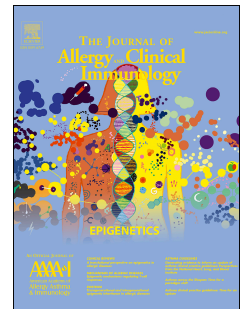


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**Ezh2 controls development of natural killer T cells that cause spontaneous
asthma-like pathology**

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Keywords: Asthma, Allergy, Inflammation, Epigenetics, Natural Killer T cells.

26 Abbreviations: natural killer T (NKT), invariant NKT (iNKT), wild-type (WT),
27 knock-out (KO), double knock out (DKO), house dust mite (HDM), airways
28 hyperreactivity (AHR), methacholine (MCh), innate lymphoid cell (ILC), double
29 negative (DN), promyelocytic leukemia zinc finger (PLZF), T-box 21 (T-bet), RAR-
30 related orphan receptor gamma (ROR γ t), interleukin (IL), immunoglobulin (Ig),
31 mesenteric, lymph nodes (MLN), bronchial-associated lymph nodes (BALN).
32
33 Word count: 9071 (including supplementary Figure Legends and Methods).

34 **Abstract:**

35 **Background:** Natural killer T (NKT) cells express a T cell receptor that recognises
36 endogenous and environmental glycolipid antigens. Several subsets of NKT cells
37 have been identified, including IFN γ -producing NKT1 cells, IL-4-producing NKT2
38 cells and IL-17-producing NKT17 cells. However, little is known about the factors
39 that regulate their differentiation and respective functions within the immune system.

40 **Objective:** We sought to determine if the polycomb repressive complex 2 protein
41 Ezh2 restrains pathogenicity of NKT cells in the context of asthma-like lung disease.

42 **Methods:** Numbers of iNKT1, iNKT2 and iNKT17 cells, and tissue distribution,
43 cytokine production, lymphoid tissue localisation and transcriptional profiles of iNKT
44 cells from wild-type (WT) and Ezh2 knock-out (KO) iNKT mice were determined.
45 The contribution of NKT cells to development of spontaneous and house dust mite
46 (HDM) induced airways pathology including airways hyperreactivity (AHR) to
47 methacholine was also assessed in WT, Ezh2 KO, and Ezh2 KO mice lacking NKT
48 cells.

49 **Results:** Ezh2 restrains the development of pathogenic NKT cells that induce
50 spontaneous asthma-like disease in mice. Deletion of Ezh2 increased production of
51 IL-4 and IL-13, and induced spontaneous AHR, lung inflammation, mucus
52 production, and IgE. Increased IL-4 and IL-13, AHR, lung inflammation, and IgE
53 were all dependent on iNKT cells. In HDM exposed animals, Ezh2 KO resulted in
54 enhanced AHR that was also dependent on iNKT cells.

55 **Conclusion:** Ezh2 is a central regulator of iNKT pathogenicity and suppresses the
56 ability of iNKT cells to induce asthma-like pathology.

Capsule Summary:

The epigenetic regulator Ezh2 suppresses development of an IL-4-expressing natural killer T cell population that causes hyper-IgE, airway inflammation and airway hyperreactivity in mice.

Key Messages:

- 1) Conditional deletion of Ezh2 in T cells results in hyper-IgE, airway inflammation and both spontaneous and house-dust-mite-induced airway hyperreactivity in mice.
- 2) Increased asthma-like pathology in mice lacking Ezh2 in T cells is dependent on invariant natural killer T (iNKT) cells.
- 3) Ezh2 suppresses the development of IL-4-producing iNKT2 cells and asthma-like pathology in mice.

Introduction:

Allergic asthma is a chronic inflammatory disease of the airways that develops due to aberrant immune responses to common environmental antigens including dust mites, pollens and fungi (1). The Type-2 cytokines IL-4, IL-5 and IL-13 contribute to the development of allergic responses including IgE production, cellular inflammation, and airway hyperreactivity (AHR) in asthmatics (2). Type-2 cytokine production by allergen-specific CD4⁺ T-helper (Th) cells promote allergic inflammation and antibody production by B cells (3), and epigenetic regulation has been shown to play an important role in the regulation of Th cell cytokine production (4). Ezh2 is a member of polycomb repressive complex 2 and is enzymatically responsible for methylating lysine 17 on histone 3, a key epigenetic modification known to repress lineage determining transcription factors during cellular differentiation (5, 6). We previously found that deficiency of Ezh2 in T cells in mice results in increased Type-2 cytokine production from CD4⁺ T cells and markedly elevated levels of serum IgE (7), one of the hallmark indicators of atopy in humans. In addition, lower expression of Ezh2 was reported in peripheral blood Th cells from allergic rhinitis patients compared to healthy controls, and Th2 cytokine production after house dust mite stimulation was found to negatively correlate with Ezh2 expression (8), indicating that Ezh2 may regulate Th2 differentiation in allergic individuals.

In addition to T cells, an innate lymphoid cell (ILC) subset called ILC2 has been recently shown to play a role in the development of allergic inflammation (9). Natural killer T (NKT) cells display similarities to both natural killer cells (a type of ILC) and adaptive T cells, and possess the ability to rapidly secrete large amounts of cytokines. Invariant NKT (iNKT) cells express an invariant TCR α chain consisting of

V α 14/J α 18 in mice or V α 24/J α 18 in humans, and account for the majority of NKT cells (10). NKT cells are activated by endogenous and environmental glycolipid ligands presented in the context of the non-polymorphic major histocompatibility complex (MHC) class 1-like antigen-presenting molecule CD1d. Fungal and bacterial antigens from *Sphingomonas* (11), *Aspergillus fumigatus* (12), and *Streptococcus pneumoniae* (13) have been shown to be recognized by NKT and are capable of inducing asthma-like symptoms in an NKT-dependent manner in mouse models. The involvement of NKT cells in protein-induced asthma is more controversial, with some studies finding that NKT cells are necessary for allergic pathology in mouse models (14, 15), and others reporting that NKT cells are not required (16). In human asthmatics, increased numbers of NKT cells in BAL samples (17-19), and an increase in NKT cells after allergen challenge (20) have been reported; however, some studies have failed to detect a difference in lung NKT cell numbers between asthmatics and non-asthmatics (21, 22), indicating that more research on NKT cells in asthma is needed.

NKT cells can be differentiated into three subsets based on cytokine production and expression of the transcription factors promyelocytic leukemia zinc finger (PLZF), T-box 21 (T-bet), and RAR-related orphan receptor gamma (ROR γ t); NKT1 cells produce IFN- γ and express high levels of T-bet (23), NKT2 cells produce IL-4 and express high levels of PLZF (24), and NKT17 cells produce IL-17 and express high levels of ROR γ t (24-26). It is also becoming evident that further significant heterogeneity exists within the NKT cell subsets (27). For example, Bcl-6-dependent follicular helper NKT (NKT_{fh}) cells have been shown to promote antibody production and provide help for B cells via production of IL-21 (28), and interfollicular NKT were recently found to be essential for initiation of antiviral B cell

immunity via early production of IL-4 (29). It was recently shown that increased numbers of PLZF-expressing iNKT cells are present in Ezh2 KO mice (30, 31), but the peripheral distribution of these cells and their functional characteristics are largely undefined.

We herein show that loss of the epigenetic regulator Ezh2 in T cells results in the development of iNKT2 cells that produce large amounts of Type-2 cytokines and drive development of AHR and allergic asthma-like lung pathology in mice.

Methods:

Mice: C57BL/6 mice were from CLEA Co. (Tokyo, Japan). Mice with loxp sites flanking the SET domain of Ezh2 ($Ezh2^{fl/fl}$) were generated as described (47) and backcrossed with C57BL/6 mice for 10 generations. These mice were bred with mice expressing transgenic constructs for Cre recombinase. CD4-Cre mice were purchased from Taconic. CD1d KO (B6.129S6-Del(3CD1d2-Cd1d1)1Sbp/J) mice were purchased from The Jackson Laboratory. $\alpha 18$ -deficient mice were generated as previously described (48). Mice used in this study are shown in Table 1. Controls were either $Ezh2^{fl/fl}$ Cre-negative or $Ezh2^{wt/wt}$ Cre-positive. Similar experimental results were obtained with control mice irrespective of Cre transgene expression.

All mice were maintained under specific pathogen free conditions and animal care was conducted in accordance with the guidelines of Chiba University.

Measurement of airway hyperreactivity: Airway hyperreactivity, quantitated by increased airway resistance, was assessed as previously described (49) by methacholine (MCh)-induced (Sigma-Aldrich) airflow obstruction in intubated and ventilated mice by forced oscillation technique using a computer-controlled small animal ventilator (SCIREQ).

House dust mice challenge model: mice were challenged with 50 μ g of HDM extract in 20 μ l phosphate buffered saline (PBS) 8 times on days 0, 1, 7, 8, 9, 14, 15, 16. On day 17 MCh-induced AHR was analysed.

Bronchoalveolar lavage and differential cell count: Bronchoalveolar lavage (BAL) was performed by intratracheal administration and recovery of 2 lavages of 1 mL PBS. Cell numbers were enumerated by counting with Turks solution. Cells were then spun onto glass slides by cytocentrifugation (Shandon) and stained with Diff-Quik (Sysmex). Differential cell counts were performed based on cell morphology using light microscopy.

Enzyme-linked immunosorbent assay (ELISA): A standard sandwich ELISA protocol was used to measure the concentrations of cytokines in cell-free culture supernatants and in BAL fluid samples. Clone BVD4-1D11 and clone BVD6-24G2-biotin were used for detection of IL-4, clone TRFK5 and clone TRFK4-biotin were used for detection of IL-5, clone R4-6A2 and clone XMG1.2-biotin were used for detection of IFN- γ , all purchased from BD Biosciences. IL-13 was measured using a DuoSet mouse IL-13 ELISA set (R&D Systems) and IgE was measured using an OptEIA mouse IgE ELISA set (BD Biosystems). ELISA for Derp1 used biotinylated Derp1 (Citeq Biologics) to detect Derp1-specific antibodies in serum. Briefly, plate coated anti-IgG1 Clone A85-3 (BD Biosciences) was used to capture total IgG1 from serum, then Derp1-specific antibodies were detected with the biotinylated Derp1 followed by streptavidin-HRP and addition of TMB substrate (Life Technologies).

ELISPOT assay: For detection of IgE-secreting B cells, MAIPSWU microplates (Multiscreen HTS; Millipore) were activated with 70% ethanol, washed with sterile water, coated with purified anti-IgE in PBS, blocked with RPMI1640/10% FCS, and 1.5×10^5 cells per well were incubated for 5 hours at 37°C followed by washing with PBS. IgE was detected with alkaline phosphatase-conjugated antibodies to mouse IgE

(Southern Biotechnology). Spots were visualized with 100 µl/well AEC solution (Sigma) and were counted manually.

Lung histology: For histological analysis, lungs were fixed in 10% (v/v) formalin in phosphate buffered saline. Lung sections were paraffin embedded, sectioned, and stained with periodic acid-Schiff (PAS) commercially. PAS stained sections were examined for pathological changes under a light microscope for the presence of mucus in the airways.

Fluorescence microscopy: Samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS. Tissue was frozen in OCT media (Sakura) and sectioned using a Cryostat. Sections were stained and mounted with Fluorescent mounting medium (DakoCytomation). A confocal laser microscope (LSM710, Carl Zeiss) was used to capture images. Sections were stained with anti-B220-FITC, anti-TCRβ-APC/Cy7, and PBS-57-Tet-PE. Images were analyzed with ImageJ software (National Institutes of Health).

Preparation of single cell suspensions from tissues: Spleens and lymph nodes were disassociated using frosted glass slides (Matsunami). Lungs were dissociated in digestion buffer (RPMI 1640 supplemented with Collagenase Type III (200 U/ml) (Worthington) and DNaseI (200 µg/ml) (Sigma-Aldrich)) with incubation for 30 minutes at 37°C with constant agitation. Liver was disassociated by passing through a 200 µm wire mesh. Lung and liver mononuclear cells were then separated by centrifugation on Percoll (GE Healthcare, UK). Spleen, lymph node, lung and liver

cell suspensions were then passed through a 70 μ m cell strainer (Greiner bio-one, JP) and red blood cells were lysed using ACK lysis buffer (Gibco).

Cell purification: For purification of CD44^{hi}CD4⁺ T cells and iNKT cells used in the functional experiments in this manuscript and for RNA-Seq analysis of iNKT cells, cells were first enriched by positive magnetic selection of T cells using biotinylated anti-CD3 ϵ (clone 145-2C11) and anti-biotin microbeads (Miltenyi Biotec), followed by staining with anti-TCR β , anti-CD4, anti-CD44 and PBS-57-loaded CD1d-tetramers (PBS-57-Tet). PBS-57 is an analogue of alpha-galactosylceramide (α GalCer). Either PBS-57 or α GalCer presented in the context of CD1d can be recognised by the invariant T cell receptor of iNKT cells. PBS-57-Tet were provided by the National Institutes for Health Tetramer Core Facility. TCR β ⁺, PBS-57-Tet⁺ iNKT cells and TCR β ⁺, CD4⁺, CD44^{hi} conventional T cells were further purified using a FACSaria cell sorter (BD Biosciences). For iNKT RNA-Seq analysis, iNKT cells from the spleens of 3 WT and 3 Ezh2 KO mice were combined to obtain 150,000 iNKT cells for each of the two RNA-Seq biological replicates (6 WT and 6 Ezh2 KO mice in total). For purification of CD44^{hi}CD4⁺ T and CD44^{lo}CD4⁺ T cells in Fig. S3 and Fig. S4, CD4⁺ T cells were obtained using a CD4⁺ T cell isolation kit with anti-CD4 microbeads (Miltenyi Biotec) and CD44^{hi}CD4⁺ T cells were then further purified using a FACSaria cell sorter (BD Biosciences). For RNA-Seq of CD44^{hi}CD4⁺ T cells, 1 x 10⁶ cells were purified by FACS from one WT and one Ezh2 KO mouse. CD44^{hi}CD4⁺ T cells and iNKT cells were purified from spleens for all experiments.

In vitro stimulation: Tissue culture plates (Costar) were pre-coated overnight with 10 µg/ml agonistic anti-TCRβ antibody (clone H57-597). For stimulation of spleen cells, 3 x 10⁶ cells were plated into wells of 24 well tissue culture plates in 1 ml culture media, and for purified iNKT cells and CD44^{hi}CD4⁺ T cells 1 x 10⁵ cells were plated into 96 well tissue culture plates in 200 µl tissue culture media. Tissue culture media consisted of RPMI supplemented with 10% foetal bovine serum (Equitech-Bio), 1mM Sodium Pyruvate, 10mM HEPES, and MEM Non-Essential Amino Acids, and 55µM 2-Mercaptoethanol (Gibco). Cells were cultured at 37°C for 16 hours before measurement of cytokine production.

RNA sequencing: RNA-seq was carried out as previously described (50). Total RNA was extracted with TRIzol reagent (Invitrogen). TruSeq RNA Sample Prep Kit v2 (Illumina) was used for cDNA library construction, according to the manufacturer's protocol. Sequencing was performed on a HiSeq 1500 sequencer (Illumina). Read sequences (50 bp) were aligned to the mm10 mouse reference genome (University of California, Santa Cruz (UCSC) December 2011) using Bowtie 2 (version 2.0.0) and TopHat (version 1.3.2). Fragments per kilobase of exon per million mapped reads (FPKM) for each gene were calculated using Cufflinks (version 2.0.2). Genes with low expression (less than 5 FPKM for all samples) were excluded from the analysis. Differential gene expression was analysed using limma. GlmFit and glmLRT were used for generalized linear modeling (51). Genes with a FDR < 0.1 were used for gene ontology enrichment analysis using DAVID (52).

Chromatin immunoprecipitation (ChIP)-sequencing: ChIP-Seq was performed as previously described (7). Polyclonal anti-Ezh2 (pAB-039-50, Diagenode) was used

for immunoprecipitation (IP). Read sequences (36 bp) with two or less mismatches were aligned to the mm9 mouse reference genome (University of California, Santa Cruz (UCSC) July 2007) using Eland software (Illumina). For visualization of Ezh2 binding, data was converted to wiggle file format at 25 bp resolution and uploaded to the UCSC genome browser (<http://genome.ucsc.edu/>).

Quantitative Real-Time PCR: Total RNA isolation, cDNA synthesis, and Quantitative real-time PCR was described previously (7). Primers and Roche Universal probes were purchased from Sigma and Roche, respectively. Gene expression was normalized to amount of hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) mRNA in each sample.

Antibodies used for cell surface and cytoplasmic staining:

Antibodies used for cell surface staining were anti-TCR β (clone H57-597), anti-CD44-APC (clone IM7), anti-CD62L-PE (clone MEL-14), and-B220-PerCP/Cy.5.5 (clone RA3-6B2) from BD Biosciences, anti-CD4 (clone RM4-5) from Biolegend. PBS-57-Tet -PE was from the National Institutes of Health tetramer facility. Antibodies used for cytoplasmic staining were: anti-IL4-PE (clone 11B11), anti-IFN- γ -FITC (clone XMG1.2), anti-PLZF-APC or FITC (clone R17-809), anti-Roryt-APC (clone Q31-378) from BD-Biosciences and anti-T-bet-BV421 (clone eBio4B10), from eBioscience.

Statistical analysis: Unless otherwise indicated, p values were calculated using Student's t tests or ANOVA with Tukey' post-tests when multiple comparisons were performed.

Accession numbers: ChIP-seq and RNA-Seq data are available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under the accession numbers (TBA) and (TBA) respectively.

Table 1: description of the mouse strains used in this study.

Genetic background	Strain	Source/reference	Description
C57BL/6	Ezh2 fl/fl/ Ezh2 ^{tm1Yugo}	Hirabayashi Y, <i>et al.</i> <i>Neuron</i> 63(5):600-613	Loxp sites flanking exons 18 and 19 of <i>Ezh2</i>
C57BL/6	CD4-Cre/ B6.Cg-Tg(CD4-cre)1Cwi N9	Purchased from Taconic	Crossed with Ezh2 fl/fl mice to generate Ezh2 KO mice
C57BL/6	CD1d KO/ B6.129S6- Del(3CD1d2-Cd1d1)1Sbp/J	Purchased from The Jackson Laboratory	Deficient in both <i>Cd1d1/Cd1d2</i> loci.
C57BL/6	J α 18 KO/ Tcra-J ^{tm1Tgi}	Cui J, <i>et al.</i> , <i>Science</i> . 1997 Nov 28;278(5343):1623-6	Deletion of the Tcra-J region results in lack of iNKT cells

Results:**Loss of Ezh2 function in CD4⁺ T cells results in spontaneous development of asthma-like pathology.**

Mice with conditional inactivation of Ezh2 in T cells (referred to herein as Ezh2 KO) develop a spontaneous increase in the levels of circulating IgE and an age-dependent increase in CD44^{hi}CD4⁺ T cells that produce the Type-2 cytokines including IL-4 and IL-13 (7). A schematic representation of the conditional inactivation of Ezh2 in cells that express CD4 is shown in Supplementary Figure 1. We examined 3-4 month old WT and Ezh2 KO mice for methacholine (MCh)-induced airway resistance, an indicator of airway constriction and airway hyperresponsiveness (AHR) using a Scireq flexivent. Ezh2 KO mice exhibited increased AHR compared to wild type (WT) mice of the same age (Fig. 1A). We also detected a significant increase in the numbers of eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage (BAL) samples from Ezh2 KO mice (Fig. 1B). We used flow cytometry to further define which lymphocytes were increased in the BAL of Ezh2 KO mice. We found a significant increase in the number of CD19⁺ B cells and no change in the number of TCRβ⁺ T cells in the BAL (Fig. S2A and S2B). Approximately one-third (7 out of 22 mice examined) showed visible signs of airways inflammation and mucus production in the lungs (Fig. 1C). In contrast, we did not detect obvious signs of inflammation or pathology in the intestines, heart, or kidneys. Using ELISPOT, we found increased numbers of IgE producing cells in the spleens of Ezh2 KO compared to WT mice (Fig. 1D).

Ezh2 regulates iNKT cell numbers *in vivo*.

Increased numbers of CD44^{hi}CD4⁺ T cells accumulated in Ezh2 KO mice (Fig. S3A), as we reported previously (7). To further characterise these cells, we performed RNA-Seq on CD44^{hi}CD4⁺ T cells purified from the spleen of WT and Ezh2 KO mice. We found that the most upregulated genes included many killer cell lectin-like receptor genes, the NK cell associated receptor gene *Cd160*, and *Zbtb16* (that encodes the transcription factor PLZF, a master regulator of NKT cell differentiation) (Fig. S3B). Increased expression of *Zbtb16* was confirmed by qPCR (Fig. S4A) and increased expression of PLZF protein confirmed by intracellular staining (Fig. S4B). We also found that PLZF expression was restricted to CD44^{hi}CD4⁺ T cells (Fig. S4C). To determine if Ezh2 directly targeted *Zbtb16* in T cells, we used chromatin immunoprecipitation with Ezh2-specific antibody and massively parallel sequencing (ChIP-Seq). We did not detect Ezh2 binding at the *Zbtb16* gene in naïve CD4⁺ T cells (Fig S4D); however, a strong peak of Ezh2 binding was evident at the *Zbtb16* gene after differentiation of CD4⁺ T cells into effector Th cells (Fig. S4D). These data show that Ezh2 directly targets and controls *Zbtb16* expression in differentiated T cells.

To confirm that the numbers of iNKT cells were increased in Ezh2 KO mice, we used CD1d tetramers loaded with the iNKT cell ligand PBS-57 (PBS-57-Tet). PBS-57 presented in the context of CD1d is specifically recognised by the invariant T cell receptor of iNKT cells. We found significantly increased frequencies of iNKT cells in both the thymus (Fig. 2A) and spleen (Fig. 2B) of Ezh2 KO mice. To further characterize the distribution of iNKT cells in the peripheral tissues of Ezh2 KO mice, we also assessed the numbers of iNKT cells in the mesenteric lymph nodes (MLN) and bronchial-associated lymph nodes (BALN), and the liver and lungs. We found that both the proportions and total numbers of iNKT cells were increased in the MLN,

BALN, and the liver, but not in the lungs themselves (Fig. S5A and S5B). The absolute numbers of iNKT cells and iNKT1, iNKT2 and iNKT17 cells were determined based on transcription factor expression (Fig. 2C). Significantly increased proportions of PLZF-expressing iNKT2 cells were detected in the thymus (Fig. 2D) and spleen (Fig. 2E) of Ezh2 KO mice. A more modest increase in T-bet-expressing iNKT1 was also evident in the spleen, but not the thymus of Ezh2 KO mice. These data indicate that Ezh2 regulates PLZF expression in T cells and that loss of Ezh2 function results in the expansion of PLZF-expressing iNKT2 cells. iNKT cells can be divided into CD4⁺ and CD4⁻CD8⁻ double negative (DN) subsets. We found that the increase in PLZF⁺ NKT2 cells was evident in both CD4⁺ and DN subsets in the spleen and thymus of Ezh2 KO mice (Fig. S5C-S5D). In contrast, the increase in T-bet⁺ iNKT1 cells was restricted to the DN iNKT subset (Fig. S5E and S5F). To determine if there was an increase in the proportion of iNKT2 cells in the lungs of Ezh2 KO mice, we digested lungs from WT and Ezh2 KO mice and measured the proportions of iNKT subsets using intracellular staining. We found that there was a switch from predominately iNKT1 cells to iNKT2 cells in the lungs of Ezh2 KO mice (Fig. 2F and 2G).

We also detected increased numbers of PLZF-expressing T cells that did not bind PBS-57-Tetramers in both the thymus and spleen of Ezh2 KO mice (Fig. S6A), as reported previously (30). To determine if these cells were non-invariant NKT cells or conventional T cells, we crossed Ezh2 KO mice with mice lacking CD1d (CD1d KO) that have no NKT cells to make Ezh2/CD1d double KO (DKO) mice. In Ezh2/CD1d DKO mice, PBS-57-Tet-negative PLZF-expressing T cells were still present in both the thymus (Fig. S6B) and spleen (Fig. S6C), showing that they are not CD1d-restricted and that they develop via a different pathway to NKT cells.

iNKT cells in Ezh2 KO mice resemble Th2 and Tfh-like cells but remain in the interfollicular zone in lymphoid tissues.

To further characterize the iNKT cells that accumulate in response to loss of Ezh2, we compared transcriptional profiles of iNKT cells purified from the spleens of WT and Ezh2 KO mice (Fig. S7A). We found that there were 184 genes upregulated and 112 genes downregulated in Ezh2 KO iNKT cells compared to WT iNKT cells. Gene ontology analysis of the differentially expressed genes showed significant enrichment in “Cytokine-cytokine receptor interaction” (KEGG pathway, $p=4.2 \times 10^{-5}$), “Cell surface” (GOTERM Cellular-Component, $p=1.3 \times 10^{-5}$), and “Transcription factor activity” (Uniprot keyword, $p=4.2 \times 10^{-5}$) genes. Enriched “Cytokine-cytokine receptor interaction” and “Cell surface” genes (Fig. 3A) and enriched “Transcription factor activity” genes (Fig. 3B) are shown. iNKT cells from Ezh2 KO mice displayed a Th2-like phenotype with increased expression of genes encoding the Th2-associated chemokine receptor *Ccr4* and the cytokine IL-4, and lower expression of genes encoding the Th1-associated proteins IFN γ , IL12R β , STAT-4, and T-bet (encoded by *Tbx21*). As expected, we found a significant increase in the expression of the gene encoding PLZF (*Zbtb16*) in Ezh2 KO iNKT cells. *Egr2* has also been shown to regulate iNKT development (32), and expression of *Egr2* was also increased in Ezh2 KO iNKT cells.

To confirm that iNKT cells from Ezh2 KO mice produced more Th2 cytokines, we stimulated spleen cells from WT and Ezh2 KO mice and analysed IL-4 and IFN γ by intracellular staining. A higher proportion of iNKT cells from Ezh2 KO mice produced IL-4 (3.5-fold increase), and a lower proportion produced IFN γ (2-fold decrease) compared to iNKT cells from WT mice (Fig 3C). To examine the amount of

IL-4 produced by iNKT cells from WT and Ezh2 KO mice, purified iNKT were stimulated *in vitro* with anti-TCR β for 16 hours followed by measurement of IL-4 production. Ezh2 KO iNKT cells produced significantly more IL-4 than WT iNKT cells, and both WT and KO iNKT cells produced much more IL-4 than CD44^{hi}CD4⁺ conventional T cells from either WT or Ezh2 KO mice (Fig. 3D).

iNKT cells from Ezh2 KO mice expressed increased amounts of several genes associated with follicular T helper (Tfh) cell identity and chemokine-induced T cell positioning within lymphoid tissues. Tfh cells express high levels of IL-21, Cxcr5 and PD1 (33), and these genes were among the most highly expressed in Ezh2 KO iNKT cells (Fig. 3A). The transcription factors Bcl6, Ascl2, Tcf7, Lef1 have been shown to regulate Tfh development (34-38), and of these Tcf7 and Lef1 were expressed more highly in Ezh2 KO iNKT cells (Fig. 3B). Bcl-6 expression was very low in both WT and Ezh2 KO iNKT cells. Ezh2 KO iNKT cells therefore share several key genes expressed by Tfh cells. Down-regulation of Ccr7 in pre-Tfh cells facilitates localization to the border between the T cell and B cell zones of lymphoid tissue (T/B border), and upregulation of Cxcr5 facilitates Tfh homing to germinal centers (33). iNKT cells from Ezh2 KO mice express increased levels of genes encoding both Cxcr5 and Ccr7 compared to WT iNKT cells (Fig. 3A). To determine if iNKT cells were present in B cell follicles, and were thus true iNKT-Tfh hybrid cells, we analyzed iNKT localization in spleen and MLN tissue. We found that iNKT cells were not within B cell follicles of Ezh2 KO mice, but instead localized in the T cell zone closely surrounding the follicles (Fig. 3E and Fig. S7C). In WT mice, iNKT cells were rarely detected. We have previously shown that Ezh2 KO mice have significantly higher production of IgE (7) and Fig. 1D. To determine if Ezh2 KO mice also have increased levels of other isotypes, we measured IgG1, IgG2c, IgA and IgM

by ELISA. We found that Ezh2 KO results in decreased amounts of IgG1 (Fig. S8A) and IgM (Fig. S8B), increased IgG2c (Fig. S8C) and no change in the amount of IgA (Fig. S8D). These data indicate that Ezh2 KO differentially alters immunoglobulin production and that Ezh2 KO does not result in a general increase in all immunoglobulin isotypes.

iNKT cells drive spontaneous development of allergic asthma-like pathology in Ezh2 KO mice.

We previously reported that CD44^{hi}CD4⁺ T cells from Ezh2 KO mice produced markedly elevated levels of the Type-2 cytokines IL-4 and IL-13 when stimulated *in vitro* (7). To determine if the increased production of Type-2 cytokines was due to the presence of iNKT cells within this population, we analysed CD44^{hi}CD4⁺ T cells from Ezh2/CD1d DKO mice. We found that deletion of CD1d returned cytokine production from Ezh2 KO CD44^{hi}CD4⁺ T cells to levels comparable to, or less than, that produced by cells from WT mice (Fig. 4A). This indicates that NKT cells may be the predominant cell type responsible for producing Type-2 cytokines in Ezh2 KO mice. To differentiate between NKT cells with the invariant V α 14/J α 18 TCR (iNKT) and non-invariant TCR NKT cells, we also generated mice lacking the gene encoding J α 18 together with Ezh2 (J α 18 KO mice lack only iNKT cells). Analysis of cytokine production by CD44^{hi}CD4⁺ T cells from Ezh2/J α 18 DKO mice produced results very similar to the Ezh2/CD1d DKO mice, indicating that iNKT cells are the predominant cell population responsible for producing Type-2 cytokines in Ezh2 KO mice (Fig. 4B). In contrast, production of IFN- γ was comparable between WT and Ezh2 KO cells (Fig. S9A and C) and IL-17 was significantly decreased from Ezh2 KO cells (Fig. S9B and D). IFN- γ was also

significantly lower than both WT and Ezh2 KO in Ezh2/CD1d DKO and Ezh2/J α 18 DKO groups (Fig. S9A and C), and IL-17 production was unchanged compared to Ezh2 KO (Fig. S9B and D). We next examined 6-7 month old mice to determine if the iNKT2 cells that accumulate in Ezh2 KO could induce a switch toward conventional Th2 cells in older mice. We sorted conventional CD44^{hi}CD4⁺ T cells (excluding iNKT cells) from the spleens of 6-7 month old WT, Ezh2 KO, and Ezh2/J α 18 DKO mice and compared cytokine production after 72 hours stimulation *in vitro*. We found that conventional CD44^{hi}CD4⁺ T cells from Ezh2 KO mice did produce more IL-4 (Fig. S9E) and IL-13 (Fig. S9F) than CD44^{hi}CD4⁺ T cells from WT mice. Moreover, this increase in IL-4 and IL-13 production was not evident in CD44^{hi}CD4⁺ T cells from Ezh2/J α 18 DKO mice, indicating that iNKT cells in Ezh2 KO mice can induce the accumulation of Th2 cells *in vivo*.

We next sought to determine if iNKT cells drove the spontaneous development of allergic asthma-like pathology that we observed in the Ezh2 KO mice. To do this we analysed lung cell numbers, AHR, and serum IgE in 3-4 month old Ezh2/CD1d and Ezh2/J α 18 DKO mice so that we could assess the contribution of all NKT cells (Ezh2/CD1d DKO), and iNKT cells (Ezh2/J α 18 DKO) to the development of allergic asthma-like pathology. We again detected a significant increase in AHR in Ezh2 KO mice. In contrast, the Ezh2 KO mice that also lacked either all NKT cells (Ezh2/CD1d DKO), or iNKT cells (Ezh2/J α 18 DKO) did not display any increase in AHR compared to WT mice (Fig. 4C). Likewise, the increase in eosinophil and neutrophil numbers in the lungs of Ezh2 KO mice were similarly dependent on NKT cells and iNKT cells (Fig. 4D). The increase in IgE in Ezh2 KO mice was also shown to be dependent on NKT (Fig. 4E).

Lastly, we examined if intranasal challenge with the known allergen house dust mite (HDM) would result in a greater increase in AHR and antigen-specific antibody production in Ezh2 KO mice compared to WT mice, and whether this increase was dependent on iNKT cells (using Ezh2/J α 18 DKO mice). For this experiment we used 8 week old mice where the Ezh2 KO group had not yet developed spontaneous AHR. We challenged mice with 50 μ g of HDM extract 8 times on days 0, 1, 7, 8, 9, 14, 15, 16 and analysed AHR on day 17. We detected a significant increase in Derp1-specific IgG1 after HDM exposure, but this was not significantly different between WT and Ezh2 KO mice. Interestingly Ezh2/J α 18 DKO mice showed a significant reduction in Derp1-specific IgG1 compared to both WT and Ezh2 KO mice (Fig. S10). We found that WT, Ezh2 KO, and Ezh2/J α 18 DKO mice all developed AHR in response to intranasal HDM exposure. Importantly, Ezh2 KO mice developed significantly increased AHR compared to WT mice and AHR in Ezh2/J α 18 KO mice was almost identical to that observed in WT mice (Fig. 5). These data show that Ezh2 KO results in increased AHR in response to HDM exposure in the airways and that this increase is dependent on the presence of iNKT cells.

Together, these data indicate that iNKT cells are capable of driving asthma-like disease in mice, and that Ezh2 is a central regulator of iNKT pathogenicity.

Discussion:

We herein show that mice lacking Ezh2 function in T cells spontaneously develop allergic-asthma like pathology. By 4 months of age, Ezh2 KO mice developed allergic-asthma-like symptoms including eosinophil and neutrophil accumulation in the lungs, AHR, and mucus production in the airways. Both airway inflammation and AHR were found to be completely dependent on iNKT cells. Moreover, AHR in response to exposure to the known allergen house dust mite (HDM) is increased in Ezh2 KO mice and this was also dependent on iNKT cells.

Increased serum IgE and Type-2 cytokine-producing CD4⁺ T cells are typically present in atopic individuals and are associated with diseases such as atopic dermatitis and allergic asthma. We previously reported that CD44^{hi}CD4⁺ T cells from Ezh2 KO mice produce large amounts of Type-2 cytokines when activated *in vitro*, and that Ezh2 KO mice have markedly increased serum IgE (7). We show here that genetic deletion of CD1d or the iNKT J α 18 TCR chain also rescues the increased Type-2 cytokines and spontaneous increase in serum IgE levels in Ezh2 KO mice. Transcriptional profiling showed that Ezh2 KO iNKT cells expressed genes normally associated with Tfh cells, including those encoding Cxcr5, PD-1, and IL-21. iNKT cells are capable of activating B cells (39), can provide help to B cells responding to CD1d-restricted antigens (40, 41), and follicular helper NKT (NKTfh) cells have been shown to provide IL-21-dependent help to antigen-specific B cells (28). In addition, NKT cells located in the interfollicular regions, similarly positioned to the iNKT cells described here, were recently found to be required for initiation of antiviral B cell immunity (29). This study also shows that iNKT positioned close to the follicular borders rapidly produce IL-4 and are required for efficient formation of germinal centres and antibody production (29). Interestingly, while we did not detect a

difference in Derp1-specific antibody production in HDM-challenged WT or Ezh2 KO mice, mice lacking both Ezh2 and iNKT cells did develop significantly less HDM specific IgG1 compared to both WT and Ezh2 KO mice. We did not detect iNKT cells within B cell follicles in Ezh2 KO mice, but rather surrounding the B cell zones. Since high levels of IgE are present in Ezh2 KO mice and IgE class switching can occur in extrafollicular locations within lymphoid tissues (42), it will therefore be important to further investigate under which conditions interfollicular Tfh-like iNKT cells can directly promote antibody production by B cells.

Allergic asthma normally develops in response to environmental stimuli such as pollen, dust mites and fungi. NKT cells have been shown to recognise a variety of fungal and bacterial antigens (11, 12, 13); however, it is not known if environmental NKT ligands are required for spontaneous development of asthma-like symptoms in Ezh2 KO mice. Increased numbers of NKT cells in BAL samples from asthmatics (14, 18, 19), and an increase in NKT cells after allergen challenge (20) have been reported; however, some studies have found no difference in NKT cell number between asthmatics and non-asthmatics (21, 22). In the present study, we did not detect a change in the numbers of iNKT cells in the lungs of Ezh2 KO mice, but rather a change from iNKT1 to iNKT2 cells in the lungs and an increase in the number of iNKT cells in lymphoid tissues. These data indicate that more detailed analysis of the iNKT phenotype in asthmatics may be necessary. The clear increase in development of HDM-induced AHR in Ezh2 KO mice that was dependent on the presence of iNKT cells indicates that iNKT cells are capable of driving increased AHR and implicate iNKT2 cells in the development of AHR in response to a known allergen.

The transcriptional regulators that govern NKT cell subset differentiation into NKT1, NKT2, or NKT17 cells are beginning to be identified. The transcription factor PLZF (encoded by *Zbtb16*) is required for differentiation of the NKT lineage (43), and *Egr1* and *Egr2* have been shown to facilitate iNKT development in the thymus (32). NKT2 cells depend on the transcription factors PLZF, *Gata3* and *Irf4* for development (24, 44). T-bet is required for differentiation of IFN γ -producing NKT1 cells (24), and *Roryt* expression is linked to production of IL-17 (45). It was also recently shown that the microRNA Let 7 controls development of NKT1 cells in the thymus (46). Expression of *Zbtb16*, *Egr1* and *Egr2* are all increased in *Ezh2* KO iNKT cells, and *Egr2* has been shown to directly bind *Zbtb16* and promote its expression (32). *Ezh2* binds to, and regulates, expression of several transcription factor genes in T cells, including *Gata3*, *Tbx21* and *Eomes* (7), and we show that *Ezh2* also binds directly to *Zbtb16* in differentiated Th cells. Interestingly, an alternative mechanism for *Ezh2*-mediated regulation of PLZF has also recently been proposed, whereby methylation of PLZF by *Ezh2* was shown to target PLZF for degradation (31). It is therefore possible that both loss of gene repression and altered PLZF protein stability contribute to the high levels of PLZF in *Ezh2* KO T cells.

In summary, our data show that iNKT2 cells may have direct roles in the development of AHR and IgE production. We find that *Ezh2* is required to negatively regulate the pro-inflammatory properties of NKT cells, and that iNKT cells are capable of causing spontaneous development of asthma-like symptoms in mice. These data indicate that further investigation of *Ezh2* expression and activity in iNKT cells in allergic patients, and examination of the relative proportions of NKT subsets in asthmatics and patients with increased IgE is required.

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Main Figure Legends:

Figure 1: Loss of Ezh2 function in T cells results in spontaneous development of asthma-like pathology in mice. Wild type (WT) or Ezh2^{fl/fl} CD4-Cre (Ezh2 KO) mice were housed under specific pathogen-free conditions and analysed at 3-4 months old for methacholine-induced airways hyperreactivity expressed as airway resistance **(A)**, total cell numbers (Total) and numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and monocyte/macrophages (Mono/Mac) in bronchoalveolar lavage samples **(B)**, lung tissue and mucus in the airways visualized by periodic acid Schiff stain **(C)**, IgE producing cells in the spleen, mesenteric lymph nodes, and bone marrow by ELISPOT **(D)**, * significantly different from WT mice p<0.05, data expressed as mean +/- SEM. **A** and **B**, n= 5 WT, 8 Ezh2 KO, **C**, representative data showing airway mucus production where 7/22 Ezh2 KO mice had visible goblet cell hyperplasia and mucus in the airways compared to 0/20 WT mice. **D**, n=4 WT, 4 Ezh2 KO.

Figure 2: Ezh2 regulates the numbers of invariant NKT cells and PLZF^{hi} CD4 T cells. Proportions of TCRβ⁺PBS-57-Tet⁺ invariant natural killer T (iNKT) cells in the thymus **(A)**, and spleen **(B)**, total numbers of iNKT1 (PLZF^{lo}T-bet^{hi}) and iNKT2 (PLZF^{hi}T-bet^{lo}) cells were identified by intracellular staining for PLZF and T-bet (left panels), and iNKT17 (PLZF^{lo}Rorγt^{hi}) cells were identified by staining for PLZF and Rorγt (right panels) **(C)**, in the thymus **(D)** and spleen **(E)** of 3-4 months old wild type (WT) or Ezh2^{fl/fl} CD4-Cre (Ezh2 KO) mice. Lung iNKT1 (PLZF^{lo}T-bet^{hi}), iNKT2 (PLZF^{hi}T-bet^{lo}), and iNKT17 (PLZF^{lo}T-bet^{lo}) cells in 3-4 month old WT and Ezh2 KO mice were identified by intracellular staining for PLZF and T-bet **(F)**, proportions of lung iNKT1, iNKT2, iNKT17 cells **(G)**, * significantly different from WT mice

p<0.05, data expressed as mean +/- SEM. **A** and **B**, n=8 WT, 12 Ezh2 KO, representative data showing intracellular staining for NKT1, NKT2, NKT17 cells in **(C)** the spleen and **(F)** the lung. **D** and **E**, n=6 WT, 7 Ezh2 KO, **G**, n=6 WT, 6 Ezh2 KO.

Figure 3: iNKT cells from Ezh2 KO mice produce IL-4 and express genes associated with Tfh cells. Transcriptional profiling by RNA-seq on purified TCR β ⁺PBS-57-Tet⁺ iNKT cells recovered from spleens of wild type (WT) or Ezh2^{fl/fl} CD4-Cre (Ezh2 KO) mice at 2 months of age, read counts are expressed as reads per kilobase of transcript per million mapped reads (blue indicates highest expression and purple indicates lowest expression for each row/gene) and fold change (Log₂, red indicates higher expression in Ezh2 KO and green indicates lower expression in Ezh2 KO), false detection rate (FDR) and raw p values are also shown for each gene (**A** and **B**). Intracellular staining for IL-4 and IFN- γ in TCR β ⁺PBS-57-Tet⁺ iNKT cells analysed by flow cytometry after 16 hours stimulation of spleen cells with anti-TCR β *in vitro* **(C)**. iNKT cells (TCR β ⁺PBS-57-Tet⁺) and CD44^{hi}CD4⁺ T cells were purified from spleens of mice and 5 x 10⁴ cells stimulated with anti-TCR β *in vitro* for 16 hours followed by measurement of IL-4 in supernatant by ELISA **(D)**. Visualisation of iNKT location in mesenteric lymph node sections from Ezh2 KO mice by fluorescence immunohistochemistry; sections were stained with PBS-57-Tet (red), anti-TCR β (purple), and anti-CD45RA/B220 (green) **(E)**. * significantly different from WT mice p<0.05, data expressed as mean +/- SEM. **C**, representative data from 3 independent experiments **D**, n=4 WT, 4 Ezh2 KO.

Figure 4: iNKT cells in Ezh2 KO mice produce large amounts of Type-2 cytokines and drive spontaneous development of airway pathology. CD44^{hi}CD4⁺ T cells were purified from the spleen of WT, Ezh2 KO, and Ezh2/CD1d DKO **(A)** or WT, Ezh2 KO, and Ezh2/J α 18 DKO **(B)** 3-4 month old mice, and stimulated *in vitro* with anti-TCR β antibody for the indicated times followed by measurement of IL-4 and IL-13 production by ELISA. Airway hyperreactivity to methacholine expressed as airway resistance **(C)**. Total numbers of eosinophils (Eos) and neutrophils (Neu) in bronchoalveolar lavage samples from WT, Ezh2 KO, Ezh2/CD1d DKO, and Ezh2/J α 18 DKO mice **(D)**. Serum IgE was measured by ELISA **(E)**. * significantly different from WT mice p<0.05, data expressed as mean +/- SEM. **A**, n=4 WT, 4 Ezh2 KO, 4 Ezh2/CD1d DKO, **B** n=4 WT, 4 Ezh2 KO, 4 Ezh2/J α 18 DKO, **C**, n7 WT, 13 Ezh2 KO, 5 Ezh2/J α 18 DKO, 5 Ezh2/CD1d DKO, **D**, n=4 WT, 6 Ezh2 KO, 7 Ezh2/J α 18 DKO, 7 Ezh2/CD1d DKO, **E**, n=6 WT, 12 Ezh2 KO, 6 Ezh2/CD1d DKO.

Figure 5: iNKT cells in Ezh2 KO mice drive increased house dust mite-induced airway hyperreactivity. Airway hyperreactivity to methacholine (MCh) expressed as airway resistance, * Ezh2 KO MCh challenged mice significantly higher than WT and Ezh2/J α 18 DKO MCh challenged mice and all phosphate buffered saline (PBS) challenged mice p<0.05, ** WT and Ezh2/J α 18 DKO MCh challenged mice higher than PBS challenged mice, data expressed as mean +/- SEM. n=5 WT MCh challenged, 4 Ezh2 KO MCh challenged, 5 Ezh2/J α 18 DKO MCh challenged, 4 WT PBS challenged, 4 Ezh2 KO Saline challenged, 3 Ezh2/J α 18 DKO PBS challenged.

Figure 1

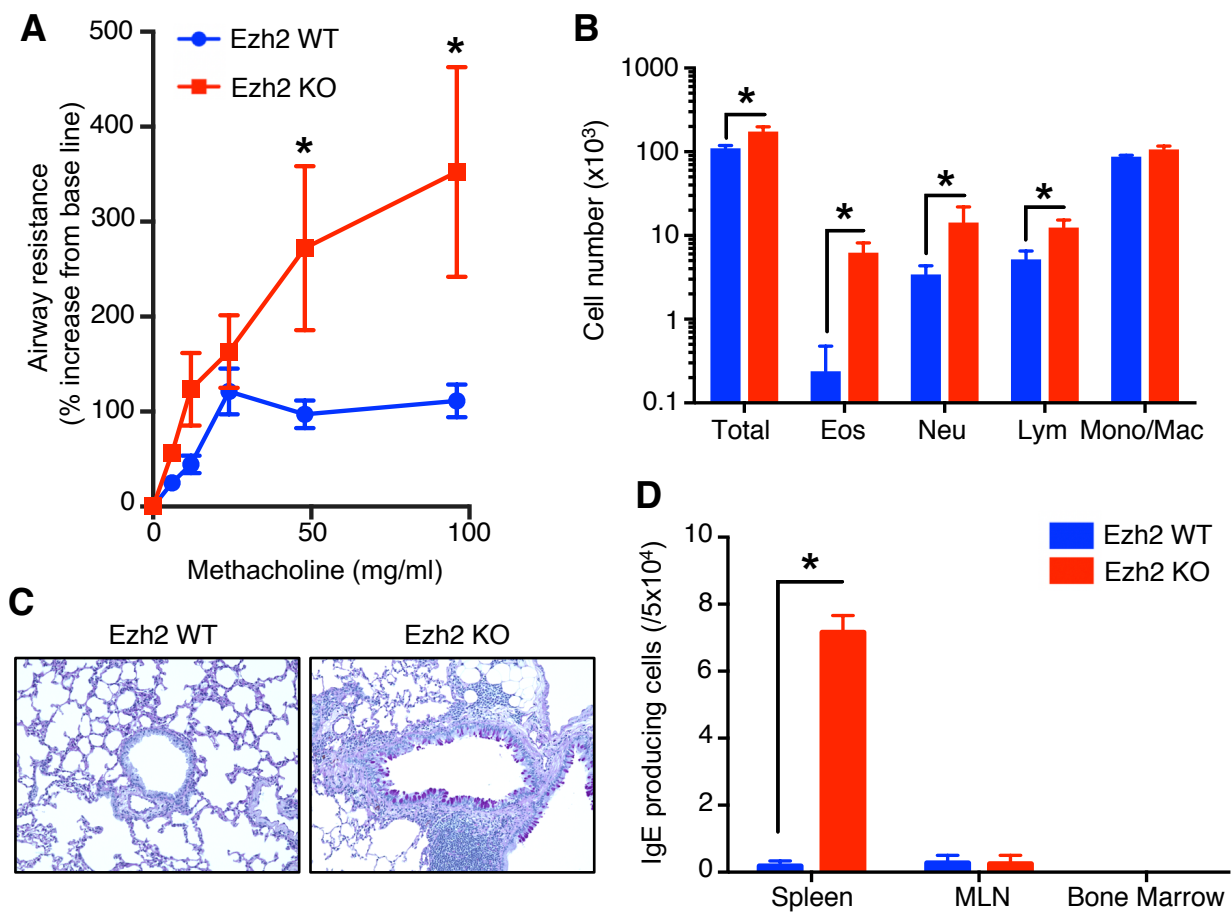


Figure 2

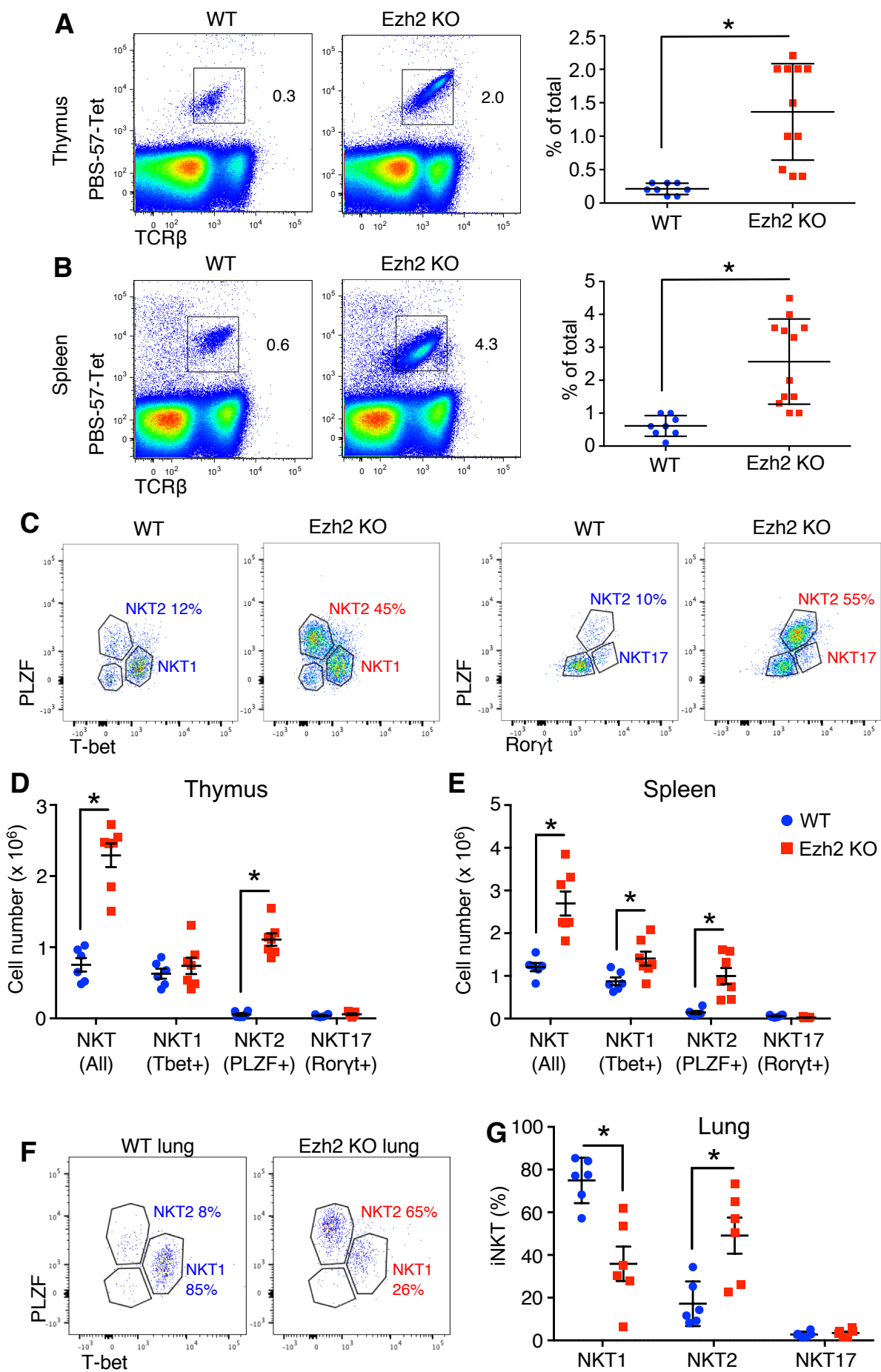


Figure 3

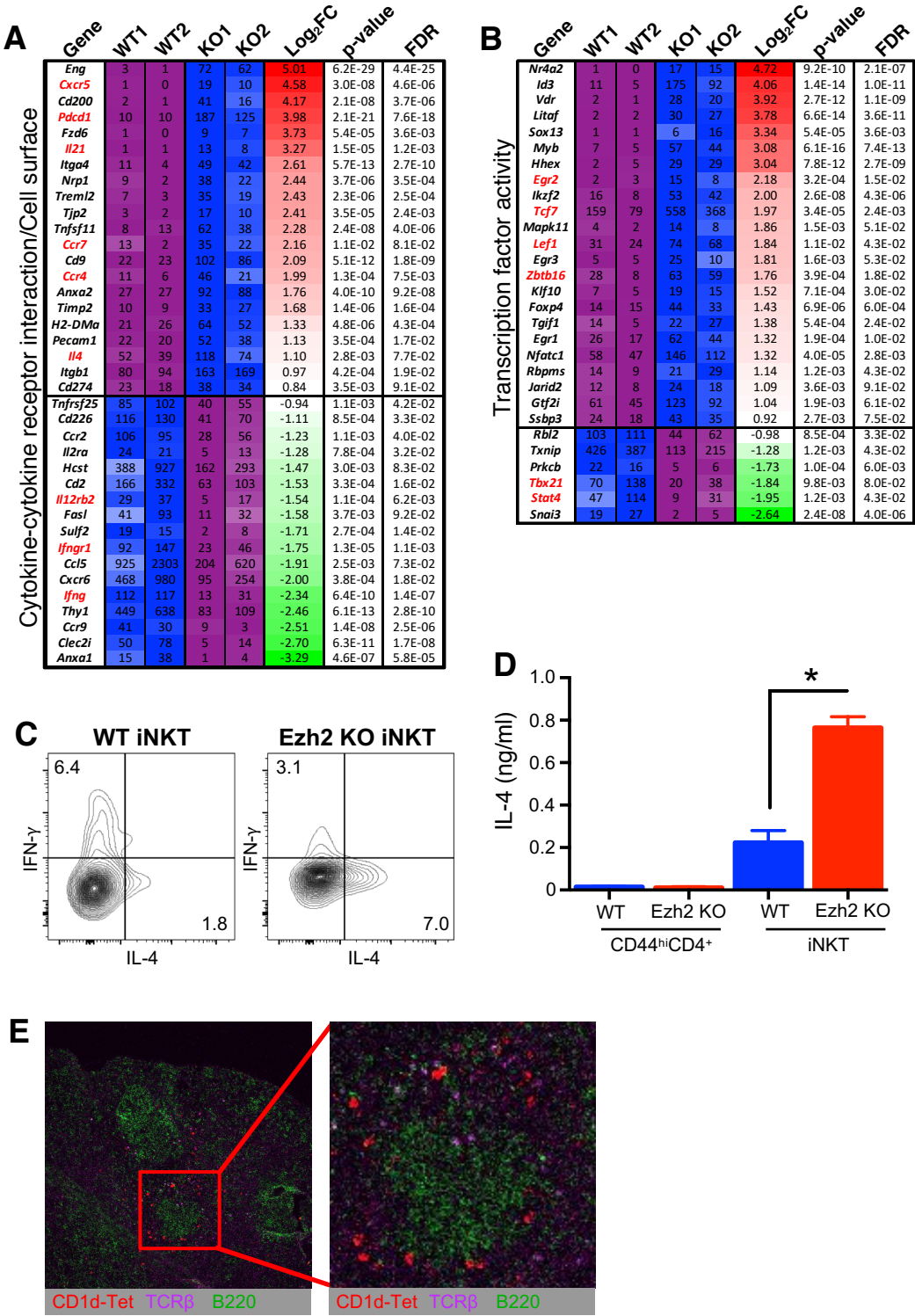


Figure 4

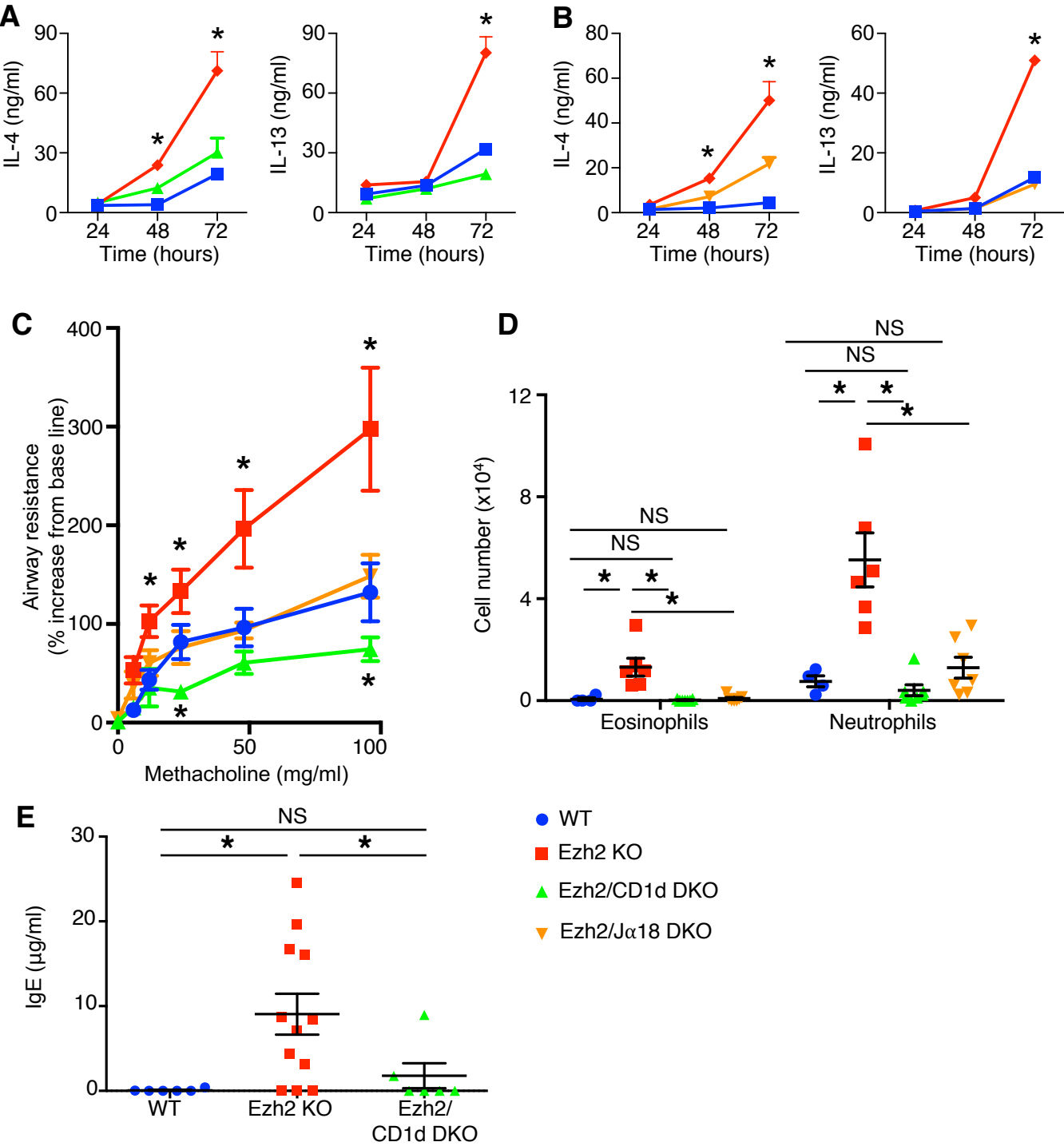
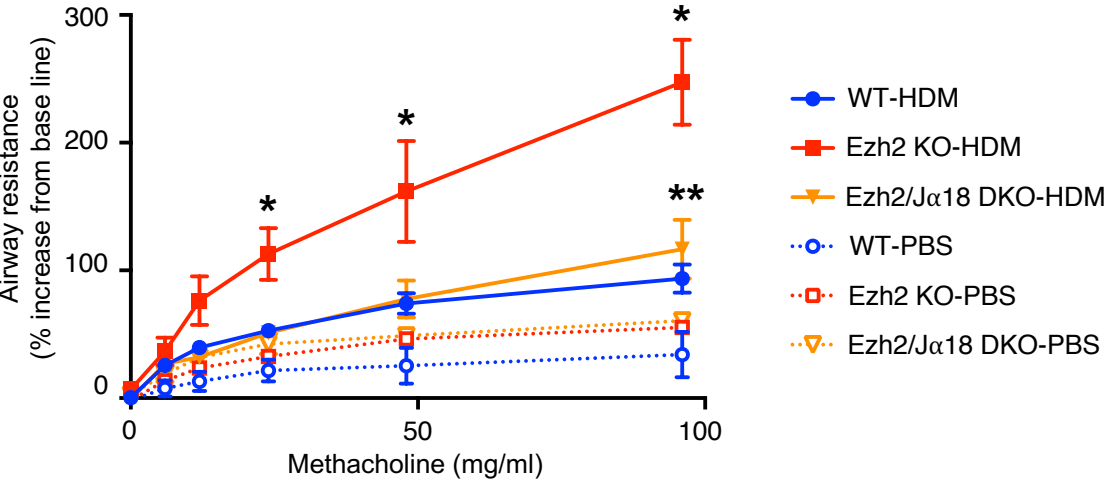


Figure 5



Supplementary Figure Legends:

Supplementary figure 1: Schematic representation of conditional inactivation of Ezh2 after crossing Ezh2^{fl/fl} mice with CD4-Cre mice. The SET domain of Ezh2 is primarily encoded by exons 18 and 19 and Loxp sites have been inserted either side of exons 18/19 in Ezh2^{fl/fl} mice. Cre-driven recombination results in deletion of exons 18/19 and inactivation of Ezh2.

Supplementary figure 2: Analysis of lymphocytes recovered by bronchiolar lavage from 3 month old WT and Ezh2 KO mice. Flow cytometry was used to determine the number of CD19⁺ B cells (**A**) and TCRβ⁺ T cells (**B**). * significantly different from WT mice p<0.05. Data expressed as mean +/- SEM, **A** and **B**, n=6 WT and 7 Ezh2 KO,

Supplementary figure 3: Flow cytometry analysis of CD44 and CD62L staining on CD4⁺ T cells from the spleen of WT and Ezh2 KO mice (**A**). RNA-seq analysis of CD44^{hi}CD4⁺ WT and CD44^{hi}CD4⁺ Ezh2 KO spleen cells purified by fluorescence activated cell sorting; data are expressed as reads per kilobase of transcript per million mapped reads (RPKM) and fold increase (Log₂) in CD44^{hi}CD4⁺ Ezh2 KO cells compared to CD44^{hi}CD4⁺ WT cells is shown (**B**).

Supplementary figure 4: *Zbtb16* expression measured in CD44^{lo}CD4⁺ T cells and CD44^{hi}CD4⁺ T cells purified from spleens of WT and Ezh2 KO mice by fluorescence activated cell sorting (**A**). Intracellular staining for PLZF (encoded by *Zbtb16*) in CD4⁺ T cells from WT (blue) and Ezh2 KO (red) mice (**B**), and staining for CD44 together with PLZF in CD4⁺ T cells from Ezh2 KO mice (**C**). Chromatin

26 Immunoprecipitation (ChIP)-seq analysis of Ezh2 binding at the *Zbtb16* gene in naïve,
 27 and differentiated Th1 and Th2 CD4⁺ T cells (**D**). **A**, * significantly different from
 28 WT mice p<0.05. Data expressed as mean +/- SEM, n=4 WT and 4 Ezh2 KO.

29

30 **Supplementary figure 5:** Proportions of iNKT cells (**A**) and total numbers of iNKT
 31 cells (**B**) in mesenteric lymph nodes (MLN), bronchial associated lymph nodes
 32 (BLN), lung tissue (Lung) and liver tissue (Liver) of WT and Ezh2 KO mice, and the
 33 total numbers of CD4⁺ (**C and D**) and CD4⁻CD8⁻ double negative (DN) (**E and F**)
 34 iNKT1, iNKT2, iNKT17 cells in the spleen (**C and E**) and thymus (**D and F**) of WT
 35 and Ezh2 KO mice. * significantly different from WT mice p<0.05, data expressed as
 36 mean +/- SEM. **A and B**, n= 4 WT, 5 Ezh2 KO mice, **C to F** n=6 WT, 7 Ezh2 KO
 37 mice.

38

39 **Supplementary figure 6:** Total numbers of NKT-like cells identified as TCRβ⁺PBS-
 40 57-Tet⁻PLZF⁺ cells in spleen and thymus of WT and Ezh2 KO mice. Proportions of
 41 iNKT and NKT-like cells in the thymus (**B**) and spleen (**C**) of WT, Ezh2 KO, and
 42 Ezh2/CD1d double KO mice shown, data are representative of 3 independent
 43 experiments. **A**, * significantly different from WT mice p<0.05, data expressed as
 44 mean +/- SEM. n= 6 WT, 7 Ezh2 KO mice.

45

46 **Supplementary figure 7:** Fluorescence activated cell sorting (FACS) of iNKT cells
 47 for RNA-seq shown in Figure 3A and B, upper panels show CD3ε⁺ enriched spleen
 48 cells and lower panels show purified iNKT cells (**A**). Cells were gated on TCRβ⁺
 49 PBS-57-Tet⁺ cells for FACS. Visualisation of iNKT location in spleen sections from

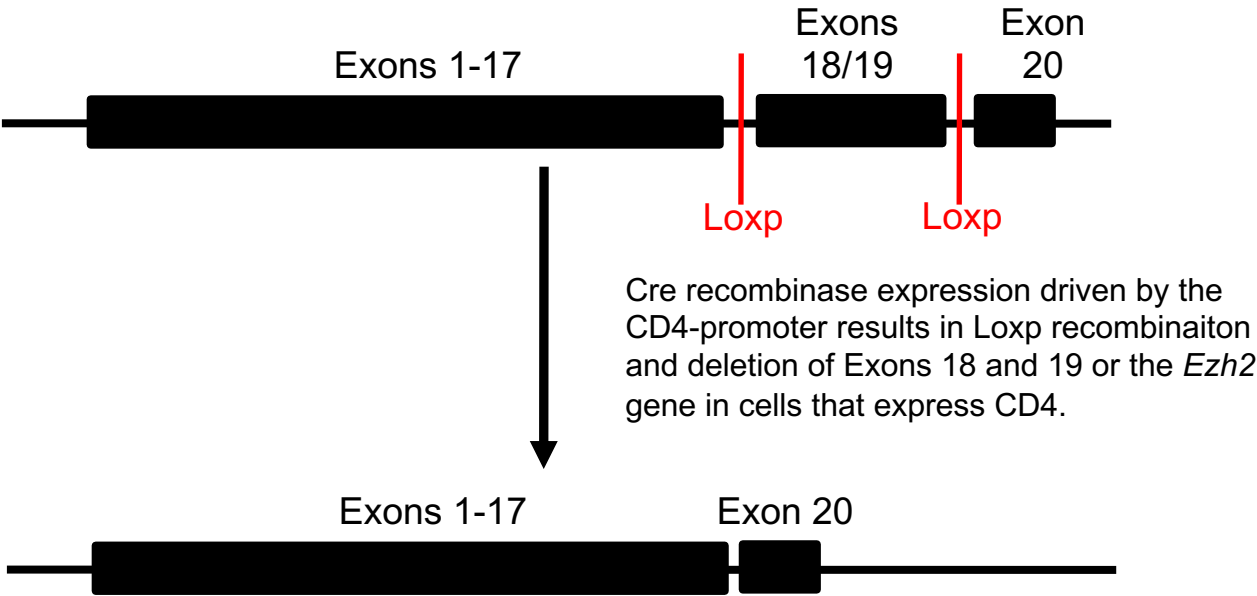
Ezh2 KO mice by fluorescence immunohistochemistry; sections were stained with PBS-57-Tet (red), anti-TCR β (blue), and anti-CD45RA/B220 (green) **(B)**.

Supplementary figure 8: Serum IgG1 **(A)**, IgM **(B)** IgG2c **(C)**, IgA **(D)**, and from 3 month old WT and Ezh2 KO mice. Serum immunoglobulins were measured by ELISA. * significantly different from WT mice $p < 0.05$. Data expressed as mean $\pm$ SEM. **A, B, D**, $n = 7$ WT and 13 Ezh2 KO. **C**, $n = 6$ WT and 13 Ezh2 KO

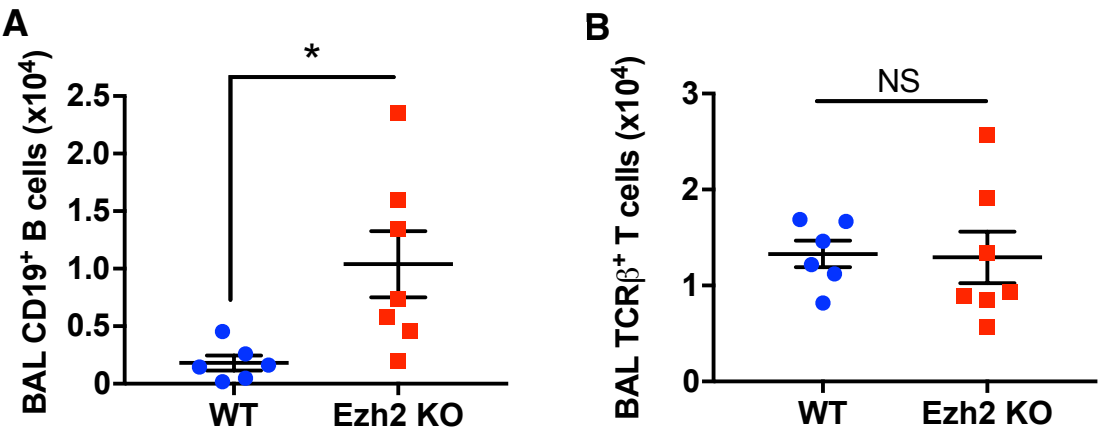
Supplementary figure 9: CD44^{hi}CD4⁺ T cells were purified from the spleen of 3-4 month old WT, Ezh2 KO, and Ezh2/CD1d DKO **(A and B)** or WT, Ezh2 KO, and Ezh2/J α 18 DKO **(C and D)** mice, and stimulated *in vitro* with anti-TCR β antibody for the indicated times followed by measurement of IFN- γ **(A and C)** and IL-17 **(B and D)** production by ELISA. CD44^{hi}CD4⁺PBS-57-Tet⁻ conventional T cells were purified from the spleen of 6-7 month old WT, Ezh2 KO, and Ezh2/J α 18 DKO **(E and F)** mice, and stimulated *in vitro* with anti-TCR β antibody for 72 hours followed by measurement of IL-4 and IL-13 production by ELISA. * significantly lower than WT and Ezh2 KO, ** Significantly higher than Ezh2 KO or Ezh2/CD1d DKO and Ezh2/J α 18 DKO, *** significantly lower than Ezh2 KO $p < 0.05$, data expressed as mean $\pm$ SEM. **A and B**, $n = 4$ WT, 4 Ezh2 KO, 4 Ezh2/CD1d DKO, **C and D**, $n = 4$ WT, 4 Ezh2 KO, 4 Ezh2/J α 18 DKO, **E and F**, $n = 4$ WT, 4 Ezh2 KO, 4 Ezh2/J α 18 DKO.

Supplementary figure 10: Serum Derp1-specific IgG1 was measured by ELISA in serum after intranasal challenge of mice with either PBS or house dust mite. * $p < 0.05$, data expressed as mean $\pm$ SEM. $n = 5$ WT, 4 Ezh2 KO, 5 Ezh2/J α 18 DKO.

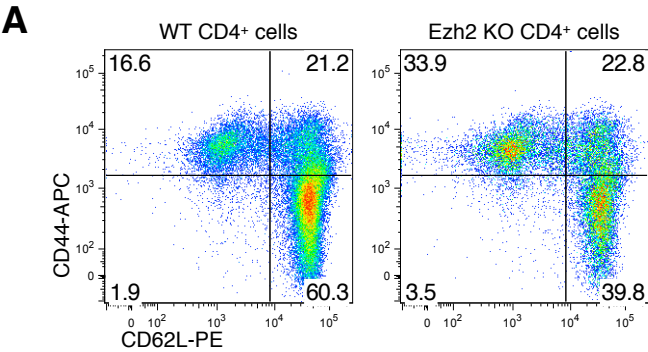
Supplementary Figure 1



Supplementary Figure 2



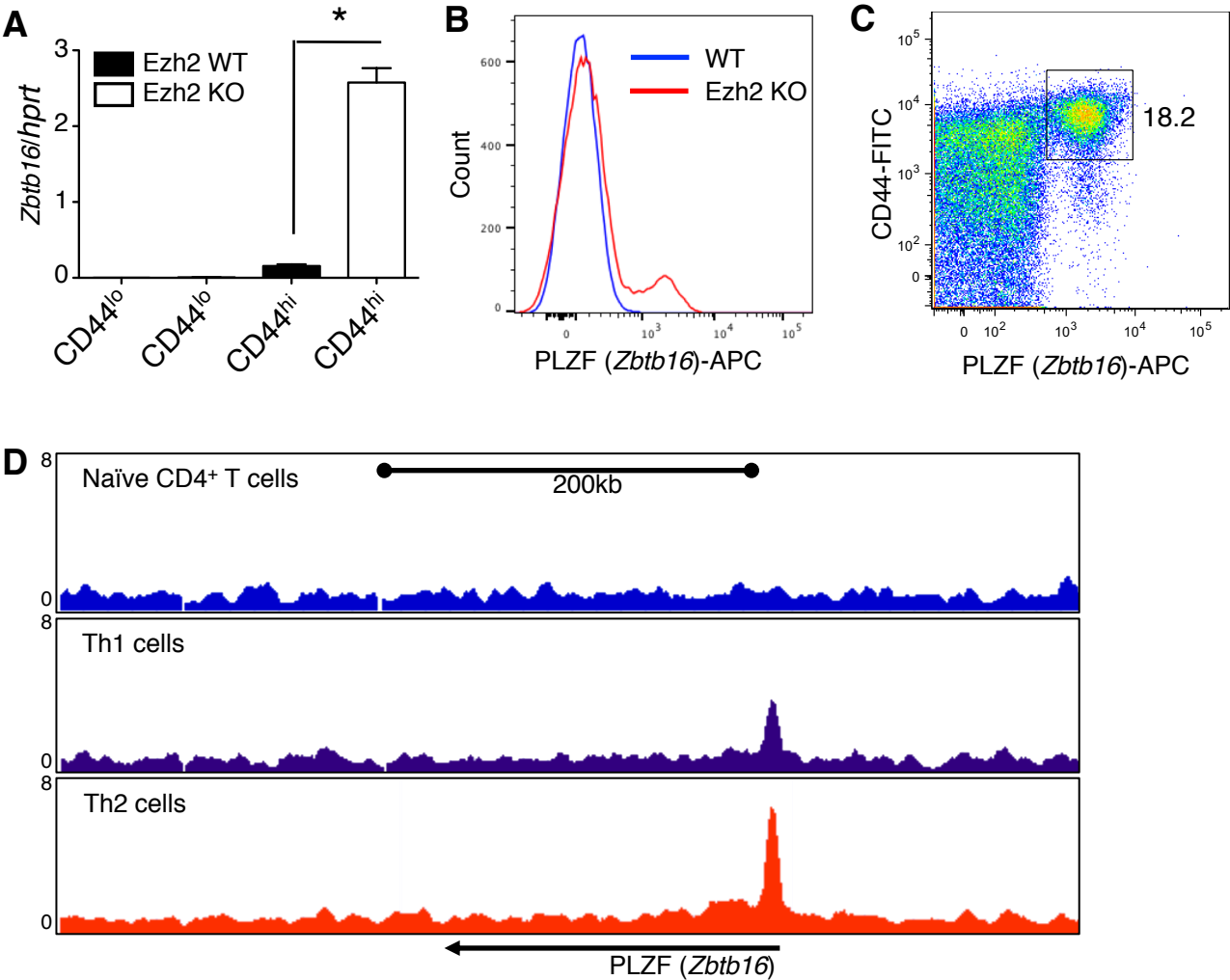
Supplementary Figure 3



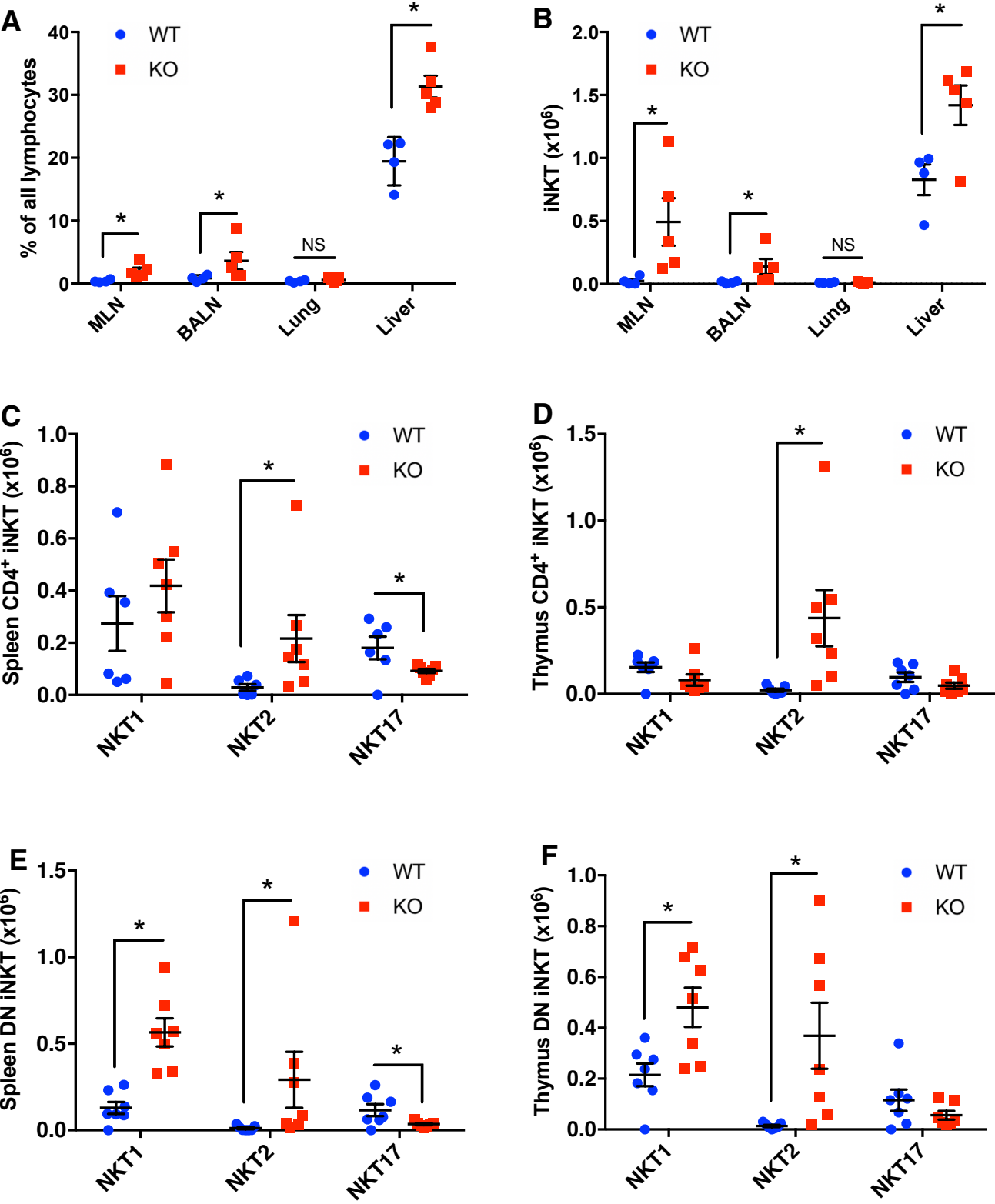
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Gene	WT CD44 ^{hi}	Ezh2 KO CD44 ^{hi}	Log ₂ FC
<i>Klra23</i>	0.0	12.2	22.5
Hba-a2	0.0	19.8	14.0
Eng	0.6	38.9	6.1
Sox13	0.1	6.3	6.1
Arhgap24	0.2	5.5	4.7
Epha3	0.4	7.4	4.4
<i>Klra3</i>	0.9	14.3	3.9
<i>Klra1</i>	0.6	8.2	3.7
Il17rb	7.5	80.9	3.4
<i>Zbtb16</i>	4.3	41.4	3.3
Atp6v0d2	3.2	30.4	3.3
Chdh	3.2	27.6	3.1
Gpnmb	1.3	10.3	3.0
Cox6a2	7.8	55.7	2.8
Fam174b	1.3	8.5	2.7
Ctsf	1.0	6.2	2.6
Spry2	1.2	6.4	2.4
<i>Klrc3</i>	1.6	8.4	2.4
Adh1	1.9	9.6	2.4
Rbpms	2.1	10.9	2.3
Hbb-b1	33.4	169.2	2.3
Ly6g5b	4.1	19.5	2.2
Stc2	1.5	6.8	2.2
Irf8	22.9	104.9	2.2
Raver2	1.5	6.8	2.2
Qpct	2.5	11.5	2.2
Hba-a1	54.5	241.8	2.2
Rnf32	1.3	5.8	2.1
Mpeg1	18.2	75.8	2.1
<i>Cd160</i>	13.2	54.5	2.0
Aqp9	3.2	13.2	2.0
<i>Klrb1c</i>	14.9	58.5	2.0
Cxxc5	5.2	19.8	1.9
<i>Klrc1</i>	13.8	49.8	1.8

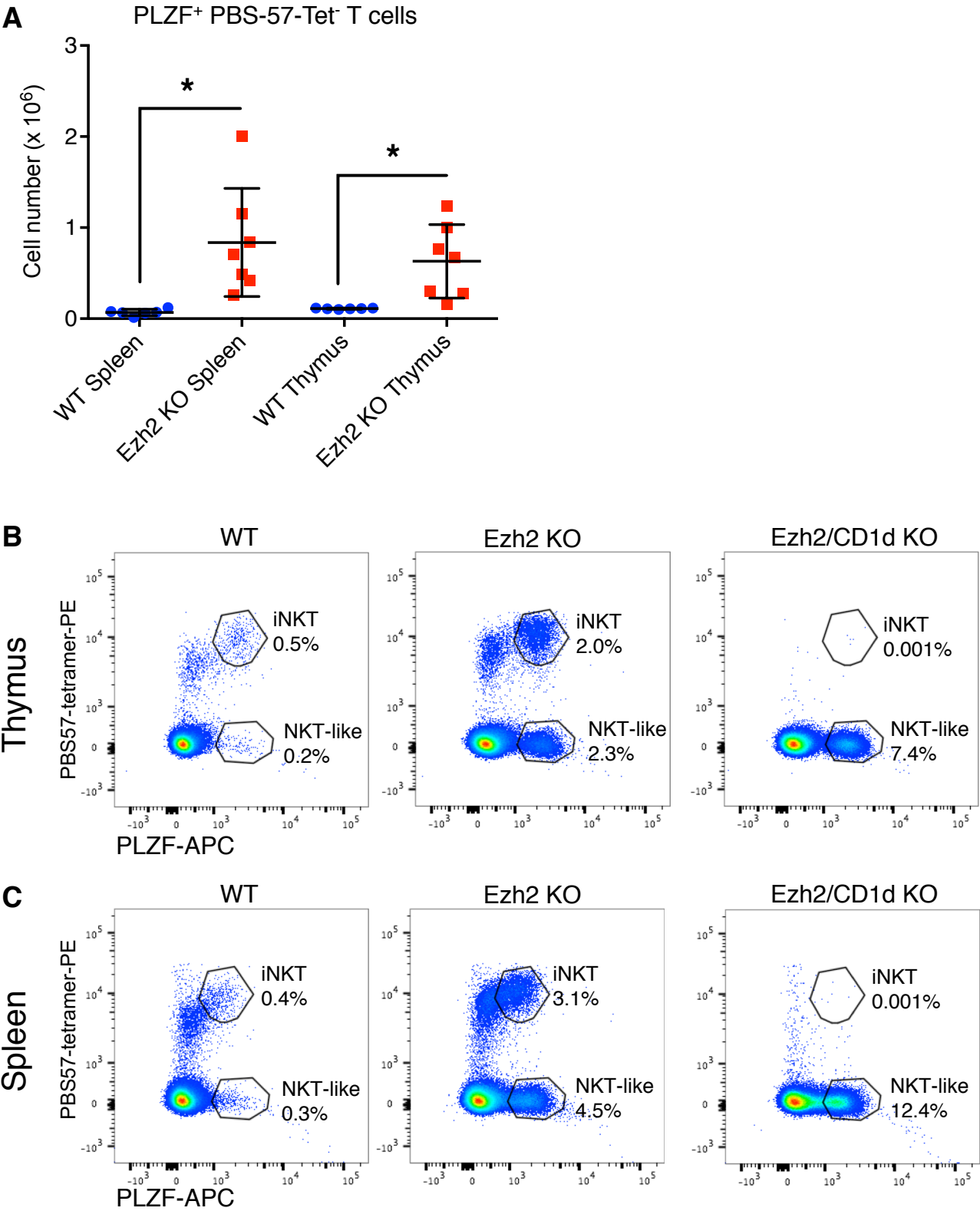
Supplementary Figure 4



Supplementary Figure 5

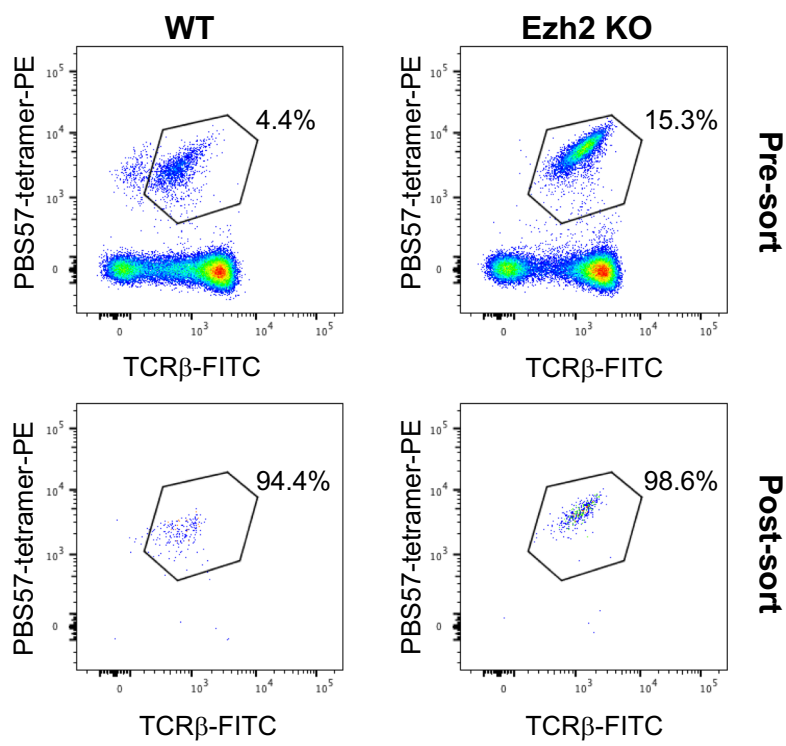


Supplementary Figure 6

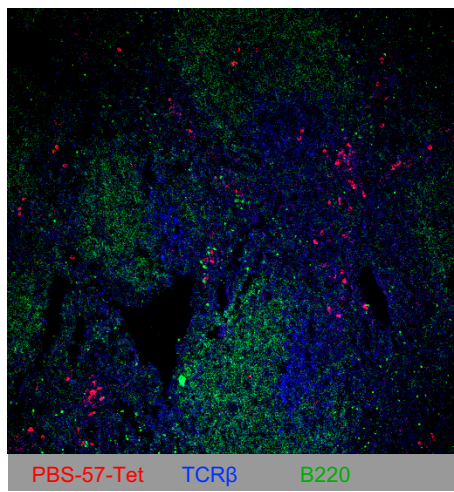


Supplementary Figure 7

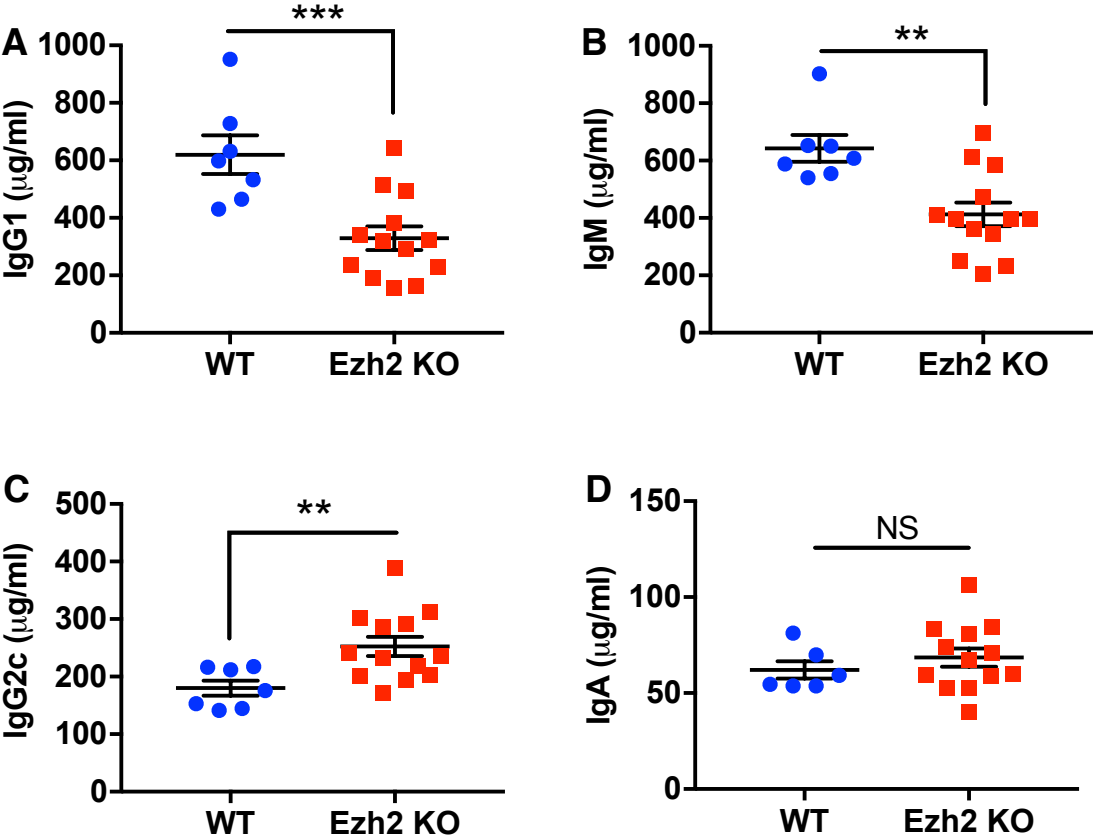
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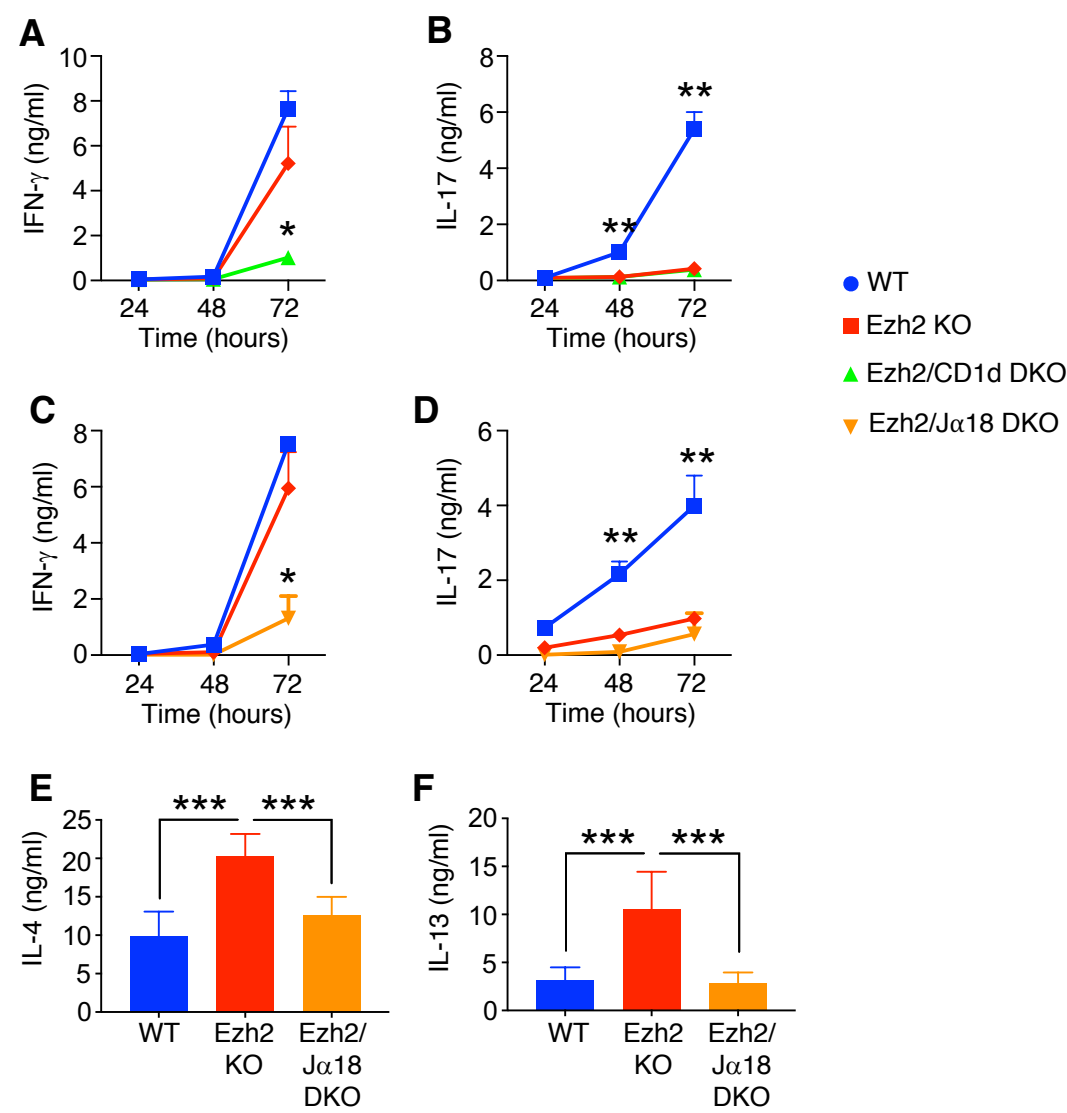
B



Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10

