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B and T Lymphocyte Attenuator Tempers Early Infection Immunity[1]

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Abstract

Co-inhibitory pathways are thought to act in later stages of an adaptive immune response, but whether co-inhibition 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*$^{-/-}$ and *BTLA*$^{-/-}$, but not *LIGHT*$^{-/-}$, mice are more resistant to listeriosis compared to 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 post-infection, implicating the initial innate response. Therefore, innate cell function in *BTLA*$^{-/-}$ mice was studied. We show that innate cells from *BTLA*$^{-/-}$ mice secrete significantly more pro-inflammatory 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.

Keywords

Bacterial Infection; Cell Surface Molecules; Cytokines; Costimulation; Knockout Mice

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Introduction

After acute antigen challenge, T cells rapidly activate, proliferate and differentiate into effector cells and then subsequently contract in number. The development and maintenance of T cell responses depends not only on antigen/MHC interaction as a first signal, but also on co-signaling molecules, including both co-stimulatory and co-inhibitory molecules, as a second signal (1-3). Several co-inhibitory molecules have been identified; these include CTLA-4, programmed cell death 1 (PD-1)[5], and B and T lymphocyte attenuator (BTLA). It is increasingly clear that co-inhibition is essential for maintaining a balanced immune response, the contraction of the T cell pool after clearance of antigen, and the homeostasis of immune cells (3). It is believed that co-stimulatory signaling may be more influential during early stages of an immune response, while co-inhibitory signaling may play a dominant role in later stages, aiding T cell contraction. Recently, the co-inhibitory PD-1 receptor has been shown to participate in restraining the CTL response at the later contraction stage of the immune response to infection, thus contributing to persistent infection (4-7). Whether co-inhibitory signaling is required during the early immune response however, has not been demonstrated.

Herpesvirus entry mediator (HVEM), a TNF receptor superfamily member (TNFR14), has been previously described as a T cell co-stimulatory receptor through interaction with its ligand LIGHT (TNFSF14). HVEM is broadly expressed on hematopoeitic cells including T cells and dendritic cells (DCs) (8-10). It was predicted that *HVEM*$^{-/-}$ T cells would be hyporesponsive to various T cell stimuli when compared to WT T cells. Unexpectedly, we observed that *HVEM*$^{-/-}$ mice exhibited hyperresponsiveness to various T cell stimuli, both *in vivo* and *in vitro*, and were more susceptible to developing autoimmune diseases (11). This revealed an alternative function of HVEM as an inhibitor of T cell responses. Indeed, recent studies have demonstrated that HVEM also serves as a unique ligand interacting with a newly defined co-inhibitory molecule, BTLA (12,13). BTLA exhibits polymorphisms of both structure and expression in mice (14). It is primarily expressed on B cells, T cells and DCs, and is highly induced in anergic CD4$^+$ T cells in murine models (15-17). HVEM can induce BTLA tyrosine phosphorylation and association of the tyrosine phosphatase, SHP-2, resulting in the repression of antigen-driven T cell proliferation (12). BTLA engagement with an agonistic mAb inhibits T cell proliferation and cytokine production (18). In addition, both HVEM- and BTLA-deficient mice share similar phenotypes, such as: increased susceptibility to experimental autoimmune encephalomyelitis (EAE) (11,15), enhanced rejection of partially mismatched allograft (19), and an increased CD8$^+$ memory T cell population (20). Together, these findings suggest that HVEM predominantly plays a negative role through interaction with its receptor BTLA in T cell responses, in stark contrast to its co-stimulatory properties delivered by LIGHT. Therefore, HVEM may play a unique dual role in T cell activation and homeostasis determined by the ligand and receptor engaged. Interestingly, a recent study demonstrated that BTLA plays a positive role in the T cell response during chronic allostimulation through maintaining CD4$^+$ T cell survival (21). Therefore, both HVEM and BTLA have the potential to either co-stimulate or co-inhibit 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 dissected.

To determine whether a co-signaling 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

[5]Abbreviations used in this manuscript: BTLA, B and T lymphocyte attenuator; DC, dendritic cell; HKLM, heat-killed *Listeria monocytogenes*; HVEM, Herpes virus entry mediator; LM, *Listeria monocytogenes*; PD-1, programmed cell death 1

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 co-inhibition 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 ($Rag^{-/-}$) 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 more than ten 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 antibodies

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

LM infection, treatment and determination of CFU

The recombinant LM strain rLM-OVA was provided by Dr. Hao 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 70°C for 3 H. For determination of bacterial load in tissues, WT and various knockout mice were infected with 5×10^5 CFU rLM-OVA, and $Rag^{-/-}$ mice were infected with 1×10^6 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 with 100 μg of anti-BTLA (6A6) or 200 to 300 μg of HVEM-Ig by i.p. injection on the same day of infection. Hamster IgG and mIgG 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 of BTLA, LN cells (2×10^5 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 hours, pulsed with 1 μCi of [^{3}H]thymidine for 18 hours, 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 2×10^9 HKLM/ml in RPMI for the indicated times, washed 3 times with RPMI, plated at 2×10^6 cells/well in round-bottomed 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, San Diego, CA). 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 (5×10^5 CFU/mouse), and measured bacterial burden four days after infection. We found that *HVEM*$^{-/-}$ mice exhibited significantly lower bacterial burden in both spleens and livers, compared to WT mice (Fig. 1A), suggesting 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 to WT mice. *BTLA*$^{-/-}$ mice, however, exhibited significantly reduced bacterial loads in both the spleen and liver, compared to 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 to control hamster IgG treatment (Fig. 1D). These data demonstrate that signaling through a co-inhibitory 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 engaging BTLA through crosslinking. 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 mIgG did not show inhibition. Hence, immobilized HVEM-Ig, able to cross-link and engage its receptor, 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 titered 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 seen, however, in BTLA-deficient T cell function at all doses tested (Fig. 2C). These data suggest that HVEM-Ig-induced suppression of T cell proliferation is by crosslinking BTLA on T cells, rather than 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 to 100% survival of the control mIgG treated mice (Fig. 3B), demonstrating that HVEM-Ig treated mice are less capable of clearing LM infection than 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 co-inhibitory signal, or by binding to LIGHT, thus blocking a co-stimulatory signal. To test the latter possibility, we treated *LIGHT*$^{-/-}$ mice with either HVEM-Ig or mIgG and infected with LM. Similarly to WT mice, HVEM-Ig treatment significantly increased LM loads in both the spleen and liver of *LIGHT*$^{-/-}$ mice, compared to mIgG treated mice (Fig. 3C), suggesting the *in vivo* effect of HVEM-Ig is independent of LIGHT. These data confirm that crosslinking 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 anti-bacterial 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, between WT and *BTLA*$^{-/-}$ mice (Fig. 4A), WT mice treated with anti-BTLA and hamster IgG (Fig. 4B), or with HVEM-Ig and mouse IgG (Fig. 4C). Moreover, differences between all groups remained significant five days after LM infection. These experiments demonstrate that the HVEM-BTLA pathway regulates host immunity very early after infection, suggesting that BTLA signaling tempers early, innate-mediated anti-bacterial immunity.

We next tested the hypothesis that BTLA signaling suppresses early infection immunity by regulating innate cell function. As differences in bacterial load in WT and *BTLA*$^{-/-}$ mice were apparent very early after infection (Fig. 4A), we decided to use HKLM in order to avoid spurious results due to differing loads of live bacteria, as opposed to intrinsic differences in innate cell function. Splenocytes from WT or *BTLA*$^{-/-}$ mice were cultured with HKLM and the supernatants from these cultures were subsequently analyzed for cytokine secretion. BTLA-deficient splenocytes secreted significantly more TNF-α, IFN-γ and IL-6, compared to WT splenocytes, in response to *in vitro* HKLM stimulation (Fig. 5A). Similarly, *BTLA*$^{-/-}$ mice injected with 1×10^9 HKLM i.v. had significantly higher levels of serum TNF-α than WT mice 6 hours after injection (Fig. 5B). Strikingly, BTLA-deficient mice appeared to take longer to recover from this HKLM bolus than WT mice (unrecorded observations), suggesting that higher TNF-α, or other pro-inflammatory cytokine, levels may be increasing septic shock and/or immunopathology responses in these mice. Accordingly, a moderately higher dose (1.5×10^9) of HKLM rapidly induced death in all of the *BTLA*$^{-/-}$ mice, while 80% (4/5) of WT

mice were able to recover from this high dose (Fig. 5C). 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. The majority of 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 antibody, this study has addressed three novel and separate concepts of major importance: 1) Co-inhibitory 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 to 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.

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*$^{-/-}$ were more resistant to listerial infection. On the contrary, *LIGHT*$^{-/-}$ mice showed increased LM load in the liver compared to WT mice, suggesting 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). Since 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), it is 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 to WT mice. As we have previously reported that the only significant difference in the cellular compositions of the lymphoid organs of these BTLA-deficient mice, compared to 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 co-

inhibitory 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 over-activation of the immune system. Indeed, data presented here indicate that the over-active innate response in *BTLA*$^{-/-}$ mice render 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 ConA, most HVEM-deficient mice produce high amounts of proinflammatory cytokines, dying rapidly of a ConA-mediated cytokine storm within 5-8 hours (11). These findings may be particularly relevant to our recent description of an inhibitory effect of T cells on innate cell function (32); BTLA signaling may play an important role in this T cell-mediated tempering of the innate response. 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 over-active 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, capable of generating an effective memory response. These complicated issues remain to be determined.

In summary, our study has revealed a critical role for co-inhibition 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 co-signaling 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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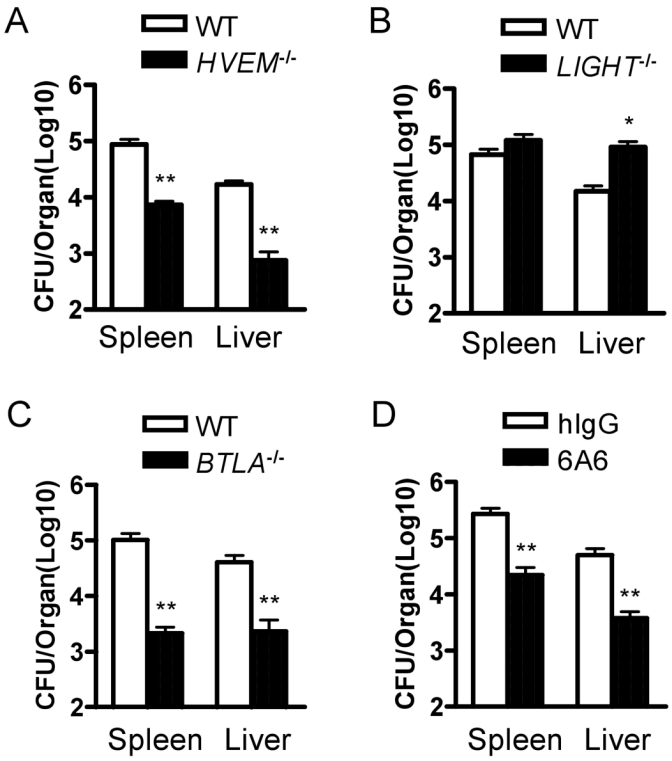

FIGURE 1.

HVEM-BTLA interactions play an essential role in regulating early LM clearance. Age and sex matched WT (Open bar) and knockout mice (Closed bar) including *HVEM*$^{-/-}$ (*A*), *LIGHT*$^{-/-}$ (*B*) and *BTLA*$^{-/-}$ (*C*) mice were infected with 5×10^5 CFU rLM-OVA by i.p. injection. (*D*) WT mice were infected with 5×10^5 CFU rLM-OVA and treated with 100 mg 6A6 (closed bar) or hamster IgG (hIgG, open bar) as control. Four days later, the bacterial burden in the spleen and liver was tested. Bar graphs depict mean±SEM of bacterial loads from six to nine mice per group.

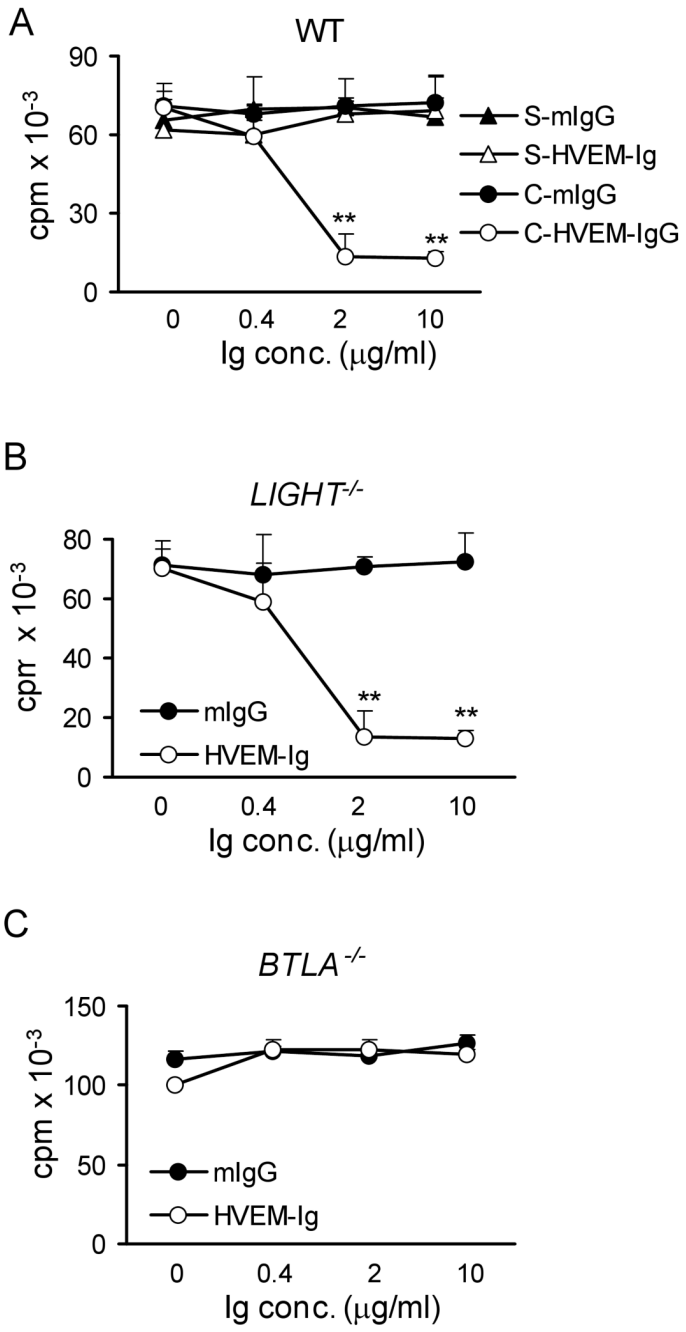

FIGURE 2.

Immobilized HVEM-Ig inhibits T cell proliferation in a BTLA-dependent but LIGHT-independent manner. LN cells isolated from WT (*A*), *LIGHT*$^{-/-}$ (*B*) or *BTLA*$^{-/-}$ mice (*C*) were stimulated with immobilized anti-CD3 (2 μg/ml) in the absence or presence of various doses of soluble mIgG (s-mIgG, closed triangle), soluble HVEM-Ig (S-HVEM-Ig, open triangle), plate bound mIgG (C-mIgG, closed circle) or plate bound HVEM-Ig (c-HVEM-Ig, open circle) for two days (*A*), or in the presence of plate bound mIgG (solid circle) or HVEM-Ig (open circle) for two days (*B* & *C*), then pulsed with [^{3}H]thymidine for 18 hours. Data are expressed as the mean [^{3}H]thymidine incorporation of triplicate cultures (+SD). The results are representative of three independent experiments.

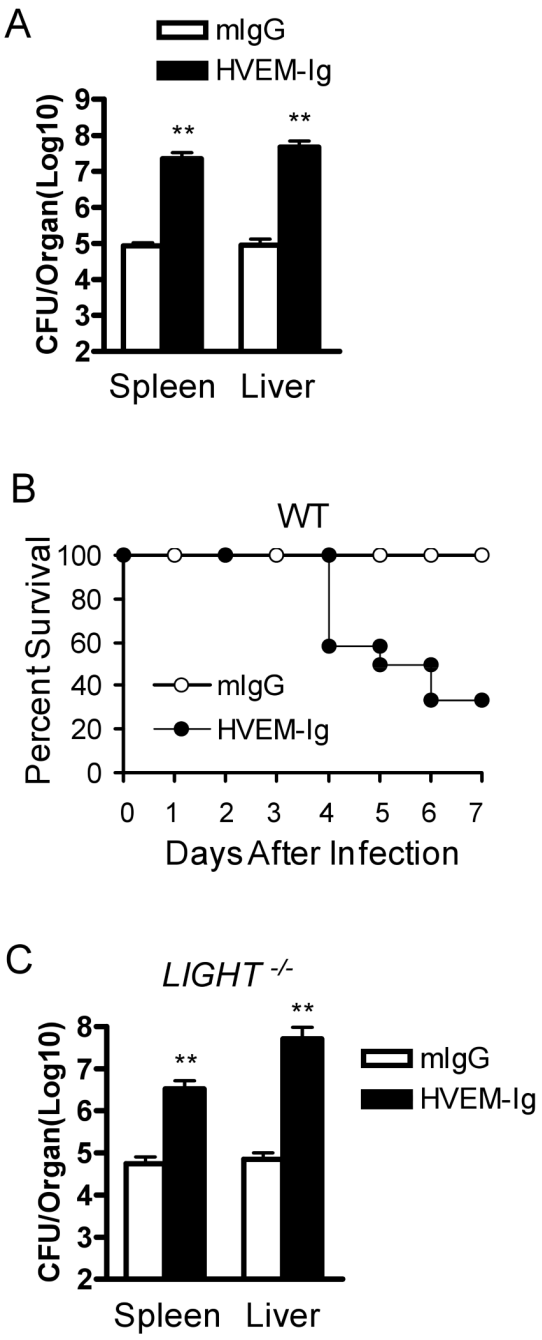

FIGURE 3.

HVEM-Ig treatment enhances host susceptibility to listeria infection in a LIGHT-independent manner. WT (*A* & *B*) and *LIGHT*$^{-/-}$ (*C*) mice were infected with 5×10^5 CFU rLM-OVA and treated with 200 to 300 μg of HVEM-Ig (closed bars) or mIgG (open bars). Four days later, bacterial burdens of spleens and livers (*A* and *C*, respectively) were tested. Survival was observed in a separate experiment (*B*). Bar graphs depict mean±SEM of bacterial load from six to twelve mice per group.

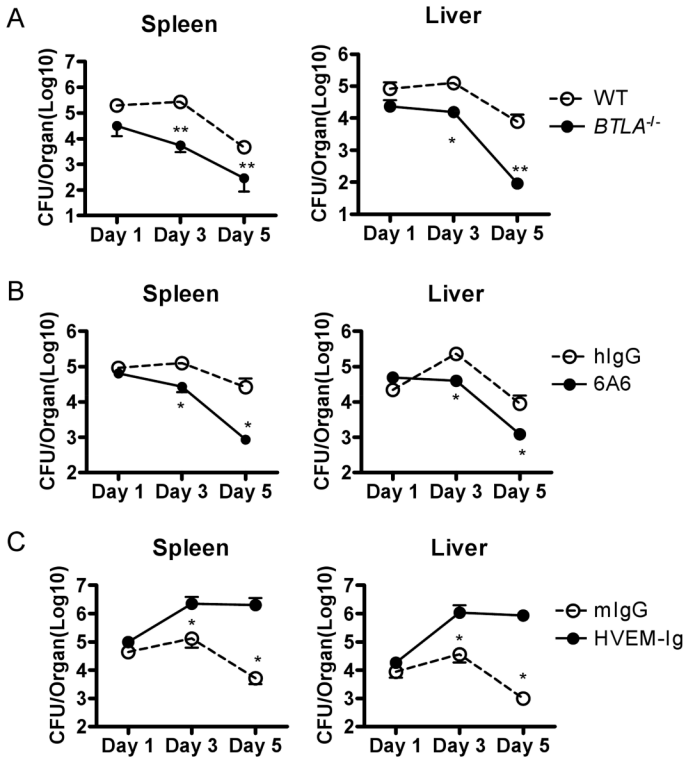

FIGURE 4.

Kinetics of LM infection in WT and *BTLA*$^{-/-}$ mice, and WT mice treated with anti-BTLA and HVEM-Ig. (*A*) WT (open circles) and *BTLA*$^{-/-}$ mice (closed circles), (*B*) WT mice treated with 100 μg of 6A6 (closed circles) or hIgG (open circles), and (*C*) WT mice treated with 200 μg of HVEM-Ig (closed circles) or mIgG (open circles), were infected with 5×10^5 CFU rLM-OVA. On days 1, 3, and 5, the bacterial burden of the spleen and liver was tested. Bar graphs depict mean±SEM of bacterial load from four to five mice per group.

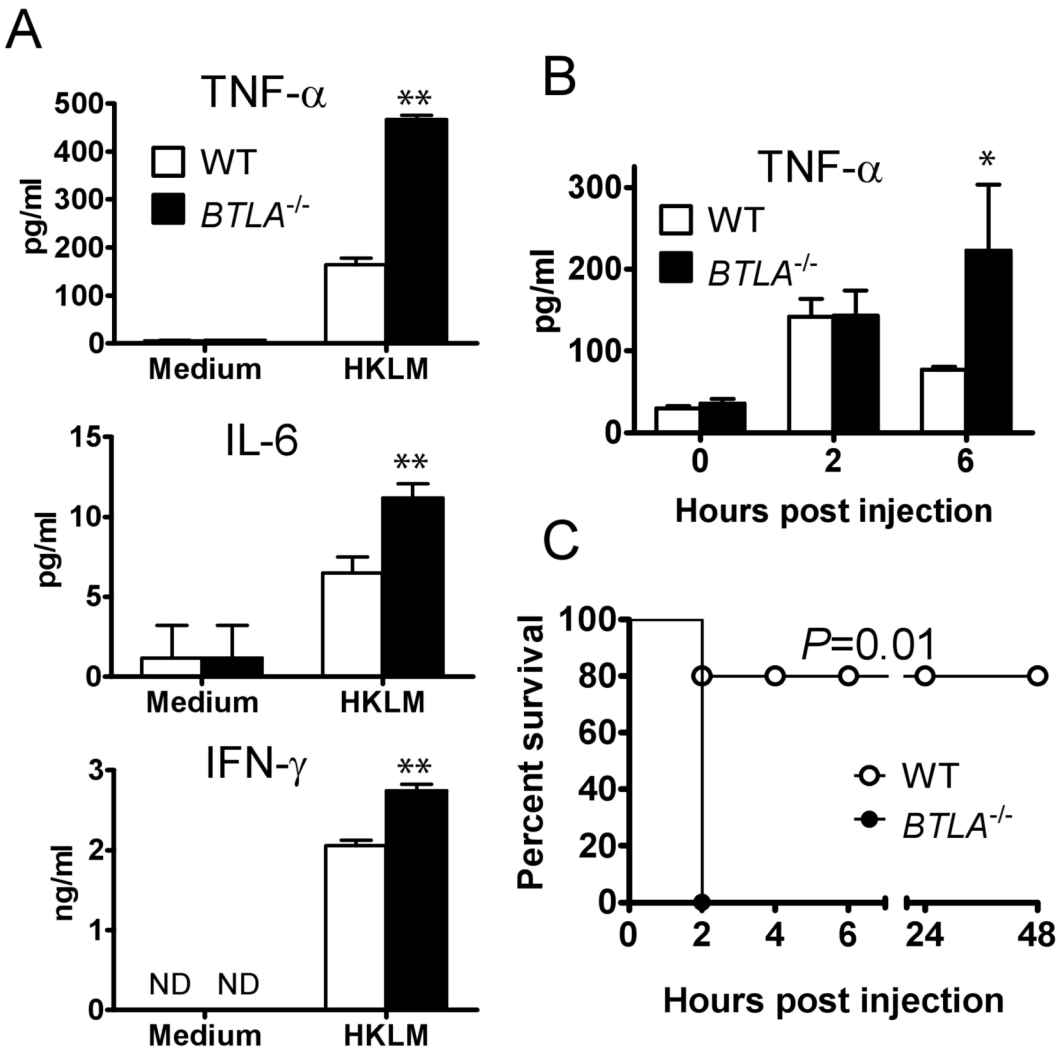

FIGURE 5.

Increased innate response to LM in the absence of BTLA. (*A*) WT and *BTLA*$^{-/-}$ splenocytes were incubated in triplicate wells with HKLM for 5 minutes (for TNF-α and IL-6 determination) or 15 minutes (for IFN-γ determination), washed, and cultured *in vitro* for 24 hours before supernatant cytokine levels were determined. (*B*) WT and *BTLA*$^{-/-}$ mice were injected i.v. with 1×10^9 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 1.5×10^9 HKLM and survival was monitored; five mice per group. Bar graphs depict mean±SEM; ND, not detected.