

Viral antigen mediated NKp46 activation of NK cells results in tumor rejection via NK-DC crosstalk

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Abbreviations: DC, dendritic cell; NK, natural killer cell; HN, haemagglutinin-neuraminidase

Natural killer (NK) cells play a critical role in antitumor immunity, their activation being regulated through NK cell receptors. Although the endogenous ligands for these receptors are largely unknown, viral ligands have been identified. We investigated the ability of an activating NK receptor ligand derived from the mumps virus, haemagglutinin-neuraminidase (HN) to enhance NK activation against tumor cells. HN-expressing B16.OVA tumor cells induced stronger activation of NK cells compared with B16.OVA cells and also promoted dendritic cell (DC) activation toward a DC1 phenotype, *in vitro*. Moreover, incubation of DCs, NK cells and HN-expressing B16-OVA cells further enhanced NK cell activation through the NK-DC crosstalk, in a cell-to-cell contact- and IL-12-dependent fashion. Immunization of mice with HN-expressing B16-OVA cells resulted in > 85% survival rate after subsequent challenge with parental B16 or B16.OVA tumor cells. Tumor rejection was dependent on both NK and CD8⁺ T cells but not on CD4⁺ T cells, demonstrating induction of an effective adaptive immune response through innate immune cell activation. Our data indicate the potential of using robust NK cell activation, which through the NK-DC crosstalk stimulates effective antitumor responses, providing an alternate vaccine strategy.

Introduction

Recognition of tumor cells by the immune system is essential for effective antitumor immune responses. Although it has been well established that the immune system is capable of recognizing tumor-specific antigens and eradicating malignant cells, the optimal method for harnessing the immune response against cancer remains elusive. The process is indeed complex and involves the orchestrated activities of innate and acquired immunity.

Natural killer (NK) cells are lymphocytes of the innate immune system which play a key role in the defense against tumors and viral infections. NK cell activation resulting in target cell lysis and/or cytokine and chemokine production is mediated by various activating receptors. These include NKp46, NKp30 and NKp44, collectively termed natural cytotoxicity receptors (NCRs).^{1,2} NCRs are unique to NK cells with NKp46 and NKp30 being expressed on both resting and activated NK cells and NKp44 being expressed only upon activation.^{1,2} Of note, only NKp46 is expressed in mice. Endogenous ligands for these activating receptors are mostly unknown, although viral ligands have been defined.³ A strong correlation between the density of NCR expression and the ability of NK cells to kill target cells, including a wide variety of tumor cells, has been identified.⁴ A role for NKp46 in antitumor immunity has been shown, as

the use of anti-NKp46 blocking antibodies inhibited the ability of NK cells to lyse targets,⁵ although the cellular ligand for NKp46 is unknown. On the other hand, influenza (A/PR/8/34) haemagglutinin (HA) and the haemagglutinin-neuraminidase (HN) of Sendai virus have been shown to trigger NKp46 signaling through binding of threonine at position 225, via α 2, 6-linked sialic acid in the membrane proximal domain of the molecule.^{3,6} NKp44 has been shown to trigger NK activation in response to the same ligands, via similar mechanisms.⁷ Viruses, in particular RNA paramyxoviridae, are being used as potential therapies for cancer. In particular, vaccines derived from viruses are being used to provide “danger” signals which would allow/enhance immune responses to tumor-associated antigens. These viral “danger” signals were found to induce both innate and adaptive immune responses, and promising antitumor activity was observed (reviewed in ref. 8).

NK activation has been shown to influence adaptive immune responses, predominantly through interaction with dendritic cells (DCs). Initial reports of the NK-DC interaction focused on NK activation by DCs. Both cytokine production by DC, which includes interleukin (IL)-12/IL-18, IL-15 and Type 1 interferons, as well as the direct contact between DCs and NK cells, have been shown to be required for DC-mediated NK cell activation.⁹ Once activated by DC, NK cells can mediate primary

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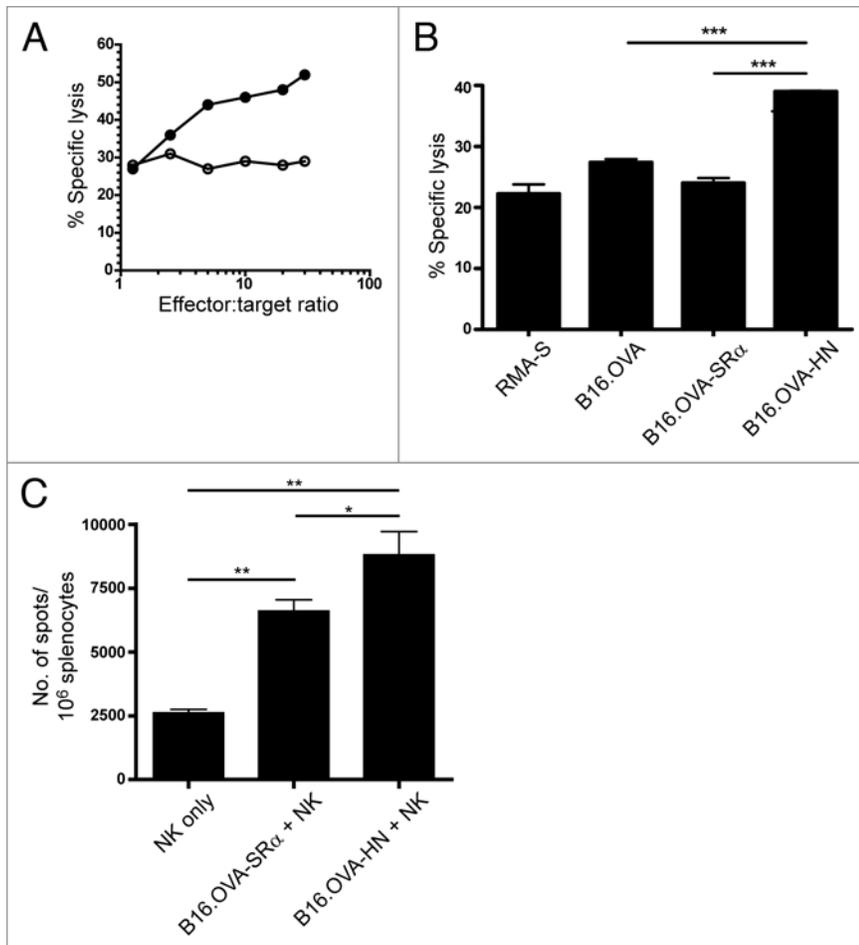


Figure 1. HN expression by B16.OVA tumor cells enhances NK cell responses. **(A)** Specific lysis of B16.OVA cells transfected with HN derived from mumps virus (+) or an empty vector (○) following incubation with syngeneic NK cells at effector:target ratios from 30:1 to 1.25:1. **(B)** Specific lysis of B16.OVA-HN, B16.OVA-SR α , B16.OVA or (Class I MHC-negative) RMA-S cells incubated with NK cells at a 10:1 effector:target ratio. *** $p < 0.001$, B16.OVA-HN cells in comparison with B16.OVA-SR α or B16.OVA cells. **(C)** The production of IFN γ was assessed by ELISpot following incubation of NK cells alone or with B16.OVA-SR α , B16.OVA-HN cells for 24 h. ** $p < 0.01$, * $p < 0.05$, B16.OVA-HN + NK cells in comparison to B16.OVA-SR α + NK cells and both compared with NK only. p values were calculated with two tailed Student's t -tests.

tumor rejections.¹⁰ The NK-DC interaction was found to be bidirectional and complex. Indeed, activated NK cells can induce DC maturation by producing cytokines including interferon γ (IFN γ) and tumor necrosis factor α (TNF α)^{11,12} and/or upon direct cell-cell contact,¹³ and can promote the generation of CD8⁺ T cell memory responses.¹⁴ Conversely, NK cells are capable of killing immature DCs by virtue of the low expression of NKG2A ligand (HLA-E) on immature DCs, and NK-cell activation via NKp30.^{15,16}

In this study we investigated the immunological effects of tumor cells expressing HN derived from mumps virus and its ability to enhance antitumor immune responses *in vivo*. Our data demonstrate that HN expression on tumor cells enhances NK cell activation and induces DC maturation. We also show that NK cell and DC activation is further stimulated through the NK-DC crosstalk, which enables the generation of robust

adaptive immune responses and provides protection to mice against subsequent challenges with cancer cells. This strategy therefore provides a strong basis for the development of novel anticancer immunotherapy protocols.

Results

HN expression on tumor cells enhances lysis by NK cells and production of IFN γ . NK cell activation through NKp46 engagement is important in mediating tumor cell lysis *in vivo*,¹⁷⁻¹⁹ however, the tumor cell targets are unknown. We therefore explored whether the expression use of viral antigens, known to engage NKp46, by tumor cells would facilitate NK cell activation and cancer cell lysis. B16.OVA tumor cells were transfected with the haemagglutinin-neuraminidase (HN) gene from the mumps virus and assessed for sensitivity to NK-cell mediated lysis. Stable transfection of B16.OVA with HN failed to generate long-term expressing clones, with HN expression being lost after 96 h despite survival in antibiotic selection medium. Therefore, transient transfection was employed. Transfection efficiency of B16.OVA was assessed by flow cytometry prior to each experiment and was typically 15–25%. NK mediated lysis of B16.OVA cells transfected with HN (B16.OVA-HN) was significantly greater than that of B16.OVA cells transfected with a vector (B16.OVA-SR α) (40% compared with 20% at 10:1 effector:target ratio; **Fig. 1A and B**), indicating the ability of HN to enhance tumor cell lysis. As an additional validation of NK activation, the ability of HN-expressing tumor cells to induce production of IFN γ by NK cells was assessed. NK cells incubated with

B16.OVA-HN cells induced a greater number of IFN γ -producing cells compared with B16.OVA-SR α (**Fig. 1C**), confirming the ability of HN to enhance NK responses to tumor cells.

NK cell activation by HN expressing tumor cells is dependent on NKp46. NK cell activation is governed by the balance between activating and inhibitory receptors, with the latter predominating in normal homeostasis. Following the increase in activation seen with HN-expressing tumor cells, we sought to identify the interactions that would be responsible for this phenomenon. A candidate NK activating receptor which responds to HN is NKp46, an NCR found only on NK cells, which has been shown to bind to viral haemagglutinin.^{3,6,20} We first investigated whether NKp46 bind HN expressed on tumor cells using immunoglobulin (Ig)-fusion constructs. NKp46-Ig incubated with B16.OVA-HN revealed binding to HN-expressing tumor cells, which was not observed when the unrelated NK receptor

KIR2DS4-Ig fusion was used (Fig. 2A and B). Interestingly, there was also low level of NKp46-Ig binding to B16.OVA cells, consistent with previous work suggesting the existence of currently unidentified NKp46 ligands on tumor cells.^{7,21} We next investigated whether the interaction between HN and NKp46 is required for the activation of NK cells. B16-OVA-HN cells incubated with NKp46-Ig were lysed by NK cells much less (~50%) than cells incubated with control Igs. The inhibition provided by NKp46-Ig was reduced as the amount of NKp46-Ig was reduced (Fig. 2C). In addition, no inhibition of NK-mediated lysis was observed when B16.OVA cells were incubated with NKp46-Ig (data not shown), indicating that the predominant mechanism underlying NK-mediated lysis was the interaction between NKp46 and HN. Failure to obtain complete inhibition with NKp46-Ig perhaps was due to the use of sub-saturating amounts of NKp46-Ig or to the involvement of other NK activating receptors.²¹

Induction of DC maturation and IL-12 production by tumor cells expressing HN. HN and other viral antigens have been shown to exert varying effects on the immune response. Thus, measles virus-derived HA inhibits DC activation²² whereas, influenza virus-derived HA incorporated into virus like particles promotes DC activation and the release of Th1 cytokines.²³ In addition, the neuraminidase activity of human parainfluenza 3 virus-derived HN induces DC maturation and activation.²⁴ Having demonstrated the effect of HN expression on NK cell activation and lysis of tumor cells, we investigated the effect of HN-expressing tumor cells on antigen-presenting cells (APCs) such as DCs. Incubation of B16-OVA-HN cells with DCs resulted in the upregulation of the activation/maturation markers Class II MHC, CD80 and CD86, whereas incubation of DC with B16-OVA cells failed to do so (Fig. 3A). To confirm DC activation, we investigated the production of the key Th1 cytokine IL-12, following incubation with B16.OVA-HN cells. Both the number of DCs producing IL-12 and the amount of IL-12 produced were significantly increased when DCs were incubated with B16.OVA-HN, while incubation with B16-OVA-SR α or B16-OVA cells resulted in no increase (Fig. 3B and C).

Co-incubation of NK Cells and DCs with HN-expressing tumor cells leads to enhanced IFN γ production. With the observation that both NK cells and DCs are activated by tumor cells expressing HN, we investigated whether HN induces a functional crosstalk between NK cells and DCs. HN or SR α -transfected tumor cells were incubated with NK cells, alone or plus DCs. The combination of NK, DC and B16.OVA-HN cells significantly increased the amount of IFN γ detected in culture supernatants to approximately twice the amount seen when NK cells and DCs were incubated with B16.OVA-SR α (Fig. 4A). We utilized trans-well plates in order to investigate the mechanisms behind this enhanced IFN γ release, and in particular to assess whether cell-to-cell contacts were important. Placing all three cell types in the same chamber

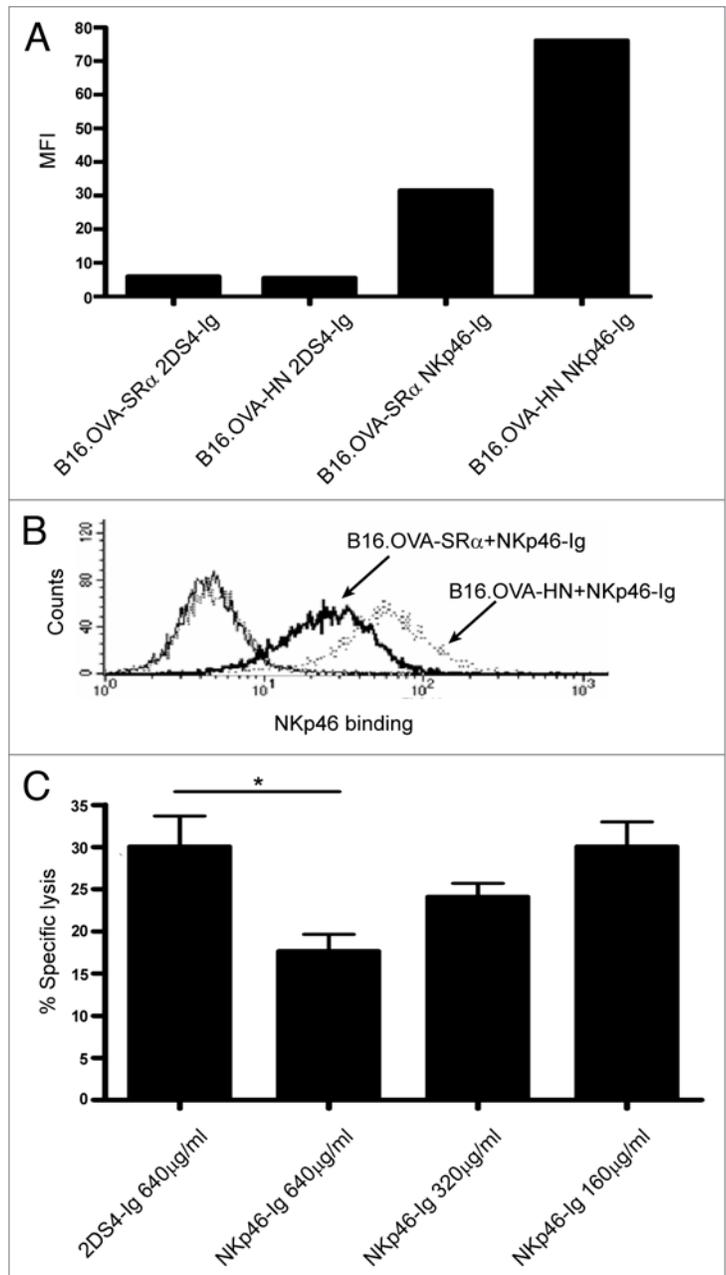


Figure 2. Enhanced NK cell activation by HN is mediated by NKp46 engagement. (A) B16.OVA-SR α or B16.OVA-HN cells were incubated with NKp46-Ig or KIR2DS4-Ig for 30 min and assessed for binding by flow cytometry using an anti-human IgG. Data are representative of 3 experiments. (B) A representative histogram of KIR2DS4-Ig binding to B16.OVA-SR α (dark gray) or B16.OVA-HN (light gray) cells and NKp46-Ig binding to B16.OVA-SR α (black) or B16.OVA-HN (dotted line) cells. (C) NK cells were incubated with B16.OVA-HN cells at a 10:1 effector:target ratio in the presence of KIR2DS4-Ig or NKp46-Ig. * $p < 0.05$, comparing KIR2DS4-Ig with NKp46-Ig at 640 mg/mL, p values were calculated with two-tailed Student's t -tests.

resulted in the highest amount of IFN γ production (Fig. 4B). By contrast, separation of the cells resulted in significantly lower IFN γ production. These results suggest that cell-to-cell contacts and synapse formed between DCs and NK cells are essential to enhance IFN γ production by NK cells. This fits

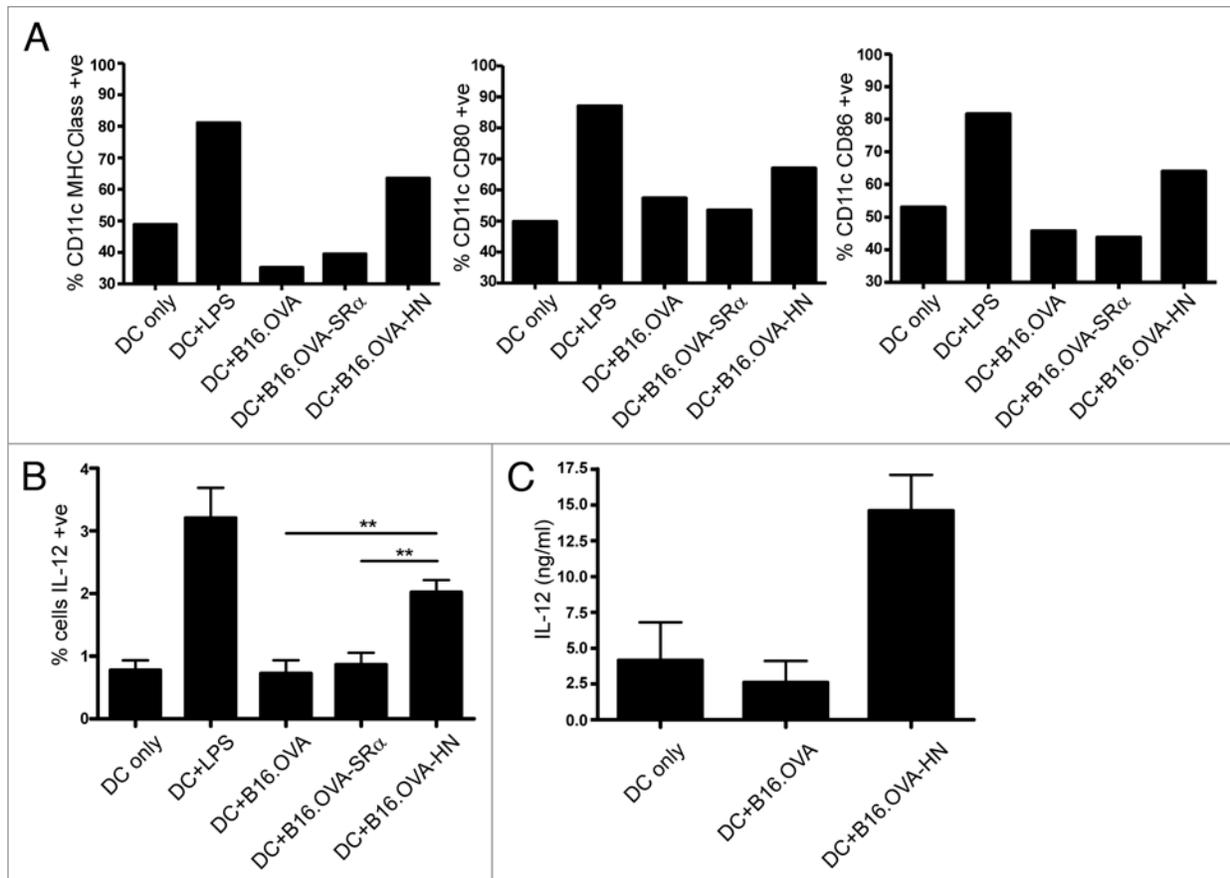


Figure 3. HN-expressing B16.OVA cells induce DC maturation. DCs were incubated alone or with LPS, B16.OVA, B16.OVA-SR α or B16.OVA-HN cells for 24 h and CD11c⁺ cells were then assessed for; (A) expression of DC maturation markers Class II MHC (left part), CD80 (center part) and CD86 (right part) by flow cytometry (data are representative of three experiments) and IL-12 production by intracellular cytokine staining (B) or ELISA (C). **p < 0.01, comparing B16.OVA-HN cells with B16.OVA-SR α or B16.OVA cells, p values were calculated with two-tailed Student's t-tests.

with the model by which transfer of pre-assembled stores of IL-12 from DCs to NK cells enhances IFN γ release by NK cells.²⁵ To assess the importance of DC-derived IL-12 in this response, we repeated the incubation of B16.OVA-HN cells, NK cells and DCs in the presence of an anti-IL-12 blocking antibody. Addition of the blocking antibody induced a significant reduction in IFN γ , to a level equivalent to that observed in the presence of NK cells and DCs only (Fig. 4C). To further confirm the role of IL-12 in enhancing NK cell activation and IFN γ production, B16.OVA-HN and B16.OVA cells were transfected with an IL-12 expression plasmid. Incubation of NK cells with these IL-12 expressing tumor cells resulted in a significant increase (20 to 25-fold) in the amount of IFN γ produced, which was greatest when B16.OVA-HN were used (Fig. 4D). This confirms the importance of IL-12 provided by DCs in enhancing NK cell activation. Taken together, these results suggest a mechanism of crosstalk between NK cells and DCs which serves to enhance NK cell cytotoxicity and cytokine production as well as DC activation following exposure to HN expressed on tumor cells.

HN-expressing tumor cells induce an increase in circulating NK cells following in vivo challenge. We next investigated whether HN-expressing tumor cells could activate NK

cells in vivo. B6 mice were vaccinated with B16.OVA-HN or B16.OVA-SR α tumor cells and the percentage of activated NK cells assessed daily using the NK cell marker DX5 and activation marker CD69. Mice challenged with B16.OVA-HN cells exhibited increased percentage of activated (DX5⁺ and CD69⁺) NK cells (> 150%) as compared with animals challenged with B16.OVA-SR α cells, peaking at day 2 after vaccination (Fig. 5). Tumor cells transfected with a control vector induced a modest increase in activated NK cells (~30%). These data confirm that HN-expressing tumor cells are able to induce more robust NK activation in vivo than tumor cells alone. We next investigated whether B16.OVA-HN cell vaccination would induce protective immunity against a subsequent challenge with B16.OVA cells. B6 mice were vaccinated with 2 × 10⁵ irradiated B16.OVA-HN or B16.OVA tumor cells on days 0 and 7. On day 14, mice were challenged with 1 × 10⁵ living B16.OVA cells. Strikingly, mice vaccinated with B16.OVA-HN tumor cells were better protected against a subsequent challenge with B16.OVA cells than mice receiving irradiated B16.OVA cells as well as than non-vaccinated mice (Fig. 6A). Since B16.OVA-HN cell vaccination enabled robust protection against B16.OVA cells, we examined which cells were important for this immunity. Depletion of NK cells during B16.OVA-HN vaccination

prevented tumor rejection and resulted in tumor development at the same rate as naïve B16.OVA cell-challenged animals (Fig. 6B). In addition, depletion of CD8⁺ T cells at the time of the B16.OVA challenge abrogated tumor protection with a median survival time similar to naïve controls (Fig. 6B). These results indicate that, in this setting, antitumor protection is mediated by both NK and CD8⁺ T cells.

B16.OVA-HN cell vaccination does not induce long-term immunity. In order to examine whether B16.OVA-HN cell vaccination induced long-term protective immunity to B16.OVA cells, mice protected from the initial B16.OVA challenge were rechallenged 4 weeks later and their ability to reject the tumor assessed. B16.OVA-protected mice challenged with either B16.OVA or B16 tumor cells showed an increase in median survival time (16.5 and 23 d respectively) compared with naïve controls (15 d) (Fig. 7A). Of note, the protection from tumor challenge was only seen in 1/8 mice, indicating that the initial B16.OVA-HN vaccination may not be sufficient to generate a long-term memory response to B16- or OVA-derived antigens. Failure to generate long-term immunity suggests that the initial anti-B16.OVA response did produce effector CD8⁺ T cells that efficiently eradicated the tumor, but not a CD8⁺ T-cell memory population. Inability to generate memory CD8⁺ T cells has been linked with the costimulatory molecule CD27 and its ligand CD70.^{26,27} DCs play a major role in both the activation of naïve CD8⁺ T cells and their differentiation into memory cells in vivo, and CD70 blockade at priming impairs memory T-cell responses.²⁸ We therefore investigated whether the activation of DCs by B16.OVA-HN tumor cells in the presence of NK cells induces CD70 expression. DCs cultured with B16.OVA-HN tumor cells plus NK cells did not upregulate expression of CD70 although CD70 expression was induced on DCs when incubated with soluble CD154 and CpG oligonucleotides (Fig. 7B). This suggests that despite inducing a robust antitumor CD8⁺ T cell response, the lack of CD70 on DCs at priming prevents the differentiation of memory T cells. The ability of DCs to prime naïve CD8⁺ T cells indicates that they were “licensed,” but not fully competent to induce memory

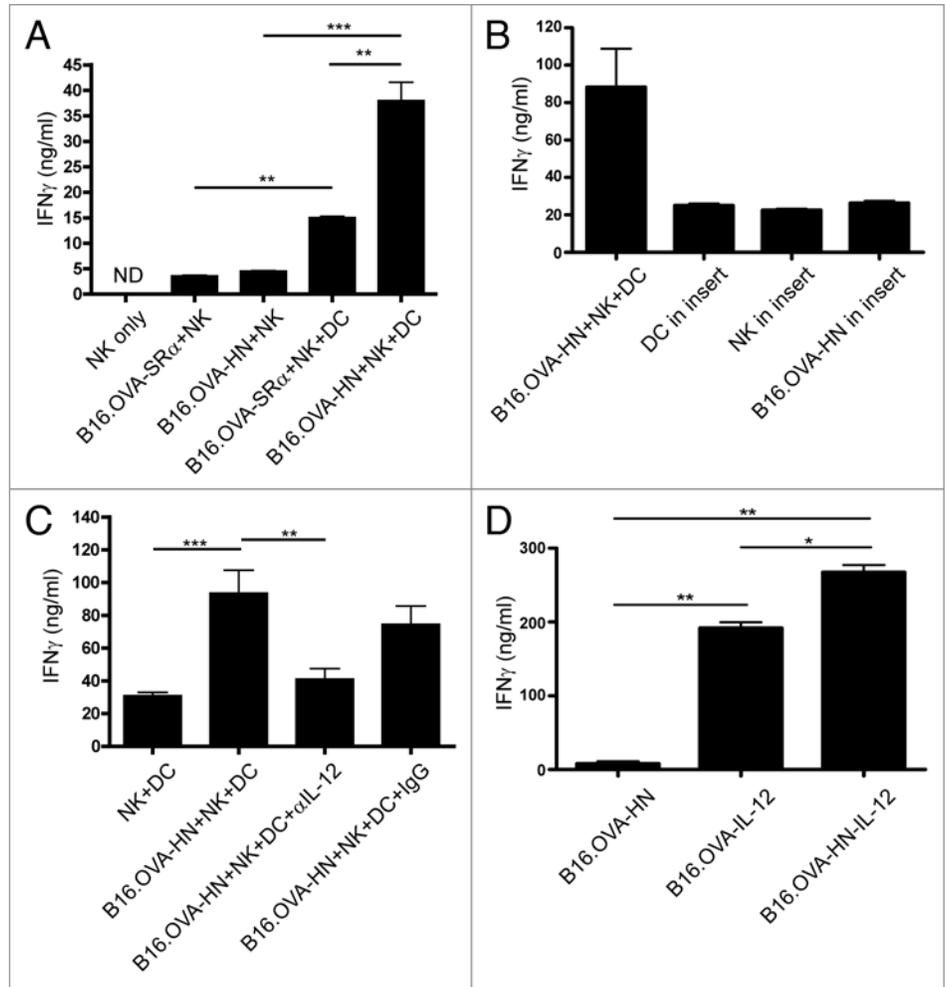


Figure 4. The NK-DC crosstalk further enhances HN-mediated activation of NK cells. (A) NK cells were incubated alone or with B16.OVA-SR_α or B16.OVA-HN cells in the presence or absence of DCs at a ratio of 2 B16.OVA cells:1 NK cell: 4 DCs for 24 h and assessed for IFN γ production. ***p < 0.001, **p < 0.01, comparing B16.OVA-HN + NK + DC with B16.OVA-HN + NK and B16.OVA-SR_α + NK + DC, or B16.OVA-SR_α + NK + DC with B16.OVA-SR_α + NK, ND = Not detectable. (B) B16.OVA-HN, NK cells and DCs were incubated in transwell plates with a different cell type placed in the insert and supernatants assessed for IFN γ . As a positive control B16.OVA-HN, cells NK cells and DCs were co-cultured. (C) NK cells and DCs were incubated with B16.OVA-HN cells in the presence or absence of a neutralizing anti-IL-12 or control antibody and assessed for IFN γ production. ***p < 0.001, **p < 0.01, comparing B16.OVA-HN + NK + DCs to B16.OVA-HN + NK + DCs + α IL-12 and NK + DCs. (D) NK cells were incubated with B16.OVA-HN cells, IL-12 expressing B16.OVA cells or IL-12 expressing B16.OVA-HN cells. The production of IFN γ was assessed in all experiments. **p < 0.01, *p < 0.05 comparing, B16.OVA-HN cells with B16.OVA-IL-12 cells or B16.OVA-HN-IL-12 cells, or comparing B16.OVA-HN-IL-12 cells with B16.OVA-IL-12 cells, p values were calculated with two tailed Student's t-tests.

T cells. As DCs are “licensed” through engagement with CD4⁺ T cells, we investigated the role of CD4⁺ T cells in the anti-B16.OVA response. Mice vaccinated with B16.OVA-HN cells and then challenged with B16.OVA cells were depleted of CD4⁺ T cells either throughout the entire experiment or at the point of tumor challenge. CD4⁺ T cell depletion did not affect the ability to induce robust anti-B16.OVA responses (Fig. 7C). This result suggests that CD4⁺ T cell responses plays a critical role neither during the priming phase (α CD4) nor during the acute effector phase (α CD4-effector).

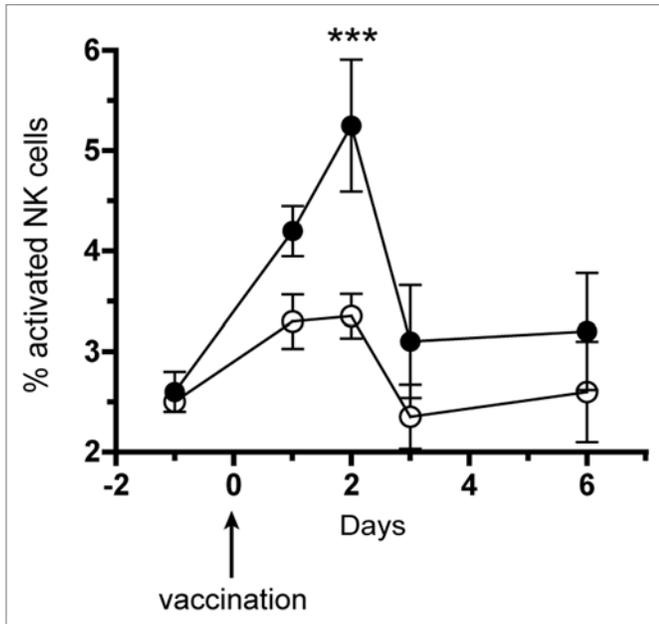


Figure 5. In vivo immunization of irradiated HN-expressing B16.OVA cells increases circulating activated NK cells. B6 mice were immunized with irradiated B16.OVA-SR α (○) or B16.OVA-HN (●) cells (n = 9/group) and the percentage of circulating activated NK cells (CD49b⁺ and CD69⁺) was assessed from tail bleeds on days -1, 1, 2, 3 and 6 by flow cytometry. Data are reported as mean percentage \pm SEM for each time point. ***p < 0.001, comparing B16.OVA-HN cells to B16.OVA-SR α cells on day 2 after vaccination using two-way ANOVA with Bonferroni correction test.

Discussion

We have examined the effect of expressing paramyxoviral (mumps) HN in a murine tumor model. We show that HN has an effect on both NK cells and DCs. HN is a ligand for the activating NK cell receptor NKp46, and we demonstrate that the interaction between HN and NKp46 results in enhanced NK-mediated killing of HN-transfected tumors and increased IFN γ production by activated NK cells. Furthermore, HN-expressing cells promote DC maturation and stimulate them to produce IL-12. When DC and NK cells are cultured with B16.OVA-HN cells, activation of NK cells is further boosted by a mechanism that requires cell-to-cell contact. Importantly, we demonstrate that vaccination with B16.OVA-HN cells protects mice against a subsequent challenge with the parental B16.OVA cells (> 85%). This protection is dependent on both innate (NK) and adaptive (CD8⁺ T cells) immune responses. These results show that the combined effects of direct NK activation by HN-expressing B16.OVA cells and enhanced NK-DC crosstalk enables protection against the parental (non-HN-expressing) tumor.

Strategies aimed at increasing or activating the NK cell compartment have been attempted in cancer patients. Initial approaches used low dose IL-2 to selectively manipulate NK cells in patients with a range of metastatic tumors, leading to preferential stimulation of NK cells.²⁹ Another approach was based on the ex vivo expansion and reinfusion of autologous NK cells. Ex vivo

expansion with IL-2 and reinfusion is well tolerated in patients and although NK cells have showed low cytotoxicity against tumor cells in early studies,³⁰⁻³² more recently strong antitumor responses have been demonstrated.³³⁻³⁵

The expression of HN by tumor cells induced activation of NK cells and DC maturation toward a DC1 profile (IFN γ and IL-12 production). Interestingly, incubation of NK cells and DCs together with HN-expressing tumor cells enhanced IFN γ production by NK cells, an effect that was IL-12-dependent and required cell-to-cell contacts. This DC/IL-12-dependent improvement in NK cell activation is consistent with the NK-DC crosstalk mechanism. The NK-DC crosstalk is bi-directional, with activated NK cells inducing DC maturation by cytokine production and direct cell-to-cell contact.³⁶ Direct interactions between DCs and NK cells also coordinate immune responses. Formation of a stimulatory synapse between activated NK cells and DCs promotes the polarized secretion of preassembled stores of IL-12 by DCs, which acts on NK cells. IL-12 is required for IFN γ production by NK cells, creating a positive feedback loop influencing cell mediated immunity.²⁵ The DC-NK synapse is also required for NK cell-mediated cytotoxicity.¹¹ The NK-DC cooperation has previously been documented in tumor models.¹⁴ T cell-mediated tumor rejection of A20 B-cell lymphoma cells was dependent on DC activation by NK cells, with IFN γ secreted during NK-cell mediated tumor rejection being critical for the generation of antitumor CTLs.

Vaccination of B6 mice with HN-expressing B16.OVA tumor cells induced robust protection against a subsequent challenge with living B16 or B16.OVA cells (> 85% survival). This indicates that the immunity induced in the initial vaccination was directed toward B16-specific rather than HN- or OVA-specific antigens. Surprisingly, despite this strong initial protection, the majority of mice were not protected against a subsequent challenge with B16 or B16.OVA tumor cells, pointing to a failure in the generation of antigen-specific memory T cells. This inability to generate strong secondary memory responses in the B16 tumor model system has previously been demonstrated. One study investigating the generation of immune responses against B16 cell-based vaccines reported a robust primary immune response, but not an effective memory response.³⁷ An effective memory response was only generated when a CD40L-expressing plasmid was included in the vaccination. This indicates that the engagement of DCs in the priming or effector phase of CD8⁺ T cells influences the differentiation of B16-specific memory T cells. The factors that allow for the induction of CD8⁺ T cell memory cells are poorly understood, but a role for epitope density has recently been shown.³⁸ CD4⁺ T-cell help is also important for the generation, survival and functional responsiveness of long-lived memory CD8⁺ T cells.³⁹ Fernandez et al. found that, depending on the nature of the initial stimulation, effective cytotoxic CD8⁺ T cells could be generated but they were short lived, and no functional memory CTLs were detected unless a source of CD4⁺ T-cell help was provided. Interestingly, the NK-DC crosstalk may serve to bypass the function of CD4⁺ T-help in CTL induction against some tumors.¹⁰ In our experiments, we have not identified a role for CD4⁺ T cells in the B16.OVA response,

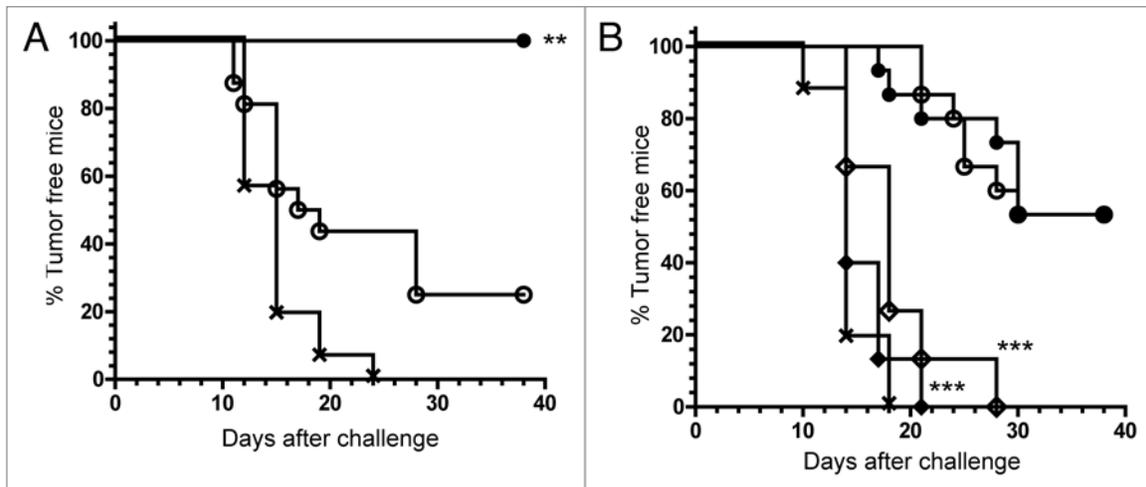


Figure 6. In vivo immunization of irradiated HN-expressing B16.OVA cells protects against B16.OVA tumor challenge. **(A)** B6 mice ($n = 15/\text{group}$) were immunized twice with irradiated B16.OVA (\circ) or B16.OVA-HN (\times) cells on days -14 and -7 or left untreated (x). On day 0 all mice were challenged with B16.OVA cells and tumor development was assessed. **(B)** B6 mice ($n = 15/\text{group}$) were immunized using only B16.OVA-HN cells. In addition, three further groups were injected with anti-NK (\blacklozenge), anti-CD8 (\diamond) or irrelevant antibodies (\circ) during vaccination, left untreated ($+$) or unimmunized (x). At day 0, all mice were challenged with B16.OVA cells and tumor development assessed. $**p < 0.01$, p values were calculated using the log-rank test comparing B16.OVA-HN-immunized with B16.OVA-immunized mice **(A)**. $***p < 0.0001$, comparing NK or CD8⁺ T-cell-depleted with control mice **(B)**.

neither in the priming nor effector stage, however, as HN expression may support the NK-DC crosstalk, this does not exclude a role for CD4⁺ T-cell help in CD8⁺ memory T-cell differentiation in other B16 vaccine protocols.

The generation of a good T-cell memory response is also dependent on proper DC activation. DCs need to receive appropriate activation signals in order to become “licensed” to elicit both the efficient expansion of antigen-specific primary CD8⁺ T cell responses as well as the generation of memory T cells. “Licensing” can occur via CD4⁺ T cells or upon recognition of pathogen associated molecular patterns (PAMP).⁴⁰ CD70 expression on DCs and subsequent ligation of CD27 is required for CD8⁺ T-cell activation and the generation of an effective memory T-cell response.²⁸ Blocking the CD70/CD27 interaction in a primary response generated CD8⁺ T cells capable of lysing target cells, but incapable to expand following secondary challenge. Interestingly, despite the induction of DC maturation by HN-expressing tumor cells, as measured by upregulation of MHC Class II, CD80 and CD86, upregulation of CD70 was not observed. It is therefore possible that the inability of HN to fully activate/mature DCs prevents the differentiation of B16-specific CD8⁺ T-cell memory cells following challenge. The use of agonistic anti-CD27 antibody in combination with the B16.OVA-HN vaccine may provide sufficient activation of naïve CD8⁺ T cells to facilitate memory T-cell differentiation. The engagement of CD70/CD27 and other co-stimulatory molecules (CD40/CD40L and 4-1BB/4-1BBL) in the generation of long-term CD8⁺ T-cell memory populations is currently under investigation.

The use of HN to enhance antitumor immunity and allow for tumor rejection provides a novel strategy for harnessing the NK-DC crosstalk against cancer. HN-expressing tumor cells induced NK activation and DC maturation. Moreover,

incubation of NK, DC and HN-expressing tumor cells substantially enhanced NK activation. The ability of these NK cells to facilitate tumor rejection in vivo indicates the benefit of HN in stimulating not only innate but also adaptive immune responses. Thus, our vaccine strategy may provide a novel adjuvant able to induce activation of both innate and adaptive immunity in vivo.

Materials and Methods

Cell analysis and transfection. For dendritic cell (DC) production, murine bone marrow cells were harvested from femurs of C57BL/6 (B6) mice and plated out in a single-cell suspension in 100 ml RPMI medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES buffer and 50 μM 2-ME) in 6 well tissue culture plates (6 ml/well). GM-CSF was added at 20 ng/ml and the cells incubated at 37°C for 7 d. NK cells were isolated from spleens taken from B6 mice using biotinylated anti-CD49b (DX5) antibody (BD biosciences, 553856) together with the CELLection Biotin Binder Kit (Life technologies, 115-33D). NK cells were resuspended at 2×10^6 cells/ml in DMEM medium (DMEM with 10% FCS, 100 U/ml penicillin and streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, 50 μM 2-ME) containing 1,000 U rIL-2/ml (Peprotech, 212-12) in a 96-well U-bottomed plate and incubated at 37°C for 4 d.

The murine melanoma stably transfected with OVA, B16.OVA, were maintained as previously described in reference 41. B16.OVA cells were transfected with pcDL-SR α 296-HN encoding recombinant HN derived from mumps virus, pcDL-SR α 296-IL-12 (encoding IL-12) or empty pcDL-SR α 296 vector (a kind gift of Dr Y. Takebe, National Institute of Infectious Diseases, Japan) using Effectene transfection reagent (Qiagen) according to the manufacturers’ protocol. Efficiency of

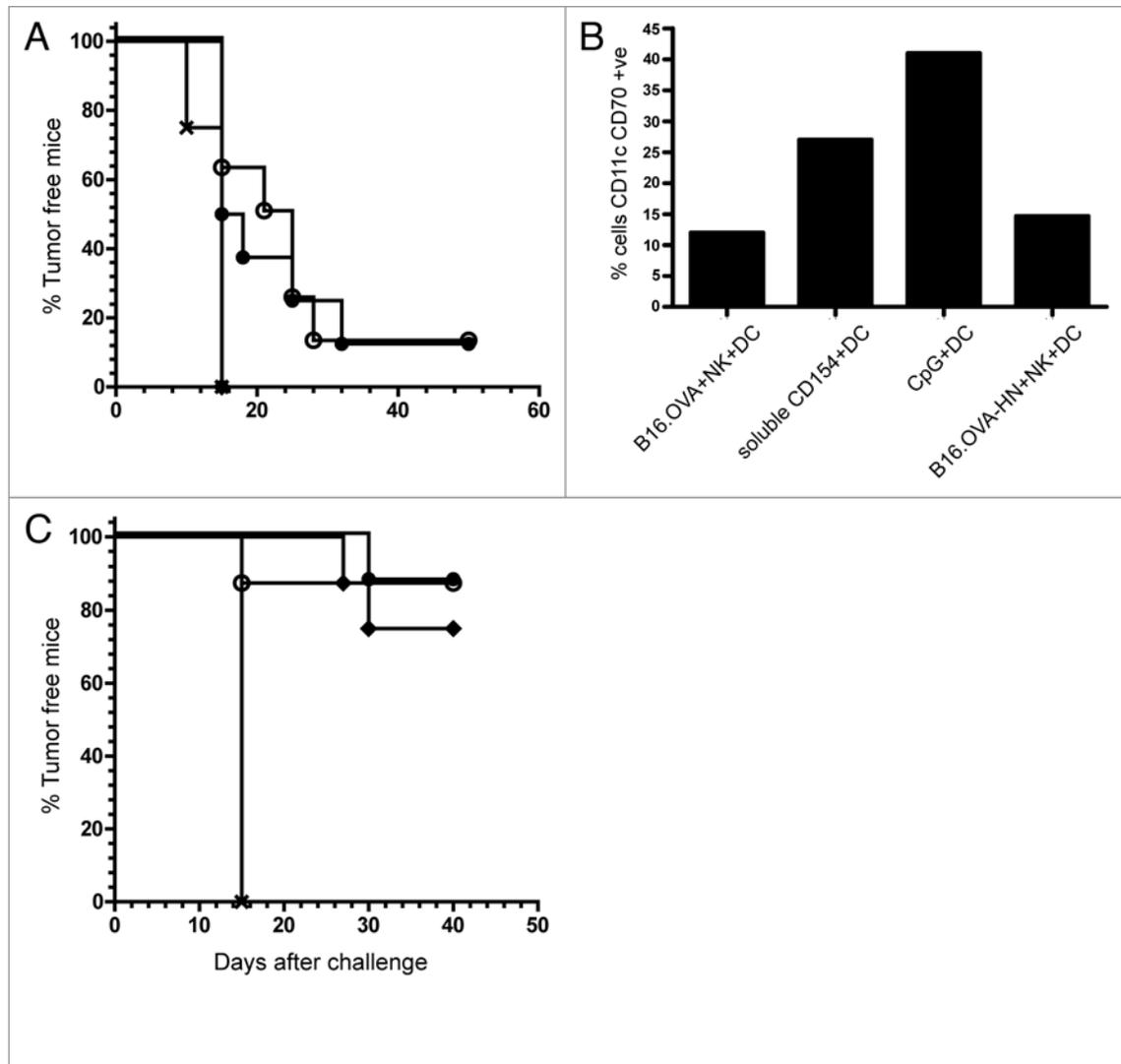


Figure 7. B16.OVA-HN immunization does not induce memory T-cell responses. **(A)** B6 mice ($n = 8/\text{group}$) that had rejected the B16.OVA tumor challenge were rechallenged with B16.OVA (\diamond) or B16 (\circ) cells. In addition, naïve B6 mice ($n = 8/\text{group}$) were challenged with B16.OVA (\blacklozenge) or B16 (\times) cells. Mice were monitored for tumor development. **(B)** DCs were incubated with B16.OVA and NK cells, B16.OVA-HN and NK cells, soluble CD154 or CpG oligonucleotides. CD11c⁺ cells were analyzed for CD70 expression by flow cytometry. Data are representative of four experiments. **(C)** B6 mice ($n = 8/\text{group}$) were immunized with irradiated B16.OVA-HN cells and challenged with B16.OVA (\diamond) cells as in Figure 6 or left untreated (\times). In addition, two groups of mice ($n = 8$) were depleted of T cells either throughout the experiment (\circ ; CD4) or at the time of B16.OVA challenge (\blacklozenge ; CD4-effector). Mice were monitored for tumor development.

transfection was assayed by flow cytometry using the HN specific antibody 3-1.⁴²

To examine DC activation by NK and tumor cells, DC were co-cultured with HN or control plasmid expressing B16.OVA for 24 hours and analyzed for the expression of activation/maturation markers CD80, CD86, MHC Class II and CD70 (BD biosciences, 553756, 553691, 553605 and 555286) with a FACSCalibur using CellQuest software (BD Biosciences). For CD70 experiments, a CD70 upregulators soluble CD154 and 5 $\mu\text{g}/\text{ml}$ CpG (oligonucleotide 1668) were used. In some assays LPS (Sigma-Aldrich) was used as a positive control for DC activation.

For transwell experiments, B16.OVA or B16.OVA-HN cells were plated out in the wells or inserts of transwell plates (VWR

international, 734-1560P) and incubated with DC and NK cells at 1:1:0.5 (B16:DC:NK) ratio. The above experiments were repeated separating NK cells and DC. Supernatant from the cultures was harvested after 24 h for analysis.

Tumor challenge. B6 mice, bred in Southampton, were used at 6–10 weeks of age. B16.OVA or B16.OVA-HN cells were irradiated (25 Gy) and 2×10^5 cells injected into the flank of B6 mice on days -14 and -7. On day 0 mice were challenged with 2×10^5 B16.OVA into the opposite flank and monitored daily for tumor development. Mice were sacrificed when mean tumor diameter was > 10 mm. Animal welfare and experimentation were conducted in accordance with the United Kingdom Coordinating Committee for Cancer Research guidelines with approval from University of Southampton Ethical Committee and under UK. Home Office License.

NK cell detection and NK, CD8 and CD4 T cell depletion. B6 mice vaccinated as described above were monitored for circulating NK cells using daily tail bleeds for 6 d. Erythrocytes were lysed using red cell lysis solution (Gentra, D-40K) and PBMCs analyzed by flow cytometry using anti-CD49b and anti-CD69 (BD biosciences, 5538567 and 553237).

For depletion of NK and cells mice were injected with anti-asialo GM1 antibody (Wako, 986-10001) or normal rabbit serum (Sigma-aldrich, R9133) as a control on days -17, -15, -8 and -1 of the B16.OVA vaccination protocol described above. The efficiency of NK cell depletion was confirmed by flow cytometry (anti-NK1.1-PE and anti-CD3-FITC; BD biosciences 553165 and 553061). For CD8⁺ and CD4⁺ T cell depletion, anti-CD8 (YTS169.4.2.1) or anti-CD4 antibodies (YTS191.1.2) were injected five times every 3 d starting at day-15. CD8 T cell depletion was confirmed by flow cytometry (anti-CD8-APC, anti-CD4-APC and anti-CD3-FITC; BD biosciences 553035 and 553051) from tail bleeds. Control rat monoclonal antibody, Mc10-6A5 (anti-BCL1 Id mAb) was kindly provided by Dr M.J. Glennie, University of Southampton.

Detection of IFN γ and IL-12 producing cells. NK cells and/or dendritic cells were incubated with B16.OVA transfected with HN or control plasmid DNA for 24 h. Blocking anti-IL-12 antibody (R&D Systems, AF-419-NA) was also added to cultures (10 μ g/ml) to assess the role of IL-12 in the response. IFN γ and IL-12 was detected in the culture supernatants by ELISA. For detection of IFN γ the capture antibody R4-6A2 and the biotinylated detection antibody XMG1.2 (BD biosciences, 551216 and 554410) were used. For IL-12, the antibodies 9A5 and biotinylated C17.8 were used for capture and detection respectively (BD biosciences, 554658 and 554476). Avidin-AP (Sigma-Aldrich, A7294) and pNPP substrate (Sigma-Aldrich, P5994) were detection and read at 405 nm (BioRad 680).

IFN γ production was also assessed by ELISpot. Multiscreen-IP 96-well plates (Millipore, S2EM004M99) were coated with capture antibody R4-6A2. NK cells were added to each well \pm dendritic cells \pm HN or control transfected B16.OVA cells. Medium only and 0.5 μ g/well of concanavalin A were used for negative and positive controls, respectively.

Following overnight incubation IFN γ was detected using biotinylated antibody XMG1.2. Streptavidin-AP (MabTech, 3310-10) and substrate BCIP/NBT (Cambridge Bioscience, BCIB-0100-01) were used for detection and spots counted with an ELISpot reader and reported as the number of spots per 10⁶ splenocytes.

For intracellular detection of IL-12, DC and HN transfected B16.OVA were incubated for 24 h and IL-12 production assessed using the Cytofix/Cytoperm Fixation/Permeabilization solution kit with Golgi Plug (BD biosciences, 555028). Anti-IL-12-FITC (BD biosciences, 560564) was used to detect IL-12 producing DC. Analyses were performed on CD11c⁺ DC populations.

Cytotoxicity assay. The cytolytic activity of NK cells against B16.OVA transfectants was assessed in a standard 4 h ⁵¹Cr-release assay in which effector cells were co-incubated with 5 \times 10³ ⁵¹Cr-labeled targets at a 10:1 E:T ratio. Spontaneous release was determined by incubation of labeled target cells with medium and maximal release determined by incubation of target cells with detergent (4% NP-40). % specific lysis was calculated as 100 \times [(cpm experimental well - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)]. For blocking experiments, recombinant mouse NKp46-Ig chimera (R&D Systems, 2225-NK-050) or human KIR construct (2DS4-Ig) were used. The Ig chimeras were included in cultures at a final concentration of 160–640 μ g/ml. To prevent NK cell interaction with Fc region of the chimeras, either anti-mouse CD16/CD32 (BD biosciences, 553141) was used (25 μ g/ml) or targets were incubated in 50% mouse serum for 1 h before being introduced into the ⁵¹Cr-release assay.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest financial or otherwise.

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