WASH maintains NKp46⁺ ILC3 cells by promoting AHR expression

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Innate lymphoid cells (ILCs) communicate with other haematopoietic and non-haematopoietic cells to regulate immunity, inflammation and tissue homeostasis. How these ILC lineages develop and are maintained is not clear. Here we show that WASH is highly expressed in the nucleus of group 3 ILCs (ILC3s). WASH deletion impairs the cell pool of NKp46⁺ ILC3s. In NKp46⁺ ILC3s, WASH recruits Arid1a to the Ahr promoter thus activating AHR expression. WASH deletion in ILC3s decreases the number of NKp46⁺ ILC3s. Moreover, Arid1a deletion impedes AHR expression and impairs the maintenance of NKp46⁺ ILC3s. Therefore, WASH-mediated AHR expression has a critical function in the maintenance of NKp46⁺ ILC3s.

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Innate lymphoid cells (ILCs) reside in mucosal surfaces to potentiate immune responses, sustain mucosal integrity and maintain tissue homeostasis. ILCs can be categorized into three groups based on their signature effector cytokines, analogous to the classification of T cell subsets\(^1\). Group 1 (ILC1) cells are characterized by their capacity to secrete interferon \(\gamma\) (IFN-\(\gamma\)) in response to interleukin 12 (IL-12), IL-15 and IL-18 (refs 2,3). Group 2 (ILC2) cells generate type 2 T helper (Th2) cell cytokines such as IL-5, IL-9 and IL-13 in response to IL-25 and IL-33 stimulation\(^4\). Group 3 (ILC3) cells produce IL-17 and IL-22 upon stimulation with IL-1\(\beta\) and IL-23 (refs 7–9). ILC3 cells can be divided into subpopulations by their expression of CD4 and NKp46 (encoded by Ncr1) receptors, such as CD4\(^+\) ILCs, NKp46\(^+\) ILCs and CD4\(^-\) NKp46\(^-\) ILC3s (DN ILC3s)\(^1\). ILC3 subsets in the fetal intestines are CD4\(^+\) or CD4\(^-\) lymphoid tissue inducer (LTi) cells, which are necessary for the development of Peyer’s patches (PPs) and lymph nodes\(^1\). In addition, ILC3s are central to the defence against bacterial infection in the intestine\(^2\),\(^1\) NKp46\(^+\) ILC3s, for example, specifically generate IL-22 but not IL-17 (refs 14,15).

ILC3s are enriched in PPs and intestinal lamina propria\(^1\). ILC3s, together with other ILCs, are derived from the earliest progenitor cells (zLPs, CXCR\(^+\) integrin \(\alpha 4\beta 7\)-expressing common lymphoid progenitor (CLPs))\(^1\), which differentiate into restricted common helper-like innate lymphoid progenitor (CHILP) cells\(^1\). Subsequently, downstream precursor ILC3s (common progenitor of ILC3s) are characterized by expression of transcription factor PLZF and can generate ILC1, ILC2, and ILC3 subsets\(^8\). ROR\(\gamma\)t (encoded by Rorc) drives differentiation of ILC3s from their precursor ILCP\(^1\)\(^9\),\(^10\). ROR\(\gamma\)t deletion causes a complete loss of ILC3s but not ILC1s or ILC2s. In addition, aryl hydrocarbon receptor (AHR) is highly expressed in ILC3s, and is required for their development and maintenance\(^2\),\(^1\)\(^–\)\(^2\)\(^3\). However, the underlying mechanism that control ILC3 development and maintenance are unclear.

WASH (Wiskott-Aldrich syndrome protein (WASP) and SCAR homologue) was originally identified as an actin-nucleating factor belonging to the WASP family\(^2\),\(^4\),\(^5\) and has an essential role in endosome sorting via promoting tubule fission by Arp2/3 activation. WASH is also located in autophagosomes that modulate autophagy induction\(^2\),\(^4\),\(^6\). We previously demonstrated that WASH is highly expressed in the nucleus of long-term hematopoietic stem cells (LT-HSCs)\(^2\). Conditional WASH deletion in the hematopoietic system causes defective blood production of the host 8 weeks after WASH deletion, leading to severe cytopenia and rapid anemia. To further explore whether WASH participates in the development and maintenance of ILCs in the intestine, we assayed compositions of ILCs in Wash\(^{flox/flox}\)Mx1-Cre mice (gene Wash1 is referred to here as Wash) 3 weeks post poly(I: C) administration, meanwhile, bone marrow (BM) hematopoiesis was not affected by WASH deletion. We noticed that WASH deletion reduced the number of ILC3s in the intestine, but not ILC1s or ILC2s (Fig. 1a,b, Supplementary Fig. 1a,b). Of note, WASH deletion caused a greater loss of NKp46 expressing ILC3s (NKp46\(^+\) ILC3s), but no impact on CD4\(^+\) ILC3s or CD4\(^-\) NKp46\(^-\) ILC3s (DN ILC3s) (Fig. 1b; Supplementary Fig. 1c). We also tested progenitors of ILC3s in the BM of Wash\(^{flox/flox}\)Mx1-Cre mice. We observed that WASH deletion did not affect cell numbers of their progenitors such as zLPs, CHILPs and ILCPs (Fig. 1c, Supplementary Fig. 1d–g). These observations suggest that WASH deficiency reduces the number of NKp46\(^+\) ILC3s rather than other ILC cell subpopulations.

We next examined mRNA expression levels of WASH in ILC subsets. We found that WASH was actually expressed in all ILC populations and displayed a higher level in NKp46\(^+\) ILC3s (Supplementary Fig. 1e). We then crossed Wash\(^{flox/flox}\) mice with Ncr1-Cre mice\(^1\) to generate mice with conditional deletion of WASH in NKp46\(^+\) ILC3s (Fig. 1d). We observed that WASH deficiency did not affect the number of cryptpatches (Fig. 1e), suggesting that WASH is not necessary for the development of LTi cells. Since ILC3s are enriched in Peyer’s patches and intestinal lamina propria\(^10\), we then isolated lamina propria lymphocyte (LPL) populations from small and large intestines to test constitutions of ILCs. We noticed that NKp46\(^+\) ILC3s were remarkably declined in the small intestine of Wash\(^{flox/flox}\)Mx1-Cre mice compared with that of Wash\(^{flox/flox}\) wild type (WT) control mice (Fig. 1f; Supplementary Fig. 1j). However, other ILC3 subsets, including CD4\(^+\) NKp46\(^-\) ILC3s (DN ILC3s) and CD4\(^-\) ILCs were unchangeable in Wash\(^{flox/flox}\)Ncr1-Cre mice (Fig. 1f; Supplementary Fig. 1j). Similar results were achieved in both large intestines and Peyer’s patches of Wash\(^{flox/flox}\)Ncr1-Cre mice. Given that Ncr1-Cre also induced WASH deletion in NKp46 expressing ILC1s, we then examined cell numbers of ILC1s in intraepithelial lymphocyte (IEL) populations from small intestines. Notably, WASH deletion in ILC1s did not impact cell numbers of this subset, suggesting that WASH is not required for the development and maintenance of ILC1s.

ROR\(\gamma\)t (encoded by Rorc) is involved in the development of all ILC3 subsets\(^1\),\(^1\)\(^2\). To further validate the specific role of WASH in the maintenance of NKp46\(^+\) ILC3s, we generated Wash\(^{flox/flox}\)Rorc-Cre mice by crossing Wash\(^{flox/flox}\) mice with Rorc-Cre mice to conditionally delete WASH in ILC3s (Fig. 1g). We observed that WASH deletion in ILC3s did not affect numbers of cryptpatches (Fig. 1h), confirming that WASH deficiency does not impact the development and maintenance of LTi cells. As expected, Wash\(^{flox/flox}\)Rorc-Cre mice significantly reduced cell numbers of NKp46\(^+\) ILC3s, but not DN ILC3s or CD4\(^+\) ILC3s (Fig. 1i; Supplementary Fig. 1k). Moreover, WASH deficiency had no significant effect on number changes of ROR\(\gamma\)t \(^+\) T cells (Supplementary Fig. 1l). Taken together, we conclude that WASH deficiency impairs the maintenance of NKp46\(^+\) ILC3s, but not their development.

Of note, Wash\(^{flox/flox}\)Ncr1-Cre mice did not impact the apoptosis of all ILC3 subsets (Supplementary Fig. 1m). To determine whether WASH affected the expansion of ILC3s, we then analysed cell cycle statuses of ILC3s. We found that Wash\(^+/−\) NKp46\(^+\) ILC3s maintained in a resting state compared with those of Wash\(^+/+\) NKp46\(^+\) ILC3s (Fig. 1j). Similar results were obtained from WASH deficient NKp46\(^+\) ILC3s in Wash\(^{flox/flox}\)Mx1-Cre mice. Of note, WASH deficient mice did not cause susceptible change to Citrobacter rodentium infection (Supplementary Fig. 1n). Finally, WASH deficiency did...
Figure 1 | WASH deficiency reduces the cell pool of NKp46⁺ ILC3s. (a) ILCs from Washfloxflox and WashfloxfloxMx1-Cre mice 3 weeks post poly(I:C) administration were immunoblotted with the indicated antibodies (left panel). Numbers of ILCs in small intestines of Washfloxflox (Wash⁺/+⁻) and WashfloxfloxMx1-Cre (Wash⁻/-⁻) mice were calculated (right panel). (b) Numbers of NKp46⁻ and NKp46⁺ ILC3s in small intestines of the indicated mice were calculated. (c) Numbers of ILC progenitor cells in BM of the indicated mice. Gating strategies: Lin⁻ cKi⁺ CD127⁺ αβ⁺ for αLP, Lin⁻ CD127⁺ αβ⁺ PLZF⁺ for ILCP, Lin⁻ CD127⁺ αβ⁺ CD25⁻ CD244⁺ Id2⁺ for CHILP. For a-c, n = 4. (d) RKp46⁺ ILC3s sorted from small intestine lamina propria lymphocyte populations (SI LPL) of Washfloxflox and WashfloxfloxNcr1-Cre mice were immunoblotted with the indicated antibodies. (e) Numbers of cryptopatch (CP) clusters in the small intestine (left panel) or large intestine (right panel) of Washfloxflox and WashfloxfloxNcr1-Cre mice. n = 6. (f) Flow cytometry analysis of SI LPL of Washfloxflox and WashfloxfloxNcr1-Cre mice. Cells were gated from CD45⁺ CD19⁻ CD3⁻ cells (left panel). Numbers of ILC3s and the indicated subpopulations of ILC3s were calculated (right panel). (g) ILC3s sorted from SI LPL of Washfloxflox and WashfloxfloxNcr1-Cre mice were immunoblotted with the indicated antibodies. (h) Representative images of SI CPs from Washfloxflox and WashfloxfloxNcr1-Cre mice staining with RORγt, NKp46 and DAPI (upper panel). Numbers of cryptopatch (CP) clusters in the small intestine (lower left) or large intestine (lower right) of Washfloxflox and WashfloxfloxNcr1-Cre mice. n = 6. Scale bar, 50 μm. (i) Small intestine sections from Washfloxflox and WashfloxfloxNcr1-Cre mice were stained with antibodies against NKp46, RORγt and CD3c, followed by nuclear staining with DAPI. Cells with NKp46 and RORγt staining were annotated with white arrow heads. Scale bar, 100 μm. (j) Live NKp46⁺ ILC3 cells were sorted from SI LPL of Washfloxflox and WashfloxfloxNcr1-Cre mice carrying RORγt-GFP reporter through identifying surface markers, followed by Hoechst33342 and pyronin Y staining (upper panel). Percentages of S/G2/M cells in the indicated cells were calculated (lower panel). n = 7. Data are shown as means ± s.d. *P < 0.05; **P < 0.01; ***P < 0.001. Data are representative of at least three independent experiments.
not affect cell numbers of liver NK cells or Nkp46+ RORγ− cells in the intestine (Supplementary Fig. 2a,b). Altogether, WASH maintains the cell pool of Nkp46+ ILC3 population via the regulation of cell expansion.

WASH intrinsically maintains Nkp46+ ILC3s. To examine whether WASH intrinsically affected the maintenance of Nkp46+ ILC3s, we transplanted Washlox/loxNcr1-Cre BM cells with an equal number of CD45.1 WT BM cells into lethally irradiated CD45.1 recipient mice for 8 weeks, followed by examination of donor chimerism of ILC3s post BM reconstitution (Supplementary Fig. 2c). ILC3s were significantly reduced in Washlox/loxNcr1-Cre BM cells transferred mice compared with those transferred with Washlox/lox control cells. By contrast, ILC1s and ILC2s did not change in Washlox/loxNcr1-Cre BM cells transferred mice compared with those transferred with Washlox/lox control cells. Additionally, we found that Nkp46+ ILC3s derived from Washlox/loxNcr1-Cre donor cells were dramatically decreased in small intestines (Fig. 2a), large intestines (Fig. 2b) and Peyer’s patches compared with those derived from Washlox/lox donor cells. However, DN ILC3s or CD4+ ILC3s derived from Washlox/loxNcr1-Cre donor cells were unchanged (Fig. 2a,b). In addition, Nkp46+ ILC3s derived from Washlox/loxNcr1-Cre donor cells sustained in a resting state compared with those from Washlox/lox donor cells (Fig. 2c; Supplementary Fig. 2d). Similar results were achieved by competitively transferring Washlox/lox Rorc-Cre BM cells or Washlox/lox BM cells into lethally irradiated CD45.1 recipient mice (Supplementary Fig. 2e–g; Fig. 2d,e). These observations indicate that WASH deletion intrinsically affects the maintenance of Nkp46+ ILC3 cells.

WASH promotes AHR expression in Nkp46+ ILC3s. To explore the molecular mechanism by which WASH regulated the maintenance of Nkp46+ ILC3s, we screened key transcription factors and surface markers involved in development and maintenance of ILCs. We noticed that Ahr was dramatically reduced in WASH deleted Nkp46+ ILC3s (Fig. 3a). We isolated ILC subsets from small intestines of Washlox/loxRorc-Cre and Washlox/lox mice and examined AHR expression. Indeed, Ahr was highly expressed in Nkp46+ ILC3s derived from Washlox/lox mice (Fig. 3b), with a modest expression in DN ILC3s and CD4+ ILC3s. These results were further validated by immunoblotting (Supplementary Fig. 2h). Notably, WASH knockout specifically impaired AHR expression in Nkp46+ ILC3s, but not in DN ILC3s or CD4+ ILC3s (Fig. 3b), suggesting WASH is involved in the regulation of AHR expression in Nkp46+ ILC3s. Through chromatin immunoprecipitation (ChIP) assays, we found that WASH bound to Ahr promoter (−400 to −200) in Nkp46+ ILC3s (Fig. 3c), but not in DN ILC3s or CD4+ ILC3s (Fig. 3d). WASH deficiency markedly suppressed AHR transcription in Nkp46+ ILC3s by a nuclear run-on assay, but not in DN ILC3s or CD4+ ILC3s (Fig. 3e). We then transplanted WASH overexpressing BM cells together with recipient BM cells into lethally irradiated CD45.1 recipient mice for reconstitution assays. We observed that WASH overexpression augmented AHR transcription in Nkp46+ ILC3s, but not in DN ILC3s or CD4+ ILC3s (Fig. 3f), suggesting other factors than WASH may be required for AHR expression in DN ILC3s or CD4+ ILC3s. These data indicate that WASH promotes AHR expression in Nkp46+ ILC3s through association with its promoter.

We previously showed that WASH acts as a transcriptional associating factor to promote transcription of target genes via its

Figure 2 | WASH affects the maintenance of Nkp46+ ILC3s in a cell-intrinsic manner. (a,b) 2 × 10^6 Washlox/loxRORγt-GFP or Washlox/loxNcr1-CreRORγt-GFP BM cells were co-transplanted with 2 × 10^6 CD45.1 BM cells into lethally irradiated CD45.1 recipient mice, followed by donor chimerism examination of the indicated ILC3 subpopulations in small intestine (a) and large intestine (b) 8 wk later through flow cytometry (left panel). Donor chimerisms were calculated (right panel). For a and b, n = 6. (c) Live Nkp46+ ILC3 cells were sorted from mice reconstituted as in a through identifying surface markers, followed by Hoechst33342 and pyronin Y staining (left panel). Percentages of S/G2/M cells in the indicated cells were calculated (right panel). n = 5. (d) Mice reconstituted with Washlox/loxRORγt-GFP or Washlox/loxRorc-CreRORγt-GFP BM cells were intraperitoneally injected with 7 mg kg⁻¹ BrdU for 16 h, followed by flow cytometry analysis of BrdU signals in the indicated ILC3 cells. Percentages of BrdU positive cells were calculated. n = 4. (e) Live ILC3 cells were sorted from mice reconstituted with Washlox/loxRORγt-GFP or Washlox/loxRorc-CreRORγt-GFP BM cells through identifying surface markers, followed by Hoechst33342 and pyronin Y staining. Percentages of S/G2/M cells in the indicated ILC3 were calculated. n = 4. Data are shown as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. Data are representative of at least three independent experiments.

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Figure 3 | WASH promotes AHR expression in NKp46⁺ ILC3s. (a) Expression levels of the indicated genes were examined in the indicated cells sorted from the indicated mice by RT-PCR analysis. (b) AHR expression levels were examined in the indicated ILC3 subsets from the indicated mice. For a and b, n = 5. (c) Sorted NKp46⁺ ILC3 cells were subjected to ChIP assay with antibody against WASH, followed by detection of Ahr promoter (right panel) with different primers shown as in the left panel. (d) The indicated ILC3 subsets were subjected to ChIP assay with anti-WASH antibody, followed by detection of Ahr promoter through PCR. (e) The indicated ILC3 subsets were subjected to nuclear run-on assay, followed by RT-PCR analysis of Ahr. (f) Vector or WASH overexpressing ILC3 subsets were subjected to nuclear run-on assay, followed by RT-PCR analysis of Ahr. For c-f, n = 5. (g) pGL3-AHR promoter expressing NK92 cells were transfected with the indicated WASH variants with or without cytochalasin D, followed by luciferase assays (upper panel). Endogenous expression levels of AHR were examined by immunoblotting with anti-AHR antibody (lower panel). (h) In situ hybridization of Ahr promoter and WASH in sorted NKp46⁺ ILC3 cells (left panel). White arrow head denotes Ahr promoters colocalized with WASH. Percentages of cells with WASH colocalized with Ahr promoter were calculated (right panel). At least 200 NKp46⁺ ILC3 cells were counted. Scale bar, 5 μm. (i-j) ChIP analysis of Ahr promoter in NKp46⁺ ILC3 cells sorted from the indicated mice with antibodies against H3K9K14ac (i) or H3K4me3 (j). (k) NKp46⁺ ILC3 nuclei of the indicated mice were treated with indicated units of DNase I. DNA was extracted and examined by PCR (lower panel). Intensities of PCR products were calculated (upper panel). (l) ChIP analysis of Ahr promoter in NKp46⁺ ILC3 cells sorted from the indicated mice with anti-H3K27me3 antibody. (m) NKp46⁺ ILC3 cells sorted from the indicated mice were subjected to nuclear run-on assay, followed by RT-PCR analysis of Ahr. For (i-m), n = 9. Data are shown as means ± s.d. *P<0.05; **P<0.01; ***P<0.001. Data are representative of at least three independent experiments.
VCA domain. We next wanted to determine whether the VCA domain of WASH was required for Ahr transcription. We then transfected full-length WASH (WASH(FL)) or VCA truncated WASH (WASH(AVCA)) into pGL3-AHR expressing NK92 cells for luciferase assays. Consistent with our previous results, WASH(AVCA) abrogated Ahr activation, whereas WASH(FL) was able to activate Ahr transcription (Fig. 3g). The actin nucleation inhibitor cytochalasin D blocked Ahr activation (Fig. 3g). Furthermore, WASH was co-localized with Ahr promoter in NKp46+ ILC3s by fluorescence staining (Fig. 3h). Furthermore, WASH deficiency repressed the acetylation of H3K9K14 and the methylation of H3K4 on Ahr promoter (Fig. 4a). Arid1a belongs to the BRG1-interactor of WASH (Fig. 4a). Arid1a together bind to the Ahr promoter region. Furthermore, Arid1a deletion abolished the interaction between WASH and other components of BAF complex (Fig. 4e), suggesting that WASH associates with BAF complex via Arid1a interaction. Similar to WASH, Arid1a bound to the same region of Ahr promoter in NKp46+ ILC3s (Fig. 4f). Additionally, other components of BAF complex were also recruited to Ahr promoter (Fig. 4g). These data suggest that WASH may recruit BAF complex to Ahr promoter for enhancing its transcription.

We generated Arid1a/flox/flox Rorc-Cre mice by crossing Arid1a/flox/flox mice with Rorc-Cre mice. Arid1a was completely deleted in ILC3s (Fig. 4h). Through nuclear run-on assays, we noticed that Arid1a knockout repressed Ahr transcription in NKp46+ ILC3s (Fig. 4i), but not in CD4+ ILC3s. We next transplanted Arid1a overexpressing BM cells together with recipient BM cells into lethally irradiated recipient mice. We observed that Arid1a overexpression caused elevated expression of AHR in NKp46+ ILC3s, but not in DN ILC3s or CD4+ ILC3s (Fig. 4j). We observed that Arid1a was expressed in three subsets of ILC3s (Fig. 5a). Moreover, Arid1a deficiency suppressed the acetylation of H3K9K14 and the methylation of H3K4 on Ahr promoter (Fig. 5b,c). Arid1a knockout also made Ahr promoter more resistant to DNase I cleavage (Fig. 5d), suggesting an inaccessible state of Ahr promoter for transcriptional activation. We next transfected full-length Arid1a (Arid1a(FL)) or two Arid1a mutants lacking WASH binding domains into pGL3-AHR expressing WASH silenced NK92 cells, followed by examination of Ahr activation through luciferase assays. We observed that Arid1a mutants failed to activate Ahr transcription in both shCtrl treated and WASH silenced cells (Fig. 5e). By contrast, Arid1a(FL) could activate Ahr transcription in shCtrl treated cells, but not in WASH silenced cells (Fig. 5e). These observations suggest that Arid1a promotes Ahr transcription in a WASH-dependent manner.

WASH knockout abrogated the co-localization of Arid1a with Ahr promoter in NKp46+ ILC3s by fluorescence imaging (Fig. 5f). In parallel, WASH deletion impaired the recruitment of Arid1a and BAF complex to Ahr promoter by ChIP assays (Fig. 5g,h). Finally, in WASH silenced cells, Arid1a mutants failed to bind Ahr promoter (Fig. 5i). Taken together, we conclude that WASH recruits the BAF complex to promote AHR expression in NKp46+ ILC3s.

Arid1a maintains NKp46+ ILC3s through AHR ILC3s. We next examined ILC3 subsets in Arid1a/flox/flox Rorc-Cre and Arid1a/flox/flox mice. We observed that Arid1a/flox/flox Rorc-Cre mice showed reduced numbers of NKp46+ ILC3s, but not other subsets of ILC3s (Fig. 6a), these observations were similar to those of WASH/flox/flox Rorc-Cre mice. In addition, Arid1a deficiency did not affect the apoptosis of NKp46+ ILC3s either. We found that Arid1a deficient NKp46+ ILC3s derived from Arid1a/flox/flox Rorc-Cre mice incorporated much less BrdU than those from Arid1a+/flox mice (Fig. 6b). In addition, Arid1a+/− / NKp46+ ILC3s maintained in a resting state compared with those of Arid1a+/− / ILC3s (Fig. 6c). Similar results were obtained when we reconstituted recipient mouse with Arid1a deficient BM cells. We next overexpressed full length or mutant Arid1a in Arid1a/flox/flox Rorc-Cre BM cells and then transplanted these BM cells together with recipient BM cells into lethally irradiated recipient mice. We found that overexpression of two Arid1a mutants lacking WASH binding ability in Arid1a/flox/flox Rorc-Cre BM cells failed to rescue donor reconstitution ratios of NKp46+ ILC3s caused by Arid1a deficiency (Fig. 6d). By contrast,
overexpression of full length Arid1a in Arid1a<sup>−/−</sup> ILC3s could rescue donor reconstitution rates of NKp46<sup>+</sup> ILC3s as well as IL-22 expressing ILC3s (Supplementary Fig. 4a, b). In addition, DKO mice dramatically reduced AHR expression in NKp46<sup>+</sup> ILC3s (Supplementary Fig. 4c), accompanied with fewer cycling cells (Supplementary Fig. 4d). Finally, DKO mice did not impact apoptosis of NKp46<sup>+</sup> ILC3s (Supplementary Fig. 4e). These observations indicate that WASH and Arid1a exert synergistic effect on Ahr expression.

We next rescued AHR in Arid1a KO or DKO NKp46<sup>+</sup> ILC3s by transplanting AHR overexpressing Arid1a<sup>−/−</sup> ILC3s or WASH<sup>−/−</sup> ILC3s into lethally irradiated recipient mice for reconstitution assays (Supplementary Fig. 4f). AHR restoration
rescued donor reconstitution ratios of NKp46+ ILC3s reduced by Arid1a KO or DKO of WASH with Arid1a (Supplementary Fig. 4g), and maintained more cycling cells (Supplementary Fig. 5e). By contrast, AHR antagonist treatment rendered NKp46+ ILC3s in resting state even in WT ILC3s (Supplementary Fig. 5e). Altogether, WASH-mediated AHR expression is required for the maintenance of NKp46+ ILC3s.

**Discussion**

Innate lymphoid cells (ILCs) are a distinct arm of the innate immune system, and can directly communicate with other hematopoietic and non-hematopoietic cells to regulate immunity, inflammation and tissue homeostasis34. However, how these ILCs...
Donor chimerism (%)

lineages develop and maintain remains largely unknown. In this study, we show that WASH is highly expressed in the nucleus of ILC3s. WASH deletion impairs the maintenance of NKp46+ ILC3s but not other ILC subsets. In NKp46+ ILC3s, WASH recruits Arid1a to Ahr promoter to activate AHR expression. WASH-mediated AHR expression is required for the maintenance of NKp46+ ILC3s. WASH deletion in ILC3s reduced the number of NKp46+ ILC3s. Notably, double knockout of WASH and Arid1a impedes AHR expression and NKp46+ ILC3s maintenance.

All ILC subsets are derived from common lymphoid progenitors (CLPs), which also differentiate into T and B cells17,34. The earliest progenitor cells specific to ILCs are CXCR3+ integrin α4β7-expressing CLPs, referred to as α-lymphoid precursor (αLP) cells, which give rise to ILC1, ILC2, ILC3, and conventional NK cells (cNKs)35,36. The common progenitor to all ILC lineages (CHILP) is identified as its Lin−CD127−I1d2−CD25−α4β7+ phenotype and differentiates to all ILC subsets, but not cNKs37. The common precursor to ILCs (ILCP) is defined by expression of transcription factor PLZF and gives rise to ILC1, ILC2, and ILC3 subpopulations38. We previously reported that conditional deletion of WASH in the hematopoietic system impairs the differentiation of HSCs and causes defective blood production of the host28. In this study, we show that WASH deletion in the hematopoietic system does not impact the development of progenitors of ILCs, including αLP, CHILP and ILCP cells. However, WASH deficiency only affects the maintenance of NKp46+ ILC3 subsets, but not other ILC lineages or ILC3 subpopulations. Our observations suggest that the development and maintenance of different ILC subsets utilize different intrinsic checkpoints.

Group 3 ILCs (ILC3) are defined by their expression of RORγt, including CCR6+ lymphoid tissue inducer (LTI) cells and CCR6− ILC3 lineage15,37. Through expression of lymphotixin (LT) γ1β2, LTI cells induce the development of lymph nodes and Peyer’s patches through LTβ receptor (LTβR) signalling38,39. Some CCR6− ILC3s express a natural cytotoxicity-triggering receptor (NCR): NKp46 in mice32 and NKp44 in humans30, and eventually develop into NKp46+ ILC3s. Unlike LTI cells, CCR6− ILC3s are developed from PLZF+ ILPC cells18, and they proliferate largely in mice mainly after birth to 3–4 weeks of age41. NKp46+ ILC3s uniquely produces only IL-22, not IL-17 (refs 14,15), and have the potential to differentiate into IFN-γ producing ILC1s17. Here we show that WASH deletion only impairs the maintenance of NKp46+ ILC3s and reduces IL-22 production. However, WASH deletion did not change the IFN-γ
secretion, suggesting that WASH specifically controls the maintenance of NKp46+ ILC3s, but not IFN-γ producing ILC1s. WASH, a member of WASP family, plays a critical role in endosomal trafficking via its actin nucleation function. We previously showed that WASH is localized at autophagosomes of mouse embryonic fibroblasts (MEFs) and regulates autophagy induction. Additionally, WASH-mediated AHR expression has no impact on the development of NKp46+. We also found that WASH is constitutively expressed in the blood cells and mainly resides in the nucleus of long-term hematopoietic stem cells (LT-HSCs). WASH deletion breaks the balance of self-renewal and differentiation of HSCs. We demonstrated that WASH promotes the differentiation of LT-HSCs through association with the NURF complex, which activates c-Myc transcription. Chromatin remodelling complexes are involved in nucleosome sliding, dissociation, or replacement using the energy derived from ATP hydrolysis. Herein we show that WASH is located in the nucleus of NKp46+ ILC3s and associates with the BAF chromatin remodelling complex through direct interaction with Arid1a. Arid1a, also known as BAF250a, is a major component of BAF complex. Arid1a deficiency impairs the pluripotency and self-renewal of embryonic stem (ES) cells, and results in early embryonic lethality. Here we showed that WASH is preferentially highly expressed in NKp46+ ILC3s. High nuclear levels of WASH in NKp46+ ILC3s could be acquired to recruit the BAF complex to Ahr promoter for promoting its transcription. WASH-mediated AHR expression controls the maintenance of NKp46+ ILC3 cell pool of the host. However, why WASH is preferentially expressed in NKp46+ ILC3s needs to be further investigated.

AHR is expressed in many cell types and thus exerts pleiotropic effects by integrating with other signalling pathways. AHR deficient mice frequently died after birth or exhibited a slower growth rate in the first few weeks of life. AHR signalling is implicated in a variety of immune responses from different cell types. Especially, AHR plays a critical role at mucosal interfaces where the host encounters other living entities and environmental agents. Of note, ILCs are preferentially distributed at mucosal surfaces, where they can perceive changes in the surrounding microenvironment primarily via cytokine receptor signalling. ILC3s express high levels of AHR and sensitively respond to AHR signalling. AHR signalling is not only needed for IL-22 production by ILC3s, but is necessary for their development and/or maintenance. AHR deficient mice displayed reduced numbers of ILC3s in small intestines and in Peyer’s patches. Here we show that WASH-mediated AHR expression controls the maintenance of NKp46+ ILC3s, but not other ILC lineages. Additionally, WASH-mediated AHR expression has no significant impact on the development of NKp46+ ILC3s. Consequently, decreased numbers of NKp46+ ILC3s substantially reduce production of IL-22. In sum, WASH-mediated AHR expression is indispensable for the maintenance of NKp46+ ILC3s in the intestine.

Methods

Antibodies and reagents. A rabbit polyclonal antibody against WASH was generated from the VCA domain of WASH protein as described previously. Commercial antibodies: Antibodies against CD3 (17A2), CD4 (GK1.5), CD8 (53–6.7), CD19 (6D5), CD20 (L27), CD45 (30-F11), and CD11b (ED7) were from BD Pharmingen. Anti-CD25 (PC61.5), anti-CD11c (N418), and anti-CD11c (RB6–8C5), and F4/80 (BM8), Ter119 (TER-119), CD27 (LG.7F9), CD90 (H9; BD Biosciences), CD45.2 (104), and anti-RORγt (AFK9S) were from Biolegend. Anti-CD4 (14-5-2C11), anti-CD8 (53–1.5), anti-CD19 (6D5), and anti-CD20 (GK1.5) were from eBioscience (San Diego, USA) and used in a 1:100 dilution for flow cytometric analysis. Polyclonal antibodies against CD3 (17A2), CD4 (GK1.5), CD19 (6D5), CD8 (53–6.7), CD11b (ED7), and CD11c (RB6–8C5) were from Sigma-Aldrich and used in a 1:2,000 dilution for western blotting and in a 1:500 dilution for ELISA. Polyclonal antibodies against CD11c (N418), Gr1 (RB6–8C5), F4/80 (BM8), Ter119 (TER-119), CD27 (LG.7F9), CD90 (H9; BD Biosciences), CD45.2 (104), and anti-RORγt (AFK9S) were from Biolegend. Anti-CD4 (14-5-2C11), anti-CD8 (53–1.5), anti-CD19 (6D5), and anti-CD20 (GK1.5) were from eBioscience (San Diego, USA) and used in a 1:100 dilution for flow cytometric analysis. Polyclonal antibodies against CD3 (17A2), CD4 (GK1.5), CD19 (6D5), CD8 (53–6.7), CD11b (ED7), and CD11c (RB6–8C5) were from Sigma-Aldrich and used in a 1:2,000 dilution for western blotting. Donkey anti-rabbit IgG secondary antibodies conjugated with Alexa-488, Alexa-594 or Alexa-405 were purchased from Molecular Probes. Donkey anti-mouse IgG secondary antibodies conjugated with Alexa-488 or Alexa-594 were purchased from Thermo Fisher Scientific. Anti-ΔGt (60C6) and anti-ΔBt (SP124) antibodies were from Sigma-Aldrich and used in a 1:2,000 dilution for western blotting. Cell culture and transfection. NK cell line NK92 (ATCC: CRL-2407) was cultured in Alpha Minimum Essential medium (α-MEM) containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM 2-mercaptoethanol, 10 mM HEPES, 500 U ml−1 recombinant IL-2, 12.5% horse serum and 12.5% FBS. For NK92 transfection, cells (1 × 10^7) were resuspended in 100 µl Nucleofector Solution buffer (Lonza) containing 5 µg DNA, followed by transfection using the NucleoFector System Y-601 on Amaxa nucleofector II device (Lonza). Cells were harvested after 24 h, solubilized in β-mercaptoethanol, and used for proteinase K, ILC3s, but not other ILC lineages. For BM cell transfection, πSN-F2-E3-RES-EGFP lentiviral vectors carrying the indicated genes were transfected into HEK293T cells (maintained by our laboratory) together with pSPAX2 and pMD2.G, followed by concentration through ultracentrifugation on 50,000 g for 2 h. Pellets of lentivirus were resuspended in serum-free α-MEM media. Donor BM cells were infected with lentviruses by centrifugation at 500 g for 1.5 h in the presence of 8 µg ml−1 Polybrene (Sigma-Aldrich) and then incubated at 37 °C for 18 h, followed by sorting for GFP+ cells that were used for transplantation into lethally irradiated recipient mice.

C. rodentium infection. C. rodentium was a gift from Dr Baoxue Ge (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences). Mice were orally infected with C. rodentium (2 × 10^7 per mouse), followed by survival rate and body change examination in the following days. Mice were sacrificed to examine colon pathology and bacterial loads on day 8 post infection.

Intestinal lymphocyte separation and flow cytometry. Intestinal lymphocytes were separated from intestines as described. Briefly, intestines were cut into pieces with 0.5 cm length post removing fat tissues and Peyer’s patches, followed by digestion in HBSS buffer containing 15 mM HEPES, 5 mM EDTA and 10% FBS. To obtain IELs, intestines were digested in the above buffer on a roller for 30 min at 37 °C for three times to collect the detached cells from the intestinal tissues. To obtain LPLs, the remaining intestinal tissues were further digested in HBSS buffer containing 10% FBS, 0.2 mg ml−1 collagenase and 0.2 mg ml−1 DNase I (Sigma-Aldrich). Detached cells were sifted through 50 µm cell strainers for further analysis. For flow cytometry, cells were stained with fluorescence-conjugated primary antibodies for 1 h on ice. Cells were then fixed and permeabilized, followed by nuclear staining of transcription factors. Cells were analysed or sorted by a cell sorter (BD AriaIII). Data were analysed using the FlowJo 7.6.1 software.

BrdU incorporation and cell cycle analysis. Mice were intraperitoneally injected with BrdU (7 mg kg−1 body weight) for 16 h, followed by flow cytometry of BrdU signals in LT-HSCs. For staining BrdU in the nucleus, cells were fixed and permeabilized in fixation/permeabilization buffer (eBioScience) according to manufacturer’s instructions. Permeabilized cells were stained with fluoresceine-conjugated anti-BrdU antibody for 2 h, and washed with diluted permeabilization buffer for three times. For cell cycle analysis using Hoechst/Pyrnonin Y staining strategy, live cells were harvested from RORγt-GFP reporter mice and resuspended in 200 µl IMDM medium containing 10 µg ml−1 Hoechst33342 for 45 min at 37 °C, followed by addition of Pyrnonin Y to a final concentration of 0.5 µg ml−1 for 15 min at 37 °C.

Histology. Colon samples were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 12 h. Fixed tissues were washed for twice using 75% ethanol and embedded in paraffin, followed by sectioning and staining with haematoxilin and eosin according to standard procedures.

Bone marrow transplantation. Bone marrow transplantation (BMT) was performed as previously described. Briefly, 2 × 10^6 BM cells were co-transplanted with 2 × 10^6 WT CD45.1 BM cells into lethally irradiated mice. For knockdown or overexpression sequences, followed by culture in HSC culture medium StemPro-34.
supplemented with cytokines for 36 h. GFP positive LT-HSCs were sorted through flow cytometer, followed by BMT of 2 × 10^6 BM cells with 2 × 10^6 WT CD45.1 BM cells into lethally irradiated mice. CD45.2 and CD45.1 C57BL/6 mice were bred in SPF conditions. Mouse experiments complied with ethical regulations and were approved by the Institutional Animal Care and Use Committees at the Institute of Biophysics, Chinese Academy of Sciences.

**Immunofluorescence assay.** Immunostaining was performed as previously described. Briefly, sorted cells were stuck on 0.01% poly-L-Lysine treated coverslips and fixed with 4% PFA for 10 min, followed by permeabilization with 0.5% Triton X-0.5% for 20 min at room temperature (RT). Primary antibodies were added for 2 h at RT post blocking with 10% donkey serum for 30 min. Samples were further stained with Alexa488-, Alexa594- or Alexa405-conjugated secondary antibodies, followed by confocal microscopy (Olympus FV1000).

**Western blot.** Cells were lysed with RIPA buffer (150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% NP40, 1 mM EDTA, 50 mM Tris (pH 8.0)), followed by separation with SDS-PAGE. Samples were then transferred to NC membrane and incubated with primary antibody in 5% BSA. After washing with TBST three times, membranes were incubated with HRP-conjugated secondary antibodies for visualization. 1 × 10^5 cells were used for a single sample for whole cell blots. See Supplementary Fig. 6 for uncorrected blots.

**Co-immunoprecipitation assay.** For co-IP experiments, 4 × 10^5 NKp46⁺ ILC3 cells (pooled from 20 RORγt-GFP reporter mice) were lysed in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) to remove cytoplasmic contents. Nuclear pellets were lysed in RIPA lysis buffer for 1 h, followed by incubation with anti-WASH antibody for 4 h post preclearance with protein A/G agarose. WASH containing complex was further precipitated by protein A/G agarose. Immunoprecipitates were washed with RIPA buffer, followed by immunoblotting with antibodies against WASH and Arid1A. 4 × 10^5 Arid1A deleted ILC3 cells sorted from Arid1afllox/flox-Rorc⁻ mice were also lysed and immunoprecipitated with anti-WASH antibody for co-IP assay as a control.

**Chromatin immunoprecipitation.** ChIP was performed using MAguify ChIP kit with manufacturers' instructions. Briefly, cells sorted from intestines were cross-linked with 1% formaldehyde at 37 °C for 10 min, and then quenched with 0.125 M glycine, followed by swelling in lysis buffer (50 mM Heps, pH 7.5, 140 mM NaCl, 0.1% Triton X-100, 0.1% NaDeoxycholate, and Protease Inhibitor Cocktail Set III) for 30 min on ice. Chromatin was sheared to a mean length of 400 bp by sonication. After being de-cross-linked by RNase, proteinase K, and heat, input genomic DNA was precipitated with ethanol and quantified in a GeneQuant 100 spectrophotometer (GE Healthcare). Chromatin was preclotted with protein A/G-agarose, followed by incubation with indicated antibodies at 4 °C overnight and further incubation with protein A/G-agarose for 2 h. Beads were washed with washing buffer (10 mM Tris, pH 8.0, 250 mM LcL, 0.5% NP-40, 0.5% Nadeoxycholate, 1 mM EDTA) three times and eluted with elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 10 mM EDTA). Eluates were de-cross-linked by RNase, proteinase K, and heat, and DNA was extracted with phenolchloroform, followed by ethanol precipitation. For each ChIP experiment, 2 × 10^4 cells were used. 5% of nuclear extracts served as inputs. Immunoprecipitated DNAs were further analysed by real-time PCR. 2 × 10^5 NKp46⁺ ILC3 cells were pooled from 4–10 WT or WASH knockout mice for each group.

**RNA interference.** RNA interference was performed according to pSUPER system instructions (Oligogene). pSUPER vectors encompassing target sequences were constructed. DNA target sequences were: #1, 5'-GGCGAGGCTGATAGAA TGAA-3'; #2, 5'-AGGCCAAGACTGG AGAAGAA-3'.

**RT-PCR assay.** Total RNA was extracted from cells using Trizol reagent and cDNA was reverse-transcribed using Superscript II (Invitrogen). RT-PCR was performed using StarScript II Two-step RT-PCR Kit (Genstar) with the following primers: WASH, forward, 5'-CTCTTGGCCACGCCTCAG-3', reverse, 5'-CTCG CAGACCGGCTCATTACG-3'; Arid1a, forward, 5'-ATCTTGGCACGGTCTG ACTCC-3', reverse, 5'-GGCATTCTGATTTC GCAGTGG-3'; Ahr, forward, 5'-AAGAAGAGGGAGGCAAGGAGC-3', reverse, 5'-CTGCCC TTGGGATCATC AAC-3'.

**Recombinant protein preparation.** cDNAs were cloned from a bone marrow cDNA library48. WASH or Arid1a was subcloned to pGEX-6 P-1 for GST-tagged protein expression or to PET-28a for His-tagged protein purification. Plasmids were transformed into E. coli strain BL21 (DE3). DE3 clones were cultured (OD600 = 0.6), followed by induction with 0.2 mM IPTG at 16 °C for 24 h. Cells were collected and lysed by sonoporation, followed by purification through Ni-NTA resins or GST resins. GST-tagged proteins were cleaved by PreScission protease to remove GST tags.

**Luciferase assay.** Luciferase reporter was constructed as described. Ahr promoter region (~ 2,000 to 0) was subcloned into pGL3 vector. Luciferase reporter vectors were cotransfected with pRL-TK (as an internal control reporter vector) into macrophages by electroporation. Luciferase assays were performed with guidelines provided by the manufacturer (Promega). For NK92 transfection, cells (1 × 10^5) were resuspended in 100 µl Nucleofector Solution buffer (Lonza) containing 5 µg DNA, followed by transfection using the Nucleofector Program Y-001 on Amnax nucleofector II device (Lonza). Cells were recovered in RPMI1640 media containing 4 mM l-glutamine, 1.5 g/l sodium bicarbonate, and 10% heat-inactivated fetal bovine serum (FBS) for 6 h, followed by flow cytometry sorting for viable cells.

**Nuclear run-on assay.** Sorted cells were harvested in buffer containing 150 mM KCl, 10 mM Tris- HCl, 4 mM MgOAc with pH 7.4, followed by centrifugation to collect cell pellets. Pellets were lysed in buffer containing 150 mM KCl, 10 mM Tris- HCl, 4 mM MgOAc, and 0.5% NP-40, followed by sucrose density gradient centrifugation to prepare crude nuclei. Crude nuclei were incubated with 10 mM ATP, CTP, GTP, BrUTP and RNase inhibitor at 28 °C for 5 min. RNAs were collected and resuspended in Trizol reagent. After users of DNAse I, RNA transcripts were immunoprecipitated with antibody against BrdU, followed by reverse transcription and RT-PCR analysis. 5 × 10^4 cells were used for a single sample.

**DNasel accessibility assay.** Nuclear were purified from cells according to the manufacturer's protocol with Nuclei Isolating Kit (NUC101-1KT, Sigma-Aldrich). Then nuclei were resuspended with DNase I digestion buffer and treated with indicated units of DNase I (Sigma-Aldrich) at 37 °C for 5 min. 2 × DNase I stop buffer (20 mM Tris Ph 8.0, 4 mM EDTA, 2 mM EGTA) was added to stop reactions. DNA was extracted and examined by PCR.

**Yeast two-hybrid screening.** Yeast two-hybrid screening was performed as described. Briefly, WASH was cloned into pGBKt7 vector (BD-WASH). Yeast AH109 cells were transfected with BD-WASH and plasmids containing a mouse BM cDNA library (Clontech/Takara) and then plated on selective SD medium. Selected clones were isolated and sequenced. X-y gal assay was carried out following manufacturer's instructions.

**Statistical analysis.** Student's t-test was used as statistical analysis by using Microsoft Excel.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

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