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Adenovirus-engineered human dendritic cells induce natural killer cell chemotaxis via CXCL8/IL-8 and CXCL10/IP-10

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Recombinant adenovirus-engineered dendritic cells (Ad.DC) are potent vaccines for induction of anti-viral and anti-cancer T cell immunity. The effectiveness of Ad.DC vaccines may depend on the newly described ability of Ad.DC to crosstalk with natural killer (NK) cells via cell-to-cell contact, and to mediate activation, polarization and bridging of innate and adaptive immunity. For this interaction to occur in vivo, Ad.DC must be able to attract NK cells from surrounding tissues or peripheral blood. We developed a novel live mouse imaging system-based NK-cell migration test, and demonstrated for the first time that human Ad.DC induced directional migration of human NK cells across subcutaneous tissues, indicating that Ad.DC-NK cell contact and interaction could occur in vivo. We examined the mechanism of Ad.DC-induced migration of NK cells in vitro and in vivo. Ad.DC produced multiple chemokines previously reported to recruit NK cells, including immunoregulatory CXCL10/IP-10 and proinflammatory CXCL8/IL-8. In vitro chemotaxis experiments utilizing neutralizing antibodies and recombinant human chemokines showed that CXCL10/IP-10 and CXCL8/IL-8 were critical for Ad.DC-mediated recruitment of $CD56^{hi}CD16^{-}$ and $CD56^{lo}CD16^{+}$ NK cells, respectively. The importance of CXCL8/IL-8 was further demonstrated in vivo. Pretreatment of mice with the neutralizing anti-CXCL8/IL-8 antibody led to significant inhibition of Ad.DC-induced migration of NK cells in vivo. These data show that Ad.DC can recruit spatially distant NK cells toward a vaccine site via specific chemokines. Therefore, an Ad.DC vaccine can likely induce interaction with endogenous NK cells via transmembrane mediators, and consequently mediate Th1 polarization and amplification of immune functions in vivo.

Introduction

Dendritic cells (DC) and natural killer (NK) cells are central components of the innate immune system that function at the interface of innate and adaptive immunity.[1-3] DC are professional antigen presenting cells (APC) with the unique ability to efficiently process and present antigenic epitopes to, and activate antigen-specific naïve T cells.[2] NK cells are spontaneously cytotoxic cells that rapidly recognize and directly eliminate virally-infected and transformed cells.[4] Both NK cells and DC secrete proinflammatory and immunoregulatory cytokines, and mediate inflammation as well as polarization and regulation of both innate and adaptive immune responses.[1,3,4]

An important feature of DC and NK cells is their crosstalk via cell-to-cell contact leading to reciprocal regulation and stimulation of type-1 polarization and cytokine secretion.[1,3,5-7] NK cells induce DC maturation and secretion of IL-12p70. Reciprocally, DC induce NK-cell activation and enhanced IFNγ secretion, tumoricidal activity and proliferation. This early cellular crosstalk is an essential immunoregulatory mechanism bridging innate and adaptive immunity, and defining the quality and magnitude of immune mechanisms that control viral infections and tumor growth in vivo.[1,3,5,8-10]

A number of strategies have been developed to utilize the ability of DC to stimulate immunity to treat various diseases in clinical settings. One involves the use of recombinant adenoviral vectors (AdV) to genetically modify DC to express a protein of interest. DC engineered with AdV (Ad.DC) are potent immune adjuvants showing promise as vaccines for viral infection and cancer prevention and therapy.[11,12] Ad.DC acquire an intermediate level of maturation and have increased ability to stimulate antigen-specific $CD4^{+}$ and $CD8^{+}$ T-cell responses.[1,2,5,6,13-15] We have recently shown that Ad.DC effectively activate resting NK cells and upregulate Type-1 cytokine secretion, proliferation, and antitumor activities via cell-to-cell contact and cooperative activities of transmembrane TNF (tmTNF) and *trans*-presented IL-15 (*trans*-IL-15).[16]

NK-cell subsets exhibit specific tissue distributions. Immunoregulatory $CD56^{hi}CD16^{-}$ NK cells express L-selectin (CD62L, a

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pivotal cell adhesion molecule for engaging lymph node high endothelial venules) and CCR7, CCR5 and CXCR3 chemokine receptors.[17,18] These molecules enable $CD56^{hi}CD16^{-}$ NK cells to preferentially localize in lymph node T-cell areas.[19-23] In contrast, cytotoxic $CD56^{lo}CD16^{+}$ NK cells are poorly represented in lymph nodes, but they comprise the large majority of NK cells in the peripheral blood, spleen, liver, inflammatory sites and cancer tissues. They express high levels of CXCR1 and CX_3CR1 chemokine receptors, which enable their preferential migration into inflamed tissues.[19,20,24,25] DC are also present in inflamed tissues and lymph node T-cell areas,[26,27] and a number of DC-produced chemokines, including CXCL9/MIG and CXCL10/IP-10, induce NK-cell chemotaxis.[19,28,29] Therefore, DC could recruit NK cells in vivo, into inflamed tissues and lymph nodes.

Based on these findings, we hypothesized that Ad.DC-based vaccine effectiveness might depend on Ad.DC ability to recruit NK cells and enable their cell-to-cell contact interaction leading to Th-1 cytokine polarization and amplification of both innate and adaptive immune functions.[30] Human DC modulate the repertoire of cytokines and chemokines produced based on external signals received (LPS, AdV, cytokines).[13,28] In the present study, we show that Ad.DC induce in vivo mobilization and migration of human NK cells toward a vaccine site (mediated largely by CXCL8/IL-8), and in vitro chemotaxis of $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells via CXCL8/IL-8 and CXCL10/IP-10, respectively. This study is the first to demonstrate in vivo mobilization and migration of human NK cells by DC, to identify CXCL8/IL-8 as a crucial chemokine by which DC recruit NK cells and to determine that Ad.DC effectively recruit both major subsets of resting NK cells.

Results

Ad.DC induce migration and attract NK cells in subcutaneous tissues. The efficiency of anticancer or antiviral vaccines may depend on their ability to induce DC-NK cell crosstalk via cell-to-cell contact and transmembrane mediators at a vaccine site and in draining lymph nodes.[7,16] The induction of cell crosstalk at a vaccine site requires that DC and NK cells are localized in close proximity or recruited from the surrounding tissues or blood circulation into the vaccination site. Under physiological conditions, the common skin vaccine site contains DC (Langerhans cells) and $CD56^{hi}CD16^{-}$ NK cells which are mostly distributed in separate tissue compartments, epidermis and dermis, respectively.[31,32] In contrast, hypodermis lacks both DC and NK cells. Therefore, a subcutaneous vaccine could induce DC-NK cell crosstalk if it could recruit DC and NK cells to the immunization site. A subcutaneously-delivered Ad.DC vaccine could induce Ad.DC-NK cell crosstalk if Ad.DC could mobilize and attract NK cells from the dermis and/or peripheral blood.

To directly evaluate the ability of human Ad.DC to attract NK cells into a vaccine injection site, we developed an IVIS test for NK-cell migration in subcutaneous tissues of mice. For this test we used NK cells and iDC labeled with different amine reactive (NHS ester) near-infrared (NIR) fluorochromes, and DC transduced with luciferase-encoding AdV (Ad.DC.Luc). Subcutaneously-injected Ad.DC.Luc or iDC and NK cells consistently showed well defined and strong transcutaneous signals for at least 72h in both immunocompetent albino BL6 (B6A) and immunodeficient NOD SCID gamma (NS.IL2R$\gamma^{-/-}$) mice. Ad.DC.Luc maintained their bioluminescence signal in NS.IL2R$\gamma^{-/-}$ mice for more than 10 d, indicating their ability for prolonged survival and functionality in vivo (data not shown).

To define the optimal site for the test, multiple injection sites were tested. Of these, the easiest, the most consistent, and the most precise measurements were obtained in the lower back. This location was chosen as the optimal site for in vivo migration tests. NK-cell migration was detected 24 h post-injection, and no additional migration was observed at later time-points (data not shown).

Ad.DC.Luc and iDC were injected subcutaneously into contralateral regions of the back. NK cells were injected distally to a DC injection. Transcutaneous localization of Ad.DC.Luc (bioluminescence), iDC and NK cells (fluorescence) was performed using a small animal IVIS at 0 h and 24 h after cell injections (**Fig. S1**). A chemotaxis experiment was considered positive if we observed at least 5% migration by at least one of the three measurements taken: distances between the DC signal focus and the proximal edge, focal point, and distal edge of an NK-cell signal. In 6/14 (43%) tested mice, NK cells migrated toward Ad.DC.Luc over a period of 24 h after the cell injections (**Fig. 1**; **Fig. S2**). In the positive Ad.DC.Luc experiments, we observed median and mean NK cell-to-Ad.DC.Luc distance decreases of 18% and 22.2% (from proximal edge of the NK-cell signal to the Ad.DC.Luc signal focus; **Fig. 1B**), 6.9% and 14.9% (between NK cell and Ad.DC.Luc signal focal points; **Fig. 1C**), and 9.9% and 13.6% (from the distal edge of NK-cell signal to the Ad.DC.Luc signal focus; **Fig. 1D**), respectively. In 4/6 assessments, NK-cell migration toward Ad.DC.Luc was evidenced by all three measurements demonstrating translocation of the entire NK-cell population. In 2/6 assessments, the NK-cell signal completely crossed the initial distance from, and made an overlap with the Ad.DC.Luc signal (**Fig. 1A**; Ad.DC-best; and data not shown). In sharp contrast, in 5/5 performed assessments, NK cells did not migrate toward iDC (**Fig. 1A–D**). These findings demonstrate the ability of Ad.DC, and inability of iDC, to attract and induce migration of NK cells in vivo, in subcutaneous connective tissues.

Ad.DC induce chemotaxis of NK cells. To determine the mechanism of Ad.DC-mediated NK-cell chemo-attraction, we examined whether Ad.DC produce soluble factors that can induce chemotaxis of freshly isolated, resting NK cells across a transwell membrane. We found that Ad.DC and mDC supernatants induced median chemotaxis of 13.5% and 16.5% of resting NK cells, respectively, while iDC supernatants had no chemoattractive activity (**Fig. 2**). The degree of DC supernatant-induced chemotaxis was donor-dependent, however the overall migration patterns were the same in all tests. These findings indicate that Ad.DC and mDC, but not iDC, produce soluble factors capable of recruiting NK cells.

Ad.DC recruit both $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK-cell subsets. Two major subsets of human NK cells, cytotoxic $CD56^{lo}CD16^{+}$ and immunoregulatory $CD56^{hi}CD16^{-}$, are differently represented in lymphoid and other normal or inflamed

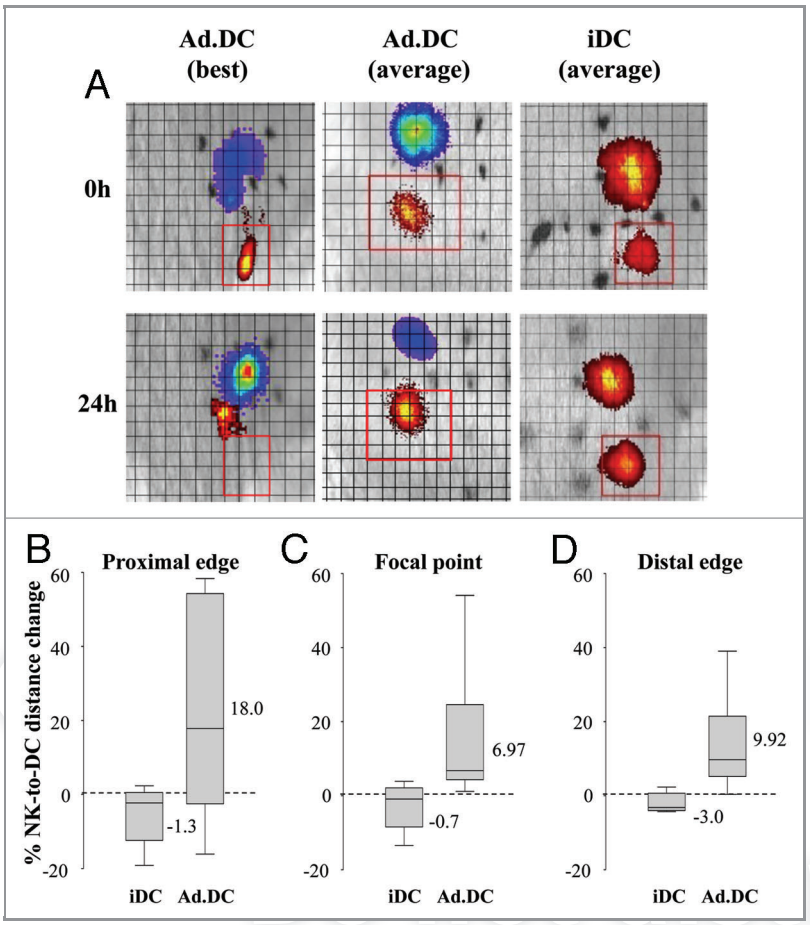

Figure 1. Ad.DC induce migration and attract NK cells in subcutaneous tissues. The in vivo NK-cell chemotaxis test was performed in seven experiments and 14 mice. Bioluminescence and fluorescence images were collected and analyzed as described in Methods and illustrated in **Figure S1**. (A) Enlarged image overlays of the best and average examples of NK-cell (red images) migration toward Ad.DC.Luc (Ad.DC; blue images), and an average example of NK-cell (red distal images) migration toward iDC (red proximal images) over a span of 24 h are shown. Whole mouse image overlays can be seen in **Figure S2**. Migration was quantified by measuring the percent of NK cell-to-DC distance changes between Ad.DC or iDC signal focus to the (B) proximal edge, (C) focal point and (D) distal edge of an NK-cell signal. The data were standardized by calculating the percent change in the determined distances over a span of 24 h after cell injections. Box-plots represent analysis of all iDC tests and only positive (6/14) Ad.DC tests.

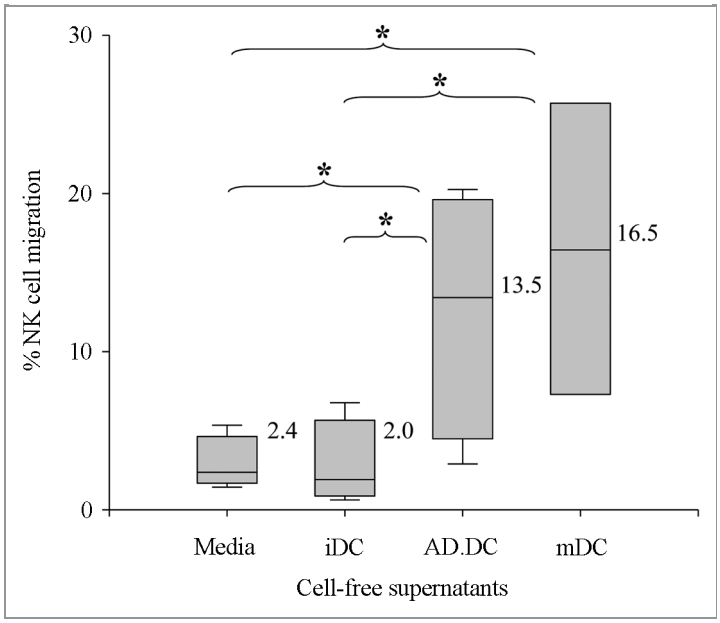

Figure 2. Ad.DC induce chemotaxis of NK cells in vitro. NK-cell migration induced by control media (Media) or iDC, Ad.DC or mDC supernatants was measured by FACS. Box-plot analysis represents distribution of percentages of NK cells that migrated from the insert into the chamber in four independent experiments. Median values are shown. * = $p < 0.05$.

tissues, and are believed to be mobilized by different chemokines. We assessed whether Ad.DC and mDC-conditioned media could mobilize both NK-cell subsets. Flow cytometric analysis showed that both $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK-cell subsets migrated toward Ad.DC and mDC supernatants, and that a similar population distribution was observed before and after NK-cell migration (**Fig. 3**). Analysis of purified NK cells prior to migration confirmed that predominantly $CD56^{hi}CD16^{-}$ cells expressed CD27 (present on mature and lymph node-infiltrating NK cells) (**Fig. S3A**).[33,34] We also confirmed that both NK-cell subsets expressed NKG2D,[35] NKp30 and NKp46[36,37] (**Fig. S3B, C and D**), but not NKp44[38] (data not shown). While NKG2D expression was consistently found at similar levels on $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells (**Fig. S3B**), NKp30 was expressed at higher levels on $CD56^{lo}CD16^{+}$ and NKp46 was expressed at higher levels on $CD56^{hi}CD16^{-}$ NK cells (**Fig. S3C and D**). The patterns of expression of all examined NK receptors remained similar on NK-cell subsets that migrated toward Ad.DC supernatant (**Fig. S3A, B, C and D**). These data demonstrate that Ad.DC are capable of recruiting both major subsets of NK cells.

Ad.DC produce NK-cell-recruiting chemokines. Our findings that Ad.DC-conditioned media induce migration of both major subsets of NK cells indicate that Ad.DC may produce chemokines capable of mobilizing the NK cells. Cell-free supernatants from iDC, Ad.DC and mDC were collected 24 h after treatment and analyzed by Luminex and ELISA for nine chemokines that have been described to play a role in NK-cell recruitment: CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL19/MIP-3β, CCL21/6Ckine, CXCL8/IL-8, CXCL9/MIG and CXCL10/IP-10.[19,28,29] Of the chemokines

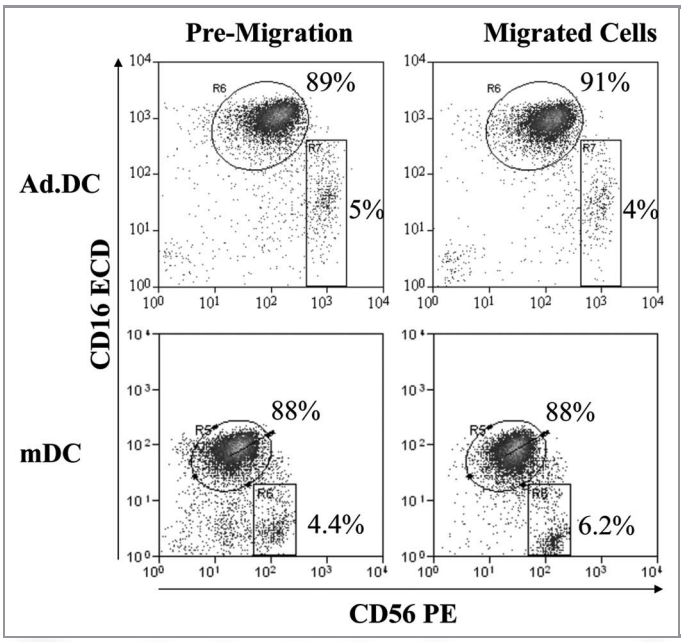

Figure 3. Ad.DC and mDC induce chemotaxis of both CD56^{lo}CD16^{+} and CD56^{hi}CD16^{-} NK cells. NK cells were evaluated for their ability to migrate in response to Ad.DC or mDC supernatant. Cells that migrated into the lower chamber (Migrated Cells) were harvested and their CD56/CD16 co-expression was examined and compared with that of NK cells prior to migration (Pre-Migration) by FACS. Percentages of positive cells relative to the whole NK cell population are presented. The results shown are representative of two experiments.

tested, only CCL21/6Ckine was not produced by tested DC types. Compared with iDC, Ad.DC produced increased levels of all chemokines except for CXCL9/MIG, which was only produced by IFN-γ/LPS-mDC (**Fig. 4**). mDC produced the highest level of all chemokines. We additionally showed that not only transfected with AdV encoding for β-galactosidase DC but also Ad.DC.Luc produced CXCL8/IL-8 at high levels, suggesting that the effects we report are AdV-mediated and not transgene-mediated (**Fig. S4**). These data indicate that AdV transduction induces a unique chemokine profile in DC that mostly quantitatively differs from that of mDC, which allows Ad.DC to effectively recruit both major subsets of NK cells.

NK-cell subsets differentially express chemokine receptors associated with NK-cell chemotaxis. Because resting NK cells were actively recruited toward chemokine-rich Ad.DC and mDC supernatants, we examined whether resting NK cells express corresponding chemokine receptors to the secreted ligands. We found that CCR7, CXCR1 and CXCR3 were consistently expressed on CD56^{lo}CD16^{+} NK cells, CCR7 and CXCR3 were expressed on CD56^{hi}CD16^{-} NK cells, while CCR2, CCR3, CCR4, and CCR5 were solely expressed on the CD56^{lo}CD16^{-} NK cells (**Table 1**; **Fig. S5**). CD56^{lo}CD16^{-} NK cells were the minor NK-cell population, representing $<$ 5% of the total NK-cell population in more than 90% of tested donors. (**Fig. 3**). Therefore, we focused our study on CD56^{lo}CD16^{+} and CD56^{hi}CD16^{-} NK cells.

Expression levels of CCR7, CXCR1, and CXCR3 on CD56^{lo}CD16^{+} and CD56^{hi}CD16^{-} NK cells were differentially modulated by different DC. CCR7, CXCR1, and CXCR3 expression levels were increasingly downregulated on both NK-cell subsets following co-incubation with iDC, Ad.DC, and mDC, respectively (**Fig. 5A, B and C**). Such increasing receptor downregulation suggested that the receptors were engaging increasing levels of respective ligands produced by iDC, Ad.DC and mDC, which in turn induced increasing receptor-ligand complex internalization.[39-41] These results suggested that CCR7, CXCR1, and CXCR3 were actively involved in NK cell recruitment by Ad.DC and mDC.

Ad.DC rapidly secrete biologically significant quantities of CXCL8/IL-8 and CXCL10/IP-10. We found in Ad.DC-conditioned media CCL19/MIP-3β, CXCL8/IL-8 and CXCL10/IP-10 chemokines, whose respective receptors CCR7, CXCR1 and CXCR3 were consistently expressed on resting NK cells. Therefore, Ad.DC might utilize CCL19/MIP-3β, CXCL8/IL-8 and CXCL10/IP-10 to attract resting NK cells. For a chemokine to be able to efficiently attract one cell type, it should be produced rapidly and in relatively large quantities by the other cell type. We examined the quantities and kinetics of CCL19/MIP-3β, CXCL8/IL-8 and CXCL10/IP-10 secreted by Ad.DC. Ad.DC started to secrete CXCL8/IL-8 after 3h, CXCL10/IP-10 after 6 h and CCL19/MIP-3β after 12 h following AdV transduction. CXCL8/IL-8 and CCL19/MIP-3β were increasingly secreted up to 48 h, while CXCL10/IP-10 up to 24 h (**Fig. 5D**). These data indicate that Ad.DC produced high, moderate and low levels of CXCL8/IL-8, CXCL10/IP-10 and, CCL19/MIP-3β, respectively. To determine the levels of the chemokines produced by Ad.DC, iDC and mDC, we evaluated cell culture-conditioned media of these DC obtained from a large cohort of healthy donors for the quantities of secreted CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β (**Fig. 5E**). We found that Ad.DC produced high (1,400 pg/ml), moderate (240 pg/ml), and low (34 pg/ml) median levels of CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β, respectively. Furthermore, Ad.DC always produced these chemokines more than iDC, but less than mDC. Overall, these results show that Ad.DC produce CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β rapidly and at increasing quantities during an extended period of time, but only produce CXCL8/IL-8 and CXCL10/IP-10 in significant quantities.

Ad.DC recruit NK cells via CXCL8/IL-8 and CXCL10/IP-10. We next tested whether CXCL8/IL-8 and CXCL10/IP-10 in Ad.DC or mDC culture-conditioned media mediate the observed NK-cell migration. Anti-CXCL10/IP-10 and anti-CXCL8/IL-8 antibodies inhibited 23% and 71%, Ad.DC culture-conditioned media-induced NK-cell migration, respectively, compared with the isotype control antibody (**Fig. 6**). Combined anti-CXCL10/IP-10 and anti-CXCL8/IL-8 caused complete inhibition of NK-cell migration (**Fig. 6A**). Similarly, CXCL10/IP-10 and CXCL8/IL-8 blockades inhibited 16% and 60% of mDC culture-conditioned media-induced NK cell migration, respectively, indicating that mDC also induced NK-cell chemotaxis mostly via CXCL10/IP-10 and CXCL8/IL-8. Combined antibody block did not inhibit NK-cell migration as completely (85.1%) as for

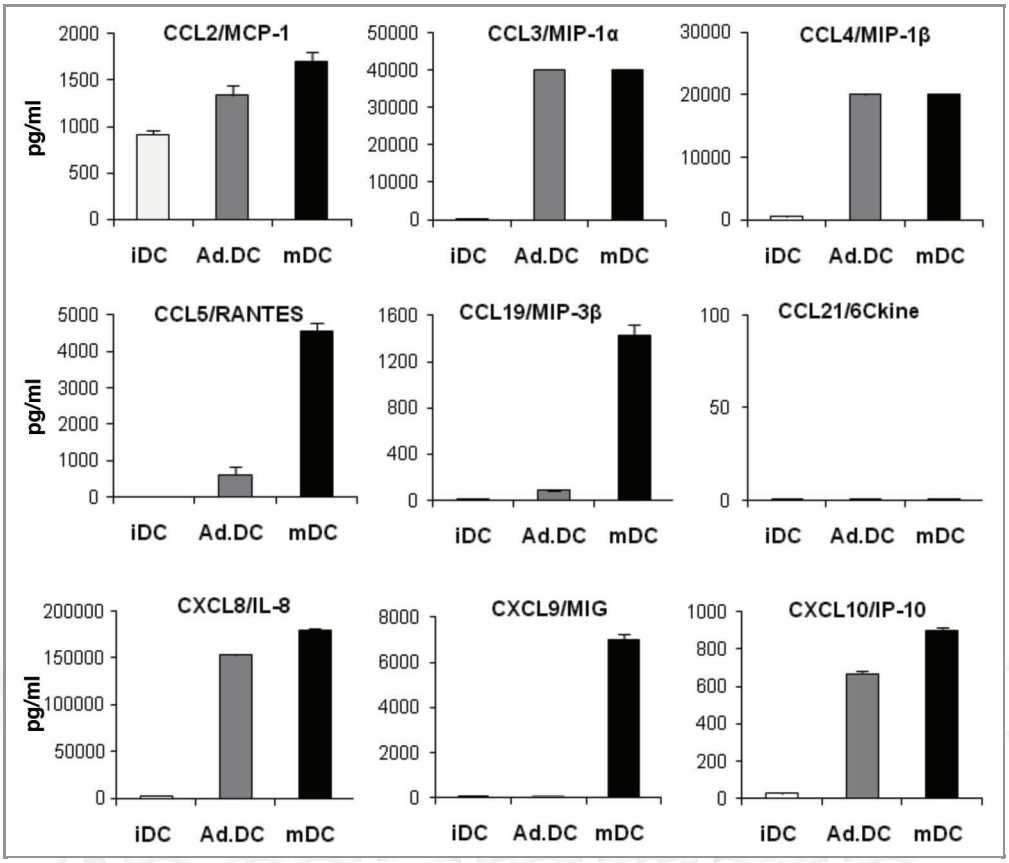

Figure 4. Ad.DC secrete chemokines that mediate NK-cell chemotaxis. iDC, Ad.DC and mDC were plated at 10^6 cells/ml. Cell-free supernatants were collected from DC cultures and tested for CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL19/MIP-3β, CCL21/6Ckine, CXCL8/IP-10, CXCL9/MIG and CXCL10/IP-10 by Luminex and ELISA assays. The results shown are representative of seven experiments.

Ad.DC, suggesting that other chemokines (e.g., CXCL9/MIG, CCL19/MIP-3β) secreted by mDC may also play a role in NK-cell recruitment (**Fig. S6**).

Since CXCL8/IL-8 was the dominant Ad.DC-secreted NK-cell chemo-attractant, which induced migration of the majority of peripheral blood NK cells in vitro, we evaluated whether it had the same importance in vivo. We performed CXCL8/IL-8 blocking experiments of Ad.DC.Luc-induced NK cell migration in vivo. In IgG control-treated mice, we observed NK-cell migration in 6/7 tests [median distance changes of 11.9% (proximal edge) and 6.15% (focal point) between NK-cell and Ad.DC.Luc signals], and only in 1/7 anti-CXCL8/IL-8-treated mice (**Fig. 6B**). These data show that CXCL8/IL-8 plays a major role in the recruitment of NK cells by Ad.DC in vitro and in vivo.

Table 1. Chemokine receptors tested on circulating NK cells by FACS

		Receptor expression on:		
Ligand	**Receptor**	**$CD56^{lo}CD16^{+}$**	**$CD56^{hi}CD16^{-}$**	**$CD56^{lo}CD16^{-}$**
CCL2/MCP-1	CCR2	-	-	+
CCL5/RANTES	CCR3	-	-	++
CCL4/MIP-1β, CCL2, CCL5	CCR4	-	-	+
CCL3/MIP-1α, CCL4, CCL5	CCR5	-	-	+
CCL19/MIP-3β, CCL21/6Ckine	CCR7	++	+	+++
CXCL8/IL-8	CXCR1	++	-	++
CXCL9/MIG, CXCL10/IP-10	CXCR3	++	+++	++

-, 0–2%; +, 2–25%; ++, 25–50%; +++, > 50%.

CXCL8/IL-8 and CXCL10/IP-10 preferentially recruit $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells, respectively. To evaluate which NK-cell subsets were recruited by which Ad.DC-secreted chemokines, titrating doses of recombinant human CXCL10/IP-10 and CXCL8/IL-8 were tested for the ability to induce migration of freshly isolated, resting $CD56^{hi}CD16^{-}$ and $CD56^{lo}CD16^{+}$ NK cells (**Fig. S7A**). At doses of 500 pg/ml or greater, CXCL8/IL-8 induced specific migration of $CD56^{lo}CD16^{+}$ NK cells, while 5,000 pg/ml of CXCL10/IP-10 induced specific migration of $CD56^{hi}CD16^{-}$ NK cells as shown by specific percentage increases in the post-migration distribution of NK cell subsets (**Fig. S7A**). The ability of $CD56^{hi}CD16^{-}$ NK cells to migrate in response to CXCL10/IP-10, but not CXCL8/IL-8,

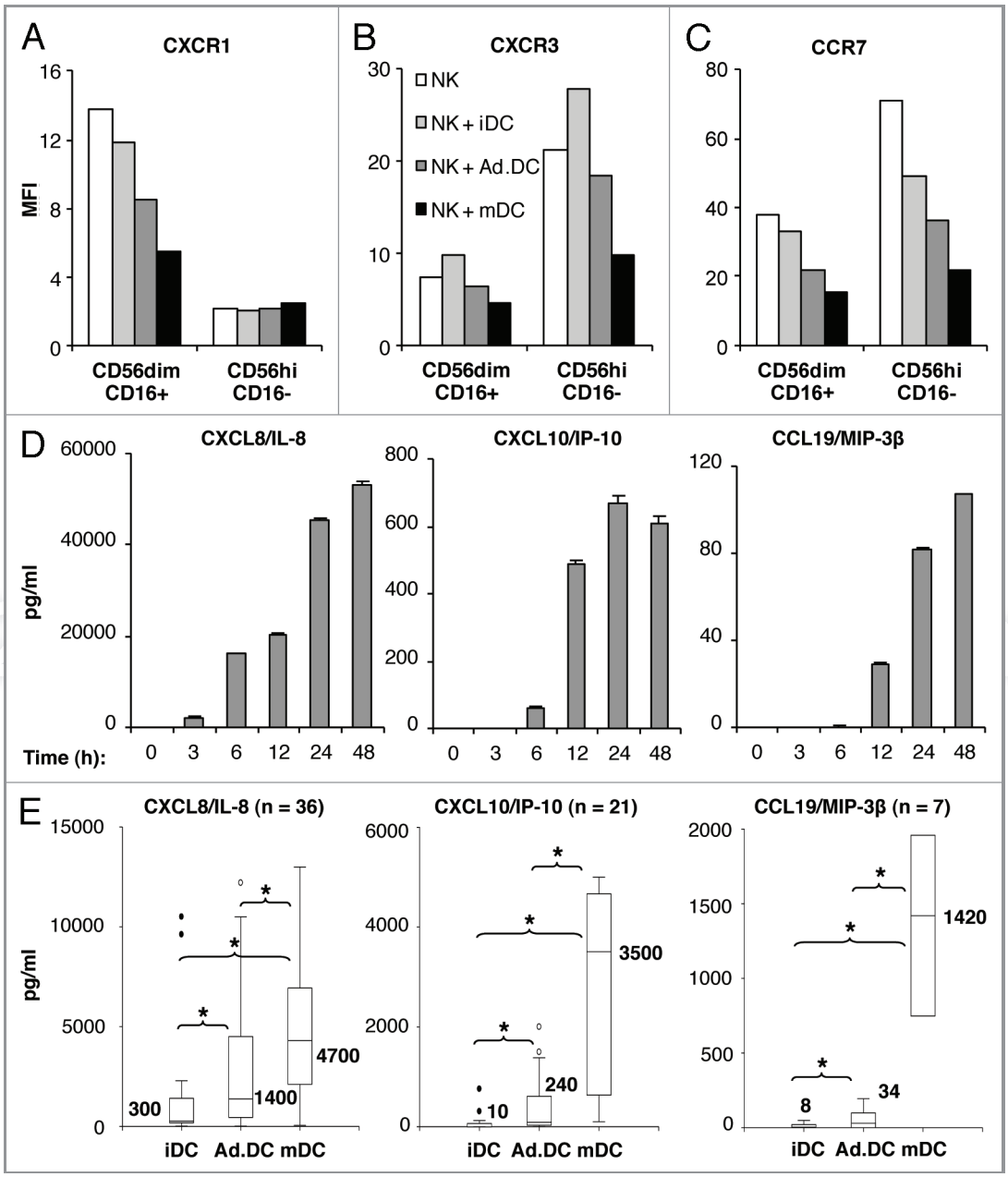

Figure 5. Expression levels of CCR7, CXCR1 and CXCR3 chemokine receptors on NK cells are decreased following co-culture with DC and Ad.DC secrete significant amounts of CXCL8/IL-8 and CXCL10/IP-10 but not CCL19/MIP-3β. NK cells were cultured alone (NK) or with iDC, Ad.DC or mDC (NK + iDC, NK + Ad.DC, and NK + mDC, respectively) for 24 h. Afterwards, (A) CXCR1, (B) CXCR3 and (C) CCR7 expression levels were measured on harvested $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells using FACS. The results shown are MFI and representative of three experiments. (D) Time courses of CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β secretion by Ad.DC were measured. (E) CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β secreted in iDC, Ad.DC and mDC-conditioned media were measured. For all tests, 10^6 DC/ml were plated and cultured. Cell culture-conditioned media were collected at different time points between 3 h and 48 h (D), or at 24 h (E) of DC cultures, and tested for CXCL8/IL-8, CXCL10/IP-10 and CCL19/MIP-3β by Luminex and ELISA assays. (D) Results shown are pg/ml and representative of three experiments. (E) Data are box-plots, medians and ranges of 7 to 36 experiments. n = number of donors tested. * = $p < 0.05$.

was additionally confirmed by using highly enriched $CD16^{-}$ NK cells (**Fig. S7B and C**). Together, these data suggest that Ad.DC produce significant amounts of biologically active CXCL8/IL-8 and CXCL10/IP-10 capable of recruiting $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells, respectively.

Discussion

DC transduced with tumor-associated antigen-encoding AdV efficiently stimulate antigen-specific adaptive $CD8^{+}$ and $CD4^{+}$ T-cell responses in vitro and in cancer patients,[12,15,42] and

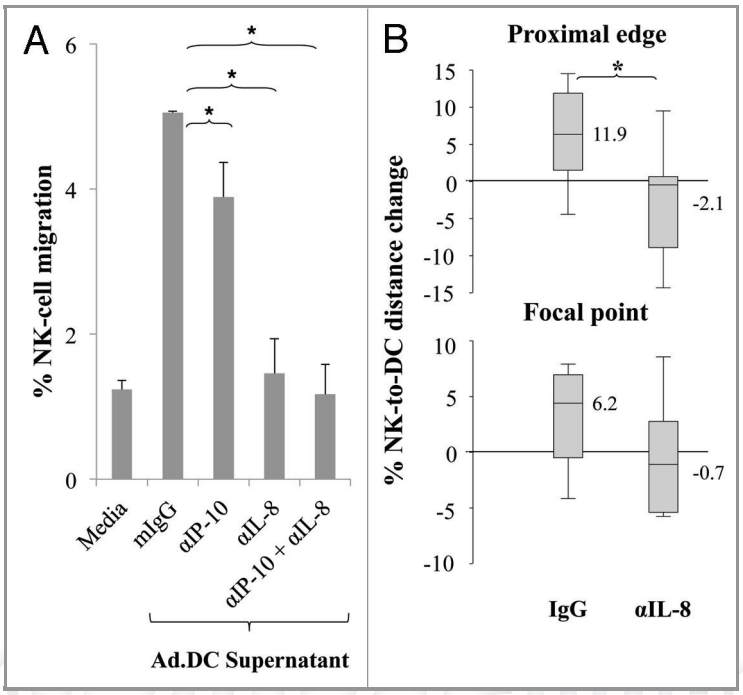

Figure 6. Ad.DC can recruit NK cells via CXCL8/IL-8 and CXCL10/IP-10. (A) Chemotaxis assay using Ad.DC supernatants and NK cells was performed as described in **Figure 2**. Control wells contained media only (Media). Specific chemokines found in Ad.DC supernatants were blocked with isotype control IgG (mIgG), anti-CXCL10/IP-10 (αIP-10), anti-CXCL8/IL-8 (αIL-8) and joint anti-CXCL10/IP-10 and anti-CXCL8/IL-8 (αIP-10 + IL-8) antibodies. Values represent means + SEM of percentages of NK cells that migrated of duplicate tests. Data are representative of seven experiments. (B) Ad.DC-induced migration of NK cells was assessed in mice following intraperitoneal injections of anti-CXCL8/IL-8 (αIL-8) or isotype control (IgG) antibodies. Four experiments were performed using eight mice. Six mice had duplicate tests in both flanks, and in total seven tests were performed for each antibody group. Migration was quantified by measuring the distance between Ad.DC signal focal point to the proximal edge and focal point of an NK-cell signal. Data were collected and standardized as described in **Figure 1**. Box-plots represent data distribution of all seven iDC and Ad.DC tests performed. * = $p < 0.05$.

represent a promising cancer vaccine.[11,12] Ad.DC also effectively stimulate innate immunity by activating NK cells and amplifying their type-1 immune functions and antitumor activities via tmTNF and *trans*-IL-15.[16] This contact-mediated Ad.DC-NK cell crosstalk may be also critical for activation and polarization of adaptive immune responses, and therefore effectiveness of Ad.DC-based vaccines. Here we examine whether Ad.DC can recruit NK cells, an essential first step that could lead to Ad.DC-NK cell contact, interaction and activation in vivo. We demonstrate for the first time that Ad.DC can induce migration of spatially distant NK cells, which could lead to Ad.DC-NK cell engagement in a subcutaneous vaccine site. Furthermore, we show that Ad.DC secrete a number of chemokines and effectively induce chemotaxis of major resting NK-cell subsets via different chemokines in vitro.

DC and NK cells are present in separate tissue compartments in the skin, and they normally do not form cell-to-cell contacts and do not crosstalk. However, under inflammatory conditions such as atopic dermatitis and autoimmune disease of the skin (psoriasis, lichen planus and pemphigus vulgaris) NK cells are recruited in large numbers to skin lesions[43] and could infrequently be seen in direct contact with DCs.[44,45] NK cells are found to deeply penetrate non-vascularized epidermis which contains Langerhans cells, demonstrating their ability to move and migrate through dense, solid tissue toward an inflamed locus containing DC.[45] Therefore, the recruitment of NK cells to an inflamed skin lesion can occur from both the peripheral blood and the dermal pool of NK cells. Ad.DC vaccine could be regarded as a rich source of proinflammatory cytokines and chemokines that can instantly create an inflammatory environment at the injection site. Our demonstration that Ad.DC can induce migration of NK cells in hypodermis suggests that an Ad.DC vaccine is capable of efficiently attracting and, likely interacting with NK cells from the neighboring dermis and peripheral blood.

We show that AdV transduction of DC leads to early and sustained production of several chemokines associated with NK-cell migration, including CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL19/MIP-3β, CXCL8/IL-8, and CXCL10/IP-10.[17-19,29] Among these chemokines, CXCL8/IL-8 and CXCL10/IP-10 induce significant chemotaxis of peripheral blood NK cells. As $CD56^{lo}CD16^{+}$ NK cells selectively express CXCR1, the CXCL8/IL-8 receptor, both Ad.DC-conditioned media and recombinant CXCL8/IL-8 induce chemotaxis of $CD56^{lo}CD16^{+}$ NK cells. On the other hand, as $CD56^{hi}CD16^{-}$ NK cells preferentially express CXCR3, the CXCL010/IP-10 receptor, both Ad.DC-conditioned media and recombinant CXCL010/IP-10 recruit $CD56^{hi}CD16^{-}$ NK cells. Of the chemokines analyzed, only production of CXCL9/MIG is restricted to LPS/IFNγ mDCs. These data support previous observations that CXCL9/MIG is an IFNγ-inducible chemokine.[46]

Another chemokine found to be produced by Ad.DC, which possibly could play a role in the recruitment of NK cells, is CCL19/MIP-3β. This chemokine is produced by Ad.DC at likely insufficient quantities to induce chemotaxis in vitro. However, we find that CCR7 (CCL19/MIP-3β receptor) expression undergoes downregulation in NK cell-DC co-cultures, suggesting that the receptor might engage its respective ligand, and as a consequence, the receptor-ligand complex is internalized. This could mean that, in close proximity, Ad.DC-produced CCL19/MIP-3β could trigger CCR7. However, because of its low levels of secretion, the role of the CCL19/MIP-3β chemokine in Ad.DC-induced recruitment of NK cells remains questionable.

DC play a crucial role in initiating the innate immune response in the periphery. DC express pattern-recognition receptors, such as Toll-like receptors (TLR), which recognize conserved pathogen-associated molecular patterns. TLR ligation and activation induce a genetic program that is critical for host defense, including the induction of certain chemokines. It has been previously shown that CXCL10/IP-10 is preferentially induced by the TLR4 agonist *Escherichia coli* LPS, while CXCL8/IL-8

is preferentially induced by the TLR2-specific agonists *Staphylococcus aureus* peptidoglycans and yeast zymosan.[29] Here we report that both of these chemokines, as well as a number of other chemokines, are induced by AdV infection, and LPS/IFNγ stimulation. To date, no AdV-associated TLR ligand has been identified, and the exact mechanism of AdV induction of DC maturation and subsequent NK-cell crosstalk is unknown. Previous studies have indicated that AdV induction of DC maturation and upregulation of antigen presenting machinery is dependent on AdV structures.[13,47,48] Therefore, viral structures could be also responsible for AdV induction of chemokine secretion by DC.

The effectiveness of Ad.DC vaccines might ultimately depend on their ability to produce chemokines, and inflammatory and immunoregulatory cytokines for extended periods of time, allowing Ad.DC to recruit, engage, and activate NK and T cells. Our in vitro data demonstrate that CXCL8/IL-8 and CXCL10/IP-10, as well as CCL19/MIP-3β, are secreted at the highest levels during the initial 24 h after AdV transduction. These data are substantiated by our in vivo experiments, which suggest that the initial 24 h post AdV infection are critical for the recruitment of NK cells, and likely other lymphocytes. Simultaneous secretion of the proinflammatory chemokine CXCL8/IL-8 and immunoregulatory chemokines CXCL10/IP-10 and CCL19/MIP-3β by Ad.DC vaccine in situ could recruit and stimulate both cytotoxic $CD56^{lo}CD16^{+}$ and immunoregulatory $CD56^{hi}CD16^{-}$ NK cells. However, as dermal NK cells are $CD56^{hi}CD16^{-}$ and spatially the closest NK cells to a subcutaneous vaccine,[31] these NK cells would be the first and likely the most numerous NK cells to be recruited by a subcutaneous Ad.DC vaccine. These NK cells could, via TNF and IFNγ secretion, further promote AdV-induced DC maturation and resistance to NK-cell mediated killing, and their ability to migrate to draining lymph nodes. $CD56^{lo}CD16^{+}$ NK cells could also be recruited from the peripheral blood to the immunization site, and, after activation by Ad.DC, they could eliminate remaining immature Ad.DC. Following their full maturation at the vaccination site, Ad.DC could migrate to a draining lymph node, where they could recruit and interact with the resident immunoregulatory $CD56^{hi}CD16^{-}$ NK cells,[49] and induce their activation and Th1 polarization, and consequently mediate a robust and appropriate adaptive T-cell response.[50] These possibilities remain to be tested, and the findings would have direct biological relevance and clinical application. They could suggest that chemokine production and NK-cell recruitment could be indications of an Ad.DC vaccine potency.[12]

In conclusion, we show that Ad.DC effectively induce migration of NK cells in vivo, and chemotaxis of major NK-cell subsets in vitro. The chemotaxis is mediated by specific chemokines produced by Ad.DC. These functions of AdV.DC coupled with their ability to crosstalk with, activate and polarize NK cells, make them potent activators of innate immunity. The study also newly defines CXCL8/IL-8 as a crucial chemokine by which DC recruit $CD56^{lo}CD16^{+}$ NK cells. As Ad.DC also potently stimulate polyclonal anti-tumor T-cell responses, they represent a highly potent vaccine, which simultaneously induces activation, polarization and bridging of innate and adaptive immunity, and consequent mounting of enhanced anti-tumor immune functions.

Materials and Methods

Reagents. NK-cell phenotype was evaluated using fluorochrome-conjugated antibodies against CD69-FITC, CD27-FITC, CD56-PE (BD PharMingen, San Jose, CA), CD3-APC, NKG2D-PE, NKp30-PE, NKp44-PE, NKp46-PE, CD16-ECD (Beckman Coulter, Fullerton, CA), CXCR1-PE, CCR1-PE, CCR2-PE, CCR5-PE (R&D Systems, Minneapolis, MN), CCR3-PE, CCR4-PE, CCR6-PE, CCR7-PE-Cy7, and CXCR3-PE (BD PharMingen). DC phenotype was tested as described.[16] Neutralization of chemokines in DC supernatants and in vivo was performed with blocking antibodies specific for human CXCL8/IL-8, and CXCL10/IP-10. Mouse IgG_1 isotype non-reactive monoclonal antibody (R&D) was used as a negative control. Recombinant human CXCL8/IL-8 and CXCL10/IP-10 (R&D) were used for chemotaxis assays.

Recombinant AdV *vectors*. AdV vectors encoding *E. coli* β-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) were obtained from the University of Pittsburgh Vector Core Facility. Ad.LacZ and Ad.Luc are E1/E3-deleted and replication-defective. Multiplicity of infection (MOI) is calculated based on plaque forming units (pfu) per ml.

Mice. Eight week old T, B and NK-cell deficient NS.IL2Rγ$^{-/-}$ and B6A female mice were obtained from the Jackson Laboratory. Mice were housed at the University of Pittsburgh Cancer Institute's AAALAC-accredited animal facility. Animal studies were performed in accordance with a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Isolation of peripheral blood mononuclear cells (PBMC). Peripheral blood was obtained from 50 healthy donors with their written consent, under an IRB-approved protocol (UPCI 04-001). PBMC were separated from blood using Ficoll-Hypaque gradient centrifugation (Cellgro; Mediatech, Inc.).

DC preparation for in vitro experiments. DC were generated from purified human peripheral blood $CD14^{+}$ monocytes as described.[16] For chemotaxis experiments, iDC were resuspended in RPMI 1640 media (Invitrogen) supplemented with 0.5% bovine serum albumin (Fisher Scientific) and were either untreated (iDC), transduced with Ad.LacZ or Ad.Luc vectors using 500 MOI (Ad.DC) or matured with IFN-γ (1,000 IU/ml; PeproTech, Inc.) and LPS (250 ng/ml; Sigma) (mDC). DC were incubated at 37°C for an additional 24–48 h. Supernatants were tested for chemokines, and chemotaxis activity.

NK-cell isolation. NK cells were purified from freshly-isolated PBMC by negative MACS selection using the human NK-cell isolation kit (Miltenyi Biotech), according to the manufacturer's protocol. $CD16^{-}$ NK cells were obtained by negative selection of purified NK cells using anti-human CD16 microbeads (Miltenyi Biotech). For chemotaxis experiments, NK cells were resuspended in RPMI 1640 media supplemented with 0.5% bovine serum albumin at a concentration of 2.5×10^{6} cells/ml.

***In vivo* NK-cell migration test.** Test was performed in NS.IL2Rγ$^{-/-}$ and B6A mice. iDC were either labeled with VivoTag® 680 XL (PerkinElmer) NHS ester NIR fluorochrome or transduced with Ad.Luc vector using 4,000 MOI for 3 h in AIM-V media. Autologous NK cells were freshly isolated, and labeled with VivoTag-S™ 750 fluorochrome (PerkinElmer). After labeling, DC and NK cells were resuspended in ice cold PBS (16.5–33 × 10^6/ml). B6A or NS.IL2Rγ$^{-/-}$ mice were anesthetized with Isoflurane (Wester Veterinary; Sterling, MA), shaved, and their skin was labeled with a Fisherbrand Marking Pen (Fisher Scientific) for guided injections and data analyses. Thirty microliters of the DC and NK-cell suspensions were injected subcutaneously into a shaved mouse back, parallel to the spine, at a mutual distance of 0.5–1 cm. For chemokine-blocking experiments, anti-CXCL8/IL-8 or isotype control antibodies were injected intraperitoneally (R&D; 100 μg/mouse) prior to cell injections. Animals were examined using the small animal in vivo imaging system (IVIS; IVIS® 200 Series) immediately after cell injections (0 h), as well as 24 h, 48 h and 72 h later. Prior to IVIS analysis, mice were intraperitoneally injected with D-luciferin potassium salt solution (200 μl/mouse of 30 mg/ml stock solution; Gold Biotechnology; St. Louis, MO). Ten minutes following the injection, the luciferin/luciferase reaction occurring in Ad.Luc-transduced DC, and resulting bioluminescence was measured using the IVIS. Subsequently, iDC and NK cell fluorescence signals were analyzed using the Cy5.5 and ICG channels, respectively. Images were obtained and analyzed with Living Image® v4 program (Caliper Life Sciences, Inc.). DC and NK-cell image overlays were constructed using the Adobe Photoshop v6.0 (Adobe Systems Inc.). To precisely measure NK-cell migration, a 20x20 mm grid was constructed, and, with the guidance of drawn skin landmarks, placed over a DC-NK cell image overlay using Microsoft Office PowerPoint 2007. A red quadrant marked the starting NK-cell location at 0 h. The same grid, with the red quadrant, was carried over to the 24 h image, and positioned the same way as for the 0 h overlay using the guiding skin labels (**Fig. S1**). NK-cell migration was examined by measuring the distances between the DC signal focus (point of highest bioluminescence/fluorescence intensity) and the proximal edge, focus, and distal edge of an NK-cell signal at 0 h and 24 h post-injection using digital calipers. The data were standardized by calculating the percent change in the DC-NK-cell distance. Migration was considered positive if we observed at 24 h more than 5% decreases of the 0 h distances between DC and NK cells by at least one of the three measurements.

***In vitro* NK-cell chemotaxis test.** Experiments were performed in 24-well plates with 5 μm-pore size polyester membrane inserts (Corning Inc.). Wells were filled with 500 μl of DC supernatants or RPMI 1640 media supplemented with 0.5% bovine serum albumin and recombinant chemokines at indicated concentrations. Control wells were filled with media only. To neutralize specific chemokines, anti-CXCL8/IL-8, CXCL10/IP-10, CCL2/MCP-1 or corresponding isotype control antibodies (R&D, 20 μg/ml) were added to Ad.DC or mDC supernatants. 2.5 × 10^5 of allogeneic NK cells were seeded in 100 μl of media in the transwell insert. After 90 min of incubation, NK cells that migrated into the well were harvested. Their numbers and phenotype were analyzed by FACS. Migration was presented as a percent of NK cells that migrated from the insert into the well. All tests were performed in duplicates and data are means ± standard error.

Cell phenotype analysis. One step staining of cellular antigens was performed using fluorochrome-conjugated primary antibodies as previously described.[13,16]

DC/NK-cell coculture. Autologous DC and NK cells were cocultured as previously described.[16]

Chemokine measurement. DC culture supernatants were assessed for CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL8/IL-8, CXCL9/MIG and CXCL10/IP-10 by Luminex arrays (Biosource, Immunologic Monitoring Lab); and ELISA for CCL19/MIP-3β and CCL21/6Ckine (R&D).

Statistical analysis. One-sided paired t-test analyses were used to estimate statistical significance of differences of obtained chemokine secretion and NK-cell migration data. p values ≤ 0.05 were considered to be statistically significant. In vivo NK-cell migration and the distribution of the chemokine amounts produced were analyzed using box-plots constructed with NCSS 2000 Statistical Software (Kaysville, UT).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/19788/

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