IL-15 AND TYPE I INTERFERON ARE REQUIRED FOR ACTIVATION OF
TUMORICIDAL NK CELLS BY VIRUS-INFECTED DENDRITIC CELLS

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Abstract

There is increasing evidence that natural killer (NK) cells play an important role in antitumor immunity following dendritic cell (DC) vaccination. Little is known, however, about the optimal stimulation of DCs that favors NK activation in tumor-bearing hosts. In this study, we demonstrate that treatment with TLR ligands or infection with a mutant vesicular stomatitis virus (VSV-ΔM51) both induced DC maturation. Further, inoculation of these DCs led to robust NK-mediated protection against tumor challenge. Strikingly, only VSV-ΔM51-infected DCs were capable of suppressing the growth of established tumors, suggesting that additional signals provided by viral infection may be required to activate tumoricidal NK cells in tumor-bearing hosts. VSV-ΔM51 infection of DCs induced greater type I interferon (IFN I) production than TLR ligand treatment, and disruption of the IFN I pathway in DCs eliminated their ability to induce NK activation and tumor protection. However, further studies indicated that IFN I alone was not sufficient to activate NK cells, especially in the presence of a tumor, and DC-derived IL-15 was additionally required for tumoricidal NK activation. These results suggest that induction of IFN I by VSV-ΔM51 allows DCs to overcome tumor-associated immunosuppression and facilitate IL-15-mediated priming of tumoricidal NK cells. Thus, the mode of DC maturation should be carefully considered when designing DC-based cancer immunotherapies.
Introduction

Dendritic cell (DC)-based cancer vaccines have been widely used to prime antigen-specific T cell responses to tumors. This approach has shown significant promise in both pre-clinical animal models and clinical trials (1-3). However, a clear relationship between the induction of antigen-specific T cell responses and clinical outcome remains to be fully established. In fact, several recent clinical trials have shown that NK cell responses following vaccination with DCs or DC-derived exosomes correlated more closely with positive clinical outcomes than T cell responses (4-6). Our own preclinical data also demonstrate that activation of NK cells is a necessary step for maximal antitumor immunity, especially in animals with established tumors (7). Furthermore, the induction of an immunosuppressive environment during tumor development and the outgrowth of antigen-loss variant tumor cells create difficult obstacles for current approaches that only aim at T cell activation (2, 8, 9). Therefore, DC-based cancer vaccines may be improved by the inclusion of strategies that activate complementary immune components and elicit a pro-inflammatory tumor microenvironment.

NK cells are important for maintenance of homeostasis and prevention of tumorigenesis (10). However, although NK cells are capable of eliminating transformed cells without prior stimulation (11), evidence from previous studies indicates that NK cells must be additionally activated to provide protection against otherwise fatal tumor challenges in several different mouse models (12-15). The cross talk between DCs and NK cells has been well documented, and reports from our group and others have demonstrated that DC vaccines can potently activate NK cells (7, 12, 13, 16). However, similar to T cells, NK
cells are also subject to regulation by tumor-associated immunosuppressive mechanisms and conceivably it would be more difficult to elicit NK activity in tumor-bearing hosts even with DC vaccines (17-20). Indeed, we have shown that LPS-matured DCs can effectively prime NK cells against tumor challenge but failed to induce NK-mediated protection in tumor-bearing animals. Interestingly, however, infection of DCs with an interferon-inducing mutant of vesicular stomatitis virus (VSV-ΔM51) (21) rendered them capable of activating NK-mediated antitumor activity in mice with pre-existing tumors (7). It appears that the mode of DC activation is critical for DC:NK interaction and that VSV-ΔM51 infection can provide additional signals enabling DC to prime NK cells in the tumor cell environment.

In this work, we extend our previous studies to confirm the superiority of VSV-ΔM51-infected DCs in activating tumoricidal NK cells and investigate the underlying mechanisms. We demonstrate that VSV-ΔM51 potently and uniquely induces high-level IFN I production, which is critical for autocrine signaling in DCs and subsequent induction of antitumor immunity in vivo. Furthermore, we revealed that IL-15, a cytokine induced by IFN I, is required for the NK activation and tumor protection generated by VSV-ΔM51-infected DCs in tumor-bearing hosts.
Materials and Methods

Mice. Female 6-8 week old C57BL/6 mice were obtained from Charles River laboratories (Wilmington, MA). IFNAR\(^{-/-}\), MyD88\(^{-/-}\), TLR4\(^{-/-}\), IL-15\(^{-/-}\), IL15R\(\alpha\)^{-/-}, IRF3\(^{-/-}\) and IRF7\(^{-/-}\) mice were bred in the McMaster central animal facility. All procedures conformed to the guidelines established by the Canadian Council on Animal Care and were approved by the McMaster Animal Research Ethics Board.

Recombinant Vesicular Stomatitis Virus. Production of recombinant vesicular stomatitis viruses (VSV-\(\Delta M51\)) has been previously described (7). VSV-\(\Delta M51\) possesses a deletion mutation in the coding region for the matrix protein, impairing its ability to inhibit the host cells’ antiviral immune response (21). VSV-\(\Delta M51\) encoding a bicistronic construct of murine IL-15 and IL-15 receptor alpha (VSV-IL15/R\(\alpha\)) was constructed by cloning murine IL-15 and murine IL15R\(\alpha\) flanking the GTX Internal Ribosome Entry Site (IRES). Production of IL-15 was confirmed using the IL-15 duoset ELISA, according to manufacturer’s instructions (R&D systems, Burlington, Canada) and IL15R\(\alpha\) surface expression was confirmed by flow cytometry (anti-IL15R\(\alpha\); clone N-19, Santa Cruz Biotechnology, CA) (data not shown).

Dendritic cell culture and activation. Culture of dendritic cells from bone marrow has been described previously (22). DCs were activated by incubation with either 10 \(\mu g/mL\) CpG (sequence 1826, DC/CpG), 10 \(\mu g/mL\) LPS (DC/LPS), 25 \(\mu g/mL\) polyI:C, (DC/polyI:C), or by infection with 25 PFU/cell VSV-\(\Delta M51\)-MT (DC/VSV) for 4 h, as described elsewhere (7). Cells were harvested and washed thrice using cold PBS prior to
experimental use. For in vivo experiments, 1x10^6 DCs were inoculated by subcutaneous (s.c.) footpad injection.

**Tumor cells and challenge model.** The growth and propagation of B16F10-OVA cells have been described (7, 12). This cell line was obtained from Dr. Kenneth Rock (University of Massachusetts Medical School), and propagated in G418 to maintain OVA expression. Intracellular staining for gp100 (clone: HMB-45) and ELISPOT using OT-1 cells as effectors have been carried out within the past 12 months to validate this cell line. To establish lung tumor nodules, 1x10^6 tumor cells were inoculated by intravenous (i.v.) tail vein injection. Tumor cells were injected either 7 days after DC inoculation (prophylactic model) or 10 days before DC injection (therapeutic model). Mice were euthanized 21 days after tumor challenge, and lungs were collected and fixed in 4% paraformaldehyde. The number of tumor nodules was counted using a dissecting microscope. In the event that individual tumor nodules could not be accurately distinguished, lungs were assigned a value of 1000, based on an attempted count of tumor burden.

**FACS analysis for surface marker expression and intracellular cytokines.** All flow cytometry reagents were obtained from BD Pharmingen (Mississauga, ON, Canada), unless otherwise noted. Following stimulations or mock treatments, DCs were treated with FcBlock prior to staining with surface antibodies: anti-CD11c (clone HL3), anti-CD40 (clone 3/23), anti-CD86 (clone GL1), and anti-MHC II (clone 25-9-17). To assess cytokine production by DCs, cells were incubated in the presence of GolgiPlug for 12 h.
after infection or treatment, then fixed and permeabilized using the cytofix/cytoperm kit from BD Pharmingen, according to manufacturer’s instructions. Intracellular stains were performed using anti-TNF-α (clone MP6-XT22) or anti-IL-12 (p40/p70) (clone C15.6) mAbs. NK cells were surface stained using anti-NK1.1 (clone PKH136) and anti-CD3 (clone 145-2C11). Intracellular staining of NK cells was completed using anti-IFNγ (clone XMG1.2). All samples were examined using a BD FACSCanto flow cytometer (BD Pharmingen) and analyzed using FlowJo 9.0.1 software (TreeStar, Ashland, OR, USA).

Type I IFN bioassay. Quantification of type I IFN production by DCs by IFN bioassay has been described (7).

Lysate preparation and western blotting. DCs were infected and harvested at the indicated timepoints, and washed using cold PBS. Cells were resuspended in RIPA lysis buffer supplemented with the protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktails I and II (Sigma-Aldrich, Mississauga, ON, Canada). Protein concentrations were quantified by Bradford assay, and proteins were separated on 7.5% acrylamide gels. Following transfer, membranes were probed using anti-phospho STAT-1 (tyr 701, Millipore, Temecula, CA) and anti-β-actin (Sigma-Aldrich). Membranes were incubated with fluorochrome-conjugated anti-rabbit and anti-mouse secondary antibodies and analyzed using the Odyssey Infrared Imaging system (LiCor, Lincoln, NB).
**NK:DC coculture.** DCs were treated with TLR ligands or infected with VSV-ΔM51 for 4 h, washed thrice and resuspended in fresh media. NK cells were purified from naïve C57BL/6 spleens using a negative selection NK cell enrichment kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. NK purity was typically >85% (data not shown). NK cells and DCs were seeded at a 1:1 ratio (5x10⁴ of each cell type) into 96-well V-bottom plates. For cocultures involving tumor cells, 1x10⁴ cells were added at the beginning of the coculture period. Cells were allowed to interact for 12 h and GolgiPlug was added to wells after the first 6 h of culture. In some experiments, a blocking antibody for the IL-15 receptor β (AbD Serotech, Raleigh, NC) or IgG2b isotype control was used to pretreat NK at 40 μg/mL at room temperature before coculture with DC/VSV.

**Statistical analysis.** Tumor burdens and DC maturation were compared using a Kruskall-Wallis non-parametric analysis, with Dunn’s post-hoc test. For all analyses, p<0.05 was considered to be statistically different from chance alone.
Results

DC/VSV uniquely activates NK cells for therapeutic anticancer activity. We (12), and others (13, 23), have demonstrated that DCs can activate NK cells for protection against a subsequent tumor challenge. Administration of ex vivo-cultured DCs by intraplantar injection leads to initial accumulation of activated NK cells in the draining lymph nodes, which subsequently circulate through the peripheral blood to spleen and lungs (12, 13). More recently, we reported that DCs infected with an interferon-inducing mutant of vesicular stomatitis virus (VSV-ΔM51), but not matured with LPS, could effect reduction of established lung metastatic tumors in vivo by activating tumoricidal NK cells (7). Depletion of NK cells fully abrogated tumor protection, even when tumor-specific T cells were activated, demonstrating that this phenomena is indeed NK-dependent (7). We have also ruled out a possible role of VSV-ΔM51 as an oncolytic virus in this model, demonstrating that VSV-ΔM51 infection was abortive in DCs and inoculation of VSV-ΔM51 alone was not sufficient to impact B16F10 tumors (7). In the present investigation, we aimed to determine whether VSV-ΔM51 infection was indeed superior to TLR ligand exposure to mature DCs for activation of antitumor immunity in vivo. To that end, we compared four strategies for DC activation for their ability to elicit tumor protection: TLR stimulation using LPS (DC/LPS), CpG (DC/CpG) or polyI:C (DC/pI:C), and infection with VSV-ΔM51 (DC/VSV). The dose of each agent was determined by its ability to induce maximal DC maturation with minimum toxicity ((7) and data not shown). As expected, each of these maturation strategies primed DCs to activate prophylactic tumor protection in vivo (p<0.05). In hosts bearing pre-existing tumors, however, DC/VSV evoked significantly greater tumor protection than DCs matured by...
TLR ligation (DC/TLR) (Figure 1A; p<0.05). To determine if this effect was attributable to enhanced maturation of DCs following VSV-ΔM51 infection, we compared prototypical markers of DC activation induced by the various stimuli. Proinflammatory cytokine production (IL-12 and TNF-α, Figure 1B) and surface markers (CD40, MHC II and CD86, Figure 1C) were upregulated by each maturation strategy. Although the absolute levels were variable among treatments, the difference for cytokine production and CD86 expression did not reach statistical significance. Higher CD40 level was induced by both VSV-ΔM51 and LPS (p<0.05), and VSV-ΔM51 induced slightly greater MHC II expression compared with all TLR ligands (p<0.05).

Since VSV-ΔM51 is a potent IFN I inducer, we also examined IFN I production induced by viral infection and TLR ligation (21). Indeed, VSV-ΔM51 stimulated 10- to 100-fold greater production of IFN I by DCs compared with TLR ligation (Figure 1D).

Tumor protection activated by DC/VSV is dependent on the IRF-3 signaling pathway.

DCs sense VSV-ΔM51 infection via both TLR4 and intracellular interferon signaling (24, 25). Therefore, the increased IFN I production and surface marker expression induced by VSV-ΔM51-infected DCs, compared with TLR ligands, may be attributable to the engagement of multiple signaling pathways. To discern the relative contributions of the TLR and interferon signaling pathways for DC activation, we derived DCs from mice deficient for IFN I induction (IRF3-/-) or TLR4 signaling (TLR4-/- or MyD88-/-). DCs were infected with VSV-ΔM51 and used to treat mice bearing 10-day established lung tumors. As shown in Figure 2A, tumor protection was lost when DCs were deficient for
IRF-3 (p<0.05), but was still generated by TLR4⁻/⁻ or MyD88⁻/⁻ DCs. Interestingly, VSV-ΔM51 infection of IRF-3⁻/⁻, TLR4⁻/⁻ or MyD88⁻/⁻ DCs still prompted upregulation of surface maturation markers and production of TNF-α and IL-12 (Figures 2B and C). LPS and CpG exposure, however, failed to induce maturation of TLR4⁻/⁻ or MyD88⁻/⁻ DCs, respectively, confirming functional impairment of these pathways (data not shown). Together, these results imply that redundant mechanisms exist for upregulation of prototypical markers of DC maturation. In contrast, IFN I production was decreased by 2 logs in IRF-3⁻/⁻ DCs compared with WT cells, but was not affected by deletion of MyD88 or TLR4 (Figure 2D). Thus, IRF-3 is non-redundant and indispensable for high-level IFN I production and antitumor activity generated by VSV-infected DCs.

Autocrine/Paracrine IFN I is required for DC activation. Our results demonstrate that IRF-3⁻/⁻ DCs produce significantly less IFN I after VSV-ΔM51 infection which correlates with loss of DC/VSV-generated antitumor activity. IRF-3 initiates IFN I production, however, maximal IFN I production requires IRF-7 activation, which is induced by autocrine/paracrine IFN I signaling (26). To determine whether IFN I signals to DCs after VSV-ΔM51 infection, we measured STAT-1 phosphorylation by western blotting. Activation of this protein was obvious by 4 h post-infection and persisted for at least 24 h, confirming that DCs both make and respond to IFN I in response to VSV-ΔM51 infection (Figure 3A). To determine whether autocrine/paracrine IFN I signaling and high-level IFN I production in DCs are relevant for priming antitumor activity, we conducted our in vivo tumor challenge model using DCs that lacked IFNAR or IRF-7. Loss of either IFN I signaling or IRF-7 activity abrogated the anti-tumor effect of the
DC/VSV (Figure 3B), and corresponded with a drastic reduction in IFN I production (Figure 3C). These results demonstrate that autocrine IFN I signaling and high-level IFN I production are critically required to generate antitumor immunity in vivo.

Tumor cells inhibit NK cell activation by IRF-3 or IRF-7 deficient DCs in vitro. Our in vivo results indicate that IRF-3, IRF-7 and IFNAR are critical for high-level IFN I production and in vivo tumor protection. To further delineate their role in DC activation of NK cells, we developed an in vitro DC:NK coculture system. After extensive analysis with different ratios and incubation times, we found that a 1:1 ratio of naïve NK cells:DC/VSV resulted in activation of IFN-γ expression within the NK cells by 12 h (Figure 4). Neither mock-treated, nor TLR ligand-activated DCs were able to activate NK cells to produce IFN-γ under the same conditions, confirming that VSV-ΔM51 infection is indeed superior to other approaches for facilitating DC-mediated activation of NK cells.

To determine whether IFN I signaling was required in this coculture system, we used IRF-3−/−, IRF-7−/− or IFNAR−/− DCs. IFNAR−/− DCs infected with VSV-ΔM51 could not activate NK cells (Figure 5A), confirming a requirement for autocrine/paracrine IFN I signaling in DCs. Surprisingly, however, IRF-3−/− and IRF-7−/− DCs, which did not produce high levels of IFN I, retained the capacity to activate NK cells in vitro, at levels similar to WT controls (Figure 5A). We reasoned that in vitro DC:NK cocultures may not be subjected to the same immunosuppression imposed in vivo by the presence of a tumor. Thus, the small amount of IFN I produced by DCs in the absence of IRF-3 or
IRF-7 could be sufficient to prime DCs for activation of NK cells in tumor-free, but not tumor-bearing conditions. To address this possibility, we repeated the in vitro experiments with tumor cells included in the co-culture. As shown in Figure 5B, the presence of tumor cells did not impede the ability of VSV-ΔM51-infected WT DCs to activate naïve NK cells in vitro. However, VSV-ΔM51-infected DCs that lacked IRF-3 or IRF-7 were compromised in their ability to activate NK cells when tumor cells were present, presumably due to the loss of high-level IFN I production. These results demonstrate that although a high concentration of IFN I may not be necessary for NK cell activation under tumor-free conditions, it is critical for activation of NK cells in the stringent environment imposed by tumor cells.

DC-derived IFN I does not directly activate NK cells. Since both our in vivo and in vitro results pointed to a critical role for IFN I production and signaling within VSV-ΔM51-infected DCs, we decided to assess whether IFN I could directly activate NK cells in culture. We first set up a coculture using the conditions described above, but separated DCs and NK cells using a transwell system. As an additional approach, we transferred the supernatant from VSV-ΔM51-infected DCs onto naïve NK cells in separate wells without a transwell membrane. In both cases, NK cells were exposed to soluble factors secreted by VSV-ΔM51-infected DCs, but the cells were not in direct contact. NK cells could not be activated across a transwell or by supernatant transferred from DC/VSV (Figure 6), demonstrating that soluble factors produced by DCs, including IFN I, are not sufficient for NK activation by DC/VSV. These results suggest that an additional signal, which is dependent on direct cell-to-cell contact, is required for DC/VSV:NK activation.
**IL-15 is required for tumoricidal NK activation.** IL-15 is a surface-bound cytokine, presented by myeloid DCs via its receptor, IL15Rα, to the β and γ receptors on NK cells (27). We hypothesized that IL-15 could be mediating the interaction between DC/VSV and NK cells because it is a potent inducer of NK cell activation, and induced by IFN I (26-28). We infected DCs derived from IL-15−/− bone marrow and used them in coculture with naïve NK cells. As shown in Figure 7A, IL-15−/− DCs elicited NK cell activation, but to a lesser extent than WT DCs. When tumor cells were included in the coculture wells, NK activation by the IL-15−/− DCs was completely abrogated. To confirm this finding with WT DCs, we pretreated naïve NK cells with an antibody to block the IL-15 receptor β chain prior to coculture. Once again, loss of IL-15 signaling abrogated NK cell activation by VSV-ΔM51-infected DCs when cultured in the presence of tumor cells (Figure 7B). Together, these findings demonstrate that IL-15 signaling is important for mediating the potent activation of NK cells by DC/VSV.

**IL-15 signaling mediates NK:DC interaction in vivo.** To determine whether IL-15 was similarly required for NK cell activation in vivo, we used VSV-ΔM51-infected WT, IL-15−/−, or IL15Rα−/− DCs for inoculation of tumor-bearing mice. As shown in Figure 7C, loss of IL-15 signaling in DCs, either through impaired cytokine production or trans presentation, abrogated tumor protection in vivo. Finally, to further confirm the critical role for IL-15 in vivo, we constructed a recombinant VSV-ΔM51 virus that co-expresses IL-15 and IL15Rα (VSV-IL15/Rα). When VSV-IL15/Rα was used to infect IL-15−/− DCs prior to inoculation into tumor-bearing mice, tumor protection was restored to the level
achieved by WT DCs infected with VSV-ΔM51. Thus, our results demonstrate that IL-15 production by DC/VSV is critical for mediating direct interaction with, and activation of, tumoricidal NK cells in the presence of tumor cells.
**Discussion**

Although most cancer vaccines are primarily designed to elicit antigen-specific T cells, increasing evidence points to a critical role for NK cells in vaccine-induced antitumor immunity (7, 12, 13, 29, 30). DC vaccines are particularly effective in activating NK cells that can modulate both innate and adaptive antitumor activities (7, 12, 13, 30). More interestingly, we have demonstrated that once activated, NK cells provide protection against tumor challenge for at least 20 days, implying that NK cells are much more related to lymphocytes than generally appreciated (12, 31-33). However, little is known about DC activation of NK cells in tumor-bearing hosts. In the present investigation, we showed that TLR ligation using CpG, LPS or poly:I:C enabled DCs to induce NK-mediated tumor protection but failed to significantly impact established tumors. However, VSV-ΔM51 infection rendered DCs capable of activating tumoricidal NK cells in cancer-bearing animals. We identified that the superiority of DC/VSV was due to high-level IFN I production and autocrine/paracrine signaling, which were critical for activation of NK cells to combat pre-existing tumors. Furthermore, IL-15, a cell-bound cytokine induced by IFN I, is essentially required to mediate the interaction between DC/VSV and NK cells in tumor-bearing environments.

It has been shown that VSV-ΔM51 infection promotes DC maturation via TLR4 and intracellular IFN I signaling (24, 25). However, these pathways appear to be redundant for DC maturation in our studies since DCs devoid of TLR4, MyD88 or IRF-3 could generate a ‘mature’ phenotype following VSV-ΔM51 infection. Immunization with either DC/TLR or DC/VSV activated prophylactic immunity, suggesting that maturation...
with TLR agonists or VSV-ΔM51 infection is sufficient to activate NK cells in the absence of the tumor. The requirement of IFN I for VSV-ΔM51-infected DCs to prime tumoricidal activity in mice with pre-existing tumors is not through further induction of DC maturation but instead that IFN I may be required as an additional mechanism to overcome tumor-induced immunosuppression.

In myeloid DCs, IFNβ production is initiated via activation of IRF-3. Activation of IRF-7 following initial IFN I signaling through IFNAR leads to production of IFNα (26, 34). Our results show that IRF-3, IRF-7 and IFNAR are required to generate antitumor activity, but soluble factors alone are insufficient to directly activate NK cells. This observation does not necessarily rule out the possibility that the IFN I produced by DC/VSV may nonetheless contribute to antitumor activity in vivo. In fact, IFN I has been used clinically for treatment of both solid and hematologic malignancies (35, 36). The effect of IFN I cancer treatment is likely attributable to a number of mechanisms. For example, IFN I can assist in maintaining T cell viability in the tumor microenvironment, reinforce NK activation, and activate endogenous DCs for tumor antigen cross-presentation (37-40). IFN I may also alter the tumor directly, for example, by inducing upregulation of MHC I molecules on tumor cells (38, 41). A recent report demonstrated that IFN I signaling in bone marrow cells elicited production of the type III IFN, IL-28, which, upon binding to tumor cells, induced upregulation of target killing ligands for NK cells (42). Finally, a number of studies have demonstrated that tumor cells engineered to produce IFN I are unable to resist T and NK cell-mediated killing, in contrast to their parental counterparts (37, 43), suggesting that IFN I may assist in altering tumor-induced immune responses.
immunosuppression. Thus, although IFN I produced by VSV-ΔM51-infected DCs does not directly mediate NK cell activation, it may contribute to the overall success of DC/VSV-mediated tumor rejection.

Our experiments demonstrate that NK activation by DC/VSV is mediated by contact-dependent IL-15 signaling, both in vitro and in vivo. IL-15 signaling is important for NK cell development (44, 45), and potently activates NK cells for IFN-γ production and cytotoxicity (46-48). IL-15Rα chaperones IL-15 and mediates its presentation on the surface of DCs (27), thus, DC:NK interactions mediated by IL-15 require cell-to-cell contact. Our observation is in agreement with recent findings by Vujanovic and colleagues, who report that membrane-bound IL-15 and TNF-α mediate activation between adenovirus-infected DCs and NK cells (48).

IL-15 is critical for clearance of several virus infections, including HSV (49) and MCMV (46). IL-15 production and NK cell activation may be a common outcome of virus infection of DCs, endorsing virus infection as a strategy to mature DCs for use as cancer vaccine platforms. Additionally, viruses can be used to transduce DCs with tumor-associated antigens to prime antigen-specific immunity (1, 7, 50), and thus, virus infection of DCs should facilitate efficient antitumor immunity by activation of both antigen-specific T cells and tumoricidal NK cells.

Our in vitro results demonstrated that IRF3−/−, IRF7−/−, IFNAR−/− and IL-15−/− DCs could activate NK cells, unless tumor cells were also present in the coculture wells. Moreover,
each of these knockout cell types showed similar IL-12 and TNF-α production and phenotypic maturation following VSV-ΔM51 infection (Figures 1, 2 and data not shown). These results demonstrated that redundant pathways exist for DC activation, but intact interferon and IL-15 signaling are critical for productive activation of NK cells in the presence of tumor cells. Although the exact mechanisms by which tumor cells suppress IFN-γ production by NK cells during NK-DC interaction remain elusive, studies from others have suggested that tumor cell-derived cytokines or exosomes decrease NK activities (17-20). Our results suggest that the inclusion of strategies, such as VSV-ΔM51 infection, to enhance innate interferon and IL-15 production may assist in developing robust DC-based therapies with the capacity to activate NK cells in tumor-bearing individuals.

Successful cancer immunotherapy will require the development of strategies capable of activating immunity in immunosuppressed tumor-bearing individuals, and which do not rely exclusively on specific tumor-associated antigen expression. Our results demonstrate that NK cells activated by DC/VSV, but not DC/TLR, can dramatically reduce the burden of pre-existing tumors, via IFN I- and IL-15-mediated mechanisms. Thus, the mode of DC activation is an important determinant to the outcome of DC-based cancer therapies. Therefore, these cancer vaccines could be improved if designed to engage and activate NK cells in addition to priming T cells.
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Conflict of Interest Statement

We declare that we have no conflict of interest.
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Figure legends

Figure 1. VSV-ΔM51-infected DCs elicit superior therapeutic antitumor activity.

(A) Mice were challenged by intravenous tail vein injection of 10^6 B16F10-OVA tumor cells either 7 days after (day -7) or 10 days before (day +10) s.c. inoculation with 10^6 differentially-matured DCs. DCs were treated by exposure to LPS, CpG or polyI:C, or infected with VSV-ΔM51, as described in materials and methods. Mice were sacrificed 21 days after tumor challenge, and lungs were fixed in 4% paraformaldehyde prior to counting using a dissecting microscope. *, significant reduction compared with untreated mice (p<0.05). Data are representative of 2 individual experiments with 3-5 mice per group, per experiment. (B) DCs were treated as described for 12 h in the presence of golgi plug. DC production of TNF-α and IL-12 were measured by flow cytometry. (C) 24 h following treatment or infection, DCs were harvested and analyzed for cell surface expression of phenotypic markers of maturation, including CD86, MHC II and CD40. *, significantly greater compared with all other groups (p<0.05). (D) Supernatants were collected from cell cultures 24 h after infection or TLR treatment, and assayed for IFN I concentrations, as described in materials and methods. *, significantly greater than all other groups (p<0.05). Data from panels B, C and D are representative of duplicate experiments, with 3 wells per treatment per experiment.

Figure 2. DC/VSV-generated tumor protection is dependent on IRF-3 signaling, but independent of toll-like receptor signaling. Mice were challenged with 10^6 B16F10-OVA cells by intravenous tail vein injection 10 days prior to inoculation with VSV-infected WT, MyD88^-/-, TLR4^-/- or IRF-3^-/- DCs. Twenty-one days following tumor cell
inoculation, mice were sacrificed, and the number of surface tumor nodules was enumerated after fixation. *, significantly different from IRF3−/− and PBS-treated mice (p<0.05). Data are representative of two individual experiments with 3-5 mice per group, per experiment. To measure DC maturation, cytokine production (B) and phenotypic maturation (C) were measured as described in Figure 1. (D) IFN I bioassay was carried out on supernatants collected 24 h after VSV infection of MyD88−/−, TLR4−/−, IRF-3−/− or WT DCs. *, significantly different from WT (p<0.05). Data for B, C and D were generated from 2 independent experiments, with triplicate wells per sample in each trial.

**Figure 3. Autocrine/Paracrine IFN I signaling is required in DCs to activate antitumor immunity.** (A) To determine if DCs were responding to IFN I, STAT-1 phosphorylation was measured at predetermined timepoints in WT DC lysates following infection with VSV-ΔM51 by western blotting. (B) Mice bearing 10-day old lung B16F10-OVA tumors were inoculated with VSV-infected DCs derived from WT, IFNAR−/− or IRF-7−/− mice. Lungs were harvested and fixed 21 days after tumor challenge, and the number of visible surface tumors was counted with the aid of a dissecting microscope. ***, significantly lower tumor burden compared with all other groups (p<0.01). Data are representative of duplicate experiments using 3-5 mice per group, per experiment. (C) IFN I production within the first 24 h after infection was quantified in the supernatants by IFN I bioassay. *, significantly different from WT (p<0.05). Data are representative of duplicate experiments.
Figure 4. VSV-ΔM51 infection activates DCs to prime their interaction with NK cells. DCs were activated by exposure to polyI:C (ii), LPS (iii) or CpG (iv.), infected with VSV-ΔM51 for 4 h (v. VSV), or left untreated (i. mock). NK and DCs were cocultured at a 1:1 ratio in 96-well V-bottom plates. After 6 h of coculture, GolgiPlug was added, and cocultures were continued for an additional 6 h. NK cell activation was measured by intracellular cytokine staining for IFN-γ. Representative FACS plots for each treatment are shown.

Figure 5. Interferon production and signaling are required to activate NK cells in the presence of tumor cells. VSV-ΔM51 infected WT, IFNAR, IRF-3−/− or IRF-7−/− DCs were cocultured with naïve WT NK cells as described in Figure 4, in the presence (A) or absence (B) of tumor cells. Representative FACS plots are shown.

Figure 6. NK activation by VSV-infected DCs is dependent on cell:cell contact. NK cells were cultured with VSV-ΔM51-infected DCs, either together, to allow for cell-to-cell contact (i) or across a transwell insert (ii). (iii) Supernatant from DCs infected with VSV-ΔM51 for 10 h was transferred to naïve NK cells. IFN-γ production was measured 6 h later. Representative FACS plots are shown.

Figure 7. NK activation by DC/VSV in tumor-bearing mice is mediated through an IL15-dependent mechanism. (A) WT or IL-15−/− DCs were cocultured with naïve NK cells in the presence or absence of tumor cells. NK cell activation was measured by IFN-γ production 12 h later. Representative FACS plots are shown. (B) DCs were cultured at
a 1:1 ratio with naïve NK cells, either with no pre-treatment (i), pretreatment with 40 
µg/mL anti-IL15Rβ (ii), or Rat IgG2a isotype control, as described in materials and 
methods. IFN-γ production was measured as an indicator of NK cell activation 12 h after 
the beginning of the coculture period. Representative FACS plots are shown. (C) Mice 
bearing 10-day old lung tumors were inoculated with VSV-ΔM51-infected DCs that had 
been derived from WT, IL-15−/− or IL15Rα−/− mice. An additional group of mice was 
treated with IL-15−/− DCs infected with VSV-ΔM51 carrying a bicistronic construct of IL-
15 and IL15Rα. Lungs were collected and fixed 21 days following tumor challenge and 
the number of tumor nodules was counted using a dissecting microscope. *, significantly 
reduced tumor burden compared with IL-15−/− DC/VSV, IL15Rα−/− DC/VSV and PBS-
treated mice (p<0.05). Data are representative of duplicate experiments with 3-5 mice 
per group.
Boudreau Figure 1

A. Surface Tumor Nodules

- # Day -7
- # Day +10

PBS | DC/poly:C | DC/LPS | DC/CpG | DC/VSV
---|---|---|---|---

B. % of CD11c+ cells

- TNFα
- IL12

C. Fold increase over mock-treated cells

- MHC II
- CD40
- CD86

D. Acid-resistant IFNα (U/mL/10^6 cells)

- mock
- pl:C
- LPS
- CpG
- VSV

Legend:
- mock
- poly:C
- LPS
- CpG
- VSV
**Figure 3**

**A**

Time post infection (h)

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<th>Time (h)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>24</th>
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</thead>
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<td>Phospho STAT-1</td>
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<tr>
<td>β-actin</td>
<td><img src="image2" alt="β-actin blots" /></td>
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</table>

**B**

# surface tumor nodules

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>WT DC/NSV</th>
<th>IRF7+/− DC/NSV</th>
<th>IFNAR+/− DC/NSV</th>
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</thead>
<tbody>
<tr>
<td># surface tumor nodules</td>
<td><img src="image3" alt="Bar chart" /></td>
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**C**

Acid-resistant type I IFN (U/mL/10⁶ cells)

<table>
<thead>
<tr>
<th>Genotype of infected DCs</th>
<th>WT</th>
<th>IRF7+/−</th>
<th>IFNAR+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-resistant type I IFN (U/mL/10⁶ cells)</td>
<td><img src="image4" alt="Acid-resistant type I IFN chart" /></td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Boudreau Figure 7

A

WT

i. without tumor cells

ii. with tumor cells

IL-15−/−

IFNγ

NK1.1

4.21

1.19

B

i. no antibody

ii. anti-IL15Rβ

iii. rat IgG2b isotype

IFNγ

NK1.1

22.9

0.01

22.9

C

# Surface Tumor Nodules

 Pretreatment

IL-15−/− DCCV+/- MT

IL-15−/− DCCV+/- MT

WT DCCV+/- MT

IL-15−/− DCCV+/- MT

*
IL-15 AND TYPE I INTERFERON ARE REQUIRED FOR ACTIVATION OF TUMORICIDAL NK CELLS BY VIRUS-INFECTED DENDRITIC CELLS

Jeanette E Boudreau, Kyle B Stephenson, Fuan Wang, et al.

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