Regulatory and Effector T Cell Activation Levels Are Prime Determinants of In Vivo Immune Regulation

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Little is known about the in vivo conditions in which CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>) play a major role in the prevention of autoimmune diseases. T<sub>reg</sub> are also involved in the down-modulation of immune responses to tumors, allergens, and infectious agents (1) and regulate allogeneic immune responses (2). Because of the multiple functions of T<sub>reg</sub> in the immune system and their therapeutic potentials, it is essential to have a better understanding of the conditions in which they exert their suppressive activity in vivo. Notably, it is important to determine these conditions at the initiation of potentially pathological autoimmune processes vs desirable inflammatory immune responses to pathogens.

In vitro experiments suggest that the level of T cell activation may be an important parameter affecting T<sub>reg</sub>-mediated suppression. For instance, T<sub>reg</sub> inhibited T cell proliferation in the presence of low but not high amounts of anti-CD3 mAb or Ag (3, 4).

In addition, T<sub>reg</sub> inhibited T cell proliferation induced by immature but not fully mature dendritic cells (DC) (4–7). Also, T<sub>reg</sub> lose their suppressive activity in a context of strong costimulation mediated by CD28 or glucocorticoid-induced TNFR family-related molecules (8, 9). These experiments suggest that highly activated T cells would be less sensitive to suppression by T<sub>reg</sub>. However, the relevance of these in vitro findings has not been directly addressed in vivo.

In vivo properties of T<sub>reg</sub> have been mostly analyzed in lymphopenic animals. However, T cell biology is significantly altered in lymphopenic animals and it has been observed that even naive T cells can have a regulatory function in this context (10). It is thus critical to evaluate the physiological activity of natural T<sub>reg</sub> in nonlymphopenic animals. In this context, we and others have demonstrated that a fraction of autoreactive T<sub>reg</sub> is continuously dividing at the steady state (11, 12). Because T<sub>reg</sub> turn on their bystander suppressive activity upon activation (13, 14), these dividing T<sub>reg</sub> likely exert basal and continuous immunosuppression. In this line, depleting endogenous T<sub>reg</sub> led to inhibition of most but not all T-dependent immune responses in reported studies (15–20). In addition, Ag-specific T<sub>reg</sub> activated during an immune response also participate in the immunosuppression (21–24). Thus, during an immune response to a given Ag, effective T<sub>reg</sub>-mediated suppression could depend on the additive effect of basal and Ag-induced immunosuppression, the latter being dependent on the frequency of pre-existing Ag-specific T<sub>reg</sub>.

In this study, we first designed an experimental strategy that allowed the evaluation of the in vivo suppressive activity of T<sub>reg</sub> in nonlymphopenic mice. We compared the suppressive activity of endogenous T<sub>reg</sub> vs transferred Ag-specific T<sub>reg</sub> in different conditions of CD4<sup>+</sup> T cell activation toward the same Ag. Our study suggests that relative activation of T<sub>reg</sub> and effector T cells is critical in the intensity and nature of T<sub>reg</sub>-mediated suppression in vivo. This conclusion is further supported by our findings showing that endogenous T<sub>reg</sub> regulate type 1 diabetes (T1D) only in young NOD mice.

Abbreviations used in this paper: T<sub>reg</sub>, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell; DC, dendritic cell; HA, hemagglutinin; LN, lymph node; T1D, type 1 diabetes.
Materials and Methods

Mice

Six- to 8-wk-old BALB/cByJ (BALB/c) mice were obtained from Charles River Laboratories. The ins-HA-transgenic mice expressing hemaggutinin (HA) of influenza virus under the control of the insulin promoter in pancreatic islet β-cells (25) were backcrossed >10 generations onto the BALB/c genetic background. The TCR-HA mouse that expresses a transgenic TCR recognizing the HA126–138 epitope presented by I-Ad (26) were backcrossed >10 generations onto the BALB/c genetic background and then bred with Thy-1.1 BALB/c congenic mice to generate Thy-1.1 TCR-HA mice. Thy-1.1 BALB/c, TCR-HA, Thy-1.1 TCR-HA, ins-HA mice, and NOD mice were bred in our animal facility under specific pathogen-free conditions. These were manipulated according to European Union guidelines.

In vivo depletion of Treg

Mice were depleted of CD25+ cells by a single i.p. injection of 100 µg (purified mAb or ascites) of the depleting anti-CD25 mAb (PC61 hybridoma). Residual CD25+ cells were revealed by flow cytometry using the 7D4 anti-CD25 mAb. In our hands, this dose induced over 80% of CD4+CD25+ cell depletion in spleen and lymph nodes (LN) for 4 wk, which then returned to normal levels of functional Treg within 2–3 wk (Ref. 27 and data not shown).

Cell preparation and adoptive transfer

CD25+ and CD25− cells were prepared as previously described (28). Briefly, brachial, axillary, cervical, and inguinal LN and spleen were mechanically dissociated. Cells, incubated with biotin-labeled anti-CD25 mAb (Miltenyi Biotec). The CD25+ cell fraction (Treg) was obtained after two consecutive runs on magnetic cell separation LS columns (Miltenyi Biotec), giving >90% of CD25+ cells with very low levels of宣布性CD4+ cells. Cells were then stained for intracellular cytokines for 30 min at 4°C with allophycocyanin-labeled anti-CD25 mAb to block FcR binding, and then stained in PBS 3% FCS buffer with saturating concentrations of the following mAbs: allophycocyanin-labeled anti-CD4, PE-labeled or CyChrome-labeled anti-Thy-1.1, biotinylated-labeled anti-CD25, revealed anti-streptavidin-PerCP or streptavidin-CyChrome (all from BD Biosciences). For intracellular cytokine staining, 6 × 10^6 LN cells were stimulated with 6 × 10^6 BALB/c splenocytes pulsed with 4 µg/ml HA126–138 peptide for 6 h at 37°C in 6 ml in the presence of 1 µl/ml GolgiPlug (BD Biosciences). Then, cell surface staining was performed as described above, followed by fixation and permeabilization of cells with CytoFix/CytoPerm buffer (BD Biosciences) for 20 min at 4°C. Cells were then stained for intracellular cytokines at 30 min for 4°C with the following mAb diluted in Perm/Wash buffer (BD Biosciences): PE-labeled anti-IFN-γ and anti-IL-2 (BD Biosciences). PE-conjugated isotypic controls mAb were used to subtract the background staining. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or FlowJo (Tree Star) software.

Statistical analyses

Statistical significances were calculated using the two-tailed unpaired Student t test with 95% confidence intervals.

Results

Experimental design

Using an adoptive transfer model of TCR-transgenic T cells specific for a peptide of influenza virus HA, we designed different immunization conditions leading to different levels of T cell activation (Table I). The lowest level of HA-specific T cell activation was obtained by injection of HA126–138 peptide emulsified in CFA (CFA condition). Abs and flow cytometry analysis

Nondraining (of brachial and axillary) and draining (popliteal for BALB/c mice, pancreatic for ins-HA mice) LNs were mechanically dissociated. For cell surface staining, cells were preincubated with the 2.4G2 mAb to block FcR binding, and then stained in PBS 3% FCS buffer with saturating concentrations of the following mAbs: allophycocyanin-labeled anti-CD4, PE-labeled or CyChrome-labeled anti-Thy-1.1, biotinylated-labeled anti-CD25, revealed anti-streptavidin-PerCP or streptavidin-CyChrome (all from BD Biosciences). For intracellular cytokine staining, 6 × 10^6 LN cells were stimulated with 6 × 10^6 BALB/c splenocytes pulsed with 4 µg/ml HA126–138 peptide for 6 h at 37°C in 6 ml in the presence of 1 µl/ml GolgiPlug (BD Biosciences). Then, cell surface staining was performed as described above, followed by fixation and permeabilization of cells with CytoFix/CytoPerm buffer (BD Biosciences) for 20 min at 4°C. Cells were then stained for intracellular cytokines at 30 min for 4°C with the following mAb diluted in Perm/Wash buffer (BD Biosciences): PE-labeled anti-IFN-γ and anti-IL-2 (BD Biosciences). PE-conjugated isotypic controls mAb were used to subtract the background staining. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or FlowJo (Tree Star) software.

Table I. Activation of HA-specific CD4+ T cells under different immunization conditions

<table>
<thead>
<tr>
<th>Immunization route</th>
<th>Analyses at Day 4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cell number (million)^d</td>
</tr>
<tr>
<td>BALB/c (n = 4)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>ins-HA (n = 13)</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>BALB/c (n = 14)</td>
<td>15.3 ± 6.3</td>
</tr>
<tr>
<td>DC2</td>
<td>12.1 ± 4.4</td>
</tr>
<tr>
<td>BALB/c (n = 22)</td>
<td>12.0 ± 4.0</td>
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</table>

^d CFSE-labeled CD25+ T cells from Thy-1.1 TCR-HA-transgenic mice were adoptively transferred in ins-HA-transgenic mice (ins-HA condition) or in BALB/c mice. The day after, BALB/c mice were immunized s.c. with DC pulsed in vitro with either 2 µg/ml HA peptide (DC2 condition), or 20 µg/ml HA peptide (DC20 condition), or 20 µg/ml HA peptide associated with i.v. injection of anti-CD40 mAb and LPS (DC20/CD40/LPS condition). Four days after activation, CD4+Thy-1.1+ donor cells were analyzed in draining LN by flow cytometry for division, expression of CD25 and intracellular IFN-γ and IL-2. Mean values ± SD are shown.

^a Analyses in pancreatic LN (ins-HA mice) or popltal LN (BALB/c mice).

^b Mix of brachial and axillary LN.

^c Absolute cell numbers of viable cells per LN excluding trypan blue.

^d Percentages of divided cells among CD4+Thy-1.1+ were determined by loss of CFSE fluorescence.

^e Percentages among CD4+Thy-1.1+ cells.
was obtained after transfer of CFSE-labeled HA-specific T cells in ins-HA-transgenic mice expressing HA under the control of the insulin promoter (ins-HA condition). In this noninflammatory environment, donor T cells were weakly activated in draining pancreatic LN as shown by low expansion and proliferation and undetectable cytokine production (Table I). The three other conditions were based on a transfer of CFSE-labeled HA-specific T cells in BALB/c mice, subsequently immunized by s.c. injection of HA-pulsed splenic mature DC. To get a gradually stronger immune response, DC were pulsed with increasing amounts of HA peptide (DC2 and DC20 conditions) with or without the administration of the proinflammatory anti-CD40 agonist mAb and LPS reagents (DC20/CD40/LPS condition). In these three different immunization conditions, high expansion of donor HA-specific T cells (Thy-1.1<sup>+</sup>CD4<sup>+</sup>) were observed compared with nonimmunized BALB/c mice. However, gradual T cell activation was attested by progressive acquisition of the CD25 activation marker on responder T cells and by the fact that compared with the DC20 condition, the addition of the proinflammatory reagents led to an increased proportion of IFN-γ- and IL-2-producing cells by a factor of 2-3 (Table I). Interestingly, lower expansion of donor T cells was observed in the DC20/CD40/LPS condition compared with the DC20 condition despite higher levels of cytokine production and CD25 expression. This suggests an increase in activation-induced cell death possibly due to exhaustion or IFN-γ-mediated cell death (29).

**T<sub>reg</sub>-mediated suppression depends on effector T cell activation level**

We then analyzed the effects of depletion of endogenous T<sub>reg</sub> by an anti-CD25 mAb on proliferation, expansion, and cytokine production of HA-specific T cells in the four different immunization conditions described above. In the ins-HA noninflammatory condition, T<sub>reg</sub> depletion induced a significant increase of the few divided HA-specific T cells in draining pancreatic LN, whereas IL-2 and IFN-γ cytokine production was not detected (Fig. 1A). In contrast, in BALB/c mice immunized with HA-pulsed DC, T<sub>reg</sub> depletion did not induce expansion of transferred HA-specific T cells, irrespective of the condition (Fig. 1A). However, in the DC2 condition, T<sub>reg</sub> depletion induced a significant augmentation of the numbers of IFN-γ- and IL-2-producing T cells, which was increased by

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**FIGURE 1.** Lack of immunosuppressive effect of endogenous T<sub>reg</sub> in high T cell activation context. ins-HA and BALB/c mice were T<sub>reg</sub> depleted or not by injection of anti-CD25 mAb. Ten days later, mice were injected with CFSE-labeled CD25<sup>+</sup> cells from Thy-1.1<sup>+</sup> TCR-HA-transgenic mice, and BALB/c mice were immunized with the indicated protocols depicted in the Table I or with HA peptide emulsified in CFA. Cells were analyzed 4 days later by flow cytometry. A, Percentages of divided CD4<sup>+</sup>Thy-1.1<sup>+</sup> T cells among LN cells and of IFN-γ- and IL-2-producing cells among divided CD4<sup>+</sup>Thy-1.1<sup>+</sup> T cells were determined in draining pancreatic (ins-HA) or popliteal (BALB/c) LN. Each symbol represents one mouse; horizontal bars represent the mean values. Data were obtained from at least three independent experiments. **, p < 0.005. B, Representative experiment showing cell division (CFSE loss) and IFN-γ (upper panels) or IL-2 (lower panels) production among draining popliteal LN CD4<sup>+</sup>Thy-1.1<sup>+</sup> cells in the indicated conditions. The percentages of cells in the four regions defined as in the first two dot plots are shown.
a factor of 2–3 compared with controls, as shown in graphs depicting all performed experiments (Fig. 1A) and in a representative experiment (Fig. 1B). In the DC20 condition, \( T_{\text{reg}} \) depletion induced a significant increase of the numbers of IFN-\( \gamma \)-producing cells that was less pronounced than in the DC2 condition. Depleting endogenous \( T_{\text{reg}} \) in the DC20/CD40/LPS high inflammatory condition did not have any effect on expansion or cytokine production of HA-specific T cells (Fig. 1). As an additional immunization protocol, BALB/c mice were immunized with HA peptide emulsified in CFA (CFA condition). Similarly, in this highly inflammatory condition, \( T_{\text{reg}} \) depletion did not significantly affect expansion or IL-2 production of HA-specific T cells (Fig. 1). In this condition, IFN-\( \gamma \) production was barely detectable as previously reported (30). Overall, these experiments show that endogenous \( T_{\text{reg}} \) efficiently regulated T cell expansion only in the ins-HA condition. In the DC2 or DC20 conditions, \( T_{\text{reg}} \) exerted selective suppressive activity affecting only T cell differentiation, i.e., cytokine production. In the DC20/CD40/LPS and CFA conditions, \( T_{\text{reg}} \) suppressed neither expansion nor cytokine production.

To assess activation of endogenous \( T_{\text{reg}} \), we analyzed their frequency and their size in the different immunization protocols. Except for the CFA condition, no significant increased proportions of CD4\(^{+}\)CD25\(^{+}\) cells were observed in draining LN compared with nondraining LN (Fig. 2A), suggesting that endogenous \( T_{\text{reg}} \) were poorly or not activated by these various treatments. This was further supported by their small size, compared with transferred HA-specific T cells, except for the ins-HA condition (Fig. 2B). To assess cell division of polyclonal \( T_{\text{reg}} \), we adoptively transferred CFSE-labeled polyclonal \( T_{\text{reg}} \) from BALB/c mice in the DC20/CD40/LPS or CFA inflammatory conditions. These transferred \( T_{\text{reg}} \) which likely behave as endogenous \( T_{\text{reg}} \) in nonlymphopenic mice (12), were not activated, as assessed by their size and their proliferation profile (Fig. 2C). In addition, as observed with endogenous \( T_{\text{reg}} \), transferred polyclonal \( T_{\text{reg}} \) did not suppress expansion and cytokine production of transferred HA-specific T cells (Fig. 2D). Altogether, our results show that polyclonal \( T_{\text{reg}} \) (endogenous or transferred) were poorly activated in the various immunization protocols.

**Ag-specific \( T_{\text{reg}} \) can mediate immunosuppression in a strong T cell activation context**

We then investigated whether cotransfer of \( T_{\text{reg}} \) from TCR-HA-transgenic mice could suppress expansion and cytokine production of CFSE-labeled HA-specific T cells in the DC (DC2, DC20, DC20/CD40/LPS) and CFA conditions. In all conditions, cotransfer of HA-specific \( T_{\text{reg}} \) inhibited expansion of HA-specific T cells in a dose-dependent manner. Transferred divided CD4\(^{+}\) T cells represented 1.8–3% of draining LN cells, which dropped to 0.2–0.6% after cotransfer of a high dose of HA-specific \( T_{\text{reg}} \) (Fig. 3A). Inhibition of the expansion of HA-specific T cells was likely due to reduced proliferation because highly divided cells (>4 divisions) represented 80% in the absence of transferred \( T_{\text{reg}} \) vs 30–50% when high numbers of HA-specific \( T_{\text{reg}} \) were cotransferred. Consequently, the proportions of undivided cells and of cells that

**FIGURE 2.** Polyclonal \( T_{\text{reg}} \) are poorly activated even in high T cell activation context. A and B, ins-HA and BALB/c mice were transferred with HA-specific CD25\(^{+}\) T cells and immunized as described in Fig. 1 and then analyzed at day 4 by flow cytometry. A, Each symbol represents the percentage of endogenous \( T_{\text{reg}} \) (CD4\(^{+}\)CD25\(^{+}\)Thy-1.1\(^{+}\) cells) in draining and nondraining LN for one mouse. B, Forward scatters (FSC) indicate the size of endogenous \( T_{\text{reg}} \) (CD4\(^{+}\)CD25\(^{+}\)Thy-1.1\(^{+}\) cells, thick line) and of transferred HA-specific T cells (CD4\(^{+}\)Thy-1.1\(^{+}\) cells, thin line) in draining LN. C and D, BALB/c mice were transferred with HA-specific Thy-1.1\(^{+}\) CFSE-labeled CD25\(^{+}\) cells alone (No \( T_{\text{reg}} \)) or cotransferred with a low or high dose of CFSE-labeled polyclonal BALB/c \( T_{\text{reg}} \) in the DC20/CD40/LPS and CFA conditions. At day 4, cells were analyzed by flow cytometry in the draining popliteal LN. C, Size (FSC, upper panels) and division (CFSE profile, lower panels) of transferred \( T_{\text{reg}} \) (CD4\(^{+}\)Thy-1.1\(^{+}\) CFSE\(^{+}\) cells, thick line) and transferred HA-specific T cells (CD4\(^{+}\)Thy-1.1\(^{+}\) cells, thin line). D, Percentage of divided CD4\(^{+}\)Thy-1.1\(^{+}\) T cells among LN cells (upper panel) and of IFN-\( \gamma \) (middle panel) and IL-2 (lower panel) producing cells among divided CD4\(^{+}\)Thy-1.1\(^{+}\) T cells was determined. Mean percentage ± SEM from four mice per group of two independent experiments is shown.
had divided less than five times were increased in the presence of HA-specific Treg (Fig. 3, B and C). Regarding cytokine production, a high dose of Treg induced a significant decrease of cells producing IFN-γ/H9253 and IL-2 as shown in a representative experiment (Fig. 4A) and graphs showing all performed experiments (Fig. 4B). In the DC2 and DC20 conditions, IFN-γ and IL-2 production decreased from 7 to 13% of divided donor CD4+ T cells in controls to 1–3% of the cells in mice injected with a high dose of HA-specific Treg. In the DC20/CD40/LPS condition, IFN-γ- and IL-2-secreting cells decreased from 23 and 15% in controls to, respectively, 4 and 7% in Treg-injected mice. Finally, in the CFA condition, HA-specific Treg also strongly inhibited IL-2 production of HA-specific CD4+ T cells (Fig. 4). Cotransfer of a lower number of HA-specific Treg resulted in an intermediate inhibition of cytokine production of donor CD4+ T cells (Fig. 4B).

To assess activation of transferred HA-specific Treg, we analyzed their proliferation and size. Compared with nonimmunized control mice, division (CFSE loss) and increase in cell size of cotransferred Treg was readily observed in all conditions of immunization (Fig. 5A). However, highly divided CFSE+ Treg could not be discriminated from the pool of endogenous CD4+ cells because they shared the same Thy-1.2 allele. We thus transferred HA-specific Thy-1.1+ Treg alone and confirmed that they divided in all conditions except in nonimmunized mice (Fig. 5B). Overall, our
results show that contrary to polyclonal T<sub>reg</sub>, HA-specific T<sub>reg</sub> were able to efficiently suppress expansion and cytokine production in strong T cell activation contexts. Importantly, this was correlated with their strong activation.

**Endogenous T<sub>reg</sub> regulate T1D of NOD mice only in young animals**

We previously observed that NOD mice genetically deficient in T<sub>reg</sub> exhibited exacerbated diabetes, showing that T<sub>reg</sub> are major players in diabetes regulation in this model (31, 32). We performed transient T<sub>reg</sub> depletion in NOD mice at various ages to reveal their role at different phases of a chronic progressive inflammatory disease. Indeed, the autoimmune process in NOD mice is progressive with the first signs of autoimmunity starting at 3 wk of age, with few APCs and lymphocytes invading the periphery of pancreatic islets. This is followed by a chronic and progressive increase of the level of inflammation and infiltrating T cells around and then inside the islets (33–35), which is associated with a decrease in the proportion of T<sub>reg</sub> in islets and draining pancreatic LN and thus a dysbalance of the T<sub>reg</sub>-to-T cell ratio (36). Eventually, mice develop clinical diabetes in 75% of females and 25% of males at 25 wk of age. Strikingly, when T<sub>reg</sub> depletion was induced at 3 wk of age in NOD mice, we observed early onset and higher incidence of diabetes. Eighty percent of treated males and females were diabetic as early as 15 wk of age and all mice were diabetic by 21 wk of age. Strikingly, when T<sub>reg</sub> depletion was induced at 3 wk of age in NOD mice, we observed early onset and higher incidence of diabetes. Eighty percent of treated males and females were diabetic as early as 15 wk of age and all mice were diabetic by 21 wk of age. Strikingly, when T<sub>reg</sub> depletion was induced at 3 wk of age in NOD mice, we observed early onset and higher incidence of diabetes. Eighty percent of treated males and females were diabetic as early as 15 wk of age and all mice were diabetic by 21 wk of age.

**Discussion**

Three major conclusions can be drawn from our experiments. 1) The efficiency of T<sub>reg</sub>-mediated basal immunosuppression is related to the level of responder T cell activation. 2) Only Ag-specific T<sub>reg</sub> can block activation of CD25<sup>+</sup> T cells in a context of strong T cell activation. 3) Depending on the experimental approach, T<sub>reg</sub> inhibit either T cell expansion or cytokine production or both.

It has been shown that T<sub>reg</sub> can efficiently suppress the effector response in some inflammatory situations. For instance, depleting T<sub>reg</sub> in vivo led to an increased immune response to various pathogens (17, 18, 38). This could be explained by an increased activity of T<sub>reg</sub> during an infectious disease (17) due to various mechanisms (39). Inflammation associated with infection could activate DC, which in turn would induce expansion of T<sub>reg</sub> (7, 40, 41). In addition, danger signals released during infection by tissue damage may also increase T<sub>reg</sub>-mediated suppression (39). Finally, T<sub>reg</sub> express multiple TLR, and their ligands expressed by pathogens can also directly induce expansion of highly suppressive T<sub>reg</sub> (42). Our study suggests that basal immunosuppression exerted by endogenous T<sub>reg</sub> on CD4<sup>+</sup> T cells in nonlymphopenic mice is overwhelmed in a context of strong T cell activation, in apparent contrast with the above observations. This discrepancy could be explained by the poor activation of polyclonal T<sub>reg</sub> as observed in our model, due to several factors. Our immunization protocols induce limited tissue damage and thus minimal danger signals would be released to activate endogenous T<sub>reg</sub>. Also, endogenous T<sub>reg</sub> would not be activated by TLR signaling in our immunization conditions.

The importance of T<sub>reg</sub> activation for efficient immunosuppression is further suggested in our experiments with transferred HA-specific T<sub>reg</sub>. In the DC and CFA conditions, these T<sub>reg</sub> strongly divided and exerted effective immunosuppression. In that line, Ag-dependent T<sub>reg</sub> immunosuppression has been correlated with proliferation in response to immunization with Ag emulsified in Freund adjuvant (22, 24). The correlation between T<sub>reg</sub>-mediated suppression and T<sub>reg</sub> division confirms that T<sub>reg</sub> activation is a key requirement for effective immunosuppression.

Altogether, our cognitive model of various immune responses to the same Ag suggests that T<sub>reg</sub>-mediated suppression is dependent on the relative activation of both T<sub>reg</sub> and effector T cells. Indeed,
T_{reg} efficiently suppressed expansion when effector T cells and T_{reg} were both poorly activated (i.e., ins-HA condition, Figs. 1 and 2). In contrast, poorly activated T_{reg} did not suppress if effector T cells were strongly activated (i.e., polyclonal T_{reg} in DC20/CD40/LPS or CFA conditions, Figs. 1 and 2). Finally, strongly activated T_{reg} efficiently suppressed strongly activated effector T cells (i.e., HA-specific T_{reg} in DC20/CD40/LPS or CFA conditions, Figs. 3–5).

Analyzing in vivo expansion and cytokine production by Ag-specific CD4+ T cells following immunization has been performed in various models of adoptive transfer of Ag-specific T cells from TCR-transgenic mice into nonlymphopenic mice. Using this experimental approach, it has been shown that T_{reg} selectively inhibit CD25- T cells at the level of cytokine production but not of their proliferation or expansion (43, 44), whereas other investigators reported an effect of T_{reg} on CD25+ T cell expansion (22, 24). The reasons for these conflicting data could be due to several experimental differences: 1) the type of immunization (Ag-pulsed DC (43, 44) vs Ag in Freund adjuvant (22, 24)); 2) the type of approach (deficit of endogenous T_{reg} (43, 44) vs cotransfer of Ag-specific T_{reg} (22, 24)); 3) the Ag specificity of the transgenic TCR. Our study compares different immunization protocols (pulsed DC vs Freund adjuvant), different approaches (T_{reg} depletion vs T_{reg} cotransfer) with T cells specific for the same HA Ag. Our findings emphasize the importance of the experimental approach on the nature of T_{reg}-mediated suppression. Endogenous T_{reg} affect cytokine production by CD25+ T cells without inhibiting their proliferation, showing that cytokine production is not directly linked to cell division (this study and others (43, 44)). In contrast, cotransfer of Ag-specific T_{reg} also affects proliferation of CD25+ T cells (this study and others (22–24)). This may rely on the fact that self-reactive endogenous T_{reg} and TCR-transgenic T_{reg} could function in an entirely different way. Whatever the mechanism, we speculate that T_{reg} may preferentially affect expansion or cytokine production of effector T cells depending on their activation level.

Previous publications showed that blocking CTLA-4 or ICOS pathways induced diabetes exacerbation in NOD mice expressing an islet Ag-specific transgenic TCR. This was observed only when performed in young animals exhibiting no or mild insulitis. Blocking these pathways may have altered T_{reg} function, suggesting that endogenous T_{reg} play a major role at the beginning of the autoimmune process (37, 45). Here, we directly tested whether T_{reg} would play a regulatory function predominantly in young NOD mice. Depleting endogenous T_{reg} led to T1D exacerbation only if performed in young (3–6 wk old) NOD mice, when inflammation in islets is still mild (33–35). At this stage, depletion of endogenous T_{reg} would remove basal immunosuppression leading to increased activation of islet-specific CD25+ T cells. As the disease progresses, T_{reg}-mediated basal immunosuppression would be overwhelmed in a context of a rising number of islet-specific effector T cells (46, 47) and a proportional decrease of T_{reg} (36). The efficiency of this basal immunosuppression would thus depend on the balance between activated effector T cells and activated T_{reg} locally in islets or draining LN, as suggested in our cognitive model using HA-specific T cells. This hypothesis is further supported by the findings that transfer of islet-specific, but not polyclonal, T_{reg} can control advanced diabetes (48–50). We cannot rule out, however, that CD25- T cells of aging NOD mice have lesser suppressive activity than the ones of young animals, or that CD25- T cells of aging NOD mice become progressively refractory to suppression by T_{reg} as reported in vitro (51, 52). Altogether, T_{reg} specific for target Ag could be envisioned for the treatment of autoimmune diseases either acute or chronic. It is now critical to design conditions for generation of high numbers of human Ag-specific T_{reg}.

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