UNIVERSITY OF CALIFORNIA SAN DIEGO

Building a Cancer Vaccine for Immunotherapy Through Transdifferentiation of Cancer Cells into Macrophage-like Cells

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by

Calvin Kaing Lee

Committee in Charge:

Professor Jack Bui, Chair
Professor Cornelis Murre, Co-Chair
Professor John Chang
Professor Li-Fan Lu

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The Thesis of Calvin Kaing Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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ABSTRACT OF THE THESIS

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Therapeutic cancer vaccination strategies aim to stimulate patient's immune system to combat disease. Approaches to generating cancer vaccines include utilizing autologous tumor cells and tumor-antigen loaded autologous dendritic cells. Recent studies demonstrate the potential to directly convert somatic cells of one lineage into others, a process called transdifferentiation. Here, we show the potential of utilizing this transdifferentiation process to convert cancer cell lines into myeloid-like cells by ectopic expression of two transcription factors, PU.1 and C/EBPα. The ectopic expression of these two transcription factors induce the expression of surface proteins characteristic of myeloid cells and these transdifferentiated cells acquired the potential to mount a partial inflammatory response to proinflammatory stimuli. Moreover, these transdifferentiated cancer cells generally lose their ability to form tumors in immune

competent mice but fail to control parental cell line tumor growth. Mice that developed tumors from the transdifferentiated cells were able to control the progression of parental cell lines better than those that had rejected the transdifferentiated cells. These findings highlight the potential of directly converting cancer cells into antigen-presenting cells as a potential therapeutic vaccination strategy for cancer.

Introduction

A promising approach to treating cancers involves utilizing a patient's own immune system to target and eliminate cancer. The success of antibodies targeting immune checkpoint receptors and ligands in the tumor microenvironment and cell-based therapies such as chimeric antigen T cells have led to their approval by the FDA for treating certain cancers such as B-cell lymphomas (Couzin-Frankel, 2013). The aim of cancer immunotherapy is to supplement or boost the ineffective host immune response against tumor cells by, for example, identifying and removing immunosuppressive factors present in the patient's tumor microenvironment (Khalil et al. 2016).

Immune checkpoint inhibitors are a class of drugs that target the expression of immune checkpoint ligands or receptors that, when activated, inhibit CD8+ T cell responses. These immune checkpoint ligands also activate regulatory T cells (Treg) which suppress the cytolytic activity of CD8+ T cells (Pardoll, 2012). Examples of these include monoclonal antibodies specific for programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) which have been approved by the Food and Drug Administration in the United States for use for certain cancers such as HNSCC (Pardoll, 2012). About 75% of melanoma and 16% of head and neck cancer patients show durable responses to these blockade therapies. It is unclear as to why other patients, despite having objective response to PD-1 therapy, show tumor progression (Zaretsky et al. 2016, Mehra et al. 2016). Moreover, it is unclear why for some patients there is no response. In a Phase 1 clinical trial, 67% of the 655 enrolled melanoma patients treated with pembrolizumab, a monoclonal antibody (mAB) targeting PD-1,

showed no objective response to the drug (Ribas et al. 2016). Similarly, in a phase III trial comparing pembrolizumab to ipilimumab, an anti-CTLA4 mAB, objective response was observed in 33% of the 834 enrolled melanoma patients (Robert et al., 2015). These results necessitate further research into what makes a cancer acquire resistance, or non-responsive, as well as approaches to co-opt the function of other immune cells to treat cancers.

The innate and adaptive immune system play a critical role in controlling the development and progression of cancer. Studies of immunocompromised mice show that these animals are more susceptible to developing various spontaneous cancers as well as chemically induced cancers (Dunn et al. 2002). In addition to being more susceptible to developing cancer, these mice have an impaired ability to control the progression of these tumors compared to immunocompetent mice (Smyth and Swann 2007). The cancer immunosurveillance hypothesis models the interaction between endogenous immune cells and tumors in three phases (Dunn et al., 2002). The first phase is the elimination phase whereby resident cytotoxic effector cells of both the innate and adaptive arm of the immune system, such as CD8+ T Cells and Natural Killer (NK) cells, detect and kill tumor cells (Dunn et al., 2002). Failure to reject the tumor leads to an equilibrium phase where immune cells impose selective pressure for less immunogenic tumor cells. The third phase is characterized by the lack of an effective immune response to the tumor and subsequent escape of the tumor, whereby the tumor can grow freely without control from the immune system (Dunn et al., 2002).

The primary immune cells that mediate direct elimination of tumor cells are CD8+ T cells of the adaptive arm as well as NK and natural killer T (NKT) cells of the innate

arm (Swann and Smyth 2007). The cytotoxic functions of CD8+, NK, and NKT cells are critically dependent upon their interaction with dendritic cells (DCs) which are specialized innate immune cells that present immune-stimulating antigens derived from pathogens, transformed cells, as well as the host's own proteins and other molecules (Moretta, 2002, Mempel et al., 2004). These cells can phagocytose pathogens and stressed cells and process them for presentation to CD4+ and CD8+ T cells to activate them. In the context of tumor surveillance, DCs can phagocytose apoptotic cancer cells and process proteins of these cancerous cells for presentation on major histocompatibility complex (MHC) I or II to stimulate an antigen-specific response in both CD4+ and CD8+ T cells. Additionally, DCs provide both cytokine stimulation and costimulatory signals that fully activate and polarize responses CD4+ and CD8+ T and NK cells such that an appropriate immune response generated (Harris and Ronchese, 1999). However, T cell and NK cell antitumor functions may be suppressed by tumor cells and tumor associated cells such as tumor-associated fibroblasts. For example, tumor and stromal cell secretion of immune regulating factors such as transforming growth factor beta (TGFβ) or down regulation of major histocompatibility complex class I (MHC I) on their cell surface are examples of strategies used to suppress or evade host immune recognition and response (Rabinovich et al., 2007).

The role of DCs in mediating effective innate and adaptive immune responses makes them prime targets for immunotherapeutic applications. DCs have been appreciated for their critical role in mediating immunity in the context of vaccination against a variety of pathogens such as the flu virus (Gamvrellis et al. 2004). Adjuvants used in vaccine formulations activate and mature DCs such that they can present the

vaccine antigen to T cells and activate B cells (Gamvrellis et al. 2004). These adaptive cells then become activated and can both mount an effector response and generate immunologic memory against those cells expressing those antigens (Gamvrellis et al. 2004). Thus, vaccines can be generated against cancer cells so long as there is an immunogenic epitope derived from a cancer-specific antigen and an adjuvant. DCs have long been considered the body's natural adjuvant and have thus been explored as a potential cell-based therapeutic vaccine. These DC vaccines are prepared from a patient's own peripheral blood mononuclear cells that are differentiated ex vivo from CD14+ monocytes or CD34+ progenitor cells in the blood and then activated with a cytokine cocktail that attempts to recapitulate the cytokine milieu of inflammation (Palucka and Banchereau, 2012). Following differentiation and maturation in ex vivo culture, the DCs are loaded with tumor-associated or tumor specific antigens peptides such that the DCs can prime an antigen-specific T cell response against the tumor. Dendritic cell-based vaccination therapies have been employed to some success for the treatment of melanoma, glioblastomas, and prostate cancer, but to a much more limited extent in other cancer types (Palucka and Banchereau, 2012). One of the major hurdles associated with producing a DC vaccine for a patient's cancer is the identification of immunogenic tumor-specific or tumor-associated antigens that can be loaded onto DCs. Advances in sequencing technology has allowed for the identification of neoantigens expressed by a patient's cancer, however, these computational approaches require empirical validation of the immunogenicity of these antigens as peptides loaded onto MHC for presentation to T cells.

One potential approach for circumventing the need to identify and validate immunogenic peptides for DC vaccines would be to differentiate a cancer cell into an antigen-presenting cell that resembles a DC through a process called transdifferentiation. Genetically reprogramming both normal and transformed cells to either a pluripotent state or directly to another cell fate has been shown to be possible by both the Graf and Majeti group who both demonstrate that B Cell lymphomas and leukemia cells can be forced to differentiate into macrophage-like cells (Rapino et al. 2013, McClellan et al. 2015). Because the cancer cells will have the full repertoire of genetic mutations that define that cancer and presumably express the same mutated proteins, the cancer cell turned dendritic cell will potentially be capable of presenting the full range of both mutated and normal peptides characteristic of that particular cancer. Although there has been little evidence demonstrating the potential to reprogram or transdifferentiate a cancer cell into a bona fide dendritic cell, many groups have shown that it is possible to transdifferentiate various terminally differentiated cell types into macrophage-like cells, which share a common progenitor with dendritic cells, by ectopically expressing two transcription factors, PU.1 and C/EBPα, critically involved in dendritic cell and macrophage development (Feil et al., 2014, Feng et al., 2008, Laiosa et al. 2006, Welner et al., 2013, Bakri et al., 2005). Moreover, it has been shown that transdifferentiation of both patient-derived and established cell lines of B cell lymphoma into macrophage-like cells abrogates the ability of those cells to form tumors in vivo (Rapino et al., 2013). These experiments suggest these cancer cells can be forced to take on a developmentally-related cell identity and importantly that a non-

developmentally related cell type such as a fibroblast can be made to take on a hematopoietic identity.

In this current study, we aimed to provide a proof-of-principle that cancer cells are capable of being transdifferentiated into antigen-presenting cells such as macrophages. We found that forced expression using a retroviral system of two transcription factors, PU.1 and C/EBPα, induces the conversion of murine and human fibroblastic and epithelial cancer cell lines to take on a macrophage-like phenotype. Cancer cell lines transduced with the two transcription factors were induced to express the hematopoietic and myeloid markers such as CD45, CD11b, and CD80. Additionally, these cells gained responsiveness to inflammatory stimuli lipopolysaccharide (LPS) and interferon γ (IFNγ). Though these cells are capable of mounting an inflammatory response, the phagocytic capacity of these transdifferentiated cells are blunted compared to macrophages as well as the parental cell lines. Furthermore, when transplanted into immune competent mice the transdifferentiated cell lines typically fail to engraft and form tumors. When challenged with the parental cell line, the same mice that rejected the transdifferentiated cells fail to control the progression of the parental tumor. However, when the transdifferentiated cell fails to reject in vivo, the mice are capable of controlling the parental tumor development. Together, these data highlight the potential of transdifferentiating cancer cells into macrophage-like cells as a potential vaccination strategy for cancer immunotherapy.

Methods

Cell Culture and Lines.

The following cell lines were used for transdifferentiation: 3-Methylcholanthrene (MCA)-induced murine sarcoma cell line, 4862, 9609, F244, F236, F535, Lewis Lung Carcinoma (LLC), A549, MDA-MB-231, and MDA-MB-468 cell lines. Cells were grown and maintained as adherent monolayers at 37°C, at 5% CO_2, in RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS). Other cells such as HEK293T, RAW 264.7, and mouse embryonic fibroblasts (MEFs) were cultured similarly as outlined above. Bone-marrow derived macrophages were isolated and derived from femurs of 2-month-old wildtype C57BL/6 mice and cultured similarly as outlined above with the addition of L929 conditioned media.

Retrovirus Production and Infection.

Retroviral constructs PU.1-GFP and C/EBPα-hCD4 were a kind gift from Thomas Graf (CRG). These plasmids encode murine transcription factors with reporter genes, GFP or human CD4 respectively, placed downstream of an IRES sequence. Empty vectors encoding GFP alone were used as control transductions. Retroviral particles were generated in HEK293T cells by transfecting either PU.1-GFP or C/EBPα-hCD4 with packaging constructs gag-pol and VSVg. Viral particles were harvested 48 and 96 hours post transfection and either used immediately or snap frozen in liquid nitrogen and stored at -80°C. To infect cells, viral particles were added to cells at 50% confluency and supplemented with 5 ug/mL Polybrene (Sigma-Aldrich). Transduction

efficiency was assessed by GFP and human CD4 expression via flow cytometry 48 hours post infection.

Gene Expression Analysis.

Total RNA was isolated using TRIzol (Invitrogen) and quantitated with a NanoDrop Spectrophotometer (ThermoFisher). cDNA was generated with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using 250 ng of total RNA. Quantitive polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's instructions with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). A total of 2.5 ng of cDNA was used per reaction. All primers were generated using Primer-Blast. Murine primers are as follows: IL-23p19 (FWD 5'-CACCAGCGGGACATATGAATCT-3', REV 5'-CACTGGATACGGGGCACATT-3'), IL-10 (FWD 5'-GGCGCTGTCATCGATTTCTC-3' REV 5'-ATGGCCTTGTAGACACCTTGG-3'), IL-6 (FWD 5'-CCAAGAGATAAGCTGGAGTCACA-3' REV 5'-CGCACTAGGTTTGCCGAGTA-3') IL-1β (FWD 5'-GCAGTGGTTCGAGGCCTAAT-3' REV 5'-CTCATCACTGTCAAAAGGTGGC-3') TGFβ (FWD 5'-GCCCGAAGCGGACTACTATG-3' REV 5'-CTCATAGATGGCGTTGTTGCG-3').

Flow Cytometry, Cell Sorting, Antibodies.

All samples were analyzed on a BD FACSCanto II (BD Biosciences). The following antibodies were used: anti-CD45 (Clone 30-F11), anti-CD11b (Clone M1/70), anti-MHC-I (Clone M5/114 15.2), anti-CD80 (Clone 16-10A1), anti-CD200R (Clone

OX110), anti-Ly6C (Clone HK1.4), anti-PD-L1 (Clone MIH5), anti-CD204 (Clone 2F8, Serotec), anti-F4/80 (Clone BM8), anti-CD172a (Clone P84, BD Biosciences), anti-TNFα (Clone MP6-XT22, eBioscience), anti-IL-6 (Clone MP5-20F3), anti-IL12p40 (Clone C15.6), anti-IL-10 (Clone JES5-16E3). Unless otherwise stated, all antibodies were purchased from BioLegend. Cells were collected and washed twice using FACS buffer (PBS + 1% FBS + 0.05% NaN_3 (Sigma-Aldrich)), then surface stained for 20 minutes at 4°C in the dark, washed three times, and then resuspended in 200 uL of FACS buffer with 1 ug/mL 7AAD prior to acquisition. Intracellular cytokine staining (ICS) was performed using Fixation/Permeabilization Solution Kit (BD Biosciences) according to manufacturer's instructions. Cells were sorted on four parameters, GFP, human CD4, CD45, and CD11b (for murine transdifferentiated cells) or CD14 (for human transdifferentiated cells) using a BD FACSARIA II (BD Biosciences).

Cell Stimulation for ICS and Gene Expression Analysis.

Cells were treated for 24 hours with either 100 U/mL IFNγ (BioLegend) or 20 ng/mL IL-4 (BioLegend) and in the final 6 hours treated with 1 ng/mL LPS (Invivogen). For ICS, cells were treated with 1 ug/mL brefeldin A (Sigma-Aldrich) and 2 μM monensin (BioLegend) in the final 6 hours. Following incubation, cells were washed three times with PBS and harvested for analysis by flow cytometry or RNA extraction.

Tumor Cell Phagocytosis.

250,000 target tumor cells were seeded in a co-culture with 50,000 effector cells (transdifferentiated 9609, 9609 GFP controls, RAW 264.7) and incubated for 24 hours

at 37°C. Following incubation, cells were washed three times with PBS and harvested for analysis by flow cytometry. Target cells were distinguished from effector cells on the basis of dsRed expression and effector cells were distinguished on the basis of one or more of the following three criteria: GFP, CD45, and/or CD11b expression.

Mice.

C57BL/6 wildtype mice were used for tumor transplantation experiments. All mouse experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee (IACUC Protocol #S06201).

Tumor Transplantation and Challenge.

Transdifferentiated 9609 cells were harvested, washed three times with sterile PBS, and suspended in sterile PBS at a concentration of $1x10^7$ cells/mL. 100 uL ($1x10^6$ cells) of cell suspension was injected subcutaneously into the left flank of wildtype C57BL/6 mice and monitored for growth every 3 days. To assess the potential of these transdifferentiated 9609 cells as vaccines we challenged the same mice with the parental cell line. 28 days after initial injection, the parental 9609 cell lines were injected subcutaneously, following the same procedures as outlined above, on the contralateral flank of the same mice and monitored for growth every 3 days.

Results

Ectopic Expression of PU.1 and C/EBPα Alter the Surface Expression and Morphology of a Subset of Cancer Cell Lines to Resemble Macrophages.

To test whether the forced expression of two murine transcription factors PU.1 and C/EBPα could induce the expression of hematopoietic surface proteins, we first transduced MEFs with the two constructs and analyzed surface expression by flow cytometry. Empty vectors encoding GFP alone were transduced in parallel as a control. Upregulation of both CD45 and CD11b, markers of hematopoietic cells and myeloid cells respectively, was observed in 6% of PU.1 and C/EBPα expressing cells, whereas GFP control cells did not upregulate either marker fourteen days post transduction (Table 1). These data recapitulated the observations in NIH3T3 cells utilizing the same constructs, albeit at a lower frequency, and confirmed the function of the two transcription factors in upregulating hematopoietic and myeloid surface markers (Feng et al. 2008).

We next sought to determine whether murine and human cancer cell lines could similarly be induced to express hematopoietic surface markers by ectopic expression of the two transcription factors. Five murine MCA sarcoma cell lines, 4862, 9609, F244, F236, F535, one murine lung carcinoma cell line, LLC, one human lung carcinoma, A549, and two human epithelial breast cancer cell lines MDA-MB-231 and MDA-MB-468 were transduced with the two transcription factors. Amongst the nine cell lines, 4862, 9609, F535, LLC, A549, and MDA-MB-231 upregulated hematopoietic markers within fourteen days, though only a fraction of transduced cells ranging from 0.5-6% were able to do so (Table 1). When gated on GFP and hCD4, murine cell lines were

induced to express CD45 alone, or both CD45 and CD11b, whereas the human cell lines A549 only expressed CD45 and MDA-MB-231 expressed CD45 and CD14, but not CD11b (Table 1 and Figure 1A). GFP control cells for each cell line were unable to upregulate any of the hematopoietic markers used (Table 1 and Figure 1A). These data extend the utility of the transdifferentiation process beyond normal fibroblasts and B cell lymphomas to include cancers of fibroblast and epithelial origin.

We next attempted to generate stable cell lines of the 9609s and LLCs, herein referred to as reprogrammed 9609 (R9609s) and reprogrammed LLC (RLLC), by FACS sorting on GFP, hCD4, CD45, and CD11b for further characterization. Because the goal is to generate a cell type that can function similar to an APC, we selected surface markers that are characteristic of myeloid cells and antigen presenting cells including MHC I, MHC II, F4/80, and costimulatory ligands such as CD80 and inhibitory receptors and ligands such as CD172a, CD200R CD204, and PD-L1. Ly6C is known to be expressed on lung epithelium as well as myeloid cells. The R9609 and RLLCs were >90% CD45+ and CD11b+ after sorting, while GFP control cells did not express these markers. Gating on CD45+ and CD11b+ cells, R9609s express CD80, CD204, F4/80, CD172a whereas GFP control cells, gated on GFP alone, did not (Figure 1B). We also observe a decrease in expression of PD-L1 from 45% on GFP control cells to 3% on R9609s, as well as a decrease in the expression of MHC I from 41% to 13% (Figure 1B). RLLCs, compared to GFP control cells, similarly express of CD80, F4/80, and CD172a (Figure 1B). Unlike R9609s, the RLLCs have increased MHC I expression from 55% to 70%, and decreased expression of CD126, Ly6C, and CD204 when compared to LLC GFP controls (Figure 1B). In all transdifferentiated cell lines and GFP controls

there was no observed induction of MHC II expression (Figure 1B). In addition to surface expression, the morphology of R9609s resemble that of the murine macrophage cell line RAW 264 (Figure 2). Together, these data indicate that ectopic expression of both PU.1 and C/EBPα in these cancer cell lines induce morphological changes and the expression of surface proteins characteristic of macrophages.

Phagocytic Capacity is Blunted in R9609 Cells

We next wanted to determine whether the R9609 cells could function like a macrophage. One key function of macrophages is to phagocytose invading pathogens and apoptotic or stressed cells. To explore the phagocytic capacity of the R9609s, we co-cultured 250,000 dsRed-expressing 4862 fibrosarcoma cells with 50,000 R9609s, 9609 GFP controls, or RAW 264.7 cells for 24 hours. In these experiments, the R9609 cells used were 25% CD45+ only, and 10% CD45+ and CD11b+, and the remaining 65% CD45- and CD11b-, but expressing GFP which allows us to track how phagocytosis changes with acquisition of hematopoietic markers. As shown in Figure 3, R9609s are less phagocytic than both 9609 GFP controls and RAW 264.7. In Figure 3B, as the R9609 cells acquire expression of hematopoietic markers phagocytic capacity decreases, while CD45-/CD11b-/GFP+ cells in the R9609 population maintained comparable phagocytic capacity as 9609 GFP control cells. These data suggest that various stages of transdifferentiation, marked by the acquisition of hematopoietic markers CD45 and CD11b, towards a myeloid-like fate they become less capable of phagocytosis at least by tumor cell uptake.

A Subset of R9609 are Capable of Generating an Inflammatory Response.

Another feature of macrophages is their capacity to respond to inflammatory stimuli and produce cytokines to modulate an immune response. Unlike the phagocytosis assay, the R9609 cells used in these experiments were >90% CD45+/CD11b+. To assess the capacity of R9609 to mount an inflammatory response, R9609 cells were stimulated with IFNγ or IL-4 for 24 hours and further stimulated after 18 hours with LPS or stimulated with LPS alone for 6 hours. At the transcript level, LPS induced the expression of IL-6, IL-1β, IL-12p40, and TNFα in R9609 cells (Figure 4). In the presence of IFNγ, IL-6 and TNFα expression is further increased and IL-10 is expressed. In the presence of IL-4, IL-6 and TNFα expression is decreased relative to LPS or LPS with IFNγ, but IL-1β expression is greater than either condition (Figure 4). In all conditions, 9609 GFP only modestly increased IL-6 expression (Figure 4). RAW 264.7 cells followed a similar trend as R9609s with the exception of TNFα remaining similar across conditions.

At the protein level, intracellular cytokine staining for IL-6, IL-10, IL-12p40, and TNFα showed that in response to IFNγ and LPS, 3-6% of CD45+/CD11b+ produced IL-6 and TNFα, but not IL-10 or IL-12p40 (Figure 5). In contrast, a small fraction of 9609 GFP cells produced IL-6 (Figure 5). Nearly all RAW 264.7 cells produced TNFα and to a much lesser extent IL-6, IL-10, and IL-12 across conditions. Together, these data suggest that at least a subset of transdifferentiated cells are capable of mounting a pro-inflammatory response to inflammatory stimuli both at the transcript and protein level.

R9609s Can Act as a Prophylactic Vaccine When Tumorigenic.

Having determined that these transdifferentiated cells are functionally similar to macrophages, we next wanted to determine whether conversion to a macrophage-like

cell could render the cancer cell lines non-tumorigenic as observed in transdifferentiated B cell malignancies. To assess this, we transplanted $1x10^6$ R9609 cells, which were >90% CD45+/CD11b+, subcutaneously into the left flank of 2-month-old male C57BL/6 mice and monitored the injection site. As shown in Figure 6, the transplanted R9609s failed to form tumors in 3 of 4 mice. In the mouse that bore the escaped R9609, growth was not detected until 26 days post-injection.

We next sought to determine whether the mice exposed to the transdifferentiated cells developed immunity to the parental cells by challenging the mice with the parental 9609 cell line. The parental 9609 cell lines engraft within 7 days and progress rapidly in wildtype C57BL/6 mice when injected subcutaneously. At 30 days post R9609 injection, $1x10^6$ parental 9609 cells were injected into the contralateral flank of each mouse subcutaneously. As shown in Figure 6, the mice that rejected the R9609s were unable to control to control the growth of the parental 9609 tumors. In contrast, the mouse with the escaped R9609 was able to effectively control the development of the parental 9609 tumor but not completely reject it. Preliminary FACS analysis of the escaped R9609 tumor showed that the GFP mean-fluorescence intensity (MFI) was 10-fold lower than that of the R9609 cells from culture which we have found in preliminary experiments to fail to convert to macrophage-like cells at lower GFP MFI (data not shown). Together these data suggest that although the transdifferentiated cancer cell lines may be less tumorigenic, they are unable to function as a vaccine when challenged with the parental cell lines. Interestingly, when these transdifferentiated cells can engraft and form tumors they can control the progression of parental cell lines when challenged suggesting that persistent or prolonged antigen stimulation may be necessary.

Discussion

The field of cellular transdifferentiation and reprogramming has largely focused on regenerative applications, such as the generation of cardiomyocytes, various neuronal cells, and pancreatic cells from fibroblasts (Fu et al. 2014). Previous reports show that normal fibroblasts can be converted into macrophage-like cells by ectopic expression of the transcription factors PU.1 and C/EBPα as well as IRF8 and MNDA (Feng et al. 2008, Suzuki et al. 2012). In this study, we aimed to provide proof-of-principle for utilizing cellular transdifferentiation as a potential cancer vaccination strategy. We posit this strategy based on the basic assumption that many cancers arise due to the accumulation of somatic mutations. We show that various human and mouse cancer cell lines of both fibroblast and epithelial origin can be converted into macrophage-like cells through ectopic expression of two transcription factors PU.1 and C/EBPα. These transdifferentiated cells typically fail to form tumors in vivo but when they do, can act as a prophylactic vaccine that allows mice to control the development of the parental tumor when challenged.

Screening of cancer cell lines that could potentially be transdifferentiated revealed that not all cell lines are capable of being converted into macrophage-like cells and that this is not an efficient process. Without enriching populations by sorting, only up to 6% of transduced cells express CD45 and CD11b. Although this is not an issue when working with cell lines, when working with limited patient samples this will severely limit the process. The fact that not all cell lines utilized with could not be transdifferentiated, this suggests that there is a safeguard on cell identity that poses a

significant roadblock to transdifferentiation. These present issues mirror what is observed by others utilizing stem cells for disease modeling and regenerative medicine where it is observed that only a small fraction of cells can be successfully converted to induced-pluripotent stem cells (iPSC) (Malik and Rao., 2014). Similarly, some cancer cell types appear to be completely resistant to iPSC formation such as various forms of leukemia due to epigenetic changes (Munoz-Lopez et al. 2016). A recent study has elucidated the role of the chromatin assembly factor-1 (CAF1) as one potential safeguard of cell identity, where knockdown of either subunit enhances iPSC formation and fibroblast to neuronal transdifferentiation (Cheloufi et a. 2015). These and other studies highlight potential roadblocks to target in future studies to further optimize this transdifferentiation process.

Vaccination approaches critically depend on the presence of antigen-presenting cells to bridge innate immune responses to the adaptive immune system. Although most somatic cells are capable of presenting antigen to adaptive immune cells, professional APCs such as macrophages and DCs have a much greater capacity to stimulate naïve T cells. Professional APCs provide three signals to T cells for activation: antigen in the context of MHC I or II, costimulation from CD80 or CD86, and cytokines such as IL-12. Non-immune cells and cancer cells generally provide only one of three signals, thus are poor stimulators of naïve T cells.

Our results show that two of the transdifferentiated cell lines, R9609 and RLLC, are capable of providing all three. CD80 was expressed on nearly all CD45+ CD11b+ R9609 and RLLC cells and thus can provide costimulatory signals to T cells on CD28. Interestingly, we observed a decrease in MHC I expression on R9609s, but not RLLCs

which instead upregulated MHC I. Downregulation of MHC I is often a mechanism to escape immune surveillance by T cells in tumors, but we did not expect expression of either transcription factors to affect MHC I expression (Dunn et al. 2002). This is problematic in the context of cancer vaccination because this suggests that a large fraction of these transdifferentiated cells are incapable of being recognized by CD8+ T cells, though may be recognized by NK cells.

The opposing effects on R9609 and RLLC on MHC I expression also raises the question of whether or not transdifferentiation to macrophage-like cells can consistently produce a good vaccination product. The surface characterization of both R9609 and RLLC cells show that different myeloid markers are induced in these cells. For example, most R9609 cells decrease their expression of PD-L1, but RLLC cells only modestly downregulate its expression. Because the goal is to have these transdifferentiated cells to function as APCs, ligation of PD-1 on T cells by these transdifferentiated cells may actually inhibit their function in the context of cancer immunity. This is particularly concerning if the non-transdifferentiated cells persist in the host, as observed in mouse 1 in figure 6. However, because these transdifferentiated cells are amenable to genetic perturbation it is likely that these can be further engineered to be better APCs such as through forced expression of IL-12.

Consistent with previous studies, the tumorigenicity of the transdifferentiated cells are largely diminished (Rapino et al. 2013). In three of four mice injected, R9609 cells failed to engraft and form tumors. In one mouse the R9609 cells managed to engraft and form a tumor palpable at day 26 post-injection, albeit slowly compared to the parental cell line which are known to form tumors within 7 days and progress

rapidly. Interestingly, when these same mice were challenged with the parental 9609 cell line only the mouse with the escaped R9609 was able to control the growth of the parental tumor. The parental 9609 tumors that grew progressed as expected in the three mice that had rejected the R9609 cells. This result suggests that either persistent antigen stimulation or greater amounts of antigen are needed to mediate control of the parental tumor. However, it is not clear in the context of cancer immunity whether persistent antigen is beneficial to antitumor immunity. In the context of peptide-based cancer vaccines, Hailemichael et al. has shown that persistence of gp100 melanoma peptide sequestered tumor-specific CD8+ T cells to the vaccination site but transient peptide did not which resulted in superior antitumor immunity (Hailemichael et al. 2013). Preliminary analysis of the R9609 tumor show that the GFP MFI is 10-fold lower than that of R9609s derived from culture (data not shown). Initial experiments showed that high PU.1 expression, as readout by GFP expression, is necessary for conversion of cells to a macrophage-like phenotype, an observation that is also corroborated in Feng et al. 2008 (data not shown). This suggests that the escaped R9609 variant consisted primarily of cells that did not undergo transdifferentiation based on GFP MFI alone. These data highlight the caveat of this approach whereby incomplete transdifferentiation may lead to escape and growth of a tumor, though this can be mitigated by rendering cells non-mitogenic through gamma irradiation

In conclusion, our results provide proof-of-principle for utilizing cellular transdifferentiation to convert cancer cell lines into macrophage-like cells, with potential to be used a cancer vaccine. This approach can potentially be extended to a variety of solid malignancies for which current FDA approved immunotherapies show little

efficacy. Furthermore, these findings may help define characteristics of an ideal cell-based vaccine which may be engineered into conventional dendritic cell vaccines in current use.

Table 1: Conversion efficiency of various human and mouse cells.
Screening PU.1 and C/EBPα transduced cells on the basis of combinations of CD45, CD11b, or CD14 expression.

Cell Line	Days Post-Transduction	Myeloid Marker Expression %
4862	6	5.3%
LLC	6	3.4%
4862 RagGP	6	9.7%
MEF	14	6.3%
F535	14	3.2%
9609	0.4	4.6%
A549	14	0.5%
MDA-MB-231	14	2.5%
F236	-	0.0%
MDA-MB-468	14	50.0%
F244	-	0.0%
PT-9	-	0.0%
HCT116	-	0.0%
T47D	-	0.0%

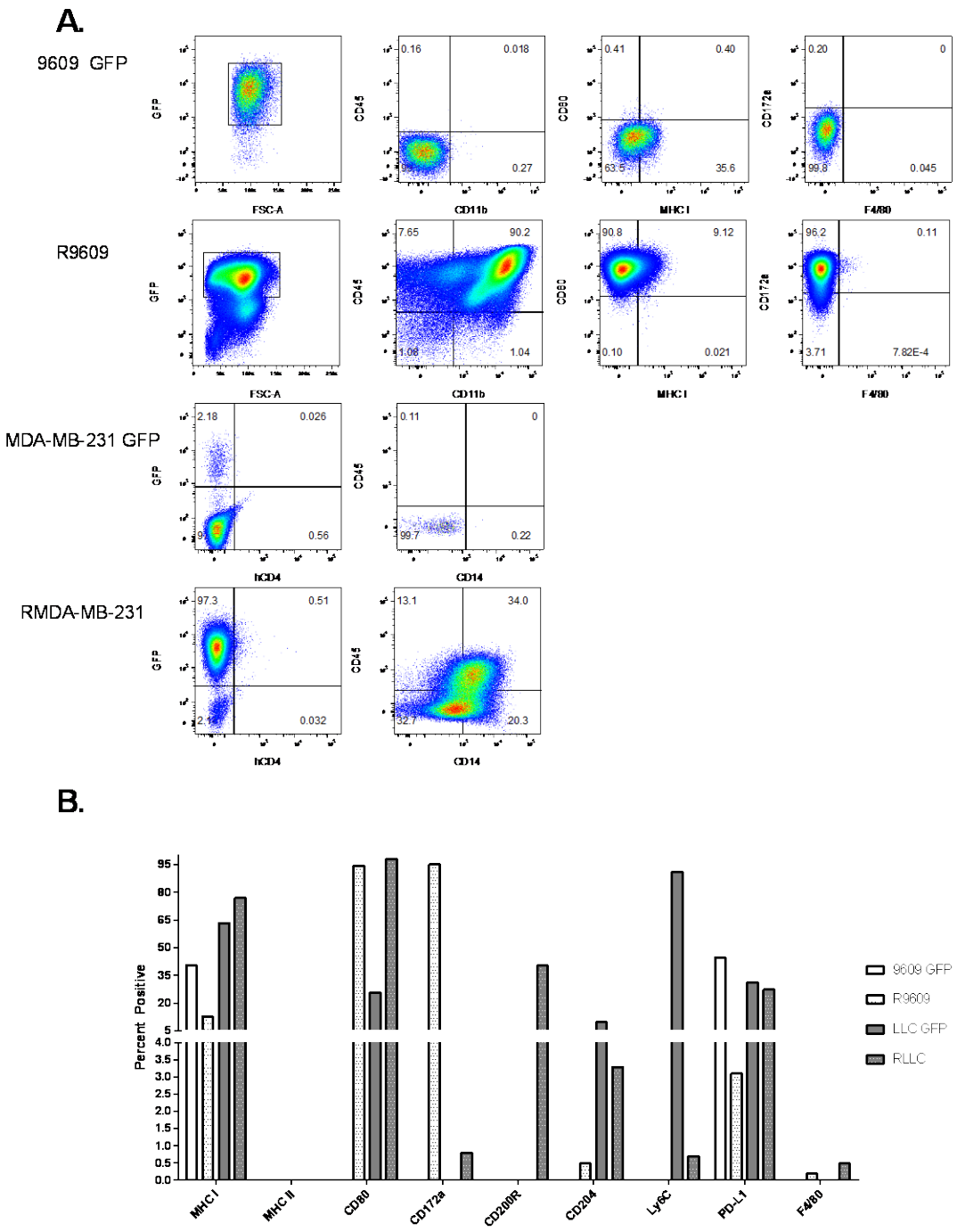
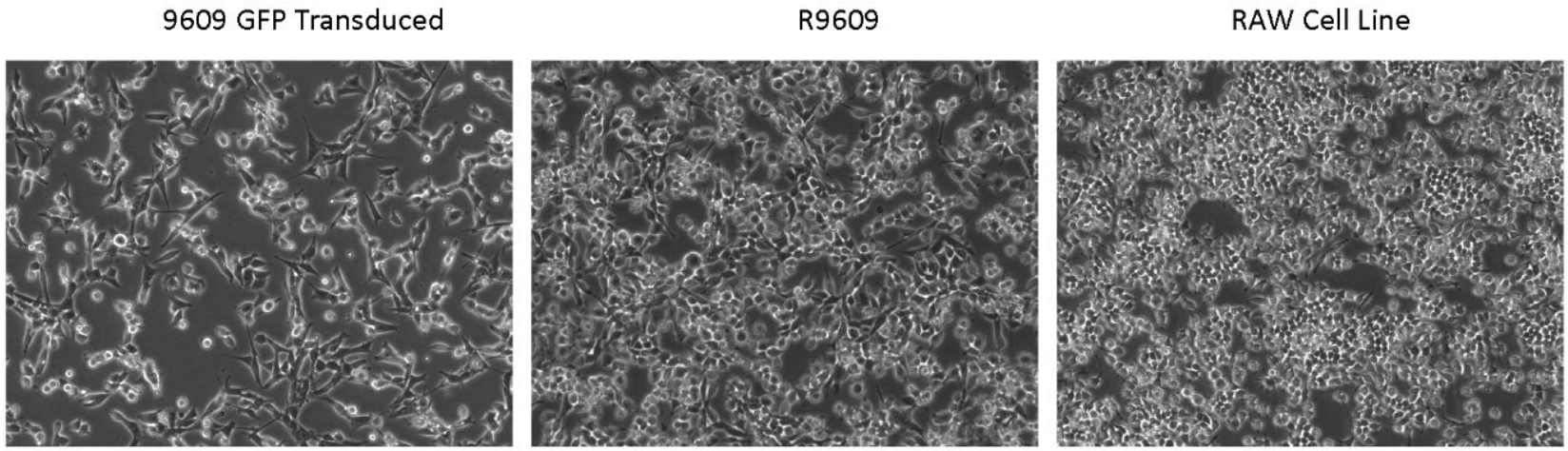
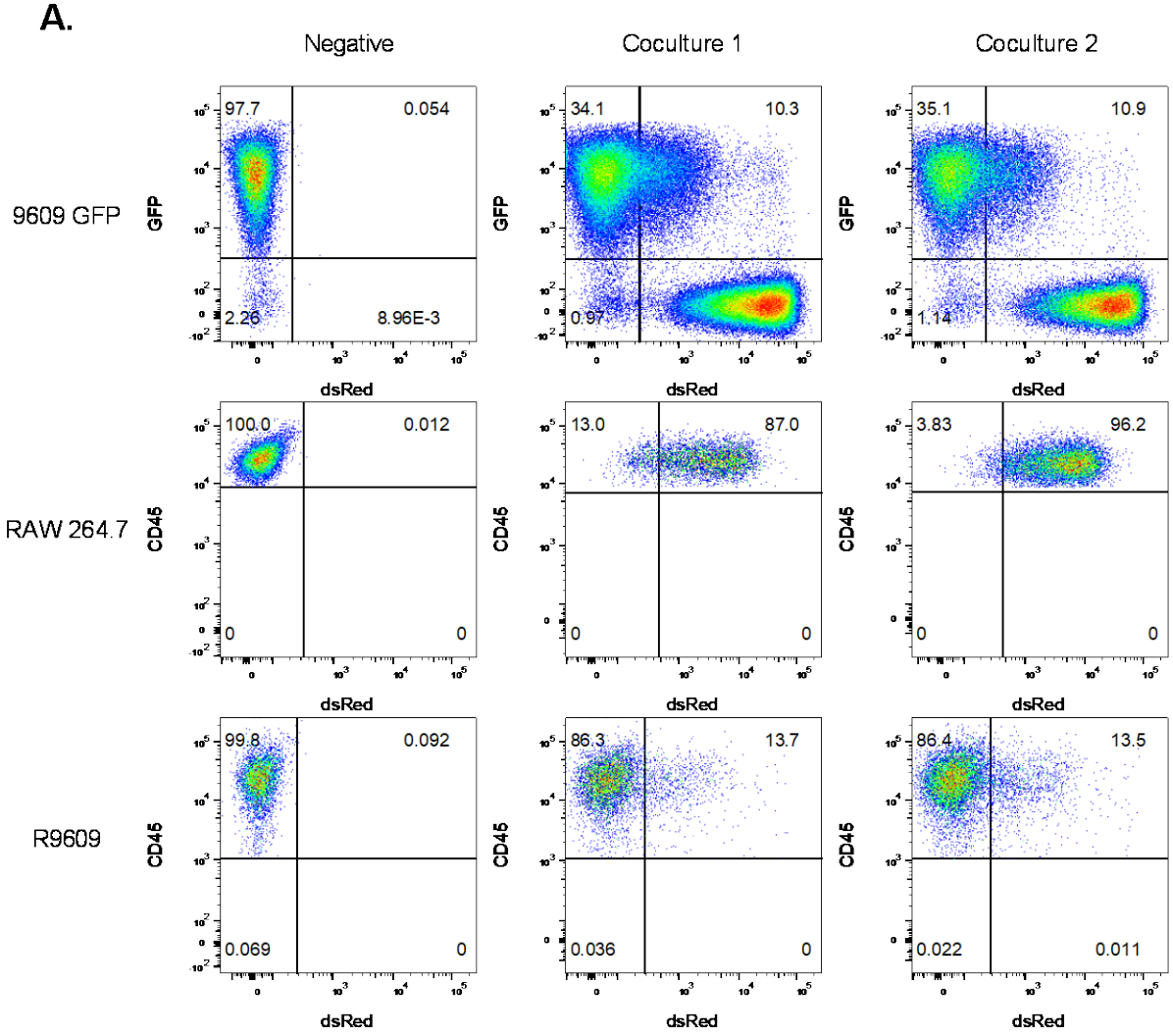


Figure 1. Cell Lines Transduced with PU.1 and C/EBPα Express Hematopoietic and Myeloid Markers. (A) Representative FACS plots of R9609 and RMDA-MB-231 and their GFP controls. (B) Plot of a range of myeloid surface proteins on both RLLCs and R9609s and their GFP controls.

Figure 2. Morphology of R9609s Resemble the Macrophage Cell Line Raw 264.7.


9609 GFP Transduced
R9609
RAW Cell Line

A.



B.

Cell Type	Population	Phagocytic Index Percentage
9609 GFP	GFP+	23.4
RAW 264.7	**CD45+/CD11b+**	**91.6**
R9609	CD45-/CD11b-/GFP+	22.5
R9609	**CD45+/CD11b-**	**20.6**
R9609	CD45+/CD11b+	13.6

Figure 3. Tumor phagocytosis in R9609 cells is blunted as myeloid markers are acquired. (A) Representative FACS plots of cocultures alone or with dsRed 4862 cells. (B) Phagocytic index of each cell type on the basis of different cell populations within each type.

Figure 4. Induction of Proinflammatory Gene Transcripts in R9609s In Response to LPS, LPS and IFNγ, or LPS and IL-4. Transcript levels of IL-6, IL-10, IL-1β, TNFα, IL-12p40, IL-23p19 and TGFβ assessed by qPCR in R9609s, 9609 GFP controls, and RAW 264.7 cell lines.

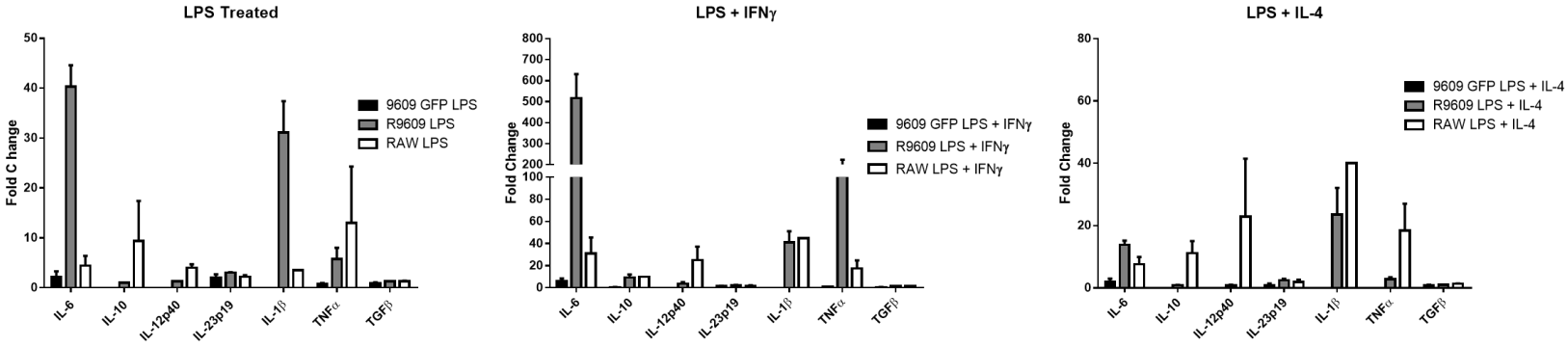
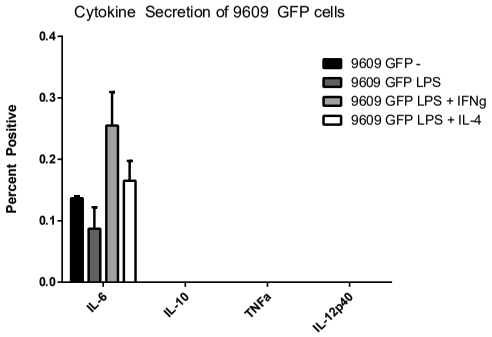
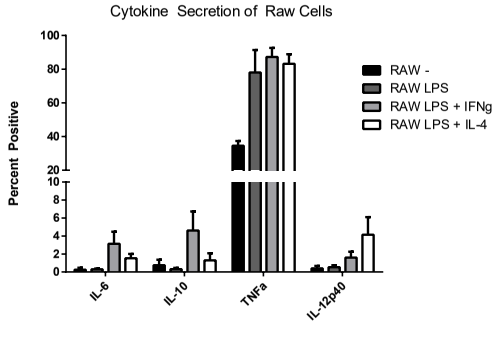
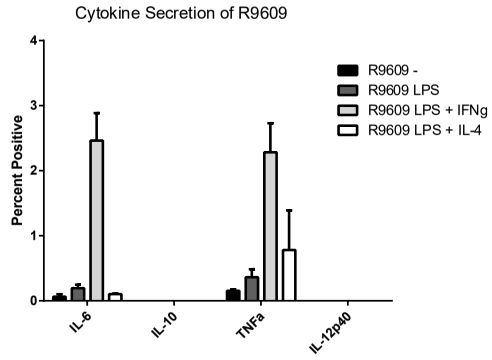

LPS Treated
Fold C hange
9609 GFP LPS
R9609 LPS
RAW LPS
IL-6
IL-10
IL-12p40
IL-23p19
IL-1β
TNFα
TGFβ
LPS + IFNγ
Fold Change
9609 GFP LPS + IFNγ
R9609 LPS + IFNγ
RAW LPS + IFNγ
LPS + IL-4
Fold Change
9609 GFP LPS + IL-4
R9609 LPS + IL-4
RAW LPS + IL-4

Figure 5. Cytokine Production of R9609s in Response to Inflammatory Stimuli. Intracellular cytokine staining of 9609 GFP, R9609, and RAW 264.7 probing for IL-6, IL-10, TNFα, and IL-12p40.


Cytokine Secretion of 9609 GFP cells
Percent Positive
0.4
0.3
0.2
0.1
0.0
9609 GFP -
9609 GFP LPS
9609 GFP LPS + IFNg
9609 GFP LPS + IL-4
IL-6
IL-10
TNFa
IL-12p40


Cytokine Secretion of Raw Cells
Percent Positive
100
80
60
40
20
10
8
6
4
2
0
RAW -
RAW LPS
RAW LPS + IFNg
RAW LPS + IL-4
IL-6
IL-10
TNFa
IL-12p40


Cytokine Secretion of R9609
Percent Positive
4
3
2
1
0
R9609 -
R9609 LPS
R9609 LPS + IFNg
R9609 LPS + IL-4
IL-6
IL-10
TNFa
IL-12p40

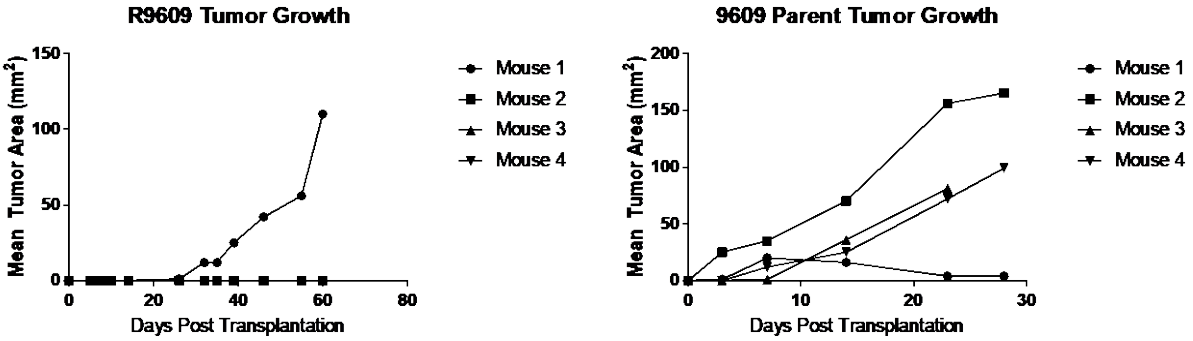


Figure 6. R9609 tumor cells permit control of 9609 parental tumors when they are tumorigenic. Measurements of R9609 tumors up to 60 days post-transplantation on left flank of C57BL/6 mice. After 30 days, 9609 parental cells were transplanted on contralateral flank of same mouse and measured.

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