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GIMAP1 is essential for the survival of naïve and activated B cells *in vivo*

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Abstract

An effective immune system depends upon regulation of lymphocyte function and homeostasis. In recent years members of the GTPases of the Immune Associated Protein (GIMAP) family have been proposed to regulate T cell homeostasis. In contrast, little is known about their function and mode of action in B cells. We have used a combination of transgenic mice and *in vivo* and *in vitro* techniques to conditionally and electively ablate GIMAP1 in resting and activated peripheral B cells. Our data suggest that GIMAP1 is absolutely essential for the survival of peripheral B cells, irrespective of their activation state. Together with recent data showing increased expression of GIMAP1 in B cell lymphomas, our work points to the possible potential of GIMAP1 as a target for manipulation in a variety of B cell-mediated diseases.

Introduction

GIMAP1 was the first identified member of the family of guanosine triphosphatases (*G*TPases) of the *im*munity-*a*ssociated *p*roteins (GIMAPs), first reported as a malaria-induced gene in mouse splenocytes (1). Since then, genetic association studies have implicated human *GIMAP* genes in autoimmune diseases including, systemic lupus erythematosus (2), Behçet's disease (3) and type I diabetes (4, 5). Furthermore, their deregulated expression has been reported in lymphomas (6-11). There are 8-9 GIMAP family members that have been identified in mammals (12). They are a family of septin-related guanine nucleotide-binding G proteins which bear strong resemblance to dynamins (13). Mammalian GIMAPs are expressed prominently within lymphoid compartments, suggesting a role in lymphocyte function (12, 14-19). *In vivo* and *in vitro* studies have implied a role for GIMAPs in lymphoid homeostasis and survival (20-30).

GIMAP5's is the most studied GIMAP family member. A mutation in *Gimap5* was found to be the cause of lymphopenia seen in the Biobreeding diabetes-prone (BB-DP) rat strain (14,

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15). In GIMAP5-deficient rats, T cell development appears to occur normally within the thymus but there are few T cells in the periphery (14, 15, 24, 31, 32). This has been attributed to spontaneous apoptosis of T cells, although the mechanism by which this occurs remains unclear (24) (32) (33). Recent work has suggested that T cell death may result from the inability of their mitochondria to sequester Ca^{2+} following capacitative entry (28). A similar paucity of peripheral T cells is seen in GIMAP5-deficient mice, which develop spontaneous colitis, resulting in early mortality (23, 26, 27). Deficiency in *Gimap5* in mice affects various haematopoietic cell types (23, 27, 34), and can lead to a progressive multilineage failure of bone marrow hematopoiesis (34). Knowledge of the extent to which these effects are cell-intrinsic awaits the use of conditional alleles in the study of *Gimap5*.

GIMAP5's close relative GIMAP1 is also required for the maintenance of peripheral T cells. Previously, we showed that conditional deletion of *Gimap1* from lymphocyte progenitors using *CD2Cre* (*Gimap1*$^{f/f}$*CD2Cre*$^{+}$ mice), resulted in normal lymphocyte development but severe reductions in peripheral T cell numbers (22). Surprisingly, we also found a profound deficit of mature peripheral B cells. This study did not address GIMAP1 function in activated B cells. To date, the role GIMAPs might play in the survival of activated lymphocytes remains unresolved. Whereas GIMAP5-deficient rat T cells can be activated successfully via their antigen receptors, GIMAP5-deficient mouse T cells were reported to be unable to proliferate in response to *ex vivo* stimulation ((24) (27) (35). More recently, other studies have suggested an important role for GIMAP1 in mature B cells, highlighting its potential role in B cell lymphomas. Diffuse large B-cell lymphomas (DLBCLs) show hypomethylation at the *Gimap* locus resulting in overexpression of GIMAP1 (10). In addition, the *Gimap* cluster is found within an early replication fragile site (ERFS) hotspot (6). ERFS hotspots are proposed to play a mechanistic role in some of the most common genome rearrangements during B cell lymphomagenesis. These studies prompted us to examine in greater depth the role GIMAP1 plays in B cell function. We have used a combination of transgenic mice in conjunction with *in vivo* and *in vitro* techniques to show that GIMAP1 is required for the maintenance of B cell numbers not only in the resting peripheral pool but also throughout mature B cell activation and differentiation.

Methods

Animals and immunisations

Mice were bred and maintained in specific pathogen-free conditions at The Babraham Institute. Husbandry and experimentation complied with existing United Kingdom Home Office and EU legislation, and local standards, as approved by the Babraham Institute Animal Welfare and Ethical Review Body. *Gimap1*$^{f/f}$ mice (described previously (22)), bearing a 'floxed' *Gimap1* allele, were crossed with *Cd79a*$^{cre/+}$ mice (obtained from Michael Reth) to generate *Gimap1*$^{f/f}$*Cd79a*$^{cre/+}$ mice, allowing conditional ablation of *Gimap1* in the B cell lineage (36). The *Gimap1*$^{f/f}$ mice were also crossed with *ER*^{T2}*Cre*$^{+}$ mice (obtained from Thomas Ludwig) to generate *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ mice, enabling conditional ablation of *Gimap1* upon administration of tamoxifen (37). To conditionally delete *Gimap1* in GC B cells, *Gimap1*$^{f/f}$ mice were crossed with *AicdaCre*$^{+}$ mice (38) (obtained from M. Busslinger) to generate *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ animals. *Gimap1*$^{f/f}$*Cd2*$^{cre/+}$

mice (previously described (22)) were crossed with Eμ-*bcl-2-36* transgenic mice expressing human Bcl2 (39) to generate $Gimap1^{f/f}Cd2^{cre/+}Bcl2^{tg}$.

Mice were immunised i.p. with 100μg of 4-hydroxy-3-nitrophenylacetyl NP-19-keyhole limpet hemocyanin (KLH; Biosearch Technologies, Nocata, CA) adsorbed to alum in saline. They were bled at indicated time points before boosting with soluble antigen. In adoptive transfer experiments lymph node cells from $Gimap1^{f/f}ER^{T2}Cre^{+}$ and $ER^{T2}Cre^{+}$ mice were stained with carboxyfluorescein succinimidyl ester (CFSE) and CellTrace™ violet (CTV; Life Technologies), respectively, and then mixed in a 1:2 ratio ($Gimap1^{f/f}ER^{T2}Cre^{+}$:$ER^{T2}Cre^{+}$) prior to i.v. injection of 5×10^6 cells/mouse into B6.SJL-*Ptprca Pepcb*/*BoyJ* mice. Mice were treated with 200μg tamoxifen per g body weight or vehicle control i.p. on days 1 and 2 following adoptive cell transfer. On day 13 after cell transfer mice were killed and the numbers of transferred cells present in peripheral blood and spleen determined on the basis of anti-CD45.1, anti-CD45.2, CFSE, CTV and anti-B220 staining.

Flow cytometry

Single cell suspensions were prepared from lymphoid tissues and peripheral blood. Antibodies directed against the following surface markers: CD93, B220, CD38, GL7, Fas, IgG1, CD138, IgM, CD45.1, and CD45.2. DAPI and biotinylated nitrophenyl-4-hydroxy-5 iodophenacetic acid (NIP) were used. Cells were analysed using a LSRII or Fortessa (BD Biosciences, Oxford, UK) and data analysed using FlowJo software (Tree Star, Ashland, OR). To purify GC and follicular B cells, splenocytes from animals immunized 7 days previously with NP-KLH in alum were first depleted of $CD43^+$ and $CD11c^+$ cells using biotinylated anti-CD43 and anti-CD11c antibodies followed by anti-biotin beads (MACS, Miltenyi), prior to depletion on an AutoMacs (Miltenyi Biotec), resulting in >95% $CD19^+$ cells. To fractionate $CD19^+$ cells into GC and follicular B cells, cells were stained with B220, GL7, and CD95 and sorted for GC cells ($B220^+CD95^+GL7^+$) and for follicular B cells ($B220^+CD95^-GL7^-$). To measure levels of apoptosis, splenocytes were stained for B220, GL7, and CD95 and active caspase-3 was determined using a Caspglow™ Fluorescein Active Caspase-3 Staining Kit (Biovision, CA, USA). For 5-ethynyl-2'-deoxyuridine (EdU) incorporation, mice were injected i.p. with 0.5ml of 2mg/ml EdU in PBS and splenocytes harvested 90 minutes later. EdU incorporation was determined using the Click-iTPlus EdU Flow Cytometry Assay Kit (Molecular Probes, CA, USA).

ELISA and ELISPOT

ELISA plates (Nunc, Paisley, UK) were coated with either NP23-bovine serum albumin (BSA) or NP2-BSA at 10μg/ml or 2μg/ml, respectively, as previously described (40). For ELISPOT, MultiScreen HA mixed cellulose ester plates (Millipore, Watford, UK) were coated with NP23-BSA or NP2-BSA in PBS, washed and blocked before applying serially diluted cells and incubating overnight. Detection was performed using biotinylated anti-IgG1 and anti-IgM (SouthernBiotech, Birmingham, AL), alkaline phosphatase-conjugated streptavidin (R&D Systems) and 5-bromo-4-chloro-3-indoyl phosphate/nitro-blue tetrazolium substrate (R&D Systems), and the number of antibody-secreting cells (ASCs) determined using a CTL ELISPOT reader and CTLImmunoSpot® Software (CA, USA).

PCR analysis

Lysates were prepared from FACS-purified GC and follicular B cells and PCR for GIMAP1 and GIMAP8 performed as previously described (22, 30).

Western blot analysis

FACS-purified GC and follicular B cells were lysed in NP-40 lysis buffer and GIMAP1 and actin protein detected by western blot as previously described (22).

iGC B cell culture

Follicular B cells were purified as described above and then cultured on fibroblasts (expressing CD40L and BAFF) with IL-4 in the presence of either vehicle control (DMSO) or 50nM 4-hydroxytamoxifen (4-OHT) for 4 days, prior to re-culture in IL-21 for a further 4 days, as described (41). Cells were counted using a Casy® cell counter (Innovatis) and stained for flow cytometric analysis as described above using anti-CD138 and anti-IgG1.

Results

Intrinsic requirement for GIMAP1 in B cells

We first determined the lineage-intrinsic nature of the B cell requirement for GIMAP1 by crossing our *Gimap1*$^{f/f}$ allele with *CD79a-Cre*, which is only active in the B cell lineage (36). Splenocytes, bone marrow, and peritoneal cells were enumerated and stained to determine the numbers of different B cells as previously described (22). The B cell phenotype of these mice mirrored that of *Gimap1*$^{f/f}$*CD2Cre*$^{+}$ mice where numbers of pro, pre, and immature B cells were normal (Figure 1A-C), but mature peripheral B cells were significantly reduced (Figure 1D-I). In addition, T2 and T3 transitional B cells were also reduced, as previously observed in *GIMAP1*$^{f/f}$*CD2Cre*$^{+}$ mice (Figure 1J-L; (22)). Previous studies have suggested that GIMAPs might regulate survival by manipulation of Bcl-2 family members (42) (29) (34) {Nitta, 2006 #33}. To address this, we crossed *Gimap1*$^{f/f}$*CD2Cre*$^{+}$ (in which *Gimap1* is ablated in the lymphoid lineage) with Eμ-*bcl-2-36* transgenic mice which over express human Bcl2 (39). To our surprise, hBcl-2 expression did not overcome the effect of *Gimap1* deletion on numbers of mature B cells in bone marrow, spleen, and peritoneum of *Gimap1*$^{f/f}$*CD2Cre*$^{+}$ mice (Figure 2A-H). More interestingly, where expression of the hBcl-2 transgene caused an increase in cell numbers in control GIMAP1 sufficient mice (*Gimap1*$^{f/f}$), this increase was not seen in GIMAP1-deficient animals (*Gimap1*$^{f/f}$*CD2Cre*$^{+}$). Signals through the BCR and BAFF receptor are also key for B cell survival. We found no differences in the expression of either of these receptors on germinal center B cells (Supplemental Figure 1).

GIMAP is required for the maintenance of the peripheral B cell pool

To examine the role of GIMAP1 in mature B cells *in vivo*, we generated mice in which the *Gimap1* gene can be inducibly deleted by crossing *Gimap1*$^{f/f}$ with mice expressing Cre recombinase under the control of the estrogen receptor, *ER*^{T2}*Cre*$^{+}$, to create *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ mice. This allowed conditional deletion of *Gimap1* upon administration of tamoxifen or its synthetic derivative, 4-hydroxytamoxifen (4-OHT). Deletion of *Gimap1*

was seen within 2 days of *in vitro* 4-OHT treatment of lymphocytes derived from *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ mice (unpublished observation). To control for Cre-mediated toxicity, *ER*^{T2}*Cre*$^{+}$ mice were used alongside *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ mice (43). In preliminary experiments we administered tamoxifen to *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ mice. We observed reductions in both T and B cells in peripheral lymphoid organs (data not shown). To eliminate the possible effect of *Gimap1*-deletion in non-lymphoid cells we went on to use an adoptive transfer model where only 5×10^{6} lymph node cells were injected into replete hosts. Using this system, only a small proportion of circulating lymphocytes would be susceptible to *Gimap1* deletion and these could be tracked by staining cells prior to transfer and by their expression of CD45.2. We adoptively transferred a mixture (in a 1:2 ratio) of *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ lymphocytes (stained with CFSE) and *ER*^{T2}*Cre*$^{+}$ lymphocytes (stained with CTV) into a replete, congenic CD45.1 host and administered tamoxifen to induce deletion of *Gimap1* in transferred *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ cells. Thirteen days later, splenocytes and peripheral blood were harvested analysed. Tamoxifen (but not vehicle) treatment resulted in the loss of nearly all *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ B cells with little effect on *ER*^{T2}*Cre*$^{+}$ B cells (Figure 3A–F). Figure 3A-B shows representative flow cytometry plots of CD45.2^{+}B220^{+} splenocytes taken on day 13 post tamoxifen or vehicle treatment where transferred *Er*^{T2}*Cre*$^{+}$ and *Gimap1*$^{f/f}$*Er*^{T2}*Cre*$^{+}$ cells are stained with CTV or CFSE, respectively. It shows the disappearance of *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ B cells and the relative retention of *Er*^{T2}*Cre*$^{+}$ B cells. Unstained cells shown in these FACSplots are transferred cells that had presumably lost their label since they were CD45.2^{+}B220^{+}. These data are representative of three independent experiments and we found no differential effect of labeling with either CTV or CFSE on lymphocyte survival since similar results were obtained when *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ cells were labeled with CTV and *ER*^{T2}*Cre*$^{+}$ cells labeled with CFSE (data not shown). This supports the view that the disappearance of *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ B cells is due specifically to the loss of GIMAP1. The percentages of input *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ *and ER*^{T2}*Cre*$^{+}$ B cells found in spleen were determined for each mouse (Figure 3C–D). The low frequency of transferred cells in the spleen is typical for this type of experiment and reflects the dispersal of lymphocytes throughout both lymphoid organs and multiple other tissues and organs of the body. We also show the ratio of recovered *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$*:ER*^{T2}*Cre*$^{+}$ B220^{+} cells from both blood and spleen following tamoxifen or vehicle treatment (Figure 3E-F). These results show that peripheral B cells intrinsically require continuous GIMAP1 expression for their maintenance.

GIMAP1 is expressed in germinal centre B cells

To date, GIMAP1 expression in germinal center B cells has not been analysed. Upon activation, antigen-specific B cells differentiate into long-lived memory B cells and plasma cells (PC), which are generated within the specialized microenvironment of the germinal center (GC). Microarray studies from the Immunological Genome Project (Immgen.org) show that GC B cells express GIMAP1 mRNA (Figure 4A). We looked at GIMAP1 protein expression in GC B cells (Figure 4B). Although mRNA levels of GIMAP1 are reduced in GC B cells compared to follicular B cells we find similar levels of GIMAP1 protein expressed in the two cell types, suggesting post-transcriptional regulation of GIMAP1 mRNA in B cells. To examine the role that GIMAP1 plays in B cell activation and differentiation, we specifically ablated *Gimap1* in activated B cells by crossing *Gimap1*$^{f/f}$

with *AicdaCre*$^{+}$ mice to generate *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice. *Aicda* is expressed within GC B cells and has been successfully used to elucidate the roles of many genes in the GC reaction (38, 44). We FACS purified GC and follicular B cells from these animals and from *Gimap1*$^{f/f}$ controls by FACS sorting, 7 days after NP-KLH immunization. Specific deletion of the *Gimap1* gene was evident only in GC B cells (defined as B220^{+ve}GL7^{+ve}Fas^{+ve}) from *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice (Figure 5A). The nearby *Gimap8* gene (analysed here as a control) was intact in both GC and follicular B cells from both strains of mice.

Severe impairment of T-dependent antibody responses in immunized *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice

In previous work we were unable to address what role GIMAP1 might play in peripheral B cell function since *Gimap1*$^{f/f}$*CD2Cre*$^{+}$ mice have very few mature B cells (22) and the lymphopenia within these mice is likely to affect B cell function. By immunizing *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice we were able to determine if GIMAP1 was required for peripheral B cell responses to a T-dependent antigen. *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice were generated and their B cells examined. Numbers and phenotypes of naïve B cells were unaffected by the transgene (Supplemental Figure 2). Mice were immunized with NP-KLH in alum and B cell responses analysed 7, 14 and 35 days later. We used binding of biotinylated NIP to detect cells bearing an NP-specific B cell receptor (BCR; Figures 5B-C). NP-specific IgG1-switched splenic B cells were identified by flow cytometry (B220^{+}IgM^{-}IgD^{-}NIP^{+}IgG1^{hi} (45) and enumerated (Figure 5B-C). *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice mounted a very poor response, with relatively few NP-specific switched B cells detectable (Figure 5B-C). We found a similar deficiency in GC B cells, defined as B220^{+}GL7^{+}CD95^{+} (data not shown). A defect in GC function in the absence of GIMAP1 was also evident when serum Ig levels in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ and *Gimap1*$^{f/f}$ mice were compared (Figure 5D). At day 7, a small reduction in NP-specific IgG1 was seen in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice compared to control mice. By day 14, this difference was much greater. This difference could reflect a slow turnover of functional GIMAP1 protein. In addition, we looked at affinity maturation by measuring antibody reactive with NP2 at 35 days post-immunization (p.i.) (Figure 5D). Again, *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice had very low titers of high affinity antibodies. The number of antibody secreting cells (ASCs) was determined by ELISPOT on day 14 and 42 p.i. (Figure 5E). As expected, no significant differences were seen in the numbers of IgM secreting cells from either the spleen or bone marrow. In contrast, numbers of IgG1 secreting cells specific for NP23 and NP2 from both spleen and bone marrow were lower in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice compared to control animals. In some mice, the number of ASCs was below the level of detection.

Failure of the germinal center to develop in the absence of GIMAP1

The impaired production of class switched and high affinity, antigen-specific antibodies and the deficit of ASCs and long-lived plasma cells in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice suggested a defect in GC B cells. We enumerated splenic GC B cells as NIP-binding, IgG1^{+} and CD38^{-}cells and looked at the development of the GC response on days 6, 8, and 10 p.i. The effect of *Gimap1* deletion was evident by day 6 p.i. (Figure 6A). By day 8 p.i. GC B cell numbers had fallen further in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice. A slight recovery was seen at day 10 p.i. (possibly reflecting the expansion of cell clones that had escaped *Gimap1* deletion).

To investigate why GC B cells failed to develop in the absence of GIMAP1, we examined their proliferation and death. EdU incorporation into newly synthesized DNA was used to measure GC B cell proliferation on day 8 p.i. (Figure 6B). There was a slight but significant decrease in the proliferation of these cells in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice compared to controls. To look at cell death in GC B cells, we measured the percentage of GC B cells expressing active caspase-3 by flow cytometry. We found a small but significant difference in the percentage of GC B cells expressing active caspase-3 in cells from *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ cells (Figure 6C).

To confirm our *in vivo* data, we made use of an *in vitro* system to generate induced GC (iGC) B cells (41). B cells were purified from spleens and cultured with fibroblasts expressing BAFF and CD40L. For the first 4 days IL-4 was added to the culture to generate iGC B cells; then IL-21 was added to induce their differentiation into PCs. B cells from *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and control *ER*^{T2}*Cre*$^{+}$ mice were cultured for 8 days in the presence of either 4-OHT or vehicle control. We were able to delete GIMAP1 by day 6 of culture and examine cell proliferation, GC development, differentiation to PCs, and cell death. Within 6 days of culture, all cells expressed GL7 and CD95, indicative of a GC-like phenotype (data not shown). The slope of the curves in vehicle treated *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ cells was similar suggesting their rate of proliferation was the same. However, 4-OHT treated *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ (but not control *ER*^{T2}*Cre*$^{+}$) cells appeared to stop proliferating between day 4 and 8 (Figure 7A). We found no difference in the percentage of cells that further differentiated into PCs (CD38^{+}IgG1^{+}; Figure 7B) showing that lack of GIMAP1 has no effect on differentiation. In addition, we looked at DAPI uptake by these cells to determine the percentage of live cells. 4-OHT-treated *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ cells had significantly fewer live (DAPI^{-}) cells at day 8. This suggests that cells initiated apoptosis (evident on day 6) and stopped proliferating, resulting in both a reduction in the number of iGC B cells and the percentage of live cells on day 8 (Figure 7C).

Failure to establish B cell memory in the absence of GIMAP1

To determine if memory B cell responses were also affected in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice, we looked at secondary responses to NP-KLH. Thirty-five days after primary immunization, *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ and control mice were boosted with NP-KLH. Memory responses were analyzed 7 days later. The numbers of antigen-specific IgG1 switched B cells were significantly reduced in *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ animals; indeed, in some animals, none could be detected (Figure 8A). NP-specific IgG1 antibody levels were also reduced, most markedly for high affinity antibody (as determined by NP2 binding; Figure 8B). When the numbers of IgG1 ASCs binding to NP23 and NP2 were determined, many *Gimap1*$^{f/f}$*AicdaCre*$^{+}$ mice had numbers below our detection limit of one in 10^{6} cells (Figure 8C).

Discussion

Regulation of lymphocyte function and homeostasis is paramount for an effective and balanced immune system. Dysregulation of this balance frequently leads to autoimmune diseases, inability to fight infection or cancer. Understanding the mechanisms maintaining

this homeostasis may offer opportunities for the selective ablation of lymphocyte pools as a therapeutic strategy. Over the last 15 years members of the GIMAP family have been implicated as important modulators of peripheral T lymphocyte homeostasis and survival. Very few studies have addressed the role of GIMAPs in B cell biology. We have shown that deletion of GIMAP8 results in a reduction in the number of recirculating B cells in the bone marrow (30). The generation and characterization of GIMAP1- and GIMAP5-deficient mouse strains has further demonstrated that peripheral B cell survival is also influenced by GIMAPs (22, 23, 27, 34). As observed for T cells, deletion of *Gimap1* appears to affect only mature cells within the B cell lineage (22). In contrast, GIMAP5-deficient mice show reductions in pro/pre-B cell progenitors resulting in a more marked reduction in immature and mature B cells. This defect is thought to be due to the effect of GIMAP5 deficiency on hematopoietic stem cells (34). In a separate study, GIMAP5-deficient mouse B cells were also reduced in numbers in the periphery and severe reductions in B cell responses to immunization were observed (27). As a caveat, there is an important methodological difference between our own studies of GIMAP1 deficiency and those of GIMAP5 in that while we employed conditional, Cre-mediated ablation of *Gimap1*, the *Gimap5*-related studies were conducted using germline mutant animals. In the absence of studies employing conditional or elective deletion of the *Gimap5* gene, it is not yet possible to assert whether the B cells changes reported in GIMAP5-deficient animals are B cell-intrinsic or mediated via effects on other cells.

We have undertaken the development of mouse strains that enable specific and elective ablation of *Gimap1* in B cells. Thus, the effects we describe cannot be attributed to a "legacy" effect of GIMAP1 (i.e. the result of a function that GIMAP1 performs during B cell development that affects the functional capacity of the cells at a later stage) or to a cell-extrinsic effect acting on B cells. Adoptive transfer of B cells followed by ablation of their *Gimap1* gene resulted in their disappearance from the periphery. This result reflects an intrinsic requirement for GIMAP1 in mature B cells, most likely to prevent their cell death in the periphery.

Importantly, our *in vivo* studies have shown that B cells further require GIMAP1 during the development of antibody responses. This is the first study to show the conditional ablation of a GIMAP protein during lymphocyte activation. Our data strongly suggest that B cells require GIMAP1 for their post-activation expansion since in our *in vitro* iGC culture, we saw differentiation of cells into iGCs and PCs but not the expected subsequent expansion of these populations. Similar to the results of our *in vitro* experiments, GIMAP1-deficient GC B cells do not go through normal expansion *in vivo*. There is also reduced antibody production and affinity maturation, recall IgM response, and an inability to generate a long-lived memory response. These data establish GIMAP1 as absolutely essential for B cell peripheral function.

The deficit of GC B cells in $Gimap1^{f/f}AicdaCre^{+}$ mice makes elucidation of the mechanism of GIMAP1 action challenging. We were able to detect an increase in active caspase-3 in GC B cells from $Gimap1^{f/f}AicdaCre^{+}$ mice, indicative of apoptosis and also observed a reduction in the percentage of proliferating GC B cells. Although these differences were relatively small the GC reaction is a rapidly evolving B cell response where small changes in

rates of proliferation and/or cell death can have a large impact on cell numbers (46). It is also likely that the few GC B cells we do detect are a mixture of GIMAP1-deficient GC B cells destined to die and GC B cells that have escaped *Gimap1* gene deletion (47).

The mode of action of GIMAP1 remains enigmatic. GIMAP5 has been reported to associate with Mcl1 and HSC70 and to promote stabilization of Mcl1 at the mitochondrial membrane (34). Mcl1 is critical for both the survival of GC B cells and PCs (48, 49). It is possible that GIMAP1 may also play a role in Mcl1 stabilisation that is not compensated by GIMAP5. However, we found no evidence of this in *Gimap1* deficient germinal center cells. Furthermore, expression of the hBcl-2 transgene did not overcome the survival defect of mature B cells in $Gimap1^{f/f}CD2Cre^{+}$ mice. A hBcl-2 transgene has been previously shown to increase numbers of immature and mature B cell subsets (50). Interestingly, deletion of GIMAP1 overcame this effect of hBcl-2, suggesting that GIMAP1-induced lymphocyte survival either uses a mechanism distinct from that of Bcl-2 family members or works downstream of Bcl-2 family members. We also looked at expression levels of IgM and BAFFR, both key mediators of B cell survival. However, no defects were detected in GIMAP1-deficient germinal center cells. In previous work we showed that GIMAP5 associates with lysosomes while GIMAP1 associates with the Golgi apparatus (51). Hence, it may be valid to speculate that B cells deficient in GIMAP1 may succumb to lysosome-related cell death: lysosomal hydrolases are delivered to late endosomes via the mannose-6-phosphate pathway from the trans-Golgi network, where GIMAP1 is located. GIMAP1 loss may compromise the structural integrity of endosomes, enabling their release into the cytosol. Indeed, apoptosis of human GC B cells has been shown to involve lysosomal destabilization resulting GC B cell death (52).

The fact that B cells can develop normally in the absence of GIMAP1 yet fail to maintain their peripheral numbers suggests that the survival mechanisms of developing and mature B cells are distinct. Many proteins involved in B cell survival are required for both developing and mature B cells. However, members of the NF-κB family show B cell knockout phenotypes similar to $Gimap1^{f/f}CD2Cre^{+}$ mice and are important for the survival of mature B cells irrespective of their activation status (53) (54) (55) (56) (57) (47). In $Gimap1^{f/f}CD2Cre^{+}$ mice, the B cell defect is first evident in the immature transitional type 2 (T2) cells found in the spleen (22) (53). This implicates a problem in either the differentiation of T1 cells into T2 cells or a failure of T2 cells to survive. A similar block is seen in mice deficient in members of the NF-κB family (53, 54). NF-κB activation is required for the differentiation of T1 cells into T2 cells, suggesting that the BCR provides a survival signal mediated by NF-κB (53) (54) (55) (56) (57). It is tempting to speculate that tonic signaling through the BCR provides a survival signal to peripheral B cells that also relies upon GIMAP1. Perturbations in NF-κB signaling are seen in GIMAP5-deficient T cells, resulting in their deregulation (58). Dysregulation of the NF-κB signaling pathway is also seen in B cell lymphomas, with DLBCLs being strongly dependent on NF-κB activity (59-61). DLBCLs also have a hypomethylated region in the *Gimap* locus, resulting in over-expression of GIMAPs 1 and 5 (10). It is tempting to speculate that GIMAPs 1 and 5 regulate cell survival via the NF-κB pathway and that manipulation of either protein can tilt the balance between lymphocyte death and survival (58).

In summary, this work shows that GIMAP1 is required for the establishment and maintenance of the peripheral B cell pool and for all stages of post-activation B cell survival. In the absence of GIMAP1 mature B cells die, irrespective of their activation status or function. Together with our previous work, this establishes GIMAP1 as a key pro-survival factor for mature B lymphocytes and a potential target for the control of B cell mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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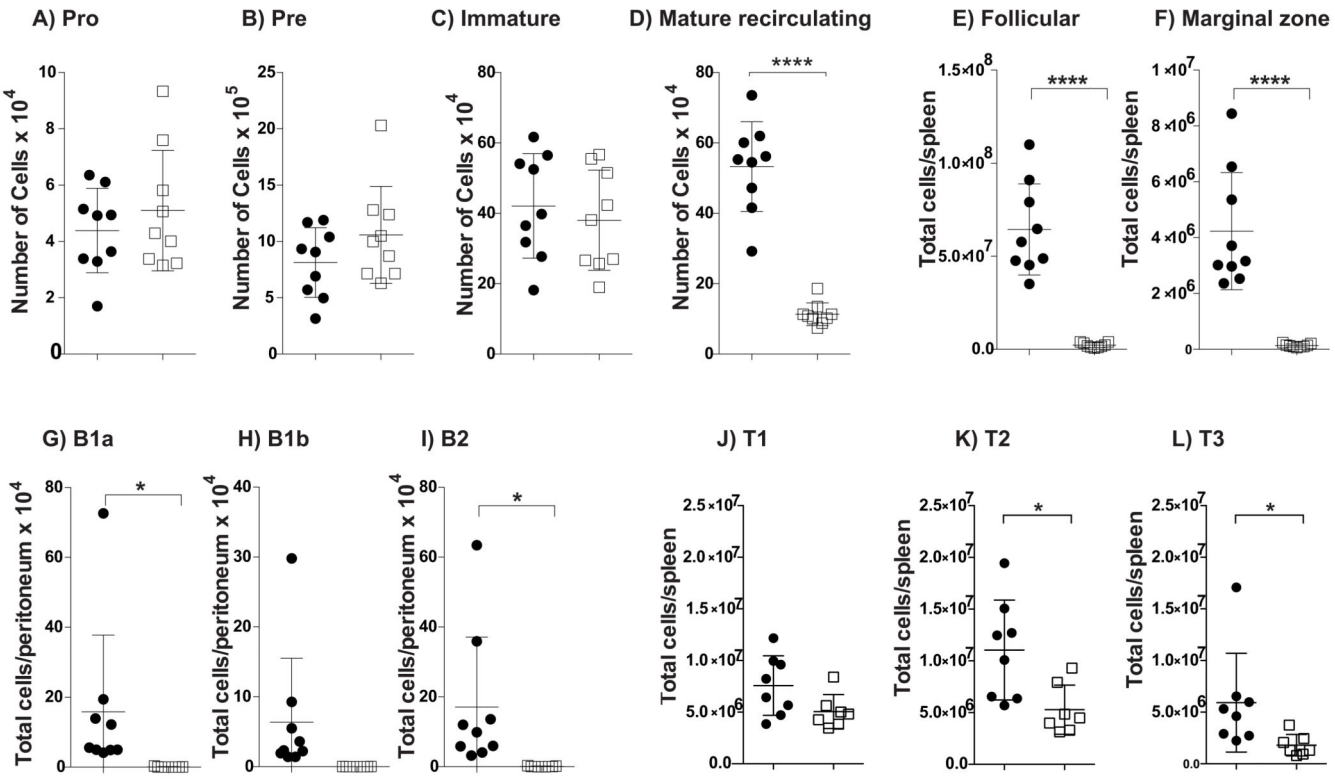

Figure 1. Cell intrinsic requirement for GIMAP1 in mature B cells

B cell subsets present in bone marrow (A-D), spleen (E-F, J-L), and peritoneal cavity (G-I) from *Gimap1*$^{f/f}$*CD79aCre*$^{+}$ mice and age- and sex-matched *Gimap1*$^{f/f}$ controls were enumerated (A-I). Gating is as previously described (22). Results show the number of cells/organ for individual mice with the mean ± S.D. (*Gimap1*$^{fl/fl}$ [●] and *Gimap1*$^{fl/fl}$*CD79aCre*$^{+}$[□]. Results show the number of cells/organ for individual mice with the mean ± S.D. *p <0.05, ****p<0.00005 (unpaired 2-tailed Student's t test).

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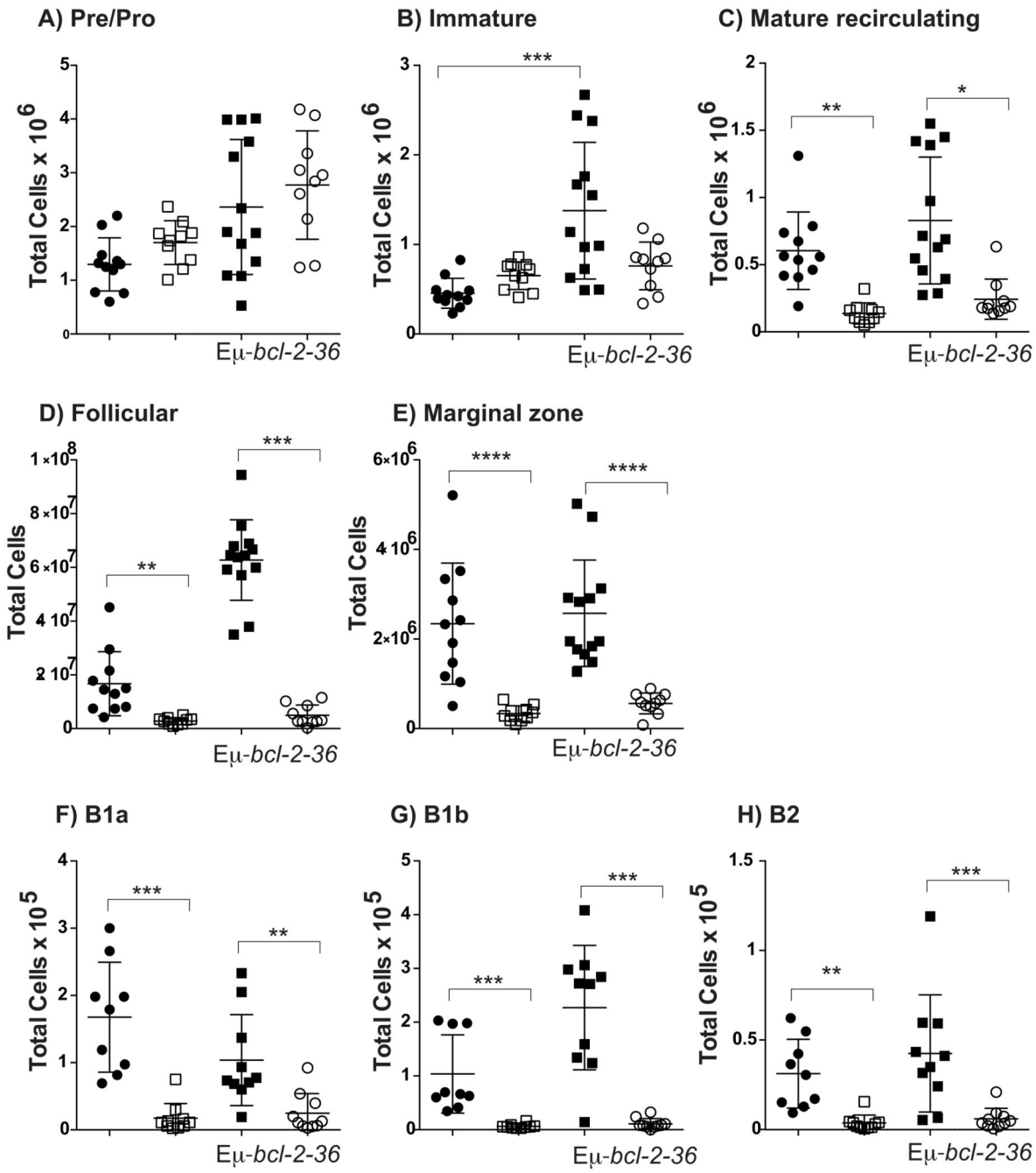

Figure 2. Bcl2 does not prevent cell death in GIMAP1-deficeint B cells

B cell subsets present in bone marrow (A-C), spleen (D-E), and peritoneal cavity (F-H) from *Gimap1*$^{fl/fl}$ [●], *Gimap1*$^{f/f}$*CD2Cre*$^{+}$ [□], *Gimap1*$^{fl/fl}$*Bcl2*^{tg} [■], and *Gimap1*$^{f/f}$*CD2Cre*$^{+}$*Bcl2*^{tg} [○] from age- and sex-matched mice were enumerated (A-H), using previously described gating (22). Results show the number of cells/organ for individual mice with the mean ± S.D. *p <0.05, **p <0.005, ***p <0.0005, ****p<0.00005 (unpaired 2-tailed Student's t test).

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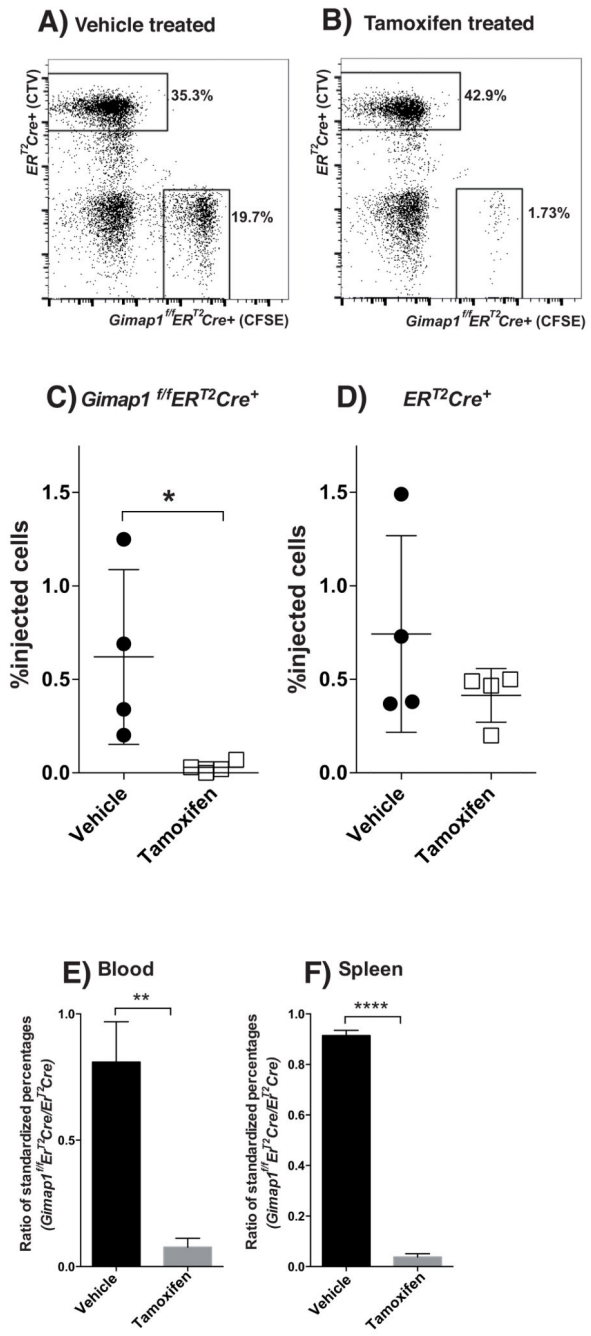

Figure 3. Mature B cells require GIMAP1 for their survival in the periphery

Lymph node cells from *Gimap1*$^{fl/fl}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ mice were stained with CFSE and CTV, respectively, mixed in a 1:2 ratio (*Gimap1*$^{fl/fl}$*ER*^{T2}*Cre*$^{+}$:*ER*^{T2}*Cre*$^{+}$) and injected into replete B6.SJL-*Ptprca Pepcb*/BoyJ (CD45.1^{+ve}) mice. Mice were treated with either vehicle control or tamoxifen and spleen and blood harvested 13 days later. (A-B) shows representative flow cytometry plots from spleen gated on transferred (CD45.2^{+ve}B220^{+ve}) cells from vehicle (A) or tamoxifen (B) treated mice. (C-D) shows the percentages of adoptively transferred *Gimap1*$^{fl/fl}$*ER*^{T2}*Cre*$^{+}$ (C) and *ER*^{T2}*Cre*$^{+}$ (D) B220^{+} cells remaining in

the spleen 13 days after vehicle or tamoxifen treatment of individual recipient mice. Each symbol represents the percentage of transferred cells remaining from an individual mouse with the mean ± S.D. shown. (E-F) shows the proportion of *Gimap1*$^{fl/fl}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ cells found in blood (E) and spleen (F) of recipient animals treated with either tamoxifen (grey bars) or vehicle control (black bars), presented as a ratio of their proportions and standardized by the percentages of *Gimap1*$^{fl/fl}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ cells injected on day 0. Each panel is representative of 3 independent experiments where 4 mice were used per group. *p <0.05, **p <0.005, ****p<0.00005 (unpaired 2-tailed Student's t test).

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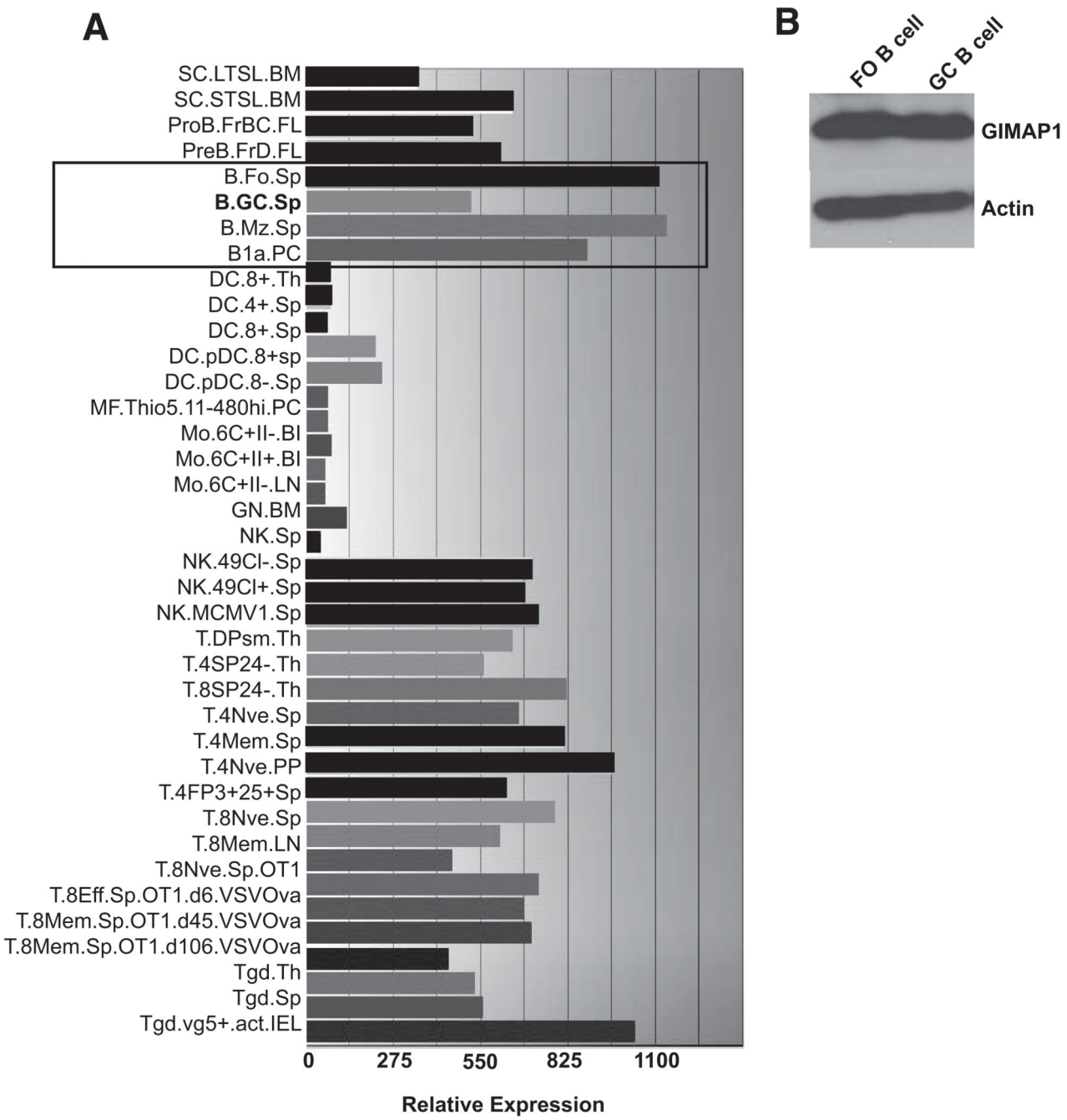

Figure 4. GIMAP1 expression in germinal center B cells

(A) Relative expression of GIMAP1 mRNA in immune cells (adapted from http://www.immgen.org/). B.Fo.Sp – splenic follicular B cells; B.GC.Sp – splenic germinal center B cells; B.Mz.Sp – splenic marginal zone B cells; B1a.PC – peritoneal cavity B1 B cells. (B) Western blot detection of GIMAP1 protein in follicular and GC B cells from wt mice.

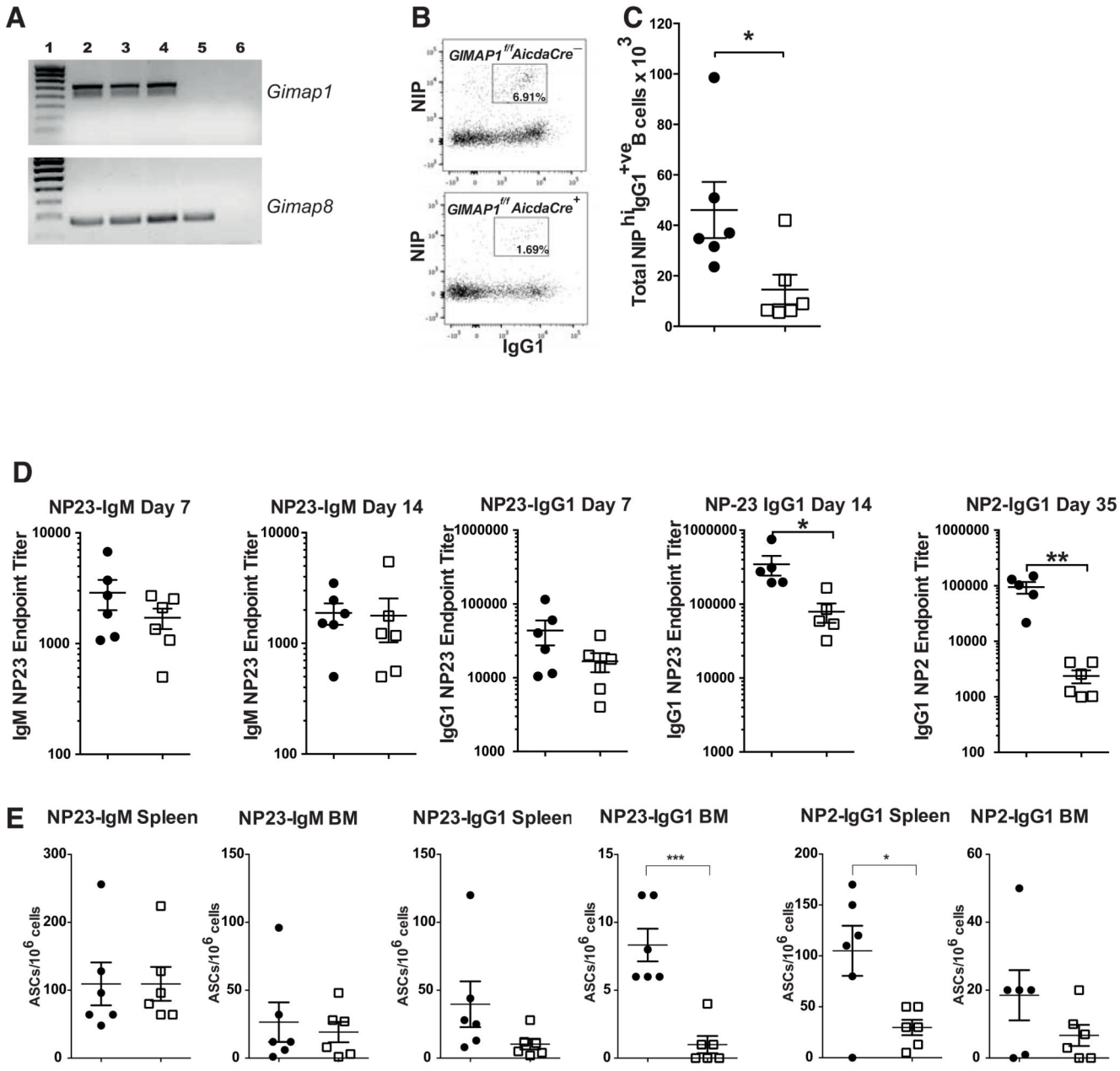

Figure 5. GIMAP1 is essential for germinal center B cell responses

(A) Conditional deletion of *Gimap1* in germinal center B cells. PCR analysis of *Gimap1* and *Gimap8* in germinal center B cells FACS-sorted from immunized *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ and *Gimap1*$^{fl/fl}$ mice. Lane 1 = 100bp DNA ladder; lane 2 = follicular B cells from control *Gimap1*$^{fl/fl}$ mice; lane 3 = germinal center B cells from control *Gimap1*$^{fl/fl}$ mice; lane 4 = follicular B cells from *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ mice; lane 5 = germinal center B cells from *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ mice; lane 6 = H_2O control. (B) Facsplots showing NIP-binding IgG1-switched B cells in *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ and *Gimap1*$^{fl/fl}$ mice on day 7 p.i. (C) Enumeration of NIP-binding IgG1-switched (B220^{+ve}IgM^{-ve}IgD^{-ve}) B cells on day 7 p.i. in *Gimap1*$^{fl/fl}$ (●) and *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (□). Results show the number of cells per spleen

for individual mice with the mean ± S.D. (D) Titres of NP23-binding (low affinity) and NP2-binding (high affinity) IgG1 and IgM antibodies on days 7 and 14 after primary immunization. Each symbol represents an individual mouse (*Gimap1*$^{fl/fl}$ (●) and *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (□) with the mean ± S.D. shown. (E) The frequency of NP-specific IgG1 and IgM ASC from *Gimap1*$^{fl/f}$ (●) and *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (□) mice 14 days p.i. as determined by ELISPOT. Each symbol represents an individual mouse with the mean ± S.D. shown. Differences were examined using an unpaired Student's *t* test and only significant differences are marked (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

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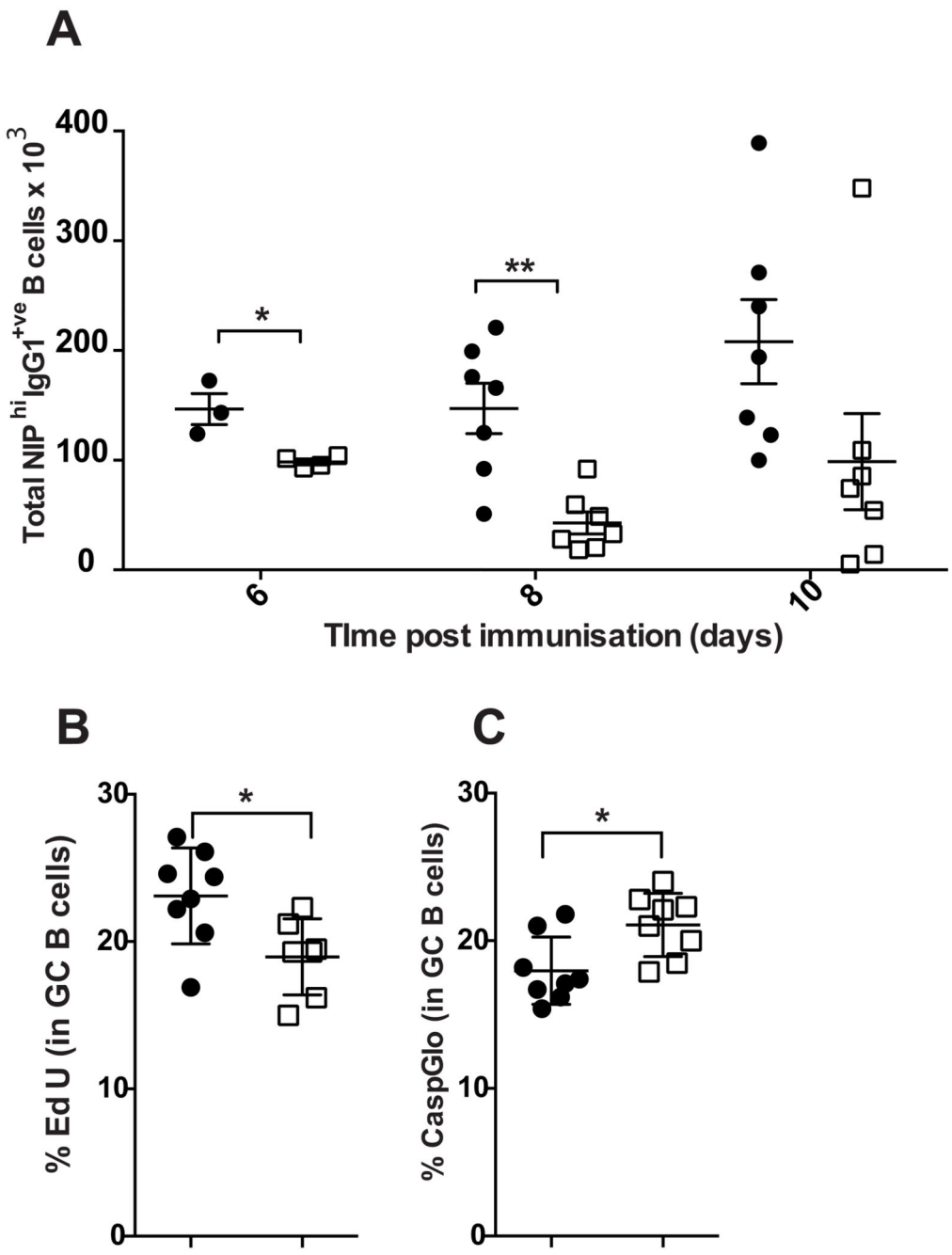

Figure 6. Germinal center responses fail to develop in the absence of GIMAP1

Results show values for individual mice with the mean ± S.D. also shown: *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (●); and control *Gimap1*$^{fl/fl}$ (□) mice. (A) NIP-binding, IgG1^{+}, CD38^{-} B cells on days 6, 8, and 10 p.i. (B) Percentages of GC cells that had incorporated EdU (data for individual mice). (C) Percentages of GC B cells that were positive for active caspase-3. Differences were examined using Student's *t* test and only significant differences are marked (* $p<0.05$, ** $p<0.005$).

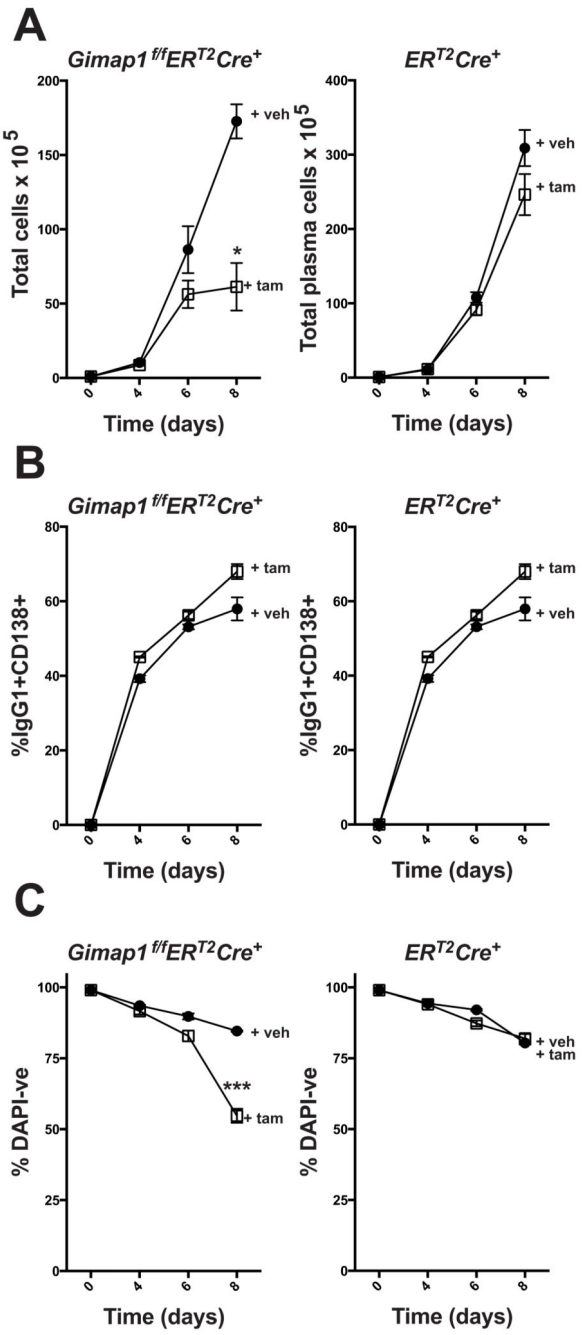

Figure 7. ***In vitro*** **generation of iGC and PCs is compromised by lack of GIMAP1**

B cells from *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ mice were cultured to induce GC and PC differentiation in the presence of either 4-OHT or vehicle control. Cells were harvested on days 4, 6, and 8, counted and stained with anti-IgG1, anti-CD138 and DAPI. Panel (A) shows the total number of PC generated from *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ B cells in the presence of 4-OHT (□) or vehicle (●). (B) Percentages of cells that had differentiated into PCs from *Gimap1*$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ B cells in the presence of 4-OHT (□) or vehicle (●). (C) Percentages of live PCs as determined by the exclusion of DAPI from

Gimap1$^{f/f}$*ER*^{T2}*Cre*$^{+}$ and *ER*^{T2}*Cre*$^{+}$ B cells in the presence of 4-OHT (□) or vehicle (●). Each symbol represents an individual mouse with the mean ± S.D. shown. Differences were examined using an unpaired Student's *t* test and only significant differences are marked (* $p<0.05$, *** $p<0.0005$).

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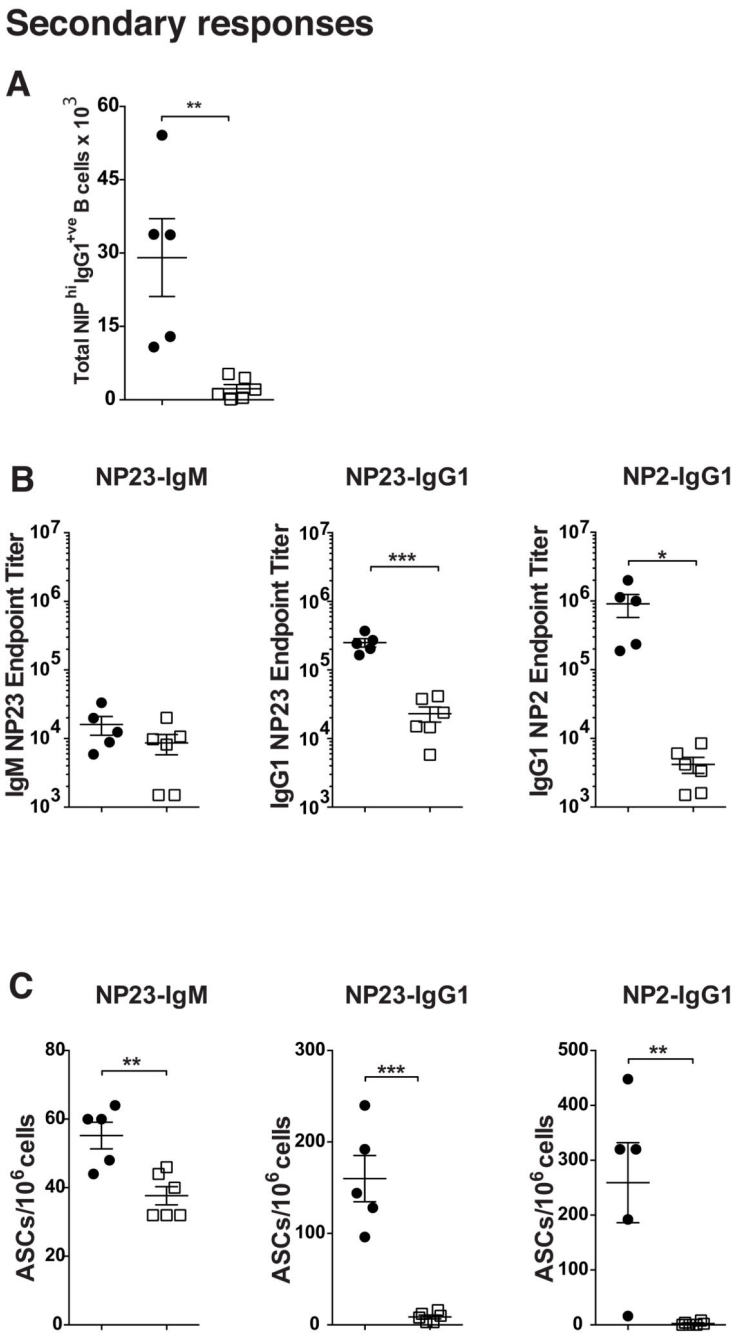

Figure 8. Failure to establish B cell memory in the absence of GIMAP1

Secondary responses in *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (□) and control *Gimap1*$^{fl/f}$ (●) mice: each symbol represents an individual mouse with mean ± S.D. also shown. (A) NIP-binding, IgG1-switched splenic B cells enumerated using flow cytometric analysis. (B) Titres of NP23-binding (total affinity) and NP2-binding (high affinity) IgG1 and IgM antibodies on day 7 of secondary immunization. (C) The frequency of NP-specific IgG1 and IgM ASC from *Gimap1*$^{fl/fl}$ (●) and *Gimap1*$^{fl/fl}$*AicdaCre*$^{+}$ (□) mice 7 days p.i. as determined by ELISPOT is shown. Each symbol represents an individual mouse with the mean ± S.D.

shown. Differences were examined using an unpaired Student's *t* test and only significant differences are marked (* $p<0.05$, ** $p<0.005$, ***<0.0005).

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