Cutting Edge: B and T Lymphocyte Attenuator Signaling on NKT Cells Inhibits Cytokine Release and Tissue Injury in Early Immune Responses

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Cutting Edge: B and T Lymphocyte Attenuator Signaling on NKT Cells Inhibits Cytokine Release and Tissue Injury in Early Immune Responses

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The role of coinhibition in an immune response is thought to be critical for the contraction of an adaptive immune response in its waning phases. We present evidence that B and T lymphocyte attenuator (BTLA) coinhibitory signaling is required to temper early inflammation. Using an in vivo Con A challenge model of acute hepatitis, we observed reduced survival and increased early serum cytokine secretion in BTLA−/− mice as compared with wild-type mice. In vitro, liver mononuclear cells from BTLA−/− mice are hyperresponsive to anti-CD3, Con A, and α-galactosylceramide stimulation and secrete higher levels of TNF-α, IFN-γ, IL-2, and IL-4. We found this was in part due to negative regulation of NKT cells by BTLA, as early cytokine inhibition from whole liver mononuclear cells or purified NKT cells depends upon BTLA signaling. Overall, our data demonstrate that coinhibition is active in early immune responses through BTLA regulation of NKT cells. The Journal of Immunology, 2009, 183: 32–36.

The immune system employs a complex system of checks and balances to allow its many mediators to respond appropriately to foreign invaders without causing damage to host tissues. One part of this system, active in T lymphocytes, is one in which cell surface receptors that regulate immune cells costimulate and/or coinhibit activation (1, 2). It is thought that stimulating receptors are used primarily in the early initiation phase of immune responses whereas inhibitory receptors are used during the waning phase, acting to contract an active immune response. Whether inhibitory molecules also play a role in controlling the immune system in the early phase of the response is still an open question in the field.

The B and T lymphocyte attenuator (BTLA) is a recently described Ig superfamily member coinhibitory molecule, similar to PD-1 and CTLA-4 (3–5), that interacts in an unusual pairing with a TNFR family member, herpes virus entry mediator (HVEM), to inhibit the activation of a BTLA-bearing cell (6, 7). BTLA is expressed on B cells, T cells, and dendritic cells in all strains of mice whereas its expression on NK cells and macrophages is limited to C57BL/6 mice (8, 9), which model human expression well (10). HVEM engagement by BTLA induces tyrosine phosphorylation and the association of BTLA with the SHP-2 tyrosine phosphatase to repress Ag-driven T cell proliferation (7). We and others have shown that the absence of BTLA-HVEM inhibitory interactions leads to increased experimental autoimmune encephalomyelitis severity (9, 11), enhanced rejection of partially mismatched allografts (12), an increased CD8+ memory T cell population (13), increased severity of colitis (14), reduced effectiveness of T regulatory cells (15), and the development of autoimmunity over time (16). These studies suggest that the BTLA signaling therefore functions to protect the host from immunopathology arising from the overstimulation of T cells over time. Its role in the early immune response, however, is still unclear.

To test whether the BTLA pathway is required to temper early immune responses, we use the Con A-mediated acute hepatitis model (17). In this study, we show that BTLA−/− mice have increased morbidity and mortality as early as 6 h after the administration of Con A. We further demonstrate that cytokine release by NKT cells is negatively regulated by BTLA signaling, implicating coinhibitory signaling in the early phase of the immune response.

Materials and Methods

Mice

C57BL/6 wild-type (WT) and CD1d−/− mice were purchased from The Jackson Laboratory. BTLA−/− mice, developed in a 129SvJ background and backcrossed to C57BL/6 for 10 generations, were provided by J. Kaye of the Scripps Research Institute, La Jolla, CA (13). All animals were on the C57BL/6 background and backcrossed to 10 generations. Female mice were used at 12 to 20 wk old, and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago, Chicago, IL.

1 Abbreviations used in this paper: BTLA, B and T lymphocyte attenuator; ALT, alanine aminotransferase; CBA, cytokine bead array; DKO, double knockout; α-GalCer, α-galactosylceramide; HVEM, herpes virus entry mediator; MNC, mononuclear cells.

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In vivo Con A or α-GalCer treatment

Mice were weighed for accurate dosing per weight and injected i.v. with 10, 16, or 20 mg/kg Con A (Sigma-Aldrich) diluted in PBS. α-Galactosylceramide (α-GalCer) (AxEssa) was injected i.v. at 2 μg/mouse.

Isolation of liver mononuclear cells (MNC)

Livers were harvested from naïve mice and MNC were isolated by mechanical disruption followed by purification with 35% Percoll (Sigma-Aldrich). RBCs were lysed. NKT cell percentage was measured using CD1d/PBS-57 (analog of α-GalCer) tetramer staining (provided by National Institutes of Health Tetramer Facility at Emory University, Atlanta, GA). BTLA expression was determined using the anti-BTLA Ab clone 6F7 (eBioscience).

NKT purification

NKT cells were purified from liver MNC on a MoFlo cell sorter (Beckman Coulter) using anti-NK1.1-1-allophycocyanin (BD Biosciences) and anti-TCRβ-FITC (e-Bioscience to sort out NK1.1⁺ TCRβ⁺ double-positive cells). TCRβ-singles positive cells were also sorted and used as the NKT-depleted T cell control.

Cytokine detection

IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were measured by mouse Th1/Th2 cytokine bead array (CBA) assay (BD Biosciences). IL-12p70, IL-6, and MCP-1 were measured by a mouse inflammation CBA assay. IL-12p70 cytokine levels were measured on liver-resident T cells early after activation.

Cell stimulation

Liver MNC cells were stimulated in flat-bottom plates at 1–3 × 10⁵ per well using Con A (5 μg/ml), α-GalCer (5 ng/ml), or immobilized anti-CD3 (clone 2C11; 2 μg/ml). Plate-bound HVEM-Ig (11) or mouse IgG isotype control (Sigma-Aldrich) was used at 5 μg/ml. Supernatant was harvested at 24 h and analyzed by CBA assay.

Serum alanine aminotransferase (ALT) detection

Serum ALT was determined using ALT measuring strips (Roche Diagnostics) and measured by a Reflotron Plus instrument (Roche Diagnostics).

Statistical analysis

Mean values were compared using an unpaired t test, Mann-Whitney U test, or log-rank test where appropriate. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad software). Statistically significant differences of p < 0.05, p < 0.01, and p < 0.001 are noted with *, **, and ***, respectively.

Results and Discussion

It is unclear whether coinhibitory pathways are involved in regulating early immune responses. To address this question, Con A-mediated acute hepatitis was used to test whether the BTLA pathway is actively involved in early T cell-mediated immune responses. Consistently among the Con A doses tested, BTLA⁻/⁻ mice had decreased survival as compared with age- and sex-matched WT mice (Fig. 1A). Decreased BTLA⁻/⁻ mouse survival so early after Con A administration indicates that BTLA may have inhibitory functions in early inflammation. Significant increases in proinflammatory cytokines at 2 and 6 h after Con A injection were observed in BTLA⁻/⁻ mice, indicating that BTLA may negatively control the release of cytokines in the early response to Con A (Fig. 1B). We therefore predicted that BTLA⁻/⁻ mice would demonstrate higher susceptibility to liver damage than WT mice. As expected, BTLA⁻/⁻ mice had significantly higher ALT liver enzyme levels than WT mice 6 h after Con A treatment (Fig. 1C). This result suggests that BTLA normally acts in the early immune response by dampening cytokine production, thereby protecting against liver injury.

One of the main mechanisms of action for Con A treatment in vivo is in targeting the liver-resident T cells (17). For this reason, liver MNC were isolated and tested in vitro for cytokine production at early time points. BTLA⁻/⁻ liver MNC produced significantly higher cytokines to Con A (Fig. 2A) or anti-CD3 (Fig. 2B) 20–24 h after stimulation. This result provides evidence that BTLA can inhibit cytokines released on liver-resident T cells early after activation.

Although NKT and conventional T cells in the liver can respond to Con A, Con A-mediated hepatitis and liver damage is...
known to be dependent on NKT cells, and IL-4 in particular (18, 19). Indeed, BTLA−/− liver MNC secreted significantly more IL-4 (Fig. 2, A–C). The observed increase in IL-4 from BTLA−/− cells raises the possibility that BTLA is critical for the regulation of NKT function, because NKT cells mainly contribute to the IL-4 released immediately after stimulation (20). NKT cells are poised to receive signals through BTLA, as BTLA is expressed constitutively on liver NKT cells as per previously published data that we have confirmed (Ref. 21 and supplemental Fig. 1). 

To test whether BTLA on NKT or other T cells is required for the inhibition of early cytokine production, we bred BTLA−/− mice with NKT-deficient CD1d−/− mice to create BTLA/CD1d double knockout (DKO) mice. This strain lacks most NKT cells, whereas all other cells are deficient in BTLA. To test whether BTLA deficiency only on NKT cells was required for the increased cytokines seen in singly deficient BTLA−/− mice at early time points after activation, WT, CD1d−/−, BTLA−/−, and BTLA/CD1d DKO liver MNC cells were stimulated by Con A or anti-CD3 in vitro. We used IL-4 as the primary readout of NKT activation, as it is the most specific for assessing NKT activation at this early time point. In response to anti-CD3 stimulation, BTLA/CD1d DKO produced low levels of IL-4 similar to those of control CD1d−/− mice (Fig. 2C). Although BTLA/CD1d DKO cells exhibited higher levels of IL-4 than control CD1d−/− mice with Con A stimulation, they nevertheless exhibited 5-fold significantly lower IL-4 levels than BTLA−/− mice (Fig. 2C). These data suggest that the negative regulation of IL-4 by BTLA in early responses is predominantly through NKT cell regulation. The significantly higher levels of IL-4 by BTLA−/− cells compared with WT cells in Fig. 2, A–C was not due to an increase in the percentage or absolute numbers of NKT cells in WT and BTLA−/− liver MNC (Fig. 2D) and thus may be intrinsic to the BTLA−/− NKT cell. Indeed, anti-CD3 in vitro stimulated BTLA−/− liver MNC consistently exhibited a 2-fold increase in the number of cells releasing IL-4 on a per cell basis as compared with WT liver MNC cells (data not shown).

It can be argued that the phenotype we observed in BTLA−/− cells can be attributed to different developmental conditions in the BTLA−/− and WT backgrounds. For example, it has been shown that the absence of BTLA-HVEM inhibitory interaction leads to an increased CD8+ memory T cell population in aged mice (13). To determine whether BTLA signaling at the time of cell activation is necessary to inhibit early cytokine release of IL-4 and other cytokines, we tested whether the HVEM-Ig fusion protein would inhibit cytokine release in liver MNC in vitro cultures from WT or BTLA−/− mice stimulated with anti-CD3. The HVEM-Ig fusion protein activates the inhibitory function of BTLA. Whereas HVEM-Ig inhibited IL-4 and IFN-γ (Fig. 3A) as well as IL-2 and TNF-α (supplemental Fig. 2) from WT cells, cytokine secretion from BTLA−/− cells was not inhibited to the extent of that from WT cells, suggesting that BTLA mediates a majority of this inhibition. These results indicate that the cross-linking of BTLA on responding cells at the time of challenge, and not the developmental differences between WT and BTLA−/− cells, is largely necessary for the inhibition of cytokine release. The residual inhibition observed in HVEM-Ig-treated BTLA−/− cells indicates the possibility

**FIGURE 2.** BTLA negatively regulates early cytokine release in vitro. A, Liver MNC were isolated from three mice, pooled, plated at 3 × 10^7/well, and stimulated with 5 μg/ml Con A. Supernatant was harvested at 24 h and cytokines were analyzed by CBA for A–C. Data are from triplicate wells. B, Liver MNC were isolated from three mice, plated at 1.5 × 10^7/well, and stimulated with 2 μg/ml anti-CD3. Data are from three individual mice in one experiment and are representative of results from four experiments. C, Liver MNC from WT, BTLA−/−, CD1d−/−, and BTLA/CD1d DKO mice were harvested and plated at 1.5–3 × 10^7 cells/well and stimulated with either 2 μg/ml anti-CD3 (α-CD3) or 5 μg/ml Con A. Each dot represents an individual well. D, Liver MNC were harvested from WT or BTLA−/− mice and labeled with a CD1d/α-GalCer tetramer and analyzed by FACS to determine the percentage of liver NKT cells. Data were compiled from 14 to 15 mice from seven separate experiments. Statistical analysis for A, B, and D, was by unpaired t test, and in C, Con A was analyzed by unpaired t test and anti-CD3 by the Mann-Whitney U test.
that HVEM-Ig is engaging another inhibitory receptor, possibly the recently described interacting partner CD160 (22), and we do not rule this out in our experimental system.

To confirm that BTLA was negatively regulating cytokine release specifically from NKT cells, we found that purified BTLA−/−NKT cells released significantly higher IL-4, IFN-γ, TNF-α and IL-2 compared with purified WT NKT cells in response to anti-CD3 stimulation in vitro (Fig. 3B and supplemental Fig. 3). This result also reveals that NKT cells themselves might be sufficient to provide HVEM to bind BTLA on their own or their neighbor’s cell surface. Cytokine release from WT NKT cells was found to be susceptible to inhibition by HVEM-Ig treatment whereas BTLA WT NKT cells was found to be susceptible to inhibition by their own HVEM-Ig as demonstrated in previous studies (23). Both in vivo and in vitro administration of α-GalCer to BTLA−/−mice or BTLA+/−isolated liver MNC, respectively, produced significantly higher levels of cytokines than WT mice early after activation (Fig. 4). The ability of an NKT-specific agonist to elicit higher cytokine release from BTLA−/−mice or cells supports the assertion that BTLA negatively controls cytokine production from NKT cells.

Previous studies that investigated the mechanisms of how coinhibitory pathways on T cells regulate the immune system have focused on their role in contracting and reducing the potency of an active, ongoing adaptive immune response. We now show that coinhibitory pathways can also dampen early activation of the immune response by regulating NKT cells. This study also gives further insights into how NKT cells are normally regulated. In the present work we identify BTLA as necessary for the negative regulation of cytokine release by these innate cells very early after stimulation, which can therefore help to regulate the delicate balance between fighting pathogens and protecting the host against immunopathology.

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Disclosures
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References


