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Overexpression of CCL-21/Secondary Lymphoid Tissue Chemokine in Human Dendritic Cells Augments Chemotactic Activities for Lymphocytes and Antigen Presenting Cells

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Abstract

Background: *Ex vivo* generated dendritic cells (DC) genetically modified to express secondary lymphoid tissue chemokine (CCL-21/SLC) have been shown to stimulate potent antitumor responses in murine models. When injected intratumorally, CCL-21 colocalizes DC and lymphocyte effector cells at the tumor site. This may improve tumor antigen presentation and T cell activation by utilizing the tumor as an *in vivo* source of antigen for DC. In order to develop DC-based cancer therapies for intratumoral injection that could promote tumor antigen uptake and presentation *in situ*, we constructed and characterized an adenoviral vector that expresses human CCL-21 (AdCCL-21).

Results: Human monocyte derived DC were cultured in GM-CSF and IL-4 for 6 days. Following AdCCL-21 transduction, CCL-21 protein production was assessed by ELISA on day 8. DC transduced with AdCCL-21 at multiplicities of infection (MOIs) of 50:1 or 100:1 produced up to 210 ± 9 ng/ml and 278 ± 6.5 ng/ml /10^6 cells/48 hours, respectively. Following transduction, an immature DC phenotype was maintained and an upregulation of the costimulatory molecule, CD86 was noted. In addition, supernatant from AdCCL-21-DC caused significant chemotaxis of peripheral blood lymphocytes and mature DC.

Conclusions: These studies demonstrate that AdCCL-21-DC generate functional levels of CCL-21 without adversely altering DC phenotype. These findings strengthen the rationale for further investigation of AdCCL-21-DC as a DC-based therapy in cancer treatment.

Background

Tumor-associated antigens are expressed by many tumors, including lung cancers, and can be presented to cytotoxic T cells by antigen presenting cells (APCs) resulting in

antitumor responses [1]. As a result of limited expression of major histocompatibility complex (MHC) antigen and costimulatory molecules, as well as production of immune inhibitory cytokines, tumor cells are ineffective APCs [2]. Therefore, recruitment of professional APCs to the tumor site may be essential for generating specific anti-tumor immune responses. Dendritic cells (DC) are potent APCs fundamental in the activation of specific immunity [3]. Advancements in the isolation and *in vitro* propagation of DC have generated interest in their use in cancer therapy. DC have been investigated as adjuvants to cancer immunotherapy to stimulate tumor-specific antigen presentation for promotion of T cell activation and anticancer immunity [4–8].

Strategies employing DC in immunotherapy have included DC pulsed with tumor antigen peptides, apoptotic tumor cells, tumor lysates, or genetic modification of DC with genes encoding tumor antigens or immunomodulatory proteins [8–17]. There is evidence that DC transduced with adenoviral vectors (AdV) have prolonged survival and resistance to spontaneous and Fas-mediated cell death [18]. This could result in the improved delivery of immunotherapy. AdV transduction can also augment the capacity of DC to induce protective antitumor immunity [19]. In addition, enhanced local and systemic anti-tumor effects have been demonstrated when AdV transduced DC expressing cytokine genes have been injected intratumorally [17,20–23].

The use of *ex vivo*-propagated DC genetically modified to express chemokines that attract DC and lymphocyte effector cells to sites of tumor may improve tumor antigen presentation and T cell activation by utilizing the tumor as an *in vivo* source of antigen for dendritic cells. Chemokines are a family of proteins involved in leukocyte chemotaxis and activation, and have been associated with the regulation of angiogenesis [24,25]. CCL-21 is a CC chemokine expressed by high endothelial venules and in T cell zones of spleen and lymph nodes that strongly attracts T cells and mature DC [26–33]. CCL-21 recruits both Th1 lymphocytes and antigen-stimulated dendritic cells into T cell zones of secondary lymphoid organs, co-localizing the immune response elements and resulting in T cell activation [26]. In plt^{-}/plt^{-} mice with undetectable levels of CCL-21, the homing of T cells and DC to secondary lymphoid organs has been shown to be significantly decreased [34]. In addition to its immunotherapeutic potential, CCL-21 has been found to have potent angiostatic effects [35], thus adding further support for its use in cancer therapy.

Based on these capacities, CCL-21 could be an important protein for evaluation in cancer immunotherapy. Studies using recombinant CCL-21 in mouse lung cancer models have shown that intratumoral injection of recombinant CCL-21 led to potent antitumor responses with complete tumor eradication in 40% of treated mice [36]. In a spontaneous lung cancer model, recombinant CCL-21 injected into the axillary lymph node region produced a marked reduction in tumor burden with extensive lymphocytic and DC infiltration of the tumors. The CCL-21 injected mice also showed increased survival [37]. Intratumoral injections of murine CCL-21 gene modified DC (murine AdCCL-21-DC) into established murine lung tumors resulted in complete tumor regression and enhanced protective immunity compared to mice treated with control vector transduced DC or DC alone [23]. Similar results have been shown in a murine melanoma model [20,21]. The anti-tumor efficacy of AdCCL-21-DC implies an important role for DC as a vehicle to deliver CCL-21 to the tumor.

Based on these preclinical results, we constructed and characterized an adenoviral vector expressing human CCL-21 (AdCCL-21) to be utilized for transduction of human monocyte-derived DC (AdCCL-21-DC). We hypothesized that this construct would effectively produce CCL-21 without compromising DC phenotype. In addition, we proposed that human DC transduced with AdCCL-21 would demonstrate enhanced chemokine-dependent biologic activity evidenced by the capacity to attract lymphocytes and APCs. Our results indicate that AdCCL-21-DC generate biologically relevant levels of CCL-21 without negatively altering DC phenotype.

Results

AdCCL-21 gene-modified human DC produce biologically active levels of CCL-21

On day 6 of culture, immature human monocyte-derived DC (at 10^6 cells/ml) were transduced with AdCCL-21 at various multiplicities of infection (MOIs, 50:1–10,000:1) for 24–192 hours. Transduction was accomplished either by the standard method (2 hours incubation at 37°C), or by the centrifugation method (2000 × g at 37°C for 2 hours) as previously described [38,39]. CCL-21 protein production was assessed by ELISA, and demonstrated higher concentrations with increasing MOIs. Protein production increased with escalating MOIs, and plateaued at MOIs greater than 100:1. Cell viability significantly decreased at MOIs greater than 500:1 (data not shown). At relatively low MOIs of 50:1 and 100:1, 210 and 278 ng/ml/10^6 cells/48 hours of CCL-21 protein was produced respectively (Table 1). Moreover, DC transduction by the centrifugation method led to 2.9 fold increase in CCL-21 protein secretion compared to the standard transduction method (Table 1). Time course experiments revealed a plateau of CCL-21 production at 48 hours with sustained protein secretion at high levels (>190 ng/10^6 cells) up to day 8 after transduction (day 14 of culture) (Figure 1).

Table 1: CCL-21 production by AdCCL-21-DC

	DC	AdCCL-21-DC MOI 50:1	AdCCL-21-DC MOI 100:1	AdCV-DC MOI 100:1
		ng/10^6 DC/48 hours		
Standard Method	0.5 ± 0.24	*71 ± 15	*91.2 ± 5	0.5 ± 0.02
Centrifugation Method	0.67 ± 0.004	*210 ± 8.9	*278.2 ± 16.5	0.78 ± 0.05
			*p ≤ 0.01; **p ≤ 0.0009	

CCL-21 protein production by AdCCL-21-DC. DC propagated *in vitro* with GM-CSF and IL-4 were transduced with human AdCCL-21 or AdCV on day 6 of culture at MOIs 50:1 and 100:1. Transduction was accomplished by the standard incubation at 37°C (a) or by the centrifugation method (b) as described in materials and methods. CCL-21 protein concentration was determined in the supernatant of AdCCL-21 DC, AdCV-DC and NTDC by ELISA 48 hours after transduction. A 2.9 fold increase in CCL-21 protein production was achieved by the centrifugation method of DC transduction compared to the standard method. Values refer to CCL-21 protein concentration expressed in nanograms per million cells and are representative of three independent experiments. P values refer to significant differences of AdCCL-21-DC with AdCV-DC and control DC.

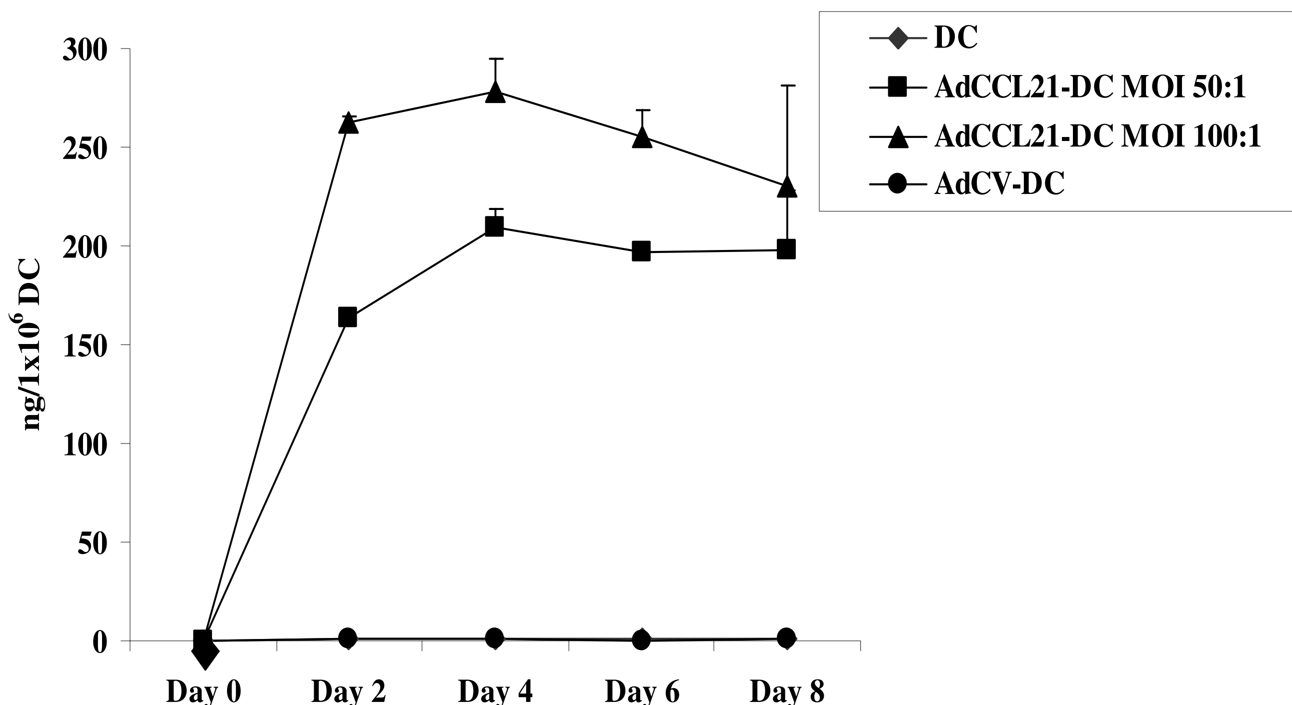

Figure 1
AdCCL-21-DC secretes CCL-21 up to 8 days after transduction. Supernatant from non-transduced DC (DC), AdCV-DC and Ad-CCL-21-DC transduced on day 6 of culture at MOIs 50:1 and 100:1 was analyzed for CCL-21 protein secretion by ELISA in a time-course experiment. Values refer to SLC protein concentration expressed in nanograms per million cells at different time-points (24, 48, 96, and 192 hours after transduction). Although very limited amounts of CCL-21 protein were detected in control DC or AdCV-DC, the levels were consistent and significantly less than the concentrations produced by AdCCL-21-DC.

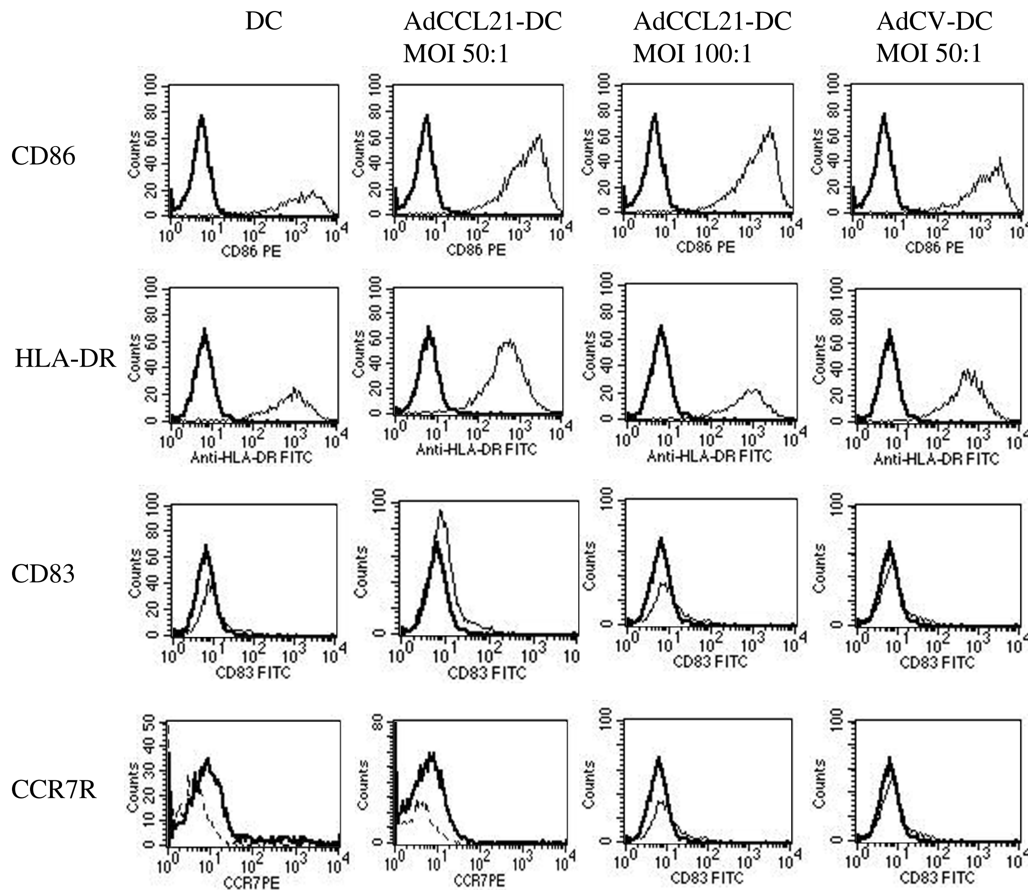

Figure 2
Phenotype of human DC transduced with AdCCL-21. DC, Ad-CCl-21-DC and AdCV-DC were characterized by flow cytometry. On day 8, cells were stained with antibodies to cell surface markers conjugated to fluorochromes listed in Materials and Methods and FACS analysis was performed. Histograms show surface expression in non-transduced DC and AdCCl-21-DC transduced at MOI 50:1 and 100:1. Control DC in the first panels; Ad-CCL-21-DC at MOI in the second column; Ad-CCL21-DC at MOI of 100:1 in the third column; and AdCV-DC in the final panels. Dotted lines represent isotype control and bold lines the surface marker staining. Results are representative of three independent experiments.

There were no significant differences noted in CCL-21 protein production when comparing LPS-matured and immature DC (data not shown). For subsequent studies, we assessed DC phenotype and function 48 hours following adenoviral transduction at MOI 50:1 and 100:1 in immature DC.

DC phenotype is maintained after transduction with AdCCL-21

DC, AdCCL-21-DC, and DC transduced with the control adenoviral construct (AdCV) were characterized by flow cytometry at 48 hours after transduction. The expression of DC surface markers was not significantly altered by transduction with AdCCL-21 or AdCV (AdCV-DC). The immature DC phenotype was preserved in AdCV-DC and AdCCL-21-DC without up-regulation of CD83, HLA-DR, or CCR7 (Figure 2). We demonstrated an increase in the expression of CD86 following transduction with either AdCCL-21 or AdCV. The latter is consistent with previous reports [19,40,41].

AdCCL-21-DC augments chemotaxis of PBL and mature DC

The migration of peripheral blood lymphocytes (PBL) to AdCCL-21-DC was assessed by a standard transwell chemotaxis assay. Supernatants from AdCCL-21-DC, AdCV-DC, and non-transduced DC (NTDC), as well as recombinant CCL-21 (rCCL-21) were used to demon-

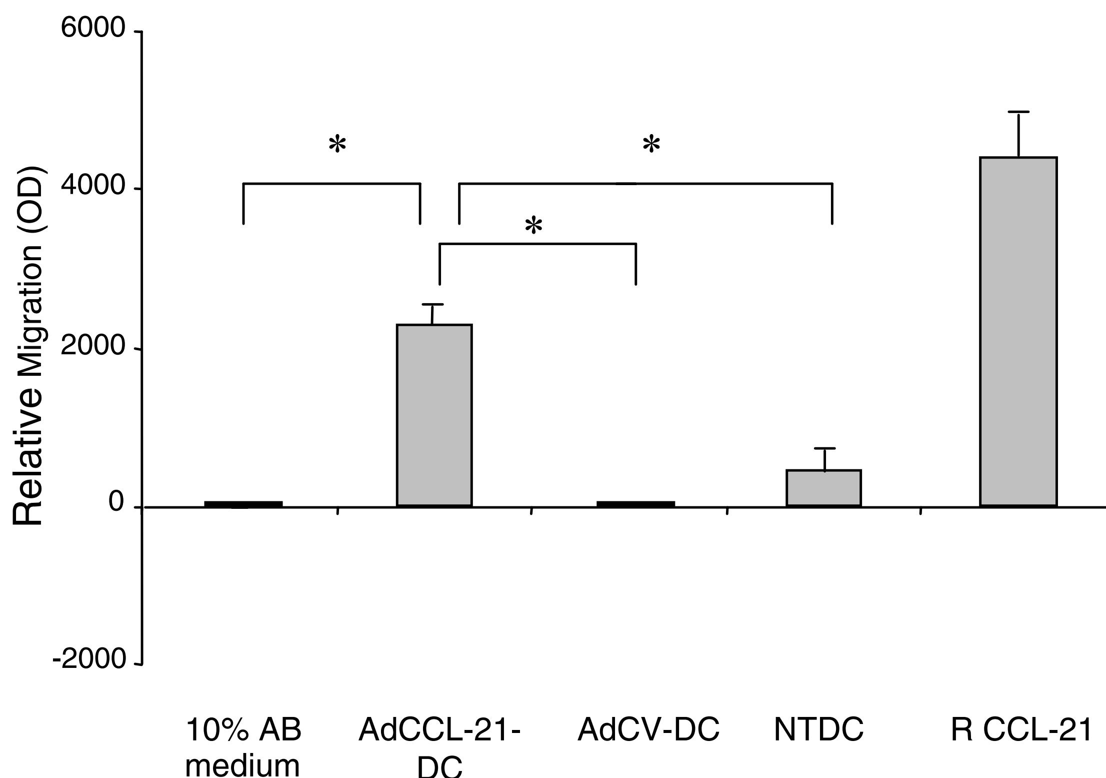

Figure 3
AdCCL-21-DC augments PBL chemotaxis. Ad-CCL-21-DC, AdCV, NTDC, and recombinant CCL-21 were used to investigate the induction of migration of PBL in response to AdCCL-21-DC. 2×10^5 freshly isolated PBL were labeled with 5 μm cell tracker™ green 5-chloromethylfluorescein diacetate and loaded in the upper chamber of a Transwell apparatus fitted with 3 μm pre size membrane inserts. Supernatant from NTDC, AdCCL21-DC (producing 20 ng/ml/10^6 cells/48 hours CCL-21 protein) and AdCV-DC were added to the lower chamber. The OD of migrated cells was measured after 2 hours incubation at 37°C by a fluorescence plate reader. Values are expressed as relative migration versus control (10% AB medium). The bar on the far right indicates chemotaxis to recombinant CCL-21 at 100 ng/ml. Results are representative of three independent experiments.

strate this integral activity. 2×10^5 freshly isolated PBL, labeled with a fluorescent probe, were allowed to migrate through 3 μm pore inserts of the transwell plate. Total cell migration was determined by measuring optical density (OD). Supernatant from AdCCL-21-DC (producing 20 ng/ml/10^6 cells) induced enhanced chemotaxis of PBL when compared to AdCV-DC supernatant, non-transduced DC supernatant alone, or control medium ($p < 0.002$) (10% AB serum) (Figure 3). Furthermore, AdCCL-21-DC promoted chemotaxis of LPS-matured DC (data not shown).

Anti-CCL-21 antibody significantly inhibits the enhanced chemotaxis stimulated by AdCCL-21-DC

We evaluated the contribution of CCL-21 to the enhanced function of AdCCL-21-DC compared to AdCV-DC or NTDC. A pre-titered neutralizing concentration of CCL-21 antibody was added to AdCCL-21-DC supernatant or recombinant CCL-21 and incubated for 30 minutes. The chemokine-dependent activity of AdCCL-21-DC was assessed by measuring the migration of T2 cells to AdCCL-21-DC and NTDC supernatants or rCCL-21 in the presence or absence of anti-CCL-21 antibody. T2 is a T/B hybridoma that lacks HLA class II antigen, but expresses

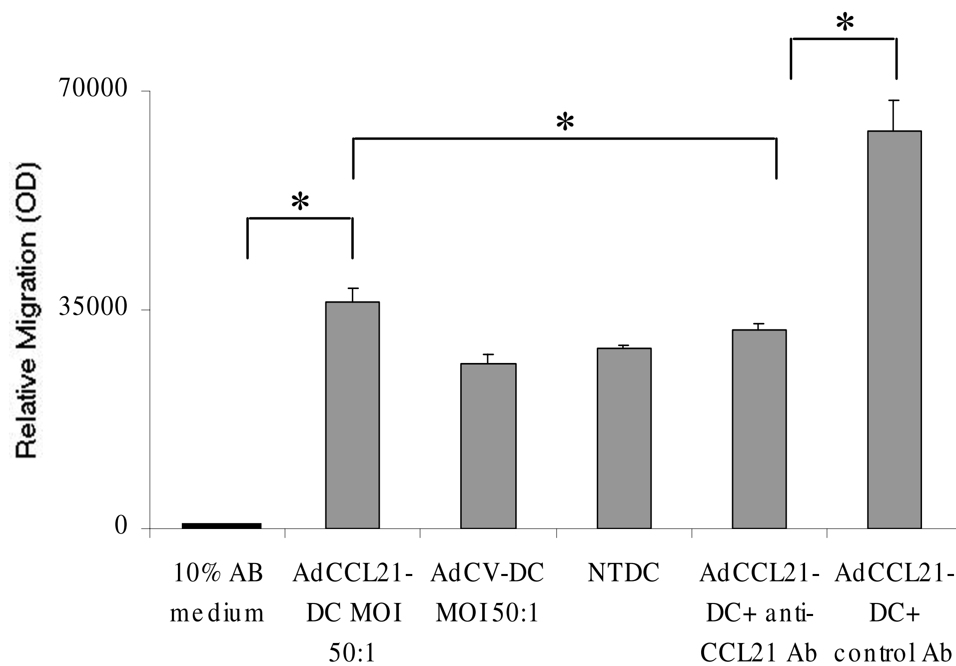

Figure 4
Chemotactic activity of AdCCL-21-DC is blocked by anti-CCL-21 neutralizing antibody. T2 cells were used as APCs in transwell Chemicon assays. 2.0 × 10^5 T2 cells in 100 μl serum free medium were added to the upper chamber of the transwell plate. 150 μl of supernatant from AdCCL-21-DC were pre-incubated for 30 minutes with anti-CCL-21 antibody or with a control antibody prior loading to the lower chamber of the Chemicon apparatus. After 2 hours incubation at 37°C, cells were lysed and stained with a green fluorescent dye according to the manufacturer's instructions. Total cell migration was determined by measuring the fluorescence of cells collected from the lower chamber and the bottom of the inserts using a fluorescence plate reader with 480/520 nm filter set. Values are expressed as relative migration versus the control (10% AB medium) and are representative of three independent experiments.

HLA.A2 molecules. These cells are often used as APCs when evaluating specific cytotoxic T lymphocyte responses, and were chosen to evaluate chemotaxis of APCs to AdCCL-21-DC. Our results showed that anti-CCL-21 antibody efficiently blocked the chemotactic activity of recombinant CCL-21 (data not shown). Moreover, the enhanced attraction of T2 cells observed with AdCCL-21-DC supernatant was significantly inhibited by the addition of anti-CCL-21 antibody. (Figure 4). Our data shows a partial reversal of the chemoattractive properties of AdCCL-21-DC using anti-CCL-21 antibody. Other proteins and chemokines with attractant capacities exist in DC supernatants, and these likely account for the incomplete inhibition of cell migration when anti-CCL-21 is added to AdCCL-21-DC. Chemotaxis of AdCCL-21-

DC was not inhibited following addition of control antibody.

AdCCL-21 transduction decreases immunosuppressive cytokine production

Previous studies have indicated that that adenoviral vector transduction of DC stimulates enhanced effector cell activity [19], in part through increased IL-12 production. We investigated the effects of AdCCL-21 and AdCV transduction on DC production of IL-12 and IL-10 after stimulation with IFNγ and LPS or LPS alone, respectively. We found that AdCV-DC and AdCCL-21-DC maintained the capacity to secrete IL-12 with decreased production of IL-10 (Figure 5). Secretion of IL-10 was decreased by 40–49% after AdCCL-21 or AdCV transduction.

Discussion

The ability of DC to capture, process, and present antigens with subsequent activation of $CD4^+$ and $CD8^+$ T lymphocytes makes them ideal adjuvants in immunotherapy approaches to cancer. In animal models, intratumoral injection of AdV transduced DC expressing cytokine genes has generated enhanced anti-tumor effects compared with NTDC or DC transduced with control vector [17,20–23]. We have characterized DC following transduction with an adenoviral vector expressing the CCL-21 gene. At the MOIs evaluated, DC maintained their immature phenotype without significantly up-regulating CD83 or CCR7 expression. Our findings that the DC expression of CD86 is increased following adenoviral transduction is consistent with prior reports [19,40,41]. These results suggest that DC transduced with adenovirus will have improved costimulatory capacities. In addition, supernatant from AdCCL-21-DC augmented the chemoattraction of peripheral blood lymphocytes and LPS-stimulated DC when compared to migration to supernatants from non-transduced DC or DC transduced with control adenoviral vector. These studies demonstrate that AdCCL-21 transduction of monocyte-derived DC yield cells with enhanced capacity to attract PBL and APCs. Thus, intratumoral administration of gene modified DC to express CCL-21 may prove to be an effective strategy of immunotherapy for achieving tumor antigen presentation utilizing the tumor as an *in vivo* source of antigen for DC [17].

CCL-21 recruits both T lymphocytes and antigen-stimulated DC into T cell zones of secondary lymphoid organs. This creates a convergence of the immune response elements to sites of CCL-21 production, resulting in T cell activation and antitumor effects [26,36]. CCL-21 acts through CCR7, a chemokine receptor belonging to a subfamily of G protein-coupled seven-transmembrane receptors [42]. CCR7 is integral in mediating the homing of DC and naïve and activated T lymphocytes to secondary lymphoid organs. This creates an environment in which co-localization of T cells and mature antigen-loaded DC can occur [43].

We found that intratumoral injection of murine AdCCL-21-DC was effective in eradicating established tumors and promoting long-term anti-tumor immunity in a murine lung cancer model [23]. We have developed the use of intratumoral administration of DC that have been modified with cytokine genes *ex vivo*, and this approach achieves tumor antigen presentation by utilizing the tumor as an *in vivo* source of antigen for DC [17]. In contrast to *in vitro* immunization with purified peptide antigen, autologous tumor has the capacity to provide the DC administered at the tumor site access to the entire repertoire of available antigens *in situ*. This may increase the likelihood of a response and reduce the potential for tumor resistance due to phenotypic modulation.

Increased IL-10 at the tumor site has been reported to suppress immune responses [44,45]. Our finding that transduction of DC with AdCV or AdCCL-21 resulted in decreased IL-10 production while maintaining IL-12 secretion when compared to non-transduced DC, is consistent with an overall immunostimulatory effect of the adenovirus upon transduction of DC [19]. The ability to polarize the immune response to a Th1 phenotype may potentiate the antitumor immune response seen with intratumoral injection of AdCCL-21-DC.

Our investigations have revealed that the transduced DC maintain their immature phenotype. This has important implications for its use as a potential intratumoral therapy in cancer because immature DC are efficient in antigen uptake. Following antigen capture, DC mature and develop improved migration, antigen presentation, and T cell stimulation [46]. Mature DC are potent APCs, expressing high levels of MHC class II, adhesion, and costimulatory molecules on their surface. Surface expression of CCR7 is also enhanced and mature DC thus gain the capacity to respond to CCL-21 [47,48]. Clinical trials employing mature DC injected by subcutaneous and intranodal routes have suggested that these cells may be effective in eliciting T cell responses [49–51]. Our approach involves the transduction of immature DC with AdCCL-21 to stimulate the migration of mature DC and T lymphocytes to the site of injection. We propose that introducing immature DC to the site of tumor will create the optimal environment for effective antigen uptake, using the tumor as an *in vivo* source of antigen [17,23]. CCL-21 expression could then promote movement of mature DC and Th1 lymphocytes to the site of tumor for potential cross-presentation and activation of T cells for potent anti-tumor immune responses.

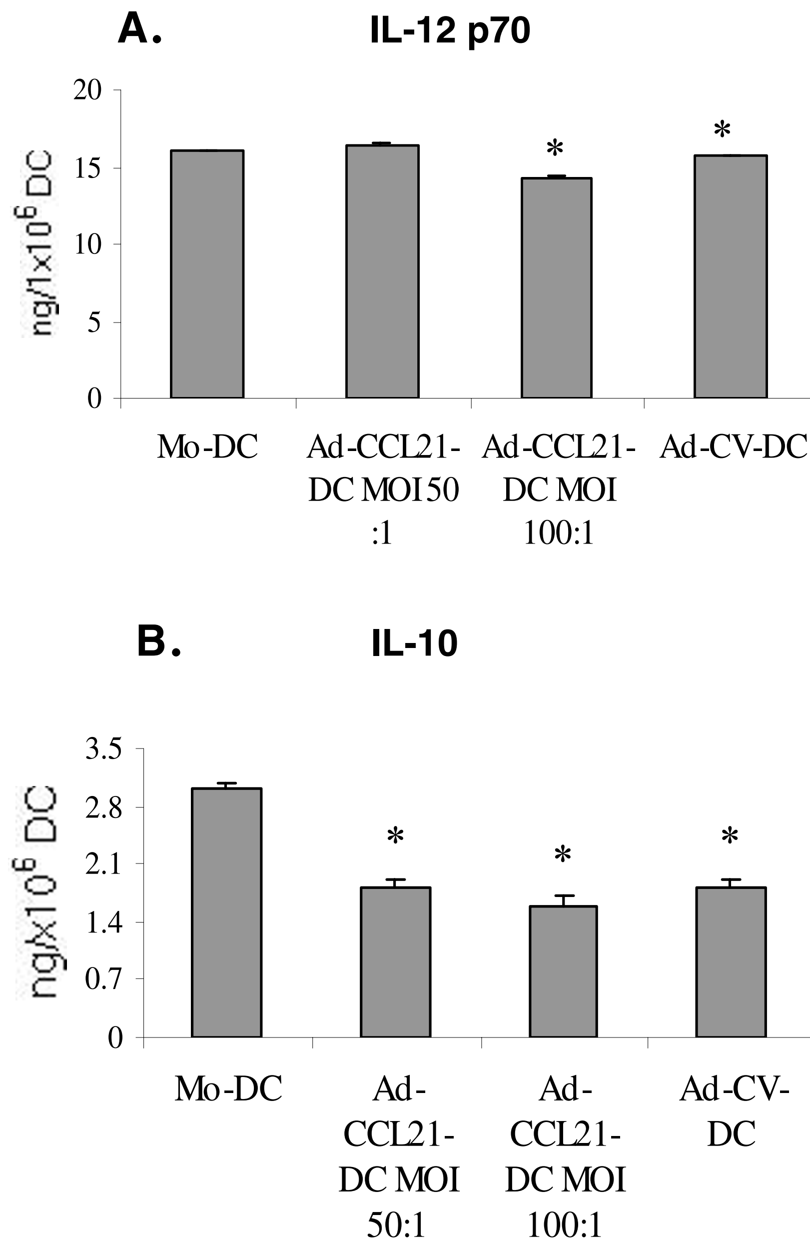

Figure 5
IL-12 p70 and IL-10 production by AdCCL-21-DC. Human monocyte-derived DC were transduced with AdCCL-21 and AdCV at an MOI of 50:1 and 100:1 at 37°C for 2 hrs by the standard method. 1.0×10^5non-transduced DC (Mo-DC), AdCCL-21-DC and AdCV-DC were plated into a 24 well plate in 1 ml of culture medium in presence or absence of the appropriate stimulation for IL-12p70 (γ-IFN and LPS) or IL-10 (LPS). After 48 hrs induction, supernatants from stimulated and non-stimulated DC (non-transduced DC, AdCCL-21-DC and AdCV-DC) were harvested and measured for IL-12p70 and IL-10 concentrations by specific ELISA. Values refer to cytokine concentrations expressed in nanograms per million cells 48 after stimulation (96 hrs after transduction) and are representative of three separate experiments.

Conclusions

AdCCL-21-DC may potentiate the induction of anti-tumor immune responses by augmenting antigen presentation and T lymphocyte activation. Thus, in this system the transferred DC expressing CCL-21 may act to enhance the generation of anti-tumor immunity because the tumor can serve as the *in vivo* source of antigen for DC. Our results demonstrate that AdCCL-21-DC generate biologically functional levels of CCL-21 without impairing DC phenotype. This data, revealing the biological activity of AdCCL-21-DC, paired with our results of intratumoral injection of AdCCL-21-DC in murine lung cancer models [23], supports further investigation of this approach as a novel genetic immunotherapy for cancer treatment.

Methods

Preparation of adenoviral vector expressing human CCL-21

The adenoviral construct (AdCCL-21) is an E1-deleted, replication-deficient adenoviral serotype 5 vector encoding a 491 bp-containing human CCL-21 cDNA (Figure 6). The coding sequence 59–453, was amplified by PCR with the following primers: 5' primer 5'-CTTGCAGCT-GCCCACCTCAC (1–20) and 3' primer 5'-TCTCCAG-GGCTCCAGGCT-GC-3 (491-472). The CCL-21 gene was amplified and cloned into pAC-CMVpLpA (originally cloned by Dr. R. Gerard, and was acquired from Dr. J. Economou at UCLA, Los Angeles, CA) to generate pAC-CMV-CCL-21pA. pAC-CMV-CCL-21pA was co-transfected with pJM-17 (plasmid containing the E1-deleted Ad-genome, obtained from Dr. J. Economou) into 293 cells (American Type Culture Collection [ATCC] Manassas, VA) to yield recombinant E1-deleted adenovirus (AdCCL-21) following homologous recombination. The control vector (AdCV) did not contain the CCL-21 cDNA insert. Clones of AdCCL-21 and AdCV were obtained by limiting dilution analysis of the ability of the medium to induce a cytopathic effect on fresh 293 cells. Clones were confirmed by CCL-21 specific ELISA. Viral stocks were then obtained by amplification of the 293 cells followed by CsCl purification, dialysis, and storage as a glycerol (10% volume/volume) stock at -80°C. The titer of each viral stock was routinely 10^{11}–10^{12} plaque forming units by plaque assay on 293 cells. Contamination with wild type recombinant adenovirus was assessed for each viral stock by plaque assay on Hela cells and was consistently negative.

Generation of dendritic cells

The use of leukocyte-enriched buffy coat from healthy donors was approved by the UCLA IRB, and informed consent was obtained from all donors. Peripheral blood mononuclear cells (PBMC) were obtained from leukocyte-enriched buffy coat from healthy donors by centrifugation with Ficoll-Paque™ Plus (Amersham Biosciences, Uppsala, Sweden) and the light density fraction from the 42.5–50% interface was recovered. The cells were resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 20 mM HEPES buffer (Cellgro, Mediatech, Herdon, VA), 100 units/ml Penicillin-Streptomycin, and 2 mM glutamine (Invitrogen, Carlsbad, CA). The cells were allowed to adhere to tissue culture flasks for 2 hours at 37°C. After incubation, the non-adherent cells were removed, and adherent cells were washed twice in Dulbecco's PBS without Ca^{2+} and Mg^{2+}(Cellgro). Cells were then cultured for 6 days in complete RPMI medium with 10% human AB serum (Gemini Bio-Products, Woodlands, CA), recombinant human GM-CSF (75 ng/ml), (Peprotech Inc., Rocky Hill, NY, specific activity $\geq 1 \times 10^7$ units/mg) and recombinant human IL-4 (75 ng/ml) (Peprotech Inc., specific activity $\geq 5 \times 10^6$ units/mg). Cytokines were added every 2–3 days during culture.

Adenoviral transduction of human DC

Monocyte-derived DC were transduced with human AdCCL-21 or AdCV expression vectors on day 6 of culture either by the standard method of incubation at 37° for 2 hours or by centrifugation method as previously described [38,39]. Briefly 1.0×10^6 DC, resuspended in 300 μl DPBS (Invitrogen, Carlsbad, CA) containing Ad-CCL-21 or AdCV at various MOIs (from 5:1 to 10,000:1) were incubated at 37°C for 2 hours or centrifuged at 2000 × g at 37°C for two hours (Microfuge Eppendorf 5415R, Fisher Scientific, Pittsburgh, PA). NTDC, AdCCL-21-DC and AdCV-DC were then washed twice, resuspended in complete medium and incubated at 37°C for 24 to 192 hours. The cumulative CCL-21 protein production was measured daily up to 8 days following transduction.

Immunophenotypic analysis and cell viability by flow cytometry

DC, AdCCL-21-DC, and AdCV-DC were characterized by flow cytometry on day 8 of culture. The following panel of monoclonal antibodies was used: HLA-DR-FITC (fluorescein isothiocyanin), CD40-PE (phycoerytrin), CD80-PE, CD86-PE, HLA-DR-FITC (BD Biosciences PharMingen, San Diego, CA), and CD83-FITC (Coulter Immunology, Hialeah, FL). All antibodies were used with appropriate isotype controls (BD Biosciences, La Jolla, CA). Evaluation of CCR7 surface expression was performed by a double step staining method using purified anti-human CCR7 (BD Biosciences PharMingen) followed by biotin-streptavidin staining. Briefly, DC were first labeled with purified mouse anti-human CCR7 monoclonal antibody for 30 minutes at room temperature. Cells were washed twice in FACS buffer (PBS, 2% FBS) and incubated with a biotin-conjugated rat anti-mouse IgM monoclonal antibody (BD PharMingen). After two washes in FACS buffer cells were stained with streptavidin PE (BD PharMingen). Viability of the cells was measured using 7-Amino-Actinomycin-D

(7-AAD) (Calbiochem, San Diego, CA). A live gate was set by forward and side scatter and 7-AAD staining. 10,000 cells with high forward scatter and high side scatter were counted. Events were acquired by FACS using a LSR flow cytometer (Becton, Dickenson, and Co. Biosciences Immunocytometry System, San Jose, CA) in the University of California, Los Angeles, Jonsson Comprehensive Cancer Center Flow Cytometry Core Facility. Data analysis was performed by CELLQuest software.

CCL-21 ELISA

CCL-21 protein concentration from NTDC, AdCCL-21-DC and AdCV-DC supernatant was determined by CCl-21-specific ELISA. Briefly, 96-well Costar (Cambridge, MA) plates were coated overnight with 2 μg/ml of anti-CCL-21 monoclonal antibody (Peprotech Inc., Rocky Hill, NY). After four washes in PBS-0.05% Tween-20, the unbound material was removed and the plate was incubated with a blocking buffer (10% fetal bovine serum, Gemini Bioproducts, Woodlands, CA) in PBS for 30 minutes. After four washes, the plate was incubated with CCL-21 standard or with the sample supernatant for one hour and excess antigen was washed off with PBS/Tween. The plate was then incubated with avidin-conjugated to horseradish-peroxidase for 20 minutes in the dark. After washing to remove all unbound reagents, the plate was incubated with a substrate solution for 20 minutes in the dark. Reactions were stopped by adding 1-M sulfuric acid and the optical density was read at 450 nm in a microplate reader (Molecular Dynamics, Sunnyvale, CA). The sensitivity of the CCl-21 assay was 156 pg/ml.

Chemotaxis assay

The biological function of supernatant derived from AdCCL-21-DC, AdCV-DC and NTDC was assessed in a standard chemotaxis assay. We measured the migration of PBL through a 24-well transwell plate fitted with 3 μm polycarbonate membrane inserts. 2×10^5 freshly isolated PBL by Ficoll-Paque density-gradient separation were labeled with 5 μm cell tracker™ green 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes Inc., Eugene, OR) for 45 minutes, washed and loaded in the upper chamber of the transwell plate in 100 μl serum-free medium. 500 μl of supernatant from 10^6 AdCCL-21-DC, AdCV-DC and NTDC were added to the lower chamber. 10% AB medium and 100 ng/ml recombinant CCL-21 (R&D Systems, Minneapolis, MN) were used as negative and positive controls, respectively. Cells were incubated for 2 hours at 37°C. The migrated cells were recovered from the lower chamber, centrifuged, and transferred into a 96-well flat-bottomed plate (Costar). Total cell migration was determined by measuring optical density (OD) of migrated cells using Wallac 1420 (Perkin Elmer, Boston, MA) fluorescent plate reader set at excitation/emission wavelengths of 485/530 nm.

Chemotaxis blocking assay

Chemotaxis blocking assays were performed using QCM™ chemotaxis 96-well plates fitted with 3 μm membrane inserts (Chemicon International, Inc., Temecula, CA) according to the manufacturers instructions. Briefly, 2×10^5 cells/ml T2 cells (a gift from Dr. J. Economou, David Geffen School of Medicine at UCLA, Los Angeles, CA), were resuspended in serum-free medium and loaded in the upper chamber of a QCM™ apparatus. 150 μl of supernatant derived from AdCCL-21-DC were pre-incubated with neutralizing concentrations of anti-CCL-21 antibody (R&D Systems) or Chrompure goat IgG whole molecule as the control antibody (Jackson Immunoresearch, Westgrove, PA) for 30 minutes at room temperature. Similarly 100–600 ng/ml recombinant CCL-21 was pre-incubated with 4–8 μg/ml anti-CCL-21 or control antibody. The neutralizing concentration of anti-CCL-21 antibody (R&D Systems) used in these studies (4 μg/ml and 8 μg/ml) was based on the ND50 (50% maximum inhibition of cytokine activity when CCL-21 is present at a concentration high enough to elicit maximum response) of goat anti-human CCL-21 as reported by the manufacturer (4 – 8 μg/ml anti-CCL-21 with 500 ng/ml rCCL-21). The pre-incubated samples were then added to the lower chamber of the QCM™ apparatus. In addition, 150 μl of supernatant without antibody from AdCCL-21-DC, 10% AB medium or 100–600 ng/ml recombinant CCL-21 (R & D Systems) were added to the lower chambers as chemotaxis controls. After 2 hours incubation at 37°C migrated cells were recovered from the lower chamber and from the inserts according to the manufacturer's instructions. Cells were lysed and stained with a green fluorescent dye (CyQuant GR dye, Molecular Probes) for 15 minutes at room temperature. Cells were transferred to a 96-well flat-bottomed ELISA microplate (Costar) and fluorescence was read at 480/525 nm using Wallac 1420 fluorescent plate reader (Perkin Elmer).

IL-10 and IL-12p70 ELISA

To induce IL-12 production, (1.0×10^5) AdCCL21-DC, AdCV-DC and control DC were primed with 50 ng/ml γ-IFN (Peprotech Inc.) for 2 hours and then stimulated with 1 μg/ml LPS (Sigma) for 48 hours. To induce IL-10 production, (1.0×10^5) AdCCL21-DC, AdCV-DC and control DC were stimulated with 1 μg/ml LPS for 48 hours. IL-12 p70 and IL-10 concentrations were measured in the supernatant of either unstimulated or stimulated DC by specific ELISA as previously described [52]. For detection of IL-12p70, antibodies and standards were purchased from R&D Systems Inc. For detection of IL-10, antibodies and standards were obtained from BD Biosciences. The sensitivity of the ELISA for IL-12 p70 and IL-10 was < 1 pg/ml.

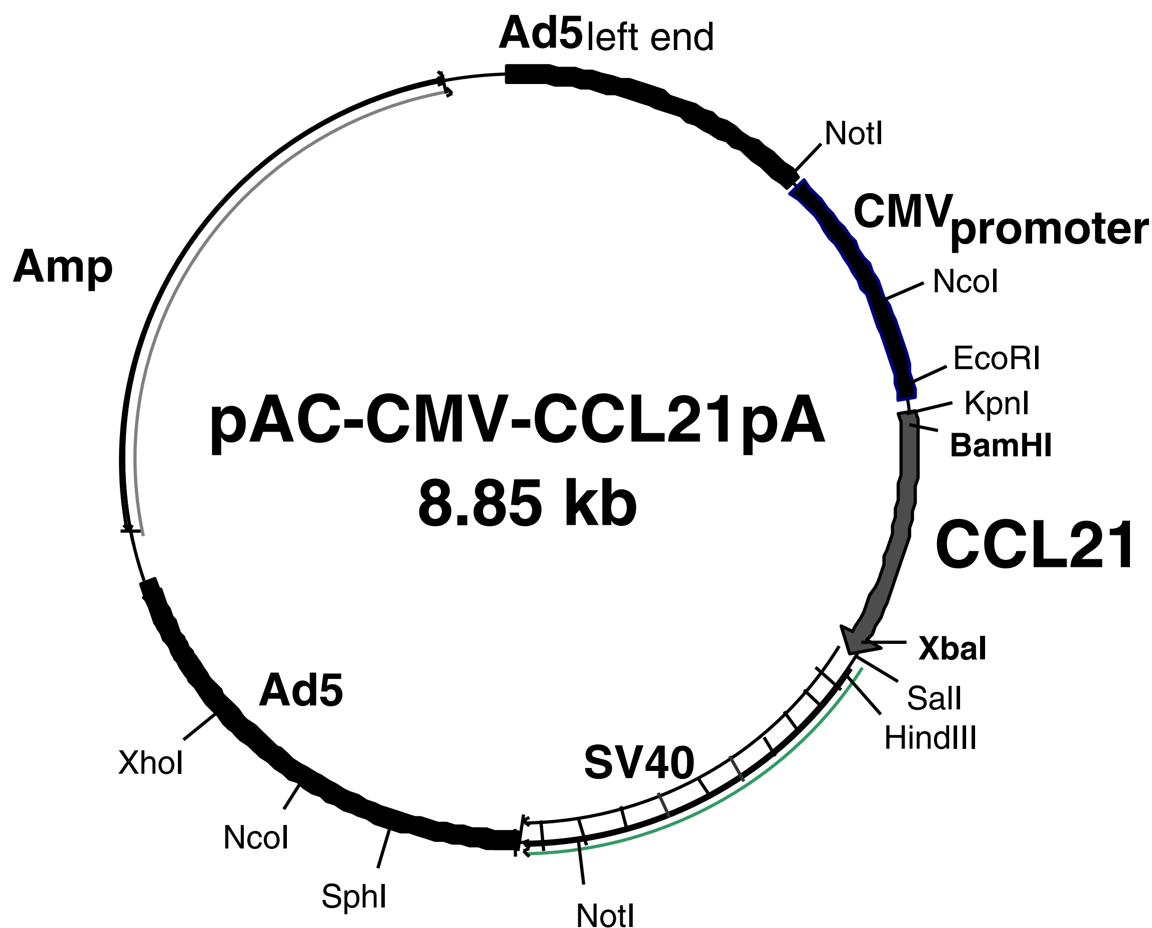

Figure 6
Adenoviral vector expressing human CCL-21. The adenoviral construct (Ad-CCL-21) is an E1-deleted, replication-deficient adenoviral serotype 5 vector encoding a 491 bp human CCL-21 cDNA. The 491 bp-containing human CCL-21 coding sequence 59–453, was amplified by PCR with the following primers: 5' primer 5'-CTTGCAGCTGCCCACCTCAC-(1-20) and 3' primer 5'-TCTCCAGGGCTCCAGGCT-GC-3 (491-472). The CCL-21 gene was amplified and cloned into pAC-CMVpLpA (to generate pAC-CMV-CCL21pA) with confirmation using restriction analysis and sequencing. pAC-CMV-CCL21pA was co-transfected with pJM-17 (plasmid containing the E1-deleted Ad-genome) into 293 cells to yield recombinant E1-deleted adenovirus (AdCCL-21) following homologous recombination.

Statistical Analysis
The unpaired two-tailed Student's t test was used to compare differences in DC and AdCCL-21-DC groups. P-values = 0.05 were considered significant.

List of abbreviations
The abbreviations used are: DC, dendritic cells; CCL-21/SLC, secondary lymphoid tissue chemokine; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MOI, multiplicity of infection; APC, antigen presenting cell; MHC, major histocompatibility complex; AdV, adenoviral vector; Th, T helper; LPS, lipopolysaccharide; PBL, peripheral blood lymphocyte; NTDC, non-transduced DC; rCCL-21, recombinant CCL-21; OD, optical density; PBMC, peripheral blood mononuclear cell; FITC, fluorescein isothiocyanin; PE, phycoerytrin; FACS, fluorescence-activated cell scanner; Ig, immunoglobulin; CMFDA, 5-chloromethylfluorescein diacetate, HLA; human leukocyte antigen.

Author's contributions
Author 1 KR and Author 2 FB contributed equally to this research. KR and FB coordinated and carried out all the experiments. Authors SCY, JL, RB, and RF contributed to the discussion of the article. Author FB together with KR performed the statistical analyses. Authors RS, SS, and SD

together with KR and FB conceived of the study, participated in its design, and drafted the manuscript.

All authors read and approved the final manuscript.

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