

REGULATION OF THYMIC T CELL PROGENITOR IMPORTATION

by

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ABSTRACT

The thymus does not contain self-renewing T cell progenitors (TCP) and therefore requires continuous importation of progenitors from the blood to sustain T cell production. Recruitment of TCP to the thymus is facilitated by a multistep adhesion cascade initiated by the interaction of PSGL-1 expressed on TCP with P-selectin expressed on thymic endothelium. Thymic TCP importation is not a steady state process but is a periodic, gated event thought to be regulated by a negative feedback signal dependent on the occupation status of intrathymic TCP niches. However the nature of this feedback mechanism remains enigmatic.

The aim of this study is to analyze the underlying mechanism that control thymic TCP importation in a temporal and quantitative manner. The study is based on the hypothesis that ingress of TCP into the thymus is controlled by the regulated expression of key molecules within the thymic adhesion cascade. Indeed, the first set of data shows that P-selectin and CCL25 expression correlate with thymic receptivity. Furthermore, this study finds that P-selectin and CCL25 are periodically expressed in thymic tissues indicating that they are essential parts of the thymic gate-keeping-mechanism. Absence of the P-selectin on thymic endothelial cells or functional P-selectin ligands on TCP significantly reduces the numbers of the earliest intrathymic TCP population and abolishes periodic expression of P-selectin and CCL25 suggesting that niche occupancy plays a role in regulating the periodicity of thymic TCP importation. The study further shows that the size of the peripheral lymphocyte pool directly affects thymic P-selectin expression and TCP receptivity indicating that extrathymic factors also control thymic receptivity. As a feedback signal that could mediate changes in the peripheral lymphocyte pool into altered thymic TCP receptivity

sphingosine-1-phosphate (S1P) was identified. These findings suggest a model whereby thymic TCP importation is controlled by both early thymic niche occupancy and the peripheral lymphocyte pool via S1P.

TABLE OF CONTENTS

ABSTRACT	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF BOXES	ix
LIST OF ABBREVIATIONS	x
ACKNOWLEDGEMENTS	xii
CHAPTER 1: INTRODUCTION	1
1.1 The thymus	1
1.1.2 Function of the thymus	2
1.1.3 The thymus is essential for T cell development	2
1.2 Structure and anatomy of the thymus	5
1.3 Development of the thymus is a highly timed and coordinated process	6
1.3.1 Ontogeny of thymic stromal compartment	7
1.3.2 Maintenance of the adult thymus	10
1.3.2.1 Maintenance of cortical epithelial cells (CEC)	10
1.3.2.2 Maintenance of medullary epithelial cells (MEC)	11
1.4 Various thymic microenvironments support different stages in T cell development	13
1.4.1 Perimedullary-cortex (PMC): Site of TCP entry and retention	13
1.4.2 Inner cortex	14
1.4.3 Outer cortex	15
1.4.4 Subcapsular zone	16
1.4.5 Positive selection in the cortex	17
1.4.6 Medulla and negative selection	18
1.5 Journey of a TCP: from bone marrow to the thymus	22
1.5.1 Progenitors leave the bone marrow niche and migrate through the blood to the thymus	22
1.5.2 Progenitor populations in the bone marrow	24
1.5.3 Progenitor populations in the blood	24
1.6 Seeding of the thymus	26
1.6.1 Tethering and rolling induced by selectins and their ligands	27
1.6.1.1 Genomic and protein structure of selectins	28
1.6.1.2 Regulation of endothelial selectin expression	29
1.6.1.3 Relevant selectin ligands	32
PSGL-1	32
MECA-79 ⁺ ligands	33

1.6.2 Activation of integrins and cell polarization	34
1.6.2.1 Chemokines	34
CCL25 – CCR9	35
CXCR4 – CXCL12 (SDF-1)	36
1.6.3 Firm adhesion	37
1.6.3.1 Integrins and integrin ligands	37
1.6.3.2 Non-integrin ligand CD44	38
1.6.3.3 Ectoenzymes	38
VAP-1 39	
Autotaxin	40
1.6.4 Transendothelial migration	40
1.7 Regulation of thymic TCP homing	43
1.8 Objective	44
CHAPTER 2: MATERIALS AND METHODS	45
2.1 Mouse strains used in this study	45
2.1.1 Congenic marker system	46
2.2 Tissue extraction	46
2.3 Flow Cytometry, fluorescent activated cell sorting (FACS) and cell counting	46
2.3.1 Thymocyte subset analysis	47
2.3.2 Analysis and sorting of thymic endothelial and epithelial cells	47
2.3.3 Analysis of P-selectin protein expression on thymic endothelial cells	50
2.4 Adoptive transfer	51
2.5 <i>In vivo</i> T cell depletion	51
2.6 Quantitative real time PCR (qrtPCR)	52
2.7 FTY720 and DOP treatment	54
2.8 Blood cell count by flow cytometry	54
2.9 S1P measurement by LC/MS/MS	54
2.10 Assessment of short-term receptivity	55
2.11 Intrathymic labeling of cells with fluorophore	56
2.12 Bone marrow transfer	56
2.12.1 Intrathymic bone marrow injection	57
2.13 TREC analysis	57
2.14 S1P dose response using the bend.3 endothelial cell line	59
2.15 Statistics	60
CHAPTER 3: RESULTS	61
3.1 Introduction and rationale	61
3.2 Results	62

3.2.1 Thymi of PSGL-1 and IL-7R deficient mice are hyper-receptive for TCP	62
3.2.2 P-selectin and CCL25 expression correlate with thymic TCP importation	67
3.2.3 P-selectin and CCL25 RNA is periodically expressed in wt but not in PSGL-1 ^{-/-} or C2GnT1 ^{-/-} thymi	72
3.2.4 Thymic niche-occupancy is not the only factor limiting thymic receptivity	80
3.2.5 Thymic emigration of SP T cells is impaired in PSGL-1 ^{-/-} mice	84
3.2.6 Lymphopenic male HY ^{tg} mice express high thymic P-selectin levels	87
3.2.7 Manipulation of peripheral lymphocyte levels affect thymic TCP receptivity	90
3.2.8 P-selectin signaling can be excluded to play a role in regulation of thymic TCP receptivity	92
3.2.9 Plasma derived substances can modulate thymic P-selectin expression	94
3.2.10 Thymic P-selectin expression correlates with plasma S1P levels	96
3.2.11 <i>In vitro</i> S1P and FTY720-P affect endothelial P-selectin expression in a dose-dependent manner	98
3.2.12 Thymic P-selectin RNA expression and TCP receptivity <i>in vivo</i> can be modulated by FTY720	100
3.2.13 Manipulation of the local S1P gradient in lymphoid tissues changes thymic P-selectin RNA expression and TCP receptivity	102
3.2.14 Manipulation of peripheral lymphocyte numbers alters S1P levels in blood	104
 CHAPTER 4: DATA DISCUSSION	 105
4.1 Data summary	105
4.2 P-selectin and CCL25 expression correlate with thymic TCP receptivity	105
4.3 P-selectin and CCL25 are periodically expressed in the wild type thymus ..	107
4.4 Internal and external feedback mechanisms regulate thymic receptivity	109
4.5 S1P as a potential mediator between periphery and thymic vascular gates ..	113
4.6 Regulation of S1P level/gradient	117
4.7 New working model	117
 CHAPTER 5: SUMMARY, MODELS AND FUTURE PERSPECTIVE	 119
5.1 Analyzing TCP entry and T cell exit <i>in vivo</i>	119
5.2 Molecules involved in thymic TCP importation	121

5.3 Model for the internal feedback mechanism	122
5.4 Model for the external feedback mechanism: how does S1P modulate expression of adhesion molecules?	123
5.5 The role of ecto-enzymes in thymic TCP importation	125
5.6 Summarized hypothetical model of internal and external feedback loops regulating TCP entry into the thymus	127
5.7 Potential involvement of P-selectin PSGL-1 interaction in S1P homeostasis	130
5.8 Physiological and clinical significance of thymic TCP importation	132
5.9 Using the cervical thymus to analyze thymic specific properties	134
5.10 Future perspective	135
 REFERENCES	 136
 APPENDIX A: ADDITIONAL DATA	 154
A.1 Thymic emigration impairment in PSGL-1 ^{-/-} mice is cell autonomous	154
A.2 E-selectin is not actively involved in the thymic adhesion cascade	155
A.3 Pregnant mice express high level of thymic P-selectin	157
A.4 PSGL-1 ^{-/-} mice have reduced numbers of naïve T cells in the blood but not in lymph nodes.	159
 APPENDIX B: ANIMAL AND BIOSAFETY PROTOCOLS	 161
 APPENDIX C: PUBLICATION LIST	 166

LIST OF TABLES

Table 1.1: Critical windows in immune-development	7
Table 1.2: T cell progenitor populations	23
Table 2.1: Relevant phenotypes of the used mouse strains	45
Table 2.2: Antibodies used for flow cytometry and FACS	49
Table 2.3: Primer used for qrtPCR	53

LIST OF FIGURES

Figure 1.1 Thymic vascular adhesion cascade	27
Figure 2.1: Formation of TRECs.....	58
Figure 3.1: Thymic P-selectin expression is reduced after transfer of wt bone marrow cells into IL-7R deficient mice.	62
Figure 3.2: Thymic receptivity in wt, Rag-1 ^{-/-} , C2GnT1 ^{-/-} , PSGL-1 ^{-/-} and IL-7R ^{-/-} mice in short- and long-term reconstitution assays.	66
Figure 3.3: Thymic P-selectin expression is increased in hyper-receptive thymi of IL-7R ^{-/-} and PSGL-1 ^{-/-} mice.	68
Figure 3.4: CCL25 RNA is expressed in thymic endothelial cells and is also highly expressed in hyper-receptive thymi of IL-7R ^{-/-} and PSGL-1 ^{-/-} mice.	70
Figure 3.5: P-selectin RNA is periodically expressed in wt thymi but not in spleen.....	73
Figure 3.6: P-selectin and CCL25 RNA are synchronously expressed in wt thymi over time.....	75
Figure 3.7: P-selectin RNA expression only partially correlates with thymic receptivity over time in wt mice.....	77
Figure 3.8: E-selectin, Chst-1 and VAP-1 are up-regulated at times of high P-selectin and CCL25 expression.	79
Figure 3.9: Niche occupancy regulates thymic receptivity in reconstituted IL-7R ^{-/-} mice.	81
Figure 3.10: Loss of functional P-selectin-PSGL-1 interaction is associated with reduced intrathymic ETP content.	83
Figure 3.11: PSGL-1 deficiency is associated with reduced thymic T cell output, intrathymic accumulation of mature T cells and peripheral T cell lymphopenia.	85
Figure 3.12: PSGL-1 deficiency leads to reduced thymic T cell exportation and reduced T cell numbers in blood.....	87
Figure 3.13: Thymic P-selectin expression is modulated by thymic T cell output. ...	89

Figure 3.14: The size of the peripheral lymphocyte pool modulates thymic P-selectin expression and thymic receptivity.....	91
Figure 3.15: P-selectin signaling is not involved in modulation of thymic P-selectin expression.....	93
Figure 3.16: Impact of plasma derived factors on thymic P-selectin RNA expression.....	95
Figure 3.17: Plasma S1P levels correlate with thymic P-selectin RNA.....	97
Figure 3.18: S1P and its analogue FTY720 modulate P-selectin RNA expression <i>in vitro</i>	99
Figure 3.19: Changes in S1P levels are associated with altered thymic P-selectin RNA expression and TCP receptivity.	101
Figure 3.20: Local tissue levels of S1P determine P-selectin expression and thymic receptivity.....	103
Figure 3.21: Plasma S1P levels are modulated by boosting/depleting the peripheral T cell pool.	104
Figure 5.1: High numbers of intrathymic TCP suppress P-selectin expression.....	128
Figure 5.2: Reduced intrathymic TCP numbers lead to increased P-selectin expression.....	129
Figure A.1: Thymic emigration impairment in PSGL-1 deficient mice is cell autonomous	155
Figure A.2: E-selectin is not actively involved in the thymic adhesion cascade ...	156
Figure A.3: Pregnant mice are lymphopenic and show increased thymic P-selectin expression.....	158
Figure A.4: PSGL-1 deficient mice have reduced numbers of naïve T cells in blood but not lymph nodes.	160

LIST OF BOXES

Box 1: VDJ recombination.....	1
Box 2: Thymocyte-TEC interaction is required for a functional thymopoiesis	12
Box 3: Sphingosine-1-phosphate (S1P)	21

LIST OF ABBREVIATIONS

APC	Allophycocyanin
ATCC	American Type Culture Collection
bm	Bone marrow
CEC	Cortical epithelial cells
Chst	N-acetylglucosamine-6-O-sulfotransferase
CLP	Common lymphoid progenitor
DC	Dendritic cell
DN	CD4, CD8 double negative thymocytes
DP	CD4, CD8 double positive thymocytes
EDTA	Ethylene-diamine-tetra acetic acid
ETP	Early T cell progenitor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
H	Hours
HDL	High density lipoproteins
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
IL-7R	Interleukin-7 receptor
Lin ⁻	Lineage marker negative
LSK	Lin ⁻ Sca-1 (Ly6A/E) ⁺ cKit (CD117) ⁺
MEC	Medullary epithelial cells
Min	Minutes
MPP	Multipotent progenitors
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin

PLC	Phospholipase C
PSGL-1	Platelet selectin glycoprotein ligand 1
RAG	Recombination activating gene
RBC	Red blood cells
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
TCP	T cell progenitors
TCR	T cell receptor
TEC	Thymic epithelial cells
TREC	T cell receptor excision circle
TSA	Tissue specific antigen
VAP-1	Vascular adhesion molecule
VCAM-1	Vascular cell adhesion molecule 1

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CHAPTER 1: INTRODUCTION

1.1 The thymus

The thymus is a primary lymphoid organ found in all jawed vertebrates. Its evolutionary appearance is thought to coincide with the emergence of VDJ recombination (see Box 1) as a novel means of somatically diversifying antigen receptors (1). The unique function of the thymus is to support the development of self-tolerant T cells expressing a diverse repertoire of T cells receptors.

Box 1: VDJ recombination

Immunoglobulins in B cells and T cell receptors (TCRs) in T cells are assembled by combinatorially recombining sub-genetic segments of the variable (V), diversity (D) and joining (J) genes. In $\alpha\beta$ T cells the TCR consists of two chains, the α - and the β -chain. Recombination occurs first in the β -chain, by joining one of the $D\beta$ gene segments to one of the six $J\beta$ gene segments. DNA between the recombined loci is deleted and remains as circular double stranded DNA - called T cell receptor circle (TRECs) - in the cells (see section 2.13). DJ-recombination is followed by $V\beta$ -DJ β recombination by joining the DJ complex with one V gene from upstream of the DJ complex thereby deleting any DNA between these two recombination sites. A primary transcript $V\beta$ - $D\beta$ - $J\beta$ - $C\beta$ is then produced including one of the two constant (C) regions while splicing out the other C regions. In addition to the described combinatorial diversity there is also a junctional diversity achieved by randomly adding or subtracting nucleotides at the joints between gene segments. Junctional diversity is mediated by recombination enzymes and terminal deoxynucleotidyl transferases. The α -chain of the TCR is rearranged after the β -chain in a similar way as the latter. One exception is that the first step in α -chain recombination is the V-J recombination step as the α -chain does not contain α D region.

1.1.2 Function of the thymus

The major biological function of the thymus is to provide a microenvironment that allows T cell progenitors to develop into mature T cells and to provide the appropriate quality control to remove self-reactive T cell clones. The Greek physician Galen von Pergamum (130-200 AD) observed that the thymus grows in size and mass until one year after birth and then constantly involutes with age (2). However, it was found that even in the elderly the thymus is still actively producing T cells (3). Until the 1960s the function of the thymus remained enigmatic. Some thought the thymus has only minor endocrine functions while others believed that the thymus was an evolutionary remnant with no functions at all (4). In the early 1960s Jacques Miller observed that removing the thymus in neonates made them incapable of rejecting grafts or fighting infections. Miller described the function of the thymus as follows: “the thymus provides an environment wherein lymphocytes (whether produced in the thymus itself or by the rest of the lymphoid complex) can proliferate much more actively than in other lymphoid organs” (5). This observation led Miller later to discover the two major arms of the adaptive immune system, which are T cells (derived from thymus) and B cells (derived from bone marrow). Miller’s observations helped to understand and identify a variety of immune deficiency disorders that are associated with the absence of a thymus or a not properly functioning thymus.

1.1.3 The thymus is essential for T cell development

From a general perspective lymphoid organs fulfill three functions. First, provide microenvironments to support lymphocyte development. Second, provide the appropriate quality control steps to ensure the formation of a self-tolerant lymphocyte pool. Third,

regulate the efficiency and precision of immune responses (6). Central or primary lymphoid organs like the bone marrow or thymus fulfill the first two functions in a non-redundant manner. The third function is executed by the peripheral or secondary lymphoid organs, which are dispensable for lymphocyte development. The spleen is part of the primary and peripheral system, because in the spleen late B cell development takes place and it also functions as a specialized “lymph node” for blood borne antigens. Until the evolutionary transition from jawless to jawed vertebrates lymphocyte antigen receptor diversity was achieved by a combinatorial system utilizing alternative RNA splicing or gene conversion of antigen receptor genes (7). This template based receptor arrangement resulted in a self-tolerant lymphocyte pool because the receptors were subjected to the evolutionary selection process. However with appearance of VDJ recombination (see Box 1) in primitive vertebrates imprecise joining of gene sections and potential inclusion of non-template nucleotides at the junctions of the V, D or J gene segments led to a truly randomized receptor assembly thereby removing direct evolutionary selection of the assembled receptor (1). While VDJ rearrangement results in huge increase of receptor repertoire compared to the combinatorial system it also produces a great number of self-reactive receptors. Since evolutionary selection cannot directly remove those receptors a “quality control” system was required to remove self-reactive and destructive receptors. It is thought that the development of the VDJ rearrangement created the selection pressure for the evolution of lymphoid organs and resulted in the development of a proto-thymus that was able to provide the required quality control (1, 7).

A recent study proposes that the pharyngeal epithelium of jawless vertebra expanded in cartilaginous fish including the incorporation of novel genes in particular genes encoding

chemokines like CCL25 (7). In jawed vertebrates this led to the evolution of novel gene regulatory networks that control chemokine receptor expression in lymphocytes, finally resulting in the emergence of Delta/Notch signaling as a prerequisite for T cell development (7).

There is evidence suggesting that B cell development is the default pathway for lymphoid progenitors, unless the progenitors encounter an environment that provides Notch signaling predominantly mediated by the delta like 4 ligand (DL4) (8-10). These observations indicate that antibody producing myelolymphoid cells could represent the primordial phenotype of vertebrate lymphocyte lineage and that the T cells lineage and VDJ rearrangement occurred at a later time point (1, 11).

Why do T cells not develop in the bone marrow as most other blood cell lineages? It is still not known, but one speculation is that the mesenchymal origin of the bone marrow stroma might not allow the developmental plasticity that is required to select a self-tolerant T cell population. Furthermore, two “safety reasons” could have required the emergence of an entirely separated organ specialized on T cell development. Firstly, the destructive potential of autoreactive T cells leaking out into the periphery from a poorly separated niche might have favored the development of a thymus (12). Secondly, the requirement for Notch-DL4 interaction in T cell development might also have supported the emergence of the thymus, because ectopic expression of DL4 in the bone marrow induces T cell development in the bone marrow and ablation of B cell development, finally resulting in lethal T cell leukemia/lymphoma (13). The tightly controlled isolated expression of DL4 in the thymic cortex thus promotes efficient thymic T cell development and allows B cell development in the bone marrow.

1.2 Structure and anatomy of the thymus

The thymus was first anatomically described by the Italian Jacopo Berengario da Carpi in the fifteenth century (1). The (thoracic) thymus is located in the upper part of the chest cavity right below the sternum. Recently it was discovered that a second thymus exists in the cervical region in humans and several animals (cervical thymus) (14, 15). The cervical thymus is smaller as the thoracic thymus, mostly mono-lobular and develops asynchronously from the thoracic thymus and produces functional T cells only after birth (12). It is not known yet whether the thoracic and the cervical thymus share a common developmental origin. However the expression of the transcription factor FoxN1 in both thymic tissues suggests that the cervical and thoracic thymi are related (14). This work will only analyze processes in the thoracic thymus and thus the term thymus will only refer to the thoracic thymus.

The thymus, separated from the surrounding by a capsule of dense fibrous tissue, consists of two lobes that are composed of several lobules joined by areolar tissue. A single thymic epithelial progenitor can give rise to one complete lobule suggesting that this structure is the smallest functional unit (12). Thus, the thymic organ can be viewed as a composite structure of several lobule units each arising from one epithelial progenitor (6). However, it is not known whether these units function autonomously or together in creating a self-tolerant T cell pool. The lobules can be distinguished into two anatomically and functionally different zones, which are the outer cortical area and medullary area in the middle. The cortex is comprised of densely packed thymocytes interspersed with a three-dimensional network of epithelial reticular cells. The cortex supports early events in thymocyte development including positive selection. The medulla has a far coarser reticulum

and a lower density of thymocytes. The medulla supports later events in T cell development particularly negative selection.

The thymus consists of non-hematopoietic and hematopoietic cells. The non-hematopoietic cells constitute the “stroma” and include epithelial and mesenchymal cells but also neurons, myocytes, fibroblasts and endothelial cells (16). The other major cellular components in the thymus are hematopoietic cells including macrophages, B cells, T cells, and dendritic cells. The phagocytic properties of the macrophages are required to remove dead thymocytes resulting from the selection processes. Dendritic cells are present in the cortex adjacent to CCL21⁺ capillary vessels (17) and more frequently in the medulla. It has been shown that cortical and medullary DCs are required to efficiently delete self-reactive thymocytes during negative selection process (18).

1.3 Development of the thymus is a highly timed and coordinated process

As for any other tissue system, the ontogeny of the hematopoietic system in mammals is a carefully orchestrated and highly regulated process. In particular for the vertebrate immune system the developmental steps are meticulously timed and coordinated. The ontogeny of the immune system is characterized by a sequence of events that have certain “critical time windows” during which they require input of other parallel processes and thus are very susceptible to interfering signals (immuno-toxins) (Table 1.1). The sequence of the events is the same in all mammals characterized so far, however the timing is different. For example the development of the immune system in rodents is delayed compared to humans. Unlike humans, peri- or neonatal rodents are not fully immuno-competent at birth (19). Several “critical windows” characterize the development and maintenance of the thymus,

especially during the time the thymus is seeded by T cell progenitors. Delay or absence of certain critical events can have dramatic consequences for the immuno-competence of the organism (Table1.1).

Critical window	Consequence when critical step is impaired or absent
Hematopoietic stem cell formation from undifferentiated mesenchymal cells	Failure of stem cell formation, abnormal hematopoiesis, partial to complete immune failure
Migration of hematopoietic stem cells to fetal liver and thymus, early hematopoiesis, and migration of macrophages to tissues	Thymic atrophy; impaired postnatal T cell function, impaired innate immunity, inflammations in organs where macrophages play a role in development (e.g. brain, testis, lung)
Establishment of the bone marrow as a primary site of hematopoiesis, and the bone marrow and thymus as primary lymphopoiesis sites for B and T cells	Increased risk of later-life cancer, autoimmunity, or allergy
Functional development and maturation of immuno competence	Shifted Th1-Th2 balance, which could result in loss of the conceptus during pregnancy, increased incidence of childhood viral infection, reduced response to vaccinations
Mature immune response and establishment of immunologic memory	Increased risk of infection (common and opportunistic) and cancer, allergy/atopy

Table 1.1: Critical windows in immune-development

Critical steps in development of immune system and the potential consequences when developmental steps do not occur in a timely fashion. Adapted from (19)

1.3.1 Ontogeny of thymic stromal compartment

In contrast to most other lymphoid tissues that are of mesenchymal origin the thymic stromal epithelial compartment is exclusively derived from the endodermal layer of the anterior foregut (20-22). The occurrence of the thymus from the inner layer of the embryonic gut ancestor is reminiscent of the development of gut associated lymphoid tissue (GALT), a

key lymphoid tissue in species prior to the appearance of a thymus. This led to the speculations that the thymus is a derivative of GALT (12). On day 10.5 of the mouse embryonic development (E10.5) commitment to a thymic cell fate takes place. The pharyngeal region shows discrete swellings called branchial arches. The inner lining of the arches consists of endodermal epithelium forming four invaginated pouches. Each of these pouches give rise to specific tissues; the Eustachian tube, tonsils, parathyroid-thymus, and ultimobranchial bodies, respectively. The common parathyroid-thymic primordium arises from a lateral outpocketing of endoderm of the third pharyngeal pouch under the influence of neural crest-derived mesoderm. The early thymic anlage thus consists of an epidermal core and a mesenchymal sheath. The thymic anlage contains an epithelial progenitor pool and differentiation of these progenitors into cytokeratin (CK) CK5⁺8⁺ cortical and CK5⁺8⁻ medullary epithelial cells is dependent on the expression of the transcription factor forkhead box N1 (FoxN1) and occurs independent of interaction with thymocytes (23). At E11.5 the thymic anlage attracts lymphoid progenitors that engage in a cross talk with the epithelial cells essential for the morphological and functional maturation of both cell compartments (Box 2).

It is yet not known which factors determine the site and size of the thymic anlage (24). FoxN1 seems to be the master transcription factor in thymic organogenesis, as absence of functional FoxN1 results in the nude phenotype where thymic development is blocked and only a non-functional thymic rudiment persists. Further important transcription factors for thymus development are Hoxa3, Pax1, Pax9 and Eya1 (25). Formation of immature cortical epithelial cells (CEC) (CK5⁺8⁺) expressing CD205 occurs independently of thymocytes already at E12 (26). These CD205⁺ CEC also express high RNA level of the cortical specific

protease subunit $\beta 5t$. Furthermore, the $CD205^+ \beta 5t^+$ CEC are highly prolific therefore most likely constitute a CEC progenitor population. After proliferation this subset increases expression of MHCII and CD40, molecules required for positive selection. In the absence of mature T cells in the early thymus lymphoid tissue inducer cells (LTi, $CD4^+CD3^-CD11c^-$) promote the formation of the medulla by interacting with immature medullary epithelial cells (MEC) via the RANK-RANKL axis supporting the maturation of immature MEC into Aire expressing mature MEC (27).

Until E15 the thymic anlage is not fully vascularized which requires that lymphoid progenitors migrate through the surrounding mesenchyme to reach the thymic anlage. It is thought that the chemokines CCL25 and CXCL12 are involved in guiding the progenitor to the thymic anlage. However, deletion of either chemokine or their receptors CCR9 or CXCR4 does not abort thymic development and seeding suggesting that several factors contribute to early thymic homing, possibly in a redundant manner (28). It has also been shown that CCL21 produced in the adjacent parathyroid-anlage provides additional cues to guide hematopoietic progenitors near the thymic anlage (29). Starting with E15-16, increased VEGF production of thymic epithelial cells induce vascularization of the thymus and lymphoid progenitors begin to enter the thymus through postcapillary vessels at the cortico-medullary junction. In mice seeding of the thymus occurs in waves and, depending of the developmental stage, by progenitors originating from the aorta-gonado-mesonephros region, fetal liver or bone marrow (30).

It has been proposed that this gated importation of TCP permits the generation and exportation of functionally discrete thymocytes resulting in specialized populations of peripheral T cells in the skin, mucosa, liver and peripheral lymphoid organs (31, 32).

1.3.2 Maintenance of the adult thymus

Thymic epithelial cells (TEC) build the stromal component of the thymus that defines the location of the thymus within the developing embryo and later in the adult recruits all other cellular components that define a functional thymus. Furthermore, the stromal cells are actively involved in creating a self-tolerant T cell population which was shown by two breakthrough discoveries: first by the finding that TEC in particular medullary epithelial cells express tissue specific antigens (33) and second by the identification of the master gene (Aire) that regulates the expression of tissue specific antigens on epithelial cells (34). Recent studies showed that cortical and medullary epithelial cells are derived from cortical and medullary precursor cells which in turn are progenitors from a common bi-potent precursor during embryonic and postnatal stages (22). However the questions whether the common thymic epithelial precursor are true self-renewing progenitor cells and could thus resemble a stem cell like phenotype still needs to be answered.

1.3.2.1 Maintenance of cortical epithelial cells (CEC)

Functional CEC can be identified by the expression of epithelial cell adhesion molecule 1 (EpCAM-1), MHCII, CD40, CD80, CD205 and Ly51. However only little is known about regulation of CEC development and how the heterogeneity of this population might reflect functional specialization. The observation that deletion of a negative regulator of the wnt signaling pathway leads to disrupted CEC development indicates an involvement of this pathway in the formation of a functional CEC (35).

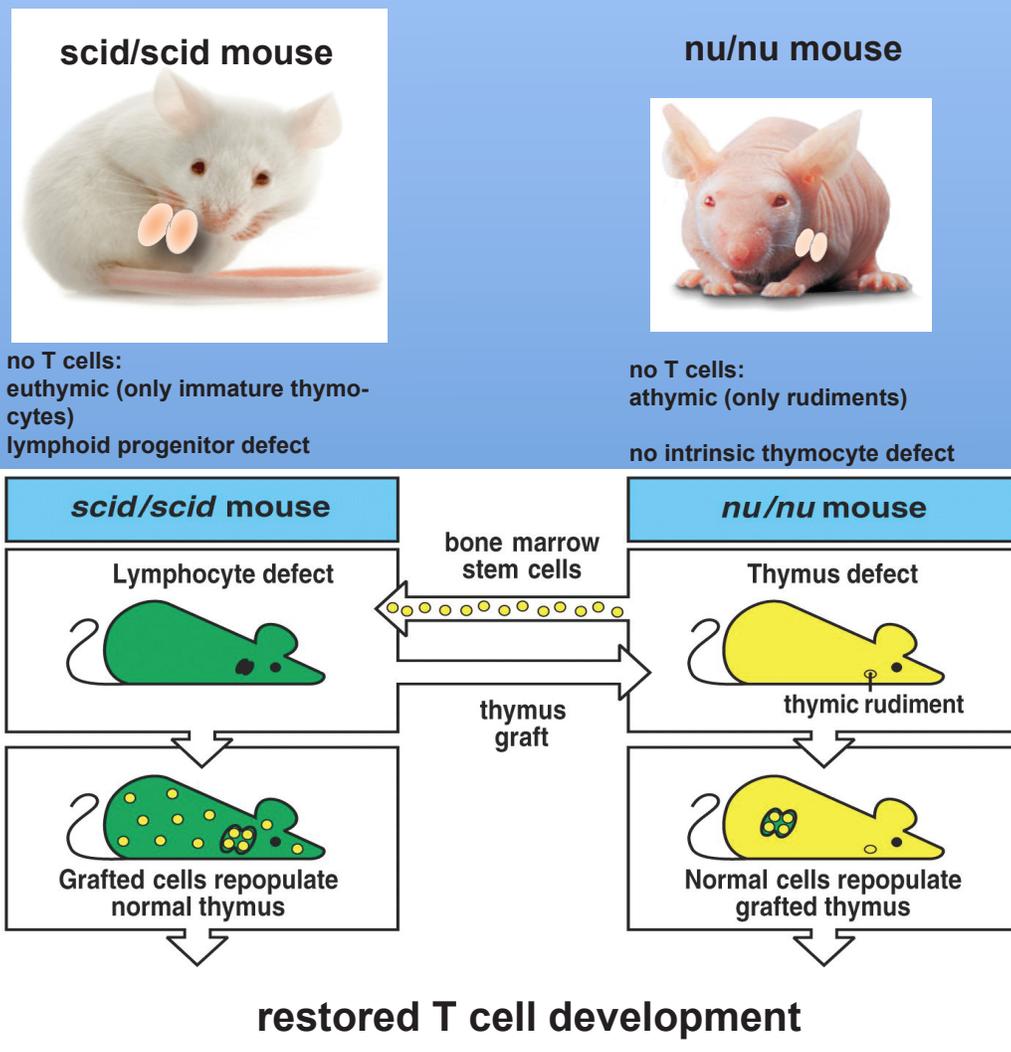
1.3.2.2 Maintenance of medullary epithelial cells (MEC)

Whereas the development of CEC remains largely unknown there were recent advances in deciphering the signaling pathways underlying MEC development. MEC arise stepwise from an MHCII⁻ Aire⁻ CD80⁻ precursor into MHCII^{lo} Aire⁻ CD80^{lo} immature MEC and then into MHCII^{hi}, CD80^{hi} Aire⁺ mature MEC. As CEC also MEC express the co-stimulatory molecules CD40, CD80 and CD86.

The exact underlying signaling pathways that lead to MEC development are not fully understood yet, however it is clear that several tumor necrosis factor superfamily members (RANK, CD40, LT β) are involved in this process. Recently it has been shown that the RANK-RANKL axis plays a dominant role in the mature MHCII^{hi} subset development whereas CD40-CD40L interaction is required for the development of the immature MHCII^{lo} subset (36). Furthermore, it was shown that RANK and CD40 co-operate to induce development of the TSA and Aire expressing MEC subset. A complementary study further showed that RANKL and CD40L are provided by lymphoid tissue inducer cells during early embryonic development and starting from around E16-17 by autoreactive CD4⁺ SP cells (37).

Box 2: Thymocyte-TEC interaction is required for a functional thymopoiesis

The necessity of thymocyte-stroma interaction for efficient T cell development is illustrated by reciprocal transplantation assays. Mice carrying the scid mutation have defective thymocytes but an intact thymic stroma. In contrast, nude mice have impaired thymic stroma development but normal thymocytes. T cells do not develop in either mouse strain. Bone marrow from nude mice transplanted into scid mice restores T cell development in scid mice. Conversely, grafting a scid thymus into nude mice also restores T cell development. Adapted from (38).



1.4 Various thymic microenvironments support different stages in T cell development

T cells arise from bone marrow derived progenitors that have the ability to home to the thymus and give rise to all T cell subsets (39, 40). One hallmark of intrathymic T cell development is that developing thymocytes migrate in and out of distinct thymic microenvironments while they mature in a highly regulated fashion. From this follows that distinct maturation stages can be mapped to certain functional microenvironments within in the thymus (reviewed in (41)). The microenvironments support lineage commitment, gene rearrangement and proliferation by direct cell-cell contact and soluble factors. These microenvironments are composed of epithelial cells but also mature hematopoietic cells (T cells, dendritic cells, macrophages, B cells, eosinophils etc.). Currently there is not a commonly used terminology to describe the thymic microenvironments and various thymocyte subsets. Throughout this study the terminology proposed by Petrie and Zúñiga-Pflücker (41) is used to describe thymic T cell development and their progress through the different “micro-anatomical” areas of the thymus.

1.4.1 Perimedullary-cortex (PMC): Site of TCP entry and retention

Due to the low numbers of TCP that enter the thymus and due to its anatomical location and properties it is difficult to track homing of cells into the thymus *in vivo* (42). Therefore, the precise location of the thymic entry sites for TCP is not known. While it is likely that large post-capillary venules found in the PMC support TCP entry (43, 44), it is believed that not all vessels are equally efficient in supporting the homing of TCP from the blood. It is thought that similar to the bone marrow microvasculature specialized entry sites

exist that are highly efficient in mediating TCP entry and are closely linked to intrathymic progenitor niches (30, 41, 45).

After passing the endothelial layer of blood vessels TCP enter the perivascular space surrounding the post-capillary vessels. While it is not known how long TCP reside in this area, it has been shown that they encounter mature T cells and TCP and possibly interact with them (46). TCP are double negative (DN) for the T cell co-receptors CD4 and CD8 and are phenotypically divided into subgroups (DN1-preDP) according to their CD25 and CD44 expression. One of the earliest intrathymic subset defined so far is the early T lineage progenitor (ETP, Lin^{low}, CD117^{hi}, Sca-1⁺, CD44⁺, CD25⁻) that resides near the entry sites in the PMC (31, 47, 48) and that is part of a bigger heterogeneous populations of DN1 cells that functions as an intrathymic “stem cell pool”. DN1 cells reside in the PMC area for an average time of about 10 days and then asynchronously migrate deeper into the cortex (49, 50). The asynchronous release of DN1 cells from the PMC area might explain why the thymus cellularity does not fluctuate in parallel to waves of seeding TCP.

The stromal environment at the PMC provides factors that promote cell survival and induce massive proliferation (up to 10 serial cell divisions) of ETP cells (cKit, IL-7, Hedgehog) and their differentiation into T cells (Notch-ligands). The PMC thus executes several functions: attraction of TCP, support of proliferation and differentiation, retention of DN1 thymocytes in the PMC and their asynchronous release (41).

1.4.2 Inner cortex

DN1 cells differentiate into highly proliferative DN2 (Lin^{lo}, CD4⁻, CD8⁻, CD44⁺, CD25⁺) cells that migrate towards the subcapsular regions on a cellular matrix of VCAM-1⁺

and E-cadherin⁺ epithelial cells (51, 52). The chemokine CXCL12 and CCL25 have been implicated in supporting the polarized migration of thymocytes at the DN2 stage. However there is no evidence that these chemokines establish a gradient spanning the cortical area and it is thus believed that these chemokines induce directed migration in the absence of a gradient, as already shown for CXCL12 (53).

IL-7 receptor signaling induces an increase in recombinase activity leading to the recombination at the *TCRγ* and *TCRδ* loci in some thymocytes (1-5%) and together with the transcription factors Sox13, Runx3 and Tbx21 results in the formation of TCRγ/δ T cells (54-57). The exact stage at which γ/δ lineage commitment occurs is not known yet, it has been shown to occur in DN2, DN3 and preDP thymocyte stages (58).

Together with Kit ligands IL-7 induces proliferation, survival and migration of DN2 cells, Notch ligands further support the specification of most DN2 thymocytes into the TCRα/β lineage (59). Recently, it was reported that subsets of DN2 thymocytes are already irreversibly committed to the T cell lineage, an event that was traditionally thought to commence with TCR rearrangement at the DN3 stage (60).

It is thought that migration across the inner cortex takes thymocytes about an average of two days (49, 50).

1.4.3 Outer cortex

While migrating from the inner to the outer cortex DN3 (Lin^{lo}, CD4⁻, CD8⁻, CD44^{lo}, CD25⁺) cells fully commit to the T cell lineage as somatic rearrangement of the T cell receptor (TCR) β, γ and δ gene loci progresses. It is thought that IL-7 signaling regulates the accessibility of the TCR β gene locus for the recombination enzymes, however the

underlying mechanisms are still not resolved (61, 62). DN3 cells continue to migrate on a cellular matrix using $\alpha 4\beta 1$ integrin to bind to stromal VCAM-1. CCL25 expressed by stromal cells is thought to induce polarity of the migration further into the capsule, however is not clarified yet whether CCL25 induces chemotactic or chemokinetic migration (63, 64). While some DN3 cells differentiate into the $\gamma\delta$ T cell lineage, the majority will develop into CD8⁺, CD4⁺ pre-double positive (pre-DP) cells losing their NK and DC lineage potential. Continued Notch signaling is required for TCR α/β lineage specification (65). The signals that support survival and proliferation of DN3 cells are not clearly defined yet, but there is evidence that IL-7 signaling promotes survival (66) and that Hedgehog signals supported by Notch and cKit signaling support the four-fold expansion of thymocytes during the DN3 stage (67, 68). During the DN2 and DN3 stages thymocytes require constant contact with the VCAM-1⁺ stromal cells thereby limiting the accessibility of binding sites for other thymocytes in these areas. Limited niche capacity leads to a competition among thymocytes for survival, proliferation and differentiation factors produced in these microenvironments. The average residence time of thymocytes in the outer cortex is estimated to be around two days (49, 50).

1.4.4 Subcapsular zone

The subcapsular zone contains predominantly cells that successfully re-arranged their TCR β locus in frame and start to express the TCR β chain together with a pre- α -chain, e.g. late DN3 (Lin^{lo}, CD4⁻, CD8⁻, CD44^{lo}, CD25^{lo}) and pre-DP (Lin^{lo}, CD4^{lo}, CD8^{lo}, CD44^{lo}, CD25⁻). Late DN3 cells start to express CD4/8 mRNA and later also low protein level of the TCR co-receptor on their surface and are then called pre-DP. These cells then undergo a

massive expansion resulting in a DP population that makes up the bulk of the total thymic cellularity. DP thymocytes initiate TCR α recombination and upon expression of a functional TCR $\alpha\beta$ undergo positive selection while migrating back towards the medulla promoted by CCL25 and possibly CCL19 and CCL21 (69). In the subcapsular zone thymocytes seem to lose their requirement for intimate contact to stromal cells via VCAM-1 and start to migrate on an extracellular matrix containing laminin-5 engaging $\alpha6\beta4$ integrins. While the role of IL-7 seems to be less important in this microenvironment, Notch signaling is required to promote continued survival signals (70). The residence time of cells in the subcapsular zone is relatively short and on average around one day or even less (41).

1.4.5 Positive selection in the cortex

During the DP-stage thymocytes transiently stop proliferating for the first time since entering the thymus. DP do not require constant interaction with a cellular matrix, thus stromal factors like Notch signaling are less important, however stromal signals like IL-7 are still required for survival. The most important stromal signals at the DP stage are likely mediated by MHC molecules and ICAM-1 molecules that together facilitate the selection process (71, 72). MHC-peptide complexes expressed by CEC are presented to thymocytes that randomly scan CEC. Most TCRs clonally expressed by DP are not capable of engaging the MHC-peptides and die by neglect whereas cells that successfully bind to a MHC-peptide complex receive a survival signal (positive selection). Since the success of the positive selection process solely depends on TCR signaling, pre-selected DP are refractory to most cytokine stimuli (73).

Positively selected DP thymocytes stop their random migration, up-regulate CCR7 and rapidly migrate towards the medulla following a gradient of CCL19 and CCL21 (17, 69). While positively selected cells migrate they lose expression of either the CD4 or CD8 co-receptors based on the MHC-restriction of their TCR during the selection process (74). However, the mechanism that controls CD4 versus CD8 lineage decision is not clear (75). Recently several reports document that negative selection also can take place in cortical areas probably mediated by dendritic cells cross-presenting auto-antigens (17, 76).

1.4.6 Medulla and negative selection

CD4 or CD8 SP cells enter the medulla as semi-mature ($CD69^+$, $CD24^{hi}$, $Lsel^-$, $Qa2^-$, $S1P_1^-$) cells and are still susceptible to apoptosis upon TCR activation, which is the basis for the negative selection process. Aire expressing medullary epithelial cells present self-antigens to the semi-mature SP thymocytes. DCs are able to cross-present antigens expressed by the stromal cells and play a crucial role mediating efficient negative selection via the CD28-CD80/CD86 co-stimulatory pathways. Negative selection can be achieved via different mechanisms. A process called TCR editing is induced in thymocytes with high affinity to self-antigens leading to a second rearrangement of the TCR α -loci resulting in a TCR with a different specificity (77, 78). Furthermore, thymocytes can be instructed to enter a state of unresponsiveness to TCR signals, a condition called anergy. However, it is thought that the contribution of anergy and TCR editing to overall negative selection is only minor (79). The major mechanisms to induce self-tolerance are clonal deletion - the induction of apoptosis in self-reactive thymocytes - and clonal diversion of self-reactive thymocytes into lineages with immuno-regulatory function, like regulatory T cells (80, 81). MEC express

high levels of IL-7 which re-installs expression of the anti-apoptotic molecule Bcl2 and thereby supports survival of SP cells. Thymic stromal lymphopoietin (TSLP) is also highly expressed in MEC and it is thought that TSLP together with TCR signaling leads to the formation of regulatory T cells.

In contrast to their immature counterparts, mature SP thymocytes (CD69⁻, CD24^{lo}, CD62L⁺, Qa2⁺, S1P₁⁺) do not induce apoptosis upon TCR activation but instead proliferate upon TCR stimulation. The mechanism that regulates the crucial step from semi-mature to mature SP thymocyte is still elusive. It has been shown that the transcription factor Krüppel-like factor-2 (KLF-2) is required to induce expression of L-selectin and sphingosine-1-phosphate receptor 1 (S1P₁) (82-84), however the signals that lead to KLF-2 expression are unknown. CD69 has been described as a “thymic retention factor” because over-expression of CD69 in thymocytes reduced emigration (85). It has been shown that CD69 forms a complex with the S1P receptor 1 (S1P₁) thereby inhibiting the pro-emigrating signal delivered by sphingosine-1-phosphate (S1P) (86).

As mentioned earlier maturing SP cells interact with the immature MEC via members of the tumor necrosis factor superfamily members (RANK, LTβ) supporting the maturation of immature MEC into mature antigen presenting MEC.

Emigration of mature SP T cells from the thymus occurs 4-5 days after the cells became SP and is controlled by the concerted action of several chemokines and the recently characterized gradient of sphingosine-1-phosphate (S1P, see box 3) (87, 88). S1P levels are approximately 25 times lower in the thymus than in blood plasma (88). The gradient is mainly maintained by the S1P degrading enzyme S1P-Lyase highly expressed in thymocytes and to a lesser extent in thymic stromal cells. Mature T cells follow the gradient to emigrate

from the thymus and enter the circulation. However, some T cell subsets seem to emigrate from the thymus in an SIP independent manner (89, 90) suggesting that thymic export might be differently regulated depending on the T cell subset (91).

It has been reported that the chemokine CCL19 and to a lesser degree also CCL21 support T cell emigration from the thymus during the neonatal period, however these results could not be extrapolated to the adult (92). In addition to chemokinetic factors, there is evidence that further factors like differential expression of adhesion molecules and cell-cell contacts are involved in regulation of the T cell emigration process. Furthermore, it is likely that homeostatic regulation of thymic size and peripheral T cell homeostasis are also important factors in regulating thymic export (reviewed in (93)). Recent studies stressed the importance of the actin regulating protein Coronin 1a in final thymocyte maturation steps (transition from semi mature to mature T cell stage) and in the thymic emigration process itself. Lack or non-functional Coronin leads to actin accumulation and motility defects in responses to chemotactic signals (94). This finding together with the observation that mDia1, another actin regulatory protein, also leads to impaired thymic emigration suggests that actin remodeling is important for T cell maturation and export from the thymus (95)

In the periphery, recent thymic emigrants (RTEs) home to lymphoid tissues where they reside for several weeks and continue their maturation process until reaching full maturation (96).

Box 3: Sphingosine-1-phosphate (S1P)

S1P is a hydrophobic, zwitterionic lysophospholipid ligand that acts in an auto- and paracrine manner and is important for many extra- and intracellular functions ranging from development and maintenance of the vascular system, regulation of cardiac rhythm, cell trafficking and adhesion to endothelial barrier integrity (97). Sphingosine derived from ceramides is phosphorylated by one of two sphingosine kinases (SphKs) resulting in S1P. The two SphKs are differently regulated and distributed: SphK1 is mostly expressed in spleen, kidney and blood whereas SphK2 is highly expressed in liver, kidney, brain and heart. SphK2 is only found intracellularly, SphK1 can be secreted and might contribute to plasma S1P level. Sphingosine kinases are highly expressed in red blood cells and endothelial cells, the two major sources for S1P. The formation of S1P can also occur in the plasma catalyzed by the enzyme autotaxin that converts sphingosyl-phosphoryl-choline into S1P. Degradation of S1P is mainly achieved by S1P-Lyase that degrades S1P irreversibly to phosphatidylethanolamine and hexadecanal. Nonspecific ecto-enzymes like lipid-phosphate phosphohydrolases rapidly and reversibly degrade S1P to sphingosine near endothelial plasma membranes. Due to its hydrophobic properties more than 98% of plasma S1P is associated with HDL and to a lesser degree with albumin providing a constitutive reservoir of S1P in circulation (98). In mammals S1P binds to five high-affinity G protein coupled receptors S1P receptors that are highly diverse in coupling with G protein partners thereby triggering different and at times opposing downstream signals. Deletion of S1PR1 is embryonic lethal at E13.5 due to impaired development of the vascular system similar to the phenotype that occurs upon deletion of both SphKs (98). S1PR signaling depends on a variety of factors and is highly complex. Many cell types also have a substantial intracellular reservoir of S1PRs within caveolae that can be rapidly externalized upon activation. Conversely, S1PRs are also rapidly internalized and either recycled or degraded depending on the S1P concentration. Many cell types co-express several S1PR suggesting functional redundancy. However, it has been shown that some receptors inhibit the action of another depending on S1P concentrations (99). For example endothelial S1PR2 and S1PR3 show opposing effects to S1PR1 regarding the regulation of endothelial barrier permeability (100).

1.5 Journey of a TCP: from bone marrow to the thymus

In adult mammals, including humans, blood cells originate from stem and progenitor cells that reside in the bone marrow. For reasons that still need to be clarified the thymus does not contain its own pool of self-renewing TCP. Hence to sustain constant T cell output the thymus imports bone marrow derived progenitors from the blood stream. Apart from cryptopatches in the small intestine, the thymus seems to be the only hematopoietic organ that selectively imports hematopoietic progenitors while at the same time excludes most mature hematopoietic cells from entering (6, 101). Furthermore, the timely fashion how the thymus imports progenitor cells also distinguishes the thymus from most other lymphoid organs (102).

1.5.1 Progenitors leave the bone marrow niche and migrate through the blood to the thymus

In the past decade huge efforts were made to identify T cell progenitors outside the thymus. Most work focused on the bone marrow where all hematopoietic progenitors derive from. Mostly based on phenotypic characteristics of different progenitor types this field of research produced a confusing amount of potential progenitor populations. T cell progenitor activity was detected in the hematopoietic stem cell (HSC) population and in several of its down-stream progenitors (103).

Progenitor	Location	Description	Phenotype	References
HSC	BM, BL	Hematopoietic stem cell, self-renewing	Lin ⁻ , Sca-1 ⁺ , cKit ^{hi} , Flt3 ⁻ , Subsets: Long-term HSC (LTHSC): CD34 ⁻ , CD150 ⁺ , CD48 ⁻ , CD244 ⁻ Short-term HSC (STHSC): CD34 ⁺	(104, 105), (106)
MPP	BM, BL	Multi-potent progenitor	Lin ⁻ , Sca-1 ⁺ , cKit ^{hi} , Flt3 ^{lo/+}	(104, 107)
ELP, LMPP	BM, BL	Early lymphoid progenitor, lymphoid specified multi-potent progenitor	Lin ⁻ , Sca-1 ⁺ , cKit ^{hi} , Flt3 ^{hi}	(108)
CLP	BM, BL	Common lymphoid progenitor	Lin ⁻ , Sca-1 ^{lo} , IL-7Ra ⁺ , cKit ^{lo} , Flt3 ⁺ , (CD27 ⁺)	(109, 110)
CLP-2 and pre-pro B cells	BM, BL?	Common lymphoid progenitor-2	Lin ⁻ , Sca-1 ^{lo} , IL-7Ra ⁺ , B220 ⁺ , CD19 ⁻ , cKit ^{-/lo}	(111)
CTP	BL	Circulating T cell progenitor	Lin ⁻ , Thy1 ⁺ , CD25 ⁻	(112)
ETP	Thymus	Early T cell progenitor	Lin ^{lo} , cKit ^{hi} , Sca-1 ⁺ , CD25 ⁻ , CD44 ⁺ , Flt3 ⁺ CCR9 ⁺ and Flt3 ^{lo} CCR9 ^{lo} subsets; CD24 ⁻ (DN1a) and CD24 ⁺ (DN1b)	(113)

Table 1.2: T cell progenitor populations

Table shows common kinds of TCP population found in bone marrow, blood and thymus. Lin, Lineage marker (CD3, CD4, CD8, CD19, GR-1, CD11b, CD11c, Ter119, NK and γ/δ TCR); BL, blood; BM, bone marrow.

1.5.2 Progenitor populations in the bone marrow

Hematopoietic stem cells are a part of the lineage negative (Lin^-) bone marrow cell population and were originally described as Lin^- , Sca-1^+ , cKit^+ (LSK) cells. Based on CD34 expression, functional properties and differences in self-renewing capacity, the LSK population is subdivided into long-term reconstituting cells (LTHSCs) and short-term reconstituting cells (STHSCs). In contrast to STHSCs that still have limited self-renewing capacity multipotent progenitors (MPPs) do not self-renew. MPPs that are distinguished from the HSC population by the expression of Flt3 lost erythroid and megakaryocyte lineage potential but retained lymphoid and myeloid lineage potential. Early lymphoid progenitors (ELP) are thought to be derived directly from MPPs, they do not self-renew and lost the myeloid lineage potential of the MPPs. Phenotypically they are distinguished from MPPs by low expression of the IL-7 receptor (IL-7R) and Rag-1 genes which are essential for B and T cell development. ELPs are thought to be a lymphoid-primed precursor population in the bone marrow and their presence in the bone marrow indicates that lymphoid lineage specification can occur early in hematopoiesis. Common lymphoid progenitors appear downstream of ELPs, they express higher level of IL-7R and show rapid and short-term lymphoid (T, B, NK cells) restricted reconstitution activity.

1.5.3 Progenitor populations in the blood

To develop into T cells, progenitor cells have to leave their bone marrow niches, travel through the blood to the thymus and enter the thymic tissue. Although multiple types of progenitors can be found in the blood the mechanism that regulate the release of these progenitors from the bone marrow are widely unknown. Furthermore it is still debated

whether progenitor mobilization from the bone marrow is a selective and regulated process. Residence times of progenitors in the blood circulation have been shown to span from seconds to minutes (114), possibly explaining the overall low frequency of progenitors in the blood.

HSCs, MPPs and ELPs have been detected in blood. The frequency of common lymphoid progenitors (CLPs) in the blood is very low and therefore these progenitors can only be detected after proliferation using the OP9-DL1 culturing system (115). Another population of progenitors that occurs only in the blood and not in the BM is the common T cell progenitor (CTP), initially identified in fetal blood (116, 117). CTPs show T cell potential but have only inefficient B, myeloid and erythroid lineage potential. They rapidly create DP cell upon transfer indicating that they might be developmentally more advanced than ELPs or CLP. However, it is not clear yet from which source CTPs arise from.

Despite the advances in delineating progenitor populations and their development there are important questions unanswered. It is still unknown which population of progenitors seeds the thymus under physiological conditions; most progenitor populations were identified using artificial settings that do not reflect physiologic circumstances. It is also still debated whether T cell lineage specification occurs before progenitors enter the thymus and if so to what degree. Additionally, it needs to be addressed whether T cell development always follows the same sequence of developmental steps or whether there are alternative routes. To confuse matters more, it has been proposed that the thymus is seeded by more than one progenitor population with different lineage potential and efficiencies to produce T cells and that the thymus selectively recruits progenitors from this heterogeneous intrathymic “stem cell pool” depending on “demand” for different kinds of T cells (41). Furthermore the

selective recruitment of progenitor potentially dynamically varies depending on intra- and extrathymic signals.

In contrast to the mobilization of progenitors from the bone marrow, there is clear evidence that the thymic seeding process is selective and regulated (63, 118-121).

1.6 Seeding of the thymus

The thymus is periodically seeded by blood borne progenitors (122). There is evidence that progenitors in the blood utilize a multistep adhesion cascade to enter the thymus similar to that used by lymphocytes leaving the circulation to reach sites of infections or secondary lymphoid organs (120, 121). As mentioned above endothelial structures in large post-capillary venules in the peri-medullary cortex that are similar to high endothelial venules found in lymph nodes are most likely the sites where progenitors enter the thymus. These structures have been shown to express a specific pattern of vascular addressins that selectively allow cells expressing the corresponding ligands and receptors to enter the thymic tissue. Based on the low numbers of progenitors that enter the thymus it is speculated that not all vessels are equally efficient in importing TCP but rather that a few highly efficient vascular gates exist that recruit the progenitors. This however makes it difficult to identify the gates and to analyze their specific expression of adhesion molecules. The adhesion molecules and chemokines so far identified to play a role in thymic homing will be described in detail in the following sections.

The thymic vascular adhesion cascade consists of several steps, including low-affinity tethering, rolling along the endothelium, high-affinity binding to the endothelium and migration across the endothelium and through the basement membrane into the perivascular

space. Although the adhesion cascade is divided into several steps it has to be stressed that each step is not independent from the others and that the adhesion cascade is a flexible and dynamic system where the functions of adhesion overlap and the different kinds of adhesion molecules engage in a crosstalk with each other to enable circulating cells to leave the blood stream in a concerted action.

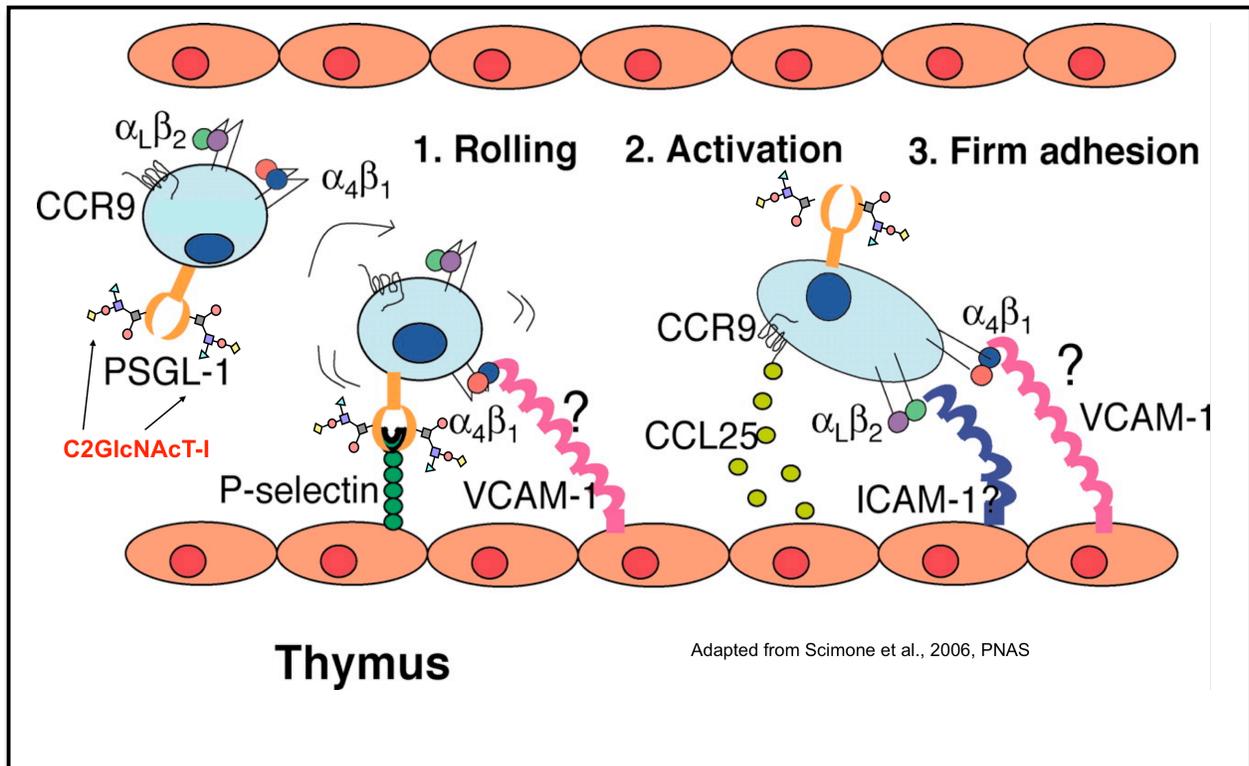


Figure 1.1 Thymic vascular adhesion cascade

Thymic TCP recruitment is mediated by a unique series of adhesion and signaling events. Progenitors that express functional PSGL-1 can initiate rolling and tethering by interacting with P-selectin expressed by endothelial cells. CCL25 signaling through CCR9 activates integrins (probably $\alpha_4\beta_1$ and $\alpha_L\beta_2$) that interact with integrin ligands (VCAM-1, ICAM-1) leading to firm adhesion and cell arrest. Adapted from (120).

1.6.1 Tethering and rolling induced by selectins and their ligands

Since P-selectin plays an important functional role in murine thymic TCP recruitment (Rossi et al. 2005) a more detailed description of this molecule and the selectins will follow.

P- and E-selectin are referred to as the “endothelial selectins”. Their original major function is to control the early stages of inflammatory response. The complex structure of the endothelial selectins allows rapid bond and dissociation rates with ligands and furthermore to absorb high translational energies transferred to them by the migrating cells necessary to induce rolling of the interacting cells along the endothelium. In contrast to the endothelial selectins L-selectin is expressed on leukocytes under non-inflammatory conditions facilitating entry of leukocytes into lymph nodes by binding to its ligands expressed on endothelial cells of high endothelial venules (HEV).

1.6.1.1 Genomic and protein structure of selectins

All three selectins are type II membrane proteins and have comparable genetic and protein structures and their extracellular domains share high homology at the nucleotide as well as amino acid level (123). All selectins are encoded on chromosome 1 in human and mouse, indicating that likely genomic duplication events led to the evolution of the three selectins. There are only a few reported isoforms that result from alternative splicing (124). Overall, the genomic organization of the selectin genes is similar with separate exons that encode for the different protein domains.

The N-terminal end of the mature selectin protein consists of the lectin domain that is also called carbohydrate recognition domain (CRD). This domain is a close homolog to the Ca^{2+} -dependent or C-type lectin (CTL) domain found on various leukocytes (125). Mutational analysis and competitive peptide binding assays revealed that the presence of the lectin domain is absolutely required for binding of P- and E-selectin to carbohydrate structures like the tetrasaccharide sialyl Lewis X (sLe^x) present on leukocytes (126, 127). The

lectin domain also determines the specificity of a given selectin for its ligands. Adjacent to the lectin domain is the epidermal growth factor (EGF) – like domain that also contributes to the binding capacity of the selectin. The EGF domain interacts with the lectin domain and the Ca^{2+} ion that is complexed within the lectin domain. Further towards the C-terminus of the selectin protein is a series of complement control protein modules (CCP)/short consensus repeats (SCR) often referred to as CR repeats that have homology to a number of complement proteins. The selectins differ in their numbers of CR repeats. In humans, P-selectin has nine, E-selectin six and L-selectin only two CR repeats. The function of the CR domains is not clear but it is assumed that they promote binding and extend the lectin domain away from the cell surface thereby increasing the accessibility of the lectin domain for their ligands (128). The cytoplasmic tails show the least homology between the three selectins but are highly conserved among different species. Deletion or mutation of the cytoplasmic tail disturbs protein trafficking, localization, internalization, recycling, degradation and rolling efficiency. Furthermore, the cytoplasmic tails in all three selectins are involved in signaling upon engagement.

Soluble forms of all selectin have been found in plasma and most likely result from shedding or cleavage by proteases (123). However the physiological roles of the soluble selectin forms are not understood yet. It is thought that they play a role in regulating inflammatory responses by blocking selectin ligands or inducing signaling events (123).

1.6.1.2 Regulation of endothelial selectin expression

Most studies analyzed induction of selectin expression under inflammatory conditions. Therefore only little is known about homeostatic selectin expression. In general,

pro-inflammatory mediators like $\text{TNF}\alpha$, $\text{IL-1}\beta$, LPS, complement components, immune complexes, oxygen radicals, hydrogen peroxide, ammonium and formaldehyde induce the rapid appearance of E- and P-selectin on the surface of endothelial cells (129, 130). However, there are major differences in signaling and regulation that lead to the expression of the two endothelial selectins. P-selectin is constitutively produced and stored in Weibel-Palade-Bodies allowing a rapid surface expression within seconds (on platelets) or minutes (endothelial cells). In contrast, E-selectin is not expressed under homeostatic conditions and surface expression requires promoter activation and transcription (123). Both selectins undergo extensive post-translational modifications including high mannose N-linked oligosaccharides. E- and P-selectin are removed from the cell surface by endocytosis in clathrin-coated pits that move through the endosomal pathway for lysosomal degradation (123). In addition, P-selectin is often recycled by re-directing the protein back to the Weibel-Palade-Bodies (123).

The transcription factors nuclear factor- κB (NF- κB) p50/p65, activating transcription factor -2 (ATF-2), c-JUN and the high mobility group I(Y) [HMG-I (Y)] have been shown to mediate the cytokine induced transcription of human E-selectin (131, 132). Once the cytokine stimulus diminishes the transcription stops and E-selectin RNA is rapidly degraded. The regulation of P-selectin transcription is less understood than that for E-selectin. The human P-selectin gene has several transcriptional start sites and lacks a TATA box signal. Several regulatory DNA elements have been identified in the 5' end of the P-selectin gene. These regulatory sites include a novel NF- κB site that only binds p50/p50 or p52/52 homodimers but not the p50/p65 heterodimers as for E-selectin, one GATA element and several Ets, two STAT6 and one HOX element (133-135). $\text{TNF-}\alpha$, IL-4, oncostatin-M or

LPS rapidly induce P-selectin transcription thereby likely saturating the sorting pathway into secretory granules leading to a direct delivery of the protein to the cell surface (136, 137). These data indicate that P-selectin expression can also be subject to direct transcriptional control in contrast to the indirect regulation under homeostatic conditions when P-selectin is constitutively produced and stored in secretory granules. It is important to note that different transcriptional regulatory pathways exist between different mammals. For instance, TNF- α and LPS are potent inducers of E-selectin in human and mouse and for P-selectin in mouse. However, these cytokines do not induce P-selectin surface expression in human.

P-selectin is constitutively expressed on the thymic endothelium (121), lung and choroid plexus microvessels (138), bone marrow microvasculature (139, 140), in post-capillary venules of the skin (141) and on peritoneal macrophages (142). The latter express P-selectin and its major ligand P-selectin glycoprotein ligand 1 (PSGL-1) at the same time (142). It is not known yet whether P-selectin expressed on thymic endothelium is subject to direct or indirect transcriptional regulation. The low affinity binding of P-selectin to its ligand PSGL-1 expressed on most T cell progenitor populations likely induces tethering and rolling thereby contributing to efficient thymic homing (121). Blocking antibodies against P-selectin reduced CLP-2 homing to the thymus by 67% indicating that P-selectin is one of the major contributors of TCP homing to the thymus (120). Furthermore, the P-selectin RNA expression correlated with intrathymic niche occupancy status as bone marrow transfer into mice with a highly receptive thymus significantly reduced the high thymic P-selectin expression suggesting that thymic P-selectin expression might be an indicator for thymic receptivity (121).

1.6.1.3 Relevant selectin ligands

Based on their importance in inflammatory processes, selectin ligands have mainly been analyzed under inflammatory conditions. This present study however tries to unravel the mechanism of thymic TCP importation under steady state conditions. Hence, only selectin ligands that are relevant under steady state conditions will be described.

PSGL-1

PSGL-1 is 240 kDa homodimer, that if properly glycosylated binds to all three selectins. PSGL-1 is the major P-selectin ligand. It is estimated that around 90% of P-selectin mediated rolling and tethering of activated T cells is based on PSGL-1-P-selectin interaction (143). The residual 10% are based on PSGL-1 independent so far unknown P-selectin ligands (144). The majority of LSK and CLP cells in blood and bone marrow as well as most LSK in the thymus highly express PSGL-1 in its functional form, which means appropriately glycosylated to be recognized by P-selectin (120, 121). Parabiosis and competitive bone marrow reconstitution experiments showed that PSGL-1 binding to P-selectin on the thymic endothelium is required for efficient thymic TCP homing (121).

For the binding of endothelial P-selectin to PSGL-1 on migrating cells, the ligand has to be modified by several glycosyltransferases: α 1-3 fucosyltransferases (FucT-IV or FucT-VII), the O-linked branching enzyme core 2 β 1-6 glycosaminyltransferase 1 (C2GnT1), a β 1-4 galactosyltransferase I (β 1-4GalT-I), a sialyl-transferases (ST3Gal) that add a sialic acid to a terminal galactose in α 2-3 linkage. High efficient binding of P-selectin to PSGL-1 additionally requires the action of at least one of two tyrosine sulfotransferases. Relative to other glycosyltransferases C2GnT1 is expressed at low levels. The fact that it acts early in the

synthesis of P-selectin ligand makes this enzyme a potential limiting enzyme for P-selectin-ligand formation (143). While initiation of E-selectin tethering and capturing is also C2GnT1 dependent, continued E-selectin mediated rolling is PSGL-1 and C2GnT1 independent. Although FucT-VII is required for ligand formation for all three selectins, it is the limiting enzyme only for E-selectin-ligand synthesis. The independent regulation of C2GnT1 and FucT-VII expression thus determines E- versus P-selectin ligand formation (143). Furthermore, the binding epitopes for P- and L-selectin on PSGL-1 are different from those for E-selectin as shown in antibody inhibition assays (145, 146).

MECA-79⁺ ligands

It has been speculated that L-selectin expressed on most progenitor types interacts with endothelial cells that express the N-acetylglucosamine-6-O-sulfotransferase (Chst6) required for the formation of the L-selectin recognition 6-sulfo sialyl Le^x carbohydrate structure (41). Chst6 is predominantly expressed on endothelial structures at the peri-medullary cortex contributing to the formation of the MECA-79 epitope (sulfated N-acetylglucosamines (GlcNAc-6-SO₄)), a marker common for L-selectin ligand structures (147). MECA-79 positive endothelial ligands are collectively known as peripheral node addressins (PNADs) and include CD34, GlyCAM-1, MAdCAM-1, podocalyxin-like protein, endomucin and endoglycan. However, it is not known which proteins on the thymic endothelium are decorated with the MECA79 structure functioning as potential ligands for L-selectin. Interestingly, Chst6 activity is up-regulated during times when the thymus is highly receptive suggesting a potential regulatory role for this enzyme in thymic TCP importation (147). Together with the finding that ligands on TCP like PSGL-1 have to be properly

glycosylated to support progenitor entry into the thymus (121) these observations suggest that post-translational modification might be important in regulating TCP importation. However, usage of blocking antibodies against L-selectin on CLP-2 T cell progenitors did not affect their ability to home to the thymus, suggesting that L-selectin is not required for thymic homing at least for this particular type of progenitor (120).

1.6.2 Activation of integrins and cell polarization

The interaction of TCP with selectins decelerates the cells and brings them in the range of action of chemotactic molecules. One major group of chemotactic molecules are chemokines. These are small peptide molecules that induce various processes in migrating cells including integrin activation, chemokinesis and chemotaxis and therefore also play an important role for the thymic seeding process.

1.6.2.1 Chemokines

Chemokines are secreted by many cells including fibroblasts, macrophages and endothelial cells in response to a variety of stimuli such as growth factors, interferons, viral transformation or bacterial products. Most members of this family of proteins have mitogenic, chemotactic or inflammatory activities. Chemokines are cationic proteins of 70 to 100 amino acid residues that share four conserved cysteine residues forming two disulphide bonds. Chemokines can be distinguished into two groups based on the distribution of the two amino-terminal cysteines. In the first group the two cysteines are separated by a single residue (C-x-C), while in the second group they are adjacent (C-C) (148). Most chemokine receptors belong to the group of seven transmembrane spanning G-proteins coupled receptors. It is thought that chemokines receptor genes emerged in the genome of the

common ancestor of vertebrates and that this event probably coincided with the appearance of lymphocytes as a distinct cell lineage (7).

The involvement of chemokines in the thymic homing process was shown by inhibiting G-protein coupled receptors by pertussis-toxin in bone-marrow cells prior to transfer (149). The chemokine expression has been extensively studied for the fetal thymus (28, 92, 150-152). It is assumed that in addition to the absence of Notch ligands, reduced or lacking expression of CCL25 and CXCL12 lead to the thymic phenotype in embryonic nude mice where hematopoietic cells are almost completely absent (28, 153, 154). However, the role of chemokines in the thymic homing process in the adult is not clear yet. It is yet not known what role the different chemokines play during the thymic homing process and whether they induce chemotaxis or rather promote chemokinesis.

CCL25 – CCR9

The chemokine CCL25 or thymus expressed chemokine (TECK) is highly produced in thymic stromal tissue, venular endothelial cells in the small intestine and endothelial cells in the liver (155, 156). In the thymus CCL25 expression is regulated by the thymic master transcription factor FoxN1 (7). The CCL25 receptor CCR9 is widely expressed on T cells and TCP and it is thought that the appearance of the CCR9/CCL25 receptor ligand pair in cartilaginous fish coincided with that of the thymus (7). The expression of CCR9 in TCP is controlled by the transcription factor bcl11b (7, 119, 120, 157). Progenitors deficient for CCR9 and CCR7 are severely impaired in colonizing the fetal prevascular murine thymus. In medaka embryos CCL25 and CXCL12 co-operate to attract lymphoid progenitors to the thymus and inhibition of CCL25/CXCL12 signaling leads to a complete block of

thymopoiesis in the fish embryos (7). However in adult CCR9 and CCR7 double deficient mice no signs of a disturbed thymopoiesis were detected (29) indicating that CCR9 and CCR7 co-operate during colonization of the fetal but not adult thymus. CCR9 deficient bone marrow cells showed a pronounced disadvantage in repopulating the thymus when competed against CCR9⁺ bone marrow (118, 120). A study that used CCR9-EGFP reporter mice found that the earliest intrathymic progenitor expressed high level of EGFP indicating that the thymic homing process selectively enriches for CCR9 expressing progenitors (158). Additionally, the usage of CCR9 neutralizing antibodies in adult mice reduced thymic homing by 52% when compared to a control antibody (120). CCR9⁺ TCP have also been shown to be more efficient in producing T cells than their CCR9⁻ counterparts in a competitive repopulation assay (119). Lastly, Pre-thymic fms-like tyrosine kinase receptor 3 (Flt3) signaling was shown to be required for the generation of CCR9⁺ progenitors demonstrating that the generation of specific thymic seeding cells occurs already in the bone marrow or blood and that the thymic homing process is selective (119). Together these data indicate that CCL25/CCR9 are important in recruiting blood borne progenitors to the adult thymus and likely contribute to the selectivity of the homing process. However, how CCL25 promotes progenitor importation is not known.

CXCR4 – CXCL12 (SDF-1)

A recent comparative genomic study suggests that the CXCR4/CXCL12 receptor-ligand pair seems to be evolutionary older than the CCR9/CCL25 pair or the emergence of a thymus (7). CXCL12 has been shown to play a major role in mediating germ cells or stem cell movement in fish, mouse and human (159-161). Similar to CCL25 the involvement of a

CXCR4/CXCL12 axis in the thymic homing process has been mostly studied for the fetal thymus. One study found that germline depletion or antibody inhibition of CXCR4 in competitive repopulation assays led to reduced thymic homing to postnatal thymi of sub-lethally irradiated recipients (159). However, possible thymic epithelial damage and vascular leakage due to irradiation make these results questionable for physiological settings.

1.6.3 Firm adhesion

1.6.3.1 Integrins and integrin ligands

Integrins are obligate heterodimers consisting of an α and β subunit. Currently, there are 19 different α and eight β subunits described (162). Integrins are adhesion and signaling molecules and they exist in two conformational stages, non-activated and activated. The transition between the two states is rapid (“switchblade-like”) and induced by chemokines, cytokines, selectin and surface receptor signaling. The involvement of integrins in the progenitor recruitment process to the fetal or postnatal thymus has been addressed by several studies. It has been shown that lack of $\alpha 4$ integrin on T cell progenitors does not affect thymic homing during fetal thymopoiesis but leads to reduced TCP entry in the postnatal thymus (163, 164). It could be argued that the lack of $\alpha 4$ integrin affects the emigration of progenitors from the bone marrow and thus only indirectly affect thymic progenitor homing. However, antibody inhibition assays targeted against $\alpha 4$ and $\beta 2$ integrin on TCP showed that these integrins are directly involved in TCP entry into the thymus (120). The ligands for $\alpha 4$ when paired with $\beta 1$ and $\beta 7$ integrin are fibronectin, MadCAM-1 and VCAM-1. These ligands are all expressed near the thymic vascular gates at the PMC (51, 120, 147, 165, 166). Inhibition studies support the notion that VCAM-1 plays a role in the thymic TCP

recruitment process (120, 147). One study also found an involvement for the integrin $\alpha 6$ and its ligand fibronectin in the recruitment process by using blocking antibodies (167).

1.6.3.2 Non-integrin ligand CD44

CD44 is expressed on all hematopoietic cells, epithelial cells, fibroblasts and endothelial cells. Due to a great number of alternative splice forms and various post-translational modifications CD44 molecules form a very heterogeneous population (168). CD44 is a pleiotropic molecule and its involvement in the thymic recruitment process has first been described in inhibition assays using pan-CD44 antibodies in combination with competitive thymic repopulation (169). The same study also showed that the canonical CD44 ligand hyaluronic acid seems not to be involved in the recruitment process suggesting that CD44 on progenitors binds in a homophilic manner to CD44 on thymic endothelial cells. Alternatively, CD44 in its form as hematopoietic cell E/L-selectin ligand (HCELL) expressed on TCP could potentially bind E-selectin expressed on the thymic vasculature similar to what has been shown for mesenchymal stem cells homing to the bone marrow (170). However, it has not been shown yet that E-selectin protein is expressed on the thymic vasculature. More recently a study found that the standard isoform of CD44 (CD44s) is important for the homing process, whereas the variant isoform CD44v6 plays an important intrathymic role by protecting DN thymocytes from apoptosis and promoting their expansion (171).

1.6.3.3 Ectoenzymes

Ectoenzymes are expressed on the surface of circulating or endothelial cells and their involvement in the extravasation cascade has only emerged in recent years (172).

Ectoenzymes can function as adhesion molecules themselves or contribute to adhesion with

their catalytic activity; a few ectoenzymes have bi-functional roles. The group of ectoenzymes is highly diverse among them nucleotidases (CD39, CD73), ADP-ribosylcyclases (CD38, CD157), ADP-ribosyl transferases (ART-2), peptidases and proteases (CD10, CD13, CD26, CD156a, b), and oxidases (VAP-1, NADPH) (173). Ectoenzymes support the adhesion and migration of circulating cells during every step of the adhesion cascade (173). Potentially relevant ectoenzymes for thymic TCP homing are vascular adhesion protein 1 (VAP-1) and autotaxin.

VAP-1

VAP-1 is an ecto-oxidase that is predominantly expressed by endothelial cells (174). Pro-inflammatory signals lead to the translocation of VAP-1 from secretory vesicles to the cell surface (175). VAP-1 is a member of the semicarbazide-sensitive amine oxidase (SSAO) family that catalyzes the deamination of primary amines present in the blood. Thymic VAP-1 expression was reported to be restricted to the CMJ postcapillary vessels suggesting a potential role for this enzyme in TCP homing (147). Current models propose that VAP-1 has a bi-functional role in the adhesion cascade. First, it acts as an adhesion molecule by itself and secondly, its catalytic activity enhances the adhesive properties of endothelial cells. It has been shown that oxidase activity of VAP-1 induces endothelial E- and P-selectin expression and thus leukocyte adhesion, probably mediated by hydrogen peroxide resulting from the enzymes activity (130). However, a direct involvement of VAP-1 in thymic TCP importation has not been shown yet.

Autotaxin

Autotaxin is a lysophospholipase D secreted by endothelial cells. Recently, it was described that autotaxin expressed in endothelial cells of HEV enhance entry of lymphocytes into lymph nodes (176). Like VAP-1 the ectoenzyme autotaxin is a bi-functional enzyme contributing to leukocyte homing through its adhesive and catalytic activity. Two products of the enzymatic reaction, lysophosphatidic acid (LPA) and sphingosine-1-phosphate, enhance the motility and chemokinesis of lymphocytes and control the barrier function of endothelial cells (177) indicating that this enzyme potentially regulates the extravasation of cells at multiple levels. Autotaxin RNA was detected in the thymus suggesting a role in thymic TCP entry (176). However, a direct role of autotaxin in the homing process of TCP to the thymus is still speculative.

1.6.4 Transendothelial migration

While migrating on the vascular endothelium, TCP search for appropriate sites to pass through the endothelial layer in a process called diapedesis. There are two routes to pass the endothelial layer: para- and trans-cellular. Cells that pass the endothelial layer on the trans-cellular route migrate through the cell body of an endothelial cell. The significance and exact mechanism of this type of migration is unclear. The para-cellular route is probably the major route for cells to pass an endothelial layer. On this route migrating cells search for sites where the junctions between two or more adjacent cells are less continuous and then start to extend pseudopods across the endothelial layer by disassembling their cytoskeletons on the luminal surface and reassembling it on the abluminal surface. It needs to be emphasized that endothelial cells do not represent a passive substrate. Like migrating cells endothelial cells

undergo dramatic morphological changes and thus rather behave like “fluids” than “static” gates. Endothelial cells interact and cross-talk with migrating cells and guide them by redistributing junctional proteins thereby supporting transmigration. For many vessels in lymphoid organs that continuously support homing of circulating cells, like HEV in lymph nodes, it is assumed that specialized endothelial “gates” exist that are very efficient in supporting transmigration of leukocytes. Similarly, it is thought that intrathymic microvascular gates support and regulate the entry of T cells into the thymus (30).

The transmigration process is mainly mediated by homophilic interaction of members of the immunoglobulin supergene family (IgSF) expressed on migrating cells and endothelial cells. Three different kinds of endothelial junctions that are characterized by location and molecule composition have been described: adherens junctions, tight junctions and gap junctions. Since a migrating cell has to pass through all three types of junctions, the transmigration process can be described as a multistep process. The principal IgSF members involved in transmigration are ICAM-1, ICAM-2, members of the junctional adhesion molecule (JAM) family, PECAM-1 (CD31) and CD99. The JAM family members are part of tight junctions that are the most apical junctions (closest to the luminal side). JAMs can bind homophilic to each other and probably also bind to the integrin LFA-1 on migrating cells (178). The ICAMs, expressed on the surface and junctions of endothelial cells, bind to LFA-1, MAC-1, VLA-4 and $\alpha 4\beta 7$ integrins expressed on migrating cells and might provide haptotactic signals to guide lymphocytes into endothelial junctions (179, 180). Engagement of the ICAMs also activates signaling events in the endothelial cells leading to cytoskeletal re-arrangements that further support diapedesis. Adherens junctions are formed predominantly by cadherins like vascular endothelial (VE) cadherin that form tight

homophilic interactions. VE-cadherin is intracellularly associated with catenin and actin and functions as a signaling molecule. VE-cadherin is physically pushed aside by transmigrating cells and is thus believed to rather contribute to vascular integrity than actively supporting transmigration (178).

PECAM-1 and CD99 are actively recruited to sites of transendothelial migration. They are excluded from tight- and gap-junctional regions and thus are mostly present in adherens junctions. Both molecules function in a homophilic manner by establishing transient bonds between themselves and family members on the migrating cell. Combined antibody blockage of PECAM-1 and CD99 completely abolishes transmigration (181). Gap-junctions are a cluster of hydrophilic molecules (connexins) that allow ion exchange between adjacent cells. The involvement of gap-junctions in the transmigration process is still not clear. It has been reported that inhibiting gap-junction formation favors migration of neutrophils but inhibits migration of monocytes suggesting that gap-junctions control the selectivity in the transmigration process (182). Although it is likely that transmigration of TCP into the thymus involves several molecules described in this section, there is no experimental confirmation for a role of any of the molecules in the importation of TCP.

As for leukocyte homing, the thymic TCP homing process seems to be a robust system stabilized by multiple redundant genes. Mice deficient for either of the above-mentioned molecules show normal thymic development and only competitive experimental set-ups revealed the role of the respective molecules.

1.7 Regulation of thymic TCP homing

Thymic TCP importation is independent of the numbers of available TCP in the blood (183). Furthermore, HSCs found in the bone marrow and circulation could not be detected in the thymus (31, 184). These data suggest that thymic TCP importation is regulated, selective and unlikely to be limited by factors associated with TCP. Additionally, thymic TCP importation is not a steady state process as the thymus alternates between non-receptive and receptive periods (122). This observation suggests that a feedback mechanism regulates expression of recruitment signals on vascular or peri-vascular cells. Further studies showed that intrathymic niche vacation by irradiation made the thymus receptive for i.v. injected bone marrow cells. In contrast, intrathymic injection of progenitors prior to an i.v. bone marrow transfer made the thymus refractive (non-receptive) for further progenitors (185). All these observations resulted in a cumulative model that proposes that TCP entry into the thymus is controlled by the limited number and the occupation status of intrathymic niches (30, 183). Using various knockout models, the Petrie laboratory showed that numbers of DN2 or DN3 thymocytes negatively correlated with thymic TCP receptivity (183). It is assumed that competition among thymocytes for a limited quantity of survival and differentiation factors provided by niches triggers a negative feedback signal (183). Thus, full thymic niches are thought to trigger a negative feedback signal to reduce TCP importation. As the DN3 thymocytes mature they leave the niches and the intensity of the feedback signal diminishes over time as more and more niches become available again. The weakening signal allows the re-expression or up-regulation of homing molecules at the thymic vascular gates promoting TCP entry (30). The only parts of the model that have been experimentally confirmed are the periodic importation of TCP and the numeric correlation between

receptivity and DN3 thymocyte numbers (122, 183). However, the nature of the proposed feedback signal and the molecular basis of how the signal is executed at the vascular gates are unknown.

1.8 Objective

The general objective of this study is to broaden the existing knowledge of how TCP entry into the thymus is regulated. Earlier data show that thymic P-selectin is required for efficient thymic seeding and that thymic P-selectin RNA expression correlates with the occupation status of intrathymic niches (121). Based on this data the present work hypothesizes that P-selectin is a part of the thymic gate keeping mechanism that regulates thymic TCP importation. Thus the objective of this study is to identify the mechanism that controls TCP entry into the thymus and evaluate the significance of P-selectin in the regulation process.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mouse strains used in this study

C57Bl/6 (CD45.2), congenic C57Bl/6 (CD45.1), PSGL-1^{-/-}, Psel^{-/-}, IL7R^{-/-} and Rag-1^{-/-} all backcrossed onto C57Bl/6 background were purchased from Jackson Laboratory. Mice deficient in C2GlcNAcT-1 (C2^{-/-}, C2GnT1^{-/-}) backcrossed on C57Bl/6 (186) (obtained from Dr. J. Marth, Howard Hughes Medical Institute, UCSD) were maintained and bred at the specific pathogen free (SPF) animal facility at the Biomedical Research Centre. All animal experiments were performed according to institutional guidelines and approved by the Animal Care Committee of the University of British Columbia. Table 2.1 gives a brief overview of the relevant phenotypes found in the used mouse strains.

Strain	Phenotype		Reference
	Periphery	Thymus	
Rag-1 ^{-/-}	No mature T and B cells	Developmental block at DN2 stage	(187)
C2GnT1 ^{-/-}	Mild neutrophilia, reduced rolling of activated lymphocytes	Reduced ETP numbers	(186), present study
PSGL-1 ^{-/-}	Neutrophilia, reduced rolling of activated lymphocytes, reduced T cell numbers in peripheral blood	Reduced ETP numbers	(144), (188), present study, phenotyping core unit consortium for functional glycomics (www.functionalglycomics.org)
IL-7R ^{-/-}	No mature T and B cells	Developmental block at DN3 stage	(189)
Psel ^{-/-}	Neutrophilia, reduced rolling of activated lymphocytes	Reduced ETP numbers	(190), (191), the present study

Table 2.1: Relevant phenotypes of the used mouse strains

2.1.1 Congenic marker system

Congenic markers like CD45.1 and CD45.2 (encoded on the *Ptprc* locus, formerly known as *Ly5*) are commonly used to track cells or to distinguish donor and recipient cells *in vivo* (192). CD45, a protein tyrosine phosphatase also known as the leukocyte common antigen, is an essential regulator of leukocyte activation and development and is expressed on all bone marrow derived cells except erythrocytes and erythroblasts (193). CD45 exists in multiple isoforms and different allelic forms like CD45.1/*Ly5.1* or CD45.2/*Ly5.2*. Specific antibodies to either of these allelic forms can be used to distinguish recipient and donor cells in *in vivo* competition studies using bone marrow or leukocytes from inbred mouse strains carrying either the CD45.1 or CD45.2 allele.

2.2 Tissue extraction

Mice were sacrificed using CO₂ and perfused with PBS, 5 mM EDTA. Single cell suspensions of peripheral lymph nodes and spleens were prepared by mincing tissues and squeezing them through a mesh. Thymic cell suspensions were obtained by gently squeezing the thymus between two frosted glass slides and washing it several times with RPMI, 2 % FCS, 2 mM EDTA. The thymocyte containing suspension fraction was filtered and kept for flow cytometry analysis. The remaining non-suspension fraction was either directly processed for qrtPCR or flow cytometry analysis or stored at -80 °C.

2.3 Flow Cytometry, fluorescent activated cell sorting (FACS) and cell counting

Cell suspensions were stained in staining buffer (PBS, 2 % FCS, 2 mM EDTA) with titrated amounts of antibodies specified (Table 2.2). Samples were analyzed on a Calibur or

LSRII flow cytometer and cell sorting was performed on a FACS Vantage or FACS Aria (all BD Bioscience). Data were analyzed with FlowJo software (Treestar). Where required, a fixed number of reference beads (Interfacial Dynamics, Invitrogen) were added per sample. The 10 μ m latex beads were distinguished from cells by forward/sideward scatter characteristics. Cell and bead numbers were determined by flow cytometry. Absolute cell numbers in the sample were calculated based on the determined cell:bead ratio and the known number of used beads and sample volume.

2.3.1 Thymocyte subset analysis

For DN thymocyte subset analysis, thymus suspension cells were stained with a lineage marker mix consisting of biotinylated antibodies (for specifications see Table 2.2) against CD19, B220, CD3, CD8, Gr-1, CD11c, CD11b, Ter119, NK1.1 and γ/δ TCR and detected by PE-Cy7-conjugated streptavidin (SA). Resolving DN subset distribution was achieved by additional staining for antibodies against CD25-FITC and CD44-APC. For ETP analysis, cells were furthermore stained with Sca-1-PE and cKit-PE-Cy5 antibodies. For mature thymocyte subset analysis, cells were stained with antibodies against CD4-PECy7, CD8-APC-Alexa750, CD24-PE, biotinylated L-selectin (detected with SA-PE-Cy5), Qa2-FITC and CD69-Alexa647.

2.3.2 Analysis and sorting of thymic endothelial and epithelial cells

For endothelial and epithelial cell analysis thymi were extracted and any remaining lymph nodes, fat or connective tissues were carefully removed using forceps. Thymi were then gently squeezed between two frosted glass slides. To remove thymocytes, thymic fragments were gently stirred in HBSS for 30 minutes at 4 °C replacing the supernatant every

10 minutes. Thymic tissue was then successively digested in collagenase D (1 mg/mL + 2 μ g/mL DNase) and collagenase/dispase (0.5 mg/mL + 10 μ g/mL DNase) (all from Roche) in HBSS at 37°C, filtered through a 70 μ m cell strainer (Nunc) and enriched for stromal and endothelial cells by density centrifugation using a discontinuous Percoll (GE Healthcare) gradient (bottom layer δ = 1.115 g/mL, middle layer δ = 1.06 g/mL, top layer PBS) as described elsewhere (194, 195). Cells recovered from the upper interface were washed and stained with antibodies against CD45, CD31, CD144, G8.8 and Ly51. Cells showing forward scatter greater than 400 and side scatter greater than 300 (large cells) were negatively gated on CD45; medullary epithelial cells were defined as large CD45⁻G8.8⁺, Ly51^{int} cells; cortical epithelial cells were defined as large CD45⁻G8.8⁺, Ly51^{hi} cells; and endothelial cells were defined as CD45⁻CD31⁺, CD144⁺ positive cells (37, 196). Cells were sorted on a FACS Vantage (BD Bioscience) and directly sorted into TrizolLS (Invitrogen) for RNA extraction. For analysis of stromal and endothelial cell numbers thymi were gently squeezed between two frosted glass slide and then agitated in HBSS for 30 minutes replacing the supernatant every 10 minutes. Thymic tissues were then subjected to enzymatic digestion as described above to obtain single cell suspensions. Cells were collected, washed, filtered through 100 μ m mesh (Nunc) and resuspended in 1 mL of staining buffer containing a fixed number of reference beads to allow calculation of absolute cell numbers (see section 2.3). Cells were then stained with the same antibodies as mentioned above and analyzed using a LSRII flow cytometer (BD Bioscience).

Target	Species	Clone	Conjugate	Source
CD19	Rat	ID3	Biotin	Hybridoma Bank/ATCC
B220	Rat	RA6B2	Biotin	Hybridoma Bank/ATCC
CD3	Rat	2C11	Biotin	Hybridoma Bank/ATCC
CD4	Rat	RM4-5	PE-Cy7	ebioscience
CD8	Rat	53.67	Biotin	Hybridoma Bank/ATCC
CD8	Rat	53.67q	APC-Alexa 750	ebioscience
Gr-1	Rat	Ly6G	Biotin	Hybridoma Bank/ATCC
CD11b	Rat	M1/70	Biotin	Hybridoma Bank/ATCC
CD11c	Hamster	HC3	Biotin	Hybridoma Bank/ATCC
Ter119	Rat	Ter119	Biotin	Hybridoma Bank/ATCC
panNK	Rat	DX5	Biotin	Hybridoma Bank/ATCC
γ/δ TCR	Hamster	eBioG13	Biotin	ebioscience
CD25	Rat	PC61	FITC/PE	ebioscience
CD44	Rat	IM7	Alexa647/FITC	Hybridoma Bank/ATCC
Sca-1	Rat	D7	PE	BD Pharmingen
CD117(cKit)	Rat	2B8	PE-Cy7	ebioscience
CD24	Rat	M1/69	PE	ebioscience
CD62L	Rat	Mel14	Biotin	Hybridoma Bank/ATCC
Qa2	Mouse	69H1-9-9	FITC	ebioscience
CD69	Hamster	H1.2F3	Alexa647	Biolegend
CD31	Rat	390	FITC	BD Pharmingen
CD144	Rat	eBioBV13	Alexa647	ebioscience
CD62P	Rat	RB40.34	Biotin	BD Pharmingen
MHCII	Rat	M5/114.15.2	Biotin	ebioscience
CD43	Rat	S11	purified	In house
Ly51	Rat	6C3	Biotin	BD Pharmingen
CD326 (EpCAM)	Rat	G8.8	purified	ebioscience
CD45.1	Mouse	A20	FITC/PE	BD Pharmingen
CD45.2	Mouse	104	FITC/PE/PECy5	BD Pharmingen
Donkey α Rat	Donkey	polyclonal	purified	ebioscience

Table 2.2: Antibodies used for flow cytometry and FACS

2.3.3 Analysis of P-selectin protein expression on thymic endothelial cells

Single cell suspensions from thymi were obtained by enzymatic digestion and enriched using a discontinuous percoll (GE Healthcare) gradient as described above (2.3.2). Cells were stained with antibodies against CD45, P-selectin, CD31, CD144 and analyzed by flow cytometry. Forward and sideward light scatter settings were adjusted to exclude dead cells and platelets. The cell population was then negatively gated on CD45 to exclude hematopoietic cells and positively gated for the endothelial markers CD31 and CD144. Double positive cells were then analyzed for the frequency of P-selectin^{high} expressing cells, thereby excluding possible skewing of the results by different thymic size and relative endothelial content.

2.3.4 Sorting and i.v. injection of TN1-3 subsets

Thymic cell suspensions from up to 8-10 thymi were pooled and labeled with lineage marker mix as described above (2.3.1). Lineage marker positive cells were then depleted with streptavidin-coupled magnetic beads using the “depletes” program on an AutoMacs system (all Milteni Biotech). Lineage marker negative cells were stained with antibodies against CD25 and CD44 and FACS-sorted on a FACS Vantage (BD Bioscience). TN1 (CD44⁺, CD25⁻), TN2 (CD44⁺, CD25⁺) and TN3 (CD44⁻, CD25⁺) were collected, counted and 5000 TN cells of either subset together with 10⁶ IL-7R^{-/-} splenocytes were i.v. injected into non-irradiated IL-7R^{-/-} recipients. The purity of the sorted population was > 95 %. 12 d after bone marrow transfer recipient mice were sacrificed, thymus and blood analyzed for donor chimerism and thymi additionally analyzed P-selectins RNA expression.

2.4 Adoptive transfer

Lymph node cell suspensions from CD45.1 congenic mice were either directly injected into recipients or enriched for T cells or B cells by negative depletion using anti rat IgG coupled magnetic beads (Dyna). For T cell enrichment cells were labeled with rat IgG antibodies against mouse CD19, MHC-II, CD11c, Gr-1 and subsequently negatively depleted using anti rat IgG coupled magnetic beads (Dyna). Accordingly, B cells were enriched from spleen suspension cells using rat IgG antibodies against mouse CD43, CD11b, MHC-II and Gr-1. Purities of target populations assessed by flow cytometry were generally > 90 %. 20×10^6 cells in HBSS were injected i.v. into recipients (CD45.2) that were 37-40 days old. Two days after adoptive transfer one cohort of recipient mice was analyzed for donor chimerism (CD45.1), S1P level and thymic P-selectin expression. The other cohort received a bone marrow transplant from CD45.1/2 congenic donors to test for thymic receptivity. Thymic chimerism was assessed three weeks after bone marrow transfer by flow cytometry.

2.5 *In vivo* T cell depletion

In vivo T cell depletion was performed by i.p. injection of 0.5 mg of anti CD4/CD8 antibodies in PBS. Control mice received PBS only. After 24 hours mice were sacrificed to analyze peripheral T cell numbers, P-selectin RNA and S1P levels. For testing thymic TCP receptivity mice received 2×10^6 congenic bone marrow transplant two days after antibody injection. Thymic chimerism was assessed three weeks after bone marrow transfer using flow cytometry.

2.6 Quantitative Real time PCR (qrtPCR)

RNA was extracted using TRIZOL (Invitrogen) solution according to the manufacturers instructions. Quality and quantity of total RNA was assessed using Nanodrop spectrometer ND-1000 (Fisher-Scientific). 1 mg total RNA was treated with DNase (Fermentas) and reverse transcribed with the high capacity cDNA reverse transcription Kit (Applied Biosystems) according to manufacturers instructions. Relative gene expression was determined using primer/probes listed in Table 2.3. qrtPCR reactions were performed using TaqMan® Gene-expression or SYBR® Green PCR master mix and run on a HT7900 real time PCR system (all Applied Biosystems). Relative mRNA expression ratios were calculated using the 7000 SDS relative quantification software (Applied Biosystems).

Primer/Probe	Sequence	Reference
Psel/primers/Fam probe	ABI Assay ID: Mm00441295_m1	
VECad/primers/Vic probe	ABI Assay ID: Mm00486938_m1	
S1P1 forward	5'-GTGTAGACCCAGAGTCCTGCG-3'	(82)
S1P1 reverse	5'-AGCTTTTCCTTGGCTGGAGAG-3'	
S1PLYase forward	5'-TCTGCTGATAGTCTGGGTGTATGAG-3'	(88)
S1PLYase reverse	5'-CCAATAAATGGCATCTCCTGATA-3'	
SphK1 forward	5'-GAGCTCCGAGCTGTTTGCA-3'	(197)
SphK1 reverse	5'-TGACACCCCGCACGTA-3'	
SphK2 forward	5'-CCACCTGAGCTCCGAGCTGT-3'	
SphK2 reverse	5'-GGCACATGAACCAGGTATGGA-3'	
ICAM-1 forward	5'-GCTGTTGAGCTGAGCGAGATCGG-3'	Designed by Probefinder 2.32 (Roche)
ICAM-1 reverse	5'-TGAGGTCCTTGCTACTTGCTGCC-3'	
VCAM-1 forward	5'-TGGTGAAATGGAATCTGAACC-3'	Designed by Probefinder 2.32 (Roche)
VCAM-1 forward	5'-CCCAGATGGTGGTTTCCT-3'	
CCL25 forward	5'-GCTTTTGCCTGCCTGGTTG-3'	(150)
CCL25 reverse	5'-TCAGTCTGAGAGTCTGAGGC-3'	
KLF2 forward	5'-TATCTGCCGTCCTTTGCCA-3'	(198)
KLF2 reverse	5'-TTAGGTCCTCATCCGTGCC-3'	
Gapdh forward	5'-TCAACCACCATGGAGAAGGC-3'	(199)
Gapdh reverse	5'-GCTAAGCAGTTGGTGGTGCA-3'	
TbP forward	5'-CCTCACCAATGACTCCTATGAC-3'	(200)
TbP reverse	5'-CAAGTTTACAGCCAAGATTCA-3'	
HPRT forward	5'-GTTGGATATGCCCTTGAC-3'	(200)
HPRT reverse	5'-CAAGTTTACAGCCAAGATTAC-3'	
TREC forward	5'-CATTGCCTTTGAACCAAGCTG-3'	(201)
TREC reverse	5'-TTATGCACAGGGTGCAGGTG-3'	
TREC Probe FAM labeled	5'-CAGGGCAGGTTTTGTAAAGGTGCTCACTT-3'	

Table 2.3: Primer used for qrtPCR

2.7 FTY720 and DOP treatment

FTY720 (Cayman chemicals) stocks were prepared in DMSO and diluted in vehicle solution (PBS, 0.1 % mouse serum) before injection. Mice received 1 mg/kg FTY720 or vehicle alone i.p. for three consecutive days and were analyzed 24 hours after the last injection. Mice received 30 mg/L DOP (4' deoxypridoxine, Sigma) in drinking water with 10g/L glucose for three days (88). After FTY720 or DOP treatment one cohort was sacrificed to analyze thymic P-selectin RNA expression, S1P levels and thymocyte subset distribution. The second cohort received a congenic bone marrow transplant to test for thymic receptivity as described above. Thymic chimerism was assessed three weeks after bone marrow injection.

2.8 Blood cell count by flow cytometry

Mice were bled from the saphenous vein and 15 μ L of peripheral blood was stained with anti CD4 and anti CD8 antibodies. 10^5 reference beads were added per sample. After red blood cells lysis samples were analyzed by flow cytometry. Cell numbers per μ L blood were calculated based on the cell:bead ratio (see section 2.3).

2.9 S1P measurement by LC/MS/MS

S1P levels in blood plasma and lymphoid tissues were assessed by LC-MS/MS as described earlier (197). Briefly, platelet poor plasma was obtained from blood drawn by cardiac puncture. The plasma samples were diluted with acetonitrile to a final concentration of 20 %. Thymus, 6 lymph nodes and spleen were homogenized in four times the volume (assuming a tissue density of 1 g/mL) of 80 % acetonitrile. All samples were processed together with 60 ng/mL of internal standard (isotope labeled S1P, D-erythro-sphingosine-1-

phosphate-¹³C₂D₂, Toronto Research Chemicals). After centrifugation supernatants were analyzed on a hybrid linear ion-trap LC-MS/MS system (Q Trap, Applied Biosystems, Framingham, MA, USA) coupled to a HPLC system (LC packings, San Francisco, CA, USA) via a Turbo Ion Spray source (Applied Biosystems). Sample separation was performed over a 1.0 x 50 mm ZORBAX Eclipse XDB-C18 MicroBore column (particle size 3.5 μm, Agilent Technologies, USA) at the flow rate of 0.2 mL/min using the following gradient: wash column with a mixture of 50 % A (water:methanol:acetic acid (69:30:1)(v:v:v) containing 5 mM ammonium acetate) and 50 % B (methanol:acetic acid (99:1)(v:v) containing 5 mM ammonium acetate) for 2 minutes, then apply a linear gradient to 100 % B over 4 minutes, hold 100 % B for 2 minutes, re-equilibrate column with 50 % B for 2 minutes. For the detection of both S1P and internal standard, on the Q Trap instrument operated in positive mode with the ion spray voltage set at 5000 V and source temperature at 400 °C. Other MS/MS parameter settings, such as declustering potential and collision energy, etc. were optimized for S1P and internal standard, respectively. The elution of S1P and internal standard were monitored using multiple reaction monitoring (MRM) mode. The MRM transitions (Q1/Q3) settings for S1P and internal standard were 380.0/264.2 and 384.0/268.2, respectively. Data were acquired and processed using Analyst 1.4.2 (Applied Biosystems).

2.10 Assessment of short-term receptivity

Short-term receptivity was determined by i.v. injection of fluorescent-labeled cells as described earlier (121). Briefly, wt bone marrow cells were labeled with 2.5 μM CFSE (Molecular Probes) for 5 minutes at room temperature in HBSS. After staining 40 x 10⁶ cells

were i.v. injected into a non-irradiated recipient. 24 hours after injection recipients were bled to determine blood cell counts and subsequently sacrificed and perfused. Thymi were removed, weighed and mechanically dissociated to obtain single cell suspensions as described under “tissue extraction”. Thymic cell suspensions were stained with antibodies against lineage markers as described above. After staining cells were resuspended in 1 mL staining buffer containing 10^5 reference beads (see section 2.3).

2.11 Intrathymic labeling of cells with fluorophore

Intrathymic fluorophore labeling was performed as described earlier (202). Briefly, 10 μ L of a 2 mg/mL fluorescein isothiocyanate (FITC) (Sigma) solution in PBS was directly injected into each thymic lobe of anesthetized mice using a Tridek Stepper (Tridek, Brookfield, CT). The incision was then closed with surgical clips and mice were left on a heat pad to recover. FITC covalently binds lysine residues on membrane proteins and thus labels cells randomly in the thymus. FITC solution was injected into the chest cavity of control mice to test whether FITC that leaked out the thymus labels mature peripheral T cells. However, no labeling of T cells was observed when FITC was not administered i.t. as already shown before (202). 36 hours after FITC injection mice were sacrificed and numbers of FITC⁺CD4⁺Lsel⁺ and FITC⁺CD8⁺Lsel⁺ positive cells were determined in thymi, peripheral lymph nodes and spleen by flow cytometry. Labeling efficiency of thymocytes was on average 76 %.

2.12 Bone marrow transfer

Bone marrow was extracted from respective congenic mice by flushing femurs and tibiae with RPMI, 2 % FCS, 2 mM EDTA. After red blood cell lysis 2×10^6 cells in HBSS

were injected i.v. into recipients. Depending on the experiment, 12 days to three weeks later thymi, lymph nodes and spleens were harvested and analyzed for chimerism based on congenic markers (CD45.1 and CD45.2).

2.12.1 Intrathymic bone marrow injection

Intrathymic bone marrow injections were performed as described elsewhere (122). 2×10^6 congenic wt bone marrow cells in 10 μ L RPMI were injected into each thymic lobe of anesthetized mice using a Tridek Stepper (Tridek, Brookfield, CT) as described in section 2.11. 10-14 days after bone marrow injection thymic chimerism was assessed by flow cytometry.

2.13 TREC analysis

As explained in Box 1, thymocytes recombine certain sub-genetic fragments during their maturation leading to the excision of the intermediate DNA. Per TCR chain there are at least two recombination events resulting in the formation of two T cell receptor excision circles (TRECs), the signal joint region containing TREC (sjTREC) and later the coding joint region containing TREC (cjTREC). As most other studies, this study uses the sjTRECs of the TCR $\alpha\delta$ locus to determine thymic T cell output. These TRECs arise from the last recombination events and are not skewed by intrathymic proliferation events like TREC arising from β -chain recombination (201, 203-205). All types of TRECs are episomal circular DNA not amplified during cell proliferation therefore directly reflecting recombination activity in the thymus. In the periphery most T cells are progenies of thymic emigrants meaning they are derived from mitosis events in the periphery. The frequency of TREC positive T cells in the periphery is therefore dependent on several variables like

recombination activity in thymus, T cell emigration rate from the thymus, intrathymic or peripheral proliferation of T cells, survival of recent thymic emigrants etc. and should therefore be used with caution when used to compare thymic output in mice from different backgrounds.

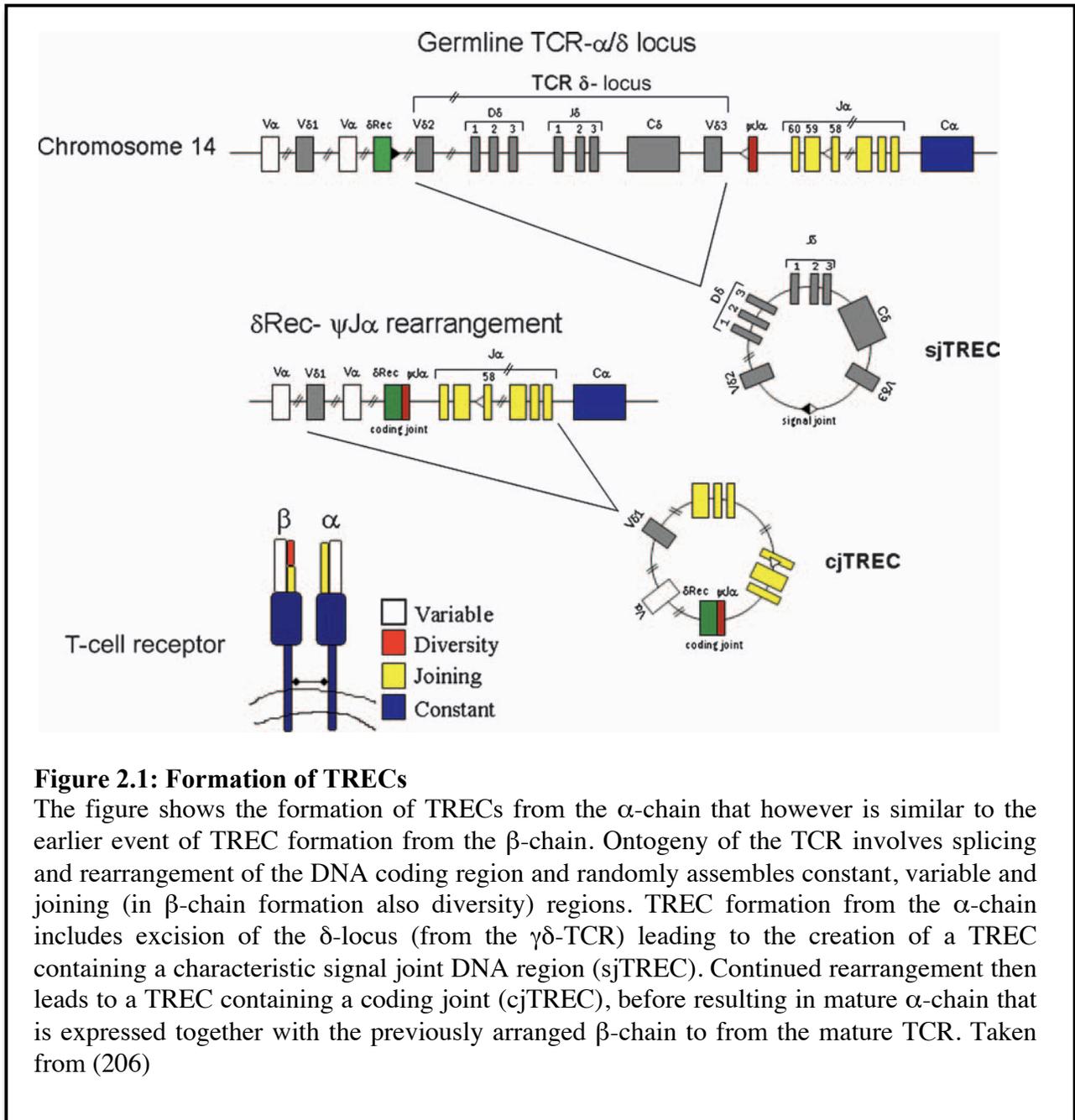


Figure 2.1: Formation of TRECs

The figure shows the formation of TRECs from the α -chain that however is similar to the earlier event of TREC formation from the β -chain. Ontogeny of the TCR involves splicing and rearrangement of the DNA coding region and randomly assembles constant, variable and joining (in β -chain formation also diversity) regions. TREC formation from the α -chain includes excision of the δ -locus (from the $\gamma\delta$ -TCR) leading to the creation of a TREC containing a characteristic signal joint DNA region (sjTREC). Continued rearrangement then leads to a TREC containing a coding joint (cjTREC), before resulting in mature α -chain that is expressed together with the previously arranged β -chain to form the mature TCR. Taken from (206)

The assessment of TRECs in this study was performed as described elsewhere (201). Briefly, T cells from thymi or spleens were labeled with biotinylated anti CD4 and anti CD8 antibodies and purified using streptavidin coupled magnetic beads (Milteni Biotech) according to the manufacturers instructions. After counting, the purified cells were put into Trizol (Invitrogen) solution and processed according to manufacturers recommendations. After removing the aqueous phase, the DNA was extracted from the remaining interphase and organic phase using back extraction buffer (4M Guanidine Thiocyanate, 50 mM Sodium Citrate, 1 M Tris). After phase separation by centrifugation the DNA in the aqueous phase was precipitated using isopropanol and the DNA pellet was washed several times with 75 % ethanol. Quantity and quality of the DNA was assessed using a Nanodrop spectrometer ND-1000. Equal amounts of DNA were then amplified with primers and detected with a fluorescent-labeled probe ((201), table 2.3) using the Probe Master Mix (Roche) on a LC480 Lightcycler system (Roche). Absolute numbers of excision circles were determined using a calibration curve that was produced with known amounts of plasmids (Topo Vector, Invitrogen) containing the target sequence.

2.14 S1P dose response using the bend.3 endothelial cell line

The murine endothelial cell line bend.3 (ATCC) has been described before to express P-selectin and the sphingosine-1-receptors S1PR1 and S1PR2 (135, 207). Cells were cultured in high glucose DMEM media, containing 1 mM sodium pyruvat, 0.1 mM non-essential aminio acids, 1000U of penicillin/streptomycin and 10 % FCS (all Gibco). For the dose response assay 50 000 bend.3 cells were seeded into each well of a 24 well plate (Falcon) and allowed to adhere overnight. Cells were washed several times with serum-free media (see

above) containing 4 % of fatty-acid free BSA (Calbiochem). Cells were then cultured for 12 hours in serum-free media (+ 4 % of fatty-acid free BSA) containing S1P or FTY-720P (Cayman Chemicals) at the indicated concentrations. Control cells were maintained in serum-free media (+4 % of fatty-acid free BSA). After 12 hours media was removed, cells were lysed using Trizol (Invitrogen) and RNA was extracted according to manufacturers instructions (see section 2.6)

2.15 Statistics.

Data are presented as mean values and error bars as s.e.m. The statistical significance of the data was assessed using unpaired, two-tailed, student's t test with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Where p values using student t-test barely reached significance (3.3a, 3.11a, 3.17a and 3.18e) ANOVA test followed by dunnet's or tukey t-test as post-hoc tests were used to confirm significance with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Statistics were calculated using the statistic software PASWStatistics 18 for Mac.

CHAPTER 3: RESULTS

3.1 Introduction and rationale

The thymus does not contain self-renewing T cell progenitors (TCP) and therefore requires continuous importation of progenitors from the blood to sustain T cell production (122, 208). Recruitment of TCP to the thymus is facilitated by a multistep adhesion cascade initiated by the interaction of PSGL-1 expressed on TCP with P-selectin expressed on thymic endothelium (120, 121). Specific N-terminal carbohydrate modifications on PSGL-1 catalyzed by glycosyltransferases are required for binding to P-selectin. In particular, the activity of C2GnT1 has been highlighted in selectin ligand formation on PSGL-1 (186). Interaction of PSGL-1 with endothelial P-selectin slows down TCP, allows them to respond to local chemokine gradients and to engage the endothelial adhesion molecules ICAM-1 and VCAM-1 via their integrins leading to a firm arrest of TCP on the thymic endothelium (120).

Thymic TCP importation is independent of the numbers of available TCP in the blood (183) and is not a steady state process as the thymus alternates between non-receptive and receptive periods (122). This suggests that a feedback mechanism regulates expression of recruitment signals on vascular or peri-vascular cells.

One factor proposed to control progenitor entry into the thymus is the limited availability of intrathymic niches for which TCP compete and thus gain access to survival and differentiation factors necessary for their development (183). Full thymic niches are thought to trigger a feedback signal to reduce TCP importation, but the nature of these feedback signals and how they influence thymic TCP entry is unknown.

3.2 Results

3.2.1 Thymi of PSGL-1 and IL-7R deficient mice are hyper-receptive for TCP

It was shown previously that P-selectin is functionally involved in thymic TCP importation (121). Thymi of mice lacking the P-selectin ligand PSGL-1 (PSGL-1^{-/-}) were found to have significant elevated levels of P-selectin RNA and an increased thymic receptivity for TCP. IL-7 receptor α -chain deficient mice (IL-7R^{-/-}), known to have high TCP receptivity (183), were also shown to have elevated thymic P-selectin RNA expression that was down-regulated after thymic reconstitution with wild type (wt) TCP (121) (Fig. 3.1). These observations suggested a feedback mechanism between thymic niche occupancy and P-selectin expression and indicated a possible regulatory role for P-selectin in the thymic gate keeping mechanism.

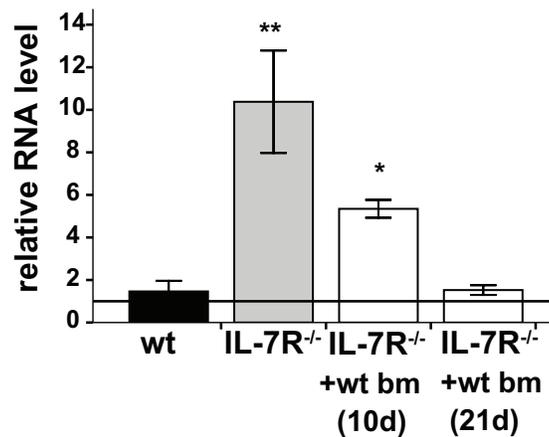


Figure 3.1: Thymic P-selectin expression is reduced after transfer of wt bone marrow cells into IL-7R deficient mice.

P-selectin RNA expression as determined by qrtPCR in thymi of wt, IL-7R^{-/-} mice and IL-7R^{-/-} that received wt bone marrow 10 or 21 days before analysis. RNA levels were normalized to VE-cadherin expression and expressed relative to wt (1, as indicated). Mice were sex-matched and 38d \pm 2d old. The experiment was repeated twice (n = 4). p-values describe significance of difference in means relative to wt.

To further characterize the relationship of thymic P-selectin expression and T cell progenitor receptivity, the analysis of thymic P-selectin RNA expression and of thymic T cell progenitor receptivity was extended in this study by including two further mouse strains: C2GnT1^{-/-} mice that are also deficient in TCP homing due to a lack of functional P-selectin ligand formation on TCP (121) and mice that lack the recombination activation gene 1 (Rag-1) required for the formation of B and T cell receptors and that were shown to have a refractory thymus (183, 187)

Initially, two complementary approaches were used to assess thymic TCP receptivity in mice deficient for Rag-1, C2GnT1, PSGL-1, IL-7R or P-selectin. Short-term homing assays in non-irradiated recipients were performed to determine the capacity of thymi to import i.v. injected CFSE labeled bone marrow cells from the blood stream. Long-term reconstitutions of non-irradiated hosts were carried out to determine the contribution of i.v. injected bone marrow cells to the host's DP thymocyte pool.

Short-term receptivity of thymi was expressed as a ratio of homed cells to thymic cell numbers (Fig. 3.2a, b) or per milligram of thymic weight (Fig. 3.2c) to eliminate a skewing of the data due to variations in thymic size in the different mouse strains analyzed (Fig. 3.2f). When expressed as ratio of number of homed CFSE⁺ cells per 2×10^6 thymocytes the data show that IL-7R^{-/-} thymi import 4-fold more CFSE⁺ cells than wt mice (Fig. 3.2a). PSGL-1^{-/-} mice show also significantly increased receptivity for CFSE⁺ bone marrow cells whereas C2GnT1^{-/-} thymi did not have an increased receptivity when compared to wt mice. Receptivity in Rag-1^{-/-} thymi was non-significantly reduced compared to wt mice.

The differences in the short-term homing data are more prominent when displayed as ratio of homed lineage marker (Lin: CD3, CD4, CD8, CD19, GR-1, CD11b, CD11c, Ter119, NK and γ/δ TCR) negative CFSE⁺ cells per 2×10^6 thymocytes. IL-7R^{-/-} mice have an almost 30-fold increased thymic receptivity compared to wt mice (Fig. 3.2b), whereas PSGL-1^{-/-} and C2GnT1^{-/-} mice have an 8- and 2-fold increased receptivity, respectively, while receptivity in Rag-1^{-/-} mice was reduced.

Similarly, IL-7R^{-/-} thymi were again found to be the most receptive with approximately 80 CFSE⁺ cells per mg thymus when data were expressed as numbers of CFSE⁺ cells per mg thymic tissue, followed by PSGL-1 and C2GnT1 deficient thymi with 60 and 40 CFSE⁺ cells per mg thymic tissue, respectively (Fig. 3.2c). Rag-1^{-/-} mice in contrast showed significantly ($p < 0.005$) reduced receptivity when compared to wt controls.

The numbers of CFSE⁺ cells per milliliter blood was similar in all mouse strains, confirming that differences in thymic importation are not due to differences of injected donor cell in the blood (Fig. 3.2d). Taken together, this set of data shows that IL-7R^{-/-} and PSGL-1^{-/-} thymi have increased receptivity, whereas C2GnT1 deficient mice showed no or only modest increased thymic receptivity. As shown before Rag-1^{-/-} thymi were found to have reduced TCP receptivity (183).

The short-term assay can not be used to determine whether thymi import functional TCP, since neither the exact nature of the TCP population in the blood that seeds the thymus under physiological conditions nor the intrathymic TCP population that contributes predominantly to T cell formation is yet known (103). Long-term thymus reconstitution experiments in non-irradiated recipients were thus used to evaluate whether the observed increased short-term receptivity in IL-7R^{-/-} and PSGL-1^{-/-} mice corresponded to an increased

contribution of injected donor bone marrow cells to DP thymocyte formation. Three weeks after transfer of congenic wild type bone marrow cells, the long-term reconstitution experiments showed that IL-7R^{-/-} mice had the highest chimerism (79x10⁶ donor derived DP). There was a significant increase in numbers of donor derived DP thymocytes in PSGL-1^{-/-} and C2GnT1^{-/-} mice (38x10⁶ and 20x10⁶ respectively) when compared to wt recipients (4.2x10⁶ donor derived DP) (Fig. 3.2e) whereas thymi of Rag-1^{-/-} mice had significantly lower numbers (4.4 x10⁵) of donor derived DP thymocytes (Fig. 3.2e). These findings are in agreement with the data from the short-term homing assays.

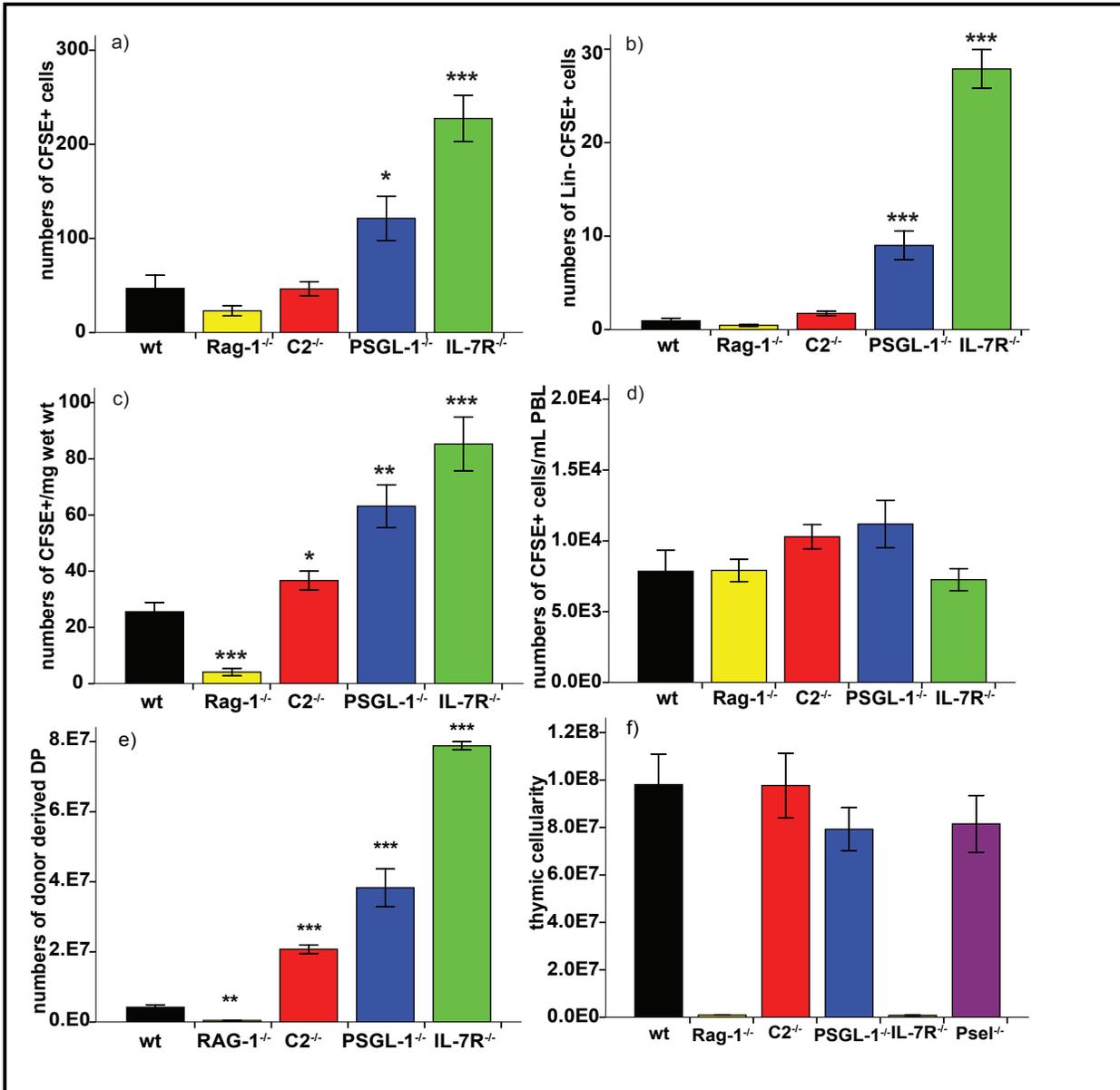


Figure 3.2: Thymic receptivity in wt, Rag-1^{-/-}, C2GnT1^{-/-}, PSGL-1^{-/-} and IL-7R^{-/-} mice in short- and long-term reconstitution assays.

(a-d) 24 hours after i.v. injection of CFSE labeled bone marrow cells, mice were analyzed for CFSE⁺ cells. (a) Absolute numbers of CFSE⁺ cells per 2x10⁶ thymocytes, (b) numbers of Lin⁻ CFSE⁺ cells per 2x10⁶ thymocytes and (c) numbers of CFSE⁺ cells per milligram thymus (wet wt) in thymi of the indicated mouse strains. (d) Numbers of CFSE⁺ cells present in one mL peripheral blood (PBL). (e) Numbers of donor-derived DP three wks after i.v. injection of congenic bone marrow cells. (f) Thymic cellularity in sex- and age-matched mice of the indicated mouse strains as determined by flow cytometry using reference beads to calculate absolute cell numbers. Data are representative for three independent experiments with at least four mice per group. Mice were 35 +/- 3 days. p-values describe significance of difference in means relative to wt. C2, C2GnT1.

3.2.2 P-selectin and CCL25 expression correlate with thymic TCP importation

To determine whether differences in thymic P-selectin expression could explain differences in thymic receptivity, P-selectin RNA expression was measured by quantitative real time PCR (qRT-PCR) on whole thymic tissues crudely depleted of thymocytes. Thymic P-selectin expression is restricted to endothelial cells (121). Thus, P-selectin RNA was normalized using the endothelial specific marker VE-cadherin to exclude a skewing of the data due to differences in thymic size and relative endothelial content. P-selectin RNA levels were high in *IL-7R^{-/-}* and *PSGL-1^{-/-}* thymi when compared to wt thymi (Fig. 3.3a). P-selectin RNA levels were also increased in *C2GnT1^{-/-}* thymi, but about 4.5-fold lower than in *PSGL-1^{-/-}* thymi, whereas *Rag-1^{-/-}* mice had significantly reduced thymic P-selectin RNA. Control experiments showed that P-selectin RNA levels in spleens were comparable in all mouse strains, demonstrating that altered P-selectin expression was thymus-specific (Fig. 3.3b).

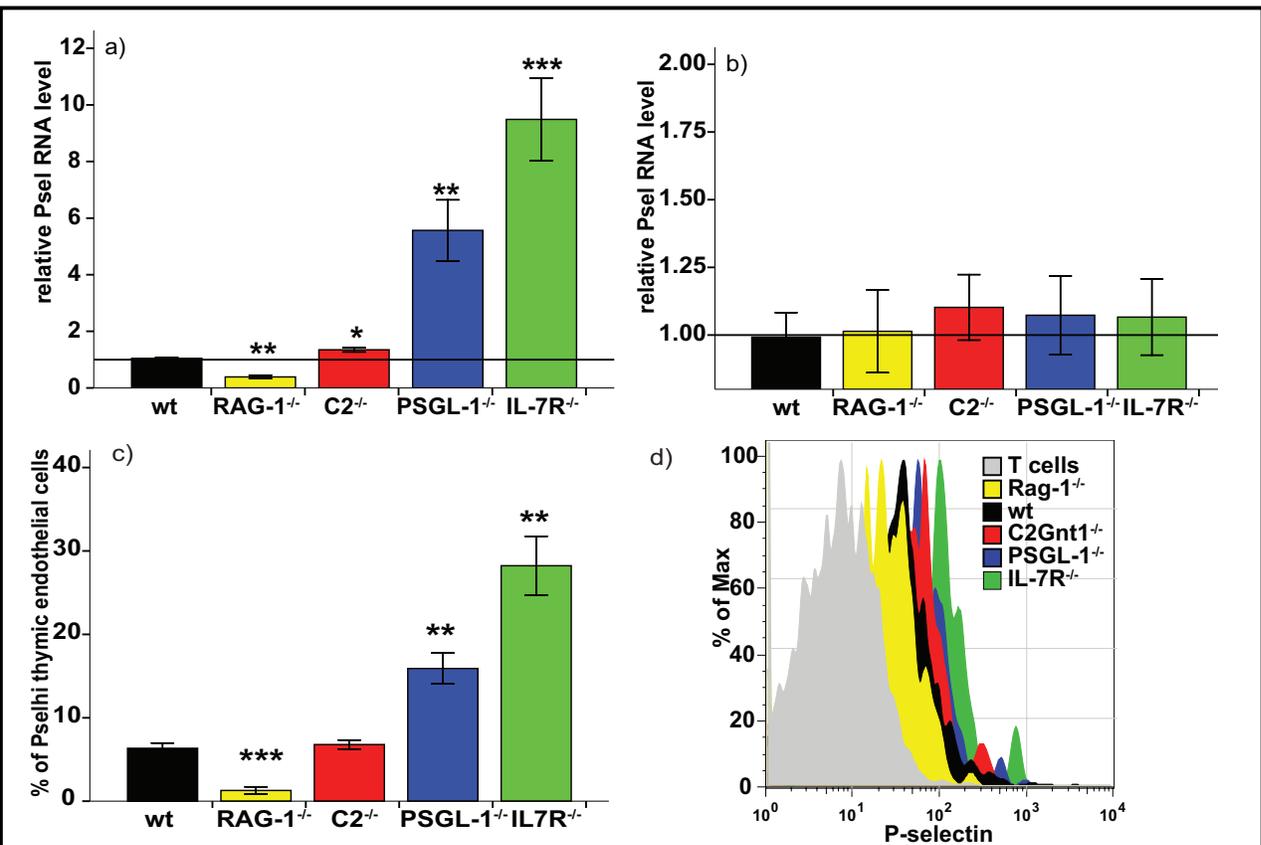
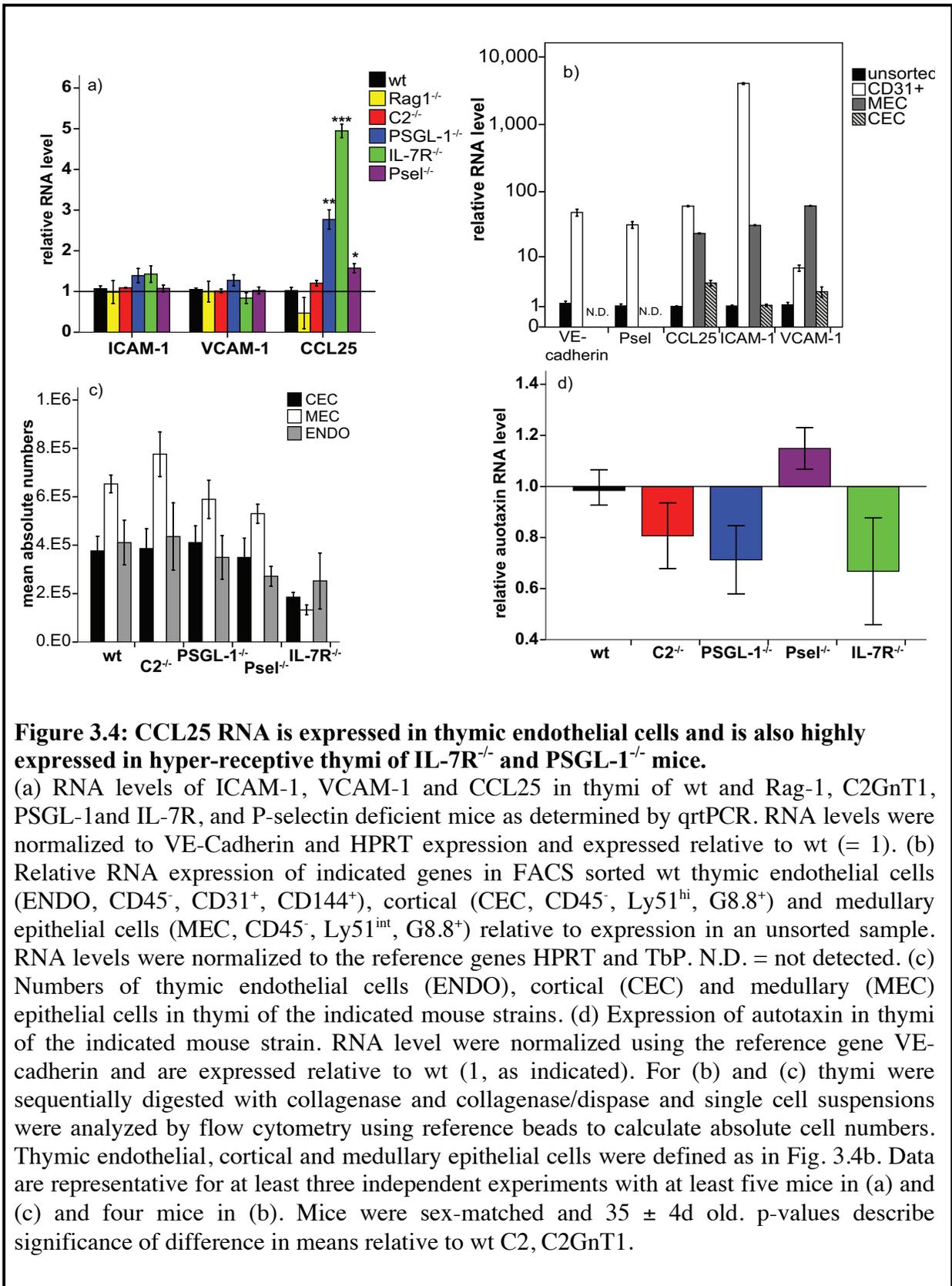


Figure 3.3: Thymic P-selectin expression is increased in hyper-receptive thymi of IL-7R^{-/-} and PSGL-1^{-/-} mice.

P-selectin RNA expression determined by qrtPCR in thymi (a) and spleens (b) of mice of indicated mouse strains. RNA levels were normalized to VE-cadherin expression and expressed relative to wt (1, as indicated). (c) Frequencies of P-selectin^{hi} expressing cells within the thymic endothelial cell population (defined as CD45⁻, CD144⁺, CD31⁺) in thymic cell suspensions derived from wt, Rag-1, C2GnT1, PSGL-1 and IL-7R deficient mice as determined by flow cytometry. (d) Representative histograms for P-selectin expression on thymic endothelial cells (defined as in c) from the indicated mouse strains. Data are representative for three independent experiments with at least six mice per group in (a-b) and at least three mice in (c). Mice were sex-matched and 37d ± 2d old. p-values describe significance of difference in means relative to wt. C2, C2GnT1.

To confirm that increased P-selectin RNA levels are associated with elevated P-selectin protein levels on thymic endothelial cells thymic endothelial cells were analyzed (CD45⁻, CD31⁺, CD144⁺) by flow cytometry. PSGL-1^{-/-} and IL-7R^{-/-} mice had a 2- and 4-fold increased frequency of P-selectin^{hi} expressing endothelial cells respectively when compared to wt controls (Fig. 3.3c). In C2GnT1^{-/-} thymi P-selectin protein expression was comparable to wt controls whereas it was significantly reduced on endothelial cells from Rag-1^{-/-} thymi. Direct comparison of P-selectin level on thymic endothelial cells derived from the different mouse strains followed the same trend with highest P-selectin levels in hyper-receptive IL-7R^{-/-} mice and the lowest in Rag-1^{-/-} mice (Fig. 3.3d).

In addition to P-selectin, the integrin ligands ICAM-1 and VCAM-1 and the chemokine CCL25 are known to support thymic T cell progenitor importation (120). To assess whether expression of these molecules is similarly regulated to P-selectin expression, RNA levels in thymi of wt and the abovementioned mouse strains were compared. Significantly increased levels of CCL25 RNA were found in thymi of IL-7R^{-/-} and PSGL-1^{-/-} mice and to a lower degree in thymi of P-selectin^{-/-} mice when compared to wt thymi (Fig. 3.4a). C2GnT1^{-/-} mice showed similar CCL25 expression as wt mice. ICAM-1 and VCAM-1 RNA levels were comparable in all tested strains.



qRT-PCR analysis carried out on sorted thymic endothelial (ENDO), cortical (CEC) and medullary epithelial (MEC) cells confirmed that P-selectin and the reference gene VE-Cadherin were exclusively expressed in thymic endothelial cells (CD45⁻, CD31⁺, CD144⁺) and absent in CEC (CD45⁻, Ly51^{hi}, G8.8⁺) or MEC (CD45⁻, Ly51^{int}, G8.8⁺) (Fig. 3.4b). Consistent with earlier literature reports, ICAM-1 and VCAM-1 expression was detected in endothelial and epithelial cells (51, 71). The highest ICAM-1 expression was found in endothelial cells, whereas the highest VCAM-1 expression was found in MEC. Furthermore, it was found that CCL25 RNA levels were highest in endothelial cells, followed by MEC and CEC. Since the numbers of endothelial cells in the thymi of the different mouse strains were comparable (Fig. 3.4c) skewing of our data due to variation in thymic cell composition can be excluded.

Recently, it was described that autotaxin expressed in endothelial cells of HEV enhance entry of lymphocytes into lymph nodes (176). Since the postcapillary venules in the thymus have been described to locally exhibit some HEV-characteristics (41) it is possible that autotaxin is also expressed in the thymus and might contribute to T cell entry. Thymic tissues of wt mice and C2GnT1, PSGL-1, P-selectin and IL-7R deficient mice expressed RNA for autotaxin (Fig 3.4d). The relative levels for autotaxin RNA were not significantly different in the tested mouse strains and did not follow the trend of thymic P-selectin or CCL25 expression. However, the thymi with increased receptivity like C2GnT1^{-/-}, PSGL-1^{-/-} and IL-7R^{-/-} mice showed a trend for reduced autotaxin RNA level whereas the thymi of P-selectin deficient mice expressed increased autotaxin RNA level when compared to the wt control.

Collectively, this set of data demonstrate that thymi with high receptivity for TCP express elevated levels of P-selectin and CCL25, supporting the idea that these molecules are not only functionally involved in thymic TCP importation, but might also be involved in regulating this process by differential expression.

3.2.3 P-selectin and CCL25 RNA is periodically expressed in wt but not in PSGL-1^{-/-} or C2GnT1^{-/-} thymi

Thymic progenitor importation periodically alternates between non-receptive and receptive phases (122). Should P-selectin and CCL25 regulate TCP entry, one would expect these molecules to be expressed in an oscillating manner. Thus thymic P-selectin and CCL25 RNA expression were determined in mice ranging in age from 12 to 68 days. Indeed, in wt mice P-selectin RNA expression was periodic with distinct phases of high and low P-selectin expression. P-selectin RNA levels showed a much more blunted periodicity over time in C2GnT1^{-/-} and PSGL-1^{-/-} mice (Fig. 3.5a, c, e). P-selectin RNA baseline levels were only slightly increased in C2GnT1^{-/-} mice (1.5-fold) compared to 20 day-old wt controls but significantly (4-fold) in PSGL-1^{-/-} thymi. Neither C2GnT1^{-/-} or PSGL-1^{-/-} mice showed similar amplitude in P-selectin expression than wt mice. P-selectin expression in spleens of wt, C2GnT1^{-/-} and PSGL-1^{-/-} mice remained relatively constant over time suggesting that the observed periodicity is specific for the wt thymus (Figs. 3.5b, d, f).

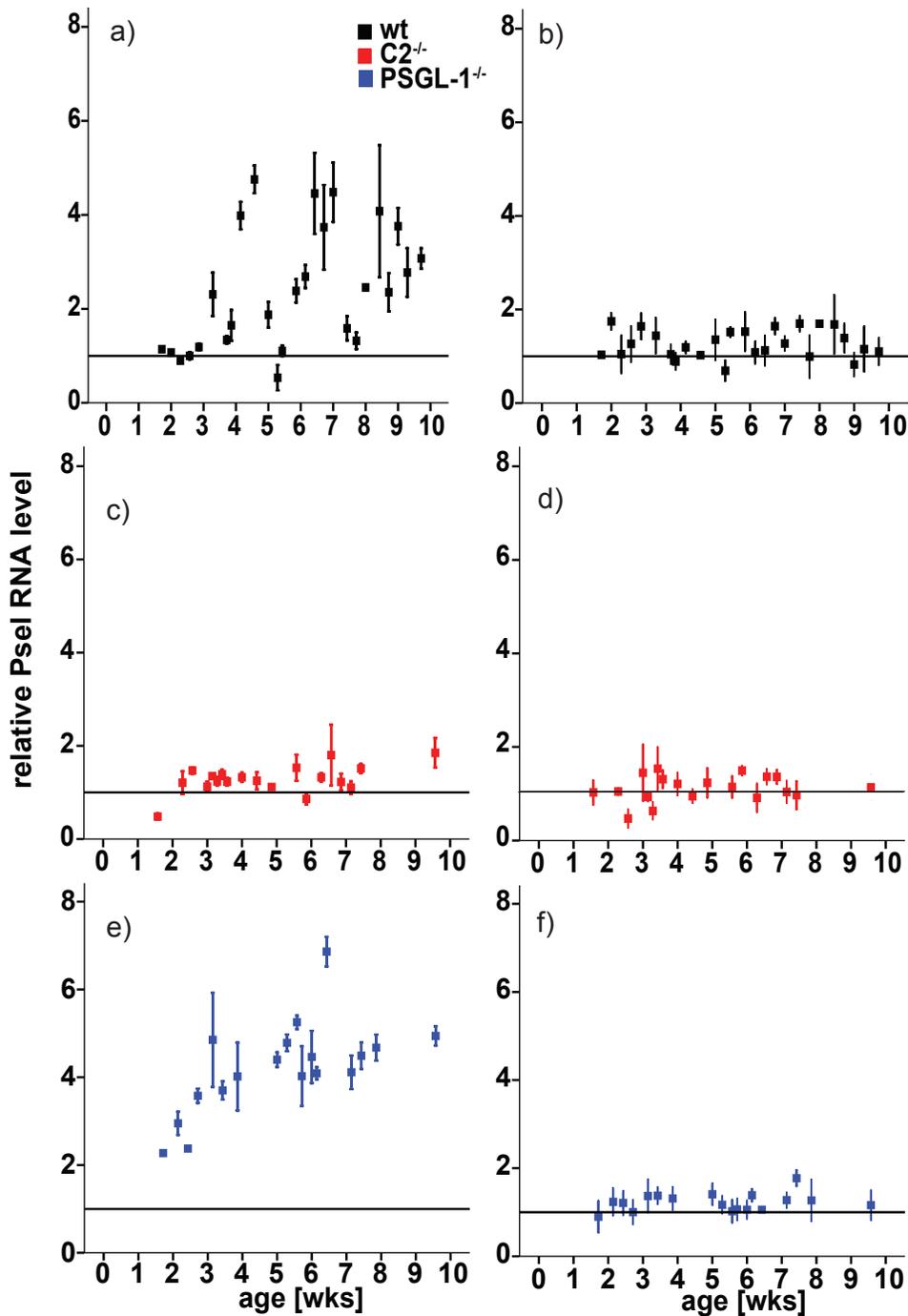


Figure 3.5: P-selectin RNA is periodically expressed in wt thymi but not in spleen.

P-selectin expression in thymi (a, c, e) and spleens (b, d, f) of wt (a, b) PSGL-1^{-/-} (c, d) and C2GnT1^{-/-} mice (e, f). P-selectin RNA levels were normalized to VE-cadherin expression and are expressed relative to wt values at age 20 days (black line). Data are representative for at least five independent experiments (n ≥ 4 per time point). C2, C2GnT1.

In wt mice, the temporal thymic expression pattern of CCL25 RNA was similar to that observed for P-selectin (Fig. 3.6a). Plotting the mean expression level of P-selectin as a function of mean CCL25 level at the same time points showed that P-selectin RNA expression highly correlated with CCL25 expression ($p < 0.001$, Pearson's $\rho = 0.607$) (Fig. 3.6b). Thymic CCL25 RNA expression again lacked periodicity in C2GnT1^{-/-} and PSGL-1^{-/-} mice where it was expressed at relatively constant levels with only minor periodic changes. Similar to the observation for thymic P-selectin, CCL25 RNA levels were considerably higher in PSGL-1^{-/-} thymi than in C2GnT1^{-/-} thymi (Fig. 3.6c).

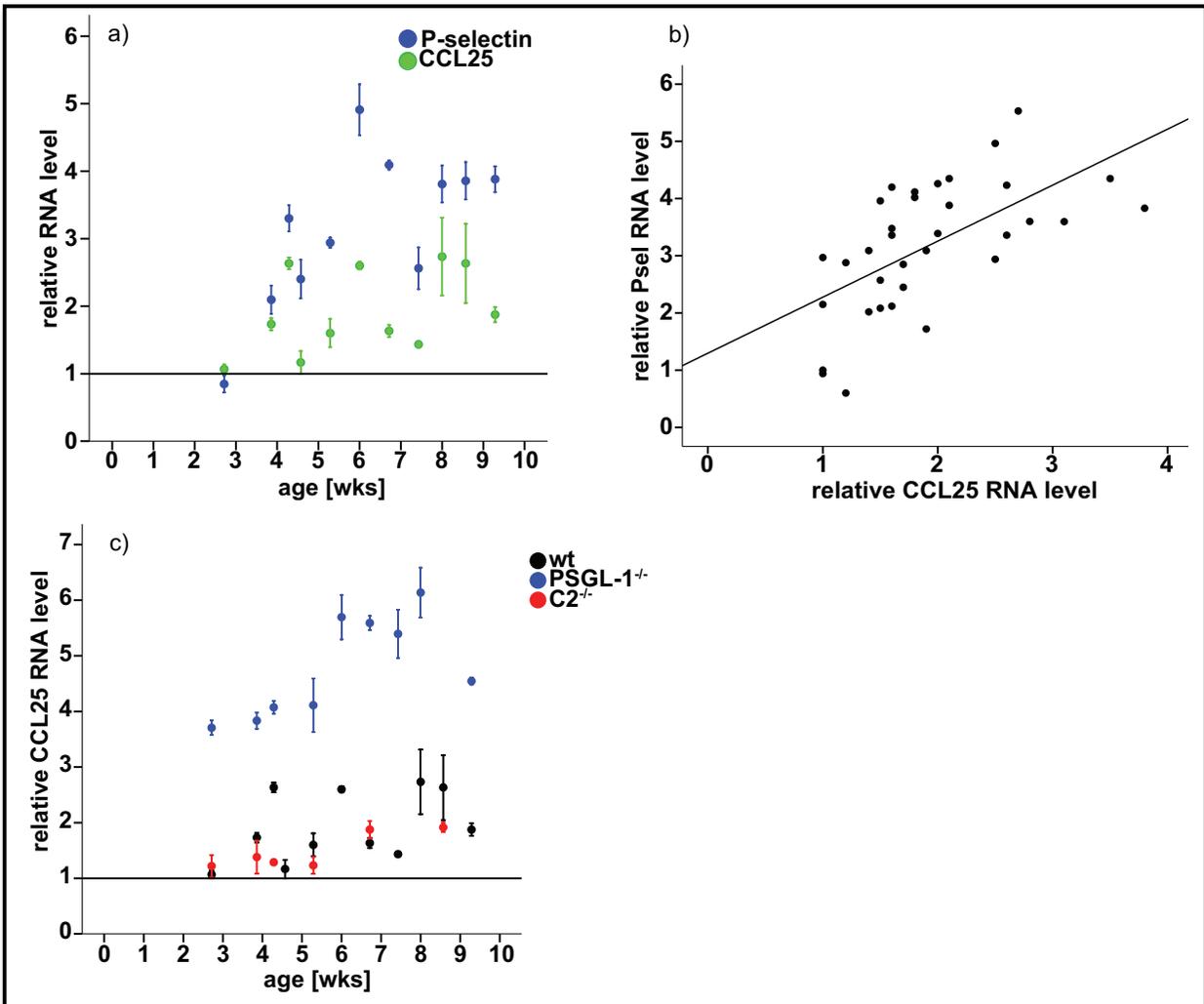


Figure 3.6: P-selectin and CCL25 RNA are synchronously expressed in wt thymi over time.

(a) Temporal P-selectin and CCL25 RNA expression in wt thymi. (b) Scatter plot showing P-selectin expression as a function of CCL25 expression with the same samples used in Fig. 3a. Line shows linear regression. Thymic P-selectin expression and CCL25 RNA show a highly significant ($p < 0.001$) correlation with Pearson's $\rho = 0.607$. (c) CCL25 expression over time in thymi of wt, PSGL-1^{-/-} and C2GnT1^{-/-} mice. P-selectin and CCL25 RNA level were normalized to the reference genes VE-cadherin and HPRT and are expressed relative to the corresponding value found in wt thymi at age 20 days (indicated). Data are representative for at least five independent experiments ($n \geq 4$ per time point). C2, C2GnT1.

To evaluate whether P-selectin expression over time correlated with thymic receptivity for TCP in wt mice, P-selectin expression was analyzed in one cohort of mice aged 3 to 10 weeks. In parallel the other cohort received congenic bone marrow i.v. on the same day P-selectin expression was analyzed. Three weeks after bone marrow transfer mice were analyzed for numbers of donor derived DP thymocytes and thymic chimerism was correlated with thymic P-selectin RNA expression (Fig. 3.7). Thymic P-selectin RNA expression and TCP receptivity measured in numbers of donor derived DP three weeks after bone marrow transfer showed only low linear correlation (Pearson's $\rho = 0.146$, $p = 0.301$) over time in wt mice (Fig. 3.7b). However, a trend was observed that times of high P-selectin expression were mostly associated with increased receptivity, in particular around the age of 6 to 7.5 weeks. Nevertheless, the high noise level in the measurement of thymic receptivity does not allow a clear conclusion. Reasons that could explain the high errors in the receptivity measurement will be discussed in chapters 4 and 5.

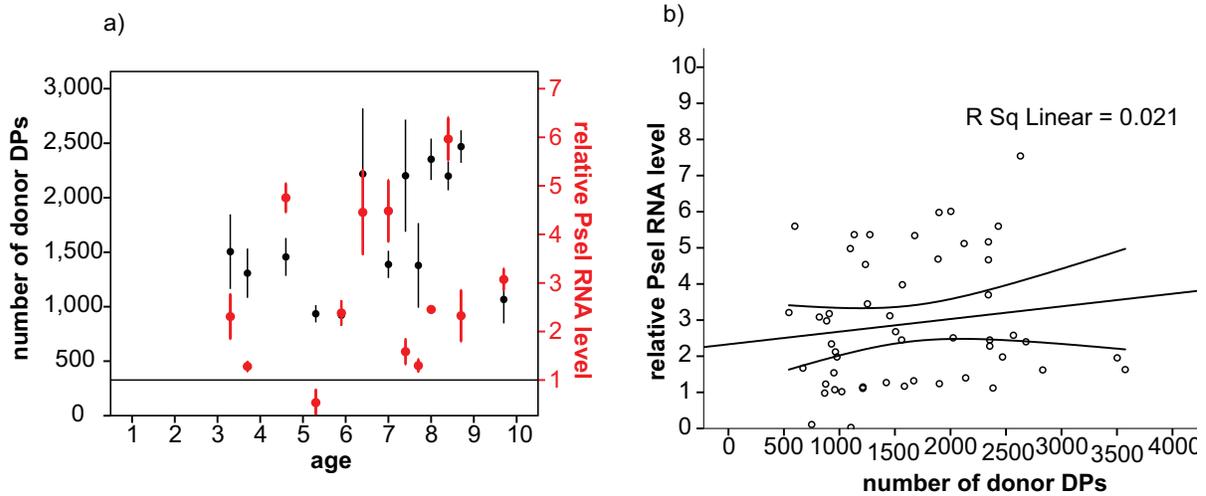


Figure 3.7: P-selectin RNA expression only partially correlates with thymic receptivity over time in wt mice.

(a) P-selectin RNA expression levels over time were determined by qrtPCR in one cohort of mice aged 3 to 10 weeks. In parallel the second cohort of mice received congenic bone marrow injections on the same day of P-selectin analysis to test for thymic receptivity. Three weeks after bone marrow injections mice were analyzed for donor DP chimerism. (b) Relative P-selectin RNA level as a function of numbers of donor derived DP found in the same thymus three weeks after bone marrow transfer. Lines show linear regression with confidential intervals (95%). P-selectin RNA levels were normalized using the reference genes VE-cadherin and are expressed relative to the corresponding value found in wt thymi at age 20 days (1, as indicated). Shown is one experiment representative for a total of three, with $n \geq 3$. Only female mice were used.

While it is known that P-selectin, ICAM-1, VCAM-1 and CCL25 have supportive roles in thymic T cell progenitor importation (120) it is speculated whether other proteins such as E-selectin, Chst-1, VAP-1 and CXCL12 might also contribute to TCP entry and regulation of TCP entry merely due to their presence on the thymic vasculature (147) (see sections 1.6.1.3 and 1.6.3.3). To evaluate whether the expression of these molecule changes in a wt steady state thymus, RNA expression of E-selectin, Chst-1, VAP-1 and CXCL12 was tested at time of low (week 4) and high P-selectin (week 6.5) expression. When compared to the RNA level found at week 4, all tested genes showed increased expression, except for CXCL12. These data confirm earlier findings (147) and point to a possible role for E-selectin, Chst-1 and VAP-1 in the regulation of TCP entry (Fig. 3.8).

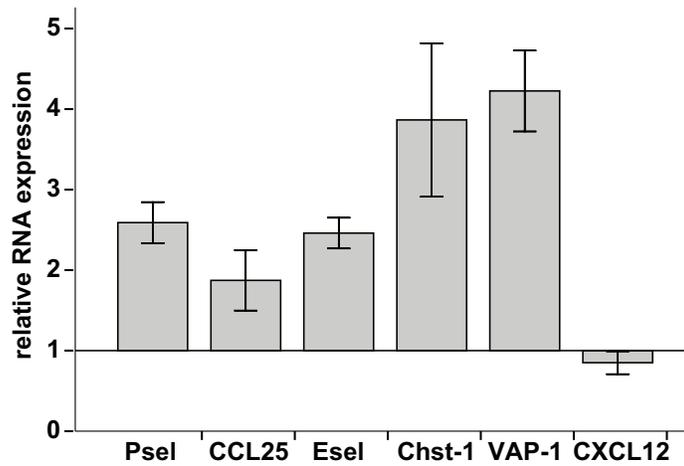


Figure 3.8: E-selectin, Chst-1 and VAP-1 are up-regulated at times of high P-selectin and CCL25 expression.

RNA expression of the indicated genes in wt thymi was measured at week 4 (Psel^{lo}) and week 6.5 (Psel^{hi}). RNA levels were normalized using the reference gene VE-cadherin and expressed relative to the expression at week 4 (1, as indicated). Data are representative for two independent experiments (n = 4).

3.2.4 Thymic niche-occupancy is not the only factor limiting thymic receptivity

To identify the feedback signals that regulate thymic P-selectin/CCL25 expression and thymic TCP receptivity thymocyte subsets, their potentials to give rise to T cells and the thymic niches were characterized.

It has been shown that competition for intrathymic niches especially at the triple negative (TN; all are Lin^{lo}CD4⁻CD8⁻ with TN1 CD44⁺CD25⁻, TN2 CD44⁻CD25⁺, pre-DP CD44⁻CD25⁻) TN2 and TN3 stages limit TCP entry into the thymus (185). To test this model non-irradiated IL-7R^{-/-} mice were reconstituted with FACS sorted TN1, TN2 or TN3 wt thymocytes and thymic P-selectin expression and chimerism in thymus and blood was measured 12 days later (Fig. 3.9a-c). Injection with each TN subset led to thymic reconstitution and the formation of peripheral T cells (Fig. 3.9 e, f) which was accompanied by a significant reduction in thymic P-selectin RNA when compared to the vehicle treated controls (Fig. 3.9a). TN3 cells did not give rise to TN1 or TN2 cells (Fig 3.9d) indicating that TN3 cells and their progeny are sufficient to control thymic receptivity. These data are consistent with the niche occupancy model and it was thus expected that differences in thymic receptivities observed in this study were caused by differences in thymic niche occupancy.

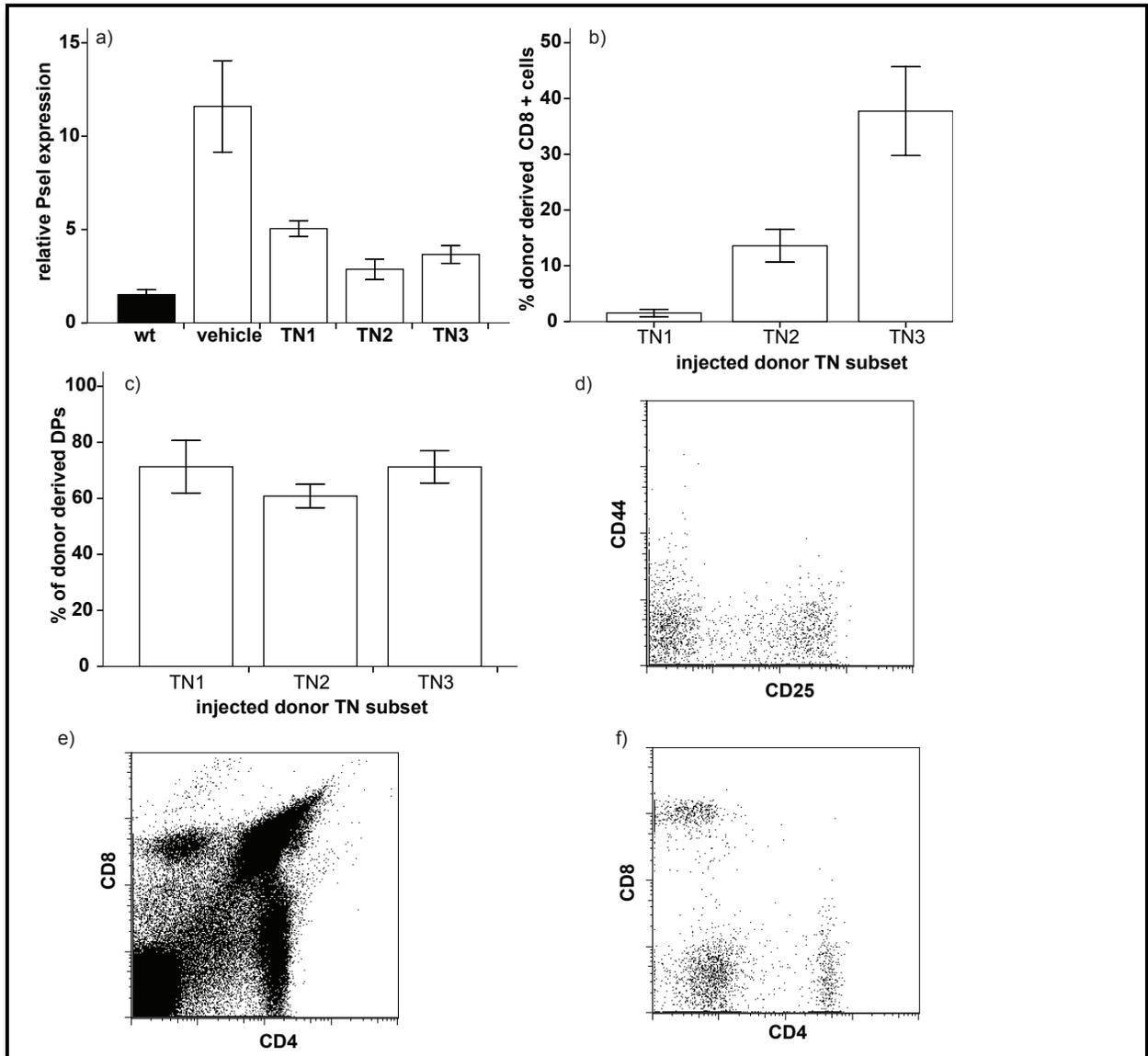
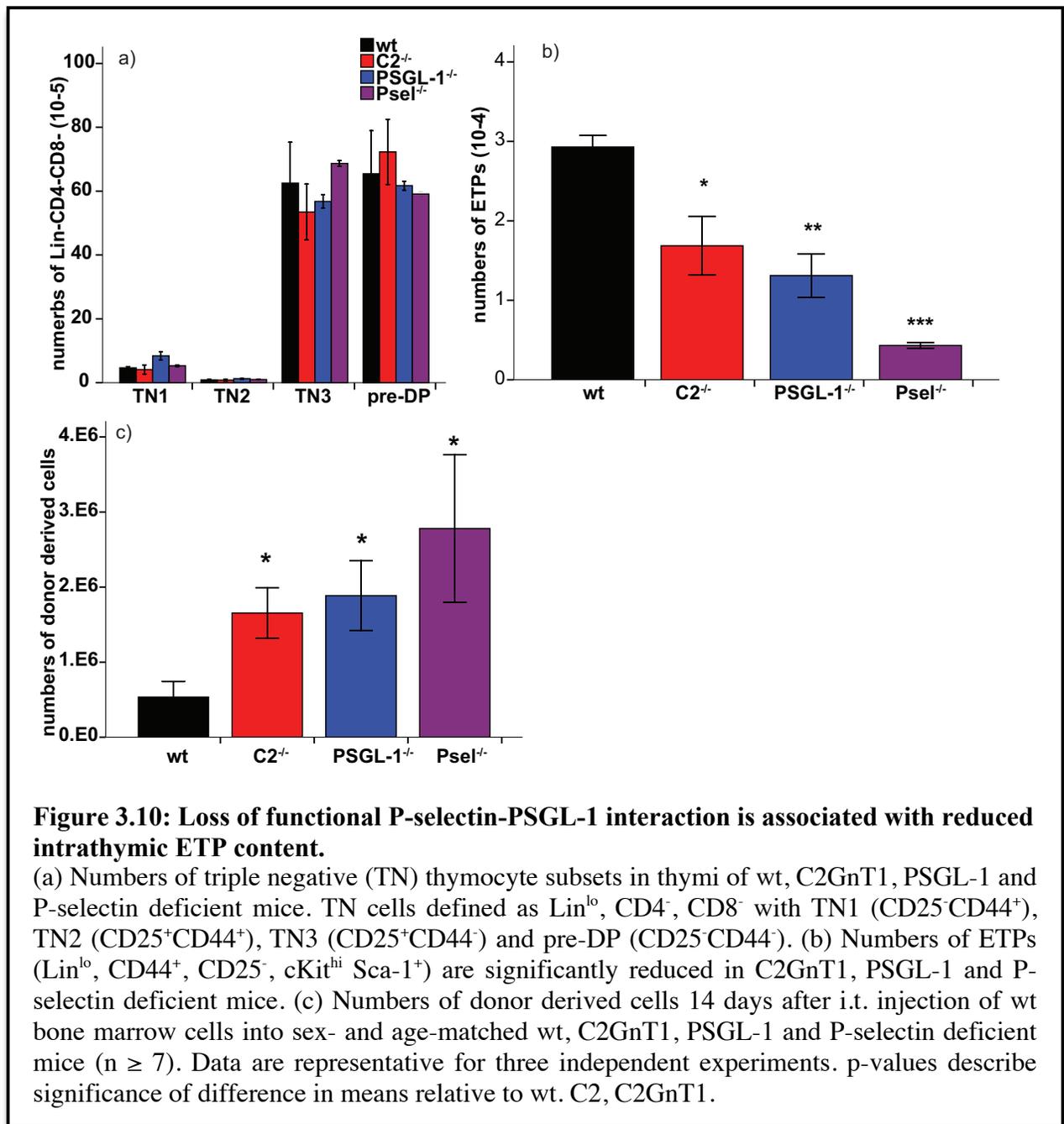


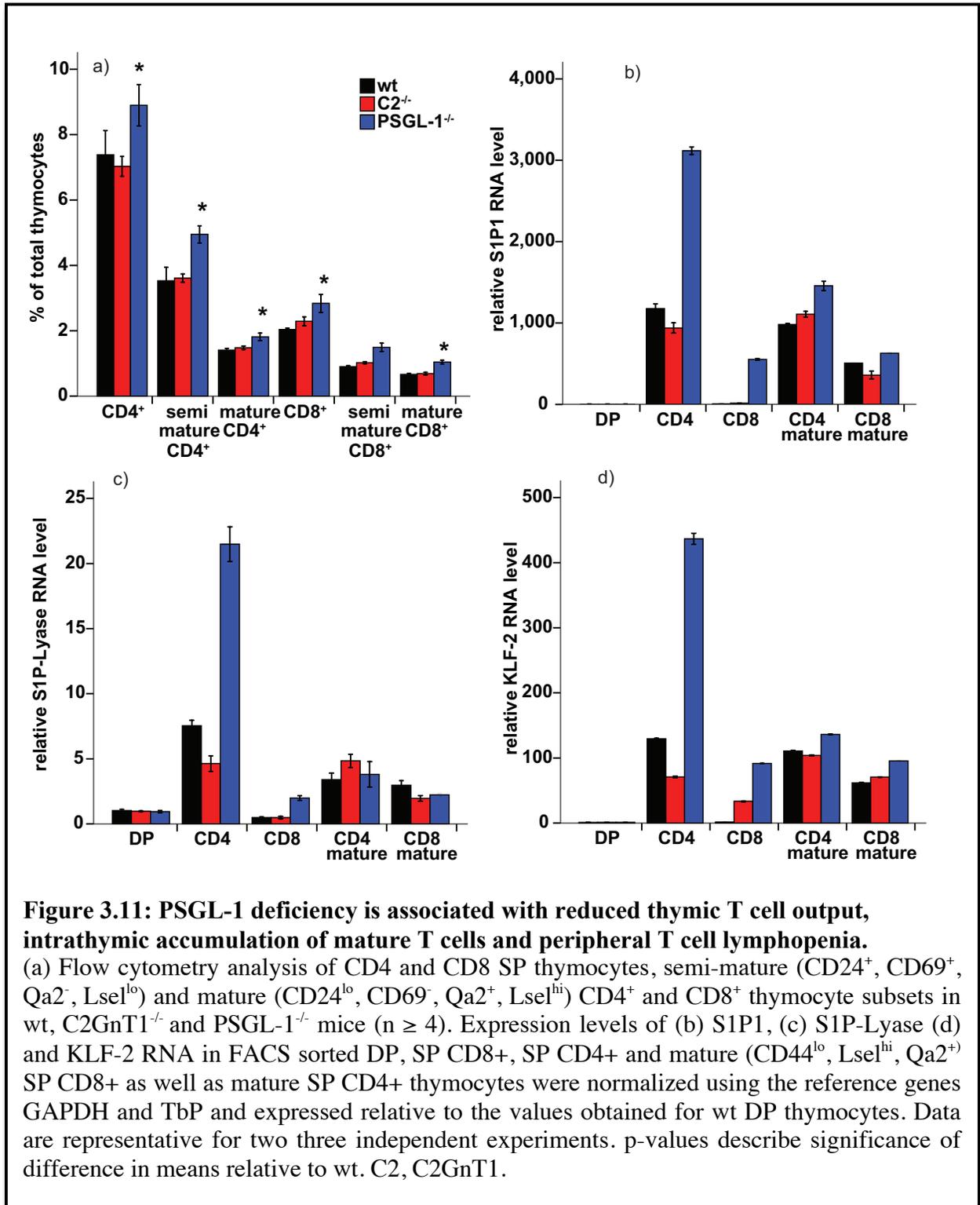
Figure 3.9: Niche occupancy regulates thymic receptivity in reconstituted *IL-7R^{-/-}* mice. (a) TN1-TN3 thymocyte subsets were purified by FACS and i.v. injected into non-irradiated *IL-7R^{-/-}* mice. The figure shows thymic P-selectin RNA level determined by qrtPCR using the reference gene VE-Cadherin 12 days post injection. P-selectin levels are expressed relative to sex- and age-matched wt mice (1, as indicated). (b, c) FACS sorted wt TN1-TN3 subsets were i.v. injected into non-irradiated *IL-7R^{-/-}* mice. 12 days after injection mice were sacrificed and (b) peripheral blood and (c) thymi were analyzed for frequencies of donor derived cells. (d) Injection of TN3 cells does not lead to formation of TN1 or TN2 thymocytes 12 days post injection. Injection of TN3 cells fully reconstitutes the *IL-7R^{-/-}* (e) thymus and leads to formation of (f) peripheral CD4 and CD8 cells 12 days after bone marrow transfer. Data are representative for three independent experiments with at least four mice. Mice were 45 +/- 4d old.

Next, the content and distribution of thymocyte subsets derived from wt, PSGL-1^{-/-}, C2GnT1^{-/-} and P-selectin^{-/-} thymi was characterized. Triple negative thymocyte subset distributions were surprisingly similar in wt and all three knockout strains (Fig. 3.10a). However, the size of the ETP (Lin^{lo}, CD117^{hi}, Sca-1⁺, CD44⁺, CD25⁻) (31) population that is believed to contain the earliest intrathymic T cell progenitors was reduced by 45-85% in thymi of C2GnT1^{-/-}, PSGL-1^{-/-} and P-selectin^{-/-} mice (Fig. 3.10b). Given that the total thymic cellularity and the numbers of thymocyte subsets of the knockout thymi are similar to wt thymi (Fig. 3.2f), it is likely that the numbers of existing intrathymic niches may be similar in wt and knockout thymi. To test whether differences in the occupation status of intrathymic niches could explain the observed differences in receptivity, mice from these mice strains were intrathymically (i.t.) injected with wt bone marrow cells. Significantly (3-5 times) increased availability of intrathymic niche space was found in mice deficient for PSGL-1, P-selectin or C2GnT1 (Fig. 3.10c). Niche availability inversely correlated with thymic ETP content and was comparable in C2GnT1^{-/-} and PSGL-1^{-/-} thymi (Fig. 3.10b, c), contrasting with the significant differences observed between these two mouse strains in short- and long-term receptivity and pointing to mechanisms in addition to niche occupancy that are involved in the regulation of thymic TCP receptivity.



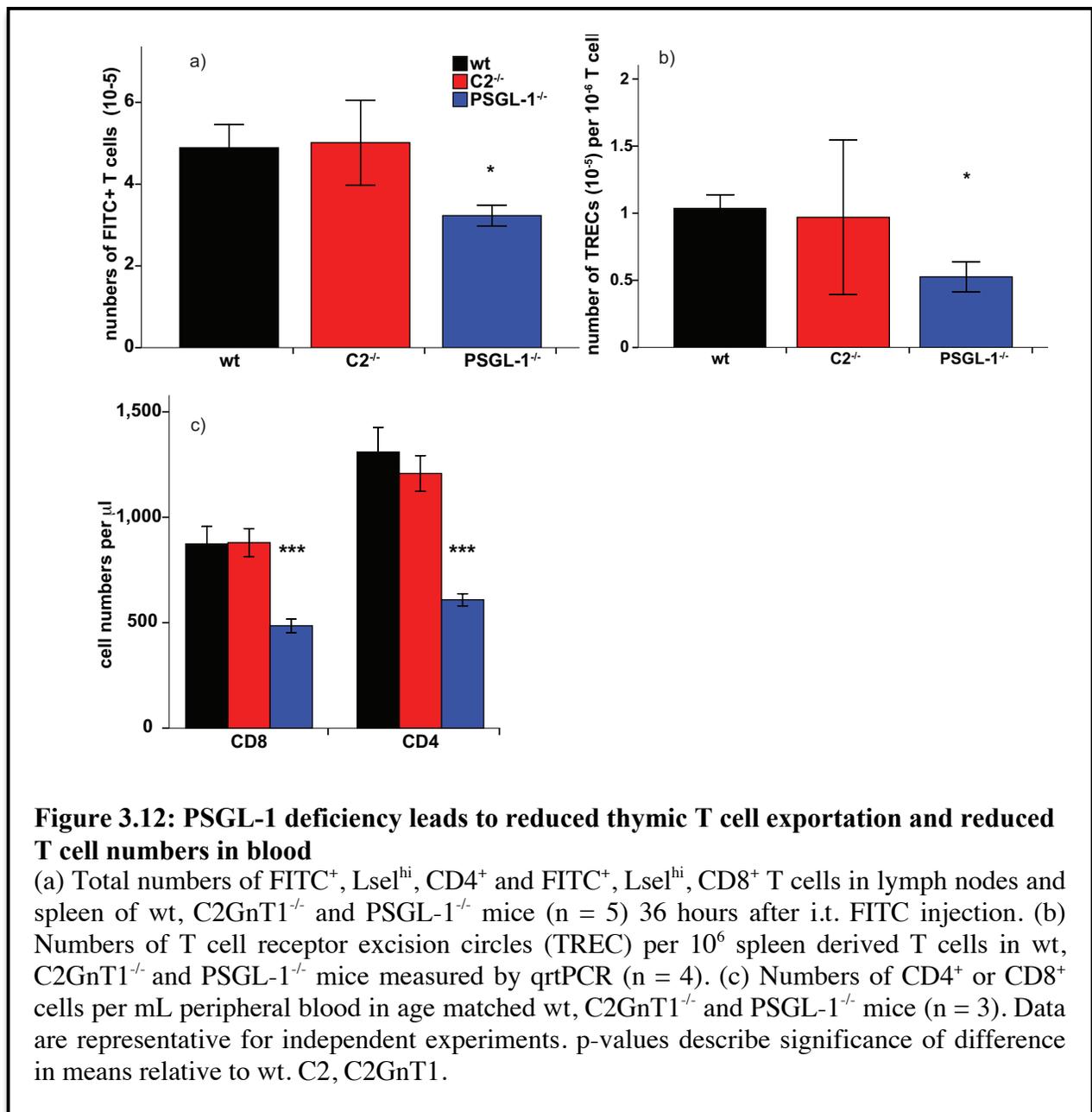
3.2.5 Thymic emigration of SP T cells is impaired in PSGL-1^{-/-} mice

In an effort to find alternative mechanisms that could explain the differences observed in thymic receptivity between PSGL-1 and C2GnT1 deficient mice, mature thymocyte subsets distribution and thymic T cell export rates were determined in wt, PSGL-1 and C2GnT1 deficient mice. Cell surface marker based analysis of maturing thymocyte subsets showed increased frequencies of semi-mature (CD24⁺, CD69⁺, CD62L^{lo}, Qa2⁻) and mature (CD24^{lo}, CD69⁻, Lsel^{hi}, Qa2⁺) (41, 84, 209) CD4⁺ and CD8⁺ thymocytes in thymi of PSGL-1^{-/-} but not in C2GnT1^{-/-} thymi when compared to wt controls (Fig. 3.11a). qrtPCR analysis of FACS sorted mature (CD44^{lo}, CD62L^{hi}, Qa2⁺) CD8⁺ and CD4⁺ thymocytes revealed a significant increase in RNA levels of T cell maturation markers (S1P-Lyase, the transcription factor KLF-2 and its target S1P₁, Fig. 3.11 b-d) confirming a high maturation status of accumulated SP thymocytes in PSGL-1^{-/-} thymi suggesting impaired thymic emigration in these mice.



To directly test whether thymic T cell emigration is impaired in PSGL-1 deficient mice i.t. FITC injections as well as quantification of T cell receptor excision circles (TREC) were performed to determine thymic exit rates. Consistent with the above observations these experiments revealed that mature PSGL-1^{-/-} T cells leave the thymus at a significantly lower rate than wt T cells, whereas C2GnT1^{-/-} T cells emigrated at a rate comparable to wt T cells (Fig. 3.12 a, b).

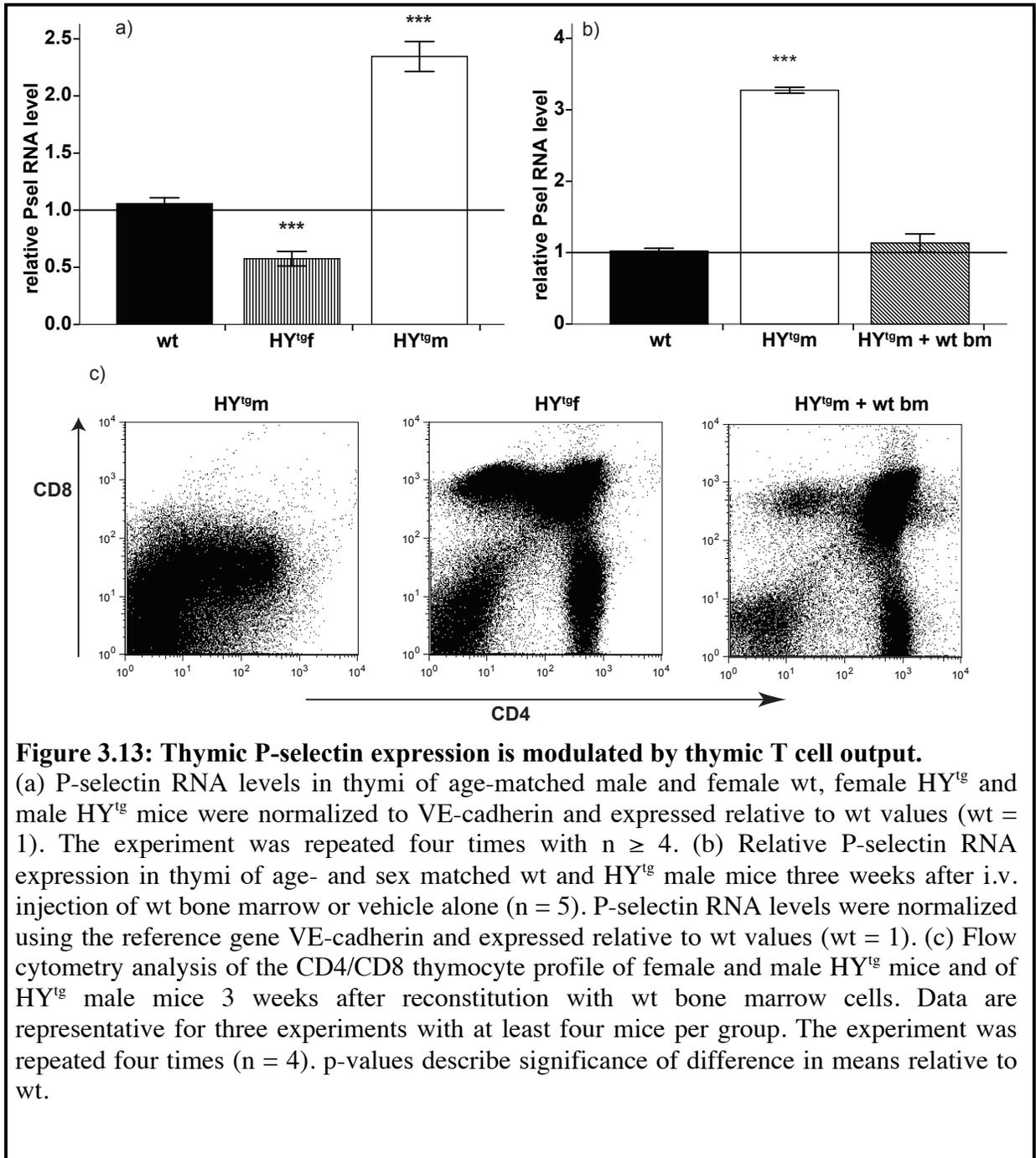
The reduced thymic output in PSGL-1^{-/-} mice was furthermore associated with decreased T cells in the periphery. PSGL-1^{-/-} mice showed significantly reduced numbers of CD4 and CD8 cells in peripheral blood whereas T cell numbers in C2GnT1^{-/-} mice were comparable to wt controls (Fig. 3.12c). Reduced thymic T cell output together with reduced numbers of peripheral T cells in PSGL-1^{-/-} mice led to the hypothesis that thymic T cell output and/or the size of the peripheral lymphocyte pool might modulate thymic P-selectin expression and thus TCP receptivity.



3.2.6 Lymphopenic male HY^{tg} mice express high thymic P-selectin levels

To test whether peripheral T cell lymphopenia modulates thymic P-selectin expression, mice transgenic for the male antigen specific T cell receptor (HY^{tg} model) were used. In this mouse model male and female transgenic mice show significant differences in thymic DP and SP content and consequentially in peripheral T cell numbers. Comparison of

numbers and distribution of DN thymocytes in male and female HY^{tg} mice has been shown to be comparable (210), indicating similar niche occupancy in male and female mice. Thymocytes in male HY^{tg} mice are deleted by negative selection causing thymic atrophy and peripheral T cell lymphopenia (210). Male HY^{tg} mice expressed increased levels of thymic P-selectin compared to wt non-HY^{tg} littermate controls (Fig. 3.13a). In contrast, female HY^{tg} mice that have an increased production of SP CD8 cells due to enhanced positive selection of transgenic CD8 cells (210) expressed reduced levels of thymic P-selectin when compared to wt non-HY littermate controls (Fig. 3.13a). Reconstitution of HY^{tg}-male mice with wt bone marrow cells led to full restoration of the DP and SP compartments three weeks after bone marrow transfer that was accompanied by a reduction of P-selectin expression to wt levels (Fig. 3.13b, c). These data support a model whereby more mature T cell or peripheral T cell levels modulate thymic P-selectin expression and thus thymic TCP importation.



3.2.7 Manipulation of peripheral lymphocyte levels affect thymic TCP receptivity

The above data suggest that peripheral T cell numbers modulate thymic P-selectin expression and TCP importation. To test whether manipulation of the peripheral lymphocyte pool affect thymic P-selectin expression and receptivity, PSGL-1^{-/-} mice were injected with 20 x 10⁶ wt lymphocytes to compensate their lack of peripheral T cells. Two days after lymphocyte transfer thymic P-selectin expression and receptivity for TCP was reduced by 50-60% when compared to saline injected controls (Fig. 3.14a, b, e).

These observations suggested that depletion of peripheral T cells might lead to increased P-selectin expression and thus increased thymic progenitor receptivity. To test this hypothesis peripheral CD4 and CD8 T cells in wt mice were depleted with anti CD4 and CD8 antibodies, whereas thymic T cell numbers remained unchanged (Fig. 3.14f). Both thymic P-selectin RNA and TCP receptivity increased nearly 3-fold within 24 hours after T cell depletion when compared to saline treated controls (Fig. 3.14c, d, e). Collectively, these data support the hypothesis that thymic TCP importation can be altered by changes in peripheral lymphocyte levels.

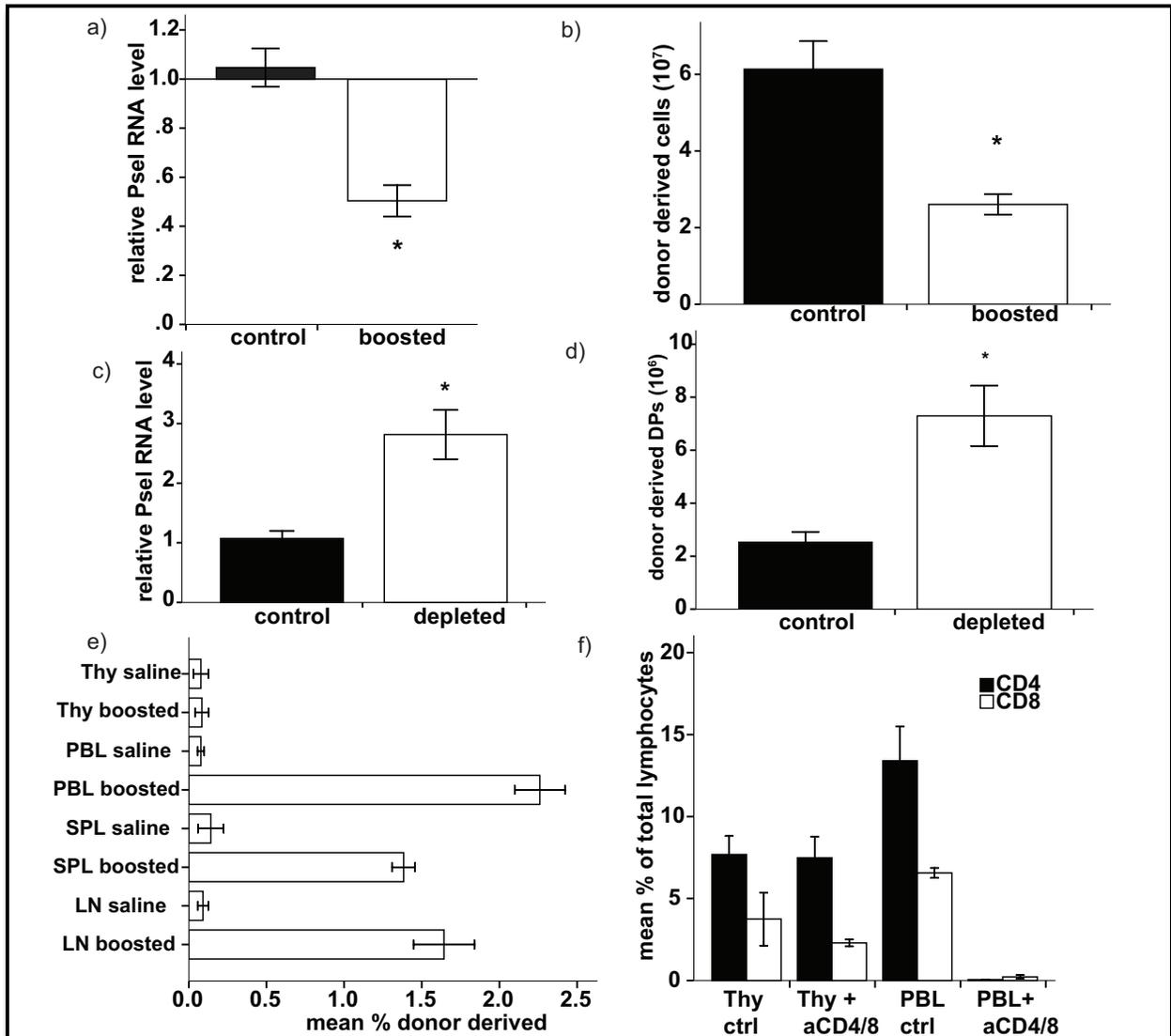


Figure 3.14: The size of the peripheral lymphocyte pool modulates thymic P-selectin expression and thymic receptivity.

(a) Thymic P-selectin RNA levels in PSGL-1^{-/-} mice determined two days after i.v. transfer of 20 x 10⁶ wt lymphocytes (boosted) or vehicle alone (control). (b) Numbers of donor derived DP thymocytes determined by flow cytometry three weeks after transfer of wt bone marrow into lymphocyte (boosted) or vehicle (control) injected PSGL-1^{-/-} mice. (c) Thymic P-selectin RNA levels in wt mice 24 hours after i.p. injection of anti CD4/CD8 antibodies (depleted) or vehicle alone (control). (d) Numbers of donor derived DP thymocytes determined by flow cytometry three weeks after bone marrow transfer into CD4/CD8 T cell depleted (depleted) or control wt mice. (e) Percentages of donor derived wt lymphocytes in PSGL-1 deficient recipient mice two days after adoptive transfer. (f) Frequencies of CD4 and CD8 T cells after in vivo depletion using anti CD4 and CD8 antibodies or vehicle alone as a control. Data shown are representative for two independent experiments with n ≥ 6. RNA levels were normalized to VE-cadherin expression and expressed relative to values from vehicle treated controls (1, as indicated).

3.2.8 P-selectin signaling can be excluded to play a role in regulation of thymic TCP receptivity

The results obtained in the previous boost experiments with PSGL-1 deficient suggest that interaction of P-selectin ligands expressed on transferred lymphocytes with endothelial P-selectin triggers signaling events that might be important for changes in P-selectin expression and therefore thymic receptivity. However, this scenario is unlikely, as resting lymphocytes do not express functional P-selectin ligands (211). Nevertheless, experiments were performed to test for an involvement of P-selectin signaling in regulating TCP entry into the thymus. PSGL-1 deficient mice were boosted with 20×10^6 lymphocytes derived from wt, C2GnT1^{-/-} or PSGL-1^{-/-} mice and two days later P-selectin RNA levels were determined. Injection of lymphocytes from either source – including wt cells expressing PSGL-1 allowing for interaction with endothelial P-selectin – led to significant down-regulation of thymic P-selectin expression. These data suggest that P-selectin engagement by transferred lymphocytes is not required to modulate thymic TCP receptivity (Fig.3.15).

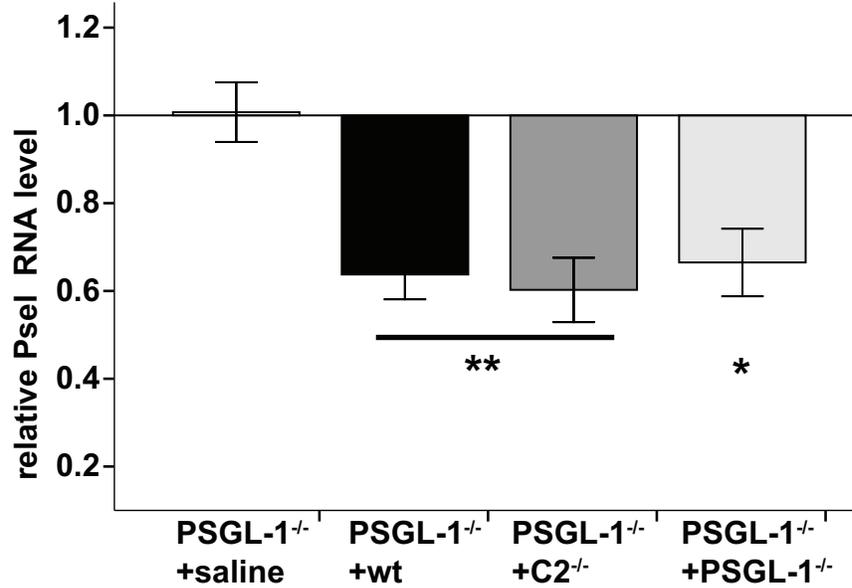


Figure 3.15: P-selectin signaling is not involved in modulation of thymic P-selectin expression.

Thymic P-selectin RNA levels in PSGL-1^{-/-} mice determined two days after i.v. transfer of 20 x10⁶ wt, C2GnT1^{-/-} or PSGL-1^{-/-} lymphocytes or vehicle alone (saline). RNA levels were normalized to VE-cadherin expression and expressed relative to values from saline treated controls (1, as indicated). The experiment was performed once with n = 4. C2, C2GnT1. p-values describe significance of difference in means relative to control.

3.2.9 Plasma derived substances can modulate thymic P-selectin expression

The previous sets of data indicate that the peripheral lymphocyte pool can induce changes in thymic P-selectin expression and thymic receptivity. It is conceivable that lymphocytes directly change thymic TCP importation by interacting with endothelium/epithelium via surface receptors or cell adhesion molecules. Alternatively lymphocytes could modulate thymic receptivity indirectly via cytokines or other soluble mediators. To determine whether soluble mediators play a role in the feedback mechanism between the periphery and the thymus plasma transfer experiments were performed. Mice deficient for PSGL-1 received six injections of wt platelet poor plasma (PPP) within six days, whereas wt mice received PPP derived from PSGL-1 mice. 12 hours after the last injection P-selectin expression was determined by qrtPCR. P-selectin expression in the PPP-injected PSGL-1 mice was significantly reduced compared to the saline injected control whereas P-selectin in the injected wt mice was increased (Fig. 3.16a). These data suggest that a soluble factor in the plasma is able to modulate thymic P-selectin expression.

One candidate soluble factor is $\text{TNF}\alpha$, a very potent inducer of P-selectin surface expression (212). However, when determined by ELISA no differences in plasma $\text{TNF}\alpha$ levels were detected in the different mouse strains used in this study making an involvement of $\text{TNF}\alpha$ in the regulation of thymic P-selectin expression unlikely (Fig. 3.16b).

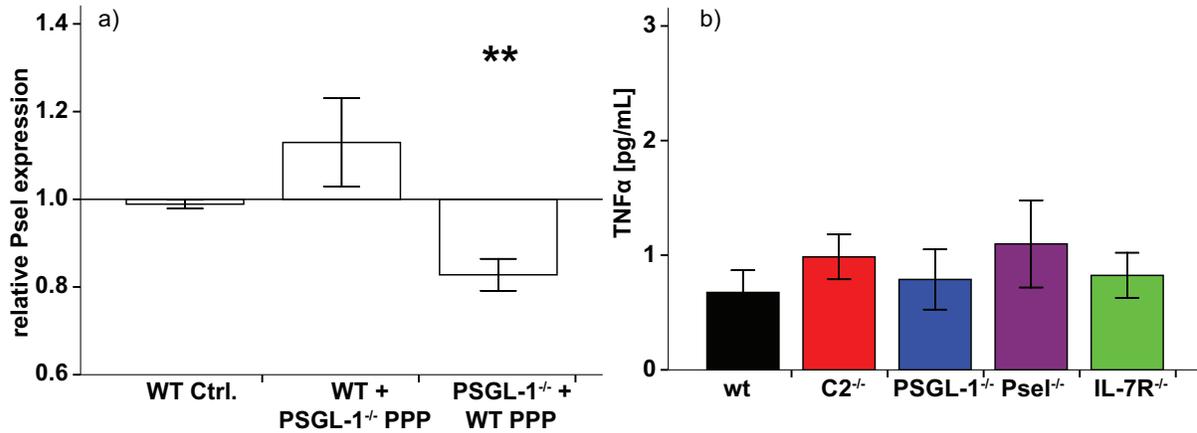


Figure 3.16: Impact of plasma derived factors on thymic P-selectin RNA expression.

(a) Thymic P-selectin RNA level determined 12 hours after the last injection of platelet poor plasma (PPP). RNA levels were normalized to VE-cadherin expression and expressed relative to values from control animals (1, as indicated). The experiment was performed three times with $n = 4$. (b) Levels of TNF α in plasma of the indicated mouse strains as determined by ELISA. The experiment was repeated once with similar results. p-values describe significance of difference in means relative to wt. C2, C2GnT1.

3.2.10 Thymic P-selectin expression correlates with plasma S1P levels

Aside from its role in lymphocyte egress from lymphoid organs S1P is involved in the regulation of endothelial barrier function and the expression of endothelial adhesion molecules including P-selectin (213-216), making this lipid metabolite another potential candidate factor to affect thymic endothelial P-selectin expression.

To test whether S1P levels vary under physiological conditions LC-MS/MS was used to determine S1P levels in plasma of wt and the abovementioned mouse strains. We found that IL-7R^{-/-} and Rag-1^{-/-} mice had S1P plasma levels comparable to wt controls. S1P was moderately (23%, $p = 0.044$) reduced in PSGL-1^{-/-} mice and slightly but not significantly reduced in C2GnT1^{-/-} mice (10%, $p = 0.349$) when compared to wt controls. In contrast, P-selectin^{-/-} mice had 26% ($p = 0.033$) increased S1P plasma levels compared to wt (Fig. 3.17a).

Given that thymic P-selectin expression is periodically expressed in wt thymi it was tested whether plasma S1P levels were also changing periodically and if so whether S1P levels correlated with thymic P-selectin RNA. S1P plasma levels varied in 19-65 day old wt mice within a range of 2-3.7 μM (Fig. 3.17b). Changes in plasma S1P concentration correlated positively and significantly ($p < 0.001$) with changes in thymic P-selectin RNA (Fig. 3.17b, c) suggesting that S1P plasma level and thymic P-selectin expression are linked.

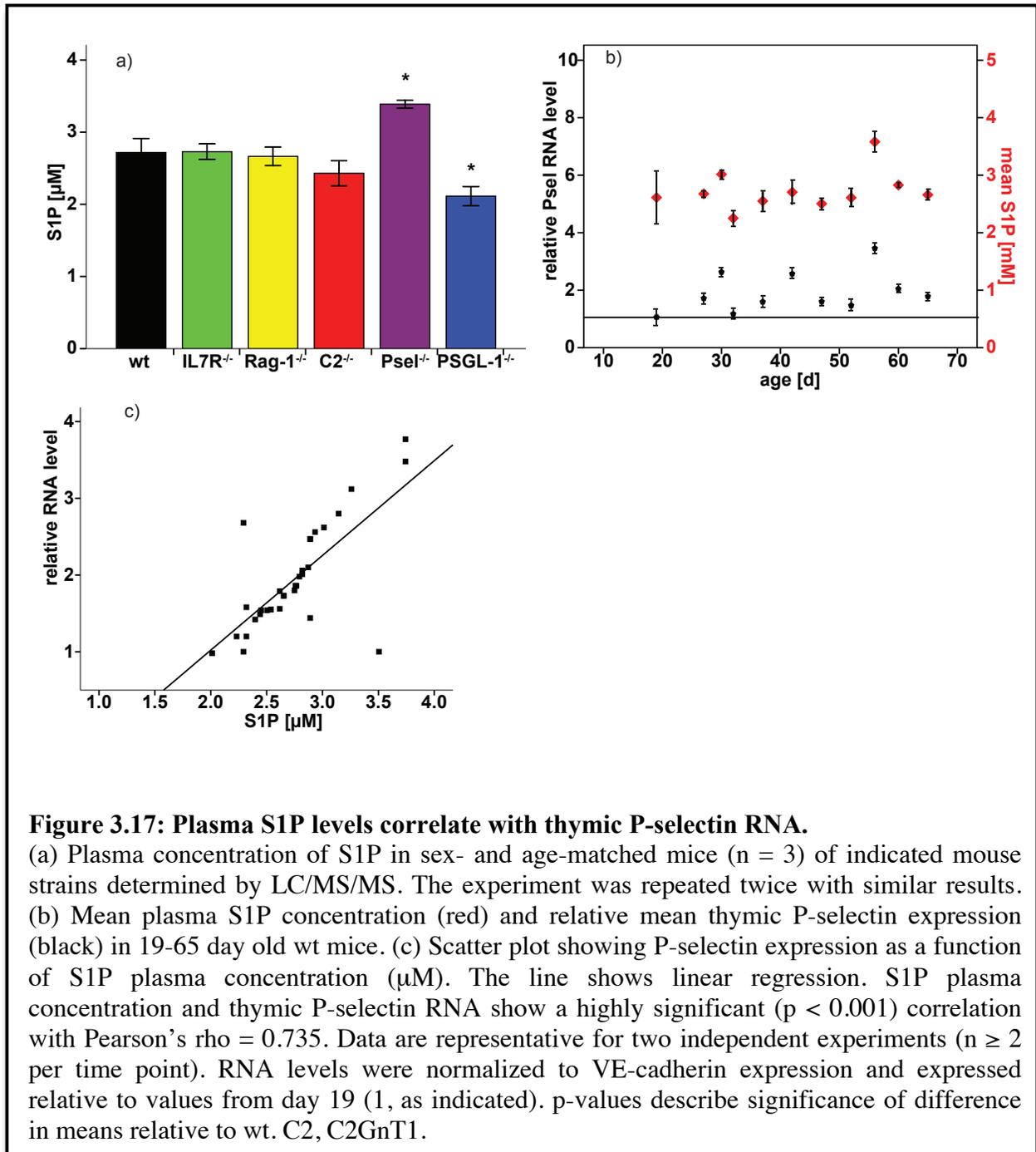


Figure 3.17: Plasma S1P levels correlate with thymic P-selectin RNA.

(a) Plasma concentration of S1P in sex- and age-matched mice ($n = 3$) of indicated mouse strains determined by LC/MS/MS. The experiment was repeated twice with similar results. (b) Mean plasma S1P concentration (red) and relative mean thymic P-selectin expression (black) in 19-65 day old wt mice. (c) Scatter plot showing P-selectin expression as a function of S1P plasma concentration (μM). The line shows linear regression. S1P plasma concentration and thymic P-selectin RNA show a highly significant ($p < 0.001$) correlation with Pearson's $\rho = 0.735$. Data are representative for two independent experiments ($n \geq 2$ per time point). RNA levels were normalized to VE-cadherin expression and expressed relative to values from day 19 (1, as indicated). p-values describe significance of difference in means relative to wt. C2, C2GnT1.

3.2.11 *In vitro* S1P and FTY720-P affect endothelial P-selectin expression in a dose-dependent manner

One hallmark of S1PR signaling is its concentration dependency; super-physiological levels of S1P often inhibit the very same processes that are activated under lower concentrations (217). The murine endothelial cell line bend.3 has been described before to express P-selectin and the sphingosine-1-receptors S1PR1 and S1PR2 (135, 207). Treatment of bend.3 cells with various doses of S1P or the S1P analogue FTY720-P showed that these molecules directly modulate P-selectin expression in endothelial cells (Figure 3.18a, b). Concentrations up to 1 μ M S1P or FTY720-P had a significant positive effect on P-selectin RNA expression when compared to control cells, whereas super-physiological concentration of S1P or FTY720-P (10 μ M) significantly reduced P-selectin expression below control values (Figure 3.18).

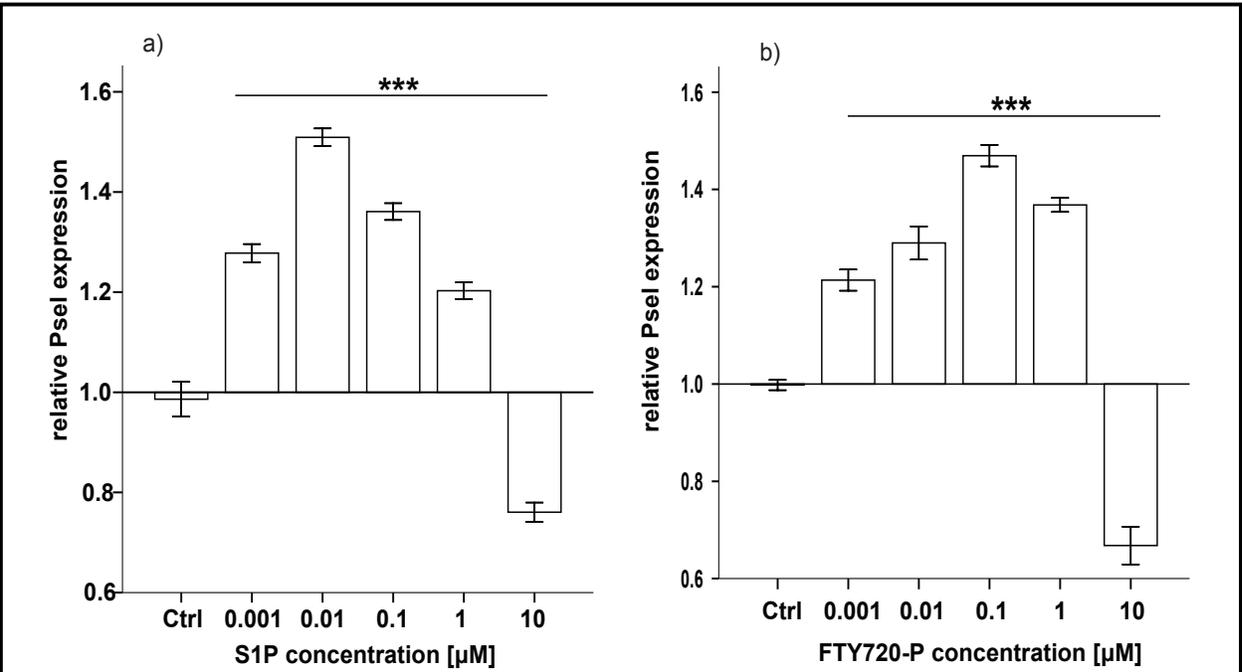


Figure 3.18: S1P and its analogue FTY720 modulate P-selectin RNA expression *in vitro*. *In vitro* dose response of P-selectin RNA expression in bend.3 cells that were cultured in indicated concentrations of S1P (a) or FTY720-P (b) for 12h. Control wells received media only (n = 8). The experiments were repeated three times (n = 4). P-selectin RNA levels were normalized using the reference gene VE-cadherin and expressed relative to the control samples (1, as indicated). p-values describe significance of difference in means relative to control.

3.2.12 Thymic P-selectin RNA expression and TCP receptivity *in vivo* can be modulated by FTY720

To determine whether increased S1P level also decreases P-selectin expression on thymic endothelium *in vivo* IL-7R^{-/-} mice, that have normal level of plasma S1P (Fig. 3.15a) and only few T and B cells, were treated for three days with the S1P analog FTY720 (218). FTY720 was used instead of S1P, as i.v. S1P injections are lethal and FTY720 has a significantly longer half-life in plasma than S1P.

Treatment of IL-7R^{-/-} mice with FTY720 significantly reduced thymic P-selectin RNA expression (Fig. 3.19a). Treatment of wt mice with FTY720 also resulted in a reduction of thymic P-selectin expression and was associated with reduced thymic TCP receptivity (Fig. 3.19b, c) suggesting that S1PR signaling is involved in the regulation of thymic P-selectin expression and thus thymic TCP receptivity. Furthermore, mice treated for 3 days with FTY720 had significantly ($p = 0.002$) reduced DN1 thymocyte numbers compared to controls (Fig. 3.19d, e). Together this set of data shows that FTY720 treatment reduces thymic TCP entry and that this effect is independent on the presence or absence of B and T cells.

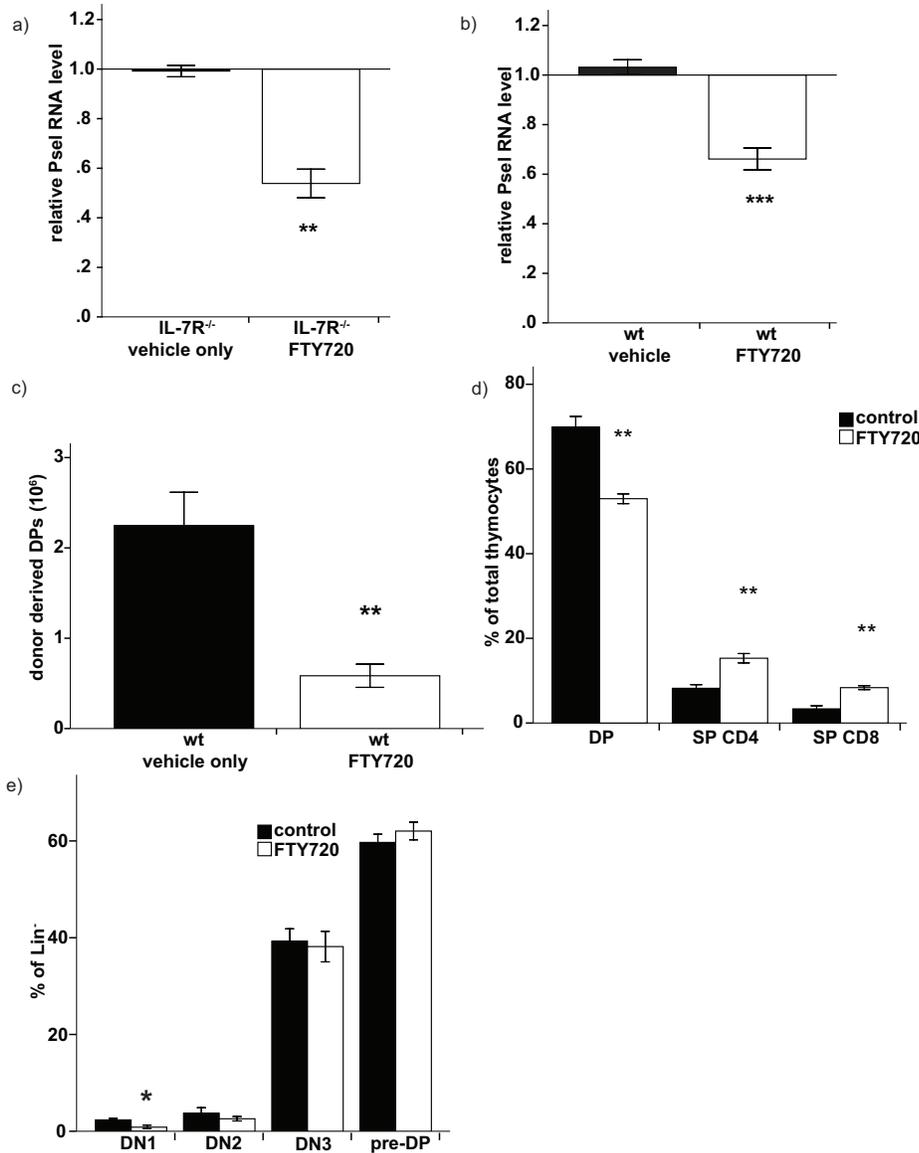


Figure 3.19: Changes in S1P levels are associated with altered thymic P-selectin RNA expression and TCP receptivity.

Thymic P-selectin RNA levels assessed by qrtPCR in $IL-7R^{-/-}$ (a) and wt (b) mice treated with FTY720 (1 mg/kg) or vehicle alone for three days. (c) Numbers of donor derived DP thymocytes three weeks after bone marrow transfer into wt recipients treated with FTY720 or vehicle alone 3d before bone marrow transfer. (d) Percentages of SP and DP thymocytes in FTY720 and vehicle treated mice (e) Numbers of DN1-preDP thymocytes in FTY720 and vehicle treated mice. Data are representative for at least three independent experiments (n = 5), mice were 42d old. RNA levels were normalized to VE-cadherin expression and expressed relative to values from vehicle-treated controls (as indicated).

3.2.13 Manipulation of the local S1P gradient in lymphoid tissues changes thymic P-selectin RNA expression and TCP receptivity

To examine whether local tissue S1P gradients or overall plasma S1P levels modulate P-selectin expression mice were treated with 2-deoxypyridoxine (DOP) to inhibit the S1P degrading enzyme S1P-Lyase, thereby increasing S1P levels only in lymphoid tissues but not in blood, disrupting the S1P gradient between lymphoid tissues and blood or lymph (88). Administration of DOP resulted in a significant reduction of thymic P-selectin RNA and TCP receptivity suggesting that disruption of the S1P gradient associated with a local increase of S1P in lymphoid tissues leads to down-regulation of thymic P-selectin and TCP receptivity (Fig. 3.20a-c). These data support the idea that the S1P gradient between periphery and thymus is more important for the regulation of thymic TCP importation than systemic plasma S1P level.

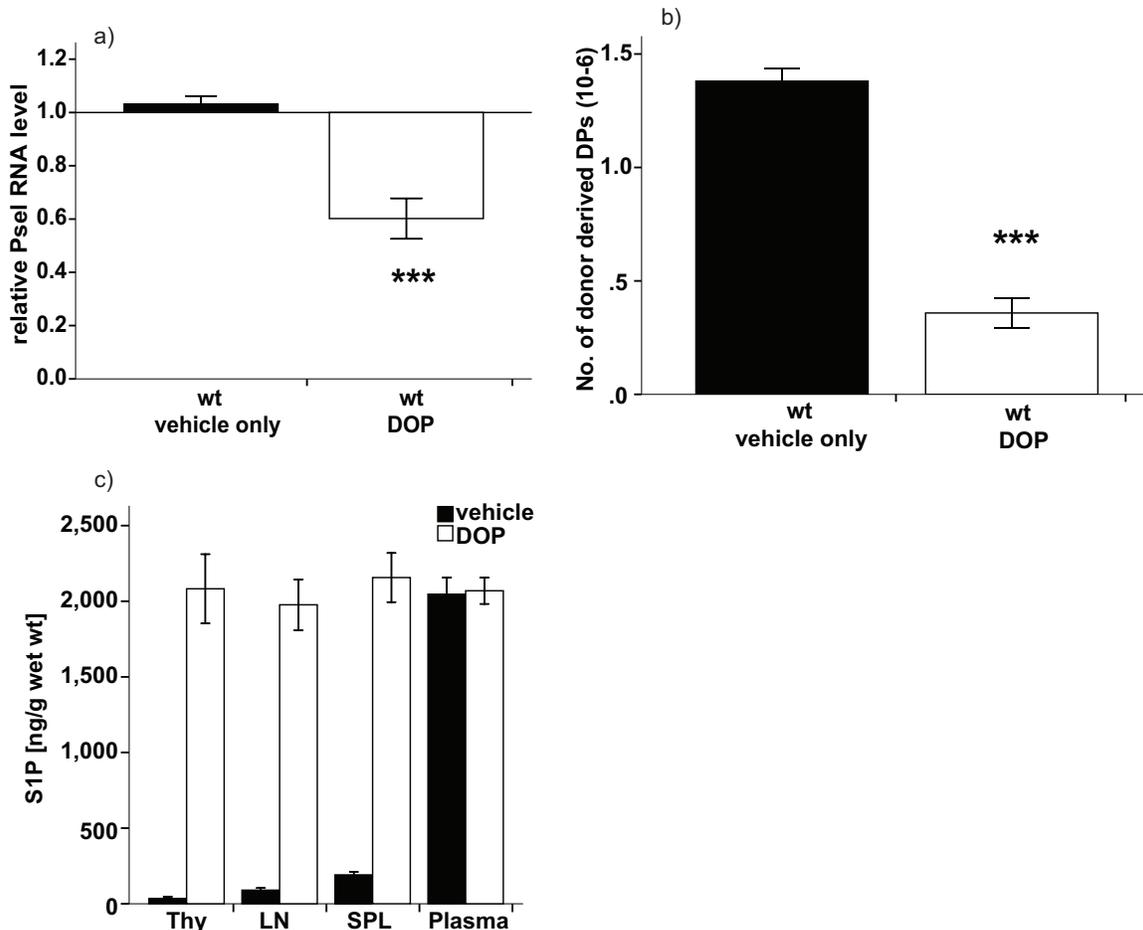
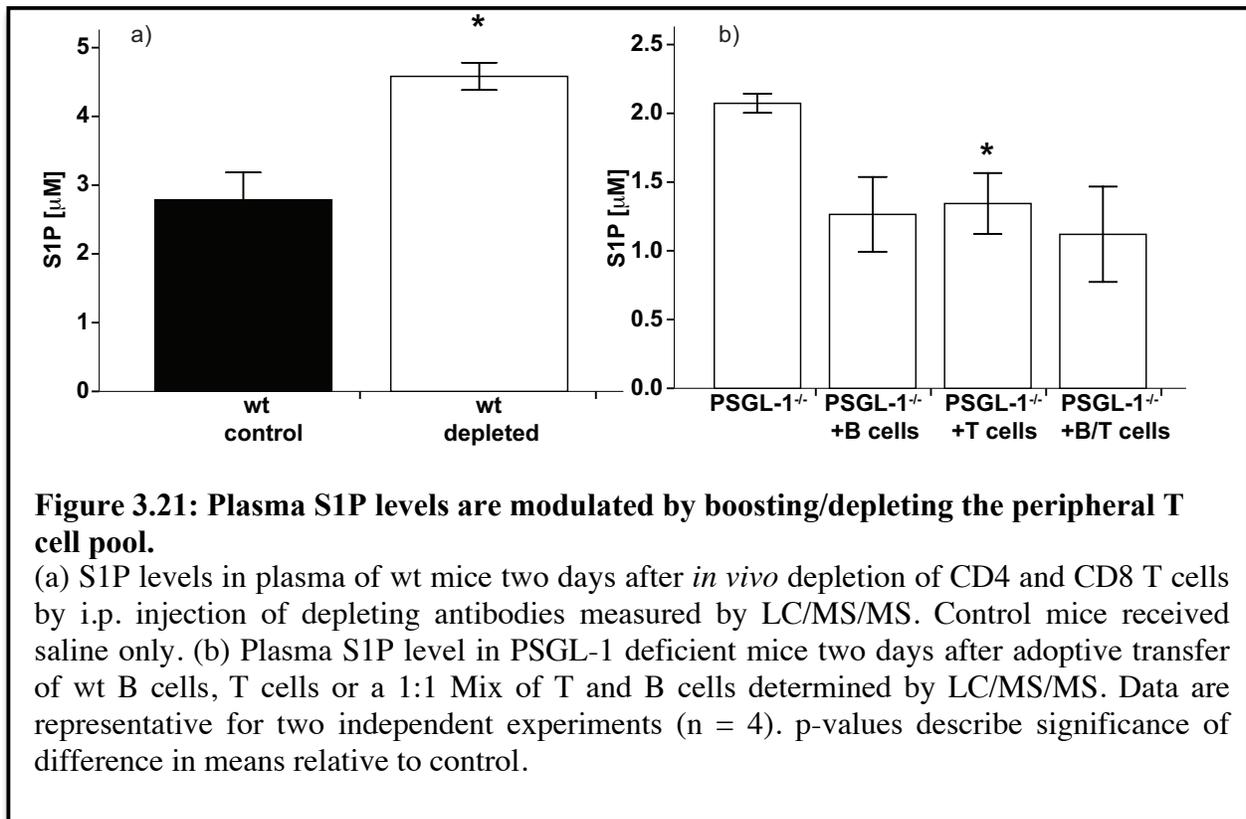


Figure 3.20: Local tissue levels of S1P determine P-selectin expression and thymic receptivity.

(a) Thymic P-selectin RNA levels in wt mice treated with 30 mg/L DOP in drinking water for three days. P-selectin expression was normalized using the reference gene VE-cadherin and expressed relative to vehicle treated control. (b) Numbers of donor derived DP thymocytes was determined by flow cytometry three weeks after bone marrow transfer into wt mice treated for three days with DOP (30 mg/mL in drinking water) or vehicle alone before bone marrow transfer. (c) S1P level in thymus (Thy), lymph nodes (LN), spleen (SPL) and plasma in wt mice treated for three days with DOP (30 mg/L in drinking water) or vehicle alone measured by LC/MS/MS. Data are representative for at least three independent experiments (n = 5).

3.2.14 Manipulation of peripheral lymphocyte numbers alters S1P levels in blood

Finally, it was analyzed whether changes in P-selectin expression after lymphocyte boost and depletion were associated with corresponding changes in plasma S1P levels. 48 hours after depletion of CD4 and CD8 T cells S1P levels were increased by 34% when compared to saline injected controls ($p = 0.052$) (Fig. 3.21a). Boosting PSGL-1^{-/-} mice with T cells, B cells or a 1:1 mixture of T and B cells was associated with a reduction in plasma S1P levels three days after cell transfer. B cells reduced S1P levels by 40%, T cells by 34% and the T and B cell mixture by 47% (Fig. 3.21b). Together this set of data shows that short-term perturbation of peripheral lymphocyte numbers can induce changes in plasma S1P levels that in turn might be linked to control of thymic P-selectin expression.



CHAPTER 4: DATA DISCUSSION

4.1 Data Summary

Thymic TCP importation is controlled by a multi-step adhesion cascade that can be summarized in three basic steps: 1) selectin mediated rolling and tethering, 2) chemokine signaling and 3) firm adhesion to endothelial cells lining the blood vessel walls mediated by integrins (120). Interaction of endothelial selectins with their corresponding ligands on the migrating cells is the first step in the adhesion cascade and thus important for all subsequent adhesion steps to occur (219). Furthermore the activity of chemokines is thought to be required to activate integrins of rolling cells that then engage integrin-ligands resulting in cell arrest and firm adhesion. TCP entry into the thymus is thus most likely controlled at each step of the adhesion cascade via modulation of the efficiency of each step.

While it has been reported that P-selectin and CCL25 functionally support TCP entry into the thymus and that thymic P-selectin expression correlates with thymic TCP receptivity (120-122, 220), this study extends the knowledge about the regulation of TCP entry into the thymus and provides evidence that P-selectin and CCL25 themselves are regulated in a temporal and quantitative manner. This study further reveals the basic processes underlying how cyclical TCP importation into the thymus is regulated and provides evidence that the thymus senses and responds to internal and external cues to regulate T cell formation.

4.2 P-selectin and CCL25 expression correlate with thymic TCP receptivity

Most TCP described so far in bone marrow, blood or thymus express functional PSGL-1 (103). Regulation of thymic P-selectin expression thus offers an effective way to control TCP importation on a general level but does not provide specificity to the thymic

recruitment process. Chemokines are likely to provide specificity and several studies have highlighted the role of CCL25 in TCP recruitment (119, 120, 157). This study shows that thymic endothelial cells as well as epithelial cells produce CCL25 RNA and that thymic CCL25 is expressed periodically in parallel with P-selectin. Hence, high expression of CCL25 should preferentially enhance the thymic homing of TCP expressing high levels of CCR9. Such CCR9^{hi} TCP have been shown to be more efficient in producing T cells than their CCR9^{lo} counterparts (157). The elevated levels of CCL25 RNA observed over time in thymi of PSGL-1^{-/-} mice are thus likely the explanation for the higher receptivity observed in PSGL-1^{-/-} mice at time points when they express similar amounts of P-selectin as the wt thymus.

Although the presence of homing molecules like P-selectin and CCL25 is important in regulating quantity and quality of TCP entering the thymus, deficiencies for these proteins can be compensated by functional redundancy among the homing molecules and potentially by intrathymic compensatory mechanisms (30). The present study shows that while PSGL-1^{-/-}, C2GnT1^{-/-} and Psel^{-/-} mice have reduced ETP numbers they have normal thymic cellularity indicating that increased intrathymic proliferation can compensate a lack in ETP numbers. Whether the T cells that result from compensatory proliferation of a reduced ETP pool are qualitatively identical to T cells that resulted from a large ETP pool remains to be investigated.

Surprisingly this study founds that C2GnT1^{-/-} mice relative to PSGL-1 deficient mice are less receptive for i.v. injected progenitors although having similar intrathymic niche occupancy and reduced numbers of ETPs than PSGL-1^{-/-} mice. The discrepancy could be

explained by residual PSGL-1-P-selectin interaction between C2GnT1^{-/-} TCP and the endothelium making the endogenous C2GnT1^{-/-} TCPs more competitive against i.v. injected wt TCP than PSGL-1 deficient TCP. The later probably cannot engage in any interaction with P-selectin therefore giving the wt TCP a nadvantage in seeding the thymus. Direct competition of C2GnT1^{-/-} with PSGL-1^{-/-} TCPs in IL-7R deficient mice also showed an advantage of C2GnT1^{-/-} TCP in seeding the thymus (121).

4.3 P-selectin and CCL25 are periodically expressed in the wild type thymus

In this study, P-selectin and CCL25 RNA levels were found to be periodically expressed in wt thymi and to a lesser extend in PSGL-1 deficient mice. The temporal expression of thymic P-selectin observed was remarkably stable for the first 3 weeks after birth and started to oscillate around the time of weaning. The increase in P-selectin and CCL25 expression coincided with the second wave of postnatal TCP that seed the thymus around days 18-22 after birth (221), a period when the neonatal immune system is highly activated and develops full independence of the maternal immune system (222). P-selectin was expressed with a periodicity of two weeks in agreement with one earlier study that found a two week wave-like pattern of filling, occupation and emptying of intrathymic niches (185). However, another study showed a periodicity for thymic receptivity of about three to four weeks (122). The discrepancy to the latter study and the lack of correlation over time between P-selectin expression and thymic TCP receptivity observed in this study could be due to the use of non-synchronized mice in the present study, whereas the earlier study used irradiation or i.t. injection of bone marrow cells to synchronize the thymi in recipient mice. The use of non-fractionated bone marrow could have led to a less sensitive read-out, because

non-TCP competed with TCP for binding spots on the thymic endothelium. Lastly, the temporal expression of P-selectin protein on the surface of thymic endothelial cells in wt mice was not tested. However, based on the good correlation between RNA level and protein surface expression one would expect that P-selectin protein expression would follow a similar temporal expression pattern as found for RNA.

Thymic receptivity in female littermates is more synchronized than in corresponding male mice. Data from several studies suggest that the changing estrogen levels during the estrous cycle in female mice cause the low deviation among littermate female mice compared to male mice. Estrogen levels were found to correlate inversely with numbers of TCP in bone marrow and thymus and consequentially with thymic size (223). It was found that ovariectomy in mice leads to reduced estrogen level and an increase in thymic size. In contrast, high estrogen level during pregnancy lead to a significant reduction in thymic size and T cell output (224-226). In this study drastically increased levels of P-selectin along with reduced thymic size and peripheral T cell lymphopenia were found in pregnant mice supporting the notion that estrogen levels might impact thymic physiology (Fig. A.3) (223, 227).

Although a linear correlation of autotaxin expression with thymic receptivity was not found in this study, an involvement of autotaxin in thymic homing cannot be excluded. Multiple effects of autotaxin on the homing process of lymphocytes to lymph nodes suggest that there is not a simple linear correlation between RNA expression and enzyme activity. Autotaxin is a lysophospholipase released by endothelium upon interaction with rolling cells (176). Autotaxin then binds to the rolling cells via integrins where it catalyzes the formation

of lysophosphatidic acid, a cell signaling molecule that increases cell motility and endothelial permeability. Further tests should aim to establish first a functional role of this enzyme in thymic homing

E-selectin, Chst-1 and VAP-1 were found to be up-regulated during periods of high thymic P-selectin expression, partly supporting earlier data (147). Due to a lack of experimental tools the significance of Chst-1 and VAP-1 in thymic TCP importation was not further functionally evaluated. The impact of E-selectin on thymic TCP homing was analyzed in competition assays. A mix of wt and PSGL-1 deficient donor TCP was injected i.v. into lethally irradiated wt or E-selectin deficient recipients. In wt mice, PSGL-1 deficient donor cells have a disadvantage over wt donor cells in seeding the thymus, which would be expected to diminish in E-selectin recipients in case E-selectin was important for TCP homing. However in both recipients, PSGL-1 deficient TCP showed the same disadvantage in seeding the thymus (Fig. A.2) making an involvement of E-selectin in thymic homing unlikely. Nevertheless, there remains the possibility that P-selectin is the dominant ligand and that E-selectin supports residual homing. Reconstitution assays in P- and E-selectin double-deficient mice would help to further address this question.

4.4 Internal and external feedback mechanisms regulate thymic receptivity

One underlying cause for the periodic expression of thymic P-selectin expression could be endothelial cell turnover. However based on the longevity of endothelial cells that is estimated to be in the range of months to years, it is unlikely that cell turnover causes the cyclical P-selectin expression observed in this study (228). It is also unlikely that fluctuations

in CCL25 expression are caused by a highly proliferative subset of medullary epithelial cells (229), as CCL25 expression was also detected in cortical epithelial and in particular in endothelial cells that produced the bulk of CCL25 RNA. Instead, the observations made in this study point to the occupation status of ETP niches as a regulator for the periodicity of homing molecule expression and thymic receptivity. Mice with permanently contracted ETP pools were found to lack the periodicity of P-selectin or CCL25 supporting a model whereby ETP numbers regulate the periodic expression of P-selectin and CCL25 via a negative feedback signal. In this model full ETP niches trigger a negative feedback to close the thymic gates and keep them closed for two weeks, which is the time required for TCP to reach the TN3 developmental stage (30, 41). ETPs reside anatomically close to their thymic entry sites in the peri-medullary cortical regions and asynchronously migrate from there deeper into the cortex (41). Given that the steady egress of cells from the ETP sites occurs at a constant rate the shrinking ETP pool size could indeed function as a ‘timer’ to prepare the TCP entry sites to up-regulate recruitment molecules enabling the next wave of TCP to enter the thymus. An earlier study showed a 2-week wave-pattern of filling, occupation and emptying of early intrathymic niches (185), which corresponds to the periodic expression of P-selectin and CCL25 in our study. Thus, in mice that lack functional P-selectin-PSGL-1 interaction TCP entry is impaired and occurs at a constant rate. Hence, ETP numbers in these mice do not change over time leaving P-selectin and CCL25 levels relatively stable.

While the regulation of the periodicity of thymic receptivity seems to be exclusively regulated by an internal negative feedback signal triggered by ETP numbers this study found that the amount of homing molecules expressed on the thymic endothelium is dynamically

regulated by internal and external feedback signals. The occupation status of intrathymic niches supporting DN3 thymocytes has been described earlier to limit entry of new TCP (183). Surprisingly C2GnT1^{-/-} and PSGL-1^{-/-} mice had comparable DN thymocyte subset distribution and intrathymic niche availability but differed greatly in thymic TCP importation. This observation revealed an additional mechanism, other than intrathymic niche occupancy, that regulates thymic P-selectin/CCL25 expression and receptivity.

It is thought that TCP enter the perivascular space surrounding thymic blood vessels before entering the thymic cortex and that mature emigrating thymocytes transit the same area to reach the blood circulation (46). The co-localization of TCP and mature, pre-emigrant T cells could therefore potentially influence entry of new TCP in a direct or indirect manner for example by cytokine release or cell:cell interaction. Thus this model favors the view that thymic T cell production would modulate entry of TCP.

Using the HY transgenic mouse model this study found that lymphopenic HY^{tg} male mice that have only a few mature thymocytes express increased thymic P-selectin, whereas HY^{tg}-female that have increased production of mature thymocytes show reduced P-selectin expression compared to wt controls. These data support the hypothesis that P-selectin expression is not exclusively regulated by numbers of early thymocytes, because HY^{tg} males have about the same numbers of DN thymocytes than female HY^{tg} mice and therefore supposedly similar niche occupancy (210). Furthermore, restoring the SP T cell compartment in HY^{tg} male mice reduced the high thymic P-selectin RNA expression to wt levels. Thus, these results point to a possible involvement of thymic T cell production in regulating thymic P-selectin expression. However, since this study used a “non-timed” HY^{tg}-model where TCR

selection sets in already in early DN stages the data could be skewed by the early deletion of DN thymocytes (230).

To confirm the findings from the HY^{tg} experiment in mouse models with less severe phenotypes, PSGL-1^{-/-} and C2GnT-1^{-/-} mice were used. The selection processes in both strains have not been reported to differ from those in wt mice (186, 188). The analysis of the mature thymocytes subsets revealed a significant increase in the frequency of mature SP thymocytes in PSGL-1^{-/-} but not C2GnT-1^{-/-} thymi. Furthermore, PSGL-1 but not C2GnT1 deficient mice had reduced thymic T cell production possibly causing T cell lymphopenia observed in the peripheral blood of PSGL-1^{-/-} mice. The PSGL-1^{-/-} phenotype is reminiscent of the phenotype found in HY^{tg} male mice.

The lymphopenic PSGL-1^{-/-}, IL-7R^{-/-} and HY^{tg} mouse models clearly demonstrated that peripheral T cell lymphopenia is associated with increased thymic TCP receptivity, suggesting that the periphery might be involved in regulating thymic TCP entry. Indeed, manipulation of the peripheral lymphocyte pool led to significant changes in the intensity of thymic TCP importation within a relatively short period of time, pointing to a rapid external feedback signal between peripheral lymphocyte pool and thymic TCP recruitment. However, such a mechanism was absent in Rag-1^{-/-} mice that are lymphopenic but have very low P-selectin/CCL25 expression levels as well as thymic receptivities. Fundamental disturbances in thymic architecture noted previously in Rag-1^{-/-} may preclude formation of proper negative feedback mechanism (37, 183). Furthermore, if the external feedback loop indeed requires the presence of peripheral lymphocytes it could not function in Rag-1^{-/-} mice, because these mice lack any lymphocytes (187, 231).

In summary, the data indicate that the peripheral lymphocyte pool is able to alter the intensity of thymic TCP receptivity. However, they do not indicate that it affects the periodicity of P-selectin/CCL25 expression. As homeostatic mechanisms maintain steady lymphocyte numbers over time (232) it is unlikely that the peripheral lymphocyte pool contributes to periodicity in thymic TCP importation.

4.5 S1P as a potential mediator between periphery and thymic vascular gates

The plasma transfer experiments indicated that a soluble factor is likely to be a part of the feedback loop between periphery and thymus. A potential role for TNF α , an inducer of P-selectin cell surface expression, as a mediator could be excluded. S1P has been shown to modulate P-selectin expression on endothelial cells (216) and to alter endothelial cell layer permeability and to generally affect endothelial adhesion molecule expression (233).

S1P is known to activate the NF-kB pathway through the S1P₃ receptor (234) and thereby to induce transcription of cytokines, chemokines and adhesion molecules including P-selectin (235, 236). It has been shown that low doses of S1P increase P-selectin on the surface of endothelial cells via PLC- γ whereas higher doses inhibit P-selectin expression by activating eNOS via the PI-3K/Akt pathway (237). Using an *in vitro* system, the dose-dependent opposing effect of S1P or FTY720-P on P-selectin RNA expression was confirmed in this study. It is likely that E-selectin RNA expression is similarly affected by changes in S1P levels as murine E- and the P-selectin promotor regions share transcription factor binding sites for NF-kappa B and activating transcription factor (ATF) (236, 238). Thus, the increased expression of E-selectin in thymi that also express high levels of P-selectin could be explained by the co-regulation of these two molecules. Despite co-

regulation with P-selectin and proposed correlation of E-selectin RNA levels with S1P levels, E-selectin does not play an important role in the thymic homing process as shown by competitive reconstitution of E-selectin deficient recipients.

Confirming the *in vitro* data presented here, *in vivo* treatment of wt mice with FTY720-P reduced P-selectin RNA levels probably via activation of eNOS (239). Reduced P-selectin expression was associated with reduced thymic TCP receptivity expanding the known pharmacological activity of FTY720. However, the physiological consequences of our finding remain to be investigated. The observation that DOP treatment also inhibits thymic TCP receptivity suggests that local S1P gradients modulate TCP importation. The temporal changes in plasma S1P observed in wt mice probably reflect local changes of S1P level in lymphoid tissues.

Different S1PR can oppose each other's effects, for instance S1PR1 signaling enhances vascular permeability whereas S1PR3 signaling preserves vascular integrity (97). Our observations of S1P altering expression of adhesion molecules in the thymic vasculature could therefore also result from interaction of various S1PR receptors that signal differently under changing S1P levels. Future experiments should therefore also focus on expression and distribution of thymic endothelial S1PR and consider the crosstalk between the receptors as a possible mechanism to regulate TCP entry into the thymus. Differential expression of S1PR could also explain why periodic P-selectin expression was not observed in spleen, although one would expect changes in expression based on fluctuating systemic S1P level.

The finding that boosting or depleting peripheral lymphocyte numbers causes rapid changes in plasma S1P levels suggests that peripheral lymphocytes and the S1P metabolism interact. However, our data also show that S1P levels in lymphopenic IL-7R^{-/-} and Rag-1^{-/-} mice were not increased compared to wt mice, which seems to contradict the data from the boost/depletion experiments. It is likely that compensatory mechanisms described previously might lead to normal S1P level in those mice (197, 233, 240). Deficiency of P-selectin or PSGL-1 is associated with altered blood S1P levels indicating that the presence of these molecules is important in S1P metabolism. It has been shown that P-selectin deficient mice have an increased level of plasma high-density lipoprotein (HDL) (241). Since HDL binds free S1P thereby protecting it from degradation (242), the increased S1P levels observed in P-selectin^{-/-} mice could be explained by increased plasma HDL. Furthermore, PSGL-1^{-/-} mice showed reduced thymic output and reduced numbers of circulating T cells but normal numbers of T cells in lymph nodes and spleens. In addition to significantly lower S1P plasma level, PSGL-1^{-/-} mice express increased S1P₁ RNA level in T cells thus resembling a phenotype similar to that of sphingosine-kinase deficient mice (197).

The observations made here also allow speculating about a vascular phenotype in P-selectin and PSGL-1 deficient mice. Lack of either of these adhesion molecules could reduce interaction between blood cells and the endothelium that - even if it would occur at very low levels individually - should have a huge overall impact, because of the additive effect based on the enormous cell numbers of blood cells that constantly circulate through the blood vessels and the large surface area presented by vascular endothelial cells. Thus, these findings suggest a possible interaction between S1P metabolism and the P-selectin-PSGL-1 axis. However, the exact nature of this mechanism needs to be further investigated.

The reduced emigration of mature T cells in PSGL-1^{-/-} mice was found to be cell autonomous and probably at least partially responsible for the peripheral T cell lymphopenia observed in these mice (Fig. A.1). Additionally, PSGL-1 deficient T cells were found to immigrate into lymph nodes less efficiently than wt lymphocytes (243). Naïve T cells require regular survival signals delivered by MHC molecules and *cy* cytokines (244). Especially, recent thymic emigrants that still undergo functional maturation in the periphery are believed to be dependent on IL-7 (209). It has been shown that fibroblastic reticular cells in lymph nodes provide the IL-7 and that lymph node resident dendritic cells express the required MHC molecules (200). The lymphopenia observed in PSGL-1 deficient mice is therefore probably caused by reduced exit from the thymus and also impaired entry into lymph nodes.

When thymic T cell export cannot compensate for peripheral T cell loss, peripheral homeostatic expansion sets in to increase peripheral T cell numbers. During peripheral homeostatic proliferation CD8⁺ T cells acquire a memory cell like phenotype (245). The shift to increased frequencies of memory cell like (CD44^{hi}) cells detected in the blood of PSGL-1 deficient mice compared to wt mice suggests that homeostatic expansion takes place in PSGL-1^{-/-} mice (Fig. A.4).

The boost/depletion experiments revealed a unexpected rapid external additional feedback mechanism in the regulation of thymic TCP entry. One could speculate that boosting or depleting the peripheral T cell pool leads to increased cell stress that in turn could trigger an acute reaction leading to altered S1P level. In support of this hypothesis are the findings that S1P levels change under stress situation and inflammation (246-248). Furthermore, the observation that hematopoietic stem and progenitor cells (HSPCs) also

respond to S1P gradients (249, 250) suggests a potential bifunctional role of S1P in modulating thymic TCP receptivity: by altering adhesion molecule expression on the thymic endothelium and by modulating the migration pattern of HSPCs including TCP.

4.6 Regulation of S1P level/gradient

How can lymphocytes, which are only marginally involved in S1P production or degradation (217, 251), induce short-term changes in S1P levels? It is conceivable that lymphocytes indirectly modulate S1P production or degradation by cytokine release or more likely by interaction with vascular endothelial cells that have been shown to express high level of enzymes involved in S1P production and particular degradation (217, 233, 240, 252). However, a direct interaction of peripheral lymphocytes with thymic endothelial cell expressed P-selectin and thus a direct signaling through thymic P-selectin can be excluded as being part of the feedback as boosting of PSGL-1^{-/-} mice with C2GnT1^{-/-} or PSGL-1^{-/-} deficient lymphocytes yielded the same results as boosting with wt lymphocytes. Furthermore, it has been shown earlier that resting peripheral lymphocytes do not express functional P-selectin ligands (143).

4.7 New working model

This study proposes a model in which thymic P-selectin/CCL25 expression and consequently thymic TCP receptivity is controlled by two feedback mechanisms. The first mechanism active under homeostatic conditions triggers a feedback signal from the thymic peri-vascular region depending on the quantity and possibly also the quality of immigrated ETPs. This intrathymic signal regulates the periodicity of thymic P-selectin and CCL25 expression. Mouse strains with reduced ETP numbers such as Rag-1^{-/-}, PSGL-1^{-/-}, C2GnT1^{-/-}

and IL-7R^{-/-} mice have no periodic expression in thymic P-selectin and CCL25. The second mechanism triggers a feedback signal depending on the size of the peripheral lymphocyte pool and is active especially under acute conditions affecting the intensity of P-selectin/CCL25 expression. Manipulation of the peripheral lymphocyte pool rapidly affected peripheral S1P levels and thymic TCP receptivity. The thymic vascular gates thus integrate both feedback signals and control the entry of new TCP into the thymus by adjusting the amount and periodicity of expression of recruitment molecules on thymic endothelial cells. This study thus reveals basic processes underlying how cyclical TCP importation into the thymus is regulated and provides evidence that the thymus senses and responds to internal and external cues to regulate T cell formation.

CHAPTER 5: SUMMARY, MODELS AND FUTURE PERSPECTIVE

There are four major results found in this study. First, thymic P-selectin and CCL25 are not only functionally involved in thymic TCP recruitment but also regulate the quantity and time of progenitor entry into the thymus. Secondly, thymic receptivity seems to be regulated internally by the occupation status of early intrathymic progenitor niches at the ETP level and, thirdly, externally by the peripheral lymphocyte pool. Fourthly, this study provides strong evidence that the external feedback is partially mediated by S1P.

It has been suggested before that the thymus responds to changes in the periphery but only during periods of acute infection or of chronic inflammation (253, 254). This present study provides first insights into how the thymus-periphery cross talk might occur.

5.1 Analyzing TCP entry and T cell exit *in vivo*

Several obstacles hamper a more detailed analysis of thymic TCP homing and of the underlying regulation mechanisms. (a) The nature of the TCP population that seeds the thymus under physiological conditions is not known. This requires the use of unfractionated bone marrow or LSK cells in reconstitution assays and results in the infusion of cells that are normally not in blood circulation and that thus could cause artefacts. Furthermore, it could be possible that more than one type of progenitor seed the thymus under normal conditions and that these different types dynamically contribute to T cell formation to various degrees. (b) The exact site where TCP enter the thymus is not known, because the anatomic location and properties of the thymus make real time *in vivo* imaging currently very challenging. (c) The low numbers of TCP in the blood and the low numbers of TCP the thymus imports – 200

progenitors per receptive period seem to be enough to maintain T cell production (111, 255) – make it difficult to test receptivity of the thymus under physiological conditions. (d) The definition of a TCP includes the ability to home to the thymus from the bone marrow, enter the thymus and contribute to T cell formation. Intravenous injection of cells and short-term homing assays test for the first two attributes, while only long-term assays looking at the contribution of progenitor to the DP thymocyte pool would allow to draw conclusions about the potential of progenitors to form T cells. Currently there is no physiological model that allows testing of all required characteristics of a TCP in the same assay. In an approach to find a better model, Benz and colleagues recently transplanted thymi from newborn mice under the kidney capsule of an adult congenic mouse (256). Apart from the surgical stress and its associated inflammatory response this model has further limitations. First, this work showed that P-selectin expression in newborn mice is very low until weaning suggesting that the newborn is different from the adult thymus in recruiting TCP from the circulation. Furthermore, newborn thymi may attract different kinds of progenitors than adult thymi and the vasculature resulting from the revascularization in the kidney could differ significantly from the vascular structure in the adult. Thus, to analyze the homing of immature and mature cells to the thymus in more detail and under physiological settings requires the development of new techniques and possibly new animal models.

Currently intravital 2-photon microscopy can only be used to analyze processes in the thymic cortex (up to 200 μm deep) (42). TCP entry into the thymus at the PMC or processes in the medulla around 500 μm below the capsule cannot be analyzed with this method on an intact thymus (257). The emergence of new light-microscopical techniques like light-sheet-microscopy or harmonic-generation-microscopy bear the potential to allow visualization of

TCP entry into the thymus *in vivo* in the future (258, 259). Although experimentally challenging, *in situ* imaging using two-photon microscopy of thymic slides derived from partial GFP-chimera could help to identify thymic vascular gates where TCP enter the thymus (42). Similarly, partial Rag2p-GFP (209) chimera that express GFP in thymocytes from the Rag2 promoter could be used to determine where mature T cells leave the thymus.

5.2 Molecules involved in thymic TCP importation

So far, not a single molecule has been described that is absolutely required for TCP importation, suggesting multiple levels of redundancy. This was to be expected, since models for recruitment of mature lymphocytes to sites of inflammation revealed that the remaining members of the adhesion cascade could compensate the absence of a certain type of molecule. Furthermore, a reduction in TCP entry into the thymus could also be compensated by increased proliferation of thymocytes, although no direct evidence for increased thymocyte proliferation has been found in this study.

To find additional molecules involved in thymic TCP homing, future studies should aim to comprehensively analyze transcript and protein levels of adhesion molecules and chemotactic receptors in various types of blood derived TCP and to compare those to their intrathymic or bone marrow derived counterparts. This approach hypothesizes that the bone marrow derived progenitor or progenitors that entered the thymus do not express the molecules required to enter the thymus and could help to identify more target molecules. Secondly, transcript analysis of micro-dissected thymic vascular endothelial cells around the PMC or single cell PCR analysis could help to identify vascular addressins expressed in the thymus. The function of candidate molecules could then be tested by using mice that carry a

conditional genetic deletion of the molecules and/or by inhibiting the activity of the candidate molecules using antibodies or other inhibitors.

5.3 Model for the internal feedback mechanism

The mechanism how the intrathymic niche occupancy level regulates thymic receptivity for TCP is still elusive. It is known that this internal feedback mechanism involves the activity of early growth response gene 1 (Egr-1), because absence of Egr-1 in hematopoietic cells seems to abort the negative feedback leading to permanently high P-selectin expression and thymic receptivity resulting in hyperthropic thymi (220). However, the downstream molecular targets of Egr-1 required to regulate thymic receptivity are not known. In contrast the up-stream events that lead to expression of Egr-1 are well studied and it has been reported that S1P promotes the expression of Egr-1 in astrocytes and in endothelial cells (260, 261). It would therefore be interesting to know whether S1P increases Egr-1 expression in the thymus in particular in cells at the PMC and whether this connection is part of the mechanism regulating thymic TCP entry.

Hematopoietic stem cells and Lin⁻Sca-1⁺cKit⁺ have been shown to express S1PLyase RNA (NCBI GEO accession numbers GDS2398, GDS1077). It would be interesting to know whether ETPs express functional S1P Lyase and whether they are involved in modulating local S1P levels. If so, one could envision a model where high numbers of ETPs reduce S1P level through their S1PLyase activity resulting in reduced P-selectin expression and thymic receptivity. In contrast reduced numbers of ETPs would then allow an increase in S1P and therefore also P-selectin RNA levels and thymic receptivity (see Fig 5).

Intrathymic injection of wt bone marrow revealed reduced niche occupancy at the ETP level in mice lacking C2GnT1, PSGL-1 or P-selectin. These data also show that a reduction in ETP numbers is not compensated by increased intrathymic proliferation of ETPs to fill up the available niche space.

Future experiments should aim to answer the question whether ETP numbers are able to change local S1P level by using ETPs derived from wt mice or S1PLyase deficient mice in reconstitution or adoptive transfer experiment. Furthermore, a more detailed characterization of the ETP niches, perivascular space and thymic vasculature would help to identify potential molecules and signaling pathways that could be involved in creating and mediating feedback signals.

5.4 Model for the external feedback mechanism: how does S1P modulate expression of adhesion molecules?

The *in vitro* data clearly show that free S1P can directly modulate P-selectin expression in endothelial cells. However, whether free S1P or HDL-bound S1P is responsible for the effect observed *in vivo* in this study cannot be answered. Furthermore, this study does not provide insights on how lymphocyte numbers might alter S1P levels. It has been shown in this and other studies that lymphocytes highly express S1PLyase suggesting an involvement in S1P degradation (197). These findings could explain the reduction in S1P levels after boosting PSGL-1 deficient mice with wt lymphocytes or the increase in S1P after depletion of lymphocytes. A transient increase of plasma S1P levels after total body irradiation has been described before supporting the data presented here (240). Furthermore, the data obtained from the DOP experiments indicate that the S1P gradient across an

endothelial layer is important for the modulation of P-selectin expression. A model integrating all the findings would require the detection of a S1P gradient across endothelial cells and thus the expression of S1PRs on the luminal and abluminal side of the endothelium. On the luminal side S1PR might be permanently saturated by tonic concentrations of plasma S1P. Slight increases in the plasma S1P level might decrease vascular barrier integrity leading to increased levels of S1P on the abluminal side and increased abluminal S1PR signaling leading to increased expression of P-selectin. Such a model should also consider the expression of different S1PR on luminal and abluminal side of the endothelium. S1PRs have are known be expressed at different levels and to trigger different sometimes opposing downstream signals. For example whereas S1PR1 and S1PR3 enhance barrier integrity of endothelial cells S1PR2 reduces it (100, 262-264). Alternatively, S1PR1 mediates free S1P induced reduction of adhesion molecule expression on activated endothelial cells, whereas S1PR3 stimulates pro-adhesive properties (213, 265, 266). Thus, one can envision a scenario whereby luminal S1PR1 signaling could constantly inhibit P-selectin expression whereas abluminal S1PR3 promotes it. Future studies should therefore analyze which S1PR are expressed in thymic endothelial cells and determine the subcellular distribution of the receptors on the cell surface.

Many basic questions regarding S1P signaling have still to be answered. S1PRs have a nanomolar affinity to S1P, yet the levels of S1P in plasma are in the micromolar range. One explanation could be that most plasma S1P is actually not bioavailable (it has been suggested that only 2 % of plasma S1P is biavailable) and that HDL function as sinks, constantly removing free S1P from the plasma (98, 267). The findings that almost all plasma S1P is bound by plasma proteins suggests that changes in plasma S1P levels probably reflect

changes in local S1P production. For example, inflammation increases local S1P production to retain T cells at the site of inflammation preventing them to enter the afferent lymphatics (268). To maintain a gradient, S1P that leaks into the lymphatic or blood circulation is taken up by HDL and carried away. HDL therefore not only regulate the amount of free or bound S1P but are also involved in the local distribution of S1P in the organism (267).

5.5 The role of ecto-enzymes in thymic TCP importation

The significance of ecto-enzymes in the recruitment of mature lymphocytes under homeostatic or inflammatory conditions has emerged in recent years. However, so far no study has analyzed the impact of these enzymes in stem cell or progenitor homing. Data shown in this work provide preliminary evidence that autotaxin and VAP-1 could be involved in TCP entry into the thymus. Autotaxin has been reported to catalyze the formation of S1P supporting cell motility and angiogenesis (269). Except for the IL-7R^{-/-} mice the expression of autotaxin measured in thymus tissues correlated with plasma S1P levels. S1P plasma levels and thymic autotaxin levels were reduced in both C2GnT1^{-/-} and PSGL-1^{-/-} but increased in P-selectin^{-/-} mice. Although one has to be cautious to extrapolate local expression data to the whole vascular system, these data might point to a systemic vascular phenotype in those mouse strains possibly including altered S1P synthesis and autotaxin expression by vascular endothelial cells, which should be further addressed in future experiments.

In contrast to the systemic effect, one could also envision a model whereby local autotaxin expression in the thymic vasculature represents a mean to rapidly alter S1P level on the luminal side of thymic vascular endothelial cells. According to this scenario the S1P

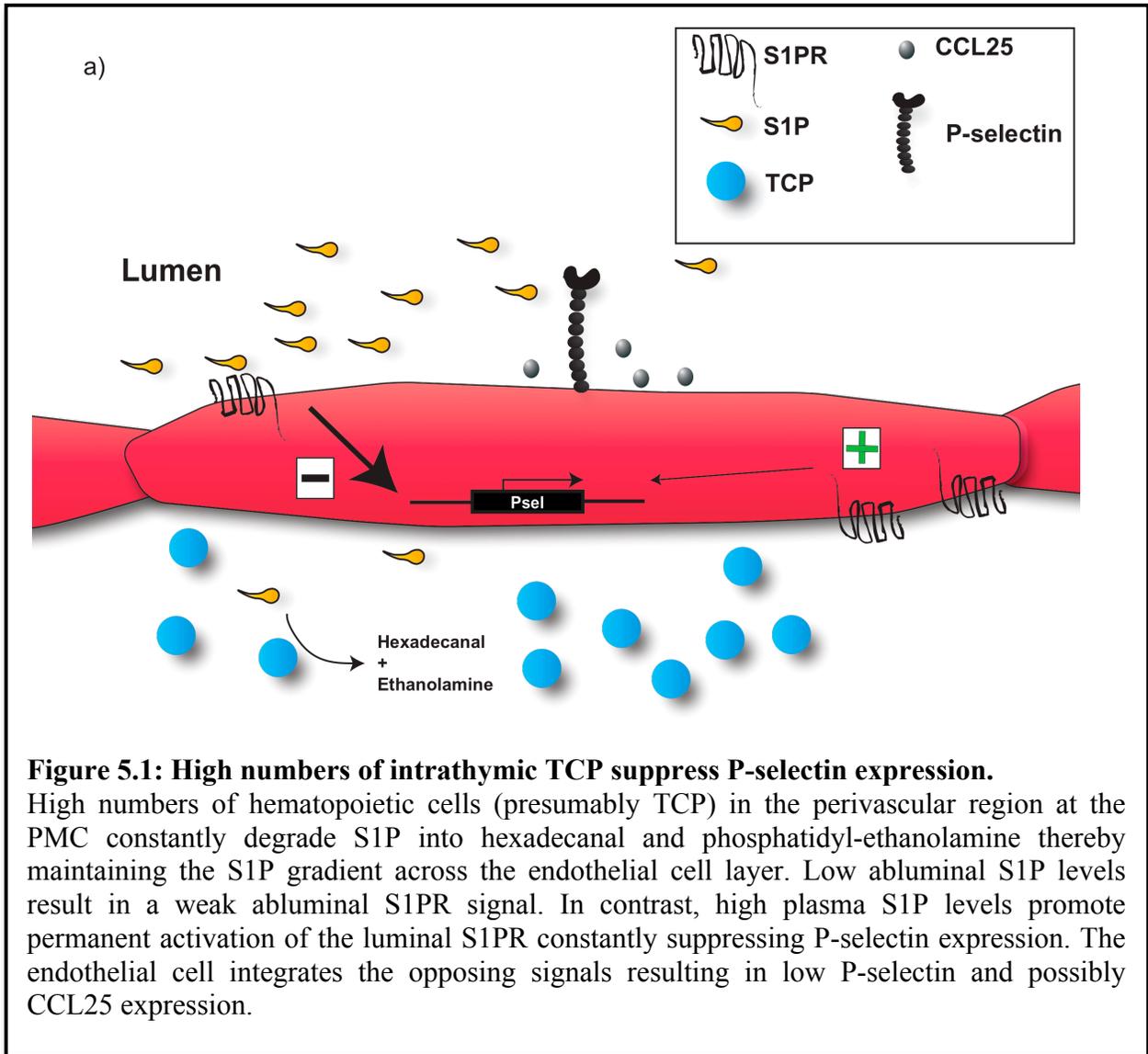
gradient across thymic vascular endothelial cells would increase with the number of rolling cells: an increased number of rolling cells expressing activated integrins that can engage autotaxin would increase the local S1P level; this in turn would increase P-selectin expression and reduce vascular integrity, resulting in increased import of cells. However, once the S1P level reaches a certain threshold P-selectin expression would be inhibited. Furthermore, the S1P gradient would collapse because a reduction in vascular integrity would also allow luminal S1P to cross the endothelial barrier possibly saturating local S1PLyases leading to a disruption of the gradient. Reduced expression of P-selectin and possibly other adhesion molecules would reduce the frequency of rolling cells and therefore local S1P production. The activity of S1PLyases on the abluminal site would then restore the S1P gradient and vascular integrity.

VAP-1 was found to be upregulated in receptive thymi (147). Furthermore, the oxidase activity of VAP-1 has been shown to induce P-selectin and E-selectin expression upon leukocyte binding, probably due to the formation of hydrogen peroxide, ammonium or formaldehyde (130). Like autotaxin VAP-1 could therefore also act as a local mediator to enhance P-selectin expression and thymic TCP receptivity dependent on the number of cells that interact with the local adhesion molecules.

Future studies should test the significance of ecto-enzymes in the importation of TCP into the thymus by using mice deficient for particular ecto-enzyme or by using soluble dominant negative forms of the ecto-enzyme (176)

5.6 Summarized hypothetical model of internal and external feedback loops regulating TCP entry into the thymus

Data from the *in vivo* DOP and the *in vitro* S1P experiments support a model whereby endothelial cells sense changes in S1P level across the endothelial cells layer. The following model assumes that TCP express functional S1PLYase and that endothelial cells express different kinds of S1PR that are expressed on the luminal and abluminal side. The luminal S1PR would be subjected to tonic S1P levels and saturated at all times. Furthermore, tonic levels of S1P would lead to a high rate of receptor internalization as has been observed for lymphocytes. This would result in a low surface expression of luminal S1PR relative to abluminal S1PR. The abluminal S1PR would detect S1P that leaked through the endothelial cell layer and was not immediately degraded by S1PLYase expressed by hematopoietic cells in the thymic perivascular region. Lastly, it is assumed that luminal and abluminal S1PR signaling antagonize each other with the luminal receptor repressing P-selectin promoter expression, and the abluminal S1PR signaling promoting expression of P-selectin. Two possible scenarios for feedback mechanisms are presented in the following figures.



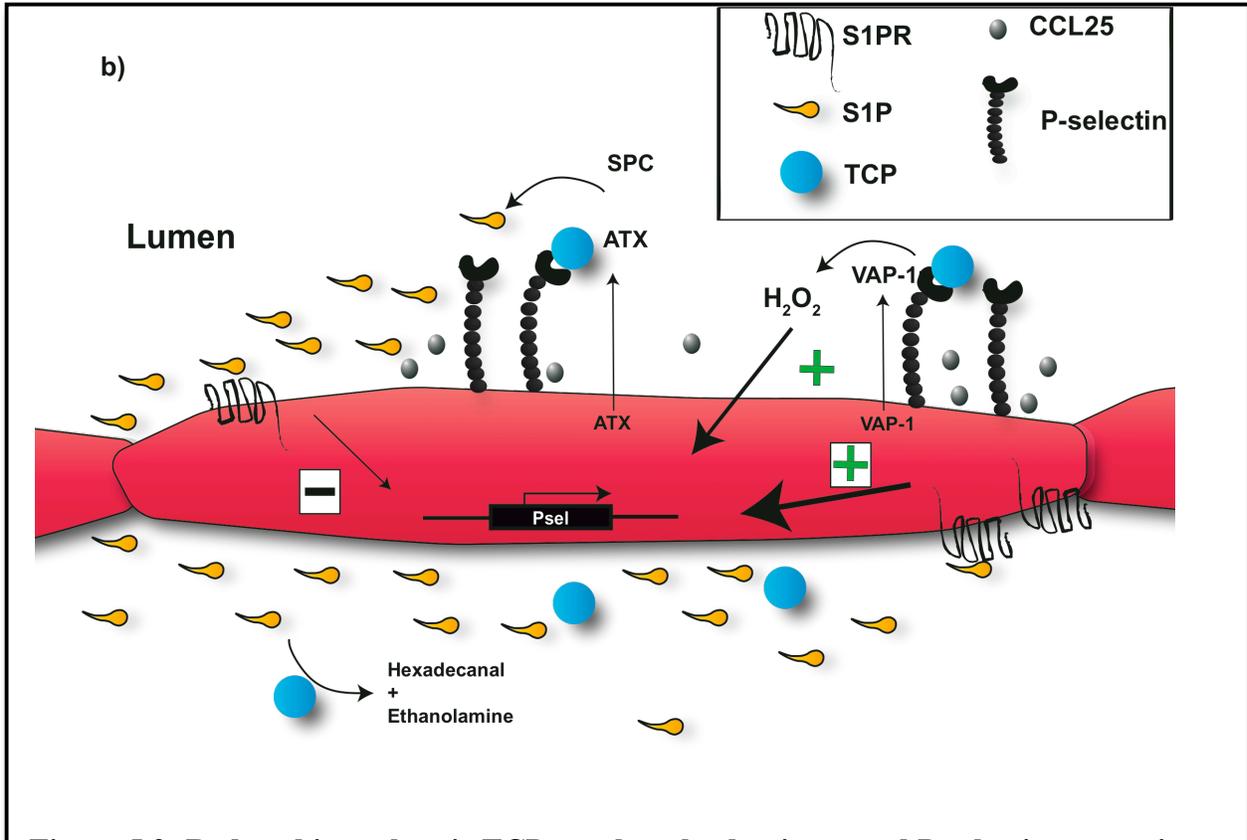


Figure 5.2: Reduced intrathymic TCP numbers lead to increased P-selectin expression.

Intrathymic TCP containing S1PLyase asynchronously migrate out of the PMC region resulting in reduced S1P degradation. Increasing perivascular levels of S1P lead to increased abluminal S1PR signaling opposing the constant luminal S1PR signaling resulting in increased P-selectin expression. Increased levels of P-selectin lead to higher frequencies of rolling cells promoting release and activation of ecto-enzymes like VAP-1 and autotaxin (ATX). VAP-1 activity leads to a local increase in hydrogen peroxide (H₂O₂), ammonium and formaldehyde level that directly promote further P-selectin expression and vascular permeability. Rolling cells also engage autotaxin that catalyzes the formation of S1P from sphingosylphosphorylcholine (SPC). Thus autotaxin locally and rapidly increases the amount of S1P depending on the number of rolling cells, likely leading to reduced luminal S1PR surface expression. S1P induced increase in endothelial barrier permeability allows more plasma S1P to enter the perivascular space possibly saturating local S1PLyases promoting a further increase in abluminal S1P levels and increased abluminal S1PR signaling. Increased abluminal S1PR signaling together with VAP-1 activity induce further P-selectin expression. Continued influx of S1P into the perivascular space eventually leads to a breakdown of the S1P gradient and to saturation and internalization of the abluminal S1PR causing reduced abluminal S1PR signaling and finally a reduction in P-selectin expression. This effect could further be enhanced by S1P induced Egr-1 expression in hematopoietic cells that then release an unknown factor that also inhibits P-selectin expression. Less P-selectin reduces the frequencies of rolling cells and reduces the activity of the ecto-enzymes. Lastly, the increased number of TCP in the perivascular space support S1P degradation and help to re-establish the S1P gradient.

Both scenarios explain the effects observed after depletion and boosting. Depletion of peripheral T cells resulted in increased S1P which could – as presented in Figure 5.2 – leak into the perivascular space and if not degraded by S1PLyases activate abluminal S1PR leading to increased P-selectin expression. In contrast boosting T cell numbers reduced S1P levels leading to less S1P crossing the endothelial layer reducing abluminal S1PR signaling. Since changes in plasma S1P level induced by boosting or depleting were mild it is expected that luminal S1PR signaling does not change much. In contrast, abluminal S1PR signaling changes rapidly, because abluminal S1P concentration are low relative to plasma levels and therefore more sensitive to the mild changes. Furthermore, abluminal S1PR signaling is dependent on endothelial barrier integrity and the presence of S1P degrading enzymes. Therefore small changes in plasma S1P level likely translate into big changes in abluminal S1PR signaling. Future studies should aim to study how the S1P gradient is maintained and how it affects S1PR signaling.

5.7 Potential involvement of P-selectin PSGL-1 interaction in S1P homeostasis

The plasma S1P data indicate that the P-selectin-PSGL-1 axis is involved in S1P homeostasis. Most plasma (> 90 %) S1P is bound by HDL and to a lesser degree by albumin. It has been shown that free S1P and bound S1P induce at times opposing effects on endothelial cells indicating that carriers are important in regulating S1P activity (reviewed in (267)). Unfortunately, most basic mechanisms regarding the loading of HDL with S1P and S1P release from HDL are not known yet.

There seems to be a negative correlation between P-selectin expression and HDL level in blood plasma: lack of P-selectin correlated with increased HDL levels whereas

artificial increased HDL level reduced lymphocyte adhesion by reducing expression of adhesion molecules including P-selectin (241, 270). In contrast to P-selectin deficient mice, PSGL-1 and C2Gnt1 deficient mice had reduced plasma S1P levels. It would be interesting to know whether HDL levels were also affected in mice deficient for PSGL-1 or C2Gnt1 and whether P-selectin or selectin ligands play a role in HDL formation and maintenance.

It has been described that endothelial cells respond to shear stress by reducing the expression of S1PLyase and S1P phosphatases and increased expression of sphingosine kinases resulting in an increased release of S1P into the plasma (240). Lack of PSGL-1 could reduce lymphocyte-endothelium interaction, which could in parts explain the reduced S1P levels in PSGL-1 deficient mice. However, the increased S1P levels observed in P-selectin deficient mice argue against such a simple mechanism and rather suggest that systemic plasma S1P level do not correlate with S1P production in a linear manner.

Activity of autotaxin requires the rolling of cells along endothelium. Lack of PSGL-1 could reduce the frequency of rolling cells leading to decreased autotaxin activity. Since autotaxin catalyzes the formation of S1P lack of adhesion molecules like PSGL-1 could therefore result in reduced local S1P production.

PSGL-1 has been shown to increase migration towards CCL21 or CCL19 gradients in naïve T cells (243). It would be interesting to know whether PSGL-1 also supports migration towards S1P. Furthermore, the finding that PSGL-1 signaling is involved in the polymerization and redistribution of F-actin in leukocytes together with recent data showing that actin remodeling is important in T cell maturation and thymic export raises the question whether PSGL-1 deficient thymocytes might have a defect in actin remodeling (94, 271). Both speculative effects – reduced response to S1P and impaired actin remodeling – could

explain the reduced thymic exit rate of mature T cells observed in PSGL-1 deficient mice which was found to be cell autonomous (Fig. A.1). Together with the reduced lymph node homing observed in PSGL-1 deficient mice reduced thymic exit could also contribute to the reduced numbers of circulating naïve T cells found in PSGL-1 deficient mice.

5.8 Physiological and clinical significance of thymic TCP importation

Knowledge of what constitutes and regulates the thymic vascular adhesion cascade might help to improve thymic seeding efficiencies. While most blood lineages are restored within 3 weeks after bone marrow transfer, restoration of the T cell lineage can take up to two years or often never fully occurs causing increased infection susceptibility, autoimmunity and disease relapse (272). The major cause for reduced T cell reconstitution is probably damage to epithelial cells by cytoablative regimes and/or graft versus host reactions resulting in ineffective support for T cell progenitors and reduced TCP importation (272). Furthermore, it has been reported that thymic donor chimerism correlated with increased tolerance towards organ transplant from the same donor, suggesting that thymic chimerism could promote central tolerance and might offer a way to induce tolerance towards allogenic organs (reviewed in (273)).

Knowledge of how the thymic vascular adhesion cascade functions and how it is regulated could help targeting T cell progenitors, therapeutic stem cells or drugs to the thymus to increase thymic reconstitution after bone marrow transplantation or inducing tolerance towards donor derived cells. The immuno-modulating drugs such as FTY720 or DOP have the potential to alter thymic receptivity and could therefore be used to manipulate thymic receptivity.

What is the physiological significance of gated importation? Given the fact that the imported progenitors also contribute to DC formation that in turn participate in selection processes, gated importation could support the development of functionally distinct T cell subsets, as observed during the peri- and postnatal periods (30, 274). Furthermore, there is accumulating evidence that mature DCs enter the thymus and that up to a third of intrathymic DCs are derived from the periphery. DCs constitutively express a form of PSGL-1 that can bind all three selectins (274-276). A recent study however using antibody inhibition assays and mice deficient for P-selectin showed that DCs require P-selectin to enter the thymus. Since blockage of L- and E-selectin did not affect DC homing it is likely that PSGL-1 is the physiological ligand for P-selectin on circulating DCs during homing to the thymus (277). Antigen-bearing DCs homing to the thymus were also able to delete antigen specific thymocytes or induce the formation of antigen specific regulatory T cells and regulatory natural killer cells (274, 277, 278). These findings indicate that re-entering DCs might contribute to establish central tolerance by gathering peripheral tissue specific antigens, presenting them to thymocytes in the thymus and inducing tolerance either by deleting reactive thymocyte or diverting reactive clones into regulatory cell types. In summary, DC progenitors and mature DCs both require P-selectin to enter the thymus suggesting that the importation of these cells is also periodic under homeostatic conditions (122, 279). The turnover of DCs in the thymus and periphery is 3-12 days, which almost corresponds to an entire refractive phase of the thymus (274, 280). Since DCs are important for the selection process each wave of newly imported DCs or DC progenitors could theoretically shape the T cell repertoire in a specific way. Under conditions of stress or inflammation upregulation of

thymic adhesion molecules like P-selectin would therefore also increase the influx of peripheral antigen loaded DCs supporting the formation of antigen specific regulatory cells (278).

It is not clear yet whether gated progenitor importation is essentially required for normal T cell production or whether it is just optimal for T cell formation (30). The data presented here would suggest that a lack of periodicity as observed in C2GnT1 or PSGL-1 deficient mice leads to a normal thymus and normal T cells. However, it is challenging to investigate whether C2GnT1 or PSGL-1 deficient T cells are functionally equivalent to wt T cells, as they lack pro-adhesive properties when activated.

5.9 Using the cervical thymus to analyze thymic specific properties

As mentioned earlier the thoracic and the cervical thymus develop asynchronously. The cervical thymus does not produce T cells before birth indicating that the cervical thymus attracts TCP at different times than the thoracic thymus and/or that it attracts different kinds of progenitors. By using the cervical thymus as a reference a comparison between cervical and thoracic thymus could thus reveal how the thoracic thymus is modulated by systemic factors, or how the thoracic thymus depends on input from other lymphoid organs like the bone marrow. Observed differences between cervical and thoracic thymus could then be used to analyze the contribution and significance of the intrathymic feedback versus the extrathymic feedback in controlling TCP importation in more detail. Lastly, this approach could also be used to assess the role of the bone marrow in controlling TCP entry into the thymus as it has been suggested before (30).

5.10 Future perspective

The study offers first insights into the mechanism of how TCP enter the thymus and how this process might be regulated. However many questions remain open and even more new question emerge from these data. This chapter offered a testable, hypothetical working model explaining how the internal and external feedback loop might regulate TCP entry into the thymus. Future studies should first aim to find out whether the assumptions made in the model prove true and then continue to test the model by analyzing the effect of the individual variables, such as S1P level, S1PLYase activity, autotaxin and VAP-1 activity, S1PR expression, S1PR distribution and S1PR effects on P-selectin expression and on thymic TCP importation.

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APPENDIX A: ADDITIONAL DATA

A.1 Thymic emigration impairment in PSGL-1^{-/-} mice is cell autonomous

To assess whether the observed thymic emigration defect in PSGL-1 deficient mice is thymocyte autonomous or dependent on external factors, competitive repopulation assays were performed in IL-7R deficient mice. In competitive homing assays, PSGL-1 deficient donor cells have a disadvantage compared to wt cells in seeding the thymus. To achieve a approximate 1:1 ratio of wt and PSGL-1 deficient cells, non-irradiated IL-7R^{-/-} recipient were first injected with 6 x10⁶ PSGL-1 bone marrow cells. Two days later recipients received 3 x 10⁶ congenic wt bone marrow cells. Three to four weeks later recipient mice were intrathymically injected with FITC. FITC⁺ cells were assessed in the periphery 36 hours after i.t. injection. To normalize for differences in reconstitution and labeling efficiency numbers of peripheral FITC⁺ cells were divided by the numbers of FITC⁺ cells of the same congenic background in the thymus. Similar to the results obtained from comparing wt with PSGL-1 deficient mice (Fig. 3.11) it was found that PSGL-1 deficient mature thymocytes leave the chimeric thymus at a significantly reduced rate than wt mature thymocytes (Fig A.1). As IL-7R deficient mice have a normal thymic environment and normal plasma S1P level, these data show that reduced thymic exit is T cell autonomous and is not caused by external factors like chemokines or S1P (183).

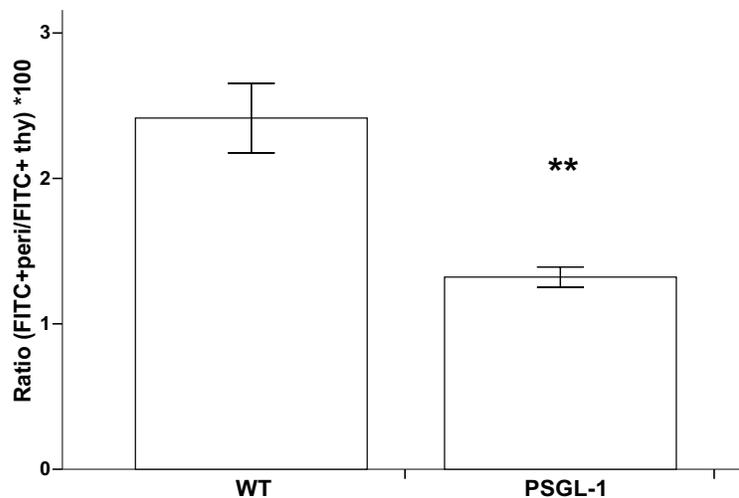


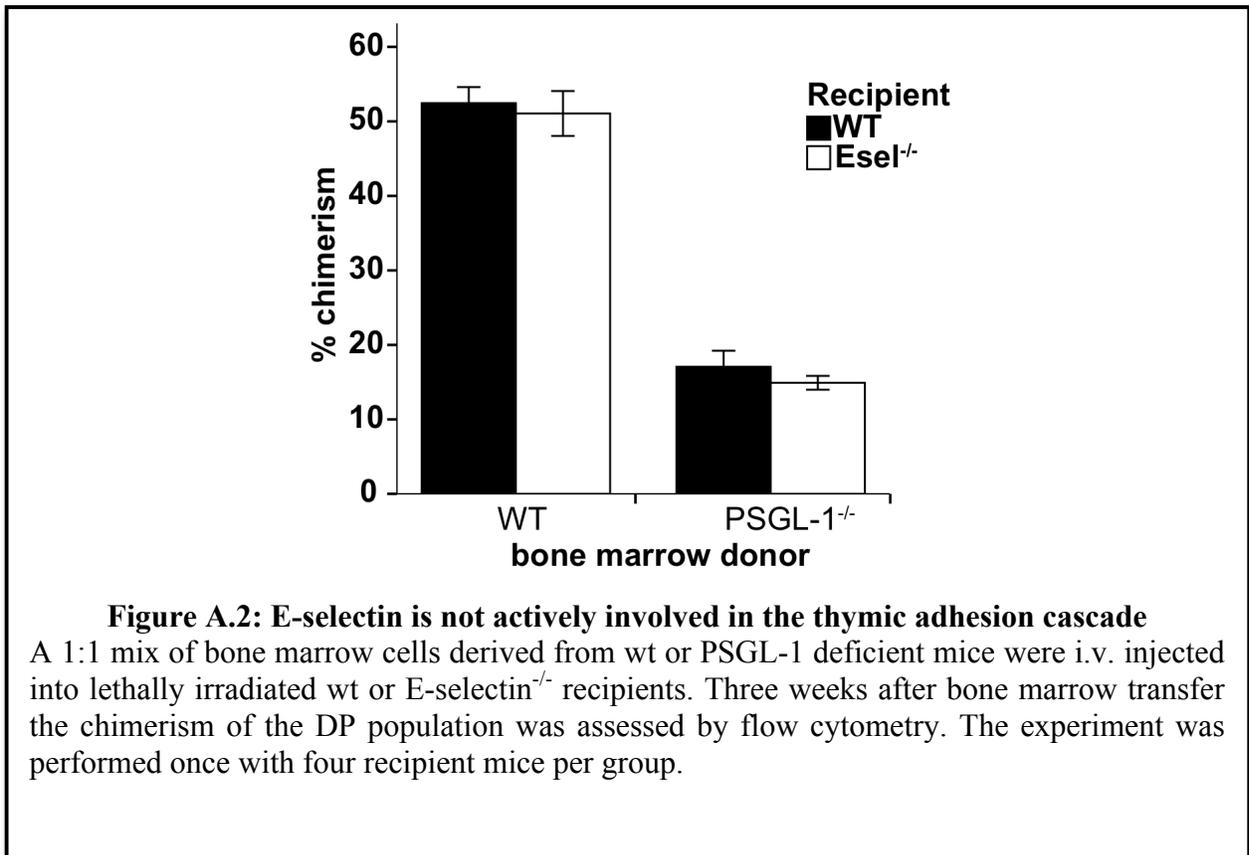
Figure A.1: Thymic emigration impairment in PSGL-1 deficient mice is cell autonomous

PSGL-1 deficient mature thymocytes show reduced exit rate when compared to wt thymocytes in the same thymus. IL-7R^{-/-} mice were reconstituted with PSGL-1 bone marrow cells and two days later with wt bone marrow cells to achieve a rough 1:1 ratio of competitor cells. After three weeks mice were i.t. injected with FITC and 36 hours later FITC⁺ T cells in the periphery were determined. To normalize for differences in reconstitution and labeling efficiency, peripheral FITC⁺ T cell numbers were divided by numbers of FITC⁺ thymocytes from the same strain (distinguished by congenic markers). Experiment was performed once with seven mice. Mice were 35 days old.

A.2 E-selectin is not actively involved in the thymic adhesion cascade

To test whether E-selectin is involved in TCP homing to the thymus competitive reconstitution assays were performed in irradiated E-selectin deficient recipients. A 1:1 mix of congenic marked bone marrow cells derived from wt or PSGL-1 deficient mice were i.v. injected into lethally irradiated E-selectin or wt mice. Three weeks later the chimerism of DP population was determined by flow cytometry. In wt mice, PSGL-1 deficient donor cells have a reported disadvantage over wt donor cells in seeding the thymus (121). This disadvantage would be expected to diminish in E-selectin recipients in case E-selectin was important for TCP homing. However, PSGL-1 deficient bone marrow cells had the same

disadvantage relative to wt bone marrow cells in repopulating thymi of E-selectin deficient recipients or wt recipients suggesting that PSGL-1 E-selectin interaction does not play an important role in TCP entry into the thymus (Fig 6.2).



A.3 Pregnant mice express high level of thymic P-selectin

It has been reported that pregnancy in mice leads to peripheral T cell lymphopenia and reduced thymic cellularity (227). To test whether the peripheral lymphopenia caused by pregnancy is associated with altered thymic P-selectin expression two-week pregnant mice were analyzed for T cell content and thymic P-selectin expression. Pregnant mice had a 50% reduction in T and B cell frequencies in the blood and significantly reduced DP cell frequencies in the thymus, confirming data from Zoller and colleagues. Furthermore, P-selectin levels in thymi of pregnant mice were about 15 times higher than in non-pregnant mice suggesting that pregnant mice are likely to be more receptive for TCP than non-pregnant mice. While these data support the hypothesis that peripheral T cell lymphopenia increases thymic P-selectin expression the physiological significance of this effect during pregnancy is not known.

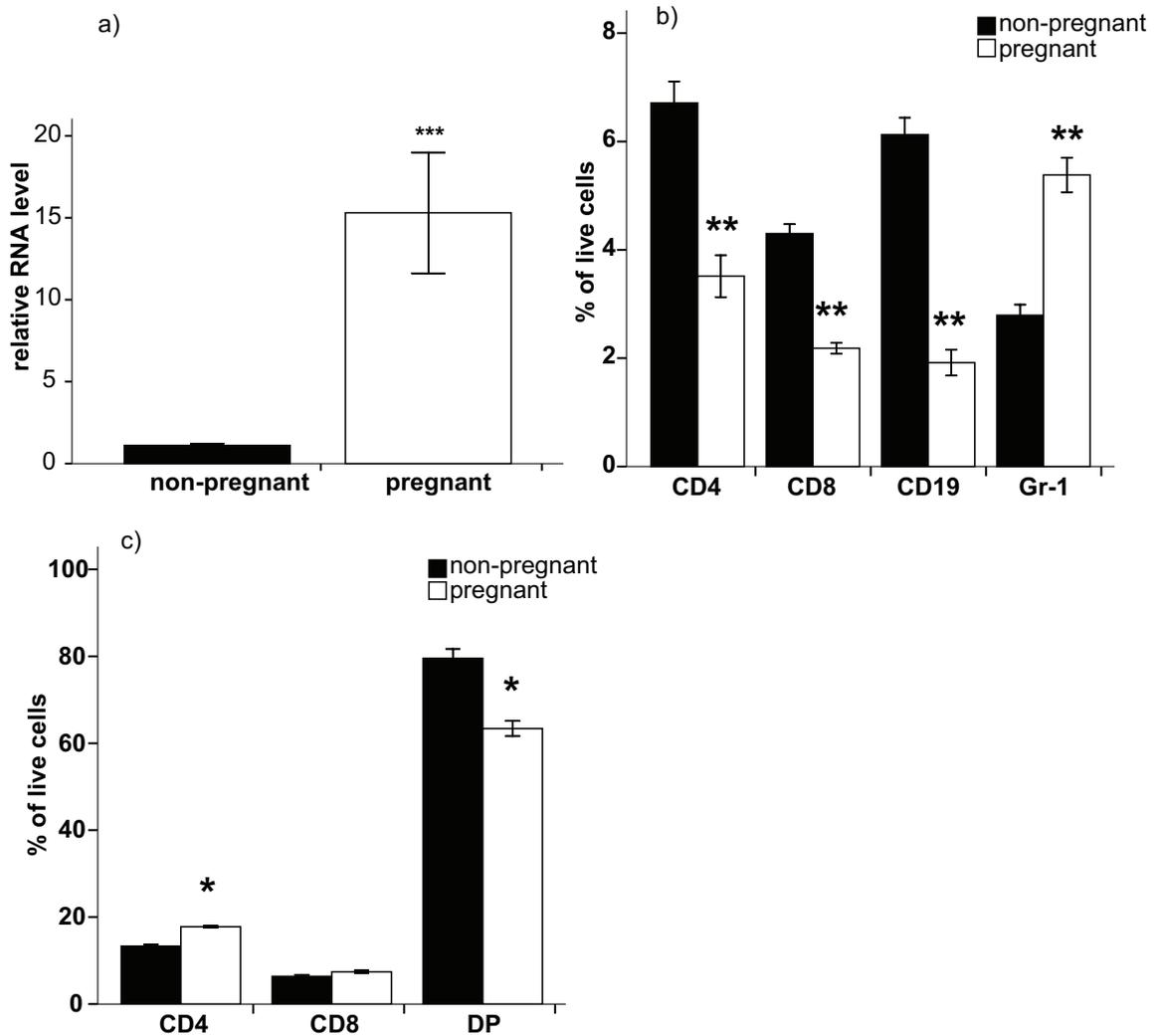


Figure A.3: Pregnant mice are lymphopenic and show increased thymic P-selectin expression.

(a) Thymic P-selectin RNA levels as determined by qrtPCR. RNA levels were normalized using the reference gene VE-cadherin and are expressed relative to the age-matched, non-pregnant control. (b) Pregnant mice show reduced frequencies of lymphocytes in peripheral blood, as determined by flow cytometry. (c) Pregnant mice show reduced frequencies of DP thymocytes and increased frequencies of SP thymocytes. The experiment was performed once with $n = 3$. Mice were pregnant for two weeks and were 49 ± 5 d old.

A.4 PSGL-1^{-/-} mice have reduced numbers of naïve T cells in the blood but not in lymph nodes.

PSGL-1 deficient T cells were found to have reduced exit rates from the thymus and to home less efficiently to peripheral lymph nodes than wt cells (243). To test whether these deficiencies are associated with differences in T cell subset distribution in peripheral blood and lymph nodes, PSGL-1^{-/-} and wt mice were analyzed for numbers of naïve (Lsel^{hi}, CD44^{lo}), central memory (Lsel^{hi}, CD44^{hi}) or effector memory (Lsel^{lo}, CD44^{hi}) T cells. Most tested subsets in the peripheral blood of PSGL-1^{-/-} mice were reduced in cell numbers compared to wt mice, while numbers were similar to wt in lymph nodes. In particular, numbers of naïve CD4 and CD8 T cells were dramatically reduced in peripheral blood of PSGL-1^{-/-} mice (Figure A.4). In contrast, numbers of effector memory cells in peripheral blood of PSGL-1^{-/-} mice were slightly increased as compared to wt controls. These data point to a homeostatic defect for circulating T cell in PSGL-1 deficient mice, but the mechanism that causes the defect is not known.

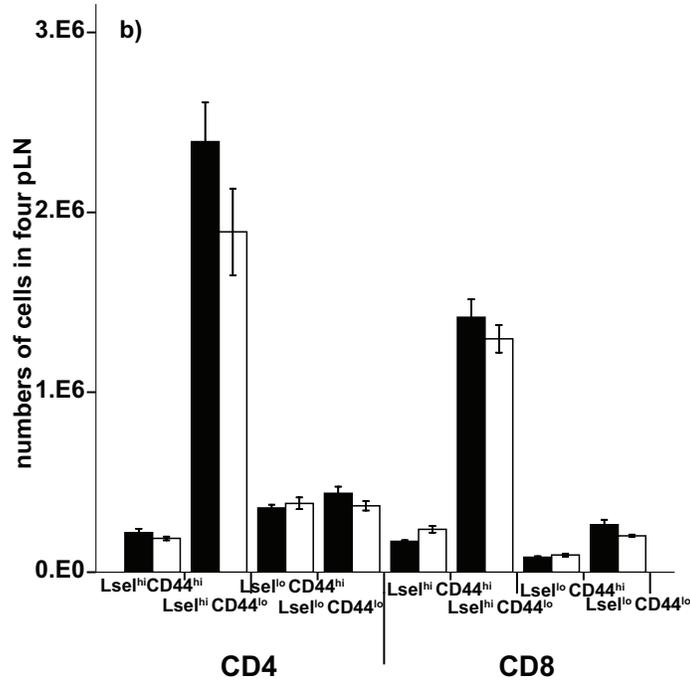
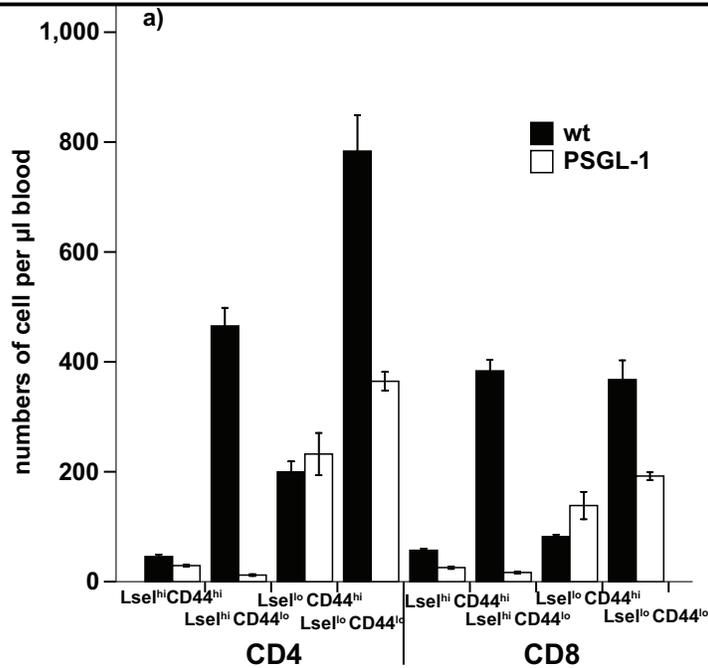


Figure A.4: PSGL-1 deficient mice have reduced numbers of naïve T cells in blood but not lymph nodes.

Numbers of the indicated CD4 or CD8 subsets per ml peripheral blood (a) or four peripheral lymph nodes (b), as determined by flow cytometry. The experiment was performed three times with $n = 4$. Mice were 42 ± 6 days old.

APPENDIX B: ANIMAL AND BIOSAFETY PROTOCOLS

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THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0061

Investigator or Course Director: Hermann J. Ziltener

Department: Medicine, Faculty of

Animals:

- Mice C2GlcNAcT-Inull 100
- Mice PSGL-1null 200
- Mice Rag-1 null 1000
- Mice E-selectin 50
- Mice HY tg 100
- Mice CD43 null 100
- Mice ADAM8 null 50
- Mice P-selectin null 100
- Mice IL7Rnull 100
- Mice C57Bl/6 1000

Start Date: April 1, 2006

Approval Date: July 7, 2006

Funding Sources:

Funding Agency: Canadian Institutes of Health Research

Funding Title: Branched o-glycan expression and lymphocyte function

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.



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Unfunded title: N/A

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APPENDIX C: PUBLICATION LIST

Rossi, F. M., Corbel, S. Y., Merzaban, J. S., Carlow, D. A., Gossens, K., Duenas, J., So, L., Yi, L., Ziltener, H. J., Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. 2005, *Nature Immunology*

Gossens, K., Naus, S., Corbel, S. Y., Lin, S., Rossi, F. M., Kast, J., Ziltener, H. J., Thymic progenitor homing and lymphocyte homeostasis are linked via SIP-controlled expression of thymic P-selectin/CCL25, 2009 *Journal of Experimental Medicine*.

Carlow, D. A., Gossens, K., Naus, S., Veerman, K. M., Seo, W., Ziltener, H. J., PSGL-1 function in immunity and steady state homeostasis, 2009, *Immunological Reviews*.