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ORIGINAL ARTICLE

Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help

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Recently, CD4+ T helper cells were shown to induce differentiation of human B cells into plasma cells by expressing interleukin (IL-)21 and CD40 ligand (CD40L). In the present study we show, that in the absence of CD40L, CD4+ T cell-derived IL-21 induces differentiation of B cells into granzyme B (GzmB)-secreting cytotoxic cells. Using fluorescence-activated cell sorting (FACS) analysis, ELISpot and confocal microscopy, we demonstrate that CD4+ T cells, activated via their T-cell receptor without co-stimulation, can produce IL-21, but do not express CD40L and rapidly induce GzmB in co-cultured B cells in an IL-21 receptor-dependent manner. Of note, we confirmed these results with recombinant reagents, highlighting that CD40L suppresses IL-21-induced GzmB induction in B cells in a dose-dependent manner. Surprisingly, although GzmB-secreting B cells did not express perforin, they were able to transfer active GzmB to tumor cell lines, thereby effectively inducing apoptosis. In contrast, no cytotoxic effects were found when effector B cells were activated with IL-2 instead of IL-21 or when target cells were cultured with IL-21 alone. Our findings suggest GzmB+ cytotoxic B cells may have a role in early cellular immune responses including tumor immunosurveillance, before fully activated, antigen-specific cytotoxic T cells are on the spot. CD40 ligand determines whether IL-21 induces differentiation of B cells into plasma cells or into granzyme B-secreting cytotoxic cells.
Immunology and Cell Biology (2012) **90,** 457–467; doi:10.1038/icb.2011.64; published online 2 August 2011

Keywords: B cell; IL-21; CD40 ligand; neoplasm; tumor; virus

Interleukin (IL)-21 is a recently discovered member of the IL-2 cytokine family.[1] Its sources include CD4+ T cells and NKT cells.[1–3] IL-21 has potent antitumor activity and is associated with early pro-inflammatory immune responses during autoimmune diseases and viral infections.[3,4] IL-21 induces upregulation of gene transcription for granzymes in cytotoxic T lymphocytes (CTL)[5,6] as well as proliferation in B cells, NK cells and T cells.[1,7,8] Furthermore, IL-21 triggers Bcl-2-interacting mediator of cell death (BIM)-dependent apoptosis in murine B cells and human malignant B cells depending on additional stimuli such as toll-like receptor or CD40 stimulation.[9–11] Importantly, IL-21 is a strong inducer of human B-cell differentiation into plasma cells. This IL-21-induced differentiation pathway is supported by CD40 ligation and IL-4, both of which are integral parts of a full CD4+ T helper cell response.[12,13] The fate of B cells stimulated with IL-21 in the absence of T helper cell-derived signals has not been investigated in detail so far.

Granzyme B (GzmB) represents a major constituent of the granules of CTL. CTL delivers GzmB to targets such as tumor or virus-infected cells, where it effectively induces apoptosis.[14] The recognition of target cells by CTL requires that dendritic cells pick up antigens, process and present them on major histocompatibility complex (MHC) molecules,[15] and migrate to draining lymph nodes, where they activate antigen-specific T cells. The processes involved are complex and time consuming. Therefore, more immediate immune response mechanisms exist that are independent of MHC presentation and that bridge the gap between the first recognition of danger signals and the establishment of an efficient CTL response. Several cell subsets belonging to the innate arm of the immune system including NK cells and NKT cells can have a role in such early immune responses.[16–18] Since B cells use their specific immunoglobulins as surface receptors, they can also recognize antigens in an immediate and MHC-independent manner. Moreover, the variety of potential antigens recognized by

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Received 3 November 2010; revised 22 June 2011; accepted 23 June 2011; published online 2 August 2011

B-cell receptors (BCRs) is larger than that recognized by T-cell receptors (TCRs) and includes nucleic acid, glycolipid, peptide and carbohydrate antigens.[19,20] Therefore, from a teleological point of view, it appears likely that B cells are also involved in early cytotoxic immune responses against tumors and viruses, before starting their terminal differentiation into antibody-secreting plasma cells.

In the present study, we show that during B-cell activation by the acute phase cytokine IL-21 and BCR engagement, CD40 ligation determines whether a B cell differentiates into a plasma cell or into a GzmB-secreting and potentially cytotoxic B cell. We demonstrate that incompletely, but not completely activated $CD4^+$ T cells express IL-21 along with low levels of CD40 ligand (CD40L) and induce GzmB in co-cultured B cells in an IL-21 receptor-dependent manner. In order to reveal a potential *in vivo* relevance of GzmB-secreting B cells, we investigated GzmB responses in B cells from healthy subjects before and after vaccination against different viruses and found that the corresponding viral antigens induced strong GzmB secretion by antigen-specific B cells only after, but not before vaccination. Furthermore, we showed that GzmB produced by B cells is secreted in its enzymatically active form and that GzmB-secreting B cells are able to deliver GzmB to and induce apoptosis in sensitive cancer cell lines. Our results demonstrate that GzmB secretion and accompanying cytotoxicity are not limited to CTL but are also found in B cells, which may thus be part of early cellular cytotoxic immune responses. Our data may have implications for the development of novel immunotherapeutic approaches for neoplastic and viral diseases, which involve the induction of GzmB-secreting cytotoxic B lymphocytes.

RESULTS

Without sufficient co-stimulation, $CD4^+$ T cells express IL-21, but not CD40L, and induce GzmB in co-cultured B cells

$CD4^+$ T cells represent a major source of the acute phase cytokine IL-21.[1] Given our previously reported findings, that IL-21 can trigger GzmB expression in virus-specific B cells,[21] we hypothesized that $CD4^+$ T cells may be capable of directly inducing GzmB in B cells. We compared two stimulation modes, namely TCR activation in the absence of co-stimulation (anti-CD3 only), and in the presence of co-stimulation (anti-CD3 and anti-CD28). In pilot experiments, we determined concentrations of anti-CD3 and anti-CD28 antibodies that induced equal activation of T cells as indicated by expression of CD69 after 1 day culture. Using these concentrations, we found that $CD4^+$ T cells activated by anti-CD3, but not by anti-CD3/CD28, acquired significant capacity to induce GzmB in co-cultured B cells (Figures 1a and b). Simultaneous incubation with an anti-IL-21R-blocking antibody inhibited GzmB induction, demonstrating its dependency on IL-21 (Figure 1b). Analyzing the phenotype of activated $CD4^+$ T cells after 24 h, we found that IL-21 was expressed at equal levels by both $CD4^+$ T cells, which received co-stimulation and $CD4^+$ T cells, which did not receive co-stimulation (Figure 1c).

In an effort to further differentiate the phenotype of IL-21-producing $CD4^+$ T cells, we isolated naive ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) $CD4^+$ T cells and used them for similar assays as done before with pan-$CD4^+$ T cells. In these experiments, we found that it was mainly memory T cells that produced IL-21 after stimulation with anti-CD3 alone (Figure 1d). In contrast, in the presence of co-stimulation with anti-CD28, naive T helper cells appeared to be stronger producers of IL-21 than memory T helper cells (Figure 1d). Of note, neither $IL\text{-}21^+$ nor $IL\text{-}21^-$ T cells expressed IL-17 at 24 h, suggesting that in our setting it was not Th17 cells, which secreted IL-21 (data not shown). Instead, we found that memory $CD4^+$ cells, and, to a lesser extent naive $CD4^+$ T cells expressed the chemokine receptor CXCR5, which is a known marker for follicular helper T cells (Figures 1e and f). Expression of CXCR5 on these cells was not influenced by stimulation of CD3 or CD3 and CD28 (Figure 1f).

Importantly, CD40L (CD154) was substantially expressed on $CD4^+$ T cells only when TCR stimulation was accompanied by co-stimulation (Figure 1g). Here, no differences were found between memory and naive T cells (data not shown). These data suggest that although CD40L has been described to have a key role for IL-21-initiated differentiation of B cells into plasma cells, its presence does not appear to be necessary for their differentiation into $GzmB^+$ cells.

IL-21 induces B-cell GzmB expression in the absence, but not in the presence of CD40 ligation

Previous studies demonstrated that IL-21 induces differentiation of B cells into plasma cells in the presence, but not in the absence of CD40 ligation[12] (Figure 2a). Based on these findings and our results above, we started investigating GzmB expression in B cells in response to IL-21, BCR engagement and CD40 ligation. We found low expression of GzmB in purified B cells after stimulation with IL-21 alone, an effect known to be synergistically enhanced in the presence of BCR engagement (Figures 2b and c). Importantly, GzmB expression induced by IL-21 was effectively suppressed by simultaneous ligation of CD40 on B cells (Figures 2a, b and c). This suppression occurred in a dose-dependent manner and was not due to induction of B-cell apoptosis (Figure 2d). We also tested the impact of IL-4 on IL-21-induced GzmB expression and found slight GzmB suppression by IL-4, but not by IL-2. Importantly, neither IL-2 nor IL-4 by itself induced GzmB secretion by B cells (Supplementary Figure 1). Recently, we demonstrated that apart from IL-21, IL-4 in combination with IL-10 also induces substantial amounts of GzmB in B cells.[21] We, therefore, wished to confirm or exclude a potential sensitization of IL-21-stimulated B cells to IL-4 and IL-10. Subsequent experiments revealed that IL-4 and IL-10 were not able to increase the amount of GzmB-positive B cells stimulated with IL-21 or with IL-21 and anti-BCR, thereby excluding a sensitization effect as hypothesized above (Supplementary Figure 2).

IL-21-stimulated B cells exhibit an activated phenotype and secrete enzymatically mature GzmB

Next, we investigated the transport of GzmB after IL-21-mediated induction in B cells. Immunofluorescence staining of sections from paraffin-embedded enriched B cells suggested GzmB was mainly accumulating in perinuclear granula (Figure 3a). By culturing purified B cells on GzmB-specific ELISpot plates, we found strong secretion of GzmB by B cells stimulated with IL-21 and anti-BCR (Figure 3b). GzmB secretion was completely abrogated in the presence of brefeldin A, indicating that an active transport from the endoplasmic reticulum to the Golgi apparatus was involved. Again, CD40 ligation strongly suppressed GzmB secretion, confirming our flow-cytometric results. Importantly, secreted GzmB was enzymatically mature, as we detected a time-dependent accumulation of proteolytically active GzmB in B-cell culture supernatants, reaching 30% of the amounts secreted by PHA (phytohemagglutinin)-stimulated non-B cells (Figure 3c). GzmB production by B cells activated with IL-21 and anti-BCR was accompanied by activation of SYK (Figure 3d) and an activated phenotype with loss of memory and naive B-cell markers, and upregulation of co-stimulatory, antigen-presenting and cell adhesion molecules (Supplementary Figure 3). Of note, activation of B cells with IL-21 did not

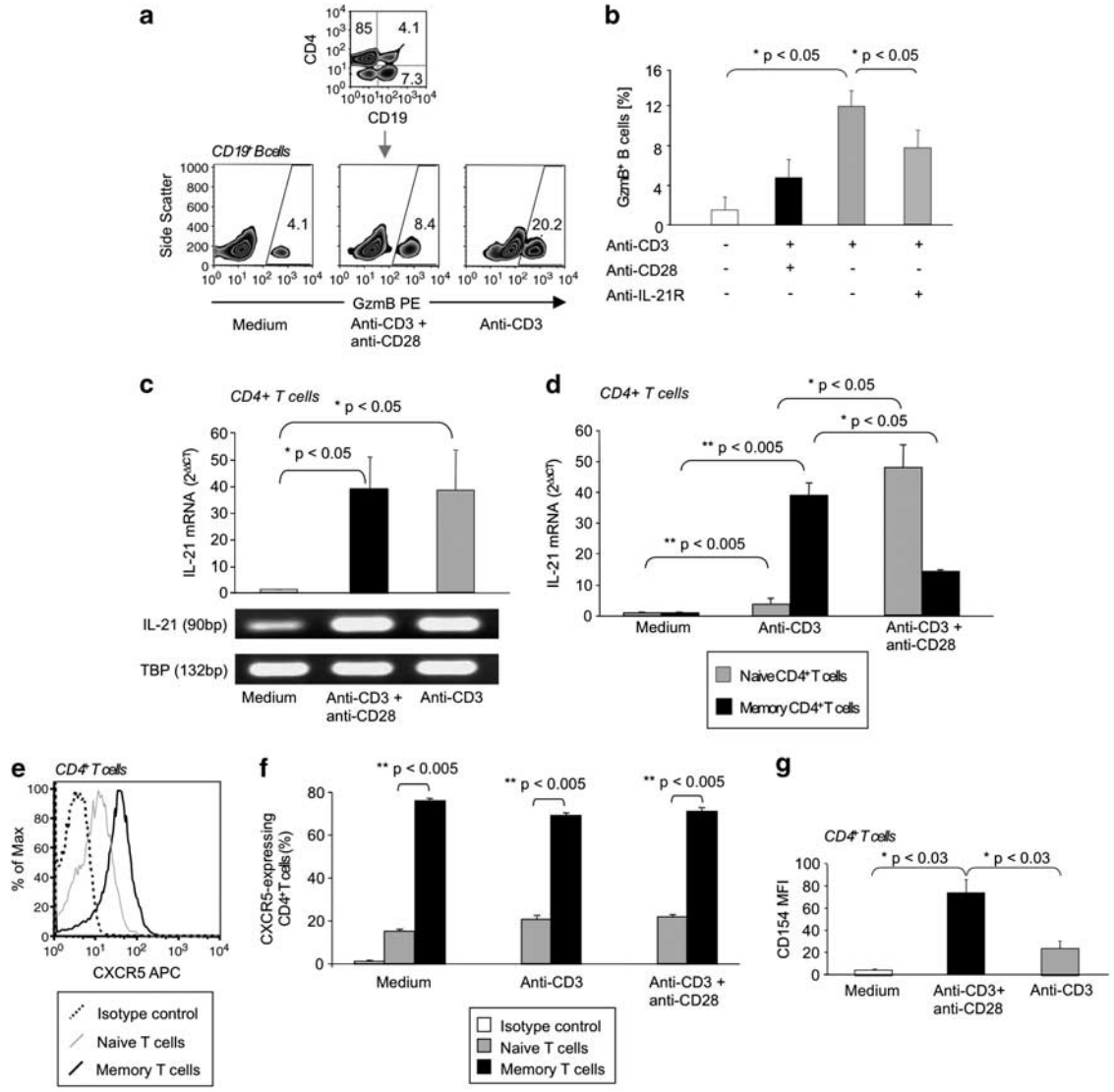
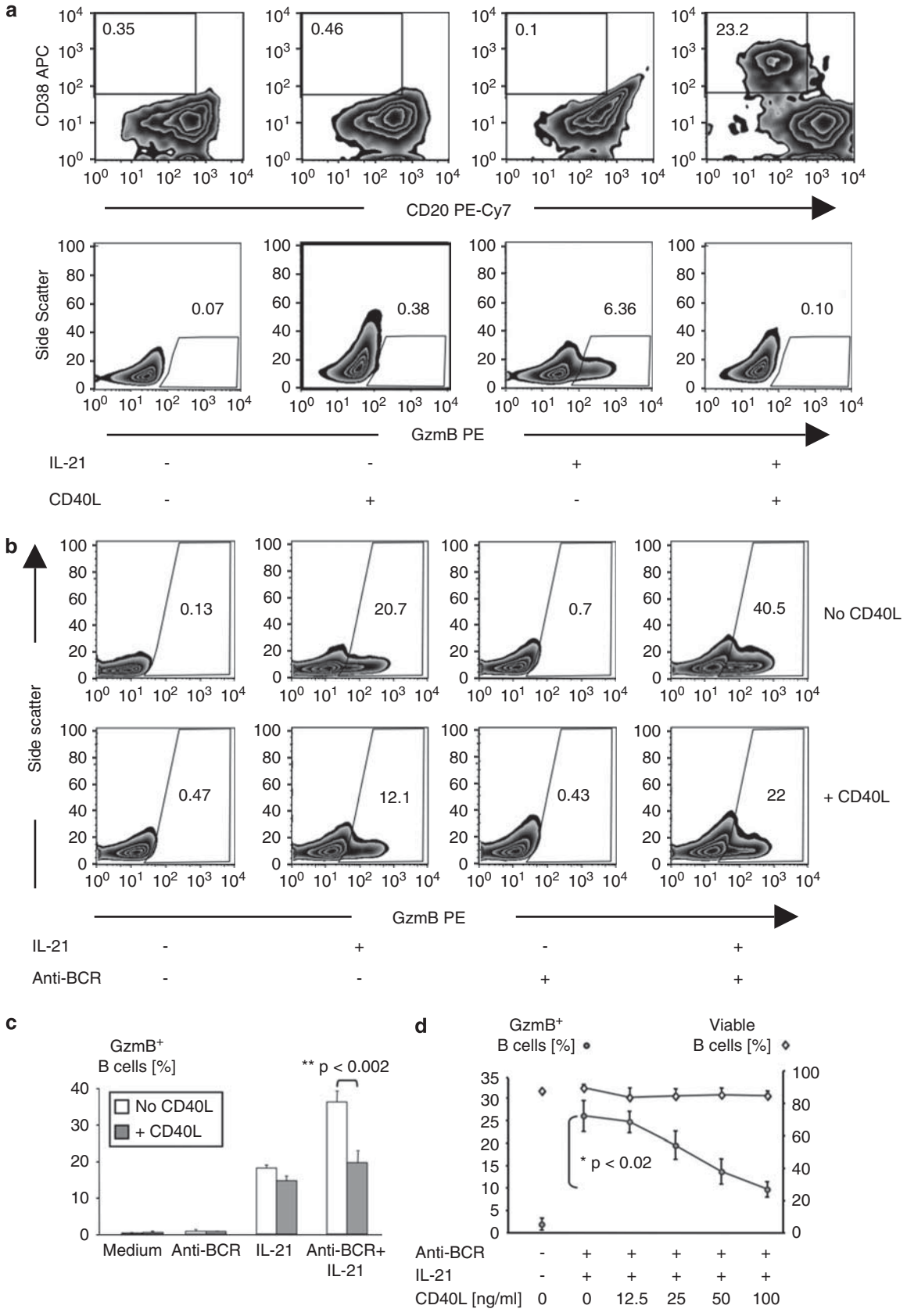


Figure 1 Expression of CD40 ligand (CD154) and IL-21 is differentially regulated in CD4$^+$ T cells and controls GzmB induction in B cells. Purified CD4$^+$ T cells (>98%) from healthy donors were cultured in the presence of stimulating antibodies to CD3 or CD3/CD28 as indicated. Anti-CD3 or anti-CD3/CD28 antibodies were used at concentrations that induced equal levels of T-cell activation based on CD69 expression. (**a**) Purified CD4$^+$ T cells stimulated with either anti-CD3 or anti-CD3/CD28, or unstimulated T cells were co-cultured with purified, anti-BCR-activated CD19$^+$ B cells at a ratio of 10:1. After 1 day co-culture, cells were harvested and stained for CD4, CD19 and intracellular GzmB. Upper zebra plot demonstrates gating strategy to analyze B cells from B cell–T cell co-cultures. Gates in lower zebra plots indicate percentages of GzmB$^+$ B cells from one representative B-cell–T-cell co-culture out of three. (**b**) Bar graphs represent average percentages of GzmB$^+$ B cells after co-culture with CD4$^+$ T cells as described above and in the presence of increasing concentrations of a neutralizing IL-21 receptor antibody (n=3). Error bars indicate s.e.m. (**c**, **d**) Pan-CD4$^+$ T cells (**c**) or from naive and memory CD4$^+$ T cells (**d**) were purified and stimulated with either anti-CD3/CD28 or anti-CD3, or left unstimulated for 1 day. Total RNA was isolated and real-time RT-PCR performed for quantification of IL-21 mRNA. Relative quantity of mRNA was determined with the comparative C_T method. Bar graphs represent the mean IL-21 mRNA expression relative to mRNA expression of the housekeeping gene TBP. Data are representative of three individual experiments for each cell population. Error bars indicate s.e.m. Bands on the gel represent mRNA reaction products from one representative donor of pan-CD4$^+$ T cells. (**e**) Line graphs represent CXCR5 expression on naive and memory CD4$^+$ T cells from one representative donor after 1 day culture. Isotype control is shown for memory T cells. (**f**) Bar graphs show averages for CXCR5 expression (median fluorescence intensities) on naive versus memory CD4$^+$ T cells from three different donors. Error bars indicate s.e.m. (**g**) Bar graphs show average CD154 expression in CD4$^+$ T cells after 1 day culture from three different donors. Error bars indicate s.e.m.

enhance B-cell apoptosis as compared with cells cultured in the absence of IL-21, suggesting that GzmB production did not directly result in activation-induced B-cell death (data not shown).

Since the antibody fragments against the BCR used above represent a polyclonal B-cell stimulus, we wished to demonstrate GzmB induction in B cells by an antigen-specific stimulus. Recently, we could show that B cells from healthy individuals vaccinated against tick-borne encephalitis virus (TBEV) exhibit a higher potential to produce GymB in response to IL-21 and viral antigens than B cells from unvaccinated individuals.[21] To expand these findings, we recruited 15 healthy donors before and 2 weeks after vaccination against various viral diseases including TBEV, rabies and hepatitis B. B cells from these donors were purified and cultured in the presence of IL-21 and the corresponding viral antigens or the carrier control at increasing concentrations.


a
CD38 APC
0.35
0.46
0.1
23.2
CD20 PE-Cy7
Side Scatter
0.07
0.38
6.36
0.10
GzmB PE
IL-21 - - + +
CD40L - + - +
b
Side scatter
0.13
20.7
0.7
40.5
No CD40L
0.47
12.1
0.43
22
+ CD40L
GzmB PE
IL-21 - + - +
Anti-BCR - - + +
c
GzmB+
B cells [%]
** p < 0.002
No CD40L
+ CD40L
Medium
Anti-BCR
IL-21
Anti-BCR+
IL-21
d
GzmB+
B cells [%]
Viable
B cells [%]
* p < 0.02
Anti-BCR - + + + + +
IL-21 - + + + + +
CD40L [ng/ml] 0 0 12.5 25 50 100

Isolated B cells from vaccinated, but not from unvaccinated individuals showed a dose-dependent induction of GzmB after direct re-stimulation with the corresponding viral antigens (Supplementary Figures 4A and B). In contrast, incubation of B cells from vaccinated or unvaccinated donors with the carrier $Al(OH)_3$ alone did not enhance GzmB expression (Supplementary Figure 4B, upper panel).

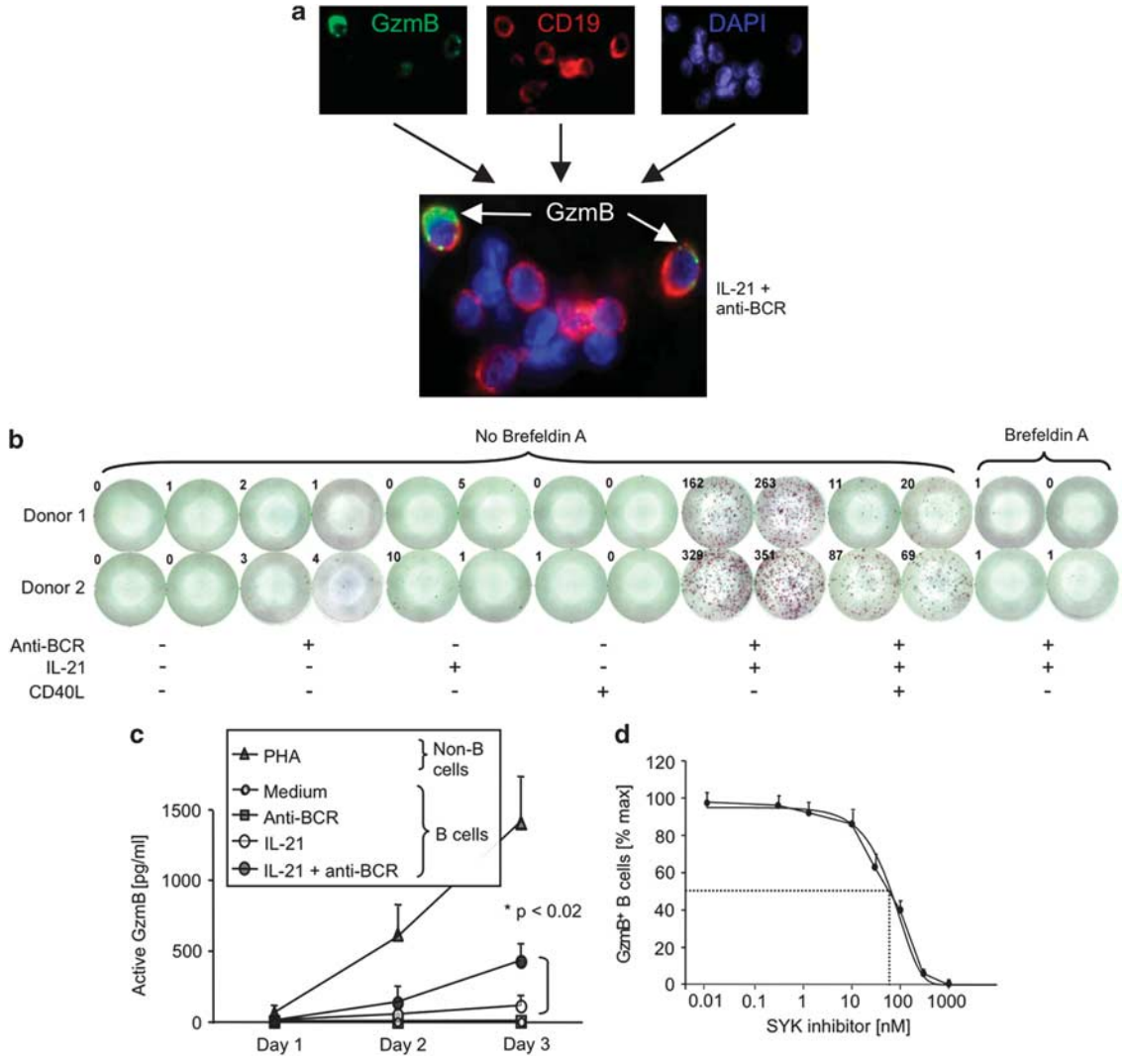


Figure 3 GzmB in B cells is enzymatically active after secretion and occurs in an SYK-dependent manner. (**a**) Enriched B cells (20%) from healthy donors were cultured for 16 h with IL-21 and anti-BCR. Brefeldin A was added for four more hours, and cells were pelleted and paraffin embedded. Immunofluorescence microscopy images from paraffin-embedded sections stained for GzmB (green), CD19 (red) and cell nuclei (DAPI, blue) are shown. Similar results were obtained in three independent experiments. (**b**) Purified B cells (>99.5%) from healthy subjects were cultured on GzmB-specific 96-well ELISpot plates at 1×10^5 B cells per well with IL-21, anti-BCR, CD40 ligand, and brefeldin A ($1\,\mu g\,ml^{-1}$) as indicated. After 16 h, plates were developed and dots counted. Each condition was run in duplicate; two representative experiments out of four are shown. (**c**) Purified B cells (>99.5%) and non-B cells from healthy individuals were cultured for 1, 2 or 3 days at 1×10^5 per well in the presence of IL-21, anti-BCR or PHA as indicated. Supernatants were collected and GzmB activity was determined using a highly sensitive GzmB activity assay. Line graphs represent average GzmB activities from three donors. Error bars indicate s.e.m. (**d**) Purified B cells were cultured for 16 h with IL-21, anti-BCR and increasing non-toxic concentrations of SYK inhibitor as indicated. Then, B cells were stained for intracellular GzmB and analyzed by FACS. The line graph shows average percentages (n=4) of GzmB$^+$ B cells. Error bars indicate s.e.m.

Figure 2 IL-21 induces strong expression of GzmB in B cells in the absence, but not in the presence of CD40 engagement. (**a**) Purified B cells (>99.5%) were cultured for 6 days (upper panels) or 16 h (lower panels) in the presence or absence of IL-21 ($50\,ng\,ml^{-1}$) and CD40 ligand ($100\,ng\,ml^{-1}$). Then, cells were stained for CD38 and CD20 (upper panels) or for intracellular GzmB (lower panels) and analyzed by FACS. Gates show percentages of CD38$^+$CD20^{low} plasma cells (upper panels) or GzmB$^+$ B cells (lower panels, n=3). (**b**, **c**) Purified B cells were cultured for 16 h in the presence or absence of IL-21, anti-BCR ($6.5\,\mu g\,ml^{-1}$) and CD40 ligand. Then, cells were stained for intracellular GzmB and analyzed by FACS (n=3). (**b**) Zebra plots with GzmB$^+$ B cells gated are shown. (**c**) Bar graphs show average percentages of GzmB$^+$ B cells. Error bars indicate s.e.m. (**d**) Purified B cells were cultured for 16 h with IL-21, anti-BCR and increasing concentrations of CD40 ligand as indicated. Cells were stained for intracellular GzmB and analyzed by FACS. Line graphs show average percentages (n=3) of GzmB$^+$ B cells (gray circles) and percentages of morphologically viable B cells (white diamonds). Error bars indicate s.e.m.

After IL-21 stimulation, B cells can deliver GzmB to tumor cells and induce tumor cell apoptosis

To investigate the potential of GzmB-secreting versus GzmB-negative B cells to induce GzmB-dependent target cell apoptosis, we tested a large variety of B-cell lines as potential cytotoxic effector cells. The ability to secrete GzmB in response to IL-21 varied considerably between B-cell lines with ARH-77 and MEC-1 showing very strong GzmB response and others like Raji showing no GzmB response at all (Figure 4a; Supplementary Table 1). As in normal B cells, the IL-21-related cytokine IL-2 did not induce GzmB in B-cell lines (Figure 4a) and B-cell lines were not able to express or secrete perforin in response to IL-21 (data not shown). Using spinning disk confocal microscopy and a fluorescent GzmB substrate, we confirmed the presence of enzymatically active GzmB in IL-21-stimulated ARH-77 cells (Supplementary Figure 5; Supplementary Video 1). To evaluate their cytotoxic potential, we co-cultured GzmB$^+$ ARH-77 cells and GzmB$^-$ Raji cells with a series of GzmB$^-$ tumor cell lines including HeLa, G-361 and K-562 for 3 days. Since neither primary B cells nor B-cell lines secrete perforin in response to IL-21,[21] we measured target cell death not by standard ^{51}Cr release assay, but rather by annexin V/propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis. This method allows detection of slowly developing apoptosis, in contrast to the ^{51}Cr release assay, which detects rapid, mainly perforin-mediated lysis. We found, that IL-21-activated ARH-77 cells, but not IL-21-activated Raji cells reduced target cell viability on average by 41% in HeLa cells (Figure 4b, left and middle panels) and by 21% in G-361 cells. Similarly, MEC-1 reduced viability on average by 32% in HeLa cells and by 36% in G-361 cells (data not shown). Importantly, GzmB-secreting primary B cells from healthy subjects also reduced viability in HeLa cells on average by 25% (Figure 4b, right panel). No pro-apoptotic effects were found when effector cells were activated with IL-2 instead of IL-21 or when target cells were cultured with IL-21 alone. To visualize the interactions between GzmB$^+$ ARH-77 cells and HeLa cells, we performed spinning disk confocal microscopy with ARH-77/HeLa co-cultures in the presence of a fluorogenic GzmB substrate. Using this technique, we demonstrated that ARH-77 cells deliver enzymatically active GzmB to HeLa cells (Figure 5a; Supplementary Video 2) and that this attack by GzmB$^+$ ARH-77 cells finally results in the apoptotic shrinkage of HeLa cells (Figure 5b; Supplementary Video 3).

DISCUSSION

IL-21 is a novel and highly promising cytokine for the treatment of neoplastic, autoimmune and infectious diseases.[3,22,23] Several phase I

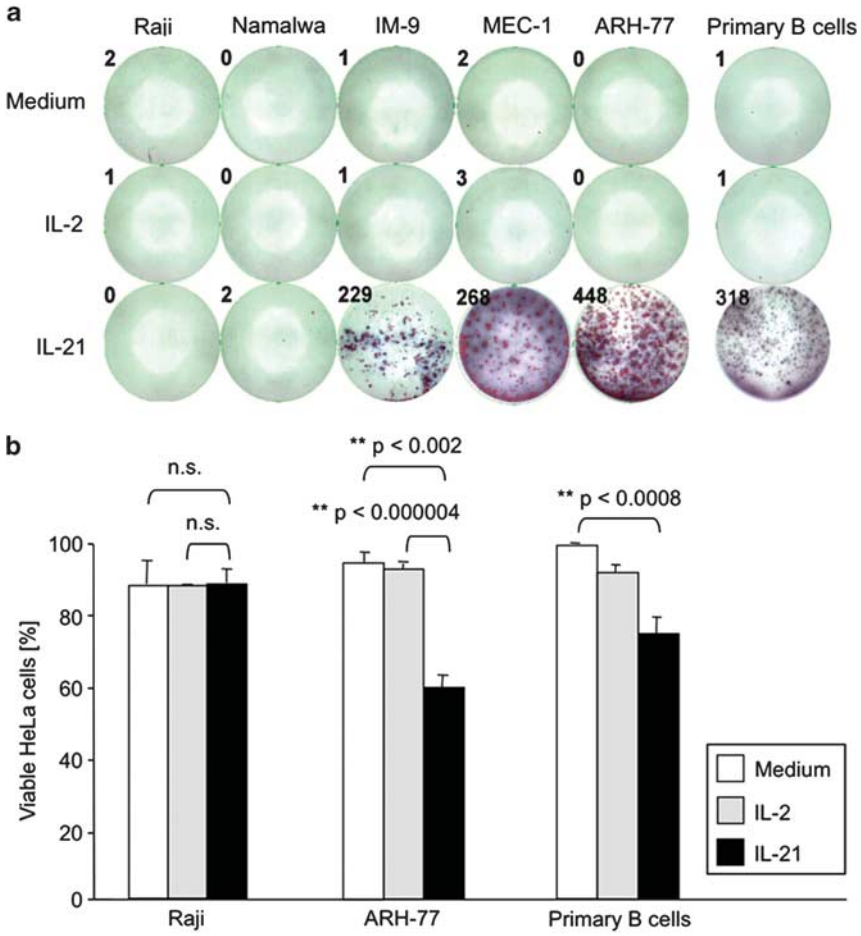


Figure 4 GzmB-secreting, but not GzmB-negative B cells can induce tumor cell apoptosis. (**a**) Various B-cell lines or primary human B cells were cultured at 37°C on GzmB-specific 96-well ELISpot plates at 1×10^5 cells per well with IL-2 (500 IU ml^{-1}) or IL-21 (50 ng ml^{-1}) as indicated. Primary B cells were stimulated with anti-BCR (6.5 μg ml^{-1}) in addition to cytokines. After 16 h, plates were developed and dots counted (n=3). (**b**) In all, 2.5×10^5 effector B cells were added to 1×10^4 PKH26-stained target cells in a final volume of 200 μl on a round-bottom 96-well plate. Target cell apoptosis was determined by annexin V/propidium iodide staining and FACS analysis after 3 days incubation with IL-2 or IL-21. Bar graphs show average percentages of viable (non-apoptotic) HeLa cells on day 3, normalized to maximum viability (n=3). Error bars indicate s.e.m and ** indicate $P<0.005$.

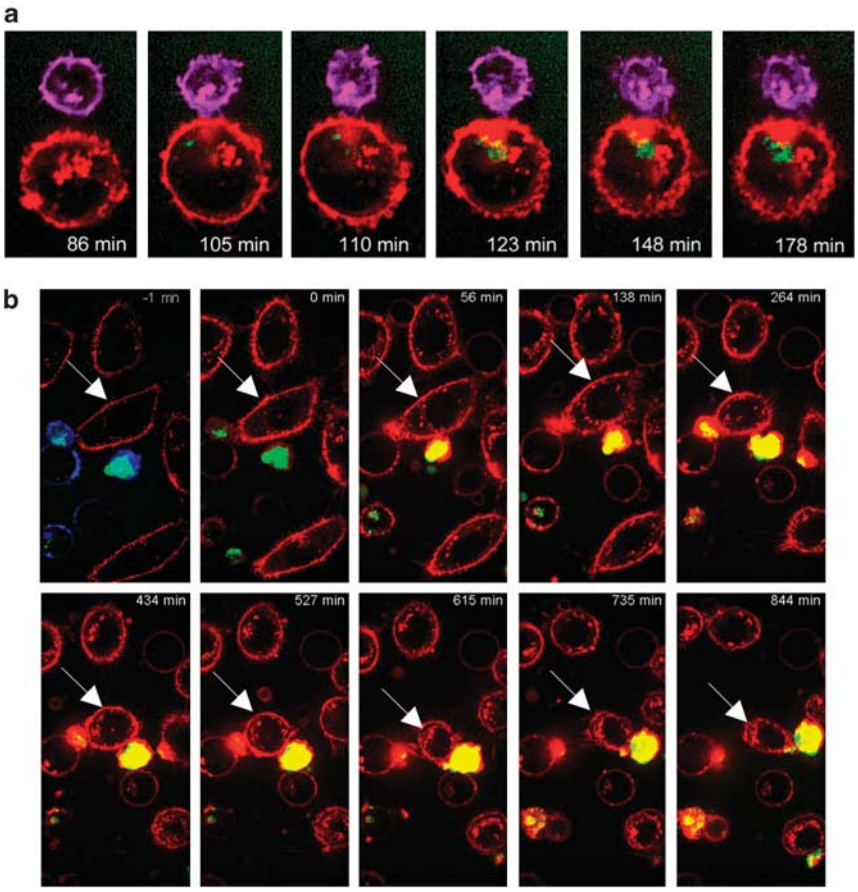


Figure 5 GzmB$^+$ ARH-77 cells attack HeLa cells by transfer of enzymatically active GzmB. (**a**) Anti-CD19 PerCP-stained ARH-77 cells were pre-incubated for 24 h in complete medium (1×10^6 per ml) in the presence of IL-21 (50 ng ml^{-1}). In all, 1×10^5 anti-CD19 PerCP-stained ARH-77 cells (purple) were added to ibiTreat chamber slides containing 2.5×10^4 immobilized HeLa cells stained with Cell Mask deep red membrane dye (red). Cells were cultured for another 16 h in the incubator, then GranToxiLux fluorogenic GzmB substrate (green) was added and confocal microscopy was performed for 3 h. (**b**) ARH-77 cells were pre-incubated for 36 h in complete medium (1×10^6 per ml) in the presence of IL-21 (50 ng ml^{-1}). In all, 1×10^5 ARH-77 cells were washed, stained with anti-CD19 PerCP (blue) and plated on ibiTreat chamber slides together with 2.5×10^4 HeLa cells stained with Cell Mask deep red membrane dye (red). Finally, GranToxiLux fluorogenic GzmB substrate (yellow) was added and confocal microscopy was performed for 14 h. White arrows indicate a HeLa cell attacked by GzmB$^+$ ARH-77 cells.

and II clinical trials with recombinant IL-21, particularly in the field of cancer immunotherapy, have been completed or are currently ongoing, providing data on its safety and efficacy in subjects suffering from a series of malignant diseases.[22] Many of the beneficial effects of IL-21 are attributed to its action on late humoral and cellular immune responses. Although IL-21 is one of the earliest cytokines occurring during viral infections,[4] less attention has been paid to its impact on early immunological events so far. Of note, up to 90% of infections are controlled by early, innate immune mechanisms, as suggested by high evolutionary conservation of the involved molecules.[24] Similarly, immunosurveillance mechanisms including the detection of highly conserved danger signals like heat-shock proteins are considered to allow for early recognition of cells in incipient malignant transformation.[25–29]

In the present study, we demonstrate that the early effects of IL-21 on B cells in the absence of CD40L differ substantially from its later effects in the presence of CD40L. We show that such early stimulation of B cells with IL-21 results in strong secretion of enzymatically active GzmB. While IL-21 is required for the observed effects, BCR engagement has a strongly supportive role, as confirmed by three independent findings. First, only antibodies (or F(ab')$_2$) directed against the BCR, but not control antibodies, enhanced IL-21-induced GzmB in B cells. Second, GzmB induction in B cells could be efficiently suppressed using a pharmacological inhibitor of the BCR-dependent kinase SYK. Finally, *ex vivo* viral re-stimulation induced a strong enhancement of IL-21-induced GzmB in B cells from correspondingly vaccinated, but not from unvaccinated donors. Our data, therefore, suggest that the type of cytotoxic B-cell response revealed in this study combines early and rapid onset with antigen specificity.

Although IL-21 and IL-2 belong to the same family of cytokines, their secretion is differentially regulated. Efficient induction of IL-2 requires T-cell activation by two signals, specifically, TCR engagement along with a co-stimulatory signal such as CD28 engagement. In contrast, induction of IL-21 occurs already in the presence of a single calcium signal[30,31] and can be achieved by mere crosslinking of the TCR in the absence of co-stimulation,[1,30] as shown in this study for CD4$^+$ T cells with a phenotype that is best compatible with that of memory type T cells (CD45RO$^+$CXCR5$^+$CD45RA$^-$). In addition to these findings, we demonstrated that incompletely (anti-CD3 stimulation only), but not fully (anti-CD3/CD28) activated CD4$^+$ T cells were

able to induce substantial levels of GzmB in co-cultured B cells in an IL-21R-dependent manner. It is known that the establishment of an efficient and antigen-specific T-cell response requires the continuous presence of a broad variety of pre-activated T cells (that is, T cells stimulated via their TCR only). On the other hand, antigen-specific T-cell responses toward foreign antigens are often initiated and supported by the recognition of early exposed self-antigens.[32–34] A situation, in which IL-21 is secreted in the absence of CD40L expression, is therefore conceivable during primary immune responses, when early exposed self-antigens are crosslinking the TCRs of a variety of low-affinity memory type T cells, at a time when antigen specific and fully activated T cells are not yet present at the site of inflammation. Our view is supported by two very recent publications showing that in freshly infected viremic HIV patients $CD4^+$ T cells are induced, that express both IL-21 and the follicular helper T-cell marker CXCR5.[35,36] Most likely these cells are important contributors to viral control in these patients, and could therefore have a crucial role for the orchestration of cytotoxic responses *in vivo*.

During the classical granule exocytosis pathway of cytotoxic lymphocytes, GzmB secretion is accompanied by perforin secretion (Figure 6b). Perforin can directly induce cell lysis *in vitro* at high (lytic) concentrations.[37] However, the induction of cell lysis *in vivo* may pose a considerable risk for the development of autoimmunity due to the uncontrolled release of auto-antigens.[37,38] Therefore, cell lysis is preceded by a controlled degradation of cellular components such as self-proteins[39] and DNA[40] by proteases including GzmB, which normally prevents extensive release of auto-antigens. Although the endosomal escape of GzmB can be supported by perforin,[40] it is widely accepted that GzmB uptake into target cells occurs independently of perforin[38,41] and its presence is not a *sine qua non* condition for GzmB to reach the target cell cytosol and to be functional. Microbial proteins like pore-forming bacterial lysins[42,43] or viral delivery proteins[44,45] as well as stress-related molecules such as heat-shock proteins expressed by tumor cells[46] can support GzmB internalization and action in the absence of perforin (Figure 6a). Therefore, the inability of GzmB-secreting B cells to co-secrete perforin may ensure that cytotoxic B-cell responses are limited to cells that are clearly associated with microbial infections or early neoplastic differentiation.

Although less known, an additional function of GzmB in the absence of perforin can be the suppression of T cells. It was previously demonstrated that regulatory T cells suppress effector T-cell function

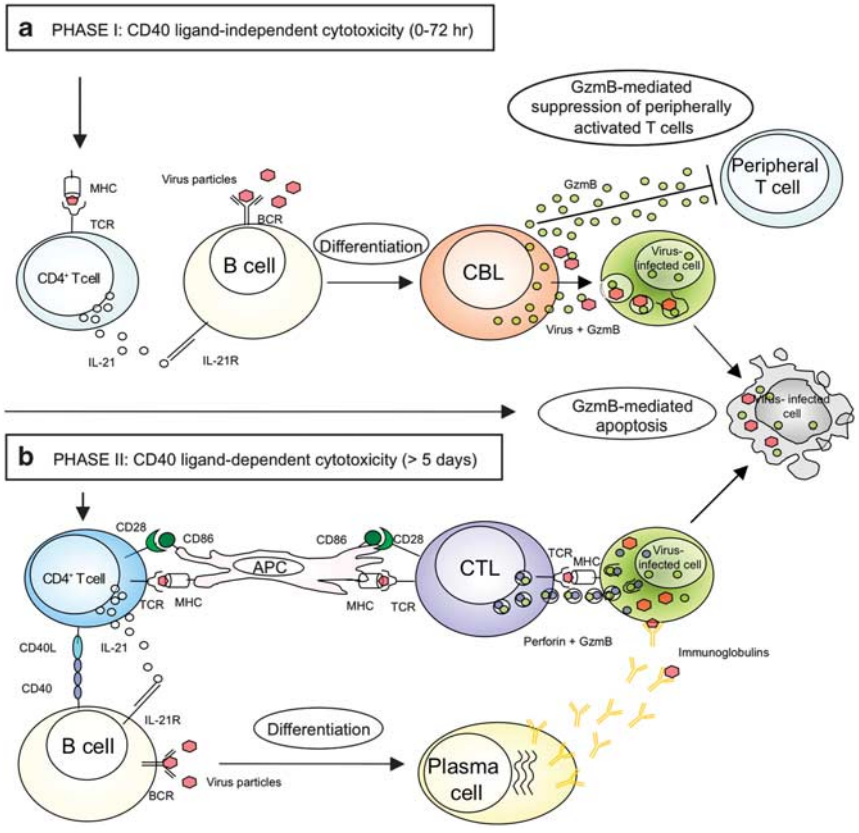


Figure 6 Model for a biphasic cytotoxic antiviral immune response involving B cells and T cells. (**a**) In the early phase (phase I) of a viral infection (0–72 h), a broad spectrum of low-affinity $CD4^+$ T cells are present that are in a *pre-activated* state secreting IL-21, but not CD40 ligand. B cells are activated by recognizing antigen in an MHC-*independent* manner. $CD4^+$ T cell-derived IL-21 in the absence of CD40 ligation may trigger differentiation of these activated B cells into GzmB-secreting CBL. Using various routes such as the endosomal infection pathway engaged by a series of viruses, GzmB may reach the target cell cytoplasm, where it may then induce apoptosis, thereby slowing down virus replication. At the same time, premature activation of non-specific peripheral T cells may be suppressed by extracellularly secreted GzmB to prevent the development of cellular autoimmune responses. (**b**) In a later phase (phase II) of viral infection (>5 days), after co-stimulation by professional APC, antigen-specific T cells arrive at the site of infection in a *fully activated* state. $CD4^+$ T cells now express both IL-21 and CD40 ligand, thereby abrogating GzmB secretion and triggering differentiation of activated B cells into plasma cells that secrete antibodies against viral antigens. Simultaneously, antigen-specific $CD8^+$ T cells recognize antigens in an MHC-*restricted* manner and induce target cell apoptosis using the classical granule exocytosis pathway.

in a GzmB-dependent, yet perforin-independent way.[47] Similarly, we could recently show that pDC are potent producers of GzmB, but not perforin, and that they can efficiently suppress T-cell proliferation in a GzmB-dependent manner as well.[48] Finally, and in line with these both observations, we have very recently found that GzmB-secreting B cells are also able to suppress T-cell proliferation (manuscript in preparation). It is, therefore, conceivable that in addition to their cytotoxic effects, GzmB-secreting B cells may also prevent premature activation of non-specific peripheral T cells to impede the development of autoimmune processes until antigen-specific and fully activated T cells from the draining lymph nodes have arrived and replaced non-antigen-specific T cells at the site of inflammation.

B cells are able to directly recognize antigen-bearing cells without the need for antigen presentation by professional antigen-presenting cell (APC). In being the first to recognize cells in incipient malignant transformation in an antigen-dependent manner, they may substantially contribute to cancer immunosurveillance. As a proof of principle, we have shown in this study that ARH-77, a B-cell line with high potential for GzmB secretion, and normal B cells from healthy subjects, but not the GzmB$^-$ B-cell line Raji, are able to induce apoptosis in sensitive cancer cell lines including HeLa and G-361. Interestingly, the CML-derived cell line K-562 appeared to be resistant to ARH-77 cell-induced apoptosis. Since K-562 cells are classically used as targets in standard ^{51}Cr release assays to demonstrate NK cell-mediated perforin-dependent lysis *in vitro*, the lack of sensitivity of K-562 cells to ARH-77 cell-induced cytotoxicity may, therefore, be due to the absence of perforin in IL-21-activated B cells. These results complement previous findings suggesting that rapid and efficient cytotoxic responses to various tumor types may require distinct subsets of cytotoxic effector cells and molecules.[16–18]

In conclusion, we provide evidence that activation of human B cells by IL-21 and BCR engagement in the absence of CD40 ligation results in their differentiation into GzmB-secreting cytotoxic cells rather than into plasma cells. Consequently, we could demonstrate that incompletely, but not fully activated CD4$^+$ T cells induce GzmB in B cells. B cell-derived GzmB was enzymatically active and GzmB-secreting B cells were able to deliver GzmB and to induce apoptosis in sensitive tumor cell lines. GzmB secretion by B cells may, therefore, contribute to cancer immunosurveillance particularly in the early phase of tumor cell differentiation and to rapid cellular immune responses at the beginning of viral infections. Our findings provide insights into a novel cytotoxic immune response mechanism and may stimulate the development of innovative immunotherapeutic approaches based on cytotoxic B lymphocytes.

METHODS

Healthy donors, cell lines and cell culture

Peripheral blood from healthy donors was collected after obtaining informed consent. For some experiments, blood from healthy individuals before and 2 weeks after vaccination against TBEV, rabies or hepatitis B was used. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, and CD19$^+$ B cells (>99% purity), total CD4$^+$ T cells, memory CD4$^+$ T cells or naive CD4$^+$ T cells (>95% purity for T cells) were magnetically purified using appropriate isolation kits from Miltenyi Biotec (Bergisch Gladbach, Germany). Cells were suspended in AIM-V medium (Gibco BRL, Grand Island, NY, USA) and incubated on round-bottom 96-well plates (1×10^6 cells per ml, 200 µl per well, if not stated otherwise). The following cell lines were used: Raji, Namalwa, IM-9, ARH-77, HeLa, and K-562 cultured in RPMI medium (PAA, Pasching, Austria). MEC-1 was cultured in DMEM medium (Gibco BRL). G361 was cultured in McCoy's 5A medium (Gibco BRL). For B-cell/T-cell co-culture experiments, purified CD4$^+$ T cells were added to purified CD19$^+$ B cells at a ratio of 10:1 and were incubated on round-bottom 96-well plates for 1 day. For B-cell/cancer cell co-culture experiments, 2.5×10^5 effector cells were added to 1×10^4 target cells in a final volume of 200 µl on a round-bottom 96-well plate for 3 days.

Reagents for functional assays

Affinity purified rabbit F(ab')$_2$ against human IgA+IgG+IgM (H+L) was used at 6.5 µg ml^{-1} as polyclonal BCR stimulus (anti-BCR, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Human recombinant IL-21 (50 ng ml^{-1}) was purchased from BioSource (Camarillo, CA, USA). Human recombinant IL-2 and IL-4 (both at 500 IU ml^{-1}) were purchased from PeproTech GmbH (Hamburg, Germany). The SYK inhibitor used in this study (BAY 61-3606, Merck, Darmstadt, Germany) is selective for SYK (IC_{50}=10 nM) with no inhibitory effects against Btk, Fyn, Itk or Lyn. CD40L was used at 100 ng ml^{-1} along with an enhancer (Axxora Deutschland GmbH, Lörrach, Germany). TBEV antigens were used as inactivated TBEV (standard concentration 10 ng ml^{-1}, strain Neudörfl) adsorbed to 0.35 mg Al(OH)$_3$ (FSME-IMMUN Erwachsene, Baxter, Heidelberg, Germany). Pure Al(OH)$_3$ was purchased from Sigma-Aldrich (standard concentration 1.5 µg ml^{-1}, Munich, Germany). Rabies antigens were used as inactivated rabies virus (standard concentration 0.5 mIU ml^{-1}, strain WISTAR PM/WI 38-1503-3M, Sanofi Pasteur MSD, Leimen, Germany). Hepatitis B antigens were used as 20 µg surface antigen purified from recombinant *Saccharomyces cerevisiae* and adsorbed to 0.35 mg Al(OH)$_3$ (standard concentration 100 ng ml^{-1}, Engerix-B Erwachsene, GlaxoSmithKline, Munich, Germany). PHA (Sigma-Aldrich, Schnelldorf, Germany) was used at 10 µg ml^{-1}. For TCR stimulation, 0.5 µg ml^{-1} of a CD3-stimulating antibody (OKT-3, Orthoclone, Janssen-Cilag, Neuss, Germany) was used according to the manufacturer's instructions. For TCR stimulation in the presence of co-stimulation, we used anti-CD3/CD28 antibody-coated beads (0.25 µl per 200 µl per well) from Dynal (Invitrogen GmbH, Karlsruhe, Germany). For inhibition of IL-21 binding to its receptor, an azide-free polyclonal goat anti-human IL-21 receptor subunit antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany) was used.

Flow cytometry

For surface marker staining, cells were harvested at the times indicated, washed in phosphate-buffered saline and stained as described previously.[49–51] FITC-, PE-, PE-Cy5-, PE-Cy7- or allophycocyanin-labeled antibodies to CD19, CD20, CD27, CD38, CD40, CD45RA, CD45RO, CD54, CD69, CD86, CD154, IgD, MHC I, MHC II and CD185 (CXCR5) were purchased from Becton Dickinson (BD) Biosciences (Heidelberg, Germany). PE-labeled antibodies to human IL-21 and FITC-labeled antibodies to human IL-17 were purchased from eBiosciences (Hatfield, UK). Functionality of the anti-IL-17 antibody was confirmed using CD4$^+$ T cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (both from Sigma-Aldrich). PE- and APC-labeled antibodies to human GzmB (clone GB12) and appropriate isotype controls were purchased from Invitrogen (Carlsbad, CA, USA). Intracellular GzmB and IL-21 were detected as described recently.[21] For detection of apoptosis, cells were stained with Annexin V (BD Biosciences) for 15 min at room temperature. Propidium iodide at 1 µg ml^{-1} was added before FACS analysis. For tracking of target cells in effector/target cell co-cultures, cells were stained with PKH26 (Sigma-Aldrich, St Louis, MO, USA) as described previously.[9]

Immunofluorescence microscopy

B cell-enriched peripheral blood mononuclear cells (20% B cells) from healthy subjects were cultured overnight on 96-well plates (5×10^6 cells per ml, 200 µl per well) in the presence of IL-21 and anti-BCR. Brefeldin A (1 µg ml^{-1}) was added, and cells cultured for four more hours. Subsequently, cells were pelleted and embedded in paraffin. Immunocytochemistry was performed on paraffin sections of pelleted cells using primary antibodies against GzmB and CD19 (DAKO Cytomation, Glostrup, Denmark) and Cy2- or Cy3-conjugated secondary antibodies specific for IgG_{2a} or IgG_1, respectively (Jackson ImmunoResearch Laboratories). Cells were examined using an Axioscope 2 fluorescence microscope (Zeiss, Göttingen, Germany).

Real-time RT-PCR for IL-21

In all, 4×10^6 CD4$^+$ T cells or, alternatively, 4×10^6 naive or 4×10^6 memory CD4$^+$ T cells were isolated from three healthy subjects and activated as

described above. After digestion with DNAse, total RNA was obtained using the RNeasy mini kit (both from Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II, oligo-dT primer (both from Invitrogen, Darmstadt, Germany) and xU RNAse Inhibitor (ABI, Darmstadt, Germany) in a GeneAmp PCR System 9700 (ABI, Darmstadt, Germany). Real-time quantitative PCR was performed using the 7300 Real-Time PCR System from Applied Biosystems (Darmstadt, Germany) in combination with the QuantiFast SYBR Green PCR Kit (Qiagen). Validated QuantiTect Primer Assays for the housekeeping gene TBP (TATA box-binding protein) (QT00000721) and IL-21 (QT00038612) were also purchased from Qiagen. All analyses were performed in duplicate and mean values were used for further calculations. The mRNA expression of IL-21 was normalized to TBP mRNA expression (endogenous control). The relative expression levels were calculated as $2^{(-\Delta\Delta Ct)}$. Four microliters of the reaction products from one representative donor was run on a 2% agarose gel along with a 50-bp ladder.

GzmB ELISpot

A human GrB ELISpot kit was purchased from Cell Sciences (Canton, MA, USA) and PVDF-bottomed 96-well plates from Millipore (Bedford, MA, USA). Cells were plated in AIM-V medium at 1×10^5 per 100 µl per well. Plates were analyzed on an Immunospot Series 1 Analyzer and counted using Immunospot 3 software (CTL Cellular Technology Ltd., Cleveland, OH, USA).

GzmB activity assay

For demonstration of enzymatic activity of secreted GzmB in the supernatants of stimulated B cells and non-B cells, we used a highly specific GzmB activity assay (SensiZyme, Sigma-Aldrich, Munich, Germany). Briefly, GzmB from supernatants was captured on a 96-well plate by an anti-GzmB antibody-based precipitation step, followed by washing with phosphate-buffered saline. Then, substrate A (a proenzyme containing the GzmB cleavage site), along with substrate B (a chromogenic substrate for active substrate A) were added to the plate and the accumulating chromogenic product was detected in a Dynatech MR 7000 ELISA reader (Dynatech, Deckendorf, Germany) at 405 nm after 16 h incubation.

Spinning disk confocal microscopy

ARH-77 cells were cultured for 16 h in the presence of IL-21 (50 ng ml^{-1}). Brefeldin A (1 µg ml^{-1}) was added for 4 h and cells were harvested, stained in FACS tubes with Cell Mask deep red membrane stain (Invitrogen, Paisley, UK) at 5 µg ml^{-1} for 15 min at 37 °C and incubated for 1 h with 25 µl GranToxiLux fluorogenic GzmB substrate in 75 µl AIM-V medium at room temperature (OncoImmunin Inc., Gaithersburg, MD, USA). For total GzmB staining, ARH-77 cells were fixed, permeabilized, blocked with FcR blocking reagent (Miltenyi Biotec), and stained with anti-GzmB biotin (Abcam, Cambridge, UK) for 15 min at room temperature. After washing, cells were resuspended in 100 µl permeabilization buffer and stained for 15 min with streptavidin labeled with Alexa Fluor 488 (Invitrogen, Paisley, UK). Finally, cells were resuspended in 200 µl phosphate-buffered saline and placed on 8-well Lab-Tek Permanox chamber slides (Nalge Nunc International, Naperville, IL, USA) coated with fetal calf serum. For ARH-77/HeLa cell co-culture experiments, ARH-77 cells were pre-incubated for the indicated periods of time in the presence of IL-21 (50 ng ml^{-1}). ARH-77 cells were stained with anti-CD19 PerCP (BD Biosciences). At the indicated time points, 2.5×10^4 HeLa cells were plated on ibiTreat chamber slides (Integrated BioDiagnostics GmbH, Munich, Germany), stained with Cell Mask deep red membrane dye and washed with phosphate-buffered saline. Finally, ARH-77 cells and 50 µl GranToxiLux fluorogenic GzmB substrate were added to the chamber slides. Fluorescence images were acquired using an inverted microscope (Axio Observer, Zeiss) with oil immersion objective (UPlanSApo ×60/1.35, Olympus, Hamburg, Germany), environmental control (PECON, Erbach, Germany), an image splitting unit (OptoSplit II, Cairn Research, Faversham, UK) and an EMCCD camera (DV-887, Andor, Belfast, UK). ImageJ software (NIH, Bethesda, MD, USA) was used for subsequent image processing.

Statistics

Data are expressed as mean values ± s.e.m. To determine statistical differences between the means of two data columns, the paired Student's *t*-test was used as appropriate. A *P*-value of <0.05 was considered statistically significant. *P*-values were corrected using the Bonferroni method where applicable.

ACKNOWLEDGEMENTS

This work received support through grant JA 1769/2-2 from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), through grant SP 07/10 from the Deutsche Jose Carreras Leukämiestiftung and through grant D/08/11870 from the Deutscher Akademischer Austauschdienst (DAAD, German Academic Exchange Service) to B Jahrsdörfer. We wish to thank HR Rodewald from the Department of Immunology for permission to use his 4-color flow cytometer and B Böhm from the Department of Medicine II for permission to use his ELISpot reader (both at Ulm University).

Author contributions: BJ generated the hypothesis and designed the research; MH, KS, KD, SB, CK, TB, SH, OL and KT performed experiments; MH, KS, KD, SB, CK, TB, SH, OL, KT, TFEB and BJ conducted data analyses; TFEB, DF, GUN, TS and HS provided key research tools; MH, KS, KD, SB, CK, TB, SH, OL and BJ prepared the figures; MH and BJ wrote the manuscript.

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)