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Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells

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Abstract

Co-signal receptors provide crucial activating or attenuating signals for T cells. The B and T lymphocyte attenuator (BTLA/CD272) is a third member of co-inhibitory receptors, which belongs to the CD28 immunoglobulin-superfamily. Using monoclonal antibodies (mAbs) against human BTLA, we show that BTLA is constitutively expressed on most $CD4^+$ and $CD8^+$ T cells and its expression progressively decreases upon T cell activation. Polarized Th1 and Th2 cells contained both BTLA-positive and BTLA-negative populations, but the extended culture diminished BTLA expression. Cross-linking BTLA with an agonistic mAb inhibited T cell proliferation and the production of the cytokines IFN-γ and IL-10 in response to anti-CD3 stimulation. BTLA-mediated inhibition of T cell activation occurred during both primary $CD4^+$ T cell responses and secondary $CD4^+$ and $CD8^+$ T cell responses, suggesting that BTLA ligation sends a constitutive "off" signal to T cells and thus might play an important role in the maintenance of T cell tolerance.

Keywords: CD28 family; Co-inhibition; Co-stimulation; Immune regulation; T cells

Optimal T cell activation requires two signals. The first is provided by the ligation of the T cell receptor (TCR) by peptide-MHC complexes on antigen-presenting cells (APCs) and the second by the binding of T cell co-stimulatory molecules by their ligands on APCs [1,2]. Among the CD28 family of receptors, three co-inhibitory receptors have been identified: cytotoxic T lymphocyte antigen-4 (CTLA-4/CD152) [3], programmed death-1 (PD-1/CD279) [4], and B and T lymphocyte attenuator (BTLA/CD272) [5].

BTLA was originally cloned from a cloned murine Th1 cell [5]. The initial mouse studies showed that BTLA was not observed on naïve T cells and that its expression was induced during Th1-/Th2- polarization of naïve $CD4^+$ T cells. BTLA mRNA expression was subsequently lost in highly polarized Th2 cells but not in Th1 cells [5]. The expression of BTLA was much higher on B cells than on T cells [6]; however, unlike in T cells, BTLA expression was decreased upon B cell activation [6,7]. BTLA expression on human cells has not been studied.

The cytoplasmic region of both human and murine BTLA, similar to that of PD-1 [8,9], contains three tyrosine residues, which comprise a Grb-2 binding site, an immunoreceptor tyrosine-based inhibitory motif (ITIM), and an immunoreceptor tyrosine-based switch motif (ITSM) [5,10]. The presence of an ITIM suggests that BTLA functions as an inhibitory receptor. Indeed, cross-linking BTLA and the TCR on murine T cells has been shown to reduce T cell proliferation and IL-2 production. In addition, T cell activation is enhanced in BTLA-deficient mice [5,7].

The way in which BTLA regulates human T cell activation and, in turn, the ways in which BTLA expression and function are regulated during T cell activation have not been studied. To address these questions, we generated monoclonal antibodies (mAbs) against human BTLA.

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The expression and function of human BTLA was compared with those of other co-inhibitory receptors, including PD-1 and CTLA-4.

Materials and methods

Transfectants. Total RNA was extracted from human peripheral blood mononuclear cells (PBMC), and BTLA cDNA [5] was amplified by RT-PCR using the following primers with Xho-I or Not-I cloning site: sense, 5′-CAG CTC GAG ACC ATG TGC AGG-3′ and anti-sense, 5′-AAA AGC GGC CGC GGT CCC TGT TGG AGT CAG-3′. The PCR product was inserted into pCR2.1 vector (Invitrogen, Carlsbad, CA), and the BTLA sequence was verified. The product was subcloned into pIRES-eGFP expression vector (Clontech, Mountain View, CA) at the franking Xho-I and Sac-II sites (BTLA-pIRES-eGFP). Murine mastocytoma P815 and fibroblast L cells were transfected with BTLA-pIRES-eGFP by electroporation and lipofectin, respectively. Transfected cells were selected as GFP-positive cells by flow cytometry as described previously [11]. For transduction of the murine myeloma cell line NSO, the BTLA cDNA was cloned into pMX retrovirus vector [12,13], and virus was produced using the Plat-E packaging cell system [14], which was kindly provided by Dr. Toshio Kitamura, Institute of Medical Science, University of Tokyo. NSO cells were infected with high-titer virus products, and the GFP-positive cells were selected by flow cytometry. P815 cells transfected with human CD28 [15], CTLA-4 [16], and PD-1 [17] were described previously.

Generation of anti-BTLA mAbs. C3H/HeN mice were immunized with BTLA-transfected L cells. Spleen cells from immunized mice were fused with P3U1 myeloma cells. After hypoxanthine-aminopterin-thymidine selection, hybridomas were selected that produced antibodies specific for the BTLA-expressing L cells, but not the parental L cells. Two hybridoma clones (MIH26 and MIH27), both of which produced IgG2b antibodies expressing κ light chains, were obtained.

MAbs and immunofluorescence. Hybridomas producing mAbs against PD-1 (MIH4, IgG1) [17], CTLA-4 (MIH8, IgG3) [18], CD28 (TN228, IgG1) [15], CD3 (OKT3, IgG2a), CD4 (OKT4, IgG2b), CD8 (OKT8, IgG2a), CD16 (3G8, IgG1), and CD14 (63D3, IgG1) were used. OKT3, OKT4, OKT8, and 63D3 hybridomas were obtained from the American Type Culture Collection (Manassas, VA). All mAbs were purified from ascites using protein G-column or caprylic acid extraction, and the purities were verified by SDS–PAGE. FITC-conjugation and biotinylation were performed according to standard protocols. FITC-conjugated anti-CD19 (HIB19, IgG1) and anti-CD45RO (UCHL1, IgG2a) mAbs, Phycoerythrin-Cyanin 7 (PE-Cy7)-conjugated anti-CD3 mAb (SK7, IgG1), and PE-conjugated anti-CD45RO, anti-CD4 (SK3, IgG1), and anti-CD8 (RPA-T8, IgG1) mAbs were obtained from BD PharMingen (San Diego, CA) or eBioscience (San Diego, CA). The biotinylated antibodies MIH4, MIH8, and MIH26 were used with PE- or allophycocyanin (APC)-conjugated streptavidin (eBioscience). Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). For intracellular cytokine staining, the cells were stained with anti-BTLA mAb, and then fixed and permeabilized with FACS lysing and permeabilizing solutions, respectively (BD Biosciences). The fixed cells were stained with FITC- or PE-conjugated anti-IL-4 (8D4-8, IgG1) and anti-IFN-γ (4S.B3, IgG1) mAbs, or with control Ig, according to the manufacturer's protocol (BD Biosciences).

Cell lines. T cell lines (Molt-4 and Jurkat), Burkitt's lymphoma cell lines (Daudi, Ramos, and Raji), and myeloid and histiocytic leukemia cell lines (THP-1, U-937, and HL-60) were obtained from the ATCC. An EBV-transformed B lymphoblastoid cell line (JY), a B-lymphoma cell line (Nalm6), and a thymoma cell line (YT2C2) were obtained from DNAX Research Institute [19]. All cells were cultured in RPMI 1640 medium with glutamine (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics.

Isolation of $CD4^+$ and $CD8^+$ T cells. PBMC were isolated from the peripheral blood of healthy volunteers by density gradient centrifugation. Monocytes were depleted by plastic adherence, and $CD4^+$ and $CD8^+$ T cells were isolated from the non-adherent cells by positive selection using anti-CD4- or anti-CD8-coated Dynabeads (M-450) and Detachabeads, according to the manufacturer's recommendation (Dynal, Oslo, Norway). The purities of the $CD4^+$ and $CD8^+$ T cells were greater than 97% and 90%, respectively.

T cell activation. PBMC were stimulated with immobilized anti-CD3 (5 μg/ml) and soluble anti-CD28 (10 μg/ml) mAbs in 24-well plates. IL-2 (100 U/ml, BD PharMingen) was added at day 5. At day 7, viable cells were isolated by density gradient centrifugation and re-stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAbs for three additional days. Th1- and Th2-polarization was performed as described previously [20]. Briefly, $CD45RO^-CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of anti-IL-4 mAb, rIFN-γ, and rIL-12 (Th1 condition), or anti-IFN-γ, anti-IL-12 mAbs, and rIL-4 (Th2 condition). For an extended culture, viable cells were re-stimulated with anti-CD3 and anti-CD28 mAbs every week under the respective polarizing conditions, and the expression of intracellular IFN-γ and IL-4 and surface BTLA was analyzed at the indicated days.

To determine the effects of BTLA cross-linking, freshly isolated $CD4^+$ T cells (2×10^5/well) were stimulated with immobilized anti-CD3 mAb (0.2, 1.0, and 5.0 μg/ml) and either anti-BTLA mAb or control IgG2b (5, 10, and 20 μg/ml) in flat-bottomed 96-well plates for 72 h. In selected experiments, soluble anti-CD28 mAb (5 μg/ml) was also added to the culture. In some experiments, PBMC were pre-activated with immobilized anti-CD3 (5 μg/ml) and soluble anti-CD28 (10 μg/ml) mAbs for 7 days. Viable cells were purified using anti-CD4- or anti-CD8-coated Dynabeads and were rested overnight. The pre-activated $CD4^+$ or $CD8^+$ T cells (2×10^5/well) were re-stimulated with combinations of immobilized anti-CD3 mAb (1 μg/ml) and either anti-BTLA mAb or control IgG2b (5, 10, and 20 μg/ml) for 48 h. All cultures were pulsed with [^{3}H]-thymidine (1 μCi/well; Perkin–Elmer, Boston, MA) for the last 18 h and harvested on a Micro 96™ Cell Harvester (Molecular Devices, Sunnyvale, CA). The incorporated radioactivity was measured using a microplate beta counter (Micro β Plus; Wallac, Turku, Finland). Supernatants from duplicate cultures were collected after 72 h for assessment of cytokine production by ELISA. Human IL-4, IL-10, and IFN-γ were measured using Ready-SET-Go! ELISA kits (eBiosicence), according to the manufacturer's instructions.

Results and discussion

BTLA expression and regulation on lymphocytes

We generated two hybridoma clones, MIH26 and MIH27, that produce mAbs against human BTLA. MIH26 reacted specifically with cell lines transfected with human BTLA, but not with the non-transfected parental cell lines (Fig. 1A). MIH26 also failed to react with the P815 cells expressing other CD28 family molecules, including CD28, CTLA-4, and PD-1. Similar results were obtained with the MIH27 mAb (data not shown). These results demonstrate that both MIH26 and MIH27 specifically recognize BTLA.

We next analyzed the cell surface expression of BTLA on human PBMC. BTLA was constitutively expressed on approximately 90% of $CD3^+$ T cells and $CD19^+$ B cells in freshly isolated PBMC (Fig. 1B). In contrast, $CD16^+$ NK cells did not express detectable BTLA (data not shown). To clarify the phenotype of the T cells lacking BTLA expression, we performed multicolor staining with CD45RO, CD45RA, CD28, CD11b, CD25, PD-1, or CTLA-4. The T cells lacking BTLA expression included both $CD45RO^+$ memory T cells and $CD45RA^+$ naïve T cells, and did not exhibit preferentially high or low