Development of regulatory T cells and induction of mucosa-specific homing

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Zusammenfassung

Regulatorische $CD4^+CD25^+$ T-Zellen (Tregs) spielen eine wichtige Rolle bei der Aufrechterhaltung des homeostatischen Gleichgewichts und der peripheren Selbst-Toleranz. In vorausgegangenen Untersuchungen unserer Arbeitsgruppe konnten Subpopulationen von $CD4^+$ Tregs identifiziert werden, die sich durch die Expression des Integrins $\alpha_E\beta_7$ auszeichnen. α_E-exprimierende Treg Subpopulationen weisen einen Effektor/Memory-ähnlichen Phänotyp auf und sind mit verschiedenen Homingrezeptoren ausgestattet, die ihre Einwanderung in entzündete Gewebe ermöglichen. Diese phänotypischen Merkmale und das darausfolgende Wanderungsverhalten der α_E^+ Treg-Populationen bilden die Grundlage für ihre Fähigkeit, eine bestehende Entzündung in nicht-lymphoiden Geweben zu unterdrücken. In der vorliegenden Arbeit wurden die Umstände, die zur Entstehung von Effektor/Memory α_E^+ Tregs *in vivo* führen, näher untersucht. Dabei konnte gezeigt werden, dass die α_E^+ Treg-Populationen unter physiologischen Bedingungen in gesunden Mäusen einen hohen Anteil sich-teilender Zellen aufweisen. Diese *in vivo* Proliferation war zum Teil abhängig von der kommensalen Mikroflora im Darm der untersuchten Tiere. Dementsprechend führte das Fehlen von mikrobiellen Stimuli zur Reduktion der absoluten Zahl von $CD4^+Foxp3^+$ Tregs, wahrscheinlich als Folge der verringerten Zellteilung von Tregs in den darm-assoziierten lymphoiden Geweben (GALT). Darüberhinaus konnte in einem adoptiven Transfermodel mit transgenen T-Zell-Subpopulationen aus DO11.10 Mäusen gezeigt werden, dass $\alpha_E^+Foxp3^+$ Effektor/Memory Tregs nicht nur durch Differenzierung naiver $CD4^+CD25^+$ Tregs entstehen, sondern, dass sie unter tolerogener Antigengabe in den GALT auch durch *de novo* Induktion aus konventionellen T-Zellen hervorgehen können. Diese Ergebnisse deuten darauf hin, dass es sich bei den α_E^+ Tregs um Effektor/Memory Zellen handelt, die eine hohe Teilungsrate aufweisen und sowohl aus konventionellen naiven T-Zellen in der Peripherie, als auch aus thymus-generierten naïven $CD4^+CD25^+$ Tregs entstehen können. Im Zusammenhang mit der beobachteten Rolle der kommensalen Mikroflora deuten diese Ergebnisse auf einen wichtigen Mechanismuns bei der Homeostase der $Foxp3^+$ Treg-Populationen im Allgemeinen und der $\alpha_E^+Foxp3^+$ Tregs im Besonderen hin. Sie legen den Schluss nahe, dass das spezifische mukosale Mikroenvironment sowohl die Expansion als auch die Konvertierung von Tregs fördert.

Desweiteren wurde untersucht in wie weit naïve $CD4^+CD25^+$ Tregs für die Ausbildung eines gewebespezifischen Homingrezeptor-Phänotyps empfänglich sind. *In vitro* Kultur-Systeme zeigten dabei, dass sich Tregs, ähnlich wie konventionelle T-Zellen, mit organspezifischen Migrationseigenschaften versehen lassen, die eine effiziente Wanderung in den Darm oder in die Haut ermöglichen. Die Aktivierung naïver $CD4^+CD25^+$ Tregs in Anwesenheit von DCs aus mesenterialen Lymphknoten oder des löslichen Faktors Retinolsäure führte zur Ausbildung von darmspezifischen $\alpha_4\beta_7^+$ Tregs, wohingegen die Aktivierung in Anwesenheit von DCs aus peripheren Lymphknoten oder IL-12 zur Entstehung hautmigrierender E/P-Selektin-Ligand$^+$ Tregs führte. Zudem konnte gezeigt werden, dass durch Expansion unter polarisierenden Bedingungen weder die Foxp3-Expression von $CD4^+CD25^+$ Tregs noch ihre suppressorische Kapazität *in vitro* verringert wurde. Diese Ergebnisse

weisen darauf hin, dass das Wanderungsverhalten von $CD4^+CD25^+$ Tregs während der Aktivierung durch das gewebespezifische Mikroenvironment und organspezifische DCs bestimmt wird. Zudem legen die Daten den Schluss nahe, dass die Herstellung von Tregs mit spezifischen Wanderungseigenschaften eine Option für therapeutische Anwendungen in der adoptiven T-Zell Therapie sein könnte.

Abstract

Regulatory $CD4^+CD25^+$ T cells (Tregs) play an important role in immune homeostasis and in the maintenance of self-tolerance. Our group has previously identified a subset of $CD4^+$ Tregs characterised by expression of the integrin $\alpha_E\beta_7$, which displays an effector/memory-like phenotype and is equipped with homing receptors allowing their migration into inflamed sites. These phenotypic and migratory characteristics are required for the capacity of α_E^+ Tregs to suppress ongoing inflammation in non-lymphoid tissues. In the present study the circumstances favouring *in vivo* generation of effector/memory-like α_E^+ Tregs were analysed. The results presented here show that α_E^+ effector/memory-like Treg subsets contain a large fraction of cycling cells under physiologic conditions in healthy mice. This *in vivo* proliferation depended, at least in part, on intestinal commensal microflora. Absence of microbial stimuli led to reduction in the total number of $CD4^+Foxp3^+$ Tregs, most likely as a result of decreased Treg proliferation within the gut-associated lymphoid tissue (GALT). Furthermore, adoptive transfer of transgenic T cell subsets from DO11.10 mice provided clear evidence that $\alpha_E^+Foxp3^+$ effector/memory Tregs not only developed by differentiation of naïve-like $CD4^+CD25^+$ Tregs, but also were generated *de novo* from naïve conventional T cells under tolerogenic conditions of antigen delivery in the GALT. These findings suggest that Tregs expressing the integrin $\alpha_E\beta_7$ are effector/memory Tregs with a high turnover rate that can develop from both naïve T cells converted into Tregs in the periphery as well as from thymic-derived naïve $CD4^+CD25^+$ Tregs differentiating into an effector/memory stage. In combination with the observed role of the commensal microflora, these results imply an important mechanism for the maintenance of $Foxp3^+$ Tregs in general and $\alpha_E^+Foxp3^+$ Tregs in particular and indicate that the mucosal microenvironment favours both expansion and conversion of Tregs.

In addition, susceptibility of naïve $CD4^+CD25^+$ Tregs to acquire tissue-specific homing receptor phenotypes was investigated. *In vitro* culture systems demonstrated that Tregs, similarly to conventional T cells, could be configured with organ-selective homing properties allowing efficient targeting into gut and skin. Priming of naïve $CD4^+CD25^+$ in the presence of either dendritic cells from mesenteric and peripheral LN, or the soluble factors retinoic acid and IL-12 induced $\alpha_4\beta_7^+$ mucosa-seeking and E/P-selectin ligand$^+$ skin-seeking Tregs, respectively. Importantly, the expansion under polarising conditions did not change Foxp3 expression and *in vitro* suppressive capacity of $CD4^+CD25^+$ Tregs. These results indicate that the tissue microenvironment and organ-specific dendritic cells shape migratory properties of naïve $CD4^+CD25^+$ Tregs during priming and suggest that generation of Tregs with specific homing properties for therapeutic purposes in adoptive T cell therapy might be a feasible option.

List of Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
BrdU	Bromodeoxyuridine
BSA	Bovine serumalbumin
CFSE	5-Carboxy-fluorescein diacetate succinimidyl ester
CTLA-4	Cytolytic T lymphocyte-associated antigen 4
Cy5	Indodicarbocyanin
d	Day
DAPI	Diamidinophenylindol
DC	Dendritic cell
DRFZ	Deutsches Rheumaforschungs Zentrum
DTH	Delayed-type hypersensitivity
E-Lig	E-selectin ligand
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein-Isothiocyanat
Foxp3	Forkhead box protein 3
FSC	Forward scatter
FucT	α(1,3)-Fucosyltransferase
GALT	Gut-associated lymphoid tissue
GMFI	Geometric mean fluorescence intensity
GFP	Green fluorscent protein
HBSS	Hanks balanced salt solution
HEV	High endothelial venule
IFA	Incomplete Freud's Adjuvant
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph node
mAb	Monoclonal Ab
MACS	Magnetic cell sorting
MAdCAM	Mucosal addressin cell adhesion molecule-1
mLN	Mesenteric LN
OVA	Ovalbumin
PBS	Phosphate-buffered salt solution

PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein
PI	Propidium Iodid
P-Lig	P-selectin ligand
pLN	Peripheral LN
PNAd	Peripheral lymph node addressin
PP	Peyer's Patch
PSGL-1	P-selectin glycoprotein ligand-1
RA	Retinoic acid
RAG	Recombinase activation gene
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SPF	Specific-pathogen-free
SSC	Side scatter
TCR	T cell receptor
tg	Transgenic
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
Treg	Regulatory T cell
wt	Wildtype

1 Introduction

The immune system is a remarkable defence mechanism, found in its most advanced form in higher vertebrates. It provides the means to make rapid, highly specific and protective responses against potentially pathogenic microorganisms including bacteria, viruses, fungi and parasites, thus, creating a state of protection from infectious disease named immunity. Examples of immunodeficiency, as seen in both genetically determined diseases and in the acquired immunodeficiency syndrome (AIDS), illustrate the central role of the immune response in protection against microbial infection. However, not only a deficient but also an excessive immune response as seen in autoimmunity and hypersensitivity reactions can lead to tissue damage and fatal outcome. Therefore, a balanced response, which is able to discriminate between self and non-self, is the prime challenge of the immune system.

Innate immunity acts as a first line of defence against infectious agents. The defences range from external physical and biochemical barriers (epithelial cells, mucous surfaces) to an internal defence involving e.g. phagocytes, dendritic cells (DC), natural killer (NK) cells and soluble mediators such as plasma proteins (e.g. complement cascade, C-reactive protein). The innate immune system relies on a set of germ-line encoded receptors named pattern-recognition receptors (PRRs), which are not specific to a particular pathogen but rather recognise conserved molecular patterns associated with pathogens (pathogen-associated molecular patterns, PAMPs) (Medzhitov and Janeway, 2000).

In contrast, the adaptive immune response is characterised by a high degree of specificity to individual pathogens by virtue of antigen-specific receptors and the ability to form a stable memory ensuring increased protection against re-infection. T cells, together with B cells, form the major part of adaptive immunity. T cells can efficiently recognise and eliminate infected cells and support the function of other cellular components of the immune system, such as B cells and macrophages. A fundamental basis for an adaptive T cell response is the large diversity of the T cell repertoire, which is generated by random rearrangement of gene sequences coding for a functional T cell receptor (TCR) during development of T cells in the thymus. However, the high degree of diversity carries the inherent danger of reactivity against endogenous structures of the organism and therefore sophisticated control mechanisms are required to ensure a state of unresponsiveness – tolerance – to self-antigens and other harmless determinants. Thus, the prime task of the immune system is to sustain the balance between immunity and tolerance. A set of central and peripheral tolerance mechanisms has evolved to ensure such discrimination at different levels, and failure can lead to autoimmune diseases like type1 diabetes, multiple sclerosis or rheumatoid arthritis (Sakaguchi, 2000).

Central tolerance is a function of the thymus and involves a combination of negative and positive selection processes, which eliminate most of the autoreactive T cell specificities (Kappler, et al., 1987; Kisielow, et al., 1988) while providing the large diversity of specificities to foreign antigen. However, elimination of self-reactive T cells during thymic development is not complete and therefore

additional, peripheral mechanisms are required to establish immunologic self-tolerance. These mechanisms include anergy and clonal deletion of autoreactive T cells as well as ignorance to self-antigen (Arnold, et al., 1993; Miller and Heath, 1993; Van Parijs and Abbas, 1998). In addition, it is increasingly recognised that dominant tolerance processes are in place, which are attributed largely to distinct subsets of T cells actively protecting from autoimmunity. The initial studies postulating the existence of T cells engaged in dominant tolerance date back to the early 1970s, when they became known as suppressor T cells (Gershon and Kondo, 1970; Ha and Waksman, 1973). However, due to lack of specific markers and the inability to identify the suppressive factor, the whole concept was discussed controversially and as a result interest gradually faded.

1.1 $CD4^+$ regulatory T cells

When Sakaguchi and colleagues identified the surface molecule CD25 as a marker for $CD4^+$ T cells with suppressive capacity, they initiated the re-emergence of the suppressor T cell paradigm and fuelled a remarkable interest in this field of research (Sakaguchi, et al., 1995). They found that 5-10% of $CD4^+$ T cells constitutively expressed the α–chain of the high affinity interleukin (IL)-2 receptor, a molecule associated with T cell activation. Using a model of adoptive transfer into immunodeficient recipient mice these authors demonstrated that $CD4^+CD25^-$ cells caused a wide spectrum of autoimmune disease in the host, while the presence of $CD4^+CD25^+$ was able to prevent the development of disease. These initial results indicated that regulatory T cells (Tregs) are present in the T cell repertoire of normal mice and that they are enriched within the small subset of $CD4^+CD25^+$ T cells. Much evidence has accumulated since to establish the importance of Tregs in various animal models of organ-specific autoimmunity, allergic responses, anti-tumor immunity, allograft rejection and immune responses to pathogens (Sakaguchi, 2004), including observations showing that Tregs control systemic homeostasis and total lymphocyte numbers (Annacker, et al., 2000). In addition, identification of human $CD4^+CD25^+$ cells with similar properties (Baecher-Allan, et al., 2001) and functional deficiency of the same observed in human autoimmune disease (Viglietta, et al., 2004), contributed to the acceptance of the proposed concept.

1.1.1 Features of $CD4^+$ Tregs

Further characterisation of $CD4^+CD25^+$ Tregs revealed several distinct features as opposed to the $CD4^+CD25^-$ T cell population. Besides CD25, Tregs were found to constitutively express other molecules associated with TCR-mediated activation such as cytolytic T lymphocyte-associated antigen 4 (CTLA-4) (Takahashi, et al., 2000) and glucocorticoid-induced TNF receptor (GITR) (Gavin, et al., 2002; McHugh, et al., 2002; Shimizu, et al., 2002). As these markers are shared with conventional T cells upon activation, their use is somewhat limited in the context of inflammatory immune responses. The search for more specific markers identifying the Treg subset produced some

potential candidates such as neuropilin-1 (Bruder, et al., 2004) and G protein-coupled receptor (GPR) 83 (Hansen, et al., 2006), however, the transcription factor forkhead box protein 3 (Foxp3) has emerged as the most reliable lineage specification marker to date. Due to the intracellular expression pattern, however, the ability to isolate pure $Foxp3^+$ cells for enhanced phenotypic characterisation and functional studies has been delayed until only recently when new tools became available such as antibodies for flow cytometric analysis and green fluorescent protein (GFP)-Foxp3 fusion-protein reporter knock in mice (Fontenot, et al., 2005).

Despite their activation-related phenotype Tregs have been characterised as anergic, as they do not respond to TCR-mediated stimulation *in vitro* and only high amounts of exogenous IL-2 can overcome their hypo-responsiveness (Thornton and Shevach, 1998). Another characteristic feature of Tregs is their ability to inhibit activation of other T cells *in vitro*. It was demonstrated that co-culture with $CD4^+CD25^+$ suppressed the proliferation of $CD4^+CD25^-$ T cells upon TCR-mediated activation by inhibiting the production of IL-2. Due to the limitations regarding Treg-specific surface markers this dominant characteristic allowed functional identification of potential Tregs and thus, has since been used widely as a defining property of Tregs in general. Their suppression was shown to depend on direct cell-cell contact and addition of exogenous IL-2 is able to abrogate the inhibition of $CD4^+CD25^-$ cells *in vitro* (Thornton and Shevach, 1998). Importantly, while the suppressive activity requires antigen-specific or CD3-mediated activation of $CD4^+CD25^+$ Tregs, once activated they inhibit both $CD4^+$ and $CD8^+$ T cell proliferation in an antigen-unspecific manner (Takahashi, et al., 1998; Thornton and Shevach, 2000).

1.1.2 Mode of action

Although many different *in vitro* and *in vivo* systems have been used to elucidate the immunosuppressive mechanism used by Tregs, no single mode of action has been identified so far. *In vitro* the contribution of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β has been largely excluded (Piccirillo, et al., 2002; Takahashi, et al., 1998), while competition for IL-2 was proposed as a possible mechanism of suppression and growth-inhibition. By virtue of their high level expression of CD25, Tregs may consume IL-2 produced by co-cultured $CD4^+CD25^-$ cells much more efficiently than the latter (De La Rosa, et al., 2004). However, recent evidence argues against the above mechnism, as peripheral $Foxp3^+$ Tregs from IL-$2R\alpha^{-/-}$ mice displayed similar suppressive capacity *in vitro* as wild-type (wt) Tregs (D'Cruz and Klein, 2005; Fontenot, et al., 2005) indicating that additional mechanisms are involved.

Conflicting data exist also concerning the role of constitutively expressed CTLA-4 in the suppressive function of Tregs. Upon blockade with specific anti-CTLA-4 antibodies suppressive activity of Tregs was abrogated *in vitro* (Takahashi, et al., 2000) and in a mouse model of colitis (Read, et al., 2000). As one possible mechanism it was proposed that Tregs initiate tryptophan catabolism in DCs through CTLA-4/B7-mediated interaction and induction of indoleamin 2,3 dioxygenase (IDO) (Fallarino, et

al., 2003), leading to deprivation of the essential amino acid tryptophan followed by growth inhibition. Furthermore, induction of TGF-β secretion after crosslinking of CTLA-4 on Tregs was reported (Chen, et al., 1998). However, others have not identified a role for CTLA-4 (Thornton and Shevach, 1998) and CTLA-$4^{-/-}$ Tregs also exhibit some suppressive function (Takahashi, et al., 2000).

Contrary to the *in vitro* results, a role for immunosuppressive cytokines *in vivo* has been observed in several studies. $CD4^+CD25^+$ were found to produce IL-10 *in vivo* (Annacker, et al., 2001; Klein, et al., 2003; Uhlig, et al., 2006) and this production was required, at least partially, for their suppressive function (Asseman, et al., 1999; Uhlig, et al., 2006). The contribution of TGF-β to the suppressive effect of Tregs is discussed more controversially, mainly because TGF-β is a highly pleiotropic cytokine, which is produced by many different cell types and has differential effects on a wide range of responder cells (Letterio and Roberts, 1998). Nevertheless, blocking of TGF-β was found to abrogate suppressive function of Tregs in experimental colitis (Liu, et al., 2003; Powrie, et al., 1996), signalling through the TGF-β receptor was required for functional activation of Tregs (Huber, et al., 2004) and $CD4^+CD25^+$ mediated suppression of cytolytic activity of $CD8^+$ T cells in tumor rejection depended on TGF-β signalling in $CD8^+$ target cells (Chen, et al., 2005). Although TGF-β mRNA expression was detected in $CD4^+CD25^+$ (Asano, et al., 1996), the above findings do not necessarily require that Tregs produce TGF-β themselves, consistent with the observation that $CD4^+CD25^+$ from TGF-$\beta^{-/-}$ mice prevented colitis in a similar way as wt Tregs and that suppression was dependent on non-Treg-produced TGF-β (Fahlen, et al., 2005).

Collectively, the available data confirm an important role for CTLA-4, IL-10 and TGF-β in suppression of autoimmune responses, while the exact mechanisms of action with regard to Tregs remain to be defined.

1.1.3 Foxp3

A major breakthrough in the field of dominant tolerance was the identification of Foxp3 as the first molecule specifically associated with the Treg phenotype. In 2001 Brunkow and colleagues laid the basis by identifying Foxp3 as a new member of the forkhead/winged-helix family of transcriptional regulators and as the defective gene in the scurfy mouse, a spontaneous X-linked recessive mouse mutant. The phenotype of scurfy mice is characterised by lethal lymphoproliferative disease similar to CTLA-$4^{-/-}$ and TGF-$\beta^{-/-}$ mice (Brunkow, et al., 2001). In addition, gene defects of the human homologue FOXP3 were found to be the cause of a lymphoproliferative disorder with a wide spectrum of autoimmune disease known as immune dysregulation, polyendocrinopathy enteropathy, X-linked syndrome (IPEX) (Bennett, et al., 2001).

Subsequent studies revealed that Foxp3 is specifically expressed by $CD4^+CD25^+$ Tregs and that both scurfy mice and Foxp$3^{-/-}$ mice exhibit similar phenotypes and are deficient in $CD4^+CD25^+$ Tregs. Finally, over-expression of Foxp3 in $CD4^+CD25^-$ cells converted them into cells with suppressive

properties similar to $CD4^+CD25^+$ Tregs (Fontenot, et al., 2003; Hori, et al., 2003; Khattri, et al., 2003). Furthermore, it was demonstrated that Foxp3 expression was required for thymic development of $CD4^+CD25^+$ Tregs (Fontenot, et al., 2003). Recent studies using GFP-Foxp3 fusion-protein reporter knock in mice have allowed more in-depths analysis of the relationship between CD25 and GFP-Foxp3 ($Foxp3^{gfp}$) expression on the single cell level and demonstrated that a significant proportion $Foxp3^{gfp+}$ Tregs expressed little or no CD25. On the other hand, a fraction of $CD25^+$ cells did not express $Foxp3^{gfp}$ and was identified as an activated/effector subset, which did not display suppressive capacity *in vitro*. Nevertheless, high levels of CD25 expression correlated well with $Foxp3^{gfp}$ (Fontenot, et al., 2005) and validated the usefulness of CD25 as a Treg marker, at least in the absence of overt inflammation.

1.1.4 Origin of $CD4^+$ Tregs

The thymus has a central role not only in generating a diverse T cell repertoire for protective immunity against pathogens, but also in preventing unwanted immune responses against self by deleting autoreactive thymocytes. In addition, the thymus is also constantly generating regulatory T cells that exert dominant tolerance mechanisms in the periphery (Seddon and Mason, 2000).

From a number of studies it is well documented that a fraction, if not all $CD4^+CD25^+$ Tregs develop in the thymus as a distinct lineage. Transfer of $CD25^+$ depleted $CD4^+CD8^-$ thymocytes into athymic mice results in various autoimmune diseases. Postnatally, $CD4^+CD25^+$ Tregs appear first in thymus before they become detectable in the periphery. They show similar phenotypic and functional properties as their peripheral counterparts (Itoh, et al., 1999). These findings clearly demonstrate that the thymus constantly produces Tregs. Foxp3 appears to be the most important single factor required for recruitment of thymocytes into the Treg pool (Fontenot, et al., 2003; Hori, et al., 2003; Khattri, et al., 2003), while the exact signals driving expression/up-regulation of this lineage-specific transcription factor are not understood. Expression of $Foxp3^{gfp+}$ was already detected at the $CD4^+CD8^+$ stage of thymocyte development, however, induction occurred preferentially at the $CD4^+CD8^-$ stage (Fontenot, et al., 2005). Current evidence suggests that high affinity TCR interaction with self-peptide/MHC ligands is a prerequisite for development of $CD4^+CD25^+$ Tregs (Jordan, et al., 2001; Kawahata, et al., 2002). Apparently, this interaction is not so high as to lead to depletion of Tregs during the negative selection process. Interestingly, in TCR-transgenic (TCR-tg) mouse models $CD4^+CD25^+$ Tregs are also present and they were found to express endogenous TCR α-chains. In contrast, $CD4^+CD25^+$ Tregs are absent in recombinase activation gene-2 (RAG-2)-deficient TCR-tg mice, indicating that thymic generation of $CD4^+CD25^+$ Tregs in TCR-tg mice is controlled by endogenous TCR gene rearrangement (Itoh, et al., 1999; Suto, et al., 2002).

Expression of self-antigen on radioresistant elements of the thymus was found to be required for thymic development of $CD4^+CD25^+$ Tregs (Apostolou, et al., 2002) and a particular role for thymic cortical eptihelial cells in the selection process has been proposed (Bensinger, et al., 2001), while

recent data from the Foxp3 reporter mouse suggest an important role for the thymic medulla in driving development of $Foxp3^{gfp+}$ thymocytes (Fontenot, et al., 2005). In addition to antigen-specific interactions accessory molecules such CD28, CTLA-4, B7 and CD40 contribute to thymic generation of Tregs (reviewed by Sakaguchi (Sakaguchi, 2004)). Further studies are required to dissect the interplay of the different signals, which determine the fate of developing thymocytes.

Apart from thymic generation of $CD4^+CD25^+$ Tregs there is accumulating evidence to suggest that regulatory T cells can be induced *de novo* from the conventional, naive $CD4^+$ T cell pool both *in vitro* and *in vivo*. Immunosuppressive cytokines like TGF-β (Chen, et al., 2003; Fantini, et al., 2004) and IL-10 (Groux, et al., 1997), as well as immature DCs (Kretschmer, et al., 2005; Mahnke, et al., 2003) have been used to induce regulatory T cells with distinct properties. Generation of Tregs was also observed with various protocols of tolerance induction, i.e. oral, nasal and i.v. antigen-administration (Apostolou and von Boehmer, 2004; Hauet-Broere, et al., 2003; Hultkrantz, et al., 2005; Thorstenson and Khoruts, 2001; Unger, et al., 2003). However, the reported phenotypes of *de novo* induced Tregs are heterogeneous, particularly with respect to the expression of the Treg marker Foxp3.

Due to the large heterogeneity of peripheral T cells with regulatory properties, thymic-derived $CD4^+CD25^+Foxp3^+$ Tregs are generally referred to as natural Tregs in order to distinguish them from those subsets that were induced in the periphery. Natural $CD4^+CD25^+$ Tregs are $Foxp3^+$ by definition, while not all peripherally generated Treg subsets express Foxp3. Among those are the Tr1 cells, which are characterised by the production of IL-10 upon repeated TCR-mediated stimulation in the presence of IL-10 (Barrat, et al., 2002; Groux, et al., 1997; Vieira, et al., 2004). Oral antigen administration results in the development of TGF-β-secreting Th3 cells (Chen, et al., 1994). They lack CD25 expression and their suppressive activity relies solely on TGF-β and thus they are distinct from natural $CD4^+CD25^+$ Tregs both with respect to their phenotype and suppressive mechanism (Zhang, et al., 2001).

Taken together, these observations indicate that the peripheral pool of Tregs is composed of various different subsets with partially overlapping functional properties. Their relationship to each other is currently not well understood.

1.1.5 Integrin $\alpha_E\beta_7$ expression on $CD4^+$ Tregs

Recent studies from our group and from others have identified the integrin α_E (CD103) as a marker for murine $CD4^+$ Tregs in secondary lymphoid organs (Banz, et al., 2003; Lehmann, et al., 2002; McHugh, et al., 2002; Zelenika, et al., 2002). Initially, expression of $\alpha_E\beta_7$ was predominantly ascribed to intraepithelial lymphocytes residing in the gut wall and other epithelial sites, i.e. skin and lung (Cerf-Bensussan, et al., 1987; Kilshaw and Murant, 1991; Picker, et al., 1994). Expression of $\alpha_E\beta_7$ is closely associated with mucosal tissues, where TGF-β, which transcriptionally regulates expression of

the α_E subunit, is abundant (Kilshaw and Murant, 1991). The ligand for $\alpha_E\beta_7$, E-cadherin, is expressed on a variety of epithelial cells such as intestinal epithelial cells and Langerhans' cells in the skin (Cepek, et al., 1994; Geiger and Ayalon, 1992). Consequently, the postulated function of $\alpha_E\beta_7$ is to provide retention within epithelial sites (Suffia, et al., 2005). This is in contrast to the closely related integrin $\alpha_4\beta_7$, which mediates interaction of T cells with the gut-associated vascular endothelium and thereby controls trafficking to the intestine (Hamann, et al., 1994; Holzmann and Weissman, 1989). However, the exact function of $\alpha_E\beta_7$ is currently not clear, studies in $\alpha_E^{-/-}$ mice have not provided conclusive results. Deficiency in $\alpha_E\beta_7$ leads to modest reduction in the number of mucosal T cells and development of an age-related skin disorder when introduced to certain genetic backgrounds (Schon, et al., 1999; Schon, et al., 2000).

Our group has previously shown that within the murine $CD4^+$ Treg compartment α_E and CD25 identify phenotypically and functionally distinct Treg subsets. Using these markers it was possible to subdivide the Treg compartment into naive-like $\alpha_E^-CD25^+$ and effector/memory-like $\alpha_E^+CD25^+$ or $\alpha_E^+CD25^-$ cells (Huehn, et al., 2004). Each of these Treg subsets expresses Foxp3 mRNA and displays suppressive capacity *in vitro* and *in vivo*. While the $\alpha_E^+CD25^+$ subset always exhibited the most potent inhibitory capability, $\alpha_E^+CD25^-$ cells were functional in preventing experimental colitis but less effective in suppressing the proliferation of naïve responder cells *in vitro* (Lehmann, et al., 2002).

Most strikinkgly, expression of α_E was found to correlate with an effector/memory phenotype. α_E^+ Tregs displayed reduced amounts of T cell recepor excision circles (TREC), expressed increased amounts of molecules such as CD44, CD54, CD29, LFA-1 and P-selectin ligand, reduced levels of CD62L and migrated in response to inflammatory chemokines *in vitro*. In contrast, $\alpha_E^-CD25^+$ Tregs displayed a naïve-like phenotype characterised by high amounts of CD62L and increased chemotactic sensitivity to CCR7 ligands. These differential phenotypic properties resulted in selective homing patterns upon adoptive transfer (Huehn, et al., 2004).

Ultimately, these distinct characteristics were directly coupled to the functional activity of the respective subsets *in vivo*, indicating that appropriate location was a prerequisite for suppressive capacity. Naïve-like $\alpha_E^-CD25^+$ Tregs suppressed proliferation of naïve antigen-specific T cells in draining lymph nodes, while α_E^+ Tregs where much more efficient in ameliorating acute skin inflammation in a model of delayed-type hypersensitivity (DTH) (Siegmund, et al., 2005).

Collectively, the above findings led to the hypothesis that there is division of labour between distinct subsets of naturally occurring Tregs. In order to suppress the sequential stages of potentially harmful immune responses appropriate localisation of Tregs is indispensable for their function (Huehn and Hamann, 2005). However, the physiologic conditions, which may favour the peripheral development of distinct subsets of natural Tregs, remained elusive.

1.1.6 Peripheral maintenance of $CD4^+$ Tregs

One prerequisite for survival and function of Tregs in the periphery appears to be the presence of their respective antigen (Garza, et al., 2000; Jordan, et al., 2001; Nishikawa, et al., 2005; Seddon and Mason, 1999; Walker, et al., 2003). As the majority of $Foxp3^+$ Tregs are selected towards high affinity recognition of self-antigen in the thymus, it is thought that the very antigen that caused their positive selection in the thymus activates them in the periphery. Consistent with this notion Tregs display remarkable proliferation *in vivo*, i.e. they respond to self-peptide presented by unique DCs in tissue-draining lymph nodes (LN) (Cozzo, et al., 2003; Fisson, et al., 2003; Klein, et al., 2003; Walker, et al., 2003; Yamazaki, et al., 2003). In addition, cytokines contribute to the maintenance of Tregs under steady state conditions. The growth factor IL-2 has been identified as the principle survival factor of peripheral Tregs. The pivotal role of this cytokine is delineated from studies showing that mice deficient for either IL-2 or the high affinity receptor IL-2R α-chain (CD25) develop generalised autoimmune diseases (Sadlack, et al., 1993; Sadlack, et al., 1995; Willerford, et al., 1995). Pathology is a consequence of dramatically reduced numbers of $CD4^+CD25^+$, reflecting the importance of IL-2 signalling in maintenance of Treg homeostasis and function (Bayer, et al., 2005; D'Cruz and Klein, 2005; Fontenot, et al., 2005; Furtado, et al., 2002; Setoguchi, et al., 2005). Furthermore, an essential role for TGF-β signalling has been identified for peripheral expansion, maintenance of Foxp3 expression and suppressive function of Tregs (Huber, et al., 2004; Marie, et al., 2005). Thus, a complex interplay between different signals received under physiologic conditions controls the survival and function of Tregs in the periphery.

1.1.7 Mucosal immune homeostasis

Apart from responses to self-antigens, constant encounters with dietary and microbial antigens at mucosal surfaces require regulatory mechanisms, which also include the action of various intestinal Treg subsets (reviewed in (Mowat, 2003; Rook and Brunet, 2005)). This state of intestinal tolerance was demonstrated in several studies showing i.e. that under non-inflammatory conditions the response to bacterial antigen is actively suppressed. Intestinal T cells did not proliferate in response to enterobacterial antigens *in vitro* (Duchmann, et al., 1996; Gad, et al., 2004; Khoo, et al., 1997), however, depletion of $CD4^+CD25^+$ T cells (Gad, et al., 2004) or exposure to heterologous bacterial antigen (Duchmann, et al., 1996) reversed unresponsiveness in these cultures. *In vivo* break of tolerance towards the intestinal microflora is believed to be the major cause of inflammatory bowel disease (Duchmann, et al., 1995). The capacity of $CD4^+CD25^+$ Tregs to prevent such inflammatory responses in the intestine has been illustrated in numerous studies of experimental colitis and clearly demonstrates a pivotal role for Tregs in the maintenance of intestinal homestasis (reviewed in (Izcue, et al., 2006)).

Studies in germfree mice demonstrate a pivotal role for the commensal flora in the development of a tolerogenic microenvironment. First of all, the absence of microbial stimuli influences the development of mucosal and systemic lymphoid tissues. In germfree mice Peyer's Patches (PP) are hypoplastic, IgA-secreting plasma cells and lamina propria $CD4^+$ T cells are greatly reduced in numbers and structural defects also affect the spleen and peripheral LN (pLN). Colonisation of germfree mice with commensal flora from SPF mice is able to reverse these abnormalities (Macpherson and Harris, 2004). Furthermore, development of oral tolerance was dependent on a complex intestinal flora (Rask, et al., 2005) and previous exposure of Tregs to bacterial antigens was required for amelioration of experimental colitis (Strauch, et al., 2005).

Recognition of pathogens by the Toll-like receptor (TLR) family is well documented (Medzhitov and Janeway, 2000). Commensal microorganisms equally express their respective ligands and therefore TLRs are similarly implicated to play a role under steady state conditions. Accordingly, absence of TLR signalling impaired normal gut epithelial homeostasis (Rakoff-Nahoum, et al., 2004) and increased allergic responses to food antigens (Bashir, et al., 2004). Some TLRs are selectively expressed by Tregs as compared to other T cells (Caramalho, et al., 2003) and particularly TLR2 was described to play a role for Treg proliferation both *in vitro* and *in vivo* (Liu, et al., 2006; Netea, et al., 2005; Sutmuller, et al., 2006)

These data provide good evidence to suggest that intestinal colonisation by commensal microbes may contribute to the continuation of immune homeostasis and protection from aberrant immune responses by providing various stimuli for expansion and maintenance of Tregs in the periphery.

1.2 Migration of T cells

T cells continuously recirculate between blood and tissue in order to exert their immunologic function (Gowans and Knight, 1964). Depending on their state of differentiation and activation they follow different migratory routes and enter different types of tissue (Butcher and Picker, 1996). Naïve T cells preferentially migrate to secondary lymphoid organs including pLN, mesenteric LN (mLN), spleen and PP, which are the sites where they get activated if cognate antigen and co-stimulatory signals are present. Upon antigen-specific activation T cells proliferate, differentiate into effector/memory T cells and acquire the ability to migrate to non-lymphoid tissue and to sites of inflammation.
In order to emigrate from the blood into lymphoid and non-lymphoid tissue T cells have to interact with specialised endothelial surfaces, either the high endothelial venules (HEVs) in secondary lymphoid tissue, such as LNs, or the postcapillary venules in non-lymphoid tissue. Molecules on the surface of T cells as well as molecules on the endothelium are involved in this interaction, which eventually leads to extravasation of T cells from the blood into tissue.

1.2.1 The multistep model

The process of transendothelial migration of T cells and other leukocytes can be divided into four sequential stages and many of the molecules that are involved in each step, have been identified (Butcher, 1991; Butcher, 1990; Springer, 1990; Springer, 1994). In the initial phase T cells are slowed down from the blood flow via labile interactions with the endothelial cells mediated by the selectin family and their ligands. Due to the particular adhesive strength of this interaction T cells do not come to a complete halt but roll along the vessel wall and sample the specific site for factors that can trigger their firm adhesion. The triggering or activation is mediated by chemokines, which are displayed on the luminal side of the endothelium. Binding to chemokine receptors on the surface of T cells leads to activation of integrins and subsequently to arrest and firm adhesion of the T cell to the vessel wall. The activation of integrins is required to allow high affinity binding to their ligands on the endothelium, which belong to the immunoglobulin (Ig)-superfamily. Firm adhesion is followed by transmigration of T cells through the endothelium into the tissue. Chemokines serve as chemotactic factors guiding the migration of T cells to their final destination within the tissue.

1.2.2 Molecules involved in the transmigration cascade

The selectin family of adhesion molecules contains three members termed E-selectin (CD62E), P-selectin (CD62P) and L-selectin (CD62L). The family members share common structural features such as N-terminal Ca^{2+}-dependent lectin homologous regions. These domains enable them to bind to carbohydrate ligands expressed on the surface of other cells (Springer, 1990). E- and P- selectin are expressed on endothelial cells, while L-selectin is exclusively expressed on lymphocytes.

The ligands for selectins are sialylated carbohydrate moieties, which are linked to mucin-like molecules. Mucins are heavily O-glycosylated serine- and threonine-rich proteins (Springer, 1994). Their glycosylation is dependent on the expression and activity of a family of enzymes, including α(1,3)-fucosyltransferases (FucT). Modification of the P-selectin glycoprotein ligand 1 (PSGL-1) by these enzymes is essential for the expression of functional ligands for P- and E-selectin (Maly, et al., 1996). Another ligand for E-selectin, the cutaneous lymphocyte-associated antigen (CLA), is a specialised isoform of PSGL-1 and is involved in tissue specific migration of a subset of memory T cells (Fuhlbrigge, et al., 1997). Selectin/selectin ligand interactions display rapid "on" and "off" rates and provide labile adhesive interaction between leukocytes and endothelial cells. Thus they have been shown to have a role in the initial slowing of leukocytes from the blood flow (Springer, 1994).

Integrins are a large family of versatile adhesion molecules. They are heterodimeric transmembrane proteins, containing non-covalently linked α and β subunits. At least 18 α chains and 8 β chains have been described forming at least 24 heterodimers. Integrins play a major role in cell adhesion to extracellular matrix proteins as well as in cell-cell interactions. They are pivotal in regulating adhesion and migration during development, wound healing and immune reactions (van der Flier and Sonnenberg, 2001). Integrins are grouped into several subfamilies, the β chain determining the family and the α chain determining the ligand specificity. T cells express β_1, β_2 and β_7 heterodimers, respectively. The individual integrin pattern is subset-specific and depends on the state of differentiation. The β_2 and α_4 containing integrin hetrodimers are mainly involved in cell-cell interaction between leukocytes and other cells. The major cellular ligands for leukocyte integrins are found within the Ig superfamily. Family members involved in the leukocyte-endothelial interaction, include intercellular adhesion molecule 1 (ICAM-1), ICAM-2, vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1). A characteristic feature of these transmembrane C2-type proteins is the presence of a variable number of Ig-like domains. MAdCAM-1 is unique in that it also contains a mucine-like region, which makes it a dual ligand both for the integrin $\alpha_4\beta_7$ as well as for L-selectin. The expression of the above Ig superfamily members on endothelial cells is either constitutive or upregulated upon stimulation with inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) (Carlos and Harlan, 1994; Springer, 1994). The distinct pattern of adhesion molecules expressed on the surface of endothelial cells is one level of regulating the selective recruitment of leukocytes across the endothelial cell layer.

Chemokines are small **chemo**attractant cyto**kines** that can be classified according to NH_2-terminal cysteine motifs. Four subfamilies are recognised comprising C, CC, CXC and CX3C chemokines. The C and CX3C subfamilies contain only one family member. The CC subfamily currently comprises 28 members and the CXC subfamily 16 members. Based on a more functional classification chemokines are grouped into "inflammatory" (also called inducible) or "homeostatic" (also termed constitutive)

chemokines. Inflammatory chemokines play a crucial role in the recruitment of various effector cells to sites of inflammation. They are produced and secreted by resident or infiltrating cells in the inflamed tissue upon pathogen contact or activation with pro-inflammatory cytokines (Moser and Loetscher, 2001). The constitutive chemokines on the other hand maintain physiological trafficking of leukocytes and are usually produced in a distinct microenvironment, e.g. lymphoid tissue or skin and mucosa. They are mainly involved in hematopoiesis, antigen sampling and immune surveillance (Moser and Loetscher, 2001). However, the division of chemokines into either group is not absolute, as several chemokines show overlapping functions.

Chemokines exert their functions via binding to a family of surface receptors, which are seven transmembrane domain G-protein-coupled receptors. Most chemokine receptors are promiscuous and bind more than one chemokine ligand. However, CC chemokines only bind to CC receptors and CXC chemokines only to CXC receptors. The expression of chemokine receptors is dependent on the state of differentiation and activation. Naive T cells, as a rather homogenous population express limited numbers of chemokine receptors. Upon antigen-specific activation the chemokine receptor pattern changes. Differential expression has been described for Th1 and Th2 effector T cells linking the distinct combination of chemokine receptor expression to differential functional properties (Sallusto, et al., 1998).

The main function of chemokines and their receptors is the control of leukocyte trafficking. They have a major role in the promotion of leukocyte migration along chemotactic gradients and in activation of integrins, thereby inducing adhesion of leukocytes to other cells and components of the extracellular matrix (Moser and Loetscher, 2001).

1.2.3 Organ-selective migration

Differential expression and distinct distribution of the molecules involved in each sequential step of transendothelial migration, provide the basis for the high level of specificity that is required to control T cell trafficking. Both, T cells and endothelium, contribute to this specificity. An area code model has been proposed, that allows specific targeting of circulating T cells based on the combination of three sequential interactions mediated by selectin/selectin ligand, chemokine/chemokine receptor and integrin/ligand binding. An important feature is that these interactions occur in sequence and not in parallel. Multiple receptors and ligands within each group provide the molecular basis for a large combinatorial diversity in regulating T cell migration (Butcher, 1991; Springer, 1994).

1.2.3.1 Migration and homing of naïve T cells to lymphoid tissue

The combined expression of the selectin family member CD62L (or L-selectin) and the chemokine receptor CCR7 mediates selective migration of naïve T cells to secondary lymphoid organs. The endothelial ligands for CD62L include glycosylation-dependent cell-adhesion molecule-1

(GlyCAM-1) and CD34, which are expressed on pLN HEVs, and MAdCAM-1 on intestinal HEVs. They all carry the peripheral lymph node addressin (PNAd) carbohydrate epitope, which mediates interaction with CD62L on T cells (Patel, et al., 2002). Trafficking of naïve T cells to gut-associated lymphoid tissue (GALT), such as mLN and PP, is controlled additionally by low-level expression of the integrin $\alpha_4\beta_7$. Rolling on PP HEVs and to a lesser extent on mLN HEVs, is supported by interaction of $\alpha_4\beta_7$ with MAdCAM-1 (Berlin, et al., 1995). The CCR7 ligand, CCL21, is expressed by HEVs (Gunn, et al., 1998) and triggers activation of LFA-1- or $\alpha_4\beta_7$-mediated adhesion to endothelial ligands (Hamann, et al., 1988). Within the LN chemotactic gradients of the other CCR7 ligand CCL19 ensure proper localisation of naïve T cells to the T cell zone, where they can interact with antigen-presenting cells (APCs).

1.2.3.2 Migration and homing of effector/memory T cells to non-lympoid tissue

In contrast to the uniform homing properties displayed by naïve T cells, the pool of circulating effector/memory T cells contains distinct subsets exhibiting tissue-selective homing behaviour. Migration to GALT, the small intestinal lamina propria and the mucosal epithelium is mediated by the integrin $\alpha_4\beta_7$ (Hamann, et al., 1994; Holzmann and Weissman, 1989). The principal endothelial ligand of $\alpha_4\beta_7$, MAdCAM, is almost exclusively expressed on mLN and PP HEVs and postcapillary venules in the intestinal lamina propria (Berlin, et al., 1993; Nakache, et al., 1989; Streeter, et al., 1988). This differential expression pattern provides the basis for selective recruitment of effectory/memory T cell subsets expressing high levels of $\alpha_4\beta_7$. In addition, expression of the chemokine receptor CCR9 has been identified on a fraction of circulating $\alpha_4\beta_7^+$ T cells (Kunkel, et al., 2000; Zabel, et al., 1999) and on the majority of freshly isolated small intestinal lamina propria lymphocytes (Papadakis, et al., 2000), consistent with high expression levels of the CCR9 ligand CCL25 by epithelial cells of the small intestine and in lamina propria venules (Papadakis, et al., 2000). Thus, expression of these two homing receptors defines gut-tropic effector/memory T cells.

In contrast, T cell trafficking to the skin is mediated by P-selectin ligands (P-Lig) and E-selectin ligands (E-Lig) (Picker, et al., 1990; Picker, et al., 1991; Tietz, et al., 1998) as well as CCR4 and CCR10 (Campbell, et al., 1999; Homey, et al., 2002; Reiss, et al., 2001). Expression of E-selectin and P-selectin on skin venules is constitutive (Weninger, et al., 2000) in contrast to other vascular endothelium, where they are transiently expressed in response to inflammatory signals (Bevilacqua, et al., 1987; Gotsch, et al., 1994). Similarly, the ligands for CCR4 (CCL17) and CCR10 (CCL27) are found on inflamed and non-inflamed skin endothelium (Campbell, et al., 1999; Homey, et al., 2002).

These two examples of peripheral tissue sites are the best studied to date and undermine the principle of organ-selective migration (Figure 1).

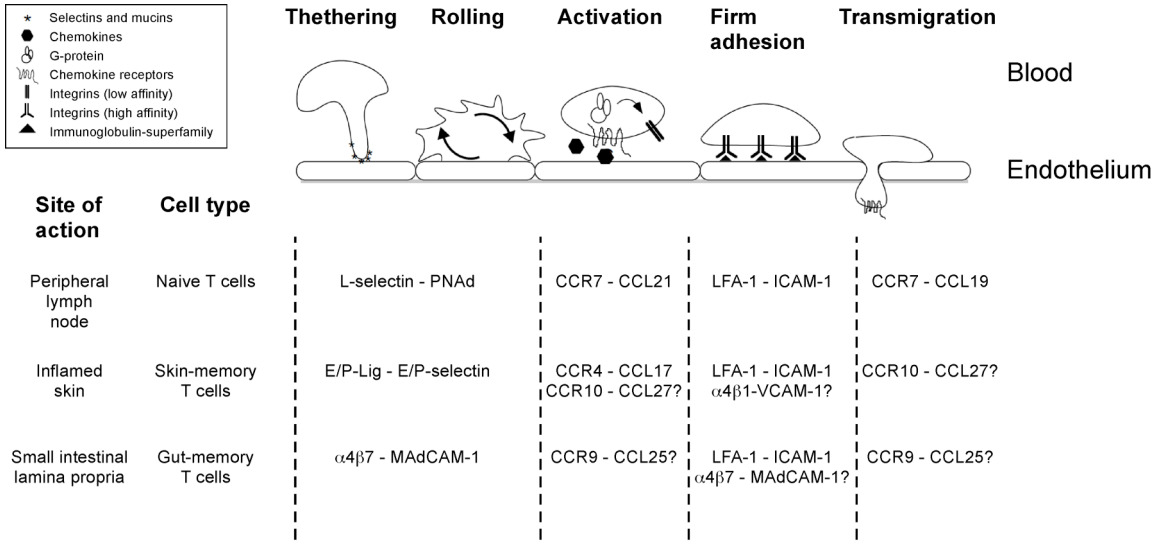


Figure 1: The multistep model in organ-selective trafficking of T cells

Sequential steps of transendothelial migration of T cells and the postulated adhesion molecules and chemokines/chemokine receptors involved in homing of naïve T cells, skin-specific and gut-specific effector/memory T cells, respectively. See text for details (1.2.1 and 1.2.3) (adapted from Campbell and Butcher (Campbell and Butcher, 2000)).

1.2.4 Regulation of organ-specific migration

Accumulating evidence suggests that the site of antigen presentation predicts the homing phenotype of the activated T cells. Early studies already indicated that activated lymphocytes isolated from the intestinal or cutaneous lymphoid tissue homed preferentially to their tissue of origin upon adoptive transfer (Griscelli, et al., 1969; Hall, et al., 1977). More recently, a number of studies have described that antigen-dependent differentiation of naïve T cells in lymphoid organs leads to the generation of effector T cells exhibiting a capacity to enter peripheral non-lymphoid tissue (Kunkel and Butcher, 2002). Effector T cells generated in different lymphoid organs display distinct tissue tropism, which is regulated by an organ-specific induction of adhesion molecules and chemokine receptors during T cell priming (Calzascia, et al., 2005; Campbell and Butcher, 2002; Dudda and Martin, 2004; Dudda, et al., 2004; Johansson-Lindbom, et al., 2003; Mora, et al., 2003; Stagg, et al., 2002; Svensson, et al., 2002). For example, T cells activated in mLN and PPs draining the gut acquire high levels of the integrin $\alpha_4\beta_7$, whereas activation in skin-draining pLN results in the up-regulation of selectin ligands such as P-Lig. Tissue-specific DCs have been shown in a number of *in vitro* and *in vivo* studies to be involved in the instruction of naïve T cells (Annacker, et al., 2005; Dudda and Martin, 2004; Dudda, et al., 2004; Johansson-Lindbom, et al., 2005; Johansson-Lindbom, et al., 2003; Mora, et al., 2003; Mora and von Andrian, 2006; Stagg, et al., 2002).

Recently, Iwata et al. reported that gut-associated lymphoid tissue DCs were capable of converting metabolites of dietary vitamin A into retinoic acid (RA), which in turn induced T cell expression of $\alpha_4\beta_7$ and CCR9 leading to the generation of gut-tropic T cells (Iwata, et al., 2004). In contrast, the

factors involved in the generation of skin-specific T cell homing *in vivo*, are largely unknown, although important roles for cytokines including IL-12 and TGF-β have been suggested from *in vitro* studies (Austrup, et al., 1997; Lim, et al., 1999; Wagers and Kansas, 2000). On the T cell side, induction of functional P/E-Lig depends on expression of the enzyme FucT-VII, which in turn is up-regulated upon TCR stimulation and further enhanced by the presence of IL-12 (White, et al., 2001).

1.3 Aims and objectives

Our group has previously shown that the markers α_E and CD25 allow subdivision of the murine $CD4^+$ Treg compartment into naïve-like and effector/memory-like Treg subsets. α_E-expression identifies Tregs with an activated state that have undergone repetitive rounds of proliferation indicative of antigen-specific expansion or differentiation in the periphery. The effector/memory phenotype of α_E^+ Tregs enabled recruitment of these Treg subsets into peripheral sites of inflammation, whereas naïve-like α_E^- Tregs preferentially migrated to secondary lymphoid organs. Ultimately, these distinct characteristics were directly coupled to the functional activity of the respective subsets *in vivo*, indicating that appropriate localisation was a prerequisite for suppressive capacity. These results initiated the hypothesis that there exists division of labour between distinct subsets of naturally occurring Tregs. However, physiologic conditions, which support peripheral development of distinct subsets of natural Tregs, had not been identified. Moreover, it was not clear whether α_E^+ Tregs originate from naïve-like $\alpha_E^-CD25^+$ Tregs differentiating into an effector/memory phenotype upon antigen-driven activation and proliferation, or whether α_E^+ Tregs can also directly develop from naïve T cells under appropriate, tolerogenic conditions. In the present study *in vivo* proliferation of various subsets of $CD4^+$ Tregs under homeostatic conditions was analysed and the influence of commensal microflora on homeostatic proliferation of $CD4^+$ Tregs was investigated. Furthermore, activation and differentiation of TCR-tg T cells in response to oral feeding of cognate antigen were examined in an adoptive transfer model.

An additional aim of the current study was to investigate how Tregs acquire the expression of homing receptors needed for tissue- or inflammation-specific migration. Although homing receptors known from conventional T cells were also found on Tregs, it was unknown, whether they respond in a similar way as conventional naïve T cells to signals instructing tissue-specific homing receptor expression and organ-selective trafficking. The present study focused on the induction of gut- and skin-specific trafficking patterns on Tregs. The susceptibility of naïve Tregs to acquire homing receptor expression under defined *in vitro* culture conditions was investigated and the functionality of the induced phenotype was tested upon adoptive transfer.

2 Materials and Methods

2.1 Material

2.1.1 Material and Reagents

all-trans-Retinoic acid (RA)	Fluka BioChemika, Buchs, Schwitzerland
β-Mercaptoethanol	Invitrogen, Karlsruhe
Bovine Serumalbumin (BSA, Fraction V)	Fluka BioChemika, Buchs, Schwitzerland
Bromodeoxyuridin (BrdU)	Sigma-Aldrich, Taufkirch
Calliper "Oditest-Schnelltaster"	Kröplin, Schlüchtern
5-Carboxy-fluorescein diacetate succinimidyl ester (CFSE)	Moleculare Probes, Eugene, USA
Cell strainer, 70 μm	Beckton Dickinson Labware, Franklin Lakes, NJ, USA
Collagenase D	Roche Diagnostic, Mannheim
Culture dishes, various sizes	Greiner, Nürtingen Corning Inc., NY, USA
Diamidinophenylindol (DAPI)	Sigma-Aldrich, Taufkirch
DNase I	Sigma-Aldrich, Taufkirch
Ethylendiaminetetraacetic acid (EDTA)	Sigma-Aldrich, Taufkirch
Fetal calf serum (FCS) inactivated for 30 min at 56°C	Sigma-Aldrich, Taufkirch
Fluoresbrite microspheres, 20 μm	Polyscience Inc., Warrington, PA, USA
Gentamycin	Biochrome AG, Berlin
Hanks balanced salt solution (HBSS)	Biochrome AG, Berlin
Hydroxyethylpiperazine ethanesulfonic acid (HEPES)	Biochrome AG, Berlin
Incomplete Freud's Adjuvant (IFA)	Sigma-Aldrich, Taufkirch
^{111}Indiumoxin	Amersham Biosciences, Freiburg
Lipopolysaccharide (LPS), from E. coli serotype 055:B5	Sigma-Aldrich, Taufkirch
MACS MicroBeads and MultiSort reagents	Miltenyi Biotec GmbH, Bergisch Gladbach
MACS® Pre-Separation Filter	Miltenyi Biotec GmbH, Bergisch Gladbach
MidiMACS™ Separator and MACS® Separation columns LS and MS	Miltenyi Biotec GmbH, Bergisch Gladbach
Mycojector-Syringe U40	BSNmedical, Eschborn
Neubauer haemocytometer	Paul Marienfeld GmbH & Co. KG, Lauda
Nycodenz	Nyegaard, Oslo, Norway
Ovalbumin peptide ($OVA_{323-339}$) Sequence: ^{323}ISQAVHAAHAEINEAGR^{339}	Institut für Biochemie, Humboldt-Universität zu Berlin
Ovalbumin (OVA) protein, Grade V	Sigma-Aldrich, Taufkirch
Penicillin-Streptomycin	Biochrome AG, Berlin
Phosphate buffered salt solution (PBS)	PAA Laboratories, Pasching, Austria

Propidium Iodide (PI)	Sigma-Aldrich, Taufkirch
Rat Immunoglobulin (IgG)	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Recombinant murine cytokines IL-12, IFN-γ, IL-2	R&D Systems, Wiesbaden
Rosewell Park Memorial Institute Medium (RPMI 1640) with GlutamaxI	Invitrogen, Karlsruhe
Sodium Pyruvate	Gibco, Karlsruhe
Syringes and needles	Braun-Melsungen, Melsungen
Trypanblue	Sigma-Aldrich, Taufkirch

2.1.2 Buffers and Media

Complete Medium	RPMI 1640, 25 mM HEPES, 10% FCS, 50 µM β-Mercaptoethanol, 1 mM Sodium-Pyruvat, 100 U/ml Penicillin, 100 U/ml Streptomycin, 50 µg/ml Gentamycin
Phosphate-buffered salt solution (PBS)	8 g/L NaCl, 0.2 g/L KH_2PO_4, 1.4 g/L $Na_2PO_4 \cdot H_2O$
PBS/BSA	PBS, 2 g/L BSA
HBSS/BSA	HBSS, 2 g/L BSA
Erythrocyte-Lysing-Buffer	10 mM $KHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA, pH 7.5

2.1.3 Instruments

autoMACS™ Separation unit	Miltenyi Biotec GmbH, Bergisch Gladbach
CO_2 -Incubator	Binder, Tuttlingen
Flow cytometer: BD FACSDiva™, BD FACSAria™, BD FACSCalibur™, BD LSRII™	Beckton Dickinson, Heidelberg
γ-Irradiator Biobeam 2000	STS Steuerungstechnik&Strahlenschutz GmbH, Braunschweig
γ-counter	Wallac, Turku, Finland
Laminar-Flow-Bench HERAsafe	Heraeus Instruments, Hanau
Microscope	Helmut Hund GmbH, Wetzlar
Centrifuges: Biofuge fresco, Biofuge 28 RS, Megafuge 1.0R	Heraeus Instruments, Hanau

2.1.4 Staining reagents and antibodies for flow cytometry

Table 1: Antibodies and reagents used for fluorescent labelling of lymphocytes

Specificity	*Clone*	*Isotype*	*Fluorochrome/Hapten*	*Origin*
Mouse CCR9	242503	Rat IgG2b	PE	R&D Systems
Mouse CD4	GK1.5	Rat IgG2b	FITC, Alexa 405	DRFZ
	RM4-5	Rat IgG2a	FITC, PerCP, Pacific Blue	BD Biosciences
Mouse CD11c	N418	Hamster IgG	PE	DRFZ
Mouse CD16/CD32	2.4G2	Rat IgG2b	pure	DRFZ
Mouse CD25	PC6.1	Rat IgG1	APC, PerCP-Cy5.5	BD Biosciences
	7D4	Rat IgM	FITC, Biotin	BD Biosciences
Mouse CD44	IM7	Rat IgG2b	Biotin	DRFZ
Mouse CD62L / L-Selectin	MEL-14	Rat IgG2a	FITC, Biotin	DRFZ
Mouse CD103 /	M290	Rat IgG2a	PE	BD Biosciences
Integrin α_E			Alexa 647, Biotin	DRFZ
Mouse Integrin $\alpha_4\beta_7$	DAKT32	Rat IgG2a	PE, Biotin	BD Biosciences
Mouse Foxp3	FJK-16s	Rat IgG2a	FITC, PE	eBioscience
Mouse OVA TCR	KJ1.26	Rat IgG2a	Biotin, Cy5	DRFZ
BrdU	n.d.	n.d.	FITC	BD Bioscience
Biotin		Streptavidin	PE, PE-Cy7	BD Bioscience
Human IgG (H+L)	polyclonal	$F(ab')_2$ donkey	PE	Jackson
		$F(ab')_2$ mouse	Cy5	ImmunoResearch
Human IgM (H+L)	polyclonal	Rabbit	Biotin	Jackson ImmunoResearch
Mouse P-selectin ligand (P-Lig)	Fusion-protein consisting of mouse P-selectin and human IgG Fc fragment			DRFZ, BD Bioscience
Mouse E-selectin ligand (E-Lig)	Fusion-protein consisting of mouse E-selectin and human IgM Fc fragment			John Lowe, Daniel J. Campbell

2.1.5 Mice

BALB/c	Bundesinstitut für Risikobewertung, Berlin
DO11.10	Bundesinstitut für Risikobewertung, Berlin
C57BL/6	Bundesinstitut für Risikobewertung, Berlin and Forschungseinrichtung für experimentelle Medizin (FEM), Charité, Berlin
C57BL/6, thymectomised	kindly provided by L. Klein Institute of Molecular Pathology, Vienna, Austria

2.2 Methods

2.2.1 Isolation of lymphocytes from mouse tissue

Spleen and various LNs were removed from mice killed by cervical dislocation. As skin-draining pLNs cervical, mandibular (neck), brachial, axilial (arm pit) and inguinal LNs were pooled. Furthermore, gut-draining mLN and liver-draining celiac LN (livLN) were usually pooled. To obtain a single cell suspension, spleen and LNs were cut into small pieces with surgical scissors and mashed through steel or nylon sieves in HBSS/BSA using a syringe plunger. Centrifugation of cell suspensions was performed at 300 x g and 4°C for 8-10 min, unless otherwise stated. Erythrocytes were lysed by incubating the cell suspension in cold Erythrocyte-Lysing buffer for 2-3 min at room temperature (RT). Subsequently, cells were washed twice with PBS/BSA and stored on ice until further use. Lymphocytes were counted in a haemocytometer in the presence of trypanblue to exclude dead cells.

2.2.2 Isolation of lymphocyte subsets by MACS and FACS

In order to isolate specific subpopulations from the heterogeneous lymphocyte population two methods were used, both based on specific labelling of surface molecules with monoclonal antibodies (mAbs). For magnetic cell sorting (MACS) mAbs coupled to 50 nm sized paramagnetic MicroBeads (Miltenyi Biotec) were used. The labelled cell suspension was applied to a steel column in a magnetic field. Thereby magnetically labelled cells were retained on the column in the magnetic field, while unlabelled cells were washed out in the flow through. In the following, removal of the column from the magnetic field allowed elution of the magnetically labelled cells from the column (Miltenyi, et al., 1990). The actual separation was performed either manually with single-use steel columns (LS and MS columns, Miltenyi Biotec) operated by gravity flow or by using an automated separation unit (autoMACS, Miltenyi Biotec) with standardised flow rates. Separation via the autoMACS allowed further fine tuning of the separation process by using different flow rates for optimised retention of weakly labelled cells at low flow or increased purity by higher flow and sequential separation via two columns. By combining different sorting strategies of depletion/removal of unwanted cells and enrichment of wanted cells by positive selection, MACS enabled isolation of cells according to a maximum of three parameters. One major advantage of the MACS technology is the processing of large cell numbers at one time i.e. up to 2 x 10^9 lymphocytes per autoMACS separation.

For fluorescence-activated cell sorting (FACS) fluorochrome-coupled mAbs were used for labelling of the cells. Similar to analytic flow cytometric analysis, stained cells are taken up via a sample port and transported to the flow chamber where they pass a laser beam of defined wavelength. The process of hydrodynamic focussing ensures that cells are lined up one by one and pass the laser as a stream of single cells. The excited fluorochromes on the labelled cells emit light according to their distinct physical properties, which is detected by specific detectors (photomultiplier tube, PMT). After passage

through the flow chamber the stream of cells is separated into single droplets by vibration of the nozzle. Statistically, each droplet contains a single cell and subsequently receives an electric charge. These charged droplets are deflected in an electric field and sorted separately into individual sample tubes. The charge of the droplet and thereby the sorting is set according to the parameters assigned by the operator and represents the labelling of the cells with a specific fluorochrome (Goettlinger, et al., 1999; Goettlinger, et al., 1999). Thereby, labelled cells can be separated from unlabelled cells. Using the FACSAria (BD Bioscience) up to 4 different cell subsets can be sorted at the same time. This method is particularly useful in order to sort subpopulations of cells, which can only be identified by combinations of several surface markers. However, one disadvantage is the fact that only up to 1 x 10^8 cells can be sorted per hour.

By combining both methods, MACS and FACS, it was possible to isolate even very small subpopulations of lymphocytes to high purity.

2.2.2.1 Isolation of naïve $CD4^+$ T cells

Single cell suspensions from pooled LNs and spleen were obtained as described above (2.2.1). In order to block unspecific binding of mAbs to Fc receptors on lymphocytes, anti-Fcγ receptor II/III mAb was added to the cell suspension at a final concentration of 20 µg/ml. Cells were stained with anti-CD4-FITC at a cell density of 2 x 10^8 cells/ml for 10 min at 6-12° C. Unbound mAb was removed by washing the cell suspension with at least 10-fold excess volume of PBS/BSA. Subsequently cells were labelled with anti-FITC MultisortBeads (Miltenyi Biotec) at a cell density of 2 x 10^8 cells/ml for 15 min at 6-12° C. Unbound MultisortBeads were removed by washing as described above. $CD4^+$ T cells were isolated using the autoMACS (program: PosselD2). In order to remove the magnetic label Release Reagent (Miltenyi Biotec) was added to the positive fraction and incubated for 10 min at 6-12°C and 20 min at RT. The cell suspension was re-applied to the autoMACS separator (Deplete) and non-magnetic $CD4^+$ T cells were collected in the negative fraction. Subsequently $CD4^+$ T cells were labelled with anti-CD62L MicroBeads and naïve $CD4^+CD62L^{high}$ T cells were purified on the autoMACS (PosselD2). Purity of the enriched cells was confirmed by flow cytometry and was usually about 98%. Naïve $CD4^+CD62L^{high}$ T cells were used for generation of Th1 cells (2.2.4.1).

In some experiments $CD25^-CD4^+CD62L^{high}$ T cells were required. Therefore, $CD25^+$ cells were removed from the initial cell suspension by magnetic labelling with anti-CD25-APC and anti-APC MicroBeads and subsequent sensitive separation on the autoMACS (DepleteS). From the remaining $CD25^-$ fraction naïve $CD4^+CD62L^{high}$ cells were isolated as described above. $CD25^-CD4^+CD62L^{high}$ cells were used as responder cells in *in vitro* proliferation assays (2.2.4.2).

2.2.2.2 Isolation of α_E^- lymphocytes for adoptive transfer

Single cell suspensions from lymphoid organs of DO11.10 mice were labelled with anti-α_E-PE and anti-PE MicroBeads and α_E^+ cells were depleted using the sensitive mode of the autoMACS (Deplete05). The α_E^- fraction was collected and labelled with biotinylated anti-CD25 and anti-Biotin MicroBeads. In the next step the cell suspension was separated into $CD25^+$ and $CD25^-$ cells on the autoMACS (DepleteS). While the negative fraction was used for isolation of naïve $CD25^-$ cells using anti-CD62L MicroBeads, the positive fraction was re-applied to the autoMACS (PosselD2) in order to improve the purity of $CD25^+$ Tregs. For adoptive transfer cells were not purified according to CD4 expression in order to minimise sample handling and cell loss. The actual number of transferred $CD4^+$ T cells was calculated from the total transferred lymphocyte count according to the frequency of $CD4^+$ T cells measured by flow cytometry. For adoptive transfer sorted cells were washed and resuspended in PBS. Purified $\alpha_E^-CD25^+$ Tregs and $\alpha_E^-CD25^-CD62L^{high}$ cells from DO11.10 mice were used for antigen-specific activation *in vivo* (2.2.5.3).

2.2.2.3 Isolation of naïve $CD4^+$ T cell subsets for homing receptor induction *in vitro*

Naïve $CD4^+CD25^+$ Tregs were isolated by a combination of MACS and FACS sorting from cell suspensions of pooled LNs and spleens from DO11.10, BALB/c or C57Bl/6 mice. Single cell suspensions were prepared as described in 2.2.1 and labelled with anti-CD25-FITC and anti-FITC MicroBeads followed by magnetic pre-enrichment of $CD25^+$ T cells using the autoMACS (PosselS). For isolation of naïve $CD4^+CD25^+$ Tregs, the positive fraction was stained with anti-CD4-PerCP and anti-CD62L-APC and $CD4^+CD25^+CD62L^{high}$ Tregs were sorted by FACS (FACSAria) to a purity of >96%. Sorted cells were resuspended in complete medium and used for *in vitro* culture under various conditions (2.2.4.3 and 2.2.4.4).

In some experiments $CD4^+CD25^-CD62L^{high}$ T cells were also sorted from the remaining cell suspension. Therefore, the negative fraction of the magnetic $CD25^+$ enrichment was labelled with anti-CD4 MicroBeads and $CD4^+CD25^-$ T cells were isolated by autoMACS separation (PosselD2). The positive fraction was stained with anti-CD4-PerCP and anti-CD62L-APC and purified by FACS sorting into $CD4^+CD25^-CD62L^{high}$ T cells. Sorted cells were resuspended in complete medium and used for *in vitro* culture under various conditions (2.2.4.3 and 2.2.4.4).

2.2.2.4 Isolation of tissue-specific DCs for homing receptor induction *in vitro*

Tissue-specific dendritic cells (DC) were isolated from pLNs and mLNs, respectively. LNs were cut into pieces and incubated in medium containing 1 mg/ml Collagenase D (Roche Diagnostics) and 50 µg/ml DNaseI (Sigma Aldrich) for 30 minutes at 37°C. Subsequently, the digested tissue was passed through 70 µm cell strainers into PBS/BSA and cells were washed twice with PBS/BSA. DCs were labelled with anti-CD11c MicroBeads (Miltenyi Biotec) and magnetically enriched on two

consecutive columns (combination of LS and MS or autoMACS "PosselD2"). Purity of $CD11c^+$ DCs was confirmed by flow cytometric analysis and was typically around 80%. Isolated DCs were washed and resuspended in complete medium. Tissue-specific $CD11c^+$ DCs were used for induction of organ-specific homing receptors on naïve $CD4^+CD25^+$ Tregs *in vitro* (2.2.4.3).

2.2.3 Flow cytometric analysis of lymphocytes

Lymphocytes stained with fluorochrome-labelled Abs as described in 2.2.3.1 were analysed on FACSCalibur or LSRII flow cytometers (BD Bioscience). Multicolour analysis and phenotyping was almost exclusively done on the LSRII, which is equipped with 3 lasers (violet diode 405 nm, Argon 488 nm, HeNe 633 nm) and allows simultaneous detection of 8 fluorescent parameters in addition to forward scatter (FCS) and side scatter (SSC) signals (Tabel 2).

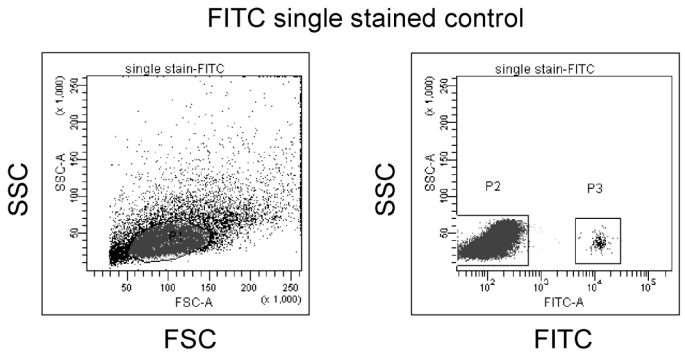
Tabel 2: Configuration of LSRII flow cytometer (laser, optical filter set up and parameters)

LSRII	*Longpass filter (nm)*	*Bandpass filter (nm)*	*Parameter/Fluorochrome*
Blue Laser (488nm)	735	780/60	PE-Cy7
	685	695/40	PerCP-Cy5.5
	635	670/14	PerCP, PE-Cy5
	550	575/26	PE
	505	539/30	FITC
	-	488/10	SSC
Red Laser (633 nm)	735	780/60	APC-Cy7
	-	660/20	APC, Alexa 647, Cy5
Violet Laser (405 nm)	505	525/50	DAPI
	-	444/40	Alexa 405, Pacific Blue

Combination of many fluorochromes requires careful compensation in order to minimise spectral overlap into neighbouring channels. The Diva Software is operated in digital mode, which allows compensation and potential correction of already acquired data. In order to set up a compensation matrix, single stained samples were prepared with each of the fluorochromes used in the respective experiment. Unstained cells and the single stained compensation samples were run on the instrument and compensation was either performed by using the automatic compensation tool of the Diva software or manually by the following method: the fluorochrome of interest was depicted in a dot plot vs. SSC. Regions were drawn on unstained and positively stained cells, respectively (Figure 2a). Using the statistics tool, the medians of the two populations were retrieved for all other fluorescent channels (Figure 2b). Spectral overlap was corrected in the compensation matrix until the median of the stained cells corresponded to the median of the unstained cells in the respective channel (Figure

2c). This was repeated for all other fluorochromes and the achieved compensation values combined for the multicolour acquisition of the actual stained samples and the appropriate controls.

a



b before compensation

Experiment Name: Exp129 260106_spleen
Specimen Name: single stain
Tube Name: FITC
Record Date: Mar 8, 2006 4:50:38 PM

Population	#Events	%Parent	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	PE-Cy7-A Median	A647-A Median
P1	100,529	80.4	121	134	79	33	36
P2	100,352	99.8	121	134	78	33	36
P3	162	0.2	12,245	5,073	622	186	33

c after compensation

Experiment Name: Exp129 260106_spleen
Specimen Name: single stain
Tube Name: FITC
Record Date: Mar 8, 2006 4:50:38 PM

Population	#Events	%Parent	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	PE-Cy7-A Median	A647-A Median
P1	100,529	80.4	118	84	64	3	33
P2	100,354	99.8	118	84	64	3	33
P3	162	0.2	12,244	86	65	4	23

Figure 2: Manual compensation of single stained samples using Diva software on LSRII

In order to set up proper compensation single stained samples of each fluorochrome were run on the LSRII. Each sample was depicted separately and spectral overlap into other channels corrected based on the median fluorescence intensity of unstained vs. stained cell subsets. (a) Representative dot plots of a FITC single stained sample. Lymphocytes were gated according to FSC and SSC properties and the gated cells were depicted in a new dot plot displaying SSC vs. FITC. Regions were drawn on unstained (P2) and stained (P3) populations, respectively. (b) Statistics were generated from the dot plot shown in (a) displaying the median of unstained and stained cells in all fluorescent channels. (c) Compensation was applied to each channel until the median of stained cells reached the median of unstained cells in each channel.

Analysis of the data files was done using the CellQuest Software (BD Bioscience). Fluorescent minus one controls (FMOC) were used in order to set the appropriate gate for the analysis of the stained samples (Roederer, 2001). FMOCs contained all fluorochrome-labelled antibodies except one, so that the spectral background/influence of expression of any other marker could be evaluated for the parameter of interest. Results of the FACS analysis were expressed either as frequency of positive cells of the marker of interest or as the geometric mean fluorescent intensity (GMFI) of the marker of interest.

2.2.3.1 Fluorescent labelling of surface molecules on lymphocytes

Lymphocytes were phenotyped by labelling with fluorochrome-coupled Abs directed against specific surface molecules followed by flow cytometric analysis of the stained cells. Single cell suspensions prepared as described in 2.2.1 or cultured cells were resuspended in 50 – 100 µl of PBS/BSA. In order to block unspecific binding of mAbs to Fc receptors on lymphocytes anti-Fcγ receptor II/III mAb was added to the cell suspension at a final concentration of 20 µg/ml. Antibodies directed against surface molecules were added at previously pre-determined titers and the suspension was incubated for 10 to 20 minutes at 6-12° C. Most of the Abs were directly coupled to fluorochrome molecules (Table 1), those conjugated to biotin were detected by fluorochrome-labelled streptavidin as secondary reagent. For staining of functional P-selectin ligands (P-Lig) and E-selectin ligands (E-Lig) on lymphocytes, fusion-proteins consisting of mouse P-selectin and human IgG heavy chain or mouse E-selectin and human IgM heavy chain were used (kindly provided by D. Vestweber and J. Lowe, respectively). Importantly, binding of the chimera depended on the presence of Mg^{2+} and Ca^{2+} and therefore the complete staining procedure was performed in HBSS/BSA. Binding of P-selectin was detected by fluorochrome-labelled anti-human IgG (Jackson ImmunoResearch) and binding of E-selectin by biotinylated anti-human IgM (Jackson ImmunoResearch) and fluorochrome-labelled streptavidin. Unbound Abs and secondary reagents were removed by washing the cell suspension with at least 10-fold excess volume of PBS/BSA or HBSS/BSA. Cells were then analysed by flow cytometry and DAPI or PI were added directly before acquisition to exclude dead cells.

2.2.3.2 Intracellular labelling of Foxp3

Intracellular staining for Foxp3 was performed using the anti-rat/mouse Foxp3 staining set from eBioscience, containing all required buffers and fluorochrome-labelled anti-Foxp3 mAb. Staining was done according to the manufacturer's protocol with minor modifications. Briefly, upon staining for surface molecules as described above, up to 5 x 10^6 cells were resuspended in 500 µl eBioscience fixation buffer and incubated for 1 to 24 h at 6-12° C. The cell suspension was centrifuged and the cell pellet washed once with 1 ml of eBioscience permeabilisation solution. In order to block unspecific binding, cells were resuspended in 25 µl eBioscience permeabilisation solution containing rat IgG (Jackson ImmunoResearch) at a final concentration of 10 µg/ml. After 15 min at 6-12° C, 25 µl of fluorochrome-labelled anti-Foxp3 mAb diluted in eBioscience permeabilisation solution were added to a final concentration of 2 µg/ml mAb and the cells were incubated for another 30 min at 6-12° C. Unbound mAb was removed by washing the cell suspension with 1 ml of eBioscience permeabilisation solution. Cells were resuspended in PBS/BSA and analysed by flow cytometry.

2.2.3.3 Intracellular staining of BrdU

Incorporation of Bromodeoxyuridine (BrdU) (2.2.5.1) was detected by using the BrdU Flow Kit (BD Bioscience) according to the manufacturer's instructions with minor modifications. All reagents required for intracellular staining were supplied in the Kit. Surface staining was performed on single cell suspensions as described above (2.2.3.1) and 1 - 10 x 10^6 cells were fixed in 200 µl of BD Cytofix/Cytoperm buffer for 20 min at RT. Cells were washed with 1 ml of BD Perm/Wash buffer. Fixed cells were resuspended in 100 µl of BD Cytoperm Plus buffer for 10 min on ice. The suspension was washed with 1 ml of BD Perm/Wash buffer and fixed again in 100 µl of BD Cytofix/Cytoperm buffer for 5 min at RT. Following another wash with 1 ml of BD Perm/Wash buffer the cells were resuspended in 65 µl of DNAseI solution (300 µg/ml) and incubated for 1 h at 37° C, in order to make the DNA accessible for detection of incorporated BrdU by a specific mAb. The incubation was stopped by washing with 1 ml of BD Perm/Wash buffer. Finally, intracellular staining was performed with FITC-labelled anti-BrdU mAb diluted 1 to 50 in BD Perm/Wash buffer for 20 min at RT. Unbound mAb was removed by washing with 1 ml of BD Perm/Wash buffer, cells were resuspended in PBS/BSA and analysed by flow cytometry. In some experiments BrdU detection was combined with intracellular staining for Foxp3. Therefore, PE-labelled anti-Foxp3 mAb (clone FJK-16s, eBioscience) was added to the staining solution containing anti-BrdU mAb and labelling was performed as described.

2.2.3.4 Enumeration of lymphocyte numbers by flow cytometry

In experiments with large sample numbers a flow cytometric-based method with Fluosbrite microspheres (20 µm, Polyscience) as counting beads was used in order to quantify lymphocyte numbers. The concentration of the Fluosbrite microspheres stock solution was determined by counting in a haemocytometer and defined amounts were then added to the lymphocyte samples. Single cell suspensions from various lymphoid organs were prepared as described in 2.2.1. After washing cell pellets were resuspended in a defined volume (200 µl – 2 ml) of PBS/BSA. In the following a defined aliquot was removed for enumeration and mixed with an equal volume of Fluosbrite microspheres. Shortly before flow cytometric analysis of these mixtures fluorochrome-labelled CD4 mAb was added. Without washing the mixed suspension was measured and at least 3000 fluorescent beads were acquired. Due to their high SCC properties Fluosbrite microspheres do not interfere with lymphocyte scatter signals. This allows direct correlation of the event number in a lymphocyte region and the event number in the Fluosbrite bead region (Figure 3). The cell number was calculated according to the events in a lymphocyte and/or CD4 gate relative to the known number of fluorescent beads using the following equation:

$$c_{lymph} = n_{lymph} / n_{Fluosbrite} \times c_{Fluosbrite}$$

$c_{Fluosbrite}$ = concentration of Fluosbrite microspheres determined by counting in haemocytometer

c_{lymph} = concentration of lymphocytes calculated using Fluosbrite microspheres

$n_{Fluosbrite}$ = event count of Fluosbrite microspheres determined by flow cytometry

n_{lymph} = event count of lymphocytes determined by flow cytometry

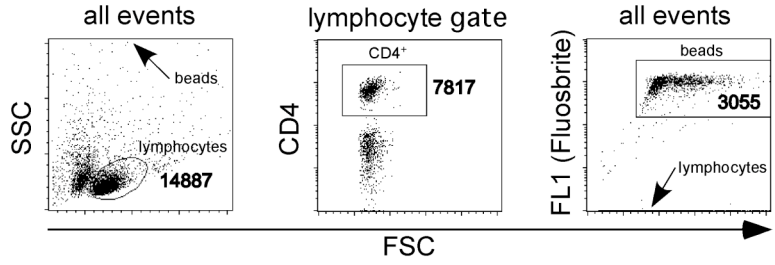


Figure 3: Enumeration of lymphocytes by flow cytometry using fluorescent beads

The first dot plot shows all acquired events. A region was drawn on lymphocytes according to known FSC and SSC properties. The second dot plot shows CD4 staining of all lymphocytes gated as shown in the first dot plot. A region was drawn on $CD4^+$ lymphocytes. The third dot plot depicts the Fluosbrite microspheres detected in channel FL1, a region was drawn in order to calculate the event number. Numbers in all dot plots indicate the event number in the respective gates. Due to the differential SSC properties, lymphocytes and Fluosbrite microspheres (beads) cannot be properly displayed in the same dot plot.

2.2.3.5 Labelling of T cells with CFSE

In order to follow proliferation of T cells both *in vivo* and *in vitro* freshly isolated T cells were labelled with the amin-reactive cell tracker dye 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE). This compound is taken up by cells and cleaved by endogenous cytoplasmic esterases, which enables covalent binding of the succinimidyl-group of the dye to amin-residues of intracellular proteins. During cell division the CFSE-labelled proteins are evenly distributed between the two daughter cells leading to a reduction of fluorescence intensity of individual cells by a factor of two. This loss of fluorescence intensity can be analysed by flow cytometry and allows identification of single generations of proliferating cells.

For labelling, T cells were washed twice in PBS and adjusted to 2 x 10^7 cells/ml in PBS. An equal volume of CFSE solution (5 nM in PBS) was added to the cell suspension and incubated for 3 min at RT. Washing with at least 10-fold excess volume of complete medium or PBS/BSA terminated the reaction. Labelled cells were subsequently activated *in vitro* by various procedures or adoptively transferred and activated *in vivo*. Analysis of proliferation was done by flow cytometry of cultured cells or re-isolated *ex vivo* cells 3-7 days after labelling. Loss of CFSE intensity was expressed as geometric mean fluorescence intensity (GMFI).

2.2.4 *In vitro* culture and activation of T cells

T cells were cultured in complete medium in a humidified CO_2- Incubator at 37° C.

2.2.4.1 Generation of Th1 cells

Th1 cells were generated by antigen-specific activation of naïve $CD4^+CD62L^{high}$ T cells (2.2.2.1) from DO11.10 mice. This mouse strain contains T cells with transgenic (tg) TCRs specific for the chicken ovalbumin$_{323-339}$ (OVA_{323-33}) peptide (Murphy, et al., 1990). Naïve DO11.10 T cells were activated under Th1 polarising conditions by co-culture with peptide-loaded antigen presenting cells (APCs). As APCs $CD90^-$ lymphocytes were prepared from spleen and LNs using anti-CD90 MicroBeads and autoMACS separation and were subsequently irradiated (γ-irradiation, 30 Gy). Sorted T cells were adjusted to 2 x 10^6 cells/ml in complete medium and activated at a ratio of 1 to 3 with $CD90^-$ APCs, together with 1 µg/ml $OVA_{323-339}$ peptide, anti-IL-4 (5 µg/ml), IFN-γ (20 ng/ml) and IL-12 (5 ng/ml). The cultures were diluted by a factor of 1 to 3 in fresh medium on day 3 and transferred to new plates. On day 6 of the culture, cells were harvested and dead cells removed by carefully layering the cell suspension on Nycodenz solution (17.1% isotonic Nycodenz, Nyegaard) and subsequent density-gradient centrifugation at 780 x g for 10 min at RT. Live cells were collected from the interface and washed twice with PBS/BSA. Th1 cells were used for the induction of a DTH reaction (2.2.5.4).

2.2.4.2 *In vitro* proliferation assay

In order to assess the functional activity of Tregs, co-culture assays with naïve CFSE-labelled $CD4^+CD25^-$ responder cells were performed. Suppressive capacity of the Treg fraction was measured indirectly by following the proliferation of the responder cells using flow cytometry.
Naïve $CD4^+CD25^-CD62L^{high}$ responder T cells were isolated and labelled with CFSE as described above (2.2.2.1 and 2.2.3.5). APCs were prepared by depletion of $CD90^+$ cells from $CD4^-$ fractions using anti-CD90 MicroBeads and were irradiated (30 Gy) prior to culture. Unlabelled Tregs were mixed with CFSE-labelled responder cells at different ratios (1:2 – 1:50). A total of 8 – 10 x 10^4 T cells were cultured with APC at a 1:2 ratio in 96-well U-bottom plates with addition of anti-CD3 mAb (1 µg/ml) for 3 days in triplicates. After incubation, cells were collected and analysed by FACS. To exclude dead cells, PI was added immediately before acquisition. Proliferation analysis is based on the CFSE GMFI of total $CFSE^+$ T cells.

2.2.4.3 Induction of homing receptors on Tregs by tissue-specific DCs

For induction of organ-specific homing receptor expression naïve $CD4^+CD25^+$ Tregs were antigen-specifically activated *in vitro* in the presence of tissue-specific DCs. 2 x 10^5 isolated Tregs (2.2.2.3) from DO11.10 mice were cultured for 4 days in 96-well U-bottom plates together with 2 x 10^4 $CD11c^+$ DCs (2.2.2.4) in the presence of 1 µg/ml $OVA_{323-339}$ peptide and 40 ng/ml recombinant IL-2 (R&D

Systems) in complete medium. Fresh medium containing IL-2 was added to the cultures on day 3 and proliferating cells were diluted by a factor of 1 to 2. After incubation, cells were collected and analysed for homing receptor expression by FACS.

2.2.4.4 Induction of homing receptors on Tregs by soluble factors

Naïve $CD4^+CD25^+$ Tregs were isolated as described above (2.2.2.3) and stimulated on anti-CD3 (6 µg/ml) and anti-CD28 (4 µg/ml) coated plates together with IL-2 (40 ng/ml). Cultures were performed either under neutral (anti-IL-12, 5 µg/ml) or under polarising conditions with RA (100 nM), anti-IL-12 (5 µg/ml) and IL-12 (5 ng/ml), respectively. On day 3 of the culture, Tregs were removed from the mAb-coated plates, transferred to fresh wells and rested for another 2-3 days before analysis of homing receptor expression by FACS. In some experiments naïve $CD4^+CD25^-$ $CD62L^{high}$ T cells were stimulated under neutral or polarising conditions as described above, except that no exogenous IL-2 was added. Cell culture and analysis were performed as described above.

2.2.5 *In vivo* experiments and adoptive transfer

Mice were kept under specific-pathogen free (SPF) conditions, unless otherwise stated. Killing was carried out by cervical dislocation under authorisation of Genehmigungs-Nr. T0085/99 and experiments with live animals were performed according to Tierversuchsgenehmigung G0202/03.

2.2.5.1 *In vivo* labelling with BrdU

In order to follow proliferation of Tregs *in vivo,* BrdU (Sigma Aldrich) was added to the drinking water of mice at 1 mg/ml and changed every 2-3 days. The thymidin analog incorporates during cell division into newly synthesised DNA. This allows specific labelling of cells that have proliferated during the period of BrdU administration (Gratzner and Leif, 1981; Miltenburger, et al., 1987; Sasaki, et al., 1989). After 10 days of BrdU feeding, mice were sacrificed and cell suspensions were prepared from different lymphoid organs. Cell surface staining was performed as described in 2.2.3.1. Incorporation of BrdU was detected by using the BrdU Flow Kit (BD Bioscience) according the procedure described above (2.2.3.3).

To discriminate thymic from peripheral proliferation, thymectomised animals were used in initial experiments. In later experiments discrimination of thymic and peripheral proliferation was based on differential BrdU staining intensity. According to an observation reported by Sprent and Tough only peripheral proliferation of T cells led to high BrdU and high CD44 staining intensities, while T cells that had proliferated in the thymus showed lower BrdU and CD44 staining intensities. Consequently, this population was found missing in mice that had been thymectomised prior to BrdU administration ((Sprent and Tough, 1994) and Figure 4). Furthermore, evaluation of thymic tissue for BrdU incorporation confirmed that the specific microenvironment of the thymus resulted in low BrdU

staining intensities (data not shown). Based on these findings, the frequency of $BrdU^{high}$ cells was taken as a measure for peripheral proliferation of the T cells subsets of interest.

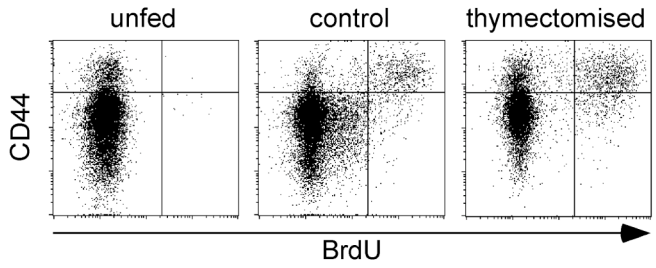


Figure 4: Evaluation of peripheral proliferation by differential BrdU staining intensity

Differential staining properties of T cells following *in vivo* labelling with BrdU and detection of incorporated BrdU by intracellular staining with anti-BrdU mAb were confirmed in an initial test experiment. Control C57BL/6 mice or thymectomised C57BL/6 mice were given BrdU in the drinking water for a period of 10 days. Staining for surface molecules (CD4, CD44) and incorporated BrdU was performed as described in 2.2.3.1 and 2.2.3.3. Lymphocytes obtained from a mouse that had not received BrdU (unfed) were subjected to the same staining protocol and used for determination of BrdU staining specificity. Dot plots show staining of CD44 vs. BrdU of CD4-gated LN cells from non-thymectomised mice (unfed and control) and a thymectomised mouse.

2.2.5.2 Depletion of commensal microflora

In some experiments commensal microflora of C57BL/6 mice was depleted. Treatment was done in collaboration with M.M. Heimesaat and O. Liesenfeld (Charité, Berlin) according to a standard protocol with minor modifications (Rakoff-Nahoum, et al., 2004). Groups of mice were given a cocktail of antibiotics (ampicillin, 1 g/L, Ratiopharm; vancomycin, 500 mg/L, Cell Pharm; ciprofloxacin, 200 mg/L, Bayer Vital; imipenem, 250 mg/L, MSD; metronidazole, 1 g/L, Fresenius) added to the drinking water *ad libitum* for 8 weeks. Mice were housed in sterile cages, which were changed daily for the first two weeks. Efficient bacterial eradication was monitored by weekly bacteriologic evaluation of faeces from individual mice. Lack of cultivable bacteria (Gram-negative rods, Gram-positive rods, Gram-positive cocci, aerobic and anaerobic cultures) for at least two consecutive weeks was taken as indication of complete removal of commensal microflora as described by Heimesaat et al. (Heimesaat, et al., 2006). These gnotobiotic mice were used for *in vivo* labelling with BrdU as described above (2.2.5.1).

2.2.5.3 Antigen-specific activation *in vivo*

In order to follow the induction of Foxp3 and α_E expression upon antigen-specific activation *in vivo,* a model of adoptive transfer was applied. α_E^- cells from DO11.10 mice were isolated, labelled with CFSE and transferred into BALB/c recipient mice. Transferred cells were activated by administration of OVA protein and analysed 5 - 7 days later by flow cytometry. This allowed analysis of phenotypic changes on the single cell level in correlation to proliferation.

Cells were sorted and labelled with CFSE as described above (2.2.2.2 and 2.2.3.5). 4 – 6 x 10^6 $\alpha_E^-CD25^-CD62L^{high}$ or 6 – 9 x 10^5 $\alpha_E^-CD25^+$ cells were injected in a volume of 200 µl PBS i.v. into the tail vein of BALB/c mice. On the next day, OVA protein (Sigma Aldrich) was administered for antigen-specific activation *in vivo*. The tolerogenic protocol involved continuous feeding of OVA protein at various doses (0.5 – 20 mg/ml) in the drinking water for 6 days and preparation of lymphoid organs on day 7. For immunogenic antigen administration recipient mice were injected once with 200 µl PBS containing 500 µg of OVA protein and 70 µg LPS (Sigma Aldrich) i.p. and mice were sacrificed on day 5. Cell surface staining was performed on single cell suspensions from different lymphoid organs, followed by fixation, permeabilisation and intracellular staining for Foxp3 as described above (2.2.3.2). Cells were analysed by flow cytometry and transferred tg cells were identified by gating on $CD4^+$ and $KJ1.26^+$ cells.

2.2.5.4 Th1-mediated DTH model

This model of an acute skin inflammation is based on the adoptive transfer of antigen-specific Th1 cells into recipient mice. One day after transfer, the DTH reaction in the footpad is triggered by s.c. injection of the cognate antigen. This leads to an acute local inflammation, which can be followed by measuring the swelling of the foot over time. The peak of the swelling/inflammation occurs 24 h after antigen application and slowly declines over a period of 2 weeks (Feuerer, et al., 2006; Siegmund, et al., 2005).

Th1 cells were generated from OVA-specific TCR-tg $CD4^+$ T cells as described above (2.2.4.1). 5 x 10^5 Th1 cells were injected i.v. into naïve BALB/c mice, and 24 h later the DTH response was induced by s.c. injection of 250 ng $OVA_{323-339}$ peptide together with incomplete Freud's Adjuvant (IFA, Sigma Aldrich) into the left footpad. As a control the right footpad was injected with PBS/IFA. The course of the swelling was followed over 2 weeks by measuring the thickness of the footpad daily using a calliper. Measurements were performed in blinded fashion. This model was used for evaluation of skin-specific migration (2.2.5.5) and *in vivo* suppressive capacity of cultured Tregs. For analysis of Treg function, Tregs cultured under various conditions (2.2.4.4) were co-transferred together with Th1 cells at a ratio of 1:2. For analysis of Treg migration DTH mice were used on day 1 after s.c. antigen application.

2.2.5.5 Homing of adoptively transferred Tregs

In order to analyse the migratory behaviour of Tregs *in vivo*, they were radioactively labelled and adoptively transferred into recipient mice. After 24 h mice were sacrificed and the distribution of transferred Tregs was analysed by detection of radioactivity in various organs. In order to generate a specific target site for migration into inflamed skin, recipient mice suffering from an acute skin-inflammation were used (Th1-mediated DTH model). Recipient mice had received Th1 cells and

antigen as described in 2.2.5.4 and were used for homing assays on day 1, at the peak of inflammation. Tregs cultured as described above (2.2.4.4) were labelled with ^{111}In (^{111}Indiumoxin; 10 µCi/ml) (Amersham Biosciences) at a cell density of 0.5 - 1x10^8 cells/ml in FCS-free medium for 20 min at RT. Labelling was terminated by washing twice with complete medium, followed by 1 h incubation at 37°C in fresh medium and removal of dead cells by Nycodenz gradient centrifugation. Labelled cells were injected i.v. into BALB/c mice, which suffered from a DTH reaction in the footpad. In all experiments a minimum of 10^4 cpm was injected. Mice were sacrificed 24 h after transfer of labelled cells and the distribution of radioactivity in different organs, serum and the remaining body was measured in a γ-counter (Wallac Counter). Homing assays were performed in cooperation with Uta Lauer, Kerstin Siegmund and Astrid Menning (Exp. Rheumatology, Charité, Berlin).

2.2.5.6 Data management and statistical analysis

The results obtained in this study were either represented as single values or as mean ± standard deviation (SD). Since normal distribution could not be assumed for the collected data, for statistical analyses non-parametric tests were used to determine significant differences. For paired observations the Wilcoxon signed rank test and for unpaired observations the Mann Whitney test were used, respectively. In order to allow analysis of data, where the sample size was lower than the minimally required number of observations for non-parametric tests, the Student's *t* test was used instead. Differences were considered statistically significant, when $p < 0.05$ and highly significant when $p < 0.01$. Statistical analysis was done using GraphPad Prism and SPSS software. Help with statistical analyses was kindly provided by D. Hüscher (DRFZ, Berlin).

3 Results

3.1 Origin and development of Tregs

This part of the project was initiated in order to analyse the relationship between various subsets of $CD4^+$ Tregs. Of particular interest were Treg subpopulations characterised by the expression of the integrin $\alpha_E\beta_7$. Here, conditions leading to the generation of effector/memory α_E^+ Tregs *in vivo* were defined and their cellular precursors as well as the sites, at which generation and expansion take place, were investigated.

3.1.1 Phenotype and *in vivo* proliferation of effector/memory-like Tregs

3.1.1.1 α_E-expression on $CD4^+Foxp3^+$ Tregs correlates with an effector/memory phenotype

Previous results from our group identified a close link between expression of α_E on naturally occurring Tregs and expression of adhesion molecules and activation markers associated with an effector/memory phenotype. At the same time RT-PCR results demonstrated similar levels of Foxp3 mRNA among α_E/CD25 Treg subsets, however a direct correlation on the single cell level was not demonstrated (Huehn, et al., 2004). Therefore, in the study presented here, the marker profile of $CD4^+Foxp3^+$ Tregs from the spleen of C57BL/6 mice was analysed in order to assess the phenotype of α_E^+ and α_E^- Treg subsets. $\alpha_E^-CD25^+$ cells contained 78.4 ± 7.7%, $\alpha_E^+CD25^+$ cells 88.2 ± 6.3% and $\alpha_E^+CD25^-$ cells 58 ± 9.6% $Foxp3^+$ cells, respectively (mean ± SD, n=17). Investigation of the co-expression of Foxp3 and CD62L or CD44 confirmed, that α_E-expression on $CD4^+Foxp3^+$ Treg subsets was tightly linked to an effector/memory-like phenotype. The majority of $\alpha_E^-CD25^+Foxp3^+$ Tregs was positive for CD62L and expressed only low levels of CD44, which corresponded to their previously described naive-like phenotype. In contrast, both $\alpha_E^+Foxp3^+$ Treg subsets expressed much higher levels of CD44 and lower amounts of CD62L, indicating a higher activation/differentiation state. These results confirmed the initial observation that α_E-expression correlates with an effector/memory-like phenotype on the single cell level for $CD4^+Foxp3^+$ Tregs.

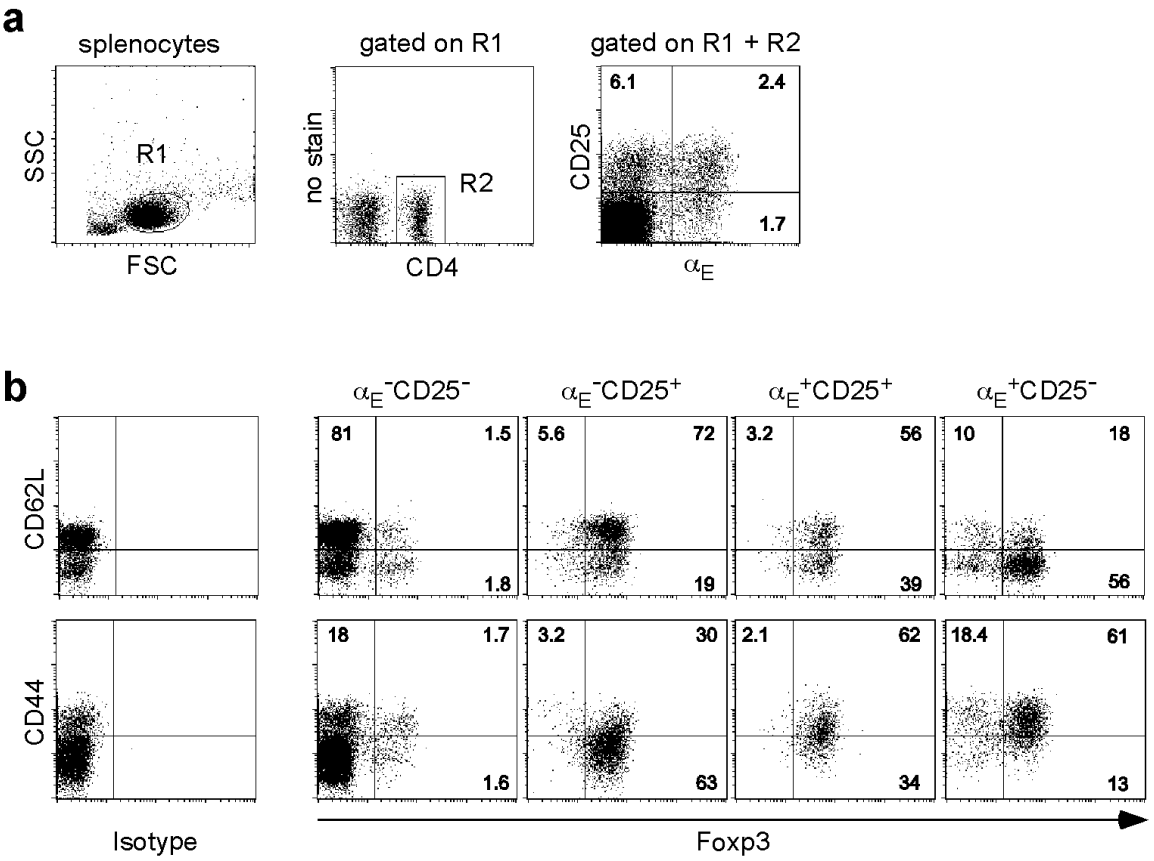


Figure 5: Effector/memory phenotype of CD4$^+$Foxp3$^+$ cells segregates with expression of α_E

Lymphocytes were isolated from peripheral lymphoid organs of mice and stained with fluorochrome-labelled antibodies to CD4, CD25, CD62L, CD44 and α_E. Cells were subsequently fixed and permeabilised for intracellular staining of Foxp3 followed by flow cytometric analysis. (a) The left dot plot shows FCS vs. SSC of all acquired events. A region (R1) was drawn on lymphocytes according to their known scatter properties excluding debris and doublets from further analysis. The middle dot plot represents CD4 staining of cells gated according to the lymphocyte region R1. A new region (R2) was drawn on all CD4$^+$ lymphocytes. The right dot plot shows staining of CD25 vs. α_E of gated CD4$^+$ lymphocytes (combined lymphocyte (R1) and CD4$^+$ (R2) gate). The quadrant subdivides the gated CD4$^+$ lymphocytes according to expression of the respective markers. (b) Flow cytometric analysis of spleen cells from C57BL/6 mice for the expression of Foxp3 and CD62L or CD44 on indicated CD4$^+$ Treg subsets. CD4$^+$ lymphocytes were gated according to expression of α_E and CD25 as shown in (a) and analysed for indicated markers. Numbers show frequency within respective quadrants. Dot plots are representative of at least two independent experiments with 2-3 individual mice each.

3.1.1.2 α_E^+ Tregs show high proliferation under homeostatic conditions

Based on the previous observation that α_E^+ Tregs harbour reduced T cell receptor excision circle (TREC) numbers, indicative of increased proliferative history (Huehn, et al., 2004), the following experiments addressed the question whether this is the consequence of peripheral proliferation. To exclude any impact of thymic proliferation, adult-thymectomised mice were used for the evaluation of peripheral proliferation by BrdU incorporation. Four weeks after thymectomy C57BL/6 mice were given BrdU in their drinking water for a period of 10 days. Mice were sacrificed on day 10 of BrdU-feeding and spleen and LNs were evaluated for BrdU$^+$ cells. Incorporation of BrdU was analysed by flow cytometry. Single cell suspensions from various lymphoid organs were stained for the expression

of surface molecules, followed by fixation and permeabilisation as described in 2.2.2. BrdU was detected using a specific mAb.

Figure 6 shows representative staining of incorporated BrdU among gated $CD4^+$ α_E/CD25 subsets from pLN cells. High frequencies of $BrdU^+$ cells were detected in all three Treg subsets, while the predominantly naïve fraction of $\alpha_E^-CD25^-CD4^+$ T cells showed only low frequencies of $BrdU^+$ cells (Figure 6a). Within the Treg subsets $\alpha_E^+CD25^-$ cells showed the highest frequency of BrdU-labelled cells in all of the tested lymphoid organs (Figure 6), indicating that a substantial number of cells had proliferated within the 10 days of BrdU administration. $\alpha_E^+CD25^+$ cells showed only slightly reduced frequencies of $BrdU^+$ cells compared to $\alpha_E^+CD25^-$ cells and the lowest frequency was detected among the $\alpha_E^-CD25^+$ Tregs.

These data correspond to the phenotype of the respective Treg subsets (Figure 5) and to the previous observation that α_E^+ Treg subsets have reduced TREC numbers, suggesting, that indeed these cells display a high degree of peripheral proliferation under steady state conditions.

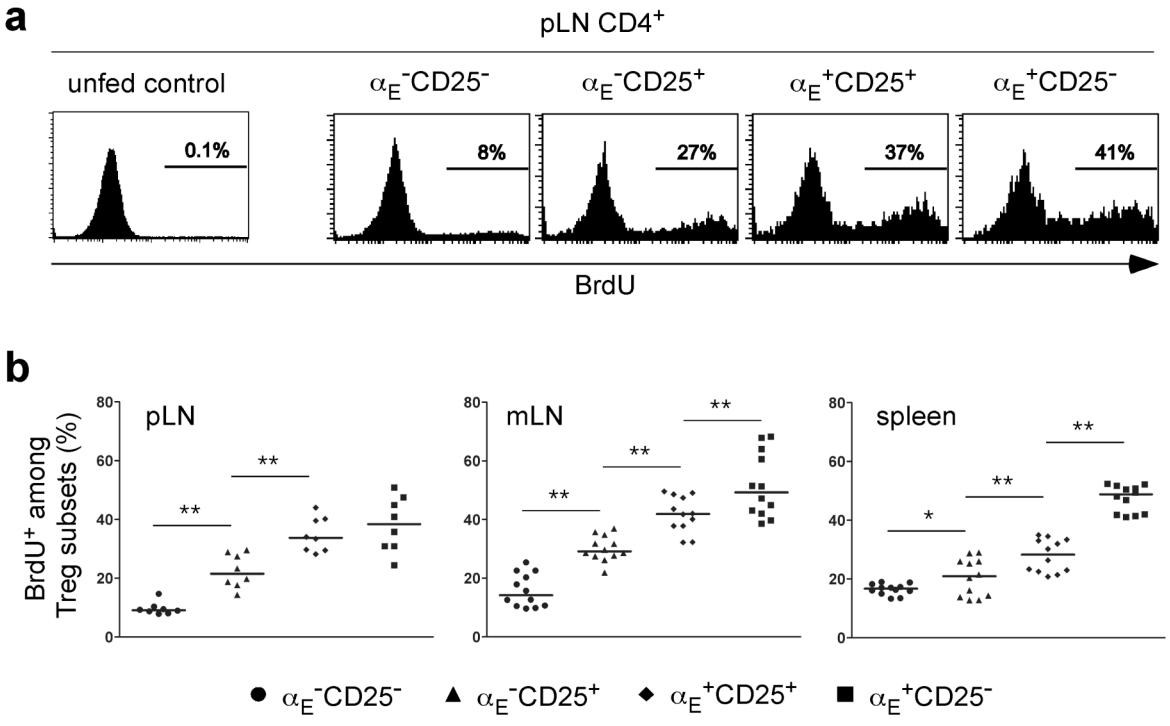


Figure 6: Evaluation of *in vivo* proliferation by incorporation of BrdU

Adult-thymectomised C57BL/6 mice were given BrdU in the drinking water for a period of 10 days. Single cell suspensions were prepared from lymphoid organs and stained for expression of CD4, CD25 and α_E. Incorporation of BrdU was detected by intracellular staining with anti-BrdU and subsequent flow cytometric analysis. Cells were gated according to the expression of CD4 and α_E/CD25 subsets as shown in Figure 5 and analysed for the frequency of $BrdU^+$ cells. (a) Control staining for BrdU of gated $CD4^+$ T cells isolated from pLN of a control mouse, which did not receive BrdU in the drinking water (unfed control). Representative staining for BrdU of gated $CD4^+$ α_E/CD25 subsets from pLN of mice after 10 days of continuous BrdU feeding (right pannel). (b) Data summarise the frequency of $BrdU^+$ cells among Treg subsets for the indicated lymphoid tissues. Data are pooled from two independent experiments and single data points represent individual mice, lines indicate the median. $*p < 0.05$; $**p < 0.01$.

3.1.1.3 α_E^+ Treg numbers are stable in the absence of thymic output

During analysis of the relative distribution of α_E/CD25 Treg subsets highly increased frequencies of α_E-expressing Tregs in thymectomised mice compared to non-thymectomised control mice were observed (Figure 7a). In addition, calculation of the total cell number according to the method described in 2.2.3.4. revealed that the number of total $CD4^+$ T cells was reduced in thymectomised animals (Figure 7b), presumably due to the lack of thymic output of newly generated T cells. In contrast, the total number of α_E-expressing Tregs in thymectomised mice was found to be similar to control mice (Figure 7b). This indicated that the high frequency was rather the result of a selective decrease of all other T cells leading to a skewed ratio between conventional $CD4^+$ T cells and α_E^+ Tregs. This shift only affected the effector/memory-like α_E^+ Tregs but not the naïve-like $\alpha_E^-CD25^+$ Tregs.

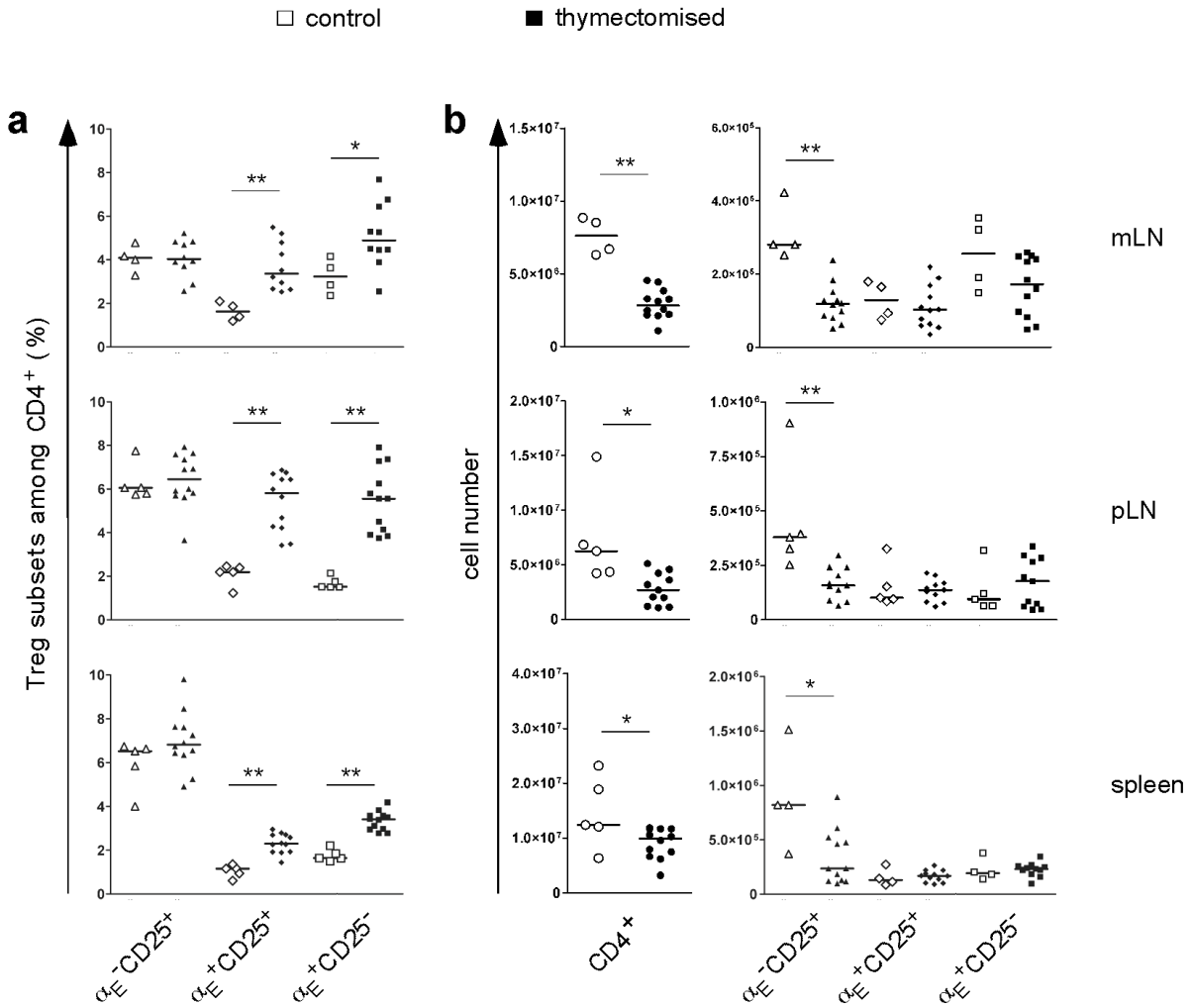


Figure 7: α_E^+ Treg numbers are unaffected by thymectomy

Adult-thymectomised C57BL/6 mice were analysed for the frequency and cell number of $CD4^+$ T cell subsets and compared to non-thymectomised age-matched control mice. Enumeration of lymphocyte numbers was done using a flow cytometric based assay (2.2.3.4) in combination with flow cytometric analysis of lymphocyte subsets stained for CD4, α_E and CD25. (a) Frequency of $CD4^+$ α_E/CD25 Treg subsets in indicated organs from control (open symbols) and thymectomised mice (filled symbols). (b) Absolute cell numbers of $CD4^+$ T cells (left) and $CD4^+$ α_E/CD25 Treg subsets (right) in indicated organs. Data are pooled from two independent experiments and single data points represent individual mice, lines indicate the median. * $p < 0.05$; ** $p < 0.01$.

3.1.1.4 Removal of intestinal microflora affects *in vivo* proliferation of CD4$^+$Foxp3$^+$ Tregs

The gut associated lymphoid tissue (GALT) favours tolerogenic immune reactions against dietary and commensal-flora derived antigen (Dubois, et al., 2005). The strong mitotic activity among α_E^+ Tregs from mLN suggested that proliferation of Tregs might be, at least in part, driven by environmental antigen acquired from the intestinal lumen. In order to investigate whether the observed proliferative activity of Tregs was somehow related to microbial stimuli derived from the intestine, comparable experiments were performed using gnotobiotic mice. These mice had been generated by treating adult specific-pathogen free (SPF) mice with a cocktail of antibiotics for a period of 8 – 10 weeks as described in 2.2.5.2. Upon successful eradication of the gut flora as judged by regular bacteriological investigation of faeces samples, BrdU was administered in the drinking water of antibiotics-treated and untreated control mice, respectively. After 10 days of continuous feeding, mice were sacrificed and CD4$^+$ Tregs from various lymphoid organs were analysed for incorporation of BrdU. In the following set of experiments intracellular staining for Foxp3 was included, allowing a more accurate evaluation of cycling Foxp3$^+$ Treg subsets. Furthermore, non-thymectomised animals were used and therefore only the frequency of BrdU^{high} cells was taken as a measure for peripheral proliferation as described in 2.2.5.1. Figure 8 shows representative co-staining for BrdU and Foxp3 of gated CD4$^+$ α_E/CD25 subsets from mLN of untreated control mice. This revealed that there is also a considerable BrdU^{high} population among α_E^-CD25$^-$Foxp3$^+$ Tregs. This subset was previously unrecognised, as no specific surface marker is known to identify it. The α_E^-CD25$^-$ Treg subset showed similar frequencies of BrdU^{high} cells as the α_E^+CD25$^-$ Treg subset in PP, mLN and spleen (Figure 8b). When comparing peripheral proliferation in control mice vs. antibiotics-treated mice, all Foxp3$^+$ α_E/CD25 subsets showed a significant reduction in the frequency of BrdU^{high} cells in PPs, while only the α_E^+CD25$^+$ Tregs displayed significantly reduced BrdU^{high} frequency in mLN. No difference was observed between control and treated mice in the spleen. Surprisingly, in pLN, the frequency of BrdU^{high} cells was even increased among α_E^+CD25$^-$ Tregs from antibiotics-treated mice.

Evaluation of absolute numbers of BrdU^{high} cells among Foxp3$^+$ α_E/CD25 Treg subsets revealed reduced numbers of cycling BrdU^{high} Tregs in all organs analysed upon treatment with antibiotics. This numerical decrease of proliferating Tregs was significant in all Treg subsets, except the α_E^-CD25$^+$ Tregs, where the reduction did not reach statistical significance.

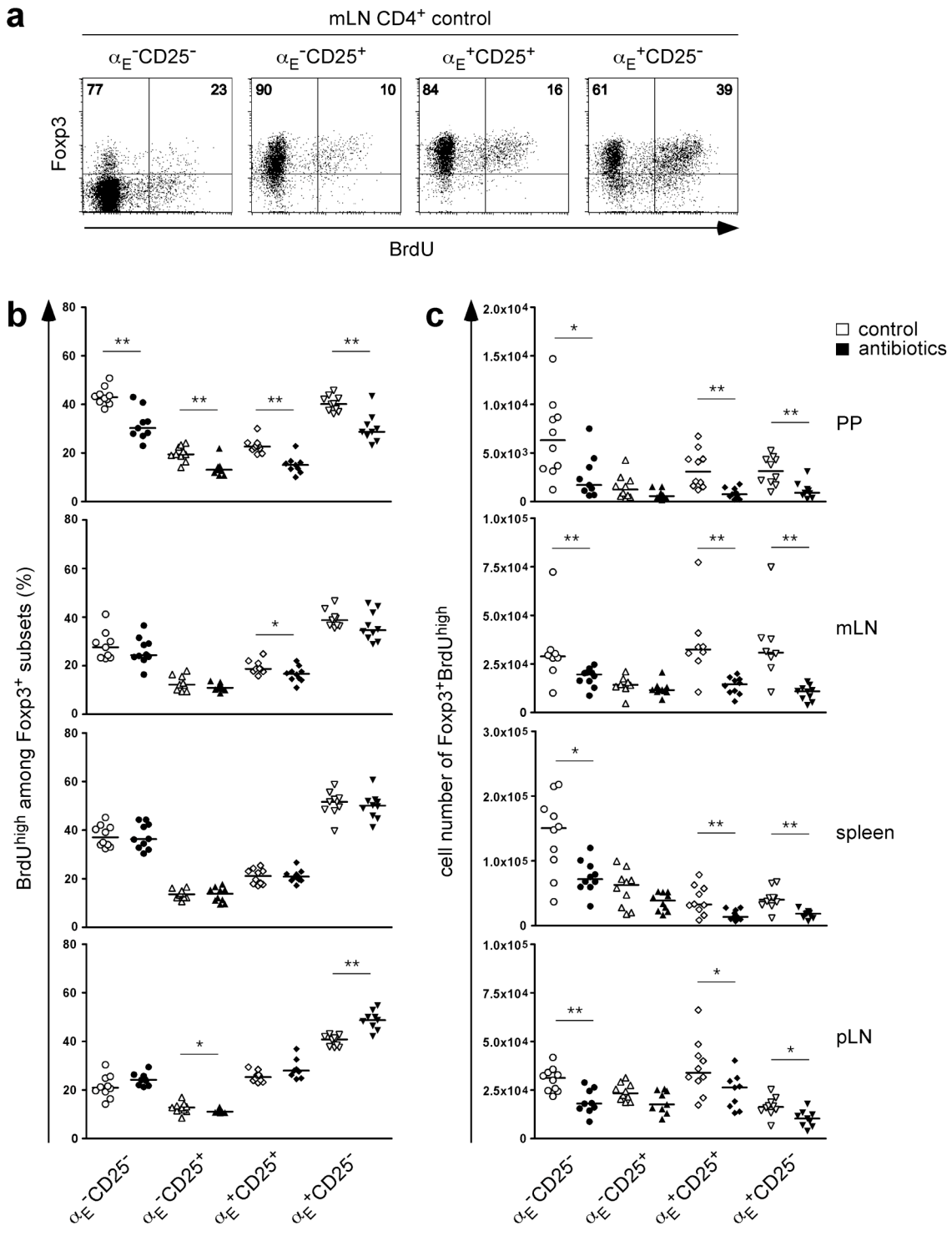


Figure 8: Microbial stimuli influence peripheral proliferation of Treg subsets

C57BL/6 mice were treated with a cocktail of antibiotics *ad libitum* for a period of 8-10 weeks. Upon successful eradication of the intestinal microflora BrdU was administered for 10 days followed by flow cytometric analysis of CD4, CD25, α_E and Foxp3 expression and BrdU incorporation. (a) Dot plots show representative intracellular staining of gated $CD4^+$ T cell subsets from mLN of control mice for Foxp3 and BrdU. Numbers indicate the frequencies of $BrdU^{high}$ cells among $Foxp3^+$ subsets (upper two quadrants). (b) Data summarise the frequencies of $BrdU^{high}$ cells among $Foxp3^+$ α_E/CD25 subsets from indicated lymphoid tissues of untreated control (open symbols) and antibiotics-treated mice (filled symbols), respectively. (c) Absolute cell number of $BrdU^{high}$ cells among $Foxp3^+$ α_E/CD25 subsets from indicated organs of untreated control (open symbols) and antibiotics-treated mice (filled symbols), respectively. Data are pooled from two independent experiments and single data points represent individual mice, lines indicate the median. * $p < 0.05$; ** $p < 0.01$.

3.1.1.5 Lack of microbial stimuli results in reduced cell numbers of Foxp3$^+$ Tregs

The overall frequency of CD4$^+$ T cells in antibiotics-treated mice was unchanged compared to untreated control mice, however, the total number of lymphocytes was decreased (Figure 9). As a consequence the absolute CD4$^+$ T cell number was reduced upon depletion of commensal microflora in all organs analysed. The CD4$^+$ T cell number of control mice vs. antibiotics-treated mice in spleen was 8.0 ± 3.7 x 10^6 vs. 5.3 ± 1.6 x 10^6; in pLN 4.0 ± 0.4 x 10^6 vs. 3.2 ± 0.9x 10^6; in mLN 3.3 ± 1.0 x 10^6 vs. 2.6 ± 0.4 x 10^6 and in PP 3.2 ± 1.9 x 10^5 vs. 1.6 ± 0.9 x 10^5 (mean ± SD, n=10). The frequency of Foxp3$^+$ Tregs in antibiotics-treated mice was similar to control mice, only in mLN a significant lower frequency was observed (Figure 9d). Consequently, the absolute cell numbers of Foxp3-expressing CD4$^+$ T cells were decreased similar to the overall lymphocyte numbers upon eradication of the microflora (data not shown).

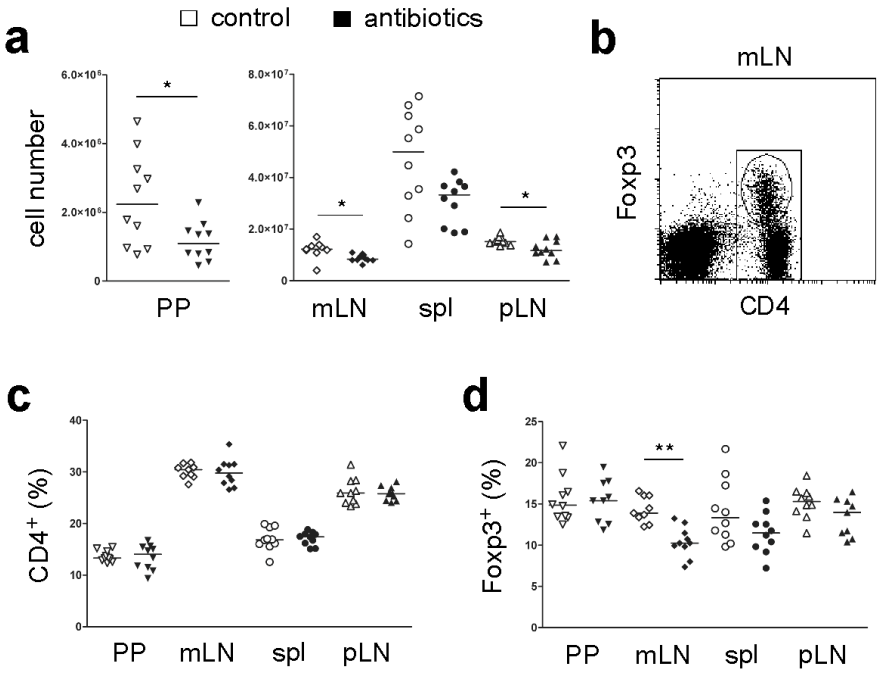


Figure 9: Reduced lymphocyte numbers upon removal of intestinal bacteria

C57BL/6 mice were treated with a cocktail of antibiotics *ad libitum* for a period of 8-10 weeks. Upon successful eradication of the intestinal microflora mice were analysed for the frequency and cell number of CD4$^+$ T cell subsets and compared to untreated control mice. Enumeration of lymphocyte numbers was done using a flow cytometric based assay (2.2.3.4) in combination with flow cytometric analysis of lymphocyte subsets stained for CD4 and Foxp3. (a) Absolute cell number of lymphocytes isolated from indicated organs of untreated control (open symbols) and antibiotics-treated mice (filled symbols), respectively. (b) Dot plot shows representative staining of lymphocytes from mLN of control mice for CD4 and Foxp3. (c) Frequency of CD4$^+$ T cells among lymphocytes and (d) Foxp3$^+$ cells within CD4$^+$ T cells from indicated organs. Data are pooled from two independent experiments and single data points represent individual mice, lines indicate the median. * $p < 0.05$; ** $p < 0.01$.

Interestingly, when the ratio of α_E/CD25 subsets among Foxp3$^+$ Tregs was compared in the two experimental groups, the frequency of naïve-like α_E^-CD25$^+$ Tregs was significantly increased upon removal of bacterial stimuli. The frequencies of the other α_E/CD25 Treg subsets were either

unchanged or even decreased under these conditions (Figure 10b and Appendix 1). Despite the overall reduction in lymphocyte numbers the reduction in $\alpha_E^-CD25^+$ Tregs did not reach statistical significance ($p = 0.1331$ in mLN; $p = 0.6607$ in PP; $p = 0.0753$ in spleen and $p = 0.4002$ in pLN), in contrast to the remaining α_E/CD25 Foxp3$^+$ Tregs (Figure 10c).

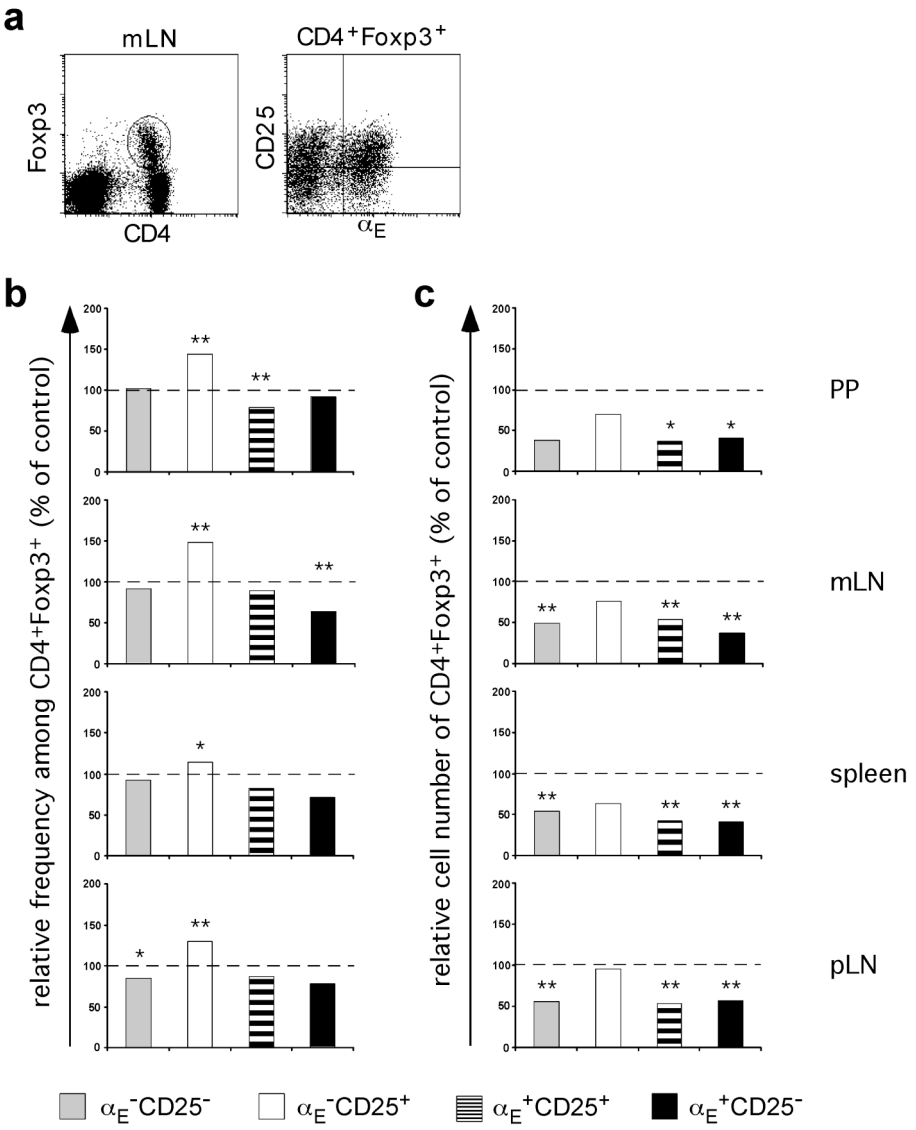


Figure 10: Microbial stimuli influence cell numbers of Foxp3$^+$ α_E/CD25 Treg subsets

C57BL/6 mice were treated with a cocktail of antibiotics *ad libitum* for a period of 8-10 weeks. Upon successful eradication of the intestinal microflora mice were analysed for the frequency and cell number of CD4$^+$ T cell subsets and compared to untreated control mice. Enumeration of lymphocyte numbers was done as described in Figure 9 and Foxp3-gated cells were analysed for the frequency and cell number of α_E/CD25 Tregs. (a) Representative dot plots showing staining of CD4 vs. Foxp3 on lymphocytes isolated from mLN of control mice (left) and staining of α_E vs. CD25 on CD4$^+$Foxp3$^+$ gated cells (right). (b) and (c) The results obtained in two independent experiments were pooled and used to calculate the median frequency and the median cell number of each Treg subset. The values obtained from untreated control animals were defined as 100 % and the relative change in antibiotics-treated mice was calculated and expressed as % of control. (b) Relative change in the frequency of α_E/CD25 Treg subsets among CD4$^+$Foxp3$^+$ cells in indicated organs from antibiotics-treated mice (% of control). (c) Relative change in cell numbers of CD4$^+$Foxp3$^+$ α_E/CD25 Tregs in indicated organs. Graphs include data from two independent experiments (n=10); dashed lines indicate 100 % of the untreated control group. Statistical analysis was performed on raw data and transferred to this graph. Single data points of frequency and cell number are displayed in Appendix 1. * $p < 0.05$; ** $p < 0.01$.

Taken together, these data from gnotobiotic mice suggest that local proliferation of $CD4^+Foxp3^+$ Treg subsets in GALT is substantially driven by microbial stimuli. Removal of these stimuli not only led to reduced proliferation but also decreased the total numbers of Tregs in GALT, spleen and pLN. Only naïve-like $\alpha_E^-CD25^+$ Tregs were less affected, primarily in their relative frequency but also in their overall cell number. These findings support the view that naïve-like $\alpha_E^-CD25^+$ Tregs are less dependent on environmental factors for their peripheral homeostasis than effector/memory-like α_E^+ and $\alpha_E^-CD25^-$ $Foxp3^+$ Treg subsets. In contrast, it suggests that differentiation into an effector/memory-like phenotype might be diminished under these conditions leading to a relative increase of this subset.

3.1.2 Generation of effector/memory-like Foxp3$^+$ Tregs by antigen-specific activation *in vivo*

The previous experiments confirmed that the effector/memory-like phenotype of α_E-expressing Tregs indeed correlated with a high degree of peripheral/homeostatic proliferation in normal, healthy mice. However, these data did not clarify whether expansion of preformed natural Tregs or *de novo* induction of Tregs from naïve T cells occurred under the investigated conditions. Given that naturally occurring Tregs are thought to originate from the thymus, where they are positively selected to recognition of self-antigen, acquisition of the effector/memory phenotype could be a consequence of activation by self-antigen in the periphery, i.e. in secondary lymphoid tissue draining the site of respective self-antigen expression (Walker, et al., 2003). In this case naïve-like α_E^- Tregs should differentiate into effector/memory-like α_E^+ Tregs upon antigen-specific stimulation *in vivo*. In order to address this question the experimental set up was switched from analysis of the polyclonal T cell pool to an antigen-specific system, where it was possible to follow the immune response to known cognate antigen.

3.1.2.1 *In vivo* conversion of naïve-like CD25$^+$ Tregs into α_E^+ Tregs

CD25$^+$ Tregs from OVA specific TCR-tg DO11.10 mice were used to analyse antigen-specific activation upon adoptive transfer. It has to be noted that CD25$^+$ Tregs in TCR-tg mice preferentially express second endogenous TCRs required for their selection in the thymus (Itoh, et al., 1999). This occurs at the expense of the tg TCR (Hurst, et al., 1997) and therefore the fraction of T cells expressing endogenous TCRs is higher among CD25$^+$ Tregs as compared to CD25$^-$ T cells (~60 % vs. ~30 % in DO11.10 mice). However, in the DO11.10 mouse model tg TCRs can be idendified with the clonotypic anti-OVA TCR mAb KJ1.26 and thus, flow cytometric analysis of antigen-specific T cell-responses is possible.

Naïve-like α_E^-CD25$^+$ Tregs were isolated from DO11.10 mice (2.2.2.2). After adoptive transfer of CFSE-labelled DO11.10 Tregs into naive BALB/c recipients the cognate antigen was administered *ad libitum* in the drinking water, a route that is known to result in tolerance induction (Mayer and Shao, 2004). Proliferation and phenotype of transferred cells were followed by multicolour flow cytometry. Transferred cells were identified among endogenous CD4$^+$ T cells based on staining with the clonotypic mAb KJ1.26 and CFSE. Continuous feeding of recipient mice with different doses of OVA protein (4 to 20 mg/ml) in the drinking water for a period of 6 days led to moderate proliferation of the antigen-specific KJ1.26$^+$CD25$^+$ Tregs in gut-associated lymphoid organs such as the celiac LN draining liver, pancreas and stomach (livLN) (Matsuno, et al., 1990) and mLN. Hardly any tg CD25$^+$ Tregs were detected in PP and spleen (data not shown). When transferred CD25$^+$ cells were analysed for their phenotype, a high degree of co-expression of α_E on previously α_E^-CD25$^+$ cells was detected upon antigen-driven proliferation (Figure 11b). The frequency of α_E on CD25$^+$ cells increased with

each division as judged by loss of CFSE and reached similar levels both in livLN and mLN (Figure 11c). Importantly, transferred $CD25^+$ Tregs maintained expression of CD25 and Foxp3 upon *in vivo* activation and proliferation (Figure 12). Together, these results support the concept that upon antigen-specific activation in the periphery naïve-like $\alpha_E^-CD25^+$ Tregs differentiate into effector/memory-like Tregs characterised by expression of α_E.

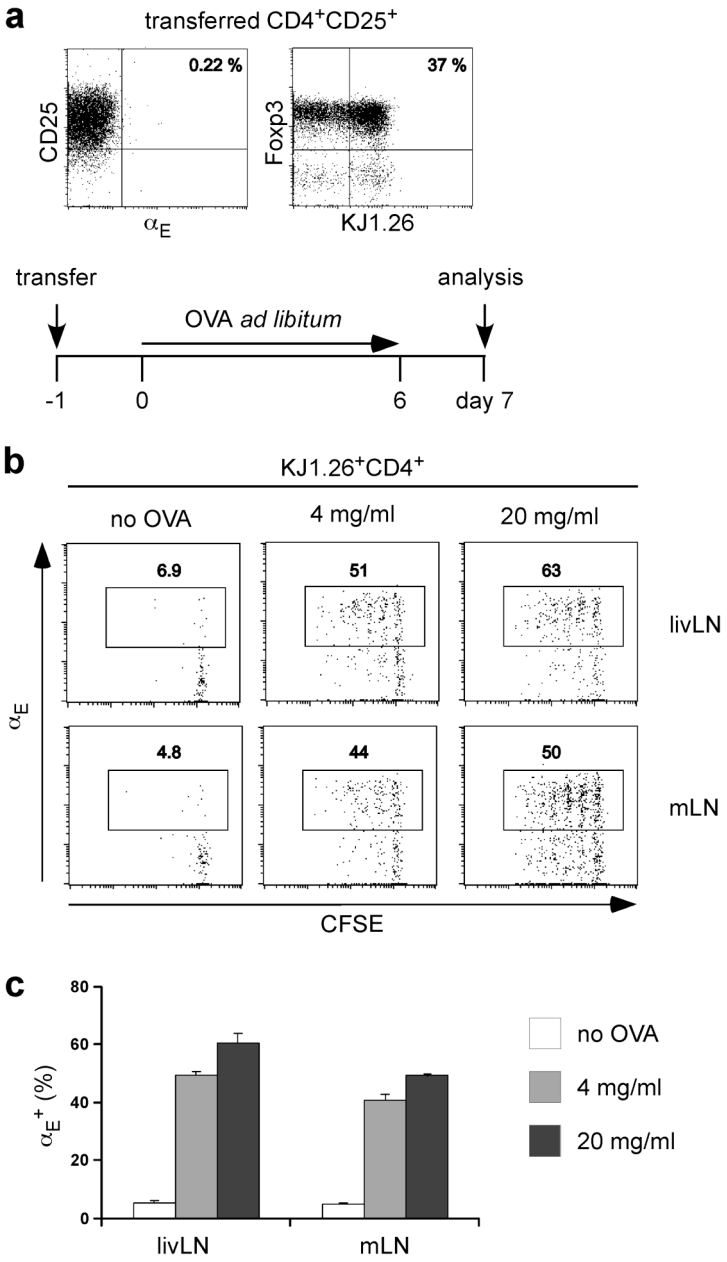


Figure 11: α_E is induced on naïve-like $CD4^+CD25^+$ Tregs upon antigen-specific activation

Pooled spleen and LN cells from DO11.10 mice were depleted of α_E^+ cells and enriched for naïve-like $CD25^+$ T cells by MACS. Purified $\alpha_E^-CD25^+$ cells were labelled with CFSE, adoptively transferred into BALB/c recipients and subsequently stimulated *in vivo* by oral application of their cognate antigen. (a) Dot plots show staining of CD25 vs. α_E and KJ1.26 vs. Foxp3 of $CD4^+CD25^+$ DO11.10 Tregs before transfer. (b) After 6 days of continuous feeding with OVA, mice were sacrificed and lymphocytes were analysed by flow cytometry. Transferred cells were identified according to $CD4^+KJ1.26^+$ staining and CFSE. Dot plots show representative analysis of α_E-expression and proliferation of re-isolated Tregs in livLNs and mLNs after feeding different doses of OVA. (c) Frequencies of α_E^+ Tregs in indicated lymphoid organs after different doses of OVA in drinking water. Data represent mean ± SD of 3 individual mice per group.

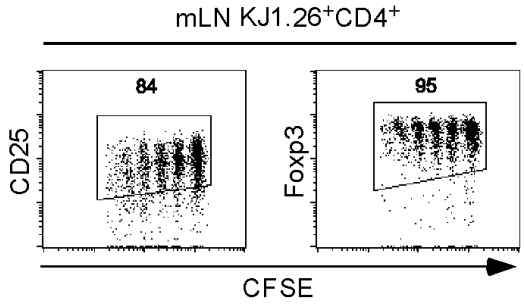


Figure 12: Naïve-like CD4$^+$CD25$^+$ Tregs remain CD25$^+$ and Foxp3$^+$ upon antigen-specific activation

Purified α_E^-CD25$^+$ Tregs were labelled with CFSE and adoptively transferred into BALB/c recipients. Recipient mice were continuously fed for 6 days with 10 mg/ml OVA in the drinking water and expression of Foxp3 and CD25 on transferred DO11.10 cells was analysed on day 7. Representative dot plots showing staining of CFSE vs. CD25 and Foxp3, respectively, on transferred CD4$^+$KJ1.26$^+$ T cells re-isolated from mLN after oral application of OVA. Numbers indicate the frequency of cells stained positive for the respective marker. Data are representative of at least two independent experiments with 2-3 mice each.

3.1.2.2 *In vivo* generation of α_E^+Foxp3$^+$ cells from conventional naïve T cells

In order to investigate whether acquisition of the α_E^+ Treg phenotype could also occur by conversion of conventional naïve CD4$^+$ T cells, corresponding experiments with adoptive transfer of naive α_E^-CD25$^-$CD62L^{high} T cells from DO11.10 mice and OVA administration via the oral route were performed. Proliferating tg KJ1.26$^+$CD4$^+$ T cells were detected in livLN, mLN, PPs and spleen at various doses of antigen (Figure 13b and Appendix 2). While slightly reduced proliferation in livLN was observed compared to the other organs (Appendix 2), a high proportion of tg Foxp3$^+$ cells re-isolated from the livLN was identified. The induction was dose-dependent and largely confined to this particular LN (Figure 13b and c), as the frequency of Foxp3$^+$ cells was much lower in mLN and PP cells (Figure 13c). When the phenotype of these induced Foxp3$^+$ cells was analysed, it was found that the vast majority of the cells co-expressed α_E (Figure 14a) showing a proliferation-dependent up-regulation of both markers on previously naïve, Foxp3$^-$ tg T cells in the livLN. Importantly, this induced Treg phenotype was only observed after tolerogenic antigen-administration. When recipient mice were immunised with OVA protein i.p. in the presence of the adjuvant LPS no significant up-regulation/induction of α_E or Foxp3 in the livLN were detected (Figure 14b), suggesting that induction of α_E^+Foxp3$^+$ Tregs was restricted to antigen-specific activation under distinct tolerogenic conditions. At the same time it indicated that the specific microenvironment of the livLN is not a bona fide site for the induction of tolerance but, in contrast, is readily able to support proliferation of naive antigen-specific T cells and their anticipated conversion into an effector Th phenotype in the presence of inflammatory stimuli.

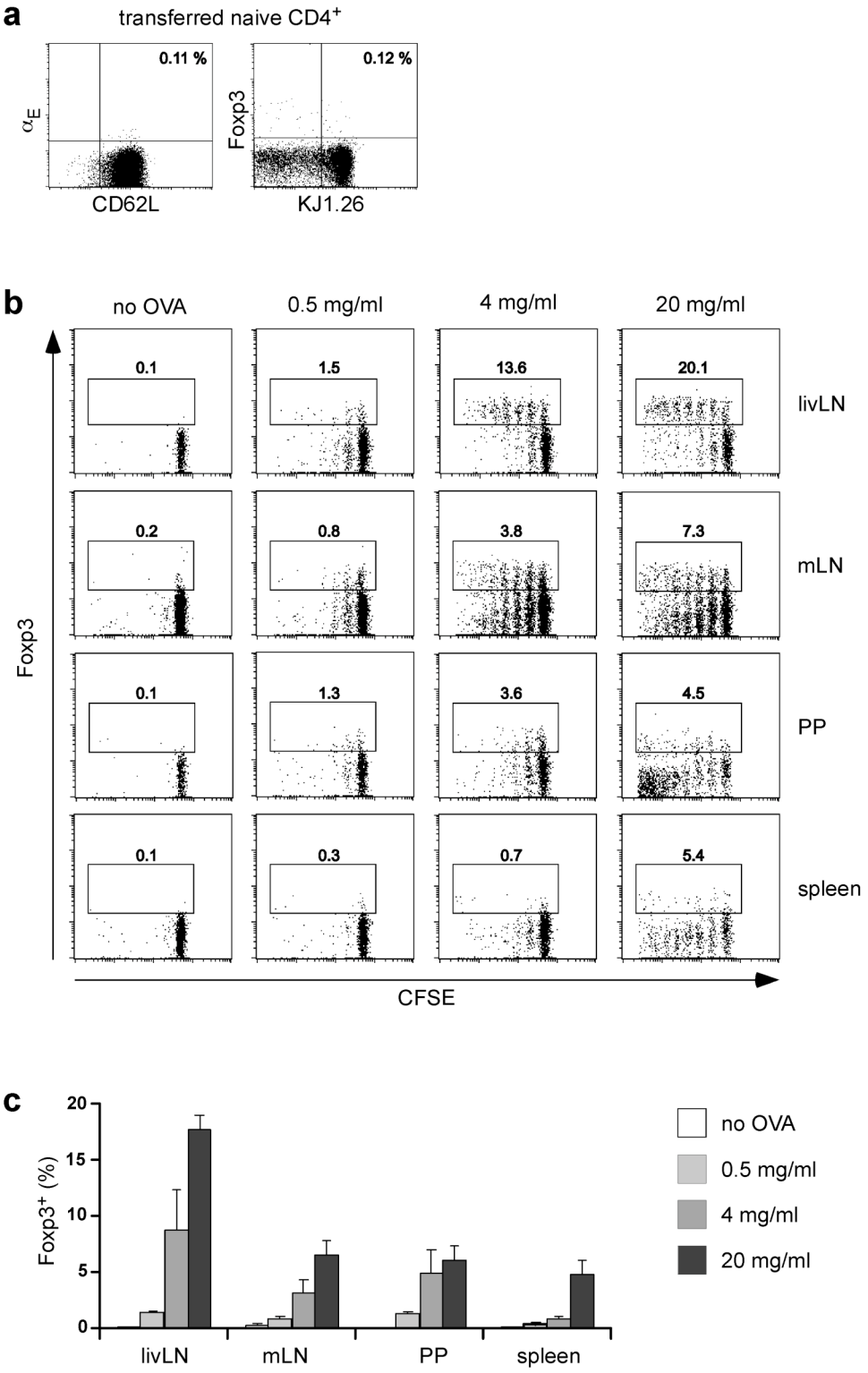


Figure 13: *De novo* induction of Foxp3$^+$ cells by antigen-specific activation in vivo

Naïve T cells were isolated from DO11.10 mice by depleting α_E^+ and CD25$^+$ cells and subsequent isolation of CD62L^{high} cells. Purified α_E^-CD25$^-$CD62L^{high} cells were labelled with CFSE, adoptively transferred into BALB/c recipients and subsequently stimulated *in vivo* by oral application of their cognate antigen. (a) Dot plots show staining of CD62L vs. α_E and Foxp3 vs. KJ1.26, respectively, of CD4$^+$ DO11.10 T cells before transfer. (b) After 6 days of continuous feeding with OVA, mice were sacrificed and lymphocytes were analysed by flow cytometry. Transferred cells were identified according to CD4$^+$KJ1.26$^+$ staining and CFSE. Dot plots show representative data of Foxp3-expression and proliferation of re-isolated DO11.10 T cells in indicated lymphoid organs after feeding of different doses of OVA. (c) Frequencies of Foxp3$^+$ cells among transferred CD4$^+$KJ1.26$^+$ in indicated lymphoid organs after different doses of OVA in drinking water. Data represent mean ± SD from 3-4 individual mice per group.

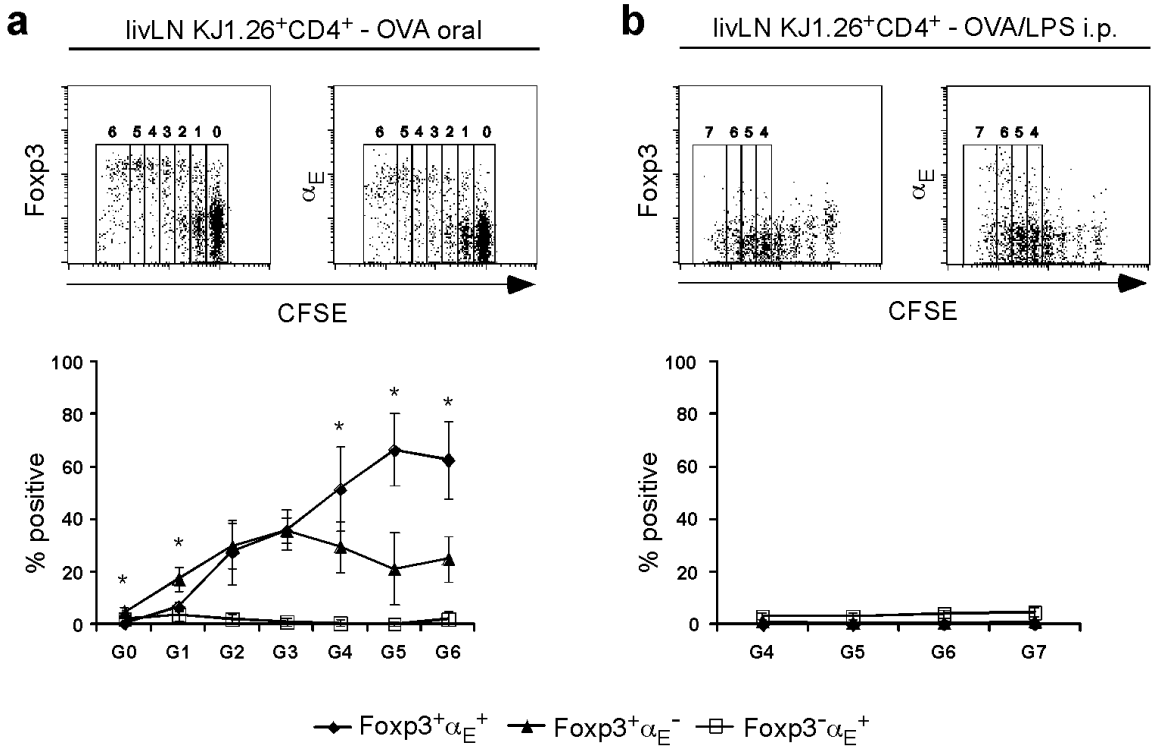


Figure 14: α_E as a marker of peripherally generated Foxp3$^+$ Tregs

Naïve α_E^-CD25$^-$CD62L^{high} DO11.10 cells were isolated, CFSE-labelled and adoptively transferred as described in Figure 13. Recipient mice were continuously fed for 6 days with 10 mg/ml OVA in the drinking water and induction of Foxp3 and α_E on transferred DO11.10 cells was analysed in livLN on day 7 (a). Alternatively, recipient mice were immunised by i.p. injection of 500 µg OVA with LPS one day after adoptive transfer of naïve α_E^-CD25$^-$CD62L^{high} DO11.10 cells and analysed for expression of Foxp3 and α_E on transferred cells in livLN on day 5 (b). Representative dot plots showing staining of CFSE vs. Foxp3 or α_E on transferred CD4$^+$KJ1.26$^+$ T cells in livLN after oral application of OVA (a) or i.p. immunisation with OVA/LPS (b). Regions indicate gating on individual generations according to CFSE-dilution. Graphs show frequency of α_E^+Foxp3$^+$, α_E^+Foxp3$^-$ and α_E^-Foxp3$^+$ in individual generations of transferred transgenic T cells. Data represent mean ± SD of 6 individual mice from two independent experiments. Significance was calculated for the difference in frequency of α_E^+Foxp3$^+$ vs. α_E^-Foxp3$^+$ in individual generations; * $p < 0.05$.

An alternative explanation for the appearance of Foxp3$^+$ Tregs after transfer of CD25$^-$ non-regulatory T cells could be selective outgrowth or survival of small numbers of contaminating Foxp3$^+$ T cells transferred together with the inoculated cells. In order to address this issue the frequency and cell number of Foxp3$^+$ Tregs among either CD25$^-$ or CD25$^+$ precursor cells transferred separately into BALB/c recipient mice was analysed before and after antigen-specific activation *in vivo*. Table 3 shows representative results indicating that a small number of Foxp3$^+$ Tregs injected along with the CD25$^-$ T cells led to a similar yield of Foxp3$^+$ Tregs in livLN and mLN as in the case of adoptive transfer of CD25$^+$ containing almost 50 times more Foxp3$^+$ cells in the initial inoculate. If selective outgrowth or survival was the cause for the emergence of Foxp3$^+$ T cells among transferred CD25$^-$ cells, this would imply a 50-fold higher recovery of Foxp3$^+$ cells compared to transfer and activation of naïve-like CD25$^+$ under the same setting and in the same anatomical microenvironment. In addition, it was observed that Foxp3 was up-regulated even on the non-divided CFSE^{high} fraction of transferred

CD25$^-$ cells, but only in the presence of cognate antigen, indicating that selective outgrowth of the pre-existing 0.12% of Foxp3$^+$ cells did not account for this population (Figure 14b). Collectively, these findings argue against emergence of Foxp3$^+$ Tregs by expansion of pre-existing CD25$^-$Foxp3$^+$ Tregs and rather support the view that Foxp3$^+$ Tregs were induced from conventional naïve CD25$^-$ T cells.

Table 3: Foxp3$^+$ cell numbers before and after antigen-specific expansion/induction by oral antigen

	Input of Foxp3$^+$ cells		Foxp3$^+$ recovered after 6 days of OVA-feeding			
	transferred		livLN		mLN	
Precursor cells	Foxp3$^+$ (%)[a]	Cell number (x 10^3)[b]	Foxp3$^+$ (%)[c]	Cell number (x 10^3)[d]	Foxp3$^+$ (%)[c]	Cell number (x 10^3)[d]
α_E^-CD25$^-$	0.19	6.2	24.6 ± 3.3	1.2 ± 0.5	9.4 ± 1.1	37 ± 6.1
α_E^-CD25$^+$	92	347	98.5 ± 0.5	2.3 ± 1.3	97.1 ± 2.0	26.6 ± 4.9

[a]Frequency of Foxp3$^+$ cells among CD4$^+$KJ1.26$^+$ DO11.10 cells transferred into BALB/c recipients.

[b]Cell number of Foxp3$^+$ cells among CD4$^+$KJ1.26$^+$ DO11.10 cells transferred into BALB/c recipients.

[c]Mean frequency ± SD of Foxp3$^+$ cells among CD4$^+$KJ1.26$^+$ recovered from BALB/c recipients (n=3-4).

[d]Mean cell number ± SD of Foxp3$^+$ cells among CD4$^+$KJ1.26$^+$ recovered per LN (n=3-4).

Taken together the present data suggest that α_E identifies Foxp3$^+$ Tregs, which have expanded in the periphery under non-inflammatory conditions. This subset of naturally occurring Tregs represents a population of heterogeneous origin containing cells differentiated from thymic-derived CD25$^+$ Tregs as well as *de novo* induced Tregs, which have converted from the conventional T cell pool.

3.2 Induction of gut-specific homing receptor expression on Foxp3$^+$ Tregs

The aim of the second part of this study was to investigate how Tregs acquire the expression of homing receptors needed for tissue- or inflammation-specific migration. It was not known whether they respond in a similar way as conventional naïve T cells to signals instructing tissue-specific homing receptor expression and organ-selective trafficking. Therefore, the susceptibility of naïve Tregs to acquire homing receptor expression under defined *in vitro* culture conditions was investigated and the functionality of the induced phenotype was tested upon adoptive transfer.

3.2.1 Induction of gut-specific homing receptors by tissue-specific DCs

Tissue-specific DCs have been shown in a number of *in vitro* and *in vivo* studies to be involved in the instruction of naïve T cells (Annacker, et al., 2005; Dudda and Martin, 2004; Dudda, et al., 2004; Johansson-Lindbom, et al., 2005; Johansson-Lindbom, et al., 2003; Mora, et al., 2003; Mora and von Andrian, 2006; Stagg, et al., 2002). In order to investigate whether organ-selective homing receptors are induced on Tregs in a similar fashion, *in vitro* culture systems were set up using freshly isolated DCs and sorted naïve CD25$^+$ Tregs. To overcome the hyporesponsiveness of Tregs, high doses of exogenous IL-2 were added during antigen-specific activation.

CD11c$^+$ DCs from mLNs and pLNs were isolated and co-cultured with naïve CD4$^+$CD25$^+$ Tregs from OVA-specific TCR-tg mice in the presence of OVA_{323-33} peptide. Naïve Tregs were used, which typically display low levels of $\alpha_4\beta_7$ and lack P-Lig expression (Huehn, et al., 2004), to exclude Tregs that already have been imprinted for these homing receptors within donor mice. On day 4 cultured CD4$^+$ Tregs were harvested and analysed by flow cytometry for expression of the gut-specific homing receptors $\alpha_4\beta_7$ and CCR9 as well as for homing receptors associated with skin-specific migration, P-Lig and E-Lig. In Figure 15 representative results from flow cytometric analyses are shown. Tregs cultured in the presence of mLN-derived DCs homogeneously expressed high levels of $\alpha_4\beta_7$, whereas only a minor fraction of Tregs cultured with pLN-derived DCs showed intermediate $\alpha_4\beta_7$ expression. CCR9 expression was not induced under these culture conditions, neither by mLN-derived DCs nor by pLN-derived Dcs. In contrast, P-Lig expression was observed on Tregs from co-cultures with both mLN- and pLN-derived DCs, while the latter induced slightly enhanced frequencies. Selective differences were observed with respect to expression of E-Lig, which was induced on a fraction of Tregs cultured in the presence of pLN-derived DCs, while no induction occurred in mLN-derived DC co-cultures (Figure 15). These results indicated that naïve CD25$^+$ Tregs responded in a similar manner to tissue-specific factors mediated by organ-specific DCs as has been previously reported for conventional T cells.

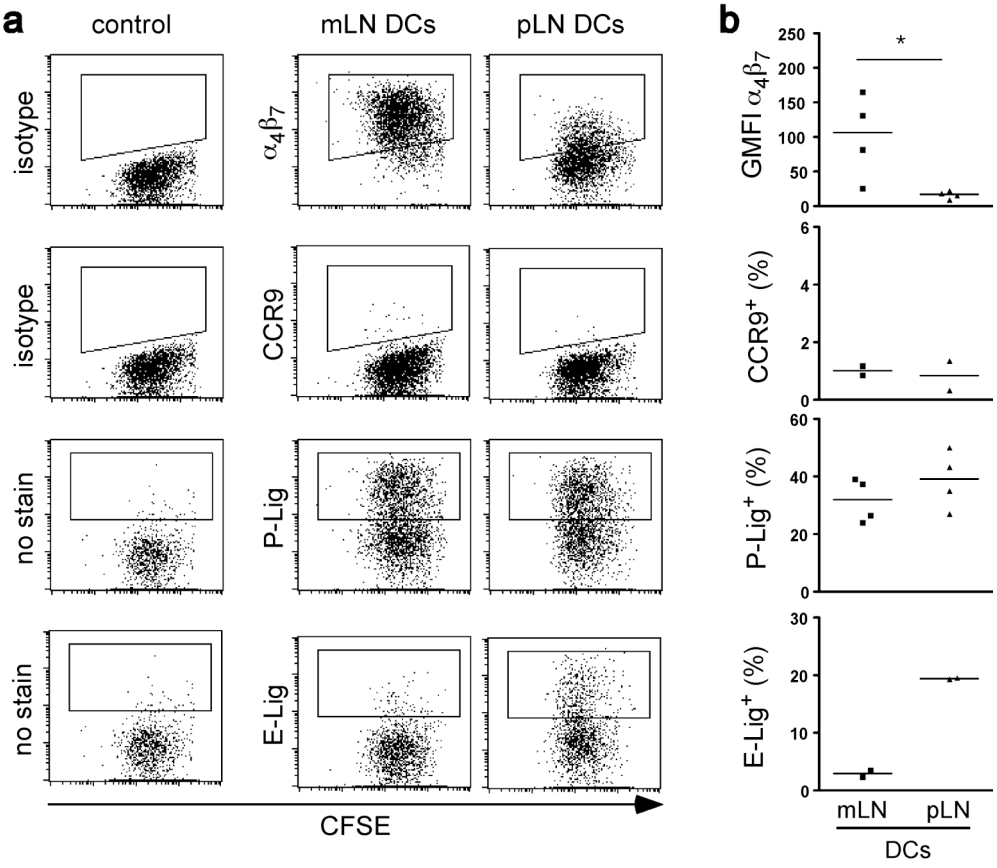


Figure 15: Induction of homing receptor expression on Tregs cultured with organ-specific DCs

Organ-specific DCs were isolated by magnetic enrichment of $CD11c^+$ cells from single cell suspensions of pLN and mLN, respectively (2.2.2.4). Naïve $CD25^+CD4^+CD62L^{high}$ were isolated from pooled LNs and spleens of DO11.10 mice by magnetic enrichment of $CD25^+$ cells and subsequent FACSorting of $CD4^+CD62L^{high}$ cells (2.2.2.3). Purified $CD4^+CD25^+CD62L^{high}$ Tregs were labelled with CFSE and stimulated for 4 days with OVA_{323-33} peptide and high dose IL-2 in the presence of $CD11c^+$ DCs derived from mLN and pLN, respectively. (a) Dot plots show CFSE vs. staining for $\alpha_4\beta_7$, CCR9, P-Lig and E-Lig (right panel) on cultured Tregs after 4 days of antigen-specific activation with tissue-specific DCs. Control stainings are depicted in the left panel. (b) GMFI of $\alpha_4\beta_7$ expression of the whole population, frequency of $CCR9^+$, $P\text{-}Lig^+$ and $E\text{-}Lig^+$ cells. Data points represent independent experiments except for E-Lig (dublicate analysis from one experiment), lines indicate the median. *$p < 0.05$.

3.2.2 Induction of gut-specific homing receptors by soluble factors

In addition to co-cultures with tissue-specific DCs, DC-free stimulation systems using plate-bound anti-CD3 and anti-CD28 mAbs were established to induce expression of organ-selective homing receptors on Tregs. As polarising compounds, RA and IL-12 were used, which have been shown to induce $\alpha_4\beta_7$ and P-Lig on activated T cells, respectively (Austrup, et al., 1997; Iwata, et al., 2004). *In vitro* activation of naïve $CD25^+$ Tregs in the presence of RA induced high $\alpha_4\beta_7$ levels on Tregs (Figure 16a). In contrast, only very weak $\alpha_4\beta_7$ levels were observed upon activation under neutral conditions or upon addition of IL-12. Similarly, expression of CCR9 was selectively induced on a fraction of Tregs in cultures containing RA, but not IL-12. A different picture was observed with respect to the expression of P-Lig. Whereas culture of naïve $CD25^+$ Tregs under neutral conditions only slightly enhanced P-Lig expression, a large fraction of cells cultured in the presence of IL-12 acquired P-Lig expression. However, also *in vitro* culture in the presence of RA led to induction of P-Lig on a significant fraction of Tregs. In contrast, in cultures of conventional $CD4^+CD25^-$ T cells P-Lig expression was differentially regulated, here only IL-12 led to significant frequencies of $P\text{-}Lig^+$ cells. Expression of E-Lig was consistently induced upon addition of IL-12 on fractions of both $CD25^+$ Tregs and $CD25^-$ T cells and was efficiently suppressed in RA cultures, as reported previously (Iwata, et al., 2004) (Figure 16a).

In summary, Tregs were found to respond by and large similar to tissue-specific signals as conventional T cells, and were sensitive to selective induction of organ-specific homing receptors in response to appropriate stimuli.

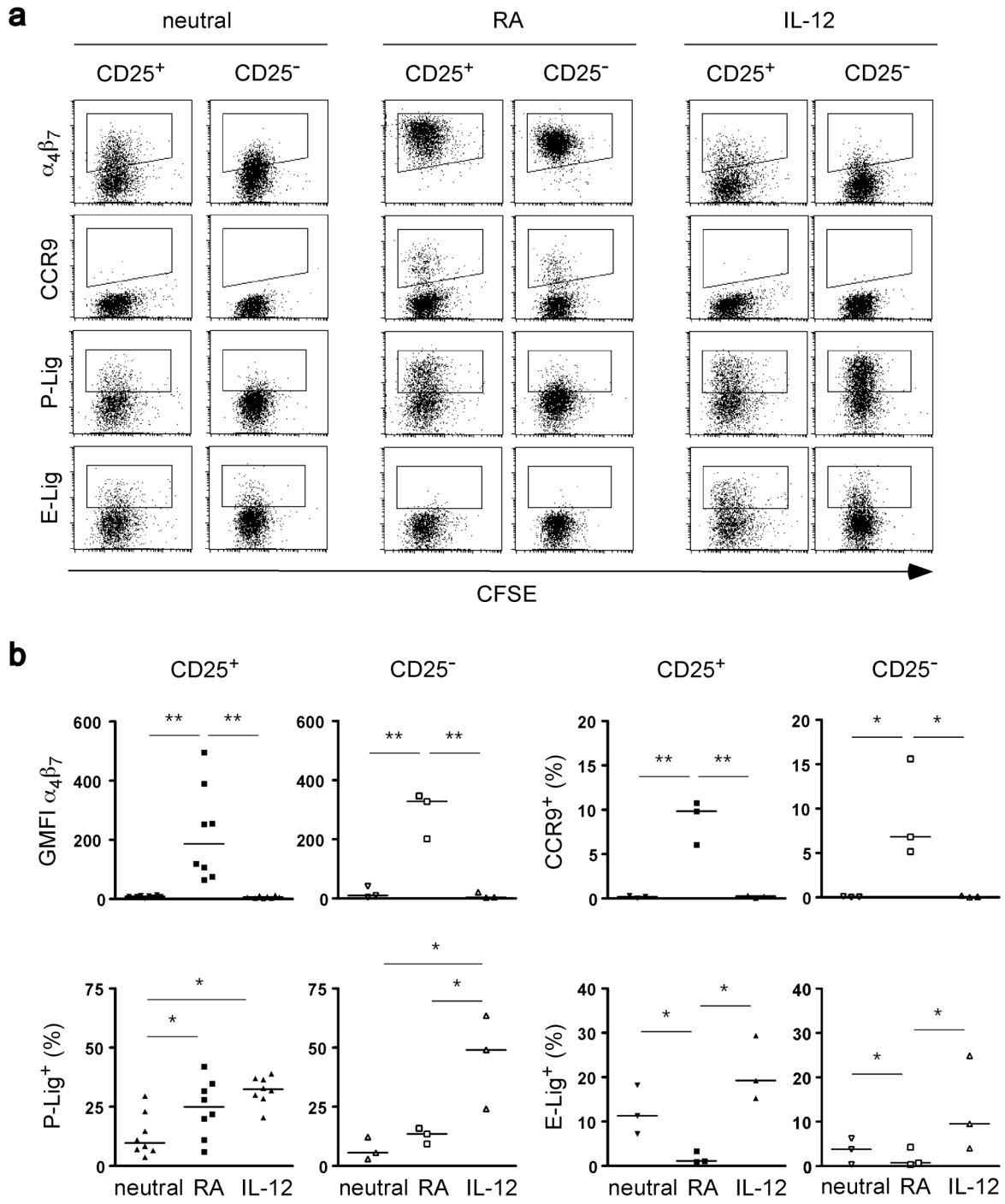


Figure 16: Induction of homing receptor expression on Tregs cultured under polarising conditions

Naïve $CD4^+CD25^+CD62L^{high}$ and $CD4^+CD25^-CD62L^{high}$ cells were isolated from pooled LNs and spleens of BALB/c mice by a combination of MACS and FACS (2.2.2.3). Purified T cells were labelled with CFSE and stimulated on anti-CD3/CD28 coated plates under neutral or polarising conditions in the presence of RA and IL-12, respectively. (a) Dot plots show representative results of CFSE vs. staining for $\alpha_4\beta_7$, CCR9, P-Lig and E-Lig on cultured Tregs ($CD25^+$) and conventional naïve T cells ($CD25^-$) after 6 days of polyclonal activation under indicated conditions. (b) GMFI of $\alpha_4\beta_7$ expression of the whole population, frequency of $CCR9^+$, $P\text{-}Lig^+$ and $E\text{-}Lig^+$ cells. Single data points represent independent experiments, lines indicate the median. $*p < 0.05$; $**p < 0.01$.

3.2.3 Phenotype and function of *in vitro* modulated Tregs

An important prerequisite for potential therapeutic use of *in vitro* expanded Tregs, is maintenance of their characteristic features. Therefore, Tregs skewed to tissue-specific homing receptor expression *in vitro* were tested for their suppressive activity. The vast majority of Tregs expanded under any condition - neutral, RA or IL-12 - maintained high frequencies of Foxp3$^+$ cells (Figure 17). The highest frequency was consistently observed within RA-cultured Tregs. More importantly, when tested in an *in vitro* proliferation assay with CFSE-labelled CD25$^-$CD4$^+$ naïve target T cells, all Tregs expanded for 6 days under different conditions similarly suppressed naïve CD4$^+$ T cell proliferation indicating that cultured Tregs maintained high suppressive capacity after the culture period (Figure 18).

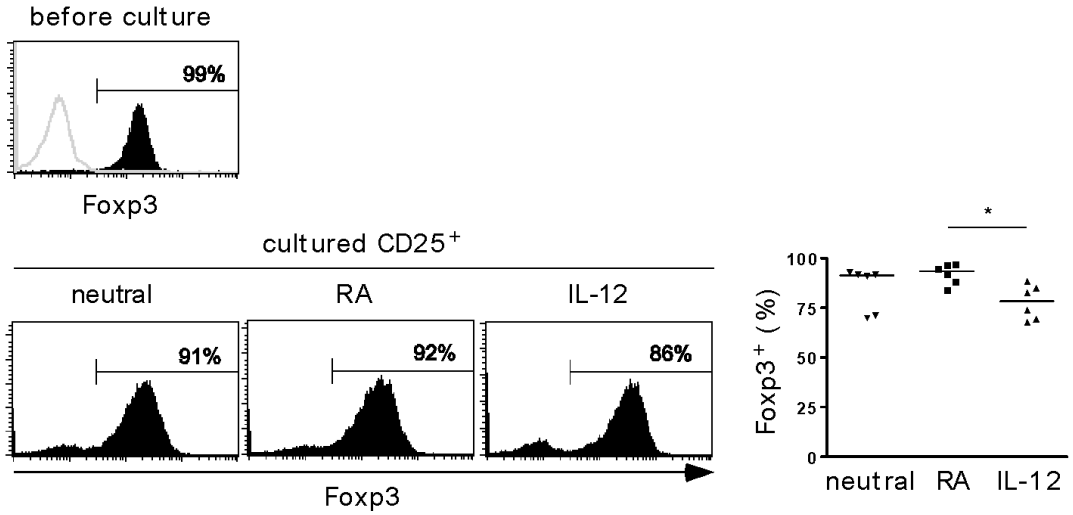


Figure 17: *In vitro* polarised Tregs maintain Foxp3 expression

Naïve CD4$^+$CD25$^+$CD62L^{high} Tregs were stimulated on anti-CD3/CD28 coated plates under neutral or polarising conditions for 6 days as described in Figure 16 and analysed for expression of Foxp3. Histograms show representative staining for Foxp3 (filled) on Tregs before and after culture under respective conditions. Numbers indicate the frequency of Foxp3$^+$ cells. As control, Foxp3 staining of naïve CD4$^+$CD25$^-$ T cells is shown (grey line, upper histogram). Data points represent the frequency of Foxp3$^+$ cells among cultured CD25$^+$ Tregs from independent experiments, lines indicate the median. * $p < 0.05$.

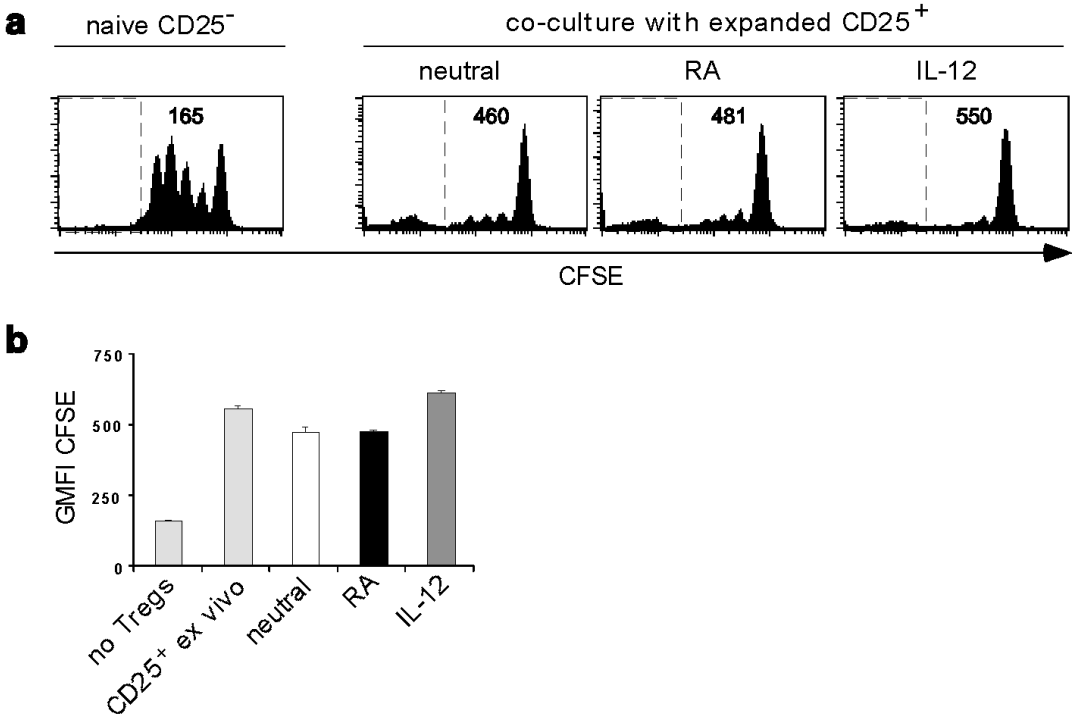


Figure 18: *In vitro* polarised Tregs maintain suppressive capacity

Naïve $CD4^+CD25^+CD62L^{high}$ Tregs were stimulated on anti-CD3/CD28 coated plates under neutral or polarising conditions for 6 days as described in Figure 16 and used for co-cultures with CFSE-labelled naïve $CD4^+CD25^-CD62L^{high}$ responder T cells at a ratio of 1:2 in the presence of CD90-depleted APCs and anti-CD3 for 3 days. Proliferation of naïve responder cells was evaluated according to CFSE dilution. (a) Histograms show CFSE-staining of responder T cells alone (left) or in the presence of cultured $CD25^+$ Tregs (right). The GMFI of the $CFSE^+$ fraction (dotted line) is indicated. (b) GMFI of $CFSE^+$ cells (mean ± SD, from triplicate measurements) from naïve responder cells cultured under indicated conditions. Representative data from one out of three independent experiments with similar results are depicted.

3.2.4 Migratory behaviour of *in vitro* modulated Tregs

Having confirmed that expansion and modulation of Tregs *in vitro* did not alter their characteristic features, while at the same time conveying selective homing phenotypes, the functionality of the newly achieved homing receptors remained to be tested. Therefore, Tregs that had been cultured under neutral or polarising conditions in the presence of RA and IL-12, respectively, were analysed for their migration behaviour *in vivo* in a DTH footpad inflammation model (Feuerer, et al., 2006; Siegmund, et al., 2005). Cultured Tregs were radioactively labelled and injected i.v. into mice, in which 24h before the DTH response had been induced. At his time point acute inflammation in the footpad had reached its maximum and thus allowed to monitor recruitment of transferred Tregs into a peripheral site of inflammation. One day after transfer, the migration of Tregs was determined by measuring the distribution of radioactivity in individual organs. Tregs cultured in the presence of RA showed preferential migration into the gut (small and large intestine) as well as into mLNs and PPs, supporting previous findings that expression of $\alpha_4\beta_7$ enabled entrance into these compartments (Hamann, et al., 1994; Holzmann and Weissman, 1989). RA-cultured Tregs were barely detected in the inflamed footpad. In contrast, Tregs that had been expanded in the presence of IL-12 efficiently migrated into

the inflamed footpad. This inflammation-seeking capacity was significantly higher compared to Tregs cultured under neutral conditions or in the presence of RA (Figure 19). Clearly, Tregs cultured in the absence of polarising factors did not display distinct organ-specific migration behaviour. They did not enter the inflamed footpad very effectively and were recruited less efficiently into the gut and GALT. Distribution of differentially cultured Tregs throughout other organs, i.e. spleen, lung and liver was similar. These findings provided evidence that *in vitro* modulation of naïve-like Tregs resulted in the induction of functional organ-selective homing receptors allowing efficient migration of polarised Tregs into mucosal and inflamed tissues, respectively.

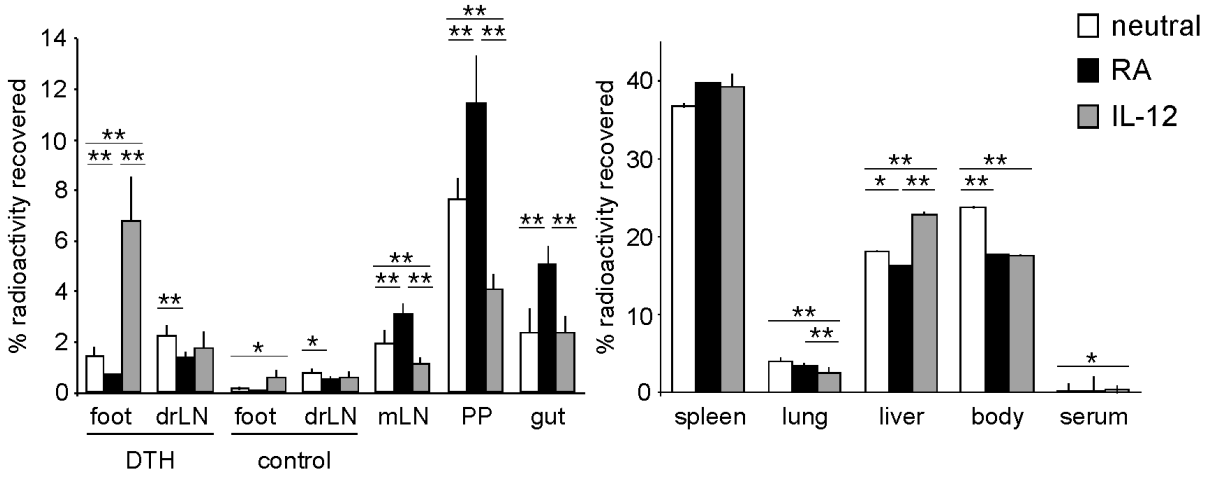


Figure 19: *In vivo* migration potential of *in vitro* polarised Tregs.

Naïve $CD4^+CD25^+CD62L^{high}$ Tregs were stimulated on anti-CD3/CD28 coated plates under neutral or polarising conditions for 6 days as described in Figure 16. Cultured Tregs were harvested, radioactively labelled with ^{111}In as described in 2.2.5.5 and adoptively transferred into recipient mice bearing an acute skin inflammation in one footpad (DTH). Distribution and migratory behaviour of Tregs cultured under the indicated conditions was analysed 24h after adoptive transfer by determining the amount of radioactivity recovered from indicated organs using a γ-counter. Bars display mean ± SD from 6 individual mice and are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$.

4 Discussion

Tregs are increasingly recognised as a major cell population involved in the regulation of numerous immune responses. Their mode of action ranges from the suppression of onset of disease in the local LNs to amelioration of established effector responses directly at the site of inflammation. Consistent with these observations and the numerous disease models describing a role for Tregs, the phenotype and function of Treg populations were found to be highly diverse. Our group focused on a distinct subset of naturally occurring Tregs characterised by the expression of the integrin α_E. At the beginning of this study it was recognised that α_E identified Tregs with an activated, highly differentiated phenotype. These α_E^+ Tregs were found to be equipped with adhesion molecules and chemokine receptors allowing their efficient migration into peripheral, non-lymphoid sites. How and where these effector/memory-like α_E^+ Tregs were generated *in vivo* was not known. Furthermore, it was not known whether Tregs could be modulated *in vitro* in order to acquire distinct sets of adhesion molecules conveying targeted migration upon adoptive transfer.

4.1 α_E identifies effector/memory-like Tregs with high proliferative activity

Expression of α_E on Foxp3$^+$ Tregs correlates with an effector/memory-like phenotype

In this study previous findings regarding the phenotype of α_E^+ Tregs were confirmed and extended by direct correlation of their phenotypic key features to intracellular expression of Foxp3. The results obtained from co-staining with Foxp3 corroborated the differential expression of CD62L and CD44 on α_E^-CD25$^+$, α_E^+CD25$^+$ and α_E^+CD25$^-$ Treg subsets, respectively, and the association of an effector/memory-like phenotype with expression of α_E.

In addition to these α_E/CD25 subsets, Foxp3$^+$ cells were also detected in a small fraction of the α_E^-CD25$^-$ population. The existence of CD25^{low}Foxp3$^+$ Tregs was first reported by Fontenot and colleagues, who generated GFP knock in mice expressing a GFP-Foxp3 fusion-protein under the control of the Foxp3 promoter. This allowed thorough analysis of the correlation between CD25 and Foxp3^{GFP+} cells. Global gene analysis of the two Foxp3$^+$ populations revealed that CD25^{low}Foxp3$^+$ expressed higher levels of genes associated with regulatory effector functions and tissue migration than CD25^{high}Foxp3$^+$. In addition, the CD25^{low}Foxp3$^+$ population was enriched for cells displaying an activated surface phenotype and for proliferating Ki67$^+$ cells (Fontenot, et al., 2003). Our data extent these findings and indicate that the observed characteristics of the CD25^{low}Foxp3$^+$ may be attributed largely to the α_E^+CD25$^-$Foxp3$^+$ subset, while both the α_E^+CD25$^+$Foxp3$^+$ and α_E^-CD25$^-$Foxp3$^+$ subsets displayed intermediate activation/differentiation levels.

Taken together, these results further support the concept of a division of labour between distinct sets of naturally occurring Foxp3$^+$ Tregs in the healthy un-manipulated host (Huehn and Hamann, 2005)

and confirm that the combined use of the markers CD25 and α_E may be helpful in identification of these different Foxp3$^+$ Treg subsets.

4.1.1 Peripheral proliferation of Tregs in thymectomised mice

One major characteristic of Tregs, observed during initial studies, was their inability to proliferate under standard *in vitro* conditions. While they required TCR-mediated activation, only addition of exogenous IL-2 was able to overcome the anergic state of Tregs (Thornton and Shevach, 1998). In contrast, strong proliferation *in vivo*, i.e. in LNs draining tissue that expresses their respective self-antigen was demonstrated in subsequent studies (Fisson, et al., 2003; Klein, et al., 2003; Walker, et al., 2003).

Here, the correlation of α_E expression and peripheral proliferation of Tregs in thymectomised mice in the steady state was analysed. Dividing cells were highly enriched in the α_E^+CD25$^-$ fraction, to a lesser extent in the α_E^+CD25$^+$ Treg subset and the lowest fraction of proliferating cells was found within the α_E^-CD25$^+$ Treg subset. These findings demonstrated a clear link between high proliferative activity and the effector/memory-like phenotype of α_E^+ Tregs as previously hypothesised (Huehn, et al., 2004). The observed proliferation indicates that a major part of Tregs is constantly cycling under steady state conditions, presumably in response to their cognate antigen. These findings are in accordance with results from Fisson and colleagues, who demonstrated rapid turnover of adoptively transferred polyclonal CD4$^+$CD25$^+$CD62L^{high} Tregs in non-lymphopenic recipients associated with acquisition of an activated phenotype (Fisson, et al., 2003). Based on the CFSE profile of transferred Tregs, these authors observed loss of CD62L expression and up-regulation of CD44 only on those cells that had undergone several rounds of division. In addition, they demonstrated that incorporation of BrdU was restricted to a major fraction of CD25$^+$CD44^{high} Tregs. Our study extends these findings by examining more closely the contribution of different naturally occurring Treg subsets to peripheral proliferation under steady state conditions. A proliferative response of CD25$^+$ Tregs *in vivo* was also observed in the presence of cognate antigen in studies using TCR-tg models. Proliferation was found to occur both under non-inflammatory conditions in response to tissue-restricted expression of the respective antigen (Cozzo, et al., 2003; Fisson, et al., 2003; Green, et al., 2002) as well as upon immunisation with cognate antigen and IFA (Klein, et al., 2003; Walker, et al., 2003), supporting the notion that Treg proliferation requires antigen-specific activation.

In the polyclonal repertoire the nature of these antigens remains elusive, although, it was shown that Tregs display a diverse TCR repertoire (Takahashi, et al., 1998), which is distinct from conventional T cells and skewed towards high affinity recognition of self-antigen (Hsieh, et al., 2004; Nishikawa, et al., 2005). Furthermore, Tregs and potentially pathogenic self-reactive T cells were found to have intersecting TCR repertoires (Hsieh, et al., 2006). Nevertheless, one recent report by Suffia and colleagues provided evidence for specific reactivity towards foreign antigen in a model of Leishmania

major infection. These authors concluded that parasite-specific recognition was essential for expansion and function of responding Foxp3$^+$ Tregs (Suffia, et al., 2006). In addition, data from gnotobiotic mice obtained in this study suggest a contribution of stimuli derived from the intestinal microflora on the proliferation of Tregs (see below).

Collectively, the above findings indicate that under steady state conditions sufficient antigen sources exist stimulating homeostatic proliferation of Tregs in healthy mice. Clearly, the results from this study show that the highest proliferative activity accumulates in the Treg subsets, which display an effector/memory-like phenotype and are characterised by the expression of α_E.

α_E^+ Treg numbers are independent of thymic output

Compared to untreated controls, thymectomised mice displayed a relative increase in the frequency of α_E-expressing Tregs, which was due to the reduced cell count of conventional CD4$^+$ T cells resulting in a mild lymphopenia of the T cell compartment. Absolute numbers of α_E^+ Treg were comparable to non-thymectomised control mice, indicating that thymic export was not necessary to maintain this Treg subset. In contrast, the α_E^-CD25$^+$ Tregs displayed an equal reduction in cell numbers following thymic ablation as the conventional, naïve T cell pool. These findings support a concept, where α_E identifies peripherally expanded effector/memory-like Tregs, which possibly have differentiated from naïve-like α_E^-CD25$^+$ Tregs. The data obtained here are in line with a previous report by Dujardin et al, who demonstrated a pronounced predominance of α_E^+CD25$^+$ Tregs in adult mice that had been thymectomised at day 3 (d3Tx) (Dujardin, et al., 2004). In newborn mice α_E^+CD25$^+$ Tregs were barely detected, while significant numbers of α_E^-CD25$^+$ Tregs had already emigrated from the thymus before thymectomy. In contrast, adult d3Tx mice showed 5-fold higher frequencies of α_E^+CD25$^+$ Tregs compared to non-thymectomised control mice. These authors suggested that the α_E^+CD25$^+$ population had expanded from thymic-derived α_E^-CD25$^+$ Tregs upon exposure to peripheral antigen. Our study using mice thymectomised at the age of three weeks, demonstrates that independence of α_E^+ Tregs from thymic output is not restricted to a situation where postnatal development of the whole T cell compartment is severely disturbed.

4.1.2 Peripheral proliferation in gnotobiotic mice

Tregs are believed to contribute constantly to the maintenance of immune homeostasis and prevention of undesired responses to self (Sakaguchi, 2004). Most importantly, the presence of the respective antigen is required for survival and function of Tregs (Garza, et al., 2000; Jordan, et al., 2001; Nishikawa, et al., 2005; Seddon and Mason, 1999; Walker, et al., 2003).

Apart from responses to self-antigens, constant encounters with dietary and microbial antigens at mucosal surfaces require regulatory mechanisms, which also include the action of various intestinal Treg subsets (reviewed in (Mowat, 2003; Rook and Brunet, 2005)). A role for Tregs in the regulation

of gut-associated immune responses has been postulated for some time (Cong, et al., 2002; Duchmann, et al., 1996; Gad, et al., 2004; Khoo, et al., 1997) and break of tolerance towards the intestinal microflora is believed to be the major cause of inflammatory bowel disease (Duchmann, et al., 1995). However, the impact of a complex intestinal microflora on the $Foxp3^+$ Treg compartment has not been sufficiently addressed to date. Here it was found that both numbers and relative proportion of $CD4^+Foxp3^+$ Tregs were substantially regulated by the presence of microbial stimuli. Under steady state conditions Tregs proliferated vigorously in GALT of SPF mice, while these responses were significantly reduced upon depletion of the commensal flora. These results suggest that local expansion in GALT influences overall Treg numbers and thereby, contributes to Treg homeostasis under non-inflammatory conditions.

$Foxp3^+$ Treg numbers are decreased upon depletion of intestinal bacteria

Upon eradication of the intestinal microflora, treated mice acquired a reduction of the whole lymphocyte compartment. The decrease also included all $Foxp3^+$ Treg subsets. This suggested that the overall number of $Foxp3^+$ Tregs was influenced by the presence or absence of intestinal flora. Whether this was mediated by direct recognition of bacterial antigen or rather a consequence of cross-presentation of self-antigen by bacterial-activated APCs, remains currently unknown. These scenarios are not mutually exclusive and would equally result in reduced proliferation of the responding Tregs upon removal of the bacterial stimuli.

It was found here that α_E-expressing Treg subsets were much more reduced in their absolute cell number by depletion of commensal bacteria than the naïve-like $\alpha_E^-CD25^+$ subset and that on the contrary, the frequency of the latter was even relatively increased among all $Foxp3^+$ Tregs. These data support the hypothesis, that maintenance of α_E^+ Tregs depends more on the presence of peripheral stimuli driving activation and proliferation of Tregs. In contrast, naïve-like $\alpha_E^-CD25^+$ Tregs appear to rely much more on other factors i.e. thymic export for homeostatic balance. The $\alpha_E^-CD25^-Foxp3^+$ Treg subset was found to display an almost similar proliferative activity as the $\alpha_E^+CD25^-Foxp3^+$ Tregs consistent with the increased frequency of $Ki67^+$ cells within $CD25^{lo}Foxp3^{gfp+}$ Tregs reported earlier by Fontenot and colleagues (Fontenot, et al., 2005). However, if and how these $CD25^-$ Treg subsets are related to each other is currently unknown. Clearly, $\alpha_E^-CD25^-$ cells exceeded $\alpha_E^+CD25^-$ cells and were similar to $\alpha_E^+CD25^+$ cells both in number and frequency among $CD4^+Foxp3^+$ Tregs in healthy mice. Taken together these data indicate that α_E-expression and/or lack of CD25-expression identifies three subsets of $Foxp3^+$ Tregs, which show enhanced proliferation and activated phenotypes compared to the naïve-like $\alpha_E^-CD25^+$ Tregs.

Antigen-specific recognition of bacterial antigen by Tregs?

As mentioned above the nature of the interaction of Tregs with commensal bacteria is unknown. Several scenarios can be envisioned: 1) Tregs, though selected in the thymus towards the recognition

of self-antigen could cross-react to environmental antigen, which as a consequence would initiate activation, expansion and differentiation of the cross-reactive Tregs as suggested by Belkaid et al. (Belkaid, et al., 2006; Suffia, et al., 2006). 2) The repertoire of Tregs may indeed be strictly self-reactive and peripheral activation may occur solely in the presence of the respective self-antigen. Nevertheless, environmental antigen could indirectly activate proliferation of Tregs when presented on activated APCs together with the respective self-antigen. Release of self-antigen would be increased during acute inflammation due to tissue-damage. 3) Response to bacterial compounds could be mediated by receptors directly sensing the presence of pathogen associated molecular patterns. Distinct Toll-like receptors (TLR) are selectively expressed by Tregs (Caramalho, et al., 2003) and recognise i.e. bacterial LPS, CpG-DNA or peptidoglycan. Ligand interaction with TLRs was shown to influence the suppressive activity of Tregs (Kubo, et al., 2004; Pasare and Medzhitov, 2003) and to induce expansion of Tregs *in vitro* even in the absence of additional TCR-stimulation (Liu, et al., 2006; Sutmuller, et al., 2006).

None of these interactions are mutually exclusive and clearly provide a basis to explain the effect of the presence or absence of intestinal bacteria on the number and the proliferative activity of $Foxp3^+$ Tregs demonstrated in this study. The impact of individual mechanisms on the peripheral expansion of Tregs will have to be dissected in future studies.

A matter of homeostatic equilibrium?

The observed decreases of both Treg numbers and *in vivo* proliferation in GALT upon depletion of commensal flora favour the view of a direct interaction between intestinal bacterial stimuli and $Foxp3^+$ Tregs, either by antigen-specific or through bystander activation. However, another interpretation of the present data could be a strict regulation of the homeostatic balance between conventional T cells and Tregs. Such regulation would imply that only as many Tregs are maintained under steady state conditions as are required for suppression of potentially harmful immune responses to peripheral antigens. Such a balance could be regulated via the production of IL-2, which is indispensable for maintenance of peripheral Tregs (Bayer, et al., 2005; D'Cruz and Klein, 2005; Fontenot, et al., 2005; Furtado, et al., 2002; Malek and Bayer, 2004; Sadlack, et al., 1995; Schorle, et al., 1991; Setoguchi, et al., 2005; Suzuki, et al., 1995; Willerford, et al., 1995). Tregs themselves are not capable to produce IL-2 and therefore crucially depend on paracrine IL-2 for their survival and expansion in the periphery. While certain DCs may represent one possible cellular source of IL-2 (Granucci, et al., 2001), more likely, activation and IL-2 production by conventional $CD4^+$ T cells supply the survival factors for Tregs (Setoguchi, et al., 2005). This provides a feed back loop, which links the number and proportion of $Foxp3^+$ Tregs directly to the number of IL-2 producing T cells as recently proposed by Almeida and colleagues (Almeida, et al., 2006). In the absence of activating signals from microbial antigen the reduction of conventional T cell produced IL-2 would then lead to reduced numbers and

proliferation of Tregs and thereby regulate the homeostatic equilibrium of these two T cell subsets to each other.

Based on the finding that IL-2 is not essential for thymic development of Foxp3$^+$ Tregs, while it is required for an adequate size of the Treg cell population in the periphery (Fontenot, et al., 2005), reduced IL-2 production by conventional T cells in the absence of intestinal stimuli should much more affect the number of Treg subsets associated with homeostatic expansion. Consistent with this idea, our finding that the proportion of naïve-like α_E^-CD25$^+$ Tregs was increased among CD4$^+$Foxp3$^+$ cells after eradication of the intestinal microflora supports the notion that this population relies more on thymic export than on homeostatic expansion/survival. In contrast, the generation of effector/memory-like α_E^+ Tregs may respond much more sensible to the presence or absence of survival factors like IL-2 leading to reduced peripheral proliferation and a decrease in population size.

4.2 Generation of effector/memory-like Tregs *in vivo*

Differentiation of naïve-like CD25$^+$ Tregs into α_E^+ Tregs

The majority of naturally occurring Foxp3$^+$ Tregs are thought to originate from the thymus, where they are generated as a distinct lineage, which is selected towards recognition of self-antigen. The absolute contribution of these thymic-derived Tregs to the peripheral pool of Foxp3$^+$ Tregs is currently unknown. In order to clarify the developmental relationship between the different naturally occurring Foxp3$^+$ Treg subsets, this part of the project aimed to identify the precursor of the α_E-expressing Treg subset. When OVA-specific naïve-like α_E^-CD25$^+$ Tregs were transferred, followed by oral antigen administration, the vast majority of CD25$^+$ Tregs previously negative for α_E up-regulated expression of this marker, suggesting that tolerogenic, antigen-specific activation of naïve-like Tregs led to proliferation, differentiation and acquisition of an effector/memory-like phenotype. Under these conditions, transferred CD25$^+$ Tregs maintained high levels of Foxp3 and CD25 expression.

Earlier studies in rats already indicated a requirement for access to the respective auto-antigen for survival and function of Tregs, suggesting self-antigen-driven expansion in the periphery (Seddon and Mason, 1999). Direct evidence for proliferation of CD25$^+$ Tregs *in vivo* upon antigen-specific activation was provided in subsequent studies (Fisson, et al., 2003; Klein, et al., 2003; Walker, et al., 2003). Proliferation depended on the presence of antigen within the local environment and consequently, was restricted to LNs draining the site of tissue-expression or antigen-uptake. The kinetics of the antigen-specific proliferative response of CD25$^+$ Tregs were found to be similar to those of CD25$^-$ cells activated under the same conditions *in vivo* (Klein, et al., 2003). While no correlation with the expression of α_E was included in these studies, the present data confirm and extend the previous findings by demonstrating a close link between tolerogenic activation of naturally occurring Tregs and induction of an effector/memory-like phenotype characterised by α_E-expression.

De novo induction of α_E^+Foxp3$^+$ Tregs

When conventional, naïve α_E^-CD25$^-$Foxp3$^-$CD62L^{high} DO11.10 T cells were transferred and subjected to the same regimen of antigen administration, induction of Foxp3$^+$ cells in a dose-dependent fashion was observed. Induction of Foxp3$^+$ cells was most prominent in the livLN, where basically all cells acquired Foxp3 expression after two rounds of division, while undivided cells only partially up-regulated Foxp3. Several studies have demonstrated the conversion of non-regulatory, conventional T cells into Tregs under distinct conditions *in vivo*, both by using TCR-tg (Apostolou and von Boehmer, 2004; Cobbold, et al., 2004; Hultkrantz, et al., 2005; Kretschmer, et al., 2005; Mucida, et al., 2005; Thorstenson and Khoruts, 2001) and polyclonal naïve precursor cells (Curotto de Lafaille, et al., 2004; Liang, et al., 2005).

One of the first studies to show the emergence of CD4$^+$CD25$^+$ upon i.v. injection of peptide or oral protein administration, employed adoptive transfer of OVA-specific DO11.10 CD25$^-$ cells from mice on a RAG-2$^{-/-}$ background (DORAG) (Thorstenson and Khoruts, 2001). In contrast to DO11.10 mice, DORAG mice do not contain CD25$^+$ Tregs (Itoh, et al., 1999) and therefore, use of these mice excluded the potential outgrowth of pre-existing CD25$^+$ Tregs and clearly demonstrated the capacity to induce Tregs *de novo* under appropriate conditions *in vivo*. Apostolou and colleagues used low dose s.c. peptide administration via implanted osmotic pumps and could demonstrate that under these conditions CD25$^+$Foxp3$^+$ Tregs were generated in thymectomised TCR HA tg RAG-2$^{-/-}$ mice in the absence of overt activation and proliferation (Apostolou and von Boehmer, 2004). Another study from this group followed a different approach by targeting DCs with a specific antibody (DEC205) coupled to the cognate antigen and demonstrated that transferred TCR HA tg RAG-2$^{-/-}$ cells acquired expression of Foxp3 in a dose- and proliferation-dependent manner (Kretschmer, et al., 2005). Mucida et al. stated that induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs was possible upon oral antigen administration in a mouse strain containing HA-monospecific B cells and OVA-monospecific T cells on a RAG-1$^{-/-}$ background (T/B monoclonal). Devoid of thymic-derived Tregs newly generated Tregs were able to prevent subsequent allergic responses towards OVA-HA immunisation (Mucida, et al., 2005). A different experimental set up used the same T/B monoclonal mice as recipients of BALB/c polyclonal CD4$^+$CD25$^-$ T cells. Lymphopenia-driven proliferation equally led to the generation of donor-derived CD4$^+$CD25$^+$ Tregs expressing high levels of Foxp3 mRNA (Curotto de Lafaille, et al., 2004). Similarly, Liang and colleagues showed induction of polyclonal CD4$^+$CD25$^+$Foxp3-mRNA$^+$ Tregs. A fraction of C57BL/6 CD4$^+$CD25$^-$ T cells transferred into congenic recipients converted into a phenotype indistinguishable from naturally occurring Tregs upon peripheral proliferation in sub-lethally irradiated and non-irradiated hosts. These authors reported that the conversion was independent of the thymus but required co-stimulation via B7 (Liang, et al., 2005).

The data from our study are in line with the above findings, however here the *de novo* induction of Foxp3 upon oral antigen administration was directly visualised on the single cell level. This provides

conclusive evidence that conversion of naïve $CD4^+$ T cells into a phenotype that is indistinguishable from natural $CD25^+Foxp3^+$ Tregs can occur in mucosal tissues under tolerogenic conditions.

Expression of α_E was analysed in some studies and detected on a fraction of converted $CD25^+$ Tregs similar to frequencies observed on naturally occurring $CD25^+Foxp3^+$ Tregs (Curotto de Lafaille, et al., 2004; Liang, et al., 2005). Hultkrantz and colleagues described an important role for the livLN in the generation of $CD25^+$ Tregs, characterised by high co-expression of α_E and suppressive capacity (Hultkrantz, et al., 2005). However, the authors did not detect Foxp3 mRNA in this subset. In contrast, in our study expression of both α_E and Foxp3 was consistently observed on the single cell level. The periportal livLN drains the liver, stomach and pancreas (Matsuno, et al., 1990) and several studies have shown an important role for the liver in generating tolerogenic immune responses to food antigen and even in transplantation settings (Knolle, 2006; May, et al., 1969). Antigen from the intestinal lumen reaches the liver directly via the portal vein and occlusion of the portal vein prevented the development of oral tolerance (Yang, et al., 1994). *De novo* induction of $Foxp3^+$ Tregs in the livLN upon oral antigen administration found here underlines a pivotal role for the livLN in the induction for periperal tolerance.

$Foxp3^+$ Tregs develop from naïve $CD4^+$ T cells

The data presented here indicated that $Foxp3^+$ Tregs could be generated by conversion of previously $Foxp3^-$ non-regulatory T cells. That this was not due to selective outgrowth or survival of small numbers of contaminating $Foxp3^+$ T cells was concluded from the following findings: 1) Up-regulation of Foxp3 occurred even on a part of the non-divided $CFSE^{high}$ fraction of transferred cells, but only in the presence of cognate antigen. This argues against selective outgrowth of the pre-existing 0.12% of $Foxp3^+$ cells. 2) The absolute cell numbers recovered after transfer of either 6.2×10^3 $CD25^-Foxp3^+$ cells or 3.5×10^5 $CD25^+Foxp3^+$ clearly contradicted the possibility that appearance of $Foxp3^+$ Tregs in recipients of $CD25^-$ cells was due to expansion from the pre-existing contaminating $CD25^-Foxp3^+$ subset. Otherwise a dramatically increased expansion and/or survival rate (30 to 70-fold) would have to be assumed compared to the situation where $CD25^+Foxp3^+$ had been transferred. Although this scenario cannot be formally excluded, it appears to be highly unlikely and was also disproved in a study by Liang and colleagues (Liang, et al., 2005). Therefore, based on these findings we concluded that $Foxp3^+$ Tregs were induced from the non-regulatory $CD62L^{high}$ T cell pool in response to the particular conditions of antigen-specific activation, predominantly in the livLN, following oral administration of cognate antigen.

A role for TGF-β in generation of $\alpha_E^+Foxp^+$ Tregs in vivo?

It is tempting to speculate that TGF-β might be a major factor driving generation of $Foxp3^+$ cells in the setting described here. Several *in vitro* data have shown induction of Foxp3 upon stimulation in the

presence of TGF- β (Chen, et al., 2003; Fantini, et al., 2004). Furthermore, TGF-β is important for maintenance of Foxp3 expression and suppressive function of Tregs *in vivo* (Huber, et al., 2004; Marie, et al., 2005). Protection from disease in NOD mice was associated with expansion of $Foxp3^+$ T cells in the draining LN following transient over-expression of TGF-β in the islets (Peng, et al., 2004). On the other hand, *de novo* generation of $CD4^+CD25^+Foxp3^+$ Tregs *in vivo* was reduced or completely inhibited by administration of blocking anti-TGF-β mAbs during tolerance induction to skin grafts under the cover of anti-CD4 mAb treatment (Cobbold, et al., 2004), when targeting $DEC205^+$ DCs (Kretschmer, et al., 2005) or during oral antigen administration (Mucida, et al., 2005). Taken together, these findings clearly indicate a role for TGF-β in the induction and maintenance of $Foxp3^+$ cells *in vivo*. Next to these recent results, it has been known for some time that TGF-β is a major factor inducing expression of α_E on $CD4^+$ T cells (Kilshaw and Murant, 1991). Initial data from our group confirmed that *in vitro* activation of naïve $CD4^+CD25^-$ T cells in the presence of TGF-β induced co-expression of both Foxp3 and α_E (data not shown). However, expression of Foxp3 upon *in vitro* stimulation was not stable and associated with poor demethylation of a conserved promoter element in the *foxp3* locus in contrast to $CD4^+CD25^+$ displaying a high degree of demethylation of the same region (Floess, et al., in press). The stability of Foxp3 expression of *in vivo* induced Tregs is currently unknown.

Taken together, induction of α_E-expression on Tregs may reflect activation in a microenvironment where local concentrations of active TGF-β were sufficient to both maintain Foxp3 expression in proliferating thymus-derived $CD25^+$ Tregs and to generate new $Foxp3^+$ cells from non-regulatory T cells.

α_E-expression in chronic infection

Expression of α_E has also been associated in a number of studies with peripherally induced/expanded Tregs in infection models. Suffia et al. demonstrated a functional role for α_E-expression in the retention of Tregs at epithelial sites, where they suppressed the local response to Leishmania major and were critically involved in the chronicity of the infection (Suffia, et al., 2005). The Tregs were parasite-specific and derived from the naturally occurring $Foxp3^+$ Treg pool. Their proliferation and survival depended on the presence of the parasite (Suffia, et al., 2006). Baumgart and colleagues demonstrated in a model of Schistosoma mansoni that during the course of the disease $Foxp3^+$ Tregs changed their phenotype and acquired increasing frequencies of α_E^+ cells within the $CD4^+Foxp3^+$ Treg population, while the overall proportion of $Foxp3^+$ Tregs remained unchanged. Again, the presence of these activated Tregs was associated with dampening of the egg-induced inflammation and thus contributed to survival of the parasite within the infected host (Baumgart, et al., 2006). A similar increase in the frequency of $\alpha_E^+Foxp3^+$ Tregs during disease progression was observed in a model of Heligmosomodes polygyrus infection (S. Hartmann, personal communication).

The factors involved in up-regulation of α_E during chronic infection are unknown, however, it was suggested that the parasites modulate the local environment to favour α_E-mediated retention of Tregs in the dermis of L. major infected skin. A direct role for TGF-β is currently merely a matter of speculation, but expression of α_E was enhanced on Tregs isolated from chronically infected mice upon culture with L. major-infected DCs *in vitro*, while the presence of pro-inflammatory stimuli (LPS) together with infected DCs down-regulated the α_E-expression on Tregs (Suffia, et al., 2005).
These findings are in line with the results obtained in our study, where tolerogenic activation of adoptively transferred naïve-like $\alpha_E^-CD25^+$ Tregs preferentially led to up-regulation of α_E on the majority of Tregs, while i.p. immunisation in the presence of LPS was much less effective in this respect (data not shown).

Origin of α_E^+ Tregs in immunocompetent mice

In summary, the data obtained here support the hypothesis that $\alpha_E\beta_7$ expression correlates not only with an effector/memory-like phenotype of $Foxp3^+$ Tregs, but in addition identifies Tregs that are constantly cycling under steady state conditions in healthy mice. The sequence of events as hypothesised here postulates that the pool of naïve-like $\alpha_E^-CD25^+$ Tregs is replenished by constant export from the thymus. In the periphery, encounter with cognate antigen (self or cross-reactive non-self) induces expansion and differentiation of these thymic-derived Tregs into an effector/memory-like phenotype characterised by expression of α_E. In the absence of thymic production an imbalance between input and transition leads to a decrease of the naïve Treg population. In contrast, upon reduction of peripheral proliferation and differentiation due to removal of environmental antigen/stimuli the balance is skewed towards a relative increase of this population. In line with these considerations are the observations made for the effector/memory-like α_E^+ Treg pool. The size of this population is maintained in the absence of thymic export but cell number and proliferation are decreased upon removal of microbial antigen/stimuli.
Furthermore, the results presented here strongly suggest, that the $\alpha_E^+Foxp3^+$ Treg pool is of diverse origin, consisting of both thymic-derived, expanded natural Tregs as well as of *de novo* generated $Foxp3^+$ Tregs, induced upon tolerogenic, antigen-specific activation in distinct anatomical sites of the body. These findings further enlarge our understanding of the fundamental aspects of Treg biology and support the concept, that $Foxp3^+$ Tregs with a phenotype indistinguishable from the naturally occurring $Foxp3^+$ Treg pool can be generated *de novo* under appropriate conditions of antigen-delivery in the periphery, while at the same time the very same conditions promote proliferation/expansion of pre-existing natural Tregs.

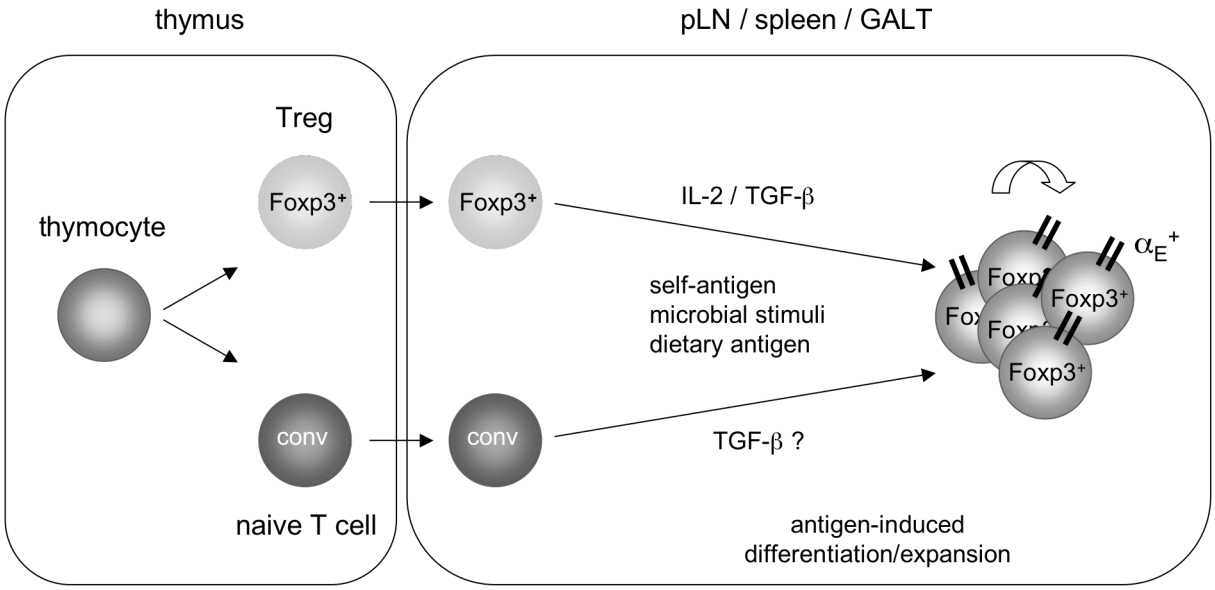


Figure 20: Origin and development of α_E^+Foxp3$^+$ Tregs

4.3 Selective induction of organ-specific homing receptors on Tregs *in vitro*

Based on the general view that suppressive function of Tregs requires direct cell-cell contact or at least close proximity, it is conclusive that Tregs should display the same migratory properties as their target cells. Further, accumulating evidence suggests that suppression of unwanted immune responses may take place not only during initiation of an immune response in secondary lymphoid tissue but also during the effector phase directly at peripheral sites of inflammation. As a consequence, appropriate migration and localisation of Tregs is a prerequisite for their function *in vivo*.

The importance of selective trafficking of Tregs might be concluded from several studies observing that naïve $CD62L^{high}CD25^{+}CD4^{+}$ Tregs, but not their $CD62L^{low}$ counterparts and/or occupancy within LNs were required for preventing development of diabetes, skin inflammation, graft rejection and GvHD (Ermann, et al., 2005; Schwarz, et al., 2004; Szanya, et al., 2002; Taylor, et al., 2004). These findings suggest that the ability to home to or reside within peripheral lymphoid tissue was important to suppress the onset of disease.

In contrast, efficiency of Tregs in established disease relied largely on distinct subsets of effector/memory-like Tregs and indicated a role for recruitment to peripheral sites of inflammation. Some studies demonstrated the importance of distinct Treg subsets selectively expressing inflammatory chemokine receptors such as CCR2 and CCR6 (Bruhl, et al., 2004; Kleinewietfeld, et al., 2005). Furthermore, previous data from our group showed that deficiency in the expression of functional skin-tropic E/P-selectin ligands on Tregs abrogated recruitment of these cells to the inflamed skin and thus, their capacity to ameliorate local inflammation (Huehn, et al., 2004; Siegmund, et al., 2005). These findings clearly indicated that tissue-specific migration of Tregs was crucial for effective function *in vivo*.

The use of Tregs for adoptive transfer in various disease models represents an attractive therapeutic option. However, due to the low numbers of Tregs that can be isolated from an individual, expansion of Tregs *in vitro* is currently tested as a procedure to reach the numbers potentially necessary for *in vivo* function of transferred Tregs. In this respect, the idea to modulate the homing receptor phenotype of isolated Tregs during *in vitro* culture presented an interesting aspect. Thus, in this part of the study, the feasibility of such an approach was tested by culturing *ex vivo* isolated naïve Tregs under distinct tissue-skewing conditions.

Tissue-specific DCs modulate homing receptor expression of $CD25^{+}$ Tregs

Extended phenotypic analysis of effector/memory Tregs not only confirmed the increased expression of various homing receptors (Huehn, et al., 2004) but indicated also that effector/memory Tregs displayed a high degree of compartmentalisation under steady state conditions. Cells expressing the mucosal homing receptor $\alpha_4\beta_7$ resided predominantly in mLN, whereas cells expressing selectin ligands were enriched in pLN (data not shown). These findings indicated a role for the tissue-specific microenvironment in the instruction of homing receptor phenotypes on Tregs, similar to results

obtained previously for conventional $CD4^+$ T cells (Campbell and Butcher, 2002). Organ-specific DCs have been shown to play a critical role for the induction of homing receptors on conventional T cells. Whereas *in vitro* T cell activation by intestinal DCs from PP and mLN is necessary and sufficient to generate a $\alpha_4\beta_7^+$ gut-homing phenotype, DCs isolated from pLNs induce higher levels of selectin ligands than intestinal DCs (Dudda, et al., 2004; Johansson-Lindbom, et al., 2003; Mora, et al., 2003; Stagg, et al., 2002).

The findings obtained in the current study using *ex vivo* isolated organ-specific $CD11c^+$ DCs to stimulate naïve Tregs *in vitro,* were largely in line with previous reports on cultures of conventional T cells in the presence of DCs isolated from either skin-draining or gut-draining lymphoid organs. Recent studies by Annacker et al. and Johansson-Lindbom et al. (Annacker, et al., 2005; Johansson-Lindbom, et al., 2005) identified a subset of mLN-DCs characterised by the expression of α_E, which selectively induced CCR9 and $\alpha_4\beta_7$ expression on T cells upon antigen-specific activation *in vitro*. In co-cultures with $CD8^+$ OT-I cells CCR9 and $\alpha_4\beta_7$ were only incuced by $\alpha_E^+CD11c^+$ DCs but not in the presence of $\alpha_E^-CD11c^+$ DCs. In contrast, cultured $CD4^+$ OT-II cells up-regulated expression of $\alpha_4\beta_7$ in presence of either DC subset, while CCR9 expression was only induced by $\alpha_E^+CD11c^+$ DCs (Johansson-Lindbom, et al., 2005). The above findings indicate that expression of $\alpha_4\beta_7$ and CCR9 is differentially regulated in $CD4^+$ T cells, while in $CD8^+$ T cells the induction of both homing receptors appeared to require similar molecular signals. The results obtained in our study showing selective induction of $\alpha_4\beta_7$, but not CCR9 on $CD25^+$ Tregs co-cultured with mLN-derived DCs may therefore reflect insufficient numbers of $\alpha_E^+CD11c^+$ among the whole mLN-derived $CD11c^+$ fraction and support the concept that expression of these two gut-specific homing receptors is differentially regulated not only in conventional $CD4^+$ T cells but also in $CD4^+CD25^+$ Tregs.

Similar to results obtained previously with $CD8^+$ T cells (Mora, et al., 2005), in the study presented here "default" induction of P-Lig in co-cultures with mLN-derived DCs was observed. Cultures in the presence of pLN-derived DCs led to only slightly increased frequencies of $P\text{-}Lig^+$ cells. This was in line with previous findings by our group, where P-Lig-expression was induced on conventional naïve T cells in the presence of mLN and PP-derived DCs, albeit to a lower degree than in the presence of pLN-derived DCs (unpublished observation).

Taken together, the results obtained during activation of naïve $CD25^+$ Tregs in co-cultures with tissue-specific *ex vivo* isolated DCs indicate that Tregs largely respond to the same stimuli as conventional T cells and acquire gut-specific and skin-specific homing receptor expression, respectively.

$CD25^+$ Tregs respond to soluble factors

Among the molecular factors that control the induction of homing receptor expression on T cells, RA has recently been shown to play a critical role for the generation of gut-tropic T cells (Iwata, et al., 2004). In a thorough study these authors identified the causative link between lack of effector/memory T cells in the gut mucosa of vitamin A deficient mice and the molecular mechanisms involved in the

induction of gut-tropic T cells. They found that only intestinal DCs from PP and mLN, but not splenic or pLN-derived DCs, expressed retinal dehydrogenase isoenzymes required for conversion of RA from the vitamin A metabolite retinal. In turn, RA that binds to nuclear receptors for RA (RAR and RXR), which function as heterodimeric ligand-inducible transcription factors, induced expression of $\alpha_4\beta_7$ and CCR9 in T cells (Iwata, et al., 2004). Consistent with a pivotal role of RA during intestinal imprinting, effector T cells that are stimulated in the presence of exogenous RA acquire a marked gut-homing phenotype irrespective of their prior tissue commitment or the nature of the activating stimulus (Mora and von Andrian, 2006). These findings suggest that RA dominantly controls the establishment of gut-specific homing.

Our data demonstrate that also Tregs are susceptible to RA and can be converted into an $\alpha_4\beta_7$-expressing gut-homing phenotype by activation in the presence of RA. Yet, induction of CCR9 was only observed on a minor fraction of Tregs in the presence of RA. Induction was dose-dependent and lower doses of RA (1 nM) did not consistently induce CCR9 expression under the experimental conditions used here (data not shown).

Similar to what was found for the co-cultures with organ-specific DCs, induction of P-Lig expression on Tregs under the influence of polarising factors was less selectively controlled, with IL-12 having only an additive effect. This was in contrast to E-Lig expression, which was suppressed upon addition of RA, as has been reported previously (Iwata, et al., 2004). Our findings suggest that distinct pathways control expression of the two functional selectin ligands in Tregs.

Blocking of residual IL-12 activity by addition of an anti-IL-12 mAb in RA and neutral cultures did not prevent P-Lig induction on Tregs, indicating that a molecular mechanism independent of IL-12 and insensitive to RA-derived signals may be involved. In line with our observation, White and colleagues demonstrated that T cell activation alone induced expression of FucT-VII, which is required for expression of functional E/P-Lig (White, et al., 2001).

Based on these findings a role for pathways independent of IL-12, but associated with TCR-stimulation may be relevant for induction of P-Lig in Tregs, at least under the culture conditions used here. Clearly, the basis of the distinct molecular mechanisms should be subject of future studies.

$CD25^+$ Tregs maintain their phenotypic characteristics upon in vitro modulation

Having established that naïve $CD25^+$ Tregs can acquire distinct homing receptor phenotypes upon culture with the appropriate signals, an important issue was to analyse the effect of such treatment on the expression of the Treg specific transcription factor Foxp3. Importantly, the vast majority of $CD25^+$ Tregs continued to express Foxp3 following the culture period. RA-cultured Tregs expressed the highest frequency of $Foxp3^+$ cells followed by Tregs cultured under neutral conditions. Addition of IL-12 led to slightly, but significantly reduced frequencies of $Foxp3^+$ cells. Another observation made during the culture experiments was the fact that only sorting for $CD25^+CD62L^{high}$ Tregs, but not for naïve-like $\alpha_E^-CD25^+$ Tregs, yielded high frequencies of Foxp3-expressing cells, suggesting that some

contaminating $\alpha_E^-CD25^+$ non-regulatory effector cells might over-grow the cultures (data not shown). A similar observation has been reported for human $CD25^+$ Tregs showing that only the naïve $CD25^+CD45RA^+$ fraction gave rise to stable Foxp3-expressing Tregs (Hoffmann, et al., 2006). In contrast, during *in vivo* activation of naïve-like $\alpha_E^-CD25^+$ consistently high frequencies of $Foxp3^+$ cells were maintained upon both oral and i.p. immunisation (Figure 12 and data not shown). These differential findings may reflect the requirement of additional factors for maintenance of Foxp3-expression i.e. TGF-β as discussed above or other cell contact dependent signals.

Nevertheless, despite the somewhat reduced frequency of $Foxp3^+$ cells, Tregs cultured in the presence of IL-12 were as effective as neutral or RA-cultured Tregs in suppressing proliferation of naïve CFSE-labelled responder cells in an *in vitro* assay. The suppressive capacity of all expanded Tregs was comparable to to freshly isolated $CD25^+$ Tregs. These results suggest that expansion of Tregs under conditions, which modulate the homing receptor phenotype, do not change their principle characteristics such as Foxp3-expression and *in vitro* suppression of T cell proliferation.

Migratory properties of in vitro modulated $CD25^+$ Tregs

Analysis of the migration behaviour of the *in vitro* polarised Tregs showed that upon adoptive transfer Tregs cultured under polarising conditions displayed selective organ-specific homing properties. Inflamed skin selectively recruited IL-12-cultured Tregs, while migration to gut and GALT was most prominent, though not exclusive, for Tregs cultured in the presence of RA. Low-level expression of $\alpha_4\beta_7$ on neutral and IL-12-cultured Tregs may have been sufficient to enable homing to the intestine and associated lymphoid tissue. In addition, although it has been repeatedly demonstrated that the integrin $\alpha_4\beta_7$ is important for migration of T cells into both the non-inflamed as well as the inflamed intestine (Berlin, et al., 1993; Hamann, et al., 1994; Holzmann and Weissman, 1989), an additional role of LFA-1 and selectins and their ligands has also been shown by others (Haddad, et al., 2003; Lefrancois, et al., 1999; Rivera-Nieves, et al., 2005) and in our own lab (unpublished data). In contrast, P-Lig expression detected on RA-cultured Tregs was not sufficient to allow trafficking to inflamed skin indicating that either E-Lig expression was crucial or alterations in expression or functional activity of other homing-related receptors contributed to the increased inflammation-seeking capacity of IL-12-treated Tregs.

These findings confirmed the functionality of the induced homing receptor phenotype and further supported the notion that Tregs are equally susceptible to the molecular factors controlling expression of adhesion molecules and chemokine receptors as conventional $CD4^+$ T cells. However, previous work with conventional T cells revealed an unexpected degree of convertibility in the migration behaviour. The results suggested that peripheral tissue commitment of conventional memory T cells was not a stable property, but apparently reflected dynamic functional states (Dudda, et al., 2005; Mora, et al., 2005). Nevertheless, results from our group observed significant stability of a $P\text{-}Lig^+$

phenotype over prolonged periods *in vivo*, at least in a subfraction of conventional effector/memory T cells (Jennrich et al., manuscript submitted (Jennrich, et al., in revision)).
Thus, it remains to be shown to what extent induced homing phenotypes can become stably imprinted and whether this also applies to Tregs.

Use of in vitro cultured Tregs for adoptive transfer

The data presented here indicate that Tregs display similar plasticity with respect to the instruction to specific homing by activation within distinct microenvironments. This suggests that a custom modulation of Tregs designed to serve therapeutic purposes in adoptive T cell therapy might be feasible and provides the option to generate Treg populations with specific homing properties. To date, only few studies have shown that adoptive transfer is a feasible therapeutic option in established disease. Suri-Payer and colleagues demonstrated that $CD4^+CD25^+$ suppressed not only the induction of autoimmune disease in thymectomised recipients, but also efficiently suppressed disease induced by cloned autoantigen-specific effector cells (Suri-Payer, et al., 1998). In models of experimental colitis cure of intestinal inflammation was achieved upon transfer of *ex vivo* isolated or activated $CD25^+CD4^+$ Tregs (Liu, et al., 2003; Mottet, et al., 2003). Tang and colleagues demonstrated that small numbers of antigen-specific *in vitro* expanded Tregs were able to reverse established diabetes (Tang, et al., 2004). It will of course be interesting to investigate the efficiency of Tregs instructed to acquire specific homing properties in ongoing immune reactions such as autoimmunity or chronic inflammation. However, preliminary experiments from our group in the DTH model of acute skin-inflammation revealed only moderate efficiency of *in vitro* expanded Tregs *per se*. Despite selective short-term recruitment of IL-12-cultured Tregs they were only marginally superior to RA-cultured Tregs in ameliorating the inflammatory response (Appendix 3). Furthermore, although they remained $Foxp3^+$ and preserved *in vitro* suppressive capacity, cultured Tregs were less effective in this model compared to *ex vivo* isolated effector/memory-like α_E^+ Tregs as shown previously (Siegmund, et al., 2005). The exact mechanisms responsible for effective suppressor function of Tregs *in vivo* are currently unknown and a role for a number of soluble and/or surface molecules has been proposed (reviewed in (von Boehmer, 2005)). In particular, it is not clear how well *in vitro* suppressive mechanisms reflect relevant *in vivo* mechanisms. Nevertheless, suppressive capacity *in vitro* seems to be a minimum requirement for potential therapeutic use of expanded Tregs.
These preliminary findings highlight the necessity for an optimised culture system aimed to preserve the intrinsic functional properties of isolated Tregs and for establishment of informative *in vitro* assays allowing a more precise prediction of *in vivo* function of cultured Tregs. Furthermore, the therapeutic effect of adoptively transferred Tregs may be different in individual disease models and thus, careful evaluation will be necessary to examine the feasibility of curative Treg transfer strategies.

4.4 Conclusion and Outlook

The results obtained in this study support the notion that multiple factors in secondary lymphoid tissue contribute to the continuation of immune homeostasis by providing various stimuli for expansion and maintenance of Tregs in the periphery. The physiological relevance of intestinal colonisation by commensal microbes is illustrated by the finding that Foxp3$^+$ Treg numbers were substantially decreased in gnotobiotic mice. The proportion and absolute number of proliferating Tregs were reduced in these mice, suggesting that local responses in GALT impact on the homeostasis of the peripheral Treg pool. It will be interesting to investigate in future studies the basis of the observed effect of intestinal microflora, particularly whether the pool of thymic-derived self-reactive Tregs harbours sufficient diversity in order to directly recognise bacterial antigen. This can be addressed using *in vitro* culture systems of GALT-derived CD25$^+$ Tregs together with DCs and bacterial protein pools. Antigen-specific recognition should result in proliferation of Tregs and up-regulation of activation markers. Alternatively, other stimuli such as TLR-mediated signalling may contribute to peripheral expansion and maintenance of Tregs. This issue can be addressed by using Tregs from e.g. TLR-2$^{-/-}$, TLR-4$^{-/-}$ or MyD88$^{-/-}$ mice that are impaired in their capacity to respond to microbial products such as peptidoglycan and LPS.

In addition, the results achieved here demonstrate that oral antigen leads to antigen-specific expansion of pre-formed thymic-derived Tregs as well as to *de novo* induction of Foxp3$^+$ Tregs. This, in combination with the observed role of the commensal microflora, provides an important mechanism for the maintenance of Tregs and indicates that the mucosal microenvironment favours both expansion and conversion of Tregs. A currently unsolved issue relates to the stability of *de novo* induced Foxp3$^+$ T cells. Re-isolation and adoptive transfer into secondary hosts can be used to address this question and to determine, whether a stable cell lineage has been generated during oral tolerance induction. In this respect the use of T cells from GFP-Foxp3 reporter mice will be extremely helpful, as it allows re-isolation and FACS sorting of oral antigen-induced GFP-Foxp3-expressing cells for further functional analysis and for survival studies. In addition, this would permit analysis of the methylation status of the *foxp3* gene in *de novo* induced Foxp3$^+$ T cells in comparison to thymic-derived Foxp3$^+$ Tregs.

With regard to the homing receptor phenotype of Tregs, the results obtained here indicate that Tregs display similar plasticity as conventional T cells with respect to the instruction to specific homing by activation within distinct microenvironments. This can be regarded as proof of principle and suggests that the option to generate Treg populations with specific homing properties for therapeutic purposes in adoptive T cell therapy might be feasible. However, various important issues remain unsolved to date, including the stability of the induced homing phenotype and *in vivo* functional properties of expanded Tregs. Adoptive transfer experiments with and without re-stimulation *in vivo* can help to determine the stability of organ-specific phenotypes on Tregs. An important issue is to determine the usefulness of *in vitro* assays with respect to the corresponding *in vivo* suppressive capacity of Tregs. Ultimately, to test their functional properties, use of expanded Tregs in prevention and cure of disease

in animal models such as experimental colitis have to be performed. They should provide further insights into the feasibility of therapeutic concepts based on adoptive transfer of expanded Tregs with selective tissue-tropism.

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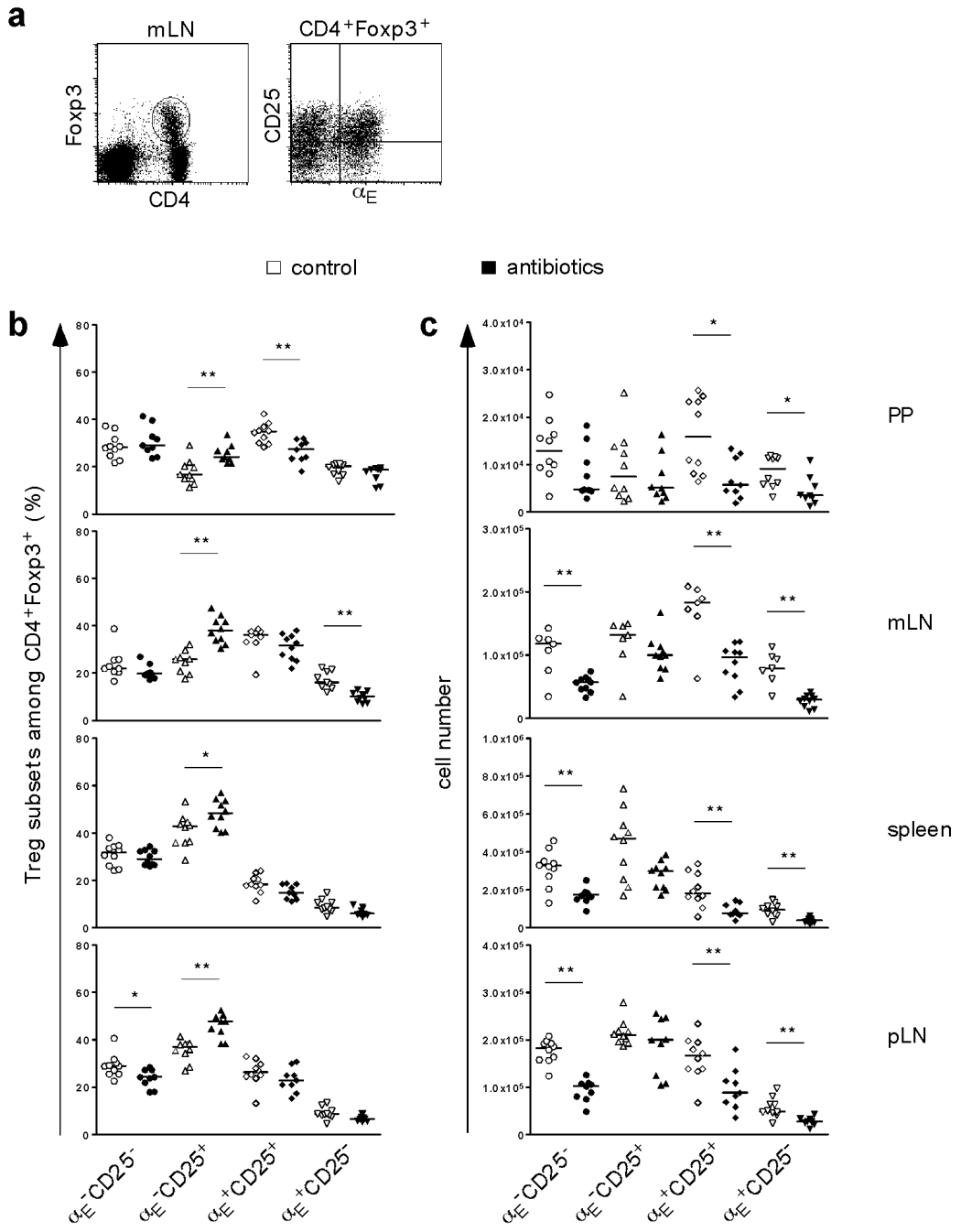
6 Appendix

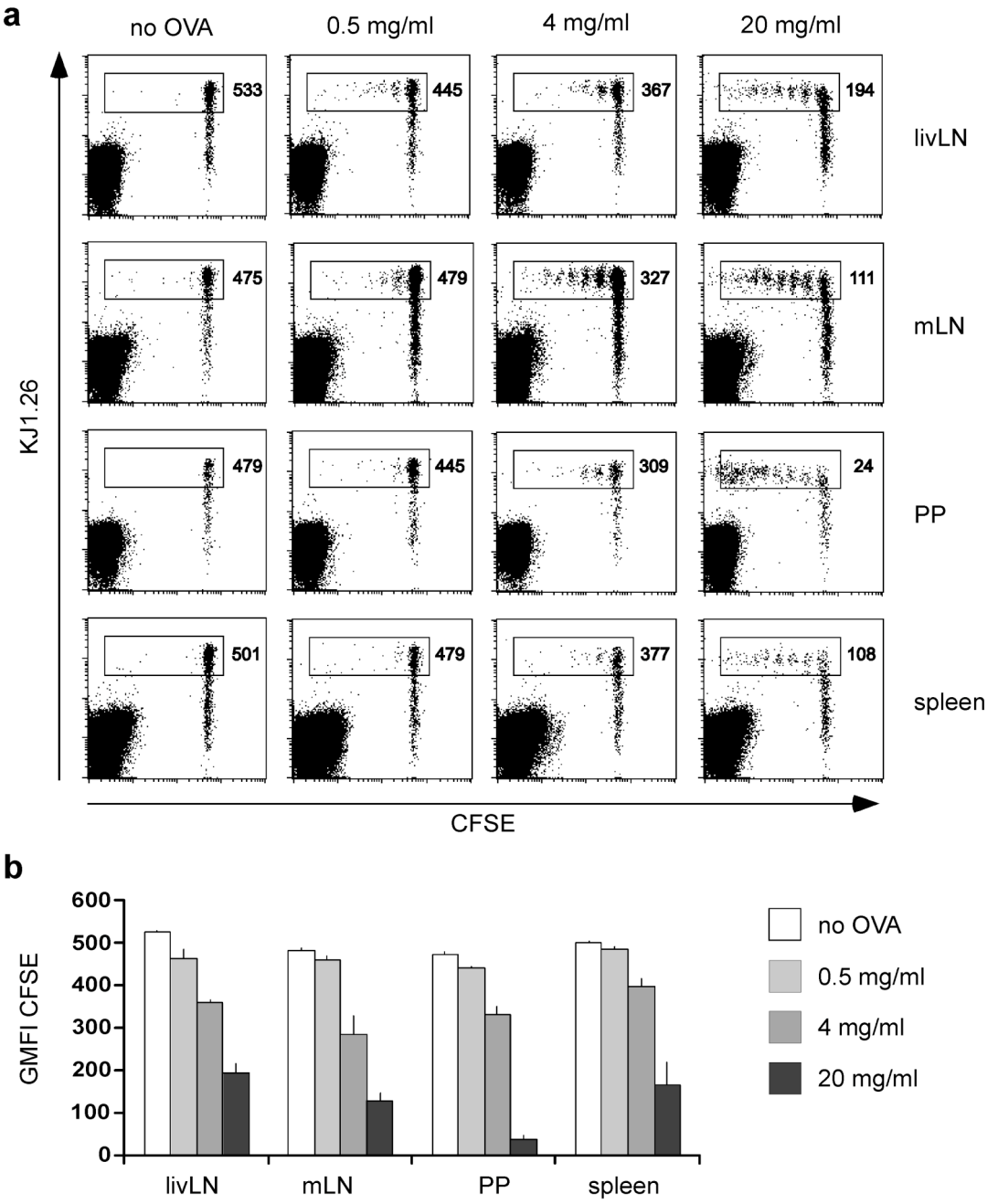
6.1 Appendix 1



Appendix 1: Microbial stimuli influence cell umbers of Foxp3$^+$ α_E/CD25 Treg subsets

C57BL/6 mice were treated with a cocktail of antibiotics *ad libitum* for a period of 8-10 weeks. Upon successful eradication of the intestinal microflora mice were analysed for the frequency and cell number of CD4$^+$ T cell subsets and compared to untreated control mice. Enumeration of lymphocyte numbers was done as described in Figure 9 and Foxp3-gated cells were analysed for the frequency and cell number of α_E/CD25 Tregs. (a) Representative dot plots showing staining of CD4 vs. Foxp3 on lymphocytes isolated from mLN of control mice (left) and staining of α_E vs. CD25 on CD4$^+$Foxp3$^+$ gated cells (right). (b) Frequency of α_E/CD25 Treg subsets among CD4$^+$Foxp3$^+$ cells in indicated organs from untreated control mice (open symbols) and antibiotics-treated mice (closed symbols). (c) Cell numbers of CD4$^+$Foxp3$^+$ α_E/CD25 Tregs in indicated organs. Graphs include data from two independent experiments, symbols represent individual mice and lines indicate the median. * $p < 0.05$; ** $p < 0.01$.

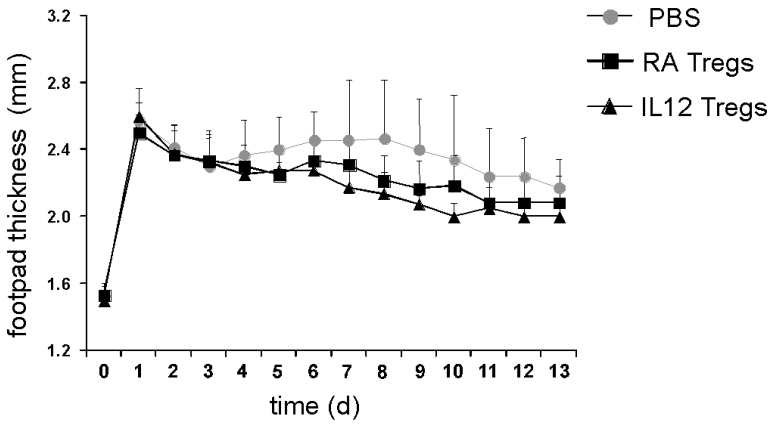
6.2 Appendix 2



Appendix 2: ***Dose-dependent proliferation of transferred naïve CD25- DO11.10 cells after oral antigen application***

Naïve T cells were isolated from DO11.10 mice by depleting α_E^+ and $CD25^+$ cells and subsequent isolation of $CD62L^{high}$ cells. Purified $\alpha_E^-CD25^-CD62L^{high}$ T cells were labelled with CFSE, adoptively transferred into BALB/c recipients and subsequently stimulated *in vivo* by oral application of their cognate antigen. After 6 days of continuous feeding with OVA, mice were sacrificed and lymphocytes were analysed by flow cytometry. Transferred cells were identified according to $CD4^+KJ1.26^+$ staining and CFSE. (a) Dotplots show representative staining of KJ1.26 and CFSE on $CD4^+$ gated cells from indicated lymphoid organs. Proliferation of re-isolated DO11.10 T cells after feeding of different doses of OVA was assessed by loss of CFSE of $KJ1.24^+$ cells and is expressed as GMFI of CFSE in gated regions. (b) GMFI of CFSE of re-isolated $CD4^+KJ1.26^+$ T cells in indicated lymphoid organs after different doses of OVA in drinking water. Mean ± SD from 3-4 individual mice per group.

6.3 Appendix 3



Appendix 3: *In vivo* suppressive potential of *in vitro* polarised Tregs.
Naïve $CD4^+CD25^+CD62L^{high}$ Tregs were isolated from DO11.10 mice and stimulated with $OVA_{323-339}$ peptide and $CD90^-$ APC under polarising conditions for 6 days. Cultured Tregs were harvested and adoptively transferred together with OVA-specific Th1 cells at a ratio of 1 : 2 (Treg : Th1). Control animals received only Th1 cells (PBS). One day later the DTH response was triggered by injecting $OVA_{323-339}$ peptide/IFA into one footpad as described in 2.2.5.4. The swelling of the footpad was followed over time and measured daily as described previously (Siegmund, et al., 2005). Graphs display mean footpad thickness ± SD from 4 to 7 individual mice per group.

7 Eidestattliche Erklärung

Hiermit erkläre ich, die von mir vorgelegte Dissertation selbständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben.

Ich habe mich nicht anderwärts um einen Doktorgrad beworben und besitze noch keinen entsprechenden Doktorgrad.

Ich erkläre zudem die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Mathematisch-Naturwisschenschaftlichen Fakultät I der Humboldt-Universität zu Berlin.

8 Lebenslauf

Persönliche Daten

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Promotion

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Berufstätigkeit

08/1994 – 09/01	Miltenyi Biotec GmbH, Bergisch Gladbach Projektleiterin in der Abteilung für Forschung und Entwicklung
03/1993 – 06/94	Klinik I für Innere Medizin der Universität zu Köln Technische Assistentin
08/1992 – 02/93	Cornell University Medical College, NY, NY, USA Department of Cell Biology and Anatomy Research Fellow
08/1988 – 07/92	Universität zu Köln Institut für Genetik Technische Assistentin

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10/2001 – 09/02	University of Birmingham, Großbritannien Postgraduierten Studium M.S.c. Immunology & Infection Thesis zu dem Thema: „A direct role for the rheumatoid synovial microenvironment in regulating T cell behaviour” Abschluss: Master of Science (Immunology) (mit Auszeichnung)
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9 Publikationsliste

Siewert C, Hamann A, Huehn J
Experience-driven development: effector/memory-like $\alpha_E\beta_7^+$Foxp3$^+$ regulatory T cells originate from both naïve T cells and naturally occurring naïve-like regulatory T cells (in Revision)

Siewert C, Menning A, Dudda J, Siegmund K, Haubold U, Floess S, Campbell DJ, Hamann A, Huehn J (2007) Induction of organ-selective CD4$^+$ regulatory T cell homing. *Eur J Immunol*. **37**:978-89

Floess S*, Jennifer Freyer J*, **Siewert C***, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Klein-Hessling S, Serfling E, Hamann A, Huehn J (2007) Epigenetic control of the *foxp3* locus in regulatory T cells. *PLos Biol.* **5**:e38 (* gleicher Beitrag)

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Alyonycheva T, Cohen-Gould L, **Siewert C**, Fischman DA, Mikawa T (1997) Skeletal muscle-specific myosin binding protein-H is expressed in Purkinje fibers of the cardiac conduction system. *Circ Res* **80**:665-72

Manuskripte in Vorbereitung:

Siewert C, Heimesaat MM, Liesenfeld O, Hamann A, Huehn J
Microbial stimuli induce expansion of Foxp3$^+$ regulatory T cells in gut-associated lymphoid tissue

10 Kongressbeiträge

Vorträge:

1st Joint Meeting of European National Societies of Immunology, 2006, Paris, France

Annual TH1/TH2 Meeting, 2006, Marburg, Germany

XVIII Congress of the International Society for Analytical Cytology, 1996, Rimini, Italy

Poster Präsentationen:

5th Annual Meeting of the Federation of Clinical Immunology Societies, 2006, Boston, MA, USA

Joint Annual Meeting of the Scandinavian and German Societies of Immunology, 2005, Kiel, Germany

1st Spring School of Immunology of the German Society of Immunology, 2005, Ettal, Germany

Joint Annual Meeting Immunology of the Dutch and German Societies of Immunology, 2004, Maastricht, Netherlands

3rd International Symposium on Minimal Residual Cancer, 2001, Hamburg, Germany

91st Annual Meeting of the American Association for Cancer Research, 2000, San Francisco, CA, USA

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