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Activated Mouse B Cells Lack Expression of Granzyme B

Magdalena Hagn,* Gabriele T. Belz,‡ Axel Kallies,‡ Vivien R. Sutton,*† Kevin Y. Thia,*† David M. Tarlinton,‡ Edwin D. Hawkins,*†,1 and Joseph A. Trapani*†,5,1

Recently, it has been reported that human B cells express and secrete the cytotoxic protease granzyme B (GrB) after stimulation with IL-21 and BCR cross-linking. To date, there are few clues on the function of GrB in B cell biology. As experimental transgenic murine systems should provide insights into these issues, we assayed for GrB in C57BL/6 B cells using an extensive array of physiologically relevant stimuli but were unable to detect either GrB expression or its proteolytic activity, even when Ag-specific transgenic BCRs were engaged. Similar results were also obtained with B cells from DBA/2, CBA, or BALB/c mice. In vivo, infection with either influenza virus or murine γ-herpesvirus induced the expected expression of GrB in CTLs, but not in B cell populations. We also investigated a possible role of GrB on the humoral immune response to the model Ag 4-hydroxy-3-nitrophenylacetyl–keyhole limpet hemocyanin, but GrB-deficient mice produced normal amounts of Ab with typical affinity maturation and a heightened secondary response, demonstrating conclusively the redundancy of GrB for Ab responses. Our results highlight the complex evolutionary differences that have shaped the immune systems of mice and humans. The physiological consequences of GrB expression in human B cells remain unclear, and the current study suggests that experimental mouse models will not be helpful in addressing this issue. The Journal of Immunology, 2012, 188: 000–000.

Granzyme B (GrB) is a 32-kDa serine protease that is a key constituent in secretory granules of NK cells and CTLs (1, 2). Five different human granzymes, seven in rats and 10 in mice, have been described to date (3, 4). Classically, GrB is released via granule exocytosis toward target cells such as tumor or virus-infected cells where it induces apoptosis by cleaving the BH3-only proapoptotic protein BID and/or processing of the effector caspase-3 (5, 6). The pore-forming molecule perforin plays a major role in this process as it facilitates delivery of granzymes into the target cell (7–9). In mice, GrB expression has to date been described in T cells, NK cells, and their thymic precursors (10). In contrast, recent evidence has linked human GrB to a broader spectrum of cells including CD34+ hematopoietic progenitor cells (11), keratinocytes (12), basophils (13), mast cells (14), plasmacytoid dendritic cells (15), and B cells (16).

In human B cells, GrB is induced by IL-21 and is strongly enhanced by simultaneous BCR cross-linking. Notably, exposure to viral Ags can substitute for artificial BCR cross-linking and enhance the IL-21–induced GrB secretion from B cells of vaccinated human donors (16). In a subsequent study, CD40L was found to play a determining role in this process: B cells secreted GrB in its absence but differentiated into plasma cells when it was present (17). Moreover, malignant human B cells (chronic lymphocytic leukemia, B-CLL) secrete low levels of GrB upon triggering by either BCR cross-linking or CpG oligodeoxynucleotide (CpG ODN) combined with IL-21. These B cells were found to induce apoptosis in bystander B-CLL cells in a GrB-dependent manner (18). Furthermore, a significant correlation between GrB and IL-21 serum levels has been found in patients with the autoimmune disease systemic lupus erythematosus. In these patients, GrB was constitutively expressed in CD5+ but not CD5− B cells (19).

These various studies have revealed a novel function for IL-21 and strongly suggest a role for GrB expression in B cells, which may contribute to immunosurveillance, early antiviral immune response, and autoimmune pathogenesis. Furthermore, we hypothesized that the induced expression of GrB in B cells upon BCR cross-linking and exposure to IL-21 might play a role in the regulation of contraction of germinal center responses by eliminating helper populations such as follicular T helper cells. At this stage, however, there is little experimental evidence for the physiological importance of GrB expression in B cells or B cell-mediated cytotoxicity. Hence, the aim of the current study was to establish a reliable mouse model further to study this interesting set of observations.

Materials and Methods

Mice, in vivo immunization, and viral infections

Mice 6 to 10 wk of age were used in all experiments and studies performed according to the institutional guidelines for animal experimentation of the Peter MacCallum Cancer Centre and The Walter and Eliza Hall Institute of Medical Research, C57BL/6, BALB/c, DBA/2, and CBA mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia). Granzyme B cluster-deficient mice (B6. GrzmB−/−) and HEL Rag SHH+/ SHL−/− mice (SW Hel) were bred...
and maintained at the Peter MacCallum Cancer Centre. In vivo immunization was administered by i.p. injection, with 50 μg of 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin (NP–KLH; The Walter and Eliza Hall Institute of Medical Research) in the molar ratio of 13:1 as described previously (20), with alum as an adjuvant. Secondary responses were generated by i.p. injection 45 d later of 10 μg NP–KLH suspended in PBS. For viral infections, mice were infected intranasally with 10^3 PFU HKx31 (H3N2) influenza virus (21) or with 3 × 10^4 PFU murine γ-herpesvirus-68–OVA (MHV–OVA) (22).

Cell culture of mouse B cells

On day 10 (NP–KLH) or day 5 and day 10 (viral infections) after immunization, spleens and mediastinal lymph nodes were harvested from euthanized mice. Mononuclear cells were prepared from lymph nodes after red cell lysis and B cells purified from the spleen. B cell enrichment (96–99%) was achieved using a negative selection with Ab-coated beads (no. 130-090-862; Miltenyi Biotec, Bergisch Gladbach, Germany). NK cells were isolated from spleens of either B6.Grzmb^−/− or C57BL/6 mice using MACS-based negative selection (Miltenyi Biotec) with subsequent separation on an autoMACS sorter. Media for primary cell culture consisted of RPMI 1640 medium supplemented with 2 mM glutamine, 50 mg/ml streptomycin, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, and 10% FBS. Cells were plated on flat-bottom 96-well plates at 100,000 cells/well/200 μl (spleen) or 25,000 cells/well/200 μl (lymph node) in the presence of reagents as follows: recombinant mouse IL–21 (100 ng/ml) and IL–4 (500 U/ml) (R&D Systems, Minneapolis, MN); CpG ODN 1668 (5′-TCCATGACGTTCCTGATGCT-3′; GeneWorks, Thebarton, SA, Australia) was used at a final concentration of 1 μM; LPS derived from Salmonella typhosa (Sigma-Aldrich, St. Louis, MO) and anti-CD40 (FGK4.5; The Walter and Eliza Hall Institute of Medical Research) were at 20 μg/ml. BCR engagement in naïve B cells was achieved by using Abs against IgM (clone b.7.6) and IgD (clone 1.19) at 10 μg/ml (kindly provided by Prof. Phil Hodgkin, The Walter and Eliza Hall Institute of Medical Research). For in vivo immunization experiments with NP–KLH, some cells were restimulated in vitro with plate-bound NP13–BSA at 5 μg/ml. In SIH2-transgenic B cell cultures, hen egg lysozyme (HEL) was used for direct ligation of the BCR (100 ng/ml; Sigma-Aldrich). NP ELISA

Serum of immunized B6.Grzmb^−/−, C57BL/6, or naive mice was collected from the retro-orbital sinus on day 10, 14, and 21 after injection or on day 8 after boost (day 53). Concentrations on NP-specific total or high-affinity Abs were determined by ELISA. Briefly, 96-well flat-bottom plates were coated with 10 μg/ml NP13–BSA and NP14–BSA (for total Ab) and NP13–BSA and NP2–BSA (for high-affinity Ab) and incubated overnight at 4°C.

FIGURE 1. Human but not mouse B cells express and secrete active GrB after stimulation of the B cell and the IL–21 receptors. (A) freshly isolated B cells from peripheral blood of healthy donors (human) or splenic B cells from C57BL/6 mice were incubated for 16 h with anti-BCR, IL–21, or the combination. After culture, cell lysates were prepared, protein amounts adjusted, and ASPase activity determined. Shown is one representative plot of two independent experiments. The error bars represent SEM of intraexperimental duplicates. (B) Purified human and mouse B cells (2 × 10^5) were cultured overnight as indicated. Total RNA was isolated and real-time RT-PCR for GrB mRNA performed. Relative quantity compared with non-B cells (upper panel) or wild-type (WT) NK cells (lower panel) was determined by the comparative CT method. Bar graphs represent means of two independent experiments for each mouse (n = 2). Error bars indicate SEM.
strategy for CD3 + T cells and B220 + B cells. The indicate baseline GrB expression of one representative naive mouse or mice after 5 d and 10 d postinfection, respectively. The upper panels (A immediately after isolation [ex vivo,) influenza virus. Mediastinal lymph nodes and spleens were harvested on day 5 (day 5 = 2) and day 10 (day 10 = 4) postinfection. Organs of two naive mice served as baseline control. Mononuclear cells (lymph nodes) and B cells (spleen, purity >98%) were isolated and cultured. Brefeldin A was added for 4 h immediately after isolation [ex vivo, (A, B)] or after stimulation for 16 h with LPS, anti-CD40 plus IL-4, and CpG ODN in the presence or absence of IL-21. (C) After culture, cells were harvested and stained for CD3, CD19, and B220 (lymph nodes) and intracellular GrB (lymph nodes and spleen). (A) Dot plots indicate baseline GrB expression of one representative naive mouse or mice after 5 and 10 d postinfection, respectively. The upper panels show gating strategy for CD3+ T cells and B220+ B cells. The middle and lower panels illustrate percentages of GrB+ cells as analyzed by FACS. (B) Line graph depicts average ex vivo GrB expression of B and T cells from lymph node and splenic B cells of two mice ± SEM. (C) Bar graphs show percentage cells with GrB expression after 16 h of culture with reagents as indicated. Error bars indicate SEM of data from two mice.

### Table I. Primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human GrB</td>
<td>F: CCATCCAGCTTAAATCTCTTA</td>
</tr>
<tr>
<td></td>
<td>R: CCGTCATGTCATCTCCACCCT</td>
</tr>
<tr>
<td>Human RPL-32</td>
<td>F: GCTTAACTGCGGGAAACCCA</td>
</tr>
<tr>
<td></td>
<td>R: TTGGAGAGCATTCGGCACCA</td>
</tr>
<tr>
<td>Mouse GrB</td>
<td>F: GTGTTTCCTCCGCACTGGCTTCA</td>
</tr>
<tr>
<td></td>
<td>R: CTGCCTGAAAACTGTCACCTGTC</td>
</tr>
<tr>
<td>Mouse RPL-32</td>
<td>F: TTCTGTTCACAAAGTCtCAAG</td>
</tr>
<tr>
<td></td>
<td>R: TGAGCGATTCACCAGCAC</td>
</tr>
</tbody>
</table>

F. forward primer; R. reverse primer.

Stanards and serum samples were added serially diluted in blocking buffer (PBS, 0.05% Tween-20, 0.6% milk, and 1% FBS) and incubated at room temperature overnight. Plates were then washed and incubated for 4 h at room temperature with a goat anti-mouse IgG, HRP secondary Ab (Southern Biotech, Birmingham, AL). After washing, tetramethylbenzidine substrate (Sigma-Aldrich) containing 0.01% H2O2 (Merck, Darmstadt, Germany) was added, the reaction stopped with 1 M H2SO4, and plates were analyzed in an ELISA plate reader (VersaMax PLUS ROM v.1.21; Molecular Devices, Sunnyvale, CA) at 450 nm.

**Human B cell culture**

Human B cells were isolated using a MACS-based kit with negative selection (no. 130-091-151; Miltenyi Biotec). B cells (purity >99%) were resuspended in AIM-V medium (Invitrogen, Carlsbad, CA) and cultured on 96-well round-bottom plates at 200,000 cells/well/200 μl if not stated otherwise. The following reagents were used as in vitro B cell stimuli: human recombinant IL-21 (50 ng/ml) was purchased from Invitrogen and affinity purified rabbit F(ab’)2 against human IgA plus IgG plus IgM (H+L) was used for polyclonal BCR stimulation at 6.5 μg/ml (anti-BCR; Jackson ImmunoResearch Laboratories). Non-B cells (= B cell-depleted fraction) were activated with a combination of 100 U/ml human recombinant IL-2 (Proleukin; National Cancer Institute, Bethesda, MD) and PHA (1 μg/ml; 23, 24).

#### Intracellular GrB staining

After stimulation, brefledin A (1 μg/ml; A.G. Scientific, San Diego, CA) was added for 4 h, and cells were harvested and stained as described previously (16). For human studies, a PE-labeled Ab to GrB (clone GB11; Sanquin, Amsterdam, The Netherlands) was used, and for mouse B cells, an Alexa Fluor 647 mouse anti-human Ab (clone GB11; BD Biosciences, San Jose, CA). To exclude a lack of sensitivity for the Ab to detect GrB in mice, in all experiments NK cells from either C57BL/6 or B6.GrzmAB–/- were activated over 4 d with IL-2 in 24-well plates at 700,000 cells/ml/well (1000 U/ml) and used as staining controls (Fig. 1B). Cell surface markers were analyzed for some mouse experiments using B220-FITC, CD3-Pacific blue, or CD138-PE (BD Biosciences). Flow cytometry was performed on a FACSCanto or LSR device (BD Biosciences) and data analyzed using FlowJo software (version 8.8.7; Tree Star, Stanford, CA).

#### GrB secretion and activity assay

C57BL/6 B cells were isolated and stimulated overnight with anti-IgM plus anti-IgD (= anti-BCR), anti-CD40 plus IL-4 plus IL-4 with or without IL-21. The next day, cells were cultured for 4 h in the presence of PMA (1 μg/ml) and ionomycin (10 μg/ml), and culture supernatants were collected. Additionally, whole-cell lysates were prepared and normalized for protein content. GrB activity was determined by the hydrolysis of synthetic peptide thiochromenyl ester substrate (Asp-ase activity, Boc-Ala-Ala-Asp-S-Bzl; SM Biochemicals), as described previously (25, 26). Supernatants of IL-2–stimulated NK cells or lysates of activated T cells from either C57BL/6 or B6.GrzmAB–/- were used as controls. T cells (2.5 × 10⁶ cells/ml in 6-well plates) were stimulated with IL-2 (100 U/ml), IL-7 (2 ng/ml), and purified anti-CD3 and anti-CD28 (100 ng/ml; BD Biosciences) for 3 d. Cells were split on day 2 when fresh IL-2 was added.

#### Real-time RT-PCR

Murine or human B cells (2 × 10⁶) were isolated and activated as described earlier. After culture, total RNA was obtained using the RNeasy mini kit (Qiagen, Doncaster, Australia) according to the manufacturer’s methods. RNA was reverse-transcribed using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant) and oligonucleotide primer (both from Promega, Madison, WI). Real-time quantitative PCR was performed using an

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**FIGURE 2.** Mouse B cells do not express GrB after in vivo infection with influenza virus. Mice (n = 4) were infected intranasally with 10⁴.5 PFU HKx31 influenza virus. Mediastinal lymph node and spleens were harvested on day 5 (day 5 = 2) and day 10 (day 10 = 2) postinfection. Organs of two naive mice served as a baseline control. Mononuclear cells (lymph nodes) and B cells (spleen, purity >98%) were isolated and cultured. Brefeldin A was added for 4 h immediately after isolation [ex vivo, (A, B)] or after stimulation for 16 h with LPS, anti-CD40 plus IL-4, and CpG ODN in the presence or absence of IL-21. (C) After culture, cells were harvested and stained for CD3, CD19, and B220 (lymph nodes) and intracellular GrB (lymph nodes and spleen). (A) Dot plots indicate baseline GrB expression of one representative naive mouse or mice after 5 d and 10 d postinfection, respectively. The upper panels show gating strategy for CD3+ T cells and B220+ B cells. The middle and lower panels illustrate percentages of GrB+ cells as analyzed by FACS. (B) Line graph depicts average ex vivo GrB expression of B and T cells from lymph node and splenic B cells of two mice ± SEM. (C) Bar graphs show percentage cells with GrB expression after 16 h of culture with reagents as indicated. Error bars indicate SEM of data from two mice.
SYTO9 green fluorescent nucleic acid stain (Invitrogen) in a Corbett Rotor Gene 6000 cycler (Corbett Life Sciences, Concorde, Australia). Primers used for PCR (all from Sigma) are presented in Table I.

Stimulated non-B cells served as a positive control for human experiments and activated C57BL/6 NK cells for mouse B cells. Samples were run in duplicate and mRNA expression of GrB normalized to RPL-32 using Rotor Gene 6000 Software (version 1.7) and Microsoft Excel 2004. The relative expression levels to positive controls were calculated as $2^{-\Delta\Delta C_t}$.

Statistics
Data are expressed as mean ± SEM. Statistical differences between the means of two data groups was determined by using the paired or unpaired Student t test, and $p$ values <0.05 were considered significant.

Results
Stimulation with IL-21 and BCR cross-linking induces GrB in human but not in murine B cells
In previous reports, we demonstrated that the IL-2 family cytokine IL-21 in combination with BCR ligation induces expression and secretion of the serine protease GrB in human B cells (16, 17, 19). These studies suggested that GrB might play a significant role in the early anti-viral immune response and the control of autoimmunity. To enable more detailed studies of the underlying mechanisms, we investigated the induction of GrB in CFSE-labeled B cells from C57BL/6 mice in response to anti-CD40 plus IL-4 and BCR stimulation in the presence or absence of IL-21. When we analyzed the cells by flow cytometry, we found that unlike human B cells, murine B cells did not express GrB (Fig. 1A), although they were activated and underwent multiple rounds of division after 48 h as demonstrated by dilution of CFSE (Supplemental Fig. 1). Furthermore, extending cultures for up to 3 d was not sufficient to induce GrB expression (data not shown). Similar results were found in B cells derived from BALB/c, CBA, or DBA/2 mice (Supplemental Fig. 2). In all experiments, IL-2–activated NK cells from either B6.Grzmb$^{-/-}$ or C57BL/6 mice were used as negative or positive staining controls, respectively (Fig. 1B). Human GrB was enzymatically active when secreted into B cell culture supernatants (17) and thus was confirmed once again by testing whole-cell lysates of activated human B cells for cleavage of the highly specific Ala-Ala-Asp substrate (Fig. 1C, upper panel); however, neither proteolytic activity of cell lysates (Fig. 1C, lower panel) nor secretion of active GrB in cell culture supernatants (Supplemental Fig. 3) was detected when murine B cells were stimulated in a similar fashion. To determine whether the GrB gene is transcribed in mouse B cells, we measured GrB mRNA in stimulated B cells. In humans, GrB mRNA is not constitutively expressed but requires IL-21 and engagement of the BCR (Ref. 16 and Fig. 1D, upper panel). In mice, however, GrB mRNA levels were not significantly different from those of GrB-deficient mice, in which the gene has been largely deleted (Fig. 1D, lower panel, Table I). Similar results were obtained for GrA (whose

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** In vivo immunization with NP–KLH does not induce GrB in mouse B cells, and GrB deficiency does not alter B cell responses. C57BL/6 ($n = 9$) and B6.Grzmb$^{-/-}$ ($n = 9$) mice were injected i.p. with the T cell-dependent Ag NP–KLH, and B cell responses assessed on day 10. B cells from immunized mice (wild-type and knockout) and one naive control were isolated from spleen. Brefeldin A was added immediately after isolation (A) or after 16 h of culture with LPS, anti-CD40 plus IL-4, CpG ODN, and plate-bound NP–KLH, with or without IL-21 (B). After culture, cells were harvested, fixed and permeabilized, and stained for intracellular GrB. Data shown are one representative set from two independent experiments. (A) B cells and non-B cells of immunized and naive mice were stained ex vivo for baseline GrB expression and analyzed by flow cytometry. Dot plots show percentage of GrB$^+$ B cells and non-B cells from immunized and naive mice were stained ex vivo for baseline GrB expression and analyzed by flow cytometry. Dot plots show percentage of GrB$^+$ B cells from one representative mouse ($n = 4$). (B) B cells were cultured for 16 h with several B cell stimuli as indicated above. Dot plots show percentage of GrB$^+$ B cells in primary response to Ag of one representative wild-type and one knockout mouse ($n = 4$). (C and D) Sera of immunized C57BL/6 and B6.Grzmb$^{-/-}$ mice were collected on day 10 ($n = 5$), day 14 ($n = 7$), and day 21 ($n = 4$) after injection with 50 µg NP–KLH (C) or on day 8 after boost (day 53 after immunization, $n = 4$) (D). Bar graphs show total Ab (left panels) and high-affinity Ab (right panels) to NP as detected by ELISA. The error bars represent SEM of data pooled from two independent experiments. One naive C57BL/6 mouse served as negative control (data not shown).
gene is located on a separate chromosome) and GrC, the gene that is tightly linked to the GrB gene (data not shown).

**Infection with influenza virus does not induce GrB in B cells**

We recently demonstrated that restimulation of Ag-specific B cells from previously vaccinated human subjects with viral Ags triggered GrB expression (16). We therefore tested GrB expression in the B cells of mice infected with influenza virus. Referring to previous studies, we hypothesized that GrB expression might bridge innate and adaptive immune responses (17). Therefore, we tested an early time point postinfection (day 5) and at a time when a T cell response would be generated (day 10). As expected, infection with influenza induced GrB in cytotoxic T cells ex vivo, most notably by day 10, but we failed to detect the presence of GrB in B cells (Fig. 2A, 2B). The effect in T cells was enhanced by adding IL-21 and various other stimuli to cell cultures (Fig. 2C and data not shown). However, no GrB was detected in B cells at any time point, irrespective of the stimuli applied. To examine if exposure to persistent viral pathogens was required for GrB expression, we tested a second model of infection, murine γ-herpesvirus. Similar to the results obtained with the influenza model, T cells expressed GrB after exposure to virus, whereas B cell populations did not (Supplemental Fig. 4).

**Ag-specific mouse B cells lack expression of GrB**

We next used the model B cell Ag NP–KLH to address the role of GrB in the humoral immune response. This model enabled us to engage both the B cell and IL-21 receptors and CD4⁺ follicular T helper cells that are known to be crucial for humoral immune responses during the germinal center reaction via the production of IL-21 (27). Wild-type C57BL/6 and B6.GrzmB−/− mice were immunized i.p. with 50 μg NP–KLH Ag and spleens harvested 10 d after immunization. We first tested baseline GrB expression by incubating freshly purified B cells for 4 h with brefeldin A and subsequent testing for intracellular GrB by flow cytometry (Fig. 3A). Next, we stimulated B cells from immunized mice for 1 to 3 d with or without IL-21 and other stimuli such as LPS, CD40 plus IL-4, and CpG ODN 1668 to cover a wide spectrum of B cell activators. Direct antigenic restimulation as a highly specific stimulus was performed with plate-bound NP–KLH (Fig. 3B, with day 1 shown as a representative time point). Although all stimuli induced typical morphological changes indicating activation of the B cells (Supplemental Fig. 1), we could not detect intracellular GrB at any time point.

**GrB is redundant for Ab responses to NP**

The role of GrB in infection, cancer, and autoimmunity has been studied extensively (28–31); however, it is still unclear whether GrB contributes to humoral immune responses, potentially by B cell-induced death of helper cells leading to the contraction of the germinal center. Thus, we collected serum from NP–KLH–immunized mice on day 10, 14, and 21 after primary immunization or on day 8 after boost (day 53 after first injection) and tested for total Ab and high-affinity Abs to NP. We found no significant difference between wild-type and B6.GrzmB−/− mice in either response (Fig. 3C, 3D).

**Direct ligation of SWHEL-transgenic BCRs fails to induce GrB in B cells**

As BCR cross-linking appears to play a fundamental role in GrB induction in human B cells (16, 17), we applied a transgenic mouse model that enabled us to test Ag-specific BCR stimulation. B cells from SWHEL-transgenic mice produce Abs of high affinity to HEL and can undergo Ig class switch recombination and somatic hypermutation (32). We bred this model onto a RAG−/− background so that all B cells expressed a specific BCR against HEL. IL-21 and simultaneous expression of CD40L has previously been shown to induce differentiation of B cells to plasma cells in a CD4⁺ T cell-dependent manner (33). We recently showed that B cells secreted GrB when stimulated by IL-21, but only if CD40

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**FIGURE 4.** GrB in B cells is not detected by direct ligation of IgHEL-transgenic BCRs. B cells from SWHEL mice were isolated from spleen, resuspended in B cell media, and cultured for 4 d with LPS, anti-CD40 plus IL-4, and CpG ODN in the presence or absence of IL-21 and HEL, or a combination of both. (A) Intracellular GrB expression was determined on day 1 to day 4 by FACS. Dot plots show one representative experiment on day 1 of four independent experiments. (B) CD138 staining was performed on day 3 and 4 as a positive control to assess engagement of the BCR. Histogram shows expression of CD138 in B cells stimulated with LPS and soluble HEL on day 3.
ligation is absent (17). Similarly, B cells from SW<sub>HEL</sub> mice differen-
tiate into plasma cells when stimulated with LPS, but not when soluble HEL is added to the culture (34, 35). We hypothe-
sized that ligation of the BCR in these cultures might contribute to the loss of plasma cells by inducing GrB-secreting B cells. B cells were purified from SW<sub>HEL</sub> mice, cultured for 4 d with LPS, anti-
CD40 plus IL-4 and CpG ODN 1668, with or without IL-21 and soluble HEL. B cells were then stained for intracellular GrB and for expression of the plasma cell marker CD138. We observed no induction of GrB expression in the presence of HEL (Fig. 4A), although engaging the BCR with soluble HEL induced the expected loss of plasma cells (Fig. 4B).

**Discussion**

To date, GrB expression in human B cells has been described in the context of viral infections (16), autoimmune disease (19), and cancer (18), suggesting a regulatory or modifying role in these diseases. Yet, exact mechanisms involved in GrB expression of B cells and the physiological relevance of these findings require further in-
vestigation. In the absence of a recognized syndrome of human granzyme deficiency, mouse models have been invaluable for our understanding of granzyme biology. For example, GrzmB<sup>−/−</sup> mice survive most experimental virus infections (36, 37), but the combined loss of GrA and GrB results in a severe immunological defect revealed by infection with poxviruses such as ectromelia (38, 39).

We reasoned that if human B cells express GrB, it is likely that certain stimuli will bring about a similar effect in murine B cells. However, we failed to detect GrB or its proteolytic activity using a variety of assays in mouse B cells stimulated with B cell activators in combination with IL-21, the most potent inducer of GrB in human B cells (16). We combined IL-21 with various other B cell stimuli including engagement of IgM and IgD in naive B cells. Subsequently, IL-4 in combination with CD40 ligation, LPS, and CpG ODN 1668 were tested to induce proliferation and isotype switching. To specifically address BCR engagement, we tested B cells with a transgenic BCR (SW<sub>HEL</sub>). We also extended our studies into several in vivo models with viral infections and immu-
unization with a specific Ag, the latter an extensively used system for studying B cell responses in vivo (40–42). Infection with either influenza or murine γ-herpesvirus did not induce GrB in B cell populations, and GrB deficiency did not alter B cell Ab production in vivo. Additionally, we tested mouse B cells in these models for constitutive expression of GrB, as we found baseline GrB expression in CD5<sup>+</sup> B cells from patients with systemic lupus erythematosus (19). All of the conditions, however, failed to induce GrB expression in B cells. We therefore conclude that the mechanisms regulating GrB expression are not conserved between humans and mice.

Human and mouse GrB have previously been shown to possess a number of important functional and phenotypic differences. For example, mouse GrB is 30 times less cytotoxic than human GrB, activates distinct apoptotic pathways (43), and exhibits signifi-
cantly different substrate specificities (44, 45). This raises the likelihood that mouse and human GrB may have developed species-specific functions in response to particular evolutionary pressures. This hypothesis is further supported by the fact that human GrB has multiple functional paralogues in the mouse suggesting that the various functions of human GrB might be split between different granzymes in rodents. For example, the down-
stream “orphan” GrC and/or GrF have reported functions in mouse CTL/NK cytotoxicity, whereas the equivalent role in humans is probably played by GrH (46–48). Furthermore, GrB and its specific inhibitor serpin PPI are found in the human testis and may play a role in reproduction (49), whereas in mice GrN (and not GrB) is found in this organ (50). GrB also shows ex-
tensive polymorphism in wild mice (51), whereas only three common human GrB alleles have been identified (52). To exclude strain differences, we also tested CBA, DBA/2, and BALB/c mice, but we found no expression of GrB.

In conclusion, the current study indicates that findings on GrB cannot be generalized from one species to another. The physio-
logical and pathological relevance of GrB expression in human B cells still remains unclear, but mouse models are unlikely to provide insight into these processes. Our study demonstrates the need to develop novel in vivo systems to study human immune responses.

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Brink for supplying HEL-transgenic mice (Garvan Institute, Sydney, NSW, Australia).

**Disclosures**

The authors have no financial conflicts of interest.

**References**

tivation by granzyme B is indirect, and caspase autoprocessing requires the re-
idence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol. Cell. Biol. 19: 8604–8615.


Supplementary Figure 1. Various B cell stimuli induce proliferation in B cell cultures
Freshly isolated B cells from C57BL/6 mice were labelled with CFSE and plated for 3 days in the presence of LPS, anti-IgM, anti-IgD and anti-CD40+IL-4, with or without IL-21. Histograms show representative examples on day 2 compared to unstimulated controls.

Supplementary Figure 2. B cells from different inbred mouse strains are negative for GrB expression after stimulation of the B cell- and the IL-21 receptors
B cells from BALB/c (H-2b), CBA (H-2k), DBA/2 (H-2d) mice were isolated and cultured for 1 to 3 days with LPS, anti-BCR (=anti-IgM+anti-IgD), anti-CD40+IL-4, CpG ODN with or without IL-21. After another 4 h of culture in the presence of Brefeldin A, B cells were harvested, fixed, permeabilized and stained for intracellular GrB. Dot plots show percentage of GrB positive B cells on day 1 as representative for three days with similar results.

Supplementary Figure 3. Mouse B cells do not secrete active GrB
C57BL/6 B cells were isolated and stimulated overnight with anti-IgM+anti-IgD (=anti-BCR) with or without IL-21. After 4 h of culture in the presence of PMA (1μg/ml) and Ionomycin (10 μg/ml), supernatants were collected and ASPase activity determined. Shown is one representative plot out of three independent experiments. The error bars represent SEM of intraexperimental duplicates. Supernatants from either C57BL/6 or B6.GrzmAB-/ NK cells served as controls.

Supplementary Figure 4. Mouse B cells do not express GrB after in vivo infection with herpes virus
Mice were infected intranasally with 3 x 10^4 PFU of MHV-OVA (n=4). Mediastinal lymph nodes and spleens were harvested on day 5 (n=2) and day 10 (n=2) after infection. Organs of two naïve mice served as a baseline control. Mononuclear (lymph nodes) and B cells (spleen, purity >98%) were isolated and cultured. Brefeldin A was added for 4 h immediately after isolation (ex vivo, (A) and (B)) or after stimulation for 16 h with LPS, anti-CD40+Il-4, CpG ODN in the presence or absence of IL-21 (C). After culture, cells were harvested and stained for CD3, CD19 and B220 (lymph nodes) and intracellular GrB (lymph nodes and spleen). (A) Dot plots indicate baseline GrB expression of one representative naïve mouse, or mice 5 days and 10 days after infection, respectively. The upper panels show gating for CD3+ T cells and B220+ B cells. The middle and lower panels illustrate percentages of GrB positive cells as analysed by FACS. (B) Line graph depicts average ex vivo GrB expression of B and T cells from lymph node and splenic B cells +SEM of two mice. (C) Bar graphs show percentage cells with GrB expression after 16 h of culture with reagents as indicated. Error bars indicate SEM of data from two mice.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3

The graph shows the absorbance (O.D. units) at 405 nm over time (min) for different samples:
- WT
- GrAB-/-
- Medium
- IL-21
- anti-BCR
- anti-BCR+IL-21

The y-axis represents absorbance values ranging from 0.0 to 2.0, and the x-axis represents time in minutes from 0 to 11.

The graph indicates an increasing absorbance over time for the B cell samples, with a distinct separation between NK cell and B cell categories.
Supplementary Figure 4