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The capacity of the TNF family members 4-1BBL, OX40L, CD70, GITRL, CD30L and LIGHT to costimulate human T cells

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Abstract

Activating signals generated by members of the tumour necrosis factor receptor (TNFR) superfamily upon interaction with their cognate ligands play important roles in T cell responses. Members of the tumour necrosis factor (TNF) family namely 4-1BBL, OX40L, CD70, GITRL, LIGHT and CD30L have been described to function as costimulatory molecules by binding such receptors on T cells. Using our recently described system of T cell stimulator cells we have performed the first study where all these molecules have been assessed and compared regarding their capacity to costimulate proliferation and cytokine production of human T cells. 4-1BBL, which we found to be the most potent molecule in this group was able to mediate sustained activation and proliferation of human T cells. OX40L and CD70 were also strong inducers of T cell proliferation, whereas the costimulatory capacity of human GITRL was significantly lower. Importantly CD30L and LIGHT consistently failed to act costimulatory on human T cells, and we therefore suggest that these molecules might be functionally distinct from the costimulatory members of this family.

Keywords

Human; T cells; Costimulation; Cell Activation

Introduction

Efficient T cell activation requires two different signals, provided by MHC-TCR interaction (signal 1) and additional receptor-ligand interactions that give a costimulatory signal (signal 2) to T cells [1]. Interaction of the immunoglobulin superfamily members CD80/86-CD28 and ICOSL-ICOS can efficiently costimulate proliferation, cytokine production and effector cell generation of T cells [2]. Another group of costimulatory molecules is comprised by

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Conflict of interest

The authors do not declare any financial or commercial conflict of interest.

members of the tumour necrosis factor receptor (TNFR) superfamily, which can costimulate TCR-signals upon interaction with their cognate ligands, members of TNF superfamily. Costimulation of T cell activation has been reported for several members of the TNFR/TNF superfamilies namely for 4-1BB – 4-1BBL, OX40 – OX40L, CD27 – CD70, GITR – GITRL, HVEM – LIGHT and CD30 – CD30L [3, 4]. Interaction of these TNF family ligands with their receptors leads to recruitment of TNFR-associated factors (TRAFs), which initiate signalling cascades that result in T cell activation. All these TNFR can recruit TRAF 2 but there are differences between these molecules in the recruitment of other TRAF proteins [5].

While there is extensive literature on costimulatory pathways involving human 4-1BBL, OX40L and CD70 few reports have described costimulatory functions for human LIGHT and CD30L [6, 7]. Signals transduced by members of the TNFR family are regarded as especially important for survival, expansion and effector function of T cells that have initially received activating signals via the CD28 receptor [4]. Consequently a number of reports have addressed the role of members of the TNFR/TNF families on antigen-experienced T cells and have demonstrated potent and important costimulatory functions for these molecules on virus-specific human T cells [8-11]. Thus blocking or enhancing costimulatory TNFR family molecule interaction has been discussed as promising therapeutic avenue in host defense, autoimmunity, cancer and transplantation associated pathologies [12-14]. However, costimulatory TNFR family members, which are either constitutively expressed on primary T cells or quickly induced upon activation can also effectively costimulate primary human T cells.

In this study we have analysed the capacity of individual members of the TNF family to costimulate human T cells derived from peripheral blood using a recently described novel system of T cell stimulators. This system is based on a cell line that expresses membrane bound anti human CD3 antibodies and thus can give “Signal 1” to human T cells [15]. By expressing accessory molecules on these cells their contribution to activation of human T cells can readily be assessed. We have generated T cell stimulators expressing the individual TNF family ligands and analyzed their capacity to induce proliferation and cytokine production on human T cells. This is the first study where the effects of the human TNF family members that have been implicated in costimulatory processes are compared side by side in the same experimental system.

Results

Expression of TNF receptors on resting and activated human T cells

In a first set of experiments we have analysed the expression of the TNFR family members 4-1BB, OX40, CD27, GITR, CD30 and HVEM on human T cells. T cells were analysed freshly or following stimulation with PMA/ionomycin for 24, 48 and 72 hours. The stage of activation was evaluated by analysing the expression of the activation marker CD25.

CD27 was expressed on primary, unstimulated CD4⁺ and CD8⁺ T cells with the exception of a small subset of CD8⁺ cells. These cells are most likely to represent terminally differentiated effector T cells [16]. *In vitro* activation resulted in the down-modulation of CD27 expression on both the CD4⁺ and the CD8⁺ T cells (Fig. 1). No specific binding of the anti-GITR, anti-4-1BB, anti-OX40 and anti-CD30 antibodies was detected on resting T cells. Up-regulation of these molecules could be detected in the CD4⁺ and the CD8⁺ T cells during the course of the experiments. While there were no significant differences in the up-regulation of GITR and CD30 between the CD4⁺ and the CD8⁺ T cell subsets, CD4⁺ cells induced higher levels of OX40 and CD8⁺ cells preferentially up-regulated 4-1BB during activation. All resting T cells expressed moderate levels of HVEM. In contrast to a previous

study that reported a strong down-regulation of this molecule on human T cells upon *in vitro* activation [17] we did not detect appreciable changes in the expression of HVEM. We also analysed expression of TNFR family members in T cells activated by co-culture with T cell stimulator cells expressing a membrane-bound anti-CD3 antibody. These experiments yielded similar results however up-regulation of CD25 on T cells was weaker as was the up-regulation of activation-induced TNFR family molecules (data not shown).

Generation of engineered T cell stimulators expressing TNF family members

We wanted to assess the costimulatory capacity of human TNF family members in the T cell activation process using a cellular system that allows stimulating human T cells regardless of their specificity. For this we used T cell stimulators, which are based on a murine cell line that expresses a membrane-bound single chain antibody to human CD3 (mb-aCD3scFv). We have previously established T cell stimulator clones that stably express high levels of mb-anti-CD3 and thus give a strong signal 1 to human T cells upon co-culture [15]. This cell line was then retrovirally transduced to generate stimulator cell lines expressing one of the human TNF family members (4-1BBL, OX40L, CD70, GITRL, LIGHT and CD30L) or were mock-transduced (control). For comparison T cell stimulators expressing the B7 superfamily members CD80 and ICOSL were also generated. Expression of the membrane-bound anti-CD3 antibody and the costimulatory molecules on the stimulator cells used in this study is shown in Figure 2. Untransduced Bw5147 cells reacted neither with the antibody used to detect expression of the membrane-bound-anti-CD3 antibody, nor with antibodies to the human costimulatory molecules used in this study (data not shown).

The costimulatory capacity of TNF family members

With the stimulator cells described above we had a system available that allowed us to evaluate the individual contribution of 4-1BBL, OX40L, CD70, GITRL, LIGHT and CD30L to human T cell activation processes. Human T cells purified from peripheral blood were co-cultured for 72 hours with stimulator cells expressing different costimulatory molecules on their surface and T cell proliferation was assessed. Figure 3A shows the results of a representative experiment. Presence of 4-1BBL, CD70, and OX40L induced strong proliferative responses in human T cells, whereas the capacity of GITRL to induce proliferation was much weaker. T cell stimulators expressing CD30L and LIGHT did not induce stronger proliferative response than mock transduced cells. The B7 molecule CD80, which can be regarded as the primary costimulatory molecule, consistently induced the strongest proliferative response, whereas ICOSL on our stimulator cells induced weaker T cell proliferation and was in its potency comparable to OX40L and CD70. Consistent results regarding the capacity of TNF superfamily members to induce proliferative responses of T cells from different donors were obtained. The results of independently performed experiments with 10 donors are summarized in Figure 3B. CD80 induced a significantly higher proliferative response than any other costimulatory molecule tested in this study. 4-1BBL was the most potent costimulatory molecule among the TNF family members and induced also higher T cell proliferation than ICOSL. OX40L and CD70 did not significantly differ in their costimulatory potential and induced stronger response than GITRL, which still induced significantly higher proliferation than control stimulator cells expressing no costimulatory molecule. There was no difference in the proliferation induced by stimulator cells expressing LIGHT and control stimulator cells. We found that stimulator cells expressing CD30L induced significantly less proliferation in human T cells than control stimulator cells but the difference between these T cell responses was very small.

We wanted to exclude the possibility that the proliferative responses to different TNF family molecules would be affected by the expression levels of these molecules on our stimulator cells. Therefore we also tested their costimulatory capacity in a system where the expression

levels of different TNF family molecules can be compared. For this we engineered expression constructs where a myc-tag sequence is inserted between the trans-membrane domain and the extra-cellular domain of the TNF family molecule (Fig. 3C). T cell stimulator cell lines expressing these constructs were generated and we employed FACS sorting in conjunction with an anti-myc antibody to generate stimulator cell lines carrying comparable levels of chimeric TNF family members on their surface (Fig. 3D). Co-culture experiments with these cell lines and human T cells confirmed the general trend regarding the costimulatory capacity obtained with stimulator cells expressing untagged TNFL in showing that 4-1BBL is the most potent costimulator of this group (Fig. 3E). Stimulator cells expressing myc-tagged CD30L did not induce stronger proliferation than control stimulator cells (Fig. 3E). An inhibitory effect of the myc-tagged CD30L on human T cell proliferation was observed when it was expressed at higher levels (data not shown). Expression of the chimeric LIGHT construct could not be detected by FACS and this molecule was therefore not included in these experiments.

Costimulation of naïve human T cells

The peripheral blood of adults contains significant amounts of antigen-experienced T cells and we have therefore also analysed the capacity of TNF family members to costimulate naïve T cells isolated from peripheral blood of adult donors and purified human umbilical cord T cells. Our results demonstrate that the TNF family members 4-1BBL, OX40L, CD70 also strongly costimulate the proliferation of naïve human T cells purified from adult donors (Fig. 4A). The results obtained with cord blood cells, which contain over 90% naïve T cells were similar to the results obtained with naïve T cells from adults with the exception of CD70, which was the most potent costimulator among the TNF family on cord blood cells (Fig. 4B). Presence of GITRL did only slightly enhance proliferation of naïve T cells and LIGHT and CD30L failed to show any costimulatory effects also on naïve peripheral T cells and on cord blood cells. (Fig. 4).

4-1BBL mediates sustained proliferation in human peripheral blood T cells

Strong T cell activation results in production of cytokines and it is well established that the presence of certain signals during T cell activation can influence the cytokine profile expressed by these cells. We wanted to compare on one hand the potency of different costimulatory molecules to induce cytokine production in human T cells, and on the other hand we were interested to analyse whether different costimulatory molecules induce different cytokine profiles in human T cells. Stimulator cells were co-cultured with peripheral blood human T cells, and the cytokine production was examined 48 hours later by measuring the cytokine levels in the supernatants of these co-cultures.

Our results show that TNF family members that strongly enhanced proliferation of human T cells were also potent inducers of cytokines. CD80 and 4-1BBL induced high levels of cytokine expression (Fig. 5A). Stimulator cells expressing CD30L or LIGHT that did not costimulate T cell proliferation also failed to induce higher levels of cytokines than stimulator cells expressing no costimulatory molecule (Fig. 5A). Whereas our results clearly show that the tested costimulatory molecules differ in their potency to induce cytokine expression in peripheral blood human T cells they do not point to the induction of a different cytokine profile by any of the costimulatory molecules analysed in this study.

Induction of IL-2 production is regarded to be especially important for sustained T cell responses. Only costimulatory signals generated by CD80 and 4-1BBL consistently induced significant levels of IL-2 in our experiments. To analyse the ability of different costimulatory signals to induce prolonged T cell proliferation we activated T cells with our set of stimulator cells and assessed ³[H]thymidine uptake in the co-cultures at later time

points. We found that 4-1BBL, like the B7 family member CD80, mediated sustained activation and proliferation of human T cells, resulting in an increase of the proliferative response during the course of the experiment (6 days). In contrast, when T cells were activated in the presence of other costimulatory TNF family members or ICOSL their proliferative responses declined after the 5th day of activation (Fig. 5B).

Stimulation of CD4⁺ and CD8⁺ T cells by TNF family members

Some costimulatory molecules have been described to be especially important for the activation of the CD4 or the CD8 subset. 4-1BB is generally regarded as a potent activator of CD8⁺ T cells [18, 19], whereas many reports describe the OX40-OX40L pathway to preferentially costimulate proliferation of CD4⁺ T cells [20, 21]. However it has been reported that CD4 and CD8 T cells can receive strong costimulatory signals via 4-1BB and OX40, respectively [22, 23]. There is little information on the capacity of other human TNF molecules to induce activation of the CD4 and CD8 subset. To analyse this we performed two types of experiments. In the first experiments we subjected purified T cells to CFSE labelling and subsequently co-cultured them with our set of stimulator cells. Following six days of co-culture, the cell cycling profile of CD4⁺ and CD8⁺ T cell were assessed by four colour flow cytometry using labelled CD4 and CD8 antibodies. Dead cells were excluded by the use of PI. In these experiments that assessed the proliferation of CD8⁺ T cells in the presence of CD4⁺ T cell help, CD8⁺ T cells were more prone to proliferate in response to co-culture with our stimulator cells than CD4⁺ T cells. In co-cultures with stimulator cells expressing no costimulatory molecule on their surface but giving Signal 1 to T cells 14.1% of the CD8⁺ cells had undergone cell division, whereas only 3.6% of the CD4⁺ cell had divided. Several independent experiments with CFSE-labelled cells were performed, and 4-1BBL constantly gave the strongest costimulatory signal of all TNF molecules analysed. When taking the lower proliferative response of the CD4 subset to stimulation in the absence of costimulation into account there was no evidence that any of the TNF ligands preferentially induced proliferation of the CD4 or CD8 T cell subset (Fig. 6A). In the second set of experiments purified CD4⁺ and CD8⁺ T cells were co-cultured with our stimulator cells and their proliferation was assessed following 72 hours of co-culture. Also in these experiments 4-1BBL induced the strongest proliferation among the TNF family members in both subsets. In contrast, compared to its effects on the CD4⁺ T cells the capacity of OX40L to enhance proliferation of CD8⁺ T cells was much weaker. Thus whereas the weaker effect of OX40L on CD8⁺ T cells is correlated with a weaker expression of OX40 on these cells, 4-1BBL strongly costimulated CD4 T cells despite a weak expression of 4-1BB on this T cell subset (Fig. 1). T cell stimulator cells expressing LIGHT or CD30L failed to induce stronger proliferation of CD4⁺ and CD8⁺ T cells than control T cell stimulators (Fig 6B).

4-1BBL but not CD80 costimulate the proliferation of CD28⁻CD8⁺T cells

Exhaustion and senescence of human T cells can lead to a complete loss of CD28 [24] and under such circumstances CD28⁻ T cells can comprise the majority of the CD8⁺ population. The inability of this T cell subset to receive activating signals through the primary and most potent costimulatory receptor might contribute to the compromised immune function in elderly or individuals suffering from chronic virus infection. We therefore analysed the capacity of different costimulatory molecules to activate such cells. CD28⁻CD8⁺ human T cells from elderly persons were isolated and co-cultured with our set of T cell stimulator cells. In contrast to CD28⁺CD8⁺ T cells from the same donors (data not shown) these cells generally showed low proliferative responses upon stimulation. CD80 failed to induce significant proliferation of CD28⁻CD8⁺ T cells indicating that triggering the TCR-complex does not reinstall CD28 expression. T cell stimulators expressing 4-1BBL induced the highest proliferation in CD28⁻ T cells whereas the effect of other TNF molecules and ICOSL was much weaker (Fig. 7).

TNF family members synergize with other costimulatory molecules

To analyse the cooperative effects between members of the B7 and the TNF family but also between different TNF family members, we generated stimulator cells expressing two costimulators on their surface. Single cell clones of stimulator cells expressing 4-1BBL or CD70 were established and were mock-transduced or transduced to express another costimulatory molecule either of the TNF superfamily, or ICOSL (Fig. 8). Activation of human T cells with stimulator cells expressing two costimulators give evidence for an enhanced costimulatory effect (Fig. 8). This was not only seen when testing combinations of 4-1BBL or CD70 with the B7 family member ICOSL but also when stimulating human T cells in the presence of two different TNF superfamily members (4-1BBL and CD70; Fig. 8). Similar results were observed when T cells were stimulated with OX40L in combination with ICOSL or 4-1BBL (data not shown). Furthermore as expected we found that the addition of soluble CD28 mAb to co-cultures of T cells with stimulator cells harbouring costimulatory TNF family members increased their proliferative response in a dose-dependent manner (data not shown).

Discussion

The immune system employs many different receptor-ligand pairs that contribute to the activation of T cells. Currently this redundancy among costimulators is incompletely understood. While numerous studies have addressed the contribution of individual costimulatory molecules, data where such molecules have been compared are much more limited [10, 11, 25]. Furthermore, as most previous studies have focused on the role of TNF/TNFR family molecules on antigen-experienced T cells and have shown important costimulatory functions on such cells much less is known on the ability of these molecules to activate primary human T cells. In this study we wanted to use the same experimental system to compare all TNF family members that have previously been implicated in costimulatory processes [4] regarding their capacity to activate human T cells. For this we have used a previously described system of T cell stimulators that is based on a cell line that was engineered to express a membrane-bound anti-human CD3 antibody fragment. By ligating the TCR complex these cells can give “Signal 1” to human T cells regardless their specificity [15]. By expressing the TNF family members 4-1BBL, OX40L, CD70, GITRL, CD30L and LIGHT on this cells lines we have created an unique tool to study the function of individual costimulatory molecules in one system. For comparison we have also analysed the B7 superfamily members CD80 and ICOSL.

4-1BBL, which induced strong proliferation and cytokine production was found to be the most potent T cell costimulator of the TNF family. Like CD80, which gave the strongest costimulatory signal it was able to induce sustained activation in human T cells. OX40L and CD70 strongly supported proliferation and cytokine production but they were found to be significantly weaker costimulators than 4-1BBL. While there are many reports on the function of 4-1BBL, OX40L and CD70 fewer studies have addressed the functional role of GITRL in human T cell activation. Although GITRL induced significantly lower proliferation than the other costimulatory members of the TNF family it consistently enhanced proliferation and cytokine production in human T cells. GITR is highly expressed on natural Tregs and addition of anti-GITR to co-cultures of Tregs and CD25⁻ T cells results in loss of suppression [26]. We observed a comparable costimulatory effect of GITRL on our stimulator cells when we activated Treg-depleted (CD25⁻) T cells (data not shown). This indicates that at least most of the costimulatory effects of GITRL obtained in our study are independent of Tregs.

In order to rule out the possibility that differences in the costimulatory effects of TNF family members are due to different expression levels we have also performed experiments where

the expression levels of these molecules can be compared. For this we generated constructs that encoded chimeric TNF family molecules harbouring a peptide-tag. By activating T cells with stimulator cell lines that express similar levels of these chimeric molecules on their surface we could show that the results obtained with the untagged TNF family members reflect the different costimulatory capacities of these molecules. The importance of costimulatory TNF family members like OX40L and 4-1BBL is often regarded to lie mainly in enhancing the responses of T cells that have initially been activated by the B7 molecules. However, we have analysed the capacity of these molecules to stimulate CD45RA⁺ T cells or cord blood T cells, which contain over 90% naïve T cells [27] and show that they can very efficiently costimulate the proliferation of these cells. This indicates that costimulatory TNF family members may also be important in the activation of naïve human T cells.

Costimulatory molecules that induced higher proliferation in T cells also induced production of high levels of cytokines. Furthermore we show that only 4-1BBL and CD80 induce significant amounts of IL-2 and this might contribute to the ability of these two costimulatory ligands to induced sustained proliferation of T cells (Fig. 4B). Importantly we found no evidence for a distinct cytokine pattern induced by any of the costimulatory molecules analyzed in this study.

The most unexpected finding of our study was that CD30L and LIGHT consistently failed to costimulate proliferation and cytokine production of human T cells. It is very unlikely that this is due to misfolding of these molecules as the integrity of our expression constructs was checked by DNA sequencing and stimulator cells expressing CD30L or LIGHT strongly bound specific antibodies to these molecules (Fig. 2). In addition, HVEM-Fc specifically and very strongly bound to LIGHT expressing stimulator cells (data not shown). We have recently described T cell stimulator cells that express very low levels of membrane-bound anti-CD3 antibodies and thus give a weak activation signal to human T cells that is not sufficient to induce proliferation or cytokine production [15]. We have also analysed the costimulatory capacity of TNF family members on such cells. Importantly LIGHT and CD30L also failed to act costimulatory in the context of a weak “Signal 1” (data not shown). Furthermore we have not observed stimulatory effects of LIGHT or CD30L on pre-activated T cells (data not shown). While there are several studies that report on costimulatory effects of LIGHT on human and mouse T cells [7, 28, 29] more recently it was shown that this molecule is completely dispensable for immune responses to influenza A virus in mice [30]. In this study the authors found that deficiency in LIGHT did neither affect primary expansion nor memory responses of T cells in their model. Furthermore it has recently been shown that 4-1BBL and CD70 but not LIGHT can costimulate cytokine production, expansion and effector function of virus-specific human CD8⁺ T cells [11]. In our study the presence of CD30L resulted in a small but statistically significant down-regulation of human T cell responses (Fig. 3B). Pleiotropic effects of CD30 are a well described phenomenon and several reports show inhibitory function of this molecules on lymphoma cells and cell lines upon interaction with CD30L [31, 32] but to our knowledge to date such a function has not been shown on T cells. However, additional studies are warranted to confirm a negative role of CD30L in the human T cell responses. Our results that show that CD30L and LIGHT consistently failed to costimulate human T cells under conditions where 4-1BBL, OX40L, CD70 and GITRL readily promoted proliferation and cytokine production strongly suggests that these two molecules might be functionally distinct from the costimulatory members of the TNF family. Importantly the lack of costimulatory functions of these molecules are not due to absence of their receptors since HVEM and CD30 are both present on activated T cells (Fig. 1).

Efficient activation and expansion of primary T cells is a prerequisite for effective immune responses and the generation of sufficient memory T cells. Furthermore the importance of

CD4⁺ T cell help for the generation of CD8⁺ T cell immunity *in vivo* is underlined by the impaired CTL responses that are associated with the decline of the CD4⁺ T cell subset during HIV infection. Thus our results that identify 4-1BBL as the most potent costimulatory TNF family member for peripheral blood human CD4⁺ and CD8⁺ T cells further underscore the significant therapeutic potential of enhancing 4-1BB signals that was deduced from previous studies on pathogen-specific human CD8⁺ T cells. In addition our results demonstrate that among the members of the TNF family 4-1BBL is the most potent activator for T cells lacking the primary costimulatory receptor CD28.

Currently there are numerous attempts to combat cancer by immunizing tumour patients with autologous monocyte-derived DC loaded with tumour antigens. Neither costimulatory TNF members nor ICOSL can be detected on the surface of these cells (our unpublished results) and there are *in vitro* data showing that human DC engineered to express TNF family ligands might induce efficient T cell responses [33, 34]. Cooperative effects between different costimulatory TNFR molecules have been demonstrated in animal models and on virus-specific human T cells [11, 25, 35] and we show here that proliferative responses to stimulation in the presence of high levels of 4-1BBL can be further enhanced by the B7 family member ICOSL but also the TNF family member CD70. Thus expressing 4-1BBL or combinations of 4-1BBL with other potent costimulatory molecules identified in this study might enhance the ability of DC-based cancer vaccines to induce efficient immune responses to tumours.

Material and Methods

Media and reagents

Cells were cultured in RPMI-1640 + L-Glutamine medium (Gibco Ltd., Paisley, Scotland) 1% Penicillin/Streptomycin (PAA, Pasching, Austria) and 0.4% Amphotericin (250 µg/ml, PAA) and 10% of fetal bovine serum (FBS) (Gibco). Propidium iodide (PI) was obtained from Sigma (Deisenhofen, Germany) and was added in a concentration of 0.1 µg/ml. CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) was purchased from Molecular Probes (Eugene, OR).

Antibodies and fluorescence staining

The mAb to CD80 (7-480), CD27 (VIT14a), CD30 (BerH2), CD25 (3G10), and the non-binding control Ab VIAP (calf intestine alkaline phosphatase specific) were produced in our laboratory. The mAbs to ICOS-L (2D3), 4-1BB/CD137 (4B4-1), 4-1BBL/CD137L (C65-485), CD30L (D2-1173), CDw70 (Ki-24), OX40 (ACT35), OX40L (ik-1), CD4 (RPA-T4; PE conjugated) and CD8 (RPA-T8; APC conjugated) were purchased from Becton Dickinson (Palo Alto, CA). GITRL m-Ab (EB11) was purchased from eBioscience (San Diego, CA). LIGHT-mAb (115520) was from R&D Systems (Minneapolis, MN) and HVEM-mAb (Clone 112) from Lab Vision Cooperation (Freemont, CA).

For flow cytometric analysis, cells ($0.5-2 \times 10^5$) were incubated with fluorochrome-conjugated mAbs or unlabeled primary antibody (10 µg/ml) for 20 minutes on ice and washed. Binding of primary antibodies was detected with PE-conjugated goat anti-mouse IgG Fcγ specific Abs (Jackson ImmunoResearch; West Grove, PA). For analysis of expression of the membrane-bound anti-CD3 antibody fragment on our stimulator cells a PE-conjugated goat anti-mouse IgG Ab from Caltag (Burlingame, CA) that reacts with the variable regions of the single chain antibody was used. Myc-tagged TNF family molecules were detected with rabbit anti-myc antibodies (10 µg/ml; Sigma) in conjunction with PE-conjugated donkey anti-rabbit-Ig-Abs (Jackson ImmunoResearch). Flow cytometric analysis was performed using a FACScalibur flow cytometer supported by CELLQUEST software

(Becton Dickinson). In all histograms the fluorescence intensity is shown on a standard logarithmic scale. CFSE labeling experiments were performed as described [36].

Generation of T cell stimulator cells

cDNAs encoding human TNF family molecules 4-1BBL, OX40L, CD70, GITRL, LIGHT and CD30L were PCR amplified from a retroviral cDNA expression library generated from human mdDC [37] or from cDNA prepared from human T cells activated using PMA/Ionomycin. PCR products were cloned into the retroviral expression vector pBMN [37]. The integrity of the resulting constructs was confirmed by DNA sequencing. Expression plasmids encoding human ICOSL or CD80 have been described [15].

Using retroviral transduction as described [15], these constructs were expressed on our system of T cell stimulator cells. The control stimulator cell line was transduced with retrovirus-containing supernatant derived from cells transfected with the pBMNZ plasmid containing the lac Z gene. T cell stimulator cells that are based on Bw5147 (referred to as Bw cells), a murine thymoma cell line expressing a membrane-bound single chain fragment of the variable regions of the anti-CD3 antibody (mb- α CD3scFv of clone OKT3 [38]) at high density have been described [15]. Expression constructs encoding myc-tagged 4-1BBL, OX40, CD70, GITRL and CD30L were made by generating PCR fragments with forward primers specific for the N-terminal end of the molecule and reverse primers specific for the myc-tag and the transmembrane domain of the TNF family molecule. A second set of PCR fragments were generated using a forward primer specific for the myc-tag and the N-terminal end of the extra-cellular domain of the TNF family molecule and the reverse primer specific for the C-terminal end of the molecule. The two sets of PCR-fragments were purified, combined by PCR employing the primers specific for the N-terminal and the C-terminal ends of the molecules. The final products were cloned into the retroviral expression vector pBMN and their integrity was confirmed by DNA sequencing. For transduction of stimulator cells to express two costimulatory molecules, single cell clones of stimulator cells expressing a human costimulatory molecule at high density were established. These cells were subsequently retrovirally transduced to express an additional costimulatory molecule or were mock-transduced. Expression of the costimulatory molecules on the stimulator cells was analysed by flow cytometry.

T cell proliferation assays and cytokine measurement

This study was approved by the ethical review board of the Medical University of Vienna and informed consent was obtained from the participating subjects. Human T cells were purified from PBMCs of healthy volunteers by MACS through depletion of CD11b, CD14, CD16, CD19, CD33 and MHC-II harbouring cells with the respective mAbs as described [36]. For the purification of CD45RA⁺ cells PBMCs were depleted from cells harbouring CD11b, CD14, CD16, CD19, CD33 MHC-II and CD45RO using MACS. The purity of T cells used in this study was 96% $\pm$ 2% as determined by FACS analysis. Cord blood T cells were purified by MACS using the same protocol. Umbilical cord blood from healthy donors was collected during full-term deliveries. Cord blood T cells used in this study were 90% CD45RA⁺ and CD45RO⁻. CD8⁺ T cells and CD4⁺ T cells were purified from monocyte-depleted PBMC using MACS in conjunction with antibodies to CD4 and CD8. CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells were enriched from PBMC derived from healthy elderly person in a series of separations using magnetic beads as previously described [39]. The purity of CD4⁺, CD8⁺ and CD8⁺CD28⁻ T cells was more than 90%. For T cell proliferation assays purified human T cells (1×10^5 /well) were co-cultured with irradiated T cell stimulator cells (2×10^4 /well) and uptake of methyl-³[H]thymidine was measured as described [15]. For cytokine measurement human T cells (1×10^5 /well) were co-cultured with irradiated T cell stimulator cells (2×10^4 /well). After 48 h the supernatants

of the co-cultures were collected and pooled from triplicate wells. Interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), interleukin 13 (IL-13), interferon- γ (IFN- γ), and tumor necrosis factor alpha (TNF- α) were measured in cell culture supernatants using the Bio-Plex Human Cytokine 8-Plex Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers instruction.

Statistics

Statistical significances were assessed using the two-tailed wilcoxon signed-ranks test. Differences were considered significant at $p < 0.01$.

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Abbreviations used in this paper

HVEM	Herpes virus entry mediator
TNFR	tumour necrosis factor receptor
TRAF	TNFR-associated factors
scFv	single chain fragment of the variable regions

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Figure 1.

Expression of TNFR family members on freshly isolated and in vitro activated human CD4⁺ (A) and CD8⁺ (B) T cells. Resting T cells or T cells activated in vitro using PMA/Ionomycin for the indicated time were analysed for expression of the activation marker CD25 or TNFR family members (grey histograms). Open histograms represent staining with isotype control antibody with T cells. anti-CD4-PE and anti-CD8-APC antibodies were used to identify CD4⁺ and CD8⁺ T cells. The experiment shown is representative for four independently performed experiments.

Figure 2.

Characterization of T cell stimulator cell lines. A Bw5147 cell clone engineered to express high levels of membrane-bound anti-CD3 antibody was retrovirally transduced to express human CD80, ICOSL or one of the human TNF family members 4-1BBL, OX40L, CD70, GITRL, CD30L or LIGHT. The resulting T cell stimulator cell lines were analysed for expression of membrane-bound anti-CD3 antibody (open histograms) and these molecules (grey histograms). The open histograms in the left middle and the right panels represent reactivity of isotype control antibodies.

Figure 3.

The capacity of TNF family members to costimulate proliferation of primary human T cells. (A) Human T cells were co-cultured for 72 hours with stimulator cell lines expressing the indicated human molecules or control stimulator cells expressing membrane-bound anti-CD3 only. T cell proliferation was determined by assessing ^3H thymidine uptake. (B) Box-blots representing independent experiments with ten donors performed as described in A. The proliferative responses to stimulator cells expressing the indicated molecules are shown as fold induction compared to the responses to control stimulator cells. Black dots represent outliers. For each stimulator cell type shown in the diagram the stimulator cells that have induced significantly higher proliferative responses are indicated: Stars above the box blots indicate that the stimulator cells listed on the left of the star induced a significantly higher ($p > 0.01$) proliferative response than the stimulator cell line represented by the box blot. (C) Schematic representation of chimeric TNF family molecule with myc-sequence inserted between transmembrane domain and c-terminal extra-cellular domain. (D) Stimulator cells expressing myc-tagged TNF family molecules were sorted for similar expression of these molecules. The resultant cell lines were probed with antibodies specific for the myc-tag of the chimeric molecules (upper panels) and with antibodies specific for the TNF family members as indicated (lower panels) The mean fluorescence intensity (MFI) is shown. (E) Human T cells were activated with stimulator cell lines expressing the indicated chimeric

molecules or with control stimulator cells. This experiment was repeated three times with similar outcome.

Figure 4. The capacity of TNF family members to costimulate the proliferation of naive T cells. (A) CD45RA⁺ human T cells were co-cultured for 72 hours with the indicated stimulator cell lines. (B) T cells purified from umbilical cord blood were co-cultured for 72 hours with the indicated stimulator cell lines. Each type of experiment was performed three times with similar outcome.

Figure 5. 4-1BBL potently costimulates cytokine production and can mediate sustained proliferation of human T cells. (A) T cells were stimulated for 48 hours with stimulator cell lines. The concentration of cytokines in the culture supernatant was determined using a multiplex assay. The experiments shown are representative for six independently performed. (B) 4-1BBL mediates strong and sustained proliferation of primary human T cells. Human T cells were co-cultured with stimulator cell lines expressing the indicated molecules for 96, 120 and 144 hours. ³[H]-thymidine uptake was measured to assess T cell proliferation. This experiment was repeated three times with similar outcome.

Figure 6.

Stimulation of CD4⁺ and CD8⁺ T cell subsets by TNF family members. (A) CFSE-labelled human T cells were co-cultured for six days with stimulator cells. CD4⁺ and CD8⁺ T cells were identified by counter-staining T cells with CD4-PE and CD8-APC antibodies. (B) Purified human CD4⁺ and CD8⁺ T cells were co-cultured for 72 hours with stimulator cell lines and proliferation was determined by three times with similar outcome.

Figure 7. 4-1BBL costimulates the proliferation of CD28⁻CD8⁺ T cells. CD28⁻CD8⁺ human T cells from elderly persons were isolated and co-cultured with stimulator cells. Proliferation was determined by measuring ³[H]thymidine uptake following 72 hours of co-culture. The inset shows expression of CD8 and CD28 by the T cells used in this experiment. One of two experiments with similar outcome is shown.

Figure 8.

TNF family members synergize with other costimulatory molecules. (A) A single cell clone of a stimulator cell line expressing 4-1BBL was transduced to express a second costimulatory molecule (ICOSL or CD70) or was mock-transduced. Expression of the costimulatory molecules was analysed by FACS (grey histograms: reactivity of antibodies to the costimulatory molecules as indicated; open histograms: reactivity of isotype control antibodies). Proliferative responses of primary human T cells co-cultured for 72 hours with stimulator cells expressing the indicated costimulatory molecules is shown (right panel). (B) A single cell clone of a stimulator cell line expressing the costimulator CD70 was transduced to express ICOSL or 4-1BBL or was mock-transduced. Expression of the costimulatory molecules was analysed by FACS as described above. The proliferative responses of primary human T cells to the resultant cell lines are shown on the right. These experiments were repeated three times with similar outcome.