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CD40 Ligand (CD154) Enhances the Th1 and Antibody Responses to Respiratory Syncytial Virus in the BALB/c Mouse

Ralph A. Tripp, Les Jones, Larry J. Anderson, and Michael P. Brown

CD40 ligand (CD40L) is a cell surface costimulatory molecule expressed mainly by activated T cells. CD40L is critically important for T-B cell and T cell-dendritic cell interactions. CD40L expression promotes Th1 cytokine responses to protein Ags and is responsible for Ig isotype switching in B cells. Respiratory syncytial virus (RSV) is an important pathogen of young children and the elderly, which causes bronchiolitis and pneumonia. Studies of mice infected with RSV suggest that a Th2 cytokine response may be responsible for enhanced pulmonary disease. To investigate the effect CD40L has on RSV immunity, mice were infected simultaneously with RSV and either an empty control adenovirus vector or one expressing CD40L or were coimmunized with plasmid DNA vectors expressing CD40L and RSV F and/or G proteins and subsequently challenged with RSV. The kinetics of the intracellular and secreted cytokine responses, the cytotoxic T lymphocyte precursor frequency, NO levels in lung lavage, rates of virus clearance, and anti-RSV Ab titers were determined. These studies show that coincident expression of CD40L enhances the Th1 (IL-2 and IFN-γ) cytokine responses, increases the expression of TNF-α and NO, accelerates virus clearance, and increases the anti-F and anti-G Ab responses. These data suggest that CD40L may have the adjuvant properties needed to optimize the safety and efficacy of RSV vaccines. The Journal of Immunology, 2000, 164: 5913–5921.

Worldwide, the most important cause of bronchiolitis and serious lower respiratory tract disease in infants and young children is respiratory syncytial virus (RSV) (1–3). Natural infection with RSV provides limited protection from reinfection and disease, as demonstrated by the recurrence of even severe RSV infections throughout life (4, 5). Moreover, the peak incidence of serious RSV disease is in the young infant who has maternally acquired neutralizing RSV Abs. Thus, the development of RSV vaccines has long been a priority for infants and young children (2, 6–9), and more recently, vaccines are being developed for older children at risk for serious complications of infection and for the elderly (10–12). RSV vaccine development has been focused toward live temperature-sensitive, attenuated vaccines and subunit vaccines based on the F and/or G glycoproteins (13, 14). In animal model systems the F glycoprotein is the most effective, and G glycoprotein is the next most effective in inducing neutralizing Abs and protective immunity, but vaccination with the G glycoprotein generally induces only limited protection (15, 16). In clinical studies, no vaccine has yet proven safe and efficacious, mainly because candidate live virus vaccines have been under- or overattenuated or have reverted to the wild-type phenotype (17). However, the recent availability of an RSV infectious clone has expanded the options in developing live virus candidate vaccine strains and renewed hope that a safe and efficacious live virus vaccine can be developed (18, 19). Development of subunit vaccines, in particular, has been hindered by concerns about the enhanced lung disease that occurred in young children who received a formalin-inactivated RSV vaccine and then had natural RSV infection (20). Although the reasons for this vaccine-augmented disease remain uncertain, evidence in the BALB/c mouse model suggests that a heightened Th2 cytokine response may be responsible in part (21–23). In these studies enhanced lung pathology in formalin-inactivated RSV-vaccinated mice challenged with live RSV was shown to be mediated by Th2-type CD4+ T cells expressing IL-4, IL-5, IL-6, and IL-10. In contrast, mice immunized and challenged with live RSV do not develop extensive lung pathology and respond with a mixed Th1/Th2 immune response (21, 24–26).

The best indicator of protection from RSV disease is high titers of neutralizing Abs, which is illustrated by the fact that passive administration of high titrated neutralizing RSV Abs can decrease the risk of serious RSV disease (27, 28). Unfortunately, both attenuated live and subunit candidate vaccines induce only modest increases in Abs (29, 30). Consequently, a variety of adjuvants are being evaluated for their ability to enhance the immune response to subunit vaccines (31–33). In addition, genes for immune-enhancing molecules could be introduced into live virus vaccine candidates using the RSV infectious clone.

One potential immune-enhancing molecule is CD40L. CD40L is critical to productive interactions between T cells and APC (34–40). A requirement for CD40L in anti-viral immune responses was shown in studies that examined the anti-adenovirus response in CD40L−/− mice. In these studies diminished CD4+ T cell priming and reduced IL-4, IL-10, and IFN-γ cytokine expression occurred in the absence of CD40L (41). The importance of CD40L expression for both the B and T cell immune responses was also shown in CD40L−/− mice challenged with lymphocytic choriomeningitis virus (LCMV) (42, 43). CD40L−/− mice infected with LCMV...
were capable of generating primary CTL responses, but had defective memory CTL responses. Furthermore, primary anti-LCMV IgG1 Ab responses were severely impaired in the absence of CD40L. The importance of CD40L expression in the development of Th1 cytokine responses was also demonstrated by Ab inhibition studies in which anti-CD40L Ab decreased Th1-mediated autoimmune diabetes in nonobese diabetic mice by reducing IL-12 secretion and slightly increasing IL-4 production (44).

We hypothesize that supplementary CD40L expression during primary RSV infection could promote Th1 over Th2 immune responses, increase the Ab response, and improve the safety and efficacy of RSV vaccines. We examined two different methods that used CD40L as an adjuvant to enhance the immune response to RSV and show that its presence has a broad immune-enhancing effect that might be beneficial for an RSV vaccine.

Materials and Methods

Animals

Four- to 6-wk-old, specific-pathogen-free, female BALB/c mice were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). The mice were housed in microisolator cages and were fed sterilized water and food ad libitum.

Viruses

RSV/A2 (A2) was grown in Vero cells (African green monkey kidney fibroblasts; CCL 81, American Type Culture Collection, Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2% heat-inactivated (56°C for 30 min) FBS (HyClone, Salt Lake City, UT), 1% l-glutamine, and 1% antibiotic/antimycotic (TCM; all from Life Technologies). Upon usually detectable cytopathic effect, the medium was decanted, replaced with a minimal volume of Dulbecco’s modified PBS (D-PBS), and frozen at −70°C. The flask was thawed, and the loosely adherent cell monolayer was scraped off using a cell scraper (Costar, Cambridge, MA) and collected. The cell lysate and supernatant were centrifuged at 2000×g for 20 min at 4°C. The resultant supernatants were collected, subdivided into aliquots, and stored at −70°C or in liquid nitrogen vapor. The titer was determined by methylcellulose plaque assay on Vero cells. Virus titer was measured by plaque assay. Virus stock was incubated for 3 h at 37°C on a Vero cell monolayer that was then overlaid with 2% carboxymethylcellulose (Sigma, St. Louis, MO) in TCM and incubated for 5–6 days at 37°C. Finally, the cells were fixed with 4% formalin containing 0.1% crystal violet dye (Sigma), and the end-point titer was determined by macroscopic counting of plaques.

Adenovirus (Ad) constructs

Recombinant replication-defective adenovirus vectors were made using an adenovirus backbone that contained deletions in E1 and E3 regions. The murine CD40L cDNA (45) was cloned into the XbaI and EcoRV sites of the pAVS6 adenoviral transfer plasmid (Genetic Therapy, Gaithersburg, MD) under the control of the Rous sarcoma virus promoter. Control (Ad-VC) and murine CD40L adenovirus vectors (Ad-CD40L) were produced as previously described (46). The empty control adenovirus vector (Ad-VC) was produced under identical conditions, except that it lacked the murine CD40L cDNA insert. Virus titer was measured by plaque assay of 293 cells. Removal of contaminating wild-type virus was achieved by triple-plaque purification, which was confirmed by two observations: 1) absent E1 DNA using a PCR-based method (47), and 2) absent cytopathic effect after supernatants from vector-infected and noncomplementing A549 cells were passaged serially on A549 cells (48).

DNA vectors and vaccination

DNA vaccines were prepared using pcDNA3.1 vector plasmids (Invitrogen, San Diego, CA) constructed with G or F gene cDNA from RSV/A2 (49) or murine CD40L cDNA (46). G gene DNA vaccine (pcDNA-G), F gene DNA vaccine (pcDNA-F), murine CD40L DNA vaccine (pcDNA40L), and control (pcDNA3.1 only) plasmid constructs were propagated in Escherichia coli SURE2 cells (Stratagene, La Jolla, CA) and purified using an EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA). For immunization, DNA plasmids were precipitated onto 1-μm gold microcarriers (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Mice were anesthetized with Avertin (2,2,2-tribromoethanol), their abdomen hair was shaved, and they were immunized in two different locations in the obliquis extremus abdominis muscle with 20 μg of DNA vaccine/immunization under helium velocity using the Helios Gene Gun (Bio-Rad). Using a similar immunization procedure, mice were boosted with a total of 40 μg of DNA vaccine every week for 3 wk. To confirm plasmid expression in vivo, serum from eye bleeds was collected weekly and analyzed by ELISA for Abs against RSV-infected and uninfected Vero cells.

Infection, treatment, and sampling

For experiments involving Ad as a vector, mice were anesthetized with Avertin and subsequently infected by i.n. administration of 10⁶ PFU/mouse of A2 immediately followed by i.p. infection with 2×10⁶ PFU/mouse with Ad construct Ad-VC or Ad-CD40L or with a PBS control. The Ad vector dose was given i.p. because of exacerbated pulmonary inflammation that occurred following i.n. administration of both Ad vector and RSV. A dose response of the Ad vectors (2×10⁴, 2×10⁵, and 2×10⁶ PFU/mouse) given i.p. suggested that optimal immune enhancement occurred in mice immunized with 2×10⁶ PFU/mouse. For DNA vaccine experiments, 1 wk following the last of three DNA boosts, mice were anesthetized with Avertin and subsequently challenged by i.n. administration of 10⁷ PFU/ml of A2. At various times following challenge, mice were anesthetized with Avertin and exsanguinated by severing the right caudal artery, and lymphoid organs and cells were removed. All organs and cells were collected, on ice, in HBSS (Life Technologies). The lung was lavaged three times using HBSS containing 0.1% BSA (Sigma). Four to six mice were used for each experiment, and experiments were repeated on three separate occasions.

Flow cytometry

Bronchoalveolar lavage (BAL) and spleen cells were examined for intracellular cytokine expression directly ex vivo. Single-cell suspensions of lymphocytes were blocked with 10% normal mouse sera (The Jackson Laboratory, Bar Harbor, ME) in D-PBS for 15 min at 4°C. The procedure used for intracellular (IC) cytokine staining was modified for microwell staining from the protocol described by PharMingen (San Diego, CA). Briefly, the cells were washed in PBS (Life Technologies), and the cell surface Ag was stained with the appropriate Ab and subsequently fixed with 4% paraformaldehyde (Ted Pella, Redding, CA) in D-PBS containing 0.1% BSA. The cells were washed in PBS, and the membrane permeabilization using 0.1% saponin (Sigma). All Abs were PE labeled and purchased from PharMingen. Anti-IL-2 (JES6-5H4), anti-IL-4 (BVD4-1D11), anti-IL-5 (TRFK5), anti-IL-6 (MP5-20F3), anti-IL-12 (C15.6), anti-IFN-γ (XMG1.2), and anti-anti-TNF-α (XMG2.2) Abs were PE labeled and purchased from PharMingen. Anti-IL-2 (JES6-5H4), anti-IL-4 (BVD4-1D11), anti-IL-5 (TRFK5), anti-IL-6 (MP5-20F3), anti-IL-12 (C15.6), anti-IFN-γ (XMG1.2), and anti-TNF-α (XMG2.2) Abs were PE labeled and purchased from PharMingen. Anti-IL-2 (JES6-5H4), anti-IL-4 (BVD4-1D11), anti-IL-5 (TRFK5), anti-IL-6 (MP5-20F3), anti-IL-12 (C15.6), anti-IFN-γ (XMG1.2), and anti-TNF-α (XMG2.2) Abs were PE labeled and purchased from PharMingen.

FlowMatrix

Screted cytokines in BAL cell-free exudate were quantitated directly ex vivo using FlowMatrix analysis (Luminex, Austin, TX) and a FACSscan (Becton Dickinson) using a modified Ab capture-sandwich assay protocol on defined latex microspheres. In brief, individual prestained red spectrum-colored microsphere bead sets (Luminex) were diluted in PBS/Tween (Sigma), and the capture Abs (anti-IL-2, JES-1A12; anti-IL-4, BDV4-1D11; anti-IL-5, TRFK5; anti-IL-6, MP5-20F3; anti-IL-12, C15.6; anti-IFN-γ, XMG1.2, and anti-TNF-α, XMG2.2) were microwell- and vial-assayed using a two-step method. A standard curve and were assayed against duplicate dilutions of cell-free BAL exudate in PBS. Capture Ab-coated microspheres were incubated with normalized 1-ml standards or unknown 1-ml samples for 30 min at room temperature. Following incubation, ALEXA-streptavidin (Molecular Probes, Eugene, OR)-conjugated biotinylated cytokine detection Abs (IL-2, JES6-5H4; IL-4, BDV6-24G2; IL-5, TRFK4; IFN-γ, XMG1.2; all from PharMingen) were incubated with the samples for 30 min at room temperature. Following incubation, the beads were pelleted by centrifugation, resuspended in PBS/TBN, and analyzed using FACSscan and FlowMatrix software.

MHC class I-restricted CTL precursor (CTLP) assays

Class I-restricted target cells used were the mouse mastocytoma line, P815 (American Type Culture Collection, TIB 64). The P815 cell lines were maintained in RPMI 1640 (Life Technologies) containing 10% FBS (HyClone) plus 1% antibiotic/antimycotic (Life Technologies). The target cells
were prepared by suspending 10^6 cells in 1.0 ml of serum-free MEM (Life Technologies) containing 10^4 PFU/ml RSV cell lysate (or a comparable dilution of uninfected cell control lysate) for 18 h at 37°C followed by addition of 1.0 ml of MEM containing 10% FBS and 200 μCi of ^51 Cr (Na_2CrO_4, Amersham, Arlington Heights, IL) and incubating for an additional 2 h at 37°C. The cells were then washed and resuspended to an appropriate concentration in TCM comprised of suspension-MEM (Life Technologies) containing 10% FBS (HyClone), 1% essential amino acids, 2% nonessential amino acids, 2% sodium pyruvate, 2% l-glutamine, 1% antibiotic/antimycotic (all from Life Technologies), and 50 μM 2-ME (Sigma).

Virus-specific CTLp prevalence was determined using a modification of a well-established limiting dilution assay (50). In brief, different dilutions of responder cells in 0.1 ml of TCM were added to wells (24 wells/dilution) of round-bottom, 96-well microtiter plates (Costar) with 0.1 ml of APCs. The APCs were syngeneic splenocytes that had been incubated in a serum-free MEM (Life Technologies) containing 10^7 PFU/ml RSV for 3 h at 37°C and resuspended at 10^7 cells/ml in TCM containing 20% EL4.IL-2 supernatant (the lymphoma cell line, EL4.IL-2 (American Type Culture Collection, TIB 181), endogenously secretes IL-2). The responder cells and APCs were incubated at 37°C for 7 days in a humidified atmosphere. The contents of individual wells were then divided in two, placed into replica plates, and incubated for 6 h with 10^4 ^51 Cr-labeled, RSV-infected or mock-infected target cells. The virus-specific CTLp frequency was estimated using linear regression and 95% confidence intervals about the slope of the regression line plotting the number of cells vs the number of nonresponding cultures. A responding well was defined as one in which the mean ^51 Cr release from RSV-infected targets plus responding cells was ≥3 SD from the mean of ^51 Cr release from control wells containing uninfected target cells plus responding CTLp. The virus-specific CTLp frequency was estimated according to the Poisson equation at the 37% nonresponding culture point (Fp) along the slope of the linear regression line. The 95% confidence intervals were used to determine significance, which is indicated by p < 0.05%.

**Nitrite determination**

Nitrite was measured by using a modified Greiss reaction. Briefly, 1 ml cell-free BAL samples were diluted 2-fold with distilled water and deproteinized with zinc sulfate to give a final concentration of 15 g/l. The samples were centrifuged at 1000 g for 15 min at 10°C, and 0.1-ml aliquots were transferred to microtiter plate wells (Costar). Greiss reagent (0.1 ml: 1 g/l sulfanilamide, 25 g/l phosphoric acid, and 0.1 g/l N-1-naphthylethylenediamine) was added to the wells, mixed by gentle pipetting, and allowed to incubate for 15 min at room temperature. The absorbance was read on a microplate reader (Titer-Tek, McLean, VA) at 540 nm. Each sample was assayed in triplicate. Background was determined by treating normalized BAL samples only with 25 g/l phosphoric acid. Sodium nitrite in distilled water was used to generate standard curves.

**Determination of anti-RSV Ab titers and Ig isotypes**

Mice were anesthetized with Avertin, and 200 μl of peripheral blood was collected from the eye capillary bed. The serum from the peripheral blood was collected and analyzed for RSV Abs by ELISA with RSV-infected or mock-infected target cells.
uninfected Vero cell lysate-coated microtiter plates and peroxidase-conjugated anti-mouse Ig (Accurate, Westbury, NY). Ig isotypes were determined using an isotyping kit according to the manufacturer’s instructions (Pierce, Rockford, IL). Specimens were tested at 2-fold dilutions from 1/100 to 1/3200. The ratio of absorbance from RSV-infected cells over the absorbance of uninfected Vero cell lysate was used in analysis of the ELISA results.

Statistical analysis
Each experiment was performed using four to six individual animals. SEMs were calculated for each experiment. The data shown represent the results from one of three separate experiments. The sample variance ($s^2$) between experiments was determined by subtracting the mean from each observation, squaring the differences obtained, and dividing the squared differences by the total number of observations minus [($s^2 = \Sigma_{i=1}^n(x_i - \bar{x})^2/n - 1$)].

Results
CD40L enhances the expression of intracellular Th1 cytokines
BALB/c mice infected i.n. with RSV were concurrently given saline (sham) treatment i.p. (Fig. 1A) or infected with empty adenovirus vector or Ad-VC (Fig. 1B) or with Ad vector containing murine CD40L cDNA, and Ad-CD40L (Fig. 1C). The kinetics of the IC cytokine response of BAL T cells is shown in Fig. 1. BAL T cells from sham-treated mice after RSV challenge expressed peak cytokine levels between days 7 and 14 p.i. (Fig. 1A). Expression of Th1 cytokine levels was generally higher than that of Th2 cytokines (Fig. 1A). Following RSV infection, BAL T cells from mice infected with Ad-VC (Fig. 1B) showed similar kinetics and patterns of cytokine expression as sham-treated mice (Fig. 1A). Peak expression levels of Th1 cytokines IL-2 (19%) and IFN-γ (17%), were higher than those of Th2 cytokines, IL-4 (11%), IL-5 (9%), and IL-6 (10%; Fig. 1B). In comparison, BAL T cells from mice simultaneously infected with Ad-CD40L and RSV had augmented expression levels of IL-2, IL-6, IL-12, IFN-γ, and TNF-α and modified cytokine expression kinetics (Fig. 1C). Maximal expression levels of IL-2 expression (38%) occurred on day 10 p.i., peak IL-6 expression (18%) occurred on day 10 p.i., peak IL-12 expression (25%) occurred on day 14 p.i., peak IFN-γ expression (36%) occurred on day 10 p.i., and peak TNF-α expression (34%) occurred on day 14 p.i. (Fig. 1C). Expression of IL-4 or IL-5 was not increased in comparison with either sham-treated (Fig. 1A) or Ad-VC-infected (Fig. 1B) mice, suggesting that coincident expression of CD40L primarily enhanced Th1-type cytokine expression.

Cytokine expression in T cells from the spleen was also analyzed after Ad vector infection and RSV challenge (Fig. 2). Although the percentage of CD3+ T cells positive for each cytokine

![Diagram](http://www.fjimmunol.org/broadenhancementoftheanti-rsvimmuneresponsebycd40l.png)
increased post-treatment compared with naive (day 0) values, the percentage of cells expressing each cytokine was lower in spleen than in BAL (Fig. 1). The Th1/Th2 cytokine profiles were similar to those observed in T cells from the BAL. Sham-treated mice that were infected with RSV alone maximally expressed Th1 (IL-2, IL-12, and IFN-γ) and Th2 (IL-4, IL-5, and IL-6) cytokines and TNF-α between days 7 and 10 p.i. (Fig. 2A). The cytokine profiles for Ad-VC-infected (Fig. 2B) and sham-treated mice were similar, except for a shift in the kinetics of the peak response. Peak cytokine expression for IL-2 (8%), IL-4 (8%), IL-6 (13%), IFN-γ (15%), and TNF-α (14%) all occurred on day 14 p.i., whereas peak expression of IL-5 (6%) and IL-12 (7%) occurred on day 10 p.i. (Fig. 2B). Infection with Ad-CD40L lead to a decrease in the expression of Th2 cytokines and IFN-γ (Fig. 2C).

**CD40L enhances the secretion of pulmonary Th1 cytokines**

The concentrations of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokines in the cell-free BAL fluid of RSV-infected, sham-treated (Fig. 3A), Ad-VC-infected (Fig. 3B), or Ad-CD40L-infected (Fig. 3C) mice were determined. The BAL cytokine responses in sham-treated and Ad-VC-infected mice were moderate compared with those in Ad-CD40L-infected mice. For sham-treated mice, IL-2 and IFN-γ concentrations ranged from 50–100 pg/ml from days 3–14 p.i., and IL-4 and IL-5 ranged from 10–25 pg/ml (Fig. 3A). Similar concentrations of secreted cytokines were detected in Ad-VC infected mice (Fig. 3B), except that higher levels of IL-2 (200–225 pg/ml) were found between days 7 and 14 p.i. (Fig. 3B). The late elevation in IL-2 may reflect an anti-adenovirus T cell response occurring in the lung; Ad given i.p. is expected to infect the lung. In contrast to Ad-VC-associated cytokine levels, much higher levels of IFN-γ were produced in BAL after infection with Ad-CD40L (Fig. 3C). In particular, 75–150 pg/ml of IL-2 and 150–300 pg/ml IFN-γ were detected between days 5–7 p.i.; levels peaked on day 10 p.i. (IL-2, 175 pg/ml; IFN-γ, 525 pg/ml). IL-4 and IL-5 were not detected in the BAL at any time point examined (Fig. 3C). These data suggest that infection with Ad-CD40L can enhance the Th1 cytokine response in the lung, especially augmenting the production of IFN-γ.

**FIGURE 3.** Quantification of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokine secretion in the cell-free BAL fluid during the immune response of mice treated simultaneously with RSV i.n. and i.p. injections of saline (sham-treated; A), Ad-VC (B), or Ad-CD40L (C). The data for each time point are given as the mean picograms per milliliter of the cytokine plus SEM of four to six individual mice.
Coincident expression of CD40L enhances pulmonary nitrite production associated with enhanced virus clearance

Pulmonary macrophages produce NO to destroy pathogens and invasive organisms. The NO reaction produces nitrite, molecular oxygen, and water; thus, nitrite levels correlate well with NO production (22). Nitrite concentrations were analyzed in cell-free BAL from sham-treated, Ad-VC-infected, and Ad-CD40L-infected mice that were challenged simultaneously with RSV (Fig. 4). BAL nitrite levels peaked between days 5 and 7 p.i. in sham-treated (90 μM), Ad-VC-infected (125 μM), and Ad-CD40L-infected (160 μM) mice. By day 10 p.i., BAL nitrite levels declined in sham-treated mice (25 μM), but remained high in Ad-VC-infected and Ad-CD40L-infected mice. By day 14 p.i., nitrite levels had dropped to the 20–35 μM range in all groups (Fig. 4).

Enhanced virus clearance was observed in mice infected with Ad-CD40L compared with sham-treated or Ad-VC-infected mice (Table I). Ad-CD40L-infected mice challenged with RSV cleared virus by day 7 p.i., whereas sham-treated and Ad-VC-infected mice cleared virus between days 10–14 p.i. (Table I). Moreover, Ad-CD40L-treated mice exhibited reduced RSV titers on day 6 p.i. compared with sham-treated or Ad-VC-infected mice. No significant differences in virus clearance were observed at any time point examined between Ad-VC-infected and PBS-treated mice. The enhanced virus clearance correlated temporally with CD40L expression, increased pulmonary NO production, and enhanced IC Th1 cytokines and pulmonary Th1 cytokine secretion.

RSV-specific MHC class I-restricted CTLp frequencies persist in CD40L-treated mice

To address the effect of coincident CD40L expression on the pulmonary RSV-specific CTLp frequency, MHC class I-restricted CTLp frequencies were measured in the BAL from sham-treated, Ad-VC-infected, and Ad-CD40L-infected mice at various times following RSV challenge (Fig. 5). The average values from all experiments (n = 9) are summarized and show that infection with Ad-CD40L helps to sustain a higher frequency (average, 1/6,000) of RSV-specific CTLp at a later time point (day 21 p.i.) in the lung than either sham treatment (average, 1/8,200) or Ad-VC-infected mice. Significant differences in virus clearance were observed at any time point examined between Ad-VC-infected and PBS-treated mice. The enhanced virus clearance correlated temporally with CD40L expression, increased pulmonary NO production, and enhanced IC Th1 cytokines and pulmonary Th1 cytokine secretion.

Table I. Titer of RSV in lungs of mice

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* Mice were i.p. immunized with 2 × 10^7 PFU Ad construct or PBS and i.n. challenged with 10^7 PFU/mouse RSV/A2 in PBS.

* Vero cell monolayers were seeded onto 24-well flat-bottom tissue culture plates. Cells were inoculated with 200 μl of 10-fold dilutions of lung lysate in PBS. Virus was allowed to absorb for 2 h at 37°C, and overlay medium was added to each well. Overlay medium consisted of RPMI 1640 + 2% FCS + 0.9% Noble agar. The cultures were incubated for 6–7 days at 37°C, fixed for 5 min with staining solution (PBS + 1% glutaraldehyde + 0.1% crystal violet), and individual plaques were counted. Virus titers were determined from three replicates/dilution. Data represent the range of dilution’s producing plaques.

**FIGURE 4.** Nitrite levels produced in the cell-free BAL of mice treated simultaneously with RSV and i.p. injections of saline (sham-treated), Ad-VC, or Ad-CD40L. The data for each time point are given as micromolar concentrations of nitrite plus SEM of four to six individual mice.

**FIGURE 5.** CTLp frequencies during the immune response of mice simultaneously challenged with RSV i.n. and given i.p. injections of saline (sham-treated), Ad-VC, or Ad-CD40L. The frequencies of class I-restricted CTLp and the ranges are shown for nine experiments. Significant differences are given by 95% confidence limits.
ratios for serum at a serum dilution of 1/3200 from five or six mice examined, no distinct Th1- or Th2-type humoral pattern emerged in relation to any of the infections. However, mice infected with Ad-CD40L and simultaneously challenged with RSV; the sera were collected and analyzed on days 14 and 21 p.i. As the same rank order of Ig isotype concentrations (IgG2a > IgG2b > IgG1 > IgM > IgG3 > IgG1) applied to all groups examined, no distinct Th1- or Th2-type humoral pattern emerged in relation to any of the infections. However, mice infected with Ad-CD40L did develop a higher titer of anti-RSV Ab, i.e., a 2-fold higher increase in titer on day 21 p.i. (1/3200) compared with sham-treated mice (1/1600) or mice infected with Ad-VC (1/1600).

To determine whether CD40L could augment the Ab response to RSV, mice were sham treated with PBS, infected with Ad-VC, or infected with Ad-CD40L and simultaneously challenged with RSV; the sera were collected and analyzed on days 14 and 21 p.i.. As the same rank order of Ig isotype concentrations (IgG2a > IgG2b > IgG1 > IgM > IgG3 > IgG1) applied to all groups examined, no distinct Th1- or Th2-type humoral pattern emerged in relation to any of the infections. However, mice infected with Ad-CD40L did develop a higher titer of anti-RSV Ab, i.e., a 2-fold higher increase in titer on day 21 p.i. (1/3200) compared with sham-treated mice (1/1600) or mice infected with Ad-VC (1/1600).

A striking 7-fold increase in the Ab response (i.e., absorbance ratio) to the G subunit vector was induced by the addition of CD40L vector alone (Fig. 6). The anti-RSV Ab titers exceeded 1/3200 for all mice except those immunized with the G subunit vector alone. The anti-RSV Ab responses were enhanced by the addition of the CD40L vector, as indicated by higher absorbance ratios at the 1/3200 dilution (Fig. 6).

Discussion

Our findings support our hypothesis that expression of CD40L during RSV infection or immunization would enhance the overall immune response and promote a Th1-type over a Th2-type response. Previous studies suggested that CD40L might enhance both humoral and cellular immune responses (35, 36, 51–54). CD40L expression has been shown to enhance T cell and APC activation and signaling, the importance of which has been revealed by studies of CD40L−/− mice (42, 55–62) and CD40L−/− humans (63). Coincident expression of CD40L has been shown to promote T cell-mediated immunity (37, 41, 64–66). Constitutive retroviral expression of CD40L restored Ag-specific cytolytic and humoral immune responses in CD40L−/− mice infected intramurally with the Hkx31 strain of influenza (66). Immunization of BALB/c mice with DNA plasmids expressing β-galactosidase and CD40L increased the Th1-type immune response to β-galactosidase (51). Infection of mice with recombinant vaccinia virus that expressed CD40L markedly enhanced viral clearance by both an IFN-γ-dependent mechanism and a novel CD40L-dependent mechanism (64). Administering anti-CD40 mAb to mice together with pneumococcal polysaccharide generated strong, isotype-switched Ab responses (67).

Our studies also revealed that CD40L expression coincident with RSV infection or coexpressed during DNA vaccination had broad immune-enhancing effects. CD40L enhanced the levels of IL-2 and IFN-γ (Figs. 1 and 3), increased pulmonary NO synthesis (Fig. 4), increased the frequency of RSV-specific CTL precursors in the lung (Fig. 5), increased the anti-RSV Ab response (Fig. 6), and decreased the RSV clearance time (Table I). The increase in pulmonary NO production associated with CD40L treatment may have contributed to the more rapid clearance of RSV. NO is believed to inhibit an early stage of viral replication and spread (68), possibly by activating APC (69) and the associated increased production of IFN-γ and activation of bystander T cells (70). CD40L expressed by Th cells is a major contributor to T cell-dependent NO production by macrophage, and reduced macrophage production in CD40L-deficient mice enhanced susceptibility to Leishmania infection (61, 62). The increased expression of TNF-α and IFN-γ and the enhanced Ab response could also contribute to the accelerated clearance of RSV from the lungs (Table I).

Although we did not observe an overall increase in RSV-specific CTLp frequency with CD40L expression, the higher CTLp frequency observed at 21 day p.i. compared with that with control treatments raises the possibility that CD40L may enhance the duration of the RSV-specific CTL response (Fig. 5). In studies of CD40L−/− mice infected with LCMV and Pichinde virus, the primary CTL response was normal, but the memory response was defective (42).

Finally, coexpression of CD40L with F and G proteins enhanced the RSV Ab response to later RSV challenge (Fig. 6). This priming effect was most pronounced for the anti-G Ab response. Because a high titer of neutralizing RSV Abs may be needed for effective protection as has been shown in passive Ab studies (28, 71), we presume that the same may also apply for RSV vaccination. If this is the case, then expression of CD40L with a RSV vaccine may enhance the RSV Ab response and thus the protective immune response.

In summary, our results indicate that supplementary expression of CD40L broadly enhances the RSV immune response and directs this response toward a Th1 phenotype. The results suggest that CD40L can enhance RSV immunity and might be a useful adjuvant for an RSV vaccine.

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