The aryl hydrocarbon receptor instructs the immunomodulatory profile of a subset of Clec4a4+ eosinophils unique to the small intestine

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C-type lectin domain family 4, member a4 (Clec4a4) is a C-type lectin inhibitory receptor specific for glycans thought to be exclusively expressed on murine CD8α− conventional dendritic cells. Using newly generated Clec4a4-mCherry knock-in mice, we identify a subset of Clec4a4-expressing eosinophils uniquely localized in the small intestine lamina propria. Clec4a4+ eosinophils evinced an immunomodulatory signature, whereas Clec4a4− eosinophils manifested a proinflammatory profile. Clec4a4+ eosinophils expressed high levels of aryl hydrocarbon receptor (*Ahr*), which drove the expression of Clec4a4 as well as other immunomodulatory features, such as PD-L1. The abundance of Clec4a4+ eosinophils was dependent on dietary AHR ligands, increased with aging, and declined in inflammatory conditions. Mice lacking AHR in eosinophils expanded innate lymphoid cells of type 2 and cleared *Nippostrongylus brasiliensis* infection more effectively than did wild-type mice. These results highlight the heterogeneity of eosinophils in response to tissue cues and identify a unique AHR-dependent subset of eosinophils in the small intestine with an immunomodulatory profile.

eosinophil | intestine | helminth | aryl hydrocarbon receptor | allergy

Eosinophils are a small subset of granulocytes with acidophilic cytoplasmic granules that are generated in the bone marrow (BM) from myeloid progenitors; circulate in the bloodstream; and reside at low numbers in hematopoietic organs, the lung, adipose tissue, and the gastrointestinal tract (1–3). Eosinophil-specific granules contain cationic proteins, such as major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (EPX), that have cytotoxic, ribonuclease, and oxidative activities (3–5). Eosinophils also produce lipid mediators, such as cysteinyl leukotriene C4, which activate cognate cysteinyl leukotriene receptors (CysLTRs) to promote vascular permeability, mucus secretion, and smooth muscle contraction (6). Moreover, eosinophils contain intracellular granules that store preformed cytokines and chemokines, which enable these leukocytes to respond to rapid stimuli and release soluble factors through classical exocytosis, piecemeal degranulation, and eosinophil cytolysis (7). Through this armamentarium of soluble mediators, eosinophils primarily contribute to host defense against helminth infections. Inappropriate recruitment, accumulation, and activation of eosinophils are associated with allergic inflammatory diseases, such as asthma, atopic dermatitis, and food allergy, as well as primary eosinophilic gastrointestinal disorders. Signals that recruit and activate eosinophils during inflammation, such as eotaxins (CCL11, CCL24, CCL26), interleukins (IL-5, IL-13, and IL-33), have been extensively investigated (8–11); however, little is known about the signals that maintain tissue eosinophils quiescent in the steady state.

Clec4a4 (C-type lectin domain family 4, member a4; also known as DCIR2) is a mouse cell surface receptor that belongs to the C-type lectin receptor (CLR) family (12, 13). The carbohydrate recognition domain of Clec4a4 specifically binds to bisecting *N*-acetylglucosamine glycans expressed on normal cells (14). The cytoplasmic domain of Clec4a4 contains an immunoreceptor tyrosine-based inhibition motif (15) that recruits SHP protein tyrosine phosphatases, which mediate a downstream inhibitory pathway (16). Clec4a4 is the cell surface molecule recognized by the monoclonal antibody 33D1 that defines CD8α− conventional dendritic cells (cDCs) (17–21), which specialize in antigen presentation by major histocompatibility complex (MHC) class II to CD4+ T cells (17, 22). Clec4a4 deficiency was shown to enhance the ability of CD8α− cDCs to produce cytokines in response to toll-like receptor ligands and activate T and B cell immune responses against specific antigens (19).

Here, we generated a Clec4a4 reporter mouse and found that Clec4a4 is expressed not only by CD8α− cDCs but also by a subset of eosinophils that are uniquely present

Significance

Eosinophils contribute to type 2 immunity against helminths and allergens. The small intestine harbors eosinophils with incompletely understood pathophysiological roles. Here, we show that intestinal eosinophils include two subsets. One expresses the inhibitory receptor Clec4a4 and the inhibitory ligand PD-L1 and is unique to the small intestine; the other manifests a proinflammatory phenotype. Both subsets are blood derived. Remarkably, Clec4a4+ eosinophils were instructed by the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that imprints many gut immune cells. Selective AHR deletion in eosinophils depleted Clec4a4+ eosinophils, augmented innate lymphocytes producing type 2 cytokines, and enhanced helminth clearance. We conclude that Clec4a4+ eosinophils have immunomodulatory functions, which could be harnessed for the therapy of food allergies and eosinophilic gastrointestinal disorders.

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in the small intestine lamina propria (siLP), where they represent the largest eosinophil population. We demonstrated that these Clec4a4+ eosinophils display a transcriptional profile that cumulatively denotes an immunomodulatory profile, as it is dominated by genes encoding inhibitory receptors, ligands for inhibitory receptors expressed on other cells, leukotriene degrading enzymes, and cholesterol biosynthetic enzymes. Conversely, the relatively minor population of Clec4a4− eosinophils selectively displayed a phenotype prone to inflammation. Parabiosis demonstrated that both subsets are blood borne. The gene expression program of Clec4a4+ eosinophils was in part driven by the aryl hydrocarbon receptor (AHR), which induced expression of Clec4a4 and other immunomodulatory molecules while suppressing proinflammatory programs. The abundance of Clec4a4+ eosinophils increased progressively with age and depended on dietary AHR ligands, but it diminished in various models of helminth infection and inflammation in parallel with an increase of Clec4a4− eosinophils. *Clec4a4*−/− mice, in which both eosinophils and CD8α− cDCs were freed from Clec4a4 inhibition, cleared *Heligmosomoides polygyrus* more efficiently than did wild-type (WT) mice in early stages of the infection. Moreover, *Eo*^{Cre} × *Ahr*$^{fl/fl}$ mice, which selectively lack AHR and AHR-induced genes (including Clec4a4) in eosinophils, cleared *Nippostrongylus brasiliensis* infection more effectively than control mice, perhaps due to increased expansion of unfettered type 2 innate lymphoid cells (ILC2s), which mediate innate antihelminth responses (23, 24). We envision that AHR and its dietary ligands imprint a subset of eosinophils residing in the siLP with an immunomodulatory program. During parasitic infections and other pathogenic insults, changes in the tissue environment reverse the ratio between AHR-imprinted eosinophils and eosinophils with an inflammatory program to elicit an effective host response. This finding may open therapeutic avenues for reprogramming intestinal eosinophils in food allergies as well as primary eosinophilic gastrointestinal disorders of unknown etiology (25).

Results

The Small Intestine Contains Clec4a4+ and Clec4a4− Eosinophil Subsets. To identify Clec4a4-expressing cells, we generated Clec4a4-mCherry knock-in (*Clec4a4*^{mCherry}) mice (Fig. 1*A*). In spleens of *Clec4a4*$^{mCherry/+}$ mice, mCherry was selectively expressed in CD11b+ dendritic cells (DCs) stained by the 33D1 monoclonal antibody, which recognizes Clec4a4 (17) (Fig. 1*B*). This result verified that mCherry messenger RNA was selectively transcribed under the control of the *Clec4a4* promoter and recapitulated the expression pattern of Clec4a4 protein. We next surveyed mCherry expression in CD45+ hematopoietic cells from various tissues of *Clec4a4*$^{mCherry/+}$ mice. A large number of mCherry+ CD45+ cells were observed in the siLP (Fig. 1*C*). These cells encompassed two subsets with distinct levels of CD11c expression (Fig. 1*D*). CD11c^{high} mCherry+ cells expressed CD11b, CD103, and MHC class II (Fig. 1*E*), thereby corresponding to the extensively characterized intestinal CD103+ CD11b+ cDCs (26). However, the CD11c^{low} mCherry+ subset expressed CD11b and SiglecF, a cell surface marker of eosinophils, suggesting expression of Clec4a4 on eosinophils. To corroborate this result, we examined mCherry expression in siLP eosinophils that were gated as side scatter (SSC)^{high}, MHC class II−, CD11b+, and SiglecF+ cells (27). Indeed, mCherry was expressed in ∼70 to 80% of siLP eosinophils, whereas the mCherry− subset represented a minor fraction (Fig. 1*F* and *SI Appendix,* Fig. S1*A*). Both

mCherry+ and mCherry− eosinophils displayed ring-shaped and segmented nuclei indicative of mature eosinophils (Fig. 1*G*). Both subsets expressed the CC chemokine receptor 3 (CCR3), a common eosinophil marker and a receptor for eotaxin (Fig. 1*H*) (1–3). Analysis of intestinal sections of *Clec4a4*$^{mCherry/+}$ mice by fluorescence microscopy at steady state showed that most of the SiglecF+ Clec4a4+ eosinophils were present in the lamina propria of the intestinal villi (Fig. 1*I*). Examination of mCherry expression in eosinophils of colon, adipose tissue, liver, lung, blood, and BM revealed that Clec4a4 expression was restricted to siLP eosinophils (Fig. 1*J*).

Since many immune cell types found in the small intestinal mucosa consist of long-term tissue-resident cells adapted to environmental cues, we sought to examine whether this is the case for Clec4a4+ eosinophils. We performed parabiosis, joining WT (CD45.1) and *Clec4a4*$^{mCherry/+}$ (CD45.2) mice for 6 wk. Analysis of CD45.1 WT mice revealed a substantial proportion of CD45.2+ eosinophils in the small intestine (Fig. 1 *K* and *L*), ∼60% of which were mCherry+ (Fig. 1*M*), suggesting that Clec4a4+ eosinophils are mainly blood borne rather than derived from a local resident population. Interestingly, although the frequency of parabiont-derived eosinophils in the small intestine was similar to that observed in blood and lung, only cells in the small intestine expressed mCherry, indicating that Clec4a4 is induced by the unique environment of the small intestine. Accordingly, mCherry was not expressed in activated lung eosinophils purified from a model of house dust mite–induced inflammation (*SI Appendix,* Fig. S1*B*). Within the small intestine, the representation of Clec4a4+ eosinophils varied in different segments, decreasing from the proximal to most distal regions (*SI Appendix,* Fig. S1*C*). Taken together, these results demonstrate that the small intestine contains Clec4a4+ and Clec4a4− eosinophil subsets in the steady state and that the Clec4a4+ subset is unique to the small intestine due to the impact of the microenvironment on blood-derived eosinophils.

Clec4a4+ and Clec4a4− Eosinophils Display Distinct Receptor Repertoires. To gain more insight into the characteristics of Clec4a4+ eosinophils, we examined genes differentially expressed between fluorescence-activated cell sorting (FACS)-sorted Clec4a4+ and Clec4a4− eosinophils by microarray analysis (the sorting strategy is shown in *SI Appendix,* Fig. S1*A*). Seventy-four transcripts were expressed at least twofold higher in Clec4a4+ eosinophils than in the Clec4a4− population, whereas 85 transcripts had the opposite pattern (Fig. 2 *A* and *B* and *SI Appendix,* Fig. S2 *A* and *B*). Clec4a4+ eosinophils abundantly expressed *Cd22, Cd68, Pecam1,* and *Cd274* (encoding PD-L1), whereas *Itgb7* (encoding the β7-integrin) was more highly expressed by Clec4a4− eosinophils. We confirmed by flow cytometry that a fraction of siLP Clec4a4+ eosinophils expressed CD22, a receptor of the sialoadhesin family that transmits inhibitory signals; all Clec4a4+ eosinophils expressed the lysosomal marker CD68, the adhesion molecule PECAM1, and CD274 (PD-L1), which is the ligand for the inhibitory receptor PD-1 (Fig. 2*C*). Conversely, Clec4a4− eosinophils expressed the lymphocyte Peyer's patch adhesion molecule 1 (LPAM1), which consists of the α4β7-integrin heterodimer. A survey of these cell surface markers in the eosinophils of colon lamina propria, adipose tissue, liver, lung, blood, and BM showed that CD22 was exclusively expressed in siLP eosinophils, corroborating a recent study (28) (Fig. 2*D*); CD68 was expressed by blood eosinophils, and PECAM1 was expressed by liver, lung, blood, and BM eosinophils. LPAM1 was expressed in all Clec4a4− eosinophils of various tissues. Overall, these data validate the identification of two
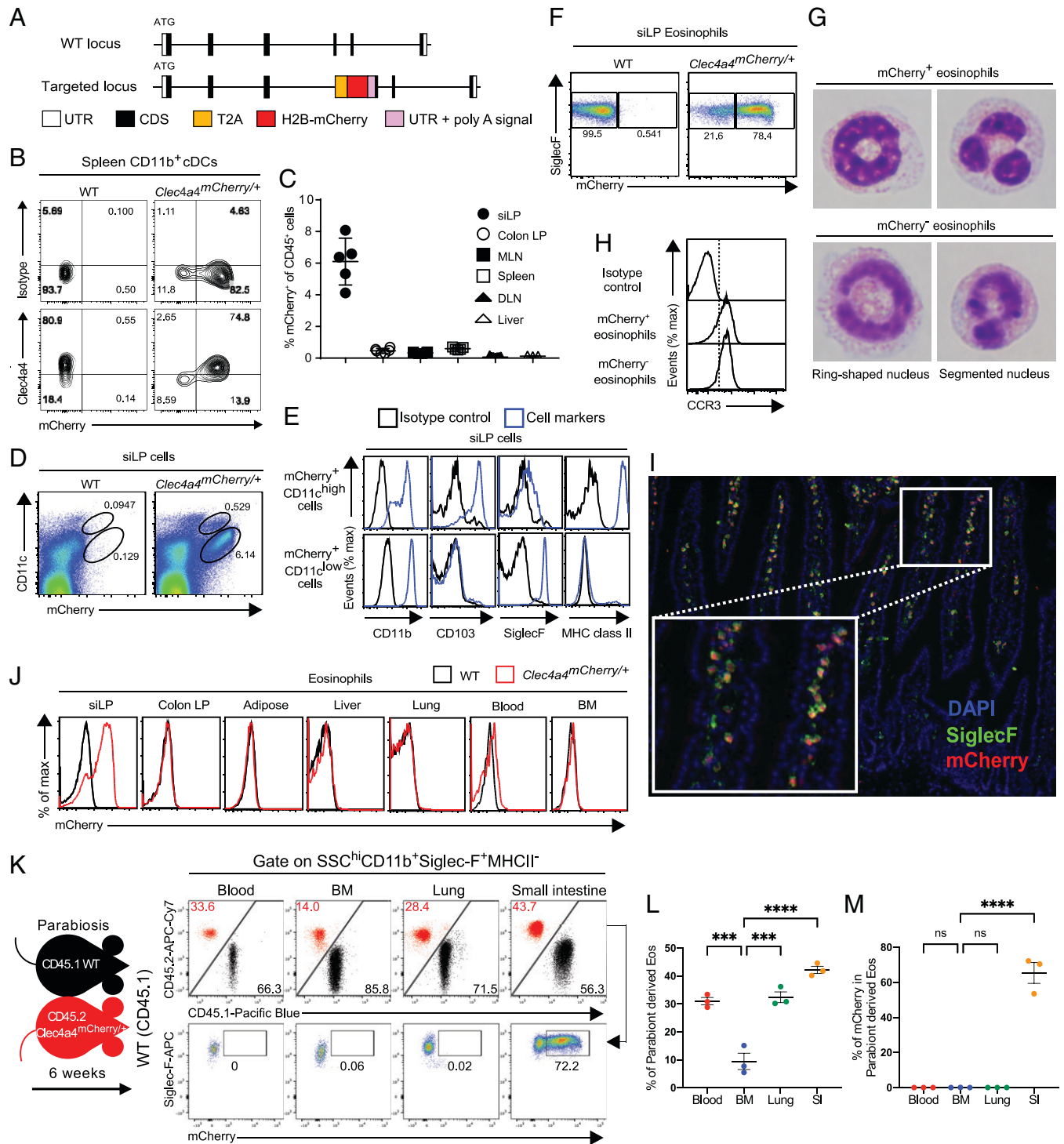
Fig. 1. A heterogeneous population of Clec4a4⁺ and Clec4a4⁻ eosinophils resides in the small intestine. (*A*) Targeting strategy to generate *Clec4a4^mCherry/+* mice. CDS, coding sequence; H2B, human histone H2B; T2A, T2A self-cleaving peptide; UTR, untranslated region. (*B*) Expression of Clec4a4 and mCherry in splenic CD11b⁺ cDCs. (*C*) Frequency of mCherry⁺ cells among CD45⁺ cells (gated on live cells) in the indicated tissues (*n* = 3 to 7). DLN, skin-draining lymph node; LP, lamina propria; MLN, mesenteric lymph node. (*D*) mCherry expression on CD11c^high and CD11c^low CD45⁺ cells derived from siLP (gated on live cells). (*E*) Expression of CD11b, CD103, Siglec-F, and MHC class II on mCherry⁺ CD11c^high CD45⁺ cells and mCherry⁺ CD11c^low CD45⁺ cells derived from siLP (gated on live cells). (*F*) mCherry expression in Siglec-F⁺ eosinophils derived from siLP (gated on SSC^high MHC class II⁻ CD11b⁺ cells). (*G*) Representative pictures of FACS-sorted mCherry⁺ and mCherry⁻ eosinophils from siLP. (*H*) CCR3 expression in mCherry⁺ and mCherry⁻ eosinophils in siLP. (*I*) Representative image showing localization of Siglec-F⁺mCherry⁺ eosinophils in siLP villi of Clec4a4^mCherry/+ mice. *Inset* is an enlargement of *I*. DAPI, 4′,6-diamidino-2-phenylindole. (*J*) mCherry expression in eosinophils in the indicated tissues. (*K*) Parabiosis of CD45.1 WT and CD45.2 Clec4a4^mCherry/+ mice indicates that eosinophils recirculate in siLP, blood, and lung but much less so in BM; Clec4A4 mCherry is only expressed by eosinophils in the siLP. (*L*) Frequency of parabiont-derived eosinophils in the indicated tissues. (*M*) Frequency of parabiont-derived mCherry⁺ eosinophils in the indicated tissues. Results are shown as mean ± SEM. *P* values were calculated using one-way ANOVA and the Tukey's multiple comparisons test or the two-tailed Student's *t* test. ns, not significant. ***$P < 0.001$; ****$P < 0.0001$.

phenotypically distinguishable subsets of siLP eosinophils. The lung was recently reported to include a subset of Siglec-F^int CD62L⁺CD101^low–resident eosinophils and a subset of

Siglec-F^hi CD62L⁻CD101^hi–recruited inflammatory eosinophils (29). However, these subsets were phenotypically distinct from Clec4a4⁺ and Clec4a4⁻ siLP subsets, as both CD62L and
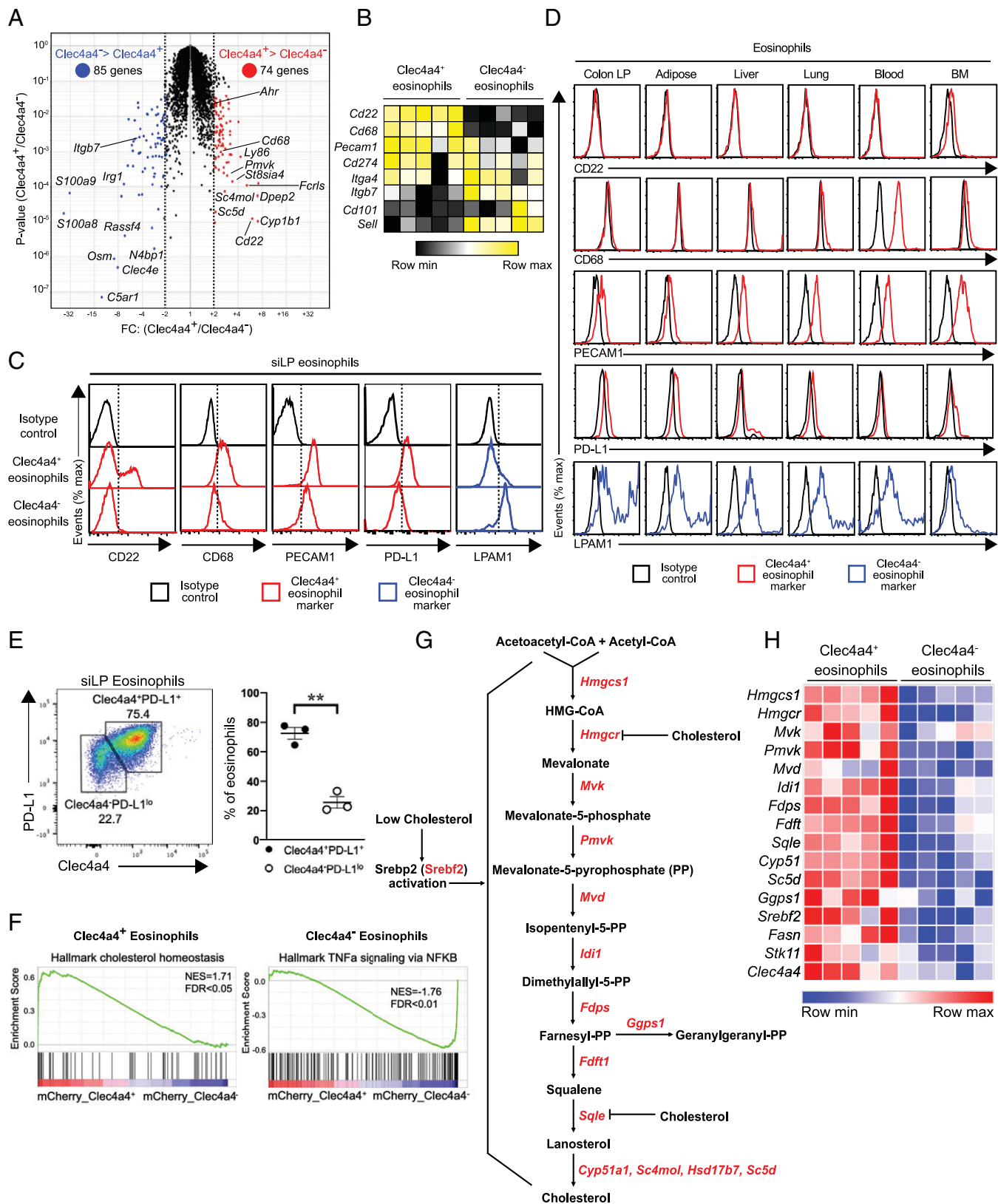
Fig. 2. Transcriptomic profiling and characterization of Clec4a4⁺ and Clec4a4⁻ eosinophil subsets. (*A*) Volcano plot of differentially expressed genes in mCherry⁺ and mCherry⁻ eosinophils (*n* = 5 replicates in each group). Transcripts with FC (fold change) > 2 and *P* < 0.05 in mCherry⁺ and mCherry⁻ eosinophils are depicted in red and blue, respectively. (*B*) Heat map of selected genes specific to Clec4a4⁺ and Clec4a4⁻ eosinophils. (*C*) Differential expression of cell surface (CD22, PECAM1, PD-L1, and LPAM1) and intracellular markers (CD68) by Clec4a4⁺ and Clec4a4⁻ siLP eosinophils. (*D*) Expression of the markers indicated in *C* by eosinophils from the indicated tissues. LP, lamina propria. (*E, Left*) Expression of Clec4a4 and PD-L1 in eosinophils derived from siLP. (*E, Right*) Frequency of Clec4a4⁺PD-L1⁺ and Clec4a4⁻PD-L1ˡᵒ eosinophils in the siLP. (*F*) Enrichment plots demonstrating up-regulated cholesterol homeostasis and TNFα signaling via NF-κB in mCherry⁺ and mCherry⁻ eosinophils, respectively. NES, normalized enrichment score; FDR, false discovery rate. (*G*) Biochemical pathways for de novo cholesterol synthesis. CoA, coenzyme A; HMG, hydroxymethylglutaryl. (*H*) Heat map of genes associated with cholesterol synthesis pathways in mCherry⁺ and mCherry⁻ eosinophils. Results are shown as mean ± SEM. *P* values were calculated using the two-tailed Student's *t* test. **P < 0.01.

CD101 were weakly expressed in siLP eosinophils (*SI Appendix*, Fig. S2*C*).

We noted that Clec4a4⁺ eosinophils expressed higher amounts of signal-regulatory protein alpha (SIRPα) (*SI Appendix*, Fig. S2*D*), a receptor that delivers intracellular inhibitory signals upon recognition of its ubiquitously expressed ligand CD47 (30, 31). Given that CD22 and Clec4a4 have also been shown to be potent inhibitory receptors specific for glycans expressed by B cells (32) and DCs (19), respectively, the concerted expression of these inhibitory receptors suggests that Clec4a4⁺ eosinophils are fairly refractory to activating stimuli. Notably, Clec4a4⁺ eosinophils expressed PD-L1 (Fig. 2 *C–E*), which inhibits ILC2, T cells, and myeloid cells expressing PD-1 (33, 34). Thus, Clec4a4⁺ eosinophils may further maintain a noninflammatory environment by inhibiting immune responses by ILC2s, T cells, and myeloid cells. Conversely, the Clec4a4⁻ subset expressed LPAM1, which interacts with mucosal cell adhesion molecule-1 expressed in vascular endothelium and is required for the homing of eosinophils into the gastrointestinal tract (2), suggesting that Clec4a4⁻ eosinophils may be recently recruited from the blood.

Clec4a4⁺ and Clec4a4⁻ Eosinophils Express Alternative Immunomodulatory and Inflammatory Programs. We next explored transcriptome profiles of Clec4a4⁺ and Clec4a4⁻ eosinophils for different functional properties. Clec4a4⁺ eosinophils selectively expressed *Dipeptidase 2* (*Dpep2*), a membrane-bound dipeptidase that hydrolyses the leukotriene D4 into leukotriene E4 (Fig. 2*A* and *SI Appendix*, Fig. S2 *A* and *B*). LTE4 is the weakest activator of CysLTR1 (6), and DPEP2 has been reported to modulate macrophage inflammation and nuclear factor kappa B (NF-κB) signaling independent of its enzymatic activity (35). Another striking feature of the Clec4a4⁺ eosinophil transcriptome was the concerted expression of many genes controlling the de novo cholesterol biosynthesis pathway (e.g., *Hmgcs1*, *Pvmk*, *Idi1*, *Fdps*, *Fdft1*, *Sqle*, *Sc5d*, *Cyp51*, *Sc4mol*) (36) (Fig. 2 *F–H*). Cholesterol synthesis may be necessary to generate membranes for efficient intracellular storage of eosinophil granules and for secretory vesicles (7).

Clec4a4⁻ eosinophils evinced a clear proinflammatory and tissue repair profile exemplified by the preferential expression of genes involved in inflammation (*S100a8*, *S100a9*, *Tnf*, *Il6*) and fibrosis (*Oncostatin M*, *Osm*) (Fig. 2*A* and *SI Appendix*, Fig. S2 *A* and *B*). The expression of *Irak3*, components of the NF-κB pathway (e.g., *Nfkb1*, *Nfkb2*, *Nfkbia*), *Fas*, and components of the inflammasome (e.g., *Nlrp3*, *Casp4*) corroborated the proinflammatory predisposition of Clec4a4⁻ eosinophils (Fig. 2*F* and *SI Appendix*, Fig. S2*E*). A similar gene expression pattern was observed in BM eosinophils stimulated by IL-33, which activates the NF-κB pathway (37). Overall, these results demonstrate that Clec4a4⁺ and Clec4a4⁻ siLP eosinophilic subsets have distinct immunomodulatory and proinflammatory programs, respectively.

AHR Drives the Profile of Clec4a4⁺ Eosinophils. We asked which transcription factors could drive the different programs of siLP eosinophils. We noted that Clec4a4⁺ eosinophils preferentially expressed the *Ahr* gene and its target *Cyp1b1*, which we confirmed by qRT-PCR analysis (Fig. 3 *A* and *B*). AHR is a member of the Per–Arnt–Sim superfamily of transcription factors that sense environmental signals. In the gut, AHR senses indole compounds that are generated by degradation of nutritional tryptophan by the microbiota or are directly introduced with certain foods, such as cruciferous vegetables (38–42). AHR signaling sustains development and function of many cell types in the intestine, including ILC3s, Th17, DCs, macrophages, intraepithelial lymphocytes (IELs), and enteric neurons (41–45). To validate the impact of AHR on Clec4a4⁺ eosinophils, we examined *Ahr*⁻/⁻ mice. *Ahr*⁻/⁻ siLP eosinophils and DCs did not express Clec4a4, while *Ahr*⁻/⁻ spleen CD11b⁺ cDCs maintained Clec4a4 (Fig. 3*C* and *SI Appendix*, Fig. S3*A*). We also noted that the absence of Clec4a4⁺ eosinophils in *Ahr*⁻/⁻ mice was paralleled by a compensatory increase in Clec4a4⁻ eosinophils, such that the total number of eosinophils was comparable in *Ahr*⁻/⁻ and WT mice (Fig. 3*D*). Thus, AHR is required for Clec4a4 expression, while the overall pool of siLP eosinophils is AHR independent in the steady state. To see whether the requirement of AHR was cell intrinsic or cell extrinsic, we performed a mixed BM chimera experiment. After lethal irradiation, CD45.1⁺ WT mice were transplanted with a 50:50 mixture of CD45.1/2⁺ WT BM and CD45.2⁺ *Ahr*⁻/⁻ BM (Fig. 3*E*). Both WT BM and *Ahr*⁻/⁻ BM successfully reconstituted splenocytes (*SI Appendix*, Fig. S3*B*) and spleen CD11b⁺ Clec4a4⁺ cDCs (Fig. 3 *F* and *G*) in about a 50:50 WT/*Ahr*⁻/⁻ ratio (Fig. 3 *F* and *G*). Small intestinal eosinophils were reconstituted at a 30:70 WT/*Ahr*⁻/⁻ ratio (*SI Appendix*, Fig. S3*B*), indicating that *Ahr*⁻/⁻ eosinophils have a competitive reconstitution advantage over WT eosinophils for reasons that remain to be determined. Notwithstanding, only WT BM reconstituted both Clec4a4⁺ and Clec4a4⁻ eosinophils, whereas *Ahr*⁻/⁻ BM could only generate Clec4a4⁻ eosinophils (Fig. 3 *F* and *G*). We conclude that the requirement of AHR for Clec4a4 expression in eosinophils is cell intrinsic.

We next asked whether AHR is just required for Clec4a4 expression or for the differentiation of the entire Clec4a4⁺ eosinophil program. Since all eosinophils were Clec4a4⁻ in *Ahr*⁻/⁻ mice, we selected genes differentially expressed between Clec4a4⁺ and Clec4a4⁻ eosinophils of WT mice and ascertained their expression in Clec4a4⁻ eosinophils of *Ahr*⁻/⁻ mice. Among the genes more abundantly expressed in WT Clec4a4⁺ than in WT Clec4a4⁻ eosinophils, 20 genes, including *Cd22*, were more highly expressed in Clec4a4⁻ eosinophils of *Ahr*⁻/⁻ mice than in Clec4a4⁻ eosinophils of WT mice, indicating AHR independency (*SI Appendix*, Fig. S3*C*). Conversely, 21 of the genes highly expressed in WT Clec4a4⁺ but not in WT Clec4a4⁻ eosinophils were expressed at low levels in Clec4a4⁻ eosinophils of *Ahr*⁻/⁻ mice, indicating AHR dependency; these genes included *Dpep2* as well as genes involved in cholesterol metabolism, including *Idi1*, *Sc5d*, *Hmgcs1*, and *Hsd17b7* (*SI Appendix*, Fig. S3*C*).

Among the genes expressed less in WT Clec4a4⁺ than in WT Clec4a4⁻ eosinophils, 17 genes were expressed at higher levels in *Ahr*⁻/⁻ eosinophils, indicating suppression by AHR (*SI Appendix*, Fig. S3*D*). These genes included many inflammatory molecules, such as *Il6*, *S100a8*, and *S100a9*. In comparison with their relatively high expression in Clec4a4⁻ eosinophils of WT mice, 10 genes were equally suppressed in Clec4a4⁺ eosinophils of WT mice and Clec4a4⁻ eosinophils of *Ahr*⁻/⁻ mice, including *Tnf*, *Nlrp3*, and *Fas* (*SI Appendix*, Fig. S3*D*). Taken together, these results demonstrate that the differentiation of Clec4a4⁺ eosinophils is both AHR dependent and independent. Genes either induced or suppressed in an AHR-independent fashion in Clec4a4⁺ eosinophils may be controlled by other transcription factors, such as NR4A2 (also called Nurr1), which is more abundantly expressed in Clec4a4⁺ than in Clec4a4⁻ eosinophils (*SI Appendix*, Fig. S3*C*) and has been shown to control inflammation and lipid metabolism (46).

Differentiation of Clec4a4⁺ Eosinophils Is Partly Dependent on Dietary AHR Ligands. Since AHR senses indole compounds generated by intestinal microbiota or introduced with certain
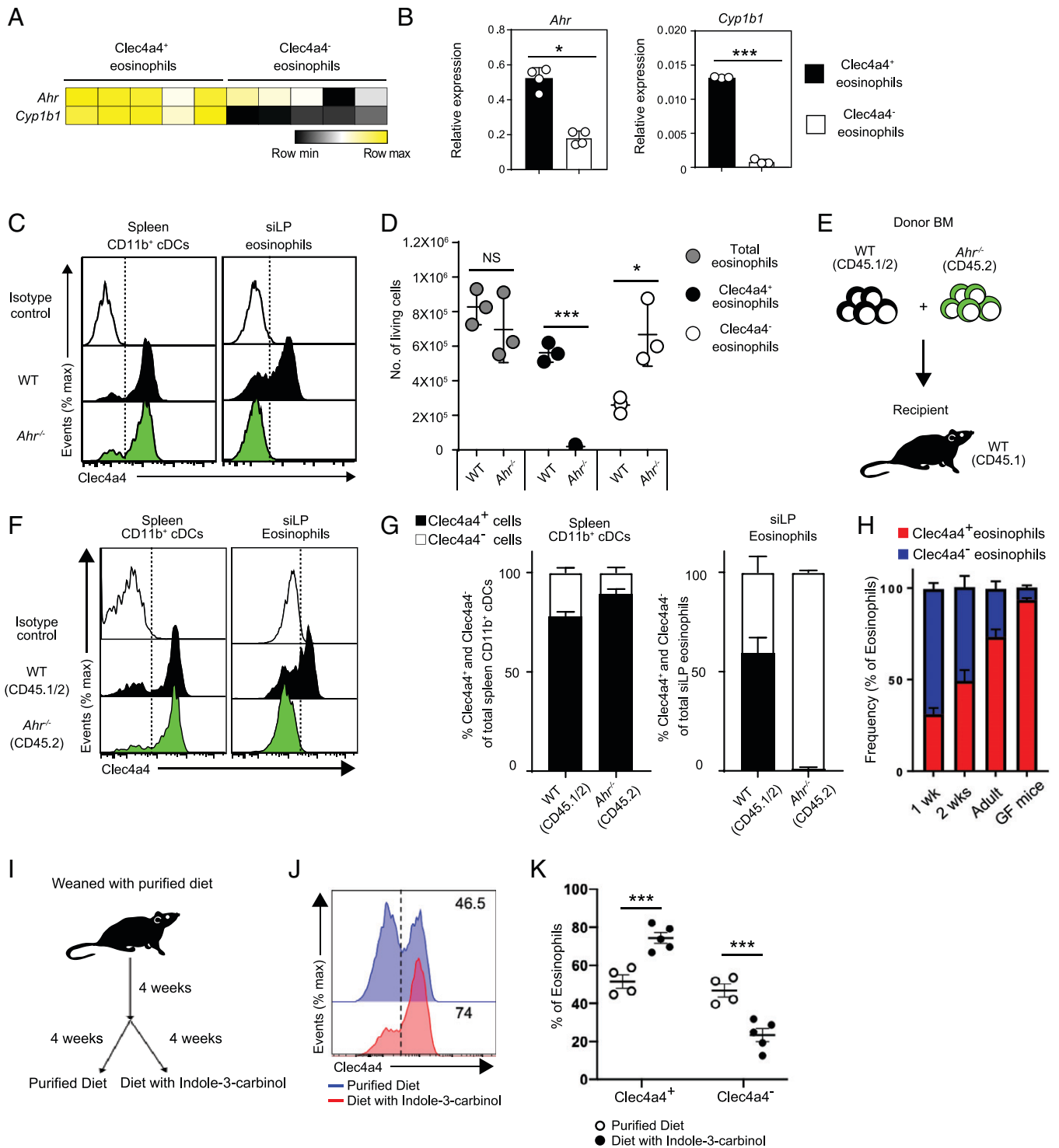
Fig. 3. Development of the Clec4a4+ eosinophil subset is dependent on aging and on the AHR pathway. (*A*) Heat map of *Ahr* and *Cyp1b1* in mCherry+ and mCherry− eosinophils. (*B*) qRT-PCR of *Ahr* and *Cyp1b1* transcripts in Clec4a4+ and Clec4a4− eosinophils (*n* = 4 replicates). (*C*) Clec4a4 expression in splenic CD11b+ cDCs and siLP eosinophils from WT and *Ahr*−/− mice. (*D*) Absolute numbers of Clec4a4+ and Clec4a4− eosinophils in WT and *Ahr*−/− mice (*n* = 3 in each group). (*E*) Experimental strategy for mixed BM chimeras. (*F*) Clec4a4 expression in splenic CD11b+ cDCs and siLP eosinophils derived from WT and *Ahr*−/− BM cells after reconstitution. (*G*) Frequency of Clec4a4+ and Clec4a4− splenic CD11b+ cDCs and siLP eosinophils in BM chimeras. (*H*) Frequency of Clec4a4+ and Clec4a4− siLP eosinophils in WT mice at 1, 2, and 10 to 12 wk of age (*n* = 3, 6, and 7 replicates, respectively) and in GF mice (*n* = 5). (*I*) Experimental time line for feeding mice with a purified diet free of AHR ligands or a purified diet containing the AHR ligand I3C. (*J*) Representative Clec4a4 expression in siLP eosinophils derived from mice fed with a purified diet free of AHR ligands or a purified diet containing AHR ligands. (*K*) Frequency of Clec4a4+ and Clec4a4− siLP eosinophils in mice fed with a purified diet free of AHR ligands (*n* = 4) or a purified diet containing AHR ligands (*n* = 5). Results are shown as mean ± SEM. *P* values were calculated using the two-tailed Student's *t* test. NS, not significant. *P < 0.05; ***P < 0.001.

nutrients, we hypothesized that Clec4a4+ eosinophils may develop upon colonization of the intestinal tract by a mature microbiota and/or introduction of a normal diet after weaning. Consistent with this hypothesis, Clec4a4+ cells within siLP

eosinophils were more abundant in young adult mice than in 1- or 2-wk-old mice (Fig. 3*H*). To directly test the impact of microbiota on siLP eosinophils, we examined germ-free (GF) mice but found that Clec4a4+ eosinophils were equally

represented in GF and control specific pathogen–free (SPF) mice (Fig. 3H). We also treated SPF mice with antibiotics and then, colonized them with *Lactobacilluls reuteri* 100-23, which produces large amounts of indole ligands when fed a tryptophan-rich diet (44, 47). However, enrichment of microbiota with indole-producing *L. reuteri* did not expand Clec4a4+ eosinophils further than that seen in mice colonized with an *L. reuteri* mutant unable to produce AHR ligands (*SI Appendix*, Fig. S3E). We hypothesized that the lack of bacterial-derived AHR ligands may be compensated by dietary ligands. To test the impact of dietary AHR ligands, weaned mice were fed a purified diet lacking AHR ligands for 4 wk and then, kept for an additional 4 wk on this diet with or without additional indole-3-carbinol (I3C), a plant-derived precursor of AHR ligands (Fig. 3I). The population of Clec4a4+ eosinophils declined in mice fed the purified diet but was restored by the I3C supplement (Fig. 3 J and K), corroborating a dependency of Clec4a4+ eosinophils on nutritional AHR ligands.

Clec4a4+ Eosinophils Wane during Intestinal Inflammation.
We sought to assess the presence of Clec4a4+ and Clec4a4− eosinophils in response to infection with *H. polygyrus*, a natural intestinal parasite of mice (48). In this experimental model, BALB/c mice are infected with *H. polygyrus* by oral gavage with infective larvae, which penetrate into the submucosa of the small intestine and develop into adult worms that return into the intestinal lumen, mate, and produce eggs that are excreted in the feces. We noted that after infection with *H. polygyrus*, Clec4a4+ eosinophils declined by day 8 postinfection, whereas Clec4a4− eosinophils expanded in compensation (*SI Appendix*, Fig. S4A). A similar decrease of Clec4a4+ eosinophils paralleled by a compensatory increase of Clec4a4− eosinophils was observed in other intestinal inflammatory conditions, such as those induced in a model of high-fat diet (*SI Appendix*, Fig. S4B) and in a model of food allergy caused by gastrointestinal intolerance to ovalbumin (*SI Appendix*, Fig. S4 C–F). These findings raise the possibility that Clec4a4+ eosinophils become Clec4a4− in pathological settings, acquiring a proinflammatory program more suitable for host responses. In addition, newly recruited Clec4a4− eosinophils may fail to acquire the AHR-induced profile.

Clec4a4 and AHR Imprinting Attenuate Host Defense against Parasites.
Given that Clec4a4 is an inhibitory receptor, we hypothesized that a deletion of Clec4a4 may augment the early responses against parasites, such as *H. polygyrus*. To test this, we infected homozygous *Clec4a4mCherry/mCherry* mice, in which both endogenous *Clec4a4* genes are disrupted by *mCherry*. Mice were infected with *H. polygyrus* and examined for the numbers of eggs in the feces on days 10, 12, 14, 16, and 18 postinfection. *Clec4a4mCherry/mCherry* mice had lower egg counts than did WT mice on days 12 and 14 postinfection (Fig. 4A), suggesting that the release of quiescent eosinophils from Clec4a4-mediated inhibition enhances early host responses against helminth infection.

Since Clec4a4 is expressed in both eosinophils and CD11b+ DCs, both of which may contribute to host response, we sought to more selectively establish the impact of Clec4a4 and possibly, other AHR-induced genes on the clearance of helminth infection. For this purpose, we crossed mice with floxed alleles of *Ahr* (*Ahrfl/fl*) with an *EoCre* deleter mice, in which Cre recombinase is driven by the endogenous promoter of *Epx* (49). A marked reduction of Clec4a4+ eosinophils was evident in the small intestine of *EoCre* × *Ahrfl/fl* mice (Fig. 4B). This finding was consistent with the cell-intrinsic requirement of AHR for

Clec4a4 expression observed in the mixed BM chimera experiment (Fig. 3F). The expression of PD-L1 on small intestine eosinophils was also markedly reduced in *EoCre* × *Ahrfl/fl* mice (Fig. 4 B and C). We next challenged *EoCre* × *Ahrfl/fl* mice with *H. polygyrus*. Fecal content of *H. polygyrus* eggs and granuloma counts were similar in *EoCre* × *Ahrfl/fl* mice and control mice (*SI Appendix*, Fig. S5). We further infected mice with *N. brasiliensis*, which elicits a more rapid gastrointestinal expulsion than *H. polygyrus*. While clearance of *H. polygyrus* infection in C57BL/6 mice requires up to 20 wk and a sustained T cell response (50), adult *N. brasiliensis* worms are normally expelled from the intestines of C57BL/6 mice by day 7 postinfection. *N. brasiliensis* counts were lower in *EoCre* × *Ahrfl/fl* mice than in control mice on day 6 postinfection (Fig. 4D). We noted that enhanced worm expulsion was paralleled by an increase in ILC2s in mesenteric lymph nodes (Fig. 4 E and F). ILC2s mediate antiparasitic responses through secretion of IL-5 and IL-13 (23, 24). Since ILC2s express the inhibitory receptor PD-1 (34) and Clec4a4+ eosinophils express PD-L1, the lack of this inhibitory interaction in *EoCre* × *Ahrfl/fl* mice may facilitate ILC2 expansion. We conclude that AHR-imprinted Clec4a4+ eosinophils attenuate intestinal parasite clearance mechanisms.

Discussion

Eosinophils contribute to host defense against helminth infections through the release of granule proteins, leukotrienes, and cytokines but can also cause allergic inflammation (1–3). Using a newly generated reporter mouse for the inhibitory receptor Clec4a4, we have identified a unique population of eosinophils exclusively found in the siLP. In addition to Clec4a4, the receptor repertoire of these cells included other inhibitory receptors, such as CD22 (28) and SIRPα (30, 31). The concomitant expression of PD-L1, which inhibits ILC2s, T cells, and myeloid cells expressing PD-1, and DPEP2, which degrades leukotrienes, indicates that Clec4a4+ eosinophils may be both refractory to activation and prone to restrain intestinal immune responses. This large population of Clec4a4+ eosinophils was accompanied by a smaller population of Clec4a4− eosinophils with a proinflammatory and tissue repair program specifying tumor necrosis factor (TNF), IL-6, and OSM among other soluble mediators. These cells were poorly represented in the steady state but expanded in inflammatory conditions, while Clec4a4+ eosinophils declined. Gut Clec4a4+ and Clec4a4− eosinophils were phenotypically distinct from other eosinophil subsets recently reported in the lung, such as lung-resident eosinophils (29) and airway-recruited inflammatory eosinophils (29). We conclude that eosinophils, like other immune cell types, exhibit a tissue diversity that reflects the influence of microenvironmental factors.

Remarkably, the phenotype of Clec4a4+ eosinophils was in part instructed by AHR, which also controls the development and function of many other gut immune cell populations, including ILC3s, Th17, DCs, macrophages, and IELs, as well as enteric neurons (41–45). AHR drove expression of Clec4a4 as well as other immunomodulatory molecules, such as DPEP2 and PD-L1. A very recent study independently corroborated that eosinophils of the gastrointestinal tract display an AHR-dependent profile (51). In the gut, AHR is activated by indoles derived from vegetables that contain tryptophan catabolic products (38–42) and microbial degradation of nutritional tryptophan (47). Accordingly, Clec4a4+ eosinophils were depleted in mice fed an AHR ligand–deprived diet but restored by a supplement of nutritional I3C; although not formally confirmed by us, I3C is known to generate the biologically active AHR
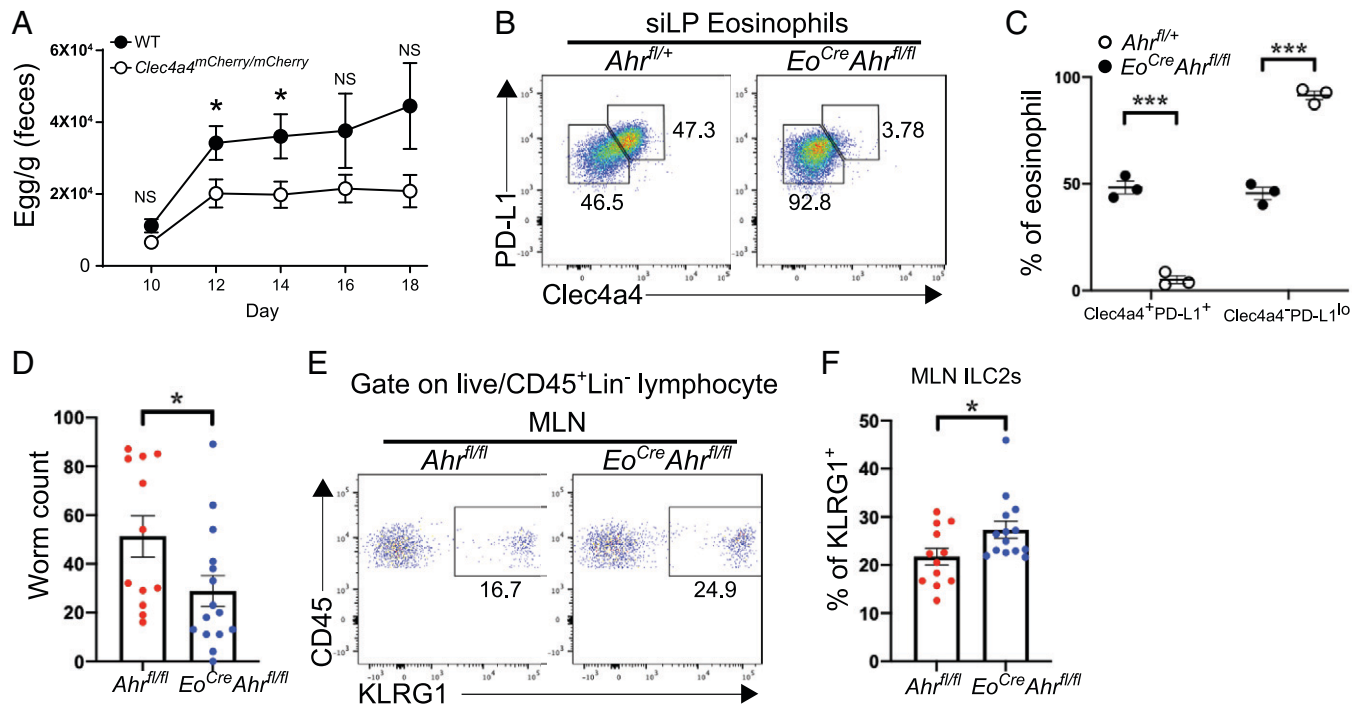
Fig. 4. Clec4a4$^+$ eosinophils modulate type 2 immunity during helminth infection. (*A*) Egg counts in feces of WT and Clec4a4-deficient (*Clec4a4$^{mCherry/mCherry}$*) mice (*n* = 13 in each group) at the indicated time points during *H. polygyrus* infection. (*B*) Expression of Clec4a4 and PD-L1 in siLP eosinophils derived from *Ahr$^{fl/+}$* and *Eo^{Cre}Ahr$^{fl/fl}$* mice. (*C*) Frequency of Clec4a4$^+$PD-L1$^+$ and Clec4a4$^-$PD-L1^{lo} siLP eosinophils in *Ahr$^{fl/+}$* and *Eo^{Cre}Ahr$^{fl/fl}$* mice (*n* = 3 each group). (*D*) Worm counts in *Ahr$^{fl/fl}$* (*n* = 12) and *Eo^{Cre}Ahr$^{fl/fl}$* (*n* = 15) mice at day 6 postinfection with *N. brasiliensis*. (*E*) Representative percentages of KLRG1$^+$ ILC2s among CD45$^+$ cells in mesenteric lymph nodes (MLNs) of *Ahr$^{fl/fl}$* and *Eo^{Cre}Ahr$^{fl/fl}$* mice. A gate was applied on lineage-negative cells. Lineage markers included CD3, CD4, CD8, CD19, and NK1.1. (*F*) Frequency of ILC2s in MLNs from *Ahr$^{fl/fl}$* (*n* = 12) and *Eo^{Cre}Ahr$^{fl/fl}$* (*n* = 14) mice at day 6 postinfection with *N. brasiliensis*. Results are shown as mean ± SEM. *P* values were calculated using the two-tailed Student's *t* test or one-way ANOVA and the Tukey's multiple comparisons test. NS, not significant. *$P < 0.05$; ***$P < 0.001$.

ligand indolo[3,2-b]carbazole in the acidic pH of the stomach. Clec4a4$^+$ eosinophils were similarly represented in GF mice and SPF mice; moreover, this population did not preferentially expand in mice with a microbiota enriched with AHR ligand–producing bacteria, suggesting redundancy between dietary and microbiota-generated AHR ligands. Future studies will be necessary to test the relative impact of dietary and microbiota-derived AHR ligands in the generation of Clec4a4$^+$ eosinophils by comparing the abundance of these cells in GF and SPF mice fed a purified diet and reconstituted with microbes either competent or unable to produce AHR ligands.

The instruction of Clec4a4$^+$ eosinophils by AHR ligands is reminiscent of the tissue imprinting previously reported in gut-resident immune cells, such as ILCs and macrophages. However, our parabiosis experiment demonstrated that Clec4a4$^+$ eosinophils are blood derived, while tissue-resident ILCs and macrophages are long-lived tissue cells that renew from local progenitors (52–54). We conclude that blood eosinophils adapt to the environment and acquire Clec4a4$^+$ features soon after reaching the small intestine. Notably, during intestinal *H. polygyrus* infection or models of inflammation, the representation of Clec4a4$^+$ eosinophils rapidly declined, paralleled by an increase of Clec4a4$^-$ eosinophils. We envision that Clec4a4$^+$ eosinophils derive from blood Clec4a4$^-$ eosinophils instructed by AHR signaling. In inflammatory conditions, this imprinting may weaken or vanish because of decreased AHR expression or less accessible AHR ligands in the altered tissue environment. Further studies are needed to test this hypothesis.

We noted that the functional impact of AHR-imprinted eosinophils in antihelminth defense was different in the two models of helminth infection in *Eo^{Cre}* × *Ahr$^{fl/fl}$* mice that we tested. Clearance of *H. polygyrus* infection was similar in *Eo^{Cre}* × *Ahr$^{fl/fl}$* and control mice, a result that was also recently reported (51).

Since *H. polygyrus* infection lasts up to 20 wk in B6 mice and requires a robust Th2 response for worm clearance, this result probably reflects the limited contribution of eosinophils to this infection model (55, 56). Conversely, *N. brasiliensis* was more effectively cleared in *Eo^{Cre}* × *Ahr$^{fl/fl}$* mice. This phenotype correlated with an increased frequency of ILC2s in the mesenteric lymph nodes, which may reflect release of PD-1$^+$ ILC2s from the inhibitory constraints imposed by PD-L1 expressed on eosinophils (34). Whether Th2 responses are also altered in this scenario remains to be established. We envision that the immunomodulatory effect of AHR-dependent eosinophils may preferentially emerge in this model because *N. brasiliensis* infection in C57BL/6 mice elicits a more rapid and robust gastrointestinal immune response in which eosinophils may play a more prominent role (57).

To date, human counterparts of gut Clec4a4$^+$ and Clec4a4$^-$ eosinophils have not been identified. In the mouse, *Clec4a4* is one of four closely linked CLR genes, *Clec4a1* to *-4* (also known as *Dcir1* to *-4*); in humans, only one DCIR molecule exists, which is expressed by CD14$^+$ monocytes, CD15$^+$ granulocytes, DC subsets, and B cells in the peripheral blood (15, 58–60). Thus, more eosinophil-specific markers should be investigated. The identification of an AHR-imprinted eosinophil subset in human intestine may open therapeutic avenues aimed at reprogramming eosinophils in food allergies and primary eosinophilic gastrointestinal disorders that are caused by abnormal eosinophil activation (25).

Materials and Methods

Experimental details on animals, cell extraction from tissues, antibody staining for flow cytometry and sorting, microarray analyses, parasitic infections, and statistical analyses for this study are described in detail in *SI Appendix, Materials and Methods*.

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