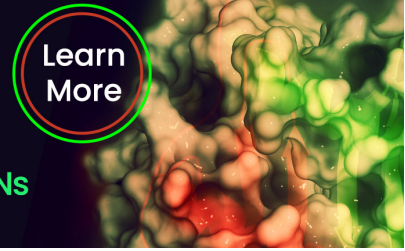


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## Superantigen-Induced Regulatory T Cells Display Different Suppressive Functions in the Presence or Absence of Natural CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells In Vivo<sup>1</sup> **FREE**

Susanna Grundström; ... et. al

*J Immunol* (2003) 170 (10): 5008–5017.

<https://doi.org/10.4049/jimmunol.170.10.5008>

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# Superantigen-Induced Regulatory T Cells Display Different Suppressive Functions in the Presence or Absence of Natural CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells In Vivo<sup>1</sup>

Susanna Grundström,<sup>2\*</sup> Lukas Cederbom,<sup>2†</sup> Anette Sundstedt,\* Peter Scheipers,\* and Fredrik Ivars<sup>3†</sup>

Repeated exposures to both microbial and innocuous Ags in vivo have been reported to both eliminate and tolerize T cells after their initial activation and expansion. The remaining tolerant T cells have been shown to suppress the response of naive T cells in vitro. This feature is reminiscent of natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. However, it is not known whether the regulatory function of in vivo-tolerized T cells is similar to the function of natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. In this study, we demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> as well as CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from mice treated with superantigen three consecutive times to induce tolerance were functionally comparable to natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, albeit more potent. The different subpopulations of in vivo-tolerized CD4<sup>+</sup> T cells efficiently down-modulated costimulatory molecules on dendritic cells, and their suppressive functions were strictly cell contact dependent. Importantly, we demonstrate that conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells could also be induced to acquire regulatory functions by the same regimen in the absence of natural regulatory T cells in vivo, but that such regulatory cells were functionally different. *The Journal of Immunology*, 2003, 170: 5008–5017.

The immune system must raise effective immune responses against foreign invaders but at the same time avoid destructive reactivity to self-components. A number of different mechanisms such as anergy, deletion, ignorance, and suppression mediated by regulatory T cells (reviewed in Refs. 1 and 2) are involved in maintaining self-tolerance.

Currently, an intensely studied subset of regulatory T cells both in human, rat, and the mouse is the natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell population (reviewed in Refs. 2–4). Natural regulatory T cells are thought to develop in the thymus (5–7) as the result of high avidity interactions with self-Ags on thymic cortical epithelial cells (8, 9). These cells suppress T cell and B cell activation in vitro by a cell contact-dependent mechanism (10, 11), and their in vivo function is probably mediated by CD152 (12, 13), TGF- $\beta$  (13, 14), and IL-10 (15). It is noticeable that these cells proliferate poorly upon Ag stimulation in vitro and are therefore considered to be anergic (10, 11). Another characteristic is that they seem to be

relatively resistant to activation-induced cell death (AICD)<sup>4</sup> (16), the basis of which is now partially understood from their unique pattern of gene expression (17).

T cell tolerance can be experimentally induced in vivo in a number of different ways. For example, repeated exposures to low doses of protein Ag either by injection or by oral or nasal administration lead to initial activation of responding T cells followed by deletion and anergy in the remaining T cells (18–21). Other investigators have used model systems in which TCR-transgenic (TG) T cells are constitutively exposed to their ligands as neo-self-Ags in vivo, which also result in tolerance in the reactive T cells (22–24). It has been demonstrated that such in vivo-tolerized CD4<sup>+</sup> T cells are not necessarily devoid of effector functions, but rather mediate active suppression of Ag-specific immune responses (22–24). Interestingly, T cells anergized in vitro have been found to adopt regulatory T cell functions as well (25–27). One common denominator of these different models is that the responding T cells encounter the Ag in the absence of adjuvants activating the APCs. Indeed, several of the in vitro anergy induction protocols made use of agents that interfere with APC functions (25).

Importantly, infections by various pathogens also induce the development of immunosuppression. Thus, it has been shown that chronic infections with viruses, such as mouse Friend virus (28), certain parasites (29), and bacteria (30) give rise to anergic T cells with regulatory functions. The regulatory T cells appearing in such infected individuals are functionally similar to those induced in the experimental models described above. This suggests that the anergic regulatory T cells induced in these various situations may represent the same T cell population. In addition, exposure of animals to bacterial or viral superantigens (SAG) in vivo mimics these infections and induces CD4<sup>+</sup> T cell deletion and anergy within the remaining T cell population (31, 32). Repeated injections of SAG within a short period of time have proven particularly efficient in inducing these phenomena (33, 34).

As outlined above, CD4<sup>+</sup> T cells tolerized by SAG in vivo might be both functionally and developmentally related to the natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. This raises the question of

\*Active Biotech Research AB, and <sup>†</sup>Section for Immunology, Department of Cell and Molecular Biology, Lund University, Lund, Sweden

Received for publication November 19, 2002. Accepted for publication March 10, 2003.

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<sup>1</sup> This work was supported in part by grants from the Swedish Research Council (Grant K2002-06X-14184-01A), Greta and Johan Kocks Stiftelser, Anna Greta Crafoords Stiftelse för Reumatologisk Forskning, Kungliga Fysiografiska Sällskapet i Lund, Petrus och Augusta Hedlunds Stiftelse, Alfred Österlunds Stiftelse (all to F.I.), and the Medical Faculty of Lund University (to L.C. and F.I.).

<sup>2</sup> S.G. and L.C. contributed equally to this work.

<sup>3</sup> Address correspondence and reprint requests to Dr. Fredrik Ivars, Section for Immunology, BMC I:13, Department of Cell and Molecular Biology, Lund University, SE-221 84 Lund, Sweden. E-mail address: Fredrik.Ivars@immuno.lu.se

<sup>4</sup> Abbreviations used in this paper: AICD, activation-induced cell death; TG, transgenic; SAG, superantigen; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; DC, dendritic cell; 3×SEA, immunized with SEA three consecutive times at 4-day intervals; 3×SEB, immunized with SEB three consecutive times at 4-day intervals; MHC-II, MHC class II; GeoMFI, geometric mean fluorescence intensity.

whether the regulatory properties of the remaining tolerant T cells are a common feature of the whole CD4<sup>+</sup> T cell population or specific for a subpopulation. It has been shown recently that the anergic state and suppressive potential of SA<sub>g</sub>-tolerized CD4<sup>+</sup> T cells are largely dependent on the regulatory activity of CD4<sup>+</sup>CD25<sup>+</sup> T cells (35). However, the CD4<sup>+</sup>CD25<sup>-</sup> T cells were partially anergic even in the absence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, suggesting that the state of anergy might be separated from regulatory effects. This also implies that tolerance due to suppression is different from intrinsic anergy. Interestingly, in another model of SA<sub>g</sub>-induced tolerance, it was concluded that cell-autonomous rather than environmental factors control T cell anergy in vivo (36). Additionally, it was demonstrated that staphylococcal enterotoxin A (SEA)-induced tolerance in *rag-2*<sup>-/-</sup> TCR-TG mice involved a combination of both clonal anergy and cytokine-mediated immunosuppression (34).

In this report, we have investigated the relationship between natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and the tolerant CD4<sup>+</sup> T cells remaining in TCR-TG mice after repeated exposures to SA<sub>g</sub> in vivo. We conclude from this study that both SA<sub>g</sub>-tolerized CD4<sup>+</sup> T cells and natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells suppress the proliferation and IL-2 production of CD4<sup>+</sup>CD25<sup>-</sup> responder T cells through a similar mechanism. Importantly, both the CD25<sup>+</sup> and CD25<sup>-</sup> fractions of the SA<sub>g</sub>-tolerized CD4<sup>+</sup> T cell population contained highly efficient regulatory T cells. Furthermore, conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells could be tolerized and acquire suppressor cell function in vivo even in the absence of natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. However, these tolerant T cells were not functionally identical with the natural regulatory T cells as they suppressed T cell responses mainly by a cell-contact-independent mechanism.

## Materials and Methods

### Animals and treatment

TCR-TG mice expressing a rearranged *TCRβ* chain gene of the 2B4 CD4<sup>+</sup> T cell hybridoma (Vβ3-TG) were kindly provided by Dr. M. Davis (Stanford University, Stanford, CA). DO11.10 TCR-TG mice (Vβ8-TG) were obtained from Dr. Y. Xue (Karolinska Institute, Stockholm, Sweden). DO11.10 TG *rag-2*<sup>-/-</sup> mice crossed to the B10.D2 background (Vβ8-TG *rag-2*<sup>-/-</sup>) were kindly provided by Drs. E. Dahlén and W. Agace (Lund University). CB.17 SCID mice were kept at our animal facility under special pathogen-free conditions. All animals were used at the age of 6–14 wk. Recombinant SEA and staphylococcal enterotoxin B (SEB) were expressed in *Escherichia coli* and purified to homogeneity as described previously (37). Vβ3-TG or Vβ8-TG mice were injected i.v. three times with 10 μg of SEA or 1 μg of SEB, respectively. The SA<sub>g</sub>s were administered in 0.2 ml of PBS supplemented with 1% normal syngeneic mouse serum at 4-day intervals. Two days after the last SA<sub>g</sub> injection, untreated or SA<sub>g</sub>-treated mice were sacrificed. Spleen and peripheral/mesenteric lymph nodes were prepared. CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>high</sup> or total CD4<sup>+</sup> T cells were purified from single-cell suspensions by positive selection (see *Cell separation*). Vβ8<sup>+</sup> T cells (7.5 × 10<sup>6</sup>) of each CD4<sup>+</sup> T cell subset were separately injected i.v. in 0.2 ml of PBS into SCID female mice. One day after cell transfer, the mice were injected with SEB, as described above. A control group of transferred SCID mice were treated only once with SEB on the day after transfer.

### Antibodies

The mAbs (NA/LE) used in cell culture experiments (anti-CD16/CD32, anti-IL-10R, anti-CTLA-4, rat IgG, mouse IgG, and hamster IgG (isotype controls)) were purchased from BD Pharmingen (San Diego, CA). Monoclonal anti-TGF-β (clone 1D11) and recombinant mouse IL-10 and TGF-β1 were from R&D Systems (Abingdon, U.K.). The Abs used for flow cytometry (anti-TCR-Vβ3 PE, anti-TCR-Vβ8.1/8.2 FITC, anti-CD4 FITC, anti-CD45RB PE, anti-CD25 FITC, anti-CD25 biotin, anti-CD80 biotin, anti-CD86 PE, anti-CD11c FITC, and anti-CTLA-4 PE mAbs) were purchased from BD Pharmingen. Anti-CD4 tricolor and anti-KJ1-26 FITC came from Caltag (Burlingame, CA).

### Cell separation

Single-cell suspensions were prepared from pooled spleens and lymph nodes. To isolate CD25<sup>+</sup> and CD25<sup>-</sup> subpopulations of CD4<sup>+</sup> T cells, the cells were first stained with anti-CD4 FITC and thereafter positively selected using the anti-FITC Multisort kit (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions yielding >95% pure CD4<sup>+</sup> T cells. The isolated CD4<sup>+</sup> T cells were released from the beads, and incubated with anti-CD25 biotin and streptavidin-microbeads (Miltenyi Biotec). Next, the CD4<sup>+</sup>CD25<sup>+</sup> T cells were positively selected, yielding >95% pure CD4<sup>+</sup>CD25<sup>+</sup> T cells, while the CD4<sup>+</sup>CD25<sup>-</sup> were recovered in the negative fraction, yielding >99% pure cells. To further select for naive CD4<sup>+</sup>CD25<sup>-</sup> T cells, the CD4<sup>+</sup>CD25<sup>-</sup> fraction was incubated with anti-CD62L microbeads (Miltenyi Biotec) and positively selected, generating >99% pure CD62L<sup>high</sup>CD45RB<sup>high</sup> T cells. Total CD4<sup>+</sup> T cells were positively selected with CD4 microbeads (Miltenyi Biotec), yielding routinely >99% pure CD4<sup>+</sup> T cells. To isolate dendritic cells (DCs), spleens were first treated for 30 min at 37°C with collagenase (1.6 mg/ml; Worthington Biochemical, Lakewood, NJ) and 1% DNase (Sigma-Aldrich, Stockholm, Sweden) in serum-free tissue culture medium. The cell suspensions were then passed once on a positive-selection column and thereafter incubated with CD11c microbeads (Miltenyi Biotec). The cells were then positively selected and were routinely >95% CD11c<sup>+</sup>.

### Cell culture conditions

Various CD4<sup>+</sup> populations prepared from either untreated or SA<sub>g</sub>-treated TCR-TG mice were cocultured at indicated ratios with CD4<sup>+</sup>CD25<sup>-</sup> T cell responders (5 × 10<sup>4</sup> cells/well) and splenic DCs (5 × 10<sup>3</sup> cells/well) from untreated TG mice. The cells were cultured in 96-well round bottom plates in 0.2 ml of R10 medium (RPMI 1640 supplemented with glutamine, HEPES, gentamicin, 2-ME, sodium pyruvate, and 10% FCS) and stimulated either with SEA (1 μg/ml), or SEB (1 μg/ml), or PMA (50 ng/ml) and ionomycin (250 ng/ml) for 72 h at 37°C as indicated. Proliferation was assessed by [<sup>3</sup>H]thymidine (1 μCi/well) uptake during the last 4 h of culture. To measure IL-2 production, supernatants were harvested after 48 or 72 h of culture, and the IL-2 content was quantified by ELISA. Transwell cultures were performed in 24-well plates in a volume of 0.5–1 ml of R10 medium or in 96-well plates in 0.2 ml of medium (the SEB model). The different regulatory CD4<sup>+</sup> T cell subsets were cocultured at a 3:1 ratio with CD4<sup>+</sup>CD25<sup>-</sup> T cell responders (2 × 10<sup>5</sup>/5 × 10<sup>4</sup> cells/well), together with DCs (2 × 10<sup>4</sup>/5 × 10<sup>3</sup> cells/well) and SEA/SEB (1 μg/ml), in the presence or absence of a semipermeable Transwell membrane (BD Biosciences). Latex beads (Interfacial Dynamics, Portland, OR) were conjugated with anti-CD3 Abs as described by the manufacturer. The beads were counted in a microscope before use in the in vitro cell cultures.

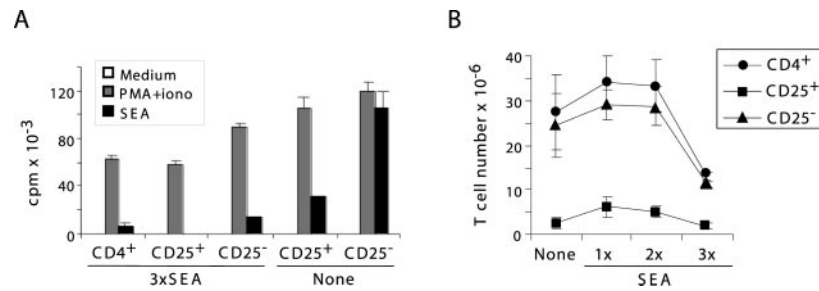
### Cytokine ELISA

The content of IL-2 in culture supernatants was determined using specific ELISAs from BD Pharmingen according to the instructions of the manufacturer. We routinely detected IL-2 concentrations of 50–80 ng/ml and 15–30 ng/ml in cultures containing SA<sub>g</sub>-activated T cells from mice immunized with SEA three consecutive times at 4-day intervals (3×SEA) and mice immunized with SEB three consecutive times at 4-day intervals (3×SEB), respectively.

## Results

### Repeated SA<sub>g</sub> exposures induce CD4 T cell deletion and anergy

Three consecutive injections of SEA at 4-day intervals induce a state of anergy in the responding CD4<sup>+</sup> T cells of Vβ3-TG mice (33, 34). The anergic state is characterized by poor proliferation and IL-2 production upon in vitro SEA challenge (33, 38). We first confirmed the anergic phenotype of CD4<sup>+</sup> T cells from 3×SEA Vβ3-TG mice and CD4<sup>+</sup>CD25<sup>+</sup> T cells from untreated Vβ3-TG mice in proliferation responses to SEA in vitro (Fig. 1A). In contrast, however, SEA induced a robust proliferation response in CD4<sup>+</sup>CD25<sup>-</sup> T cells from untreated Vβ3-TG mice. As expected, the potent mitogenic combination of PMA and ionomycin induced proliferation in all of the CD4 populations.



**FIGURE 1.** Repeated SEA exposure induces anergy and deletion of  $V\beta 3^+CD4^+$  T cells. **A**, Indicated  $CD4^+$  T cell populations ( $5 \times 10^4$  cells/well) isolated from 3 $\times$ SEA or untreated  $V\beta 3$ -TG mice were cocultured with splenic DCs ( $5 \times 10^3$  cells/well) for 72 h either in medium alone, with PMA (50 ng/ml) plus ionomycin (250 ng/ml), or with SEA (1  $\mu$ g/ml). Proliferation was studied by pulsing the cells with [<sup>3</sup>H]thymidine for the last 4 h of culture. **B**, Single-cell suspensions of spleen and lymph nodes from untreated, 1 $\times$ SEA, 2 $\times$ SEA, or 3 $\times$ SEA  $V\beta 3$ -TG mice were stained with CD4 and CD25 Abs and analyzed by FACS. The SEA-treated mice were analyzed 2 days after the last injection. The mean calculated absolute number  $\pm$  SD (four mice per group) of indicated  $CD4^+$  T cell populations is depicted. One representative experiment of three performed is shown.

Repeated *in vivo* SAg exposures induce massive deletion of SAg-reactive  $CD4^+$  T cells. In concordance with previous data (31, 39, 40), the number of total  $CD4^+$  T cells in  $V\beta 3$ -TG mice increased after SEA injection, while repeated exposures to SEA significantly decreased the number of  $CD4^+$  T cells (Fig. 1B).  $CD4^+CD25^-$  T cells followed the same pattern of expansion and deletion (Fig. 1B). The number of  $CD4^+CD25^+$  T cells also increased after a single SEA injection but did not decrease beyond the initial number of cells after three injections. Previously, it was demonstrated that natural regulatory  $CD4^+CD25^+$  T cells are not deleted by repeated SAg exposures, suggesting that this population is resistant to AICD (16). Our data support the view that a substantial fraction of the  $CD4^+CD25^-$  population is vulnerable to deletion during a chronic immune response, whereas the  $CD4^+CD25^+$  population is less sensitive.

#### *In vivo*-tolerized $CD4^+$ T cells are potent regulatory T cells

Previous observations from other laboratories have shown that tolerant  $CD4^+$  T cells, which were induced in animals repeatedly exposed to Ag, inhibit the immune response of naive  $CD4^+$  T cells to that same Ag (22–24, 35, 41, 42). Because the tolerant  $CD4^+$  T cells from 3 $\times$ SEA  $V\beta 3$ -TG mice contained a mixture of both  $CD4^+CD25^+$  T cells and  $CD4^+CD25^-$  T cells, we wanted to investigate the regulatory properties of these different T cell subpopulations.

Accordingly, we tested whether the SEA-tolerized  $CD4^+$  T cells would suppress SEA-induced proliferation and IL-2 production of responder  $CD4^+CD25^-$  T cells from untreated  $V\beta 3$ -TG mice. These are suppressive effector functions previously ascribed to the  $CD4^+CD25^+$  population (10, 11). The  $CD4^+$  T cells from 3 $\times$ SEA mice were on a per-cell basis  $\sim$ 3-fold more potent suppressors of SEA-induced responder T cell proliferation and IL-2 production than natural  $CD4^+CD25^+$  regulatory T cells from untreated  $V\beta 3$ -TG mice were (Fig. 2A). In addition, both the  $CD25^+$  ( $\sim$ 30-fold) and the  $CD25^-$  fractions ( $\sim$ 3-fold) of the SEA-tolerized  $CD4^+$  T cells were also more potent regulatory T cells, providing evidence that the regulatory function of total  $CD4^+$  T cells cannot exclusively be ascribed to the  $CD25^+$  fraction of this population.

#### *Suppression by tolerant $CD4^+$ T cells is cell contact dependent and involves down-modulation of costimulatory molecules on APCs*

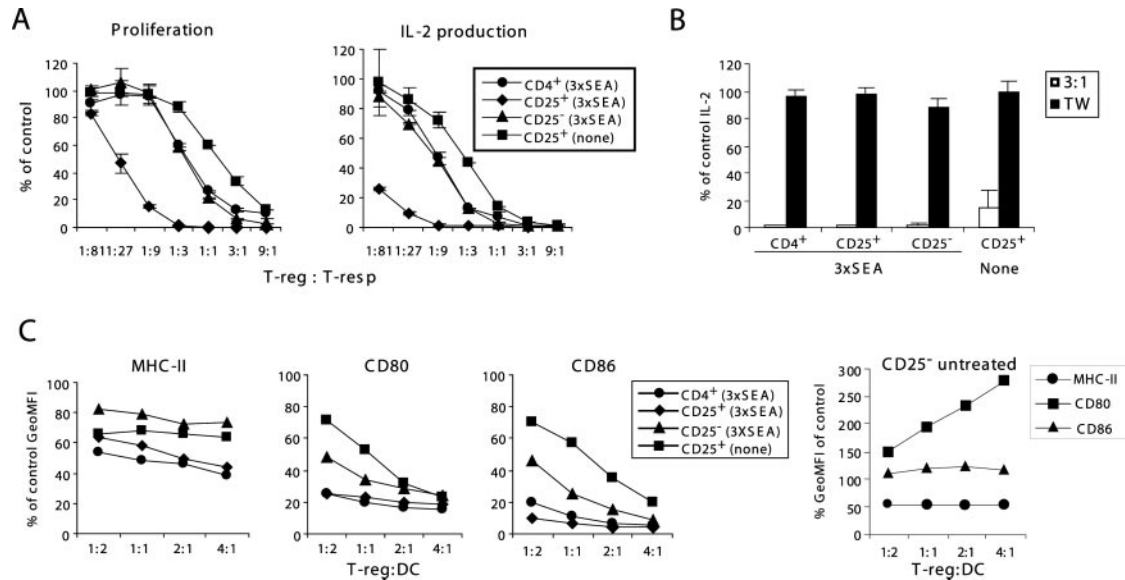
There are two principally different ways by which regulatory T cells can mediate suppression, either through cell contact-dependent mechanisms or through secretion of soluble factors such as IL-10 or TGF- $\beta$  (reviewed in Refs. 2–4). It has been demonstrated that natural  $CD4^+CD25^+$  regulatory T cells suppress responder T

cell proliferation in a cell contact-dependent way *in vitro* (10, 11). Transwell culture experiments were used to test whether the suppression by the SEA-tolerized  $CD4^+$  T cells would also be cell contact dependent. Upon cell contact between regulatory and responder T cells, the IL-2 production was efficiently suppressed by all of the subpopulations tested. However, when the various populations of effector  $CD4^+$  T cells were cultured with SEA-pulsed DCs separated from the responder  $CD4^+CD25^-$  T cells by the Transwell membrane, the IL-2 response was not inhibited (Fig. 2B). Thus, the suppression mediated by the tolerant  $CD4^+$  T cells from 3 $\times$ SEA mice was clearly cell contact dependent.

We have previously shown that natural regulatory  $CD4^+CD25^+$  T cells down-modulate the expression of CD80 and CD86 molecules on cocultured APCs (43).<sup>5</sup> Likewise, the SEA-tolerized  $CD4^+$  T cell population as well as the  $CD4^+CD25^-$  and  $CD4^+CD25^+$  subpopulations efficiently down-modulated the costimulatory molecules CD80 and CD86 on cocultured splenic DCs (Fig. 2C). Again, the  $CD4^+$  T cells from 3 $\times$ SEA mice were, on a per-cell basis, more potent regulatory cells than the natural  $CD4^+CD25^+$  regulatory T cells from untreated  $V\beta 3$ -TG mice were. In contrast,  $CD4^+CD25^-$  T cells from untreated mice rather enhanced the expression of CD80 while sustaining the expression of CD86 on DCs. In accordance with the suppressive efficiency on responder T cell proliferation and IL-2 production, the  $CD25^+$  fraction of the SEA-tolerized  $CD4^+$  T cells was considerably more potent than the  $CD25^-$  subpopulation (Fig. 2, A and C) was. The viability of the DCs was similar in cultures established with the various  $CD4^+$  T cells populations, and the observed down-modulation is therefore not due to selective elimination of DCs expressing high levels of CD80 and CD86. Furthermore, the different  $CD4^+$  T cell populations, including the  $CD25^-$  cells from untreated mice, down-modulated the level of MHC class II (MHC-II) molecules on the DCs (Fig. 2C). Interestingly, anergic alloreactive  $CD4^+$  T cell clones were also reported to down-modulate MHC-II on APCs (44). T cell activation by SEA and anergic alloreactive T cell clones have in common that they both engage the MHC-II molecules on the APCs, which might cause this down-modulation. Collectively, these results demonstrate that the different subpopulations of SEA-tolerized  $CD4^+$  T cells contained regulatory cells that were functionally similar, albeit more potent, compared with natural regulatory  $CD4^+CD25^+$  T cells.

<sup>5</sup> L. Cederbom, A. Makowska, C. Oderup, C. M. Cilio, and F. Ivars. The functional role of CD152-dependent down-modulation of costimulatory molecules in  $CD4^+CD25^+$  regulatory T cell-mediated suppression. *Submitted for publication.*





**FIGURE 2.** Tolerant CD4<sup>+</sup> T cells are potent regulatory cells. *A*, Indicated CD4<sup>+</sup> T cell subsets isolated from 3×SEA or untreated Vβ3-TG mice were cocultured for 72 h with CD4<sup>+</sup>CD25<sup>-</sup> responder T cells (5 × 10<sup>4</sup> cells/well) from untreated Vβ3-TG mice at the indicated ratios, together with splenic DCs (5 × 10<sup>3</sup> cells/well) and SEA (1 μg/ml). Proliferation was assayed as above. After 48 h, supernatants from these cultures were collected and analyzed for IL-2 content. The results are presented as the percentage of proliferation or IL-2 production of responder T cells stimulated in the absence of regulatory T cells. *B*, CD4<sup>+</sup> T cell subsets (3 × 10<sup>5</sup> cells/well) were cocultured with responder T cells (1 × 10<sup>5</sup> cells/well), splenic DCs (1 × 10<sup>4</sup> cells/well), and SEA (1 μg/ml). In parallel cultures, responder T cells were cocultured with DCs, separated from the regulatory T cells and DCs in the upper chamber of a Transwell (TW) membrane. After 48 h of culture, supernatants were collected from triplicate cultures and analyzed for IL-2 content. The results are presented as the percentage of the IL-2 production by responder T cells stimulated in the absence of regulatory T cells. *C*, The same T cell subsets as in *A* were cocultured for 18 h with splenic DCs (1 × 10<sup>5</sup> cells/well) at the indicated ratios, in the presence of SEA (1 μg/ml). The cells were thereafter stained with CD11c, MHC-II, CD80, and CD86 Abs and analyzed by FACS. The log geometric mean fluorescence intensities (GeoMFI) of the indicated markers on CD11c<sup>+</sup> cells were determined, and the results are presented as the percentage of the GeoMFI of DCs cultured in the absence of T cells. One representative experiment of four performed is shown.

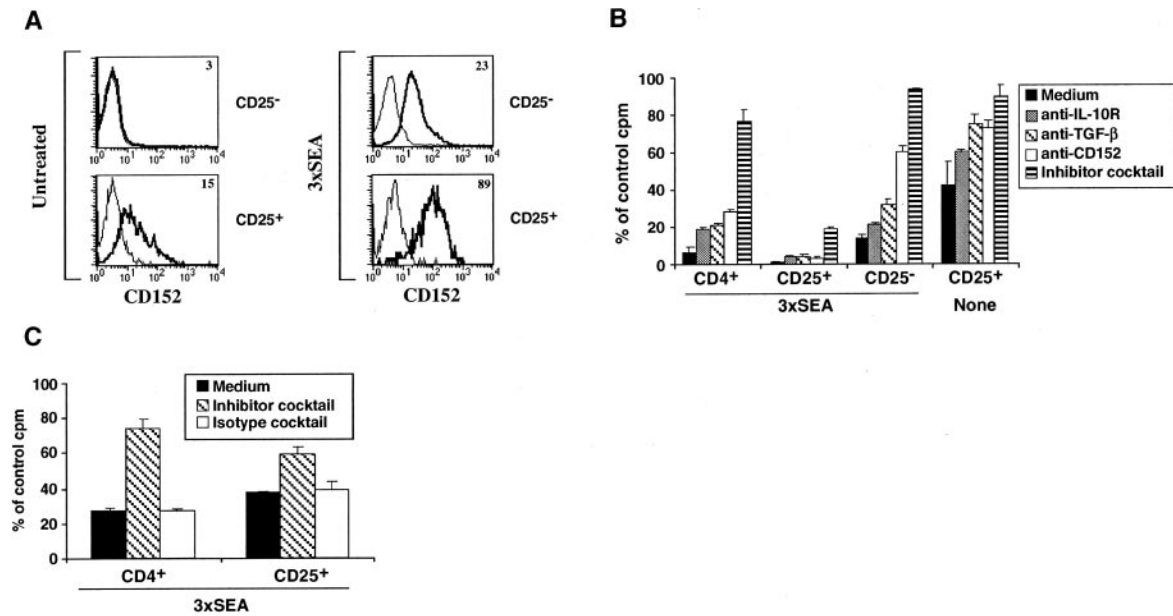
*In vivo-tolerized CD4<sup>+</sup> T cells and natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells express elevated levels of CD152 and suppress T cell proliferation in a similar way*

As expected (12, 13, 45, 46), natural regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells, but not naive CD4<sup>+</sup>CD25<sup>-</sup> T cells, from untreated Vβ3-TG mice constitutively expressed high levels of CD152 (Fig. 3A). Activation increases the expression level of CD152 on both CD4<sup>+</sup>CD25<sup>-</sup> T cells and natural regulatory T cells (12, 13), but the kinetics of expression are much faster in the latter population (L. Cederbom, unpublished observations). The CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells from 3×SEA mice had ~6-fold higher CD152 expression than the corresponding populations from untreated mice did (Fig. 3A), suggesting that the remaining CD4<sup>+</sup> cells in the 3×SEA mice had all been activated by the SEA treatment.

The Transwell experiments (Fig. 2B) suggested that SEA-tolerized CD4<sup>+</sup> T cells suppressed CD4<sup>+</sup>CD25<sup>-</sup> responder T cells in a cell contact-dependent way. However, both TGF-β and IL-10 have been implicated in the effector function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in vivo (13–15). Furthermore, these cells carry TGF-β and IL-10 mRNA (11, 47) and produce both TGF-β and IL-10 after in vitro activation (48). In addition, previous studies using the SEA-induced tolerance model had demonstrated that high amounts of IL-10 are produced both in vivo after tolerance induction (38) and by tolerant CD4<sup>+</sup> T cells in vitro (34). To further compare the effector functions of the tolerant CD4<sup>+</sup> T cells from 3×SEA mice and natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, experiments using high concentration of blocking Abs directed against IL-10R, TGF-β, or CD152 were performed. Similar to re-

sults from previous studies (12, 48), anti-CD152 or anti-TGF-β partially inhibited natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell-mediated suppression of responder T cell proliferation (Fig. 3B). In addition, these Abs also partially blocked the suppression of responder T cell proliferation by the CD4<sup>+</sup>CD25<sup>-</sup> and total CD4<sup>+</sup> T cells from 3×SEA mice. In contrast, the suppression by the SEA-tolerized CD4<sup>+</sup>CD25<sup>+</sup> T cells was only slightly inhibited. Well in line with previously published data (10, 11, 48), anti-IL-10R Abs only marginally blocked the suppression of responder T cell proliferation in vitro (Fig. 3B).

In parallel cultures, a mixture of anti-IL10R, anti-TGF-β, and anti-CD152 was added. The fact that suppression mediated by natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells or CD4<sup>+</sup>CD25<sup>-</sup> and total CD4<sup>+</sup> T cells from 3×SEA mice was nearly completely reversed in this case (Fig. 3B), implies that the blocked molecules may play complementary roles in suppression. Furthermore, the data demonstrate that these cells suppressed proliferation of CD4<sup>+</sup> T cells in a similar way. Due to the superior suppressor cell potency of the CD4<sup>+</sup>CD25<sup>+</sup> fraction of 3×SEA mice, we performed a second round of experiments using these cells at a lower effector to responder ratio (1:3) giving comparable suppression to the one obtained with total CD4<sup>+</sup> cells at a ratio of 3:1. As shown in Fig. 3C, the suppressor activity of both these populations was partially reversed by the Ab mixture. However, 3×SEA CD4<sup>+</sup>CD25<sup>+</sup> T cells were much less sensitive to the inhibition than ex vivo CD4<sup>+</sup>CD25<sup>+</sup> T cells from untreated mice. It might be that these cells are less sensitive to blocking due to their activated state, or alternatively, they might possess additional mechanisms of suppression that have been amplified by the activation.



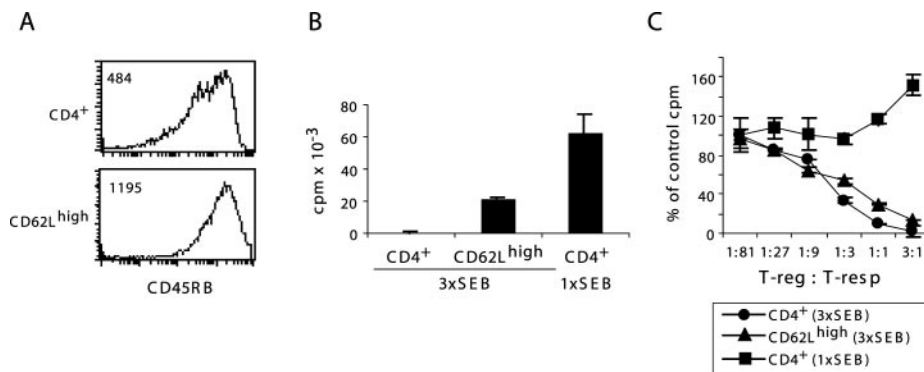
**FIGURE 3.** In vivo-tolerized  $CD4^+$  T cells are more potent but functionally similar to natural regulatory T cells. *A*, Single-cell suspensions of pooled spleen and lymph nodes from 3 $\times$ SEA or untreated  $V\beta 3$ -TG mice were stained with anti-CD4 and anti-CD25 on the membrane and in the cytoplasm with anti-CD152 (bold line) or isotype control (thin line). Indicated  $CD4^+$  T cell subsets were gated, and the CD152 expression and GeomFI value are shown in the histograms. *B*, Indicated  $CD4^+$  T cell subsets from 3 $\times$ SEA or untreated  $V\beta 3$ -TG mice ( $1.5 \times 10^5$  cells/well) were cocultured for 72 h with responder T cells ( $5 \times 10^4$  cells/well) as above, splenic DCs ( $5 \times 10^3$  cells/well), and SEA ( $1 \mu\text{g/ml}$ ). Anti-IL-10R ( $100 \mu\text{g/ml}$ ), anti-TGF- $\beta$  ( $100 \mu\text{g/ml}$ ), or anti-CTLA-4 ( $100 \mu\text{g/ml}$ ) blocking mAbs, or a mixture of these was included in indicated cultures. *C*, Cultures were set up as in *B* at suppressor to responder T cell ratios of 3:1 (left bars) and 1:3 (right bars) in the presence of the inhibitor mixture. A mixture of the same concentrations of isotype control Abs was added to parallel cultures. Proliferation was assayed as above and is presented as the percentage of proliferation of responder T cells in the absence of regulatory T cells. One representative experiment of three performed is shown.

#### $CD4^+CD45RB^{\text{high}}$ T cells repeatedly exposed to SAg become anergic regulatory T cells

Previous reports had suggested that conventional  $CD4^+CD25^-$  T cells tolerized by SAg in vivo might become regulatory T cells (34). To formally test this possibility, we used a cell-transfer system in which purified T cells from SEB-responsive DO11.10 TCR-TG ( $V\beta 8$ -TG) mice were transferred to CB.17 SCID recipient mice, which are devoid of T cells. The  $CD62L^{\text{high}}$  fraction of purified  $CD4^+CD25^-$  T cells was isolated to enrich for naive

$CD45RB^{\text{high}}$  cells (Fig. 4A). This was done to limit the presence of a possible  $CD25^-$  population of natural regulatory T cells in the  $CD4^+$  T cell inoculum (49). The SCID mice were reconstituted with either total  $CD4^+$  or  $CD4^+CD25^-CD62L^{\text{high}}$  T cells and thereafter challenged with SEB once or three times using the same protocol as described above.

The  $CD4^+$  T cells isolated from 3 $\times$ SEB recipients of  $CD4^+CD25^-CD62L^{\text{high}}$  T cells were more responsive to SEB restimulation in vitro compared with cells isolated from SCID mice



**FIGURE 4.** Repeated SAg stimulation induces anergy and regulatory T cell function in  $CD4^+CD45RB^{\text{high}}$  naive T cells. *A*,  $CD4^+CD25^-CD62L^{\text{high}}$  or total  $CD4^+$  T cells were purified from  $V\beta 8$ -TG mice (see *Materials and Methods*), stained with CD4 and CD45RB Abs, and analyzed by FACS. The GeomFI of the CD45RB expression is indicated in the histograms. *B*,  $CD4^+CD25^-CD62L^{\text{high}}$  or total  $CD4^+$  T cells ( $7.5 \times 10^6$ ) purified from  $V\beta 8$ -TG mice were transferred to syngeneic CB.17 SCID mice and injected once or repeatedly with SEB ( $1 \mu\text{g}$ ) (see *Materials and Methods*). Two days after the last SEB treatment,  $CD4^+$  T cells were isolated from pooled spleen and lymph nodes of recipients and cultured ( $5 \times 10^4$  cells/well) for 72 h in the presence of SEB ( $1 \mu\text{g/ml}$ ) and splenic DCs ( $5 \times 10^3$  cells/well). Proliferation was assayed as above and represents the mean  $\pm$  SD of triplicate cultures. *C*, The  $CD4^+$  T cell subsets in *B* were cocultured for 72 h with responder T cells ( $5 \times 10^4$  cells/well) as above at the indicated ratios together with splenic DCs ( $5 \times 10^3$  cells/well) and SEB ( $1 \mu\text{g/ml}$ ). Proliferation was assayed in triplicate cultures as above, and the results are presented as the percentage of responder T cell proliferation in the absence of additional T cells. One representative experiment of three performed is shown.

receiving total CD4<sup>+</sup> T cells (Fig. 4B). This difference might be due to the differential number of natural regulatory CD4<sup>+</sup> T cells in the two inocula. However, both CD4<sup>+</sup> T cell populations from 3×SEB SCID mice were clearly anergic as compared with CD4<sup>+</sup> T cells isolated from SCID mice treated only once with SEB (Fig. 4B).

Next, we investigated whether these CD4<sup>+</sup> T cells, which had been tolerized by repeated SEB exposure in SCID mice, would inhibit responder T cell proliferation in vitro. Evidently, both CD4<sup>+</sup> T cells isolated from the 3×SEB recipients of CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>high</sup> and of total CD4<sup>+</sup> T cells were equally efficient regulatory T cells (Fig. 4C). In contrast, CD4<sup>+</sup> T cells from SCID mice reconstituted with the total CD4<sup>+</sup> T cell population and treated once with SEB did not suppress the response of the CD4<sup>+</sup>CD25<sup>-</sup> responder T cells (Fig. 4C). Collectively, these results indicate that naive CD4<sup>+</sup>CD25<sup>-</sup> T cells can be both anergized and induced to regulatory functions by repeated SA<sub>g</sub> exposure in vivo independently of natural regulatory T cells, because these are not present in the SCID host. However, it could be argued that the repeated SEB stimulation in the above cell transfers would strongly select for any remaining natural regulatory T cells due to their relative resistance to AICD (6, 16).

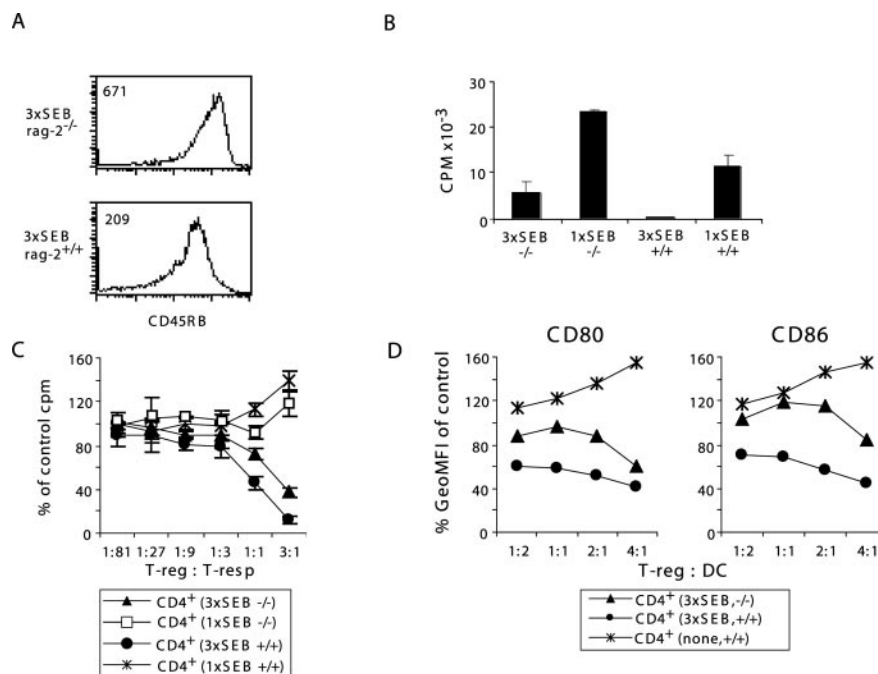
#### Differential regulatory cell function of CD4<sup>+</sup> T cells from Vβ8 TG *rag-2*<sup>-/-</sup> and *rag-2*<sup>+/+</sup> mice treated repeatedly with SA<sub>g</sub>

To gain additional insight into the regulatory function of in vivo-tolerized CD4<sup>+</sup>CD25<sup>-</sup> T cells, we used Vβ8-TG mice crossed to either a *rag-2*<sup>-/-</sup> or a *rag-2*<sup>+/+</sup> genetic background. Because the former strain does not harbor natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (7, 8, 50, 51), this approach enabled us to study the effector

functions of tolerized bona fide CD4<sup>+</sup>CD25<sup>-</sup> T cells in the absence of natural regulatory T cells. Furthermore, these cells could be compared functionally to tolerized CD4<sup>+</sup> T cells that had been induced in the presence of natural regulatory T cells (in *rag-2*<sup>+/+</sup> Vβ8-TG mice), similarly to the above cell transfer model. Because we could recover only very few cells from the reconstituted SCID mice, we took advantage of the *rag-2*<sup>-/-</sup> model to facilitate such analyses.

The Vβ8-TG *rag-2*<sup>-/-</sup> and *rag-2*<sup>+/+</sup> mice were injected three times with SEB to induce tolerance or once to induce T cell activation. The CD45RB expression was remarkably higher on SEB-tolerized CD4<sup>+</sup> T cells from *rag-2*<sup>-/-</sup> as compared with *rag-2*<sup>+/+</sup> mice (Fig. 5A). In addition, fewer of the CD4<sup>+</sup> cells from *rag-2*<sup>-/-</sup> mice expressed the CD25 marker (2.1 ± 0.7%; *n* = 7), as compared with the *rag-2*<sup>+/+</sup> cells (8.8 ± 1.3%; *n* = 6). Importantly, both these CD4<sup>+</sup> T cell populations were hyporesponsive to SEB restimulation in vitro, while CD4<sup>+</sup> T cells from mice treated only once with SEB exhibited a robust proliferation (Fig. 5B). Furthermore, the in vitro SEB response of CD4<sup>+</sup> T cells from *rag-2*<sup>-/-</sup> mice was relatively stronger compared with the SEB response of CD4<sup>+</sup> T cells from *rag-2*<sup>+/+</sup> mice (Fig. 5B). Thus, these proliferation data were fully consistent with the results obtained using the SCID transfer model (Fig. 4B).

The suppressive effects of the SEB-tolerized CD4<sup>+</sup> T cells from Vβ8-TG *rag-2*<sup>-/-</sup> or *rag-2*<sup>+/+</sup> mice were studied as above using CD4<sup>+</sup>CD25<sup>-</sup> responder T cells from untreated *rag-2*<sup>+/+</sup> mice. CD4<sup>+</sup> T cells from both 3×SEB *rag-2*<sup>-/-</sup> and *rag-2*<sup>+/+</sup> mice efficiently suppressed responder T cell proliferation (Fig. 5C). In contrast, increased T cell proliferation was observed in cocultures



**FIGURE 5.** CD4<sup>+</sup> T cells tolerized in the presence or in the absence of natural regulatory T cells in vivo are phenotypically and functionally different. *A*, CD4<sup>+</sup> T cells were purified from Vβ8-TG *rag-2*<sup>-/-</sup> or *rag-2*<sup>+/+</sup> mice, treated once or repeatedly with SEB, and analyzed for CD45RB expression by FACS. The GeoMFI value of CD45RB expression is indicated in the histograms. *B*, The indicated CD4<sup>+</sup> T cells ( $5 \times 10^4$  cells/well) were cultured for 72 h in the presence of splenic DCs ( $5 \times 10^3$  cells/well) and SEB (1 μg/ml). Proliferation was assayed in triplicate cultures as above and represent the mean cpm ± SD. *C*, The same T cell subsets as in *B* were cocultured at the indicated ratios for 72 h with responder T cells ( $5 \times 10^4$  cells/well) from untreated *rag-2*<sup>+/+</sup> mice, together with splenic DCs ( $5 \times 10^3$  cells/well) and SEB (1 μg/ml). Proliferation was assayed in triplicate cultures as above, and the results are presented as the percentage of the proliferation of responder T cells stimulated in the absence of additional T cells. *D*, The same T cell subsets as in *B* were cocultured for 18 h with splenic DCs ( $1 \times 10^5$  cells/well) at the indicated ratios in the presence of SEB (1 μg/ml). The cells were thereafter stained with CD11c, CD80, and CD86 Abs analyzed by FACS. CD80 and CD86 expression by CD11c<sup>+</sup> cells is presented as the percentage of the GeoMFI of DCs cultured in the absence of T cells. One representative experiment of three performed is shown.

with CD4<sup>+</sup> T cells from mice treated once with SEB (Fig. 5C). Taken together, these results show that, similar to the CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>high</sup> T cell populations tolerized by repeated SEB treatments in SCID mice, the CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from 3×SEB Vβ8 TG *rag-2*<sup>-/-</sup> mice were both anergic and possessed regulatory functions upon in vitro culture.

To study whether the SEB-tolerized CD4<sup>+</sup> T cells from Vβ8 TG *rag-2*<sup>+/+</sup> or *rag-2*<sup>-/-</sup> mice would down-modulate costimulatory molecules on APCs, cocultures were conducted at various effector T cell to DC ratios in the presence of SEB. As expected, CD4<sup>+</sup> T cells from *rag-2*<sup>+/+</sup> mice efficiently down-modulated both CD80 and CD86 on the DCs (Fig. 5D). However, the CD4<sup>+</sup> T cells from *rag-2*<sup>-/-</sup> mice down-modulated CD86 expression only at the highest T cell:DC ratio and down-modulated CD80 expression only modestly (Fig. 5D). CD4<sup>+</sup>CD25<sup>-</sup> T cells from untreated Vβ8 TG mice rather increased the expression of both CD80 and CD86 on the surface of DCs. These results suggest that CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from SEB-tolerized *rag-2*<sup>-/-</sup> mice were not functionally identical with the CD4<sup>+</sup> T cells from SEB-tolerized *rag-2*<sup>+/+</sup> mice.

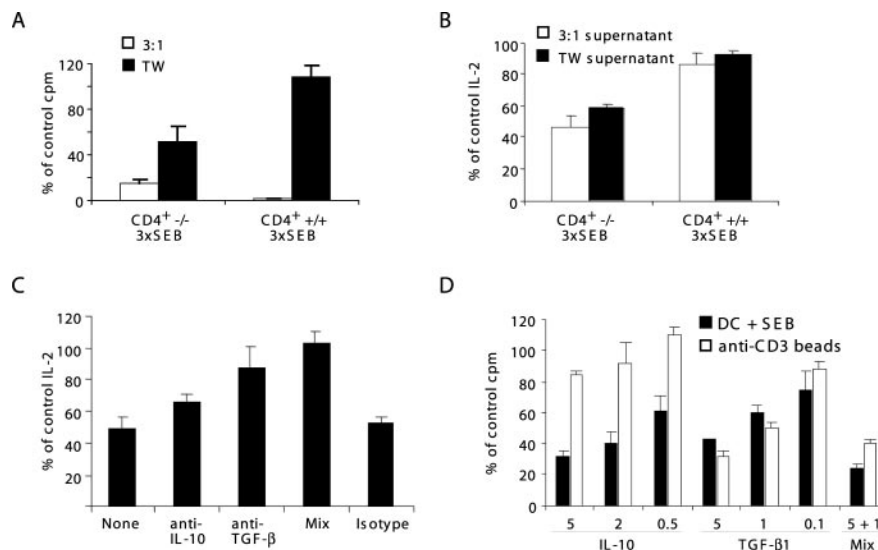
#### Suppression by in vivo-tolerized CD45RB<sup>high</sup>CD4<sup>+</sup> *rag-2*<sup>-/-</sup> T cells is mainly cytokine mediated

To search for further differences in the regulatory functions of CD4<sup>+</sup> T cells from tolerized *rag-2*<sup>-/-</sup> and *rag-2*<sup>+/+</sup> Vβ8-TG mice, we studied their cell-contact dependency using the Transwell culture system. The suppression of responder T cell proliferation was only partially inhibited by physically separating SEB-tolerized *rag-2*<sup>-/-</sup> CD4<sup>+</sup> T cells from the responders by the Transwell membrane (Fig. 6A). In contrast, the suppression mediated by

SEB-tolerized *rag-2*<sup>+/+</sup> CD4<sup>+</sup> T cells was completely inhibited. The supernatants from these Transwell experiments were collected and added to secondary cultures. Consistent with the relative cell contact independence of CD4<sup>+</sup> T cells from *rag-2*<sup>-/-</sup> mice, these supernatants reduced both IL-2 production (Fig. 6B) and proliferation (data not shown) of the responder T cells, whereas supernatants collected from cocultures containing SEB-tolerized CD4<sup>+</sup> T cells from *rag-2*<sup>+/+</sup> mice did not. The same observations were made using supernatants collected from conventional suppression cultures (Fig. 6B). These results indicate that suppression by tolerant CD4<sup>+</sup> T cells from *rag-2*<sup>-/-</sup> mice was largely dependent on soluble factors.

Next, we addressed whether the suppressive factors in the supernatants from the tolerant *rag-2*<sup>-/-</sup> T cells could be neutralized with IL-10 and/or TGF-β blocking Abs. Either IL-10 or TGF-β Abs were able to partially abrogate suppression, whereas a combination of these Abs eliminated most of the suppressive activity of the supernatants (Fig. 6C). According to this result, the cell contact independent suppression by the tolerant *rag-2*<sup>-/-</sup> CD4<sup>+</sup> T cells was mediated by these two cytokines. The addition of either recombinant IL-10 or TGF-β1 to parallel cultures demonstrated that these cytokines were indeed potent inhibitors of T cell IL-2 production (Fig. 6D) and proliferation (data not shown). Interestingly, IL-10 was suppressive only in the presence of APC, whereas TGF-β inhibited IL-2 production both in the presence and in the absence of APC (Fig. 6D).

We conclude that CD4<sup>+</sup>CD45RB<sup>high</sup> T cells tolerized with SAG in *rag-2*<sup>-/-</sup> mice are functionally different from natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in that they suppress responder T



**FIGURE 6.** Suppression mediated by SAG-tolerized CD4<sup>+</sup>CD25<sup>-</sup> T cells is largely cytokine dependent. **A**, CD4<sup>+</sup> T cells purified from 3×SEB Vβ8-TG *rag-2*<sup>-/-</sup> or *rag-2*<sup>+/+</sup> mice ( $1.5 \times 10^5$ /well) were cocultured for 72 h with responder T cells ( $5 \times 10^4$  cells/well) as in Fig. 5, splenic DCs ( $5 \times 10^3$  cells/well), and SEB (1 μg/ml). In parallel cultures, responder T cells were cocultured with DCs, separate from the regulatory T cells and DCs in the upper chamber of a Transwell (TW) membrane. Proliferation was assayed in triplicate cultures as above, and the results are presented as the percentage of responder T cell proliferation in the absence of regulatory T cells. **B**, After 48 h, supernatants were collected from the cultures in **A** and added (1:1 ratio with fresh medium) to secondary cultures containing responder T cells, splenic DCs, and SEB as described in **A**. IL-2 production was assayed in triplicate cultures after 72 h as above. The results are presented as the percentage of IL-2 concentration in cultures of responder T cells stimulated in the presence of control supernatants from CD4<sup>+</sup>CD25<sup>-</sup> T cells cultured 48 h in the absence of additional T cells. **C**, Cocultures were prepared as in **A**, and supernatants collected after 48 h were added to secondary cultures as in **B**. These secondary cultures were incubated in the presence or absence of the indicated neutralizing Abs (100 μg/ml) or isotype control. The results are presented as in **B**. **D**, Responder T cells from Vβ8-TG mice ( $5 \times 10^4$  cells/well) were cultured with either splenic DCs ( $5 \times 10^3$  cells/well) and SEB (1 μg/ml) or anti-CD3-coated latex beads ( $5 \times 10^4$  beads/well). Various concentrations of recombinant IL-10 or TGF-β1 were added as indicated. After 72 h, supernatants were collected from triplicate cultures and analyzed for IL-2 content. The results are presented as the percentage of IL-2 production by responder T cells stimulated in the absence of IL-10 or TGF-β1. One representative experiment of three performed is shown.



cells mainly in a cytokine-dependent way and without causing efficient down-modulation of CD80 and CD86 molecules on APCs.

## Discussion

A number of recent reports demonstrate that chronic *in vivo* Ag stimulation of CD4<sup>+</sup> T cells induces a state of poor *in vitro* responsiveness or anergy to that Ag (22–24, 35, 41, 42). Intravenous or mucosal administration of peptides in the absence of adjuvants induces a similar state of unresponsiveness in responding CD4<sup>+</sup> T cells (52, 53). In several cases, anergic T cells have also been shown to inhibit Ag-specific immune responses by naive CD4<sup>+</sup> T cells, suggesting that the anergic population contained regulatory T cells (23, 24, 34, 35, 42). Interestingly, CD4<sup>+</sup> T cells with similar regulatory functions have been detected during various chronic pathogen infections (28–30). In the present study, we have investigated the functional relationship between CD4<sup>+</sup> T cells tolerized by repeated SAg exposures *in vivo* and natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.

We confirm that repeated exposures of CD4<sup>+</sup> T cells to the bacterial SAg SEA induce expansion followed by deletion and anergization of the responding T cells in Vβ3-TG mice. In the subsequent functional analyses, we found that the SEA-tolerized CD4<sup>+</sup> T cells were similar in function to natural regulatory T cells isolated from untreated mice. Thus, the tolerant CD4<sup>+</sup> T cells suppressed SEA-induced IL-2 production by responder T cells in a cell contact-dependent way, inhibited responder T cell proliferation, and down-modulated CD80 and CD86 on DCs. However, the SEA-tolerized CD4<sup>+</sup> T cells were considerably more efficient suppressors on a per-cell basis than *ex vivo* natural regulatory T cells were.

A major observation from the initial functional analyses was that both the CD25<sup>+</sup> and CD25<sup>−</sup> fractions of the SEA-tolerized CD4<sup>+</sup> T cells possessed regulatory functions. Indeed, both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> T cell populations have previously been reported to contain regulatory T cells (13, 17, 54–59). It is possible that the CD4<sup>+</sup>CD25<sup>+</sup> T cells from 3×SEA mice represent activated natural regulatory T cells, because activation has been shown to dramatically improve their functional potency (48, 60). Furthermore, repeated Ag exposure may specifically spare these cells from deletion (6, 16).

The origin of the regulatory CD4<sup>+</sup>CD25<sup>−</sup> T cells from 3×SEA mice is unknown. It has been demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T cells may lose the CD25 marker *in vivo*, even though they maintain their regulatory potential (17, 55). Thus, some of these cells might represent bona fide natural regulatory T cells. Another possibility was that the chronic SAg exposure had tolerized and induced regulatory function in CD4<sup>+</sup>CD25<sup>−</sup> T cells. We took two approaches to formally test this possibility. Our first approach involved the transfer of CD4<sup>+</sup> T cells depleted of natural regulatory T cells to recipient SCID mice. The second approach involved the use of TCR-TG mice crossed to the *rag-2*<sup>−/−</sup> genetic background. It has been established that natural regulatory T cells do not develop in such mice (7, 8, 50, 51) unless they are exposed to a high-avidity ligand during thymic development (8, 9, 57). Using these two approaches, we demonstrate that CD4<sup>+</sup>CD25<sup>−</sup> T cells could be both tolerized and induced to acquire regulatory functions by repeated exposure to SAg *in vivo*, independently of natural regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells. In this line, it was recently shown that CD4<sup>+</sup>CD25<sup>−</sup> T cells could be tolerized and acquire regulatory T cell function upon continuous exposure to their peptide ligand on peripheral hemopoietic cells (57). Our present results extend those observations by demonstrating that regulatory T cells are produced also in experimental situations mimicking chronic bacterial infections.

Interestingly, our experiments revealed functional differences between the tolerant CD4<sup>+</sup> T cells recovered from 3×SEA *rag-*

*2*<sup>−/−</sup> and *rag-2*<sup>+/+</sup> Vβ8-TG mice. The former T cell population was less severely anergized. Furthermore, even though CD4<sup>+</sup> T cells from 3×SEA *rag-2*<sup>−/−</sup> mice suppressed both responder T cell proliferation and IL-2 production (Fig. 5C and data not shown), they did not down-modulate costimulatory molecules on APCs as efficiently as the SEA-tolerized CD4<sup>+</sup> T cells from *rag-2*<sup>+/+</sup> mice did. Most importantly, the suppressive activity of the tolerant CD4<sup>+</sup>CD25<sup>−</sup> T cells from *rag-2*<sup>+/+</sup> mice required cell contact (Fig. 2B), whereas the suppression by the corresponding T cells from *rag-2*<sup>−/−</sup> mice was mainly cytokine-mediated and less dependent on cell contact (Fig. 6, A–C). As previously observed in several different models (reviewed in Ref. 2), the cytokine-mediated suppression involved IL-10 and TGF-β.

There might be several reasons for these functional differences. First, in the *rag-2*<sup>−/−</sup> animals, the CD4<sup>+</sup>CD25<sup>−</sup> T cells were tolerized in the absence of natural regulatory T cells and B cells. One appealing possibility would be that the natural regulatory T cells, in consistency with the phenomenon of infectious tolerance (61), might induce similar effector functions in CD4<sup>+</sup>CD25<sup>−</sup> cells responding to the tolerogenic Ag, whereas the absence of such natural regulatory T cells would lead to tolerant T cells with a different regulatory phenotype. Second, putative CD25<sup>−</sup> natural regulatory T cells might be both functionally and phenotypically dominant in the CD4<sup>+</sup>CD25<sup>−</sup> population of the *rag-2*<sup>+/+</sup> animals. Whatever the explanation to this phenomenon is, our current data demonstrate functional differences between CD4<sup>+</sup>CD25<sup>−</sup> T cells tolerized in the presence and absence of other lymphocytes.

Two reports have previously studied the induction of tolerance in CD4<sup>+</sup> T cells from TCR-TG *rag*<sup>−/−</sup> mice either by repeated exposure to SAg (34) or *i.v.* or oral Ag exposure (62). In both these cases, the tolerant CD4<sup>+</sup> T cells were shown to contain regulatory T cells capable of inhibiting IL-2 production by responder T cells. In the latter report, a fraction of the tolerant T cells was found to express the CD25 marker (62), which was also found in similar experiments by others (57). Thus, the possibility was raised that some CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells may develop in the periphery (57, 62). However, these previous reports did not perform further functional comparison of natural regulatory T cells and the *in vivo*-tolerized T cells as was done in this study. The CD4<sup>+</sup> T cells recovered from 3×SEA *rag-2*<sup>−/−</sup> Vβ8-TG mice expressed significantly higher levels of CD45RB than the cells recovered from the *rag-2*<sup>+/+</sup> Vβ8-TG mice (Fig. 5A). Additionally, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells was considerably lower in SEA-tolerized *rag-2*<sup>−/−</sup> than in *rag-2*<sup>+/+</sup> mice.

It has been suggested that the *in vivo* function of natural regulatory T cells may be dependent both on IL-10 and TGF-β as well as CD152 (12–15). However, the *in vitro* function of these cells is strictly cell contact dependent, and the role of IL-10 and TGF-β in suppression *in vitro* is controversial (reviewed in Refs. 2 and 63). We found that a mixture containing high concentrations of IL-10R, TGF-β, and CD152 Abs almost completely inhibited the suppression of T cell proliferation mediated by natural regulatory T cells. Both the CD25<sup>−</sup> fraction and the total CD4<sup>+</sup> population of SEA-tolerized cells were similarly inhibited. The CD25<sup>+</sup> fraction could represent chronically activated natural regulatory T cells, but the Ab mixture only marginally inhibited the suppression mediated by these cells. We do not know the reason for the differential sensitivity of *ex vivo* and SEA-tolerized CD4<sup>+</sup>CD25<sup>+</sup> T cells to Ab blocking. However, it could be that these repeatedly stimulated cells are less sensitive to blocking due to their activated state, or alternatively, they might have acquired other suppressive functions. From the data in Fig. 2, one can calculate that 50% inhibition of responder T cell proliferation was obtained using ~1800 CD4<sup>+</sup>CD25<sup>+</sup> T cells from SEA-tolerized mice. Using the total

CD4<sup>+</sup> population ( $2.5 \times 10^4$  cells), which contains  $\sim 1200$  CD25<sup>+</sup> cells, was required to attain the same degree of suppression. Taken together with the differential sensitivity of the two populations to Ab blocking, it thus appears as if the CD25<sup>+</sup> cells have only a minor impact on the suppression mediated by the total CD4<sup>+</sup> population. This could, for example, be due to competition for responder T cells by the 20-fold excess of CD25<sup>+</sup> cells in the CD4<sup>+</sup> population or perhaps due to inhibition of the CD25<sup>+</sup> cells. Analysis of CD4<sup>+</sup> T cells from 3 $\times$ SEB V $\beta$ 8-TG *rag-2*<sup>+/+</sup> and *rag-2*<sup>-/-</sup> mice provided similar results, because the regulatory potency of these populations was similar (Fig. 5). Importantly, however, even though the presence of natural regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells did not detectably influence the regulatory potency of the CD4<sup>+</sup> population, their presence during repeated stimulation clearly influenced the effector functions of the total population (Figs. 5 and 6). The recent studies by Kullberg et al. (58) and Sundstedt et al. (59) reported a major role of IL-10-producing CD4<sup>+</sup>CD25<sup>-</sup> regulatory T cells in tolerance to intestinal flora and in peptide-induced tolerance, respectively (58, 59). Thus, even though natural regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells have been shown to be efficient regulatory T cells in vivo (3), CD25<sup>-</sup> T cells can in a variety of models be induced by in vivo Ag stimulation to acquire potent regulatory T cell function.

One should take into account that, when studying the function of natural regulatory T cells in vitro, the experimental approach is almost always inhibition of mitogen-induced responder T cell proliferation. This inhibition can potentially be achieved at many different levels of the T cell activation cascade. Furthermore, the suppressed responder T cells are themselves anergized (64–66) and, at least in the human system, these cells themselves also acquire suppressor cell functions. Thus, inhibition of proliferation might not only involve cell contact-dependent interactions (10, 11) and signal delivery between the natural regulatory T cells and the responder T cells (67) but might also involve interactions among the responder T cells themselves. Other important parameters might be that not only do IL-10 and TGF $\beta$ -1 both inhibit T cell proliferation and APC functions (68, 69), but they are also functionally connected (70). In such circumstances, a combination of Abs, as the one used in this study, might be effective, even though the individual Abs appear to have no or limited impact on suppression. Indeed, the suppressor function of human CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from thymus was also found to be effectively inhibited by a combination of CD152 and TGF- $\beta$  Abs, whereas either Ab alone was without effect (71).

Both TGF- $\beta$  and IL-10 have been implicated in the functions of other kinds of regulatory T cells. Tolerance induced through oral administration of Ag results in the differentiation of TGF- $\beta$ -secreting Th3 cells (reviewed in Ref. 72). In addition, IL-10-producing T regulatory type 1 cells suppress Ag-specific immune responses both in vivo and in vitro (reviewed in Ref. 73). The relationship between the Th3 and T regulatory type 1 cells, the in vivo-tolerized CD4<sup>+</sup>CD25<sup>-</sup> T cells, and natural regulatory T cells is unknown at present. However, the T regulatory type 1 cells can be derived under anergizing conditions in the presence of APCs and IL-10 (25), and it is therefore tempting to speculate that they might represent tolerant CD4<sup>+</sup>CD25<sup>-</sup> T cells similar to the ones in this study (73).

As outlined above, other laboratories have previously shown that CD4<sup>+</sup> T cells tolerized by chronic exposure to viral and bacterial SAgS as well as during other situations of chronic Ag exposure acquire regulatory T cell functions. However, our study is the first thorough comparison of the functions of such tolerant CD4<sup>+</sup> T cells and natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. As revealed by the chronic stimulation of CD4<sup>+</sup> T cells with SAgS, there are

most probably several different subsets of regulatory T cells. The CD25<sup>-</sup> subpopulation of the remaining CD4<sup>+</sup> T cells in SEA-tolerized mice has acquired T cell functions similar to those of the natural regulatory T cells, maybe due to interactions with those cells. Another, at least partially, functionally and phenotypically different subset was revealed when CD4<sup>+</sup> T cells were tolerized in the absence of natural regulatory T cells in *rag-2*<sup>-/-</sup> TCR-TG animals. Because regulatory T cells with similar functions could not be detected in *rag-2*<sup>+/+</sup> animals, this observation suggests that natural regulatory T cells influence the function of the induced CD4<sup>+</sup>CD25<sup>-</sup> regulatory T cells. As discussed above, these induced CD4<sup>+</sup>CD25<sup>-</sup> T cells appear to constitute the dominant regulatory T cells in the chronically Ag-stimulated CD4<sup>+</sup> T cell population.

## Acknowledgments

We thank Drs. Mark M. Davis and Yintong Xue for the kind gifts of TCR-TG mice. We also thank Dr. Francisco Salgado for critical reading of the manuscript.

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