Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation

Laurel A Monticelli1, Michael D Buck2, Anne-Laure Flamar1, Steven A Saenz1,6, Elia D Tait Wojno1,6, Naomi A Yudanin1, Lisa C Osborne1,6, Matthew R Hepworth1,6, Sara V Tran1, Hans-Reimer Rodewald3, Hardik Shah4, Justin R Cross4, Joshua M Diamond5, Edward Cantu5, Jason D Christie5, Erika I Pearce2 & David Artis1

Group 2 innate lymphoid cells (ILC2s) regulate tissue inflammation and repair after activation by cell-extrinsic factors such as host-derived cytokines. However, the cell-intrinsic metabolic pathways that control ILC2 function are undefined. Here we demonstrate that expression of the enzyme arginase-1 (Arg1) during acute or chronic lung inflammation is a conserved trait of mouse and human ILC2s. Deletion of mouse ILC-intrinsic Arg1 abrogated type 2 lung inflammation by restraining ILC2 proliferation and dampening cytokine production. Mechanistically, inhibition of Arg1 enzymatic activity disrupted multiple components of ILC2 metabolic programming by altering arginine catabolism, impairing polyamine biosynthesis and reducing aerobic glycolysis. These data identify Arg1 as a key regulator of ILC2 bioenergetics that controls proliferative capacity and proinflammatory functions promoting type 2 inflammation.

The innate lymphoid cell (ILC) family orchestrates immunity, inflammation, metabolic homeostasis and tissue repair in multiple tissues, including intestine, liver, adipose, skin and lung. Group 2 ILCs (ILC2s) are the dominant ILC population in human and murine lung, where they act as key initiators of allergen- and non-allergen-induced type 2 inflammation and promote airway remodeling. ILC2s are activated by cell-extrinsic environmental cues such as the cytokines interleukin-25 (IL-25), IL-33 and thymic stromal lymphopoietin 1,2. However, the cell-intrinsic pathways that regulate ILC2 effector function remain poorly characterized. In particular, regulation of cell metabolism is a critical determinant of adaptive lymphocyte development and function, although whether cell-intrinsic metabolic signals influence ILC biology is unknown. The enzyme arginase-1 (Arg1) has been identified as a marker of fetal intestinal ILC precursors and adult lung ILC2s, although the functional significance of Arg1 enzymatic activity in ILC2s remains unclear. Arg1 metabolizes the amino acid l-arginine to generate urea and ornithine, which is further metabolized into the downstream metabolites proline and polyamines that drive collagen synthesis and bioenergetic pathways critical for cell proliferation, respectively. Although homeostatic l-arginine metabolism occurs primarily in the liver to complete the urea cycle, immune cells can serve as critical extrahepatic sites of Arg1 activity during infection and tissue inflammation. Particularly in the context of cancer or type 2 cytokine–driven inflammation in the intestine, liver and skin, Arg1 activity is a key signature of alternatively activated macrophages (AAMacs). AAMac-derived Arg1 acts primarily extrinsically, promoting wound healing and tissue fibrosis by triggering collagen synthesis by fibroblasts or by limiting T cell responses via nutrient deprivation of l-arginine. In contrast, evidence supporting the functional significance of AAMac-derived Arg1 enzymatic activity in the lung remains controversial. For example, models targeting macrophage-specific Arg1 have failed to recapitulate the effects observed in studies using global inhibition of Arg1 to dampen airway inflammation, which suggests that other cell populations might be responsible for Arg1’s ability to promote the development of lung disease. We demonstrate here that Arg1 has a critical cell-intrinsic role in regulating ILC2 metabolism and the development of type 2 inflammation.

RESULTS
Constitutive Arg1 expression in precursor and mature ILC2s
Arg1 expression has been reported in a population of ILC precursors in the fetal intestine and in mature ILC2s in the lung. Whether Arg1 is differentially expressed in distinct adult ILC precursors and...
Constitutive Arg1 expression is a conserved trait of precursor and mature ILC2s across diverse tissue sites. (a) Flow cytometric analysis of Arg1-YFP expression in bone marrow HSCs (Lin−CD90−CD127+CD25−C57Bl/6J WT, black), CLPs (Lin−CD90−CD127+CD25−C57Bl/6J WT, blue), and CHILPs (Lin−CD90−CD127+CD25−CD3−CD19−F4/80−MHCII+), eosinophils (Lin−CD90−CD127+CD25−CD3−CD19−F4/80+MHCI+), NK cells (Lin−CD90−CD127+CD25−CD3−CD19−CD4−), and B cells (Lin−CD90−CD127+CD25−CD3−CD19−CD4+). (b) Flow cytometric analysis of Arg1-YFP expression in immature ILC2Ps (Lin−CD90−CD127+CD25−*α4β1−Flt3−Sca-1+c-Kit−) and mature ILC2s (Lin−CD90−CD127+CD25−CD3−CD19−CD4−) from C57Bl/6J WT mice. (c) Flow cytometric analysis of intracellular Arg1 expression or Arg1-YFP expression in mature ILC2s (Lin−CD45−CD90−CD25−IL-33R+) from naive lung tissue of C57Bl/6J WT mice. (d) Flow cytometric analysis of Arg1-YFP expression in mature ILC2s (Lin−CD45−CD90−CD127+IL-33R+) from naive spleen (SP), mesenteric lymph node (MLN), small intestine (SI), and epididymal white adipose tissue (EWAT) from C57Bl/6J WT mice. (e) Sequential flow cytometric gating in naive lung tissue from Arg1-YFP mice. Lineage-negative gating (Lin−) includes markers for CD3, CD4, FcεR1, NK1.1, CD11b, B220, and CD11c. Numbers indicate the frequency of flow cytometric events. (f) Quantification of the relative frequency of Arg1-YFP+ ILC2s (solid circles) versus that of all other cell populations (non-ILC2) (open circles) in naive lung tissue of Arg1-YFP mice. *P < 0.001, unpaired Student’s t-test. (g) Representative flow cytometry plots of Arg1-YFP expression in ILC2s (Lin−CD45−CD90−CD25−IL-33R+), alveolar macrophages (CD11b+CD11c+CD169+), dendritic cells (DCs) (CD11c+CD11b+), eosinophils (CD11b+Siglec-F+CD11c+), NK cells (CD3−NK1.1+), CD4+ T cells (CD3+CD4+), and B cells (CD3−CD19+). WT, wild-type. All data are representative of three independent experiments with similar results. N = 3 mice per group except in e,f, for which n = 8 mice. Data shown represent flow cytometric events.
Figure 2  ILC2s are a main source of Arg1 in the lung during type 2 inflammation. (a) Arg1 mRNA expression in lung tissue of wild-type mice treated with 30 μg of papain or PBS intranasally (i.n.) for 5 d and analyzed on day 6. Values shown were determined by RT-PCR and are relative to those for PBS-treated control mice. (b–g) Analysis results for wild-type and Arg1<sup>YFP</sup> mice treated with 30 μg of papain or PBS (i.n.) for 5 d and analyzed on day 6. (b) Representative flow cytometry plots of lung ILC2 frequencies, pregated on Lin−CD45+ cells. (c) Arg1<sup>YFP</sup> expression in lung ILC2s from Arg1<sup>YFP</sup> mice. (d,e) Frequency (d) and number (e) of Arg1<sup>YFP</sup>− ILC2s at day 6. (f) Sequential flow cytometric gating of Arg1<sup>YFP</sup> expression in lung tissue from papain-treated Arg1<sup>YFP</sup> mice. Lineage-negative gating (Lin) includes markers for CD3, CD5, NK1.1, CD11b, B220 and CD11c. Numbers indicate the frequency of flow cytometric events. (g) Quantification of the relative frequency of Arg1<sup>YFP</sup>− ILC2s versus that of non-ILC2 populations in lung tissue of papain-treated Arg1<sup>YFP</sup> mice. All data are representative of three independent experiments with similar results. N = 3 mice per group (a–e) or 5 mice per group (f,g). Data shown are the mean ± s.e.m. *P < 0.001 as determined by unpaired Student's t-test.

with increased frequencies of ILC2s in the lung parenchyma (Fig. 2b). The ILC2s continued to express high amounts of Arg1<sup>YFP</sup> after allergen exposure (Fig. 2c), resulting in elevated frequencies and total numbers of Arg1-expressing ILC2s compared to those in PBS-treated control mice (Fig. 2d,e). Further, unbiased analysis of total Arg1<sup>YFP</sup>− cells showed that ILC2s constituted a major source of Arg1 expression in the inflamed lung (Fig. 2f,g). These data suggest that ILC-intrinsic expression of Arg1 influences the development and/or progression of lung inflammation.

Human ILC2s express Arg1 during lung disease
Elevated expression of Arg1 and dysregulation of arginine metabolism have been reported in people diagnosed with asthma<sup>25–28</sup>, chronic obstructive pulmonary disease<sup>29,30</sup> (COPD) or idiopathic pulmonary fibrosis<sup>37</sup> (IPF). However, the cellular sources of the enzyme in human lung disease are incompletely understood and presumed to be limited to the myeloid lineage<sup>11,12,31</sup>. Using primary lung tissue obtained from individuals diagnosed with COPD or IPF, we next sought to determine whether Arg1 is expressed by ILCs during inflammatory lung disease in humans. Flow cytometric analysis of Lin−CD127<sup>+</sup> ILCs identified all group 1 ILC (ILC1), ILC2 and group 3 ILC (ILC3) populations in human explant tissues (Fig. 3a), although the total ILC frequency and subset distribution did not seem to differ significantly between disease states (Supplementary Fig. 1a,b). However, examination of intracellular Arg1 expression showed a pattern of differential expression among ILC subsets in which Arg1 expression by IL-33R<sup>+</sup> ILC2s was comparable to that by CD14<sup>+</sup>CD16<sup>+</sup> myeloid cells (Fig. 3b,c). Arg1 expression in ILC2s did not differ significantly between people diagnosed with COPD and those diagnosed with IPF (Supplementary Fig. 1c), which suggests that Arg1 expression might be a more generalized signature of these innate cells during inflammatory conditions and could represent a novel target for clinical therapies modulating arginine metabolism.

Deletion of ILC-intrinsic Arg1 dampens airway inflammation
To investigate the role of ILC2-derived Arg1, we generated a genetic mouse model that deletes Arg1 from ILCs using a Cre recombinase targeting cells expressing the IL-7R<sub>α</sub> chain (Il7r<sup>Cre<sup>+<sup></sup></sup></sub>). Fate-mapping analysis using Il7r<sup>Cre<sup>+<sup></sup></sup></sub> mice crossed to Rosa26<sup>FloxSTOP-eYFP</sup> mice showed that all lung ILC2s were marked with a history of IL-7R<sub>α</sub> expression (Fig. 4a). Notably, although CD4<sup>+</sup> T cells, B cells and NK cells were also marked by a history of IL-7R expression (Supplementary Fig. 2), none of those cells expressed Arg1<sup>YFP</sup> under steady-state conditions or during papain-induced lung
inflammation (Supplementary Fig. 3). Therefore, use of the IL-7Rα Cre recombinase in combination with Arg1<sup>flox/flox</sup> mice generates a mouse that lacks Arg1 specifically in ILCs (referred to as Arg1<sup>ILC</sup> here). Transcriptional (Fig. 4b) and flow cytometric (Fig. 4c) analysis confirmed efficient deletion of Arg1 expression in lung ILCs.

To test whether ILC-intrinsic Arg1 has a role in the development of lung inflammation, we treated Arg1<sup>flox/flox</sup>, Arg1<sup>flox/flox</sup>/Il7r<sup>Cre/+</sup> control and Arg1<sup>ILC</sup> mice with PBS or papain and assessed them for immunologic and pathologic parameters of type 2 lung inflammation. All groups treated with PBS had equivalent frequencies of lung ILC2s (Fig. 4d,e), which suggests that despite constitutive expression of Arg1, the enzyme was not required for the development, maturation or maintenance of ILC2s. Although exposure to papain resulted in increased frequencies (Fig. 4d,e) and total numbers (Fig. 4f) of ILC2s in the lungs of Arg1<sup>flox/flox</sup> and Arg1<sup>flox/flox</sup>/Il7r<sup>Cre/+</sup> mice compared with PBS-treated controls, loss of ILC-intrinsic Arg1 expression severely diminished papain-induced ILC2 responses in Arg1<sup>ILC</sup> mice (Fig. 4d-f). The lack of an ILC2 response correlated with decreased Arg1 expression in lung homogenates from Arg1<sup>ILC</sup> mice compared to Arg1<sup>flox/flox</sup> and Arg1<sup>flox/flox</sup>/Il7r<sup>Cre/+</sup> mice (Fig. 4g), consistent with ILC2s being a critical regulator of Arg1 in the inflamed lung.

ILC2s are the primary source of the type 2 cytokines IL-5 and IL-13 during acute papain-induced inflammation, and these cytokines are critical for driving eosinophilia and the type 2 helper T (T<sub>H2</sub>) response. Using RT-PCR, we determined that although exposure to papain resulted in clusters of CD45<sup>+</sup> immune cells (primarily eosinophils) in the bronchoalveolar lavage fluid (BALF) at day 6 after treatment, determined by RT-PCR and normalized to β-actin levels (Fig. 4c-f). Representative flow cytometry histograms show that although exposure to papain resulted in clusters of CD45<sup>+</sup> immune cells (primarily eosinophils) in the BALF at day 6 after treatment, determined by RT-PCR and normalized to β-actin levels (Fig. 4c-f). Representative flow cytometry plots in which numbers indicate the frequency of flow cytometric events (d), total frequencies (e) and cell numbers (f) of lung ILC2s. Key in e applies to e-i.m. (g-i) Expression of Arg1 (g), Il5 (h) and Il13 (i) mRNA in lung tissue, determined by RT-PCR and presented relative to levels in PBS-treated Arg<sup>flox/flox</sup> mice. (j,k) Representative flow cytometry plots in which numbers indicate flow cytometric events (j) and frequencies (k) of lung ILC2s expressing IL-5 and IL-13. ILC2s gated as live, Lin<sup>−</sup>CD45<sup>−</sup>CD90<sup>−</sup>CD25<sup>−</sup>IL-33R<sup>+</sup> from Arg<sup>flox/flox</sup> and Arg<sup>ILC</sup> mice treated with 30 µg of papain or PBS i.n. for 5 d and assessed on day 6. (d-f) Representative flow cytometry plots in which numbers indicate the frequency of flow cytometric events (d), total frequencies (e) and cell numbers (f) of lung ILC2s. Key in e applies to e-i.m. (g-i) Expression of Arg1 (g), Il5 (h) and Il13 (i) mRNA in lung tissue, determined by RT-PCR and presented relative to levels in PBS-treated Arg<sup>flox/flox</sup> mice. (j,k) Representative flow cytometry plots in which numbers indicate flow cytometric events (j) and frequencies (k) of lung ILC2s expressing IL-5 and IL-13. ILC2s gated as live, Lin<sup>−</sup>CD45<sup>−</sup>CD90<sup>−</sup>CD25<sup>−</sup>IL-33R<sup>+</sup> from the indicated mice. (l,m) Total cell numbers of CD45<sup>+</sup> immune cells (l) and eosinophils (CD11b<sup>+</sup>Siglec-F<sup>−</sup>CD11c<sup>−</sup>) (m) in bronchoalveolar lavage fluid (BALF) at day 6 after treatment. (n) Periodic acid–Schiff (PAS) staining of lung sections in PBS- or papain-treated mice.
of immune cell infiltrates and severe goblet cell hyperplasia in Arg1<sup>Δ</sup>/floxed mice relative to PBS-treated controls, deletion of ILC-intrinsic Arg1 effectively blocked the development of airway inflammation (Fig. 4n). Collectively, these results define an essential role for ILC-intrinsic Arg1 expression in promoting acute type 2 inflammation in the lung.

**Arg1 regulates ILC2 responses in chronic lung inflammation**

In addition to their role in regulating the development of acute lung inflammation, ILC2s have been implicated in the progression or resolution of chronic lung inflammation, including in IPE32 and helminth-parasite-induced lung remodeling33,34. We next sought to test the involvement of ILC-derived Arg1 during chronic lung inflammation. Infection with the helminth parasite *Nipponstrongylus brasiliensis* leads to the development of chronic lung inflammation characterized by increased amounts of type 2 cytokines and heightened Arg1 expression35,36. Although Arg1<sup>Δ</sup>/floxed mice exhibited a twofold increase in the frequency of ILC2s in the inflamed lung 1 month after infection, compared to naive controls, deletion of ILC-intrinsic Arg1 prevented the generation of ILC2 responses (Supplementary Fig. 4a,b) and was associated with decreased Arg1 expression in the inflamed lung (Supplementary Fig. 4c). Similarly, in a mouse model of elastase enzyme instillation that leads to chronic damage to the alveolar septa partially mimicking the emphysemaous pathology observed in COPD37,38, we found that absence of ILC-intrinsic Arg1 prevented the generation of robust ILC2 responses (Supplementary Fig. 4d,e), was associated with decreased Arg1 expression in lungs (Supplementary Fig. 4f) and reduced the severity of emphysemaous lung pathology (Supplementary Fig. 4g). Taken together, these data highlight a conserved role for ILC-intrinsic Arg1 in controlling ILC2 responses in both acute and chronic models of lung inflammation.

**ILC3 function is independent of ILC-intrinsic Arg1**

Expression of Arg1 is not restricted to the ILC2 lineage, as fetal RORγ<sup+t</sup> lymphoid-tissue-inducer (LTi) cells and a proportion of adult intestinal RORγ<sup+t</sup> LTi-like ILC3s have been reported to express Arg1-YFP39. Therefore, we next sought to test whether ILC-intrinsic Arg1 can regulate ILC3 responses in the steady state or in the context of bacteria-induced intestinal inflammation. Examination of gut-associated RORγ<sup>t</sup> ILCs and GATA3<sup>hi</sup> ILC2s in *Arg1<sup>Δ</sup>/floxed* and *Arg1<sup>Δ</sup>/Δ* mice showed equivalent total ILC frequencies (Supplementary Fig. 5a,b) and ILC3/ILC2 ratios (Supplementary Fig. 5c,d), which suggests that deletion of ILC-intrinsic Arg1 does not impair the development or homeostasis of ILC3s. To test whether the functionality of ILC3s is affected by the absence of ILC-intrinsic Arg1, we infected mice with the intestinal bacterium *Citrobacter rodentium*. ILC3-derived IL-22 responses are critical mediators of host protection in *C. rodentium* infection39. Whereas mice lacking RORγ<sup>t</sup> cells (RORγ<sup>t</sup><sup>ΔΔ</sup>/Δ<sup>Δ</sup>) or innate and adaptive lymphocytes (Rag2<sup>−/−</sup>H2<sup.RequestMethod: GETt</sup>arg<sup>−/−</sup> and RAG1<sup>−/−</sup>) succumbed to infection within 2 weeks after the bacterium was introduced, *Arg1<sup>Δ</sup>/floxed* and *Arg1<sup>Δ</sup>/Δ* mice did not show any evidence of *Citrobacter*-induced inflammation or disease and survived infection (Supplementary Fig. 5e). Collectively, these data demonstrate that Arg1 activity controls optimal ILC2, but not ILC3, responses.

**Lung inflammation is independent of myeloid Arg1**

In addition to its association with ILCs, Arg1 expression is an effector signature of AAMacs, although the functional significance of myeloid-derived Arg1 activity during type 2 inflammation in the lung remains controversial19–23. Fate-mapping analysis of Ily<sup>Cre</sup>/+Rosa26<sup>ΔΔ</sup>/Δ<sup>Δ</sup> mice showed that a minor population of macrophages showed a history of IL-7Rα expression (Fig. 5a), and only a
A small proportion of macrophages expressed Arg1-YFP during acute papain-induced inflammation (Fig. 5b). Flow cytometric analysis did not show any significant difference in macrophage frequencies (Fig. 5c), and we observed equivalent expression of macrophage-intrinsic Arg1 mRNA (Fig. 5d) and protein (Fig. 5e) in the inflamed lungs of Arg1floxflox and Arg1Il7cIlc mice after papain exposure, indicating that AAMac accumulation and Arg1 expression are not impaired in Arg1Il7cIlc mice.

To test whether myeloid sources of Arg1 can act extrinsically to influence the magnitude of ILC2 responses and/or the development of papain-induced airway inflammation, we deleted Arg1 expression in macrophages and neutrophils using Cre recombinase under control of the LysM (LysMc) promoter19,40 (Arg1LysMcre). Absence of Arg1 in LysMc myeloid cells did not prevent the generation of robust lung ILC2 responses to papain (Fig. 5f,g), nor did it influence the development of airway eosinophilia (Fig. 5h) or lung inflammation (Fig. 5i). Taken together, these results indicate a selective requirement for ILC-intrinsic Arg1 in the development of acute airway inflammation, revealing an unexpected degree of cellular specificity with respect to the role of this enzyme in the lung.

**Arg1 controls optimal ILC2 proliferation**

The lack of a robust ILC2 response in the absence of cell-intrinsic Arg1 metabolic function could be due to impaired cell survival and/or reduced proliferative capacity. We found that deletion of Arg1 had no significant effect on the frequencies of 7AAD annexin V apoptotic ILC2s in the lungs of either PBS-treated or papain-treated mice (Supplementary Fig. 6a–c), which suggests that the absence of ILC-intrinsic Arg1 does not influence ILC2 survival under homeostatic conditions or during acute lung inflammation. To test whether loss of Arg1 affects ILC2 proliferation, we examined intracellular expression of the proliferation marker Ki67 in ILC2s isolated ex vivo during acute papain-induced lung inflammation. A high proportion of lung ILC2s from Arg1floxflox and Arg1Il7cIlc mice were Ki67+ cells (38–44%) (Fig. 6a,b), indicating that those mice mounted a strong effector response to the papain allergen. In contrast, deletion of ILC-intrinsic Arg1 severely restrained ILC2 proliferation, resulting in significantly decreased frequencies (Fig. 6a,b) and cell numbers (Fig. 6c) of Ki67+ ILC2s compared to Arg1floxflox and Arg1Il7cIlc mice. To test directly whether cell-intrinsic Arg1 enzymatic activity is required for regulation of ILC2 proliferation, we sort-purified activated ILC2s from wild-type mice treated with recombinant murine IL-33 (rmIL-33) and tracked cellular division in the presence or absence of the selective Arg1 inhibitor Nω-hydroxy-nor-arginine (nor-NOHA) (Fig. 6d). Within 48 h, nearly 80% of the ILC2s treated with dimethylsulfoxide (DMSO; controls) had undergone at least one division, and more than 20% had undergone three to five divisions (Fig. 6e). Compared to controls, cells subjected to Arg1 enzymatic inhibition showed greatly decreased division, with more than 45% of the cells remaining undivided (Fig. 6e,f). Consistent with our *in vivo* papain studies, the lack of division by the nor-NOHA-treated cells was not associated with increased cell death (Supplementary Fig. 6d). Collectively, these studies demonstrate an essential role for Arg1 enzymatic activity in controlling the maximal proliferative capacity, but not survival, of activated ILC2s.

**Arg1 inhibition disrupts amino acid metabolism**

Arginine can be catabolized through several enzymatic pathways to yield a diverse set of metabolic intermediates that serve as substrates for downstream cellular and bioenergetic processes (Supplementary Fig. 7a and refs. 11,31,41,42). We hypothesized that the lack of proliferation in Arg1-deficient ILC2s might be associated with an imbalance among these metabolic substrates due to the disruption of Arg1 activity and possible compensation by the other enzymatic pathways to catabolize the excess arginine. To examine this, we first cultured IL-33-activated ILC2s in the presence or absence of nor-NOHA and measured the relative abundance of metabolites using liquid chromatography coupled with mass spectrometry (Supplementary Fig. 7b). As expected, inhibition of Arg1 resulted in increased amounts of arginine in nor-NOHA-treated cells (Fig. 7a), confirming that ILC2s express functional Arg1. Whereas DMSO-treated cells showed high abundance of the immediate downstream products ornithine and proline, the amounts of these metabolites were comparatively severely diminished in cells subjected to Arg1 inhibition (Fig. 7b,c). We did not observe any corresponding increase in metabolites associated with two other main arginine catabolic pathways that would generate either citrulline-argininosuccinate via nitric oxide synthase (Fig. 7d) or creatine-creatine biosynthesis via L-arginine-glycine amidinotransferase (Fig. 7e), which suggested that excess arginine is probably not diverted through these pathways to compensate for the absence of Arg1.
Figure 7 Inhibition of cell-intrinsic Arg1 disrupts the balance of amino acid metabolites and impairs polyamine synthesis in ILC2s. (a–e) Analysis of sort-purified lung ILC2s from rmIL-33-treated wild-type mice cultured with rmIL-2, rmIL-7 and rmIL-33 in DMSO or nor-NOHA for 24 h. Results of liquid chromatographymass spectrometry analysis for abundance of (a) arginine, (b) ornithine, (c) proline, (d) citrulline and (e) creatine. (f–k) Analysis of sort-purified lung ILC2s from rmIL-33-treated wild-type mice cultured with U-13C-l-arginine (13C L-arg) with rmIL-2, rmIL-7, rmIL-33 and DMSO or nor-NOHA for 18 h. Incorporation kinetics and relative abundance of the U-13C-l-arginine isotope tracer into (f,g) ornithine, (h,i) proline and (j,k) spermidine, as determined by liquid chromatographymass spectrometry isotope-tracing analysis. Data are representative of two independent experiments with similar results. N = 3 biological replicates per condition. (f,h,j) Data represent individual biological replicates (circles) and means (curves) where N = 3 biological replicates. Data shown are the mean and s.d. for a–e,g,i,k. *P < 0.05, **P < 0.001, unpaired Student’s t-test (a–c) or two-way ANOVA with Bonferroni post-test correction (f,h,j).

Changes in the size of a metabolite pool can result from either altered production or an altered consumption rate within the cell. Therefore, to further examine the metabolic fate of arginine in activated ILC2s, we traced the incorporation of uniformly enriched U-13C-l-arginine isotope in ILC2s in the presence or absence of Arg1 activity. Whereas we observed rapid and almost complete entry of 13C-l-arginine into ornithine (Fig. 7f,g) and proline (Fig. 7h,i) in DMSO-treated ILC2s, with saturation kinetics being quickly achieved, this process was severely diminished in cells treated with Arg1 inhibitor, indicating that arginine is the primary precursor for both molecules in activated ILC2s. Further, inhibition of Arg1 activity also resulted in severely diminished entry of 13C-l-arginine into biosynthesis of the ornithine-derived polyamine metabolite spermidine (Fig. 7j,k). Notably, reversible interconversion between proline and ornithine metabolite pools is a well-appreciated reaction in mammalian cells, supporting the conclusion that ornithine-derived polyamines, rather than ornithine and proline themselves, are a primary fate of l-arginine catabolism in ILC2s.

Taken together, these data indicate a model in which disruption of Arg1 in ILC2s is not associated with enhanced metabolism of nitric oxide synthase or l-arginine:glycine amidinotransferase (Supplementary Fig. 7a) but does prevent the generation of l-arginine-derived polyamines (Supplementary Fig. 7a), molecules that are known to regulate cell growth and survival.

Arg1 regulates maximal ILC2 glycolytic capacity

Proteins are just one component of a diverse set of nutrient substrates that immune cells can use to fuel their growth, raising the question of whether Arg1 metabolism influences bioenergetic pathways in addition to amino acid catabolism. In particular, the induction of aerobic glycolysis is a hallmark of effector lymphocyte metabolism, although whether this reprogramming is used by ILCs during activation has never been examined. First, to characterize the bioenergetic profile of ILC2s during activation, we sort-purified lung IL-33R+ ILC2s and Th2 cells from mice treated with rmIL-33 (Fig. 8a) and measured the cellular oxygen-consumption rate (OCR) and extracellular acidification rate (ECAR), indicators of mitochondrial respiration and aerobic glycolysis, respectively. ILC2s showed both elevated spare respiratory capacity (Fig. 8b,c) and high ECAR (Fig. 8d,e) in comparison to Th2 cells, suggesting that ILC2s possess the capacity to augment mitochondrial respiration above basal conditions compared to Th2 cells and strongly engage aerobic glycolysis. To test specifically whether Arg1 enzymatic activity can influence the respiratory or glycolytic capacity of ILC2s, we activated lung ILC2s with rmIL-33 in vivo and treated the cells ex vivo with nor-NOHA to inhibit Arg1 activity during measurement of ECAR and OCR. ILC2 spare respiratory capacity was not substantially affected by Arg1 inhibition (Fig. 8f,g), which indicates that this enzyme does not have a notable effect on oxidative phosphorylation in ILC2s. In contrast, inhibition of Arg1 resulted in severely diminished maximal glycolytic capacity (Fig. 8h,i), which indicates a critical regulatory role for Arg1 in influencing glucose metabolism in activated ILC2s.

Together with the trace analysis of arginine metabolites, these data provide evidence for a previously unrecognized aspect of this biochemical pathway in which Arg1 enzymatic activity acts on multiple biosynthetic and bioenergetic pathways to function as a metabolic checkpoint that controls ILC2 proliferation and effector function driving type 2 inflammation.
DISCUSSION

ILC2s can promote pathologic inflammation or host-protective tissue repair in the lung after activation by cell-extrinsic environmental cues such as alarmins and cytokines. However, the cell-intrinsic metabolic pathways that regulate ILC2 effector function are undefined. Here we demonstrate a selective and essential role for cell-intrinsic Arg1 expression in regulating the proliferation and effector function of ILC2s required to promote acute and chronic type 2 inflammation. The identities of the sources of human Arg1 expression must be induced via AAMac polarization, a process that is partially dependent on ILC2-derived type 2 cytokines, thus afflicting ILC2 responses or dampening papain-induced airway inflammation. Although further analysis using additional models of myeloid cell depletion is required to determine the reasons for this cellular compartmentalization of Arg1 function, it may provide a rationale for reconciling paradoxical reports of the role of Arg1 during type 2 lung inflammation. Previous studies using deletion of myeloid-cell-intrinsic Arg1 failed to recapitulate the effects observed when global chemical inhibition or absence of hematopoietic-cell-restricted Arg1 diminishes airway inflammation. The data presented here demonstrate that deletion of ILC2-intrinsic Arg1 acts to limit the magnitude of the ILC2 response itself, thereby diminishing the cells’ proinflammatory capacity and preventing the development of acute and chronic type 2 inflammation in the lung. Notably, myeloid-cell-intrinsic Arg1 expression must be induced via AAMac polarization, a process that is partially dependent on ILC2-derived type 2 cytokines, thus revealing an additional, indirect mechanism by which ILC2s control Arg1 metabolism during inflammatory conditions.

Elevated ILC2 responses have been reported in a number of human inflammatory disorders of the intestine, skin and lung barrier sites. Particularly in the lung, dysregulation of L-arginine metabolism and Arg1 expression has been observed in people diagnosed with inflammatory diseases such as asthma and COPD, IPF. Further, dietary supplementation with l-arginine and chemical inhibition of Arg activity are being evaluated as clinical therapies for the treatment of asthma. Despite these clinical correlations, the mechanisms by which this enzyme regulates lung inflammation are incompletely understood and are assumed to be largely due to a reciprocal effect of increased nitric oxide and smooth muscle contractility in the absence of Arg1 (refs. 26, 30). Further, the identities of the sources of human Arg1 are controversial and incompletely defined. Using primary lung tissue from people with COPD and IPF, we observed selective expression of Arg1 by ILC2s and IL2 populations but not by other ILC2 subsets. Although the significance of this restricted expression pattern is not yet clear, IL-33 expression is strongly enhanced on human ILC2s in response to cytokine signals and therefore may represent a more activated state that ILC2s gain in inflamed tissue. Taken together with
the mouse mechanistic data, our studies suggest that Arg1 metabolic control of ILC2 function is a critical determinant of both acute and chronic type 2 inflammation in the lung and thus may represent a previously unrecognized axis for improvement of targeted therapeutics.

Despite constitutive expression of Arg1 in bone marrow ILC2 progenitors, the absence of Arg1 did not seem to affect ILC2 development or maturation. Instead, Arg1 activity profoundly influenced the cells’ ability to proliferate and exert their proinflammatory function after activation. This suggests a clear demarcation in the role of this enzyme in regulating the inflammatory, but not the homeostatic, state of ILC2s, perhaps because of the differential metabolic requirements of proliferating cells compared to those at rest. Inhibition of Arg1 and the subsequent decreased catabolism of arginine into ornithine resulted in an imbalance of amino acid metabolites, especially reduced synthesis of polyamines from l-arginine. Polyamine metabolites have been linked to diverse biological processes such as proliferation, apoptosis and cellular stress responses31,41, and further biochemical studies will be required to investigate the fates of these molecules in ILC2 biology.

Amino acids are one of a large array of nutrients used for cell growth22,41, provoking the question of whether Arg1 influences other aspects of ILC2 metabolic programming. We found that the effect of Arg1 was not limited to arginine catabolism—it also resulted in severe inhibition of the ability of these cells to engage in maximal rates of aerobic glycolysis (also known as Warburg metabolism22,44) to fuel proliferation. Although the effects of Arg1 on arginine and glucose metabolism might not be directly linked and probably involve a complex imbalance of many metabolic intermediates, it is remarkable that disruption of a single urea-cycle enzyme profoundly influences such distinct metabolic pathways, and this may lead to a new understanding of the importance of this enzyme beyond the classical urea cycle. As both a constitutive and an inflammatory source of arginine, ILC2s seem to occupy a unique functional niche mimicking both Arg1-dependent homeostatic hepatocyte function and inflammation-induced myeloid cell function. Critically, however, mammalian survival depends on hepatocyte-intrinsic Arg1 expression above any hematopoietic source of the enzyme12,21, perhaps reflecting an evolutionary compartmentalization of arginase activity that fulfills the primary biological need of mammals for urea production while also limiting the proinflammatory consequences of Arg1-driven ILC2 activation. Understanding how Arg1 metabolism is integrated into the broader metabolic networks that govern the ability of ILCs and other innate immune cells to meet the biosynthetic and bioenergetic demands created during tissue inflammation may reveal new therapeutic targets for the treatment of numerous human inflammatory and metabolic diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.A.M. carried out mouse experiments with technical assistance from A.-L.F., N.A.Y., L.C.O., M.R.H. and S.Y.T. M.D.B. and E.L.P. carried out Seahorse metabolic assays. H.S. and J.R.C. carried out liquid chromatography–mass spectrometry experiments. J.M.D., E.C. and J.D.C. provided human tissue samples. L.A.M., S.A.S. and E.D.T.W. carried out experiments involving human tissue samples. H.-R.R. provided Il15-def mice. L.A.M. and D.A. conceived of the study, designed experiments, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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For isolation of cells from lymphoid tissues, tissues were pressed through 70-µm cell strainers and washed with DMEM. The adipocyte layer was removed, and remaining red blood cells were lysed with ACK buffer. Cells were frozen in liquid nitrogen and thawed before use. All protocols were approved by both the University of Pennsylvania and the Weill Cornell Medicine Institutional Animal Care and Use Committees.

**Models of lung and intestinal inflammation.** For induction of acute type 2 airway inflammation, we anesthetized mice with Isothesia isoflurane, 1% t-glutamine (Gibco), 1% penicillin–streptomycin (Gibco), 25 mM HEPES buffer, and 55 mM 2-β-mercaptoethanol (Sigma-Aldrich) with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich), 500 ng/ml IL-5–phycoerythrin (eBioscience). Cells were subsequently surface-stained with a combination of the antibodies listed above, fixed and permeabilized using a commercially available kit (BD Cytofix/Cytoperm, BD Biosciences), and stained with IL-13–Alexa Fluor 488 (eBioscience) and IL-5–phycoerythrin (eBioscience).

For measurement of intracellular arginine, cells were isolated directly from lung tissue and stained with antibodies to surface antigens, fixed and permeabilized according to the manufacturer’s instructions (BD Biosciences) and stained with sheep anti-human or anti-mouse Arg1 conjugated with FITC or phycoerythrin (R&D Systems). For analysis of transcription factor expression, cells were isolated directly from lung tissue, stained with antibodies to surface antigens, fixed and permeabilized according to the manufacturer’s instructions (BD Biosciences) and stained with sheep anti-human or anti-mouse Arg1 conjugated with FITC or phycoerythrin (R&D Systems).

**Flow cytometry.** Single-cell suspensions were stained with a combination of fluorescently conjugated monoclonal antibodies. All antibodies were from Affymetrix eBioscience and used at 1:200 dilution unless otherwise specified. For mouse studies, we used antibodies to the following mouse proteins: αββ (DATK32), Flt3 (A2F10), KLRG1 (2F1), Ly6G (1A8), F4/80 (BM8), FcRγ (MAR-1), CD3 (145-2C11), CD4 (GK1.5), CD5 (53-73), CD11b (M1/70), CD11c (N418), CD19 (eBio1D3), CD25 (PC6.5), CD35 (40-F11), CD127 (A7R34), CD90.1 (HL51), CD90.2 (53-2-1), IL-33R (Db1, MD Bioproducts), NK1.1 (PK136), Silyc-F (E50-2440), CD103 (2E7) and MHCII (MS/114.15.2).

**Isolation of cells from mouse tissue.** For isolation of cells from lymphoid tissues, tissues were pressed through 70-µm cell strainers using the plunger of a 3-ml syringe and washed with DMEM wash media (Dulbecco’s modified Eagle’s medium with 10% FBS (Denville Scientific)), 1% l-glutamine (Gibco), 1% penicillin–streptomycin (Gibco), and 20 µg/ml collagenase D (Roche) and 20 µg/ml DNase I (Roche) at 37 °C with shaking at 200 r.p.m., and staining the digested tissue through a wire mesh sieve. Liquid was passed through 70-µm cell strainers, underlaid with Ficoll-Paque (GE Healthcare), and centrifuged at 1,600 r.p.m. for 20 min at room temperature with the brake off. The white lymphocyte interface layer was removed, and remaining red blood cells were lysed with ACK buffer. Cells were frozen in 90% FBS, 10% DMSO in a Nalgene Mr. Frosty container (Thermo Scientific). For flow cytometric analysis, samples were thawed and stained as a single cohort to ensure consistency.

**Isolation of cells from human lung tissue.** Explanted human lung tissue was collected from patients undergoing lung transplantation who elected to participate in the Prospective Registry of Outcomes in Patients Electing Lung Transplantation (PROPEL) study at the Hospital of the University of Pennsylvania. University of Pennsylvania Institutional Review Board approval and informed written consent from patients were obtained before recruitment. We prepared single-cell suspensions of lung by chopping tissue into pieces with scissors, incubating the finely chopped tissue for 1 h with 2 mg/ml collagenase D (Roche) and 20 µg/ml DNase I (Roche) at 37 °C with shaking at 200 r.p.m., and washing the digested tissue through a wire mesh sieve. Liquid was passed through 70-µm cell strainers, underlaid with Ficoll-Paque (GE Healthcare), and centrifuged at 1,600 r.p.m. for 20 min at room temperature with the brake off. The white lymphocyte interface layer was removed, and remaining red blood cells were lysed with ACK buffer. Cells were frozen in 90% FBS, 10% DMSO in a Nalgene Mr. Frosty container (Thermo Scientific). For flow cytometric analysis, samples were thawed and stained as a single cohort to ensure consistency.
RNA isolation and real-time quantitative PCR. For analysis from lung tissue, lungs were perfused as described above, and two small representative pieces from the right lobe were placed in RNAlater (Qiagen). Tissue was later homogenized in RLT buffer, and RNA was isolated using an RNeasy mini kit according to the manufacturer’s instructions (Qiagen). For analysis of purified immune cells, Lin−CD45+CD25−IL-33R+ ILC2s or CD45+CD11c+F4/80+ macrophages were sort-purified from wild-type mice treated with 30 µg of papain for 5 d. Cells were sorted directly into TRIZol, and RNA was isolated via chloroform extraction. For both methods, cDNA was generated via Superscript reverse transcription (Invitrogen). Real-time quantitative PCR (qRT-PCR) was carried out on cDNA with SYBR Green master mix (Applied Biosystems) and commercially available primer sets (Qiagen). Reactions were run on a real-time PCR system (ABI7500, Applied Biosystems). Samples were normalized to β-actin, and results are shown as a fold induction relative to expression levels in PBS-treated or naive tissue as indicated.

Tissue histological sections. Left lobes of lungs were inflated, fixed with 4% paraformaldehyde (bioWORLD) and embedded in paraffin, and 5-µm sections were collected by centrifugation at 5,000 r.p.m. for 5 min and prepared for RNA isolation and real-time quantitative PCR. For both methods, cDNA was generated via Superscript reverse transcription (Invitrogen). Real-time quantitative PCR (qRT-PCR) was carried out on cDNA with SYBR Green master mix (Applied Biosystems) and commercially available primer sets (Qiagen). Reactions were run on a real-time PCR system (ABI7500, Applied Biosystems). Samples were normalized to β-actin, and results are shown as a fold induction relative to expression levels in PBS-treated or naive tissue as indicated.

Liquid chromatography–mass spectrometry. Metabolite pool analysis. C57BL/6/J mice were treated with 100 ng of rmIL-33 i.n. every 3 d for 2–3 weeks. Lin−CD45+CD90+CD25+IL-33R+ ILC2s were sort-purified from the lung and rested for 18 h in DMEM complete media with 10% FBS (Gibco), 1% l-glutamine, 1% penicillin–streptomycin, 25 mM HEPES buffer, 55 µM 2-β-mercaptoethanol, 0.80 mM lysine and 0.4 mM U-13C-l-arginine (Cambridge Isotopes). Cells were collected by centrifugation at 5,000 r.p.m. for 5 min and prepared for LC-MS analysis as described above. Extracted ion chromatograms for all mass isotopomers were generated with Agilent MassHunter software and corrected for natural abundance with IsoCor software.

Statistical analysis. Results represent the mean ± s.e.m. unless otherwise indicated. Statistical significance was determined by p value in figure legends. Statistical analyses were done with Prism GraphPad software v5.0. Researchers were not blinded to experimental groups. Exclusion criteria were not applied, as no experimental samples were excluded from analysis.