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Hierarchical IL-5 Expression Defines a Subpopulation of Highly Differentiated Human Th2 Cells

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Each of the three Th2 cytokine genes, IL-4, IL-5, and IL-13, has different functions. We hypothesized that Th2 heterogeneity could yield Th2 subpopulations with different cytokine expression and effector functions. Using multiple approaches, we demonstrate that human Th2 cells are composed of two major subpopulations: a minority IL-5+ (IL-5+, IL-4+, IL-13+) and majority IL-5− Th2 (IL-5−, IL-4−, IL-13−) population. IL-5− Th2 cells comprised only 20% of all Th2 cells. Serial rounds of in vitro differentiation initially yielded IL-5− Th2, but required multiple rounds of differentiation to generate IL-5− Th2 cells. IL-5− Th2 cells expressed less CD27 and greater programmed cell death-1 than IL-5− Th2 cells, consistent with their being more highly differentiated, Ag-exposed memory cells. IL-5− Th2 cells expressed greater IL-4, IL-13, and GATA-3 relative to IL-5− Th2 cells. GATA-3 and H3K4me3 binding to the IL5 promoter (IL5p) was greater in IL-5− relative to IL-5− Th2 cells, whereas there was no difference in their binding to the IL4p and IL13p. Conversely, H3K27me3 binding to the IL5p was greater in IL-5− Th2 cells. These findings demonstrate Th2 lineage heterogeneity, in which the IL5 gene is regulated in a hierarchical manner relative to other Th2 genes. IL-5− Th2 cells are phenotypically distinct and have epigenetic changes consistent with greater IL5p accessibility. Recurrent antigenic exposure preferentially drives the differentiation of IL-5− Th2 cells. These results demonstrate that IL-5− and IL-5− Th2 cells, respectively, represent more and less highly differentiated Th2 cell subpopulations. Such Th2 subpopulations may differentially contribute to Th2-driven pathology. The Journal of Immunology, 2011, 187: 3111–3120.
Materials and Methods

Subjects

Subjects were 18–60 y of age. Clinical and immunological details on allergic eosinophil-associated gastrointestinal disorder (EGID) and healthy nonallergic control subjects used in this study were previously reported (15). Allergic asthmatic subjects had a minimum 1-y history of episodic bronchospasm relieved by β-agonist medications and three or more positive skin test responses (1 mm) out of 10 aeroallergens. Written informed consent was obtained from all subjects. The study was approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board approved the clinical protocols used for this study. All subjects signed informed consent.

Cells and culture

Subjects underwent lymphapheresis (National Institutes of Health Clinical Center Department of Transfusion Medicine), and PBMCs were isolated using density gradient separation (Lymphocyte Separation Media-1077; MP Biomedicals, Aurora, OH), washed twice in HBSS (Invitrogen, Carlsbad, CA), and cryopreserved in liquid nitrogen. Cell cultures were carried out in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids (all Invitrogen), 1 mM Na-pyruvate, 10 mM HEPES (Cellgro, Herndon, VA), and 50 μM/10-2 ME (Sigma-Aldrich, St. Louis, MO). PBMCs were cultured with the below noted stimuli at 2 × 10^6 cells/ml in 24-well plates in a 5% CO2 incubator for 6 h and then fixed for intracellular cytokine staining (ICCS). Stimuli included: washed THP-1 (100 μM/ml; ALK, Round Rock, TX), TNF α (4 μg/ml; Indoor Biotechnologies, Charlottesville, VA), staphylococcal enterotoxin B (SEB; 10 μg/ml; Toxin Technology, Sarasota, FL) or 20 ng/ml PMA, and 1 μM ionomycin (EMD Chemicals, Gibbstown, NJ).

Secreted cytokine staining and cell separation was performed using the MACS Cytokine Secretion Assay IL-4 and IL-5 Cell Enrichment and Detection Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Cells were activated with SEB for 6 h, cytokine-producing cells enriched with beads, stained with 7-aminocyanin D (Invitrogen) and CD4, and viable CD4 cytokine-producing subpopulations sorted (FACS Aria; BD Biosciences). For dual color cytokine sorts, cells were bead enriched for IL-4 and stained with IL-4-PE and IL-5 allophycocyanin.

Polycryl T cell lines were generated from cytokine-stored cells after culture with 40 units/ml IL-2 (Chiron, Emeryville, CA) at 5000 cells/well in a 96-well plate for 1 wk. Th2 clones were generated by seeding cytokine-stored cells in 96-well U-bottom plates at limiting dilution with 1 × 10^4 3000 rad-irradiated allogeneic PBMC and IL-2 40 U/ml. After 1 wk, 100 μg/ml media was replaced with fresh media and 8 U IL-2; after 2 wk, positive clones were further expanded in additional media and IL-2; and after 3 wk, clones were activated with PMA/ionomycin/brefeldin A (BFA) for 6 h and analyzed by ICCS. Cloning efficiency was 4–18%.

Dust mite Ag-specific cell lines were generated from allergic asthmatic subjects by activating PBMC with dust mite Ag for 6–8 h, after which IL-2 (100 U/ml) was added and the resulting cell line propagated for an additional 10–12 d and finally restimulated with PMA/ionomycin/BFA for 6 h and analyzed by ICCS. PBMC from EGID subjects were thawed, washed twice in RPMI 1640, labeled with 8 μM CFSE (Invitrogen) in RPMI 1640 at 37°C for 10 min, quenched by adding five times the volume of ice-cold PBS/1% BSA, and washed twice in RPMI. Cells were then resuspended at 5 × 10^5 cells/ml in RPMI with 10% autologous serum and cultured with 100 μg/ml crude peanut extract in 24-well plates. After 4 to 5 d, 1 ml culture supernatant was replaced with fresh media. After 7 d, cells were activated with PMA/ionomycin/BFA for 6 h and analyzed by ICCS.

For Th2 differentiation, naive CD4 T cells were obtained from PBMC by first using the naive CD4+ T cell isolation kit (Miltenyi Biotec), then sorting for viable CD4+ CD31+CD45RO- cells. Naive cells were Th2 polarized according to published methods (18) using plate-bound anti-CD3 (10 μg/ml, clone OKT3; BioLegend), soluble anti-CD28 (2 μg/ml clone CD28.2; BioLegend), IL-4 (12.5 ng/ml; R&D Systems), anti–IFN-γ (10 μg/ml clone B27; BD Biosciences), anti–IL-10 (5 μg/ml, clone JES3-9D7; BD Biosciences), and IL-2 (40 U/ml). After 4 d, cells were removed from the plate and expanded with additional IL-2 for 3 d. Th2-polarization cycles were repeated at weekly intervals. At each time point, cells were activated with PMA/ionomycin/BFA for 6 h and analyzed by ICCS.

Quantitative PCR and TCR excision circle quantitation

RNA was extracted from cell pellets, and quantitative RT-PCR analysis was performed as previously published (19). All four FAM-MGB-labeled TaqMan probe and primer sets for IL-4 (Hs00174122_m1), IL-5 (Hs00174200_m1), IL-13 (Hs00174379_m1), and GATA-3 (Hs00221122_m1) were purchased from Applied Biosystems (Invitrogen). The eukaryotic translation initiation factor 3 subunit B gene was selected as the endogenous control based on its constitutive expression across various biological conditions as determined from pilot experiments using human Th2 cells; probe and primer sets are detailed in Supplemental Table I. Relative mRNA expression was calculated as the ratio of target gene to eukaryotic translation initiation factor 3. TCR excision circle (TREC) quantification was performed using PCR as previously published (20).

ICCs and flow cytometry

ICCs was performed according to previously published methods (15, 21). Briefly, after activation, cells were washed once with cold PBS, labeled on ice with Live/Dead Fixable Violet Dead Cell Stain (Invitrogen), washed, and fixed in 4% paraformaldehyde (Sigma-Aldrich). Cells were then resuspended in PBS with 10% DMSO (Sigma-Aldrich) and cryopreserved at −80°C. The following eight-color panel was used for the Boolean analyses: Violet LIVE/DEAD, CD3 Qdot605 (clone UCHT1), CD8 PE/TR (clone 3B5) (all Invitrogen), CD4 FITC (clone Q4120; Sigma-Aldrich), IL-4 4allophycocyanin (clone 2D5), IL-5 PE (clone JES1-39D10), IFN-γ Alexa Fluor 700 (clone B27) (all BD Biosciences), and IL-13 PE/Cy7 (clone JES10-5A2, custom conjugate). Samples were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). The data were exported to Excel (Microsoft, Redmond, WA) for the generation of the gating strategy. Mean fluorescence intensities (MFIs) were identified by flow cytometry: CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height. After gating on viable CD3*, CD4*, and CD8* cells, data were plotted for various cytokine combinations. Statistical markers were based on the staining of unactivated media control samples. Boolean analysis was performed with FlowJo software (Tree Star), and the data were exported to Excel (Microsoft, Redmond, WA) for the generation of the MFI. The differentiation of the cultures was calculated by gating forward versus side scatter, and cell doublets were excluded using forward scatter area versus height.
cytokines: IL-4, IL-5, and IL-13. A variety of stimuli and culture systems were used, including 6-h ex vivo activation with either polyclonal activators or allergens and short-term TH2 cell lines. A common finding across these diverse systems and patient populations was that IL-5 was always expressed by a minority subset of TH2 cells (Fig. 1A), whereas IL-4 and IL-13 were highly coexpressed (Fig. 1B). Similar patterns of coexpression were found when anti-cytokine mAb fluorochromes were swapped (data not shown). IL-5 expression was consistently restricted to a minority TH2 subpopulation over a 2–23-h time course (Supplemental Fig. 1), demonstrating that this finding is not simply due to differences in cytokine expression kinetics.

To quantitate all potential combinations of TH2 cytokines at the single-cell level, we simultaneously stained for all three TH2 cytokines and performed Boolean analysis according to the cytokine subpopulation color scheme noted (Fig. 1C) (21, 22). Polyclonal activation with PMA/ionomycin was used to assess the entire TH2 compartment. For the purposes of this study, TH2 cells were operationally defined as cells expressing at least one TH2 cytokine, but not IFN-γ. Remarkably, in nonatopic healthy controls, IL-5 expression was restricted to only 17% of all TH2 cells (arc, Fig. 1D). We further examined TH2 responses in subjects with allergic EGID, an eosinophilic form of food allergy associated with increased CD4 T cell IL-5 expression (15, 23). Even in these highly TH2-skewed EGID subjects, IL-5 expression was restricted to only 23% of the total TH2 population (Fig. 1E). Similar highly restricted IL-5 expression was found in SEB-activated samples (data not shown). In contrast, IL-4 and IL-13 were each expressed by 70–80% of TH2 cells, with most TH2 cells expressing both cytokines. Examination of individual subjects confirmed this overall trend (Supplemental Fig. 1). The majority of TH2 cells were one of two cytokine phenotypes: IL-4+IL-5+IL-13+ triple-positive or IL-4+IL-5-IL-13+ double-positive cells. The fraction of TH2 cells with the IL-5+IL-4+IL-13+ (red) phenotype was greater in EGID versus nonatopic healthy control subjects (18 versus 12%, respectively, \( p = 0.06 \); Fig. 1D versus 1E). Both allergen-specific TH2 cells identified 6 h ex vivo and short-term IFN-54-sorted TH2 cell lines demonstrated similar coexpression patterns and dominant TH2 subpopulations (Fig. 1F, 1G).

There was a reciprocal relationship between the frequency at which a given cytokine was expressed within the TH2 lineage and the proportion of cytokine-expressing cells that were of the IL-5+ IL-4+IL-13+ triple-positive phenotype (Supplemental Fig. 2). For example, whereas IL-5 was expressed by only 20% of all TH2 cells, 79% of IL-5+ cells were of the triple-positive phenotype. In contrast, IL-4 and IL-13 were, respectively, expressed by 74 and 79% of all TH2 cells, but only 21 and 17% of IL-4+ and IL-13+ cells were triple positive. In both allergen-specific TH2 cells and IL-4-sorted TH2 cell lines, 98–100% of IL-5+ TH2 cells had the triple-positive phenotype (Fig. 1F, 1G).

In sum, these results demonstrate that IL-5 expression is highly restricted to a minority subpopulation of TH2 cells with an IL-4+ IL-5+IL-13+ phenotype, whereas the majority of TH2 cells express IL-4 and IL-13, but not IL-5.

We next determined if these different TH2 subpopulations were associated with differences in per-cell cytokine expression. Both IL-4 and IL-13 MFIs demonstrated a significant and incremental decrease progressing from IL-4+IL-5+IL-13+ triple-positive, to double-positive, to single-positive TH2 cells (Fig. 1F, 1H; \( p < 0.001 \) for both). Within a given TH2 subpopulation, there was no significant difference in IL-4 or IL-13 MFI between EGID and nonatopic control subject groups (\( p > 0.05 \)).

**TH2 subpopulations retain their respective phenotype**

To establish that the coexpression patterns obtained using ICCS reflected actual cytokine secretion, cells were activated and stained for secreted IL-4 and IL-5. Notably, cytokine secretion assay staining showed the same pattern of dichotomous IL-5 expression, confirming the results obtained using ICCS (Fig. 2A). We next sought to determine whether differential IL-5 expression was a
hereditable and durable property of these Th2 subpopulations. To this end, cells were sorted for IL-4+IL-5+ or IL-4+IL-5- populations (Fig. 2A), expanded in vitro for 7 d, and analyzed for Th2 cytokine coexpression. IL-4+IL-5+ sorted cells yielded a population that was almost wholly IL-4+IL-5+IL-13+ triple-positive, whereas IL-4+IL-5- sorted cells yielded a mix of IL-5+ and IL-5+ Th2 cells (Fig. 2B, 2C versus 2D, 2E). In contrast, IL-4 and IL-13 were uniformly coexpressed in both sorted cell lines (Fig. 2C, 2E). Using Boolean analysis, there are seven potential combinations of Th2 cytokine expression (Fig. 2H); however, of those seven, only two major Th2 subpopulations were noted; IL-4+IL-5+IL-13+ and IL-4+IL-5+IL-13+ cells. Notably, IL-5+ sorted cells yielded a significantly greater frequency of IL-5+ Th2 cells than did IL-5- sorted cells (Fig. 2F versus 2G, 76 versus 36%; p = 0.008, n = 5 independent experiments). Conversely, IL-5- sorted cells maintained the IL-5- Th2 phenotype relative to IL-5+ sorted cells (44 versus 16%; p = 0.008, n = 5).

**Clonal inheritance of Th2 cytokine expression**

To examine the inheritance, durability, and pattern of Th2 cytokine expression at the single-cell level, IL-5+ and IL-4+IL-5- Th2 cells were sorted and cloned at limiting dilution. Such Th2 clones demonstrated the same two dominant Th2 subpopulations previously found in polyclonal systems (Fig. 3A, 3B). Although many Th2 clones expressed a uniform IL-4+IL-5+IL-13+ or IL-4+IL-5-IL-13+ phenotype (Fig. 3A, 3B, left and rightmost panels), most were IL-4+IL-13- but had bimodal IL-5 expression (Fig. 3A, 3B, three center panels). Each clone was then analyzed using the above Boolean gating approach. In agreement with the polyclonal data, a significantly greater fraction of IL-5- sorted clones were of the IL-4+IL-5-IL-13+ phenotype relative to IL-5- sorted clones (Fig. 3C versus 3D, 81 versus 52%; p < 0.0001). IL-5- sorted clones largely had bimodal IL-5 expression. When the IL-5+ and IL-5- Th2 subpopulations within each clone were compared, IL-4 and IL-13 expression was significantly greater in the IL-5+ Th2 population (p < 0.0001; Fig. 3E, 3F).

Th2 clones representing the extreme IL-5+ and IL-5- phenotypes (percent IL-5+ cells by ICCS, 97.8 versus 7.8%, respectively) were selected according to their ICCS staining (Fig. 3G) and further analyzed by quantitative PCR (qPCR) (Fig. 3H–J). As expected, these two populations exhibited a significant difference in IL-5 expression (154 versus 5.8 relative expression; p < 0.0001). Additionally, IL-5+ clones expressed greater IL-13 relative to the IL-5- Th2 clone (96 versus 62 relative expression; p = 0.04), which was not found for IL-4 (2.1 versus 1.95 relative expression).

In sum, these results using both polyclonal and clonal analysis demonstrate that the dichotomous IL-5 expression found in Th2 cells is a durable property of the respective Th2 subpopulations. These results further establish that Th2 cells are composed of two major subpopulations, IL-4+IL-5-IL-13+ and IL-4+IL-5-IL-13+ cells.

**Generation of IL-5+ Th2 cells requires multiple rounds of differentiation**

To examine the single-cell dynamics of Th2 differentiation, naive CD4 T cells were differentiated in vitro under Th2 conditions using serial cycles of TCR activation. After two cycles, IL-13 was expressed by 73% of cells, whereas IL-5 was limited to only 5.7%, but progressively increased in subsequent rounds (Fig. 4A). Similarly, Boolean analysis demonstrated a consistent and markedly delayed acquisition of the IL-4+IL-5-IL-13+ triple-positive Th2 phenotype relative to IL-5- Th2 cells (Fig. 4B). Similar to the results in Fig. 1I, per cell, IL-13 expression was significantly greater in IL-4+IL-5+IL-13+ cells versus either double- or single-positive Th2 cells (p = 0.0066) and increased with multiple rounds of Ag-driven differentiation (p < 0.0001; Fig. 4C). Similarly, IL-4 was more highly expressed in IL-4+IL-5-IL-13+ cells versus IL-4+ IL-5+IL-13+ double-positive cells (Fig. 4D).

GATA-3 is the major Th2 lineage-specific transcription factor and binds to sites in the Th2 locus control region as well as the IL5p and IL13p (9, 24). To determine the relationship between Th2 heterogeneity and GATA-3 expression, we next examined GATA-3 expression during serial rounds of Th2 differentiation. After one to two rounds of Th2 differentiation, GATA-3 was expressed by the majority of CD4 T cells, whereas IL-5 and IL-13 were limited to a small fraction of the cells (Fig. 4E–G). qPCR analysis similarly demonstrated early upregulation of GATA3 and delayed acquisition of IL5 expression during Th2 differentiation (Fig. 4H).

Because the acquisition of IL-5 expression in vitro requires a greater number of rounds of differentiation, we examined whether in vivo-differentiated IL-5+ Th2 cells similarly had undergone more rounds of cell division. To this end, IL-5- and IL-5+ Th2 cells were sorted and analyzed for TREC. IL-5+ Th2 cells had...
generally lower numbers of TREC and undetectable TREC, relative to IL-5$^+$ Th2 cells (Supplemental Fig. 3). In sum, these data indicate that differentiation of cells to the IL-5$^+$ Th2 phenotype requires a greater number of rounds of antigenic stimulation, relative to IL-5$^-$ Th2 cells.

IL-5$^+$ Th2 cells are highly differentiated T cells

The delayed acquisition of the IL-5$^+$ Th2 phenotype implies that IL-5$^+$ Th2 cells are more highly differentiated than IL-5$^-$ Th2 cells. To further examine this question, Th2 subpopulations were identified using ICCS and assessed for their expression of memory T cell markers. Both Th2 subpopulations had a CD45RO$^+$ CD45RA$^-$ phenotype (data not shown). CD27 is a member of the TNFR superfamily, for which expression is lost on highly differentiated memory CD4 T cells (25, 26). CD27 expression was significantly lower in IL-5$^+$ versus IL-5$^-$ Th2 cells (Fig. 5B,5C; $p = 0.03$). Programmed cell death-1 (PD-1), a CD28 family member that is expressed on chronically activated or exhausted T cells (27), was significantly greater in IL-5$^+$ versus IL-5$^-$ Th2 cells (Fig. 5B,5C; $p = 0.03$). In contrast, CD57, which has been reported as a marker of polyfunctional and/or terminally differentiated CD8 and Th1 cells (26, 28), was no different between these Th2 subpopulations. In sum, these results demonstrate that IL-5$^+$ Th2 cells have a phenotype consistent with highly differentiated memory Th2 cells.

Th2 subpopulations display epigenetic changes consistent with differential IL-5p activation

The sequential transition from naive to IL-5$^-$ Th2 to IL-5$^+$ Th2 cells (Fig. 4) suggests stepwise chromatin remodeling, first opening up the IL4 and IL13 loci and ultimately opening up the IL5 gene. To this end, we first assessed total GATA-3 expression in IL-5$^+$ and IL-5$^-$ Th2 cells. As expected, all IL-5$^+$ and IL-13$^+$ cells expressed GATA-3, whereas Th1 cells did not (Fig. 6A). GATA-3 immunofluorescence was significantly greater in the IL-5$^+$ Th2 versus the IL-5$^-$ Th2 subpopulation (Fig. 6B; $p = 0.03$). Similarly, as shown in Fig. 6C, GATA-3 expression as determined by qPCR for IL5, IL4, and IL13, respectively. Horizontal bars represent the median values. Statistical significance was determined using the Mann–Whitney U test.
dust mite-specific cell lines from allergic asthmatic subjects (Fig. 6D) and analyzed by ChIP. IL-5+ Th2 cells had greater GATA-3 binding to the IL5p relative to IL-5 Th2 cells (Fig. 6E, 6F). In contrast, GATA-3 binding to the IL4p and IL13p was similar between the Th2 subpopulations. These results were confirmed with a second anti–GATA-3 mAb clone (data not shown). To further assess the mechanism for differential gene expression in these Th2 subpopulations, we performed ChIP for the methylated histones H3K4me3 and H3K27me3, which are, respectively, associated with active and repressed chromatin. H3K4me3 demonstrated greater IL5p binding in IL-5+ versus IL-5 Th2 cells. Conversely, H3K27me3 demonstrated less IL5p binding in IL-5+ versus IL-5 Th2 cells. In sum, these data demonstrate an open chromatin configuration in the IL5p specific to IL-5+ Th2 cells, which are consistent with differential IL-5 expression by these Th2 subpopulations.

**Discussion**

We report two human Th2 subpopulations, both expressing IL-4 and IL-13, but differing in IL-5 expression: a minority IL-5+ Th2 (IL-4+IL-5+IL-13+) and the majority IL-5 Th2 (IL-4+IL-5 IL-13+) subpopulation. The generalizability of this finding is underscored by its ubiquity across the wide range of experimental systems and disease entities used in the study. IL-5 expression was restricted to a small minority of Th2 cells in both nonallergic controls and Th2-skewed eosinophilic subjects. These data are most consistent with a hierarchical model of Th2 differentiation and gene expression characterized by sequential transcriptional regulation in which IL5 activation represents the most highly differentiated stage. IL-5+ Th2 cells demonstrated greater chromatin accessibility at the IL5p and greater overall GATA3 expression, relative to IL-5 Th2 cells, suggesting that hierarchical differentiation is due to sequential epigenetic changes. Our findings demonstrate that IL-5 Th2 cells are highly differentiated Th2 cells for which differentiation requires multiple rounds of antigenic stimulus. These results suggest a possible mechanism whereby Th2-dominant disease states characterized by recurrent Ag exposure would thus favor the generation of IL-5+ Th2 cells, which may preferentially drive eosinophilic inflammation.

**FIGURE 4.** The generation of IL-5+ Th2 cells requires multiple rounds of differentiation. A–H. Naive CD4 T cells underwent serial rounds of in vitro Th2 differentiation and were analyzed for Th2 cytokine expression by ICCS at each time point noted. A, IL-5 versus IL-13 plots from a representative experiment. B, Boolean analysis of combined results of cytokine expression. For each Th2 subpopulation and time point, IL-13 (C) and IL-4 (D) MFI ratio was determined. At the indicated time points, cells were stained for IL-5 versus IL-13 (E) or GATA-3 (F) and the percentage of positive staining cells (IL-5, IL-13, and GATA-3) or MFI (GATA-3) determined. Combined results of ICCS (G) and qPCR (H) for the indicated time points and analytes; results in H were normalized to the day 40 values. I, Subpopulations in B–D are according to the color scheme shown in I. Results in B–D, G, and H are median values from three independent experiments; results in A, E, and F are representative of three independent experiments. Statistical significance in C was determined using a two-way ANOVA test.
We have previously reported the disease association of IL-5+ Th2 cells with allergic EGID (15). However, this current report provides a more thorough characterization of these cells as being true subpopulations. The generalizability of these findings to all human Th2 responses is underscored by the presence of these subpopulations across a wide variety of experimental approaches, including multiple culture systems (6 h ex vivo, Th2 lines, in vitro-differentiated Th2 cells), Ags (polyclonal activators, allergens), assay systems (ICCS, cytokine secretion staining, PCR), and subject populations (allergic asthma, EGID, nonatopic controls) (Fig. 1). The hereditable and durability of these Th2 subpopulations in vitro, their different phenotypic and epigenetic signatures, and their disynchronous appearance in Th2-differentiation cultures all support the thesis that these are distinct entities. These data, together with established T cell biology (2, 8), suggest that these subpopulation differences are intrinsic to the cells rather than due to heterogeneity of activation conditions resulting in different cytokines being expressed.

Notably, evidence for IL-5 expression restricted to a minority Th2 subgroup within the larger IL-5+ Th2 population (Figs. 1–4, Supplemental Fig. 2). First, IL-5+ Th2 cells were almost exclusively IL-4+IL-5+IL-13+ triple-positive cells within the larger Th2 population (Figs. 1–4, Supplemental Fig. 2). Second, this hierarchy is apparent in the greater per-cell IL-4 and IL-13 expression as Th2 cells progressed from single-positive to double-positive to triple-positive (Figs. 1H, 1I, 4C). Third, both phenotyping and in vitro-differentiation studies indicate that IL-5+ Th2 cells are more highly differentiated (Figs. 4, 5). Lastly, IL-5+ Th2 cells demonstrate epigenetic changes and greater GATA-3 expression, consistent with greater chromatin accessibility at the Th2 locus (Fig. 6). A dichotomous lineage model would generate clones that were either wholly IL-5+ or IL-5-, whereas sorted Th2 clones displayed a range of IL-5 expression, indicating a continuum of differentiation states (Fig. 3D). The polyfunctional phenotype (37) is conferred by greater expression of effector genes in any combination, whereas our findings clearly indicate a hierarchy of Th2 cytokine expression with IL-5 at the extreme. Intriguingly, IL-17A and IL-17F are coexpressed in a similar pattern to IL-5 and IL-4/13 (38), suggesting that comparable hierarchical expression may occur within the Th17 lineage.

The findings in this current work also support a probabilistic model of cytokine expression, in which the chromatin configuration and transcription factor binding to a given allele confer a probability of that gene being expressed (39, 40). The finding that clones derived from the IL-4/13 IL-5+ sort exhibit a wide range of IL-5 expression (Fig. 3) is most consistent with IL-5 being expressed in a probabilistic manner. In this model, a single cell for which IL5 loci have an intermediate probability of expression clonally expands into a population with intermediate IL-5 expression. In contrast, the high coexpression of IL-4 and IL-13, most dramatically exhibited in the Th2 cytokine secretion-sorted cells (Figs. 2, 3), suggests probabilities of IL-4 and IL-13 gene expression approaching 100%. Th2-differentiation cultures initially yielded predominantly IL-5+ Th2 cells, but required multiple additional rounds of differentiation to generate IL-5+ Th2 cells (Fig. 4). This suggests the possibility that Th2-dominant diseases characterized by chronic Ag exposure may preferentially drive IL-5+ Th2 cell differentiation and eosinophilic inflammation. This mechanism is further supported by our previous findings of IL-5+ and IL-5- dominant food Ag-specific Th2 responses in EGID and peanut anaphylaxis, respectively (15). Because EGID patients generally do not have anaphylaxis to foods, they may ingest greater amounts of allergenic foods, presumably driving the differentiation of IL-5+ Th2 cells. In contrast, peanut-allergic patients avoid peanuts, likely leading to less Ag exposure, resulting in their having less differentiated IL-5- Th2 cells. Recent sporadic cases of EGID in peanut and milk anaphylactic patients undergoing oral immunotherapy may possibly reflect this enhanced differentiation of IL-5+ Th2 cells upon repeated food Ag exposure (41–44). Similarly, the association of asthma with perennial allergen sensitization (e.g., house dust mites, cockroach) may reflect the generation of IL-5+ Th2 cells upon recurrent allergen inhalation (45).

Although these results do not directly address Th2 plasticity, our finding that ≥90% of IL-4+ sorted cells retain the Th2 phenotype after 1–3 wk in culture provides evidence of both the durability of the Th2 phenotype and the relative rarity of IL-4+IFN-g-Tho-like cells in vivo. Less differentiated or early Th2 cells are more plastic to Th1 differentiation than highly differentiated Th2 cells (5). If IL-5+ Th2 cells are more highly differentiated, then one would expect them to be less plastic than IL-5- Th2 cells to cross-differentiation toward Th1, Th17, or Th9.

FIGURE 5. IL-5+ Th2 cells are highly differentiated T cells. PBMC were activated for 6 h ex vivo with PMA/ionomycin, and viable CD4 T cells were simultaneously stained for IL-5, IL-13, and IFN-γ and the indicated T cell-differentiation markers. A, After gating on IL-5+IL-13+ (thick black line), IL-5-IL-13+ (thin black line), or IFN-γ-IL-13+ cells (thick gray line), CD27, PD-1, and CD57 histograms for each subset were generated and displayed using the same symbols (B). C, Combined results from five subjects showing CD27 and PD-1 expression by IL-5+IL-13+ (filled squares) and IL-5-IL-13+ (open circles) Th2 cells; bars represent median values. Statistical significance was determined using a Mann–Whitney U test.
The molecular mechanisms underlying the hierarchical expression of IL-5 by Th2 cells are of particular interest. Chromatin accessibility at the IL5p is differentially regulated relative to either IL-4 or IL-13 (Fig. 6). These data are most consistent with a model of Th2 differentiation characterized by an ordered sequence of transcriptional regulation in which increased chromatin accessibility at the IL5p is the last step. Our findings of greater overall GATA-3 expression in IL-5+ Th2 cells suggest that this difference may be due to IL5 gene expression requiring greater GATA-3 concentrations than those required for Th2 differentiation. Notably, IL-13, which is also highly GATA-3 dependent, was expressed at greater levels in IL-5+ versus IL-5− Th2 cells (Figs. 1, 3, 4). Previous reports demonstrating a GATA-3 gene dose effect in GATA-3 heterozygotes indicate that a 2-fold difference in GATA-3 concentration is sufficient to affect Th2 differentiation and cytokine expression (46, 47). Similarly, a recent report demonstrating epigenetic changes at the GATA-3 promoter in more highly differentiated Th2 cells further supports a critical role for GATA-3 (48). Alternatively, hierarchical IL5 expression could be due to a limiting transcription factor other than GATA-3 that is uniquely required for IL-5 gene activation or to the IL5p itself being more resistant to chromatin remodeling. The clinical relevance of these epigenetic changes is further underscored by these studies having been performed in house dust mite-specific cell lines, each derived from a different allergic asthmatic subject.

Luster and colleagues (51) recently reported a subpopulation of CCL8-responsive CCR8+ Th2 cells with enhanced IL-5 expression, which may represent the same IL-5+ Th2 cells characterized in this current work. In the previous report, the generation of CCL8-responsive murine Th2 cells required multiple rounds of in vitro differentiation, analogous to our findings in Fig. 4. Notably, we found IL-5 expression was almost entirely limited to IL-4−IL-5+IL-13+ triple-positive Th2 cells, whereas in the previous work, CCL8-responsive Th2 cells were largely characterized as IL-4−IL-5+. This discrepancy may alternatively reflect differences in Th2-differentiation methods, human versus mouse Th2 gene regulation, or Th2 ICCS methods.

This work has not specifically addressed the respective function of these Th2 subpopulations or their contribution to eosinophilic inflammation. Presumably, through their greater expression of IL-5, IL-5+ Th2 cells may have a proeosinophilic inflammatory function (13, 14). The disease association of IL-5+ Th2 cells with EGID provides additional evidence in that direction (15, 23). Notably, IL-5+ Th2 cells expressed greater per-cell quantities of IL-4 and IL-13 than did IL-5− Th2 cells (Figs. 1, 3, 4) and as such may have greater overall Th2 function. Notably, this study was limited to the three classic Th2 cytokines. It is possible that IL-5+ Th2 cells differentially express other genes in addition to IL-5 (e.g., CCR8) (51) that confer additional functional properties to this subpopulation.

In summary, these findings demonstrate a fundamental and generalizable heterogeneity in the Th2 lineage in which the IL5 gene is regulated in a hierarchical manner relative to other Th2 genes. These findings establish that IL-5+ and IL-5− Th2 cells, respectively, represent more and less highly differentiated Th2 cell subpopulations, with each having distinct phenotypic and epigenetic features. A potential consequence of this heterogeneity is that specific Th2 subpopulations may differentially contribute to...
Th2-driven immunopathology and as such may represent distinct therapeutic targets.

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Disclosures
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