Effector T Cells Boost Regulatory T Cell Expansion by IL-2, TNF, OX40, and Plasmacytoid Dendritic Cells Depending on the Immune Context

Audrey Baeyens, David Saadoun, Fabienne Billiard, Angéline Rouers, Sylvie Grégoire, Bruno Zaragoza, Yenkel Grinberg-Bleyer, Gilles Marodon, Eliane Piaggio and Benoît L. Salomon

*J Immunol* published online 29 December 2014

http://www.jimmunol.org/content/early/2014/12/28/jimmunol.1400504

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/12/28/jimmunol.1400504.DCSupplemental

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Effector T Cells Boost Regulatory T Cell Expansion by IL-2, TNF, OX40, and Plasmacytoid Dendritic Cells Depending on the Immune Context

Audrey Baeyens,¹ David Saadoun,¹ Fabienne Billiard, Angéline Rouers, Sylvie Grégoire, Bruno Zaragoza, Yenkel Grinberg-Bleyer, Gilles Marodon, Eliane Piaggio, and Benoît L. Salomon

CD4⁺CD25⁺Foxp³ regulatory T (Treg) cells play a major role in peripheral tolerance. Multiple environmental factors and cell types affect their biology. Among them, activated effector CD4⁺ T cells can boost Treg cell expansion through TNF or IL-2. In this study, we further characterized this effector T (Teff) cell–dependent Treg cell boost in vivo in mice. This phenomenon was observed when both Treg and Teff cells were activated by their cognate Ag, with the latter being the same or different. Also, when Treg cells highly proliferated on their own, there was no additional Treg cell boost by Teff cells. In a condition of low inflammation, the Teff cell–mediated Treg cell boost involved TNF, OX40L, and plasmacytoid dendritic cells, whereas in a condition of high inflammation, it involved TNF and IL-2. Thus, this feedback mechanism in which Treg cells are highly activated by their Teff cell counterparts depends on the immune context for its effectiveness and mechanism. This Teff cell–dependent Treg cell boost may be crucial to limit inflammatory and autoimmune responses. The Journal of Immunology, 2015, 194: 000–000.

When it functions properly, the immune system is able to induce inflammation and activation of innate and adaptive immune responses while maintaining tissue integrity for the long term. This requires complex cellular and molecular networks with multiple feedbackforward and feedback mechanisms to amplify and control various responses, respectively. The balance between Foxp³⁺CD4⁺CD25⁺ regulatory T (Treg) cells and effector T (Teff) cells determines the level of the adaptive immune response (1, 2). Seminal recent works have shown that the homeostatic and suppressive activity of Treg cells are highly influenced by environmental cues, which give rise to diverse Treg cell subsets having different phenotypes and functions (3–5). Some of these signals are provided by activated Teff cells indexing Treg cell–mediated suppression to Teff cell activation (6–8).
Cell preparation and adoptive transfer

Cells were prepared as previously described (14). Briefly, LN (brachial, axillary, cervical, and inguinal) and spleen were mechanically dissociated. Cells, incubated with biotin-labeled anti-CD25 mAb (7D4; BD Biosciences), were coated with anti-biotin microbeads (Miltenyi Biotec). The CD25-depleted (Teff cells) was obtained after two consecutive runs on magnetic cell separation LS columns (Miltenyi Biotec) or with auto-MACS, reaching 85% purity of CD25+ cells and >90% were Foxp3+. The CD25-depleted cells (Teff cells) were harvested from the flowthrough. This fraction contained 30% CD4+ T cells and 0.5% residual CD25+ cells. To obtain in vitro preactivated HA111−Teff cells, the CD25+ fraction from TCR-HA111 mice was stimulated in vitro for 3−4 d by CD11c+ splenic DC (purified using anti-CD11c-coupled Miltenyi Biotec microbeads) pulsed with 10 μg/ml HA111 peptide in the presence of 10 ng/ml GM-CSF (R&D Systems).

Before i.v. injection, Teff or Treg cells were labeled with CFSE at 2.5 μM for 5 min in serum-free PBS at room temperature and were washed twice in PBS. When mice were challenge with a new cohort of Teff cells (Fig. 1C, 1D), cells were labeled with 2 μM violet cell trace dye for 20 min in serum-free PE or 37°C and were washed twice in PBS. Mice received 1 × 10^6 Treg cells with or without 1 × 10^6 Teff cells or 1 × 10^6 Treg cells alone except in Fig. 3, for which some mice were injected with 10 × 10^6 or 30 × 10^6 Treg cells, and in Fig. 4, for which mice received 2 × 10^6 T cells from chimeric IL-2−deficient mice because of the twice lower proportion of HA-specific T cells.

Immunization protocols in WT mice

Mice were immunized the day after the transfer of HA−Treg cells and HA−Teff cells. Mice were immunized by s.c. injection in the rear footpad with 2 μg HA126 peptide and/or 2 μg HA111 peptide (or 0.02 μg HA111 peptide when indicated) emulsified in CFA. Alternatively, mice were immunized by s.c. injection of DC pulsed overnight with 20 μg/ml HA126 peptide as previously described (1). In vivo treatment

For blockade of TNF, we performed i.p. injections of 0.5 mg soluble TNFR-Fc (etanercept) or i.v. injections of 0.5 mg anti-TNF mAb (XT3.11; Bio X Cell) at days −1, 1, and 3 after cell transfer. For blockade of OX40, mice received i.p. injections of 0.5 mg anti-OX40 mAb (RM 134.1; Bio X Cell) at days −1, 1, and 3 after cell transfer in ins-HA mice and daily from days 1 to 3 in WT mice. For blockade of IL-2, WT mice received 150 μg neutralizing anti-IL-2 mAb (S4B6; Bio X Cell) at days −1, 1, and 3 after cell transfer for pDC depletion, mice received daily p.i. injections of the anti-BST2 mAb (120G8; Bio X Cell) at 0.2 mg from days −1 to 5 in ins-HA mice and at 0.2 or 0.3 mg from days −1 to 3 in WT mice. For IL-2 (25,000 IU Proleukin from Novartis) and TNF (2 μg/ml TNF, Ghent University) treatments, mice were injected in the rear footpads 4 h before sacrifice and then the draining popliteal LN (pop-LN) was analyzed to determine 3 CHARACTERIZATION OF THE Teff-DEPENDENT Treg BOOST

We previously described the phenomenon of the Teff→Treg boost in transgenic mice expressing the model HA Ag under the control of the insulin promoter (ins-HA mice) (7). In these mice, adaptively transferred Treg cells specific for the HA111 peptide (HA111−Treg cells) poorly proliferated in the draining pancreatic LN at day 7. The cotransfer of HA111−Teff cells induced a dramatic increase of the proliferation and expansion of functionally suppressive HA111−Treg cells in these LN (7). This initial characterization of the Teff→Treg boost phenomenon was observed in a context of low inflammation with T cells reacting to a foreign Ag (hereafter called “low inflammatory context”). We thus wondered whether similar findings would occur in a context of high inflammation with T cells reacting to a foreign Ag (hereafter called “high inflammatory context”). Treg cells specific for the exogenous HA126 peptide (HA126−Treg cells) were transferred in WT mice and activated by their cognate peptide emulsified in CFA (CFA/HA126) in the rear footpad. When transferred alone, HA126−Treg cells weakly proliferated in the draining pop-LN at day 4 (Fig. 1A), and not in nondraining LN (not shown). The cotransfer of HA126−Teff cells induced a weak but significant increase of HA126−Treg cell expansion (Fig. 1B). A Teff→Treg boost was also observed when Treg and Teff cells recognize different epitopes, namely HA126 presented by MHC class II A4 for Teff cells and HA111 presented by MHC class II E3 for Treg cells. Indeed, the cotransfer of HA126−Treg cells and HA111−Teff cells, followed by immunization with HA126 and HA111 peptides emulsified in CFA (CFA/HA126+111) induced a significant increase of HA126−Treg cell proliferation and expansion, compared with mice transfused with HA126−Treg cells alone (Fig. 1A, 1B).

We then assessed whether HA126−Treg cells boosted by HA111−Teff cells were suppressive in vivo. WT mice were first injected with HA126−Treg cells alone or cojected with HA111−Teff cells, or they were left un.injected (controls). Some control mice were not injected with cells. Then mice were immunized with CFA/HA126+111. At day 5, they were challenged with a new cohort of HA126−Teff cells, and we then measured their expansion in the draining LN 3 d later, as a readout of in vivo suppression. Compared to control mice (no cell injection at day 0), proliferation of HA126−Teff cells was highly suppressed in the two groups of mice initially transferred with HA126−Treg cells alone or cotransferred with HA111−Treg cells (Fig. 1C, 1D). The level of suppression was even higher in the latter compared with the former group of mice. Altogether, Teff cell activation enhanced expansion of functionally suppressive Treg cells.

The Teff→Treg boost is observed only when Treg cells poorly proliferate on their own

We next studied different parameters to further characterize the conditions required for a Teff→Treg boost in the inflamed context. Both Teff and Treg cells had to be activated by their cognate Ag because the Treg cell boost was no longer observed in mice cotransferred with either HA126−Treg cells and polyclonal Teff cells or polyclonal Treg cells and HA126−Teff cells, followed by CFA/HA126 immunization (conditions 3 and 4 in Fig. 2A, 2B). Then, we assessed whether a Teff→Treg boost was observed in situations in which Treg cells highly proliferated when transferred alone. This was obtained in mice transfused with HA126−Treg cells and immunized with HA126−pulsed DC (DC/HA126) or in mice transfused with HA111−Treg cells and immunized with CFA/HA111 (conditions 5, 6, and 7 in Fig. 2B). In these two settings, the
cotransfer of HA111–Treg cells or HA126–Teff cells did not further increase donor Treg cell expansion in draining LN at day 4 (Fig. 2A). These data suggest that a Teff→Treg boost was obtained only when Treg cells weakly proliferated on their own. To confirm this hypothesis, we repeated condition 7 (cotransfer of HA111–Treg cells and HA126–Teff cells, followed by CFA/peptide immunization), except that HA111–Treg cells were stimulated with a 100-fold lower dose of HA111 peptide (0.02 versus 2 μg previously) to decrease their activation level. In this condition 8, Treg cells weakly proliferated when transferred alone (Fig. 2B). Their proliferation was significantly increased when Teff cells were cotransferred (Fig. 2C, 2D). Thus, when Treg cells were highly activated, Teff cells no longer increased their expansion.

The Teff→Treg boost required that Teff and Treg cells were activated in the same LN

We then assessed whether the Teff→Treg boost was observed when Treg and Teff cells were activated in different LN. Mice, cotransferred with HA126–Treg cells and HA111–Teff cells were immunized with CFA/HA126 in one rear footpad and with CFA/HA111 in the other rear footpad (Fig. 3A). HA126–Treg cell proliferation was assessed in LN draining the HA126 peptide. As expected, these cells weakly proliferated when transferred alone, but their activation was not increased when HA111–Teff cells were cotransferred, even when high numbers of cells were injected (up to 30-fold more) (Fig. 3B, 3C). Interestingly, activated HA111–Teff cells were readily observed in this LN owing to recirculating cells primarily activated in the contralateral LN (Fig. 3D, 3E).

Taken together, these experiments showed that a Teff→Treg boost was observed in an inflammatory environment induced by CFA in the presence of cognate peptides. However, when Treg cells highly proliferated when transferred alone, adding activated Teff cells did not further increase their expansion. Also, a Teff→Treg boost required that both Treg and Teff cells were stimulated in the same LN. For the rest of this study, we analyzed and compared molecular and cellular mechanisms involved in the Teff→Treg boost newly described in the present study in a high inflammatory condition, and in a low inflammatory context in ins-sa mice that we previously described (7). In the former context, we performed experiments only in mice transferred with HA126–Treg cells and HA111–Teff cells and immunized with CFA/HA126+111 (condition 2 in Fig. 2A).

TNF and IL-2 are involved in the Teff→Treg boost in the high inflammatory condition

In the low inflammatory context, we showed that TNF, but not IL-2, was involved in the Teff→Treg boost (7). We thus tested these two factors in the Teff→Treg boost observed in a high inflammatory condition (condition 2 in Fig. 2A). The role of IL-2 was assessed using IL-2–deficient HA111–Teff cells. Importantly, the expansion of IL-2–sufficient and IL-2–deficient Teff cells was similar in the draining LN (Fig. 4A). In control mice, the cotransfer of IL-2–sufficient HA111–Teff cells increased expansion of HA126–Treg cells, as shown above. Interestingly, the Teff→Treg boost was significantly reduced when IL-2–deficient HA111–Teff cells were cotransferred (Fig. 4B). Because IL-2–deficient mice spontaneously develop a massive lymphoproliferation (16), one may argue that the function of IL-2–deficient Teff cells may be altered, hampering their capacity to boost Treg cells. This was unlikely because when IL-2–deficient mice were crossed with TCR-HA111 transgenic mice, the lymphoproliferative disease was significantly delayed (17). Indeed, IL-2–deficient TCR-HA111 mice were free of disease at 6–7 wk of age, when we sacrificed them for experiments (data not shown). To definitively rule out a putative undetected intrinsic defect of IL-2–deficient HA111–Teff cells, besides their incapacity to produce IL-2, we generated bone marrow chimeric mice composed of a 1:1 ratio of cells from IL-2–deficient TCR-HA111 mice and WT mice. These chimeric mice did not develop any sign of lymphoproliferation (data not shown).

The cotransfer of Teff cells from these mice did not increase the
expansion of HA126–Treg cells, confirming that IL-2 produced by Teff cells was necessary to boost Treg cells in this setting (Fig. 4B). However, was IL-2 sufficient? To address this question, we injected IL-2 in mice transferred with HA126–Treg cells alone and immunized with CFA/HA126. The addition of IL-2 at a dose that induced biological effects in another setting (18) was not sufficient to increase expansion of HA126–Treg cells (Fig. 4C), suggesting that other factors were involved.

The role of TNF in the inflammatory context (condition 2 in Fig. 2A) was assessed by administration of a soluble TNF receptor or anti-TNF mAb to neutralize the cytokine. When TNF was neutralized, the Teff→Treg boost was not observed anymore be-

FIGURE 2. The Teff→Treg boost is observed only when Treg cells poorly proliferated on their own. WT mice were injected with CFSE-labeled Thy-1.1+ HA126–Treg cells, HA111–Treg cells or polyclonal Treg cells alone or cotransferred with HA126–Teff cells, or HA111–Teff cells or polyclonal Teff cells as indicated. Then, mice were immunized with peptide (2 μg each) in CFA or peptide-pulsed DC as indicated. Donor Treg cell expansion was analyzed at day 4 in the draining pop-LN. (A) The level of Treg cell boost (fold increase in Treg cell expansion) was determined by measuring the ratio of divided donor Treg cells (CFSEdimCD4+Thy1.1+Foxp3+) in mice cotransferred with Treg and Teff cells compared with mice transferred with the Treg cells alone. Data were obtained from 4 to 17 mice per group from at least two independent experiments. (B) Representative CFSE profile from three independent experiments of donor Treg cells (CD4+Thy1.1+Foxp3+) when transferred alone. (C and D) WT mice were transferred with HA111–Treg cells alone or with HA126–Teff cells and were immunized with HA126 and 2 or 0.02 μg HA111 peptide in CFA. Representative CFSE profile of donor Treg cells (CD4+Thy1.1+Foxp3+) (C) and absolute number of divided donor Treg cells (CFSEdimCD4+Thy1.1+Foxp3+) (D) are shown. Each symbol represents one mouse, and bars show the means from three independent experiments. **p < 0.01, ***p < 0.001.
cause the proliferation (Fig. 5A) and expansion (Fig. 5B) of donor Treg cells were similar to control mice transferred with Treg cells alone. Importantly, proliferation (Fig. 5C) and expansion (Fig. 5D) of donor Teff cells were unaffected by neutralizing TNF. In this high inflammatory condition, both IL-2 and TNF are necessary for the Teff→Treg boost, and inhibiting only one is sufficient to block the boost. This suggests that either the two cytokines act simultaneously and independently using different signaling pathways or additively using the same cascade of events. The latter mechanism would imply that either TNF impacts somewhere along the IL-2 signaling pathway–dependent Treg cell boost or, vice versa, that IL-2 impacts along the TNF signaling–dependent Treg cell boost. To address this challenging question, we used a simplified in vivo assay in which we injected s.c. a high dose of either IL-2 or TNF, with both cytokines promoting Treg cell expansion in the draining LN (data not shown). We first analyzed the expression of the receptors of the two cytokines, CD25 for IL-2 and TNFR2 for TNF, because it has been previously reported that the TNF-dependent Treg cell boost was TNFR2 mediated (19). Interestingly, TNF increased the expression of CD25 (Supplemental Fig. 1A), as previously observed (20). This may indicate that TNF induced a Treg cell boost by increasing IL-2 responsiveness. Because it is well described that the effect of IL-2 on Treg cells is mediated by STAT5 activation (21), we addressed whether TNF induced phosphorylation of STAT5 in Treg cells. Injection of TNF did not increase the proportion of p-STAT5, whereas in control mice injected with IL-2 this signaling pathway was strongly activated (Supplemental Fig. 1B). The absence of either IL-2 or TNF, with both cytokines promoting Treg cell expansion in the draining LN (data not shown). We first analyzed the expression of the receptors of the two cytokines, CD25 for IL-2 and TNFR2 for TNF, because it has been previously reported that the TNF-dependent Treg cell boost was TNFR2 mediated (19). Interestingly, TNF increased the expression of CD25 (Supplemental Fig. 1A), as previously observed (20). This may indicate that TNF induced a Treg cell boost by increasing IL-2 responsiveness. Because it is well described that the effect of IL-2 on Treg cells is mediated by STAT5 activation (21), we addressed whether TNF induced phosphorylation of STAT5 in Treg cells. Injection of TNF did not increase the proportion of p-STAT5, whereas in control mice injected with IL-2 this signaling pathway was strongly activated (Supplemental Fig. 1B). The absence
of a TNF effect on p-STAT5 in Treg cells was confirmed during the Teff→Treg boost in the high inflammatory condition. Indeed, neutralizing TNF did not decrease the proportion of p-STAT5 among Treg cells, contrary to the effect of blocking IL-2 (Supplemental Fig. 1C). We could not analyze whether IL-2 affects the signaling pathway involved in the TNF-dependent Treg cell boost because this pathway is not yet characterized. In conclusion, we showed that the Teff→Treg boost in the high inflammatory context involved both TNF and IL-2. The detailed mechanism of the additive or synergistic activity of the two cytokines on the Treg cell boost requires further characterization.

The OX40/OX40L signaling pathway is involved in the Teff→Treg boost in the low inflammatory condition

In the low inflammatory context in ins-HA mice, the Teff→Treg boost was only partially dependent on TNF (7). We thus wondered whether other members of the TNFR family would be involved. Because OX40 costimulation led to increased Treg cell expansion (22), we assessed the role of this pathway in the Teff→Treg boost described in low and high inflammatory contexts using a neutralizing anti-OX40L mAb (23). When we blocked the OX40 pathway using a neutralizing anti-OX40L mAb (23), we assessed the role of this pathway in the Teff

Because OX40L is expressed on APCs rather than T cells (24), we wondered whether the former cells would be involved in the Teff→Treg boost. pDC were an interesting candidate because they express OX40L (25) and are able to promote Treg cell expansion (26, 27). We thus tested the role of this DC subset by injecting a mAb that depletes pDC (28). In the high inflammatory context (condition 2 in Fig. 2A), depleting pDC (Fig. 7A) had no impact on Treg cell proliferation or expansion (Fig. 7B, 7C) or Teff cell proliferation or expansion (Fig. 7D, 7E). In contrast, in the low inflammatory context, pDC depletion (Fig. 7F) was associated with a significant decrease of the Teff→Treg boost (Fig. 7G, 7H). Importantly, the proliferation and expansion of donor Teff cells were not significantly affected by pDC depletion (Fig. 7I, 7J). Thus, pDC were involved in the Teff→Treg boost observed in the low, but not the high, inflammatory context.

Treg cells boosted by Teff cells have a phenotype of effector Treg cells in both low and high inflammatory contexts

It was recently described that the Treg cell population can be discriminated in two major subsets. Central Treg (cTreg) cells, defined by the CD62LhighCD44low phenotype, expressed high levels of CCR7 and CD25, and effector Treg (eTreg) cells, defined by the CD62LlowCD44high phenotype, expressed high levels of ICOS and CXCR3. Both Treg cell subsets were present in lym-
phoid tissues whereas eTreg cells were found predominantly in nonlymphoid tissues (29). At steady-state, survival of cTreg cells depended on IL-2 whereas eTreg cells were maintained owing to interaction with ICOS and DC. In this study, we showed that the Teff → Treg boost in the high and low inflammatory contexts depended on IL-2 and pDC, respectively. This may suggest that boosted Treg cells in the high and low inflammatory conditions were related to cTreg and eTreg cells, respectively. To address this hypothesis, we further characterized the phenotype of boosted Treg cells. Before that, we first verified the presence of cTreg and eTreg cells among recipient cells. We could identify a population of cTreg cells that had the CD62LhighCD44lowCCR7highICOSlowCXCR3low phenotype and of eTreg cells that had the CD62LlowCD44highCCR7lowICOShighCXCR3high phenotype (Fig. 8). These two Treg cell subsets were present in all our contexts in draining and nondraining LN (Fig. 8). We then analyzed the phenotype of boosted Treg cells, defined as donor divided Treg cells (CD4+Foxp3+Thy1.1+CFSElow), and compared it with the one of donor nondivided (CFSEhigh) Treg cells, as well as of endogenous cTreg and eTreg cells, as internal controls. In the low inflammatory condition, divided donor Treg cells expressed higher levels of ICOS and CXCR3 and lower levels of CCR7 when compared with nondivided donor Treg cells and endogenous cTreg cells, and they also expressed high levels of CD44 (Fig. 8B). This suggests that these boosted Treg cells also belonged to the eTreg cell population, although they still maintained a relative high level of CD62L expression. Altogether, Treg cells boosted in the low and high inflammatory conditions expressed a phenotype that resembled the one of eTreg cells, although it was not exactly identical.

**Discussion**
The proper functioning of the immune system relies on complex interactions between immune cells. We and others have described that Teff cells are able to help or boost Treg cell activation (7, 8, 30). In this work, we further characterized this phenomenon in two directions. We described immune conditions required for a Teff → Treg boost and we studied its mechanism, revealing the involvement of IL-2, TNF, OX40, or pDC depending on the context.

**Conditions for a Teff → Treg boost**
What did we learn from our present work on conditions required for a Teff → Treg boost? One critical parameter was the level of Treg cell expansion when they were activated on their own. We identified three conditions of low-level Treg cell expansion in which cotransfer of Teff cells induced a Treg cell boost: 1) in pancreatic LN of ins-HA mice injected with HA111→Treg cells, 2) in pop-LN
of WT mice injected with HA126–Treg cells followed by a CFA/HA126 immunization, and 3) in pop-LN of WT mice injected with HA111–Treg cells followed by immunization with low-dose HA111 peptide in CFA. Alternatively, we identified two conditions of a high level of Treg cell expansion in which cotransfer of Teff cells had no impact: 1) in pop-LN of WT mice injected with HA126–Treg cells followed by DC/HA126 immunization, and 2) in pop-LN of WT mice injected with HA111–Treg cells followed by immunization with a high dose of the HA111 peptide in CFA. Thus, a Teff→Treg boost phenomenon was observed only when Treg cells poorly proliferated on their own.

We also learned from our study that a Teff→Treg boost was observed when both Treg and Teff cells were activated by their cognate Ag, even when they were specific to different Ags. However, this phenomenon required that both cell types were activated in the same LN. Interestingly, it has been reported that high and systemic activation of Teff cells induced a burst of IL-2 led to an increased proliferation of recipient polyclonal Treg cells (8). This Treg cell boost was IL-2–dependent and comparable to the one obtained after systemic administration of high-dose IL-2 (31, 32). A systemic activation of endogenous Treg cells was not observed in our various immune conditions likely because only low numbers of Treg cells were activated due to local immunization. Thus, a Teff→Treg boost was observed when both Treg and Teff cells were activated in the same LN by their cognate Ag, with the latter being either the same or different.

**Molecular mechanisms of the Teff→Treg boost**

We showed in the present study that molecular mechanisms involved in the Teff→Treg boost vary depending on the immune context. In the ins-HA mouse setting, we identified a role of TNF and OX40L, whereas in the WT mouse setting, we identified a role of TNF and IL-2. This could result from the fact that environmental cues, such as signals from the tissues or various inflammatory factors, affect Treg cell biology, giving rise to distinct Treg cell subsets (4). Thus, different factors may be involved in the survival and proliferation of Treg cells depending on their environment, as recently shown for Treg cells of lymphoid and non-lymphoid tissues (29). The role of IL-2 was expected because this cytokine plays a critical role in survival and proliferation of Treg cells (9, 18, 32). The role of TNF in the Teff→Treg boost was previously described in the ins-HA mouse setting (7). Our data are in line with the ones of Oppenheim and colleagues (19, 20), which showed a direct effect of TNF on Treg cells via its TNFR2, inducing an increase of their proliferation and survival. Regarding OX40, it has been shown that OX40 agonist administration in-

**FIGURE 6.** The Teff→Treg boost is OX40-dependent in the low inflammatory condition. (A–D) WT mice were injected with CFSE-labeled HA126–Treg cells alone or coinjected with HA111–Teff cells and immunized with CFA/HA126+111. In some mice, OX40 was inhibited using an anti-OX40L mAb. Cells were analyzed at day 4 in pop-LN. (E–H) Ins-HA mice were injected with CFSE-labeled Thy-1.1+ HA111–Treg cells alone or with HA111–Teff cells with or without blocking OX40 (anti-OX40L). Cells were analyzed at day 7 in pancreatic LN. (A and E) Representative CFSE profile of donor Treg cells (CD4+Thy-1.1+Foxp3+). (B and F) Absolute number of divided donor Treg cells (CFSEdimCD4+Thy-1.1+Foxp3+). (C and G) Representative CFSE profile of donor Teff cells (CD4+6.5+Foxp3–). (D and H) Absolute number of divided donor Teff cells (CFSEdimCD4+6.5+Foxp3–). Each symbol represents an individual mouse, and bars show the means pooled from three to four independent experiments. *p < 0.05, **p < 0.001. n.s., not significant.
FIGURE 7. pDC are involved in the Teff–Treg boost in the low inflammatory condition. (A–E) WT mice were transferred with CFSE-labeled HA126–Treg cells alone or coinjected with HA111–Teff cells and immunized with CFA/HA126+111. Some mice were daily injected with a mAb depleting pDC (anti-pDC). Cells were analyzed in pop-LN at day 4. (F–J) Ins-HA mice were injected with CFSE-labeled Thy-1.1+ HA111–Treg cells alone or with HA111–Teff cells with or without a daily treatment depleting pDC (anti-pDC). Some mice were daily injected with a mAb depleting pDC (anti-pDC). Cells were analyzed in the pancreatic LN at day 7. (A and F) pDC depletion was assessed by quantification of Siglec H+ cells among CD45+ cells in mice cotransferred with Treg and Teff cells. (B and G) Representative CFSE profile of donor Treg cells (CD4+Thy-1.1+Foxp3+). (C and H) Absolute number of divided donor Treg cells (CFSEdimThy1.1+Foxp3+CD4+). (D and I) CFSE profile of donor Teff cells (CD4+6.5+Foxp32). (E and J) Absolute number of divided donor Teff cells (CFSEdimCD4+6.5+Foxp32). Each symbol represents an individual mouse, and bars show the means from four to five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
duced an increase of Treg cell proliferation and that OX40-deficient Treg cells had reduced survival capacity in inflamed tissues (33, 34). Interestingly, blocking both TNF and OX40L in ins-HA mice did not fully inhibit the Teff → Treg boost (data not shown), suggesting that other molecules were involved. Some of them may belong to the TNFR family, such as death receptor 3, death receptor 5, or glucocorticoid-induced TNFR family–related protein because these molecules are able to promote Treg cell expansion (35–39). Thus, molecular mechanisms of the Teff → Treg boost are diverse and differ depending on the tissue and the type of inflammation.

Role of pDC in the Teff → Treg boost

Part of the Teff → Treg boost may be due to a direct effect of Teff cells on Treg cells. IL-2 or TNF, which are both produced by activated Teff cells at high levels, may directly boost Treg cells that express at a high level the high-affinity IL-2 receptor and TNFR2. In support of this mechanism, these two cytokines are able to directly promote survival and proliferation of Treg cells (35–39). Thus, molecular mechanisms of the Teff → Treg boost are diverse and differ depending on the tissue and the type of inflammation.

FIGURE 8. Phenotypic characterization of Treg cells boosted in the high and low inflammatory conditions. Ins-HA mice were coinjected with CFSE-labeled Thy-1.1+ HA111–Treg cells and HA111–Teff cells (A) and WT mice were coinjected with CFSE-labeled Thy-1.1+ HA126–Treg cells and HA111–Teff cells and immunized with CFA/HA126+111. (B) Expressions of different molecules by donor CFSEhigh and CFSElow Treg cells (CD4+Foxp3+Thy-1.1+) as well as recipient cTreg cells (defined by CD4+Foxp3+CD62LhighCD44low expression) and eTreg cells (defined by CD4+Foxp3+CD62LlowCD44high expression) were analyzed in pancreatic draining LN (D-LN) 7 d later (A) and in draining and nondraining LN (ND-LN) 5 d later (B). CFSE profiles (upper panels) and mean fluorescent intensities (MFI, lower panels) are shown. Each symbol represents an individual mouse, and bars show the means from two to three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

eTreg and cTreg cells

In the low inflammatory context, boosted Treg cells resemble cTreg cells that were recently described (29) because the two Treg cell types expressed a similar phenotype and were not dependent on IL-2 for their proliferation. In the high inflammatory condition, boosted Treg cells exhibited features of cTreg cells based on their high expression of CD62L and their IL-2 dependency. However, at the same time, they had clearly an eTreg cell phenotype considering the expression of CD44, ICOS, CCR7, and CXCR3. Importantly, the cTreg/eTreg cell dichotomy was initially defined at steady-state (29). When inflammation was induced by injection of LPS or CFA, cTreg cells acquired characteristic of eTreg cells (29). A similar switch may occur in our high inflammatory condition because boosted Treg cells, which depended on IL-2 for their proliferation, acquired characteristics of eTreg cells, confirming the plasticity of cTreg and eTreg cell populations.

Physiological implications

Importantly, the Treg cells boosted in the low and high inflammatory conditions were suppressive. Compared to mice that received Treg cells alone, the cotransfer of Teff cells even generated a more suppressive environment in draining LN. Thus, we may envisage physiological roles and implications of the Teff → Treg boost phenomenon. The Treg cell boost observed in the low in-
flammary and autoreactive context, illustrated in the present study in ins-HA mice, may take place at the beginning of an autoimmune process or during chronic mild inflammation. In this situation, low background levels of Teff cell activation may maintain relatively high numbers of autoreactive Treg cells due to a Teff → Treg boost, perpetuating stable and low-grade inflammation. Immunopathology. Indexing the level of Treg cell activation to the high risk of tissue damage. This is why a Teff inflammation may not be appropriate to stimulate Treg cells, raising Treg cell survival or expansion (19, 20, 36–38, 48, 49). This situation may be related to high inflammatory conditions in which we observed high Treg cell expansion even without further help from Teff cells, illustrated in this study in conditions 5, 6, and 7 in Fig. 2. In other high inflammatory contexts, the nature of inflammation may not be appropriate to stimulate Treg cells, raising a high risk of tissue damage. This is why a Teff → Treg boost would be crucial to increase their numbers, as observed in the present study (conditions 1, 2, and 8 in Fig. 2). A too weak or dysfunctional Teff → Treg boost may favor the development of an immunopathology. Indexing the level of Treg cell activation to the level of Teff cell activation may be a feedback regulatory mechanism to limit sustained pathogenic inflammation. Our study may also reveal new therapeutic targets aiming to enhance Treg cell activity and control inflammatory and autoimmune responses.

Acknowledgments

We thank Christelle Enond, Flora Issert, Olivier Bregerie, and Bocar Kane, all from the Centre d’Exploration Fonctionnelle, Université Pierre et Marie Curie in Paris, for expert care of the mouse colony. We are grateful to Bertrand Dubois and Bart Lambrecht for providing the mAb to deplete pDC.

Disclosures

The authors have no financial conflicts of interest.

References


