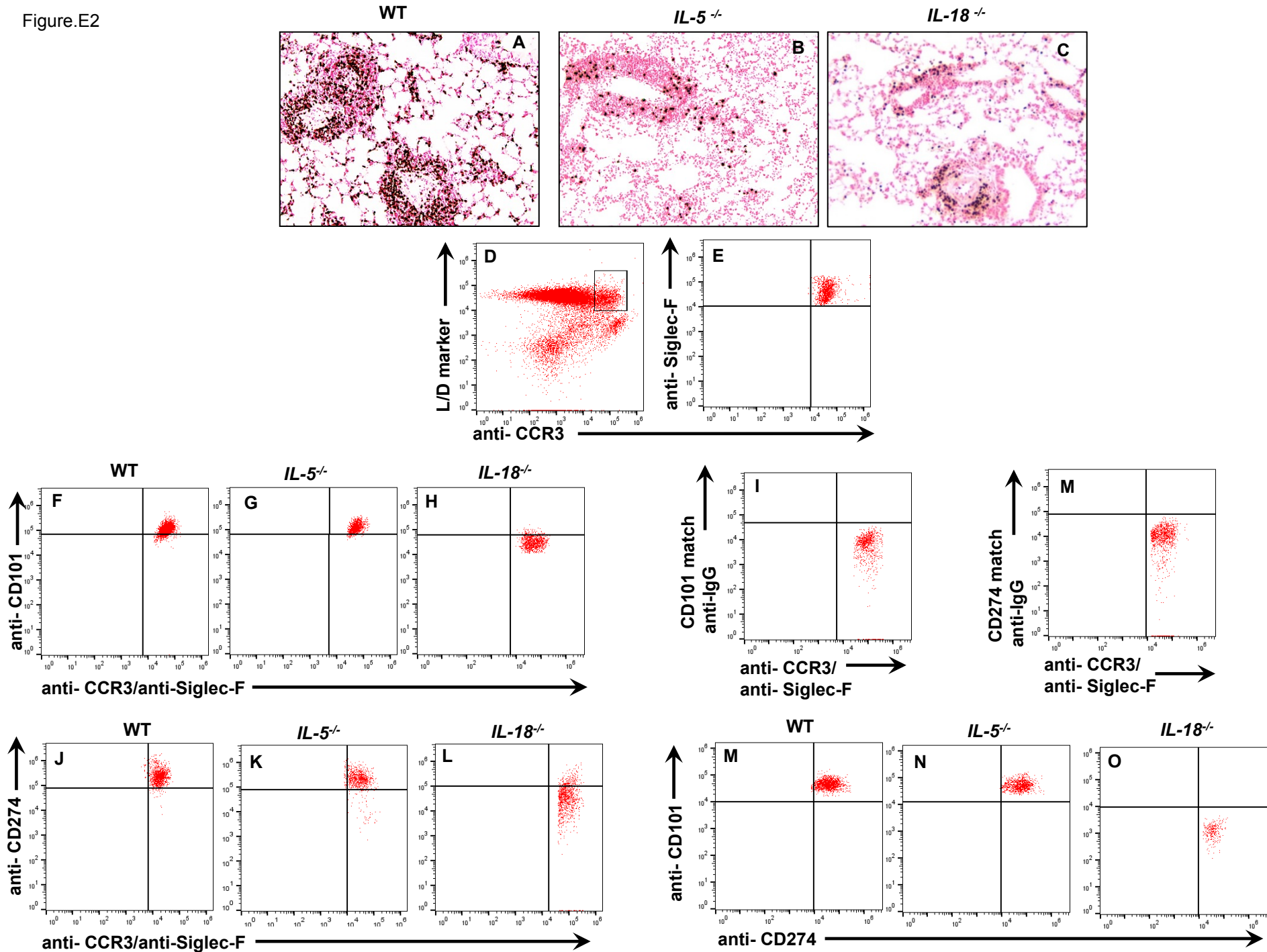


Figure.E2



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.

1 **Supplementary Material**

2 **Material and Methods**

3 **Mice.**

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

12

13 **Mouse bone marrow-derived eosinophils.**

14 Bone marrow cells were collected from the femurs and tibiae of WT mice, *IL-5*
15 gene-deficient mice (*IL-5^{-/-}*) and *IL-18* gene-deficient mice (*IL-18^{-/-}*) by flushing the
16 cut opened bones with IMDM medium (Invitrogen). RBCs were lysed using RBC
17 lysing buffer (Sigma-Aldrich). The RBCs lysed bone marrow cells were washed
18 with PBS containing 0.1% BSA and low-density eosinophil bone marrow cells
19 (LDEBM) precursors obtained via Percoll density gradient were cultured at 10^6 /ml
20 in medium containing RPMI 1640 (Invitrogen) with 20% FBS (Cambrex Corp), 100
21 IU/ml penicillin and 10 µg/ml streptomycin (Cellgro), 2 mM glutamine (Invitrogen),
22 25 mM HEPES and 1x nonessential amino acids and 1 mM sodium pyruvate (Life
23 Technologies), and 50 µM 2-ME (Sigma-Aldrich) supplemented with 100 ng/ml

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

32

33 **Allergic patient characteristics and eosinophil subsets analysis.**

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

42

43 **Flow cytometer analysis.**

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

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

53

54 **Confocal microscopy.**

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

64

65 **Electron microscopy.**

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

69 2 days at 60°C. Sections were cut, mounted and examined with a Zeiss EM 912
70 electron microscope.

71

72 **RT-PCR analysis.**

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

86 *mMBP*, F- 5' AACTTGCCTAGGGATGCAGA-3'; R-5'-
87 GAGGTAGCGACAGGTCTTGC-3'; *mEPO*, F-5'-ATGGAGACAGATTCTG GTGG-
88 3'; R-5'-AGTATTGTTCGCATACAATC C-3'; *mECP* F-5'-CCGTGGACCCAC
89 AGTGACAGC-3'; R-5'-TCTGGGAAGGGGTTGGTCGCT-3'; *mEDN*, F-5'-ATTGAC
90 CCCCTCCCGGTGGTTT-3'; R-5'-TGTGTAACGTTAACGGCCCGCAT-3';
91 *mCD101*, F-5'-CAGGGTAACCTTCGGCTCTG-3'; R5'-
92 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).**

116 The mice were euthanized by CO₂ inhalation. Immediately thereafter, a midline
117 neck incision was made, and the trachea was cannulated. The lungs were lavage
118 2 times with 1.0 ml PBS containing 1% FCS and 0.01 mM EDTA. The recovered
119 BALF was centrifuged at 400g for 5 minutes at 4°C and re-suspended in PBS
120 containing 1% FCS. Total cell numbers were counted with a hemocytometer.
121 Cytospin preparations of 5 x 10⁴ cells were stained with Giemsa-Diff-Quick (Dade
122 Diagnostics), and differential cell counts were determined. The BALF eosinophil
123 counts were expressed as an indication of lung eosinophilia.

124 **Lung tissue eosinophils analysis.**

125 5-µm tissue paraffin sections of mouse lung sections were immunostained with
126 antiserum against the mouse eosinophil major basic protein (anti-MBP) as per the
127 method described previously. ^{E3}

128

129

130 **Statistical analysis.**

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

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138 **Supplementary References**

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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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