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Distinct Activation Signals Determine whether IL-21 Induces B Cell Costimulation, Growth Arrest, or Bim-Dependent Apoptosis

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IL-21 costimulates B cell proliferation and cooperatively with IL-4 promotes T cell-dependent Ab responses. Somewhat paradoxically, IL-21 also induces apoptosis of B cells. The present study was undertaken to more precisely define the expression of the IL-21R, using a novel mAb, and the circumstances by which IL-21 promotes B cell growth vs death. The IL-21R was first detected during T and B cell development, such that this receptor is expressed by all mature lymphocytes. The IL-21R was further up-regulated after B and T activation, with the highest expression by activated B cells. Functional studies demonstrated that IL-21 substantially inhibited proliferation and induced Bim-dependent apoptosis for LPS or CpG DNA-activated B cells. In contrast, IL-21 induced both costimulation and apoptosis for anti-CD40-stimulated B cells, whereas IL-21 primarily costimulated B cells activated by anti-IgM or anti-IgM plus anti-CD40. Upon blocking apoptosis using C57BL/6 Bim-deficient or Bcl-2 transgenic B cells, IL-21 readily costimulated responses to anti-CD40 while proliferation to LPS was still inhibited. Engagement of CD40 or the BCR plus CD40 prevented the inhibitory effect by IL-21 for LPS-activated B cells. Collectively, these data indicate that there are three separable outcomes for IL-21-stimulated B cells: apoptosis, growth arrest, or costimulation. We favor a model in which IL-21 promotes B cell maturation during a productive T cell-dependent B cell response, while favoring growth arrest and apoptosis for nonspecifically or inappropriately activated B cells. The Journal of Immunology, 2004, 173: 657–665.

Interleukin-21 is a member of the common γ-chain (γc)1-dependent cytokine family that includes IL-2, IL-4, IL-7, IL-9, and IL-15 (1–6) and is produced primarily by activated T lymphocytes (7, 8). The gene for IL-21 is closely linked to the IL-2 and the IL-15 gene loci on human chromosome 4q26-q27 (8). The IL-21R is a member of the cytokine receptor superfamily that most closely resembles IL-2Rβ and IL-4Rα (7, 8). The gene for the IL-21R is very closely linked to the IL-4Rα locus and is located on human chromosome 16p11. This close proximity suggests that these two genes arose from gene duplication during evolution. A functional IL-21R, consisting of the IL-21R subunit and γc, activates Jak-1, Jak-3, STAT1, STAT3, and STAT5 in several cell lines (4, 5, 7). Based on mRNA analysis, IL-21R expression is restricted to lymphoid tissues. By using biotinylated IL-21, IL-21R is weakly detected on primary resting B cells and several NK and T cell lines (7, 8). However, the absence of a mAb to the IL-21R has prevented a more refined analysis of expression of this protein, including determining the extent the IL-21R is regulated during lymphocyte development and activation.

On its own, IL-21 does not play an obvious mandatory role in lymphocyte development or function, because IL-21R-deficient mice have a normal complement of developing and mature T and B cells and lack severe immunopathology (9, 10). However, based primarily on in vitro studies, IL-21 exhibits a broad capacity to regulate lymphoid cell functions. For T lymphocytes, IL-21 somewhat costimulates proliferation by anti-CD3-activated T cells, augments allospecific CTL activity by CD8+ T cells, and promotes the differentiation of Vγ9/Vδ2 T cells into central memory CD45RO+ T cells (8, 9, 11). IL-21 is preferentially produced by Th2 cells and in turn inhibits the development of IFN-γ-producing Th1 cells, in part by decreasing the responsiveness of developing Th cells to IL-12 (12). More recent studies indicate that IL-21 also regulates dendritic cell function by inhibiting their activation and maturation (13, 14). For NK cells, IL-21 has been shown to promote their maturation and activation in conjunction with IL-15, Ftl-3 ligand, and stem cell factor (8, 15). However, IL-21 limited the survival and expansion of activated NK cells while augmenting their lytic function (9).

With respect to B lymphocytes, IL-21 delivers both positive and negative regulatory signals. Initially, IL-21 was shown to costimulate human B cell proliferation induced by anti-CD40, but suppress proliferation stimulated by anti-IgM and IL-4 (8). However, more recent studies demonstrated that IL-21 did not costimulate anti-CD40-induced proliferation in mouse B cells, but rather regulated B cell homeostasis by inducing apoptosis of resting and activated B lymphocytes (16). Although the mechanism of apoptosis was not established, overexpression of Bcl-xL or Bcl-2 prevented IL-21-induced B cell death. With respect to Ab responses, IL-21 appears to be a negative regulator of mouse IgE production. IL-21 directly blocked IgE production by IL-4-stimulated B cells through the inhibition of germline IgCe transcription, and IL-21R-deficient mice exhibited enhanced T cell-dependent IgE production (10, 17).

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2 Address correspondence and reprint requests to Dr. Thomas R. Malek, Department of Microbiology and Immunology, University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101. E-mail address: tmalek@med.miami.edu
3 Abbreviations used in this paper: γc, common γ-chain; PI, propidium iodide; BocD, β-butoxy-carbonyl-Asp; int, intermediate; Fas, Fas ligand; Tg, transgenic.
In contrast, IL-21R-deficient mice also exhibited lower production of IgG, indicating a positive role for IL-21 in Ig production (10). Strikingly, upon immunization, IL-21R/IL-4 double-knockout mice were characterized with dysgammaglobulinemia primarily due to absent IgG responses (10). These latter experiments directly implicate IL-21, in a redundant fashion with IL-4, in delivering essential signals during B activation that promote differentiation into Ab-secreting cells.

With the importance of IL-21 in regulating B cell function, the present study was undertaken to more precisely define the expression of the IL-21R on B lineage cells and the context in which IL-21 promotes B cell activation vs apoptosis. Besides characterizing the developmental expression of the IL-21R using a novel mAb, we show that this cytokine receptor is most extensively expressed on activated B cells. After activated B cells were stimulated with IL-21, apoptosis, growth arrest, or costimulation occurred, depending on the nature of activating signals. These findings support a model in which IL-21 promotes B cell survival and maturation during a productive T cell-dependent B cell response, whereas this cytokine favors apoptosis and growth arrest for B cells that were nonspecifically or inappropriately activated. We further established that IL-21 induces apoptosis through a Bim-dependent mechanism.

Materials and Methods

Mice

C57BL/6, BALB/c, Fas-deficient (lpr) and Bc1-2 transgenic (Tg) (36Wehi) mice, both on the C57BL/6 genetic background, were purchased from The Jackson Laboratory (Bar Harbor, ME). Fas ligand (Fasl)-deficient (gld) mice on C57BL/6 genetic background and TNFRI- or TNFRII-deficient mice on BALB/c genetic background were provided by Dr. R. Levy (University of Miami). Bim-deficient mice on C57BL/6 genetic background were provided by Dr. A. Strasser (Walter and Eliza Hall Medical Research Institute, Melbourne, Australia) via Drs. M. Wang and P. Marrack (Howard Hughes Medical Institute, National Jewish Research and Medical Center, Denver, CO). IL-21R-deficient mice were provided by Drs. M. Collins (Wyeth Research, Cambridge, MA) and M. J. Grusby (Harvard School of Public Health, Boston, MA). Normal mice were used at 2–4 mo of age, whereas transgenic or gene-defective mice were used at 1–2 mo of age.

Production of mAb to IL-21R

Mouse cDNA encoding a truncated IL-21R, expressing the leader, extracellular, and transmembrane domains but only 2 aa of the cytoplasmic tail, was prepared by RT-PCR using total RNA from C57BL/6 thymocytes and 5'–AGTAGGTCTGGGACACAGCATGC–3' and 5'–GATCTTCAGACCCATGAAAACCAG–3' as the forward and reverse PCR primers. The cDNA was directionally subcloned into the HindIII/Apal sites in the eukaryotic expression vector pCDNA3.1/V5-His-TOPO followed by a 3' V5 epitope tag (Invitrogen Life Technologies, Carlsbad, CA). The construct was transfected into a rat YB2/0 hybridoma cell line by electroporation as previously described (18). Transfected cell lines expressing a high level of the IL-21R were identified by Western blot analysis using the anti-V5 Ab to the epitope tag and maintained in complete medium containing G418 (1 mg/ml; Invitrogen Life Technologies). Lewis rats were immunized and boosted twice with IL-21R-transfected YB2/0 cells. Three days after the last immunization, spleen cells from immunized rats were fused to the SP2/0 hybridoma, as previously described (18).

Cell purification

B cells were purified from spleens with magnetic anti-CD19 beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instruction. These cells were typically 99%/B220/CD4−CD8− cells with <0.5% CD11b+ cells, when determined by the multicolor FACS analysis. The purified B cells were not activated by this procedure, because the culture of these cells in medium did not result in [3H]thymidine incorporation 24–48 h later or induction of activation markers such as CD69 when analyzed 8–16 h later. To enrich NK cells, B and T cells were reduced from spleen cells by panning on anti-Ig-coated plates (19) followed by depletion of T cells using magnetic anti-Thy1.2 beads (Miltenyi Biotec) according to the manufacturer’s instruction. The flow-through fraction was enriched ~10-fold for NK cells, i.e., 20% of the cells were NK1.1+TCRβB220−. The remaining cells were primarily B cells (~25% B220−) and T cells (~55% TCRβ+).

Cell culture

Cells were cultured in complete medium containing 5% FCS as described (20). Purified B cells were placed either in 96-well plates (1 × 105/well) for [3H]thymidine incorporation and apoptosis assays or in 24-well plates (1 × 105/well) for FACS, cell cycle, or molecular analyses. B cells were stimulated with LPS (1 μg/ml; Escherichia coli 026:B6; Sigma-Aldrich, St. Louis, MO), anti-CD40 mAb (HM40-3; 1 μg/ml; BD Pharmingen, San Diego, CA), anti-μ-F(ab')2 mAb (1 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA), or CpG-containing nucleoside-resistant phosphorothioate oligodeoxynucleotides 1826 (0.3 μM; CpG ODN-1826; 5′-CCATGACGTTCTGACCTGT-3′) in the absence or presence of mouse IL-21 (30 ng/ml; R&D Systems, Minneapolis, MN) for the indicated time. To measure cell proliferation, [3H]thymidine (1 μCi) was added into each well during the last 4–6 h of cultures, and the incorporation of radioactive into DNA was determined as previously described (20). Splenocytes (1 × 106/well) were cultured in 24-well plates with either anti-CD3 (145-2C11; 5% supernatant) or IL-21 (30 ng/ml; R&D Systems, Minneapolis, MN) for the indicated period. We established that IL-21 expression was assessed by a two- or three-step staining protocol. Two-step staining consisted of incubation with biotinylated 4A9 followed by either PE- or CyChrome-streptavidin. As a control, this staining was specifically blocked by a preincubation of cells with unlabeled 4A9. In some cases, a more sensitive three-step staining method was used, which consisted of an initial incubation with 4A9, a second incubation with biotinylated anti-rat Ig, and a third incubation, which first included quenching of the unreactive anti-rat Ig sites by addition of rat IgG, followed 10 min later by the addition of PE- or CyChrome-streptavidin. In the control in this case was to omit the first-step staining with 4A9. Multicolor FACS analysis for cell surface marker expression was determined as previously described (21) using a LSR analyzer and CellQuest software (BD Biosciences, Mountain View, CA). Typically, 5,000–20,000 viable cells were analyzed based on forward- vs side-scatter gating.

For cell cycle analysis, cultured B cells were washed with HBSS, fixed in 70% cold ethanol at 4°C overnight, and then washed, treated with RNase (1 mg/ml), and stained with propidium iodide (PI; 50 μg/ml; Sigma-Aldrich) in the dark for 1 h followed by FACS analysis. For cell viability analysis, cultured B cells were washed and stained with PI for 10 min in the dark. Dead cells were enumerated as the fraction of PI-positive cells by FACS analysis.

Molecular analysis

The caspase inhibitor r-butoxy-carbonyl-Asp (BocD)-fluoromethylketone (20 μM) was obtained from Enzyme Systems Products (Dublin, CA). The protein synthesis inhibitor cycloheximide (1 μg/ml) and the transcriptional inhibitor actinomycin D (30 ng/ml) were obtained from Sigma-Aldrich.

For mRNA analysis, total RNA was extracted from the indicated cell population using TRizol (Invitrogen Life Technologies). cDNA was prepared by reverse transcription reaction and hybridized to the GEArray apoptosis gene expression arrays (SuperArray Bioscience, Frederick, MD) according to the manufacturer’s instruction. The resulting hybridization signal was visualized by chemiluminescence. Data were subjected to densitometric analysis using Scion Image software (Scion, Frederick, MD). RNA levels were expressed as the relative OD after normalizing the hybridization signals to β-actin.

Western blot analysis was performed essentially as previously described (22). For immunoprecipitation, protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) containing 4A9 were incubated with the indicated Nonidet P-40 lysates (5 × 105/sample) overnight at 4°C. The washed immunoprecipitates or unfractionated Nonidet P-40 lysates (30 μg/sample) were separated by 10–12% SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride nitrocellulose membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk, incubated with mouse anti-V5 mAb (Invitrogen Life Technologies),
Results

Generation of a rat mAb to the mouse IL-21R

Lewis rats were immunized with IL-21R-transfected YB2/0, and after fusion of immunized spleen cells to SP2/0, the resulting hybridomas were screened for selective staining of the IL-21R-transfected vs untransfected YB2/0. One highly reactive hybridoma, 4A9, secreting a rat IgG2a mAb, was identified and subcloned twice by limiting dilution. FACS analysis showed that the cloned 4A9 mAb selectively bound to the IL-21R-transfected YB2/0, but not to untransfected YB2/0 (Fig. 1A). By using the anti-V5 mAb to the epitope-tagged IL-21R, material of 41 and 53 kDa was detected by Western blot analysis from unfracionated lysates or 4A9 immunoprecipitates from only IL-21R-transfected YB2/0 (Fig. 1B). Such bands were not detected after control immunoprecipitation using anti-γ (4G3)-coated beads (not shown). The calculated $M_e$ for the truncated IL-21R is ~28 kDa. Therefore, it is likely that the 41-kDa band represents an initial intracellular glycosylated form of the IL-21R, whereas the 53-kDa band likely represents a heavy and variable glycosylated mature IL-21R. This type of $M_e$ heterogeneity has been observed for other members of the IL-21R family (Sigma-Aldrich), as appropriate, for 1 h. The Ag-Ab reactions were detected by ECL (Pierce, Rockford, IL).

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Type</th>
<th>IL-21R</th>
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<tbody>
<tr>
<td>BW5157</td>
<td>Thymoma</td>
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<tr>
<td>EL-4</td>
<td>Thymoma</td>
<td>−</td>
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<td>2B4</td>
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<tr>
<td>L1STRA</td>
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<td>+</td>
</tr>
<tr>
<td>YAC-1</td>
<td>T lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>CTLL-1</td>
<td>Cytotoxic T</td>
<td>+</td>
</tr>
<tr>
<td>DAP3</td>
<td>Fibroblast</td>
<td>−</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Fibroblast</td>
<td>−</td>
</tr>
<tr>
<td>S9</td>
<td>Stromal cell</td>
<td>−</td>
</tr>
<tr>
<td>P815</td>
<td>Mastocytoma</td>
<td>−</td>
</tr>
<tr>
<td>P33D1</td>
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<td>−</td>
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<tr>
<td>WEHI3</td>
<td>Myelomonocytic</td>
<td>+</td>
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<tr>
<td>SP2/0</td>
<td>Myeloma</td>
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</table>

*IL-21R expression was determined by staining the cells with biotinylated 4A9 followed by fluorescent-conjugated streptavidin and FACS analysis. Staining was determined to be specific by inhibition with unlabeled 4A9.
and a high level of IL-21R expression was evident on most immature B220<sup>lo</sup>IgM<sup>lo</sup> and all mature B220<sup>hi</sup>IgM<sup>hi</sup> B cells. Very few B220<sup>−</sup> cells in the bone marrow expressed the IL-21R. In the spleen (Fig. 2C), IL-21R expression was detected on essentially all naive B cells. Somewhat higher staining was observed on activated spleen or NK-enriched cells (Fig. 2D). In both A and C, concentration of IL-21R expression on activated B cells. Additionally, the expression of the IL-21R was substantially and similarly up-regulated after B cells were activated with LPS (Fig. 2D), or either CpG DNA or anti-CD40 (not shown). Thus, although the IL-21R is broadly detected on lymphoid cells, this cytokine receptor is most highly expressed on activated T and B lymphocytes, with consistently higher expression on activated B cells.

Regulation of B cell growth, death, and cell cycle progression by IL-21

Given the importance of IL-21 in contributing to Ig production in vivo (10) and the conflicting reports concerning the role of IL-21 as a B cell costimulatory vs proapoptotic cytokine in vitro (8, 16), we investigated the effect of IL-21 on B cell growth, death, and cell cycle progression in the context of B cell activation in vitro using two mouse strains, C57BL/6 and BALB/c, that generate somewhat distinctive B cell responses. For B cells stimulated through TLR4 and -9 using LPS or CpG DNA, proliferative responses (Fig. 3A) were inhibited and cell death (B) was increased by IL-21 when assayed 48 h after culture initiation. Time course studies for B cells from both strains indicated that cell death by IL-21 was first detected at 8–12 h after culture initiation. However, optimal detection of cell death and growth arrest was observed at 48 h. Similar suppressive effects were seen after B cells from C3H/HeJ mice, a LPS-hyporesponsive strain, were activated by CpG DNA, excluding the possibility that this effect was due to LPS that contaminated the CpG DNA (data not shown). Cell cycle analysis (Fig. 3C) 24 h after activation with LPS confirmed that IL-21 reduced the fraction of B cells in S-G<sub>2</sub>M phases of the cell cycle by 2-fold and increased apoptotic B cells with hypodiploid DNA by ~2-fold. A somewhat lower level of apoptotic cells was noted when dead cells were measured after 24 h, either by containing hypodiploid DNA (Fig. 3C) or staining with PI (not shown), when compared with cells cultured for 48 h (B). Thus, IL-21 down-regulated B cell responses to LPS and CpG DNA at two levels, cell cycle arrest and apoptosis, which was more striking for LPS-activated B cells.

In contrast, for anti-CD40-stimulated B cells, IL-21 functioned as a costimulatory signal because this cytokine increased the fraction of cycling cells and proliferative responses (Fig. 3, B and C). However, IL-21 concomitantly increased the percentage of dead B cells as measured by the fraction of cells that stained with PI (Fig. 3B) or contained hypodiploid DNA (C). IL-21 also costimulated B cell proliferation in conjunction with anti-IgM or anti-IgM and anti-CD40 (Fig. 3, A and B). In these cultures, IL-21 did not enhance B cell apoptosis, but instead improved the survival of these cells. C57BL/6 B cells were consistently more susceptible to IL-21-mediated cell death after stimulation with either LPS, CpG DNA, or anti-CD40, whereas BALB/c B cells typically generated much greater IL-21-dependent costimulation to anti-CD40 or anti-IgM. Therefore, these data demonstrate the magnitude of IL-21-dependent apoptosis or costimulation is dependent on the mouse strain from which the B cells were derived and the nature of the activating signals.

Molecular analysis of IL-21-induced apoptosis

To further characterize the molecular basis of IL-21-mediated apoptosis, we primarily examined the effect of this cytokine on LPS-activated C57BL/6 B cells because this situation typically generated the greatest cell death. IL-21 failed to induce B cell death after blocking either mRNA or protein synthesis using actinomycin D (Fig. 4A) and cycloheximide (B), respectively, when examined 8 h after culture initiation. At this time, substantial cell death was already apparent for LPS-activated B cells in the presence of IL-21. In agreement with a previous study on naive C57BL/6 B cells (16),...
the general caspase inhibitor BocD, also prevented IL-21-mediated cell death for LPS-activated B cells (Fig. 4C). In all cases, these inhibitors did not exhibit toxicity toward unstimulated B cells.

To identify potential molecular targets for IL-21-induced apoptosis, mRNA probes were prepared from C57BL/6 B cells stimulated with LPS in the presence or absence of IL-21 for 4 h, and these probes were hybridized to a mouse apoptosis gene array that includes 96 genes implicated in controlling apoptosis. Only 41 of 96 genes were detected, of which the expression of 34 genes was similar between both samples. Interestingly, IL-21 up-regulated the mRNAs for proapoptotic Bim and Apaf-1, and down-regulated the mRNAs for antiapoptotic molecules Bcl-xL and Bfl-1 (Fig. 5, A and B). Western blot analysis demonstrated that B cells cocultured with IL-21 and LPS had higher expression of the large and short isoforms of Bim and somewhat lower expression of Bcl-xL when compared with B cells solely stimulated by LPS. The expression of Bcl-2 and actin was essentially unchanged between these two experimental groups (Fig. 5C). Thus, these results suggest that IL-21 may induce apoptosis by regulation of genes required for the mitochondrial cell death pathway and this process requires de novo mRNA and protein synthesis.

**Bim is required for IL-21-induced B cell apoptosis**

In other systems, B cell apoptosis depends upon direct engagement of cell surface death receptors. However, IL-21 readily induced apoptosis of LPS-activated B cells derived from mice deficient in either Fas, Fasl, TNFR1, or TNFR2 (Fig. 6A). These data rule out that IL-21 might indirectly cause B cell death by inducing one of these death receptors or ligands. In marked contrast, this type of B cell apoptosis was essentially not observed for the Bim-deficient and Bcl-2 Tg B cells (Fig. 6A). The response to LPS and the expression of the IL-21R for B cells from these two mouse strains were comparable to that observed for wild-type B cells (data not shown). When compared with wild-type B cells, IL-21-mediated apoptosis after activation by CpG DNA (Fig. 6B) or anti-CD40 (C) was also reduced for Bcl-2 Tg B cells. An identical reduction in IL-21-mediated apoptosis for CpG DNA and anti-CD40 was noted in a single experiment using Bim−/− mice (not shown). Thus, these data indicate that IL-21 induces B cell apoptosis through a Bim-dependent mitochondrial cell death pathway.

**Regulation of B cell proliferation by IL-21 in the absence of apoptosis**

One explanation for the low proliferation to anti-CD40 and LPS in the presence of IL-21, especially by C57BL/6 B cells, was simply the induction of apoptosis. To test this notion, the effect of IL-21 on such proliferation was examined when cell death was prevented by using Bim-deficient or Bcl-2 Tg C57BL/6 B cells. For the anti-CD40 response (Fig. 7A), IL-21 functioned as a potent costimulating agent, because the proliferation by the genetically modified mice increased ~3- to 5-fold. Thus, the modest costimulation of the anti-CD40 response by IL-21 for C57BL/6 B cells is largely accounted for by their relatively high susceptibility to apoptosis rather than an intrinsic defect to proliferate. However, in the case of LPS (Fig. 7B), IL-21 still substantially blocked the proliferation for wild-type, Bim-deficient, or Bcl-2 Tg C57BL/6 B cells. Therefore, IL-21-induced growth arrest is separable from apoptosis of the LPS responses.
groups containing IL-21 in the presence or absence of metabolic inhibitors.

BCR and T cell helper signals in the regulation of LPS-activated B cell response to IL-21

The preceding experiments demonstrate two important outcomes of IL-21 signaling in B cells, i.e., to negatively regulate LPS and CpG DNA responses and to enhance proliferation after engaging the BCR and/or CD40. Because the initiation of an Ab response to an infectious agent often occurs in the presence of LPS and CpG DNA, it is not clear whether the net effect of IL-21 is to promote or inhibit a T cell-dependent B cell response. To assess this issue, we examined the effect of IL-21 on proliferation (Fig. 8A) and apoptosis (B) for C57BL/6 B cells costimulated with LPS, anti-IgM, and/or anti-CD40, the latter two as surrogates for BCR engagement and T cell help. When cocultured with LPS, anti-IgM or anti-CD40 slightly reduced the proliferative response, whereas B cell viability remained relatively high (~80–90%). IL-21 inhibited proliferation and induced apoptosis for B cells activated solely with LPS or LPS plus anti-IgM. Importantly, IL-21 did not inhibit B cell proliferation or induce apoptosis after costimulation by LPS and anti-CD40 or anti-IgM plus anti-CD40. Thus, these data indicate that IL-21 mediates growth arrest and apoptosis for B cells solely stimulated with LPS or LPS and anti-IgM, whereas this cytokine does not prevent B cell responses if they receive signals through the BCR and CD40.

Discussion

There is a growing body of data that IL-21 regulates T, B, NK, and dendritic cell responses in vitro (3, 14). However, with the exception of B cells, there is little data concerning the precise role for IL-21 in regulation of immunity in vivo. IL-21R-deficient mice exhibited a very modest phenotype, with no obvious problems in lymphocyte development and immune responses, other than higher IgE and lower IgG1 production (9, 10). Importantly, immunized IL-21R/IL-4 double-deficient mice failed to mount IgG responses, demonstrating an essential role for these two cytokines in B cell maturation leading to Ig production (10). Somewhat paradoxically, IL-21 has been shown to deliver proapoptotic signals and variably supports B cell costimulation in vitro (8, 16).

Our study helps to resolve this conundrum and other seeming inconsistencies concerning the function of IL-21 in the immune system. The production of a mAb to the mouse IL-21R has permitted a more precise definition of the tissue distribution of the IL-21R. This cytokine receptor was detected on developing T and B lymphocytes after commitment to each of these lineages. The lack of expression of the IL-21R in early pro-T and -B cells indicates that this cytokine does not contribute to early steps in lymphopoiesis, which is consistent with the observation that T and B cell development is normal in IL-21R-deficient mice (9, 10). The IL-21R remains expressed at relatively high levels on essentially all mature and activated T and B lymphocytes and was detected on many activated NK cells. Consistent with the importance of IL-21 in regulating B cell responses in vivo, the IL-21R was most highly expressed on activated B cells. Furthermore, γc expression was coordinately up-regulated on activated T, B, and NK cells (data not shown), indicating that the number of functional IL-21R increased on each of these activated cell populations. Although IL-21R was not readily detected on virtually all other lymphoid and myeloid lineage cells, it remains to be determined whether specific activation signals might induce expression on some of these cells.

One somewhat controversial area is the expression of IL-21R on NK cells because this receptor has been variably detected on human NK cells (8). Our data clearly indicate that the vast majority of NK cells in normal mice do not express the IL-21R, even when using a sensitive three-step staining procedure. However, nearly 50% of NK cells expressed the IL-21R after activation with IL-15 or IL-2. Therefore, some of the variability previously noted may simply reflect that there are more activated NK cells in the peripheral blood of some human subjects. This finding is also consistent

![Image](65x145 to 281x358)

**FIGURE 4.** Effect of metabolic inhibitors on IL-21-induced B cell apoptosis. Purified B cells were stimulated for 8 h with LPS in the presence or absence of IL-21 and actinomycin D (A), cycloheximide (B), and BocD (C). Percentage of dead cells was assessed by PI staining and FACS analysis. Data shown are derived from the mean ± SD of three independent experiments. *, p < 0.05; or **, p < 0.01; nonpaired Student’s t test when comparing experimental groups containing IL-21 in the presence or absence of metabolic inhibitors.

![Image](148x650 to 455x742)

**FIGURE 5.** Regulation of pro- and antiapoptotic molecules in activated B cells by IL-21. The mouse apoptosis gene arrays were hybridized with cDNAs probes that were derived from B cells stimulated by LPS or LPS plus IL-21 for 4 h. A, Representative hybridized arrays. B, Relative RNA levels of selected mRNAs normalized to β-actin expression. Data shown are the mean ± range of two independent experiments. C, Western blot analysis after a reducing 12% SDS-PAGE for the indicated proteins using cell lysates (30 μg/sample) from fresh purified B cells or B cells cultured as indicated for 8 h. Data shown are representative of three experiments.
with several reports that IL-21 regulates NK cells only in conjunction with other activation signals (8, 16).

As observed in other studies (8, 16), we also found that B cells were susceptible to IL-21-induced costimulation or apoptosis. We found two major reasons for the distinct outcomes of IL-21 signaling in B cells. First, the magnitude of IL-21-mediated B cell costimulation or apoptosis differed between B cells from C57BL/6 and BALB/c mice. This was especially striking for anti-CD40-activated B cells, where both an increase in cycling and apoptotic cells were noted. These data indicate that there is polymorphism with respect to the outcome in IL-21 signaling in B lymphocytes. This polymorphism is primarily due to higher sensitivity of C57BL/6 B cells to IL-21-induced apoptosis because C57BL/6 B cells generate very robust IL-21-dependent costimulatory responses in the presence of anti-CD40 when apoptosis was prevented by using B cells from Bim-deficient or Bcl-2 Tg mice. Thus, the past failure to note IL-21-mediated costimulation of B cell proliferation (16) is likely because these investigators used C57BL/6 B cells for all of their experiments.

Besides the strain difference, the second feature that favors apoptosis by IL-21 is the context by which the B cells were activated. Thus, IL-21-driven apoptosis and inhibition of proliferation was dominant when B cells were activated through TLRs 4 and 9 via LPS and CpG DNA. Costimulation and lower apoptosis occurred for B cells activated with anti-IgM or anti-IgM plus anti-CD40. However, IL-21 regulation of the response to anti-CD40 appears distinct in that both apoptosis and costimulation were detected. The reason for this at the present is not known but raises the possibility that subsets of B cells differentially respond to IL-21. Importantly, IL-21-mediated apoptosis was prevented if LPS-stimulated B cells were further activated with anti-CD40 or anti-CD40 and anti-IgM. Collectively, these findings are consistent with the notion that IL-21 favors B cell responses in the context of BCR activation and cognate T cell help, whereas apoptosis is dominant for B cells that are solely activated through TLRs.

From these observations, we favor a model in which IL-21 represents an important checkpoint for a productive B cell response. On the one hand, IL-21 delivers apoptotic signals for B cells solely stimulated through LPS, CpG, or anti-CD40. IL-21-mediated apoptosis occurred even in the presence of IL-4 (Ref. 16; H. Jin and T. R. Malek, unpublished data), which on its own promotes B cell survival of naive, or LPS- and CpG DNA-activated B cells. In the presence of sufficient level of LPS or other innate signals such as CpG DNA, polyclonal nonspecific B cell activation occurs by engaging TLRs (27–29). This potentially leads to Ig production by Ag-nonspecific B cells, including potentially self-reactive B cells, which has been proposed to induce or exacerbate autoimmune diseases as a consequence of infection (30–34). In this situation, the presence of IL-21 from bystander Th cells, presumably activated by T-dependent Ags associated with the infectious agent, is predicted to induce growth arrest or Bim-dependent apoptosis of the nonspecifically activated B cells. If bystander T-B CD40L-CD40 interactions actually occur in vivo, in some cases IL-21 may also lead to apoptosis of these B cells. However, those LPS-activated B cells that engage their BCR to other Ags and receive cognate T help through CD40 may be protected from the negative regulation of IL-21. Furthermore, under this circumstance, IL-21 is predicted to promote maturation and class switching of these B cells.

![FIGURE 6. IL-21-induced B cell apoptosis is dependent upon Bim. Purified B cells from the indicated strains of mice were stimulated for 48 h with LPS (A), CpG DNA (B), or anti-CD40 (C) in the presence or absence of IL-21. Percentage of apoptotic B cells was assessed by PI staining and FACS analysis. Data shown are derived from the mean ± SD of two to three experiments for each genotype. ***, p < 0.01; or ***, p < 0.005, nonpaired Student’s t test when comparing wild-type vs genetically altered mouse strains in the presence of IL-21.](http://www.jimmunol.org/)

![FIGURE 7. Regulation of B cell proliferation by IL-21 after blockade of cell death. Purified B cells from the wild-type (WT), Bim+/-, or Bel-2 transgenic (Tg) mice were stimulated for 48 h with anti-CD40 (A) or LPS (B) in the presence or absence of IL-21, and B cell proliferation was determined. Shown is the relative change in the proliferative responses in the presence of IL-21 for each mouse strain. Data shown are the results of the mean ± SD of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 8. T cell-derived CD40 helper signals and BCR engagement prevent the suppressive effect of IL-21 on LPS-activated B cells. Purified C57BL/6 B cells were stimulated for 48 h with LPS and IL-21 in the absence or presence of anti-IgM and/or anti-CD40, as indicated, and B cell proliferation (A) or cell death (B) were assayed. Data shown in A are derived from the mean ± SD of triplicate samples from one representative experiment of three. Data shown in B are the mean ± SD of three independent experiments.](http://www.jimmunol.org/)
latter notion is in line with the redundant and essential role of IL-4 and IL-21 for B cell functional maturation in vivo (10). This model provides an explanation for the potent positive and negative effects of IL-21 on B cells and a framework for future studies.

Fas/FasL (CD95/CD178) has been implicated as one important mechanism eliminating low-affinity and self-reactive B cells that may contribute to the affinity maturation of the Ab response and maintenance of self-tolerance, respectively (35–39). Past analysis of IL-21-dependent B cell apoptosis demonstrated that it was prevented after the overexpression of Bcl-2 (16). However, both the death receptor and intrinsic mitochondrial death pathways are suppressed by overexpression of Bcl-2 (39–42). Our experiments establish that IL-21-dependent apoptosis is independent of Fas/FasL or TNF/TNFFRs. Furthermore, by using B cells from Bim-deficient or Bcl-2 Tg mice, we directly demonstrated that the mitochondrial pathway is involved in the proapoptotic function of IL-21. With respect to B cells, Bim-dependent apoptosis contributes to eliminating autoreactive B cells (43). Our data raise the possibility that IL-21 might function in maintaining self-tolerance by activation of Bim-dependent apoptosis of autoreactive B lymphocytes.

Current studies indicate Bim induces intrinsic cell death by its translocation from microtubules to the mitochondrial outer membrane, where Bim inhibits antiapoptotic Bcl-2 and Bcl-xL, and promotes activation of Bax and/or Bak, with ensuing apoptosis-mediated cell death (44, 45). Although we have not followed the cellular traffic of Bim, we have identified IL-21 as a trigger of Bim activation. Based on examining steady-state mRNA and protein levels and the susceptibility to inhibitors of transcription and translation, IL-21 appears to induce apoptosis by increasing the level of Bim and decreasing the amount of Bcl-xL, whereas the levels of Bcl-2 were largely unaffected. This finding does not exclude regulation of other molecules within the mitochondrial pathway, and in fact, some increase in Apaf-1 mRNA was also noted. Importantly, this proapoptotic activity of IL-21 is unique for the γ chain-dependent cytokine family and indicates that an extrinsic cytokine signal can lead to activation of the intrinsic mitochondrial cell death pathway.

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References
5. Habib, T., S. Sannaheera, K. Weinberg, and K. Kaushansky. 2002. The common γ chain (γc) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAKS. Biochemistry 41:8725.


