Early Infection Immunity

B and T Lymphocyte Attenuator Tempers

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**B and T Lymphocyte Attenuator Tempers Early Infection Immunity**

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Cochinhibitory pathways are thought to act in later stages of an adaptive immune response, but whether coinhibition contributes to early innate immunity is unclear. We show that engagement of the newly discovered co-inhibitory receptor B and T lymphocyte attenuator (BTLA) by herpesvirus entry mediator (HVEM) is critical for negatively regulating early host immunity against intracellular bacteria. Both HVEM<sup>−/−</sup> and BTLA<sup>−/−</sup>, but not LIGHT<sup>−/−</sup>, mice are more resistant to listeriosis compared with wild-type mice, and blockade of the BTLA pathway promotes, while engagement inhibits, early bacterial clearance. Differences in bacterial clearance were seen as early as 1 day postinfection, implicating the initial innate response. Therefore, innate cell function in BTLA<sup>−/−</sup> mice was studied. We show that innate cells from BTLA<sup>−/−</sup> mice secrete significantly more proinflammatory cytokines upon stimulation with heat-killed *Listeria*. These results provide the first evidence that a co-inhibitory pathway plays a critical role in regulating early host innate immunity against infection.

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**Abbreviations used in this paper:** BTLA, B and T lymphocyte attenuator; DC, dendritic cell; HKLM, heat-killed *Listeria monocytogenes*; HVEM, herpesvirus entry mediator; LM, *Listeria monocytogenes*; WT, wild type.

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and BTLA have the potential to either costimulate or coinhibit T cell immunity, depending on the nature of the ongoing immune response. In addition to its impact on T cells, the HVEM-BTLA pathway also regulates the homeostasis of certain DC subsets (22). However, the role of this pathway in regulating early innate immunity against infection has not been determined.

To determine whether a cosignaling pathway plays a critical role in early infection, we studied the potential functions of the HVEM-BTLA negative signaling pathway during the early response to infection. We used *Listeria monocytogenes* (LM), a Gram-positive facultative intracellular bacterium broadly used to study both the innate and adaptive immune responses against infection, as an infection model. Murine resistance to LM includes an early innate cell-mediated nonspecific phase, followed by a T cell-mediated specific immune phase, resulting in sterile clearance of bacteria from the host (23–25). Effective innate immunity is necessary to control pathogen replication during the early days of infection. Here, we reveal for the first time that HVEM-BTLA interactions are necessary and sufficient to inhibit early host immunity against bacterial infection, demonstrating that coinhibition unexpectedly plays an essential role in tempering the initial, innate immune response.

**Materials and Methods**

**Mice**

C57BL/6J (B6, referred to as WT) and Rag-1-deficient mice (Rag-1−/−) on the B6 background were purchased from The Jackson Laboratory. LIGHT-deficient (LIGHT−/−) (26), HVEM-deficient (HVEM−/−) (11), and BTLA-deficient (BTLA−/−) (16) mice were generated as previously described and backcrossed to the B6 background for >10 generations. All mice were maintained under specific pathogen-free conditions. All procedures were approved by the University of Chicago’s Institutional Animal Care and Use Committee.

**Fusion protein and Abs**

Mouse HVEM-Ig fusion protein (27) and blocking anti-BTLA mAb (clone 6A6, hamster IgG) (17) were generated as previously described. Mouse IgG and hamster IgG (Sigma-Aldrich) were used as controls for HVEM-Ig and 6A6, respectively.

**LM infection, treatment, and determination of CFUs**

The recombinant LM strain rLM-OVA was provided by Dr. H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) (28). rLM-OVA was grown in brain-heart infusion broth (Difco Laboratories). Heat-killed LM (HKLM) was derived by growing log-phase rLM-OVA at 37°C for 3 h. For determination of bacterial load in tissues, WT and various knockout mice were infected with 5 × 10⁶ CFU rLM-OVA, and Rag-1−/− mice were infected with 1 × 10⁶ CFU rLM-OVA, by i.p. injection. The bacterial dose was verified by plating dilutions of the inoculum on brain-heart infusion agar plates. To block or engage the BTLA pathway, mice were administered 100 μg of anti-BTLA (6A6) or 200–300 μg of HVEM-Ig by i.p. injection on the same day of infection. Hamster IgG and mlgG were used as controls, respectively. At indicated times after infection, mice were sacrificed and specimens of spleen and liver were examined for bacterial titers. In brief, organs were homogenized and lysed in sterile water with 0.5% Triton X-100, serial dilutions of homogenates were plated on brain-heart infusion agar plates, and colonies were counted after incubation at 37°C for 24 h. For in vivo stimulation with HKLM, mice were injected i.v. with the indicated doses of HKLM in PBS.

**In vitro proliferation and cytokine detection assays**

To test whether HVEM-Ig inhibits T cell proliferation dependent on BTLA, lymph node cells (2 × 10⁶ per well) isolated from WT, LIGHT−/−, or BTLA−/− mice were stimulated with immobilized anti-CD3 (2 μg/ml) in the absence or presence of either soluble or plate-coated control murine IgG or HVEM-Ig at various doses for 48 h, pulsed with 1 μCi of [³H]thymidine for 18 h, and harvested for liquid scintillation counting.

To determine the secretion of cytokines by WT and BTLA−/− cells after HKLM stimulation, splenocytes from either strain of mice were incubated with or without 2 × 10⁶ HKLM/ml in RPMI 1640 for the indicated times, washed three times with RPMI 1640, plated at 2 × 10⁶ cells per well in round-bottom 96-well microtiter plates, and incubated for 24 h at 37°C. The plates were then centrifuged, and the supernatants were collected and the cytokine concentrations determined with the mouse inflammation cytokine bead array kit (BD Biosciences).

**Statistical analysis**

Mean values of bacterial loads in spleen and liver were compared using the unpaired Mann-Whitney *U* test. Mean values of proliferation and cytokine levels were compared using the unpaired Student’s *t* test. All statistical analyses were performed using GraphPad Prism (GraphPad Software). Statistically significant differences of *p* < 0.05 and *p* < 0.01 are noted with + and **, respectively.

**Results**

**Both HVEM and BTLA play essential roles in regulating protective immunity against early infection**

To determine the role of HVEM in early infection, we infected WT and HVEM−/− mice with a sublethal dose of LM (5 × 10⁷ CFU/mouse) and measured bacterial burden 4 days after infection. We found that HVEM−/− mice exhibited significantly lower bacterial burden in both spleens and livers compared with WT mice (Fig. 1A), suggesting that HVEM is involved in suppressing bacterial clearance. Since HVEM interacts with both LIGHT and BTLA, we also tested the susceptibility of LIGHT−/− and BTLA−/− mice to LM infection to determine which binding partner of HVEM is essential for this suppression. In contrast to HVEM−/− mice, LIGHT−/− mice did not manifest reduced bacterial burden in either the spleen or liver (Fig. 1B). Rather, LIGHT−/− mice displayed an increased LM load in the liver compared with WT mice. BTLA−/− mice, however, exhibited significantly reduced bacterial loads in both the spleen and liver compared with WT mice (Fig. 1C), similar to HVEM−/− mice. These data suggest that HVEM-BTLA interactions, and not HVEM-LIGHT interactions, play an essential role in regulating early bacterial clearance.

To rule out the possibility that the reduced susceptibility of HVEM−/− and BTLA−/− mice to LM infection is due to an intrinsic difference between WT and deficient mice, such as an increased number of memory CD8+ T cells in both HVEM−/− and BTLA−/− mice compared with WT mice (20), WT mice were treated with an antagonist anti-BTLA mAb (6A6) upon LM infection. In congruence with HVEM−/− and BTLA−/− mice, administration of anti-BTLA mAb, which blocks BTLA signaling, reduced the bacterial load in spleens and livers compared with control hamster IgG treatment (Fig. 1D). These data demonstrate that signaling through a coinhibitory molecule plays an essential, inhibitory role in the early immune response against infection, and that blocking such a pathway enhances immunity to infectious agents.

**BTLA engagement by HVEM is sufficient to inhibit early LM clearance**

HVEM-Ig is a chimeric soluble protein comprised of the extracellular domain of mouse HVEM fused to the Fc portion of mouse IgG2a (27). HVEM is known to interact with both LIGHT and BTLA to modulate T cell function. HVEM-Ig may inhibit an immune response by either blocking LIGHT-HVEM interaction or by engaging BTLA through cross-linking. To address this issue, we tested the roles of both soluble and plate-bound HVEM-Ig on T cell proliferation. We found plate-bound HVEM-Ig dramatically inhibited T cell proliferation in response to anti-CD3 stimulation in a dose-dependent manner (Fig. 2A). However, soluble HVEM-Ig did not show inhibition at concentrations up to 10 μg/ml, and both plate-bound and soluble control mlgG did not show inhibition. Hence, immobilized HVEM-Ig, able to cross-link and engage its receptor, as opposed to soluble HVEM-Ig that blocks HVEM interaction with its ligand or receptor, is capable of delivering an
inhibitory signal. To define the ligand or receptor involved in HVEM-Ig-induced suppression, we tested the proliferation of LIGHT- and BTLA-deficient T cells in the presence of anti-CD3 and titrated doses of plate-bound HVEM-Ig. The results showed that plate-bound HVEM-Ig could inhibit LIGHT-deficient T cell proliferation similarly to WT T cells (Fig. 2B). No suppression was however, seen in BTLA-deficient T cell function at all doses tested (Fig. 2C). These data suggest that HVEM-Ig-induced suppression of T cell function is by cross-linking BTLA on T cells, rather than by blocking the interaction of endogenous HVEM and LIGHT.

We then further examined the importance of the HVEM-BTLA pathway in early LM infection by testing whether engaging BTLA with HVEM-Ig was sufficient to inhibit bacterial clearance. We treated WT mice with HVEM-Ig or control mIgG on the same day as LM infection. Four days later, bacterial burdens in the spleen and liver were analyzed. We found that HVEM-Ig-treated mice exhibited significantly increased bacterial titers in both organs compared with mIgG-treated mice (Fig. 3A). Importantly, only 30% of HVEM-Ig-treated mice survived this normally sublethal dose of LM infection, compared with 100% survival of the control mIgG-treated mice (Fig. 3B). These data suggest that HVEM-Ig-treated mice are less capable of clearing LM infection than are WT mice. Since HVEM-Ig can bind to both BTLA and LIGHT, its biological function in vivo could be due to either engaging BTLA, providing a coinhibitory signal, or by binding to LIGHT, thus blocking a costimulatory signal. To test the latter possibility, we treated LIGHT−/− mice with either HVEM-Ig or mIgG and infected them with LM. Similarly to WT mice, HVEM-Ig treatment significantly increased LM loads in both the spleen and liver of LIGHT−/− mice, compared with mIgG-treated mice (Fig. 3C), suggesting the in vivo effect of HVEM-Ig is independent of LIGHT. These data confirm that cross-linking of BTLA by HVEM can negatively regulate host resistance to early LM infection. Therefore, our data suggest that the HVEM-BTLA pathway plays a necessary and sufficient role in regulating early host immunity against LM infection.

BTLA signaling suppresses innate cell function

Various innate and adaptive immune cells participate in LM clearance at different stages of infection. To determine at which stage of infection the BTLA pathway regulates antibacterial immunity, we
followed the kinetics of bacterial clearance early after infection. Differences between treated groups were evident as early as the first day after infection, and by day 3 we found consistently significant differences, in both spleens and livers, compared with WT mice, and BTLA blockade with anti-BTLA mAb strongly indicated that HVEM-BTLA interactions regulate host immunity very early after infection. These experiments demonstrate that the HVEM-BTLA pathway regulates host immunity early after infection, suggesting that BTLA signaling tempers the early phase of infection is largely unknown. Most studies thus far have focused on negative regulation during the contraction process of T cells of the adaptive immune system (5–7). Using genetic knockout of BTLA, HVEM, and LIGHT, as well as HVEM-Ig and anti-BTLA Ab, this study has addressed three novel and separate concepts of major importance: 1) coinhibitory molecules play a role in limiting the immune response at the early phase, 2) the HVEM-BTLA pathway is important in negatively regulating early immunity to infection, and 3) BTLA signaling suppresses innate cell function. This study reveals an unappreciated role of the BTLA signaling pathway in regulating early immunity after infection. Our data demonstrated that both HVEM- and BTLA-, but not LIGHT-, deficient mice were more resistant to early LM infection than did WT mice 6 h after infection (Fig. 5B). Strikingly, BTLA-deficient mice appeared to take longer to recover from this HKLM bolus than did WT mice (our unpublished observations), suggesting that higher TNF-α, or other proinflammatory cytokine, levels may be increasing septic shock and/or immunopathology responses in these mice. Accordingly, a moderately higher dose (1.5 × 10^5) of HKLM rapidly induced death in all of the BTLA−/− mice, while 80% (four of five) of WT mice were able to recover from this high dose (Fig. 5C). Taken together, these data indicate that BTLA signaling suppresses the innate cell response.

**Discussion**

The timing and scope of negative regulation of host immunity in the early phase of infection is largely unknown. Most studies thus far have focused on negative regulation during the contraction process of T cells of the adaptive immune system (5–7). Using genetic knockout of BTLA, HVEM, and LIGHT, as well as HVEM-Ig and anti-BTLA Ab, this study has addressed three novel and separate concepts of major importance: 1) coinhibitory molecules play a role in limiting the immune response at the early phase, 2) the HVEM-BTLA pathway is important in negatively regulating early immunity to infection, and 3) BTLA signaling suppresses innate cell function. This study reveals an unappreciated role of the BTLA signaling pathway in regulating early immunity after infection. Our data demonstrated that both HVEM- and BTLA-, but not LIGHT-, deficient mice were more resistant to early LM infection compared with WT mice, and BTLA blockade with anti-BTLA promoted, while BTLA engagement with HVEM-Ig inhibited, bacterial clearance very early after infection. These experiments strongly indicate that HVEM-BTLA interactions regulate early host protective immunity by suppressing innate cell function.
vented LM clearance in both WT and system is under investigation. However, our data definitively show HVEM interact (29); the role of HVEM-CD160 interactions in our for human HVEM, and also demonstrated that murine CD160 and 

...HVEM-Ig inhibited cytokine levels were determined. A recent study has revealed that CD160 is a natural receptor by guest on September 15, 2017 http://www.jimmunol.org/ Downloaded from

FIGURE 5. Increased innate response to LM in the absence of BTLA. A, WT and BTLA−/− splenocytes were incubated in triplicate wells with HKLM for 5 min (for TNF-α and IL-6 determination) or 15 min (for IFN-γ determination), washed, and cultured in vitro for 24 h before supernatant cytokine levels were determined at indicated times (five to ten mice per group). B, WT and BTLA−/− mice were injected i.v. with 1 × 10⁹ HKLM and serum cytokine levels were determined at indicated times (five to ten mice per group). C, WT and BTLA−/− mice were injected i.v. with 1.5 × 10⁹ HKLM and survival was monitored (five mice per group). Bar graphs depict means ± SEM; ND, not detected.

In our model, HVEM-Ig binds to both BTLA and LIGHT. However, our data support the idea that the HVEM-BTLA interaction is dominant in regulating immunity for several reasons. First, T cell proliferation in response to anti-CD3 was only inhibited by plate-bound HVEM-Ig, capable of engaging the BTLA receptor, and not soluble HVEM-Ig, which would block the LIGHT ligand interaction with the HVEM receptor. Second, and more directly, HVEM-Ig inhibited LIGHT−/− but not BTLA−/− T cell proliferation. A recent study has revealed that CD160 is a natural receptor for human HVEM, and also demonstrated that murine CD160 and HVEM interact (29); the role of HVEM-CD160 interactions in our system is under investigation. However, our data definitively show that HVEM-Ig-mediated inhibition of T cell proliferation is largely BTLA-dependent. Third, administration of HVEM-Ig in vivo prevented LM clearance in both WT and LIGHT−/− mice. Fourth, HVEM−/− but not LIGHT−/− mice showed increased LM load in the liver compared with WT mice, suggesting that LIGHT may play a positive role in LM clearance in the liver. Therefore, it is likely that HVEM-BTLA signaling plays a key role in tempering the early immune response to Listeria clearance.

LM is broadly used as a model of cell-mediated immunity (24, 25, 30), and both innate and adaptive immune cells participate in protection against LM infection (23, 24, 31). Innate immunity plays a critical role in protection against early intracellular bacterial infection (24, 25), both HVEM and BTLA are detected on innate cells (14), and it has been shown that the HVEM-BTLA pathway negatively regulates DC subset homeostasis (22). Thus, it seemed likely that BTLA signaling was acting to suppress innate cells during an immune response. Indeed, our data show that cytokine secretion, both in vitro and in vivo, in response to listerial stimulation is significantly higher in BTLA−/− mice as compared with WT mice. As we have previously reported that the only signficant difference in the cellular compositions of the lymphoid organs of these BTLA-deficient mice, compared with WT mice, is an increased number of memory CD8 T cells (16, 20), it is likely that the innate cell response is tempered by BTLA signaling, rather than BTLA−/− mice harboring an increased ratio of innate cells. Whether BTLA signals directly on innate cells during the immune response to infection is currently being determined. The biological significance of negatively regulating immunity at such an early phase of infection is an intriguing issue. Based on the timing and nature of the HVEM-BTLA coinhibitory interaction from this study, it is possible that one important function of tempering the early immune response is to aid host survival by reducing mortality and morbidity from overactivation of the immune system. Indeed, data presented herein indicate that the overactive innate response in BTLA−/− mice renders them more susceptible to death from acute immunostimulation. In additional support of this concept, we recently observed that while WT mice survive an i.v. injection of a sublethal dose of Con A, most HVEM-deficient mice produce high amounts of proinflammatory cytokines, dying rapidly of a Con A-mediated cytokine storm within 5–8 h (11). These findings may be particularly relevant to our recent description of an inhibitory effect of T cells on innate cell function (32); indeed, BTLA signaling may play an important role in this T cell-mediated tempering of the innate response. Taken together, these studies further indicate that negative signaling is important in tempering strong immune responses at an early stage of infection, thereby protecting the host against an overactive immune system. On the other hand, during a mild infection, negative regulatory mechanisms may temper the quick clearance of pathogen by the innate immune system, allowing for a robust adaptive immune response that is capable of generating an effective memory response. These complicated issues remain to be determined. In summary, our study has revealed a critical role for coinhibition in the early immune response to infection, and implicates the HVEM-BTLA pathway as a mechanism for this effect. It remains to be determined how BTLA signaling regulates innate immunity, and whether other negative signaling molecules are also critically involved in the early immune response. Further study of cosignaling molecules at various stages of immune responses will allow for the rational design of vaccinations and aid future immunotherapies of various inflammatory diseases, including infectious disease, cancer, and autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

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