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B and T Lymphocyte Attenuator Exhibits Structural and Expression Polymorphisms and Is Highly Induced in Anergic CD4+ T Cells

Michelle A. Hurchla,* John R. Sedy,* Maya Gavrielli,* Charles G. Drake,‡ Theresa L. Murphy,* and Kenneth M. Murphy**‡

B and T lymphocyte attenuator (BTLA) was initially identified as expressed on Th1 cells and B cells, but recently reported to be expressed by macrophages, dendritic cells, and NK cells as well. To address this discrepancy we generated a panel of BTLA-specific mAbs and characterized BTLA expression under various activation conditions. We report the existence of three distinct BTLA alleles among 23 murine strains, differing both in Ig domain structure and cellular distribution of expression on lymphoid subsets. The BALB/c and MRL/lpr alleles differ at one amino acid residue, but C57BL/6 has nine additional differences and alters the predicted cysteine bonding pattern. The BALB/c BTLA allele is also expressed by B cells, T cells, and dendritic cells, but not macrophages or NK cells. However, C57BL/6 BTLA is expressed on CD11b+ macrophages and NK cells. Finally, in CD4+ T cells, BTLA is expressed most highly following Ag-specific induction of anergy in vivo, and unlike programmed death-1 and CTLA-4, not expressed by CD25+ regulatory T cells. These results clarify discrepancies regarding BTLA expression, suggest that structural and expression polymorphisms be considered when analyzing BTLA in various murine backgrounds, and indicate a possible role in anergic CD4+ T cells. The Journal of Immunology, 2005, 174: 3377–3385.

A g-presenting cells express ligands for receptors on T cells during primary activation that provide both positive and negative costimulatory signals (1, 2). CD28 provides important positive costimulation for T cells, whereas CTLA-4 provides negative regulation (3, 4), and each interact with B7-1 and B7-2 expressed by APCs (1). Additional costimulatory molecules have been identified, such as ICOS expressed by T cells (5), providing an activating signal during T cell priming (6, 7) upon interaction with B7h expressed by APCs and somatic cells (8–10). A second inhibitory receptor, programmed death-1 (PD-1) (11) is expressed by both activated CD4+ and CD8+ T cells and LPS-activated B cells (12), and interacts with a pair of ligands, PD-L1 and PD-L2 (13, 14).

We recently identified a receptor expressed on lymphocytes, B and T lymphocyte attenuator (BTLA) (15), which shared features with these other costimulatory receptors. Similar to CD28, ICOS, CTLA-4, and PD-1, BTLA contains a single Ig domain and is expressed exclusively by lymphoid cells. Similar to PD-1, BTLA contains ITIMs, and like PD-1 and CTLA-4 associates with Src homology domain 2-containing protein tyrosine phosphatase 1 (SHP-1) and SHP-2 (15, 16). BTLA+/− lymphocytes showed increased proliferation (15, 17), and BTLA−/− mice showed enhanced susceptibility rather than resistance to peptide-induced experimental allergic encephalomyelitis (15), suggesting an inhibitory activity. Distinct from CTLA-4 and PD-1, BTLA contains another tyrosine-based motif similar to those interacting with the Grb2 adaptor molecule (18). Finally, we recently showed that the ligand for BTLA is the TNFR family member herpesvirus entry mediator (19).

Independently, Kaye and colleagues (17) recently characterized BTLA expression and analyzed behavior of BTLA−/− T cells, confirming our interpretation of BTLA as an inhibitory receptor. In addition, that study reported BTLA expression on additional lymphoid cells including dendritic cells and macrophages (17) and noted differences in the Ig domain between two mouse strains, BALB/c and C57BL/6. We had also noted polymorphisms in the sequence of BTLA cDNA isolated from murine cell lines derived from distinct backgrounds. Therefore, the purpose of this study was to resolve discrepancies regarding the pattern of BTLA expression by lymphoid subsets, and to characterize the basis of the observed sequence polymorphisms in murine BTLA.

Our approach involved the generation of a panel of mAbs to BTLA capable of distinguishing between allelic variants expressed by different strains. Such reagents provided the means to directly test for potential expression level polymorphisms between alleles of BTLA. In addition, these regents were used to further characterize BTLA expression and regulation in CD4+ T cells during activation and induction of anergy. We find that murine BTLA exhibits both structural and expression polymorphisms between various murine genetic backgrounds, and is selectively induced to high levels on anergic T cells rather than on CD25+ T regulatory cells. These data suggest that BTLA may play a complex regulatory role during the immune response that is distinct from the general inhibitory role mediated by CTLA-4 and PD-1.
Materials and Methods

Reagents

The following Abs used for FACS analysis were from BD Pharmingen: CD4-CyChrome (RM4-5), CD8-FITC (53-6.7), B220-allophycocyanin (RA3-6B2), CD11b-FITC (M1/70), CD11c-FITC (HL3), DX5-FITC, I-A<sup>PE</sup> (AMS-32.1), I-A<sup>PE</sup> (AF-6-120.1), IgM-PerCP Cy5.5 (R6.60.2), CD21/CD25-FITC (7G6), CD25-allophycocyanin (PC61), CD62 ligand-FITC (MEL14), Thy-1-PerCP (OX-1), Thy-1-PerCP, anti-mouse IgG-PF, mouse anti-Arabian/Syrian hamster IgG-PF (mixture), Streptavidin (SA)-PE, SA-Chrome, and SA-allophycocyanin. KJ1-26 TriColor, hamster IgG-biotin, and murine IgG1-biotin were from Caltag Labs. All FACS analysis included an initial incubation with 2.4G2 (anti-CD16/CD32); BD Pharmingen) to block Fc receptor interactions. DO11.10 TCR transgenic analysis included an initial incubation with 2.4G2 (anti-CD16/CD32; BD Pharmingen) to block Fc receptor interactions. DO11.10 TCR transgenic analysis included an initial incubation with 2.4G2 (anti-CD16/CD32; BD Pharmingen) to block Fc receptor interactions.

Sequenceing of BTLA and PD-1 Ig domains

Exon 2 of BTLA or PD-1, encompassing the Ig domain, was amplified by PCR from genomic DNA from a panel of mouse strains previously described (23) using Easy-A High Fidelity PCR Cloning Enzyme (Stratagene) and the following intronic primers: BTLA (sense) AGTCTGGTCTCAAGAAGTGAC (antisense) ATAGATGTTGCCTGGGATGAC and PD-1 (sense) CAGGCTCTCCTCCACAGC (antisense) CTAAGGGTCCTGGACGAC. PCR products were cloned into the pGem-T Easy vector (Promega) and inserted from at least three individual subclones from each strain were sequenced using the T7 universal primer.

Generation of soluble BTLA Ig domain

The Ig domain of C57BL/6 BTLA was PCR amplified from cDNA using the following primers: BTLA (sense) CAGGCCAGGAAAGCTACTAAGAGGAA-3<sup>tail</sup> and BTLA (antisense) CGGGATCCCTGAAGAGTTTTGAGTCCTTTTC-3<sup>tail</sup>. This product was subcloned into the pET28c vector (Novagen) that had been modified to contain a BirA biotinylation sequence (GGGLNDIFQAEKQIEWHE) onto the C terminus of the BTLA Ig domain (24). Proteins were expressed as insoluble inclusion bodies in BL21 (DE3) Codon Plus RIIL (Strategene) and refolded as described (24).

Production of mAbs to BTLA

Armenian hamsters or BALB/c background BTLA<sup>−/−</sup> mice were immunized with 100 µg of refolded C57BL/6 BTLA Ig domain protein in CFA, boosted biweekly with 100 µg of protein in IFA, and received a final i.v. boost 3 days before fusion. Splenocytes were cultured with 10 µg/ml PMSF and 100 µg/ml myeloma, and hybridoma supernatants screened for binding to IBAB cells expressing either C57BL/6 or BALB/c BTLA Ig domains as GFP fusion proteins. The BTLA-GFP chimera was prepared by splicing by overlap PCR of C57BL/6 and BALB/c BTLA Ig domains as described (23) using Easy-A High Fidelity PCR Cloning Enzyme (Stratagene). This fragment was then cloned into the pCT302-AGA2d vector (25) to create an HA-tagged fusion to the Aga2 peptide. QuickChange mutagenesis was used to introduce mutations into the BTLA cDNA (amino acids 323–339) and irradiated (2000 rad) BALB/c splenic APCs as stimulators. Th0 cells were differentiated in 100 U/ml IL-4 and 3 µg/ml anti-IL-12 (TOSH), and IL-2. Cells were restimulated with Ag and APCs on days 7 and 14. Th1/Th2 phenotypes were confirmed on days 7 and 14 by intracellular cytokine staining for IFN-γ and IL-4.

Gene microarray

Anergic T cells were isolated by adoptively transferring 2.5 × 10<sup>5</sup> Thy1.1<sup>+</sup> HA-specific T cells to recipient mice (C3-HA<sup>+/+</sup>) as previously described (21, 26, 27). After 4 days in vivo, animals were sacrificed via CO<sub>2</sub> asphyxiation. Spleens were harvested, and subjected to ACK lysis. Adoptively transferred HA<sup>+</sup> T cells were sorted using a FACSVantage equipped with the CellQuest program (Becton Dickinson) to find the subset of cells with high binding to the BTLA-GFP chimera (see Materials and Methods). Sorted T cells were enriched using fluorescence-based cell sorting on a FACSVantage TurboSort (BD Biosciences). The resulting populations were between 95 and 99% pure.

 Yeast display mapping

The Ig domain of the C57BL/6 BTLA allele was amplified from cDNA using the primers 5′-GGAATTCATCTGAGCAAGCAGAGCTCCGCTG-3′ and 5′-CATGCTAGGCGAGAGAAGCTAAGAGGAAAGA-3′ and subcloned into the Ndel and the Nhel sites of the pCT302-AGA2d vector (25) to create an HA-tagged fusion to the Aga2 peptide. QuickChange mutagenesis was used to introduce additional mutations into the BTLA cDNA. Western blot analysis was conducted as previously described (16). Conditions used to induce Th1 and Th2 development were as previously described (22).

CD4<sup>+</sup> T cell activation and expression analysis

DO11.10 TCR transgenic cells were activated with 0.3 µM OVA peptide (amino acids 323–339) and irradiated (2000 rad) BALB/c splenic APCs as described (20). Th1 conditions consisted of heat-killed Listeria monocytogenes, IL-2 (20 U/ml; Takeda Chemical Industries), and 10 µg/ml anti-IL-4 (1B11). Th2 cells were differentiated in 100 U/ml IL-4, 3 µg/ml anti-IL-12 (TOSH), and IL-2. Cells were restimulated with Ag and APCs on days 7 and 14. Th1/Th2 phenotypes were confirmed on days 7 and 14 by intracellular cytokine staining for IFN-γ and IL-4.

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Assessment of energy by proliferation

On indicated days following transfer of HA-TCR transgenic T cells, 20 × 10⁶ splenocytes were incubated with increasing doses of HA peptide. Proliferation was assayed after 48 h, with a [³H]thymidine pulse in the final 12 h.

BTLA expression by naive, activated, and anergic CD⁴⁺ T cells

HA-TCR transgenic T cells were enriched by depletion of CD⁸⁺ and B220⁺ cells as earlier described. Cells were CFSE-labeled as previously described (29) before adoptive transfer of 2.5 × 10⁶ clonotypic cells via tail vein injection. Cells were stained with anti-Thy1.1 PerCP and the anti-BTLA Ab 6F7-biotin, followed by SA-PE.

Purification and activation of CD⁴⁺CD25⁺ T regulatory cells

Splenocytes and lymph node cells from BALB/c mice were isolated. Following erythrocyte lysis, B220⁺ cells were depleted by magnetic separation with anti-B220 Microbeads (Miltenyi Biotec). The negative fraction was stained with CD25-PE (BD Pharmingen) and anti-PE Microbeads (Miltenyi Biotec) and magnetically separated into CD25⁺ and CD25⁻ fractions. Enrichment was assessed by FACS as shown (see Fig. 7D). Contaminating non-CD⁴⁺ cells were mainly B220⁻ or CD8⁺. To activate T cells, 1 × 10⁶ cells/ml of each fraction were cultured on flat-bottom plates coated with 10 µg/ml 2C11 (anti-CD3; BD Pharmingen) for 48 h. Cells were pulse with 1 µCi/well [³H]thymidine for an additional 12 h.

Ab response to NP-Ficoll

Eight-week-old BTLA⁻/⁻ and BTLA⁺⁻ littermate mice on the 129SvEv background were immunized i.p. with 50 µg of nitrophenyl (NP)-Ficoll (Biosearch Technologies) in Imject alum (Pierce). Sera were collected on day 14, and the titers of anti-NP were determined by ELISA using NP²⁵-BSA (Biosearch Technologies) for Ab capture and the Southern Biotechnology clonotyping/HRP kit for IgG subclass-specific ELISA (Southern Biotechnology Associates).

Results

Allelic polymorphisms in BTLA

We previously generated BTLA cDNA from several sources, including from the cell line WEHI 231, a commercial murine C57BL/6 splenocyte cDNA library, and 129SvEv mice (15), finding several polymorphisms within the BTLA Ig domain coding sequence. To determine the basis of differences, we sequenced the coding region for the BTLA Ig domain from genomic DNA of several inbred and wild mouse strains (23) (Fig. 1). Among 23 strains, we identified three distinct alleles of BTLA, differing in their predicted amino acid sequence and potential predicted disulfide bonding pattern (Fig. 1A). The allele represented by BALB/c was present in CBA/J, SJLJ, New Zealand White (NZW), BXSB, C3H/J, New Zealand Black (NZB/BinJ), NOD, 129SvEv, and 129Sv/J (Fig. 1B). A second allele, represented by the strains MLR/lpr, AKR, SWR, CALB/RK, and DBA/2J, differed from the BALB/c allele at only one amino acid, containing histidine rather than arginine at residue 38 of the BTLA protein. These two alleles each have five cysteine residues within the Ig domain, predicting two disulfide bonds and one unpaired cysteine. The third allele, represented by C57BL/6, was also present in B10.PL and several wild-derived inbred strains, and differed from the BALB/c and MLR/lpr alleles at 10 and 11 amino acid residues, respectively (Fig. 1A). Notably, the C57BL/6 allele has a cysteine at amino acid residue 49, making six total cysteine residues with three predicted disulfide bonds in the BTLA Ig domain. As a control, we found no sequence polymorphisms in the PD-1 Ig domain from BALB/c, MLR/lpr, and C57BL/6 (data not shown).

Generation of allele-specific mAbs to murine BTLA

To generate anti-BTLA mAbs, we immunized Armenian hamsters and BTLA⁻/⁻ BALB/c mice with recombinant Ig domain of the C57BL/6 BTLA allele. To allow the identification of Abs that could potentially recognize either the BALB/c or C57BL/6 allele of BTLA, hybridoma supernatants were screened for binding to BJAB cells expressing either the C57BL/6 or BALB/c allele of BTLA as a GFP fusion protein. One hamster anti-BTLA Ab, 6A6, was identified that reacted only with the C57BL/6 allele, but not the BALB/c allele of BTLA (Fig. 2A). The majority of the murine anti-BTLA Abs reacted with both the C57BL/6 and BALB/c BTLA alleles, including 6F7, 6G3, 8F4, and 3F9.D12 (Fig. 2B). One murine Ab, 3F9.C6, reacted only with C57BL/6 BTLA, and not with BALB/c BTLA. Another Ab, 6H6, reacted with both alleles, but stained the C57BL/6 allele more highly than the BALB/c allele. For each of these Abs, staining was observed on wild type splenocytes, but not splenocytes of BTLA⁻/⁻ mice (Fig. 2C, and data not shown), suggesting that these Abs in fact recognize BTLA, and react with native BTLA as well.

To further assess how these Abs interact with BTLA, we characterized their behavior in IP and Western blot analysis (Fig. 2, D and E). The pan-specific Abs 6F7 and 6G3 each specifically immunoprecipitated both the C57BL/6 and BALB/c allele of BTLA as a GFP fusion protein. One hamster anti-BTLA Ab, 6A6, was identified that reacted only with the C57BL/6 allele, but not the BALB/c allele of BTLA (Fig. 2A). The majority of the murine anti-BTLA Abs reacted with both the C57BL/6 and BALB/c BTLA alleles, including 6F7, 6G3, 8F4, and 3F9.D12 (Fig. 2B). One murine Ab, 3F9.C6, reacted only with C57BL/6 BTLA, and not with BALB/c BTLA. Another Ab, 6H6, reacted with both alleles, but stained the C57BL/6 allele more highly than the BALB/c allele. For each of these Abs, staining was observed on wild type splenocytes, but not splenocytes of BTLA⁻/⁻ mice (Fig. 2C, and data not shown), indicating that the allelic specificity observed by FACS analysis extends to its behavior in IP Western blot analysis. Also, these interactions seen in IP Western blot analysis were specific because no BTLA was immunoprecipitated using mouse or hamster IgG1 as an isotype control (Fig. 2D, lanes 7–10).

Notably, although equivalent amounts of each BTLA allele were immunoprecipitated when assessed by immunoblotting for the GFP epitope of the fusion proteins, detection of the Ig domain by IP Western blot analysis was not equally efficient. Following immunoprecipitation, the C57BL/6 BTLA Ig domain was detected much more strongly than the BALB/c allele by 6G3 and 6F7, both pan-specific anti-BTLA Abs, (Fig. 2D, top panel, lanes 1, 2, and 4–6). These results may indicate differential sensitivity between alleles for recognition or detection of the Ig domains, even using pan-specific Abs, which could result from differential sensitivity to denaturation of the antigenic epitope. Whatever the cause, it is necessary to consider this fact when using IP Western blot analysis in comparing BTLA from varying allelic backgrounds. Finally, certain Abs allow coimmunoprecipitation of BTLA-associated
proteins. For example, IP Western blot analysis using 6A6 reproduces the known (15) specific and inducible coassociation of SHP-2 with BTLA following pervanadate treatment (Fig. 2E).

Mapping antigenic epitopes recognized by anti-BTLA Abs

To map which of the polymorphic residues differing between BALB/c and C57BL/6 BTLA were involved in strain-specific reactivity of 6A6 and 3F9.C6, we used yeast display technology (30). We first expressed the BTLA Ig domain as an Aga2 fusion protein, and then generated a series of mutant BTLA Ig domains with single amino acid substitutions at the polymorphic residues, replacing BALB/c residues into the C57BL/6 allele one residue at a time (Fig. 3). This series of wild type and mutant BTLA proteins were then analyzed for reactivity with pan-specific anti-BTLA mAbs and two B6-specific Abs, 6A6 and 3F9.C6 (Fig. 3). As a positive control, we confirmed that the pan-specific anti-BTLA mAb 6F7 recognized the wild type C57BL/6 BTLA Ig domain, and also recognized each of the single residue substitutions of BTLA (Fig. 3, left column), as expected for pan-specific reactivity. In contrast, the two C57BL/6-specific Abs recognized some, but not all of BTLA mutants. Specifically, 6A6 showed a very selective loss of reactivity only with the Q27E, C49W, and Q66R substitutions, indicating that these residues are involved in the strain-specific recognition of BTLA. A distinct pattern of reactivity was observed with 3F9.C6, with a selective loss of reactivity with the R107W substitution and reduced reactivity with the Q27E substitution. Also, whereas 6A6 reactivity is sensitive to the C49W substitution, which disrupts one of three predicted disulphide bonds, 3F9.C6 reactivity remains in this substitution. These results indicate that the C57BL/6 specificity of these two Abs derive from interactions with the distinct, but polymorphic, region of the BTLA Ig domain.

In summary, at least two of the BTLA alleles can be distinguished by their antigenic structure, as shown by two C57BL/6-specific anti-BTLA Abs. Importantly, we also identified several pan-specific anti-BTLA Abs, which now allow direct comparisons of the fine specificity of tissue expression of native BTLA expression between various murine strains.

Distribution and expression of murine BTLA

In our previous studies (15, 16), we were restricted to analyzing BTLA expression either by mRNA expression or by using epitope-tags because we lacked Abs to native BTLA. Conceivably, we failed to detect low but physiologically important levels of BTLA.
on certain lymphocytic subsets for this reason. Thus, we examined BTLA surface expression on various lymphoid subsets again, using both allele-specific Ab 6A6 and pan-specific Ab 6F7 (Fig. 4).

First, BTLA was expressed uniformly on B cells at levels that were similar for C57BL/6 and BALB/c mice (Fig. 4A). CD4⁺ and CD8⁺ T cells expressed lower levels of BTLA compared with B cells, but again, at levels that were similar for C57BL/6 and BALB/c mice. For 6A6, we found that a subpopulation of CD11b⁺ cells, CD11c⁺ dendritic cells, and DX5⁺ cells were positive for BTLA expression, and again identified only in C57BL/6 mice as expected (Fig. 4A, middle row). Using the pan-specific 6F7 Ab, we found that B cells express the highest levels of BTLA, again at levels similar between C57BL/6 and BALB/c mice, with lower levels expressed in CD4 and CD8 T cells (Fig. 4A, lower row). Interestingly, using the pan-specific reagent 6F7, we found that BTLA was expressed on CD11c⁺ BALB/c cells at levels similar to CD11c⁺ C57BL/6 cells, but that BTLA was only expressed on CD11b⁺ macrophages and DX5⁺ NK cells from C57BL/6 mice, but not in BALB/c mice (Fig. 4A, lower row). The fact that 6F7 detects BTLA expression on B cells, T cells, and CD11c⁺ cells from both BALB/c and C57BL/6 mice serves as a control for its ability to bind BTLA from both strains. Thus, the selective binding of 6F7 to DX5⁺ and CD11b⁺ cells only in C57BL/6, not BALB/c mice, indicates a difference between these strains for BTLA expression by these cell types. Thus, these strains appear to have a distinct difference in the cell types expressing detectable BTLA, explaining the differences between BTLA expression reported previously (15, 17).

We also examined BTLA expression in splenic B cell populations (Fig. 4B). BTLA expression was detected at the highest levels on follicular B cells (IgM⁺CD21⁺CD35⁺), and at reduced levels on marginal zone B cells (IgM⁺CD21⁻CD35⁺) and transitional B cells (IgM⁺CD21⁻CD35⁻) (Fig. 4B). Notably, because the 6F7 pan-specific Ab was used for analysis, we can also conclude that the levels on each subpopulation of B cells are similar between C57BL/6 and BALB/c mice (Fig. 4B).

We next examined BTLA expression in thymocyte and B cell development (Fig. 5). In thymus, BTLA was expressed at highest levels on mature CD4⁺ T cells, and at slightly reduced levels on CD8⁺ T cells (Fig. 5A). BTLA expression on immature CD4⁻CD8⁻ T cells or CD4⁺CD8⁺ double positive T cells was nearly undetectable (Fig. 5A). In bone marrow, BTLA was expressed at the highest levels on B220⁺ IgM⁺ mature B cells (Fig. 5B), and was detected at relatively low levels on B220⁺IgM⁺ immature B cells. BTLA expression was undetectable on B220⁺ IgM⁻ pro-B cells and pre-B cells. Further, we found no differences between C57BL/6 or BALB/c mice for the levels of BTLA expression on the thymocyte and bone marrow populations.

Finally, we examined the BTLA expressed on CD4⁺ T cells under various conditions of activation and polarization by cytokines (Fig. 6A). BTLA surface expression on resting CD4⁺ T cells was induced by 10-fold on day 2 following activation with Ag and APCs, decreased by day 4, and was nearly undetectable by day 7 after activation (Fig. 6A). The rapid increase in BTLA expression by day 2 on Ag-activated CD4⁺ T cells occurred both in Th1-inducing or Th2-inducing conditions (Fig. 6A). Upon secondary T cell activation, BTLA was again highly induced 2 days following activation, again in both Th1 and Th2 cultures (data not shown). However, tertiary activation of T cells revealed selective induction in the Th1 cultures, but not in the Th2 cultures (Fig. 6A). These results suggest that BTLA expression on CD4⁺ T cells is initially controlled primarily by T cell activation and not by factors governing Th1 or Th2 differentiation. The delayed loss of BTLA inducibility in Th2 cells might suggest a silencing process rather than a Th1-specific pathway for induction, which would be consistent with our initial finding that BTLA expression is not dependent on Stat4 or Stat1 (15). Finally, the rapid modulation of BTLA...
expression, peaking on day 2 and extinguished by day 7, suggests that it may act in the mid-phases of T cell activation following interactions with APCs.

In contrast to the activation-dependent expression of BTLA seen in CD4\(^{+}\) T cells, BTLA expression on B cells was maintained at high levels throughout activation by LPS or anti-IgM stimulation (Fig. 6B). These results differ slightly from the reported 3- to 10-fold decrease in BTLA expression following LPS activation of B cells (17). Nonetheless, our results agree with that report in the finding of high levels of BTLA expression on B220\(^{+}\) B cells in the periphery, and to some degree, the constitutive nature of its expression.

Selective induction of BTLA on anergic T cells

Previously, a method of anergy induction for naive CD4\(^{+}\) T cells was developed that involves adoptive transfer of Ag-specific CD4\(^{+}\) T cells into recipients expressing Ag on somatic tissues (21, 33). FIGURE 4. BTLA shows broad and allelic-specific expression on lymphoid cell populations. A, Four-color FACS analysis was conducted on splenocytes from C57BL/6 (solid histogram) or BALB/c (dotted histogram). Two-color histograms (upper row) of the indicated markers used to gate cells for single-color histograms of 6A6 (middle row) or 6F7 (lower row) staining are shown. In the columns one, two, and three, cells were stained with anti-B220 allophycocyanin, anti-CD4 CyChrome, anti-CD8 FITC, and either biotinylated b-6A6 or b-6F7 followed by SA-PE secondary. In columns four, five, and six, cells were stained with anti-I-A\(^{d}\) PE (BALB/c cells) or anti-I-A\(^{d}\) PE (C57BL/6 cells), and anti-CD11b FITC (fourth column), CD11c-FITC (fifth column), or anti-DX-5 FITC (sixth column), and b-6A6 or b-6F7 followed by SA-CyChrome secondary. Shaded histograms are staining of a mixture of C57BL/6 and BALB/c splenocytes using isotype controls of biotinylated hamster IgG (middle row) and mouse IgG1 (lower row). The numbers shown in top panels are the percentage of live cells within the indicated gate. The identity of the gated population is indicated in the panel. B, C57BL/6 and BALB/c splenocytes were stained with Abs to identify the following B cell populations: follicular B cells (FO), IgM\(^{+}\)CD21/CD35\(^{int}\); marginal zone (MZ), IgM\(^{hi}\)CD21/CD35\(^{hi}\); transitional (TR), IgM\(^{+}\)CD21/CD35\(^{wm}\). Staining with the pan-BTLA-specific Ab 6F7 revealed equivalent BTLA levels between strains for all subsets.

FIGURE 5. BTLA is expressed during late stages of B and T lymphocyte development. A, Thymocytes from C57BL/6 (solid histogram) or BALB/c (dotted histogram) mice were stained with a combination of markers, anti-B220 FITC, anti-CD11c FITC, anti-CD11b FITC, anti-GR-1 FITC, anti-DX-5 FITC, CD4-CyChrome, CD8-PE, and either biotinylated (b)-6A7 or b-mouse IgG1, and SA-allophycocyanin. The two-color histogram (first panel) is gated on marker (FITC)-negative live cells, and the numbers indicate the percentage of cells in the indicated gates. Single-color histograms for each gate are shown for b-6F7/SA-allophycocyanin staining for CD4\(^{+}\)CD8\(^{+}\) double negative (DN), CD4\(^{+}\)CD8\(^{+}\) double positive (DP), CD4\(^{+}\) single positive (CD4 SP), or CD8\(^{+}\) single positive (CD8 SP) populations. Shaded histograms are staining the b-mouse IgG1 isotype control. B, Bone marrow cells were stained with anti-B220 allophycocyanin, anti-IgM PerCp Cy5.5, either b-6F7 or murine IgG1-biotin, and SA-PE. The numbers are the percentage of live gated cells within the three numbered gates. BTLA expression is shown in the single-color histograms for each gate; gate 1, Pre-B cells and Pro-B cells (IgM\(^{+}\) B220\(^{hi}\)); gate 2, Immature B cells (IgM\(^{+}\) B220\(^{hi}\)); gate 3, Mature B cells (IgM\(^{+}\) B220\(^{hi}\)). Shaded regions are mouse IgG1 isotype control staining.
FIGURE 6. BTLA expression during CD4⁺ T cell activation and Th1 polarization. A, DO11.10 transgenic T cells were purified by cell sorting (22) and activated with 0.3 μM OVA peptide 324–336 under Th1 or Th2 conditions (see Materials and Methods). Cells were harvested either before activation (Day 0) or on the indicated day following primary activation, and stained with KJ1-26 TriColor, b-6F7, and SA-PE. T cells were restimulated with OVA peptide on day 7 and day 14. B, BALB/c splenocytes were stimulated with 10 μg/ml anti-IgM and 5 μg/ml anti-CD40 (left) or 1 μg/ml LPS (right). Single-color histograms of B220⁺ cells (anti-B220-FITC) are shown for b-6F7/SA-PE staining on day 0 (dotted histogram) and day 2 (solid histogram) after activation. Shaded histograms are the biotinylated mouse IgG1 isotype control.

Specifically, clone 6.5 transgenic T cells, reactive to HA peptide 110–120 presented by I-Ad, become anergic when transferred into recipient mice expressing a membrane bound form of HA targeted for expression on lung and prostate tissue (27). We analyzed BTLA expression following T cell transfer on various days after transfer using Affymetrix gene arrays and FACS (Fig. 7, A and B). We found that BTLA mRNA was highly induced in these anergic CD4⁺ T cells in this system, compared with CD4⁺ T cells activated by Ag-expressing vaccinia virus (Fig. 7A). At 2 days after transfer, BTLA expression by T cells undergoing anergy induction was twice the level of naive T cells, and significantly higher than activated T cells. This induction was even more evident by day 3 and day 4 following transfer, with BTLA expression ~3-fold higher than in naive T cells. By contrast, BTLA levels were substantially reduced in fully activated T cells compared with naive or anergic T cells at these times (Fig. 7A). As a control, myosin VIIa, a constitutive “housekeeping” gene, showed essentially no change in these three conditions over these times. Thus, BTLA mRNA appears to decline more rapidly than BTLA surface protein in activated T cells because activated T cells express peak BTLA surface levels at day 2 (Fig. 6), but show reduced BTLA mRNA (Fig. 7B). These observations are consistent with the reduced BTLA surface expression by day 4 and the essentially undetectable BTLA expression by day 7.

We next measured BTLA expression by FACS under conditions of anergy induction or activation (Fig. 7B). Notably, the highest levels of BTLA surface expression coincided with induction of anergy in vivo. Specifically, 6 days after transfer, anergic T cells expressed ~10-fold higher BTLA than naive T cells, and ~3-fold higher than in vivo-activated T cells (Fig. 7B). We verified that the CD4⁺ T cells transferred into HA-expressing recipients did become anergic as defined by lack of proliferation (Fig. 7C), consistent with previous reports (26). For comparison, we also wished to evaluate BTLA expression on conventional naive CD4⁺ T cells (CD4⁺CD25⁻) T cells or T regulatory cells (CD4⁺CD25⁺) either as resting cells ex vivo or after in vitro activation with anti-CD3 (Fig. 7D). As expected, BTLA was expressed at low levels on naive T cells, and was induced ~10-fold 36 h after anti-CD3 treatment. Freshly isolated T regulatory cells expressed similar levels of BTLA as freshly isolated naive CD4⁺ T cell, but showed only a slight increase after treatment with anti-CD3 (Fig. 7D). As a control, we confirmed that T regulatory cells, but not naive T cells, expressed PD-1, consistent with previous reports (31). As a further control, we showed that the isolated CD25⁺ T regulatory cells failed to proliferate in vitro, in contrast to the robust proliferation of freshly isolated naive T cells (Fig. 7E). In summary, BTLA shows a pattern of expression that is somewhat distinct from that of CTLA-4 and PD-1 in terms of its response to anergy induction and expression by T regulatory cells.

Role of BTLA in T cell-independent Ab responses

Our initial analysis of BTLA was motivated by consideration of its role in T cell activation (15). However, the fact that B cells express the highest level of BTLA, and the constitutive nature of this expression, motivated a second examination of its effect on Ab production. In our study, we examined T cell-independent Ab responses using immunization with NP-Ficoll in wild-type mice or BTLA⁻/⁻ 129SvEv mice, which express the BALB/c allele of BTLA. We immunized cohorts of mice with one injection of NP-Ficoll in alum and measured production of anti-NP Abs of specific isotypes on day 14 (Fig. 8). For the isotypes IgM, IgG1, IgA, we found no specific changes in levels of anti-NP Abs. For IgG2a or IgG2b, we found only slight increases in anti-NP Abs in the BTLA⁻/⁻ compared with wild-type mice. However, for Abs of the IgG3 isotype, which is primarily associated with T-independent responses, we found an ~2-fold increased in anti-NP-specific Abs in BTLA⁻/⁻ mice compared with wild-type mice. The size of this difference is consistent with the relatively modest increases in B cell and T cell proliferation responses described for BTLA⁻/⁻ cells previously by both our report (15) and by others (17), and consistent with an inhibitory rather than activating role of BTLA. However, the relatively modest magnitude of this effect could also be an indication that BTLA expression by B cells may serve a purpose other than cell-intrinsic signaling, such as perhaps delivery of a signal toward cells expressing ligands for BTLA.

Discussion

We initially identified BTLA as a novel transcript selectively expressed in Th1 cells, although we did recognize that high levels were also present in resting splenic B cells (15). Having identified it from a screen of novel Th1-specific genes, our initial study emphasized actions of BTLA in T cell-dependent responses, although we did examine limited features of Ab responses as well. The features of BTLA signaling identified in our report suggested an inhibitory role in T lymphocytes. Our suggestion of an inhibitory action of BTLA was recently confirmed independently by Kaye and colleagues (17), who also generated mAbs to murine BTLA. That study reported a distinct pattern of BTLA expression, in that
FIGURE 7. BTLA is induced on anergic CD4+ T cells, but not CD4+CD25+ regulatory T cells. A, HA-TCR T cells were transferred into and subsequently harvested from B10.D2 mice (naive), C3-HA<sup>eq</sup> mice (anergized) or B10.D2 mice infected with vaccinia-HA (activated) on days 2, 3, 4, or 7 after transfer as indicated. After harvest, T cells were isolated using combined magnetic bead and fluorescence sorting (27), and cDNA probe prepared and hybridized to Affymetrix microarrays M174A, M174B, and M174C. Relative BTLA expression intensity was determined using a probe prepared and hybridized to Affymetrix microarrays M174A, M174B, and M174C. A latin-squares approach in Affymetrix Microarray Suite, version 5.1. software. Expression of myosin VIa gene is shown as a control. B, CFSE-labeled HA-TCR T cells were adoptively transferred into B10.D2 mice (naive), C3-HA<sup>eq</sup> mice (anergized), or B10.D2 mice immunized with vaccinia-HA (activated), and harvested on day 6 as in A. Cells were stained with anti-CD4 allophycocyanin, anti-Thy1.1 PerCP, and either biotin-6F7 or murine IgG1-biotin, and SA-PE. BTLA expression is shown as single-color histogram for CFSE<sup>+</sup> (naive) or CFSE<sup>+</sup> (activated) and anergized for CD4<sup>+</sup>Thy1.1<sup>+</sup> donor cells. C, Splenocytes harvested from recipients as in A were restimulated with HA peptide and proliferation measured on day 2. D, Splenocytes and lymph node cells from BALB/c mice were enriched for CD25-negative and CD25-positive populations using anti-CD25-PE and magnetic beads as described in Materials and Methods, and stained with anti-CD4-Cy-chrome, and biotin-conjugated 6F7, or biotin-IgG1. Two-color dot plots are shown for CD25 and CD4<sup>+</sup> cells as assayed directly ex vivo. In contrast in C57BL/6, BTLA is expressed on macrophages, NK cells, and dendritic cells, as reported by Kaye and colleagues (17), and on B cells and T cells as we previously described (15). However, using pan-specific mAbs that react with both C57BL/6 and BALB/c mice, we can directly demonstrate that the BALB/c allele is expressed by B cells, T cells, and a population of dendritic cells, but not by macrophages or NK cells as assayed directly ex vivo. In contrast in C57BL/6, BTLA is expressed by macrophages or NK cells assayed directly ex vivo.

We previously used cells from the BALB/c background to characterize BTLA tissue distribution because we had included analysis of DO11.10 TCR transgenic T cells in our study, and this line was maintained on the BALB/c background. Further, we did not initially characterize BTLA expression on CD11c<sup>+</sup> dendritic cells. Thus, our initial characterization of BTLA expression was accurate for the strain we examined, and the discrepancy between our results (15) and those of Kaye and colleagues (17) may largely be due to an unexpected BTLA expression polymorphism. The basis of this expression polymorphism is not clear, and could be due either to actual differences in genetic regulatory elements directly controlling BTLA, or alternately to differences in the state of physiologic activation operating in vivo in unmanipulated mice. In any case, this difference, and the structural differences in the Ig domain that may impact in vivo additional lymphoid subsets were identified to express BTLA expression than initially we described.

Moreover, we have reconciled these differences by generating Abs to murine BTLA that either distinguish between allelic variants of BTLA, or recognize BTLA expressed on all laboratory mouse strains. Kaye and colleagues (17) used two Abs, PK3 and PK18, which recognize only the BTLA allele expressed by C57BL/6 strain, but not BALB/c, thus being able to examine BTLA expression on only a subset of mouse strains. With our pan-specific BTLA Abs, we could directly compare BTLA expression on different lymphoid cell types between several strains of mice. Surprisingly, we find polymorphic tissue distribution between C57BL/6 and BALB/c mice in BTLA expression on particular lymphoid cells assayed directly ex vivo. Specifically, using our C57BL/6-specific Ab, 6A6, we confirm that BTLA is expressed on macrophages, NK cells, and dendritic cells, as reported by Kaye and colleagues (17), and on B cells and T cells as we previously described (15). However, using pan-specific mAbs that react with both C57BL/6 and BALB/c BTLA, we can directly demonstrate that the BALB/c allele is expressed by B cells, T cells, and a population of dendritic cells, but not by macrophages or NK cells as assayed directly ex vivo. In contrast in C57BL/6, BTLA is expressed by macrophages or NK cells assayed directly ex vivo.

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![FIGURE 8.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
activity of BTLA, should be considered in ongoing analysis of the functional role of BTLA in the immune system.

The present study describes interesting features regarding the regulation of BTLA expression. BTLA expression by mature B cells appears constitutive, and is acquired during the later stages of B cell development in the bone marrow. This pattern contrasts with a more complicated expression pattern on T cells. In the thymus, BTLA is undetectable in double negative and double positive thymocytes, and expressed at low levels on mature CD4+ and CD8+ T cells, which are maintained in the periphery. Following Ag-specific activation of CD4+ T cells, BTLA is rapidly induced, with peak surface expression by day 2, quickly diminishing by day 4, and nearly undetectable by day 7. Similarly, secondary reactivation of T cells leads to rapid BTLA induction. However, upon tertiary reactivation, BTLA induction is maintained only in Th1, and not Th2, culture conditions. We have not exhaustively examined subsequent regulation of BTLA induction in vitro, but we have noted long-term Th1 clones to express BTLA.

This study has uncovered an additional and unique aspect of BTLA expression in CD4+ T cells, in that it is most highly expressed following anergic induction. We have studied a system of anergy in which CD4+ T cells encounter Ag expressed by somatic tissues, proceed through an activated effector phase, followed by the development of an anergic phenotype (27). In examining gene expression in this system, we found BTLA to be one of the most highly induced genes selectively expressed in anergic CD4+ T cells. We confirmed this observation using FACS analysis, again finding the highest level of BTLA surface expression on anergic T cells compared with naive or normally activated CD4+ T cells. By contrast, BTLA was not highly expressed on naturally occurring CD4-CD8- double negative and double positive thy-mocytes, and expressed at low levels on mature CD4+ and CD8+ T cells, which are maintained in the periphery. Following Ag-specific activation of CD4+ T cells, BTLA is rapidly induced, with peak surface expression by day 2, quickly diminishing by day 4, and nearly undetectable by day 7. Similarly, secondary reactivation of T cells leads to rapid BTLA induction. However, upon tertiary reactivation, BTLA induction is maintained only in Th1, and not Th2, culture conditions. We have not exhaustively examined subsequent regulation of BTLA induction in vitro, but we have noted long-term Th1 clones to express BTLA.

This study also demonstrated a modest enhancement in the BTLA-deficient mouse of T cell-independent IgG3 isotype responses, again suggesting an inhibitory role of BTLA in B cells. We note that the magnitude of this effect is only ~2- to 3-fold. Although it is conceivable that such small effects could contribute to fine-tuning of the Ab response, and that this effect is the reason d’età of BTLA, we would suggest instead that there might be circumstances showing greater differences between wild type and BTLA-/-, such as in models of transplantation and graft-vs-host disease (data not shown). Thus, in pursuing BTLA function in various experimental models, it will be necessary to keep in mind the role that polymorphisms in BTLA structure and expression might play, as well as distinct regulation of BTLA in T cells that may differ between the various strains of mice used in these experimental models.

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Disclosures
The authors have no financial conflict of interest.

References
CORRECTIONS


An error was made in the grant information. The correct footnote is shown below.

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The third author’s last name is misspelled. The correct name is Maya Gavrieli.


The first author’s last name is misspelled. The correct name is Isabelle Béatrice Bekeredjian-Ding. The error has been corrected in the online version, which now differs from the print version as originally published.


In References, an author’s name was omitted from Reference 17. The correct citation is shown below.