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Altered Th1 Cell Differentiation Programming by CIITA Deficiency

Dipak R. Patel,* Mark H. Kaplan,† and Cheong-Hee Chang‡‡

CD4 T cell differentiation is a complex process affected by many transcription factors interacting in a tightly regulated manner. We have previously shown that CIITA-deficient mouse Th1 cells expressed Th2-type cytokines, while IFN-γ expression was normal. In this study, we show that CIITA-deficient Th1 cells contain three distinct populations: cells secreting IL-4 alone, IFN-γ alone, and both IL-4 and IFN-γ together. This novel phenotype is stable over multiple rounds of stimulation in the presence of Th1-inducing factors. CIITA-deficient Th1 cells require TCR-mediated signaling to express Th2 cytokines, and this occurs with similar kinetics as wild-type Th2 cells. Both GATA-3 and IL-4 appear to be required for CIITA-deficient Th1 cells to express Th2-type cytokines. Interestingly, however, CIITA-deficient Th1 cells can produce IL-4 in the absence of exogenous IL-4. Introducing either CIITA or antisense GATA-3 during Th1 differentiation partially reduces Th2-type cytokine expression. With the exception of Th2-type cytokine expression, Th1 differentiation occurs normally in the absence of CIITA, as measured by expression of T-bet, IL-12Rβ2, IL-18Ra, and IFN-γ. Therefore, CIITA plays a key role to repress Th2-type cytokine expression as naive CD4 T cells differentiate toward the Th1 lineage. The Journal of Immunology, 2004, 173: 5501–5508.

Upon activation, naive CD4 T cells proliferate and differentiate to a Th1 or a Th2 effector phenotype. Many factors, including the extracellular cytokine environment, APCs, method of Ag delivery, and costimulation, influence the phenotype to which naive cells differentiate (reviewed in Ref. 1). However, the extracellular cytokine environment is generally considered to be the primary determinant of Th1/Th2 fate. Development of a Th1 phenotype is favored by IL-12, and Th1 cells secrete IFN-γ, IL-2, and lymphotoxin to mediate a cellular immune response capable of clearing intracellular pathogens. IL-4 promotes a Th2 phenotype, and Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 to mediate a humoral immune response capable of clearing extracellular pathogens. The development of an appropriate CD4 T cell-mediated immune response to a particular pathogenic challenge is essential to clearing infections and avoiding autoimmune or allergic reactions.

CD4 T cell differentiation is regulated by multiple transcription factors, though T-bet and GATA-3 are considered primary regulators of Th1 and Th2 development, respectively. T-bet is a Th1-specific transcription factor activated by IFN-γ signaling via STAT1 (2). T-bet then transactivates IFN-γ expression and up-regulates IL-12Rβ2 to reinforce Th1 development. Retroviral T-bet expression induces Th1 development from differentiating Th2 cells as well as from Th2 clones (3). T-bet-deficient mice lack normal Th1 immune responses and improperly express Th2 cytokines in response to Leishmania major infection (4, 5). GATA-3 is a Th2-specific transcription factor that is activated by IL-4 signaling in a Stat6-dependent and -independent manner (6–9). GATA-3 then cooperates with other factors, including c-maf and NF-AT, to reinforce IL-4 expression and subsequent signaling (10, 11). GATA-3 expression, either as a transgene or a retroviral vector, activates Th2 cytokine expression from developing Th1 cells (12, 13). Conversely, either antisense or dominant negative GATA-3 reduces Th2 cytokine expression from differentiated Th2 clones (14).

The MHC CIITA is the master regulator of MHC class II expression (15, 16). CIITA is required for MHC class II expression, as well as that of other proteins, such as invariant chain and H-2M, necessary for MHC class II-mediated Ag presentation (17, 18). CIITA-deficient (CIITA−/−) mice do not express MHC class II on peripheral APCs, and very low levels of MHC class II expression are detected in the thymus (19–21). As a result, CIITA−/− mice lack peripheral CD4 T cells and therefore exhibit a severe immunodeficiency. The effects of CIITA deficiency on CD4 T cell function have been studied in CIITA−/− mice that express the I-E MHC class II molecule as a transgene under the control of a MHC class I promoter (CIITA−/−IE) (22). CD4 T cells from CIITA−/−IE mice expressed comparable levels of TCR and other T cell surface molecules, indicating a normal phenotype (22). However, CIITA−/− Th1 cells secreted high levels of IFN-γ and, surprisingly, IL-4 as well. CIITA−/− Th2 cells produced IL-4 at levels equivalent to that of control Th2 cells (22). These results were obtained from naïve (CD45RBhighCD44low) CD4 T cells sorted by flow cytometry and then differentiated in vitro. Therefore, it is highly unlikely that either Th2 memory cells or NKT cells could be responsible for the phenotype. This suggested that CIITA participates in the process of CD4 T cell differentiation by regulating the expression of Th2 cytokines. However, it is still unclear whether CIITA functions through an intrinsic mechanism, or whether it alters CD4 T cell programming extrinsically by changing the selective environment in vivo.

Though mouse CD4 T cells do not express MHC class II, CIITA has been detected by PCR-based techniques in both naïve and activated cells (22–24). In naïve CD4 T cells, CIITA is expressed at levels 10–20 times lower than those in mouse B cells (24). It has

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been estimated that mouse B cells express only five CIITA transcripts per cell (25), implying that a fraction of naïve CD4 T cells express only one transcript per cell. Upon activation, Gourley et al. (22, 23) demonstrated CIITA expression in naïve and Th1, but not Th2 cells. In contrast, Otten et al. (24) observed CIITA primarily in naïve CD4 T cells, but not in either Th1 or Th2 cells. CIITA expression has not been reported by Western blot in either mouse B cells or CD4 T cells, most likely due to low protein levels unsuitable for detection by Western blotting.

In this study, we further characterized Th-specific gene expression in CIITA+/- Th1 cells. CIITA+/- Th1 cells produced Th2 cytokines with kinetics similar to that of control Th2 cells, and their phenotype was maintained even after multiple rounds of stimulation in the presence of Th1-inducing factors. Expression of many Th1-restricted genes was normal. Surprisingly, CIITA+/- Th1 cells contained subpopulations that secrete IFN-γ, IL-4, or both cytokines simultaneously. Introducing CIITA into developing CIITA-/- Th1 cells reduced Th2-type cytokines. The IL-4 gene, but not exogenous IL-4, was required by CIITA-/- Th1 cells expressing Th2 cytokines with kinetics similar to that of control Th2 cells, and their phenotype was maintained even after multiple rounds of stimulation in the presence of Th1-inducing factors. Expression of many Th1-restricted genes was normal. Surprisingly, CIITA+/- Th1 cells contained subpopulations that secrete IFN-γ, IL-4, or both cytokines simultaneously. Introducing CIITA into developing CIITA+/- Th1 cells reduced Th2-type cytokines. The IL-4 gene, but not exogenous IL-4, was required by CIITA+/- Th1 cells to express IL-5 and IL-13. Finally, Th2 cytokine expression from CIITA+/- Th1 cells is partly due to the presence of GATA-3, because their levels were reduced when antisense GATA-3 was expressed during Th1 differentiation.

Materials and Methods

Mice

CIITA+/-IE and IL-4+/- mice were previously described (22, 26). C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine (Indianapolis, IN) in accordance with institutional guidelines.

Preparation and stimulation of CD4 T cells

Total CD4 T cells were isolated (>90% purity) from the spleens and lymph nodes of mice using magnetic-positive selection with anti-CD4 beads according to manufacturer protocols (Miltenyi Biotec, Auburn, CA). Cells were cultured with 5 μg/ml plate-bound anti-CD3 (145-2C11), 1 μg/ml anti-CD28 (37.51), and 50 U/ml IL-2 (Roche, Indianapolis, IN) for 5 days. To induce Th1 differentiation, 3.5 ng/ml IL-12 and 10 μg/ml anti-IL-4 Ab (11B11) were added to the cultures. For Th2 differentiation, 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ Ab (R4-6A2) were added. Live cells were then isolated on a Ficol gradient (ICN Biomedical, Aurora, OH), washed, and restimulated for either ELISA or intracellular cytokine staining analysis.

Recombinant cytokines were purchased from BD Pharmingen (San Diego, CA), and neutralizing Abs were purified from hybridoma supernatants. For long-term cultures, cells received the primary stimulations described above. Cells were then restimulated with 1 μg/ml plate-bound anti-CD3, as well as IL-2 and the Th1/Th2-inducing factors at the same concentrations used for primary stimulation.

Cytokine assays

Cells received an overnight secondary stimulation of 5 μg/ml plate-bound anti-CD3 for ELISA analysis. Supernatants were analyzed for IFN-γ, IL-4, IL-5, and IL-13 using cytokine-specific mAbs (BD Pharmingen; and R&D Systems, Minneapolis, MN) according to manufacturer protocols. For intracellular cytokine staining, cells were stimulated at 1–1.5 × 10^6/ml with 50 ng/ml PMA and 1.5 μM ionomycin (Calbiochem, San Diego, CA) for 4 h. A total of 3 μM monensin (Sigma-Aldrich, St. Louis, MO) was added during the final 2 h of stimulation. Cells were stained for surface CD4, fixed with 4% paraformaldehyde, and permeabilized with 0.15% saponin (Sigma-Aldrich). Cells were then stained with anti-IFN-γ (XMG1.2) and anti-IL-4 (11B11; BD Pharmingen). Cells were analyzed on a FACSCalibur with CellQuest software (BD Biosciences, Mountain View, CA). IL-12Rβ2 was stained with hamster anti-mouse IL-12Rβ2, followed by fluorochrome-labeled anti-hamster Ab (BD Pharmingen). IL-18Rα was stained with biotinylated anti-mouse IL-18Rα (R&D Systems), followed by fluorochrome-labeled avidin (BD Pharmingen).

RNA preparation and quantitative real-time PCR

RNA was prepared with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Quantitative real-time PCR was performed as previously described (23). Primer sequences for IFN-γ, IL-4, IL-5, IL-13, and GAPDH were previously described (23, 27). Cytokine transcript levels in each sample were first normalized by subtracting GAPDH transcript levels from the same sample. Normalized transcript levels from each sample were then expressed as fold induction compared with normalized levels from the control Th1 (IFN-γ) or the control Th2 (IL-4, IL-5, and IL-13) samples.

Western blot

Protocols for the preparation of total cell lysates and immunoblotting were previously described (28). Abs used are as follows: anti-GATA-3 (H-33), anti-T-bet (4B10), anti-NF-ATc1 (7A6), anti-NF-ATc2 (4G6-G5) (all from Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (AC-15, Sigma-Aldrich).

Retroviral transduction

Bicistronic constructs encoding GFP and GFP with CIITA were described previously (23). The antisense GATA-3 construct was generated from a full-length (2.1 kb) murine GATA-3 cDNA kindly provided by Dr. W.-P. Zheng (University of Rochester, Rochester, NY). The cDNA was cloned into the bicistronic GFP retroviral vector, and the antisense orientation was verified by restriction analysis. The protocol for retroviral transduction was described previously (23). Briefly, CD4 T cells were stimulated 20–28 h under Th1-inducing conditions. Cells were spin infected with retroviral supernatant and cultured an additional 4–6 days in the presence of Th1-inducing cytokines. GFP+ CD4 T cells were sorted by FACS, expanded overnight with 50 U/ml IL-2 and 3.5 ng/ml IL-12, and then stimulated overnight with 5 μg/ml anti-CD3 for ELISA analysis.

Results

CIITA+/- Th1 cells exhibit a normal phenotype, and also express Th2-type cytokines

Previously, we reported that naïve CIITA+/- CD4 T cells secrete IL-4 when differentiated in vitro to a Th1 phenotype, and that this is due to transcription of the IL-4 gene (22). To better understand the molecular mechanisms for these phenomena, we analyzed CIITA+/- Th1 cells in greater detail. A mature CD4 T cell population does not exist in CIITA+/- mice, but expressing the I-E MHC class II transgene (CIITA+/-IE) restores the CD4 T cell compartment (22). Therefore, we used CIITA+/-IE mice as the source of CIITA-/- CD4 T cells. CIITA+/-IE CD4 T cells are referred to as CIITA-/- CD4 T cells in all experiments.

Consistent with the previous data (22), CIITA-/- Th1 cells produced both IFN-γ and Th2-type cytokines after secondary stimulation (Fig. 1A). This was due to transcription of Th2-type cytokine genes, as shown by quantitative real time RT-PCR (Fig. 1B). The level of IFN-γ gene transcripts was similar between the CIITA-/- and control Th1 cells. However, CIITA-/- Th1 cells transcribed IL-4, IL-5, and IL-13 at levels within 50% of control Th2 cells, while control Th1 cells produced negligible Th2 cytokine transcripts. There was at least a 10-fold increase in Th2 cytokine gene transcripts from CIITA-/- Th1 cells, compared with control Th1 cells. We next asked whether the cell surface molecules IL-12Rβ2 and IL-18Rα, which are up-regulated on Th1 cells, were properly expressed in CIITA-/- Th1 cells (29, 30). Cell surface levels of both IL-12Rβ2 and IL-18Rα were increased on CIITA-/- Th1 cells, relative to Th2 cells (Fig. 1C). These data indicate that, with the exception of Th2 cytokine expression, CIITA-/- CD4 T cells are capable of differentiating into otherwise normal Th1 cells. NKT cells also express IFN-γ and Th2 cytokines. Because all of our experiments use total CD4 T cells, our phenotype could be due to NKT cells (31, 32). However, percentages of CD4+NK1.1+ cells in either the spleens or pooled lymph nodes were comparable between individual wild-type and CIITA+/-IE mice (Fig. 1D). This, in addition to our previous observation that Th1 cells derived from sorted naïve CD4 T cells express Th2 cytokines (22), make
both control and shown in Fig. 2 differentiated under Th1- and Th2-inducing conditions. We then restimulated these, CD4 T cells from control and whether they are already committed to express IL-4. To address this, cell surface expression of IL-12Rβ2 and IL-18Rα was analyzed by flow cytometry. Filled and open histograms represent Th1 and Th2 cells, respectively. Histograms were gated on CD4⁺ cells. Spleens or pooled lymph nodes were harvested from control or CITA⁻/⁻/IE mice. Cell surface expression of CD4 and NK1.1 was analyzed by flow cytometry. Each bar represents either the spleen or pooled lymph nodes from an individual mouse. Data shown are representative of at least three independent experiments.

FIGURE 1. CITA⁻/⁻ Th1 cells secrete Th2 cytokines. A, CD4 T cells from CITA⁻/⁻/IE and control mice were differentiated to a Th1 or Th2 phenotype and restimulated as described in Materials and Methods. Supernatants were collected for ELISA (A) and were lysed to isolate RNA for quantitative real-time PCR (B). Cytokine transcript levels in each sample were expressed as fold induction compared with transcript levels from control Th1 (IFN-γ) or the control Th2 (IL-4, IL-5, and IL-13) samples, as described in Materials and Methods. C. Cells were cultured as in A, but not restimulated. Cell surface expression of IL-12Rβ2 and IL-18Rα was analyzed by flow cytometry. Each bar represents either the spleen or pooled lymph nodes from an individual mouse. Data shown are representative of two independent experiments.

FIGURE 2. CITA⁻/⁻ Th1 cells express IL-4 with similar kinetics as control Th2 cells. A, CD4 T cells were cultured as in Fig. 1. During primary stimulation, cells were harvested each day as indicated and restimulated overnight. Supernatants were collected for ELISA. B, CD4 T cells were cultured as in Fig. 1 for 6 days. At the end of each round of stimulation, cells were restimulated using the same differentiation conditions. Round 3 represents cells that have undergone three cycles of stimulation. Data are representative of two independent experiments.

it unlikely that NKT cells are responsible for the observed CITA⁻/⁻ Th1 phenotype.

CITA⁻/⁻ Th1 cells express Th2 cytokines during primary stimulation, and after multiple rounds of stimulation Typically, IL-4 and IFN-γ are not readily detectable by ELISA unless cells have undergone Th1 and Th2 differentiation for 2–3 days (33). Thus, we asked whether developing CITA⁻/⁻ Th1 cells require a similar length of time to express Th2-type cytokines, or whether they are already committed to express IL-4. To address this, CD4 T cells from control and CITA⁻/⁻/IE mice were differentiated under Th1- and Th2-inducing conditions. We then restimulated cells every day and measured cytokine production. As shown in Fig. 2A, both control and CITA⁻/⁻ Th1 cells acquired the ability to secrete IFN-γ at day 3. Control Th2 cells did not secrete significant levels of IFN-γ at any point during the differentiation. Control Th2 cells, as well as CITA⁻/⁻ Th1 cells, required ~3 days of differentiation to secrete detectable levels of IL-4. Expression of IL-5 and IL-13 followed the same pattern observed with IL-4 (data not shown). These data demonstrate that Th2 cytokine expression from CITA⁻/⁻ Th1 cells is not likely due to existing Th2 memory cells, which are capable of secreting high levels of IL-4 immediately upon stimulation (reviewed in Ref. 31).

We next asked whether the production of Th2-type cytokines by CITA⁻/⁻ Th1 cells is transient and can therefore be changed by continuous restimulation in the presence of Th1-inducing factors. To test this, CD4 T cells from the control and CITA⁻/⁻/IE mice were restimulated every 6 days in the presence of Th1-inducing factors for a total of three rounds of stimulation. Cells were cultured for three rounds because it has been shown that Th1 cells lose the ability to switch between Th1 and Th2 phenotypes after three rounds of restimulation (34). Despite multiple rounds of such stimulation, CITA⁻/⁻ Th1 cells maintained their phenotype by producing high levels of Th2-type cytokines, indicating a stable phenotype (Fig. 2B).

CITA⁻/⁻ Th1 cells express both IFN-γ and IL-4 simultaneously Th2 cytokines produced by CITA⁻/⁻ Th1 cells could be the result of a minor subpopulation secreting high levels of IL-4, or of the entire population producing low levels of IL-4. To address this issue, CD4 T cells from control and CITA⁻/⁻/IE mice were differentiated under Th1-inducing conditions. IFN-γ and IL-4 production from individual cells was assessed by intracellular cytokine staining. Control Th1 cells showed the typical profile, with a dominant population of IFN-γ-producing cells (Fig. 3, upper left panel). On the contrary, CITA⁻/⁻ Th1 cells exhibited a very different pattern. First, cells producing IFN-γ alone were present, but at a reduced level compared with the control. Second, significant numbers of cells producing IL-4 alone were present. Third, a population of cells produced both IFN-γ and IL-4 simultaneously. Thus, our data revealed that IL-4 production by CITA⁻/⁻ Th1 cells was contributed by two cell populations, IL-4 single-positive
The proportions of IL-4 remained rather constant, indicating a stable phenotype (Table I). Stable phenotype of cells (IL-4) and IL-4/IFN-γ double-positive cells (IL-4+/IFN-γ+). Similarly, two different cell types, IFN-γ+ and IL-4+/IFN-γ+ cells, produced IFN-γ, and the total percentage of IFN-γ-producing cells was comparable between the control and CITATA−/− Th1 cells. Data from control Th2 cells are included for comparison.

We wondered whether the IL-4+/IFN-γ+ cells represent an intermediate stage of cells that have not been fully differentiated. If so, multiple rounds of stimulation would lead them to produce only one type of cytokine (IL-4 or IFN-γ), instead of both cytokines simultaneously. When we tested CITATA−/− Th1 cells over multiple rounds of stimulation, the proportion of IL-4+/IFN-γ+ cells remained rather constant, indicating a stable phenotype (Table I). The proportions of IL-4+ cells also remained comparable upon multiple restimulations. Only the IFN-γ+ cells increased steadily in proportion over the three rounds of stimulation.

Expressing CITATA in CITATA−/− Th1 cells reduces Th2-type cytokine production

The expression of Th2-type cytokines from CITATA−/− Th1 cells suggests that CITATA represses these cytokines in primary Th1 cells. If this hypothesis is correct, introducing CITATA into differentiating CITATA−/− Th1 cells should restore their cytokine profile to a normal state. Bicistronic retroviral vectors encoding either GFP or GFP with CITATA were transduced into CITATA−/− CD4 T cells cultured under Th1-inducing conditions. At the end of the primary stimulation, GFP CD4 T cells were sorted, expanded, and then restimulated to test cytokine production by ELISA. CITATA−/− Th1 cells transduced with CITATA, but not with GFP alone, expressed the I-Aβ MHC class II molecule on their surfaces (Fig. 4A). This indicates the retroviral CITATA gene product is functional in transduced cells. CITATA-transduced cells produced much lower levels of IL-4, IL-5, and IL-13, with the most dramatic reduction observed with IL-13 (Fig. 4B). These data further support the hypothesis that CITATA down-regulates Th2-type cytokine production in Th1 cells.

If the unique distribution of cytokine-producing cells observed in Fig. 3 is due to the deficiency of CITATA, the phenotype should be reversed by CITATA expression. When CITATA−/− Th1 cells were transduced with CITATA, the percentages of IL-4+ and IL-4+/IFN-γ+, but not IFN-γ+ cells, were reduced (Fig. 4C, upper panels). The percentage of IFN-γ+ cells increased, keeping the total number of IFN-γ-producing cells similar. Cytokine expression from GFP+ CD4+ T cells in the same cultures was essentially unchanged (Fig. 4C, lower panels).

CITATA−/− Th1 cells require IL-4 to express IL-5 and IL-13

IL-4 is known to be the most critical cytokine directing cells to the Th2 lineage (reviewed in Ref. 1). Thus, we asked whether the production of Th2-type cytokines by CITATA−/− Th1 cells requires IL-4. To answer this, we used two systems. First, we added increasing amounts of anti-IL-4 neutralizing Ab to the CITATA−/− IE CD4+ T cells as they differentiated under Th1-inducing conditions. Increased doses of the anti-IL-4 neutralizing Ab had little effect on Th2 cytokine production by CITATA−/− IE Th1 cells, suggesting that IL-4 expression from CITATA−/− IE Th1 cells is not inhibited by the anti-IL-4 neutralizing Ab (Fig. 5A). Control Th2 cells failed to produce IL-4, IL-5, and IL-13 in the presence of 10 μg/ml neutralizing Ab, despite the addition of rIL-4 (Fig. 5A). These data imply that CITATA−/− Th1 cells do not require exogenous IL-4 to express Th2 cytokines. Second, we generated IL-4−/− CITATA−/− mice and studied cytokine production by their Th1 cells. As shown in Fig. 5B, IFN-γ production was similar between control, CITATA−/−, and IL-4−/− CITATA−/− Th1 cells. CITATA−/− Th1 cells produced Th2 cytokines, consistent with data previously shown in Fig. 1. However, IL-4−/− CITATA−/− Th1 cells did not produce IL-5 or IL-13. These data suggest the IL-4 gene is required by CITATA−/− Th1 cells to express Th2 cytokines.

CITATA−/− Th1 cells express GATA-3

The transcription factors T-bet and GATA-3 are essential regulators of Th1 and Th2 differentiation, respectively. Th1 cells lose GATA-3 expression as they differentiate, resulting in the absence of Th2 cytokine production (35). Expressing GATA-3 as a transgene during Th1 differentiation induces Th2 cytokine production (13). Therefore, Th2-type cytokine expression from CITATA−/− Th1 cells could be due to the absence of GATA-3. To test this, Th1 and Th2 cells were prepared from control and CITATA−/− IE mice, and total cell lysates were analyzed by Western blot. As shown in Fig. 6A, T-bet was expressed at equivalent levels between CITATA−/− and control Th1 cells. Similarly, both Th2 populations expressed GATA-3 protein at comparable levels. However, CITATA−/− Th1 cells expressed GATA-3 protein, while control Th1 cells did not. The expression patterns of NF-ATc1 and NF-ATc2, which also participate in Th1 and Th2 differentiation, were similar (11, 36–38).

We next wanted to determine whether CITATA−/− Th1 cells require GATA-3 to express Th2 cytokines. Because GATA-3 deficiency results in embryonic lethality (39), and because complementation systems lack normal T cell development (40), we used an antisense approach. Antisense GATA-3 was transduced into differentiating CITATA−/− Th1 cells and the levels of cytokines were measured. As shown in Fig. 6B, CITATA−/− Th1 cells expressing antisense GATA-3 produced lower levels of Th2-type cytokines relative to cells transduced by GFP alone. Thus, GATA-3 expression in CITATA−/− Th1 cells is at least partly responsible for the production of Th2-type cytokines.

Table I. Stable phenotype of CITATA−/− Th1 cells after multiple rounds of stimulation

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<th>Round No.</th>
<th>Control Th1</th>
<th>CITATA−/− Th1</th>
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<tbody>
<tr>
<td>IFN-γ+</td>
<td>53</td>
<td>69</td>
</tr>
<tr>
<td>IL-4+/IFN-γ+</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IL-4+</td>
<td>1</td>
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* Cells were cultured as in Fig. 2B, except that cells were restimulated with PMA and ionomycin. Numbers indicate the percentages of each population as determined by flow cytometry.
FIGURE 4. Expressing CIITA in CITA−/− Th1 cells represses Th2-type cytokine production. A, CITA−/− CD4 T cells were cultured under Th1-inducing conditions overnight and then transduced with retrovirus-encoding GFP (RV-GFP) or GFP with CIITA (RV-CIITA), as described in Materials and Methods. Cells were then cultured an additional 4–6 days in the presence of Th1-inducing conditions. MHC class II expression was assessed by flow cytometry, and histograms were gated on CD4+GFP+ cells. B, Cells were cultured as in A, sorted for CD4+GFP+ cells, rested 1 day in the presence of IL-2 and IL-12, and restimulated overnight with anti-CD3. Supernatants were collected for ELISA. C, CITA−/− Th1 cells were transduced as in A and analyzed by intracellular cytokine staining. Plots were gated on CD4+GFP+ (upper panels) or CD4+GFP− (lower panels) cells. Data shown are representative of two independent experiments.

Discussion

We previously reported CITA−/− Th1 cells produce Th2-type cytokines, while CITA−/− Th2 cells exhibit a normal phenotype (22). Based on these observations and others (28), we proposed that CIITA is a negative regulator of Th2-type cytokine expression during Th1 differentiation. To better characterize CIITA’s role in CD4 T cell differentiation, we analyzed Th cell-specific gene expression more thoroughly in CITA−/− Th1 cells.

Three distinct populations of cytokine-producing cells, IFN-γ+, IL-4+, and IL-4+/IFN-γ+, were generated when CITA−/− CD4 T cells were directed to the Th1 lineage. IL-4 production was contributed by both the IL-4+ and the IL-4+/IFN-γ+ cells. Likewise, both IFN-γ+ and IL-4+/IFN-γ+ cells produced IFN-γ. This distribution of cytokine-producing cells persisted over multiple rounds of stimulation in the presence of Th1-inducing conditions, indicating that the IL-4-producing phenotype of CITA−/− Th1 cells is stable.

Naive CD4 T cells produce both IFN-γ and IL-4 (41). In this regard, CITA−/− IL-4+/IFN-γ− Th1 cells resemble naive cells. Normally, naive cells produce either IFN-γ or IL-4 after differentiation, leading to the disappearance of IL-4+/IFN-γ− cells. In the absence of CIITA, developing Th1 cells cannot completely repress IL-4 production, resulting in a significant and stable IL-4+/IFN-γ− population. Disrupting the Mbd2 gene results in a similar, though much milder, phenotype in Th1 cells (42), while expressing Hlx as a transgene results in a comparable IL-4+/IFN-γ− phenotype in Th2 cells (43). Knocking out other genes known to affect Th1/Th2 differentiation, such as T-bet, NF-ATc1/c2, or c-maf, does not result in a double-cytokine-positive phenotype (37, 38, 44, 45).

It is tempting to speculate that the IL-4+/IFN-γ− cells represent an intermediate in the CD4 T cell differentiation process, ultimately leading to either IL-4+ or IFN-γ+ cells. Kamogawa et al. (46) first showed that effector cells producing either IL-4 or IFN-γ originated from a common precursor that expresses the IL-4 gene. If CD4 T cells progress through an IL-4+/IFN-γ− state during differentiation, it would be expected that the proportion of IL-4+/IFN-γ− cells should decrease after multiple rounds of stimulation as all of the surviving cells become committed. Our data demonstrate otherwise. However, this could be due to replenishment of the IL-4+/IFN-γ− compartment from the uncommitted (IL-4−/IFN-γ−) population as IL-4+/IFN-γ− cells differentiate to either IL-4+ or IFN-γ+ cells. In this regard, CD4 T cell differentiation may be similar to thymocyte development, where precursor T cells go through a CD4+CD8− stage before they become either CD4+ or CD8+. Further investigation of the IL-4+/IFN-γ− cells is necessary to have a better understanding of the progression from naive IL-4+/IFN-γ− cells to either IFN-γ+ Th1 or IL-4+ Th2 cells.

The origin of the IL-4− cells is puzzling. They may have originated from the IL-4+/IFN-γ− cells, or directly from uncommitted cells. Regardless, the IL-4− cells must have received different signals to fully differentiate into Th2 cells. Perhaps, the GATA-3 gene is turned on in a subset of uncommitted cells by an unknown mechanism, which then causes them to become Th2 cells. Attempts to isolate the individual cytokine-secreting cell populations for evaluation of GATA-3 and T-bet protein levels were not successful. We expect both the IFN-γ+ and IL-4+/IFN-γ− cells to express T-bet, as T-bet expression from unfractionated CITA−/− Th1 cells was similar to control Th1 cells. We also expect the IL-4− cells to express GATA-3, but not T-bet. Given the low level of GATA-3 detected from total CITA−/− Th1 cells, it is difficult to predict GATA-3 expression in the IL-4+/IFN-γ− cells. The amount of GATA-3 in IL-4+/IFN-γ− cells, if any, and its ratio relative to that of T-bet, has yet to be determined.

Though ectopic MHC class II expression is most likely not responsible for the CITA−/− Th1 phenotype, our data do not exclude the possibility that other extrinsic factors are relevant. An intriguing possibility is that CIITA deficiency alters thymic development, independent of MHC class II-mediated effects, in such a way that mature CITA−/− CD4 T cells are committed to Th2 cytokine production. If this were the case, CITA−/− Th1 cells...
would express IL-4, IL-5, and IL-13 even in the presence of Th1-inducing factors. The concept that Th fate can be determined during thymic development has not yet been reported but it is an attractive model to test. Another possibility is that CIITA regulates other molecules that affect Th1/Th2 differentiation, and thus CIITA−/− Th1 cells express Th2 cytokines due to an indirect effect. For example, the effects of Notch signaling by APCs on Th differentiation have recently been studied (47–50). If CIITA regulates the expression of Notch ligands, alterations in Notch signaling during thymic development in CIITA−/− mice could be responsible for the observed CIITA−/− Th1 phenotype. However, Notch-mediated Th cell differentiation is independent of IL-4/STAT6, suggesting that different mechanisms may be operated in CIITA-deficient cells (50). Our laboratory is currently investigating both possibilities in our system.

Although these possibilities exist, it is unlikely that CIITA−/− CD4 T cells are precommitted to produce Th2 cytokines, for several reasons. First, we did not observe enhanced histone acetylation of the IL-4 promoter region in freshly isolated CIITA−/− CD4 T cells compared with control CD4 T cells (D. Patel and C.-H. Chang, unpublished result). Second, GATA-3 mRNA levels in freshly isolated CIITA−/− CD4 T cells were comparable to those in control cells (D. Patel and C.-H. Chang, unpublished result). Third, CIITA−/− Th1 cells require IL-4 to express IL-5 and IL-13 (Fig. 5), and GATA-3 also appears to be critical for Th2-type cytokine production by CIITA−/− Th1 cells (Fig. 6B). Therefore, the classic IL-4-mediated pathway (reviewed in Ref. 1) for Th2 cytokine production seems to be required by CIITA−/− Th1 cells. It appears that CIITA deficiency results in the loss of inhibition of Th2 cytokine production during Th1 differentiation. Noben-Trauth et al. (51, 52) demonstrated that CD4 T cells produce low levels of IL-4 upon primary stimulation, and this amount of IL-4 is sufficient to generate Th2 cells. Therefore, an initial burst of IL-4, even under Th1 conditions, could push CIITA−/− CD4 T cells toward Th2 cytokine production. This also explains the difference between IL-4 neutralizing Ab treatment and IL-4 gene-deficiency shown in Fig. 5, perhaps due to Ab access in the autocrine scenario. Unfortunately, this cannot be quickly tested without an experimental system deficient in IL-4, IL-4R, and CIITA.

Recently, Otten et al. (24) reported that Th1 cells from CIITA transgenic mice produced Th2-type cytokines, similar to that of CIITA−/− Th1 cells. This is an unexpected finding, if in fact CIITA is a negative regulator of Th2-type cytokine expression.

FIGURE 5. CIITA−/− Th1 cells require IL-4 to express IL-5 and IL-13. A, CD4 T cells from CIITA−/− mice were cultured as in Fig. 1, and the indicated doses of anti-IL-4 neutralizing Ab were added at days 0, 2, 3, and 4 of the differentiation. Equal numbers of cells were then restimulated for ELISA analysis. Data shown are representative of two independent experiments. B, CD4 T cells from control, CIITA−/−, and IL-4−/− CIITA−/− mice were cultured as in Fig. 1. Cells were then restimulated for ELISA analysis. Data shown are representative of three independent experiments.

FIGURE 6. GATA-3 is required to produce Th2-type cytokines by CIITA−/− Th1 cells. A, Total cell lysates were prepared from cells cultured as in Fig. 1. Western blot analysis was performed as described in Materials and Methods. B, CIITA−/− CD4 T cells cultured under Th1-inducing conditions overnight were transduced with retrovirus-encoding GFP (GFP) or antisense GATA-3 (A-S), as described in Fig. 4A. CD4+ GFP+ cells were then sorted, rested, and restimulated for ELISA analysis. Data shown are representative of three independent experiments.
Based on their observations, Otten et al. (24) proposed that the expression of MHC class II and other genes related to Ag presentation is responsible for the phenotypic alteration in Th1 cells, not the CIITA deficiency itself. This implies that the Th1 phenotype is due to the extrinsic effects of alterations in both CD4 T cell selection in the thymus as well as activation in the periphery. We do not believe this is the case, as several observations contradict this hypothesis. First, re-expressing CIITA in CITA⁻/⁻ Th1 cells reduced the level of Th2-type cytokines, though the cells expressed MHC class II (Fig. 2). Second, AB⁻/⁻IE transgenic Th1 cells behave identically to control Th1 cells (22). Finally, CITA⁻/⁻ Th1 cells express GATA-3 (Fig. 6).

It is surprising that both CIITA deficiency (Ref. 22, and this report) and transgenic expression of CITA (24) result in Th2-type cytokine expression from Th1 cells. Based on this study and others, we propose the following to explain the available data. First, Otten et al. (24) used the viral SRα promoter to express CIITA in multiple cell types in vivo. This system most likely cannot properly replicate the expression profile of endogenous CIITA during CD4 T cell development and differentiation, either in the amount of protein or in the timing of protein expression. In contrast, expressing CITA with a retroviral long terminal repeat promoter in differentiating CITA⁻/⁻ Th1 cells does result in significant Th2 cytokine repression (Fig. 4). CITA had similar effects in differentiating control Th2 cells (D. Patel and C.-H. Chang, unpublished results). Second, we suspect that CD4 T cells do not express the B cell-specific isoform of CIITA that is commonly used in T cell studies (22, 24, 53). We have not been able to detect the 5′ end of the known CIITA isoform transcripts in Th1 cells, though exons common to all isoforms were detectable (D. Patel and C.-H. Chang, unpublished results). This implies the existence of a B cell-specific isoform of CIITA, which could have different effects from the B cell-specific isoform on Th2 cytokines. A system that better mimics the in vivo expression profile of the endogenous CITA gene is warranted to conclusively define the role of CIITA in CD4 T cell differentiation.

Despite these limitations, it is worth noting that Th2 development is not affected in mouse CD4 T cells when CITA is either deficient (22) or expressed as a transgene (24). This indicates that CITA’s role in CD4 T cell differentiation is restricted to the Th1 lineage. Moreover, CITA⁻/⁻ Th1 cells behave similarly to control Th1 cells with regards to IFN-γ gene expression, T-bet protein level, and surface expression of both IL-12R and IL-18Rα. Taken together, CIITA seems to be required to block Th2 cytokine production as naive cells differentiate to the Th1 lineage.

In conclusion, we defined a role for CIITA in repressing Th2 cytokine expression in Th1 cells. Although the exact mechanism by which CIITA regulates Th cell differentiation is not yet clear, our data strengthen the hypothesis that proper CIITA expression is important for appropriate Th1-restricted cytokine expression.

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