Conditional Disruption of Hem-1 Results in Impaired B Cell Development and Aberrant Antibody Production

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Abstract

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Hematopoietic protein-1 is a hematopoietic cell specific member of the actin-regulatory WAVE complex, which acts downstream of multiple immune receptors to stimulate F-actin polymerization. Loss-of-function mutations in the gene encoding Hem-1, *NCKAP1L*, have recently been found to result in Primary Immunodeficiency Disease in humans, characterized by recurrent bacterial and viral respiratory infections, asthma, skin infections, bacteremia, atopy, and autoimmunity. However, the cellular and molecular mechanisms of how loss of Hem-1 results in PID are not known. In this study, we generated constitutive and B cell specific *Nckap1l* knockout mice using the Cre-LoxP system to dissect the importance of Hem-1 in B cell development and functions. We found that mice with B cell specific disruption of Hem-1 lack mature recirculating B cells in the bone marrow and lymph nodes, along with reduced peripheral B cell populations such as B1, follicular and marginal zone (MZ) B cells due to the decreased capability to efficiently migrate to different lymphoid tissues. Immunization with the T-independent antigens NP-ficoll and heat-killed *S. pneumoniae* (HKSP), failed to elicit antibody production due to a lack of the innate-like B1 and MZ B cell populations. Immunization with HKSP failed to produce antibody titers sufficient to protect Hem-1 deficient mice from a challenge with a lethal dose of the bacteria Streptococcus pneumoniae. In contrast to the poor antibody response to T-independent antigens, Hem-1 deficient mice produced pronounced IgM and IgG2c antibody titers following immunization with the T-dependent antigen NP-KLH. Hem-1 deficient B cells demonstrated hyperreactive states as shown by: decreased surface IgM expression, increased and sustained calcium influx following IgM stimulation, increased

expression of activation markers and higher expression of phosphorylated molecules downstream of the BCR before and after IgM stimulation. These changes in Hem-1 deficient B cells are permissive to the formation of the unique T-bet+CD11c+ B cell population that form autoantibodies. These collective findings suggest that Hem-1 is essential for normal development of B cells, B cell migration and homing and regulating BCR signaling thus providing a mouse model that may provide further insight into the mechanism of autoimmunity in children with mutations in NCKAP1L.

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LIST OF ABBREVIATIONS

ABI – ABL interactor 1

ARP – actin related protein

BCR – B cell receptor

BSA – bovine serum albumin

CXCL12 – C-X-C motif chemokine 12

CXCR-3 – C-X-C motif chemokine receptor 3

CXCR-4 – C-X-C motif chemokine receptor 4

dsDNA – double-stranded deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

F-actin – Filamentous actin

GTP – guanosine triphosphate

Hem-1 - Hematopoietic protein-1

HKSP – heat-killed Streptococcus pneumoniae

HSPC – hematopoietic stem/progenitor cell protein

KLH – Keyhole Limpet Hemocyanin

MZ – marginal zone

MRF – mature recirculating follicular

Nckap1l – Nck-associated protein1-like

NP – (4-hydroxy-3-nitrophenyl)-acetyl

pAkt – phosphorylated protein kinase B

pERK – phosphorylated extracellular signal-regulated kinase

pS6R – phosphorylated S6 ribosomal protein

PC – phosphorylcholine

PID – primary immunodeficiency disease

Rac1/2 – Ras-related C3 botulinum toxin substrate ½

Sra1 – Rac-associated protein

S. pneumoniae – Streptococcus pneumoniae

WASP – Wiskott-Aldrich syndrome protein

WAVE – WASP-family verprolin homologous protein

WT – wild-type

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DEDICATION

I dedicate this work to Kayleigh whose love and support was invaluable, our girls Aspen and Luna, and my mother who instilled in me an appreciation and love of the animals I am now privileged to serve.

INTRODUCTION

B cells constitute a portion of the adaptive arm of the host immune system. These cells are responsible for producing immunoglobulins that neutralize viruses, opsonize bacteria, and mark malignant cells for destruction. The function of B cells is mediated through the initial recognition of a specific antigen by the B cell receptor (BCR), which induces B cell proliferation, maturation, and subsequent antibody production. Signaling through the B cell receptor is mediated in part by the actin cytoskeleton; the importance of the actin cytoskeleton is demonstrated in patients with Wiskott-Aldrich syndrome resulting in primary immunodeficiency disease (PID) (Pfajfer et al. 2017). In these patients, the Wiskott-Aldrich syndrome protein (WASP) fails to regulate actin polymerization rendering B cells incapable of migrating toward lymphocyte specific chemokines, proliferating in response to antigen, and normal antibody production (Pfajfer et al. 2017, Antón et al. 2002, Westerberg et al. 2005).

Further studies elucidate the role of the actin cytoskeleton as it pertains to BCR signaling. In inactive B cells, the BCR is restricted to confinement zones and diffusion is prevented by activity of the actin cytoskeleton (Treanor et al. 2010). Activation of the BCR leads to actin cytoskeleton depolymerization through the dissociation of filamentous actin (F-actin) from the cell membrane and assembly of F-actin in the periphery of the cell which promotes further interaction of the cell with the antigen during remodeling (Freeman et al. 2011). Remodeling of the actin cytoskeleton is key to many of the activities of B cells, such as formation of the immunological synapse, adhesion and integrin clustering, migration, and antibody production (Meyer-Bahlburg et al. 2008, Westerberg et al. 2005). The importance of the actin cytoskeleton in regulating BCR signaling is further demonstrated by the fact that disruption of the cytoskeleton is enough to initiate downstream signaling that is equivalent to BCR cross-linking (Mattila et al. 2013).

BCR signaling promotes the formation of F-actin polymerization through the membrane associated Rho family GTPases (Henderson et al. 2010, Tybulewicz and Henderson 2009). Specifically, signaling mediated through the Rac1 and the hematopoietic specific Rac2 promotes B cell development, antigen response and activation, differentiation, and migration (Tybulewicz and Henderson 2009, Walmsley et al. 2003, Croker et al. 2002). Activity of the Rac1 and Rac2 proteins is mediated through the WASP-family verprolin homologous protein (WAVE) complex to activate cell motility and signaling through its interaction with the actin related protein (ARP) 2/3 promoting the formation of F-actin from monomeric G-actin (Croker et al. 2002, Yan et al. 2003). The complex is composed of 5 subunits that include: Rac-associated protein 1 (Sra1), Nck-associated protein 1-like (*NCKAP1L*), ABL interactor 1 (ABI1), WAVE2, and hematopoietic stem/progenitor cell protein 300 (HSPC300) (Campellone and Welch 2010). Loss of *Nckap1l* expression, which encodes for hematopoietic protein 1 (Hem-1), is shown to cause defects in migration and phagocytic capabilities of innate immune cells and developmental disruption in lymphocytes in mice (Park et al. 2008, Millius et al. 2009).

Hem-1 is a scaffolding protein that is exclusively expressed in cells of the hematopoietic lineage (Hromas et al. 1991). In normal functioning neutrophils, Hem-1 remains diffuse throughout the cytosol. Upon stimulation, Hem-1 translocates to the periphery of the cell and gives the cell polarity by forming the leading edge (Weiner et al. 2006). The formation of F-actin by Hem-1 also proves important in the phagocytic capabilities of neutrophils and macrophages, as cells deficient in Hem-1 have decreased phagocytic capabilities (Park et al. 2008). When Hem-1 is defective, lymphocytes and neutrophils lose the ability to polymerize G-actin into F-actin which leads to the degradation of the WAVE complex (Park et al. 2008, Weiner et al. 2006).

Recent reports of 4 unrelated human kindreds show that patients with loss-of-function mutations in the *NCKAP1L* gene present with clinical features consistent with severe immunodeficiencies (Comrie et al. 2019). As Hem-1 is expressed almost exclusively in the cells of the hematopoietic cell lineage, its loss has a profound effect on multiple cell types. Cells from children with loss-of-function mutations in *NCKAP1L* had high mortality likely due to multiple dysfunctions of the immune system such as: impaired T cell activation and subsequent proliferation; impaired migration of T cells and neutrophils in response to a chemoattractant gradient; immune hyperreactivity; increased IgM, IgG and IgE production with poor specificity for the immunogen; and autoantibody production. These dysfunctions of the cells lead to clinical manifestations such as: recurrent bacterial and viral infections, septic arthritis, bacteremia, meningitis, atopy, and immune complex glomerulonephritis.

Mouse models with loss-of-function point-mutations in the *Nckap1l* gene reproduce the findings of human patients (Park et al. 2008). In Hem-1 deficient mice, neutrophils failed to migrate efficiently in the presence of a chemoattractant gradient. Additionally, T cells had impaired capabilities to proliferate and adhere properly as well as increased release of cytokines. Because mice have characteristics like those of children with mutations in *NCKAP1L*, they are a useful model to study Hem-1 and the consequences of its dysregulation. Here we utilized a Cre-*LoxP* approach by crossing mice with *Nckap1l* LoxP flanked alleles to mice expressing a Cre-recombinase early in development or in early B cell development to study the effects of Hem-1 deficiency in B cells specifically. The role of Hem-1 in B cells has yet to be elucidated; however, because B cells can play a role in autoantibody production, preventing recurrent infections, and atopy observed in children with mutations in *NCKAP1L*, it is important to understand the function of Hem-1 in B cell activity and development. Here we demonstrate that B cells deficient

in Hem-1: have impaired development; fail to adequately migrate to lymphoid tissues; fail to produce protective antibody against T-independent antigens; are predisposed to increased IgM and IgG2c antibody production to the T-dependent antigen NP-KLH; have increased basal levels of downstream IgM-signaling and are susceptible to becoming Tbet+CD11c+ age-associated B cells that produce autoantibody.

RESULTS

Constitutive deletion of Hem-1 severely disrupts central and peripheral B cell development. To model how a mutation in *NCKAP1L* in children affects B cell development, we generated mouse models with constitutive knock-out *Nckap1l* alleles using the Cre-*LoxP* system. We bred *Nckap1l* floxed mice (*Hem1*$^{fl/fl}$) to mice expressing Cre-recombinase under the control of *Meox2*, which is expressed early in mesodermal development (Tallquist and Soriano 2000). The early expression of *Meox2* affects all hematopoietic cell types by deleting exons 12 through 16 in *Nckap1l* resulting in Hem-1 deficiency. Offspring from these mice were screened for deletion by PCR amplification for the *Nckap1l* alleles; mice with constitutive *Nckap1l* deletions are henceforth referred to as *Hem-1*$^{-/-}$ mice. The disruption of *Nckap1l* in *Hem-1*$^{-/-}$ mice has profound effects on the development of lymphocytes, granulocytes and red blood cells as previously reported in mice with a point mutation in *Nckap1l* (Tallquist and Soriano 2000, Park et al. 2008). Examination of bone marrow B cell populations from mice aged 6 – 12 weeks of age demonstrated severe lymphopenia starting at the earliest stage of B cell development, the pro-B cell (B220+CD43+CD24-BP1-) stage through the mature recirculating follicular (MRF) B cell (B220hiCD43-IgM+) stage (Figure 1A and 1B). Consequently, peripheral B cell populations in the spleens of *Hem-1*$^{-/-}$ mice demonstrated severe lymphopenia beginning at the transitional T0 (B220+CD93+IgM+IgD-CD23-), T1 (B220+CD93+IgM+IgD+CD23-), and T2 (B220+CD93+IgM+IgD+CD23+) stages. Follicular B cells were reduced (B220+CD93-CD21+CD23+), with the most pronounced loss occurring in the marginal zone (MZ) B cell (B220+CD93-CD21hiCD23-) population (Figure 1C and 1D). Furthermore, examination of the long-lived fetal-liver derived peritoneal B1 B cells also demonstrated severe lymphopenia (Figure 1E and F).

B cell specific deletion of Hem-1 inhibits the development of transitional, marginal zone, and mature recirculating follicular B cells. To determine the B cell specific function of Hem-1 in B cell development, we bred *Hem1*$^{fl/fl}$ to *Mb1Cre* mice, which express Cre-recombinase under the control of the B cell specific *Mb1* promoter that initiates expression at the pro-B cell stage (Hobeika et al. 2006). These crosses resulted in the development of *Hem1*$^{fl/fl}$*Mb1-Cre* mice. Analysis of bone marrow from *Hem1*$^{fl/fl}$*Mb1-Cre* mice revealed that Hem-1 deficient B cells develop normally through the immature B cell (B220loCD43-IgM+) stage but lack MRF B cells (Figure 2A and 2B). During their development, the B cells from *Hem1*$^{fl/fl}$*Mb1-Cre* undergo normal B cell development phases, such as light chain rearrangement, in similar fashion to WT B cells (Figure 2C). Because Hem-1 has been shown to be important for migration in other cells of the hematopoietic lineage such as neutrophils, we sought to answer whether the transitional B cells in the bone marrow were capable of efficiently egressing from the bone marrow and into the blood to complete their development in the spleen. Further analysis revealed an accumulation of transitional T0 B cells in the bone marrow of *Hem1*$^{fl/fl}$*Mb1-Cre* mice (Figure 2D).

Due to a potential decrease in transitional T0 B cells efficiently egressing from the bone marrow, we analyzed all transitional B cells in the blood to determine if T0 B cells were also arrested in the blood of *Hem1*$^{fl/fl}$*Mb1-Cre* mice. We found that the T0 B cells were overly represented in the transitional B cell populations in the blood of *Hem1*$^{fl/fl}$*Mb1-Cre* mice. Subsequently, the proportions of later stages, such as T1 and T2, were reduced in the blood of *Hem1*$^{fl/fl}$*Mb1-Cre* mice (Figure 2E).

We next asked if T0 B cells migrate into the white pulp and continue their development. Analysis of the spleens of *Hem1*$^{fl/fl}$*Mb1-Cre* mice revealed B cell lymphopenia throughout all stages of development (Figure 3A and 3B). The total number of transitional T0, T1, T2 and T3 B

cells were all decreased in *Hem1$^{fl/fl}$Mb1-Cre* mice; however, T0 B cells made a smaller proportion of the total B cell population in the spleens of *Hem1$^{fl/fl}$Mb1-Cre* mice (Figure 3C). As was observed with the *Hem-1$^{-/-}$* mice, FO B cells were reduced and MZ B cells were almost completely depleted (Figure 3B). Analysis of the MRF B cells in the lymph nodes of the *Hem1$^{fl/fl}$Mb1-Cre* mice showed severe lymphopenia (Figure 3D). Long-lived resident B1 B cells of the peritoneum were also decreased in *Hem1$^{fl/fl}$Mb1-Cre* mice, thus demonstrating the importance of Hem-1 in the development of the B cell populations we examined (Figure 3E).

We subsequently crossed *Hem1$^{fl/fl}$Mb1-Cre* mice to mice with the reporter gene *TdTomato*, which becomes active when the stop codon is removed upstream of *TdTomato* by the activity of a Cre-recombinase (Madisen et al. 2010). This system allows for the analysis of B cells that have active Cre-recombinase activity and subsequent targeted deletion, in this case deletion of *Nckap1l*. The use of this system showed activity in >90% of all cell types following the pro-B cell stage where *Mb1* is only starting its expression (Figure 4A – 4D).

Disruption of Hem-1 inhibits the capability of B cells to migrate in vitro and in vivo. Actin polymerization and F-actin formation have been shown to be important for the generation of filopodia, which drive cell migration (Svitkina 2018). We hypothesized that disruption of Hem-1 results in impaired migration to and from essential lymphoid niches, such as the active migration of immature B cells from the bone marrow to the spleen, where developing B cells receive essential growth factors for continued maturation, therefore, the loss of migratory capabilities may explain the developmental abnormalities observed in *Hem1$^{fl/fl}$Mb1-Cre* mice (Loder et al. 1999). To understand the mechanism in a controlled environment, we utilized an *in vitro* approach utilizing a transwell migration plate and the chemokine, C-X-C motif chemokine 12 (CXCL-12), which acts to attract and retain B cells to the appropriate lymphoid niche. This

approach showed that Hem-1 deficient B cells, and MRF B cells in particular, exhibited decreased capability to migrate in response to CXCL-12 (Figure 5A). To rule-out a potential defect in the surface expression of C-X-C motif chemokine receptor 4 (CXCR-4), the receptor for CXCL-12, leading to a migratory defect, we analyzed the expression of CXCR4 on the surface of B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice. From this we found that there was no difference in the mean fluorescence intensity between B cells from *Hem1$^{fl/fl}$Mb1-Cre* and WT mice, indicating failure to migrate is likely due to an inability to respond to the stimulus rather than an inability to recognize CXCL-12 (Figure 5B).

We next explored the role of Hem-1 in the migration of B cells into different B cell niches, such as the bone marrow, spleen, and lymph nodes. To this end, we utilized a competitive *in vivo* transfer approach whereby *Hem1$^{fl/fl}$Mb1-Cre* and WT positively selected B cells were labeled with the fluorescent dyes cell trace violet and CFSE, respectively, mixed 1:1 and transferred via intravenous injection to recipient WT mice. Twenty-four hours post-transfer, analysis of the fluorescently labeled B cells in the blood of recipient mice showed that B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice made up a larger proportion of B cells than WT B cells in the blood of the recipient mice (Figure 6C). In contrast, the proportion of *Hem1$^{fl/fl}$Mb1-Cre* B cells vs. WT B cells found in lymphoid tissues including bone marrow, spleen, inguinal lymph nodes (LN), mesenteric LN, and submandibular LN were greatly decreased relative to peripheral blood consistent with a decrease in migration efficiency in B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice.

Disruption of Hem-1 inhibits T-independent antibody production and increases susceptibility against Streptococcus pneumoniae infection. Innate-like MZ and B1 B cells are important for early antibody responses to T-independent antigens such as those found in encapsulated bacteria. Because *Hem1$^{fl/fl}$Mb1-Cre* mice have decreased MZ and B1a and B1b B cells, we hypothesized

that the lack of these populations in *Hem1$^{fl/fl}$Mb1-Cre* mice would impair T-independent antibody production following immunization. To understand the role of Hem-1 in T-independent antibody production, we immunized *Hem1$^{fl/fl}$Mb1-Cre* and WT mice with the T-independent antigen NP-Ficoll. Six days following immunization sera and tissues were collected. We measured antibody titers using an ELISA assay which tests for antibody specific against a portion of the immunogen, NP-BSA$_{30}$.We found that *Hem1$^{fl/fl}$Mb1-Cre* produced little to no antibodies against the NP molecule, whereas WT mice responded normally (Figure 6A). Although total plasma cells were represented normally in *Hem1$^{fl/fl}$Mb1-Cre* mice, NP-specific plasma cells had about a six-fold decrease relative to WT mice (Figure 6B). These results suggest that B cell specific disruption of Hem-1 greatly impairs antibody responses to T-independent antigens and are consistent with the reduction in MZ and B1 B cells in *Hem1$^{fl/fl}$Mb1-Cre* mice.

Because children with mutations in *NCKAP1L* fail to produce antibody following vaccination against pneumococcal pneumonia, we hypothesized that *Hem1$^{fl/fl}$Mb1-Cre* mice would also fail to produce antibody against the T-independent antigen, heat-killed *Streptococcus pneumoniae* (HKSP). Using an ELISA assay with a component of the *S. pneumoniae* cell surface, PC-BSA as the capture antigen, we found that *Hem1$^{fl/fl}$Mb1-Cre* mice fail to produce antibody against HKSP (Figure 6C). To probe the effect this lack of antibody response would have on susceptibility against bacterial infection, we challenged HKSP-immunized mice with a lethal dose of *S. pneumoniae* serotype 2 strain D39. To closely mimic the susceptibility of children with a mutation in *NCKAP1L*, we elected to immunize and infect *Hem-1$^{-/-}$* for this portion of the study, as well as WT and *Hem1$^{fl/fl}$Mb1-Cre* mice.

As antibodies were noted to be formed acutely following immunization in other studies and in our previous experiment, we chose to immunize WT, *Hem1$^{fl/fl}$Mb1-Cre* mice and *Hem-1$^{-/-}$* three days prior to bacterial challenge. Throughout the course of bacterial infection, mice were monitored for change in body weight, which was compared to the weight obtained on the day of infection. This day 0 weight was a baseline throughout the course of infection and used to measure how susceptible the mice were to the infection based on how much weight they lost. We found that all mice showed signs of infection and lost weight on day 1 post-infection (Figure 6E). On day 2 post-infection, all *Hem-1$^{-/-}$* mice, 3 *Hem1$^{fl/fl}$Mb1-Cre* and unimmunized WT mice succumbed to infection and required euthanasia (Figure 6D). The *Hem-1$^{-/-}$* and *Hem1$^{fl/fl}$Mb1-Cre* mice were more susceptible to infection as they lost a significant amount of weight compared to immunized WT who were beginning to recover from infection on day 2 post-infection, thus illustrating the importance of Hem-1 for T-independent immunization and susceptibility to bacterial infection (Figure 6E).

Deficiency of Hem-1 causes aberrant IgM and IgG2c antibody production following immunization with the T-dependent antigen NP-KLH and infection with Influenza virus.

Unlike the B cells in the peritoneum or in the marginal zone, follicular B cells require interaction with T cells to produce antibody against T-dependent antigens such as (4-hydroxy-3-nitrophenyl)-acetyl conjugated to Keyhole Limpet Hemocyanin (NP-KLH). Because the actin cytoskeleton plays a role in influencing the availability and signaling functions of BCRs on the cell surface, we hypothesized there would be poor interaction between Hem-1 deficient B cells and T cells leading to a poor T-dependent antibody response. Ten days post-immunization with NP-KLH in alum, an adjuvant meant to stimulate the immune response, we collected sera and spleens from both *Hem1$^{fl/fl}$Mb1-Cre* and WT mice and found that they both produced similar

proportions of B cells expressing germinal center (B220+CD95+GL7+) B cells (Figure 7A). To test T-dependent antibody response, we serially bled the immunized mice on a weekly basis for four weeks. By measuring antibody titers using an ELISA assay with NP-BSA_{30} as the capture antigen, we interestingly found that *Hem1$^{fl/fl}$Mb1-Cre* initially produced similar levels of IgM specific to the NP molecule following immunization that greatly increased on weeks 3 and 4 relative to WT mice (Figure 7B). As part of antibody production, class switching is expected from IgM to different IgG subclasses. *Hem1$^{fl/fl}$Mb1-Cre* mice produced similar quantities of IgG1 (data not shown) and IgG3 (Figure 7B). The most pronounced class switch was to IgG2c in *Hem1$^{fl/fl}$Mb1-Cre* mice, as they produced significantly higher quantities of IgG2c as early as one-week post-immunization and remained higher than control mice up through week 4 (Figure 7B). Although Hem-1 deficient mice produced large amounts of IgG2c, the ratio of high affinity NP-$BSA_{2:}$ low affinity NP-BSA_{30} antibodies did not increase even at 4-weeks post-immunization, indicating that affinity maturation is reduced following disruption of Hem-1 (Figure 7C).

To further test the ability of *Hem1$^{fl/fl}$Mb1-Cre* mice to produce T-dependent antibodies during viral infection, we infected WT and *Hem1$^{fl/fl}$Mb1-Cre* mice with the mouse adapted PR/8 influenza virus. Ten days following infection, all infected mice lost nearly 30% of their body weight and we did not demonstrate a substantial difference in susceptibility to the influenza virus (data not shown). At 10 days post-infection, mediastinal lymph nodes were collected and analyzed for germinal center (B220+ CD95+ GL7+), plasmablast (B220+IgDintCD138int) and plasma cell (B220+IgDintCD138hi) differentiation (Burbage et al. 2015). This analysis found that within the mediastinal lymph nodes, that drain the lungs, there was a strong capability of the *Hem1$^{fl/fl}$Mb1-Cre* B cells to form germinal centers and differentiate into plasmablasts and plasma cells (Figure 7D). In addition, bronchoalveolar lavage fluid and serum were collected to

determine the ability of these mice to produce antibody. Interestingly, sera from the *Hem1$^{fl/fl}$Mb1-Cre* mice were able to produce similar levels of IgM when compared to WT; however, their ability to produce IgM within the lumen of the pulmonary tree was decreased, suggesting that B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice are unable to migrate to the proper location to effectively produce antibody where the infection occurs (Figure 7 E).

Deficiency in Hem-1 promotes an increase in plasma cell formation following boost-immunization with NP-KLH. Because *Hem1$^{fl/fl}$Mb1-Cre* mice responded to immunization with a robust antibody response, we wanted to determine the longevity of these cells following immunization by assessing antigen-specific memory (B220+CD19+IgD-CD38+NP+) B cells. To test this, we boosted WT and *Hem1$^{fl/fl}$Mb1-Cre* mice with NP-KLH in PBS via an intraperitoneal injection. Eight days following the boost immunization, the spleens were collected and memory B cells were assessed (Jones, Wilmore, and Allman 2015). We found that both WT and *Hem1$^{fl/fl}$Mb1-Cre* mice had similar numbers of antigen-specific memory B cells following boost immunization (Figure 8A). Most striking was the high number of NP-specific plasma cells that differentiated in response to boost-immunization with NP-KLH (Figure 8C).

Deficiency in Hem-1 causes a decrease in surface expression of IgM and an increase in B cell activation following IgM stimulation. We had previously noted that B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice had decreased expression of IgM on the cell surface throughout B cell development (Figure 9A). We hypothesized that this was due to either a defect in the actin cytoskeleton and its role in maintaining the BCR on the cell surface or decreased IgM as a mechanism to avoid deletion due to excessive signaling and subsequent negative selection. Consistent with the latter hypothesis, expression of the BCR isotype IgD was increased in transitional, FO, MZP and MZ B cells (Figure 9 B and C). IgD has previously been shown to deliver lower signal strength

following ligation and increased expression of IgD over IgM has been proposed as a mechanism for B cells to be more tolerant of self-antigen and prevent BCR-mediated apoptosis (Noviski et al. 2018).

To test the role of the Hem-1 dependent actin cytoskeleton modulation of surface IgM upon IgM stimulation, we stimulated B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice with anti-IgM for various time points. This was done to determine their capability to internalize the BCR following stimulation thus controlling BCR signaling. Contrary to other models of actin dysregulation, we found that B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice internalized surface IgM at a significantly faster rate than those from WT at the time points measured (Figure 10A). Subsequently, we wanted to determine if the increased rate of surface IgM downregulation would modulate the downstream BCR signaling pathways, therefore we performed a calcium influx assay. We found that *Hem1$^{fl/fl}$Mb1-Cre* B cells displayed a higher influx and duration of intracellular calcium levels following stimulation (Figure 10B).

Following these discoveries, we hypothesized that the decrease in IgM surface expression and increased IgD was due to *Hem1$^{fl/fl}$Mb1-Cre* B cells trying to control excessive downstream signaling through the BCR and prevent activation induced cell death (Noviski et al. 2018). To test our hypothesis that B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice have increased basal BCR signaling relative to WT mice, we collected and analyzed B cells for the expression of CD25, a lymphocyte activation marker, and found that B cells from experimentally naïve *Hem1$^{fl/fl}$Mb1-Cre* mice had a significantly higher level of CD25 expression (Figure 10C). We then isolated B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice and stimulated them with anti-IgM for 16-hours and analyzed the level of expression for both CD69 and CD25, an early and late marker of activation for lymphocytes, respectively (Zikherman, Parameswaran, and Weiss 2012). We found that

following activation with anti-IgM, B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice had a significantly higher surface expression of both activation markers (Figure 7D). Western blots for phosphorylated, and thus activated, molecules downstream of the BCR following stimulation with anti-IgM revealed that *Hem1$^{fl/fl}$Mb1-Cre* B cells exhibited activated mTORC2 (p-AKT^{473}), mTORC1(p-S6R) and ERK (p-ERK) prior to stimulation (Figure 10E). Following stimulation, the relative intensity of mTORC2 (p-AKT^{473}), mTORC1(p-S6R) and ERK (p-ERK) increased to higher levels than B cells from WT mice. These results further support earlier findings that *Hem1$^{fl/fl}$Mb1-Cre* B cells are hyperresponsive to IgM stimulus.

Hem1fl/flMb1-Cre mice produce autoantibody and have a higher proportion of age-associated Tbet+CD11c+ B cells. Because *Hem1$^{fl/fl}$Mb1-Cre* B cells appear to have tendencies one would expect from autoreactive cells such as hyperresponsiveness following IgM stimulation, decreased surface IgM and higher surface IgD expression, we hypothesized that *Hem1$^{fl/fl}$Mb1-Cre* mice would produce increased levels of autoantibody relative to WT controls. Using an ELISA with serum from *Hem1$^{fl/fl}$Mb1-Cre* and WT mice up to six-months of age, we found that *Hem1$^{fl/fl}$Mb1-Cre* mice produced significantly more anti-dsDNA and anti-snRNP autoantibodies (Figure 11 A).

It has been previously shown that IgG2c is associated with autoimmunity in both humans and mice, and that IgG2c expression is driven by the transcription factor T-bet. In addition, a unique population of B cells that express T-bet and CD11c are known to expand following chronic stimulation and are elevated in a number of autoimmune diseases such as systemic lupus erythematous (Karnell et al. 2017). Given that the B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice show increased basal stimulation prior to anti-IgM stimulation and increased IgG2c in our previous experiments, we hypothesized that *Hem1$^{fl/fl}$Mb1-Cre* mice would have this unique T-

bet+CD11c+ B cell population. Indeed, splenocytes from *Hem1*$^{fl/fl}$*Mb1-Cre* mice had a higher proportion of the T-bet+CD11c+ B cells, as well as B cells expressing CXCR3, a known transcriptional target of T-bet. These results support our hypotheses that constant stimulation experienced by *Hem1*$^{fl/fl}$*Mb1-Cre* B cells likely contribute to autoimmunity observed in humans with mutations in *NCKAP1L* (Figure 11B) (Piovesan et al. 2017, Serre et al. 2012).

DISCUSSION

Children with PID due to loss-of-function mutations in the *NCKAP1L* gene which encodes for Hem-1 suffer from a variety of clinical manifestations including recurrent bacterial and viral infections, pneumonia and autoimmunity that result in high mortality. Studies from *Hem1*$^{pt/pt}$ mice demonstrated presentations similar to the human patients, such as T cell lymphopenia, dysregulated cytokine production, defective migration and phagocytosis by neutrophils and autoimmunity thus demonstrating the importance and utility of mouse models to study the consequences of Hem-1 deficiency (Park et al. 2008). However, to best understand the mechanisms of how Hem-1deficiency results in recurrent infections and autoimmunity, it is important to individually dissect the cell autonomous role of Hem-1 in specific immune cells. In this study, we chose to investigate the B cell specific roles of Hem-1 in protective immunity, in part because Hem-1 deficient humans fail to respond efficiently pneumococcal immunization and because PIDs often lead to autoimmunity due to failed B cell tolerance and/or alterations in B cell signaling mechanisms (Lehman 2015). Our results demonstrate that B cells from *Hem1*$^{fl/fl}$*Mb1-Cre* mice have defects in their ability to: migrate and home to lymphoid tissues; produce protective antibodies against T-independent antigens; properly regulate BCR activation and limit excessive signaling; and prevent autoreactivity (Peng, Szabo, and Glimcher 2002).

The migration of transitional B cells from the bone marrow, to the blood, to the red pulp and subsequently the white pulp are critical steps in normal B cell maturation and central tolerance (Loder et al. 1999). In particular, the activity of the Rac GTPase, which activates the WAVE complex in response to immune receptor activation, has previously been shown to be important for the migration of T0 B cells from blood to the splenic white pulp to allow for continued development (Henderson et al. 2010). Using a transwell migration plate and the chemokine

CXCL12, we found that migration is defective in splenic B cells, particularly those of the follicular B cell stage. Consistent with the finding of poor migration is the higher proportion of T0 B cells within the transitional B cell population in the blood of *Hem1*$^{fl/fl}$*Mb1-Cre* mice, suggesting that these cells also fail to migrate into the appropriate niche to continue maturation. Interestingly, there is a higher number of total T0 B cells in the bone marrow of *Hem1*$^{fl/fl}$*Mb1-Cre* mice suggesting that Hem-1 has a role in B cell egression from the bone marrow. Despite accumulating in the bone marrow, the increase of T0 B cells in the blood may be explained by poor migration to the spleen. This explanation is supported by the fact that the proportion of T0 B cells is decreased in the spleens of *Hem1*$^{fl/fl}$*Mb1-Cre* mice when compared to WT mice, indicating that once Hem-1 deficient T0 B cells enter the appropriate niche they are able to progress through the transitional B cell stages with similar proportions to WT mice. The poor migration of B cells from *Hem1*$^{fl/fl}$*Mb1-Cre* mice is likely not attributed to a lack of the chemokine receptor, CXCR4, on the surface of B cells from WT and *Hem1*$^{fl/fl}$*Mb1-Cre* as B cells from both genotypes had similar mean fluorescence intensities for CXCR4 on the cell surface thus demonstrating that the poor migration is due to a lack of proper migration mediated by Hem-1.

We observed that the cellularity of mature B cells in the lymph nodes and bone marrow of *Hem1*$^{fl/fl}$*Mb1-Cre* mice was reduced relative to WT mice, which we hypothesized was because MRF B cells failed to migrate to the lymph nodes and back to the bone marrow following maturation in the spleen. Consistent with this notion, transfer of WT and *Hem1*$^{fl/fl}$*Mb1-Cre* B cells into recipient C57BL/6J mice demonstrated the poor capability of the *Hem1*$^{fl/fl}$*Mb1-Cre* B cells to home to lymphoid tissues, as a higher proportion of *Hem1*$^{fl/fl}$*Mb1-Cre* B cells remained in the blood of the recipient mice and reduced numbers entered lymphoid tissues. Because an equal

number of B cells were injected into recipient mice, and both WT and *Hem1$^{fl/fl}$Mb1-Cre* mice have similar proportions of follicular B cells, this assay largely demonstrated the inability of follicular B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice to home to lymphatic tissues. This is also supported by our demonstration using the anti-CD19 PE injection strategy that MRF B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice failed to migrate into the bone marrow parenchyma and remained in the sinusoids of the bone marrow (Figure 2F).

The loss of Hem-1 in *Hem1$^{fl/fl}$Mb1-Cre* mice resulted in the near absence of the innate-like MZ and B1 B cell populations. The MZ and B1 B cells are two subsets that are located in the MZ of the spleen and the peritoneal cavity, where they are poised to rapidly respond and produce antibodies against blood-borne and peritoneal pathogens independent of T cells (Cunningham et al. 2014). *Hem1$^{fl/fl}$Mb1-Cre* mice failed to produce IgM in response to immunization with the T-independent antigen, NP-Ficoll. The NP molecule has the advantage of allowing the user to determine the proportion of antigen-specific clones. Because of this advantage, we determined that the lack of IgM is likely due to the failure of MZ and B1 B cells from *Hem1$^{fl/fl}$Mb1-Cre* mice to expand and differentiate into great enough numbers in response to the NP antigen to produce adequate levels of IgM.

The lack of MZ and B1 B cells in *Hem1$^{fl/fl}$Mb1-Cre* mice may be the mechanism as to why children with mutations in Hem-1 fail to respond properly to immunization. For example, several patients studied failed to produce antibody against the T-independent vaccine, Pneumovax®, and are susceptible to infection by *Streptococcus pneumoniae* despite immunization. Because MZ and B1 B cells are necessary for the early response against *S. pneumoniae,* we immunized WT and *Hem1$^{fl/fl}$Mb1-Cre* mice and measured their antibody titers *(Martin, Oliver, and Kearney 2001, Haas et al. 2005).* Similar to the results from the NP-Ficoll immunization, we found that

Hem1$^{fl/fl}$*Mb1-Cre* mice, but not WT controls, failed to produce IgM against heat-killed *S. pneumoniae* (HKSP). Lethality was prevented in WT mice vaccinated with HKSP 3 days prior to being challenged with a lethal dose of *S. pneumoniae*. Specifically, WT mice were able to produce protective levels of IgM against a lethal dose of *S. pneumoniae* and survived and recovered from infection, whereas *Hem1*$^{-/-}$, *Hem1*$^{fl/fl}$*Mb1-Cre* and immunized control mice succumbed to infection within three days following bacterial challenge thus implicating the need for Hem-1 to produce protective antibodies against T-independent antigens.

Loss of function in a component of the WAVE complex, NCKAP1L, as well as the *RAC2* GTPase which activates the WAVE complex in response to immune stimulation, are known to cause PIDs and autoimmunity in humans (Alkhairy et al. 2015, Comrie et al. 2019). Decreased expression of IgM and increased expression of IgD on the B cell surface are found in autoreactive B cells (Zikherman, Parameswaran, and Weiss 2012). In this study we demonstrate that a B cell specific deletion of Hem-1 in mice causes phenotypes that are highly suggestive of anergic B cells, such as the downregulation of IgM and upregulation of IgD throughout development in the spleen to the follicular B cell stage. The upregulation of IgD throughout development is considered beneficial as it is less sensitive than IgM in recognizing endogenous antigens, but still sufficient to drive germinal center formation as we observed in the capability to produce germinal centers in response to the T-dependent antigen NP-KLH (Noviski et al. 2018). However, despite the downregulation of IgM on the cell surface of *Hem1*$^{fl/fl}$*Mb1-Cre* B cells, these cells have a higher level of basal activation and are hyper-responsive to IgM stimulation. Western blots of WT and *Hem1*$^{fl/fl}$*Mb1-Cre* B cells demonstrate that *Hem1*$^{fl/fl}$*Mb1-Cre* B cells have a higher basal level of activation as demonstrated by increased p-AKT^{473}, p-S6R and p-Erk. Furthermore, in experimentally naïve *Hem1*$^{fl/fl}$*Mb1-Cre* mice there is a higher proportion of

follicular B cells that express the lymphocyte activation marker CD25 and both CD25 and CD69 levels are increased in Hem-1 deficient B cells following anti-IgM stimulation

Hem-1 and the WAVE regulatory complex have been shown to be important for initiating actin polymerization. We hypothesize that loss of Hem-1 results in the loss of the cortical actin network which restrains diffusion of BCRs and promotes the formation of BCR nanocluster which activate BCR signaling. For example, it has previously been shown that disruption of the actin cytoskeleton is sufficient to initiate BCR signaling due to its role in maintaining the BCR in confinement zones and preventing interaction with CD19 (Treanor et al. 2010, Mattila et al. 2013). The BCR is maintained on the cell surface in lipid raft bound nanoclusters; however, when the actin cytoskeleton depolymerizes BCR nanoclusters are free to diffuse into microclusters, resulting in BCR-induced calcium influx. (Hao and August 2005). The phenomenon of prolonged BCR-induced calcium influx is noted when actin depolymerization is prolonged, resulting in a phenotype similar to what we observed in *Hem1*$^{fl/fl}$*Mb1-Cre* B cells. Perhaps this observation suggests that the loss of Hem-1 prevents efficient repolymerization of the actin cytoskeleton following IgM stimulation and allows for continued calcium influx. Interestingly, most mouse models demonstrate that mutants with defective actin polymerization result in a delay in IgM internalization. However, here we demonstrate that *Hem1*$^{fl/fl}$*Mb1-Cre* B cells will internalize IgM quicker than WT, perhaps in response to hyperactive BCR signaling.

In addition to the downregulated levels of IgM on the surface of *Hem1*$^{fl/fl}$*Mb1-Cre* B cells, the abundant amounts of IgG2c following immunization with NP-KLH in alum is also consistent with what is found in autoimmunity (Peng, Szabo, and Glimcher 2002). The administration of alum is known to stimulate CD8+ T cells to produce interferon-γ, promoting the switch to IgG2a/c antibody production under the control of the transcription factor T-bet (Mohr et al.

2010). Indeed, the overexpression of T-bet has been implicated in autoimmunity in a class of B cells known as age-associated B cells or T-bet+CD11c+ B cells, which are known to form under chronic stimulation (Karnell et al. 2017). We found that *Hem1*$^{fl/fl}$*Mb1-Cre* mice have a higher proportion of CD11c+ B cells that are also T-bet positive, and have increased expression of CXCR3, a known target gene of T-bet (Barnett et al. 2016, Zhang et al. 2019). Given that these cells are more likely to produce IgG2c, this T-bet+CD11c+ B cell population in naïve *Hem1*$^{fl/fl}$*Mb1-Cre* mice may be primed to start producing IgG2c, when directed by IFN-γ that is produced is response to alum. T-bet signaling is further implicated in autoimmunity because the loss of T-bet in mice caused the loss of autoimmunity features such as immune-complex renal disease (Peng, Szabo, and Glimcher 2002). In addition, in *Hem1*$^{fl/fl}$*Mb1-Cre* mice no older than 6 months, there was a higher amount of IgM raised against double-stranded DNA and snRMP, further strengthening the current thought that Hem1 is important in proper B cell function and preventing autoimmunity.

MATERIALS AND METHODS

Mice

Nckap1l floxed mice (*Hem1*$^{fl/fl}$) were bred to mice expressing Cre-recombinase under the control of the *Meox2* or *Mb1* promoter to generate B cell specific deletion on Hem1 (ref). *Hem1*$^{fl/fl}$*Mb1-Cre* mice were screened and maintained by genomic PCR analysis, following amplification with *Hem1floxed* forward (5′- -3′) and reverse (5′- -3′) oligonucleotides, *Mb1*cre forward (5'-3') and reverse (5'-3') primers. Mice were housed under Specific Pathogen-Free conditions. Mouse procedures were approved by the Institutional Animal Care and Use Committees of the University of Washington.

In vivo migration assay

Spleens from *Hem1*$^{fl/fl}$*Mb1-Cre* and WT mice were collected from donor mice and serve as donors. B lymphocytes were purified by positive selection with CD45R (B220) microbeads (Miltenyi Biotec) according to the manufacturer's procedure. B lymphocytes from WT and Hem1fl/flMb1cre were stained using CFDA/SE (Invitrogen) and Cell Trace Violet (Invitrogen) respectively. Dye-labelled cells were mixed at a 1:1 ratio prior to injection. C57BL6/J recipient mice were injected with 10^6 B lymphocytes via tail vein injection. 24hrs later, spleens, femurs, inguinal, submandibular, and mesenteric lymph nodes were collected. Donors cells were tracked and assessed by flow cytometry.

In vitro migration

Total splenic cells from *Hem1*$^{fl/fl}$*Mb1-Cre* and WT mice were prepared. Transwell plates (5-μm pore, Costar) loaded at the lower chamber with media (RPMI 1640, 2 mM glutamine, and 0.5% BSA) were warmed at 37°C one hour prior to the addition of splenocytes. Chemotactic reagent,

CXCL-12 (0.2mg/ml), was added to the lower chamber prior to the addition of $5x10^5$ cells/well to the upper chamber. Three hours later, cells were collected from the top and bottom of the chamber and analyzed by flow cytometry. The percentage of migrating B cells was calculated by dividing the absolute number of cells recovered in the lower well divided by the sum of the absolute number of cells recovered in the upper and lower chambers.

In vivo tracking sinusoidal B cell populations

Labeling of sinusoidal cells was performed by injecting *Hem1$^{fl/fl}$Mb1-Cre* and WT mice with 0.1 μg/mouse PE-labeled anti-CD19 antibody via retro-orbital injection (clone 1D3, BD Biosciences) while mice were under anesthetization using 5% isoflurane in an induction chamber and maintained on 0.5 – 2% isoflurane via a nose cone. Two minutes after injection mice were euthanized immediately to collect bone marrow and spleen. Cells were analyzed with flow cytometry

Bacteria and pneumococcal infection model

Frozen *Streptococcus pneumoniae* serotype 2 Strain D39 (10^8cfu) were heat-killed by incubation in a water bath (60°C) duration one hour. Bacterial viability was confirmed by plating 50 uL of the bacterial stock on blood agar plates overnight. The heat-killed bacteria were washed and resuspended in PBS. *Hem1$^{fl/fl}$Mb1-Cre* and WT mice were anesthetized within an isoflurane chamber and received *S. pneumoniae (*10^7 cfu) via retro- orbital or intraperitoneal injection. Sera was collected from mice prior to and 5 days after heat-killed *S. pneumoniae* administration to measure antibody titer. For pneumococcal infection, mice were immunized with heat-killed *S. pneumoniae* (10^7 cfu) either i.p. or i.v. Three days later, mice were challenged with *S. pneumoniae* (10^7 cfu) at lethal dose via an intranasal route. Mice were monitored and weighed

daily for 10 days and were euthanized when respiratory distress or body weight loss (≥20%) was observed.

T-dependent and independent antibodies production

Mice between 6 – 10 weeks of age were immunized with 100 μg/mouse NP-KLH (Biosearch Technologies) emulsified with Imject alum adjuvant (Thermo Scientific) or 50ug/ml NP-Ficoll (50 μg/mL) intraperitoneally. For NP-KLH immunization, sera were obtained prior to immunization and then each week for up to 4 weeks following immunization. 8 weeks later, mice were boosted with 20 μg/mL NP-KLH intraperitoneally. For NP-Ficoll immunization, sera were obtained prior to immunization and 6 days post-immunization.

ELISA

NUNC Maxisorb plates were coated with PC BSA (50 ug/ml) (Biosearch Technologies), NP BSA_2 or NP BSA_{30} (10ug/ml) (Biosearch Technologies), overnight at 4°C. Sera from immunized mice were serially diluted and incubated overnight at 4°C Sera from unimmunized mice was used as a background control. Antibodies titers were measured by Spectrophotometer at optical density 405 using horseradish peroxidase conjugated isotype antibodies (SouthernBiotech).

IgM internalization assay

Splenic cells (2×10^6 cells/well) were plated and were incubated with biotinylated anti-IgM antibody in cell culture media on ice for 30 minutes. After then IgM internalization were tracked and measured for 0, 2, 5, 10, 15 and 20 minutes at 37°C. The presence of cell surface IgM was measured by flow cytometry after staining with streptavidin conjugated PE in combination with fluorescent conjugated B220.

B lymphocyte activation

Splenocytes (2 x 10^6 cells/well) were stimulated with 10 μg/mL anti-IgM antibody (Invitrogen) for overnight. Splenocytes were stained with fluorescent conjugated anti CD25, anti CD69, anti B220, and were analyzed by flow cytometry.

Immunoblot analysis

Immunoblot analyses were performed as previously described (Iritani et al. 1997) using rabbit polyclonal antibodies or mouse monoclonal antibodies specific for βActin, Akt, P-Ser473 Akt, P-Ser240/244 S6 Ribosomal Protein, P-Erk, α-rabbit IgG HRP (Promega), and others were purchased from Cell Signaling.

Flow cytometry

Bone marrow (BM) and spleen were stained with fluorescent-conjugated antibodies specific for B220, CD93, CD19, CD21, CD23, CD24, CD62L, CD25, CD43, IgD, IgM, Igλ, Igκ, BrdU, Caspase3/7, Ghost dye™, NP-PE (Biosearch Technologies) and analyzed by flow cytometry.

Calcium influx assay

Intracellular Ca^{2+} was measured with a cell-permeable form of indo1-AM (Invitrogen). Splenic B cells (10^7 cells) washed with Ca^{2+}-free buffer and resuspended in 2 ml cell loading media (RPMI, 2% FCS, 25mM HEPES pH 7.4) containing 1.5mM Indo1 AM. Fluorescence of cell suspensions was detected with LSRII flow cytometry at an excitation wavelength of 350 nm (UV) and Indo1-AM fluorescence detection filter wavelengths of 395nm. Collection of a

baseline measurement was followed by stimulation with 10 μg/ml anti-μ (F(ab′)2 fragment), and ratiometric Ca^{2+} values (DAPI:Indo) were plotted as a function of time. Ionomycin (1mg/ml) stimulation was used for positive control.

Statistical analysis

Data were analyzed using the Student's one or two-tailed *t*-test using Prism 6. $P<0.05$ were considered as significant values.

FIGURES

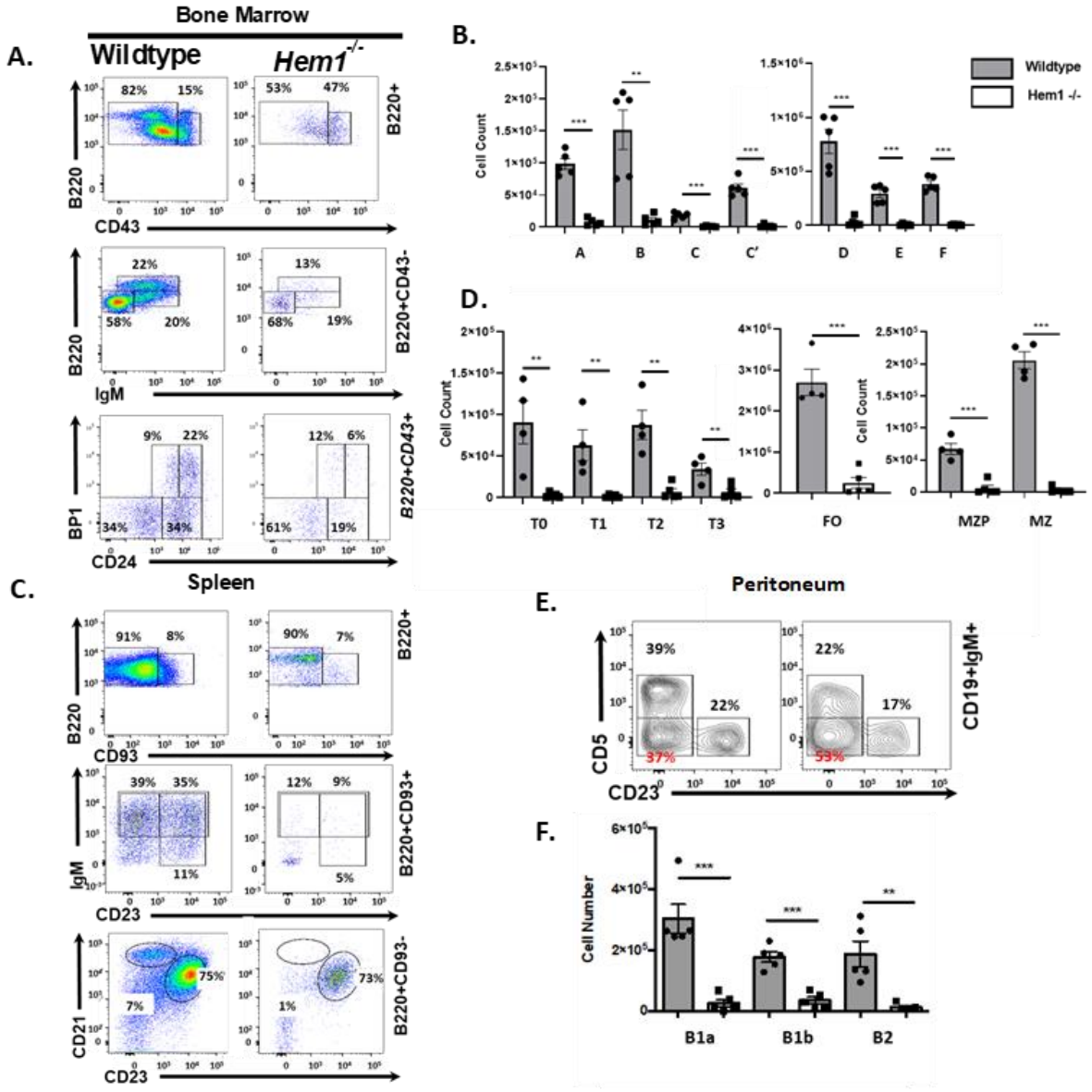

Figure 1. Constitutive deletion of Hem1 results in central and peripheral B cell lymphopenia. (A - B) Flow cytometry and quantification of B cell populations within the BM of 6 – 12-week-old WT and *Hem1*$^{-/-}$ mice. (C- D) Flow cytometry and quantification of B cell populations within the spleen of WT and *Hem1*$^{-/-}$ mice. (E - F) Flow cytometry and quantification of B cells in the peritoneum of WT and *Hem1*$^{-/-}$ mice. Data presented in mean ± SEM and was analyzed via unpaired Student's t-test. *p<0.05, **p<0.01, *** p<0.001

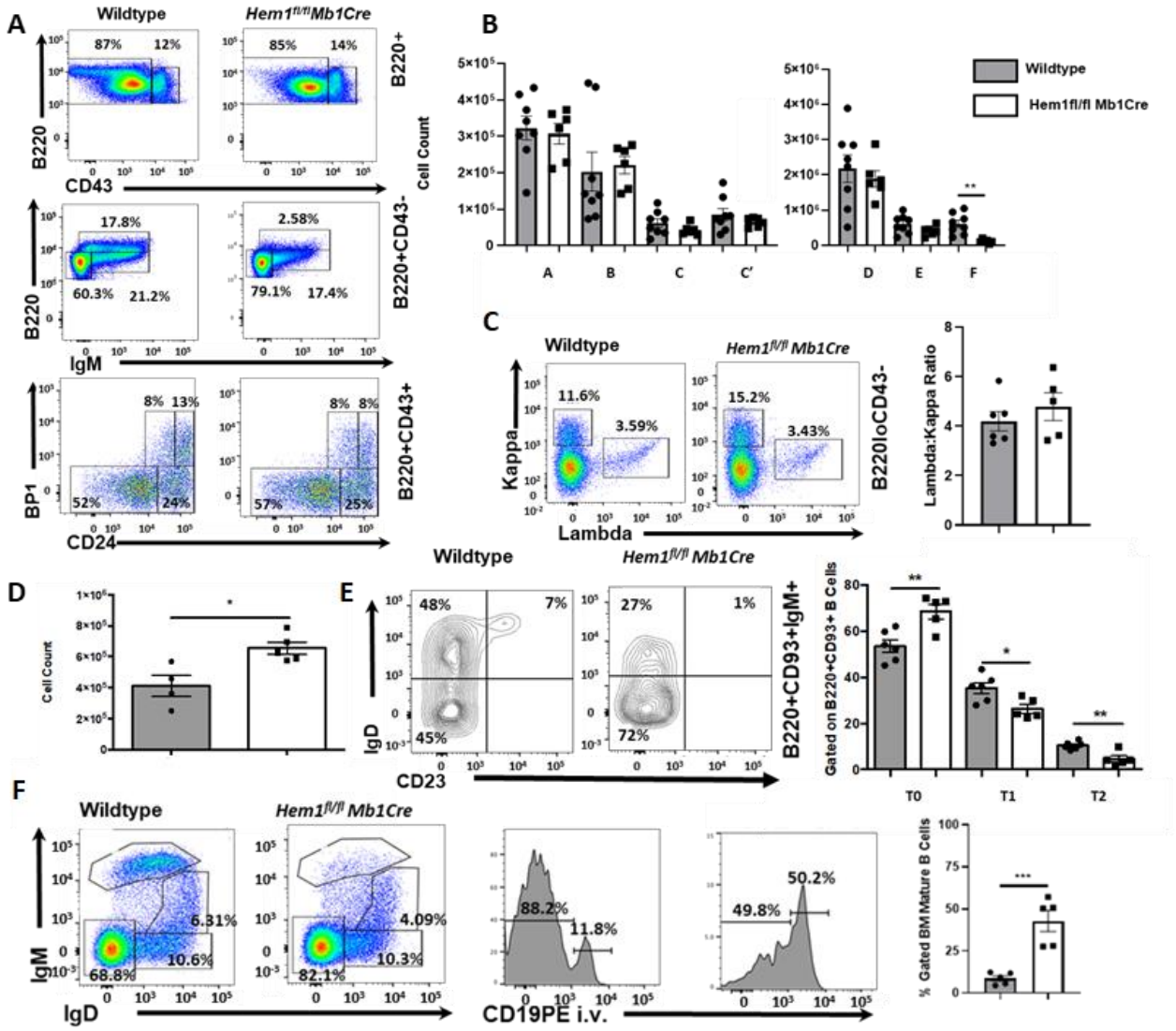

Figure 2. Conditional deletion of Hem1 in B cells results in central mature recirculating B cell lymphopenia. (A - B) Flow cytometry and quantification of B cell populations within the BM of 6 – 12-week-old WT and *Hem1$^{fl/fl}$Mb1-Cre* mice. (C) Flow cytometry of light chain (κ and λ) rearrangement in immature (B220loCD43-) B cells with graphical representation of λ and κ ratio. (D) Quantification of T0 (B220+CD93+IgM+IgD-CD23-) B cells in the bone marrow of WT and *Hem1$^{fl/fl}$Mb1-Cre* mice. (E) Flow cytometry of and graphical representation of the proportions of transitional B cells in the blood of WT and *Hem1$^{fl/fl}$Mb1-Cre* mice. (F) *In vivo* labeling with anti-CD19PE of cells in the BM after 2 minutes post-injection to demonstrate the proportions of mature recirculating follicular B cells (B220+IgD+, IgM+) in the BM sinusoids. Data presented in mean ± SEM and analyzed via unpaired Student's t-test or paired Student's t-test in (F). *p<0.05, **p<0.01, *** p<0.001

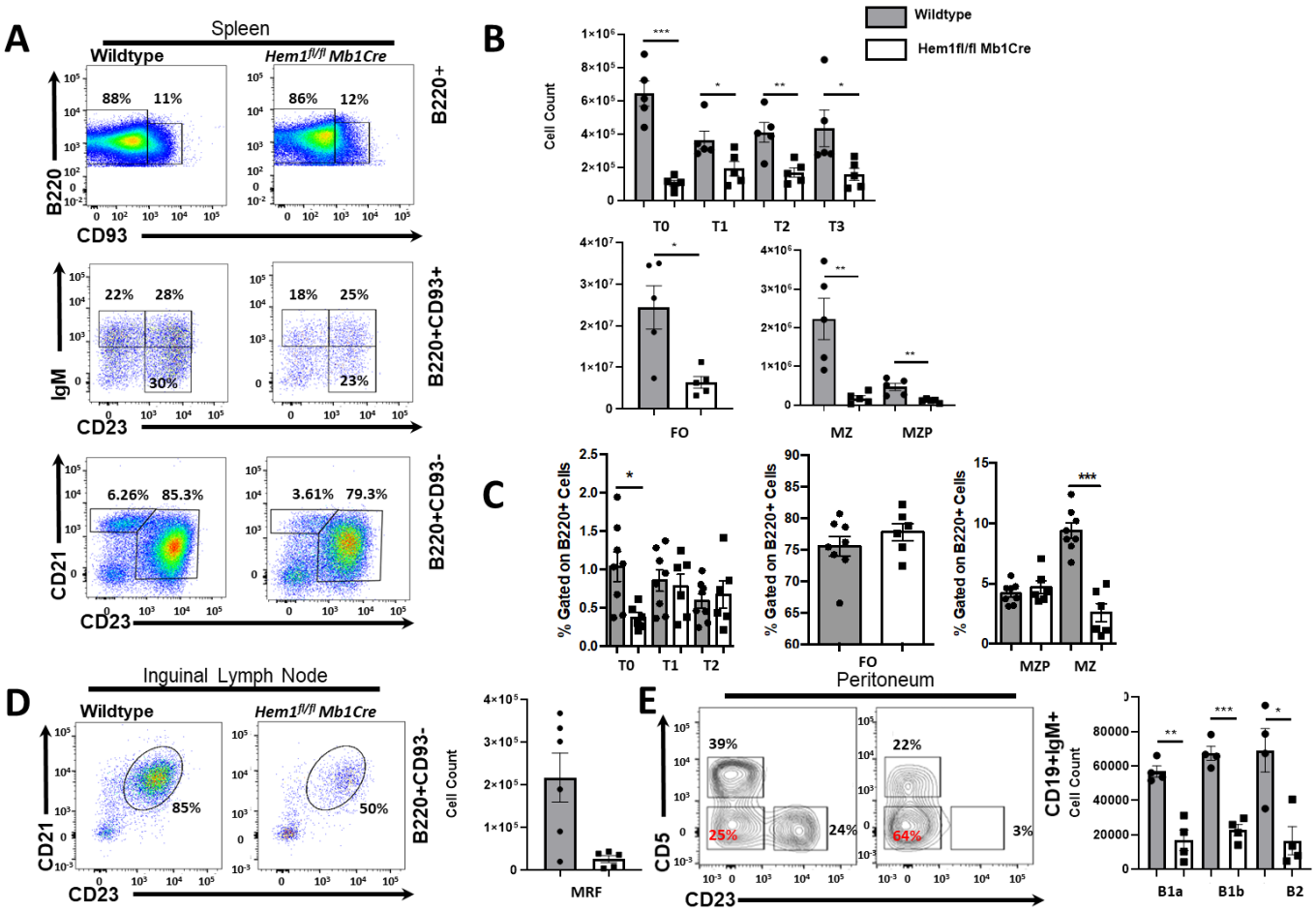

Figure 3. Conditional deletion of Hem1 in B cells results in peripheral B cell lymphopenia. (A - B) Flow cytometry and quantification of B cell populations within the spleen of 6 – 12-week-old WT and *Hem1*$^{fl/fl}$*Mb1-Cre* mice. (C) Flow cytometry and graphs of the proportions of each B cell type based on B220+ gating (D) quantification of MRF B cells in inguinal LN (E) Flow cytometry and quantification of B cells in the peritoneum. (Data presented in mean ± SEM and analyzed via unpaired Student's t-test or paired Student's t-test in (F). *p<0.05, **p<0.01, *** p<0.001

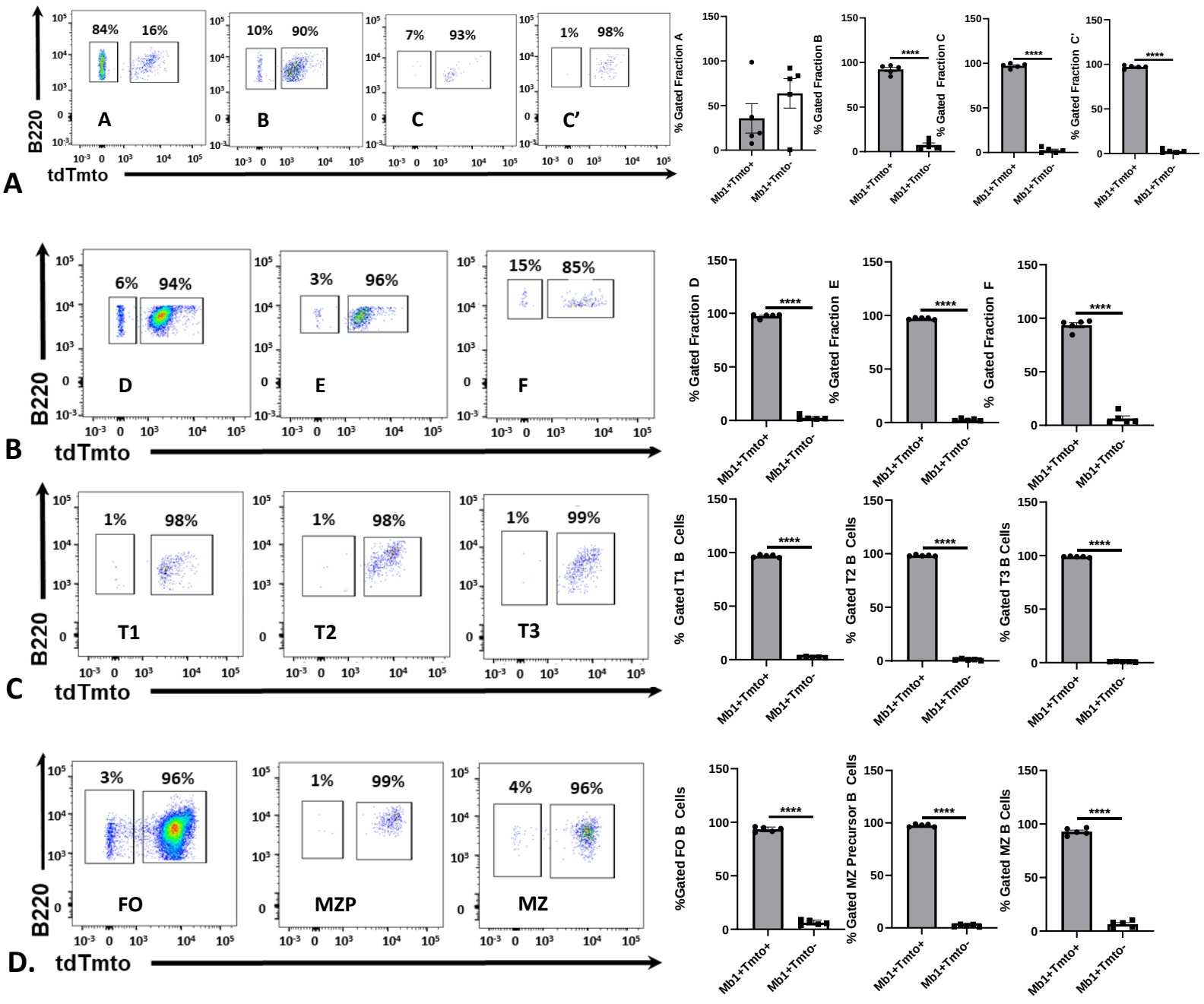

Figure 4. Use of reporter TdTmto demonstrates Cre-recombinase activity in all stages of B cell development in *Hem1$^{fl/fl}$Mb1-Cre* mice. (A) Expression of tdTmto in B220+CD43+ B cells from the bone marrow of *Hem1$^{fl/fl}$Mb1-Cre* mice. (B) Expression of tdTmto in B220+Cd43- B cell populations in the BM of *Hem1$^{fl/fl}$Mb1-Cre* mice. (C) Expression of tdTmto in B220+CD93+ B cell populations in the spleen of *Hem1$^{fl/fl}$Mb1-Cre* mice. (D) Expression of tdTmto in B220+CD93- B cell populations in the spleen of *Hem1$^{fl/fl}$Mb1-Cre* mice. Data presented in mean ± SEM and analyzed via paired Student's t-test
*p<0.05, **p<0.01, *** p<0.001

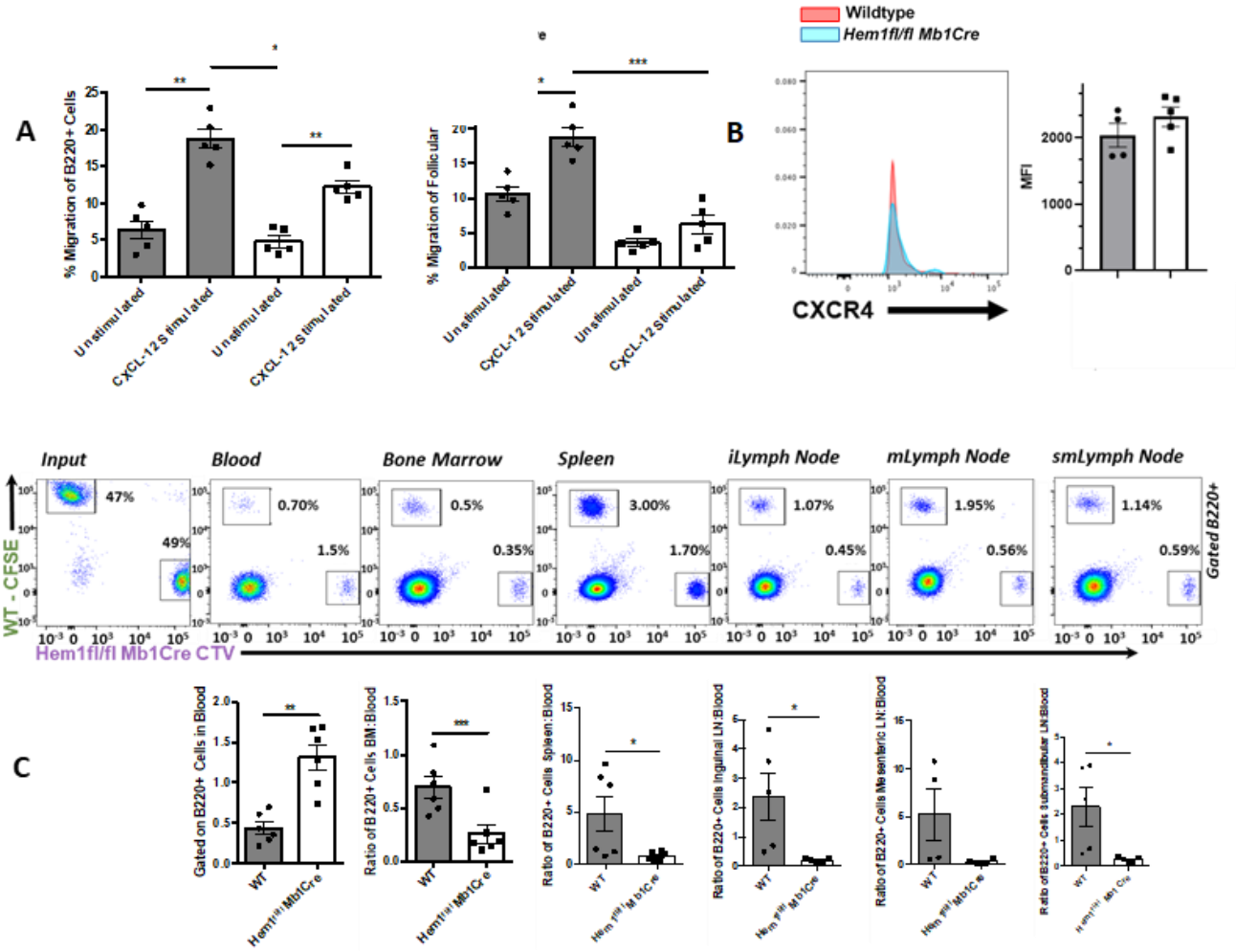

Figure 5. Conditional deletion of Hem1 in B cells results in disrupted migratory response of CXCL-12 and homing to lymphoid tissues. (A) *In vitro* migration through a 3 μm transwell plate following stimulation with 0.2 μg/mL CXCL-12 of total B220+ cells and follicular B cells from the spleens of WT and *Hem1*$^{fl/fl}$*Mb1-Cre* mice. (B) Mean fluorescence intensity for the expression of CXCR4 on the cell surface. (C) Flow cytometry of the donor cells combined at a 1:1 ratio and the subsequent ratios in the blood and lymphoid tissues. Also included is the ratio of B220+ cells in each tissue and those found in the blood of recipient mice. Data presented in mean ± SEM and analyzed via paired Student's t-test in (F). *p<0.05, **p<0.01, *** p<0.001

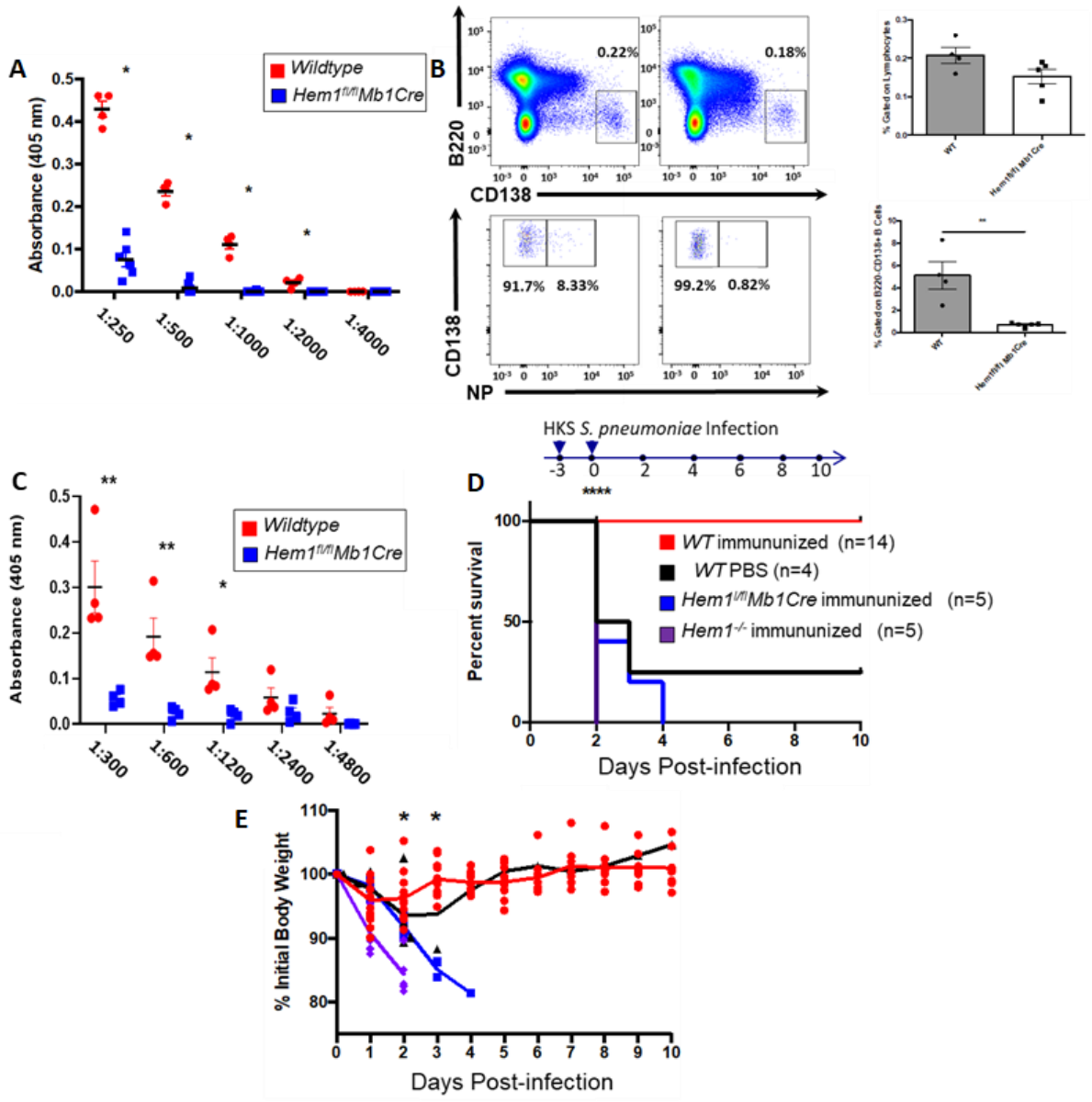

Figure 6. Hem1 is required for T-independent antibody production and protection against *Streptococcus pneumoniae* infection. (A) Antibody production six-days post i.p. immunization of NP-ficoll as measured by ELISA. (B) The ratio of plasma cells (B220-CD138+) in mice immunized with NP-ficoll and determination of expansion of NP-specific plasma cells. (C) IgM production five-days post i.v. immunization of heat-killed *Streptococcus pneumoniae* as measured by ELISA. Unimmunized WT and immunized WT, *Hem1*$^{-/-}$ and *Hem1*$^{fl/fl}$*Mb1-Cre* mice were challenged with 10^7 cfu *S. pneumoniae* and (D) were monitored for morbidity and mortality and (D) weighed daily. Data presented in mean ± SEM and analyzed via unpaired Student's t-test. *p<0.05, **p<0.01, *** p<0.001

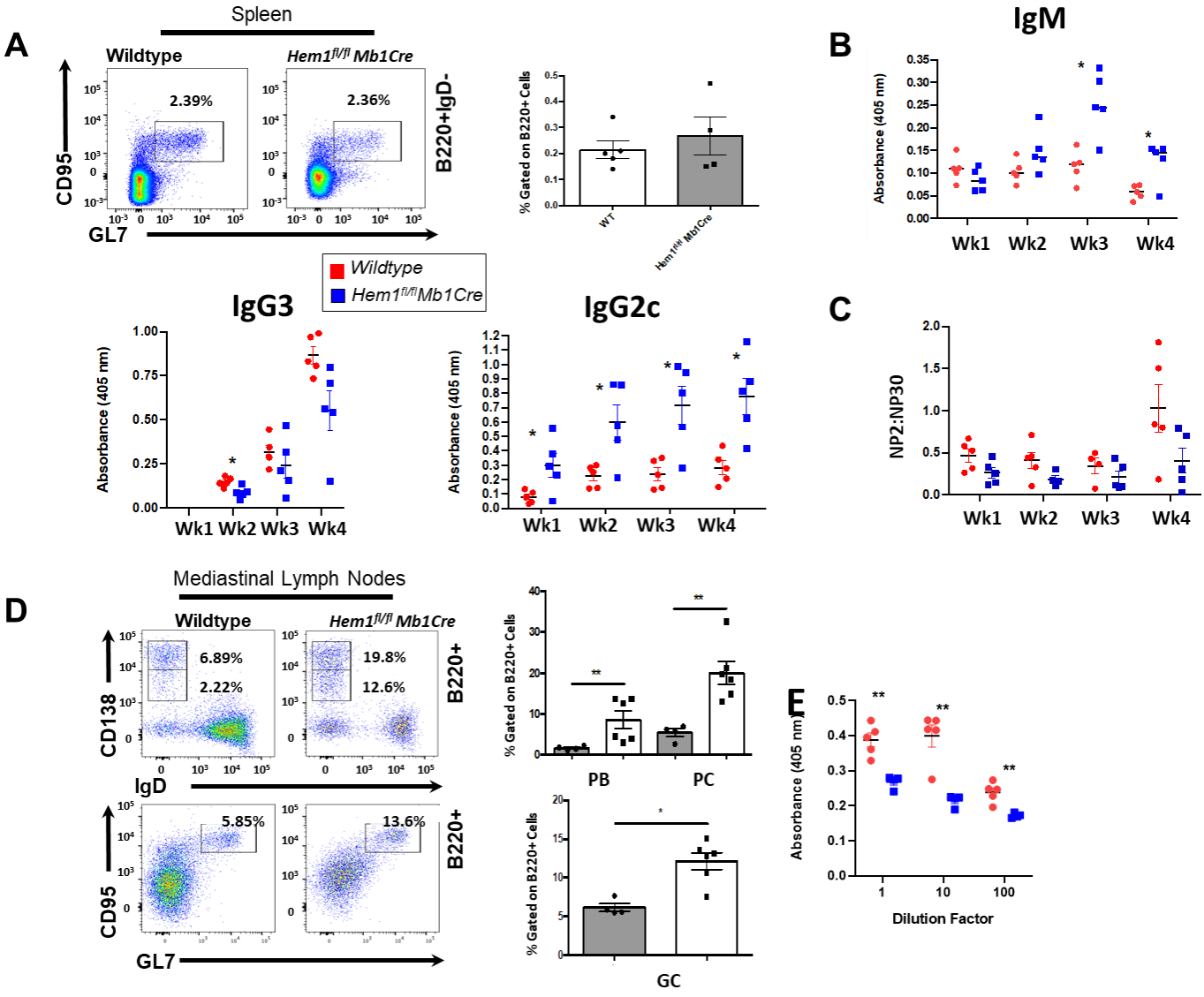

Figure 7. Conditional deletion of Hem1 in B cells results in increased IgG2c antibody production. Eight to ten-week-old mice were immunized with 100 ug/mouse NP-KLH in alum. (A) Expansion of germinal center in the spleens of WT and *Hem1$^{fl/fl}$Mb1-Cre* mice 10 days post-immunization. Antibody production following immunization with NP-KLH in alum for (B) IgM, IgG3 and IgG2c. Affinity maturation as determined by ratio of antibody binding to high-affinity NP-2 and low-affinity NP-30. (D) Plasma cell (PC) and plasmablasts (Pb) and germinal center (GC) formation 10 days post-infection with graphical representation of their proportions. (E) IgM measured from collected bronchoalveolar lavage fluid via ELISA. Data presented in mean ± SEM and analyzed via unpaired Student's t-test. *p<0.05, **p<0.01, *** p<0.001

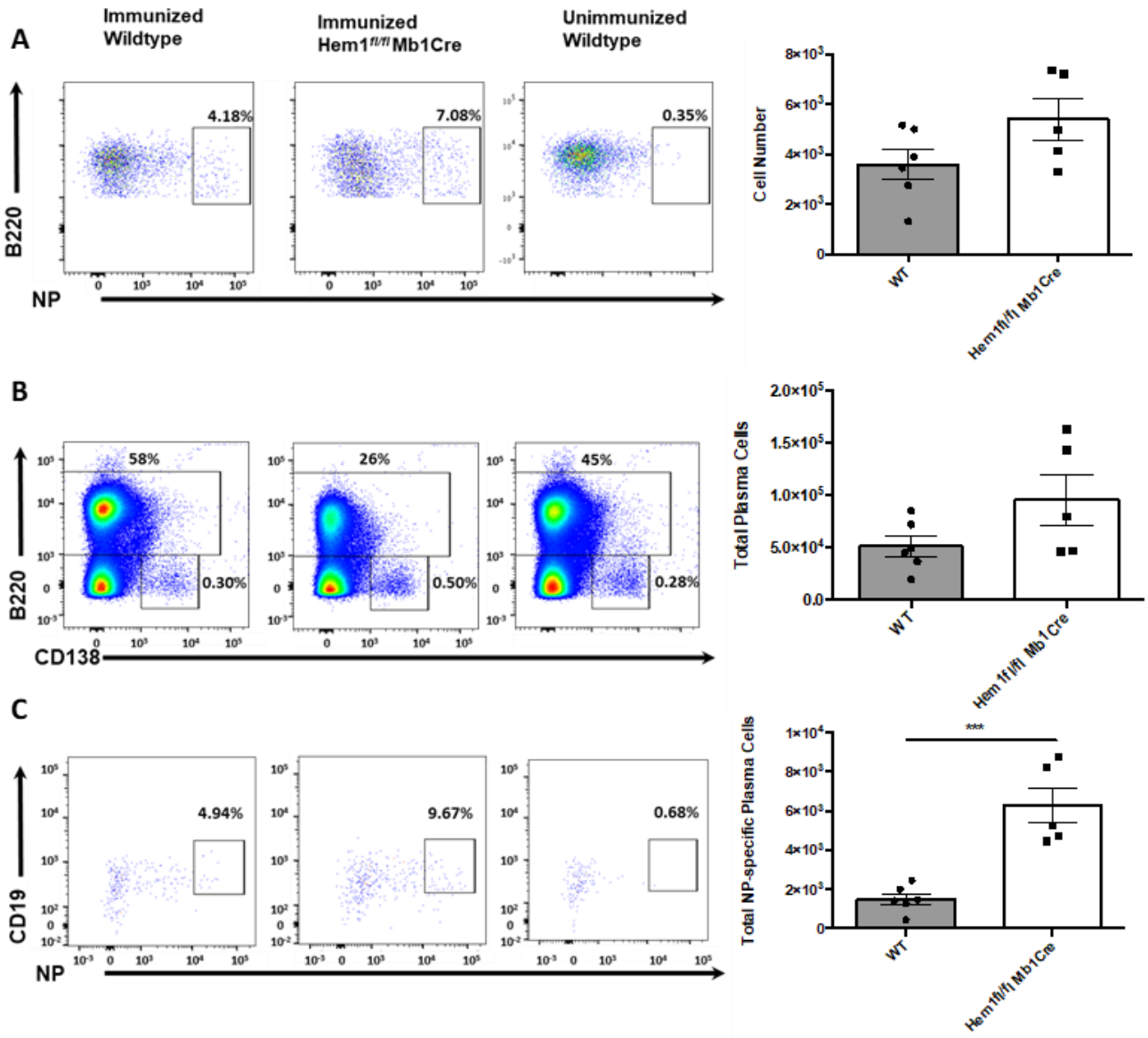

Figure 8. Conditional deletion of Hem1 favors the expansion NP-specific plasma cells following boost-immunization. Four weeks following initial immunization with NP-KLH in alum, mice were boosted with 20 ug/mouse NP-KLH in PBS. (A) Memory B cell (B220+IgD-CD38+CD19+NP+) quantification 7 days post—boost immunization from immunized WT, and *Hem1*$^{fl/fl}$*Mb1-Cre* mice and an unimmunized control. (B) Number of plasma cell (B220-CD138+) quantification from immunized WT, and *Hem1*$^{fl/fl}$*Mb1-Cre* mice and an unimmunized control. (C) NP-specific plasma cell (B220-CD138+CD19+NP+) quantification from immunized WT, and *Hem1*$^{fl/fl}$*Mb1-Cre* mice and an unimmunized control. Data presented in mean ± SEM and analyzed via unpaired Student's t-test. *p<0.05, **p<0.01, *** p<0.001

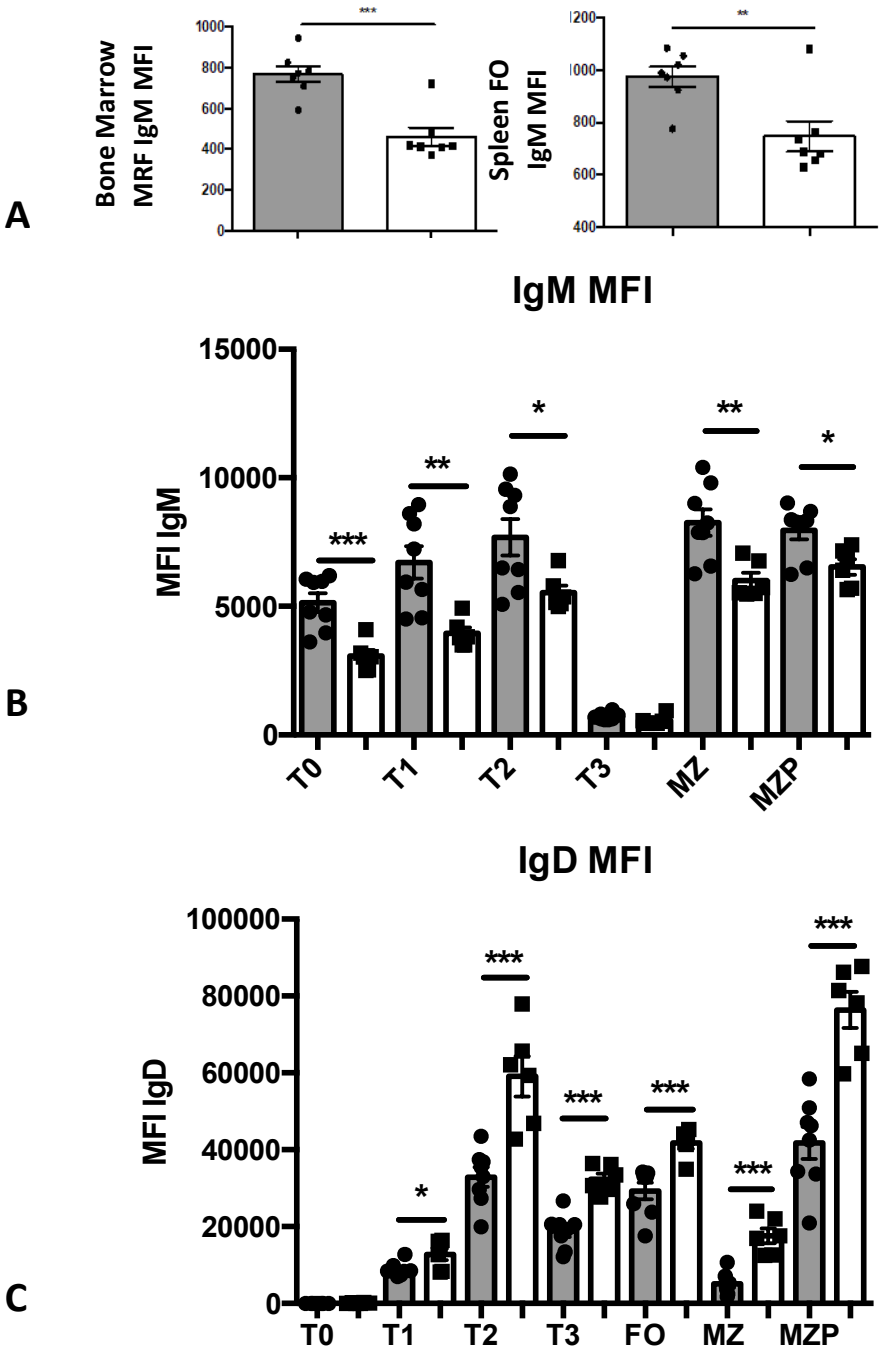

Figure 9. Surface expression of IgM is decreased on *Hem1$^{fl/fl}$Mb1-Cre* B cells. (A) MFI of surface IgM expression on mature recirculating follicular B cells in the bone marrow and follicular B cells in the spleen. (B) MFI of surface IgM expression throughout development in the spleen. (C) MFI of surface IgD expression throughout development in the spleen. Data presented in mean ± SEM and analyzed via unpaired Student's t-test). *p<0.05, **p<0.01, *** p<0.001

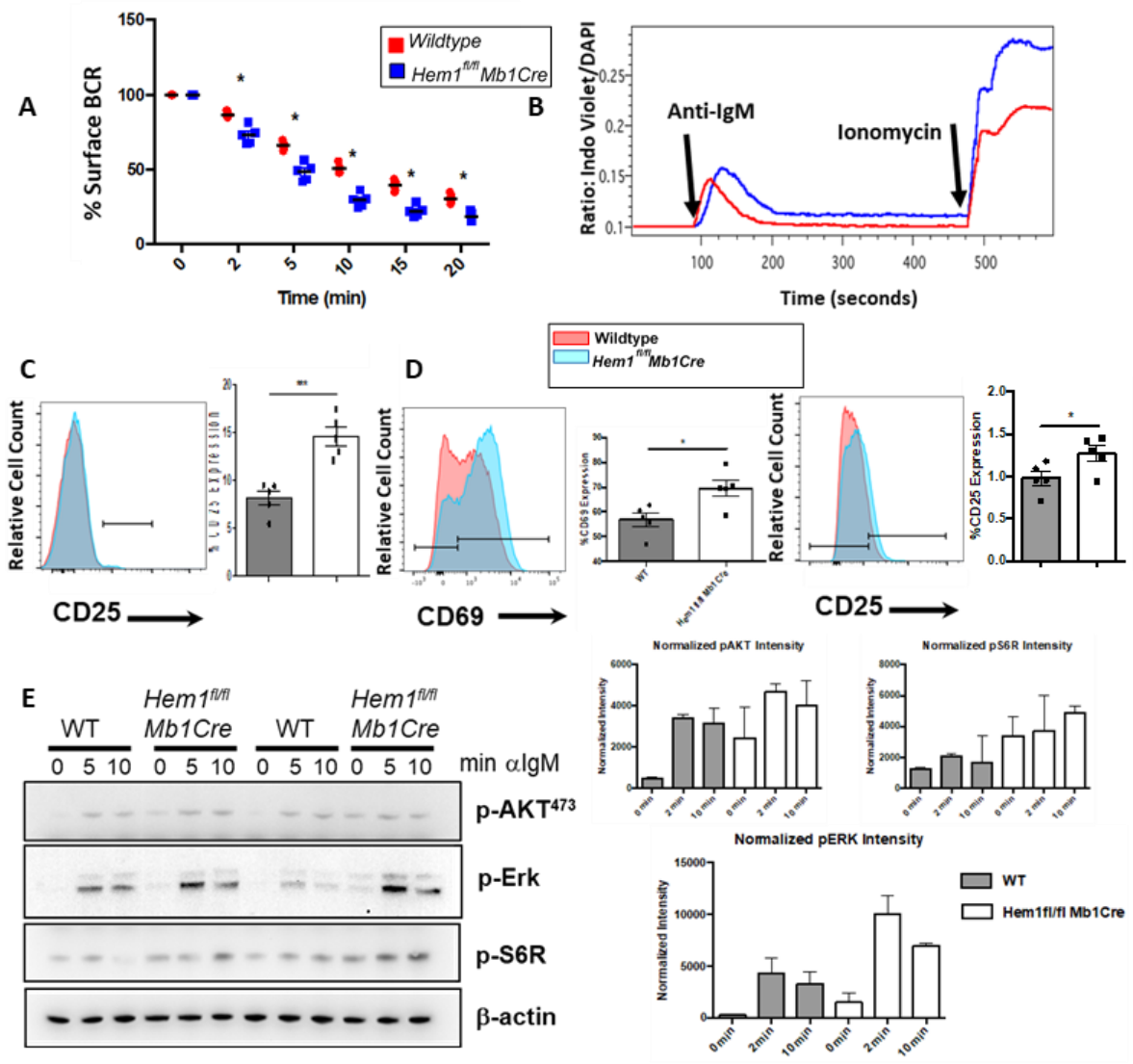

Figure 10. Conditional deletion of Hem1 in B cells results in hyper-response against IgM activation and a higher basal level of activation downstream of IgM. (A) B cells were treated with anti-IgM on ice then moved to warmed media to allow for IgM internalization; the MFI at each time point was compared to that of 0 minutes. (B) B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice were stimulated with anti-IgM followed by ionomycin to determine BCR induced calcium influx. (C) Mature B cells (B220+CD93-CD21+CD23+) from experimentally naïve WT and *Hem1$^{fl/fl}$Mb1-Cre* mice were assessed for CD25 MFI. (D) B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice were treated with 20 ug/mL anti-IgM for 16 hours and the two activation markers were assessed. (E) B cells from WT and *Hem1$^{fl/fl}$Mb1-Cre* mice were stimulated for 0, 5, and 10 minutes and activation downstream of IgM was determined through Western blot as determined from two independent individuals. Relative intensity determined through Image J. Data presented in mean ± SEM and analyzed via unpaired Student's t-test. *p<0.05, **p<0.01, *** p<0.001

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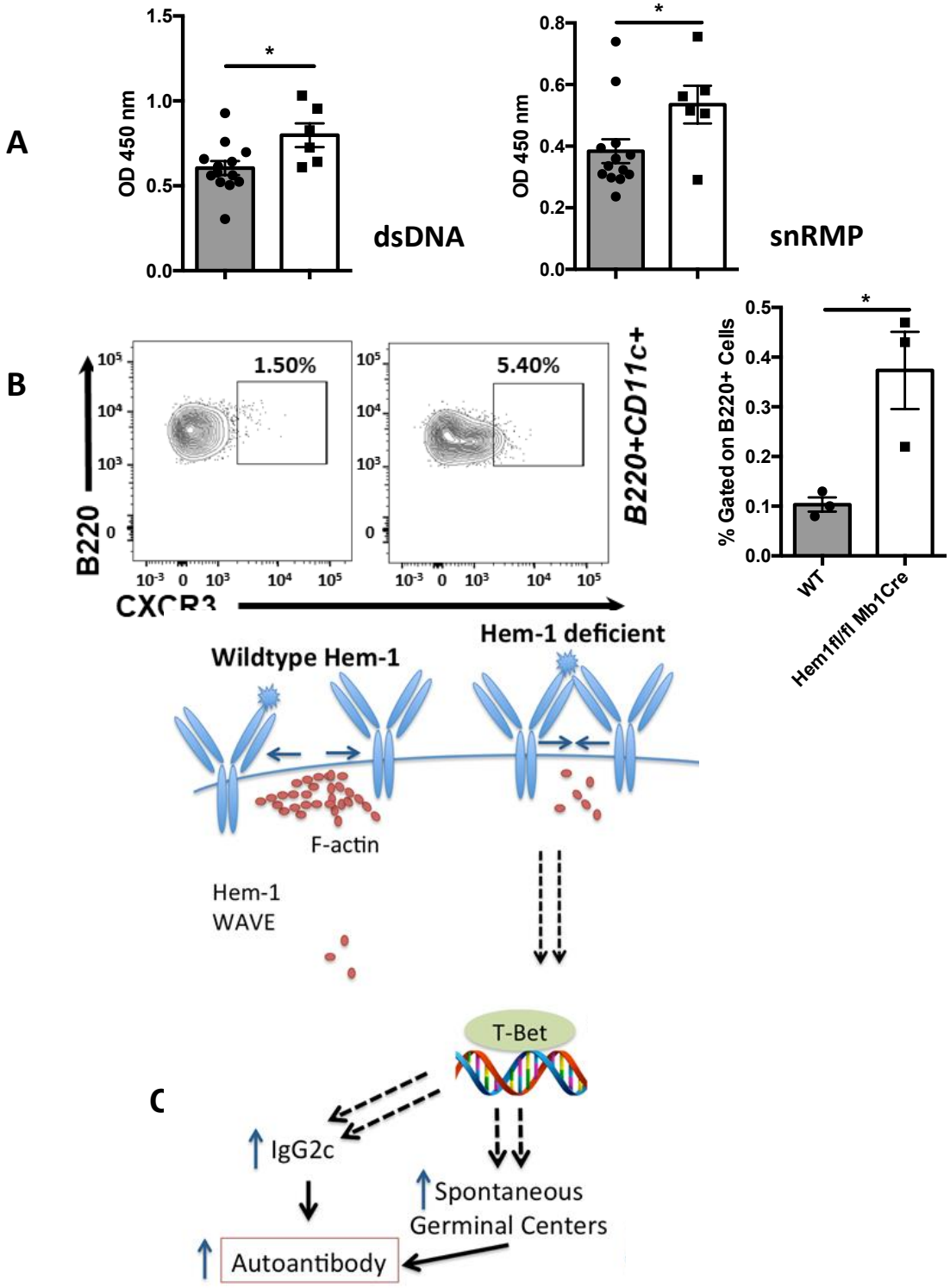

Figure 11. *Hem1$^{fl/fl}$Mb1-Cre* B cells have a higher proportion of age-associated B cells and produce auto-antibody. (A) *Hem1$^{fl/fl}$Mb1-Cre* mice of six-months of age and varying sexes produce auto-antibodies against dsDNA and snRMP. (B) *Hem1$^{fl/fl}$Mb1-Cre* mice have T-bet+CD11c+ B cells known as age-associated B cells; these age associated B cell have a higher proportion of cells expressing CXCR3, a marker directly downstream of the transcription factor T-bet. (C) Chronic stimulation of the BCR is permissive for the formation of T-bet+CD11c+ B cells which lead to higher IgG2c antibody production and autoantibody production. Data presented in mean ± SEM and analyzed via unpaired Student's t-test or paired Student's t-test in (F). *p<0.05, **p<0.01, *** p<0.001

References

Alkhairy, O. K., N. Rezaei, R. R. Graham, H. Abolhassani, S. Borte, K. Hultenby, C. Wu, A. Aghamohammadi, D. A. Williams, T. W. Behrens, L. Hammarström, and Q. Pan-Hammarström. 2015. "RAC2 loss-of-function mutation in 2 siblings with characteristics of common variable immunodeficiency." *J Allergy Clin Immunol* 135 (5):1380-4.e1-5. doi: 10.1016/j.jaci.2014.10.039.

Antón, I. M., M. A. de la Fuente, T. N. Sims, S. Freeman, N. Ramesh, J. H. Hartwig, M. L. Dustin, and R. S. Geha. 2002. "WIP deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation." *Immunity* 16 (2):193-204. doi: 10.1016/s1074-7613(02)00268-6.

Barnett, B. E., R. P. Staupe, P. M. Odorizzi, O. Palko, V. T. Tomov, A. E. Mahan, B. Gunn, D. Chen, M. A. Paley, G. Alter, S. L. Reiner, G. M. Lauer, J. R. Teijaro, and E. J. Wherry. 2016. "Cutting Edge: B Cell-Intrinsic T-bet Expression Is Required To Control Chronic Viral Infection." *J Immunol* 197 (4):1017-22. doi: 10.4049/jimmunol.1500368.

Burbage, M., S. J. Keppler, F. Gasparrini, N. Martínez-Martín, M. Gaya, C. Feest, M. C. Domart, C. Brakebusch, L. Collinson, A. Bruckbauer, and F. D. Batista. 2015. "Cdc42 is a key regulator of B cell differentiation and is required for antiviral humoral immunity." *J Exp Med* 212 (1):53-72. doi: 10.1084/jem.20141143.

Campellone, K. G., and M. D. Welch. 2010. "A nucleator arms race: cellular control of actin assembly." *Nat Rev Mol Cell Biol* 11 (4):237-51. doi: 10.1038/nrm2867.

Comrie, William A., M. Cecilia Poli, Sarah A. Cook, Morgan Similuk, Andrew J. Oler, Aiman J. Faruqi, Douglas B. Kuhns, Sheng Yang, Alexandre F. Carisey, Benjamin Fournier, D. Eric Anderson, Susan Price, Wadih Abou Chahla, Alexander Vargas-Hernandez, Lisa R. Forbes, Emily M. Mace, Tram N. Cao, Zeynep H. Coban-Akdemir, Shalini N. Jhangiani, Donna M. Muzny, Richard A. Gibbs, James R. Lupski, Jordan S. Orange, Geoffrey D.E. Cuvelier, Moza Al Hassani, Nawal AL Kaabi, Zain Al Yafei, Soma Jyonouchi, Nikita Raje, Jason W. Caldwell, Yanping Huang, Janis K. Burkhardt, Sylvain Latour, Baoyu Chen, Gehad ElGhazali, V. Koneti Rao, Ivan K. Chinn, and Michael J. Lenardo. 2019. "Genetic immunodeficiency and autoimmune disease reveal distinct roles of Hem1 in the WAVE2 and mTORC2 complexes." *bioRxiv*:692004. doi: 10.1101/692004.

Croker, B. A., D. M. Tarlinton, L. A. Cluse, A. J. Tuxen, A. Light, F. C. Yang, D. A. Williams, and A. W. Roberts. 2002. "The Rac2 guanosine triphosphatase regulates B lymphocyte antigen receptor responses and chemotaxis and is required for establishment of B-1a and marginal zone B lymphocytes." *J Immunol* 168 (7):3376-86. doi: 10.4049/jimmunol.168.7.3376.

Cunningham, A. F., A. Flores-Langarica, S. Bobat, C. C. Dominguez Medina, C. N. Cook, E. A. Ross, C. Lopez-Macias, and I. R. Henderson. 2014. "B1b cells recognize protective antigens after natural infection and vaccination." *Front Immunol* 5:535. doi: 10.3389/fimmu.2014.00535.

Freeman, S. A., V. Lei, M. Dang-Lawson, K. Mizuno, C. D. Roskelley, and M. R. Gold. 2011. "Cofilin-mediated F-actin severing is regulated by the Rap GTPase and controls the cytoskeletal dynamics that drive lymphocyte spreading and BCR microcluster formation." *J Immunol* 187 (11):5887-900. doi: 10.4049/jimmunol.1102233.

Haas, K. M., J. C. Poe, D. A. Steeber, and T. F. Tedder. 2005. "B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae." *Immunity* 23 (1):7-18. doi: 10.1016/j.immuni.2005.04.011.

Hao, S., and A. August. 2005. "Actin depolymerization transduces the strength of B-cell receptor stimulation." *Mol Biol Cell* 16 (5):2275-84. doi: 10.1091/mbc.e04-10-0881.

Henderson, R. B., K. Grys, A. Vehlow, C. de Bettignies, A. Zachacz, T. Henley, M. Turner, F. Batista, and V. L. Tybulewicz. 2010. "A novel Rac-dependent checkpoint in B cell development controls entry

into the splenic white pulp and cell survival." *J Exp Med* 207 (4):837-53. doi: 10.1084/jem.20091489.

Hobeika, E., S. Thiemann, B. Storch, H. Jumaa, P. J. Nielsen, R. Pelanda, and M. Reth. 2006. "Testing gene function early in the B cell lineage in mb1-cre mice." *Proc Natl Acad Sci U S A* 103 (37):13789-94. doi: 10.1073/pnas.0605944103.

Hromas, R., S. Collins, W. Raskind, L. Deaven, and K. Kaushansky. 1991. "Hem-1, a potential membrane protein, with expression restricted to blood cells." *Biochim Biophys Acta* 1090 (2):241-4. doi: 10.1016/0167-4781(91)90109-y.

Iritani, B. M., K. A. Forbush, M. A. Farrar, and R. M. Perlmutter. 1997. "Control of B cell development by Ras-mediated activation of Raf." *EMBO J* 16 (23):7019-31. doi: 10.1093/emboj/16.23.7019.

Jones, D. D., J. R. Wilmore, and D. Allman. 2015. "Cellular Dynamics of Memory B Cell Populations: IgM+ and IgG+ Memory B Cells Persist Indefinitely as Quiescent Cells." *J Immunol* 195 (10):4753-9. doi: 10.4049/jimmunol.1501365.

Karnell, J. L., V. Kumar, J. Wang, S. Wang, E. Voynova, and R. Ettinger. 2017. "Role of CD11c." *Cell Immunol* 321:40-45. doi: 10.1016/j.cellimm.2017.05.008.

Lehman, H. K. 2015. "Autoimmunity and Immune Dysregulation in Primary Immune Deficiency Disorders." *Curr Allergy Asthma Rep* 15 (9):53. doi: 10.1007/s11882-015-0553-x.

Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. "B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals." *J Exp Med* 190 (1):75-89. doi: 10.1084/jem.190.1.75.

Madisen, L., T. A. Zwingman, S. M. Sunkin, S. W. Oh, H. A. Zariwala, H. Gu, L. L. Ng, R. D. Palmiter, M. J. Hawrylycz, A. R. Jones, E. S. Lein, and H. Zeng. 2010. "A robust and high-throughput Cre reporting and characterization system for the whole mouse brain." *Nat Neurosci* 13 (1):133-40. doi: 10.1038/nn.2467.

Martin, F., A. M. Oliver, and J. F. Kearney. 2001. "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." *Immunity* 14 (5):617-29. doi: 10.1016/s1074-7613(01)00129-7.

Mattila, P. K., C. Feest, D. Depoil, B. Treanor, B. Montaner, K. L. Otipoby, R. Carter, L. B. Justement, A. Bruckbauer, and F. D. Batista. 2013. "The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling." *Immunity* 38 (3):461-74. doi: 10.1016/j.immuni.2012.11.019.

Meyer-Bahlburg, A., S. Becker-Herman, S. Humblet-Baron, S. Khim, M. Weber, G. Bouma, A. J. Thrasher, F. D. Batista, and D. J. Rawlings. 2008. "Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis." *Blood* 112 (10):4158-69. doi: 10.1182/blood-2008-02-140814.

Millius, A., S. N. Dandekar, A. R. Houk, and O. D. Weiner. 2009. "Neutrophils establish rapid and robust WAVE complex polarity in an actin-dependent fashion." *Curr Biol* 19 (3):253-9. doi: 10.1016/j.cub.2008.12.044.

Mohr, E., A. F. Cunningham, K. M. Toellner, S. Bobat, R. E. Coughlan, R. A. Bird, I. C. MacLennan, and K. Serre. 2010. "IFN-{gamma} produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine." *Proc Natl Acad Sci U S A* 107 (40):17292-7. doi: 10.1073/pnas.1004879107.

Noviski, M., J. L. Mueller, A. Satterthwaite, L. A. Garrett-Sinha, F. Brombacher, and J. Zikherman. 2018. "IgM and IgD B cell receptors differentially respond to endogenous antigens and control B cell fate." *Elife* 7. doi: 10.7554/eLife.35074.

Park, H., K. Staehling-Hampton, M. W. Appleby, M. E. Brunkow, T. Habib, Y. Zhang, F. Ramsdell, H. D. Liggitt, B. Freie, M. Tsang, G. Carlson, S. Friend, C. Frevert, and B. M. Iritani. 2008. "A point mutation in the murine Hem1 gene reveals an essential role for Hematopoietic protein 1 in

lymphopoiesis and innate immunity." *J Exp Med* 205 (12):2899-913. doi: 10.1084/jem.20080340.

Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. "T-bet regulates IgG class switching and pathogenic autoantibody production." *Proc Natl Acad Sci U S A* 99 (8):5545-50. doi: 10.1073/pnas.082114899.

Pfajfer, L., M. G. Seidel, R. Houmadi, J. Rey-Barroso, T. Hirschmugl, E. Salzer, I. M. Antón, C. Urban, W. Schwinger, K. Boztug, and L. Dupré. 2017. "WIP deficiency severely affects human lymphocyte architecture during migration and synapse assembly." *Blood* 130 (17):1949-1953. doi: 10.1182/blood-2017-04-777383.

Piovesan, D., J. Tempany, A. Di Pietro, I. Baas, C. Yiannis, K. O'Donnell, Y. Chen, V. Peperzak, G. T. Belz, C. R. Mackay, G. K. Smyth, J. R. Groom, D. M. Tarlinton, and K. L. Good-Jacobson. 2017. "c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses." *Cell Rep* 19 (3):461-470. doi: 10.1016/j.celrep.2017.03.060.

Serre, K., A. F. Cunningham, R. E. Coughlan, A. C. Lino, A. Rot, E. Hub, K. Moser, R. Manz, A. Ferraro, R. Bird, K. M. Toellner, J. Demengeot, I. C. MacLennan, and E. Mohr. 2012. "CD8 T cells induce T-bet-dependent migration toward CXCR3 ligands by differentiated B cells produced during responses to alum-protein vaccines." *Blood* 120 (23):4552-9. doi: 10.1182/blood-2012-03-417733.

Svitkina, T. 2018. "The Actin Cytoskeleton and Actin-Based Motility." *Cold Spring Harb Perspect Biol* 10 (1). doi: 10.1101/cshperspect.a018267.

Tallquist, M. D., and P. Soriano. 2000. "Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function." *Genesis* 26 (2):113-5. doi: 10.1002/(sici)1526-968x(200002)26:2<113::aid-gene3>3.0.co;2-2.

Treanor, B., D. Depoil, A. Gonzalez-Granja, P. Barral, M. Weber, O. Dushek, A. Bruckbauer, and F. D. Batista. 2010. "The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor." *Immunity* 32 (2):187-99. doi: 10.1016/j.immuni.2009.12.005.

Tybulewicz, V. L., and R. B. Henderson. 2009. "Rho family GTPases and their regulators in lymphocytes." *Nat Rev Immunol* 9 (9):630-44. doi: 10.1038/nri2606.

Walmsley, M. J., S. K. Ooi, L. F. Reynolds, S. H. Smith, S. Ruf, A. Mathiot, L. Vanes, D. A. Williams, M. P. Cancro, and V. L. Tybulewicz. 2003. "Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling." *Science* 302 (5644):459-62. doi: 10.1126/science.1089709.

Weiner, O. D., M. C. Rentel, A. Ott, G. E. Brown, M. Jedrychowski, M. B. Yaffe, S. P. Gygi, L. C. Cantley, H. R. Bourne, and M. W. Kirschner. 2006. "Hem-1 complexes are essential for Rac activation, actin polymerization, and myosin regulation during neutrophil chemotaxis." *PLoS Biol* 4 (2):e38. doi: 10.1371/journal.pbio.0040038.

Westerberg, L., M. Larsson, S. J. Hardy, C. Fernández, A. J. Thrasher, and E. Severinson. 2005. "Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response." *Blood* 105 (3):1144-52. doi: 10.1182/blood-2004-03-1003.

Yan, C., N. Martinez-Quiles, S. Eden, T. Shibata, F. Takeshima, R. Shinkura, Y. Fujiwara, R. Bronson, S. B. Snapper, M. W. Kirschner, R. Geha, F. S. Rosen, and F. W. Alt. 2003. "WAVE2 deficiency reveals distinct roles in embryogenesis and Rac-mediated actin-based motility." *EMBO J* 22 (14):3602-12. doi: 10.1093/emboj/cdg350.

Zhang, W., H. Zhang, S. Liu, F. Xia, Z. Kang, Y. Zhang, Y. Liu, H. Xiao, L. Chen, C. Huang, N. Shen, H. Xu, and F. Li. 2019. "Excessive CD11c." *Proc Natl Acad Sci U S A* 116 (37):18550-18560. doi: 10.1073/pnas.1901340116.

Zikherman, J., R. Parameswaran, and A. Weiss. 2012. "Endogenous antigen tunes the responsiveness of naive B cells but not T cells." *Nature* 489 (7414):160-4. doi: 10.1038/nature11311.