Adenoviral vectors transduce alveolar macrophages in lung cancer models

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ABSTRACT

Adenoviral vectors expressing Cre recombinase are commonly used to initiate tumor formation in murine lung cancer models. While these vectors are designed to target genetic recombination to lung epithelial cells, adenoviruses can infect additional cell types that potentially influence tumor development. Our goal was to explore the consequences of adenoviral-mediated alveolar macrophage (AM) transduction in a Kras-initiated lung tumor model. As expected, treatment of animals harboring the Kras<sup>LSL-G12D</sup> allele and an inducible green fluorescence protein (GFP) tracking allele with an adenoviral vector expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad5-CMV-Cre), caused GFP-positive lung adenocarcinomas. Surprisingly, however, up to 70% of the total GFP<sup>+ </sup>cells were AM, and AM could be detected 6 months after tumor initiation, and transduced AM demonstrated Kras activation and increased proliferation. In contrast, recombination was not detected in other immune cell populations and AM recombination could be eliminated by tumor initiation with an adenovirus expressing Cre recombinase under the control of the surfactant protein C (SPC) promoter. In addition, AM isolated from Kras<sup>LSL-G12D</sup> animals and transduced by Ad5-CMV-Cre displayed prolonged survival in vitro and increased the growth of murine lung adenocarcinoma CMT/167 cells when co-injected in an orthotopic flank model. Given the importance of the immune system in tumor development and progression, inadvertent AM transduction by Ad5-CMV-Cre merits careful consideration during lung cancer model selection particularly if studies evaluating the tumor-immune interactions are planned.

Introduction

Animal models are critical to understanding basic cancer biology and testing potential therapeutic approaches in vivo. With the advent of immunotherapy, models that accurately recapitulate tumor-immune interactions are critical to understanding basic mechanisms of action of these agents and developing rational combinations of immunotherapeutic drugs with other treatment modalities. One of the most common methods of inducing lung tumor formation involves intratracheal or intranasal delivery of adenovirus that expresses Cre recombinase into animals harboring Cre-inducible oncogenes and/or alleles that allow the conditional deletion of tumor suppressors. This approach has been used to generate animal models of all the major lung cancer subtypes. While these models clearly produce epithelial derived carcinomas, adenoviruses can also transduce other cell types including macrophages, which can potentially affect both tumor growth and the immune tumor microenvironment (TME).

Alveolar macrophages (AM) are resident lung macrophages that are an important component of the innate immune system. Tumor associated macrophages (TAMs) can be derived from AM or from recruited circulating monocytes; these populations may play distinct roles during lung tumorogenesis. The role of TAMs and macrophage polarization in lung cancer is increasing appreciated. In human lung cancer an increased proportion of alternatively activated macrophages is associated with worse prognosis, while in animal models macrophage...
depletion protects against urethane-induced carcinogenesis, orthotopic lung tumor formation, and lung metastasis.\textsuperscript{9,11}

In the process of evaluating the immune TME in a well-established lung adenocarcinoma model, we found that while adenovirus expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad5-CMV-Cre) induced lung tumor formation in Kras\textsuperscript{LSL-G12D} mice, it also transduced a substantial fraction of AM. This was not seen when tumors were initiated with adenovirus expressing Cre recombinase under the control of the surfactant protein C (SPC) promoter (Ad5-SPC-Cre) which limits Cre recombinase expression to type II alveolar epithelial cells. That Ad5-CMV-Cre-transduced macrophages have prolonged survival \textit{in vitro} and accelerate the growth of murine lung cancer cells \textit{in vivo}, suggests that oncogene activation in immune cells affects tumor growth and illustrates the importance of model system selection for studies of the immune TME.

**Results**

We initiated lung tumor formation in Kras\textsuperscript{LSL-G12D} mice using Ad5-CMV-Cre or Ad5-SPC-Cre, mixed 1:1 with murine CMT/167 cells, or intrapulmonary Ad5-SPC-Cre, respectively (Fig. 3A). Although all animals harbored GFP+ tumors (see Fig. 1), flow cytometry demonstrated that a large fraction of GFP+ cells in Ad5-CMV-Cre treated animals were SiglecF\textsuperscript{+}/CD11c\textsuperscript{+} AM (73% with intratracheal instillation and 44% with intrapulmonary injection, Fig. 3B). In contrast, no GFP+ CD11b\textsuperscript{+}/Ly6G\textsuperscript{+} neutrophils or GFP+ CD3\textsuperscript{+} T cells were observed (Fig. 3B). Interestingly, no GFP+ immune cells were detected after treatment with a virus harboring a promoter that restricts Cre recombinase expression to lung epithelial cells (Ad5-SPC-Cre) even though this virus initiates tumor formation in Kras\textsuperscript{LSL-G12D} mice and causes recombination at a level similar to Ad5-CMV-Cre when delivered by the same approach (Fig. 3A). When we compared the GFP+ populations of tumors initiated by direct injection of Ad5-CMV-Cre and Ad5-SPC-Cre, we confirmed that Ad5-CMV-Cre initiated tumor bearing lungs have a large population of GFP+ CD11c\textsuperscript{+} AM that is absent in Ad5-SPC-Cre initiated tumors (Fig. 3C).

When we evaluated the immune responses to tumors initiated by Ad5-CMV-Cre and Ad5-SPC-Cre we found that, similar to previous reports,\textsuperscript{15} tumors initiated by intratracheal Ad5-CMV-Cre demonstrated significantly more AM (Fig. 3D), however, this was less pronounced in tumors initiated by direct injection of virus into the left lung (Fig. 3D). Whether this is related to tumor burden, time from tumor initiation, or reduced adenoviral mediated transduction of AM is unclear. To investigate potential mechanisms of adenoviral mediated transduction, we isolated AM, neutrophils, T cells, and epithelial cells by flow cytometry and then assessed expression of the high affinity coxsackie/adenovirus receptor (CAR) by qPCR (Fig. 3E). That AM do not express CAR suggests that AM transduction is CAR-independent.

To further investigate the functional significance of adenoviral-mediated AM transduction, we harvested AM from Kras\textsuperscript{LSL-G12D}.mT/mG animals then transduced them \textit{ex vivo} with Ad5-CMV-Cre or Ad5-SPC-Cre. Consistent with our \textit{in vivo} observations, Ad5-CMV-Cre, but not Ad5-SPC-Cre, efficiently mediated genetic recombination in AM in a dose dependent manner (Fig. 4A). Ad5-CMV-Cre treated AM also demonstrated improved survival \textit{in vitro} compared to Ad5-SPC-Cre treated or untreated AM (Fig. 4B), potentially secondary to Kras\textsuperscript{G12D} activation. To assess the effect of Kras\textsuperscript{G12D} activation in AM on tumor growth, AM harvested from Kras\textsuperscript{LSL-G12D}.mT/mG mice were transduced \textit{ex vivo} with Ad5-CMV-Cre or Ad5-SPC-Cre, mixed 1:1 with murine CMT/167 cells and transplanted into the flanks of immunocompetent C57BL/6 hosts. In this system, Ad5-CMV-Cre treated AM from Kras\textsuperscript{LSL-G12D}.mT/mG mice increased the growth of CMT/167 flank tumors (Fig. 4C-D) suggesting that Kras\textsuperscript{G12D} activation in AM promotes tumor growth. In addition, rare GFP+ AM could be detected in these tumors 4 weeks after transplant (Fig. 4E).

To evaluate adenoviral-mediated AM transduction at time points shortly after viral transduction we assessed AM harvested by bronchoalveolar lavage (BAL) as this is a simple method to obtain \textasciitilde 95% AM.\textsuperscript{19} One week after intratracheal Ad5-CMV-Cre in Kras\textsuperscript{LSL-G12D}.mT/mG mice, \textasciitilde 37% of BAL...
AM expressed mGFP by direct fluorescence (Fig. 5A). At this time point, essentially all AM also expressed mTom, presumably from residual mTom expression, the degradation of which can vary in a tissue dependent manner.\textsuperscript{16} To determine whether other adenoviruses used to initiate lung tumor formation in mouse models are similarly capable of transducing macrophages, we examined the lungs of mice treated intratracheally with adenovirus expressing FLAG-tagged Cas9 and guide RNAs capable of mediating the echinoderm microtubule-associated protein like 4 (EML4)-anaplastic lymphoma kinase (ALK) gene rearrangement (Ad5-EA).\textsuperscript{20} Three days after Ad5-EA treatment, we could detect FLAG expression in a subset of CD107b\textsuperscript{+} AM (Fig. 5B), suggesting that Ad5-EA can also infect AM.

**Discussion**

Tracheal or intranasal Ad5-CMV-Cre is one of the most frequently used methods for initiating lung tumor formation in animals harboring inducible (“knock-in”) oncogenes...
and conditional ("floxed") tumor suppressor deletions and this approach has been used to create mouse models of all major lung cancer subtypes.1,2 Our studies clearly demonstrate that adenoviral vectors with strong ubiquitous promoters can also induce genetic recombination in AM and that this can impact both tumor growth (Fig. 4C-D) and the immune TME (Fig. 3D), presumably because these transduced AM can persist for months after tumor initiation (Figs. 1–3). While the precise consequences of inadvertent AM targeting on tumor growth and progression likely depend on the oncogenic context, these findings have significant implications for models where detailed studies of tumor-immune interactions are planned.

AM are derived from fetal monocytes early in development and have the capacity to proliferate both at baseline and in response to various stimuli.21-24 Our data suggest that KrasG12D activation promotes AM survival in vitro (Fig. 4B). That transduced AM can be detected months after Ad5-CMV-Cre exposure is consistent with the long life span and slow turnover of these cells25,26 and suggests that the transduced AM seen months after Ad5-CMV-Cre treatment were present at the time of viral exposure or were derived from these AM. While

Figure 2. Ad5-CMV-Cre is capable of transducing AM. (A) Immunostaining for CD107b (red) and GFP (green) in a KrasLSL-G12D.mT/mG animal after Ad5-CMV-Cre treatment demonstrating co-localization of GFP in a subset of CD107b+ AM. Arrows indicate GFP+/CD107b+ AM. In each panel isotype controls are shown as insets, the tumor margin is highlighted in yellow, and the scale bar is 100 μm. (B) Triple immunostaining for TTF-1 (red), GFP (green), and CD107b (blue) illustrating GFP+/TTF+ tumor cells and a GFP+/CD107b+ AM (white arrow). (C) Dual immunostaining for CD107b (red) and KrasG12D (green) illustrating KrasG12D expression in both tumor cells and CD107b+ AM. Arrows indicate KrasG12D+/CD107b+ AM. A KrasG12D- negative AM is shown at bottom left. (D) Triple immunostaining for CD107b (red), GFP (green), and PCNA (blue) showing PCNA staining in GFP+/CD107b+ AM (yellow cells with blue nuclei in right panel). Five sections from 4 Ad5-CMV-Cre initiated lung tumors were quantified. A tumor infiltrating macrophage is shown in the right panel (arrow at top right); additional images of tumor infiltrating macrophages are shown as Supplemental Fig. S3.
our in vivo experiments cannot eliminate the possibility that GFP+ AM have simply engulfed GFP+ tumor cells, our in vitro experiments showing that AM can be directly infected by adenovirus are highly consistent with prior reports, and would argue against this hypothesis. While we did not examine the effect of other oncogenic events in AM, it seems likely that the activation of other oncogenes or deletion of tumor suppressors would also alter AM behavior as well as the global immune TME.

In mouse adenocarcinoma models, macrophage numbers typically increase during tumorigenesis and increased immunosuppressive, M2 polarized macrophages are associated with tumor progression, however, the role of macrophage subsets may be complex with macrophage populations having distinct roles in tumor initiation and tumor progression. Consistent with previous reports, we saw increased macrophages in the tumor bearing lungs of Ad5-CMV-Cre treated KrasLSL-G12D mice, though this was less pronounced with direct injection of...
Ad5-CMV-Cre or Ad5-SPC-Cre (Fig. 3D). This suggests that increased macrophage numbers may be partially mediated by widespread adenoviral-induced Kras\textsuperscript{G12D} activation in AM after Ad5-CMV-Cre exposure (Fig. 3B) in addition to the well described pro-inflammatory effects of Kras activation in tumor epithelial cells.\textsuperscript{9,27,28}

Despite inconsistent reports of CAR expression on macrophages,\textsuperscript{3,29,30} adenoviral-mediated infection of human and mouse macrophages has been repeatedly described and ascribed to CAR-independent mechanisms.\textsuperscript{19,31-37} Although we were unable to detect significant CAR expression in AM (Fig. 3E), consistent with the notion that CAR does not mediate adenoviral transduction of murine AM, our observations are highly consistent with a prior report clearly demonstrating the rapid uptake of labeled adenovirus by murine AM in vivo.\textsuperscript{19} Thus, while it is perhaps not surprising that we observed adenoviral-mediated AM transduction in lung cancer models, this has not been previously described.

Although ectopic Kras expression can cause oncogene induced senescence (OIS) in fibroblasts,\textsuperscript{38} OIS depends on both oncogene dose and cellular context.\textsuperscript{14,39} Our observation that Kras\textsuperscript{G12D} knock-in leads to enhanced survival of AM in vitro is consistent with the ability of this allele to promote proliferation and partial transformation in mouse embryonic fibroblasts\textsuperscript{14} as
well as the ability of low level ras activation to promote proliferation during mammary tumorigenesis.\textsuperscript{39}

In conclusion, we found that the adenoviral vectors commonly used to initiate tumor formation can also infect AM. When these vectors employ strong ubiquitous promoters, oncogenic events can be inadvertently targeted to AM and this can impact both the immune TME and tumor growth. Given the critical role of macrophages in lung tumor progression and metastasis,\textsuperscript{10,11} it may be preferable to use transgenes or viral vectors that restrict Cre recombinase expression to lung epithelial cells to avoid this issue, particularly when studying interactions between specific oncogenic events and the immune system.\textsuperscript{18,27}

Materials and methods

Animal models: All studies were IACUC approved. Mice harboring the oncogenic Kras\textsuperscript{LSL-G12D} allele (JAX #8179) and the mTomato/mGFP (mT/mG) tracking allele (JAX #7576) were obtained from Jackson Laboratory (Bar Harbor, ME). Ad5-CMV-Cre and Ad5-SPC-Cre were purchased from the University of Iowa Viral Vector Core (Iowa City, IA). Ad5-EA was purchased from Viraquest (North Liberty, IA). Mice were treated with tracheal instillation of Ad5-CMV-Cre (30\,\mu \text{lof10}^9\text{PFU/ml}) or Ad5-EA (30\,\mu \text{lof5} \times 10^8\text{PFU/ml}) or by direct intrapulmonary injection of Ad5-CMV-Cre (2\,\mu \text{lof10}^7\text{PFU/ml}) or Ad5-SPC-Cre (2\,\mu \text{lof10}^{10}\text{PFU/ml}).\textsuperscript{1,12}

Immunofluorescence: Immunostaining was performed as previously described\textsuperscript{40} with primary antibodies against GFP (1:1000; ab13970, Abcam), Kras\textsuperscript{G12D} (1:50; GTX132407, GeneTex), CD107b (1:200; 550292, BD Biosciences), PCNA (1:250; ab18197, Abcam), E-Cadherin (1:200; 3195, Cell Signaling), TTF1 (1:200; ab76013, Abcam), FLAG (1:50; F4049, Sigma-Aldrich), and species-appropriate secondary antibodies: Alexa Fluor 594 (1:200; A11007 & A11012, Invitrogen), Alexa Fluor 488 (1:300; A11006, A11008 & A11039, Invitrogen), Alexa Fluor 405 (1:200; ab175652, Abcam) and Alexa Fluor 350 (1:200; A21093, Invitrogen). Isotype controls were performed with excess amounts of IgG (31235; Thermo Fisher), rat IgG1 (14-4301-81; Invitrogen), and chicken IgY (AB-101-C, BD Biosciences) antibodies. After DAPI counterstaining, images were acquired with a Nikon Eclipse 80i microscope equipped with a Nikon Intensilight C-HGFI illuminator and Nikon DS-Ri1 digital camera. Image processing was performed with Adobe Photoshop CS3 software.

Flow cytometry: Single cell suspensions were prepared as previously described.\textsuperscript{6} Prior to staining, Fc\gammaR was blocked with anti-CD16/CD32 (553142, BD Biosciences) for 10 min, then cells were stained for 60 min at 4°C with the following antibodies (1:100): CD3-PE-Cy7 (clone 145-2C11; 100319, Biolegend), CD8-APC (clone 53-6.7; 100711, BioLegend), CD4-APC-Cy7 (clone GK1.5; 100414, BioLegend), Ly6G-Alexa Fluor 700 (clone 1A8; 127621, BioLegend), CD64-APC (clone M1/70; 101215, BioLegend), Ep-CAM-APC (clone G8.8; 118213, BioLegend), CD3-FITC (clone 145-2C11; 100306, BioLegend). Cells were analyzed at the University of Colorado Cancer Center Flow Cytometry Core Facility using a Gallios 561 Flow Cytometer (Beckman Coulter). Cell sorting was performed on a MoFlo XDP70.
(Beckman Coulter). The analysis strategy involved excluding debris and cell doublets by light scatter and dead cells by 1 μg/ml DAPI (62247, Thermo Scientific). Data were analyzed using Kaluza Software (Beckman Coulter).

qRT-PCR: RNA was extracted by the RNeasy Micro Kit (74004, Qiagen) then qPCR with a GAPDH internal control and Cxadr (CAR) probe (Mm00438355_m1, 4448892, Life Technologies) was performed and analyzed as previously described.41

Cell line: CMT167 cells derived from a spontaneous lung adenocarcinoma in C57BL/6 mice were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, L-glutamine, and sodium pyruvate (11885-084, Gibco), supplemented with 10% (v/v) fetal bovine serum (16000-044, Gibco) and 100 μg/ml primocin (ANT-PM2, Invivogen) at 37°C in a humidified atmosphere of 5% carbon dioxide. Cells in the exponential growth phase with over 95% viability were used in all experiments.

AM harvest, analysis, and viral transduction: Murine AM were harvested from KrasLSL-Cre/mT/mGFP expressing mice. For analysis of mTomato and mGFP expression in live cells, pelleted BAL cells were re-suspended in 200 μl PBS, then 10 μL of this suspension was placed on a slide, heat-fixed (5 min at 80°C) and counterstained with DAPI. For in vitro viral transduction studies, pelleted AM were re-suspended with 1 ml of 1x RBC lysis buffer (00-4333-57, ebioscience) at room temperature for 2 min. After incubation, 2 ml of complete DMEM was added and the suspension was centrifuged. Viral treatments with Ad5-CMV-Cre or Ad5-SPC-Cre were performed in ultra-low attachment, polystyrene cell culture plates (3471, Corning) overnight at 37°C in a humidified atmosphere of 5% carbon dioxide.

Macrophage survival assay: AM (5000/well) were plated in duplicate into 96-well tissue culture plates (CC7682-7596, CytoOne) and transduced with Ad5-CMV-Cre or Ad5-SPC-Cre as described above. The following day media was replaced with 100 μL DMEM; thereafter, media was then replaced weekly. Images were taken with an IncuCyte ZOOM live-cell analysis system (2016B, Essen Bioscience) using a dual color filter module (4459, Essen Bioscience) and then analyzed with IncuCyte Control software. The fraction of macrophages remaining were determined by comparing cell counts from day 6 of each week to counts from day 6 of the first week.

Orthotopic tumor/AM co-injections: Following viral transduction cells were washed twice with PBS and trypsinized, then 20,000 AM were mixed 1:1 with CMT167 cells in 50 μl DMEM containing 13% matrigel (354234, Corning) and then injected into the flank of C57BL/6 recipients. Once palpable, tumors were measured twice weekly with calipers and weighed at the conclusion of the experiment.

Statistical analysis: Results are presented as mean ± standard error of mean. Differences between groups were compared with two-tailed unpaired t tests. P < 0.05 was considered statistically significant.

Financial support

D.D.T was supported by the NIH/NCI under a Ruth L. Kirschstein National Research Service Award (T32 CA174648). J.P. was supported by NIH/NCI (R01 CA162226 and P50 CA058187). H.L. was supported by NIH/NCI (P50 CA058187), VA (IK2BX001282), and the Cancer League of Colorado. H.S. was supported by Ruth L. Kirschstein National Research Service Awards (T32 CA17468 and T32 AG000279). J.D. was supported by NIH/NCI (R01 CA157850 and P50 CA058187). R.A. N. was supported by the NIH/NCI (R01 CA162226 and P50 CA058187) and The Gift of Life and Breath.

S.P.M. was supported by the NIH/NCI (R21 CA194662, P50 CA058187, and P30 CA046934).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Andrea Ventura (Memorial Sloan Kettering) for the use of the Ad5-EA virus. The Flow Cytometry Shared Resource at the University of Colorado is supported by P30 CA046934 and P30 AR057212.

Funding

HHS | NIH | National Cancer Institute (NCI), T32 CA174648 HHS | NIH | National Cancer Institute (NCI), P30 CA046934 HHS | NIH | National Cancer Institute (NCI), P30 CA046934 The Gift of Life and Breath, HHS | NIH | National Cancer Institute (NCI), R21 CA194662 HHS | NIH | National Cancer Institute (NCI), P50 CA058187 HHS | NIH | National Cancer Institute (NCI), R01 CA162226 HHS | NIH | National Cancer Institute (NCI) HHS | NIH | National Cancer Institute (NCI) HHS | NIH | National Cancer Institute (NCI) The Cancer League of Colorado U.S. Department of Veterans Affairs (VA), IK2BX001282 HHS | NIH | National Cancer Institute (NCI), T32 CA17468 HHS | NIH | National Cancer Institute (NCI), T32 AG000279 HHS | NIH | National Cancer Institute (NCI), R01 CA157850 HHS | NIH | National Cancer Institute (NCI), R50 CA058187.

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