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Enhanced Production of IL-10 by Dendritic Cells Deficient in CIITA

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Dendritic cells (DC) are professional APCs that play a critical role in regulating immunity. In DC, maturation-induced changes in MHC class II expression and Ag presentation require transcriptional regulation by CIITA. To study the role of CIITA in DC, we evaluated key cell functions in DC from CIITA-deficient (CIITA−/−) mice. The ability to take up Ag, measured by fluid phase endocytosis, was comparable between CIITA−/− and control DC. Although CIITA−/− DC lack MHC class II, they maintained normal expression of costimulatory molecules CD80, CD86, and CD40. In contrast, CIITA−/− DC activated with LPS or CpG expressed increased IL-10 levels, but normal levels of TNF-α and IL-12 relative to control. Enhanced IL-10 was due to greater IL-10 mRNA in CIITA−/− DC. Aβ−/− DC, which lack MHC class II but express CIITA normally, had exhibited no difference in IL-10 compared with control. When CIITA was cotransfected with an IL-10 promoter-reporter into a mouse monocyte cell line, RAW 264.7, IL-10 promoter activity was decreased. In addition, reintroducing CIITA into CIITA−/− DC reduced production of IL-10. In all, these data suggest that CIITA negatively regulates expression of IL-10, and that CIITA may direct DC function in ways that extend beyond control of MHC class II.


Abbreviations used in this paper: β-gal, β-galactosidase; DC, dendritic cell; qRT-PCR, quantitative real-time RT-PCR; ΔCt, comparative threshold cycle; BLS, bare lymphocyte syndrome.

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Materials and Methods

Mice and cells

CIITA <−/− mice were as previously described (17). β<sup>2</sup>-<sub>M<sup>−/−</sub></sup> and C57BL/6 mice were purchased from Taconic Farms and The Jackson Laboratory, respectively. All mice were maintained under specific pathogen-free conditions at the Indiana University School of Medicine animal facility.

Bone marrow-derived DC were prepared as previously described (19). Briefly, total bone marrow cells were extracted from mouse femurs and tibiae and depleted of erythrocytes, T cells, B cells, and MHC class II-positive cells. Bone marrow precursors were then cultured for 5 days in RPMI 1640 supplemented with 5% FBS, 10 ng/ml recombinant mouse GM-CSF (BD Pharmingen) and 20 μg/ml gentamycin. After 48 h of culture, nonadherent cells were removed carefully and fresh media were added. After 5 days of culture, immature DC were disaggregated by pipetting and mature DC were generated by REPLATING 1 × 10<sup>5</sup> cells/ml in the presence of 2.5 μg/ml LPS (E. coli O55:B5 serotype; Sigma-Aldrich) or 2 μg/ml CpG (cytosine/phosphate/guanine dinucleotide-rich sequence: TC CATGACGGCTCTGGATGCTC) for 2 days. Cells cultured for 7 days were used as immature DC. Splenic DC were isolated by positive selection with CD11c magnetic microbeads (Miltenyi Biotech). Following isolation, splenic DC were cultured at a density of 5 × 10<sup>5</sup> cells/ml in RPMI 1640 supplemented with 5% FBS and activated for 24 or 48 h in the presence of 2.5 μg/ml LPS.

Splenic B cells were enriched by positive selection with B220 magnetic microbeads (Miltenyi Biotech). B cells cultured at a density of 3 × 10<sup>5</sup> cells/ml in RPMI 1640 with 10% FBS and stimulated for 24 or 72 h with 5 ng/ml recombinant mouse IL-4 (BD Pharmingen), and 4 μg/ml LPS (Sigma-Aldrich).

Phoenix-ECO cells were maintained in DMEM (Invitrogen Life Technologies) with 10% FBS and 100 mM HEPES. RAW 264.7 cells were maintained in DMEM with 10% FBS and 100 mM HEPES (Sigma-Aldrich).

Cytofluorometric analysis

For cell surface staining, Abs specific for mouse CD11c (integrin α<sub>m</sub> and α<sub>m</sub> chains, clone HL3), CD11b (Mac-1/intergxin α<sub>m</sub> chain, clone M1/70), CD45R (B220, clone RA3-6B2), CD40 (clone 3/23), CD54 (ICAM-1, clone 3E2), CD80 (B7-1, clone 16-10A1), CD86 (B7-2, clone GL-1), MHC class I (H-2K<sup>B</sup>, clone AF6-88.5), and MHC class II (I<sub>A</sub><sup>β</sup>/I<sub>A</sub><sup>α</sup>, clone AF6-120.1) were obtained from BD Biosciences. Flow cytometric analysis was performed using a FACSscan or FACSCalibur and analyzed using CellQuest software (BD Biosciences).

FITC-dextran uptake

DC from C57BL/6 or CIITA<−/− mice were differentiated as described above, except for maturation stimulus. LPS (250 ng/ml), recombinant mouse TNF-α (10 ng/ml; BD Pharmingen), and IL-4 (10 ng/ml; BD PharMingen), to obtain mature DC. DC were collected by pipetting, and 2.5 × 10<sup>5</sup> cells were resuspended in 250 μl of media containing 25 μg/ml FITC-dextran (40 kDa; Sigma-Aldrich). After 90 min of incubation with the FITC-dextran at 4°C or 37°C, cells were washed three times with ice-cold PBS with 1% FBS, then stained for cell surface CD11c, and analyzed by FACS. FSC-high and CD11c-positive cells were gated, and the fraction of FITC-positive cells was calculated. Values were normalized to the C57BL/6 immature DC sample (set as 1). Within each experiment, triplicates of each sample were incubated and stained independently.

Cytokine assays

ELISAs were used to assay for IL-6, IL-10, IL-12 p40/70, IL-12 p70, and TNF-α in culture supernatants as previously described (6). Purified anti-mouse capture and biotinylated detection Abs were as follows: IL-10: JES5-2A5, SXC-1; IL-12 p40/70: C15.6, C17.8; IL-12 p70: 9A5, C17.8; IL-6: P5-20F3, MPS-32C11; TNF-α: G281-2626, MP6-X3 (BD PharMingen). Tertiary detection was performed with avidin-conjugated alkaline phosphatase (A7294; Sigma-Aldrich) and p-nitrophenyl phosphate substrate (104-0; Sigma-Aldrich). A standard curve for each assay was generated with a known concentration of murine recombinant cytokines (BD PharMingen).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was prepared using TRIzol (Invitrogen Life Technologies), and cDNA was prepared as described (20). Quantitative real-time PCR of mouse and IL-10 and IL-6 was performed by the comparative threshold cycle (ΔC<sub>T</sub>) method and normalized to mouse GAPDH as described (11). Mouse IL-10 primer sequences and concentrations were as follows: forward: 5′-ATTGGAATCTCCGGTGAAG-3′ (300 nM), reverse: 5′-CACAGGGGGAATCTGATGCA-3′ (300 nM); product size, 75 bp. Mouse IL-6 primer sequences and concentrations were as follows: forward: 5′-GGCCCTCTCCTACTCACAAG-3′ (300 nM), reverse: 5′-ATTCTCA CGATTTCCAGAG-3′ (300 nM); product size, 125 bp.

DNA transfections for IL-10 reporter assays

The following DNA constructs were as described previously: FLAG-tagged type I and type III CIITA, and the expression vector pcDNA3 (15); CIITA domain mutants encoding residues 1–331, 408–857, and 990–1130 (7); the D1 luciferase reporter construct containing the mouse IL-10 promoter (802 bp) in pG-L-3 Basic vector (21, 22); CMV promoter-driven β-galactosidase (β-gal) (6).

For functional studies of promoter activity, 7.5 × 10<sup>5</sup> RAW 264.7 cells were transiently transfected with Lipofectamine and Lipofectamine Plus reagents (Invitrogen Life Technologies) using 1 μg of IL-10 promoter, 1 μg β-gal reporter and 1 μg type I or type III CIITA, or vector. Three hours after transfection, media was changed and cells were pooled together, then split into two samples. Cells were rested for 3 h, then left in culture (unstimulated) or stimulated with 2.5 μg/ml LPS for 20 h. Cell lysates were prepared and used for luciferase and β-gal assays as described (6). Relative luciferase activity was calculated using the luciferase activity of cells transfected with reporter DNA alone. Each transfection was performed in duplicate and values represent the average of at least three transfections.

Preparation of retroviruses and retroviral transduction of primary DC

Retroviral constructs expressing eGFP (RV-GFP) and eGFP with type III CIITA (RV-GFP/type III CIITA) in the MSCV2.2 vector were generated as described (11). To generate type I CIITA-expressing retrovirus (RV-GFP/type I CIITA), the type I isoform of CIITA (15) was cloned into the Xhol site of the modified MSCV2.2 vector containing the internal ribosome entry site (IRES) of eGFP. Retrovirus was generated in Phoenix-Eco packaging cells as previously described (11). Briefly, Phoenix-Eco cells were transiently transfected with RV-CIITA/GFP (type I or type III) or RV-GFP using the CaPO<sub>4</sub> method (23). The transduced Phoenix-Eco cells were cultured at 37°C for 12–18 h, then at 32°C for an additional 24 h to allow efficient viral production. The supernatants containing either the GFP or CIITA/GFP viruses were filtered through a 0.45-μm syringe filter (Millipore) and used immediately to transduce differentiating primary DC at day 1 of DC culture.

Retroviral transduction was performed by adapting a previously described method (24, 25). Virus was supplemented with 8 μg/ml polybrene (hexadimethrine bromide; H-9268; Sigma-Aldrich) and added to DC after 1 day of DC culture. Differentiation of BM-derived DC was performed by REPLATING 1 × 10<sup>5</sup> cells/ml in RPMI 1640 with 5% FBS and rested for 12–14 h in culture. DC were then matured for 48 h by the addition of 2.5 μg/ml LPS. After 2 days of stimulation, supernatants were analyzed by ELISA.

Results

Expression of cell surface molecules by CIITA<−/− DC

To analyze CIITA<−/− DC, we first examined DC from the bone marrow (BM) of CIITA<−/− mice. Bone marrow-derived DC from control (C57BL/6) or CIITA<−/− mice were differentiated for 5 days in the presence of GM-CSF, matured for 2 days with LPS, then analyzed for expression of cell surface markers, including costimulatory molecules. As shown in Fig. 1A, the CIITA<−/− DC had similar populations of CD11c<sup>+</sup>, CD11b<sup>+</sup>, and CD8α<sup>+</sup> DC as control DC, suggesting that GM-CSF-mediated differentiation of BM-derived DC can occur despite the absence of CIITA. CIITA<−/− DC also expressed wild-type levels of MHC class I, the costimulatory molecules CD40, CD80 (B7-1), and CD86 (B7-2), and CD54 (ICAM-1). MHC class II was absent from CIITA<−/− DC.
Ag uptake by CIITA^{−/−} DC

Before presenting exogenous Ags on MHC class II, DC must first internalize them. Thus, we next tested fluid-phase endocytosis of FITC-labeled dextran as a measurement of Ag uptake. Immature and mature bone marrow-derived DC from control (C57BL/6) or CIITA^{−/−} mice were incubated with FITC-dextran, with incubation at 4°C used as a baseline for nonspecific binding. We found uptake of FITC dextran by immature CIITA^{−/−} DC at 37°C occurred at the same level as that of immature control DC (Fig. 1, A and C). DC exhibit a characteristic decrease in Ag uptake upon maturation. We also observed this decrease in both control and CIITA^{−/−} DC, suggesting that CIITA^{−/−} DC are also capable of down-regulating Ag uptake function during the phenotypic shift from immature to mature DC.

Cytokine production by CIITA^{−/−} DC

Cytokine production by DC is an important component of T cell activation and immune signaling. Cytokines produced by DC can serve to activate T cells and direct the course of T cell differentiation and immune responses. To determine whether the cytokine profile of CIITA^{−/−} DC differs from that of wild type, we measured the amount of IL-10 and TNF-α released by LPS-stimulated bone marrow-derived DC from control (C57BL/6) or CIITA^{−/−} mice. Although comparable levels of TNF-α were observed for control and CIITA^{−/−} DC, the CIITA^{−/−} DC produced about twice as much IL-10 protein (Fig. 2A, top panels). Because production of IL-10 has been associated with decreased IL-12 production (26, 27), we also examined production of IL-12 (p40 subunit...
and assembled p70). Similar levels of IL-12 protein were produced by control and CIITA<sup>−/−</sup> DC (Fig. 2A, bottom panels).

Because CIITA is required for transcription of MHC class II, CIITA<sup>−/−</sup> mice also lack MHC class II (17). To determine whether the increased IL-10 production arises directly from the absence of CIITA or is a secondary effect of MHC class II deficiency, we also compared cytokine production by DC from AB<sup>−/−</sup> mice, which lack MHC class II but express normal levels of CIITA. The levels of IL-10, IL-12, and TNF-α protein produced by AB<sup>−/−</sup> DC were similar to those from control DC (Fig. 2A). Thus, the increase in IL-10 protein is associated with the absence of CIITA, not MHC class II.

To determine whether differences in IL-10 protein could be attributed to differences in mRNA, we also compared IL-10 mRNA levels in BM-derived DC from control (C57BL/6) and CIITA<sup>−/−</sup> mice. Using qRT-PCR, we observed a 2-fold increase in IL-10 mRNA from CIITA<sup>−/−</sup> DC relative to control (Fig. 2B). In contrast, IL-6 mRNA levels were similar in both samples (Fig. 2B). Thus, the increased IL-10 protein correlates with an increase in IL-10 mRNA.

So far, our data suggest that CIITA down-regulates IL-10 gene expression. If this is the case, an inverse correlation between CIITA and IL-10 gene expression in normal DC is expected. When we compared IL-10 and CIITA mRNA levels between immature and mature DC, the amount of IL-10 mRNA was increased upon maturation while CIITA expression declined.

**CIITA<sup>−/−</sup> DC express increased IL-10 upon stimulation with either LPS or CpG**

Maturation of DC is triggered by the recognition of pathogenic markers through pattern recognition receptors, the TLR, which are specific for unique products of bacteria and other pathogens (28). The quantity and combination of cytokines produced by mature DC can vary depending on the maturation stimulus received (29). We wished to determine whether CIITA<sup>−/−</sup> DC would also express increased IL-10 when matured with another activation stimulus, CpG. In contrast to LPS, a product of Gram-negative bacteria recognized by TLR4, unmethylated CpG motifs are present in viral and bacterial DNA and are recognized by TLR9 (29). As with LPS-stimulated DC, CIITA<sup>−/−</sup> DC stimulated by CpG exhibited enhanced production of IL-10 (Fig. 3). The relative increase between CIITA<sup>−/−</sup> DC and control was 2- to 3-fold for DC matured with either LPS or CpG. Both control and CIITA<sup>−/−</sup> DC matured with CpG exhibited decreased IL-10 and IL-6, but increased IL-12, relative to LPS-matured DC.

**Splenic DC from CIITA<sup>−/−</sup> mice do not express increased IL-10**

DC include several subsets that can be divided by developmental origin, cell surface phenotype, functional capacities, and localization within the body (29, 30). Bone marrow–derived DC are in vitro-differentiated cells and consist primarily of CD11c<sup>+</sup> CD11b<sup>+</sup> B220<sup>−</sup> DC of myeloid origin (30). In contrast, DC isolated from the spleen have differentiated in vivo and also include CD11b<sup>−</sup> and B220<sup>+</sup> populations of lymphoid origin (30). Having observed differences in IL-10 expression in CIITA<sup>−/−</sup> bone marrow–derived DC, we next asked whether other populations of CIITA<sup>−/−</sup> DC possessed the same phenotype. Splenic DC from CIITA<sup>−/−</sup> mice showed similar myeloid (CD11c<sup>+</sup>CD11b<sup>+</sup>) and lymphoid (CD11c<sup>+</sup>CD11b<sup>−</sup>) DC compared with control (Fig. 4, A–C). However, when we examined B220 expression in the two subpopulations, CIITA<sup>−/−</sup> spleen had consistently fewer CD11c<sup>+</sup>CD11b<sup>−</sup> B220<sup>−</sup> cells (Fig. 4B).

To assess IL-10 production by splenic DC, CD11c<sup>+</sup> cells were enriched and activated with either LPS or CpG. The total amount of IL-10 and IL-6 produced by splenic DC was much less than with the BM-derived DC (Fig. 4D). In contrast to BM-derived DC, splenic DC produced greater IL-10 when stimulated with CpG than with LPS, and CpG-stimulated splenic DC from CIITA<sup>−/−</sup> mice produced IL-10 at levels comparable to control (Fig. 4D). IL-10 from LPS-stimulated DC fell near or below the threshold of detection by ELISA and could not be accurately quantified (data not shown).

**CIITA<sup>−/−</sup> B cells also express increased IL-10**

Because CIITA<sup>−/−</sup> DC produce greater IL-10, we next wished to determine whether other types of APC would also express increased IL-10 in the absence of CIITA. Because B cells express IL-10 and CIITA, we tested production of IL-10 in CIITA<sup>−/−</sup> B cells. B220<sup>+</sup> cells were enriched from CIITA<sup>−/−</sup> or control (C57BL/6) splenocytes using magnetic microbeads and stimulated with LPS and IL-4 for 3 days. We found that the CIITA<sup>−/−</sup> B cells produce 2- to 3-fold more IL-10 protein than control B cells (Fig. 5A). Levels of IL-6 were comparable between the CIITA<sup>−/−</sup> and control B cells (Fig. 5A). To determine whether this difference correlated with changes in levels of IL-10 mRNA, we activated CIITA<sup>−/−</sup> or control B220<sup>+</sup> cells with LPS and IL-4 for 24 h, then assessed IL-10 mRNA levels using qRT-PCR. We observed a 2-fold increase in IL-10 mRNA in the CIITA<sup>−/−</sup> B cells relative to control (Fig. 5B). Greater IL-10 protein and mRNA were also expressed in CIITA<sup>−/−</sup> B cells activated with LPS alone, although the overall expression of IL-10 was much lower (data not shown). Overall, we observed enhanced expression of IL-10 in CIITA<sup>−/−</sup> B cells, with a fold increase on the same order as with DC.

To assess whether B cells also exhibit an inverse correlation between IL-10 and CIITA gene expression upon activation by LPS, we measured mRNA levels of IL-10 and CIITA using freshly isolated splenic B cells as well as activated B cells. Consistent with DC, IL-10 expression was induced but the CIITA gene was down-regulated in activated B cells (Fig. 5C).

**CIITA decreases IL-10 promoter activity**

We wanted to find out whether the difference observed in IL-10 expression could be attributed to the regulation of IL-10 promoter activity by CIITA. To study this, we used a luciferase reporter driven by 802 bp of the IL-10 promoter. For the transfection experiments, we chose the RAW 264.7 mouse monocyte/macrophage cell line, which has been used for previous studies with this
Il-10 reporter (21). When RAW 264.7 cells were transfected with the IL-10 reporter and type III CIITA, luciferase activity at the IL-10 reporter was decreased (Fig. 6). Cotransfection with type I or type III CIITA, luciferase activity at the IL-10 reporter was decreased (Fig. 6). Cotransfection with type I CIITA was capable of repressing the IL-10 promoter activity. Cotransfection of the acidic domain but not others inhibited IL-10 promoter activity (Fig. 6C).

Reintroduction of CIITA into CIITA−/− DC reduces IL-10 production

To provide further evidence in support of CIITA as a negative regulator of IL-10, we used a retroviral system to reintroduce CIITA into CIITA−/− DC. Bone marrow precursors from control or CIITA−/− mice were differentiated to DC and transduced with retroviruses expressing GFP alone, GFP and type III CIITA, or GFP and type I CIITA. Transduced DC were then sorted based on GFP and CD11c expression, matured with LPS, and analyzed. As shown in Fig. 7A, efficiency of transduction was comparable between the CIITA and the control viruses. Cells transduced with virus expressing CIITA were MHC class II positive, indicating that transducing CIITA was capable of inducing MHC class II expression (Fig. 7B). Consistent with our previous findings (15), we observed that transduction with the type I CIITA virus resulted in higher expression of cell surface MHC class II in mature cells. The difference in MHC class II levels was not observed in immature

FIGURE 4. Splenic DC from CIITA−/− mice express normal IL-10. A, Comparison of subpopulations in control and CIITA−/− splenic DC. CD11c+ DC were enriched from control (C57BL/6) or CIITA−/− mouse splenocytes using magnetic selection, then analyzed for CD11c+CD11b− and CD11c+CD11b+ subpopulations. Labels indicate each subpopulation as a percentage of live DC. B and C, Relative expression of B220 in subpopulations of control and CIITA−/− splenic DC. Control and CIITA−/− splenic DC from A were gated on CD11c+CD11b− (B) or CD11c+CD11b+ (C) cells and were analyzed for expression of cell surface B220. Labels indicate B220− cells as a percentage of gated DC. D, Comparison of IL-10 and IL-6 production by control and CIITA−/− splenic DC. CD11c+ splenic DC from control (C57BL/6) and CIITA−/− mice were enriched using magnetic selection, then plated at a density of 1 × 10^6 cells/ml and stimulated with CpG (2 μg/ml). After 1 day of stimulation, supernatants were harvested and analyzed by cytokine ELISA for IL-10 and IL-6. Data are the mean and SD of three independent experiments.

FIGURE 5. CIITA−/− B cells express increased IL-10 protein and mRNA. A, Comparison of IL-10 and IL-6 production by control and CIITA−/− B cells. B220+ B cells selected from control (C57BL/6) or CIITA−/− mouse splenocytes were enriched using magnetic selection, then plated at a density of 3 × 10^6 cells/ml and stimulated with LPS (4 μg/ml) and IL-4 (5 ng/ml). After 3 days of stimulation, supernatants were harvested and analyzed by cytokine ELISA for IL-10 and IL-6. Data are the mean and SD of three independent experiments. B, Comparison of IL-10 mRNA in control and CIITA−/− B cells. B220+ B cells were isolated as in A, stimulated with LPS (4 μg/ml) and IL-4 (5 ng/ml) for 24 h, and used to prepare RNA for qRT-PCR. IL-10 signals were normalized to GAPDH using the ΔCt method and expressed relative to control. Data are the mean and SD of two independent experiments. C, IL-10 induction correlates with decreased CIITA expression upon activation of splenic B cells. B220+ B cells from control mice were prepared as in B, then used to isolate RNA for qRT-PCR of IL-10 and CIITA. Expression was normalized to GAPDH and is shown relative to immature cells. Data are the mean and SD of two independent experiments.
cells, probably because DC sequester MHC class II protein within the cell until maturation.

We next tested IL-10 production to determine whether transduced CIITA would affect expression of IL-10. IL-10 production by CD11c<sup>GFP</sup>/H<sup>11001</sup> cells was reduced in DC infected with either of the CIITA viruses, relative to control (Fig. 7C, left panel). To confirm that this change was specific to IL-10, we also compared levels of IL-6 and IL-12 (p40/70) protein. IL-6 was comparable among all samples, while IL-12 was slightly increased with the CIITA-transduced DC (Fig. 7C). Nontransduced (CD11c<sup>GFP</sup>/H<sup>11002</sup>) DC populations showed similar levels of IL-10 production regardless of virus (data not shown).

**Discussion**

We observed that CIITA<sup>−/−</sup> DC were phenotypically similar to wild-type DC for certain indicators of maturation (expression of costimulatory molecules and production of cytokines other than IL-10). However, expression of IL-10 was increased in CIITA<sup>−/−</sup> BM-derived DC matured with either LPS or CpG, as well as in CIITA<sup>−/−</sup> B cells. Increased IL-10 production has many potential implications for the behavior of CIITA<sup>−/−</sup> DC. IL-10 is an immunomodulatory cytokine that plays a key role in down-regulating inflammatory responses (31). DC and other APC exposed to exogenous IL-10 during differentiation can exhibit altered maturation responses and decreased ability to stimulate T cells (32, 33). Although we were not able to test T cell activation by CIITA<sup>−/−</sup> DC
because of the lack of MHC class II, we were able to study cytokine production by BM-derived DC. In mature DC, IL-10 can inhibit production of IL-12 (27) and negatively affect DC capacity for T cell activation (31, 34, 35). Although we did not observe a difference in IL-12 production from CIITA<sup>−/−</sup> DC, we did find that CIITA<sup>−/−</sup> DC transduced with CIITA, which had decreased IL-10, produced slightly greater IL-12.

In addition to activating T cells, DC also regulate immunity by influencing polarization of T cells to Th1 or Th2, or by inducing a suppressor phenotype. Expression of IL-10 by DC can direct the polarization of naive T cells to T regulatory cells, as well as inhibit cytokine production and T cell proliferation (33, 36). Thus, it is possible that the increased IL-10 production by CIITA<sup>−/−</sup> DC could promote induction of regulatory T cells by DC, or produce altered cytokine production by interacting T cells.

Like CIITA<sup>−/−</sup> DC, CIITA<sup>−/−</sup> CD4<sup>+</sup> T cells exhibit aberrant expression of specific cytokines. In the case of CD4<sup>+</sup> T cells, this appears as increased production of Th2 cytokines by Th1-differentiated cells (6). It does not seem likely that the expression observed in T cells can be attributed to the effects of IL-10 production by CIITA<sup>−/−</sup> DC on activated T cells, because IL-10 alone is not capable of directing T cells toward a Th2 phenotype and no large differences were observed in expression of other cytokines. However, CIITA<sup>−/−</sup> APC also play an important role much earlier in the T cell life cycle when interacting with developing T cells in the thymus. Alternatively, it is also possible that the kinds of mechanisms produce abnormal expression of different cytokines in the two different cell types; that is, that the differences in cytokine production by T cells and DC represent different manifestations of the same underlying phenomenon.

Individuals with a genetic defect in the CIITA gene exhibit immunodeficiency primarily due to the lack of MHC class II and CD4<sup>+</sup> T cells and are known as bare lymphocyte syndrome (BLS) patients. Although it is not clear whether BLS patients produce more IL-10, it is possible that increased IL-10 partly contributes to immunosuppression that is often associated with BLS patients. However, it would be difficult to assess the role of IL-10 in the context of host immunity because Ag presentation function is impaired in BLS patients.

Splenic DC from CIITA<sup>−/−</sup> mice exhibited a slightly smaller subpopulation of CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup> DC, considered to be murine plasmacytoid DC (37, 38). The significance of this finding remains to be investigated. Differences in B220 expression by DC may indicate some differences in developmental origin caused by CIITA deficiency. Plasmacytoid DC express increased IFN-α and decreased IL-12; they are also poor T cell activators (30, 37–39). Like CIITA<sup>−/−</sup> DC, we did find that CIITA<sup>−/−</sup> APC also exhibited aberrant expression of specific cytokines.

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Splenic DC from CIITA<sup>−/−</sup> mice exhibited a slightly smaller subpopulation of CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup> DC, considered to be murine plasmacytoid DC (37, 38). The significance of this finding remains to be investigated. Differences in B220 expression by DC may indicate some differences in developmental origin caused by CIITA deficiency. Plasmacytoid DC express increased IFN-α and decreased IL-12; they are also poor T cell activators (30, 37–39). It is not clear whether the change in this population in CIITA<sup>−/−</sup> DC is related to aberrant cytokine expression, or represents an alteration in DC development.

Although CIITA<sup>−/−</sup> DC derived from bone marrow produced greater IL-10, splenic DC did not. This may reflect a developmental difference, because the BM-derived DC were cultured in vitro while splenic DC had differentiated in vivo. Origin of DC precursors may also account for the differences, because splenic DC represent multiple populations of DC from both myeloid and lymphoid origins. Indeed, a recent report showed that plasmacytoid DC differ from the rest of DC subsets with respect to expression of CIITA and MHC class II (40). Unlike other DC subsets, they do not down-regulate the CIITA gene upon maturation. Moreover, CpG treatment enhanced CIITA gene expression. Therefore, it is not surprising to see different mechanisms of IL-10 regulation between splenic DC and BM-derived DC. The limited number of DC obtained from spleen precludes studies of IL-10 expression by individual subsets, but it would be interesting to study cytokine production in the different populations.

Multiple means for regulating expression of IL-10 have been reported. On the level of transcriptional regulation, transcription of IL-10 requires binding of the ubiquitous transcription factors Sp1 and Sp3 to recognition sites in the IL-10 promoter (21, 41). The 3′-untranslated region of the IL-10 mRNA contains destabilizing sequences that negatively affect mRNA stability and production (21, 22). Additionally, multiple studies have found that inducing cAMP and protein kinase A can enhance production of IL-10 (42–44). We observed that the increase in IL-10 protein in CIITA<sup>−/−</sup> DC correlated with greater expression of IL-10 mRNA, which could be attributed to increased transcription of IL-10, greater message stability, or a combination of the two.

The two CIITA isoforms primarily expressed in DC, type I and type III, equally inhibited activity at the IL-10 promoter, shown in the transient transfection assay. Both isoforms decreased IL-10 expression in retrovirally transduced DC, with type I CIITA having slightly a greater effect. Interestingly, the effects of CIITA on IL-10 differ from CIITA-mediated repression of cathespin E, which can be mediated by type III but not type I CIITA (45). Thus, inhibition by CIITA seems to be mediated by a different mechanism depending on the target gene.

It is unclear whether the phenotype observed in CIITA<sup>−/−</sup> DC arises from specific regulatory effects of CIITA on IL-10 or as the result of altered DC development in the absence of CIITA. In the case of IL-4 and collagen α<sub>2</sub>, two of the other genes repressed by CIITA, inhibition is mediated by competing key coactivators away from the promoter (7, 8). Regulation of IL-10 could potentially arise from a more generalized regulation of the IL-10 locus; however, no such function has yet been reported for CIITA.

Overall, we found that while CIITA<sup>−/−</sup> DC express most of the characteristics of normal DC, they also exhibit a key difference, increased production of IL-10, which may affect how DC shape immune responses. We believe further understanding of how the absence of CIITA results in greater IL-10 expression and affects DC development will provide valuable insight into the function of CIITA, the processes that regulate IL-10, and the physiology of DC.