

Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells

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Interleukin (IL)-4 is known to be the most potent cytokine that can initiate Th2 cell differentiation. Paradoxically, IL-4 instructs dendritic cells (DCs) to promote Th1 cell differentiation. We investigated the mechanisms by which IL-4 directs CD4 T cells toward the Th1 cell lineage. Our study demonstrates that the IL-4-mediated induction of Th1 cell differentiation requires IL-10 production by DCs. IL-4 treatment of DCs in the presence of lipopolysaccharide or CpG resulted in decreased production of IL-10, which was accompanied by enhanced IL-12 production. In IL-10-deficient DCs, the level of IL-12 was greatly elevated and, more importantly, the ability of IL-4 to up-regulate IL-12 was abrogated. Interestingly, IL-4 inhibited IL-10 production by DCs but not by B cells. The down-regulation of IL-10 gene expression by IL-4 depended on Stat6 and was at least partly caused by decreased histone acetylation of the IL-10 promoter. These data indicate that IL-4 plays a key role in inducing Th1 cell differentiation by instructing DCs to produce less IL-10.

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DCs, specialized APCs derived from BM precursors, carry out important functions during both innate and adaptive immune responses (1, 2). On exposure to stimuli such as infectious agents, immature DCs located in peripheral tissues capture the antigen and migrate to secondary lymphoid organs during maturation (3). Mature DCs express high levels of MHC class II and costimulatory molecules, and they are potent professional APCs to prime naive CD4 T cells (4). Activated DCs secrete both inflammatory and antiinflammatory cytokines, which regulates the immune response. Depending on the pattern of cytokines secreted during the interaction between DCs and T cells, the immune response can polarize toward either a cellular or a humoral response mediated by Th1 or Th2 cells, respectively (5, 6).

IL-4 is a pleiotropic immunomodulatory cytokine produced by Th2 lymphocytes, mast cells, and eosinophils (7). IL-4 is the key cytokine for generating Th2 effector cells from naive CD4 T cells (8). In addition, IL-4 activates B cells but inhibits macrophage activation, which results in humoral immunity (9, 10). Although IL-4, together with GM-CSF, is commonly used to differentiate DCs from BM precursors, the effect of IL-4 on DC maturation remains largely unknown. Studies have demonstrated that IL-4 enhances the production of IL-12p70 by DCs (11, 12). Because IL-12 is a potent cytokine directing Th1 cell differentia-

tion (13), IL-4 is suggested to have a positive role for Th1 cell differentiation via DCs. Indeed, it was reported that IL-4 can instruct DCs to promote the Th1 response, which depends on IL-12 (14). However, it is unclear how IL-4 induces IL-12 production in DCs that leads to Th1 cell differentiation. A possible mediator for this process is IL-10 because IL-10 inhibits IL-12 production by DCs (15). However, it remains unknown whether IL-10 is responsible for IL-4-mediated induction of the Th1 response.

We investigated the molecular mechanisms by which IL-4 directs Th1 cell differentiation. Our data showed that IL-4 up-regulates IL-12 while inhibiting IL-10 production by DCs, but not by B cells. The inhibition of IL-10 by IL-4 appears necessary for the induction of Th1 cell differentiation because the ability of IL-4 to induce IL-12 was lost in IL-10-deficient DCs. IL-4 inhibits IL-10 promoter activity, which depends on Stat6 and involves histone deacetylation of the IL-10 promoter. As a result, IL-4 plays a key role for Th1 cell differentiation by inhibiting IL-10 gene expression to up-regulate IL-12 production by DCs.

RESULTS AND DISCUSSION

IL-4 inhibits IL-10 while enhancing IL-12p70 production by BM-derived DCs (BM-DCs)

To examine the effects of IL-4 on DC maturation, we cultured BM cells with GM-CSF for

5 d, followed by stimulation with LPS in the presence or absence of IL-4 for 2 d. IL-4-treated DCs had similar populations of CD11c⁺CD11b⁺CD8 α ⁻B220⁻ DCs and expressed comparable levels of MHC II and the costimulatory molecules CD40, CD80, and CD86 as controls (unpublished data). When cytokine production was examined, DCs stimulated in the presence of IL-4 produced less IL-10 but more IL-12p70 (Fig. 1 A). IL-6 and TNF- α levels were not altered by IL-4 (Fig. 1 A). IL-4 treatment showed a similar effect on cytokine production from DCs stimulated by CpG (unpublished data), indicating that culture with IL-4 affects stimulation by both TLR4 and TLR9 ligands (16). Thus, IL-4-treated BM-DCs produce less IL-10 and more IL-12p70.

To determine whether the changes in protein levels correlate with their mRNA levels, BM-DCs were stimulated by LPS with or without IL-4 for 24 h, and the mRNA levels of IL-10 and IL-12 were measured using quantitative real-time RT-PCR (qRT-PCR). As shown in Fig. 1 B, IL-4 had little effect on the basal level, but considerably inhibited LPS-inducible IL-10 gene expression. In addition, the IL-12p35, but not the IL-12p40, mRNA level was enhanced by IL-4, which is consistent with a previous study (11). IL-6 expression was comparable with or without IL-4. Therefore, IL-4

reduces IL-10 and induces IL-12 production by regulating IL-10 and IL-12p35 mRNA expression.

Induction of Th1 cell differentiation by IL-4-treated DCs is IL-10 dependent

IL-10 is known to inhibit IL-12 expression (15). Therefore, it is possible that the induction of IL-12 by IL-4 is caused by the reduction of IL-10 production. To test this, we examined cytokine production by *IL-10*^{-/-} DCs. *IL-10*^{-/-} BM-DCs secreted more IL-12 and expressed higher IL-12p35 mRNA than control DCs on LPS stimulation (Fig. 2, A and B). More importantly, the effect of IL-4 on IL-12 production and IL-12p35 mRNA expression was abolished in *IL-10*^{-/-} DCs (Fig. 2, A and B). These data indicate that the up-regulation of IL-12 by IL-4 correlates with decreased IL-10 levels.

To confirm the role of IL-10 in IL-4-mediated Th1 cell differentiation, we tested the ability of DCs to differentiate CD4 T cells. Total BM cells were prepared from BALB/c WT and *IL-10*^{-/-} mice and cultured for 5 d in the presence of GM-CSF to generate BM-DCs. BM-DCs at day 5 were stimulated for 6 h with LPS alone or LPS with IL-4 and washed extensively to eliminate cytokines in the culture. CD4⁺ T cells from DO11.10 mice were enriched and dif-

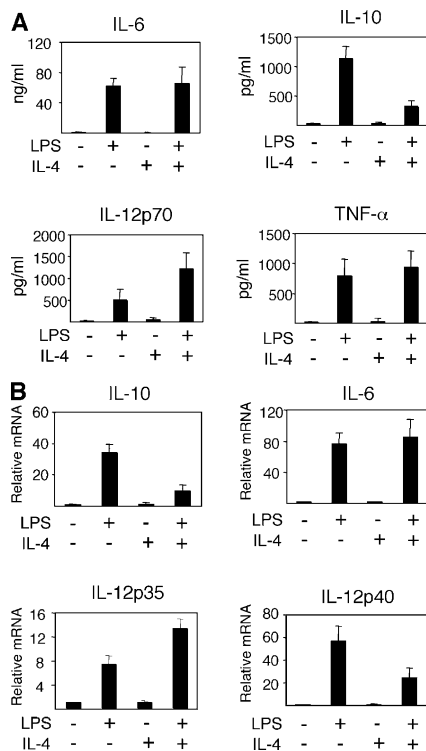


Figure 1. IL-4 inhibits IL-10 while enhancing IL-12 expression in BM-DCs. (A) BM-DCs from C57BL/6 mice were stimulated by LPS with or without IL-4 for 2 d. ELISA was performed to measure IL-6, IL-10, IL-12p70, and TNF- α production. (B) BM-DCs were stimulated for 24 h as indicated. qRT-PCR was performed to assess the amount of mRNA for IL-10, IL-6, IL-12p35, and IL-12p40. The relative mRNA levels were normalized to the GAPDH gene. Data are means \pm SE of at least three independent experiments.

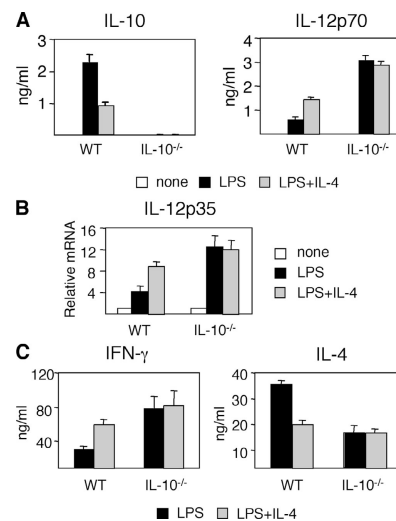


Figure 2. IL-4 up-regulates IL-12 and promotes Th1 cell differentiation by inhibiting IL-10. (A) BM-DCs were prepared from BALB/c WT and *IL-10*^{-/-} mice and stimulated with LPS alone or LPS with IL-4 for 1 d. ELISA was performed to detect IL-10 and IL-12p70 production. (B) BM-DCs from BALB/c WT and *IL-10*^{-/-} mice were activated for 6 h with the indicated stimuli. IL-12p35 mRNA expression was measured by qRT-PCR. (C) BM-DCs were generated from BALB/c WT and *IL-10*^{-/-} mice and treated with LPS alone or LPS with IL-4 for 6 h. 2×10^4 BM-DCs were then washed and added to 2×10^5 CD4⁺ T cells enriched from splenocytes of DO11.10 TCR-transgenic mice together with 0.01 μ M OVA peptide and 50 U/ml rIL-2. 5 d later, CD4 T cells were restimulated overnight with plate-bound anti-CD3 antibody. Supernatants were collected to measure IL-4 and IFN- γ production by ELISA. Data are means \pm SE of three independent experiments.

differentiated by providing OVA peptides as antigens and stimulated BM-DCs as APCs. 5 d later, live cells were restimulated overnight by anti-CD3 antibody, and IL-4 and IFN- γ production was measured.

Two interesting observations emerged. First, CD4 T cells primed by IL-4-treated BM-DCs from WT mice produced more IFN- γ and less IL-4 than cells primed by DCs treated without IL-4 (Fig. 2 C). In contrast, this effect of IL-4 was not observed when DCs from *IL-10*^{-/-} mice were used as APCs to prime CD4 T cells. Second, *IL-10*^{-/-} DCs were more potent to direct Th1 cell differentiation than WT DCs. This finding may be caused by the elevated IL-12 production by *IL-10*^{-/-} DCs, as shown in Fig. 2 A.

IL-4 inhibits IL-10 production by DCs but not by B cells

IL-10 is produced not only by DCs but also by other APCs, including B cells (17). In addition, IL-4 activates B cells and induces Ig isotype switching (7). Therefore, we asked whether IL-10 production by B cells is also suppressed by IL-4. Splenic B cells were purified and cultured in the presence of LPS alone or LPS with IL-4 for 2 d. As a control, we also stimulated purified splenic DCs with LPS or CpG in the presence or absence of IL-4. Consistent with BM-DCs, splenic DCs produced less IL-10 if IL-4 was added to the culture (Fig. 3 A). On the contrary, IL-4 did not inhibit IL-10 production by B cells. Rather, the IL-10 level was increased in the presence of IL-4 (Fig. 3 B). Thus, the inhibitory effect of IL-4 on IL-10 production is specific for DCs and the molecular mechanisms governing IL-10 expression must be distinct between DCs and B cells.

Stat6 is required for IL-4-mediated inhibition of IL-10

To further ascertain how IL-4 regulates IL-10 gene expression in DCs, we tested the role of Stat6, which is a primary molecule mediating the IL-4 signal (18, 19). *Stat6*^{-/-} BM-DCs produced an equivalent level of IL-10 protein, as well as mRNA, in response to LPS (Fig. 4, A and B). However, unlike DCs from the control mice, IL-4 was unable to inhibit IL-10 expression in *Stat6*^{-/-} DCs (Fig. 4, A and B). Therefore, Stat6 is required for the inhibitory effect of IL-4 in DCs.

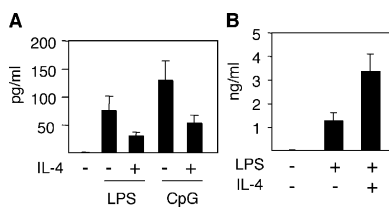


Figure 3. IL-4 inhibits IL-10 production by splenic DCs, but not by splenic B cells. CD11c⁺ DCs (A) and B220⁺ B cells (B) were enriched from the spleen of C57BL/6 mice using magnetic selection and stimulated with LPS or CpG in the presence or absence of IL-4, as described in Materials and methods. ELISA was performed to detect IL-10 production. Data are means \pm SE of three independent experiments.

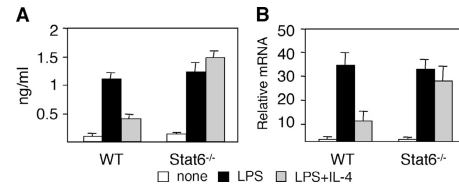


Figure 4. IL-4 inhibits IL-10 gene expression in a Stat6-dependent manner. BM-DCs from C57BL/6 WT and *Stat6*^{-/-} mice were stimulated by LPS with or without IL-4 for 24 h. ELISA and qRT-PCR were performed to detect IL-10 protein (A) and mRNA (B), respectively. Data are means \pm SE of three independent experiments.

IL-4 down-regulates IL-10 promoter activity by decreasing histone acetylation

There are at least two possibilities that can explain the decrease of IL-10 mRNA by IL-4: decreased mRNA stability or transcription. To distinguish these possibilities, we first measured IL-10 mRNA half-lives and found that there was no difference in IL-10 mRNA half-life with and without IL-4 (Fig. 5 A). We next examined IL-10 promoter activity in the DC cell line DC2.4 using a transient transfection method. DC2.4 cells were transfected with the luciferase reporter driven by the IL-10 promoter, followed by stimulation with LPS in the presence or absence of IL-4. Luciferase activity was enhanced by LPS but not by LPS together with IL-4, indicating that IL-4 down-regulates IL-10 promoter activity in DCs (Fig. 5 B).

To confirm that IL-4 has a similar effect on the endogenous IL-10 promoter, we examined the histone acetylation status of the IL-10 promoter by chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 5 C, histone acetylation of the IL-10 promoter was enhanced in LPS-treated BM-DCs. The induction of histone acetylation by LPS was compromised by IL-4 in WT DCs, but not in *Stat6*^{-/-} DCs (Fig. 5 C).

IL-4 inhibits the LPS-inducible, but not the basal, level of IL-10 expression (Fig. 1 B and Fig. 5 B), suggesting cross talk between IL-4-induced and LPS-mediated signaling pathways. Mitogen-activated protein kinase and NF- κ B signals have been demonstrated to be involved in DC activation and cytokine production (16). However, IL-4 did not affect LPS-mediated mitogen-activated protein kinase or NF- κ B activation in BM-DCs (unpublished data). This finding is not surprising because IL-4 did not have a global inhibitory effect on cytokine production by DCs. It is not clear how IL-4 down-regulates IL-10 gene expression via Stat6. We suspect that IL-4-activated Stat6 may compete with other transcription factors and/or cofactors that are essential for LPS-inducible expression of the IL-10 gene in DCs. Whatever the mechanisms might be, IL-4-mediated regulation of IL-10 in DCs could play an important role in mounting a proper Th response. However, we cannot rule out the possibility that IL-4 may affect additional DC functions that have not been investigated.

A recent study has demonstrated that IL-4 instructs Th1 responses, which in turn protects mice from *Leishmania major*

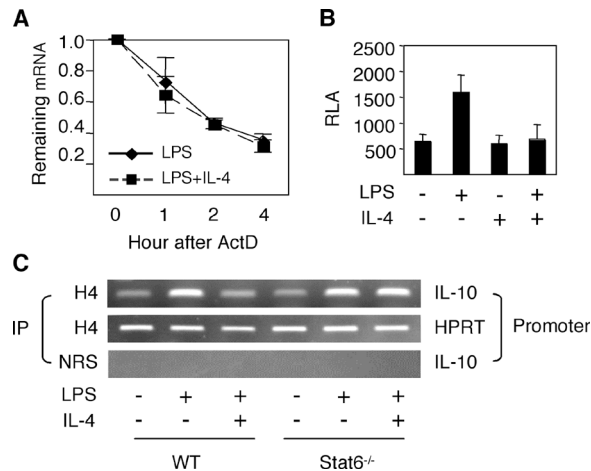


Figure 5. IL-4 inhibits LPS-inducible IL-10 gene expression.

(A) BM-DCs from C57BL/6 mice were stimulated 6 h with LPS alone or LPS with IL-4, followed by a 5- μ g/ml actinomycin D treatment to stop RNA synthesis. The remaining IL-10 mRNA levels were measured by qRT-PCR at the indicated time points. (B) DC2.4 cells were transfected with the IL-10 promoter-driven luciferase reporter, stimulated by 2.5 μ g/ml LPS with or without 10 ng/ml IL-4 overnight, and harvested to assess luciferase activity. Relative luciferase activity was normalized by protein concentrations. (C) BM-DCs from C57BL/6 and *Stat6*^{-/-} mice were stimulated by LPS with or without IL-4 for 90 min. ChIP was performed using Abs specific for the acetylated histone H4 or normal rabbit serum. Purified DNA fragments were amplified using primers specific for the IL-10 or the HPRT promoter, as described in Materials and methods. Data are means \pm SE (A and B) or representative (C) of at least three independent experiments.

infections (14). Interestingly, the authors elegantly showed that the effect of IL-4 in eliciting the Th1 response is limited to the initial stage of infection, although the mechanisms for this observation were not provided. Similarly, other studies also have shown initial Th1 responses during the early stage of infection with *L. amazonensis* or *Schistosomiasis mansoni* in *IL-10*^{-/-} mice (20, 21). Despite this early response, parasites persisted in chronic lesions with normal Th2 responses in the absence of IL-10 (20, 21). We showed that IL-4 inhibits IL-10 production by both splenic and BM-DCs, but not by B cells, on LPS stimulation (Fig. 3). This suggests that all DCs share common regulatory mechanisms to produce IL-10. Given the potency of DCs as APC-activating naive CD4 T cells, the presence of IL-4 during the initial phase of the immune response would favor the generation of Th1 cells. B cells primarily reside in the secondary lymphoid organs, which prevents them from encountering antigens early in the response. If an infection progresses, B cells would be activated and be able to present antigens to naive CD4 T cells. In B cells, however, IL-4 does not inhibit IL-10 production. Instead, IL-4 would help B cells to produce more IL-10 and skew the immune response toward Th2 cells. Therefore, a shift from Th1 to Th2 responses by IL-4 is at least partly caused by a change in the cell type presenting antigens to T cells.

With this scenario, one would expect to see a dominant Th1 response in the absence of B cells. Indeed, DCs produced

more IL-12 in the absence of B cells, resulting in Th1 cell deviation (22). This study also showed that B cells regulate the capacity of DCs to promote IL-4 secretion, further enhancing the Th2 response. Therefore, there is a negative feedback loop of cytokine production controlled by different APCs. In addition, B cell-deficient mice did not show recovery from disease in a Th1 cell-dependent model of experimental autoimmune encephalomyelitis (23). Experimental autoimmune encephalomyelitis recovery was dependent on IL-10 production by B cells. In the absence of B cells, DCs would be the primary APC-activating CD4 T cells, generating pathogenic Th1 cells and enhancing the severity of the disease. It is likely that the presence of both DCs and B cells is important to balance Th1 and Th2 cell generation and, moreover, proper temporal regulation of cytokine production for an effective immune response.

In conclusion, we showed that IL-4 directs DCs to produce less IL-10, resulting in more IL-12 production to promote the Th1 response. Therefore, IL-4 has a dual role for Th cell differentiation, and the type of APCs present during the initial activation of CD4 T cells appears critical for the CD4 T cell effector function.

MATERIALS AND METHODS

Mice and cells. C57BL/6, BALB/c, and IL-10-deficient (*IL-10*^{-/-}) mice were purchased from the Jackson Laboratory. *Stat6*^{-/-} mice were as previously described (18). All mice were maintained under specific pathogen-free conditions at the Indiana University School of Medicine animal facility.

BM-DCs were prepared as previously described (24). In brief, total BM cells depleted of T and B cells were cultured for 5 d in RPMI 1640 with 5% FBS and 10 ng/ml murine rGM-CSF (BD Biosciences). These cells were considered immature DCs. Immature DCs were replated at 10⁶ cells/ml and matured in the presence of 1 μ g/ml LPS (*Escherichia coli* O55:B5 serotype; Sigma-Aldrich) or 2 μ g/ml CpG (TCCATGACGTTCTGATGCT) with or without 10 ng/ml murine rIL-4 (BD Biosciences) for up to 2 d.

Splenic DCs and B cells were isolated by positive selection with anti-CD11c and anti-B220 magnetic beads (Miltenyi Biotec), respectively. Splenic DCs were activated for 24 h using the same conditions as BM-DCs. Splenic B cells were cultured at a density of 10⁶ cells/ml in RPMI 1640 with 10% FBS and stimulated for 48 h by 4 μ g/ml LPS with or without 5 ng/ml rIL-4.

FACS analysis. Abs used for flow cytometry, CD11c (clone HL3), CD11b (clone M1/70), CD8 α (clone 53-6.7), CD45R (B220; clone RA3-6B2), CD40 (clone 3/23), CD80 (B7-1; clone 16-10A1), CD86 (B7-2; clone GL-1), and MHC class II (I-A^b; clone AF6-120.1), were obtained from BD Biosciences. Flow cytometric analysis was performed using FACS-Calibur and analyzed using CellQuest software (BD Biosciences).

ELISA. Cytokine concentrations in the culture supernatants were detected by ELISA as previously described (25). Purified anti-mouse capture and biotinylated detection Abs were IL-6 (P5-20F3, MP5-32C11), IL-10, (JES5-2A5, SXC-1), IL-12p70 (9A5, C17.8), TNF- α (G281-2626, MP6-XT3), IFN- γ (R4-6A2, XMG1.2), and IL-4 (11B11, BVD6-24G2). All Abs were obtained from BD Biosciences.

qRT-PCR. Total RNA was prepared using Trizol (Invitrogen), and cDNA was prepared as previously described (26). qRT-PCR was performed by the comparative threshold cycle (Δ C_T) method and normalized to GAPDH. The primers used for IL-10, IL-6, and GAPDH were as described previously (24). The primers used for IL-12p35 were 5'-CCTCAGTTTG-GCCAGGGTC-3' and 5'-CAGGTTTCGGGACTGGCTAAG-3' and for IL-12p40 were 5'-GGAAGCACGGCAGCAGAATA-3' and 5'-AACT-TGAGGGAGAAGTAGGAATGG-3'.

Transient transfections and luciferase assays. DC2.4 cells were maintained in RPMI 1640 with 10% FBS, 50 μ M 2-ME, 2 mM L-glutamine, and 100 μ g/ml penicillin and streptomycin. 10^6 cells were transiently transfected with the 802-bp IL-10 promoter-driven luciferase reporter plasmid (provided by M. Tone, University of Pennsylvania, Philadelphia, PA, and H. Waldmann, University of Oxford, Oxford, UK; reference 27) using the Lipofectamine Plus reagents (Invitrogen). 4 h after transfection, cells were divided into four wells and rested 3 h before receiving different treatments overnight. Cell lysates were prepared and used for luciferase assays as previously described (25). Relative luciferase activity was normalized by protein concentration.

ChIP assay. ChIP assays were performed essentially according to Upstate Biotechnology's protocol. In brief, 10^7 cells were treated for 10 min with 1% formaldehyde to cross-link DNA binding proteins to the DNA and were lysed in SDS-containing buffer. Cell extracts were sonicated to shear DNA to \sim 500 bp and immunoprecipitated overnight with Ab specific for acetylated histone H4 (Upstate Biotechnology). The recovered protein-nucleic acid complexes were incubated for 4 h with 0.4 M sodium chloride at 65°C to reverse cross-links. Purified DNA fragments were amplified 30 cycles using PCR and analyzed on 1.5% agarose gels. Immunoprecipitations with normal rabbit serum served as a negative control and PCR for the proximal promoter of the mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) gene was used as an internal control. The primers used for the IL-10 promoter were 5'-GGCAC-CAGAACTCTCCTCTG-3' and 5'-TGGGTTGAACGTCGGATATT-3' and for the HPRT promoter were 5'-CTGCCTCTGCCTCCTAAATG-3' and 5'-CTCCAGAGGATTCCCAGAT-3'.

In vitro antigen-specific CD4⁺ T cell priming. CD4⁺ T cells were enriched from total splenocytes of DO11.10 TCR transgenic mice using anti-CD4 magnetic beads (Miltenyi Biotec). BM-DCs from BALB/c WT and *IL-10*^{-/-} mice were matured by LPS with or without IL-4 for 6 h and extensively washed before adding to enriched CD4⁺ T cells with 0.01 μ M OVA₃₂₃₋₃₃₉ peptide (Peptides International) and 50 U/ml human rIL-2. After 5 d, cells were restimulated with plate-bound anti-CD3 (145-2C11) antibody overnight. ELISA was performed to detect IFN- γ and IL-4 production.

We thank Drs. Masahide Tone and Herman Waldmann for providing the mouse IL-10 promoter-reporter plasmid. We also thank Brian McCarthy for technical help and Kevin Nickerson for helpful discussions.

C.-H. Chang was supported by National Institutes of Health grants AI45811 and AI56097.

The authors have no conflicting financial interests.

Submitted: 10 February 2005

Accepted: 26 April 2005

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