

Andrea M. Coverstone, MD
Brad Wilson, MA
Dana Burgdorf, RN
Kenneth B. Schechtman, PhD
Gregory A. Storch, MD
Michael J. Holtzman, MD
Mario Castro, MD, MPH
Leonard B. Bacharier, MD
Kaharu Sumino, MD, MPH

From the Washington University School of Medicine in St Louis, St Louis, Mo. E-mail: ksumino@dom.wustl.edu.

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A critical role for IL-18 in transformation and maturation of naive eosinophils to pathogenic eosinophils



To the Editor:

Eosinophils have very distinct roles in maintaining innate immunity in healthy subjects and are also involved in the pathogenesis of allergic diseases, autoimmune diseases, and

allograft rejection.^{1,2} IL-5 is a well-established differentiation, growth, and survival factor for eosinophils, and earlier reports indicate that IL-5 and eotaxins regulate baseline resident eosinophils.^{1,3} Most recently, we and others reported a distinguishable subset of CD101-expressing⁴ and CD274-expressing⁵ eosinophils, similar to the other immune cell populations that exhibit differences in their phenotype and function.

CD101 expressing eosinophils have been described as IL-5-dependent lung-specific inflammatory eosinophils in a house dust mite (HDM)-induced mouse model of asthma, which is different from the IL-5-independent residential eosinophils detected in healthy mouse lungs.⁴ Previously, we also reported CD101- and CD274-expressing eosinophils that are induced in the blood of patients with eosinophilic esophagitis (EoE), and their numbers increase with disease severity and decrease after treatment.⁵

Here we validate our previous findings of CD101- and CD274-expressing eosinophils in patients with EoE and further report that both these subsets are also present in the blood of patients with other allergic diseases, such as asthma (Fig 1, A-D).

Interestingly, we observed that most CD101-expressing blood eosinophils are not CD274-expressing eosinophils (Fig 1, E and F) and that their percentages and absolute numbers increase in the blood of allergic patients (Fig 1, G and H). This observation indicates that both of these eosinophil subsets are either not similar or in a different developmental stage. Most importantly, we report that the tissue-accumulated eosinophils in the nasal lavage fluid of asthmatic patients and esophageal biopsy specimens of patients with EoE are CD101 and CD274 double-positive (CD101⁺CD274⁺) eosinophils (Fig 1, I-N). These observations confirm that CD101- and CD274-expressing eosinophils present in the blood are indeed intermediate-stage eosinophil that mature into CD101⁺CD274⁺ double-positive pathogenic eosinophils after accumulation in tissue in patients with allergic diseases. CD101-expressing eosinophils have been shown to be pathogenic in adoptive transfer experiments in a mouse model of asthma.⁴ Detailed clinical characteristics of patients are provided in Table E1 in this article's Online Repository at www.jacionline.org.

Mechanistically, to understand eosinophil subset development, we first analyzed IL-5-responsive eosinophils of CD2-IL-5 transgenic mice. This analysis showed that in both spleen (Fig 2, A-F) and blood (Fig 2, G-J) most eosinophils do not express CD101 or CD274 in CD2-IL-5 transgenic mice. These observations are inconsistent with recent reports of CD101-expressing eosinophils as IL-5 dependent,⁴ and therefore we investigated the factors that promote CD101 and CD274 expression on eosinophils. Several cytokines (IL-5, IL-13, IL-15, IL-18, IL-21, and IL-33) have been implicated in eosinophil-associated allergic disease.^{6,7} Thus we investigated the role of IL-13, IL-15, IL-18, IL-21, and IL-33 in the eosinophil differentiation and transformation of both the CD101 and CD274 eosinophil subsets. We show that CD2-IL-5 transgenic mouse eosinophils become CD101- and CD274-expressing eosinophils kinetically only after *ex vivo* exposure to IL-18. No other cytokine was able to transform IL-5-generated eosinophils into CD101- or CD274-expressing eosinophils (data not shown), indicating that IL-18 is critical in this transformation (Fig 2, K-R). Notably, IL-18 is reported in almost all allergic

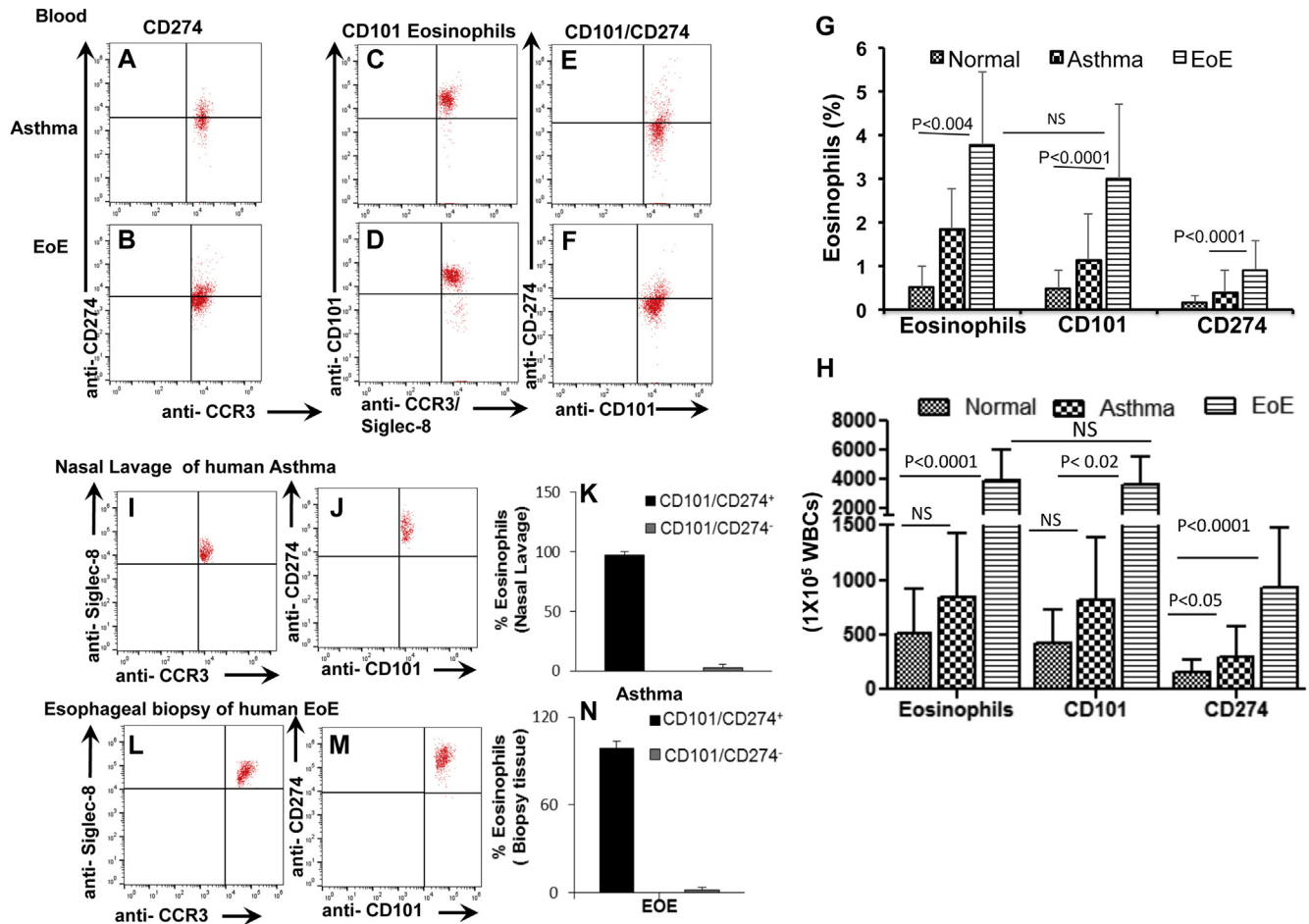


FIG 1. Human blood and tissue CD101 and CD274 expression on eosinophils in healthy subjects and allergic patients. **A–D**, Representative flow cytometric analysis was performed by selecting live CCR3⁺ and anti-Siglec-8 double-positive eosinophils for identifying CD101- and CD274-expressing eosinophils in the blood of healthy subjects, asthmatic patients, and patients with EoE. **E and F**, Analysis shows that all blood CD101-expressing eosinophils do not express CD274. **G and H**, Percentages and absolute numbers of both CD101- and CD274-expressing CCR3⁺ Siglec-8⁺ eosinophils in the blood of patients with EoE and asthmatic patients compared with healthy subjects are shown in Fig 1, **G** and **H**, respectively. **I–N**, Nasal lavage fluid from asthmatic patients (Fig 1, **I–K**) and esophageal biopsy specimens from patients with EoE (Fig 1, **L–N**) showed only double-positive CD101⁺CD274⁺ eosinophils. All data presented are expressed as means $\pm$ SDs (healthy subjects, n = 10; asthmatic patients, n = 14; patients with EoE, n = 16). NS, Not significant.

diseases, including an HDM antigen-induced mouse model of asthma.⁸ These mechanistic eosinophil transformation data indicate that because of the lack of understanding on the role of IL-18, the investigators of recently reported studies concluded that CD101-expressing eosinophils in the lungs are IL-5 dependent in asthmatic mice.⁴

Here we also show that IL-18 is capable of eosinophil differentiation apart from IL-5. The low-density CD34⁺ mouse bone marrow (LDEBM) precursors exposed for 2 weeks *ex vivo* rIL-18 differentiate approximately 6% to 7% eosinophils regardless of mouse genotype compared with rIL-5 (approximately 45% to 52%)-generated eosinophils (see Fig E1, **D** and **E**, in this article's Online Repository at www.jacionline.org). This indicates that IL-5-independent eosinophils develop in response to IL-18. In addition, we also observed IL-5 is required for the growth and survival of IL-18-generated eosinophils (see Fig E1, **F**). No other cytokine (IL-13, IL-15, IL-18, IL-21, and IL-33) alone is able to differentiate eosinophils from LDEBM.

These observations prompted us to characterize further both IL-5- and IL-18-differentiated *ex vivo* eosinophils, including their size, nuclear shape, cytoplasmic/nuclear ratio, granular contents, and expression of differentially regulated genes. Confocal microscopic images of anti-MBP-stained IL-5- and IL-18-differentiated eosinophils from LDEBM indicated differences in their nuclear shape and cytoplasm/nuclear ratio (Fig 3, **A–C**). Furthermore, electron microscopic analysis shows intact eosinophil granules in IL-5-generated eosinophils compared with the degranulated eosinophils found among IL-18-generated eosinophils (Fig 3, **D**). Finally, differences in eosinophil size and granularity of IL-18- and IL-5-generated eosinophils were established by performing flow cytometric analysis, which indicated that IL-18-derived eosinophils are more granular and larger in size (Fig 3, **E**). Similar electron microscopic characteristics were reported recently for CD101-expressing lung eosinophils of asthmatic mice, which we observed in IL-18-differentiated eosinophils.⁴ Interestingly, microarray analysis also identified highly induced CD274

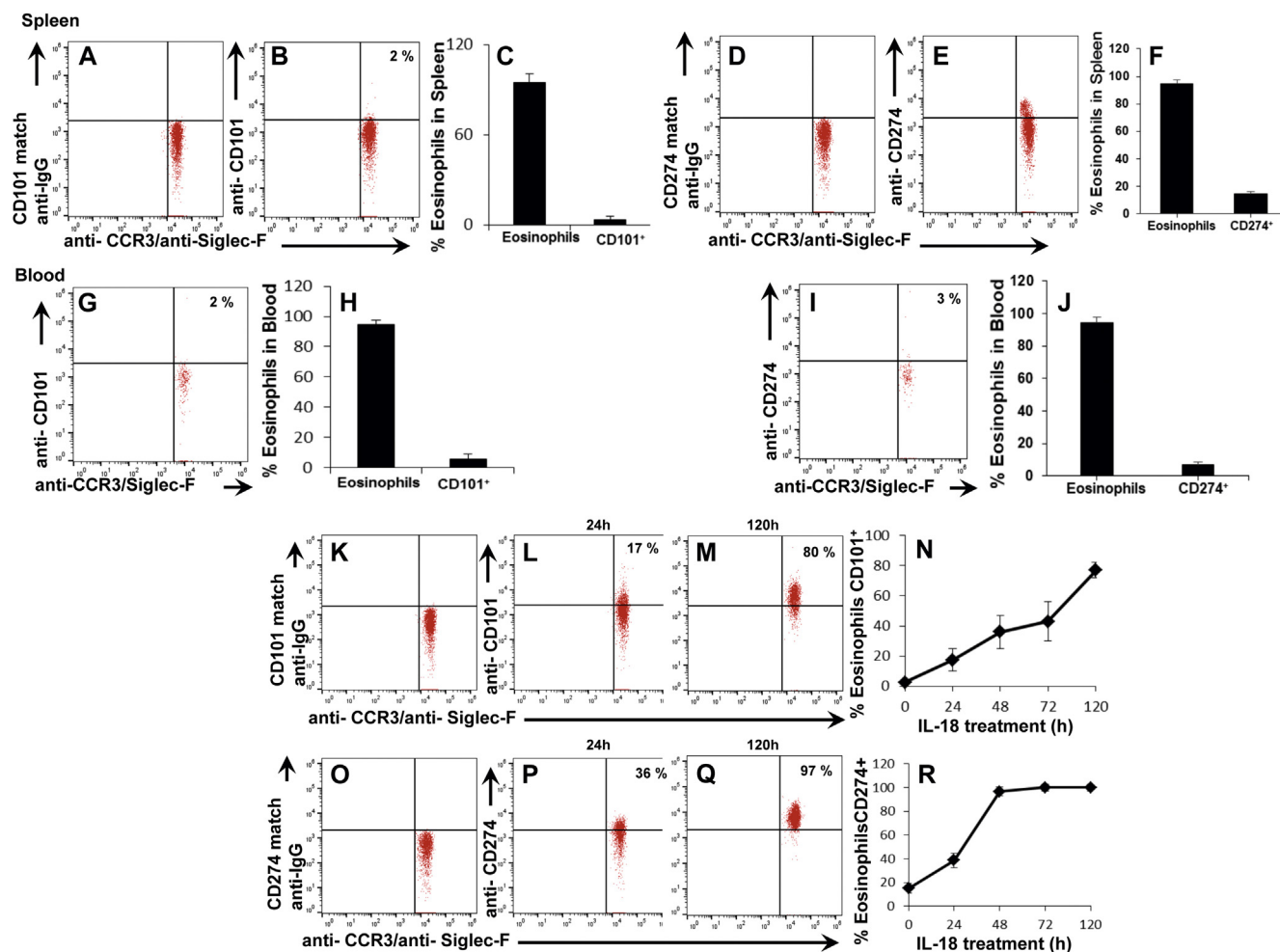


FIG 2. IL-18 is critical for the development of CD101 and CD274 eosinophil subsets. **A-F**, A representative flow cytometric analysis of eosinophil surface expression on 7-aminoactinomycin D (7AAD)⁺/CCR3⁺ leukocytes selected for detection of CD101 and CD274 on anti-CCR3 and anti-Siglec-F double-positive eosinophils of CD2-IL-5 transgenic mouse splenocytes and blood based on CD101 and CD274 isotype-matched anti-IgG. **G-J**, Very few (>2%) CD101-expressing (Fig 2, **G** and **H**) and CD274-expressing (Fig 2, **I** and **J**) eosinophils (CCR3⁺ Siglec-F⁺) were detected in the blood. **K-R**, CD2-IL-5 transgenic mouse splenocytes exposed to IL-18 *ex vivo* showed a time-dependent kinetic increase in expression of CD101 on CCR3⁺ Siglec-F⁺ eosinophils (Fig 2, **K-N**) and CD274 on CCR3⁺ Siglec-F⁺ eosinophils (Fig 2, **O-R**). Data are expressed as means $\pm$ SDs ($n = 3$ individual experiments).

transcript expression in IL-18-differentiated eosinophils (Fig 3, **F**) compared with IL-5-differentiated eosinophils that was further confirmed by performing quantitative PCR analysis (Fig 3, **G**). Notably, CD101 expression was not detected by using microarray analysis, and a very low and comparable level of CD101 expression was found in IL-5- and IL-18-generated eosinophils (Fig 1, **H**).

A detailed gene expression list of IL-5- and IL-18-differentiated transcripts is provided in Table E2 in this article's Online Repository at www.jacionline.org. These transcript profiles further confirm that IL-18-generated eosinophils are indeed a distinct subset of eosinophils. Despite high expression of CD274 transcript, a distinct hierarchical clustering characteristic also revealed that several proinflammatory genes, such as *CLEC4D*, *PTGS2*, *IL15*, *PF4*, *NT5C3b*, *Saa3*, *CXCL2*, *Spp1*, and *Rsad2*, are also found to be highly induced in IL-18-derived eosinophils compared with IL-5-derived eosinophils (Fig 3, **F**).

The role of IL-18 in eosinophil maturation is further supported by the detection of multiple-fold increase in eosinophil granules (eosinophil cationic protein and eosinophil-derived neurotoxin) in IL-18-differentiated eosinophils compared with IL-5-generated eosinophils (Fig 3, **I**). The enhanced levels of major basic protein, eosinophil-derived neurotoxin, and eosinophil cationic protein granules were reported earlier in a number of patients with allergic diseases, including asthma, EoE, and colitis.⁹ The details of the methods used to identify and characterize these subsets of eosinophils are reported in the Methods section in this article's Online Repository at www.jacionline.org.

Furthermore, the significance of IL-18 in the development of both CD101 and CD274 expression on eosinophils *in vivo* was also examined in the mouse model of *Aspergillus* allergen-induced experimental asthma. We show that asthmatic mice also have CD101- and CD274-expressing double-positive eosinophils (CD101⁺CD274⁺) in wild-type mice that were not detected in the absence of endogenous IL-18 in mice (see Fig E2, **A-M**, in

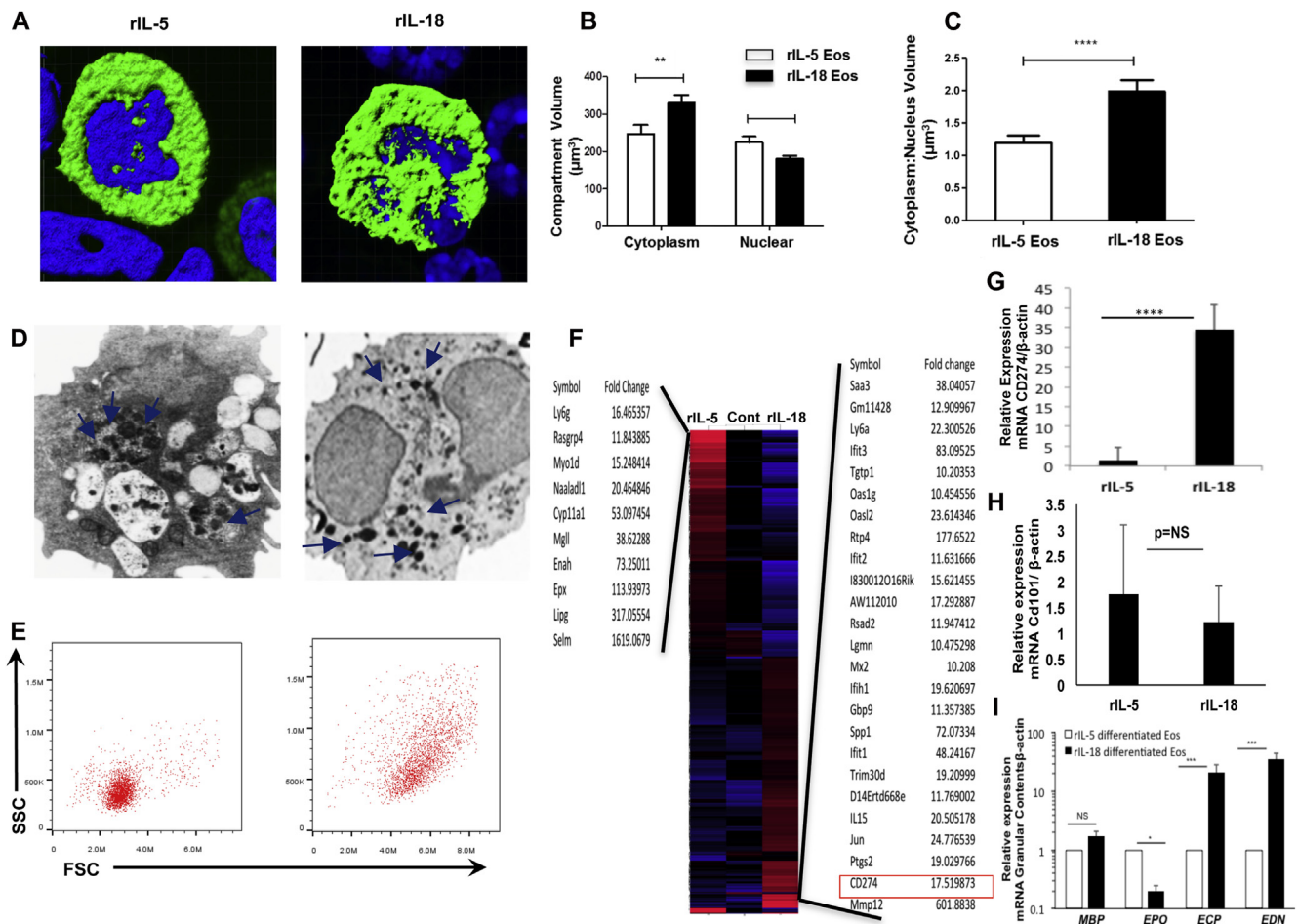


FIG 3. Confocal, electron microscopic, flow cytometric, and microarray analyses of eosinophils derived from LDEBM precursors in response to rIL-5 and rIL-18. **A**, Representative confocal photomicrographs of anti-MBP and anti-IgG-fluorescein isothiocyanate (FITC; green)-stained and 4'-6-diamidino-2-phenylindole dihydrochloride-stained (blue) rIL-5 and rIL-18 differentiated eosinophils. **B** and **C**, Total computerized quantitative area of the compartmentalized nucleus and cytoplasm and their ratio analyzed by using IMARIS software presented (mean $\pm$ SD, $n = 30$) from rIL-5- and rIL-18-differentiated eosinophils (Fig 3, **B** and **C**). **D**, Electron photomicrographs of rIL-5-differentiated eosinophils (original magnification $\times 24,000$) and rIL-18-differentiated eosinophils (original magnification $\times 1,800$, $n = 15$ eosinophils analyzed). **E**, Representative flow cytometric analysis ($n = 5$) of eosinophil granularity and size analysis on side-scatter (SSC) and forward-scatter (FSC) plots for rIL-5- and rIL-18-treated eosinophils. **F**, Microarray analysis of CD34⁺ LDEBM precursors treated with FLT3L for 4 days (control), rIL-5-derived eosinophils, and rIL-18-differentiated eosinophils purified by means of fluorescence-activated cell sorting. Overexpressed genes are presented in red, and downregulated genes are presented in blue. Each column represents combined experimental data sets analyzed by using multiple independently generated eosinophil RNAs, and a gene list of genes with a greater than 10-fold change in response to IL-18 and IL-5 are presented. **G** and **H**, Highly expressed CD274 (PDL1) mRNA relative expression along with CD101 was also examined by using real-time PCR. **I**, Comparative real-time PCR analysis data of the transcript levels (fold change) in IL-5- and IL-18-generated eosinophil granules (MBP, EPO, eosinophil cationic protein [ECP], and eosinophil-derived neurotoxin [EDN]) are presented and demonstrate more granular content in IL-18-differentiated eosinophils compared with IL-5-generated eosinophils, except EPO. Arrows demonstrate eosinophil granules in the eosinophils. Data are expressed as means $\pm$ SDs ($n = 3$). * $P < .04$, ** $P < .01$, *** $P < .0001$, and **** $P < .00001$. NS, Not significant.

this article's Online Repository at www.jacionline.org). This further confirms that IL-18 has a critical role in the development of CD101⁺CD274⁺ pathogenic eosinophils in the lungs of allergic mice, similar to observed in the tissues of human allergic diseases.

We observed approximately 8% eosinophils in *Il18*^{-/-} mice seem to be weakly positive for CD274, which might be due to

the influence of allergen-induced IFN- γ . IFN- γ induction in the lungs of *Aspergillus* allergen-challenged asthmatic mice was reported recently,¹⁰ and it has been reported that CD274 expression on immune cells is influenced by IFN- γ . Therefore, the possibility exists that in the absence of *in vivo* endogenous IL-18, allergen-induced IFN- γ influences weak CD274 expression on lung eosinophils. Notably, even though few

eosinophils from *IL18*^{-/-} mice show weak expression of CD274, they are not double-positive eosinophils (CD101⁺CD274⁺), as observed in *IL5*^{-/-} and wild-type mice (see Fig E2, M-O). This further confirms that IL-18 has a critical role in the development of CD101⁺CD274⁺ pathogenic eosinophils in the lungs of allergic mice that are also present in the tissues of patients with allergic diseases.

We have shown the significance of IL-18 in eosinophil differentiation, maturation, activation, and transformation from naive to pathogenic eosinophils. Additionally, these data authenticate that both the CD101-expressing and CD274-expressing eosinophil subsets are not IL-5 dependent, as reported earlier.⁴ These investigations also indicate that IL-5-independent hidden eosinophils identified in an earlier report are immature residential eosinophils in normal lungs that mature after HDM challenge in asthmatic mice.⁴

The mature and activated pathogenic eosinophils that accumulate in the tissues of patients with allergic diseases are CD101⁺CD274⁺. These double-positive pathogenic eosinophils are observed in the tissue of both mice and human subjects under allergic conditions. Moreover, this study also shows some discrepancy in expression levels of CD101 in the blood of healthy human subjects and mice, which might be due to the well-controlled conditions of age, environment, and food provided to the mice. In addition, analyzed healthy human subjects have some limitations, such as the analyzed age group (14-48 years), different food habits, and environmental conditions. The range of CD101 expression levels in blood eosinophils of healthy subjects clearly denote this possibility and might be the reason for this observed discrepancy. This CD101 expression discrepancy also explains the wisdom of earlier reported CD101-expressing eosinophils in allergic mice but not in allergic human subjects compared with the respective healthy subjects.⁴

In conclusion, for the first time, the presented data authenticate a critical role for IL-18 in the development and transformation of distinct pathogenic eosinophils in patients with allergic diseases.

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Sathisha Upparahalli Venkateshaiah, PhD^{a,*}

Akanksha Mishra, BS, MA^{a,e}

Murli Manohar, PhD^a

Alok K. Verma, PhD^a

Priya Rajavelu, PhD^d

Rituraj Niranjana, PhD^d

Laurianne G. Wild, MD^b

Nereida A. Parada, MD^a

Uwe Blecker, MD^c

Joseph A. Lasky, MD^d

Anil Mishra, PhD^a

From the Department of Medicine, ^aSection of Pulmonary Diseases, Tulane Eosinophilic Disorders Center (TEDC), ^bSection of Allergy and Immunology, and ^cSection of Pediatric Gastroenterology, Tulane University School of Medicine, New Orleans, La; ^dAllergy and Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; and ^eElite Biosciences, Cleveland, Ohio. E-mail: amishra@tulane.edu.

*These authors contributed equally to this work.

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Innate lymphoid cells 3 induce psoriasis in xenotransplanted healthy human skin



To the Editor:

Recent studies have demonstrated that the number of group 3 innate lymphoid cells (ILC3s) is increased in peripheral blood, lesional skin, and nonlesional skin of patients with psoriasis.^{1,E1,E2} However, definitive evidence is as yet entirely missing that these innate immunocytes, which produce IL-22 and IL-17, but not TNF-α or IFN-γ, and lack cytotoxic effectors,^{E2} are functionally involved in the induction of psoriatic lesions in human skin. Hence, the present study aimed to determine whether human ILC3s (ie, CD3⁻ RORγt⁺ NKp44⁺ cells)^{E1} suffice to induce psoriatic lesions in healthy human skin *in vivo*.

This was tested in a well-established humanized mouse model of psoriasis, in which skin lesions that are indistinguishable from the human disease are induced in healthy human skin xenotransplants by intradermal injection of autologous, preactivated PBMCs.^{2,3,E3-E5}

Normal donor skin from healthy female volunteers aged 46 ± 10 years was transplanted onto severe combined immunodeficient/beige mice and 20 mL of venous blood was obtained from the same donors as described.^{E3} The study was approved by the Institutional Ethics Committee of Rambam Medical Center, Haifa, Israel. First, ILC3s were purified from cultured PBMCs and characterized for subsequent injection into the xenotransplants. To expand the population of RORγt⁺ cells, that is, a characteristic of both ILC3 and T_H17 cells,^{4,E6} PBMCs were cultured with high-dose IL-2 and the aryl hydrocarbon receptor (AhR) agonist, 6-formylindolo carbazole (see Fig E1 in this article's Online Repository at www.jacionline.org).^{E7} Gated on Lin⁻ cells resulted in 94% RORγt⁺ cells, and 92% cells

Supplementary Material

Material and Methods

Mice.

Pathogen-free BALB/c and IL-18 gene-deficient (*IL-18^{-/-}*) C57 background mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-5 gene-deficient (*IL-5^{-/-}*) mice were kindly provided by Marc Rothenberg, MD, PhD (Cincinnati Children's Hospital Medical Center, Cincinnati OH). Mice were maintained in a pathogen-free barrier facility. All experiments were conducted with gender-matched, 6-8 week old mice. The Tulane Institutional Animal Care and Use Committee (IACUC) approved the animal protocol in accordance with National Institute of Health (NIH) guidelines.

Mouse bone marrow-derived eosinophils.

Bone marrow cells were collected from the femurs and tibiae of WT mice, *IL-5* gene-deficient mice (*IL-5^{-/-}*) and *IL-18* gene-deficient mice (*IL-18^{-/-}*) by flushing the cut opened bones with IMDM medium (Invitrogen). RBCs were lysed using RBC lysing buffer (Sigma-Aldrich). The RBCs lysed bone marrow cells were washed with PBS containing 0.1% BSA and low-density eosinophil bone marrow cells (LDEBM) precursors obtained via Percoll density gradient were cultured at 10^6 /ml in medium containing RPMI 1640 (Invitrogen) with 20% FBS (Cambrex Corp), 100 IU/ml penicillin and 10 μ g/ml streptomycin (Cellgro), 2 mM glutamine (Invitrogen), 25 mM HEPES and 1x nonessential amino acids and 1 mM sodium pyruvate (Life Technologies), and 50 μ M 2-ME (Sigma-Aldrich) supplemented with 100 ng/ml

stem cell factor (SCF; PeproTech) and 100 ng/ml FLT3 ligand (FLT3-L; PeproTech) from days 0 to 4. On day 4, the medium containing SCF and FLT3-L was replaced with medium containing 10 ng/ml mouse rIL-5 or rIL-18 (R&D Systems) as per the method described earlier.^{E1} On day 7, all non-adherent cells were removed and supplemented with 10 ng/ml rIL-5 or rIL-18. The medium with rIL-5 or rIL-18 was replaced on every 3rd day for 3-4 weeks. A flow cytometer using 5×10^5 cells was performed for eosinophil analysis following staining the cells with a combination of anti-CCR3, anti-Siglec-F, and anti-CD274 antibodies.

Allergic patient characteristics and eosinophil subsets analysis.

Asthma and eosinophilic esophagitis (EoE) patients, along with normal patients, were selected without regard to age, gender or race in collaboration with the Tulane Eosinophilic Disorder Center (TEDC). The CD101 and CD274 expressing eosinophils analysis will be performed by flow cytometer analysis in the blood and nasal lavage, tissues biopsies of allergic and non-allergic patients using anti-CCR3, anti-siglec-8, anti-CD274 and anti-CD101 antibodies with their respective isotype controls obtained from BD Biosciences, BioLegend, and eBiosciences. Detailed subject characteristics are provided in the supplementary Table 1.

Flow cytometer analysis.

Mouse bone-marrow derived eosinophils and CD-2 IL-5 tg mice spleen eosinophils were stained with the following combination of antibodies along with a live/dead cell marker and different fluorochrome-labeled: anti-mCCR3, anti-mSiglecF, anti-

mCD274 and anti-mCD101 with their respective isotype controls obtained from BD Biosciences, BioLegend, eBiosciences and Miltenyi Biotec. A live/dead marker (BioLegend) was used to exclude dead cells. Data were acquired with a BD Accuri flow cytometer or FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software version 7.1 (Tree Star). Positive cells were identified by comparison to the appropriately conjugated isotype controls.

Confocal microscopy.

FACS purified rIL-5 and rIL-18 generated eosinophils were fixed in 4% paraformaldehyde followed by ice-cold methanol. Anti-MBP and rat IgG-Alexa Fluor 647 antibody (Molecular Probes) were applied for the immunofluorescence staining of the eosinophils followed by 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) mounting. Images were collected using a confocal microscope (Leica Microsystems). FITC was excited using an argon laser at 488 nm, and DAPI, nuclear stain, was excited using a 405 nm diode laser (Coherent) and eosinophil cytoplasm and nucleus images were taken and analyzed by IMARIS software (Biplane AG).

Electron microscopy.

Cultured eosinophils were fixed in 3% glutaraldehyde and Cacodylate buffer overnight at 4°C followed by 1% osmic acid in Cacodylate buffer for 1 hour and dehydrated in ethanol. They were embedded in LX 112 resin and polymerized for

2 days at 60°C. Sections were cut, mounted and examined with a Zeiss EM 912 electron microscope.

RT-PCR analysis.

The rIL-5 and rIL-18 derived eosinophils were suspended in RNazol B (Tel-Test) at a concentration of 1ml/10⁶ cells and extraction proceeded as per the manufacturer's instructions. The precipitated RNA was harvested by centrifugation, washed in 70% ethanol, dried, and suspended in sterile diethyl pyrocarbonate (DEPC)-treated water. RNA (1µg) prepared as described was subjected to DNase I treatment (Invitrogen) and reverse transcribed using a First Strand cDNA Synthesis Kit for RT-PCR (avian myeloblastosis virus reverse transcriptase; Roche Diagnostics). cDNA (1µl) was subjected to TaqMan (Q) PCR using a FAM-labeled probe and primers for each eosinophil granular genes (*MBP*, *ECP*, *EDN*, and *EPO*). All experiments included a no-reverse transcriptase and no-template controls and mouse β -actin was used as the endogenous control. Transcripts at each time point are normalized to β -actin. Values were expressed in relative expression; fold change (mean $\pm$ SD). The primers that were used in the study:

mMBP, F- 5' AACTTGCCTAGGGATGCAGA-3'; R-5'-GAGGTAGCGACAGGTCTTGC-3'; *mEPO*, F-5'-ATGGAGACAGATTCTG GTGG-3'; R-5'-AGTATTGTCGCATACAATC C-3'; *mECP* F-5'-CCGTGGACCCAC AGTGACAGC-3'; R-5'-TCTGGGAAGGGGTTGGTCGCT-3'; *mEDN*, F-5'-ATTGAC CCCCTCCCGGTGGTTT-3'; R-5'-TGTGTAACGTTAACGGCCCGCAT-3'; *mCD101*, F-5'-CAGGGTAACCTTCGGCTCTG-3'; R5'-GATGCGGTACCCTGGGAATTA-3'. *mCD274*, F-5'-TCACAGCCTGCTGTCACTT-

93 3'; R-5'-TAAGGTCCTCCTCTCCTG CC-3'; β -Actin, F-5'-
94 CGATGCCCTGAGGCTCTTTTCC-3'; R-5'-CATCCTGTCA GCAATGCCTGGG-
95 3'.

96 **Microarray Analysis.**

97 The Agilent Bioanalyzer was used to test the RNA quality (2100 Hewlett Packard)
98 using the RNA 6000 Pico Assay and Ovation Pico WTA System v2 (Nugen) was
99 used to synthesizing cDNA target from 1 -10 ng of total RNA. Further, the Encore
100 Biotin Module (NuGEN) was used to both chemical fragment and biotin – label the
101 cDNA target. The samples are hybridized to a standard Probe Array Cartridge
102 (GeneChip Mouse Gene 1.0 ST Array, Affymetrix) in the GeneChip Hybridization
103 Oven 640 (Affymetrix). Probe arrays were washed and stained using the Fluidics
104 Station 450 (Affymetrix). The arrays were scanned with the Affymetrix GeneChip
105 Scanner 3000 7G. Command Console and Affymetrix operating software program
106 was used to create raw data files.

107

108 **Experimental allergic airway murine model of asthma.**

109 A mouse model of allergic asthma was established using methods described
110 previously.^{E2} In brief, mice were lightly anesthetized with isoflurane (Iso-Flo;
111 Abbott Laboratories), and 100 μ g (50 μ l normal saline) of *Aspergillus fumigatus*
112 (Greer Laboratories) or 50 μ l of normal saline alone was given intranasal using a
113 micropipette with the mouse held in the supine position.

114

115 **Bronchoalveolar Lavage Fluid (BALF).**

The mice were euthanized by CO₂ inhalation. Immediately thereafter, a midline neck incision was made, and the trachea was cannulated. The lungs were lavage 2 times with 1.0 ml PBS containing 1% FCS and 0.01 mM EDTA. The recovered BALF was centrifuged at 400g for 5 minutes at 4°C and re-suspended in PBS containing 1% FCS. Total cell numbers were counted with a hemocytometer. Cytospin preparations of 5 x 10⁴ cells were stained with Giemsa-Diff-Quick (Dade Diagnostics), and differential cell counts were determined. The BALF eosinophil counts were expressed as an indication of lung eosinophilia.

Lung tissue eosinophils analysis.

5-µm tissue paraffin sections of mouse lung sections were immunostained with antiserum against the mouse eosinophil major basic protein (anti-MBP) as per the method described previously.^{E3}

Statistical analysis.

The nonparametric Mann–Whitney *U*-test was employed for comparison of data between two groups, and Krustal–Wallis for comparison of more than two groups. Parametric data were compared using *t*-tests or analysis of variance. Values are reported as mean ± S.D. *P*-values < 0.05 were considered statistically significant.

Supplementary References

- E1. Dyer KD, Moser JM, Czapiga M, Siegel SJ, Percopo CM, Rosenberg HF. Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J Immunol* 2008; 181:4004-9.
- E2. Rajavelu P, Rayapudi M, Moffitt M, Mishra A, Mishra A. Significance of para-esophageal lymph nodes in food or aeroallergen-induced iNKT cell-mediated experimental eosinophilic esophagitis. *Am J Physiol Gastrointest Liver Physiol* 2012; 302: G645-54.
- E3. Venkateshaiah SU, Zhu X, Rajavelu P, Niranjana R, Manohar M, Verma AK, et al. Regulatory effects of IL-15 on allergen-induced airway obstruction. *J. Allergy Clin Immunol* 2017.

164 **Supplementary T 1: Patient clinical Characteristics.**

	Age (Year)	Gender	Diagnosis	Treatment
Healthy Individual				
1	32	F	Normal	None
2	29	M	Normal	None
3	50	F	Normal	None
4	24	F	Normal	PPI
5	37	F	Normal	PPI
6	35	M	Normal	None
7	31	F	Normal	None
8	64	M	Asthma/COPD	INH GCS
9	33	F	Asthma/sarcoid	INH GCS
10	47	F	Asthma	Prednisone
11	28	F	Asthma	INH GCS
12	29	F	Asthma	Prednisone, INH GCS, Nasal steroid
13	54	F	Asthma	Xolair
14	52	M	Asthma	INH GCS
15	14	M	Asthma	INH GCS, Omeprazole
16	60	F	Asthma	Prednisone, INH GCS, Nasal steroid
17	54	F	Asthma/ rhinitis	Xolair
18	29	F	Asthma/ rhinitis	Prednisone, INH GCS, Nasal steroid
19	33	F	Asthma/ rhinitis	INH GCS, Nasal steroid
20	34	M	Asthma/ rhinitis	Prednisone, INH GCS, Nasal steroid
21	36	F	Asthma/ rhinitis	Prednisone, INH GCS, Nasal steroid
22	53	F	GERD/ EoE	Famotidine
23	18	M	EoE	None
24	20	F	GERD/ EoE	Milk and wheat elimination
25	48	F	Dysphagia/EoE	Nasal steroid
26	58	F	GERD/Dysphagia	Omeprazole
27	16	M	GERD/EoE	Protonix

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Gene Symbol	Fold change	Hpgds	1.9841925	Epstii	3.393537	H2-T24	1.5814703	Cd97	-1.4712859	Gm15922	-1.7750001
Itpr1	1.5977836	Ctsl	20.004852	AF251705	2.4778054	Ubl4	-1.5257152	Def6	-1.8332913	Pip5k1b	-2.396449
Parp4	-1.2108622	Mx1	3.9526718	Plxna1	1.7617814	Gm10320	-1.5535886	Csgnalc2	-2.0309918	Vcl	-2.4301975
Ccnd2	1.6703563	Gm6166	5.084049	Gnb21	-1.4399115	Ppp2r5c	-1.5187649	Dstrn	-1.4871391	Paqr7	-2.0677285
Pdcd4	1.3898603	Ifi272la	5.4732757	AC133103.2	1.572595	Tkt	-1.9188772	Diap1	-1.6124411	Gm6517	-2.9642704
Ctla2a	1.4508667	Ifi2	11.631666	Peli1	1.5920957	Junb	1.3013182	Hk2	-1.8108288	Pygl	-1.8751411
Ms4a4c	4.0642414	Thbs1	2.9893436	Rabf5	1.527117	Acot7	-1.6113366	Sipa13	-1.6871387	Ikzf2	-2.3662126
Trps1	2.006977	Tlr1	2.3581104	Rel	1.9529502	Adk	-1.5886397	Zxdc	-1.7815939	F630111L10Rik	-1.9037659
Alpk1	1.647012	Cd36	3.0662777	Cyb5r3	1.5066248	Mfsd10	-1.5169681	Cbara1	-1.7736956	Tob1	-1.692371
AI504432	1.5917554	Parp14	2.166224	Usp18	7.8723907	Fryl	-1.6886102	Gse1	-1.7454322	C1rl	-2.3206272
Mnd1	1.2185938	Cxcl16	1.970302	Med9	1.521023	Zfp36	2.0194001	Ppxo	-1.6222917	Accs1	-2.8179855
Ptprj	1.9493752	I830012016Rik	15.621455	Ccdc86	1.7083136	Fos	5.027671	Nomo1	-2.061887	Cib1	-2.6545062
Cd93	1.5982546	Prdx4	2.0452628	Sepp1	2.6875854	Fam108c	-1.5891843	Hcfc2	-1.6870457	Accs2	-2.1321233
Map4k4	1.6367196	Cd69	2.445385	Ly96	2.5889127	BC037034	-1.9473037	Tmem2	-1.4963788	Gfod1	-2.60839
Sap30	1.9946864	Apobec1	2.7568455	Cln8	2.0609653	Men1	-1.5465966	Ncoa7	-1.560232	Ctsh	-2.0736647
Gm12699	1.4532923	Gstm1	1.8203621	Anxa4	2.8591878	Spcs3	-1.6081345	Dnpep	-1.6616567	Nuch1	-2.097302
Ndufa2	1.4074521	Gm12699	8.79289	Oasl1	4.075188	Ier3	2.0692282	Ier2	-1.09599	Axin2	-2.256172
Gm9766	1.3354632	Stat1	3.0624382	Ttcl	1.7353148	Ints4	-1.6848999	Lta4h	-1.921311	Tmem164	-2.8008623
Tlr2	1.8701211	Fam49a	1.999489	Gbp4	7.4034925	Asxl1	-1.6510412	Tmem208	-1.8208532	Sia	-2.761573
Ms4a6c	2.0411248	Ms4a7	9.70932	Trim30d	19.20999	Tmem40	-1.6089977	Lrrfp2	-1.49742	Atp2a3	-2.5959554
Rbm47	2.0544372	Eif2ak2	2.615751	Mdfic	2.1775117	Scap	-1.5101694	Chd7	-1.6990633	Prkca	-2.6650074
Trim30a	4.5958433	AW112010	17.292887	Ripk3	1.6081029	Yif1b	-1.5571955	Limd2	-1.8764482	41153	-2.4064417
Cd14	2.0183291	Herc6	4.325467	Ptms	1.8795764	Gm8995	1.3895621	Rml2481	-1.6043015	Cnr2	-2.2237759
Ifi203	3.46930694	Gm7984	1.5757091	Lgals8	2.9053535	Plagl2	-1.5618947	Gab27a	-1.9380895	Myk	-2.3797207
BC147527	2.3367753	Zeb2	1.6093024	Strn	1.5706496	Tcp11l2	-1.5822999	Tcp11l2	-1.7039105	Pdlim7	-2.7596233
Lpcat1	1.4403828	Tubb6	2.0245323	Mmp12	8.512985	S1pr4	601.8838	S1pr4	-1.6158768	Il1rl1	-2.857577
Cd180	1.7696785	Aim2	1.6162112	Tbc1d9b	1.825419	Stim1	-1.5107901	Stim1	-1.8123893	Nbeal2	-3.096066
Ly6c1	2.066024	Alcam	1.9731284	Uqcrl	-1.4238309	Adipor1	-1.7609415	Adipor1	-1.4647444	Npl	-2.3963356
1700034H14Rik	1.2264786	Abi3	1.6878129	Arl8b	2.0313382	Mtmr12	-1.6600503	Ngly1	-1.9669100	Ntc5c	-1.9857137
Saa3	38.04057	Rsa2d	11.947412	Gmip	-1	Gm9034	-1.6130192	Gm9034	-1.5897671	Rnf125	-2.5210266
Ms4a6d	4.2108917	Igsf6	1.7265439	Gngt2	2.0476813	Abca7	-1.5264024	Myl12b	-1.6752338	Glipr2	-2.3607166
Ifitm3	2.536096	Dnajc13	1.6322272	Lbr	-1.5130674	Bri3bp	-1.7257515	Acsl4	-1.6573985	Hck	-2.597085
Slnf5	3.679039	Lgmn	10.475298	Igf1	9.080402	Reep5	-1.5449005	Rcsd1	-1.7468522	Rnd1	-3.2602549
Srx30	1.530436	Snx5	1.5547254	Gm7582	1.893151	Fam118b	-1.5715077	Orai2	-1.7930597	Itgb2	-3.0201294
Msr1	6.787488	Ly9	1.586905	Isg20	2.0548358	Stk11	-1.5361586	Rgs14	-2.2524917	5330426P16Rik	-2.3279762
AI607873	4.034886	Arhgap26	1.5419935	Wwp1	2.0136096	Zwint	-1.62781	Adrbk1	-2.1084225	Cox7a1	-2.2780817
Ifi44	5.9145155	Phf11	4.417819	Myo1e	2.5291796	Rin3	-1.5096706	Kif3b	-1.6142254	Mboat1	-3.141216
Cd68	2.3707697	Appf2	2.319764	Gm16026	2.0666752	Mtmr3	-1.5180544	Ptpn7	-2.4368784	Il5ra	-3.0260603
Gm11428	12.909967	Trim34a	2.883817	B2m	1.9421402	Alas1	-1.9266717	Camk2g	-2.1510744	Rab37	-3.1478179
Pyhin1	5.2381287	Fnbp1l	3.0818894	Galnt7	1.6674182	Eif4a2	-1.7619184	Arid3a	-1.8358113	Arap3	-2.8308287
Srsf3	1.2443994	Mx2	10.208	Ccnl1	2.0313452	Exoc6	-1.6927879	Pmm1	-1.7191224	Gf1b	-3.6903653
Ly6a	22.300526	A530032D15Rik	1.9416376	Gm4070	2.1340377	Kcnab2	-1.8443729	Alox5ap	-1.664606	Kirb1f	-3.5296092
Marc3s	3.954168	Oas1a	8.057089	Phyh	1.8854586	Nfyb	-1.6428541	Rps6ka1	-2.415727	Pglyrp1	-3.07828
Ms4a6b	7.0100217	Plscr1	2.03441	Il15	20.505178	Lypla2	-2.0106568	Dennnd4a	-1.6555367	Tspan5	-3.6101093
Oas2	5.130382	Fundc1	1.5792446	Jun	24.776539	Tagln2	-1.7500385	Mprl41	-2.1595097	Cln3	-3.73777
Ifi3	83.09525	Zbp1	8.348387	Gbp7	2.7920806	Wbp2	-1.7273291	Pdlim1	-2.5039122	Nfe2	-4.1209526
Tubb2a	1.9485376	Nup210	-1.1503824	Gbp1	3.9991295	Plin2	2.8494685	Pyhin9	-1.7557539	Pla2g3	-3.4533517
Ifi204	9.950549	Traf1d1	2.0318599	Pln2	2.8494685	Heatr5a	-1.8025247	Der1i	-2.1947746	4632428N05Rik	-3.5165362
Clec4a3	2.7477698	Srgap2	1.7607462	BC006779	1.9156773	Sykb	-1.343742	Nqo2	-1.8265634	Adra2a	-3.6569436
Tgtp1	10.20353	Slc31a1	1.854515	Reep4	-1.5094303	Amfr	-1.6828454	Trerf1	-1.8164843	Cnn2	-4.8107405
Ifi202b	3.372781	Zufsp	2.2652657	Sp100	2.7064753	Megf9	-1.5186616	Sec24a	-2.4293616	Upp1	-3.3261774
Taf1d	1.4182425	Clec4e	3.990909	G530011006Rik	7.261815	Surf4	-1.9854017	Rsu1	-2.0358117	Rab44	-4.3490653
Lgals3bp	3.1869497	Ifi1h	19.620697	Nmi	1.9290936	Tmed4	-1.3150674	Idl1	-1.7393795	F2rl2	-3.7633297
Mpeg1	1.8945024	Gm5582	1.5670313	Gm4951	7.056487	Cdk2ap2	-1.5101179	Hyal3	-1.7204657	Grina	-3.0084548
Cops7b	-1.1929224	Gm15026	1.5919882	SNORA76	-1.5688239	Traf3ip3	-1.9486462	Hist1h1c	-1.3317913	Spns3	-5.970436
Tpm4	1.976	Oas3	5.4294505	Ptgs2	19.029766	Ccni	-1.7309895	Mboat7	-2.269088	Tuba8	-3.967948
PI4	3.4205542	Elk3	1.6321967	Znfx1	2.4855525	Nrm	-1.934019	Gba2	-2.0378454	Cot1l	-4.6846633
Lztf1l	1.4622722	Lpp	1.9768475	Rrbp1	1.5798213	Grk6	-1.7189924	St3gal4	-2.3724773	Cd55	-4.6093554
Klf23	1.2860837	Mmp8	5.1751037	Daxx	1.8969448	Gm11868	-1.5353901	Vasp	-2.299901	lqsec1	-3.725634
Zfp187	1.4285791	Cd86	2.448642	Nlrc5	3.7413154	Atg9a	-1.4084349	Prr13	-1.9195843	Anxa11	-4.9368744
Npy	6.7258596	Abhd12	1.510997	Ccl4	7.764238	Rabif	-1.4196776	Ltb4r1	-2.037916	Neu1	-4.461438
Clec4d	3.5702777	Gpr137b-ps	1.9402564	n-R5s1	1.4611309	Fes	-1.6357335	Mfsd5	-1.9127399	Ar5b	-5.342794
Osgsep	1.3689857	Sh3glb1	2.8271368	Dxd58	3.005823	41167	-1.6868688	Pnp1a7	-1.8462437	Bhlhe40	-3.2740574
Slnf8	6.2340865	C230081A13Rik	1.8339324	Cdca5	-1.530798	Ing1	-1.4901292	Flot1	-2.1492026	Lpcat3	-4.7379913
Gm13768	1.9032629	Sgpl1	2.37972	Hist1h1a	-1.629668	Gss	-1.2931271	Itga2b	-2.2351594	Lilra6	-4.4367967
Oas1g	10.454556	Lipa	1.5414226	CD274	17.519873	Ankrd44	-2.1265647	Itga2b	-2.2351594	Tesc	-5.558256
Ifi205	6.5848107	Lip	1.5414226	Anxa1	1.8857256	Pik3cb	-1.3274628	Ccdc109a	-2.1741357	Siglec 5(F)	-5.692438
Casp4	2.322265	Rin2	1.7562006	Mkl1	1.7992138	Cisd2	-1.6667881	Eef2k	-2.7202473	Perp	-5.7366967
Macf1	1.4001329	Gbp9	11.357385	Rbm38	-1.6857207	Kdm5b	-1.4688559	Ckap4	-1.8515227	Padi4	-5.543704
Slnf1	6.998337	Isg15	5.915503	H2-Q8	1.545866	Add1	-1.6443403	Klf21b	-2.0265846	Aqp9	-6.228664
Rnf213	3.1720839	Parp12	2.5202184	Il18bp	1.6569332	G6pdx	-1.6915126	Ston2	-1.7715594	Suox	-7.910738
Oas12	23.614346	Atf3	4.752628	Ccl12	8.664734	Ptk2b	-1.5647969	Akna	-2.1130443	Gaint6	-8.33286
Zchhc11	1.7631749	Emp1	2.4648194	Mpp1	1.7258685	Stim2	-1.6033244	Btk	-2.309672	Adora3	-7.8491
Apoe	3.0230672	Spp1	72.07334	Pdpn	3.034531	Atrn	-1.5403583	St14	-2.2296848	Offm4	-8.865338
Gp5	1.2778836	Dennd1a	1.5679296	Gla	2.6233373	Eno1	-1.3115609	Atxn1	-1.7044345	Gm9847	-7.881515
Dpep2	1.7120758	Casp1	3.1738133	n-R5s4	2.1668146	B4galt3	-1.763353	Alidh2	-2.78948	Ly6g	-16.465357
AI530040E14Rik	1.9734762	Vegfa	1.61019	Ada	1.6585665	Sh3bgrl3	-1.5216616	Unc13d	-2.1860797	Rasgrp4	-53.097454
Lpl	3.8852677	H2-Q6	2.2867665	Lamp2	1.3534625	Mknk1	-1.7818584	Ubac2	-2.6121612	Cish	-9.985856
Il7r	4.769913	Fniip2	2.6148233	Mrps28	-1.5213538	Lrmp	-1.6776307	St3gal1	-2.742279	Myo1d	-15.248414
Rtp4	177.6522	Atp7a	1.7834997	H2-D1	1.3600413	Sbf1	-1.7682605	Cat	-2.19086	Naaladl1	-20.464846
Xaf1	7.7757535	Slamf9	1.5775619	Mbn13	-1.6823198	Tbc1d10c	-1.9262218	Cdcd109a	-2.1741357	Cyp11a1	-53.097454
Zfp361	4.3627143	Psme2b-ps	2.0942347	Mapk42	-1.5072157	Cd37	-2.1850789	Man2b1	-1.8768756	Mgll	-38.62288
Ltn1	-1.2647562	Abca1	2.172051	Fosb	5.6951118	Ndel1	-1.4616419	2310001A20Rik	-2.7356675	Enah	-73.25011
		Slnf10-ps	3.5452497	H2-T22	3.588243	Acadslb	-2.066481	Taldo1	-2.5140522	Exp	-113.93973
		Dtx3l	3.0860522	Prkag1	-1.534945	Ccng2	-1.6520759	Cd300lb	-1.8606455	Lipg	-317.05554
		Ifi1t	48.24167					Gpx4	-1.9749722	Selm	-1619.0679

Supplementary Figure Legends

Figure E1. *Ex vivo* generation and proliferation of endogenous IL-5-independent IL-18-differentiated eosinophils. The schematic protocol used to generate eosinophils from CD34⁺ LDEBM myeloid precursors in response to rIL-5 or rIL-18 **(A)**. Differentiated CCR3 positive live cells were selected **(B)**, based on isotype matched Siglec-F and CCR3 anti-IgG stained cells **(C)** to detect CCR3⁺/Siglec-F double positive eosinophils in response to rIL-5 and rIL-18 from the LGEBM of *IL-18*^{-/-}, *IL-5*^{-/-} and WT mice **(D)**. The average percentage of eosinophils differentiated from multiple independent experiments in response to rIL-5 and rIL-18 are presented as the mean ± SD, n=5 **(E)**. A representative flow cytometer analysis of one-week rIL-18 differentiated from CD34⁺ LDEBM in response to another week with rIL-5, n=3 **(F)**.

Figure. E2. CD101⁺CD274⁺ eosinophils are not detected in IL-18 gene deficient mice following the induction of *A. fumigatus*-induced experimental asthma. A representative photomicrograph of anti-MBP antibody immunostained lung sections shows eosinophils in *IL-5*^{-/-}, *IL-18*^{-/-} mice and WT mice **(A-C)** following allergen-induced experimental asthma (n=6 mice/group). A representative flow cytometer dot blot analysis of BALF CCR3 positive leukocytes were selected for the detection of CCR3⁺Siglec-F⁺ eosinophils **(D, E)**. The CCR3⁺Siglec-F⁺ eosinophils of *IL-5*^{-/-} *IL-18*^{-/-} and WT mice were analyzed for the expression of CD101 and CD274. Representative flow cytometer analysis detected high expression of CD101⁺ **(F-H)** or CD274⁺ **(J-L)** in eosinophils from BALF detected based on isotype matched anti-IgG of CD101 or CD274 **(I, M)**, n=4 independent experiments.

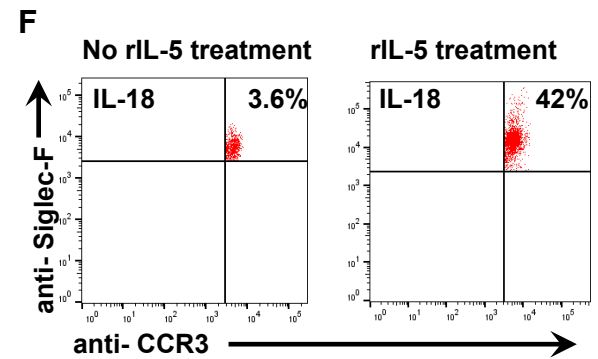
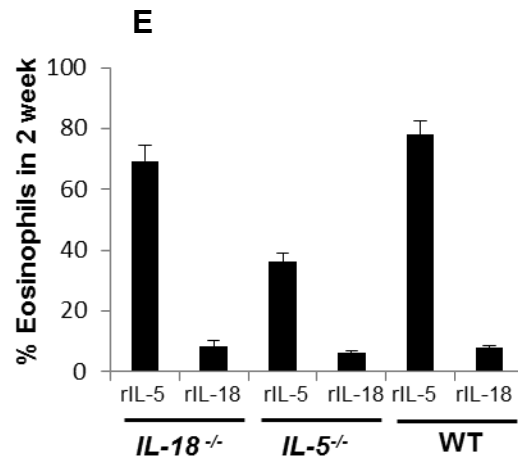
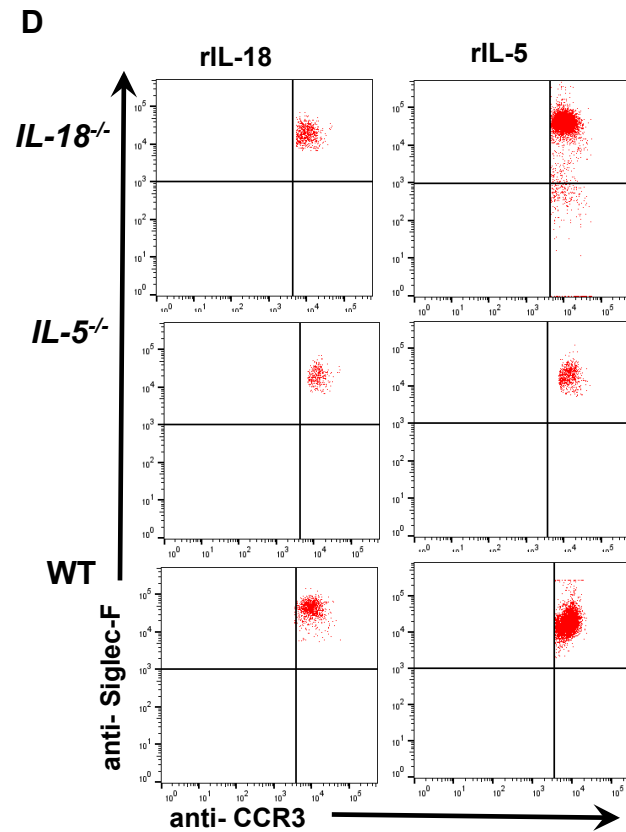
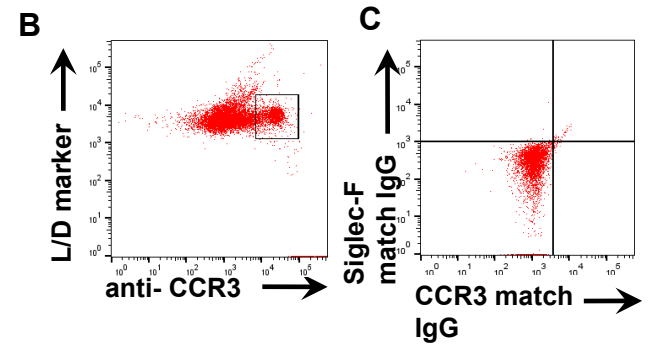
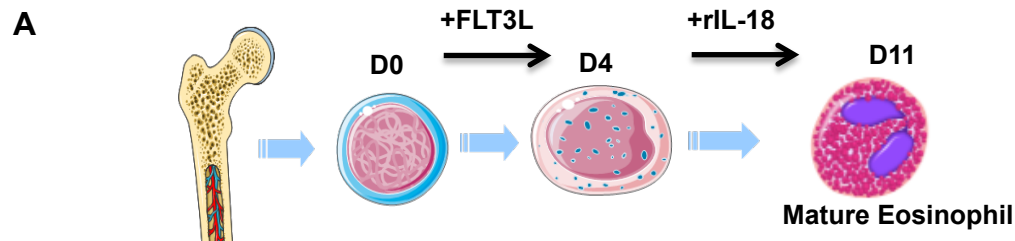


Figure.E2

