

## ORIGINAL ARTICLE

# Significant alterations of biodistribution and immune responses in Balb/c mice administered with adenovirus targeted to CD40(+) cells

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CD40 ligation has been shown to promote antigen-presenting functions of dendritic cells, which express CD40 receptor. Here we reported significantly altered biodistribution and immune responses with the use of CD40-targeted adenovirus. Compared with unmodified adenovirus 5, the CD40-targeted adenovirus following intravenous administration (*i.v.*) resulted in increased transgene expressions in the lung and thymus, which normally do not take up significant amounts of adenovirus. Intradermal injection saw modified adenovirus being mainly processed in local draining lymph nodes and skin. Following intranasal administration (*i.n.*), neither unmodified nor targeted viruses were found to be in the liver or spleen, which predominantly took up the virus following *i.v.* administration. However, inadvertent infection

of the brain was found with unmodified adenoviruses, with the second highest gene expression among 14 tissues examined. Importantly, such undesirable effects were largely ablated with the use of targeted vector. Moreover, the targeted adenovirus elicited more sustained antigen-specific cellular immune responses (up to 17-fold) at later time points (30 days post boosting), but also significantly hampered humoral responses irrespective of administration routes. Additional data suggest the skewed immune responses induced by the targeted adenoviruses were not due to the identity of the transgene but more likely a combination of overall transgene load and CD40 stimulation.

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## Introduction

A variety of viruses, such as adenovirus (Ad) and poxvirus, have been explored as vaccine and gene delivery vectors for the prevention and/or treatment of various human diseases.<sup>1,2</sup> Among the viral vectors, recombinant Ad is a preferred and promising delivery system<sup>1</sup> since the virus exhibits relatively low cytotoxicity, large cloning capacity, replication to high titres and ability to infect both dividing and undividing cells. Recently, adenoviral vectors were found to be very effective for mucosal vaccination due to their natural tropism for the mucosal surfaces.<sup>2</sup> It is noted, however, that the main cellular receptor for Ad, coxsackievirus and adenovirus receptor (CAR), has been readily detected in a wide range of human tissues, which may potentially have undesirable consequences.<sup>3–5</sup> These

findings prompted several groups to conduct various genetic modifications of the Ads to improve the delivery efficiency and/or minimize the relatively random distribution of the injected Ads by re-targeting the Ads to dendritic cells (DCs).

DCs are antigen-presenting cells playing crucial roles in establishing antigen-specific adaptive immune response and therefore stimulate potent immune responses. The possibilities of DC-based strategies for immunotherapy and immunoprotection of cancer, infectious disease and transplantation have been vigorously investigated during the past several years.<sup>1</sup> However, DCs are deficient in expression of the CAR receptor, presenting a challenge for effective transduction of these cells by Ads.<sup>1</sup> In addition, there is also a concern that the cytopathic effect of high-dose Ad on DCs could compromise clinical value of recombinant Ads.<sup>3</sup> To overcome these limitations, re-targeting delivery strategies via modification of the tropism of the virus have been developed for better transduction into DCs. One approach involves genetic modification of the fiber/knob proteins of Ad to target the surface intergrins of DCs.<sup>6,7</sup> Another approach is to target the CD40 surface receptor of DCs<sup>8,9</sup> since the CD40 receptor may play essential

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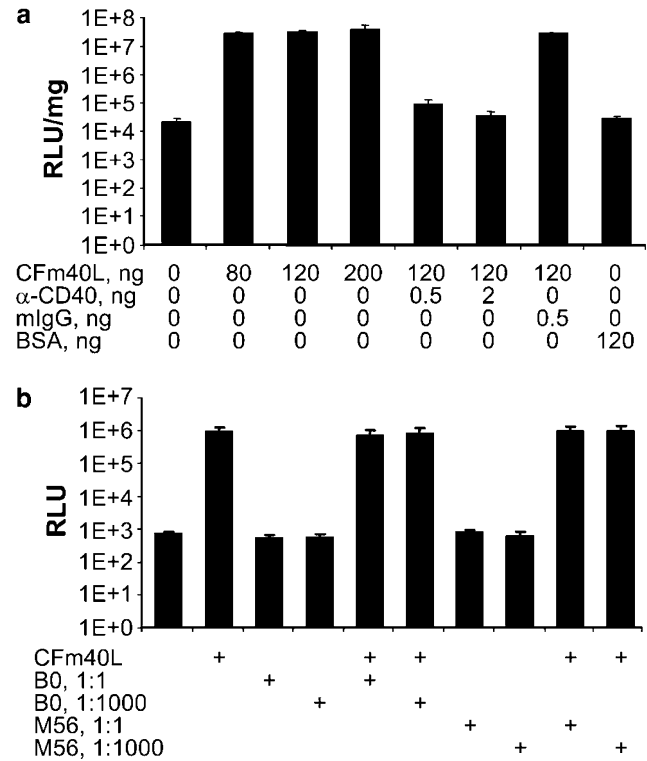
roles in promoting both DC activation and antigen presentation.<sup>10–14</sup> To this end, the viruses were usually complexed with bi-specific adaptor molecules comprised of either anti-Ad single-chain antibody or the extracellular domain of CAR with the targeting CD40 ligand (CD40L).<sup>8,9</sup> While marked improvement of transduction efficiency and enhanced immune responses against the transgenes delivered by various targeted Ad vectors have been reported by others and us, systematic analyses of biodistribution, toxicity and immune responses following various administration routes remains poorly documented. Uncertainties also remain with respect to other forms of delivery (DNA- and protein-based system). For instance, even though CD40L has been consistently shown to enhance Th1 (cellular) immune response, its role in Th2 (humoral) response remains unclear, with some reports suggesting an increased antibody response and others reporting no adjuvant effect of CD40L.<sup>15–19</sup> Lack of knowledge in these areas could substantially hinder the transition from laboratory investigations to clinical application of CD40-targeted gene delivery and genetic vaccination.

This study focused on the *in vivo* analyses of the biodistribution and immunogenicity of a modified Ad complexed with an adaptor protein (CFm40L), a fusion protein comprised of human Ad receptor CAR fused to mouse CD40L via a trimerization motif.<sup>9</sup> We reported previously that CFm40L can significantly enhance *in vitro* gene transfer to DCs expressing CD40.<sup>9</sup> Here we demonstrate that the modified Ad, denoted as tAd in this report, irrespective of the route of administration, can profoundly alter the patterns of biodistribution and immune responses against the transgene. The potential mechanisms underlying these differences between the unmodified and targeted vectors are discussed.

## Results

### CFm40L facilitates Ad transduction of CD40(+) DCs in the presence of Ad-neutralizing antibodies

We showed previously that CFm40L could direct Ad into CD40(+) DCs.<sup>9</sup> In addition, we have also found that the mouse bone marrow-derived DCs stimulated by Ad-CFm40L complex resulted in elevated levels of maturation markers (CD11c, CD11c, CD54, CD40, CD80, CD86 and I-A<sup>d</sup>).<sup>9</sup> Prior to the initiation of the biodistribution study in Balb/c mice, we first tested whether anti-CD40 mAb could prevent *in vitro* gene transfer by tAd-Luc (Ad5 expressing luciferase gene and conjugated with CFm40L). To this end, Ad-Luc conjugated with CFm40L as described previously<sup>9</sup> was used to transduce human CD40(+) DCs in the presence or absence of anti-CD40L. As compared with the unmodified Ad, the relative luciferase activity for the CFm40L-complexed Ads is 1307-fold higher with the addition of 80 ng CFm40L, 1491-fold with 120 ng and 1799-fold with 200 ng (Figure 1a), respectively. Each reaction had four replicates. The experiments were repeated three times with similar findings. Importantly, the addition of 0.5 and 2.0 µg of anti-CD40L mAb into the pre-complexed Ads largely prevented the enhanced luciferase activities, while pre-mixing with 2.0 µg of normal mouse immunoglobulin (Ig) G did not show any inhibition of luciferase activity. Also as a control, Ads pre-complexed with 120 ng of



**Figure 1** Enhanced entry of tAd into DCs in the presence of Ad-neutralizing antibodies. (a) Determination of tAd entry into CD40(+) DCs *in vitro*. Ad-Luc denotes Ad-5-expressing luciferase, tAd-Luc-Ad-Luc conjugated with CFm40L. CD40(+) DCs were cultured in 24-well plates ( $1 \times 10^5$  cells per well) for 4 days in RPMI-1640 medium containing rh-IL-4 and rhGM-CSF. tAd-Luc ( $2.5 \times 10^6$  PFU or  $2.5 \times 10^7$  VP) was prepared by incubating Ad-Luc with various amount of CFm40L at 80, 120 and 200 ng, respectively and used to infect cultured DCs at MOI of 50. After 2 days of infection, luciferase activities were determined. The experiments were repeated three times, with results being presented as relative light units (RLU) normalized for protein concentration. Bars represent mean (four replicates)  $\pm$  s.d. (same below). (b) The effects of Ad-neutralizing antibodies on CFm40L mediated DCs transduction. Human DCs were infected with CD40-targeted Ad-encoding luciferase reporter in the presence of human ascites fluids 'B0' (Ad Ab negative) and 'M56' (Ad Ab positive) at 1:1 and 1:1000 dilutions. Luciferase activity in cell lysates was assessed 36 h post transduction.

bovine serum albumin (BSA) did not result in enhancement of luciferase activity in the transduced cells. These results further confirmed that the enhanced gene transduction into both human and murine DCs by Ads complexed with CFm40L is mediated by the CD40 receptor. We next determined whether tAd could still transduce DCs in the presence of Ad-neutralizing antibodies. Towards this end, we infected human peripheral blood-derived DCs with Ad-CFm40L complexes in the presence of Ad-negative and Ad-positive ascites fluids collected from ovarian cancer patients from a phase I gene therapy clinical trial. The ascites M56 (patient 'M', day 56 after Ad administration) has been shown to have high ( $>1:16\ 000$ ) Ad-neutralizing antibody titres, and the ascites B0 (patient 'B', day 0 of Ad administration) to have none.<sup>20</sup> Furthermore, the ability of the ascites M56 to neutralize Ad infectivity and the lack of neutralizing capability by the ascites B0 was previously documented in the same studies.<sup>20</sup> We again

confirmed the absence of anti-Ad antibodies in ascites B0 and their presence in ascites M56 with the titre of 1:65 610 in Ad enzyme-linked immunosorbent assay (ELISA; Supplementary Figure S1). Then Ad vector-encoding luciferase was pre-complexed with CFm40L (50 ng per  $1.25 \times 10^6$  PFU per  $2.5 \times 10^4$  cells) mixed with the ascites at 1:1 and 1:1000 dilutions and applied to DCs. Luciferase activity was measured in cell lysates 36 h post transduction. The results (Figure 1b) indicate that neutralizing antibodies had no inhibitory effects on CFm40L mediated Ad-Luc transduction of human DCs.

*CFm40L directs more rapid capturing of the vector by the liver, thymus and lung following i.v. administration*

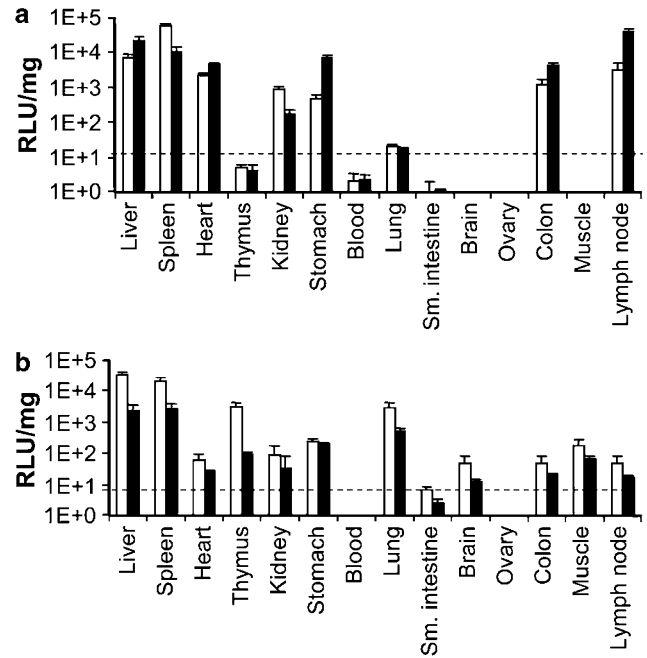
The tAds encoding firefly luciferase reporter gene (tAd-Luc) were then injected into Balb/c mice intravenous (i.v.) for the analysis of tissue distribution. A total of  $1 \times 10^{10}$  VP (viral particle) of Ad was chosen for Ad and tAd since excess amount of Ad ( $>1-2 \times 10^{10}$ ) could saturate tissue macrophages in mice following systemic administration, resulting in non-receptor-mediated process.<sup>21</sup> For tAd preparation,  $1 \times 10^9$  PFU ( $\sim 1 \times 10^{10}$  VP) of Ads were complexed with 16  $\mu$ g of CFm40L, equivalent to 80 ng of CFm40L for  $5 \times 10^6$  PFU of Ad. We chose this ratio between Ad and CFm40L since 80 ng of CFm40L is sufficient to complex  $2.5 \times 10^6$  Ad for enhanced transduction *in vitro* (Figure 1a). In agreement with other reports with i.v. administration,<sup>14,22-25</sup> the Ad was detected after 3 and 7 days in a wide range of tissues, with the liver and spleen expressing the highest luciferase activities on day 3, followed by mesenteric lymph nodes (a visible cluster of lymph nodes inside the peritoneal cavity), heart, colon and others (Figure 2a). Yet, a noticeably altered biodistribution was observed with tAd-Luc (Figure 2b), that is in addition to the predominant disposition to the liver and spleen, the viruses were also effectively captured by thymus and the lung on day 3 (Figure 2b).

*Localized expression of transgenes following i.d. administration*

Following intradermal (i.d.) administration, high levels of luciferase expression were detected in the skin surrounding the injection site and in the local draining lymph nodes (inguinal) for both Ad-Luc and tAd-Luc and, as expected, the luciferase activity with tAd-Luc administration was significantly lower than that with Ad-Luc ( $\sim 300$  times lower) since tAd-Luc could only get into CD40(+) cells and express the luciferase gene (Figures 3a and b), consistent with results shown in Figure 1a. Clearly, no virus was detected in other organs and tissues, suggesting the viruses were processed near the injection site sites and local draining lymph nodes, with little spillover of the virus into the circulation.

*CF40mL prevented inadvertent infection of the brain and attenuated local inflammatory reaction in the lung following i.n. administration*

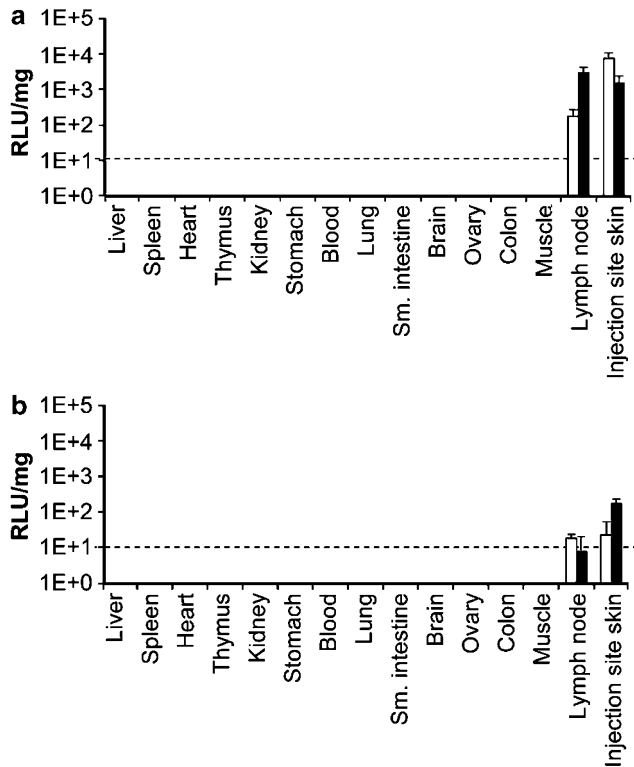
Following intranasal (i.n.) delivery of Ad-Luc, there was gene transfer with the Ad in various organs/tissues with the i.n. injection (Figure 4a). It is of note that the expression level of luciferase in the brain is high following Ad-Luc administration (Figure 4a), only second to the one detected in the lung. Clearly, the



**Figure 2** Biodistribution of Ad-Luc and tAd-Luc (Ad-Luc pre-complexed with CFm40L) following i.v. administration (tail vein). Five mice were injected with  $1 \times 10^{10}$  VP of either Ad-Luc (a) or tAd-Luc (b). Three and seven days post-injection the indicated tissues were isolated, homogenized and measured for luciferase activity. Values below 10 RLU (relative light units) per mg, as indicated by the dashed lines in the figures, are background readings (same below). Open bars and filled bars represent 3 and 7 days after injection, respectively.

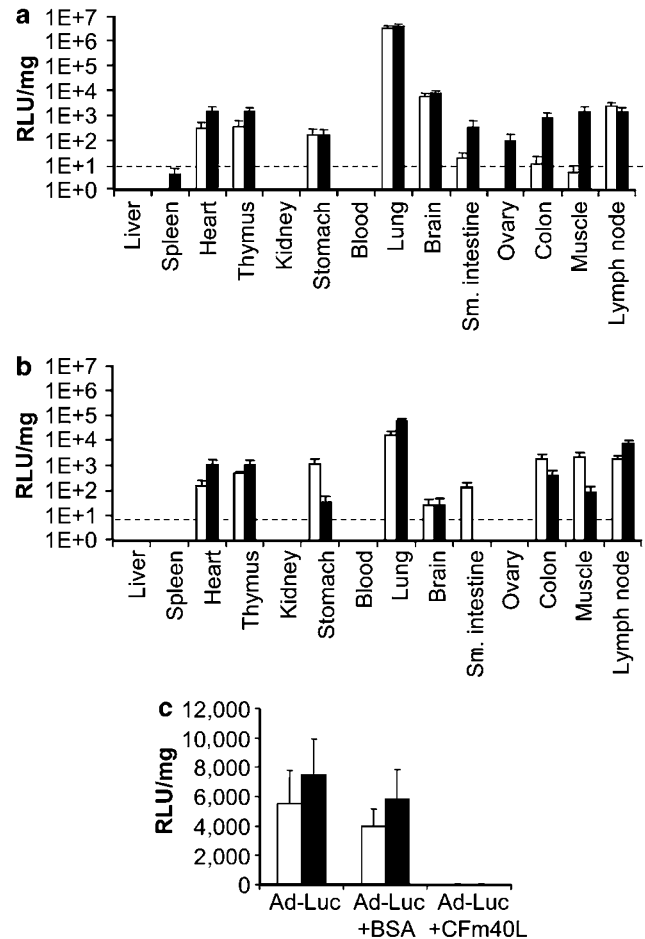
infection of the brain was largely ablated with the use of tAd-Luc, but not the infection of other tissues such as lung, heart, thymus, colon, muscle and mesenteric lymph nodes (Figures 4a and b), suggesting CAR-mediated inadvertent infection of the brain by non-modified Ad. This interpretation is further supported by the observation that pre-complex of Ad-Luc with BSA did not prevent brain infection (Figure 4c). Interestingly, no significant difference of virus deposition between tAd-Luc and Ad-Luc in the heart was observed following i.n. administration (Figures 4a and b), an observation that differs from that following i.v. injection, which shows the heart took up more Ad-Luc than tAd-Luc following i.v. administration (Figure 2). We have no definite explanation for these findings. It is plausible, however, that since the heart is in close proximity of the lung, tAd-Luc could gain access more easily to the heart following i.n. delivery than with the i.v. route (tail vein).

The high level of luciferase activity in the lung following i.n. administration prompted us to conduct histological examination of the lung. As compared to the control mice injected with buffers only (Figure 5, panel a), i.n. delivery of Ad-Luc resulted in moderate accumulations of mononuclear leukocytes in many peribronchial and perivascular areas, typical of inflammatory reactions (Figure 5, panel b). Following the i.n. administration of Ad-NP expressing the nucleocapsid protein (NP) of SARS-CoV (SARS-CoV NP was used here as a model antigen), however, there are much more significant accumulations of mononuclear leukocytes in the peribronchial and perivascular areas throughout the entire histological section and infiltration of mononuclear cells



**Figure 3** Biodistribution of Ad-Luc and tAd-Luc administrated i.d. (inguinal). (a) Mice that were injected with  $1 \times 10^{10}$  VP of Ad-Luc in 50  $\mu$ l PBS. (b) Mice that were injected with  $1 \times 10^{10}$  VP of tAd-Luc. Three (open bar) and seven days (filled bar) post injection the indicated tissues were isolated, homogenized and measured for luciferase activity.

in the alveolar spaces (Figure 5, panel c). In the severely affected area, the airway epithelial cells demonstrate mild-to-moderate damage and sloughing, revealing an ‘exaggerated’ inflammatory reaction in the lung (Figure 5, panels c and e). The reason for the visible damage to the airway epithelial cell layers by Ad-NP but not by Ad-Luc could be due to the intrinsic property of NP, a strong immunogen in severe acute respiratory syndrome (SARS) patients, as was suggested (see below for discussion). It is of note that with the addition of CFm40L (denoted as tAd-NP), the excessive infiltration of inflammatory cells and the damage of epithelial cells were abolished (Figure 5, panel d). Taken together, a significantly altered pattern of biodistribution of Ad complexed with CFm40L was observed following i.v. and i.n. administrations. Attenuation of inadvertent infection of the brain and aggravated local inflammation has been demonstrated in the airway of the lung following i.n. administration. Finally, none of the Ad preparations (unconjugated or conjugated, irrespective of administration route) resulted in detectable signs of systematic toxicity following analyses of various blood biomarkers (blood chemistry) pertinent to vital organs such as kidney, heart and liver (data not shown), even though visible damages to the airway epithelial cells were found following i.n. administration of Ad-NP. We also conducted immunohistological examinations of available lung tissues using antibodies against both luciferase and CD11c, one of the surface markers for DCs. Preliminary results suggest that the tAd-Luc was

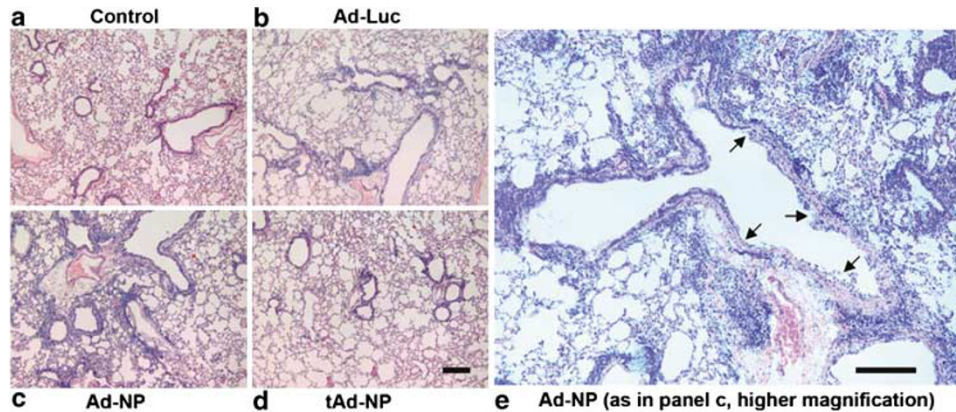


**Figure 4** Biodistribution of Ad-Luc and tAd-Luc administrated i.n.. (a and b) Results from studies with mice administrated i.n. with Ad-Luc and tAd-Luc, respectively. The mice were injected with  $1 \times 10^{10}$  VP of virus in 100  $\mu$ l PBS. Three (open bar) and seven days (filled bar) post injection the indicated tissues were isolated, homogenized and analysed for luciferase activities. (c) CFm40L abolishes inadvertent infection of the brain by the virus: Ad-Luc was conjugated with either CFm40L or bovine serum albumin (BSA) and injected (i.n.) into the mice. RLU were determined from the brain tissues of mice injected with  $1 \times 10^{10}$  VP of Ad-Luc pre-complexed either with CFm40L or BSA. Open bar depicts 3 days post administration, while filled bar designates 7 days post administration.

mainly found in CD11c(+) cells, while Ad-Luc were not restricted to CD11c(+) cells (Supplementary Figure S2). Clearly, more vigorous investigations will be needed to identify all cell types that also express CD40.

#### CFm40L elicited a more sustained Ag-specific cellular immune response

To determine the adjuvant and targeting effects of CFm40L, Ad expressing the SARS-CoV NP protein (Ad-NP) was compared with the same virus complexed with CFm40L (tAd-NP) following either i.d. or i.n. administration, with tAd-Luc serving as the baseline control. We chose the same amount of virus used in the above biodistribution experiments. Thirty days post-prime, the mice were boosted once. The splenocytes were isolated on days 0, 15 and 30 post boosting for the determination of interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2



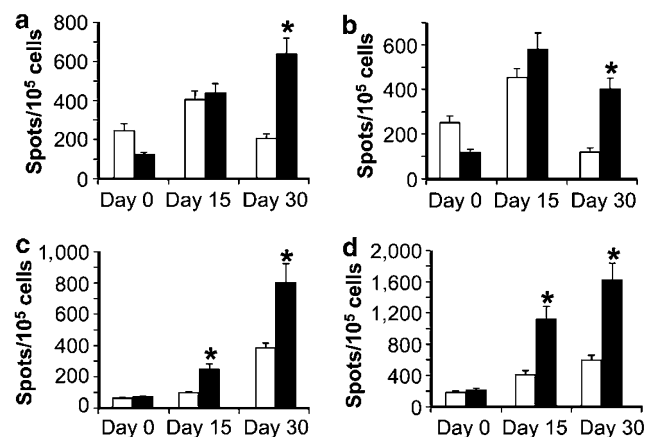
**Figure 5** Histological examination of the lungs in mice administrated i.n. with various preparations of Ads. The lung tissues from mice were collected and snap frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ . The tissue sections were prepared and stained by hematoxylin and eosin. (a) Normal lung from a mouse which received PBS only; (b) Lung from mouse which received Ad-Luc; (c) Lung from a mouse which received Ad-NP (Ad-5 expressing the NP of SARS-CoV); (d) Lung from a mouse which received tAd-NP (Ad-N complexed with CFm40L). Panel e represents panel c at high magnification. Significant accumulations of mononuclear leukocytes in the peribronchial and perivascular areas throughout the entire section and infiltration of mononuclear cells in the alveolar spaces were observed as shown in (b) and (c), with (c) demonstrating signs suggestive of a much stronger local inflammatory reaction. In the severely affected area of lungs from mice, which received Ad-NP, layers of airway epithelial cells display sloughing (indicated by arrows in (e)). These data are representative photos among at least five vision fields from >three mice. Scale bar:  $100\ \mu\text{m}$ .

(IL-2). As shown in Figures 6a and b, following i.d. administration, both IFN- $\gamma$  and IL-2 specific for SARS-CoV NP, have been increased in mice immunized with either Ad-NP or tAd-NP compared with the control (mice receiving Ad-Luc). However, it is of note that on day 30 after boosting, the levels of IFN- $\gamma$  and IL-2 in mice receiving tAd-NP were substantially higher than those immunized with Ad-NP, suggesting a more sustained Th1 response with the use of CFm40L (Figures 6a and b). Furthermore, such an enhancement of Ag-specific host immune response by CFm40L has also been observed with the i.n. injection route (Figures 6c and d). Collectively, these data revealed that CFm40L elicited a more sustained Ag-specific cellular immune response ( $P < 0.01$ ).

#### CFm40L delayed Ag-specific humoral responses and reduced IgG 1/IgG 2a ratio

Specific IgG and IgA antibodies against the NP protein were determined by ELISA to measure the antibodies present in the sera or the trachea and lung (mucosal antibodies). Surprisingly, the levels of IgG and IgA in the sera following tAd-NP administration (i.d.) was significantly lower than that with Ad-NP immunization via the i.d. administration route ( $P < 0.01$ ) (Figures 7a and b).

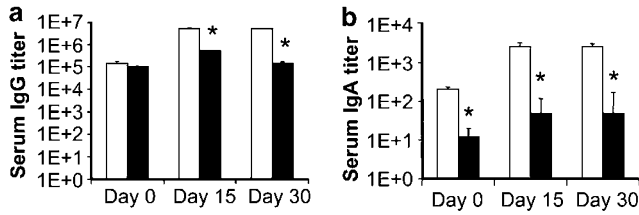
Next we determined the titres of IgG and IgA present in the sera and trachea/lung lavage following i.n. administration and found that the humoral responses were similar to that with the i.d. route. As shown in Figure 8, both the IgG and IgA titres in mice immunized with tAd-NP were significantly lower than those with Ad-NP, especially on Days 0 and 15 post-boosting ( $P < 0.01$ ). It is of note, however, that the antibody titres did reach similar levels 30 days post-boosting following i.n. administration (Figure 8). In addition, similar findings in terms of Ag-specific antibody responses against a different transgene (luciferase) were also obtained with the use of tAd-Luc (Supplementary Figure S3). These data collectively suggest that the reduction of



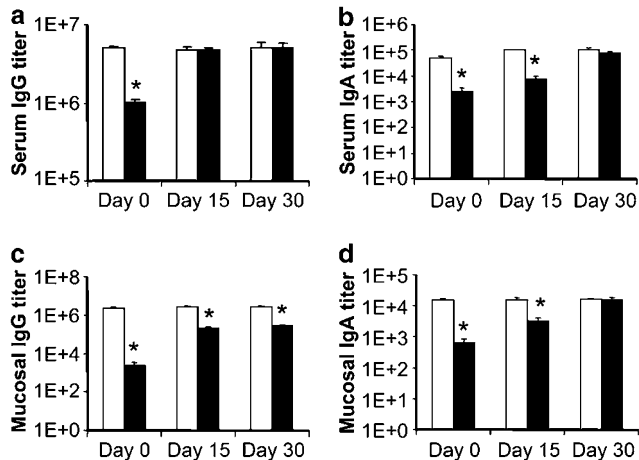
**Figure 6** Determination of interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2 (IL-2) in mice receiving either Ad-NP or tAd-NP. The mice were administrated with either Ad-NP (open bar) or tAd-N (filled bar) and boosted 4 weeks later. The splenocytes were then harvested on days 0, 15 and 30 for the analyses of Ag-specific IFN- $\gamma$  or IL-2 using ELISPOT as described in the 'Materials and methods' section. Data represent spots per  $10^5$  splenocytes stimulated by  $10\ \mu\text{g ml}^{-1}$  of NP. (a) IFN- $\gamma$  levels in mice injected with either Ad-NP or tAd-NP (i.d. route); (b) IL-2 levels in mice injected with either Ad-NP or tAd-NP (i.d. route); (c) IFN- $\gamma$  levels in mice injected with either Ad-NP or tAd-NP through the i.n. route; (d) IL-2 levels in mice injected with either Ad-NP or tAd-NP (i.n. route). All data represent the means  $\pm$  s.d. from four mice. Asterisks (\*) indicate significant difference between Ad-NP and tAd-NP (same below). Statistical comparisons were conducted with the use of a two-tailed *t*-test, with  $P < 0.05$  being considered significant. Open bar depicts Ad-NP, while filled bar designates tAd-NP.

Ag-specific humoral responses is not linked to the identity of the transgene but to the use of CFm40L in conjunction with adenoviral vectors.

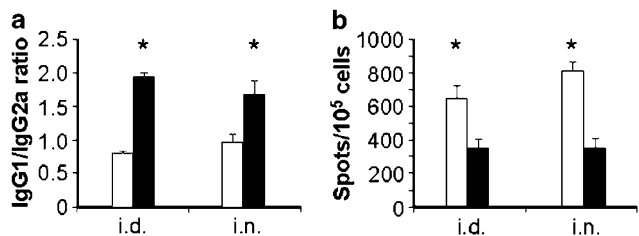
We then determined the relative ratio of IgG1 and IgG2a delivered by either unmodified or CD40-targeted vectors. To this end, horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 and IgG2a were used in place of HRP-conjugated anti-mouse IgG in ELISA.<sup>26</sup>



**Figure 7** Ag-specific antibody responses in mice following i.d. administration of either Ad-NP or tAd-NP. Mice immunized (i.d. route) with either Ad-NP (open bar) or tAd-NP (filled bar) were analysed for Ag-specific antibody levels using ELISA. (a) Serum immunoglobulin G (IgG) levels in mice following i.d. administration; (b) serum IgA levels in mice following i.d. administration. \* $P < 0.05$ .

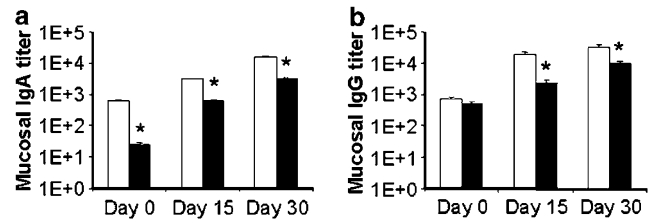


**Figure 8** Ag-specific antibody responses in mice following i.n. administration of either Ad-NP or tAd-NP. Mice immunized (i.n. route) with either Ad-NP (open bar) or tAd-NP (filled bar) were analysed for Ag-specific antibody levels using ELISA assays. (a) Serum immunoglobulin (Ig) G levels in mice; (b) serum IgA levels in mice; (c) mucosal IgG levels (antibodies present in the trachea and lung); (d) mucosal IgA levels (antibodies present in the trachea and lung). \* $P < 0.05$ .



**Figure 9** Determination of transgene-specific immunoglobulin (Ig) G1/IgG2a ratio and IL-4 level. Panel a represents IgG1/IgG2a ratio while panel b depicts IL-4 level. HRP-conjugated anti-mouse IgG1 and IgG2a were used to substitute the HRP-conjugated anti-mouse IgG in ELISA as described.<sup>26</sup> The serum samples were derived from mice 30 days post boosting. Similar results were obtained from 15 days after the boosting (data not shown). \* $P < 0.05$ .

As shown in Figure 9a, a reduced ratio of IgG1 and IgG2a was observed with the use of the targeted vector. We also observed lower levels of IL-4 (Figure 9b). These data, in addition to the increased levels of Th1 cytokines (IL-2 and IFN $\gamma$ ; Figure 6), suggest that the CD40-targeted immunization might have elicited a Th1-skewed immune response.



**Figure 10** Detection of antibodies against Ad proteins in lung lavage in mice receiving i.n. administration of tAd-NP. The lung lavage was collected from the same group of mice as in Figure 7 and analysed for the level of antibodies against adenoviral proteins by ELISA. The antigens used in the ELISA are adenoviral lysate from Ad-E (an irrelevant Ad virus expressing a different transgene), which were purified by sucrose gradient. (a) Immunoglobulin (Ig) A titres, while (b) describes IgG levels from mice which received either Ad-NP or tAd-NP. Open bar depicts Ad-NP, while filled bar designates tAd-NP. \* $P < 0.05$ .

### CFm40L attenuates anti-Ad vector antibody responses

We then determined whether conjugation of Ad with CFm40L could affect generation of anti-Ad antibody responses. To this end, we analysed Ad-specific IgA in lung lavage collected from the same mice immunized with tAd-NP (i.n. administration) by ELISA. We used sucrose gradient-purified Ad viral lysate derived from Ad-E (Ads expressing a different exogenous gene) as antigens in place of the NP protein in the ELISA. As shown in Figure 10, the antibody responses against the other Ad proteins were markedly suppressed in mice immunized with tAd-NP compared with that following immunization with Ad-NP. Furthermore, these data also provided additional evidence that the decreased Ag-specific antibody responses were linked to the use of CFm40L/adenoviral vectors not to the identity of the transgene, in agreement with Figures 7, 8 and Supplementary Figure S3.

## Discussion

A variety of macromolecules with immunostimulatory or immunomodulatory properties have been explored in recent years as molecular adjuvants to enhance the efficiency of genetic vaccination and/or gene therapy in pre-clinical and clinical settings.<sup>1,27</sup> CD40L, a member of the tumor necrosis factor superfamily, is a very attractive candidate since it can potentially activate DCs, which express CD40 and are well known to be professional antigen-presenting cells.<sup>27–32</sup> Numerous studies reported in the literatures revealed that administration of the model antigens together with CD40L or DNA constructs expressing fusion proteins comprised of the antigen and CD40L resulted in substantial enhancement of cellular (type I) and humoral (type II) immune responses.<sup>15–17,33–36</sup> Three lines of evidences prompted us to conduct the current studies. First, it was noted that data are lacking from systematic analyses of CD40L/viral vector in terms of biodistribution, toxicity and immunogenicity. Second, although enhanced immune responses were observed, knowledge remains poor regarding the outcome following an alternative route, that is i.n. administration. It is noteworthy that Ad has been found to be effective for mucosal delivery due to the natural tropism of Ads for mucosal surfaces.<sup>2</sup> Third, although cellular immune responses appeared to be markedly improved with the

use of CD40L as adjuvant, conflicting results have been observed for humoral responses with the use of protein- and DNA-based administrations. In this report, a head-to-head comparison between the unmodified Ad5 (Ad) and CD40-targeted Ad5 (tAd) was conducted in terms of biodistribution and immune responses.

#### *Biodistribution of tAd following intravascular administration*

As expected from *in vitro* transduction assay, the adaptor protein CFm40L, comprised of human Ad receptor CAR fused to mouse CD40L, noticeably altered the pattern of tissue distribution of the viruses *in vivo*, with predominant deposition of the viruses detected in the liver, spleen, thymus and lung (Figure 2). Such an altered biodistribution pattern is unlikely due to detargeting of CAR since modified adenoviral vectors ablated for CAR have been shown to have an overall reduced efficiency of gene expression in tissues but not a change of biodistribution pattern.<sup>36,37</sup> In contrast, we found the expression levels of transgenes delivered by the two vectors were comparable in certain tissues such as liver and spleen (*i.v.* administration). Furthermore, we observed the thymus and lung took up more tAd than Ad, a fact that also differs from the detargeting of CAR following *i.v.* administration.<sup>36,38</sup> These data suggest that the altered biodistribution pattern is due to CD40 targeting rather than detargeting of CAR.

The higher expression of tAd in lung may suggest the likely presence of abundant CD40(+) cells in these organs. It has been shown that epithelial/endothelial cells and antigen-presenting cells (DCs, activated monocytes and B cells) are CD40(+).<sup>39</sup> The presence of these CD40(+) cells in the lung may suggest that the innate host defences are well in place to fend off invading pathogens from the air. It is interesting that under normal circumstances the lung is not the major tissue/organ that takes up unmodified Ad following intravascular administration (Figure 2a). The pulmonary intravascular macrophages, however, can take up unexpectedly high amounts of the Ad5 virus in animals with hepatic disorders, such as cirrhosis, resulting in severe pulmonary pathology.<sup>5</sup> Clearly, better understanding of the biodistribution of both unmodified and modified Ad vector is of importance, given that such knowledge helps understand the clearance of virus vector as well as identify the tissues/organs, which could be predisposed to adverse events during the course of gene therapy.<sup>5,40</sup>

#### *Biodistribution of tAd following i.n. administration*

As a result of comparison between Ad and tAd following *i.n.* administration, it appears that the inadvertent infection of central nervous system (CNS) is mediated by the CAR receptor but not other receptors, such as  $\alpha_v\beta$  integrin, and heparin sulphate glycosaminoglycans, which were shown to mediate the entry of Ads.<sup>36,41,42</sup> As discussed above, inadvertent infection of the CNS by live viral vectors could be a safety concern if the vectors are given *i.n.*<sup>43,44</sup> Moreover, we observed visible lesions of the lungs, especially the airway epithelial cells, in mice receiving unmodified Ad-NP. Such deleterious effects of Ad-NP were apparently caused by strong local inflammatory reactions as suggested by a massive infiltration of

lymphocytes and macrophages. Yet, no such exaggerated local inflammatory reactions were observed in mice receiving tAd-NP. At this time, we have no definite explanation for the marked differences of local inflammatory reactions between Ad-NP and tAd-NP. Yet, it is interesting to note that we and others reported that the SARS-CoV NP protein itself, used in this study as model antigen, could alter cellular signal transduction pathways, stimulate pro-inflammatory cytokine production and/or even cell death.<sup>45,46</sup> Therefore, the excessive inflammatory reactions or toxicity induced by Ad-NP are likely due to the combined immunostimulatory or immunopathologic effects of Ad infection and NP protein. This notion is also supported by the observation that Ad-Luc failed to induce the same magnitude of local inflammatory reactions (Figure 5b).

#### *Ag-specific immune responses*

The substantially increased production of Ag-specific IL-2 and IFN- $\gamma$  with the use of tAd-NP at later time points was largely expected since ligation of CD40 has been suggested to promote the activation and antigen-presenting functions of DCs, which express CD40 and are well known to initiate immune response.<sup>39</sup> Interestingly, at early time points the levels of Ag-specific IL-2 and IFN- $\gamma$  were rather comparable between the unmodified and targeted vector groups. At later time points, however, the levels of these two cytokines remain elevated with the use of targeted vectors, accompanied by the lower level of IL-4 and the reduced ratio of IgG1/IgG2a. Whether the more sustained IL-2 and IFN- $\gamma$  responses with the use of targeted vectors were due to a better development of memory T-cell compartment would require further investigation. It is of note, however, that CD40 stimulation has been shown to facilitate the development of memory T-cell compartment<sup>39,47-49</sup> under other experimental conditions.

The markedly delayed Ag-specific mucosal and systemic antibody responses with the use of CFm40L adaptor proteins were somewhat surprising, given the fact that most reports in the literature suggest that CD40L activates both cellular and humoral immune responses against antigens delivered in the form of proteins or DNA constructs.<sup>15-19</sup> However, our findings are in partial agreement with two studies demonstrating CD40L lacks stimulatory effects on the production of Ag-specific antibodies.<sup>17,50</sup> It is unlikely that the biased immune responses reported here were linked to the identity of the transgene as similar observations (more sustained IL-2 and IFN- $\gamma$  but reduced antibody titres at later time points) were observed with both tAd-NP and tAd-Luc (Supplementary Figure S3). In addition, the use of tAd resulted in decreased antibody levels against other adenoviral proteins (Figure 10). However, since both transgenes in this current study (SARS-CoV NP and firefly luciferase) were intracellular proteins, it remains to be determined whether tAd encoding a secreted transgene would result in a similar pattern of immune responses. It is noted, however, others recently reported in a different system (soluble proteins) that CD40-targeted secreted proteins failed to generate a stronger antibody response compared with the untargeted secreted proteins.<sup>17</sup> At this time, we think a variety of factors could be ascribed to the differences of results with respect to the patterns of immune responses among

various groups. One of them might be due to the differences in the vector systems. It is understood that ours is the only study employing live viral vector in conjunction with CD40L, while other systems were designed to deliver the antigens in the form of DNA plasmid, soluble proteins or virus-like particles.<sup>15–19</sup> Second, the biased immune response might be due to a much lower level of transgene expression with the use of tAd vector at the site of the body receiving the viral vectors. For instance, as compared with Ad, ~300-fold less transgene expression in the injection site skin (Figures 3a and b) was observed following i.d. injection of tAd, while about 180-fold less in the lungs 3 days after i.n. administration (Figures 4a and b). In general, Ag-specific humoral responses are not preferentially elicited in response to smaller amounts of antigens as compared with cellular immune responses. Third, the tAd-induced biased immune responses could be tAd interacting with B cells. There are previous reports documenting that CD40 ligation could lead to largely positive growth outcomes for resting B cells, but inhibited the growth and Ig production of activated B cells which are also known to be CD40(+).<sup>39,51–53</sup> Given these findings, caution appears to be warranted when considering CD40L as targeting moiety for adenoviral vectors in genetic immunization when induction of humoral immune response is crucial, that is, neutralizing antibodies against pathogens. Nevertheless, therapeutic manipulation to facilitate biased or skewed immune responses could be beneficial in clinical applications. For instance, type 2-biased immune responses (predominant antibody reactions) may be associated with certain immunopathologic complications, such as allergy, asthma and autoimmune diseases. Thereby, redirection of the immune response or therapeutic manipulation towards a biased immune response, that is suppression of antibody responses, could help reverse medical conditions related to certain immunopathologic complications.<sup>38,54</sup> Furthermore, enhanced cellular immune responses are well known to play critical roles eliminating or containing certain intracellular pathogens and cancers.<sup>10–12,28,41</sup> Finally, as preliminary data presented in this report, the efficient *in vitro* gene transduction by tAd even in the presence of anti-Ad neutralizing antibodies and lower levels of antibodies against Ad viral proteins *in vivo* might also help allay one of the major concerns associated with pre-existing immunity against Ad. We are currently conducting more in-depth *in vivo* studies to address these issues.

## Materials and methods

### Reagents

Anti-CD40 mAb, rh-IL-4, recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), the ELISPOT kits with various controls, including IFN- $\gamma$  (EL485) and IL-2 (EL402), were purchased from R&D systems, Minneapolis, MN, USA. Bright-Glo luciferase assay system was obtained from Promega, Madison, WI, USA. Unless specified, other antibodies and peroxidase-conjugated secondary antibodies were purchased from Cedarlane Labs (Burlington, Ontario, Canada).

### Production, purification and characterization of CFm40L adaptor protein

The adaptor protein, CFm40L, is a fusion protein that consists of human Ad receptor CAR fused to mouse CD40L via a trimerization motif. CFm40L was constructed and used to target Ad vectors to mouse DCs expressing CD40.<sup>9</sup> CFm40L was purified from the supernatants in stable HEK293 cells transfected with the expression vector. The production and purification of CFm40L was described previously.<sup>9</sup> Briefly, the stable cell line producing CFm40L as a secreted protein was cultured in DMEM:F12 (1:1) medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100  $\mu\text{g ml}^{-1}$  G<sub>418</sub> and 40  $\mu\text{g ml}^{-1}$  gentamicin sulphate. The medium from the CFm40L-expressing 293 cell cultures were collected and the proteins were precipitated by addition of an equal volume of cold-saturated ammonium sulphate. The precipitates were then collected by centrifugation and dissolved in 1/20 of the original medium volume in phosphate-buffered saline (PBS), followed by dialysis against PBS. The recombinant CFm40L protein was purified from the dialysed protein solution by immobilized metal affinity chromatography using cobalt-immobilized TALON affinity resin (Clontech, Mountain View, CA, USA). The purity of the adaptor protein was confirmed by Coomassie blue staining and western blotting.<sup>9</sup>

### Construction and production of recombinant Ads

All transgenes were cloned into adenoviral transfer vector, Ad5.<sup>9</sup> The vector expressing SARS-CoV NP<sup>45</sup> was used here as a model antigen. The isogenic control vector expressing the luciferase gene was constructed similarly. Replication defective recombinant Ads were generated from the constructed transfer vectors as described.<sup>9</sup> Transgene expression by recombinant Ads was confirmed by luciferase activity assay or by western blot analysis with anti-SARS N-protein antibody. Ad particles were purified by CsCl gradient centrifugation and titrated as plaque-forming units (PFU) per ml using Adeno-X Rapid Titer kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

### DC culture, Ad infection and luciferase activity assay

Myeloid DCs were obtained from AICELLS, Emeryville, CA, USA. Over 80% of these monocyte-derived cells are CD40(+) in addition to other surface markers including CD1a, CD11c, CD86 and HL-DR. We previously reported elevated levels of maturation markers (CD11c, CD54, CD40, CD80, CD86) in DCs treated with CFm40L-targeted Ad,<sup>9</sup> consistent with other studies which show that CD40 stimulation could facilitate DC maturation.<sup>1,39</sup> The DCs were cultured in RPMI-1640 medium containing rh-IL-4 and rhGM-CSF ( $1 \times 10^5$  cells per well in 24-well plates) for 4 days prior to being subjected to treatments with Ads. The preparation of the tAd-Luc (Ad-5 encoding luciferase gene and conjugated with CFm40L) and infection of DCs *in vitro* were described previously.<sup>9</sup> In brief, Ad expressing luciferase genes (Ad-Luc) was pre-mixed with the CFm40L adaptor protein and incubated at 37 °C for 30 min and then used to infect DCs at 50 PFU per cell. After 2 days of infection, the cells were harvested to measure luciferase activity using Bright-Glo luciferase assay system.



### *Biodistribution and pathological examination*

Female Balb/c mice were purchased from Charles River Lab, Montreal, Quebec, Canada. All animal experiments were conducted in accordance with the guidelines and protocols for animal experiments at Health Canada. The mice were maintained in a laminar airflow cabinet under pathogen-free conditions and used at 6–7 weeks of age. A total of  $1 \times 10^9$  PFU ( $\sim 1 \times 10^{10}$  viral particles) of Ads suspended in 100  $\mu$ l of PBS per mouse was administered using a syringe with a 29-gauge needle by i.v. (tail vein), intradermal (i.d.) (inguinal) or i.n. routes. Control mice were injected with 100  $\mu$ l of PBS. The preparation of Ads complexed with CFm40L was described previously.<sup>9</sup> In brief, the same amounts of Ads was mixed with 16  $\mu$ g CFm40L (that is 80 ng CFm40L/ $5 \times 10^6$  PFU Ads) in 100  $\mu$ l of PBS and incubated at 37 °C for 30 min. The mice were killed on days 3 and 7 after administration of Ads, with tissues collected for subsequent analyses of biodistribution. The levels of luciferase activity, normalized by protein concentration, were determined. Five mice were used in each group. Pathological examination of tissues using hematoxylin and eosin staining method was conducted using a standard procedure.

### *Isolation of splenocyte and ELISPOT assay for the measurement of cytokines*

To determine the adjuvant effects of CFm40L, non-modified Ad expressing the SARS-CoV NP protein (Ad-NP) was compared with the same virus complexed with CFm40L (tAd-NP) following either i.d. or i.n. administration, with tAd-Luc serving as the baseline control. We chose the same amount of virus as in the above biodistribution experiments. For immunization, five mice were primed with  $1 \times 10^{10}$  VP of Ad-NP, tAd-NP or tAd-Luc (negative control). Thirty days post administration, the mice were boosted once. The splenocytes were isolated on days 0, 15 and 30 post boosting for the determination of IFN- $\gamma$  and IL-2. Splenocytes were isolated from mouse spleens by grinding them between the frosted ends of two microscope slides. The slides were rinsed with 5 ml of RPMI 1640 medium to collect the cells. Afterwards, the cell suspension was passed through a fine nylon mesh to obtain a single cell suspension. The spleen pieces were then gently mashed with the rubber end of a syringe plunger. A second filtration was then performed by pipetting the 5 ml suspensions of dispersed cells through a fresh 70- $\mu$ m nylon mesh cell strainer into a 50-ml conical tube (on ice). This procedure was repeated with additional washes of 5 ml medium until the splenic capsules turned white (3–4 more times). The total volume of cell suspension was brought up to 20 ml with medium. Cell pellets were then obtained by centrifugation at 400 g for 5 min at 4 °C. About 1 ml of 0.84% NH<sub>4</sub>Cl solution was used to lyse the red blood cells. The tubes were gently rolled by hand for 2 min at room temperature, followed by the addition of 30 ml of chilled RPMI medium. After passing the cells through another layer of 70- $\mu$ m nylon mesh, cells were collected by centrifugation at 400 g for 5 min at 4 °C. The pellet was then gently resuspended to  $2 \times 10^6$  cells per 100  $\mu$ l ( $2 \times 10^7$  cells per ml) in RPMI 1640 containing 10% heat-inactivated fetal bovine serum in the presence of 10 purified SARS-CoV NPs (10  $\mu$ g ml<sup>-1</sup>) to stimulate Ag-specific cytokine.<sup>43</sup> As stimulation with NP of

splenocytes derived from the control animals receiving Ad-Luc did not result in any detectable production of cytokines (background), all subsequent experiments were conducted in the presence of 10  $\mu$ g ml<sup>-1</sup> of NP. These cells were then used at 100  $\mu$ l per well for ELISPOT assays using procedures as provided by the supplier, R&D system.

### *Determination of Ag-specific antibody responses and IgG 1/IgG 2a ratio*

ELISA was conducted to determine the titre of antibodies against the SARS-CoV NP protein. To this end, the Nunc 96-well plates were coated with the 100  $\mu$ l of NP protein at 5  $\mu$ g ml<sup>-1</sup> in 50 mM carbonate buffer (pH 8.6) and incubated at 4 °C overnight. The wells were then washed five times with PBS, 0.05% Tween-20, followed by the addition of blocking buffer comprised of PBS, 0.05% Tween-20 and 5% BSA. After incubation at 37 °C for 1 h, the blocking buffer was removed, followed by the addition of samples of serum or washing fluids of the lung from the mice. The plates were incubated again at 37 °C for 1 h. Afterwards, secondary antibodies (peroxidase-conjugated goat anti-mouse IgG, IgM or IgA) were added at concentrations recommended by the supplier (Cedarlane Labs). Following an additional incubation at 37 °C for 1 h, the plates were washed five times before *o*-phenylenediamine dihydrochloride (OPD) was added for colorimetric development. The positive controls (mouse anti-SARS monoclonal antibodies) were purchased from BD Pharmingen, San Diego, CA, USA). The cut-off was defined as mean of five negative samples (from un-immunized control) plus two s.d. For the determination of relative levels of transgene (NP)-specific IgG subclasses, anti-mouse IgG1 and IgG2a conjugated with HRP (Cedarlane Labs) were substituted for anti-mouse IgG-HRP prior to the addition of OPD for colorimetric development.<sup>26</sup>

### *Statistical analysis*

Results are presented with the means  $\pm$  s.d. Statistical comparisons were conducted with the use of a two-tailed Student's *t*-test, with  $P < 0.05$  being considered to be significant.

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## References

- Noureddini SC, Curiel DT. Genetic targeting strategies for adenovirus. *Mol Pharm* 2005; **2**: 341–347.
- Santosuosso M, McCormick S, Xing Z. Adenoviral vectors for mucosal vaccination against infectious diseases. *Viral Immunol* 2005; **18**: 283–291.
- Okada N, Masunaga Y, Okada Y, Mizuguchi H, Iiyama S, Mori N et al. Dendritic cells transduced with gp100 gene by RGD fiber-mutant adenovirus vectors are highly efficacious in generating anti-B16BL6 melanoma immunity in mice. *Gene Therapy* 2003; **10**: 1891–1902.
- Cichon G, Schmidt HH, Benhidjeb T, Loser P, Ziemer S, Haas R et al. Intravenous administration of recombinant adenoviruses causes thrombocytopenia, anemia and erythroblastosis in rabbits. *J Gene Med* 1999; **1**: 360–371.
- Smith JS, Tian J, Muller J, Byrnes AP. Unexpected pulmonary uptake of adenovirus vectors in animals with chronic liver disease. *Gene Therapy* 2004; **11**: 431–438.
- Rea D, Havenga MJ, van Den Assem M, Suttmuller RP, Lemckert A. Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells. *J Immunol* 2001; **166**: 5236–5244.
- Worgall S, Busch A, Rivara M, Bonnyay D, Leopold PL, Merritt R et al. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. *J Virol* 2004; **78**: 2572–2580.
- Brandao JG, Scheper RJ, Lougheed SM, Curiel DT, Tillman BW, Gerritsen WR et al. CD40-targeted adenoviral gene transfer to dendritic cells through the use of a novel bispecific single-chain Fv antibody enhances cytotoxic T cell activation. *Vaccine* 2003; **21**: 2268–2272.
- Pereboev AV, Nagle JM, Shakhmatov MA, Triozzi PL, Matthews QL, Kawakami Y et al. Enhanced gene transfer to mouse dendritic cells using adenoviral vectors coated with a novel adapter molecule. *Mol Ther* 2004; **9**: 712–720.
- Mackey MF, Gunn JR, Maliszewsky C, Kikutani H, Noelle RJ, Barth Jr RJ. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J Immunol* 1998; **161**: 2094–2098.
- Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998; **393**: 480–483.
- Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic T-cell responses is mediated by CD40 signalling. *Nature* 1998; **393**: 478–480.
- Shayakhmetov DM, Eberly AM, Li ZY, Lieber A. Deletion of penton RGD motifs affects the efficiency of both the internalization and the endosome escape of viral particles containing adenovirus serotype 5 or 35 fiber knobs. *J Virol* 2005; **79**: 1053–1061.
- Nakamura T, Sato K, Hamada H. Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. *J Virol* 2003; **77**: 2512–2521.
- Tripp RA, Jones L, Anderson LJ, Brown MP. CD40 ligand (CD154) enhances the Th1 and antibody responses to respiratory syncytial virus in the BALB/c mouse. *J Immunol* 2000; **164**: 5913–5921.
- Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. *Vaccine* 2002; **20**: 3123–3129.
- Stone GW, Barzee S, Snarsky V, Kee K, Spina CA, Yu XF et al. Multimeric soluble CD40 ligand and GITR ligand as adjuvants for human immunodeficiency virus DNA vaccines. *J Virol* 2006; **80**: 1762–1772.
- Ito D, Ogasawara K, Iwabuchi K, Inuyama Y, Onoe K. Induction of CTL responses by simultaneous administration of liposomal peptide vaccine with anti-CD40 and anti-CTLA-4 mAb. *J Immunol* 2000; **164**: 1230–1235.
- Lu Z, Yuan L, Zhou X, Sotomayor E, Levitsky HI, Pardoll DM. CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. *J Exp Med* 2000; **191**: 541–550.
- Hemminki A, Wang M, Desmond RA, Strong TV, Alvarez RD, Curiel DT. Serum and ascites neutralizing antibodies in ovarian cancer patients treated with intraperitoneal adenoviral gene therapy. *Hum Gene Ther* 2002; **13**: 1505–1514.
- Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM et al. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* 2001; **3**: 28–35.
- Zinn KR, Douglas JT, Smyth CA, Liu HG, Wu Q, Krasnykh VN et al. Imaging and tissue biodistribution of 99mTc-labeled adenovirus knob (serotype 5). *Gene Therapy* 1998; **5**: 798–808.
- Wood M, Perrotte P, Onishi E, Harper ME, Dinney C, Pagliaro L et al. Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther* 1999; **6**: 367–372.
- Groot-Wassink T, Aboagye EO, Wang Y, Lemoine NR, Reader AJ, Vassaux G. Quantitative imaging of Na/I symporter transgene expression using positron emission tomography in the living animal. *Mol Ther* 2004; **9**: 436–442.
- Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev* 2005; **207**: 166–183.
- Kim JJ, Nottingham LK, Sin JI, Tsai A, Morrison L, Oh J et al. CD8 positive T cells influence antigen-specific immune responses through the expression of chemokines. *J Clin Invest* 1998; **102**: 1112–1124.
- Calarota SA, Weiner DB. Enhancement of human immunodeficiency virus type 1-DNA vaccine potency through incorporation of T-helper 1 molecular adjuvants. *Immunol Rev* 2004; **199**: 84–99.
- Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 1998; **393**: 474–478.
- Dubois B, Vanbervliet B, Fayette J, Massacrier C, Van Kooten C, Briere F et al. Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J Exp Med* 1997; **185**: 941–951.
- Clark EA. Regulation of B lymphocytes by dendritic cells. *J Exp Med* 1997; **185**: 801–803.
- Fayette J, Dubois B, Vandenabeele S, Bridon JM, Vanbervliet B, Durand I et al. Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. *J Exp Med* 1997; **185**: 1909–1918.
- Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* 1996; **184**: 747–752.
- Mendoza RB, Cantwell MJ, Kipps TJ. Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) on gene immunization. *J Immunol* 1997; **159**: 5777–5781.
- Gurunathan S, Irvine KR, Wu CY, Cohen JI, Thomas E, Prussin C et al. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J Immunol* 1998; **161**: 4563–4571.
- Staveley-O'Carroll K, Schell TD, Jimenez M, Mylin LM, Tevethia MJ, Schoenberger SP et al. *In vivo* ligation of CD40 enhances priming against the endogenous tumor antigen and promotes CD8+ T cell effector function in SV40 T antigen transgenic mice. *J Immunol* 2003; **171**: 697–707.

- 36 Koizumi N, Kawabata K, Sakurai F, Watanabe Y, Hayakawa T, Mizuguchi H. Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alpha V integrin, and heparan sulfate binding reduce *in vivo* tissue transduction and toxicity. *Hum Gene Ther* 2006; **17**: 264–279.
- 37 Leissner P, Legrand V, Schlesinger Y, Hadji DA, Van Raaij M, Cusack S *et al*. Influence of adenoviral fiber mutations on viral encapsidation, infectivity and *in vivo* tropism. *Gene Therapy* 2001; **8**: 49–57.
- 38 Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum* 2004; **34**: 501–537.
- 39 van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol* 2000; **67**: 2–17.
- 40 Manickan E, Smith JS, Tian J, Eggerman TL, Lozier JN, Muller J *et al*. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. *Mol Ther* 2006; **13**: 108–117.
- 41 Hong SS, Karayan L, Tournier J, Curiel DT, Boulanger PA. Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J* 1997; **16**: 2294–2306.
- 42 Dehecchi MC, Melotti P, Bonizzato A, Santacatterina M, Chilosi M, Cabrini G. Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J Virol* 2001; **75**: 8772–8780.
- 43 Lemiale F, Kong WP, Akyurek LM, Ling X, Huang Y, Chakrabarti BK *et al*. Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system. *J Virol* 2003; **77**: 10078–10087.
- 44 Franklin R, Quick M, Haase G. Adenoviral vectors for *in vivo* gene delivery to oligodendrocytes: transgene expression and cytopathic consequences. *Gene Therapy* 1999; **6**: 1360–1367.
- 45 He R, Leeson A, Andonov A, Li Y, Bastien N, Cao J *et al*. Activation of AP-1 signal transduction pathway by SARS coronavirus nucleocapsid protein. *Biochem Biophys Res Commun* 2003; **311**: 870–876.
- 46 Nicholls JM, Butany J, Poon LL, Chan KH, Beh SL, Poutanen S *et al*. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. *PLoS Med* 2006; **3**: e27.
- 47 Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M *et al*. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J Exp Med* 2001; **194**: 769–779.
- 48 Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H *et al*. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* 2004; **199**: 815–824.
- 49 Schjetne KW, Fredriksen AB, Bogen B. Delivery of antigen to CD40 induces protective immune responses against tumors. *J Immunol* 2007; **178**: 4169–4176.
- 50 Skountzou I, Quan FS, Gangadhara S, Ye L, Vzorov A, Selvaraj P *et al*. Incorporation of glycosylphosphatidylinositol-anchored granulocyte-macrophage colony-stimulating factor or CD40 ligand enhances immunogenicity of chimeric simian immunodeficiency virus-like particles. *J Virol* 2007; **81**: 1083–1094.
- 51 Rothstein TL, Wang JK, Panka DJ, Foote LC, Wang Z, Stanger B *et al*. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature* 1995; **374**: 163–165.
- 52 Majlessi L, Bordenave G. Role of CD40 in a T cell-mediated negative regulation of Ig production. *J Immunol* 2001; **166**: 841–847.
- 53 Erickson LD, Durell BG, Vogel LA, O'Connor BP, Cascalho M, Yasui T *et al*. Short-circuiting long-lived humoral immunity by the heightened engagement of CD40. *J Clin Invest* 2002; **109**: 613–620.
- 54 Schilizzi BM, Boonstra R, The TH, de Leij LF. Effect of B-cell receptor engagement on CD40-stimulated B cells. *Immunology* 1997; **92**: 346–353.

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