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T-cell tolerance induced by repeated antigen stimulation: Selective loss of Foxp3$^-$ conventional CD4 T cells and induction of CD4 T-cell anergy

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Repeated immunization of mice with bacterial superantigens induces extensive deletion and anergy of reactive CD4 T cells. Here we report that the *in vitro* proliferation anergy of CD4 T cells from TCR transgenic mice immunized three times with staphylococcal enterotoxin B (SEB) (3 × SEB) is partially due to an increased frequency of Foxp3$^+$ CD4 T cells. Importantly, reduced number of conventional CD25$^-$ Foxp3$^-$ cells, rather than conversion of such cells to Foxp3$^+$ cells, was the cause of that increase and was also seen in mice repeatedly immunized with OVA (3 × OVA) and OVA—peptide (OVAp) (3 × OVAp). Cell-transfer experiments revealed profound but transient anergy of CD4 T cells isolated from 3 × OVAp and 3 × SEB mice. However, the *in vivo* anergy was CD4 T-cell autonomous and independent of Foxp3$^+$ Treg. Finally, proliferation of transferred CD4 T cells was inhibited in repeatedly immunized mice but inhibition was lost when transfer was delayed, despite the maintenance of elevated frequency of Foxp3$^+$ cells. These data provide important implications for Foxp3$^+$ cell-mediated tolerance in situations of repeated antigen exposure such as human persistent infections.

Key words: Immune regulation · Tolerance · Treg

Introduction

Immunization of mice with bacterial superantigens (SAg) causes the deletion of SAg-reactive CD4 T cells [1–3]. The extent of deletion is dose-dependent and single high doses or repeated low doses of the SAg causes deletion of a larger fraction of the reactive CD4 T cells [4, 5]. The remaining SAg-reactive CD4 T cells in the immunized animals are anergic, as they fail to respond to the immunizing SAg both *in vivo* and *in vitro* [4–8]. The anergic CD4 population in SAg-immunized mice has been extensively studied. Again, depending on the immunization protocol, the unresponsiveness of the anergic cells varies and in some instances, the cells even fail to respond to IL-2 [9–11].

Reports from several laboratories have demonstrated that the anergic CD4 T-cell population induced in mice repeatedly immunized with SAg, in addition to inherently non-responsive T cells, also contains Treg that can suppress an immune response to the antigen both *in vivo* and *in vitro* [10, 12–16]. The Treg were induced in experimental models involving repeated stimulation with either bacterial or viral SAg [10, 12–16] and therefore active suppression by antigen-specific Treg seems to be a common component that might contribute to the anergic phenotype observed at the T-cell population level. Interestingly, the Treg induced by repeated stimulation were functionally similar either to natural CD4$^+$CD25$^+$ Treg [12, 13, 15] or to Tr-1 cells (IL-10-Treg) [10, 14–16].

Persistent infections involve repeated contacts between the microbe and the immune system of the host. Such infections often correlate with an increased fraction of regulatory Foxp3$^+$ CD4$^+$ cells in blood (reviewed in [17, 18]). The increased fraction of Foxp3$^+$ cells has been taken as evidence for the induction of development of these cells in the infected individual. We have previously observed that repeated immunization with SAg caused the efficient induction of both CD4$^+$25$^-$ and CD4$^+$CD25$^+$ Treg in mice [15], a scenario that may mimic the situation during chronic infections. That conviction prompted us to further investigate the nature of the Treg induced in our model.

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We report here that repeated antigen stimulation increases the proportion of Foxp3$^+$ Treg by decreasing the number of antigen-reactive conventional CD4 T cells, rather than induction of *de novo* development of Foxp3$^+$ Treg. Antigen-specific T-cell proliferation was potently inhibited in the repeatedly immunized mice and cells from such mice were anergic upon transfer to naïve recipients. We show that *in vitro* and *in vivo* T-cell anergy in this model is differentially dependent on Foxp3$^+$ cells.

Results

Increased frequency of Foxp3$^+$ cells in repeatedly immunized mice

Our previous results demonstrated that both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells isolated from mice repeatedly immunized with a bacterial SAg suppressed SAg-induced T-cell proliferation and IL-2 production *in vitro* [15]. As the two T-cell populations were functionally similar to natural Treg, we speculated that the CD4$^+$CD25$^-$ T-cell population might contain natural Treg that failed to express the CD25 marker.

To address this hypothesis, we immunized DO11.10 TCR-transgenic (TCR-TG) mice three times with staphylococcal enterotoxin B (3× SEB). Spleen (SPL) cells from the 3× SEB TCR-TG mice responded poorly to SEB *in vitro* as compared with cells from control animals, confirming the induction of T-cell anergy by the repeated SEB immunization (Fig. 1A). As expected [19–21], both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from control mice expressed the Foxp3 protein, but immunization significantly increased the frequency of Foxp3$^+$ cells in the splenic CD4$^+$CD25$^-$ population (Fig. 1B). The number of splenic Foxp3$^+$ CD4 T cells was slightly increased in 3× SEB mice, but the increase did not reach significance in repeated experiments (Fig. 1D). Rather, a pronounced reduction in the number of Foxp3$^-$ CD4$^+$CD25$^-$ T cells (Fig. 1C) caused a twofold increased frequency of splenic CD4$^+$ Foxp3$^+$ cells in 3× SEB mice, as compared with cells from non-immunized TCR-TG mice (Fig. 1E). Similar results were obtained from analyses of cells from MLN (not depicted), suggesting that the immunization protocol induced systemic changes in the CD4 T-cell compartment. Thus, although it is well-established that antigen-exposure can convert peripheral naïve CD4 T cells to Foxp3$^+$ Treg [22, 23], we conclude that the main cause of the increased frequency of Foxp3$^+$ cells in this model is a selective reduction in the number of CD4$^+$CD25$^-$ conventional T cells.

These changes in the CD4 T-cell repertoire are not peculiar to the 3× SEB model. Thus, OT-II TCR-TG mice immunized with OVA–peptide (OVAp; 3× OVAp mice) or OVA protein (3× OVA mice) (Fig. 2), using a similar immunization protocol [16, 24] provided analogous data. The number of CD4$^+$CD25$^-$ T cells was also reduced in MLN and SPL of these mice (Fig. 2A and D) and the fraction of Foxp3$^+$ CD4 T cells was increased in a corresponding way (Fig. 2B and E). Further, MLN CD4 T cells (Fig. 2C and F) and SPL cells (not depicted) from these mice were anergic upon stimulation with OVAp *in vitro*.

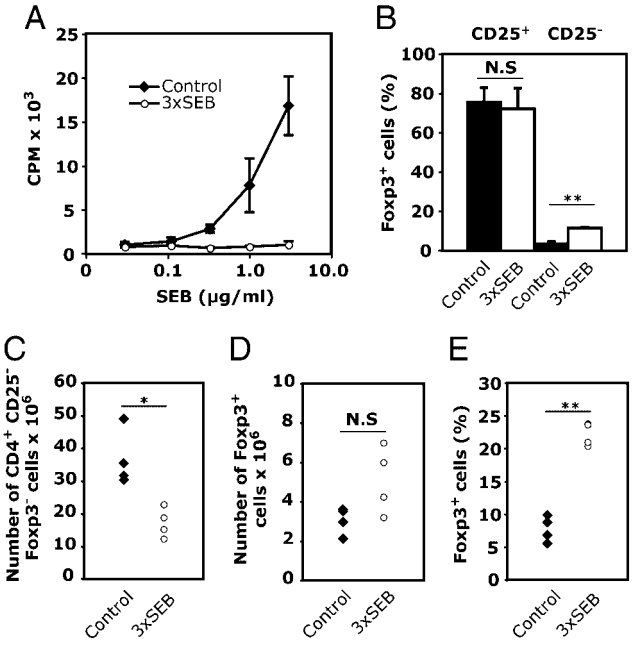

Figure 1. Induction of anergy in 3× SEB mice correlates with increased frequency of Foxp3-expressing CD4$^+$CD25$^-$ T cells. (A) SPL cells (1×10^5/well) from 3× SEB (circles, $n=4$) or non-immunized DO11.10 TCR-TG mice (diamonds, $n=3$) were cultured *in vitro* either in medium alone or in the presence of indicated concentrations of SEB. Proliferation as determined by tritiated thymidine incorporation was assayed on day 3 of culture. The results represent mean ± SD of triplicate cultures. (B) SPL cells from control ($n=3$) or 3× SEB TCR-TG mice ($n=4$) were stained with antibodies to CD4, CD25 and Foxp3 and analyzed by flow cytometry. Cells were gated on the CD4$^+$ population, the results represent mean ± SD. (C, D) Absolute numbers of splenic CD4$^+$Foxp3$^-$CD25$^-$ cells and CD4$^+$Foxp3$^+$ cells, respectively, in the same animals as in (B). (E) Frequency of Foxp3$^+$ cells in the CD4$^+$ population in the same animals as in (B). Data are representative of four independent experiments. N.S = not significant; *p<0.05, **p<0.01, Student's t-test.

In the immunized mice, the percentage of splenic CD4$^+$ Foxp3$^+$ cells expressing CD62L was reduced (45±5%, $n=3$) as compared with control mice (57±0.5%, $n=3$; $p=0.01$). The CD62L expression level was also reduced (3× SEB MFI: 647±6, control MFI: 1916±305; $p=0.002$), suggesting that the cells had been activated [25, 26]. Similarly, splenic CD4$^+$Foxp3$^+$ cells in 3× OVA mice had down-regulated CD62L (MFI: 786±118; $n=3$) compared with controls (MFI: 1992±120; $n=3$ $p=0.0002$) and up-regulated CD44 expression (MFI: 2202±146 *versus* MFI: 1326±55; $p=0.0006$).

Taken together, repeated immunization with SEB as well as other protein antigens increases the proportion of CD4$^+$ Foxp3$^+$ T cells by reducing the number of conventional CD4 T cells and induces CD4 T-cell anergy.

Repeated immunization does not increase *foxp3*-expression

Previous reports have shown that CD4 T cells in *rag*-deficient (*rag*$^{-/-}$) TCR-TG mice fail to express *foxp3* [19], but that

tolerogenic antigen stimulation can convert such cells to *foxp3*$^+$ Treg [22, 23]. We therefore used *rag-2*$^{-/-}$ DO11.10 TCR-TG mice to determine whether our immunization protocol can convert conventional CD4 T cells to *foxp3*$^+$ Treg. T-cell anergy was readily induced in 3× SEB *rag-2*$^{-/-}$ animals (Fig. 3A), thereby confirming our previous results [15]. We next analyzed *foxp3*-expression in mRNAs from individual mice using quantitative RT-PCR. *Foxp3*-expression was detectable but low as compared with that of control cells from *rag*-sufficient DO11.10 mice (Fig. 3B). However, *foxp3*-expression was not significantly increased in the immunized mice. In support of this result, analysis of Foxp3-expression by FACS revealed only a minor, not significant, increase in frequency of Foxp3-expressing cells (Fig. 3C). These data suggest that conversion of conventional CD4 T cells to Foxp3$^+$ cells does not contribute significantly to the increased frequency of Foxp3$^+$ cells seen in *rag*-sufficient mice in the above experiments (Fig. 1).

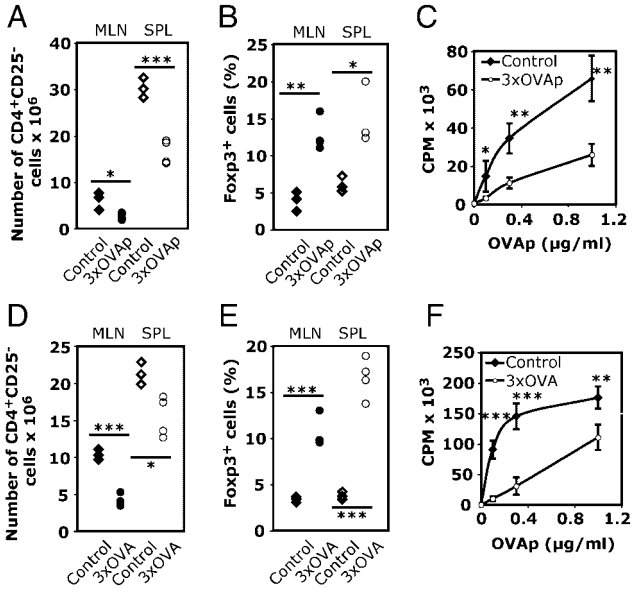

Figure 2. Increased frequency of Foxp3$^+$ cells in TCR-TG mice repeatedly immunized with OVAp or OVA. MLN and SPL cells from control (diamonds) or 3× OVA mice (circles) were analyzed by flow cytometry. The diagrams show the number of CD25$^-$ cells (A, D) and the percentage of Foxp3$^+$ cells in the CD4$^+$ population (B, E). (C, F) T-cell anergy in OTII-SJL mice repeatedly immunized with OVAp or OVA. MLN cells (10^5/well) were cultured in the presence of various concentrations of OVAp. Proliferation on day 3 of culture is shown. The results represent mean ± SD of triplicate cultures and data are representative of three experiments performed. *p<0.05, **p<0.01, ***p<0.001, Student's t-test.

Transient inhibition of T-cell proliferation in repeatedly immunized mice

Repeated immunization with bacterial SAg was previously shown to increase the systemic IL-10 level [8, 14, 27]. Furthermore, cell-transfer experiments have suggested the establishment of suppression in 3× SEB mice [16]. Using cell-transfer experiments, we confirm here that SEB-induced proliferation of CFSE-labeled naïve CD4 T cells was profoundly inhibited in 3× SEB mice (Fig. 4A). Consistently, with the similar induction of T-cell anergy and changes in CD4 T-cell repertoire in 3× OVAp mice, proliferation of transferred naïve CFSE-labeled CD4 T cells was also efficiently inhibited in 3× OVAp recipients (Fig. 4B). The inhibitory mechanism was only transiently active as there was no significant reduction of T-cell proliferation in repeatedly immunized recipients when cell transfer was delayed 1–2 wk after the last immunization with antigen (Fig. 4A and B). Importantly, the percentage of CD4$^+$Foxp3$^+$ cells remained elevated in the day 15 recipient mice (MLN 5±0,2 *versus* 11±2%; $p = 0.006$ / SPL 7±0,1 *versus* 21±3%; $p = 0.002$).

Transient *in vivo* anergy of repeatedly stimulated T cells

SAg-induced T-cell anergy has been extensively studied (reviewed in [28]). However, most of the previous studies

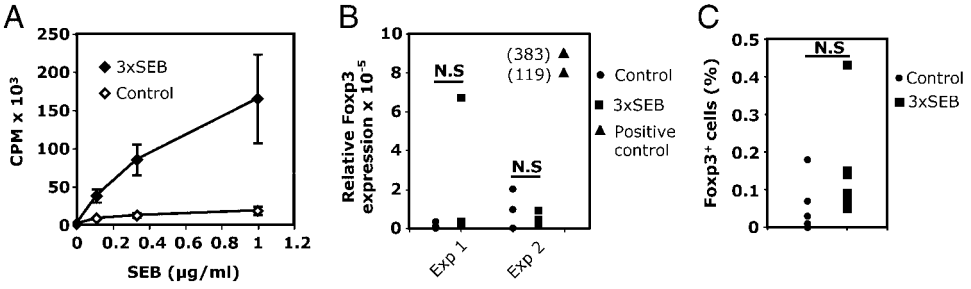

Figure 3. 3× SEB-treated *rag-2*$^{-/-}$ TCR-TG CD4 T cells are anergic, but do not display increased Foxp3-expression. (A) SPL cells from control ($n = 3$) or 3× SEB DO11.10 TCR-TG *rag-2*$^{-/-}$ mice ($n = 3$) were cultured (10^5/well) in the presence of indicated concentrations of SEB and proliferation determined on day 3 of culture. Data from one out of two experiments are shown. The results represent mean± SD of triplicate cultures. (B) Relative *foxp3*-expression in mRNA from SPL cells of control and 3× SEB DO11.10 TCR-TG *rag-2*$^{-/-}$ mice was determined using quantitative RT PCR (see the *Materials and methods*). Splenic mRNA from DO11.10 *rag*-sufficient mice was used as positive control in each experiment (relative *foxp3*-expression is shown in brackets). Data from two independent experiments are shown. Various amounts of cDNA were analyzed on three independent occasions with similar results. (C) SPL cells from control ($n = 6$) and 3× SEB mice ($n = 6$) were stained with CD4, CD25 and Foxp3 antibodies and analyzed for percentage of Foxp3$^+$ cells in the CD4 population using flow cytometry. Data were pooled from two independent experiments. Student's t-test was used.

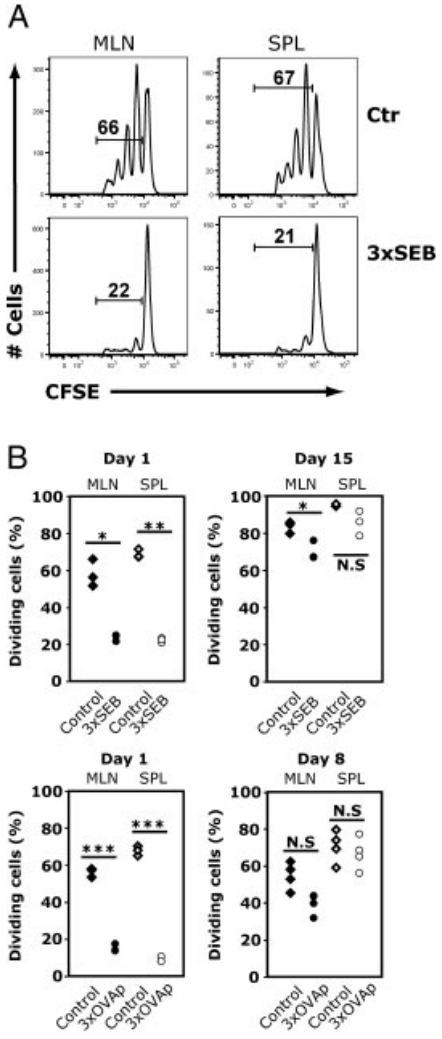

Figure 4. Transient suppression of CD4 T-cell proliferation in 3× SEB and 3× OVAp mice. DO11.10 CD4 T cells or OT-II CD4 T cells were labeled with CFSE and transferred (5×10^6/mouse) i.v. to control or immunized recipient mice. (A) Representative CFSE dilution profiles of transferred CFSE-DO11.10 cells in MLN and SPL from recipient control and 3× SEB DO11.10 mice (summary data shown in B top left panel). (B) Top panels: transfer to control DO11.10 (diamonds) or 3× SEB DO11.10 recipients (circles) either 1 (left panel, $n = 3$) or 15 days (right panel, $n = 3$) after the last immunization. Bottom panels: transfer to control OT-II (diamonds) or 3× OVAp OT-II-SJL recipient mice (circles) either 1 (left panel, $n = 3$) or 8 days (right panel, $n = 4$) after the last immunization. The day after cell transfer, recipient mice were immunized with either SEB or OVAp (10 and 20 μg/mouse i.p., respectively). CFSE-dilution in transferred cells was analyzed by FACS in recipient MLN (filled symbols) and SPL cells (open symbols) 3 days thereafter. Percentage dividing cells was determined as shown in (A). Data are representative of 2–3 experiments. N.S = not significant; *$p<0.05$, $p<0.01$, $p<0.001$, Student's *t*-test.

involved *in vitro* analyses of T cells derived from the tolerized mice. We wanted to determine whether anergy induced by repeated immunization also operates *in vivo*. Therefore, MLN cells from repeatedly immunized mice and controls were labeled with CFSE and transferred to normal recipient mice. CD4 T cells derived from 3× OVAp mice proliferated poorly and were clearly anergic as compared with cells from non-immunized mice (Fig. 5A). Transfer experiments performed with MLN cells isolated from 3× SEB DO11.10 mice and 3× OVA OTII-SJL mice were also anergic (not depicted). Further, the anergy was a property of the CD4 T-cell population, as cell-transfer experiments performed using purified MLN CD4 T cells yielded similar results (Fig. 6 and data not depicted). However, the *in vivo* anergy of the 3× OVAp cells was only transient, as the *in vivo* proliferative capacity of the cells had recovered 20 days (Fig. 5C) after the last immunization. Despite the gradual loss of anergy, there was a persistent increase in frequency of $Foxp3^+$ CD4 T cells both at day 8 (Fig. 5B, right panel) and at day 20 (Fig. 5C, right panel). Proliferation analysis of the transferred cells *in vitro* revealed anergy of the cells at day 8 but loss of anergy at day 20 (not depicted). Thus, the T-cell anergy in this model is CD4 T-cell autonomous, but only transient.

The role of $Foxp3^+$ cells in T-cell anergy

$CD4^+$ T cells from 3× SEA mice potently suppressed *in vitro* T-cell proliferation [15]. We therefore expected that the increased frequency of $Foxp3^+$ cells observed in repeatedly immunized mice (Figs. 1B, 2B and E) might contribute to the T-cell anergy by suppressing proliferation of conventional antigen-reactive T cells present in the anergic population. To address a possible role of $Foxp3^+$ cells in anergy, we depleted CD4 T cells from repeatedly immunized mice of cells expressing CD25, CD103 and glucocorticoid-induced TNF receptor (GITR) [29–31] by magnetic cell sorting. The Foxp3-cell-depleted 3× SEB CD4 T cells (denoted $Foxp3^-$) remained partially anergic as compared with depleted control cells (Fig. 6A). This purification protocol, however, failed to remove all $Foxp3^+$ cells (not depicted). We therefore FACS-sorted $CD25^-CD103^-GITR^-$ CD4 T cells expressing a high level of CD45RB ($CD45RB^{hi}$), which more efficiently removed the $Foxp3^+$ cells (Fig. 6B). While sorted 3× OVAp CD4 T cells (denoted CD4tot) were profoundly anergic when stimulated by OVAp, proliferation was partially restored in $CD45RB^{hi}$ cells depleted of $Foxp3^+$ cells (Fig. 6C). Despite the more rigorous depletion of $Foxp3^+$ cells, the response of these cells remained lower than that of depleted cells from control mice (denoted $Foxp3^-$ control in Fig. 6C). These data suggest that the *in vitro* anergy of the 3× SEB CD4 T-cell population was partially due to suppression by $Foxp3^+$ T cells.

The observation that *in vivo* anergy was lost despite the persistent increase in $Foxp3^+$ cells in repeatedly immunized animals (Fig. 5B and D) suggested that the *in vivo* T-cell anergy might not be dependent on $Foxp3^+$ cells. As shown in Fig. 6D, *in vivo* proliferation of 3× SEB CD4 T cells in naïve recipients was significantly reduced. Most cells were only dividing twice, as compared with control CD4 T cells where most cells divided 3–4 times during the 3 days *in vivo* response. Depletion of $Foxp3^+$ cells by cell sorting did not change the proliferation capacity of 3× SEB CD4 T cells in the adoptive host. We conclude that the

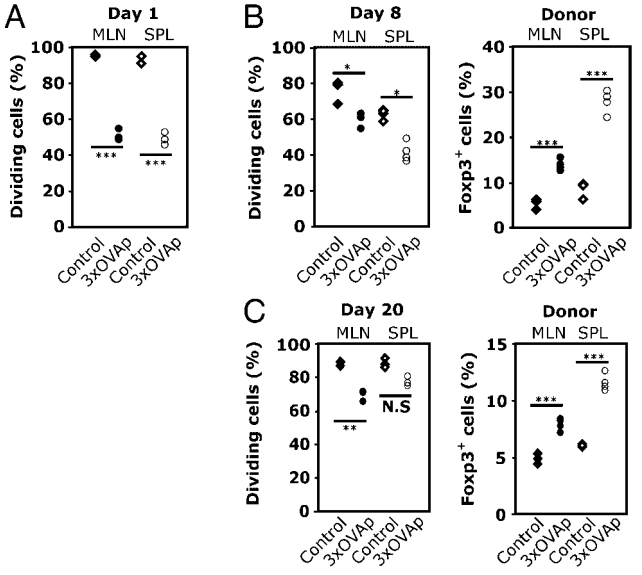

Figure 5. *In vivo* anergy of CD4 T cells from 3 × OVAp mice. (A–C) MLN cells (4×10^6/mouse) from 3 × OVAp OTII-SJL TCR-TG mice were isolated on various days after the last OVAp immunization as indicated. These cells and MLN cells from control OTII-SJL TCR-TG mice were CFSE-labeled and transferred i.v. to C57BL/6 recipients ($n = 3$–4). Recipient mice were immunized with OVAp (20 μg/mouse i.p.) on the day after cell transfer. CFSE-dilution of transferred cells was analyzed by FACS in MLN and SPL cells from recipient mice 3 days thereafter (A, left panels in B and C). The right panels in (B) and (C) show the percentage of $Foxp3^+CD4^+$ cells in the MLN cells transferred on day 8 and day 20, respectively. Data are representative of 2–3 experiments. N.S = not significant, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Student's *t*-test.

in vivo anergy of repeatedly stimulated CD4 T cells is independent of $Foxp3^+$ T cells.

Discussion

Several mechanisms have been reported to contribute to the CD4 T-cell anergy induced by repeated immunization of mice with bacterial SAg. Thus, the anergic population has been shown to contain an elevated fraction of $CD4^+CD25^+$ T cells that, based on their functional characteristics, most likely represented $Foxp3^+$ natural Treg [12, 13, 15]. Further, the population has also been shown to contain cells with IL-10-Treg activity [10, 14, 16] and *bona fide* anergic cells [10, 11] with an inherent inability to respond to the antigen. Classically, T-cell anergy is defined as the failure of the $CD4^+$ population to proliferate or produce IL-2 in response to the immunizing SAg.

We show that repeated immunization with either SEB or conventional protein antigens in two TCR-TG models increased the frequency of Foxp3-expressing cells in the $CD4^+CD25^-$ population, largely by reducing the number of $Foxp3^-$ cells in that population. This phenotypic change was detected both in peripheral blood and in all peripheral lymphoid organs studied and persisted for several weeks in MLN and SPL. Further, when using i.p. immunization, the deletion was in general more pronounced in MLN than in SPL. This may explain why the increased frequency of $Foxp3^+$ CD4 T cells is variable in different studies using similar protocols of repeated immunization [24, 32].

It is well-established that CD4 T cells from $rag^{-/-}$ TCR-TG mice fail to express Foxp3. A fraction of these cells can be converted to $Foxp3^+$ Treg during *in vivo* exposure to their cognate peptide ligand, but the efficiency of conversion varies extensively between different experimental models [22, 33–35]. In our model, we detected only a minor increase in frequency of $Foxp3^+$ cells in 3 × SEB CD4 T cells of TCR-TG $rag^{-/-}$ mice and no significant increase in *foxp3*-expression. Because our immunization protocol does not significantly increase the number of $Foxp3^+$ cells in $rag^{+/+}$ mice, it remains possible that a low number of $Foxp3^+$ cells induced by a first injection of SEB in the $rag^{-/-}$ mice might not be detectable in the repeatedly immunized $rag^{-/-}$ mice either. To address this possibility in a direct way, we transferred CD4 T cells depleted of natural Treg to normal BALB/c mice. The number of $Foxp3^+$ cells remained the same in 3 × SEB-treated and -untreated recipient mice (our unpublished data). We propose therefore, that reduction in the number of $CD4^+CD25^-$ T cells is the main cause for the increased proportion of $Foxp3^+$ CD4 T cells seen in secondary lymphoid organs of the repeatedly immunized mice. A recent study indicates that this deletion might be due to Bim-dependent apoptosis [36].

The differential Foxp3-expression in the anergic CD4 T cells induced in 3 × SEB TCR-TG $rag^{+/+}$ and $rag^{-/-}$ mice may explain why we previously found these populations to be functionally different [15]. Thus, the suppressor function of anergic $rag^{-/-}$ CD4 T cells was found to be partially cell-contact-independent and mediated by IL-10 and TGF-β-1. This effector function is reminiscent of IL-10-Treg. Interestingly, $rag^{-/-}$ TCR-TG mice repeatedly immunized intra-nasally developed peptide-specific tolerance mediated by IL-10-Treg [37, 38]. Similar to our results, the peptide-induced IL-10-Tregs in that model lacked Foxp3-expression [38].

Papiernik *et al*. have studied the *in vivo* immune response to a virus-encoded SAg [12, 39]. Similar to our data, the authors showed that the chronic stimulation by the SAg in infected mice selectively eliminated reactive $CD4^+CD25^-$ T cells whereas $CD4^+CD25^+$ T cells were maintained. The same laboratory subsequently showed that $CD4^+CD25^+$ T cells were more resistant to CD95 ligand (CD95L)-induced cell death than $CD4^+CD25^-$ T cells [40]. Interestingly, $CD4^+CD25^-$ T cells were also selectively deleted in septic shock patients [41]. Further, an increased fraction of $CD4^+CD25^+$ natural Treg is often observed during chronic and persistent viral infections [17, 18] and the selective cell death of non-Treg CD4 T cells might contribute to that increase. Stimulation of CD4 T cells with peptide antigens also induces deletion [42–45].

We show that the repeated antigen stimulation in this system caused potent *in vivo* T-cell anergy. Thus, CD4 T cells from repeatedly immunized mice proliferated poorly in response to antigen challenge in an adoptive host. The anergy was most potent in cells transferred on the day after the last immunization. When cell transfer was delayed, anergy was gradually lost. Previous results from other laboratories have suggested that

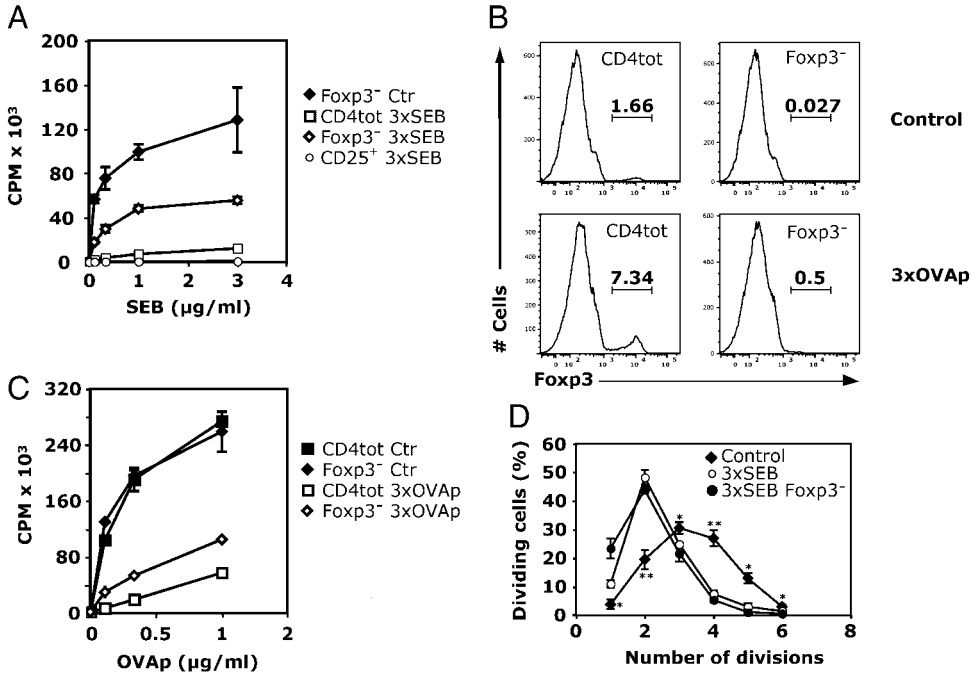

Figure 6. *In vitro* T-cell anergy is partially mediated by Foxp3$^+$ cells. (A) SPL cells from control and 3 × SEB mice were depleted of Foxp3$^+$ Treg by magnetic cell sorting (see the *Materials and methods*). These cell populations, denoted Foxp3$^-$ Ctr (filled diamonds) and Foxp3$^-$ 3 × SEB (open diamonds) were cultured (2.5×10^4/well) in the presence of splenic DC (5×10^3/well) and indicated concentrations of SEB. Cells bound to the column (CD25$^+$ 3 × SEB) and total CD4 T cells from 3 × SEB mice (CD4tot 3 × SEB) served as controls. Proliferation was assayed on day 3 of culture. Data represents mean ± SD of triplicate cultures. (B) CD45RB^{hi} CD25$^-$CD103$^-$GITR$^-$ cells were isolated by FACS sorting (see the *Materials and methods*) from pooled SPL and MLN cells of control (Foxp3$^-$ Ctr) and 3 × OVAp mice (Foxp3$^-$ 3 × OVAp). Total CD4 cells were sorted and used as controls (denoted CD4tot Ctr and CD4tot 3 × OVAp, respectively). Sorted cells were analyzed for Foxp3-expression by flow cytometry. (C) Proliferation of the sorted populations in the presence of indicated concentrations of OVAp was determined as described in (A). (D) *In vivo* anergy is independent of Foxp3$^+$CD4 T cells. MLN CD4 T cells from 3 × SEB mice were depleted of Foxp3$^+$ cells by FACS sorting (as in B) and labeled with CFSE. These cells and CFSE-labeled total CD4 T cells from immunized and untreated control mice (2×10^6 cells/mouse) were injected into normal BALB/c recipients ($n = 3$ *per* group). The recipient mice were immunized with OVA (200 μg/mouse) on the next day and CFSE dilution analyzed flow cytometrically 3 days later. Data represents percentage of dividing CFSE-labeled cells in various division cycles (1–6 divisions). Error bars represent SD. Data are representative of 2–3 experiments. *p<0.05, **p<0.01, ***p<0.001, Student's t-test.

maintenance of *in vivo* anergy is antigen-dependent [44, 46, 47], suggesting that the loss of anergy in OVAp-immunized mice observed here might be due to loss of the antigen [44]. The changes in the CD4 T-cell repertoire induced by repeated immunization, *i.e.* reduced number of CD4 T cells and increased frequency of Foxp3$^+$ cells remained, despite the loss of *in vivo* anergy. This suggested that the *in vivo* anergy did not directly correlate with these changes in the CD4 T-cell repertoire. Depletion of Foxp3$^+$ cells from the CD4 T-cell population of 3 × SEB mice confirmed this hypothesis and indicated that our immunization protocol induced T-cell intrinsic anergy similar to what had previously been observed in other models [35, 48]. In contrast, the *in vitro* anergy may partially be mediated by suppression as elimination of Foxp3$^+$ cells reduced the anergy of the CD4 population.

Abbas and coworkers and Schwartz and coworkers have, in a series of papers, studied the *in vivo* T-cell adaptation to cognate antigen upon transfer of naïve TCR-TG T cells to antigen-expressing TG recipients. The adapted cells in both models raised poor proliferation responses to the antigen *in vitro*. However, the *in vivo* responsiveness to antigen varied. In one model, the adapted T cells proliferated in response to the systemic antigen when re-transferred to an antigen-expressing recipient [47], whereas in the other, T-cell anergy was maintained [49]. Further, the adaptation in the Schwartz model did not involve suppression [35, 47]. The studies of Schwartz and coworkers used *rag*$^{-/-}$ TCR-TG cells and these failed to express Foxp3 after the adaptation to self antigen [35], whereas in the Abbas model [34], an increased fraction of activated Foxp3$^+$ cells was present amongst the antigen-experienced CD4 T cells. Thus, tolerance is based on both T-cell anergy and suppression, but the relative contribution of these may vary depending on the experimental model used.

Braun and coworkers have previously shown that proliferation of naïve CD4 T cells is partially suppressed in normal BALB/c and C57BL/6 mice repeatedly immunized with SEB [14, 16]. We have confirmed that observation in here and extend it by showing that CD4 cell proliferation was also inhibited in mice repeatedly immunized with OVAp. A recent report showed that mice immunized using a similar protocol carry an increased frequency of IL-10-expressing Foxp3$^+$ cells both in MLN and SPL [24]. Further, Noel *et al.* showed that anti-IL-10 antibodies could block suppression of an *in vivo* immune response (SAg-induced

lethality) mediated by SPL cells from 3 × SEB mice [14]. These results imply a role of IL-10 in suppression mediated by T cells from 3 × SEB mice. In our model, there was only transient inhibition of the proliferation of transferred CD4 T cells, despite an elevated frequency of Foxp3$^+$ cells in the recipients. These results suggest that the Foxp3$^+$ cells may not play a major role in this particular inhibition mechanism. However, that does not exclude that the Foxp3 cells may efficiently regulate an antigen-induced immune response.

It has been shown that OVAp, albeit after i.v. injection of higher amount than in here, is maintained for 5–10 days in a T-cell stimulatory way [44]. This correlates well in time with the recovery of the capacity of the 3 × OVAp recipient mice to support proliferation of transferred naïve CD4 T cells (Fig. 4). Further, antigen-primed T cells can compete with the stimulation and reduce the expansion of naïve antigen-specific T cells [50]. We speculate that competition may be one mechanism operating in the 3 × OVAp mice. Our preliminary data show that the proliferation of OT-I CD8$^+$ T cells is not inhibited in 3 × OVAp recipients, which is consistent with a competition mechanism of inhibition, as activated host OVA-specific CD4 T cells would not be expected to compete with the activation of class I MHC-restricted OT-I T cells.

Materials and methods

Animals and treatment

DO11.10 TCR-TG mice crossed to the BALB/c background, OT-II TCR-TG, OT-II-SJL (OT-II TCR-TG mice crossed to B6-SJL (Ly5.1$^+$)), were bred under special pathogen free conditions at the BMC animal facilities at Lund University. C57BL/6, BALB/c and DO11.10 *rag-2*$^{-/-}$ mice crossed to the BALB/c background, were bought from Taconic (Ry, Denmark). Recombinant SEB was expressed in *Escherichia coli* and purified to homogeneity as described previously [51]. In initial experiments, we used the immunization protocol of our previous study [15]. In brief, mice were injected i.v. with 5 μg of recombinant SEB in PBS, three times at 4 days interval. The animals were sacrificed 2 days after the last injection. For convenience, we changed the protocol to involve peritoneal (i.p.) injections on days 0, 2 and 4 of 20 μg SEB (Sigma Aldrich, Sweden AB) and sacrificed 1 day thereafter. The two protocols yielded similar results. The latter protocol was also used for the immunization of OT-II or OT-II–SJL mice with the $OVA_{323–336}$ peptide (Schafer-N, Copenhagen Denmark; 20 μg/injection) or OVA (200 μg/injection).

Antibodies and flow cytometry

Antibodies for flow cytometry (CD25-FITC, CD25-biotin, CD103-biotin, CD4-APC) were bought from BD Pharmingen (San Diego, CA). Foxp3-PE, Foxp3-APC, CD45RB-PE, GITR-biotin, CD62L-PE/Cy7 and CD4-PE/Cy5 were bought from eBioscience (Nordic Biosite, Täby, Sweden). Streptavidine-Alexa 488 was bought from Molecular Probes (Eugene, OR). Dead cells were excluded using 7-amino-actinomycin D or propidium iodide, bought from Sigma Aldrich. Biotin-conjugated goat anti-GITR antibodies were bought from R&D Systems (Abingdon, UK). This reagent was used in combination with biotin-conjugated donkey-anti-goat antibodies (Jackson ImmunoResearch). Anti-CD4-Alexa 647 and anti-FcRII/III (2.4G2) were prepared in our laboratory. Intracellular staining was performed using the Foxp3 staining set (eBioscience). Stained cells were analyzed on FACSCalibur or FACSAria flow cytometers (BD Pharmingen) and analyzed using the CellQuest or FlowJo softwares.

Cell separation

Single-cell suspensions were prepared from pooled SPL and LN. The cell suspension was depleted of B cells by panning on anti-mouse Ig coated plates (DAKO Cytomation, Glostrup, Denmark). In initial experiments, we depleted CD4 T cells of CD25, CD103 and GITR-expressing cells using magnetic cell sorting essentially as described before [52]. To deplete Foxp3$^+$ cells by FACS, cells were first depleted of B cells by panning. The cells were thereafter incubated with biotin-conjugated anti-CD25, anti-CD103 and anti-GITR antibodies, followed by anti-CD45RB-PE and anti-CD4-Pacific blue and streptavidine Alexa-488. Dead cells were excluded using propidium iodide and doublets excluded by FSC-A/FSC-H gating. CD45RB^{hi} CD25$^-$CD103$^-$GITR$^-$ and total CD4 T cells were sorted. Splenic DC were isolated as previously described in detail [53].

Cell transfer

CD4 T cells were purified as described above. The cells were labeled with 2 μM CFSE (Molecular Probes) at room temperature for 5 min, and thereafter washed three times in complete RPMI. The cells were injected i.v. ($2–5 \times 10^6$ cells in 200μL PBS) in recipient mice. On the following day, the recipients were challenged with either SEB (20μg i.p.) or $OVA_{323–339}$ peptide (20μg i.p.) or OVA protein (200μg i.p). Three days after antigen challenge, recipients were sacrificed and SPL and MLN cells isolated for FACS analysis.

Cell culture conditions

SPL or MLN cells were cultured in U-bottom microplates (Becton Dickinson Labware, Franklin Lakes, NJ) in 200 μL RPMI 1640 medium (Gibco-Invitrogen, Paisley, Scotland) supplemented with 50 μM 2-mercaptoethanol, antibiotics and 10 mM HEPES buffer (all supplements from Gibco) and 10% FCS (Sigma Aldrich). Cultures of sorted T cells were supplemented

with 10^4 splenic DC. To detect proliferation, 1 μCi of ^{3}H-Thymidine (Amersham Biosciences, UK) was added to the cultures 4 h before harvesting the cells. Cells were activated by addition of various concentrations of SEB, $OVA_{323–339}$ peptide or OVA protein to the cultures. In all proliferation experiments, anti-CD3 (1 μg/mL) was added to parallel cultures as a positive control for T-cell responsiveness (data not shown).

Quantitative RT-PCR analysis

RNA was isolated from sorted cell populations using TRIZOL (Life Technologies, Täby, Sweden) and subsequently treated with Turbo DNA-free kit (Ambion, Austin, TX). Transcriptor cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) was used to prepare cDNA. Quantitative RT-PCR was performed using the Platinium SYBR Green qPCR supermix-UDG kit (Invitrogen, Carlsbad, CA) using an iCycler instrument (BioRad, Hercules, CA). The PCR products were analyzed on 2% agarose gels. The following primers were used: β-actin-S 5′-CCACAGCTGAGAGGGAAATC-3′, β-actin-AS 5′- CTTCTCCAG GGAGGAAGAGG -3′, Foxp3-S 5′- CCCAGGAAAGACAGCAACCTT-3′ and Foxp3-AS 5′-TTCTCACAACCAGGCCACTTG-3′ (Invitrogen). The amplifications were performed using the following program: 2 min at 50°C, 10 min at 96°C followed by 45 cycles 10 s at 96°C, 30 s at 60°C. Ct values were determined and relative Foxp3-expression determined with the formula $2^{-(\text{Foxp3 (Ct)}-\beta\text{-actin (Ct)})}$.

Statistical analyses

Statistical analyses were performed using Student's *t*-test.

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Abbreviations: **GITR:** glucocorticoid-induced TNF receptor · **OVAp:** ovalbumin peptide · **SAg:** superantigen · **SEB:** Staphylococcal enterotoxin B · **SPL:** spleen

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