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E-Protein Inhibition in ILC2 Development Shapes the Function of Mature ILC2s during Allergic Airway Inflammation

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E-protein transcription factors limit group 2 innate lymphoid cell (ILC2) development while promoting T cell differentiation from common lymphoid progenitors. Inhibitors of DNA binding (ID) proteins block E-protein DNA binding in common lymphoid progenitors to allow ILC2 development. However, whether E-proteins influence ILC2 function upon maturity and activation remains unclear. Mice that overexpress ID1 under control of the thymus-restricted proximal *Lck* promoter (ID1^{tg/WT}) have a large pool of primarily thymus-derived ILC2s in the periphery that develop in the absence of E-protein activity. We used these mice to investigate how the absence of E-protein activity affects ILC2 function and the genomic landscape in response to house dust mite (HDM) allergens. ID1^{tg/WT} mice had increased KLRG1⁺ ILC2s in the lung compared with wild-type (WT; ID1^{WT/WT}) mice in response to HDM, but ID1^{tg/WT} ILC2s had an impaired capacity to produce type 2 cytokines. Analysis of WT ILC2 accessible chromatin suggested that AP-1 and C/EBP transcription factors but not E-proteins were associated with ILC2 inflammatory gene programs. Instead, E-protein binding sites were enriched at functional genes in ILC2s during development that were later dynamically regulated in allergic lung inflammation, including genes that control ILC2 response to cytokines and interactions with T cells. Finally, ILC2s from ID1^{tg/WT} compared with WT mice had fewer regions of open chromatin near functional genes that were enriched for AP-1 factor binding sites following HDM treatment. These data show that E-proteins shape the chromatin landscape during ILC2 development to dictate the functional capacity of mature ILC2s during allergic inflammation in the lung. *The Journal of Immunology*, 2022, 208: 1–14.

Group 2 innate lymphoid cells (ILC2s) are sentinel cells that are enriched at barrier sites (1, 2) and belong to the lymphoid lineage but lack Ag-specific receptors (3). ILC2s respond to factors that signal damage by pathogens or allergens, including allergens in house dust mite (HDM) extracts (3, 4). IL-25 and IL-33 produced by epithelial and stromal cells (5–7) activate ILC2s, which express receptors for these factors in tissue-specific patterns (3). The lung ILC2 population in mice is heterogeneous, with ILC2 subsets delineated by surface marker expression and function (8). Lung-resident ILC2s express the IL-33 receptor subunit ST2 (8). Following IL-25 or IL-33 treatment or infection with the rodent helminth *Nippostrongylus brasiliensis*, KLRG1^{hi} inflammatory ILC2s

also infiltrate the lung (8, 9), are potent producers of IL-5 and IL-13, and are functionally plastic (8, 10). KLRG1 expression is not unique to infiltrating inflammatory ILC2s, as lung-resident ILC2s can also upregulate KLRG1 in response to IL-33 during inflammation (9, 10). The exact function of KLRG1 on ILC2s is not clear, though it may be inhibitory (11).

Previous genomic analyses of lung ILC2s during *N. brasiliensis* infection demonstrate that ILC2s maintain open chromatin surrounding functional genes in the absence of inflammation, allowing for rapid transcription of genes, including *Il5* and *Il13*, in response to insult (12). This adaptation, which is not seen in naive CD4⁺ T cells, likely facilitates rapid ILC2 responses in tissues (12). However, how

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out experimental work. J.J.L. and C.G.D. assisted in ATAC-seq analysis. X.-H.S. provided ID1^{tg/WT} mice and provided input on data interpretation. J.v.M. and C.G.D. supervised some of the research. L.M.W. and E.D.T.W. designed the project and supervised the research. E.D.T.W. conceived the project.

The sequencing data presented in this article have been submitted to the Gene Expression Omnibus under accession number GSE185950 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185950>).

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Abbreviations used in this article: AP-1, activator protein 1; ATAC-seq, assay for transposase-accessible chromatin sequencing; BM, bone marrow; C/EBP, CCAAT-enhancer-binding protein; CLP, common lymphoid progenitor; ETP, early T cell precursor; GO, gene ontology; HDM, house dust mite; ID, inhibitor of DNA binding; i.n., intranasal; ILC2, group 2 innate lymphoid cell; ILC2p, group 2 innate lymphoid cell progenitor; lin, lineage; MHC II, MHC class II; PCA, principal component analysis; scRNA-seq, single-cell RNA-sequencing; TF, transcription factor; Treg, regulatory T cell; WT, wild-type.

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chromatin accessibility and transcription factor (TF) families coordinate to regulate ILC2 gene expression in the lung in response to airway allergens has not been fully defined.

The E-protein TFs E12/E47 (also known as E2a) and Heb, encoded by *Tcf3* and *Tcf12* genes, respectively, drive T cell development in the thymus from lineage-committed lymphoid precursors (13–15). Inhibitor of DNA binding (ID) proteins, specifically ID2, drive ILC development from common lymphoid progenitors (CLPs) by binding to and inhibiting E-protein function (16). ID2 deletion inhibits the development of ILC precursors that differentiate into ILC1–3 (16), whereas potentiation of E-protein function in CLPs most significantly impacts the development of ILC2s compared with other ILC subsets (17), highlighting that E-protein inhibition is a prerequisite for effective ILC2 development. E-proteins are also active in mature lymphocytes, promoting naive B cell activation and class switching (18) and inhibiting regulatory gene expression in FOXP3⁺ regulatory T cells (Tregs), the latter function being reversible with ID protein overexpression (19). E- and ID proteins thus function antagonistically (20), with E-protein knockout or forced ID1/2 overexpression in thymic lymphoid progenitors causing early T cell precursors (ETPs) to turn off T cell maturation and instead promote ILC2 development at this site. This leads to increased ILC2s in the periphery, particularly the lung, where these thymus-derived ILC2s displayed decreased KLRG1 expression (21), yet still promoted acute airway type 2 inflammation driven by papain or *N. brasiliensis* infection (21–23). Together, these data suggest that the E–ID protein axis may regulate ILC2 functional capacity in the lung in addition to its role in ILC2 development. However, it is not yet known whether E proteins are active in mature ILC2s and how E-proteins shape ILC2 function remains unclear.

To clarify the role of E-proteins in shaping ILC2 function, we used mice that overexpress ID1 under control of the thymus-restricted proximal *Lck* promoter (ID1^{tg/WT}), which have a large pool of primarily thymus-derived ILC2s in the periphery that develop in the absence of E-protein activity (24). Using these mice allowed us to investigate how the absence of E-protein activity affects ILC2 phenotype, function, and genomic landscape in response to HDM allergens. Following HDM treatment, ID1^{tg/WT} mice had increased KLRG1⁺ ILC2s in the lung with diminished capacity to produce type 2 cytokines, suggesting a role for E-proteins in regulating ILC2 function following HDM treatment. However, analysis of wild-type (WT) ILC2 accessible chromatin showed that putative E-protein binding motifs were scarce at HDM-driven gained accessible chromatin near inflammation-responsive genes (those that display increased or decreased expression following HDM treatment), and instead, activator protein 1 (AP-1) and CCAAT-enhancer-binding protein (C/EBP) motifs were strongly associated with ILC2 inflammatory gene programs. In contrast, E-protein binding sites were enriched at such functional genes in mature lung ILC2s prior to inflammation, indicating that E-proteins could act during development to prime future functional capacity of mature ILC2s that is governed by other TFs. Consistent with this idea, analysis of accessible chromatin in ID1^{WT/WT} and ID1^{tg/WT} ILC2s responding to HDM revealed that AP-1–driven regulation of the ILC2 inflammatory response was largely depleted in ID1^{tg/WT} mice, whereas E-proteins appeared to play a minimal role. These findings highlighted that E-proteins shape ILC2 functional capacity by acting primarily during the development of mature lung ILC2s from precursors. This functional programming was associated with a lung ILC2 chromatin landscape in which AP-1 TFs control the expression of effector genes during type 2 inflammation. Thus, E-proteins set the functional capacity of ILC2s during the developmental process, suggesting that genomic regulation during ILC2 development governs their future functional capacity during allergic airway inflammation.

Materials and Methods

Mice and treatments

ID1^{tg/tg} and ID1^{tg/WT} mice were as described (25). For ID1^{tg/WT} mice, ID1^{tg/tg} mice were crossed with C57BL/6 WT mice (The Jackson Laboratory, Bar Harbor, ME) and bred in-house. Cohoused experimental controls were ID1^{WT/WT} male or female littermates in specific pathogen-free conditions at Cornell University or the University of Washington, in accordance with protocols approved by the respective Institutional Animal Care and Use Committees. For induction of HDM-driven allergic airway inflammation, animals were administered 80 µg HDM (Stallergenes Greer) in 40 µl PBS or PBS alone intranasally (i.n.), every 7 d for 3 wk. Tissues were collected 3 d after the final treatment.

Tissue preparations

Lungs were perfused with PBS through the heart. The middle, caudal, and accessory lobes of the lungs were collected for flow cytometry, and the cranial left and right lobes were collected for ELISA, RNA isolation, and histology. For flow sorting, the entire lung was collected for tissue digestion. For flow cytometry and cell sorting, lungs were digested with 20 µg/ml DNase (Roche) and 2 mg/ml collagenase D (Roche) or 1 U/ml Liberase TL (Roche) at 37°C. Single-cell suspensions were mashed through a 40- (lung) or 70-µm (thymus) strainer. Lungs were RBC lysed with ACK lysis buffer (Lonza).

Flow cytometry and cell sorting

Single-cell suspensions were incubated with Aqua Live/Dead Fixable Dye (Thermo Fisher Scientific) and then stained with fluorochrome-conjugated mAbs against mouse CD4 (RM4-5), CD11b (M1/70), CD11c (N418), CD19 (eBio1D3), CD25 (PC61.5), CD44 (1M7), CD45 (30-F11), CD62L (MEL-14), CD64 (X54-5/7.1), CD127 (A7R34), CD90.2 (53-2.1 or 30-H12), c-KIT (2B8), I-A/I-E MHC class II (MHC II) (M5/114.15.2), KLRG1 (2F1), Ly6C (HK1.4), Ly6G (1A8-Ly6g), MERTK (DS5MMER), NK1.1 (PK136), SiglecF (E50-2440), ST2 (RMST2-2), TCRβ (H57-597), and/or TCRγδ (eBioGL3) (from Thermo Fisher Scientific, BD Biosciences, or BioLegend). Surface staining was performed with 10% rat serum (Jackson ImmunoResearch Laboratories) and 1 µg/ml FcR block (anti-CD16/32, 2.4G2; BioXCell). For intranuclear staining, cells were fixed and permeabilized with eBioscience fixation/permeabilization buffer/permeabilization buffer (Thermo Fisher Scientific) and then stained for FOXP3 (FJK-16s) and GATA3 (TWAJ) (Thermo Fisher Scientific). For intracellular staining, cells were incubated for 3 h with 10 µg/ml brefeldin A (Sigma-Aldrich) without any other stimulation. Cells were fixed in 2% paraformaldehyde, permeabilized using BD Perm/Wash buffer (BD Biosciences), and stained with mAbs against GM-CSF (MP1-22E9), IL-13 (eBio13A), and IL-5 (TRFK5). Samples were run on a four- or five-laser LSR II (BD Biosciences), a four-laser Attune NxT (Thermo Fisher Scientific) flow cytometer, or a five-laser Symphony A3 (BD Biosciences), and FlowJo 10 (Tree Star) was used to analyze data. Gates were set using fluorescence minus one or isotype controls. For ILC2, CD4⁺ T cell, and T cell precursor purification, single-cell suspensions were sorted using a four-laser FACS Fusion (BD Biosciences) (WT ILC2 and CD4⁺ T cell assay for transposase-accessible chromatin sequencing [ATAC-seq]) or a four-laser and three-laser FACSaria (BD Biosciences) (ID1^{WT/WT} versus ID1^{tg/WT} ILC2 ATAC-seq and assessment of ID1 transgene expression in precursors and lung immune cells) with an 85-µm nozzle. All leukocyte populations were gated as singlet, live (Live/Dead Aqua⁺) CD45⁺ leukocytes (by forward light scatter area/side scatter area), ILC2s were gated as lineage (lin) (CD3/CD5orTCRβ/CD11b/CD11c/CD19/NK1.1/TCRγδ)[−]CD90.2^{+/−}CD127⁺KLRG1^{+/−}ST2^{+/−}, and CD4⁺ T cells were gated as TCRβ⁺, lin (CD11b/CD11c/CD19/NK1.1/TCRγδ)[−]CD4⁺, further subdivided as FOXP3⁺ Tregs, FOXP3[−]CD44^{hi}CD62L^{lo} activated, and FOXP3[−]GATA3⁺, or ST2⁺ Th2 cells. For assessment of ID1 transgene expression in precursors and lung immune cells, thymic T cell progenitors were gated as lin (CD3/CD5/CD11c/CD19/NK1.1/F4/80/TCRγδ)[−]ST2[−]c-KIT^{+/−}CD25^{+/−} cells to include both DN1 and DN3 T cell progenitors (23) and monocytes in the lung as lin[−]Ly6C⁺CD11b⁺.

ELISA and real-time PCR

Cytokine levels in tissue lysates were assessed using standard sandwich ELISA for IL-4, IL-5, IL-13, and IL-33 (Thermo Fisher Scientific or R&D Systems). For real-time PCR, RNA was isolated from lung lysates using TRIzol extraction (Invitrogen). Real-time PCR was performed on cDNA generated using a Superscript II reverse transcription kit (Life Technologies) with SYBR Green Master Mix (Applied Biosystems) and Qiagen QuantiTect primer sets and run on the ABI 7500 or the Viia 7 real-time PCR system (Thermo Fisher Scientific). The ID1 transgene was identified in thymocytes and lung immune cells using the following primers: ID1 #18 (5′-CAG TGG

CAG TGC CGC AGC CGC TGC AGG-3') and 3hGH2 (5'-CCA CAG GAC TCC GAG TGG TTC G-3').

Histology

At necropsy, a lung section was fixed in Methacarnoy's solution (60% absolute methanol, 30% chloroform, and 10% acetic acid) and stored in 100% ethanol before paraffin embedding, and 5- μ m sections were stained with periodic acid–Schiff/Alcian blue. Image acquisition was performed using an Axio Observer A1 inverted microscope (Zeiss). Samples were analyzed and scored for pathology based on perivascular and bronchoalveolar immune cell infiltrate, epithelial cell hyperplasia, and goblet cell numbers. A researcher blinded to sample and group identity performed the enumerations at $\times 20$ objective, with representative images at $\times 10$ original magnification.

Single-cell RNA-sequencing analysis from published data

To investigate the ILC2 transcriptome, analysis was performed on published single-cell RNA-sequencing (scRNA-seq) data of murine ILC2s from different tissue sites (accession code GSE117568) (26). Analysis of published scRNA-seq data was also used to delineate the impact of HDM treatment on the transcriptome of lung ILC2s (accession code GSE102299) (27). Mice were sensitized with HDM on day 0 and challenged a week later before sacrifice on day 10, along with PBS-treated controls. Cells were prepared for sorting and purified for scRNA-seq analysis as previously described (26, 27). Counts underwent quality control in Seurat (v3.2.1) and were normalized using LogNormalize, linear transformed, and assessed by principal component analysis (PCA). Cells were clustered via Seurat's RunUMAP function with the dim parameter set to the selected number of principal components. For functional pathway analysis of bulked scRNA-seq data, g:Profiler-identified biological process gene ontology (GO) terms associated with genes upregulated in lung ILC2s from HDM-treated mice compared with lung ILC2s from PBS-treated mice were clustered in Cytoscape v3.8 using EnrichmentMap.

ATAC-seq library preparation and sequencing

Following flow sorting, ILC2s or CD4⁺ T cells were resuspended in cold lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, and 0.1% Nonidet P-40 Lysis Buffer [IGEPAL] in H₂O) with 1 $\times$ protease and 2 U/ml SUPERase-In RNase (Thermo Fisher Scientific) inhibitors. Permeabilized cells were resuspended in storage buffer (50 mM Tris-HCl [pH 8.3], 5 mM MgCl₂, 40% glycerol, 0.1 mM EDTA [pH 8], plus 40 U RNase inhibitor in H₂O) and stored at -80°C . Permeabilized cells were washed in PBS and then incubated in Transposition Mix (Nextera DNA Sample Preparation kit; Illumina), as previously described (28). Following transposition, WT CD4⁺ T cells and ILC2 samples from PBS- or HDM-treated mice were purified using the Monarch Gel Extraction Kit (New England Biolabs). Libraries were amplified using Nextera PCR oligos (Illumina) and Next High-Fidelity PCR Master Mix (New England Biolabs) for 12–14 cycles, cleaned up using the Monarch Gel Extraction kit and AMPure beads (Beckman Coulter), and sequenced with 2 $\times$ 41 nt paired-end reads on the NextSeq500 (Illumina). For ID1^{WT/WT} and ID1^{tg/WT} ILC2 analysis from HDM-treated mice, following transposition, samples were purified using the Qiagen MinElute PCR Purification Kit. All libraries were amplified using PCR oligos as described previously (29) and NEBNext High-Fidelity PCR Master Mix (New England Biolabs) for 11 cycles. Libraries were purified using the Qiagen MinElute PCR Purification Kit and treated with the Free Adapter Blocking Reagent (Illumina) with 1.8 $\times$ Agencourt AMPure XP bead size selection (Beckman Coulter). Sequencing was performed with a target of 50 M reads/library using 59 base paired-end reads on a P2 flowcell on the NextSeq2000 (Illumina). Data are publicly available on the Gene Expression Omnibus under accession number GSE185950.

ATAC-seq mapping and analysis

ATAC raw data were mapped to the *Mus musculus* 10 (mm10) genome using the Burrows-Wheeler Alignment-mem algorithm (30), and peaks were called using MACS2 (Ref. 31 and J.M. Gaspar, manuscript posted on bioRxiv, DOI: 10.1101.496521). For PBS- versus HDM-treated WT ILC2 ATAC-seq, libraries from KLRG1⁺ST2⁺, KLRG1⁺, and ST2⁺ ILC2s were made separately and combined for the purpose of comparison with other WT cell populations. For ID1^{WT/WT} and ID1^{tg/WT} ILC2 ATAC-seq libraries, all lung ILC2s were collected together, regardless of subset (to maximize cell yield). Peaks were assigned to genes based on proximity to the nearest promoter as provided by Gencode M25. As changes in accessibility of both promoters and distal enhancers are expected to affect target gene expression, we did not set a maximal distance cutoff for the nearest gene assignments. The distribution of ATAC peak-promoter distances in our data was well within the median distance between mouse topological-associated domain boundaries (880 kb) (32), with 90% of the ATAC peaks falling within 50 kb

and 99% falling within 200 kb of the nearest promoter. TF binding motifs at ATAC peaks were called using MotifScan (33). The fraction of ATAC peaks containing TF motifs identified by the JASPAR binding motif database (34) was calculated across all genes or at peaks of interest, and the z-score was calculated across cell types/treatments. For TF-specific GO enrichment analysis of the WT mice ILC2 HDM-responsive peaks, genes nearby ATAC peaks containing TF motifs were mapped to GO biological processes using PANTHER (35) (Fig. 5D, Supplemental Fig. 3B), and pathway fold enrichment was calculated by assessing the likelihood that the biological process would be picked up randomly. This was plotted against specific enrichment for the biological process, measured as the proportion of the biological process-mapped genes out of all genes with inflammation-gained putative TF binding relative to the overall incidence of genes mapped to that biological process. For GO analysis of genes nearby ATAC peaks that were ID1^{WT/WT}-specific and ID1^{tg/WT}-specific, we used the clusterProfiler R package by running the enrichGO function with the following parameters: OrgDb = "org.Mm.eg.db"; ont = "BP"; pAdjustMethod = "BH"; pvalueCutoff = 0.01; and qvalueCutoff = 0.05. Plotting of the results was done by running the dotplot function on the enrichGO output, showing the 10 most enriched biological processes in each set. For comparison with *Pde4b* ATAC-seq signal, the chromatin immunoprecipitation sequencing H3K27ac signal was from published data of small intestine ILC2s and LN CD4⁺ T cells (36, 37). Published ATAC-seq data from CLPs and ILC2 progenitors (ILC2ps) were used in Fig. 5 (12).

Statistics

Statistical analysis of all nonsequencing data was carried out using JMP software (SAS) and analyzed using linear mixed-effects models with a fixed effect of experimental group and a random effect of experiment day. Model assumptions of normality and homogeneous variance were assessed by a visual analysis of the raw data and the model residuals. Right-skewed data were log or square root transformed. The experimental group was considered statistically significant if the fixed effect *F* test *p* value was ≤ 0.05 . Post hoc pairwise comparisons between experimental groups were made using the Tukey honest significant difference multiple-comparison test. Statistical outliers were identified using the extreme studentized deviate method and omitted prior to the mixed model analysis. Graphs of results were shown as mean $\pm$ SEM of untransformed data using Prism version 6 (GraphPad).

For combined analysis of ATAC-seq and scRNA-seq data, Fisher exact test was used to test for the effect of predicted TF binding on gene expression (Fig. 4D) by analyzing the fold enrichment of the TF motif nearby overlapping peaks at inflammation-driven up- and downregulated genes against the likelihood that a gene up- or downregulated in response to HDM would overlap with an ATAC peak containing or lacking that TF binding motif versus the likelihood that these outcomes would occur for all detected genes. For analysis of E-protein TF binding motif enrichment compared across the developmental/maturation axis of ILC2s (Fig. 5A), the Fisher exact test was carried out to test the statistical significance of the number of ATAC peaks overlapping with E-protein motifs versus peaks not overlapping with E-protein motifs in cells in the developmental/activation state of interest compared with the number of ATAC peaks overlapping with E-protein motifs versus peaks not overlapping with E-protein motifs in the preceding developmental/activation stage. A one-sample *t* test was used to test the significance of inflammation-induced expression changes in genes that gained/lost nearby ATAC peaks that contained E-protein binding motifs in areas of chromatin that gained accessibility in development from bone marrow (BM) ILC2p to mature lung ILC2 (ILC2s PBS) or displayed no change in chromatin state during maturation (Fig. 5B), under the null hypothesis that the mean log₂(fold change) of the genes is zero.

Results

E-protein inhibition in thymic progenitors alters the mature lung ILC2 population in response to HDM

To begin to investigate how E-protein activity affects the phenotype and function of ILC2s following HDM challenge, we used ID1^{tg/WT} mice, in which an ID1 transgene is expressed under control of the proximal *Lck* promoter to inhibit E-protein activity in thymic lymphoid progenitors (21–23) (Fig. 1A). Previous work has highlighted that the proximal *Lck* promoter drives *Lck* protein expression in ETPs in the thymus, but not in mature T cells in the periphery (38). In agreement with this, we found that expression of the ID1 transgene was restricted to DN1/DN3 thymocytes and was not detectable in mature lung ILC2s in ID1^{tg/WT} mice (Supplemental Fig. 1A). Thus,

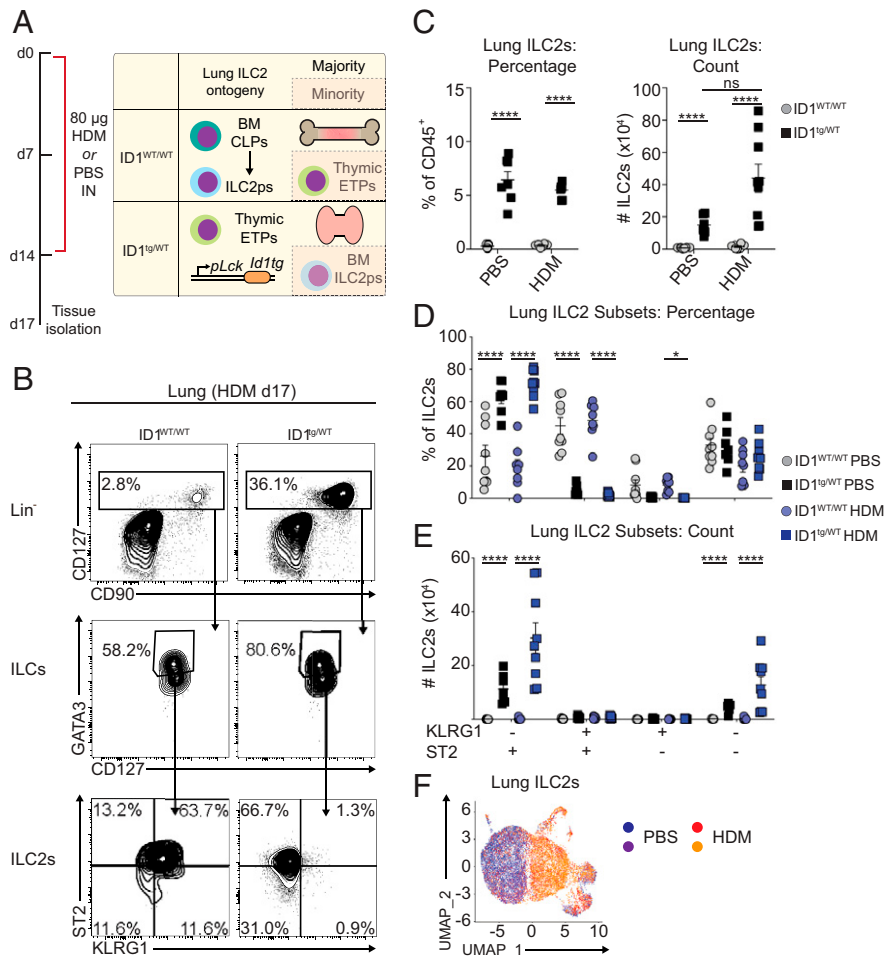


FIGURE 1. ID1^{tg/WT} mice display altered lung ILC2 populations in the steady state and following HDM-driven allergic airway inflammation. **(A)** Schematic of ID1^{WT/WT} mice, which have the majority of lung ILC2s derived from BM ILC2ps and a smaller number of thymic-derived ILC2s that develop in the presence of physiological levels of ID and E-protein function; and ID1^{tg/WT} mice, which overexpress an ID1 transgene in ETPs, leading to large numbers of thymus-derived ILC2s in the lung. Mice were treated i.n. on days 0, 7, and 14 with PBS or 80 µg of HDM in PBS, before tissue isolation on day 17. **(B)** Representative plots of lung ILC2 analysis following HDM treatment by flow cytometry in ID1^{WT/WT} (left) and ID1^{tg/WT} (right) mice. **(C)** Total GATA3⁺ ILC2s in the lung as a percentage of CD45⁺ cells and total counts in ID1^{WT/WT} and ID1^{tg/WT} mice treated with PBS or HDM. Subpopulations of lung ILC2s defined by KLRG1 and ST2 expression in ID1^{WT/WT} and ID1^{tg/WT} mice in response to PBS or HDM, as a percentage of ILC2s **(D)** or cell numbers **(E)** in the lung. **(F)** UMAP plot representing ILC2s from the lungs of mice treated i.n. with HDM or PBS analyzed by scRNA-seq. Scatter plots (C–E) depict mean ± SEM. *p < 0.05, ****p < 0.0001, analyzed using a linear mixed-effect model with pairwise comparison; all data from four experiments pooled, n = 8 to 9/group in total.

these mice allow us to test how the absence of E-protein activity in thymic progenitors affects ILC2 phenotype, function, and genomic landscape in response to HDM allergens.

We chose to study responses to HDM, as it drives innate and adaptive cell-mediated lung inflammation in mice and chronic asthma in humans (39), with ILC2s playing a key role (40, 41). Homozygous ID1^{tg/tg} mice display T cell ablation, ILC2 differentiation from thymic progenitors, and spontaneous autoimmune activation (21–23). To assess ILC2 responses to HDM in the absence of autoimmunity, we focused our studies on heterozygous ID1^{tg/WT} mice. ID1^{tg/WT} and ID1^{WT/WT} mice were sensitized and challenged with HDM or PBS i.n. every 7 d for 3 wk (Fig. 1A), and ILC2 populations in the lung were assessed 3 d after the final treatment. There was a dramatic increase in ILCs in the lungs (Fig. 1B, 1C) in both PBS- and HDM-treated ID1^{tg/WT} compared with ID1^{WT/WT} mice, the majority of which were GATA3⁺ ILC2s (Fig. 1B, 1C, isotype controls, Supplemental Fig. 1B) and likely derived from thymic ETPs, that represent up to 80% of the lung ILC2 population following E-protein inhibition (21–23). There was no significant change

in the proportion of CD45⁺ cells that were ILC2s or total numbers of ILC2s in the lungs of ID1^{WT/WT} or ID1^{tg/WT} mice following HDM treatment compared with PBS-treated controls (Fig. 1C).

Strikingly, although ILC2s in the lung of ID1^{tg/WT} mice were almost entirely KLRG1⁻ST2⁺, regardless of treatment, ID1^{WT/WT} ILC2s expressed high levels of KLRG1 and ST2 in the steady state and following HDM treatment, with no significant effect of HDM treatment on the makeup of ILC2 populations in either ID1^{WT/WT} or ID1^{tg/WT} mice (Fig. 1D, 1E). Similarly, analysis of a previously published scRNA-seq data set (Fig. 1F) showed that the majority of WT lung ILC2s expressed *Klrk1* and *Il1rl1* (ST2) after HDM treatment (Supplemental Fig. 1C). Notably, there were small differences in gene expression in *Klrk1*⁺ ILC2s compared with *Klrk1*⁻ cells in WT mice treated with HDM (Supplemental Fig. 1D), and *Klrk1*⁻ versus *Klrk1*⁺ ILC2s did not emerge as distinct subsets or clusters (Supplemental Fig. 1E). These data suggest that there are phenotypic differences between a lung ILC2 pool in which the majority are generated in the enforced inhibition of E-protein activity during development and the pool of ILC2s generated in WT animals. A

loss of KLRG1 expression in ILC2s in ID1^{tg/WT} mice is potentially due to their ontogeny or their environment.

Given the central role of E-proteins in T cell development (21), as well as the known function of CD4⁺ T cells in the development of HDM-driven type 2 inflammation (4), we also characterized the CD4⁺ T cell compartment within the lungs and thymus of ID1^{tg/WT} mice. Despite the impact of ID1 overexpression on thymic T cell development (21), ID1^{tg/WT} mice did not display a significant deficit in CD4⁺ T cell accumulation in the lung under any condition (Fig. 2A). In ID1^{tg/WT} compared with ID1^{WT/WT} mice, there was a significant increase in the percentage of activated CD44^{hi}CD62L^{lo} CD4⁺ T cells in PBS- and HDM-treated lungs (Fig. 2B) and total numbers of these cells in HDM-treated lungs (Supplemental Fig. 1F), consistent with previous findings (42). There was an increase in the frequency of lung GATA3⁺CD4⁺ Th2 cells, as well as an increase in activated CD44^{hi}CD62L^{lo}GATA3⁺ST2⁺CD4⁺ T cells, in PBS- but not HDM-treated ID1^{tg/WT} compared with ID1^{WT/WT} mice (Fig. 2C, 2D). This finding was not associated with an increase in baseline levels of activated CD4⁺ T cells or Th2 cells in the thymus or a Treg deficiency in the lung or thymus under any condition in ID1^{tg/WT} mice (Fig. 2E, Supplemental Fig. 1G). Indeed, there was actually a significant increase in the frequency of FOXP3⁺ Tregs in both the thymus and lung of untreated or PBS-treated ID1^{tg/WT} compared with ID1^{WT/WT} mice (Fig. 2E, Supplemental Fig. 1G). There was no significant difference in GATA3-expressing FOXP3⁺ Tregs in the lungs of ID1^{tg/WT} compared with ID1^{WT/WT} mice under any condition (Fig. 2F). Together, these data show that overexpression of ID1 in thymic progenitors during ILC2 and CD4⁺ T cell development results in an

expanded ILC2 compartment and enhanced CD4⁺ T cell activation in the lung in response to HDM, with increased activation of CD4⁺ T cells emerging in the periphery.

ILC2s that developed in the absence of E-protein activity have impaired cytokine production capacity

Because ID1^{tg/WT} mice had large numbers of ILC2s and increased CD4⁺ T cell activation in the lung, we hypothesized that these animals would have an exacerbated response to HDM treatment. Although ID1^{tg/WT} mice did have significantly increased lung eosinophilia following HDM treatment compared with ID1^{WT/WT} controls (Fig. 3A) (21), other inflammatory infiltrating cells were not similarly affected (Supplemental Fig. 1H, 1I). In addition, HDM exposure led to comparable increases in other tissue responses driven by type 2 inflammation, including *Muc5ac* expression, epithelial hyperplasia, and total pathology score, in the lungs of ID1^{tg/WT} versus ID1^{WT/WT} mice (Fig. 3B, 3C, Supplemental Fig. 1J). Together, these findings highlight that ID1 overexpression in thymic ETPs does alter the inflammatory microenvironment in the lung in the steady state and in response to HDM but has a minimal impact on overall pathological outcome following HDM exposure.

To determine what underlies comparable pathology following HDM treatment in ID1^{tg/WT} and ID1^{WT/WT} mice (Fig. 3B, 3C, Supplemental Fig. 1I) despite increased ILC2 numbers (Fig. 1C) and enhanced CD4⁺ T cell activation (Fig. 2B), we investigated ILC2 and CD4⁺ T cell cytokine responses, as these responses are drivers of pathology following HDM treatment (4). There was no significant increase in IL-4, IL-5, IL-13, or IL-33 gene transcripts or total protein levels in the lungs of ID1^{tg/WT} compared with ID1^{WT/WT} mice treated with PBS or HDM (Supplemental Fig. 2A, 2B). In addition, the ID1 transgene had little impact on Th2 cell cytokine output (Supplemental Fig. 2C–E). Strikingly, however, ID1^{tg/WT} mice treated with HDM had significantly decreased frequencies of ILC2s that were positive for IL-5, IL-13, and GM-CSF compared with ID1^{WT/WT} mice treated with HDM (Fig. 3D). There were still elevated total numbers of cytokine-producing lung ILC2s in ID1^{tg/WT} compared with ID1^{WT/WT} mice (Fig. 3E) due to the large increase in total ILC2s (Fig. 1C); however, on a per-cell basis, cytokine-producing ILC2s in ID1^{tg/WT} mice made less IL-13 than cytokine-producing ILC2s in ID1^{WT/WT} mice following HDM treatment (Supplemental Fig. 2F). The decrease in the frequency of IL-5 and IL-13 producers in ID1^{tg/WT} compared with ID1^{WT/WT} mice after HDM treatment largely held true for KLRG1⁺ST2⁺ and KLRG1⁺ST2⁺ ILC2s (Fig. 3F) but not KLRG1⁺ST2⁺ ILC2s (Supplemental Fig. 2G), though there was no significant difference in GM-CSF production between ID1^{tg/WT} and ID1^{WT/WT} lung ILC2s when broken down into subsets (Fig. 3F, Supplemental Fig. 2G). Thus, ID1^{tg/WT} ILC2s demonstrated impaired capacity to produce effector cytokines in response to HDM, but the sheer numbers of ILC2s in the lungs of ID1^{tg/WT} mice likely translate into relatively similar levels of lung pathology in ID1^{tg/WT} and ID1^{WT/WT} mice. These data show that ILC2s that develop in mice with E-protein inhibition in thymic progenitors have an altered functional capacity, both in bulk and in the ST2-expressing subsets, compared with control ILC2s, suggesting a role for E-proteins in shaping ILC2 functional profiles.

E-proteins have a limited role in directing gene expression at regions of open chromatin in mature lung ILC2s during airway inflammation

Our in vivo studies suggested a role for E-proteins in regulating ILC2 function in addition to lineage specification, but it remained unclear whether these effects were fixed during the developmental

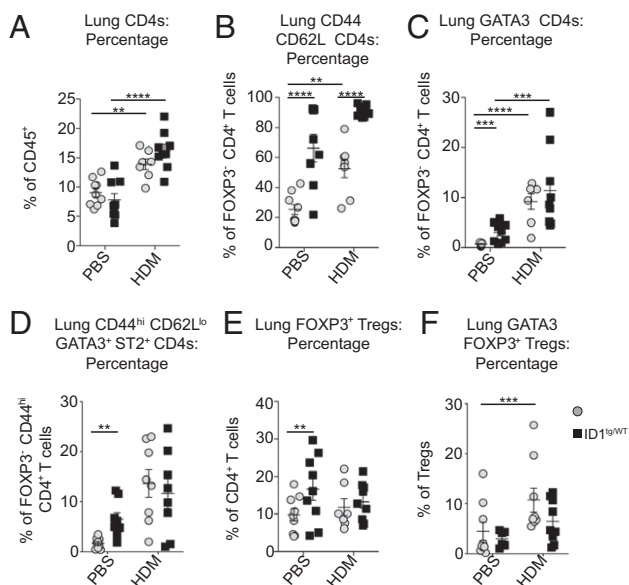


FIGURE 2. ID1^{tg/WT} mice display an increase in activated lung CD4⁺ T cells. (A) CD4⁺ T cells as a percentage of all CD45⁺ immune cells in the lung of ID1^{WT/WT} and ID1^{tg/WT} mice treated with PBS or HDM i.n. FOXP3⁺CD44^{hi}CD62L^{lo} activated (B), GATA3⁺ Th2 CD4⁺ T cells (C), and FOXP3⁺CD44^{hi}CD62L^{lo} activated GATA3⁺ST2⁺ Th2 CD4⁺ T cells (D) as a percentage of total FOXP3⁺CD4⁺ T cells or CD44^{hi}CD62L^{lo} activated CD4⁺ T cells from the lung of ID1^{WT/WT} and ID1^{tg/WT} mice treated i.n. with PBS or HDM. Lung FOXP3⁺ CD4⁺ Tregs (E) and GATA3⁺FOXP3⁺CD4⁺ Tregs (F) as a percentage of total CD4⁺ T cells from ID1^{WT/WT} and ID1^{tg/WT} mice treated i.n. with PBS or HDM. Mean ± SEM. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, analyzed using a linear fixed-effect model with pairwise comparison; data from three to four experiments pooled, *n* = 6–15/group in total.

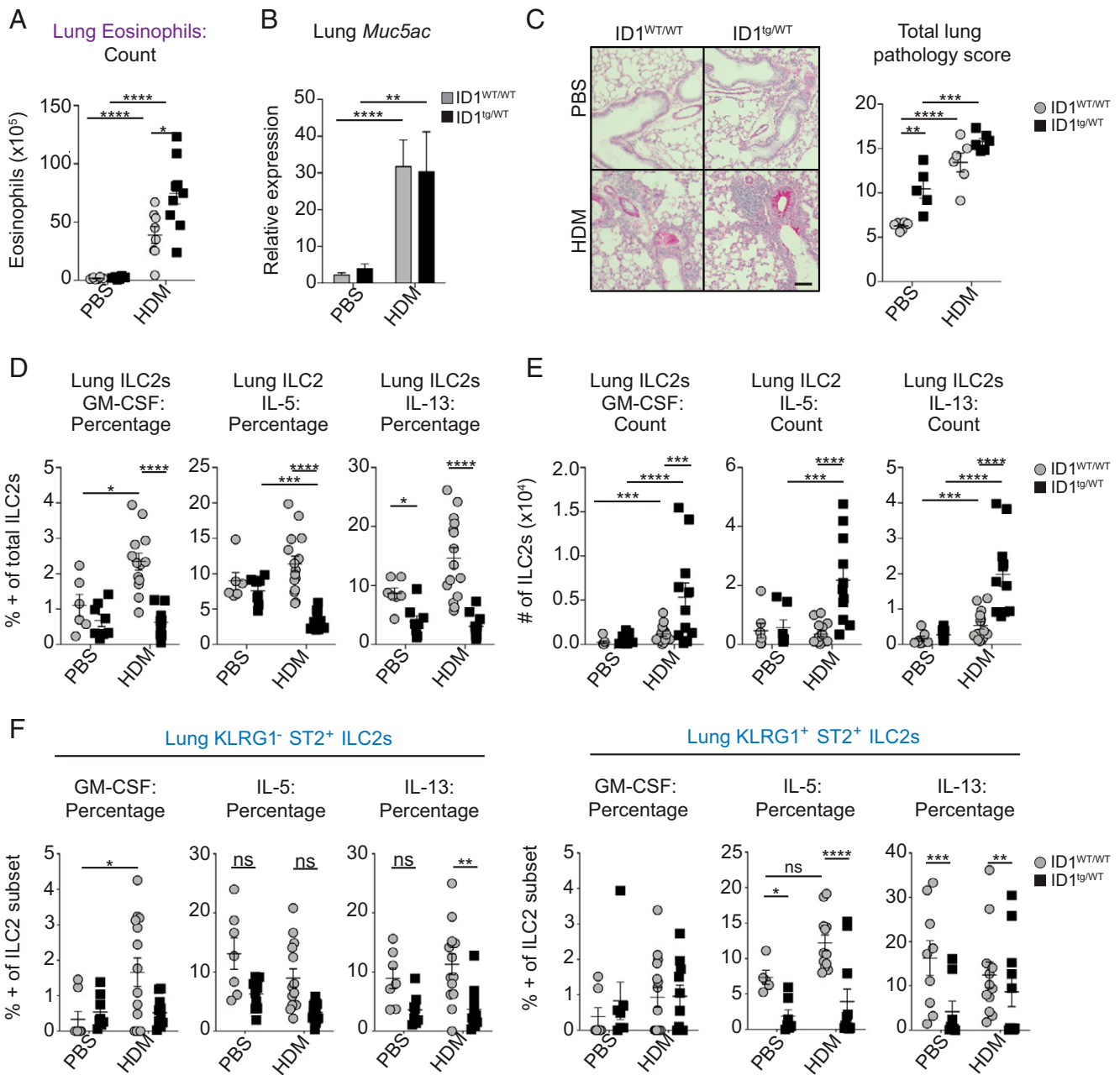


FIGURE 3. An increase in thymic-derived ILC2s in ID1^{tg/WT} mice has minimal impact on HDM-driven pathology, associated with diminished ILC2 cytokine production. **(A)** Total numbers of eosinophils in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice treated with PBS or HDM. **(B)** Relative expression of *Muc5ac* in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice following PBS or HDM treatment. **(C)** Representative images of periodic acid-Schiff/Alcian blue-stained lung sections. Scale bar, 100 μ m. **(D)** Total pathology scores for ID1^{WT/WT} and ID1^{tg/WT} mice exposed to PBS or HDM are shown at right. Proportion of GM-CSF⁺, IL-5⁺, and IL-13⁺ of total ILC2s **(D)** or total numbers of cytokine⁺ ILC2s **(E)** in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice following PBS or HDM treatment identified by intracellular cytokine staining. **(F)** Proportion of GM-CSF⁺, IL-5⁺, and IL-13⁺ of KLRG1⁺ST2⁺ and KLRG1⁺ST2⁺ lung ILC2 subsets from ID1^{WT/WT} and ID1^{tg/WT} mice exposed to PBS or HDM following intracellular cytokine staining. Mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, analyzed using a linear fixed-effect model with pairwise comparison; data from two (C) or three to four (A, B, D-F) experiments pooled, n = 5 to 6/group in total (C) or n = 6–15/group in total (A, B, D–F).

process when ID1 was overexpressed or whether they occurred in mature ILC2s. Although the proximal *Lck* promoter that drives ID1 overexpression is largely restricted to the thymus (24), it remained possible that residual ID1 pools could continue to inhibit E-proteins in mature ILC2s in the lung. One way to assess TF regulation of gene expression in ILC2s and other cell types is to identify putative TF binding sites in areas of accessible chromatin using ATAC-seq (28). Indeed, previous studies have shown that ILC2s in the lung

have poised open chromatin that allow them to quickly begin transcribing certain effector and cytokine genes (12). Thus, to understand how our findings in ID1^{tg/WT} mice reflected the importance of the E-ID protein axis in regulating ILC2 function, we next identified E-protein binding sites adjacent to open chromatin in steady state and HDM-stimulated lung ILC2s from WT mice, testing whether E-proteins played a key role in gene regulation in mature ILC2s as they responded to HDM. To do so, we assessed ILC2 chromatin

accessibility and E-protein binding motifs in the wider context of transcriptional control using ATAC-seq performed on total ILC2s (lin[−]CD127⁺CD90^{+/lo} cells that were ST2⁺ and/or KLRG1⁺) from the lungs of mice following treatment with HDM or PBS, with bulk CD4⁺ T cells or ST2⁺ Th2 CD4⁺ cells used as a comparison (Fig. 4A).

In agreement with published work (12), ILC2s had a large proportion of unchanged ATAC peaks between the PBS- and HDM-treated conditions compared with CD4⁺ T cells (Fig. 4B). However, the total number of genome-wide changes in chromatin accessibility were similar in Th2 cells and ILC2s responding to HDM (Fig. 4B), indicating that there are significant changes in accessible chromatin in ILC2s following HDM treatment. This is consistent with the idea that TFs (10, 43), potentially including E-proteins, regulate ILC2 gene expression during maturity as they responded to HDM. Thus, we sought to identify which TFs were associated with HDM-driven changes in ILC2s and Th2 cells. To do so, we quantified the fraction of ATAC peaks that contained known TF binding motifs in regulatory elements near gene promoters in open chromatin, displaying these data in a heat map of normalized z-scores across cell types and conditions by individual TF or TF family (Fig. 4C, Supplemental Fig. 3A). This analysis revealed that AP-1 and C/EBP TF binding sites were enriched in both lung ILC2s and Th2 cells from HDM-

compared with PBS-treated mice, with the HDM-induced increase in binding sites for these TFs particularly stark in ILC2s (Fig. 4C, Supplemental Fig. 3A). These data are consistent with previous findings that have highlighted a role for these TF families in regulating activated ILC2 gene expression (10). In contrast, binding motif abundance for E-proteins in areas of open chromatin was under-represented in lung ILC2s from HDM- compared with PBS-treated mice (Fig. 4C, Supplemental Fig. 3A, Supplemental Table I). These data suggest that although mature ILC2 gene expression is regulated by TF binding to accessible chromatin that opens in response to HDM, E-proteins likely play a minimal role in this process.

Consistent with this idea, using a previously published scRNA-seq data set of ILC2s purified from various tissue sites (Fig. 4D), we observed minimal *Tcf3* or *Tcf12* expression detected in mature lung ILC2s, regardless of tissue (Fig. 4E). Similarly, minimal *Tcf2* or *Tcf3* expression was detectable in lung ILC2s responding to PBS or HDM treatment (Fig. 1F, Fig. 4E). In addition, we observed generally higher proportions of ILC2s expressing genes encoding AP-1 TFs and C/EBP β compared with E-proteins in both PBS- and HDM-treated mice, and the proportion of E-protein-expressing ILC2s did not dynamically change in response to HDM treatment (Supplemental Fig. 3B). We also found that C/EBP and AP-1 but not E-protein motifs were enriched near inflammation-responsive

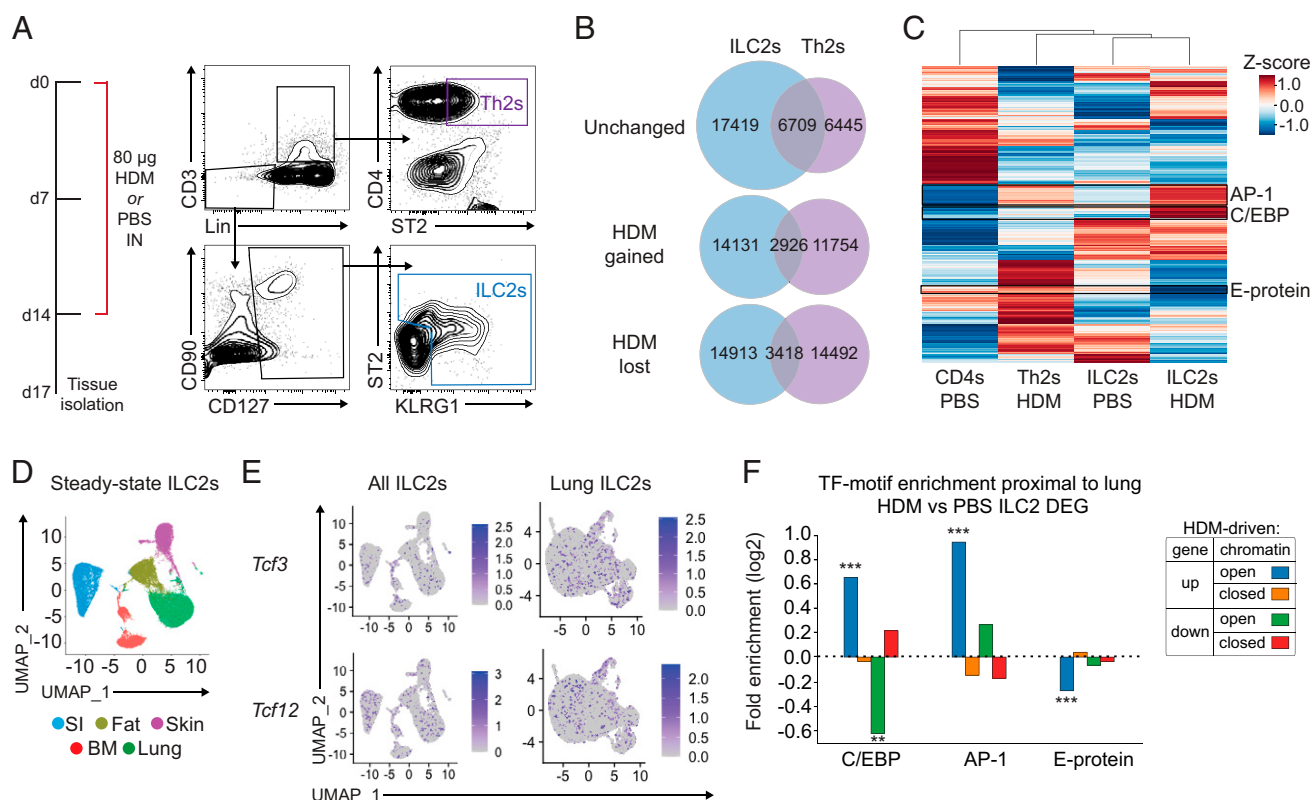


FIGURE 4. HDM drives major changes in the chromatin accessibility landscape of lung ILC2s. **(A)** C57BL/6 WT mice were treated i.n. on days 0, 7, and 14 with PBS or HDM, before tissue isolation on day 17, when total lung CD4⁺ T cells (PBS-treated mice, gate not shown), Th2 cells (HDM-treated mice, purple), and ILC2s (blue) were isolated for ATAC-seq. **(B)** Venn diagram of ATAC peaks that were unchanged, gained, or lost that were unique or shared between lung ILC2s and Th2 cells from HDM-treated mice compared with cells from PBS-treated controls. **(C)** Heat map of TF family motifs identified in ATAC-seq data from lung CD4⁺ T cells and ILC2s from PBS- or HDM- treated mice, reported by normalized z-score across conditions/cell type, with the samples organized by hierarchical clustering. **(D)** UMAP plot representing ILC2s from steady-state mouse BM, small intestine (SI), fat, skin, and lung analyzed by scRNA-seq. **(E)** UMAPs of normalized relative expression of *Tcf3* and *Tcf12* transcripts in steady-state ILC2s across tissues and lung ILC2s from PBS- and HDM-treated mice analyzed using scRNA-seq. **(F)** Bar plot showing fold enrichment of representative AP-1, C/EBP, and E-protein motifs in chromatin open or closed near up- or downregulated genes in lung ILC2s from HDM-treated mice compared with PBS-treated controls. ****p* < 0.001, analyzed using Fisher exact test. ATAC-seq data representative of two samples per group, collected from three experiments. ILC2 scRNA-seq data representative of at least two individual biological replicates per sample.

genes, up- or downregulated, as detected by scRNA-seq analysis (27) following HDM treatment in areas of gained open chromatin (Fig. 4F, Supplemental Table I). C/EBP and AP-1 motifs were enriched near HDM-upregulated genes in peaks gained in ILC2s following HDM treatment, whereas C/EBP motifs were largely absent from peaks near downregulated genes, in agreement with prior studies (10) (Fig. 4F, Supplemental Table I). Ontology mapping of genes proximal to C/EBP motifs with HDM-driven gained accessibility (Supplemental Table I) showed enrichment for genes involved in the regulation of cardiac muscle function (Supplemental Fig. 3C), including genes that regulate smooth muscle contraction, a feature of chronic pulmonary allergic disease (44). One such gene, *Pde4b*, encoding a phosphodiesterase enzyme that regulates airway smooth muscle development and proliferation (45), displayed HDM-dependent changes in accessibility uniquely in ILC2s at regulatory regions that overlapped with AP-1 and C/EBP binding motifs (Supplemental Fig. 3D). Together, these data suggest a limited role for E-proteins in directly regulating mature ILC2 gene expression changes and function during type 2 inflammation, with a dominant role for AP-1 and C/EBP TFs in this setting.

E-protein activity during ILC2 development promotes the activation of gene pathways integral to ILC2 function

Defective ID1^{tg/WT} ILC2 cytokine-producing capacity during type 2 inflammation (Fig. 3D) but a limited enrichment for E-protein binding sites at chromatin sites proximal to inflammation-responsive genes (Fig. 4F) led us to hypothesize that E-proteins act in ILC2 development to shape the functional capacity of mature ILC2s. This hypothesis aligned with our data and published findings highlighting that ILC2s maintain accessibility in large regions of their genome in the steady state that remain open following the onset of type 2 inflammation (12). To identify how E-proteins could impact functional gene expression throughout the ILC2 developmental arc, we quantified putative E-protein binding sites at accessible chromatin that was gained across the ILC2 developmental trajectory, starting with BM CLPs, to committed BM ILC2ps (12), to steady-state lung ILC2s, and finally to HDM-elicited lung ILC2s. There was a smaller proportion of E-protein motifs in accessible chromatin regions in committed BM ILC2ps compared with CLPs, as well as in HDM-activated ILC2s compared with steady-state ILC2s (Fig. 5A). However, there was increased E-protein binding site enrichment in accessible chromatin in mature steady-state ILC2s (PBS ILC2s) compared with ILC2ps (Fig. 5A, Supplemental Table I), suggesting that at this time point in ILC2 maturation, E-proteins could regulate gene expression.

We next sought to determine how E-protein binding in accessible chromatin during ILC2 development might alter gene expression in mature ILC2s. To do so, we quantified E-protein binding sites in accessible chromatin gained or lost in the transition from BM ILC2ps to steady-state lung ILC2s that were associated with inflammation-responsive genes as detected using bulked scRNA-seq data. The majority of genes that gained E-protein binding during ILC2 maturation were downregulated in response to HDM, including *Id2* (Fig. 5B, 5C), as well as genes that were still associated with an accessible E-protein binding motif (red), and many more that lost their nearby E-protein binding peaks in inflammation (green) (Fig. 5C). However, there were several genes that gained E-protein binding during maturation from ILC2ps to steady-state ILC2s that were upregulated in response to HDM as well. These data indicate that E-protein peaks that are altered during development are proximal to inflammation-responsive genes in ILC2s from PBS- compared with HDM-treated mice.

To elucidate the role of E-proteins in dictating mature ILC2 function during the developmental process, we performed pathway

analysis of bulked scRNA-seq data from lung ILC2s from PBS- and HDM-treated WT mice to identify biological processes that are activated in lung ILC2s during inflammation. Identified pathways featured inflammation-responsive genes involved in Ag-driven responses to damage, insult, and pathogens (7, 46, 47), IFN functional pathways, and pathways integral to ILC2 function, including cytokine-driven responses, Ag presentation, interaction with T cells, and activation of cytokine production (Supplemental Fig. 3E) (48). To elucidate which of these processes were dictated by E-protein function in development, we then performed GO mapping of genes that gained accessible E-protein binding motifs in the development of mature steady-state lung ILC2s. This analysis showed that E-proteins may regulate ILC2 genes in JAK/STAT signal transduction pathways, including *Jak1* and *Jak2*, *Fer*, *Mapk14*, and *Zfp36*; the cellular response to IL-3, including *Jak2*, *Gsk3a*, *Jak1*, *Gsk3b*, and *Sh2b3*; and MHC II-dependent Ag presentation, including *Cd74*, *H2-Eb1*, *H2-Ab1*, and *H2-DMb2* (Fig. 5C, 5D, Supplemental Table I). Some JAK/STAT effectors and regulators that gained proximal E-protein motifs during maturation were downregulated following HDM treatment based on scRNA-seq data, including *Lif*, *Nr4a1*, *Socs1*, and *Zfp3611*, whereas others were upregulated, including *Socs3* (Fig. 5C, 5E). In contrast, expression of most MHC II Ag presentation machinery genes was upregulated upon HDM treatment in scRNA-seq data (Fig. 5C, 5F), suggesting that E-protein binding to accessible chromatin in the development of ILC2ps to mature ILC2s may promote certain aspects of ILC2 function in response to HDM (while dampening other aspects). Thus, in ILC2s in which E-proteins are inhibited during development, as in thymic-derived ILC2s in ID1^{tg/WT} mice, specific functional pathways may be inhibited by the loss of E-protein activity. Taken together, these findings suggest that E-proteins can act on key functional genes during ILC2 development to control ILC2 effector functions during type 2 inflammation.

Inhibition of E-proteins during ILC2 development limits accessibility of functional genes associated with AP-1 TF binding sites in ILC2s following HDM treatment

To further understand how inhibition of E-protein activity during ILC2 development might regulate the function of mature lung ILC2s in response to allergic airway inflammation, we performed a side-by-side ATAC-seq analysis of total lung ILC2s from ID1^{tg/WT} and ID1^{WT/WT} mice following HDM treatment. PCA showed a clear difference in overall genome accessibility between ID1^{tg/WT} and ID1^{WT/WT} lung ILC2s following HDM exposure (Fig. 6A). As our comparison between steady-state and HDM-treated ILC2s in WT mice highlighted AP-1 and C/EBP TFs as best candidates to regulate the ILC2 response to inflammation (Fig. 4), we compared the fractions of ATAC peaks that overlapped with motifs of the main representatives of these TF families, as well as those of TCF3 and TCF12 (Fig. 6B). In line with a marginal role for E-proteins in mature ILC2 responses to HDM, there was virtually no change in E-protein motifs represented in the ATAC peaks between genotypes. A similar lack of change was observed for C/EBP TF motifs, suggesting that their effect during inflammation is not altered when E-proteins are inhibited during development. However, four of the five tested TF motifs of the AP-1 family (BATF, FOS, JUND, and JUNB) displayed a consistently lower representation in ATAC peaks of lung ILC2s from ID1^{tg/WT} compared with ID1^{WT/WT} mice (Fig. 6B).

To ask whether the effect of ID1 overexpression on AP-1-regulated genes in ILC2s is central for the differences between ID1^{tg/WT} and ID1^{WT/WT} ILC2s, we curated a set of ATAC peaks unique to ID1^{WT/WT} and ID1^{tg/WT} ILC2s (ID1^{WT/WT}-specific and ID1^{tg/WT}-specific, respectively). These included ATAC peaks detected in all three biological replicates of one genotype and in none of the

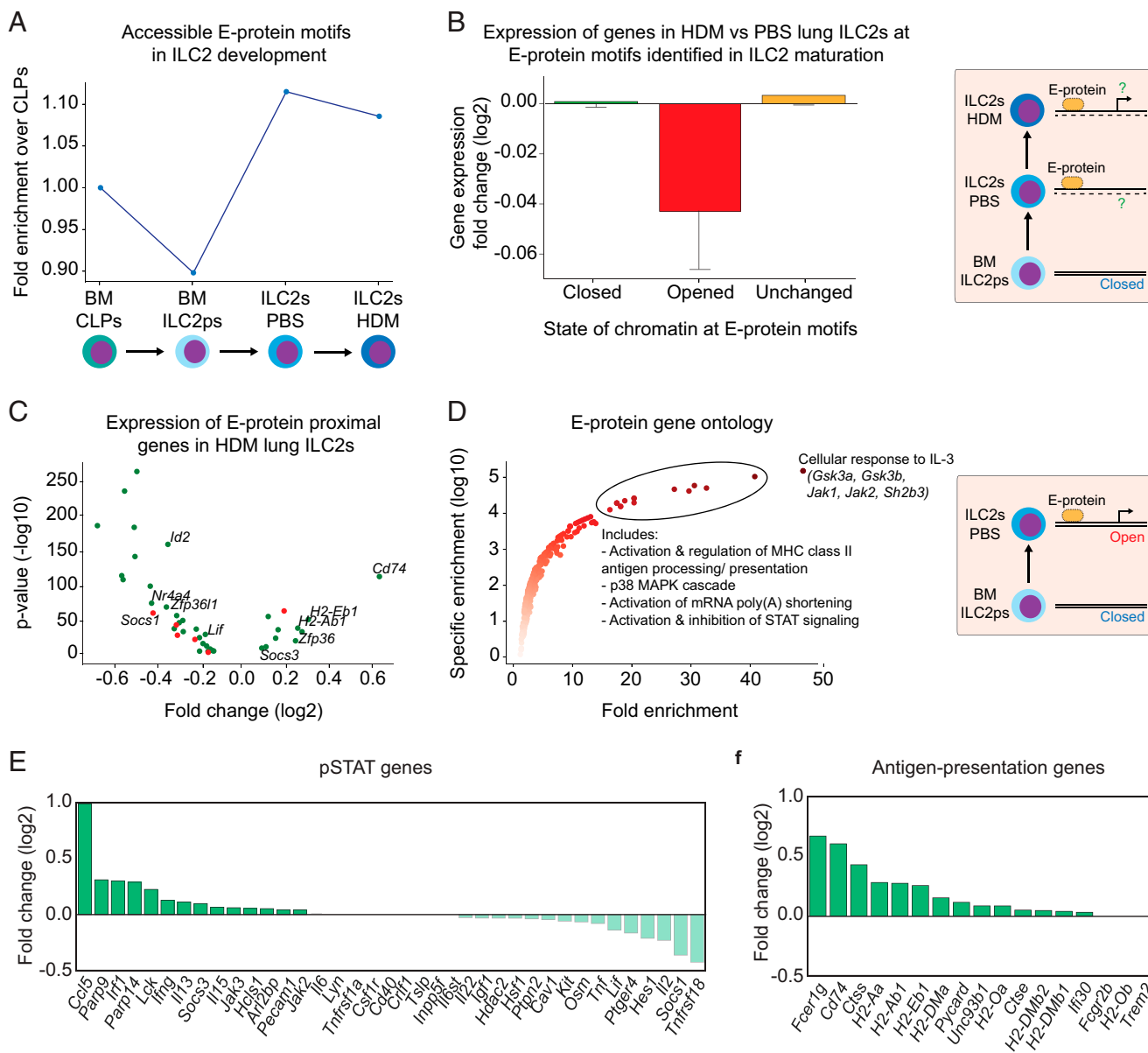


FIGURE 5. E-protein binding at accessible chromatin in developing ILC2s is associated with altered functional gene expression in mature lung ILC2s in response to HDM. **(A)** Changes in E-protein motif enrichment at ATAC peaks in specific developmental/activation stages of WT ILC2s, compared with the proceeding developmental/activation stage, from BM CLPs to ILC2ps from a published data set to lung ILC2s from PBS- and HDM-treated mice. **(B)** Fold change in expression of genes in HDM- versus PBS-treated lung ILC2s that are proximal to putative E-protein binding sites that are lost, gained, or unchanged upon ILC2 maturation from ILC2p. **(C)** Volcano plot showing HDM-driven expression level changes (from bulked scRNA-seq data) of lung ILC2 genes that gained E-protein binding during ILC2 maturation from ILC2ps, with chromatin accessibility retained (red) or lost (green) during inflammation, with key gene names depicted. **(D)** Fold versus specific enrichment of GO biological processes (left) associated with upregulated genes proximal to open chromatin containing E-protein motifs gained in mature lung ILC2s compared with ILC2ps (schematic, right), with the most enriched pathways highlighted, including upregulation genes associated with cellular responses to IL-3. Fold change in expression of genes associated with STAT phosphorylation **(E)** and Ag presentation pathways **(F)** of lung ILC2s from HDM- compared with PBS-treated mice identified in scRNA-seq analysis. Genes shown are proximal to E-protein binding motifs that were gained during ILC2 maturation. ATAC-seq data representative of two samples per group, collected from one to three experiments. ILC2 scRNA-seq data representative of at least two individual biological replicates/sample.

samples from the other genotype. We found 10,378 ID1^{WT/WT}-specific and 4,562 ID1^{tg/WT}-specific peaks, out of a total 364,131 ATAC peaks found in all samples (Fig. 6C) and focused on the peaks that were ID1^{WT/WT}-specific and thus lost in ID1^{tg/WT} ILC2s in HDM-treated mice. Comparison of these unique ATAC peaks to total ATAC peaks within each genotype revealed a clear enrichment for putative AP-1 TF binding ID1^{WT/WT}-specific but not ID1^{tg/WT}-specific peaks, whereas none of the tested C/EBP motifs showed a

similar ID1^{WT/WT}-specific enrichment (Fig. 6D). Supportive of our hypothesis that E-proteins act early in the developmental axis, rather than during response to allergens, E-protein motif overlap was similarly absent in both ID1^{WT/WT}- and ID1^{tg/WT}-specific peaks from lung ILC2s from mice treated with HDM (Fig. 6D). ID1^{WT/WT}-specific peaks were preferably distributed near genes mapped to ontologies associated with immune and inflammatory responses (Fig. 6E), indicating that this relatively small fraction of unique peaks represents

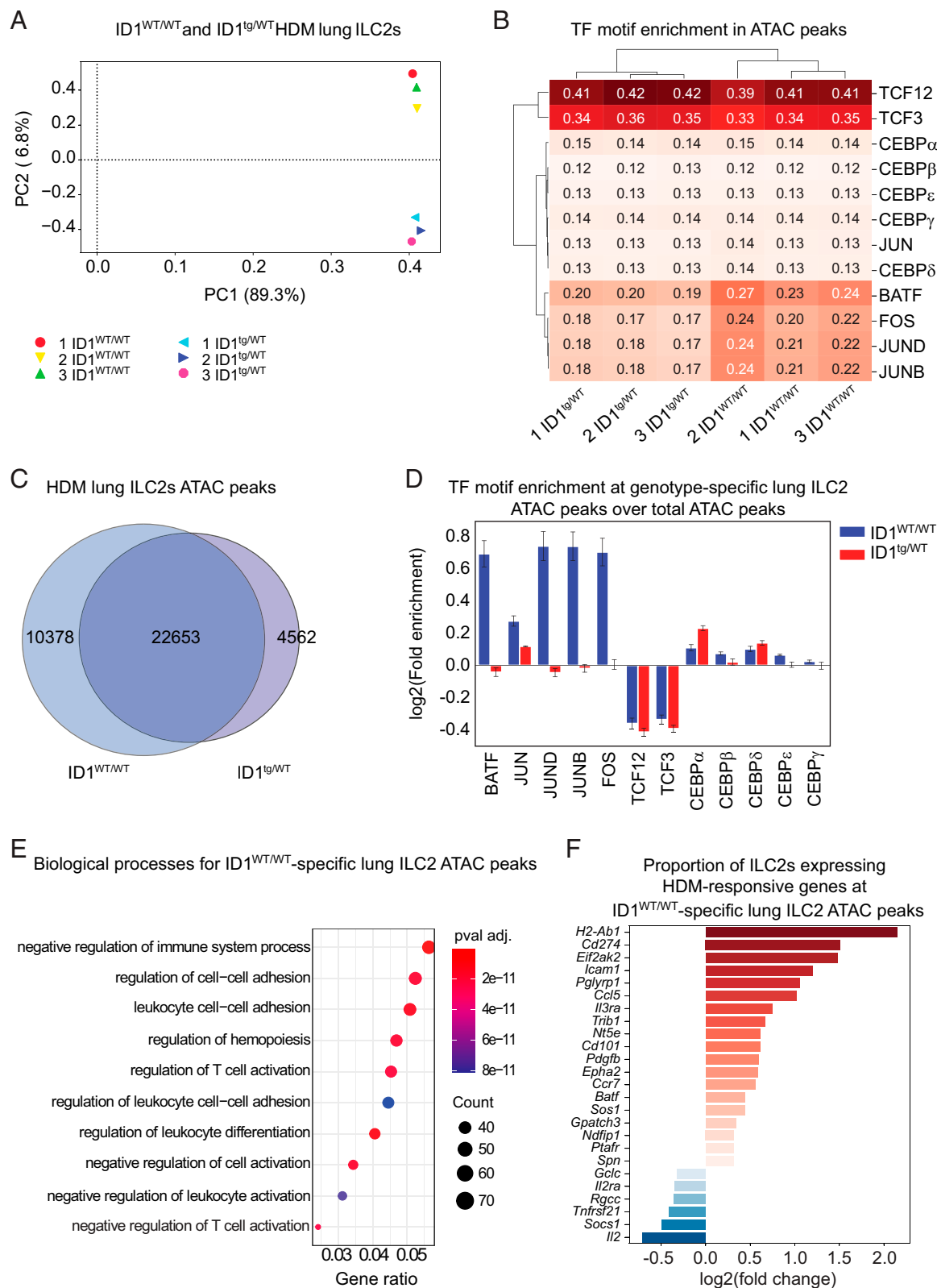


FIGURE 6. Inhibition of E-protein function during ILC2 development limits accessibility of AP-1 binding sites proximal to functional genes that are active during HDM-driven inflammation. **(A)** PCA plot of ATAC-seq data from total lung ILC2s isolated from ID1^{WT/WT} and ID1^{tg/WT} mice following i.n. HDM treatment. **(B)** Heat map showing relative frequency of E-protein, C/EBP, and AP-1 TF binding motifs in ATAC peaks of ID1^{WT/WT} and ID1^{tg/WT} mice treated i.n. with HDM. **(C)** Venn diagram of ATAC peaks that were unique to lung ILC2s from ID1^{WT/WT} and ID1^{tg/WT} mice treated with HDM or that were shared by mice from both genotypes. Only ATAC peaks present in all three replicate samples of that genotype were included in analysis. **(D)** Bar graph to show enrichment of E-protein, C/EBP, and AP-1 TF binding motifs in ATAC peaks that were unique to HDM-treated lung ILC2s from ID1^{WT/WT} or ID1^{tg/WT} mice, compared with all ATAC peaks. **(E)** Top 10 enriched GO biological processes associated with genes near ATAC peaks that contained an AP-1 binding motif present in lung ILC2s from HDM-treated ID1^{WT/WT} mice but not ID1^{tg/WT} mice. **(F)** Bar plot to show fold change in expressing cell fraction of inflammation-responsive genes that were specific to ID1^{WT/WT} ILC2s isolated from the lung following HDM i.n. treatment. ATAC-seq data representative of three independent biological replicates of two to three pooled mice (ID1^{WT/WT}) or a single mouse (ID1^{tg/WT})/group, collected from a single experiment.

important functional differences between the two genotypes. Consistent with this idea, ILC2 functional genes that are responsive to HDM as detected in scRNA-seq data were dynamically regulated, with many upregulated, including *H2-Ab1*, *Cd274*, and *Batf*, and some downregulated (Fig. 6F). Taken together, our data suggest that E-proteins act early in the development of ILC2s to shape the lung ILC2 inflammatory response that is mediated by AP-1 family TFs (Fig. 7).

Discussion

E- and ID proteins play a key role in regulating development of innate and adaptive immune cells, with E- and ID proteins functioning antagonistically to drive the development of ILCs and T cells (13–15, 21–23, 49). However, whether E-proteins regulate mature ILC2 gene expression and how that might dictate their function has not previously been explored. In this study, we combine analysis of lung ILC2 gene expression and genomic landscape with studies in mice with enforced E-protein inhibition during ILC2 development following HDM allergen exposure, identifying a novel role for E-proteins acting during ILC2 development to regulate lung ILC2 function in maturity (Fig. 7).

Initial studies of the genomic landscape of ILC2s by ATAC-seq demonstrated that, unlike naïve $CD4^+$ T cells, ILC2s maintain

chromatin around important functional genes in an open state (12). Thus, ILC2s can rapidly activate transcription of functional genes upon inflammation. Importantly, our analysis using immune cells from the lung for all conditions identified this pattern as well, as the previous study used control ILC2s from the intestine and splenic naïve T cells, in contrast to our study (12). However, although we also observed that large numbers of ATAC peaks are conserved in ILC2s from PBS- and HDM-treated mice, ILC2s responding to HDM do have significant changes in accessible chromatin genome-wide (Fig. 4B, 4C). These findings suggested that functional pathways integral to ILC2 inflammatory activation are modulated by changes in chromatin accessibility and altered gene transcription during the developmental process and during inflammation.

Specific TFs appear to be key in regulating gene expression in these two contexts. AP-1 and C/EBP family TFs emerged in our analysis as critical in regulating gene expression in mature ILC2s in the lung that responded to inflammation, as motifs for these factors were highly enriched in areas of open chromatin in lung ILC2s from HDM-treated mice (Figs. 4C and 7). BATF, an AP-1 family member with a dominant-negative effect on other members of this family, and C/EBP have previously been implicated in regulating $CD4^+$ T cell IL-4 production and Th2 cell function (50–52) and in upregulating genes in $KLRG1^+$ but not $KLRG1^-$ ILC2s in the lung during *N. brasiliensis* infection (10). AP-1 and C/EBP motifs appear

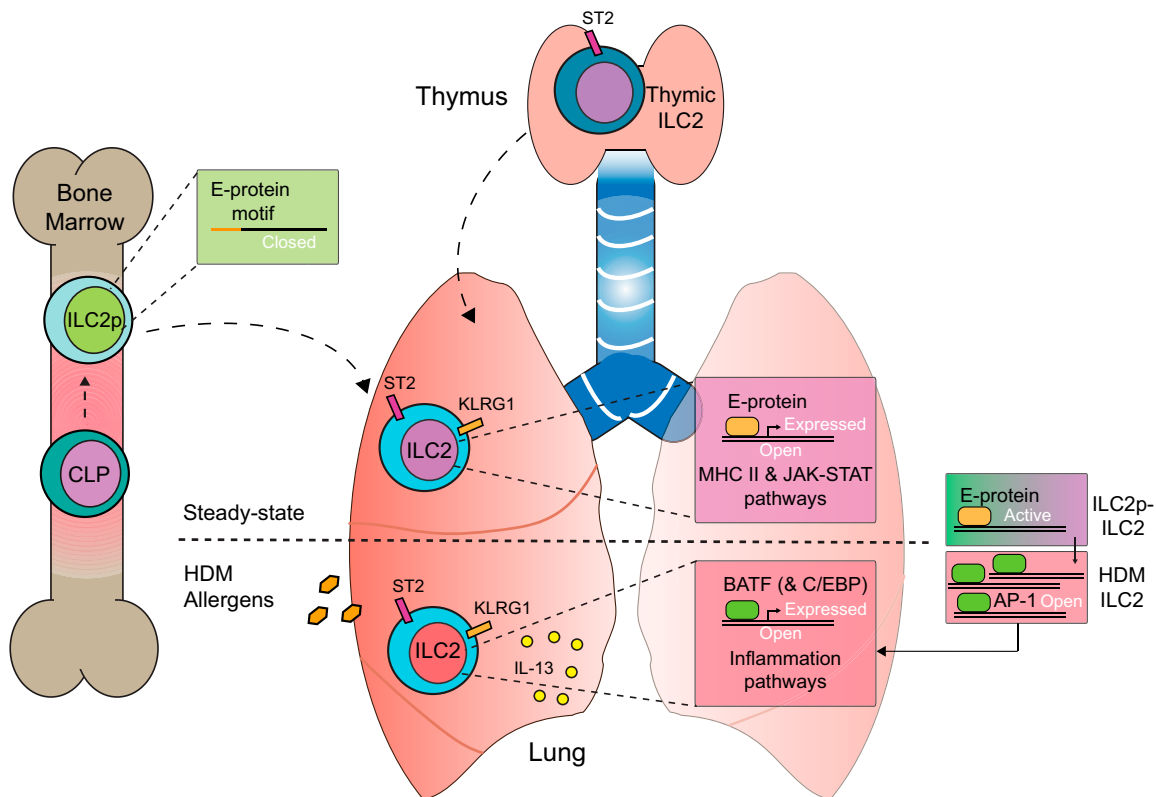


FIGURE 7. Proposed model of how E-protein, AP-1, and C/EBP TFs activate functional pathways in lung ILC2s during development or upon allergen challenge. BM ILC2ps that develop from CLPs have limited accessible E-protein binding sites in their chromatin. However, WT lung ILC2s, largely $KLRG1^+ST2^+$, in the steady state derived from BM ILC2ps have regions of open chromatin near functional genes. These include regions with binding motifs for E-protein TFs proximal to JAK/STAT signaling and MHC II-dependent Ag presentation genes that promote transcriptional changes downstream of cytokine receptors, both pathways that drive ILC2 activation, function, and cytokine production in type 2 inflammation. The lungs also contain a small population of $KLRG1^-ST2^+$ ILC2s that develop in the thymus from T cell precursors. Following exposure to airway allergens, such as HDM, lung ILC2s undergo changes in chromatin accessibility, losing some accessible E-protein motifs and gaining motifs that bind the AP-1 TF BATF and C/EBP TFs. The activity of E-protein TFs during ILC2 development from ILC2p to steady-state lung ILC2s promotes increased chromatin accessibility at AP-1 binding sites during type 2 inflammation, including sites proximal to genes that drive ILC2 functionality, such as Ag presentation and cell adhesion. In this inflammatory environment, BATF and C/EBP promote expression of genes that promote ILC2 inflammatory responses, including production of IL-13 and other type 2 cytokines.

to have a reciprocal pattern of accessibility in lung ILC2s compared with E-proteins, with AP-1 and C/EBP motifs enriched, whereas accessible E-protein motifs decreased in response to HDM, compared with cells from PBS-treated controls (Fig. 4C, Supplemental Fig. 3A). Analysis of scRNA-seq data from HDM-treated and untreated lung ILC2s showed an increase in the proportion of *Batf*-expressing cells following HDM treatment and a large proportion of ILC2s expressing various AP-1 family members, with both of these findings not seen for E-proteins (Supplemental Fig. 3B). These data suggest that AP-1 family members but not E-proteins are good candidates to be the primary TFs that regulate changes in gene expression following ILC2 inflammatory responses, consistent with previous reports (10).

Although AP-1 factors appeared to be important in regulating mature lung ILC2 responses to HDM, our findings suggested a role for E-proteins in regulating future ILC2 function during their development from ILC2ps to mature lung ILC2s. In ID1^{tg/WT} mice, in which it was possible to study the function of a large pool of ILC2s that had emerged in the context of E-protein inhibition in thymocytes (21, 22), we observed that ID1^{tg/WT} ILC2s had deficient cytokine-producing capacity in response to HDM (Fig. 3D, Supplemental Fig. 2F). This response was associated with a pathological outcome in response to HDM very similar to that seen in control ID1^{WT/WT} animals (Fig. 3C, Supplemental Fig. 1J), despite the large numbers of ILC2s in the ID1^{tg/WT} lung (Fig. 1C), though it is important to point out that we do not identify the key cellular players in pathology in these animals, and CD4⁺ T cells, in particular, may play a role. In addition, our ATAC-seq analysis across the ILC2 developmental axis highlighted that despite a decrease in accessible E-protein sites in ILC2ps compared with CLPs (Fig. 5A), mature lung ILC2s had increased accessibility of E-protein binding sites compared with CLPs and, by extension, also ILC2ps, and these were largely maintained following exposure to HDM (Fig. 5A). We also identified genes with E-protein motifs in open chromatin gained in ILC2 maturation that were associated with mature ILC2 function and activation. These genes included those associated with MHC II-dependent Ag presentation (largely upregulated) and STAT signaling pathways downstream of cytokine receptor activation (upregulated and downregulated) (Figs. 5C–F and 7), suggesting that E-protein binding acts to open chromatin during ILC2 development and promotes MHC II-related functions in mature ILC2s but plays a more nuanced role in shaping how mature ILC2s respond to activating cytokines (Fig. 5C–F).

Taken together, these data suggest a model in which E-proteins act at an earlier stage in the ILC2 developmental arc and must make way for or set the stage for the action of other TFs like AP-1 and C/EBP family members (Fig. 7). Indeed, analysis of TF motif accessibility in ILC2s with E-protein overexpression *in vitro* led to a loss of ATAC peaks over time, many containing AP-1 binding sites, whereas deletion of *Tcf3/12* in thymocytes resulted in increased *Batf* expression (53). Thus, although E-protein motifs are more accessible in steady-state mature ILC2s than in ILC2s from HDM-treated mice (Fig. 5A), a decrease in E-protein motif accessibility and activity during HDM-driven inflammation may ease AP-1 inhibition to activate expression of AP-1-dependent genes, many of which are associated with ILC2 function (10). The mechanisms that underlie E-protein inhibition during HDM treatment are not elucidated in our current study, though it is possible that exposure to type 2 cytokine signals drives ID protein activity to repress E-protein function, as has been shown previously in T cells following TCR stimulation (54). However, the decrease in *Id2* gene expression in ILC2s from HDM- compared with PBS-treated mice (Fig. 5C) suggests a potentially more complex network of regulation.

Our ATAC-seq analysis of lung ILC2s from ID1^{tg/WT} and ID1^{WT/WT} mice sheds additional light on how E-proteins function during development to shape specifically AP-1-associated ILC2 responses during type 2 inflammation. This analysis highlighted AP-1 TF binding as the main source of differences between ID1^{tg/WT} and ID1^{WT/WT} ILC2s (Fig. 6B, 6D). Notably, genes nearby ATAC peaks specific to ID1^{WT/WT} mice, which were preferentially adjacent to AP-1 motifs (Fig. 6D), were enriched for type 2 inflammation-associated pathways (Fig. 6E), including genes involved in the JAK/STAT cascade and the regulation of the MHC II machinery that was responsive to HDM, including *Ccl5*, *Il2*, *Socs1*, and *H2-Ab1* (Fig. 6F). Genes in similar pathways were also shown to gain nearby E-protein ATAC peaks during development of WT ILC2s (Fig. 5C–F). These data indicate that E-proteins play a critical role in setting the stage during ILC2 development for AP-1 family TF regulation of the lung ILC2 inflammatory response.

Thus, E-proteins may regulate pathways that control ILC2 activation status and functional capacity as regards cytokine production, albeit indirectly. In line with this idea, lung ILC2s from ID1^{tg/WT} mice displayed augmented cytokine production *in vitro* in response to IL-25 and suppressed production in response to IL-33 (17). In contrast, ID1^{tg/WT} thymic ILC2s from naive mice and thymus-derived lung ILC2s from papain-treated mice expressed more IL-5 and IL-13 compared with WT control cells (21, 23). In our study, ILC2s from HDM-treated ID1^{tg/WT} mice displayed decreased type 2 cytokine output compared with ID1^{WT/WT} cells (Fig. 3D, Supplemental Fig. 2F). Future studies are required to determine how the effect of E-protein binding to open chromatin during development shapes ILC2 STAT signaling- and MHC II-dependent functions in response to HDM. MHC II-mediated interactions with CD4⁺ T cells impact the ability of ILC2s to produce cytokine (55), and ILC2 responsiveness to cytokines like IL-25, IL-33, TSLP, and others is critical for shaping their function (56). In the future, it will be important to determine how E-protein and AP-1 activity during development and maturity shapes the regulation of genes involved in JAK/STAT signaling cascades and the MHC II machinery in response to HDM.

Our work highlights a previously unappreciated role for E-protein TFs in regulating key ILC2 functional pathways during development or prior to inflammation (Fig. 7). These data complement our understanding of the importance of E-protein inhibition by ID2 to guide ILC2 and T cell development. Additionally, E-proteins may function as important pioneer TFs during ILC2 maturation to activate functional pathways, with other TFs activated in response to inflammation, further promoting the expression of effector genes. Indeed, E12/E47 can bind to nucleosome-associated DNA (57), a key feature of pioneer TFs that facilitates increased accessibility of previously closed genes (58), allowing binding of other TFs—in this case, potentially an AP-1 family member—that promote gene expression. Future studies that leverage inducible deletion or overexpression of E-proteins as well as chromatin immunoprecipitation sequencing studies to assess E-protein and AP-1 occupancy at key functional genes throughout the ILC2 development and activation arc will be required to determine exactly where and when E-proteins function in the ILC2 regulome to promote poised functionality, changes in chromatin accessibility, and gene expression in response to inflammation. Genome editing of E-protein, AP-1, and C/EBP binding sites near functional genes are needed to test for a direct effect on gene expression levels and ILC2 function. As E-proteins are also critical regulators of T cell responses, parallel studies targeting CD4⁺ T cells are required to fully understand how E-proteins function in lymphocytes to regulate gene expression and function. Our findings demonstrate that these important TFs control ILC2

functional capacity, with significant implications for our understanding of how the regulation of gene expression underpins lung ILC2 effector functions that drive type 2 inflammation.

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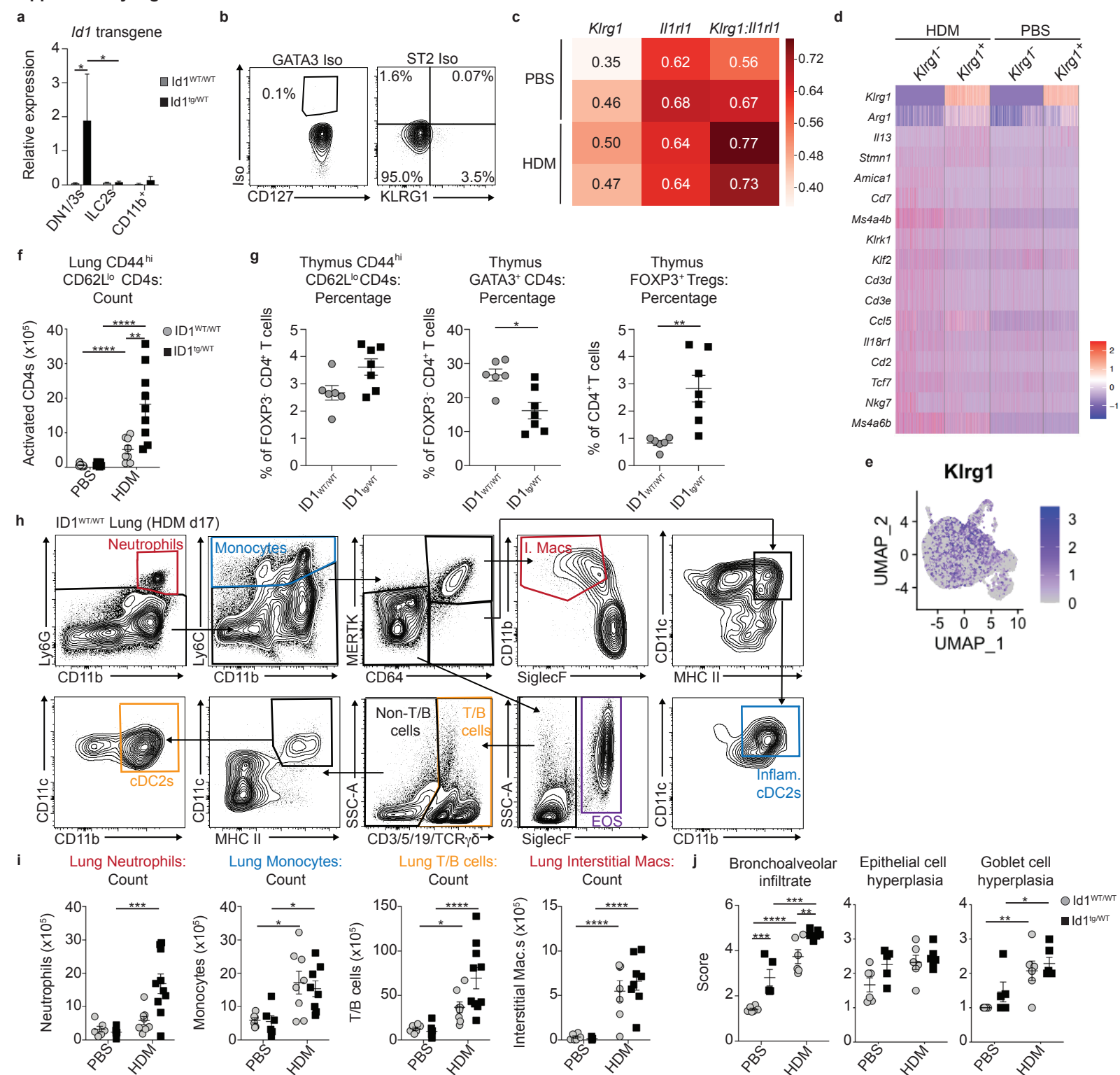
Disclosures

The authors have no financial conflicts of interest.

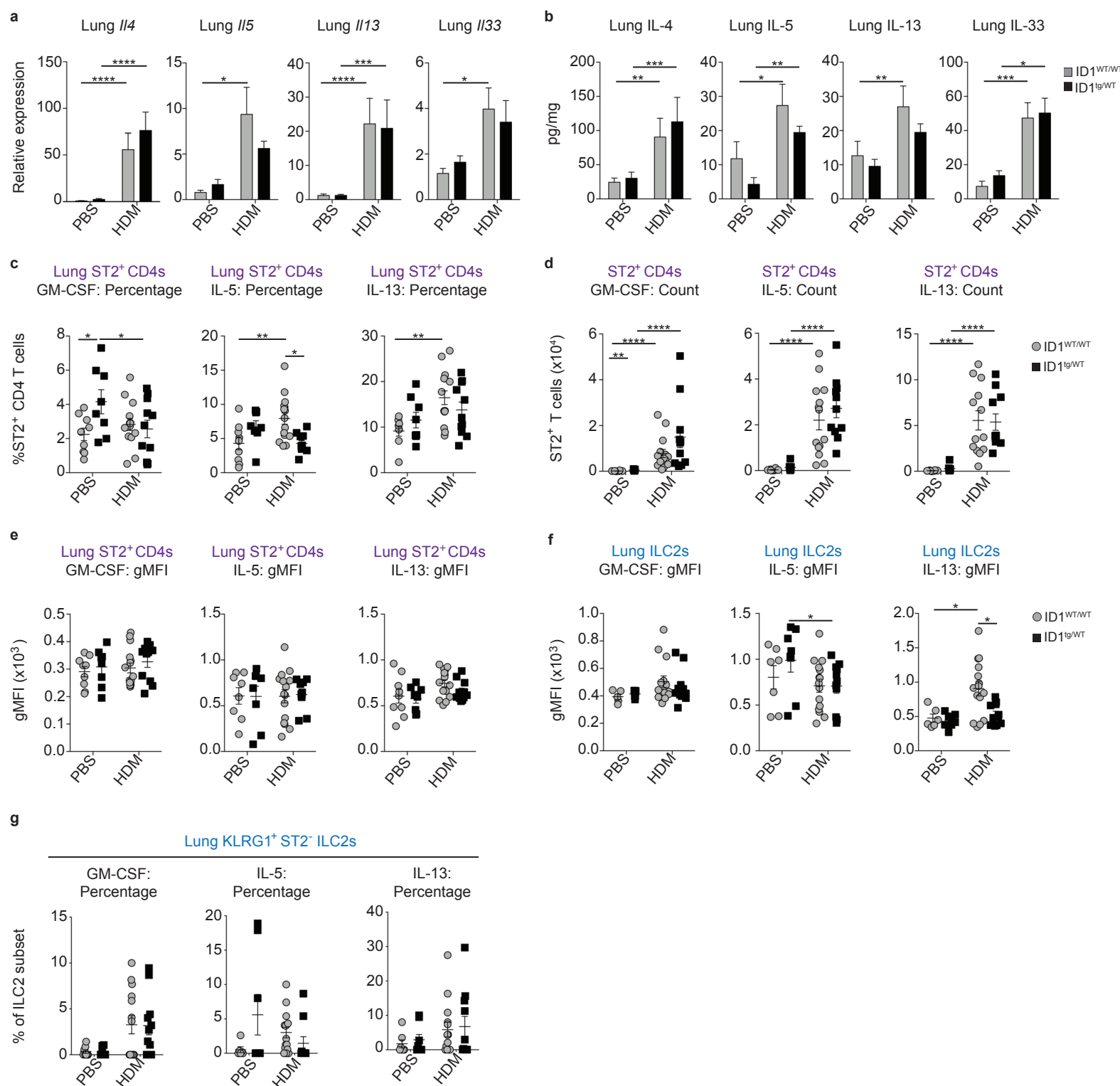
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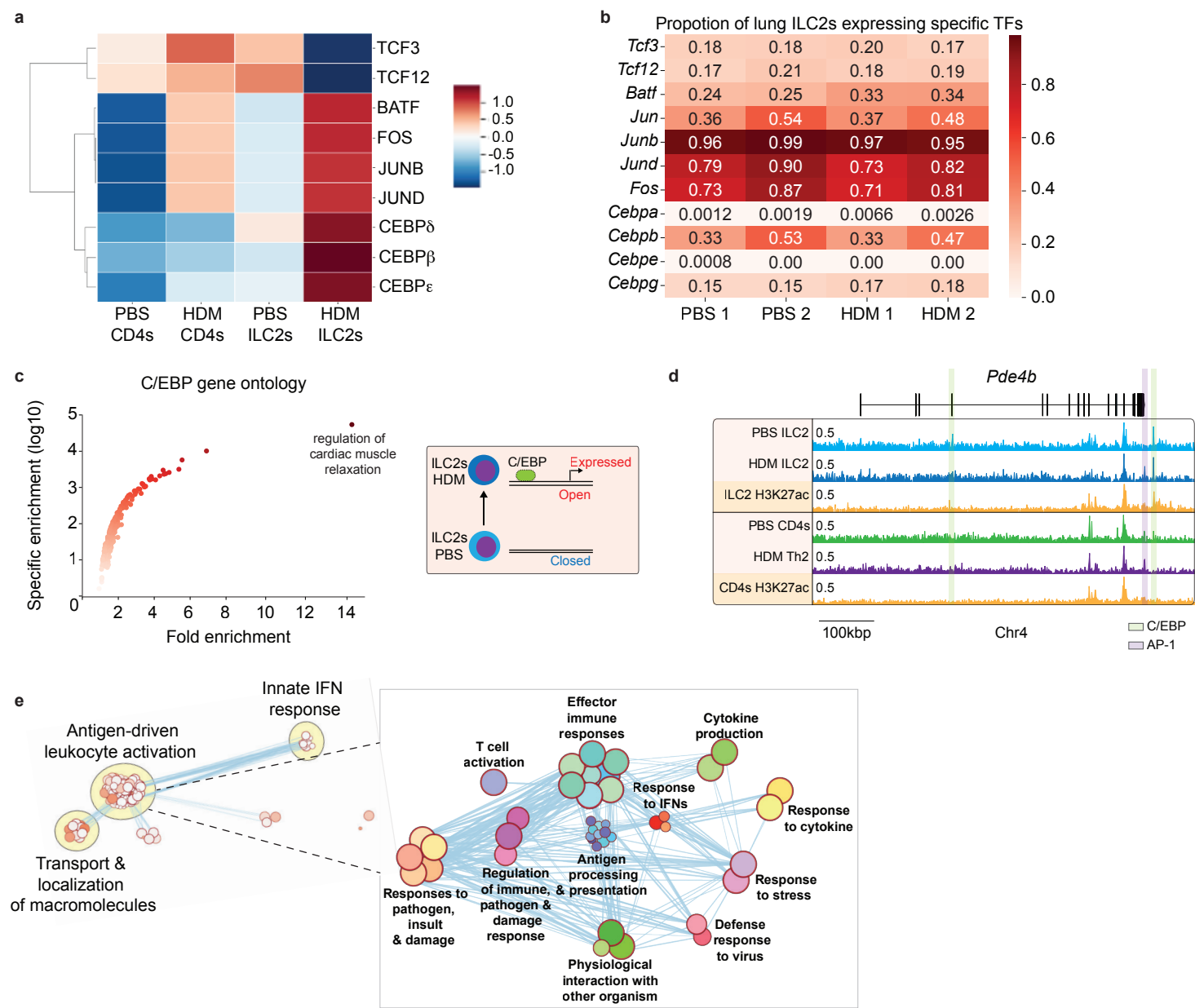
Supplementary Fig. 1. Lung immune cell populations of ID1^{WT/WT} and ID1^{tg/WT} mice following HDM exposure. (a) Relative expression of ID1 transgene by RT-PCR in thymic DN1 and DN3 T cell progenitors, lung ILC2s and CD11b⁺ cells of ID1^{WT/WT} and ID1^{tg/WT}. (b) Isotype controls for GATA3 and ST2 in lung ILC2 staining depicted in Fig. 1. (c) Proportion of lung ILC2s from WT HDM-treated mice expressing *Klrg1* and *Il1rl1* (ST2 gene) and ratio of *Klrg1*⁺ over *Il1rl1*⁺ cells, from scRNA-seq data. (d) Heatmap of genes differentially expressed by *Klrg1*⁺ versus *Klrg1*⁻ lung ILC2s isolated following PBS or HDM treatment analyzed by scRNA-seq. (e) UMAP of normalized relative expression of *Klrg1* transcripts in steady-state ILC2s from PBS- and HDM-treated mice analyzed using published scRNA-seq data. (f) Total numbers of CD44^{hi} CD62L^{lo} CD4⁺ T cells in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice following PBS or HDM treatment. (g) Proportion of FOXP3⁺ CD44^{hi} CD62L^{lo} activated and GATA3⁺ CD4⁺ T cells, and Foxp3⁺ Tregs in the thymus of steady-state ID1^{WT/WT} and ID1^{tg/WT} mice. (h) Gating strategy for lung CD45⁺ immune cell populations. (i) Total numbers of lung-infiltrating inflammatory cells of ID1^{WT/WT} and ID1^{tg/WT} mice. (j) Pathology scores of ID1^{WT/WT} and ID1^{tg/WT} mice. Mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; analyzed using a linear mixed effect model with pairwise comparison; data from 3-4 (b,h,i) or 2 (a,g,j) experiments pooled, data representative of n=4-6 (a,g,j) or n=5-15/ (b,f-i) group. (c-e) scRNA-seq data representative of 2 pooled biological replicates/ sample.



Supplementary Fig. 2. Induction of HDM-driven type 2 inflammation in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice.

(a-b) Type 2 cytokine levels in the lung tissue of PBS- and HDM-treated ID1^{WT/WT} and ID1^{tg/WT} mice by analysis of (a) relative gene expression by realtime PCR and (b) protein levels by ELISA. (c) Proportion and (d) total numbers of GM-CSF⁺, IL-5⁺ and IL-13⁺ ST2⁺ Th2 cells in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice identified by intracellular cytokine staining from PBS- or HDM-treated mice. (e) gMFI of GM-CSF, IL-5 and IL-13 staining of combined IL-5⁺IL-13⁺/IL-5⁺IL-13⁻IL-13⁺ lung ST2⁺ Th2 cells and (f) ILC2s from PBS- and HDM- treated ID1^{WT/WT} and ID1^{tg/WT} mice. (g) Proportion of GM-CSF⁺, IL-5⁺ and IL-13⁺ KLRG1⁺ST2⁻ lung ILC2s in the lungs of ID1^{WT/WT} and ID1^{tg/WT} mice following PBS or HDM treatment. Mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; analyzed using a linear mixed effect model with pairwise comparison, data from 4-6 experiments pooled, n=5-15/ group in total.

Supplementary Figure 3



Supplementary Fig. 3. Genomic analyses of lung ILC2s in the steady state or responding to intranasal HDM allergen challenge.

(a) Heat-map of TF E-protein, AP-1 and C/EBP family motifs identified in ATAC-seq data from lung CD4⁺ T cells and ILC2s from PBS- or HDM- treated WT mice, reported by normalized z-score across conditions/ cell type. **(b)** Proportion of lung ILC2s expressing specific TF genes following intranasal (IN) treatment with PBS or HDM, analyzed from scRNA-seq data. **(c)** Fold vs. specific enrichment of gene ontology (GO) biological processes associated with upregulated genes proximal to open chromatin containing C/EBP motifs gained in lung ILC2s in response to HDM (schematic, right), with the most enriched pathway highlighted. **(d)** ATAC-seq signal in innate and adaptive cells from the lungs of PBS- and HDM- treated mice, and H3K27ac ChIP-seq signal from small intestine ILC2s and LN CD4⁺ T cells, at the *Pde4b* locus. Putative C/EBP (green-shaded) and AP-1 (purple-shaded) binding motifs are highlighted. **(e)** Clustering of gene pathway GO terms activated in ILC2s following exposure to HDM, with zoomed in gene networks from antigen-driven leukocyte activation, filtered on nodes with an FDR<10⁻⁸ identified in bulked scRNA-seq analysis of lung ILC2s. **(a,c,d)** ATAC-seq data representative of 2 samples/ group, collected and pooled from 3 experiments. **(b,c,e)** scRNA-seq data representative of 2 pooled biological replicates/ sample.