Interleukin 21–Induced Granzyme B–Expressing B Cells Infiltrate Tumors and Regulate T Cells

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Abstract
The pathogenic impact of tumor-infiltrating B cells is unresolved at present, however, some studies suggest that they may have immune regulatory potential. Here, we report that the microenvironment of various solid tumors includes B cells that express granzyme B (GrB, GZMB), where these B cells can be found adjacent to interleukin (IL)-21–secreting regulatory T cells (Treg) that contribute to immune tolerance of tumor antigens. Because Tregs and plasmacytoid dendritic cells are known to modulate T-effector cells by a GrB-dependent mechanism, we hypothesized that a similar process may operate to modulate regulatory B cells (Breg). IL-21 induced outgrowth of B cells expressing high levels of GrB, which thereby limited T-cell proliferation by a GrB-dependent degradation of the T-cell receptor ζ-chain. Mechanistic investigations into how IL-21 induced GrB expression in B cells to confer Breg function revealed a CD19⁺CD38⁺CD1d⁻IgM⁺CD147⁺ expression signature, along with expression of additional key regulatory molecules including IL-10, CD25, and indoleamine-2,3-dioxygenase. Notably, induction of GrB by IL-21 integrated signals mediated by surface immunoglobulin M (B-cell receptor) and Toll-like receptors, each of which were enhanced with expression of the B-cell marker CD5. Our findings show for the first time that IL-21 induces GrB⁺ human Bregs. They also establish the existence of human B cells with a regulatory phenotype in solid tumor infiltrates, where they may contribute to the suppression of antitumor immune responses. Together, these findings may stimulate novel diagnostic and cell therapeutic approaches to better manage human cancer as well as autoimmune and graft-versus-host pathologies. Cancer Res; 73(8); 2468–79. ©2013 AACR.

Introduction
It is widely recognized that certain B-cell subpopulations exhibit potent regulatory properties and are involved in immune pathologies including autoimmune and malignant diseases (1, 2). In murine autoimmune models, adoptive B-cell transfer can ameliorate chronic inflammatory responses even when the disease is already established (1, 3). Similarly, in human patients with rheumatoid arthritis, disease activity is lower in patients with high peripheral B-cell counts (4). One of the factors produced by regulatory B cells (Breg) is the immunosuppressive cytokine interleukin (IL)-10, which has recently been identified in mouse models (1, 5–7) and humans (1, 2, 8, 9). Nevertheless, although some clinical observations clearly support the concept of human B cells contributing to the limitation of autoimmune diseases, in other cases they are known to have rather aggravating potential (10). To better understand the development of regulatory versus pathogenic B cells, a more comprehensive knowledge of factors inducing and mediating Breg functions is necessary.

A previously discovered cytokine with pleiotropic effects on a variety of immune cells is IL-21 (11). The effects of IL-21 on B cells strongly depend on additional signals including Toll-like receptors (TLR) agonists, B-cell receptor (BCR) stimulation and CD40 ligation (12). Recently, CD40 ligand (CD40L) was shown to determine whether IL-21 induces differentiation of B cells into plasma cells (13, 14), or, in its absence, into B cells secreting the serine protease granzyme B (GrB; refs. 15, 16).
Importantly, GrB is characterized not only by its classical function as cytotoxic protease, but also exhibits immunosuppressive properties such as in regulatory T cells (Treg refs. 17–19) and dendritic cells (20). In line with these findings, IL-21 has been described to mediate expression of IL-10 by murine cytotoxic cells and B cells (21, 22), supporting its involvement in immune regulation.

Several inflammatory conditions including systemic lupus erythematosus (SLE) and acute viral infections are characterized by both elevated GrB (23, 24) and IL-21 serum levels (15, 25, 26). Moreover, we recently showed that GrB+ B cells can be found in patients with SLE (15) and in subjects vaccinated against viral infections (16). An association of GrB+ B cells with solid tumors has not been reported so far. However, while infiltration of certain tumors with B cells can be associated with a good prognosis (27–30), in others the presence of B cells support a tumor-protective environment (31–33). We therefore hypothesized that GrB-expressing B cells may phenotypically represent Breg and may be present in the microenvironment of human tumors.

In the current work, we show that IL-21 induces a regulatory phenotype in human B cells with expression of immunoregulatory molecules including GrB, IL-10, indoleamine-2,3-dioxygenase (IDO), and CD25. Moreover, we show that GrB+ B cells suppress T-cell proliferation by GrB-dependent degradation of the T-cell receptor (TCR) ζ-chain, a known GrB substrate (34). Importantly, we show that GrB+ B cells as well as IL-21–providing T cells are present in the tumor microenvironment of certain solid tumors. Of note, CD5+ B cells from human cord blood possess an enhanced capacity to express GrB, supporting the concept that CD5+ B cells may play a particular role in immune regulation, as suggested by various mouse models (5–7).

In summary, our findings suggest IL-21 is a key cytokine for the generation of human Bregs under certain environmental contexts, and GrB represents a central immunomodulatory molecule expressed by such Breg. GrB+ Breg may play a so far unappreciated role in human pathologies by infiltration of tumors or by modulation of inflammatory processes. Our study also suggests IL-21–induced Breg may prove useful as innovative cell-therapeutic tool for the management of undesired immune activation in autoimmune diseases and GVHD.

Materials and Methods

Human subjects and cell culture

The use of blood from human subjects was approved by the Ethics Committee at Ulm University (Ulm, Germany). Blood samples were collected after informed consent had been given. Peripheral blood mononuclear cells (PBMC) or mononuclear cells from umbilical cord blood (cord blood mononuclear cells) were isolated by Ficoll density gradient centrifugation. CD19+ B cells (>99% purity) and CD4+ T cells (>95% purity) were magnetically purified using appropriate negative selection kits according to the manufacturer’s protocol (Miltenyi Biotec). Cells were suspended in AIM-V medium (Gibco BRL) and incubated on U-bottom 96-well plates at 1 × 10^5 cells/mL and 200 μL/well, if not stated otherwise. For B-cell/T-cell coculture experiments, CD4+ T cells were added to CD19+ B cells at a 1:1 ratio and incubated for the time indicated. Reagents used for functional assays are outlined in the article Supplementary Data.

Reagents for functional assays

For BCR stimulation, affinity purified rabbit F(ab’)2 against human IgA+IgG+IgM (H+L) was used at 6.5 μg/mL (anti-BCR, Jackson ImmunoResearch Laboratories). Human recombinant IL-21 (50 ng/mL) was purchased from BioSource. Human recombinant IL-2 (100 IU/mL) was obtained from PeproTech GmbH. CpG ODN 2243 was obtained from Coley Pharmaceutical Group. IRS 661 and IRS 869 were purchased from Bioner. Cycloheximide and brefeldin A (both at 1 μg/mL) were from Sigma-Aldrich. Tick-borne encephalitis virus (TBEV) antigens were used as inactivated TBEV vaccine (standard concentration 100 ng/mL, strain Neudoerfl) adsorbed to 0.35 mg Al(OH)3 (FSME-IMMUN Erwachsene; Baxter). For TCR stimulation, anti-CD3/CD28 antibody-coated beads (0.02 μL/200 μL-well) from Dynal (Invitrogen) were used. For GrB inhibition, GrB inhibitor IV (Ac-IEPD-CHO) at 5 μmol/L (American Peptide Company) or a carrier- and preservative-free goat anti-human GrB polyclonal antibody [immunoglobulin G (IgG)] at 10 μg/mL (R&D Systems) was used.

Flow cytometry

Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy5-, PE-Cy7-, or allophycocyanine (APC)-labeled antibodies to CD1d, CD3, CD4, CD5, CD19, CD20, CD25, CD27, CD38, CD58, CD69, CD70, CD86, CD107a, IgD, and IgM were purchased from BD Biosciences. FITC-labeled antibodies to CD10 and PE-labeled antibodies to CD24 were purchased from EXBIO, FITC-labeled antibodies to CD58, CD147, Annexin V, and PE-labeled antibodies to IL-10 from Immunotools. FITC-labeled antibodies to CD154 from BioLegend, Alexa Fluor 488–labeled antibodies to human IDO from R&D Systems, carboxyfluorescein succinimidyl ester (CFSE) from Sigma-Aldrich, and PE- and APC-labeled antibodies to human GrB (clone GB12) from Invitrogen. Appropriate isotype controls were used. For intracellular GrB, CD10, and IDO staining, cells were incubated with brefeldin A (1 μg/mL) during the last 4 hours of incubation and analyzed as recently described (16). For apoptosis detection, cells were stained with Annexin V for 15 minutes at room temperature. Propidium iodide (PI; 1 mg/mL) was added and samples immediately analyzed by fluorescence-activated cell sorting (FACS). Flow-cytometric analyses were conducted on a FACScan or a FACS-Calibur (BD Immunocytometry Systems). Data were analyzed using FlowJo (version 9.3.1; Tree Star).

CFSE staining and proliferation assay

For proliferation experiments, 1 × 10^5 cells purified CD4+ T cells were resuspended in 10 mL PBS containing 0.1% bovine serum albumin (PBS/BSA). CFSE was added to a final concentration of 1 μmol/L and cells were incubated at 37°C for 10 minutes. Incubation was stopped by adding 40 mL ice-cold culture medium and suspension was incubated for 5 minutes
on ice before 3 additional washing steps with PBS/BSA. Then, CD4+ T cells were cocultured for 6 days with autologous or allogeneic purified B cells in the presence of anti-CD3/28 beads, IL-2, IL-21, anti-BCR, GrB inhibitors, or combinations of these as indicated. After 3 days of culture, 100 μL medium was exchanged.

**GrB ELISpot**

Human GrB ELISpot kits were purchased from Gene-Probe Di clone SAS and polystyrylde difluoride (PVDF)-bottomed 96-well plates from Millipore. ELISpot assays were conducted as recently described (15). Briefly, cells were plated in AIM-V medium at 1 × 10⁶ per 100 μL per well for 16 hours. Then, plates were developed, read on an Immunospot Series 1 Analyzer and spots counted using Immunospot 3 software (CTL Cellular Technology Ltd.).

**ELISA for enzymatically active GrB**

For the detection of enzymatically active, secreted GrB in the supernatants of stimulated B cells, we used a highly specific GrB activity assay according to the manufacturer’s protocol (SensiZyme; Sigma-Aldrich).

**Spinning disk confocal microscopy**

Purified CD19+ B cells were cultured for 16 hours in the presence of IL-21 and anti-BCR. Purified CD4+ T cells at 1 × 10⁶ cells/mL were cultured separately in the presence of anti-CD3/28 beads. Then, 1 × 10⁶ T cells were harvested, put on ibiTreat chamber slides (ibidi GmbH) for 30 minutes for immobilization, stained with Cell Mask deep red membrane dye (Invitrogen) at 5 μg/mL for 15 minutes at 37°C and washed 3 times with PBS. A total of 1 × 10⁶ B cells in 50 μL of PBS and 25 μL of GranToxiLUX fluorogenic GrB substrate (OncoImmun) were added. Fluorescence images were acquired using a spinning disk confocal microscope and the acquisition software Andor iQ I.6 as described previously (20).

**Western immunoblotting**

Purified B cells (>99%) from healthy individuals were stimulated with anti-BCR ± IL-21 overnight. Autologous purified CD4+ T cells (>98%) were simultaneously stimulated with anti-CD3/28 beads in a separate culture. After removal of beads and treatment with 1 μg/mL cycloheximide, CD4+ T cells were cocultured at a 1:1 ratio with prestimulated B cells for 24 hours in the presence or absence of anti-GrB antibodies. Then, cells were resuspended in lysis buffer (10 mmol/L HEPES, 10 mmol/L NaCl, 1 mmol/L KH₂PO₄, 5 mmol/L NaHCO₃, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 5 mmol/L EDTA in 1 mM) containing a protease inhibitor (protease inhibitor cocktail set III; Calbiochem) for 15 minutes on ice. Protein content was measured using Pierce BCA colorimetric protein assay kit (Thermo Scientific). Then, proteins were separated on 18% SDS-PAGE and transferred onto 0.45-μm PVDF membranes. After washing and blocking, membranes were incubated with primary monoclonal antibodies against human TCR-ζ (16 hours, 4°C; Biologend) or β-actin (30 minutes, room temperature; Thermo Scientific). Then, membranes were washed 3 times and incubated with a horseradish peroxidase–conjugated secondary antibody (goat anti-mouse IgG; Santa Cruz Biotechnology) for 1 hour at room temperature. Finally, membranes were developed and band intensities quantified using ImageJ.

**Cytometric bead array**

For quantification of IL-2, supernatants from B cells and T cells cocultured at a 1:1 ratio for 3 days were analyzed using a BioPlex-9 Plex Cytokine Assay Kit (BioRad Laboratories) according to the manufacturer’s instructions. All incubations were conducted at room temperature. Briefly, the anti-cytokine bead solution was diluted and 50 μL were placed on 96-well Durapore membrane plates (Millipore). After washing by vacuum filtration, 50 μL of undiluted supernatants and standards were placed into wells and incubated for 30 minutes on a shaker. After washing, 25 μL detection antibody was added and incubated for another 30 minutes while shaking. After washing, streptavidin–PE was added for 10 minutes. Finally, plates were washed, resuspended in assay buffer and read on a flow cytometer (Luminex). Data were analyzed using Bio-Plex Manager software (Bio-Rad).

**Immunofluorescence microscopy**

Formalin-fixed, paraffin-embedded cancer tissue sections (1 μm) from various solid epithelial cancers including breast, cervical, ovarian, colorectal, and prostate carcinomas were obtained from the local department of pathology (Table 1). In compliance with the German law for correct usage of archival tissue for clinical research the blocks were anonymized. Double immunofluorescent staining of formalin-fixed, paraffin-embedded cancer tissue sections against GrB, IL-21, or IL-10 as first antigens and against CD19 or CD3 as second antigens was conducted as follows: tissue sections were deparaffinized in xylol and alcohol and antigens retrieved by 20-minute incubation in 10 mM citric acid (pH 6.0) in a pressure cooker. Then, sections were incubated with primary antibodies against GrB (mouse anti-human GrB; Dako Cytomation), IL-21 (rabbit anti-human IL-21; Acris Antibodies), or IL-10 (rabbit anti-human IL-10; Acris Antibodies) for 30 minutes in a moist chamber and rinsed with PBS. Subsequently, sections were incubated with secondary biotinylated antibodies (anti-mouse or anti-rabbit; Dako Cytomation) for 30 minutes. For GrB and IL-21 staining, this was followed by a 30-minute incubation with streptavidin–Alexa Fluor 488–conjugate (Molecular Probes), for IL-10 staining with a DyLight 488–conjugated anti-rabbit secondary antibody (Dianova). To enable double immunohistochemistry with antibodies from the same host as used for the first antigen, sections were placed on a heating plate for 4 minutes at 90°C in H₂O to denature open binding sites on the primary antibodies. Then, slides were incubated with primary mouse anti-human CD19 or CD3 antibodies (Dako Cytomation) for 30 minutes, followed by incubation with a Cy3-conjugated anti-mouse antibody (Dianova) for 30 minutes. Internal controls included exclusion of nonspecific binding of Cy3-conjugated secondary antibodies as well as premature denaturation of antibody binding sites by placing the heating step directly after binding of the primary anti-GrB and anti-IL-21 antibodies, respectively. In this case, no binding
of secondary biotinylated antibodies could be observed. For analysis, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), sections were mounted in Vectashield mounting medium (Vector Laboratories Inc.) and visualized using an Axioscope 2 fluorescence microscope (Zeiss).

Statistics
Data are expressed as mean ± SEM unless stated otherwise. Statistical differences between the means of 2 data columns were assessed using the unpaired and paired Student t test as appropriate. Results with P values less than 0.05 were considered statistically significant. P values were corrected using the Bonferroni method where applicable.

Results
Interleukin 21–activated B cells produce granzyme B and suppress CD4+ T-cell proliferation
Recently, we found that the IL-2 family cytokine IL-21 can trigger B cells to secrete the serine protease GrB (15, 16).

Table 1. Characteristics of screened tumor sections

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Characteristics</th>
<th>Age</th>
<th>Tumor stage</th>
<th>Tumor grade</th>
<th>(%_0/00) GrB⁺ CD19⁺ B cells</th>
<th>(%_0/00) IL-21⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary carcinoma</td>
<td>Highly differentiated, invasive and infiltrating growth</td>
<td>77</td>
<td>pT2, pN1a</td>
<td>2</td>
<td>10 ± 1</td>
<td>53 ± 19</td>
</tr>
<tr>
<td></td>
<td>Intermediately differentiated, ductal invasive growth</td>
<td>61</td>
<td>pT1b, pN0</td>
<td>2</td>
<td>19 ± 5</td>
<td>24 ± 4</td>
</tr>
<tr>
<td></td>
<td>Differentiated, invasive and infiltrating growth</td>
<td>78</td>
<td>pT2, pN1</td>
<td>2</td>
<td>27 ± 13</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>Poorly differentiated serous carcinoma</td>
<td>51</td>
<td>pT3c</td>
<td>1</td>
<td>13 ± 3</td>
<td>23 ± 0</td>
</tr>
<tr>
<td></td>
<td>Intermediately differentiated mucinous adenocarcinoma</td>
<td>39</td>
<td>pT1c</td>
<td>2</td>
<td>6 ± 1</td>
<td>57 ± 8</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated serous papillary carcinoma</td>
<td>71</td>
<td>pT3c</td>
<td>3</td>
<td>9 ± 2</td>
<td>53 ± 19</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>Poorly differentiated invasive cervical squamous cell carcinoma</td>
<td>42</td>
<td>pT2b</td>
<td>3</td>
<td>18 ± 7</td>
<td>43 ± 11</td>
</tr>
<tr>
<td></td>
<td>Intermediately differentiated cervical squamous cell carcinoma</td>
<td>70</td>
<td>pT2b</td>
<td>2</td>
<td>5 ± 1</td>
<td>35 ± 5</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated cervical squamous cell carcinoma</td>
<td>41</td>
<td>pT1b1</td>
<td>3</td>
<td>6 ± 2</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>Intermediately differentiated tubular adenocarcinoma</td>
<td>82</td>
<td>pT1pN0pMx</td>
<td>1-2</td>
<td>7 ± 2</td>
<td>35 ± 9</td>
</tr>
<tr>
<td></td>
<td>Mucinous adenocarcinoma, colon ascendens</td>
<td>79</td>
<td>pT3pN0pMx</td>
<td>3</td>
<td>0 ± 0</td>
<td>30 ± 12</td>
</tr>
<tr>
<td></td>
<td>Intermediately differentiated adenocarcinoma, infiltrating growth</td>
<td>78</td>
<td>pT3N0L0V0R0</td>
<td>3</td>
<td>10 ± 6</td>
<td>46 ± 14</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>Differentiated, tubular, cribriform and solid adenocarcinoma</td>
<td>73</td>
<td>pT2c</td>
<td>3±4 = 7³</td>
<td>5 ± 3</td>
<td>7 ± 4</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>57</td>
<td>pT3b</td>
<td>4±5 = 9³</td>
<td>5 ± 2</td>
<td>17 ± 4</td>
</tr>
<tr>
<td></td>
<td>Acinar carcinoma</td>
<td>64</td>
<td>pT2c</td>
<td>3±3 = 6³</td>
<td>4 ± 2</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

NOTE: Listed are clinical characteristics including tissue type, tumor characteristics, patient age, tumor stage and grade, as well as the frequency of CD19⁺ GrB⁺ cells and IL-21⁺ cells. Given is the average frequency per field with 60-fold magnification \(\%_0/00 ± SEM\).

aGleason score.
Because expression of GrB by B cells is not accompanied by perforin expression, they may exhibit perforin-independent functions (23), such as the immunosuppressive capacity of Treg (17–19). To test the hypothesis that GrB+ B cells may exhibit similar functions, we isolated B cells and preactivated them with IL-21 and BCR stimulation. IL-21 rapidly induced expression and secretion of enzymatically active GrB in a number of B cells, an effect strongly enhanced by simultaneous BCR stimulation (Fig. IA and B and Supplementary Fig. S1). We then isolated CD4+ T cells and induced proliferation using by activating CD3 and CD28 (CD3/CD28).

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Supplementary Fig. S2) or activated B cells. T-cell proliferation was strongly suppressed in the presence or absence of differentially activated B cells. T-cell proliferation was induced by anti-CD3/CD28 beads. After 6 days, cell cultures were harvested, stained for CD4, and analyzed by FACS. C–F, healthy PBMC were cultured for 48 hours in the presence of indicated reagents. Cells were harvested, stained for CD19, fixed, permeabilized, and stained for GrB. A, dot plots show GrB expression in CD19+ B cells from 1 representative experiment. Gates represent GrB+ B cells. B, bar graphs show mean percentages of GrB+ B cells from 5 individual experiments. C–F, healthy CD4+ T cells were stained with CFSE and cocultured with B cells at a 1:1 ratio in the presence of IL-2, IL-21, anti-BCR, and GrB inhibitors as indicated. T-cell proliferation was induced by anti-CD3/CD28 beads. After 6 days, cell cultures were harvested, stained for CD4, and analyzed by FACS. C, dot plots show CFSE-stained CD4+ T cells in the presence of autologous B cells prestimulated as indicated. Data are from 1 representative experiment out of 7 with similar results. Gated are proliferated T cells. D, bar graphs show mean percentages of proliferated T cells from 7 individual experiments. E, dot plots show CFSE-stained CD4+ T cells in the presence of autologous B cells prestimulated with anti-BCR ± IL-21 and GrB inhibitors as indicated. Proliferated T cells are gated. One representative experiment out of 3 with similar results is shown. F, bar graphs show mean percentages of proliferated T cells from 3 individual experiments.

**Inhibition of T-cell proliferation by B cells involves transfer of active GrB to T cells and degradation of the T-cell receptor ζ-chain but not induction of T-cell apoptosis**

Next, we characterized the underlying interactions between GrB+ B cells and T cells. First, we tested whether B cell–derived GrB is delivered to T cells in its active form. To this end, we preincubated B cells with or without IL-21 and anti-BCR.
Granzyme B⁺ Regulatory B Cells Infiltrate Tumors

Figure 2. B cells transfer active Grb to T cells and induce Grb-dependent degradation of the TCRζ-chain. A, healthy B cells were isolated and activated using IL-21 and anti-BCR overnight. In parallel, CD4⁺ T cells were isolated and separately cultured with anti-CD3/CD28 beads. Then, T cells were cultured with Cell Mask deep red membrane dye and cocultured with B cells on a chamber slide. Grb-specific fluorogenic substrate was added for cellular detection of active Grb and live cell imaging conducted for 6 hours. Shown are 9 video frames at different time points. Images are from 1 representative experiment out of 3 with similar results. B, purified B cells from healthy individuals were stimulated with anti-BCR ± IL-21 overnight. Autologous purified CD4⁺ T cells were simultaneously stimulated with anti-CD3/28 beads in a separate culture. After removal of beads and treatment with 1 μg/mL cycloheximide, CD4⁺ T cells were cocultured at a 1:1 ratio with prestimulated B cells for 24 hours in the presence or absence of anti-GrB antibodies. Then, cells were lysed, protein contents of samples equalized, and TCRζ expression analyzed. C, relative band intensities of TCRζ and β-actin were determined using ImageJ and ratios were calculated. Bar graphs show the ratios between TCRζ and β-actin band intensities.

and then started coincubating them with CD4⁺ T cells in the presence of a Grb-specific fluorogenic substrate on microscopy dishes. Spinning disk confocal microscopy showed that after 2 to 3 hours T cells began to internalize B cell–derived Grb into the cytoplasm (Fig. 2A and Supplementary Video S1). No active Grb was observed in T cells in the presence of unstimulated, Grb⁻ B cells (data not shown).

A potential impact of Grb on T cells may be induction of apoptosis by caspase cleavage. Nevertheless, using FACS analysis, we excluded that B cells stimulated in the presence of IL-21 ± anti-BCR were able to induce significant T-cell apoptosis (Supplementary Fig. S3A and S3B). A more recently identified Grb substrate is the ζ-chain of the TCR (TCRζ), which plays a role in delivering activation and growth signals to T cells (34). To test the impact of B cell–derived Grb on TCRζ, we coincubated CD3/CD28-activated CD4⁺ T cells in the presence or absence of B cells activated with IL-21 ± anti-BCR. B cells stimulated with IL-21 and anti-BCR, but not B cells stimulated with anti-BCR only, were able to strongly suppress TCRζ expression in coincubated CD4⁺ T cells (Fig. 2B and C). This effect was not seen in the absence of B cells (data not shown). More importantly, TCRζ expression in T cells was partly rescued by a Grb-neutralizing antibody (Fig. 2B and C).

Grb expression by IL-21–stimulated B cells is part of a regulatory B cell phenotype

As outlined earlier, we primarily described IL-21–induced Breg as cells able to express and secrete Grb (Fig. 1), a capacity they seem to share with Tregs (17–19) and regulatory dendritic cells (20). To define in more detail the phenotype of IL-21–induced Grb⁺ B cells, we tested a variety of surface antigens described in the past to characterize Breg (5, 6, 8, 9, 35). These antigens include activation markers, molecules of the immune superfamily, costimulatory molecules, enzymes, and adhesion molecules. We found significant upregulation of several of these molecules in IL-21–stimulated Grb⁺ B cells including CD38, CD1d, IgM, CD86, CD154, CD10, and CD20 (Fig. 3). CD70 showed a higher expression in Grb⁺ B cells as compared with unstimulated B cells, whereas CD24, CD27, and IgD exhibited no increase or even a decrease in Grb⁺ B cells (Fig. 3).
A frequently reported characteristic of Breg is the expression of IL-10, with such B cells often referred to as B10 cells (2, 5, 6). Here, we show that healthy peripheral B cells stimulated with IL-21/anti-BCR also responded with the development of a small but significant population of B cells expressing IL-10 (Fig. 4A and B). This population did not occur in the presence of IL-2 or in the absence of cytokines. Moreover, costimulation of B cells with IL-21/anti-BCR was able to induce IDO, another regulatory molecule previously described in dendritic cells (36). Again, this effect was small but significant, and most pronounced in the presence of IL-21, but not with IL-2 or without cytokines (Fig. 4C and D). Importantly, after stimulation of B cells with IL-21 and anti-BCR, GrB not only was the first regulatory molecule expressed (Fig. 1), but all additional regulatory molecules tested were primarily expressed on GrB+ but not on GrB−/CD5− B cells (Supplementary Fig. S4).

Because IL-21–activated GrB+ B cells seem to express characteristic Breg markers, we also tested Treg-specific antigens. Tregs typically express high levels of CD25 and it was hypothesized that part of their inhibitory effect is effector T-cell deprivation of IL-2 (37, 38). IL-21–stimulated B cells indeed significantly upregulated surface CD25 (Fig. 4E and F). Nevertheless, we were not able to rescue the proliferative response of cocultured T cells by retitrating recombinant IL-2 (data not shown). CD5+ B cells exhibit an enhanced potential to express GrB as compared with CD5− B cells

CD5 is another marker described on murine Breg (5, 6), although it is not expressed on IL-21–induced GrB+ peripheral B cells (data not shown). Recently, however, we found that CD5+ B cells from patients with SLE constitutively express GrB, in contrast to CD5− B cells from normal healthy donors (15). To directly compare the GrB potential of CD5+ versus CD5− B cells, we isolated B cells from cord blood samples and from adult healthy donors. We found that the GrB response of CD5+ B cells to IL-21 was more than 2-fold stronger than the response of CD5− B cells from healthy adult donors (Fig. 5A–C). Moreover, we previously showed that B cells from healthy volunteers vaccinated against viral infections such as TBEV respond with stronger GrB expression to IL-21 stimulation than B cells from unvaccinated donors (16). Therefore, we compared the GrB response of CD5+ and CD5− B cells from normal donors to IL-21 in the presence of TBEV vaccines instead of anti-BCR. Here, the GrB response of CD5− B cells was up to 10-fold stronger (Fig. 5D and E), suggesting an innate potential of CD5+ B cells to express GrB in the presence of danger signals such as viral stimuli.

IL-21–induced GrB expression by B cells depends on both BCR and TLR signaling pathways

IL-21–induced GrB expression by peripheral B cells is strongly enhanced in the presence of BCR stimulation, an effect suppressed by SYK inhibition (16). However, the induction of GrB in antigen-unexperienced CD5+ B cells by viral antigens (Fig. 5D and E) suggests the additional involvement of TLR such as TLR7 or TLR9. Indeed, we found that inhibition of both TLR7 and TLR9 using specific inhibitory...
ODN (40) suppressed IL-21–induced GrB expression in B cells in a dose-dependent manner. This effect was found in both CD5− and CD5+ B cells (Supplementary Fig. S6).

**GrB**-expressing B cells and IL-21–expressing T cells reside in the microenvironment of various solid tumors

Although the regulation of immunity is important, its suppression may be undesired when the establishment of a robust immune response, for example against a tumor, is required. To evaluate whether B cells with a regulatory phenotype could play a role in the modulation of antitumor immune responses, we screened a variety of tumor tissue sections for GrB− B cells. We found that GrB-expressing B cells reside within the microenvironment of different tumor types including breast, ovarian, cervical, colorectal, and prostate carcinomas (Fig. 6A–F and Table 1). Because we identified IL-21 as key cytokine for differentiation of B cells into GrB− B cells, we tested whether IL-21–expressing cells are also present in the tumor tissue. As expected, IL-21–expressing CD3+ T cells could be identified in the vicinity of B cells in these tissue sections (Fig. 6G and H). Importantly, we also detected IL-10–expressing CD19− B cells in the same tumor tissues that contained GrB− B cells, suggesting that B cells with a regulatory phenotype in this tumor microenvironment may express both IL-10 and GrB (Supplementary Fig. S7). Nonetheless, the frequency of IL-10− B
cells in tumor tissue was significantly lower than the frequency of GrB+ B cells, which is in line with our in vitro data showing that the predominant regulatory molecule expressed by IL-21–induced human Breg is GrB (Supplementary Fig. S4).

Discussion

B cell–mediated immune regulation seems to be a fundamental property of the immune system (1, 2). In the present study, we identified IL-21 as key cytokine for the induction of human Bregs. Their phenotype includes surface markers such as CD1d, CD38, IgM, CD10, CD86, and CD154 (5, 6, 8, 9, 35) but also molecules with actual regulatory functions including GrB as CD1d, CD38, IgM, CD10, CD86, and CD154 (5, 6, 8, 9, 35) but human Bregs. Their phenotype includes surface markers such as CD1d, CD38, IgM, CD10, CD86, and CD154 (5, 6, 8, 9, 35) but

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The exact phenotype of Breg is not clearly defined and multiple discrepancies exist between different studies (1, 2). A common finding in mice seems to be IL-10 secretion and expression of CD1d and CD5 (5–7). These data were only partly confirmed in humans (8, 9). A recent study described a human CD19+CD24+CD38+ B-cell population with regulatory potential, which is functionally impaired in patients with SLE (8). This B-cell population secreted IL-10 but did not express CD5. Of note, its regulatory capacity was dependent on CD86 expression, but only partially on IL-10 secretion, so that the authors postulated the existence of further soluble factors with regulatory potential. These results are complementary to our findings, and recent data showing that CD86 is strongly upregulated in IL-21–induced human Breg GrB may be a common effector molecule of human regulatory cells in general. Apart from the GrB effect identified in our study, namely TCR-ζ degradation, GrB may exhibit further regulatory functions such as TGF-β release from soluble β-glycans (44) or induction of apoptosis in certain immune cells. In IL-21–induced human Breg GrB not only is the first regulatory molecule expressed, but all other regulatory molecules tested are expressed primarily on GrB+ B cells. This suggests that GrB represents a predominant regulatory molecule and an important novel marker of human Breg.

Although the existence of tumor-infiltrating B cells has been previously described (27, 45), their significance for solid tumors remains unclear. For example, in ovarian cancers some publications correlate the presence of B cells in the tumor microenvironment with a better prognosis (28), whereas others state that their presence is associated with a worse outcome (33). To detect the "smoking gun" of B cells with a GrB+ regulatory phenotype directly in tumor tissue, we therefore started screening various human tumors for CD19+ B cells. This screening revealed that GrB-expressing B cells in solid tumors secreted IL-10 and CD25 suggests mechanistic similarities between GrB-expressing Breg and Treg. Given previous findings in Treg and in pDC (17–20), we hypothesize that particularly GrB may be a common effector molecule of human regulatory cells in general. Apart from the GrB effect identified in our study, namely TCR-ζ degradation, GrB may exhibit further regulatory functions such as TGF-β release from soluble β-glycans (44) or induction of apoptosis in certain immune cells. In IL-21–induced human Breg GrB not only is the first regulatory molecule expressed, but all other regulatory molecules tested are expressed primarily on GrB+ B cells. This suggests that GrB represents a predominant regulatory molecule and an important novel marker of human Breg.

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suggest IL-21–induced GrB+ B cells may indeed play an immunosuppressive role in certain tumors, possibly with similar impact on T-cell responses as Treg. Nevertheless, while the presence of IL-21+ cells provides a valid explanation for the induction of GrB+ B cells in tumor tissue, a variety of cofactors such as CD40L, TLR ligands, and tumor-derived danger signals may influence the function of IL-21. Moreover, local secretion within a tumor may allow IL-21 to interact with different cell types than its systemic occurrence. It is therefore possible that IL-21 supports an immunosuppressive environment within a tumor, whereas its systemic application may allow activation of cytotoxic T cells and natural killer cells with antitumor activity at tumor-distant sites, eventually even resulting in the establishment of a robust antitumor immune response as recently described (47). Altogether, it likely depends on the activation status of infiltrating B cells, and the presence of additional cofactors whether an efficient antitumor immune response is suppressed or supported by infiltrating B cells.

In conclusion, we have shown for the first time that IL-21 is a key cytokine for the induction of human Breg and have identified GrB as predominant immunomodulatory molecule and phenotypic marker of human Breg. IL-21–mediated induction of GrB is significantly stronger in CD5− as compared with CD5+ B cells, and integrates both BCR- and TLR-dependent signals. GrB plays a central role for the regulatory function of Breg, as they can efficiently suppress T-cell proliferation by GrB-dependent TCR-ζ degradation. GrB+ B cells exhibit a CD19+CD38+IgM+IgD− phenotype and further regulatory molecules including IL-10, CD25, and IDO. Most importantly, GrB+ B cells can infiltrate the microenvironment of various solid tumors, where they are observed adjacent to IL-21–providing T cells. Our findings strongly suggest GrB+ B cells may contribute to the modulation of cellular adaptive immune responses by Treg-like mechanisms, possibly allowing the escape of certain tumors from an efficient antitumor immune response. On the other hand, the induction and use of GrB+ Breg may be evaluated as an innovative cell therapeutic approach to the management of immune pathologies including autoimmune diseases and GVHD.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Granzyme B+ Regulatory B Cells Infiltrate Tumors


Interleukin 21–Induced Granzyme B–Expressing B Cells Infiltrate Tumors and Regulate T Cells

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