IL-6 Receptor α Defines Effector Memory CD8⁺ T Cells Producing Th2 Cytokines and Expanding in Asthma

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Abstract

Rationale: Cytokine receptors can be markers defining different T-cell subsets and considered as therapeutic targets. The association of IL-6 and IL-6 receptor α (IL-6R α) with asthma was reported, suggesting their involvement in asthma.

Objectives: To determine whether and how IL-6R α defines a distinct effector memory (EM) CD8⁺ T-cell population in health and disease.

Methods: EM CD8⁺ T cells expressing IL-6R α (IL-6R α^{high}) were identified in human peripheral blood and analyzed for function, gene, and transcription factor expression. The relationship of these cells with asthma was determined using blood and sputum.

Measurements and Main Results: A unique population of IL-6R α^{high} EM CD8⁺ T cells was found in peripheral blood. These cells that potently proliferated, survived, and

produced high levels of the Th2-type cytokines IL-5 and IL-13 had increased levels of GATA3 and decreased levels of T-bet and Blimp-1 in comparison with other EM CD8⁺ T cells. In fact, GATA3 was required for IL-6R α expression. Patients with asthma had an increased frequency of IL-6R α^{high} EM CD8⁺ T cells in peripheral blood compared with healthy control subjects. Also, IL-6R α^{high} EM CD8⁺ T cells exclusively produced IL-5 and IL-13 in response to asthma-associated respiratory syncytial virus and bacterial superantigens.

Conclusions: Human IL-6R α^{high} EM CD8⁺ T cells is a unique cell subset that may serve as a reservoir for effector CD8⁺ T cells, particularly the ones producing Th2-type cytokines, and expand in asthma.

Keywords: IL-6 receptor; CD8⁺ T cells; Th2 cytokines; asthma; human

The primary function of CD8⁺ T cells is to kill target cells. However, CD8⁺ T cells can also produce cytokines and contribute to the development of inflammation (1). Memory CD8⁺ T cells generated from

naive CD8⁺ T cells by antigenic stimulation rapidly expand on reencountering the same antigen later. Based on the expression of the lymphoid tissue homing chemokine receptor CCR7, memory T cells are divided

into central and effector memory T cells (CM and EM), which can migrate to secondary lymphoid tissues and infected or inflamed peripheral tissues, respectively (2). The cytokine IL-7 and its receptor IL-7

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At a Glance Commentary

Scientific Knowledge on the

Subject: Cytokines and cytokine receptors can be markers defining different subsets of T cells with distinct functions and considered as therapeutic targets for various immune-mediated inflammatory disorders.

What This Study Adds to the

Field: Here we identified effector memory (EM) CD8⁺ T cells expressing IL-6 receptor α (IL-6R α) in human peripheral blood that have unique cellular characteristics including differentiation, proliferation, survival, and cytokine production with a distinct expression profile of transcription factors. Human EM CD8⁺ T cells expressing IL-6R α have a potent capacity to proliferate, survive, and selectively produce Th2-type cytokines with the expression of the transcription factor GATA3. IL-6R α ⁺ EM CD8⁺ T cells expand in peripheral blood of patients with asthma, suggesting the possible biologic relevance of this cell subset in asthma.

receptor α (IL-7R α) are essentially involved in maintaining memory CD8⁺ T cells by promoting cell survival (3, 4). Indeed, EM CD8⁺ T cells in human peripheral blood contain two subsets with high and low levels of IL-7R α expression (IL-7R α ^{high} and -7R α ^{low}) (5). The two cell subsets have distinct characteristics with respect to cell survival and proliferation (5, 6), suggesting a role for cytokine receptors like IL-7R α in identifying different subsets of EM CD8⁺ T cell.

IL-6 is a potent proinflammatory cytokine involved in host defense and inflammatory tissue injury (7). The association of IL-6 and IL-6 receptor α (IL-6R α , CD126) with asthma was reported, suggesting a potential involvement of these molecules in the development of asthma (8–12). IL-6 can affect T-cell functions including cytokine production, cell proliferation, and survival (8, 13–16). IL-6 binds to the IL-6R α in complex with the signal transducing protein gp130 (17). Although gp130 is expressed by most cells in the body, IL-6R α is primarily expressed on leukocytes and hepatocytes (17, 18).

In T cells, IL-6R α -expressing cells were mostly naive and CM T cells in humans and mice (19). In mice, a subset of activated CD8⁺ T cells with high levels of IL-6R α expression survived better compared with the same cells with low levels of IL-6R α expression (20). Although these findings suggest a potential role of IL-6R α in defining a unique cell subset of CD8⁺ T cells, it is unknown whether human EM CD8⁺ T cells express different levels of IL-6R α and whether such subsets have distinct cellular characteristics in health and disease. Addressing these questions is important given recent advances in immunosuppressive therapy targeting cytokine receptors, including IL-6R. Here we found a novel subset of EM CD8⁺ T cells with the expression of IL-6R α (referred to as IL-6R α ^{high} cells) in human peripheral blood and showed its relationship with Th2 cytokines and asthma.

Methods

Human Subjects

This study was approved by the institutional review committee of Yale University. Peripheral blood was obtained from healthy adult donors and patients with asthma after obtaining informed consent (see the online supplement METHODS) (5, 21).

Flow Cytometric Analysis

Peripheral blood mononuclear cells were isolated from blood by Ficoll-Hypaque gradient method and analyzed for surface and intracellular molecules by flow cytometry. Additional detail on the method for making these measurements, including antibodies, is provided in the online supplement.

Cell Purification, Culture, and Analysis

EM CD8⁺ T-cell subsets were sorted using a FACSaria (BD Biosciences, San Jose, CA) (see Figure E2 in the online supplement for gating strategy). Cells were cultured for 5 or 7 days in complete RPMI 1640 media (Life Technologies, Grand Island, NY) with various stimuli (see the online supplement METHODS and figure legends) and analyzed by flow cytometry. Culture supernatants were analyzed for cytokines using a Bioplex Pro Human Cytokine Assay kit (Bio-Rad, Hercules, CA).

Microarray and Gene Knockdown

Duplicate experiments were performed for each condition. Total RNA was extracted, amplified, and hybridized to the Illumina HumanHT-12 v4.0 BeadChip (Illumina, San Diego, CA) at the Keck Biotechnology Resource Laboratory of Yale Medical School. Additional detail on microarray analysis is provided in the online supplement. The data were deposited in the Gene Expression Omnibus database (GSE34562).

Quantitative reverse transcriptase polymerase chain reaction and GATA3 gene knockdown were done as previously described with some modifications (see the online supplement METHODS) (22).

Site-directed Mutagenesis and Reporter Gene Assay

The GATA3 binding sequence in the promoter of human *IL6RA* gene was mutated using a site-directed mutagenesis kit (Invitrogen, Carlsbad, CA). The *IL6RA* promoter with the wild-type or mutant GATA3 binding sequence was cloned into pGL3 basic vector (Promega, Madison, WI) and cotransfected into the HEK 293T cell line with pCMV6-XL5 (control vector; Origene, Rockville, MD) or pCMV6-XL5-GATA3 (GATA3-expression vector; Origene) using Lipofectamine Plus (Invitrogen). After 24 hours, the luciferase activity was measured and normalized by *Renilla* activity using a dual-luciferase assay kit (Promega).

Immunofluorescence Staining

The paraffin-embedded asthmatic and normal lung tissues were analyzed for the expression of CD3, CD8, and IL-6R α using immunofluorescent staining (see the online supplement METHODS).

Statistical Assay

The one-way analysis of variance, Student *t* test, and Spearman correlation were done as appropriate using Prism 6.0 (GraphPad Software, La Jolla, CA). *P* values less than 0.05 were considered statistically significant.

Results

Identification of an EM CD8⁺ T-Cell Subset That Expresses IL-6R α

We analyzed the expression of IL-6R α on CD8⁺ T-cell subsets in human peripheral blood. Most naive (CD45RA⁺CCR7⁺) and CM (CD45RA⁺CCR7⁺) CD8⁺ T cells

expressed high levels of IL-6R α (see Figure E1). Although a large number of EM (CD45RA^{+/−}CCR7[−]) CD8⁺ T cells did not express IL-6R α at high levels, a subset of EM CD8⁺ T cells with high levels of IL-6R α expression was identified based on isotype control staining (Figure 1A). This cell subset accounts for about 10% of EM CD8⁺ T cells in peripheral blood of healthy subjects (Figure 1B). Western blot and quantitative polymerase chain reaction analyses also showed the increased expression of IL-6R α protein and gene by IL-6R α ^{high} EM CD8⁺ T cells (Figures 1C and 1D). This cell subset that also expressed high levels of IL-7R α was found in both CD45RA⁺ and CD45RA[−] EM CD8⁺ T cells (Figure 1E). IL-6R α ^{high} EM CD8⁺ T cells

had increased expression of the signal transducing protein gp130 compared with IL-6R α ^{low} EM CD8⁺ T cells (Figure 1F).

IL-6R α ^{high} EM CD8⁺ T Cells Potently Proliferate, Survive, and Produce High Levels of the Th2-Type Cytokines

We studied the characteristics of IL-6R α ^{high} EM CD8⁺ T cells. Compared with other cell subsets, IL-6R α ^{high} EM CD8⁺ T cells produced higher levels of the Th2-type cytokines IL-5 and IL-13 as well as IFN- γ in response to 5 days of anti-CD3/CD28 antibody stimulation (Figure 2A). Although all three EM CD8⁺ T-cell subsets could produce substantial amounts of IFN- γ , IL-5 and IL-13 were produced selectively by IL-6R α ^{high} EM CD8⁺ T cells (Figure 2A).

IL-4 was barely produced only by the latter subset. IL-6R α ^{high} EM CD8⁺ T cells potently proliferated (Figures 2B and 2D), which likely stemmed from increased IL-2 production (Figure 2C). Indeed, adding anti-IL-2 antibodies substantially decreased their proliferation (Figure 2D). In accordance with the finding of our previous study (6), IL-7R α ^{low} EM CD8⁺ T cells had impaired cell proliferation in response to anti-CD3/CD28 stimulation. Also, the survival capacity of activated IL-6R α ^{high} EM CD8⁺ T cells was greater than that of other EM cell subsets (Figure 2E). Overall, our findings indicate that IL-6R α ^{high} EM CD8⁺ T cells have the capacity to potently proliferate, survive, and produce high levels of IL-5 and IL-13 in addition to IFN- γ .

Figure 1. Identification of effector memory (EM) CD8⁺ T cells with high levels of IL-6 receptor α (IL-6R α) expression in healthy human peripheral blood. (A) Flow cytometric analysis of IL-6R α and IL-7R α expression on EM (CD45RA^{+/−}CCR7[−]) CD8⁺ T cells in peripheral blood of a healthy donor. (B) Frequency of IL-6R α ^{high}, IL-6R α ^{low}IL-7R α ^{high}, and IL-6R α ^{low}IL-7R α ^{low} cells in EM CD8⁺ T cells of healthy donors ($n = 12$) as measured by flow cytometry. (C) Western blot analysis of IL-6R α expression by fluorescence-activated cell sorter (FACS) sorted IL-6R α ^{high}, IL-6R α ^{low}IL-7R α ^{high}, and IL-6R α ^{low}IL-7R α ^{low} EM CD8⁺ T cells. (D) *IL6RA* gene expression in FACS-sorted IL-6R α ^{high} and IL-6R α ^{low}IL-7R α ^{high} EM CD8⁺ T cells from healthy donors ($n = 9$) as measured by quantitative polymerase chain reaction. (E) Flow cytometric analysis of CD45RA on IL-6R α ^{high} and IL-6R α ^{low}IL-7R α ^{high} EM CD8⁺ T cells. (F) Flow cytometric analysis of gp130 expression on IL-6R α ^{high}, IL-6R α ^{low}IL-7R α ^{high}, and IL-6R α ^{low}IL-7R α ^{low} cells in EM CD8⁺ T cells. FACS sorting and flow cytometry were done using FACSAria and LSRII, respectively. Representative data from more than 10 (A, E, F) or 4 (C) independent experiments. Bars and error bars indicate mean $\pm$ SEM. P value was obtained by Student t test.

Figure 2. IL-6R α^{high} effector memory (EM) CD8⁺ T cells selectively produce high levels of IL-5 and IL-13 as well as potentially proliferate and survive in response to T-cell receptor triggering. (A–E) Peripheral blood mononuclear cells were obtained from healthy donors. (A) Multiplex cytokine assay of culture supernatants from fluorescence-activated cell sorter (FACS) purified naive, IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8⁺ T cells that were stimulated for 5 days with anti-CD3/CD28 antibody (Ab)-coated beads or phosphate-buffered saline (unstimulated). (B) Frequency of proliferating cells in FACS-purified naive, IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8⁺ T cells that were stained with CFSE and stimulated for 5 days with or without anti-CD3/CD28 Ab-coated beads. The frequency of proliferating cells was determined by flow cytometry. (C) IL-2 measurement of culture supernatants from cells stimulated as in A. (D) Flow cytometric analysis of proliferating cells in FACS-purified IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8⁺ T cells that were stained with CFSE and stimulated for 5 days with or without anti-CD3/CD28 Ab-coated beads in the presence or absence of anti-IL-2 Abs. (E) Frequency of live cells (Annexin V⁻ and 7AAD⁻) in FACS-purified IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8⁺ T cells that were stimulated for 5 days with or without anti-CD3/CD28 Ab-coated beads. The frequency of live cells was determined by staining with Annexin V and 7AAD. FACS sorting and flow cytometry were done using FACSARIA and LSRII, respectively. Numbers in dot plots indicate the frequency of live cells. Bars and error bars indicate mean (n = 5–8 donors) $\pm$ SEM (A–C). Representative data from two independent experiments with two donors (D and E). *Undetectable levels of cytokines. P values were obtained by one-way analysis of variance. ** $P < 0.05$.

IL-6R α^{high} EM CD8⁺ T Cells Have a Distinct Gene Expression Profile

To investigate why IL-6R α^{high} EM CD8⁺ T cells have unique cellular characteristics, we profiled the gene expression and molecules associated with CD8⁺ T-cell

differentiation. A total of 3,279 genes were differentially expressed in IL-6R α^{high} EM CD8⁺ T cells compared with other EM CD8⁺ T cells as measured by microarray (Figure 3A). Cluster analysis of the differentially expressed genes showed that

these genes are mainly involved in the processes related to T-cell differentiation and cell death (Figure 3B). With advanced differentiation, CD8⁺ T cells up-regulate perforin and granzyme B and natural killer cell-associated molecules KLRG1 and 2B4

Figure 3. IL-6R α^{high} effector memory (EM) CD8 $^{+}$ T cells have a distinct gene expression profile with the less differentiated cell phenotype compared with other EM CD8 $^{+}$ T cells. (A–G) Peripheral blood mononuclear cells were obtained from healthy donors. (A) Heatmap displaying six clusters categorized based on their expression patterns of 3,279 differentially expressed genes in IL-6R α^{high} EM CD8 $^{+}$ T cells (red population in Figure 1A) compared with IL-6R α^{low} IL-7R α^{high} (IL6RH/IL6RL, green population in Figure 1A) or IL-6R α^{low} IL-7R α^{low} (IL6RH/IL7RL, blue population in Figure 1A) EM CD8 $^{+}$ T cells. The red and green colors indicate increased and decreased gene expression, respectively. (B) Differentially expressed genes associated with T-cell differentiation and cell death were selected according to gene ontology biologic process using DAVID (Database for Annotation, Visualization and Integrated Discovery) software (National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD). Data were from two healthy donors (A and B). (C) Flow cytometric analysis of perforin, granzyme B, 2B4, T-bet, and eomesodermin expression by IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8 $^{+}$ T cells. (D) *KLRG1* gene expression by fluorescence-activated cell sorter (FACS) purified IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8 $^{+}$ T cells of healthy donors ($n = 6$) as measured by quantitative polymerase chain reaction. Bars and error bars indicate mean $\pm$ SEM. * $P < 0.05$ by one-way analysis of variance. (E) Western blot analysis of Blimp-1 expression by FACS-purified IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8 $^{+}$ T cells of a healthy donor. Representative data from two independent experiments with two different donors. (F) Flow cytometric analysis of IFN- γ expression by IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8 $^{+}$ T cells stimulated for 4 hours with or without phorbol myristate acetate (PMA) and ionomycin. (G) Flow cytometric analysis of CD27, CD28, and CD57 expression by IL-6R α^{high} , IL-6R α^{low} IL-7R α^{high} , and IL-6R α^{low} IL-7R α^{low} EM CD8 $^{+}$ T cells. FACS sorting and flow cytometry were done using FACSAria and LSRII, respectively. Gene expression array from two donors (A and B). Representative data from 10 (C and G) and five (F) independent experiments with 10 (C and G) and five (F) donors.

and the transcription factors T-bet and eomesodermin (Eomes) (23–27). Among EM CD8 $^{+}$ T cells, IL-6R α^{high} cells had the lowest expression of these molecules

(Figures 3C and 3D), implying that IL-6R α^{high} EM CD8 $^{+}$ T cells are not terminally differentiated memory cells. The latter notion is further supported by the low

level of expression of the transcription repressor Blimp-1, which promotes terminal differentiation effector CD8 $^{+}$ T cells in mice (Figure 3E) (28–30).

Although IL-6Rα^{high} EM CD8⁺ T cells had decreased expression of T-bet, which promotes IFN-γ expression, we noticed high levels of IFN-γ production from this cell subset after 5 days of stimulation compared with other cell subsets (Figure 2A). This unexpected outcome was likely secondary to the strong proliferative capacity of IL-6Rα^{high} EM CD8⁺ T cells with the accumulation of IFN-γ in the culture supernatant over 5 days. In fact, after 4-hour stimulation, the frequency of

IFN-γ-producing cells was much lower in IL-6Rα^{high} EM CD8⁺ T cells compared with other cell subsets, which is in line with decreased T-bet expression (Figure 3F). With the differentiation of EM CD8⁺ T cells, the costimulatory molecules CD27 and CD28 are down-regulated, while the natural killer cell-associated molecule CD57 increases (23, 24). CD27 and CD28 but not CD57 were highly expressed on IL-6Rα^{high} EM CD8⁺ T cells (Figure 3G). Overall, our findings indicate that IL-

6Rα^{high} EM CD8⁺ T cells do not have the traits of terminally differentiated memory CD8⁺ T cells.

GATA3 Is Highly Expressed and Required for the Expression of IL-6Rα in IL-6Rα^{high} EM CD8⁺ T Cells

The transcription factor GATA3 promotes Th2 cell differentiation (31). IL-6Rα^{high} CD8⁺ T cells expressed high levels of GATA3 compared with other cell subsets, whereas the expression of T-bet, which

Figure 4. GATA3 is required for the expression of IL-6Rα in IL-6Rα^{high} effector memory (EM) CD8⁺ T cells. Quantitative polymerase chain reaction (A) and Western blot (B) analyses of GATA3 and TBX21 (*T-bet*) gene and protein expression by fluorescence-activated cell sorter (FACS) purified IL-6Rα^{high}, IL-6Rα^{low}IL-7Rα^{high}, and IL-6Rα^{low}IL-7Rα^{low} EM CD8⁺ T cells from healthy donors ($n = 7$ and 2 for quantitative polymerase chain reaction and Western blot, respectively). Bars and error bars indicate mean $\pm$ SEM. (C) Western blot analysis for GATA3 and IL-6Rα by FACS-purified IL-6Rα^{high} EM CD8⁺ T cells that were nucleofected with control siRNA or GATA3 siRNA and then harvested after 48 hours. Representative data from two independent experiments. (D) Location of the wild-type (WT) or mutant GATA3-binding consensus sequence in the promoter region of the human *IL6RA* gene. (E) The promoter activity of the *IL6RA* gene containing the WT or mutant GATA3-binding consensus sequence (D) was analyzed in the presence or absence of GATA3 overexpression in HEK 293T cells (see details in METHODS). Data represent the mean $\pm$ SD from five independent experiments. GATA3 overexpression in HEK 293T cells was confirmed by Western blot analysis. (F) Venn diagram depicting the relationships of the differentially expressed genes in IL-6Rα^{high} versus IL-6Rα^{low}IL-7Rα^{high} EM CD8⁺ T cells in this study (see Figure 3A) and potential target genes of GATA3. This overlap (277) is highly significant ($P < 1 \times 10^{-5}$). FACS sorting was done using FACS Aria (A–C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, all by one-way analysis of variance.

suppresses Th2 cell differentiation, was low (Figures 4A and 4B). These findings account for the increased production of IL-5 and IL-13 from IL-6R α^{high} EM CD8 $^{+}$ T cells. A recent study reported the role of GATA3 in regulating IL-7R α expression in mice (32). We thus explored the possible role of GATA3 in up-regulating IL-6R α expression. IL-6R α^{high} EM CD8 $^{+}$ T cells

treated with GATA3 small interfering RNA (siRNA) to knock down expression of GATA3 had reduced IL-6R α expression compared with cells treated with control siRNA (Figure 4C). We identified a known GATA3-binding consensus sequence in the promoter of the human *IL6RA* gene (Figure 4D). The overexpression of GATA3 increased the promoter activity of the

IL6RA gene, whereas mutating the GATA3-binding sequence decreased the promoter activity (Figure 4E). These data provide the explanation for the coexpression of IL-6R α and Th2 cytokines by human IL-6R α^{high} EM CD8 $^{+}$ T cells. In fact, there was a highly significant overlap ($P < 1 \times 10^{-5}$) between the differentially expressed genes in IL-6R α^{high} EM CD8 $^{+}$ T cells and the

Figure 5. The frequency of IL-6R α^{high} effector memory (EM) CD8 $^{+}$ T cells increases in the peripheral blood of patients with asthma. (A) Flow cytometric analysis of IL-6R α^{high} EM CD8 $^{+}$ T cells in peripheral blood of healthy donors ($n = 19$) and patients with asthma ($n = 19$). Peripheral blood mononuclear cells were purified and then stained with CD3, CD8, CD45RA, CCR7, IL-7R α , and IL-6R α . IL-6R α^{high} EM CD8 $^{+}$ T cells were identified by gating on EM CD8 $^{+}$ T cells as in Figure 1A. (B) Correlation of percent predicted FEV $_1$ and frequency of peripheral IL-6R α^{high} EM CD8 $^{+}$ T cells in patients with asthma ($n = 34$). Correlation of eosinophil (C) or neutrophil (D) percent in sputum from patients with asthma and frequency of peripheral IL-6R α^{high} EM CD8 $^{+}$ T cells in patients with asthma ($n = 26$). (E) The induction of intercellular adhesion molecule (ICAM) 1 on human eosinophils by culture supernatant of T cell–receptor–triggered IL-6R α^{high} EM CD8 $^{+}$ T cells. Fluorescence-activated cell sorter purified IL-6R α^{high} or IL-6R α^{low} IL-7R α^{high} EM CD8 $^{+}$ T cells were stimulated for 5 days with anti-CD3/CD28 antibody-coated beads. The culture supernatants of individual cell subsets were collected and added to eosinophils purified from a healthy donor (2.5% final concentration of culture supernatant). After 4 hours of incubation, eosinophils were collected and then analyzed for ICAM-1 expression by flow cytometry. Representative data from three independent experiments with three healthy donors, respectively. (F) Flow cytometric analysis of CCR4, CCR5, and CCR6 expression by EM CD8 $^{+}$ T-cell subsets. Representative data from five independent experiments with five healthy donors. P values were obtained by Student t test (A) and Spearman correlation analysis (B–D).

genes with putative GATA3-binding sites as identified by MetaCore (Thomson Reuters, New York, NY) data mining and pathway analysis (Figure 4F) (33). This further supports the role of GATA3 in regulating the cellular traits of IL-6Rα^{high} EM CD8⁺ T cells.

The Frequency of IL-6Rα^{high} EM CD8⁺ T Cells Increases in the Peripheral Blood of Patients with Asthma

Recent data support the role of CD8⁺ T cells and the cytokines IL-5 and IL-13 in the pathogenesis of asthma (1, 34–42). To elucidate a possible biologic relevance of IL-6Rα^{high} EM CD8⁺ T cells, we determined the frequency of this cell subset in the peripheral blood of healthy individuals and patients with asthma. The frequency of IL-6Rα^{high} cells in the EM CD8⁺ T-cell population was higher in patients with asthma compared with age- and sex-matched healthy control subjects (Figure 5A) (characteristics of patients and control subjects in Table 1 and Table E2, respectively). IL-13-producing cells were found primarily in IL-6Rα^{high} EM CD8⁺ T cells in patients with asthma after 4 hours of phorbol myristate acetate/ionomycin stimulation, whereas IFN-γ-producing cells were found in all of the three subsets (see Figure E3). The percent

predicted FEV₁ is an indicator of asthma severity; the lower the percent predicted FEV₁, the higher the disease severity. Of interest, the frequency of IL-6Rα^{high} cells in the peripheral EM CD8⁺ T cells moderately correlated with percent predicted FEV₁ in patients with asthma, indicating the inverse correlation of this cell subset with disease severity (Figure 5B). IL-5 and IL-13 are known to activate eosinophils, which are frequently increased in the sputum of patients with asthma (34).

The frequency of IL-6Rα^{high} cells in the peripheral EM CD8⁺ T cells subset also had a moderate inverse correlation with the percentage of eosinophils but not neutrophils in the sputum of patients with asthma (Figures 5C and 5D). The cell culture supernatant of T-cell receptor-stimulated IL-6Rα^{high} EM CD8⁺ T cells potently activated eosinophils as measured by the expression of intercellular adhesion molecule 1, the eosinophil activation marker (Figure 5E) (43). IL-6Rα^{high} EM CD8⁺ T cells expressed high levels of CCR4, which is known to play a key role in recruiting T cells into the airways of patients with asthma (Figure 5F) (44). Overall, our data show expansion of IL-6Rα^{high} CD8⁺ T cells with the potent capacity to produce IL-5 and IL-13 in the peripheral EM CD8⁺ T cells of patients with asthma.

A Combination of IL-6 and IL-15 Potently Induces Proliferation of IL-6Rα^{high} EM CD8⁺ T Cells That Produce High Levels of IL-5 and IL-13 in Response to Respiratory Syncytial Virus

Increased levels of IL-6 were reported in the sputum and peripheral blood of patients with asthma (10–12, 45). Thus, we determined whether IL-6 could induce expansion of IL-6Rα^{high} EM CD8⁺ T cells. Although IL-6 alone could not induce the proliferation of these cells, adding IL-6 and IL-15, a cytokine known to promote CD8⁺ T-cell expansion (4, 6), synergistically enhanced their proliferation (Figure 6A). IL-7 alone or a combination of IL-7 and IL-6 barely induced proliferation of IL-6Rα^{high} EM CD8⁺ T cells (see Figure E4). Studies suggest the potential involvement of viral infections, such as respiratory syncytial virus (RSV), and CD8⁺ T cells recognizing superantigens in asthma

(39, 46–51). We analyzed cytokine production from IL-6Rα^{high} and other EM CD8⁺ T-cell subsets in response to RSV and bacterial superantigens. IL-5 and IL-13 were produced only from IL-6Rα^{high} EM CD8⁺ T cells in the presence of RSV antigen (Figure 6B). Similarly, the same cytokines were highly produced from IL-6Rα^{high} EM CD8⁺ T cells in response to the bacterial superantigens SEB and TSST-1 (Figures 6C and 6D).

Discussion

We have identified a novel subset of EM CD8⁺ T cells with high levels of IL-6Rα expression in human peripheral blood. IL-6Rα^{high} EM CD8⁺ T cells actively proliferated, survived, and produced high levels of IL-5 and IL-13. Such traits were likely governed by the distinct expression of genes including GATA3. In fact, GATA3 was required for the expression of IL-6Rα in this cell subset. Of interest, the frequency of IL-6Rα^{high} cells in the EM CD8⁺ T-cell population increased in the peripheral blood of patients with asthma, suggesting a biologic relevance of IL-6Rα^{high} EM CD8⁺ T cells to this disease where CD8⁺ T cells and Th2-type cytokines can be involved in disease pathogenesis (1, 34–39). Having the capacity to potently proliferate and produce cytokines, including the Th2-type cytokines IL-5 and IL-13, human IL-6Rα^{high} EM CD8⁺ T cells can be a reservoir for effector CD8⁺ T cells, which expands on immune stimulation, such as in asthma.

The unique characteristics of IL-6Rα^{high} EM CD8⁺ T cells can be explained by the differential expression of several transcription factors including GATA3, T-bet, Eomes, and Blimp-1. GATA3 promotes Th2 response, whereas T-bet suppresses it (52). Mice deficient of GATA3 had defective T-cell response to viral infection and alloantigen with impaired peripheral maintenance of CD8⁺ T cells and decreased proliferative capacity with low levels of IL-7Rα (32, 53). In our study, IL-6Rα^{high} EM CD8⁺ T cells with high levels of GATA3 had increased cell proliferation compared with other EM CD8⁺ T cells with low levels of GATA3 expression. We also found that GATA3 was required for IL-6Rα expression and its promoter activity in IL-6Rα^{high} EM CD8⁺ T cells.

Table 1. Clinical Characteristics of Patients with Asthma

Age, yr*	49.2 ± 18.2
Sex, male/female	13:21
FEV ₁ , L/s*	2.44 ± 1.03
Predicted % FEV ₁ *	76.5 ± 22.21
Patients with history of tobacco smoking	
n	12
Pack-years*	5.6 ± 2.93
Patients with history of rhinitis, n	30
Medications [†]	
Steroid inhalers	29
Oral steroids	6
Long-acting β-agonist	25
Short-acting β-agonist	33
Anticholinergics	8
Leukotriene inhibitors	20
Antihistamine	17
Omalizumab	3

n = 34.

*Values are mean ± SD.

[†]Numbers indicate the numbers of patients taking individual medications among 34 patients.

Figure 6. A combination of IL-6 and IL-15 potently induces proliferation of IL-6R α ^{high} EM CD8⁺ T cells that produce high levels of IL-5 and IL-13 in response to respiratory syncytial virus (RSV) and bacterial superantigens. (A) The frequency of proliferating cells in fluorescence-activated cell sorter (FACS) purified IL-6R α ^{high} EM CD8⁺ T cells that were cultured for 7 days in the presence or absence of IL-6 (25 ng/ml) and/or

These findings can account for the mechanism underlying the coexpression of the Th2-type cytokines and IL-6 α by human IL-6 α^{high} EM CD8 $^{+}$ T cells. With advanced differentiation, CD8 $^{+}$ T cells increase T-bet, Eomes, and Blimp-1 (23–27, 54, 55). Low levels of T-bet, Eomes, and Blimp-1 expression in IL-6 α^{high} EM CD8 $^{+}$ T cells supports that they are not terminally differentiated memory CD8 $^{+}$ T cells. It is possible that IL-6 α^{high} EM CD8 $^{+}$ T cells have an alternative lineage that is largely Th2-like polarized. Nevertheless, the ontogeny of IL-6 α^{high} EM CD8 $^{+}$ T cells and their ontogenetic relationship with other EM CD8 $^{+}$ T cells are yet to be determined.

We found an increased frequency of IL-6 α^{high} CD8 $^{+}$ T cells in the peripheral EM CD8 $^{+}$ T-cell population of patients with asthma compared with healthy control subjects. In fact, these cells were the only subset of EM CD8 $^{+}$ T cells that produced high levels of IL-5 and IL-13 in response to RSV and bacterial superantigens, which have been associated with asthma (39, 46–51). Mouse and human studies supported the involvement of CD8 $^{+}$ T cells and of the cytokines IL-5 and IL-13 in the pathogenesis of asthma and allergy (1, 34–39, 56). A recent study reported that patients with severe asthma had a transcript expression profile associated with an activated phenotype in circulating CD8 $^{+}$ T cells compared with healthy control subjects (57). In a mouse model of asthma, deleting CD8 $^{+}$ T cells reduced allergic airway inflammation, whereas reconstituting CD8 $^{+}$ T cells in these mice with EM CD8 $^{+}$ T cells increased airway hyperresponsiveness (AHR) and IL-13 levels in bronchoalveolar lavage (37). Mice that were adoptively transferred with allergen-specific CD8 $^{+}$ T cells producing high levels of IL-4 had increased numbers of eosinophils and the induction of AHR (58). The transfer of CD8 $^{+}$ T cells from mice deficient of IL-13 to CD8 $^{+}$ T-cell knockout mice could not

increase AHR and inflammation (38). This finding is supported by a human study showing increased IL-13-producing CD8 $^{+}$ T cells in bronchoalveolar lavage of patients with asthma (42). IL-5 and IL-13 can have multiple effects on eosinophils, mast, B, epithelial, and airway smooth muscle cells, which are implicated in the pathogenesis of asthma (34). Specifically, these cytokines can promote the activation, recruitment, and survival of eosinophils, a cellular hallmark of asthma (1, 34).

In our study, the expansion of peripheral blood IL-6 α^{high} EM CD8 $^{+}$ T cells in patients with asthma had a moderate inverse correlation with disease severity. A possible explanation for this observation could be the migration of this cell subset to the asthmatic lung tissue given its expression of CCR4, the Th2 cell-associated chemokine receptor (44). We noticed CD8 $^{+}$ T cells expressing IL-6 α in the biopsied lung tissue of a patient with asthma (see Figures E5A and E5B). However, additional studies are warranted to find the exact role of IL-6 α^{high} EM CD8 $^{+}$ T cells in the pathogenesis of asthma, although this may not be simple in the human system. We analyzed the frequency of peripheral IL-6 α^{high} EM CD8 $^{+}$ T cells in patients with and without a history of rhinitis. Both groups had similar frequencies of these cells (mean [%] $\pm$ SEM, 19.7 ± 1.87 vs. 17.7 ± 3.34 ; $P = 0.712$). Similarly, there was no difference in the frequency of this cell population between patients with asthma with and without a history of smoking (mean [%] $\pm$ SEM, 21.6 ± 2.79 vs. 18.4 ± 2.13 ; $P = 0.371$). We recognize that IL-6 α^{high} EM CD8 $^{+}$ T cells also produced IFN- γ in particularly after 5 days of stimulation. In fact, this cell population seems to have several subpopulations in that cells producing IFN- γ and IL-13 or IL-5 are largely distinct as measured by intracellular cytokine analysis (see Figure E6A), although some cells coproduce IL-13, IL-5, and/or IL-2 (see Figure E6B). Th2-type cytokines are a major pathogenic player in asthma and have a reciprocal relationship

with Th1-type cytokines. The involvement of IFN- γ in AHR was reported in mice (59, 60). Also, IFN- γ -producing CD8 $^{+}$ T cells were found in the airway of patients with asthma (35). However, the significance of this production of IFN- γ in asthma is yet to be determined.

The unique production of the asthma-associated Th2 cytokines by IL-6 α^{high} EM CD8 $^{+}$ T cells and the expansion of such cells in patients with asthma raise the possibility of testing a therapeutic approach depleting IL-6 α^{high} EM CD8 $^{+}$ T cells by using dual specific antibodies targeting IL-6 α and CD8 $^{+}$ T cells simultaneously. This notion is supported by the clinical use of anti-IL-6 α antibody therapy for other inflammatory conditions and by the improvement of allergic airway inflammation in mice treated with anti-IL-6 α antibodies (8, 61, 62).

Taken together, we show the presence of a novel subset of EM CD8 $^{+}$ T cells with high levels of IL-6 α expression in human peripheral blood. IL-6 α^{high} EM CD8 $^{+}$ T cells had the potent capacity to proliferate, survive, and produce high levels of the Th2-type cytokines IL-5 and IL-13. The distinct cellular traits of IL-6 α^{high} EM CD8 $^{+}$ T cells are likely driven by the differential expression of the transcription factors including high levels of GATA3 along with low levels of T-bet and Blimp-1. The frequency of these cells increased in the peripheral EM CD8 $^{+}$ T cells of patients with asthma, although the exact biologic significance of this cell subset in asthma remains to be elucidated. With the unique cellular characteristics, IL-6 α^{high} EM CD8 $^{+}$ T cells may serve as a reservoir for the effector CD8 $^{+}$ T cells, especially the ones producing Th2-type cytokines, which expands on immune stimulation, such as in asthma. ■

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Figure 6. (Continued). IL-15 (5 ng/ml) or with anti-CD3/CD28 antibody-coated beads. (B, C, and D) Multiplex cytokine assay of culture supernatants from FACS-purified IL-6 α^{high} , IL-6 α^{low} /IL-7 α^{high} , and IL-6 α^{low} /IL-7 α^{low} EM CD8 $^{+}$ T cells that were incubated for 7 days with RSV lysate (B), staphylococcal enterotoxin B (SEB) (C), toxic shock syndrome toxin-1 (TSST-1) (D), or phosphate-buffered saline (control) in the presence of mitomycin C-treated autologous peripheral blood mononuclear cells as antigen-presenting cells (APCs). FACS sorting was done using FACSaria. Representative data from two (A) and four (B, C, and D) independent experiments with two and four healthy donors, respectively. *Undetectable levels of cytokines.

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