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

Author(s):
Muri, Jonathan; Thut, Helen; Bornkamm, Georg W.; Kopf, Manfred

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

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

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.

Authors

Jonathan Muri, Helen Thut, Georg W. Bornkamm, Manfred Kopf

Correspondence

manfred.kopf@ethz.ch

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

Jonathan Muri,1 Helen Thut,1 Georg W. Bornkamm,2 and Manfred Kopf1,3,*

1Institute of Molecular Health Sciences, ETH Zurich, 8093 Zürich, Switzerland
2Institute of Clinical Molecular Biology and Tumor Genetics, Helmholtz Zentrum München, 85764 Neuherberg, Germany
3Lead Contact
*Correspondence: manfred.kopf@ethz.ch
https://doi.org/10.1016/j.celrep.2019.10.070

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 antioxidant 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 xc−, 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), hepatocytes 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) Gpx4fl/fl;Cre-ERT2 mice and control Gpx4fl/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 Gpx4fl/fl; Cd19-Cre and Gpx4fl/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).

(legend continued on next page)
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 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\textsuperscript{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\textsuperscript{fl/fl};Cre-ERT2 mice led to a significant reduction in total CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the spleen (Figure 1A). To our surprise, however, total CD19\textsuperscript{+} IgM\textsuperscript{+} B cell numbers in the bone marrow and the percentage of CD19\textsuperscript{+} B cells in the blood were comparable in TAM-treated Gpx4\textsuperscript{fl/fl};Cre-ERT2 and control (Gpx4\textsuperscript{fl/fl}) mice (Figures 1B and 1C). We additionally verified that Cre-mediated deletion in both T and B cells of Gpx4\textsuperscript{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 T and B lymphocytes, we generated Gpx4\textsuperscript{fl/fl};Cd19-Cre mice. The deletion of Gpx4 was expectedly complete at the mRNA level in both IgM\textsuperscript{+} and IgM\textsuperscript{−} CD19\textsuperscript{+} B220\textsuperscript{+} B cells in the bone marrow and in total CD19\textsuperscript{+} B cells in the spleen of Gpx4\textsuperscript{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/pre-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\textsuperscript{fl/fl};Cd19-Cre compared to Gpx4\textsuperscript{fl/fl} littermate control mice (Figure 1F). Furthermore, numbers of mature IgM\textsuperscript{+}IgD\textsuperscript{−}CD19\textsuperscript{+}B220\textsuperscript{+} 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\textsuperscript{+}CD45.2\textsuperscript{−}) were reconstituted with an equal ratio of congenically marked donor bone marrow cells from Gpx4\textsuperscript{fl/fl};Cd19-Cre mice (CD45.2\textsuperscript{−}) and WT (CD45.1\textsuperscript{−}) 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\textsuperscript{+} 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\textsuperscript{fl/fl};Cd19-Cre and Gpx4\textsuperscript{fl/fl} control mice with replication-defective Qb-virus-like particles (Qb-VLPs) containing E. coli single-stranded RNA (ssRNA) and monitored Qb-VLP-specific antibody levels on days 7, 18, and 32 post-infection. Gpx4\textsuperscript{fl/fl};Cd19-Cre and Gpx4\textsuperscript{fl/fl} mice mounted comparable Qb-VLP-specific immunoglobulin M (IgM) (Figure 2A) and class-switched IgG2b (Figure 2B) antibody responses. Qb-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 Qb-VLPs. GC B cells are generally defined by the marker profile B220\textsuperscript{+}CD38\textsuperscript{high}Fat− and can be further subdivided into CXCR4\textsuperscript{−} dark zone (DZ) and CD86\textsuperscript{−} light zone (LZ) cells (Vic tora et al., 2010). Interestingly, Gpx4\textsuperscript{fl/fl};Cd19-Cre mice showed a higher frequency in GC B cells compared to the Gpx4\textsuperscript{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) Expression of CD62L on CD19+ B cells from the spleen (I) and lungs (J); 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\textsuperscript{fl/fl};Cd19-Cre BM cells expressing the congenic markers CD45.1 and CD45.2, respectively. After reconstitution, the contribution of Gpx4\textsuperscript{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\textsuperscript{α}+ 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\textsuperscript{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 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 ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 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 Gpx4fl/fl;Cd19-Cre mice, we fluorescence-activated cell sorted (FACS) B220+CD38lowFas+ GC B cells from both Gpx4fl/fl;Cd19-Cre and Gpx4fl/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 Gpx4fl/fl;Cd19-Cre and Gpx4fl/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+B0 cells were comparable in Gpx4fl/fl;Cd19-Cre and Gpx4fl/fl mice, whereas CD21/35CD23+ 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-deficient 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-T 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+ cells in the peritoneal cavity of Gpx4fl/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) (Ghosn et al., 2008). We found that both CD11b+B1a and CD11b+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 Gpx4fl/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 reconstructed mainly by WT cells (Figure 3O), consistent with the reduced MZ B cell numbers we observed in naïve Gpx4fl/fl;Cd19-Cre mice compared to Gpx4fl/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 reconstructed by bone-marrow precursors upon steady-state perturbations, such as B1-cell depletion due to irradiation (Duber 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 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\textsuperscript{fl/fl};Cd19-Cre and Gpx4\textsuperscript{fl/fl} littermate control mice via flow cytometry.
(A) Gating strategy of Fo (CD23\textsuperscript{+}) and MZ (CD21/35\textsuperscript{+}CD23\textsuperscript{–}) B cells in the spleen (pre-gated on live/CD45\textsuperscript{+}/CD11b\textsuperscript{–}/TCR\textsuperscript{hi} and gated on B220\textsuperscript{hi}CD5\textsuperscript{–}CD19\textsuperscript{hi} CD11b\textsuperscript{–}CD23\textsuperscript{–}).

(legend continued on next page)
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 inoculated 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 Gpx4fl/fl;Cd19-Cre control group mounted a strong anti-PC IgM response, while the Gpx4fl/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 Gpx4fl/fl;Cd19-Cre compared to Gpx4fl/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, 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 4A–4C), 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.
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-BODIPY581/591 (n = 5–6). Representative FACS plots (A) and quantification (B) of lipid ROS in B2 (CD19+B220+CD43–CD23+) and B1 (CD19+B220lowCD43+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+B220lowCD43+CD23–), and MZ B (CD19+B220+CD21/35+CD23–) cells in the spleen.

(E and F) Gpx4fl/fl;Cd19-Cre and Gpx4fl/fl littermate control mice were intravenously injected with BODIPY FL C 16 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 Gpx4fl/fl;Cd19-Cre and Gpx4fl/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.
reprogramming and switching to aerobic glycolysis to produce metabolic intermediates for sustainment 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 development 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 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 Tirosi, 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 former, 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

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.jcelrep.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. and M.K.; Visualization, J.M.; Supervision, M.K.; Funding Acquisition, M.K.; Resources, G.W.B.; Supervision, M.K.

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_893323 |
| 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_89334 |
| 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 CD6, 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 2811/CXCR4 | BD Biosciences | Cat#561966; 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#127972; RRID: AB_2562567 |
| Anti-mouse NK1.1, PE, clone PK136 | Thermo Fisher Scientific | Cat#12-5941-82; RRID: AB_466050 |
| Anti-mouse TCR, BV421, clone H57-597 | Biolegend | Cat#109229; RRID: AB_10933326 |
| Anti-mouse TCR, 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 |
(Continued on next page)
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 |
| Qβ-Virus-like particles (VLPs) | provided by Martin Bachmann, University of Bern | N/A |
| Bacteria: Streptococcus pneumoniae (strain D39) | provided by Benjamin Marsland, Monash University | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Ferrostatin-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<sup>fl/fl</sup> | 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/6/J | The Jackson Laboratory | Cat#JAX:000664; RRID: IMSR_JAX:000664 |
| Mouse: B6.SJL-Ptgcr<sup>p</sup>Pepcb<sup>Cb</sup>/BoyJ | The Jackson Laboratory | Cat#JAX:002014, RRID:IMSR_JAX:002014 |
| **Oligonucleotides** | | |
| Gpx4-FWD: 5’-CTGTGGAAATGGATGAAAG-3’ | This paper | N/A |
| Gpx4-REV: 5’-TCAATGAAAGCTTGGATTAAG-3’ | This paper | N/A |
| Tbp-FWD: 5’-TTGACCTAAGGACATGGACTTC-3’ | This paper | N/A |
| Tbp-REV: 5’-TTCCTCATGATGACTGACGACAA-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 Veterinärsamt Zürich) and were performed according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG). Gpx4fl/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 Gpx4fl/fl;Cd19-Cre, Gpx4fl/fl;Cre-ERT2 and Gpx4fl/fl;Cd4-Cre mice, Gpx4fl/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-Ptprc8Pepcb/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% Borgal, 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 Gpx4fl/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 1x10^8 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. Bone marrow cells were flushed from femurs and tibia, and then directly passed through the 70 μm cell strainer. ACK buffer (homemade) 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 (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, 100x10⁶ cells/ml were stained with microbeads (1:10 dilution) for 15 min at 4°C. After washing, 100x10⁶ cells were resuspended in 500 µL 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 (10⁵/well) were seeded in IMDM + GlutaMAX, 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM β-mercaptoethanol (all GIBCO) into 96 well plates and stimulated in the presence of AffiniPure F(ab)₂ Fragment Goat anti-mouse IgM (10 µg/mL; Jackson ImmuneResearch) and LPS from Escherichia coli 0111:B4 (1 µg/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 µM; 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 µg/mL streptomycin, 50 µM β-mercaptoethanol (all GIBCO) into 96 well plates pre-coated with anti-CD3 (4 µg/ml; 145-2C11; home-made) and anti-CD28 (2 µg/ml; 37.51; home-made) for stimulation (10⁵/well). To analyze cell death by ferroptosis, T cells were stimulated in the presence of ferrostatin-1 (Fer-1; 10 µM; Sigma-Aldrich) for the indicated times.

Antibody measurement by ELISA

To assess the IgM response to phosphorylcholine, plates were coated with 25 µg/ml of phosphorylcholine conjugated to bovine serum albumin (PC-BSA, Biosearch Technologies) in PBS. For determination of the antibody response to Qβ-VLP in PBS, plates were coated with 1 µg/ml Qβ-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 ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001 for Student’s t test; *, p ≤ 0.0332; **, p ≤ 0.0021; ***, p ≤ 0.0002; ****, p ≤ 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.