CTMI (2005) 293:89–114

Migration Rules: Functional Properties of Naive and Effector/Memory-Like Regulatory T Cell Subsets

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Abstract Suppressor T cells were first described in the early 1970s, but since the hypothetical soluble suppressor factor could not be identified on a molecular level and since appropriate cellular markers were lacking, the suppressor T cell concept vanished for a long time. The discovery by Sakaguchi and co-workers, that the adoptive transfer of $CD25^+CD4^+$-depleted T cells induced several organ-specific autoimmune diseases in immunodeficient recipients, put the suppressor T cell model back into the focus of many immunologists. $CD25^+CD4^+$ T cells were named regulatory T cells (Treg) and since then have been intensively characterized by many groups. It has now been well documented in a variety of models that $CD25^+CD4^+$ Tregs, in addition to cell-intrinsic peripheral tolerance mechanisms such as anergy induction and peripheral deletion, play indispensable roles in the maintenance of natural self-tolerance, in averting autoimmune responses as well as in controlling inflammatory reactions. However,

a number of fundamental questions concerning their origin, mechanism of action, and the sites of suppression remain elusive and are currently a matter of debate. Notably, the potential heterogeneity of Tregs with respect to phenotype and function deserves attention and is a major issue discussed in this review.

1 Introduction

Suppressor T cells have first been described in the early 1970s (Gershon and Kondo 1970; Gershon 1975), but since the hypothetical soluble suppressor factor could not be identified on a molecular level and since appropriate cellular markers were lacking, the suppressor T cell concept vanished for a considerable time. The discovery made by Sakaguchi and coworkers, that the adoptive transfer of $CD25^+CD4^+$-depleted T cells induced several organ-specific autoimmune diseases in immunodeficient recipients, put the suppressor T cell model back into the focus of many immunologists (Sakaguchi et al. 1995).

$CD25^+CD4^+$ T cells were named regulatory T cells (Treg) and since then have been intensively characterized by many groups (reviewed by Maloy and Powrie 2001; Shevach 2002; Sakaguchi 2004). It has now been well documented in a variety of models that $CD25^+CD4^+$ Tregs, in addition to cell-intrinsic peripheral tolerance mechanisms such as anergy induction and peripheral deletion, play indispensable roles in the maintenance of natural self-tolerance, in averting autoimmune responses, as well as in controlling inflammatory reactions.

However, a number of fundamental questions concerning their origin, mechanism of action, and the sites of suppression remain elusive and are currently a matter of debate. Notably, the potential heterogeneity of Tregs with respect to phenotype and function deserves attention and is a major issue discussed in this review.

2 Characterization of Tregs

2.1 $CD25^+CD4^+$ Tregs Can Suppress a Variety of Immune Reactions

$CD25^+CD4^+$ Tregs are pluripotent suppressors modulating many different immune reactions. They efficiently inhibit naive $CD4^+$ T cell proliferation and differentiation (Thornton and Shevach 1998; Oldenhove et al. 2003),

prevent cytotoxic activity of $CD8^+$ T cells both in vitro and in vivo (Piccirillo and Shevach 2001; Murakami et al. 2002; Suvas et al. 2003; Dittmer et al. 2004), suppress the activation and antibody production of B cells (Sakaguchi et al. 1995; Bystry et al. 2001) and limit the activity of cells from the innate immune system such as NK cells, neutrophils, and monocytes (Maloy et al. 2003). Moreover, $CD25^+CD4^+$ Tregs can efficiently limit the stimulatory capacity of antigen-presenting cells (APCs) by downregulating cell surface expression of costimulatory molecules such as CD80 and CD86 (Cederbom et al. 2000).

2.2 Suppressor Mechanisms

The precise molecular mechanisms by which $CD25^+CD4^+$ Tregs suppress the different target cells are currently controversial and under intense investigation. In vitro studies have shown that Tregs have to be activated via their T cell receptor (TCR) to exert their suppressive activity, but once they have been activated they can suppress antigen nonspecifically. Under these in vitro conditions, $CD25^+CD4^+$ Tregs suppress their target cells in a cell–cell contact-dependent and cytokine–soluble factor-independent manner (Takahashi et al. 1998; Thornton and Shevach 1998). This in vitro suppressive cell contact was not due to killing of the responder T cell population via the Fas/FasL- or TNF/TNF receptor-dependent pathway (Takahashi et al. 1998). However, killing of antigen-presenting B cells by $CD25^+CD4^+$ Tregs in vitro in a Fas/FasL-dependent manner has recently been reported (Janssens et al. 2003).

In contrast to the in vitro situation, the mechanisms via which Tregs regulate immune responses in vivo seem to be far more complicated, and several immunosuppressive cytokines such as IL-10 and TGFβ have been implicated in Treg suppressor function. A critical contribution of IL-10 to $CD25^+CD4^+$ Treg-mediated suppression was initially shown in the adoptive transfer colitis model (Asseman et al. 1999; Annacker et al. 2001) as well as in models of transplantation tolerance, graft-versus-host disease, chronic parasite infection and other autoimmune models (reviewed in Hori et al. 2003b). However, Tregs from IL-10-deficient mice, although not capable of suppressing colitis induction, are fully capable of suppressing autoimmune gastritis in the same mouse, indicating a tissue-specific role for this cytokine (Suri-Payer and Cantor 2001).

The contribution of TGFβ to $CD25^+CD4^+$ Treg-mediated suppression remains controversial as its cellular sources are numerous and may include effector T cells, nonlymphoid tissues such as epithelium, which are targets of autoimmune attack or in the process of healing, or even Tregs themselves (Asano et al. 1996; Prud'homme and Piccirillo 2000). TGFβ blockade has been

shown to abrogate suppression in the induced SCID colitis model (Powrie et al. 1996; Read et al. 2000), and Nakamura and colleagues reported that activated $CD25^+CD4^+$ Tregs, but not $CD25^-CD4^+$ T cells, expressed functional TGFβ in a cell surface-bound manner (Nakamura et al. 2001). However, in a different study a functional role of TGFβ for the suppressive capacity of $CD25^+CD4^+$ Tregs could not be observed (Piccirillo et al. 2002).

Another molecule that has been implicated in the function of $CD25^+CD4^+$ Tregs is CTLA-4. In contrast to conventional naive $CD25^-CD4^+$ T cells, Tregs in normal mice constitutively express CTLA-4 and several studies using neutralizing antibodies indicate a critical role for this molecule in Treg-mediated suppression (Read et al. 2000; Salomon et al. 2000; Takahashi et al. 2000). These results collectively indicated that signals through CTLA-4 may activate $CD25^+CD4^+$ Tregs to exert suppression and that blockade of the signal lead to a failure in their activation and thereby to attenuation of the Treg-mediated suppression. However, another suppressor mechanism involving CTLA-4 has been suggested involving a direct T–T interaction and reverse signaling through the costimulatory molecules CD80/CD86 being expressed on activated target T cells leading to their inhibition (Gavin and Rudensky 2003). So far, it is unknown whether $CD25^+CD4^+$ Tregs can use all these different suppressor mechanisms concomitantly or whether specialized subsets exist that exert suppression just via one mechanism.

2.3 Identification of Activation-Independent Treg Markers

In addition to CD25 and CTLA-4, another molecule named GITR has been shown to be constitutively expressed on $CD25^+CD4^+$ Tregs (Shimizu et al. 2002; McHugh et al. 2002). However, the usage of these molecules to identify Tregs is problematic, because their expression is strongly dependent on the activation status of the cell (Table 1). CD25 expression, for example, is transiently upregulated on activated cells and therefore is not sufficient to discriminate between recently activated T cells and Tregs constitutively expressing CD25.

The best marker currently known for $CD4^+$ Tregs seems to be the recently identified transcription factor Foxp3, a forkhead family transcriptional regulator, which has been shown to be expressed almost exclusively in $CD25^+CD4^+$ Tregs and to be essential for both the generation and function of $CD25^+CD4^+$ Tregs (Hori et al. 2003a; Khattri et al. 2003; Fontenot et al. 2003). Most importantly, activation of $CD25^-CD4^+$ T cells or differentiated Th1/Th2 cells failed to induce Foxp3 expression (Hori et al. 2003a; Fontenot et al. 2003). Strikingly, retroviral transfection of $CD25^-CD4^+$ T cells with Foxp3 induces Treg-like cells both phenotypically and functionally, indicating that the tran-

Table 1 $CD4^+$ Treg markers[a]

	Induced upon activation on naive conventional $CD4^+$ T cells	Proposed functional involvement in the suppressor mechanism
CD25	Yes	IL-2 competition
CTLA-4	Yes	Costimulatory signals and *trans*-signaling
GITR	Yes	Ligation abrogates suppressive activity
$\alpha_E\beta_7$	No	Retention of Tregs at inflamed sites
Foxp3	No	Required for generation and effector function
Neuropilin-1	No	Immunological synapse formation

[a]This table summarizes molecular markers currently used to identify $CD4^+$ T cells with suppressive capacity and states whether these markers are capable of discriminating between Tregs and recently activated T cells. Moreover, putative functions of these molecules in the action of Tregs are listed. For details and references see text.

scription factor itself is sufficient to induce Tregs (Hori et al. 2003a; Khattri et al. 2003; Fontenot et al. 2003). In humans, mutations in the Foxp3 gene have been shown to be associated with the development of several autoimmune diseases (Gambineri et al. 2003).

Although Foxp3 so far is widely accepted as the best marker to identify Tregs, its intracellular expression does not allow isolating Foxp3-expressing Tregs, as it is possible for the cell surface markers CD25, CTLA-4, or GITR. A comprehensive attempt to find better Treg cell surface markers recently screened $CD25^+CD4^+$ Tregs for molecules, which are specifically and stably expressed on $CD25^+CD4^+$ Tregs and which are not induced on $CD25^-CD4^+$ T cells upon activation. Neuropilin-1 was identified as a novel activation-independent cell surface Treg marker, which might even be involved in the suppressive function (Bruder et al. 2004).

2.4 Further Subsets with Suppressive Capacity

In addition to $CD25^+CD4^+$ Tregs, other T cell subsets bearing suppressive capacity have been described (reviewed by Jonuleit and Schmitt 2003). Among those the most prominent were Tr1 and Th3 cells, which have been shown to be peripherally induced as a consequence of antigen exposure under certain tolerogenic conditions and which are characterized by the production of the immunosuppressive cytokines IL-10 and TGFβ, respectively (Roncarolo et al. 2001; Weiner 2001).

Tr1 cells were first described by Groux and colleagues and, in contrast to $CD25^+CD4^+$ Tregs, were shown to solely depend on the expression of IL-10 to exert their suppressive capacity (Groux et al. 1997). Th3 cells were originally identified in mice after oral tolerance induction to an autoantigen, preferentially produced TGFβ and established a state of systemic tolerance that prevented the development of autoimmunity and that was reversible by neutralizing antibodies against TGFβ (Chen et al. 1994). Although the contribution of TGFβ to $CD25^+CD4^+$ Treg-mediated suppression is still discussed controversially, it cannot be excluded that cells described as Th3 cells in fact might belong to the population of $CD25^+CD4^+$ Tregs.

Moreover, there is a substantial amount of data that $CD45RB^{low}$ T cells in the $CD25^-CD4^+$ T cell population in normal naive rodents bear similar suppressive activity in vivo and in vitro as their $CD25^+$ counterparts (Stephens and Mason 2000; Read et al. 2000; Annacker et al. 2001; Kullberg et al. 2002; Hori et al. 2002b). Recent efforts to further characterize these Tregs in the $CD25^-CD45RB^{low}CD4^+$ T cell population in terms of Foxp3 expression and in vitro suppressive activity have revealed that they are $CTLA\text{-}4^+$ and $GITR^{high}$, similar to $CD25^+CD4^+$ Tregs (Sakaguchi 2004). This indicates that expression of CD25 on $CD4^+$ T cells is not sufficient to identify a cell as Treg, and that a significant heterogeneity among cells with suppressive function might exist.

2.5 In Vivo Behavior of Tregs

Numerous in vitro studies suggested that $CD25^+CD4^+$ Tregs own an anergic phenotype with poor proliferation upon TCR triggering as well as growth dependence on exogenous IL-2 (Asano et al. 1996; Thornton and Shevach 1998; Papiernik et al. 1998). However, recent publications demonstrated that $CD25^+CD4^+$ Tregs display a completely different behavior in vivo, showing a high homeostatic as well as antigen-induced proliferation in different systems (Hori et al. 2002c; Klein et al. 2003; Oldenhove et al. 2003; Walker et al. 2003; Yamazaki et al. 2003; Fisson et al. 2003; Cozzo et al. 2003). These findings suggest that, in normal naive mice, a fraction of $CD25^+CD4^+$ Tregs is slowly proliferating without exogenous antigenic stimulation, presumably by recognizing self-antigens in the periphery (Fisson et al. 2003; Cozzo et al. 2003; Sakaguchi et al. 2003). Thus, $CD25^+CD4^+$ Tregs show antigen-specific expansion and consequently augment antigen-specific suppression with each successive exposure to a particular antigen.

Another important aspect regarding the in vivo suppressive capacity of $CD25^+CD4^+$ Tregs has only very recently been addressed and concerns the localization and migratory behavior of Tregs. Whereas in vitro data can only

give limited information about the suppressive capacity of Treg subsets per se, the in vivo situation might be completely different, as the Tregs need the ability to migrate to the sites where suppression is required. The impact of the migratory behavior for the in vivo suppressive capacity of $CD25^+CD4^+$ Tregs will be discussed in more detail below.

3 Origin of Tregs: Thymus and Peripheral Induction

A number of findings provide ample evidence that the majority of $CD25^+CD4^+$ Tregs are produced within the thymus in the process of thymic selection as a functionally distinct and mature subpopulation of T cells. It is currently not known whether Tregs develop from a unique lineage precursor, or whether any $CD4^+CD8^-$ thymocyte can differentiate into a Treg under particular conditions.

In a normal thymus, 2%–5% of $CD4^+CD8^-$ thymocytes express CD25, and these cells are functionally competent, as illustrated by their ability to suppress naive T cell activation in vitro and to protect mice from developing autoimmunity upon adoptive transfer (Itoh et al. 1999). Similar to their peripheral counterparts, $CD25^+CD4^+CD8^-$ thymocytes express Foxp3, and in Foxp3-deficient mice both populations are lacking (Hori et al. 2003a; Fontenot et al. 2003). Furthermore, an earlier study reported that neonatal day 3 thymectomy results in the development of organ-specific autoimmunity due to the diminished number of $CD25^+CD4^+$ Tregs in the periphery (Sakaguchi et al. 1995). These studies indicate that the normal thymus is continuously producing potentially pathogenic self-reactive $CD25^-CD4^+$ T cells as well as functionally mature $CD25^+CD4^+$ Tregs. Thus, the thymus contributes to the maintenance of self-tolerance not only by deleting or inactivating the majority of self-reactive T cells during the process of negative selection but also by producing $CD25^+CD4^+$ Tregs.

The repertoire of antigen specificities of $CD25^+CD4^+$ Tregs is thought to be as broad as that of naive T cells, and they conceivably are capable of recognizing a wide array of both self- and non-self-antigens, thus enabling them to control various immune responses (Sakaguchi et al. 2003; Takahashi et al. 1998; Romagnoli et al. 2002; Pacholczyk et al. 2002; Hori et al. 2002b). There are accumulated findings indicating that the thymic development of $CD25^+CD4^+$ Tregs requires unique interactions of their TCRs, with self-peptides being presented on MHC molecules expressed on thymic stromal cells. In a double-transgenic model in which mice expressing a transgenic TCR of known specificity are crossed with mice that express the corresponding antigen in the thymus,

antigen-specific Treg cells appear to require high-affinity antigen recognition in the thymus to develop (Jordan et al. 2001). Under these circumstances, if high-affinity antigen-specific thymocytes recognize their antigen being expressed on thymic epithelial cells, the vast majority of these thymocytes developed into $CD25^+CD4^+$ Tregs (Apostolou et al. 2002; Kawahata et al. 2002; Jordan et al. 2001).

In addition to the thymic generation of $CD25^+CD4^+$ Tregs, a number of reports have shown that $CD4^+$ T cells bearing suppressive capacity can be induced in the periphery from conventional $CD25^-CD4^+$ precursors upon antigen exposure under tolerogenic conditions. Two major types of these induced Tregs have already been mentioned and were described as Tr1 (Roncarolo et al. 2001) and Th3 cells (Weiner 2001). However, even $CD25^+CD4^+$ Tregs showing the same characteristics as thymus-derived $CD25^+CD4^+$ Tregs could be peripherally induced from antigen-specific $CD25^-CD4^+$ T cells by either intravenous or oral administration of low-dose peptide antigen or by adoptive transfer of naive transgenic T cells to antigen-expressing transgenic mice (Thorstenson and Khoruts 2001; Apostolou et al. 2002). This induction process neither requires an intact thymus nor the presence of thymus-derived $CD25^+CD4^+$ Tregs as observed in a skin allograft model (Karim et al. 2004).

Recent data from von Boehmer's group support these findings, showing that continuous systemic low-dose antigen delivery by osmotic pumps induced long-lived highly potent $CD25^+CD4^+$ Tregs from TCR-transgenic $CD25^-CD4^+$ T cells or even from the endogenous T cell pool (Apostolou 2004). However, it remains to be determined whether these peripherally generated Tregs are de novo induced from naive T cells or derived from Treg-precursors ($Foxp3^+$CTLA-4^+GITR^{high}) present in the $CD25^-CD4^+$ T cell population. Further comprehensive studies are required to compare the phenotypic and functional properties of such apparently de novo induced Tregs with thymic-derived $CD25^+CD4^+$ Tregs.

In order to classify the diverse subtypes of suppressive T cells, Bluestone and Abbas recently suggested the nomenclature of "natural" and "adaptive" Tregs (Bluestone and Abbas 2003). In their terminology, natural Tregs comprise those Tregs that develop as $CD25^+CD4^+$ Tregs within the thymus and that are specialized to regulate immune homeostasis and to maintain self-tolerance. In contrast, those suppressive T cells that develop in the periphery either from naive T cells or from natural Tregs upon antigen-induced differentiation/expansion under certain tolerogenic conditions were named adaptive Tregs, and this type of Tregs also includes Tr1 and Th3 cells.

4 The Integrin $\alpha_E\beta_7$ Is a Marker for Peculiar Treg Subsets

Recently, several groups have identified the integrin $\alpha_E\beta_7$ as a marker for murine $CD4^+$ Tregs isolated from secondary lymphoid organs (Lehmann et al. 2002; Zelenika et al. 2002; Gavin et al. 2002; McHugh et al. 2002; Banz et al. 2003). So far, the integrin $\alpha_E\beta_7$ has been well known as a marker for intraepithelial lymphocytes (IEL) residing in the gut wall and other epithelial compartments such as skin or lung, and its expression was shown to be largely controlled by TGFβ (Cerf-Bensussan et al. 1987; Kilshaw and Murant 1991). However, only very limited knowledge exists on the function of the integrin $\alpha_E\beta_7$ on $CD4^+$ T cells.

In contrast to the related integrin $\alpha_4\beta_7$, which acts as a homing receptor for mucosa-seeking lymphocytes by recognizing the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Hamann et al. 1994), $\alpha_E\beta_7$ seems not to play a role in the migration of lymphocytes into mucosal or epithelial sites (Austrup et al. 1995). Instead, it has been suggested that the interaction between $\alpha_E\beta_7$ and its ligand E-cadherin, which is expressed on epithelial cells but not on endothelium (Cepek et al. 1994), might be involved in the retention of lymphocytes within epithelial compartments. This assumption is supported by the phenotype of α_E-deficient mice, which showed a reduction in the number of mucosal T lymphocytes (Schon et al. 1999). Furthermore, these mice developed mild cutaneous inflammatory disorders (Schon et al. 2000), which led to the suggestion that the integrin might be important for the control of autoimmunity in the skin.

In addition to intraepithelial lymphocytes, the integrin $\alpha_E\beta_7$ is expressed on a small subpopulation of about 5–6% $CD4^+$ T cells from secondary lymphoid organs. Initial characterization of this subset in our laboratory revealed that the majority of this subpopulation co-expresses CD25 and is localized within the $CD45RB^{low}$ compartment (Lehmann et al. 2002). The integrin not only subdivides the $CD25^+$ compartment into CD25 single positive ($\alpha_E{}^-CD25^+$) and $\alpha_E{}^+CD25^+$ cells, but also identifies CD25-negative α_E single positive cells ($\alpha_E{}^+CD25^-$). Since CD25 and $CD45RB^{low}$ have been described as Treg markers, we analyzed whether the α_E-expressing subsets exhibit suppressive capacity (Fig. 1).

Functional studies both in vitro (suppression of naive T cell proliferation) and in vivo (inhibition of induced SCID colitis) revealed that $\alpha_E{}^+$ T cell subsets irrespective of their CD25 expression showed regulatory activity (Lehmann et al. 2002; Banz et al. 2003). Throughout all settings, $\alpha_E{}^+CD25^+$ cells turned out to be the most potent suppressors. In vitro, the $\alpha_E{}^+CD25^-$ subpopulation displayed only moderate suppressive activity comparable to total $CD45RB^{low}$

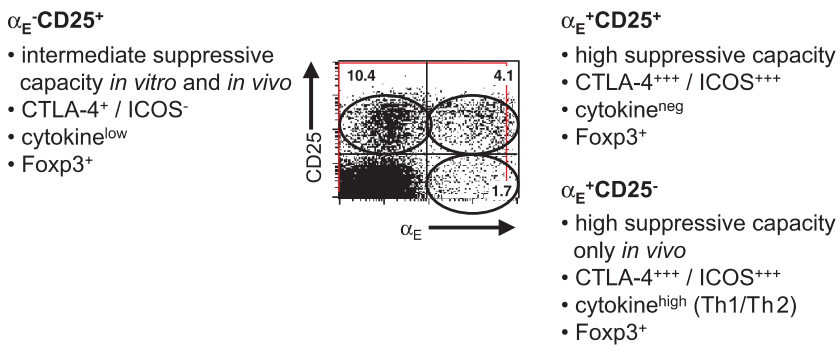

Fig. 1 Phenotype of a_E^- and a_E^+ Treg subsets. For details see text

$CD4^+$ cells, but strikingly α_E single positive cells were potent regulators in vivo, being as effective as CD25 single positive cells in inhibiting the development of SCID colitis (Lehmann et al. 2002). Furthermore, Foxp3 mRNA was present in all three Treg subsets with a similar expression level in α_E single positive cells compared to both CD25 expressing subsets (Huehn et al. 2004). This latter finding underlines the regulatory function of $\alpha_E^-CD25^+$, $\alpha_E^+CD25^+$ as well as $\alpha_E^+CD25^-$ $CD4^+$ T cells in the murine system.

However, the integrin $\alpha_E\beta_7$ does not account as a marker for Tregs from the human peripheral blood as $\alpha_E^+CD25^+$ cells are absent in the peripheral blood $CD4^+$ T cell compartment and the small subset of $\alpha_E^+CD25^-$ $CD4^+$ T cells does not contain any suppressive capacity (unpublished observations; Iellem et al. 2003; Stassen 2004).

Further characterization of α_E-expressing as well as $\alpha_E^-CD25^+$ Tregs revealed striking differences between these subsets, supporting the concept that a high degree of heterogeneity exists within the suppressor T cell pool (Fig. 1). Whereas $\alpha_E^-CD25^+$ and especially $\alpha_E^+CD25^+$ cells fulfilled the hallmark of Tregs by expressing only low frequencies of both proinflammatory and Th2-type cytokines upon restimulation, the $\alpha_E^+CD25^-$ subset showed a rather peculiar cytokine expression pattern, producing high levels of both Th1- and Th2-type cytokines (Lehmann et al. 2002). Additionally, the $\alpha_E^-CD25^+$ subset showed only minor frequencies of $CTLA\text{-}4^+$ cells, whereas both α_E-expressing subsets expressed this immunomodulatory molecule at high frequencies (Lehmann et al. 2002). However, we did not observe any differences with regard to GITR expression, as all three subsets showed a comparable high expression of this molecule (unpublished observation). Nevertheless, the considerable heterogeneity in the Treg pool with respect to suppressive capacity, cytokine secretion, and expression of CTLA-4 suggests distinct functional profiles of these subsets.

5 Are α_E-Expressing Tregs Prototypes of Peripherally Induced or Expanded Adaptive Tregs?

To get a more comprehensive picture of molecular differences between α_E^+ and α_E^- Treg subsets, we performed global gene expression analyses. The results suggested a fundamental dichotomy with regard to phenotype and developmental stage, allowing the differentiation into naive- and effector/memory-like Tregs (Table 2; Huehn et al. 2004).

CD25 single positive cells, although expressing CD45RB at low levels, displayed a rather naive-like phenotype with high expression levels of CD62L as well as expression of functional CCR7. Their CD62L expression was comparable to α_E^-CD25$^-$ control cells, which largely are composed of conventional naive T cells bearing a CD45RB^{high} phenotype.

In contrast, both α_E-expressing subsets and especially the α_E^+CD25$^-$ cells showed an activated effector/memory-like phenotype with low expression levels of CD45RB and CD62L combined with high levels of certain effector/memory markers (CD44, ICOS, CD29, LFA-1). Additionally, other markers known to characterize antigen-experienced or recently activated CD4$^+$

Table 2 Phenotypic characteristics and functional properties of α_E^+ and α_E^- Treg subsets (for details see text)

	α_E^-CD25$^+$	α_E^+CD25$^+$	α_E^+CD25$^-$
Effector/memory-like phenotype	–	++	+++
Naive-like phenotype	+++	+	–
TREC content	+++	++	+
CD62L expression	+++	++	+
E/P-selectin ligand expression	–	++	+++
Chemokine responsiveness			
CCR7 ligand	+++	++	+
CXCR3 ligand	+	++	+++
CCR4 ligand	+	++	+++
CCR6 ligand	+	+++	++
In vivo suppression			
Induced SCID colitis	++	+++	+++
Antigen-induced arthritis	–	+++	++
Skin inflammation	+	+++*	+++*

* in this model only total α_E^+ Tregs were analyzed in comparison to α_E^-CD25$^+$ Tregs

T cells including CD69, Ki67 (Brown and Gatter 2002), granzyme B (Jacob and Baltimore 1999) and CD8 (Nascimbeni et al. 2004) were also upregulated within the α_E-expressing Treg subsets.

The highly differentiated effector/memory-like phenotype of α_E-expressing Tregs suggested that these cells have been activated in the periphery upon contact with their cognate antigen, leading to their differentiation and expansion. Indeed, when we were analyzing the TREC (T cell receptor excision circles) content of the Treg subsets, which is an indicator of their expansion after recombination of the TCR, we observed a relatively high TREC numbers in CD25 single positive cells, similar to the predominantly naive $\alpha_E^-CD25^-$ control cells. This reflects a limited proliferative activity of CD25 single positive cells during development. In contrast, both α_E-expressing subsets and especially $\alpha_E^+CD25^-$ cells showed a strongly reduced TREC content indicating that these cells have undergone repetitive cell divisions after maturation within the thymus (Huehn et al. 2004). Our data on the proliferative history of α_E-expressing Treg subsets support recently published observations from different groups showing a strong in vivo proliferative capacity of $CD25^+CD4^+$ Tregs (reviewed in von Boehmer 2003).

Interestingly, Fisson and colleagues have postulated that the $CD25^+CD4^+$ Treg compartment is composed of two Treg subsets showing distinct phenotypes and homeostasis under steady-state conditions (Fisson et al. 2003). Upon adoptive transfer of natural $CD62L^{high}CD25^+CD4^+$ Tregs, which largely corresponds to the $\alpha_E^-CD25^+$ Treg subset, a substantial fraction of these cells underwent repetitive cell divisions upon contact with tissue self-antigen. Strikingly, those cells that were dividing extensively acquired an effector/memory-like phenotype ($CD44^{high}$ $CD69^+$ $CD134^+$ $CD62L^{low}$). Whether these cells also express the integrin $\alpha_E\beta_7$ was not addressed in that study, but our own data would suggest that these cells become α_E^+ Tregs, indicating that adaptive effector/memory-like Tregs can develop from natural $CD25^+CD4^+$ precursors.

However, as already mentioned above, von Boehmer and colleagues have shown in an antigen-specific adoptive transfer model that continuous peripheral low-dose delivery of specific peptide antigen by osmotic pumps generates $CD25^+CD4^+$ Tregs from conventional naive $CD25^-CD4^+$ T cells. Interestingly, when analyzing the phenotype of these de novo induced Tregs through gene array technology, not only the key Treg marker Foxp3, but also the integrin α_E was found to be strongly upregulated in the antigen-specific T cells (H. von Boehmer and J. Buer, personal communication).

Together these data suggest that α_E-expressing effector/memory Tregs can be derived either from conventional naive $CD25^-CD4^+$ T cells or from

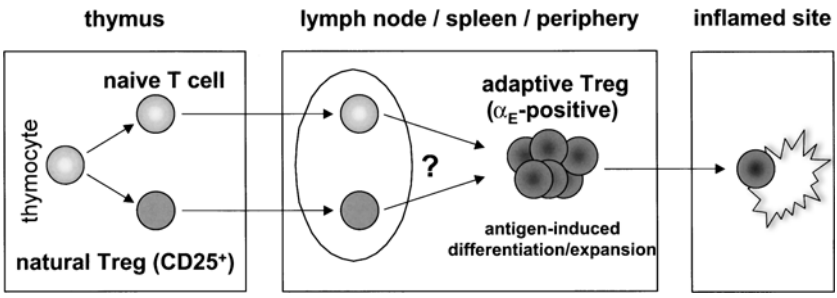

Fig. 2 CD25 single positive cells represent natural Tregs, whereas a_E-expressing subsets are prototypes of adaptive Tregs

natural naive-like $CD25^+CD4^+$ Tregs, strengthening our current view that the expression of the integrin $\alpha_E\beta_7$ on $CD4^+$ Treg subsets is indicative of their antigen-specific differentiation and expansion in the periphery (Fig. 2). Where in the organism the conversion into adaptive Tregs takes place has not been addressed sufficiently yet. However, it is very likely that this scenario involves secondary lymphoid organs as the precursors, either naive conventional $CD25^-CD4^+$ T cells or naive-like natural $CD25^+CD4^+$ Tregs, both express high levels of CD62L and CCR7 and therefore display remarkable tropism for these sites (Mackay et al. 1990; Moser and Loetscher 2001).

Despite these numerous findings supporting the hypothesis of the peripheral generation of α_E-expressing Treg subsets, the inter-relationship between $\alpha_E{}^+CD25^+$ and α_E single positive cells remains largely unknown and needs further investigation. It is unlikely that $\alpha_E{}^+CD25^-$ Tregs merely represent a nonactivated and thereby CD25-negative precursor state of $\alpha_E{}^+CD25^+$ cells, as the α_E single positive cells display the most differentiated phenotype with respect to the expression of cytokines, effector/memory markers, and reduced TREC numbers (Lehmann et al. 2002; Huehn et al. 2004). On the other hand, α_E single positive cells express relatively high levels of CD25 mRNA and rapidly acquire CD25 expression upon activation, suggesting some flexibility in the phenotypes with regard to this marker (Lehmann et al. 2002).

6 Distinct Migratory Behavior of Treg Subsets Correlates with Their Suppressive Capacity in Certain Models

The gene array based analysis of $\alpha_E{}^+$ and $\alpha_E{}^-$ Treg subsets not only revealed differences in the expression of effector/memory markers, unraveling a dichotomy within the Treg compartment with respect to antigen experience and

developmental stage. Striking differences were also observed with respect to the expression of certain adhesion molecules and chemokine receptors, suggesting that in vivo α_E^+ and α_E^- Treg subsets are not equally distributed throughout the body and that specialized Treg subsets exist that can patrol through distinct sites of the body (Table 2; Huehn et al. 2004).

These observations fit to our current concept that α_E-expressing Tregs have acquired their effector/memory-like phenotype in the periphery, as it is known that antigen recognition in secondary lymphoid tissues not only results in clonal expansion and differentiation into effector/memory cells with distinct functional properties, but also induces a change in the expression of adhesion molecules and chemokine receptors that allows the exit from the lymph nodes and the migration into distinct effector sites (Mackay et al. 1990; Austrup et al. 1997; Tietz et al. 1998; Masopust et al. 2001; Campbell and Butcher 2002).

Indeed, migration studies of α_E^+ and α_E^- Treg subsets revealed that the observed phenotypic differences between the Treg subsets precisely correspond to their behavior in vivo (Huehn et al. 2004). Naive-like CD25 single positive cells efficiently migrated into lymph nodes fitting to their combinatorial high CD62L expression and high responsiveness toward CCR7 ligands. Both molecules have been shown to be a prerequisite for the entry into lymph nodes (Gallatin et al. 1983; Forster et al. 1999; Luther et al. 2000). In contrast, both α_E-expressing Treg subsets and especially α_E single positive cells showed increased frequencies of E/P selectin ligand expression combined with a substantial downregulation of CD62L, high expression levels for LFA-1, β_1-integrin, and ICAM-1, as well as a high responsiveness toward a number of inflammatory chemokines. Corresponding to these phenotypes, effector/memory-like Treg subsets displayed only a rather poor capacity to migrate into lymph nodes, but, in contrast, efficiently entered inflamed sites.

Overall, the in vivo migration data identified naive-like $\alpha_E^-CD25^+$ as a recirculating subset, whereas the effector/memory-like α_E-expressing Tregs proved to be rather inflammation-seeking (Huehn et al. 2004). The observed migration behavior of α_E^+ and α_E^- Treg subsets largely corresponds to findings in a number of recent publications studying adhesion molecule expression or chemokine responsiveness of Treg subsets (Iellem et al. 2001; Iellem et al. 2003; Goulvestre et al. 2002; Colantonio et al. 2002; Sebastiani et al. 2001; Gavin et al. 2002; Fu et al. 2004). Most strikingly, Szanya et al., by separating the $CD25^+CD4^+$ compartment into $CD62L^{high}$ and $CD62L^{low}$ cells, which largely correspond to CD25 single positive and $\alpha_E^+CD25^+$ cells, respectively, were able to show enhanced expression of CCR7 on the $CD62L^{high}$ subset, whereas levels of CCR2, CCR4, and CXCR3 were much higher on $CD62L^{low}$ cells (Szanya et al. 2002).

The migratory phenotype of Treg subsets is discussed more controversially in the human system. Iellem et al. have observed an enrichment in E-selectin-binding and $CCR4^+$ cells among $CD25^+CD4^+$ Tregs accompanied by a paucity of gut-homing ($\alpha_4\beta_7{}^+$, $CCR9^+$) cells, suggesting that these cells most likely would home into the skin as well as inflamed sites (Iellem et al. 2003). This report contrasts with a recent finding from Jonuleit and colleagues who have shown that 15%–30% of human $CD25^+CD4^+$ Tregs expressed the integrin $\alpha_4\beta_7$, suggesting that these cells would preferentially migrate into the mucosa (Stassen 2004). However, in the murine system we did not observe a preferential migration of any of the analyzed Treg subsets into both noninflamed and inflamed mucosal tissues, although all subsets expressed the integrin $\alpha_4\beta_7$ at reasonable frequencies (30%) (Huehn et al. 2004 and unpublished observations). These findings indicate that Tregs similar to conventional effector/memory T cells display a heterogenous migration pattern, which is not biased toward a single tissue.

Despite accumulating knowledge on the chemokine responsiveness of certain Treg subsets, only limited data exist on where distinct Treg subsets localize in vivo. Whereas it has been shown that the $CD62L^{high}$ subset of $CD25^+CD4^+$ Tregs preferentially migrates into peripheral lymph nodes (Fisson et al. 2003), the phenotype of those Tregs that have been isolated from effector sites such as synovial fluid of rheumatoid arthritis patients (Cao et al. 2003), lung tumors (Woo et al. 2002), transplants (Graca et al. 2002), skin lesion of *Leishmania major*-infected mice (Belkaid et al. 2002), lungs of *Pneumocystis carinii*-infected mice (Hori et al. 2002a), islets of Langerhans in a diabetes model (Lepault and Gagnerault 2000; Green et al. 2002), or the inflamed intestine in the induced colitis model (Mottet et al. 2003) remains largely unknown. However, it is tempting to speculate that those Tregs largely correspond to adaptive effector/memory-like Tregs displaying an activated phenotype.

Interestingly, Tregs from the inflamed intestine showed strong signs of proliferation (Mottet et al. 2003) and those Tregs that were recovered from the inflamed islets in the diabetes model were mainly $CD62L^{low}$ (Lepault and Gagnerault 2000), indicating some similarities with α_E-expressing Tregs. This latter finding was recently supported by observations of Mathis and colleagues who analyzed the phenotype of Tregs isolated directly from the inflamed pancreatic islets in BDC2.5/NOD mice using gene array technology. Interestingly, these inflammation-derived Tregs showed an enhanced expression of the integrin $\alpha_E\beta_7$ and also displayed increased mRNA for the inflammatory chemokine receptors CCR2, CCR5, and CXCR3 (A. Herman, C. Benoist and D. Mathis, personal communication), fitting to our own observations that only α_E-expressing Tregs, which show an enhanced migratory response toward inflammatory chemokines, can enter such inflamed sites (Huehn et al. 2004).

Moreover, α_E-expressing Tregs have also been observed in the aforementioned *Leishmania major* infection model. In this model, antigen-specific Tregs could be isolated from skin lesions of infected mice and have been shown to play a crucial role in the immune response against the parasite (Belkaid et al. 2002). Belkaid and colleagues now have observed in a follow-up study that up to 80% of Tregs from the skin lesions stained positive for the integrin $\alpha_E\beta_7$. Strikingly, using a monoclonal antibody that blocked the interaction of the integrin $\alpha_E\beta_7$ with its ligand E-cadherin, they could show that the number of Tregs in the chronic site of infection were significantly reduced, leading to the hypothesis that the expression of the integrin $\alpha_E\beta_7$ on the skin-resident Tregs has a functional role mediating the retention of the Tregs within the skin lesions of infected mice (Y. Belkaid, personal communication).

However, a general functional role for the integrin $\alpha_E\beta_7$ in the retention of effector/memory-like Tregs at any site of acute inflammation could not be supported by Annacker et al., who did not observe a significant difference in the suppressive capacity of $CD25^+CD4^+$ Tregs derived from wild-type or α_E-deficient mice in the induced SCID colitis model (Annacker 2003). This latter finding corresponds to data obtained with $CD4^+$ or $CD8^+$ effector T cells, for which a role of $\alpha_E\beta_7$ in homing into or retention within epithelial sites could not be demonstrated (Austrup et al. 1995; Lefrancois et al. 1999).

The conspicuous different migration phenotypes of $\alpha_E{}^+$ and $\alpha_E{}^-$ Treg subsets also turned out to be of functional significance when the suppressive capacities of these subsets were compared in an inflammation model, the antigen-induced arthritis. Strikingly, only the α_E-positive cells, which efficiently migrated into the inflamed site, could significantly reduce acute knee joint swelling as well as signs of chronic inflammation. In contrast, CD25 single positive cells, which showed no preferential migration to the inflamed knee joint, lacked suppressive activity under these acute inflammatory conditions (Huehn et al. 2004).

In order to generalize the concept that the suppressive capacity of Treg subsets is correlated with their in vivo migration behavior, we have established another inflammation model affecting the skin. This model is based on the adoptive transfer of in vitro generated, fully differentiated TCR-transgenic Th1 cells, and the inflammation is elicited by the subsequent injection of the cognate antigen into the footpad. Similar to the antigen-induced arthritis model, CD25 single positive cells, which showed no migration into the inflamed site, were not effective in suppressing the acute inflammatory response, whereas $\alpha_E{}^+$ Tregs, which efficiently entered the inflamed skin, significantly suppressed the Th1-mediated footpad swelling (unpublished observations).

Recently, another group published data supporting our view of the functional relevance of the appropriate localization of Tregs for their in vivo

suppressive capacity using a contact hypersensitivity model. In this model, hapten-specific Tregs induced by ultraviolet radiation were capable of inhibiting the induction phase of the skin inflammation, but showed no suppressive capacity during the effector phase (Schwarz et al. 2004). These hapten-specific Tregs expressed high levels of CD62L, but not the ligands for the skin-homing receptors E- and P-selectin. This phenotype most likely allows the migration into lymph nodes, the site of the induction phase, but not into the skin, where the effector response takes place. However, if these hapten-specific Tregs were injected directly into the effector site they could even suppress the challenge reaction. This finding indicates that hapten-specific Tregs, although in principle able to inhibit T effector cells, do not suppress the effector phase, because they obviously do not migrate into the skin (Schwarz et al. 2004).

The observation that effector/memory-like Tregs express certain adhesion molecules and chemokine receptors that allow their efficient migration into inflamed sites has important implications for the use of such molecules as targets for anti-inflammatory therapies. Inhibition of migratory functions might not only prevent the infiltration of effector cells but also that of highly effective adaptive Tregs.

This issue was addressed in a recent publication demonstrating a crucial role of CCR2-expressing Tregs in a model of collagen-induced arthritis (Bruhl et al. 2004). Whereas blockade of CCR2 using monoclonal antibodies during the disease initiation phase markedly improved the signs of arthritis, blockade during the later phase of arthritis progression significantly aggravated clinical and histological signs of arthritis (Bruhl et al. 2004). The authors postulate that this latter effect was most likely due to the interference with the proper in vivo localization of $CCR2^+CD25^+CD4^+$ Tregs, which seemed to be essential for the control of the inflammatory response. Interestingly, CCR2 expression on $CD25^+CD4^+$ Tregs strongly correlated with the expression of the integrin $\alpha_E\beta_7$ (Bruhl et al. 2004), supporting our view that α_E-expressing effector/memory-like Tregs are important for the control of already ongoing inflammatory reactions (Fig. 2).

In contrast to the inflammation models, in which naive-like $\alpha_E{}^-CD25^+$ Tregs showed almost no suppressive activity, recent reports demonstrated potent suppressive capacity of CD62L-expressing $CD25^+CD4^+$ Tregs in other models. Strikingly, $CD62L^{high}$ but not $CD62L^{low}$ $CD25^+CD4^+$ Tregs were capable of preventing the development of autoimmunity in the NOD diabetes model, indicating that the homeostatic regulation by naive-like Tregs is of major importance under conditions where the initiation of the immune response has to be controlled (Herbelin et al. 1998; Lepault and Gagnerault 2000; Szanya et al. 2002). Similar results were observed in the murine model of allogeneic bone marrow transplantation, in which only adoptive transfer of

donor $CD62L^{high}CD25^{+}CD4^{+}$ Tregs protects recipient mice from lethal acute graft-versus-host disease (aGVHD) induced by donor $CD25^{-}CD4^{+}$ T cells (J. Ermann, personal communication).

Most studies report a similar in vitro suppressive capacity of $CD62L^{high}$ and $CD62L^{low}$ Treg subsets (Kuniyasu et al. 2000; Thornton and Shevach 2000; Szanya et al. 2002). Only one recent report showed increased in vitro suppressive activity within the $CD62L^{high}$ subset (Fu et al. 2004). Thus, the differential regulatory capacities of the $CD62L^{high}$ and $CD62L^{low}$ subsets in the diabetes model most likely reflect differences in homing properties rather than suppressor potential per se. As adoptive Treg transfer in the NOD model was performed before the onset of diabetes, the control of the induction of autoimmunity apparently takes place in the lymph node, where antigen-specific T cells get activated and become effector cells. Since only $CD62L^{high}CD25^{+}CD4^{+}$ Tregs efficiently can enter lymph nodes (Fisson et al. 2003; Huehn et al. 2004), the development of these effector cells and thereby the induction of autoimmunity could only be prevented by $CD62L^{high}$ Tregs.

Results supporting this hypothesis were again obtained from the aGVHD model, in which early after transplantation a higher number of donor-type Treg cells could be recovered from host spleen and mesenteric lymph nodes when $CD62L^{high}CD25^{+}CD4^{+}$ Tregs were transferred as compared to the $CD62L^{low}$ subset, suggesting that the ability of Treg cells to efficiently enter secondary lymphoid organs is a prerequisite for their protective function in this model (J. Ermann, personal communication). Finally, these data were supported by Oldenhove and colleagues, showing that $CD25^{+}CD4^{+}$ Tregs limit the development of Th1 cells directly within the lymph node draining the site of antigen injection, suggesting that $CD62L^{high}$ Tregs were involved in this part of immune regulation (Oldenhove et al. 2003).

7 Division of Labor Between Distinct Treg Subsets?

Phenotype and localization properties let suggest a subdivision of the Treg compartment into distinct lineages or differentiation stages according to the aforementioned model of Bluestone and Abbas proposing the existence of so-called natural and adaptive Tregs (Bluestone and Abbas 2003). CD25 single positive cells might be good candidates for these natural Tregs, as they preferentially migrate into lymph nodes where they control the priming phase of immune responses. In contrast, α_E-expressing Tregs subsets bearing an effector/memory-like, inflammation-seeking phenotype seem to be good candidates for the adaptive Tregs specialized for the suppression of already

ongoing immune reactions. These α_E-expressing Treg subsets are thought to be tasked when the lymph node-residing natural Tregs have failed or when immune reactions are going out of control.

However, as these types of immune reactions take place at peripheral effector sites this "fail-safe" system of peripheral tolerance absolutely requires specialized Tregs, which harbor the capacity to enter inflamed effector sites. Therefore, to allow the suppression of such established immune reactions, Tregs do not merely require extraordinarily potent inhibitory mechanisms but also need to efficiently enter the hot spots of the inflammatory reaction. We assume that the localization of Treg subsets is of equal importance as their direct suppressive capacity as exemplified by α_E single positive cells having only poor in vitro, but high in vivo suppressive potential (Lehmann et al. 2002, Huehn et al. 2004). Therefore, considerations on the migratory capacities of Treg populations have to be taken into account when future therapeutic strategies based on the adoptive transfer of Treg subsets become attractive.

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