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B7-H3 is a potent inhibitor of human T cell activation: No evidence for B7-H3 and TREML2 interaction

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Abstract

B7-H3 belongs to the B7 superfamily, a group of molecules that costimulate or down-modulate T cell responses. Although it was shown that B7-H3 can inhibit T cell responses, several studies - most of them performed in murine systems - found B7-H3 to act in a costimulatory manner. In this study we have specifically addressed a potential functional dualism of human B7-H3 by assessing the effect of this molecule under varying experimental conditions as well as on different T cell subsets. We show that B7-H3 does not costimulate human T cells. In presence of strong activating signals, B7-H3 potently and consistently down-modulated human T cell responses. This inhibitory effect was evident when analyzing proliferation and cytokine production and affected naïve as well as pre-activated T cells. We furthermore demonstrate that B7-H3 - T cell interaction is characterized by an early suppression of IL-2 and that T cell inhibition can be reverted by exogenous IL-2.

Since TREML2 has been recently described as costimulatory receptor of murine B7-H3 we have extensively analysed interaction of human B7-H3 with TREML2 (TLT2). In these experiments we found no evidence for such an interaction. Furthermore our data do not point to a role for murine TREML2 as a receptor for murine B7-H3.

Keywords

costimulatory molecules; immune regulation; T cells

Introduction

For fine-tuning the immune response several costimulatory and coinhibitory signals are needed in addition to signal 1 provided via the peptide-MHC/TCR-complex interaction. CD80 (B7-1) and CD86 (B7-2) serve as primary costimulatory ligands. Recently, additional

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members of the B7 family – the so-called B7 homologs - have been identified [1]. The functional role of several of these B7 homologs is still controversially discussed. One of these molecules is B7-H3, which was originally described as a potent costimulatory molecule and inducer of IFN-γ in human T cells [2]. In contrast Ling et al. found human B7-H3 to strongly down-regulate T cell proliferation and cytokine production [3]. It was suggested that presence of two B7-H3 receptors with different functions could explain these divergent results [3]. Recent data that showed opposing effects of B7-H3 on resting and cytokine-activated T cells as well as contradicting results on the function of murine B7-H3 would also be in support for such a constellation [4-7]. Such receptor molecules could either be differentially regulated on T cells or be expressed on different T cell subsets. Depending on the experimental system used the effects of the costimulatory or the inhibitory receptor could prevail and explain the discrepancies in different studies.

Here we have specifically addressed a potential functional dualism of B7-H3 by studying B7-H3 effects under varying experimental conditions as well as on different subsets of human T cells. Our results point to a potent and consistent inhibitory role of human B7-H3 in T cell activation and give no evidence for a costimulatory function of this molecule. Recently, the triggering receptor expressed on myeloid cells like transcript 2 (TREM-like transcript 2, TLT-2, TREML2) has been reported to act as a costimulatory B7-H3 receptor on murine T cells and it was shown that overexpression of this molecule renders T cells more responsive to B7-H3 mediated costimulation [8]. We have therefore also extensively analysed a potential interaction of B7-H3 with TREML2. We demonstrate in binding and functional studies that human TREML2 does not serve as a costimulatory receptor for human B7-H3. Furthermore we do not find any evidence for a role of murine TREML2 as B7-H3 receptor.

Results

B7-H3 does not costimulate a weak signal 1 in human T cells

In contrast to its first description as a costimulatory molecule and potent inducer of IFN-γ [2], several studies have found B7-H3 to inhibit T cell responses [3, 5, 7]. To specifically assess a potential functional dualism of human B7-H3 in T cell activation we analysed its role under different conditions at different time points and on different T cell subsets.

Costimulatory functions are best seen in context of a weak signal 1 since in this case T cell proliferation takes only place in the presence of a second signal. We therefore stimulated human T cells with different amounts of plate bound anti-CD3 antibody in presence of B7-H3-Ig, ICOS-L-Ig or control-Ig (for characterization of fusion proteins see supplementary information Fig. 1A). In these experiments in absence of any costimulatory signal (control-Ig) 0.1 μg/ml of plate bound anti-CD3 antibodies were needed to induce T cell proliferation whereas in presence of ICOS-L-Ig T cell proliferation could already be observed at anti-CD3 antibody concentrations of 0.01 μg/ml and was strongly enhanced at higher concentrations. In contrast B7-H3-Ig failed to lower the threshold of anti-CD3 induced T cell proliferation and moreover we observed an inhibitory effect of this fusion protein at high concentrations of anti-CD3 antibody (Fig. 1A). Similar results were obtained when stimulating T cells with our system of T cell stimulator cells (described previously [9]) that is based on the murine thymoma cell line Bw5417 (short designation Bw; for characterization of T cell stimulator cells used in this study see supporting information Fig. 1B and 1C): Presence of ICOS-L but not B7-H3 costimulated proliferation of human T cells on our Bw-aCD3^{low} T cell stimulators expressing anti-CD3 antibody fragments at a level that is not sufficient to induce significant T cell proliferation in absence of costimulatory signals (Fig. 1B).

B7-H3 inhibits T cell activation

In a next set of experiments we stimulated T cells with high amounts of plate-bound anti-CD3 antibodies sufficient to induce T cell proliferation. In this setting B7-H3-Ig constantly inhibited T cell proliferation (median inhibition 74%, $p<0.001$, n=9; Fig. 2A). In line with these results presence of B7-H3 on Bw-CD3^{high} T cell stimulator cells expressing high amounts of membrane-bound anti-CD3 antibody and thus inducing T cell proliferation also in absence of costimulation also strongly reduced T cell proliferation compared to control stimulator cells ($p<0.001$, n=14; Fig. 2B) [9, 10]. In contrast to B7-H3 we found that ICOS-L significantly increased T cell activation in both types of experiments.

Upon its identification B7-H3 has been described as a molecule containing 2Ig like domains [2]. However, subsequent reports have demonstrated that in humans and other primates this molecule contains 4Ig like domains whereas mice and other rodents express a 2Ig molecule [3, 6, 11]. To exclude the possibility that the different forms exert different functions in the process of T cell activation, we stimulated human T cells in the presence of our 4Ig B7-H3-Ig (short designation of 4Ig B7-H3 in this paper B7-H3) and a 2Ig B7-H3-Ig. As shown in Figure 2C, 4Ig B7-H3 and 2Ig B7-H3-Ig induced comparable inhibition of T cell activation, excluding the possibility that different functions apply to the two forms. Furthermore, since there was no difference in the T cell inhibitory properties of the commercially available 2Ig B7-H3 fusion protein and the 4Ig B7-H3 fusion protein produced in our laboratory, these experiments also rule out that the observed inhibitory effect of the 4Ig B7-H3 is due to the production procedure.

Since B7-H3 is not only expressed on tumour cells and peripheral tissues but also on professional APC, like DC which harbour a plethora of potent accessory molecules we tested if costimulatory signals abrogate the inhibitory effect of B7-H3. We therefore analysed the effects of B7-H3-Ig in presence of anti-CD28 antibodies and found that B7-H3 also strongly inhibited proliferation of human T cells receiving a costimulatory signal via CD28 ($p<0.01$, n=8; Fig. 2D). Since previously also stimulatory functions have been reported for human B7-H3 we assessed if using B7-H3-Ig at different concentrations would reveal such an activity in our test system. However, when analyzing the effects of B7-H3-Ig immobilized at different levels we observed a dose-dependent inhibition of human T cell proliferation with relatively high levels of B7-H3-Ig required for significant T cell inhibition. Importantly, also the results obtained with concentrations too low to inhibit T cell proliferation (0.1 μg/ml) did not point to costimulatory effects of B7-H3 (supporting information Figure 2).

B7-H3 reduces cytokine production of human CD4$^+$ and CD8$^+$ T cells

B7-H3 has originally been described as a potent inducer of IFN-γ production [2] whereas Ling et al. reported reduction of cytokine production in the presence of B7-H3 fusion protein [3]. We did not find evidence for the specific induction of IFN-γ regardless if CD4$^+$ and CD8$^+$ T cells were stimulated with B7-H3-Ig in the presence of plate-bound anti-CD3 antibodies or with B7-H3 expressing T cell stimulator cells (Fig. 3 and data not shown). In contrast, we found B7-H3 to inhibit IFN-γ production. Moreover, and in line with the results obtained when measuring T cell proliferation, we found that B7-H3 profoundly reduced the levels of IL-2, IL-10 and IL-13 in the culture supernatants of both, CD4$^+$ and CD8$^+$ T cells (Fig. 3). The inhibitory effect of B7-H3 was also evident upon analysis of cytokine mRNA levels by quantitative PCR (data not shown).

B7-H3 inhibits proliferation of CD4$^+$, CD8$^+$, naïve and pre-activated T cells

To determine the effects of B7-H3 on CD4$^+$ and CD8$^+$ T cells we performed two types of experiments. First we stimulated CFSE-labelled T cells with control stimulator cells or with

stimulator cell expressing B7-H3 for 5 days. Subsequently, cells were stained for CD4 or CD8 expression and analysed by FACS. In these experiments presence of B7-H3 led to a comparable reduction of proliferation in both subsets (Fig. 4A). However upon analysis of purified $CD4^+$ and $CD8^+$ T cells we found B7-H3 to strongly reduce the proliferation of $CD4^+$ cells ($p<0.001$, n=9) whereas $CD8^+$ cells were less responsive to direct inhibition mediated via B7-H3 ($p<0.01$, n=9; Fig. 4B). Thus it appears that the strong inhibition of $CD8^+$ T cell proliferation observed in the first type of experiments is mainly due to reduced help by $CD4^+$ T cells.

Recently it was reported that the functional effects of B7-H3 expressed on fibroblast-like synoviocytes depend on the activation state of T cells: Cytokine pre-activated T cells appeared to show increased cytokine production upon interaction with B7-H3 whereas resting T cells were inhibited [6]. To address the influence of the activation state of human T cells on the functional effects of B7-H3 we analysed on one hand naïve T cells (T cells depleted from $CD45RO^+$ cells) from adult donors. Although these cells showed lower proliferation upon anti-CD3 stimulation than purified T cells ($CD45RO^+$ and $CD45RO^-$) from the same donors we found B7-H3-Ig to reduce the proliferation of both T cell subsets to a similar extent (Fig. 4C). Furthermore, we found that human cord blood T cells that contain over 90% naïve cells were also strongly inhibited by B7-H3-Ig ($p<0.001$, n=9; Fig. 4D). On the other hand to evaluate the effect of B7-H3 on pre-activated $CD4^+$ and $CD8^+$ T cells stimulated with anti-CD3/CD28 antibodies for 9 days were restimulated in presence or absence of B7-H3-Ig. These experiments show that B7-H3 down-regulates also the proliferation of pre-activated $CD4^+$ and $CD8^+$ T cells (Fig. 4E). Taken together our results do not point to a significant influence of the activation state of human T cells on the functional effects of B7-H3.

B7-H3 mediated T cell inhibition is characterized by early suppression of IL-2

In order to test the kinetics of B7-H3-mediated T cell inhibition we stimulated T cells in the presence of B7-H3-Ig or control fusion protein and assessed their proliferation at several time points. In these experiments methyl-^{3}[H]-thymidine uptake started at 32 hours of activation and peaked at 72 hours. B7-H3-Ig strongly reduced methyl-^{3}[H]-thymidine uptake and its inhibitory effect was evident throughout the course of the experiment (Fig. 5A).

We could observe that upon anti-CD3 stimulation of human T cells low levels of IL-2 are detectable in the cultures within a few hours (Fig. 5B). We therefore monitored the concentration of this cytokine in T cell cultures to determine the effects of B7-H3 on T cell activation at early time points. In the presence of B7-H3 the IL-2 concentration in the culture supernatant was strongly reduced. Importantly the B7-H3 mediated reduction of IL-2 was already evident after 4 hours of activation. This points to an interaction of B7-H3 with a receptor, which is either constitutively expressed or rapidly induced following activation. Furthermore since IL-2 is an essential growth factor for T cells, the reduced availability of this cytokine in the early phase of T cell activation might explain the profound inhibition of T cell proliferation by B7-H3. In support for this we found that IL-2 added immediately to stimulation assays could revert B7-H3 mediated T cell inhibition, whereas IL-2 supplemented at later time points showed minimal reversion of this inhibitory effect (Fig. 5C).

No evidence for interaction of human B7-H3 with TREML2

Taken together all our results point to an interaction of B7-H3 with inhibitory receptors on human T cells. The identification of receptors would greatly aid the understanding of human B7-H3 – T cell interaction. Recently TREML2 (TLT-2) was reported to serve as a costimulatory receptor for murine B7-H3 [8]. To test whether human TREML2 might be a

receptor for human B7-H3 we cloned and expressed it on the Bw cell line. In spite of very high expression of TREML2 we could not observe specific interaction with B7-H3-Ig: Neither the commercially available 2Ig B7-H3 nor the B7-H3 fusion protein produced in our laboratory bound to human cells expressing TREML2, strongly suggesting that on human T cells this molecule does not serve as a receptor for B7-H3 (Fig. 6A). In contrast ICOSL-Ig bound to ICOS expressing cells and CD80-Ig strongly and specifically interacted with human CD28, CTLA-4 and also with PD-L1, as recently reported [12, 13]. Importantly the interaction of ICOSL-Ig and CD80-Ig with their receptors were detectable at concentration that were 100-fold lower than the highest concentration of B7-H3-Ig used for binding studies with TREML2 transductants, excluding the possibility that our binding assay might not be sufficiently sensitive. Experiments where we generated and tested a fusion protein representing the extracellular domain of human TREML2 (TREML2-Ig; for characterization of TREML2-Ig see supporting information Fig. 3) further excluded an interaction of this molecule with human B7-H3: TREML2-Ig did not bind to cells expressing high levels of B7-H3 whereas fusion proteins representing CTLA-4 or PD-1 strongly bound to cells expressing their ligands (Fig. 6B). Hashiguchi et al. reported that mouse T cells overexpressing TREML2 were rendered more responsive for B7-H3 costimulation [8]. We have previously found the Jurkat cell line to be unresponsive to B7-H3 mediated inhibition of activation. Thus this cell line seems not to express an inhibitory B7-H3 receptor that could interfere with a costimulatory signal putatively provided via B7-H3-TREML2 interaction. However we found that overexpressing TREML2 in a Jurkat reporter cell line [14] did not induce enhanced IL-2 promotor activity upon interaction with B7-H3 expressing T cell stimulator cells, compared to control Jurkat reporter cells. In contrast we found that upon expression of the costimulatory receptor 4-1BB these cells were strongly costimulated by 4-1BBL expressed on T cell stimulator cells (Fig. 6C; for characterization of TREML2 and 4-1BB Jurkat reporter cells see supporting information Fig. 4). Stimulation of Jurkat-4-1BB with 4-1BBL also leads to enhanced induction of the activation marker CD69 whereas Jurkat-TREML2 did not up-regulate CD69 upon interaction with B7-H3 (data not shown). Thus in line with binding studies our functional analysis did also not point to an interaction of TREML2 with human B7-H3.

Mouse TREML2 does not serve as receptor for mouse B7-H3

Since receptor-ligand interactions are generally conserved between mice and humans we also analysed the interaction of mouse B7-H3 with mouse TREML2. For this we generated a fusion protein representing the extracellular domains of mouse B7-H3 (mB7-H3-Ig; for characterization of mouse B7-H3-Ig see supporting information Fig. 5) to analyse its interaction with cells expressing high levels of mouse TREML2 (mTREML2). In contrast to Hashiguchi et al. we did not observe evidence for an interaction of these molecules (Fig. 7). Importantly independently performed experiments using a previously described B7-H3-Ig [15] did also not point to a specific interaction of murine B7-H3-Ig with TREML2 (supporting information Fig. 5).

Discussion

For several of the new members of the B7 superfamily, the so-called B7 homologs, contradictory results regarding their role in the regulation of T cell responses have been reported. PD-L1 (B7-H1) and PD-L2 (B7-DC) bind the inhibitory receptor PD-1 and in line with this a number of studies describe a down-modulation of T cell response via PD-ligands [9, 16-20]. However several reports found PD-L1 and 2 to act costimulatory on T cells, pointing to additional receptors for these molecules [21-25]. B7-H3 is another member of the B7 family that was described as a costimulatory but also as an inhibitory ligand: it was first reported to be a potent inducer of proliferation and INF-γ production in human T cells

and additional studies supported a costimulatory function for this molecule [2, 4, 26]. In contrast, other authors have described inhibitory functions for human and murine B7-H3 and B7-H3 deficient mice were found to have enhanced T cell responses in vivo and in vitro [3, 5, 7]. One possible explanation for these discrepant findings is that B7-H3 has two receptors and the contrasting results are due to different experimental conditions that preferentially lead to the engagement of either a costimulatory or an inhibitory receptor on T cells. The identification of a B7-H3 receptor would therefore not necessarily resolve the controversy regarding the function of B7-H3 as it could not rule out the existence of additional receptors with opposing roles.

In this study we specifically addressed a potential functional dualism of human B7-H3 by analysing the consequences of B7-H3 - T cell interaction using several different experimental conditions. Costimulatory functions of accessory molecules are best studied in the context of a weak signal 1 since under such conditions T cell proliferation only takes place in the presence of a second signal. In our experiments this was observed for ICOS-L that significantly lowered the concentration of anti-CD3 that was required to induce T cell proliferation. In contrast B7-H3 failed to act costimulatory under such conditions and at high anti-CD3 level an inhibitory effect of co-immobilized B7-H3 fusion protein was evident (Fig. 2). We found B7-H3 to negatively regulate the activation of naïve as well as pre-activated T cells and show that it down-regulates proliferation of $CD4^+$ and $CD8^+$ T cells. In contrast ICOS-L, a well established costimulatory member of the B7 family, consistently enhanced proliferation and cytokine production excluding a bias towards revealing inhibitory effects in our experimental systems. The effect of B7-H3 was also evident when we analysed the levels of cytokines but our results did not point to a function of B7-H3 signals in skewing T cells towards the expression of a distinct cytokine pattern. Instead we observed a strong reduction of both, Th1 and Th2 type cytokines but also of IL-10, a pleiotropic cytokine, which was shown to have potent immunosuppressive functions. B7-H3 does not result in T cell apoptosis nor does it induce anergy as T cell stimulated in the presence of B7-H3 are not impaired in their ability to respond to secondary stimuli (data not shown). Furthermore co-culture experiments of T cells stimulated in the presence of B7-H3 did not reveal evidence that B7-H3 induces a suppressor phenotype in human T cells (data not shown).

IL-2 is generally regarded to be essential for efficient T cell activation and we detected IL-2 in culture supernatants very early after the initiation of T cell activation cultures. Furthermore we found that presence of B7-H3 led to strongly reduced IL-2 concentrations and it is likely that down-regulation of IL-2 production contributes to the strong anti-proliferative effect that is exerted by B7-H3. The inhibitory effect was already observed when we analysed culture supernatants 4 hours following T cell activation. This indicates that B7-H3 receptors are either present on resting T cells or quickly induced in these cells upon activation. Exogenous IL-2 could fully revert the inhibition of T cell by B7-H3 when present during initiation of T cell activation but was less effective when added at later time points. In line with the work of Ling et al. this study shows that B7-H3 strongly down-regulates proliferation and cytokine production of human T cells [3]. We extend their findings by demonstrating that analysis of B7-H3 under different conditions and on different T cell subsets does not yield any evidence for a costimulatory function of this molecule. Originally B7-H3 has been described as a molecule with 2 Ig-like domains but our previous data and work by others strongly suggest that B7-H3 is comprised of two highly homologous V and IgC2-like domains (4Ig B7-H3) [3, 6, 11]. Although in this study we have primarily focussed on the 4Ig B7-H3 we have also analysed a commercially available B7-H3 fusion protein representing a 2Ig form and found this molecule to have the same functional effects on T cell proliferation than the full-length molecule (Fig. 2C). Furthermore, very similar results were obtained when we compared the functional effects of

2Ig B7-H3 and 4Ig B7-H3 on our stimulator cells (data not shown). Thus the costimulatory effects for B7-H3 that were described in the initial report can not be explained by the fact that these authors used a short form of B7-H3 in their experiments [2].

The identification of receptors would greatly aid the understanding of human B7-H3 – T cell interaction. We put extensive efforts in identifying B7-H3 receptors using different approaches: On one hand we are trying to identify such molecules by retroviral expression cloning and on the other hand we use bioinformatics to identify proteins homologous to the CD28 superfamily members. We have analysed human molecules that have similarities to this family including IGSF6 (DORA), SIRPB1 (CD172B), HAVCR1 (TIMD1), GPA33 (Glycoprotein A33) and CD300LG (TREM4). However none of these molecules bound B7-H3-Ig (data not shown). Recently, TREML2 (TLT-2) was described as costimulatory receptor for murine B7-H3 using a similar approach [8]. Interestingly, although the human TREML2 protein has over 50% sequence identity with its mouse orthologue, it was not identified in our screens for molecules with similarity to the group of known receptors for B7-family members (PSI-BLAST or HMM search of candidates with matching domain structure; not shown). To experimentally test whether TREML2 might be a receptor for human B7-H3 we analysed the interaction of immunoglobulin fusion proteins representing human B7-H3 and TREML2 with cells expressing high levels of human TREML2 and B7-H3, respectively. In these experiments we could not detect specific interaction between these molecules. Importantly fusion proteins representing human CTLA-4, PD-1, BTLA, CD80, PD-L1, PD-L2 and ICOSL that were produced in our laboratory using the same methodology strongly and specifically bound to cells expressing their respective ligands (Fig. 6 and data not shown). It is therefore very unlikely that our B7-H3 and TREML2 fusion proteins were not functional. In addition a commercial fusion protein representing human B7-H3 did also not bind to cells expressing high levels of human TREML2 (Fig. 6). Finally in showing that a human T cell line overexpressing TREML2 is not activated by B7-H3 we also provide functional evidence that human B7-H3 is not a costimulatory ligand for TREML2. Moreover in experiments performed in two laboratories with independently generated reagents we did also not find any evidence for an interaction of mouse TREML2 with mouse B7-H3 (Fig. 7 and supporting information Fig. 5).

Although we cannot completely rule out the existence of costimulatory B7-H3 receptors our data indicate that the net effect of B7-H3 T cell interaction results in a profound down-modulation of T cell responses. B7-H3 is widely expressed on peripheral tissues including different tumours, which are known to express numerous surface molecules and soluble factors that are able to subvert the immune system [27]. This also points to an immunosuppressive role for B7-H3 as presence of costimulatory molecules on such cells is a rare phenomenon. Moreover B7-H3 expression in human prostate and non-small lung cancer was found to be a predictor for reduced survival [28, 29]. Data from other groups and the results of this study strongly indicate that blocking human B7-H3 might be a promising strategy to enhance natural or therapeutically induced immune responses to B7-H3 expressing tumours.

Material and Methods

Antibodies, cell culture and FACS staining

293T cells, the mouse thymoma cell line Bw5147 (short designation within this work Bw), and AKR1.G.1 cells (ATCC: TIB-232; designation within this work AK) were cultured as described [9, 14, 30]. Jurkat clone 41-19 expressing an IL-2 promoter-driving luciferase (designation in this work Jurkat reporter cells) was cultured as described [14]. The ethical review board of the General Hospital and the Medical University of Vienna approved the human studies performed within this work and informed consent was obtained from the

donors. PBMC were isolated from heparinised whole blood of healthy volunteer donors by standard density centrifugation with Ficoll-Paque (Amersham Bioscience, Roosendaal, Netherlands). Human T cells were obtained through depletion of CD11b, CD14, CD16, CD19, CD33 and MHC-class II bearing cells with the respective mAbs by MACS. $CD8^+$ T cells, $CD4^+$ T cells and $CD45RA^+$ T cells were purified from human T cells using MACS in conjunction with antibodies to CD4, CD8 or CD45RO. Umbilical cord blood from healthy donors was collected during full-term deliveries and cord blood T cells were purified by MACS using the antibody pool described above. Cord blood cells in this study were ≥ 90% $CD45RA^+$ and $CD45RO^-$.

The mAbs to B7-H3 (13-I-241), CD11b (VIM12), CD14 (VIM13), CD33 (4D3), CD4 (VIT4), CD8 (VIT8), MHC-class II (1/47), CD80 (7-480), B7-H1 (PD-L1, 5-272) and the non-binding control antibody VIAP (calf intestine alkaline phosphatase specific) were produced at our institute. The mAbs to CD14 (MEM-18) was purchased from An der Grub (Kaumberg, Austria), CD3 mAb from Ortho Pharmaceutical Corporation (Raritan, NJ), CD28 (28.2), ICOS-L (2D3/B7-H2), 4-1BB (4B4-1), ICOS (DX29) and CTLA-4 (BN13) mAb from BD Pharmingen (Palo Alto, CA), CD19 mAb (BU12) from Ancell (Bayport, MN), CD45RO beads were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany) and mAb PD-L2 (MIH18) from eBioscience (San Diego, CA). Goat-anti-human TREML2 Ab was from R&D (Minneapolis, MN). This antibody was also used to measure expression of mouse TREML2 since we found it to strongly cross-react with the mouse orthologue. Coating antibodies for proliferation assays: goat-anti-mouse IgG H+L antibodies were obtained from Caltag (Caltag; Burlingame, CA) and the goat-anti-human IgG-Fcγ-specific antibodies from Jackson ImmunoResearch (West Grove, PA).

FACS analysis was performed as described previously [10]. Binding of primary antibodies was detected with PE-conjugated goat anti-mouse IgG-Fcγ specific Abs or PE-conjugated donkey anti-goat IgG Abs. Binding of Immunoglobulin (Ig)-fusion proteins was detected using PE or APC-conjugated goat anti-human IgG-Fcγ specific Abs (all from Jackson ImmunoResearch). Flow cytometric analysis was done using a FACScalibur flow cytometer supported by CELLQUEST software (Becton Dickinson). Fluorescence intensity is shown on a standard logarithmic scale. CFSE labelling was performed as described [31].

For Western blotting goat-anti-mouse IgG-Fcγ-HRP (Jackson ImmunoResearch) and rabbit-anti-goat Ig-HRP (Dako, Glostrup, DK) were used.

Generation of immunoglobulin fusion proteins

cDNAs encoding CD80, ICOS-L, B7-H3, TREML2, PD-1 and CTLA-4 were PCR amplified from a retroviral cDNA expression library derived from mature and immature human dendritic cells [11] or from cDNA prepared from PMA/Ionomycin activated human T cells. cDNA encoding for murine B7-H3 was PCR amplified from PMA/Iono activated C5Bl6 spleenocytes. PCR products were cloned into a modified pEAK12 expression vector (Edge Biosystems, Gaithersburg, MD). The resultant expression constructs encode the extra-cellular domains of CD80 (aa 1-262), ICOS-L (aa 1-135), 4Ig B7-H3 (aa 1-462), TREML2 (aa 1-223), CTLA-4 (aa 1-161), PD-1 (aa 1-202) or murine B7-H3 (aa 1-250) fused to the hinge region, the C_H2 and C_H3 domains of human IgG_1 (short designation within this work: CD80-Ig, ICOS-L-Ig, B7-H3-Ig, TREML2-Ig, CTLA-4-Ig, PD-1-Ig and mB7-H3-Ig). A control fusion protein consisting of the CD5 leader fused to the hIgG1Fc part was also generated (Co-Ig). The integrity of the constructs was confirmed by DNA sequencing. The Ig fusion protein constructs were transiently transfected into the 293T cell line. Cell culture supernatant was collected 48 and 96 hours after transfection. For protein purification the HiTrap rProtein A FF column (Amersham Bioscience) was used. The human 2Ig B7-H3-Ig

fusion protein was purchased from R&D and an additional mouse B7-H3-Ig also used in this study has been described [15].

Retroviral transduction - Generation of T cell stimulators, AK, Bw and Jurkat-transductants

The system of T cell stimulators expressing high or low levels of mb anti-CD3 single chain antibodies has been described previously [9]. Expression plasmids encoding, CD80, ICOS-L, B7-H3, TREML2, PD-L1, PD-L2, CTLA-4, CD28, ICOS, 4-1BB and mouse TREML2 (mTREML2) were retrovirally transduced in our system of T cell stimulators, AK, Bw or Jurkat cells as described [11]. Stimulator cells expressing 4-1BBL have been described [10].

T cell proliferation assays

All T cell proliferation assays were done in triplicates, means and SD are shown.

For T cell proliferation assays with plate-bound anti-CD3 mAb (OKT3) and human immunoglobulin fusion proteins (Ig-fusion proteins) 96 well ELISA plates were coated with anti-mouse IgG (final concentration: 3 μg/ml) and anti-human IgG Fcγ-specific Abs (final concentration: 10 μg/ml) in sterile PBS over night at 4°C. Anti-CD3 mAb and Ig-fusion proteins were immobilized at concentrations of 1 μg/ml and 10 μg/ml, respectively unless indicated otherwise. Human T cells (1×10^5/well) were added and cultured for 4 days. In some proliferation assays CD28 mAb or IL-2 (R&D) was added at the indicated final concentrations. Co-culture experiments of human T cells with the T cell stimulator cells were described previously [9].

To assess T cell proliferation methyl-^{3}[H]-thymidine (final concentration: 0.025 mCi; MP Biomedicals; Heidelberg, Germany) was added for the last 18 hours except for proliferation kinetic experiments where methyl-^{3}[H]-thymidine was added 12 hours prior harvesting of the cells. Methyl-^{3}[H]-thymidine uptake was measured as described [9].

In restimulation experiments purified $CD4^+$ and $CD8^+$ T cells were stimulated with Dynabeads T cell expander (Invitrogen) for 9 days. Subsequently, the T cells were harvested and restimulated using plate-bound anti-CD3 mAb (1 μg/ml) and human Ig-fusion proteins (10 μg/ml) and soluble CD28mAb (10 ng/ml) for 48 hours (10^5 cells/well).

Jurkat luciferase assay activity

Non-transduced Jurkat reporter cells and Jurkat reporter cells retrovirally transduced to express human TREML2 or 4-1BB (1×10^5/well) were co-cultured with T cell stimulators (2×10^4/well) for 6 hours. Cells were lysed according to the luciferase assay system protocol (Promega, Madison, WI, USA). Luciferase activity was assayed as described [30, 32].

Cytokine measurement

For cytokine measurement supernatants of T cell stimulation experiments were collected at the indicated time-points and pooled from triplicate wells. IL-2, -10, -13 and IFN-γ were measured in cell culture supernatants using the Luminex 100 system (Luminex Corporation, Texas, USA).

Bioinformatics-based search for candidate B7-H3 receptors

We screened for molecules with similarity to CD28, CTLA-4 (CD152), ICOS (CD278) and PD-1 (CD279). These were studied using local and global alignments as well as profile sequence analysis methods [33, 34]. In a liberal, high-sensitivity screen, we accepted

potential V-domains with an E-value less than 10 and rejected sequences only if they had a high-confidence C2-domain (E<0.001). These sequences were then searched with PSI-BLAST using a query alignment [35] of the four known receptors and the default search parameters. Hits that had an E<1 value and were not isoforms or fragments of the known B7 family receptors were expressed for binding studies with the B7-H3-Ig.

Statistics

Two-tailed Student-t test was used to assess significances. Differences were considered significant at p<0.01. The error bars indicate the SD of three replicates from one experiment, and the data are representative of three independent experiments, unless indicated otherwise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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abbreviations

APC	allo-phycoerythrin
BTLA	B-and T lymphocyte attenuator
Bw-anti-CD3	Bw 5417 mouse thymoma cell line expressing membrane bound αCD3 antibody fragments
ICOS-L	inducible costimulator ligand
PD-1	programmed death 1
PD-L	programmed death ligand
TREML2/TLT2	triggering receptor expressed on myeloid cells like transcript 2

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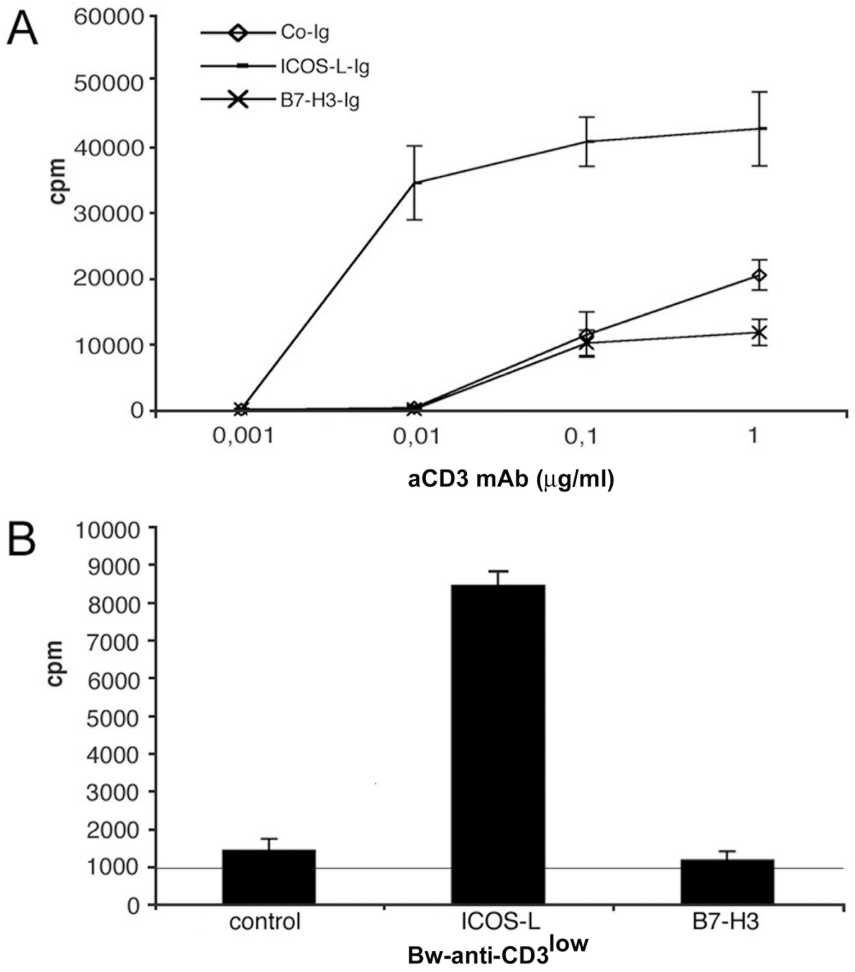

Figure 1. B7-H3 does not costimulate a weak signal 1

(A) Human T cells were incubated with plate-bound anti-CD3 mAb immobilized at the indicated concentrations in the presence of control-Ig (Co-Ig), ICOS-L-Ig or B7-H3-Ig (immobilized at 10 μg/ml). (B) Human T cells were co-cultured with control Bw-anti-$CD3^{low}$ stimulator cells and stimulator cells expressing ICOS-L and B7-H3. The thin black line indicates the mean methyl-^{3}[H]-thymidine incorporation of the irradiated stimulator cells in absence of human T cells. T cell proliferation was determined by assessing methyl-^{3}[H]-thymidine uptake (cpm: counts per minute) on day 4 (A) or on day 3 (B) of culture. Data show mean ± SD of triplicates from one experiment and are representative of three independent experiments.

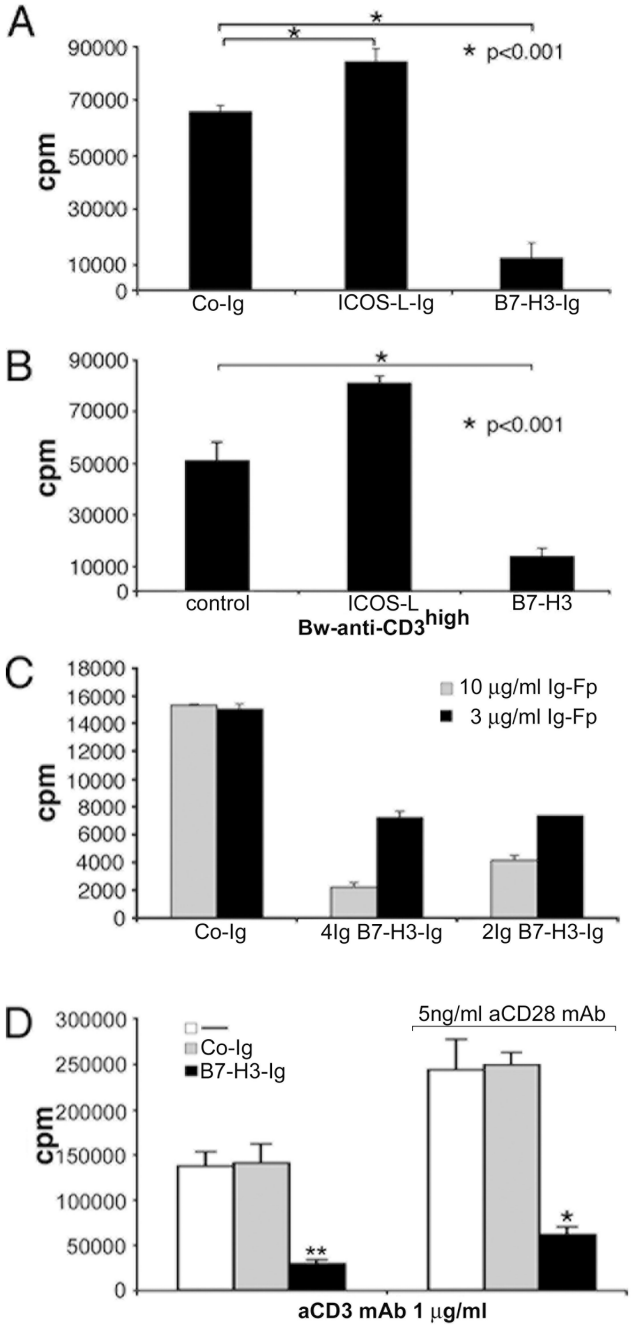

Figure 2. B7-H3 inhibits human T cell activation

(A) T cells were stimulated with plate bound anti-CD3 mAb in presence of control-Ig (Co-Ig), ICOS-L-Ig or B7-H3-Ig. Presence of B7-H3 significantly inhibits T cell proliferation ($p<0.001$, n=9) whereas ICOS-L strongly enhanced T cell proliferation ($p<0.001$, n=7). (B) T cell stimulator cells expressing ICOS-L or B7-H3 and control Bw-anti-CD3^{high} stimulator cells were incubated with T cells. Differences in T cell proliferation induced by control stimulator cells and stimulator cells expressing B7-H3 were statistically significant ($p<0.001$, n=14). (C) 4Ig and 2Ig B7-H3 inhibit proliferation of human T cells to a similar extent. Human T cells were incubated with plate-bound anti-CD3 mAb in presence of Co-Ig, 4Ig B7-H3-Ig or 2Ig B7-H3-Ig immobilized at the indicated concentrations. (D) T cells were

stimulated with plate-bound anti-CD3 mAb in the presence of Co-Ig or B7-H3-Ig without ($p<0.001$, n=8) or in the presence of anti-CD28 mAb (final concentration of 5 ng/ml; $p<0.01$, n=8). Proliferation was measured on day 3 (B) or on day 4 (A, C, D) of culture. Data show mean ± SD of triplicates from one experiment. *Number of experiments indicated for each panel separately*. Two-tailed Student-t test was used to assess significances.

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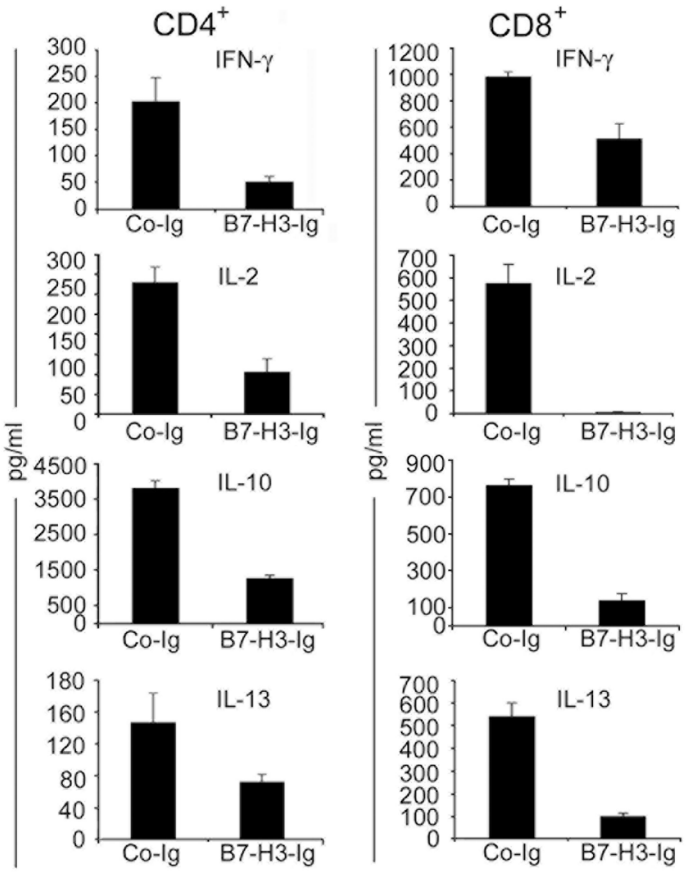

Figure 3. B7-H3 inhibits cytokine production

Human $CD4^+$ and $CD8^+$ T cells were stimulated with plate-bound anti-CD3 mAb in the presence of control-Ig (Co-Ig) or B7-H3-Ig. Culture supernatant was harvested after 72 hours and subjected to multiplex cytokine measurement. Data show mean ± SD of triplicates from one experiment and are representative of three independent experiments.

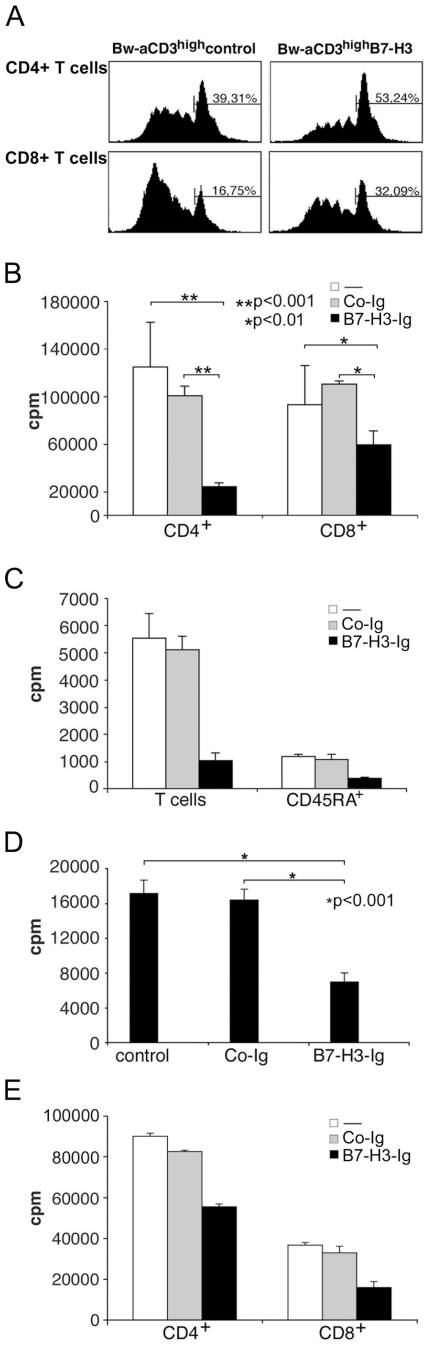

Figure 4. B7-H3 inhibits proliferation of CD4$^+$, CD8$^+$, naïve and pre-activated T cells

(A) CFSE-labelled T cells were co-cultured with Bw-anti-CD3^{high}-control and Bw-anti-CD3^{high}-B7-H3 stimulator cells for 5 days. Cell cycling was analysed by FACS using CD4- and CD8-specific antibodies. The data are representative for four independent experiments. (B) Human CD4$^+$, CD8$^+$ T cells, (C) CD45RA$^+$ human T cells and (D) human umbilical cord blood T cells were incubated with plate-bound anti-CD3 mAb in absence or presence of control-Ig (Co-Ig) or B7-H3-Ig. T cell proliferation was measured on day 4. Differences in proliferation induced in the presence or absence of B7-H3-Ig were statistically significant (B) CD4$^+$ ($p<0.001$, n=9), CD8$^+$ T cells ($p<0.01$, n=9); (D) human umbilical cord blood T cells ($p<0.001$, n=9). (E) CD3/CD28 stimulated CD4$^+$ and CD8$^+$ cells were restimulated

using plate-bound anti-CD3 mAb in presence of Co-Ig and B7-H3-Ig. Proliferation was measured after 48 hours. Data show mean ± SD of triplicates from one experiment and are representative of three independent experiments. (B, C, D) Two-tailed Student-t test was used to assess significances.

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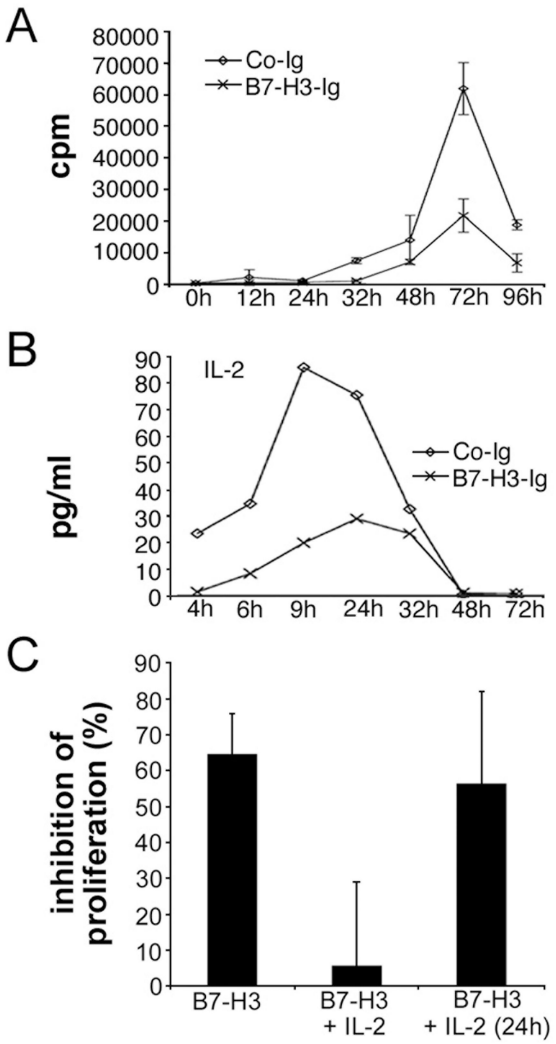

Figure 5. B7-H3 mediated T cell inhibition is characterized by early suppression of IL-2

Human T cells were stimulated with plate-bound anti-CD3 mAb in presence of control-Ig (Co-Ig) or B7-H3-Ig. (A) T cell proliferation was measured at the time points indicated. (B) Culture supernatant was harvested at the time points indicated and its IL-2 content was measured by a Luminex-based assay. Data show mean ± SD of triplicates from one experiment and are representative of three independent experiments. (C) IL-2 (final concentration 50 units) was added to co-culture as indicated. Inhibition (mean ± SD of three independently performed experiments) of T cell proliferation in presence of B7-H3 is shown.

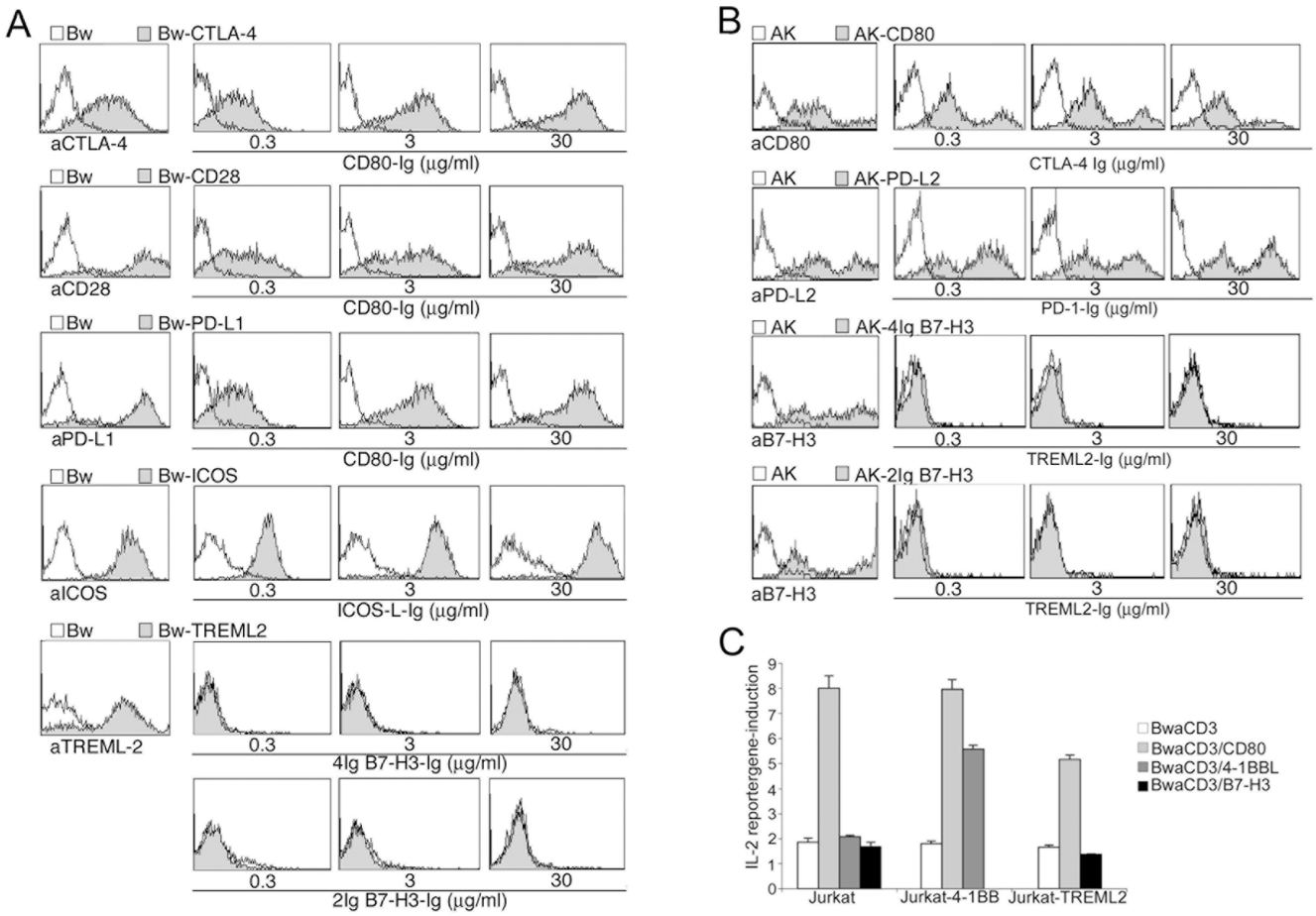

Figure 6. Human TREML2 does not serve as a costimulatory receptor for B7-H3

(A) Fusion proteins representing human B7 family members were analysed for binding to their receptors or TREML2 (grey histograms) and control cells (open histograms). (B) Fusion proteins representing receptors for B7 family members and human TREML2 were analysed for binding to their ligands or B7-H3 (grey histograms) and control cells (open histograms). (A+B) Left panels show expression of indicated molecules. Binding experiments were repeated four times with similar outcome. (C) Non-transduced Jurkat reporter cells and Jurkat reporter cells expressing 4-1BB or TREML2 were co-cultured with T cell stimulators expressing the indicated molecules. Following 6 hours of co-culture IL-2 promotor activity was analysed in a luciferase assay. Results are representative for five independent experiments.

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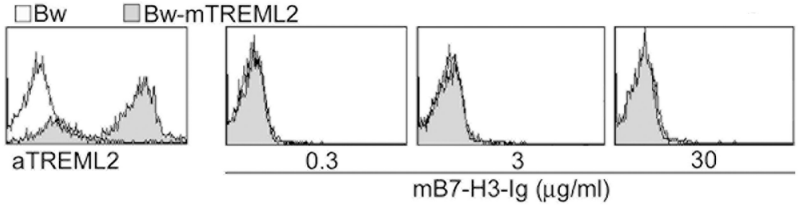

Figure 7. Mouse B7-H3-Ig does not bind to cells expressing TREML2

A fusion protein representing murine B7-H3 was analysed for binding to Bw cells expressing murine TREML2 (Bw-mTREML2, grey histograms) and control cells (Bw, open histograms). Left panel show interaction of αTREML2 antibody with cell lines as indicated. Results are representative for three independent experiments.