Materials and Methods

The Helix-Loop-Helix Factor Id3 Regulates IL-5 Expression and B-1a B cell proliferation

Perry, et.al.

Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. *Apoe*$^{-/-}$ male mice were purchased from Jackson Laboratory. *Id3*$^{-/-}$ and *Id3*$^{fl/fl}$ mice were a generous gift of Dr. Yuan Zhang (Duke University). *Id3*$^{-/-}$ mice were bred to the *Apoe*$^{-/-}$ background to obtain *Id3*$^{-/-}$*Apoe*$^{-/-}$ as previously described [1]. *CD19*$^{cre/+}$ mice were gifted by Timothy Bender (University of Virginia). *Id3*$^{fl/fl}$ *mice* [2] *were bred to the Apoe*$^{-/-}$ mouse, and then to *CD19*$^{cre/+}$ mice to generate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$. These mice were then bred to *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice to generate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ and littermate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice. All mice were purchased from Jackson on a pure C57BL/6J background or mice were backcrossed 10 generations to pure C57BL/6J. All mice were fed a standard chow diet (Tekland 7012) and euthanized at 8 to 10 weeks of age by CO_2 inhalation.

ELISA

Whole blood was harvested from mice by right ventricular puncture and peritoneal fluid was harvested by lavage with 1 mL PBS at the time of sacrifice. Determination of mouse E06 was determined as previously described in detail [3, 4]. For IL-5 levels (BD OptEIA™, BDbiosciences), mice were i.p injected with 125 ng of mIL-33 (CF, R&D) reconstituted in PBS every three days for 7 days. IL-33 was measured undiluted by the mouse/rat Quantikine ELISA kit (R&D).

FACS

Aortas were harvested and digested as previously described [1] except periaortic adipose tissue was left intact with all lymph nodes carefully removed. Peritoneal cells, splenocytes, or digested aortas were first blocked for Fc receptors (FCR-4G8, invitrogen), then stained for cell surface or intracellular markers and a live/dead stain as previously described [1]. Mesenteric cells were prepared as described elsewhere [5]. Briefly, mice were transcardially perfused with 20 U mL^{-1} heparin after CO_2 euthanasia. Mesenteric fat was carefully removed of mesenteric lymph nodes and separated from intestines, and finely chopped in 5 mL of DMEM with 2 mg mL^{-1} Collagenase I (Worthington Biochemical Co.) and 4% BSA (Sigma), then digested at 37°C in a shaking incubator for 45 min. The supernatant containing adipocytes after centrifugation was aspirated. After washing the cells in HBSS with 10% HI-FBS, cells were strained through a 40 µm filter and stained. For intracellular staining, the FIX & PERM® kit (Invitrogen) was used as per manufacturer's instructions. Flow cytometry antibodies CD3ε (500A2), CD4 (GK1.4, RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD43 (S7) CD44 (IM7), CD45 (30F11), CD45R/B220 (RA3-6B2), CD49b (DX5), CD90 (53-2.1), CD117 (ACK2), CD196 (29-2L17), FcεR1α (MAR-1), IgM (II/41), IFNγ (XMG1.2), IL-4 (BVD6-24G2), IL-5 (TRFK5), Ly6G (RB6-8C5), NK1.1 (PK136), Sca-1 (D7), TCRβ (H57-597), TCRγδ (GL3) and Ter119 (TER119) were purchased from eBioscience or BD Bioscience and T1/ST2 (DJ8) from MDbioscience. Live/dead discrimination was always determined by LIVE/DEAD® fixable yellow cell staining (Invitrogen) or dapi. Cells were run on a CyAN ADP (Beckman Coulter) or sorted on a Reflection Cell Sorter (iCyt) and analyzed with FlowJo software (Tree Star Inc) using fluorescence minus one (FMO) controls for gate determination.

Counting beads were used for quantification (CountBright™ Absolute Counting Beads, Molecular Probes).

Real-time PCR

Total RNA was isolated from FACS purified peritoneal B cells using the RNeasy Plus Micro kit with gDNA elimination columns (Qiagen). RNA (1 µg) was reversed transcribed with SuperScript III First-Strand Synthesis System by Oligo d(T) tailing (Invitrogen). E06 gene expression was normalized by the ΔΔCq method to 18S as determined by TaqMan real-time PCR (SsoFast™ Probes Supermix, Bio-Rad). Id3 in NH cells (10,000 to 20,000 cells from 4 to 5 pooled mice) was also determined by TaqMan real-time PCR (Cells to Ct kit^{TM}, Invitrogen) as per manufacturers instructions. Secreted IgM or Id3 in FACS-sorted B cells was normalized by the ΔΔCq method to 18S or cyclophilin with SYBR® Green real-time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad). Primers were used as follows: E06 IgHV, forward primer (5'-CTG TGC AAG AGA TTA CTA CGG TAG-3') flanked the E06 IgH V and D junction, the reverse primer (5'-AGG ACT GAC TCT CTG AGG AGA CG-3') flanked the JH and mu chain junction for amplifying E06 IgM, but not T15 IgA. The fluorescent probe (6FAM CGC CCC AGA CAT CGA AGT ACC AG TAMRA, Applied Biosystems) matched to the E06 IgH D and J junction; sIgM, forward primer (5'-GGA GAG ACC TAT ACC TGT GTT GTA GG-3') and reverse primer (5'-TGA GCG CTA GCA TGG TCA ATA GCA G-3'); 18S forward primer (5'-CGG CTA CCA CAT CCA AGG AA-3'), reverse primer (5'-AGC TGG AAT TAC CGC GGC-3') and probe (6FAM TGC TGG CAC CAG ACT TGC CCT C TAMRA, Applied Biosystems); Id3 forward primer (5'-TGC TAC GAG GCG GTG TGC TG-3') and reverse primer (5'-TGT CGT CCA AGA GGC TAA GAG GCT-3'); cyclophilin forward primer (5'-TGC CGG AGT CGA CAA TGA T
-3') and reverse primer (5'-TGG AGA GCA CCA AGA CAG ACA-3').

In vivo proliferation

1.5 mg of BrdU was administered i.p. and peritoneal cells or spleens were harvested and stained for BrdU as per manufacturer's kit instructions (BD Pharmingen).

Apoptosis assay

Peritoneal lavage cells were washed in cold PBS and suspended in binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCL, 2.5 mM $CaCl_2$). PerC cells were then incubated with Annexin V (BD Pharmigen, 556420) for 15 min at room temperature. After washing with binding buffer, cells were incubated with dapi and analyzed by flow cytometry.

Western blot analysis

Splenic B cells were isolated using CD19 microbeads (Miltenyi Biotech) with >99% pure CD19+ B cells. 10 x 10^6 cells were resuspended in 200 uL lysis buffer (1% DOC, 1% NP40, 50mM Tris pH 7.6, 1 mM EDTA, 150mM NaCl, 0.1% SDS with protease inhibitor cocktail), incubated at room temperature for thirty minutes, and centrifuged for 10 minutes at 11,600 x g, 4C. The infranatants were separated from the pellet. Samples were assayed for protein concentration with the Bio-Rad DC protein assay (catalog #500-0111), adjusted to equal concentration with lysis buffer, and

supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad). Equal protein loading was confirmed by staining the membrane with immunodetection of β-tubulin. Western blotting was carried out using an antibody to Id3 (0.1 µg/ml, CalBioreagents, catalog M100, clone 17-3) or β-tubulin (16 µg/ml, Cell Signaling Technology, Inc. catalog #2146S) followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL reagent (Amersham Pharmacia Biotech). Densitometry was analyzed by ImageJ.

Cell culture

Mesenteric cells were isolated as above and plated at 2 x 10^5 cells per well in a 96 well plate with 200 µL of complete RPMI 1640 (cRPMI, RPMI-1640 medium) containing 10% HI-FBS, 50 mM 2-mercaptoethanol, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, 1X nonessential amino acids, 10 mM HEPES, 1 mM sodium pyruvate, and 10 ng mL^{-1} mrIL-33 (CF, R&D) for 4 days without any golgi transport inhibitor.

For Th2 cells, 7 x 10^5 splenic CD4+ cells isolated by macs column purification with CD4 microbeads (Miltenyi Biotech) were stimulated with soluble anti-CD3/anti-CD28 Dynabeads® (GIBCO) and a combination of 10 ng mL^{-1} mIL-4 (peprotech), 5 ng mL^{-1} and mIL-2 (R&D), 5 ng mL^{-1} for four days. Cells were then stimulated for six hours with 50 ng mL^{-1} PMA and 1 µg mL^{-1} ionomycin. During the last three hours, golgiplug (BDbioscience) was added to the culture. Cells were then harvested for flow cytometry and polarization was confirmed with intracellular staining of IL-4.

Mast cells and basophils were derived by culturing bone marrow harvested from femurs with 20% WEHI-3b conditioned media. After 7 days, cells in suspension were collected and plated with cRPMI and 10 ng mL^{-1} mrIL-33 for 5 days. On day 6, golgiplug was added to the culture for the last 4 hours, and cells were harvested flow cytometry and stained for mast cells ($Fc\varepsilon R1\alpha^+$, $CD49b^-$, $CD117^+$) and basophils ($Fc\varepsilon R1\alpha^+$, $CD49b^+$, $CD117^-$) and intracellular IL-5 and IFNγ.

Eosinophils were derived from bone marrow as described by others [6, 7]. Briefly, bone marrow was harvested from femurs and seeded for 5 days in cRPMI with mrIL-33 (CF, R&D) with 10 ng mL^{-1} mrIL-33. On day 6, cells were harvested for intracellular IL-5 and IFNγ detection by flow cytometry as above.

IL-5 Promoter-Reporter Assay

BJAB cells co-electroporated with 2 ug of human IL-5 promoter-luciferase construct, pLightSwitch-IL5 (pLS-IL5, Switchgear Genomics) and 10 ug of empty pEF4 or pEF4 human Id3 expression vector previously described [8]. GFP was used as a electroporation efficiency control. After 24 hours, cells were harvested for luciferase chemiluminescence and normalized to total protein.

Statistics

To test if data sets fit a Gaussian distribution, a D'Agostino-Pearson omnibus normality test was used. If data was normal and had equal variance, a student's t-test was performed. If data sets had unequal variance, a t-test with Welch's correction was

used. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean only or mean ± SEM.

References:

1. Doran AC, Lipinski MJ, Oldham SN, et al. B-cell aortic homing and atheroprotection depend on id3. *Circ Res*. 2012;110:e1-12
2. Guo Z, Li H, Han M, Xu T, Wu X, Zhuang Y. Modeling sjogren's syndrome with id3 conditional knockout mice. *Immunology letters*. 2011;135:34-42
3. Tsimikas S, Palinski W, Witztum JL. Circulating autoantibodies to oxidized ldl correlate with arterial accumulation and depletion of oxidized ldl in ldl receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:95-100
4. Merki E, Graham MJ, Mullick AE, Miller ER, Crooke RM, Pitas RE, Witztum JL, Tsimikas S. Antisense oligonucleotide directed to human apolipoprotein b-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein b-100 particles in lipoprotein(a) transgenic mice. *Circulation*. 2008;118:743-753
5. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, Furusawa J, Ohtani M, Fujii H, Koyasu S. Innate production of t(h)2 cytokines by adipose tissue-associated c-kit(+)sca-1(+) lymphoid cells. *Nature*. 2010;463:540-544
6. Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D, Liew FY. Il-33 exacerbates eosinophil-mediated airway inflammation. *J Immunol*. 2010;185:3472-3480
7. Dyer KD, Garcia-Crespo KE, Percopo CM, Bowen AB, Ito T, Peterson KE, Gilfillan AM, Rosenberg HF. Defective eosinophil hematopoiesis ex vivo in inbred rocky mountain white (irw) mice. *Journal of leukocyte biology*. 2011;90:1101-1109
8. Doran AC, Lehtinen AB, Meller N, Lipinski MJ, Slayton RP, Oldham SN, Skaflen MD, Yeboah J, Rich SS, Bowden DW, McNamara CA. Id3 is a novel atheroprotective factor containing a functionally significant single-nucleotide polymorphism associated with intima-media thickness in humans. *Circ Res*. 2010;106:1303-1311

Supplemental Material

The Helix-Loop-Helix Factor Id3 Regulates IL-5 Expression and B-1a B cell Proliferation

Perry, et.al.

Supplemental Figures:

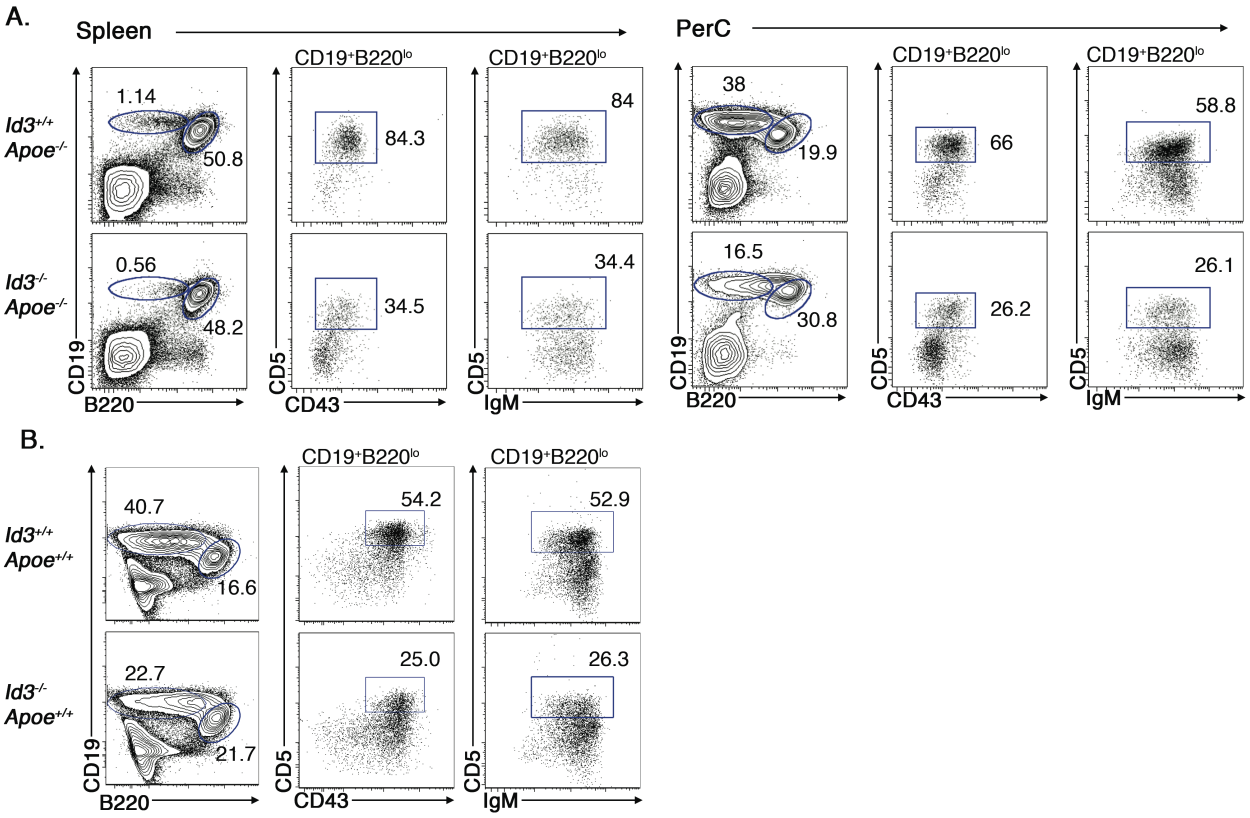

Figure I Representative flow cytometry plots of **(A)** B-2 ($CD19^{+}B220^{hi}$) and B-1a ($CD19^{+}B220^{lo}CD5^{+}CD43^{+}IgM^{hi}$) B cells in the spleen and PerC of *Id3*$^{+/+}$*Apoe*$^{-/-}$ or *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice or **(B)** B-1a B cells in the PerC of wild-type, C57BL/6, (*Id3*$^{+/+}$*Apoe*$^{+/+}$) or *Id3*$^{-/-}$*Apoe*$^{+/+}$ mice at 8 weeks of age.

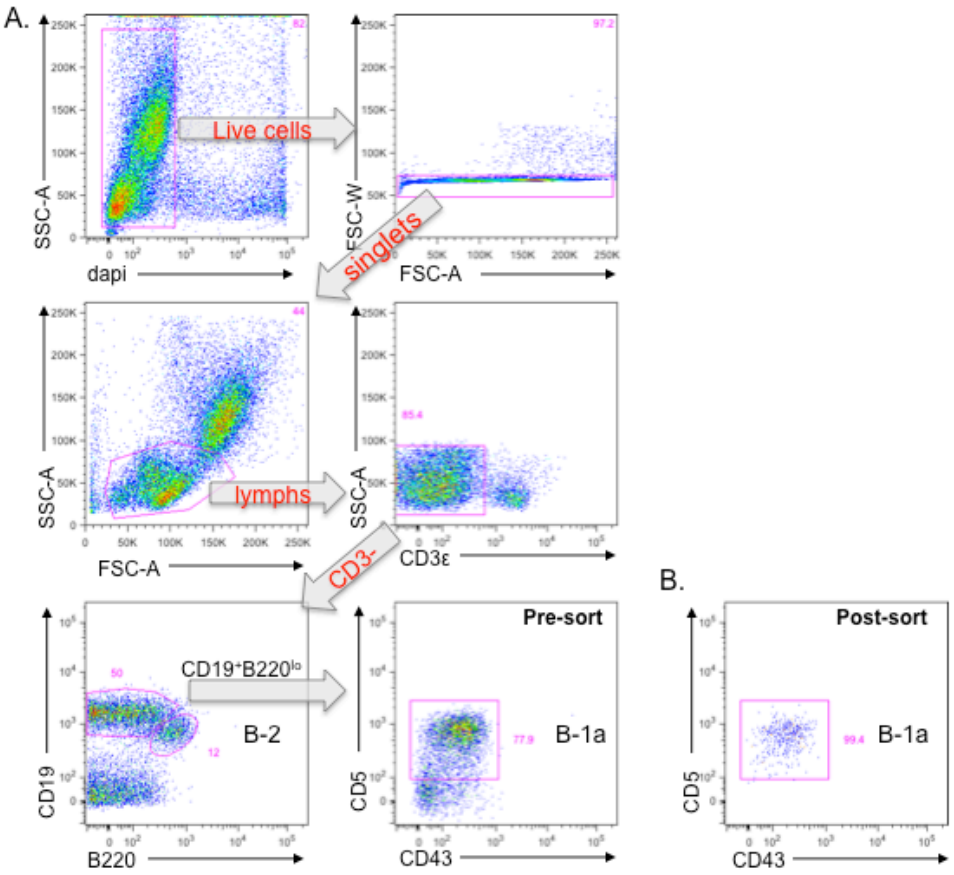

Figure II Gating strategy for FACS purification of B-1a and B-2 B cells. Peritoneal lavage cells from 5 *Id3*$^{+/+}$*Apoe*$^{-/-}$ or 5 *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were pooled and stained for FACS with dapi, CD3ε, CD19, B220, CD43 and IgM. (A) Gating strategy used to sort B cells (dapi-, singlets, lymphocytes (lymphs), CD3ε-, B-2: CD19^{+}B220^{hi}, B-1a: CD19^{+}B220^{lo}, CD5+. (B) Post-sort purity of B-1a cells.

Supplemental Table I. Average random serum lipid profiles of *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice at 8 to 10 weeks of age**.**

	Id3$^{+/+}$*Apoe*$^{-/-}$	*Id3*$^{-/-}$*Apoe*$^{-/-}$
Cholesterol (mg/dL)	314.2 ± 23.9	376.0 ± 34.5
HDL-C (mg/dL)	35.63 ± 3.6	36.75 ± 1.4
Non HDL-C (mg/dL)	252.6 ± 13.9	303.4 ± 33.6
Triglycerides (mg/dL)	184.3 ± 31.7	215.1 ± 20.7

Values are the average ± SEM. No comparisons are statistically significant.

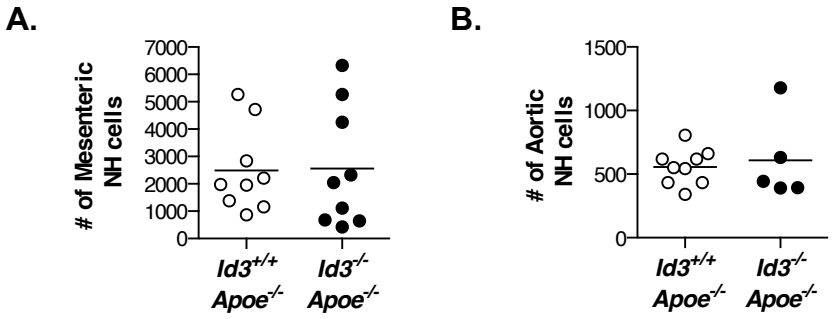

Figure III *Id3*$^{-/-}$*Apoe*$^{-/-}$ and *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice have equal numbers of NH cells in the mesentery and adventitia/PVAT. Tissue was processed, stained with fluorochrome conjugated MAbs and analyzed by flow cytometry to detect NH cells in the (A) mesentery or (B) adventitia/PVAT.