Co-stimulation with 4-1BB ligand allows extended T-cell proliferation, synergizes with CD80/CD86 and can reactivate anergic T cells

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Abstract

Activation of T cells requires co-stimulation, in addition to signals through the antigen–receptor complex. Antigen encounter without adequate co-stimulation results in T-cell desensitization or anergy, a mechanism of peripheral tolerance and an apparent obstacle to cancer immunotherapy. One important co-stimulatory pathway involves CD28 engagement by CD80 or CD86. However, other ligand–receptor pairs can also provide co-stimulation and may have important functions modulating the immune response. Previous reports indicated that co-stimulation using 4-1BB ligand (4-1BBL) or agonistic anti-4-1BB antibodies could prolong T-cell responses, avoid activation-induced cell death and promote anti-tumour responses in mice. To further investigate the potential for cancer immunotherapy, we studied the effects of CD80/CD86 and 4-1BBL in repeated stimulation of human T cells and asked whether 4-1BBL might be capable of reversing anergy. We expressed CD80, CD86 and 4-1BBL in A549 lung carcinoma cells using adenovirus vectors and co-cultured these with human T cells stimulated with anti-CD3 antibody. Proliferation co-stimulated by CD80 or CD86 was transient; however, 4-1BBL-co-stimulated cultures continued to proliferate for up to 5 weeks, with repeated stimulation. Combined co-stimulation with CD80/CD86 and 4-1BBL also allowed continuous proliferation at a faster rate than either signal alone. Co-stimulation with 4-1BBL did not suppress expression of the inducible, inhibitory CD80/CD86R, CTLA-4. Significantly, we show that T cells that had become non-responsive to anti-CD3, either alone or together with CD80/CD86 co-stimulation, and thus were anergic, could be reactivated to proliferate when costimulated with 4-1BBL, either alone or combined with CD80/CD86.

Introduction

Activation of T cells requires both engagement of the TCR with a cognate peptide–MHC complex and an additional co-stimulatory signal. The best known co-stimulatory ligands are members of the B7-family, B7-1 (CD80) or B7-2 (CD86), that are expressed on professional antigen-presenting cells (APCs) such as dendritic cells (DCs); these act through their receptor CD28 on T cells (1). CTLA-4 (CD152) is a second receptor for CD80 and CD86, which is not expressed on most resting T cells but is induced upon T-cell activation. The interaction of CD80/CD86 with CTLA-4 has higher affinity than that with CD28, and down-regulates T-cell activation (2). Thus, CTLA-4 effectively competes with CD28 for CD80/CD86 at later stages of the immune response to suppress the activation and bring about activation-induced non-responsiveness (AINR) and/or activation-induced cell death (AICD) (3). Down-regulation of T-cell function by CTLA-4 engagement appears to play a key role in development of T-cell tolerance or anergy towards self- or tumour antigens (4-6), while antigenic stimulation in the absence of co-stimulatory signals is also tolerogenic (7-9).

A number of alternative ligand–receptor pairs capable of providing co-stimulatory signals to T cells have been identified, including 4-1BB ligand (4-1BBL). 4-1BBL is a member of the tumour necrosis factor family (10-12). Its receptor...
4-1BB (CD137) is absent from resting T lymphocytes but rapidly expressed upon antigenic stimulation. 4-1BB can transmit co-stimulatory signals to T cells, which appear to modulate the magnitude and duration of the immune response and the size of subsequent immune memory compartments (13, 14). In mice, 4-1BB engagement was shown to inhibit AICD following T-cell stimulation by superantigen (13). In view of the role of CTLA-4 in curtailing T-cell activation, this raises the possibility that one mechanism by which signalling via 4-1BB exerts its effects might be to down-regulate expression of CTLA-4.

Early studies implied that 4-1BB stimulation acted principally upon CD8* lymphocytes (15); however, others reported equivalent effects on both CD4* and CD8* subsets (16). Studies using knockout mice found that 4-1BB deficiency only caused a modest reduction in the primary CD4* T-cell response and had no effect on the magnitude of primary responses of CD8* T cells, although co-stimulation via 4-1BB could substitute for CD28 signalling in CD28-deficient mice (17, 18). In contrast, the secondary response of CD8* T cells was highly dependent upon 4-1BB, which was also the case for some, though not all secondary CD4* T-cell responses (14, 17–19).

Co-stimulation via 4-1BB permits responses at lower levels of signalling through the TCR-CD3 complex and CD28 and promotes Th1 differentiation/cytokine profiles in CD4* cells (20, 21). This and the increased duration and magnitude of immune responses co-stimulated via 4-1BB would appear to be important in the context of cancer immunotherapy, and indeed stimulation of this pathway in mice can elicit large, established, poorly immunogenic tumours (22–25). Immune stimulation via 4-1BB promoted eradication of tumours when CD80 was ineffective (26); this required only CD8* cells, although CD4* cells were necessary for a protective, memory response (27). In such assays, 4-1BB acted synergistically with either CD80 (23) or IL12 (28, 29), or an agonistic peptide (25). The efficacy of 4-1BB or agonistic anti-4-1BB antibodies in promoting immune rejection of poorly immunogenic tumours could be ascribed simply to enhancing the co-stimulatory signal to naive, tumour-specific T cells (25). However, the efficacy even in a diversity of models involving pre-established tumours suggests that 4-1BB co-stimulation may be able to overcome anergy, to reactivate tumour-specific T cells that have been tolerized through prior, poorly immunogenic encounter with tumour antigens.

The effects of 4-1BB co-stimulation on T-cell activation, and on the magnitude and longevity of response, as well as the encouraging therapeutic activity in murine tumour models, suggest that manipulation of 4-1BB co-stimulation could be beneficial for immunotherapy of human cancer. In this paper, we report the establishment of an in vitro system to study the effects of 4-1BB and other co-stimulatory ligands on human T-cell responses, using replication-defective adenoviruses to express the ligands on A549 lung carcinoma cells. This system could be extended to investigate the effects of 4-1BB and other co-stimulatory signals on antigen-specific responses, including tumour-specific T cells from cancer patients, and potentially also to therapeutic applications. Localized expression of immunostimulatory ligands using gene transfer approaches may reduce the risk of excessive, generalized immune activation seen in some clinical trials of systemically delivered antibodies (30, 31). Here, we investigate the proliferative response of normal human T cells to co-stimulation in vitro with CD80, CD86 and 4-1BBL. We show that 4-1BBL can synergize with CD80 or CD86 to increase T-cell proliferation and permits extended T-cell proliferation continuing over several weeks. Importantly, we demonstrate for the first time that co-stimulation with 4-1BBL can reactivate T cells that have been anergized by prior, sub-optimal antigenic stimulation.

Methods

Recombinant adenoviruses for expression of co-stimulatory proteins

The E1-deleted, replication-defective adenoviruses (Ad-5) expressing human CD80 (Ad-CD80) or enhanced green fluorescent protein (GFP, used as a control) were described previously (32). The coding region of human 4-1BBL was obtained by reverse transcription–PCR from RNA from a lymphoblastoid cell line (LCL) and that for human CD86 using RNA from DCs. Both were initially cloned into the plasmid pXlNCX (32) and the correct sequence confirmed, before insertion into adenovirus vectors essentially similar to Ad-CD80, but with a larger E1 deletion (between bp 357 and 3525 of Ad5). Viruses were grown in HEK293 cells and purified by CsCl density gradient ultracentrifugation. The concentration of virus particles was determined by DNA assay using the fluorescent dye PicoGreen (Molecular Probes, Invitrogen, Paisley, UK).

The lung carcinoma cell line A549 was infected with the recombinant viruses at a multiplicity of infection (MOI) of 300 virus particles (vp) per cell (for co-infection with Ad-CD80 or Ad-CD86 and Ad-4-1BBL, 300 vp per cell of each virus), before plating in 96- or 24-well plates, depending on the experiment. Infections were performed 2 days before the A549 cells were required for co-culture with lymphocytes, to allow time for expression of the co-stimulatory molecules.

Lymphocyte cultures

Venous blood was obtained with informed consent from healthy human volunteers, and PBMCs were isolated by Ficoll density gradient centrifugation (Life Technologies, Paisley, UK). The isolated PBMCs (10⁵ cells ml⁻¹) were incubated in tissue culture flasks in complete medium (RPMI 1640 with 7% FCS and 2% human AB serum, 2 mM glutamine, 100 IU penicillin and 100 μg ml⁻¹ streptomycin) at 37°C to allow attachment of plastic-adherent cells. The non-adherent cells were harvested after 2 h for use in the experiments.

³H-thymidine incorporation assay

A549 cells were infected 2 days previously as above with recombinant adenoviruses to express GFP, CD80, 4-1BBL or CD80 + 4-1BBL and irradiated at 80 Gy (Cs-137 Irradiator CIS IBL 437, France), before plating 5 × 10⁴ cells per well in a 96-well plate. Lymphocytes were added (10⁵ per well), in the presence of 0, 10 or 100 ng ml⁻¹ OKT3 anti-CD3 mAb (JANSSEN-CILAG, High Wycombe, UK), and the plates
were stained with isotype control antibodies. Depending on the different co-stimulation conditions required, some wells were pre-seeded 2 days previously with A549 cells infected with recombinant adenoviruses expressing GFP, CD80, 4-1BBL, CD80 + 4-1BBL, CD86 or CD86 + 4-1BBL. At intervals as specified in the figure legends, the lymphocytes were re-suspended, an aliquot was mixed 1:1 with trypan blue stain and the cell density was determined using a haemocytometer. The density of lymphocytes was re-adjusted, either by dilution or by combining cells from more than one well, to 5 × 10^5–1 × 10^6 cells per well, when the remaining lymphocytes were transferred in fresh, OKT3-containing medium to new wells pre-seeded with A549 cells, expressing the appropriate co-stimulatory molecules or GFP as control. The cumulative expansion or shrinkage of lymphocyte populations over the extended cultures was calculated taking into account the fold increase or decrease in cell number between passages.

**Results**

**Expression of CD80 and 4-1BBL on A549 cells following adenoviral gene transfer**

In order to investigate the effects of native, cell-associated CD80 and 4-1BBL on T-cell responses, we used replication-defective adenovirus vectors to express these co-stimulatory ligands on A549 lung carcinoma cells. Uninfected A549 cells did not express significant levels of these ligands, but infection with 300 virus particles per cell of Ad-CD80 (Fig. 1A) or Ad-4-1BBL (Fig. 1B) resulted in expression of CD80 or 4-1BBL, respectively, on most of the cells. In subsequent experiments (Supplementary Figure 1 available at *International Immunology* Online), expression of the co-stimulatory proteins on infected A549 cells was compared with that on in vitro-matured DCs (36) and an LCL (activated B cells). The mean level of CD80 expression on DCs was ~3-fold higher than on A549 cells at this MOI, and the distributions overlapped. Expression of 4-1BBL was higher on A549 cells infected with Ad-4-1BBL than on DCs or the LCL.

**CD80 and 4-1BBL enhance response to anti-CD3 stimulation**

PBMCs were obtained from healthy human volunteers, and to minimize possible co-stimulation by monocytes, they were depleted of plastic-adherent cells before use in all experiments. Initial experiments compared the rates of lymphocyte proliferation in response to 0, 10 or 100 ng ml⁻¹ OKT3 anti-CD3 antibody, when co-cultured with irradiated A549 cells that expressed CD80, 4-1BLB, both ligands or GFP (as a control). Proliferation was assessed by incorporation of ^3^H-thymidine after 3, 7 and 14 days of co-culture (Fig. 1C–E). On day 3, the proliferation of lymphocytes co-stimulated with 4-1BLB was very similar to the low, anti-CD3-dependent background of the control cultures with no co-stimulation.
was now lower than cultures co-stimulated with 4-1BBL alone, while CD80 + 4-1BBL still resulted in greatest proliferation.

At all time points, there was minimal thymidine incorporation in cultures without anti-CD3, confirming that the co-stimulatory signals cannot stimulate lymphocyte proliferation in the absence of an antigenic signal and indicating that the allogeneic response to A549 cells was minimal. Furthermore, since significant lymphocyte proliferation was only obtained by co-culture with A549 cells expressing CD80 and/or 4-1BBL, it is clear that the level of co-stimulation provided by B cells present in the PBMC (or by trace contamination with other APCs not removed by prior plastic adherence) is insignificant in this assay. In subsequent experiments, co-stimulation with CD86 also resulted in lymphocyte proliferation, with a similar magnitude and timecourse to that obtained using CD80, and CD86 also demonstrated similar cooperativity with 4-1BBL (results not shown).

Dual co-stimulation increases the proportion of cells undergoing multiple divisions

To provide greater insight into the proportion of lymphocytes that proliferate, and the number of divisions they are induced to undergo, lymphocytes were re-labelled with the fluorescent dye CFSE before stimulation with 100 ng ml\(^{-1}\) anti-CD3 and co-culture with A549 cells expressing GFP or 4-1BBL, it is clear that the level of co-stimulation provided by B cells present in the PBMC (or by trace contamination with other APCs not removed by prior plastic adherence) is insignificant in this assay. In subsequent experiments, co-stimulation with CD86 also resulted in lymphocyte proliferation, with a similar magnitude and timecourse to that obtained using CD80, and CD86 also demonstrated similar cooperativity with 4-1BBL (results not shown).

Calculation (34) of the number of input cells that gave rise to those in the peaks corresponding to one or more divisions at day 7 indicates that 30% of the input lymphocytes

Fig. 1. Expression of CD80 and 4-1BBL on A549 cells can provide co-stimulation to co-cultured lymphocytes. A549 cells were infected with 300 vp of Ad-CD80 (A) or Ad-4-1BBL (B). After 2 days, expression of the co-stimulatory proteins on the cells was determined by flow cytometry after staining with the respective FITC-labelled antibody (filled area) or an isotype control antibody (solid line). Dotted line—staining of uninfected cells for CD80 or 4-1BBL. Lymphocytes were co-cultured with irradiated A549 cells expressing GFP, CD80, 4-1BBL or CD80 + 4-1BBL and stimulated with 0, 10 or 100 ng ml\(^{-1}\) anti-CD3. Incorporation of \(^{3}H\)-thymidine into lymphocyte DNA was determined after (C) 3, (D) 7 and (E) 14 days, as an indicator of proliferation. Data show mean and standard deviation of triplicate wells.
underwent one or more divisions in response to anti-CD3 combined with CD80 co-stimulation; 16% of input lymphocytes responded to anti-CD3 + 4-1BB; while 45% responded to anti-CD3 + CD80 + 4-1BB. (These calculations assume there was no lymphocyte death before day 7.) Despite the near additivity of these proportions, the number of cells that underwent three or more divisions in response to dual co-stimulation clearly greatly exceeds the sum of the responses to the individual ligands (Fig. 2). Therefore, the signals from CD80 and 4-1BB must interact cooperatively on at least some of the responding cells to increase the number of lymphocytes that undergo three or more divisions, which constituted 68% of the culture by day 7 and a still greater proportion by day 14.

The receptor 4-1BB (CD137) is not expressed on resting T cells, but is induced upon anti-CD3 stimulation (11), peaking at up to ~15% positive cells after 3 days in our experiments (data not shown). In an analysis of receptor expression at day 4, co-stimulation with CD80 alone did not appear to influence CD137 expression, although in the cultures with CD80 + 4-1BB, the CD137+ population expanded in line with their proliferation. Reciprocally, while co-stimulation with CD80 down-regulated CD28 expression from ~98 to ~20% of CD4+ cells and from ~40 to ~15% of CD8+ cells by day 4,
T-cell co-stimulation with CD80/CD86 and 4-1BBL

A

GFP

CD80

4-1BBL

CD80 + 4-1BBL

CD4 (%) vs. time (days)

CD8 (%) vs. time (days)

B

Total

CD4-depleted

CD8-depleted

GFP

CD80

4-1BBL

CD80 + 4-1BBL

Events vs. CFSE
4-1BBL did not appear to affect the expression of CD28 (Habib-Agahi, unpublished data).

**Similar and independent initial proliferative responses of CD4+ and CD8+ cells**

The proportion of CD4+ and CD8+ lymphocytes was monitored in the CFSE-labelled cultures. This showed broadly similar responses of CD4+ and CD8+ cells under all co-stimulation conditions at day 7, with just a slight tendency for the CD8+ cells to undergo more divisions. By day 14, this trend was more pronounced particularly in the cultures co-stimulated with both CD80 and 4-1BBL, where the modal number of divisions for activated CD4+ cells was four to five, while that for CD8+ cells was seven (data not shown). Figure 3(A) summarizes the proportions of CD4+ and CD8+ lymphocytes from five donors, stimulated with 100 ng ml⁻¹ anti-CD3 under the different co-stimulation conditions for 3, 7 and 14 days. While at day 7 the proportions of both CD4 and CD8 cells tended to increase in all experimental conditions, indicating expansion of both populations relative to other PBMC, by day 14 there was an apparent tendency for the proportion of CD8+ cells to increase, and CD4+ cells to decline, in cultures co-stimulated with 4-1BBL alone or in combination with CD80.

In order to determine whether both CD4+ and CD8+ cells respond directly and independently to both CD80 and 4-1BBL, similar co-cultures were established after immunomagnetic depletion of either CD4+ or CD8+ cells. The CD4+ cells were depleted from 70 to 0.2% of lymphocytes and CD8+ cells from 31 to 0.5% (data not shown). Analysis of the CFSE-labelled cells on day 5 after stimulation (Fig. 3B) again showed very similar responses of CD4+ and CD8+ cells, and of the undepleted non-adherent cells, to stimulation with anti-CD3 and CD80, 4-1BBL or both, confirming that during the early stages of the response, each subtype responds similarly and independently to the co-stimulatory signals, in agreement with the 7-day time point in Fig. 3(A).

**Analysis of activation markers and CTLA-4 expression**

A selection of T-cell activation markers was monitored on lymphocytes stimulated with anti-CD3 and the different conditions of co-stimulation. Lymphocytes stimulated with anti-CD3 but without co-stimulation showed little change in the expression of CD45RA or -RO of CD25 or of CD62L (Supplementary Figure 2A–D is available at International Immunology Online). Co-stimulation with CD80, 4-1BBL or both reduced the proportion of CD45RA, and increased the proportion of CD45RO-positive cells, reflecting the levels of proliferation in these conditions. Up-regulation of CD25 was apparent particularly with dual co-stimulation. There appeared little change in CD62L expression, apart from a drop from 73 to 29% CD62L+ cells between days 7 and 14, in the cultures co-stimulated with both CD80 and 4-1BBL.

We also monitored expression of the inducible, inhibitory receptor CTLA-4 (2, 3, 37) (Supplementary Figure 2E–G is available at International Immunology Online). Prior to stimulation, only ~5% of CD4+ T cells and 1% of CD8+ T cells showed positive staining for CTLA-4. Anti-CD3 stimulation increased the frequency of CTLA-4-positive cells to ~35–70% after 3 days. Notably, neither the levels of CTLA-4 induced nor the proportion of CTLA-4-positive cells showed any significant reduction in cultures co-stimulated with 4-1BBL (with or without CD80) relative to cultures co-stimulated with CD80 alone.

**Co-stimulation with 4-1BBL prolongs T-cell response**

In the above experiments, the A549 cells expressing GFP or co-stimulatory ligands were added to the cultures on day 0; however, in the co-stimulated cultures, the A549 cells were largely eliminated during the first week, possibly due to activation of CTLs. To investigate the effects of repeated co-stimulation on the T-cell response, similar lymphocyte cultures were passaged at intervals, and fresh A549 cells expressing GFP or the co-stimulatory ligands were added, along with 100 ng ml⁻¹ anti-CD3. Viable lymphocytes were counted at intervals, and Fig. 4 charts the cumulative expansion/contraction of the lymphocyte populations. Stimulation with anti-CD3 either alone or in the presence of GFP-expressing A549 cells led to a progressive decline in the number of viable lymphocytes. Co-stimulation with CD80 led to a 2.8-fold increase in total lymphocyte number by day 8. Re-stimulation at this time did not result in any further change in cell number by day 17, after which the number of lymphocytes decreased despite further re-stimulation, dropping below the starting level between days 23 and 31. As expected, cultures co-stimulated with 4-1BBL initially lagged behind those with CD80; however, by day 8 the lymphocyte number exceeded that with CD80 alone, and continued to increase up to day 31, reaching a 19-fold expansion from the initial lymphocyte number. Cultures co-stimulated with both CD80 and 4-1BBL showed the greatest cell number at all time points and expanded continuously to 92 times the starting cell number after 31 days. Thus, whereas CD80 co-stimulation allows a transient activation followed by non-responsiveness and cell death, co-stimulation with 4-1BBL allows a more sustained activation lasting >4 weeks. Combined co-stimulation with 4-1BBL and CD80 allows a continuous response that greatly exceeds that obtained with either ligand individually.

**4-1BBL reactivates non-responsive, anergic T cells**

Exposure of T cells to antigenic ligands in the absence of co-stimulatory signals can induce a state of unresponsiveness or clonal anergy (8, 9, 37, 38). Although co-stimulation via CD80/CD86 allows initial response to an antigen, this...
can nonetheless also lead to anergy, either as a result of inhibitory signals mediated via CTLA-4 or in some situations by CTLA-4-independent mechanisms (5, 6, 9, 39).

Figure 5(A) shows an example of a culture of non-adherent PBMC stimulated with anti-CD3 alone that expanded by ~30% during the first week, before becoming unresponsive and declining to one-third the initial number of cells after 3 weeks, despite weekly replenishment of the medium containing anti-CD3. After 3 weeks, some of the remaining cells were placed in co-culture with A549 cells expressing either 4-1BBL or CD80 + 4-1BBL, still in the presence of anti-CD3. Over the following week, the rate of decline was slowed by 4-1BBL co-stimulation, and after 2 weeks the cell number had increased to >25% more than when transferred to 4-1BBL co-stimulation. More dramatically, lymphocytes transferred after 3 weeks of anti-CD3 exposure without co-stimulation, to cultures with anti-CD3, CD80 and 4-1BBL, had expanded by ~25% within 1 week, and the cultures expanded 4.6-fold after 2 weeks of dual co-stimulation. At this time, the parallel culture maintained with anti-CD3 alone had declined by a further factor of ~16 to ~2% of the starting cell number.

As shown in Fig. 5(B), in the same experiment, lymphocytes initially activated using anti-CD3 and CD80 co-stimulation showed an initial expansion of ~70%, but subsequently became unresponsive to these stimuli and the cultures declined to below the input cell number by 3 weeks, despite weekly re-stimulation with anti-CD3 and CD80. Switching some of these lymphocytes to co-cultures with A549 cells expressing 4-1BBL, with or without CD80, resulted in a doubling of cell number within 1 week and a 6- to 8-fold increase in cell number after 2 weeks. This contrasts with the cultures that continued to receive anti-CD3 and CD80 co-stimulation alone, which declined 8-fold over the same 2 weeks, to below 10% of the input cell number. Similarly, as shown in Fig. 5(C), the proliferation induced by anti-CD3 and CD86 co-stimulation was also transient, with the cells becoming unresponsive and diminishing in number after the first few days, despite weekly restimulation. Again, switching to 4-1BBL co-stimulation was able to halt the decline and initiate further proliferation, and more rapid proliferation was obtained by dual co-stimulation with CD86 + 4-1BBL. Thus, exposure to 4-1BBL was able to reactivate T cells from an...
unresponsive, anergic state brought about by treatment with anti-CD3 antibody either alone or with CD80/CD86 co-stimulation, and the rate of proliferation of these reactivated cells could be further increased by dual co-stimulation with both CD80/CD86 and 4-1BBL.

Discussion
The recognition that T-cell activation required two signals—one antigenic, the other costimulatory—was an important step in understanding how the immune system can maintain peripheral tolerance to self-antigens, while allowing robust responses towards foreign or pathogen-associated antigens. The main pathway of co-stimulation involves the engagement of CD28 on T cells with B7-family ligands on APCs (1). Delivery of antigenic signals alone was shown to cause T-cell unresponsiveness or anergy, whereas simultaneous engagement of CD28 on T cells with B7-1/CD80 allowed optimal T-cell proliferation and IL-2 secretion (8). Using a similar model system involving adenovirus-mediated gene transfer to A549 lung carcinoma cells, we have confirmed that co-stimulation of human T cells with CD80 (or the related B7-2/CD86) allows them to proliferate in the presence of anti-CD3 antibody, whereas there was little or no response to anti-CD3 alone. In the typical experiment shown in Fig. 2, ~30% of the input lymphocytes went through between one and seven divisions, and the responding fraction expanded by ~7-fold, resulting in a 2.8-fold increase in total lymphocyte number. DNA synthesis was much reduced by day 7, and there was no significant further increase in cell number by day 14. Even in cultures replenished with fresh media and re-stimulated with anti-CD3 and CD80-expressing A549 cells, the lymphocytes ceased to proliferate and, after 2–3 weeks in culture, the number of viable cells declined (Fig. 4). These observations are consistent with the reported up-regulation of the inhibitory CD80R, CTLA-4/CD152 upon T-cell activation, bringing about AINR and AICD (3–6, 37). Thus, while exposing T cells to an antigenic signal alone can cause unresponsiveness and anergy, simultaneous co-stimulation with CD80/CD86 leads only to a transient activation and proliferation, followed by AINR and AICD. The importance of CTLA-4 in the natural down-regulation of the T-cell response was illustrated by the lethal lymphoproliferation in CTLA-4-deficient mice (40) and by the induction of autoimmunity in human cancer patients treated with CTLA-4-blocking antibodies (30).

Engagement of the tumour necrosis factor receptor (TNFR)-family receptor 4-1BB (CD137) was shown to enhance the expansion and long-term survival of superantigen-activated T cells (13), suggesting this pathway can modulate the magnitude and duration of an immune response. Indeed, 4-1BB signalling has been shown to regulate cell cycle progression of CD8+ T cells by increasing expression of cyclins D2, D3 and E, while down-regulating expression of the CDK-inhibitor p27kip1 (41) and to promote T-cell survival via NFκB-mediated up-regulation of the anti-apoptotic genes bcl-xL and bfl-1 (42). The ability of 4-1BBL to provide a co-stimulatory signal in the absence of ligands for CD28 was evident in our model system. At early times, these cultures lagged behind those co-stimulated with CD80, which may be explained by the fact that CD28 is constitutively expressed on a high proportion of resting T cells, allowing an immediate response, whereas 4-1BB/CD137 only becomes expressed on a proportion of T cells upon stimulation (11). In our experiments, CD137+ cells increased from <1 to ~12% of PBMC with 24 h, peaking at ~15% after 3 days of anti-CD3 stimulation (data not shown). Importantly, co-stimulation with 4-1BBL allowed a continuous proliferative response to anti-CD3 stimulation, showing a further substantial increase in lymphocyte number between 7 and 14 days after initial stimulation (Fig. 2), a period during which cultures co-stimulated with CD80 (or CD86) were static or showing onset of AICD. In cultures that were passaged and re-stimulated (Fig. 4), co-stimulation with 4-1BBL allowed continuous expansion of the T cells for as long as the cultures were maintained (at least 4–5 weeks).

At all time points, simultaneous co-stimulation with both CD80 (or CD86) and 4-1BBL allowed greater levels of T-cell proliferation than either signal alone. After 7 days, the number of cells that had undergone three or more divisions clearly exceeded the sum of the responses to CD80 or 4-1BBL individually, and the trend continued to day 14 (Fig. 2). Thus, the results cannot be explained by different populations of cells responding independently to either CD80 or 4-1BBL; a significant proportion of T cells must respond more strongly to both co-stimulatory signals than to either alone. With passage and restimulation, cultures with both CD80 + 4-1BBL expanded continuously, corresponding to a ~40- to 90-fold increase from the starting lymphocyte population after 4–5 weeks (Fig. 4). Thus, these results are consistent with previous observations that 4-1BB-mediated co-stimulation can function independently of CD80 (17, 43) or synergize with this pathway (20, 23). Many studies have relied upon 3H-thymidine incorporation to compare relative rates of lymphocyte proliferation in different conditions; however, this does not directly indicate the actual increase in cell number. Our flow cytometric analysis of CFSE-labelled lymphocytes provides a more detailed picture of the fraction of responding cells and the number of divisions undergone over 7–14 days of stimulation. Because we have monitored the actual number of viable cells in the extended, re-stimulated cultures, tracking the cumulative expansion (or shrinkage) of the lymphocyte population, our results particularly highlight the extended timescale over which the responses can continue and the large expansion of the responding cell population that can result from dual co-stimulation with CD80 and 4-1BBL. Our recent preliminary results (data not shown) using autologous fibroblasts to express co-stimulatory ligands and display peptide antigens have shown similar cooperativity between CD80 and 4-1BBL for expanding antigen-specific T cells, supporting the validity of the above conclusions drawn using anti-CD3 to provide the TCR signal.

We found that both CD4+ and CD8+ lymphocytes initially showed similar responses to anti-CD3 stimulation with CD80, 4-1BBL co-stimulation or both, in either unfractionated or magnetically depleted cultures, as previously reported (16, 44). However, by day 14 following stimulation, there appeared a distinct trend for the frequency of CD4+ cells to decline and for CD8+ cells to increase in cultures co-stimulated with 4-1BBL alone or with CD80, consistent with
While it has been shown that signalling via another TNFR family
may allow reactivation of T cells that have become anergic.
(51) suggested a further possibility that 4-1BB engagement
(50). However, the efficacy even against established tumours
i.e. enhancement of T-cell expansion and inhibition of AICD
roles of 4-1BB-mediated co-stimulation discussed above,
rejection in mice. These effects have been attributed to the
signalling can promote tumour-specific immunity and tumour
lymphocyte proliferation observed in their system.
thus provide a much stronger signal through the TCR com-
mAb to be bound at the surface of the 'artificial-APCs' and
proach of Suhoski
pendency on co-stimulation. One key difference in the ap-
provided by natural tumour antigens and increase the de-
this could be more representative of the weak stimulation
and 4-1BBL, this down-regulation was unaffected by 4-1BBL
(Habib-Agahi, unpublished results). The anti-apoptotic func-
tions of 4-1BBL (42) could support the continued survival of
T cells with down-regulated CD28, while also co-stimulating
their proliferation.
While this manuscript was in preparation, another study has reported up to 10^4-fold expansion of CD8\(^+\) T-cell cul-
tures within 3 weeks, by incubation with K562 cells trans-
duced with lentiviral vectors to express CD80 and 4-1BBL,
combined with anti-CD3 stimulation (47). That study sup-
ports the benefits of combing these two co-stimulatory
ligands, as reported here. In our work, we chose to use a rela-
tively low concentration (100 ng ml\(^{-1}\)) of soluble anti-CD3
to provide a weak, sub-optimal signal through the TCR since
this could be more representative of the weak stimulation
provided by natural tumour antigens and increase the de-
pendency on co-stimulation. One key difference in the ap-
proach of Suhoski et al. (47) is that the K562 cells were also
engineered to express Fc receptors, allowing the anti-CD3
mAb to be bound at the surface of the 'artificial-APCs' and
thus provide a much stronger signal through the TCR com-
plex. This may account for the even greater magnitude of
lymphocyte proliferation observed in their system.
A number of studies have shown that activation of 4-1BB
signalling can promote tumour-specific immunity and tumour
rejection in mice. These effects have been attributed to the
roles of 4-1BB-mediated co-stimulation discussed above,
.i.e. enhancement of T-cell expansion and inhibition of AICD
(48–50); or to polarization towards a Th1 immune response
(50). However, the efficacy even against established tumours
(51) suggested a further possibility that 4-1BB engagement
may allow reactivation of T cells that have become anergic.
While it has been shown that signalling via another TNFR family
member, OX40, can overcome peripheral tolerance in CD4\(^+\)
T cells (52, 53), to our knowledge it has not been reported
whether 4-1BB signalling can reactivate anergic T cells.
T-cell anergy is a mechanism of peripheral tolerance, by
which non-immunogenic antigen encounter results in a state
of hypo-responsiveness, although the T cell remains viable
for extended periods (i.e. longer than the 8–24 h characteris-
tic of cells committed to apoptosis) (9). Thus, provision of
'signal 1' for T-cell activation, in the form of antigen or anti-
CD3 antibodies and in the absence of costimulation, results
in clonal anergy of the lymphocytes (7–9). Although co-
stimulation via CD28 allows initial response to antigen and ap-
parent prevention of anergy in some models (7, 8), in other
situations it appears that anergy can be induced despite
CD80 co-stimulation, depending upon signalling through
TNF-α (4–6, 39). The inability of our lymphocyte cultures
stimulated at weekly intervals with anti-CD3 and CD80 (or
CD86) to expand after the first week indicates that non-
responsiveness or anergy has been induced and would be
consistent with a CTLA-4-dependent mechanism. Since co-
stimulation with 4-1BBL, either alone or together with CD80/
CD86, allowed continuous proliferation in response to anti-
CD3 for at least 4–5 weeks (Fig. 4), this clearly prevented
the induction of anergy that occurred in parallel cultures,
identical but for the absence of 4-1BBL. We therefore asked
whether co-stimulation with 4-1BBL might be able to reverse
the anergy that had been established in cultures lacking
4-1BBL. Our results clearly show that T cells that had become
non-responsive either to anti-CD3 antibody alone or to anti-
CD3 + CD80/CD86 co-stimulation, and continued to show
progressive AICD when re-treated with these same ligands,
could be rescued from the unresponsive, anergic state and
reactivated to further proliferation when co-stimulated with
4-1BBL (Fig. 5). As with the primary stimulation, combining
4-1BBL co-stimulation with either CD80 or CD86 resulted in
a greater level of proliferation than obtained with 4-1BBL
alone. Over the 2 weeks following the switch to co-stimulation
with 4-1BBL + CD80, the treated lymphocyte populations
expanded by ~4–8-fold relative to the cell number at the time
the co-stimulation was switched or ~50-fold higher than the
number of lymphocytes in parallel cultures that were main-
tained without 4-1BBL co-stimulation. We conclude that
4-1BBL is able to reactivate T cells from an anergic state
and propose that the anti-tumour effects of 4-1BB ligation
observed in a number of mouse tumour models (23, 49, 50),
particularly those involving pre-established tumours (51),
are likely to be due at least in part to reactivation of anergic,
tumour-specific T cells.
This study reinforces the role of 4-1BBL as an important
modulator of T-cell immune responses. Our demonstration
that co-stimulation with 4-1BBL is capable not only of allow-
ging greatly extended proliferation of T cells but also of reac-
tivating unresponsive, anergised T cells, strengthens the
rationale for developing applications in the immunotherapy
of human cancer. The model system we have developed in
this study is being extended to analyse the effects of
4-1BBL on antigen-specific immune responses, including
studies of tumour antigen-specific responses of lymphocytes
from cancer patients.

**Supplementary data**

Supplementary data are available at *International Immunol-
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Abbreviations
Ad adenovirus
AIDC activation-induced cell death
AINR activation-induced non-responsiveness
APC antigen-presenting cell
4-1BBL 4-1BB ligand
CFSE carboxy fluorescein diacetate succinimidyl ester
DC dendritic cell
GFP green fluorescent protein
LCL lymphoblastoid cell line
MOI multiplicity of infection
TNFR tumour necrosis factor receptor
vp virus particles

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