Experience-Driven Development: Effector/Memory-Like $\alpha_E^{+}$Foxp3$^+$ Regulatory T Cells Originate from Both Naive T Cells and Naturally Occurring Naive-Like Regulatory T Cells

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Experience-Driven Development: Effector/Memory-Like α^E_+Foxp3^+ Regulatory T Cells Originate from Both Naive T Cells and Naturally Occurring Naive-Like Regulatory T Cells

Christiane Siewert,* Uta Lauer,* Sascha Cording,* Tobias Bopp,† Edgar Schmitt,† Alf Hamann,* and Jochen Huehn2*

Naturally occurring Foxp3^+CD25^+CD4^+ regulatory T cells (Treg) have initially been described as anergic cells; however, more recent in vivo studies suggest that Tregs vigorously proliferate under both homeostatic as well as inflammatory conditions. We have previously identified a subset of murine CD4^+ Tregs, which is characterized by expression of the integrin α_Eβ_7, and which displays an effector/memory-like phenotype indicative of Ag-specific expansion and differentiation. In the present study, the α^E_+ Treg subset was found to contain a large fraction of cycling cells under homeostatic conditions in healthy mice. Using an adoptive transfer system of Ag-specific T cells, we could demonstrate that the vast majority of transferred natural, naive-like CD25^+CD4^+ Tregs acquired expression of the integrin α_Eβ_7 upon tolerogenic application of Ag via the oral route. In addition, using the same system, Foxp3^+ Tregs could be de novo induced from conventional naive CD25^-CD4^+ T cells, and this conversion was associated with concomitant expression of α_E. These findings suggest that Tregs expressing the integrin α_E are effector/memory Tregs with a high turnover rate that can develop in the periphery upon Ag contact under tolerogenic conditions, both from thymic-derived CD25^+CD4^+ Tregs with a naive-like phenotype as well as from conventional naive T cells. The Journal of Immunology, 2008, 180: 146–155.

The CD4^+ regulatory T cells (Treg) are powerful regulators of immune responses in organ-specific autoimmunity, allergic responses, allograft rejection, and host responses against pathogens (1). In addition, they control systemic homeostasis and total lymphocyte numbers (2). The transcription factor Foxp3 appears to be the most specific marker identifying naturally occurring CD25^+ and CD25^-CD4^+ Tregs generated as a distinct lineage in the thymus (3). We have recently shown that within the murine CD4^+ Treg compartment, the markers CD25 and α_E may serve to identify phenotypically and functionally distinct Treg subsets. Using these markers we were able to subdivide the Treg compartment into naive-like α_E^-CD25^+ and effector/memory-like α_E^+CD25^+ or α_E^-CD25^- cells (4). The differential phenotypes of α_E^- and α_E^+ Treg subsets resulted in distinct homing patterns, which were directly coupled to the functional activity of the respective subsets in vivo, indicating that appropriate localization was a prerequisite for suppressive capacity (5).

Initially, Tregs had been characterized as anergic cells, as they do not respond to TCR-mediated stimulation in vitro, and only high amounts of exogenous IL-2 can overcome their hyporesponsiveness (6). Later, it has been demonstrated that Tregs show proliferation in vivo, i.e., they respond to self-peptide presented by unique dendritic cells in tissue-draining lymph nodes (LN) (7–12). These cells display an activated phenotype and proliferate in normal unmanipulated mice (7, 13). However, a clear correlation between peripheral proliferation of Tregs and acquisition of α_E expression has not been established in much detail yet (13).

Apart from the peripheral expansion of thymic-derived Tregs, there is also ample indication that Tregs can be induced de novo from the conventional naive CD4^+ T cell pool, both in vitro and in vivo. Immunosuppressive cytokines like TGF-β (14–16) and IL-10 (17), as well as immature dendritic cells (18, 19), have been used to induce Tregs with distinct properties. Various protocols of tolerance induction, i.e., oral, nasal, and i.v. Ag-administration in transfer models of Ag-specific T cells, were also shown to result in the generation of Tregs (16, 20–24). However, the reported phenotypes of de novo induced Tregs are heterogeneous, particularly with respect to the expression of the Treg marker Foxp3.

The memory phenotype of α_E-expressing Tregs suggests Ag-driven induction or expansion in the periphery. However, it was not clear so far whether α_E^- Tregs originate from naive-like α_E^-CD25^+ Tregs differentiating into an effector/memory phenotype upon Ag-driven activation and proliferation, or whether α_E^+ Tregs can also directly develop from conventional naive T cells under appropriate tolerogenic conditions.

In the present study, we analyzed proliferation of the various Treg subsets and of naive T cells under homeostatic conditions and upon oral feeding of Ag. Adoptive transfer of Ag-specific TCR-transgenic T cell subsets provided clear evidence that α_E^- effector/memory Tregs can develop both from conventional naive T
Figure 1. αE+ Tregs preferentially express an effector/memory-like phenotype. Spleen cells from C57BL/6 mice were surface-stained for CD4, CD25, αE, and CD62L or CD44, followed by intracellular staining for Foxp3. Cells were analyzed by flow cytometry. A, Representative dot plots showing expression of CD25 and αE among CD4+ T cells (left) and CD4+Foxp3+ T cells (right). B, CD4+ T cells were gated according to the expression of CD25 and αE as displayed in A. Dot plots show expression of Foxp3 and CD62L or CD44 on indicated subsets. Isotype control staining for Foxp3 is shown (left dot plots). Data are representative of at least two independent experiments with 2–3 individual mice each. Numbers indicate frequency of positive cells among indicated CD4+ T cell subsets.

Materials and Methods

Animals

BALB/c, C57BL/6, and OVA-specific TCR-transgenic D011.10 mice were obtained from the breeding facility BIR (Bundesinstitut für Risikobewertung). Thymectonized C57BL/6 mice were provided by L. Klein (Research Institute of Molecular Pathology, Vienna, Austria). D011.10 × Rag-1−/− mice were from the breeding facility of the University of Mainz. Mice were 6–10 wk of age unless otherwise stated. All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines.

Abs, staining, and sorting reagents

The following Abs were produced in our laboratory: anti-CD3 (145–2C11), FITC- and Alexa 405-labeled anti-CD4 (GK1.5), biotinylated anti-OVA-TCR (KJ1.26), biotinylated and Alexa 647-labeled anti-αE (CD103; M290), FITC-labeled anti-CD44 (IM7), and FITC-labeled and biotinylated anti-CD25 (Mel-14). The following Abs and secondary staining reagents were purchased from BD Pharmingen: FITC-labeled anti-CD4 (RM4–5), allophycocyanin- and PerCP-Cy5.5-labeled anti-CD25 (PC6.1), biotinylated anti-CD25 (7D4), PE-Cy7-labeled streptavidin, PE-labeled anti-CD44 (IM7), and appropriate isotype controls. The following Abs were purchased from eBioscience: allophycocyanin-Alexa750-labeled anti-CD4 (RM4–5) and Pacific Blue-labeled anti-CD62L (Mel-14). For intracellular detection of BrdU incorporation, the BrdU Flow kit (BD Biosciences) was used. Intracellular staining for Foxp3 was performed using the Foxp3 staining set with PE- or PE-Cy5-labeled anti-mouse Foxp3 (FJK-16s) from eBioscience. All MicroBeads were purchased from Miltenyi Biotec.

Cell preparation and flow cytometry

For ex vivo analysis of Treg subsets, single-cell suspensions were prepared from indicated, secondary lymphoid organs. Erythrocyte-depleted cells were stained using fluorochrome-labeled reagents, and multicolor analysis was performed using a LSRII (BD Biosciences) and the CellQuest software. Cell preparation and flow cytometry were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines.

Evaluation of homeostatic proliferation by BrdU incorporation

C57BL/6 mice thymectomized at the age of 3 wk were used 4 wk later for the experiment. BrdU (Sigma-Aldrich) was added to the drinking water at 1 mg/ml and changed every 2–3 days. On day 10 after the start of BrdU feeding, mice were killed and cell suspensions were prepared from different lymphoid organs. Cell surface staining was performed, and subsequently cells were fixed, permeabilized, and stained with FITC-labeled anti-BrdU Ab according to the manufacturer’s instructions. Cells were analyzed on a LSRII.

Ag-specific activation in vivo

For Ag-specific activation, lymphocyte subsets from OVA-specific, TCR-transgenic D011.10 mice were isolated by MACS and transferred into Foxp3-negative recipient mice. Single-cell suspensions of the lymphoid organs of D011.10 mice were depleted of αE+ cells, subsequently CD25+ cells were isolated with biotinylated anti-CD25 and anti-Biotin MicroBeads. From the MACS-negative fraction naive cells were isolated using anti-CD62L MicroBeads. Sorted cells were labeled with CFSE and 4–6 × 105 αE CD25+CD62Llo or 6–9 × 105 αE CD25+ cells were injected i.v. into BALB/c mice. One day after adoptive transfer, OVA protein (Sigma-Aldrich) was administered via different routes. The tolerogenic protocol involved continuous feeding of OVA protein at indicated doses in the drinking water for 6 days and preparation of lymphoid organs on day 7. For the immunogenic-Ag administration, recipient mice were injected once with 500 μg OVA protein and 70 μg LPS (Sigma-Aldrich) i.p.; mice were sacrificed on day 5 after immunization. Cell surface staining was performed on single-cell suspensions from different lymphoid organs, followed by fixation, permeabilization, and intracellular staining for Foxp3 according to the manufacturer’s instructions. Cells were analyzed by FACS.

In vitro suppression assay

αE+Foxp3+CD4+ T cells were de novo induced as described above and isolated from the celiac LN draining liver (livLN), pancreas, and stomach. Thereto, single-cell suspensions from 12 to 17 livLNs were pooled, stained for CD4 and KJ1.26, and sorted for CFSElow KJ1.26CD4+ T cells using a FACS Aria (BD Biosciences). CD25+ control Tregs, CD62Lhi CD25+CD4+ naive responder T cells, and CD90−APCs were isolated from lymphoid organs of BALB/c mice. Briefly, CD25+ cells were isolated with biotinylated anti-CD25 and anti-Biotin MicroBeads. From the MACS-negative fraction, CD4+ T cells were enriched using anti-CD4-FITC and anti-FITC MultisortBeads. After release of the beads according to the manufacturer’s instructions, naive CD62LhiCD25+CD4+ T cells were sorted using anti-CD4-FITC MicroBeads. APCs were prepared from the CD4+ fraction by depletion of CD90+ cells using anti-CD90 MicroBeads and were irradiated (30 Gy) before culture.

A total of 1 × 106 CD62LhiCD25+CD4+ naive responder T cells were cultured with 2 × 106 APCs in round-bottom microtiter plates with addition of anti-CD3 (1 μg/ml), and unstimulated cells were taken as controls. Cell culture was done with RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich). Cells were incubated for 48 h, followed by addition of 1μCi of methyl-[3H]thymidine (Amersham Pharmacia Biotech) per well for 20 h to measure proliferation. In coculture assays, 2–3 × 105 CFSElow KJ1.26CD4+ T cells or CD25− control Tregs were added to naive responder cells and APCs and treated as described above.

Statistical analysis

The results were either represented as single values representing individual mice or as mean ± SD. For statistical analyses, nonparametric tests were used to determine significant differences. For paired observations, the Wilcoxon signed-rank test was applied.

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signed rank test and for unpaired observations, the Mann-Whitney U test were used, respectively. Differences were considered statistically significant when \( p < 0.05 \) and highly significant when \( p < 0.01 \).

### Results

\( \alpha_i^+ \)Foxp3\(^+\) Tregs preferentially express an effector/memory-like phenotype

Our group has previously described a dichotomy within the naturally occurring CD4\(^+\) Treg population based on thorough phenotyping of \( \alpha_i^+ \) Tregs. Expression of \( \alpha_i \) was found to be highly associated with an effector/memory-like status of Tregs (4). In this study, we confirmed and extended these previous findings regarding the phenotype of \( \alpha_i^+ \) and \( \alpha_i^- \) Treg subsets by analyzing the subset composition of CD4\(^+\) T cells and Foxp3\(^+\) CD4\(^+\) Tregs from the spleen of C57BL/6 mice (Fig. 1a). Gating on CD4\(^+\) T cells revealed that \( \alpha_i^- \) CD25\(^+\) cells contained 78.4 \pm 7.7\%, \( \alpha_i^+ \) CD25\(^+\) cells 88.2 \pm 6.3\%, and \( \alpha_i^- \) CD25\(^-\) cells 58 \pm 9.6\% Foxp3\(^+\) cells (mean \pm SD, \( n = 17 \)), respectively. Conversely, among gated Foxp3\(^+\) CD4\(^+\) T cells, the frequency of \( \alpha_i^- \) CD25\(^+\) cells was 47.3 \pm 9.3\%, \( \alpha_i^+ \) CD25\(^+\) cells 16.6 \pm 4.4\%, and \( \alpha_i^- \) CD25\(^-\) cells represented 7.7 \pm 2.8\% (mean \pm SD, \( n = 17 \)), respectively.

Investigation of the coexpression of Foxp3 and CD62L or CD44 confirmed that \( \alpha_i \) expression on Foxp3\(^+\) Treg subsets is tightly
from DO11.10 mice were depleted of CD4+ T cells from DO11.10 mice, the frequency of CD4+ T cells among gated CD4+ T cells was 42.7 ± 1.0%, CD25- cells 17.0 ± 1.2%, and CD25+ cells represented 10.8 ± 0.2% (mean ± SD, n = 5), respectively. Among gated KJ1.26+ CD4+Foxp3+ T cells from DO11.10 mice, the frequency of CD25+ cells was 32.6 ± 5.2%, CD25- cells 18.7 ± 2.5%, and CD25+CD4+ cells represented 13.0 ± 2.7% (mean ± SD, n = 5), respectively. Pooled spleen and LN cells from DO11.10 mice were depleted of CD4+ cells and enriched for naive-like CD25+ T cells by MACS. Purified CD4+CD25+ cells were labeled with CFSE, adaptively transferred into BALB/c recipients, and subsequently stimulated in vivo by oral application of their cognate Ag. Dot plots show staining of CD62L and CD44 expression of re-isolated Tregs in indicated lymphoid organs after different doses of OVA in drinking water. Mean ± SD corresponds to three individual mice per group from one of two independent experiments with similar results.

**FIGURE 4.** αE+ expressing peripheral Treg subsets show high proliferation under homeostatic conditions

Based on our previous observation that αE+ Tregs harbor reduced TCR excision circle numbers, indicative of increased proliferative history (4), we wanted to address the question whether this is the consequence of peripheral proliferation. To exclude any impact of thymic proliferation, we used thymectomized mice for the evaluation of peripheral proliferation by BrdU incorporation. C57BL/6 mice were thymectomized at the age of 3 wk, when the immune system is thought to have fully evolved. Four weeks later, we started to feed the mice with 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. Surprisingly, we found high frequencies of BrdU+ cells in all Treg subsets, indicating that a substantial number of cells had proliferated within the 10 days of BrdU administration. In contrast, the fraction of αE+ CD25+CD4+ T cells, which largely consists of conventional naive cells, showed only low frequencies of BrdU+ cells as expected (Fig. 2). Within the Treg subsets, the αE+CD25+ cells showed the highest frequency of BrdU-labeled cells in all of the tested lymphoid organs (Fig. 2). αE+CD25+ cells showed only slightly reduced frequencies of BrdU+ cells compared with αE+CD25- cells, and the lowest frequency was detected among αE-CD25+ Tregs. These data correspond to the phenotype of the respective Treg subsets (Fig. 1b) and to our previous observation that αE+ Treg subsets have reduced TCR excision circle numbers (4), suggesting that indeed these cells display a high degree of peripheral proliferation under steady-state conditions.

**αE+ Treg numbers are stable in the absence of thymic output**

When we analyzed the relative distribution of αE/CD25 Treg subsets, we observed highly increased frequencies of αE-expressing Tregs in thymectomized mice compared with non-thymectomized control mice (Fig. 3a). At the same time, the size of the total CD4+ compartment was reduced in all organs analyzed (Fig. 3b), presumably due to the lack of thymic output of newly generated T cells. Analysis of the respective cell number of αE/CD25 Tregs associated with an effector/memory phenotype (Fig. 1b). The majority of αE+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 αE+Foxp3+ Treg subsets expressed much higher levels of CD44 and lower amounts of CD62L, indicating a higher activation/differentiation state. These results confirmed our initial correlation of αE expression with an effector/memory-like phenotype on the single cell level for Foxp3+ Tregs.
(Fig. 3b) revealed that the total number of αE-expressing Tregs in thymectomized mice was similar to control mice and 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 αE⁺ Tregs. Interestingly, this shift only affected the effector/memory-like αE⁺ Tregs but not the naive-like αE⁺ CD25⁺ Tregs, suggesting that the latter subset relies much more on newly generated, thymic-derived Tregs to maintain stable cell numbers in the absence of thymic output, whereas the αE⁺ Tregs kept a stable population size.

In vivo conversion of naive-like CD25⁺ Tregs into αE⁺ Tregs

The previous experiments confirmed that the activated phenotype of αE-expressing Tregs indeed correlates with a high degree of peripheral/homeostatic proliferation in normal, healthy mice. Given that naturally occurring Tregs are thought to originate from the thymus, where they are positively selected to recognition of self-Ag (1), we speculated that acquisition of the effector/memory
phenotype could be a consequence of activation by self-Ag in the periphery, i.e., in secondary lymphoid tissue draining the site of respective self-Ag expression (8). In this case, naive-like $\alpha_E^{-}$ Tregs should differentiate into effector/memory-like $\alpha_E^{+}$ Tregs upon Ag-specific stimulation in vivo. To address this question, we switched from the analysis of the polyclonal T cell pool to an Ag-specific system, where we were able to follow the immune response to known cognate Ag. We chose the OVA-specific system and first compared the distribution of Treg subsets in TCR transgenic DO11.10 mice with that of age-matched BALB/c mice. Besides a weak reduction in the frequency of $\alpha_E^{-}$ CD25$^+$Foxp3$^+$ Tregs, no gross differences were observed (Fig. 4a). Next, we isolated naive-like $\alpha_E^{-}$ CD25$^+$ Tregs from DO11.10 mice (Fig. 4b). After adoptive transfer of the Ag-specific Tregs into naive BALB/c recipients, we administered the cognate Ag via the oral route, which is known to result in tolerance induction (25), and followed proliferation and phenotype of transferred cells by multicolor FACS analysis (Fig. 4c). When we continuously fed the recipient mice with different doses of OVA protein (4–20 mg/ml) in the drinking water for a period of 6 days, we observed moderate proliferation of the Ag-specific KJ1.26$^+$CD25$^+$ Tregs in gut-associated lymphoid organs such as the celiac LN draining liver, pancreas, and stomach (liv LN) (26) and mesenteric LNs (mLNs). Hardly any OVA-specific CD25$^+$ Tregs were detected in Peyer’s Patches (PP) and spleen (data not shown). We analyzed the transferred CD25$^+$ Tregs for the expression of $\alpha_E$, and could detect a high degree of coexpression of $\alpha_E$ on previously $\alpha_E^{-}$ CD25$^+$ cells after Ag-driven proliferation (Fig. 4c). The frequency of $\alpha_E$ expression on OVA-specific CD25$^+$ Tregs increased with each cell division, as judged by loss of CFSE, and reached similar levels both in liv LN and mLN (Fig. 4d). Moreover, converted Tregs displayed down-regulated CD62L levels and up-regulated CD44 expression when compared with nonconverted $\alpha_E^{-}$ CD25$^+$ Tregs (Fig. 4e). These results support the concept that upon Ag-specific activation in the periphery, naive-like $\alpha_E^{-}$ CD25$^+$ Tregs differentiate into effector/memory Tregs, characterized by expression of $\alpha_E$.

**Induction of $\alpha_E^{-}$Foxp3$^+$ cells from conventional naive T cells**

To answer the question whether, in addition to the aforementioned differentiation of naive-like $\alpha_E^{-}$ CD25$^+$ Tregs, de novo induction of Foxp3$^+$ Tregs from conventional naive T cells might contribute to the pool of $\alpha_E$-expressing effector/memory-like Tregs, we performed corresponding experiments with adaptive transfer of naive $\alpha_E^{-}$ CD25$^+$CD62L$^+$ T cells from DO11.10 mice and OVA
administration via the oral route (Fig. 5a). Proliferating transgenic KJ1.26\(^+\)CD4\(^+\) T cells were detected in livLN, mLN, PPs, and spleen at various doses of Ag (Fig. 5b). Although we observed slightly reduced proliferation in livLN compared with the other organs (Fig. 5b and data not shown), intriguingly, we identified a high proportion of Foxp3\(^+\) cells among transgenic cells re-isolated from the livLN, predominantly, but not exclusively, on proliferating cells. The induction was dose-dependent and largely confined to this particular LN (Fig. 5, a and b), as the frequency of Foxp3\(^+\) cells was much lower in mLN and PP cells (Fig. 5c). When we isolated CFSE\(^{\text{low}}\)KJ1.26\(^+\)CD4\(^+\) T cells from the livLN, which largely were composed of Foxp3\(^+\) cells (data not shown), and tested their suppressive capacity, we observed that these de novo induced Foxp3\(^+\) cells were equally efficient in suppressing naïve T cell proliferation as ex vivo isolated CD25\(^{\text{high}}\)CD4\(^{\text{high}}\) Tregs, identifying them as bona fide Tregs (Fig. 5d).

Phenotyping of the induced Foxp3\(^+\) Tregs revealed that the vast majority of these cells coexpressed αE (Fig. 6a), showing a proliferation-dependent up-regulation of both markers on previously naïve Foxp3\(^-\) transgenic T cells in the livLN. Importantly, the induced Treg phenotype was only observed after tolerogenic Ag administration. When we immunized the recipient mice with OVA, we observed de novo induction of Foxp3\(^+\) cells among CD4\(^+\) T cells derived from DO11.10 \times RAG-1\(^{-/-}\) mice, which lack natural Foxp3\(^+\) Tregs (27, 28), and fed the recipient mice with OVA, we observed de novo induction of Foxp3\(^+\) expression in a substantial fraction of transferred cells (Fig. 6d). Viewed as a whole, these findings argue against emergence of Foxp3\(^+\) Tregs by expansion of pre-existing CD25\(^{\text{high}}\) Tregs and rather support the view that Foxp3\(^+\) Tregs were induced from conventional naïve CD25\(^{\text{+}}\) T cells.

### Discussion

Despite initial difficulties to drive Tregs into proliferation under standard in vitro conditions, strong proliferation in vivo, i.e., in LNs draining tissue that expresses their respective self-Ag, was demonstrated in subsequent studies (7–9). In this study, we analyzed the correlation of αE expression and peripheral proliferation of Tregs in thymectomized mice in the steady state. Dividing cells were highly enriched in the αE\(^{\text{+}}\)CD25\(^{\text{+}}\) fraction, to a lesser extent in the αE\(^{\text{+}}\)CD25\(^{\text{−}}\) Treg subset, and the lowest fraction of proliferating cells was found within the αE\(^{\text{−}}\)CD25\(^{\text{+}}\) Treg subset. These findings demonstrate a clear link between a high proliferative activity and the effector/memory-like phenotype of αE\(^{\text{+}}\) Tregs, as previously hypothesized (4). In this study, we defined conditions leading to the generation of effector/memory αE\(^{\text{+}}\) Tregs in vivo as well as the sites where generation and expansion take place.

The observed proliferation indicates that a major part of Tregs is constantly cycling under steady-state conditions, presumably in response to their cognate Ags. Our findings are in accordance with results from Fisson and colleagues (7), who demonstrated rapid turnover of transferred polyclonal CD25\(^{\text{+}}\)CD4\(^{\text{+}}\) Tregs in nonlymphopenic recipients associated with acquisition of an activated phenotype. A proliferative response of CD25\(^{\text{+}}\) Tregs toward the presence of their cognate Ag in vivo could also be observed in studies using TCR-transgenic models (8, 9), supporting the notion that Treg proliferation requires Ag-specific activation.

In the polyclonal repertoire, the nature of the Ags recognized by Tregs remains elusive; however, it was shown that Tregs display a diverse TCR repertoire (29), which is distinct from conventional T cells and skewed toward high affinity recognition of self-Ag (30, 31). Furthermore, recent findings demonstrated that Tregs and potentially pathogenic self-reactive T cells have intersecting TCR repertoires (32). However, Tregs were also reported to recognize Ags from infectious agents such as *Leishmania major* (33), and unpublished data from our own group suggest a contribution of

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### Table I. Foxp3\(^+\) cell numbers before and after Ag-specific expansion/induction by oral Ag

<table>
<thead>
<tr>
<th>Precursor Cells</th>
<th>Input of Foxp3(^+) Cells</th>
<th>Foxp3(^+) Recovered after 6 Days of OVA-Feeding</th>
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<td>Foxp3(^+) (%E)</td>
<td>Cell number (×10(^3))</td>
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<tr>
<td>αE(^{\text{+}}) CD25(^{\text{−}})</td>
<td>0.19</td>
<td>6.2</td>
</tr>
<tr>
<td>αE(^{\text{−}}) CD25(^{\text{+}})</td>
<td>92</td>
<td>347</td>
</tr>
</tbody>
</table>

* 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).
stimuli derived from the intestinal microflora to the proliferation of Tregs in specific pathogen-free mice. Taken together, these findings indicate that under steady-state conditions, sufficient Ag sources exist stimulating homoeostatic proliferation of Tregs in healthy mice. Clearly, the highest proliferative activity accumulates in the Treg subsets, which displays an effector/memory-like phenotype and is characterized by the expression of αe.

In thymectomized mice, we observed a relative increase in the frequency of αe-expressing Tregs, which was due to the reduced cell count of conventional CD4+ T cells resulting in a mild lymphopenia in the T cell compartment. Absolute numbers of αε− Tregs were comparable to non-thymectomized control mice, indicating that thymic output was not necessary to maintain this Treg subset. In contrast, the αε− CD25+ Tregs displayed a comparable reduction in cell numbers following thymic ablation, as the conventional naive T cell pool. Constant numbers of αε− Tregs upon thymectomy appear to contradict the higher sensitivity toward apoptosis of αε+ CD25+ Tregs as compared with αε− CD25+ Tregs, which has recently been described by Stephens and colleagues (13) and which we could confirm using Treg subsets from Foxp3-GFP-reporter mice (data not shown). The observation that numbers of αε+ Tregs are maintained in the absence of thymic export despite a high propensity for apoptosis, therefore, suggests that the fate of αε− Tregs is well balanced by a constant peripheral expansion of this population of Tregs.

Our findings support a concept where αε identifies peripherally expanded effector/memory-like Tregs with an activated phenotype, which possibly have differentiated from αε CD25+ Tregs. Our data are consistent with a previous report by Dujardin et al. (34), who demonstrated a pronounced predominance of αε− CD25+ Tregs in adult mice that had been thymectomized at day 3 (d3Tx). In the periphery of newborn mice, these authors observed significant numbers of αε− CD25+ Tregs emigrated from the thymus before thymectomy, but not of αε− CD25+ Tregs, while a 5-fold higher frequency of αε− CD25− Tregs was found in adult d3Tx mice. Our study using mice thymectomized at the age of 3 wk, a time point when the T cell compartment has fully evolved, further demonstrates that independence of the αε+ Treg subset from thymic output is not restricted to the aforementioned situation in d3Tx mice, where postnatal development of the whole T cell compartment is severely disturbed. Furthermore, when we compared in vivo proliferation in non-thymectomized control mice to thymectomized mice, no differences were observed regarding the frequency of BrdUhigh cells, which are indicative of proliferation in the periphery among the various Treg subsets (data not shown). In summary, the above findings clearly indicate that post-thymic proliferation contributes to the development of αε− Treg subsets. The data suggest that αε− Tregs keep proliferating, yet we cannot formally exclude the alternative hypothesis that only αε− CD25+ Tregs proliferate and cease to cycle once αε-expression has been acquired.

To clarify the developmental relationship between the different naturally occurring Treg subsets, we aimed to identify potential precursors of the αε-expressing Treg subset. When we transferred OVA-specific naive-like αε− CD25+ Tregs followed by oral Ag administration, the vast majority of CD25+ Tregs previously negative for αε up-regulated expression of this marker, suggesting that tolerogenic, Ag-specific activation of naive-like Tregs leads 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 (data not shown). The degree of proliferation of formerly αε− CD25+ Tregs under these tolerogenic conditions was modest as compared with an immunogenic activation in the presence of LPS (data not shown). Nevertheless, it remains elusive how already converted αε− CD25+ Tregs would respond to Ag-specific activation in this setting. Indirect evidence for a profound proliferative response of αε− Tregs comes from recent data obtained in a polyclonal transfer model, demonstrating that total CD4−CD25+ cells, but not αε-depleted CD4−CD25+ cells, displayed loss of CFSE within 7 days after transfer into congenic recipient mice (13).

When we transferred conventional naive αε− CD25+ Foxp3− mice and subjected them to the same regimen of oral Ag administration, we observed induction of Foxp3+ cells in a dose-dependent fashion. 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 nonregulatory, conventional T cells into Tregs under distinct conditions in vivo (19, 20, 24, 35, 36), both by using TCR-transgenic and polyclonal naive precursor cells. In line with our findings, recent data investigating the homeostatic proliferation of transferred CD4+Foxp3eGFP+ in RAG−1− recipient mice revealed that those cells that had converted in the GALT into Foxp3eGFP+ cells were positive for αε-expression (16).

Hultkrantz and colleagues (23) first described an important role for the livLN in the generation of CD25+ Tregs, characterized by high levels of αε coexpression and suppressive capacity. However, these authors did not detect Foxp3 mRNA in this subset. This is in contrast to our finding, where we consistently observed expression of Foxp3 on the single cell level as well as suppressive activity of CFSE−KJ1.26+ CD4+ cells re-isolated from the livLN. The perivascular livLN drains the liver, stomach, and pancreas (26), and several studies have shown an important role for the liver in generating tolerogenic immune responses to food Ag and even in transplantation settings (37, 38). Ag from the intestinal lumen reaches the liver directly via the portal vein, and occlusion of the portal vein prevented the development of oral tolerance (39). De novo induction of Foxp3+ Tregs in the livLN, upon oral Ag administration, found in this study underlines the pivotal role of the livLN in the induction for peripheral tolerance. At the same time, immunization with OVA protein i.p. in the presence of LPS did not result in the induction of Foxp3+ Tregs, revealing that the specific microenvironment of the livLN is tolerogenic only in the absence of inflammatory stimuli.

The data presented in this study indicated that Foxp3+ Tregs could be generated by conversion of previously Foxp3− non-Tregs. 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 nondivided CFSEhigh fraction of transferred cells, but only in the presence of cognate Ag. 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 × 105 CD25− Foxp3− cells or 3.5 × 105 CD25+ Foxp3+ cells 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 with 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 (36). In addition, our data obtained upon adoptive transfer of CD4+ T cells from DO11.10×RAG−/− mice followed by oral Ag administration confirmed de novo induction of Foxp3+ cells. Therefore, based on these cumulative findings, we concluded that Foxp3+ Tregs were induced from the nonregulatory CD62Lhigh T cell pool in response to
the particular conditions of Ag-specific activation, predominantly in the livLN, following oral administration of cognate Ag.

It is tempting to speculate that TGF-β might be a major factor driving generation of Foxp3+ cells in our setting. Several in vitro data have shown induction of Foxp3 upon stimulation in the presence of TGF-β (14, 15). Furthermore, TGF-β is important for maintenance of Foxp3 expression and suppressive function of Tregs in vivo (40, 41). Next to these recent findings, it has been known for some time that TGF-β is a major factor inducing expression of αE on CD4+ T cells (42). Therefore αE expression may reflect activation in a microenvironment where local concentrations of active TGF-β were sufficient to both maintain Foxp3 expression in proliferating naive-like CD25+ and to generate new Foxp3+ cells from conventional naive T cells. Whether retinoic acid is critically involved in the de novo generation of αE+ Tregs, as recently suggested for the generation of Foxp3+ Tregs (43), remains to be shown.

Expression of αE has also been associated in a number of studies with peripherally induced/expanded Tregs in infection models. Suffia et al. (44) demonstrated a functional role for αE expression in the retention of Tregs at epithelial sites, where αE+ Tregs suppressed the local response to Ag-delivery in the periphery, while at the same time the very same conditions promote proliferation/expansion of pre-existing Tregs with a phenotype indistinguishable from the naturally occurring Foxp3+ Treg pool. Their proliferation and survival depended on the presence of the parasite (33). In other infection models, such as Schistosoma mansoni (45) and Heligmosomoides polygyrus (S. Hartmann, personal communication), Foxp3+ Tregs displayed increasing frequencies of αE+ Tregs during the course of disease progression.

In summary, our data support the hypothesis that αE expression is not only preferentially associated 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. Furthermore, our results strongly suggest that the αE+ Foxp3+ Treg pool is of diverse origin, consisting of both thymic-derived, expanded/differentiated natural Tregs as well as de novo induced Foxp3+ Tregs, generated upon tolerogenic, Ag-specific activation in distinct anatomical sites of the body. Our 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 Ag-delivery in the periphery, while at the same time the very same conditions promote proliferation/expansion of pre-existing natural Tregs.

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Disclosures
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References


