B and T Lymphocyte Attenuator Restricts the Protective Immune Response against Experimental Malaria

Guido Adler, Christiane Steeg, Klaus Pfeffer, Theresa L. Murphy, Kenneth M. Murphy, Jean Langhorne and Thomas Jacobs

_J Immunol_ 2011; 187:5310-5319; Prepublished online 12 October 2011;
doi: 10.4049/jimmunol.1101456
http://www.jimmunol.org/content/187/10/5310

References
This article cites 40 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/187/10/5310.full#ref-list-1

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Errata
An erratum has been published regarding this article. Please see next page or:
/content/188/2/923.full.pdf
BTLA and T lymphocyte attenuator (BTLA) is a recently discovered inhibitory receptor that shares structural and functional similarities with CTLA-4 and programmed death 1 (1). Recently, the interaction partner of BTLA, named herpesvirus entry mediator (HVEM), which is a member of the TNF receptor superfamily, has been identified (2). HVEM is expressed by most cells of hematopoietic origin but also by epithelial and endothelial cells (3–6). HVEM also binds to the receptor “homologous to lymphotoxins, inducible expression, containing protein tyrosine phosphatase 1 and 2 via immunoreceptor tyrosine-based inhibitory/switch motifs of BTLA. In accordance with the role of BTLA as a negative receptor, BTLA knockout (KO) T cells are hyperresponsive to Ag-specific activation (1). In acute allergic airway inflammation, BTLA contributes to the maintenance of Th2-mediated immune response, because mice lacking these receptors show prolonged lung inflammation (11). Moreover, an inhibitory function of BTLA has been demonstrated in in vivo transplantation models using MHC-mismatched cardiac allografts (12). BTLA-KO mice are also more susceptible to experimental autoimmune encephalomyelitis (1) and Con A-induced hepatitis (13). Triggering BTLA or deleting BTLA highly positive T cells also might have therapeutic significance. In a model of cerebral malaria, we have shown that an agonistic Ab can prevent experimental cerebral malaria (14) and others have shown that anti-BTLA can induce delayed onset of diabetes (15), decreased T cell-induced colitis (6), and prolonged allograft survival (15, 16).

Plasmodium falciparum malaria remains one of the leading causes of morbidity and mortality, especially in sub-Saharan Africa (17). The infection starts when infected mosquitoes inoculate sporozoites into the host during a blood meal. A few sporozoites enter the blood stream and reach the liver where they infect hepatocytes. In the liver, in particular, CD8+ T cells that recognize antigenic peptides on MHC class I can mediate pro-inflammatory (18). However, an effective immune response is hampered by several circumstances: 1) only very little antigenic material is delivered, 2) only very few cells in the liver are infected, 3) the liver stage is very short, and 4) the liver is a tolerogenic organ.
Thus, during this stage, the immune system has to be tuned to the highest sensitivity, vigor, and speed. The demands made on the immune response by the subsequent blood stage are very different, because many erythrocytes are infected that are either in the circulation or sequester in different organs, such as spleen, liver, lung, and brain. This leads to an enormous amount of antigenic material in the infected host, including parasitic material that is capable of triggering innate immunity by binding to TLR2 and TLR9 (19, 20). Consequently, the blood stage of malaria is accompanied by strong activation of T cells producing proinflammatory cytokines, such as IFN-γ and TNF-α. These cytokines are central mediators of protection but also are implicated in pathology, which often is associated with the blood stage of malaria infection (21). After repeated infection, individuals in hyperendemic areas often develop a state of “clinical immunity.” This stage is characterized by high Ab titers against blood-stage Ags, which control the parasitemia on a low level. Thus, a successful immune response against the blood stage of malaria relies on different arms of the immune system that have to be tightly controlled and coordinated to ensure rapid and strong activation of innate and adaptive immune mechanisms while avoiding overreaction that may cause severe inflammation.

We used the blood-stage infection of mice with nonlethal *Plasmodium yoelii* as a model to study the impact of BTLA on the regulation of the immune response against malaria blood stage. Infection of C57BL/6 mice causes a transient parasitemia that is cleared within 3 wk. Protection in this model is not dependent on a single immune mechanism, but instead it has been shown that early innate immune mechanisms mediated by macrophages but also T and B cells are needed to eliminate *P. yoelii* parasites (22).

Using BTLA-KO mice, we demonstrate that BTLA restricts the protective immune response during experimental malaria, because BTLA-KO mice display strongly reduced parasitemia, which correlated with increased cytokine production by T cells without inducing pathology. We also show that BTLA restricts the production of *P. yoelii* strain 17NL (PyNL)-specific IgG2a Abs in a B cell-intrinsic way. Furthermore, our data substantiate that not only the adaptive immune response is enhanced but also innate immune mechanisms in BTLA-KO mice contribute to decreased parasitemia, which is attributable to BTLA deficiency of innate cells themselves. Collectively, these data provide evidence that BTLA can modulate the various arms of the immune system throughout an immune response against infectious diseases.

Materials and Methods

**Ethics statement**

All of the experiments were conducted in accordance with the local animal ethics committee regulations (Ministry of Science and Health, Hamburg, Germany). All efforts were made to minimize suffering.

**Mice and parasites**

The following mice were used in the experiments: C57BL/6 (Charles River, Sulzfeld, Germany), BTLA-KO (The Jackson Laboratory, Bar Harbor, ME), HVEM-KO (K. Pfeffer, University of Duesseldorf, Duesseldorf, Germany), RAG1-KO and CD45.1 congenic C57BL/6 mice (both from the animal facility of the University Hospital of Hamburg, Hamburg, Germany), and JHT mice (Minka Breloer, Bernhard Nocht Institute, Hamburg, Germany). Double-knockout mice (JHT-BTLA-KO and RAG1-BTLA-KO) were generated by cross-mating the single-knockout strains, interrupting the resulting F1 generation, and then screening the F2 offspring for the absence of B cells and BTLA-JHT-BTLA-KO) or for the absence of B cells, T cells, and BTLA (RAG1-BTLA-KO) by flow cytometry. The absence of BTLA was verified further by PCR on genomic DNA for RAG1-BTLA-KO mice.

A stabilate of the nonlethal PyNL was prepared by freezing aliquots of highly parasitized blood in a solution of 0.9% NaCl, 4.6% sorbitol, and 35% glycerol. Mice were infected by i.p. injection of 200 μl of thawed stabilate diluted in PBS containing 2 × 10⁶ parasitized RBCs. Parasitemia was quantified by microscopic examination of Giemsa-stained blood smears.

**Depletion experiments**

For depletion of CD4⁺ cells, 300 μg anti-CD4 Ab (clone GK1.5) per mouse was injected i.p. at days −3, 0, 3, 9, and 15 relative to the day of infection (day 0). The absence of CD4⁺ cells was verified by flow cytometric analysis of blood leukocytes stained with anti-CD4-FITC Ab clone RM4-5, which is not blocked by clone GK1.5 in 100-fold excess in vitro (data not shown).

**Flow cytometry**

Splenocytes and blood leukocytes were stained with the following Abs after blockade of FcRs with anti-CD16/CD32 cell culture supernatant: anti-CD4-allophycocyanin (RM4-5), anti-CD4-V500 (RM4-5), anti-CD8-PerCP-Cy5.5 (53-6.7), anti-B220-allophycocyanin (RA3-6B2), anti-CD11b-PerCP-Cy5.5 (M1/70), anti-CD25-PE (3C7), anti-CD62L-PE (MEL-14), anti-CD69-PE (H1.2F3), anti-BTLA-PE (6F7), anti-HVEM-PE (H1), anti-CD45.1-PerCP (A20), anti-IL-2-allophycocyanin (JES5-5H4), anti-IL-4-allophycocyanin (11B11), anti-IFN-γ-allophycocyanin (XMG1.2), and anti-TNF-α-allophycocyanin (MP6-XT22). Analysis was performed on a FACSCalibur or LSRII cytometer.

**Analysis of liver transaminase levels in serum**

Blood was collected from the tail with lithium heparinate capillaries and analyzed with a Reflotron Plus system (Roche).

**Analysis of cytokine levels in serum**

Serum cytokine levels were determined with either the Th1/Th2 or the inflammatory cytokine bead array kit analyzed on a FACSCalibur flow cytometer.

**Quantification of PyNL-specific Ab titers in serum**

Ab-naive *P. yoelii*-parasitized RBCs were purified by magnetic separation. Blood from Ab-deficient JHT mice was taken by cardiac puncture 8 d postinfection with *P. yoelii*. Parasitemia was determined to be 20%. Heparinized blood was diluted with three parts of buffer (PBS, 0.5% BSA, and 2 mM EDTA) and passed over a MACS LS column (Miltenyi Biotec) in a magnetic field. After being washed twice with 3 ml buffer, parasitized RBCs were eluted outside the magnetic field. The eluate contained >90% parasitized RBCs. Parasitized RBCs were incubated for 90 min with 30 μl serum and bound Abs were quantified with biotinylated anti-isotype Abs and streptavidin-allophycocyanin (BD 554067) measured by flow cytometry. Data were analyzed and compared using the median fluorescence intensity.

**Generation of mixed bone marrow chimeric mice**

Six-week-old recipient mice were subjected to 9 Gy of cardiac puncture 8 d postinfection with *P. yoelii*. Parasitemia was determined to be 20%. Heparinized blood was diluted with three parts of buffer (PBS, 0.5% BSA, and 2 mM EDTA) and passed over a MACS LS column (Miltenyi Biotec) in a magnetic field. After being washed twice with 3 ml buffer, parasitized RBCs were eluted outside the magnetic field. The eluate contained >90% parasitized RBCs. Parasitized RBCs were incubated for 90 min with 30 μl serum and bound Abs were quantified with biotinylated anti-isotype Ab and streptavidin-allophycocyanin (BD 554067) measured by flow cytometry. Data were analyzed and compared using the median fluorescence intensity.

**Results**

**BTLA hampers immunity to *P. yoelii* NL blood-stage infection**

In previous studies, we demonstrated marked attenuation of the antiparasitic immune response mediated by the coinhibitory receptor CTLA-4 (23). However, due to its role as a master immune regulator, modulation of CTLA-4 signaling can have devastating immunopathological side effects. We therefore analyzed the influence of abrogating BTLA signaling on the antiparasitic immune response, because BTLA deficiency does not lead to pronounced immune pathology in the absence of infection and therefore can be regarded as a more subtle immune regulator. In the absence of BTLA signaling, mice suppressed parasitemia to low levels over the whole course of infection and completely cleared parasitemia.
3–6 d earlier (Fig. 1A). To assess whether the enhanced immune response was accompanied by immune pathology, serum levels of liver transaminases as hallmarks of immune-mediated liver damage were analyzed on day 6 postinfection, when parasitemia was already markedly lower in BTLA-KO mice than in wild-type mice (Fig. 1B). With baseline transaminase levels and no weight loss during the infection (data not shown), BTLA-KO mice did not display signs of immune pathology.

**BTLA-KO mice exhibit increased expression of HVEM**

Because BTLA is not the only ligand of HVEM, which also binds to LIGHT and CD160, altered expression levels of HVEM could influence these regulatory signaling pathways in BTLA-KO mice. We therefore quantified HVEM expression levels on different cell populations in BTLA-KO and wild-type mice. CD4+ and CD8+ T cells expressed significantly more HVEM in the absence of BTLA (Fig. 2A). Enhanced immune reactivity in BTLA-KO mice therefore could be a compound effect of two factors: 1) lack of attenuation due to lack of inhibitory BTLA signaling; 2) bidirectionally augmented costimulatory signals into HVEM- and LIGHT-expressing cells due to augmented HVEM expression. The latter effect may be enhanced further by the absence of BTLA–HVEM *cis* interactions that interfere with the HVEM–LIGHT interaction in *trans* (24).

**Phenotype of HVEM-KO mice suggests a protective role for the HVEM–LIGHT interaction**

To assess whether the enhanced immune response to *P. yoelii* in BTLA-KO mice is due to a lack of inhibitory BTLA signaling or enhanced HVEM–LIGHT signaling, we analyzed the infection in HVEM-KO mice. Parasitemia was unaltered by a lack of HVEM expression until day 12 postinfection, but at later time points HVEM deficiency caused significantly lower parasitemia and earlier parasite clearance (Fig. 2B). Because in HVEM-KO mice both the inhibitory BTLA–HVEM interaction and the costimulatory HVEM–LIGHT interaction are abolished, the comparable levels of parasitemia until day 12 postinfection in wild-type as well as in HVEM-KO mice suggest an influence of the costimulatory HVEM–LIGHT interaction on parasitemia in wild-type mice. Because in the absence of HVEM the coinhibitory HVEM–CD160 interaction also is abrogated, the influence of the costimulatory HVEM–LIGHT interaction even may be underestimated in this setting. Taken together, these data suggest a beneficial influence of the costimulatory HVEM–LIGHT interaction, especially early in the infection, and a disadvantageous influence of the coinhibitory BTLA–HVEM interaction over the whole course of the infection.

**Expression kinetics of BTLA and HVEM during infection**

In vitro studies demonstrated that upon activation CD4+ T cells quickly upregulate BTLA expression, whereas B cell expression of BTLA decreases slightly (10, 25). Consistent with these findings during *P. yoelii* infection, BTLA expression on CD4+ T cells increased between days 2 and 4 postinfection and remained increased over the course of the infection, albeit with considerable fluctuation (Fig. 3A). BTLA expression of B cells however increased between days 6 and 9 postinfection and slowly decreased to naive levels afterward. HVEM expression of B cells was not regulated substantially, but CD4+ T cells showed an increase of HVEM expression within the first 2 d postinfection with peak levels on day 4 postinfection and elevated expression levels over the whole course of infection Fig. 3B. This is in agreement with previous studies showing that Ag-specifically activated Th1 and Th2 CD4+ T cells upregulate HVEM expression within a week of culture (26). High expression of the BTLA ligand on CD4+ T cells during the infection may allude to an important role for CD4+ T cells in attenuating the immune response against *P. yoelii* infection via the BTLA–HVEM pathway.

**CD4+ T cells as well as early innate immune mechanisms contribute to decreased parasitemia in BTLA-KO mice**

CD4+ T cells are known to be central mediators of protection against *P. yoelii* infection in C57BL/6 mice (27). To assess whether BTLA deficiency of CD4+ cell populations would enhance the immune response sufficiently to render CD4+ cells dispensable, we depleted CD4+ cells by administration of an Ab. Of blood lymphocytes, <1% were CD4+ on days 0, 3, 6, 9, and 12 postinfection and <3.3% were CD4+ on day 15 postinfection (data not shown). During the first 6 d of infection, BTLA deficiency suppressed parasitemia to its full extent, irrespective of CD4+ cell depletion (Fig. 4A and inset). The early occurrence of this difference suggests that the causative cell population is part of the innate immune system. Later than 6 d postinfection, depletion of CD4+ cells abrogated protection despite BTLA deficiency in other compartments (Fig. 4A). Therefore, BTLA-deficient non-CD4+ cells cannot compensate for the lack of CD4+ cells completely. The central role of CD4+ T cells in protection is attributed to coordination of effector mechanisms of the innate as well as the adaptive immune system by cytokine secretion. In the first approach, we analyzed the serum levels of cytokines associated with the capacity of CD4+ T cells to coordinate immune responses as a potential mediator of differential parasitemia in BTLA-KO and wild-type mice, but no statistically significant differences were found (Fig. 4B). However, any direct comparison of immune parameters in BTLA-KO and wild-type mice during the infection suffers two major drawbacks. First, due to the broad expression of BTLA in many cell types, any
parameter measured on a particular cell population cannot be attributed to BTLA expression of that cell population alone but may be influenced by interaction with other cell populations and their BTLA expression. Thus, cell type-intrinsic effects of BTLA are masked. Second, the amount of antigenic material that the immune systems of BTLA-KO and wild-type mice are confronted with during the infection differs greatly, but the contribution to this difference of any particular cell type may be small. This may obfuscate immune correlates of protection in any single cell type, which would be measurable in response to identical parasitemia.

BTLA modulates infection-induced cytokine production in a CD4⁺ T cell-intrinsic way

To analyze the CD4⁺ T cell-specific and intrinsic effects of BTLA in response to uniform levels of parasitemia, we used mixed bone marrow chimeric mice. These mice harbor BTLA-KO and wild-type CD4⁺ T cells in similar proportions, distinguishable by the expression of congenic alleles of CD45. Thus, each CD4⁺ T cell is situated in identical mixed BTLA-KO/wild-type environments before and during the infection, ruling out any influence of BTLA expressed on non-CD4⁺ T cell populations on CD4⁺ T cell cytokine expression. Additionally, both types of CD4⁺ T cells are subjected to the same amount of antigenic material, providing for an undistorted comparison of the cytokine response (Fig. 5A). A higher proportion of BTLA-deficient than wild-type CD4⁺ T cells produced cytokines in response to *P. yoelii* infection (Fig. 5B). This was particularly distinctive regarding IL-2 production, which was induced very early, with peak levels on day 3 postinfection. At this time point, the proportion of cells producing IL-2 was more than twice as high in the BTLA-KO compartment than that in the wild-type compartment. Induction of TNF-α was seen 6 d postinfection and also was twice as frequent in CD4⁺ T cells lacking BTLA. The IFN-γ response did not differ substantially, but IL-4 production also was more frequent in BTLA-deficient cells 6 d postinfection. These data show that BTLA modulates the cytokine response of CD4⁺ T cells to an infection and thereby may impede the induction and maintenance of an efficient and well-coordinated immune response, including innate and humoral effector mechanisms.
Innate immunity to PyNL is hampered by BTLA

Interfering with adaptive immunity by CD4+ T cell depletion produced evidence for an immunoregulatory role for BTLA in the innate immune system, albeit in an indirect way (Fig. 4A). To directly demonstrate attenuation of innate immune responses by BTLA expressed in this compartment, we generated RAG1-BTLA double-deficient mice lacking BTLA as well as mature T and B cells. Mice without an adaptive immune system infected with PyNL fail to control parasitemia and have to be sacrificed before day 12 postinfection due to fulminant parasitemia. BTLA deficiency in the innate compartment partially rescues the ability to control parasitemia in the absence of adaptive immunity (Fig. 6A).

To further verify if BTLA has an immunoregulatory role in the innate compartment, we compared cytokine concentrations in sera of infected RAG1-deficient and infected RAG1-BTLA double-deficient mice. In the latter, we found increased levels of TNF-α at day 9 postinfection (Fig. 6B). These data substantiate our finding that BTLA also has an inhibitory function in the innate compartment.

BTLA expressed on B cells influences the Ab response to PyNL infection

B cells have been shown to be essential for the clearance of parasitemia in C57BL/6 mice infected with PyNL (28). To analyze whether effector mechanisms of non-B cells enhanced by the lack of BTLA could compensate for the lack of B cells, we generated B cell and BTLA double-deficient mice (JHT-BTLA-KO). In PyNL infection, parasitemia was as uncontrolled in these mice as in BTLA-sufficient JHT mice, demonstrating that B cells remain essential even if B cell-independent effector mechanisms are improved by a lack of BTLA expression (Fig. 7A). This finding prompted the analysis of the role for B cell-expressed BTLA in the humoral immune response to PyNL infection.

Evidence for the influence of B cell-expressed BTLA on the adaptive humoral response has been elusive so far, despite the fact that B cells express ~10-fold more BTLA than T cells. Studies on this matter have been hampered by the lack of a cell population-specific deletion of BTLA and the concurrent inability to delineate effects of B cell-expressed BTLA from the influence of BTLA expressed on other cell types during an immunization scheme or immune response. BTLA-KO 129SvEv mice were shown to produce substantially more IgG1, IgG2a, and IgG2b after T cell-dependent immunization with 4-hydroxy-3-nitrophenyl...
acetyl-keyhole limpet hemocyanin, but this was regarded as an effect of enhanced T cell help in the absence of T cell-expressed BTLA (1).

In response to T cell-independent immunization with 4-hydroxy-3-nitrophenyl acetyl-Ficoll, BTLA-deficient 129/SvEv mice showed hardly any alterations of IgM, IgG1, IgG2a, IgG2b, and IgA responses. Only IgG3 levels were enhanced modestly (25). Although direct T cell help is not required in this setting, an influence of the BTLA-deficient environment cannot be ruled out.

For the analysis of the influence of B cell-expressed BTLA on the humoral immune response to *Py*NL, we generated mixed bone marrow chimeric mice that harbor wild-type and BTLA-KO B cells in approximately equal proportions (58 and 42%, respectively). These B cells are situated in the identical mixed BTLA-KO/wild-type environment before and during the infection, ruling out any influence of BTLA expressed on non-B cells on Ab production. Because BTLA-KO and wild-type B cells expressed congenitally marked alleles of the Ig heavy chains, Abs produced by BTLA-KO and wild-type B cells in these chimeras can be detected independently and quantified by allotype-specific secondary Ab pairs. Sera from these mice taken 12 d postinfection were screened for *Py*NL-specific Abs by a novel flow cytometric method utilizing Ab-naive parasitized RBCs as target for Ab binding. IgG2a Abs produced by BTLA-KO B cells showed 3-fold higher binding activity compared with that of IgG2a Abs from wild-type B cells, with binding activity being a compound parameter of Ab concentrations and avidities. No differences were found for IgG1 and IgM isotypes (Fig. 7B). These data

---

**FIGURE 5.** BTLA deficiency facilitates a stronger cytokine response of CD4+ T cells to infection. Mixed bone marrow chimeric mice generated by transfer of 60% wild-type and 40% BTLA-KO bone marrow were infected i.p. with *Py*NL 10 wk after transfer. A, Splenocytes from infected mice at days 3 and 6 postinfection and from naive mice were stained with PE-labeled anti-BTLA, PerCP-labeled anti CD45.1, and V500-labeled anti-CD4 Ab. After permeabilization, cells were stained with allophycocyanin-labeled anti-cytokine or isotype control Ab and analyzed by flow cytometry. B, The percentage of cytokine-producing BTLA-KO and wild-type CD4+ T cells of individual mice is shown. Data were analyzed with paired Student t test (naive, *n* = 5; days 3 and 6 postinfection, *n* = 7). Data are representative of two independent experiments except for analysis of IL-2. Statistical significant differences are indicated with asterisks (*p* < 0.05, **p** < 0.01, ***p*** < 0.001).
demonstrate a strictly B cell-intrinsic effect of BTLA on Ab production.

**Discussion**

In the current study, we analyzed the function of BTLA on different cell populations in the control of *P. nyae* blood-stage infection. BTLA-KO mice displayed strongly reduced parasitemia over the whole course of the infection and cleared parasites earlier than control mice. This is consistent with recent publications describing BTLA as a negative regulator of the immune response in different models (29). We also have shown recently that a blockade of CTLA-4 enhances the immune response during experimental malaria infection, leading to lower peak parasitemia (23). Interestingly, this was accompanied by a strong inflammatory

![FIGURE 6. BTLA hampers innate immunity to *P. nyae* infection. A. RAG1-KO and RAG1-BTLA-KO mice were infected i.p. with *P. nyae* and parasitemia was monitored over the course of the infection. B. Cumulative data from two independent experiments analyzed with repeated-measures two-way ANOVA with the Bonferroni post test are shown as mean ± SEM (RAG1-BTLA-KO, *n* = 7; RAG1-KO, *n* = 8). At day 9 postinfection, sera of both groups of mice (*n* = 6) were analyzed for the indicated cytokines using a cytometric bead array. Data are expressed as mean ± SEM and were analyzed using the unpaired Student *t* test.](http://www.jimmunol.org/)

![FIGURE 7. BTLA attenuates the humoral immune response to *P. nyae* in a B cell-intrinsic way. A. JHT mice and JHT-BTLA-KO mice were infected i.p. with *P. nyae* and parasitemia was monitored over the course of the infection. Cumulative data from two independent experiments analyzed with repeated-measures two-way ANOVA with the Bonferroni post test are shown as mean ± SEM (*n* = 7). B. Mixed bone marrow chimeric mice generated by transfer of 65% wild-type and 35% BTLA-KO bone marrow were infected i.p. with *P. nyae* 10 wk after transfer. Ab production of wild-type and BTLA-KO B cells in the same bone marrow chimeric mouse in response to *P. nyae* infection was compared by quantifying binding of Abs from immune sera from the chimeric mice 12 d postinfection on Ab-naive parasitized RBCs. Data expressed as median fluorescence intensity were analyzed using the paired Student *t* test (naive, *n* = 11; *P. nyae* IgM, *n* = 4; *P. nyae* IgG2a, *n* = 3; *P. nyae* IgG1, *n* = 4). Statistical significant differences are indicated with asterisks (*p* < 0.05).](http://www.jimmunol.org/)
BTLA exerts its function upon binding to HVEM (26). Interestingly, we found that BTLA-KO mice express significantly enhanced levels of HVEM, which might indicate that HVEM expression and BTLA expression are tightly interconnected. Because HVEM also binds to LIGHT, which is a costimulatory interaction in both directions (31), it has to be taken into account that BTLA-KO mice not only lack the coinhibitory BTLA–HVEM interaction but also might have an enhanced costimulatory pathway due to the increased expression of HVEM, which might augment signaling via HVEM–LIGHT. The absence of BTLA also liberates HVEM from cis interactions that block costimulatory engagement in trans, potentially further increasing costimulatory effects. To get more insight into the function of the HVEM–LIGHT interaction during malaria, we analyzed the course of infection in HVEM-KO mice. These mice exhibit a phenotype that was in between that of BTLA-KO and wild-type mice, because they do exhibit lower parasitemia than wild-type mice at later time points but are more susceptible overall than BTLA-KO mice. This might indicate that a costimulatory HVEM–LIGHT interaction augments immune mechanisms, especially early in infection, because the absence of this interaction partially counterbalances the immune-enhancing effect of BTLA deficiency. In contrast, malaria-induced immune pathology was found to be independent of HVEM–LIGHT interactions using the P. berghei ANKA model of experimental cerebral malaria (14).

PyNL infection is an attractive model to study the immune modulatory mechanisms of BTLA, because in this article innate as well as T cell- and B cell-mediated effector mechanisms are implicated in protection and studies already have shown that BTLA does affect innate mechanisms (32) as well as T cell-mediated mechanisms (33). Attenuation of BCR signaling by BTLA has been shown (34); however, a B cell-intrinsic effect on B cell effector mechanisms has been elusive so far. To understand how BTLA modulates the immune system, we first analyzed the kinetics of expression during infection. Both CD4+ T cells and CD8+ T cells (data not shown) upregulated BTLA and HVEM early postinfection. In contrast, B cells, expressing higher levels of BTLA than T cells in naive mice, further upregulated BTLA during infection, but only a slight increase in HVEM expression was found. Interestingly, BTLA and HVEM were induced homogenously in the whole T cell compartment during PyNL infection, whereas only a fraction of 30–40% of T cells in the blood or spleen express activation markers as we have shown in a previous study (23). This indicates that the induction of BTLA and HVEM is mediated by systemic factors such as cytokines without TCR engagement.

Using B cell-deficient JHT mice, we demonstrated that B cells remain central for protection even if the immune response is enhanced by a lack of BTLA on all of the other cells. We analyzed the PyNL-specific humoral response of BTLA-KO and wild-type B cells in identical in vivo environments by means of PyNL-infected mixed bone marrow chimeras. The novel approach of analyzing the binding of serum Abs from these chimeras not to denatured and concentrated, intra- and extracellular crude plasmodial Ag in ELISA but to fully intact, Ab-naïve parasitized RBCs ensured the relevance of the results for the immune response in vivo. Given the identical environment that the B cells were analyzed in, differences can be attributed to B cell-intrinsic effects of BTLA, excluding, for example, the recently found influence of BTLA on T follicular helper cells (35). BTLA-KO B cells produced significantly more IgG2a Abs directed against parasitized RBCs than wild-type mice. IgG2a has been shown to be the Ig isotype most important for protection against PyNL (36). IgG1 production was not found to be significantly augmented by a lack of BTLA on B cells when measured 12 d postinfection; however, IgG1 production is known to peak considerably later in infection than IgG2a, at least in other mouse strains (37, 38). Therefore, analysis at later time points possibly could unveil augmented IgG1 production. Because IgM production of BTLA-KO versus wild-type B cells was equal if not lower, it is tempting to speculate that the increased production of IgG2a Abs by BTLA-KO B cells is not due to overall higher B cell reactivity but may be caused by improved germinal center reaction in the absence of BTLA on B cells.

It is known that CD4+ T cells contribute to protection, whereas CD8+ T cells are virtually dispensable (27, 39, 40). By depleting CD4+ T cells, we found that from day 9 postinfection the resistant phenotype of BTLA-KO mice was abrogated, which provided evidence that BTLA expression on CD4+ T cells hampers the induction of adaptive immunity. Interestingly, until day 6 postinfection, BTLA-KO mice were more resistant even in the absence of CD4+ T cells. Thus, BTLA expressed on CD4+ cells modulates early effector mechanisms during malaria infection. This is consistent with findings in a model of Listeria monocytogenes infection where also an effect of BTLA deficiency on innate immunity was demonstrated (32). To further evaluate which innate immune responses were modulated by BTLA, we used BTLA-KO mice on RAG1-KO background. Although these mice lack all B and T cells, BTLA deficiency facilitates better control of parasitemia. Because NK cells and granulocytes, cell populations potentially involved in parasite control, express only marginal levels or no BTLA at all (10), their contribution to the observed enhanced parasite clearance in BTLA-RAG1 double-deficient mice is unlikely. Instead, it is more likely that macrophages, which express considerable levels of BTLA in wild-type mice (10), contribute to the enhanced innate response seen in BTLA-deficient animals. This is corroborated further by our finding that BTLA deficiency of RAG1-KO mice was accompanied by higher production of TNF-α. Due to the fact that all of the cells of the innate immune system, including macrophages, express HVEM, binding to BTLA might occur either in trans or in cis. Until now, a cis interaction of HVEM–BTLA was only demonstrated on naive T cells and it was suggested that BTLA in cis competes with BTLA in trans, whereas only the latter is capable of triggering HVEM-dependent NF-κB activation (24). If such a scenario is likely to occur on innate immune cells is not known presently. It was shown recently that BTLA-KO mice exhibit enhanced innate immunity upon infection with L. monocytogenes (32). In this model, splenocytes from BTLA-KO mice secrete more cytokines compared with their wild-type counterparts when incubated with heat-killed listeria in vitro, indicating that BTLA deficiency leads to increased production of cytokines by immune cells. The considerably decreased parasitemia in BTLA-KO mice is mediated by multiple cell populations alleviated of BTLA attenuation. This raises problems in finding immune correlates of enhanced protection in any single cell population, because parasitemia is lowered cooperatively to such an extent in BTLA-KO mice that enhanced effector mechanisms of any single cell population may be confounded by the overall lower immune activation called for by lower parasitemia. Therefore, alterations of immunological parameters between wild-type and BTLA-KO mice
BTLA DAMPENS PROTECTION AGAINST MALARIA

The authors have no financial conflicts of interest.

CD45.1

marked cells, CD45.1+ wild-type cells could be delineated from the sequence of BTLA function. Because we transferred congenitally in this study, the differences in effector mechanisms are a direct consequence of BTLA. Interestingly, even in the absence of infection, BTLA-KO T cells produce proinflammatory cytokines in significantly higher frequencies compared with their wild-type counterparts within the identical host. This, again, demonstrates that BTLA is a negative regulator of T cell function and thus might act synergistically with other negative regulators such as programmed cell death or CTLA-4.

In summary, our findings provide evidence that BTLA dampens infection, without influencing immune polarization. T cell pool in this model but restricts the production of cytokines in the absence of infection, BTLA expression. This, again, demonstrates that BTLA is a negative regulator of T cell cell elimination of malaria from hepatocytes. Science. 2004; 305(5691):1078–1081.


Corrections


In Fig. 2A, the flow cytometry contour plot intended to depict HVEM expression of CD8<sup>+</sup> cells in BTLA-deficient mice is a duplicate of the plot above, showing HVEM expression of CD4<sup>+</sup> cells in the same mouse. The replacement figure represents data obtained from the same experiment and performed at the same time as the rest of the data in Fig. 2A. The figure has been replaced in the online version of the article, which now differs from the printed version as originally published.

The corrected figure is shown below. The figure legend was correct as published and is shown below for reference.

FIGURE 2. HVEM expression is increased in the absence of BTLA and modulates parasitemia by engaging multiple ligands. A, Splenocytes of non-infected wild-type and BTLA-KO mice were stained with PerCP-labeled anti-CD8, allophycocyanin-labeled anti-CD4, and PE-labeled anti-HVEM or isotype control Ab and were analyzed by flow cytometry. Representative results of HVEM expression on CD4<sup>+</sup> and CD8<sup>+</sup> cells are shown as contour plots (left panels) and histograms (middle panels). The expression of HVEM as a summary of six mice per group expressed as mean fluorescence intensity is depicted in the right panels. Data were analyzed with the unpaired Student *t* test. B, Wild-type and HVEM-KO mice were infected i.p. with *P. yoelii* and parasitemia was monitored over the course of the infection. Cumulative data for three independent experiments are shown analyzed by repeated-measures two-way ANOVA with the Bonferroni post test and depicted as mean ± SEM (*n* = 18). Statistical significant differences are indicated with asterisks (**p < 0.01, ***p < 0.001).