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Evaluation of OX40 Ligand as a Costimulator of Human Antiviral Memory CD8 T Cell Responses: Comparison with B7.1 and 4-1BBL

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CTL are important effectors of antiviral immunity. Designing adjuvants that can induce strong cytotoxic T cell responses in humans would greatly improve the effectiveness of an antiviral vaccination or therapeutic strategy. Recent evidence suggests that, in addition to its well-established role in costimulation of CD4 T cell responses, OX40L (CD134) can directly costimulate mouse CD8 T cells. In this study, we evaluated the role of OX40L in costimulation of human antiviral CD8 T cell responses and compared it with two other important costimulators, B7.1 (CD80) and 4-1BBL (CD137L). Delivery of OX40L to human monocytes using a recombinant replication-defective adenovirus led to greater expansion, up-regulation of perforin, enhanced cytolytic activity, and increased numbers of IFN-γ- and TNF-α-producing antiviral memory CD8 T cells in cultures of total T cells. Synergistic or additive effects were observed when OX40L costimulation was combined with 4-1BBL (CD137L) or B7.1 (CD80) costimulation. In total T cell cultures, at low Ag dose, 4-1BBL provided the most potent costimulus for influenza-specific CD8 T cell expansion, followed by B7.1 (CD80) and then OX40L. For isolated CD8 T cells, 4-1BBL was also the most consistent costimulator, followed by B7.1. In contrast, OX40L showed efficacy in direct activation of memory CD8 T cells in only one of seven donors. Thus, OX40L costimulates human antiviral memory CD8 T cell responses largely through indirect effects and can enhance anti-influenza, anti-EBV, and anti-HIV responses, particularly in combination with 4-1BBL or B7. The Journal of Immunology, 2005, 175: 6368–6377.

The CD8 CTL are important effectors of antiviral and antitumor immunity (1). They play a key role in containing viral diseases such as HIV. For example, it has been reported that increases in HIV-specific CTL correlate with decreases in viral load (2). However, progression of HIV leads to reduced CTL function, characterized by reduced perforin, reduced proliferation, and reduced maturation of the HIV-specific CTL (3–5). Designing a therapeutic strategy that targets CTL (specifically, improving their effector function) would be invaluable for immunosuppressive viral diseases such as HIV. This may be possible through the modulation of costimulatory signals.

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OX40-OX40L signals have been shown to costimulate T cell effector functions, increasing proliferation and cytokine responses, enhancing T cell survival (20–24), and even reversing T cell anergy (25). Most studies have demonstrated a bias for OX40 in the activation of CD4 T cells (9). However, recent data using murine models suggest that OX40 may play a role in CD8 responses, controlling the survival of proliferating CD8 T cells, but not their cytotoxic or functional capacities (26). Furthermore, in the same study, agonistic anti-OX40 Abs were shown to decrease tumor growth in a CD4-deficient environment, suggesting a direct role for OX40 on CD8 T cells. OX40 was also shown to synergistically enhance CD8 T cell expansion when combined with 4-1BB stimulation, resulting in the rejection of a murine sarcoma in a CD4 T cell-independent manner (27).

In this study, we investigate a possible role for OX40-OX40L signaling in the costimulation of human antiviral memory CD8 T cell responses, using an adenoviral system to deliver OX40L to donor monocytes. Moreover, we investigate the potential benefit of dual costimulation offered by OX40L in combination with either B7.1 or 4-1BBL for augmenting anti-influenza and anti-HIV-1 responses. We demonstrate that OX40L on human monocytes acts as an adjuvant, enhancing the expansion and effector function of Ag-specific CD8 T cells. In addition, dual costimulation by OX40L in combination with B7.1 or 4-1BBL leads to improved expansion and effector function of CTL over costimulation with individual costimulatory molecules. Experiments with isolated CD8 T cells indicate that these effects of OX40L are dependent on the presence of CD4 T cells.

Materials and Methods

Tetramers, Abs, and peptides

MHC I class I–peptide tetramers were produced, as previously described (19, 28). The following peptides were used: influenza HLA-A2-restricted GILGFVFTL (M1, 58-66) and EBV HLA-A2-restricted GLCTLVAML (BMLF1 280-288) were from the Alberta Peptide Institute; HIV HLA-A2-restricted SLYNTVATL (gag, 77-85), HIV HLA-B7-restricted IPRQG (env 843-851), and HIV HLA-B8-restricted FLKEKGGL (inf 90-97) were purchased from the Sheldon Biotechnology Center.

Anti-human CD8 and CD4 were purchased from eBioscience. Anti-human OX40L was obtained from R&D Systems. Anti-human perforin, IFN-γ, TNF-α, IL-2, B7.1, 4-1BBL, and OX40 were obtained from BD Pharmingen. Anti-human OX40L was obtained from R&D Biotechnologies. Anti-human Bcl-xL was purchased from Southern Biotechnology Associates.

Recombinant adeno viruses

Replication-defective adenovirus 5 expressing 4-1BBL (4-1BBL-adv) or B7.1 (B7.1-adv) or no transgene as a control (control-adv) were generated using the two-plasmid rescue method, as described previously (19). The OX40L-adv (this study) was generated by the same method. Transgene expression was confirmed by FACS analysis using adenovirus-infected 293 cells and human monocytes. Large scale virus purification was done using virus-infected 293N3S cells. Following cell lysis, viruses were purified by cesium chloride gradient ultracentrifugation. Virus titers were measured by plaque assay.

Sample collection

All healthy donors gave informed consent, as approved by the University of Toronto human subjects review board. A total of 100 ml of blood was obtained from healthy volunteers by venal puncture. PBMC were isolated by Ficol-Paque density gradient centrifugation and frozen in 10% DMSO in 50/50 FCS/medium mixture at −150°C. Donors were screened for HLA-A2 by FACS analysis. For all donor samples used in this study, an influenza- and/or EBV tetramer-positive population was detectable in unstimulated PBMC ranging from 0.05 to 0.3% of CD8+ T cells.

HIV-infected subjects gave informed consent, as approved by the University of Toronto, the University of Montreal, and the McGill University Health Care Centre ethics committees. Leukaphoresis samples were obtained from HIV-infected patients, and PBMC were isolated and frozen, as above. Patients were HLA typed using the amplification-resistant mutation system (ARMS-PCR; Pel-Freez Clinical Systems), and reactivity to HIV peptide was established by using IFN-γ ELISPOT.

Costimulation assays

Freshly thawed PBMC were suspended at 8 × 106 cells/ml medium and plated at 0.5 ml/well of a 48-well plate, or 0.1 ml/well of a 96-well plate. PBMC were incubated for 1 h at 37°C to allow monocytes to adhere to the plastic, and nonadherent cells were removed and kept overnight at 37°C. OX40L-adv, 4-1BBL-adv, B7.1-adv, or control-adv were added to the purified monocytes at a multiplicity of infection (MOI) of 50, followed by centrifugation at 37°C at 3000 rpm for 1 h. Peptides were added to the monocytes postcentrifugation. Optimum peptide concentrations were determined for each HIV subject in trial experiments to ensure low background due to peptide alone. In healthy donors, influenza peptide was used at a concentration of 0.5 μM, unless otherwise indicated. Following an overnight incubation, the APC were washed twice to remove excess adenovirus and peptide. T cells were added from HLA-A2-restricted CD8 T cell line using the pan T cell-negative selection kit from Miltenyi Biotec (MACS). T cell purity was routinely better than 99.5% by flow cytometry. Purified T cells were added to the APC at a concentration of 1 × 105 cells/well of a 48-well plate, and 2 × 105 cells/well of a 96-well plate. T cells and APC were cocultured for 7–8 days in the absence of any exogenous cytokines. We also monitored the cultures from HIV+ donors by p24 ELISA (AIDS reagent and reference program) at the end of the stimulation period; however, no virus was detected.

CD8 T cells were purified from adherent cell-depleted PBMC by negative selection using the Human CD8+ T Cell Isolation Kit II (Miltenyi Biotec; MACS). The purity of the CD8 T cells was on average ~92%. Based on pilot experiments in which we titrated IL-2 at different Ag doses (to find a range in which we saw Ag-dependent and costimulation-depen- dent T cell expansion), IL-2 was added to the CD8 T cell cultures at 0.04 U/ml. Higher doses resulted in increased background responses. Total T cell cultures did not include any added cytokines.

Flow cytometry

Samples were stained with tetramers at 37°C for 15 min and washed in cold buffer. All subsequent Ab stains were done on ice. For intracellular cytokine staining, samples were restimulated with 5 μM peptide for 5 h in the presence of GolgiPlug or GolgiStop (BD Biosciences). Following restimulation, cells were first stained for surface markers, then fixed and permeabilized using the CytoFix/CytoPerm kit (BD Biosciences), followed by intracellular staining with anti-cytokine Abs. In all figures, the indicated gates were set based on staining with isotype control Ab for each sample (data not shown). Cells were analyzed by flow cytometry using a FACS-Calibur (BD Biosciences), and data were analyzed using FlowJo (Tree Star).

CTL assays

The HLA-A2+ cell line T2 or HLA-matched autologous EBV-transformed B-lymphoblastoid cell line were used as target cells for the CTL assays. T2 cells were pulsed with 5 μM HIV, influenza, or EBV peptides, or irrelevant melanoma control peptide overnight. Targets were then labeled with 200 μCi of Na251CrO4 for 1 h, washed thoroughly, and incubated with T cells for 4 h. Supernatants were analyzed for the release of radioactive chromium. Nonspecific chromium release was subtracted from each reading, and data were expressed as a percentage of maximum chromium release. Lysis observed with the irrelevant melanoma peptide control-pulsed targets was used to correct for nonspecific lysis. B-lymphoblastoid cell line targets were incubated with 5 μM peptide and labeled with Na251CrO4 concurrently over 1 h.

Statistical analysis

Data are presented as means with SDs, unless otherwise stated. Statistical significance was assessed using paired two-tailed Student’s t test.

Results

Expression of OX40L on APC and OX40 on T cells

To examine the effect of OX40L costimulation on T cell antiviral responses, we used replication-defective recombinant adenoviruses to deliver OX40L to donor monocytes. Forty-eight hours postinfection with recombinant adenoviruses carrying the OX40L

Abbreviations used in this paper: MOI, multiplicity of infection; CI, confidence interval.
gene, human monocytes expressed OX40L on their surface, as assessed by flow cytometry using an anti-human OX40L mAb (Fig. 1A). Control adenovirus-infected monocytes had no detectable OX40L expression. We observed a dose response for expression, leveling off at ~50 MOI and reaching a maximum of ~35–50% positive at 100 MOI. Similar results were obtained when 4-1BBL-adv and B7.1-adv were used (19) (data not shown). Titration of the dose of OX40L, 4-1BBL, and B7.1-adv showed that, for each molecule, maximal stimulatory effects were also obtained at an MOI of 50–100 (data not shown). Therefore, an MOI of 50 was used for all subsequent experiments to maximize expression and function, while minimizing the amount of virus used.

In most healthy individuals, resting T cells do not express OX40. To test whether OX40 expression is induced in donor T cells during the stimulation period, we analyzed T cells by flow cytometry for expression of OX40, at 48 h postexposure to peptide-loaded adenovirus-infected monocytes. OX40 surface expression was evident on both CD8 and CD4 T cells (Fig. 1B). OX40 was detected independently, of which recombinant adenovirus was used to infect the monocytes, and was costimulation independent. Thus, the adenovirus-modified monocyte model results in expression of OX40L on the APC and Ag-dependent induction of OX40 on the T cells.

**OX40L costimulates antiviral memory CD8 T cell responses**

To test the effect of OX40L on memory responses to viruses, we incubated T cells from healthy donors with syngeneic adherent monocytes infected with either OX40L-adv plus influenza M1 peptide, control-adv plus influenza M1 peptide, or OX40L-adv plus irrelevant melanoma peptide. OX40L costimulation significantly enhanced expansion of influenza-specific tetramer \(^+\) T cells as compared with controls (Fig. 2A, first and second row, and B). The average expansion over peptide alone (control-adv) was 3.29-fold (95% confidence interval (CI) = 2.3–4.27). Similar results were observed when the EBV peptide BMLF1 was used to measure EBV responses (data not shown). Results are reported as percentage of tetramer positive, but conversion to total T cells gave similar results.

To investigate the effector function of the expanded T cells, we restimulated the T cells with influenza peptide at the end of the 8-day stimulation assay. We observed a consistently higher number of IFN-γ- and TNF-α-producing CD8 T cells in cultures costimulated with OX40L compared with controls (Fig. 2A, third and fourth row). The number of cytokine-producing cells was proportional to the number of tetramer \(^+\) T cells, meaning that the conditions that gave maximal expansion of tetramer \(^+\) T cells also showed the highest proportion of cytokine-producing cells. Very few, if any, IL-2-producing CD8 T cells were observed under any of the stimulatory conditions (Fig. 2A, bottom row).

We also analyzed perforin levels in the tetramer \(^+\) T cell population. Perforin levels were higher in OX40L-stimulated T cells as compared with control (Fig. 2C). OX40L-stimulated T cells also showed enhanced ability to kill influenza peptide-pulsed target cells in a chromium release assay (Fig. 2D). This increased killing appears to correlate more closely with the increased frequency of tetramer \(^+\) T cells (Fig. 2A) rather than with the level of perforin per cell (Fig. 2C).
OX40L, like other costimulatory members of the TNF superfamily, has been shown to enhance the survival of T cells by regulating the antiapoptotic factors Bcl-xL and Bcl-2 (23). Bcl-xL levels were increased in OX40L-costimulated cultures in three of six experiments, with an average increase over control of $32 \pm 12.7\%$ (see Fig. 2E for an example).

**FIGURE 2.** OX40L augments influenza-specific recall CD8 T cell responses. Human monocytes from healthy donors were infected with OX40L-adv or control-adv, coated with influenza M1 or control peptide, and cocultured with autologous T cells for 7–8 days, as described in Materials and Methods. A, Postculture cells were analyzed for CD8 and binding of HLA-A*0201/influenza M1 tetramer by flow cytometry (first and second row). Data shown are gated on CD8$^+$ events. The number indicates the percentage of tetramer$^+$ T cells. Cytokine production was assessed by intracellular staining after 5 h of restimulation with 5 μM influenza peptide in the presence of GolgiPlug. Cells were analyzed for CD8 and IFN-γ (third row), TNF-α (fourth row), or IL-2 (fifth row). Data shown are gated on CD8$^+$ events. The number indicates the percentage of cytokine-positive cells. Representative data are shown. Similar data were obtained from six donors (three donors for IL-2). Experiments with each donor were repeated one to three times. B, A summary of percentage of influenza tetramer$^+$ CD8 T cells from 10 experiments (performed as described in A) is shown. Data were normally distributed, and statistical significance was assessed by paired Student’s $t$ test. C, Intracellular perforin levels were assessed by flow cytometry. OX40L-adv-costimulated cells are shown in black, control-adv in gray, and isotype control in black dots. Cells are gated on tetramer$^+$ CD8$^+$ cells, as shown in A. Representative data are shown. Similar data were obtained from six donors. D, Killing of influenza peptide-coated T2 target cells by T cells costimulated for 8 days is shown. OX40L-adv-costimulated cells are shown in gray, control-adv in black slashes, and irrelevant peptide in dots. Representative data are shown. Similar data were obtained from four donors, with experiments repeated one to two times. E, Intracellular Bcl-xL levels were assessed by flow cytometry. OX40L-adv-costimulated cells are shown in black. Control-adv-costimulated cells are shown in gray, and isotype control in black dots. Cells are gated on tetramer$^+$ CD8$^+$ cells, as shown in A. Representative data are shown. As described in Results, six experiments were performed and Bcl-xL levels were higher in the OX40L-adv-costimulated cultures in three of six experiments.
Thus, OX40L enhanced both influenza- and EBV-specific antiviral memory CTL responses, as evidenced by greater expansion of tetramer+ T cells, with enhanced cytokine production, and cytotoxic capacity.

**Comparison of OX40L with 4-1BBL and B7.1**

B7.1 is the classic costimulatory ligand, acting with its receptor CD28 to provide the second signal required for T cell activation (6). 4-1BBL is a TNF superfamily member, like OX40L, and has been shown to costimulate primarily CD8 responses, both in mouse and human, but also CD4 responses (9). Thus, it was of interest to compare the efficacy of OX40L in costimulation of CD8 T cells with that of 4-1BBL and B7.1.

Previous results have shown that 4-1BBL lowers the effective peptide dose for the expansion of influenza-specific CD8 T cells by as much as 100-fold (19). Therefore, to compare the efficacy of the different costimulatory molecules, we conducted peptide titrations at a fixed MOI of adenovirus for several donors with demonstrated HLA-A2-restricted responses to influenza M1 peptide (Fig. 3). For all donors tested, at low peptide concentrations, 4-1BBL was clearly superior to B7.1 and OX40L in augmenting Ag-specific memory CD8 T cell expansion. At higher Ag doses, B7.1 approached 4-1BBL in efficacy, whereas OX40L was the weakest of the three costimulators overall. These data suggest that the order of efficacy of the three costimulatory molecules for activation of memory CD8 T cell expansion in cultures of total T cells is 4-1BBL > B7.1 > OX40L.

**Role of OX40L, 4-1BBL, and B7.1 on purified CD8 T cells**

Most donors have probably been exposed to adenovirus in the environment (29). Thus, although we add only a minimal CD8 T cell epitope to the cultures, CD4 T cells could contribute to the response both by responding to adenovirus components delivered to the APC or through regulatory effects. Therefore, it was of interest to test the role of these costimulatory molecules in a pure CD8 T cell plus monocyte culture. Due to the limited amount of IL-2 produced by CD8 T cells, we added IL-2 to these cultures. Initial experiments showed that IL-2, even at 1 U/ml, resulted in higher backgrounds and/or Ag-dependent CD8 memory T cell expansion independently of costimulation (data not shown). However, reduction of the added IL-2 to 0.04 U/ml resulted in costimulation-dependent, Ag-dependent CD8 T cell responses, and this dose was used in all subsequent experiments with purified CD8 T cells.

Of eight A2-positive donors tested, seven showed an Ag-specific CD8 T cell response to influenza virus. Seven of seven responders showed enhanced responses when 4-1BBL was included, and five of seven showed enhanced responses with B7.1. In contrast, OX40L was a very weak costimulator for isolated human CD8 T cells and stimulated an Ag-specific response above control adenovirus-treated monocytes in only one of seven donors (Fig. 4, lower right panel). The 4-1BBL was more effective than B7.1 in four of seven donors and equivalent to B7.1 in three of seven donors (see Fig. 4 for representative examples of each type of response). Thus, OX40L appears to function in human Ag-specific memory CD8 T cell expansion only in the presence of CD4 T cells, whereas B7.1 and 4-1BBL can function to directly stimulate human memory CD8 T cells. Subsequent analysis of OX40L in combination with other costimulators was therefore done with total T cell cultures.

**Additive effects of combining OX40L costimulation with either B7.1 or 4-1BBL in total T cell cultures**

To determine whether dual costimulation through OX40L and either B7.1 or 4-1BBL would result in enhanced memory CD8 T cell expansion and activation, we stimulated healthy donor T cells for 8 days with monocytes infected with OX40L-adv in combination with either B7.1-adv or 4-1BBL-adv and coated with influenza M1 peptide. Overall, dual stimulation with OX40L and B7.1 resulted in a significant enhancement in influenza-specific tetramer+ memory T cell expansion over stimulation with either molecule alone (Fig. 5, A and B). In some (7 of 14) experiments, effects of dual costimulation showed a synergistic trend (that is, the values for dual costimulation were on average 38% greater than additive; range 11–92% greater). Strictly additive effects were observed in 4 of 14 (28.6%) experiments.

Dual stimulation with OX40L and 4-1BBL also resulted in a significant overall enhancement in tetramer+ T cell expansion over single molecule stimulation (Fig. 6, A and B). We observed a synergistic trend (on average 48% greater than additive; range 20–93%) in 7 of 14 (50.0%) experiments and additive stimulatory effects in 6 of 14 (42.9%) experiments.

![Comparison of costimulatory effects of OX40L, 4-1BBL, and B7.1 on anti-influenza CD8 responses in cultures of total T cells.](http://www.jimmunol.org/DownloadedFrom/)
In a subset of experiments, we investigated the effector function of T cells exposed to dual vs single costimulation, by restimulating with peptide at the end of the 8-day stimulation period. Dual costimulation leading to enhanced tetramer$^+$ T cell expansion correlated with a higher number of IFN-$\gamma$- and TNF-$\alpha$-producing CD8$^+$ T cells (data not shown). The additive effects of combining OX40L with either B7.1 or 4-1BBL were also evident when assessing the cytotoxic activity of these T cells using the chromium release assay. Dual costimulation leading to enhanced tetramer$^+$ T cell expansion (Figs. 5A and 6A) correlated with an enhancement of cytotoxic activity against peptide-loaded target cells (Figs. 5C and 6C). This enhanced effector function appears to be due to an enhanced number of activated T cells, rather than an enhanced effector function per T cell.

Thus, dual costimulatory signals provided by OX40L in combination with a second TNF family member 4-1BBL or the CD28 family member B7.1 resulted in a significant enhancement of antiviral memory cytotoxic T cell responses over costimulation with the individual costimulatory molecules.

**OX40L costimulates HIV-specific CTL response: effects of dual costimulation**

To determine whether the costimulatory effects of OX40L we observed with influenza and EBV extended to HIV-specific responses, we tested four chronically infected HIV-1$^+$ subjects in the in vitro costimulation assay. Stimulation with OX40L-adv-infected, peptide-loaded monocytes resulted in higher expansion of Ag-specific (tetramer$^+$) CD8 T cells over peptide alone (control-adv) in all experiments performed ($n = 7$; Fig. 7A). The mean increase with OX40L costimulation over peptide alone was 2.9-fold (95% CI = 1.0 – 4.8). In comparison with OX40L, the mean increase over peptide alone for B7.1 costimulation was 2.0-fold (95% CI = 0.50 – 3.6), and for 4-1BBL costimulation was 2.9-fold (95% CI = 1.6 – 4.2). OX40L stimulation also enhanced the number of CD8$^+$ T cells producing TNF-$\alpha$ and IFN-$\gamma$ in response to peptide restimulation (Fig. 7B), including the number of TNF-$\alpha$ and IFN-$\gamma$ double-positive T cells, a subset shown to be better effectors in the context of HIV infection (30). Consistent with the enhancement in tetramer$^+$ T cell expansion seen with OX40L stimulation, we also observed higher perforin levels (data not shown) and enhanced cytotoxic activity assessed by chromium release assay over controls (Fig. 7C).

Dual costimulation with OX40L in combination with B7.1 resulted in improved tetramer$^+$ T cell expansion over either molecule alone in three of four HIV$^+$ subjects (Fig. 7A), with an overall mean increase over peptide alone of 6.1-fold (95% CI = 1.7 – 10.4). OX40L in combination with 4-1BBL resulted in improved tetramer$^+$ T cell expansion in two of four HIV$^+$ subjects (Fig. 7A), with an overall mean increase over peptide alone of 4.2-fold (95% CI = 2.0 – 6.4). Each subject was tested in duplicate, with similar results observed in both experiments.

Dual costimulation leading to enhanced tetramer$^+$ T cell expansion (Fig. 7A) correlated with an enhancement of cytotoxic activity, especially when OX40L stimulation was combined with B7.1 (Fig. 7C, right panel). Although OX40L in combination with 4-1BBL resulted in an enhancement of cytotoxic activity, the level of cytotoxicity was similar to that seen with 4-1BBL alone (Fig. 7C, left panel).

In all subjects in which dual costimulation improved tetramer$^+$ T cell expansion (Fig. 7), it also caused an enhancement in the number of CD8$^+$ T cells producing TNF-$\alpha$ and IFN-$\gamma$, including the number of TNF-$\alpha$ and IFN-$\gamma$ double-positive T cells (Fig. 7B). Dual costimulation also resulted in higher TNF-$\alpha$ production in the one patient not showing enhanced tetramer$^+$ T cell expansion with OX40L and B7.1 costimulation, and in one of the two patients not showing enhanced tetramer$^+$ T cell expansion with OX40L and 4-1BBL costimulation.

**Discussion**

Generating a strong cell-mediated immune response, which includes a specific and effective CD8 T cell response, should be the goal of any vaccine designed to control viral infections. Replica-tion-deficient adenoviruses incorporating viral Ags have been used in preclinical trials, in mice and nonhuman primates, to elicit effective CTL immune responses against viral diseases such as HIV/SIV (31, 32). In fact, an adenovirus-based vaccine strategy for HIV-1 is currently in phase II trials in North America. Several approaches have been used to boost the immune response to these vaccine candidates, including the combination of adenovirus-based vaccines with DNA or poxvirus vaccines in prime-boost strategies. Incorporation of costimulatory molecules in adenovirus vaccine constructs may serve as adjuvants to boost immune responses.
In this study, we investigate the role of OX40L costimulation on human antiviral CD8 T cell responses, using recombinant replication-deficient adenoviruses to deliver OX40L to human monocytes. The data show that in cultures of total T cells, OX40L enhances Ag-specific expansion of memory antiviral CD8 T cells. OX40L costimulation also enhanced effector function, as evidenced by higher levels of perforin on the gated tetramer-HLA-M1 tetramer binding by flow cytometry. Data shown are gated on CD8^+ events. The number indicates the percentage of tetramer^+ T cells. Data shown are representative of 14 experiments performed using 6 donors. A, A summary of percentage of influenza tetramer^+ CD8 T cells from all 14 experiments is shown. Data were normally distributed, and statistical significance was assessed by paired Student’s t test. Not shown in the figure: OX40L-adv, B7.1-adv, and OX40L + B7.1-adv are statistically significantly different from control-adv (p < 0.05). The mean percentage of tetramer positive for each costimulatory condition is shown below the x-axis labels. Percent specific lysis of influenza peptide-coated T2 target cells by T cells costimulated for 8 days is shown. OX40L-adv-costimulated cells are shown in gray triangles, B7.1-adv in □, B7.1 + OX40L-adv in ▪, control-adv in black slashes, and irrelevant peptide in black dots. Representative data from one donor are shown. Similar data were obtained from three donors, with experiments repeated one to two times per donor.

In this study, we investigate the role of OX40L costimulation on human antiviral CD8 T cell responses, using recombinant replication-deficient adenoviruses to deliver OX40L to human monocytes. The data show that in cultures of total T cells, OX40L enhances Ag-specific expansion of memory antiviral CD8 T cells. OX40L costimulation also enhanced effector function, as evidenced by higher levels of perforin on the gated tetramer^+ T cells, compared with control-adv and Ag-stimulated cells. There was also better killing of peptide-pulsed targets compared with controls and higher numbers of Ag-specific IFN-γ and TNF-α-producing CD8 T cells, correlating with increased numbers of Ag-specific T cells. Bcl-x<sub>L</sub> expression was also increased over controls in a portion of donors tested. We observed similar effects of OX40L costimulation for influenza-, EBV-, and HIV-1-specific memory CD8 T cell responses.

We previously showed, using a similar experimental model, that 4-1BBL costimulation increases influenza and EBV CD8 T cell recall responses, leading to improved expansion and effector function (19). Thus, it was of interest to determine which costimulatory molecules for costimulation of CD8^+ T cell expansion (Fig. 3), consistent with evidence that 4-1BBL is an important costimulatory molecule for CD8 T cell responses to viruses in mice (9). In isolated CD8 T cell cultures, both B7.1 and 4-1BBL showed strong costimulatory effects in five of seven and seven of seven donors, respectively. However, OX40L stimulated a direct response on CD8 T cells in only one of seven responders tested. Thus, B7.1 and 4-1BBL can costimulate human CD8 T cells directly, whereas OX40L effects on human CD8 T cells require the presence of CD4 T cells, suggesting that OX40L-dependent costimulation of CD4 T cells contributes to the CD8 T cell responses, possibly via cytokine production.

Previous studies have shown that HIV-specific CD8 T cells from subjects with nonprogressive disease have greater proliferative capacity (4). Recent evidence suggests that the presence of IL-2/IFN-γ-producing CD8 T cells correlates with this improved proliferative capacity and allows CD4-independent CD8 T cell expansion (33). In the present study, although OX40L improved T cell expansion and effector function, particularly in combination with B7.1 or 4-1BBL, there was no direct effect of OX40L on...
generation of IL-2-producing CD8 T cells (Fig. 2A). This is consistent with findings in mouse, in which OX40L was found to primarily effect survival rather than proliferation by CD4 and CD8 T cells (23, 26). In contrast, 4-1BBL can induce IL-2 production in the absence of CD28 (34, 35). Furthermore, costimulation of human anti-influenza memory CD8 T cells by Ag plus adenovirus-delivered 4-1BBL resulted in the appearance of IL-2-producing influenza-specific CD8 T cells that were not observed with Ag plus control adenovirus-treated cultures (19). Recent evidence suggests that highly purified CD8 T cells from some recently or chronically infected HIV donors can respond to 4-1BBL or B7.1 costimulation in the absence of added IL-2 (42). Thus, 4-1BBL costimulation, but not OX40L costimulation, may be sufficient for generation of CD4-independent IL-2-producing CD8 T cells.

The role of OX40L in humans has been investigated in human antitumor CD8 T cell responses. In one study, OX40L transferred to human leukemic B cells was unable to expand tumor-reactive CD8 T cells unless paired with CD40L (36). In a second study, human culture-derived dendritic cells transfected with OX40L mRNA used to stimulate autologous PBMC were shown to be better at inducing tumor Ag-specific CD8 T cell IFN-γ production and killing activity, compared with dendritic cells lacking OX40L (37). In that study, the role of OX40 in direct stimulation of CD8 T cells was not tested.

The finding that 4-1BBL was markedly superior to B7.1 or OX40L in enhancing Ag-specific CD8 T cell responses at limiting Ag doses (Fig. 3) is consistent with the observations that 4-1BBL-deficient mice have decreased antiviral T cell responses in the CD8, but not CD4 T cell compartment (15–17, 38), whereas OX40- or OX40L-deficient mice primarily have deficiencies in the CD4 compartment (20–22, 38). However, in the presence of a strong adjuvant or with supraphysiological stimulation with agonistic Abs, OX40 ligation can clearly promote murine CD8 T cell expansion in vivo (26, 27). In the present study, which analyzed human antiviral CD8 T cell responses, the effects of OX40L on CD8 T cell expansion were found to be largely due to indirect effects requiring the presence of CD4 T cells. The effect of OX40L on isolated CD8 T cells was minimal at best.

We previously attempted to amplify costimulatory signals by combining 4-1BBL with B7.1. However, the combination did not result in any increased CD8 T cell expansion compared with stimulation with the individual costimulatory molecules in healthy donors (19). Recent evidence suggests that this is due to excessive IFN-γ production in cultures of total T cells from healthy donors.
stimulated with 4-1BBL and B7.1 in combination (42). In contrast, when we combined OX40L with either 4-1BBL or B7.1, we observed a significant enhancement of peptide-specific antiviral CD8 T cell responses over costimulation by either molecule alone. This was observed not only in the expansion of tetramer$^+$ T cells, but in their ability to produce cytokines and their cytotoxic activity. Our data are in agreement with two previous studies done in mice, showing that coadministration of anti-4-1BB and anti-OX40 Abs resulted in enhanced effector CD8 T cell accumulation (27, 39).

The data presented in this study suggest that incorporating dual costimulation provided by OX40L in combination with either 4-1BBL or B7.1 could further improve the effectiveness of an adenovirus vaccine. This is especially relevant in the context of HIV, in which we observed dual costimulation leading to higher levels of T cells producing both TNF-$\alpha$ and IFN-$\gamma$ (Fig. 7), a subset previously demonstrated to be more effective against HIV (30).

In summary, we present data suggesting that delivery of OX40L individually, but especially in combination with either 4-1BBL or B7.1, using a recombinant replication-defective adenovirus, is an effective way to deliver costimulatory signals leading to enhanced Ag-specific expansion and effector function of antiviral memory CD8 T cells. Replication-defective adenovirus-based vaccines are a promising therapeutic strategy for improving immune responses to infectious agents and tumors (32, 40, 41). Incorporation of OX40L, particularly with other costimulatory molecules, as part of an adenovirus-based vaccine strategy may provide sufficient adjuvant effects to not only improve the immune response to the vaccine, but to also allow for a reduction in the quantity of adenovirus needed to be delivered for a therapeutic dose, thus reducing toxicity concerns.

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Disclosures
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