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*J Immunol* 2006; 177:40-44; doi: 10.4049/jimmunol.177.1.40
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Cutting Edge: Induction of B7-H4 on APCs through IL-10: Novel Suppressive Mode for Regulatory T Cells

Ilona Kryczek, * Shuang Wei, * Linhua Zou, * Gefeng Zhu, † Peter Mottram, ‡ Huanbin Xu, ‡ Lieping Chen, † and Weiping Zou2*

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. *The Journal of Immunology, 2006, 177: 40–44.*

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 (Bly6), and CD14-allophycocyanin-Cy7 (MΦP9) 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 276707; R&D Systems) as indicated.

**Mouse experiments**

C57BL wild-type and IL-10−/− 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 CD11b 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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Received for publication November 8, 2005. Accepted for publication May 3, 2006.

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1 This work was supported by National Cancer Institute Grants CA092562, CA100227, and CA99985 (to W.Z.) and CA97085 (to L.C.).

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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 CD4^+^ 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 × 10^6^/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 237/38, 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 CCCAGGGAACCATG) (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 polyethyleneimine (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 (n = 7; *, p < 0.04) (Fig. 1D). The production of TNF-α, but not IL-10, was higher in the culture with CD4^+^CD45RO^-^CD25^-^ T cells (632 ± 87 pg/ml) than with Treg cells (91 ± 22 pg/ml) and CD4^+^CD25^-^ T cells (132 ± 53 pg/ml) (n = 4; p < 0.01), whereas there was no significant difference in IL-1β production among the three groups. These data suggest that Treg cells, but not conventional T cells, may selectively trigger high levels of IL-10 production.

We detected little IL-10 in the culture supernatants when monocytes were omitted. Addition of monocytes resulted in significantly higher levels of IL-10 production in Treg cell and monocyte coculture (n = 7; *, p < 0.001) (Fig. 1D). Similar results were observed when monocytes were replaced by MDCs and MDDCs. Altogether, our data demonstrate that Treg cells condition APC-dependent, high levels of IL-10 production.

Treg cell suppressive capacity is reduced in the absence of IL-10 in APCs

To determine the importance of the high levels of IL-10 triggered by Treg cells, we sorted CD11b^+^ monocytes, Treg cells, and CD4^+^CD25^-^ T cells from wild-type and IL-10^-/-^ mice. We cocultured autologous Treg cells with T cells and CD11b^+^ cells in the presence of anti-CD3 mAb. As expected, IL-10^-/-^ and IL-10^-/-^/B7-H4^-/-^ Treg cells exhibited a dose-dependent 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^-/-^/B7-H4^-/-^ mice (n = 5; p < 0.05) (Fig. 2A). 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^-/-^/B7-H4^-/-^ 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 and data not shown). When the ratio between Treg cells (2 × 10^7^/ml) and CD4^+^CD25^-^ T cells (2 × 10^7^/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.

IL-10^-/-^ Treg cells or IL-10^-/-^ conventional T cells slightly affected Treg cell-mediated suppression (p > 0.05) (Fig. 2B). Our data suggest that APC-dependent, but not Treg cell-dependent IL-10, implicates in Treg cell-mediated suppression.

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

![Figure 1](http://www.jimmunol.org/)
Treg cells suppressive activity (Fig. 2). We asked whether Treg cell-conditioned APCs are distinct from conventional T cells. To this end, we incubated CD14+ 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+ cells pretreated with Treg cells, but not CD4+CD25+ T cells and medium, significantly suppressed T cell proliferation (Fig. 2A; $p < 0.05$) (Fig. 2A, C–E). These data suggest that Treg cells enable APC suppressive activity.

Induction of APC B7-H4 expression (Fig. 3). B7-H4 expression also was expressed by histogram (E). The data indicate that Treg cells enable APC suppressive activity.

To study the role of IL-10 in T cell suppression mediated by Treg cell-conditioned APCs, we incubated Treg cells, CD4+CD25+ T cells, or CD11b+ cells with anti-human IL-10 receptor. After being conditioned with Treg cells, CD4+CD25+ T cells and medium, significantly suppressed T cell proliferation (Fig. 3A; $n = 5$; $p < 0.05$) (Fig. 3A). The data indicate that Treg cells enable APC suppressive activity.

To determine whether Treg cells stimulate B7-H4 in different APC subsets, we cocultured Treg cells with MDCs or MDDCs. We showed that Treg cells significantly stimulated B7-H4 expression on MDCs and MDDCs (Fig. 3, D and E). The data indicate that Treg cells can stimulate B7-H4 expression on multiple APC subsets.

Induction of APC B7-H4 is IL-10-dependent (Fig. 4). We hypothesized that IL-10 may contribute to B7-H4 induction on APCs. To test this, we analyzed B7-H4 expression on CD14+ cells during coculture with Treg cells in the presence of neutralizing Ab against IL-10. Anti-human IL-10 partially but significantly decreased CD14+ 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. 4, B and C). Further, low concentrations of IL-10

![FIGURE 2](image1.png)

**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–/– APCs. Treg cells, CD4+CD25+ T cells and CD11b+ cells were sorted from wild-type and IL-10–/– mice. Variable concentration of Treg cells were cocultured with 2 × 10^5/ml CD4+CD25+ T cells and CD11b+ 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–/– vs IL-10+/+ mice. B, Treg-mediated suppression in different combinations of cells from IL-10–/– and IL-10+/+ mice. Treg-mediated suppression was analyzed with different combinations of cells sorted from IL-10–/– and IL-10+/+ 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% - $100 \times cpn$ values with Treg cells/cpn values without Treg cells. Different concentrations of Treg cells were used. Results were shown at 4 × 10^5/ml Treg cells. IL-10–/–, cells from IL-10–/– mice; W, cells from wild-type mice.

![FIGURE 3](image2.png)

**FIGURE 3.** Human Treg cells render CD14+ cells immunosuppressive. A, Treg cell-conditioned monocytes are suppressive. Fresh CD14+ cells were cultured with autologous Treg cells or CD4+CD25+ T cells or medium in the presence of soluble anti-CD3 and anti-CD28 mAbs for 72 h. CD14+ cells were sorted from this coculture. Different concentrations of the sorted CD14+ cells were further cultured with fresh CD4+CD25+ 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+ cells were initially incubated with anti-human IL-10 receptor. After being conditioned with Treg cells, CD14+ cells (4 × 10^5/ml) 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+CD25+ 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+ cells (C and D) and CD11c+ cells (D). B7-H4 expression also was expressed by histogram (E). Treg cell concentration was 1 × 10^6/ml for D and E.

![FIGURE 4](image3.png)

**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+ 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+ cells. B, IL-10 induces B7-H4 expression on APCs. 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 mAb 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 oligos 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 immunosuppressive 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

FIGURE 5. Suppressive activity of Treg-conditioned APCs is B7-H4 dependent. A, B7-H4 blocking oligos block monocyte B7-H4 mRNA. Blood monocytes were exposed to B7-H4 blocking oligos or control oligos for 24 h with or without IL-10. B7-H4 mRNA was detected by RT-PCR. n = 9. B, B7-H4 blocking oligos block IL-10-induced monocyte B7-H4 protein. Blood monocytes were exposed to B7-H4 blocking oligos and control oligos with IL-10 (10 ng/ml). Cell surface B7-H4 was detected by FACS analysis. Results were expressed as the mean percentage of positive cells ± SEM. C, B7-H4 blocking oligos block Treg-induced monocyte B7-H4 expression. Blood monocytes were exposed to B7-H4 blocking oligos, control oligos, and medium and were cocultured with Treg cells as described. B7-H4 expression was detected by FACS by gating on CD14+ cells. Results were expressed as the mean percentage of positive cells ± SEM. D, Oligos exposed, Treg-conditioned CD14+ cells were sorted and further subject to stimulating autologous CD4+CD25+ T cells as described. T cell proliferation was measured by thymidine incorporation. Results were expressed as the mean cpm values ± SEM.
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.

Acknowledgments

We thank Andrew S. Flies for technical assistance.

Disclosures

The authors have no financial conflict of interest.

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