Fas ligand mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas

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Loss of function of the tumour suppressor *PRDM1/BLIMP* or deregulated expression of the oncogene *BCL6* occurs in a large proportion of diffuse large B cell lymphoma (DLBCL) cases. However, mutation of either gene leads to only slow and infrequent development of malignant lymphoma, and despite frequent mutation of *BCL6* in activated B cells of healthy individuals lymphoma development is rare. Here we show that Blimp1-deficiency or overexpression of Bcl6 in the B cell lineage does not result in overt lymphoma in the presence of T cells. Impairment of T-cell control, however, results in rapid development of DLBCL-like disease, which can be eradicated by polyclonal $CD8^+$ T cells in a T cell receptor, CD28 and Fas-ligand dependent manner. Thus, malignant transformation of mature B cells requires mutations that impair intrinsic differentiation processes and permit escape from T cell-mediated tumour surveillance.

Non-Hodgkin lymphoma is a heterogeneous group of lymphoid malignancies, the most common type being DLBCL[1,2]. DLBCL represents 30-40% of cases of non-Hodgkin lymphoma and despite improved treatment almost half of the DLBCL patients still succumb to their disease[3,4]. A detailed understanding of DLBCL pathogenesis is therefore needed to develop new therapeutic approaches. DLBCL comprises a number of distinct disease entities that are distinguished by differences in oncogenic driver mutations, gene expression signatures and responses to therapy, and include germinal centre B cell (GCB) – DLBCL and activated B cell (ABC) – DLBCL[5-7]. Inactivating mutations of *PRDM1/BLIMP1*, a transcription factor required for plasma cell differentiation[8,9], are prevalent in human ABC-DLBCL[10] and loss of Blimp1 was shown to cause ABC-DLBCL-like malignancy in mice[10,11]. The transcriptional regulator BCL6 was originally identified by its involvement in non-Hodgkin B cell lymphomas[12], and mutations that deregulate its expression occur frequently in GCB- and ABC-DLBCL[12,13]. Later BCL6 was shown to be required for the differentiation of T cell dependent GC B cells[14]. GC B cells express the enzyme activation induced cytidine deaminase (AID), which catalyses mutation in the immunoglobulin gene (*Ig*) loci required for affinity maturation and class

switching in B cells during the immune response[15]. AID activity is also known to mediate DNA damage and transformation of B cells through off-target mutation of other genes, including oncogenes such as *BCL6*[16-18]. However, despite the presence of oncogenic mutations in activated B cells of healthy individuals, lymphoma development is rare[16-18]. While this reflects the requirement for multiple oncogenic changes for complete transformation, it is also consistent with the idea that the immune system has the capacity to recognise and eradicate transformed cells thereby inhibiting the cancer development. Consistent with this idea, B cell lymphomas show a strong association with immune suppression[19], and it has been postulated that defects that result in a break-down of immune surveillance due to immune deficiency, treatment with immuno-suppressive drugs or acquisition of additional mutations can promote the development and progression of B cell lymphomas and other malignancies[20]. In support of this notion, 75% of all DLBCL show mutations that lead to the loss or mis-expression of MHC class I on lymphoma cells[21], and low expression or lack of MHC class II strongly correlates with shorter overall survival[22], suggesting strong selection for lymphoma cells that escape T cell recognition. Several studies have provided evidence that ageing mice lacking the lymphocyte cytotoxic protein perforin are susceptible to the development of spontaneous B cell lymphomas[23,24]. In these studies, a number of different effector lymphocytes were implicated, but the mechanism for the escape from immune surveillance was not established, nor was it shown that T cells prevented spontaneous B cell lymphoma. Thus, despite ample circumstantial evidence, there is currently no direct proof for a critical role of T cells in the suppression of DLBCL.

Here we show that Blimp1-deficiency or overexpression of Bcl6 in the B cell lineage does not result in overt lymphoma in the presence of a functioning T cell compartment. Impairment of T-cell control, however, results in the rapid development of DLBCL-like pathology, which can be eradicated by polyclonal $CD8^+$ T cells in a T cell receptor, CD28 and Fas-ligand dependent manner. Therefore, malignant transformation of mature B cells requires mutations that impair intrinsic differentiation processes and escape from T cell-mediated tumour surveillance.

RESULTS

Accumulation of pre-plasmablasts in the absence of Blimp1

To understand the pathogenesis of ABC-DLBCL and the role of immune surveillance in more detail we aimed to develop a model of accelerated lymphoma development. To this end, we infected mixed bone marrow chimaeric mice containing congenically marked wild-type and Blimp1-deficient B cells (*Blimp1*$^{gfp/gfp}$: Ly5.1)[9] and mice with a B cell-specific ablation of Blimp1 (*Blimp1*$^{fl/fl}$*Cd79aCre*) with the murine γ-herpes virus - 68 (γHV). This establishes a persistent infection that, similar to infection of humans with the related Epstein Barr-Virus (EBV)[25], leads to increased incidence of B cell lymphoma in mice[26]. γHV infection induced the development of plasma cells, characterised by high expression of CD138 and down-regulation of CD19, from wild-type B cells but as expected, not from Blimp1-deficient B cells (**Fig. 1a**). In contrast, Blimp1-deficient B cells showed a robust and progressive increase in the proportions of Fas^{+}Cxcr4^{+} germinal centre (GC)-like cells (**Fig. 1a, b, Supplementary Fig. 1a**). While *Blimp1* expression is usually restricted to CD138^{+} antibody secreting cells (**Supplementary Fig. 1b**), a large but variable fraction of Blimp1-deficient CD19^{+} B cells expressed GFP from the *Blimp1* locus (**Fig. 1c, Supplementary Fig. 2a**). Blimp1-deficient GFP^{+} B cells were large, blast-like cells that did not express CD138, had down-regulated B220 and CD23, and expressed the Pax5 target gene *Embigin*, consistent with their arrest at the pre-plasmablast stage of plasma cell differentiation[9] (**Fig. 1c, Supplementary Fig. 2a, b**). Similar results were obtained in mice immunised with the model antigen nitrophenol coupled to keyhole limpet haemocyanin (**Supplementary Fig. 2c**). However, despite pronounced accumulation of pre-plasmablasts, malignant B cell lymphoma development was not observed over the course of 6 months. Given the established role of Blimp1 as a tumour suppressor in B cells, this was a surprising result, which suggested that strong, possibly extrinsic mechanisms restrained the lymphomagenic potential of Blimp1-deficient B cells.

Accelerated lymphoma development in *Blimp1*-mutant T cell deficient mice

Consistent with the hypothesis that immune surveillance of incipient B cell lymphomas by T cells may play a role in preventing malignant disease that is driven by the loss of Blimp1, we found that aged (12-15 months) *Blimp1*$^{gfp/gfp}$: Ly5.1 chimaeric mice had dramatically reduced populations of splenic B cells (25.5±22.4%) in comparison to control chimaeras (65.4±5.3%) (**Fig. 1d**, upper panel and data not shown). Transfer of Blimp1-deficient B cells from these chimaeras into mice lacking T cells (*Rag1*$^{-/-}$), but not transfer into wild-type control mice, resulted in most cases in splenomegaly with dramatic expansion of mutant B cells (**Fig. 1d**, lower panel and data not shown). This suggested that T cells actively suppressed the expansion of Blimp1-deficient pre-malignant B cells.

To stringently test this hypothesis we generated *Blimp1*-mutant mice on a T cell deficient background (*Blimp1*^{gfp}*Cd3e*$^{-/-}$). Strikingly, all T-cell deficient mice homozygous for the *Blimp1*^{gfp} allele (i.e. Blimp1 deficient) developed severe splenomegaly and succumbed to malignant lymphoma within 230 days (median latency ~130 days, **Fig. 2a, b**). These mice also frequently showed enlarged livers and signs of hind limb paralysis with concurrent lymphocytic infiltration into the spinal cord (**Fig. 2b** and data not shown). Histological examination revealed loss of splenic architecture and infiltration of large lymphoid cells resembling human DLBCL (**Fig. 2b**). T cell sufficient mice that were heterozygous for the *Blimp1*^{gfp} allele (*Blimp1*$^{+/gfp}$) showed no immune deficiencies and did not develop B cell lymphomas[27,28]. Remarkably, loss of one copy of *Blimp1* in the absence of T cells led to development of B cell lymphomas in ~80% of the mice within the observation period (300 days; **Fig. 2a**). In contrast, mice with a B cell specific deletion of *Blimp1* on a T cell sufficient background did not develop B lymphomas within this time frame (**Fig. 2a**), consistent with other reports using a similar model[10,11]. Thus, our data demonstrate that ongoing T cell surveillance is critical for the prevention of spontaneous lymphoma development caused by loss or haplo-insufficiency of the tumour suppressor *Blimp1*.

Lymphomas that developed in *Blimp1*-mutant mice showed considerable heterogeneity. In 52% of affected mice tested (n=26), splenocytes consisted almost exclusively of

$CD19^+B220^+$ cells (termed here type A lymphoma), while the remainder (48%, n=24) had only a small population of $CD19^+$ cells (2.5-27%) with heterogeneous B220 expression (type B lymphomas) (**Fig. 2c**). All lymphomas tested were IgM^+ but lacked the mature B cell markers IgD, CD23 and CD21, and some type B lymphomas expressed low amounts of CD138 and were $CD43^+$ (**Supplementary Fig. 3a**). Lymphomas that developed in *Blimp1*$^{+/gfp}$ mice were of a similar phenotype but showed more varied expression of Blimp1/GFP^+ and surface markers (**Fig. 2d**). Thus, while all lymphomas had an activated B cell phenotype, a spectrum of different stages of plasmacytic differentiation was observed. Analysis of *Ig* gene rearrangements demonstrated that the lymphomas were largely of clonal origin (**Supplementary Fig. 3b**) and, in line with their characterization as DLBCL-like lymphomas, clonal Ig (paraprotein) was not detected in the sera of most sick mice examined (**Supplementary Fig. 3c**). Importantly, in all cases transfer of splenic $CD19^+$ cells from sick mice of either genotype into *Rag1*-deficient recipients resulted in the development of secondary lymphomas of a phenotype similar to the primary lymphoma; however, transfer into wild-type recipients did not (**Fig. 2c**, and data not shown).

AID^+Bcl6^+ B cell lymphomas can arise in the absence of germinal centres

To further characterise the *Blimp1*-mutant lymphomas, we examined the expression of key transcription factors that are critical for plasma cell differentiation and are used to classify B cell lymphomas. Western blot analysis revealed that all lymphomas tested (n=23) expressed high amounts of IRF4 (**Fig. 3a, b**), consistent with their classification as ABC-DLBCL like lymphoma[5,7]. Similar to human ABC-DLBCL[10], *Blimp1* heterozygote lymphomas showed loss or decreased expression of the wild-type allele (**Fig. 3a**). Interestingly, we detected high expression of Bcl6 in 54.5% of the lymphomas, almost all of which were classified as type A by surface marker expression (**Fig. 3a**). Some lymphomas also showed expression of *Aicda* (**Fig. 3b, Supplementary Fig. 3d**), encoding AID, which is involved in the pathogenesis of lymphoma[29]. This was surprising as all lymphomas had arisen in the absence of a functioning T cell compartment, and thus in the absence of any GC activity. In line with this observation, 4 out of 6 lymphomas

examined showed signs of somatic hypermutation of *Ig* genes (**Supplementary Fig. 3e**). Taken together these results provide evidence that a diverse array of ABC-DLBCL-like malignancies, including Bcl6^{+} and AID^{+} lymphomas, can develop in mice as a consequence of *Blimp1* mutations, independent of GC formation.

Bcl6 overexpression in the absence of T cells drives lymphoma development

To examine whether T cell mediated suppression of B lymphoma development is also important in an oncogene driven model of DLBCL, we crossed the B cell-specific *Bcl6* transgene from IμHABcl6 mice[30], onto the *Blimp1*^{gfp}*Cd3e*$^{-/-}$ background. Strikingly, all *Bcl6*-transgenic (*Bcl6*^{Tg}) mice on a T cell deficient background developed lymphoma within 8-12 (10.3±1.6) weeks after birth independent from the *Blimp1* allele (**Fig. 4a**). In contrast, and in agreement with earlier reports[29,30] T cell sufficient *Bcl6*^{Tg} mice developed disease at low frequency and with long latency, with none of the mice developing lymphoma within 200 days, and fewer than 40% within 500 days (**Fig. 4a**). All lymphomas examined from *Bcl6*^{Tg} mice (n=43) consisted of CD19^{+}B220^{+} cells representing type A lymphoma and most (12/14) showed an active *Blimp1* locus, indicating progression towards the pre-plasmablastic stage (**Fig. 4c**). Analysis of *Bcl6*^{Tg} mice before the development of B cell lymphoma revealed the presence of an expanded population of B cells with pre-GC characteristics, including upregulation of Bcl6 specifically in mice deficient for T cells (**Supplementary Fig. 4a**). In line with the notion that T cells can directly recognize incipient B lymphoma cells, T cells of aged *Bcl6*^{Tg} mice showed signs of acute activation as evidenced by expression of CD69 (**Supplementary Fig. 4b**). Thus, T cells are crucial to prevent development of malignant B cell lymphomas that result from an intrinsic block in plasma cell differentiation.

Polyclonal CD8^{+} T cells can eradicate B lymphoma cells

To determine which T cell populations were responsible for preventing emergence of malignant B cell lymphoma, we co-cultured B lymphoma cells derived from *Cd3*$^{-/-}$ mice with polyclonal CD8^{+} or CD4^{+} T cells purified from wild-type mice and measured

expression of the activation markers CD69 and CD25 (**Fig. 5a**). As a positive control, both T cell populations were activated homogenously by CD3 antibody stimulation. Notably, a large fraction of both $CD8^+$ (~10%) and $CD4^+$ (~18%) T cells were activated in the presence of B lymphoma cells, but not in response to non-transformed activated splenic B cells (**Fig. 5a, Supplementary Fig. 5a**). Only $CD8^+$ T cells, however, proliferated and differentiated into $IFN\gamma^+$, granzyme B^+ effector cells in co-culture with B lymphoma cells (**Fig. 5a, Supplementary Fig. 4a** and data not shown). The relatively large fraction of T cells activated in this assay suggested a polyclonal T cell response rather than activation of rare clones specific to select tumour antigens. This inference was supported by the analysis of the T cell receptors (TCR) of the responding $CD8^+$ T cells, which revealed a broad spectrum of TCR variable β chain usage, similar to that seen in naïve or CD3 antibody treated T cells (**Supplementary Fig. 5b**).

To directly test whether polyclonal populations of T cells could control lymphoma growth, we injected lymphoma cells derived from *Blimp1*-mutant or $Bcl6^{Tg}$ mice into $Rag1^{-/-}$ mice, either alone or together with purified $CD4^+$ or $CD8^+$ T cells. While all mice injected with B lymphoma cells alone and most mice co-injected with lymphoma cells and $CD4^+$ T cells developed splenomegaly and succumbed to malignant disease within 3-6 weeks, $CD8^+$ T cells efficiently prevented lymphoma growth for at least 12 weeks (**Fig. 5b, Supplementary Fig. 5c**). $CD8^+$ T cells isolated from healthy $Blimp1^{gfp/+}$ or $Bcl6^{Tg}$ mice were equally capable of controlling the growth of B lymphoma cells derived from T cell deficient $Blimp1^{gfp/+}$ or $Bcl6^{Tg}$ mice, respectively, indicating that it was not GFP or the HA derived epitopes that were recognised by $CD8^+$ T cells (**Supplementary Fig. 5d**). Furthermore, naïve and memory polyclonal $CD8^+$ T cells that had developed in response to unknown non-tumour antigens and were isolated from healthy wild-type mice were similarly capable of controlling lymphoma growth (**Supplementary Fig. 5e**). Taken together these results indicated that $CD8^+$ T cells with a range of different TCRs are activated in response to B lymphoma cells and control the growth of ABC-DLBCL like lymphomas.

$CD8^+$ T cells recognize B lymphoma cells in a TCR and CD28-dependent manner

Molecules that participate in T cell recognition are frequently altered in B cell lymphoma, including MHC-I, MHC-II, CD80 and CD86, suggesting that TCR signals and co-stimulation are involved in tumour surveillance[21,22,31]. All lymphomas tested in this study expressed high amounts of MHC molecules as well as CD80 and CD86 (n=14), both of which are usually restricted to activated B cells and GC B cells[32,33] (**Fig. 5c**). We therefore reasoned that their expression might contribute to activating T cell mediated immune surveillance. Indeed, T cells deficient in CD28, a receptor for CD86 and CD80 that provides co-stimulatory signals required for full T cell activation, failed to control B lymphoma cells *in vivo* (**Fig. 5d**). Similarly, $CD8^+$ T cells with a TCR specific for an ovalbumin-derived peptide (OT-I) or $CD8^+$ T cells lacking the transcription factor IRF4, which is required for TCR-stimulation induced clonal expansion and development of effector function in $CD8^+$ T cells[34], were unable to control lymphoma growth, demonstrating that TCR-mediated recognition of self or tumour antigen and co-stimulation are required for lymphoma suppression (**Fig. 5e**).

$CD8^+$ T cells target B lymphoma cells using the FasL-Fas death pathway

To uncover the mechanisms of T cell mediated control of B cell lymphoma development, we tested the ability of $CD8^+$ T cells deficient in one or several molecules linked to the cytotoxic activity of T cells to eradicate lymphoma cells. Unexpectedly, neither loss of perforin, TRAIL, IFNγ, the transcription factor T-bet, which controls many aspects of $CD8^+$ T cell function[35], nor the combined loss of granzymes A, B and M impaired the control of B cell lymphoma growth in recipient mice (**Fig. 6a**). In contrast, $CD8^+$ T cells deficient in membrane Fas ligand (FasL), which is essential for Fas-induced apoptosis, were impaired in their ability to control the growth of B cell lymphomas (**Fig. 6a**). This correlated with high levels of Fas expression on B cell lymphomas that had arisen in T cell deficient mice (**Fig. 6b**). In contrast, B cell lymphomas that had formed in T cell sufficient mice expressed low levels or no Fas (**Fig. 6b, c**) and were resistant to killing by recombinant FasL (**Fig. 6d, Supplementary Fig. 6**). Strikingly, B lymphomas derived from immune-competent mice were largely resistant to control by FasL-deficient $CD8^+$ T cells (**Fig. 6e**). This reveals that FasL-Fas dependent mechanisms constitute a major non-

redundant component of T cell mediated immune surveillance of B lymphoma whose loss cannot be readily compensated for.

DISCUSSION

The concept of immune surveillance was proposed long ago[36] but few studies have conclusively tested the cellular and molecular requirements for this process directly[20,37]. A recent report showed that the EBV derived antigen LMP1 expressed under the control of B cell specific regulatory elements can directly activate T cell mediated immune surveillance, leading to efficient deletion of LMP1 transgenic B cells, thereby preventing development of B lymphoma[38]. While that study demonstrated the critical role of T cells in the control of lymphomas driven by a viral antigen, it did not clarify the role of immune surveillance in the pathogenesis of spontaneous B cell lymphomas that do not express foreign or pathogen associated antigens. Our study unambiguously and for the first time demonstrates the ability of T cells to prevent spontaneous lymphoma development triggered by mutations that block an intrinsic cellular differentiation pathway.

Our study also sheds light on the potential precursors of DLBCL. Surprisingly, a large proportion of Blimp1 mutant lymphomas, all of which corresponded to the ABC-DLBCL type lymphoma, expressed high amounts of Bcl6 and *Aicda*, both of which are typically associated with GC B cells. This was unexpected, as all lymphomas had arisen in the absence of a functioning T cell compartment, and hence in the absence of any GC activity. While AID expression was thought to be restricted to GC B cells[39], more recent reports suggested that, similar to Bcl6, it might already be expressed in pre-GC B cells[40-42]. This would be in line with the observation that Bcl6-deficient B cells can undergo Ig isotype switching despite being unable to give rise to GC B cells[43]. Thus, our data provide further evidence for a role of AID outside of germinal centres and indicate that at least in mice a diverse array of ABC-DLBCL-like lymphomas, including $Bcl6^+$ and AID^+ lymphomas, can develop from non-GC B cells.

The mechanisms by which T cells can prevent tumour growth are diverse and the molecular basis for successful adoptive T cell mediated tumour eradication is not fully

understood[44]. Direct killing through cytolytic granules and cytokine-mediated cytotoxicity have been implicated in tumour surveillance[45,46]. However, our data do not support a unique role for either of these pathways in the control of DLBCL. In contrast our results indicate that the FasL-Fas-dependent death receptor pathway is critically important for immune-surveillance of incipient B cell lymphomas. This is in agreement with studies that implicated the same pathway in the prevention of plasmacytoid tumors[47] and in the Rituximab induced death of lymphoma cells[48]. Although recent whole exome sequencing of DLBCL samples did not provide evidence for *FAS* mutations[49,50], alterations of the *FAS* gene locus or other mutations that impair the FasL-Fas apoptotic pathway have been reported to be prominent in DLBCL and other post-GC B cell malignancies and associated with poor prognosis[51-55]. These results suggest that mutations, which impact on Fas expression, are more likely to occur in *FAS* gene regulatory regions, or in the signalling pathways downstream of Fas. Therefore, more lymphoma genome sequencing data are required to accurately estimate the impact and frequency of such mutations in DLBCL. Importantly, down-modulation of FasL expression during drug-induced immune suppression is a likely mechanism of escape from immune surveillance.

An intriguing question that remains is, how the polyclonal $CD8^+$ T cell population can recognize transformed B cells. As B lymphoma cells themselves are antigen-presenting cells, our data thereby support a model in which T cells bearing low-avidity TCRs for self-antigens have the capacity to eliminate incipient B lymphoma cells that are identified based on their uncontrolled expression of MHC molecules presenting self-peptides in conjunction with high levels of co-stimulatory molecules and Fas. Importantly, the TCR signalling threshold for FasL-Fas mediated killing of target cells is substantially lower than for perforin-mediated cytotoxicity[56-59] suggesting that even partial agonists or self-antigen may be sufficient to trigger this process for lymphoma cell killing. As B cells responding to antigen, even in healthy individuals, harbour significant numbers of AID-mediated mutations in a variety of genes, including oncogenes[16-18], this process of T cell mediated immune surveillance may have evolved as a mechanism to deal with the dangerous 'side-effects' of immunoglobulin class switch recombination and affinity maturation during humoral immune responses.

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Author contributions

S.A.S., D.Z. and N.B. performed and analysed the majority of the experiments; A.K.S., L.R., A.E.A. and J.W. conducted and analysed experiments; F.M. and G.T.B. did infection experiments; L.M.C., L.O.R., A.S., M.J.S., R.J. contributed to the design of experiments; D.M.T. and S.L.N. contributed to the design of the study and the writing of the manuscript; A.K. designed the study, analysed data and wrote the manuscript.

Figure Legends

Figure 1. Expansion of a pre-plasmablast population in the absence of Blimp1. (**a-c**) Mixed bone marrow chimaeras (*Blimp1*$^{gfp/gfp}$: Ly5.1) containing Blimp1-deficient and wild-type B cells and control chimaeras (*Blimp1*$^{gfp/+}$: Ly5.1) were infected with murine γ herpes virus 68 and analysed at acute (d13-14) or persistent (d34-56) time points. (**a**)

Flow cytometric analysis of chimaeras, gated on wild-type ($Ly5.1^+$) or $Blimp1^{gfp/gfp}$ ($Ly5.2^+$) total splenic cells (upper panel) or $CD138^-CD19^+$ splenic B cells (lower panel). (**b**) Proportions of $Cxcr4^+Fas^+$ B cells in the wild-type or $Blimp1^{gfp/gfp}$ compartments. Values for individual mice are indicated by symbols, horizontal line indicates the mean ±S.D. P values compare groups as indicated (*P<0.05, **P<0.016, ***P<0.004, NS, not significant). (**c)** Flow cytometric analysis of $Ly5.2^+CD19^+$ B cells from a control ($Blimp1^{gfp/+}$: Ly5.1) and two individual $Blimp1^{gfp/gfp}$: Ly5.1 chimaeric mice (as in a) at persistent time points. Data are representative of two experiments, each containing 6 mice. (**d**) Flow cytometric analysis of aged (12-16 months) Ly5.1 mice reconstituted with $Blimp1^{gfp/gfp}$ foetal liver cells. Plots are gated on $Ly5.2^+$ splenic leukocytes. B cells were isolated from each of these mice (upper panel) and transferred into $Rag1^{-/-}$ recipients, in which they caused splenomegaly and pathology consistent with B lymphoma (lower panel). Data show 4 individual mice representative of a total of 12 mice with similar results.

Figure 2. Accelerated lymphoma development in *Blimp1*-mutant T cell deficient mice. (**a)** Kaplan-Meier survival graph of *Blimp1*-mutant and control mice on a T cell deficient ($Cd3e^{-/-}$) background. Numbers of mice in each cohort are indicated in brackets. (**b)** Pathology in Blimp1-mutant $Cd3e^{-/-}$ mice. Images show enlarged spleen and liver in a $Blimp1^{gfp/gfp}Cd3e^{-/-}$ mouse (upper left), splenomegaly of two individual $Blimp1^{gfp/gfp}Cd3e^{-/-}$ mice in comparison to a wild-type spleen as indicated (lower left, scale bars 5 mm), and histology of spleens from a $Blimp1^{gfp/gfp}Cd3e^{-/-}$ mouse or a $Cd3e^{-/-}$ mouse wild-type for the *Blimp1* allele as indicated (right, scale bars 200 μm). (**c)** Flow cytometric analysis of two primary (prim.) and the corresponding secondary (sec., after transfer into $Rag1^{-/-}$ mice) B cell lymphomas from $Blimp1^{gfp/gfp}Cd3e^{-/-}$ mice. **(d)** Flow cytometric analysis of a secondary lymphoma from a $Blimp1^{gfp/+}Cd3e^{-/-}$ mouse.

Figure 3. Molecular profiles of B cell lymphomas. (**a)** Western Blot analysis of transcription factors in individual lymphomas as indicated. t-Blimp1 indicates the truncated Blimp1 protein expressed from the $Blimp1^{gfp}$ allele. (**b)** Quantitative RT-PCR for *Aicda* in individual lymphomas. Data show the mean ±S.D. of gene expression relative to *Hprt.* FACS sorted follicular (FO) and germinal centre (GC) B cells were used

as controls. *denotes lymphomas from *Blimp1*$^{gfp/+}$*Cd3e*$^{-/-}$ mice, all others were from *Blimp1*$^{gfp/gfp}$*Cd3e*$^{-/-}$ mice.

Figure 4. Bcl6 overexpression in the absence of T cells drives rapid B cell lymphoma development. (**a)** Kaplan-Meier graph of *Bcl6*-transgenic mice on a T cell deficient (*Cd3e*$^{-/-}$) or sufficient (*Cd3e*$^{+/-}$ or *Cd3e*$^{+/+}$) background. Numbers of mice in each cohort are indicated in brackets. (**b)** Histology of spleens (upper panels) and livers (lower panels) showing destruction of the splenic architecture and infiltration of large blasting cells in the *Bcl6*^{Tg}*Cd3e*$^{-/-}$ samples in comparison to sections from a *Cd3e*$^{-/-}$ mouse without the *Bcl6*-transgene as indicated (scale bars 400 μm). **(c)** Representative flow cytometric analysis of B cell lymphomas from two *Bcl6*-transgenic *Blimp1*$^{gfp/+}$*Cd3e*$^{-/-}$ mice from the cohort described in (a).

Figure 5. Polyclonal CD8$^+$ T cells can eradicate B lymphoma cells in a TCR and CD28-dependent manner. **(a)** CD8$^+$ T cells, labelled with cell division tracker (CTV) and cultured in the presence of B lymphoma cells or wild-type activated splenic B cells with and without CD3 antibody. Flow cytometric analysis of TCRβ$^+$CD8$^+$ T cells for expression of activation markers and granzyme B (GzmB) on day 1 and 3 after start of the culture. Numbers in plots are mean % ±S.D., from five experiments using three different B lymphomas. (**b**) Frequency of lymphoma development in *Rag1*$^{-/-}$ recipients after transfer of B lymphoma cells with or without purified CD4$^+$ or CD8$^+$ T cells. **(c**) Flow cytometric analysis of the indicated molecules on normal B and plasma cells (d7 after immunization with NP-KLH) and on B lymphoma cells. Naïve B cells were B220$^+$CD138$^-$Blimp/GFP$^-$, plasma cells and plasmablasts B220^{low}CD138$^+$Blimp/GFP$^+$. Data are representative of one of 3 mice and 6-8 individual lymphomas. (**d**) Flow cytometric analysis of *Rag1*$^{-/-}$ mice co-injected with B lymphoma cells and CD8$^+$ T cells from either wild-type or *Cd28*$^{-/-}$ mice as indicated, 4 weeks post injection. Data are representative of 10 mice in three experiments using four individual *Blimp1* mutant lymphomas. (**e**) Frequencies of lymphoma development in *Rag1*$^{-/-}$ recipients after transfer of *Blimp1* mutant B lymphoma cells with or without purified CD8$^+$ T cells of the indicated genotypes. Data in (b) and (e) are the combined results from 3-6 experiments

each performed using 2-3 mice with different individual lymphomas. Statistical analysis was performed with Bonferroni Corrected two-tail Fisher's Exact Test.

Figure 6. $CD8^+$ T cells eradicate B lymphoma cells by activating the FasL-Fas apoptotic pathway. (**a**) Frequencies of lymphoma development in $Rag1^{-/-}$ recipients after transfer of *Blimp1* mutant B lymphoma cells with or without purified $CD8^+$ T cells of the indicated genotypes, measured as in Figure 5b. Data present the combined results from 3 experiments each performed using 2-3 mice with different individual lymphomas. (**b**) Representative flow cytometric analysis of individual *Bcl6* transgenic B lymphomas that had developed in T cell sufficient ($Cd3e^{+/+}$, #266, #PM23) or T cell deficient ($Cd3e^{-/-}$, #66, #186) as indicated. (**c-d**) *Bcl6* transgenic B cell lymphomas from T cell sufficient ($Cd3e^{+/+}$) or T cell deficient ($Cd3e^{-/-}$) mice as indicated were analysed by flow-cytometry for Fas expression (c, MFI, mean fluorescence index) and survival (d) in the presence of recombinant Fc-FasL. Values for individual samples are indicated by symbols, horizontal line indicates the mean ±S.D. (**e**) Lymphoma incidence measured as in a after transfer of *Bcl6*-transgenic B cell lymphomas that had developed in T cell sufficient mice, with or without T cells of the genotypes as indicated. Graph represents 6-8 individual lymphomas transferred in replicates. Statistical analysis in (a) and (e) was performed with Bonferroni Corrected two-tail Fisher's Exact Test.

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Online Methods

Mice. *Blimp1*$^{gfp/+}$ and *Blimp1*$^{fl/fl}$ mice were generated and maintained on a pure C57BL/6 (Ly5.2) background as described previously[27,60]. *Bcl6* transgenic mice (IμHABcl6)[30], *Blimp1*^{gfp}Eμ-v-abl transgenic mice[28,61], *Cd79aCre* mice[62], *Cd3e*$^{-/-}$ mice[63], and mice deficient in CD28[64], granzymes A, B and M[65], perforin[66], Trail[67], IFNγ[68], mFasL[69], T-bet[70] or Irf4[71] were described earlier. *Rag1*$^{-/-}$ mice were obtained from The Jackson Laboratory. OT-I mice[72] on a *Rag1*-deficient background were provided by Taconic. *Blimp1*$^{gfp/gfp}$ mice were generated as described[9]. *Blimp1*$^{gfp/gfp}$: Ly5.1 mixed bone marrow chimaeras were generated by reconstituting lethally irradiated Ly5.1 recipients with a mixture of bone marrow cells isolated from *Blimp1*$^{gfp/gfp}$ and wild-type Ly5.1 mice. Chimaeric mice were analysed after a minimum of 6 weeks. Mouse cohorts were monitored twice weekly and euthanized when signs of tumour development (splenomegaly, hind limb paralysis or wasting) occurred. All mice were bred and maintained on a C57BL/6 background at The Walter and Eliza Hall Institute of Medical Research or the Peter MacCallum Cancer Centre, Melbourne. Animal experiments were undertaken according to Animal Experimental Ethics Committee guidelines and approval.

Antibodies and flow cytometry. The following monoclonal antibodies to mouse proteins were from eBioscience or BD Biosciences Pharmingen and were used in multi-parameter flow cytometric analysis: CD4 (L3T4), CD8 (53-6.7), CD25 (PC61), Ly5.2

(104), Ly5.1 (A20), CD69 (H1.2F3), B220 (RA3-6B2), CD19 (eBio1D3), CD23 (B3B4), CD43 (S7), CD80 (16-10A1), CD86 (GL1), CD138/Syndecan-1 (281-2), IgM (II/41), IgD (II-26c), MHC-I (M1/42), MHCII (M5/114.15.2), Fas (Jo2), Cxcr4 (2B11), Cxcr5 (2G8), TCRβ (H57-597), Granzyme B (GB12). Peanut agglutinin (PNA) was from Vector Laboratories; anti-Bcl6 (in-house clone 7D1-10) was conjugated to A647 (Molecular Probes). FcgRII/III (24G2; supernatant) and normal rat serum was used for blocking. The TCRVβ Screening Panel was from BD Biosciences Pharmingen. Biotinylated mAbs were revealed with streptavidin Cy5 or streptavidin PerCPCy5.5. Streptavidin Cy5 was from Southern Biotech and the remaining reagents were from BD Biosciences Pharmingen or eBioscience. The anti-mouse embigin monoclonal antibody was described earlier[73]. Viable cells were identified by propidium iodide or SytoxBlue (Invitrogen) exclusion. For intracellular staining, cells were fixed, permeabilized, and stained using the protocol in the Foxp3 Staining Buffer Set (eBioscience). Cells were analyzed on FACS Canto II cytometers (Becton Dickinson) and cell sorting was performed on MoFlo (Beckman Coulter) or Aria cytometers (Becton Dickinson). Data was processed using Flowjo software.

Histology. Organs were harvested and fixed overnight in 10% neutral buffered formalin at 4°C. After fixation, organs were progressively dehydrated in 70%, 95% and 100% ethanol, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Images were acquired with a Zeiss Axioplan-2 microscope.

Western blotting. Total protein extracts were produced from equivalent numbers of cells with DISC buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol and Complete Protease Inhibitor (Roche)). Rat monoclonal anti-Pax5 (1H9)[9], anti-Blimp1 (5E7)[27], anti-Bcl6 (7D1) and anti-IRF4 (3E4) were generated in-house. Equal loading of lanes with proteins was confirmed by probing with horseradish peroxidase–conjugated anti-β-actin (I-19; Santa Cruz Biotechnology).

Cell isolation and culture. $CD4^+$ and $CD8^+$ cells were enriched from pooled spleens and lymph nodes by magnetic-activated cell sorting (MACS) using MACS beads (Miltenyi) according to the manufacturer's protocol. In mixed cultures, T cells were cultured in the presence of B lymphoma cells or LPS and IL-4 activated control B cells at a ratio of 1:5

in the presence of recombinant mouse IL-2 (100 U/ml, R&D Systems). Anti-CD3 (145-2C11) (5 μg/mL) was added to cultures as indicated. The CellTrace™ Violet Cell Proliferation Kit (Invitrogen) was used according to the manufacturer's protocol.

Cell transfer. Recipient mice were injected with 1-2x10^6 lymphoma cells. In some experiments, lymphoma cells were mixed prior to injection with the same number of isolated T cells. Recipient mice were monitored for signs of disease and palpated for the development of splenomegaly twice weekly. Mice were analysed when disease had developed or 6-8 weeks after the recipients of B lymphoma cells without T cells had developed malignant disease.

Real-time PCR analysis. Total RNA was prepared using the RNeasy kit (Qiagen). cDNA was synthesized from total RNA with random hexamers and SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen). Analyses were performed in triplicate and the mean normalized expression calculated using the Q-Gene application with *Hprt* serving as a reference gene. Primer sequences were as follows, *Aicda*: CCGGCACGTGGCTGAGTTT, GATGCGCCGAAGTTGTCTGGTTAG; *Hprt*: TCCAACACTTCGAGAGGTCC, GGGGGCTATAAGTTCTTTGC.

Viral infections and immunization experiments. Mice were anaesthetised with methoxyflurane and then infected intra-nasally with 3×10^4 PFU recombinant murine γ-herpes virus 68 (γHV)[74]. Immunizations were done with a single i.p. injection of 100 μg nitrophenol (NP) coupled to keyhole limpet haemocyanin (KLH) in the ratio of 13:1. The antigen was precipitated onto alum and washed extensively before injection.

Clonality analysis and Ig gene mutation analysis. DNA was extracted from individual B cell lymphomas by standard methods and VDJ recombination of the *IgH* gene locus was tested by PCR as described[75]. For paraprotein and serum Ig analysis 0.5 - 1.0 μL serum isolated from lymphoma bearing mice was used as described[61]. Somatic mutation at the *Ig* heavy chain locus was determined by sequencing 260 bp 3' of the JH segment used in the heavy chain VDJ rearrangement, previously determined by differential PCR. The revealed sequence was compared to its germline equivalent from C57BL/6 mice and differences recorded as mutations per 100 bp.

***In vitro* Fas ligand killing assay.** MACS purified B lymphoma cells were incubated for 16 h with 10,000 units recombinant Fc-FasL (ACRP-FasL)[76] as described. Proportions of live cells were determined by propidium iodide exclusion using flow cytometry.

Statistics. If not stated otherwise a paired or unpaired student t test as appropriate was performed to test for statistical significance.

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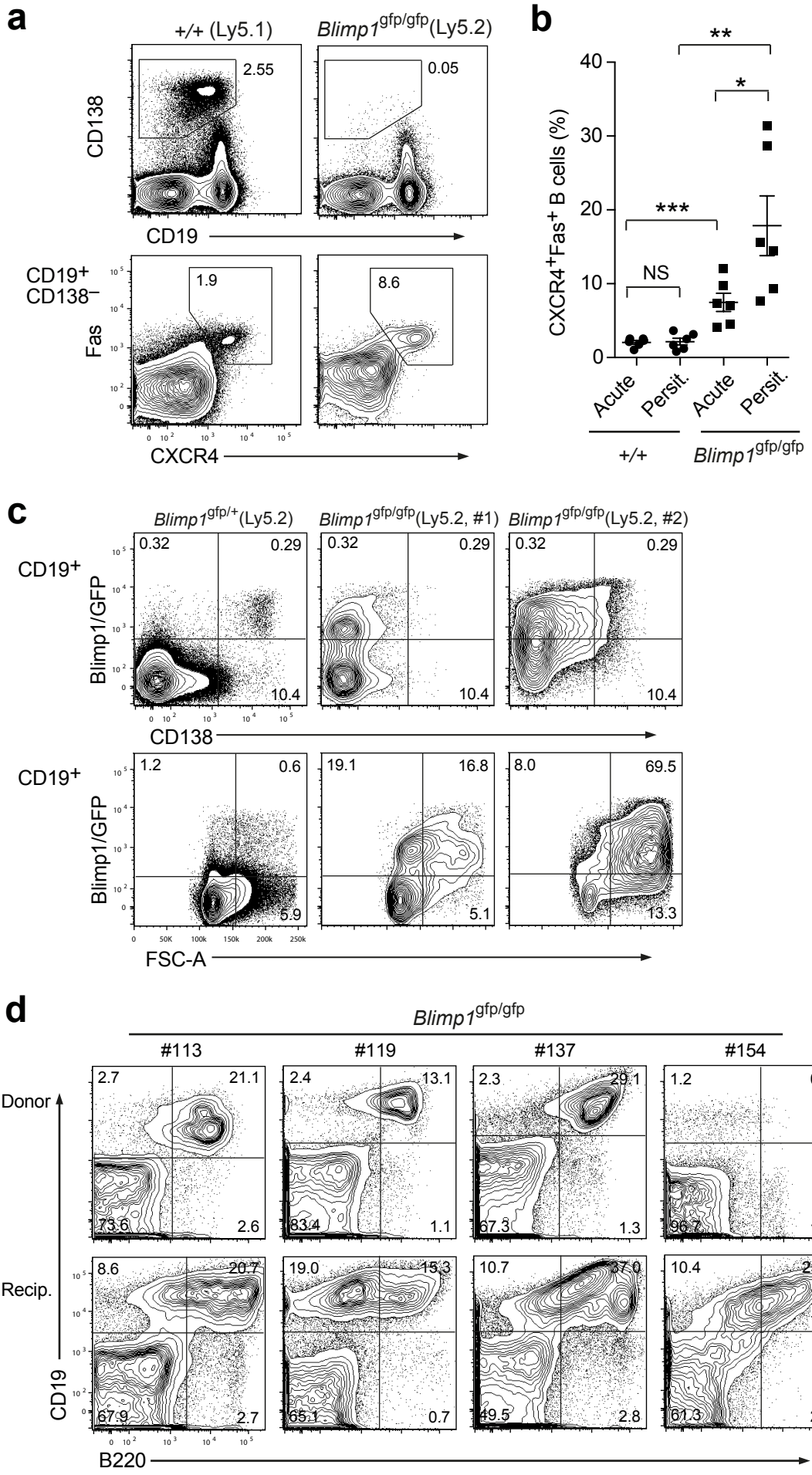
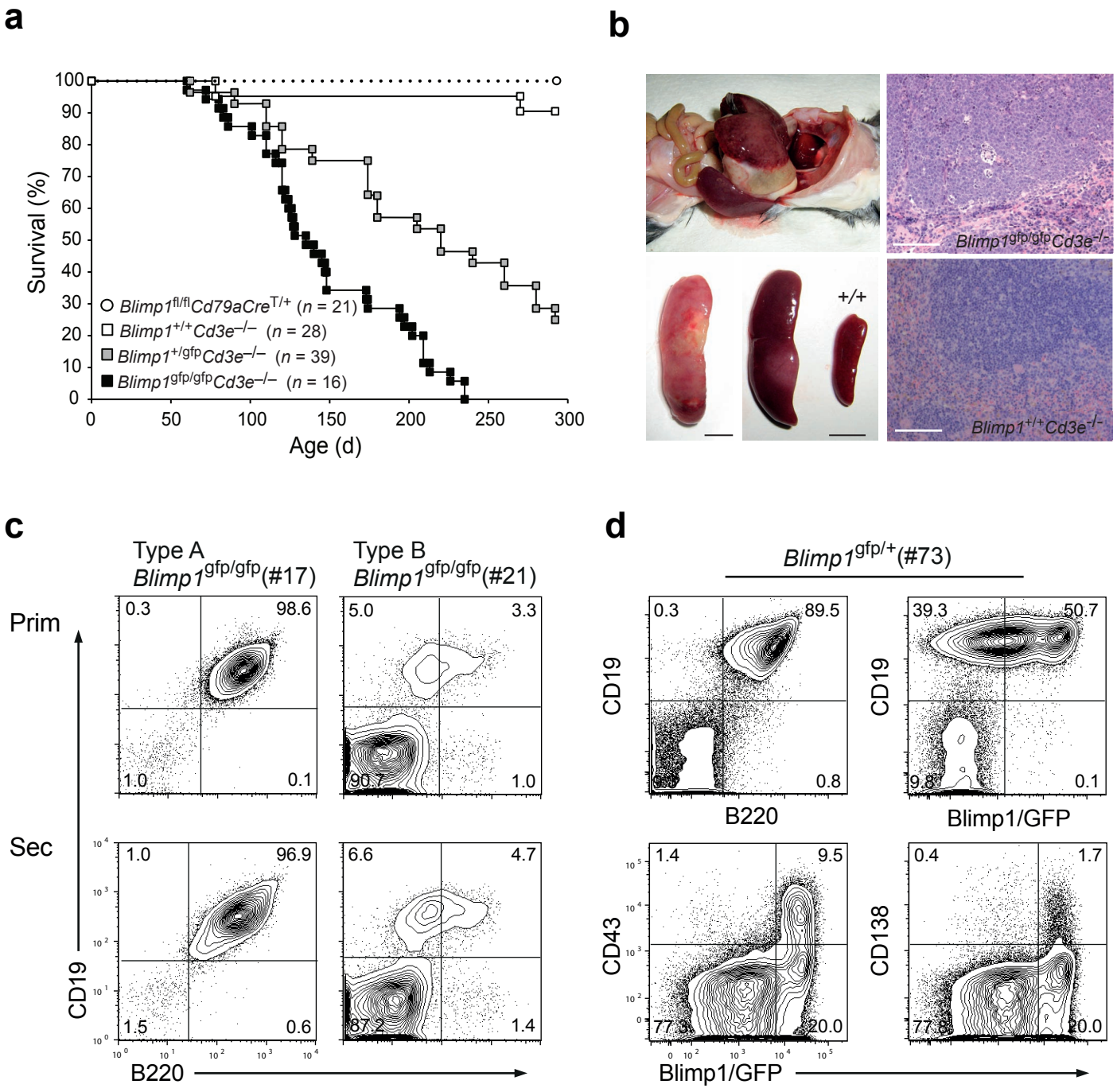


Sterle et al. Fig. 1



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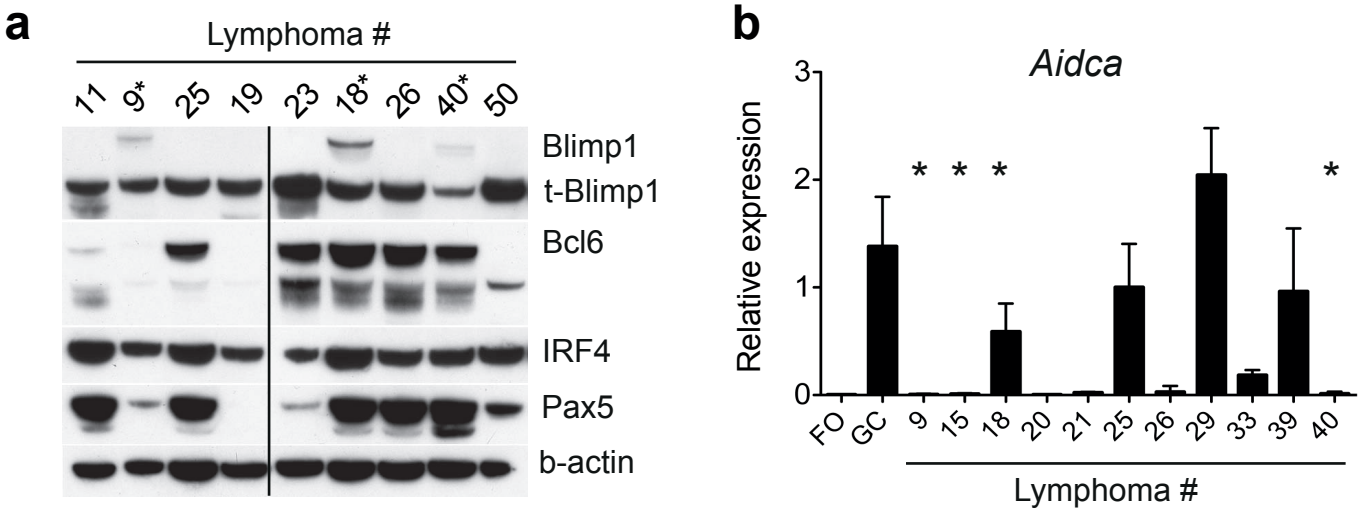

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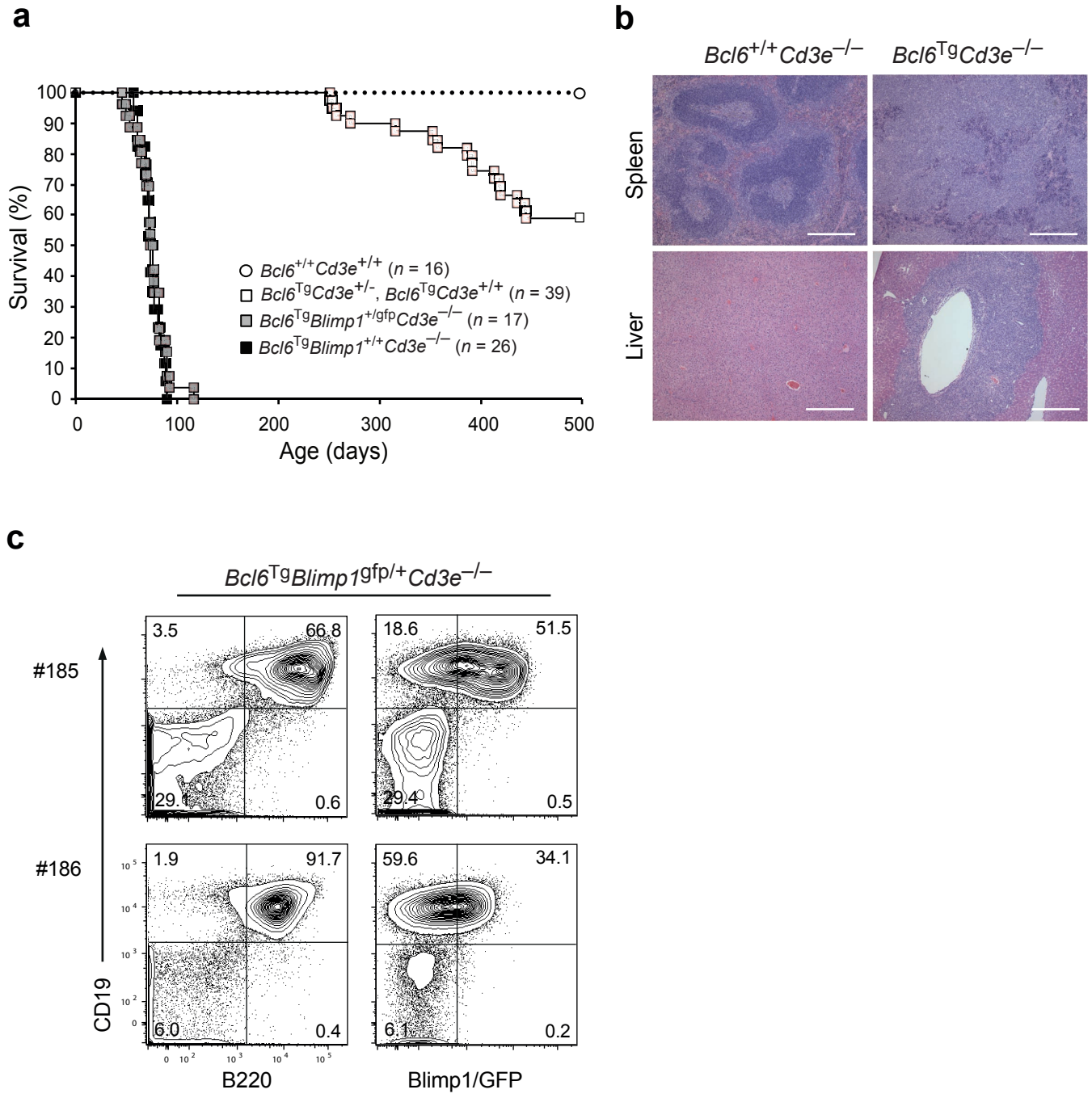

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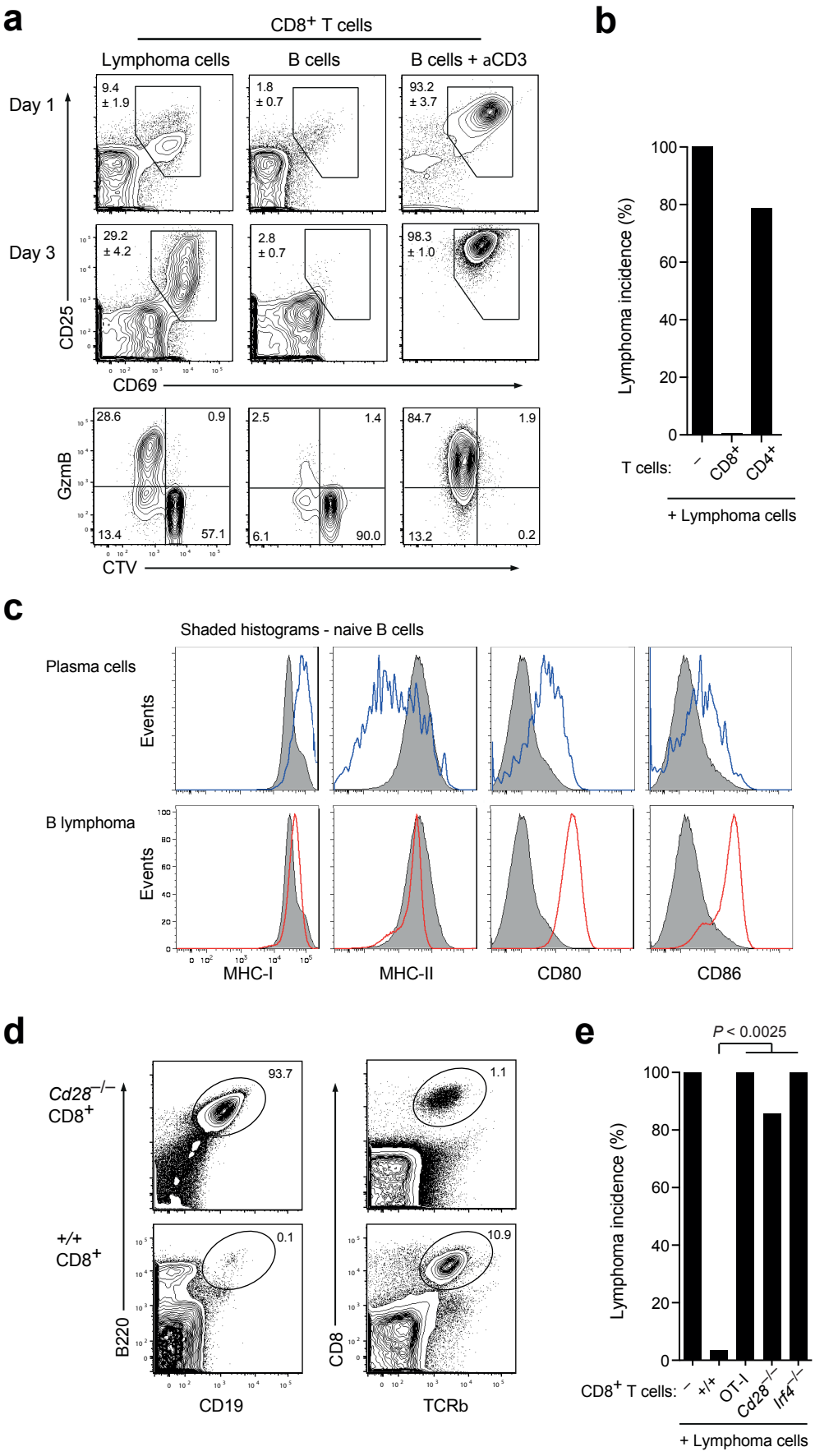

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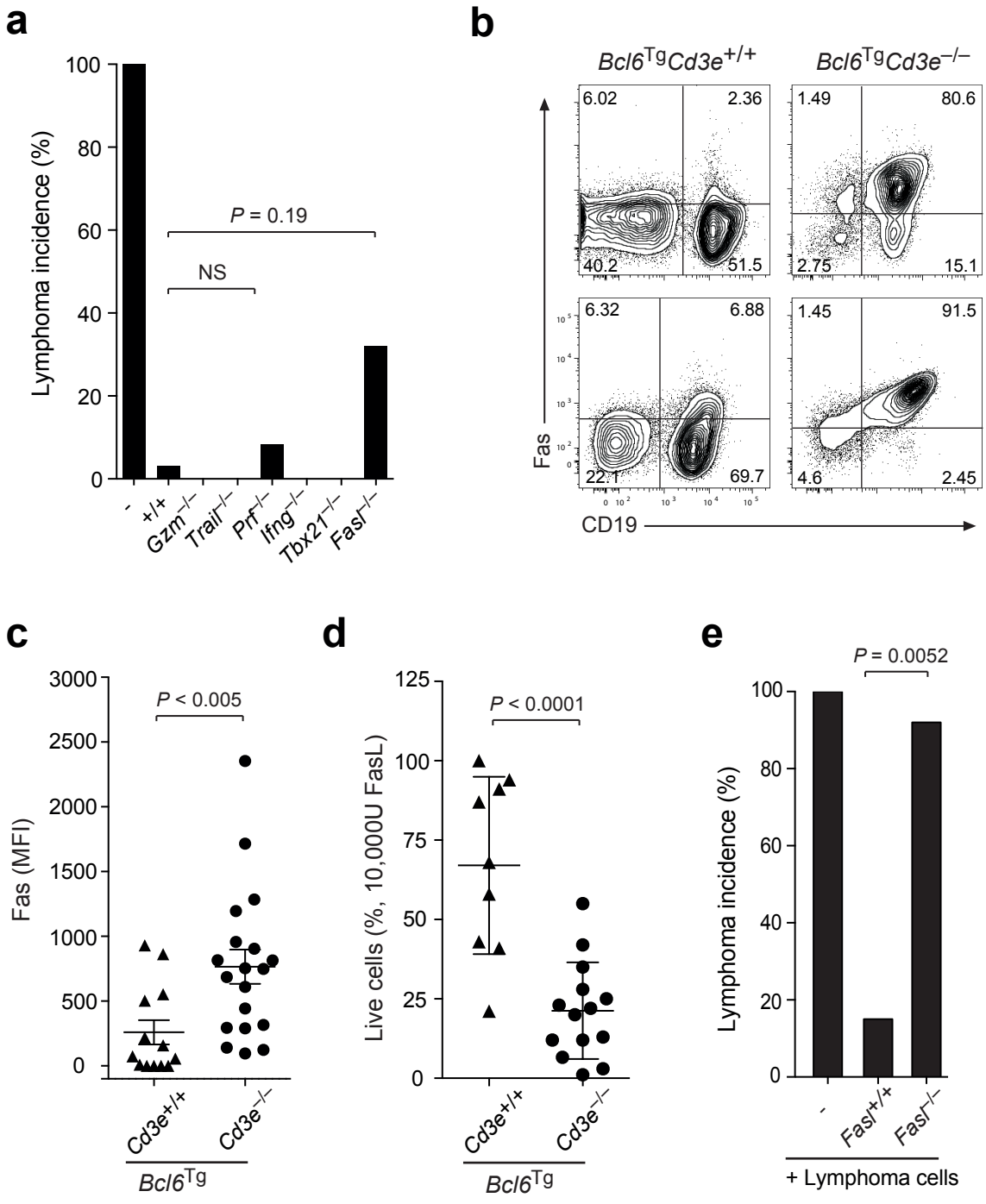


Sterle et al. Fig. 6

Supplementary Figures

Supplementary Figure 1. a, Flow cytometric analysis of mice with a B cell specific inactivation of the *Blimp1* gene (*Blimp1*$^{fl/fl}$*Mb1Cre*$^{T/+}$) or control mice (*Blimp1*$^{+/+}$*Mb1Cre*$^{T/+}$) 34 days post infection with γHV. Gated CD19^{+} splenic B lymphoid cells are shown. Data are representative for three individual mice of a group of 16 mice. **b,** Flow cytometric analysis of Blimp1/GFP expression in different B cell compartments of a *Blimp1*$^{gfp/+}$ mouse 7 days after immunisation with the model antigen nitrophenol coupled to keyhole limpet haemocyanin in alum (NP-KLH). Naïve (shaded), germinal centre B cells (bold line) and plasma cells and plasmablasts (fine line) were gated as indicated. Data are representative of at least 9 mice from three independent experiments.

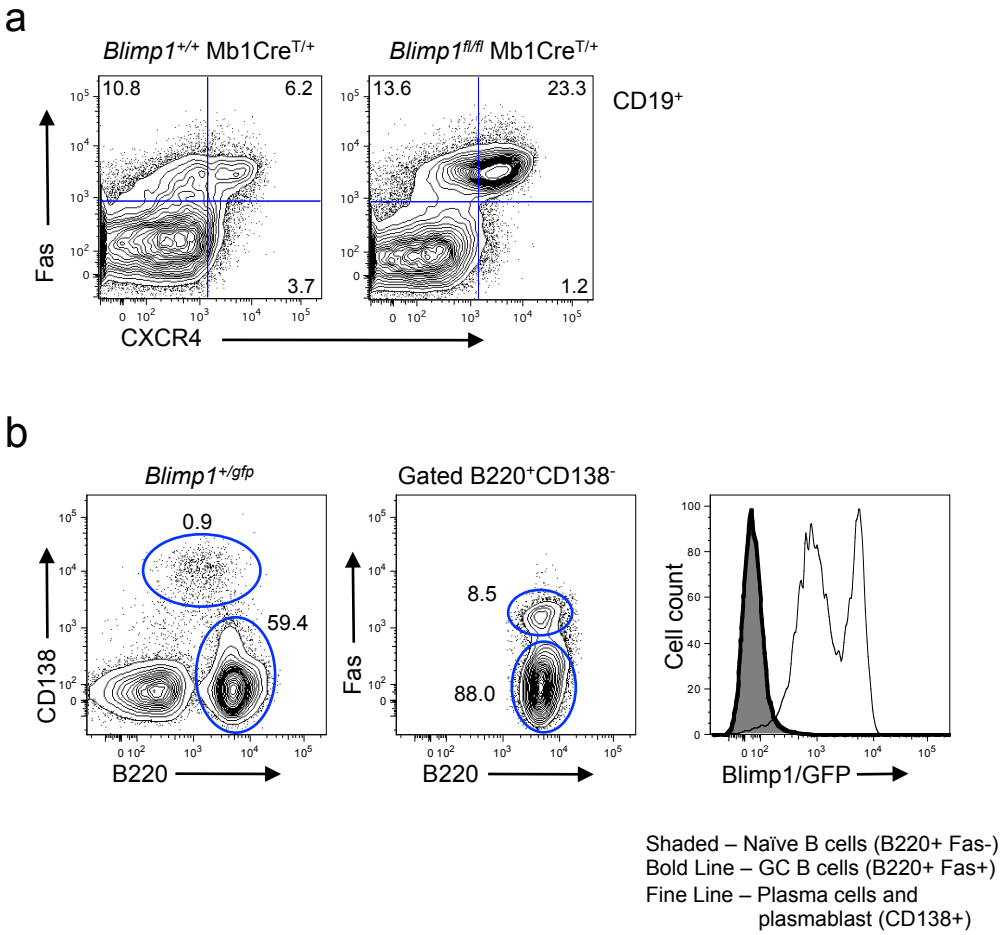
Supplementary Figure 2. a, Flow cytometric analysis of Ly5.2^{+}CD19^{+} splenic B cells from two individual *Blimp1*$^{gfp/gfp}$/Ly5.1 mixed chimaeras infected with γHV and analysed 34 days post infection. Data are representative for two experiments each containing 6 mice. **b,** Flow cytometric analysis of mice with a B cell specific inactivation of the *Blimp1* gene (*Blimp1*$^{fl/fl}$*Mb1Cre*$^{T/+}$) or control mice (*Blimp1*$^{+/+}$*Mb1Cre*$^{T/+}$). Data are representative of three individual mice out of a group of 16 γHV-infected mice. **c,** Flow cytometric analysis of mixed bone marrow chimaeras as in (a) 14 days after immunisation with NP-KLH in alum. Plots are gated on wild-type (Ly5.1^{+}) or *Blimp1*$^{gfp/gfp}$ (Ly5.2^{+}) CD19^{+} splenic B cells as indicated. Graph shows proportions of CXCR4^{+}Fas^{+} B cells in the wild-type or *Blimp1*$^{gfp/gfp}$ compartments. Values for individual mice are indicated by dots, the horizontal line indicates the mean ±S.D. Data are representative of two experiments each containing 4-5 mice.

Supplementary Figure 3. a, Representative flow cytometric analysis of two individual B cell lymphomas as indicated from *Blimp1*$^{gfp/gfp}$*Cd3e*$^{-/-}$ mice. **b,** Clonal *Igh* gene rearrangements of individual B cell lymphomas from *Blimp1*$^{gfp/gfp}$ and *Blimp1*$^{gfp/+}$*Cd3e*$^{-/-}$ mice. Splenic B cells were used as a positive control. **c,** Analysis of serum Ig from *Rag1*$^{-/-}$ recipients that developed lymphoma after injection with lymphoma cells from *Blimp1*$^{gfp/gfp}$ or *Blimp1*$^{gfp/+}$*Cd3e*$^{-/-}$ mice. Serum from an *Eμ-v-abl* transgenic mouse bearing a plasmacytoma and from a healthy control mouse, were used as controls. Clonal Ig (paraprotein) is marked by an arrow.

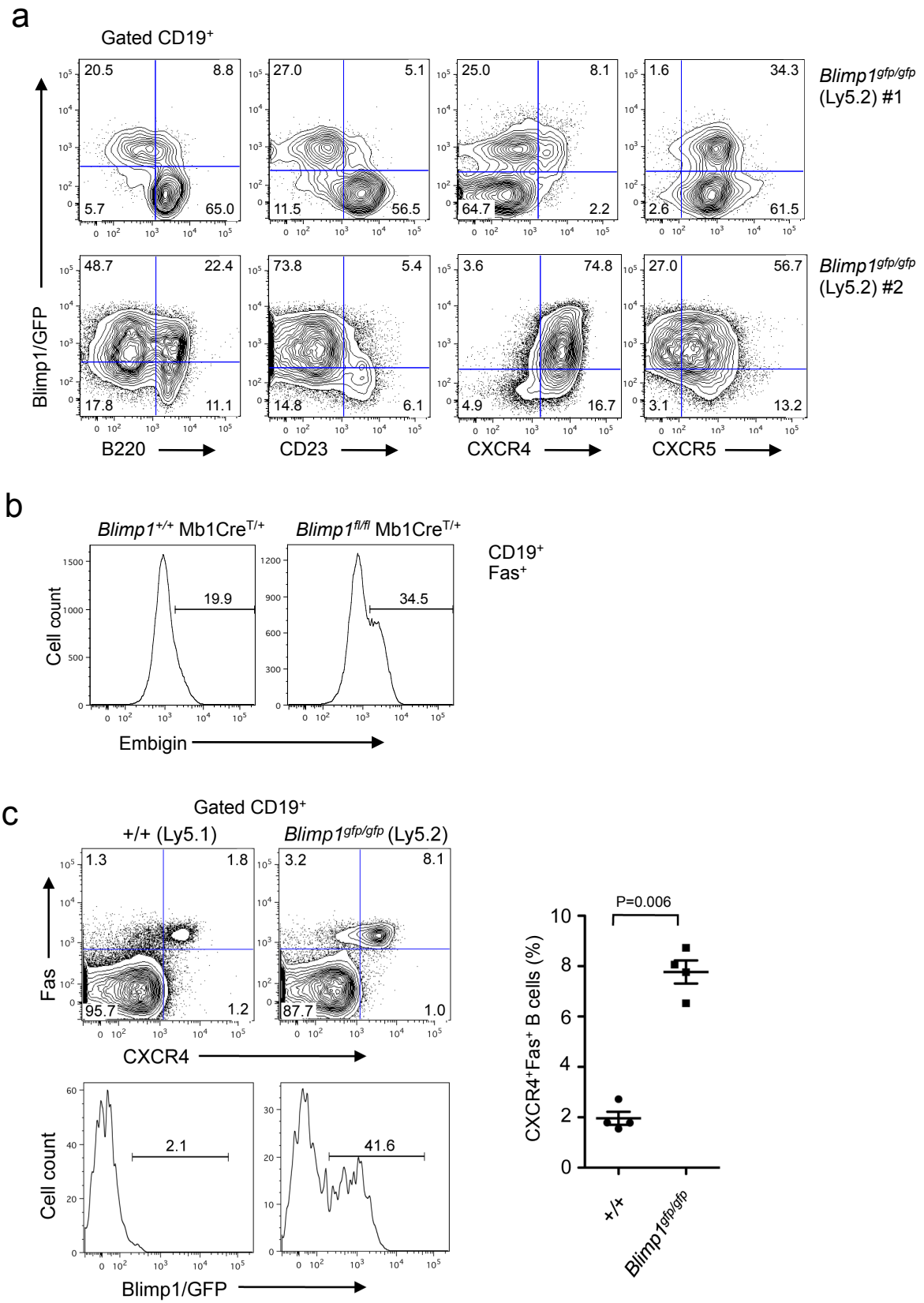
Supplementary Figure 4. a, Flow cytometric analysis of spleen cells from healthy *Bcl6*-transgenic mice and control mice as indicated. $Bcl6^{Tg}Cd3e^{-/-}$ and $Bcl6^{+/+}Cd3e^{-/-}$ mice were analysed at 6 weeks of age; the remaining mice were 6 months old. **b,** Flow cytometric analysis of spleen cells from lymphoma bearing T cell sufficient *Bcl6*-transgenic mice. Numbers indicate the proportion of $CD8^+$ and $CD4^+$ T cells (left) and the proportion of activated T cells (middle and right).

Supplementary Figure 5. a, $CD4^+$ T cells are activated but do not proliferate in response to B lymphoma cells *in vitro*. Flow cytometric analysis of $TCR\beta^+CD4^+$ T cells labelled with cell division tracker (CTV) and cultured in the presence of B lymphoma cells or, as a control, with wild-type splenic B cells with and without CD3 antibody as indicated. Plots show activation markers (CD25 and CD69) and cell division as measured by loss of CTV on day 1 and 3 as indicated. **b,** Polyclonal $CD8^+$ T cell response in the presence of B lymphoma cells from *Blimp1*-mutant $Cd3e^{-/-}$ mice. Flow cytometric analysis of TCRvβ chain expression on $CD8^+$ T cells freshly isolated (shaded) or $CD8^+$ T cells that had divided (CTV^{low}) in co-culture with B lymphoma cells 3 days after the start of the culture. Data are representative of two experiments performed with two individual lymphomas. **c,** Flow cytometric analysis of $Rag1^{-/-}$ recipients injected with B lymphoma cells with or without purified $CD4^+$ or $CD8^+$ T cells as indicated. Data are representative of 9-17 mice in 3-6 experiments. **d-e,** Lymphoma incidences in $Rag1^{-/-}$ mice after transfer of B lymphoma cells with or without $CD8^+$ T cells as indicated. **d,** Total $CD8^+$ T cells from healthy mice transferred along with B lymphoma cells of the indicated genotypes. Data represent 4-5 mice and at least two individual lymphomas. **e,** Naïve T cells were FACS sorted as $CD8^+CD62L^{high}$ and $CD44^{low}$, memory T cells were $CD8^+CD44^{high}$. Data represent at least 6 mice and three individual lymphomas.

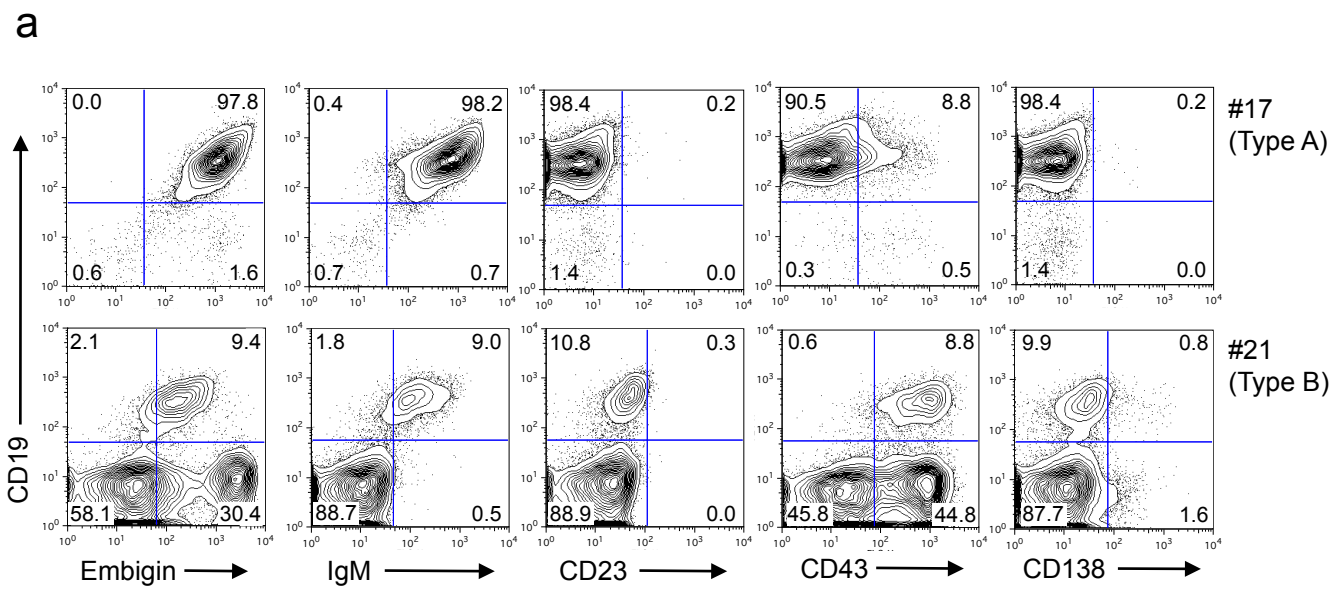
Supplementary Figure 6. Flow cytometric analysis of B lymphoma cells after over-night culture in the presence or absence of 10,000 U recombinant Fc-FasL as indicated. Gates indicate frequencies of $CD19^+PI^-$ live cells.

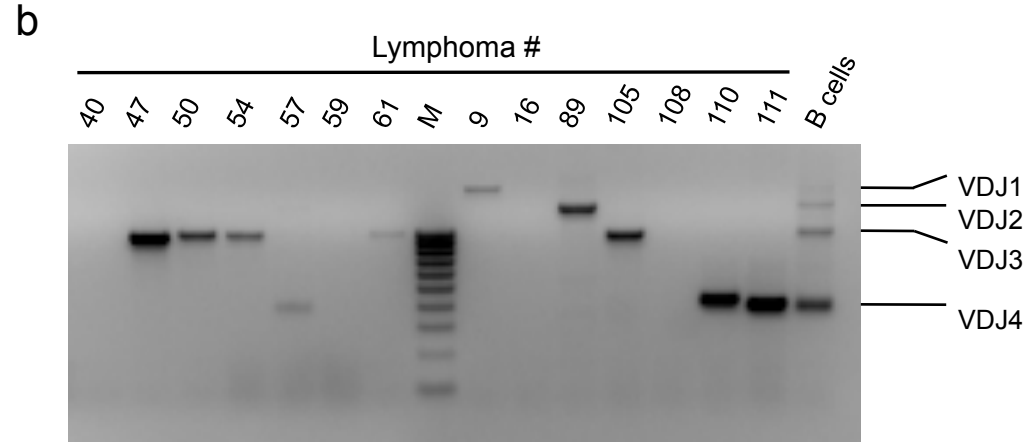


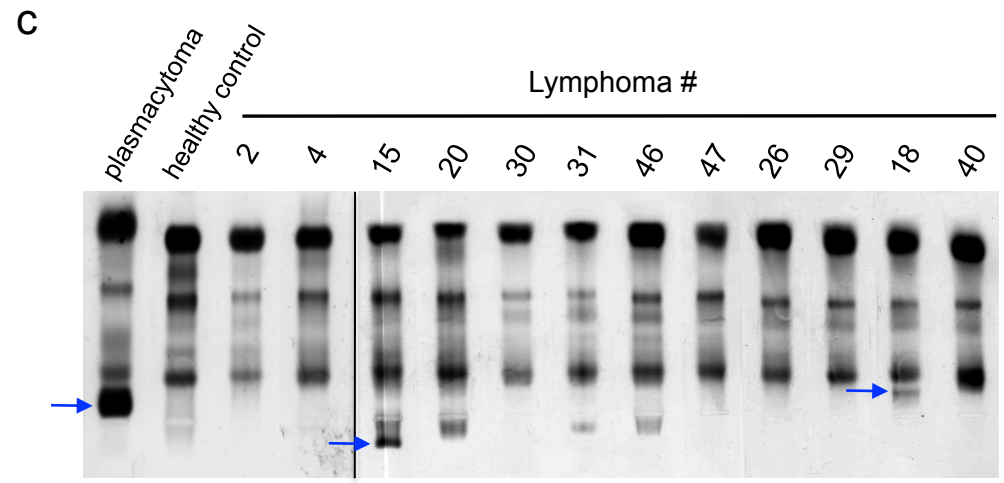
Sterle et al. Suppl. Fig. 1



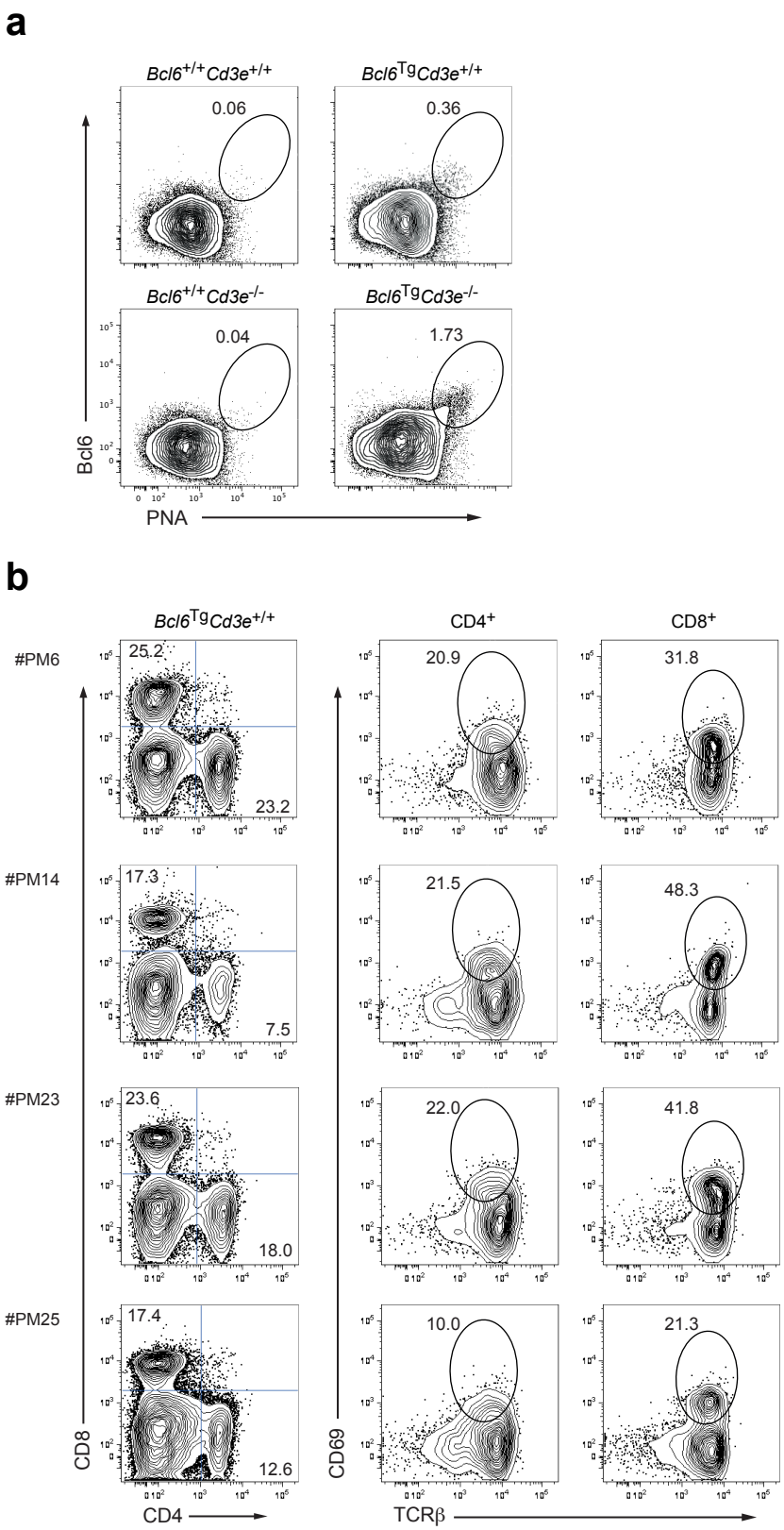
Sterle et al. Suppl. Fig. 2



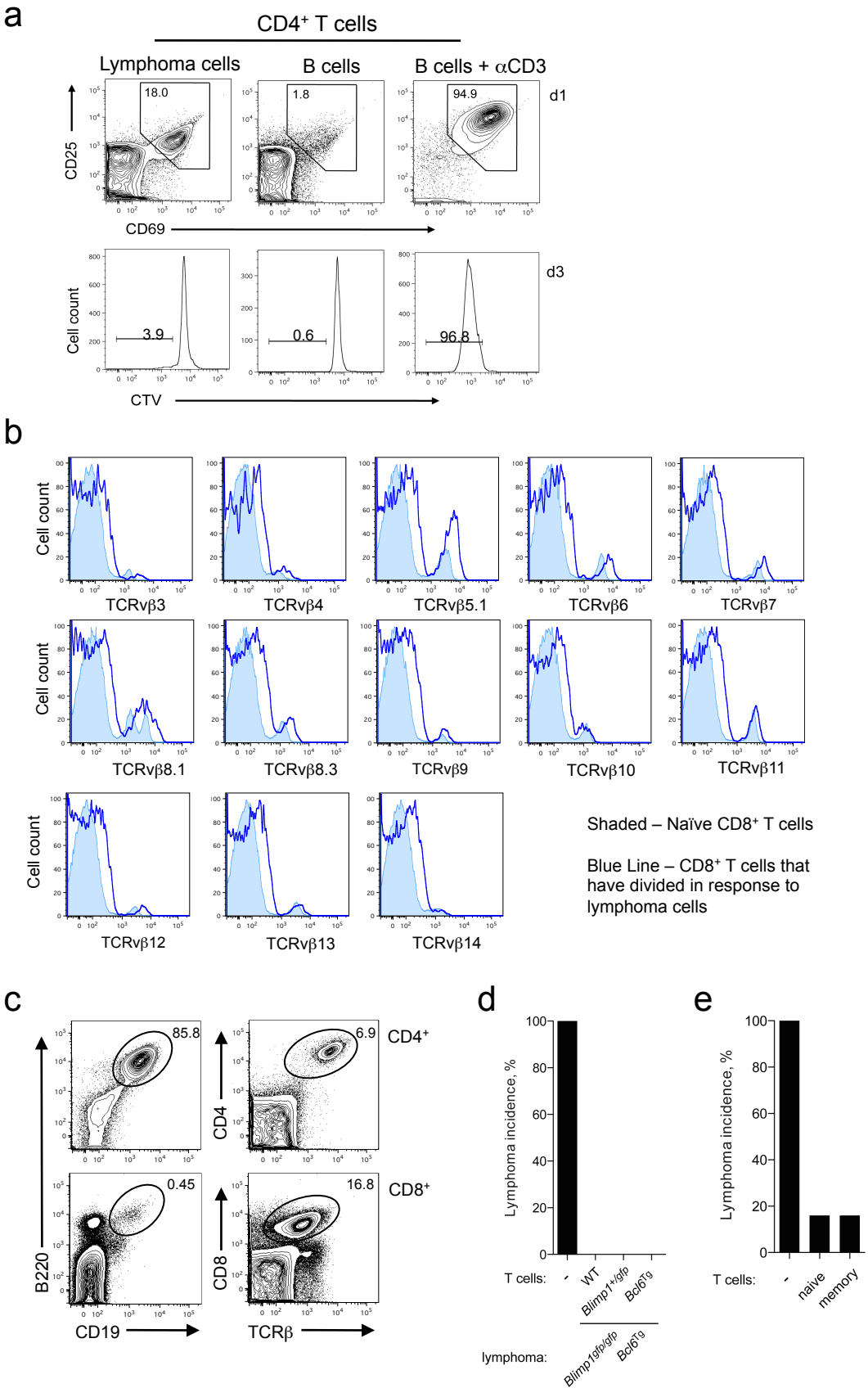




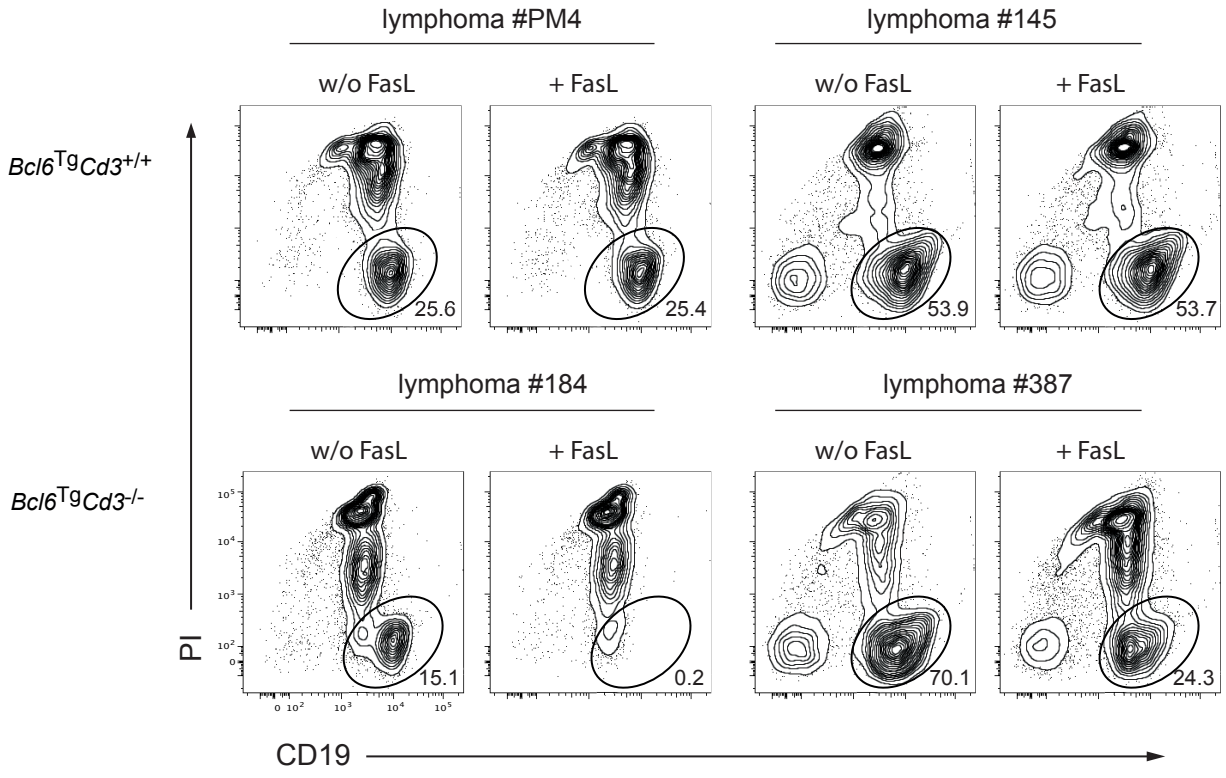
Sterle et al. Suppl. Fig. 3



Sterle et al. Suppl. Fig. 4



Sterle et al. Suppl. Fig. 5



Sterle et al. Suppl. Fig. 6