B and T Lymphocyte Attenuator Is Highly Expressed on CMV-Specific T Cells during Infection and Regulates Their Function

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BTLA was highly induced on CMV-specific CD8+ T cells immediately following their differentiation from naive cells. After control and is progressively downregulated in memory and differentiated effector-type cells. During primary CMV infection, BTLA expression went down on memory CD8+ cells. Engagement of BTLA by mAbs blocked CD3/CD28-mediated T cell proliferation and Th1 and Th2 cytokine secretion. Finally, in vitro blockade of the BTLA pathway augmented, as efficient as anti–PD-1 mAbs, allogeneic as well as CMV-specific CD8+ T cell proliferation. Thus, our results suggest that, like PD-1, BTLA provides a potential target for enhancing the functional capacity of CTLs in viral infections. The Journal of Immunology, 2010, 185: 3140–3148.

Immune responses are controlled by cosignaling molecules that regulate T lymphocyte activation either positively or negatively. The B7/CD28 family of negative receptors include CTLA4, programmed cell death 1 (PD-1) (also called CD279), and a recently identified molecule known as B and T lymphocyte attenuator (BTLA) (1, 2). In mice, PD-1 is found on activated, but not resting, T and B cells (3). PD-1 has two ligands called PD-L1 (also called B7-H1 and CD274) and PD-L2 (also called B7-DC and CD273), which have distinct patterns of expression. Recently, it has been reported that PD-1 is highly expressed on exhausted murine T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection and that it is upregulated on human HIV and HCV-specific CD8+ T cells. Blocking of PD-1/PD-1 ligand interactions reinvigorates exhausted T cells, allowing them to expand and produce effector cytokines (4–10), raising the possibility that this pathway has been exploited by a variety of viruses to establish chronic infection.

BTLA (CD272) is the most recently described member of the CD28 family. BTLA was first identified as a transcript highly specific to Th1 cells (11), but it was later shown to also be expressed by thymocytes (12). BTLA interacts with herpes virus-entry mediator (HVEM), a member of the TNFR family (13). The interaction of BTLA, an Ig superfamily member, and HVEM, a costimulatory receptor homologous to TNFR, is quite unique in that it defines a cross talk between these two families of structurally distinct receptors (14). BTLA is expressed on a broad array of murine immune cells, including B cells, CD4+ T cells, CD8+ T cells, NK cells, macrophages, and dendritic cells (DCs).

Like PD-1, BTLA contains a membrane proximal ITIM and membrane distal immunoreceptor tyrosine-based switch motif (ITSM). Disruption of either the ITIM or ITSM abrogated the ability of BTLA to recruit either Src homology region 2 domain-containing phosphatase (SHP) 1 or SHP2 (15), suggesting that BTLA recruits SHP1 and SHP2 in a manner distinct from PD-1 and that both tyrosine motifs are required to block T cell activation. The human BTLA cytoplasmic region also contains two Grb-2 binding sites, YDN and YSN (16).

PD-1 and BTLA are both implicated in the negative regulation of immune responses. PD-1, moreover, may not only to be involved in peripheral tolerance control, but also in immune exhaustion in chronic infections and cancer. The role of BTLA appears to be limited to lymphocyte activation as shown in BTLA-deficient mice, which have normal lymphoid organ development. However, these mice display hyperproliferative T cell responses to TCR-mediated activation (11, 17). Furthermore, loss of BTLA function leads to increased susceptibility to experimental autoimmune encephalomyelitis and MHC-mismatched allograft rejection, supporting a role for BTLA in modulating T cell activation and effector responses (11, 18). In contrast to PD-1, BTLA is not upregulated...
in the LCMV murine model of chronic infection, and consequently, it is not supposed to play a prominent role in the function of antiviral-specific CD8 T cells, at least during chronic infection (19). Yet, its role in the specific human antiviral T cell responses has not been demonstrated.

Hence, we have performed a comprehensive study of human BTLA expression on the subsets of CD4+ and CD8+ T cells and compared its expression to that of its relative PD-1. In addition, we have analyzed BTLA expression in the course of CMV-specific responses in vitro.

Materials and Methods

Generation of anti-human, PD-1, and BTLA mAbs

All mAbs were produced similarly. Female BALB/c mice were immunized by i.p. injection with 10 μg human Ig fusion protein with 250 μl Freund adjuvant. Immunization was repeated three times at 2-wk intervals; the fourth immunization was made by i.v. injecting with 10 μg Ig fusion protein. Three days later, spleen cells were fused with X63Ag8 myeloma cells with PEG 1500 (Roche, Basel, Switzerland) and cloned with HAT selection (Sigma-Aldrich, St. Louis, MO) and hybridoma cloning factor (Origen, Igen, Rockville, MD). The hybridoma supernatants were screened by cell surface staining of human PD-1 and BTLA-transfected COS cell lines, respectively, and for lack of reactivity with untransfected COS cells. Anti–PD-1 (PD1 3.1, IgG2b; PD1 6.4, IgG1) and anti–BTLA (BTLA 7.1, IgG2b; BTLA 8.2, IgG1) were selected as reagents for FACS analysis and functional studies. In addition, these reagents have already been described elsewhere (20, 21).

Ethics statement

All subjects gave written informed consent, and the medical ethics committee of the Academic Medical Center, Amsterdam, The Netherlands, approved the study.

Subjects

Healthy volunteers were obtained from Marseille’s Blood Bank (Établissement Français du Sang, Marseille, France). Two CMV-seronegative renal transplant recipients of a CMV-seropositive kidney were studied longitudinally. Heparinized PBMCs were isolated at regular intervals using standard density gradient centrifugation techniques and subsequently cryopreserved. Both recipients were treated with basic immunosuppressive therapy (cyclosporine A, prednisolone, and mycophenolate mofetil). Patient 1 also received CD25 mAb induction therapy. The HLA typing of patient 1 was A1/11 B8/45 DR3/12 and patient 2 was A2/24 B51/39 DR1/11. Quantitative PCR for CMV was performed in EDTA whole-blood samples, as described (22). To determine CMV serostatus, anti-CMV IgG was measured in serum using the AxSYM microparticle enzyme immunoassay. All subjects gave written informed consent, and the medical ethics committee of the Academic Medical Center, Amsterdam, The Netherlands, approved the study.

Tetramer complexes

The following HLA–peptide tetramer complexes were used: HLA-A1 tetramer loaded with the CMV pp65-derived YSEHPTFTSQY peptide (CMV A1 pp65 YSE allophycocyanin), HLA-A2 tetramer loaded with the CMV pp65-derived NLVPMVATV peptide (CMV A2 pp65 NLV allophycocyanin), the CMV immediate-early (IE)-derived VLEETSMVL peptide (CMV A2 IE VLE allophycocyanin), HLA-B8 tetramer loaded with the CMV IE-derived QVRVRDMVY peptide (CMV B8 IE QIK allophycocyanin), or the CMV IE-derived ELRRKMMYM peptide (CMV B8 IE ELR allophycocyanin) (Sanquin, Amsterdam, The Netherlands). All used tetramers were allophycocyanin conjugated.

Immunofluorescence staining and flow cytometry

PBMCs were obtained from healthy donors and isolated by fractionation over Lymphoprep gradients (Abcys, Paris, France). mAbs to PD-1 were 488-conjugated anti-BTLA mAbs and a corresponding lineage-specific mAb anti-CD3–ECD (Beckman Coulter, Paris, France), anti-CD27–allophycocyanin-Alexa 750 (Invitrogen), anti-CD4-Pacific Blue, anti-CD8–Alexa 700, or anti-CD45RA–PE–Cy7 (all from Becton Dickinson). Dead cells were eliminated using aqua LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen), and nonstained cell populations were excluded from the analysis by the use of anti-CD14 and anti-CD19–PC5 mAbs (Beckman Coulter). Cells were washed twice in cold PBS with 2% FCS and 0.02% sodium azide, fixed in 4% paraformaldehyde, and incubated with an appropriate concentration of tetrameric complexes in a small volume for 30 min at 4˚C protected from light. Fluorescence-labeled mAbs were then added and incubated for 30 min at 4˚C, protected from light, at concentrations according to the manufacturer’s instructions. For surface marker expression analysis, the following Abs were used: PE–CD27, CD28–allophycocyanin–Alexa Fluor 750 (eBioscience, San Diego, CA), CD28–Alexa Fluor 700 (Biolegend, San Diego, CA), CD8–PE–Alexa Fluor 610 (Invitrogen), HLA-DR–PerCP-C5.5, and CD45RA–PE–Cy7 (BD Biosciences, San Jose, CA). Cells were washed and measured on an FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Preparation of monocyte-derived DCs

PBMCs were obtained from healthy donors and isolated by Lymphoprep (Abcys) density gradient centrifugation. CD14++ monocytes were then immunomagnetically purified with CD14 mAb-conjugated microbeads (Miltenyi Biotec, Le Pont De Claix, France) and analyzed with FlowJo (Tree Star, Ashland, OR). PBMCs from transplant recipients were washed in PBS containing 0.01% (vol/vol) Na3VO4 and 0.5% (vol/vol) BSA. One million PBMCs were incubated with an appropriate concentration of tetrameric complexes in a small volume for 30 min at 4˚C protected from light. Fluorescence-labeled mAbs were then added and incubated for 30 min at 4˚C, protected from light, at concentrations according to the manufacturer’s instructions. For surface marker expression analysis, the following Abs were used: PE–CD27, PE–PD-1, PE–BTLA, PE–CD28–allophycocyanin–Alexa Fluor 750 (eBioscience, San Diego, CA), CD28–Alexa Fluor 700 (Biolegend, San Diego, CA), CD8–PE–Alexa Fluor 610 (Invitrogen), HLA-DR–PerCP-C5.5, and CD45RA–PE–Cy7 (BD Biosciences, San Jose, CA). Cells were washed and measured on an FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Artificial APCs and T cell assays

Human CD4+ and CD8+ T cells were purified by negative selection from PBMCs using magnetic beads (Miltenyi Biotec, Paris, France) and were routinely >97% CD3+ and >98% CD4+ for CD4+ T cell isolation, and >95% CD8+ for CD8+ T cell isolation as determined by flow cytometry. T cells were stimulated with artificial APCs (aAPCs) at a ratio of 3:1 (cells/beads) comprised of magnetic beads (Dynabeads M-450 Epoxy, DYNABECH, Invitrogen) coated with the following Abs: anti–CD3 (OKT3), anti-human CD28 (CD28.2), anti-human PD-1 (PD1 6.4), anti-human BTLA (BTLA 7.1), and anti-MHC class I (MHC I) (Y4). As previously described (23), these aAPCs were coated with suboptimal anti–CD28 mAb (5%), suboptimal levels of anti–CD28 mAb (10%), and anti-MHC class I mAb (CD28/32/MHC I), anti–PD-1 mAb (CD3/28/PD-1 plus MHC I), anti–BTLA mAb (CD3/28/BTLA plus MHC I), or anti–PD-1 plus anti–BTLA (CD3/28/PD-1 plus BTLA), constituting the remaining 85% of protein added to the bead. T cells (1.5 × 105 cells/well) were stimulated in round-bottom 96-well plates. Supernatants were harvested at 48 h to analyze cytokine secretion with a human Th1/Th2 cytokine kit (BD Cytometric Bead Array, BD Biosciences). Proliferation was measured at day 5 by tritiated thymidine incorporation for the last 18 h.

Allogeneic stimulation of T cells with monocyte-derived DCs

T cells were separated from PBMCs by means of a Pan T isolation kit II (Miltenyi Biotec) according to the manufacturer’s protocol. T cells were then labeled with 0.5 μM CFSE (Invitrogen) for 10 min at 37˚C. The labeled T cells (2 × 105 cells/well) were washed and cultured in 96-well flat-bottom plates in RPMI 1640 medium containing 10% FCS (Life Technologies, Paisley, U.K.) and supplemented on days 0, 3, and 5 with 100 ng/ml GM-CSF and 20 ng/ml IL-4 (both cytokines from Abcsy). Immature DCs (iDCs) were harvested at day 5, and their maturation was accomplished by coculturing them for 2 d with 50 Gy-irradiated CD40L-transfected cells (2 × 105/well).

Allogeneic stimulation of T cells with monocyte-derived DCs

T cells were separated from PBMCs by means of a Pan T isolation kit II (Miltenyi Biotec) according to the manufacturer’s protocol. T cells were then labeled with 0.5 μM CFSE (Invitrogen) for 10 min at 37˚C. The labeled T cells (2 × 105 cells/well) were washed and cultured in 96-well flat-bottom plates in RPMI 1640 medium containing 10% FCS in the presence of blocking Fab to PD-1 (PD-1.3.1), BTLA (8.2), or a matched isotype Fab, with immature or mature allogeneic DCs (2 × 105 cells/well) in triplicate. After 5 d of culture, cells were stained with CD25–allophycocyanin, CD8–PE–Cy7, and CD4–PE. Data were acquired on an LSR II SORP 4 lasers flow cytometer (BD Immunocytometry Systems, Le Pont De Claix, France) and analyzed with FlowJo software (Tree Star).
Induction of specific anti-pp65 CD8+ T cells
The iDCs and mature DCs (mDCs) were pulsed for 2 h at 37°C in RPMI 1% FCS with a 10 μg/ml CMV pp65 NLVPVMATV peptide. After two washes, peptide-pulsed DCs (2 × 10^6/well) were cultured with autologous CFSE-labeled T cells (2 × 10^5/well) in the presence of anti–PD-1, anti-BTLA–blocking mAbs, or isotype control. On day 7, T cells were harvested and restimulated with 10 μg/ml CMV pp65 NLVPVMATV peptide for 6 h. Cells were stained with CMV-pp65-allophycocyanin and CD8-PC5. After gating of CMVpp65*CD8+ T cells, proliferation was measured by CFSE dilution and expressed as a division index calculated with the proliferation tool of FlowJo (Tree Star). For intracellular cytokine staining, cells were permeabilized using Cytofix/Cytoperm kit (Becton Dickinson) and stained with IFN-γ–PE (Becton Dickinson) mAb.

Statistical analysis
All data were analyzed using GraphPad Prism version 5.00 for Macintosh (GraphPad, San Diego, CA). The Wilcoxon matched pairs test was used to examine the variations of PD-1 and BTLA expression on T lymphocyte subsets of differentiation. Comparisons were made between either the previous subset of differentiation according to a linear differentiation pathway (in Fig. 1), between CMV positive T cells and total T CD8 as indicated (in Fig. 2), or between different conditions of stimulation (in Figs. 3, 4). Differences were considered as statistically significant when \( p < 0.05 \).

Results
Distinct expression of BTLA and PD-1 on different differentiation subsets of T lymphocytes
T cell populations can be divided into several subsets based on the expression of CD45RA and CD27 (24, 25). The putative differentiation pathway proceeds from the naive (CD45RA+CD27-) via the central memory (CM; CD45RA+CD27+) to the late effector memory (EM) stage (CD45RA-CD27+). A distinct subset of cells expressing a CD45RA^highCD27^ phenotype is found predominantly in the CD8 T cell population and is called the effector memory CD45RA- (TEMRA) subset. To analyze the expression of BTLA and PD-1 on CD8+ and CD4+ T cell subpopulations, seven-color FACS analyses were performed on six healthy PBMC donors. The gating strategy allowed us to study BTLA and PD-1 expression and coexpression in three CD4+ T cell and four CD8+ T cell subpopulations (Fig. 1A). The expression of PD-1 and BTLA within CD8+ and CD4+ T cell subpopulations showed different patterns (Fig. 1B–D). In CD4+ T cells, no difference in expression of BTLA among differentiation subsets was seen, whereas in CD8+ T cells, BTLA expression in the naive subset decreased significantly in CM and EM subsets (\( p = 0.03 \)) and is even lower in TEMRA subsets (\( p = 0.03 \)). PD-1 expression was almost undetectable on naive CD4 and CD8 T cells and appeared in CM and EM cells (\( p = 0.03 \)). Expression levels dropped upon differentiation of CD8 to the TEMRA stage. Similar profiles were obtained with different mAb clones and fluorochrome conjugates (Fig. 1C, 1D).

Thus, BTLA expression on CD8+ and CD4+ T cells seems to follow different rules. Whereas BTLA expression was maintained at almost similar level along the CD4+ T cell differentiation pathway, it appeared to be regulated on CD8 T cells. In contrast, PD-1 regulation appeared to be very similar along both CD4+ and CD8+ T cell differentiation pathways: it increased after the priming of naive cells and reached a maximum of expression at the EM stages. However, PD-1 is both higher and more frequently expressed on CD8+ than CD4+ T cells (Fig. 1C–E). Only a limited number of CD4+ and CD8+ cells coexpressed both markers, and CD8 PD-1+ and BTLA+ subpopulations appeared to be specifically excluded (Fig. 1E). High levels of expression of both markers on separate cell populations might be related to the different regulation of their expression along the differentiation pathways of CD4+ and CD8+ cells.

Unlike BTLA, which is constitutively expressed on CD4+ and CD8+ T cells, the expression of PD-1 is low on resting T cells. In vitro, PD-1 is upregulated on activated CD4+ and CD8+ T lymphocytes, and maximal expression is observed after 48 h (3, 26, 27). To compare the expression profile of BTLA upon T cell activation, purified CD4 and CD8 T cells were stimulated with plate-bound anti-CD3 (OKT3) and soluble anti-CD28 (CD28.2) and stained every day with anti-BTLA mAbs. A progressive decrease in the intensity of BTLA expression was demonstrated on CD8+ but not CD4+ T cells through 48 h (data not shown).

BTLA and PD-1 expression during primary CMV infection
We next followed the expression of BTLA on CD8+ CMV-specific T cells during posttransplantation primary infection in two patients using three distinct tetramers identifying responses against peptides derived from pp65 and IE-1 of CMV. These three distinct tetramers together with CD8, CD45RA, and CD27 staining permitted us to analyze the differentiation of CMV-specific T cell populations during the course of primary CMV infection. Viremia, monitored by levels of CMV DNA copies in blood, allowed us to identify acute and controlled phase of CMV infection (Fig. 2). We have provided data corresponding to the analyzed patients (Fig. 2B). In addition, because of the low number of tetramer-positive cells, especially during the early stages of infection, the data obtained from both patients tested with three tetramers at several time points were grouped according to the sampling performed during the acute or the controlled phase of infection (Fig. 2C) and compared with the total CD8+ T cell population of patients at an early time postinfection.

The analysis was performed as depicted in Fig. 2A. As shown in Fig. 2B, viremia was detected during wk 9 in patient 1 and 2, although in this latter patient, it disappeared between wk 9 and 12 to reappear at wks 12–16. During the follow-up period postinfection, in both patients 1 and 2, the CMV-tetramer^+ CD8+ cells progressively moved from a naive and CM phenotype to become EM and TEMRA (Fig. 2B, 2C, left panels). BTLA and PD-1 expression were upregulated at early times postinfection and decreased at wk 25–30 (Fig. 2B). Interestingly, in patient 2, the decreased viremia during wk 9–12 was associated with the concurrent decrease expression of both BTLA and PD-1 as well as the activation marker HLA-DR on tetramer-positive cells. The expression levels of BTLA and PD-1 during the acute phase of infection were significantly increased as compared with the total CD8 population (\( p = 0.0007 \) and \( p = 0.05 \), respectively). In the controlled phase of infection, BTLA decreased to reach lower expression levels significantly different from the acute phase (\( p = 0.03 \)), whereas PD-1 remained at a high level not significantly different from the acute phase (Fig. 2C, middle panel). At the early time points, tetramer-positive cells are readily activated, as shown by the higher expression levels of the activation marker HLA-DR as compared with the total CD8 population (\( p = 0.002 \)) that decreases during the controlled phase of infection (\( p = 0.03 \) (Fig. 2B, left bottom panel, 2C).

Because the analyzed patients were under immunosuppressive treatment, cyclosporin A could contribute to BTLA or PD-1 regulation. Therefore, we analyzed the effect of cyclosporin A on the expression of PD-1 and BTLA in stimulated T cells of healthy donors (Fig. 3). Consistent with previously reported results (28), cyclosporin A inhibited 45% of the expression of BTLA and 30% of the expression of PD-1 on T cells. Thus, the elevated levels of BTLA on the patient cells in the acute phase of infection (Fig. 2C) might be underestimated to a larger extent than that of PD-1.

PD-1 and BTLA are potent inhibitors of CD3/CD28 costimulation
Previous studies have described inhibition of T cell responses using anti-human PD-1 and anti-human BTLA mAbs (26, 29). However, the relative efficiency of both systems has not been investigated.

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BTLA is downregulated in the course of CD8 but not CD4 T cell differentiation. CD8+ and CD4+ T cells from PBMCs of healthy volunteers (n = 6) were analyzed by flow cytometry for expression of PD1 and BTLA. A. Gating strategy for identification of T cell subsets. PBMCs were gated according to their size then into singlets and postexclusion of dead cells (Live/dead+); live cells were gated further by exclusion of CD14+CD19+ cells into CD3+ T cells then into CD3+CD4+ and CD3+CD8+ populations. CD4+ and CD8+ T cells were analyzed for CD45RA and CD27 expression resulting in the following subsets of T lymphocytes: naive CD45RA+CD27+, CM CD45RA-CD27+, EM CD45RA-CD27+, and TEMRA CD45RA-CD27-. B. Representative histograms of BTLA and PD-1 expression in CD8+ and CD4+ T cell differentiation subsets. BTLA and PD-1 expression on T CD8+ and T CD4+ subsets of differentiation determined by means of homemade mAbs (C) and commercially available reagents (Becton Dickinson) (D). The median, minimum, and maximum values of mfi are shown. Each population is compared with the previous subset of differentiation in the sequence naive, CM, CM, EM, and TEMRA.
We have made anti-BTLA and PD-1 mAbs that are described in Fig. 4A. Both BTLA 7.1 and BTLA 8.2 mAbs efficiently inhibited the binding of HVEM-Ig to BTLA expressing COS 7 cells. PD-1 3.1 but not PD-1 6.4 inhibited the binding of both PD-L1 and PD-L2 to PD-1-expressing COS 7 cells. To investigate whether PD-1 and BTLA exert similar functions on T cells, we used aAPCs (23). Purified CD4+ or CD8+ T cells were stimulated with beads coated with anti-CD3 and anti-CD28 mAbs together with anti-human PD-1, anti-human BTLA, or both. PD-1 and BTLA significantly inhibited to a similar level (3–7 times) both CD4+ and CD8+ T cell CD3/CD28-induced proliferation (Fig. 4B). The inhibitory effect of PD-1 or BTLA on CD3/CD28-induced proliferation of T cells was highly significant, and no additive or synergistic effects were detected between the two molecules. Both PD-1 and BTLA inhibited also CD3/CD28-mediated upregulation of IFN-γ and TNF-α in CD4+ T cells and CD8+ T cells (Fig. 4B). IL-4 and IL-5 were also strongly inhibited by PD-1 and BTLA engagement on both CD4+ and CD8+ T cells (data not shown). BTLA had a stronger effect on T cell proliferation (p = 0.0001) and inhibited more efficiently cytokine secretion (p ≤ 0.004) than PD-1 (Fig. 4B, middle and bottom panels). Using suboptimal T cell activation, PD-1 and BTLA mAbs strongly inhibited T cell proliferation and Th1/Th2 cytokine secretion, and again, no functional cooperation was detected between the two molecules (data not shown).

**Blockade of PD-1 or BTLA pathway enhances both allogeneic and CMV-specific CD8+ T cell proliferation**

We investigated the functions of PD-1 and BTLA in stimulated populations of T lymphocytes in which their ligands PD-L1, PD-L2, and HVEM have been expressed using T cell allogeneic responses against DCs presenting weak (iDCs) or strong (mDCs) stimulatory capacity. Purified CFSE-labeled T cells were cultured for 5 d with allogeneic iDCs or mDCs in the presence of blocking Fab specific to PD-1 and BTLA or isotype Fab control. Proliferation was quantified as the percentage of proliferating CD8+ T cells calculated by CFSE dilution (Fig. 4C, top panel). Activation was quantified by CD25 expression on total T cells (Fig. 4C, middle panel), and IFN-γ release in supernatants was quantified by ELISA (Fig. 4C, bottom panel). In both iDC and mDC allogeneic stimulations, the blockade of BTLA or PD-1 resulted in a significant increase in proliferation, CD25 expression, and IFN-γ release. Differences between the effects of the blockade of BTLA and PD-1 were observed, depending on the strength of the stimulation. In a weak allostimulation condition (iDCs), the effects of the blockade of BTLA on proliferation (p = 0.03), CD25 expression (p = 0.0025), and IFN-γ release (p = 0.015) were significantly lower than the effects of the blockade of PD-1. In a stronger allostimulation condition (mDCs), the effects of the blockade of BTLA were similar to PD-1, except for IFN-γ release, in which a lower effect of the blockade of BTLA was observed (p = 0.013).

The expression of BTLA and PD-1 on CMV-specific T cells prompted us to analyze their function in the activation of CMV-specific CD8+ T cells. To address this issue, we compared the effect of PD-1 and BTLA pathways on CMVpp65+–specific CD8+ T cell proliferation and function (Fig. 4D). To this end, we blocked PD-1 or BTLA by soluble forms of Ab in autologous cocultures of T cells with iDCs or mDCs pulsed with pp65 CMV peptide. Anti–PD-1 (PD1.3.1) or anti-BTLA (BTLA 8.2)-blocking Abs were selected for their ability to inhibit the binding of PD-1 to its ligands PDL-1 and PDL-2 and BTLA to HVEM (Fig. 4A), and their effect was controlled by an isotypic mAb control. Proliferation of CMVpp65+-specific CD8+ T cells was analyzed after 7 d and calculated as a division index resulting from the CFSE dilution. The percentage of CMVpp65+ T cells and the percentage of IFN-γ producing cells were determined. Both mAbs in a soluble form enhanced T cell proliferation to a similar extent independently of the maturation of DCs used for stimulation, suggesting a potential role of these molecules in controlling CTL expansion (Fig. 4D, lower panel). The percentage of CMVpp65+ T cells among the CD8 population showed a slight decrease that suggests a similar apoptosis of proliferating cells. We did not detect a significant increase in the production of IFN-γ (Fig. 4D, lower panel) and TNF-α (data not shown) by intracellular staining. These data are compatible with recent demonstration that PD-1 mAbs do not affect IFN-γ production in short-term in vitro stimulation (30). The trend toward enhancement was generally greater post-exposure of CD8+ T cells to a weaker stimulating iDC population than to strongly stimulating mDCs, as shown by allogeneic stimulation results (Fig. 4C). The enhancing effect of BTLA and PD-1 on CMV-specific T cells was similar in both weak and strong DC stimulation conditions. This could be related to the differentiated phenotype of CMV-specific T cells.

**Discussion**

We describe in this study for the first time the dynamic expression of the negative regulator of T cell activation BTLA on human CD8+ T cells in the course of primary CMV infection and have determined its function in the control of memory T cell expansion. The differentiation of human naïve T cells into effector and memory cells is accompanied by changes in the expression of surface molecules and effector molecules. This topic has been recently extensively debated (31). Several markers include receptors involved in T cell activation, costimulation, chemokine receptors, adhesion molecules, and, more recently, expression of the cosignaling molecule PD-1 (5, 6, 32–35). Interestingly, PD-1 expression depends on two settings in vivo. PD-1 is upregulated early postactivation, but it is downregulated at late stages of differentiation. However, it is also linked to T cell activation because PD-1 overexpression occurs along with the upregulation of activation markers.

To understand how BTLA could affect the function of CD4+ and CD8+ T cells, we first examined the expression of BTLA on different T cell subsets and compared it to PD-1, which has been extensively studied during viral infections in humans. As already shown, PD-1 is barely detected on naive T cells and upregulated on effector, EM, and CM CD8+ T cells, as well as memory-type CD4+ T cells from healthy donors. In contrast, BTLA expression is stable on different CD4+ T cell subsets, but it is downregulated on effector and CM CD8+ T cells as compared with naive cells. These observations were reproduced in vitro because T cell activation induced progressive PD-1 upregulation on CD4+ and CD8+ T cells. In contrast, BTLA was downregulated upon activation of CD8+ but not CD4+ T cells. It would suggest a differential regulation of BTLA expression in CD4+ and CD8+ T cells. The basis for this differential decreased expression is unclear. The only available data on the regulation of BTLA expression show that it is induced by phorbol esters and calcium ionophores, but it is

EM, and TEMRA, and the significant p values are indicated as *p < 0.05 (Wilcoxon test). E, BTLA and PD1 coexpression on CD4 and CD8 T cells. PBMCs from healthy donors were stained and analyzed using the gating strategy described in A. An example of BTLA and PD-1 coexpression on CD4+ CD3+ and CD8+CD3+ T cells is shown.
FIGURE 2. BTLA and PD-1 expression during CMV infection. A, Gates used for the analysis of patient CD8+CMV-tetramer+ T cells. We show the gating of CD8+ T cells on patient 1, wk 3, and CMV-tetramer+CD8+ T cells on patient 1, wk 145, after gating on viable cells (not shown). Gates for subsets of differentiation according to CD45RA and CD27 expression are set on CD8+ T cells (patient 1, wk 3). Gates for BTLA, PD-1, and HLA-DR expression are set on negatively expressing populations within CD8+ T cells (patient 1, wk 3), respectively, as TEMRA for BTLA and naive for PD-1 and HLA-DR. B, Follow-up of markers expressed on CD8+CMV-tetramer+ T cells and CMV viremia in the two transplanted patients over time. Left panel shows raw data of CD45RA/CD27 coexpression, BTLA/PD-1 coexpression, and HLA-DR expression for each blood sampling in a representative CD8+CMV-tetramer+ T cell population (IE-elr for patient 1 and pp65 for patient 2). The number of CD8+CMV-tetramer+ T cells analyzed ranged from 50–20,000 for patient 1 and 50–100,000 for patient 2 from early to late time points. Right panel shows median, maximal, and minimum mfi values of BTLA, PD-1, and HLA-DR for the three CMV tetramers plotted over time for each patient. Viremia is represented as a gray zone and scored positive when >1 DNA copy/ml that defines the acute phase of infection. Viremia <1 DNA copy/ml defines the controlled phase of infection and is not shown on graphs. C, Global analysis of CD8+CMV-tetramer+ T cell differentiation phenotype and BTLA, PD-1, and HLA-DR expression in acute and controlled phases of infection in the two transplanted patients. Acute and controlled phases of infection are defined in C. Median, maximum, and minimum values measured for the two patient cells tested with...
downregulated by cyclosporin A (28). This report indicates that multiple events, some being dependent on calcineurin, are involved. However, these data do not explain the downregulation of BTLA expression and the differences between CD8⁺ and CD4⁺ cells.

Although the downregulation of BTLA upon interaction with its ligand HVEM has not been reported, a similar mechanism is reported for various receptors, including internalization of HVEM itself (36). Moreover, this does not explain the differences between CD8⁺ and CD4⁺ cells. CMV encodes the UL144 protein that was shown to interact with BTLA and to decrease T cell activation (37). Our observation demonstrating that BTLA was upregulated during the acute phase of CMV infection would make BTLA a likely candidate to be targeted because of its inhibitory role in T cell activation during CMV (re)activation. Whether the decreased expression of BTLA on CD8⁺ T cells is due to the internalization, masking of the binding site, or transcriptional regulation will deserve further investigation. In addition, because the data were obtained in transplanted patients, the immunosuppressive treatment may lower BTLA and PD-1 overexpression, as cyclosporin A was shown to regulate the promoters of both molecules and underestimat their increase.

The expression and the role of negative regulators of immune functions are instrumental for the understanding of the basis of the T cell responses during the course of viral infections. This study demonstrates that the negative regulator BTLA is highly expressed during the acute phase of infection on CMV-specific T cells when the population is constituted by naive, CM, or TEMRA T cells and that expression of BTLA decreases when viremia is controlled, and CMV population is constituted in a large majority by CM, EM, or TEMRA T cells. In this regard, BTLA expression follows closely the expression of the activation marker HLA-DR, which is highly upregulated on CMV-specific T cells during the acute phase of infection and returns to lower levels when viremia is controlled. PD-1 has also been described as regulated by activation (35). However, a striking difference in expression is found when comparing PD-1 and BTLA expression.

Whereas both PD-1 and BTLA are upregulated on CMV-specific cells during acute infection, PD-1 alone remains significantly overexpressed when viremia is controlled. This finding would indicate differences in the regulation of the expression of PD-1 and BTLA molecules. What are the likely roles of the expression of negative regulators on Ag-specific cells? The upregulation of PD-1 was proposed to be a likely mechanism for the control of proliferation and apoptosis of activated cells (6). Based upon the recent publications in mice using BTLA⁻/⁻ cells, two roles have been proposed. First, BTLA was initially identified as having inhibitory actions on T cell proliferation and cytokine production in vitro (11, 12). Inhibitory actions were observed in vivo in models of experimental allergic encephalomyelitis and allergic airway inflammation (11, 38). However, a prosurvival role of BTLA was also proposed (39). Hurchla et al. (39) tested BTLA function in a mice in vivo model of chronic allostimulation using BTLA⁻/⁻ cells. They proposed that, under chronic allostimulation, BTLA is acting in a prosurvival manner and is necessary for the maintenance, rather than the initiation, of the effector T cell response. In contrast, Deppong et al. (40), also using BTLA-deficient mice, had increased survival in comparison with wild-type mice in the asthma inhaled-allergen model. In humans, our in vitro results suggest that BTLA exerts mainly inhibitory function on CMV-specific memory T cells and controls the expansion of the CMV-specific T cells. Additional roles, such as a role in survival, will have to be analyzed.

Recently, Blackburn et al. (4–8) investigated the different inhibitory receptors that might be involved in the coregulation of CD8⁺ T cell exhaustion in the murine LCMV model, including the already identified receptor PD-1. These results suggest that in addition to PD-1, LAG-3 and 2B4 were upregulated on exhausted CD8⁺ effectors (19). In contrast, BTLA expression was not modified in naive, memory, and exhausted CD8⁺ T cells. Additional information regarding the expression of the inhibitory receptors including BTLA during the acute phase of the infection was not included. Our data indicate that during reactivation of human CMV, BTLA is highly regulated on the CD8⁺ subsets. In contrast, when the patients had controlled CMV viremia, the expression of BTLA diminished, suggesting that BTLA is more involved in the control of acute than controlled infection. Further studies will be necessary to assess a putative role of BTLA in T cell inhibitory/exhaustion during HIV, HCV, and other viral infections.

Our data suggest that PD-1 and BTLA cross-linking elicits similar inhibitory functions on T cell function regarding CD4 and CD8 proliferation and cytokine production. This demonstrates that upon appropriate ligation, they both are strong inhibitors of T cell responses.

Like PD-1, BTLA contains in its cytoplasmic domain two motifs: an ITIM and an ITSM, both requiring the tyrosine phosphatases SHP1 and SHP2 (11, 12, 15). At present, the targets of SHP1 and SHP2 recruited to BTLA are unknown. It is possible that they also have a role in dephosphorylation of signaling intermediates downstream of Ag receptors in lymphocytes (11, 15, 41) or in specifically targeting the PI3K-PKB pathway, as proposed for PD-1. This would be in line with the fact that they were neither additive nor synergistic in their function using CD3⁺/CD28 costimulation. In addition, PD-1 and BTLA mAb increased both allogeneic and CMV-specific T cell proliferation. The likely mechanisms rely on the inhibition of the interaction between BTLA and HVEM because the mAbs were selected

The different tetramers are plotted. **Left panel** shows the repartition of CD8⁺ CMV-tetramer⁺ T cells into the four subsets of differentiation based on CD45RA and CD27 expression. **Middle panel** shows BTLA and PD-1 mfi in CMV pp65 tetramer⁺ T cells and in total CD8⁺ T cells of both patients at an early time point (wk 3 and wk 5, respectively). **Right panel** shows HLA-DR expression. All p values are calculated with the Wilcoxon paired test. *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 3.** Inhibition of BTLA and PD-1 expression on activated T CD4⁺ cells by CsA. Purified CD4⁺ T lymphocytes were stimulated by means of beads coated with CD3 and CD28 mAbs in the presence or not of CsA (100 nM). Expression of BTLA and PD-1 was determined 24 h poststimulation on CD4⁺CD25⁺ T cells and represented as a ratio of mfi to isotypic staining control. Median, maximum, and minimum values are shown. The percentage of reduction in expression in the presence of CsA is shown. The p values were calculated between both stimulation conditions using the Wilcoxon test. *p < 0.05. CsA, cyclosporin A.
for their efficient inhibition of the interaction on BTLA with HVEM. This hypothesis would be in line with the role of HVEM in the inhibition of T cell functions via HVEM (13, 42). However, agonist effects of BTLA mAbs cannot be ruled out.

Memory maintenance against CMV has been recently revisited. Snyder et al. (43) have studied the host–virus balance in the murine CMV model. In this model, some CD8 T cell populations may be sustained or increase over time in tissues. This accumulation of virus-specific CD8+ T cells is called memory inflation. In this model, Snyder et al. (43) suggest that large pools of differentiated cells found in the blood and tissues are turning over continuously with rapid resupply from self-renewing memory

The Journal of Immunology 3147
pools as well as naive precursors. This question is important to keep large functional pools of effectors. Altogether, these data add new information on the pathways of viral-specific T cell responses regulation and on the role of negative regulators of T cell activation during human CMV reactivation.

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Disclosures

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References