

Steroidogenic enzyme Cyp11a1 regulates Type 2 CD8⁺ T cell skewing in allergic lung disease

Yi Jia^a, Joanne Domenico^a, Katsuyuki Takeda^a, Junyan Han^a, Meiqin Wang^a, Michael Armstrong^b, Nichole Reisdorph^b, Brian P. O'Connor^c, Joseph J. Lucas^a, and Erwin W. Gelfand^{a,1}

^aDivision of Cell Biology, Department of Pediatrics, ^bDepartment of Immunology, and ^cCenter for Genes, Environment, and Health, National Jewish Health, Denver, CO, 80206

Edited* by Philippa Marrack, Howard Hughes Medical Institute, National Jewish Health, Denver, CO, and approved April 4, 2013 (received for review September 26, 2012)

Allergic asthma is a heterogeneous inflammatory disorder of the airways characterized by chronic airway inflammation and airway hyperresponsiveness. Numbers of CD8⁺IL-13⁺ T cells are increased in asthmatics and during the development of experimental asthma in mice. In an atopic environment rich in IL-4, these CD8⁺ T cells mediate asthmatic responses, but the mechanisms regulating the conversion of CD8⁺ effector T cells from IFN- γ - to pathogenic IL-13-producing effector cells that contribute to an asthma phenotype have not been defined. Here, we show that cholesterol side-chain cleavage P450 enzyme, Cyp11a1, is a key regulator of CD8⁺ T-cell conversion. Expression of the gene, protein, and enzymatic activity of Cyp11a1 were markedly increased in CD8⁺ T cells differentiated in the presence of IL-2 plus IL-4 compared with cells differentiated in IL-2 alone. Inhibition of Cyp11a1 enzymatic activity with aminoglutethimide or reduction in the expression of Cyp11a1 using short hairpin RNA prevented the IL-4-induced conversion of IFN- γ - to IL-13-producing cells without affecting expression of the lineage-specific transcription factors T-box expressed in T cells (T-bet) or GATA binding protein 3 (GATA3). Adoptive transfer of aminoglutethimide-treated CD8⁺ T cells into sensitized and challenged CD8-deficient recipients failed to restore airway hyperresponsiveness and inflammation. We demonstrate that Cyp11a1 controls the phenotypic conversion of CD8⁺ T cells from IFN- γ to IL-13 production, linking steroidogenesis in CD8⁺ T cells, a nonclassical steroidogenic tissue, to a proallergic differentiation pathway.

Asthma has increased dramatically over the past 50 y and now affects 5–10% of the population in many developed countries (1). National and international guidelines recommend the use of inhaled corticosteroids as the first step in controlling airway inflammation and symptoms in persistent asthma (2, 3). However, it has been demonstrated that 45% of steroid-naïve asthmatic patients do not respond to inhaled corticosteroids. Corticosteroid insensitivity has been adopted as a principal criterion for characterizing asthma severity (4). Increased numbers of CD8⁺ T cells, which are more resistant than CD4⁺ T cells to corticosteroids (5), have been detected in steroid-insensitive asthmatics (6) and have correlated with lower lung function (7). We and others also found that numbers of CD8⁺IL-13⁺ cells were increased in experimental asthma models in mice (8–10) as a result of their activation by IL-4-producing CD4⁺ T cells (11). CD8⁺ T cells can be polarized to effector subsets with cytokine profiles similar to those found in CD4⁺ T cells (12–14). Both in vivo and in vitro, IL-4 was capable of triggering CD8⁺ T-cell differentiation from a predominant IFN- γ -producing cell to one producing IL-13. This conversion was associated with suppression of T-box expressed in T cells (T-bet) and induction of GATA binding protein 3 expression and was characterized by enhanced activating histone modifications and RNA polymerase (Pol) II recruitment to the GATA3 and IL-13 loci. IL-13 transcription only occurred at a later stage following T-cell receptor (TCR) stimulation, indicating that IL-4-induced GATA3 recruitment poised the IL-13 locus for TCR-mediated transcription (15).

Transcriptional profiling identified cholesterol side-chain cleavage P450 enzyme (Cyp11a1) transcripts as one of the most highly up-regulated during the differentiation of CD8⁺ T lympho-

cytes to a T-cell type 2 (Tc2) phenotype, that is, a CD8⁺ T-cell capable of IL-13 production. Cyp11a1 or P450_{scc} is a mitochondrial side-chain cleavage enzyme required for the conversion of cholesterol to pregnenolone. It catalyzes the initial and rate-limiting step in the synthesis of steroid hormones in tissues with steroidogenic potential (16, 17). Induction of the Cyp11a1 promoter by epidermal growth factor involves a ras/MEK1/AP-1-dependent pathway (18). Mutations in the Cyp11a1 gene result in steroid hormone deficiency and can cause the rare and a potentially fatal condition, lipoid congenital adrenal hyperplasia (19, 20).

In the present study, the role of Cyp11a1 in controlling IL-4-mediated CD8⁺ T-cell conversion in vitro and in vivo was examined. It was demonstrated that mRNA transcript levels, protein levels, and the enzyme activity of Cyp11a1 in CD8⁺ T cells were all increased dramatically following differentiation in the presence of IL-2 plus IL-4 (IL-2+IL-4) compared with IL-2 alone. Furthermore, the Cyp11a1 enzyme inhibitor aminoglutethimide (AMG) or knock down of Cyp11a1 protein levels using a specific shRNA blocked the functional conversion of CD8⁺ T cells from IFN- γ - to IL-13-producing cells. Expression of the lineage-specific transcription factors T-bet or GATA3 was not affected by inhibition of Cyp11a1 activity, indicating that it was downstream of expression of these master regulatory transcription factors. Adoptive transfer of AMG-treated CD8⁺ T cells, in contrast to untreated CD8⁺ T cells, failed to restore airway hyperresponsiveness (AHR) and inflammation in sensitized and challenged CD8-deficient mice. These studies identified Cyp11a1 as a key regulator of CD8⁺ Tc2-cell differentiation and plasticity.

Results

Cyp11a1 mRNA, Protein Levels, and Enzymatic Activity Are Increased in CD8⁺ T Cells Differentiated in the Presence of IL-2+IL-4. CD8⁺ T cells were differentiated in vitro in the presence of IL-2 or IL-2+IL-4 (Fig. 1A). Following culture for 6 d, total RNA was extracted, cDNA was prepared and quantitative real-time PCR was performed. As illustrated in Fig. 1B, Cyp11a1 mRNA levels were significantly higher in cells differentiated in the presence of IL-4. Similarly, Cyp11a1 protein levels were elevated in these cells (Fig. 1C), as determined by densitometric quantification of immunoreactive bands on autoradiographs. Cells differentiated in IL-2 alone expressed little Cyp11a1 mRNA or protein. Immunohistochemical analysis for Cyp11a1 also showed a dramatic increase in the numbers of positively stained cells in cultures treated with IL-2+IL-4 compared with IL-2 alone (Fig. 1D).

Author contributions: Y.J., K.T., N.R., B.P.O., J.J.L., and E.W.G. designed research; Y.J., J.D., M.W., and M.A. performed research; J.D. and J.H. contributed new reagents/analytic tools; Y.J., N.R., J.J.L., and E.W.G. analyzed data; and Y.J., K.T., B.P.O., J.J.L., and E.W.G. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

¹To whom correspondence should be addressed. E-mail: gelfande@njhealth.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1216671110/-DCSupplemental.

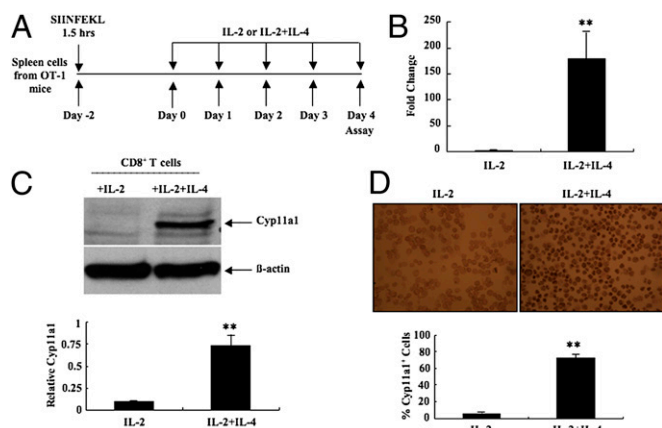


Fig. 1. Cyp11a1 expression in CD8⁺ T cells generated in the presence of IL-2 or IL-2+IL-4. (A) Protocol for differentiation of CD8⁺ T cells in IL-2 or IL-2+IL-4 in vitro. (B) Cyp11a1 mRNA expression as detected by quantitative RT-PCR in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4. (C) Cyp11a1 protein levels as detected by immunoblot analysis and densitometry of autoradiographs in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4. (D) Immunohistochemical staining for Cyp11a1 in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 in vitro. (Magnification: $\times 200$.) Quantitative analysis was performed by counting Cyp11a1-positive cells under the microscope. Data (means $\pm$ SEM) are from at least three independent experiments. ** $P < 0.01$ compared with the IL-2 group.

The enzymatic activity of Cyp11a1 was assessed using an ELISA for detection of pregnenolone levels in cell culture supernatants (21). As shown in Fig. 2A, levels of pregnenolone were increased in cultures of cells differentiated in IL-2 alone (1.8 ± 0.5 pg/mL) to 424.8 ± 35.5 pg/mL in the cells differentiated in IL-2+IL-4.

AMG Inhibits the Enzymatic Activity of Cyp11a1 Without Affecting Protein Expression. AMG is known to inhibit Cyp11a1 enzymatic activity at the initial step of conversion of cholesterol to pregnenolone in tissues such as the adrenals (22, 23). In cells differentiated in IL-2+IL-4, addition of AMG decreased pregnenolone levels in cell supernatants from 424.8 ± 35.5 to 96.4 ± 35 pg/mL (Fig. 2A). In contrast, the addition of AMG did not prevent IL-4-induced increases in Cyp11a1 protein levels, with

or without restimulation with the SIINFEKL peptide [ovalbumin (OVA)_{257–264}], as determined by immunoblot analysis (Fig. 2B). In fact, levels of protein were increased in AMG-treated cells. These data suggested that the changes in pregnenolone levels were restricted to the regulation of Cyp11a1 enzymatic activity and not attributable to changes in Cyp11a1 protein levels or cell toxicity.

Cyp11a1 Enzymatic Activity Is Essential for the Functional Conversion of CD8⁺ T Cells from IFN- γ - to IL-13-Producing Cells.

CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 were restimulated with SIINFEKL and analyzed for cytokine production by flow cytometry. CD8⁺ T cells differentiated in IL-2 alone were predominantly IFN- γ -producing with almost no IL-13-producing cells. In contrast, CD8⁺ T cells differentiated in IL-2+IL-4 were predominantly IL-13-producing, with fewer cells producing IFN- γ (Fig. 2C and Table S1). To assess the importance of the enzymatic activity of Cyp11a1 in the functional conversion of CD8⁺ T cells, the effect of addition of the Cyp11a1 enzyme inhibitor AMG on these events was determined. CD8⁺ T cells were differentiated in IL-2 or IL-2+IL-4 in the presence or absence of AMG and activated through the TCR with SIINFEKL. When CD8⁺ T cells were cultured with SIINFEKL and IL-2+IL-4 in the presence of AMG, there was a dramatic dose-dependent decrease in the percentage of IL-13-positive cells and an increase in IFN- γ -positive cells (Fig. 2C). In the presence of 500 μ M AMG, the percentage of IL-13-single-positive cells decreased from $35.7 \pm 8.2\%$ to $14.7 \pm 8.9\%$, and the percentage of IFN- γ -single-positive cells increased from $14.5 \pm 5.8\%$ to $42.4 \pm 11.5\%$; the percentage of IFN- γ - and IL-13-double-positive cells increased slightly from $8.8 \pm 2.3\%$ to $14.7 \pm 5.5\%$ (Fig. 2C and Table S1). As shown previously (15), CD8⁺ T cells differentiated in IL-2+IL-4 showed an increase in IL-4 and IL-5 production but to a lower extent than IL-13 production. Differentiation of cells in IL-2+IL-4 in the presence of AMG showed a similar level of reduction in numbers of single-positive IL-4-producing (from $10.1 \pm 1.6\%$ to $6.7 \pm 2.1\%$; $n = 8$) and IL-5-producing (from $5.3 \pm 0.8\%$ to $3.4 \pm 1.1\%$; $n = 8$) cells as seen with IL-13-producing cells. The increased numbers of IFN- γ -positive cells in the cultures indicated that the drug did not have an overall suppressive or toxic effect and that Cyp11a1 enzymatic activity was indeed required for the functional conversion of the cells to IL-13 production.

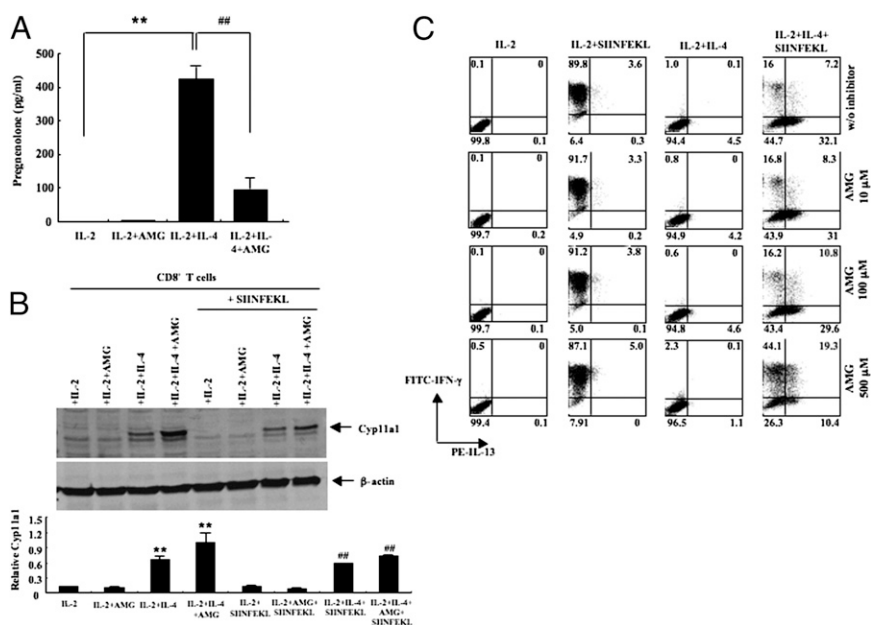


Fig. 2. Cyp11a1 enzymatic activity regulates the functional conversion of CD8⁺ T cells from IFN- γ - to IL-13-producing cells. (A) Pregnenolone levels determined by ELISA in supernatants from CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 in the presence or absence of AMG (500 μ M). ** $P < 0.01$ compared with the IL-2 group; ## $P < 0.01$ compared with the IL-2+IL-4 group. (B) Cyp11a1 protein levels detected by immunoblot analysis and densitometry of autoradiographs in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 with 500 μ M AMG. ** $P < 0.01$ compared with the IL-2 group; ## $P < 0.01$ compared with the IL-2 plus SIINFEKL group. (C) Flow-cytometric analysis of cytokine expression in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 and treatment with different concentrations of AMG. Data are from at least seven independent experiments.

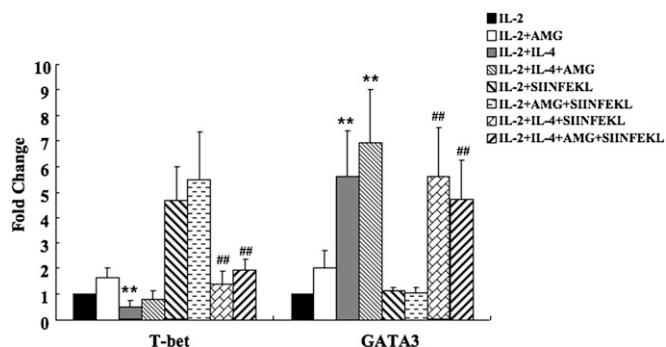


Fig. 4. Lineage-specific transcription factor expression in CD8⁺ T cells. T-bet and GATA3 expression was detected by quantitative RT-PCR in CD8⁺ T cells differentiated in IL-2 or IL-2+IL-4 with or without SIINFEKL in the presence or absence of 500 μ M AMG. Data (means $\pm$ SEM) are from at least eight independent experiments. ** P < 0.01 compared with the IL-2 group; ## P < 0.01 compared with the IL-2 plus SIINFEKL group.

phenotype in allergic disease (30). We previously demonstrated an important role for Tc2 CD8⁺ T cells in the development of experimental asthma as a result of their activation by IL-4-producing CD4⁺ T cells (11). Increased expression of leukotriene B4 receptor 1 (BLT1) on the surface of CD8⁺ T cells leads to their increased accumulation in the lungs (10). Numbers of IL-13⁺BLT1⁺CD8⁺ T cells in the airways of asthmatics were increased and correlated with reduced lung function and basement membrane thickening (31). Both human (5) and mouse (27) CD8⁺ T cells demonstrate an insensitivity to corticosteroids not seen in CD4⁺ T cells, supporting the notion that CD8⁺ T cells are at the root of the failure of asthmatics to respond to corticosteroids and may be responsible for persistent AHR and airway inflammation (6). In asthmatics, numbers of CD8⁺ T cells in the airways have correlated with lower airway function (7).

The data presented above and in previous studies indicate that CD8⁺ T-cell conversion to a Tc2 phenotype requires both IL-4 and specific antigen stimulation. Th2 cytokines can also be produced by other lymphocytes such as B cells and natural killer T (NKT) cells in addition to conventional CD4⁺ Th2 cells. Harris

et al. (32) showed that naive B cells became IL-4-producing cells when cocultured with Th2 cells. IL-4-expressing B cells in a nematode infection model were dependent on both T cells and IL-4R α -mediated signals. Akbari et al. (33) showed that IL-4 and IL-13 produced by NKT cells were essential for the development of allergen-induced airway hyperactivity. Nuocytes are a more recently described innate effector cell population shown to be important in triggering type 2 immunity through the release of IL-13 predominantly (34). Such Th2 cytokine-producing cells could conceivably contribute to the skewing of CD8⁺ T cells into a Tc2 phenotype.

Current therapies for allergic asthma have been fairly restricted, with few new drugs introduced into the clinical setting in the last decade. Inhaled corticosteroids have remained the main anti-inflammatory agent for asthma. Indeed, upwards of 40–50% of asthmatics fail to respond to inhaled corticosteroids with changes in forced expiratory volume in the first second (FEV1) (4). Moreover, corticosteroids may also enhance disease pathogenesis, especially amplifying responses in the steroid-insensitive population of CD8⁺ T cells (27). Corticosteroids may induce Th2 cytokine production while suppressing the production of Th1 cytokines. A combination of steroid insensitivity and plasticity of CD8⁺ T cells may be major contributors to the failure of some patients to respond to corticosteroids. IL-13⁺BLT1⁺CD8⁺ T cells have been proposed to be a primary cause of the airway inflammation and AHR seen in asthma (9, 10). However, the mechanism underlying the conversion of CD8⁺ T cells from IFN- γ -producing cells to IL-13-producing cells remains unclear.

CD8⁺ T cells have been primarily associated with production of IFN- γ ; however, in the presence of IL-4, CD8⁺ T cells were skewed to differentiate into IL-13-producing cells. This differentiation was associated with increases in Cyp11a1 and GATA3, and decreases in T-bet expression and cytokine production was dependent on antigen signaling through the TCR. Chtanova et al. (35) previously reported that Cyp11a1 was highly up-regulated in IL-4-differentiated CD4⁺ and CD8⁺ T cells but did not investigate the functional significance following T-cell polarization. Genome-wide transcriptional profiling done by Horiuchi et al. (36) identified Cyp11a1 transcripts as one of the most highly up-regulated during the differentiation of T lymphocytes to Th2/Tc2 phenotypes, but the functional significance of this transcriptional change was not defined. Here, we demonstrated that although IL-4 triggered the up-regulation of

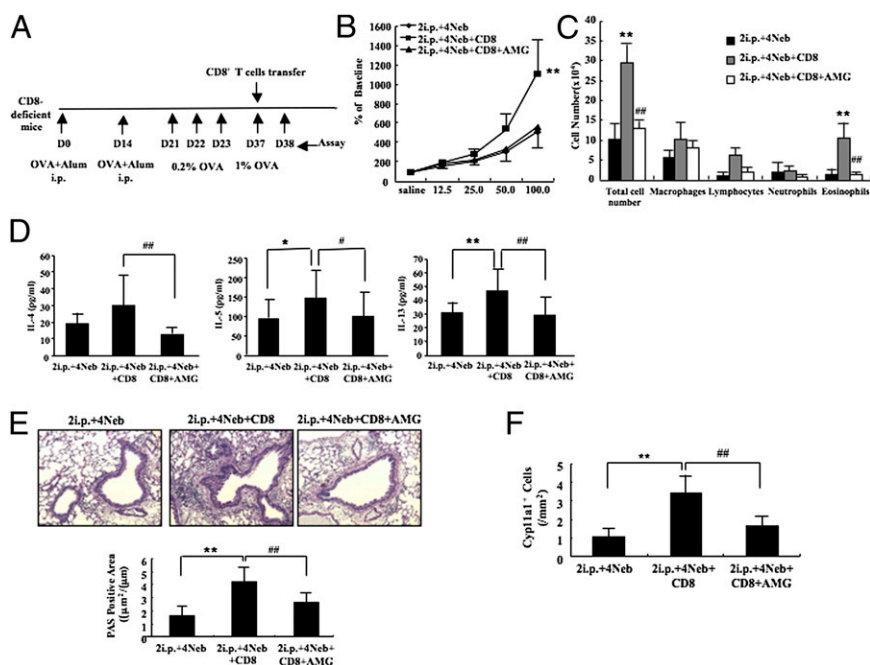


Fig. 5. Treatment of CD8-deficient recipients with CD8⁺ T cells differentiated in IL-2 and AMG (500 μ M) fails to restore AHR and inflammation. (A) Experimental protocol. (B) Changes in RL. (C) Cell composition in BAL fluid. (D) Cytokine levels in BAL fluid. (E) Representative photomicrographs of lung histology. (Magnification: $\times$ 200). Quantitative analysis of goblet cells was as described in *Materials and Methods*. (F) Number of Cyp11a1-positive cells in the lung. Data (means $\pm$ SEM) were from at least 6–10 mice. * P < 0.05 and ** P < 0.01 compared with secondary challenged CD8-deficient recipients; # P < 0.05 and ## P < 0.01 compared with secondary challenged CD8-deficient recipients of 5×10^6 IL-2-differentiated CD8⁺ T cells. 2i.p., two intraperitoneal injections; 4 nebs, four nebulized allergen challenges.

Cyp11a1 mRNA, protein, and enzymatic activity, the function of Cyp11a1 enzymatic activation was downstream of GATA3 and T-bet transcriptional events because their expression levels were unaffected by blocking Cyp11a1 activity with AMG. Because addition of SIINFEKL was also required for IL-13 cytokine production (Fig. 2C), it, thus, appeared that TCR signaling and activation of Cyp11a1 enzymatic activity were both required for the later stages in CD8 skewing to a Tc2 (IL-13) phenotype.

The extent of AMG-mediated inhibition of enzymatic activity or shRNA silencing of *Cyp11a1* was not complete, suggesting that there were both limitations to the drug efficacy, as evidenced by small amounts of residual pregnenolone production, and incomplete shRNA transfection. It is also possible that other effects, in addition to reductions in IL-13 production by CD8⁺ T cells, were associated with AMG treatment or shRNA silencing of *Cyp11a1*. A number of reports have examined the capacity of CD8⁺ T cells to suppress or enhance allergic inflammation (37–39). Tang et al. demonstrated that IFN- γ -producing T cells can alter the ability of dendritic cells (DCs) within an allergic lung to polarize T cells to a Th1 phenotype, at least *ex vivo* (38). Moreover, in accordance with our results, IFN- γ -deficient CD8⁺ T cells were capable of IL-13 production and enhancement of lung allergic responses. *In vitro*, the IL-4-induced conversion of CD8⁺ T cells to IL-13 production was associated with reduced IFN- γ production and independent of DCs (15). In adoptive transfer experiments, IFN- γ -producing CD8⁺ T cells were converted to IL-13 producers, dependent on recipient IL-4 production. Here, AMG inhibition of Cyp11a1 enzymatic activity was carried out *in vitro*, before transfer, avoiding effects in the recipients. Enomoto et al. suggested that the perforin-dependent cytotoxic function of allergen-specific CD8 T cells was essential to their ability to suppress airway eosinophilia and mucus production (39). Following IL-2+IL-4-induced differentiation of CD8⁺ T cells, we demonstrated an increase in numbers of perforin and IL-13-expressing cells with enhancement of lung allergic responses, suggesting that, at least in our model, perforin was not associated with suppression of lung allergic responses, including AHR.

Cyp11a1 is abundantly expressed in the adrenal gland, testis, ovary, and placenta, where it has been best studied and has only been found in tissues with steroidogenic potential (16). As a key regulator of steroidogenesis, catalyzing the conversion of cholesterol to pregnenolone, Cyp11a1 enzymatic activation is the first and rate-limiting step in the biosynthesis of all steroid hormones. Activation of Cyp11a1 results in a spectrum of steroid hormones, including glucocorticoids that are known to play a role in T-cell function (16, 17). Several of the gonadal steroids have been shown to have important immune effects on T cells that express their cognate receptors. T cells express receptors for androgen and estrogen, and receptor activation can impact cytokine gene transcription. These studies have related sex bias to differences in the response of CD4, CD8, and T-regulatory cells (40, 41). T cells also express many of the steroid metabolic enzymes (40). It is likely that one of the metabolites of the steroidogenesis pathway, in concert with TCR activation, regulates IL-13 transcription factor expression and IL-13 production. Determination of the precise pathway(s) and steroid metabolites that affect the phenotypic conversion of CD8⁺ T cells is now in progress.

Taken together, the data presented here establish that the steroidogenic enzyme Cyp11a1 plays a direct role in the polarization of CD8⁺ T cells from an IFN- γ - to an IL-13-producing effector cell and, as a result, is a critical regulator of the development of lung allergic responses. Cyp11a1 thus represents a pivotal enzyme linking steroidogenesis in T cells to proallergic differentiation pathways.

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 (OVA_{257–264}). CD8-deficient mice were generated by targeting the CD8 α -chain gene in C57BL/6 mice (26, 27). Animal experiments in this study were conducted under a 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 described previously (9, 10, 15). In brief, mononuclear cells were harvested from the spleens of OT-1 mice, followed by stimulation with 1 μ g/mL SIINFEKL peptide for 1.5 h. Two days after culture, living cells were reisolated using Histopaque (Sigma) and cultured in complete RPMI medium 1640 that contained recombinant mouse IL-2 (20 ng/mL) (R&D Systems) or IL-2+IL-4 (20 ng/mL) (Peprotech). The cells differentiated in IL-2+IL-4 were characterized as expressing increased levels of GATA3 and decreased levels of T-bet, eomesodermin, and runt-related transcription factor 3 (Runx3) compared with cells differentiated in IL-2 alone (15). Percentages of perforin-positive cells were higher among cells differentiated in IL-2+IL-4 ($32 \pm 4.7\%$) than among those differentiated in IL-2 alone ($19.1 \pm 1.2\%$). For some experiments, AMG was added into the medium together with IL-2 or IL-2+IL-4. Medium with cytokines was changed every day for a further 4 d. The cells were then restimulated with 1 μ g/mL SIINFEKL in medium containing 2 μ M monensin (Calbiochem) for 4 h.

RNA Preparation and Analysis. Total RNA was extracted from 5×10^6 differentiated CD8⁺ T cells using the RNeasy Mini kit (Qiagen); 1 μ g of total RNA was converted into cDNA using iScript cDNA Synthesis kit (Bio-Rad). Quantitative RT-PCR was performed using Cyp11a1 primers and probe obtained from Applied Biosystems (catalog no. Mm00490735_m1). Fold changes were determined using the $2^{-\Delta\Delta Ct}$ method, with normalization to expression of mouse GAPDH.

ELISA for Pregnenolone Measurements. CD8⁺ T cells generated in the presence of IL-2, IL-2+IL-4, IL-2 plus AMG, or IL-2+IL-4 plus AMG were cultured in six-well plates at 5×10^6 /mL for 24 h. Supernatants were collected. Pregnenolone levels were measured using the Pregnenolone ELISA kit (ALPCO Diagnostics).

Immunoblot Analysis. CD8⁺ T cells (5×10^6) were lysed with RIPA buffer containing Halt protease and phosphatase inhibitor mixture (Thermo Scientific) on ice for 30 min. Samples were analyzed by SDS/PAGE and transferred to nitrocellulose membranes. The membranes were blocked using buffer containing 2% (wt/vol) BSA and 0.5% sodium azide in tris-buffered saline with tween (TBST) for 1 h and incubated with rabbit polyclonal Cyp11a1 antibody (Lifespan Biosciences) overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) was used to detect Cyp11a1 protein. Mouse monoclonal anti- β -actin antibody (Sigma) was used as an internal control. Immunoreactive bands of Western blots were quantified by densitometric quantification of autoradiographs using ImageJ (National Institutes of Health) and expressed as relative Cyp11a1 normalized by β -actin.

CD8⁺ T-Cell Transfection. CD8⁺ T cells were transfected with a construct encoding an shRNA specific for mouse Cyp11a1 in the pGFP-V-RS vector (Origene) using an Amaxa mouse T-cell Nucleofactor kit (Amaxa/Lonza). A sequence encoding a noneffective 29-mer scrambled shRNA in the GFP-V-RS vector was used as control. Transfection was performed as directed by the manufacturer (Amaxa/Lonza) using 4 μ g of plasmid and Nucleofactor Program X-001. Forty-eight hours after transfection, cells were harvested and stimulated with SIINFEKL (1 μ g/mL) in medium containing 2 μ M monensin for 4 h and then harvested for flow-cytometric analysis.

Flow-Cytometric Analysis. For intracellular staining, 1×10^6 cells per milliliter were washed twice with PBS containing 1% BSA and stimulated with 1 μ g/mL SIINFEKL in the presence of 2 μ M monensin at 37 °C for 4 h. After fixation with 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) and permeabilization with 0.1% saponin (Sigma), cells were washed twice with PBS containing 1% BSA, then incubated with anti-mouse CD16/CD32 (2.4G2; BD Bioscience) at 4 °C for 5 min, and then stained with FITC-labeled anti-mouse IFN- γ (XMG 1.2; eBioscience) or PerCP-Cy5.5-labeled anti-mouse IFN- γ (XMG 1.2; eBioscience) and PE-labeled anti-mouse IL-13 (eBio13A; eBioscience). For some experiments, fixed cells were stained with biotin labeled rabbit anti-Cyp11a1/P450cc polyclonal antibody (Bioss), followed by PE-Cy5-labeled streptavidin (PE-Cy5-SAv; eBioscience). Cell staining was monitored on a FACSCalibur (BD Bioscience) and analyzed using Flowjo software (Tree Star).

Secondary Allergen-Challenge Model and Adoptive Transfer. The experimental protocol for sensitization and challenge to OVA was as described previously (26, 27), with some modification. CD8-deficient mice were sensitized with 20 μ g of OVA (Calbiochem) emulsified in 2.25 mg of alum (Alumimject; Pierce) on days 0 and 14 by i.p. injection. Mice were challenged with 0.2% OVA for 20 min on days 21, 22, and 23 using an ultrasonic nebulizer (model NE-U07; Omron Healthcare). To address the effect of Cyp11a1 inhibitor on CD8⁺ T cell-mediated AHR, CD8⁺ T cells (5×10^6) generated in medium containing IL-2 or IL-2 plus AMG were injected into OVA-sensitized CD8-deficient mice i.v. on day 37. Two hours after transfer, mice were challenged (secondary) with 1% OVA for 20 min. Airway function was measured and samples were collected on day 38.

Assessment of Airway Function. Airway function was assessed as described previously (26, 27) by measuring changes in airway resistance (RL) in response to increasing doses of inhaled methacholine (Sigma). Data were presented as percentage changes from the baseline RL values after saline inhalation. Baseline RL values were not significantly different among the various groups.

BAL Analysis. After measurement of AHR, lungs were lavaged via the tracheal tube with 1 mL of HBSS. The supernatants were collected, and IL-4, IL-5, and IL-13 (eBioscience) levels were measured by ELISA as described previously (26, 27). Total leukocyte numbers were counted and differentiated as described previously (26, 27).

Immunohistochemistry Staining. CD8⁺ T cells generated in the presence of IL-2 or IL-2+IL-4 were collected on slides. After fixing in 4% (vol/vol) paraformaldehyde, the slides were stained with anti-human Cyp11a1 antibody (Abcam).

Mouse lungs were isolated and fixed in 10% formalin and then embedded in paraffin and cut into 5- μ m-thick tissue sections. Sections were stained with PAS, and mucus-containing cells were quantitated as described previously (26, 27). For some experiments, lung tissue expression of Cyp11a1 was identified by immunohistochemistry staining using anti-human Cyp11a1 antibody.

Statistical Analysis. Data from in vivo experiments were representative of at least three independent experiments, with four mice per group. For the remaining studies, the number of replicates is indicated. Results were expressed as means $\pm$ SEM. Student's 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. Nonparametric analysis using the Mann-Whitney *U* test or Kruskal-Wallis test was also used to confirm that the statistical differences remained significant even if the underlying distribution was uncertain. The *P* values for significance were set to 0.05 for all tests.

ACKNOWLEDGMENTS. We thank Diana Nabighian for assistance in preparation of the manuscript. This work was supported by National Institutes of Health Grants AI-77609, HL-36577, HL-61005, and UL1 RR 025780.

- Holgate ST (2012) Innate and adaptive immune responses in asthma. *Nat Med* 18(5): 673–683.
- National Asthma Education and Prevention Program (National Heart Lung and Blood Institute) Third Expert Panel on the Management of Asthma (2007) *Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma* (National Center for Biotechnology Information, US National Library of Medicine, Bethesda), Available at www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=asthma3.TOC&depth=2.
- From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2012. Available at <http://www.ginasthma.org>.
- Martin RJ, et al.; National Heart, Lung, and Blood Institute's Asthma Clinical Research Center (2007) The predicting response to inhaled corticosteroid efficacy (price) trial. *J Allergy Clin Immunol* 119(1):73–80.
- Li LB, Leung DY, Strand MJ, Goleva E (2007) ATF2 impairs glucocorticoid receptor-mediated transactivation in human CD8⁺ T cells. *Blood* 110(5):1570–1577.
- Gelfand EW, Dakhama A (2006) CD8⁺ T lymphocytes and leukotriene B₄: Novel interactions in the persistence and progression of asthma. *J Allergy Clin Immunol* 117(3):577–582.
- van Rensen EL, et al.; AMPUL Study Group (2005) Bronchial CD8 cell infiltrate and lung function decline in asthma. *Am J Respir Crit Care Med* 172(7):837–841.
- Hamelmann E, et al. (1996) Requirement for CD8⁺ T cells in the development of airway hyperresponsiveness in a murine model of airway sensitization. *J Exp Med* 183(4):1719–1729.
- Miyahara N, et al. (2004) Contribution of antigen-primed CD8⁺ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J Immunol* 172(4):2549–2558.
- Miyahara N, et al. (2005) Leukotriene B₄ receptor-1 is essential for allergen-mediated recruitment of CD8⁺ T cells and airway hyperresponsiveness. *J Immunol* 174(8): 4979–4984.
- Koya T, et al. (2007) CD8⁺ T cell-mediated airway hyperresponsiveness and inflammation is dependent on CD4⁺IL-4⁺ T cells. *J Immunol* 179(5):2787–2796.
- Mosmann TR, Coffman RL (1989) TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145–173.
- Seder RA, et al. (1992) CD8⁺ T cells can be primed in vitro to produce IL-4. *J Immunol* 148(6):1652–1656.
- Croft M, Carter L, Swain SL, Dutton RW (1994) Generation of polarized antigen-specific CD8 effector populations: Reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* 180(5):1715–1728.
- Jia Y, et al. (2013) Step-wise epigenetic and phenotypic alterations poised CD8⁺ T cells to mediate airway hyperresponsiveness and inflammation. *J Immunol* 190(8):4056–4065.
- Lavoie HA, King SR (2009) Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and HSD3B. *Exp Biol Med* (Maywood) 234(8):880–907.
- Shih MC, et al. (2008) Mutation of mouse Cyp11a1 promoter caused tissue-specific reduction of gene expression and blunted stress response without affecting reproduction. *Mol Endocrinol* 22(4):915–923.
- Pestell RG, et al. (1996) Stimulation of the P-450 side chain cleavage enzyme (CYP11A1) promoter through ras- and Ets-2-signaling pathways. *Mol Endocrinol* 10(9):1084–1094.
- al Kandari H, Katsumata N, Alexander S, Rasoul MA (2006) Homozygous mutation of P450 side-chain cleavage enzyme gene (CYP11A1) in 46, XY patient with adrenal insufficiency, complete sex reversal, and agenesis of corpus callosum. *J Clin Endocrinol Metab* 91(8):2821–2826.
- Kim CJ, et al. (2008) Severe combined adrenal and gonadal deficiency caused by novel mutations in the cholesterol side-chain cleavage enzyme, P450_{sc}. *J Clin Endocrinol Metab* 93:696–702.
- Robel P, et al. (1995) Biosynthesis and assay of neurosteroids in rats and mice: Functional correlates. *J Steroid Biochem Mol Biol* 53(1-6):355–360.
- Slominski A, et al. (2006) An alternative pathway of vitamin D metabolism. Cytochrome P450_{sc} (CYP11A1)-mediated conversion to 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂. *FEBS J* 273(13):2891–2901.
- Oka H, Emori Y, Hayashi Y, Nomoto K (2000) Breakdown of Th cell immune responses and steroidogenic CYP11A1 expression in CD4⁺ T cells in a murine model implanted with B16 melanoma. *Cell Immunol* 206(1):7–15.
- Sundrud MS, Nolan MA (2010) Synergistic and combinatorial control of T cell activation and differentiation by transcription factors. *Curr Opin Immunol* 22(3):286–292.
- Amsen D, Spiliakakis CG, Flavell RA (2009) How are T(H)1 and T(H)2 effector cells made? *Curr Opin Immunol* 21(2):153–160.
- Miyahara N, et al. (2004) Effector memory CD8⁺ T cells mediate airway hyperresponsiveness, inflammation and remodeling. *Nat Med* 10:865–869.
- Ohnishi H, et al. (2008) Corticosteroids enhance CD8⁺ T cell-mediated airway hyperresponsiveness and allergic inflammation by upregulating leukotriene B₄ receptor 1. *J Allergy Clin Immunol* 121(4):864–871, e4.
- Ohnishi H, et al. (2009) Mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2-dependent pathways are essential for CD8⁺ T cell-mediated airway hyperresponsiveness and inflammation. *J Allergy Clin Immunol* 123(1):249–257.
- Lee NA, Gelfand EW, Lee JJ (2001) Pulmonary T cells and eosinophils: Coconspirators or independent triggers of allergic respiratory pathology? *J Allergy Clin Immunol* 107(6):945–957.
- Coyle AJ, et al. (1995) Virus-specific CD8⁺ cells can switch to interleukin 5 production and induce airway eosinophilia. *J Exp Med* 181(3):1229–1233.
- Dakhama A, et al. (2013) IL-13-producing BLT1-positive CD8 cells are increased in asthma and are associated with airway obstruction. *Allergy*, 10.1111/all.12135.
- Harris DP, Goodrich S, Mohrs K, Mohrs M, Lund FE (2005) Cutting edge: The development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *J Immunol* 175(11):7103–7107.
- Akbari O, et al. (2003) Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperactivity. *Nat Med* 9(5):582–588.
- Barlow JL, et al. (2012) Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperactivity. *J Allergy Clin Immunol* 129(1):191–198, e1–e4.
- Chtanova T, Kemp RA, Sutherland AP, Ronchese F, Mackay CR (2001) Gene microarrays reveal extensive differential gene expression in both CD4(+) and CD8(+) type 1 and type 2 T cells. *J Immunol* 167(6):3057–3063.
- Horiuchi S, et al. (2011) Genome-wide analysis reveals unique regulation of transcription of Th2-specific genes by GATA3. *J Immunol* 186(11):6378–6389.
- Takeda K, et al. (2009) Vaccine-induced CD8⁺ T cell-dependent suppression of airway hyperresponsiveness and inflammation. *J Immunol* 183(1):181–190.
- Tang Y, et al. (2012) Antigen-specific effector CD8 T cells regulate allergic responses via IFN- γ and dendritic cell function. *J Allergy Clin Immunol* 129(6):1611–1620, e4.
- Enomoto N, et al. (2012) Allergen-specific CTL require perforin expression to suppress allergic airway inflammation. *J Immunol* 188(4):1734–1741.
- Samy TS, et al. (2001) Divergent immune responses in male and female mice after trauma-hemorrhage: Dimorphic alterations in T lymphocyte steroidogenic enzyme activities. *Endocrinology* 142(8):3519–3529.
- Pottratz ST, Bellido T, Mocharla H, Crabb D, Manolagas SC (1994) 17 β -Estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J Clin Invest* 93(3):944–950.

Supporting Information

Jia et al. 10.1073/pnas.1216671110

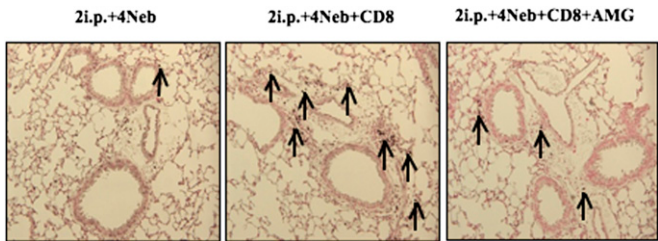


Fig. S1. Representative photomicrographs of immunohistochemical staining for cholesterol side-chain cleavage P450 enzyme (Cyp11a1)-positive cells in the lung. (Magnification: $\times 200$.) The lungs were from secondary challenged CD8-deficient recipients, secondary-challenged CD8-deficient recipients of 5×10^6 IL-2-differentiated CD8⁺ T cells, and secondary-challenged CD8-deficient recipients of 5×10^6 IL-2-plus-aminoglutethimide (AMG)-differentiated CD8⁺ T cells.

Table S1. IFN- γ and IL-13 expression in CD8⁺ T cells differentiated in IL-2 or IL-2 plus IL-4 (IL-2+IL-4) in the presence or absence of AMG

Cell type	IL-2	IL-2 plus AMG	IL-2 plus SIINFEKL	IL-2 plus AMG plus SIINFEKL	IL-2+IL-4	IL-2+IL-4 plus AMG	IL-2+IL-4 plus SIINFEKL	IL-2+IL-4 plus AMG plus SIINFEKL
IFN- γ -single-positive cells, %	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	85.6 $\pm$ 3.8	78.3 $\pm$ 8.3	0.6 $\pm$ 0.2	0.9 $\pm$ 0.5	14.5 $\pm$ 5.8	42.4 $\pm$ 11.5**
IL-13-single-positive cells, %	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2	0.5 $\pm$ 0.5	0.1 $\pm$ 0.1	3.4 $\pm$ 1.4	2.3 $\pm$ 1.5	35.7 $\pm$ 8.2	14.7 $\pm$ 8.9**
IFN- γ +IL-13 ⁺ double-positive cells, %	0	0	5.2 $\pm$ 1.1	9.0 $\pm$ 2.7	0	0.1	8.8 $\pm$ 2.3	14.7 $\pm$ 5.5

Intracellular staining of IFN- γ and IL-13 in CD8⁺ T cells with or without 1 μ g/mL OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) or 500 μ M AMG treatments. Data (means $\pm$ SEM) showing percentages of positive cells were from at least four independent experiments.

** $P < 0.01$ compared with the IL-2+IL-4 + SIINFEKL group.