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ORIGINAL ARTICLE

B and T lymphocyte attenuator interacts with CD3ζ and inhibits tyrosine phosphorylation of TCRζ complex during T-cell activation

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B and T lymphocyte attenuator (BTLA) is an important negative regulator of T-cell activation. T-cell activation involves partitioning of receptors into discrete membrane compartments known as lipid rafts and the formation of an immunological synapse (IS) between the T cell and antigen-presenting cell (APC). Here we show that after T-cell stimulation, BTLA co-clusters with the CD3ζ and is then involved in IS, as determined by a two-photon microscope. BTLA can interact with the phosphorylated form of T-cell receptor (TCR) within the lipid raft, which is associated with the T-cell signaling complex. Coligation of BTLA with the TCR significantly decreased the amount of phosphorylated TCR-related signal accumulation in the lipid raft during T-cell activation. These results suggest that BTLA functions to regulate T-cell signaling by controlling the phosphorylated form of TCRζ accumulation in the lipid raft.
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T-cell activation can be profoundly altered by the cell surface-expressing co-inhibitory and co-stimulatory molecules. These co-signaling pathways play overlapping and distinct regulatory roles at various stages of T-cell response or in different subsets of lymphocytes, so that the immune responses process in a proper manner and intensity.[1–8] B and T lymphocyte attenuator (BTLA) is a lymphoid-specific cell surface receptor that is expressed by B cells, T cells, dendritic cells (DCs), macrophages and NK cells in C57BL/6 mice.[9] Functional analysis has suggested that BTLA exerts inhibitory actions, indicating a role more similar to cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) than activating receptors of the CD28/B7 family.[9,10] BTLA engagement resulted in a remarkable downregulation of T-cell activation, and with mice deficient in BTLA, there is a significantly increase in the incidence and severity of autoimmune disorders.[11] Co-ligation of BTLA partially inhibits anti-CD3 monoclonal antibody (mAb)-induced secretion of interleukin (IL)-2, suggesting that BTLA exerts an inhibitory, rather than activating influence on T cells.[12,13] BTLA is expressed highly on activated T cells, as well as both developing Th1 and Th2 cells. BTLA is subsequently lost from highly polarized Th2 cells, but is retained by Th1 cells.[14] Thus, it may exclusively regulate ongoing immune responses and later the balance-governing immune tolerance in the periphery.

BTLA inhibits CD3 induction, but not chemically (such as using by phorbol 12-myristate 13-acetate (PMA) plus ionomycin or Con A) induced T-cell activation and IL-2 production, suggesting that BTLA might impair the early events of T-cell activation.[11] BTLA is a type I transmembrane glycoprotein with an extracellular single immunoglobulin V-like domain, a transmembrane region and a cytoplasmic region.[11,15] The cytoplasmic domain of murine BTLA contains three conserved tyrosine-based signaling motifs, including a Grb-2 recognition consensus and two immunoreceptor tyrosine-based inhibitory motifs (ITIMs).[15] Phosphorylation of the cytoplasmic domain of BTLA induced the association with the protein tyrosine phosphatases SHP-1 and -2.[11]

During antigen presentation, the interface between the T cell and antigen-presenting cell (APC) membranes forms the hot spot for T-cell activation, a highly organized ultrastructure, the immunological synapse (IS), where signaling, adhesion and cytoskeleton molecules are concentrated within lipid raft microdomains following TCR co-aggregation.[16,17] The membrane lipid rafts which are biochemically characterized as detergent insoluble glycosphingolipid-enriched microdomains act as platforms that compartmentalize key components involved in signaling in different regions of the plasma membrane.[18]

CTLA-4 is recruited to the lipid raft during negative signaling and forms a molecular complex with phosphorylated CD3ζ within the lipid raft.[19–22] However, BTLA and CTLA-4 are significantly different in both structure and binding mode than what had been expected

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from the amino-acid sequence.[15,23] Thus BTLA signaling must be activated in a different manner from that of CTLA-4 or CD28.[23] Here, we report that BTLA co-clusters with CD3ζ and its involvement in the formation of IS during mouse $CD4^+$ T-cell activation. Furthermore, BTLA forms a molecular complex with phosphorylated TCRζ and negatively regulates the tyrosine phosphorylation of TCRζ in the lipid raft.

RESULTS AND DISCUSSION

Co-localization of BTLA and CD3ζ molecules on activated T cells and co-clustering to the IS

It has been reported that BTLA expression on T cells was significantly upregulated during T-cell activation, whereas naive T cells expressed low or undetectable levels of BTLA.[11] This upregulation of BTLA expression on T cells during activation was confirmed in our laboratory.[24] Consistently, naive TCR transgenic DO11.10 or wild-type B6 $CD4^+$ T cells expressed undetectable levels of BTLA as determined by flow cytometry, where significantly higher levels of BTLA were expressed on activated DO11.10 or B6 $CD4^+$ T cells.[24]

BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1.[11,12] BTLA inhibited CD3-induced, but not chemically (such as PMA plus ionomycin or Con A) induced T-cell activation and IL-2 production, suggesting that BTLA might impair the early events of T-cell activation.[11] The open question is whether or not BTLA is involved in IS. Thus, the distribution of BTLA and its relationship with CD3ζ on activated $CD4^+$ T cells were assessed with immunofluorescence assay. The two molecules can be detected and distributed equably on the membrane of the resting $CD4^+$ T cells, although, BTLA co-clustered with CD3ζ on activated T cells (Figure 1). Sorted $CD4^+$ T cell, cultured with the purified DCs, that were pre-matured with ovalbumin (OVA) peptide for the formation of IS, found both BTLA and CD3ζ molecules were recruited to the IS locations after $CD4^+$ T cells interacted with DCs for 30 min at 37°C (Figure 2a). We did not find recruitment of BTLA and CD3ζ reorganization in resting T cell–DC and activated T cell–DC conjugates formed in the absence of OVA peptides (Figure 2a). Thus, BTLA may participate in the early events of sensitized T cells during activation. CD45 was used as the control because it is not involved in the conformation of the IS. The immunofluorescence results showed that CD45 did not join in the conformation of IS, while CD3ζ and BTLA could relocalize to the IS (Figures 2b and c). The percentages of cell conjuates displaying CD3ζ and BTLA in T cells are summarized in Figures 2d–f.

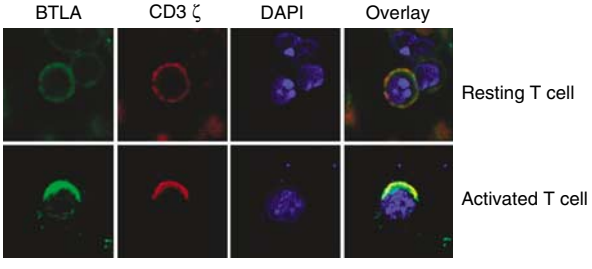

Figure 1 Co-clustering of BTLA and CD3ζ on activated $CD4^+$ T cells. Distribution of BTLA and CD3ζ on resting B6 mouse $CD4^+$ T cells and activated DO.11.10 mouse $CD4^+$ T cells. Cells were first fixed and permeabilized, and then stained with anti-BTLA pAb and anti-CD3ζ mAb. Nucleus was stained with DAPI. Second antibody used anti-goat IgG-FITC and anti-mouse IgG1-TRITC especially. The images were analyzed by a two-photon microscope. More than 50 individual $CD4^+$ T cell–DCs conjugates obstained in three independent experiments were assessed. BTLA, B and T lymphocyte attenuator; DAPI, 4′,6-diamidino-2-phenylinodole; FITC, fluorescein isothiocynate; mAb, monoclonal antibody; pAb, polyclonal antibody; TRITC, tetramethylrhodamine isothiocynate.

The interaction of BTLA with CD3ζ and the association with the tyrosine phosphorylated TCRζ signals in the lipid raft

Since BTLA co-localization with CD3ζ in activated T cells (Figure 1), we hypothesized that BTLA may negatively regulate lipid raft signaling. As the first step in addressing this issue, we examined the distribution of BTLA within the lipid rafts. TCR-transgenic DO11.10 mouse $CD4^+$ T cells were used for this study. Lysates from stimulated $CD4^+$ T cells were subjected to sucrose gradient fractionation so that the lipid raft could be separated as reported before.[25] For the identification of lipid raft, Lck was detected in the lipid raft fraction (fractions 3 and 4), whereas other fractions exclude the bottom fraction (fractions 10–12) did not show detectable levels of Lck (Figure 3), which was consistent with the previous reports.[25,26] BTLA and CD3ζ can be detected both in the lipid raft parts and in the bottom parts. And control molecule (CD45) was not involved in lipid raft.[27,28] In subsequent experiments, fraction 4 of lipid raft isolation fractions was used as lipid rafts after determination each time. Immunoprecipitation of fractions 4 and 12 and total cell lysates with anti-BTLA polyclonal antibody (pAb) (Figure 4a) resulted in significant levels of CD3ζ protein could be detected by western blot using anti-CD3ζ mAb, indicating that BTLA might interact with CD3ζ both in lipid rafts and bottom fractions (Figure 4a). Immunoprecipitation assay was repeat with anti-CD3ζ mAb and western blotting detection were done with anti-BTLA pAb. Results displayed that anti-CD3ζ antibody could also immunoprecipitation BTLA (Figure 4a). Goat serum and mouse IgG were used as negative control. It should be noted that the fractions were solubilized by *n*-octyl β-D-galactopyranoside to ensure that associations were due to protein–protein interactions and not co-presence within membrane vehicles. Furthermore, tyrosine phosphorylation of anti-BTLA pAb-mediated immunoprecipitated proteins were determined from both the lipid raft fractions and the bottom fractions (Figure 4b). The pTyr bands co-precipitated by anti-BTLA mAb mainly migrated at 59, 56, 23 and 21 kDa. The identity of the 59 and 56 kDa bands are Fyn and Lck, respectively (data not shown).[26] Goat serum also was used as negative immunoprecipitation control here (data not shown). These results suggest that BTLA may be involved in lipid raft and the BTLA/CD3ζ interaction may regulate T-cell signaling, even if BTLA cannot form a periodic repeating array at the IS.[15]

Cross-linking of BTLA significantly decreased the phosphorylated protein accumulation in the lipid raft

BTLA is a negative co-stimulatory molecule for T cells in mice and humans.[14] It has been demonstrated that BTLA significantly inhibited T-cell proliferation and the production of IL-2.[11,29] To confirm whether or not our ligation system is working, the effects of BLTA ligation on anti-CD3 and anti-CD28 mAbs-induced proliferation of activated T cells were observed as described in Methods. Ligation of BTLA by mAb significantly inhibited the proliferation of T cells induced by either anti-CD3 mAb or the combination of anti-CD3 and anti-CD28 mAbs as determined by ^{3}H-thymidine incorporation which has been reported before (Figure 5).[14] Therefore, this culture system was used for the coming studies.

The biochemical techniques used for rafts analysis have shown that many lipid-modified signaling proteins, such as tyrosine kinases of the Src family, GPI-linked proteins, and adaptor proteins, are

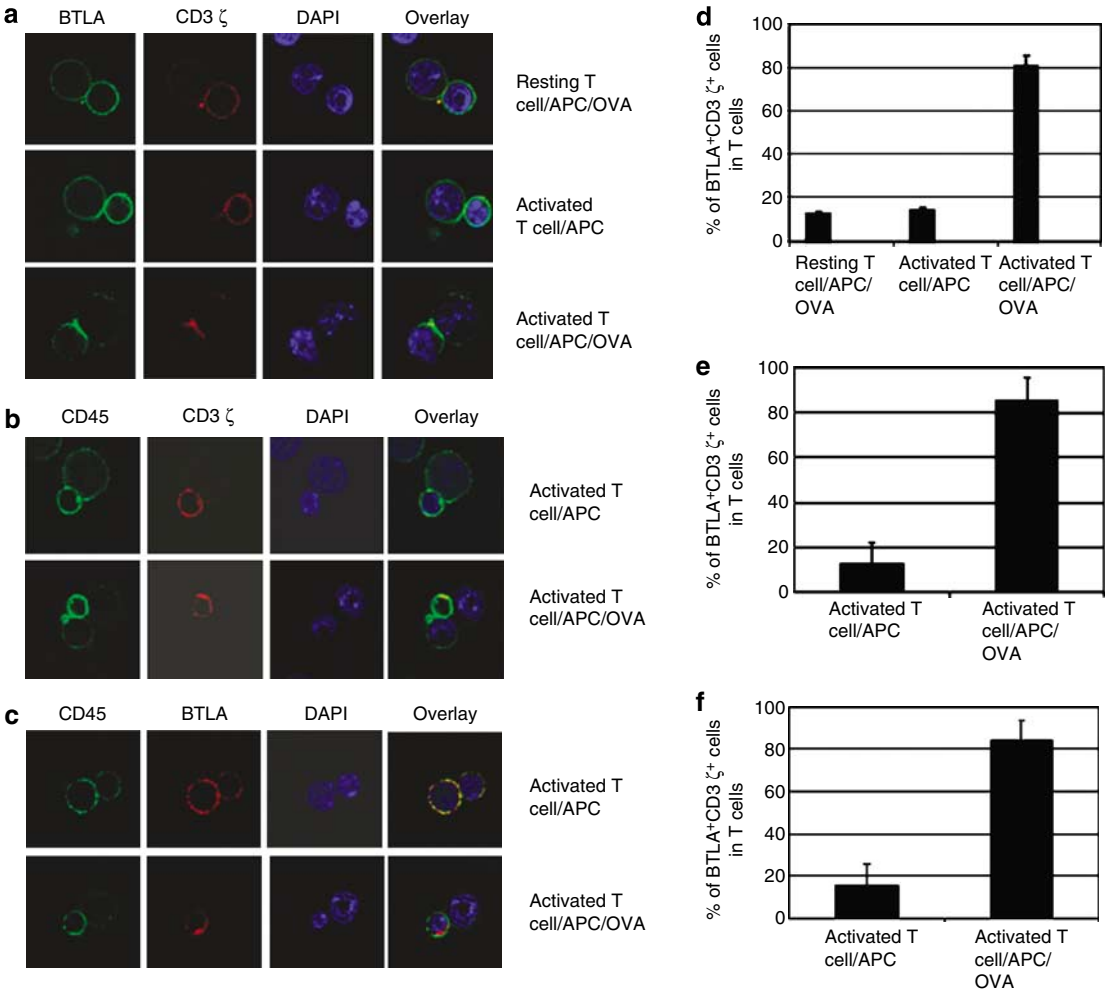

Figure 2 Co-localizaiton of BTLA and CD3ζ at the IS. Sorted CD11c$^+$ cells cultured in the presence of 100 μg/ml of OVA protein and 1 μM of OVA323-339 peptide for 10 h were allowed to form conjugates with CD4$^+$ T cells. After incubating at 37°C for 30 min, cells were fixed and permeabilized, and then stained with anti-BTLA pAb and anti-CD3ζ mAb. Nucleus was stained with DAPI. Second antibody used anti-goat IgG-FITC and anti-mouse IgG1-TRITC especially. (**a**) Co-localization of BTLA and CD3ζ at the IS of activated T cells. Pre-sensitized DO.11.10 mouse CD4$^+$ T cells and mature splenic CD11c$^+$ OVA protein or peptide-pulsed DCs were cultured together and the reaction stopped at 30 min. Resting T cell–DC conjugate and actived T cell–DC conjugate without OVA stimulating were used as negative control. (**b** and **c**) Distribution of CD45 molecules on the T cell at the formation of IS. Anti-CD45 mAb/anti-mouse IgG-FITC were used to detect CD45. One representative of three independent experiments is shown. (**d**–**f**) Quantification of the number of cell conjugates displaying BTLA and/or CD3 ζ at the IS. The images were analyzed by two-photon microscope. More than 50 individual CD4$^+$ T cell–DCs conjugates obtained in three independent experiments were assessed. BTLA, B and T lymphocyte attenuator; DAPI, 4′,6-diamidino-2-phenylinodole; DC, dendritic cell; FITC, fluorescein isothiocynate; mAb, monoclonal antibody; pAb, polyclonal antibody; TRITC, tetramethylrhodamine isothiocynate.

concentrated in raft domains.[30] This selective confinement of signaling molecules in membrane subdomains has suggested that lipid rafts could function as platforms for the formation of multicomponent signaling transduction complexes. After ligation of sensitized CD4$^+$ T cells, the lysates was subjected to sucrose gradient separation of lipid rafts as reported before.[31] No Anti-CD3 antibody activation and mouse IgG1κ ligation were used as control. TCR tyrosine phosphorylation levels in the anti-CD3 mAb-immunoprecipitated proteins in the lipid raft fraction or the bottom fraction were determined by western blot analysis. Significant tyrosine phosphorylated form of TCRζ complex was decreased by BTLA cross-linking (Figure 6). The accumulation of CD3ζ in the lipid raft was not markedly inhibited by BTLA cross-linking as determined by anti-phosphotyrosine blotting (Figure 6a). Strikingly, the p16ζ band in the rafts disappeared after BTLA cross-linking. Similar results in the bottom fraction were observed (Figure 6b). By contrast, CTLA-4 and BTLA interact with CD3ζ, but do not affect CD3ζ interaction with lipid raft. These findings suggested that the ligation of BTLA could downregulate Tyrosine phosphorylated formation of TCRζ complex in the lipid raft, but not CD3ζ accumulation in the lipid raft.

Stimulation of T cells by APCs induces formation of a highly organized complex of receptors, intracellular signaling molecules and F-actin at the contact site between T cells and APCs, or the so-called IS or supramolecular activation complex.[32,33] Studies designed to dissect the molecular mechanism of BTLA-mediated immune regulation have been reported by Dr Murphy's lab.[11] BTLA relies on dual ITIMs for its association with the phosphatases SHP-1 and -2. In this study, we tried to examine the hypothesis that BTLA is involved in lipid raft during T-cell activation and associated with tyrosine phosphorylated CD3ζ. CD3ζ is an important molecule in TCR signaling. During T-cell

activation, phosphorylated Fyn and Lck firstly phosphorylate CD3ζ SH2 domain, and then phosphorylate other recruited adaptor proteins by phosphorylated CD3ζ SH2 domain, such as LAT, PI3K, ZAP70 and PKCθ,[34–36] all of which are involved in lipid raft, an essential component of the IS.[34,37]

In conclusion, we found that BTLA co-clustered with CD3ζ, as observed by immunofluorescence assays. It appears that the two molecules co-localized in the IS, and BTLA might interact with CD3ζ in the lipid raft. Cross-linking BTLA significantly reduced tyrosine phosphorylation of TCRζ signaling components.

METHODS

Animals

Balb/c (H-2^d) and C57BL/6 (B6, H-2^b) mice were purchased from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China). Balb/c DO11.10 mice,[38] which are transgenic for a TCR specific for the immunodominant epitope of OVA peptide 323-339, were offered by Shanghai Animal Facility (Shanghai, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

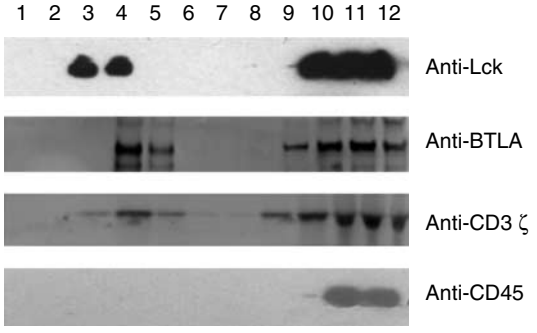

Figure 3 The presence of BTLA and CD3ζ in lipid raft of activated CD4$^+$ T cells. Purified DO11.10 CD4$^+$ T cells (5×10^7) were activated with OVA323-339 peptide, and then were lysed and fractionated by sucrose density gradient. Lipid raft parts were identified by anti-Lck mAb. Expression of BTLA and CD3ζ were detected by western blotting in the fractions. CD45 was used as negative control that does not be related with lipid raft. BTLA, B and T lymphocyte attenuator.

mAb and reagents

Anti-mouse BTLA mAb (clone 6F7) was purchased from eBioscience (San Diego, CA, USA). Anti-mouse CD3 mAb (clone 145-2C11), anti-mouse CD28 mAb (clone 37.51) and purified IgG isotypic control antibody were purchased from BD eBiosciences PharMingen (San Diego, CA, USA). Anti-mouse CD3ζ mAb (clone 6B10.2) and anti-Fyn mAb (clone Fyn3) were purchased from Santa Cruz Biotechnology (Palo Alto, CA, USA). Anti-pTyr mAb (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal rabbit anti-mouse BTLA Ab (pAb) was purchased from R&D Systems Inc. (Minneapolis, MN, USA). *n*-Octyl β-D-galactopyranoside was purchased from Sigma (Stlovis, MO, USA). OVA was obtained from Sigma-Aldrich (St Louis, MO, USA). Mitomycin C ($C_{15}H_{18}N_4O_5$) was obtained from Kyowa Hakko Co. Ltd (Tokyo, Japan).

For mouse T-cell culture, Dulbecco's modified Eagle's medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovinc serum, 10 mM HEPES, nonessential amino acids (Biosource International, Camarillo, CA, USA), 55 μM 2-mercaptoethanol, 100 μg/ml penicillin and 100 U/ml streptomycin was used as medium.

Preparation of CD4$^+$ T cells

Spleens and lymph nodes of DO.11.10 or Balb/c mice were harvested and the tissues were gently minced in Hanks' balanced salt solution supplemented with 5% fetal bovine serum (Hyclone Inc., Logan, Utah, USA). Cells were then passed through a cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA), and red blood cells were lysed with ACK lysing buffer (BioWhittaker Inc., Walkersville, MD, USA). The cells were stimulated with peptide OVA323-339 for 4 days. CD4$^+$ T-cell populations were isolated using a CD4$^+$ T Cells Isolation Kit with MidiMACS Separator according to the manufacturer's protocols (Miltenyi, Bergisch Gladbach, Germany). Briefly, cells were incubated with a biotin-antibody cocktail against CD8α (Ly2), CD11b (Mac-1), CD45R

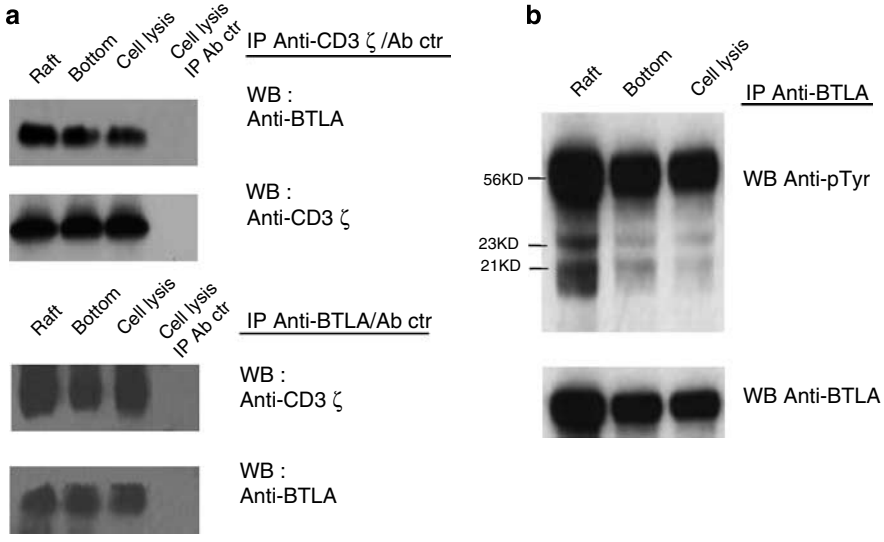

Figure 4 BTLA associates with tyrosine phosphorylated CD3ζ in the lipid raft. (**a**) The levels of CD3ζ in anti-BTLA Ab-immunoprecipitated raft fraction were detected by anti-CD3ζ mAb. Fraction 4 used as lipid raft, and fraction12 used as bottom part. Anti-BTLA pAb and anti-CD3ζ were used to immunoprecipitate the lipid raft part, bottom part and total cell lysates. Goat serum and mouse IgG-κ were used as control to immunoprecipitate from cell lysates. (**b**) The phosphotyrosine levels in anti-BTLA Ab-immunoprecipitated raft fraction were detected by anti-phosphotyrosine mAb. BTLA, B and T lymphocyte attenuator; pAb, polyclonal antibody.

(B220), CD49B (DX5) and Ter-119 for 20 min at 4°C, and then with microbead-conjugated anti-biotin mAb (Bio318E7.2). The cell suspension was loaded on a LD column, and then placed in magnetic field of a magnetic-activated cell sorting (MACS) separator. The remaining fraction in the column is the enriched $CD4^+$ T cells. The purity of the resultant cell populations was >95% as determined by fluorescence-activated cell sorting.

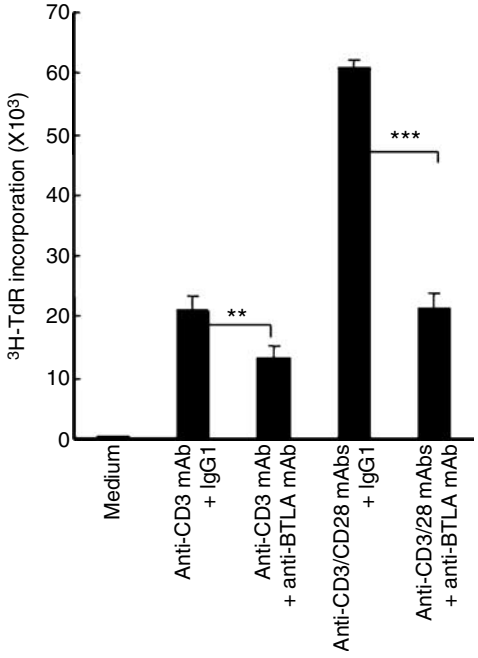

Figure 5 Ligation of BTLA significantly inhibited the proliferation of T cells induced by anti-CD3 and/or anti-CD28 mAbs. Resting B6 mouse $CD4^+$ T cells were stimulated with anti-CD3, anti-CD3/BTLA, anti-CD3/CD28 and anti-CD3/CD28/BTLA mAbs in the coated 96-well plate for 48 h and were pulsed with ^{3}H-thymidine for 18 h. Data are shown as mean±s.d. $^{**}P<0.01$, $^{***}P<0.001$ compared between the indicated groups. BTLA, B and T lymphocyte attenuator; mAb, monoclonal antibody.

T-cell proliferation assay

Separated T cells were cultured in the presence of plate-bound antibodies including 0.5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 mAbs with or without 10 μg/ml anti-BTLA mAb for 72 h at 37°C. For the last 12 h, 0.5 μCi [^{3}H]thymidine (radioactivity, 185 GBq/mmol; Atomic Energy Research Establishment, China) was added. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckman Instruments, Fullerton, CA, USA). Values are expressed as counts per minute (c.p.m.) from triplicate wells.

In vitro antibody cross-linking assay

B6 $CD4^+$ T cells separated by MACS-negative selection were stimulated by plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs for 60–70 h. The live cells were then harvested, washed and plated in T-175 culture flasks in the presence of fresh medium and 20 U/ml recombinant human IL-2. After 6 days of culture, the live cells were enriched by Ficoll density gradient. An aliquot of 10^8 cells was used for each experimental group. T cells were rested for 5 h in complete culture medium at 37°C. These cells (10^7 cells/well) were then added into wells that were pre-coated with mAbs including 0.5 μg/ml anti-CD3, 10 μg/ml anti-CD28 and 10 μg/ml anti-BTLA mAbs overnight at 4°C. The plates were centrifuged for 30 s, and incubated for 5 min 37°C.

Distribution of CD3ζ and BTLA on the surface of activated $CD4^+$ T cells and during the formation of IS

Thirty-five millimeter of glass bottom microwell dishes (MatTek Corp, Cades, SC, USA) were coated overnight with poly-L-lysine (Sigma-Aldrich). DCs were purified by digesting the spleens of Balb/c mice with collagenase followed by MACS-positive selection. Sorted $CD11c^+$ cells were cultured in the presence of 100 μg/ml of OVA protein or 1 μM of OVA323-339 peptide for 10 h to induce their maturation. The sensitized DO11.10 mouse $CD4^+$T cells (1.0×10^6) were added to the above dishes, sedimented by centrifugation to optimize interaction between the two cell types, incubated at 37°C for the 30 min and the reaction stopped by fixation with paraformaldehyde (the co-cluster assay in $CD4^+$ T cell used sorted $CD4^+$ T cell only).[39] Dishes were washed twice with phosphate-buffered saline plus 1% fetal calf serum, permeabilized with 0.5% sapoin buffer and stained with anti-BTLA pAb/anti-goat IgG mAb-FITC and anti-CD3ζ/anti-mIgG mAb-tetramethylrhodamine isothiocyanate. Image capture was done with a two-photon microscope (Carl Zeiss Inc, Oberkochen, Germany) and analyzed with the LSM 510 software (Carl Zeiss Inc.).

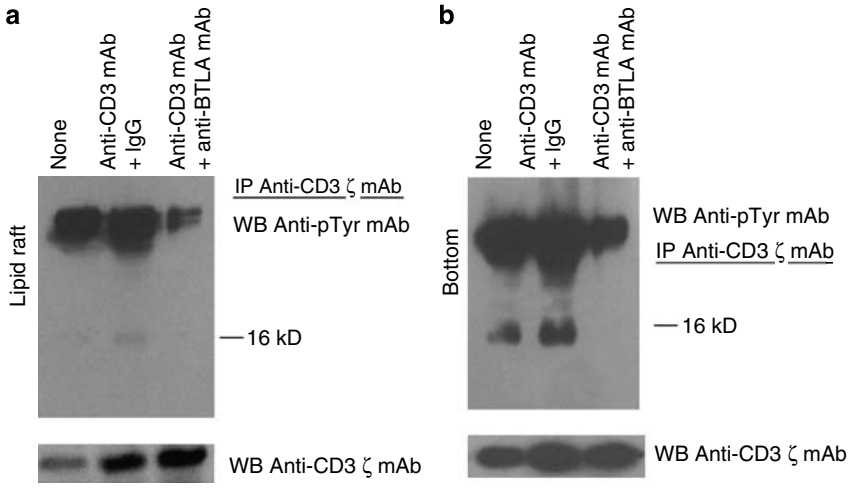

Figure 6 Ligation of BTLA inhibits TCR association to the lipid raft. Purified DO11.10 $CD4^+$ T cells (5×10^7) pre-activated with OVA323-339 peptide were left untreated or stimulated with anti-CD3 (3 μg/ml) plus mouse IgG1-κ (20 μg/ml) or anti-BTLA mAb (20 μg/ml). The lysates were fractionated by sucrose density gradient centrifugation. The raft fractions were immunoprecipitated with anti-CD3ζ mAb. The levels of phosphotyrosine in anti-CD3 mAb-immunoprecipitated part (**a**) or the detergent soluble non-raft fractions (**b**, bottom) were detected by western blot with anti-pTyr mAb. Here, mouse IgG-κ was used as control IgG. TCR, T-cell receptor.

Isolation of the lipid raft by sucrose density gradient

Sorted $CD4^+$ T cells were stimulated as described above and suspended with 500 μl of ice-cold lysis buffer (containing 0.2% Triton X-100, 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice.[4] The lysates were disrupted with 10 strokes using a Dounce cell homogenizer and mixed with an equal volume of sucrose as an 85% solution in TNE buffer (20 mM Tris (pH 8.0), 150 mM NaCl and 50 mM EDTA). The suspension was transferred into an ultracentrifuge tube (Kendro Laboratory Products, Newtown, CO, USA), where it was overlaid with 3 ml of 30% sucrose and 1 ml of 5% sucrose both suspended in TNE. The tube was then filled to the top with TNE buffer. These gradients were spun for 12–16 h at 40 000 r.p.m. in a SW-40 Ti swinging bucket rotor at 4°C in a Beckman ultracentrifuge as reported before.[25] After removing top TNE buffer, the gradients were harvested into six 0.8-ml fraction from top to bottom. The glycosphingolipid-enriched membrane domainss were identified by western blotting with anti-Lck mAb.[31] The individual fractions were mixed with 0.1 volume of 0.6 M *n*-octyl β-D-galactopyranoside (final 60 mM), incubated for 2 h to solubilize the rafts, and subjected to immunoprecipitation and western blot analyses, as previously reported.[26]

ABBREVIATIONS

APCs, antigen-presenting cells; BTLA, B and T lymphocyte attenuator; CTLA-4, cytotoxic T lymphocyte associated antigen-4; IS, immunological synapse; ITIMs, immunoreceptor tyrosine-based inhibitory motifs; TCR, T cell receptor

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