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Differential Control of T Regulatory Cell Proliferation and Suppressive Activity by Mature Plasmacytoid versus Conventional Spleen Dendritic Cells

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Anergy and suppression are cardinal features of CD4$^+$CD25$^+$Foxp3$^+$ T cells (T regulatory cells [Treg]) which have been shown to be tightly controlled by the maturation state of dendritic cells (DC). However, whether lymphoid organ DC subsets exhibit different capacities to control Treg is unclear. In this study, we have analyzed, in the rat, the role of splenic CD4$^+$ and CD4$^-$ conventional DC and plasmacytoid DC (pDC) in allogeneic Treg proliferation and suppression in vitro. As expected, in the absence of exogenous IL-2, Treg did not expand in response to immature DC. Upon TLR-induced maturation, all DC became potent stimulators of CD4$^+$CD25$^+$ T cells, whereas only TLR7- or TLR9-matured pDC induced strong proliferation of CD4$^+$CD25$^+$Foxp3$^+$ T cells in the absence of exogenous IL-2. This capacity of pDC to reverse Treg anergy required cell contact and was partially CD86 dependent and IL-2 independent. In suppression assays, Treg strongly suppressed proliferation and IL-2 and IFN-γ production by CD4$^+$CD25$^+$ T cells induced by mature CD4$^+$ and CD4$^-$ DC. In contrast, upon stimulation by mature pDC, proliferating Treg suppressed IL-2 production by CD25$^+$ cells but not their proliferation or IFN-γ production. Taken together, these results suggest that anergy and the suppressive function of Treg are differentially controlled by DC subsets. The Journal of Immunology, 2008, 180: 5862–5870.

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blood and rodent lymphoid organs (14). So-called conventional DC (cDC) can be separated in lymphoid organ-resident DC subsets and peripheral tissue DC (Langerhans cells and interstitial DC), which can migrate to lymph node, but not spleen. Plasmacytoid DC (pDC), also known as IFN-producing cells, are found in blood and secondary lymphoid organs but not peripheral tissue in the steady state. Three DC subsets can be defined in rat spleen: conventional OX62CD11bCD4+ DC (hereafter referred to as CD4+ DC) and CD4− DC (hereafter referred to as CD4− DC) (15) and pDC which are OX62CD11bCD4+CD45R− (16). In the steady state, CD4+ DC are typically located at the periphery of T cell areas, CD4− DC are found in T cell areas, marginal zones as well as red pulp, whereas pDC are found primarily in T cell areas (Ref. 17 and our unpublished observations). Both subsets of CDC express a large repertoire of TLR but only CD4+ DC produced high levels of IL-12 (18), whereas pDC selectively express TLR7 and TLR9 and produce large amounts of type I IFN as well as IL-12p40. In the steady-state in vivo, the phenotype of pDC is immature and their role in T cell stimulation remains controversial (19). However, pDC can be recruited in inflamed lymph node and once mature, pDC can efficiently stimulate naive T cells (16).

Thus, because T cell areas contain several subsets of resident and migratory DC (14), an important question is whether in vivo expansion of Treg is dependent on a specific DC subset (20). Moreover, the manner in which Treg homeostasis and functions are regulated by DC in vivo is mostly unknown. One hypothesis is that Treg functions are mainly regulated by the DC maturation state inasmuch as Treg are strongly suppressive when DC are immature, whereas DC maturation leads to a blockade of the suppressive effect of Treg (21). However, conceptually it might be more important that Treg can regulate when immunity has been triggered, i.e., when DC are mature, rather than during a resting state, i.e., when DC are immature. In addition, it is possible that Treg expansion and functions are also regulated by the DC subtype involved in T cell stimulation.

In this study, we have analyzed the capacity of rat spleen DC subsets to induce allogeneic Treg proliferation and suppressive function in vitro. We found that mature pDC, but not conventional DC, were able to reverse Treg anergy in an IL-2-independent manner. Furthermore, we show that whereas Treg strongly suppressed proliferation and IL-2 and IFN-γ production by CD4+CD25+ T cells in the presence of allogeneic conventional mature DC, they suppressed IL-2 production but not the proliferation or IFN-γ production by CD4+CD25− T cells in the presence of mature pDC.

Materials and Methods

Animals

Sprague Dawley (SPD) and Lewis and Brown Norway (BN) rats were obtained from the Centre d’Elevage Janvier and were used when 6–10 wk old. The study was approved by our institutional review board.

Reagents

Poly(I:C) and LPS were obtained from Sigma-Aldrich. The phosphodiester oligonucleotide containing the CpG motif (CpG oligodeoxynucleotide) 2006 was synthesized by Sigma-Genosys. Loxoribine was purchased from InvivoGen. The murine CD40 ligand (L)-human CD80 fusion molecule was provided by Prof. Y. Choi (Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA). CFSE and 7-hydroxy-9H(1,3-dichloro-9,9-dimethylacridin-2-one succinimidyl ester (DDAO-SE) were purchased from Molecular Probes.

Antibodies

The following mouse anti-rat mAbs obtained from the European Collection of Cell Culture were used for cell depletion, cytofluorometric studies, and cell sorting after coupling if necessary to FITC or Alexa Fluor 647 (Molecular Probes): OX35 (CD4), R7.3 (TCRαβ), OX42 (CD11b/c), 3.2.3 (NKR-P1A), OX62 (interferon γ, chain or CD103), and OX39 (CD25, IL-2R chain). HIS24-FITC and -PE (CD45R), OX35-PE (CD4), OX39-PE and OX6-allophycocyanin cyanin 7 (Cy7; MHC class II (MHCII)) were obtained from BD Pharmingen. Anti-rat Foxp3-allophycocyanin mAb was purchased from eBioscience. Rat IL-2-specific and blocking polyclonal goat IgG were purchased from R&D Systems. A blocking mouse anti-rat CD25 mAb (clone ART-18) was provided by Prof. Y. Jacques (Institut National de la Santé et de la Recherche Médicale Unité 601, Nantes, France).

Dendritic cells

Spleens were minced and digested in 2 mg/ml collagenase D (Roche Diagnostics) in RPMI 1640/1% FCS for 15 min at 37°C. EDTA at 10 mM was added for the last 5 min and the cell suspension was then pipetted up and down several times and filtered. Cells were separated into high-density cells (containing most of the pDC) and low-density cells (containing most...
were used to stimulate allogeneic CD25
lated DC (immature) or DC stimulated overnight with ligands to TLR3
pDC (mented with allogeneic CpG-stimulated
assessed at day 4. Similar results were obtained in five independent exper-
cells at a ratio of 1:5 in the absence of exogenous IL-2. Proliferation was
pDC with 1:5 in 10% FCS or
or allogeneic (T/CpG-stimulated
4.

of exogenous IL-2. Proliferation and
DC (F/CpG-stimulated
5:1 in the absence

pDC at a ratio of 5:1 in 10% FCS or

for 24 h at 5 × 10^5/ml in the presence of poly(I:C) (50 μg/ml), LPS (0.5 μg/ml), CpG2006
(10 nM), loxoribine (100 μg/ml), or soluble murine CD40L-human CD8 fusion molecule (1/1000 supernatant dilution). Cells were recovered and

Staining of exogenous IL-2.

were stimulated with increasing num-

DC were cultured with allogeneic CD4+ DC. Sorted DC subsets were either used immature or stimulated for 24 h at 37°C

Multi-well plates were washed three times in complete medium before use in MLR.

Purity was routinely ≥99%. Cells were then stained with CD8-PE and

CD-Fluor 647 mAb and sorted on a FACSARia after excluding FITC+ cells. Purity was rou-
tinely >97% and >98%, respectively, for CD4- and CD4+ DC. pDC were
isolated from high-density spleen cells after removal of RBC. T and partial

B cell depletions were first performed by incubating cells with anti-TCRβ and γδ mAbs (clones R7.3 and V65, respectively), followed by a mixture of
an anti-mouse and anti-rat Ig-coated magnetic beads (Dynal). Cells were
then stained with TCRβ-FITC (clone R7.3), CD45RA-FITC (clone OX32), CD45RB-FITC (clone HI24) and
CD4-Alexa Fluor 647 (clone OX35) mAbs and CD45RB-CD4high were then sorted on a FACSARia after excluding FITC+ cells (see Fig. 2A). Purity was routinely >97.5%.

Lymph node CD4+ T cells were obtained by negative selection of CD11b/-c-, NKR-P1A+, and CD8+ cells using specific mAbs (clones OX42, 3.2.3, and
OX8), followed by anti-mouse IgG-coated magnetic beads (Dynal). Purity was routinely ≥99%. Cells were then stained with CD8-PE and
CD25-Alexa Fluor 647 mAb and sorted on a FACSARia, after gating out
CD8+ cells, in CD25low, and/or CD25high cells (see Fig. 1).

DC stimulation

Sorted DC subsets were either used immature or stimulated for 24 h at 5 × 10^5/ml in the presence of poly(I:C) (50 μg/ml), LPS (0.5 μg/ml), CpG2006
(10 nM), loxoribine (100 μg/ml), or soluble murine CD40L-human CD8 fusion molecule (1/1000 supernatant dilution). Cells were recovered and

washed three times in complete medium before use in MLR.

MLR and suppression assays

DC were cultured with allogeneic CD4+ T cell subsets in round-bottom
96-well plates in a final volume of 200 μl of complete RPMI 1640. The
numbers of DC and T cells were titrated in preliminary MLR experiments
and we found that using 4 × 10^4 DC to stimulate 2 × 10^5 T cells (ratio
DC:T of 1:5) was optimal. These experimental conditions were used in all
of the MLR experiments described, unless indicated. After 4 days at 37°C
in 5% CO2, cultures were pulsed for the last 8 h with 0.5 μCi of [3H]Tdr
(GE Healthcare) per well. The cells were then harvested onto glass fiber
filters and [3H]TdR incorporation was measured using standard scintilla-
tion procedures (Packard Institute). In Transwell experiments, MLR were
performed in flat-bottom, 96-well Transwell plates (Corning). Wells were
pulsed with 0.5 μCi of [3H]Tdr, and cells of the upper and lower com-
partments were then harvested onto glass fiber filters for measurement of
[3H]Tdr incorporation (Packard Institute).

FIGURE 3. CpG-stimulated pDC, but not OX62-DC, reverse anergy of
CD4+CD25high T cells in the absence of exogenous IL-2. A). CD4+CD25-
(left) and CD4+CD25high (right) T cells (2 × 10^5/wells in triplicates)
were stimulated with increasing numbers of allogeneic CpG-stimulated
pDC (●), CD4- DC (▲), or CD4+ DC (■) in the absence of exogenous
IL-2. Proliferation was assessed at day 4. B). CD4+CD25- (left) and CD4+
CD25high (right) T cells were stimulated with allogeneic CpG-stimulated
pDC (●), CD4+ DC (▲), or CD4- DC (■) at a ratio of 5:1 in the absence
of exogenous IL-2. Proliferation and IL-2 production were assessed from
days 2 to 5. C). CD4+CD25high T cells were stimulated with syngeneic (□)
or allogeneic (■) CpG-stimulated pDC at a ratio of 1:5 in 10% FCS or
2% normal rat serum supplemented culture medium. Proliferation was as-
essed on day 3.
**Flow cytometry**

**Proliferation assays.** T cells were labeled with CFSE (5 μM) or DDAO-SE (5 μM) for 5 min. at room temperature and then washed twice with complete RPMI 1640. Proliferation of T cells induced by allogeneic DC was assessed by the dilution of either CFSE (excitation, 488 nm; detection, 530/30 nm) or DDAO-SE (excitation, 633 nm; detection, 630/20 nm) on a LSR II cytometer (BD Biosciences), thus enabling proliferation of both CD4 CD25 DC and CD4 CD25 DC (CFSE) to be assessed simultaneously. Culture conditions (cell numbers, ratio) were the same for thymidine incorporation experiments. Before analysis, cells were stained with MHCII-allophycocyanin-Cy7 mAb, the MHCIIph cells (DC) were gated out and dead cells were excluded by 4′-diamidino-2-phenylindole.

**Foxp3 staining.** Intracellular Foxp3 expression was assessed by flow cytometry on resting CD4 T cells or stimulated and CFSE-labeled T cells according to the manufacturer’s recommendations (eBioscience).

**Cell enumeration.** The numbers of cells in the MLR wells were evaluated by flow cytometry using Cytocounts number-calibrated microbeads (DakoCytomation).

**Cytokine production**

The production of IL-2 (R&D Systems), IL-10, and IFN-γ (BD Biosciences) in the supernatants was assessed by ELISA. Culture conditions were the same as those used for thymidine incorporation experiments.

**Statistical analysis**

Statistical analyses were performed using the Student t test.

**Results**

**Mature plasmacytoid but not conventional rat DC subsets reverse in vitro anergy of CD4 CD25 Foxp3 T cells in the absence of exogenous IL-2**

In the rat, 10% of lymph node CD4 T cells expressed Foxp3 (Fig. 1). CD4 T cell subsets were separated by FACS into CD25low, CD25high, and CD25low subsets and Foxp3 expression was analyzed on sorted cells. More than 70 and 85% of CD25low and CD25high cells, respectively, expressed Foxp3, whereas only a small subset (2.6%) of CD25low cells expressed Foxp3 (Fig. 1). We next sought to determine whether splenic DC subsets had a differential ability to stimulate the proliferation of allogeneic CD25 and CD4 CD25 T cells in vitro. We have previously described three DC subsets in the rat spleen: OX62 CD11b CD4 DC (CD4 DC), OX62 CD11b CD4 DC (CD4 DC) which are both conventional DC and pDC (OX62 CD11b CD4 high CD45R) (15, 16). DC were sorted by FACS and used, either in their immature state (freshly isolated) or after maturation induced by various TLR ligands or CD40L, to stimulate allogeneic T cells (ratio of 1:5). As shown previously (15, 16), freshly isolated CD4 DC but not CD4 DC and pDC induced low-level proliferation of allogeneic CD4 CD25 T cells (Fig. 2A). All of the immature DC subsets induced no or very low-level proliferation of CD4 CD25 T cells (Fig. 2B). Following stimulation by TLR ligands, all DC could become strong stimulators of CD4 CD25 T cells (Fig. 2A). The lack of stimulatory activity of poly(I:C) on pDC and loxoribine on CD4 (Fig. 2A) correlates with the absence or very low expression of TLR3 and TLR7 by pDC and CD4 DC, respectively (18). Unlike in human, rat pDC express low levels of TLR4 and exhibit in vitro response to LPS (18). Interestingly, only pDC stimulated with either TLR7 or TLR 9 ligands (loxoribine and CpG2006, respectively) were able to promote strong proliferation of CD4 CD25 T cells (Fig. 2B). Of note, this proliferation occurred in the absence of exogenous IL-2. LPS-matured bone marrow-derived rat DC generated with GM-CSF and IL-4 were also very poor inducers of allogeneic Treg proliferation along all DC:T cell ratios tested in the absence of exogenous IL-2 (data not shown).

In most of the following experiments, we focused on TLR9-stimulated DC. Indeed, all DC subsets express TLR9 mRNA (18) and exhibit a response to PolyI:C on pDC and LPS-matured bone marrow-derived rat DC generated with GM-CSF and IL-4 were also very poor inducers of allogeneic Treg proliferation along all DC:T cell ratios tested in the absence of exogenous IL-2 (data not shown).

**FIGURE 5.** The effect of exogenous IL-2 on the proliferation of CD25 and CD4 CD25 T cells stimulated by allogeneic mature DC. CD4 CD25 (left) and CD4 CD25 T cells were stimulated with allogeneic CpG-stimulated pDC (■), CD4 DC (▲), or CD4 DC (●) at a ratio of 5:1 in the absence or presence of increasing concentrations of exogenous IL-2. Proliferation was assessed at day 4. Similar results were obtained in three independent experiments.
expression on stimulated DC (18). Moreover, all Cpg2006-stimulated DC subsets induced strong proliferation of CD4^+CD25^- allogeneic T cells (no statistical difference in the mean proliferation of >10 experiments). We confirmed that Cpg-stimulated pDC induced significant and dose-dependent higher proliferation of CD4^+CD25^{high} allogeneic T cells than cDC across all DC:T cell ratios tested (Fig. 3A, right panel); CD4^- DC were always unable to promote proliferation of CD4^+CD25^{high} T cells and CD4^- DC induced no or only modest proliferation. These differences held true from days 2-5 of the MLR (Fig. 3B). A similar profile was actually also observed with CD4^+CD25^{low} T cells (data not shown). To confirm that pDC-induced T proliferation was alloantigen specific, we compared the proliferation of CD4^+CD25^{high} T cells to syngeneic or allogeneic Cpg-stimulated DC in either FCS or normal rat serum-containing culture medium (Fig. 3C). In both FCS and normal rat serum, the proliferation of Treg induced by pDC was much stronger in allogeneic than syngeneic conditions.

An important issue that needed to be addressed was whether mature pDC induced proliferation of Foxp3-expressing Treg rather than contaminating CD25^-Foxp3^- T cells that account for 10-13% of CD4^+CD25^{high} T cells (Fig. 1). For this purpose, T cells were labeled with CFSE after sorting, stimulated with Cpg-matured DC for 4 days, and then stained for Foxp3. As shown in Fig. 4, the three DC subsets induced similar levels of CD4^+CD25^- T cell proliferation. Importantly, we did not observe any significant differences in the percentage of Foxp3^+ cells in these conditions, indicating that allogeneic DC-mediated stimulation did not induce CD25^- T cells to express de novo Foxp3. In our hands, after 4 days of culture in medium alone, Foxp3 expression was no longer detected in the few surviving isolated CD4^-CD25^{high} T cells (data not shown). However, 50-60% of these cells were found to express high levels of Foxp3 after stimulation by mature pDC, of which >90% had divided. Although 18 and 7% of CD4^-CD25^{high} T cells also appeared to express Foxp3 after stimulation with CD4^+ and CD4^- DC, respectively, in Fig. 4, the absolute numbers of T cells in these wells after 4 days was extremely low as compared with wells containing pDC and Treg (see Fig. 8). It should be noted that the same number of cells (10,000) was acquired for each dot plot in Fig. 4.

We next assessed whether Treg anergy in response to allogeneic CD4^+ and CD4^- DC could be overcome by exogenous IL-2. Controls performed with CD4^-CD25^- T cells showed that, as expected, their proliferation was enhanced by exogenous IL-2, whatever the nature of the APC (Fig. 5A). Although exogenous IL-2 also led to CD4^- and CD4^- DC promoting Treg proliferation, at least 1000 U/ml IL-2 was required to induce the same level of Treg proliferation as observed with pDC in the absence of exogenous IL-2. Intriguingly, no significant effect of IL-2 on the proliferation of Treg induced by pDC was observed (Fig. 5B).

Therefore, in the absence of exogenous IL-2, mature pDC, but not conventional CD4^- and CD4^+ DC, reversed the so-called anergic state of Treg in vitro. This function of pDC is probably dependent on the maturation state of pDC rather than on a specific effect of TLR9 stimulation, as similar activity was found after TLR7-induced maturation. Additional experiments showed that the stimulatory effect of pDC on Treg was dominant since Treg anergy was reversed by pDC even in the presence of allogeneic CD4^+ or CD4^- DC (data not shown).

**Mature pDC-induced Treg proliferation is contact and costimulation dependent but IL-2 independent**

To analyze the role of soluble vs membrane-bound molecules in the stimulatory activity of pDC on Treg, we performed experiments in which the different cell subsets were separated by a semi-permeable membrane (Transwell). The results presented in Fig. 6 indicate that the stimulatory activity of pDC on Treg is dependent on cell contact. In addition, the lack of proliferation of Treg when stimulated with CD4^- DC could not be overcome by soluble factors derived from a MLR between Treg and pDC (data not shown). Because mature but not immature pDC promoted Treg proliferation, we assessed whether CD80 and CD86 were involved. As shown in Fig. 7A, mature pDC-induced Treg, as well as CD4^-
CD25⁺ T cell proliferation was significantly inhibited to the same extent by CTLA4-Ig (up to 80% inhibition; p < 0.01). Treg and CD4⁺CD25⁻ T cell proliferation induced by pDC was mostly CD80 independent (NS) but partially CD86 dependent (up to 60% inhibition; p < 0.01) (Fig. 7, B and C). There were no significant differences in the effect of CTLA4-Ig, anti-CD80, and CD86 mAbs between CD4⁺CD25⁻ T cell and Treg proliferation induced by allogenic mature pDC.

We next assessed the role of IL-2. Several groups have shown that Treg cell proliferation in vitro is dependent on IL-2 produced by contaminating Foxp3⁺ or CD4⁺CD25low T cells. Upon stimulation by CpG-stimulated DC subsets, CD4⁺CD25⁻ T cells produced substantial amounts of IL-2, with a peak of production at day 4 (Fig. 3B). In contrast, hardly any IL-2 was detectable in the supernatant of CD4⁺CD25high T cells, even when stimulated by pDC and therefore undergoing proliferation (Fig. 3B). The proliferation of CD4⁺CD25high T cells induced by allogeneic mature pDC was not significantly inhibited by a neutralizing rat IL-2 polyclonal Ab, which strongly inhibited in a dose-dependent fashion CD4⁺CD25⁻ T cell proliferation (up to 60% inhibition at 20 μg/ml; p < 0.01) (Fig. 7D).

A similar inhibitory effect of IL-2 blockade was observed following CD4⁺CD25⁺ T cell proliferation induced by CD4⁺ or CD4⁻ DC (data not shown). Additional experiments performed with the blocking rat CD25 ART18 mAb also suggested that pDC-induced Treg proliferation was mostly CD25 independent (data not shown). Taken together, these data indicate that mature pDC induce IL-2-independent Treg cell proliferation in vitro.

**FIGURE 8.** Differential suppressive activity of Treg on effector T cells upon stimulation by various mature DC subsets. A–C, CD25⁻ and CD4⁺CD25⁰ T cells were stimulated with allogeneic CpG-matured pDC (■), CD4⁺ DC (○), or CD4⁻ DC (□) at a ratio 1:5. Suppression assays (right-hand part of each graph) were performed by adding decreasing numbers of CD4⁺CD25⁰ T cells to a constant number of CD25⁻ cells. After 4 days, the proliferation (A), production of IL-2 (B), and IFN-γ (C) was assessed. Results represent the mean ± SD of five, five, and two experiments for proliferation, IL-2 and IFN-γ, respectively. D–F, FACS-sorted CD25⁻ and CD25⁰ CD4⁺ T cells were labeled with DDAO-SE and CFSE, respectively, and stimulated with allogeneic CpG-matured pDC (D), CD4⁺ DC (E), or CD4⁻ DC (F) at a ratio of 5:1. Suppression assays were performed as in A–C. After 4 days, cells were harvested, stained with MHCII-allophycocyanin-Cy7 mAb and the percentages of undivided and divided CD25⁻ and CD25⁰ live T cells were assessed after excluding MHCII⁺ cells by FACS by the mean dilution of DDAO-SE or CFSE, respectively. The absolute numbers of DDAO-SE- and CFSE-labeled cells in each sample were assessed simultaneously using fluorescent-calibrated microbeads. Each bar represents the absolute numbers of either CD25⁻ (left part) or CD25⁰CD4⁺ (right part) T cells and is divided in undivided and divided cells. Note the very low levels of suppression of CD25⁻ cell proliferation by Treg upon stimulation with allogeneic pDC. Similar results were obtained in three independent experiments.
mature DC subsets in MLR. Upon stimulation by CpG-matured CD4+ or CD4− DC, Treg did not proliferate and did suppress the proliferation of CD25+ cells in a dose-dependent fashion (Fig. 8A). Because both CD25− and CD25(high) CD4+ T cells proliferated in response to allogeneic mature pDC, the 3H incorporation assay could not be used to clearly determine the proliferation of either population (Fig. 8A). Therefore, we analyzed the proliferation of CD25− and CD25(high) CD4+ T cells by FACS by the mean of CFSE and DDAO-SE probe dilutions, respectively, at day 4 (Fig. 8, D–F). The absolute numbers of undivided and divided CD25− and CD25(high) live cells was determined simultaneously in each tube using calibrated fluorescent beads. This experiment confirmed the 3H incorporation data obtained with CD4+ and CD4− DC and showed that, in the presence of CpG-stimulated pDC, proliferating Treg poorly suppressed CD4+CD25− T cell proliferation (<50% suppression at a 1:1 ratio).

The suppressive activity of Treg was also analyzed according to the level of cytokine production by CD4+CD25− T cells. CD25−, but not CD4+CD25(high) T cells, produced substantial amounts of IL-2 (Fig. 8B) and IFN-γ (Fig. 8C) upon stimulation by allogeneic mature DC. Treg strongly suppressed IL-2 and IFN-γ production by CD25− cells when stimulated by allogeneic CD4+ or CD4− DC. In contrast, when stimulated by pDC, Treg proliferated and strongly suppressed the production of IL-2 but not IFN-γ production by CD25− T cells. The data depicted in Fig. 7 were obtained at day 4 of culture, which corresponded to the peak of IL-2 production. However, both the absence of IL-2 production by pDC-stimulated Treg and the suppression of IL-2 production by Treg was observed from days 2 to 7 of culture (data not shown).

Discussion

Both anergy and suppressive activity of Treg have been proposed to be tightly controlled by the maturation state of DC. However, multiple DC subsets endowed with a specialized phenotype, function, and location have been described in lymphoid organs and tissues and whether these DC subsets differentially control Treg activity is mostly unknown. In this study, we have addressed this issue in the rat and have shown that Treg anergy was reversed in vitro by mature allogeneic pDC but not conventional splenic DC, the effect of pDC on Treg being IL-2 independent. Moreover, the suppressive activity of Treg was strongly down-modulated when stimulated by mature pDC as compared with cDC.

Previous studies have examined the APC requirement for Treg expansion in vitro. In humans, mature monocyte-derived DC could not reverse CD4+CD25− T cell anergy in the absence of exogenous cytokines (22, 23). In contrast, mature but not immature murine bone marrow-derived DC (BMDC) expanded monoclonal or polyclonal CD4+CD25− T cells in the absence of exogenous IL-2 (20, 24–26). Fewer studies have been published on the capacity of murine lymphoid organ DC subsets to stimulate Treg. In a study by Yamazaki et al. (20), mature CD8+ and CD8− spleen DC subsets induced very poor mononuclear Treg proliferation, as compared with BMDC. The same group further reported that splenic CD11c(high) DC were unable to promote allogeneic Treg expansion in the absence of exogenous IL-2 (27), confirming previous data by Brinster et al. (26) obtained with LPS or CpG-stimulated CD11c+ spleen DC. Fisson et al. (28) reported that freshly isolated spleen CD8+ DC were more effective at inducing monoclonal CD4+CD25− T cell proliferation than CD8− DC and pDC, although this expansion required exogenous IL-2 and the function of DC following maturation was not assessed. Nevertheless, these studies did not assess whether proliferating CD4+CD25− T cells were actually Foxp3+, neither did they directly compare mature conventional DC subsets and pDC. In the rat, we found that mature CD4− and CD4+ spleen OX62+ DC, which are likely the counterparts of conventional CD8+ and CD8− murine DC (15, 18), respectively, did not reverse Treg anergy in MLR in the absence of exogenous IL-2. Splenic pDC, in contrast, promoted Treg expansion under the same conditions, a capacity that was dependent on a strong maturation signal provided through TLR7 or TLR9.

Mature pDC-induced Treg expansion required cell contact and was partially dependent on CD86, despite all DC tested expressing similar levels of this molecule. A similar CD86-dependency of Treg expansion was observed previously with murine BMDC (20).

Interestingly, the effect of pDC we observed on Treg proliferation was dominant, i.e., it was not inhibited by the presence of cDC. These data are consistent with the hypothesis that the capacity of mature DC to promote Treg expansion is related to specific expression by pDC of costimulatory molecules. Two candidates are GITR-L and ICOSL that were found to be expressed by human pDC (29, 30). Interestingly, ICOSL was involved in the capacity of pDC to induce the differentiation of IL-10-producing Treg (29, 30). However, we could not detect expression of ICOSL nor GITRL on CpG-activated pDC and blockade of these molecules using ICOS-Fc or GITR-Fc, respectively, during MLR did not affect rat pDC-induced Treg proliferation in vitro (data not shown). TGF-β was also found to play a role in rat Treg expansion in vivo (31–33); however, we could not detect any effect of TGF-β blockade on pDC-induced Treg proliferation (data not shown). IDO is known to be expressed in mature DC (34) and in a subset of pDC endowed with tolerogenic properties, especially toward tumor (35). A recent study indicates that IDO-expressing pDC directly activates Treg in vivo in tumor-bearing mice (36). Although CpG-activated rat pDC indeed strongly up-regulated IDO mRNA, blocking IDO activity during MLR did not modify the capacity of pDC to promote Treg expansion (data not shown). Additional experiments are needed to determine the molecular mechanism by which mature pDC reverse Treg anergy in vitro.

Results obtained in mice suggest that the suppressive activity of Treg is mainly controlled by the maturation state of DC (21). Mature BMDC were shown to reverse the CD4+CD25− T cell anergy without reversing their capacity to suppress IL-2 production by effector cells (26). We found that Treg can suppress effector cell proliferation and cytokine production induced by cDC matured by CpG or LPS (data not shown). In the presence of mature pDC, however, proliferating Treg still efficiently suppressed IL-2 production by effector cells but not their proliferation and IFN-γ production. These data suggest that mature pDC can induce IL-2-independent effector T cell proliferation and that Treg are unable to inhibit pDC-induced Th1 differentiation. The fact that exogenous IL-2 did not affect Treg expansion induced by pDC challenges the possibility that Treg consumed IL-2 rather than suppressed its production by effector T cells when stimulated by pDC. The mechanisms that control Treg-mediated suppression are not fully understood but TCR signals (37), costimulation (8), inflammatory cytokines such as IL-6 (21) and members of the TNF superfamily such as 4-1BB (38) or GITR (39) have been implicated and need to be addressed in our system. Importantly, both the intrinsic suppressive activity of Treg and the responsiveness of effector T cells to Treg suppression can be targets of these control mechanisms. TLR ligands also appear to modulate Treg anergy and function, independently of their action on APC (40–43); however, it is unlikely that the effect we observed is related to a direct effect of CpG on Treg since DC were extensively washed before use and because the effects of DC subsets differed.

Several studies suggest that the suppressive effect of Treg on effector T cells might act through DC inhibition (44–46). In fact, adaptively transferred Treg interact preferentially with CD11c+...
CD8\(^+\) DC in mice (47). Interestingly, Houot et al. (48) recently showed in humans that TLR-activated CD11c\(^+\) DC, but not pDC, were actually sensitive to the inhibitory effect of Treg in vitro. Whether such a contrasting effect of Treg on conventional DC vs pDC might explain our observation needs to be addressed.

A role for pDC in the induction of regulatory cells has been suggested. In vitro, human CD40L- and CpG-stimulated pDC induced the generation of IL-10-producing CD8\(^+\) (49) and CD4\(^+\) (30, 50) regulatory T cells, respectively (30). In these studies, the induction of a regulatory phenotype requires activation and maturation of pDC. In fact, DC can induce in the presence of TGF-\(\beta\) the in vitro differentiation of Foxp3\(^+\) CD4\(^+\) Treg from Foxp3\(^-\) precursors as soon as day 3 after stimulation (51). In our hands, the stimulation of CD4\(^+\)DC25\(^+\) T cells with TLR9-stimulated pDC did not induce their differentiation into CD4\(^+\)Foxp3\(^+\) regulatory cells. In addition, adoptive transfer of Ag-presenting CpG-matured pDC in mice did not induce tolerance but rather immunity (52).

Our results suggest a new function of pDC in which they could be recruited in inflammatory lymph nodes (56) and favor T cell activation by down-modulating the suppressive activity of Treg. At the same time, pDC could facilitate the clonal expansion of autoantigen-specific Treg that could later control the T cell-mediated inflammatory response in tissue during the effector phase of the immune response.

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Disclosures

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