CD40 Ligand Preferentially Modulates Immune Response and Enhances Protection against Influenza Virus

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*J Immunol* 2014; 193:722-734; Prepublished online 13 June 2014;
doi: 10.4049/jimmunol.1300093
http://www.jimmunol.org/content/193/2/722

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/06/13/jimmunol.1300093.DCSupplemental

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CD40L, a key regulator of the immune system, was studied as both a targeting ligand and a molecular adjuvant in nucleoprotein (NP)-based host defense against influenza in mouse models with different genetic backgrounds. Adenoviral vectors secreting NP-CD40L fusion protein (denoted as rAd-SNP40L) afforded full protection of immunocompetent and immunocompromised mice (CD40L−/− and CD4−/−) against lethal influenza infection. Mechanistically, rAd-SNP40L preferentially induced early and persistent B cell germinal center formation, and accelerated Ig isotype-switching and Th1-skewed, NP-specific Ab response. Moreover, it drastically augmented primary and memory NP-specific CTL activity and polyfunctional CD8+ T cells. The markedly enhanced nonneutralizing Abs and CTLs significantly reduced viral burdens in the lungs of mice upon lethal virus challenge. Data generated from CD40L−/− and CD4−/− mice revealed that the protection was indeed CD40L-mediated but CD4+ T cell independent, demonstrating the viability of the fusion Ags in protecting immunodeficient hosts. Notably, a single dose of rAd-SNP40L completely protected mice from lethal viral challenge 4 mo after immunization, representing the first report, to our knowledge, on NP in conjunction with a molecular adjuvant inducing a robust and long-lasting memory immune response against influenza. This platform is characterized by an increased in vivo load of CD40-targeted Ag upon the secretion of the fusion protein from adenovirus-infected cells and may represent a promising strategy to enhance the breadth, durability, and potency of Ag-specific immune responses. The Journal of Immunology, 2014, 193: 722–734.
or retargeting the Ag-delivery vector to CD40 on APCs (21–30). Although enhanced immune responses against various pathogens and tumors were observed, little is known about in vivo B cell responses, functional roles of CD4^+^ and CD8^+^ T cells during induction phase, or the contributions of Abs and CD8^+^ T cells in protection.

Influenza is a highly contagious virus that causes respiratory tract infection, which represents a serious burden to public health. Current influenza vaccines largely induce strain-specific neutralizing Abs against the two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). However, the high antigenic variability of both HA and NA necessitates annual vaccine formulation and evaluation, reviewed by Hashem et al. (31). In contrast, internal proteins such as nucleoprotein (NP) are highly conserved among all influenza A subtypes but proven to elicit limited protection in animals (32–37). Although CD40L was found to enhance immune response against the highly variable HA protein (26, 30), only partial protection was obtained even with multiple-dose immunizations.

In this article, we determined the potential of CD40L as both an Ag-targeting ligand and a molecular adjuvant to enhance NP-induced host defense against influenza. Our results clearly show that targeting influenza NP via CD40L resulted in CD4^+^ T cell–induced host defense against influenza. Notably, we show for the first time, to our knowledge, that a single dose of recombinant adenosine virus (rAd) encoding secreted NP-CD40L (SNP-CD40L) fusion protein can induce robust and long-lasting protective memory immune response against influenza.

### Materials and Methods

**Cell lines and influenza virus**

NIH-3T3 (ATCC: CRL-1658), MDCK (ATCC: CCL-34), and QBI-HEK 293A cells were cultured as described previously (38). Mouse-adapted influenza A/Puerto Rico/8/34(H1N1) and A/Jingke/30/95(H3N2) viruses were used for viral challenge.

**Generation of rAds**

Constructs were designed to express influenza A/duck/Yokohama/aq10/03 (H5N1) NP (GenBank accession no. AB212281, http://www.ncbi.nlm.nih.gov/entrez) intracellularly (rAd-NP), a secreted form of NP (rAd-SNP) consisting of influenza NP and 23 aa (MLLA VLYCLLWSFQTSAGHF-43), and ectodomain of CD40L (nt 361–795) was PCR amplified in a separate reaction using NP-3 and NP-R1 primers, to generate tAd-SNP40L. PCR product was reamplified to add 29 bp of the trimerization motif, which contains the internal HindIII restriction site, using CD40L-2 and CD40L-R primers, and to generate tAdΔCD40L. Finally, both fragments, SNP40L and ΔCD40L, were gel purified, digested with HindIII, ligated, and PCR amplified using NP-3 and CD40L-R primers. SNP40L fragment was digested and inserted into the Tad vector. Fusion gene was amplified from tAd-SNP40L using NP-1 and CD40L-R primers, and inserted into the Tad vector, to generate rAd-ΔNP40L. Cloning was confirmed by DNA sequencing and restriction enzyme digestion. rAds were then generated, purified, and titrated as described previously (37).

**Protein expression and secretion in cell culture**

Generated rAds were used to infect confluent NIH-3T3 cells in six-well plates at a multiplicity of infection of 100. Forty-eight hours later, supernatants were collected and immunoprecipitated with polyclonal anti-NP Abs using either protein G agarose beads or Pierce Crosslink Magnetic IP/Co-IP Kits (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Cells were also washed with PBS and lysed using SDS-lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, and Complete Mini PMSF protease inhibitors (Roche, Indianapolis, IN). Protein expression and secretion were confirmed by Western blot as described previously (40) using rabbit polyclonal anti-NP Abs.

**Animal studies**

Six- to 8-wk-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Eight- to 10-wk-old female C57BL/6J, B6.129S2-Cd4tm1Mak/J (CD4 deficient), B6.129S2-Cd8tm1Jat/J (CD8 deficient), B6.129S2-Cd4tm1Jat/J (CD4 deficient), and B6.129S2-Cd8tm1Jat/J (CD8 deficient) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal experiments were conducted in accordance with Health Canada institutional guidelines and the approval of the Animal Care and Use Committee.

The immunization dose was 1 × 10^9^ PFU of each rAd virus. Blood samples or cells from spleens and/or draining lymph nodes (LNs) were collected as described previously (23). BALB/c and C57BL/6J mice were intranasally challenged with 10^3^–10^9^ PFU of either influenza A/Puerto Rico/8/34(H1N1) or A/Jingke/30/95(H3N2) viruses, whereas CD40L^-/-^ mice were challenged with 5×10^6^ CFU of influenza A/Puerto Rico/8/34(H1N1) virus at time points indicated in the figure legends. Survival rates are presented as a percentage of surviving animals at each time point compared with the initial number of animals in each group. Weight change of animals was monitored weekly to account for their initial body weight. Clinical scoring was performed according to the following grading scale: healthy, 0; barely ruffled fur, 1; ruffled fur but active, 2; ruffled fur and inactive, 3; ruffled fur, inactive and hunched, 4; dead, 5, as described previously (41).

**ELISA**

The end-point titer of each anti-NP Ab isotype (IgG1, IgG2a, IgG2b, or IgM) from immunized mice was determined by ELISA as described previously (37) with minor modifications. In brief, 96-well plates coated with purified SNP protein were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 for 1 h at 37°C. Then 100 μl/well of each serum sample from immunized mice at the indicated time points was added in a 2-fold serial dilution starting from 1:100 to 1:1000 dilution in blocking buffer and incubated at 37°C for 1 h. After washing, appropriate HRP-conjugated rabbit anti-mouse Abs, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG2c, or anti-IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), were added at 1:2000 dilution in blocking buffer for 1 h at 37°C. IgG2a was detected in BALB/c mice, whereas IgG2c isotype was detected in C57BL/6J mice, we refer to IgG2c as IgG2a/c as previously described (42). Tetramethylbenzidine substrate (Cell Signaling Technology, Danvers, MA) was added for 30 min at room temperature for colorimetric development, and the reaction was stopped with 0.1 M sulfuric acid. Absorbance was read spectrophotometrically at 450 nm. Endpoint Ab titers were expressed as the reciprocals of the final detectable dilution with a cutoff defined as the mean of prebleed samples plus 3 SD.

**CD8^+^ T cell intracellular cytokine staining**

Primary and memory CD8^+^ T cell IFN-γ, TNF-α, and IL-2 responses were evaluated at 4 wk after primary and secondary immunizations as previ-
usually described (37) in BALB/c mice. In brief, spleenocytes (1 × 10^7) were cultured in RPMI 1640 medium with 10% FBS in the presence of 5 μg/ml synthetic NP MHC class I-restricted peptide (TQYRALTLY) (restricted to H-2Kd) for ex vivo restimulation and 1 μg/ml GolgiPlug for 6 h. Stimulated cells were stained with allophycocyanin-eFluor 780-conjugated anti-mouse CD8α (clone 53-6.7; eBiosciences), PerCP-Cy5.5-conjugated anti-mouse IFN-γ (clone XMG1.2; eBiosciences); PE-conjugated anti-mouse TNF-α (clone MP6-XT22; eBiosciences), and Pe-Cy7-conjugated anti-mouse IL-2 (clone JES6-5H4; eBiosciences). Results for IFN-γ, TNF-α, and IL-2 were calculated as a percentage of CD8+ T cells.

Polynuclear CD8+ T cells were analyzed using Boolean gate analysis as described previously (43). A BD LSRII flow cytometer was used for data acquisition, and analysis was completed with FlowJo, Version 8.8.4 (Tree Star, Ashland, OR). Unstained cells and single-stained compensation beads (BD Biosciences, Mississauga, ON) were used as controls for background fluorescence and false-positives because of fluorochrome bleaching.

**CTL assay**

CTL assay was conducted using lactate dehydrogenase release assay as previously described (37). In brief, 3 × 10^6 spleenocytes from immunized BALB/c mice were restimulated in vitro to generate effector CTLs. After 5 d of culture, cytotoxic activity was measured by lactate dehydrogenase release using NP peptide-pulsed P815 targets (H-2d). The percentage of specific lysis was calculated as (experimental release – effector spontaneous release)/target spontaneous release) × 100%.

**B cell flow cytometry**

Single-cell suspensions of LNs or spleens from immunized BALB/c mice were washed twice with FACS buffer (PBS with 5% FBS) and stained with Pe-Cy7–conjugated anti-mouse CD19 (clone ID3; eBiosciences), allophycocyanin-eFluor 780–conjugated anti-mouse CD45RB/B220 (clone RA3-6B2; eBiosciences), PerCP-conjugated anti-mouse IgG (Jackson ImmunoResearch), allophycocyanin-conjugated anti-mouse IgD (clone 11-26; eBiosciences), Alexa Fluor 488–conjugated anti-mouse Ly76/77GL7 (clone GL-7; eBiosciences), and PE-conjugated anti-mouse Syndecan-1/CD138 (clone 281-2; BD Biosciences) for 30 min at 4˚C. In another experiment, cells were stained with PE-conjugated anti-mouse CD19 (clone 1D3; eBiosciences), FITC-conjugated anti-mouse B7.1/CD80 (clone 16-10A1; eBiosciences), and Pe-Cy5–conjugated anti-mouse CD40 (clone 1C10; eBiosciences). Stained cell samples were fixed with 1% paraformaldehyde. A BD LSRII flow cytometer was used for data acquisition, and analysis was completed with FlowJo as described earlier.

**CD8**+ **T cells adoptive and serum passive transfer**

CD8+ T cells were purified from spleenocytes obtained from immunized BALB/c mice by negative selection using the Dynal Mouse CD8 Negative Isolation Kit (Life Technologies, Burlington, ON). Purity of CD8+ T cells was determined using PE-conjugated anti-mouse CD8α (clone 53-6.7; eBiosciences) and PE-Cy5–conjugated anti-mouse CD3 (clone 145-2C11; eBiosciences), and found to be >95% pure. Naïve BALB/c mice were injected i.v. via the tail vein with 1 × 10^7 CD8+ T cells using a 27-gauge needle. Three days later, they were intranasally challenged with 10^5 LD_{50} of influenza A/Porto Rico/8/34(H1N1) virus. In another experiment, naïve BALB/c mice were injected i.p. with 300 μl donor serum from BALB/c mice immunized with the different rAd constructs on days −3 to +1 relative to infection. On day 0, mice were intranasally challenged with 10^5 LD_{50} of influenza A/Porto Rico/8/34(H1N1) virus. Lungs were harvested from mice 6 d postchallenge and used for viral titration by plaque assay in MDCK cells as described previously (44). Titeres were expressed as log_{10} PFU/g tissue.

**Data analysis**

Statistical analysis was conducted using either one-way or two-way ANOVA when appropriate. Gehan–Breslow–Wilcoxon test was used for survival curves. Bonferroni posttest was used to adjust for multiple comparisons between the different groups. All statistical analysis was conducted using GraphPad Prism software (San Diego, CA).

**Results**

**rAd construction and in vitro protein expression and secretion**

Our goal was to determine whether CD40L, as an adjuvant and targeting molecule, could enhance specific immune response against NP, a highly conserved but much less effective protective immunogen compared with the surface glycoproteins (32–34, 36, 37). We generated rAd vector expressing NP fused to an S at the N terminus and a trimerized form of murine CD40L ectodomain at the C terminus (rAd-SNP40L) (Fig. 1A). rAd-SNP40L can infect various cell types in vivo, which subsequently would secret NP-CD40L fusion protein to activate CD40+ APCs. The controls include rAd encoding, a nonsecreted form of NP-CD40L (rAd-NP40L); rAd expressing nontargeting but secreted-NP (rAd-SNP); rAd-NP expressing nonsecreted NP intracellularly; and an empty vector control.

**FIGURE 1.** rAd constructs and in vitro protein expression and secretion. (A) Schematic representation of the generated rAd constructs. The rAd-SNP40L expresses secreted NP-CD40L fusion protein. The rAd-NP40L encodes the fusion protein missing the S. rAd-NP and rAd-SNP express NP and secreted NP, respectively. The rAd-Control is an “empty” control vector. Numbers indicate the expected m.w. of the proteins from each construct. (B) In vitro protein expression in cell lysates collected from NIH/3T3 cells 48 h postinfection with rAds at multiplicity of infection of 100. (C) In vitro protein secretion in cell culture supernatant collected 48 h postinfection. Protein expression and secretion were confirmed by Western blot using anti-NP Abs. F, trimerization motif from the T4 bacteriophage fimbria; NP, influenza A/duck/Yokohama/aq10/03(H5N1) NP.
Before animal immunizations, NP proteins expressed from the transgenes were confirmed. As shown in Fig. 1B, Western blot confirmed the expression of NP, SNP, NP40L, and SNP40L in cell lysates. Supernatant from infected cells was immunoprecipitated using anti-NP Abs and analyzed by Western blot, to confirm protein secretion by rAd-SNP and rAd-SNP40L (Fig. 1C). Protein secretion was confirmed from both rAd-SNP (Fig. 1C, lane 2) and rAd-SNP40L (Fig. 1C, lane 6). The detection of NP from rAd-NP

![FIGURE 2. Targeting NP via CD40L provides heterosubtypic protection in wild-type BALB/c and C57BL/6J mice. Survival curves and lung viral titer of (A) BALB/c and (B) C57BL/6J mice immunized with two doses of 10^9 PFU of the indicated rAd constructs and challenged with 10^3 LD_{50} of mouse-adapted influenza A/Puerto Rico/8/1934(H1N1) or A/Jingke/30/95(H3N2) viruses 4 wk after secondary immunization are shown. Data are shown from one of two experiments with n = 10 mice per treatment group in survival curves and from one experiment with n = 3 mice per treatment group in lung viral titers.](http://www.jimmunol.org/)

![FIGURE 3. Single dose of rAd-SNP40L confers enhanced protection against influenza infection in wild-type BALB/c mice. Survival curves, body weight loss, and clinical score of BALB/c mice immunized with a single dose of the indicated rAd constructs and challenged with 10^3 LD_{50} of influenza A/Puerto Rico/8/34(H1N1) virus either (A) 4 wk or (B) 4 mo postimmunization. Data are shown from one of two experiments with n = 10 mice per treatment group. ***p < 0.001, **p < 0.01, *p < 0.05 (two-way ANOVA with Bonferroni posttest for clinical score and Gehan–Breslow–Wilcoxon test for survival curves).](http://www.jimmunol.org/)
In supernatant (Fig. 1C, lane 1) was expected because small amounts of extracellular NP can be observed in cell culture supernatant (45).

Immunization with rAd-SNP40L provides heterosubtypic protection in BALB/c and C57BL/6J mice

We next evaluated the protective efficacy of these constructs in BALB/c and C57BL/6J mice against lethal H1N1 and H3N2 influenza virus challenges. Of note, initial pilot experiments showed that rAd-NP40L was unable to induce any detectable Ab response or to confer any protection in BALB/c mice; thus, this construct was excluded from all subsequent studies (see Discussion). Moreover, as shown in Fig. 2, immunization with two doses of rAd-SNP40L conferred enhanced protection against both viral strains in both mouse models compared with other constructs. This was accompanied by markedly reduced viral replication in the lungs of the mice immunized with rAd-SNP40L (Fig. 2).

Single dose of rAd-SNP40L provides durable protection against lethal challenge

We then evaluated the protective efficacy of a single dose of these constructs in BALB/c mice against a lethal challenge. When immunized mice were challenged with H1N1 influenza strain 4 wk after a single immunization with either rAd-SNP40L or rAd-NP, 14-d survival rates were 70 and 40%, respectively (Fig. 3A). Furthermore, rAd-SNP40L–immunized BALB/c mice were completely protected against H1N1 influenza strain when challenged 4 mo postimmunization compared with rAd-NP, which resulted in 70% protection, indicating an overall enhanced recall response in these mice (Fig. 3B). None of the mice immunized with rAd-SNP survived when challenged 4 wk postimmunization (Fig. 3A), and only 20% of the rAd-SNP–immunized mice were protected when challenged 4 mo postimmunization (Fig. 3B), indicating that secreting influenza NP (without CD40L targeting) is a less effective strategy for vaccination. Overall, these results highlight the strength of NP-based recall responses and particularly confirm that targeting Ags via CD40L could provide enhanced effective and long-lasting protection in mice.

CD40L preferentially elicits Th1-skewed, NP-specific Abs in BALB/c and C57BL/6J mice

We next determined the differences in NP-specific Ab isotypes in immunized mice. In this article, we observed that rAd-NP induced NP-specific Abs from all isotypes in both BALB/c (Fig. 4A and 4B) and C57BL/6J mice (Fig. 5A and 5B) 4 wk after primary or secondary immunization. In contrast, rAd-SNP induced up to 1-fold

FIGURE 4. CD40 targeting preferentially induces Th1 Ab isotypes against influenza NP in wild-type BALB/c mice. Ag-specific Ab titers at 4 wk after priming (A) or boosting (B) are shown for IgG1, IgG2a, and IgG2b isotypes. (C) IgG1/IgG2a ratio was calculated at 4 wk after priming and boosting to determine the type of immune response (Th2 versus Th1) induced by the various constructs. Numbers on columns indicate the mean ratio. BALB/c mice were boosted at 4 wk after primary immunization. Data are shown as mean titer ± SEM from two independent experiments, with n = 10 mice per treatment group in each experiment. ***p < 0.001, **p < 0.01, *p < 0.05 (one-way ANOVA with Bonferroni posttest).
higher NP-specific IgG1 Abs and markedly reduced the titers of IgG2a and IgG2b isotypes by 3- to 6- and 4- to 10-fold, respectively, in both mouse models. Targeting the secreted NP to CD40 via CD40L (rAd-SNP40L) altered the isotype ratios of NP-specific Abs by preferentially inducing IgG2a and IgG2b. Compared with rAd-NP, rAd-SNP40L reduced IgG1 levels by 10- to 20-fold and 5- to 8-fold in BALB/c (Fig. 4A and 4B) and C57BL/6J mice (Fig. 5A and 5B), respectively. As expected, empty vector (rAd-Control) failed to elicit any anti-NP Ab response. Regardless of the genetic background of BALB/c and C57BL/6J mice, which could affect the phenotype of the induced immune response (46), targeting NP via CD40L consistently elicited higher levels of IgG2b and IgG2a, and very low levels of IgG1, indicating Th1 bias, as demonstrated by the reduced IgG1/IgG2a ratios compared with rAd-NP and rAd-SNP in both mouse models (Figs. 4C and 5C).

**CD40L induces early and persistent B cell GC formation and Ig isotype switching in draining LNs in BALB/c mice**

Given the Th1-skewed, NP-specific Abs in sera, we next determined whether rAd-SNP40L regulates GC formation and isotype switching in draining LNs differently compared with other constructs in BALB/c mice. We first noted that rAd-SNP40L substantially altered the magnitude and the kinetics of NP-specific IgM Abs in serum. As shown in Fig. 6A, NP-specific IgM Abs in rAd-SNP40L-immunized mice peaked at day 28, albeit to low titers, and declined to low or undetectable levels by day 42, in contrast with other groups that showed significantly higher titers up to 4 wk after secondary immunization. These data suggested that rAd-SNP40L accelerated Ig isotype switching and memory B cell induction.

To further investigate the B cell development, we conducted flow cytometric analysis of B cells in draining LNs, which revealed no differences in GL7+ B GC cells between the different groups until day 14 (Fig. 6B and 6C). By day 28, rAd-SNP40L–immunized mice had significantly higher frequencies of GL7+CD19+ GC B cells (25.9% of B cells) than other groups, which showed low levels of total GL7+CD19+ GC cells (3.46–5.55% of B cells). It was not until day 56 that mice in remaining groups started to show an increase in GC formation, although at lower levels compared with rAd-SNP40L–immunized mice (Fig. 6B and 6C). Further analysis also revealed significantly higher numbers of isotype-switched (IgG1 IgD−GL7+) memory B cells at days 28 (5.6% of B cells) and 42 (7.8% of B cells) in the rAd-SNP40L–immunized mice in contrast with other groups in which <1% of CD19+ cells showed isotype switching (Fig. 6D). By day 56, the number of isotype-switched memory B cells in rAd-SNP40L–immunized mice was still higher than that in other groups (Fig. 6D).
Furthermore, there was an overall upregulation of IgG and the activation markers CD40 and CD80, and a downregulation of IgD on B cells in LNs from mice immunized with CD40-targeted NP relative to other groups, particularly postsecondary immunization (Supplemental Fig. 1B and 1C). Notably, targeting influenza NP via CD40L upregulated the plasma cell marker CD138 on B cells in both the LN and spleen (Supplemental Fig. 1D and 1E) to higher levels compared with other constructs. These CD138+ cells were B220+ and CD19+, typical of plasmablasts, confirming activation and differentiation of B cells into Ab-secreting cells in local lymphoid organs. Collectively, these data strongly suggest that there was an early and persistent induction of GC formation, as well as IgG+ memory B cell generation in mice immunized with rAd-SNP40L.

**Enhancement of NP-specific cellular immune response by CD40L in BALB/c mice**

Polarization of immune response toward Th1 phenotype using CD40L (Figs. 4 and 5) suggests an induction of CTL response. Thus, we investigated the effects of CD40 targeting on NP-specific primary and secondary CD8+ T cell immune responses compared with other immunogens in BALB/c mice. Upon stimulation with H-2Kd–restricted immunodominant peptide of influenza NP, we found a significant enhancement of NP-specific IL-2 (5.13%), IFN-γ (4.9%), and TNF-α (3.74%) producing CD8+ T cell frequencies in spleens from mice immunized with rAd-SNP40L at 4 wk postsecondary immunization (Fig. 7A–C). In contrast, other groups showed 3- to 5-fold less cytokines producing CD8+ T cells. Enhanced cytokines production was also observed 4 wk after primary immunization (Supplemental Fig. 2A–C). Because CD8+ T cells capable of producing multiple cytokines (polyfunctional CD8+ T cells) are a better indicator of cell-mediated immune response than single cytokine-secreting CD8+ T cells (47–49), we determined the levels of these polyfunctional CD8+ T cells among the different groups. We found significantly higher levels of NP-specific triple, double, and single cytokine producing CD8+ T cells in rAd-SNP40L–immunized mice compared with other groups at 4 wk after primary and secondary immunization (Fig. 7D and Supplemental Fig. 2D).

To measure effector functions of NP-specific CTLs, we tested splenocytes isolated from vaccinated BALB/c mice for their ability to directly kill target cells. As shown in Fig. 7E and Supplemental Fig. 2E, rAd-SNP40L significantly increased NP-specific, cell-mediated cytotoxicity of target cells (P815 cells pulsed with MHC class I-restricted peptide). This increase in Ag-specific, cell-mediated cytotoxicity was observed as early as 2 wk after primary vaccination (data not shown). These results indicated that CD40L had not only increased specific CD8+ T cell responses qualitatively, but also improved the quantity and activity of CD8+ T cells as demonstrated by the significant increase in NP-specific polyfunctional CD8+ T cells and CTL responses.

**Both NP-specific CD8+ T cells and Abs contribute to the enhanced protection in BALB/c mice**

Given the findings presented earlier, we reasoned that both the NP-specific CD8+ T cells and the Th1-skewed anti-NP Abs induced by CD40 targeting have contributed to the improved protection. Indeed, adoptive transfer of CD8+ T cells from either rAd-NP– or rAd-SNP40L–immunized BALB/c mice significantly reduced viral replication in lungs of challenged mice by one and two logs, respectively. Notably, mice that received CD8+ T cells from rAd-SNP40L–immunized group had greater reduction of virus loads in the lung compared with those that received CD8+ T cells from rAd-NP–immunized mice (Fig. 8A). Moreover, after we had
passively transferred sera from vaccinated wild-type BALB/c mice into naive mice, we observed that mice that received pooled sera from rAd-SNP40L–immunized mice showed significantly lower viral titers (more than one log) in their lungs after lethal viral challenge compared with other groups (Fig. 8B). These results are consistent with a model in which rAd-SNP40L immunization enhanced protection by augmenting the roles of both CD8+ T cells and NP-specific Abs.

Enhanced protection is mediated through CD40 targeting

To confirm that the enhanced rAd-SNP40L protection observed in the animal studies is indeed mediated through CD40-CD40L

FIGURE 7. Immune responses and cell-mediated cytotoxicity in wild-type BALB/c mice. Splenocytes isolated from immunized BALB/c mice 4 wk after secondary vaccination were restimulated ex vivo with synthetic NP peptide. CD8+ T cells were then stained for intracellular IL-2, IFN-γ, and TNF-α cytokines. Graphs represent frequencies of (A) IL-2–, (B) IFN-γ–, and (C) TNF-α–producing CD8+ T cells. Flow cytometry plots are representatives from one out of two independent experiments. (D) Analysis of single-, double-, and triple-cytokine–producing CD8+ T cells 4 wk after boosting. Bar graphs represent frequencies of various combinations of cytokine-producing cells normalized to represent cytokine-producing cell numbers per 100,000 cells. (E) Splenocytes were cultured ex vivo with synthetic NP peptide to generate effector cells. NP-specific cytotoxic activity was measured at different E:T ratios, and the percentage of specific lysis was calculated. Data are shown as mean ± SEM from two independent experiments, with n = 2–3 mice per treatment group in each experiment. ***p < 0.001, **p < 0.01, *p < 0.05 [one-way ANOVA in (A)–(D), and two-way ANOVA in (E) with Bonferroni posttest for both].

FIGURE 8. Targeting influenza NP to CD40+ cells improves the protection afforded by both CD8+ T cells and NP-specific Abs in wild-type BALB/c mice. Naive BALB/c mice (n = 4 per group) received (A) 1 × 107 purified CD8+ T cells or (B) serum from vaccinated donor mice was challenged with 103 LD50 of influenza A/Puerto Rico/8/34(H1N1) virus. Lung viral titer was measured on day 6 postchallenge. Data are shown as mean titer ± SEM. ***p < 0.001 (one-way ANOVA with Bonferroni posttest).
ligation, we investigated the protective efficacy of rAd-SNP40L in a CD40L<sup>−/−</sup> mouse model. As expected, all vaccinated mice, regardless of the type of rAd, generated weak NP-specific IgM Abs and barely detectable IgG Abs (Supplemental Fig. 3), supporting the role of CD40L in promoting Ig isotype switching. However, when these mice were challenged with influenza virus, all mice immunized with rAd-SNP40L were protected from lethal viral challenge, which was accompanied by significantly less weight loss and better clinical scores (Fig. 9A). Noticeably, even though CD40L does not seem to reconstitute the immune system in this mouse model for Ig isotype switching (see Discussion), these data demonstrate that the improved protective immune response against influenza is largely mediated through CD40–CD40L interaction.

**CD40L induces CD4<sup>+</sup> T cell–independent immune response and protection against influenza**

Next, we examined the role of, and the need for, CD4<sup>+</sup> T cells during immune response induction in rAd-SNP40L immunization. To this end, CD4<sup>−/−</sup> mice were used in these animal experiments. When immunized mice were challenged with 5× LD<sub>50</sub> of influenza virus, only 20–25% of rAd-NP or rAd-SNP vaccinated mice survived, whereas all mice immunized with rAd-SNP40L survived the challenge and maintained their body weight with less severe clinical symptoms (Fig. 9B). Mechanistically, CD4<sup>−/−</sup> mice failed to mount a significant Ab response to rAd-NP or rAd-SNP vaccination (Fig. 10A and 10B). Specifically, IgG2a/c and IgG2b isotypes in rAd-NP immunized CD4<sup>−/−</sup> mice were lower by 65- to 70- and 40- to 52-fold, respectively, after primary or secondary immunization compared with C57BL/J6 wild-type mice (Table I). Reduction in Ab response in CD4<sup>−/−</sup> was most prominent in the IgG1 isotype upon immunization with either rAd-NP or rAd-SNP, as shown in Table I, suggesting a critical role for CD4<sup>+</sup> T cells in IgG1 production. As expected, the difference in IgG2a/c and IgG2b Abs was less pronounced in rAd-SNP immunized mice because rAd-SNP vaccination induces mainly IgG1 and very low levels of these two isotypes in wild-type C57BL/J6 mice (Fig. 5). In sharp contrast, immunization with rAd-SNP40L induced high levels of anti-NP Abs, comparable with those observed in wild-type mice (Fig. 10A and 10B), with only a 3- to 15-fold difference between all the isotypes (Table I). Interestingly, the Ab isotypes in CD4<sup>−/−</sup> mice were skewed toward Th1 response (IgG2a/c and IgG2b) in a similar manner to that of wild-type C57BL/J6 mice. Taken together, these data indicate that CD40 targeting via CD40L induced CD4<sup>+</sup> T cell–independent Th1-skewed immune response and afforded full protection against lethal influenza challenge in CD4<sup>−/−</sup>-immunodeficient animals.

**CD8<sup>+</sup> T cells are indispensable in NP-based protection against influenza**

Finally, we tried to investigate the importance of CD8<sup>+</sup> T cells in the observed rAd-SNP40L–mediated protection. Interestingly, none of the constructs conferred any protection in the immunized mice, as shown in Fig. 11. All vaccinated mice suffered severe clinical symptoms similar to the control group and died within 7–9 d postchallenge. These results are consistent with previous reports and the important role of CD8<sup>+</sup> T cells in NP-based protection against influenza. They also suggest that even though both NP-specific Abs and CD8<sup>+</sup> T cells are involved in rAd-SNP40L–mediated protection, CD8<sup>+</sup> T cells are the main players in the observed protection (Fig. 11).

**FIGURE 9.** Immunization with rAd-SNP40L enhances protection in CD40L<sup>−/−</sup> and CD4<sup>−/−</sup> mice. Survival curves, body weight loss, and clinical score were presented in (A) CD40L<sup>−/−</sup> and (B) CD4<sup>−/−</sup> mice. Animals were challenged with 5× LD<sub>50</sub> of influenza A/Puerto Rico/8/34(H1N1) virus 4 wk after secondary immunization. Both knockout mouse models are on C57BL/6J genetic background. Data are shown from one experiment with n = 4–5 mice per treatment group. ***p < 0.001, **p < 0.01, *p < 0.05 (two-way ANOVA with Bonferroni posttest).
**Discussion**

Although several studies have used CD40L as a molecular adjuvant codelivered with Ags of interest using plasmid DNA or viral vectors, or as an adaptor protein to alter the tropism of delivery vectors, several reasons have prompted us to investigate the effect of CD40L on influenza NP-induced immunity. First, although CD40L was reported to enhance the humoral immune response against HA, it provided only partial protection with multiple doses (26, 30). Second, the internal protein NP, albeit a much more conserved protein (>94% homology), is a much weaker protective Ag compared with HA because NP immunization cannot prevent infection or the occurrence of mild symptoms. Thus, NP would represent a good candidate Ag to investigate the potential of CD40L on NP-specific immune response. Third, we wished to dissect the functional roles of the nonneutralizing Abs and T cell subsets in protection.

The transgene was designed to encode a secreted fusion protein targeted to CD40 receptors on APCs to achieve these goals. Initial experiments showed that the construct missing the S, designated as rAd-NP40L, is unable to induce any immune response or afford any protection against influenza challenge, underscoring the importance of the secretion of the fusion protein. It could be envisaged that because many cell types are susceptible to adenovirus infection including proliferating and nonproliferating cells (23, 27), gene products with S produced by the adenovirus-infected cells would increase the Ag loads in vivo (Fig. 12). Furthermore, because the fusion protein is targeted to CD40 on APCs, immune response against NP is expected to be amplified because of CD40-CD40L ligation. Although APCs themselves are relatively refractory to adenovirus infection because of their lack of its main receptor, coxsackie-adenovirus receptor (CAR), direct infection of these cells through different receptors or alternative mechanisms (50) can not only promote their own maturation, but can also result in secretion of the fusion protein into regional LNs and direct activation of T and B cells. Thus, CD40L can act as an adjuvant and targeting molecule in both the peripheries (sites of injection) and/or the secondary lymphoid organs, as illustrated in Fig. 12.

The finding that the three constructs (rAd-NP, rAd-SNP, and rAd-SNP40L) elicited differently skewed immune responses is noteworthy. Secreted NP after rAd-SNP injection resulted in Th2-skewed responses, as suggested by increased levels of NP-specific IgG1 Abs and reduced levels of the other isotypes. However, targeting the secreted NP to CD40+ cells via CD40L significantly induced Th1-biased, NP-specific Ab response. This dramatic shift from Th2 to Th1 was observed in both MHC haplotypes H-2d (BALB/c) and H-2b (C57BL/6J), and manifested by increased IgG2a/c and IgG2b, and reduced IgG1 isotype levels. Moreover, rAd-SNP40L preferentially altered the kinetics of B cell responses by promoting early and long-lasting GC formation, plasma cells induction, and isotype-switched memory B cell generation in draining LNs by day 28. In addition, targeting secreted NP via CD40L enhanced T cell responses as demonstrated

### Table I. Fold difference in Ab response between wild-type C57BL/6J and B6.129S2-Cd4tm1Mak/J (CD4^-/-) mice

<table>
<thead>
<tr>
<th>Ab Isotype</th>
<th>Primary Immunization</th>
<th>Secondary Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rAd-NP</td>
<td>rAd-SNP</td>
</tr>
<tr>
<td>IgG1</td>
<td>132</td>
<td>878</td>
</tr>
<tr>
<td>IgG2a/c</td>
<td>70</td>
<td>53</td>
</tr>
<tr>
<td>IgG2b</td>
<td>52</td>
<td>33</td>
</tr>
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See also Figs. 3 and 10.
by robust NP-specific CTL response as early as 14 d after primary immunization. It also improved the quality of CD8+ T cells by significantly increasing the frequencies of polyfunctional CD8+ T cells, which are known to be superior in mediating protection against infections and as a surrogate marker for the quality of vaccine-induced T cells responses (47–49). Taken together, the immune response was markedly enhanced in rAd-SNP40L-immunized mice at all levels, which explains the conferred heterogeneous protection in BALB/c and C57BL/6J mice, and the long-lasting protection even after a single dose of rAd-SNP40L. To our knowledge, there has been no report of NP-based vaccination capable of inducing such a strong protection against influenza.

The observation that both CD8+ T cells and Abs can reduce viral replication in the recipient mice in the adoptive and passive transfer studies provided additional mechanistic insight into the rAd-SNP40L vaccination. Although experiments involving depletion of CD8+ T cells or B cells in vaccinated mice may shed light on the importance and the role of these two arms of the immune system in protection, the fact that none of the rAdS used in this study was able to confer any protection in CD8-/- mice (Fig. 11) suggests that CD8+ T cells are indispensable in NP-based protection against influenza. Notably, passive transfer of anti-NP sera from rAd-NP- or rAd-SNP-immunized groups failed to convey protection to recipient mice, consistent with the suggestion that normal mice, not like B cell–deficient (µMT-/-) mice, require high levels of anti-NP Abs for protection (51). In contrast, sera from rAd-SNP40L immunized mice significantly reduced lung viral load, suggesting a correlation between the shift in Ab isotypes and the inhibition of viral replication. Indeed, Th1-related Abs, IgG2a/c and IgG2b, are associated with effector mechanisms such as FcRs binding, complement-fixation, or complement-mediated lysis of infected cells (52, 53), even though NP-Abs are not neutralizing Abs.

Vaccination of CD40L-/- mice with rAd-SNP40L elicited NP-specific IgM response, accompanied with very low levels of IgG2b isotype, which is very similar to rAd-NP- and rAd-SNP-immunized groups (Supplemental Fig. 3). These results suggest that the levels of CD40L produced by the transient expression from rAd-SNP40L–infected cells is insufficient to restore isotype-switching in these mice. This is consistent with previous studies showing that constitutive expression of CD40L (54) or coadministration of high doses of rCD40L (50 mg given 3 times/week), but not lower doses (5 mg given 3 times/week) (55), can only induce low to intermediate levels of Ag-specific isotype-switched Abs in CD40L-/- mice. Nevertheless, rAd-SNP40L immunization completely protected CD40L-/- mice against influenza challenge, most likely through CTL response, as our data suggest that CD8+ T cells play critical roles in protection (Figs. 8 and 11).

The role of CD4+ T cells was critical because rAd-NP- and rAd-SNP–immunized CD4-/- mice failed to mount a strong Ab response against influenza. In contrast, rAd-SNP40L–immunized CD4+/- mice showed increased production of all isotypes, including IgG1 Abs, to levels very similar to those observed in wild-type mice with a 4- to 7-fold difference only (Table I). Interestingly, like in the wild-type C57BL/6J mice, rAd-SNP40L induced a Th1-polarized response in CD4-/- mice. This observation is different from a study that showed Th2-skewed response in CD4-depleted mice and balanced Th1/Th2 response in CD4+/- mice.

FIGURE 11. CD8+ T cells are crucial in NP-based protection against influenza. Survival curves of CD8+/− mice immunized with two doses of 10^2 PFU of the indicated rAd constructs and challenged with 5× LD50 of mouse-adapted influenza A/Puerto Rico/8/1934(H1N1) virus 4 wk after secondary immunization are shown. Data are shown from one experiment with n = 5 mice per treatment group.

FIGURE 12. Schematic model of immune response induction using CD40-targeted Ag. Subcutaneous immunization with rAd-encoding SNP-CD40L fusion protein results in infection of proliferating and nonproliferating cells; thus, secreted gene products increase the Ag loads in vivo at the site of injection. The fusion protein then binds to CD40 on APCs via CD40L to initiate immune response induction and amplification. DCs can also be directly infected with rAd, leading to their maturation into professional APCs and subsequent secretion of the fusion protein into regional LNs to directly activate B cells. Thus, CD40L can act as an adjuvant and targeting molecule in both the peripheries and/or the secondary lymphoid organs. In the LNs, the CD40L-activated DCs activate Ag-specific CD8+ T cells to induce enhanced CTL activity and multiple cytokines producing cytotoxic T cells. Meanwhile, secreted CD40L-targeted Ags activate B cells and elicit accelerated Ig isotype switch, early and persistent GC formation, and Th1-skewed Ab response.
(17). Although we cannot fully explain the difference between the two studies at this time, such a discrepancy could be because of the difference in immunization regimens used in both studies. Specifically, whereas we used two doses of rAd to target the encoded Ag as a fusion protein directly to CD40\(^{\circ}\) APCs via CD40L, Zheng and colleagues (17) codelivered CD40L with Ag through a coimmunization or coexpression using DNA prime/Ad boost approach. Nonetheless, data reported by Zheng et al. (17) clearly show that using CD40L as an adjuvant can effectively induce significant levels of protective Abs and CD8\(^{+}\) T cells in a CD4\(^{+}\) T cell–independent manner. Furthermore, Auten et al. (20) showed that coexpression or coimmunization of CD40L together with mycobacterial protein Ag 85B in a plasmid DNA prime/Ad boost regimen can restore vaccine-induced circulating and mucosal CD4\(^{+}\) and CD8\(^{+}\) T cell responses in CD4\(^{+}\) T cell–depleted mice. Although we only examined Ab titers, but not CD8\(^{+}\) T cell response, in immunized CD4\(^{+/-}\) mice, the observation that none of the constructs generated in this study was able to confer any protection in CD8\(^{+/-}\) mice (Fig. 11) indicate that both humoral and cellular responses are involved and can be induced by using CD40L in CD4\(^{+}\) mice (Fig. 12).

The significance of these results is paramount in regard to immunodeficiency. There is a growing need to develop new strategies to elicit CD4\(^{+}\)-independent immune responses or at least to partially substitute for CD4\(^{+}\) T cell loss in immunocompromised individuals with defective CD4\(^{+}\) T cells or CD40L deficiency. For example, in HIV infection, the gradual loss of virus-specific CD4\(^{+}\) T cells is associated with significant reduction in CD40L expression (56, 57). Similarly, it was shown that age-related reduction in CD40L levels is associated with the defects in cognate CD4\(^{+}\) T cell function, thus weakening humoral response in aged individuals (58). Such defects do not only result in impaired immune responses and increased risk for opportunistic infections but also in drastically reduced efficacy of vaccination (59). Although it would be of great interest to test this vaccine in an aged mouse model in future studies, our data demonstrate that targeting Ags via CD40L may overcome such defects and induce protective immune response in hosts with either CD4\(^{+}\) T cell or CD40L immunodeficiency.

Current influenza vaccines mostly induce immune responses against the highly variable regions of the HA and NA (60, 61). Therefore, these strategies may not lead to heterosubtypic protection against the divergent influenza subtypes. However, the highly conserved internal proteins such as NP have been shown to induce effective immune responses and provide protection against various influenza A subtypes in several animal models (32–34, 62–64). Yet, NP-based vaccination has been shown to be associated with limited protection in other studies (35–37). Noticeably, Roy et al. (65) showed that a single i.m. immunization with human adenosine receptor 5 expressing influenza A/Puerto Rico/8/34(H1N1) NP can elicit a strong T cell response and 90–100% protection against a homologous challenge. However, significant heterosubtypic protection (40–80%) was observed only against influenza A/Vietnam/1203/04 (H5N1) virus, but not influenza A/Hong Kong/483/97(H5N1). In our study, we found that using CD40L as adjuvant and targeting molecule can enhance the breadth, potency, and durability of NP-specific immune responses, as well as the protection not in normal mice only but also in CD4\(^{+/-}\) and CD40L\(^{-/-}\) mice. Although we focused on vaccination using influenza NP, this work points toward a broadly applicable, effective means of inducing durable immune response and protection. Further studies are currently ongoing to determine the potential of this platform to improve the immunogenicity and protective efficacy of other Ags. To our knowledge, this is the first report demonstrating that a single dose of NP-based immunization can elicit strong and long-lasting protective immune responses against influenza involving both CD8\(^{+}\) T cells and anti-NP Abs.

Acknowledgments

We thank Louise Larocque, Marsha Russell, Emily Chomyshyn, and Michelle Lemieux for technical assistance. We are so grateful to Dr. Martha Navarro and all technicians in the animal facility for help. Drs. Aaron Farnsworth and Daryl Smith (Health Canada) are acknowledged for their critical review of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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