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Stepwise Epigenetic and Phenotypic Alterations Poise CD8+ T Cells To Mediate Airway Hyperresponsiveness and Inflammation

Yi Jia,* Katsuyuki Takeda,* Junyan Han,* Anthony Joetham,* Roland A. Marcus,† Joseph J. Lucas,* Brian P. O’Connor,‡,† and Erwin W. Gelfand*,†

The functional plasticity of CD8+ T cells in an atopic environment, encompassing a spectrum from IFN-γ- to IL-13-producing cells, is pivotal in the development of allergic airway hyperresponsiveness and inflammation, and yet remains mechanistically undefined. We demonstrate that CD8+ T cell IL-13 induction proceeded through a series of distinct IL-4/GATA3-regulated stages characterized by gene expression and epigenetic changes. In vivo, CD8+ T cells exposed to an environment rich in IL-4 displayed epigenetic changes at the GATA3 and IL-13 promoter indicative of transcriptional activation and IL-13 production. In vitro, IL-4 triggered the stepwise molecular conversion of CD8+ T cells from IFN-γ to IL-13 production. During the initial stage, IL-4 suppressed T-bet and induced GATA3 expression, characterized by enhanced activating histone modifications and RNA polymerase II (Pol II) recruitment to the GATA3 locus. Notably, recruitment of GATA3 and RNA Pol II to the IL-13 promoter was also detected at this initial stage. However, enhanced IL-13 transcription only occurred at a later stage after TCR stimulation, indicating that IL-4-induced GATA3 recruitment poises the IL-13 locus for TCR-mediated transcription. Thus, both in vivo and in vitro, an atopic (IL-4) environment poises CD8+ T cells via stepwise epigenetic and phenotypic mechanisms for pathogenic conversion to IL-13 production, which is ultimately triggered via an allergen-mediated TCR stimulus.

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The role of CD4+ T cells and the production of Th2-related cytokines in asthma have been substantiated in many studies and different species (1–4). In contrast, the role of CD8+ T cells is less clear, with conflicting results showing a protective (5, 6), enhancing (7–9), or dual role (10–12) depending on environmental conditions. In asthmatics, CD8+ T cells have been correlated with reduced lung function (7). In mice, CD8+ T cell depletion resulted in reduced airway responses (13). A unique subset of CD8+ T cells, CD8 effector memory T cells, played a critical role in experimental asthma (14–16). Unlike central memory CD8+ T cells, they expressed a high-affinity receptor for leukotriene B4, BLT1, which was essential for their recruitment and accumulation in the lung (17). The central role of CD8+BLT1+ T cells in the lung was attributed to their capacity for IL-13 production (9). Effector CD8+ T cells are typically defined by their ability to secrete IFN-γ and to produce molecules involved in cytolytic such as perforin and granzyme B (18, 19). The mechanism(s) by which effector CD8+ T cells can be converted into pathogenic IL-13–producing cells in the allergic lung has not been defined.

Th cell lineage commitment and differentiation have been viewed largely in a unidirectional manner with nonreversible terminal end points having distinct effector functions. Recognition of T cell subsets beyond Th1 and Th2 has prompted a re-examination of this concept. It appears that subsets are more “plastic,” with instability of lineage-specific transcription factor expression providing flexibility in differentiation options (20). Most studies have focused on the plasticity of CD4+ T cells, demonstrating a capacity for redirecting their functional programs. These differentiation decisions are dictated primarily by cytokines in the microenvironment; for example, reciprocal involvement of IL-4 and IL-12/IFN-γ in CD4+ T cell polarization has been well characterized (21, 22). In contrast, little has been reported on the functional plasticity of CD8+ T cells (23, 24).

Epigenetic mechanisms regulate CD4+ T cell lineage differentiation from Th0 to Th1, Th2, regulatory T cells, and Th17 subsets (25–27). Th2 polarization is regulated by association of the permissive histone modification trimethylation of lysine 4 of histone 3 (H3K4me3) with the IL-4 promoter and repressive trimethylation of lysine 27 of histone 3 (H3K27me3) with the IFN-γ promoter (26). Alterations in H3K4me3, H3K27me3, and histone acetylation regulate CD8+ T cell memory development (27–29), and permissive histone modifications poise cytokine gene promoters for rapid transcription after stimulation. Little is known about the interrelationships of histone regulation, asthma pathophysiology, and CD8+ T cell plasticity in Tc1 and Tc2 phenotypes.

In this study, we examined the mechanisms controlling IL-4–mediated CD8+ T cell conversion to an asthma-associated phe-
Adoptive transfer of IFN-γ–producing CD8+ T cells into an atopic environment demonstrated their plasticity via an acquired ability in vivo to produce IL-13 and to induce airway hyperresponsiveness (AHR) and inflammation in sensitized and challenged recipients. Histone modifications and changes in recruitment of polymerase II (Pol II) at lineage-specific effector cytokine and transcription factor loci characterized both the in vitro and in vivo lineage conversion of CD8+ T cells. These studies begin to define the mechanisms through which CD8+ T cells contribute to AHR and inflammation, and suggest novel ways to interfere with these processes in airway allergic diseases.

Materials and Methods

Animals

OT-1 TCR transgenic (OT-1) mice and homozygous CD8-deficient mice were bred in the animal facility at National Jewish Health (Denver, CO). OT-1 mice (C57BL/6-strain) express a transgenic TCR specific for SIINFEKL peptide (OVA257-264) as analyzed by FACS staining of peripheral blood cells with Abs against Vα2 and Vβ5 subunits. CD8-deficient mice were generated by targeting the CD8α-chain gene in C57BL/6 mice (14). Animal experiments in this study were conducted under the protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

CD8+ T cell culture

CD8+ effector memory T cells were generated in vitro, as previously described (14). Spleens were obtained from OT-1 mice and processed into mononuclear cells using Histopaque (Sigma, St. Louis, MO). A total of 1 μg/ml SIINFEKL peptide (OVA257-264) was used to stimulate cells for 1.5 h. Two days later, living cells were reisolated using Histopaque and cultured in complete RPMI 1640 medium that contained recombinant mouse IL-2 (20 ng/ml; R&D, Minneapolis, MN), IL-2+IL-4 (20 ng/ml; PeproTech, Rocky Hill, NJ), or IL-2+IL-5 (20 ng/ml; PeproTech). Medium with cytokines was changed every day for a further 4 d. The cells were then restimulated with 1 μg/ml SIINFEKL in medium containing IL-2 (20 ng/ml) and 2 μM monensin (Calbiochem, La Jolla, CA) for 4 h (Supplemental Fig. 1A).

Sensitization and airway challenge

CD8-deficient mice were sensitized with 20 μg OVA (Calbiochem, La Jolla, CA) emulsified in 2.25 mg alum (AlumIject; Pierce, Rockford, IL) on days 1 and 14 by i.p. injection (14). Mice were challenged with 1% OVA for 20 min on days 28, 29, and 30 using an ultrasonic nebulizer (model NE-U07; Omron Healthcare, Kyoto, Japan). Airway function was measured and samples were collected on day 32.

Adoptive transfer

CD8+ T cells (1 × 106) generated in medium containing IL-2 or IL-2+IL-4 were injected into OVA-sensitized CD8-deficient mice i.v. on day 28, followed by three challenges on days 28, 29, and 30. CD8+ T cells (5 × 106) generated in medium containing IL-2 were injected as a positive control (Supplemental Fig. 1B).

Anti-mouse IL-4 Ab administration

Anti-mouse IL-4 (200 μg) was injected i.v. into sensitized CD8-deficient mice on days 26–30. Control, rat IgG Ab was administered in the same manner. Sensitized mice received 5 × 106 CD8+ T cells on day 28 i.v. followed by three challenges on days 28, 29, and 30. Airway function was measured and samples were collected on day 32 (Supplemental Fig. 1C).

Assessment of airway function

Airway function was assessed as described previously (14, 15) by measuring changes in airway resistance (RL) in response to increasing doses of inhaled methacholine (Sigma). Data were presented as percentage change from the baseline RL values after saline inhalation.

Bronchoalveolar lavage analysis

After measurement of AHR, lungs were lavaged via the tracheal tube with 1 ml HBSS. The supernatants were collected and IFN-γ, IL-4, IL-5, and IL-13 (eBioscience, San Diego, CA) levels were measured by ELISA (11, 14). Total leukocyte numbers were counted and differentiated (11, 14).

Lung histology

Lungs were isolated and fixed in 10% formalin, then embedded in paraffin and cut into 5-μm-thick tissue sections. Sections were stained with periodic acid–Schiff (PAS). Mucous-containing cells were quantitated as previously described (30).

Recovery of and in vitro stimulation of adoptively transferred CD8+ T cells

Lung cells from sensitized and challenged CD8-deficient mice that received CD8+ T cells were isolated by collagenase (Life Technologies, Carlsbad, CA) digestion and enriched using nylon wool columns (14). CD8+ T cells were further purified using MACS beads (Miltenyi Biotec, Auburn, CA). Isolated CD8+ T cells (1 × 107/ml) were stimulated with 50 ng/ml PMA (Calbiochem, La Jolla, CA) and 1 μM ionomycin (Calbiochem, La Jolla, CA) in the presence of 2 μM monensin for 4 h. Cells were collected and washed twice with PBS containing 1% BSA for IFN-γ and IL-13 intracellular staining as described later.

Flow cytometric analysis

For surface staining, cells were washed with PBS containing 1% BSA twice, then incubated with anti-mouse CD16/CD32 (2.4G2; BD Bioscience, San Jose, CA) at 4°C for 5 min and stained with PE-labeled anti-mouse IL-4Rα (mIL4R-M1; BD Bioscience) or PE-labeled anti-mouse IL-5Rα (T21; BD Bioscience). Isotype-matched Abs were used as controls. For intracellular staining, 1 × 106/ml cells were washed with PBS containing 1% BSA twice, then stained with 1 μg/ml SIINFEKL in the presence of 2 μM monensin at 37°C for 4 h. After fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and permeabilization with 0.1% saponin (Sigma), cells were stained with FITC-labeled anti-mouse IFN-γ (XMG 1.2; eBioscience), PE-labeled anti-mouse IL-13 (eBio13A; eBioscience) or Alexa Fluor 647–labeled anti-mouse Eomes (Dan1Imag; eBioscience). Cell staining was monitored on a FACSCalibur (BD Bioscience) and analyzed using FlowJo software (Tree Star, Ashland, OR).

RNA preparation and analyses

Total RNA was extracted from 5 × 106 differentiated CD8+ T cells using the RNeasy Mini kit (Qiagen, Valencia, CA). For RT-PCR, 1 μg total RNA was converted into cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Fold changes were determined using the 2-ΔΔCt method, with normalization to expression of mouse GAPDH.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed according to manufacturer protocols (31) with ChIP assay kit reagents (Active Motif, Carlsbad, CA). In brief, chromatin was cross-linked by the addition of methanol-free formaldehyde to target cells at a final concentration of 1% at room temperature. Cross-linking was stopped by the addition of 0.125 M glycine at room temperature. After cell lysis, the cross-linked chromatin was sheared using a Covaris S2 focused energy isothermal sonicator to an average size of 300–500 bp, with highest density at 500 bp. ChIP was performed according to manufacturer’s protocols with magnetic beads and the following Abs: anti-H3K4me3, anti-H3K27me3, anti-AR-R Pol II (Abcam, Cambridge, MA), anti-RNA Pol II pS5 (Abcam), and anti-GATA3 (Santa Cruz Biotech, Santa Cruz, CA). The immunoprecipitated genomic DNA was analyzed via SYBR green quantitative PCR (qPCR). A matched isotype Ab was used as a negative control. The following promoter specific primers were used: GATA3, (forward) 5′-GGGTTGCAGTCTTTCTTGAGCT3′, (reverse) 5′-CGACGCACATTAAAGGAGGTTG3′; T-bet, (forward) 5′-AACCTTCTGAGGGAGGAAGAAA-3′, (reverse) 5′-GAAATTCCTTTTGTGAGGAG-3′; IFN-γ, (forward) 5′-TCCATGTGGTGGGAGAAATC-3′, (reverse) 5′-TCCTGTTGTGGTGGCAAT-3′; GATA3, (forward) 5′-TCCCGTGGAGGAGATCTG-3′, (reverse) 5′-TACCTGGATCGAAGGGCCTC-3′. qPCR data were analyzed via the 2-ΔΔCt methodology: [2^([ΔCt of total input – ΔCt of specific IP]) × 100]. ChIP genomic DNA from anti-GATA3 IP was also examined using IL-13 promoter–specific primers via end-point PCR following agarose gel electrophoresis. The ChIP-qPCR data were analyzed via ImageJ densitometry.

Statistical analysis

All data, unless otherwise indicated, were representative of at least two to three independent experiments with three to four mice per group. Results...
were expressed as the mean ± SEM. Student two-tailed \( t \) test was used to determine the level of difference between two groups. ANOVA was used to determine the levels of difference among more than three groups. The Mann–Whitney \( U \) and \( t \) statistical tests were performed for analysis of the ChIP data, taking into account variability in the assays across multiple biological repeats.

**Results**

**IL-4 promotes CD8\(^+\) T cell–mediated AHR and inflammation in vivo**

CD8-deficient mice develop lower AHR and eosinophilia than WT mice after sensitization and challenge in asthma models (14), and adoptive transfer of \( 5 \times 10^6 \) CD8\(^+\) T cells restores the full extent of AHR, eosinophilia, and goblet cell metaplasia (14). Thus, in response to asthma-related stimuli, skewed CD8\(^+\) T cells contribute to AHR and inflammation in vivo. To define the role of IL-4, a prototypical asthma-associated cytokine, in CD8\(^+\) T cell–mediated regulation of AHR and inflammation, \( 1 \times 10^6 \) CD8\(^+\) T cells differentiated in IL-2 alone or IL-2+IL-4 in vitro (Supplemental Fig. 1A) were transferred into sensitized CD8-deficient mice before airway challenge (Supplemental Fig. 1B). The results demonstrated that \( 1 \times 10^6 \) CD8\(^+\) T cells differentiated in IL-2+IL-4 fully restored AHR and airway inflammation (Fig. 1A, 1B). In contrast, \( 1 \times 10^6 \) CD8\(^+\) T cells differentiated in IL-2 alone could not restore these responses, although a higher dose of \( 5 \times 10^6 \) cells was sufficient. IFN-\( \gamma \)-levels were significantly lower, and IL-4, IL-5, and IL-13 levels were significantly higher in the bronchoalveolar lavage (BAL) fluid of mice that received CD8\(^+\) T cells differentiated in IL-2+IL-4 compared with those that received CD8\(^+\) T cells differentiated in IL-2 alone (Figs. 1C–F). Lung sections were analyzed by PAS staining and showed that the recipients of CD8\(^+\) T cells differentiated in IL-2+IL-4, but not IL-2 alone, had significantly increased numbers of PAS\(^+\) mucous-containing goblet cells and an increased accumulation of inflammatory cells (Fig. 1G). Thus, IL-4+IL-2, but not IL-2 alone, was sufficient to convert CD8\(^+\) T cells to a proasthmatic phenotype.

**IL-4 is essential for the functional conversion of transferred CD8\(^+\) T cells in sensitized and challenged recipients**

Given the ability of IL-4 to skew CD8\(^+\) T cells in vitro, we examined the role of IL-4 in mediating in vivo CD8\(^+\) T cell conversion. Anti-mouse IL-4 or isotype control was administered to sensitized CD8-deficient mice before transfer of \( 5 \times 10^6 \) CD8\(^+\) T cells (differentiated in IL-2 alone) as outlined in Supplemental Fig. 1C. Mice treated with anti–IL-4 had lower AHR and less eosinophilia in BAL fluid, similar to levels observed with sensi-

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**FIGURE 1.** Limiting numbers of CD8\(^+\) T cells differentiated in IL-2+IL-4 restore AHR and inflammation in CD8-deficient recipients. CD8-deficient mice were sensitized and challenged, and received no cells, \( 1 \times 10^6 \), or \( 5 \times 10^6 \) CD8\(^+\) T cells differentiated in IL-2 alone or IL-2+IL-4. (A) Changes in RL were measured in response to increasing concentrations of methacholine. (B) Cell composition in BAL fluid. Cytokine levels in BAL fluid. IFN-\( \gamma \) (C), IL-13 (D), IL-4 (E), and IL-5 (F) levels. (G) Representative photomicrographs of lung histology (original magnification \( \times 200 \)). Quantitative analysis of goblet cells was as described in Materials and Methods. Data (mean ± SEM) are from two to three experiments with three to four mice per experiment. \( *p < 0.05 \), \( **p < 0.01 \); both compared with sensitized and challenged CD8-deficient recipients of \( 5 \times 10^6 \) IL-2–differentiated cells. \( \#\#p < 0.01 \) compared with sensitized and challenged CD8-deficient recipients of \( 1 \times 10^6 \) IL-2–differentiated cells.
tized and challenged CD8-deficient mice that did not receive any cells (Fig. 2A, 2B). Levels of IL-4, IL-5, and IL-13 in BAL fluid were lower and IFN-γ was significantly higher in recipient mice treated with anti–IL-4 (Fig. 2C–F). Histological examination showed that numbers of PAS+ mucous-containing goblet cells were significantly lower in the recipients that received anti–IL-4 (Fig. 2G).

Lung CD8+ T cells were recovered after challenge of CD8-deficient mice that had received CD8+ T cells and were treated with anti–IL-4. The cells were stimulated with PMA and ionomycin in the presence of monensin for 4 h. The number of IL-13 single-positive cells was dramatically lower and IFN-γ single-positive cells were higher in the CD8+ T cells recovered from the lungs of mice treated with anti–IL-4 compared with those treated with isotype control Ab or mice not treated with Ab (Fig. 2H). Thus, IL-4 was required for the in vivo conversion of CD8+ T cells to an IL-13-secreting Tc2 phenotype.

Transfer of Tc1 skewed CD8+ T cells to an allergen-sensitized host engenders their Tc2 epigenetic conversion.

IL-13 expression is regulated, in part, by the lineage-specific transcription factor GATA3 (32). However, the role of IL-4 in regulating GATA3-mediated IL-13 expression by asthma-promoting CD8+ T cells remains unexplored. Using ChIP-qPCR, we compared histone modifications at the promoter regions of IL-13, IFN-γ, GATA3, and T-bet in CD8+ T cells differentiated in vitro with IL-2 or recovered from the lungs after adoptive transfer (of 5 × 10^6 IL-2-treated cells) to allergen-sensitized and -challenged recipients, as illustrated in Fig. 3A–D. Association of the permissive H3K4me3 histone modification at the IL-13 promoter increased 2-fold in recovered lung CD8+ T cells, whereas the reciprocal repressive H3K27me3 histone modification decreased 11-fold compared with pretransfer cells (Fig. 3A). Association of the permissive H3K4me3 histone modification at the GATA3 promoter also increased 3-fold in recovered lung CD8+ T cells compared with pretransfer CD8+ T cells, whereas reciprocal repressive H3K27me3 modification decreased 9-fold (Fig. 3C). In contrast, reciprocal changes in the permissive and repressive histone modifications were not observed at the IFN-γ and T-bet promoters. Although H3K27me3 decreased at the IFN-γ and T-bet promoters in recovered lung cells versus pretransfer, no change in H3K4me3 was observed (Fig. 3B, 3D, respectively). These data indicate that in vivo Tc2 plasticity of CD8+ T cells was associated with epigenetic regulation of the key induced, lineage-specific, promoter loci.

FIGURE 2. Anti–IL-4 treatment of CD8-deficient recipients of CD8+ T cells prevents restoration of AHR and inflammation. (A) Changes in RL were measured in response to increasing concentrations of methacholine. (B) Cell composition in BAL fluid. Cytokine levels in BAL fluid. IFN-γ (C), IL-13 (D), IL-4 (E), and IL-5 (F) levels. (G) Representative photomicrographs of lung histology (original magnification ×200). Quantitative analysis of goblet cells was as described in Materials and Methods. (H) IFN-γ and IL-13 expression in recovered lung CD8+ T cells. Data (mean ± SEM) were from at least three independent experiments (n = 9–12). *p < 0.05, **p < 0.01; both compared with sensitized and challenged CD8-deficient recipients of 5 × 10^6 IL-2–differentiated CD8+ T cells group. ***p < 0.01 compared with sensitized and challenged CD8-deficient recipients of 5 × 10^6 IL-2–differentiated CD8+ T cells treated with an isotype control.
IL-4 alters the phenotype of CD8+ T cells for IL-13 production in vitro

We used a staged in vitro differentiation system to more closely define the mechanisms through which IL-4 regulates CD8+ T cell conversion. The protocol for differentiation of CD8+ T cells in vitro is illustrated in Supplemental Fig. 1A. Approximately 25% of the cells were positive for IL-4R on day 0 and increased on days 2 and 4 in the IL-2–treated group (Table I, Supplemental Fig. 2A). In the presence of IL-2+IL-4, the percentage of IL-4R+ cells was further increased. As a control, there was little change in the number of IL-5R+ cells in any of the groups (Supplemental Fig. 2B).

CD8+ T cells were differentiated in the presence of IL-2, with or without IL-4 or IL-5 as a control. After 6 d in culture, cells were restimulated with SIINFEKL in the presence of monensin for 4 h. As shown in Fig. 4A and Table II, virtually all of the cells were negative for both IFN-γ and IL-13 in the IL-2–treated group without Ag restimulation; ~90% of the cells were identified as IFN-γ–producing after SIINFEKL restimulation. Few cells were IL-13–producing after SIINFEKL restimulation. Among cells cultured in medium containing IL-2+IL-4, few cells (~1%) were IFN-γ–producing, and small numbers (~6%) produced IL-13. After SIINFEKL restimulation, there was a significant decrease in the numbers of IFN-γ–producing cells compared with that seen in IL-2–treated cultures, and ~40% of the cells were now IL-13 producing. A substantial fraction of cells were identified as double negatives in the IL-2+IL-4–treated group even after restimulation with SIINFEKL. CD8+ T cells that were differentiated in medium containing IL-2+IL-5 showed a similar phenotype as IL-2+IL-4–treated cells (data not shown).

Cells from each group were assessed by quantitative RT-PCR. IFN-γ mRNA levels were significantly lower in cells treated with IL-2+IL-4 compared with cells treated with IL-2 alone. After restimulation with SIINFEKL for 4 h, IFN-γ mRNA levels remained lower in IL-2+IL-4–treated cells (Fig. 4B). In contrast, IL-13 mRNA levels were significantly higher in cells treated with IL-2+IL-4 compared with cells treated with IL-2 alone, and the cells showed the same pattern after restimulation with SIINFEKL for 4 h (Fig. 4C). IFN-γ and IL-13 protein levels were determined by ELISA. IFN-γ levels were below limits of detection in cells treated with either IL-2 or IL-2+IL-4. After restimulation with SIINFEKL, IFN-γ levels produced by IL-2–treated cells were significantly increased. IFN-γ production was significantly lower in the cells differentiated in the presence of IL-2+IL-4 (Fig. 4D). In contrast, IL-13 levels were significantly higher in cultures of cells grown with IL-2+IL-4 compared with IL-2 alone. The same pattern of increased IL-13 production was seen after restimulation with SIINFEKL for 4 h, except that the levels produced by cells grown in IL-2+IL-4 were much higher after Ag restimulation (Fig. 4E). IL-4 and IL-5 protein levels were also evaluated, and the data showed that both were increased in cultures treated with IL-2+IL-4 compared with IL-2 alone (data not shown). Overall, CD8+ T cells shifted their cytokine profile from exclusively IFN-γ producing to predominantly Th2 cytokine producing in the presence of IL-4.

Table I. Kinetics of IL-4R and IL-5R expression in CD8+ T cells differentiated in IL-2 or IL-2+IL-4 in vitro.

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
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<tr>
<td></td>
<td>IL-2</td>
<td>IL-2+IL-4</td>
</tr>
<tr>
<td>%IL-4Rα</td>
<td>24.5 ± 1.6</td>
<td>38.2 ± 3.8</td>
</tr>
<tr>
<td>%IL-5Rα</td>
<td>11.4 ± 1.7</td>
<td>15.3 ± 3.3</td>
</tr>
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Data shown are representative of at least four independent experiments (n = 16).

*p < 0.01 compared with the IL-2 group on day 2. **p < 0.01 compared with the IL-2 group on day 4.
IL-4 alters the levels of lineage-specific transcription factor expression in CD8\(^+\) T cells in vitro

Expression of IFN-\(\gamma\) and IL-13 in T cells is regulated by the transcription factors T-bet and GATA3, respectively (33, 34). Repressor of GATA (ROG) is a transcription factor that controls the expression of GATA3 (35, 36). CD8\(^+\) T cells cultured with IL-2+IL-4 expressed significantly lower levels of T-bet and ROG, and significantly higher levels of GATA3 compared with cells cultured with IL-2 alone both before and after restimulation with SIINFEKL (Fig. 5A).

Runx3 augments Th1 and downmodulates the Th2 phenotype by attenuating GATA3 expression (37). Runx1 inhibits the differentiation of naïve CD4\(^+\) T cells into the Th2 lineage by repressing GATA3 expression (38). Runx3, but not Runx1, expression levels were significantly lower in cells treated with IL-2+IL-4 compared with cells treated with IL-2 alone, with or without restimulation by SIINFEKL (Fig. 5B).

Eomesodermin (Eomes) is a member of the same subfamily of T-box factors as T-bet and regulates IFN-\(\gamma\) expression in CD8\(^+\) T cells (39, 40). The addition of IL-4 to the IL-2–containing medium resulted in a lower percentage of cells stained positive for Eomes, both before and after restimulation with either SIINFEKL (Supplemental Fig. 3).

Thus, the changes induced in the CD8\(^+\) T cell cytokine profile by IL-4 were accompanied by changes in the levels of transcription factors known to be “lineage-specific” differentiation factors.

### IL-4 regulates GATA3 recruitment, histone modifications, and RNA Pol II recruitment in CD8\(^+\) T cells

As described earlier (Fig. 3), T cell lineage conversion is associated with posttranslational modifications to histone proteins at the
IL-13, IFN-γ, GATA3, and T-bet promoters during in vivo Tc2 conversion (25, 26, 41). ChIP-qPCR assays were performed using the stepwise differentiation model of CD8+ Tc2 conversion, which enabled a multistage examination of IL-4–regulated reprogramming of CD8+ T cells for IL-13 production (Supplemental Fig. 1A). The analysis revealed a 30-fold increase in GATA3 recruitment to the IL-13 promoter in IL-2+IL-4–treated CD8+ T cells versus IL-2 alone (Fig. 6A). Moreover, a significant decrease in IL-13 promoter–associated GATA3 recruitment was observed in IL-2+IL-4 cells after treatment with SIINFEKL, suggesting temporal, spatial, and/or TCR-mediated regulation of the promoter region. In contrast, no significant recruitment of GATA3 to the IL-13 promoter was observed in cells that had been differentiated in IL-2 alone with or without SIINFEKL. This dynamic GATA3 recruitment was confirmed by ChIP-PCR followed by gel electrophoresis (Supplemental Fig. 4). We next examined epigenetic regulation of the promoter regions of IL-13 and IFN-γ at various stages of in vitro CD8+ T cell differentiation. Association of the permissive histone modification H3K4me3 at the IL-13 promoter increased 5-fold in IL-2+IL-4–treated CD8+ T cells versus IL-2 alone (Fig. 6B), corresponding to the period of GATA3 promoter recruitment and association (Fig. 6A). ChIP was also used to analyze recruitment of the RNA Pol II complex given the evidence for Pol II–histone methylation interaction and regulation (42, 43). IL-2+IL-4 treatment increased recruitment of Pol II and Pol II with a phosphorylated serine 5 of the C-terminal domain (Pol IIpCTD; the initiation complex) to the IL-13 promoter ~5-fold versus IL-2 alone (Fig. 6B), despite low levels of IL-13 transcription and very low protein production at this stage (Fig. 4), suggesting that IL-2+IL-4 treatment poised the IL-13 locus for active transcription, which then occurred after TCR stimulation. No significant differences in histone modifications or Pol II recruitment were observed at the IFN-γ promoter in IL-2+IL-4–treated CD8+ T cells versus IL-2 alone (Fig. 6C).

We also examined the GATA3 and T-bet promoter regions via ChIP. IL-2+IL-4 treatment increased association of permissive H3K4me3 at GATA3 by >4-fold compared with IL-2 alone (Fig. 6D). A similar pattern was observed after SIINFEKL stimulation. IL-2+IL-4 also induced a 16-fold decreased association of repressive H3K27me3 at GATA3 compared with IL-2 alone (Fig. 6D). No significant differences were detected in H3K27me3 between the treatment groups after SIINFEKL stimulation. IL-2+IL-4 treatment increased Pol II recruitment >2-fold and increased recruitment of Pol IIpCTD >6-fold compared with IL-2 treatment alone (Fig. 6D). A similar pattern was observed after SIINFEKL stimulation. Although the recruitment of Pol II and Pol IIpCTD at the T-bet promoter was greater in IL-2– versus IL-2+IL-4–treated cells, the differences in histone modifications between the treatment groups were not significant (Fig. 6E). Thus, histone modifications were correlated with Pol II recruitment at the IL-13 and GATA3 promoters, but not the IFN-γ or T-bet promoters. We observed that IL-4–regulated GATA3 recruitment coincided with histone modifications at the IL-13 promoter, although significant IL-13 protein production required SIINFEKL stimulation.
to IL-4 in vivo. What remains unclear is whether these cells have lost the ability to produce IFN-γ, and thus are clearly “converted” to IL-13 production, or rather emerge from CD8⁺ IFN-γ⁺ cells.

As reviewed by O’Shea and Paul (22), it is still unclear what is meant by the terms lineage stability and plasticity/conversion, and how they can be distinguished. Moreover, such findings do support arguments for flexibility in cytokine production, and less likely a strict and fixed lineage commitment model in CD8⁺ T cell differentiation. Currently, because of technologic issues, it remains difficult to ascertain the frequency and origin of converted cells.

Based on our earlier findings of the role of CD4⁺IL-4⁺ T cells in the initial activation of CD8⁺ T cells (14), we examined the role of IL-4 during the functional differentiation of CD8⁺ T cells in a carefully regulated and staged in vitro system, and identified mechanisms controlling the plasticity of CD8⁺ T cells, focusing initially on the functional development of CD8⁺ T cells into IFN-γ or IL-13 producers in vitro. To examine the mechanisms involved in this “stepwise” conversion, we monitored expression of the lineage-specific master regulatory transcription factors that control T cell differentiation. Before TCR restimulation, but after IL-4 treatment, GATA3 expression levels significantly increased in T cells, whereas T-bet and repressor of GATA3 levels decreased. Differences in Runx3 and Eomes expression were also observed. Significantly, changes in lineage-specific transcription factors required only IL-4 and were not dependent on TCR restimulation. In contrast, IL-4-mediated induction of IL-13 RNA and protein expression required TCR restimulation. Thus, IL-4 appeared to play...
different roles at the stages of CD8+ T cell differentiation and activation. IL-4 alone was sufficient to alter lineage-specific transcription factor levels during CD8+ T cell differentiation, poised these cells to produce cytokines after TCR engagement.

We observed IL-4-induced epigenetic programming of CD8+ T cells that poised the IL-13 locus for transcription after TCR restimulation in vitro or after adoptive transfer and allergen challenge in vivo (28, 29, 47). H3K4me3 at the GATA3 and IL-13 promoters was dependent on IL-4 treatment and occurred in the absence of TCR restimulation, whereas H3K4me3 histone modifications at the T-bet and IFN-γ promoters were not regulated by IL-4. Interestingly, loss of the repressive histone mark H3K27me3 was observed at the GATA3, IL-13, T-bet, and IFN-γ promoters in CD8+ T cells recovered after adoptive transfer in vivo. However, only the GATA3 promoter exhibited a significant loss of H3K27me3 in the parallel in vitro comparisons, IL-2 versus IL-2 + IL-4 and TCR restimulation. Addition of IL-4 in the absence of TCR restimulation did result in significant loss of the repressive H3K27me3 mark at the GATA3, IL-13, and T-bet promoters, but not at the IFN-γ promoter. The differences observed between the in vivo and in vitro histone modification patterns likely reflect the inherent complexities of in vivo inflammation, which is characterized by multiple cytokine pathways and other microenvironment signals. It is interesting to note that although the IFN-γ promoter remained intransigent to the effects of IL-4 in vitro, maintenance of permissive and loss of repressive histone marks in vivo suggested plasticity of CD8+ T cell effector function in the in vivo setting. Nevertheless, the in vivo and in vitro data demonstrated that IL-4 alone, in the absence of TCR restimulation, resulted in epigenetic poising of the IL-13 locus through gain of permissive and loss of repressive histone modifications, which was coregulated with recruitment of Pol II (48).

IL-4 was also required for GATA3 expression in CD8+ T cells, and we observed an IL-4–dependent recruitment of GATA3 protein to the IL-13 promoter. In a previous study of in vitro–polarized CD4+ T cells, GATA3 binding throughout the genome was dependent on lineage development, was associated with histone modifications, and was not always associated with transcriptional changes (32). Monitoring the stepwise CD8+ T cell skewing reported in this article, we observed that IL-4–mediated GATA3 promoter recruitment coincided with histone modifications and Pol II recruitment, but was temporally separated from the enhancement of IL-13 transcription and protein production. A SINFEKL TCR stimulus was required for IL-13 protein production, but interestingly, GATA3 was not associated with the IL-13 promoter during the TCR restimulation stage of the in vitro system. This observation required application of the staged in vitro system and could not have been made with end-point in vitro polarized cells or in vivo recovered, differentiated cells, given the complexity of the interactions. Wei et al. (32) demonstrated that the activity of GATA3 at a gene-specific level was dependent on the lineage-identity of CD4+ T cells. Their study revealed that the specific GATA3 genomic binding loci differed greatly between Th1, Th2, Th17, and inducible regulatory T cells, and that the binding of GATA3 to a specific gene locus was not always associated with a change in transcription (32). As expected, deletion of GATA3 did affect gene expression in their study, but none of the genes regulated by GATA3 was shared among any of the CD4+ T cell lineages (32). In contrast, GATA3 did regulate methylation of the histone lysines H3K4 and H3K27 at many of its gene targets, including IL-13, T-bet, and IFN-γ in CD4+ T cells (32). Evaluation of similar events using the staged in vitro system enabled the demonstration that GATA3 activity is also dependent on the temporal activation phase of T cells, albeit with the recognition that Tc1 and Tc2 CD8+ T cell lineages were examined. Similar to CD4+ T cells, the binding of GATA3 at the IL-13 locus in CD8+ T cells did correspond with changes in histone methylation. Binding of GATA3 was associated with significantly enhanced permissive H3K4me3 and significantly decreased repressive H3K27me3 histone marks at the IL-13 locus in CD8+ T cells treated with IL-4. However, during the TCR restimulation stage, when GATA3 was no longer associated with the IL-13 promoter, only the enhanced H3K4me3 permissive mark remained significant. This may imply that in CD8+ T cells, the presence of GATA3 is required for the inhibition of repressive H3K27me3 but is not required for sustaining the permissive H3K4me3 mark. Thus, enhanced IL-13 transcription and protein production in CD8+ T cells were temporally associated with sustained permissive H3K4me3, but not necessarily sustained loss of repressive H3K27me3. We propose a model in which IL-4–mediated GATA3 recruitment regulates histone modifications at the promoter, which then poised the IL-13 locus for transcription after a TCR stimulus. In the absence of IL-4, GATA3 was not recruited to the IL-13 promoter, and subsequently, TCR restimulation did not result in cytokine production. As a result, an allergic inflammatory lung microenvironment containing IL-4 supports asthma pathogenesis through epigenetically poising CD8+ T cells for Tc2 conversion via differential histone modifications at key promoters. Our observations indicate that the Tc2 pathway requires an extrinsic stimulus, IL-4, whereas the Tc1 pathway may represent a default pathway, because addition of IL-4 did not greatly affect the histone modifications at T-bet or IFN-γ. This represents an obvious difference from the Th2 lineage pathway characterized by GATA3-mediated epigenetic repression of T-bet and IFN-γ in CD4+ T cells (32), emphasizing the cell-type–intrinsic activities of GATA3 and histone modifications. This may also suggest that Tc2 conversion as observed in this study reflects functional plasticity in the gain of an epigenetically permissive IL-13 locus rather than a permanent repression of the IFN-γ locus. Further understanding of the molecular mechanisms by which IL-4 and GATA3 regulate the plasticity and/or stable conversion of CD8+ T cells should suggest novel therapeutic strategies and new targets for the treatment of asthma.

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Disclosures
The authors have no financial conflicts of interest.

References


Step-Wise Epigenetic and Phenotypic Alterations Poise CD8\(^+\) T Cells to Mediate Airway Hyperresponsiveness and Inflammation

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Figure Legends

**Figure S1. Experimental protocols.** (A) Protocol for differentiation of CD8^+^ T cells in IL-2 or IL-2+IL-4 *in vitro*. (B) CD8^+^ T cells differentiated in IL-2+IL-4 were transferred into sensitized CD8-deficient mice followed by challenge. (C) Anti-mouse IL-4 or rat IgG was given intravenously to CD8-deficient mice beginning two days before the first challenge for five consecutive days.

**Figure S2. Kinetics of IL-4 receptor and IL-5 receptor expression on CD8^+^ T cells differentiated in IL-2 or IL-2+IL-4 *in vitro*.** (A) IL-4 receptor expression on CD8^+^ T cells differentiated in IL-2 or IL-2+IL-4 on days 0, 2, and 4. (B) IL-5 receptor expression on CD8^+^ T cells differentiated in IL-2 or IL-2+IL-4 on days 0, 2, and 4. The data shown are representative of at least 4 independent experiments (n=16).

**Figure S3. The expression of Eomes in CD8^+^ T cells differentiated in IL-2 or IL-2+IL-4.** Intracellular staining of Eomes in CD8^+^ T cells differentiated in IL-2 or IL-2+IL-4 with or without SIINFEKL re-stimulation. The data shown are representative of at least 3 independent experiments (n=12).

**Figure S4. ChIP-PCR analysis of GATA3 binding capability in the IL-13 promoter.** Recruitment of GATA3 to the IL-13 promoter was examined via ChIP-PCR. Four groups of stimulated CD8^+^ T cells were analyzed for the anti-GATA3 IP: IL-2 alone, IL-2+IL-4, IL-2+SIINFEKL (TCR stimulus), IL-2+IL-4+SIINFEKL. The ChIP isolated genomic DNA was
Figure S1

A

- SIFNEKL 1.5 hrs
- Spleen cells from OT-1 mice
- Day -2

B

- CD8+ T cells (IL-2 or IL-2+IL-4)
- CD8-deficient mice
- D0
- D14
- OVA+Alum i.p.
- OVA+Alum i.p.

C

- CD8+ T cells
- CD8-deficient mice
- D0
- D14
- OVA+Alum i.p.
- OVA+Alum i.p.
- 1% OVA challenge
- Assay
- Anti-IL-4 Ab or Rat IgG
Figure S2

A

Day 0  | Day 2  | Day 4  
---|---|---
0 | 0.5 | 0.6
0 | 99.5 | 99.4
0 | 23.8 | 78.3
0 | 76.2 | 21.7
0 | 23.8 | 89.0

Isotype Control

IL-2

IL-2+IL-4

B

Day 0  | Day 2  | Day 4  
---|---|---
0 | 1.7 | 1.3
0 | 98.3 | 98.7
0 | 12.2 | 13.5
0 | 87.8 | 86.5
0 | 12.2 | 13.6

Isotype Control

IL-2

IL-2+IL-4
Fig S3

<table>
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<th>Isotype</th>
<th>No re-stimulation</th>
<th>SIINFEKL</th>
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<tr>
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Alexa 647 Eomes

SSC
Fig S4

[Image: Gel electrophoresis and bar chart showing % of Total Input Densitometry Signal for IL-2, IL-2+IL-4, IL-2+TCR, IL-2+IL-4+TCR]

- GATA3 IP
- Total Input

IL-2, IL-4, TCR