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Intravenous Transfusion of BCR-Activated B Cells Protects NOD Mice from Type 1 Diabetes in an IL-10-Dependent Manner

Shabbir Hussain* and Terry L. Delovitch2*†

Although B cells play a pathogenic role in the initiation of type 1 diabetes (T1D) in NOD mice, it is not known whether activated B cells can maintain tolerance and transfer protection from T1D. In this study, we demonstrate that i.v. transfusion of BCR-stimulated nodular spleen B cells into NOD mice starting at 5–6 wk of age both delays onset and reduces the incidence of T1D, whereas treatment initiated at 9 wk of age only delays onset of T1D. This BCR-activated cell-induced protection from T1D requires IL-10 production by B cells, as transfusion of activated B cells from nodular IL-10−/− mice does not confer protection from T1D. Consistent with this result, severe insulitis was observed in the islets of NOD recipients of transfused nodular IL-10−/− BCR-stimulated B cells but not in the islets of NOD recipients of transfused BCR-stimulated nodular B cells. The therapeutic effect of transfused activated nodular B cells correlates closely with the observed decreased islet inflammation, reduced IFN-γ production and increased production of IL-4 and IL-10 by splenocytes and CD4+ T cells (1–3). Although the onset of T1D is associated with a Th1-biased immune response, the cellular mechanisms that are causal to or regulate T1D are only partially understood (1, 3, 4). CD4+ Th1 cells promote cell-mediated immunity by producing increased amounts of IL-2 and IFN-γ, whereas CD4+ Th2 cells trigger and sustain humoral immune responses by producing more IL-4 and IL-10. Thus, shifting the paradigm of a Th1 cytokine-rich environment toward a Th2-polarized environment was proposed as a mode of therapy to prevent T1D (1–4). This hypothesis has been widely tested by using various agents to induce Th2 responses in the NOD mouse model of T1D. Such studies have achieved variable success in protection from T1D, depending on the nature of the therapeutic agent, e.g., IL-10 (5, 6), and the stage of insulitis at which treatment was initiated (7). This variability suggests that the Th1/Th2 paradigm underestimates the complexity of the pathogenesis of T1D, in part due to the ability of Th1 and Th2 cytokines to modulate the function of not only Th cells but also many other cell types (7). Thus, despite the important role of Th1 cells in the pathogenesis of T1D, Th1/Th2 modulation may be insufficient as a therapy for T1D, as exemplified by the recent demonstration of the roles of other types of T cells including CD4+CD25−FoxP3− regulatory T cells (Tregs) (8), CD4+ invariant NKT cells (9–12) and CD4+ T regulatory type 1 (Tr1) cells (13, 14) that each modulate inflammation and protect from T1D. Tregs (8) and Tr1 cells (13, 14), but not invariant NKT cells (15), can mediate protection from T1D by an IL-10-dependent mechanism.

IL-10 is a pleiotropic cytokine with well-known anti-inflammatory and immunosuppressive properties (16). In addition to blocking inflammatory responses via the inhibition of IFN-γ, IL-1β, IL-2, IL-6, IL-12, and TNF-α production by T cells, NK cells, and monocytes and macrophages, IL-10 can also induce T cell anergy (16, 17). Thus, polarization of an immune response toward a Th2 phenotype and regulation of the effector function of self-reactive T cells may represent important mechanisms of prevention of T1D (18, 19).

Systemic IL-10 expression via IL-10 gene transfer protects NOD mice from T1D (5, 6, 18, 19). Nonetheless, the potential health hazard of viral gene transfer and practicality of using recombinant IL-10 to deviate an immune response is limited in part by the relatively short plasma half-life (t1/2 = 2 min) of IL-10 in vivo (20, 21). Systemic administration of IL-10 for T1D protection also requires the repeated injection of nonphysiological doses of IL-10, which may give rise to adverse immune responses (20, 21). These findings suggest that a physiological source of IL-10 that polarizes an immune response toward a Th2 phenotype and induces anergy in self-reactive T cells would be advantageous for protection from T1D, and raise the possibility that this protection

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2 Address correspondence and reprint requests to Dr. Terry L. Delovitch, Laboratory of Autoimmune Diabetes, Robarts Research Institute, 100 Perth Drive, London, Ontario N6A 5K6, Canada. E-mail address: del@robarts.ca

3 Abbreviations used in this paper: T1D, type 1 diabetes; DC, dendritic cell; BGL, blood glucose level; Treg, regulatory T cell; Tr1, T regulatory type 1; EAE, experimental autoimmune encephalomyelitis; FoxP3, FoxP3 ligand.
may be achieved by activated B cell therapy. Because B cells activated by either LPS or BCR stimulation produce IL-10 and survive in a host for 2–3 wk after transfusion (22, 23), it is possible that a large number of B cells can be obtained from human peripheral blood and then activated in vitro before reinfusion into a susceptible individual. The result that activated B cells also express a membrane-bound form of TGF-β that can enhance tolerance induction (24) further supports the notion that activated B cells may provide a therapy for T1D in humans.

In this study, we investigated the role of BCR-stimulated B cells in the protection of NOD mice from T1D and the contribution of IL-10 to this protection. We found that short-term treatment of NOD mice by i.v. transfusion of NOD-derived BCR-stimulated B cells before the establishment of insulitis delays the onset and reduces the incidence of T1D. Protection from T1D was associated with the up-regulation of IL-10 production by BCR-stimulated B cells and CD4+ T cells in NOD recipients of NOD-derived BCR-stimulated B cells. In contrast, donor BCR-stimulated B cells from NOD.IL-10−/− mice, which are as susceptible to T1D as wild-type NOD mice (25), did not transfer protection from T1D into NOD recipients. Our results demonstrate that transfused BCR-stimulated B cells can maintain long-term tolerance and protect NOD mice from T1D by an IL-10-dependent mechanism. These findings suggest that i.v. transfusion of human subjects at high risk for T1D is a prophylactic treatment for T1D.

Materials and Methods

Mice

NOD/Scid and NOD.Scid mice were bred in specific pathogen-free barrier facilities at the University of Western Ontario (London, Ontario, Canada). NOD.IL-10−/− mice were obtained from The Jackson Laboratory, and then bred in specific pathogen-free barrier facilities at the University of Western Ontario (London, Ontario, Canada). In our colony of female NOD mice, islet infiltration begins at 4–6 wk of age, and progression to destructive insulitis and overt diabetes occurs by 4–6 mo of age.

Cell isolation and purification

Splenocytes were prepared by pressing spleens through a 40-μm nylon strainer. Spleen B cells were purified (purity ≥98%) using a B cell-enrichment mixture according to the manufacturer’s instructions (StemCell Technologies). T cells were enriched by positive selection using CD4+ T cell columns (R&D Systems), respectively, as described (23).

B cell stimulation, purification, and transfection

B cells were stimulated by culturing splenocytes in vitro with an anti-IgM F(ab′)2 Ab (10 μg/ml; Jackson ImmunoResearch Laboratories) for 48 h and then purified by using a B cell-enrichment mixture (StemCell Technologies), as described (23). Bacterial endotoxin (LPS) contamination in the anti-IgM F(ab′)2 Ab preparation used was shown to be below the limits of detection (<0.1 EU/ml) as determined using a Limulus amebocyte lysate assay (BioWhittaker), according to the manufacturer’s instructions. The purity of these B cell preparations was estimated to be ≥98%, as assessed by flow cytometry performed using an FITC anti-B220 mAb (BD Biosciences). Female NOD mice were i.v. transfused with 1.2×10^6 or 5×10^6 purified activated B cells starting at 5–6 or 9 wk of age. Activated B cells were administered three times, once every 2–3 wk during a period of 6–9 wk. The incidence of T1D was determined by measuring blood glucose levels (BGL) every wk starting at 12 wk of age. BGL over 11 mm for two consecutive readings was considered positive for T1D (23).

Flow cytometry

Splenocytes were incubated for 15 min at 4°C with a blocking agent (anti-CD16/32) to reduce the nonspecific binding of test Abs. The cells were then stained at 4°C for 45 min with FITC anti-B220, PE anti-CD86, and PE-anti-CD40 mAbs. All Abs were obtained from BD Biosciences. The cells were washed three times with PBS containing 0.1% sodium azide plus 2% FCS, and analyzed by flow cytometry using BD CellQuest software.

Histopathology

To determine the severity of insulitis, pancreata were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 5-μm intervals, stained with H&E, and analyzed as described (26). A minimum of 30 islets from each mouse was observed, and insulitis scores were determined as follows: 0, normal; 1, peri-insulitis (mononuclear cells surrounding islets and ducts but no infiltration of the islet architecture); 2, moderate insulitis (mononuclear cells infiltrating, <50% of the islet architecture); and 3, severe insulitis (>50% of the islet tissue infiltrated by lymphocytes).

Cell proliferation

Splenocytes and purified T cells (2×10^6/well) were stimulated with plate-bound anti-CD3 (2 μg/ml), and purified B cells (10^7/well) were stimulated with an anti-IgM F(ab′)2 Ab (10 μg/ml) for 64 h in a 96-well tissue culture plates in complete RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mmol/l HEPES buffer, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 μmol/l 2-ME (Invitrogen Life Technologies) at 37°C in 5% CO2. [3H]Thymidine (1 μCi/well) was added during the last 18 h of culture, and the cells were harvested and assayed for [3H]thymidine incorporation in cpm (23).

Cytokine secretion and quantification

Splenocytes and purified spleen CD4+ T cells (2×10^5/ml) from PBS-treated control NOD mice and NOD recipients of BCR-stimulated NOD or NOD.IL-10−/− B cells were stimulated with plate-bound anti-CD3 (2 μg/ml) for 48 h. To determine IL-10 production by B cells, an equal number of purified NOD B cells and B cell-depleted splenocytes from NOD.IL-10−/− mice were cocultured (4×10^5/ml) in the presence or absence of anti-CD3 (2 μg/ml) and anti-IgM F(ab′)2 Ab (10 μg/ml) for 48 h. Cell supernatants were collected and frozen at −70°C until use. The concentrations of IL-2, IL-4, IL-10, and IFN-γ were determined using mouse Quantikine ELISA kits from R&D Systems according to the manufacturer’s instructions (23).

Adoptive transfer of splenocytes into NOD.Scid mice

NOD.Scid mice (6– to 7-wk-old) were i.v. injected with splenocytes (15×10^6 or 5×10^6) from NOD mice previously treated with PBS or transfused with BCR-stimulated B cells from either NOD or NOD.IL-10−/− mice. The incidence of T1D was determined by measuring BGL twice weekly starting at 2 wk posttransfer, as described.

Statistical analysis

Each experiment was repeated three times. Statistical analyses of the data were performed using a two-sided Student’s t test and log rank test where appropriate. Values for p < 0.05 was considered significant. Data are presented as the mean value ± SD.

Results

BCR-stimulated B cells produce IL-10

In addition to the ability of IL-10 to stimulate B cell proliferation and growth (27), IL-10 can inhibit T cell function and augment natural Treg differentiation (28). In addition to the ability of IL-10 to stimulate B cell proliferation and growth (27), IL-10 can synergize in vitro with IL-2, IL-4, IL-10, and IFN-γ. We determined using mouse IL-4, IL-6, IL-10, and IFN-γ were determined using mouse Quantikine ELISA kits from R&D Systems according to the manufacturer’s instructions (23).

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Results

BCR-stimulated B cells produce IL-10

In addition to the ability of IL-10 to stimulate B cell proliferation and growth (27), IL-10 can synergize in vitro with IL-2, IL-4, and IL-5 to enhance B survival and activation (16). These various effects of IL-10 on B cell function coupled with its capacity to modulate Ag presentation by dendritic cells (DCs) and macrophages, inhibit T cell function and augment natural Treg differentiation have rendered IL-10 an interesting cytokine to target in the modulation of autoimmune disease (28, 29). Because it was of interest to analyze the ability of BCR-stimulated B cells to protect NOD mice from T1D, we initially assayed the level of IL-10 production by BCR-stimulated NOD B cells. To mimic T-B cell interactions required for B cell cytokine production in vivo, NOD.IL-10−/− B cell-depleted splenocytes were cocultured with purified NOD B cells in vitro for 48 h in the presence or absence of plate-bound anti-CD3 (2 μg/ml) mAb and anti-IgM F(ab′)2 Ab to stimulate T cells and B cells, respectively. Assays of IL-10 concentrations in these culture supernatants determined by ELISA indicate that BCR-activated NOD B cells produced the greatest amount of IL-10 in the presence of NOD.IL-10−/− T cells (Fig. 1). As expected, the latter T cells did not secrete IL-10. These results suggest that BCR-activated NOD B cells secrete detectable levels of
IL-10 in the presence of activated T cells, although the concentration of IL-10 detected was only about one-third of that detected in splenocyte cell cultures.

**IL-10 is required to induce BCR-stimulated B cell-mediated protection of NOD mice from T1D**

To determine whether BCR-stimulated IL-10-producing B cells protect NOD mice from T1D, NOD mice were i.v. transfused with BCR-stimulated syngeneic B cells in PBS starting at 5–6 wk of age (before development of invasive insulitis) or at 9 wk of age (before development of destructive insulitis). Control groups of NOD mice received PBS or unstimulated NOD B cells. Transfusions were repeated every 2–3 wk and a total of three transfusions were administered over 6–9 wk. In NOD mice that received NOD BCR-stimulated B cells beginning at 5–6 wk of age, the onset of T1D occurred at 25 wk representing a delay of 7–9 wk compared with control NOD mice that received either PBS or unstimulated B cells from NOD.IL-10 mice (Fig. 2A). A significantly lower incidence of T1D (30–40%) was observed at 30 wk of age in NOD recipients of BCR-stimulated B cells than in control NOD recipients of PBS-transfused NOD B cells (75%) or NOD.IL-10 BCR-stimulated B cells (85–90%). In contrast, when NOD BCR-stimulated B cell treatment was initiated at 9 wk of age, only a 5-wk delay (from 13 to 18 wk of age) in the onset of T1D was observed compared with PBS-treated control mice or NOD recipients of nonactivated syngeneic B cells (Fig. 2B). In addition, the incidence of T1D was similar (90%) at 30 wk of age in NOD recipients of NOD BCR-stimulated B cells, NOD nonactivated B cells, or PBS. These results indicate that protection from T1D occurs if transfusion of IL-10-producing syngeneic BCR-activated B cells is initiated before the development of invasive insulitis.

To further investigate the role of IL-10-producing BCR-activated B cells in protection from T1D, we analyzed whether splenocytes from NOD recipients of BCR-stimulated B cells from different donor mice can adoptively transfer protection from T1D to NOD.Scid mice. NOD.Scid recipients of splenocytes (15 × 10⁶) from NOD mice previously transfused with BCR-stimulated B cells from NOD, NOD.IL-10, or PBS-treated control mice obtained 5 days after the last treatment were monitored for the onset of T1D. The incidence of T1D was similar in NOD.Scid recipients of splenocytes from NOD, NOD.IL-10, and PBS-treated mice (Fig. 3A), but the onset of hyperglycemia was delayed in NOD.Scid recipients of splenocytes from BCR-stimulated NOD mice compared with recipients of splenocytes from NOD.IL-10 mice. Comparable results were obtained when splenocytes (5 × 10⁶) from NOD mice previously transfused with BCR-stimulated B cells from NOD, NOD.IL-10, or PBS-treated control mice obtained 21 days after the last treatment were monitored for their incidence of T1D (Fig. 3B). However, in the latter case, the incidence of T1D reached 100% at 75–90 days (Fig. 3B) rather than at 35–50 days (Fig. 3A), likely due to the fewer B cells transferred.

**BCR-stimulated B cells from NOD and NOD.IL-10 mice exhibit a similar phenotype and functional response**

To determine why BCR-stimulated B cells from NOD but not NOD.IL-10 mice provide protection from T1D, we compared phenotypic and functional responses of BCR-stimulated B cells from NOD and NOD.IL-10 mice. We did not find any difference in the up-regulation of expression of the CD86 and CD40 costimulatory molecules on BCR-stimulated B cells from NOD and NOD.IL-10 mice (Fig. 4A). Similarly, functional analyses of B cell proliferation following α-IgM F(ab’)_2 (10 μg/ml) stimulation did not reveal any difference in the level of proliferation between NOD and NOD.IL-10 B cells (Fig. 4B). These results suggest that despite the similar level of expression of costimulatory molecules and proliferative response to BCR stimulation, BCR-activated B cells from NOD and NOD.IL-10 mice differ in their ability to transfer protection from T1D.
BCR-activated B cells attenuates islet inflammation in NOD mice

Our finding that protection from T1D results when transfusion of IL-10-producing BCR-activated B cells is initiated before invasive insulitis suggested that this protection may be associated with a reduced severity of insulitis. To test this possibility, NOD recipients of BCR-stimulated B cells from NOD or NOD.IL-10−/− mice beginning at 5 wk of age or PBS-treated control NOD mice were sacrificed at 13 wk of age. Histological examination of the pancreas indicated that insulitis is more severe in mice that received BCR-stimulated B cells from NOD.IL-10−/− mice compared with NOD recipients of BCR-stimulated NOD B cells (Fig. 5). Although the levels of insulitis in NOD recipients of BCR-stimulated NOD B cells and PBS-treated NOD mice appear to be similar, note that a grade 3 severe insulitis was detected only in the PBS-treated NOD mice (Fig. 5A). These data suggest that transfusion of BCR-stimulated B cells protects NOD mice from T1D in part by attenuating the severity of islet inflammation, and may have a greater role in protection from T1D.

Transfusion of BCR-stimulated B cells protects NOD mice from T1D but does not overcome T cell hyporesponsiveness to TCR stimulation

Loss of protection from T1D in NOD recipients of BCR-stimulated B cells from NOD.IL-10−/− mice compared with NOD recipients of BCR-stimulated B cells from syngeneic NOD mice prompted us to investigate the functional responses of recipient spleen cells. We found that splenocytes obtained from NOD recipients 3 wk after the last transfusion of syngeneic BCR-stimulated B cells display a hyporesponsiveness to anti-CD3 stimulation compared with splenocytes from PBS-treated control NOD mice (p < 0.05) or NOD recipients of BCR-stimulated B cells from NOD.IL-10−/− mice (p < 0.001) (Fig. 6A).

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FIGURE 3. Transfusion of splenocytes from PBS-treated control NOD and NOD recipients of BCR-stimulated NOD or NOD.IL-10−/− B cells yields a similar incidence but different kinetics of onset of T1D in NOD.Scid recipients. A, Splenocytes (15 × 10⁶) from PBS-treated NOD or NOD recipient of BCR-stimulated NOD or NOD.IL-10−/− B cells (n = 8) were prepared 5 days after the last treatment and transfused i.v. into 6- to 7-wk-old NOD.Scid mice. B, Splenocytes (5 × 10⁶) from PBS-treated NOD or NOD recipients of BCR-stimulated NOD or NOD.IL-10−/− B cells (n = 8) were prepared 21 days after the last treatment and i.v. transfused into 6- to 7-wk-old NOD.Scid mice. The incidence of T1D was monitored by measurement of the BGL twice weekly starting at 2 wk (A) or 4 wk (B) posttransfer. The cumulative incidence of T1D was determined as a percentage of the total number of recipient mice that developed T1D at each time point.

BCR-activated B cells display a hyporesponsiveness to anti-CD3-stimulation

Recipient spleen cells from NOD recipients of BCR-stimulated B cells display a hyporesponsiveness to anti-CD3 stimulation compared with splenocytes from control NOD mice (p = 0.001). The data indicate that activated B cells from NOD recipients of BCR-stimulated B cells display a hyporesponsiveness to anti-CD3 stimulation, which is similar to that observed in activated T cells from NOD recipients of BCR-stimulated B cells (Fig. 6B).

Transfusion of BCR-stimulated B cells suppresses anti-CD3-induced splenocyte proliferation in NOD recipient mice but does not overcome T cell hyporesponsiveness to TCR stimulation

The data were obtained from NOD recipients of BCR-stimulated B cells from NOD or NOD.IL-10−/− mice beginning at 5 wk of age or PBS-treated control NOD mice. The mice were sacrificed at 13 wk of age. Histological examination of the pancreas indicated that insulitis is more severe in mice that received BCR-stimulated B cells from NOD.IL-10−/− mice compared with NOD recipients of BCR-stimulated NOD B cells (Fig. 5). Although the levels of insulitis in NOD recipients of BCR-stimulated NOD B cells and PBS-treated NOD mice appear to be similar, note that a grade 3 severe insulitis was detected only in the PBS-treated NOD mice (Fig. 5A). These data suggest that transfusion of BCR-stimulated B cells protects NOD mice from T1D in part by attenuating the severity of islet inflammation, and may have a greater role in protection from T1D.

Transfusion of BCR-stimulated B cells protect T1D in NOD recipients of BCR-stimulated B cells from NOD.IL-10−/− mice compared with NOD recipients of BCR-stimulated B cells from syngeneic NOD mice prompted us to investigate the functional responses of recipient spleen cells. We found that splenocytes obtained from NOD recipients 3 wk after the last transfusion of syngeneic BCR-stimulated B cells display a hyporesponsiveness to anti-CD3 stimulation compared with splenocytes from PBS-treated control NOD mice (p < 0.05) or NOD recipients of BCR-stimulated B cells from NOD.IL-10−/− mice (p < 0.001) (Fig. 6A).

FIGURE 4. BCR-stimulated B cells from NOD and NOD.IL-10−/− mice exhibit a similar phenotype and functional response. A, Splenocytes from NOD and NOD.IL-10−/− mice (n = 4) were stimulated with anti-IgM F(ab′)2, Ab (10 μg/ml) for 48 h. The percentage of B220+ cells that express CD86 and CD40 was determined by FACS analysis. B, Purified spleen B cells (10⁵/well) from NOD and NOD.IL-10−/− mice were stimulated with anti-IgM F(ab′)2, Ab (10 μg/ml) in 96-well tissue culture plates for 64 h. Cell proliferation was determined by [3H]thymidine incorporation. Results of triplicate cultures are expressed as the mean ± SD, and data from one of three representative and reproducible experiments are shown.
Previously, we reported that a decreased percentage of B7-2<sup>+</sup>/H<sub>11001</sub>B<sup>+</sup> cells in NOD mice (23) before the onset of invasive and destructive insulitis may elicit a CD4<sup>+</sup>/H<sub>11001</sub>CD25<sup>+</sup>/H<sub>11001</sub>Treg deficiency (30, 31) and T cell hyporesponsiveness to TCR stimulation (32, 33). In addition, we found that the ability of BCR-stimulation in vivo in NOD.<sub>Scid</sub> mice is associated with ability of BCR-activated B cells from NOD mice to costimulate T cells and enhance their proliferation and expansion (23). Therefore, in this study, we investigated whether the requirement of IL-10 production by BCR-activated B cells to protect NOD mice from T1D is mediated by the ability of T cells to overcome their hyporesponsiveness to anti-CD3 stimulation. Interestingly, we observed a similar level of anti-CD3 induced proliferation of purified spleen T cells from NOD recipients of BCR-stimulated B cells from NOD or NOD.<sub>IL-10</sub>−/− mice or from PBS-treated control NOD mice (Fig. 6B). These data suggest that IL-10 production by BCR-activated B cells is not required to overcome T cell hyporesponsiveness to TCR stimulation in NOD mice, consistent with our previous reports that T cell hyporesponsiveness is associated with the development of insulitis and not T1D (23, 34).

Transfusion of BCR-stimulated B cells polarizes CD4<sup>+</sup> T cell responses toward a Th2 phenotype

To analyze whether and how BCR-stimulated B cell treatment influences T cell-mediated responses, the cytokine (IL-2, IL-4, IL-10, and IFN-γ) secretion profiles of splenocytes and purified spleen CD4<sup>+</sup> T cells from PBS-treated control NOD mice and NOD recipients of BCR-stimulated syngeneic NOD or NOD.<sub>IL-10</sub>−/− B cells were assayed following stimulation in vitro with an anti-CD3 mAb for 48 h. Although IL-4 and IL-10 secretion by activated splenocytes and CD4<sup>+</sup> T cells from NOD recipients of syngeneic BCR-stimulated B cells were significantly increased relative to that observed for cells from PBS-treated control NOD mice, the levels of IFN-γ secretion by activated splenocytes and CD4<sup>+</sup> T cells from NOD recipients of syngeneic BCR-stimulated B cells were significantly decreased compared with that secreted by cells from PBS-treated control NOD mice (Fig. 7). In addition, a lower amount of IL-10 secretion was detected in activated splenocytes from NOD recipients of BCR-activated B cells from NOD.<sub>IL-10</sub>−/− mice compared with NOD recipients of BCR-activated B cells from NOD mice. A similar reduction in IL-2 secretion was detected for CD4<sup>+</sup> T cells but not splenocytes from NOD mice transfused with NOD BCR-activated B cells vs PBS-treated control mice. Thus, transfusion of NOD mice with syngeneic BCR-stimulated B cells activates and polarizes the T cells of these recipients toward a Th2 phenotype.

Discussion

B cells possess diverse immunological functions and can play both regulatory and pathogenic roles in autoimmune disease (35–42). This study investigates the regulatory role of BCR-stimulated B cells in protection from T1D in NOD mice. Although BCR-stimulated and LPS-stimulated B cells both produce IL-10 and B cells from lupus mice can produce more IL-10 when stimulated by

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**FIGURE 6.** Transfusion of BCR-stimulated B cells suppresses anti-CD3-induced splenocyte proliferation in NOD recipient mice but does not overcome T cell hyporesponsiveness to TCR stimulation. Splenocytes (A) and purified spleen T cells (B) from PBS-treated NOD or NOD recipients of NOD or NOD.<sub>IL-10</sub>−/− BCR-stimulated B cells were activated with plate-bound anti-CD3 mAb (2 μg/ml) for 64 h. Cell proliferation was determined by [3H]thymidine incorporation. Results of triplicate cultures are expressed as the mean ± SD, and data from one of three representative and reproducible experiments are shown.

**FIGURE 7.** Transfusion of BCR-stimulated B cells modulates cytokine production in splenocytes from recipient NOD mice. Cytokine concentrations (IL-2, IL-4, IL-10, and IFN-γ) in supernatants of cultures (48 h) of splenocytes or purified CD4<sup>+</sup> T cells stimulated by plate-bound anti-CD3 mAb were assayed by ELISA. Results of triplicate cultures are expressed as the mean ± SD. Data are from one of three representative and reproducible experiments. *, p < 0.05; **, p < 0.001 for values of cells from PBS-treated control and BCR-activated B cells from NOD.<sub>IL-10</sub>−/− recipients vs BCR-stimulated NOD B cell recipients.
TLR4 (LPS) and TLR9 (CpG oligodeoxynucleotides) ligands than BCR stimulation (43, 44), we analyzed the activity of BCR-stimulated rather than TLR4 or TLR9 activated B cells in protection from T1D for the following reasons. First, IL-10 production by B cells from lupus mice was assayed after BCR stimulation by intact anti-IgM Abs (43, 44). This suggests that the significantly reduced amount of IL-10 production observed in comparison to that detected after TLR4 and TLR9 stimulation was due to an FcγRII-mediated inhibition of B cell activation. In contrast, both in our previous studies (23) and in this study, we used an anti-IgM F(ab\(^{-}\)b\(_{2}\)) to activate B cells via the BCR to avoid FcγRII-mediated inhibition of B cell activity. Second, BCR stimulation by an anti-IgM F(ab\(^{-}\)) induces B7-2 but not B7-1 expression on B cells (45, 46), whereas LPS-stimulation via TLR4 enhances both B7-1 and B7-2 expression on B cells (47). This distinction between BCR and TLR4 stimulation is important because we previously found a correlation between a decreased percentage of B7-2\(^{+}\) spleen B cells and an impaired homeostasis of CD4\(^{+}\)CD25\(^{+}\) Tregs in NOD mice, and that these deficiencies in B7-2\(^{+}\) B cells and Treg homeostasis were overcome upon stimulation with an anti-B7-2 mAb (23). We reasoned therefore that it might be advantageous to stimulate B cells via the BCR to increase the frequency of B7-2\(^{+}\) spleen B cells, correct the impaired homeostasis of CD4\(^{+}\)CD25\(^{+}\) Tregs and prevent T1D in these mice. Third, human B cells do not express TLR4 (43), which would preclude the possibility of treating individuals at risk for T1D with a TLR4 ligand to attempt to prevent T1D. Fourth, TLR9 responds to bacterial DNA and treatment of individuals at risk for autoimmune disease with TLR9 can cause severe kidney disease (48, 49).

Our results demonstrate that i.v. transfusion of syngeneic BCR-stimulated B cells protect NOD mice from T1D. This protection depends on the up-regulation of IL-10 production by BCR-stimulated B cells, as BCR-stimulated B cells from NOD.IL-10\(^{-}\/\) mice do not transfer protection from T1D into NOD recipients (Fig. 2). Up-regulation of IL-10 production by BCR-stimulated B cells in the recipient NOD mice supports a role for IL-10 in this B cell-mediated protection from T1D (Fig. 7).

A previous report that i.v. transfusion of LPS-stimulated B cells protects NOD mice from T1D showed that these LPS-stimulated B cells do not produce IL-10 but rather secrete TGF-\(\beta\) and express Fas ligand (FasL) on their cell surface (50). Thus, protection from T1D was proposed to be mediated by the killing of Fas\(^{+}\) autoreactive T cells via Fas-FasL interaction and the immunosuppressive effect of TGF-\(\beta\) (50). Nonetheless, our results rule out the possibility of apoptosis of Fas\(^{+}\) autoreactive T cells by FasL\(^{+}\) B cells through Fas-FasL interaction as we previously reported that BCR-stimulated B cells fail to express FasL on their surface (36).

Rather, our data indicate that B cell-mediated protection from T1D is mediated by increased IL-10 production by B cells in the presence of host immune cells. B cells transduced with retroviruses expressing the glutamic acid decarboxylase Gad65-IgG or (Pro) insulin-IgG fusion proteins can also protect from T1D in young NOD female mice via a CD4\(^{+}\)CD25\(^{+}\) Treg-dependent mechanism (51). A regulatory role of B cells in other models of Th1-mediated autoimmune diseases has also been described (38–41). The severity of experimental autoimmune encephalomyelitis (EAE) is greater in B cell-deficient mice (41). Bone marrow chimeras, in which B cells but not T cells or professional APCs are deficient in IL-10 production, develop a severe nonremitting form of EAE (38). However, transfer of B cells from normal mice that had recovered from EAE could rescue this defect, suggesting a role for IL-10 produced by B cells in remission from EAE. In a collagen-induced arthritis model, transfer of CD40-activated B cells obtained from arthritic mice to collagen-immunized recipients inhibited the development of arthritis (40). This inhibitory effect of the B cells activated via CD40 stimulation in vitro on arthritis development was also attributed to increased IL-10 production by these B cells. Importantly, the subset of regulatory B cells that produce IL-10 and protect from EAE (38) and arthritis (40) are dependent on T cell help for IL-10 production. Similarly, we have shown that the ability of B cells activated by their BCR to produce IL-10 require T cell help (Fig. 1). Because marginal zone B cells produce IL-10 in the absence of T cell help (43, 44, 52), our findings suggest that BCR activated IL-10-producing B cells that mediate the transfer of protection from T1D in NOD mice are not marginal zone B cells. Thus, our results together with the mentioned studies support the notion that IL-10 produced by B cells is important for the inhibition of Th1-mediated autoimmune diseases.

IL-10 is secreted by a variety of cell types including B cells (40, 50), macrophages (53), DCs (54), mast cells (55), and T cells (56), and IL-10 production is regulated by IL-12 secreted by macrophages and DCs in the presence or absence of B cells. In a mouse model of EAE, CD4\(^{+}\) T cells from anti-IL-12-treated mice fail to produce IL-10 when cultured alone but IL-10 production is restored after coculturing CD4\(^{+}\) T cells with B cells (57). This suggests that B cells not only produce IL-10 but also enhance IL-10 production by CD4\(^{+}\) T cells. The latter idea may explain our finding of increased IL-10 production by splenocytes from BCR-stimulated B cell-treated mice (Fig. 7).

Our results indicate that i.v. transfusion of B cells into NOD mice can polarize T cell responses of these recipients toward a Th2-like phenotype as evidenced by the increased secretion of IL-4 and IL-10 by their splenocytes and CD4\(^{+}\) spleen T cells (Fig. 7). B cells can regulate the development of a Th2 response, as CD11c\(^{+}\) spleen DCs from B cell-deficient mice produce higher levels of IL-12 upon CD40 stimulation and display an impaired ability to induce IL-4 secretion by T cells, most likely due to decreased levels of IL-10 production by splenocytes (58). Furthermore, spleen DCs from IL-10-deficient mice display properties similar to DCs from B cell-deficient mice, and treatment of DCs from B cell-deficient mice with IL-10 restores the generation of IL-4-producing cells in vivo (58). A preferential Th1 cytokine profile observed in patients with X-linked (Bruton’s) agammaglobulinemia, who lack peripheral circulating B cells due to Btk mutations (59), further suggests a role for B cells in the development of a Th2 response. Collectively, these observations support our findings that IL-10 produced by B cells is important in the generation of a Th2 response by increasing IL-4 production by CD4\(^{+}\) T cells.

Notwithstanding, given that IL-10 is produced by many cell types including IL-10-producing Tr1 cells that can also protect NOD mice from T1D (13, 14), polarization toward a Th2 response may be a necessary but not sufficient requirement for protection from T1D achieved by the transfer of BCR-activated B cells. For example, a combined rapamycin plus IL-10 treatment efficiently protects from T1D and induces long-term immune tolerance in the absence of chronic immunosuppression in NOD mice (13). This protection and tolerance are mediated by the ability of rapamycin to promote the accumulation of CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\) Tregs in the pancreas and of IL-10 to activate Tr1 cells that reside in the spleen and prevent migration of diabetogenic T cells to the draining pancreatic lymph nodes. These results indicate that the site of localization and activation of Tregs and Tr1 cells is important, as systemic overexpression of IL-10 in 4-wk-old NOD female mice ameliorates T1D by the induction of Tr1 cells (18) whereas transgenic NOD mice expressing IL-10 in the islets display severe insulitis and accelerated onset of T1D (60). Thus, Tregs and Tr1 cells can cooperate to protect against T1D (13) in a manner similar to that we recently described for the cooperation of Tregs and...
invariant NKT cells in protection from T1D (61). Whether protection of NOD mice from T1D by the transfection of IL-10-producing BCR-stimulated B cells is mediated by the enhanced activity of Tr1 cells, Tregs and/or invariant NKT cells is being addressed in ongoing studies.

Finally, it is important to consider the potential therapeutic value of our studies. In humans, IL-10 is produced by both Th1 and Th2 cells (62) and can suppress IgE production by B cells (63). Based on its ability to suppress cell-mediated and Ab-mediated responses, IL-10 is now considered to be a major immunosuppressive cytokine with potential as a therapy for various inflammatory diseases. For example, it was recently shown that IL-10-producing B cells can protect neonatal mice from acute inflammation by the down-regulation of proinflammatory cytokine secretion by placymyoid and conventional DCs (64). Our results build on this idea and raise the intriguing possibility that transfection of immunomodulatory IL-10-producing BCR-activated B cells may provide even greater therapeutic value in the clinic than IL-10 itself for protection from not only T1D but also other Th1-mediated autoimmune diseases. This strategy depends on the use of an anti-IgM F(ab')2 biologic reagent to activate and expand syngeneic peripheral immune diseases. This strategy depends on the use of an anti-IgM F(ab')2 biologic reagent to activate and expand syngeneic peripheral

References

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Disclosures

The authors have no conflicts of interest.

References


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