

Putative GTPase GIMAP1 is critical for the development of mature B and T lymphocytes

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The guanosine triphosphatases (GTPases) of the immunity-associated protein (GIMAP) family of putative GTPases has been implicated in the regulation of T-lymphocyte development and survival. A mouse conditional knockout allele was generated for the immune GTPase gene *GIMAP1*. Homozygous loss of this allele under the influence of the lymphoid-

expressed *hCD2-iCre* recombinase transgene led to severe (> 85%) deficiency of mature T lymphocytes and, unexpectedly, of mature B lymphocytes. By contrast there was little effect of *GIMAP1* deletion on immature lymphocytes in either B or T lineages, although in vitro studies showed a shortening of the survival time of both immature and mature

CD4⁺ single-positive thymocytes. These findings show a vital requirement for GIMAP1 in mature lymphocyte development/survival and draw attention to the nonredundant roles of members of the GIMAP GTPase family in these processes. (*Blood*. 2010;115(16):3249-3257)

Introduction

The guanosine triphosphatases (GTPases) of the immunity-associated proteins (GIMAPs) are a family of guanosine nucleotide-binding proteins implicated in the regulation of T-lymphocyte development and survival.¹ They are encoded in humans, rats, and mice by a cluster of 7 to 8 autosomal genes. They are expressed strongly in lymphoid tissues but are also readily detectable in some other tissues such as kidney, lungs, and heart.²⁻⁴ Recent evidence indicates that the *GIMAP* genes are targets of Notch regulation.⁵ With the exception of *GIMAP5* and *GIMAP4*, there is little information on the in vivo function(s) of these proteins. *GIMAP5*, which has prosurvival properties in T lymphocytes, is the best-studied family member as a result of a spontaneous recessive mutation within the rat gene orthologue. This mutation predisposes to autoimmunity and gave rise to the BB rat model of type 1 diabetes and, more recently, to a model of T-helper 2 (Th2)-type inflammatory bowel disease.^{2,6-8} Furthermore, autoimmunity-related traits in humans have been associated with a *GIMAP5* gene variant.^{9,10} The mutation in rat *GIMAP5* causes peripheral T lymphopenia (reviewed in Ramanathan and Poussier¹¹), but thymic abnormalities in the mutant animals are limited and largely confined to the mature CD4⁺ and CD8⁺ single-positive (SP) compartments.¹² No effect on B-lymphocyte populations has been reported in these rats. Recently, a similar T-cell deficiency has been reported for a *GIMAP5* knockout mouse strain,¹³ although deficiency in a minor population of B cells, the splenic marginal zone (MZ) B cells, was also detected. Short hairpin RNA knockdown of *GIMAP5* and *GIMAP3* expression in mouse fetal thymic organ culture has implicated these genes in, respectively, the generation of CD4⁺CD8⁺ double-positive (DP) thymocytes and T cell-positive selection.¹

In contrast with *GIMAP5*, *GIMAP4* has “prodeath” properties. Knocking out the *GIMAP4* gene had no deleterious effect on in

vivo lymphocyte development; instead, ex vivo, T lymphocytes exhibited a slowing of the apoptotic process when subjected to a number of death-inducing treatments.¹⁴ In combination, these data imply diverse but related roles for the *GIMAP* genes in T-cell development and survival.

GIMAP1 is a close relative of *GIMAP5* and lies adjacent to it within the *GIMAP* gene cluster. Like *GIMAP5*, it encodes a protein that contains a putative transmembrane domain at its carboxyl terminus, indicative of targeting to intracellular membranes. *GIMAP1* mRNA has been found in all stages of T-cell development, without major variations in level,^{1,14,15} although differential expression of human T cell *GIMAP1* under conditions of Th1 or Th2 polarization in vitro has recently been reported.¹⁶ We now report on the novel phenotype of mice in which *GIMAP1* has been knocked out conditionally in lymphocytes. Our data show critical requirements for this gene in lymphocyte development, not only in the T-cell lineage but also, unexpectedly, in the B-cell lineage.

Methods

Animals

Mice were bred and maintained in specific pathogen-free conditions at The Babraham Institute. Husbandry and experimentation complied with existing United Kingdom Home Office licenses and local standards, as approved by the Babraham Institute Animal Welfare, Ethics, and Experimentation Committee.

Generation of mice bearing a conditional *GIMAP1* allele

A 129 mouse P1 artificial chromosome library¹⁷ was screened for the presence of *GIMAP* genes. Recombineering¹⁸ was used to develop a targeting vector for *GIMAP1* exon 3, using P1 artificial chromosome no.

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RP21-609H15 as the source of the relevant DNA sequences. Exon 3 encodes all but the first 14 residues of the predicted GIMAP1 polypeptide, so the conditional removal of this exon was expected to abolish the function of the gene. In the final targeting construct, *LoxP* sites flanked *GIMAP1* exon 3 and, 3' of that, a neomycin-resistance cassette was located between FRT sites (Figure 1A). The construct was electroporated into embryonic day 14 embryonic stem (ES) cells.¹⁹ After antibiotic selection with G418, ES cell colonies were picked, expanded and analyzed for the targeting event by Southern blotting of *SpeI*- and *BamHI*-digested DNA. Correctly targeted ES cells were injected into C57BL/6*Tyr*^{-/-} blastocysts that were transferred to pseudopregnant recipients. Chimeras generated in this way were bred to obtain germline transmission, confirmed by analysis of tail biopsy DNA. Polymerase chain reaction A (PCR-A) distinguishes between wild-type *GIMAP1* and the floxed allele. Mice positive for the *GIMAP1* floxed allele were paired with mice expressing the FlpE recombinase to remove the Frt-flanked *Neo^R* cassette and this was assessed with the use of PCR-B (Figure 1A). Mice with this *neo*-less conditional *GIMAP1* allele were then bred with animals carrying the *hCD2-iCre* transgene²⁰ to produce *GIMAP1^{fl/fl},hCD2iCre^{+/-}* mice. The mice used for the analyses reported here were on a mixed 129-C57BL/6 background. The PCR primers for PCR-A and PCR-B were as follows: PCR-A forward, 5'-CCA-GTCTAGAGTGAGCTTCACTCATCAGG-3'; PCR-A reverse, 5'-GG-ATTCTCTGTGCACCTGCAGATGCTCCTG-3'; PCR-B forward, 5'-GATTCACAGTGCAGTGACTTGGCATCAACT-3'; PCR-B reverse, 5'-CTGAGTCGACGTGTGTTCTTTGCGCTGTGA-3'.

Flow cytometric analysis

Single-cell suspensions from thymus, spleen, lymph nodes (axillary, brachial, and inguinal), bone marrow (BM), and peritoneal flushings were stained for flow cytometry. For peritoneal cells and splenocytes, cells were first incubated with Fc block (2.4G2; eBioscience). Biotinylated or fluorochrome-conjugated antibodies against CD3, CD4, CD8, CD19, CD21, CD23, CD24, CD25, CD93, T-cell receptor β (TCR β), B220, immunoglobulin M (IgM), c-kit, Thy1.2, and Qa2, as well as fluorochrome-conjugated streptavidin as a second-stage reagent (eBioscience; BD Biosciences) were used. After staining, cells were washed and resuspended in 25 ng/mL 4',6-diamidino-2-phenylindole before analysis on an LSRII Flow Cytometer (BD Biosciences). Analysis was performed using FlowJo (TreeStar Inc) software.

Intracellular staining

For pSTAT5 analysis, cells were first stimulated with culture medium containing 10% interleukin-7 (IL-7) supernatant at 37°C for 30 minutes. Cells were then fixed with 2% paraformaldehyde at 37°C. Subsequently, cells were permeabilized on ice with the use of 90% methanol in phosphate-buffered saline. After washing twice in fluorescence-activated cell sorting (FACS) buffer, cells were then stained for 1 hour with anti-pSTAT5-PE before analysis on a LSRII Flow cytometer (BD Biosciences).

Fluorescence-activated cell sorting

Single-cell suspensions of thymocytes, BM cells, or splenocytes were stained as described under "Flow cytometric analysis," and cell populations were sorted using a FACSaria (BD Biosciences). Purities were between 90% and 99.9%.

Cell survival assays

FACS-purified CD4 SP thymocytes were washed and resuspended in RPMI (10% fetal calf serum; with or without IL-7 supplementation) and plated at 5 to 7 \times 10⁵/well in 96-well plates and incubated at 37°C. At set times, cells were harvested and stained with Annexin V, anti-Qa2, and anti-CD24 antibodies before analysis by flow cytometry.

Western blotting of cell lysates

Cell lysates were prepared, and Western blots were performed essentially as described elsewhere.⁴ For the analyses in Figure 7A and B, 10⁵ cell equivalents were run per track. For Figure 7C, 5 \times 10⁵ cell equivalents were used. Details of the derivation and characteristics of the anti-GIMAP1 monoclonal antibodies used are given in Table 1.

Results

A conditional knockout of the mouse *GIMAP1* gene

GIMAP genes are expressed most strongly in lymphoid cells and tissues, but expression of some family members is also detectable elsewhere, eg, in lung, kidney, and heart. For this reason, a conditional targeting approach to the reverse genetic analysis of *GIMAP1* was selected. In addition, the availability of a conditional allele of this gene would provide flexibility in future studies of its function in different cell lineages.

A conditional targeting vector for exon 3 of mouse *GIMAP1* was developed and used to generate mouse ES cells with a modified *GIMAP1* allele (see "Methods"; Figure 1A). Mice bearing the modified allele were successfully derived and then crossed with mice bearing the *hCD2-iCre* transgene. This transgene allows Cre recombinase expression in the T-cell lineage from the CD4⁻CD8⁻ double-negative 1 (DN1) thymocyte stage onward (and possibly also in prethymic precursors); in the B lineage it is expressed from at least the late pro-B stage onward.²⁰ Efficient *hCD2-iCre*-mediated excision within the targeted *GIMAP1* gene was shown by PCR analysis of thymus DNA (Figure 1B). Western blots detected no GIMAP1 protein in the thymus of *GIMAP1^{fl/fl},hCD2iCre^{+/-}* mice (Figure 1C), in which T and B lineage-specific excision of *GIMAP1* was expected to occur. These mice are designated "GIMAP1/CD2-cKO" in the rest of the text.

Table 1. Derivation and characteristics of the anti-GIMAP monoclonal antibodies

GIMAP no.	MAb name	Rat γ isotype	Rat strain	φ/δ	Fusion partner	Immunogen	Vector/bacteria	Immunogen preparation
1	MAC420*	2a	LOU/C	φ	Y3Ag1.2.3†	GST-mGIMAP1aa1-238	pDEST15/RosettaDE3	Glutathione bead-eluted/gel-purified
4	MAC417‡	2b	LOU/C	φ	Y3Ag1.2.3	GST-mGIMAP4aa1-310	pGEX.4T1/Rosetta2	Glutathione bead-eluted
5	MAC421	2a	LEW	φ	Y3Ag1.2.3	GST-mGIMAP5aa1-270	pDEST15/RosettaDE3	Glutathione bead-eluted
6	MAC432	2b	DA	δ	IR983F§	GST-mGIMAP6 full length	pGEX.4T1/Rosetta	Inclusion bodies
9	MAC433	2a	DA	δ	Y3Ag1.2.3	GST-mGIMAP9 full length	pGEX.4T1/Rosetta	Inclusion bodies

*See Saunders et al.⁴

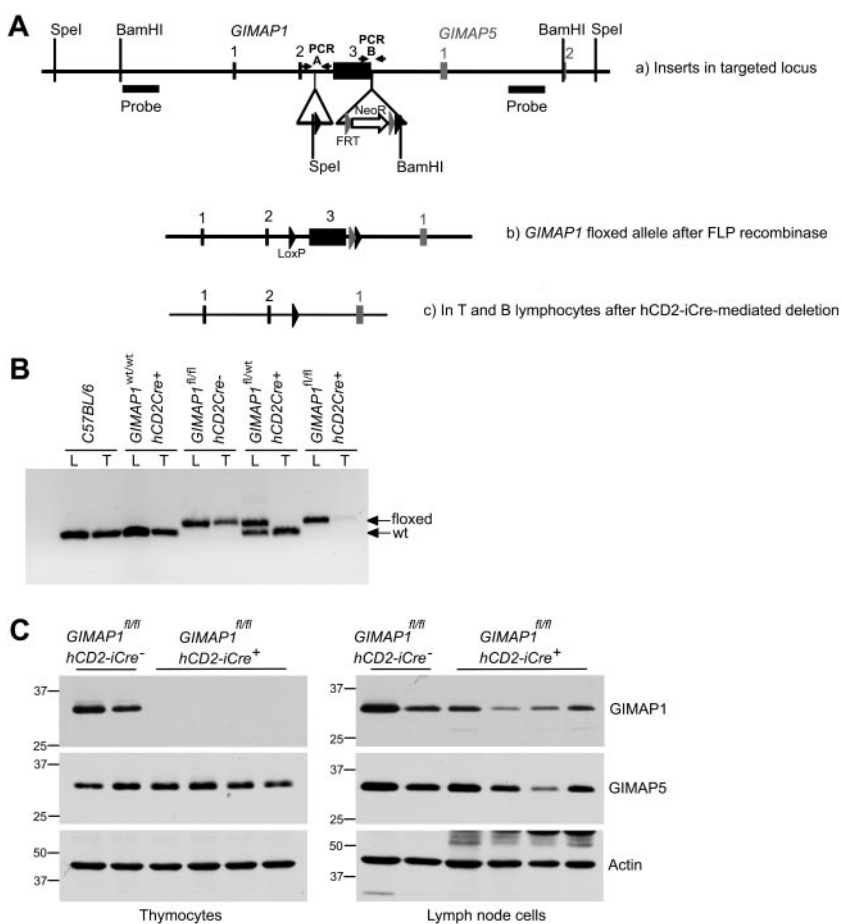
†See Galfre et al.²¹

‡See Carter et al.²²

§See Bazin.²³

||This denotes the mGIMAP polypeptide whose predicted N-terminal aa sequence is MAEPS, not the second of the 2 hGIMAP7-like polypeptides in mice which begins MAGQG.

Figure 1. Conditional deletion of the “floxed” *GIMAP1* allele with the use of the hCD2-iCre transgene. (A) Gene targeting strategy. The targeting vector introduced *LoxP* sites (black arrowheads) to flank *GIMAP1* exon 3. A neomycin resistance cassette flanked by FRT sites (gray arrowheads) was located downstream of the *LoxP* sites. Southern probes used to determine correct targeting of ES cells after *SpeI* and *Bam*HI DNA digests are indicated. (B) PCR analysis to detect the floxed and wild-type alleles of *GIMAP1* in genomic lung (L) and thymus (T) DNA. (C) Western blot analysis of GIMAP1 and GIMAP5 expression in thymocytes and lymph node cells.



Because of the compact nature of the *GIMAP* gene region, the modifications to the *GIMAP1* gene were in close proximity to the neighboring *GIMAP5* gene (Figure 1A). For this reason, and because some (rare) transcripts of *GIMAP5* including exon 1 of *GIMAP1* have been reported,²⁴ Western blots were also performed to assess the expression of the GIMAP5 protein. GIMAP5 expression was intact in the thymus of *GIMAP1/CD2-cKO* mice (Figure 1C), indicating that the gene targeting had not affected the expression of the *GIMAP5* gene.

Some GIMAP1 protein was detected in peripheral lymphoid tissues of *GIMAP1/CD2-cKO* mice (eg, lymph nodes; Figure 1C) but at lower levels than in controls. Some of this may have been expressed by myeloid cells (in which the Cre recombinase would not be expressed), but part was due to lymphocytes, and this is discussed later.

Profound lymphopenia in *GIMAP1/CD2-cKO* mice

When the lymphoid tissues of *GIMAP1/CD2-cKO* mice were analyzed, the outstanding feature was a massive deficiency of lymphoid cells in the peripheral compartments (Figure 2). Given the T cell-selective effects of *GIMAP5* mutations described previously,^{11,13} we were surprised that the lesion in *GIMAP1/CD2-cKO* mice affected not only T cells but also B cells (Figure 2C). In the case of the spleen, despite the lymphopenia, the overall cellularity was reduced by only approximately 40% in the conditional knockout (Figure 2B) because of increases in both neutrophils (Gr1⁺CD11b⁺) and monocyte/macrophages (Gr1⁻CD11b⁺; data not shown; evident in the forward scatter vs side scatter plots in Figure 2A). The cellularity of lymph nodes, in which a large-scale

expansion of myeloid cell numbers did not occur, was more severely reduced (Figure 2C).

The T-cell lineage in *GIMAP1/CD2-cKO* mice. Both spleen and lymph nodes exhibited reductions of 90% or greater in the numbers of CD4⁺ and CD8⁺ T cells enumerated with the use of flow cytometry (Figure 2B-C). Severe T lymphopenia was also evident in the peritoneum (Figure 5C) and in the blood (data not shown), the latter finding ruling out a defect affecting lymphocyte emigration from the blood to lymph nodes or spleen as the cause of the phenotype.

By contrast, thymi from *GIMAP1/CD2-cKO* mice were of broadly normal size and appearance. Detailed analysis, however, showed important effects of the conditional *GIMAP1* deletion (Figure 3). The total number of cells in *GIMAP1/CD2-cKO* thymi tended to be lower than controls, although this trend did not reach statistical significance. When thymocyte subsets were enumerated, however, a significant reduction was seen in the CD8 SP subset (Figure 3B). Investigation of maturation markers within the SP subsets also indicated a numerical reduction in the most mature (Qa2^{hi}CD24^{lo}) subsets of CD4 SP and CD8 SP cells (Figure 3C), although the proportions of these within the overall CD4 SP and CD8 SP compartments, respectively, were not significantly affected. Thus, the main effect of *GIMAP1* loss on the T lineage takes effect from the late thymic stage onward, as has been found for the *GIMAP5*-deficient models.^{11,13} Additional evidence for the functional maturity of *GIMAP1*-deficient SP thymocytes came from their high level of expression of CD5 (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), a property that is

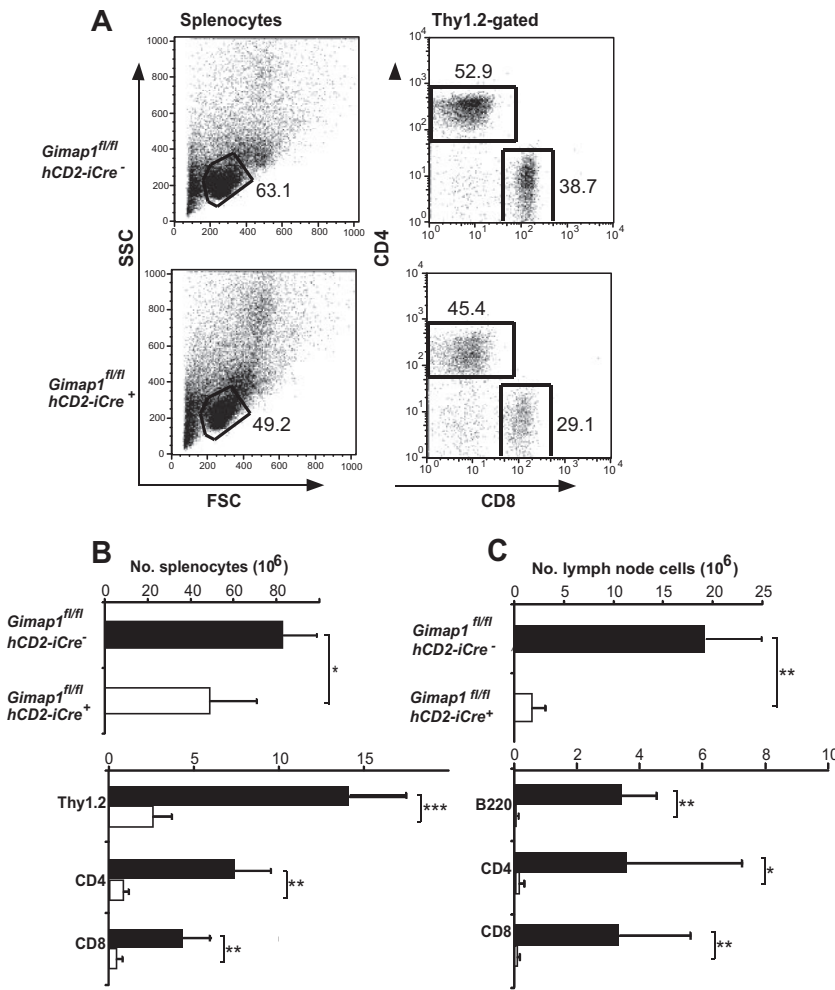


Figure 2. Defective peripheral lymphoid compartment in GIMAP1/CD2-cKO mice. Lymphoid subsets from spleen (A-B) and lymph nodes (C) of *GIMAP1^{fl/fl}hCD2-iCre⁻* and *GIMAP1^{fl/fl}hCD2-iCre⁺* mice were enumerated with the use of flow cytometry. (A) Representative FACS plots of splenocytes for each genotype. (B-C) Mean numbers (\pm SD) of cell subsets positive for the indicated surface markers (*GIMAP1^{fl/fl}hCD2-iCre⁻*, n = 9; *GIMAP1^{fl/fl}hCD2-iCre⁺*, n = 5). **P* < .05; ***P* < .01; ****P* < .001 (unpaired 2-tailed Student *t* test).

regulated developmentally by engagement of the $\alpha\beta$ TCR at positive selection,^{25,26} and from their up-regulation of CD40L in vitro in response to stimulation through the TCR with the anti-CD3 antibody²⁷ (with or without anti-CD28 costimulation; supplemental Figure 1B) both of which were equivalent to wild-type controls.

The B-cell lineage in GIMAP1/CD2-cKO mice. Severe reductions in B-cell numbers were seen in both lymph nodes (> 90%) and spleen (~ 85%; Figure 4A-B). Flow cytometric analysis for the markers CD93, IgM, CD21, and CD23 was used to enumerate B-cell subsets in GIMAP1/CD2-cKO and control spleens.

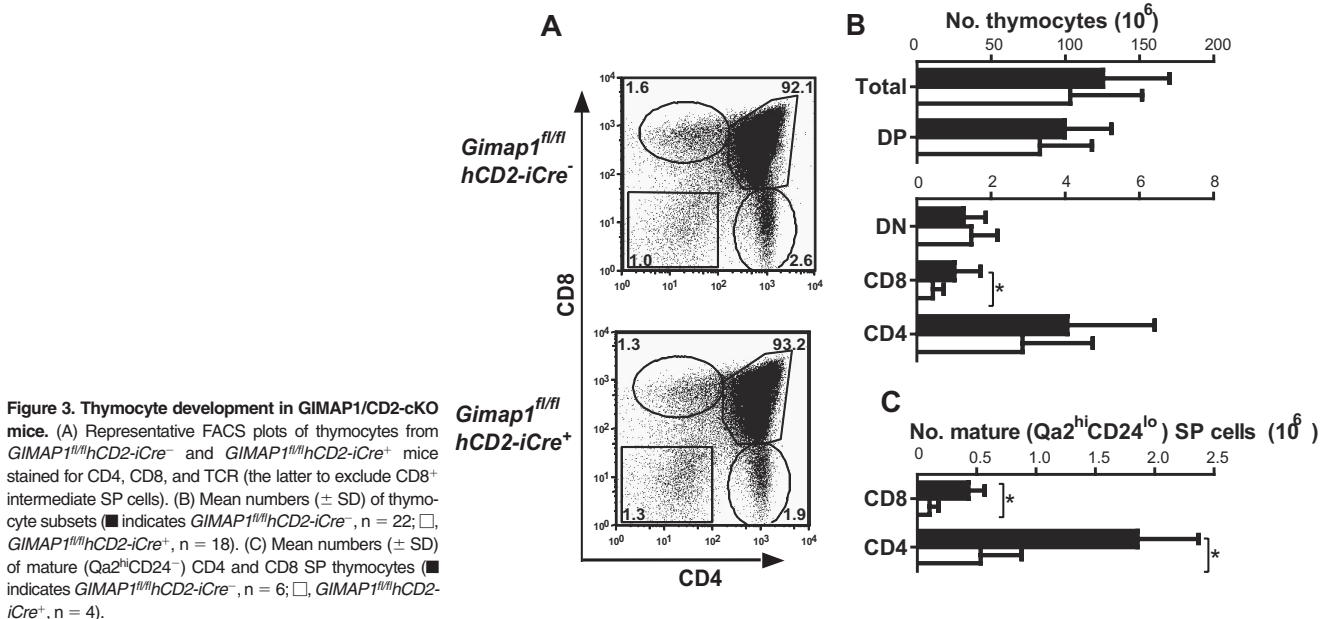
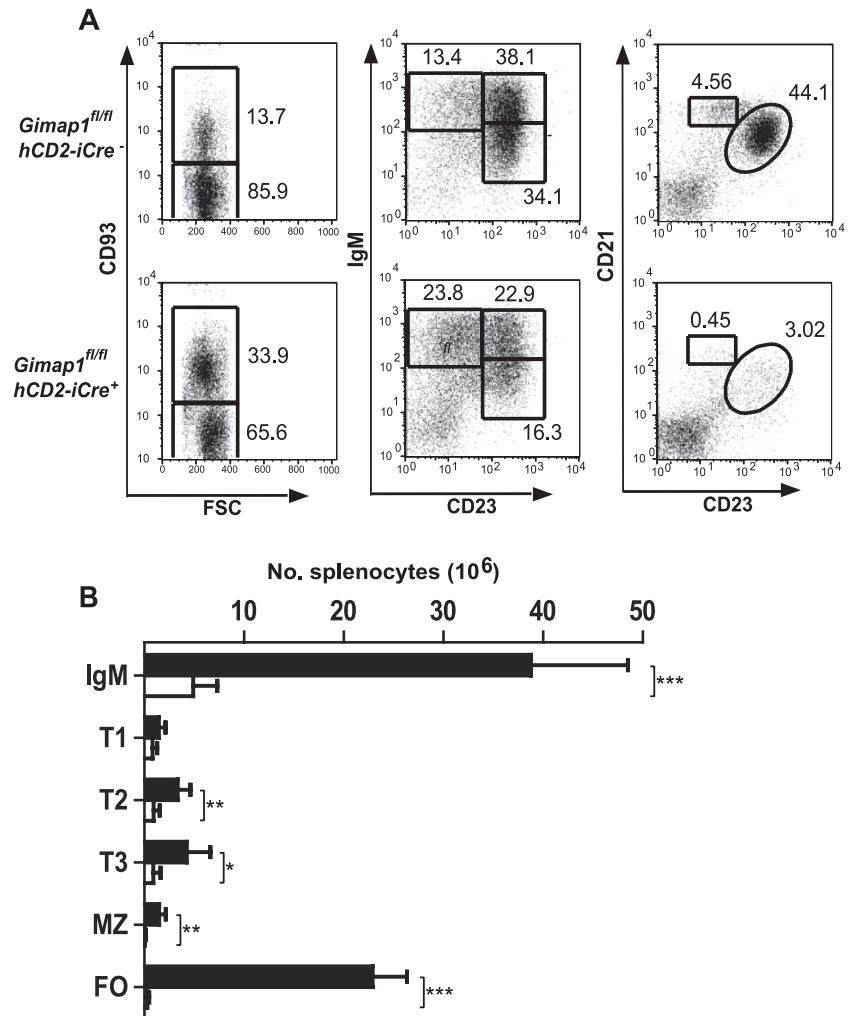


Figure 3. Thymocyte development in GIMAP1/CD2-cKO mice. (A) Representative FACS plots of thymocytes from *GIMAP1^{fl/fl}hCD2-iCre⁻* and *GIMAP1^{fl/fl}hCD2-iCre⁺* mice stained for CD4, CD8, and TCR (the latter to exclude CD8⁺ intermediate SP cells). (B) Mean numbers (\pm SD) of thymocyte subsets (■ indicates *GIMAP1^{fl/fl}hCD2-iCre⁻*, n = 22; □, *GIMAP1^{fl/fl}hCD2-iCre⁺*, n = 18). (C) Mean numbers (\pm SD) of mature (Qa2^{hi}CD24^{lo}) CD4 and CD8 SP thymocytes (■ indicates *GIMAP1^{fl/fl}hCD2-iCre⁻*, n = 6; □, *GIMAP1^{fl/fl}hCD2-iCre⁺*, n = 4).

Figure 4. Peripheral B-cell analysis. Splenocytes from *GIMAP1^{fl/fl}hCD2-iCre⁻* and *GIMAP1^{fl/fl}hCD2-iCre⁺* mice were stained for flow cytometric analysis to identify B-cell subsets. (A) Representative FACS plots for each genotype. The numbers given inside the component panels in A are the percentages of lymphocyte events contained within the indicated gates. (B) Mean numbers (\pm SD) of B-cell subsets (\blacksquare indicates *GIMAP1^{fl/fl}hCD2-iCre⁻*, n = 6; \square , *GIMAP1^{fl/fl}hCD2-iCre⁺*, n = 6). **P* < .05; ***P* < .01; ****P* < .001 (unpaired 2-tailed Student *t* test). FSC indicates forward scatter; and FO, follicular.



In the CD93⁻ fraction, the normally predominant follicular B-cell population (CD21^{int}CD23^{hi}) was massively reduced (by > 95%) in the conditional knockout as were the MZ B cells (CD21^{hi}CD23^{int}). Immature splenic B cells of the transitional T1, T2, and T3 stages were identified in the CD93⁺ population by the markers surface IgM and CD23. T2 and T3 populations were significantly reduced in *GIMAP1*/CD2-cKO mice relative to controls, whereas any reduction in the T1 population did not reach statistical significance.

B-lineage cells were also analyzed in the BM (Figure 5A-B). IgM versus B220 FACS plots showed a severe deficit of mature, recirculating B cells (IgM⁺B220^{hi}). No statistically significant differences were seen between *GIMAP1*/CD2-cKO and control mice in the number of IgM⁻B220^{int}CD25⁻ (pro-B/pre-BI) cells, IgM⁻B220^{int}CD25⁺ (pre-BII) cells, or immature (IgM⁺B220^{int}) B cells. The number of B cells in the peritoneum was also strongly reduced, and this was true of both conventional B2 B cells (CD19⁺B220^{hi}CD5⁻) and the B1a (CD19⁺B220^{int}CD5⁺) and B1b (CD19⁺B220^{int}CD5⁻) subsets (Figure 5C).

Thus, the main effect on B lymphocytes of the deletion of *GIMAP1* takes effect from the late BM stage onward, with all the peripheral B-cell types tested being affected, with the possible exception of the splenic T1 B cells. It should be noted that a substantial proportion of the few B (and T) cells found in the periphery of *GIMAP1*/CD2-cKO mice express *GIMAP1* protein and so are probably from rare clones in which the Cre-mediated ablation of *GIMAP1* has failed to occur²⁸ (Figure 1C; data not

shown). Hence, the severity of the lymphopenia recorded here probably underestimates the dependence of peripheral lymphocytes on *GIMAP1*.

GIMAP1 ablation reduces the in vitro survival capacity of CD4⁺ SP thymocytes

The most dramatic effect of *GIMAP1* loss in the T-cell lineage is seen from the late thymic stage onward. One of the characteristics acquired by developing thymocytes during the medullary stage, as CD4⁺CD8⁻ or CD4⁻CD8⁺ SP cells, is the acquisition of improved longevity/survival capacity,²⁹ a property also characteristic of peripheral, naive, T cells. This is reflected in a relatively long average survival of SP thymocytes in tissue culture. When *GIMAP1*/CD2-cKO CD4⁺CD8⁻ SP thymocytes were assayed for this property, they performed significantly worse than their *GIMAP1*-sufficient counterparts; this difference applied to both “immature” (Qa2^{lo}CD24^{hi}) and “mature” (Qa2^{hi}CD24^{lo}) cells within the CD4⁺CD8⁻ SP compartment (Figure 6A). Although cell survival could be improved by supplementing the tissue culture medium with IL-7, the difference in CD4⁺CD8⁻ SP thymocyte survival because of the *GIMAP1* deletion remained. When similar in vitro experiments were conducted to examine the survival of CD4⁻CD8⁺ SP thymocytes, the cells from *GIMAP1*/CD2-cKO mice again appeared to perform worse than wild type, but in this case the effect did not reach statistical significance (supplemental Figure 2A).

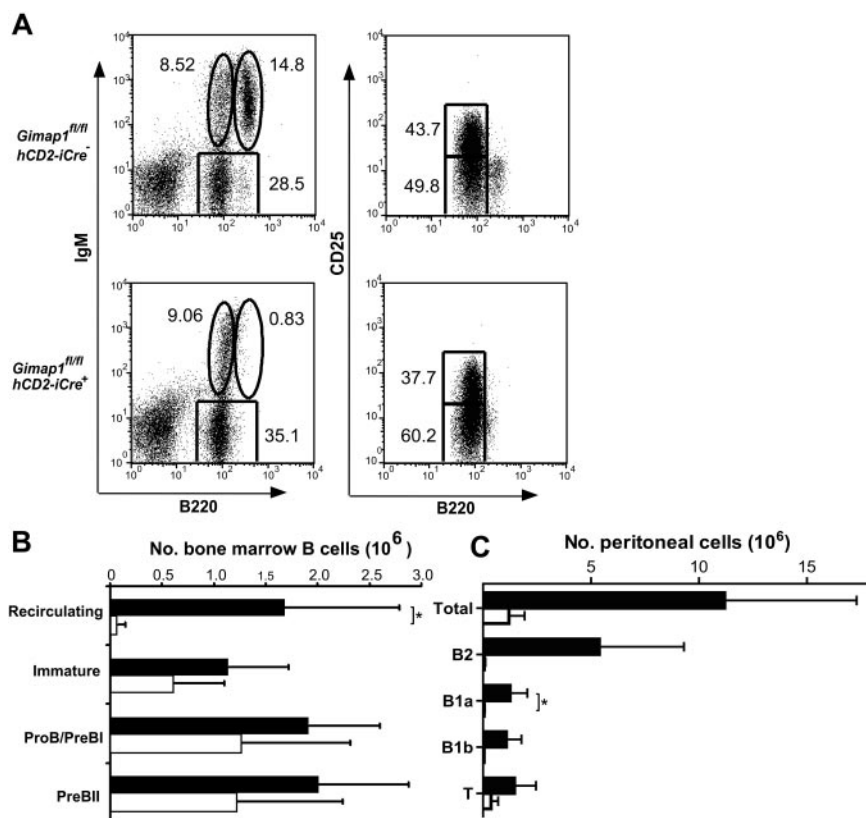


Figure 5. BM and peritoneal cell analysis. BM (A-B) and peritoneal (C) cells from *GIMAP1^{fl/fl}hCD2-iCre^{-/-}* and *GIMAP1^{fl/fl}hCD2-iCre^{+/+}* mice were stained for flow cytometric analysis to identify B-lineage subsets. (A) Representative FACS plots for each genotype. The numbers given inside the component panels in A are the percentages of lymphocyte events contained within the indicated gates. (B) Mean numbers (\pm SD) of B-lineage subsets in BM (■ indicates *GIMAP1^{fl/fl}hCD2-iCre^{-/-}*, n = 6; □, *GIMAP1^{fl/fl}hCD2-iCre^{+/+}*, n = 6). (C) Mean numbers (\pm SD) of lymphocyte populations in peritoneum (■ indicates *GIMAP1^{fl/fl}hCD2-iCre^{-/-}*, n = 6; □, *GIMAP1^{fl/fl}hCD2-iCre^{+/+}*, n = 4). **P* < .05 (unpaired 2-tailed Student *t* test).

Ex vivo GIMAP1/CD2-cKO splenic T cells showed increased Annexin V binding, indicative of impaired survival properties of the few peripheral T cells that were present (data not shown).

The improved *in vitro* survival of GIMAP1/CD2-cKO CD4⁺8-SP thymocytes brought about by IL-7 supplementation indicated that signaling through the IL-7 receptor was intact in the mutant thymocytes, and this was confirmed by flow cytometric analysis showing normal expression of IL-7R α (Figure 6B). In addition, GIMAP1/CD2-cKO and wild-type thymocytes exhibited similar downstream phosphorylation of STAT5 after *in vitro* exposure to IL-7 (Figure 6B). A further indication that IL-7 signaling was intact in GIMAP1/CD2-cKO SP thymocytes was their undiminished level of Bcl-2 expression (data not shown), a property that is under the control of IL-7 signaling.³⁰

GIMAP1/CD2-cKO B cells express BAFF-R

Given the potent effect of *GIMAP1* loss on mature B-cell survival *in vivo*, we investigated the expression of the key cytokine receptor involved in B-cell maintenance, namely the B cell-activating factor receptor (BAFF-R).^{31,32} This receptor was found expressed at approximately normal levels in GIMAP1/CD2-cKO immature B and splenic B cells (supplemental Figure 2B). This result requires cautious interpretation (particularly in the case of the splenic B cells) because, as described under "The B-cell lineage in GIMAP1/CD2-cKO mice," some of the B lymphocytes in GIMAP1/CD2-cKO spleen are rare survivors in which GIMAP1 expression has not been extinguished. Nevertheless, the flow cytometric profile did suggest that BAFF-R was being expressed by all of the splenic B cells, ie, potentially both GIMAP1-expressing and nonexpressing cells, and therefore that its expression was not disrupted by *GIMAP1* gene loss. This conclusion was supported by normal BAFF-R expression in the immature BM B cells.

Expression of GIMAP proteins in the T and B lineages

The results described indicate parallel behavior in the T- and B-cell lineages in response to *GIMAP1* loss. In both cases there are, at most, only minor effects apparent on the earlier developmental stages examined but major effects on the mature cell populations in the periphery. This points to a critical, nonredundant role for GIMAP1 in the transition between primary and secondary lymphoid stages. To what extent does this dependence of cells on *GIMAP1* match the expression of this gene and its product? Previously, we reported real-time PCR studies of *GIMAP* gene expression in the B-cell lineage.⁴ For 7 of the 8 mouse *GIMAP* genes low expression in the early BM stages was followed by a sharp rise in expression at either the immature CD25⁻B220⁺IgD⁻IgM⁺ B-cell stage (for GIMAP1, GIMAP5, GIMAP9) or the mature CD25⁻B220⁺IgD⁺IgM⁺ stage (for GIMAP3, GIMAP4, GIMAP6, GIMAP8). This indicates a generally good correlation with the stage(s) at which B-lineage cells display sensitivity to *GIMAP1* loss (see Figures 4,5).

We were interested to confirm this relationship at the protein level. Using monoclonal antibodies against 5 of the mouse GIMAPs, we found that in cells from normal C57BL/6 mice the levels of all these proteins were low in pre-BII and immature B cells and then rose sharply (eg, at the T1 or T2 stages) to high levels in the more mature B-cell stages (Figure 7A-B). A similar analysis was performed on subsets of thymocytes, including division of DP cells into 3 populations on the basis of TCR expression (Figure 7C). In the case of GIMAP4, it was already known that, after a sharp rise at the DN3 to DN4 stage, expression is extinguished in DP thymocytes and reappears at the mature SP stage.^{14,33} A similar, but less absolute, pattern was seen for most of the GIMAP family, with DP thymocytes showing low expression before a substantial rise in the mature SP subsets. GIMAP1,

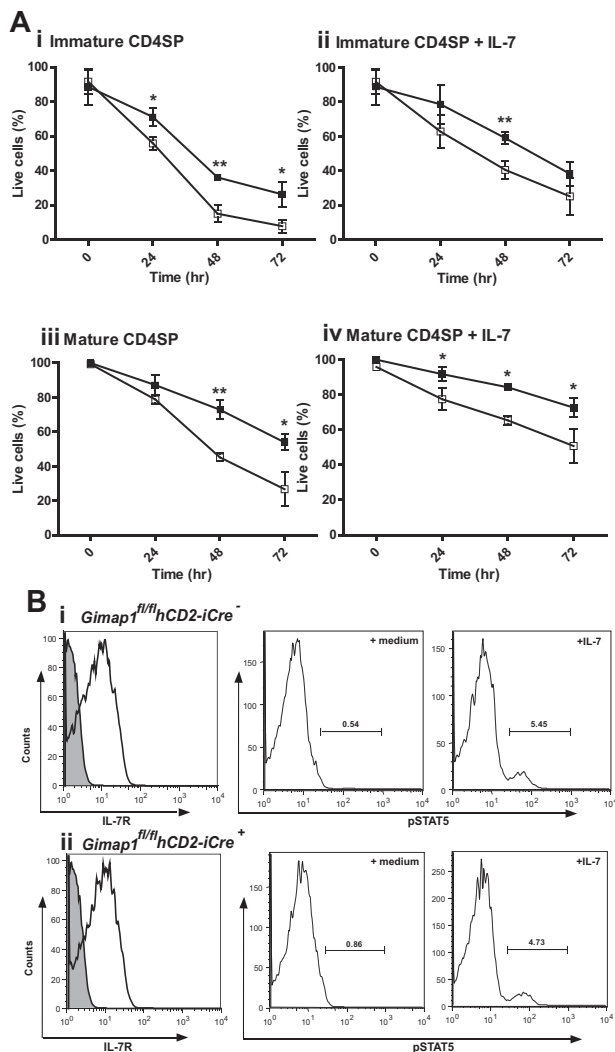


Figure 6. In vitro analysis of CD4 SP thymocyte survival. (A) CD4 SP thymocytes from *GIMAP1^{fl/fl}hCD2-iCre⁻* (■) and *GIMAP1^{fl/fl}hCD2-iCre⁺* (□) mice were FACS-purified and cultured with (ii,iv) or without (i,iii) IL-7. Immature (i,ii) and mature (iii,iv) CD4 SP cells were defined on the basis of Qa2 and CD24 expression. Live cells were determined by annexin V staining. Plots show mean percentage of live cells (± SD) for 3 mice of each genotype. **P* < .05; ***P* < .01; ****P* < .001 (unpaired 2-tailed Student *t* test). (B) IL-7 receptor expression and function in thymocytes. (Left) Expression of IL-7R (CD127; white histogram) compared with control stain (gray histogram) on CD4 SP thymocytes (as determined by flow cytometry) from (i) *GIMAP1^{fl/fl}hCD2-iCre⁻* and (ii) *GIMAP1^{fl/fl}hCD2-iCre⁺* mice. (Right) Responses of total thymocytes to IL-7 as determined by pSTAT5 induction. Total thymocytes were incubated at 37°C in medium with or without IL-7 for 30 minutes before fixing, permeabilizing, staining with anti-phospho-STAT5 antibody and analyzing by flow cytometry.

however, was an exception, displaying a roughly constant level of expression throughout, and this confirmed previous findings.⁴

These data suggest 2 hypotheses: (1) that the GIMAP proteins are under a degree of coordinate regulation bringing about high expression at the critical stages of the late thymocyte and the transitional B cell, and their subsequent entry into the mature peripheral T- or B-lymphocyte pools; and (2) that, in the thymus (but not the BM), GIMAP1 may have an ongoing function relevant at all developmental stages.

Discussion

Investigations of the GIMAP GTPases have led to the proposal that they are important regulators of T-lymphocyte development and

survival.¹ The data presented here, however, crucially extend the scope of interest in the GIMAPs to include corresponding processes in the B-cell lineage, in which the phenotype of *GIMAP1/CD2-cKO* mice diverges dramatically from its *GIMAP5*-deficient forerunners (Figures 2-3,5). The T-lineage phenotype displayed by the *GIMAP1/CD2-cKO* mice described here is similar to that seen previously in *GIMAP5*-deficient rats and mice. Features in common include (1) severe peripheral T lymphopenia in combination with a thymus that is normal to initial inspection, and (2) effects on cells at the later stages of intrathymic development, including reductions in the number of SP cells and failure of these cells to acquire a long-lived phenotype measurable in in vitro survival assays (Figures 2,4).¹² *GIMAP1/CD2-cKO* mice, however, exhibit, in addition, severe reductions in all types of mature B cells studied: follicular, mature recirculating, MZ, peritoneal B1a and B1b. No deficit of B cells has ever been reported in the *GIMAP5*-mutant rat model, and only MZ B cells showed a deficit in the *GIMAP5* knockout mouse.¹³ Paralleling the effects in the T-cell lineage, the earlier stages in B-cell development in the BM of *GIMAP1/CD2-cKO* mice were apparently unaffected (Figure 3).

Our analysis of the expression patterns of various GIMAP proteins during lymphocyte development raised the interesting possibility that the predominant role of GIMAP family proteins may be to act in a coordinated fashion to regulate lymphocyte survival from a late intrathymic (or intra-BM) stage onward. The analysis of intrathymic GIMAP expression also highlighted the expression of family members in DN thymocytes, a phenomenon first noticed for *GIMAP4*.³³ An effect on the cellular events after β -selection would explain the reported effect of *GIMAP5* loss on DP thymocyte numbers/production.¹

It is unclear why inactivation of the *GIMAP1* gene should have more extensive effects (ie, including all mature B cells) than observed for inactivation of its close relative *GIMAP5*. It seems possible from our data that the T-cell phenotype in *GIMAP1/CD2-cKO* mice is more severe than that of *GIMAP5*-mutant mice or rats (bearing in mind that a proportion of the remaining peripheral lymphocytes are cells in which Cre-mediated deletion of *GIMAP1* may have failed to occur). This might indicate that GIMAP1 is more “central” to the survival processes regulated by this GTPase family (and that its loss may therefore be capable of tilting the balance of survival signals in B cells as well as T cells). Note here that germline *GIMAP1* loss appears to be lethal soon after birth (data not shown), which is, again, a more severe phenotype than that described previously for germline loss of *GIMAP5* in mice.¹³ Peripheral T lymphopenia in *GIMAP5*-mutant rats is due to the deleterious effect of *GIMAP5* loss on the thymic SP compartment, resulting in reduced thymic T-cell output and reduced survival of thymic emigrants. Thus, the peripheral T-cell pool consists largely of recent thymic emigrants and additional T cells of unusual “activated” phenotype(s) with a paucity of recognizable resting, naive T cells and a defect in survival in the overall population. It is probable that a similar general description will apply to the *GIMAP1/CD2-cKO* phenotype.

The mechanism by which *GIMAP5* promotes T-lymphocyte survival has been the subject of diverse proposals,^{1,26,34-37} including hypotheses relating to mitochondrial permeability, mitogen-activated protein kinase and nuclear factor κ B (NF- κ B) signaling, interaction with Bcl-2 family members, regulation of calcium signaling and the endoplasmic reticulum stress pathway. A weakness intrinsic to a number of these conclusions is their basis in data from the *GIMAP5*-mutant rat, in which the few residual peripheral T cells are intrinsically short-lived and of unusual phenotype. Conditional models for *GIMAP* gene deletion should allow comparisons of normal lymphocytes before and after elective *GIMAP* gene

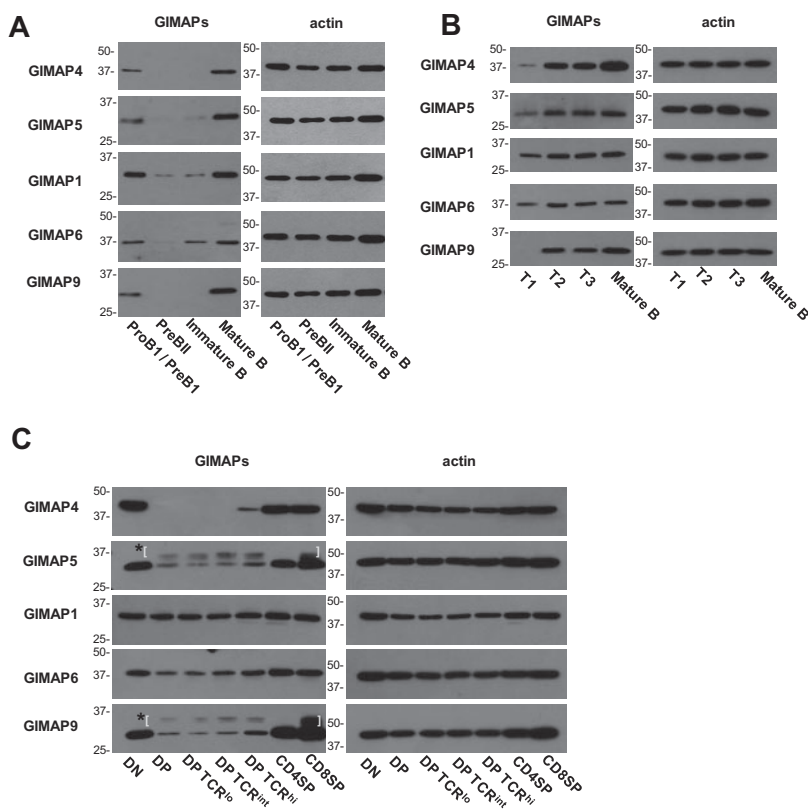


Figure 7. Analysis of GIMAP protein expression in B- and T-cell development. BM cells (A), splenic B cells (B), and thymocytes (C) from C57BL/6 mice were FACS-purified into subsets as described in "Fluorescence-activated cell sorting." Expression of GIMAP4, GIMAP5, GIMAP1, GIMAP6, and GIMAP9 was examined by Western blot, with β -actin as control. *Parentheses surround nonspecific bands caused by the secondary anti-rat IgG reagent.

deletion and hence better controlled investigations of the mechanism(s) by which GIMAP GTPases regulate lymphocyte survival.

In principle, there are 2 classes of survival process in which GIMAP proteins may participate. The first is protection from an endogenous mediator or process that is potentially cytotoxic/proapoptotic but normally present at subthreshold levels. Endogenous molecules known to have cytotoxic potential include inflammatory mediators such as tumor necrosis factor³⁸ and nicotinamide adenine dinucleotide.³⁹ Cell-autonomous processes such as the endoplasmic reticulum stress response and atrophy-apoptosis modulation would also fit into this category.

The second class of survival process is constitutive survival signaling. Continuing survival of recirculating lymphocytes requires stimulation via their antigen receptors^{40,41} as well as through specific cytokine receptors (BAFF-R for B cells; IL-7R for T cells).^{32,42} Appropriate stimulation through these receptors activates a nexus of downstream pathways which maintains cell growth and restrains apoptosis. A central role is played by the canonical and noncanonical NF- κ B pathways, alongside the PI3-kinase/Akt/mTOR pathway, the Pim2 pathway, and the Erk pathway, which may themselves contain targets of NF- κ B transcriptional control.^{43,44} The phenotypes of several spontaneous or engineered mouse mutants in components of these pathways (eg, NEMO, IKK, TAK1, NIK, Pim2) attest to their importance for establishing and maintaining the mature lymphocyte repertoire.⁴⁵ That the phenotypes of GIMAP1- and GIMAP5-deficient rodents are focused around the same critical processes is highly suggestive. The fact that *GIMAP1* loss effects both B and T lineages tends to favor common pathways, such as NF- κ B signaling, against lineage-specific pathways (BAFF-R, IL-7R), and this is supported by our demonstration of intact IL-7 signaling and responsiveness (Figure 6).

Nitta et al¹ have reported that GIMAPs 3, 4, and 5 are capable of interacting with members of the bcl-2 protein family; a role for the GIMAPs in the modulation of survival signaling might provide a layer

of fine control to the regulation of apoptosis in lymphoid lineages. In work to be submitted elsewhere, we have established that GIMAP1 and GIMAP5 occupy separate intracellular locations, indicating that the processes in which these GTPases participate span at least 2 cellular compartments. This result may also serve to explain the absence of redundancy between these 2 closely related proteins.

In conclusion, our findings have shown a major, nonredundant, role for GIMAP1 in the development of mature lymphocytes. This has uncovered an unexpected involvement of this GTPase family in B-cell development.

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Authorship

Contribution: A.S. and L.M.C.W. designed and conducted research, analyzed data, and wrote the paper; M.L.J. and C.C. designed and conducted research; A.H., J.P., N.P., and G.M. conducted research; M.T. designed research; and G.W.B. designed research and wrote the paper.

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