Supplementary Methods and Figures for:

C3a is required for ILC2 function in allergic airway inflammation

Naina Gour[1,2,*], Ursula Smole[1,3*], Hwan-Mee Yong[1], Ian P. Lewkowich[4], Nu Yao[1], Anju Singh[1], Edward Gabrielson[5], Marsha Wills-Karp[1] and Stephane Lajoie[1]

Affiliations

[*]contributed equally to this manuscript

[1]Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

[2]Solomon H. Snyder Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD

[3]Institute of Immunology, Center for Pathophysiology, Infectiology, and Immunology, Medical University of Vienna, Vienna, Austria.

[4]Department of Immunobiology, Cincinnati Childrens Hospital Medical Center, Cincinnati, OH

[5]Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, United States

Correspondence to:

Stephane Lajoie, Ph.D.

Department of Environmental Health and Engineering

Johns Hopkins Bloomberg School of Public Health

615 N. Wolfe St., Rm E6612

Baltimore, MD 21205

Ph: (410)-955-5930

Email: slajoie1@jhu.edu

Supplementary Methods

Analysis of ImmGen Consortium data

C3ar1 and *C5ar1* expression data from lymphoid-lineage cells, as previously published[55], was obtained from the ImmGen Consortium (www.immgen.org).

EdU assay

Seven to ten week old male BALB/c and *C3ar1*$^{-/-}$ mice were given PBS (30 ul) or IL-33 (0.5 ug/30 ul) i.n. on days 0, 2, and 4. On day 6, mice were injected i.v. with 0.5 mg EdU (Thermo) 2h prior to sacrifice. EdU incorporation in lung ILC2 was analyzed by flow cytometry (Click-It Plus EdU Alexa Fluor 647 Flow Cytometry Assay kit, Thermo).

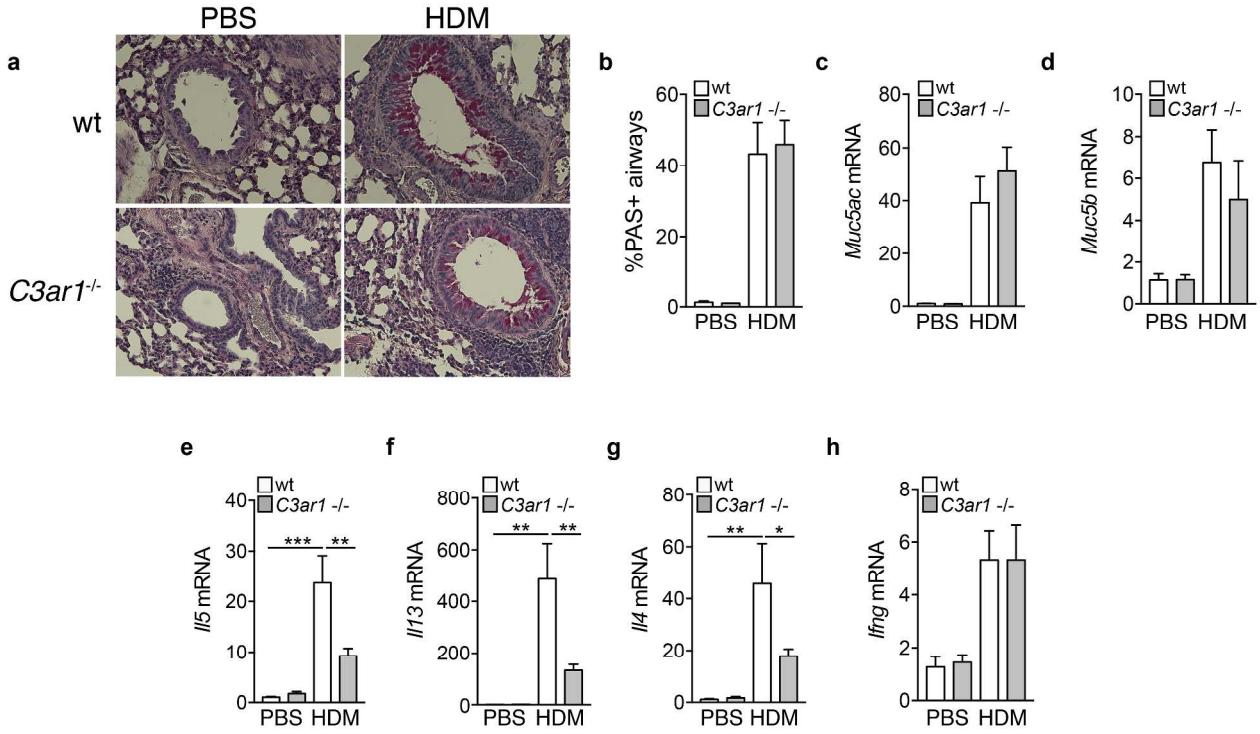
Flow cytometry

Lung cells were isolate as in the main Methods section, and stained to identify MHCII+, CD80+ and CD86+ ILC2. Lung cells were stained with PerCP-Cy5.5-conjugated lineage antibodies (CD11b, CD11c, Gr1, γδTCR, FceR1, TER119, CD49b, CD19, B220, CD3, αβTCR, CD4, CD8a- see main Methods for clone numbers), FITC-anti-IL-33R (T1/ST2, MD Bioproducts), Brilliant Violet 786 ICOS, Alexa Fluor 700 anti-CD45 (30-F11, BioLegend), APC-Cy7 anti-I-A/I-E (M5/114.15.2, BioLegend), Brilliant Violet 421 anti-CD80 (16-10A1, BioLegend), and Brilliant Violet 605 anti-CD86 (GL-1, BioLegend). For viability determination in transfer Rag2-/-Il2rg-/- transfer experiments, cells were stained with the Aqua live/dead dye, then with PE-Cy7-anti-CD45 (30-F11, BioLegend), PerCP-Cy5.5-anti-CD11b (M1/70, BioLegend), Alexa Fluor 700 anti-CD11c (N418, BioLegend), APC-Cy7-anti-Ly-6G (1A8, BioLegend), PE-CF594-anti-Siglec-F (E50-2440, BD Biosciences), BV421-anti-CD3 (17A2, BioLegend), BV605-anti-CD4 (RM4-5, BioLegend), BV786-anti-ICOS (7E.17G9, BD Biosciences), FITC-anti-IL-33R (DJ8, MD Bioproducts).

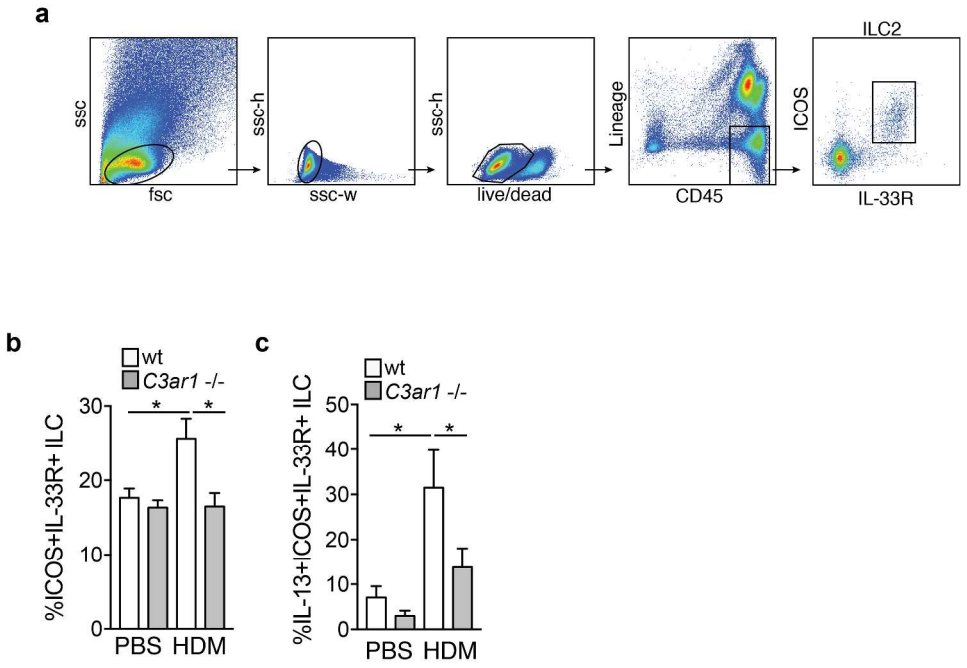
PAS staining

To assess the effects of allergen on mucus production from the airway epithelial cells, lungs were excised and fixed in 10% neutral buffered formalin, processed, paraffin embedded and sectioned. Sections were stained with Periodic Acid Schiff (PAS). Slides were read in a blinded fashion.

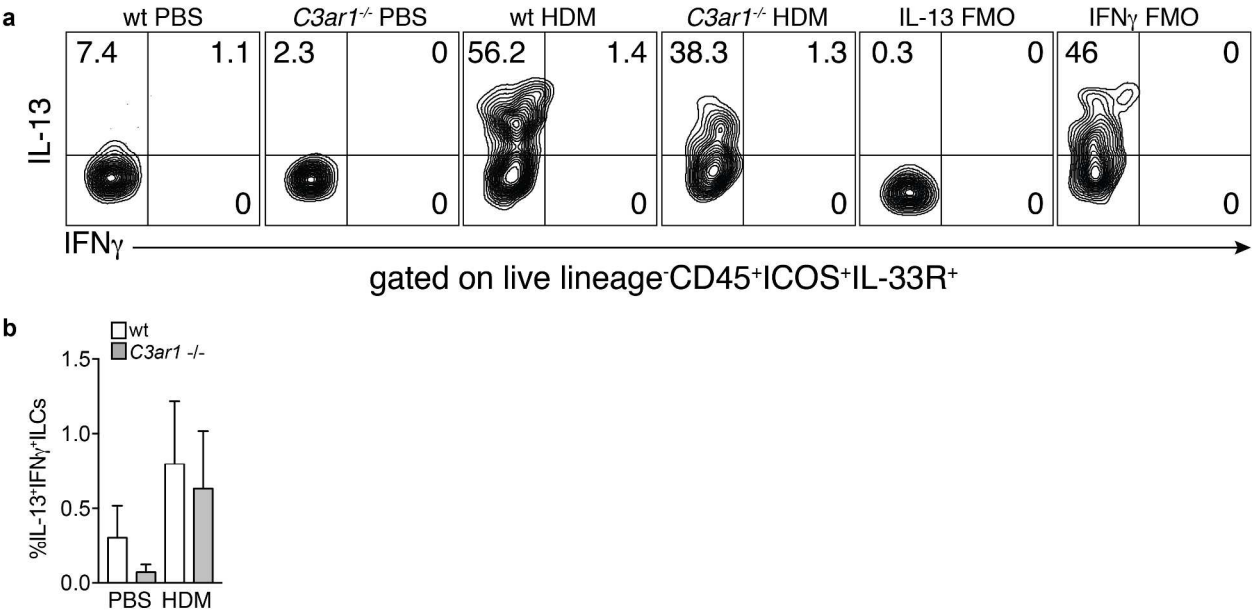
For quantifying mucus producing airway epithelial cells, % of PAS- positive cells in the left lobe of the lung were determined using a light microscope. Results are presented as mean + SE for 2–5 mice per group.



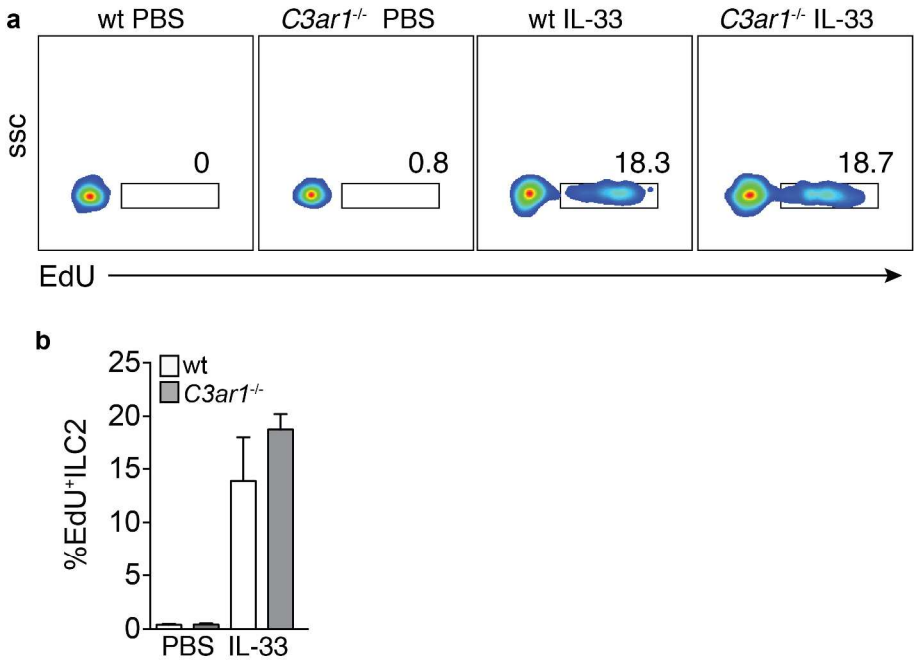
Supplementary Figure 1. C3a signaling is required to develop allergen-driven type 2 immunity. Wildtype BALBc/J and *C3ar1*$^{-/-}$ mice were exposed to PBS or HDM (100 ug) i.t. on days 0 and 14. On day 17, lungs were harvested and quantified for mucus production by PAS staining (**a**,**b**) and levels of (**c**) *Muc5ac*, (**d**) *Muc5b*, (**e**) *Il5*, (**f**) *Il13*, (**g**) *Il4*, and (**h**) *Ifng* mRNA were determined. Data represents means+SEM. Data is representative from 2 independent experiments *p<0.05, **p<0.01



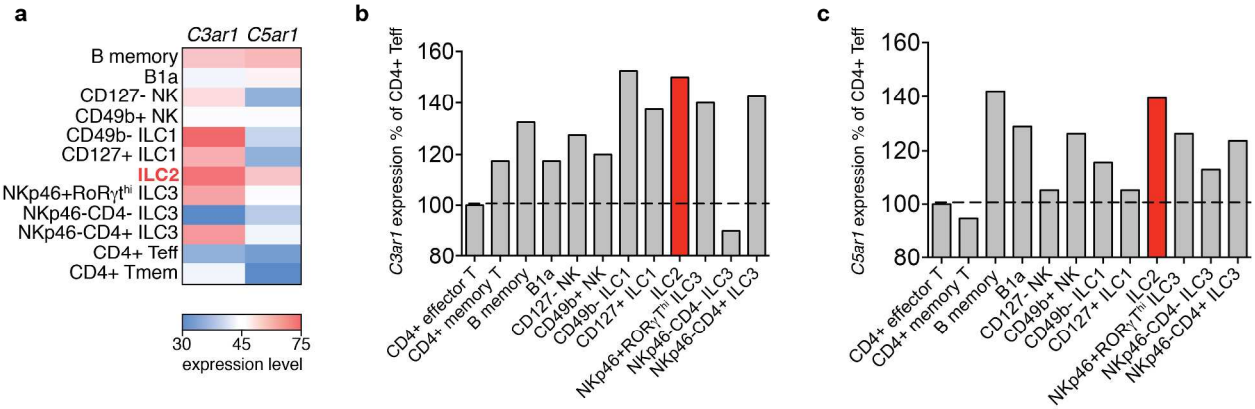
Supplementary Figure 2. Identification of lung ICOS$^+$IL-33R$^+$ ILC2. (**a**) Lung ILC2 were identified as lineage$^-$ (CD11b, CD11c, Gr1, TER119, FceR, CD19, TCRgd, CD49b, TCRb, CD4, CD8, CD3) CD45$^+$ICOS$^+$IL-33R$^+$. Frequency of lung (**b**) ICOS$^+$IL-33R$^+$ ILC2 and (**c**) IL-13$^+$ICOS$^+$IL-33R$^+$ ILC2 in wildtype and *C3ar1*$^{-/-}$ mice that were exposed to PBS or HDM (100 ug) i.t. on days 0 and 14, and analyzed on day 17. Data is representative from 2 independent experiments *p<0.05



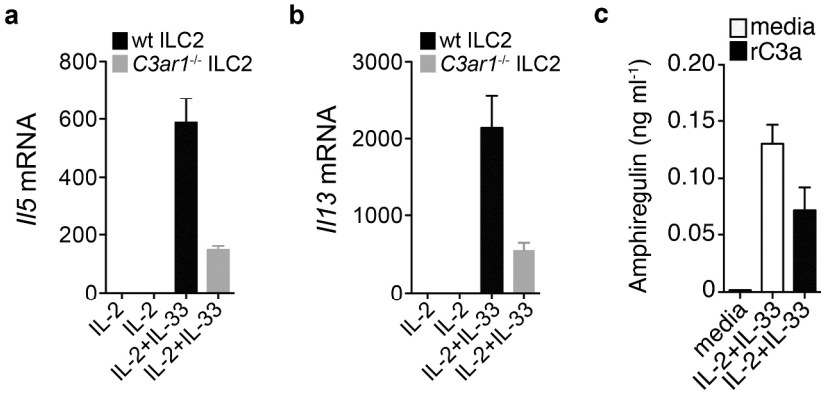
Supplementary Figure 3. C3a signaling has no effect on IFNγ production by lung ILC2. (**a,b**) Flow cytometric determination of IL-13 and IFNγ production by HDM-induced lung ILC2 (Lin^- (CD11b, CD11c, Gr1, B220, CD19, TCRb, TCRgd, CD49b, CD4, CD8, FcER1)$ICOS^+$IL-$33R^+$). Data is representative from 2 independent experiments.



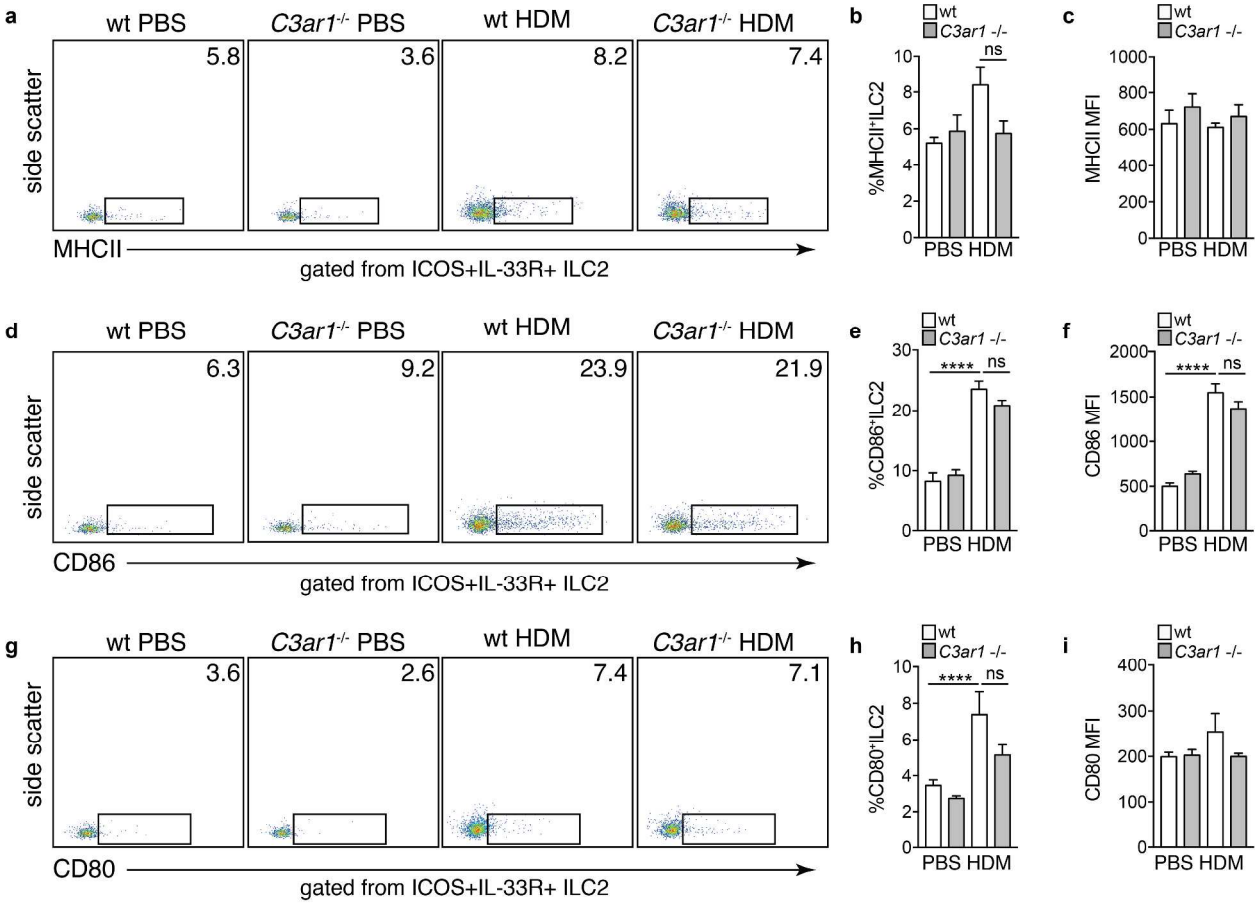
Supplementary Figure 4. C3a signaling does not enhance ILC2 proliferation. (**a**) Flow cytometric identification of EdU+ lung Lin$^-$ (CD11b, CD11c, Gr1, B220, CD19, TCRb, TCRgd, CD49b, CD4, CD8, FcER1) ICOS$^+$IL-33R$^+$ ILC2 (**b**) Levels of lung EdU$^+$ ILC2 in wildtype and *C3ar1*$^{-/-}$ mice were exposed to PBS or IL-33. Data represents means+SEM. Data is representative from 2 independent experiments.



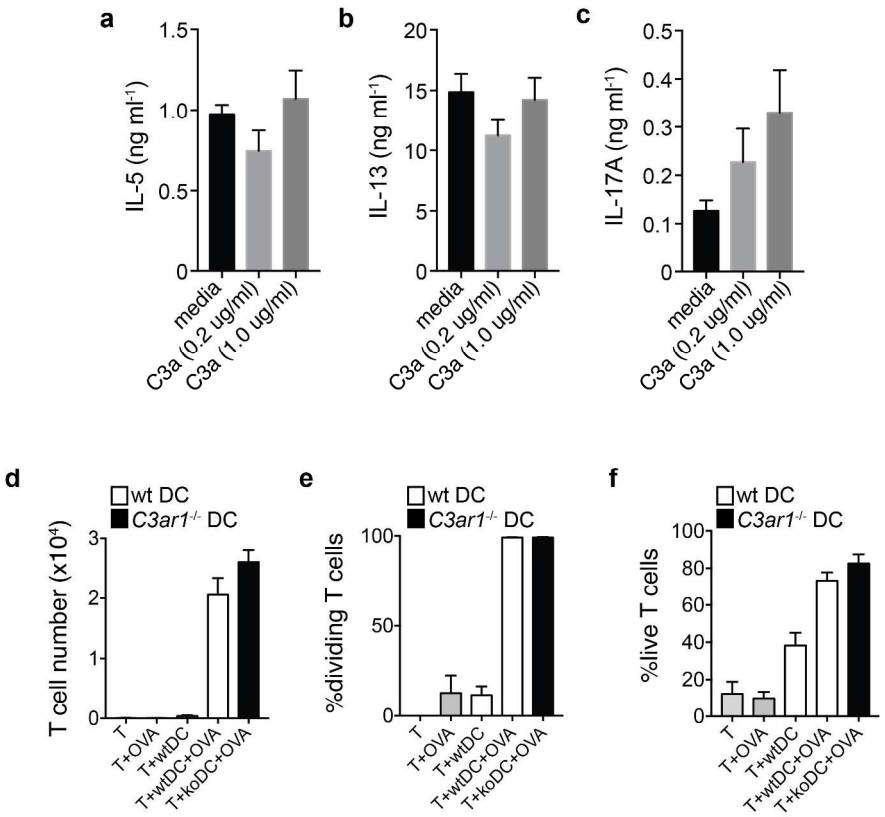
Supplementary Figure 5. ImmGen Consortium *C3ar1* and *C5ar1* expression in lymphoid lineage cells. (**a**) Data assembled by the ImmGen Consortium was analyzed for *C3ar1* and *C5ar1* expression in cells of lymphoid origins, including ILC2 ($Lin^-CD45^+CD127^+Sca\text{-}1^+IL\text{-}33R^+KLRG1^+$). *C3ar1* and *C5ar1* expression across lymphoid cells was expressed as percent of (**b**) *C3ar1* and (**c**) *C5ar1* expression in $CD4^+$ T effector cells.



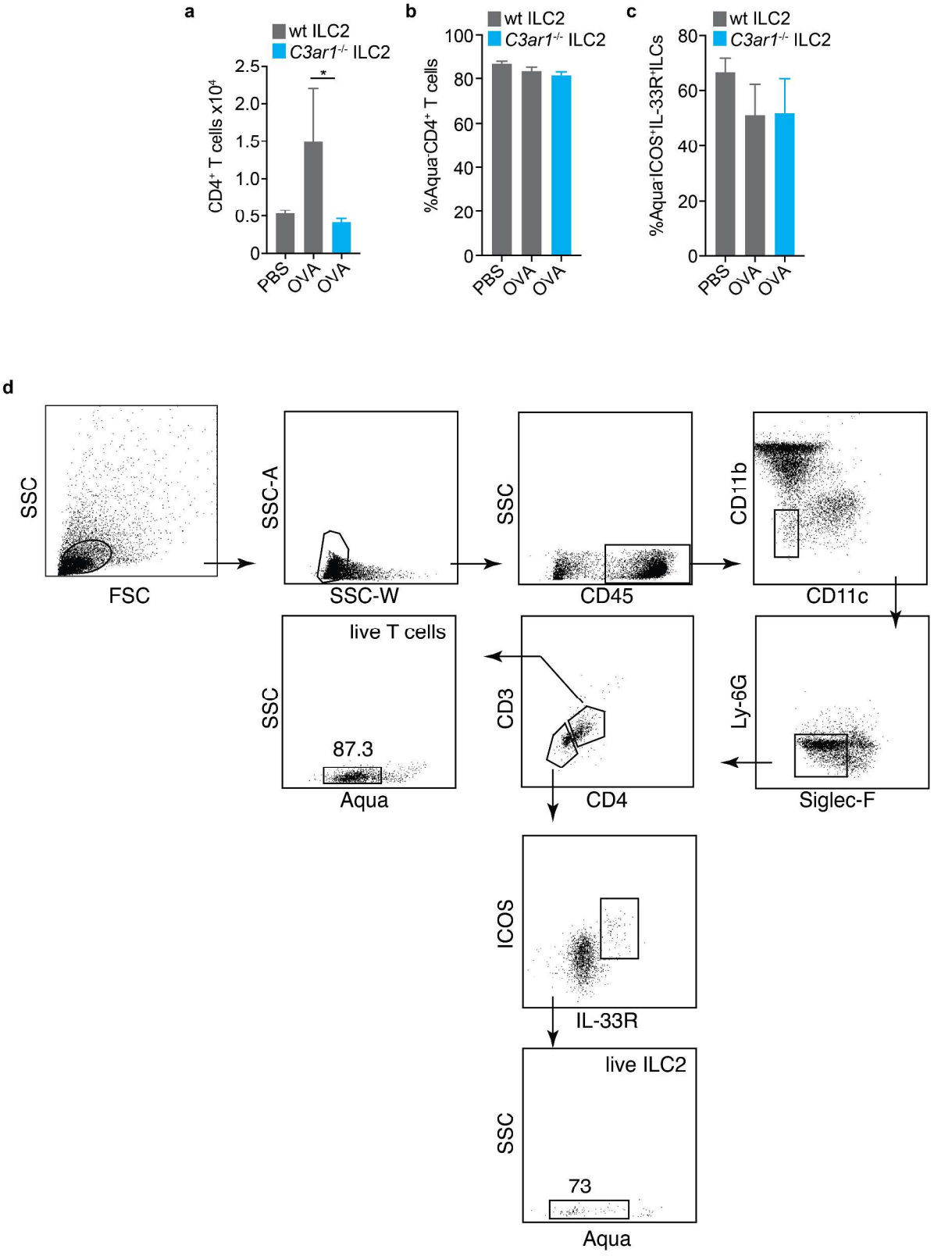
Supplementary Figure 6. C3a signaling promotes type 2 cytokines from ILC2. Flow-sorted ILC2 (Lin$^-$CD45$^+$ICOS$^+$IL-33R$^+$) were cultured with IL-2 (10 ng/ml) or IL-2 (10 ng/ml)+IL-33 (10 ng/ml) for 5 days, and levels of (**a**) *Il5* mRNA, (**b**) *Il13* mRNA were determined by real-time PCR. (**c**) Alternatively, ILCs were cultured in IL-2 in the presence of IL-33 and recombinant C3a, 5 days after, levels of amphiregulin in the supernatant were determined by ELISA. Data represents means+SEM. Data is representative of 2 independent experiments with 3-4 replicate wells, **p<0.01.



Supplementary Figure 7. C3a signaling does not significantly influence ILC2-expressed MHCII, CD80 or CD86. Wildtype or *C3ar1*$^{-/-}$ mice were given PBS or HDM (100 ug) i.t. on days 0 and 14. 48h after, lungs were analyzed for ILC2 (Lin^{-}CD45^{+}ICOS^{+}IL-33R^{+}) by flow cytometry. (**a**,**b**) Frequency of MHCII^{+}ILC2 and (**c**) MHCII MFI. (**d**,**e**) Frequency of CD86^{+}ILC2 and (**f**) CD86 MFI. (**g**,**h**) Frequency of CD80^{+}ILC2 and (**i**) CD86 MFI. Data represents means+SEM. Data is representative of 2 independent experiments with 4 mice/group.



Supplementary Figure 8. Effect of C3a signaling on DC-OVATg CD4$^+$ T cells co-cultures. Levels of (**a**) IL-5, (**b**) IL-13, and (**c**) IL-17A in the supernatant of CD11c+ DC co-cultured with DO11.10 OVATg CD4+ T cells. Cells were cultured with 2 ug/ml OVA peptide in addition to 0.2 ug/ml or 1.0 ug/ml recombinant mouse C3a for 5 days. (**d**) CD4$^+$ T cells numbers (**e**) frequency of dividing cells (CFSE^{lo}) and (**f**) frequency of live CD4$^+$ T cells in co-cultures of lung CD11b$^+$CD11c$^+$ DCs and CFSE-labeled lymph node DO11.10 OVATg CD4$^+$ T cells cultured with 2 ug/ml OVA peptide for 5 days. Data represents means+SEM. Data is representative of 2 independent experiments with 3 replicate wells.



Supplementary Figure 9. C3a is necessary for CD4$^+$ T cell responses to OVA, but does not impact CD4$^+$ T cell or ILC2 survival *in vivo*. 2x10^5 flow-sorted DO11.10 OVATg T cells (CD3$^+$CD4$^+$) were transferred with 1x10^4 flow-sorted lung IL-33R$^+$ ILC2 from wildtype or *C3ar1*$^{-/-}$ mice i.v. to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipient mice. 24h later mice were challenged i.t. with 40 ug OVA 323-339 peptide. 3 days after OVA challenge, (**a**) CD4$^+$ T cells were enumerated by flow cytometry, and frequency of surviving (Aqua$^-$, a dye non-permeant to live cells) transferred (**b**) CD4$^+$ T cells

and (**c**) ILC2 collected from the lungs, and (**d**) representative flow gating scheme. Data is means+SEM, *p<0.05.