DISSERTATION

The role of costimulatory pathways during the activation of human T cells

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Abstract

It is well established that full activation of T cells requires the interaction of the TCR complex with the peptide-MHC complex (Signal 1) and additional signals (Signal 2). These second signals are generated by the interaction of accessory molecules expressed on antigen presenting cells (APC) with their receptors on T cells. Numerous costimulatory and coinhibitory pathways have been described, but many aspects of these pathways are still incompletely understood. By acting as potent regulators of host-protective as well as pathological processes, T cell costimulatory pathways play a pivotal role in immunity and thus are promising therapeutic targets. It is evident that a better understanding of the function of T cell costimulatory molecules is a prerequisite for the development of efficient therapeutic strategies.

Since APC harbor a plethora of stimulating and inhibitory surface molecules, the contribution of individual costimulatory molecules is difficult to assess on these cells. Thus, we have developed a system, called T cell stimulator cells, that can give signal 1 to human T cells via a membrane-bound anti-CD3 antibody fragment. By expressing human costimulatory/coinhibitory ligands on these cells, their role in T cell activation processes can readily be analyzed. Here, we showed that our system of T cell stimulator cells is an excellent tool to evaluate the functional role of ligands implicated in T cell activation processes and to investigate the effect of immunomodulatory drugs on T cell activation.

In detail, we assessed the functional dualism of B7-H3, a recently identified B7 homolog, in human T cell activation, since activating as well as inhibitory roles have been ascribed to this molecule. Based on various experimental conditions we found that B7-H3 potently down-modulated human T cell responses.

Using T cells that received distinct costimulatory signals, we investigated the interplay between costimulation and immunosuppression. We found that CD28 signals, but not costimulation via CD2, 4-1BB, ICOS or LFA-1 greatly increased the IC_{50} (mean inhibitory concentration) for Cyclosporine A. By contrast, the inhibitory effects of azathioprine were not influenced by this T cell costimulatory signals.

Studies on individual costimulatory pathways can complement investigations using experimental systems employing natural human APC or animal studies to get a better insight into the intricate processes that govern human T cell responses.

Structure of this thesis

Chapter 1 provides a brief introduction on the complex theme of T cell activation and the aim of this thesis.

Chapter 2 gives a general overview of receptors and ligands implicated in human T cell costimulatory processes is given. The resulting manuscript was published in *IMLET 2010 Feb 16;128(2):89-97*.

Chapter 3 describes the experimental system we used. To assess the contribution of individual costimulatory and coinhibitory molecules on human T cell activation, we developed a system called T cell stimulator cells. These cells can deliver signal 1 to human T cells via a membrane-bound anti-CD3 antibody fragment. By expressing human costimulatory ligands on these cells, their role in T cell activation processes can readily be analyzed. The resulting manuscript was published in *JIM 2010 Oct 31;362(1-2):131-41*.

Chapter 4 specifically addresses 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. The resulting manuscript was published in *EJI 2009 Jul ;39(7):1754-64*.

Chapter 5 deals with the interplay between costimulation and the immunosuppressive agents cyclosporine A and azathioprine during the *in vitro* activation of human T cells. The resulting manuscript was submitted to *IMLET*.

Chapter 6 outlines the essential points and provides a synopsis of this thesis.

Introduction

It is well established that two signals are required for an efficient T cell activation. Signal 1 is delivered via the MHC-peptide complex to the T cell receptor complex expressed on T cells. The second signal, also called costimulatory signal, is generated by accessory molecules on antigen presenting cells (APC) interacting with their receptors on T cells [1]. Lack of the second signal leads to T cell anergy, a state of unresponsivness to antigenic stimulation.

Besides, these costimulatory signals numerous inhibitory signaling pathways have been described. These negative costimulatory pathways, have important regulatory function such as attenuation of T cell responses, maintenance of peripheral tolerance and termination of immune responses after clearance of infections [2]. Consequently, costimulatory pathways are prime therapeutic targets in diseases that are associated with aberrant T cell responses, such as autoimmune diseases, allergy or organ transplant rejection [3,4]. Moreover, blocking inhibitory signals and enhancing costimulatory pathways is a promising strategy to ameliorate persistent virus infections and to improve anti-tumor responses[5,6].

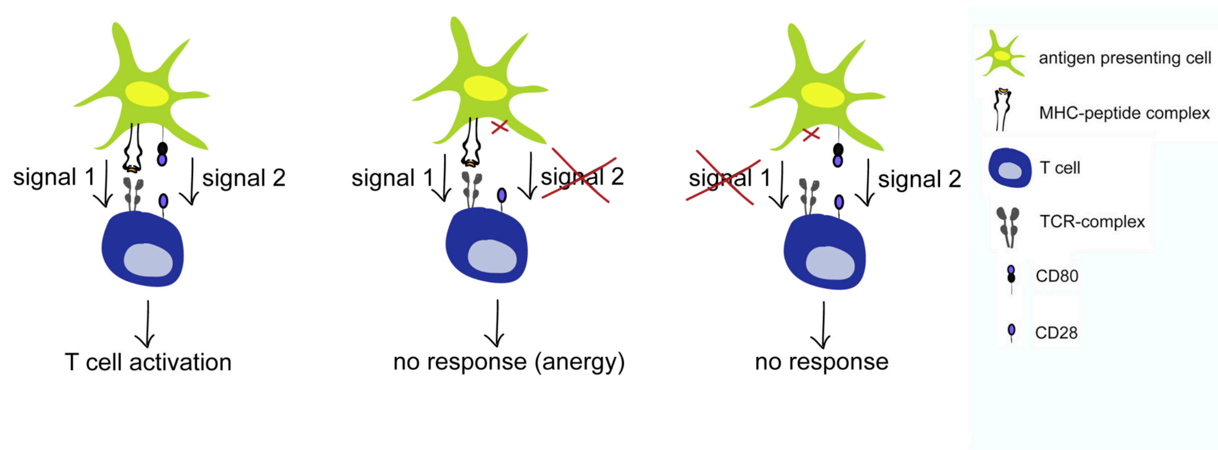

Figure 1: 2-signal hypothesis of T cell activation.

To date, numerous costimulatory and coinhibitory molecules have been described [7]. Among them the CD80/CD86 – CD28/CTLA-4 pathway of the B7/CD28 superfamily is regarded to play the most prominent role in T cell activation [8]. In the last few years additional members of this family have been identified, the so-called B7-homologs [9]. The second major group of T cell costimulatory ligands comprises members of the TNF-superfamily, which interact with their cognate receptors of the TNF-receptor superfamily [7,10,11]. However, a large number of molecules that do not belong to these families have been reported to be involved in the generation of T cell costimulatory and coinhibitory signals. A detailed overview is given in chapter 2 [7].

Since APC harbor a plethora of stimulating and inhibitory surface molecules, the contribution of single molecules to T cell activation processes is difficult to assess on these cells. Furthermore, studies on costimulatory pathways on human cells are hampered by several circumstances. First, studies on individual costimulatory pathways are mostly based on the use of immobilized antibodies. Such antibodies might differ from the natural ligands regarding their binding site and affinity. Secondly, the crosslinking of receptors by immobilized antibodies generates signals that might not exactly reflect the effects of interaction of costimulatory ligands with their receptors. In addition, there are numerous molecules that have been categorized to act costimulatory based solely on their ability to generate a second signal when ligated with antibodies [7]. Recombinant proteins representing the extracellular domains of costimulatory ligands are valuable and widely used tools to study T cell activation processes. However, their generation is time consuming and costly and they might differ from their membrane resident natural counterparts regarding their capability to modulate T cell responses [12]. There was a demand for a cellular experimental system, which can be used to assess the contribution of individual costimulatory and coinhibitory ligands on T cell activation. We have developed such a well defined system, called T cell stimulator cells and described it in detail in chapter 3). Our systems of T cell stimulator cells is an excellent tool to study various aspects of T cell activation, such as evaluation of ligands implicated in T cell costimulation.

For several accessory molecules there is still limited information about their function on T cell regulatory processes or divergent results concerning their functional role in these processes have been described. One of these molecules is B7-H3, a recently described B7-homolog [13]. Human B7-H3 consists of 4 immunglobulin like domains and has about 25% amino acid homology in its extracellular part [14-16]. In initial reports, Chapoval et al. described human B7-H3 as 2 Ig form and to act costimulatory on T cell activation [13]. However, there are several recent reports, which implicate an inhibitory role of B7-H3 in T cell activation [17,18]. These contradictary results could be explained by the existence of two receptors with different functions. To shed some light on the functional role of human B7-H3 we focused on a potential functional dualism of B7-H3 in human T cell activation, as described in detail in chapter 4[19].

T cell responses play a pivotal role in allograft rejection, graft versus host diseases and autoimmune pathologies. In most cases the clinical management of these conditions requires the extensive use of immunosuppressive agents. These drugs limit and down-modulate T cell activation by targeting different cellular processes. Briefly, drugs like Cyclosporine A (CsA), tacrolimus, rapamycine or AEB071 directly interact with T cell signaling pathways [20-22].

By contrast, other agents, such as azathioprine, interfere with DNA-syntheses and thus finally lead to cell cycle arrest [23,24]. Moreover, there is also the possibility of blocking or enhancing coinhibitory or costimulatory pathways, respectively using immunoglobulin fusionproteins or monoclonal antibodies. For instance, several studies showed that monoclonal antibodies to CTLA-4 enhanced antitumor response in melanoma patients [25-27]. Furthermore, several successful clinical trials showed that CD28 costimulation blockade by Belatacept, a CTLA-4 fusionprotein derivative, is an emerging treatment modality to prevent acute rejection and protect renal function in kidney transplant recipients [28-30].

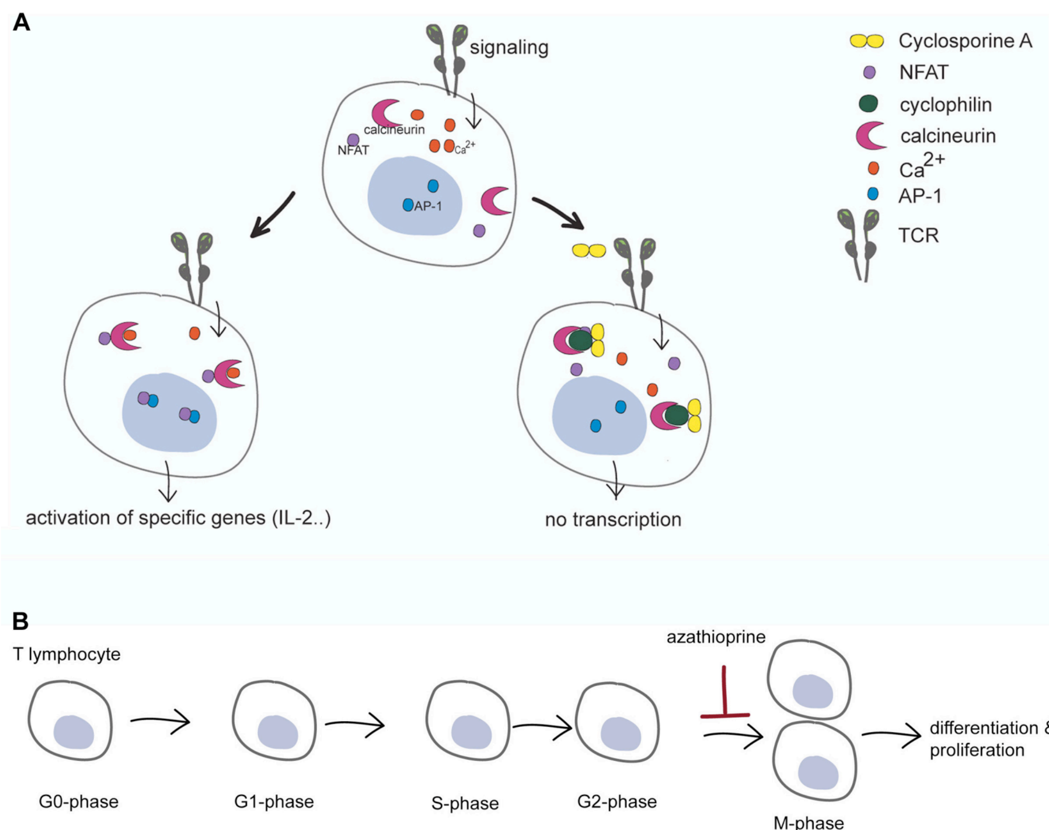

Figure 2: Scheme outlining the influence of Cyclosporine A (A) and azathioprine (B) on T cell activation.

Since many different signals can contribute to T cell activation processes, the interplay between such signals and immunosuppressive agents might have differential effects on the outcome of T cell responses. The interaction of CD80/CD86 with CD28 is generally regarded as the primary and most potent T cell costimulatory pathway[8]. However, there are many alternative costimulatory ligand-receptor pairs that potently enhance the proliferation, differentiation and cytokine production of T cells that recognize antigens [7,9,10]. Among these the CD58 - CD2, 4-1BBL - 4-1BB, ICOS-L - ICOS and CD54 - LFA-1 (CD11a/CD18) pathways are well documented to generate strong and consistent costimulatory effects in human T cells [7,12,31]. Costimulatory receptors belong to different molecule-families.

Consequently, they can induce signaling events that are distinct from the engagement of the CD28 costimulatory pathway. Previous studies have shown that engagement of the CD28 receptor greatly reduces the sensitivity of T cells to the immunosuppressive effect of CsA [32,33]. By contrast, it is not known whether triggering alternative costimulatory receptors has similar effects. Furhtermore, there is limited knowledge how different costimulatory signals affect the immunosuppressive effects of other drugs in clinical use. In chapter 5, we focused on the interplay of the immunpsuppressive drugs, Cyclosporine A and azathiporine, acting via different costimulatory receptors namely, CD28, CD2, 4-1BB, ICOS and LFA-1, during human T cell activation [34].

It is evident that a better understanding of the function of T cell costimulatory molecules is a prerequisite for the development of efficient therapeutic strategies. Studies on individual costimulatory pathways can complement investigations using experimental systems employing natural human APC or animal studies to get a better insight into the intricate processes that govern human T cell responses.

Aim of this thesis

The aim of this thesis was to address the functional role of different costimulatory and coinhibitory pathways in human T cell activation. These pathways play a decisive role in regulating T cell responses. Furthermore, many aspects of these pathways are currently incompletely understood. Moreover, costimulatory and coinhibitory pathways are prime therapeutic targets in diseases that are associated with aberrant T cell responses. Furthermore, enhancing costimulatory receptors or blocking inhibitory pathways might aid the clearence of pathogens and improve tumor immunity.

In this thesis we assessed the functional dualism of B7-H3, a recently identified B7 homolog, in human T cell activation. In addition, the impact of the immunsuppressive drugs, Cyclosporine A and azathioprine, acting via different costimulatory ligands namely, CD80, CD58, 4-1BBL, ICOS-L and CD54, on human T cell activation was analyzed.

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Receptors and ligands implicated in human T cell costimulatory processes

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ABSTRACT

It is well established that full activation of T cells that recognize antigens requires additional signals. These second signals are generated by the interaction of costimulatory ligands expressed on antigen presenting cells with their receptors on T cells. In addition, T cell activation processes are negatively regulated by inhibitory costimulatory pathways. Interaction of members of the B7 and the TNF-superfamilies with members of the CD28 and TNF-R-superfamilies play major roles in costimulatory processes. However, a large number of molecules that do not belong to these families have been reported to be involved in the generation of T cell costimulatory signals. In addition to well-defined costimulatory pathways, where both receptors and ligands are known, there are many T cell surface molecules that have been described to generate a second signal under certain experimental conditions, f. i. when ligated with antibodies. Furthermore, there are several ligands that have been shown to positively or negatively modulate T cell activation by interacting with as of yet unknown T cell receptors. Here, we give a comprehensive overview of molecules that have been implicated in human T cell activation processes and propose criteria that define genuine T cell costimulatory pathways.

key words: T cell, human, costimulation, T cell activation, costimulatory receptors, inhibitory receptors

abbreviations: ADA: adenosin deaminase, ALCAM: activated leukocyte cell adhesion molecule, BTLA: B-and T lymphocyte attenuator, BTNL2: butyrophilin-like 2, DAF: decay accelerating factor, DC-SIGN: dendritic cell-specific ICAM-3-grabbing nonintegrin, DR3: death receptor 3, Eph: erythropoetin-producing heaptocyte, FAP: fibroblast activation protein, GITR: glucocorticoid-induced tumor necrosis factor receptor, GPI: glyco-phosphatidyl-inositol, HCV: Hepatitis C virus, HSPG: heparan sulfat proteoglycane, HVEM: herpes virus entry mediator, IAP: integrin-associated protein, Ig-SF: immunglobulin-superfamily, LAG-3: lymphocyte activation gene, LAMP-3: lysosomal associated membrane proteine 3, LFA: lymphocyte-function associated antigen, LPAM-1: lymphocyte Peyer's patch HEV adhesion molecule, LT: lymphotoxin, MAdCAM: mucosal cell adhesion molecule-1, MCP: membrane cofactor of proteolysis, NKG2D: natural killer group 2 member D, PADGEM: platelet activation dependent granule-external membrane protein, PD-1: programmed death-1, PSG-17: pregnancy-specific glyoprotein 17, PtdSer: phosphatidylserine, SEMA: semaphorin, SIGLEC: sialic acid binding Ig-like lectin, SIRP: signal regulatory protein alpha - possibly also beta and gamma, SLAM: Signaling lymphocytic activation molecule, SRCR: receptors with scavenger receptor cyteine-rich domains, TAPA-1: target of anti-proliferative antibody-

1, TIM: T-cell immunoglobulin domain and mucin domain, TL1A: TNF-like ligand 1A, TM4-SF: transmembrane 4 – superfamily, TM7-SF: transmembrane 7 – superfamily, TNFRSF: tumor necrosis factor receptor superfamily, TNFSF: tumor necrosis factor superfamily, TRAMP: TNF-receptor-related-apoptosis-mediated-protein, TREML2: triggering receptor expressed on myeloid cells like transcript 2, VCAM: vascular cell adhesion molecule, VLA: very late antigen, VSIG4: V-set and immglobulin containing 4

INTRODUCTION

The current interpretation of the two signal hypothesis of T cell activation is a useful approximation of the stimuli that T cells require to achieve unmitigated activation under physiological conditions. It proposes that in addition to the antigen-specific signal that consists of the cognate interaction of the T cell receptor complex with the peptide-MHC complex (Signal 1), T cells depend on additional – costimulatory – signals (Signal 2) to achieve full activation. Although soluble factors like cytokines can also efficiently enhance the activation of T cells, that receive stimuli via their T cell receptor complex, the term costimulation usually describes the modification of T cell activation processes by the interaction of membrane-bound ligands with their T cell expressed receptors.

T cell costimulatory signals are important regulators of host-protective as well as immune-pathological processes. Positive costimulatory signals are mandatory for the initiation of effective immunity and the absence of costimulatory signals results in abortive T cell response and T cell anergy [1]. Negative costimulatory – coinhibitory – pathways afford an additional layer of control that play important regulatory functions thereby contributing to the maintenance of peripheral tolerance as well as to the termination of immune responses after clearance of infections [2]. Consequently costimulatory pathways are attractive therapeutic targets in diseases that are associated with aberrant and harmful immune responses e.g. autoimmune conditions and responses to allergens or organ grafts [3-5]. Furthermore, blocking inhibitory pathways and enhancing stimulating pathways is a promising strategy to ameliorate persistent virus infections and to enhance spontaneous or therapeutically induced immune responses to tumors [6, 7].

Interaction of the B7 molecules B7.1 (CD80) and B7.2 (CD86) with CD28 and CTLA-4, are generally regarded as the primary costimulatory pathways and additional members of the extended B7-family - the so-called B7-homologs - can also convey potent activating or inhibitory signals to T cells. The second major group of T cell costimulatory ligands comprises members of the TNF-superfamily, which interact with their cognate receptors belonging to the TNF-receptor superfamily. Intensive research has focused on

costimulatory pathways involving B7 or TNF family members and results obtained in these studies have been summarized in excellent reviews [8-12].

It is however well-established that there are numerous potent costimulatory pathways involving interaction of molecules that do not belong to these molecule families, and the list of different molecules that have been reported to mediate T cell costimulation is long. Such pathways might play important roles in immune responses especially under conditions where activation of T cells via CD28 the primary costimulatory receptor, is impaired e.g. in CD28 negative CD8 T cells or in patients treated with CTLA-4 fusion proteins.

In addition to receptors expressed on T cells that have been shown to act costimulatory upon interaction with their natural ligands expressed on antigen presenting cells (APC), a large number of T cell molecules have been categorized as costimulatory based solely on their ability to generate a second signal when ligated with antibodies. Although the effects of antibodies targeting such molecules could be therapeutically exploited for the modulation of immune responses, their physiological role as costimulatory receptors remains to be established.

Members of the B7-CD28 superfamily

The B7-CD28 superfamily is the primary group of costimulatory molecules involved in T cell costimulatory and coinhibitory processes.

The B7-CD28 superfamily comprises following receptor/ligand pairs: CD28/CTLA-4:CD80/CD86, ICOS:ICOS-L and PD-1:PD-L1/PD-L2. In addition there are two members of the B7 superfamily for which no human receptors have been identified: B7-H3 and B7-H4 [8]. The CD28/CTLA-4:CD80/CD86 pathway is the best characterized T cell costimulatory pathway. CD80 and CD86 have dual specificities for the stimulatory receptor CD28 and the inhibitory higher affinity receptor CTLA-4 [13]. CD28, which can be regarded as the most potent costimulatory receptor, promotes IL-2 production, activation of naive T cells, T cell survival and entry into the cell cycle. Engagement of CTLA-4, which is upregulated on activated T cells counterbalances the activating effects of CD28 and leads to inhibition of cell cycle progression and IL-2 production. Recently it was shown that CD80 binds also to PD-L1 and that this interaction can down regulate murine T cell responses [14]. Specific interaction between human CD80 and PD-L1 has also been demonstrated [14-16] but to date functional consequences of this interaction have not been reported.

The ICOS-L:ICOS signaling pathway induces little IL-2 but promotes T helper cell differentiation and effector function through production of T_H1, T_H2 and T_H17 cytokines (IL-10, IL-4, IFN-γ, IL-17 and IL-21) [17-21]. Furthermore, ICOS:ICOS-L interaction is crucial

for T cell dependent B cell response, B cell differentiation, germinal center formation and memory B cell development. ICOS engagement leads to upregulation of CD40L, a molecule critically involved in immunoglobulin isotype switching [22, 23].

PD-L1 and PD-L2 deliver a coinhibitory signal via PD-1 to T cells thereby strongly inhibiting T cell proliferation and cytokine production [24-29]. The PD1:PD-L1/2 pathway may also play an inhibitory role in $CD4^{+}CD25^{+}$ regulatory T cells and was shown to contribute to peripheral tolerance and might have a role in preventing autoimmunity [30-34]. PD-L1 bearing tumor cell lines could be associated with increased apoptosis of $CD4^{+}$ and $CD8^{+}$ T cell clones in vitro. However, there are also reports suggesting a costimulatory role for both PD-1 ligands [35-37]**.** Thus the existence of putative second receptors for PD-L1 and PD-L2 with costimulatory function on T cells has been suggested. All data suggesting an activating function of PD-L2 stem from experiments with murine T cells whereas costimulatory effects of human PD-L2 have not been described. In line with this we found that there is no evidence for a stimulatory receptor for PD-L2 on human T cells [38].

B7-H3, another member of the B7 superfamily, was originally reported to function as a T cell costimulator [39], but we and others find that B7-H3 does not enhance T cell activation and plays an important role as coinhibitory molecule [16, 40-42]. Recently TREML2 was reported to serve as a costimulatory receptor for B7-H3 on murine T cells [43]. We have analyzed the interaction of fusion proteins representing human B7-H3 and TREML2 with cells expressing high levels of TREML2 and B7-H3, respectively, and found no evidence for a specific interaction of these molecules [16]. Furthermore, independent experiments performed in two laboratories could also not confirm a role of B7-H3 as a ligand for murine TREML2 [16, 44]. Thus B7-H3 has still to be regarded as an orphan ligand.

Whereas several independent studies on B7-H4 (B7S1; B7x), another member of the extended B7 family report coinhibitory functions for murine B7-H4 [45-47], published data on the function of human B7-H4 are rare. In line with data obtained in mouse studies, Kryczek and coworkers reported that B7-H4 expression identifies a suppressive macrophage population in human ovarian carcinoma and show that ectopic expression of B7-H4 an human monocytes inhibits their T cell stimulatory capacity [48]. In the first report on human B7-H4 it was however found that B7-H4 fusion proteins have a higher capacity to costimulate human T cell activation than fusion proteins representing CD80 [49].

Butyrophilins and butyrophilin-like molecules are distantly related to the B7-family and among these molecules butyrophilin-like 2 (BTNL2) was shown to have a close structural homology to B7-1 (CD80) [50]. Murine BTNL2 was shown to act as an inhibitory

costimulatory ligand [51, 52] and Arnett et al. demonstrated that immobilized BTNL2-fusion proteins also inhibited anti-CD3 induced proliferation of human T cells.

The macrophage complement receptor CRIg (Z39Ig) was recently identified as B7 family related protein and reported to negatively costimulate the activation of human T cells [53].

Cellular receptors that mediate T cell inhibitory effects of B7-H4, BTNL-2 or CRIg on T cells have not been identified to date.

Members of the TNF-R/TNF superfamilies

Members of the TNFR superfamily comprise the second major group of T cell costimulatory receptors. Signals from the large family of TNF-R family members have important roles, many of which are not related to immune functions. There are six receptor-ligand pairs which are generally regarded as being involved in T cell costimulatory processes: 4-1BB/4-1BBL; OX40/OX40L; CD27/CD70; GITR/GITRL; CD30/CD30L and HVEM/LIGHT [9]. Extensive studies support a costimulatory function of 4-1BB, OX40, CD27 but few reports have described such a function for human LIGHT and human CD30L. We have recently compared the capacity of TNF-family members to costimulate human T cells. We found human 4-1BBL to have the most potent T cell costimulatory effects in this group. Furthermore, whereas OX40L, CD27L and GITRL readily costimulated the proliferation and cytokine production of human T cells, CD30L and LIGHT consistently failed to do so [18]. An independent study has demonstrated that 4-1BBL and CD70 but not LIGHT can costimulate cytokine production and effector function of virus specific human $CD8^{+}$T cells [54]. Thus we suggest that CD30 and HVEM might be functionally distinct from the costimulatory members of the TNF-R-family. More recently several studies in murine systems have demonstrated that TL1A is a costimulatory ligand for DR3 (TRAMP) [55-57] and a costimulatory function for human TL1A has been reported as well [55, 58].

The TNFR family member HVEM appears to have a central position in the network of stimulatory and inhibitory interactions: In addition to its role as a receptor for LIGHT and lymphotoxin(LT)-alpha it has been demonstrated to serve as a ligand for two receptors expressed on T cells: the immunoglobulin superfamily members BTLA and CD160, which both have been demonstrated to convey negative signals into human T cells upon engagement [59, 60].

The interaction of DC-expressed TNF-R family member CD40 with CD40L (CD154) on activated CD4 T cells can profoundly enhance T cell responses, since CD40 signals result in the up-regulation of MHC molecules and costimulatory molecules in DC [61]. Thus the

major role of CD40L in T cell costimulation is an indirect one: by inducing T cell costimulatory ligands like CD80 and CD86 on APC. However, several studies report that ligation of CD40L transduces costimulatory signals into human T cells [62, 63].

Members of the CD2 Superfamily

Like the B7- and the CD28 families, the CD2 family of receptors is part of the large immunoglobulin (Ig) superfamily. Activation of the T cell receptor CD2 (LFA-2) via CD58 (LFA-3), another member of the CD2 superfamily, was one of the first T cell costimulatory pathways to be identified [64, 65].

In addition to this interaction, which can transduce very potent costimulatory signals into human T cells, CD48, which also belongs to the CD2 family, has also been reported to costimulate human T cells via CD2 [66]. Recently it was demonstrated that T cell expressed CD48 cooperates with CD2 in the establishment of the TCR signalosome in a human T cell line [67]. Human CD48 has a much lower affinity to CD2 than CD58, whereas murine CD48 is the major ligand for murine CD2. The GPI-linked molecule CD59 has also been described to serve as a costimulatory ligand for CD2 [68] but these findings have been disputed [69].

Signaling lymphocytic activation molecule (SLAM; CD150) is expressed on activated human T cells and ligation of this receptor by antibodies was shown to costimulate anti-CD3 mediated T cell activation resulting in enhanced proliferation and production of IFN-γ [70, 71]. SLAM is a selfligating receptor but this interaction is of very low affinity (200 μM). Human T cell costimulatory effects mediated by SLAM/SLAM interaction have not been reported.

Integrins

Integrins are widely expressed on leukocytes and mainly involved in controlling and maintaining cell-cell as well as cell-extracellular matrix interaction. Integrins are noncovalently linked αβ heterodimers, which are diveded in 3 families defined by their β-subunits, which associate with multiple α-chains. The principal integrins expressed on naïve T cells are LFA-1, αβ2 integrins and VLA-4, -5 and -6. Integrin function is regulated via conformational changes. These changes can be on one hand primarily induced in the cytoplasmatic tail of the integrins e.g. by chemokines or cell differentiation and then transmitted to the extracellular domain, i.e. “inside-out” signaling. One the other hand “outside-in” signaling occurs upon ligand binding and leads to conformational changes that allow for signaling through the cytoplasmic domains [72].

LFA-1 (β2 integrin; CD11a/CD18) is the best known costimulatory member of the integrin family. LFA-1 binds to ICAM-1, -2 and -3 and ligation of LFA-1 with mAbs as well as with

its ligands in context with a signal 1 enhanced T cell proliferation and cytokine production [73, 74]. LFA-1 signaling is especially effective in context of a weak signal 1. LFA-1 is part of the immunological synapse and furthers cell-cell contact thereby allowing prolonged T cell/APC interaction. However, studies in mice have shown that LFA-1 does not only function as enhancer of TCR signaling but activates true costimulatory pathways similar to CD28 [75].

The VLA subfamily is defined by a common β-chain, β1 (CD29). VLA integrins mainly mediate interaction with the extracellular matrix. A costimulatory function has been described for VLA-4 (α4β1 integrin, CD49d/CD29) and VLA-5 (α5β1, CD49e/CD29) when interacting with fibronectin as well as VLA-6 (α6β1, CD49f/CD29) that binds to laminin [76]. VLA-4 has a second, cellular interaction partner VCAM-1 (CD106), interaction with whom leads to enhanced cytokine production and T cell proliferation [77]. VCAM-1/VLA-4 interaction inhibits actin dependent centralization of SLP 76 thereby promoting sustained signaling [78].

Another costimulatory integrin is the α4β7 integrin (LPAM-1), which is especially expressed on gut homing memory T cells and interacts with MAdCAM-1 [79].

Tetraspanins

Tetraspanins are considered to serve as organisator structures for molecular assembly on the cell membrane and to be involved in cell-cell adhesion, cell-cell fusion, signal transduction and activation processes [80]. CD9, CD37, CD63, CD81, CD82 and CD151 have been studied in the context of T cell activation. Although for some of the tetraspanins (e.g. CD9: pregnancy-specific glycoprotein 17; CD81: HCV-E2) interaction partners have been identified, their role in T cell activation has been analyzed by ligation with mAbs or by studying T cell activation in animals deficient for different tetraspans (e.g. CD37) [81]. CD9 has been reported to deliver a costimulatory signal to Jurkat cells [82], however in mice it has been shown to induce T cell activation followed by enhanced apoptosis [83]. An immobilized mAb interacting with CD63 was shown to provide a potent costimulatory signal leading to sustained activation of human T cells [84]. Costimulatory functions have also been shown for CD81 and CD82 in mice and men [85, 86]. For CD81 antibodies a superagonistic potential as well as Th2 polarization capacity have been described [87]. CD37 and CD151 however have been found to play a regulatory role in T cell activation since CD37 and CD151 knock-out mice show hyperresponsive T cells but also hyperstimulatory DC, and human T cells displayed reduced proliferation when stimulated in presence of CD37 mAbs [81, 88].

The TIM family

The T cell immunoglobulin domain and mucin domain (TIM) family, consisting of 3 human members (TIM-1, TIM-3, TIM-4) has been implicated in regulation of autimmunity, allergy and transplantation immunity [89, 90]. TIM-1, the receptor for the hepatitis A virus, is highly polymorphic and certain variants were shown to be associated with protection against allergic asthma. TIM-4 is primarily expressed on APC and has been shown to serve as a ligand for TIM-1 in mice [91]. Depending on the experimental conditions used, TIM-4 was found to costimulate or inhibit the proliferation of murine T cells activated with CD3 and CD28 antibodies. In humans TIM-1 has been shown to potentiate TCR signaling, since overexpression as well as crosslinking TIM-1 by mAbs leads to enhanced cytokine secretion [92]. TIM-3, in contrast, has been originally identified as marker of Th1 cells in mice and ligates to galectin 9. TIM-3 expression is associated with human T cell activation and TIM-3 antibodies have been shown to enhance Th1 and Th17 cytokine secretion but not proliferation of human T cells [93]. Interaction of TIM-4 and galectin 9 with human TIM-1 and TIM-3, respectively, has not been demonstrated to date.

Receptors harboring SRCR domains

CD5 (Leu 1) and CD6 both belong to the superfamily of receptors that harbor scavenger receptor cysteine-rich (SRCR) domains. CD5 is associated with the TCR complex and antibodies to CD5 augment anti-CD3 induced proliferation of human T cells [94, 95]. Cellular ligands for CD5 have not been identified, since a report that CD5 interacts with CD72 could not be substantiated [96, 97].

Antibody engagement of human CD6 enhances T cell proliferation induced via the TCR-complex, and there are numerous studies supporting a role of the CD6-ligand activated leukocyte cell adhesion molecules (ALCAM; CD166) in enhancing T cell responses via CD6 [98-100].

Other molecules implicated to serve as costimulatory receptors

There are numerous additional human molecules that have been described to serve as costimulary T cell receptors (Table 1a). Cellular ligands have currently not been described for many of these molecules and most studies have used immobilized antibodies to demonstrate that these molecules can mediate augmented activation of human T cells that are stimulated via the TCR-complex. Table 1 does not show receptors that have been described to act costimulatory in murine T cells for which such functions have yet to be confirmed in human T cells.

Orphan costimulatory ligands

There are several molecules that have been reported to modulate human T cell responses by interacting with as of yet unidentified receptors on T cells (Table 1b). Identification of T cell-expressed binding partners for such orphan ligands is a prerequisite to better understand their functional role in the activation of T cells.

CONCLUSION

The immune system employs many different receptor-ligand pairs that contribute to the activation of T cells. The complexity of the regulation of T cell responses by activating and inhibitory costimulatory signals underlines the necessity for a tight control of T cell immunity to ensure protective responses to pathogens with limited harm to host tissues. Currently the redundancy among costimulators is incompletely understood but since different costimulatory receptors induce distinct signaling events in T cells, the concert of costimulatory processes might afford a fine tuning of T cell activation processes. A better knowledge of the temporal and spatial expression patterns of human costimulatory molecules and intracellular signaling events triggered by costimulatory receptors will improve our understanding regarding the regulation of human T cell responses *in vivo*.

The advent of transgenic and gene-deleted animals along with the enormous possibilities resulting from in vivo studies of experimentally induced diseases has spawned the interest in mouse immunology. The current emphasis on the murine immune system is demonstrated by the human costimulatory ligand CD58. It is well established that interaction of CD58 with human CD2 results in a potent costimulatory signal [64, 65] and we have found that the T cell costimulatory potency of human CD58 is only surpassed by the B7 molecules (unpublished results). However since CD58 lacks a murine orthologue, CD2 and CD58 are not discussed in most recent reviews on costimulatory pathways. Murine studies have brought valuable insights on the regulation of T cell responses *in vivo*. Since there are many considerable differences between the human and murine immune system it is nevertheless necessary to carefully evaluate data generated in animal studies regarding their impact on humans T cells.

Furthermore, there are large differences between the experimental systems that have been used to demonstrate costimulatory functions on human T cells for the molecules described in table 1. In this context it should be pointed out that for a significant number of T cell molecules that have been described to transduce costimulatory signals upon crosslinking with antibodies no “natural” ligands have been identified to date. We suggest however that the term costimulatory receptor should mainly designate T cell molecules that have been shown to modulate Signal 1 upon interaction with natural ligands. Thus from our point of view, the identification of functional membrane-bound ligands for such molecules is a prerequisite to establish novel costimulatory pathways. It should be stressed that the strong crosslinking of receptors by plate-immobilized antibodies might induce events in T cells that cannot be re-capitulated upon their interaction with natural ligands expressed on the surface of antigen presenting cells. Thus we propose criteria that should be met by receptor-ligand

pairs to qualify them as genuine human T cell costimulators (Box 1) and such molecules are shown in Figure 1.

Box 1:

Criteria for well-established human T cell costimulatory pathways:

1. both receptor and ligand are known
2. the costimulatory functions have been described by independent investigators
3. costimulatory signals can be generated upon interaction of the costimulatory ligand with the receptor
4. the costimulatory ligand is a cell surface molecule
5. the costimulatory receptor can interact with the intracellular signaling machinery – either directly via intracellular signaling motifs or via (*in cis*) interaction partners that harbor such motifs
6. there is good evidence that the functional role of the receptor and ligand lies mainly in the activation of lymphocytes
7. under physiological conditions the receptor signal does only modulate Signal 1 – no significant signaling in absence TCR-complex triggering

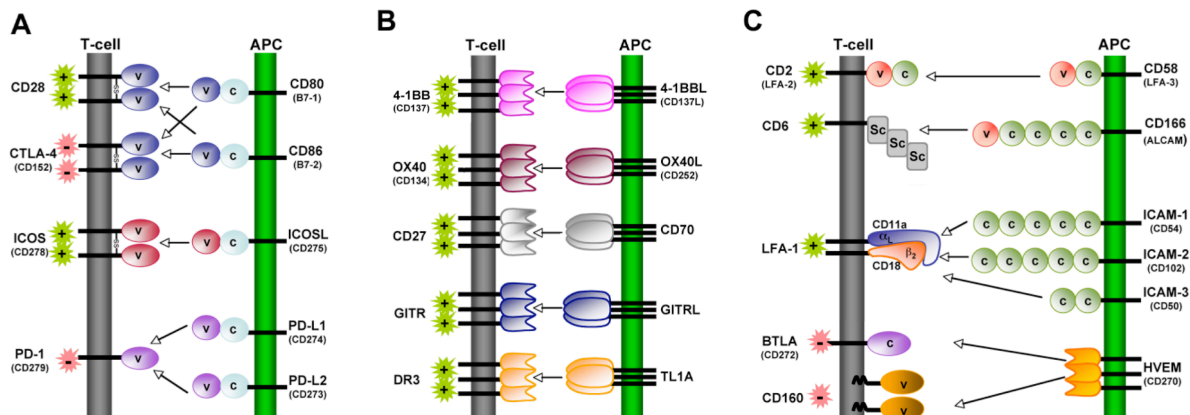

Figure 1: Receptor ligand pairs that are well established to play a role in the costimulation of human T cells. (A) Members of the B7-CD28 superfamily, (B) members of the TNF- TNF-R superfamily and (C) additional costimulatory pathways.

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AUTHORS' CONTRIBUTION

JL made tables and figures and wrote the paper with KG-P and PS.

				positive costimulation			negative costimulation		
Receptor	**Molecule family**	**Ligand**	**Molecule family**	**pos.**	**Shown with natural ligands**	**Refs.**	**neg.**	**Shown with natural ligands**	**Refs.**
CD28	CD28-SF	CD80 (B7.1), CD86 (B7.2)	B7-SF	✓	✓				
CTLA-4 (CD152)	CD28-SF	CD80 (B7.1), CD86 (B7.2)	B7-SF				✓	✓	[8, 13]
ICOS (CD278)	CD28-SF	ICOSL (CD275, B7-H2, B7h, B7RP-1)	B7-SF	✓	✓	[8, 22, 23, 102]			
PD-1 (CD279)	CD28-SF	PD-L1 (CD274, B7-H1), PD-L2 (CD273, B7-DC)	B7-SF				✓	✓	[8, 24, 26-29, 38]
CD27 (TNFRSF7)	TNFR-SF	CD70	TNF-SF	✓	✓	[9, 18, 54, 103-105]			
4-1BB (CD137, TNFRSF9)	TNFR-SF	4-1BBL (CD137L, TNFSF9)	TNF-SF	✓	✓	[9, 18, 54, 106]			
OX40 (CD134, TNFRSF4)	TNFR-SF	OX40L (CD252, TNFSF4)	TNF-SF	✓	✓	[9, 107]			
GITR (TNFRSF18)	TNFR-SF	GITRL (TNFSF18)	TNF-SF	✓	✓	[9]			
CD30 (TNFRSF8)	TNFR-SF	CD30L (CD153, TNFSF8)	TNF-SF	✓	✓	[9, 108]			
HVEM (CD270,TNFRSF14)	TNFR-SF	LIGHT (CD258, TNFSF14)	TNF-SF	✓	✓	[9]			
DR3 (TNFRSF25)	TNFR-SF	TL1A (TNFSF15)	TNF-SF	✓	✓	[55-57]			
LIGHT (CD258, TNFSF14)	TNF-SF	TR6	TNFR-SF	✓	✓	[109]			
CD154 (CD40L, TNFSF5)	TNF-SF	CD40 (TNFRSF5)	TNFR-SF	✓	✓	[62, 63]			
CD2 (LFA-2)	CD2-SF	CD58, CD48 (LFA-3)	CD2-SF	✓	✓	[64-67]			
CD150 (SLAM)	CD2-SF	CD150 (SLAM)	CD2-SF	✓		[70, 71]			
LFA-1 (CD11a/CD18)	IntegrinSF	CD54 (ICAM1), CD102 (ICAM2), CD50 (ICAM3)	Ig-SF	✓	✓	[73-75]			
VLA-4 (CD29/CD49d)	IntegrinSF	VCAM-1 (CD106), fibronectin	Ig-SF	✓	✓	[76-78]			
VLA-5 (CD29/CD49e)	IntegrinSF	fibronectin		✓	✓	[76]			
VLA-6 (CD29/CD49f)	IntegrinSF	laminin, invasin, merosin		✓	✓	[76]			
LPAM-1 (a4b7-integrin)	IntegrinSF	MAdCAM-1	Ig-SF	✓	✓	[79]			
CD9 (Tspan-29, MRP-1, p24)	TM4-SF	PSG-17*		✓		[82, 110]			
CD37	TM4-SF						✓		[81, 88]
CD63 (LAMP-3)	TM4-SF			✓		[84]			
CD81 (TAPA-1)	TM4-SF	HCV-E2		✓		[85, 87]			
CD82 (R2, C33)	TM4-SF			✓		[86]			
TIM-1 (HAVCR1, KIM-1)	TIM	TIM-1, TIM-4, IgAl, PtdSer		✓		[89, 90, 92]			
TIM-3	TIM	galectin 9*				[93, 111]	✓		
CD5 (Ly1, Leu-1)	SCRC-domain receptor	CD72 (Lyb-2)	c-type lectin	✓		[94-97]			
CD6 (T12)	SCRC-domain receptor	CD166 (ALCAM)	Ig-SF	✓		[99, 100]			
CD7 (gp40, Tp41)	Ig-SF			✓		[112-114]	✓		[115, 116]
CD47 (IAP)	Ig-SF	SIRP (CD172), Thrombospondin 1		✓		[117]			
CD50 (ICAM-3)	Ig-SF	LFA-1, DC-SIGN (CD209)	c-type lectin	✓		[118]			
CD54 (ICAM-1)	Ig-Sf	LFA-1	Integrin-SF	✓		[119]			
CD147 (M6, EMMPRIN, Basigin)	Ig-SF						✓		[120, 121]
CD160 (BY55, NK1, NK28)	Ig-SF	HVEM (CD270, TNFRSF14)	TNFR-SF				✓	✓	[60]
BTLA (CD272)	Ig-SF	HVEM (CD270, TNFRSF14)	TNFR-SF				✓	✓	[59]
LAG-3 (CD223)	Ig-SF	MHC-class II	MHC				✓	✓	[122, 123]
CD26 (dipeptidly peptidase IV)	polyoligo peptidase family	Caveolin 1, ADA, fibronectin, collagen, FAP, HIV tat protein		✓	✓	[124-127]			
CD38 (ADP-ribosyl cyclase)		CD31	Ig-SF	✓		[128-130]			
CD43 (Leukosialin, Sialophorin)	cell surface mucin family	Siglec1 (CD169, Sialoadhesin), CD54 (ICAM-1), MHC-I, P-selectin (CD62P, PADGEM)		✓		[131]			
CD46 (MCP)	RCA-family	C3b, C4b, pathogens		✓		[132, 133]			
CD55 (DAF)	RCA-family	CD97	TM7-SF	✓	✓	[134]			
CD52 (Campath-1)				✓		[135]			
CD59	Ly-6-SF			✓		[136]			
CD73 (L-VAP-2)	GPI-linked 5'-nucleotidase			✓		[137-139]			
CD99 (MIC2, E2)	mucin	CD99 (MIC2, E2)	mucin	✓		[140, 141]			
CD100 (SEMA4D)	semaphorin family	CD72	c-type lectin	✓		[142]	✓		[142]
CD101 (V7)	Ig-SF			✓		[143]	✓		[143, 144]
EphB6	Eph kinase	EphrinB2	Ephrins	✓	✓	[145]			
NKG2D	c-type lectin	MIC-A	MHC-like	✓	✓	[146]			
Syndecan-2	HSPG	EM, cytokines					✓		[147]
Syndecan-4	HSPG	EM, cytokines					✓		[147]

Table 1a: Human T cell receptors that have been reported to play a role in T cell costimulatory processes. *) only shown in mice

		positive costimulation		negative costimulation	
Ligand	**Molecule family**	**pos.**	**Refs.**	**neg.**	**Refs.**
B7-H3 (CD276)	B7-SF	✓	[39]	✓	[16, 40, 41]
B7-H4 (B7x, B7S1)	B7-SF	✓	[49]	✓	[48]
Z39Ig (VSIG4)	Ig-SF			✓	[53]
CD31 (PECAM-1)	Ig-SF			✓	[148, 149]
CD83 (HB15)	Ig-SF	✓	[150]		
BTNL2	butyrophilin			✓	[51, 52]
Siglec1 (CD169)	SIGLECs			✓	[151]

Table 1b: Orphan human ligands implicated to play a role in T cell costimulatory processes.

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T cell stimulator cells, an efficient and versatile cellular system to assess the role of costimulatory ligands in the activation of human T cells

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ABSTRACT

It is well established that full activation of T cells requires the interaction of the TCR complex with the peptide-MHC complex (Signal 1) and additional signals (Signal 2). These second signals are generated by the interaction of costimulatory ligands expressed on antigen presenting cells with activating receptors on T cells. In addition, T cell responses are negatively regulated by inhibitory costimulatory pathways. Since professional antigen presenting cells (APC) harbor a plethora of stimulating and inhibitory surface molecules, the contribution of individual costimulatory molecules is difficult to assess on these cells. We have developed a system of stimulator cells that can give signal 1 to human T cells via a membrane-bound anti-CD3 antibody fragment. By expressing human costimulatory ligands on these cells, their role in T cell activation processes can readily be analyzed. We demonstrate that T cell stimulator cells are excellent tools to study various aspects of human T cell costimulation, including the effects of immunomodulatory drugs or how costimulatory signals contribute to the *in vitro* expansion of T cells. T cell stimulator cells are especially suited for the functional evaluation of ligands that are implicated in costimulatory processes. In this study we have evaluated the role of the CD2 family member CD150 (SLAM) and the TNF family member TL1A (TNFSF15) in the activation of human T cells. Whereas our results do not point to a significant role of CD150 in T cell activation we found TL1A to potently costimulate human T cells. Taken together our results demonstrate that T cell stimulator cells are excellent tools to study various aspects of costimulatory processes.

key words: T cell activation, costimulation, TL1A, CD150

INTRODUCTION

The two signal hypothesis of lymphocyte activation proposes that T cells that receive Signal 1 via their T cell receptor (TCR) complex depend on concomitant triggering of costimulatory receptors to achieve full activation [1, 2]. T cell activation is also modulated by inhibitory costimulatory receptors that are able to attenuate TCR-signals. By acting as potent regulators of host-protective as well as pathological processes, T cell costimulatory pathways play a pivotal role in immunity [3-5]. Consequently, such pathways are prime therapeutic targets in diseases that are associated with aberrant T cell responses [6, 7]. Likewise, tumor patients or individuals suffering from chronic viral infection might benefit from therapies that enhance costimulatory pathways or block inhibitory receptors [8]. In this context it is evident that a more complete understanding regarding the function of human T cell costimulatory molecules is a prerequisite for the development of efficient therapeutic strategies.

Studies on costimulatory pathways on human cells are hampered by several circumstances. Antigen presenting cells (APC) harbour a plethora of activating and inhibitory ligands with overlapping and redundant functions, which complicate the assessment of the contribution of single molecules to T cell activation processes. Studies on individual costimulatory pathways often rely on the use of immobilized antibodies. Such antibodies might differ from the natural ligands regarding their binding site and affinity. Furthermore, the crosslinking of receptors by immobilized antibodies generates signals that might not accurately reflect the effects of interaction of costimulatory ligands with their receptors. However, there are numerous molecules that have been categorized as costimulatory based solely on their ability to generate a second signal when ligated with antibodies [9]. Recombinant proteins representing the extracellular domains of costimulatory ligands are valuable and widely used tools to study T cell activation processes. However, their generation is time consuming and costly and they might differ from their membrane resident natural counterparts regarding their capability to modulate T cell responses.

We have developed a simple cellular system to assess the role of costimulatory ligands in the activation of human T cells. This system, which we have designated T cell stimulator cells, is based on the murine thymoma cell line Bw5417 that expresses membrane-bound anti-human CD3 single chain antibody fragments at high or low densities. Upon retroviral expression of human costimulatory ligands on these cells their contribution to the activation of human T cells can readily be determined. In this study we describe this system in detail and demonstrate that T cell stimulator cells are an efficient and versatile tool to study various aspects of human T cell costimulatory processes.

MATERIAL AND METHODS

Antibodies, cell culture and FACS staining

293T cells and the mouse thymoma cell line Bw5147 (short designation within this work Bw) were cultured as described [10, 11]. 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 (Miltenyi Biotech, Bergisch Gladbach, Germany). The mAbs to CD11b (VIM12), CD14 (VIM13), CD33 (4D3), MHC-class II (1/47), CD80 (7-480), CD58 (1-456) 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), CD19 mAb (BU12) from Ancell (Bayport, MN), 41BB-L and CD150/SLAM (A12) from Biolegend (San Diego, CA). Goat anti-human TL1A/TNFSF15 antibodies were obtained from R&D (Minneapolis, MN). FACS analysis was performed as described previously [10]. Briefly, binding of primary antibodies was detected with PE-conjugated goat anti-mouse IgG-Fcγ specific Abs or donkey anti-goat IgG (H+L) (both Jackson ImmunoResearch, West Grove, PA). Expression of membrane-bound anti-CD3 antibody fragment was detected via APC-conjugated goat-anti-mouse IgG (H+L) Abs, which reacts with the variable regions of murine antibodies (Jackson ImmunoResearch). Fluorescence intensity is shown on a standard logarithmic scale.

Double immunflourescence of T cell and stimulator cell co-cultures

Human T cells were CFSE-labeled as described in detail [12]. Irradiated T cell stimulator cells ($2x10^6$/ml) were incubated with 0,5 μM working solution of CellTracker™ Orange CMTMR (5-and 6 (4-chloromethyl-benzoyl-amino-tetramethylrhodamine) mixed isomers for 30 minutes at 37°C in a CO_2 incubator. The reaction was stopped by washing once with pre-warmed medium. For double-immunflourescence CMTMR-labeled stimulatorcells ($8x10^4$/well) and CFSE-labeled T cells ($4x10^5$/well) were co-cultured in a 24-well cell culture plate in phenolred-free cell culture medium for 24h or 48h. To visualize the stimulator cell – T cell interaction at a higher magnification, cells were co-cultured for 24h, fixed in 4% paraformaldehyde and washed once with medium. Subsequently, cells were analyzed by laser scanning microscopy (LSM 410, ZEISS) [13]. CellTrace™ CFSE and CellTracker™ Orange CMTMR were both purchased from Molecular Probes (Eugene, OR).

Generation of expression constructs encoding membrane-bound anti-CD3 single chain fragments

cDNA derived from hybridoma cells producing the anti-human CD3 antibody OKT3 (ATCC, Manassas, VA) was subjected to PCR amplification using primer pairs specific for the variable regions of the heavy chain (VH-for 5' GGAATTCGCTAGCCCAGGTCCAGCTGCAGCAGTCT 3' , VH-rev 5' GGGGATCCGGTGACCGTGGTGCCTTGGCCCCAGTA 3') and light chain (VL-for 5 GGAATTCGAGCTCCCAAATTGTTCTCACCCAGTCTCCA 3' and VL-rev 5 GGGATCCCCACCGCCCCGGTTTATTTCCAACTTTGT 3'). The resulting PCR products were digested with Nhe I plus BstE II (V_H) and Sac I plus BamH I (V_L) and joined via a Sac I to BstE II fragment encoding a $(G_4S)_3$-linker by ligation. Two distinct DNA-fragments were generated by employing additional PCR and ligation steps: CD5L-OKT3scFv-CD28 encoded the OKT3-single chain antibody fragment flanked by the CD5 leader sequence and a BamH I to Not I fragment encoding the transmembrane and intracellular domains of human CD28, which was amplified using the primer pair (5' CGCGGGGGATCCCCCAAGTCCCCTATTTCCCGG 3' and 5' GCGCCCGCGGCCGCTTTAGGAGCGATAGGCTGCGAAGT 3'), whereas CD5L-OKT3-CD14 encoded the OKT3-single chain antibody fragment flanked by the CD5 leader peptide and the leaderless human CD14 molecule generated by fusing a CD14 BamH I to Nhe I fragment, which was amplified using the primer pair (5' CGCGGGGGATCCCACCACGCCAGAACCTTGTGA 3' and 5' CCTTGAGGCGGGAGTACGCT 3') to the Nhe I to Not I fragment of CD14 cDNA. Both constructs were cloned into the retroviral expression vector pMMP and the integrity of the synthetic expression constructs was confirmed by DNA-sequence analysis.

The nucleotide sequences encoding the surface expressed anti-CD3 antibody fragments have been submitted to GenBank accession ns. **HM208751** – CD5L-OKT3-scFv-CD28 (protein_id ADN42858) and **HM208750** – CD5L-OKT3-scFv-CD14 (protein_id ADN42857).

Generation of T cell stimulators

Bw5147 cells were retrovirally transduced to express the CD5L-OKT3-scFv-CD28 or the CD5L-OKT3-scFv-CD14 constructs. Tranduction with the OKT3::CD28 yielded Bw5147 cells expressing anti-CD3 antibodies at low density; the Bw-anti-CD3^{low} stimulator cells. Transduction with the OKT3::CD14 construct resulted in Bw5147 cells expressing high levels of membrane-bound anti-CD3 antibody-fragment on their surface and were thus termed Bw-anti-CD3^{high} stimulator cells. Single cell clones were obtained from both Bw lines and cell clones expressing homogenous amounts of membrane-bound anti-CD3 antibodies were

selected for further use. cDNAs encoding human CD80, CD58, CD54, CD150, TL1A, 41BB-L and ICOS-L were PCR amplified from a human dendritic cell library and cloned into the retroviral expression vector pCJK2 generated in our laboratory. Integrity of these expression plasmids was confirmed by DNA sequencing. Using retroviral transduction these molecules were expressed on the T cell stimulator cells as described [14]. Control stimulator cell lines expressing no human molecule were generated by treating T cell stimulator cells with supernatants derived from retroviral producer cell lines transfected with empty vector DNA or a vector encoding GFP.

T cell proliferation assays

All T cell proliferation assays were done in triplicates, means and SD are shown. For T cell proliferation assays human T cells ($1x10^5$/well) were co-cultured with irradiated (6000 rad) T cell stimulator cells ($2x10^4$/well) for 72 hours. In some experiments Adalimumab (Humira, Abbott Laboratories, Chicago, IL) or Beriglobin P as control (CSL Behring GmbH, Marburg, Germany), was added at a final concentration of 10 μg/ml at the onset of culture. To assess T cell proliferation methyl-^{3}[H]-thymidine (final concentration: 0.025 mCi; Perkin Elmer/New England Nuclear Coorporation, Wellesley, MA) was added for the last 18 hours prior harvesting of the cells. Methyl-^{3}[H]-thymidine uptake was measured as described [15].

In vitro expansion of human T cells

Purified human T cells ($5x10^5$/well) were co-cultured in 1 ml medium with $1.2x10^5$ irradiated anti-CD3^{high} T cell stimulator cells expressing human costimulatory molecules as indicated. Following 7 days of culture, T cells were harvested, counted and analyzed for CD8$^+$ expression. $5x10^5$ T cells were re-cultured with $1.2x10^5$ irradiated stimulator cells as described above. Five rounds of stimulation were performed. For each round of stimulation the T cell expansion factor was calculated by dividing the starting cell number by the cell number obtained after 7 days of stimulation.

Cellular cytotoxicity assay

Cytotoxic activity of expanded T cells was measured using a europium release assay kit (Delfia, Perkin Elmer) following the manufacturer's protocol. Briefly, expanded T cells ($1x10^5$/well) were incubated with the labelled target cells ($5x10^3$/well; Bw-anti-CD3^{high} cells or Bw cells not expressing anti-CD3 as control) for 2 hours at 37°C. For detection of cell lysis-associated europium release 20 μl of supernatant was transferred to a 96-well flat bottom plate and 200 μl enhancement solution was added. Fluorescence was measured using a time-resolved fluorometer (Victor; Perkin Elmer). The percentage of specific cytotoxicity was

calculated as described using the formula: (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100 [16].

Cytokine measurement

For cytokine measurement supernantants of T cell proliferation assays were collected after 48h and pooled from triplicate wells. IFN-γ, IL-10 and IL-13 were measured in the supernatants using the Luminex System 100 (Luminex, Texas, USA).

Statistics

Two-tailed Student-t test was used to assess significance. IMB® SPSS statistics software was used for Box plot and for analysis of variance (ANOVA) in Figure2.

RESULTS

Generation of T cell stimulator cells

mAbs that trigger the T cell receptor complex by interacting with CD3 molecules are widely used to study the activation of T cells. We aimed to establish a cellular system that can give "Signal 1" to human T cells. In a first step we generated synthetic retroviral expression constructs that encode a CD5 leader peptide and a single chain antibody fragment of the anti-human CD3 antibody OKT3 fused to DNA sequences encoding the transmembrane and intracellular domains of human CD28 (CD5L-OKT3-scFv-CD28) or the leaderless human CD14 (CD5L-OKT3-scFv-CD14) molecule (Fig. 1a). These constructs were expressed on the murine thymoma line Bw5147. Their expression was assessed by flow cytometry using an anti-mouse IgG antibody that reacts with the variable regions of the anti-CD3 antibody. Whereas Bw cells expressing the CD5L-OKT3-scFv-CD14 construct displayed high levels of membrane-bound OKT3 antibody fragment on their surface (Bw-aCD3^{high}), the CD5L-OKT3-scFv-CD28 molecule was expressed at much lower density (Bw-aCD3^{low}; Fig. 1a). Single cell clones that expressed homogeneous levels of membrane-bound anti-CD3 were established from both cell lines. Subsequently, both T cell stimulator cell lines were transduced to express human CD80 (Bw-aCD3^{high}-CD80; Bw-aCD3^{low}-CD80) or treated to express empty retroviral vector (Bw-aCD3^{high}-control, Bw-aCD3^{low}-control; Fig. 1b). In order to assess the T cell stimulatory capacity of these cell lines they were irradiated and co-cultured with purified human T cells. We found that T cell stimulator cells expressing low amounts of membrane-bound anti-CD3 antibody (mb-aCD3) and no human costimulatory molecules did not induce significant proliferation of purified human T cells. The low levels of cellular ^{3}[H]-thymidine incorporation that was measured in these co-cultures is the result of residual uptake by the irradiated T cell stimulator cells since similar incorporation was observed in cultures of irradiated T cell stimulator cells where no human T cells were present. This indicates that the murine thymoma line Bw5147 that was used for the generation of our T cell stimulator cells does not harbour accessory molecules that can costimulate human T cells. By contrast T cell stimulator cells that co-express low levels of anti-CD3 antibody fragments and human CD80 elicited strong proliferative responses in human T cells. T cell stimulator cells expressing membrane-bound anti-CD3 antibodies at high density induced moderate proliferation in human T cells even in the absence of human costimulatory molecules and as expected T cells activated with stimulator cells harbouring high levels of anti-CD3 in combination with human CD80 showed the highest proliferative response (Fig. 1c). To visualize the interaction of human T cells and stimulator cells, we performed co-culture experiments using CFSE-labeled T cells and CMTMR-labeled stimulator cells. Large clusters of T cells and stimulator cells

expressing CD80 can be observed whereas much smaller clusters are formed when T cells were activated by stimulator cells expressing anti-CD3 but no human costimulatory molecule (Fig. 1d).

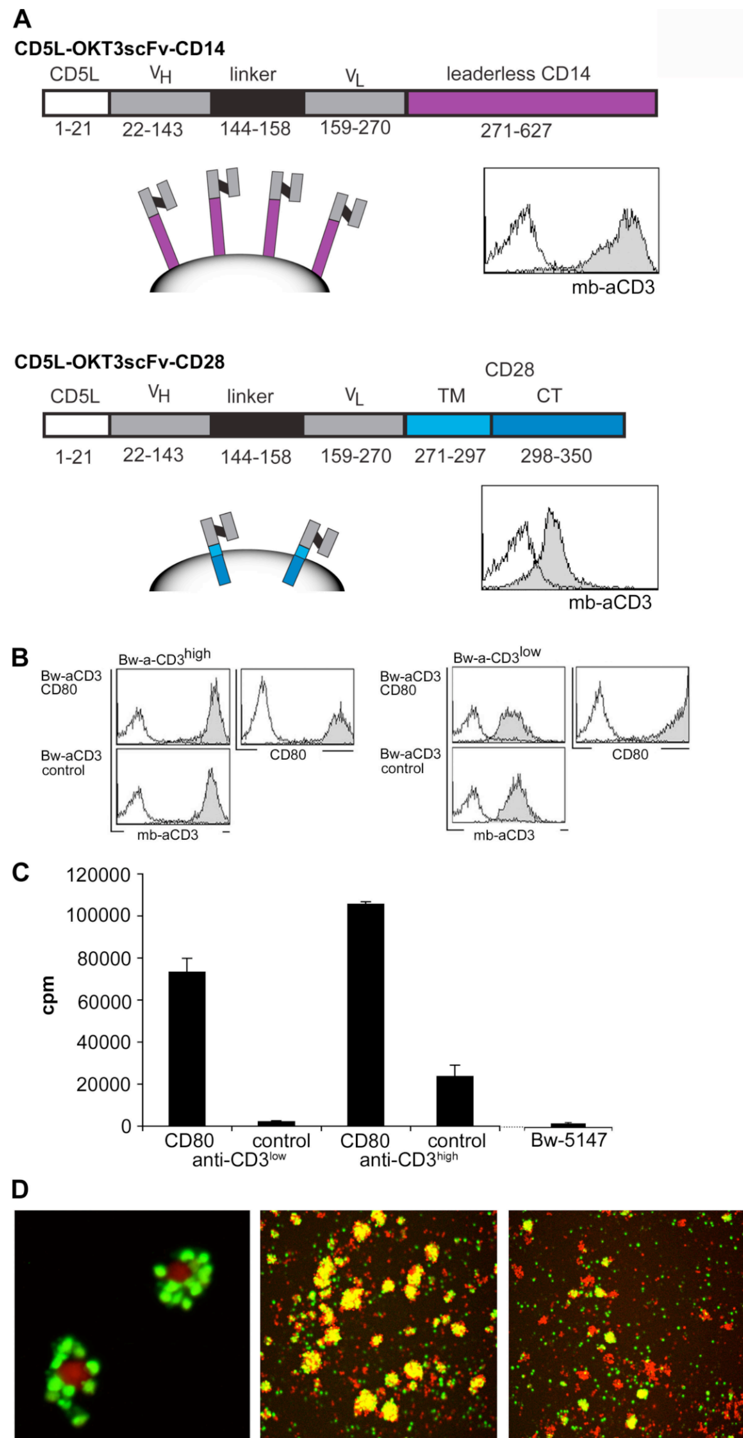

Figure 1: Generation and characterisation of T cell stimulator cells.
(A) Schemes of expression constructs encoding membrane-bound anti-CD3 (mb-aCD3) single chain fragments. FACS analysis of Bw cells expressing the constructs are shown on the right. Open histograms: control cells; filled histograms: Bw cells expressing membrane-bound anti-CD3 single chain fragment. CD5L: CD5 leader; V_H: variable domain of the heavy chain; V_L: variable domain of the light chain; TM: transmembrane domain; CT: cytoplasmic domain.
(B) Generation of T cell stimulator cells co-expressing anti-CD3 at high and low levels (Bw- anti-CD3^{high} and Bw-anti-CD3^{low}) and the costimulatory molecule CD80. Open histograms: control transduced cells; filled histograms: stimulator cells expressing CD80 or mock treated stimulator cells (control).
(C) Human T cells were stimulated with T cell stimulator cells expressing anti-CD3^{high} and anti-CD3^{low} with and without CD80. ^{3}[H]-thymidine uptake of irradiated Bw5147 cells in the absence of T cells is also shown (far right).
(D) Confocal microscopy of CFSE-labeled T cells (green) co-cultured with CMTMR-labeled T-cell stimulator cells (red). Interaction of T cells and T cell stimulator cells (left). Cluster formation between T cells and stimulator cells expressing CD80 (middle) or stimulator cells expressing no costimulatory molecule (right).

Side by side comparison of different costimulatory molecules

T cell stimulator cells transduced to express different costimulatory molecules are excellent tools to compare these ligands regarding their capacity to activate human T cells. We have generated stimulator cell lines retrovirally expressing different costimulatory molecules at high levels (Fig. 2). The resultant cell lines were used to stimulate purified T cells isolated from different healthy donors and T cell proliferation was assessed. As shown in Figure 2b stimulation of human T cells in presence of the costimulatory molecules used in this study (CD80, ICOSL, CD58, CD54 and 4-1BBL) significantly enhanced T cell proliferation compared to T cells co-cultured with stimulator cells expressing no human costimulatory molecule. Furthermore, our data show that CD80 was the strongest costimulatory ligand tested in these experiments and demonstrate that among the other molecules analyzed CD58 is the most potent inducer of T cell proliferation.

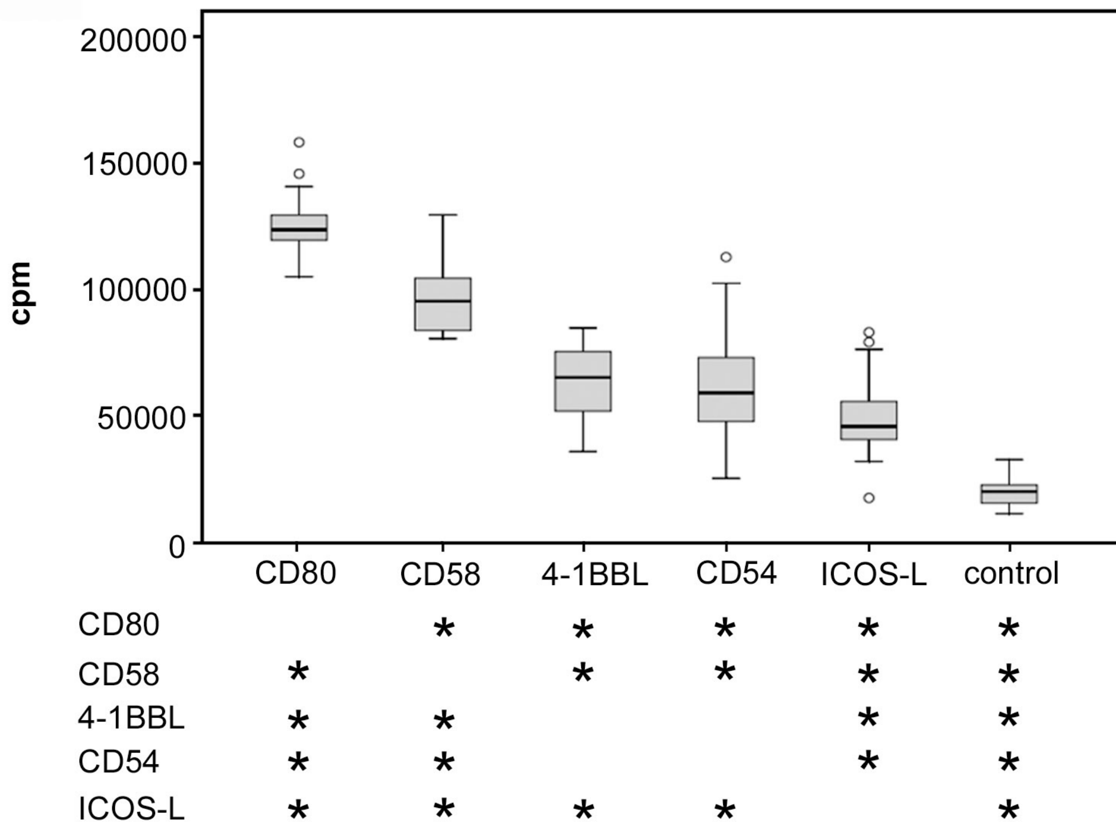

Figure 2: Comparison of different costimulatory ligands. T cell stimulator lines expressing high levels of anti-CD3 antibodies and the indicated costimulatory ligands or no costimulatory molecule (control) were co-cultured with purified human T cells. ^{3}[H]-thymidine uptake was assessed following 3 days of co-culture. Box plots show the results of 8 independent experiments with T cells from different donors. Circles indicate outliers. Stars indicate significant difference ($p<0.05$, $n=8$) from the stimulator cell type listed on the left.

The use of T cell stimulator cells to assess the role of immunomodulatory drugs in T cell activation

There is an increasing number of immunosuppressive and immunomodulatory drugs for treatment of patients suffering from autoimmune diseases and recipients of hematopoietic stem cells or solid organs. Many of these drugs target fast dividing cells whereas others specifically suppress T cells or counteract inflammatory processes. Antibodies or receptor fusion proteins that block the cytokine TNF-α are successfully used in patients suffering from psoriasis, rheumatoid arthritis and various other autoimmune diseases [17-19]. TNF-α is a pleiotrophic cytokine and the beneficial effects of TNF-α blockade are mainly ascribed to their capacity to prevent and down-modulate proinflammatory processes. Whereas other members of the TNF-family have been shown to act as potent costimulatory molecules, few studies have addressed the ability of TNF-α to directly contribute to T cell activation processes. We found that expressing TNF-α on T cell stimulator cells enhances their ability to induce proliferation in purified human T cells (Fig. 3a). Furthermore, we show that Adalimumab, a humanized therapeutic antibody that targets TNF-α, reduced proliferation of T cells activated via different costimulatory molecules (Fig. 3b). Taken together these results indicate that TNF-α gives a costimulatory signal to human T cells and that TNF-α blockade reduces human T cell responses independently of accessory cells.

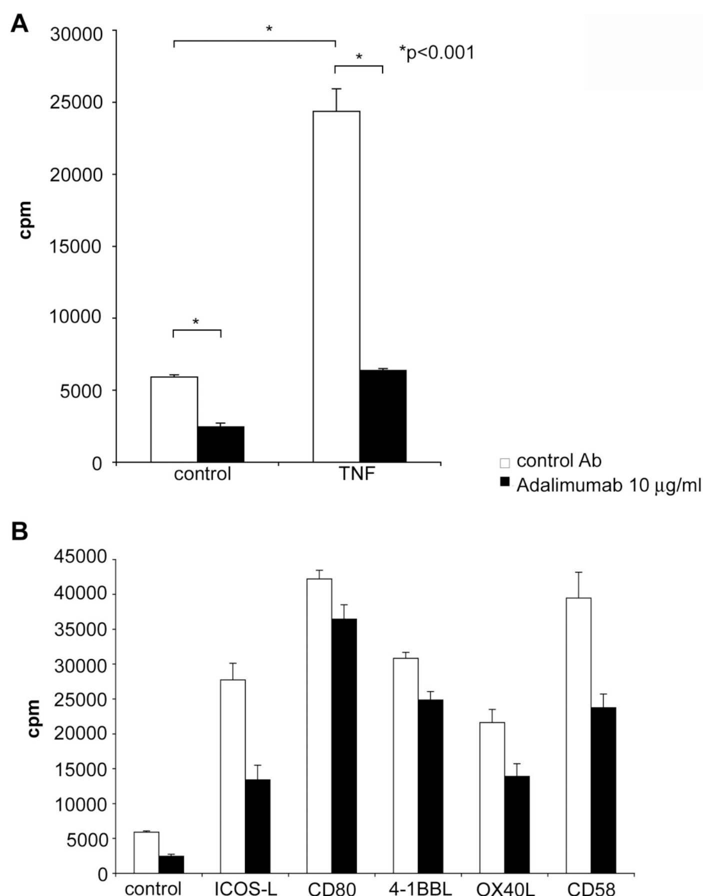

Figure 3: The therapeutic TNF-α blocking antibody Adalimumab down-modulates human T cell responses. (A) T cell stimulator cells expressing anti-CD3 antibodies at high levels (control) and T cell stimulator cells expressing anti-CD3 antibodies and human TNF-α were used to stimulate purified human T cells in the presence of control antibodies (control Ab) or the therapeutic anti-TNF-α antibody Adalimumab. (B) T cell stimulator cells expressing the indicated costimulatory molecules were used to stimulate purified human T cells in the presence of control antibodies or Adalimumab (10 μg/ml). Data shown are representative for at least 5 independently performed experiments.

The use of T cell stimulator cells to assess the role of different costimulatory molecules in the in vitro expansion of human T cells

Adoptive T cell transfer is a promising therapeutic strategy in the treatment of malignancies, and to combat virus infections [20-22]. Such approaches often depend on the efficient *in vitro* expansion of antigen specific T cells. We used T cell stimulator cells expressing individual costimulatory molecules or combinations thereof to assess their capacity to expand human T cells *in vitro*. In line with previous data we found that 4-1BB signals enhance the expansion of T cells costimulated via CD28 [23]. Furthermore, our results demonstrate that costimulation via CD2 can also potently increase the expansion of human T cells. Stimulator cells co-expressing CD80, CD58 and 4-1BBL induced significantly stronger T cell expansion compared to stimulator cells not expressing CD80. This underlines the importance of CD28 signals and suggests that the combination of CD80, CD58 and 4-1BBL might be especially suited for the expansion of human T cells (Fig. 4). Importantly, we found that during 5 rounds of stimulation in the presence of these costimulatory ligands their effector function was retained as the expanded T cells were able to efficiently kill target cells expressing anti-CD3 antibodies as surrogate antigen (Fig. 4d).

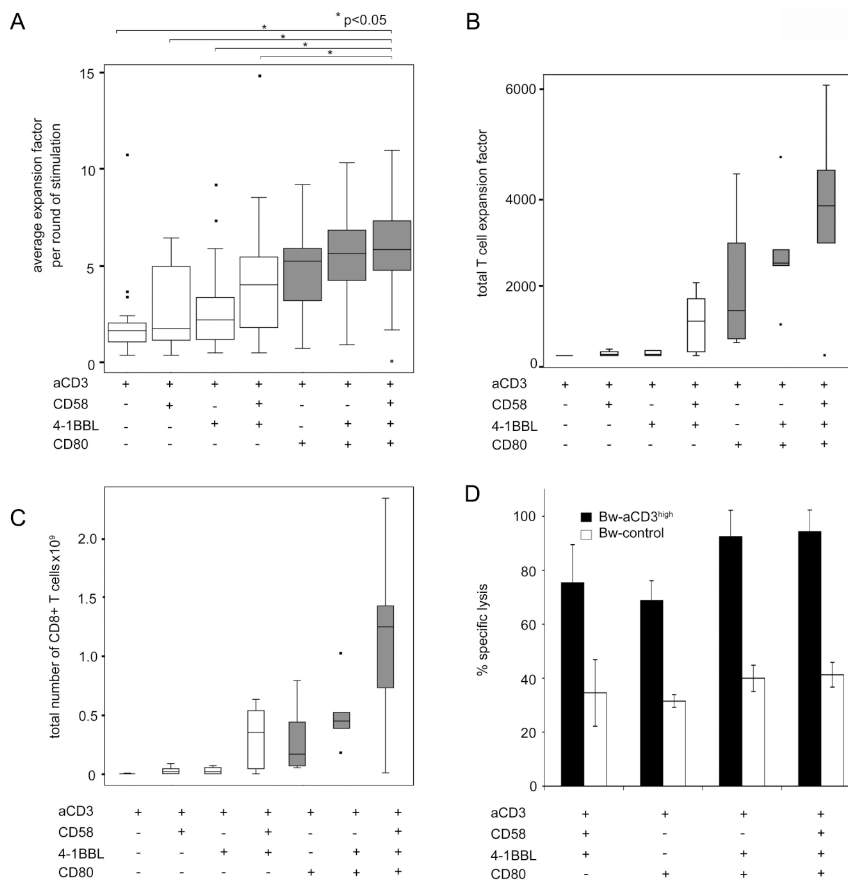

Figure 4: Costimulatory signals and the *in vitro* expansion of human T cells. T cells were subjected to five rounds of expansion using T cell stimulator cells expressing high levels of the indicated molecules. (A) Box plots representing the average fold expansion per round of stimulation. T cell stimulator cells co-expressing CD80, CD58 and 4-1BBL induced significantly higher T cell expansion than the indicated T cell stimulator cells ($p<0.05$). The total expansion of T cells during five rounds of stimulation (B) and the calculated $CD8^+$ T cell number that would have been obtained from $5x10^5$ T cells (C) is also shown. (D) Effector function of T cells expanded for 5 rounds in presence of the indicated costimulatory molecules was analyzed by europium release assay. T cell stimulator cells expressing high levels of anti-CD3 (black bars) were used as target cells and Bw cells expressing no anti-CD3 served as control cells (white bars). Percentage of specific lysis is shown.

The use of T cell stimulator cells to assess the role of CD150 and TL1A in the activation of human T cells

There is a large number of human molecules that were described to costimulate T cell activation [9]. Although for several of these molecules such a role is well established, there are still some ligands where a limited number of studies have addressed their function in T cell stimulation. We have selected two such molecules, TL1A and CD150, to study their function in T cell activation using our system of stimulator cells (Fig. 5a). For comparison T cell stimulator cells expressing CD58, a member of the CD2 superfamily, and 4-1BBL, a member of the TNF-SF, which are well established costimulatory ligands were also used.

TL1A (TNF-like molecule 1A), the newest member of the TNF-superfamily, is described to costimulate murine and human T cell proliferation via interaction with its receptor death receptor 3 (DR3, TRAMP) [24-26]. In our experiments T cell stimulator cells expressing high levels of anti-CD3 and TL1A strongly enhanced the proliferation of human T cells (Figure 5b). This costimulatory effect was observed with $CD4^+$ and $CD8^+$ T cells (Figure 5d). In line with previous studies TL1A stimulation resulted in the induction of IFN-γ [27]. In addition we obtained elevated levels of IL-10 and IL-13 in supernatants of TL1A stimulated T cell cultures (Fig. 5c).

CD150 (SLAM, signaling lymphocyte activating molecule) is a CD2 family member, expressed on activated human T cells. All previous studies that reported a costimulatory role of this molecule were based on the use of monoclonal antibodies to trigger the CD150 molecule on T cells [28-30]. CD150 is a self-ligating molecule and no other binding partners have been described. Thus, we wanted to analyse whether the costimulatory effect was also observed upon engagement of T cell-expressed CD150 with its natural ligand. Therefore, we generated stimulator cells expressing CD150 in conjunction with anti-CD3. When co-culturing these stimulator cells with human T cells, no significant contribution of this interaction to T cell proliferation and cytokine production was observed (Fig. 5b,c). In some of our experiments reduced proliferation rates of human T cells were observed in presence of human CD150 but additional experiments are required to confirm that CD150 can function as negative regulator of T cell responses.

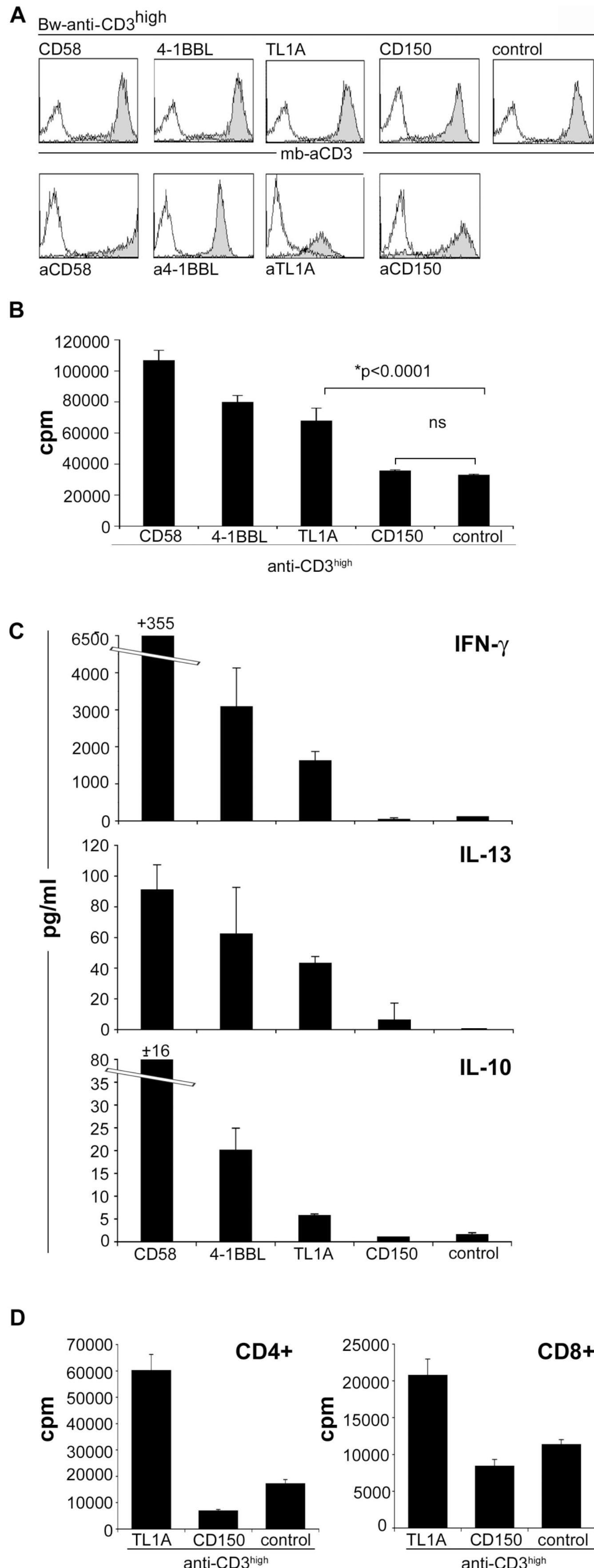

Figure 5: The role of CD150 and TL1A in the activation of human T cells.

(A) Characterisation of T cell stimulator cells expressing anti-CD3^{high} in conjunction with CD58, 4-1BBL, TL1A, CD150 or control stimulator cells expressing no costimulatory molecule by FACS. Upper panel filled histograms: expression of mb-anti-CD3 antibodies on stimulator cells; open histograms: control Bw cells. Lower panel: T cell stimulator cells expressing CD58, 4-1BBL, TL1A, CD150 were probed with antibodies specific for these molecules (filled histograms). Open histograms: reactivity of the indicated antibodies with control Bw cells.

(B) Human T cells were stimulated with stimulator cells expressing high levels of anti-CD3 in conjunction with CD58, 4-1BBL, TL1A, CD150 or control stimulator cells. Proliferation was assessed by measuring ^{3}[H]-thymidine uptake. Presence of TL1A significantly enhanced T cell proliferation ($p<0.0001$, $n=9$), whereas CD150 had no effect on human T cells proliferation (ns, not significant). Data show +/- SD of triplicates from one experiment. The experiment shown is representative for 9 independently performed.

(C) Human T cells were stimulated with stimulator cells expressing high levels of anti-CD3 in conjunction with CD58, 4-1BBL, TL1A, CD150 or no costimulatory molecule (control). Culture supernatant was harvested after 72h and subjected to multiplex cytokine measurement. The experiment shown is representative for 3 independently performed.

(D) Purified human CD4^{+} and CD8^{+} T cells were co-cultured with stimulator cells expressing anti-CD3^{high} in conjunction with TL1A, CD150 or control. Data show +/- SD of triplicates from one experiment. The shown experiment was repeated with similar outcome.

DISCUSSION

During APC-T cell interaction a complex interplay of numerous cell surface molecules modulates cellular immune responses by either enhancing or inhibiting T cell receptor complex signaling. Thus, assessing the function of individual costimulatory ligands using natural APC is a difficult task. With our T cell stimulator cells we have generated an experimental tool for studying individual costimulatory ligands in a cellular system, but detached from the context of numerous other molecules involved in the regulation of T cell activation that are expressed on professional APC. Whereas similar cellular systems that have been termed artificial APC (aAPC) use cells engineered to express Fc-γ receptors (CD32 or CD64) and depend on the addition of anti-CD3 antibodies [31-33] we used cell lines that stably express membrane-bound anti-CD3 antibody fragments. Using different anti-CD3 expression constructs we have generated two cell clones that stably express different levels of anti-CD3 antibody-fragments: A construct where the anti-CD3 antibody fragments are linked to the transmembrane domain of human CD28 molecules yielded Bw-aCD3^{low} stimulator cells that give a weak signal 1 to human T cells, whereas a construct encoding anti-CD3 antibody fragments fused to the human CD14 molecule was used to generate cells expressing high levels of GPI-anchored anti-CD3 antibody fragments (Bw-aCD3^{high}; Fig. 1). The GPI-anchored anti-CD3 antibody fragment is efficiently targeted to lipid rafts and has also successfully been used for the stimulation and manipulation of human T cells with immunosomes - virus-like particles decorated with TCR/CD3 ligands, costimulatory molecules and modified cytokines [34-37]. Another important difference between aAPC and stimulator cells is the cell type that is used as a scaffold: the former is based on the human K562 cell line and T cell stimulator cells are derived from the murine thymoma line Bw5147. Whereas K562 cells contains surface molecules that enhance T cell-APC interactions [31], Bw cells appear to be devoid of molecules that promote the proliferation of human T cells that receive a weak signal 1 (Fig. 1b). Thus, T cell stimulator cells are especially suited to study molecules that exert weak costimulatory effects. Furthermore, with this system it is also possible to compare different accessory molecules regarding their capacity to costimulate activation and proliferation of human T cells. Experiments where we have performed a side by side comparison of ligands belonging to different molecule families demonstrated a potent ability of CD58 to costimulate the activation of human T cells (Fig. 2).

In addition, to the numerous different immunosuppressive drugs that are already used in the clinic to down-modulate T cell responses there are many additional compounds or biologics that are currently tested regarding their efficacy and safety for human use. Especially in the case of antibodies that often have limited or no reactivity with the non-

human orthologues of their target antigens, extensive *in vitro* testing in human systems is highly warranted. Since costimulators govern the activation of T cells, their interplay with T cell suppressive antibodies and drugs is of great interest. Here, we have used our system of T cell stimulator cells to analyze the effect of Adalimumab, a therapeutic antibody to TNF-α, on T cell activation. We show that TNF-α has a costimulatory effect on human T cells and that TNF-α blockade reduces the proliferation of T cells, independent of accessory cells (Fig. 3). Adalimumab reduced T cell responses, regardless of the molecules used for their activation. However, we have observed that the capacity of some therapeutic antibodies and immunosuppressive drugs to diminish T cell proliferation and cytokine production is potently modulated by different costimulatory signals (our unpublished results).

The efficient *in vitro* expansion of antigen specific T cells crucially depends on appropriate costimulatory signals to ensure the generation of large amounts of potent effector cells. Different combinations of costimulatory ligands can be readily expressed on stimulator cells. The resultant stimulator cell lines can be tested in parallel to identify combinations of stimulatory molecules that potently drive expansion of human T cells *in vitro*. Our results indicate that concomitant stimulation via their CD28, CD2 and 4-1BB receptors leads to an efficient expansion of T cells, which retain their effector function during several rounds of stimulations (Fig. 4). These results, together with our findings summarized in Figure 2, underline the potency and importance of the CD2 - CD58 pathway for the activation of human T cells. CD2 was one of the first T cell costimulatory receptors identified [38] and despite its importance this pathway is currently receiving limited attention. This is in part due to the fact that CD58 lacks a murine orthologue and demonstrates the current emphasis on mouse model systems to study the costimulatory pathways.

There is an ever growing number of ligands that have been implicated to play a role in T cell costimulatory processes and contradictory results have been reported for several of these molecules [9]. We believe that T cell stimulator cells are especially suited to assess the function of accessory molecules during T cell activation since they allow analysing human T cell responses under conditions that only differ regarding the presence of the molecules of interest. We have recently used stimulator cells expressing PD-L2 and B7-H3, two members of the extended B7 family, to address their function during the activation of human T cells [10, 39]. In these studies we could show that these molecules consistently inhibited T cell responses and our experiments did give any evidence for positive costimulatory functions for human PD-L2 and B7-H3. The CD2 superfamily member CD150 and the TNF-SF member TL1A have both been described to costimulate T cell activation. CD150 is a self-ligating

receptor, whereas TL1A binds to DR3 a member of the TNFR-SF. However, few studies on these molecules have directly analyzed the consequences of the interaction of CD150 or TL1A with human T cells. In the present study we have generated T cell stimulator cell lines expressing CD150 and TL1A and used them to stimulate purified human T cells. Our results demonstrate that presence of TL1A during T cell activation significantly costimulates their proliferation and production of cytokines, whereas T cells stimulated in presence of stimulator cells expressing CD150 did not show enhanced proliferation and cytokine production. Previous studies that have described a positive costimulatory function for CD150 have used antibodies to crosslink the CD150 molecules on T cells [28, 29]. In contrast, we have used T cell stimulator cells expressing its natural ligand CD150, to assess the role of CD150-CD150 interaction in the activation of T cells. Our results, which suggest that CD150 does not function as a classical T cell costimulatory molecule, underline the importance of using natural ligands to study the functional consequences of receptor-ligand pairs implicated in T cell activation processes. The homophilic interaction of CD150 is of particular low affinity (K_d 200 mM; [40]), which might explain the different outcome of our experiments compared to studies that used antibodies. It has previously been suggested that antibody-induced SLAM/CD150 activation may not fully mimic the physiological role of CD150, because mice deficient in this molecule exhibit essentially normal levels of IFN-γ, whereas the ligation of T cell expressed CD150 by antibodies promotes strong IFN-γ secretion [41]. Further studies are required to address the physiological role of CD150 during human T cell activation. Since T cells that express costimulatory ligands can receive potent costimulatory signals (“autocostimulation”) it is also possible that homotypic interaction of CD150 *in cis* plays a role during human T cells activation [42].

Taken together our results demonstrate that the system of T cell stimulator cells is a useful tool to assess the function of costimulatory ligands. In particular they are suited to compare the function of individual costimulatory molecules and analyze their effect on different T cell subsets and in context of a strong or weak signal 1. Since professional APC like DC harbour stimulatory as well as inhibitory ligands, the interplay of positive and negative signals determine the outcome of T cell responses. We have previously shown that combinations of costimulatory molecules can be expressed and analyzed on T cell stimulator cells [12]. We are currently using our system of stimulator cells to analyze the interplay of defined costimulatory and coinhibitory molecules during the activation of human T cells. Studies on individual costimulatory pathways can complement investigations using experimental systems employing natural human APC or animal studies to get a better insight

into the complex interplay of the numerous accessory surface molecules that govern human T cell responses.

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AUTHORS' CONTRIBUTION

JL designed and performed the majority of the experiments and analyzed data. WK and RW performed experiments. OM provided essential reagents. EK performed the LSM experiments together with JL. KG-P, GZ, WFP and PS analyzed and interpreted data. JL and PS wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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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 analyzed 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.

key words: costimulatory molecules, immune regulation, T cells

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;

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 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 analyzed 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.

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 x 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 x 10^5/well) were co-cultured with T cell stimulators (2 x 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.

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 analyzed 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 analyzed 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).

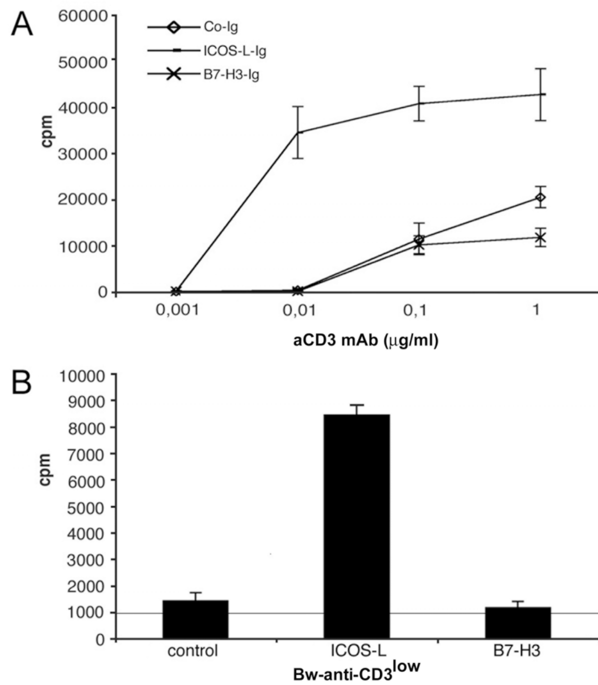


Figure 1. B7-H3 does not costimulate a weak signal 1. (A) Human T cells were incubated with plate-bound anti-CD3 mAb immobilized at the indicated concentrations in the presence of control-Ig (Co-Ig), ICOS-L-Ig or B7-H3-Ig (immobilized at 10 µg/ml). (B) Human T cells were co-cultured with control Bw-anti-CD3^{low} 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.

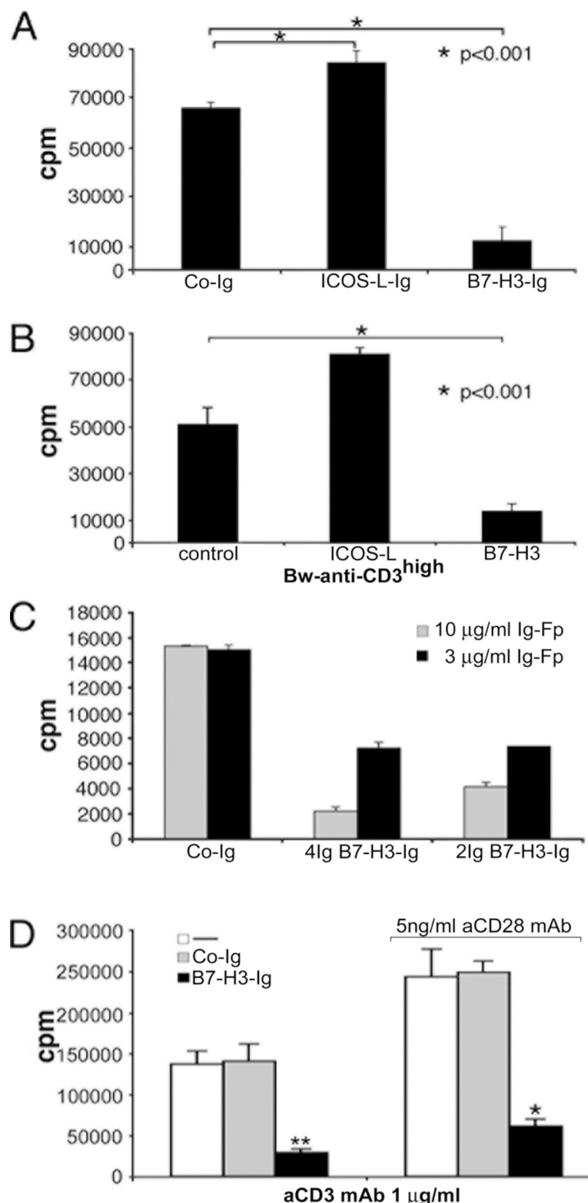


Figure 2. B7-H3 inhibits human T cell activation. (A) T cells were stimulated with plate bound anti-CD3 mAb in presence of control-Ig (Co-Ig), ICOS-L-Ig or B7-H3-Ig. Presence of B7-H3 significantly inhibits T cell proliferation ($p<0.001$, n=9) whereas ICOS-L strongly enhanced T cell proliferation ($p<0.001$, 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.

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).

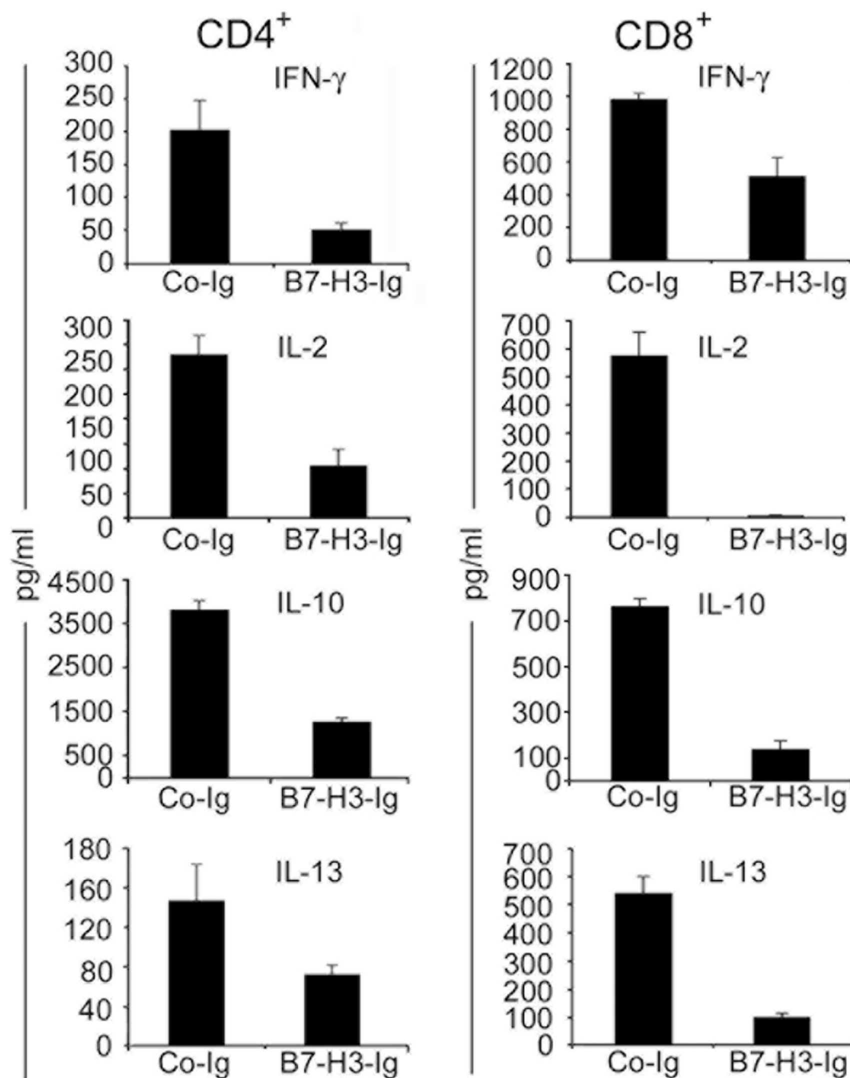


Figure 3. B7-H3 inhibits cytokine production. Human $CD4^+$ and $CD8^+$ T cells were stimulated with plate-bound anti-CD3 mAb in the presence of control-Ig (Co-Ig) or B7-H3-Ig. Culture supernatant was harvested after 72 hours and subjected to multiplex cytokine measurement. Data show mean ± SD of triplicates from one experiment and are representative of three independent experiments.

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 analyzed 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 analyzed 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.

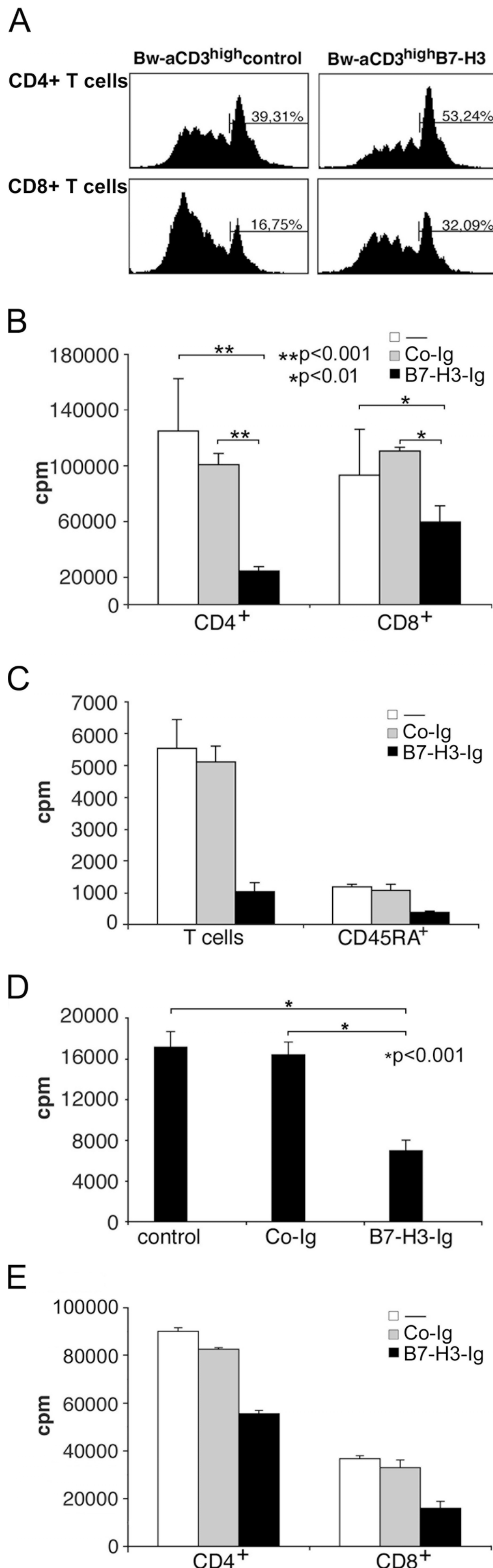


Figure 4. B7-H3 inhibits proliferation of CD4^{+}, CD8^{+}, naïve and pre-activated T cells.

(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 analyzed 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.

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).

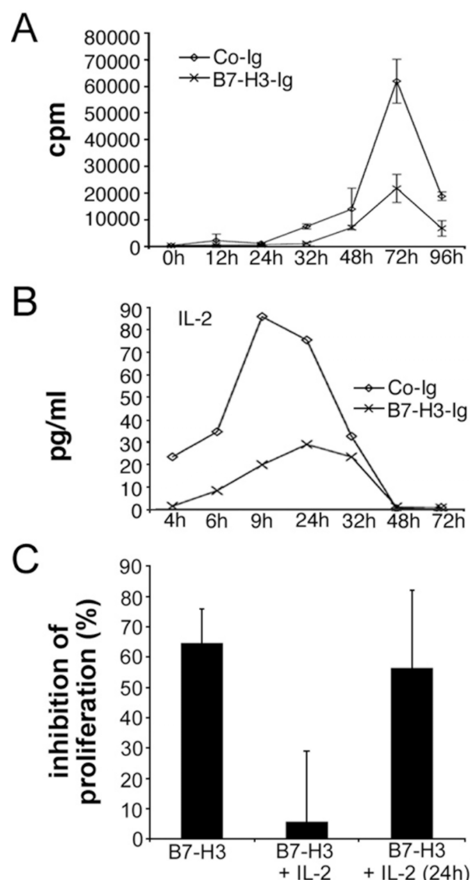


Figure 5. B7-H3 mediated T cell inhibition is characterized by early suppression of IL-2.
Human T cells were stimulated with plate-bound anti-CD3 mAb in presence of control-Ig (Co-Ig) or B7-H3-Ig. (A) T cell proliferation was measured at the time points indicated. (B) Culture supernatant was harvested 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.

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.

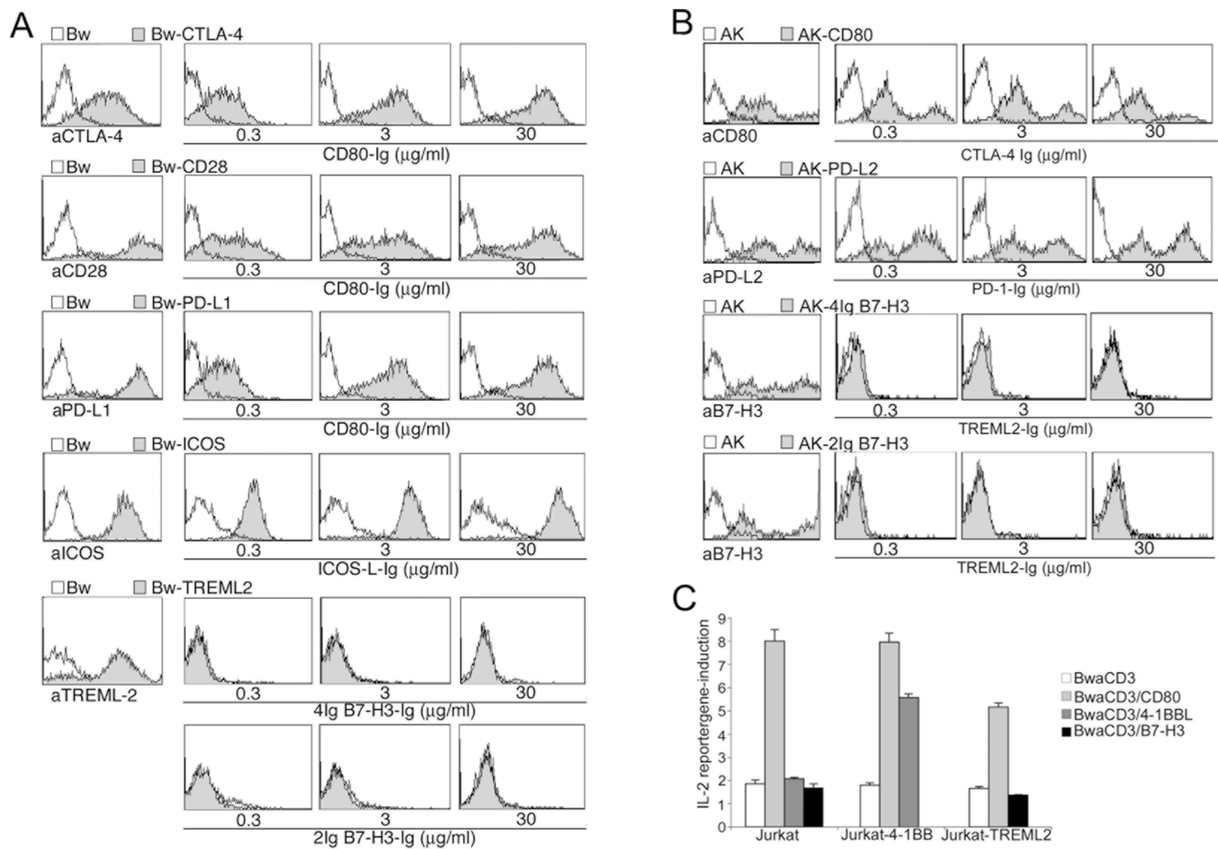


Figure 6. Human TREML2 does not serve as a costimulatory receptor for B7-H3. (A) Fusion proteins representing human B7 family members were analyzed for binding to their receptors or TREML2 (grey histograms) and control cells (open histograms). (B) Fusion proteins representing receptors for B7 family members and human TREML2 were analyzed 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 analyzed in a luciferase assay. Results are representative for five independent experiments.

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

Since receptor-ligand interactions are generally conserved between mice and humans we also analyzed 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).

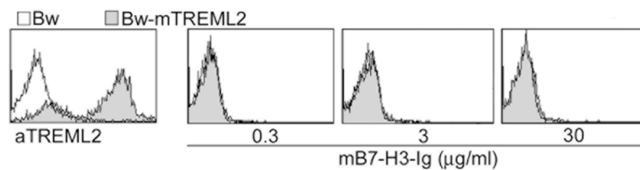
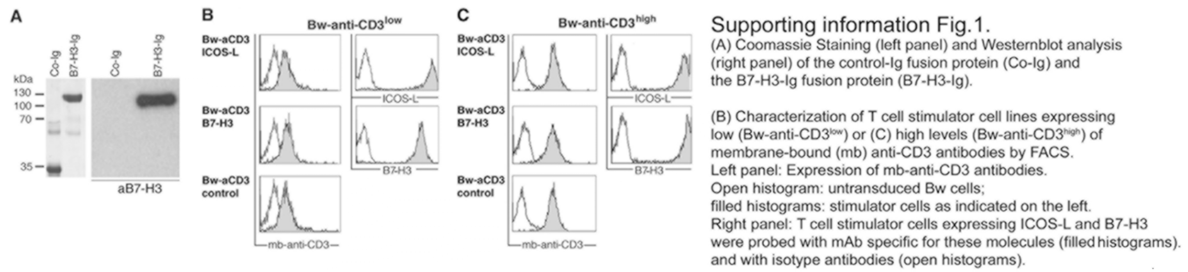


Figure 7. Mouse B7-H3-Ig does not bind to cells expressing TREML2. A fusion protein representing murine B7-H3 was analyzed for binding to Bw cells expressing murine TREML2 (Bw-mTREML2, grey histograms) and control cells (Bw, open histograms). Left panel show interaction of αTREML2 antibody with cell lines as indicated. Results are representative for three independent experiments.

SUPPORTING INFORMATION

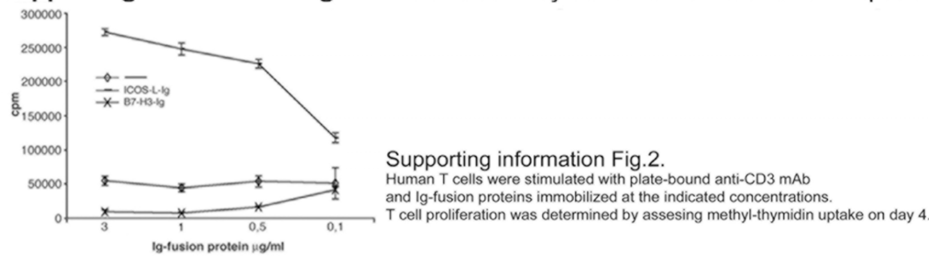
Supporting information Figure 1. Generation of B7-H3-Ig and T cell stimulator cells.



Supporting information Fig.1.
(A) Coomassie Staining (left panel) and Westernblot analysis (right panel) of the control-Ig fusion protein (Co-Ig) and the B7-H3-Ig fusion protein (B7-H3-Ig).

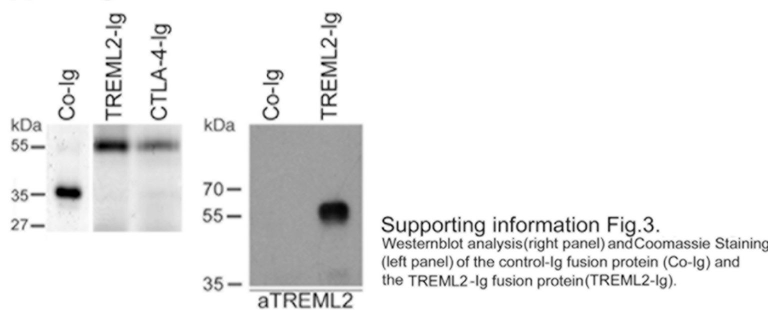
(B) Characterization of T cell stimulator cell lines expressing low (Bw-anti-CD3^{low}) or (C) high levels (Bw-anti-CD3^{high}) of membrane-bound (mb) anti-CD3 antibodies by FACS.
Left panel: Expression of mb-anti-CD3 antibodies.
Open histogram: untransduced Bw cells;
filled histograms: stimulator cells as indicated on the left.
Right panel: T cell stimulator cells expressing ICOS-L and B7-H3 were probed with mAb specific for these molecules (filled histograms). and with isotype antibodies (open histograms).

Supporting information Figure 2. The inhibitory effect of B7-h3 is dose-dependent.



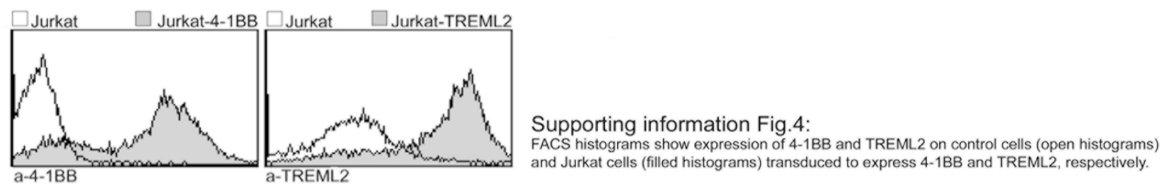
Supporting information Fig.2.
Human T cells were stimulated with plate-bound anti-CD3 mAb and Ig-fusion proteins immobilized at the indicated concentrations.
T cell proliferation was determined by assesing methyl-thymidin uptake on day 4.

Supporting information Figure 3. Characterization of the TREML2-Ig.



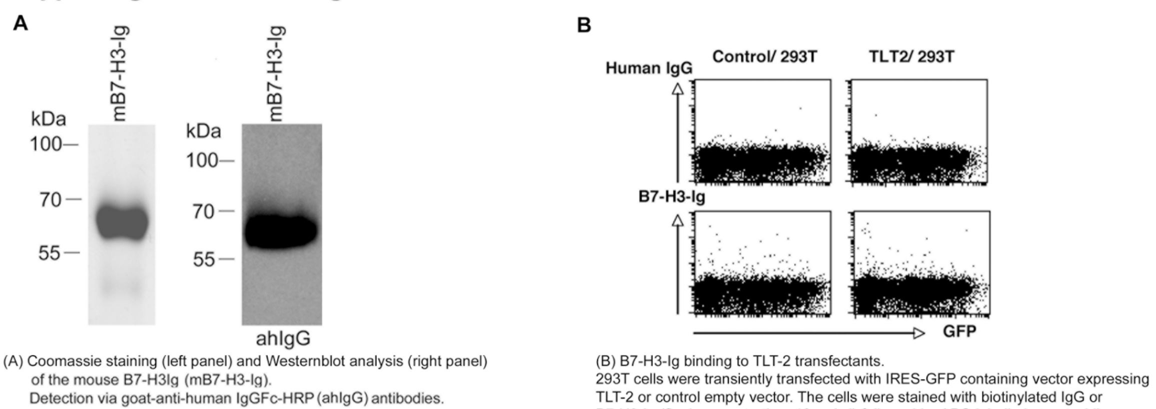
Supporting information Fig.3.
Westernblot analysis(right panel) and Coomassie Staining (left panel) of the control-Ig fusion protein (Co-Ig) and the TREML2-Ig fusion protein(TREML2-Ig).

Supporting information Figure 4. Characterization of Jurkat-4-1-BB and Jurkat-TREML2 reporter cell by FACS.



Supporting information Fig.4:
FACS histograms show expression of 4-1BB and TREML2 on control cells (open histograms) and Jurkat cells (filled histograms) transduced to express 4-1BB and TREML2, respectively.

Supporting information Figure 5. Mouse B7-H3-Ig does not bind to cells expressing TREML2.



(A) Coomassie staining (left panel) and Westernblot analysis (right panel) of the mouse B7-H3Ig (mB7-H3-Ig).
Detection via goat-anti-human IgGFc-HRP (ahIgG) antibodies.

(B) B7-H3-Ig binding to TLT-2 transfectants.
293T cells were transiently transfected with IRES-GFP containing vector expressing TLT-2 or control empty vector. The cells were stained with biotinylated IgG or B7-H3-Ig (final concentration: 10 µg/ml) followed by APC-labelled streptavidin.

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 analyzed 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 analyzed 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 analyzed 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 analyzed 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 analyzed 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.

ACKNOWLEDGEMENTS

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AUTHORS' CONTRIBUTION

JL performed the majority of the experiments. CK performed experiments. AFB and DPK performed the bioinformatics-based search. CD and TY provided data for supporting information. OM and WFP provided essential reagents. JL, KP, WFP, JS, GZ, and PS analyzed and interpreted data. JL, KP and PS wrote the paper. All authors critically revised the manuscript and approved the final version.

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The effects of Cyclosporine A and azathioprine on human T cells activated by different costimulatory signals

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ABSTRACT

Immunosuppression is an important treatment modality in transplantation and human diseases that are associated with aberrant T cell activation. There are considerable differences regarding the cellular processes targeted by the immunosuppressive drugs that are in clinical use. Drugs like azathioprine (Aza) mainly act by halting proliferation of fast dividing cells, whereas others like cyclosporine A (CsA) specifically target signaling pathways in T cells. Since the outcome of T cell responses critically depends on the quality and strength of costimulatory signals, this study has addressed the interplay between costimulation and the immunosuppressive agents CsA and Aza during the *in vitro* activation of human T cells.

We used an experimental system that allows analyzing T cells activated in the presence of selected costimulatory ligands to study T cells stimulated via CD28, CD2, LFA-1, ICOS or 4-1BB. The mean inhibitory concentrations (IC_{50}) for Aza and CsA were determined for the proliferation of T cells receiving different costimulatory signals as well as for T cells activated in the absence of costimulation.

CD28 signals but not costimulation via CD2, 4-1BB, ICOS or LFA-1 greatly increased the IC_{50} for CsA. By contrast, the inhibitory effects of Aza were not influenced by T cell costimulatory signals.

Our results might have implications for combining standard immunosuppressive drugs with CTLA-4Ig fusion proteins, which act by blocking CD28 costimulation.

key words: T cell costimulation, immunosuppression, Cyclosporine A, azathioprine

abbreviations: Aza, azathioprine; CsA, Cyclosporine A; APC, antigen presenting cells, IC_{50}, mean inhibitory concentration

INTRODUCTION

T cells have important roles in allograft rejection, graft versus host diseases and autoimmune pathologies. In most cases the clinical management of these conditions requires the extensive use of immunosuppressive agents to control aberrant T cell responses. Such drugs limit and down-modulate T cell activation by targeting different cellular processes. Drugs like azathioprine (Aza) mainly act by halting proliferation of fast dividing cells, whereas others like Cyclosporine A (CsA) more specifically target T cells by interfering with signaling pathways in these cells. Since many different signals can contribute to T cell activation processes, the interplay between such signals and immunosuppressive agents might have differential effects on the outcome of T cell responses. Especially costimulatory signals generated by interaction of APC-expressed ligands with their T cell-expressed receptors have a crucial role in the efficient activation of T cells that recognize antigen. The interaction of CD80/CD86 with CD28 is generally regarded as the primary T cell costimulatory pathway [1]. However, there are many alternative costimulatory ligand-receptor pairs that potently enhance the proliferation, differentiation and cytokine production of T cells that recognize antigens [2-4]. Among these the CD58 - CD2, 4-1BBL - 4-1BB, ICOS-L - ICOS and CD54 - LFA-1 (CD11a/CD18) pathways are well documented to generate strong and consistent costimulatory effects in human T cells [2,5]. Costimulatory receptors belong to different molecule-families and consequently they can induce signaling events that are distinct from the pathways induced by CD28 ligation. Previous studies have shown that engagement of the CD28 costimulatory pathway greatly reduces the sensitivity of T cells to the immunosuppressive effect of CsA [6,7]. By contrast, it is not known whether triggering alternative costimulatory receptors has similar effects. Furthermore, currently there is limited knowledge how different costimulatory signals affect the immunosuppressive effects of other drugs in clinical use.

We have previously developed a cellular system termed T cell stimulator cells that allows analyzing the effect of different costimulatory signals on human T cells [5,8,9]. This system is based on cell lines engineered to express membrane-bound anti-human-CD3 antibody-fragments that trigger the TCR-complex on human T cells upon co-culture. By expressing high levels of human costimulatory ligands of interest on the T cell stimulator cells it is possible to analyze and compare human T cells that receive distinct costimulatory signals. In this study we used T cell stimulator lines expressing CD80, CD58, 4-1BBL, ICOS-L. CD54 and T cell stimulator lines expressing anti-CD3 antibody-fragments but no costimulatory molecules to activate T cells purified from healthy individuals. Using this

system we determined the mean inhibitory concentrations (IC_{50}) for CsA and Aza for the proliferation of human T cells receiving different costimulatory signals.

MATERIAL AND METHODS

Antibodies, cell culture and FACS staining

293T cells and the mouse thymoma cell line Bw5147 (short designation within this work Bw) were cultured as described [9]. 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). Untouched 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 (Miltenyi Biotech, Bergisch Gladbach, Germany). The mAbs to CD11b (VIM12), CD14 (VIM13), CD33 (4D3), MHC-class II (1/47), CD80 (7-480), CD58 (1-456) and CD54 (5-216) were produced at our Institute. The mAbs to CD14 (MEM-18) was purchased from An der Grub (Kaumberg, Austria), CD19 mAb (BU12) from Ancell (Bayport, MN), 4-1BBL from Biolegend (San Diego, CA) and ICOS-L (2D3/B7H2) from BD Pharmingen (Palo Alto, CA). FACS analysis was performed as described previously [10]. Briefly, binding of primary antibodies was detected with PE-conjugated goat-anti-mouse IgG-Fcγ specific Abs (Jackson ImmunoResearch, West Grove, PA). Expression of membrane-bound anti-CD3 antibody fragment was detected via APC-conjugated goat-anti-mouse IgG (H+L) Abs, which reacts with the variable regions of murine antibodies (Jackson ImmunoResearch). Fluorescence intensity is shown on a standard logarithmic scale.

T cell activation in the presence of different costimulatory molecules

Human T cells ($1x10^5$/well) were co-cultured for 72 hours with irradiated (6000 rad) T cell stimulator cell lines ($2x10^4$/well) expressing high levels of membrane-bound anti-CD3 antibody-fragments and one of the following costimulatory ligands: CD80, CD58, 4-1BBL, ICOS-L, CD54 or control T cell stimulator cells expressing anti-CD3 but no human costimulatory molecules as described [5]. Cyclosporine A (CsA; Sandimmun®, Novartis Pharma, Basel, Swiss) and azathioprine (Aza; Imurek®, GlaxoSmithKline, Greenford, GB) were added at the indicated final concentrations. To assess T cell proliferation methyl-^{3}[H]-thymidine (final concentration: 0.025 mCi; Perkin Elmer/New England Nuclear Coorporation, Wellesley, MA) was added for the last 18 hours prior harvesting of the cells. Methyl-^{3}[H]-thymidine uptake was measured as described [8]. All T cell proliferation assays were done in triplicates, means and SD are shown.

Cytokine measurement

For cytokine measurement supernatants of T cell activation assays were collected after 48h and pooled from triplicate wells. IFN-γ, IL-2, IL-10 and IL-13 were measured in the supernatants using the Luminex System 100 (Luminex, Texas, USA).

Statistical analyses

The half maximal inhibitory concentration (IC_{50}) for CsA and Aza was calculated for each experiment by blotting the percentage of proliferation (methyl-^{3}[H]-thymidine uptake in the presence of inhibitor x 100/methyl-^{3}[H]-thymidine uptake without inhibitor) against the log concentration of the immunosuppressive drug (mg/ml) using Graph-pad PRISM. Differences between the IC_{50} values for CsA and Aza of T cells stimulated in presence of different costimulatory ligands were calculated using ANOVA for repeated measures. IMB® SPSS statistics software was used for analysis and generation of Box plot graphs. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

T cell proliferation in the presence of different costimulatory ligands

We have previously generated a cellular system called T cell stimulator cells that allows for analysing T cells receiving distinct costimulatory signals [5]. This system is based on Bw cells, a murine thymoma line, that has been engineered to express membrane-bound anti-CD3 antibody-fragments and can thus generate “Signal 1” in human T cells by triggering their TCR-complex. By co-expressing human costimulatory ligands on these stimulator cells their contribution to T cell activation processes can readily be studied. Importantly, with this system we can analyze the net effects of individual costimulatory ligands in the absence of other accessory molecules that also regulate T cell activation on human antigen presenting cells (APC).

For this study we have generated and used T cell stimulator lines expressing CD80, CD58, 4-1BBL, ICOS-L, CD54 and control stimulator cells expressing anti-CD3 antibodies but no costimulatory molecules. Those molecules were chosen, because their role as costimulatory molecules is well described and moreover they represent different molecule families. Flow cytometric analysis of the resulting T cell stimulator lines demonstrate similar amounts of membrane-bound anti-CD3 and high expression levels of the respective costimulatory ligands (Fig. 1). In Figure 2 the proliferative response of T cells stimulated with T cell stimulator lines expressing the indicated costimulatory molecules or with T cell stimulators expressing no costimulatory molecule is shown. The costimulatory molecules tested in these experiments differed in their potency to induce proliferation. Importantly, all T cell stimulator cells harbouring costimulatory ligands induced a stronger proliferation of T cells than control the T cell stimulator cell line expressing no human costimulatory molecules (Fig. 2).

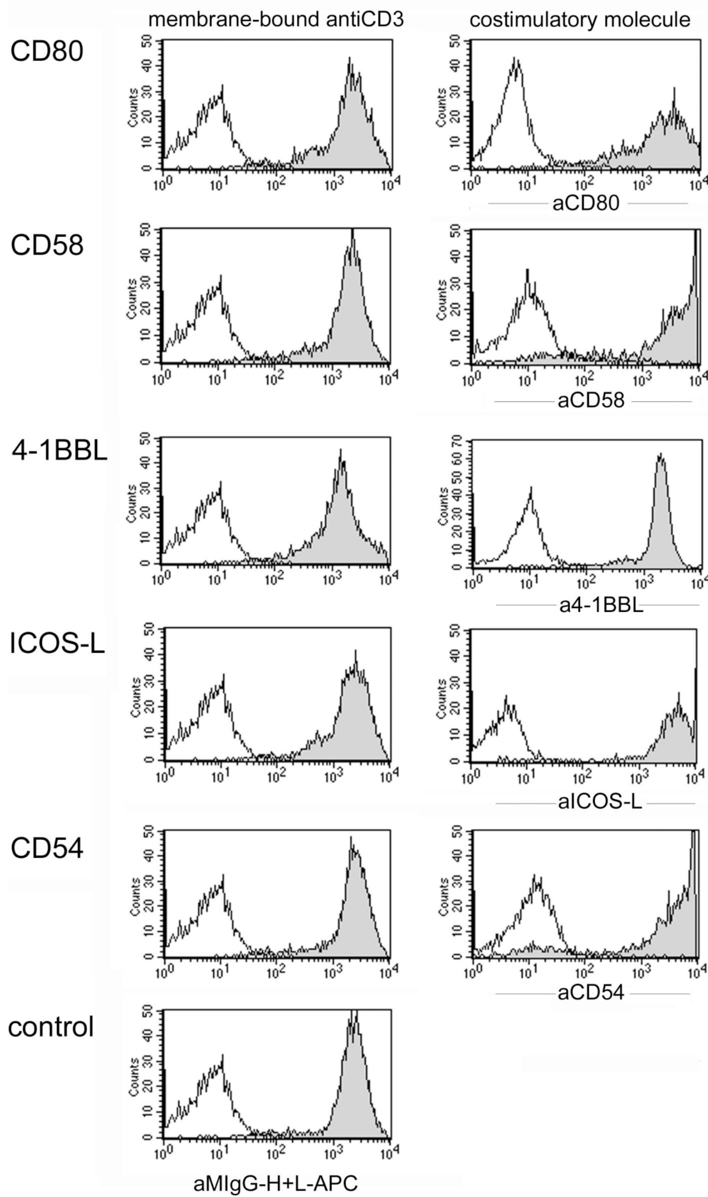


Figure 1. Characterisation of the T cell stimulator cells used in this study. Characterisation of T cell stimulator cells expressing anti-CD3^{high} in conjunction with CD80, CD58, 4-1BBL, ICOS-L, CD54 or no costimulatory molecule (control) by FACS. Left panel: expression of mb-anti-CD3 antibodies on stimulator cells; open histograms: parental Bw cells. Right panel: T cell stimulator cells expressing CD80, CD58, 4-1BBL, ICOS-L or CD54 were probed with antibodies specific for these molecules (filled histograms); open histograms: reactivity of the indicated antibodies with parental Bw cells.

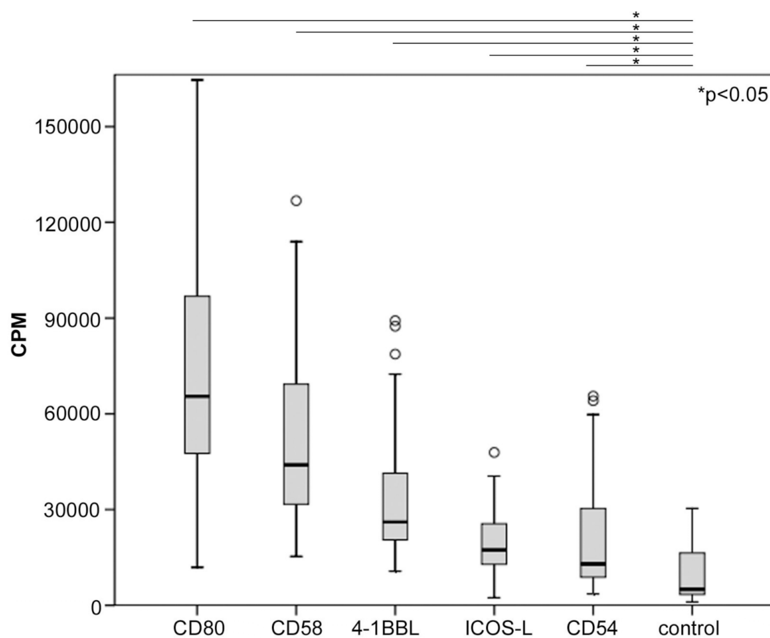


Figure 2. Proliferative response of T cells activated in presence of different costimulatory signals. Stimulator cells expressing high levels of membrane-bound anti-CD3 in conjunction with CD80, CD58, 4-1BBL, ICOS-L, CD54 or no human costimulatory molecules were co-cultured with human T cells. ^{3}[H]-thymidine uptake was assessed following 3 days of co-culture (cpm, counts per minute). Circles indicate outliers. Box plots show the results of 10 independent experiments with T cells from different healthy donors.

Impact of Cyclosporine A and azathioprine on human T cell proliferation acting via different costimulatory ligands

We have previously demonstrated that our system of T cell stimulator cells is an excellent tool to study the influence of immunmodulatory drugs on T cell activation [5].

In this study we addressed whether different costimulatory signals can influence the ability of the immunosuppressive drugs CsA and Aza to inhibit human T cell proliferation. T cell stimulator cells expressing high levels of anti-CD3 in conjunction with CD80, CD58, 4-1BBL, ICOS-L, CD54 as well as T cell stimulator cells expressing no costimulatory molecules were co-cultured with human T cells in presence of different concentrations of CsA and Aza. As shown in Figure 3, CsA and Aza led to a dose-dependent inhibition of T cell proliferation. Importantly, the sensitivity of CD28 costimulated T cells to the anti-proliferative effects of CsA was dramatically reduced. In contrast, the T cell-inhibitory effects of Aza were not modulated by costimulatory signals (Fig. 3b).

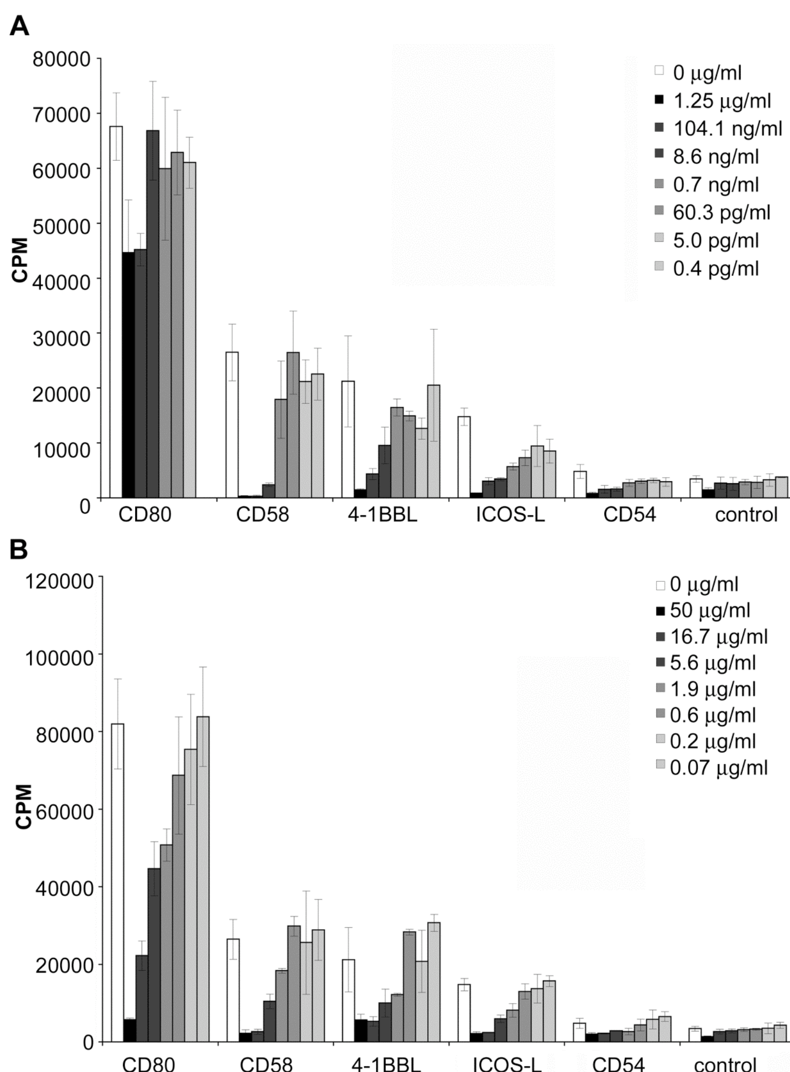


Figure 3. The impact of CsA and Aza on the proliferation of human T cells receiving different costimulatory signals. Human T cells were stimulated with stimulator cells expressing high levels of anti-CD3 in conjunction with CD80, CD58, 4-1BBL, ICOS-L, CD54 or control stimulator cells. On the onset of co-culture (A) Cyclosporine A (CsA) or (B) Azathioprine (Aza) were added at the indicated final concentrations. ^{3}[H]-thymidine uptake was assessed following 3 days of co-culture (cpm, counts per minute). Data show +/- SD of triplicates from one experiment. The experiment shown is representative for 10 independently performed.

IC_{50} values of CsA depend on the costimulatory signal

The half maximal inhibitory concentrations (IC_{50}) for CsA and Aza was calculated from dose-inhibition curves for T cells activated in the presence of T cell stimulator cells expressing the indicated costimulatory ligands. Representative dose-inhibition curves for each costimulatory molecule are shown in figure 4.

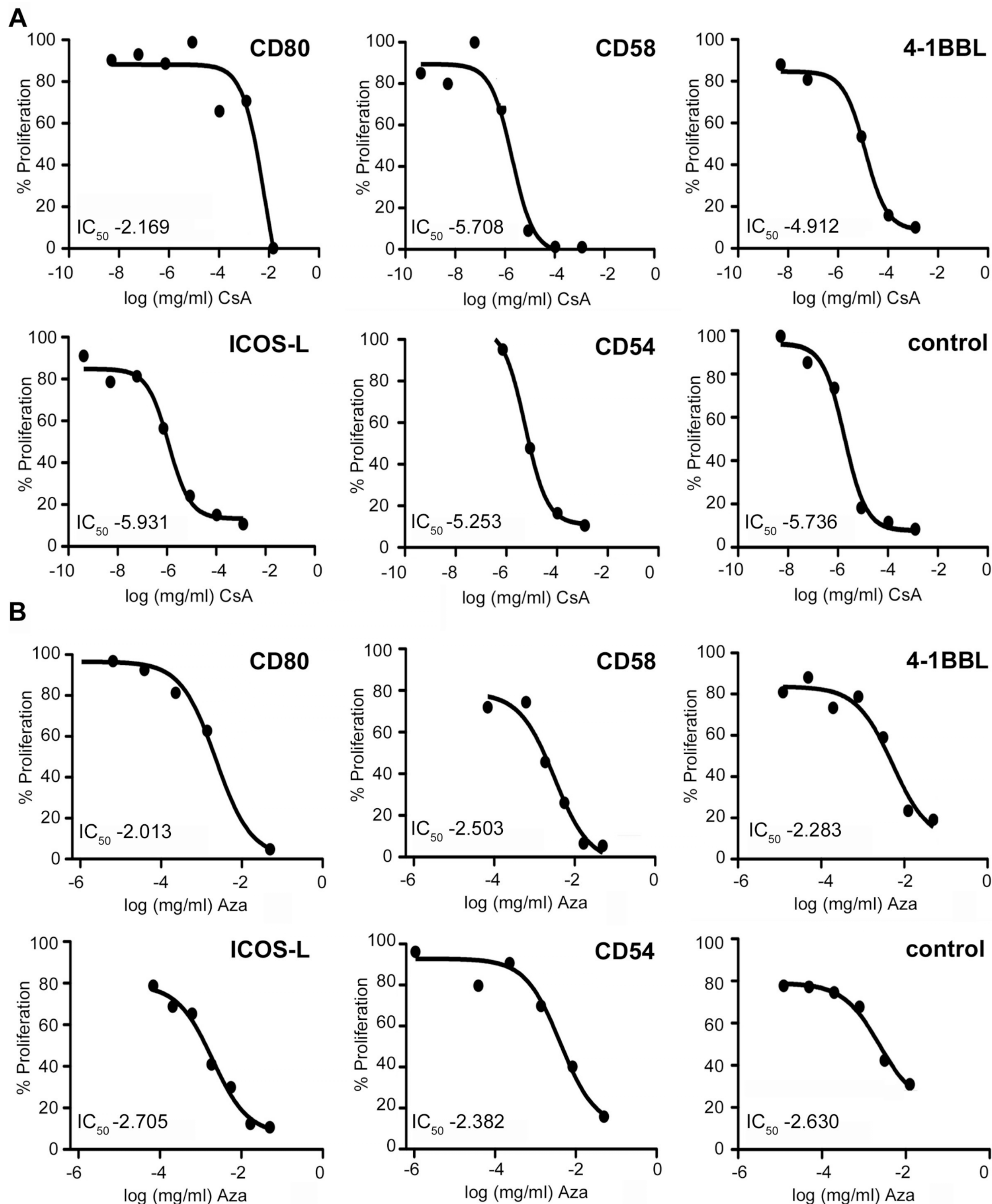
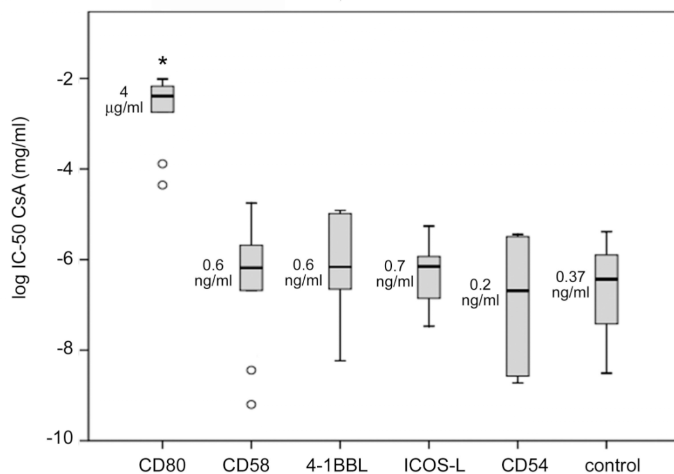


Figure 4. Dose inhibition curves for CsA and Aza.
Dose inhibition curves for CsA (A) or Aza (B) for T cells stimulated in the presence of different costimulatory ligands and for T cells activated in absence of costimulatory signals (control). The calculated IC_{50} values (half maximal inhibitory concentrations) are indicated. Each curve shown is representative for ten independently performed experiments.

Cumulative results of the IC_{50} values obtained with T cells from 10 healthy donors are shown in Figure 5. In the case of CsA the median half maximal inhibitory concentration for T cells stimulated via CD28 compared to T cells receiving no costimulatory signal was increased more than 10000-fold (median IC_{50} values 4 µg/ml versus 0.37 ng/ml; $p \leq 0.05$, n=10). Importantly, our results clearly demonstrate that decreased sensitivity to the immunosuppressive effects of CsA is an unique property of CD28 signals, since T cells stimulated via alternative pathways were inhibited by CsA to the same extent as T cells receiving no costimulatory signals (Fig. 5a). CD28 costimulated T cells were also less sensitive to CsA mediated inhibition of cytokine production (appendix supplementary figure 1 and data not shown).

Furthermore, statistical analysis of our data clearly showed that CD28 signals are completely ineffective in reducing Aza mediated T cell suppression and the IC_{50} values obtained for this drug were not influenced by any of the costimulatory signals investigated in this study (Fig. 5b).

A



B

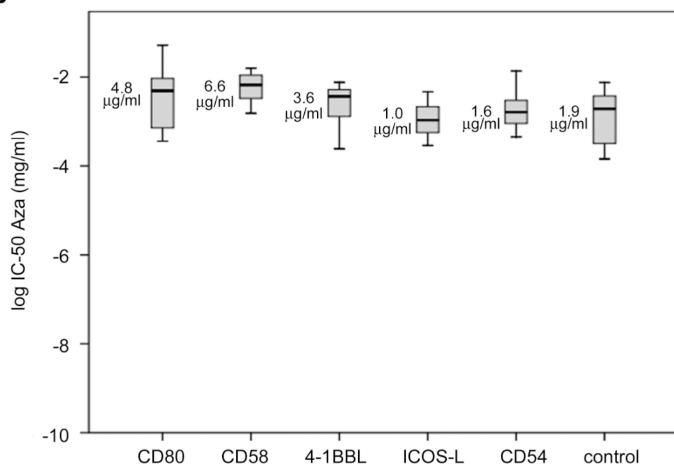
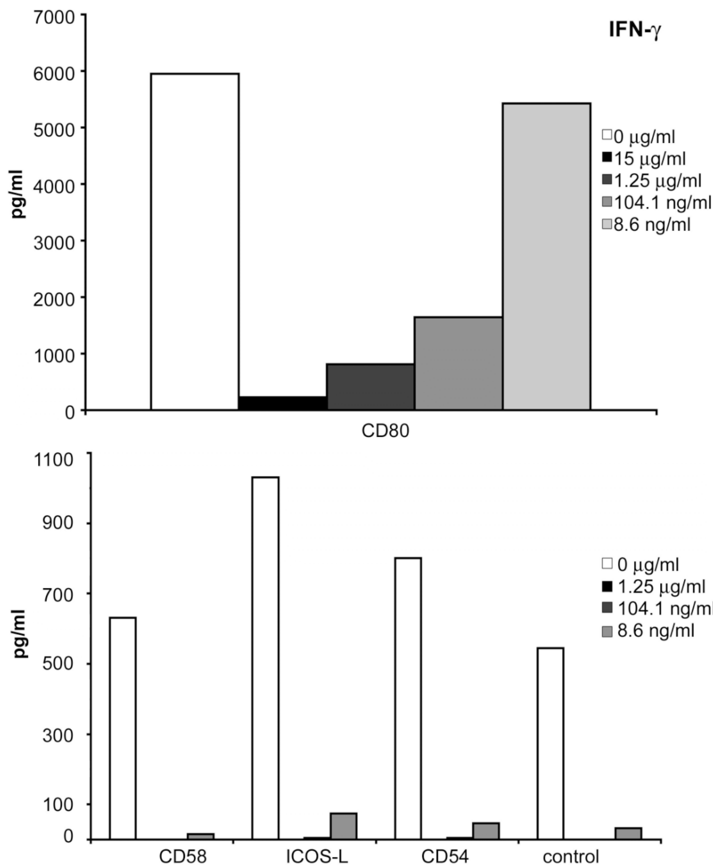


Figure 5. IC_{50} values for T cell proliferation in presence of different costimulatory signals.
The cumulative results of the IC_{50} values for (A) CsA or (B) Aza treated human T cells activated in the presence of different costimulatory ligands. Circles indicate outliers. Box plots represent data from ten experiments independently performed. Stars indicate significant differences ($p \leq 0.05$, n=10). Median values are indicated.

Appendix. Supplemental data.



Supplementary figure 1. Inhibition of IFN-γ production in the presence of CsA.
T cells were stimulated in the presence of indicated costimulatory signals. The influence of CsA on the IFN-γ production was assessed by measuring the IFN-γ concentrations in the culture supernatants using Luminex-technology.

DISCUSSION

CD28 is generally regarded as the primary and most potent costimulatory receptor on T cells and consequently many studies have investigated how CD28 signals contribute to T cell activation and differentiation processes. Several earlier studies have demonstrated that CD28 triggering greatly reduces the inhibitory effects of CsA on the proliferation and cytokine production of human T cells [6,7]. However, triggering of numerous alternative receptors can generate potent costimulatory signals in T cells and animal studies demonstrate that productive immune responses occur in the absence of CD28 signals [11,12]. Our study is the first to address whether alternative costimulatory signals can also interfere with CsA-mediated T cells suppression. Since activated APC express a plethora of accessory molecules, it is difficult to study the role of distinct costimulatory pathways in T cells stimulated by natural APC. We thus relied on a previously described cellular system, which allows studying human T cells that were activated in presence of individual costimulatory ligands of choice [5,9,10].

Our results clearly show that decreased sensitivity to the anti-proliferative effects of CsA is a unique feature of CD28 signals, since it was not observed with T cells activated by alternative costimulatory pathways. Although CD28 costimulation induced the strongest proliferative response we suggest that this is a qualitative effect. In support for this hypothesis we observed that IC_{50} values obtained for CD2 costimulated T cells, which also had very strong proliferative responses, were even slightly lower than those obtained for T cells stimulated via ICOS, which induced much less T cell proliferation and cytokine production (Fig. 2, 5 and data not shown). CsA acts by forming a complex with immunophilin which inhibits the Ca^{2+}/calmodulin-dependent serine-threonine phosphatase calcineurin. Inactive calcineurin is unable to activate the nuclear factor of activated T cells (NFAT). Previous work by Ghosh and coworkers has demonstrated that NFAT can be activated in a CsA-resistant pathway that is independent of calcineurin [13]. Furthermore, the authors showed that this pathway is induced via CD28, which would explain why CsA inhibition of calcineurin signaling can be overcome by CD28 costimulation. The exact mechanisms for this phenomenon have not been defined, but okadaic acid sensitive serine/threonine phosphatases have been demonstrated to mediate CsA-resistant transactivation of the IL-2 promotor presumably by targeting NFAT [14,15].

Importantly, our data demonstrate that CD28 costimulation does not result in a general insensitivity to immunosuppressive agents, since Aza reduced the proliferation of T cells stimulated via CD28 or alternative costimulatory molecules to a similar extent.

Successful clinical trials established CD28 costimulation blockade mediated by the CTLA-4Ig derivative Belatacept as emerging treatment modality to prevent acute rejection and protect renal function in kidney transplant recipients [16-18]. The results of our study suggest synergistic effects during the combined use of CTLA-4Ig and CsA, which target CD28 and alternative costimulatory pathways, respectively. In standard immunosuppressive regimens high doses of CsA might be required to overcome the low effectivity of this drug to block the activation of T cells through the CD28 pathway. Since very low concentrations of CsA are sufficient to inhibit T cells that do not receive CD28 signals, upon CD28 blockade the adverse effects of CsA could be largely avoided by administering this drug at greatly reduced doses. Current treatment regimens in renal graft recipients aim to achieve CsA-plasma levels of 40-250 ng/ml. Based on the results of our *in vitro* experiments these concentrations are 100 times higher than the median IC_{50} values determined for human T cells activated in absence of CD28 costimulation, which ranged from 0.6 ng/ml to 0.2 ng/ml. Moreover, these concentrations are clearly insufficient to inhibit T cells that receive strong costimulation via CD28 as their IC_{50} value was found to be in the μg-range (Fig. 5A). Importantly, such synergistic effects can not be expected when CTLA-4Ig is combined with Aza and possibly other immunosuppressive agents, which halt the proliferation of T cells regardless of the costimulatory signals they receive.

In some experimental transplant models the administration of CTLA-4Ig was suggested to induce tolerance and promoted long term survival [19,20]. There are studies that found concomitant use of CsA to be detrimental to tolerance induction since it prevented activation-induced cell death (ACID) of effector T cells [21-24]. However, in these studies high concentrations of CsA were used. Moreover, other studies have not observed impairment of long-term graft survival by combined treatment with CTLA-4Ig and CsA [25] and additive effects of CTLA-4Ig and CsA were also described [26,27].

In nonhuman primate models CTLA-4Ig has not been shown to induce indefinite graft survival or tolerance [28,29], which might be due to an important role of CD28 independent T cell activation pathways. In human or primate T cells, the CD58-CD2 interaction functions as a second major T cell activation axis that is much more potent than other non-CD28 costimulators [2,5,30-32]. This pathway is not operative in murine T cells, since mice lack CD58. Furthermore, following chronic stimulation human but not murine $CD8^+$ T cells loose CD28 expression and thus become dependent on alternative costimulatory pathways for activation [33]. Therefore, in humans and primates alternative costimulatory pathways are likely to have more important contributions to T cell activation processes than in rodents and

consequently the efficacy of immunosuppressive drugs in blocking human T cells activated by such signals is of high interest.

CONCLUSION

The efficacy of immunosuppressive drugs in blocking human T cells, activated by different costimulatory signals, is of high interest especially under conditions where standard immunosuppressive drugs are co-administered with agents that specifically block selected costimulatory pathways. The interplay between costimulatory molecules and immunosuppressive agents should be considered when combining these drugs with costimulation blockers.

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AUTHORS' CONTRIBUTION

KD and JL performed experiments and analyzed data. WFP, OM and GZ provided reagents and critically read the manuscript. JL and PS wrote the paper and designed research. All authors critically revised the manuscript and approved the final version.

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Synopsis

It is well established that for efficient T cell activation two signals are required. Signal 1 is generated upon cognate interaction between the MHC-peptide complex with the T cell receptor complex on T cells. Signal 2 is delivered by accessory molecules, expressed on antigen presenting cells (APC), interacting with their receptors on T cells. This second signal is important for regulation, fine tuning and in the case of inhibitory pathways also for the attenuation of T cell responses. Moreover, costimulatory and coinhibitory pathways are potential therapeutic targets in diseases that are associated with aberrant T cell responses. In addition, enhancing costimulatory signals or blocking inhibitory receptors might aid the clearence of pathogens and improve immune responses against tumors. Numerous costimulatory and coinhibitory surface molecules have been implicated in playing important roles in enhancing and downmodulating T cell responses. At the beginning of this work an overview about the hitherto described human activating and inhibitory receptor-ligand pairs is given.

The aim of this thesis was to elucidate the role of different costimulatory and coinhibitory pathways in human T cell activation. In detail, we wanted to evaluate the functional role of human B7-H3 in T cell costimulation. Furthermore, the impact of the immunsuppressive drugs, Cyclosporine A and azathioprine, acting via different costimulatory ligands namely, CD80, CD58, 4-1BBL, ICOS-L and CD54, on human T cell activation was analyzed.

Since APC harbour a plethora of costimulatory ligands, it is difficult to assess the contribution of single molecules to T cell activation. Thus, we have developed an experimental system, called T cell stimulator cells, that allows for analyzing T cells receiving distinct costimulatory signals during the in vitro activation of human T cells. Chapter three provides a detailed description of this system. Importantly, with this system we can assess the net effects of individual costimulatory molecules in the absence of other accessory molecules that also regulate T cell activation on human antigen presenting cells. This T cell stimulator cells can deliver signal 1 to T cells via a membrane-bound anti-CD3 fragment. By expressing human costimulatory and coinhibitory ligands on these cells, their role in T cell activation processes can readily be analyzed. Therefore, T cell stimulator cells are especially suited for the functional evaluation of ligands implicated in costimulatory/coinhibitory processes and the side by side comparison of different costimulatory molecules and. In addition, this system could be used to investigate the contribution of costimulatory signals to the in vitro expansion

of human T cells. Furthermore, these T cell stimulator cells can be used to assess the effects of immunomodulatory drugs acting on T cells stimulated by different costimulatory pathways. In chapter 4 of this study, we have specifically addressed a potential functional dualism of B7-H3 in human T cell activation, since previous reports describe activating and inhibitory functions of this molecule. Using different experimental settings and different T cell subsets we could demonstrate that B7-H3 is a potent inhibitor of human T cell proliferation and cytokine production. Since, murine TREML2 (triggering receptor expressed on myeloid cells like transcript 2) was previously described to serve as a receptor for murine B7-H3, we tested if human TREML2 might also be the receptor for human B7-H3. However, we found that TREML2 neither serves as a receptor for human nor for murine B7-H3.

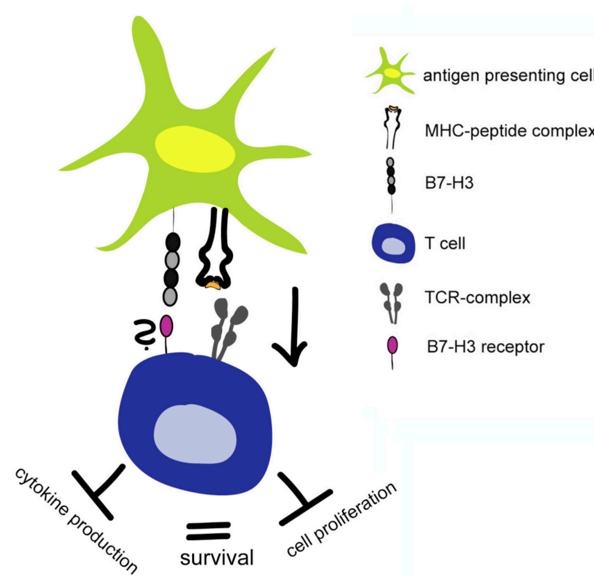

Figure 1: Functional consequences of B7-H3 interaction.

In chapter 5, we showed whether different costimulatory signals could influence the ability of the immunosuppressive drugs Cyclosporine A and azathioprine to inhibit human T cell proliferation. We found that CD28 signals, but not costimulation via CD2, 4-1BB, ICOS or LFA-1 greatly increased the IC_{50} (mean inhibitory concentration) for cyclosporine A. By contrast, the inhibitory effects of Aza were not influenced by the T cell costimulatory signals, mentioned above.

Taken together, T cell activation involves processes governed by complex interplay between numerous activating and inhibitory molecules. Redundancy and differences between costimulatory pathways is still incompletely understood. To assess the role of individual costimulatory ligands it is required to analyze them in a defined system detached from the complex content of APC. In this study, we developed such an approach, designated T cell stimulator cells, and demonstrated that it is an excellent suited tool to study various aspects of

T cell activation, for instance, the effect of immunomodulatory drugs on T cell costimulation or evaluation of ligands implicated in costimulatory processes. Here, we extensively analyzed the functional role of B7-H3 in human T cell activation. However, the receptor(s) for B7-H3 are still not known. It is evident that identification of its receptor(s) would provide a better understanding of its function and furthermore open the field for therapeutic manipulation of this potent T cell inhibitory pathway. In addition, we used our sytem of T cell stimulator cells to investigate the influence of the immunomodulatory drugs azathioprine and cyclosporine A in human T cell activation. The interplay between costimulatory molecules and immunosuppressive agents should be considered when standard immunosuppressive drugs are combined with costimulation blockers. Thus, our results might have implications for combining standard immunosuppressive drugs with CTLA-4Ig fusion proteins, which act by specifically blocking CD28 signaling.

Finally, studies on individual costimulatory pathways, using well defined systems, can complement investigations using experimental systems employing natural human APC or animal studies to get a better insight into the complex interplay of the numerous accessory surface molecules that govern human T cell responses.

Zusammenfassung

Für eine effiziente T-Zellaktivierung sind zwei Signale notwendig. Signal 1 entsteht bei der Interaktion zwischen dem MHC-Peptid Komplex und dem T-Zellrezeptorkomplex. Signal 2, auch als kostimulatorisches Signal bezeichnet, wird von akzessorischen Molekülen, welche auf antigenpräsentierenden Zellen (APC) exprimiert sind, auf deren Rezeptoren, die sich auf T-Zellen befinden, übertragen. Diese Signale spielen eine wichtige Rolle in der Regulation der T-Zellantwort. Überdies stellen diese Signalwege, bei Erkrankungen die mit unerwünschter T-Zellantwort verbunden sind, hervorragende therapeutische Ziele dar.

Eine Verstärkung von kostimulatorischen Signalen kann auch zu verbesserten Immunantworten gegen Tumore und Pathogene führen. In Tiermodellen aber auch in klinischen Studien wurde vielfach gezeigt, dass agonistische und antagonistische Antikörper oder Fusionsproteine hervorragende Werkzeuge zur Manipulation von kostimulatorischen oder koinhibitorischen Signalwegen darstellen. Bis heute ist eine Vielzahl verschiedener kostimulatorischer und koinhibitorischer Liganden beschrieben. Ein Überblick darüber wird im ersten und zweiten Teil dieser Dissertation gegeben.

Das Ziel dieser Arbeit war es, die Rolle von verschiedenen kostimulatorischen und koinhibitorischen Signalwegen in der Aktivierung humaner T-Zellen zu untersuchen. Dafür sollte ein experimentelles System entwickelt werden mit dem es möglich ist die Rolle einzelner kostimulatorischer Signalwege in der T-Zellaktivierung zu evaluieren.

Aufgrund der Tatsache, dass APC eine Vielzahl von Oberflächenmolekülen exprimieren, ist es schwierig den Effekt einzelner Liganden auf die T-Zellaktivierung zu erfassen. Basierend auf einem von uns entwickelten experimentellen System (beschrieben in Teil drei dieser Arbeit) wurde die Funktion ausgewählter Signalwege analysiert. Dieses System, die sogenannten T-Zellstimulatorzellen, basiert auf einer murinen Thymomzelllinie die ein single-chain anti-CD3 Fragment exprimiert und auf diese Weise Signal 1 in T-Zellen induziert. In diesem System kann jedes beliebige kostimulatorische oder koinhibitorische Molekül koexprimiert werden, das dann Signal 2 an die T-Zelle vermittelt. T-Zellstimulatorzellen sind ein ausgezeichnetes Werkzeug um verschiedene kostimulatorische Moleküle miteinander zu vergleichen und die Funktion von Liganden, die eine Rolle bei kostimulatorischen Prozessen spielen, zu evaluieren. Weiters kann damit untersucht werden, welche Effekte immunmodulatorische Medikamente auf T-Zellaktivierung haben.

Der vierte Teil der Arbeit analysiert die Funktion von B7-H3 in der humanen T-Zellaktivierung. In dieser Studie konnten wir in verschiedenen experimentellen Systemen und in verschiedenen T-Zellsubpopulationen zeigen, dass das von B7-H3 vermittelte Signal stark

inhibitorisch auf die T-Zellproliferation und Zytokinproduktion wirkt. Weiters konnten wir TREML2 (triggering receptor expressed on myeloid cells like transcript 2), kürzlich als Rezeptor für das murine B7-H3 beschrieben, als Rezeptor für humanes und murines B7-H3 ausschließen.
In Teil fünf dieser Arbeit wird der Einfluss einzelner kostimulatorischer Signale auf die Wirkung der immunsuppressiven Medikamente Azathioprine und Cyclosporin A behandelt. Wir konnten zeigen, dass die IC_{50} (mittlere inhibitorische Hemmkonzentration) Konzentration für Cyclosporin A durch Signale über CD28 deutlich erhöht wird, während Signale über CD2, 4-1BB, ICOS oder LFA-1 seine inhibitorische Wirkung nicht wesentlich beeinträchtigen. Im Gegensatz dazu wurde der inhibitorische Effekt von Azathioprine durch unterschiedliche kostimulatorische Signale nicht beeinflusst.

Die Aktivierung von T-Zellen wird durch das komplexe Zusammenspiel verschiedenster kostimulatorischer und inhibitorischer Moleküle reguliert. In dieser Arbeit wird ein ausführlicher Überblick über die gegenwärtig beschriebenen kostimulatorischen und koinhibitorischen Signalwege in der humanen T- Zellaktivierung geboten. Weiters entwickelten wir ein experimentelles System, mit dem man hervorragend den Beitrag einzelner Moleküle zur T-Zellproliferation erfassen kann. Mit Hilfe dieses Systems und anderer methodischer Ansätze, wurde eine umfassende Evaluierung der Rolle von B7-H3 in der T-Zellaktivierung durchgeführt.
Darüber hinaus konnten wir zeigen, dass kostimulatorische Signalwege die Wirkung von bestimmten immunsuppressiven Medikamenten stark herabsetzen, während sie andere nicht beeinflussen. Diese Ergebnisse können möglicherweise Einfluss auf Behandlungsstrategien, beispielsweise Kombination von herkömmlichen immunsuppremierenden Medikamenten mit CTLA-4 Fusionsproteinen, welche spezifisch den CD28 Signalweg targeten, haben.

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