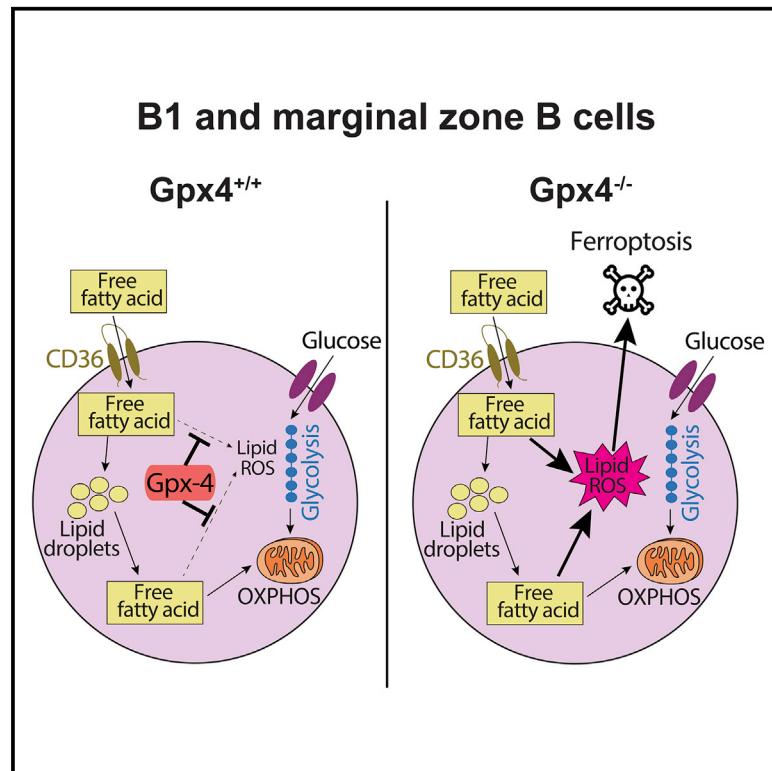


B1 and Marginal Zone B Cells but Not Follicular B2 Cells Require Gpx4 to Prevent Lipid Peroxidation and Ferroptosis

Graphical Abstract



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In Brief

Muri et al. demonstrate that B1 and marginal zone (MZ) B cells but not follicular (Fo) B2 cells require Gpx4 during homeostasis and antibody responses. Mechanistically, B1 and MZ B cells display increased lipid metabolism, sensitivity to lipid peroxidation, and ferroptosis in comparison to Fo B cells.

Highlights

- Gpx4 is required for the maintenance and antibody responses of B1 and MZ B cells
- Gpx4^{-/-} Fo B cells can develop and undergo normal germinal-center reactions
- Gpx4 prevents lipid peroxidation and ferroptosis in B1 and MZ B cells
- Gpx4 allows increased lipid metabolism in B1 and MZ B compared to Fo B2 cells



B1 and Marginal Zone B Cells but Not Follicular B2 Cells Require Gpx4 to Prevent Lipid Peroxidation and Ferroptosis

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SUMMARY

Aerobic organisms need to maintain cellular redox homeostasis. Glutathione peroxidase-4 (Gpx4) has the unique ability to protect cells against lipid peroxidation. Here, we show that Gpx4 is absolutely required to prevent ferroptosis during development, maintenance, and responses of innate-like B cells, namely, the B1 and marginal zone (MZ) B cells. In contrast, Gpx4 is dispensable for the development, germinal center reactions, and antibody responses of follicular B2 cells. Mechanistically, we show increased lipid metabolism and sensitivity to lipid peroxidation and ferroptosis in B1 and MZ B cells compared to follicular B2 cells, consistent with the requirement of Gpx4 in innate-like B cells. This high sensitivity to ferroptosis of innate-like B cells may be used to therapeutically target Gpx4 in certain forms of B cell malignancies involving B1 cells.

INTRODUCTION

Regulation of the cellular redox state is crucial for cell function and survival. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, which are generated by aerobic organisms as a result of normal cellular metabolism. The main sources of endogenously produced ROS include NADPH oxidases and electron transport in the mitochondrial respiratory chain (D'AuTréaux and Toledano, 2007). At low concentrations, ROS are critically required for physiological cellular processes, where they regulate cell proliferation, differentiation, and death. By contrast, high concentrations of ROS can damage cellular macromolecules such as lipids, nucleic acids, and proteins, thereby leading to cell death and contributing to the physiology of aging (Birben et al., 2012; Finkel, 2011; Finkel and Holbrook, 2000; Ray et al., 2012). The imbalance between ROS and antioxidants in favor of ROS, termed as oxidative stress, has been described to contribute to various diseases, such as cancer (Toyokuni et al., 1995), asthma (Andreadis et al., 2003; Comhair et al., 2005), diabetes (Brownlee, 2001), and neurological disorders (Jenner, 2003; Lyras et al., 1997).

Aerobic organisms have evolved a sophisticated cellular anti-oxidant defense network to keep ROS at homeostatic levels in the cell. Part of this system is made up by glutathione peroxidases (Gpx), an enzyme family composed of 8 enzymes with the biological role to protect the organism from oxidative damage. They utilize glutathione (GSH) as a source of electrons to reduce hydrogen peroxide or organic peroxides to water or the corresponding alcohols, respectively (Brigelius-Flohé and Maiorino, 2013). Interestingly, only a sole member of the Gpx family, Gpx4, possesses the ability to directly reduce phospholipid hydroperoxides and oxidized lipoproteins, thereby protecting cells against membrane lipid peroxidation (Roveri et al., 1994; Thomas et al., 1990). A systemic deletion of Gpx4 is embryonically lethal, which highlights the critical role of this protein isoform for development and cellular function (Yant et al., 2003).

Recently, it has been established that Gpx4 is critical to prevent cells from ferroptosis, an iron-dependent oxidative form of cell death associated with increased lipid ROS and impaired capacity to scavenge lipid peroxides (Dixon et al., 2012). Ferroptosis can be triggered by physiological conditions (e.g., increased extracellular concentrations of glutamate), by small molecules that block the Cys₂/glutamate antiporter system x_c⁻, or by the genetic ablation of Gpx4 (Dixon, 2017; Dixon et al., 2012; Friedmann Angeli et al., 2014; Maiorino et al., 2018; Matsushita et al., 2015). Indeed, Gpx4 has a central role in preventing ferroptosis by converting toxic lipid-associated hydroperoxides to harmless organic alcohols. It has been now increasingly recognized that, in some cases, metabolic reprogramming of cancer cells is associated with higher sensitivity to ferroptosis, therefore establishing novel anti-cancer strategies (Friedmann Angeli et al., 2019; Seibt et al., 2019).

Whereas the absence of Gpx4 has been demonstrated to cause renal failure (Friedmann Angeli et al., 2014), hepatocyte degeneration (Carlson et al., 2016), and neuroinflammation (Hambright et al., 2017), the understanding of Gpx4 in distinct cell types has not been rigorously studied. We have previously described the importance of Gpx4 for prevention of lipid peroxidation and ferroptosis upon T cell activation, thus allowing efficient immunity to infection (Matsushita et al., 2015). However, the role of Gpx4 in other immune cells has not been addressed so far. Here, we report that unlike T cells follicular (Fo) B2 cells do not require Gpx4 for development, homeostatic maintenance, germinal center (GC) reactions, and antibody responses.



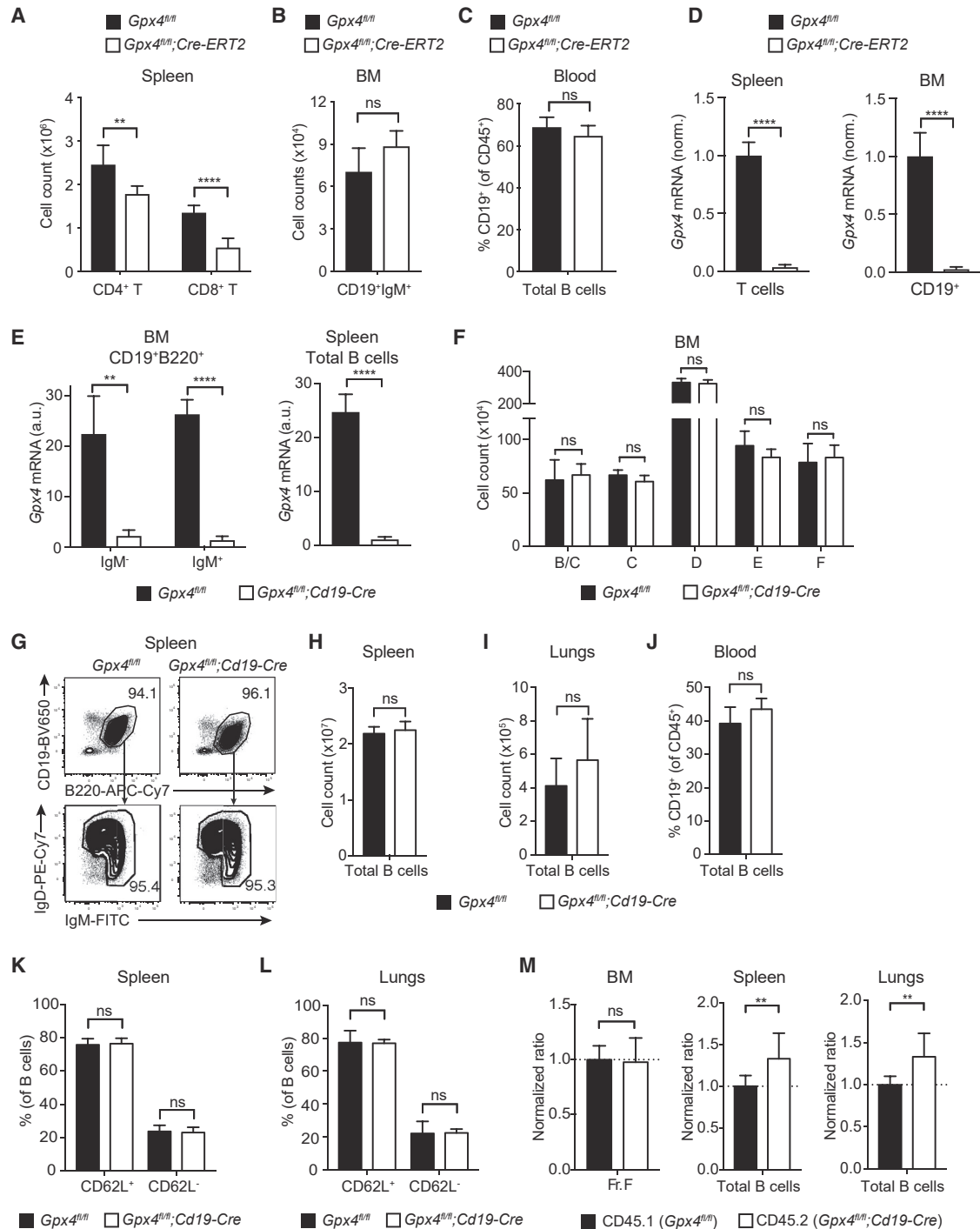


Figure 1. Gpx4 Is Dispensable for B Cell but Not T Cell Development and Homeostasis

(A–D) *Gpx4^{fl/fl};Cre-ERT2* mice and control *Gpx4^{fl/fl}* littermates were injected with TAM to delete the *Gpx4* gene and analyzed by flow cytometry 1 week later. (A–C) Total numbers of splenic CD4⁺ and CD8⁺ T cells (A) and of CD19⁺IgM⁺ B cells in the bone marrow (BM; B) and percentage of CD19⁺ B cells in the blood (C); *n* = 4–5. (D) Analysis of *Gpx4* mRNA in MACS-enriched CD90⁺ T cells in the spleen (left) and CD19⁺ B cells in the BM (right) by real-time PCR (*n* = 4). (E–L) Analysis of the different B cell populations in naive *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* littermate control mice. (E) Analysis of *Gpx4* mRNA in FACS-sorted IgM⁻ and IgM⁺ CD19⁺B220⁺ populations in the BM (left) and in splenic CD19⁺B220⁺ B cells (right) by real-time PCR (*n* = 3–4). (F) The quantifications of the indicated Hardy B cell fractions are shown (*n* = 6).

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By contrast, we found that Gpx4 is essential in B1 and marginal zone (MZ) B cells during development, maintenance, and for antibody responses to *Streptococcus pneumoniae*. Indeed, B1 and MZ B cells lacking Gpx4 accumulate lipid peroxidation and die by ferroptosis due to their high uptake of fatty acids to sustain metabolic functions. These results consequently suggest that therapeutically targeting Gpx4 might be beneficial in forms of leukemia where B1 cells are known to be involved.

RESULTS

Gpx4 Is Dispensable for the Development and Maintenance of Fo B2 Cells

We have previously described the crucial role of Gpx4 in protecting activated T cells from accumulation of lipid peroxides and cell death by ferroptosis (Matsushita et al., 2015). In this context, we now aim to investigate the importance of Gpx4 in the other main type of lymphocytes, the B cells. To address this, we generated *Gpx4^{fl/fl};Cre-ERT2* mice by crossing mice with loxP-flanked *Gpx4* alleles to mice carrying tamoxifen (TAM)-inducible *Cre-ERT2*. As expected from our previous study (Matsushita et al., 2015), TAM administration to *Gpx4^{fl/fl};Cre-ERT2* mice led to a significant reduction in total CD4⁺ and CD8⁺ T cells in the spleen (Figure 1A). To our surprise, however, total CD19⁺IgM⁺ B cell numbers in the bone marrow and the percentage of CD19⁺ B cells in the blood were comparable in TAM-treated *Gpx4^{fl/fl};Cre-ERT2* and control (*Gpx4^{fl/fl}*) mice (Figures 1B and 1C). We additionally verified that Cre-mediated deletion in both T and B cells of *Gpx4^{fl/fl};Cre-ERT2* mice was complete at the mRNA level upon TAM administration (Figure 1D). Together, these results suggest a distinct requirement of the Gpx4 antioxidant pathway in T and B lymphocytes.

To further study this interesting difference in the requirement of Gpx4 between B and T lymphocytes, we generated *Gpx4^{fl/fl};Cd19-Cre* mice. The deletion of *Gpx4* was expectedly complete at the mRNA level in both IgM⁺ and IgM⁺ CD19⁺B220⁺ B cells in the bone marrow and in total CD19⁺ B cells in the spleen of *Gpx4^{fl/fl};Cd19-Cre* mice (Figure 1E). We next studied B cell development via flow cytometry using the Hardy classification system, which subdivides distinct B cell developmental stages into fraction A (pre/pro-B cells), fraction B (pro-B cells), fraction C (large pre-B cells), fraction D (small pre-B cells), fraction E (immature B cells), and fraction F (mature B cells) (Hardy et al., 1991). All of these fractions were intact in *Gpx4^{fl/fl};Cd19-Cre* compared *Gpx4^{fl/fl}* littermate control mice (Figure 1F). Furthermore, numbers of mature IgM⁺IgD⁺CD19⁺B220⁺ B cells in the spleen (Figures 1G

and 1H), inguinal lymph nodes (iLNs; Figure S1A), lungs (Figure 1I), and their percentage in the blood (Figure 1J) were not affected by *Gpx4* deficiency. We additionally verified that the activation status of B cells as determined by measurement of CD62L surface expression also remained unaffected in these organs in the absence of *Gpx4* (Figures 1K and 1L; Figure S1B). To further confirm our findings, we investigated the ability of *Gpx4*-deficient B cells to refill the hematopoietic compartment of lethally irradiated hosts in a competitive situation with wild-type (WT) cells. Lethally irradiated WT C57BL/6 mice (CD45.1⁺CD45.2⁺) were reconstituted with an equal ratio of congenically marked donor bone marrow cells from *Gpx4^{fl/fl};Cd19-Cre* mice (CD45.2⁺) and WT (CD45.1⁺) mice. *Gpx4*-deficient B cells were able to contribute similarly to Fraction F in the bone marrow as well as to the peripheral B cell compartments in the spleen, lungs, iLNs, and in the blood (Figure 1M; Figure S1C), as WT B cells. Overall, these data demonstrate that, in contrast to an important role for T cell homeostasis (Matsushita et al., 2015), *Gpx4* is dispensable for the development and maintenance of Fo B2 cells.

Gpx4 Is Dispensable for B Cell Antibody Responses and GC Reactions

With the help of follicular helper CD4⁺ T cells (Tfh), Fo B cells undergo GC reactions to give rise to high-affinity antibody responses. Since we have shown that *Gpx4* is completely dispensable for the development and homeostatic maintenance of Fo B cells, we next sought to understand whether *Gpx4* plays a role in the production of antibodies during an *in vivo* response. Thus, we immunized *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* control mice with replication-defective Q β -virus-like particles (Q β -VLPs) containing *E. coli* single-stranded RNA (ssRNA) and monitored Q β -VLP-specific antibody levels on days 7, 18, and 32 post-infection. *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* mice mounted comparable Q β -VLP-specific immunoglobulin M (IgM) (Figure 2A) and class-switched IgG2b (Figure 2B) antibody responses. Q β -VLPs not only trigger vigorous antibodies responses but also induce GC reactions, such as hypermutations, affinity maturation, and B cell memory (Bachmann et al., 1995; Bachmann and Zinkernagel, 1997; Jennings and Bachmann, 2008). Therefore, we also analyzed B cells in the GC by flow cytometry on day 7 post-infection with Q β -VLPs. GC B cells are generally defined by the marker profile B220⁺CD38^{low}Fas⁺ and can be further subdivided into CXCR4⁺ dark zone (DZ) and CD86⁺ light zone (LZ) cells (Vic-tora et al., 2010). Interestingly, *Gpx4^{fl/fl};Cd19-Cre* mice showed a higher frequency in GC B cells compared to the *Gpx4^{fl/fl}* controls (Figures 2C and 2D), and no difference in distribution between

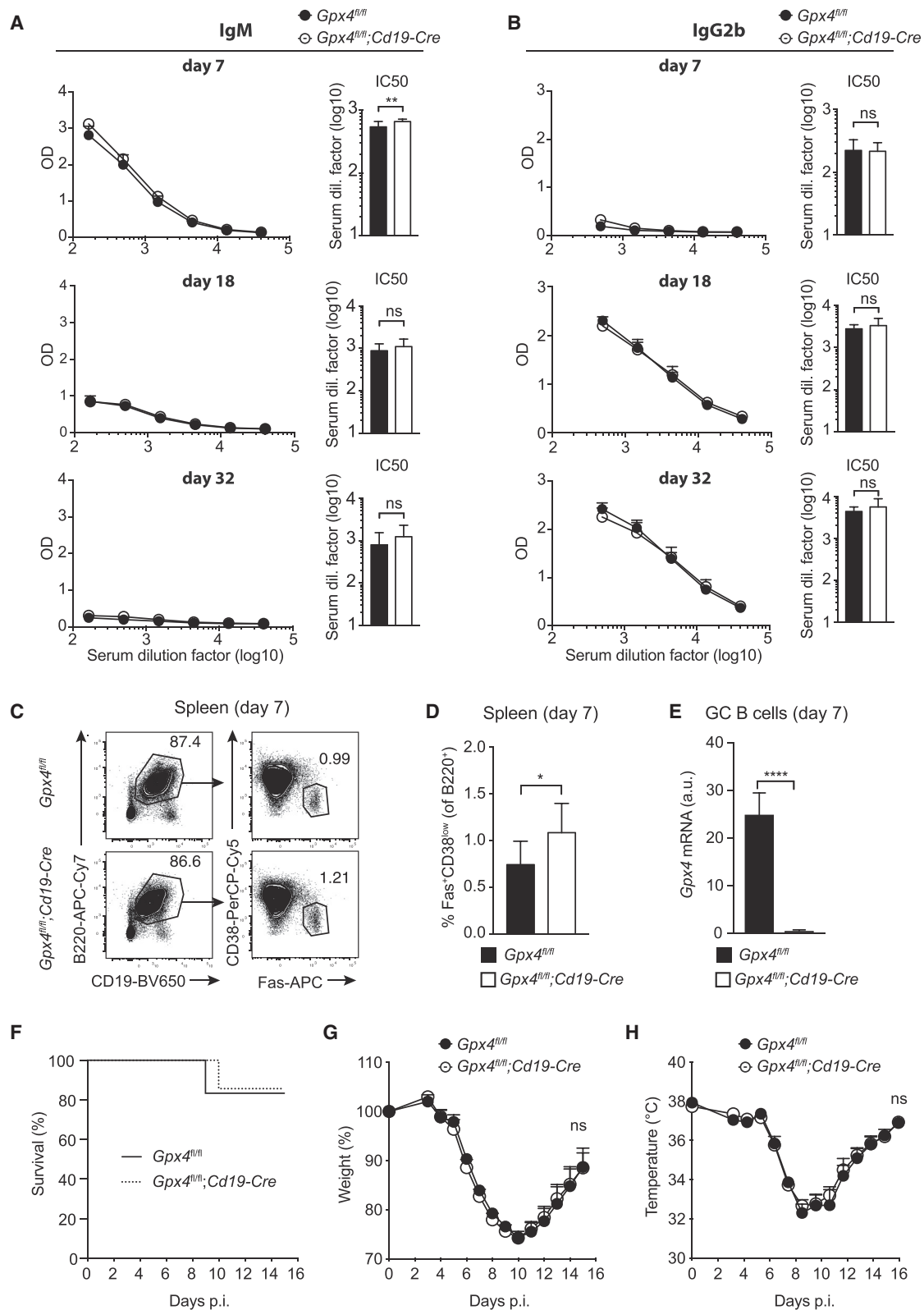
(G and H) Representative FACS plots (G) and quantification (H) of total mature B cells in the spleen (n = 6).

(I and J) The total B cell counts in the lungs (I) and percentage of B cells in the blood (J) are depicted. (n = 6).

(K and L) Expression of CD62L on CD19⁺ B cells from the spleen (K) and lungs (L); n = 6).

(M) Lethally irradiated WT mice were reconstituted with a 1:1 mixture of WT and *Gpx4^{fl/fl};Cd19-Cre* BM cells expressing the congenic markers CD45.1 and CD45.2, respectively. After reconstitution, the contribution of *Gpx4^{fl/fl};Cd19-Cre* to the indicated populations in the BM (left), spleen (middle), and lungs (right) was assessed. Values were normalized to non-Cre expressing congenic marker-matched TCR β ⁺ T cells, followed by normalization such that CD45.1⁺ WT cell contribution to the respective population equals 1. Values <1 or >1 indicate reduced or higher contribution of *Gpx4^{fl/fl};Cd19-Cre* cells to the B cell population relative to WT cells, respectively (n = 10). Bar graphs show mean $\pm$ SD. Numbers "n" represent individual mice. Numbers in the FACS plots indicate the percentage of the depicted gates. Data are representative of two (A–E and M) and three (F–L) independent experiments. Student's t test (two-tailed, unpaired) was used for the comparison of two groups (A–F and H–M): *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001; ****p $\leq$ 0.0001; ns, not significant.

See also Figures S1 and S5.



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LZ and DZ cells was observed between both groups (Figures S2A and S2B). Since the *Cd19-Cre* system is notoriously leaky and a few escapees might seed the GC in *Gpx4^{fl/fl};Cd19-Cre* mice, we fluorescence-activated cell sorted (FACS) B220⁺CD38^{low}Fas⁺ GC B cells from both *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* groups and verified that *Gpx4* deletion was complete at the mRNA level (Figure 2E).

We further confirmed the dispensability of *Gpx4* in B cells in a more potent mouse model of infectious disease by infecting *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* control mice with the replicating influenza A virus PR8. Survival, temperature and weight loss in the course of disease were comparable in both groups of mice (Figures 2F–2H), which was additionally reflected in the comparable PR8-specific antibody response kinetics (Figures S2C and S2D). Taken together, *Gpx4* is dispensable for development, homeostasis, and antibody responses of Fo B cells.

B1 and MZ B Cells Require *Gpx4* for Development and Homeostasis

B cells can be divided into several subclasses that differ in ontogeny, homeostasis, and functionality. B1 cells can be subdivided into B1a and B1b cells, while B2 cells encompass Fo and MZ B cells. While the predominant Fo B cells are important for immune responses against thymus-dependent antigens, B1 and MZ B cells rapidly respond to blood-borne antigens with antibody production independent of T cell help (Baumgarth, 2011; Pillai and Cariappa, 2009). We found that the numbers and frequencies of splenic CD23⁺ Fo B cells were comparable in *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* mice, whereas CD21⁺CD23[−] MZ B cells were significantly reduced in the absence of *Gpx4* (Figures 3A and 3B). We next investigated whether the different developing splenic B cell subsets (Allman and Pillai, 2008), which give rise to the mature MZ B cells, also exhibited a similar reduction in numbers due to *Gpx4* deficiency (Figures S3A and S3B). However, we observed similar numbers of transitional T1 and T2 cells, Fo type I and type II cells, and MZ precursors (MZPs) in *Gpx4*-sufficient and -deficient mice, thus suggesting that the steady-state maintenance of MZ B cells rather than their maturation in the spleen is impaired in the absence of *Gpx4* (Figure 3C; Figure S3C). Furthermore, *Gpx4* deletion also led to reduced frequency and numbers of total CD19⁺CD43⁺CD23[−] B1 cells in the spleen (Figures 3D and 3E). The predominant B1 subset CD5⁺ B1a was strikingly affected, although a significant reduction was also observed in the minor

CD5[−] B1b subset (Figures 3D, 3F, and 3G). Since B1 cells are highly enriched in the peritoneal cavity, we next investigated the effect of *Gpx4* deficiency in this compartment. We found significantly reduced numbers and frequencies of total CD19⁺B220⁺ B cells in the peritoneal cavity of *Gpx4^{fl/fl};Cd19-Cre* compared to WT control mice (Figures 3H and 3I). In line with the results in the spleen, the frequency of B1 cells was also reduced in the peritoneal cavity, and, as a consequence, we observed a higher percentage of B2 cells in the absence of *Gpx4* (Figures 3J and 3K). Importantly, *Gpx4* deletion led to drastically reduced numbers of B1a and B1b cells, while the B2-cell compartment remained unaffected (Figures 3L and 3M). To further analyze the various stages of peritoneal B1 cells, we next utilized the CD11b marker, which was shown to be expressed by nearly half of the cells in each of the peritoneal B1 populations (B1a and B1b) (Ghosh et al., 2008). We found that both CD11b⁺ and CD11b[−] B1a and B1b cell subsets are significantly reduced in numbers in the absence of *Gpx4* (Figures 3J and 3N). Together, while *Gpx4* is dispensable for Fo B cells, these data indicate that it is required for the development and homeostasis of B1 and MZ B cells.

To further study this differential requirement of *Gpx4* by distinct B cell subsets in a competitive situation, WT C57BL/6 mice (CD45.1⁺CD45.2[−]) were lethally irradiated and reconstituted with an equal ratio of congenically marked donor bone marrow cells from *Gpx4^{fl/fl};Cd19-Cre* mice (CD45.2⁺) and WT (CD45.1⁺) mice. While the Fo B cell pool was similarly reconstituted by both *Gpx4*-deficient and WT cells, MZ B cells were reconstituted mainly by WT cells (Figure 3O), consistent with the reduced MZ B cell numbers we observed in naive *Gpx4^{fl/fl};Cd19-Cre* mice compared to *Gpx4^{fl/fl}* controls (Figure 3B). Furthermore, B1 cells (both B1a and B1b) were also outcompeted to a higher proportion by WT cells compared to B2 cells (Figures 3P and 3Q), also in line with the results observed in non-chimeric mice (Figures 3K–3M). Here, it is important to note that, despite being fetal in origin, the B1-cell pool can be reconstituted by bone-marrow precursors upon steady-state perturbations, such as B1-cell depletion due to irradiation (Düeber et al., 2009; Holodick et al., 2009). However, this process is less efficient, as it can be observed by the fact that B1 cells, which generally are the predominant B cell population in the peritoneal cavity at the steady-state, represent only 10% of total peritoneal B cells after bone-marrow reconstitution of lethally irradiated mice (Figure S3D). Overall, these data demonstrate

Figure 2. *Gpx4* Is Not Required for B Cell-Mediated Antibody Responses and GC Reactions

(A–E) *Gpx4^{fl/fl};Cd19-Cre* mice and *Gpx4^{fl/fl}* littermate control mice were immunized with Q β -VLPs containing *E. coli* ssRNA. (A and B) Mice were bled at day 7 (top), 18 (middle), and 32 (bottom) after immunization to determine Q β -VLP-specific IgM (A) and IgG2b (B) antibody responses via ELISA (n = 6). Each panel depicts plots of OD_{405nm} against serum dilutions (left) and IC₅₀ (right) for Q β -VLP-specific antibodies. (C and D) GC B cells in the spleen as defined by the expression profile Fas⁺CD38^{low} were analyzed by flow cytometry on day 7 post-immunization (n = 5–7). Gating strategy of Fas⁺CD38^{low} B cells in the spleen (pre-gated on live/CD45⁺/TCR β [−]/F4/80[−]/Ly-6G[−]) (C). Percentage of Fas⁺CD38^{low} B cells in the spleen (D). (E) Analysis of *Gpx4* mRNA in FACS-sorted B220⁺CD38^{low}Fas⁺ GC B cells by real-time PCR (n = 4). (F–H) *Gpx4^{fl/fl};Cd19-Cre* mice and *Gpx4^{fl/fl}* littermate control mice were infected with 100 plaque-forming unit (PFU) PR8 influenza A virus (n = 4). Graph represents the survival curve (F). Weight (G) and temperature (H) were monitored as depicted in the graphs. Dot plots represent mean $\pm$ SEM (A, B, G, and H). Bar graphs show mean $\pm$ SD (A, B, D, and E). Numbers “n” represent individual mice. Numbers in the FACS plots indicate the percentage of the depicted gates (C). Data are representative of two (A, B, and E–H) and three (C and D) independent experiments. Student’s t test (two-tailed, unpaired) was used to compare *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* groups (A, B, D, and E): *p $\leq$ 0.05; **p $\leq$ 0.01; ****p $\leq$ 0.0001; ns, not significant. Two-way ANOVA adjusted by Bonferroni’s multiple comparison test was used in (G) and (H): *p $\leq$ 0.0332; ns, not significant. See also Figures S2 and S5.

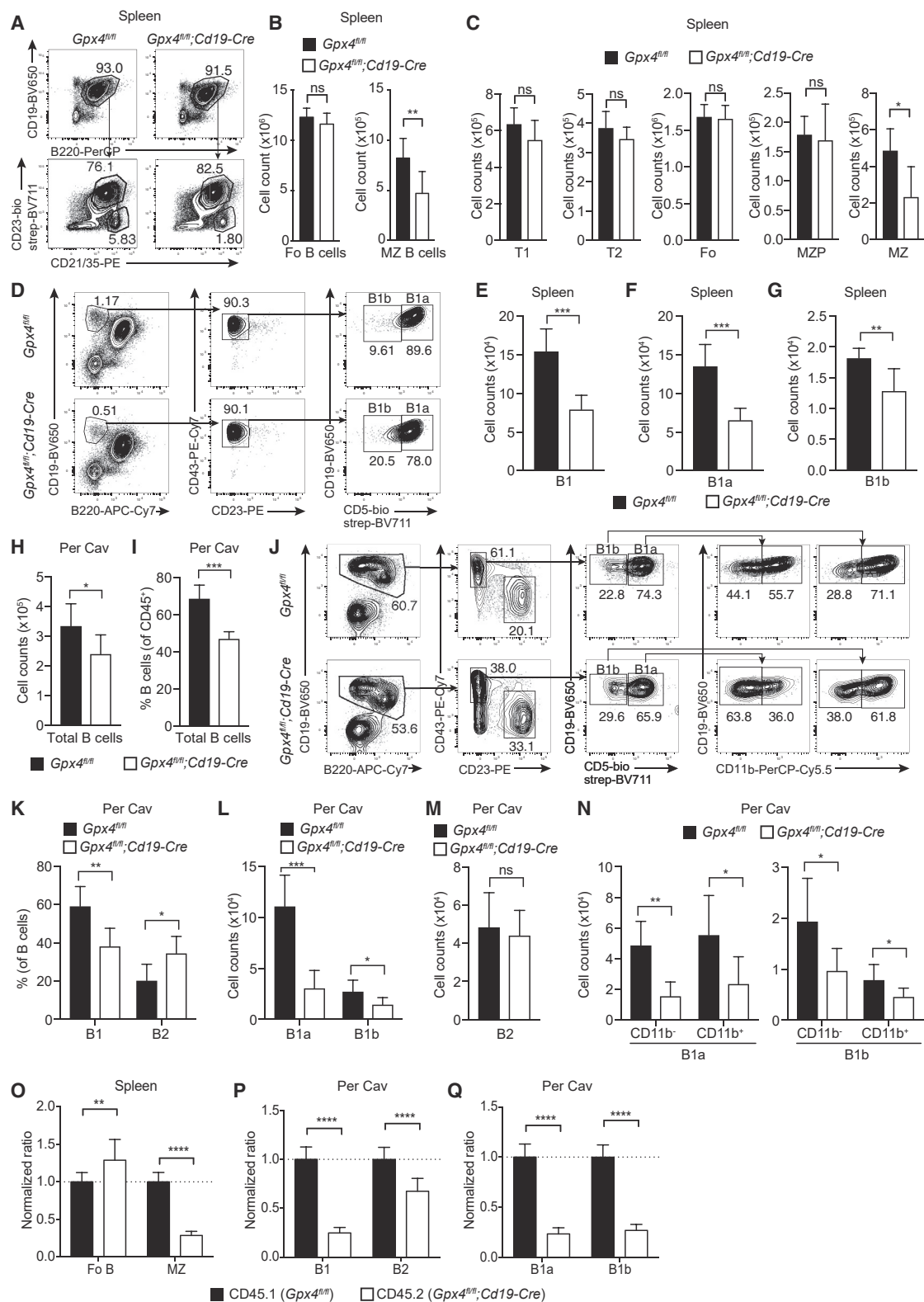


Figure 3. Gpx4 Is Critical for the Homeostasis of B1 and MZ B Cells but Not Fo B2 Cells

(A–N) Analysis of the indicated B cell populations in naive *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* littermate control mice via flow cytometry. (A) Gating strategy of Fo (CD23⁺) and MZ (CD21/35⁺CD23⁺) B cells in the spleen (pre-gated on live/CD45⁺/CD11b⁺/TCRβ⁻).

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the pivotal role of the Gpx4 antioxidant pathway in B1 and MZ B cells and imply distinct requirements of Gpx4 in the B2 and B1/MZ B cell populations.

Gpx4 Is Required to Mount an Efficient Antibody Response to *Streptococcus pneumoniae*

B1 and MZ B cells are indispensable for mounting low-affinity phosphorylcholine (PC)-specific IgM antibody responses to *Streptococcus pneumoniae* reaching the blood (Baumgarth, 2011; Martin et al., 2001; Tanigaki et al., 2002). Since we observed reduced numbers of both B1 and MZ B cells in the absence of Gpx4, we next aimed at studying the consequences of the reduction of these B cell populations in the context of antibody responses to *Streptococcus pneumoniae*. Therefore, we intravenously immunized mice with heat-inactivated bacteria and assessed the PC-specific IgM antibody response 5 days after immunization (Figure 4A). On day 0, PC-specific IgM antibody levels were low in the blood (Figure 4B). Five days after immunization, the Gpx4^{fl/fl} control group mounted a strong anti-PC IgM response, while the Gpx4^{fl/fl};Cd19-Cre mice had strikingly lower PC-specific IgM titers (Figures 4C and 4D). Together, these findings show that the defective development and homeostasis of B1 and MZ B cells in the absence of Gpx4 translates into an impaired antibody response to *Streptococcus pneumoniae*.

Gpx4 Prevents Lipid Peroxidation in B1 and MZ B Cells

Gpx4 has been described as a unique antioxidant enzyme for its ability to directly protect cells against membrane lipid peroxidation (Sattler et al., 1994; Thomas et al., 1990). Thus, we next wondered whether the B cell subsets, which were reduced in numbers in the absence of Gpx4, accumulated cellular lipid peroxides to a higher degree compared to unaffected subsets. To test this, we took advantage of the C11-BODIPY_{581/591} probe to stain for lipid ROS. Interestingly, we observed that a higher proportion of Gpx4-deficient B1 cells accumulated lipid peroxides in comparison to B2 cells in the peritoneal cavity (Figures 5A and 5B). Similarly, splenic B1 cells also accumulated lipid ROS to a greater extent compared to splenic B2 cells in the absence of Gpx4 (Figures 5C and 5D). Finally, we also detected

an increased percentage of MZ B cells with lipid peroxidation in Gpx4^{fl/fl};Cd19-Cre compared to Gpx4^{fl/fl} control mice (Figures 5C and 5D). Together, these results suggest that B1 and MZ B cells are reduced in the absence of Gpx4 due to toxic effects caused by the accumulation of lipid peroxides.

It has been recently described that B1a cells engage a metabolic program distinct from Fo B2 cells, which is characterized by increased glycolysis and fatty acid synthesis, as well as by the uptake of lipids and their storage in lipid droplets (Clarke et al., 2018). We thus hypothesized that these metabolic differences among the distinct B cell subsets might reflect their differential requirement of Gpx4. To expand on this possibility, we intravenously injected WT mice with the fluorescently labeled long-chain fatty acid palmitate (BODIPY FL C₁₆) and sacrificed them for analysis 1 h later. We observed that B1 cells have a strikingly increased fatty acid uptake compared to B2 cells in the peritoneal cavity (Figure 5E), in line with the recent report mentioned above (Clarke et al., 2018). In contrast to B1 and Fo B cells, little is known about the metabolic phenotype of MZ B cells. Therefore, we next compared fatty acid uptake between B1, Fo, and MZ B cells in the spleen. Interestingly, we found that splenic B1 cells take up the highest levels of fatty acids, while MZ B cells displayed an intermediate uptake rate still significantly higher than the one of Fo B cells (Figure 5F). Due to this similarity in the uptake of fatty acids between B1 and MZ B cells, we further analyzed expression of genes from the metabolic gene transcription signature of B1a cells (Clarke et al., 2018) using the ImmGen database. Consistent with the high rate in lipid uptake, both B1 and MZ B cells express higher levels of the fatty acid transporter protein CD36 in comparison to Fo B2 cells (Figure 5G). Moreover, they both have increased expression of *Plin3* [Perilipin-3] (Figure 5H), which has been described to participate in lipid droplet formation in B1a cells (Clarke et al., 2018). We next found that, similarly to B1 cells, MZ B cells also display increased levels of *Myc* [c-Myc], *Hk2* [Hexokinase 2], *Ldha* [Lactate dehydrogenase A], and *Gapdh* [Glyceraldehyde 3-phosphate dehydrogenase] (Figure 5I; Figure S4A–S4C) compared to Fo B2 cells, indicating increased levels of glycolysis at the steady state. Overall, these data suggest that both B1 and MZ B cells have similar metabolic features

(B) Shown are the total cell counts of Fo (left) and MZ (right) B cells in the spleen (n = 6).

(C) Shown are the total cell counts of developing splenic B cell subsets, including T1/T2 transitional cells, follicular type II (Fo-II) cells, and MZ precursors (MZPs). The reduction in mature MZ B cells is also depicted (n = 6).

(D) Gating strategy of splenic B1 cells, which can be further subdivided into B1a (CD5⁺) and B1b (CD5[−]) cells (pre-gated on live/CD45⁺/TCRβ⁺).

(E–G) Cell numbers of total B1 (E), B1a (F), and B1b (G) cells in the spleen are shown (n = 6).

(H and I) The absolute cell numbers (H) and frequencies (I) of total B cells (CD19⁺B220⁺) in the peritoneal cavity (Per Cav) was assessed (n = 6).

(J) Gating strategy of B1 (CD43⁺CD23[−]) and B2 (CD43[−]CD23⁺) cells in the peritoneal cavity (pre-gated on live/CD45⁺/F4/80[−]). B1 cells are further subdivided into B1a (CD5⁺) and B1b (CD5[−]) cells, and each of these B1 subsets is subsequently tested for the expression of the CD11b marker.

(K–N) Depicted are the percentages of B1 and B2 cells (K) and the cell numbers of B1a and B1b cells (L), of total B2 cells (M), and of the CD11b⁺ and CD11b[−] populations among the B1a and B1b cell subsets (N) in the peritoneal cavity (n = 6).

(O–Q) Lethally irradiated WT mice were reconstituted with a 1:1 mixture of WT and Gpx4^{fl/fl};Cd19-Cre BM cells expressing the congenic markers CD45.1 and CD45.2, respectively. Upon reconstitution, contribution of WT and Gpx4^{fl/fl};Cd19-Cre cells to the indicated B cell populations in the spleen (O) and peritoneal cavity (P and Q) was assessed. Values were normalized to non-Cre expressing congenic marker-matched TCRβ⁺ T cells, followed by normalization such that CD45.1⁺ WT cell contribution to the respective population equals 1. Values <1 or >1 indicate reduced or higher contribution of Gpx4^{fl/fl};Cd19-Cre cells to the B cell population relative to WT cells, respectively (n = 10).

Bar graphs show mean ± SD. Numbers “n” represent individual mice. Numbers in the FACS plots indicate the percentage of the depicted gate. Data are representative of two (A–G and N–Q) and three (H–M) independent experiments. Student’s t test (two-tailed, unpaired) was used to compare two groups for statistical differences (B, C, E–I, and K–Q): *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001; ns, not significant.

See also Figures S3 and S6.

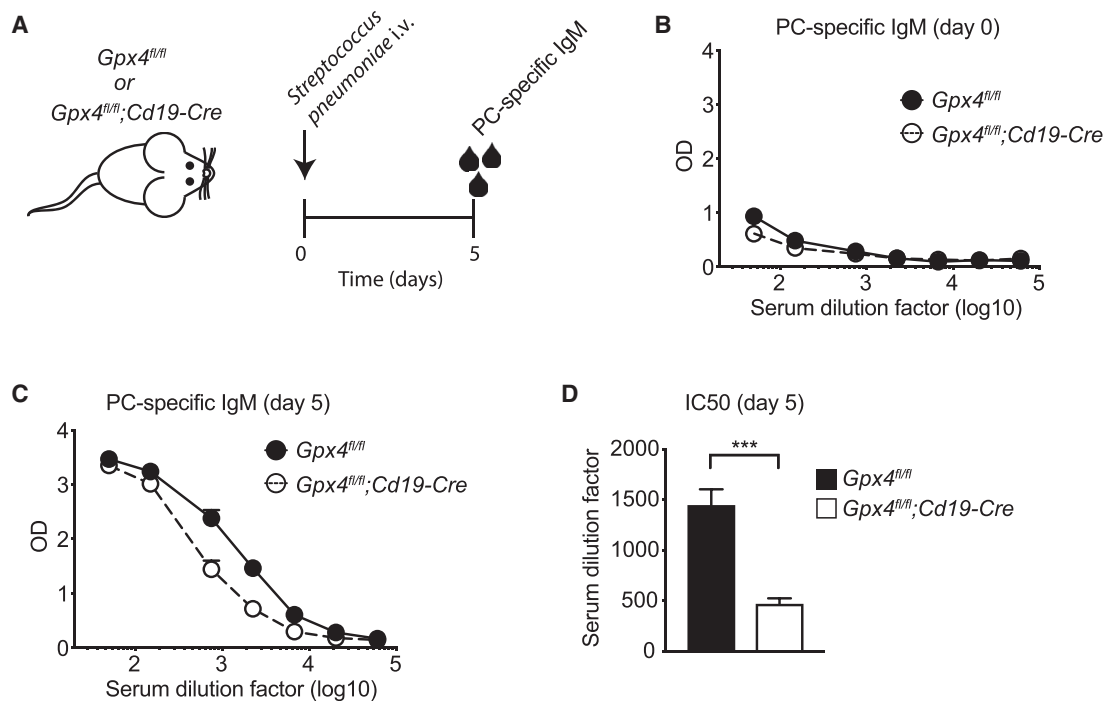


Figure 4. Gpx4 Is Required for IgM Antibody Responses to *Streptococcus pneumoniae*

Gpx4^{fl/fl};Cd19-Cre and *Gpx4^{fl/fl}* littermate control mice were infected with heat-inactivated *Streptococcus pneumoniae*, and mice were bled to determine phosphorylcholine (PC)-specific IgM titers by ELISA at day 0 and 5 after immunization (n = 4–5).

(A) Diagram showing the experimental design of the infection.

(B and C) Optical density (OD) values at day 0 (B) and 5 (C) upon *Streptococcus pneumoniae* immunization. Each panel depicts plots of OD_{405nm} (OD) against serum dilutions for PC-specific IgM antibodies.

(D) IC₅₀ for PC-specific IgM response 5 days after immunization. Dot plots and bar graphs represent mean ± SEM (B–D). Numbers “n” represent individual mice. Data are representative of two independent experiments. Student’s t test (two-tailed, unpaired) was used to compare *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* groups (D): ***p ≤ 0.001.

and that Gpx4 allows high lipid uptake by preventing lipid peroxidation.

B1 and MZ B Cells Die by Ferroptosis in the Absence of Gpx4

Accumulation of lipid peroxidation is well known to trigger ferroptosis, an iron-dependent, oxidative form of non-apoptotic cell death (Dixon, 2017; Dixon et al., 2012). We have previously described the role of Gpx4 in protecting activated T cells from the accumulation of lipid ROS and consequential ferroptosis (Matsushita et al., 2015). Thus, we next aimed to compare the survival of T and B cells lacking Gpx4. As expected from our previous study (Matsushita et al., 2015), *Gpx4*-deficient CD8⁺ and CD4⁺ T cells underwent massive cell death in the first 3 h after incubation independently of T cell receptor (TCR) stimulation (Figures 6A and 6B; data not shown). Notably, addition of the ferroptosis inhibitor ferrostatin-1 (Fer-1) completely abolished the cell death of *Gpx4*-deficient T cells, confirming cell death by ferroptosis (Figures 6A and 6B). In this study, we showed that Fo B2 cells did not require Gpx4 for development, homeostatic maintenance, and responses (Figures 1 and 2). In line with this, we observed that unlike T cells, splenic B cells survived normally in the absence of Gpx4 regardless of whether they were stimulated or not (Figures 6C and 6D). Together, these results confirm

the different requirement of the Gpx4 pathway in T and B lymphocytes.

We next wondered whether B1 and MZ B cells die by ferroptosis *ex vivo*. To test this, we FACS-sorted Fo B2 and MZ B cells from the spleen, and B1 and B2 cells from the peritoneal cavity, stimulated them with LPS and anti-IgM, and monitored cell survival. In contrast to splenic Fo B2 cells, we found that MZ B cells lacking *Gpx4* die by ferroptosis *ex vivo*, since the cell death was completely prevented in the presence of Fer-1 (Figure 6E). Moreover, *Gpx4*-deficient B1 cells from the peritoneal cavity also underwent ferroptosis to a much greater extent in comparison to peritoneal B2 cells (Figure 6F), indicating that protection from ferroptosis by Gpx4 in metabolically active B1 cells is not a consequence of the particular environment (i.e., peritoneum). Overall, these data demonstrate that the accumulation of lipid ROS in *Gpx4*-deficient B1 and MZ B cells leads to cell death by ferroptosis.

DISCUSSION

A breakthrough in the immunometabolism field was achieved through studies in T cells showing that the activation of naive T cells, which mainly rely on oxidative phosphorylation (OXPHOS) for their metabolic requirements, leads to metabolic

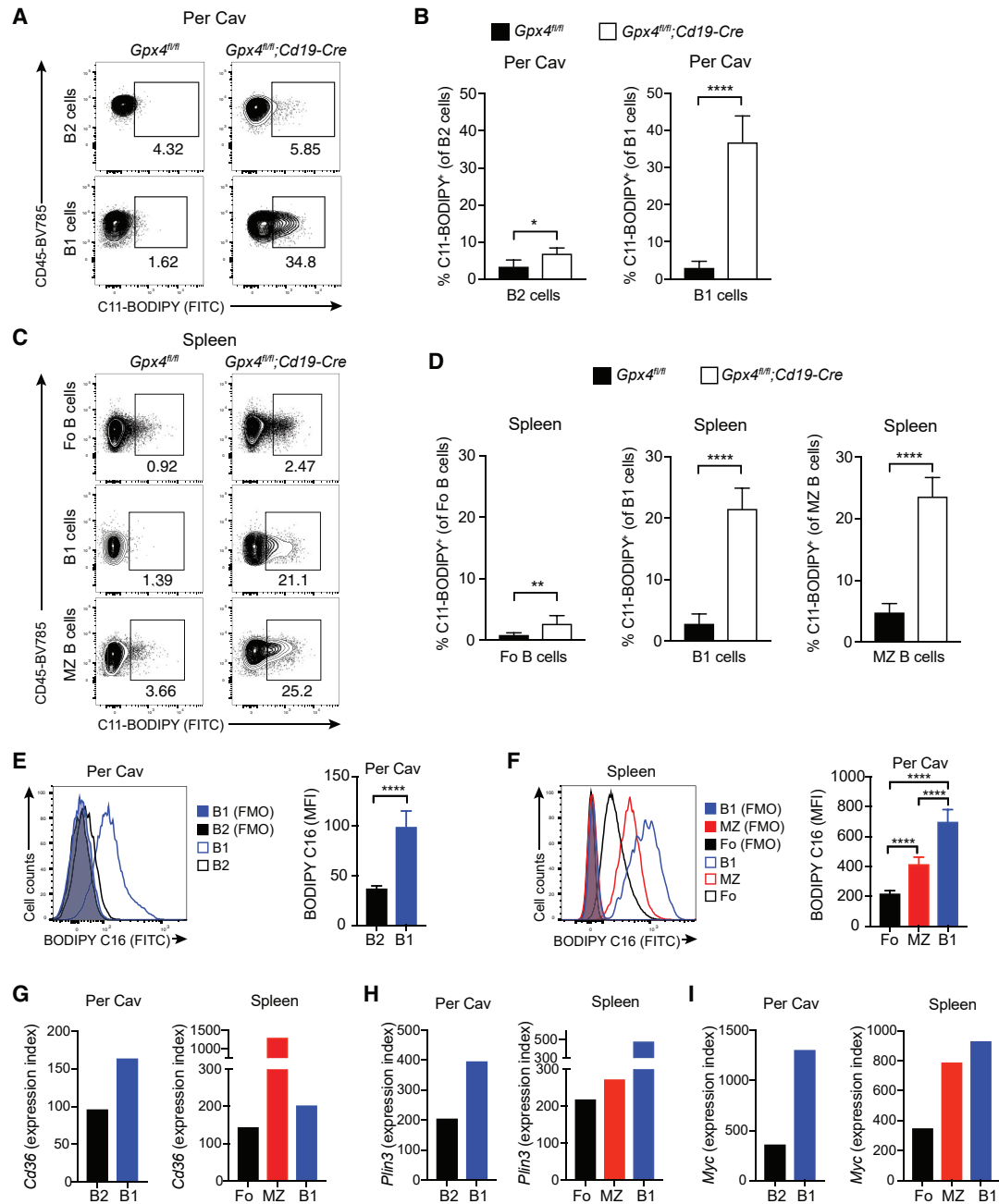


Figure 5. *Gpx4* Prevents Lipid ROS in B1 and MZ B Cells but Not in Fo B2 Cells

(A–D) The accumulation of lipid peroxidation in the indicated *Gpx4*-sufficient and -deficient B cell populations was determined by staining with the C11-BODIPY_{581/591} (n = 5–6). Representative FACS plots (A) and quantification (B) of lipid ROS in B2 (CD19⁺B220⁺CD43⁺CD23⁺) and B1 (CD19⁺B220^{low}CD43⁺CD23⁺) cells from the peritoneal cavity (Per Cav). Representative FACS plots (C) and quantification (D) of lipid peroxidation in Fo B2 (CD19⁺B220⁺CD43⁺CD23⁺), B1 (CD19⁺B220^{low}CD43⁺CD23⁺), and MZ B (CD19⁺B220⁺CD21/35⁺CD23⁺) cells in the spleen.

(E and F) *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* littermate control mice were intravenously injected with BODIPY FL C₁₆ and then analyzed 1 h later. Shown are example distributions of fluorescence (left) and quantification (right) from peritoneal B1 and B2 cells (E) and splenic Fo, MZ B, and B1 cells (F). B1, B2, and MZ B cells were gated as described in (A)–(D) (n = 7).

(G–I) Shown are the mean expression values of *Cd36* (G), *Plin3* (H), and *Myc* (I) in peritoneal B1 and B2 cells (left) and splenic Fo, MZ B, and B1 cells (right). The expression data were obtained from the Immgen database (mean values).

Bar graphs show mean ± SD (B and D–F). Numbers “n” represent individual mice. Numbers in the FACS plots indicate the percentage of the depicted gate. Data are representative of two independent experiments (A–F). Student’s t test (two-tailed, unpaired) was used to compare *Gpx4^{fl/fl};Cd19-Cre* and *Gpx4^{fl/fl}* groups (B, D, and E): *p < 0.05; **p < 0.01; ****p < 0.0001. One-way ANOVA adjusted by Tukey’s multiple comparison test was used in (F): ****p < 0.0002.

See also Figures S4 and S6.

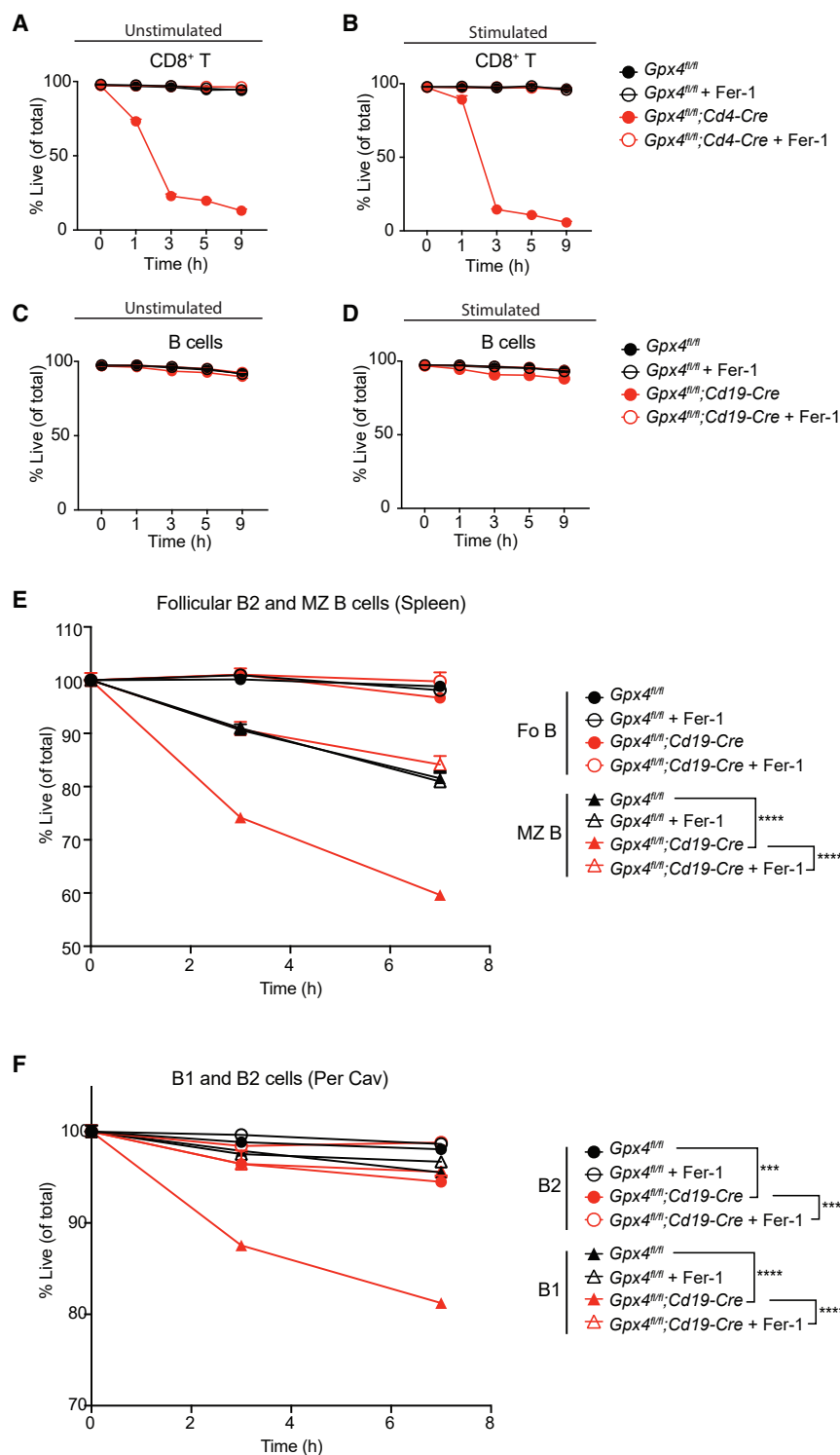


Figure 6. *Gpx4*-Deficient B1 and MZ B Cells Die by Ferroptosis Ex Vivo

(A–D) Viability of splenic MACS-enriched CD8⁺ T cells from *Gpx4*^{fl/fl}; Cd4-Cre and *Gpx4*^{fl/fl} mice (A and B) and of total MACS-enriched B cells from the spleen of *Gpx4*^{fl/fl}; Cd19-Cre and *Gpx4*^{fl/fl} littermate control mice (C and D) cultured in the presence or absence of the ferroptosis inhibitor Fer-1 was assessed. CD8⁺ T cells were left unstimulated as a control (A) or stimulated with anti-CD3 and anti-CD28 (B) for the indicated time. Total B cells were left unstimulated as a control (C) or stimulated with LPS and anti-IgM (D) as indicated. Cell viability was assessed via flow cytometry by eFluor780 staining (n = 3).

(E and F) Viability of the indicated B cell populations was assessed over time in stimulated conditions (LPS/anti-IgM) in presence or absence of Fer-1 via flow cytometry by eFluor780 staining. Splenic MZ (CD19⁺B220⁺CD21/35⁺CD23⁺) and Fo (CD19⁺B220⁺CD21/35⁺CD23⁺) B cells (E), and B1 (CD19⁺B220^{low}CD43⁺CD23⁺) and B2 (CD19⁺B220⁺CD43⁺CD23⁺) cells from the peritoneal cavity (F) were FACS sorted from *Gpx4*^{fl/fl}; Cd19-Cre and *Gpx4*^{fl/fl} littermate controls (n = 2). Graphs show mean ± SD. Numbers “n” represent biological replicates.

Data are representative of two independent experiments. Two-way ANOVA adjusted by Bonferroni’s multiple comparison test was used in (E) and (F), and the statistical significance (****p ≤ 0.0002; ****p ≤ 0.0001) is shown for the time point of 7 h. See also Figure S6.

opment and GC reactions (Akkaya and Pierce, 2019; Jellusova, 2018). Unlike Fo B2 cells, B1 cells reside in close proximity to potential invading microbes and secrete IgM antibodies before encountering pathogens. Moreover, they undergo increased homeostatic proliferation due to their maintenance by self-renewal rather than by *de novo* development (Baumgarth, 2011, 2016). To ensure this, B1 cells thus display increased metabolic activity (Akkaya and Pierce, 2019; Jellusova, 2018). For instance, a recent report demonstrated that B1a cells display a strikingly higher expression profile of glycolytic genes, high fatty acid synthesis, and uptake of lipids, which are stored in lipid droplets with the potential involvement of *Plin3* (Clarke et al., 2018). Although the metabolic requirements of MZ B cells have not been yet

reprogramming and switching to aerobic glycolysis to produce metabolic intermediates for sustenance of cell growth and proliferation (Brand, 1985; Buck et al., 2015; Wang and Green, 2012). Similar to T cells, naive B cells also drastically increase their metabolic activity to sustain rapid proliferation during devel-

extensively studied, here we additionally found that they also have increased expression of glycolytic genes and the transcription factor Myc, which is known to control metabolic reprogramming in T cells (Wang et al., 2011). These data are consistent with previous reports showing higher expression of the glucose

transporter Glut1 and consumption of glucose by MZ B cells compared to Fo B2 cells (Jayachandran et al., 2018; Jellusova et al., 2017). Moreover, we here demonstrated that MZ B cells take up high levels of fatty acids and display higher expression of *Cd36* and *Plin3* than Fo B2 cells, thus suggesting that MZ B cells might have similar metabolic requirements as B1 cells at the steady state.

Lipolysis is the hydrolytic process by which fatty acids are liberated from lipid droplets. Liberated fatty acids not only are precursors of membrane synthesis but can also be utilized as energy substrates. This requires acyl-CoA synthetases (ACSLs), which have the function to activate and shuttle fatty acids into the mitochondria for energy generation by β -oxidation. The fact that innate-like B cells display higher OXPHOS and fat uptake and storage (Clarke et al., 2018) compared to Fo B2 cells is consistent with an active breakdown of lipid droplets to fuel mitochondrial Krebs cycle to generate ATP. Indeed, the release of free fatty acids by autophagy was shown to be critical in B1 cells to sustain mitochondrial OXPHOS (Clarke et al., 2018). In addition to participate in the breakdown of fat droplets, ACSL-mediated activation of fatty acids is also critically required for lipid-droplet formation (Wilfling et al., 2014). Interestingly, the ACSL family member 4 (ACSL4), which enriches cellular membranes with long polyunsaturated omega-6 fatty acids, was described to be an essential component for ferroptosis execution (Doll et al., 2017). Thus, activation of ACSL4 for lipid-droplet formation and lipolysis may facilitate ferroptosis in *Gpx4*-deficient B1 and MZ B cells. Moreover, since high levels of intracellular free fatty acids are known to cause oxidative stress (Hauck and Bernlohr, 2016), innate-like B cells absolutely require *Gpx4* to prevent the potential lipid-peroxidation events that may occur. Therefore, our results suggest that the described distinct lipid metabolism between Fo B2 cells and B1/MZ B cells dictates the requirement of *Gpx4* to scavenge lipid ROS and inhibit ferroptosis. Interestingly, it has been recently reported that B2 cells can differentiate into functional B1 cells upon acquisition of a B1 cell-typical self-reactive B cell receptor through proliferation (Graf et al., 2019). In this regard, it would be interesting to test whether the conversion of B2 into B1 cells also changes their metabolic features and influences their requirement of *Gpx4*.

Gpx4 has a critical role in T cells to prevent lipid peroxidation and ferroptosis during T cell-mediated immune responses *in vivo*. *Gpx4*-deficient CD8⁺ and CD4⁺ T cells failed to expand and to provide protection to viral and parasite infections (Matsushita et al., 2015). By contrast, here we surprisingly found that Fo B2 cells undergo normal development, homeostatic maintenance, GC reactions, and antibody responses in the absence of *Gpx4*. Interestingly, we have previously described that the other main cellular antioxidant pathway, namely, the thioredoxin-1 (Trx1) system, is also strikingly required in T cells but largely dispensable in Fo B2 cells (Muri et al., 2018, 2019). Importantly, however, the absence of Trx1 does not affect lipid peroxidation in T cells as it is the case for the deletion of *Gpx4*, since our previous study pointed out the exclusive requirement of Trx1 to donate reducing equivalents to ribonucleotide reductase for DNA biosynthesis (Muri et al., 2018). By contrast, Fo B2 cells do not require the Trx1 system due to their capacity to tap the GSH-glutaredoxin-1 (Grx1) pathway to ensure adequate reducing power for the

generation of nucleotides (Muri et al., 2019). Therefore, the requirements of two major antioxidant systems, namely, Trx1 and *Gpx4*, is T but not Fo B cell specific, despite the existence of two distinct underlying mechanisms (nucleotide biosynthesis for Trx1 and inhibition of lipid peroxidation for *Gpx4*). We believe that this stronger antioxidant potential of Fo B2 cells, as suggested by their dispensability of both Trx1 and *Gpx4*, is at least partially required to allow secretion of the enormous amounts of antibodies, which are released by B cells after activation and differentiation to plasma cells. Indeed, the assembly of immunoglobulins involves numerous disulfide reactions in the endoplasmic reticulum, leading to ROS cascades and oxidative stress (Aronov and Tirosh, 2016). Consistent with this, other reports showed that the cellular antioxidant Nrf2 pathway is increased during plasma cell differentiation (Bertolotti et al., 2012, 2010). Expression of NADPH oxidases (NOX) in B cells but not T cells and their importance in B cell responses might also further explain the requirement of a stronger antioxidant power in B rather than T cells (Bertolotti et al., 2012). In addition to the stronger antioxidant potential and compensatory redox pathways of Fo B cells in comparison to T cells, other causes might explain the variable sensitivity to ferroptosis between different cell populations. For instance, certain metabolic features, such as the synthesis and availability of vitamin E, which completely reversed the effect of *Gpx4* deficiency in T cells (Matsushita et al., 2015), could dictate the sensitivity to ferroptosis. Moreover, the different expression of lipid-modifying enzymes (i.e., ACSL4; Dixon et al., 2015; Doll et al., 2017; Kagan et al., 2017) or the diverse phospholipid metabolism between different types of lymphocytes might also be the reason for the observed differences in sensitivity to ferroptosis.

GSH is a cellular antioxidant required to buffer ROS and prevent cellular damage. Deficiency of GSH abolishes the metabolic reprogramming that switches T cell metabolism toward increased glycolysis and glutaminolysis due to compromised activation of the mammalian target of rapamycin-1 (mTOR) and of the transcription factors NFAT and Myc (Mak et al., 2017). In addition to its direct ROS scavenging role, GSH also critically provides reducing equivalents to *Gpx4* in T cells in order to prevent lipid ROS and ferroptosis (Matsushita et al., 2015). However, it is not clear yet whether GSH has also a similar antioxidant role in B cell metabolism. Our previous work showed that the reducing power of GSH fuels the Grx1 pathway in B2 cells to sustain nucleotide biosynthesis during proliferation (Muri et al., 2019). By contrast, B1 and MZ B cells do not utilize the reducing power of GSH to sustain the Grx1 system, since this pathway is not efficiently active and cannot sustain cell proliferation (Muri et al., 2019). In keeping with this, we here show that the reducing equivalents of GSH sustain *Gpx4* function in B1 and MZ B cells. Therefore, GSH is differentially utilized in innate-like B1 and MZ B cells and in Fo B2 cells to sustain their metabolic requirements. In the formers, GSH mainly donates electrons to *Gpx4* to allow increased lipid uptake by preventing ferroptosis, whereas in the latter it sustains the biosynthesis of nucleotides downstream of the pentose phosphate pathway via reduction of Grx1.

Ferroptosis has been recently recognized to play a relevant role in cancer biology. Indeed, in some cases the metabolic

reprogramming of cancer cells has been shown to render them more sensitive to ferroptosis. However, it is not yet clear how oncogenic mutations modulate ferroptosis sensitivity and how they influence the oxidation of polyunsaturated fatty acids, which is the most well-characterized downstream event during ferroptosis execution. Thus, a deep understanding of these processes might ultimately contribute to the establishment of novel anti-cancer treatments (Friedmann Angeli et al., 2019; Seibt et al., 2019). It has been described that several forms of leukemia display a CD5⁺ phenotype and that the early generation of B1 cells contributes to the progression of chronic lymphocytic leukemia in mice, although this remains controversial in humans (Gough et al., 2017; Hayakawa et al., 2016). Therefore, exploiting the here-reported increased sensitivity of B1 cells to ferroptosis by targeting Gpx4 function or by modulating levels of cysteine and GSH might have profound implications for therapy of B cell leukemia.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.10.070>.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.M. and M.K.; Methodology, J.M.; Investigation, J.M. and H.T.; Writing – Original Draft, J.M. and M.K.; Writing – Review & Editing, J.M.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse B220, APC-Cy7, clone RA3-6B2	Biolegend	Cat#103224; RRID: AB_313007
Anti-mouse B220, PerCP, clone RA3-6B2	Biolegend	Cat#103234; RRID: AB_893353
Anti-mouse CD19, APC-Cy7, clone 6D5	Biolegend	Cat#115530; RRID: AB_830707
Anti-mouse CD19, BV650, clone 6D5	Biolegend	Cat#115541; RRID: AB_11204087
Anti-mouse CD21/35, PE, clone 7E9	Biolegend	Cat#123409; RRID: AB_940411
Anti-mouse CD23, Biotin, clone B3B4	Biolegend	Cat#101604; RRID: AB_312829
Anti-mouse CD23, PE, clone B3B4	Thermo Fisher Scientific	Cat#12-0232-83; RRID: AB_465594
Anti-mouse CD24, BV421, clone M1/69	Biolegend	Cat#101825; RRID: AB_10901159
Anti-mouse CD3e, PE, clone 145-2C11	Thermo Fisher Scientific	Cat#12-0031-82; RRID: AB_465496
Anti-mouse CD4, APC, clone GK1.5	BD Biosciences	Cat#553051; RRID: AB_398528
Anti-mouse CD11b, PE, clone M1/70	Biolegend	Cat#101208; RRID: AB_312791
Anti-mouse CD11b, PerCP-Cy5.5, clone M1/70	Biolegend	Cat#101228; RRID: AB_893232
Anti-mouse CD16/32, clone 2.4G2	This paper	N/A
Anti-mouse CD38, PerCP-Cy5.5, clone 90	Biolegend	Cat#102721; RRID: AB_2563332
Anti-mouse CD43, PE-Cy7, clone S11	Biolegend	Cat#143210; RRID: AB_2564349
Anti-mouse CD45, BV785, clone 30-F11	Biolegend	Cat#103149; RRID: AB_2564590
Anti-mouse CD45.1, PerCP-Cy5.5, clone A20	Biolegend	Cat#110728; RRID: AB_893346
Anti-mouse CD45.1, FITC, clone A20	Biolegend	Cat#110706; RRID: AB_313495
Anti-mouse CD45.2, APC, clone 104	Thermo Fisher Scientific	Cat#17-0454-82; RRID: AB_469400
Anti-mouse CD45.2, BV785, clone 104	Biolegend	Cat#109839; RRID: AB_2562604
Anti-mouse CD5, Biotin, clone 53-7.3	Thermo Fisher Scientific	Cat#13-0051-82; RRID: AB_466339
Anti-mouse CD62L, BV421, clone MEL-14	Biolegend	Cat#104435; RRID: AB_10900082
Anti-mouse CD8, FITC, clone 53-6.7	Thermo Fisher Scientific	Cat#11-0081-82; RRID: AB_464915
Anti-mouse CD8, PerCP-Cy5.5, clone 53-6.7	Biolegend	Cat#100734; RRID: AB_2075238
Anti-mouse CD86, FITC, clone GL-1	BD Biosciences	Cat#553691; RRID: AB_394993
Anti-mouse CD93, APC, clone AA4.1	Biolegend	Cat#136509; RRID: AB_2275879
Anti-mouse CD95 (Fas), APC, clone SA367H8	Biolegend	Cat#152604; RRID: AB_2632899
Anti-mouse CXCR4, PE, clone 2B11/CXCR4	BD Biosciences	Cat#551966; RRID: AB_394305
Anti-mouse F4/80, BV421, clone BM8	Biolegend	Cat#123132; RRID: AB_11203717
Anti-mouse Gr-1, PE, clone RB6-8C5	Biolegend	Cat#108408; RRID: AB_313373
Anti-mouse IgD, PE-Cy7, clone 11-26c.2a	Biolegend	Cat#405720; RRID: AB_2561876
Anti-mouse IgM, FITC, clone II/41	Thermo Fisher Scientific	Cat#11-5790-85; RRID: AB_465246
Anti-mouse Ly-6G, BV421, clone 1A8	Biolegend	Cat#127628; RRID: AB_2562567
Anti-mouse NK1.1, PE, clone PK136	Thermo Fisher Scientific	Cat#12-5941-82; RRID: AB_466050
Anti-mouse TCR β , BV421, clone H57-597	Biolegend	Cat#109229; RRID: AB_10933263
Anti-mouse TCR β , PE-Cy7, clone H57-597	Biolegend	Cat#109222; RRID: AB_893625
Anti-mouse TER-119, PE, clone TER-119	Thermo Fisher Scientific	Cat#12-5921-81; RRID: AB_466041
Anti-mouse IgM, AffiniPure F(ab) ₂ Fragment Goat	Jackson ImmuneResearch	Cat#115-005-020; RRID: AB_2338450
Anti-mouse CD3, clone 145-2C11	This paper	N/A
Anti-mouse CD28, clone 37.51	This paper	N/A
Anti-mouse IgM, human ads-AP	SouthernBiotech	Cat#1020-04; RRID: AB_2794200
Anti-mouse IgG2b, human ads-AP	SouthernBiotech	Cat#1090-04; RRID: AB_2794520

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Influenza virus: A/Puerto Rico/8/34, H1N1	provided by Jovan Pavlovic, University of Zurich	N/A
Q β -Virus-like particles (VLPs)	provided by Martin Bachmann, University of Bern	N/A
Bacteria: <i>Streptococcus pneumoniae</i> (strain D39)	provided by Benjamin Marsland, Monash University	N/A
Chemicals, Peptides, and Recombinant Proteins		
Ferostatin-1	Sigma-Aldrich	Cat#SML0583-5MG
Tamoxifen	Sigma-Aldrich	Cat#T5648-1G
Dnase I	Sigma-Aldrich	Cat#4716728001
Collagenase IV	Worthington	Cat#LS004189
LPS	InvivoGen	Cat#tlrl-3pelps
eFluor® 780	Thermo Fisher Scientific	Cat#65-0865-14
Zombie Aqua	Biolegend	Cat#423102
Streptavidin-BV711	BD Biosciences	Cat#563262
Trizol	Thermo Fisher Scientific	Cat#15596018
pNPP	Sigma-Aldrich	Cat#N2765-5
C11-BODIPY581/591	Thermo Fisher Scientific	Cat#D3861
BODIPY FL C16	Thermo Fisher Scientific	Cat#D3821
PC-BSA	Biosearch Technologies	Cat#PC-1011-10
Critical Commercial Assays		
KAPA SYBR® FAST Bio-Rad iCycler® Kit	Sigma-Aldrich	Cat#KK4608
GoScript Reverse Transcriptase Kit	Promega	Cat#A5003
Experimental Models: Organisms/Strains		
Mouse: Gpx4 ^{fl/fl}	Seiler et al., 2008	N/A
Mouse: Cd19-Cre	Rickert et al., 1997	N/A
Mouse: Cd4-Cre	Lee et al., 2001	N/A
Mouse: Cre-ERT2	Hameyer et., 2007	N/A
Mouse: C57BL/6J	The Jackson Laboratory	Cat#JAX:000664; RRID: IMSR_JAX:000664
Mouse: B6.SJL-Ptprc ^a Pepc ^b /BoyJ	The Jackson Laboratory	Cat#JAX:002014, RRID:IMSR_JAX:002014
Oligonucleotides		
Gpx4-FWD: 5'-CTGTGGAATGGATGAAAG-3'	This paper	N/A
Gpx4-REV: 5'-TCAATGAGAACTTGGTAAAG-3'	This paper	N/A
Tbp-FWD: 5'-TTGACCTAAAGACCATTCACCTTC-3'	This paper	N/A
Tbp-REV: 5'-TTCTCATGATGACTGCAGCAAA-3'	This paper	N/A
Software and Algorithms		
FlowJo Software (version 10.4.2)	Three Star	https://www.flowjo.com/
Prism 8 (version 8.0.0)	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Adobe Illustrator CS6 (version 16.0.4)	Adobe	https://www.adobe.com/
Excel	Microsoft	https://products.office.com/en/excel

LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Manfred Kopf (Manfred.Kopf@ethz.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mice were maintained at the ETH Phenomics Center (EPIC; Zurich, Switzerland) in individually ventilated cages under specific pathogen free conditions. About 6–10 week-old age- and sex-matched mice (either female or male) were used for the experiments and littermates were utilized as controls. The animals presented a healthy status, and none of the mice used in our experiments had been previously used for other procedures. All animal experiments were approved by the local animal ethics committee (Kantonales Veterinärdepartement Zürich) and were performed according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG). *Gpx4^{fl/fl}* mice (Seiler et al., 2008) (originally provided by M. Conrad, Helmholtz Zentrum, Munich, Germany) were backcrossed for more than eight generations to C57BL/6. To obtain *Gpx4^{fl/fl};Cd19-Cre*, *Gpx4^{fl/fl};Cre-ERT2* and *Gpx4^{fl/fl};Cd4-Cre* mice, *Gpx4^{fl/fl}* mice were crossed with *Cd19-Cre* (Rickert et al., 1997), *Cre-ERT2* (Hameyer et al., 2007) and *Cd4-Cre* (Lee et al., 2001) mice, respectively. C57BL/6J (CD45.2) and B6.CD45.1 (B6.SJL-Ptprca^aPeprca^b/BoyJ) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA).

METHOD DETAILS

Bone marrow chimeras

WT C57BL/6 (CD45.1⁺ CD45.2⁺) recipients were lethally irradiated (9.5 Gy in a RS 2000 [Rad Source Technologies Inc., Alpharetta, USA]). The following day, mice were reconstituted by intravenous injection of bone marrow cells from hind legs of donor mice, treated with antibiotics (0.024% Borgeal, MSD Animal Health, in the drinking water) for 6 weeks, and used for experiments 9 weeks after reconstitution.

Tamoxifen administration

For deletion of the *Gpx4* gene in *Gpx4^{fl/fl};Cre-ERT2* mice, animals were intraperitoneally injected with 2 mg TAM (Sigma-Aldrich) on two consecutive days and used for experiments at least 7 days later.

Virus infection

Influenza virus strain (A/Puerto Rico/8/34, H1N1) was originally provided by J. Pavlovic, University Zurich. Mice at age of 8 weeks were intratracheally infected with 100 plaque-forming units. Mice were bled on day 7 and on day 10 after infection to determine antibody titer in the blood serum. The body temperature and weight of the mice were monitored daily, and animals were euthanized if they fulfilled severity criteria predefined in the approval of these experiments (ZH135/15) by the local animal ethics committee.

Immunizations

For the analysis of GC reactions and antibody responses, mice were intraperitoneally injected with 10 µg of Qβ virus-like particles (Qβ-VLP) containing *Escherichia coli*-derived RNA. The particles were provided by Martin Bachmann and further information about their preparation can be found elsewhere (Cielens et al., 2000; Storni et al., 2002). Mice were bled to assess IgM and IgG2b antibody titers as indicated in each figure. To investigate the antibody response to *Streptococcus pneumoniae*, mice were intravenously injected with 1×10⁸ colony-forming units of heat-inactivated pneumococci (strain D39) and subsequently bled on day 5 to measure IgM antibody responses to phosphorylcholine by ELISA.

BODIPY C₁₆ uptake in vivo

Mice were intravenously injected with 50 µg BODIPY FL C₁₆ (Thermo Fisher Scientific) in 200 µL PBS and sacrificed for analysis after 1h. BODIPY FL C₁₆ fluorescence was then determined in the FITC-channel by flow cytometry.

Cell suspension preparations

Mice were mostly sacrificed by CO₂ asphyxiation. When lungs were harvested for analysis, mice were instead sacrificed by an intraperitoneal overdose of sodium pentobarbital. Organs were removed and processed according to the following procedure. Lungs were digested for 45 min at 37°C in IMDM medium (Life Technologies) containing 2 mg/ml of type IV collagenase (Worthington) and 0.02 mg/ml DNaseI (Sigma-Aldrich). All other organs were directly disrupted and passed through a 70 µm cell strainer (Corning). Bone marrow cells were flushed from femurs and tibia, and then directly passed through the 70 µm cell strainer. ACK buffer (home-made) was used for erythrocyte lysis for all organs.

Flow cytometry

Cells were first stained with the Zombie Aqua Fixable Viability kit (Biolegend) or with eFluor780 (eBioscience) to exclude dead cells. For surface staining, cells were resuspended in FACS buffer (PBS +2% FCS), briefly incubated with anti-CD16/CD32 antibody (2.4G2, homemade) to block Fc gamma receptors, and subsequently stained at 4°C with the relevant fluorescently labeled surface antibodies for 20 min. For the detection of lipid peroxidation, cells were stained with 2 µM of the lipid peroxidation sensor C11-BODIPY_{581/591} (Thermo Fisher Scientific) according to the manufacturer's instructions. The signal of the oxidized C11 (FITC channel, green

emission at 530 ± 30 nm) was then monitored, and the percentage of the FITC⁺ population was calculated. A complete list of all antibodies used in this study can be found in the KEY RESOURCES TABLE. Cells were acquired on LSRFortessa or on FACSCanto II, or sorted on FACSaria III (BD Bioscience). Data were analyzed in FlowJo software (Tree Star). All the gating strategies used for flow cytometry plots are shown in [Figures S5 and S6](#).

Magnetic cell sorting

CD19⁺ B cell and total T cell enrichment was achieved by positive selection using a MACS system with microbeads (MACS, Miltenyi Biotec) conjugated to monoclonal anti-mouse CD19 and anti-mouse CD90.2, respectively, following the manufacturer's instructions. Briefly, 100×10^6 cells/ml were stained with microbeads (1:10 dilution) for 15 min at 4°C. After washing, 100×10^6 cells were resuspended in 500 μ L of buffer, and the cell suspension was applied to LS columns and positively sorted in the magnetic field of a MACS separator.

In vitro B cell cultures

MACS-sorted total splenic B cells (CD19⁺) or FACS-sorted B cell populations from the spleen and peritoneal cavity (10^5 /well) were seeded in IMDM + GlutaMAX, 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M β -mercaptoethanol (all GIBCO) into 96 well plates and stimulated in the presence of AffiniPure F(ab)₂ Fragment Goat anti-mouse IgM (10 μ g/ml; Jackson ImmuneResearch) and LPS from *Escherichia coli* 0111:B4 (1 μ g/ml; InvivoGen). To test the involvement of ferroptosis in B cell survival, the stimulation of B cells was performed in the presence of ferrostatin-1 (Fer-1; 10 μ M; Sigma-Aldrich) as indicated in the figure legend.

In vitro T cell cultures

MACS-sorted T cells (CD90.2⁺) were seeded in IMDM + GlutaMAX, 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M β -mercaptoethanol (all GIBCO) into 96 well plates pre-coated with anti-CD3 (4 μ g/ml; 145-2C11; home-made) and anti-CD28 (2 μ g/ml; 37.51; home-made) for stimulation (10^5 /well). To analyze cell death by ferroptosis, T cells were stimulated in the presence of ferrostatin-1 (Fer-1; 10 μ M; Sigma-Aldrich) for the indicated times.

Antibody measurement by ELISA

To assess the IgM response to phosphorylcholine, plates were coated with 25 μ g/ml of phosphorylcholine conjugated to bovine serum albumin (PC-BSA, Biosearch Technologies) in PBS. For determination of the antibody response to Q β -VLP in PBS, plates were coated with 1 μ g/ml Q β -VLP. For measurement of influenza virus-specific antibodies, plates were coated with UV-inactivated influenza virus (PR8) in PBS. ELISAs were performed according to standard protocols using alkaline phosphatase (AP)-conjugated secondary antibodies: goat anti-mouse IgM, goat anti-mouse IgG2b (Southern Biotech). After removal of antibodies and a washing step, the alkaline phosphatase p-nitrophenyl phosphate substrate (pNPP; Sigma-Aldrich) was added into each well. Absorbance was then read at 405 nm.

RNA analysis by RT-quantitative PCR

Total RNA was extracted using TRIzol (Life Technologies), followed by reverse transcription using GoScript Reverse Transcriptase (Promega) according to the manufacturer's instructions. Real-time quantitative PCR (RT-PCR) was performed using Brilliant SYBR Green (Sigma-Aldrich) on an i-Cycler (Bio-Rad Laboratories) according to manufacturer's protocol. Expression was normalized to the housekeeping gene *Tbp* for mRNA expression. The sequences of all used primers are listed in the KEY RESOURCES TABLE.

QUANTIFICATION AND STATISTICAL ANALYSES

Statistical significance was determined by either a Student's t test (two-tailed, unpaired), or one-way ANOVA followed by Tukey's corrections, or two-way ANOVA followed by Bonferroni's corrections using Prism software (GraphPad, version 8.0.0). The Student's t test was used to analyze data from two groups, while ANOVA was used for > 2 comparison groups. The method of statistical evaluation is described in each figure legend. Asterisks in figure legends denote statistical significance (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ for Student's t test; *, $p \leq 0.0332$; **, $p \leq 0.0021$; ***, $p \leq 0.0002$; ****, $p \leq 0.0001$ for one-way and two-way ANOVA). The data are represented as mean + standard deviation or as mean + standard error of mean as indicated in each figure legend. The number of biological replicates and mice is also defined in the figure legends. The presented data are representative of at least two independent experiments.

DATA AND CODE AVAILABILITY

This study did not generate any datasets.

Supplemental Information

IRF-7 Is a Critical Regulator of Type 2 Innate

Lymphoid Cells in Allergic Airway Inflammation

Juan He, Qiong Yang, Qiang Xiao, Aihua Lei, Xing Li, Pan Zhou, Ting Liu, Lijuan Zhang, Kun Shi, Quan Yang, Junchao Dong, and Jie Zhou

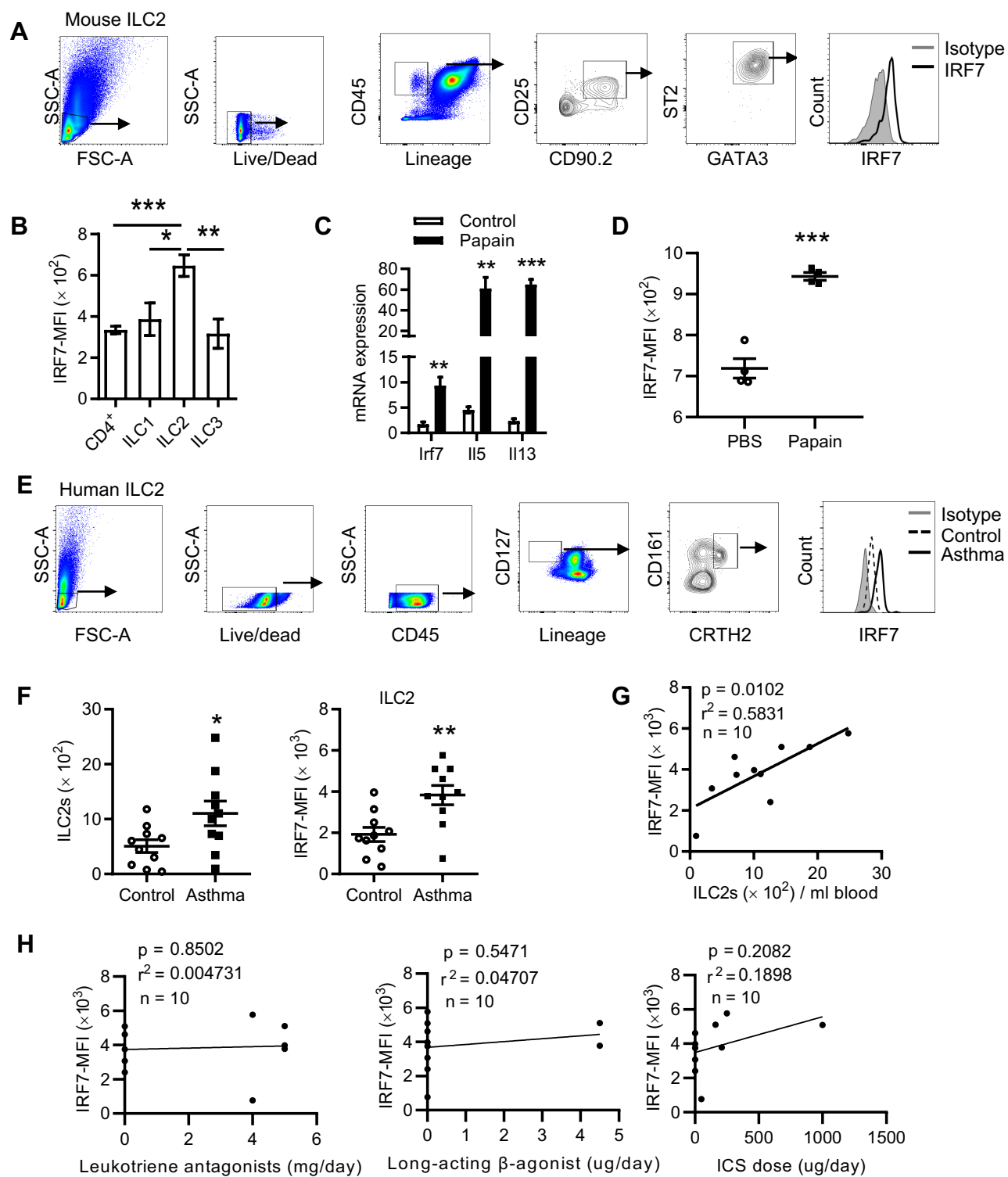


Figure S1

Figure S1. IRF7 expression in mouse and human ILC2 cells. Related to Figure 1.

A. Representative gating strategy of mouse lung ILC2 (CD45⁺Lin⁻CD90⁺CD25⁺GATA3^{high}ST2⁺) and the expression of IRF7 on ILC2s of WT mice lung. Dead cells excluded by a viability stain. **B.** Flow cytometry of IRF7 expression in mouse ILC1 (Lin⁻CD45⁺CD127⁺NKP46⁺NK1.1⁺), ILC2 and ILC3(Lin⁻CD45⁺CD90⁺CD127⁺Roryt⁺). The data shown are representative Mean fluorescent intensity (MFI) of IRF7 from two independent experiments (n=5 mice per group). **C.** The mRNA expression of IRF7 in lung tissue after papain treatment, IL-5 and IL-13 are used as positive control. **D.** The protein level of IRF7 in lung ILC2 from mice treated with papain (i.n.) was determined by flow cytometry. **E.** Representative gating strategy of human ILC2 (CD45⁺Lin⁻CD127⁺CRTH2⁺CD161⁺) and the expression of IRF7 on asthma and healthy control. Dead cells excluded by a viability stain. **F.** Left panel shows flow cytometric analysis of human ILC2 cells in PBMC of asthma patients, compared with controls, while right panel shows IRF7 expression in human ILC2 cells between asthma patients and healthy controls (n=10). **G.** Correlation between human ILC2 number and IRF7 expression in asthma patients (n=10). **H.** Correlation between IRF7 expression and the medication of asthma patients (ICS (inhaled corticosteroid), Long-acting β -agonist and leukotriene antagonists). Data are representative of two independent experiments (n=3-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001 by unpaired Student's t test.

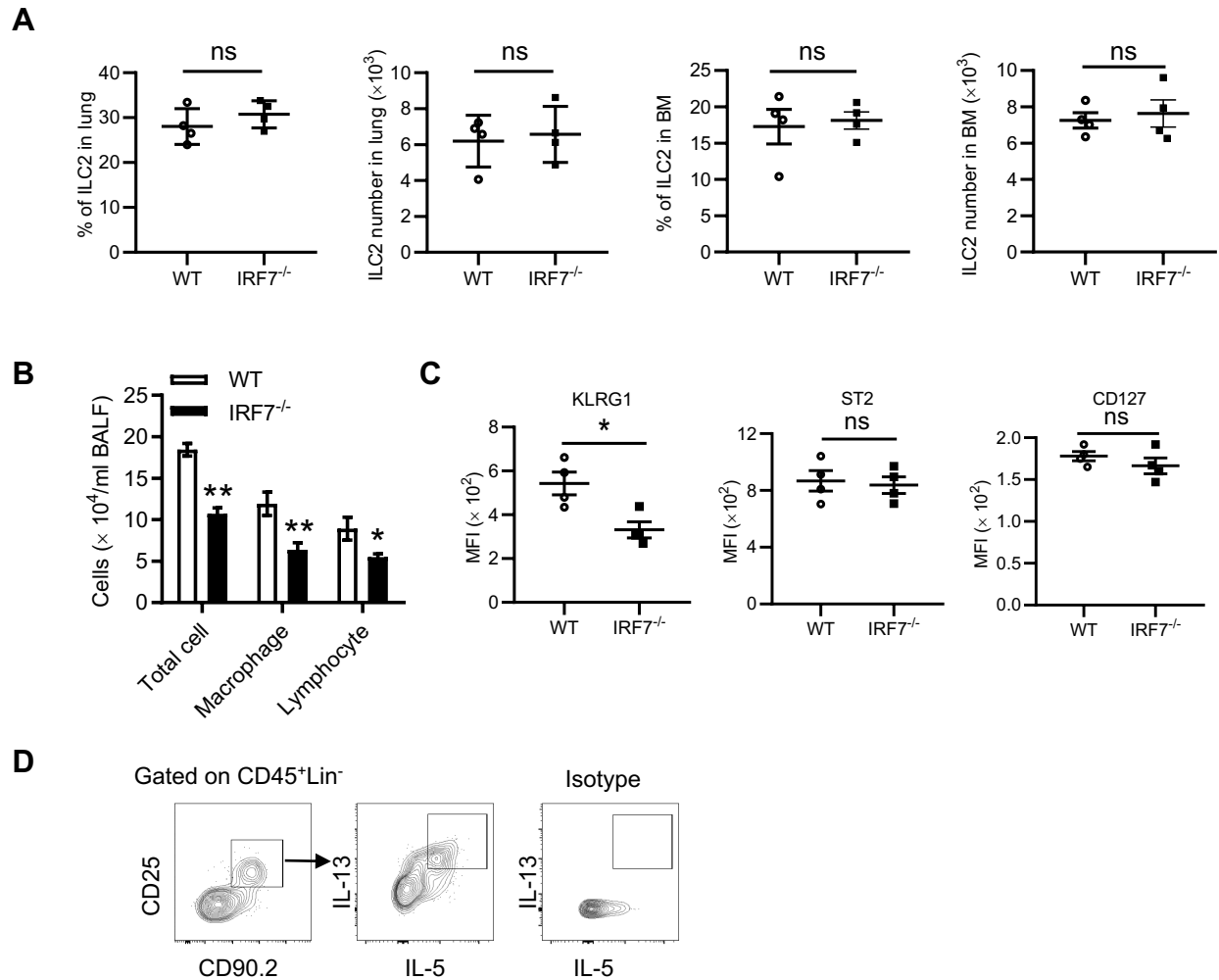


Figure S2. IRF7 deficiency causes remission of ILC2-driven airway inflammation. Related to Figure 1.

A. Flow cytometric analysis of ILC2 in lung (left) and bone marrow (BM, right) of WT and IRF7^{-/-} mice under steady-state condition (n=4). The frequency and total numbers of ILC2 are shown. Data are mean ± SEM from three independent experiments. **B.** Total cells in BALF (including macrophages and lymphocytes) from papain challenged WT and IRF7^{-/-} mice. Data are representative of two independent experiments (n=6 mice per group). **C.** Flow cytometric analysis of KLRG1, ST2, CD127 expression in lung ILC2s from WT and IRF7^{-/-} mice after challenge with papain (n=4). **D.** The representative flow cytometry analysis of IL-13⁺IL-5⁺ILC2 in lungs. Data are representative of two independent experiments. Error bars show mean ± SEM. *, p<0.05 by unpaired Student's t test. ns, not significant.

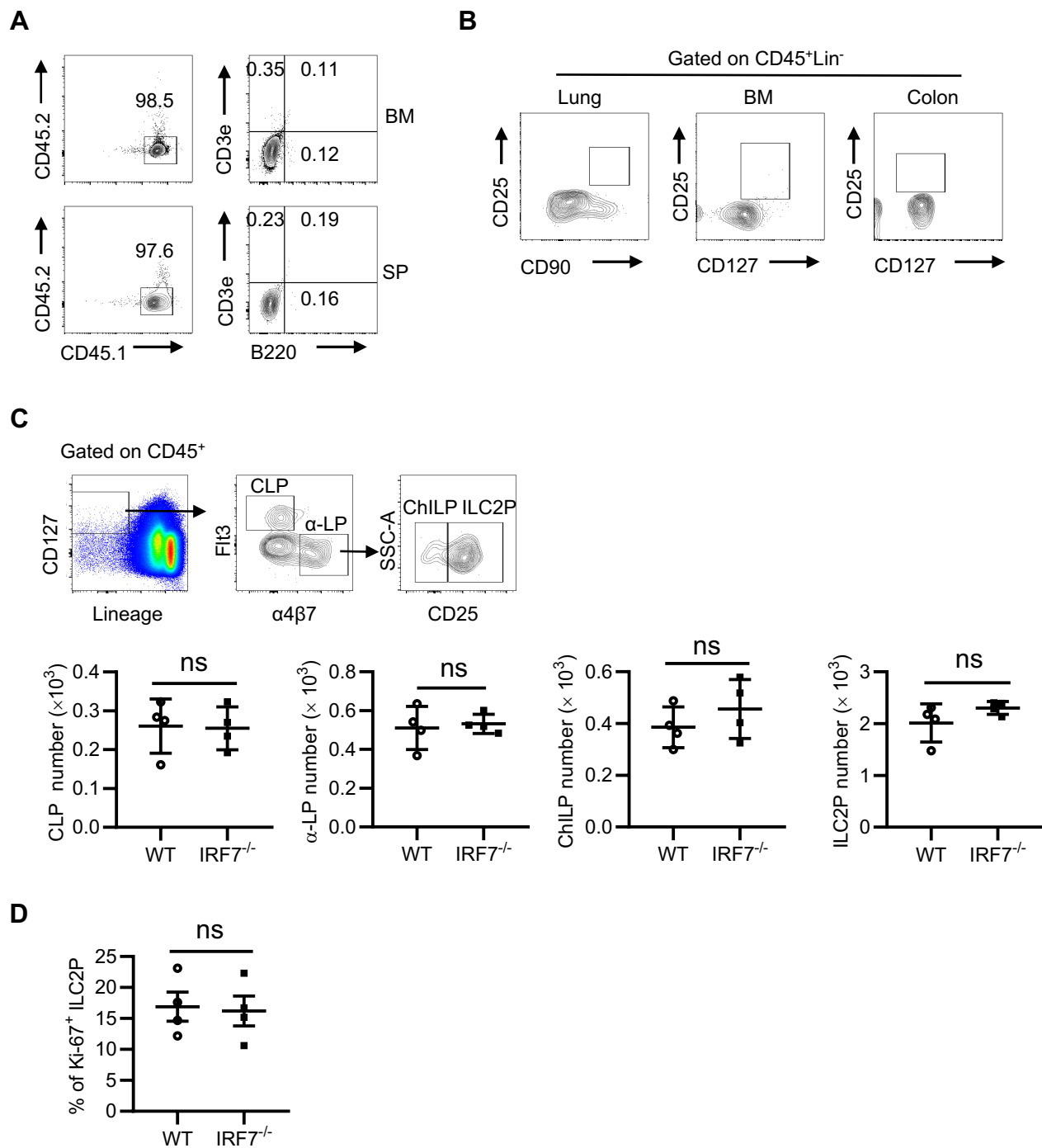


Figure S3: The cellular characteristics of NCG mice and the reconstitution of ILC2 progenitors in mixed BM chimeras. Related to Figure 4.

A. Flow cytometric plots showing percentages of B220⁺ and CD3e⁺ cells in the BM and SP from NCG mice. **B.** Flow cytometric plots showing percentages of ILC2 cells in the lung, BM and colon from NCG mice. **C.** The representative gating strategy of ILC2 progenitor cells in the BM of adult mice, pregated on live cells (upper), and their reconstitution from donor cells in recipients BM at steady-state (lower) (n=4). **D.** The proliferation of BM ILC2P from donor cells in recipients BM at steady-state (n=4). CLP: Common lymphoid progenitors, α-LP: α4β7⁺ lymphoid progenitors, ChILP: Common helper-like ILC progenitors, ILC2P: ILC2 precursor. Data are representative of two (A-B) or three (C-D) independent experiments. Error bars show mean ± SEM. ns, not significant. Numbers within flow plots indicate the percentages of cells.

Figure S3

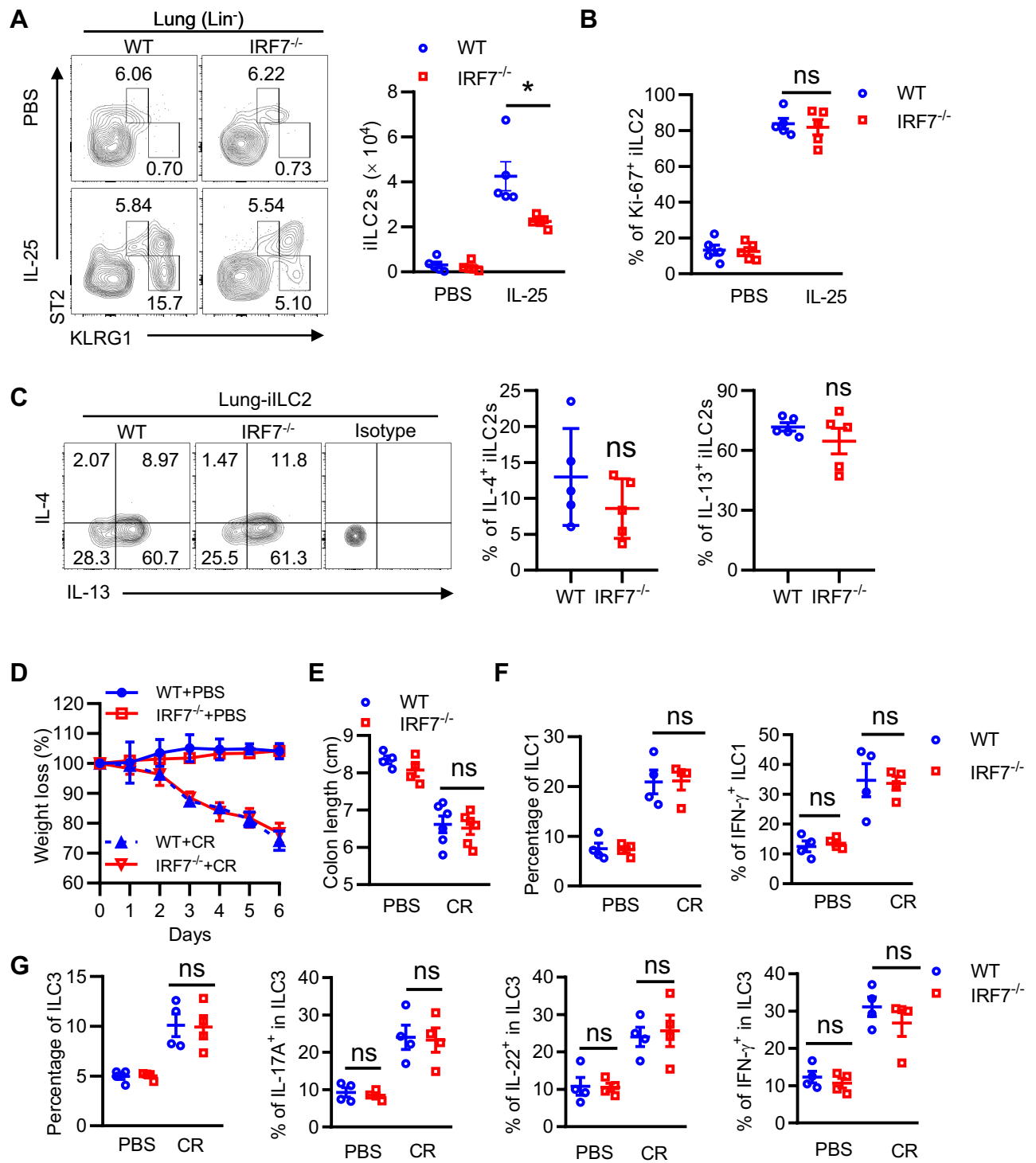


Figure S4

Figure S4. IRF7 did not affect the function of iILC2, ILC1 and ILC3. Related to Figure 4.

(A-C) WT and IRF7^{-/-} mice were administered with IL-25 (I.P. 200 ng/mouse/day) for 3 days (n=5). **A.** Flow cytometric analysis of iILC2s (Lin⁻ST2⁺KLRG1^{hi}) and their absolute numbers in lung. **B.** The proliferation of iILC2s. **C.** Flow cytometric analysis of IL-4⁺ and IL-13⁺iILC2s in lung. **(D-G)** WT and IRF7^{-/-} mice were administered with *citrobacter rodentium* (CR) (n=4-6 per group). Mice were sacrificed 7 days later. Body weight loss (**D**) and colon length (**E**) were shown. **F.** Flow cytometric analysis of ILC1 (CD45⁺Lin⁻CD127⁺NKP46⁺NK1.1⁺) and their production of IFN- γ in colon. Lineage cocktail contains anti-CD3, anti-CD45R/B220, anti-CD11b, anti-Ly-6G, anti-Erythroid marker (TER-119), anti-CD11c, anti-CD4, anti-CD5, anti-CD8a, anti-TCR β , and anti-TCR $\gamma\delta$. **G.** Flow cytometric analysis of ILC3s (CD45⁺Lin⁻CD90.2⁺CD127⁺Roryt⁺) in colon, as well as their production of IL-17A, IL-22, and IFN- γ . Data are representative of two independent experiments. Error bars show mean $\pm$ SEM. *,p<0.05 by unpaired Student's *t* test. ns, not significant. Numbers within flow plots indicate the percentages of cells gated.

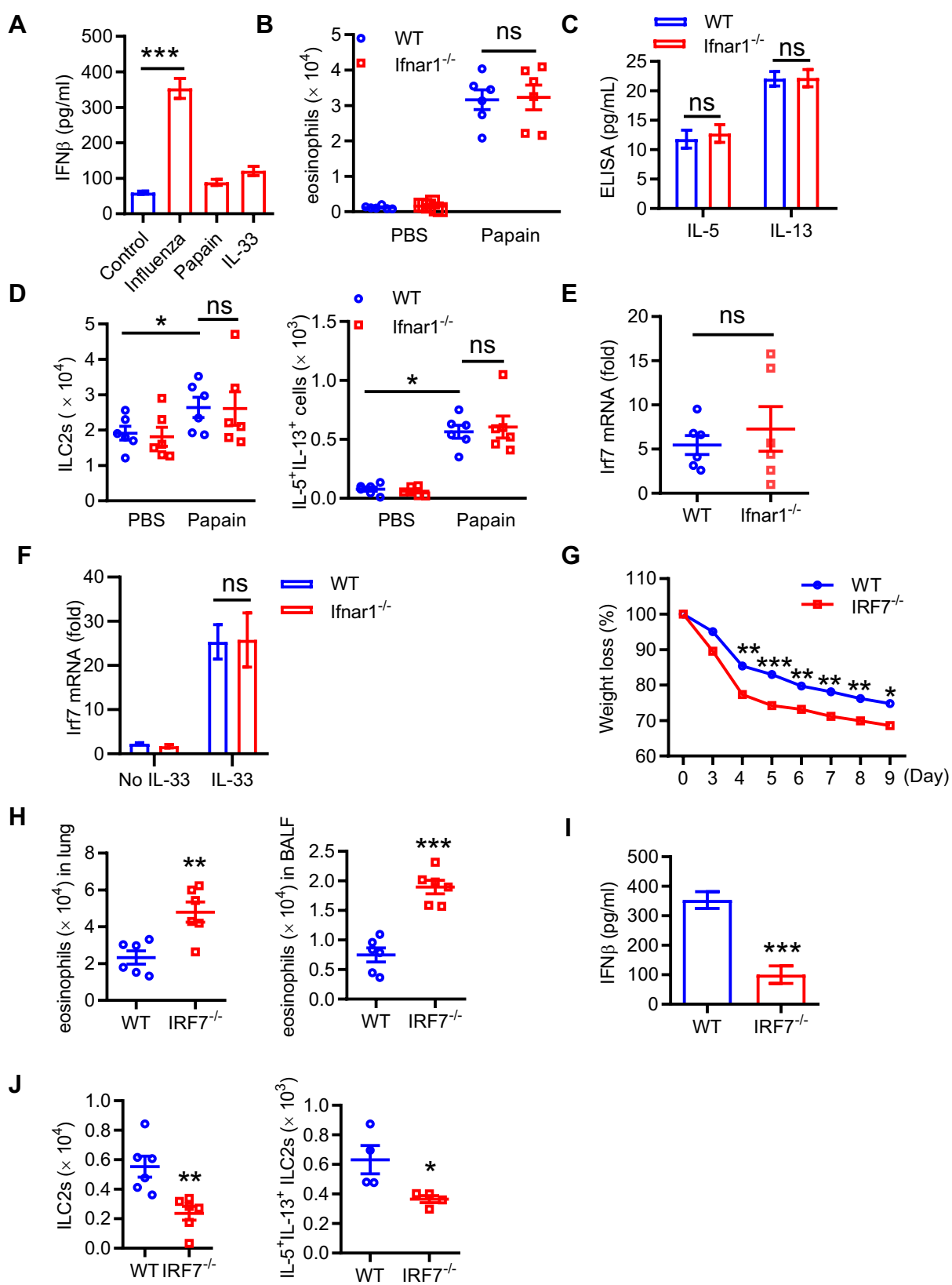


Figure S5

Figure S5: IFN does not affect the regulation of ILC2 by IRF7 in vivo. Related to Figure 6.

A. Analysis of the content of IFN β in different models (Influenza, papain, IL-33) by ELISA. **B.** Absolute numbers of eosinophils in bronchoalveolar lavage fluid between *Ifnar1*^{-/-} and WT mice after papain treatment. **C.** Bronchoalveolar lavage fluid from mice was tested for IL-5 and IL-13 by ELISA. Samples were tested in duplicate, with each experiment using six samples. **D.** Absolute numbers of lung-ILC2 and cytokines (IL-5 and IL-13) derived from lung-ILC2s between *Ifnar1*^{-/-} and WT mice after papain treatment. **E.** The mRNA expression of IRF7 in lung tissue between *Ifnar1*^{-/-} and WT mice. **F.** Lung ILC2s were sorted from *Ifnar1*^{-/-} and WT mice, followed by IL-33 stimulation in vitro. After 24h, mRNA expression of IRF7 was measure by qRT-PCR. **G.** *IRF7*^{-/-} and control mice were infected intranasally with influenza virus A/PR8. Mice were sacrificed at day 9 after infection. The body weight of control and *IRF7*^{-/-} mice was monitored daily after infection. **H.** Absolute numbers of eosinophils in lung and bronchoalveolar lavage fluid between *IRF7*^{-/-} and control mice after influenza virus infection. **I.** Analysis of the content of IFN β in the plasma of *IRF7*^{-/-} and control mice after influenza virus infection by ELISA. **J.** Absolute numbers of lung-ILC2 and cytokines (IL-5 and IL-13) derived from lung-ILC2s. Data are representative of two independent experiments (n=4-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001 by unpaired Student's t test. ns, not significant.

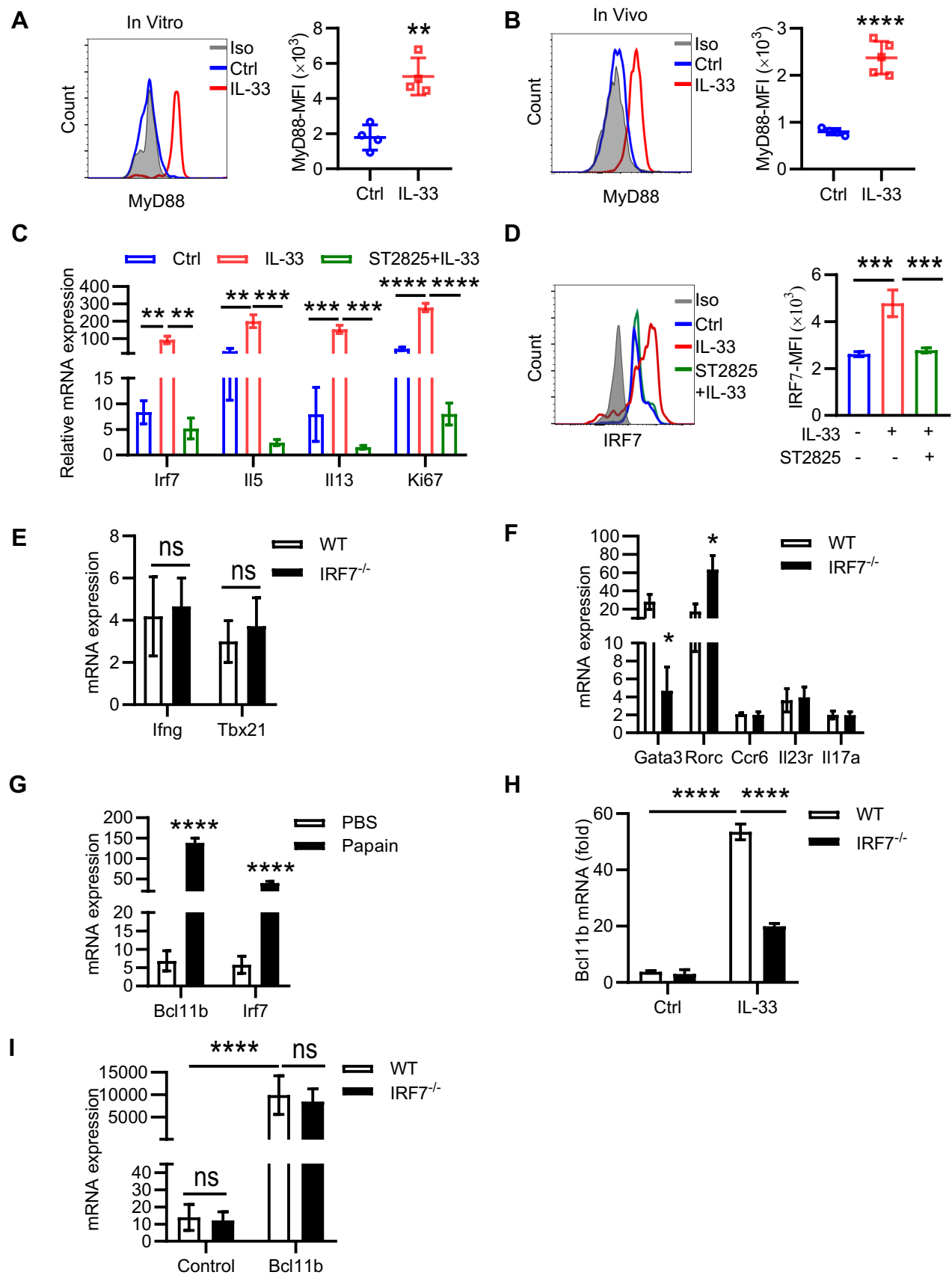


Figure S6

Figure S6: Bcl11b is induced during airway inflammation in vivo and in vitro. Related to Figure 6.

A. Flow cytometric analysis of MyD88 expression in ILC2 cells after IL-33 stimulation for 3 days in vitro. **B.** Flow cytometric analysis of MyD88 expression in lung ILC2 after mice were challenged with IL-33 for 3 days. **C.** Lung ILC2s were treated with ST2825 (20 μ M) with or without IL-33 for 24 h. The mRNA expression of *Irf7*, *Ils5*, *Ils13* and *Ki67* was determined by qRT-PCR. **D.** Lung ILC2s were treated with ST2825 (20 μ M) with or without IL-33 for 72 h. The MFI of IRF7 was measured by flow cytometry. **E.** The mRNA expression of ILC1 related genes in lung ILC2s from WT and IRF7^{-/-} mice under papain model. **F.** The mRNA expression of ILC3 related genes in lung ILC2s from WT and IRF7^{-/-} mice under papain model. *Gata3* was used as a positive control. **G.** The mRNA expression of *Bcl11b* in lung ILC2 in papain model was measured by qRT-PCR. *Irf7* was used as the positive control. **H.** Naïve lung ILC2s sorted from WT and IRF7^{-/-} mice were cultured in the presence of IL-2, IL-7 with or without IL-33. After 24 h, the mRNA expression of *Bcl11b* was measured by qRT-PCR. **I.** ILC2 cells from WT and IRF7^{-/-} mice were infected with retrovirus of Bcl11b-Migr1 or vector control. The mRNA expression of *Bcl11b* was measured by qRT-PCR after 72 h. Data are representative of two (E-I) or three (A-D) independent experiments (n=4-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001; ****,p<0.0001 by unpaired Student's t test. ns, not significant.

Table S1: Asthma subject characteristics. Related to Figure S1.

Characteristic	Asthma	Healthy Control
No. of subjects	10	10
Age (years)	8.2 $\pm$ 1.81 (6-11)	8.10 $\pm$ 1.91 (6-12)
Gender (M:F)	7:3	6:4
ICS dose (ug/day)	167 $\pm$ 308.37 (0-1000)	0
Oral corticosteroids (%)	0	0
Long-acting β -agonist (%)	20	0
Leukotriene antagonists (%)	50	0
FEV1 (liters)	1.46 $\pm$ 0.34 (1.08-2.00)	-
FEV1 (% predicted)	89.2 $\pm$ 13.83 (67-110)	-
FEV1/FVC ratio (%)	76.8 $\pm$ 6.11 (67-87)	-
FeNO (ppb)	30.47 $\pm$ 14.35 (10-50)	-

Results are expressed as means $\pm$ SD (range).

ICS dose is the equivalent of inhaled fluticasone.

FeNO: fractional concentration of exhaled nitric oxide.

ppb: parts per billion.

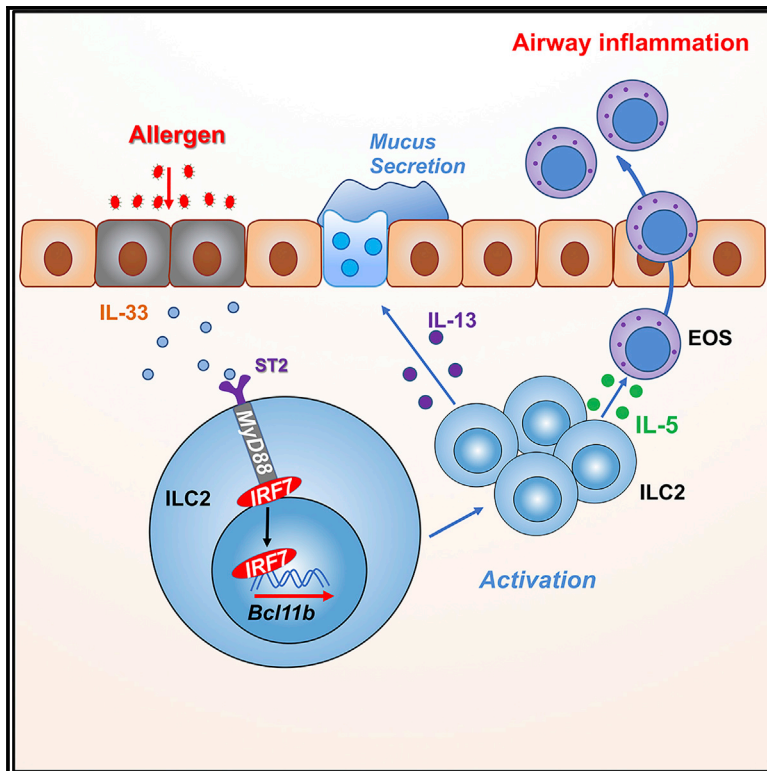
Table S2: Sequences of primers used in this study. Related to Figure 6, S1, S5 and S6.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
<i>Il5</i>	CGTGGGGGTACTGTGGAAAT	AATCCAGGAACTGCCTCGTC
<i>Il13</i>	TGCCATCTACAGGACCCAGA	CTCATTAGAAGGGGCGGTGG
<i>Gata3</i>	AGGCAAGATGAGAAAGAGTGCCTC	CTCGACTTACATCCGAACCCGGTA
<i>Irf7</i>	AAACCATAGAGGCACCCAAG	CCCAATAGCCAGTCTCCAAA
<i>Gfi1</i>	AGGAGGCACCGAGAGACTCA	GGGAGGCAGGGAAGACATC
<i>Tox</i>	CTTGAGGCTCTCCCAAGTCC	CGTTCGAAAATGCGCTCCAG
<i>PLZF</i>	AGCTGCCTCACCAACCTTTC	ATCGAGCCTTACGGTCTCT
<i>Tcf1</i>	CCCACAGAGCTTGACTAGTGG	CCCATCGTCATCCGTGTCAT
<i>Nfil3</i>	GAGCAGAACCACGATAACCCA	AGATGCAACTTCCGGCTACC
<i>Ets1</i>	TCCGCCAAGTGCCAACCTC	CGGAGTTAACAGCGGGACAT
<i>Bcl11b</i>	CCCTCCCACCTCCGTGATTA	CCATGTGTGTTCTGTGCGTG
<i>Rora</i>	GTGGAGACAAATCGTCAGGAAT	TGGTCCGATCAATCAAACAGTTC
<i>Ki67</i>	CCTGTGAGGCTGAGACATGG	CCCTCACTCTTGTCAGGGTC
<i>Rorc</i>	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT
<i>Ccr6</i>	CCTGGGCAACATTATGGTGGT	CAGAACGGTAGGGTGAGGACA
<i>Il23r</i>	TTCAGATGGGCATGAATGTTTC	CCAAATCCGAGCTGTTGTTCTAT
<i>Il17a</i>	TTTAACTCCCTTGCGCAAAA	CTTCCCTCCGCATTGACAC
<i>Ifng</i>	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
<i>Tbx21</i>	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
<i>β-actin</i>	CGTGCGTGACATCAAAGAGAAG	CGTTGCCAATAGTGATGACCTG
Bcl11b chip site-1	GAGGGGTCAGCCTCCGAGAAAGAA	CGCCCCCTCAAAAACCCAAA
Bcl11b chip site-2	GTGCTTTCTTGTTCTCTTACAGGG	GGACGTGACGTTTAATCTGC
Bcl11b chip site-3	CCCCACCCATGGCTTTCAGG	GATTGGCGAGGCTGCAAGGC

Cell Reports

IRF-7 Is a Critical Regulator of Type 2 Innate Lymphoid Cells in Allergic Airway Inflammation

Graphical Abstract



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In Brief

Allergic asthma is a highly prevalent airway inflammatory disease. He et al. demonstrate that IRF7 represents an important regulator of type 2 innate lymphoid cells (ILC2s) in lung. Mice with IRF7 deficiency display impaired responses and effector function in ILC2s, thereby attenuating airway inflammation.

Highlights

- IRF7 expression in ILC2s is induced during allergic inflammation
- IRF7 deficiency leads to remission of allergic airway inflammation
- Regulation of ILC2s by IRF7 is cell intrinsic and mediated by Bcl11b
- IRF7 expression is positively correlated with ILC2 number in asthmatic patients



IRF-7 Is a Critical Regulator of Type 2 Innate Lymphoid Cells in Allergic Airway Inflammation

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SUMMARY

Allergic asthma is a highly prevalent airway disease triggered by hyperresponsiveness to inhaled allergens. Interferon regulatory factor 7 (IRF7) has been shown to be highly expressed in nasal aspirates from children with asthma. Type 2 innate lymphoid cells (ILC2s) represent the major player in allergic airway inflammation. The role of IRF7 in ILC2-driven asthma remains to be explored. Here, we report that IRF7 expression in murine lung ILC2s is dramatically induced upon papain or interleukin-33 (IL-33) stimulation. ILC2s from asthma patients display a much higher level of IRF7 than those from healthy donors. Deficiency of IRF7 in mice significantly impairs the expansion and function of lung ILC2s in multiple models of allergic asthma. Furthermore, the regulation of ILC2s by IRF7 is cell intrinsic and mediated by the transcription factor Bcl11b. These observations identify IRF7 as a regulator of lung ILC2s, which may have immunotherapeutic value in allergic asthma.

INTRODUCTION

Both type 2 helper T (Th2) cells and type 2 innate lymphoid cells (ILC2s) are major driver of allergic airway inflammation by producing pro-inflammatory type 2 cytokines (Klein Wolterink et al., 2012; Licona-Limón et al., 2013; Walker and McKenzie, 2018). In contrast to the antigen-specific response of Th2 cells, the function of ILC2s is independent of antigen stimulation (Robinet et al., 2015). In response to inhaled allergens, airway epithelial cells produce and release thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25), and IL-33, which are strong inducers and activators of lung ILC2s (Divekar and Kita, 2015; Huang et al., 2015; Kim et al., 2013; Oboki et al., 2010). The acti-

vated lung ILC2s secrete type 2 cytokines IL-13 and IL-5, which results in eosinophil infiltration, mucus hyper-production, and airway hyperreactivity, but not immunoglobulin E (IgE) production (Fort et al., 2001; Klein Wolterink et al., 2012). In addition to initiation of innate immunity during airway inflammation, ILC2s could also promote the activation of Th2-mediated adaptive immunity (Halim et al., 2014; Liu et al., 2015; Mirchandani et al., 2014). ILC2s therefore play an important role in linking allergen-induced epithelium cytokine production and activation of type 2 adaptive immunity during allergic responses (Martinez-Gonzalez et al., 2015). The clinical significance of ILC2s in airway inflammation has also been demonstrated in patients with asthma, who displayed higher levels of ILC2s in both peripheral blood and bronchoalveolar lavage fluid (BALF) when compared with healthy individuals (Bartemes et al., 2014; Smith et al., 2016). The molecular mechanisms underlying the functions of lung ILC2s remains to be fully understood, although some important regulators have been identified (Hoyler et al., 2012; Lei et al., 2018; Loering et al., 2019; Vivier et al., 2018).

Interferon regulatory factor 7 (IRF7) is one of the master regulators of anti-viral responses that dictate the production of interferon (IFN) (Honda et al., 2005). IRF7 deficiency enhanced the vulnerability of mice to influenza A virus infection due to defect in IFN production (Ning et al., 2011). Literatures have suggested that IFN plays a pleiotropic role in airway inflammation. It was reported that IFN could suppress allergic inflammatory diseases through multiple mechanisms, including blocking IL-4-mediated IgE isotype switch and suppressing Th2 responses (Monticelli et al., 2011; Simon et al., 2003). Recently, IFN was demonstrated to be a negative regulator of ILC2s (Duerr et al., 2016; Moro et al., 2016). Interestingly, downregulation of IFN signaling was observed in asthmatic patients (Edwards et al., 2013). Patients with asthma who were infected with HCV (hepatitis C virus) and treated with IFN therapy showed a reduction in symptoms and severity of asthma (Gonzales-van Horn and Farrar, 2015). These studies indicate a negative regulation between asthma and IFN signaling. We therefore were interested to



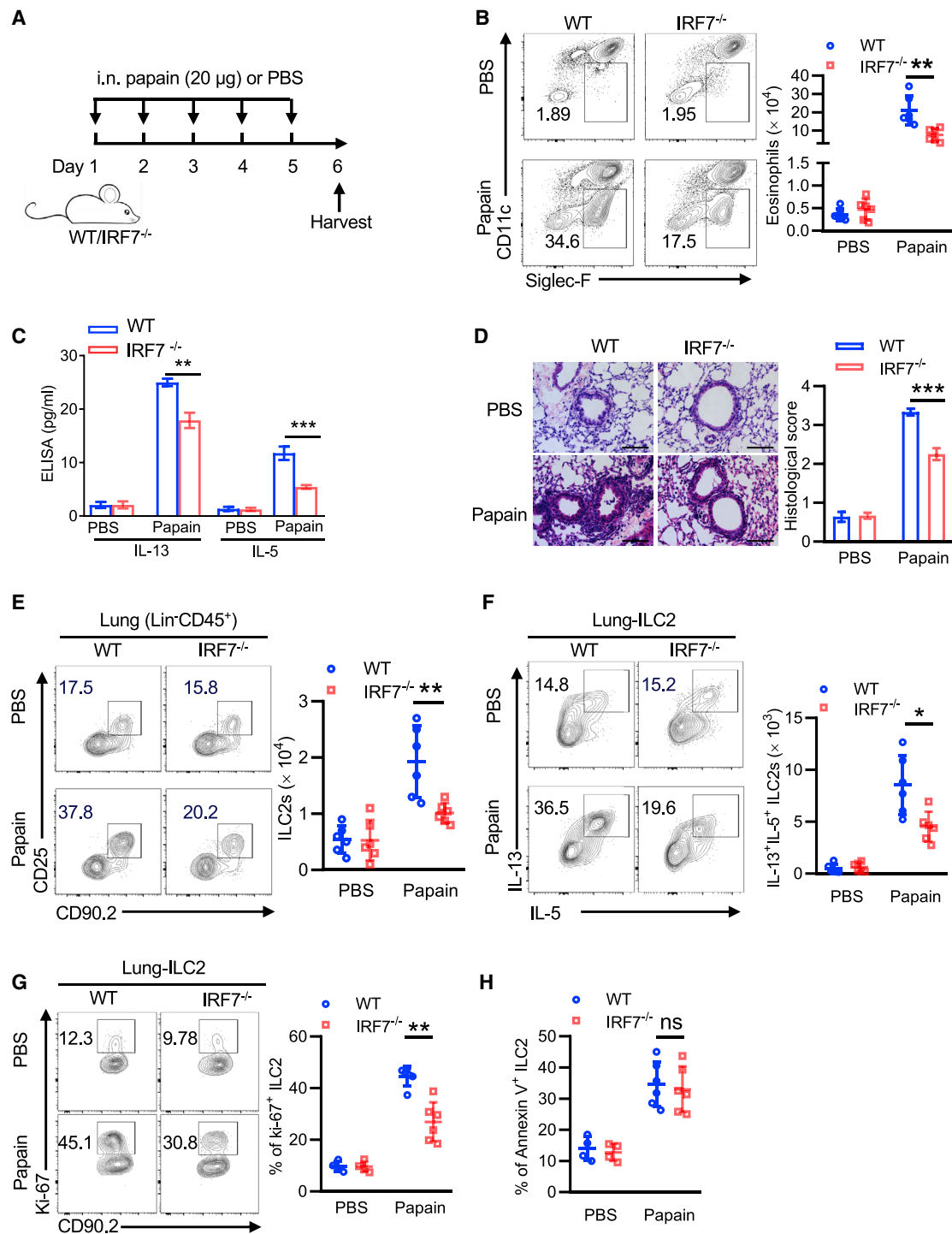


Figure 1. IRF7 Deficiency Causes Remission of ILC2-Induced Lung Inflammation (Papain)

(A) IRF7^{-/-} and WT littermate mice were intranasally challenged with papain on days 1–5, and mice were sacrificed 24 h after the last challenge (n = 4–6 per group).

(B) Flow cytometric analysis of eosinophils (CD45⁺Siglec-F⁺CD11c⁻) in BALF. Both representative results (left) and mean $\pm$ SEM from all mice analyzed (right) are included.

(C) The amounts of IL-13 and IL-5 in BALF were determined by ELISA (n=6 per group).

(D) Representative H&E staining of lung sections from four independent experiments (bars, 100 μ m) and the inflammation was determined by semiquantitative scoring.

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investigate the potential roles of IRF7 in ILC2-mediated airway inflammation.

In this study, we found that IRF7 was highly expressed in ILC2s and was induced by papain or IL-33 stimulation. IRF7 expression was positively correlated with ILC2 levels in patients with asthma. IRF7 deficiency in mice significantly impaired ILC2 responses in lung during asthma, which was independent of IFN signaling. Regulation of ILC2s by IRF7 was cell intrinsic and mediated by transcription factor Bcl11b. Collectively, this study reveals the potential therapeutic value of IRF7 in allergic asthma.

RESULTS

IRF7 Deficiency Causes Remission of ILC2-Driven Airway Inflammation

To investigate the role of IRF7 in ILC2s, its expression in ILC subsets was first evaluated. Flow cytometric analysis of murine lung ILC2s (CD45⁺Lin[−]CD90⁺CD25⁺GATA3^{high}ST2⁺) (Lei et al., 2018; Monticelli et al., 2016) is shown in Figure S1A. Results showed that ILC2 highly expressed IRF7, when compared with other ILC subsets and CD4⁺ T cells (Figure S1B). Administration of papain, a well-known activator of lung ILC2s, caused dramatic induction of IRF7 expression in mouse ILC2s (Figures S1C and S1D). Importantly, ILC2s from patients with asthma displayed much higher levels of IRF7 than those from healthy donors (Figures S1E and S1F). IRF7 expression was positively correlated with ILC2 numbers in the peripheral blood of asthma patients (Figure S1G). IRF7 expression in ILC2s was not affected by medications used in patients (Figure S1H). These observations collectively indicate a potential role of IRF7 in ILC2s during asthma.

We next evaluated the levels of ILC2s in IRF7-deficient (IRF7^{−/−}) mice. Flow cytometric analysis showed that IRF7 deficiency did not affect the frequencies of ILC2 in the lung and bone marrow (BM) under steady-state conditions (Figure S2A). Murine models of allergic airway inflammation were next performed. Wild-type (WT) and IRF7^{−/−} mice were administered intranasally with papain for 5 days (Figure 1A). Results showed that the infiltration of eosinophils (Figure 1B), as well as the amounts of type 2 effector cytokines (IL-13 and IL-5) (Figure 1C) in BALF, were dramatically diminished in IRF7^{−/−} mice upon papain administration when compared with the corresponding WT controls. Similar changes were observed for macrophages and lymphocytes in BALF (Figure S2B). The remission of lung inflammation in IRF7^{−/−} mice was further evidenced by H&E staining, as represented by fewer infiltrated cells and decreased airway wall thickness (Figure 1D). Notably, papain exposure resulted in increased numbers of lung ILC2s in WT mice, which was severely diminished in IRF7^{−/−} mice (Figure 1E). The level of ILC2 activation marker KLRG1 and the production of IL-13 and IL-5 effector cytokines by ILC2 (IL-13⁺IL-5⁺ILC2) were significantly

decreased in IRF7^{−/−} mice when compared with papain-treated WT controls (Figures S2C, S2D, and 1F). The proliferation of ILC2s was clearly lower in IRF7^{−/−} mice (Figure 1G), whereas their apoptosis was not affected (Figure 1H). These observations indicate that IRF7 deficiency impairs the responses of lung ILC2 during allergic inflammation.

For further confirmation, *Alternaria alternata*, a clinically relevant allergen, was used to induce lung inflammation (Castanheira et al., 2015; Maazi et al., 2015) (Figure 2A). Consistent with the observations from the papain model, the levels of eosinophils in BALF (Figure 2B) and the amount of IL-13 and IL-5 in BALF (Figure 2C) were dramatically decreased in IRF7^{−/−} mice compared with WT controls. The remission of airway inflammation was evidenced by histological staining of lung tissues (Figure 2D). The levels of lung ILC2s and their production of effector cytokines (IL-13⁺IL-5⁺) (Figure 2E), as well as their proliferation (Figure 2F), were dramatically decreased in IRF7^{−/−} mice when compared with WT littermates. Collectively, these observations suggest that IRF7 deficiency causes remission of ILC2-driven airway inflammation.

IRF7 Deficiency Impairs ILC2 Function in Response to IL-33 Challenge

Epithelial-derived cytokine IL-33 is known to play a crucial role in the activation of ILC2s during allergic inflammation (Barlow et al., 2013). Consistent with the observations after papain stimulation, IRF7 expression was induced in ILC2s in response to IL-33 stimulation at both the mRNA and protein levels (Figure 3A). IL-33 was next intranasally administered to induce airway inflammation in WT and IRF7^{−/−} mice (Figure 3B). As expected, administration of IL-33 dramatically induced an influx of eosinophils in BALF (Figure 3C), and enhanced the amounts of IL-13 and IL-5 cytokines in BALF (Figure 3D) in both WT and IRF7^{−/−} mice. However, the fold increase was significantly lower in IRF7^{−/−} mice than in WT littermates (Figures 3C and 3D). Consistently, the frequencies and absolute numbers of lung ILC2s (Figure 3E) and their secretion of IL-13 and IL-5 (Figure 3F) were also diminished in IRF7^{−/−} mice upon IL-33 challenge. The amelioration of inflammation was further confirmed by H&E staining of lung tissues (Figure 3G). When lung ILC2s were cultured in the presence of IL-7 and IL-33 *in vitro*, IRF7^{−/−} cells produced much lower amounts of IL-13 and IL-5 (Figure 3H) and displayed a lower level of proliferation (Figure 3I) than WT controls. These results indicated that ILC2s lacking IRF7 are functionally compromised.

For further confirmation, equal numbers of lung ILC2s from IL-33-challenged WT and IRF7^{−/−} mice (CD45.2⁺) were adoptively transferred into immunodeficient NCG (NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju) (CD45.1⁺) mice, which lacked B cells, T cells, and ILC2s (Figures S3A and S3B) (Cao et al., 2017; Lei et al., 2018). NCG recipients were then intranasally administered with IL-33 (Figure 4A). Recipients of IRF7^{−/−} ILC2s displayed

(E and F) Flow cytometric analysis of lung ILC2s (CD45⁺Lin[−]CD90⁺CD25⁺GATA3^{high}ST2⁺) (E) and their secretion of IL-13 and IL-5 (F). Both representative results (left) and mean $\pm$ SEM (right) are shown.

(G) The proliferation of lung ILC2s from papain-treated mice was evaluated by Ki-67 staining. Both representative results (left) and mean $\pm$ SEM (right) are shown. (H) Apoptosis of lung ILC2s from papain-treated mice was evaluated by Annexin V staining. Data are representative of three independent experiments. Error bars show mean $\pm$ SEM.

*p < 0.05; **p < 0.01, ***p < 0.001 by unpaired Student's t test. ns, not significant. Numbers within flow plots indicate the percentages of cells.

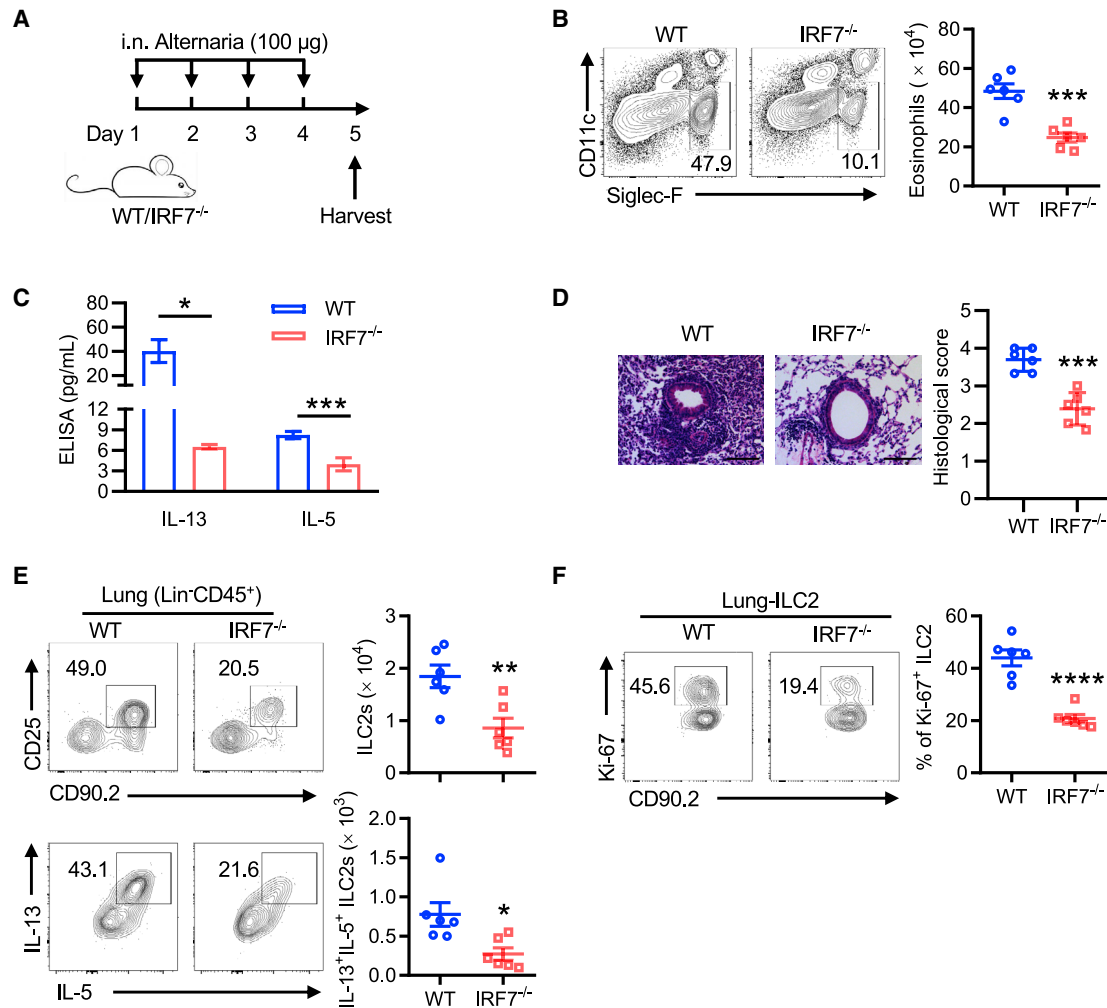


Figure 2. IRF7 Deficiency Causes Remission of ILC2-Induced Lung Inflammation (*Alternaria alternata*)

(A) Schematic diagram for intranasal challenge of *Alternaria alternata* in IRF7^{-/-} and WT mice.

(B) Flow cytometric analysis of eosinophils (CD45⁺Siglec-F⁺CD11c⁻) in BALF. Both representative results (left) and mean ± SEM from all mice analyzed (right) are included.

(C) The amounts of IL-13 and IL-5 in BALF were determined by ELISA.

(D) Representative H&E staining of lung sections (bars, 100 µm); inflammation was determined by semiquantitative scoring.

(E) Flow cytometric analysis of lung ILC2s (top) and their cytokine production (IL-13 and IL-5) (bottom).

(F) The proliferation of lung ILC2s was evaluated by Ki-67 staining.

In (E) and (F), both representative results (left) and mean ± SEM (right) are shown. Data are representative of three independent experiments (n = 6 mice per group). Error bars show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by unpaired Student's t test. Numbers within flow plots indicate the percentages of cells.

decreased levels of lung inflammation compared with those receiving WT ILC2s, which was represented as lower eosinophil influx (Figure 4B), lower levels of IL-13 and IL-5 in BALF (Figure 4C), and relieved lung histology (Figure 4D). These observations support that deletion of IRF7 impairs the function of ILC2s in response to IL-33.

We next investigated whether the regulation of ILC2s by IRF7 is cell intrinsic. Mixed BM chimeras were generated by mixing BM cells from WT (CD45.1⁺) and IRF7^{-/-} (CD45.2⁺) mice at a 1:1 ratio, followed by transfer into irradiated CD45.1/2⁺ recipients. Recipients were intranasally challenged

with IL-33 to induce lung inflammation after 6-week BM reconstitution (Figure 4E). Flow cytometric analysis showed that both the reconstitution of ILC2s in lung and their production of effector cytokines from IRF7^{-/-} donor cells were significantly lower than those of WT donor controls (Figures 4F and 4G). The reconstitution of ILC2s in BM failed to display any differences, and spleen CD3e⁺ cells equally derived from WT and IRF7^{-/-} donor cells were used as an internal control (Figures 4F and 4G). The reconstitution of ILC2 progenitors in BM, as well as the proliferation of ILC2P (ILC2 precursor), did not differ between WT and IRF7^{-/-} donor cells

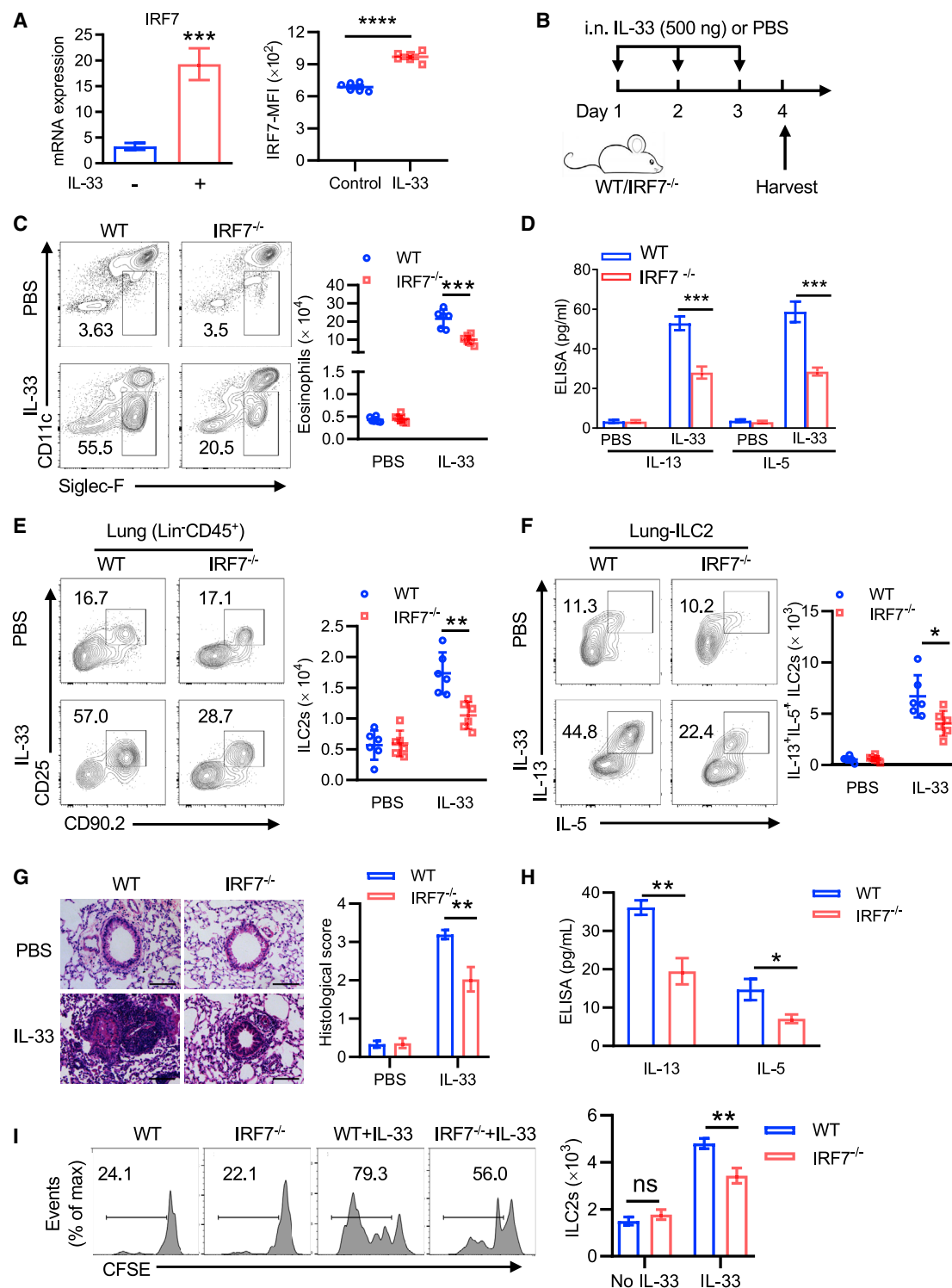


Figure 3. IRF7 Deficiency Impairs ILC2 Function in Response to IL-33 Challenge

(A) Naive lung ILC2s were cultured with IL-2 and IL-7 in the presence or absence of IL-33, and IRF7 expression was evaluated by qRT-PCR (left). The protein level of IRF7 in lung ILC2s from mice treated with IL-33 (intranasally [i.n.]) was determined by flow cytometry. Three independent experiments were performed. (B) Schematic diagram for intranasal challenge with IL-33 in IRF7^{-/-} and WT littermate mice (n = 6 per group). (C) Flow cytometric analysis of eosinophils in BALF. Both representative results (left) and mean $\pm$ SEM from all mice analyzed (right) are included. (D) The amounts of IL-13 and IL-5 in BALF were determined by ELISA. Mean $\pm$ SEM from 6 mice are shown.

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(Figures S3C and S3D), indicating that the lower reconstitution of lung ILC2s from IRF7^{-/-} donor cells was not caused by the developmental defect of their progenitors. These observations indicate that regulation of ILC2s by IRF7 is cell intrinsic.

In addition to IL-33, IL-25 is also a strong inducer of ILC2s (Huang et al., 2015). ILC2s induced by IL-25 are termed “inflammatory” ILC2s (iILC2s) that are generated under inflammation or infections (Huang and Paul, 2016; Saunders et al., 2014). When WT and IRF7^{-/-} mice were intraperitoneally (i.p.) injected with recombinant IL-25 for 3 days, the absolute numbers of iILC2s (Lin⁻ST2⁺KLRG1^{hi}) in lung were significantly decreased in IRF7^{-/-} mice when compared with WT control (Figure S4A). However, no statistically differences were observed in their proliferation and effector cytokine production (Figures S4B and S4C). These observations indicate that deletion of IRF7 may not affect the function of iILC2s induced by IL-25.

To investigate whether IRF7 affects other ILC subsets, including ILC1 and ILC3, a *Citrobacter rodentium* (CR)-induced murine colitis model was used. Consistent with a previous report (Yu, 2009), IRF7^{-/-} mice displayed disease severity comparable to WT controls, as indicated by body weight loss and shortened colon length (Figures S4D and S4E). Flow cytometric analysis showed that the levels of ILC1s in colon and their production of IFN- γ (Figure S4F), as well as the levels of ILC3s in colon and their production of IL-17A, IL-22, and IFN- γ cytokines (Figure S4G), displayed no noticeable differences between WT and IRF7^{-/-} mice. These observations indicate that IRF7 deficiency may not affect the function of other ILC subsets.

Regulation of ILC2s by IRF7 Is Independent of CD4⁺ T Cells

Both Th2 cells and ILC2s are major drivers of type 2 airway inflammation, and cross-regulation between Th2 cells and ILC2s has been reported (Halim et al., 2014; Mirchandani et al., 2014). To exclude the potential effect of CD4⁺ T cells on the regulation of ILC2s by IRF7, IRF7^{-/-} mice were cross-bred with Rag1^{-/-} mice (which lack mature T cells and B cells) to generate IRF7^{-/-}Rag1^{-/-} double-knockout (DKO) mice. Papain or PBS vehicle was administered intranasally to DKO and Rag1^{-/-} control mice (Figure 5A). As expected, papain administration caused clear lung inflammation in both Rag1^{-/-} and DKO mice, including enhanced infiltration of eosinophils (Figure 5B) and increased type 2 cytokine production (Figure 5C) in BALF; however, these effects were significantly relieved in DKO mice compared to Rag1^{-/-} controls (Figures 5B and 5C). The remission of ILC2-driven inflammation was further evidenced by H&E staining of lung tissues (Figure 5D). As expected, the numbers of ILC2s in lung (Figure 5E), and their secretion of

IL-13 and IL-5 effector cytokines (Figure 5F), were significantly lower in DKO mice upon papain administration. These observations indicate that the regulation of ILC2s by IRF7 is independent of CD4⁺ T cells.

Bcl11b Mediates the Regulatory Effect of IRF7 on ILC2s

IRF7 plays a critical role in the transcriptional regulation of type I IFN. Recent studies have shown that type I IFN negatively regulates the activation of ILC2s (Duerr et al., 2016; Moro et al., 2016). To investigate whether IFN participates in the regulation of ILC2s by IRF7, IFN- β production in the lungs was measured in different mouse models of airway inflammation. In contrast to clear induction of IFN- β production in the lungs upon influenza infection, both papain and IL-33 stimulation failed to display any noticeable effects on IFN- β production (Figure S5A). Furthermore, mice deficient in IFNAR1 (interferon alpha and beta receptor 1) (Ifnar1^{-/-}) showed similar levels of airway inflammation as WT controls in the papain model, including the infiltration of eosinophils (Figure S5B) and amount of type 2 cytokines (Figure S5C) in BALF. Importantly, no noticeable differences were observed in the frequencies of ILC2s and their production of effector cytokines between Ifnar1^{-/-} mice and WT controls (Figure S5D). In addition, IRF7 expression in lung was comparable between Ifnar1^{-/-} mice and WT controls both *in vivo* and *in vitro* (Figures S5E and S5F). IFNAR1 deficiency did not affect the induction of IRF7 induced by IL-33 stimulation (Figure S5F). These results indicate that type I IFN was not involved in papain or IL-33-induced airway inflammation.

IRF7^{-/-} and WT mice were further infected with influenza A virus, in which IFN is known to be critically important. As expected, IRF7^{-/-} mice were more vulnerable than WT mice, as evidenced by more severe loss of body weight (Figure S5G) and higher influx of eosinophils (Figure S5H). As expected, IFN- β production in IRF7^{-/-} BALF was less than 30% of that measured in WT controls (Figure S5I). In contrast to the higher inflammatory response and lower levels of IFN- β , both lung ILC2s and their production of effector cytokines were significantly decreased in IRF7^{-/-} mice compared to WT mice (Figure S5J). Considering the well-known negative effect of type I IFN on ILC2s (Califano et al., 2018; Duerr et al., 2016; Moro et al., 2016), these observations further exclude the possibility of type I IFNs in IRF7-mediated ILC2 function.

MyD88 is a signaling adaptor molecule that participates in IFN regulatory factor (IRF) 7 transcription factor-mediated type I IFN signaling in plasmacytoid dendritic cells (Yu et al., 2016). It was reported that deficiency of MyD88 could impair the expansion and proliferation of ILC2 in multiple tissues upon IL-33 challenge (Li et al., 2018; Molofsky et al., 2015).

(E and F) Flow cytometric analysis of lung ILC2s (E) and their cytokine production (F). Both representative results (left) and mean $\pm$ SEM (right) are included.

(G) Representative H&E staining of lung sections (bars, 100 μ m); inflammation was determined by semiquantitative scoring.

(H) Purified lung ILC2s were cultured in medium containing IL-2, IL-7, and IL-33 for 3 days. The amount of IL-13 and IL-5 in the supernatants was determined by ELISA. Mean $\pm$ SEM from four independent experiments are shown.

(I) Lung ILC2s sorted from WT or IRF7^{-/-} mice were labeled with 4 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE), followed cultured in the presence of IL-2 (10 ng/mL) and IL-7 (20 ng/mL), with or without IL-33 (100 ng/mL), in 96-well round plates (10,000 cells/well) for 72 h. The numbers of ILC2s were counted, and the proliferation of ILC2s was analyzed by flow cytometry after culture. Both representative results and mean $\pm$ SEM from three independent experiments are shown. Data are representative of three independent experiments. Error bars show mean $\pm$ SEM.

*p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001 by unpaired Student's t test. ns, not significant. Numbers within flow plots indicate the percentages of cells.

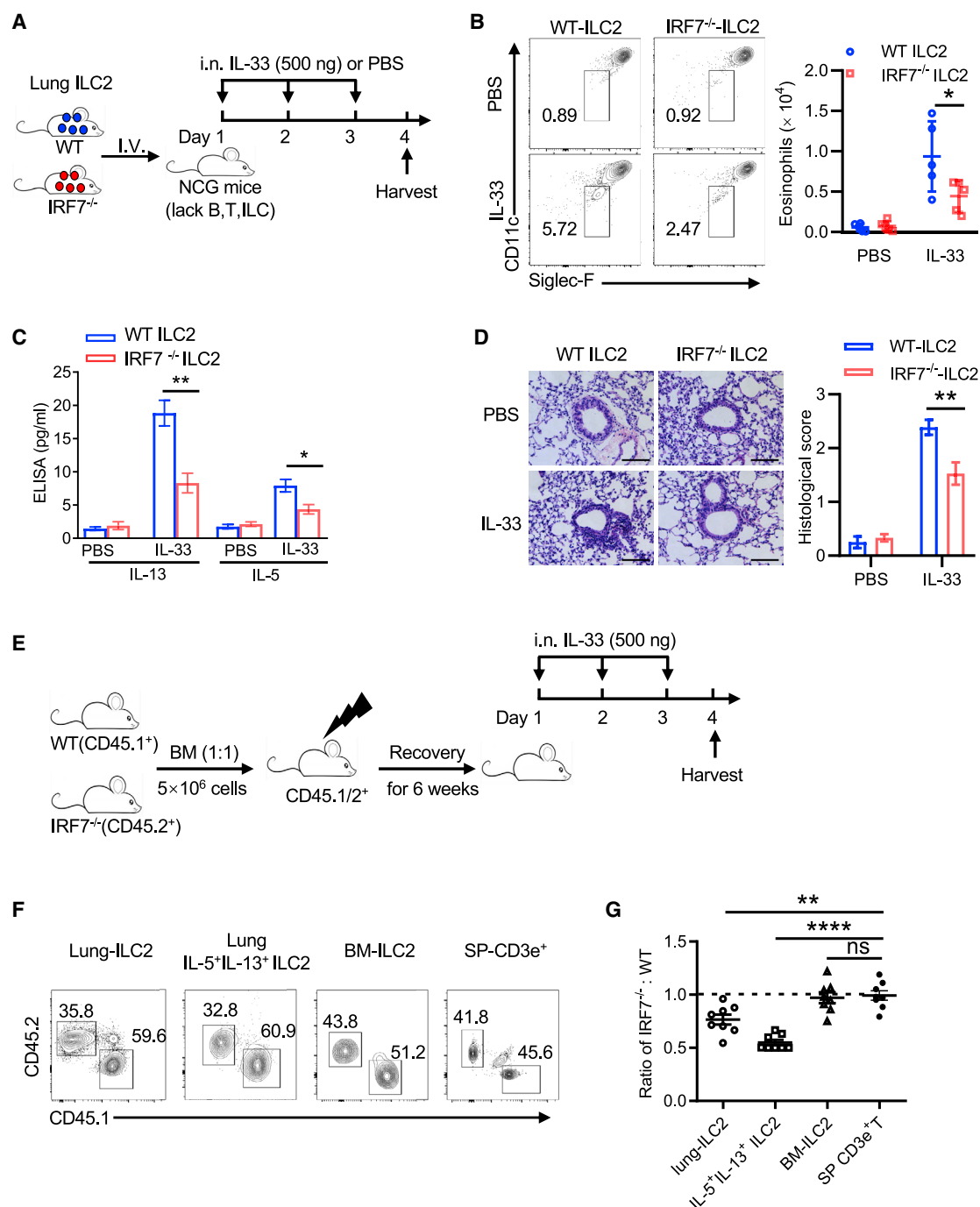


Figure 4. IRF7 Deficiency Impairs ILC2 Function in Response to IL-33 Challenge

(A) Schematic diagram for ILC2 transfer into NCG mice. An equal number of lung ILC2s (1.5×10^4) from WT or IRF7^{-/-} mice were intravenously injected into NCG mice, followed by intranasal challenge with IL-33 or PBS for 3 days ($n = 5$ per group). Mice were sacrificed on day 4.

(B) Flow cytometric analysis of eosinophils in BALF. Both representative results (left) and mean $\pm$ SEM (right) are included.

(C) The amount of IL-13 and IL-5 in BALF was determined by ELISA.

(D) Representative H&E staining of lung sections (bars, 100 μ m); inflammation was determined by semiquantitative scoring.

(E) Schematic diagram for mixed BM chimeras. BM cells from WT (CD45.1⁺) and IRF7^{-/-} (CD45.2⁺) mice were mixed at a 1:1 ratio, followed by transfer into CD45.1/2⁺ recipients. Mice were intranasally challenged with IL-33 after 6-week reconstitution.

(F) Flow cytometric analysis of ILC2s in lungs and BM and IL-13⁺IL-5⁺ ILC2 in lungs, derived from WT (CD45.1⁺) or IRF7^{-/-} (CD45.2⁺) donor cells; splenic CD3e⁺ T cells were used as an internal control.

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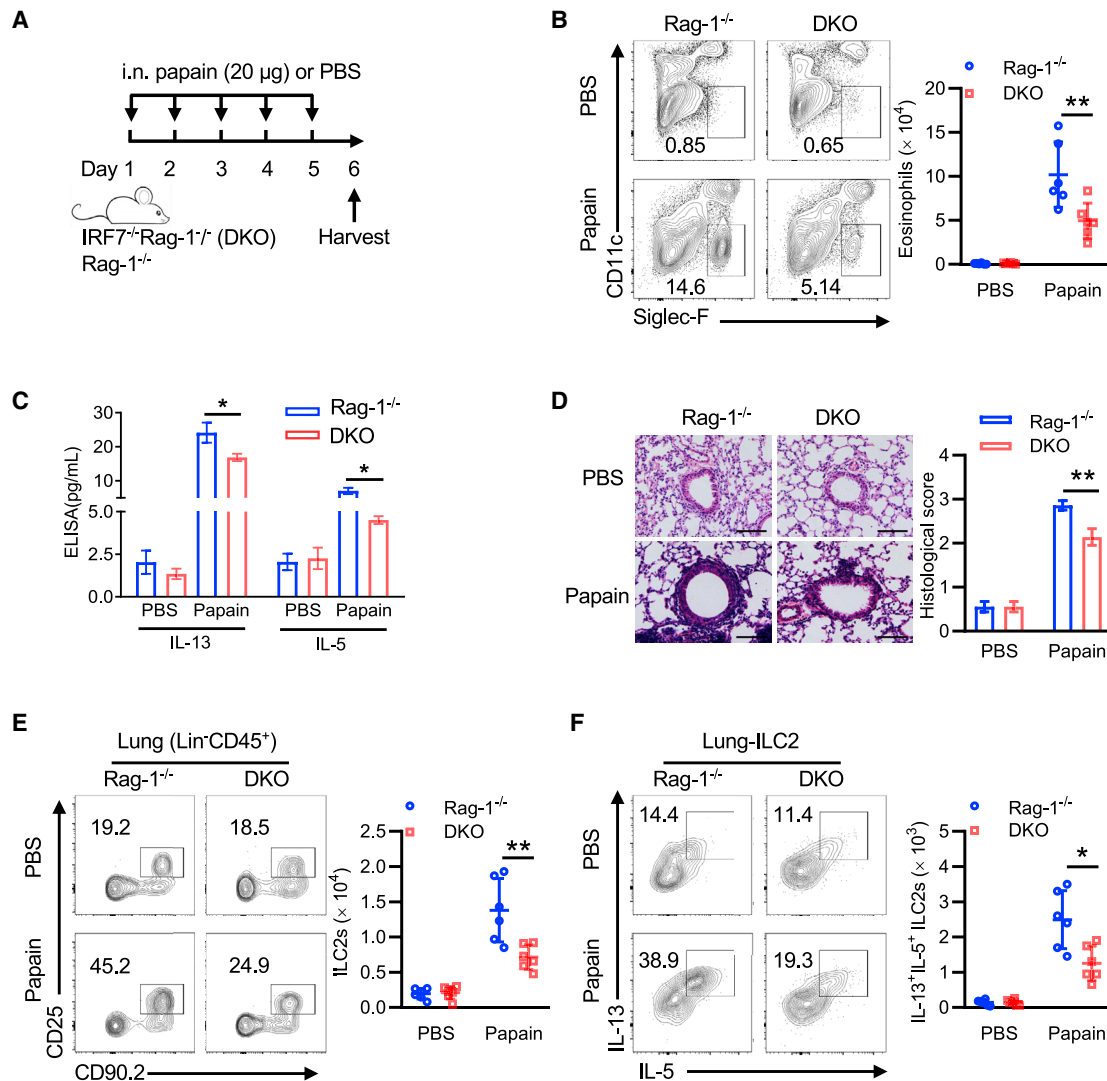


Figure 5. Regulation of ILC2s by IRF7 Is Independent of CD4⁺ T Cells

(A) IRF7^{-/-} Rag-1^{-/-} (DKO) and Rag-1^{-/-} mice were intranasally challenged with papain and sacrificed 24 h after the last challenge (n = 6 per group).

(B) Flow cytometric analysis of eosinophils in BALF. Both representative results (left) and mean ± SEM (right) are included.

(C) The amount of IL-13 and IL-5 in BALF was determined by ELISA.

(D) Representative H&E staining of lung sections (bars, 100 µm); inflammation was determined by semiquantitative scoring.

(E and F) Flow cytometric analysis of lung-ILC2s (E) and their cytokine production (F). Both representative results (left) and mean ± SEM from all mice analyzed (right) are included. Data are representative of three independent experiments. Error bars show mean ± SEM.

*p < 0.05; **p < 0.01 by unpaired Student's t test. Numbers within flow plots indicate the percentages of cells.

We therefore investigated the possibility that MyD88 participates in IL-33-induced IRF7 expression. It was found that MyD88 was significantly upregulated after IL-33 stimulation both *in vitro* and *in vivo* (Figures S6A and S6B). Pretreatment of ILC2s with the MyD88 inhibitor ST2825 significantly decreased IRF7 expression upon IL-33 stimulation at both the mRNA and protein levels (Figures S6C and S6D). IL-33-

induced type 2 cytokine expression was also abrogated by ST2825 (Figure S6C). These observations indicate that MyD88 may be upstream of IRF7 in ILC2s in response to IL-33, which deserves further investigation.

The expression of some transcription factors known to be important for ILC2s was next profiled by qRT-PCR. In order to obtain enough lung ILC2s, mice were challenged with IL-33 to

(G) The ratios of IRF7^{-/-}/WT donor cells among the indicated cell types (CD3e⁺ T cells were used as an internal control) are shown (mean ± SEM). Dashed line indicates predicted reconstitution ratio in the absence of genotype bias. Data are representative of three independent experiments (n = 8 per group). Error bars show mean ± SEM.

*p < 0.05; **p < 0.01; ****p < 0.0001 by unpaired Student's t test. ns, not significant. Numbers within flow plots indicate the percentages of cells.

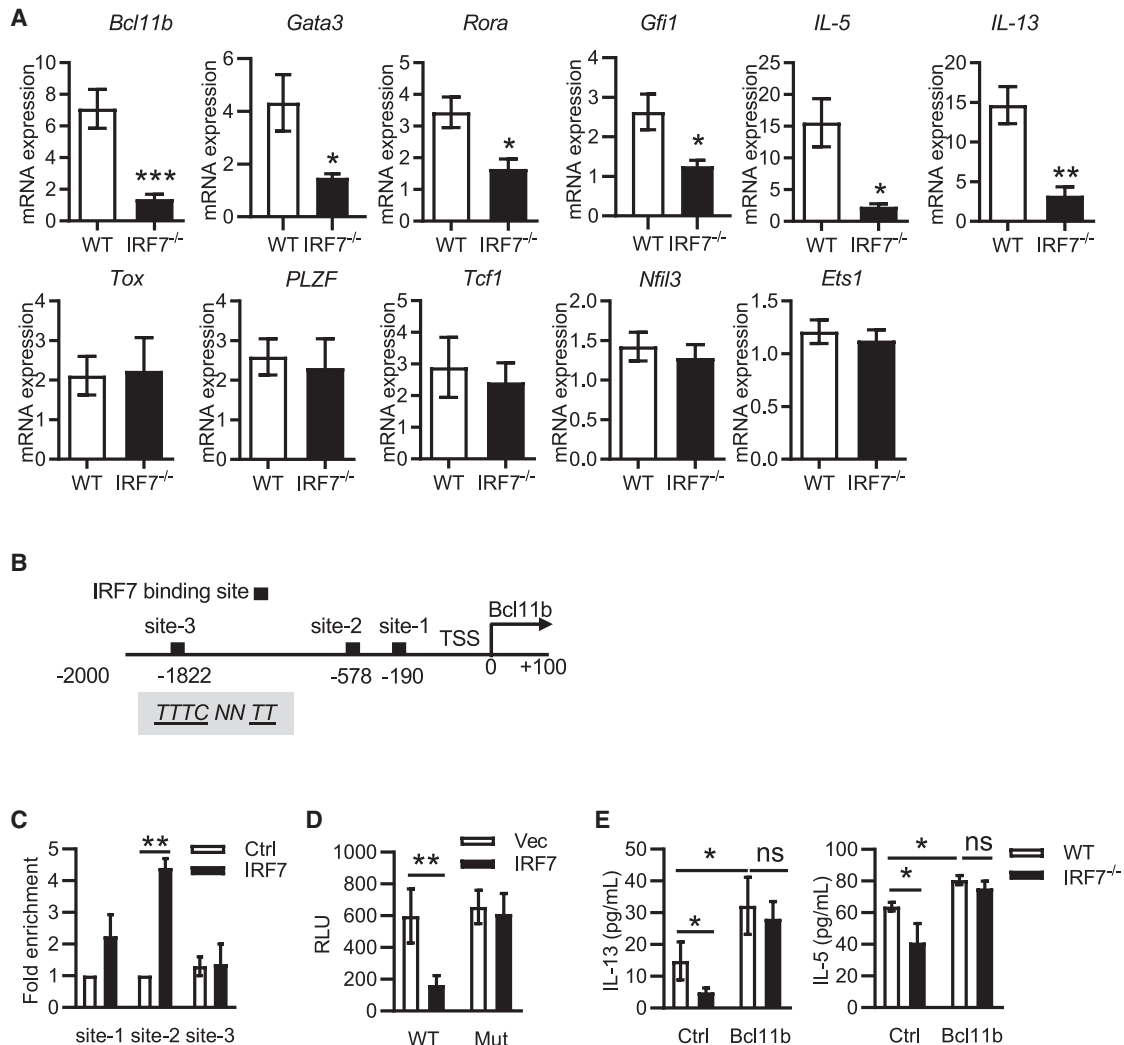


Figure 6. Bcl11b Mediates the Regulatory Effect of IRF7 on ILC2s

(A) Lung ILC2s were sorted from IL-33 i.p.-treated WT and IRF7^{-/-} mice by flow cytometry, and expression of the indicated genes was determined by qRT-PCR. Mean $\pm$ SEM from four independent experiments are shown.

(B) Sequence analysis of Bcl11b promoter region; three potential IRF7-binding sites are indicated: site 1 (approximately -190 to -198), site 2 (approximately -578 to -586), and site 3 (approximately -1,822 to -1,830).

(C) ChIP assay was performed on cultured lung lineage-negative cells using anti-IRF7 or anti-IgG antibodies, and the presence of Bcl11b promoter harboring the potential IRF7-binding sites was measured by qPCR. Data were normalized against input DNA and presented as fold increase over IgG control. Three independent experiments were performed.

(D) 293T cells were co-transfected with Bcl11b reporter (WT or mutant) and plasmid expressing IRF7 or empty vector, and luciferase activity was measured 48 h post-transfection. RLU, relative luciferase unit. Data are presented as mean $\pm$ SEM from four independent experiments.

(E) Lineage-negative cells from lungs of IRF7^{-/-} and WT mice were infected with retrovirus plasmid overexpressing Bcl11b or empty vector Migr-1 in media containing IL-2, IL-7, and IL-33 for 3 days. The amount of IL-13 and IL-5 in the supernatants was determined by ELISA. Data are presented as mean $\pm$ SEM from three independent experiments. Error bars show mean $\pm$ SEM.

*p < 0.05; **p < 0.01, ***p < 0.001 by unpaired Student's t test.

expand ILC2s before flow cytometric sorting. Results revealed a marked reduction in mRNA levels of Bcl11b, Gata3, Rora, and Gfi1 in IRF7^{-/-} ILC2s compared to those in WT controls (Figure 6A). The expression of other ILC2 regulators, including Tox, PLZF, Tcf1, Nfil3, and Ets1, was not noticeably different (Figure 6A). No differences were observed in the expression of genes related to ILC1 (Irfng and Tbx21) and ILC3 (Ccr6, Il23r, and Il17a)

between IRF7^{-/-} ILC2s and WT controls (Figures S6E and S6F). Rorc expression was increased in IRF7^{-/-} ILC2s compared with WT controls (Figure S6F). It has been reported that Bcl11b regulates the function of ILC2s via Gfi1 and Gata3 (Califano et al., 2015). Bcl11b expression was clearly induced by either papain (*in vivo*) or IL-33 stimulation (*in vitro*) (Figures S6G and S6H), and deficiency of IRF7 clearly reduced Bcl11b induction in

ILC2s upon IL-33 stimulation (Figure S6H). Further analysis of the regulatory region of Bcl11b revealed three potential IRF7-binding sites (at approximately –190 to –2,000 bp upstream of the transcriptional start site) (Figure 6B). A chromatin immunoprecipitation (ChIP) assay confirmed the binding of IRF7 protein to one of the loci (site 2: approximately –578 to –586) (Figure 6C). Ectopic overexpression of IRF7 enhanced the reporter activity of Bcl11b in the 293T cell line, and mutation of the IRF7-binding site abrogated this effect (Figure 6D). Furthermore, overexpression of Bcl11b by retroviral infection almost completely rescued the cytokine production in IRF7^{–/–} ILC2s to the levels of WT controls (Figures S6I and 6E). These observations indicate that Bcl11b mediates the effects of IRF7 on ILC2s.

DISCUSSION

ILC2s represent an important player in airway hyperreactivity and inflammation by producing type 2 effector cytokines in an antigen-independent manner (Ebbo et al., 2017). Mechanistic study about the regulation of ILC2 benefits the immunotherapy of asthma. Here, we report that IRF7 positively regulates the function of lung ILC2s, which is mediated by Bcl11b. The significance of ILC2 regulation by IRF7 was demonstrated in multiple murine models of airway inflammation.

A potential link between IRF7 and airway inflammation has been indicated in previous reports. Lewis et al. showed that IRF7 was upregulated in nasal aspirates from asthmatic children during natural colds (Lewis et al., 2012). Induced IRF7 expression was also observed in nasal epithelial scrapings from adults infected with HRV-16 (Proud et al., 2008). However, IRF7 deficiency in mice has no effect on house dust mite (HDM)-induced airway inflammation (Marichal et al., 2010). In this study, it was found that IRF7 was preferentially expressed in ILC2s. Upregulation of IRF7 expression in ILC2s was observed not only in murine airway inflammation models but also in patients with asthma, indicating a potential role of IRF7 in ILC2 biological function. In the absence of IRF7, both the levels and function of lung ILC2s were significantly diminished upon challenge with papain or *Alternaria alternata*. KLRG1, a marker specific for mature ILC2s (Huntington et al., 2007), was found to be downregulated in ILC2s lacking IRF7. This is consistent with the impaired capability of IRF7^{–/–} ILC2s in the production of effector cytokines and cell proliferation in response to IL-33 or papain than WT controls. Adoptive transfer of ILC2s into immunodeficient NCG mice further demonstrated that IRF7 is essential for the activation of lung ILC2s upon IL-33 administration. Mixed BM chimeras demonstrated that the regulation of lung ILC2s by IRF7 is cell intrinsic. These observations collectively reveal the importance of IRF7 in ILC2-driven innate immunity during airway inflammation.

Considering the critical role of IRF7 in the regulation of IFN- α/β production (Honda et al., 2005), it was initially considered that IFN signaling may be involved in ILC2 regulation by IRF7. In contrast to the influenza infection model, no noticeable changes were found in IFN- α/β production in either IL-33- or papain-induced airway inflammation. Furthermore, IFNAR1 deficiency displayed no effect on the levels of ILC2 and tissue inflammation in lung. These results indicated that IFN- α/β did not participate in ILC2-driven asthma in the papain or IL-33 models used in this

study. In the influenza infection model, mice with IRF7 deficiency exhibited a more severe disease phenotype, including body weight loss and enhanced influx of eosinophils in BALF, which is attributable to the impaired IFN- α/β production. We consistently observed decreased levels of ILC2s and defects in ILC2 function in IRF7-deficient mice in the influenza model. Considering that previous reports have demonstrated a negative regulatory effect of IFN- α/β on the responses of ILC2s (Duerr et al., 2016; Moro et al., 2016), these results exclude the possibility of IFN's participation in the IRF7-mediated ILC2 response.

Although expression of IRF7 was clearly induced in ILC2s in either the papain model or *in vitro* IL-33 stimulation, the mechanism of IRF7 induction remains to be investigated. IRF7 expression in ILC2s was comparable between IFNAR1-deficient mice and WT controls upon stimulations, indicating that it is IFN independent. MyD88 can regulate IRF7-mediated type I IFN signaling in plasmacytoid dendritic cells (Yu et al., 2016), and it is a shared adaptor for Toll-like receptor (TLR) and IL-1 family signaling and is required for IL-33 signaling (Molofsky et al., 2015). It was also reported that MyD88 could interact with IRF7 to form a complex in the cytoplasm, which provides the foundation for the TLR9-dependent activation of IFN genes (Honda et al., 2004). MyD88 also participates in IL-33-induced ILC2 responses (Li et al., 2018; Molofsky et al., 2015). We showed that MyD88 was significantly upregulated after IL-33 stimulation, and the MyD88 inhibitor ST2825 abrogated the induction of IRF7 caused by IL-33. These observations indicate that MyD88 may be upstream of IRF7 in ILC2s, which deserves further investigation. Meanwhile, although Bcl11b was identified to be regulated by IRF7 in ILC2s during allergic inflammation, other downstream target genes of IRF7 in ILC2s still warrant further investigation. Gene expression profiling of ILC2s with a deficiency or sufficiency of IRF7 would provide detailed information for the downstream programming of IRF7 in ILC2s.

The exact role of ILC2s in influenza-induced lung inflammation may be more complicated than that in other experimental airway inflammation models. In addition to their pro-inflammatory effect, a beneficial role of ILC2s in enhancing tissue repair after acute lung injuries such as influenza infection has also been reported (Monticelli et al., 2011). Depletion of ILCs caused defects in lung function and delayed airway remodeling, which could be restored by the administration of ILC2-derived amphiregulin (Areg) (Monticelli et al., 2011). Interestingly, it was found that mice with IRF7 deficiency showed lower levels of Areg expression upon influenza infection than control mice (data not shown), which may contribute to the more severe disease observed in IRF7-deficient mice.

It is well established that Th2 cells and ILC2s share a highly similar regulation mechanism, and cross-regulation between Th2 cells and ILC2s has been reported (Halim et al., 2014; Mirchandani et al., 2014). Marichal et al. showed that IRF7 is dispensable in HDM-induced airway inflammation, in which Th2 responses play a dominant role (Mirchandani et al., 2014). Consistent observations of ILC2 defects in Rag1^{–/–}IRF7^{–/–} mice excluded the effect of Th2 cells on IRF7-mediated ILC2 responses. Adoptive transfer of ILC2s into immunodeficient NCG mice also support that the regulation of ILC2s by IRF7 is cell intrinsic. These observations indicate the effect of IRF7 on ILC2s is independent of other immune cells.

In conclusion, our study demonstrates that IRF7 is required for the function of lung ILC2s and may have therapeutic value in airway inflammatory disease.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.10.077>.

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AUTHOR CONTRIBUTIONS

J.H., Qiong Yang, and Q.X. performed experiments, analyzed data, and participated in figure organization and manuscript writing; A.L. and T.L. performed the mixed BM chimera; X.L. and K.S. assisted with the experiments involving human asthma samples; P.Z., L.Z., and Quan Yang participated in mouse strain breeding and flow cytometry; J.D. provided suggestions for experimental design; and J.Z. designed the overall study, supervised experiments, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45R/B220 (clone RA3-6B2) Biotin	Thermo Fisher Scientific	Cat# 13-0452-86; RRID: AB_466451
Anti-mouse CD3e (clone 17A2) Biotin	Thermo Fisher Scientific	Cat# 13-0032-82; RRID: AB_2572762
Anti-mouse Ly-6G (clone RB6-8C5) Biotin	Thermo Fisher Scientific	Cat# 13-5931-82; RRID: AB_466800
Anti-mouse TER-119 (clone TER-119) Biotin	Thermo Fisher Scientific	Cat# 13-5921-82; RRID: AB_466797
Anti-mouse CD11b (clone M1/70) Biotin	Thermo Fisher Scientific	Cat# 13-0112-82; RRID: AB_466359
Anti-mouse TCR β (clone H57-597) Biotin	BioLegend	Cat# 109203; RRID: AB_313426
Anti-mouse TCR γ/δ (clone GL3) Biotin	BioLegend	Cat# 118103; RRID: AB_313827
Anti-mouse CD4 (clone GK1.5) Biotin	BioLegend	Cat# 100403; RRID: AB_312688
Anti-mouse CD11c (clone N418) Biotin	BioLegend	Cat# 117303; RRID: AB_313772
Anti-mouse CD5 (clone 53-7.3) Biotin	BioLegend	Cat# 100603; RRID: AB_312732
Anti-mouse CD8a (clone 53-6.7) Biotin	BioLegend	Cat# 100703; RRID: AB_312742
Anti-mouse NK1.1 (clone PK136) Biotin	BioLegend	Cat# 108703; RRID: AB_313390
Anti-mouse Siglec F (clone 1RNM44N) PE	Thermo Fisher Scientific	Cat# 12-1702-82; RRID: AB_2637129
Anti-mouse Siglec F (clone 1RNM44N) PerCP-eFluor 710	Thermo Fisher Scientific	Cat# 46-1702-82; RRID: AB_2573723
Anti-mouse CD11c (clone N418) FITC	Thermo Fisher Scientific	Cat# 11-0114-82; RRID: AB_464940
Anti-mouse CD45 (clone 30-F11) APC-eFluor 780	Thermo Fisher Scientific	Cat# 47-0451-82; RRID: AB_1548781
Anti-mouse CD25 (clone PC61.5) PE-Cyanine7	Thermo Fisher Scientific	Cat# 25-0251-82; RRID: AB_469608
Anti-mouse CD25 (clone PC61.5) Alexa Fluor 488	Thermo Fisher Scientific	Cat# 53-0251-82; RRID: AB_763472
Anti-mouse CD90.2 (clone 53-2.1) PE-Cyanine7	Thermo Fisher Scientific	Cat# 25-0902-82; RRID: AB_469642
Anti-mouse CD90.2 (clone 53-2.1) eFluor450	Thermo Fisher Scientific	Cat# 48-0902-82; RRID: AB_1272200
Anti-mouse CD127 (clone A7R34) Brilliant Violet 421	BioLegend	Cat# 135027; RRID: AB_2563103
Anti-mouse GATA3 (clone TWAJ) Percp-eFluor 710	Thermo Fisher Scientific	Cat# 46-9966-42; RRID: AB_10804487
Anti-mouse ROR γ t (clone B2D) PE	Thermo Fisher Scientific	Cat# 12-6981-82; RRID: AB_10807092
Anti-mouse ROR γ t (clone B2D) APC	Thermo Fisher Scientific	Cat# 17-6981-82; RRID: AB_2573254
Anti-mouse IL33R/ST2 (clone DIH9) PE	BioLegend	Cat# 145304; RRID: AB_2561915
Anti-mouse IL33R/ST2 (clone DIH9) APC	BioLegend	Cat# 145306; RRID: AB_2561917
Anti-mouse Ki-67 (clone SolA15) FITC	Thermo Fisher Scientific	Cat# 11-5698-82; RRID: AB_11151330
Anti-mouse CD3e (clone eBio500A2) PE	Thermo Fisher Scientific	Cat# 12-0033-82; RRID: AB_842786
Anti-mouse CD45R/B220 (clone RA3-6B2) PE-Cyanine7	Thermo Fisher Scientific	Cat# 25-0452-82; RRID: AB_469627
Anti-mouse CD45.1 (clone A20) Brilliant Violet 421	BioLegend	Cat# 110732; RRID: AB_2562563
Anti-mouse CD45.2 (clone 104) Alexa Fluor 700	BioLegend	Cat# 109822; RRID: AB_493731
Anti-mouse IL-5 (clone TRFK5) APC	BioLegend	Cat# 504306; RRID: AB_315330
Anti-mouse IL-13 (eBio13A) PE	Thermo Fisher Scientific	Cat# 12-7133-82; RRID: AB_763559
Anti-mouse IRF7 (clone MNGPKL) PE	Thermo Fisher Scientific	Cat# 12-5829-82; RRID: AB_2572629
Anti-mouse KLRG1 (clone 2F1) PE	Thermo Fisher Scientific	Cat# 12-5893-82; RRID: AB_10596642
Anti-mouse KLRG1 (clone 2F1) PerCP-eFluor 710	Thermo Fisher Scientific	Cat# 46-5893-82; RRID: AB_10670282
Anti-mouse NKP46 (clone 29A1.4) FITC	BioLegend	Cat# 137606; RRID: AB_2298210
Anti-mouse NKP46 (clone 29A1.4) Brilliant Violet 605	BioLegend	Cat# 137619; RRID: AB_2562452
Anti-mouse NK1.1 (clone PK136) PerCP-Cyanine5.5	Thermo Fisher Scientific	Cat# 45-5941-82; RRID: AB_914361
Anti-mouse IL-17A (clone TC11-18H10.1) PE	BioLegend	Cat# 506904; RRID: AB_315464
Anti-mouse IL-17A (clone TC11-18H10.1) APC	BioLegend	Cat# 506916; RRID: AB_536018
Anti-mouse IL-22 (clone 1H8PWSR) Percp-eFluor 710	Thermo Fisher Scientific	Cat# 46-7221-82; RRID: AB_10598646
Anti-mouse IFN γ (clone XMG1.2) FITC	BioLegend	Cat# 505806; RRID: AB_315400

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-mouse Flt3 (clone A2F10) APC	Thermo Fisher Scientific	Cat# 17-1351-82; RRID: AB_10717261
Anti-mouse $\alpha 4\beta 7$ (clone DATK32) PE	Thermo Fisher Scientific	Cat# 12-5887-82; RRID: AB_657803
Anti-mouse Sca1 (clone D7) APC	Thermo Fisher Scientific	Cat# 17-5981-82; RRID: AB_469487
Anti-mouse Sca1 (clone D7) PerCP-Cyanine5.5	Thermo Fisher Scientific	Cat# 45-5981-82; RRID: AB_914372
MyD88 Monoclonal Antibody (clone 4D6)	Thermo Fisher Scientific	Cat# MA5-16231; RRID: AB_2537749
Human Hematopoietic Lineage Antibody Cocktail (APC)	Thermo Fisher Scientific	Cat# 22-7776-72; RRID: AB_2043865
TCR $\alpha\beta$ (clone IP26) Biotin	BioLegend	Cat# 306704; RRID: AB_314632
TCR $\gamma\delta$ (clone B1) Biotin	BioLegend	Cat# 331206; RRID: AB_1089216
CD11c (clone 3.9) Biotin	BioLegend	Cat# 301612; RRID: AB_493021
FceR1 alpha (clone AER-37(CRA1)) Biotin	Thermo Fisher Scientific	Cat# 13-5899-82; RRID: AB_466786
Anti-human CD45 (clone HI30) APC-eFluor 780	Thermo Fisher Scientific	Cat# 47-0459-42; RRID: AB_1944368
Anti-human CD127 (clone EBioRDR5) eFluor 450	Thermo Fisher Scientific	Cat# 48-1278-42; RRID: AB_1963566
Anti-human CRTH2 (clone BM16) PE	Thermo Fisher Scientific	Cat# 12-2949-42; RRID: AB_2572601
Anti-human CD161 (clone HP-3G10) PE-Cyanine7	Thermo Fisher Scientific	Cat# 25-1619-42; RRID: AB_10807086
Anti-human IRF7 (clone 12G9A36) Alexa Fluor 488	BioLegend	Cat# 656005; RRID: AB_2563528
Bacterial and Virus Strains		
Citrobacter rodentium strain DBS100	ATCC	Stock# 51459
Influenza A/PR/8/34 (H1N1)	Charles River Laboratories	Material # 10100374
Chemicals, Peptides, and Recombinant Proteins		
Brefeldin A	Thermo Fisher Scientific	Cat# 00-4506-51
Ionomycin calcium salt	Sigma Aldrich	Cat# I0634-1MG
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich	Cat# P8139-1MG
Recombinant Mouse IL-33 (carrier-free)	BioLegend	Cat# 580506
Recombinant Mouse IL-2 (carrier-free)	BioLegend	Cat# 575404
Recombinant Mouse IL-7 (carrier-free)	BioLegend	Cat# 577804
Recombinant Mouse IL-25 (carrier-free)	BioLegend	Cat# 587304
ST2825	MedChemExpress	Cat# HY-50937
Papain	Sigma Aldrich	Cat# P3125
Alternaria alternata	Greerlabs	Cat# My1
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific	Cat# 11668019
PEG 6000	MERCK Millipore	Cat# 528877
MacConkey Agar	Thermo Fisher Scientific	Cat# CM0007B
Critical Commercial Assays		
LIVE/DEAD Fixable Green Dead Cell Stain Kit, for 488 nm excitation	Thermo Fisher Scientific	Cat# L23101
Fixable Viability Dye eFluor 450	Thermo Fisher Scientific	Cat# 65-0863-14
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	Thermo Fisher Scientific	Cat# L34965
Streptavidin PE-Cyanine7 Conjugate	Thermo Fisher Scientific	Cat# 25-4317-82
Streptavidin APC Conjugate	Thermo Fisher Scientific	Cat# 17-4317-82
Streptavidin FITC Conjugate	Thermo Fisher Scientific	Cat# 11-4317-87
CellTrace CFSE Cell Proliferation Kit	Thermo Fisher Scientific	Cat# C34554
IL-13 Mouse Uncoated ELISA Kit with Plates	Thermo Fisher Scientific	Cat# 88-7137-22
IL-5 Mouse Uncoated ELISA Kit with Plates	Thermo Fisher Scientific	Cat# 88-7054-22
Mouse IFN-beta ELISA Kit	R&D Systems	Cat# 42400-2
Experimental Models: Organisms/Strains		
Mouse: IRF7 KO	The RIKEN BioResource Centre	Cat# RBRC01420
Mouse: IFNAR1 KO	The Jackson Laboratory	Cat# 32045-JAX
Mouse: Rag1 KO	The Jackson Laboratory	Cat# 002216

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: NCG (NOD-Prkdc ^{em26Cd52} Il2rg ^{em26Cd22} /Nju)	Model Animal Research Center of Nanjing University	Cat# T001475
Mouse: C57BL/6J	Guangdong Medical Laboratory Animal Center	Cat# GDMLAC08
Oligonucleotides		
Bcl11b reporter primer (WT)-Forward: AAAGGTACC CCGGTACCGGCGAGTGTGTGTGAG	This paper	N/A
Bcl11b reporter primer (WT)-Reverse: AAA CTCGAGGG CTCGAGAGCCTCCACATGGTCA	This paper	N/A
Bcl11b reporter primer (Mutant)-Forward: AATGTTAAAAAGCCCCGCTAGTCGCC	This paper	N/A
Bcl11b reporter primer (Mutant)-Reverse: CCGCTGATCGCCCCGAAAAATTGTAA	This paper	N/A
Bcl11b coding sequence primer- Forward: CTAGCTAGCATGTCCCGCCGAAACAG	This paper	N/A
Bcl11b coding sequence primer- Reverse: GGGACCGGTTTAGCTCCTCTCAGCCTG	This paper	N/A
Primers for gene expression, see Table S2	This paper	N/A
Recombinant DNA		
Plasmid: Bcl11b-MIGR1	This paper	N/A
Software and Algorithms		
FlowJo V10.4 software	FlowJo, LLC	https://www.flowjo.com/solutions/flowjo
GraphPad Prism 8	GraphPad	https://www.graphpad.com/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jie Zhou (zhoujie@tmu.edu.cn).

All unique and stable reagents generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

IRF7^{-/-} mice were originally obtained from the RIKEN BioResource Centre (Ibaraki, Japan), maintained on a C57BL/6J background. Ifnar1^{-/-} mice and Rag1^{-/-} mice were originally obtained from The Jackson Laboratory (Bar Harbor, USA), maintained on a C57BL/6J background. NCG (NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju) mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). IRF7^{-/-}Rag1^{-/-} mice were generated by crossing IRF7^{-/-} mice with Rag1^{-/-} mice. C57BL/6J mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). C57BL/6J-Ly5.1 (CD45.1) mice were kindly provided by Dr. Haikun Wang from Institute Pasteur of Shanghai, Chinese Academy of Sciences (Shanghai, China). CD45.1/2⁺ mice were obtained by cross breeding of C57BL/6J-Ly5.1 (CD45.1⁺) and C57BL/6J mice (CD45.2⁺). All mice were maintained in specific pathogen-free condition room in the animal facility at the Sun Yat-sen University and 8 to 10 weeks old female mice were used for experiments. Littermates of the same sex were randomly assigned to experimental groups. Mouse experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Human samples

Human subjects, including asthma patients (aged 6–11 years, n = 10) and healthy controls (aged 6–11 years, n = 10), were recruited by Guangzhou Women and Children's Medical Center (Guangzhou, China), and written informed consent was obtained from all participants or their legal guardians. Inclusion criteria for allergic asthma was performed by physician-diagnosed asthma and The Global Initiative for Asthma (GINA) Program. The characteristics of asthma patients were summarized in Table S1. The peripheral blood from all participants was collected and the levels of ILC2 were analyzed by flow cytometry. The protocol was approved by Human Ethics Committees of Guangzhou Women and Children's Medical Center.

Microbe strains

Citrobacter rodentium strain DBS100 (ATCC 51459; American Type Culture Collection) was cultured in LB broth (Sigma-Aldrich) for 16 h and bacterial concentration was determined by measuring the optical density at 600 nm (OD600). 100 μ L bacteria (OD600 = 0.35 - 0.5) was plated on MacConkey agar plates (Thermo Fisher Scientific) and then *C. rodentium* colonies were identified based on morphology (characteristic pink center surrounded by a white rim) after 24 h of incubation at 37°C.

Primary cell cultures

Lung ILC2s from female IRF7^{-/-} or WT control mice were sorted and cultured in 200 μ L of RPMI-1640 media in the presence of IL-2 (10 ng/mL, BioLegend), IL-7 (20 ng/mL, BioLegend) and IL-33 (100 ng/mL, BioLegend) in 96-well round plates with a density of 10,000 cells per well. Cells were cultured in a 37°C incubator with 5% CO₂. After 3 days the cultured supernatants were collected and the amounts of IL-13 and IL-5 were measured by ELISA (Thermo Fisher Scientific) (Verhoef et al., 2016).

METHOD DETAILS

Lung inflammation models

For papain-induced allergic inflammation, mice were challenged intranasally with papain for 5 days (20 μ g in 40 μ L PBS, daily) (Sigma-Aldrich, from papaya latex), PBS was used as vehicle control (Monticelli et al., 2016). For *Alternaria alternata*-induced allergic inflammation, mice were administered intranasally with 100 μ g *Alternaria alternata* (Greerlabs, Lenoir, NC) in 40 μ L PBS for 4 consecutive days (Maazi et al., 2015). For IL-33-induced allergic inflammation, mice were intranasally administered with 500 ng recombinant IL-33 in 40 μ L PBS (500 ng/mouse, BioLegend, CA) for 3 consecutive days (Maazi et al., 2015). Mice were sacrificed 24 hours after the last challenge, lung and BALF were collected for analysis.

Lung histology

Lung tissues were fixed in 4% phosphate-buffered formaldehyde solution for 24 h. Tissue sections were prepared and stained with hematoxylin-eosin (H&E) to evaluate inflammation. Inflammation was determined by semiquantitative scoring (0–4: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe). An increment of 0.5 was used when the inflammation fell between two levels. Three fields were selected randomly for scoring using a Leica microscope by two treatment-blind pathologists independently (Dubin and Kolls, 2007; McKay et al., 2004).

Mixed bone marrow chimera

To generate mixed bone marrow chimeras, we followed the procedure described earlier (Shi et al., 2014). Briefly, a total of 5×10^6 bone marrow cells from IRF7^{-/-} mice (CD45.2⁺) and congenic WT mice (CD45.1⁺) were mixed at a ratio of 1:1 in 200 μ L sterilized 1 $\times$ PBS, followed by intravenously transfer into lethally irradiated (9 Grays in 2 bursts: 4.5-Gy/dose administered 3 hours apart) recipient mice (CD45.1/2⁺). After 6-week reconstitution, mice were intranasally administered with IL-33 for 3 days, and sacrificed at day 4 for analysis.

ILC2 adoptive transfer into NCG mice

IRF7^{-/-} and WT mice were i.p. injected with 500 ng recombinant murine IL-33 for 3 days. Lung ILC2s were sorted at day 4 by flow cytometry. After 2 days rest with RPMI 1640 complete medium in the presence of IL-2 (10 ng/ml), 1.5×10^4 ILC2s were adoptively transferred into NCG recipient mice, followed by intranasal administration with IL-33 for 3 days (500 ng/per mouse). Mice were sacrificed 24 hours after the last challenge, BALF and lung samples were harvested for analysis.

Flow cytometric analysis and sorting

Single-cell suspensions were prepared and were stained with fluorochrome-conjugated antibodies. Data were collected on a BD LSRFortessa™ flow cytometer (BD Biosciences) and analyzed with FlowJo V10.4 software (FlowJo, OR, USA). Data were acquired as the fraction of labeled cells within a live-cell gate set for at least 100,000 events for mouse or at least 300,000 events for human samples. For flow cytometric sorting, cells were stained with specific antibodies and isolated on a BD FACSAriaIII cell sorter (BD Bioscience). The antibodies used for flow cytometry were listed in Key Resources Table under STAR Methods.

The following antibodies were used to identify mouse lung ILC2s: Lineage⁻ (CD3e, CD45R/B220, CD11b, TER-119, Ly-6G, CD11c, NK1.1, CD4, CD5, CD8a, TCR β and TCR $\gamma\delta$), and positive for CD45, CD90.2, CD25, GATA3 and ST2. Eosinophils were identified as CD45⁺Siglec-F⁺CD11c⁻. For isolation of lung ILC2s, lung leukocytes were stained with biotinylated anti-mouse lineage, bound to streptavidin particles (BD Biosciences). The enrichment of Lineage⁻ fraction was then purified by CD45⁺CD90.2⁺CD25⁺ST2⁺. For human ILC2s, PBMCs were labeled with Lineage⁻ (CD2, CD3, CD14, CD16, CD19, CD56, CD235a, TCR $\alpha\beta$, TCR $\gamma\delta$, FCeR1 α , CD94, and CD11C), and positive for CD45, CD127, CRTH2 and CD161. For intracellular cytokine staining, lung leukocytes were stimulated with PMA (50 ng/mL, Sigma-Aldrich), Ionomycin (1 μ g/mL, Sigma) and Brefeldin A (1 μ g/mL, Thermo Fisher Scientific) for 4 hours at 37°C, then stained for ILC2 markers, followed by IC Fixation Buffer (Thermo Fisher Scientific) and stained with anti-IL-13 and anti-IL-5 antibodies.

In vivo amplification and in vitro treatment of ILC2s

For *in vivo* amplification of ILC2s, mice were intraperitoneally (i.p.) injected with recombinant IL-33 (500ng per mouse per day in 200 μ L PBS) daily for 4 days. Then lung ILC2s were sorted by flow cytometry.

For MyD88 inhibitor-ST2825 (MedChemExpress, USA) treatment, sorted lung ILC2s were cultured with or without ST2825 (20 μ M) (Li et al., 2018) in the presence of IL-2 (10 ng/mL, BioLegend) and IL-7 (20 ng/mL, BioLegend) for 2h. After 2h, the cell supernatants were added with IL-33 (100 ng/mL). The mRNA levels and protein levels were respectively detected at 48h and 72h.

CFSE staining

Lung ILC2s sorted from female WT control or IRF7^{-/-} mice were labeled with 4 μ M CFSE (carboxyfluorescein diacetate succinimidyl ester, Thermo Fisher Scientific), followed by cultured in the presence of murine IL-2 (10 ng/ml), IL-7 (20 ng/ml), with or without IL-33 (100 ng/ml) in 96-well round plates (10,000 cells/well) for 72 h in a 37°C incubator with 5% CO₂. The number of ILC2s were counted and the proliferation of ILC2s were analyzed by flow cytometry.

Quantitative real-time PCR (qRT-PCR)

Semiquantitative RT-PCR was performed following the procedures described earlier (Qin et al., 2013). The RNA was isolated from lung ILC2s or lung tissue. RNA was extracted using TRIzol (Thermo Fisher Scientific) and reverse transcribed with cDNA synthesis kit (Takara, Otsu, SH). Gene expression was analyzed by qRT-PCR. The primers used were listed in Table S2.

Influenza virus infection

To perform influenza infections, we followed the procedure described before (Liu et al., 2014; Morita et al., 2013). Mice were anesthetized with 1.5% isoflurane and then infected intranasally (i.n.) with influenza virus A/Puerto Rico/8 (PR8, H1N1, Charles River Laboratories, USA) using a dose of 50% of the virus lethal dose (LD₅₀) in 50 μ L PBS per mouse or same volume of PBS. Mice were monitored daily and the weight loss was recorded every 24 h post-infection. On day 9 after virus infection, mice were sacrificed. The frequency of eosinophils in lung and bronchoalveolar lavage fluid, as well as lung ILC2s and their cytokine production were analyzed by flow cytometry. The amounts of IFN β in plasma was measured by ELISA (R&D Systems). All infection experiments with PR8 were performed under biosafety level (BSL) 2 conditions.

Citrobacter Rodentium infection model

Mice were orally gavaged with 200 μ L of PBS containing 1 $\times$ 10⁹ colony-forming units (CFUs) of *citrobacter rodentium* (CR) strain DBS100 (ATCC 51459; American Type Culture Collection). CR strain was prepared by culturing in LB broth overnight and bacterial concentration was determined by measuring the optical density at 600 nm (OD₆₀₀). Body weights were monitored daily. Mice were sacrificed on day 7, and colon length were measured and flow cytometric analysis of ILC1 and ILC3 were performed. CR strain was kindly provided by Dr. Ju Qiu from Shanghai Institutes of Nutrition and health, Chinese Academy of Sciences.

Inflammatory ILC2 (iILC2) induction and analysis

Female IRF7^{-/-} and WT control mice were treated intraperitoneally (i.p.) with PBS or recombinant IL-25 (200 ng per mouse daily in 200 μ L PBS, BioLegend) for 3 days. Mice were sacrificed 24 h after the last challenge. The population of iILC2s was defined as Lin⁻ ST2⁻ KLRG1^{hi} cells as described before (Huang et al., 2015). The leukocytes in lung were isolated and analyzed by flow cytometry. For cytokine production by iILC2, lung leukocytes were stimulated with PMA (50 ng/mL), Ionomycin (1 μ g/mL) and Brefeldin A (1 μ g/mL) for 6 hours at 37°C, before staining with iILC2 markers and antibodies against IL-4 and IL-13.

Retroviral transduction

The full-length coding sequence of Bcl11b was amplified and cloned into retroviral plasmid MIGR1(MSCV-IRES-GFP). The construct was confirmed by sequencing. Primers used were listed in Key Resources Table under STAR Methods. For Retroviral packaging was performed according to the protocol described previously (Lu et al., 2003; Wang et al., 2014). Human embryonic kidney HEK293T cells were seeded at 5 million/100-mm plate in DMEM and grown for 24h. Plasmid DNA MIGR1-Bcl11b-GFP was co-transfected with the retroviral capsid protein producing plasmid pHIT60 and the envelope protein producing plasmid pVSV-G using Lipofectamine 2000 according the manufacturer's specifications (Thermo Fisher Scientific). The retrovirus-containing medium was collected 48 h later, filtered through a 0.45 μ m Millipore filter and concentrated using ultracentrifugation. The supernatants were mixed with 50% PEG 6000 (MERCK Millipore), 4M NaCl and 1 $\times$ PBS and stored at 4°C overnight. The next day centrifugation (4°C, 7000 rcf., 15 min) was performed and the precipitate was dissolved in 300 μ L fresh medium. Retrovirus titers (transduction units (TU)) were determined and adjusted to 1 $\times$ 10⁸ TU/ml for subsequent infection experiment. For retrovirus infection, Lineage negative cells from lung were infected with freshly concentrated retrovirus supernatants by spin infection (32°C, 3,000 rpm for 90 min) and then incubated in the presence of 8 μ g/mL polybrene (Santa Cruz Biotechnology, Dallas, TX) for additional 6 hours. Cells were then washed and resuspended in fresh complete medium with 10 ng/ml of IL-2, IL-7 and IL-33. 3 days later, the amounts of cytokine in supernatants were measured by ELISA (Yang et al., 2014).

Luciferase Assay

We followed the procedures described earlier (Cao et al., 2019). The fragment of mouse Bcl11b promoter (–2000 to +100 bp) was cloned into pGL3 plasmid (Promega, Madison, WI). Mutation of IRF7-binding site was generated by PCR using site-directed mutagenesis kit (Stratagene, La Jolla, CA). Reporter plasmid was transfected into human embryonic kidney HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific) and the activity of luciferase was measured 48 h post-transfection. The sequences of primer used were listed in [Key Resources Table](#) under [STAR Methods](#).

Chromatin immunoprecipitation (ChIP) assay

Lineage negative cells were sorted from lung and cultured in the presence of IL-2, IL-7 and IL-33 for 3 days. ChIP was conducted as previously described (Yang et al., 2017). In brief, cultured Lineage negative cells were fixed with a 1% formaldehyde solution, lysed, and sheared by sonication. Cell lysates were precleared with protein G-agarose and immunoprecipitated with anti-IRF7 or an anti-IgG control. Antibody-chromatin complexes were collected by incubation with protein G-agarose. After DNA purification, quantitative PCR was performed using primers (Table S2). 10% of the lysate before immunoprecipitation was used as input control. Amplification of β -actin from the input was used as a loading control for normalization. Data were normalized against input DNA and presented as fold increase over IgG control.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed using GraphPad Prism version 8.0 software (GraphPad Software, La Jolla, CA). Two-tailed unpaired Student's *t* test and one-way ANOVA with Bonferroni post-test were used to confirm most comparisons. Correlations between different parameters were analyzed using a Spearman's rank correlation test. All data were expressed as the mean $\pm$ SEM except for supplemental Table S1 expressed as the mean $\pm$ SD, and significance was defined as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.001.

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.

Supplemental Information

IRF-7 Is a Critical Regulator of Type 2 Innate

Lymphoid Cells in Allergic Airway Inflammation

Juan He, Qiong Yang, Qiang Xiao, Aihua Lei, Xing Li, Pan Zhou, Ting Liu, Lijuan Zhang, Kun Shi, Quan Yang, Junchao Dong, and Jie Zhou

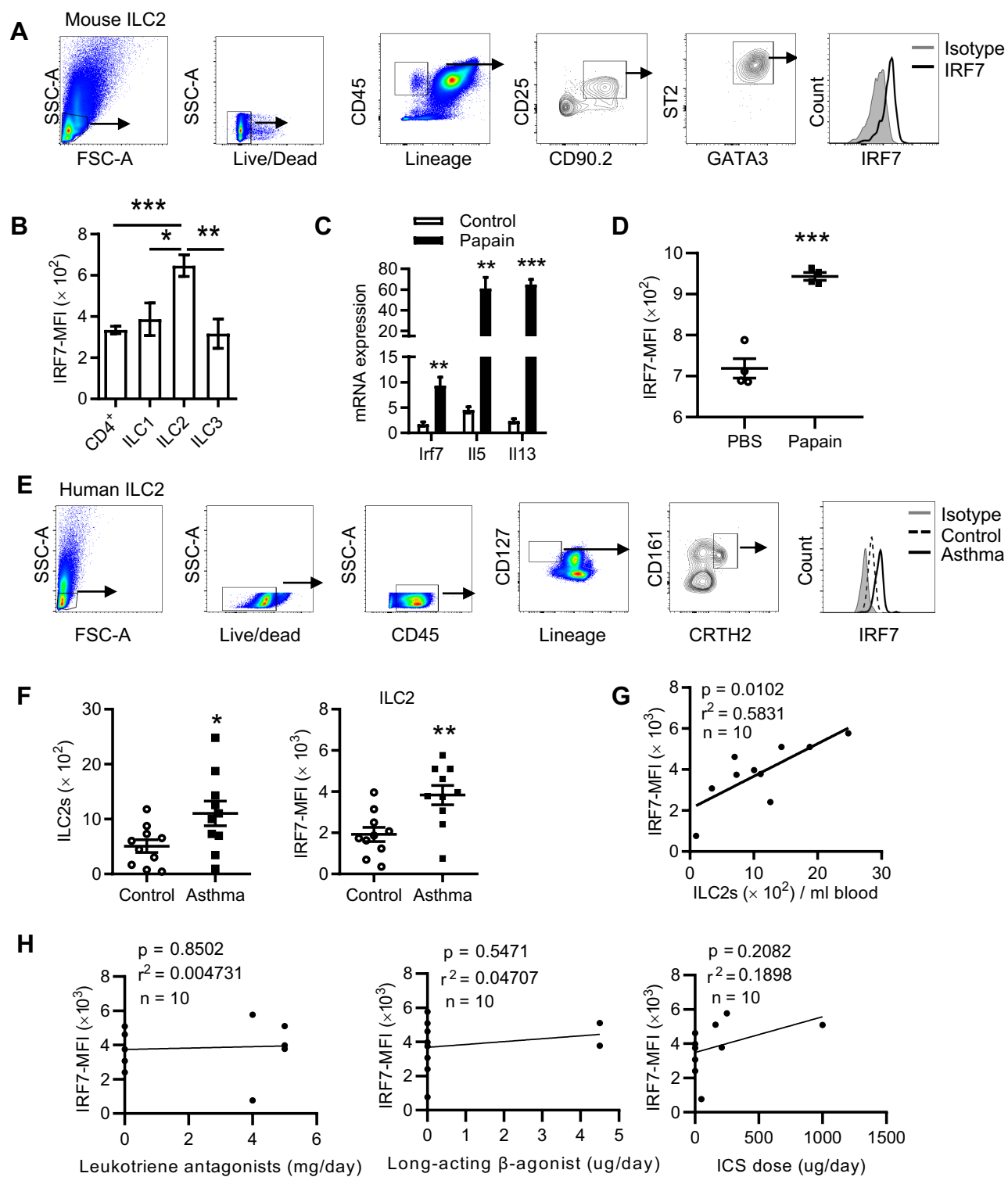


Figure S1

Figure S1. IRF7 expression in mouse and human ILC2 cells. Related to Figure 1.

A. Representative gating strategy of mouse lung ILC2 (CD45⁺Lin⁻CD90⁺CD25⁺GATA3^{high}ST2⁺) and the expression of IRF7 on ILC2s of WT mice lung. Dead cells excluded by a viability stain. **B.** Flow cytometry of IRF7 expression in mouse ILC1 (Lin⁻CD45⁺CD127⁺NKP46⁺NK1.1⁺), ILC2 and ILC3(Lin⁻CD45⁺CD90⁺CD127⁺Roryt⁺). The data shown are representative Mean fluorescent intensity (MFI) of IRF7 from two independent experiments (n=5 mice per group). **C.** The mRNA expression of IRF7 in lung tissue after papain treatment, IL-5 and IL-13 are used as positive control. **D.** The protein level of IRF7 in lung ILC2 from mice treated with papain (i.n.) was determined by flow cytometry. **E.** Representative gating strategy of human ILC2 (CD45⁺Lin⁻CD127⁺CRTH2⁺CD161⁺) and the expression of IRF7 on asthma and healthy control. Dead cells excluded by a viability stain. **F.** Left panel shows flow cytometric analysis of human ILC2 cells in PBMC of asthma patients, compared with controls, while right panel shows IRF7 expression in human ILC2 cells between asthma patients and healthy controls (n=10). **G.** Correlation between human ILC2 number and IRF7 expression in asthma patients (n=10). **H.** Correlation between IRF7 expression and the medication of asthma patients (ICS (inhaled corticosteroid), Long-acting β -agonist and leukotriene antagonists). Data are representative of two independent experiments (n=3-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001 by unpaired Student's t test.

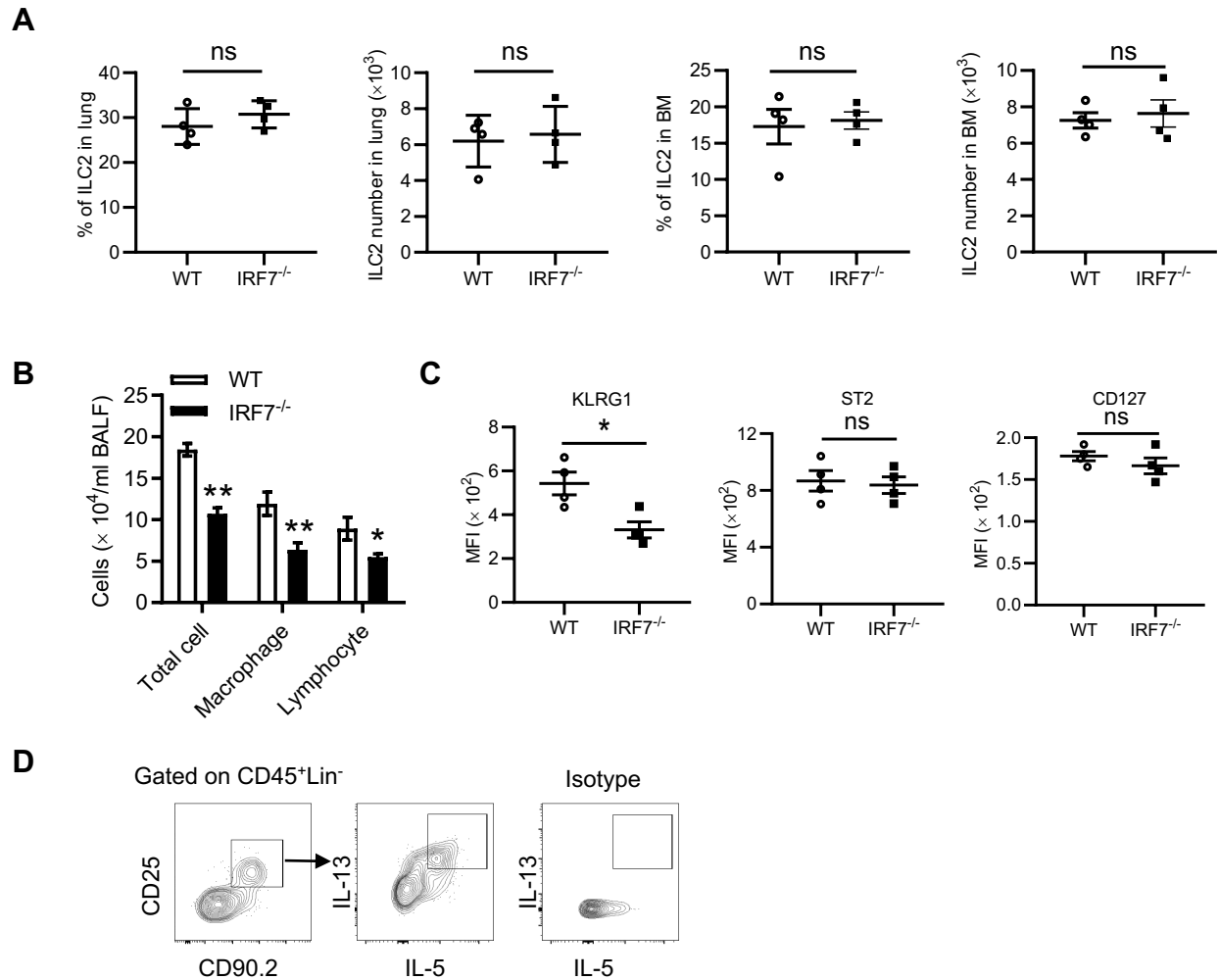


Figure S2. IRF7 deficiency causes remission of ILC2-driven airway inflammation. Related to Figure 1.

A. Flow cytometric analysis of ILC2 in lung (left) and bone marrow (BM, right) of WT and IRF7^{-/-} mice under steady-state condition (n=4). The frequency and total numbers of ILC2 are shown. Data are mean $\pm$ SEM from three independent experiments. **B.** Total cells in BALF (including macrophages and lymphocytes) from papain challenged WT and IRF7^{-/-} mice. Data are representative of two independent experiments (n=6 mice per group). **C.** Flow cytometric analysis of KLRG1, ST2, CD127 expression in lung ILC2s from WT and IRF7^{-/-} mice after challenge with papain (n=4). **D.** The representative flow cytometry analysis of IL-13⁺IL-5⁺ILC2 in lungs. Data are representative of two independent experiments. Error bars show mean $\pm$ SEM. *, p<0.05 by unpaired Student's t test. ns, not significant.

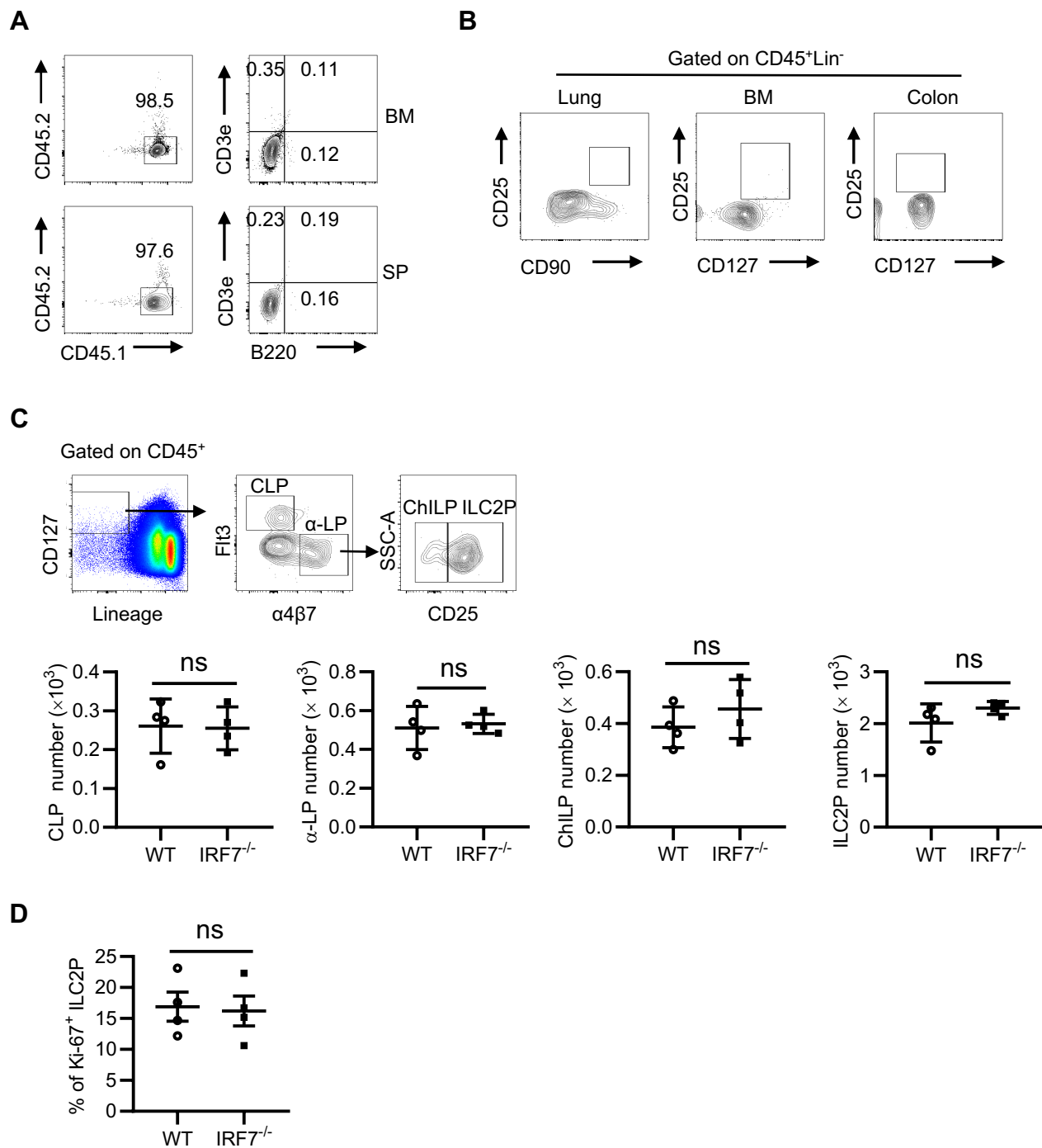


Figure S3: The cellular characteristics of NCG mice and the reconstitution of ILC2 progenitors in mixed BM chimeras. Related to Figure 4.

A. Flow cytometric plots showing percentages of B220⁺ and CD3e⁺ cells in the BM and SP from NCG mice. **B.** Flow cytometric plots showing percentages of ILC2 cells in the lung, BM and colon from NCG mice. **C.** The representative gating strategy of ILC2 progenitor cells in the BM of adult mice, pregated on live cells (upper), and their reconstitution from donor cells in recipients BM at steady-state (lower) (n=4). **D.** The proliferation of BM ILC2P from donor cells in recipients BM at steady-state (n=4). CLP: Common lymphoid progenitors, α-LP: α4β7⁺ lymphoid progenitors, ChILP: Common helper-like ILC progenitors, ILC2P: ILC2 precursor. Data are representative of two (A-B) or three (C-D) independent experiments. Error bars show mean ± SEM. ns, not significant. Numbers within flow plots indicate the percentages of cells.

Figure S3

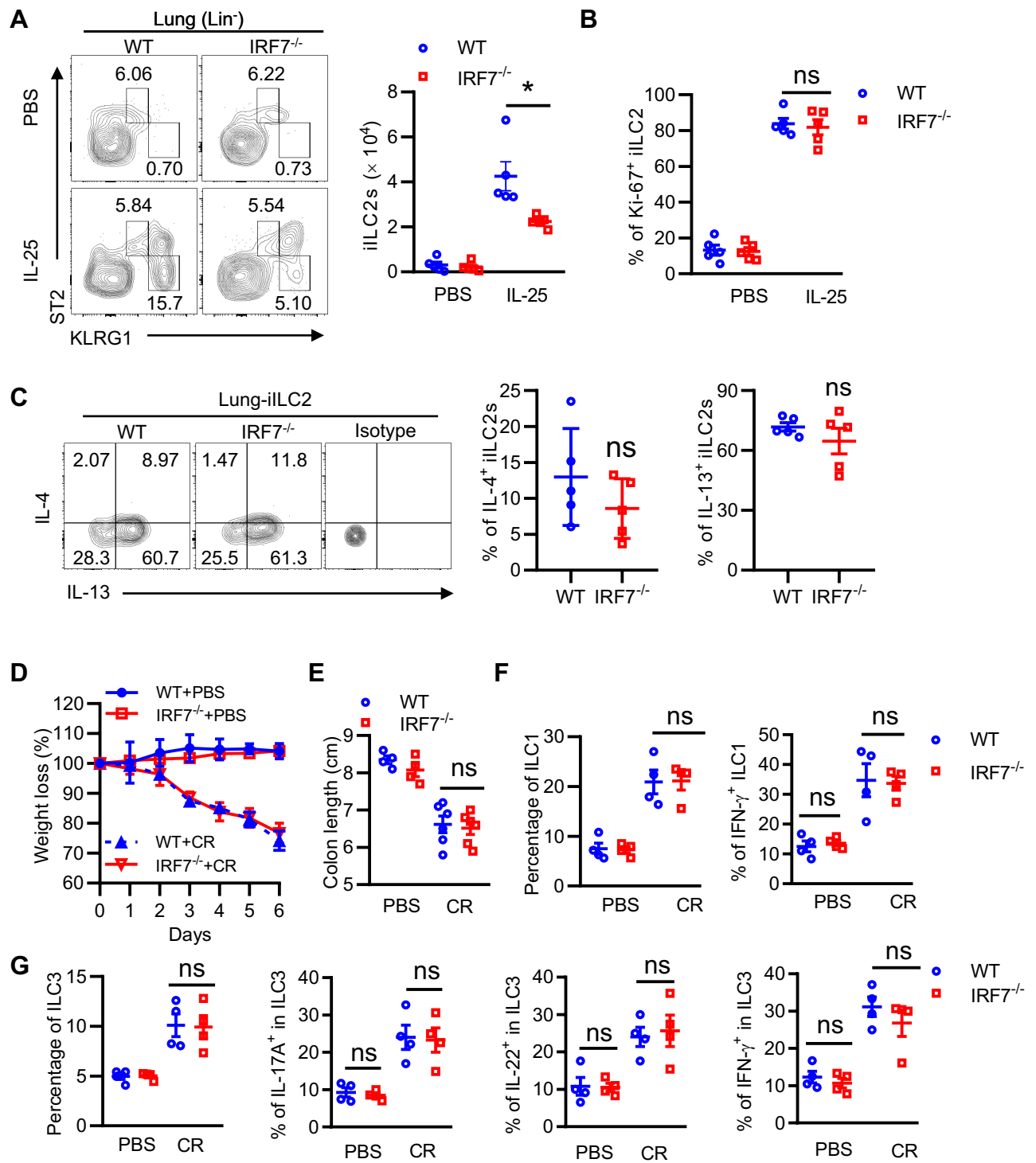


Figure S4

Figure S4. IRF7 did not affect the function of iILC2, ILC1 and ILC3. Related to Figure 4.

(A-C) WT and IRF7^{-/-} mice were administered with IL-25 (I.P. 200 ng/mouse/day) for 3 days (n=5). **A.** Flow cytometric analysis of iILC2s (Lin⁻ST2⁺KLRG1^{hi}) and their absolute numbers in lung. **B.** The proliferation of iILC2s. **C.** Flow cytometric analysis of IL-4⁺ and IL-13⁺iILC2s in lung. **(D-G)** WT and IRF7^{-/-} mice were administered with *citrobacter rodentium* (CR) (n=4-6 per group). Mice were sacrificed 7 days later. Body weight loss (**D**) and colon length (**E**) were shown. **F.** Flow cytometric analysis of ILC1 (CD45⁺Lin⁻CD127⁺NKP46⁺NK1.1⁺) and their production of IFN- γ in colon. Lineage cocktail contains anti-CD3, anti-CD45R/B220, anti-CD11b, anti-Ly-6G, anti-Erythroid marker (TER-119), anti-CD11c, anti-CD4, anti-CD5, anti-CD8a, anti-TCR β , and anti-TCR $\gamma\delta$. **G.** Flow cytometric analysis of ILC3s (CD45⁺Lin⁻CD90.2⁺CD127⁺Roryt⁺) in colon, as well as their production of IL-17A, IL-22, and IFN- γ . Data are representative of two independent experiments. Error bars show mean $\pm$ SEM. *,p<0.05 by unpaired Student's *t* test. ns, not significant. Numbers within flow plots indicate the percentages of cells gated.

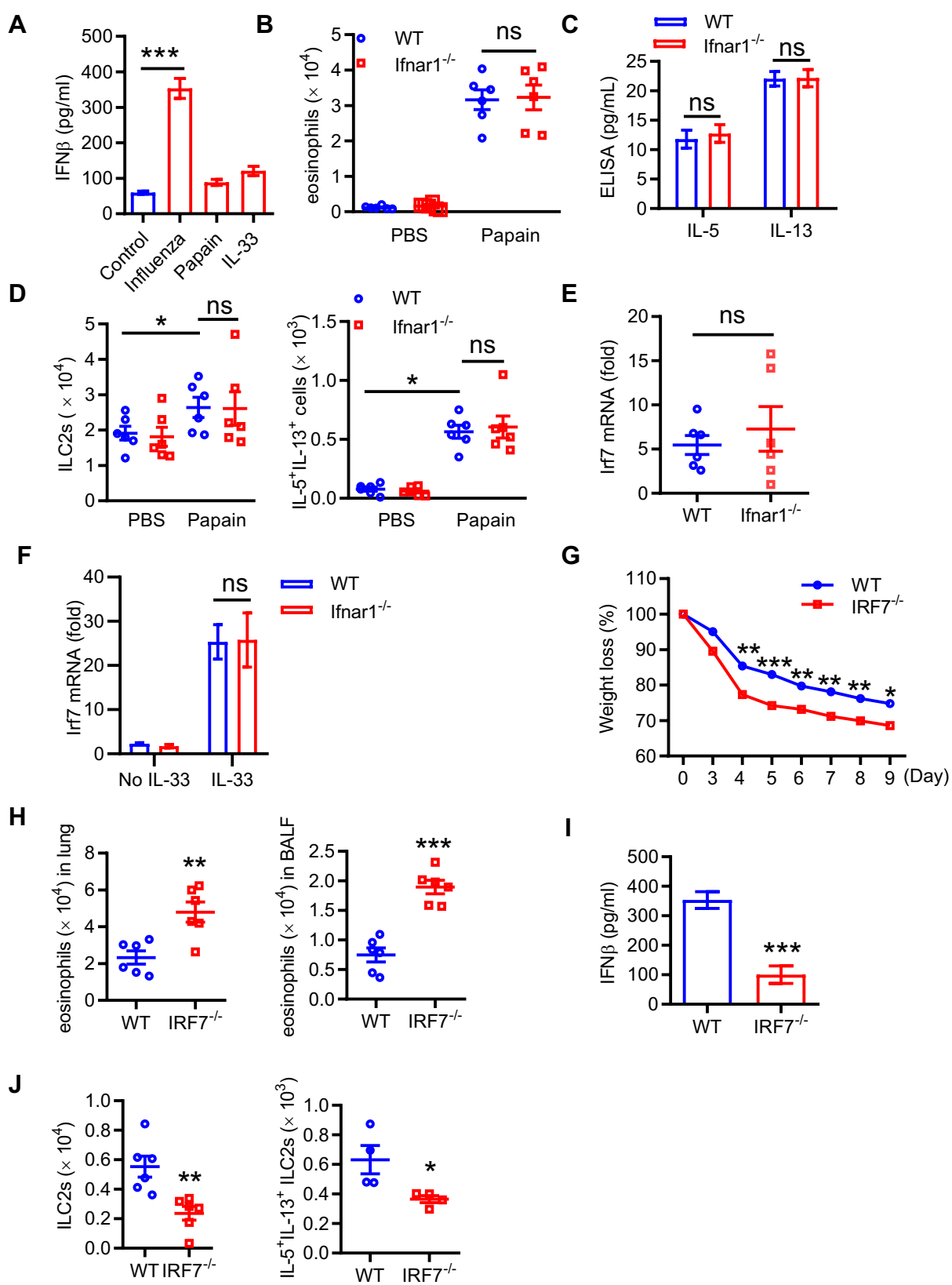


Figure S5

Figure S5: IFN does not affect the regulation of ILC2 by IRF7 in vivo. Related to Figure 6.

A. Analysis of the content of IFN β in different models (Influenza, papain, IL-33) by ELISA. **B.** Absolute numbers of eosinophils in bronchoalveolar lavage fluid between *Ifnar1*^{-/-} and WT mice after papain treatment. **C.** Bronchoalveolar lavage fluid from mice was tested for IL-5 and IL-13 by ELISA. Samples were tested in duplicate, with each experiment using six samples. **D.** Absolute numbers of lung-ILC2 and cytokines (IL-5 and IL-13) derived from lung-ILC2s between *Ifnar1*^{-/-} and WT mice after papain treatment. **E.** The mRNA expression of IRF7 in lung tissue between *Ifnar1*^{-/-} and WT mice. **F.** Lung ILC2s were sorted from *Ifnar1*^{-/-} and WT mice, followed by IL-33 stimulation in vitro. After 24h, mRNA expression of IRF7 was measure by qRT-PCR. **G.** *IRF7*^{-/-} and control mice were infected intranasally with influenza virus A/PR8. Mice were sacrificed at day 9 after infection. The body weight of control and *IRF7*^{-/-} mice was monitored daily after infection. **H.** Absolute numbers of eosinophils in lung and bronchoalveolar lavage fluid between *IRF7*^{-/-} and control mice after influenza virus infection. **I.** Analysis of the content of IFN β in the plasma of *IRF7*^{-/-} and control mice after influenza virus infection by ELISA. **J.** Absolute numbers of lung-ILC2 and cytokines (IL-5 and IL-13) derived from lung-ILC2s. Data are representative of two independent experiments (n=4-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001 by unpaired Student's t test. ns, not significant.

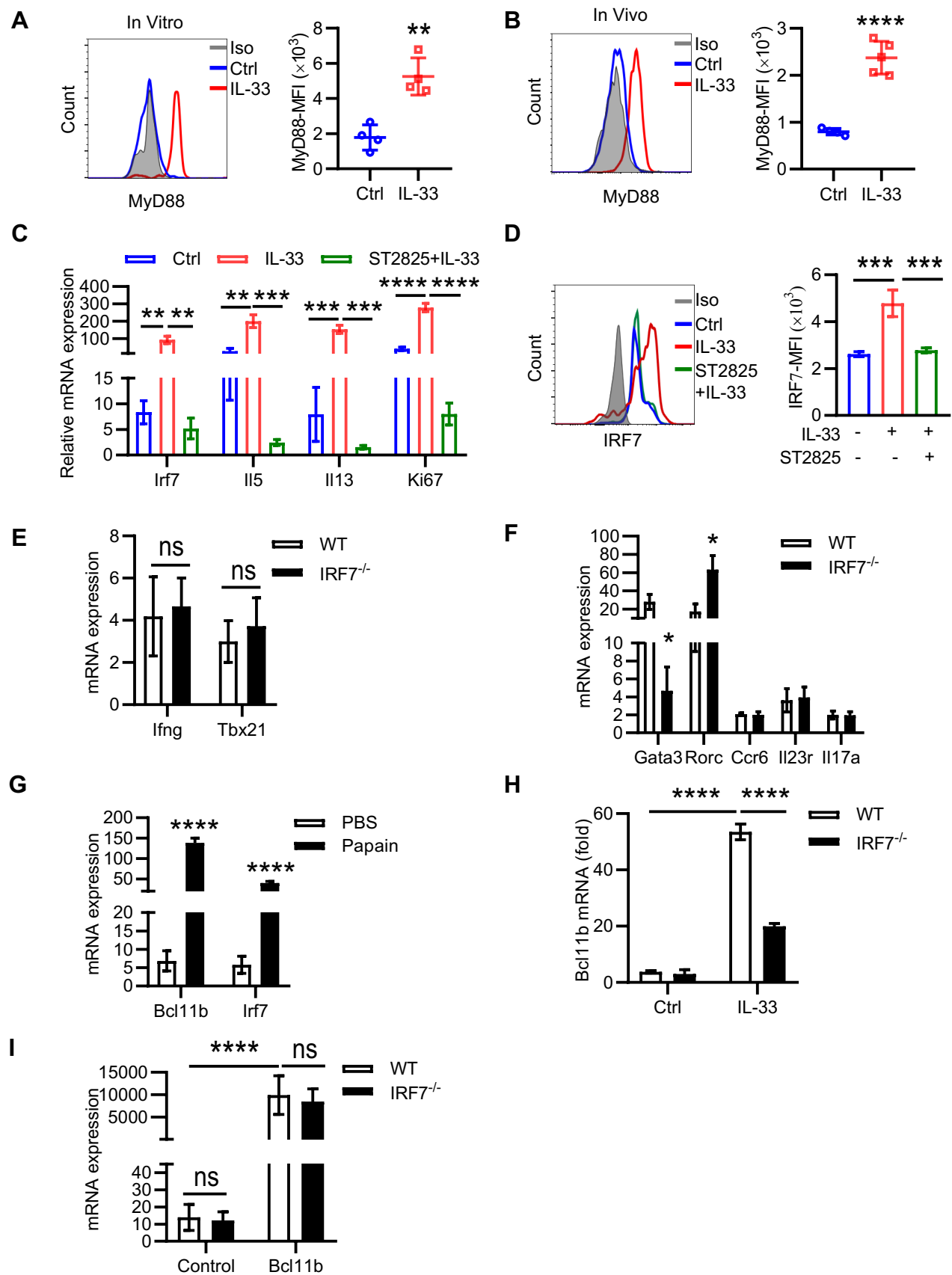


Figure S6

Figure S6: Bcl11b is induced during airway inflammation in vivo and in vitro. Related to Figure 6.

A. Flow cytometric analysis of MyD88 expression in ILC2 cells after IL-33 stimulation for 3 days in vitro. **B.** Flow cytometric analysis of MyD88 expression in lung ILC2 after mice were challenged with IL-33 for 3 days. **C.** Lung ILC2s were treated with ST2825 (20 μ M) with or without IL-33 for 24 h. The mRNA expression of *Irf7*, *Il5*, *Il13* and *Ki67* was determined by qRT-PCR. **D.** Lung ILC2s were treated with ST2825 (20 μ M) with or without IL-33 for 72 h. The MFI of IRF7 was measured by flow cytometry. **E.** The mRNA expression of ILC1 related genes in lung ILC2s from WT and IRF7^{-/-} mice under papain model. **F.** The mRNA expression of ILC3 related genes in lung ILC2s from WT and IRF7^{-/-} mice under papain model. *Gata3* was used as a positive control. **G.** The mRNA expression of *Bcl11b* in lung ILC2 in papain model was measured by qRT-PCR. *Irf7* was used as the positive control. **H.** Naïve lung ILC2s sorted from WT and IRF7^{-/-} mice were cultured in the presence of IL-2, IL-7 with or without IL-33. After 24 h, the mRNA expression of *Bcl11b* was measured by qRT-PCR. **I.** ILC2 cells from WT and IRF7^{-/-} mice were infected with retrovirus of Bcl11b-Migr1 or vector control. The mRNA expression of *Bcl11b* was measured by qRT-PCR after 72 h. Data are representative of two (E-I) or three (A-D) independent experiments (n=4-6 mice per group). Error bars show mean $\pm$ SEM. *,p<0.05; **,p<0.01; ***,p<0.001; ****,p<0.0001 by unpaired Student's t test. ns, not significant.

Table S1: Asthma subject characteristics. Related to Figure S1.

Characteristic	Asthma	Healthy Control
No. of subjects	10	10
Age (years)	8.2 $\pm$ 1.81 (6-11)	8.10 $\pm$ 1.91 (6-12)
Gender (M:F)	7:3	6:4
ICS dose (ug/day)	167 $\pm$ 308.37 (0-1000)	0
Oral corticosteroids (%)	0	0
Long-acting β -agonist (%)	20	0
Leukotriene antagonists (%)	50	0
FEV1 (liters)	1.46 $\pm$ 0.34 (1.08-2.00)	-
FEV1 (% predicted)	89.2 $\pm$ 13.83 (67-110)	-
FEV1/FVC ratio (%)	76.8 $\pm$ 6.11 (67-87)	-
FeNO (ppb)	30.47 $\pm$ 14.35 (10-50)	-

Results are expressed as means $\pm$ SD (range).

ICS dose is the equivalent of inhaled fluticasone.

FeNO: fractional concentration of exhaled nitric oxide.

ppb: parts per billion.

Table S2: Sequences of primers used in this study. Related to Figure 6, S1, S5 and S6.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
<i>Il5</i>	CGTGGGGGTACTGTGGAAAT	AATCCAGGAACTGCCTCGTC
<i>Il13</i>	TGCCATCTACAGGACCCAGA	CTCATTAGAAGGGGCGGTGG
<i>Gata3</i>	AGGCAAGATGAGAAAGAGTGCCTC	CTCGACTTACATCCGAACCCGGTA
<i>Irf7</i>	AAACCATAGAGGCACCCAAG	CCCAATAGCCAGTCTCCAAA
<i>Gfi1</i>	AGGAGGCACCGAGAGACTCA	GGGAGGCAGGGAAGACATC
<i>Tox</i>	CTTGAGGCTCTCCCAAGTCC	CGTTCGAAAATGCGCTCCAG
<i>PLZF</i>	AGCTGCCTCACCAACCTTTC	ATCGAGCCTTACGGTCTCT
<i>Tcf1</i>	CCCACAGAGCTTGACTAGTGG	CCCATCGTCATCCGTGTCAT
<i>Nfil3</i>	GAGCAGAACCACGATAACCCA	AGATGCAACTTCCGGCTACC
<i>Ets1</i>	TCCGCCAAGTGCCAACCTC	CGGAGTTAACAGCGGGACAT
<i>Bcl11b</i>	CCCTCCCACCTCCGTGATTA	CCATGTGTGTTCTGTGCGTG
<i>Rora</i>	GTGGAGACAAATCGTCAGGAAT	TGGTCCGATCAATCAAACAGTTC
<i>Ki67</i>	CCTGTGAGGCTGAGACATGG	CCCTCACTCTTGTCAGGGTC
<i>Rorc</i>	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT
<i>Ccr6</i>	CCTGGGCAACATTATGGTGGT	CAGAACGGTAGGGTGAGGACA
<i>Il23r</i>	TTCAGATGGGCATGAATGTTTC	CCAAATCCGAGCTGTTGTTCTAT
<i>Il17a</i>	TTTAACTCCCTTGCGCAAAA	CTTTCCCTCCGCATTGACAC
<i>Ifng</i>	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
<i>Tbx21</i>	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
<i>β-actin</i>	CGTGCGTGACATCAAAGAGAAG	CGTTGCCAATAGTGATGACCTG
Bcl11b chip site-1	GAGGGGTCAGCCTCCGAGAAAGAA	CGCCCCCTCAAAAACCCAAA
Bcl11b chip site-2	GTGCTTTCTTGTTCTCTTACAGGG	GGACGTGACGTTTAATCTGC
Bcl11b chip site-3	CCCCACCCATGGCTTTCAGG	GATTGGCGAGGCTGCAAGGC