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

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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: 999–1010.

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 feedforward 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 homeostasis 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).

Materials and Methods

Mice

All mice used in this study were on a BALB/cBy background. Wild-type (WT) mice were obtained from Charles River Laboratories France or Janvier. Ins-HA transgenic mice expressed the hemagglutinin (HA) of influenza virus under the control of the insulin promoter (ins-HA mice) (10). The TCR-HA126 and TCR-HA111 mice expressed a transgenic TCR recognizing the HA126 peptide presented by I-Ad (11) and the HA111 peptide presented by I-Eβ (12), respectively. Some of these mice were crossbred with Thy-1.1 congenic mice or with IL-2 knockout mice (13). To obtain IL-2 chimeric mice, WT mice were lethally irradiated and engrafted with a mix of bone marrow cells from IL-2–deficient TCR-HA111 transgenic mice and WT mice at a 1:1 ratio. Experiments were performed at least 6 wk after bone marrow transplantation. Mice were bred in our animal facility under specific pathogen-free conditions in accordance with current European legislation. All protocols were approved by the Regional Ethics Committee.
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 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 at 37˚C and were washed twice in PBS. Mice received 1 × 106 Treg cells with or without 1 × 106 Teff cells or 1 ×106 Teff cells alone except in Fig. 3, for which some mice were injected with 10 × 106 or 30 ×106 Teff cells, and in Fig. 4, for which mice received 2 ×106 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 (XRT31.11; Bio X Cell) at days −1, 1, and 3 after cell transfer. For blockade of OX40, mice received 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 5 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 i.p. 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 (200 μg, PfGmbH) treatments, mice were injected in the rear footpads 4 h before sacrifice and then the draining popliteal LN (pop-LN) was analyzed to determine treatments, mice were injected in the rear footpads 4 h before sacrifice and then the draining popliteal LN (pop-LN) was analyzed to determine treatments, mice were injected in the rear footpads 4 h before sacrifice and then the draining popliteal LN (pop-LN) was analyzed to determine .

Abs and flow cytometry analysis

The following mAbs from BD Biosciences, eBioscience, and BioLegend were used: allophycocyanin-PerCP-Cy5.5–labeled anti-CD4, PE– or PerCP-labeled anti-Thy-1.1, PE–Cy7– or PerCP-labeled anti-CD25, allophycocyanin-labeled anti–ICOS, PE–labeled anti-Siglec H, Alexa Fluor 700–labeled anti–CD45, PE–labeled anti–IL-2, PE–labeled anti–TNFR1, allophycocyanin-Alexa Fluor 750–labeled anti–CD62L, PE–Cy7– or PE–labeled anti–CD44, PE–Cy7– or Alexa Fluor 700–labeled anti–CCR7, PE–labeled anti–CXCR3, and Alexa Fluor 647–labeled anti–p–STAT5. The 6.5 anti-clonotypic mAb is specific to the TCR expressed by the HA111+ T cells. It was revealed by a biotin-labeled anti-rat IgG2b (SouthernBiotech) followed by streptavidin–allophycocyanin (BD Biosciences) staining. Alternatively, we used PE–labeled 6.5 mAb. The PE–labeled anti–Foxp3 staining was performed using the eBioscience kit and protocol. For intracellular IL-2 staining, cells were restimulated by HA111–pulsed splenocytes for 6 h in the presence of GolgiPlug (BD Biosciences). After cell surface staining, intracellular staining was performed using the Cytofix/Cytoperm kit and protocol (BD Biosciences). Detection of p–STAT5 was performed as previously described (15). Cells were acquired on a FACSCalibur or an LSR II (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or FlowJo (Tree Star) software.

Statistics

Statistical data were calculated using the two-tailed unpaired Mann–Whitney test. A p value <0.05 was considered significant. For all graphs, error bars represent SD.

Results

Teff cells enhanced Treg cell proliferation in high inflammatory condition

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, adoptively 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 Treg cells and HA111 presented by MHC class II E8 for Teff 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/HA111) induced a significant increase of HA126–Treg cell proliferation and expansion, compared with mice transferr 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 coinjected with HA111–Teff cells, or they were left un.injected (controls). Some control mice were not injected. Then mice were immunized with CFA/ HA126/HA111. 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–Teff cells (Fig. 1C, 1D). The level of suppression was even higher in the latter compared with the former group of mice. Altogether, T eff 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 boost cell 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 transferred with HA126–Treg cells and immunized with HA126–pulsed DC (DC/HA126) or in mice transferred 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–Teff 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 HA26–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 HA26–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-HA 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 T cell 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 lymphoproliferative disease (16), one may argue that the function of IL-2–deficient T 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+Thy-1.1+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+Thy-1.1+Foxp3+) (C) and absolute number of divided donor Treg cells (CFSEdimCD4+Thy-1.1+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).
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→Treg boost observed in the low inflammatory context.

pDC are involved in the Teff→Treg boost in low inflammatory condition

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. 7I, 7J) was associated with a significant decrease of the Teff→Treg boost observed in the low 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 cTreg 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 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 (8, 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 HA_{126}→Treg cells alone or coinfected with HA_{111}→Teff cells and immunized with CFA/HA_{126+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^{+} HA_{111}→Treg cells alone or with HA_{111}→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^{+}CD4^{+}). (C and H) Absolute number of divided donor Treg cells (CFSE_{dimThy1.1^{+}Foxp3^{+}CD4^{+}}). (D and I) CFSE profile of donor Teff cells (CD4^{+}6.5^{+}Foxp3^{2}). (E and J) Absolute number of divided donor Teff cells (CFSE_{dimCD4^{+}6.5^{+}Foxp3^{2}}). 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/\rightarrow 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/\rightarrow Treg boost are diverse and differ depending on the tissue and the type of inflammation.

Role of pDC in the Teff/\rightarrow Treg boost

Part of the Teff/\rightarrow 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 (9, 18–20). Alternatively and not exclusively, Teff cells may modulate or activate another cell population that itself boosts Treg cells. In the present study, we showed that pDC play a role in this indirect Teff/\rightarrow Treg boost. This result is interesting with regard to the known tolerogenic properties of pDC, partly due to their capacity to induce or activate Treg cells (40). Indeed, mature pDC and pDC localized in inflamed tumors, inflamed LN, or the lamina propria were able to promote a high level of Treg cell expansion (26, 27, 41, 42). It will be interesting to assess whether the acquired capacity of pDC to activate Treg cells in these latter settings relies on Teff cell activation and involves similar mechanisms as in the Teff/\rightarrow Treg boost that we describe in the present study.

eTreg and cTreg cells

In the low inflammatory context, boosted Treg cells resemble eTreg 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/\rightarrow Treg boost phenomenon. The Treg cell boost observed in the low in-
flammary and autoimmune 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. During acute inflammation or relapse of an autoimmune disease, high levels of inflammatory factors and pathogenic Teff cells may severely damage tissue integrity, especially as inflammation may transiently inhibit Treg cell function or stability (43). However, after few days, Treg cells may actively expand, explaining why they are found in high numbers in various inflammatory contexts (44–47), limiting tissue damage. Such Treg cell expansion may be due to an increased release of self-reactive Ags activating autoreactive Treg cells or to the presence of cytokines or costimulation molecules that are able to promote 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 of Treg cell survival or expansion (19, 20, 36–38, 48, 49). This situation may also reveal new therapeutic targets aiming to enhance Treg cell activity and control inflammatory and autoimmune responses.

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Disclosures

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References


**Supplemental Figure 1. TNF does not activate the STAT5 pathway in Treg cells.** (A) WT mice were treated with PBS, IL-2 or TNF at day 0, 2 and 4 and expression of CD25 and TNFR2 by Treg cells (CD4 Foxp3) was analyzed in the draining LN at day 6. (B) Four hours after injection of PBS, IL-2, TNF or the 2 cytokines in WT mice, the proportion of pSTAT5+ cells among Treg cells (CD4 Foxp3) was determined in the draining LN. (C) WT mice were co-injected with CFSE-labeled HA126 Treg cells and HA111 Teff cells and immunized with CFA/HA126+111 and then were treated or not with anti-TNF drugs (TNFR-FC) or anti-IL-2 neutralizing antibody. Proportion of pSTAT5+ cells among donor Treg cells (Thy1.1 CD4 Foxp3) was determined in the draining LN at day 4. (A, B, C) Representative (left panels) and cumulative (right panels) data from 2 or 3 independent experiments were depicted. Each symbol is an individual mouse.