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Cutting Edge: A Critical Role of B and T Lymphocyte Attenuator in Peripheral T Cell Tolerance Induction

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T cell activation and tolerance are delicately regulated by costimulatory molecules. Although B and T lymphocyte attenuator (BTLA) has recently been shown as a negative regulator for T cell activation, its role in peripheral T cell tolerance induction in vivo has not been addressed. In this study, we generated a novel strain of BTLA-deficient mice and used three different models to characterize the function of BTLA in controlling T cell tolerance. In an oral tolerance model, BTLA-deficient mice were found resistant to the induction of T cell tolerance to an oral Ag. Moreover, compared with wild-type OT-II cells, BTLA−/− OT-II cells were less susceptible to tolerance induction by a high-dose OVA peptide administered i.v. Finally, BTLA−/− OT-I cells caused autoimmune diabetes in RIP-mOVA recipient mice. Our results thus demonstrate an important role for BTLA in the induction of peripheral tolerance of both CD4+ and CD8+ T cells in vivo. The Journal of Immunology, 2009, 182: 4516–4520.

T cell activation and tolerance are tightly regulated by costimulatory molecules, especially those in the B7 and CD28 superfamilies (1, 2). Some of these costimulatory molecules deliver critical negative signals that control the extent of T cell responses. For example, CTLA-4 inhibits T cell responses and regulates peripheral T cell tolerance (3). Also, PD-1 (programmed cell death 1), possibly by interacting with its ligands PD-L1 and PD-L2, has been shown to be a negative regulator of T cell activation and is crucial for maintaining immune tolerance (2).

The B and T lymphocyte attenuator (BTLA)3 has recently joined the CD28 family of negative regulators (4, 5), which is broadly expressed on hemopoietic cells including CD4+ cells, CD8+ cells, B cells, dendritic cells, macrophages, and NK cells (6, 7). In the peripheral lymphoid tissue, mature T cells expressed low level of BTLA and TCR ligation increased BTLA expression. Two groups independently identified herpesvirus entry mediator (HVEM) as the unique ligand for BTLA (8, 9). Similarly as for BTLA, HVEM was also broadly expressed on cells of the immune system such as T and B lymphocytes, NK cells, and dendritic cells, but it was also expressed on endothelial cells (10).

BTLA-deficient CD4+ and CD8+ T cells were previously shown to be hypersensitive to TCR stimulation (5, 7). BTLA-deficient mice have persistent inflammation of the lung in a model of acute allergic airway inflammation (11). Also, targeting BTLA or HVEM prompted rapid rejection in a cardiac transplant study (12). These results indicate an inhibitory effect of BTLA in controlling T cell activation.

In the current study, we examined the function of BTLA in peripheral tolerance by using a novel strain of BTLA knockout (KO) mice and several in vivo tolerance-induction models. We show a crucial role for BTLA in controlling peripheral T cell tolerance.

Materials and Methods

Mice

C57BL/6J, OT-I, and OT-II transgenic mice were purchased from The Jackson Laboratory. Rat insulin promoter (RIP)-membrane-bound OVA (mOVA) mice were kindly provided by W. Heath of the Walter and Eliza Hall Institute of Medical Research (Parkville, Australia). BTLA KO mice on a C57BL/6 background were crossed with OT-I or OT-II mice to get BTLA−/− OT-I and BTLA−/− OT-II mice. All mice were housed in the specific pathogen-free animal facility at M. D. Anderson Cancer Center, and the animal experiments were performed with protocols approved by Institutional Animal Care and Use Committee. Eight- to 12-wk-old mice were used in the experiments.
Reagents for flow cytometry
CD4–Percy5.5, CD25–PE, CD44–allophycocyanin, CD62L–FITC, CD8–Percy5.5, Vε2–PE, IFN-γ–allophycocyanin, and IL-2–allophycocyanin Abs were from BD Biosciences. PD1–FITC and BTLA–PE Abs and isotype controls were from eBioscience. The OT-I tetramer was synthesized as described (13). Cells were analyzed on a FACS Calibur cytometer (BD Biosciences).

Induction and assessment of oral tolerance
Wild-type (WT) and BTLA KO mice daily received intragastrically 2 mg of chicken OVA protein (grade V; Sigma-Aldrich) dissolved in PBS for a total of five times. Control mice were given PBS alone. Seven days after the last treatment, all mice were immunized s.c. with 100 μg of OVA protein emulsified in CFA. One week later, spleens were obtained from the mice and splenocytes were restimulated with OVA protein to measure IL-2 production, T cell proliferation, and effector cytokine production.

Diabetes induction and measurement
CD8 cells from WT and BTLA−/− OT-I mice were purified using anti-CD8 Miltenyi beads and an autoMACS cell separator (Miltenyi Biotec). Five million cells were i.v. injected into RIP-mOVA recipient mice. Mice were monitored for diabetes induction, all mice were immunized s.c. with 100 μg of OVA protein emulsified in CFA. One week later, spleens were obtained from the mice and splenocytes were restimulated with OVA protein to measure IL-2 production, T cell proliferation, and effector cytokine production.

Results and Discussion
BTLA is highly expressed on tolerant T cells
We previously reported that T cells activated in the absence of CD28 and ICOS costimulation become tolerant (14). In our preliminary microarray analysis, we found that these tolerant T cells have significant up-regulated expression of BTLA compared with effector and naive T cells (data not shown). We examined BTLA levels by real-time PCR, and confirmed that tolerant T cells expressed the highest BTLA mRNA in comparison with naive and effector cells (Fig. 1A). Furthermore, the surface expression of BTLA proteins was also up-regulated in tolerant cells by flow cytometry (Fig. 1B). Because BTLA is highly up-regulated on tolerant T cells, we speculated that the BTLA signal might participate in T cell tolerance induction.

Generation and characterization of BTLA KO mice
To be able to examine the function of BTLA in vivo, we generated a BTLA KO mouse by removing part of the promoter as well as the complete exon 1, which had the signal peptide necessary for transmembrane integration. By Northern Blot analysis, we confirmed the complete absence of BTLA mRNA expression in our BTLA-deficient mice (data not shown). The BTLA-deficient mice had no obvious defects. We observed increased memory CD8+ cells in the KO mice (data not shown) as reported previously (15). There was no difference in the percentages of regulatory T cells in the thymus and spleen, and both WT and KO regulatory T cells suppressed naive T cell activation in vitro to the same level (data not shown). In keeping with previous findings (5, 7), BTLA-deficient T cells showed a heightened response to anti-CD3 stimulation. However, both WT and KO naive T cells could be suppressed similarly by regulatory T cells (data not shown). These data argued that the hypersensitivity of T cells was not due to the dysfunction of regulatory T cells in KO mice.

Resistance to oral tolerance by BTLA KO mice
Oral tolerance is a form of peripheral tolerance in which Ag-specific T cell tolerance is induced against oral Ags (16). We thus examined the function of oral tolerance induction in both WT and KO mice. We found that spleen cells from OVA-fed WT mice exhibited reduced production of IL-2, reduced proliferation, and reduced secretion of IFN-γ upon restimulation as compared with the PBS-fed group (Fig. 2A), indicating that profound T cell tolerance had been induced on the OVA protein. In contrast, T cells from KO mice produced significant more amounts of IL-2 and IFN-γ and proliferated more robustly than those from WT mice (Fig. 2A). More importantly, T cells from OVA- and PBS-fed KO mice produced similar amounts of IL-2 and IFN-γ and proliferated to the same extent upon Ag stimulation. These data demonstrated that BTLA was essential for the induction and/or maintenance of oral tolerance and that deficiency in BTLA impairs tolerance induction through the oral tract.

BTLA deficiency is required for peptide-induced tolerance in CD4+ T cells
To examine the relevance of BTLA in CD4+ T cell tolerance, we used a peptide-induced tolerance model with OT-II TCR transgenic mice (17). We found that OVA-treated WT OT-II cells failed to proliferate and produced little IL-2 and IFN-γ (Fig. 2B). In contrast, OVA-treated BTLA−/− OT-II cells displayed considerable IL-2 production and cell proliferation after anti-CD3 restimulation (Fig. 2B). Despite this significant difference, BTLA−/− OT-II cells from OVA peptide-treated mice still exhibited greatly reduced proliferation and IL-2 expression, although they produced greater amounts of IFN-γ than those
from PBS-injected mice, indicating that a molecule or molecules other than BTLA are also required for the induction of Ag-specific CD4+ T cell tolerance in this model. Moreover, after similar OVA peptide treatment, BTLA−/− OT-II cells transferred into Ly5.1 congenic mice exhibited enhanced IL-2 production compared with WT OT-II cells in the same type of recipients (data not shown), indicating an important role for BTLA in T cells in tolerance induction.

**BTLA protects against CD8+ T cell-mediated autoimmune**

To study the role of BTLA in peripheral tolerance in CD8+ T cells, we used the RIP-mOVA diabetic model (18). BTLA KO were bred with OT-I TCR transgenic mice, and the resulting cells, we used the RIP-mOVA diabetic model (18). BTLA KO mice were fed five times with OVA protein or PBS and subsequently immunized with OVA protein in CFA. Seven days later, splenocytes were stimulated with OVA protein. Data are representative of two individual experiments. Each experimental group consisted of five mice. WT OT-II and BTLA−/− OT-II mice were injected i.v. with OVA peptide or PBS twice. On day 10, CD4+ T cells were isolated and stimulated in vitro with CD3. IL-2 were measured at 24 h, IFN-γ and proliferation were measured at 72 h with [3H]thymidine added at the last 7 h of culture. The data shown are representative of two individual experiments. p values were calculated with Student’s t test. *, p < 0.05 and **, p < 0.01 for WT-PBS vs WT-OVA (or WT-OT-II); #, p < 0.05 and ##, p < 0.01 for KO-PBS vs KO-OVA (or KO-OT-II); $, p < 0.05 and $$, p < 0.01 for WT-OVA (or WT-OT-II) vs KO-OVA (or KO-OT-II).

**BTLA regulates oral tolerance and peptide-induced tolerance.**

FIGURE 2. BTLA regulates oral tolerance and peptide-induced tolerance. A, WT and BTLA KO mice were fed five times with OVA protein or PBS and subsequently immunized with OVA protein in CFA. Seven days later, splenocytes were stimulated with OVA protein. Data are representative of two individual experiments. Each experimental group consisted of five mice. B, WT OT-II and BTLA−/− OT-II mice were injected i.v. with OVA peptide or PBS twice. On day 10, CD4+ T cells were isolated and stimulated in vitro with CD3. IL-2 were measured at 24 h, IFN-γ and proliferation were measured at 72 h with [3H]thymidine added at the last 7 h of culture. The data shown are representative of two individual experiments. p values were calculated with Student’s t test. *, p < 0.05 and **, p < 0.01 for WT-PBS vs WT-OVA (or WT-OT-II); #, p < 0.05 and ##, p < 0.01 for KO-PBS vs KO-OVA (or KO-OT-II); $, p < 0.05 and $$, p < 0.01 for WT-OVA (or WT-OT-II) vs KO-OVA (or KO-OT-II).
those exhibiting the highest numbers of cell division, which suggested a role for BTLA in controlling T cell activation programs. In summary, these results indicate that BTLA signaling controls the activation and proliferation of OT-I cells during the priming phase. Subsequently, these effector cells activated in the absence of BTLA exhibited an advantage in expansion and/or survival. Together, BTLA plays an important role in the peripheral tolerance of CD8\textsuperscript{+} T cells.

It is interesting to note that in all of these three tolerance models, BTLA deficiency exerts its influence differently to a certain extent, indicating that BTLA is not simply a negative regulator (dampening signaling in general) but rather affects the expression of cytokines differentially and thus might fine tune immune responses. Indeed, the only ligand for BTLA is HVEM (8, 9). However, HVEM can bind to BTLA, LIGHT/LT\(\beta\) (4), and CD160 (21), which adds another layer of complexity to the regulation of BTLA function.

The CD28 family negative costimulatory molecules BTLA, CTLA-4, and PD-1 thus all play roles in tolerance and autoimmunity control. These molecules may play nonredundant roles at various stages of T cell activation. For example, CTLA4 regulates naive T cell activation and PD1 becomes expressed after T cell activation (1). BTLA, however, is expressed on both naive and activated T cells, also highly on tolerant cells, and potentially regulates all phases of T cell activation. In contrast, BTLA may cooperate with CTLA-4 and PD-1 to control T cell tolerance and autoimmunity. Although at this stage, it is unclear about their specificity or redundancy, these inhibitory pathways may be targeted for controlling chronic infection or boosting tumor immunity.

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Disclosures
The authors have no financial conflict of interest.

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