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4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine

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SUMMARY

4-1BB (CD137) is a tumour necrosis factor receptor (TNFR) family member, expressed primarily on CD8 T cells after activation. Signalling through 4-1BB has been reported to enhance CD8 T-cell expansion and to protect activated CD8 T cells from death, resulting in an enlarged memory population. Although stimulating 4-1BB has been shown to significantly improve the immune response to weak immunogens such as tumours, little is known about its effect on the CD8 T-cell response to a powerful viral vector such as vaccinia. To test 4-1BB's ability to improve the murine CD8 T cell response to a DNA prime, poxvirus boost vaccine, similar to those used for human immunodeficiency virus and simian immunodeficiency virus vaccines, we administered 4-1BB agonist antibody at the time of the poxvirus boost. 4-1BB stimulation increased the number of functional memory CD8 T cells by two- to fourfold. However, we saw a similar enhancement at the peak of the response and in the memory phase, thus we found no evidence in the context of virus infection that 4-1BB stimulation could increase the percentage of CD8 T cells that survive the acute activation phase to become memory cells. OX40 (CD134) is an analogous TNFR family member expressed primarily on activated CD4 T cells. OX40 stimulation increased the number of antigen-specific CD4 T cells approximately threefold. Stimulating both 4-1BB and OX40 enhanced the CD8 T-cell response more than 4-1BB alone. Thus stimulating these receptors can improve the response to a powerful virus vector, and may be useful in vaccine development.

Keywords T lymphocytes, costimulation, viral

INTRODUCTION

CD8 T cells play a major role in containing human immunodeficiency virus (HIV) and simian immunodeficiency virus infection, and in consequence, the CD8 T-cell response is considered an important component of a vaccine for HIV. Using heterologous viral vectors, the task of achieving high numbers of memory CD8 T cells is challenging. A common strategy is to prime a response using plasmid DNA expressing the viral antigen, followed by a boost with a recombinant poxvirus expressing the same antigen.[1] Cytokines and costimulatory molecules added to either the prime or boost phase have been used to increase the number of responding CD8 T cells with some success[2] but improvement is still needed.

There has been recent interest in two costimulatory molecules of the tumour necrosis factor receptor (TNFR) superfamily expressed on T cells, 4-1BB (CD137) and OX40 (CD134), which are thought to function primarily on CD8 T cells and CD4 T cells, respectively. 4-1BB and OX40 expression is induced on T cells after T-cell receptor (TCR) triggering and these receptors are expressed transiently for 1–5 days.[3–5] Their natural ligands, 4-1BBL and OX40L, respectively, are transmembrane TNF homologues that are expressed on activated APCs.[6–9] It is clear that 4-1BB signalling is necessary for optimal CD8 T-cell activation *in vivo* because 4-1BBL$^{-/-}$ mice have reduced CD8 T-cell

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responses after infection with lymphocytic choriomeningitis virus (LCMV) or influenza virus, and following peptide vaccination.[10–13] After CD3 cross-linking *in vitro*, 4-1BB stimulation has been shown to enhance both proliferation and the development of a functional phenotype.[4,14,15] Using TCR transgenic T cells, Cannons *et al*. showed that while CD28 plays the major role in initial activation, 4-1BB stimulation can act later to enhance proliferation, cytokine expression and survival of both CD4 and CD8 T cells.[4] OX40 shares sequence homology with 4-1BB and plays an analogous role in CD4 T-cell function. *In vivo*, OX40 blockade ameliorates autoimmune disease and OX40 stimulation increases CD4 T-cell proliferation.[9,16]

From a vaccine perspective, an attractive feature of 4-1BB and OX40 was that they have been shown to protect activated T cells from death in a number of model systems.[3,17–20] This was observed most dramatically after superantigen stimulation *in vivo*, in which anti-4-1BB stimulation caused a 10-fold increase in the number of cells that escaped deletion to become memory cells.[20] In response to viral infection, a vigorous CD8 T-cell response usually ensues. As viral antigen is cleared, 90–95% of activated CD8 T cells die, and the remainder survive to become a stable memory population.[21] Because the memory CD8 T-cell population is the most relevant for vaccine development, we were particularly interested in determining whether 4-1BB and OX40 stimulation would have a beneficial effect specifically on the size of the memory CD8 T-cell population.

Many of the above studies were performed *in vitro*. Importantly though, the potential of these molecules to enhance vaccine efficacy *in vivo* has been shown in several tumour vaccine models.[22–26] However, in each of these cases the vaccine immunogen was tumour cells. Tumours are inherently weak immunogens, partly because many tumour antigens are in fact self antigens with only low affinity TCRs available to recognize them, but more importantly because they do not elicit a powerful innate immune response. At the time we commenced these experiments, it was not clear whether these molecules could improve the response to a powerful immunogen such as vaccinia virus. More recently, 4-1BB stimulation was reported to augment the acute CD8 T-cell response to influenza virus in the lungs[27] but there have been no reports of the effect of these molecules on the memory phase of the T cell response to a viral immunogen.

We therefore wanted to determine whether these molecules could be exploited to improve a DNA prime, poxvirus boost vaccine strategy. A practical prophylactic vaccine strategy for stimulating these receptors would probably need to encode their ligands in the plasmid DNA and/or the recombinant poxvirus. However, as a proof-of-principle test, we used monoclonal antibodies that have previously been shown to enhance T-cell responses *in vivo*, in combination with a standard DNA-prime recombinant poxvirus boost vaccination protocol. Here we report that a two- to fourfold increase in antigen-specific CD8 T cells is achieved by stimulating 4-1BB *in vivo*. Furthermore, we show that engagement of 4-1BB and OX40 at the same time can have an additive effect on both CD4 and CD8 T cell priming *in vivo*.

MATERIALS AND METHODS

Mice

Six- to 12-week-old female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Simonsen Laboratories (Gilroy, CA) and housed in the OHSU animal care facility.

Plasmid DNA constructs

Plasmid pTH.HM[28] was a gift from Tomas Hanke, (Oxford, UK). pTH.HM encodes a string of CD8 T cell epitopes behind the human cytomegalovirus (HCMV) immediate-early (IE) promoter, including the H-$2D^d$-restricted murine epitope RGPGRAFVTI from HIV-1 envelope glycoprotein and the H-$2K^d$-restricted murine epitope SYIPSAEKI from *Plasmodium berghei*. In a preliminary experiment we saw a CD8 T-cell response to the HIV env epitope but not the *P. berghei* epitope. Therefore in experiments involving pTH.HM, only the CD8 T-cell response to HIV env was measured. Plasmid ovalbumin (pOVA) was constructed from the pIRES plasmid (Invitrogen, Carlsbad, CA), which contains two multiple-cloning sites (MCS) separated by an internal ribosomal entry site (IRES). Gene expression is controlled by the HCMV IE promoter. The full-length OVA reading frame, which encodes the H-$2K^b$-restricted CD8 T-cell antigen SIINFEKL, was isolated by polymerase chain reaction (PCR) and cloned into the 'A' site. The B site contains the Ad5E4 ORF4 gene in reverse orientation, resulting in a non-functional open reading frame (ORF; this plasmid is used as a control plasmid for other studies).

Milligram quantities of plasmid DNA were obtained using Qiagen Megaprep columns (Qiagen, Valencia, CA) according to the manufacturer's protocols, quantified by UV absorbance, and the purity verified by $A_{260/280}$ and agarose gel electrophoresis.

Recombinant viruses

Recombinant modified vaccinia virus Ankara (MVA) containing the same murine CD8 T-cell antigens as pTH.HM, MVA.HM, was a gift from Tomas Hanke, and has been previously described.[29] Recombinant vaccinia virus (VV) containing the full-length ovalbumin ORF, rVV-OVA, was a gift from J. Yewdell (NIH, Bethesda, MA), and has been previously described.[30]

Antibodies

Rat immunoglobulin G (IgG) was obtained from Sigma (St. Louis, MO). Anti-4-1BB stimulating monoclonal antibody (mAb) from the 3H3 hybridoma[31] and anti-OX40 stimulating mAb from the OX86 hybridoma (European Cell Culture Collection, Porton Down, UK) were purified on a Protein-G sepharose column (Pharmacia, Piscataway, NJ).

Vaccinations

DNA was injected intramuscularly (i.m). Mice were briefly anaesthetized with isoflurane. The lower hind legs were

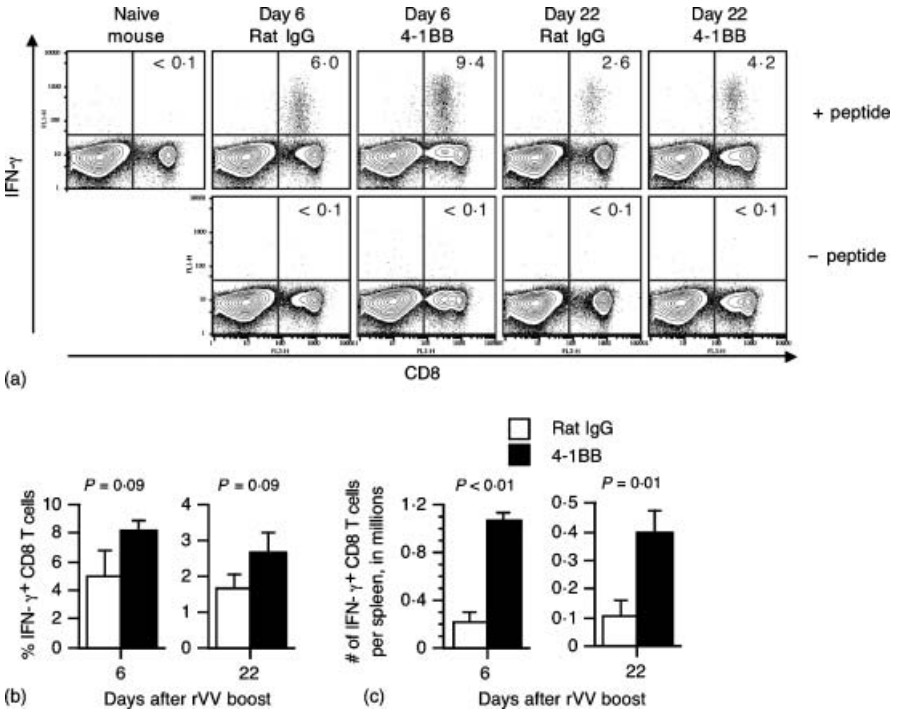

Figure 1. 4-1BB enhances CD8 T-cell activation and memory. BALB/c mice were primed with 100 μg of plasmid pTH.HM twice, 1 week apart. Two weeks later, mice were boosted with 1×10^6 pfu of MVA.HM and received 200 μg of either purified anti-4-1BB mAb or rat IgG i.p. with the boost. At the indicated number of days later, unfractionated spleen cells were cultured directly *ex vivo* for 6 hr with brefeldin A, in the presence or absence of 1 μg/ml RGPGRAFVTI peptide, then stained for CD8 and intracellular IFN-γ. One representative is shown from each group (a). Each point represents the average percentage (b) and number (c) of antigen-specific CD8 T cells $\pm$ SEM (day 6, $n = 3$ mice per group; day 22, $n = 4$ mice per group). A second experiment gave similar results.

shaved and 50 μg of plasmid DNA was injected into the calf muscle of each leg (100 μg total DNA per mouse each time vaccinated). Recombinant viruses were injected intraperitoneally (i.p) using either 1×10^6 pfu of MVA.HM or 2×10^5 pfu of rVV-OVA. All antibodies were injected as a single dose i.p. on the same day as mice were injected with virus. For the experiments shown in Figs 1 and 2, 200 μg of mAb were injected per mouse. In the experiment shown in Fig. 3, each animal received a total of 400 μg of antibody: (1) 400 μg rat IgG; (2) 200 μg 3H3 and 200 μg rat IgG; (3) 200 μg OX86 and 200 μg rat IgG; or (4) 200 μg 3H3 and 200 μg OX86.

The rationale for this protocol is as follows. The *in vivo* half-life of another mAb, 1D8, which is also specific for 4-1BB and is the same isotype (rat IgG2a) as the 3H3 mAb, is approximately 7·5 days.[31] Because 4-1BB is only expressed for a few days after T-cell activation and then returns to a state of low or no expression, we administered a single dose of anti-4-1BB mAb at the same time as antigen, and assumed that maximal 4-1BB stimulation would be achieved at the time of 4-1BB expression on CD8 T cells over the following 3–4 days.

Because rat IgG induces an anti-rat IgG antibody response in mice, we were limited to administering one dose of antibody. We assumed that stimulating 4-1BB would rescue activated CD8 T cells from death, so we chose to administer the antibody at the time of the poxvirus boost, when the maximum number of activated antigen-specific CD8 T cells were obtained. We performed one pilot experiment administering anti-4-1BB mAb at the time of the DNA prime and found that the final result was similar to that observed when administered at the time of the poxvirus boost (data not shown).

Intracellular cytokine staining

Spleen cells were separated through a nylon mesh, washed twice in Hank's balanced salt solution containing 1% fetal calf serum, counted, then resuspended in complete medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 200 mM L-glutamine, 50 μM 2-mercaptoethanol and penicillin/streptomycin). Spleen cells were cultured in 200 μl of complete medium in 96-well round-bottom plates at a density of $2–4 \times 10^6$ cells/well. For analysis of peptide-specific CD8 T cells, spleen cells were cultured for 5–6 hr in the presence of brefeldin A (GolgiPlug, BD Pharmingen, San Diego, CA) with or without 1 μg/ml synthetic peptide (Genemed Synthesis, Inc., South San Francisco, CA). For analysis of OVA-specific CD4 T cells, spleen cells were cultured with or without 400 μg/ml ovalbumin protein

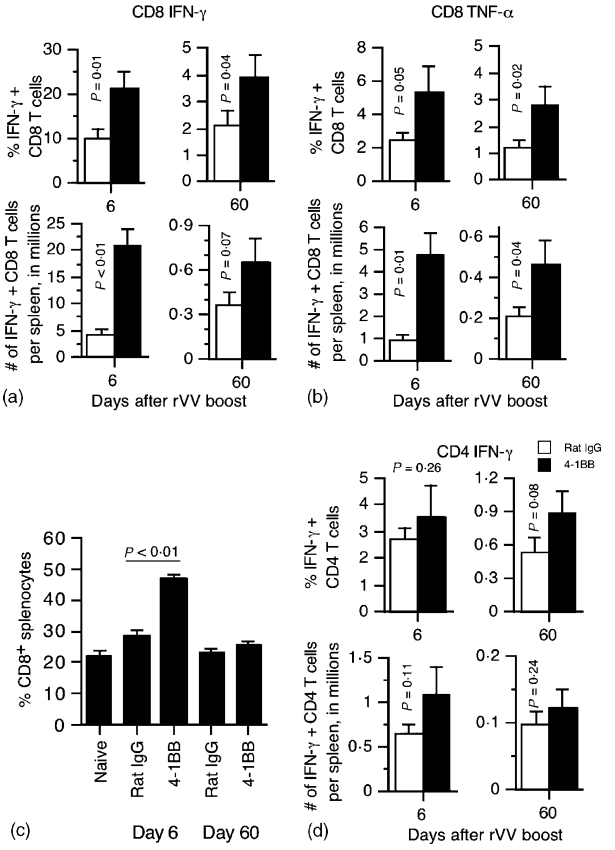

Figure 2. 4-1BB improves long-term CD8 T-cell memory. C57Bl/6 mice were vaccinated with 100 μg of plasmid pOVA twice, two weeks apart. Thirty days later, mice were vaccinated with 2×10^5 pfu of rVV-OVA and received 200 μg of either purified anti-4-1BB mAb or rat IgG i.p. (a) At the indicated number of days later, unfractionated spleen cells were cultured directly *ex vivo* for 6 hr with brefeldin A, in the presence or absence of 1 μg/ml SIINFEKL peptide, then stained for CD8 and intracellular IFN-γ or (b) TNF-α. (c) The percentage of total spleen cells that were $CD8^+$. (d) OVA-specific CD4 T cells were quantified in parallel to SIINFEKL-specific CD8 T cells. Unfractionated spleen cells were cultured directly *ex vivo* in the presence or absence of 400 μg/ml whole ovalbumin protein for 10 hr, stimulated for an additional 8 hr in the presence of brefeldin A, then stained for CD4 and intracellular IFN-γ. Data shown are the average ± SEM (day 6, $n = 6$ mice per group; day 60, $n = 10$ mice per group). A second experiment gave similar results.

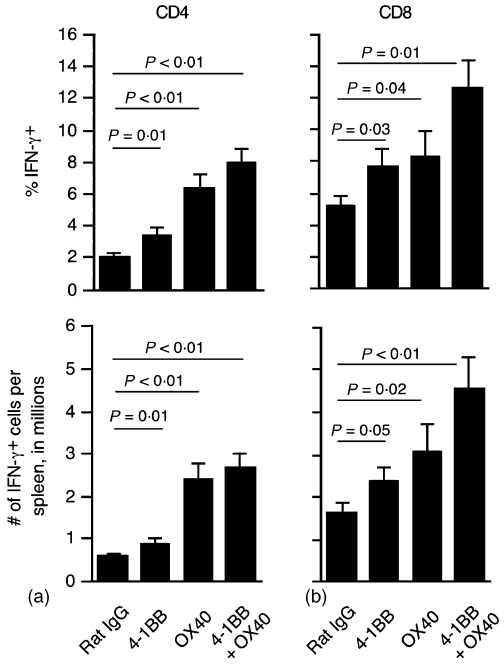

Figure 3. 4-1BB and OX40 cooperate to enhance CD8 T and CD4 T cells. C57Bl/6 mice were vaccinated with 100 μg of plasmid pOVA twice, 2 weeks apart. Thirty days later, mice were vaccinated with 2×10^5 pfu of rVV-OVA i.p. At the time of the rVV boost, mice were divided into four groups and received either 200 μg of anti-4-1BB mAb + 200 μg rat IgG, 200 μg of anti-OX40 mAb + 200 μg rat IgG, 200 μg anti-4-1BB mAb + 200 μg anti-OX40 mAb, or 400 μg of rat IgG. Five weeks later, spleen cells were examined for antigen-specific CD4 and CD8 T-cell responses. (a) Unfractionated spleen cells were cultured directly *ex vivo* in the presence or absence of 400 μg/ml whole ovalbumin protein for 10 hr, stimulated for an additional 8 hr in the presence of brefeldin A, then stained for CD4 and intracellular IFN-γ. OVA-specific CD4 T cells are shown as both a percentage of $CD4^+$ T cells and as the number per spleen. (b) Unfractionated spleen cells were cultured directly *ex vivo* for 6 hr with brefeldin A, in the presence or absence of 1 μg/ml SIINFEKL peptide, then stained for CD8 and intracellular IFN-γ. SIINFEKL-specific CD8 T cells are shown as both a percentage of $CD8^+$ T cells and total number per spleen. Data shown are the average ± SEM ($n = 10$ mice per group). A second experiment gave similar results.

(Sigma) for 18 hr total, the last 8 hr in the presence of brefeldin A.

Cells were washed in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS), pH 7·4, 1% fetal bovine serum, 0·1% NaAzide) and surface stained with Tri-Color-conjugated anti-CD8α (clone CT-CD8α, CalTag Laboratories, Burlingame, CA), CyC-conjugated anti-CD8α (clone 53–6·7, BD Pharmingen) or CyC-conjugated anti-CD4 (clone H129·19, BD Pharmingen). Cells were then washed twice, fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's protocol, and stained with fluoroscein isothiocyanate (FITC)-conjugated anti-mouse interferon-γ (IFN-γ) (clone XMG1.2, BD Pharmingen) or anti-mouse TNF-α (clone MP6-XT22, BD Pharmingen). All FACS samples were acquired on a FACSCalibur machine (BD Pharmingen) using CellQuest software. Data were analysed using FloJo software (TreeStar, Inc., San Carlos, CA). To calculate the total number of antigen-specific CD8 T cells in a mouse spleen, the total number of spleen cells was multiplied by the percentage of spleen cells that were $CD8^+$, which was multiplied by the percentage of CD8 T cells that were antigen-specific.

Statistical tests

P-values were determined using a one-tailed Student's *t*-test.

RESULTS

An anti-4-1BB mAb enhances CD8 T-cell expansion

Protocols incorporating a DNA-prime followed by a recombinant poxvirus boost are frequently used to generate a CD8 response in experimental vaccine protocols.[1] Because stimulation through 4-1BB has been reported to enhance CD8 T-cell proliferation, and to selectively rescue activated CD8 T cells from death, we wanted to determine if engagement of 4-1BB *in vivo* during a DNA-prime poxvirus boost strategy would improve the number of activated CD8 T cells and the number that survived after resolution of the primary response.

BALB/c mice were injected twice, 1 week apart, with plasmid encoding the H-2D^d-restricted HIV-1 envelope glycoprotein peptide (RGPGRAFVTI). Two weeks after the second injection, mice were boosted with MVA expressing the same antigen (MVA.HM), and given a single dose of agonist anti-4-1BB mAb (see Materials and methods for rationale) or control rat IgG. CD8 T-cell responses were assessed 6 days and 22 days later by direct *ex vivo* intracellular cytokine staining (ICS) for IFN-γ. Figure 1(a) shows typical intracellular IFN-γ staining for this experiment; one representative mouse is shown from each group. Figure 1(b) shows the results for the entire experiment, with antigen-specific CD8 T cells expressed as a percentage of total CD8 T cells. 4-1BB stimulation caused a small increase in the frequency of antigen-specific CD8 T cells, but it was not statistically significant: 6 days after the MVA.HM boost, anti-4-1BB treated mice had 8·2% IFN-γ + CD8 T cells versus 5·0% in controls ($P = 0{\cdot}09$), and at 22 days the respective percentages were 2·7% versus 1·7% IFN-γ + CD8 T cells ($P = 0{\cdot}09$; Fig. 1b).

However, when we compared the total number of antigen-specific CD8 T cells per spleen, rather than the frequency, a larger difference was seen. Six days after the MVA.HM boost, control mice had an average of $2{\cdot}2 \times 10^5$ IFN-γ + CD8 T cells per spleen, while the mice treated with anti-4-1BB mAb averaged $10{\cdot}5 \times 10^6$ IFN-γ + CD8 T cells per spleen, a greater than fourfold increase ($P < 0{\cdot}01$; Fig. 1c). Twenty-two days after MVA.HM, control mice had $1{\cdot}1 \times 10^5$ IFN-γ + CD8 T cells per spleen, while mice treated with anti-4-1BB mAb had $4{\cdot}0 \times 10^5$ IFN-γ + CD8 T cells per spleen, a difference of nearly fourfold ($P = 0{\cdot}01$). The greater effect of anti-4-1BB on total numbers than on the frequency of antigen-specific CD8 T cells reflects the fact that mice receiving the 3H3 antibody developed a more profound splenomegaly than mice that received control antibody.

We conclude from these initial experiments that by administering stimulating anti-4-1BB mAb in a vaccine setting, we could significantly increase the total number of antigen-specific CD8 T cells at both the peak of the response, day 6, and after the primary response had resolved, at day 22. 4-1BB stimulation has been reported to rescue cells from activation-induced cell death. However, we observed a similar enhancement at day 6 compared to day 22, which did not suggest a preferential survival of antigen-specific CD8 T cells after 4-1BB engagement *in vivo*. These experiments were limited by the small number of animals per group, which made it difficult to reach statistical significance. In addition, at the 22-day time point used to detect memory cells the mice still had somewhat enlarged spleens, suggesting that a true memory homeostasis had not yet been reached.

Anti-4-1BB mAb increases long-term CD8 T-cell memory

Although CD8 T-cell memory is known to be stable after clearance of a viral infection, no data exist as to whether the 4-1BB-enhancement would maintain an increased level of long-lived memory T cells. To assess long-term memory, we measured the immune response at 6 and 60 days after the poxvirus boost. For these experiments, we used as our model antigen chicken OVA, for which the immunodominant K^b-restricted epitope SIINFEKL has been well characterized. Another advantage of this model was that both the DNA prime (pOVA) and the recombinant vaccinia virus boost (rVV-OVA) expressed the whole OVA gene, allowing us to also quantify OVA-specific CD4 T cells by intracellular staining for IFN-γ.

C57BL/6 mice (10 mice per group) were injected twice with the plasmid pOVA, 2 weeks apart. Four weeks after the second DNA injection, mice were boosted with rVV-OVA together with either anti-4-1BB mAb or control antibody. CD8 and CD4 T cell responses were measured directly *ex vivo* either 6 days later, at the height of expansion, or 60 days later, to evaluate stable long-term memory. The frequency of IFN-γ + CD8 T cells was twice as high in mice given anti-4-1BB mAb with the rVV-OVA boost compared to control mice at day 6 ($P = 0{\cdot}01$; Fig. 2a, upper left) and at day 60 ($P = 0{\cdot}04$; Fig. 2a, upper right). SIINFEKL-specific TNF-α production was also assessed by ICS and was similarly affected by 4-1BB stimulation (Fig. 2b, upper panels).

When we compared the numbers rather than the frequency of SIINFEKL-specific CD8 T cells, a fourfold increase was seen at day 6 ($P < 0{\cdot}01$; Fig. 2a, lower left), and an approximately twofold difference at day 60 ($P = 0{\cdot}07$; Fig. 2a, lower right). This was accounted for by a disproportionate increase in the percentage of CD8 T cells induced by 4-1BB at day 6, compared to control IgG (Fig. 2c). We believe that this is most likely caused by a 4-1BB stimulated increase in CD8 T cells responding to the vaccinia vector. Again, similar results were obtained regardless of whether SIINFEKL-specific CD8 T cells were quantified using IFN-γ ICS (Fig. 2a, lower panels) or TNF-α ICS (Fig. 2b, lower panels).

We also analysed the CD4 T-cell response to OVA, using whole ovalbumin protein as antigen, and analyzing IFN-γ + CD4 T cells by ICS (Fig. 2d). All vaccinated animals developed a detectable OVA-specific CD4 response, which was strongest at day 6 but still readily detectable at day 60. However, treatment with anti-4-1BB mAb did not significantly increase the OVA-specific CD4 T-cell response, whether assessed as a percentage of total CD4 T cells or as absolute numbers.

We conclude from this data that anti-4-1BB mAb increased the frequency and total number of SIINFEKL-specific CD8 T cells at both day 6 and day 60 after giving the rVV-OVA boost. As in Fig. 1, we saw no evidence that activated cells were selectively rescued into the memory compartment. We further conclude that there was no significant effect of 4-1BB stimulation on CD4 T-cell expansion or commitment to memory in this experiment.

Anti-4-1BB mAb and anti-OX-40 mAb increase the number of OVA-specific CD4 T cells, with OX-40 having a more potent effect

The previous experiment indicated that stimulating 4-1BB with anti-4-1BB mAb has a stronger effect on CD8 T cells than CD4 T cells, which was consistent with most previous studies.[10,12,20] Conversely, OX40 is thought to primarily affect CD4 T cells.[32] Based on this we postulated that: (1) stimulating OX40 at the time of vaccination would lead to an enhanced CD4 T cell response; and (2) because CD4 help is often able to enhance CD8 T-cell responses, the combination of anti-OX40 and anti-4-1BB mAb would lead to an enhanced CD8 T-cell response, above 4-1BB stimulation alone.

In order to test this, we again used the OVA system because we could quantify both CD4 and CD8 T-cell responses. C57BL/6 mice were injected twice with plasmid pOVA, 2 weeks apart. Thirty days later, mice were vaccinated with rVV-OVA and divided into four groups. At the time of the boost, each group was injected with either anti-4-1BB mAb, anti-OX40 mAb, both of these mAb together, or control antibody. Five weeks later, the CD4 T-cell response to OVA and the CD8 T-cell response to SIINFEKL were quantified directly *ex vivo* by intracellular IFN-γ staining.

OX40 stimulation caused a threefold increase in the frequency of splenic CD4 T cells that were OVA-specific ($P << 0{\cdot}01$; Fig. 3a, top). 4-1BB stimulation caused a small but significant increase in OVA-specific CD4 T cells ($P = 0{\cdot}01$; Fig. 3a, top). Mice receiving a combination of both anti-OX40 mAb and anti-4-1BB mAb had the most OVA-specific CD4 T cells, fourfold more than controls but not significantly better than anti-OX40 mAb alone ($P = 0{\cdot}11$; Fig. 3a, top). In this experiment, there was no significant difference in the average number of spleen cells between any two groups of mice (data not shown). Therefore, changes in the total number of OVA-specific CD4 T cells were similar to the changes in frequency (Fig. 3a, bottom). Thus in this experiment both OX40 and 4-1BB stimulation increased the OVA-specific CD4 T-cell response, but OX40 stimulation was more effective than 4-1BB.

Anti-4-1BB mAb and anti-OX-40 mAb additively enhance CD8 T cells

In the same experiment, the SIINFEKL-specific CD8 T-cell response was also quantified. 4-1BB stimulation with the rVV-OVA vaccine increased the frequency of SIINFEKL-specific CD8 T cells by 1·5 fold ($P = 0{\cdot}03$; Fig. 3b, top), as did OX40 stimulation ($P = 0{\cdot}04$; Fig. 3b, top). As predicted, mice receiving both anti-OX40 mAb and anti-4-1BB mAb had significantly more SIINFEKL-specific CD8 T cells, 12·7%, than mice receiving either mAb alone ($P = 0{\cdot}01$, $P = 0{\cdot}03$). As with CD4 T cells, the changes in total numbers of SIINFEKL-specific CD8 T cells were similar to the changes in SIINFEKL-specific CD8 T-cell frequency (Fig. 3b, bottom).

This data further confirmed that 4-1BB stimulation is able to improve a vaccine-induced CD8 T-cell response, and that this effect is further enhanced by OX40 stimulation.

DISCUSSION

4-1BB stimulation has proved highly effective in augmenting the immunogenicity of tumours, resulting in effective antitumour immunity.[22–26] We wanted to determine if we could improve a T-cell vaccine by administering antibodies that stimulate 4-1BB and OX40 together with a DNA prime, poxvirus boost vaccine. We found that anti-4-1BB mAb was able to increase the number of antigen-specific CD8 T cells induced by vaccination by about two- to fourfold. These CD8 T cells were functional and produced IFN-γ and TNF-α in response to antigen. This was true even though vaccinia virus is already an extremely potent natural immunogen that might have been considered already optimal for CD8 T-cell activation. This stimulation was able to increase CD8 T-cell activation, but we found no evidence for preferential protection from death: the increase in memory CD8 T-cell numbers achieved by 4-1BB stimulation was either proportional to or slightly less than the increase seen in the spleen during the acute response. Furthermore, we found that administering anti-OX40 mAb alone enhanced CD4 T cells, and that administering anti-OX40 mAb and anti-4-1BB mAb together increased the numbers of CD8 T cells more than anti-4-1BB mAb alone.

The most significant effect of 4-1BB stimulation was seen at the peak of the response. 4-1BB stimulation increased the total number of CD8 T cells, as well as the number of antigen-specific CD8 T cells. Similar results have been reported in an influenza model; stimulating 4-1BB using a different agonist mAb led to a two- to threefold increase in the number of influenza-specific CD8 T cells in infected lungs during the acute infection.[27] These cells were highly active, and showed enhanced cytotoxicity directly *ex vivo*. Thus, in both that study and ours, stimulating 4-1BB in the presence of an already strong stimulus, acute virus infection, caused an increase in absolute numbers of activated antigen-specific CD8 T cells at the peak of the response. In our study, there was an increase in total splenic CD8 T cells not accounted for by antigen-specific CD8 T cells. Although not explicitly demonstrated, we believe that the majority of this is caused by CD8 T cell responses to epitopes from the viral vectors that were used for the boost.

Because we were primarily interested in prophylactic vaccine development, we were particularly interested in the

potential of 4-1BB to enhance the memory response. In studies using a variety of cleared pathogens, the number of CD8 T cells that become memory cells is directly proportional to the number of CD8 T cells seen at the peak of the response, ~5–10%.[21] Because of the evidence that 4-1BB engagement can rescue activated cells from death[3,20] we predicted that 4-1BB stimulation would increase the percentage of activated cells that became memory cells. We were therefore surprised when our data failed to support a role for 4-1BB in protection from death. However, Hurtado *et al.*[3] examined T-cell death *in vitro*, which may be controlled by different factors than T-cell death *in vivo*. Furthermore, Takahashi *et al.*[20] stimulated T cells with superantigen, but T-cell activation by superantigen is different from viral antigen stimulation in that the apoptotic signal is dominant over survival (i.e. fewer T cells are present after the response resolves than were initially present), and the kinetics of cell death are also much more rapid. We repeated the experiments performed by Takahashi *et al.* and also found that 4-1BB stimulation increased the survival of superantigen-activated CD8 T cells (data not shown). We conclude therefore that the difference between the effect reported by Takahashi *et al.* and the results reported here lies in a difference between T-cell activation by superantigen and virus infections, rather than lab-to-lab variation. Our data suggest that 4-1BB stimulation does not increase survival of CD8 T cells activated by VV.

There are a growing number of studies that have looked at the role of 4-1BB in T-cell expansion and activation state, particularly CD8 T cells. At the same time, the role of OX40 in CD4 T-cell function is also being examined in increasing detail. Although OX40 has been previously shown to enhance CD8 T-cell activation, this is the first study we are aware of to examine the ability of 4-1BB and OX40 to cooperate in both CD8 and CD4 T-cell function.

Although our results demonstrate that under some circumstances 4-1BB stimulation alone affects CD4 T cells, there was some variability between experiments, and statistical significance was not always reached. We also observed differences in splenomegaly between experiments, which may have been either random or from some difference in antibody preparations. The bottom line though is that while some aspects of our results were less robust than others, we report here the range that we saw and our main conclusions are based only on reproducible results.

In summary, we found that 4-1BB stimulation increased the size of the CD8 T-cell response induced by a vaccine both at the height of the response and in the memory phase. However, there was no preferential survival of activated CD8 T cells into the long-term memory population. We also found that OX40 stimulation was able to increase CD4 T-cell memory, and that when OX40 was combined with 4-1BB, the CD8 T-cell response was further improved. These experiments, using stimulating antibody, were designed as an initial proof-of-principle. 4-1BBL and OX40L, encoded within recombinant DNA and viral vectors, may be able to enhance the memory CD8 T-cell response to a prophylactic antiviral vaccine. However, the ability of these molecules to improve the response to a strong viral vaccine vector is likely to be less than has been seen with superantigen and tumour immunogens.

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