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Cutting Edge: Induction of B7-H4 on APCs through IL-10: Novel Suppressive Mode for Regulatory T Cells

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Multiple modes of suppressive mechanisms including IL-10 are thought to be implicated in CD4+CD25+ regulatory T (Treg) cell-mediated suppression. However, the cellular source, role, and molecular mechanism of IL-10 in Treg cell biology remain controversial. We now studied the interaction between Treg cells and APCs. We demonstrate that Treg cells, but not conventional T cells, trigger high levels of IL-10 production by APCs, stimulate APC B7-H4 expression, and render APCs immunosuppressive. Initial blockade of B7-H4 reduces the suppressive activity mediated by Treg cell-conditioned APCs. Further, APC-derived, rather than Treg cell-derived, IL-10 is responsible for APC B7-H4 induction. Therefore, Treg cells convey suppressive activity to APCs by stimulating B7-H4 expression through IL-10. Altogether, our data provide a novel cellular and molecular mechanism for Treg cell-mediated immunosuppression at the level of APCs, and suggest a plausible mechanism for the suppressive effect of IL-10 in Treg cell-mediated suppression.

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Treg cells comprise 5–10% of the circulating CD4+ T cell population and play a crucial role in different pathological settings (1–4). Multiple suppressive modes are proposed to explain the suppressive mechanisms of regulatory T (Treg) cells (5). Some in vitro experimental data suggest a minor role of IL-10 in Treg cell-mediated suppression (6, 7). However, numerous in vivo models have demonstrated a nonredundant role of IL-10 in Treg cell-mediated suppression (8–10). Nonetheless, the source and molecular suppressive mechanisms of IL-10 in the context of Treg cell biology remain elusive.

B7-H4 is a recently discovered B7 family member. B7-H4 negatively regulates T cell responses in vitro (11–13). Treg cells (2) and suppressive B7-H4+ macrophages (14) were localized in ovarian tumor. In this report, we studied the interaction between Treg cells and APCs. Our study reveals a previously unappreciated mechanistic relationship among IL-10, B7-H4, Treg cells, and APCs and demonstrates a novel molecular and cellular suppressive mechanism for Treg cell-mediated immunosuppression.

Materials and Methods

Human cells

Peripheral blood CD14+ cells, CD4+CD25− T cells, CD4+CD25high T cells (Treg cells), CD4+CD45RO+CD25− T cells, and lin− HLA-DR−CD11c+ primary myeloid dendritic cells (MDCs) were sorted with FACSaria (BD Biosciences) with purity >98% (15). Monocyte-derived DCs (MDDCs) were obtained as described (16). Cells were stained with mAbs and analyzed on a LSR II (BD Biosciences). Mouse anti-human Abs, including CD4-FITC (SK3), CD25-PE (MA251), HLA-DR-PerCP (L243), CD11c-allophycocyanin (B-ly6), and CD14-allophycocyanin-Cy7 (M09P9) were obtained from BD Biosciences.

Human FOXP3 detection

RT-PCR was conducted for FOXP3 (2). Results were expressed as fold differences relative to GAPDH (2). FOXP3 protein was detected by intracellular staining with rat anti-human FOXP3 Ab (clone PCH101; eBioscience).

Human T cell immunosuppressive assay

T cell immunosuppression was tested in a coculture system. CD4+CD25− T cells (2 × 10^5/ml) were stimulated with 2.5 μg/ml anti-human CD3 (clone UCHT1), 1.2 μg/ml anti-human CD28 (clone CD28.2) (BD Biosciences), and fresh monocytes (2 × 10^5/ml) in the presence of different concentrations of Treg cells or different concentrations of conditioned CD4+ cells as indicated. Seventy-two hours after coculture, T cell proliferation was evaluated by thymidine incorporation. In some cases, CD14+ cells were incubated with mouse anti-human IL-10 receptor (0.5 μg/ml, mouse IgG1, clone 7E6; R&D Systems) as indicated.

Mouse experiments

C57BL wild-type and IL-10−/− (5) mice (The Jackson Laboratory) were maintained in specific pathogen-free conditions. Eight-week-old female mice were used in all the experiments. Mouse CD11b+ cells, CD4+CD25− cells, and CD4+CD25+ T cells were enriched from spleen cells with mouse CD14 selection kits (StemCell Technology) and sorted with high purity (> 95%). Mouse T cell immunosuppression was tested in a coculture system. CD4+CD25− T cells (2 × 10^5/ml) were stimulated with 2.5 μg/ml anti-mouse CD3 (clone 145-2C11; BD Biosciences) and fresh CD11b+ cells (2 × 10^5/ml) in the presence of different concentrations of CD4+CD25high T cells (Treg cells). Seventy-two hours after coculture, T cell proliferation was evaluated by thymidine incorporation.

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3 Abbreviations used in this paper: Treg, regulatory T; MDC, primary myeloid dendritic cell; MDDC, monocyte-derived DC.
Expression and regulation of human APC B7-H4 expression

Blood CD14+ cells, MDCs, and MDDCs (1 × 10^6/ml) were cultured for 72 h with human IL-10 (R&D Systems) or with different concentrations of autologous CD4+ CD25+ T cells, Treg cells (0–1 × 10^5/ml) in the presence of anti-human CD3, and anti-human CD28. In some cases, cytokines were detected by ELISA (R&D Systems) in the culture supernatants. Neutralizing mAb against human IL-10 (clone 23738, 50 ng/ml; R&D Systems) was used as indicated. B7-H4 mRNA was detected by RT-PCR (17). To detect B7-H4 protein, cells were initially incubated with human AB serum to block nonspecific binding and then stained with mouse anti-human-B7-H4 (hH4.1, IgG1, 4 μg/ml) (11), further stained with goat anti-mouse Ab (BD Biosciences). Additional controls were cells stained with medium, primary mAb, second mAb with or without isotypes. B7-H4 surface protein was analyzed by flow cytometry.

**Blockade of human B7-H4 induction**

Antisense morpholino oligonucleotide specific for B7-H4 (GAGGATCTGC CCCAGGAAAGCATG) (B7-H4 blocking oligos), and the inverted oligonucleotide (control oligos) were produced by GeneTools. To block B7-H4 induction, monocytes were incubated for 3 h with 0.6 μM oligos in serum-free medium supplemented with 0.2 μM ethoxylated polyethylenimine (GeneTools). Cells were washed twice and used for additional experiments.

**Statistical analysis**

Differences in cell surface molecule expression were determined by χ^2 test, and in other variables by unpaired t test, with p < 0.05 considered significant.

**Results**

**Treg cells, but not conventional T cells, trigger APC-dependent, high levels of IL-10**

CD4+ CD25^bright T cells, but not CD4+ CD25^- T cells, highly expressed FOXP3 mRNA (n = 12; *, p < 0.001) (Fig. 1A). Treg cells are thought to be enriched in CD4+ CD25^bright T cell population (17). FOXP3+ T cells were largely found in CD4+ CD25^bright T cell population (Fig. 1B). CD4+ CD25^bright T cells inhibited T cell proliferation in a dose-dependent manner (n = 10; *, p < 0.01, **p < 0.0001) (Fig. 1C). Therefore, we sorted CD4+ CD25^bright T cells (Treg cells) for our experiments.

We studied the interaction between APCs and Treg cells. Treg cells or CD4+ CD25^- T cells or CD4+ CD45RO^- CD25^- T cells were cocultured for 72 h with autologous monocytes in the presence of anti-human CD3 and CD28. We detected higher levels of IL-10 in the culture supernatants with Treg cells than conventional T cells (24; CD25^- T cells and CD4+ CD25^- T cells; Control, no cell input. B, CD4+ CD25^- T cells express FOXP3 protein. In the absence of monocytes, Treg cells are not able to induce APC suppressive activity in vitro. However, the levels of suppression were significantly higher in the group with all the cells from IL-10^-/- mice than from IL-10^-/- mice (n = 5; p < 0.05) (Fig. 2). Thus, although IL-10 is not essential for Treg cell suppressive activity, the presence of IL-10 profoundly enhances Treg cell-mediated suppression.

To determine which population is responsible for IL-10 production, we used various combinations of cells from IL-10^-/- and IL-10^-/- mice (Fig. 2B). We showed that when the ratio between Treg cells and CD4+ CD25^- T cells was <1:1, the addition of IL-10^-/- CD11b^- cells resulted in significant reduction of Treg cell-mediated suppression (n = 5; *, p < 0.05, compared with IL-10^-/- CD11b^- cells) (Fig. 2B data not shown). When the ratio between Treg cells (2 × 10^6/ml) and CD4+ CD25^- T cells (2 × 10^6/ml) was 1:1, the levels of Treg-mediated suppression reached the plateau and were comparable among different cellular combinations. It suggests that APC-derived IL-10 is critical for Treg cell suppressive activity.

**Treg cells enable APC suppressive activity**

Our human studies suggest that Treg cells triggered APC-dependent IL-10 production (Fig. 1D). The experiments with IL-10^-/- mice indicated that APC-derived IL-10 implicates in...
Treg cells suppressive activity (Fig. 2). We asked whether Treg cell-conditioned APCs are distinct from conventional T cell-conditioned APCs. To this end, we incubated CD14<sup>+</sup> cells with Treg cells or conventional T cells in the presence of anti-human CD3 and anti-human CD28. Seventy-two hours later, we sorted these monocytes and tested their capacity of activating T cells. Strikingly, CD14<sup>+</sup> cells pretreated with Treg cells, but not CD4<sup>+</sup>CD25<sup>-</sup> T cells and medium, significantly suppressed T cell proliferation (Fig. 2A). The data indicate that Treg cells enable APC suppressive activity.

Induction of APC B7-H4 is IL-10-dependent

Treg cells trigger high levels of IL-10 production (>1 ng/ml) (Fig. 1D). We hypothesized that IL-10 may contribute to B7-H4 induction on APCs. To test this, we analyzed B7-H4 expression on CD14<sup>+</sup> cells during coculture with Treg cells in the presence of neutralizing Ab against IL-10. Anti-human IL-10 partially but significantly decreased CD14<sup>+</sup> cell B7-H4 expression (n = 6; *p < 0.001) (Fig. 4A). In support of this, rIL-10 stimulated B7-H4 expression on APCs (n = 6; p < 0.001) (Fig. 4B and C). Further, low concentrations of IL-10 induce B7-H4 expression on multiple APC subsets.

FIGURE 2. Mouse Treg suppressive capacity is reduced in the absence of IL-10 in APCs. Mouse Treg suppressive capacity is reduced in the presence of IL-10<sup>+/−</sup> APCs. Treg cells, CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD11b<sup>+</sup> cells were sorted from wild-type and IL-10<sup>+/−</sup> mice. Variable concentration of Treg cells were cocultured with 2 x 10<sup>5</sup> cells/ml CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD11b<sup>+</sup> cells were cocultured for 72 h in the presence of soluble anti-mouse CD3 mAbs. T cell proliferation was determined by thymidine incorporation. A, Treg-mediated suppression in IL-10<sup>+/−</sup> vs IL-10<sup>+/+</sup> mice. B, Treg-mediated suppression in different combinations of cells from IL-10<sup>+/−</sup> and IL-10<sup>+/+</sup> mice. Treg-mediated suppression was analyzed with different combinations of cells sorted from IL-10<sup>+/−</sup> and IL-10<sup>+/+</sup> mice as indicated. Treg-mediated suppressive activity was expressed as the mean percentage of suppression ± SEM. The percent of suppression was calculated with the formula: 100% - 100 x cpm values with Treg cells/cpm values without Treg cells. Different concentrations of Treg cells were used. Results were shown at 4 x 10<sup>5/ml</sup> Treg cells. IL-10<sup>+/−</sup>, cells from IL-10<sup>+/−</sup> mice; W, cells from wild-type mice.

FIGURE 3. Human Treg cells render CD14<sup>+</sup> cells immunosuppressive. A, Treg cell-conditioned monocytes are suppressive. Fresh CD14<sup>+</sup> cells were cultured with autologous Treg cells or CD4<sup>+</sup>CD25<sup>+</sup> T cells or medium in the presence of soluble anti-CD3 and anti-CD28 mAbs for 72 h. CD14<sup>+</sup> cells were sorted from this coculture. Different concentrations of the sorted CD14<sup>+</sup> cells were further cultured with fresh CD4<sup>+</sup>CD25<sup>+</sup> T cells for 3 days in the presence of anti-CD3 and anti-CD28 mAbs. T cell proliferation was detected by thymidine incorporation. Results are expressed as the mean cpm ± SEM. B, IL-10 is crucial for Treg cell-conditioned monocyte-mediated T cell suppression. In the above culture system, CD14<sup>+</sup> cells were initially incubated with anti-human IL-10 receptor. After being conditioned with Treg cells, CD14<sup>+</sup> cells (4 x 10<sup>5/ml</sup>) were subject to T cell activation in the presence of anti-human IL-10 receptor. Results are expressed as the mean percentage of T cell proliferation ± SEM. C–E, Treg cells stimulate B7-H4 expression on APCs. APC subsets were cocultured with different concentrations of Treg cells or CD4<sup>+</sup>CD25<sup>+</sup> T cells for 3 days in the presence of anti-CD3 and anti-CD28 mAbs. B7-H4 expression was analyzed by FACS. B7-H4 expression was expressed as the mean percentage of positive cells ± SEM in gated CD14<sup>+</sup> cells (C and D) and CD11b<sup>+</sup> cells (D). B7-H4 expression also was expressed by histogram (E). Treg cell concentration was 1 x 10<sup>4/ml</sup> for D and E.

FIGURE 4. Induction of APC B7-H4 by Treg cells is IL-10-dependent. A, Induction of APC B7-H4 by Treg cells is IL-10-dependent. Autologous Treg cells were cultured with CD14<sup>+</sup> cells with anti-CD3 and anti-CD28 mAbs for 3 days in the presence of anti-human IL-10 or and control mAb. B7-H4 expression was analyzed on CD14<sup>+</sup> cells. B, IL-10 induces B7-H4 expression on APC subsets. APC subsets were cultured for 72 h in medium or different concentrations of IL-10. B7-H4 expression was analyzed by FACS and expressed as the mean percentage of positive cells ± SEM (A and B) or histogram (C).
(0.1–1 ng/ml) efficiently stimulated B7-H4 expression on APCs (Fig. 4B), but not CD40, CD80, and CD86 expression (data not shown). Thus, Treg cells trigger APC B7-H4 expression at least partially through IL-10.

**Blocking B7-H4 reduces the suppressive activity of Treg cell-conditioned APCs**

We next determined whether APC B7-H4 is involved in the Treg cell-suppressive capacity. Specific neutralizing anti-human B7-H4 mABs is not available. We designed the B7-H4 blocking oligos and control oligos.

We initially studied IL-10-stimulated normal monocytes. The B7-H4 blocking oligos, but not control oligos, significantly inhibited basal and IL-10-induce B7-H4 mRNA expression by at least 1000-fold (Fig. 5A). B7-H4 blocking oligo expression was also blocked B7-H4 protein induced by IL-10 (n = 6; *p < 0.05) (Fig. 5B). Neither B7-H4 blocking oligos nor control oligos affected macrophage MHC class I, MHC class II, CD16, CD32, CD80, or CD86 expression (data not shown).

We next examined whether B7-H4 blocking oligo would block Treg cell-induced B7-H4 expression on monocytes. Normal blood monocytes were initially exposed to B7-H4 blocking oligos or control oligos and then cocultured with Treg cells. The B7-H4 blocking, but not control oligos, significantly reduced monocyte B7-H4 expression induced by Treg cells, whereas control oligos had no significant effects on B7-H4 expression (n = 6; *p < 0.05) (Fig. 5C). These data indicate that B7-H4 blocking oligos specifically block Treg-induced monocyte B7-H4 expression.

We further evaluated the role of B7-H4 in T cell suppression mediated by Treg-conditioned monocytes as we described (Fig. 3A). To this end, we initially exposed normal monocytes to B7-H4 blocking oligos or control oligos or medium. These monocytes were subsequently incubated with Treg cells for 72 h. We then sorted these monocytes for testing their capacity of activating T cells. As expected, T cell suppression was significantly reduced in the presence of Treg-conditioned monocytes exposed to B7-H4 blocking oligos, compared with control oligos and medium (n = 6; *, p < 0.01) (Fig. 5D). These data indicate that B7-H4 contributes to the suppressive activity mediated by Treg cell-conditioned APCs.

**Discussion**

In this study, we document that Treg cells trigger high levels of IL-10 production by APCs and, in turn, stimulate APC B7-H4 expression in an autocrine manner and render APCs immuno-suppressive via B7-H4.

Inconsistent with some reports (6, 18), we show that IL-10–/– Treg cells are suppressive. Hence, Treg-derived IL-10 is not essential for Treg-mediated suppression. However, in a typical immunosuppressive assay (6), Treg suppressive capacity is reduced when Treg cells, conventional T cells, and APCs are all from IL-10–/– mice. It indicates that IL-10 participates in Treg-mediated suppression. In support of this, when the ratio between Treg cells and conventional T cells is <1:1, the suppressive capacity of IL-10–/– and IL-10+/+ Treg cells is equally and profoundly reduced when IL-10–/– APCs are added. The data indicate that APC, but not Treg-derived IL-10, is crucial for Treg-mediated suppression.

Many in vitro suppressive assays are conducted in the absence of APCs, or APCs are substituted by irradiated whole spleen cells. It is not a surprise that these assays cannot define the role of APCs in Treg-mediated suppression, including APC-derived IL-10. APCs actively process Ags and present Ags to T cells in vivo (8–10). The ratio between Treg cells and T cells may rarely reach 1:1 in vivo. Thus, APC-derived IL-10 would likely involve in Treg-mediated T cell suppression in vivo.

Treg cells, but not conventional T cells, trigger high levels of IL-10 production. It is unknown how and why Treg cells do so. IL-10 has long been thought as an immunosuppressive cytokine, but it remains elusive how IL-10 implicates in Treg-mediated suppression. Inhibition is thought to be mediated mainly by effects on APCs (7, 19). However, very high concentrations of IL-10 (≥20–40 ng/ml) are essential to alter DC phenotypes (19). This concentration may not be physiologically relevant in vivo. We now show that as low as 0.1 ng/ml IL-10 can profoundly stimulate APC B7-H4 expression. Treg cells can trigger APC IL-10 production, which in turn stimulates B7-H4 expression and renders APCs suppressive through B7-H4. Thus, our data provide a plausible mechanism for the suppressive effect of IL-10. Because Treg cells are a small population, Treg-to-T cell contact-dependent suppressive mechanism may not ensure an efficient suppression in vivo. It is postulated that Treg cells may inhibit APC function. Induction of suppressive B7-H4 on APCs provides a novel molecular and cellular basis for Treg-mediated suppression in the level of APCs.

Our data mechanistically link IL-10, B7-H4, Treg cells, and APCs. B7-H4 is a newly identified B7 family member (11–13). Although mouse B7-H4 ligation of T cells has a profound inhibitory effect T cell activation (11, 12), the regulatory mechanisms and function of B7-H4 remain to be defined. We show...
that IL-10 stimulates B7-H4 expression on different APC subsets. More importantly, Treg-conditioned APCs strongly suppress T cell activation via B7-H4 induction. Our findings thus provide three pieces of novel information: 1) IL-10 is capable of inducing B7-H4 expression on human APCs; 2) similar to murine B7-H4 fusion protein (11–13), human APC B7-H4 negatively regulates T cell responses; and 3) human Treg cells enable suppressor activity to APCs via triggering B7-H4 expression. Thus, as suppression partially relies on Treg-triggered, APC-dependent IL-10, our observation reconciles the apparent contradiction in previous studies regarding the role and source of IL-10 in Treg cell biological activity.

In summary, our data demonstrate a novel molecular and cellular suppressive mechanism for Treg cells and suggest a plausible suppressive mechanism for the suppressive effect of IL-10 in Treg biology. Therefore, one can expect that targeting B7-H4 may be a novel strategy to revert Treg-mediated suppression in vivo.

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Disclosures
The authors have no financial conflict of interest.

References
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