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Regulatory T Cell Suppression and Anergy Are Differentially Regulated by Proinflammatory Cytokines Produced by TLR-Activated Dendritic Cells

Takekazu Kubo,*$ Robin D. Hatton,* James Oliver,* Xiaofen Liu,* Charles O. Elson,† and Casey T. Weaver2*‡

CD25⁺CD4⁺ regulatory T cells (Tregs) are required for the maintenance of peripheral tolerance to certain self Ags. In this study, the requirements for murine Treg-suppressive activity and proliferation were examined in the context of the maturation of myeloid dendritic cells (DCs). We find that the suppressive function of Tregs is critically dependent on immature DCs and is readily reversed by the maturation of DCs induced by GM-CSF, but does not require TLR activation of either DCs or Tregs. In contrast, reversal of Treg anergy is dependent on TLR activation of DCs, and involves the potentiation of Treg responsiveness to IL-2 by cooperative effects of IL-6 and IL-1, both of which are produced by TLR-activated, mature DCs. Thus, proinflammatory cytokines produced by TLR-activated, mature DCs are required for reversal of Treg anergy, but are not required to overcome Treg suppression. The Journal of Immunology, 2004, 173: 7249–7258.

The CD25⁺CD4⁺ regulatory T cells (Tregs)³ comprise 5–10% of the circulating CD4⁺ T cell population, and are required for the maintenance of self-tolerance. A deficiency of CD25⁺CD4⁺ Tregs that results from neonatal thymectomy or spontaneous and induced mutations that prevent their development leads to multiorgan autoimmune disease (1–7). The adoptive transfer of CD25⁺CD4⁺ Tregs can prevent the development of autoimmune reactions in several mouse models, establishing a dominance of these cells in suppressing normally self-reactive specificities (7–12). CD25⁺CD4⁺ Tregs develop during positive selection in the thymus through recognition of tissue-specific, self Ags (13–16). Insights into the molecular mechanisms that control the development of CD25⁺CD4⁺ Tregs have been gained through studies that linked an aggressive autoimmune syndrome in humans, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), or XLAAAD (X-linked autoimmune-allergic dysregulation syndrome) (17, 18), with defects in the gene that encodes the Foxp3 transcription factor (5, 19). Mice with spontaneous or induced mutations of the Foxp3 gene that impair Foxp3 expression fail to develop CD25⁺CD4⁺ Tregs and exhibit a syndrome with similarities to the human disease (4, 5), implicating a central role for this factor in CD25⁺CD4⁺ Treg development.

CD25⁺CD4⁺ Tregs are so-called because they constitutively express CD25, the high affinity component of the IL-2R complex (IL-2R α-chain) (1, 8, 9). CD25 serves not only as a marker for this subset, but is also required for its regulatory function (8, 9, 20). These cells also constitutively express the inhibitory receptor CTLA-4, signaling through which appears to be required for their suppressive activity (7, 21). A principal characteristic of CD25⁺CD4⁺ Tregs is that they are anergic to TCR stimulation ex vivo, and are able to render responder T cells anergic through their suppression of IL-2 production and/or availability (22, 23). Exogenous IL-2 breaks the anergic phenotype of CD25⁺CD4⁺ Tregs and reverses their suppressive activity (22), although it may also be required to activate Treg function (24). The suppressive activity of Tregs can also be blocked by signaling through the glucocorticoid-induced TNF receptor (GITR), through a mechanism not yet defined (25, 26). Furthermore, TLR stimulation of dendritic cells (DCs) has been reported to reverse the suppressive activity of CD25⁺CD4⁺ Tregs, acting via IL-6 expressed by DCs (27). Accordingly, mature DCs can induce Treg proliferation (28). Collectively, these studies indicate that multiple mechanisms may exist whereby activation of cells of the innate immune system can override the activity of CD25⁺CD4⁺ Tregs, thus permitting adaptive immune responses to antimicrobial Ags to supersed the suppressive response to self Ags.

Although the foregoing studies have identified mechanisms by which the suppressive activity of Tregs might be overcome, the precise relationship of Treg activity to DC maturation and TLR-induced activation remains incompletely defined. In this study, we examine the suppressive and proliferative activities of CD25⁺CD4⁺ Tregs along a continuum of maturation and TLR-induced activation of bone marrow-derived myeloid DCs to better define the characteristics of DCs that either promote or impede the activity of this regulatory subset. In agreement with reports by others (28), we find that Treg activity is contingent upon the maturation and activation state of DCs, progressing along a continuum from dominant suppression of naive CD4⁺ T cells in the context of immature DCs, to reversal of suppression that is accompanied by active proliferation in the context of mature, LPS-stimulated DCs. Importantly, however, the reversal of Treg suppression and Treg anergy...
can be dissociated contingent upon the TLR-induced activation of DCs. Thus, mature, but non-TLR-activated DCs, reverse the suppressive activity of Tregs without reversing their anergic state, whereas TLR-induced DC activation is required to recruit robust proliferation of Tregs. The production of the proinflammatory cytokines, IL-6 and IL-1, by TLR-activated DCs plays an important role in reversing Treg anergy by potentiating responsiveness to IL-2, but does not appear to be required for reversing Treg-suppressive function.

**Materials and Methods**

**Mice**  
BALB/c mice and TLR4 mutant mice on the BALB/c background (C3-Tlr4−/−) were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our specific pathogen-free facility, and were used at 6–10 wk of age. All mice were housed and treated according to National Institutes of Health guidelines under the auspices of the UAB Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Abs and reagents**  
Purified neutralizing anti-IL-2 (S4B6), anti-IL-6 (MP5-20F3), purified blocking or FITC-labeled anti-CD86 (HL3), biotin-conjugated anti-CD25 (7D4), anti-CD69 (H1.2F3), anti-CD80 (16-10A1), and anti-I-Ad (AMS-32.1) mAbs and allophycocyanin-conjugated anti-CD4 mAb (LE2) or streptavidin were purchased from BD Pharmingen (San Diego, CA). Purified neutralizing anti-IL-1α (161.1) and anti-IL-1β (B122.9.9.26) Abs were kindly provided by D. Chaplin (University of Alabama). Anti-CD3 mAb (145-2C11) was purified from ascites by R. Lallone (Brookwood Biotech, Birmingham, AL). IL-2, IL-1, and IL-6 were purchased from R&D Systems (Minneapolis, MN). GM-CSF was purchased from Sigma-Aldrich (St. Louis, MO). The CD25 micro bead kit, which includes PE anti-CD25 mAb (7D4) and magnetic microbead-conjugated anti-PE mAb, was obtained from Miltenyi Biotec (Auburn, CA). CFSE was obtained from Molecular Probes (Eugene, OR).

**Purification of CD25+CD4+ Tregs and bone marrow-derived DCs (BMDCs)**  
CD4+ T cells were purified from spleen and lymph nodes of BALB/c mice by positive sorting using anti-CD4 magnetic beads (Dynal Biotech, Lake Success, NY). In the course of CD4+ T cell purification, CD4+ T cell-depleted splenic and lymph node cells (SPL) were isolated in a negative fraction for use as APCs. CD25+CD4+ Tregs were isolated from CD4+ T cells by magnetic sorting on CD25-conjugated beads, according to the manufacturer’s protocol (Miltenyi Biotec), and were 95–98% pure by flow cytometric analysis. CD25+CD4+ responder T cells were obtained as a negative fraction during the course of the CD25+CD4+ purification and were typically 90–95% pure. We found that, compared with flow cytometric cell sorting, magnetic sorting yielded two to three times as many CD25+CD4+ T cells with no compromise of functional activity (data not shown).

BMDCs were prepared by culture with GM-CSF (20 ng/ml; Sigma-Aldrich), following the protocol of Lutz et al. (30), and were used 5–6 days after incubation. In indicated experiments, BMDCs cultured for 10–11 days were treated with 1 μg/ml LDS (Sigma-Aldrich) 24 h before harvest for further activation (day 10–11 BMDC/LPS).

**Functional assays of CD25+CD4+ Tregs**  
For proliferation assays, 5 × 104 CD25+CD4+ responder T cells were seeded into each well of round 96-well polypropylene plates with or without 5 × 104 CD25+CD4+ Tregs in RPMI 1640 medium supplemented with nonessential amino acid, penicillin, streptomycin, glutamine, sodium pyruvate, 2-ME, and 10% heat-inactivated FBS. After irradiation with 3000 rad, 4 × 105 S11, or 1 × 105 day 5–6 BMDC, day 10–11 BMDC, or day 10–11 BMDC/LPS were included in the cultures as APCs, and anti-CD3 (2.5 μg/ml) was added as a TCR stimulator. After incubation for 72 h, each culture was pulsed with 1 μCi of [3H]thyminde (Tdr) for 16–18 h to assess proliferative activity. For CFSE labeling, CD25+CD4+ responder T cells or CD25−CD4+ Tregs were washed twice with PBS and suspended at 1 × 106 cells/ml in PBS containing 5 μM CFSE. After 7 min at room temperature, the staining was stopped by washing the cells with RPMI 1640 containing 10% FBS. CFSE-labeled CD25+CD4+ responder T cells (3 × 105) were cultured in 48-well plates with or without 3 × 105 CFSE-unlabeled CD25−CD4+ Tregs, or vice versa, in the presence of 5 μg/ml anti-CD3 and irradiated 2 × 105 S11, 1 × 105 day 5 BMDC, 1 × 105 day 11 BMDC, or 1 × 105 day 11 BMDC/LPS in each well. After 72 or 96 h, cells were collected, stained with appropriate mAbs, and analyzed by flow cytometry. To determine the suppressive function of CD25+CD4+ Tregs expanded by day 10 BMDC with IL-6 or day 10 BMDC/LPS, 1 × 105 CD25+CD4+ T cells were maintained with the 5 × 105 irradiated DCs in the presence of 5 μg/ml anti-CD3 in 24-well plates for 6 days, and viable CD25+CD4+ T cells were collected by centrifugation on density gradients (Lympholyte-M; Cedarlane Laboratories, Hornby, Canada). The collected CD25+CD4+ T cells were then cultured with freshly isolated CD25+CD4+ responder T cells and SP, in the presence of anti-CD3 to examine the suppressive effects, as described.

**Flow cytometric analysis**  
Cells were stained for flow cytometry with FITC-, PE-, or biotin-conjugated mAbs indicated in the appropriate figure legends, as described (31). Cells labeled with biotinylated primary Ab were detected with allophycocyanin-conjugated streptavidin. For analytical flow cytometry, at least 10,000 events with forward and side scatter properties of lymphocytes were collected on a BD Biosciences FACSCalibur and analyzed using CellQuest software (BD Biosciences, San Jose, CA).

**Cytokine ELISA**  
Secreted IL-2 was quantified by capture ELISA, as previously described (32). Secreted IL-1α, IL-1β, and IL-6 in culture supernatants were measured with an immunoassay kit from R&D Systems and BioSource International (Camarillo, CA), respectively, following the manufacturer’s protocol.

**Statistical analyses**  
Statistical significance was calculated by unpaired Student’s t test or Mann-Whitney U test using Prism software (GraphPad, San Diego, CA). All p values ≤0.05 are considered significant.

**Results**  
**BMDCs derived from short-term culture with GM-CSF support suppression by CD25+CD4+ Tregs, whereas BMDCs derived by long-term culture do not**

To identify the APC requirements for Treg activity, BMDCs were used to examine Treg function. BMDCs were prepared by culture in GM-CSF for 5–11 days, with or without activation by LPS during the last 24 h of culture (Fig. 1). BMDCs produced by short-term culture (5 days; termed day 5 BMDC) were comparable to spleen/lymph node-derived DCs in supporting the suppressive activity of CD25+CD4+ Tregs (Fig. 1A, and data not shown). In contrast, BMDCs derived by longer-term culture with GM-CSF (11 days; day 11 BMDC) did not support suppression by Tregs, even at a 1:1 ratio of CD25− and CD25+CD4+ T cells (Figs. 1A and 2A). Furthermore, day 11 BMDCs activated with LPS (day 11 BMDC + LPS) not only supported the reversal of proliferative suppression, but also induced significantly augmented proliferation. Comparable results were obtained using BMDCs derived from day 6 and day 10 cultures, respectively (Fig. 3 and data not shown). Thus, BMDCs derived by short-term culture (day 5–6 BMDCs) were permissive for suppression by CD25+CD4+ Tregs, whereas BMDCs derived by long-term culture (day 10–11 BMDCs) were not. Activation of day 10–11 BMDCs by a TLR ligand (LPS) further enhanced T cell proliferation, suggesting either augmented responses of the CD25+CD4+ responders and/or recruitment of CD25+CD4+ Treg proliferation.

As shown in Fig. 1B, day 6 BMDCs exhibited a heterogeneous maturation phenotype indicated by variable expression of CD11c and MHC class II, as did BMDCs derived by culture for 5 days (data not shown). These populations also included a small fraction of contaminating non-DC myeloid cells (30) (data not shown). CD80 (B7-1) was expressed at low levels by a fraction of day 5 and day 6 CD11c+ DCs (~50%), whereas expression of CD86 (B7-2) was more limited (<20% of CD11c+ DCs). In contrast,
80% of day 10–11 BMDCs expressed both CD11c and MHC class II; 50% were CD80 positive and 30% CD86 positive. Pretreatment with LPS induced heightened and more uniform expression of MHC class II, CD80, and CD86 by day 10–11 BMDCs. Collectively, these results indicate that the day 5–6 BMDC population includes primarily immature DCs with some contaminating granulocytes and myeloid precursors (Fig. 1, and data not shown); day 10–11 BMDCs contain DCs with an intermediate phenotype (increased expression of CD80 and MHC class II with some CD86-positive cells); and LPS-pretreated day 10–11 BMDCs are mature DCs (30).

LPS activation of BMDCs promotes the reversal of anergy of CD25+CD4+ Tregs

The enhanced proliferation induced by LPS-activated BMDCs in cultures containing CD25+CD4+ T responders and CD25+CD4+ Tregs suggested possible recruitment of Treg proliferation by this APC population. The proliferative responses of CD25+CD4+ Tregs induced by different DCs were therefore determined directly, either in the absence or presence of CD25+CD4+ responders (Fig. 2). In agreement with previous reports (22, 23), Tregs were anergic to anti-CD3 stimulation by splenic APCs (Fig. 2A).

FIGURE 1. CD25+CD4+ Treg function is contingent upon DC maturation. A, Isolated CD25+CD4+ responder T cells (5 × 10⁴) were cultured with or without CD25+CD4+ Tregs (5 × 10⁴) in 96-well plates in the presence of anti-CD3 (2.5 µg/ml) and BMDCs derived by culture with GM-CSF (20 ng/ml) for 5 or 11 days in vitro (day 5 BMDC, 1 × 10⁴, or day 11 BMDC, 1 × 10⁵, respectively) or day 11 BMDC pretreated with LPS (1 µg/ml) for 24 h (day 11 BMDC + LPS, 1 × 10⁵). All of the APC populations were irradiated at 3000 rad before cultivation. After incubation for 3 days, [³H]thymidine incorporation was assessed after cultivation for additional 16–18 h. Data represent means ± SEM (n = 3). B, Surface phenotype of day 5 BMDCs was defined by staining the cells with PE anti-CD11c, biotin anti-I-A^d, biotin anti-CD80, and FITC anti-CD86 mAbs. Top panel, day 5 BMDCs; middle panel, day 11 BMDCs; bottom panel, LPS-activated day 11 BMDCs.

FIGURE 2. Reversal of Treg anergy by LPS-activated BMDCs; differential recruitment of Treg and T responder cell division by distinct DC populations. A, CD25+CD4+ Tregs (5 × 10⁴) were stimulated in 96-well plates with anti-CD3 (2.5 µg/ml) presented by irradiated SPL (4 × 10⁴), day 5 BMDCs (1 × 10⁴), day 11 BMDCs (1 × 10⁴), or LPS-activated day 11 BMDCs (1 × 10⁴) for 3 days. Proliferation was determined, as described in Fig. 1. Data represent means ± SEM (n = 3–4). B, CFSE-labeled CD25+CD4+ T responders (3 × 10⁵) or CD25+CD4+ Tregs were cultured with or without CFSE-unlabeled CD25+CD4+ Tregs or CD25+CD4+ T responders, respectively, in the presence of anti-CD3 (5 µg/ml) and irradiated day 11 BMDC (1 × 10⁵) or LPS-activated day 11 BMDCs (1 × 10⁴) in 48-well plates. BMDCs and unlabeled T cell populations were derived from CD45.1 congenic donors to permit gating only on CD45.2 CFSE-labeled T cells. After incubation for 4 days, flow cytometry was performed to examine the dilution of CFSE to assess cell division in each labeled T cell population.
Similarly, both day 5 BMDCs and day 11 BMDCs induced only limited proliferation of Tregs. In contrast, LPS-activated BMDCs supported markedly enhanced proliferation of Tregs, suggesting that TLR activation was required for reversal of Treg anergy.

Flow cytometric analysis of CFSE-labeled T cell populations was used to further examine the responses of Tregs and T responders, either separately or in cocultures (Fig. 2B). In agreement with the studies shown in Fig. 1, Tregs stimulated by day 10 BMDCs underwent limited cell division, both in the absence and presence of T responders, while the latter divided well despite the presence of Tregs. In contrast, Tregs stimulated by LPS-activated BMDCs were recruited to markedly increased cell divisions in the absence of T responders, which were further augmented by coculture with CD25+CD4+ responders. Thus, Tregs divide poorly even in the face of reversal of their suppressive effect on CD25+CD4+ T responders by day 10 BMDCs, but are recruited to active cell division by LPS-activated BMDCs.

Reversal of Treg suppression by BMDCs does not require TLR signaling

It was recently reported that DCs activated via TLR signaling could overcome suppression by CD25+CD4+ T cells (27). However, our observation that Treg suppression was reversed by day 10–11 BMDCs that were not activated by exogenous LPS suggested that TLR signaling might not be required for reversal of Treg suppression. Nevertheless, we could not exclude the possibility that contaminating endotoxins might activate day 10–11 BMDCs even in the absence of exogenous addition of LPS. To address this, Treg function was examined using BMDCs derived from TLR4-deficient mice, which are not activated by endotoxins.
PRESSIVE AND PROLIFERATIVE ACTIVITY IN RESPONSE TO LPS (33). BECAUSE INCLUDING TLR4, AND HAVE BEEN REPORTED TO EXHIBIT ENHANCED SUPPRESSION, CD4+ T CELLS (34). THIS CONFIRMS THAT TREG PROLIFERATION PROMOTED BY IL-6 AND OTHER PROINFLAMMATORY CYTOKINES IN OUR SYSTEM. SIGNIFICANT AMOUNTS OF IL-6, IL-1α, AND IL-1β WERE DETECTED IN ALL CULTURES CONTAINING LPS-ACTIVATED BMDCS, AND CYTOKINE LEVELS WERE SIMILAR INDEPENDENT OF THE T CELL POPULATIONS PRESENT OR ANTI-CD3 STIMULATION (TABLE I). IMPORTANTLY, BOTH DAY 6 AND 11 BMDCS PRODUCED UNDETECTABLE OR BARELY DETECTABLE LEVELS OF IL-6 OR IL-1 UNDER ALL CONDITIONS, DESPITE THE FUNCTIONAL REVERSAL OF TREG SUPPRESSION BY THE LATTER. THEREFORE, IT WAS UNLIKELY THAT DAY 10–11 BMDCS USED IL-6 OR IL-1 TO BREAK THE SUPPRESSION OF CD25+ CD4+ T CELLS.

TO FURTHER SUPPORT THIS CONCLUSION, NEUTRALIZING ANTI-IL-6 OR EXOGENOUS IL-6 WAS ADDED TO CULTURES OF DIFFERENT BMDC POPULATIONS AND RESPONDER T CELLS, WITH OR WITHOUT ADDITION OF TREGS. AS SHOWN IN FIG. 4, DAY 11 BMDCS DID NOT SUPPORT SUPPRESSION OF TREGS IN THE PRESENCE OF NEUTRALIZING AB TO IL-6, INDICATING THAT IL-6 PRODUCTION BY DAY 10–11 BMDCS WAS NOT INVOLVED IN THE REVERSAL OF TREG SUPPRESSION BY THIS APC POPULATION. FURTHERMORE, ADDITION OF EXOGENOUS IL-6 DID NOT REVERSE THE SUPPRESSION SUPPORTED BY DAY 6 BMDCS, INDICATING THAT IL-6 COULD NOT REVERSE THE SUPPRESSIVE ACTIVITY OF TREGS SUPPORTED BY THIS APC POPULATION. SIMILAR RESULTS WERE FOUND USING NEUTRALIZING ABS TO IL-1α AND IL-1β (DATA NOT SHOWN). THUS, NEITHER IL-6 NOR IL-1 APPEARED TO FUNCTION IN THE REVERSAL OF TREG SUPPRESSION.

Reversal of Treg suppression by day 10–11 BMDCs is IL-6 and IL-1 independent

Although it was recently reported that DCs stimulated via Toll-like pathways blocked the suppression of Tregs by secreting IL-6 and an unidentified factor (27), our data indicated that TLR activation was not required for blockade of Treg suppression. We therefore examined the involvement of IL-6 and other proinflammatory cytokines in our system. Significant amounts of IL-6, IL-1α, and IL-1β were detected in all cultures containing LPS-activated BMDCs, and cytokine levels were similar independent of the T cell populations present or anti-CD3 stimulation (Table I). Importantly, both day 6 and 11 BMDCs produced undetectable or barely detectable levels of IL-6 or IL-1 under all conditions, despite the functional reversal of Treg suppression by the latter. Therefore, it was unlikely that day 10–11 BMDCs used IL-6 or IL-1 to break the suppression of CD25+ CD4+ T cells.

To further support this conclusion, neutralizing anti-IL-6 or exogenous IL-6 was added to cultures of different BMDC populations and responder T cells, with or without addition of Tregs. As shown in Fig. 4, day 11 BMDCs did not support suppression of Tregs in the presence of neutralizing Ab to IL-6, indicating that IL-6 production by day 10–11 BMDCs was not involved in the reversal of Treg suppression by this APC population. Furthermore, addition of exogenous IL-6 did not reverse the suppression supported by day 6 BMDCs, indicating that IL-6 could not reverse the suppressive activity of Tregs supported by this APC population. Similar results were found using neutralizing Abs to IL-1α and IL-1β (data not shown). Thus, neither IL-6 nor IL-1 appeared to

(Fig. 3, and data not shown). In agreement with the results in Fig. 1, day 6 BMDC from TLR4-deficient mice supported the suppressive function of Tregs similarly to wild-type (WT) day 6 BMDCs. Furthermore, TLR4-deficient day 10 BMDCs did not support Treg suppression, either in the presence or absence of LPS pretreatment (Fig. 3A). This confirms that in the absence of LPS-induced TLR activation, day 10–11 BMDCs can overcome suppression of CD25+ CD4+ T cells. In contrast to WT BMDCs, TLR4-deficient day 10 BMDCs pretreated with LPS did not induce proliferation of Tregs, confirming that this activity is due to activation of the TLR4 pathway in BMDCs by LPS (Fig. 3B).

Although the foregoing experiments eliminated the possibility that activation of DCs via TLR4 is required to obviate Treg function, they did not preclude a possible role for direct TLR effects on Tregs as a mechanism for modulation of Treg activity. CD25+ CD4+ Tregs have been reported to express several TLRs, including TLR4, and have been reported to exhibit enhanced suppressive and proliferative activity in response to LPS (33). Because the TLR4 mutation does not eliminate LPS binding (34), it was conceivable that LPS could be presented to Tregs following DC pretreatment. This possibility was addressed using CD25+ CD4+ Tregs and CD25+ CD4+ T responders isolated from TLR4-deficient mice. As shown in Fig. 3C, the suppressive activity of TLR4-deficient Tregs was comparable to that of WT Tregs (Figs. 1 and 3A). Thus, TLR4-deficient Tregs suppressed the proliferation of TLR4-deficient responder T cells in the presence of WT day 6 BMDCs, but not in the presence of WT day 10 BMDCs, either with or without LPS pretreatment of the DCs. These results established that LPS actions on the Treg or T responder populations were not responsible for the observed effects. Furthermore, TLR4-deficient Tregs showed comparable proliferative responses to anti-CD3 stimulation by LPS-activated WT day 10 BMDCs as WT T cells (Fig. 3D). This confirms that Treg proliferation promoted by LPS-activated day 10–11 BMDC is indirect, via TLR activation of DCs, and not via direct activation of T cells by TLR signaling.

Table I. Proinflammatory cytokine production by distinct APC populations

<table>
<thead>
<tr>
<th>APCs</th>
<th>T Cells Anti-CD3 Cytokine (pg/ml)</th>
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<tbody>
<tr>
<td>SPL</td>
<td>Day 6 BMDC Day 11 BMDC Day 11 LPS/BMDC</td>
</tr>
<tr>
<td>CD25+CD4+</td>
<td>None IL-6 6 ± 2 3 ± 1 5 ± 4 251 ± 5</td>
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<tr>
<td></td>
<td>IL-1α ND ND ND ND</td>
</tr>
<tr>
<td></td>
<td>IL-1β ND ND ND ND</td>
</tr>
<tr>
<td>CD25+CD4+</td>
<td>5 µg/ml IL-6 28 ± 1 5 ± 0 6 ± 1 289 ± 4</td>
</tr>
<tr>
<td></td>
<td>IL-1α ND 9 ± 6 ND 140 ± 31</td>
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<tr>
<td></td>
<td>IL-1β ND 11 ± 13 ND 42 ± 7</td>
</tr>
<tr>
<td>CD25+CD4+</td>
<td>5 µg/ml IL-6 27 ± 1 5 ± 0 14 ± 0 372 ± 4</td>
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<tr>
<td></td>
<td>IL-1α 3 ± 2 ND 3 ± 3 134 ± 35</td>
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<td></td>
<td>IL-1β ND ND ND 37 ± 7</td>
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<td>5 µg/ml IL-6 38 ± 7 6 ± 1 14 ± 0 374 ± 1</td>
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<tr>
<td></td>
<td>IL-1α ND 6 ± 4 ND 130 ± 29</td>
</tr>
<tr>
<td></td>
<td>IL-1β ND 9 ± 10 ND 40 ± 6</td>
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</table>

*Day 6 BMDCs (×10⁵), day 11 BMDCs (1 × 10⁵), or LPS-activated day 11 BMDCs (1 × 10⁵) were cultured with CD25+ CD4+ T responders (3 × 10⁵), CD25+ CD4+ T cells (3 × 10⁵) or both in the presence of anti-CD3 (5 µg/ml) in 48-well plates. Twenty hours following initiation of cultures, supernatants were collected for measurement of the indicated cytokines by ELISA (see Materials and Methods). Data represent means ± SD (n = 3–4). Detection limits of ELISA assays: IL-6, 2.0 pg/ml; IL-1α, 2.5 pg/ml; IL-1β, 3.0 pg/ml.

ND, Not detected (value below sensitivity of assay).

FIGURE 4. Blockade of Treg suppression is IL-6 independent. CD25+ CD4+ responder cells (5 × 10⁴) and CD25+ CD4+ Tregs (5 × 10⁴) were mixed in 96-well plates with anti-CD3 (2.5 µg/ml) in the presence of day 6 BMDCs (1 × 10⁶), day 11 BMDCs (1 × 10⁶), or LPS-activated day 11 BMDCs (1 × 10⁶). Recombinant IL-6 (25 ng/ml) or anti-IL-6 Ab (10 µg/ml) was included when the cultures were established. After 3 days, proliferation of each culture was assayed (see Fig. 1). Data represent means ± SEM (n = 3).
play a significant role in the disparate capacities of day 5–6 and day 10–11 BMDCs to support or block Treg-suppressive function, respectively. Collectively, these data indicate that production of IL-6 and IL-1 by TLR-activated DCs is not required for reversal of Treg-suppressive function in our system.

**IL-6 and IL-1 act cooperatively to reverse Treg anergy and potentiate responsiveness to IL-2**

Although we found no role for IL-6 or IL-1 in reversing Treg suppression, we did find that the addition of anti-IL-6 significantly inhibited the augmented proliferation contributed by the addition of Tregs to cultures of T responders and LPS-activated BMDCs (Fig. 4), suggesting that the production of IL-6 induced by TLR activation might play a role in the reversal of Treg anergy. We therefore examined the effects of IL-6 on Treg proliferation in the absence of T responders. As shown in Fig. 5A, the addition of exogenous IL-6 did, in fact, reverse the anergic phenotype of Tregs activated by day 11 BMDCs, and to a lesser extent, that of day 6 BMDCs. Interestingly, exogenous IL-6 also augmented the proliferation induced by LPS-activated day 11 BMDCs, reflecting the higher levels of exogenous vs endogenous IL-6 (25 vs ~0.4 ng/ml; Table 1), while anti-IL-6 only modestly inhibited proliferation induced by LPS-activated BMDCs.

Given the modest inhibition of day 11 LPS/BMDC-induced Treg proliferation by anti-IL-6, we examined a possible role for IL-6 and IL-1 as important cofactors for reversal of Treg anergy, they did not address the well-established role for IL-2 in this phenomenon (9). Because Tregs are poor IL-2 producers, one possible mechanism by which IL-6 and IL-1 might reverse Treg anergy is via induction of enhanced IL-2 production by Tregs. We therefore examined the levels of IL-2 in cultures containing different APC populations and either Tregs, T responders, or both (Fig. 6A). As expected, each of the APC populations induced significant levels of IL-2 production by T responders activated with anti-CD3 in the absence of Tregs, although the highest IL-2 levels were induced by LPS-activated BMDCs. Furthermore, Tregs markedly suppressed IL-2 induction by SPL or day 6 BMDCs (7.2- and 5.1-fold, respectively), compared with modest suppression of IL-2 induction by day 11 BMDCs (1.4-fold), and no significant suppression of IL-2 by Tregs in cultures with LPS-activated BMDCs. Importantly, and in agreement with previous studies (9, 22), there was minimal IL-2 produced by Tregs, irrespective of the APC population used for stimulation. Thus, although LPS-activated BMDCs produced significant levels of IL-6 and IL-1 (Table 1), they did not induce enhanced levels of IL-2 by Tregs compared with unactivated BMDCs that did not produce IL-6 or IL-1. Hence, IL-6 and IL-1 do not appear to reverse Treg anergy by inducing Treg production of IL-2.

To explore this further, the proliferation of Tregs induced by unactivated or LPS-activated BMDCs was compared with or without neutralization of endogenous IL-2, or in the presence of exogenous IL-2 (Fig. 6B). In agreement with the lack of requirement for IL-2 in the proliferation of Tregs stimulated by IL-6 and IL-1, neutralization of endogenous IL-2 had little effect on the significant proliferation induced by LPS-activated BMDCs. Notably, however, the addition of relatively high levels of exogenous IL-2 (2.5 ng/ml) strongly augmented Treg proliferation by both unactivated and LPS-activated BMDCs. In contrast, although the anergy of Tregs could be reversed by LPS-activated BMDCs through an IL-2-independent mechanism, high levels of IL-2 strongly augmented Treg proliferation in the context of either TLR-activated or unactivated BMDCs.

**FIGURE 5.** IL-1 and IL-6 act cooperatively to reverse Treg anergy. A, CD25⁺CD4⁺ Tregs (5 × 10⁴) were cultured in 96-well round-bottom plates with anti-CD3 (2.5 µg/ml) and day 6, day 11, or LPS-activated day 11 BMDCs (1 × 10⁵) in the absence or presence of rIL-6 (25 ng/ml) or anti-IL-6-neutralizing Ab (10 µg/ml). Proliferation of each culture was assayed after 3 days, as in Fig. 1. Data represent means ± SEM (n = 3). B, CD25⁺CD4⁺ Tregs (5 × 10⁴) were cultured in 96-well round-bottom plates with anti-CD3 (2.5 µg/ml) and day 11 BMDC (1 × 10⁵) in the presence of IL-1α (25 ng/ml) and IL-6 (25 ng/ml) (left panel). CD25⁺CD4⁺ Tregs (5 × 10⁴) were cultured under the same conditions with LPS-activated day 11 BMDCs (1 × 10⁵) in the presence of the indicated combinations of neutralizing Abs to IL-1α (33 µg/ml), IL-1β (33 µg/ml), and IL-6 (10 µg/ml) (right panel). After 3 days, proliferation of each culture was assayed (see Fig. 1; maximal response, 29,570 cpm). Data represent means ± SEM (n = 3).
Taken together, the foregoing studies indicated that while IL-6 and IL-1 could act cooperatively to induce Treg proliferation independently of IL-2, nonetheless, IL-2 was a more potent growth factor for Tregs when available at high levels (2.5 ng/ml). Given that cocultures of Tregs and T responders stimulated with LPS-activated BMDCs were associated with marked recruitment of Treg proliferation compared with unactivated BMDCs (Figs. 1 and 2), and that the levels of IL-2 available in the former cultures were only ~3.5-fold greater (1.43 vs 0.39 ng/ml; Fig. 6A), we speculated that a more important role for IL-1 and IL-6 might be their potentiation of Treg responsiveness to IL-2 under conditions of limiting or physiologic amounts of IL-2. To test this, Tregs were stimulated with saturating doses of IL-6, IL-1α, or both, in the presence of graded doses of IL-2. These experiments were performed using plate-bound anti-CD3 in the absence of APCs to permit direct assessment of effects on Tregs without the possibility of indirect effects contributed by APCs. As shown in Fig. 7A, addition of either IL-1α or IL-6 alone significantly increased the proliferation of anti-CD3-stimulated Tregs at all doses of IL-2, and IL-1α induced modest proliferation in the absence of IL-2. The effects of IL-1α and IL-6 on augmentation of IL-2 effects were additive at all doses of IL-2, and in the absence of exogenous IL-2.

Importantly, at doses of IL-2 below 300 pg/ml, IL-6/IL-1 supplementation was required to reverse Treg anergy.

To further address this, we compared the proliferation of Tregs elicited by different doses of exogenous IL-2 and either unactivated or LPS-activated day 10 BMDCs (Fig. 7B). Addition of the lower dose of IL-2 (0.1 ng/ml) induced robust Treg proliferation only by LPS-activated BMDCs. At higher doses of IL-2 (1.0 ng/ml), Treg anergy was strongly reversed even by unactivated BMDCs, although significantly enhanced proliferation was stimulated by LPS-activated BMDCs. In accord with the absence of production of IL-1 and IL-6 by unactivated BMDCs, neutralization of these cytokines had no effect on Treg proliferation induced by unactivated BMDCs at either the low or high dose of IL-2. In contrast, neutralization of IL-1 and IL-6 produced by LPS-activated BMDCs significantly inhibited Treg proliferation at the lower dose of IL-2 (0.1 ng/ml), and had a more modest, but reproducible, inhibition at the higher dose of IL-2 (1.0 ng/ml). Taken together, these data indicate that IL-6 and IL-1 can act directly on TCR-stimulated Tregs to break anergy and promote proliferation; perhaps more importantly, these proinflammatory cytokines significantly potentiate proliferative responses of Tregs in the presence of limiting amounts of IL-2. Thus, reversal of Treg anergy by

**FIGURE 6.** Effects of CD25+CD4+ Tregs on the production of IL-2 by CD25+CD4+ responder T cells. A, CD25+CD4+ T cells (3 x 10^5) were cultured in 48-well plates with or without CD25+CD4+ T cells (3 x 10^5) in the presence of anti-CD3 (5 μg/ml) and irradiated APC (SPL 2 x 10^6), day 6 BMDCs (1 x 10^5), day 11 BMDCs (1 x 10^5), or LPS-activated day 11 BMDCs (1 x 10^5). After 20 h of culture, supernatants were collected and assayed for IL-2 by ELISA. Data represent means ± SEM (n = 3–4). B, CD25+CD4+ Tregs (5 x 10^5) were cultured in 96-well round-bottom plates with anti-CD3 (2.5 μg/ml) and day 11 BMDC (1 x 10^5) or day 11 BMDC/LPS (1 x 10^5) in the absence or presence of rIL-2 (2.5 ng/ml) or anti-IL-2-neutralizing Ab (10 μg/ml) for 3 days. After 3 days, proliferation of each culture was assayed (see Fig. 1). Data represent means ± SEM (n = 3).
DISCUSSION

In this study, we examined the suppressive and proliferative activities of CD25⁺CD4⁺ T cells in the context of myeloid DC maturation and activation induced by LPS-induced TLR signaling. We also examined the relative contribution of TLR activation of DCs and Tregs in modulating Treg-suppressive function. Our findings support several conclusions. First, in agreement with a recent study by Steinman and coworkers (28), only immature DCs (day 5–6, bone marrow derived) were permissive for the suppressive activity of CD25⁺CD4⁺ Tregs. Maturation of DCs induced by prolonged culture in GM-CSF (day 10–11, bone marrow derived) reversed the suppressive activity of Tregs without a requirement for TLR-mediated activation, but LPS activation of DCs was required to reverse Treg anergy. Furthermore, the effects of TLR activation on reversal of the suppressive activity and recruitment of proliferation by Tregs were exerted through activation of the DC; direct effects on regulatory or responder T cells were not required (33). Finally, in contrast to a previous report (27), the production of TLR-induced cytokines by DCs was not required to block Treg suppression in our system. Instead, IL-6 and IL-1 produced by TLR-activated DCs acted in concert to enhance the proliferative responsiveness of Tregs to limiting amounts of IL-2, thereby inducing a reversal of the anergic state of Tregs. This highlights a requirement for help from CD25⁺CD4⁺ T cells, in the form of IL-2, to efficiently recruit Treg proliferation induced by TLR-activated DCs. These results support a model of immunomodulation by CD25⁺CD4⁺ T cells in which Treg activity is contextual along a continuum of DC maturation and TLR-induced activation, and mechanisms contributing to the reversal of Treg suppression and anergy are separable, and independently modulated by proinflammatory cytokines produced by DCs.

Since the original description of CD25⁺CD4⁺ Tregs, a central issue has concerned mechanisms by which Treg activity might be overcome in the face of immune responses against pathogens. A recent report by Pasare and Medzhitov (27) showed that TLR activation of DCs plays a critical role in blocking the suppressive activity of Tregs. Importantly, unlike DCs isolated from WT mice, DCs from MyD88-deficient mice did not reverse Treg suppression following activation with the TLR ligands (LPS or CpG), despite essentially normal expression of B7 costimulators (CD80 and CD86) and CD40. This deficiency could be rescued by addition of conditioned medium from LPS-activated WT DCs; IL-6, but not IL-12 or TNF-α, could reverse Treg suppression. The effect of IL-6 appeared to act in concert with additional factors derived from TLR-stimulated DCs, and was shown to act at the level of the CD25⁺CD4⁺ responders, not the CD25⁺CD4⁺ Tregs (27). DC production of the common γ-chain cytokines IL-2, IL-15, and IL-7 could not mediate this effect, nor could the recently described ligand for GITR (26). A model was proposed wherein TLR-induced IL-6 and an unidentified soluble factor(s) produced by DCs acted on CD25⁺CD4⁺ responders to block their susceptibility to suppression by Tregs, supporting a dual role for TLR activation in the initiation of naïve T cell responses: up-regulation of costimulator expression and induction of proinflammatory cytokines (27).

In the current study, the use of BMDCs permitted distinction between DC activities that blocked the suppressive activity of CD25⁺CD4⁺ Tregs and those that induced proliferation of this population. Importantly, in contrast to the recent report of Pasare and Medzhitov (27), we found that in our system proinflammatory factors such as IL-6 were not required to block the suppressive activity of Tregs. Several observations support this conclusion. First, the blockade of Treg suppression induced by mature (day 10–11), GM-CSF-derived bone marrow DCs was not associated with detectable levels of IL-6, IL-1α, or IL-1β. Second, DCs derived from TLR4-deficient donors could reverse the suppressive activity of Tregs through a TLR-independent mechanism. Finally, addition of neutralizing Abs to IL-6 (and IL-1α, IL-1β) could not reverse the blockade of suppression induced by mature DCs that were not LPS activated. Taken together, these data indicate that proinflammatory cytokines such as IL-6 and IL-1 are not required for reversal of Treg-suppressive activity under the conditions used in the current study, although they could clearly augment Treg proliferation and might contribute to blockade of Treg suppression when they are produced by TLR ligation. Although the basis for the discrepant results between our study and that of Pasare and Medzhitov is unclear, an important point of difference concerns the populations of DCs used. Our analyses were performed using only myeloid DCs derived by ex vivo differentiation with GM-CSF, whereas Pasare and Medzhitov used splenic DCs likely to contain a more heterogeneous mix of DC subpopulations that might differ in their requirements for and response to TLR activation.
Although BMDCs generated by prolonged culture in GM-CSF could reverse the suppression of Tregs without a requirement for TLR activation, TLR signaling was required for effective reversal of Treg anergy. This is in agreement with recent reports that have demonstrated reversal of Treg anergy by mature DCs in both murine and human systems (28, 36, 37). At least one component of this activity was induction of the proinflammatory cytokines IL-6 and IL-1. Thus, addition of IL-6 and IL-1 reversed the anergy of Tregs in response to mature DCs not activated by TLR signaling. Conversely, neutralization of IL-6 and IL-1 activity produced by LPS-stimulated, mature BMDCs inhibited proliferation of CD25⁺ CD4⁺ Tregs. Importantly, the proliferative effects of IL-6 and IL-1 on CD25⁺ CD4⁺ T cells could be induced in the absence of CD25⁺ CD4⁺ T cells. Thus, although our data do not preclude actions of TLR-induced proinflammatory cytokines on CD25⁺ CD4⁺ T cells, there are clearly direct effects of these cytokines on Treg proliferation that act independently of CD25⁺ CD4⁺ T cells.

The basis for the differential capacity of BMDCs differentiated short-term (5–6 days) or long-term (10–11 days) to support Treg-suppressive activity is not yet defined. Clearly, the prolonged culture of bone marrow precursors with GM-CSF produces a larger fraction of CD80- and CD86-positive DCs, and more uniform expression of MHC class II. One attractive explanation for the resistance of day 10–11 BMDCs to Treg suppression is that the enhanced Ag presentation and costimulatory function of this population of BMDCs might override the suppressive activity of Tregs, perhaps by facilitating IL-2 production by T responders. However, in preliminary studies using BMDCs derived from mice with deficiencies of CD80, CD86, or both, we have not established a simple association between B7 expression by DCs and Treg-suppressive function (our unpublished results). Additional studies will be needed to identify the mechanistic basis for the observed resistance of more mature DCs to suppression.

It has been reported previously that IL-2 can block the suppressive activity of Tregs and reverse their anergic state (22, 23). Importantly, and in agreement with the current study, Yamazaki et al. (28) found that the addition of IL-2 to CD25⁺ Tregs failed to induce their proliferation in the absence of mature, CD86-positive DCs. Our data indicate that IL-6 and IL-1 produced by LPS-activated (CD86-positive) DCs may induce limited proliferation of Tregs in the absence of IL-2, but that IL-2 significantly enhances Treg proliferation over and above that induced by proinflammatory cytokines alone. Conversely, IL-6 and IL-1 strongly potentiated the proliferative response of Tregs to limiting amounts of IL-2. Taken together, these results support a central role for proinflammatory cytokines in potentiating the responsiveness of Tregs to IL-2. Because we and others have found very limited IL-2 production by Tregs, even in response to LPS-stimulated DCs, we suggest that IL-2 produced by responder T cells that have escaped Treg suppression is the dominant source of IL-2 used by Tregs as a growth factor, suggesting that CD25⁺ CD4⁺ T cells perform a helper function for recruitment of robust Treg proliferation under conditions resulting in TLR-induced activation of mature DCs.

In previous reports, it was found that the recruitment of robust Treg proliferation induced by TLR-activated mature DCs did not alter Treg-suppressive activity following proliferation (28, 38). Similarly, Treg proliferation induced by IL-6-supplemented, non-TLR-activated DCs did not alter Treg function (data not shown). This suggests that under circumstances in which Treg suppression is broken in vivo, typically in the context of antipathogenic responses associated with TLR-induced DC maturation and activation, Tregs that would otherwise suppress foreign- or self-reactive clones are recruited to active proliferation in parallel with naïve CD4⁺ T cells undergoing clonal expansion and effector cell differentiation. Given that clones reactive to foreign pathogens are driven by availability of Ags derived from those pathogens, it stands to reason that as T effectors are generated following the breaking of Treg suppression and the pathogen is cleared by T effector responses, the expanded populations of Tregs would be poised to regain dominance as the antipathogenic response wanes. Thus, the linkage of anergy reversal of Tregs to TLR-based activation of mature DCs represents an important homeostatic mechanism that balances the competing requirements for self-tolerance and antipathogenic responses. Furthermore, because GM-CSF, IL-6, and IL-1 are typically components of the acute phase response and are thus produced in quantities that generate circulating levels, it is feasible that these factors might potentiate Treg clonal expansion to endogenous tissue Ags systemically, i.e., distant to sites of active inflammation, without compromising Treg suppression. This could represent an important global mechanism to enhance self-tolerance in the face of an ongoing antipathogenic immune response.

Based on these considerations, we propose the following model. In the steady state (i.e., absence of foreign pathogens), DCs that migrate to secondary lymphoid tissues are charged with self Ags derived from senescent cells from peripheral tissues. Upon arrival in draining lymphoid tissues, these DCs interact preferentially with CD25⁺ CD4⁺ Tregs that recognize tissue Ags, thereby blunting subsequent DC maturation that might recruit the proliferation and effector differentiation of self-reactive CD25⁻ CD4⁺ T cells. The absence of proinflammatory cytokine production by these steady state DCs and their limited expression of class II and costimulatory molecules favor Treg suppression due to limited induction of IL-2 production by T responders. In contrast, during antipathogen responses in which TLR ligands are abundant, DCs arriving at secondary lymphoid tissues would be mature and TLR activated so as to produce proinflammatory cytokines (e.g., IL-6 and IL-1), increased densities of costimulators (e.g., CD86), and other factors (GITR ligand?) that both obviate Treg suppression and reverse Treg anergy. We propose that proinflammatory cytokines contributed by mature, TLR-activated DCs potentiate the responsiveness of Tregs to IL-2 produced by naïve, antipathogen-reactive T responders, thereby inducing proliferation of the former and perhaps proliferation and effector differentiation of the latter. As the effector response matures and primes innate immune cells for clearance of the pathogen, bystander recruitment of Treg proliferation via proinflammatory factors would lead to restored dominance of Treg suppression through recognition of self Ags presented by nonactivated DCs. Thus, modulation of Treg activity and proliferation that is contextual with respect to DC maturation and TLR activation provides a homeostatic mechanism to shift dominance between self-reactive CD25⁺ CD4⁺ Tregs or antipathogen-reactive naïve T cells as needed.

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