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Role of B cell receptor signaling in IL-10 production by normal and malignant B-1 cells

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Abstract

B-1 cells are considered innate immune cells that produce the majority of natural antibodies. B-1 cell responses to B cell receptor (BCR) and Toll-like receptor (TLR) ligation are tightly regulated owing to the cross-reactivity to self-antigens. CD5 has been shown to play a major role in down regulation of BCR responses in B-1 cells. Here, we provide evidence for another mechanism by which BCR response is regulated in B-1 cells. B-1 cells, as well as their malignant counterpart B cell chronic lymphocytic leukemia (CLL) cells, produce interleukin-10 (IL-10) constitutively. IL-10 secretion by normal B-1 cells downregulates their proliferation responses to BCR ligation. However, we found that CLL cells appear to be unique in not responding to IL-10-mediated feedback-suppressive effects in comparison to normal B-1 cells. In addition, we describe a novel role of the B cell receptor signaling pathway in constitutive IL-10 secretion by normal and malignant B-1 cells. We found that inhibition of Src family kinases, spleen tyrosine kinase, Syk, or Bruton's tyrosine kinase (Btk) reduces constitutive IL-10 production by both normal and malignant B-1 cells.

Keywords

B-1 cell; B cell receptor; Toll-like receptor; chronic lymphocytic leukemia; IL-10

Introduction

B-1 cells are a unique subset of B cells that have been identified in several species, including mice and humans. Studies using transgenic or gene-targeted mice indicate that B cell receptor (BCR) signaling plays an important role in B-1 cell development, survival, and

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proliferation. Mutations that disrupt BCR signaling result in decreases of the B-1 cell subset, while mutations or transgenes that enhance BCR signaling result in increases of the B-1 cell compartment.¹ Thus, mice with a mutation or deficiency of Bruton's tyrosine kinase (Btk), a key enzyme in the BCR signaling pathway, have a severe reduction in B-1 cells.² However, mice deficient in SHP-1, CD22, or Siglec G, proteins that negatively regulate BCR signaling, have a dramatic increase in B-1 cells. Transgenic mice that express certain B cell receptors (e.g., VH11 and VH12 families) have an increase in B-1 cell numbers.^{3,4} Despite this dependence of B-1 cells on BCR signaling, it is now clear that B-1 cells form a distinct lineage, as unique precursors for both B-1 and B-2 subsets have been identified by Montecino-Rodriguez *et al.* and Ghosn *et al.*^{5,6} B-1 cells are important for natural antibody production, for mucosal immune responses, and for production of antibodies against conserved epitopes on microbes. Early B-1 cell response appears to reduce viral load in influenza infection, whereas B-1 cell-derived antibodies are crucial for protection against certain bacterial and parasitic infections.⁷⁻⁹ Interestingly, chronic lymphocytic leukemia (CLL) cells from humans and mice express CD5, suggesting their possible origin from B-1 cells. In addition, B-1 cells expressing the *BCR-ABL* oncogene give rise to acute lymphocytic leukemia and do so more rapidly than their B-2 counterparts expressing the same oncogene.¹⁰ B-1 cells constitutively produce interleukin 10 (IL-10), an immunoregulatory cytokine. Here, we investigated the relation between BCR signaling and IL-10 production by normal and leukemic B-1 cells.

B-1 cells respond poorly to B cell receptor and TLR Ligands

The BCRs on B-1 cells exhibit polyreactivity, which allow B-1 cells to respond to conserved epitopes on microbes, but also to have cross-reactivity with self-antigens.¹¹ Indeed B-1 cell levels are increased in certain autoimmune states in mice and humans, even though a causal role of B-1 cells in autoimmunity is not well established.¹² B-1 cell responses to BCR and Toll-like receptor (TLR) ligation are tightly regulated in order to limit the possibility of cross-reactivity to self-antigens. This tight regulation and the underlying mechanisms have been studied extensively.¹³ For example, it is well known that engagement of BCR on B-2 cells leads to a strong intracellular calcium mobilization and proliferation, whereas BCR ligation on B-1 cells induces modest calcium mobilization, little or no proliferation, and increased apoptosis.^{14,15} Many key molecules have been described that negatively regulate BCR and TLR signaling in B-1 cells, including CD5, SHP-1, CD22, Siglec G, and IL-10.¹³ CD19 signaling is also deficient in B-1 cells.¹⁶ Although most studies do not distinguish among B1 cells from various anatomical sites, it was found that splenic B-1a cells may be different from their peritoneal counterparts, as they do not express CD11b but do exhibit differences in expression of CD5, IgM, B7.1, and Notch, as well as differ in responsiveness to phorbol myristate acetate (PMA) (but not anti-IgM).¹⁷

Interestingly, splenic B-1a cells are important for the natural IgM in the serum, which requires interferon response factor (IRF) 4, whereas peritoneal B-1 cells secrete IgM in an IRF4-independent fashion.¹⁸ Furthermore, spontaneous IgM secretion was found to be higher in CD138⁺ B-1a cells than in CD138⁻ B-1a cells of the spleen.¹⁹

B-1 cells produce IL-10 constitutively and IL-10 has autoregulatory function in TLR responses

Peritoneal B-1 (B-1P) cells were shown early on to have the ability to produce IL-10 constitutively.²⁰ A recently identified human CD11b⁺ B-1 cell subset was also found to constitutively secrete IL-10.²¹ The constitutive nature of IL-10 production distinguishes B-1 cells from the newly described B10 subset, which can produce IL-10 but requires further activation to do so.^{22,23} IL-10 is a cytokine that has a role in immunoregulation and inflammation;²⁴ it downregulates the expression of T_H1 cytokines, MHC class II antigens, and co-stimulatory molecules on dendritic cells and macrophages, inhibiting antigen presentation;²⁴ it inhibits pro-inflammatory cytokine production by innate immune cells.²⁴ Among the different subsets of peritoneal B-1 cells, B-1a cells produced the highest amount of IL-10 constitutively, followed by B-1b cells.²⁵ Splenic B-1a cells produced much less IL-10 than peritoneal B-1 cells but more than splenic B-2 cells.²⁵ This IL-10 production is enhanced by TLR stimulation.²⁵ In response to TLR-4 ligation, B-1 cells from IL-10 gene knockout mice proliferate significantly more than wild-type B-1 cells both *in vitro* and *in vivo*.²⁵ This led us to propose that IL-10 has autoregulatory effects in peritoneal B-1 cells through a feedback-inhibitory effect on their proliferation. Similar results were found with ligation of TLR2, TLR3, TLR7, and TLR9 receptors.²⁵ Splenic B-1 cells did not exhibit this autoregulation, as their TLR responses were not enhanced by anti-IL-10R antibodies.²⁵ The autoregulatory effect extends to the differentiation response of B-1 cells. Moreover, the autoregulation could be demonstrated in an infection model. Control of bacterial growth in mice infected with *Borrelia hermsii* has previously been shown to require antibodies made by B-1 cells (in particular B-1b) B cells.²⁶ The IL-10-mediated autoregulation appears to dampen this B-1 cell response, as IL-10 gene knockout B-1 cells were found to be better than wild-type B-1 cells in controlling the growth of these bacteria.²⁵ Interestingly, such autoregulation was not seen in response to CD40 ligation.²⁵ This appears to be related to the fact that IL-10 regulates B-1 cell response to TLR by inhibiting classical NF- κ B signaling, whereas CD40 is known to be able to signal via the alternate NF- κ B pathway.²⁵

Results and discussion

Autoregulation of BCR responses of B-1 cells by IL-10

Here we tested if the well-known hyporesponsiveness of B-1 cells to BCR signaling is in part due to such feedback-inhibitory effects of B-1 cell-derived IL-10. BCR ligation enhanced IL-10 production by peritoneal B-1 but not splenic B-2 (B-2S) cells (Fig. 1A). BCR stimulation failed to induce measurable proliferation of the cells at this 24-h time point, suggesting that the increase in IL-10 is not due to an increase in B-1 cell number. Addition of anti-IL-10 receptor (IL-10R) antibody enhanced B-1 but not B-2 cell proliferation response to BCR ligation (Fig. 1B). The absence of enhancement of B-2 BCR response by anti-IL-10R antibody was not due to lack of IL-10R on B-2 cells, as their robust proliferation response to BCR ligation was strongly suppressed by exogenous IL-10 (Fig. 1C). The role of IL-10 regulation was further supported by the finding that *Il10*^{-/-} B-1 cells proliferated better than wild-type B-1 cells upon BCR ligation, even though both types of B-1 cells responded equally well to CD40 ligation, whether tested at the optimal dose (Fig. 2A) or several doses of anti-IgM (Fig 2B). Responses to CD40 ligation were equivalent

between wild-type and *Il10*^{-/-} B cells (Fig. 2A). CFSE dilution assays supported the findings from tritiated thymidine incorporation assays (Fig. 2C). The effect of IL-10 is mainly at the G₁-S transition, as cells in S/G₂/M phase were particularly reduced in BCR-ligated B-1 cells from wild-type mice compared to *Il10*^{-/-} B-1 cells (Fig. 2D). p38 mitogen-activated protein kinase (MAPK) has been shown to be important for IL-10 production by macrophages.²⁷ BCR-induced IL-10 production by B-1 cells required activity of this enzyme (Fig. 3A). Additionally, p38 MAPK inhibitor enhanced BCR-induced B-1 cell proliferation (Fig. 3B). Furthermore, enhancement of BCR response by a combination of p38 MAPK inhibitor and anti-IL-10R antibody was no greater than with either alone, suggesting that p38 MAPK-dependent IL-10 production has a major role in the reduced proliferation of B-1 cells in response to BCR ligation (Fig. 3C). Moreover, levels of p38 MAPK protein were nearly twice as high in B-1 B cells compared to B-2 cells (Fig. 3D). Consistent with significant constitutive IL-10 production by B-1 cells, p38 MAPK was constitutively phosphorylated in B-1 cells (Fig. 3D).

B cell chronic lymphocytic leukemia and the Eμ-Tcl1 mouse model

B cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in the Western world. B-CLL accounts for one-third of the new cases of leukemia, according to the American Cancer Society.²⁸ B-CLL results in an accumulation of CD5⁺CD19⁺ B cells (B-1) in peripheral blood and multiple other tissues. The Eμ-*Tcl1* transgenic mouse is an excellent model to study the development of CLL. *Tcl1* is an oncogene cloned from a translocation in T cell leukemia;^{29, 30} *Tcl1* is an important activator of Akt, a key kinase required for cell survival.²⁹ Transgenic expression of *Tcl1* under the Eμ enhancer results in CLL when the Ig-VH promoter is used (Eμ-*Tcl1*).³¹ This mouse model closely resembles human B-CLL in its slow development (leukemia is not fully established until 10–15 months of age) and in its responsiveness to chemotherapeutic drugs used in humans.^{31,32} Expanded CD5⁺IgM⁺ B cell populations can be seen in the peritoneum as early as 2 months of age, which became evident in the spleen by 4 months and the bone marrow by 8 months.³¹ Since the Eμ-*Tcl1* mice require 10–15 months to develop disease, we have established an adoptive transfer model in which CLL cells from the Eμ-*Tcl1* mice are injected into naive C57/BL6 mice via the intravenous route. Adoptive-transfer mice develop disease within 3–5 weeks, allowing CLL analysis in a short duration. Zanesi *et al.* reported that the CLL cells from such adoptive transfer mice were very similar to the *de novo* CLL cells in the Eμ-*Tcl1* mice.³³ We have characterized our adoptive transfer CLL cells through surface expression of CD5, CD19, IgM, IgD, B220, and CD23, activation of BCR signaling, and other functional assays, which also showed that these adoptive transfer CLL cells behave similarly to the primary Eμ-*Tcl1* CLL cells.

Similarities and differences in IL-10 production by Eμ-Tcl1 CLL cells and B-1 cells

The normal B-1 B lymphocytes and the Eμ-*Tcl1* B-CLL cells exhibit several similarities. In addition to similar surface marker expression and activation status of certain molecules, CD5⁺CD19⁺ B-1 cells from spleens of Eμ-*Tcl1* mice share a similar functional phenotype with peritoneal CD5⁺CD11b⁺ B-1 cells in that they both secrete IL-10 constitutively (Fig. 4A). Since the majority of normal B-1 cells are derived from the peritoneal cavity, we also tested cells derived from the peritoneal cavity of Eμ-*Tcl1* mice for

their ability to secrete IL-10. We found that peritoneal B-CLL cells also secrete IL-10 constitutively (Fig. 4A). Finally, we demonstrated that LPS enhances IL-10 production by B-CLL cells, another property shared with normal B-1 cells (data not shown).

As shown above, IL-10 secretion by normal B-1 cells regulates their proliferation responses to TLR stimulation as well as BCR ligation. Hence, we tested if the constitutively produced IL-10 is important for CLL cell survival. Neutralization of IL-10 using anti-IL-10 antibodies or anti-IL-10R antibody did not affect the survival of the CLL cells (Fig. 4B). Thus, the CLL cells appear to be unique in not responding to IL-10-mediated suppressive effects *in vitro*, in comparison to normal B-1 cells. These results differ from a previous report of IL-10 requirement for the growth of a malignant B-1 cell clone, LNC, isolated from NZB mice and adapted for *in vitro* culture.³⁴ Although LNC was of B-1 cell origin, it does not represent CLL, as LNC but not E μ -*Tcl1* cells undergo extensive proliferation *in vitro*. Moreover, these studies use cell proliferation as IL-10 response, whereas current studies here focus on survival, as CLL cells do not proliferate extensively in tissue culture.

IL-10 production by both B-1 cells and CLL cells is dependent on BCR signaling

B cell receptor signaling is known to be essential in normal B cell development and survival as well as for B cell malignancies. Our laboratory has shown that BCR signaling through Src family kinases (SFKs) and Syk and JNK kinases is critical for survival of a number of mature B cell malignancies,^{36–38} now CLL (unpublished observations). Therefore, we asked whether BCR signaling could be involved in IL-10 production by both normal and malignant B-1 cells. We tested this hypothesis by using inhibitors specific for Src family kinases, Syk family kinase, and Btk, which all play significant roles in the BCR signaling pathway. We found that inhibition of any of these enzymes reduced IL-10 secretion by both normal B-1 cells and E μ -*Tcl1* CLL cells at inhibitor concentrations that failed to significantly affect the viability of the cells (Table 1). Requirement for Btk activity for IL-10 production is in agreement with a report from Contreras *et al.* that low levels of Btk were insufficient to support IL-10 production by B-1 cells.³⁹

Mechanisms by which BCR signaling is involved in IL-10 production by E μ -*Tcl1* CLL cells

Quantitative real-time PCR analysis of RNA showed that inhibition of BCR signaling with the Syk kinase inhibitor reduced IL-10-specific mRNA, suggesting that IL-10 may be transcriptionally regulated in the E μ -*Tcl1* CLL cells (Fig. 5). We then used lysates of E μ -*Tcl1* CLL cells treated with the Syk inhibitor BAY 61-3606 to investigate signaling molecules and transcription factors that have been known to be involved in IL-10 production by other immune cells. As mentioned previously, p38 MAPK has been shown to be important for IL-10 production by macrophages.²⁷ Surprisingly inhibition of Syk did not reduce the levels of phosphorylated p38 MAPK or total p38 MAPK protein levels in the CLL cells (Fig. S1A). STAT3 has been proposed to bind to and transactivate *Il10* in macrophage and T cell lines of mouse or human origin.⁴⁰

Western blotting revealed no changes in STAT3 activity after Syk inhibitor treatment (data not shown). However, we found that phosphorylation of Akt, an enzyme shown to be involved in IL-10 production by macrophages,⁴¹ was reduced after inhibition of Syk,

suggesting that IL-10 production by E μ -Tcl1 CLL cells may depend on the PI3-kinase/Akt pathway (Fig. S1B). Furthermore, following TLR stimulation, activation of extracellular signal-regulated kinase (ERK) regulates IL-10 expression. Thus, IL-10 secretion is decreased in the presence of chemical inhibitors of ERK or in ERK-deficient dendritic cells upon TLR-activation.^{42–44} There is constitutive phosphorylation of ERK1/2 in the E μ -Tcl1 CLL cells, which is reduced after inhibition of Syk (Fig. S1B).

E μ -Tcl1 CLL cells exhibit clonal variation in their basal levels of IL-10

During the course of this study we examined a number of E μ -Tcl1 spleens from different mice (each of which may have different B cell clones developing into CLL) for IL-10 production. Interestingly, we observed a clonal variation in the basal levels of IL-10 produced by the different clones. Thus, CLL cells from some mice produced high levels of IL-10 (641 pg/ml), while others produced a small amount (24 pg/ml) in comparison to normal B-1 cells. Multiple studies that measured serum IL-10 derived from CLL patients also reported such clonal variation in IL-10 production by human CLL cells. For example, one study reported a range of undetectable to 74 pg/ml of IL-10 from 151 CLL patients.⁴⁵ Since BCR signaling is distinct in mutated and unmutated types of CLL, and since we showed that BCR signaling is required for IL-10 production, it will be interesting to know if the clonal heterogeneity in IL-10 production in human and the mouse model relates to the VH mutational status of the BCR.

Concluding remarks

B-1 cells express polyreactive BCRs that could lead to cross-reactivity to self-antigens. There are multiple mechanisms by which B-1 cell responses are regulated that may have role in preventing production of autoantibodies. In this review, we demonstrated that IL-10 is a negative regulator of BCR signaling in normal B-1 cells in controlling the BCR-induced expansion of self-reactive B-1 cells. This IL-10-mediated negative regulation is lost in malignant B-1 cells *in vitro*, as neutralizing IL-10 or blocking IL-10R in E μ -Tcl1 cells did not affect the survival of these cells. Since E μ -Tcl1 cells appear to be unique in not responding to IL-10-mediated suppressive effects in comparison to normal B-1 cells, we hypothesize that *in vivo* IL-10 could be playing a role in the microenvironment of CLL cells by providing an immunosuppressive niche allowing the cells to proliferate. We are currently investigating this hypothesis by evaluating the growth rate of E μ -Tcl1 cells in the presence and absence of IL-10 signaling. Moreover, the mechanisms by which B-1 cells produce IL-10 remain elusive. In this study, we found that constitutive IL-10 production by both normal and malignant B-1 cells is dependent on BCR signaling and that there is clonal heterogeneity in IL-10 production.

Materials and methods

Mice and reagents

C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and Jackson Laboratory (Bar Harbor, ME). *Il10*^{-/-} mice were obtained from Jackson Laboratory. LPS and anti- β -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). The polyclonal goat anti-IgM F(ab')₂ was obtained from ICN/MP Biomedicals (Irvine, CA) or Jackson ImmunoResearch

Labs (West Grove, PA). The anti-IL-10R (Clone 1B1.3a) was obtained from BD Biosciences (San Diego, CA). Anti-CD40 (1C10 clone) was a gift from Dr. Maureen Howard and was used as ascites fluid. Syk Inhibitor IV (BAY 61-3606) and SB-203580 were obtained from Calbiochem (San Diego, CA). Ibrutinib (PCI-32765) was obtained from Selleckchem (Houston, TX). Dasatinib was obtained from Bristol-Myers Squibb Company (New York City, NY). Anti-p-Syk, anti-Syk, anti-p-p38 MAPK, anti-p38/MAPK, anti-p-p44/42 MAPK, anti-p-Akt, and anti-Akt antibodies were obtained from Cell Signaling (Danvers, MA). Anti-ERK1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Purification of B cells

Normal peritoneal cells were obtained from 2- to 3-month-old C57BL/6 mice by peritoneal lavage with Hank's buffered salt solution. Anti-B220/CD45RB (APC), anti-CD11b (PE) and anti-CD5 (FITC) antibodies (BD Pharmingen, San Jose, CA) were used to identify and sort B-1a B cells from the peritoneum. Wild-type C57BL/6 mice were used for splenic B-2 cells. E μ -Tcl1 spleens were obtained from our collaborators at Ohio State University. E μ -Tcl1 cells were stained with anti-CD5 (FITC) and anti-CD19 (PE or PE-CY7) to determine purity. Peritoneal E μ -Tcl1 cells were obtained via our adoptive transfer model mice. In the adoptive transfer model, single-cell suspension of 10^7 E μ -Tcl1 splenocytes were injected into naive C57/BL6 mice via the intravenous route. Mice were bled weekly after 3 weeks from injection to determine the percentage of B-1 cells in peripheral blood. Once the percentage of B-1 cells (CD5⁺CD19⁺) was 90%, mice were euthanized and peritoneal and splenic CLL cells were obtained.

In vitro cell proliferation assay and cytokine analysis

For proliferation analysis, splenic B-2 cells or peritoneal B-1 cells ($1-2.5 \times 10^5$) were stimulated with polyclonal goat anti-IgM F(ab')₂ (50 μ g/ml) in the presence or absence of anti-IL-10R antibody (1 μ g/ml). Peritoneal B-1 cells from *Il10*^{-/-} mice were cultured with anti-IgM F(ab')₂ (0-50 μ g/ml) or 1:1000 dilution of anti-CD40 (1C10) ascites. Peritoneal B-1 cells were cultured with SB-203580 (2 μ M or as indicated). Cultures were pulsed with ³[H] thymidine for 4 h on days 1, 2, or 3 and then harvested (Packard, Meriden, CT); the incorporated radioactivity was then measured using a Matrix 96 β -counter (Packard, Downers Grove, IL). E μ -Tcl1 cell proliferation was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay. Cells ($1-2.5 \times 10^5$) were cultured in triplicate in the presence or absence of anti-IL-10R antibody (1 μ g/ml) or anti-IL-10 antibody (1 μ g/ml). After 48 h, cells were incubated with MTT for 4 h, followed by solubilization in acidic isopropanol and spectrophotometric measurement at 560 and 690 nm. The final optical density was obtained as a difference between the two readings.

For cytokine analysis, B-1P, B-2S, and E μ -Tcl1 cells ($1-2.5 \times 10^5$) cells were cultured in triplicate for 24 h with anti-IgM F(ab')₂ (25 μ g/ml) with or without chemical inhibitors as indicated. IL-10 levels in the supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) with OptEIA kits (PharMingen, San Diego, CA). Results are presented as mean $\pm$ SD of triplicate measurements for triplicate cultures. Statistical significance of differences in response was evaluated by unpaired Student's *t*-test.

CFSE labeling and cell cycle assays

CFSE labeling was performed as described previously.²⁵ Briefly, B-1P cells from wild-type or *Il10*^{-/-} mice (resuspended at 10⁷/ml in PBS/0.1% BSA, 10 μM CFSE) were incubated at 37 °C for 20 min, and then washed with IF-12 medium (L-glutamine, gentamycin, 2-mercaptoethanol, apotransferrin, progesterone, and 10% FBS). CFSE-labeled cells (2.5 × 10⁵) were cultured in the presence of anti-IgM F(ab')₂ (50 μg/ml) or anti-CD40 (1C10) ascites (1:1000 dilution) for 48 h at 37 °C.

B-1P cells from wild-type or *Il10*^{-/-} mice (2.5 × 10⁵) were cultured with 50 μg/ml anti-IgM or 1:1000 dilution of anti-CD40 for 48 h. Cells were fixed in 70% (v/v) ethanol for at least 1 h at 4 °C, after which the cells were incubated in a mixture of 1 μg/ml propidium iodide (PI) (Sigma-Aldrich) and 25 μg/ml RNase A (Sigma-Aldrich) at 37 °C for at least 30 min. The level of PI fluorescence was measured with a flow cytometer. Cell populations at subG₁, G₁, S, and G₂/M phase were calculated using the program ModFit.

Quantitative real-time polymerase chain reaction (PCR)

Eμ-*Tcl1* cells (1 × 10⁶ cells/ml) were treated with 5 μM of Bay 61-3606 (Syk inhibitor IV) for 6 h. Total RNA was isolated using TRIZOL reagent according to the manufacturer's instructions. The isolated RNA was quantified by UV absorption with a NanoDrop. Single-stranded cDNA was synthesized using qScript cDNA SuperMix according to the manufacturer's instructions (Quanta). Levels of mRNA expression were quantified by RT-PCR using the Applied Biosystems StepOnePlus Instrument. All measurements were performed in triplicate. Expression of gene transcripts was normalized to the housekeeping gene 18S rRNA. IL-10 mRNA expression levels were measured by quantitative RT-PCR and normalized to the 18S reference gene and were analyzed using the 2^{-CT} method described by Livak and Schmittgen.⁴⁶ The *Il10* primer sequences for the forward primer was ACTGGCATGAGGATCAGCAG and for the reverse primer was AGAAATCGATGACAGCGCCT.

Western blot analysis

Eμ-*Tcl1* cells (1 × 10⁶ cells/ml) were treated with 5 μM of Bay 61-3606 (Syk inhibitor IV) for 15 min. Cell pellets were lysed using cell lysis buffer (Cell Signaling) in the presence of protease and phosphatase inhibitors (Roche Diagnostic). Proteins were separated by SDS-PAGE and processed for western blotting as described before.¹⁵ For re-probing, membranes were stripped using a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 50 min. Quantification of proteins in each lane was performed using the ImageJ program and expressed as a ratio of band intensities (phosphorylated to total protein or total protein to β-actin) to correct for unequal loading of proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hyporesponsiveness of peritoneal B-1 cells to BCR ligation is due to high IL-10 production. (A) Culture supernatants of B-2S and B-1P cells were collected after stimulation with (Fab)₂ goat anti- μ for 24 h and assayed by ELISA for IL-10. At this time point, BCR stimulation did not increase the number of either B-1P or B-2S cells recovered, indicating that the increase in IL-10 levels by B-1P cells is not due to an increase in cell number. The asterisk denotes the statistical significance ($P < 0.05$) of differences between the responses of B-1P and B-2S cells. (B) B-1P (left panel) or B-2S (right panel) cells were cultured with anti- μ antibody in the presence or absence of anti-IL-10R antibody, and proliferation was measured by ³[H]-thymidine incorporation. The P -values ($* = P < 0.05$) indicate the statistical significance of the differences between proliferation responses with and without anti-IL-10R antibody. (C) B-2S cells were cultured with exogenous IL-10 and proliferation was measured by thymidine incorporation. The asterisk ($P < 0.05$) indicates that the difference between proliferation responses with and without exogenous IL-10 is statistically significant. ND, not detectable.

Figure 2.

IL-10 inhibits B-1P cell response by arresting the cells in the G_1/S phase of cell cycle. (A) B-1P cells from C57BL/6 and $Il10^{-/-}$ mice were cultured with anti-IgM or anti-CD40, and proliferation response was measured by ^3H -thymidine incorporation. *** indicates statistical significance ($P = 0.0005$) of the differences in response between wild-type (C57BL/6) B-1P and $Il10^{-/-}$ B-1P cells. (B) IL-10 mediated inhibition of wild-type and $Il10^{-/-}$ B-1P cell proliferation responses to multiple doses of anti-IgM. The P -values ($* = P = 0.05$) denote the statistical significance of differences between the responses of B-1P from wild-type and $Il10^{-/-}$ mice. (C) CFSE-labeled B-1P cells from C57BL/6 and $Il10^{-/-}$ mice were cultured with anti-IgM and analyzed for CFSE dilution. The percentage of cells in the M1 regional gate represent CFSE dilution from the second and subsequent divisions of cells based on the ModFit analysis of CFSE fluorescence. (D) B-1P cells from C57BL/6 and $Il10^{-/-}$ mice were cultured with anti-IgM or anti-CD40 and cell cycle analysis was performed by propidium iodide staining. The fraction of cells in S/ G_2 /M was determined and plotted. The statistical significance of difference in response between wild-type and $Il10^{-/-}$ B-1P cells in response to anti-IgM is shown by $* = P = 0.005$.

Figure 3.

BCR-induced IL-10 production by B-1P cells requires p38 MAPK. (A) B-1P cells were stimulated with anti- μ and treated with SB-203580, a p38 MAPK inhibitor. Culture supernatants were assayed by ELISA for IL-10. The P -values (* = $P < 0.05$) indicate the significance of differences between IL-10 levels with or without P38 MAPK inhibitor. (B) Dose dependence of cell proliferation responses to anti- μ in the presence or absence of SB-203580 (2 μ M) was measured by 3 [H]-thymidine incorporation. (C) Proliferation responses of B-1P cells were measured after treatment with p38 MAPK inhibitor or anti-IL-10R antibody alone or together. (D) B-1P and B-2S cell lysates were probed with the indicated antibodies. Phospho-protein levels were normalized to total protein levels and total protein levels were normalized to β -actin.

Figure 4.

IL-10 production by both peritoneal and splenic Eμ-*TcII* cells. (A) Normal B-1 cells and peritoneal and splenic Eμ-*TcII* cells (10^5 cells) were cultured in 200 μl of medium for 24 h. IL-10 was measured in the supernatant of the cells by ELISA. Values represent mean $\pm$ SE of triplicate cultures. (B) Eμ-*TcII* cells (10^5 cells) were cultured in the presence or absence of anti-IL-10R antibody or anti-IL-10 antibody and the survival was measured by MTT assay.

Figure 5.

IL-10 may be transcriptionally regulated in the E μ -Tcl1 CLL cells. Primary E μ -Tcl1 CLL cells were cultured with 5 μ M BAY 61-3606, a Syk specific inhibitor, for 6 h. IL-10 mRNA expression levels were measured by qRT-PCR and normalized to 18S reference gene to control the variability in expression levels and were analyzed using the 2^{-CT} method described by Livak and Schmittgen.⁴⁶ The *P*-values (* = *P* < 0.05) indicate the significance of differences between IL-10 mRNA levels with or without Syk inhibitor.

Table 1

Constitutive IL-10 production by normal and malignant B-1 cells is dependent on BCR signaling

	IL-10 (pg/ml) (% viable cells)	
	Normal B-1 cells	E μ -Tcl1 cells
Media	141 (99%)	572 (97%)
BAY 61-3606 (2 μ M)	9 (92%)	250 (90%)
Dasatinib (2 μ M)	5 (89%)	222 (86%)
Ibrutinib (2 μ M)	ND (87%)	134 (85%)

Note: Constitutive IL-10 levels by normal B-1 cells and E μ -Tcl1 cells in the presence or absence of specific inhibitors of the BCR signaling pathway were measured after 24 h of culture. Values in parentheses represent viability of the cells after 24 h of treatment with the inhibitors. BAY 61-3606 is a Syk family kinase-specific inhibitor. Dasatinib is a Src family kinase inhibitor.

Ibrutinib (or PCI-32765) is a Btk specific inhibitor. ND, not detectable.