak on day 17.

Their reconstitution of the body weight seems to be faster than that of their counterparts, but did not reach the original weight within the 23 days of observation.

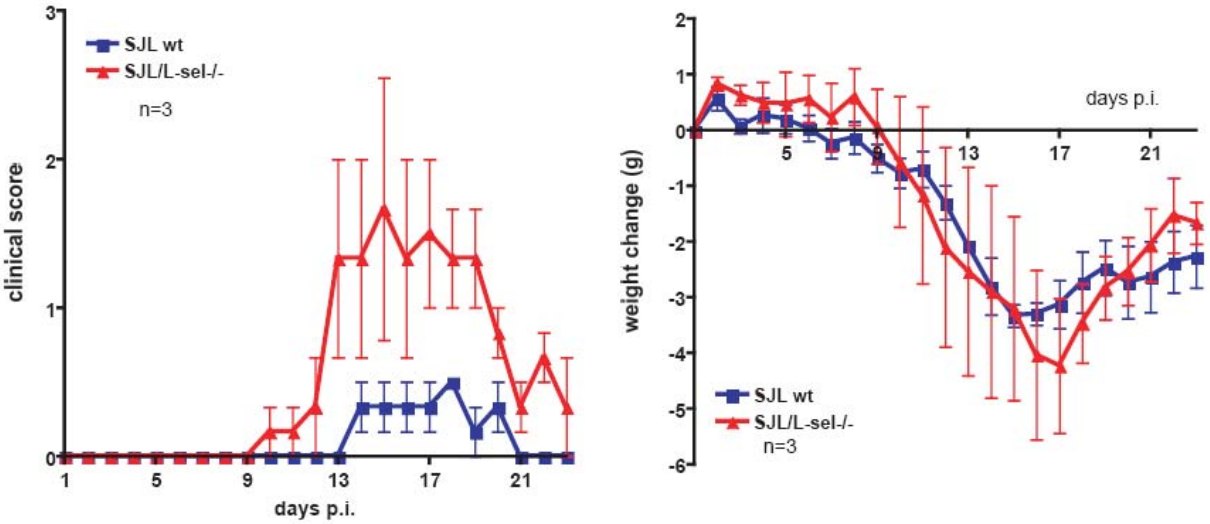

Figure III.5.7. SJL.PLP50 primary T cell blasts induce tEAE in SJL wild type and SJL/L-sel$^{-/-}$ mice. SJL.PLP50 T cell blasts induce more severe tEAE in SJL/L-sel$^{-/-}$ mice than in SJL wt littermates, which rapidly recover completely. After injection, both mouse groups slightly gain body weight, but together with the appearance of the clinical symptoms, mice loose weight and are not able to reach their original body weight within the observation time. Data are presented as mean values of the day of onset of disease and weight change ± SD. One experiment of four.

A summary of the tEAE induce by SJL wild type PLP-specific T cell blasts is shown in Table III.5.1. Only transfer of SJL.PLP50 and SJL.PLP57 PLP-specific primary T blasts induced EAE in SJL wild type and SJL/L-selectin-deficient mice. The day of the onset of the disease, in SJL wt mice, was delayed upon injection of SJL.PLP57 primary blasts compared to the disease onset detected upon injection of SJL.PLP50 blasts. Only SJL.PLP50 blasts induced tEAE in SJL/L-selectin-deficient mice. SJL wild type mice developed mild disease when injected with SJL.PLP50 and SJL.PLP57 primary T cell blasts. SJL.PLP50 blasts transferred a severe disease in SJL/L-sel$^{-/-}$ mice.

In conclusion, transfer of EAE upon injection of primary SJL wild type PLP-specific T cell blasts produced contradictory results. On the one side, only two out of four different primary cell lines induced disease in SJL wild type and in SJL/L-selectin-deficient mice. On the other side, only in one experiment (SJL.PLP50 primary T cells) SJL/L-sel$^{-/-}$ mice developed a significantly higher severity of disease compared to the SJL wild type littermates.

Primary PLP-specific SJL wild type T cell blasts injected	Number of mice per group	Day of onset of disease		Severity of disease	
		SJL wt mice	SJL/L-sel$^{-/-}$ mice	SJL wt mice	SJL/L-sel$^{-/-}$ mice
SJL.PLP50	3	14.33±0.57	10.33±0.51 *	0.58±0.14	2.0±1.0 *
SJL.PLP52	3	No disease		No disease	
SJL.PLP57	4	18.0±1.73	No disease *	0.40±0.40	No disease *
SJL.PLP58	4	No disease		No disease	

Table III.5.1. tEAE comparison in SJL wild type and SJL/L-sel$^{-/-}$ mice induced by SJL wild type PLP-specific primary T cell blasts. Mice developed disease only upon transfer of SJL.PLP50 and SJL.PLP57 primary T cell blasts. Disease was more severe and developed earlier in mice injected with SJL.PLP50 blasts. Data are presented as mean values of the day of onset of disease and severity of disease ± SD. * indicates significant difference.

Five SJL/L-selectin PLP-specific primary T cell lines were prepared to be injected in SJL wild type and SJL/L-sel$^{-/-}$ mice. Two experiments in relation to SJL/L-sel.PLP20 primary T blasts are presented here. In Figure III.5.8 a graph is shown representing the specificity and the proliferation of SJL/L-sel.PLP20 primary blasts. In the presence of their specific antigen PLP$^{139\text{-}151.}$ T cell blasts have a high proliferation in response to the positive control, the mitogen ConA. SJL/L-sel.PLP20 primary T cells have an elevated baseline proliferation as was measured at beta counter for cells plated in the absence of antigens. Higher, but not significantly different, is the proliferation upon stimulation with the specific antigen PLP$^{139\text{-}151}$ at the concentration of 10µg/mL. The counts measured in the presence of PPD, the non specific antigen, were relatively high, as was also seen during the titration experiments for PLP$^{139\text{-}151}$ antigen. This experiment was reproduced for each newly generated primary T cell line and discrepant data about ConA were only obtained in two cases, which did not induce high proliferation

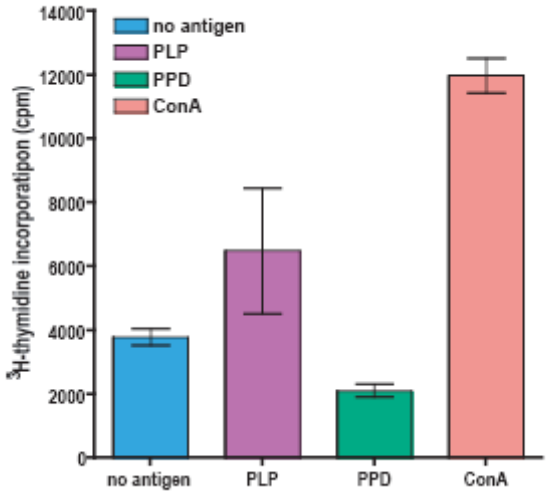

Figure III.5.8. ^{3}H-Thymidine incorporation and proliferation of the primary SJL/L-sel.PLP20 T cell blasts. SJL/L-sel.PLP20 T cell blasts massively proliferate when stimulated with the mitogenic factor Concanavalin A. Cells have an elevated baseline spontaneous proliferation. Upon PLP$^{139-151}$ stimulation, and compared to PPD, cells have a high proliferation. Data are presented as mean values of the ^{3}H-dT incorporation measured as count per million (cpm) ± SD.

tEAE induced with SJL/L-sel.PLP20 primary T cell blasts is shown in Figure III.5.9. SJL wild type and SJL/L-selectin-deficient mice develop disease on day 8-9 post injection and the peak of disease is on day 12 for both mouse groups. In SJL wild type mice, clinical symptoms scored decreased rapidly and mice completely recovered within 27 days of observation. SJL wild type mice, instead, recovered more gradually within the observation period of 35 days. Following T cell blasts injection, as shown in the weight change graph, mice slightly loose their body weight and within three days they reconstitute it. Together with the appearance of the clinical symptoms, mice rapidly have lost body weight and the peak is on day 12, like the peak of severity of disease. Gradually both mouse groups gained body weight, but they did not fully recover within the observation period.

Five different SJL/L-selectin-deficient primary cell lines were injected in SJL wild type and SJL/L-sel$^{-/-}$ mice and results were summarized in Table III.5.2. As for tEAE induced by primary SJL wild type PLP-specific T cell blasts, the results obtained were not conclusive. In fact, only two SJL/L-selectin-deficient primary T cell lines were able to transfer disease in SJL wild type and SJL/L-sel$^{-/-}$ mice. After the adoptive transfer of SJL/L-sel.PLP20 T cell blasts, SJL wild type mice developed EAE earlier than after injection of SJL/L-sel.PLP11 and SJL/L-sel.PLP21 blasts. SJL/L-selectin-deficient mice developed a delayed EAE when injected with SJL/L-sel.PLP11 T blasts compared to

SJL/L-sel.PLP20 primary T blasts, and SJL/L-sel.PLP21 T blasts did not transfer EAE in SJL/L-sel$^{-/-}$ mice. Concerning the severity of the disease, SJL/L-sel.PLP20 T blasts induced a more severe disease than SJL/Lsel.PLP11 and SJL/L-sel.PLP21 T blasts in SJL wt littermates. The disease score in SJL/L-selectin-deficient mice upon transfer of SJL/L-sel.PLP20 primary T blasts was higher compared to the disease scored after injection of SJL/L-sel.PLP11 blasts.

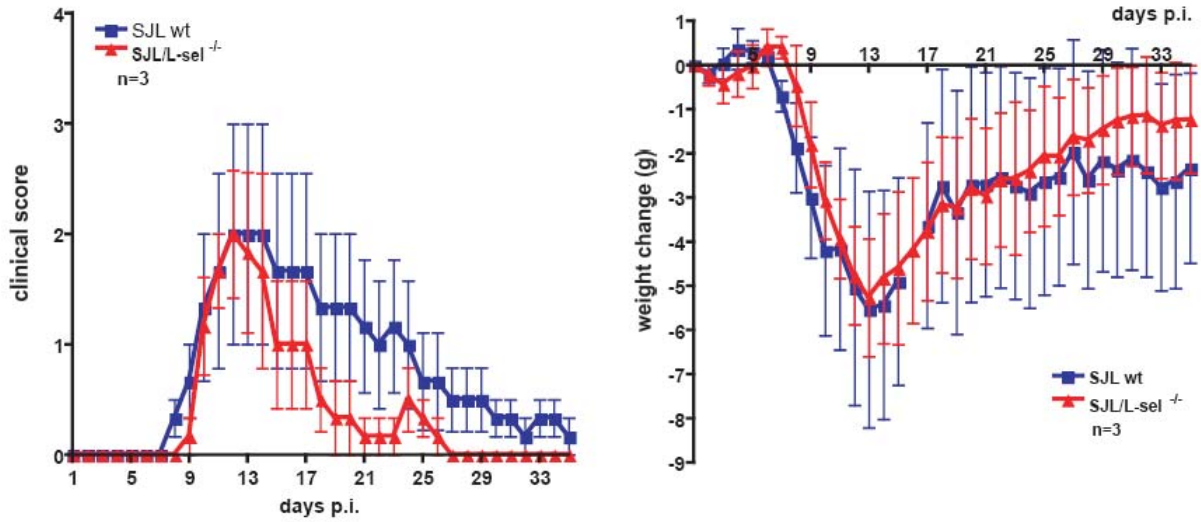

Figure III.5.9. SJL/L-sel.PLP20 primary T cell blasts induce tEAE in SJL wild type and SJL/L-sel$^{-/-}$ mice. SJL/L-sel.PLP20 T cell blasts induce equally severe tEAE in SJL wt and in SJL/L-sel$^{-/-}$ littermates. After injection, together with the appearance of the clinical symptoms, mice loose weight and are not able to reach their original body weight within the observation period. Data are presented as mean values of the day of onset of disease and weight change ± SD. One experiment of four.

Primary SJL/L-sel$^{-/-}$ primary PLP-specific T cell blasts injected	Number of mice per group	Day of onset of disease		Severity of disease	
		SJL wt mice	SJL/L-sel$^{-/-}$ mice	SJL wt mice	SJL/L-sel$^{-/-}$ mice
SJL/L-sel.PLP11	4	12.0±4.24	12.0±1.15	1.25±1.06	0.87±0.25
SJL/L-sel.PLP13	3	No disease		No disease	
SJL/L-sel.PLP18	3	No disease		No disease	
SJL/L-sel.PLP20	3	8.0±0.0	9.66±0.75 *	3.0±1.0	2.0±1.0
SJL/L-sel.PLP21	4	14.5±2.12	No disease *	1.50±0.70	No disease *

Table III.5.2. tEAE comparison in SJL wild type and SJL/L-sel$^{-/-}$ mice induced by SJLL-sel$^{-/-}$ PLP-specific primary T cell blasts. Mice developed disease only upon transfer of SJL/L-sel.PLP11 and SJL/L-sel.PLP20 primary T cell blasts. Data are presented as mean values of the day of onset of disease and severity of disease ± SD. * indicates significant difference.

To summarize, it was not possible to obtain reproducible data from tEAE experiments performed injecting SJL wild type or SJL/L-sel$^{-/-}$ PLP-specific T cell blasts. Some of the primary cell lines generated did not induce the development of EAE in SJL wt and/or SJL/L-selectin-deficient littermates. In addition, concerning the severity of disease scored, there is also variability: in fact SJL.PLP50 induced SJL/L-selectin-deficient mice to develop a more severe disease than their counterparts SJL wild type, and SJL/L-sel.PLP20 cells induced higher severity of disease in SJL wild type mice than in SJL/L-selectin-deficient animals.

During the experiment, because they developed a too severe tEAE, or at the end of the observation period, mice were sacrificed and perfused to isolate the CNS (brain and spinal cord). In Figure III.5.10 the massive infiltration of CD45 positive cells into the meningeal compartment of SJL wild type and SJL/L-sel$^{-/-}$ mice injected with SJL/L-sel.PLP20 primary T cell blasts is shown. CD45 positive cells also massively infiltrate the spinal cord in SJL wild type and SJL/L-selectin-deficient mice (Figure III.5.11) Mice were perfused on day 11 p.i. because they developed a severe disease (clinical score 3, paraparesis and incontinence). In Figure III.5.12 are shown CD45 positive infiltrates in SJL wt and SJL/L-sel$^{-/-}$ mice perfused on day 35 post injection with SJL/L-sel.PLP20 primary blasts. CD45 positive cells massively accumulated in perivascular spaces in meningeal spaces and in cerebellum, and only few cells invaded the CNS parenchyma. No difference was seen in SJL wild type mice compared to SJL/L-selectin-deficient mice. In fact, in both mouse strains analyzed, CD45 positive T cells form inflammatory cuffs and accumulate in perivascular spaces. The same results were observed in the spinal cord of SJL wild type and SJL/L-selectin-deficient mice. In addition, as shown in Figure III.5.12, CD45 inflammatory cuffs were still detectable even if mice had fully recovered from disease (see Figure III.5.9). In conclusion, no apparent differences were identified when immunohistochemistry analyses performed on brain and spinal cord section of SJL wild type and SJL/L-selectin-deficient mice were compared. Furthermore, CD45 positive cells detection in mice sacrificed during the acute phase of disease (day 11) or during the phase of recover from disease (day35), did not present any difference in terms of cuffs formation and cell infiltration.

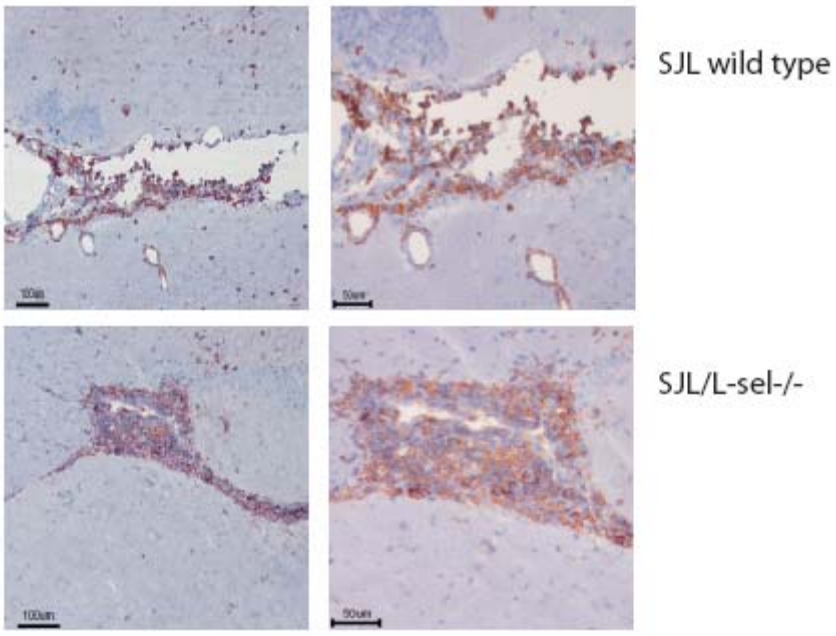

Figure III.5.10. Inflammatory CD45 positive cell infiltrates in brain. SJL wild type and SJL/L-selectin-deficient mice injected with primary SJL/L-sel.PLP20 T cell blasts were sacrificed on day 11 because they developed too severe a disease (clinical score 3). CD45 positive cells massively infiltrate CNS in SJL wild type and SJL/L-selectin-deficient mice (meningeal compartment). On the right side an enlargement of the pictures on the left is shown. CD45 positive cells were detected by the monoclonal antibody M1/9.

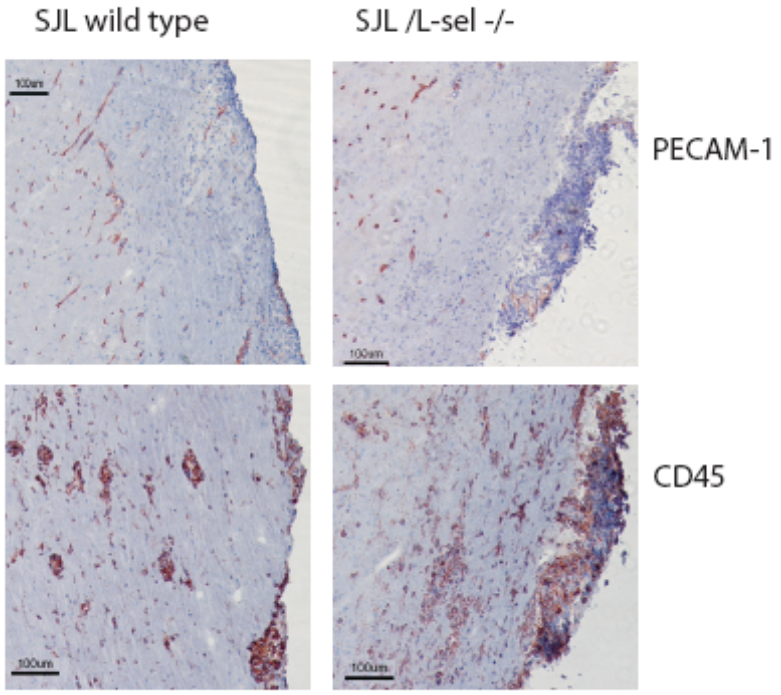

Figure III.5.10. Inflammatory CD45 positive cell infiltrates in spinal cord. SJL/L-sel.PLP20 primary T cells blasts induced high disease in SJL wt and SJL/L-sel$^{-/-}$ mice, which were perfused on day 11 (clinical score 3, foreleg paraplegia and incontinence) CD45 positive cells massively infiltrates CNS in SJL wild type and SJL/L-selectin-deficient mice (meningeal compartment). CD45 positive cells were detected by the monoclonal antibody M1/9; PECAM-1 positive cells were detected by the monoclonal antibody Mec13.3.

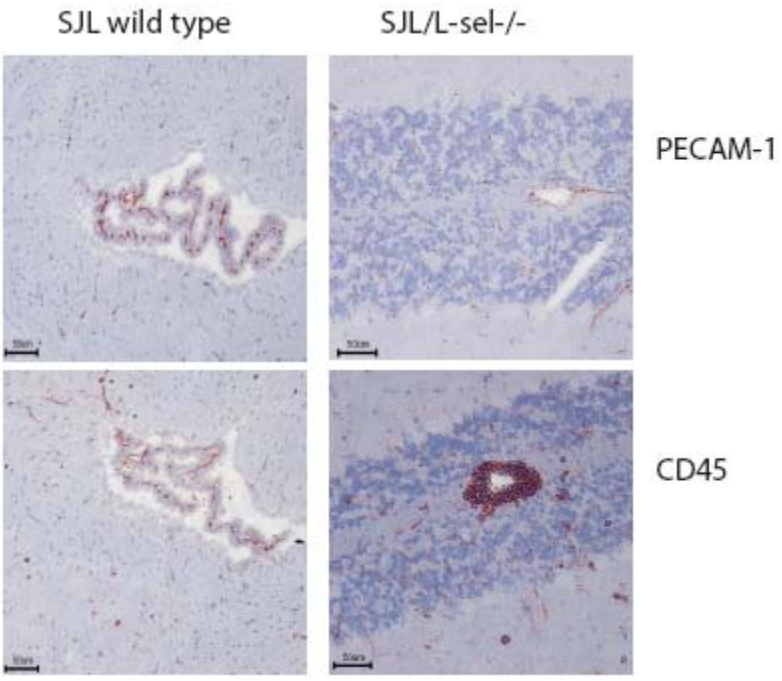

Figure III.5.12. Inflammatory CD45 positive cell infiltrates on day 35 p.i.. CD45 positive cells infiltrate plexus choroideus of SJL wild type and cerebellum of SJL/L-sel$^{-/-}$ mice injected with primary SJL/L-sel.PLP20 T cell blasts and form perivascular inflammatory cuffs as demonstrated by the colocalization with PECAM-1, which stains endothelial cells. CD45 positive cells were detected by the monoclonal antibody M1/9; PECAM-1 positive cells were detected by the monoclonal antibody Mec13.3.

In conclusion, SJL/L-sel$^{-/-}$ T cells could be generated, but when they are restimulated in the presence of their specific antigen PLP$^{139-151}$, there are still difficulties to obtain a sufficient number of blasts to inject and transfer EAE in SJL wild type and SJL/L-selectin-deficient mice. For this reason, primary PLP-specific T cells from SJL wt and SJL/L-sel$^{-/-}$ mice were generated. As was shown in this thesis, contradictory results were obtained from tEAE tests performed using primary PLP-specific SJL wild type and SJL/L-sel$^{-/-}$ T cell blasts. In fact, the SJL wt primary blasts SJL.PLP52 and SJL.PLP58, and the SJL/L-selectin-deficient blasts SJL/L-sel.PLP13 and SJL/L-sel.PLP18 did not induce disease in recipients. In addition, the primary blasts SJL.PLP57 and SJL/L-sel.PLP21 transferred disease in SJL wild type mice, but not in SJL/L-sel$^{-/-}$ littermates. When analyzed by immunohistochemistry, SJL wild type and SJL/L-selectin-deficient mice were positive for the presence of CD45 cells into the CNS, either when they had been injected with SJL wt PLP-specific primary blasts or SJL/L-sel$^{-/-}$ PLP-specific primary blasts. By flow cytometry, it was seen that on SJL wt and SJL/L-sel$^{-/-}$ PLP-specific primary blasts were not detectable CD25 and CD122, and only few cells were positive for CD132, the IL-2R common gamma-chain. α_4 integrin was slightly positive

on T cell blasts, and β_1 and β_7 integrins were not detectable. High levels of PSGL-1 were detected on SJL wt and SJL/L-sel$^{-/-}$ primary T cells. When the costimulatory molecules present on the surface of SJL wild type and SJL/L-selectin-deficient T cells were investigated, CD28 positive cells were detectable in SJL wt primary T cells and only intermediate values in SJL/L-sel$^{-/-}$ cells. Intermediate levels of CD152 and CD154, other two costimulatory molecules investigated and shown in this thesis, were detectable on both cell type analyzed, SJL wt and SJL/L-sel$^{-/-}$ primary T cells on day 2 post gradient.

III.6. Functional *in vitro* assays with SJL wild type and SJL/L-selectin-deficient PLP-specific T cells: Trans-Endothelial Migration (TEM) assay

After the phenotype of PLP-specific SJL wild type and SJL/L-sel$^{-/-}$ T cells was determined and after it was tested *in vivo* the ability of SJL/L-sel$^{-/-}$ T cell blasts to transfer EAE and the susceptibility of SJL/L-selectin-deficient mice to develop EAE, the goal was to test the migratory efficiency of SJL/L-selectin-deficient T cells across and endothelium. Although is well know that L-selectin is not involved in lymphocyte migration across the BBB endothelium, although it has already been exhaustively demonstrated that the capture of circulating lymphocytes at the BBB is mediated by α_4 integrin and ICAM-1, the migration rate of SJL/L-sel$^{-/-}$ PLP-specific T cells in comparison to SJL wild type T cells still had to be clarified. Trans-Endothelial Migration (TEM) assays were performed to answer this question. As described in Material and Methods, brain endothelial cells bEnd5 were plated on laminin coated Transwell® inserts for two days until they formed a confluent monolayer. At this point, 10^5 SJL wt and SJL/L-sel$^{-/-}$ primary T cells per insert were plated on the top of endothelial cells and allowed to migrate through the endothelium. After 4 hours, migrated cells were collected and counted at CASY® cell counter. First the efficiency of SJL.PLP46 and SJL/L-sel.PLP7 primary T cells to migrate across an endothelium stimulated for 16h with the TNFα or across a non stimulated bEnd5 monolayer was assessed (Figure III.6.1). 45-55% of T cells migrated across the endothelium and SJL/L-sel.PLP7 primary T cells migrated slightly more efficiently than SJL.PLP46 T cells. In particular, the SJL/L-sel.PLP7 T cells migrated across a non stimulated bEnd5 monolayer significantly more than SJL.PLP46 cells. The same results were obtained when endothelial cells were stimulated with TNFα: SJL/L-sel.PLP7 cells migrated significantly more than their counterparts SJL.PLP46 primary T cells. No differences in the number of migrated T cells were seen when SJL/L-sel.PLP7 were added on the top of stimulated and non stimulated endothelial cells. Also the number of SJL.PLP46 primary T cells migrating across the bEnd5 monolayer did not differ in the presence or in the absence of TNFα stimulation. This experiment was repeated three times, but it was not possible to obtain reproducible data. In fact, when the migration of SJL wt and SJL/L-sel$^{-/-}$ T cells was tested in another test, different results were obtained (Figure III.6.2). SJL.PLP52 T cells migrated in general significantly more efficiently than SJL/L-sel.PLP14 cells.

Significantly higher was also the number of lymphocytes migrating across TNFα stimulated bEnd5 monolayer compared to non stimulated, in the presence or in the absence of antibodies specific for endothelial cell surface molecule. In this assay, the antibody MJ7/18, which binds to endoglin and does not affect the lymphocyte migration was used as a control. Thus, although blocking endoglin was not impaired the migration of SJL.PLP52 or SJL/L-sel.PLP14 primary T cells across the endothelium, blocking ICAM-1 using YNI a strong reduction in cell migration was observed (as has already been published in Reiss et al., 1999). Surprisingly, SJL.PLP52 T cells migrated better across non stimulated endothelium than through TNFα stimulated bEnd5 cells.

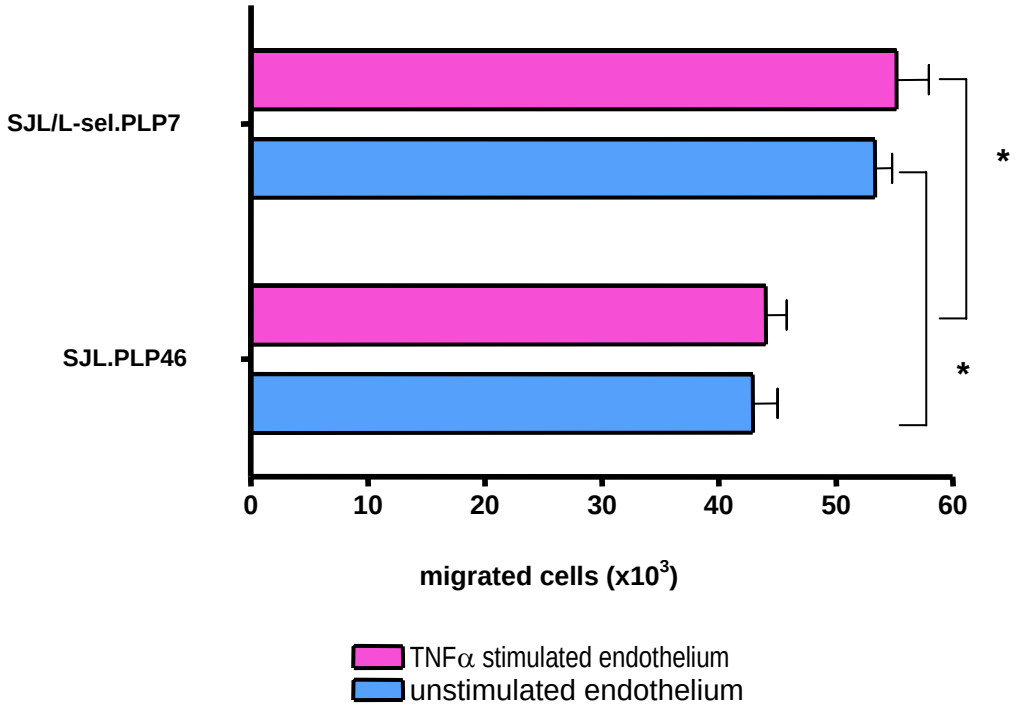

Figure III.6.1. Trans-Endothelial Migration of SJL.PLP46 and SJL/L-sel.PLP7 primary T cells. SJL/L-sel.PLP7 primary T cells migrated significantly more efficiently than SJL.PLP46 T cells across stimulated and non stimulated bEnd5 monolayer. SJL/L-sel.PLP7 and SJL.PLP46 T cells did not migrate more efficiently across TNFα stimulated endothelial cells compared to non stimulated bEnd5 cells. Data is represented as mean values of migrated cells ± SD. Data relative to TNFα stimulated endothelial cells are shown in pink; data relative to non stimulated inserts is shown in blue. One experiment of four. * indicates statistical difference.

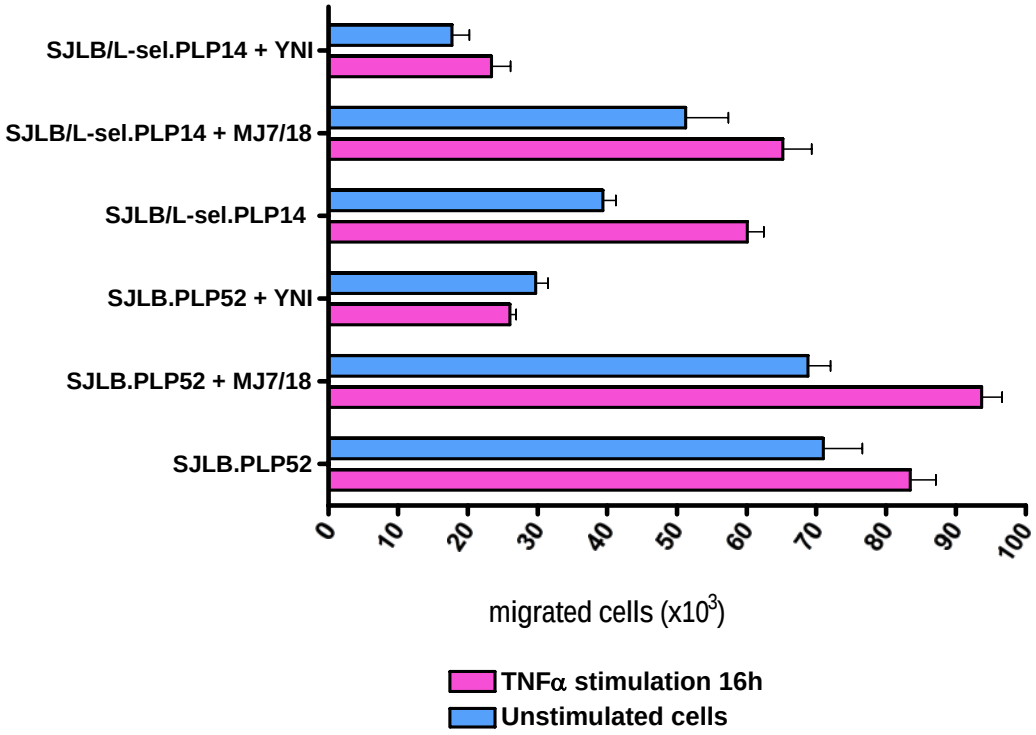

Figure III.6.2. Trans-Endothelial Migration of SJL.PLP52 and SJL/L-sel.PLP14 primary T cells. SJL.PLP52 T cells migrated more efficiently across stimulated and non stimulated endothelium. Blocking endoglin using the monoclonal antibody MJ7/18 did not impair the T cell migration efficiency. Blocking ICAM-1 with the monoclonal antibody YNI impaired the number of cells migrating through the endothelium. Data is represented as mean values of migrated cells ± SD. Data relative to TNFα stimulated endothelial cells are shown in pink; data relative to non stimulated inserts is shown in blue. One experiment of three.

In conclusion, the data relative to the migration of SJL wild type and SJL/L-selectin-deficient across an endothelium were not reproducible. In fact, SJL/L-sel.PLP7 and SJL/L-sel.PLP14 primary T cells migrated more efficiently than SJL.PLP46. When the SJL.PLP52 T cells were tested, was detected a high migration of cells also across the non stimulated endothelium (70%). Accordingly to what has already been published (Reiss et al., 1999), blocking ICAM-1 with the monoclonal antibody YNI there was a reduced migration of both SJL wt and SJL/L-sel$^{-/-}$ primary T cells across a TNFα stimulated and unstimulated bEnd5 monolayer. MJ7/18 was used as a control. In fact, the blocking of endoglin did not affect T cell migration, but rather this antibody was used to verify that the presence of an antibody on endothelial cell surface does not impair lymphocyte migration because of steric problems.

To summarize, SJL/L-selectin-deficient mice were generated backcrossing the original L-selectin-deficient mice (kindly provided by Prof. Dr. Mark Siegelman, Dallas, Texas, USA) into the EAE susceptible SJL mouse strain. The phenotype of SJL/L-sel$^{-/-}$ mice was investigated first by a comparison of the number of cells populating spleen, blood, peripheral and mesenteric lymph nodes. As was demonstrated in this thesis, SJL/L-selectin-deficient mice were characterized by splenomegaly and by a reduction in lymphocytes into the PLN compared to SJL wild type littermates. The lymphocyte number in blood and MLN was not affected by the lacking of L-selectin on the lymphocyte surface. The phenotype of SJL/L-selectin-deficient mice was then investigated by flow cytometry and compared to the phenotype of SJL wild type and L-selectin-deficient mice. The aim was to investigate the presence of the molecules involved in lymphocyte proliferation (IL-2 receptor), in lymphocyte rolling, adhesion and transmigration, such as integrins and PSGL-1, and was also investigated the presence of CD45RB on cell surface, to distinguish between naïve (CD45RB high) and effector/memory cells (CD45RB low). It showed a general impairment in detectable levels of CD25 and CD122 on the surface of SJL wt, SJL/L-sel$^{-/-}$ and L-selectin-deficient splenocytes and also on the surface of PLN and MLN lymphocytes. With the exception of β_1 integrin, which was only present on the surface of SJL wt splenocytes and PLN lymphocytes, only intermediate levels of the integrin α_4, $\alpha_4\beta_7$, LFA-1 and β_7 and were detectable on lymphocyte surface. High levels of CD45RB (naïve lymphocytes) and PSGL-1, which is involved in lymphocyte rolling on HEV, were detectable in all three mouse strains investigated, SJL wild type, SJL/L-sel$^{-/-}$ and L-selectin-deficient mice. As expected, L-selectin was present only on the surface of cells from SJL wild type mice.

After the phenotype of SJL/L-selectin-deficient lymphocytes was determined by flow cytometry, experimental autoimmune encephalomyelitis was actively induced (aEAE) to investigate if L-selectin plays a role in the immunopathogenesis of disease and in the recruitment of inflammatory cells across the BBB. aEAE developed also in SJL/L-sel$^{-/-}$ mice, indicating that L-selectin is not required on effector cells for the development of EAE. Furthermore, SJL/L-sel$^{-/-}$ mice had a disease, in terms of severity of disease, comparable to SJL wild type littermates. By immunohistochemistry the presence of inflammatory CD45 positive cells within the CNS of EAE affected mice was investigated. In SJL wild type and in SJL/L-sel$^{-/-}$ mice, it was possible to identify massive perivascular inflammatory cuffs, in the brain and in the spinal cord. Next the ability of in vitro activated T cell blasts to transfer disease (tEAE) in mice lacking L-selectin was

investigated. tEAE was first induced by the passive transfer of T cell blasts of established SJL wt PLP-specific T cell lines. Again SJL/L-selectin-deficient mice developed disease as SJL wild type littermates. The establishment of SJL/L-selectin-deficient PLP-specific T cells lines failed. It was shown that these cell lines were difficult to cultivate in Germany and exhibited impairment in proliferation. The addition of mouse recombinant IL-2 and IL-15, which are cytokines involved in T cell proliferation, was not successful. It was shown in this thesis that the presence of mr IL-2 and mr IL-15 in cell culture medium did not increase the presence on cells surface of CD25 and CD122, which are components of the IL-2 receptor. The second part of the project was conducted in Switzerland where, for reasons still not clear, it was possible to generate and maintain SJL/L-selectin-deficient PLP-specific T cells in cultures in normal media, without the addition of mouse recombinants IL-2 and IL-15. SJL/L-sel$^{-/-}$ PLP-specific T cells were analyzed by flow cytometry and compared to SJL wt T cells. T cells were stained to detect the presence of IL-2 receptor molecules (CD25, CD122 and CD132) on their surface, the presence of integrins and PSGL-1, which are involved in lymphocyte rolling, adhesion and transmigration, and the presence of CD45RB, to distinguish naïve and memory cells. It was possible to detect CD25 and CD122 on few cells, and CD132 was detectable only using a particular antibody (TUGm2). It was shown that SJL/L-sel$^{-/-}$ primary T cells were only slightly positive for α_4 integrin, but did not present β_1 integrin on their surfaces. Both cell types were PSGL-1 high, but some differences were seen in CD45RB detection. In fact, the SJL wild type PLP-specific cells shown here were CD45RB high, naïve cells, whilst their counterparts SJL/L-sel$^{-/-}$ were effector/memory CD45RB low cells. Primary T cells were also investigated for the expression of costimulatory molecules. Only intermediate levels of CD152 and CD154 were present on the cell surface, CD28, instead, was better detectable on SJL wild type primary T cells. Consequently, SJL/L-selectin-deficient PLP-specific primary T cells were generated and restimulated in the presence of their specific antigen PLP aa 139-151. Unfortunately, cell proliferation was not successful and for this reason it was decided to transfer EAE using newly generated primary T cells. SJL wild type and SJL/L-sel$^{-/-}$ primary PLP-specific cells were generated and were reactivated *in vitro* and injected into syngenic recipients. Only some of the primary cells generated were able to induce disease in SJL wild type and SJL/L-selectin-deficient mice. When mice developed disease, it was not possible to obtain reproducible data. In fact, there was a high variability in the severity of the disease score and in the day of the onset of the disease. Immunohistochemistry investigations

have clearly shown that the presence of massive CD45 positive cell infiltrates into the CNS (brain and spinal cord) of SJL wt and SJL/L-sel$^{-/-}$ EAE affected mice. Thus, when it was evident that mice lacking L-selectin develop EAE and also T cells lacking CD62L on their surface can transfer disease in syngenic recipients, it was assessed, in comparison to SJL wt T cells, the ability of SJL/L-sel$^{-/-}$ primary T cells to cross the endothelium *in vitro*. Trans-Endothelial Migration (TEM) assays were then performed and primary T cells were induced to migrate through an endothelium, which had been previously stimulated or unstimulated with TNFα, a proinflammatory molecule. T cells were able to migrate through the endothelium and it was shown that blocking ICAM-1 using an antibody (YNI), the migration of SJL wt and SJL/L-selectin-deficient primary T cells was reduced compared to the control antibody (MJ7/18).

Finally, it is possible to conclude that L-selectin is not required for the recruitment of encephalitogenic cells across the BBB and for the immunopathogenesis of EAE. Furthermore, it can also be concluded that L-selectin is not required on the surface of CD4 positive T cells to transfer disease in recipient mice.

IV. DISCUSSION

IV.1. Generation of SJL/L-selectin-deficient mice

In order to investigate the role of L-selectin during the immunopathogenesis of experimental autoimmune encephalomyelitis (EAE) SJL/L-selectin-deficient mice were generate by backcrossing the L-selectin-deficient mice into the EAE susceptible SJL mouse strain. The first aim was to investigate the phenotype of the newly generated SJL/L-selectin-deficient mice. In the present study it could be demonstrated that in comparison with the SJL wild type littermates, the SJL/L-sel$^{-/-}$ mice have an increased splenic cellularity and a reduced number of lymphocytes homing to the peripheral lymph nodes. This confirms the phenotype for the L-selectin-deficient mice already published, showing an impairment in the number of lymphocytes in the peripheral lymph nodes (PLN) (Arbones et al., 1994; Catalina et al., 1996) and an increase in splenic cellularity (Steeber et al., 1996). In fact, lymphocytes lacking L-selectin on their surface cannot bind their specific ligands on high endothelial venules (HEV) and they cannot tether and roll on HEV in the peripheral lymph nodes (PLN). Interestingly, it is shown in this work that lymphocytes did not accumulate in the blood of SJL/L-sel$^{-/-}$ mice. The number of lymphocytes in the blood of SJL/L-sel$^{-/-}$ mice ($2.2 \cdot 10^7$ lymphocytes/ml blood) did not differe compared to the amount of lymphocytes in the blood of SJL wild type littermates ($2.5 \cdot 10^7$ lymphocytes/ml blood). It is shown that the lymphocytes accumulate in the spleen of SJL/L-selectin-deficient mice ($5.1 \cdot 10^8$ cells) and their amount was significantly higher compared to the number of lymphocytes counted in the spleen of SJL wild type mice ($2.3 \cdot 10^8$ cells). Within the spleen, circulating T cells extravasate and, driven by chemokines, they accumulate in the white pulp (Gunn et al., 1999; Forster et al., 1999). In the white pulp, T lymphocytes can encounter the antigen presenting cells and become activated (reviewed by Mebius and Kraal, 2005). This demonstrates that backcrossing L-selectin-deficient mice into another mouse strain does not alter the main characteristics of the L-selectin-deficient mice, namely increased splenic cellularity and a reduced number of lymphocytes homing to the PLN. Next, lymphocytes from SJL/L-selectin-deficient mice were investigated by flow cytometry and compared to SJL wild type and L-selectin-deficient mice. In particular, cell surface molecules involved in lymphocyte proliferation (IL-2 receptor) were investigated, as well as adhesion and transmigration of lymphocytes

through the endothelium (PSGL-1 and integrins). Interleukin (IL)-2 is a cytokine secreted by activated T cells and, via an autocrine pathway, binds to its receptor and induces T cell growth and proliferation. The CD4 positive activated T cells transiently express the high affinity IL-2 receptor, which is composed of the alpha-chain CD25, the beta-chain CD122 and the common gamma-chain CD132 on their surface (Minami et al., 1993). The expression of CD25 allows to distinguish between activated T cells, non-activated T cells and regulatory T cells, which are involved in the immunologic self tolerance. In fact, the high affinity IL-2 receptor is only shortly present on the cell surface of activated T cells after antigen presentation, whilst it is constitutively present on the surface of regulatory T cells (Almeida et al., 2002). Non-activated T cells, in contrast, do not express CD25 on their surface, but show the intermediate affinity IL-2R, which is composed by beta- and common gamma-chain (Robb et al., 1981). In this thesis it is shown that only a few (1-5%) percentage of freshly isolated naïve Thy1.2 positive T cells isolated from spleen and PLN of SJL wild type, SJL/L-sel$^{-/-}$ and L-selectin-deficient stain positive for CD25 and CD122 on their surface. The IL-2 receptor beta-chain CD122, is shown to be present on the surface of a few percentage of cells, but high levels of CD132, the common gamma-chain, are detectable on Thy1.2 positive T cells in all cell populations analyzed. The low levels of CD25, compared to CD122 and CD132, suggest that the Thy1.2 positive cells analyzed are naïve cells and present the intermediate affinity IL-2 receptor on their surface. Together with the molecules involved in T cell proliferation, the molecules involved in lymphocyte rolling, such as PSGL-1 were also investigated. In addition, molecules mainly involved in adhesion and transmigration, such as integrins were studied. PSGL-1, which is a L-selectin ligand and mediates leukocyte-endothelium and leukocyte-leukocyte interactions *in vivo* (Sperandio et al., 2003), was shown to mediate the rolling of activated T cells on the vascular endothelium (Atarashi et al., 2005). In fact, whilst naïve lymphocytes express L-selectin on their surface, activated lymphocytes do not present L-selectin on their surface, they require other molecules to roll along the endothelium. Furthermore, it was shown that also E- and P-selectin do not mediate lymphocyte rolling along the blood-brain barrier (BBB) during inflammation (Engelhardt et al., 1997), but rather α_4 integrin and PSGL-1 are required to mediate lymphocyte rolling (Kerfoot and Kubes, 2002). The α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) and the leukocyte-function associated antigen-1 (LFA-1) form stable bonds with their respective ligands on the endothelium and allow the circulating lymphocytes to firmly adhere to the endothelium (Lawrence et al., 1991; Berlin et al.,

1995). In fact, the chemokines CCL19 and CCL21, which are released by the BBB endothelium, were shown to bind to their G-coupled receptor (CCR7) on the surface of the rolling lymphocytes *in vitro* (Alt et al., 2002). By intravital microscopy it was demonstrated that G-protein mediated signalling is required for the firm arrest of encephalitogenic T cells in the spinal cord (Vajkoczy et al., 2001). Via an inside-out signalling, the chemokine receptor CCR7 activates the integrins on the lymphocyte surface and integrins are now able to mediate the firm adhesion of lymphocytes to the endothelium.

In this thesis it is shown that high levels of PSGL-1 are detected on the surface of the SJL wild type, SJL/L-selectin-deficient and L-selectin-deficient lymphocytes, in particular on the surface of PLN lymphocytes. Staining for the integrins is positive, suggesting that breeding L-selectin-deficient mice into the SJL wild type mouse strain did not alter the lymphocyte characteristics and the expression of surface molecules. In general, the newly generated SJL/L-selectin-deficient mice resemble the peculiarities of the original L-selectin-deficient mice reported in the literature (Arbones et al., 1994; Catalina et al., 1996).

IV.2. active experimental autoimmune encephalomyelitis (aEAE)

Experimental autoimmune encephalomyelitis is an animal model for the study of the pathogenesis of multiple sclerosis, an inflammatory demyelinating autoimmune disease of the central nervous system (CNS) in humans. As it is shown in this thesis, after active immunization with the myelin component proteolipid protein (PLP), SJL wild type and SJL/L-selectin-deficient mice develop aEAE, which is triggered by autoaggressive PLP-specific CD4 positive T cells. It is also shown that the severity of the disease in the SJL/L-selectin-deficient mice is comparable to the one scored in the SJL wild type mice. In the SJL mouse strain, EAE is induced by active immunization with the myelin component proteolipid protein (PLP). In particular, the encephalitogenic determinant of PLP was shown to be localized at the amino acids 139-151 (Tuohy et al., 1989). After immunization, naïve lymphocytes are activated in the secondary lymphoid organs and become effector/memory T cells (Krakowski and Owen, 2000). Effector/memory autoaggressive T cells accumulate at the blood-brain barrier (BBB), where they induce

inflammation, and accumulate in the CNS perivascular spaces (Cross et al., 1990). There, the autoaggressive CD4 positive T cells encounter their specific antigen presented by the perivascular macrophages and are re-activated (reviewed by Engelhardt and Ransohoff, 2005). Migratory effector/memory T cells display a distinct phenotype. It was shown that the lymphocytes involved in the early stages of aEAE are CD4 positive effector/memory cells (CD45RB low), which present high levels of CD44 and high levels of LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$) integrins on their surface (Zeine and Owens, 1992; Engelhardt et al., 1995; Engelhardt et al., 1998). Using an anti VLA-4 (very late antigen-1) antibody it could be demonstrated that the binding of lymphocytes to brain vessels of EAE affected rats *in vitro* and accumulating in the CNS *in vivo* was inhibited (Yednock et al., 1992). Based on those observations, it was thought that blocking lymphocyte surface molecules with specific antibodies could be used to interfere with the regular onset of disease. Later on, the anti α_4 integrin antibodies were tested in EAE affected mice and were able to reduce the severity of the disease (Brocke et al., 1999). Antibodies blocking LFA-1 (leukocyte-function associated antigen-1) were also developed and could be used to interfere with the development of EAE. The results were not conclusive. In fact, on the one side some studies have shown the efficacy of the antibodies directed against LFA-1 (Gordon et al., 1995; Lin et al., 1998), on the other side it was demonstrated that the severity of the disease increased upon treatment with anti LFA-1 antibodies (Welsh et al., 1993). The selectin family proteins were also investigated to elucidate their role in inflammation and during the development of EAE. It was shown that E- and P-selectin could not be detected on the BBB endothelium and that antibodies directed against E- and P-selectin failed to prevent the recruitment of inflammatory cells across the BBB and the development of EAE (Engelhardt et al., 1997). In contrast, there is another study where the presence of P-selectin on the BBB endothelium was demonstrated (Kerfoot and Kubes, 2002). Kerfoot and Kubes, in fact, have shown by blocking P-selectin with a specific antibody, that it was possible to reduce the trafficking of encephalitogenic lymphocytes across the BBB. The role of L-selectin in inflammation was first investigated, *in vivo*, by the administration of anti L-selectin (CD62L) antibodies. It was shown that, blocking L-selectin in a peritonitis model, there was a reduced migration of neutrophils, monocytes and lymphocytes within the inflamed peritoneum (Kunkel and Ley, 1996). Using a specific anti CD62L antibody for rats, the role of L-selectin diring the development of active EAE in Lewis rats was investigated (Archelos et al., 1998). Blocking L-selectin reduced the migration of lymphocytes to the secondary lymph nodes

and it was therefore reduced the lymphocyte activation. Thus, the administration of an L-selectin antibody resulted in an amelioration of the severity of the disease and in a reduction of the inflammatory cuffs within the CNS in Lewis rats. In order to be effective, the antibody therapy requires continuous administration of antibodies during the observation time. In addition, the antibody titre needs to be high enough in the blood to be efficient and bind to its target. With the generation of L-selectin-deficient mice, the experimental procedure is simplified and the administration of antibodies *in vivo* is avoided. The generation of L-selectin-deficient mice provides important informations about the role of L-selectin during inflammation. In fact, it was demonstrated that L-selectin-deficient mice have an impairment in monocyte, neutrophil and lymphocyte migration into the chronically inflamed peritoneum (Tedder et al., 1995). Furthermore, it was shown that the L-selectin deficient mice, because of the reduced lymphocyte migration into the PLN, had a significantly reduced mortality upon lipopolysaccharide (LPS) administration compared to the wild type mice (Tedder et al., 1995). In delayed-type hypersensitivity (DTH), an altered cytokine production, a reduced footpad and ear swelling and an impaired T cell proliferation in L-selectin mice was reported, suggesting that L-selectin plays an active role in the generation of primary immune responses during inflammation (Tedder et al., 1995; Catalina et al., 1996). Also, the existence of L-selectin-independent compensatory mechanisms responsible for the priming and the activation of lymphocytes are suggested (Xu et al., 1996). In addition, the L-selectin-deficient mice, compared to wild type littermates, were demonstrated to have a reduced migration of neutrophils, monocytes and lymphocytes into inflammatory sites during contact hypersensitivity (Catalina et al., 1996). In an animal model of asthma performed in L-selectin-deficient mice, it was shown that L-selectin takes part in lymphocyte migration to the lungs during inflammation (Keramidaris et al., 2001).

In this thesis it is shown that SJL/L-selectin-deficient mice develop active EAE which is indistinguishable from the disease developed by SJL wild type mice. In fact, no differences were seen in the day of onset of the disease and in the severity of disease in SJL/L-sel$^{-/-}$ compared to wild type mice. This might suggest that in the absence of CD62L there are L-selectin-independent compensatory mechanisms playing a role in the priming and in the activation of lymphocytes. The compensatory mechanismslead to the activation of L-selectin-deficient naïve lymphocytes and they can become autoaggressive T cells.

However, it was previously reported that C57Bl/6-CD62L-deficient MBP-TCR transgenic mice did not develop EAE following active immunization with MBP (myelin basic protein) peptide (Grewal et al., 2001). These mice were additionally transgenic for the myelin basic protein T cell receptor (MBP-TCR) known to induce severe EAE shortly after immunization with MBP (Lafaille et al., 1994). Furthermore, it was shown that in C57Bl/6-CD62L-MPB-TCR transgenic mice the CNS was not damaged and, unusually for the C57Bl/6 mouse strain, the myelin structure was intact. It is known, in fact, that the CNS in the C57Bl/6 mouse strain is characterized by demyelination and damage upon immunization with MPB peptide. In the SJL/L-selectin-deficient EAE affected mice, in this study, it was possible to find inflammatory cells not only in the perivascular spaces, but also in the CNS parenchyma. On the opposite site, in the MBP-TCR transgenic C57Bl/6-CD62L-deficient mice the CD45 positive inflammatory cells were only detected in the perivascular spaces, while in the MBP-TCR transgenic C57Bl/6 wild type mice CD45 positive cells could be detected in the CNS parenchyma (Grewal et al., 2001). Taken together, it is evident that the SJL and the C57Bl/6 mice have different peculiarities and differences, which generate completely opposite results. In addition, it is clear that the lack of L-selectin on the lymphocyte surface of L-selectin-deficient mice has not the same effect as the antibody inhibition of L-selectin in Lewis rats (Archelos et al., 1996). In rats, an amelioration of the severity of the disease and a reduction in the formation of inflammatory cuffs upon antibody treatment was seen. In the MBP-TCR transgenic C57Bl/6-CD62L-deficient mice EAE did not develop upon immunization with MBP peptide while in contrast, the SJL/L-selectin-deficient mice develop EAE, as their wild type counterparts, following immunization with PLP peptide.
Thus, it is necessary to further investigate the role of L-selectin in the development of active EAE. In fact, is still necessary to verify whether L-selectin is required for the onset of EAE and where lymphocytes are primed and become autoaggressive T cells. It was shown that L-selectin-deficient mice have a reduced number of lymphocytes homing the PLN (Arbones et al., 1994; Catalina et al., 1996) and an increased splenic cellularity (Steeber et al., 1996), thus the spleen seems to be the location where naïve cells are activated and differentiate.

IV.3. Passive transfer of experimental autoimmune encephalomyelitis (tEAE)

In this thesis, it is shown that *in vitro* activated PLP-specific T cell blasts are able to induce disease not only in SJL wild type mice, but also in SJL/L-selectin-deficient littermates. It is shown that mice developed tEAE in 8-13 days upon injection of T cell blasts.

From the literature it is known that, after activation of encephalitogenic T cells *in vitro* and subsequent injection into mice in order to transfer EAE, inflammatory cells do not need to enter the lymph nodes to be activated, but rather, encephalitogenic T cells can directly circulate within the blood, reach the BBB and induce inflammation (Wekerle et al., 1986; Hickey and Kimura, 1988). It was shown that the inflamed CNS produces chemokines (CCL19 and CCL21) which, attracting CD45 positive cells, contribute to the maintenance of neuroinflammation and to the development of EAE (Columba-Cabezas et al., 2003). In Lewis rats, it was demonstrated that there are two waves of autoreactive T cells accumulating in the CNS following adoptive transfer (Flügel et al., 2001). CD4 positive autoaggressive MBP-specific T cells, transgenic for green fluorescent protein (GFP), were injected into Lewis rats and tracked during their migration through the BBB into the CNS. Alexander Flügel has shown that the first group of encephalitogenic CD4 positive MBP-GFP T cells penetrates the CNS few hours after adoptive transfer and accumulates in the CNS parenchyma and in the meningeal compartment. These cells induce CNS inflammation and prepare the environment for the second wave of cells. The second wave of autoreactive T cells is composed by lymphocytes which, upon transfer, recirculated through the lymph nodes, acquired migratory capacity and re-expressed CCR7 on their surface. With this characteristics, these T cells can easily accumulate in the CNS, amplify the neuroinflammation and induce EAE development (Flügel et al., 2001). The phenotype of *in vitro* activated encephalitogenic CD4 positive T cells was extensively investigated in the past. It was shown that murine CD4 positive infiltrating cells display a typical effector/memory phenotype, which is CD45RB low, LFA-1 high, VLA-4 high and L-selectin low (Engelhardt et al., 1998). It was as well shown that L-selectin is not expressed on the surface of activated encephalitogenic T cells at the time of injection (Laschinger and Engelhardt, 2000) because it is cleaved from the cell surface upon activation (Jung et al., 1988). In addition, it was demonstrated that, *in vivo*, encephalitogenic T cells can re-express L-selectin on their surface within three days after injection (C.Alt, PhD thesis, 2003). Thus, the possible existence of L-selectin-

independent mechanisms for T cell activation and the re-expression of L-selectin *in vivo* within three days following injection, could explain how SJL/L-selectin-deficient mice develop EAE upon adoptive transfer of SJL wild type PLP-specific encephalitogenic T cell blasts. In addition, from the literature it is known that blockade of L-selectin with the monoclonal antibody MEL-14 was not successful in the attenuation of tEAE in (PLxSJL)F_1 mice (Brocke et al., 1999). It was shown that even after several injections of MEL-14, the (PLxSJL)F_1 mice developed disease comparable to the control mice. It has to be taken in consideration that in the literature it was also reported that MBP-TCR transgenic C57Bl/6-CD62L-deficient mice did not develop EAE upon passive transfer of *in vitro* activated T cell blasts (Grewal et al., 2001). In fact, it was demonstrated that MBP-TCR transgenic C57Bl/6-CD62L-deficient mice did not develop tEAE either following wild type encephalitogenic T cell blasts transfer, or upon injection of L-selectin-deficient autoreactive T blasts. Thus, Grewal et al., concluded that L-selectin is required on non-lymphocytes. As it is shown in this thesis, *in vitro* activated SJL wild type T cell blasts were able to induce disease in SJL/L-selectin-deficient mice, suggesting that in the SJL background L-selectin is not required for the recruitment of encephalitogenic T cells across the BBB.

IV.4. Generation of SJL/L-selectin-deficient PLP-specific T cell lines

While our laboratory was still located in Germany, it was not possible to establish and cultivate SJL/L-selectin-deficient PLP-specific T cell lines. For reasons that are not understood, in Switzerland SJL/L-selectin-deficient PLP-specific T cells can be maintained in culture, but, compared to the SJL wild type T cell lines, they have an impaired proliferation and it is not possible to perform a tEAE test with these cells.

The first hypothesis was that CD4 positive T cells had an impairment in the proliferation pathway. In the literature, an impairment in cytokine production and T cells response during delayed-type hypersensitivity (DTH) in L-selectin-deficient mice was reported (Xu et al., 1996). In a short term DTH assay, it was demonstrated that interleukin (IL)-2 secretion and T cell proliferation were compromised in L-selectin-deficient mice. In fact, due to the reduced migration of lymphocytes to the PLN, T cells were less activated in L-

selectin-deficient mice compared to wild type animals, and therefore they were only able to secrete low levels of IL-2. IL-2 is known to be a cytokine secreted by CD4 T cells and that stimulates the proliferation of CD4 T cells depending on their activation state (Ashwell et al., 1986). Furthermore, it was demonstrated that IL-2 plays a role in the development and in the survival of memory CD4 positive cells (Dooms et al., 2004). It was shown that CD4 positive cells can undergo apoptosis in the absence of IL-2, and that the IL-2 producing T cells diminish in aged individuals (Haynes et al., 1999). Mouse recombinant IL-2 and IL-15 were reported to stimulate proliferation and survival of activated T cells *in vivo* and *in vitro* (Seder, 1996; Li et al., 2001). IL-15 (Grabstein et al., 1994) has its own specific alpha-chain, but it shares CD122 and CD132 (intermediate affinity IL-2 receptor) with IL-2 (Giri et al., 1995). In agreement with the literature, in the present study SJL/L-selectin-deficient PLP-specific T cells cultivated in the presence of mouse recombinant IL-2 and IL-15 could survive longer in culture than cells cultivated in the absence of those recombinants. It was also shown that on SJL/L-sel$^{-/-}$ T cells, which were cultivated in the presence of mouse recombinant IL-2 and IL-15, CD25 and CD122 were only slightly detectable, suggesting that T cells did not properly proliferate. In contrast, it was shown that in MBP-TCR transgenic C57Bl/6-CD62L-deficient mice the proliferative response of CD4 positive T cells was normal (Grewal et al., 2001). It was shown that the frequency of responding T cells and the T cell specificity for the MBP antigen *in vitro* was not defective in C57Bl/6-CD62L-deficient compared to wild type mice. However, despite their ability to proliferate in response to their specific antigen *in vitro*, these cells were not able to transfer disease in syngeneic recipients. In the present work, instead, it is shown that, in the presence of their specific antigen PLP (10µg/ml), the proliferation of SJL/L-selectin-deficient T cells, derived from the spleen of PLP-primed mice, is reduced compared to cells from wild type mice.

Thus, it is clear that in both SJL/L-selectin-deficient mice and in MBP-TCR transgenic C57Bl/6-CD62L-deficient mice there is an immune response and the activation of T cells might depend on L-selectin-independent mechanisms.

IV. 5. tEAE induced with primary PLP-specific T cell blasts

In the present study it is shown that SJL/L-selectin-deficient T cell lines are established, their proliferation is impaired compared to the SJL wild type PLP-specific T cells. Due to this fact, EAE was induced by adoptive transfer of primary PLP-specific T blasts. The presence of IL-2 receptor molecules and costimulatory molecules on the surface of SJL/L-selectin-deficient PLP-T cells was investigated. Interleukin (IL)-2 is a cytokine responsible for the proliferation of T cells. Following the binding of the T cell receptor (TCR) with its antigen presented by MHC class II molecules on antigen presenting cells (APC), IL-2 is produced and secreted by T cells. Via an autocrine pathway, IL-2 binds to its receptor, which is composed by three chains: CD25, CD122 and CD132. CD122 (beta-chain) and CD132 (common gamma-chain) are constitutively present on the T cell surface. CD25 (alpha-chain) expression, instead, is enhanced upon T cell activation. Binding of TCR and MHC class II molecules is not sufficient to fully activate T cells and to trigger the expression of CD25 on the T cell surface. In fact, a costimulatory signal, which is delivered by the CD28-B7 interaction, is necessary (Cerdan et al., 1992; Fraser et al., 1992). CD28 is a costimulatory molecule on the surface of T cells and interacts with the B7 molecules on the APC. The interaction CD28-B7 was shown to amplify the signal generated by TCR and APC (Viola and Lanzavecchia, 1996; Wulfing and Davis, 1998). In fact, it could be shown that the TCR-mediated stimulation in the absence of CD28 mediated costimulation induces T cell anergy and results in the production of a small amount of IL-2 (Powel et al., 1992; Harding et al., 1992). The antagonist of CD28 is CD152 or CTLA-4 (cytotoxic T lymphocyte associated protein-4). CTLA-4, which is also present on the surface of T cells, was demonstrated to have inhibitory effects on IL-2 production and T cell proliferation (Bluestone, 1997). In addition, CD152 was shown to be sequestered intracellularly and to be exposed on the surface of T cells following activation (Perkins et al., 1996). The role of CD28 and CD152 was investigated by the administration of specific antibodies *in vivo* in EAE. It was shown that the blockade of CD28 during *in vitro* activation of encephalitogenic T cells or during the onset of EAE resulted in an attenuation of the severity of the disease in C57Bl/6 mice (Perrin et al., 1999). In addition, C57Bl/6-CD28-deficient mice were seen to be protected from the development of active EAE (Chang et al., 1999). It has to be mentioned that recently a violent reaction to the monoclonal anti CD28 antibody in humans was reported. In March this year, during a clinical trial, the reaction against a new CD28 antibody developed by healthy volunteers so dramatic, that the trial was promptly interrupted and the affected

volunteers were kept at the hospital. Blocking CTLA-4, instead, resulted in the opposite effect. In C57Bl/6 mice, the administration of anti CD152 antibodies exacerbates the disease and enhances the mortality (Perrin et al., 1996), whilst in SJL mice it was shown to enhance T cell reactivity (Karandikar et al., 2000). In the literature, no impairment in the expression of CD28 or CTLA-4 on the T cell surface in the absence of L-selectin is reported. However, in the present thesis it is shown that intermediate levels of CD152 were detected on SJL/L-selectin-deficient and SJL wt T cells, while the presence of CD28 was slightly reduced on the surface of SJL/L-selectin-deficient T cells compared to the wild type, but this did not interfere with their ability to induce tEAE. In fact, it is shown that *in vitro* activated SJL/L-selectin-deficient T cells could induce EAE in syngeneic recipients and massive CD45 positive cell infiltrates were detected in the brain and in the spinal cord of SJL/L-selectin-deficient mice. However, in a previous study, it was demonstrated that C57Bl/6-CD62L-deficient mice did not develop EAE upon adoptive transfer of MBP-specific C57Bl/6 wild type or L-selectin-deficient T cell blasts (Grewal et al., 2001). To test whether the expression of L-selectin on T cells is required to induce EAE, Grewal and Flavell transferred *in vitro* activated T cells in sublethally irradiated non MBP-TCR transgenic C57Bl/6 wild type or CD62L-deficient mice. Wild type C57Bl/6 mice developed tEAE even when injected with C57Bl/6-CD62L-deficent T cells, indicating that L-selectin is not required on T cells to transfer the disease. The C57Bl/6-CD62L-deficent mice, instead, did not develop EAE symptoms, suggesting that L-selectin might be required on other cells than T cells. Therefore, spleens from C57Bl/6-CD62L-deficent mice were homogenized and depleted of their T cell population and injected into MBP-TCR transgenic C57Bl/6 wild type and C57Bl/6-CD62L-deficent mice. It was shown that, following injection of antigen presenting cells (APC), MBP-TCR transgenic C57Bl/6-CD62L-deficent mice developed EAE. To identify which APC transferred the disease in the recipient mice, C57Bl/6-CD62L-deficent mice were reconstituted with macrophages from wild type C57Bl/6 mice before aEAE was induced by immunization with MBP peptide. It could be demonstrated that C57Bl/6-CD62L-deficient MBP-TCR transgenic mice develop acute tEAE following the injection of wild type activated macrophages and, by immunohistochemistry, CNS demyelination was detected. Grewal and Flavell have also shown that macrophages from C57Bl/6-CD62L-deficient mice did not induce EAE symptoms and demyelination in syngeneic recipients. When the effector functions of C57Bl/6-CD62L-deficient macrophages were tested *in vitro*, any abnormality could be seen. In fact, C57Bl/6-CD62L-deficient macrophages

produced proinflammatory cytokines and chemokines *in vitro* as effective as the wild type macrophages. Thus, it was suggested that the absence of EAE symptoms in C57Bl/6-CD62L-deficient mice was due to local events involving L-selectin and was not due to the impaired activation of effector cells.

In summary, it was not possible to obtain conclusive results from the data obtained using SJL/L-selectin-deficient mice, which were shown in this thesis, and from the data obtained by Richard Flavell using C57Bl/6-CD62L-MBP-TCR transgenic mice,. In addition, the opposing results obtained indicate that further investigation are required to elucidate the role of L-selectin during the development of tEAE.

IV.6. Functional *in vitro* assays with SJL wild type and SJL/L-selectin-deficient PLP-specific T cells: Trans-Endothelial Migration

It was demonstrated *in vivo* that SJL/L-selectin-deficient mice are able to develop aEAE and that T cells, generated from SJL wt or from SJL/L-sel$^{-/-}$ mice, can transfer the disease to syngeneic recipients. The next question addressed was the migration efficiency of SJL/L-selectin-deficient primary T cells across a brain endothelium *in vitro*.

The pivotal role of ICAM-1 in the diapedesis of lymphocytes across the endothelium in the periphery and in the CNS was demonstrated *in vitro* using rat endothelial cells (Adamson et al., 1999) and mouse brain endothelial cells (Reiss et al., 1998; Lyck et al., 2003). The central role of LFA-1 in the transmigration of encephalitogenic T cells across the BBB was shown by intravital microscopy in healthy SJL mice (Laschinger et al., 2002). In addition, ICAM-1 was seen *in vivo* to be involved in lymphocyte interaction with the CNS endothelium during inflammation, such as EAE (Steffen et al., 1994) and MS (Sobel et al., 1990), and *in vitro* (Reiss et al., 1998; Reiss and Engelhardt, 1999). In particular the intracellular domain of ICAM-1 is essential for the lymphocyte migration and ICAM-1 mediates intracellular signalling which facilitates the transmigration of CD4 positive T cells across the brain endothelium (Greenwood et al., 2003; Lyck et al., 2003). In fact, it was shown that ICAM-1 intracellularly activates Rho-A *in vitro* (Adamson et al., 1999), and that blockade of the Rho-A dependent pathway inhibits the recruitment of lymphocytes into the inflamed CNS in vivo in Biozzi mice (Walters et al., 2002).

In this thesis SJL/L-selectin-deficient primary PLP-specific T cells were shown to migrate *in vitro* across an endothelial monolayer, even in the absence of proinflammatory stimuli such as TNF-α. Because of the importance of ICAM-1 in the migration of lymphocytes across the endothelium, the endothelial monolayer was pre-treated with an antibody directed against ICAM-1. According with the results already reported in the literature, in the presence of anti ICAM-1 antibodies only 20-30% of the T cells used in the assay migrated across the endothelium in comparison to the control in the absence of antibodies. This suggests that the absence of L-selectin does not interfere with the migration of lymphocytes nor with the presence of ICAM-1 on their surface. In addition, it was already shown *in vivo* that SJL/L-selectin-deficient lymphocytes can cross the BBB and penetrate the CNS.

To summarize, the exact role of L-selectin during CNS inflammation still needs to be clarified. In the past, the role of L-selectin in the pathogenesis of EAE was only addressed using antibodies blocking L-selectin and the results were not conclusive. In fact, anti L-selectin antibodies could only prevent the development of aEAE (Archelos et al., 1998), but not the onset of tEAE (Brocke et al., 1999). The administration of a specific anti L-selectin antibody after the induction of aEAE could significantly delay and suppress the disease in Lewis rats (Archelos et al., 1998). In addition, it was shown that the same antibody, called HLR3, caused a dramatic reduction in lymphocyte homing to the draining popliteal lymph nodes and a reduced T cell activation compared to the control rats. The administration of the HRL3 antibody during the complete observation time only a partially reduced the severity of the disease in Lewis rats upon adoptive transfer of encephalitogenic *in vitro* activated MBP-specific T cells. It was assumed that the inhibition of the disease was caused by the absence of L-selectin on the lymphocyte surface. In fact, blockade of L-selectin impaired lymphocyte trafficking to the PLN. Thus, it was demonstrated that, in Lewis rats, L-selectin is involved in the pathogenesis of EAE. In contrast, in (PLxSJL)F_1 mice the blockade of L-selectin with the monoclonal antibody MEL-14 did not alter the course of the disease (Brocke et al., 1999). While antibodies directed against α_4 integrin and CD44 were able to reduce the infiltration of inflammatory cells in the CNS and prevented the generation of inflammatory cuffs in the

CNS parenchyma, *in vivo* administration of MEL-14 could not prevent the development of adoptive transfer of EAE. In fact, continuous injections of MEL-14 following passive transfer of encephalitogenic T cells could not interfere with the disease progression in (PLxSJL)F_1 mice. Furthermore, antibodies against α_4 integrin and CD44 directly blocked the migration of autoaggressive T cells into the CNS in (PLxSJL)F_1 mice. It was hypothesised that these antibodies blocked not only the first influx of T cells, which occurs in the first few hours after the injection of *in vitro* activated encephalitogenic T cells into syngeneic recipients, but could block also the second influx of activated lymphocytes in the CNS. Therefore, from the data obtained in (PLxSJL)F_1 mice it was concluded that L-selectin has no effect in the immunopathogenesis of EAE and is not involved in the secondary recruitment of lymphocytes in the CNS (Brocke et al., 1999). Afterwards, the role of L-selectin in the development of EAE was studied using L-selectin-deficient mice. Richard Flavell and his collaborators, using C57Bl/6-CD62L-deficient mice transgenic for the myelin basic protein-T cell receptor (MBP-TCR), have shown that L-selectin is required on the effector cells for the development of EAE, and it is required for the entry of inflammatory cells into the CNS parenchyma and for the demyelination of the CNS (Grewal et al., 2001). They have shown that the absence of L-selectin prevented the development of EAE in their mouse model, but did not affect the development, the activation and the differentiation of encephalitogenic T cells. In fact, even if MBP-TCR transgenic C57Bl/6-CD62L-deficient mice did not develop EAE symptoms, inflammatory cuffs were detected in the perivascular spaces of the CNS. So, they concluded that the lack of EAE cannot be due to an inhibition of the lymphocyte migration to the CNS perivascular spaces, but is rather due to the lack of autoimmune T cell migration in the CNS parenchyma. Richard Flavell suggested three hypotheses to explain the fact that MBP-TCR transgenic C57Bl/6-CD62L-deficient mice did not develop EAE. The first hypothesis is that the lack of L-selectin could be responsible for the differentiation of T cells into Th2 cells and not into Th1 cells. However, it was shown that MBP-TCR transgenic C57Bl/6-CD62L-deficient mice developed encephalitogenic T cells, which were able to proliferate and to produce cytokines and to differentiate into Th1 cells as good as their wild type counterpart. The second hypothesis is that the lack of L-selectin could suppress the immune response. The data, by contrast, demonstrated that the immune response in MBP-TCR transgenic C57Bl/6-CD62L-deficient mice *in vivo* and *in vitro* was not impaired compared to their wild type littermates. The last hypothesis suggests that the absence of CD62L inhibits the homing of lymphocytes to the CNS and

their ability to induce demyelination. A precise immunohistochemical analysis revealed that in MBP-TCR transgenic C57Bl/6-CD62L-deficient mice, as in the wild type littermates, the infiltrating perivascular cells are T cells, B cells and macrophages. Thus, it was clear that the failure of MBP-TCR transgenic C57Bl/6-CD62L-deficient encephalitogenic T cells to migrate within the CNS parenchyma prevented the development of EAE, not a general deficiency in lymphocyte migration to the CNS. To explain why encephalitogenic T cells do not migrate within the CNS parenchyma in the absence of L-selectin, Richard Flavell suggested that this is due to the absence of an unknown L-selectin ligand on oligodendrocytes. In fact, lymphocytes were demonstrated to bind to the CNS white matter *in vitro* in a Stamper-Woodruff assay (Huang et al., 1991; Huang et al., 1994). Thus, the binding of L-selectin to its ligand in the CNS can be responsible for the homing of activated lymphocytes to CNS and for the development of inflammation.

In this thesis, in contrast, it is shown that the absence of L-selectin is not affecting the priming and the expansion of inflammatory cells *in vivo*. SJL/L-selectin-deficient mice are able to develop EAE following active immunization with the antigen proteolipid protein. These results confirm that, even in the absence of L-selectin, naïve T cells can enter the lymph nodes and can be activated in response to the PLP antigen, before they migrate across the BBB and induce inflammation in the CNS. Furthermore, CD45 positive inflammatory cells were detected not only in the perivascular spaces, but are also detected in the CNS parenchyma, and no difference between SJL/L-selectin-deficient mice and SJL wild type littermates is seen. In the present study it is also shown that *in vitro* activated CD4 positive encephalitogenic T cells can induce EAE in recipient mice. Despite the results obtained by the group of Richard Flavell, the adoptive transfer of *in vitro* activated established PLP-specific T cell blasts, which originated from SJL wild type T cell line, induce the development of tEAE in SJL/L-selectin-deficient mice. In addition, it is also shown that *in vitro* activated primary T cell blasts generated from SJL/L-selectin-deficient mice are able to induce the development of tEAE in syngeneic recipients.

The contradictory results are due to the different characteristics of SJL and C57Bl/6 mice. In fact, it is known that SJL mice develop a relapsing-remitting EAE, which is characterized by development of the disease followed by a remission of the disease and the disease is accompanied by edema formation. C57Bl/6 mice, instead, develop a chronic disease and the CNS is demyelinised. Besides, the group of Richard Flavell used

mice which were transgenic for the myelin basic protein T cell receptor (MBP-TCR). Because of the constitutive expression of the T cell receptor specific for MBP on the lymphocyte surface, the MBP-TCR transgenic mice are known to develop severe disease shortly after immunization with MBP peptide (Lafaille et al., 1994). In this study, instead, mice were only deficient for L-selectin expression on lymphocytes.

To conclude, the differences detected in this thesis compared to the data obtained by the group of Richard Flavell suggest that the role of L-selectin in the pathogenesis of EAE and in the recruitment of inflammatory cells across the BBB needs to be further investigated. In fact, from the study with C57Bl/6-CD62L-deficient mice it was concluded that the expression of L-selectin on effector cells is important for the demyelination of the CNS in EAE. From the study performed with SJL/L-selectin-deficient mice, instead, it is possible to conclude that L-selectin is not required for the immunopathogenesis of EAE and for the recruitment of inflammatory cells across the BBB. In addition, a lot of discrepancies were also detected in the studies performed by blocking L-selectin with monoclonal antibodies (Archelos et al., 1998; Brocke et al., 1999) compared to the investigations made using L-selectin-deficient mice. It was suggested the existence that in the absence of L-selectin, lymphocytes are primed in the spleen. Priming and activation of naïve lymphocytes in the spleen could explain why SJL/L-selectin-deficient mice developed EAE. Furthermore, it still has to be further investigated whether oligodendrocytes present a novel L-selectin ligand on their surface. The existence of this ligand was shown *in vitro* on frozen sections of EAE affected mice (Huang et al., 1991), but at present the ligand was not yet characterized.

Hence, it is shown that the molecules and the mechanisms involved in the migration of encephalitogenic T cells across the BBB into the CNS are far to be completely understood. This means that the field is open for further investigations.

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A. SUMMARY

The role of adhesion molecules in the recruitment of inflammatory cells across the blood-brain barrier (BBB) and in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS), was already demonstrated. The role of L-selectin in the immunopathogenesis of EAE is still not clarified. In fact, previous investigations were not conclusive. To address whether L-selectin, the homing receptor for lymphocytes to peripheral lymph nodes (PLN), is involved in the development of EAE, SJL/L-selectin-deficient mice were generated. After the characterization of SJL/L-selectin-deficient mice, which were generated backcrossing L-selectin-deficient mice into the EAE susceptible SJL mouse strain, EAE was induced by active immunization (aEAE) with proteolipid protein (PLP) in complete Freund's adjuvant (CFA). The disease symptoms in SJL/L-selectin-deficient mice and in SJL wild type mice were indistinguishable, and in the CNS of EAE affected mice massive CD45 positive cell infiltrates were detected. EAE was also induced by adoptive transfer (tEAE) of encephalitogenic T cell blasts in SJL/L-selectin-deficient and SJL wild type mice. By transfer of SJL wild type PLP-specific T cell blasts in both mouse strains EAE symptoms were detected. It was not possible to generate stable SJL/L-selectin-deficient PLP-specific T cell lines, thus by transfer of primary SJL/L-selectin-deficient and SJL wild type T cells in syngeneic recipients tEAE was induced. Primary PLP-specific SJL/L-selectin-deficient and SJL wild type T cells induced disease in both mouse groups, and in the perivascular spaces and in the parenchyma CD45 positive cell infiltrates were detected. The migration efficiency of primary PLP-specific SJL wild type and SJL/L-selectin-deficient T cells across an endothelium was evaluated *in vitro* by Trans-Endothelial Migration (TEM) assay. SJL/L-selectin-deficient primary PLP-specific T cells migrated across the endothelium and their migration in the presence of anti ICAM-1 antibodies, compared to the isotype control, was reduced. Taken together, it is possible to conclude that L-selectin is not involved in lymphocyte recruitment across the BBB during EAE, but still further investigations are required. In fact, the development of EAE in SJL/L-selectin-deficient mice suggests that lymphocytes can be primed in other organs than PLN, such as in spleen, and suggests the existence of L-selectin ligands in the CNS, which are not yet identified.

B. LIST OF ABBREVIATIONS

^{3}H-dT	^{3}H-deoxy Thymidine
aa	amino acid
aEAE	active experimental autoimmune encephalomyelitis
AEC	3-amino-9-ethyl-carbazol
APC	antigen presenting cells
BBB	blood-brain barrier
bp	base pair
CFA	complete Freund's adjuvant
CNS	central nervous system
ConA	Concanavalin A
ConAS	Concanavalin A supernatant
ddH_2O	double distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTPs	mixture of dATP, dCTP, dGTP, dTTP
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene-diamide-tetra-acetic acid
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocynate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-pipirazineethansulfonic acid
HEV	high endothelial venules
HRP	horseradish peroxidase
IL-2	interleukin-2
L-sel$^{-/-}$	L-selectin-deficient
LPS	lipopolysaccharide
MAM	migration assay medium

MBP	myelin basic protein
MEM	
MESPA	3-aminopropyltrymethoxysylane
MHC	major histocompatibility complex
min	minute
MOG	myelin oligodendrocytic glycoprotein
MS	multiple sclerosis
NK cells	naural killer cells
NMS	normal mouse serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PD	petri dishes
PFA	paraformaldehyde
PLP	proteolipid protein
PPD	purified protein derivate from *Mycobacterium tuberculosis*
pymT	polyoma middle T oncogene
RPMI	Roswell Park Memorial Institute
rpm	rounds per minute
RT	room temperature
SA	streptavidin
SA/HRP	streptavidin / horseradish peroxidase
Taq	*Termophilus aquaticus*
tEAE	transfer experimental autoimmune encephalomyelitis
TCGF	T cell growth factor medium
TEM	trans-endothelial migration
TNF-α	tumor necrosis factor-α
TJ	tight junctions
TWEEN	polyoxyethylene (20) sorbital manolaurate
wt	wild type

C. LIST OF FIGURES

D. LIST OF TABLES

E. CURRICULUM VITAE

Chiara Uboldi

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G. Ehrenwörtliche Erklärung

Hiermit versichere ich, dass ich bisher noch keinen Promotionsversuch unternommen habe.
Hiermit versichere ich, dass ich die vorgelegte Dissertation selbst und ohne unerlaubte Hilfe angefertigt, alle in Anspruch genommen Quellen und Hilfsmittel in der Dissertation angegeben habe und die Dissertation nicht bereits anderweitig als Prüfungsarbeit vorgelegen hat.