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Nicotinic Receptor $\beta 2$ Determines NK Cell-Dependent Metastasis in a Murine Model of Metastatic Lung Cancer

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Abstract

Cigarette smoke exposure markedly compromises the ability of the immune system to protect against invading pathogens and tumorigenesis. Nicotine is a psychoactive component of tobacco products that acts as does the natural neurotransmitter, acetylcholine, on nicotinic receptors (nAChRs). Here we demonstrate that natural killer (NK) cells strongly express nAChR $\beta 2$. Nicotine exposure impairs the ability of NK cells to kill target cells and release cytokines, a process that is largely abrogated by nAChR $\beta 2$ deficiency. Further, nicotinic suppression of NF- κ B-induced transcriptional activity in NK cells is dependent on nAChR $\beta 2$. This nAChR subtype also plays a large role in the NK cell-mediated control of melanoma lung metastasis, in a murine lung metastasis model exposed to nicotine. Our findings suggest nAChR $\beta 2$ as a prominent pathway for nicotine induced impairment of NK cell functions which contributes to the occurrence of smoking-related pathologies.

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Introduction

Smoking related disorders such as infection and tumorigenesis have been linked to the compromised functions of the immune system in smokers [1,2]. Among the multiple immune-modifying components of tobacco smoke, nicotine has been shown to have a profound impact on a number of nicotinic acetylcholine receptor (nAChR)-bearing leukocytes from both innate and adaptive immune systems. Expression of nAChR $\alpha 7$ on macrophages and monocytes, and its ability to inhibit the immune response during systemic inflammation and in organ-specific diseases have been relatively well described [3,4,5,6,7,8]. Results suggest that nicotine regulates the intensity of endotoxemia and sepsis [3,4,5], and attenuates $\alpha 7$ -specific autoimmune responses in an nAChR $\alpha 7$ -dependent manner [6,7,8]. On the other hand, it has recently been demonstrated that other nAChR subtypes may play a role in nicotine's anti-inflammatory effects [3,4,5,6,7,8]. In this context, the expression profile of additional nAChRs on leukocytes and their role in disease are relatively less explored.

NK cells are large, granular lymphocytes that operate through cytolytic activity and cytokine secretion. These two functions empower NK cells in innate host defense against certain microbial agents and cells undergoing malignant transformation. Several studies have shown that NK cell numbers and activities are decreased in smokers compared with non-smokers [1,2]. Exposure to cigarette smoke attenuates the cytotoxic activity and cytokine

production of NK cells in humans and mice [9,10,11], thereby linking NK cell defects to increased infection and cancer. Smoking has been particularly associated with the highly malignant small cell lung cancer. Even after surgical removal at an early stage, nearly half of patients die from a secondary tumor metastasis. It is postulated that this is due in part to defective NK cell-mediated immune surveillance because aberrant NK cell function in smokers increases the re-emergence of cervical cancer metastasis [12].

Here, we comprehensively examined the cellular and molecular effects of nicotine as one of the components of cigarette smoke on NK cells. We profiled nAChR expression on NK cells and identified nAChR $\beta 2$ as a key determinant for nicotine-mediated impairment of NK cell functions. Further, we demonstrate that nicotinic inhibition of NK cell functions via nAChR $\beta 2$ significantly increases melanoma metastasis in a xenogeneic model.

Materials and Methods

Animals

Female C57BL/6 mice (6–8 wk old), RAG2^{-/-}, RAG2^{-/-} γ_c ^{-/-}, all on a C57BL/6 background, were purchased from Taconic Farms. $\alpha 7$ and $\beta 2$ KO mice [13], also crossed to C57BL/6 background, were kindly provided by Dr Allan C. Collins. Mice were maintained under pathogen-free conditions. The Animal

Research Ethics Board of Tianjin Medical University and St. Joseph's Hospital and Medical Center approved all experiments described in this study.

mRNA Purification and Reverse-transcription PCR

mRNA was purified from fresh acutely-isolated cells ($\sim 1.5 \times 10^6$ cells per sample) by using the μ MACS mRNA isolation kit (Miltenyi Biotec), as per the supplied protocol. Reverse transcription was performed with the SuperScript III First Strand cDNA Synthesis kit (Invitrogen, USA) by following the supplied protocol. Oligo-dT sequences were used to prime the reverse transcriptase. PCR was then performed following established protocols, using a variety of primers that are specific to each target mRNA (Table 1). Primer pairs were designed with the use of PubMed's "Primer-blast" tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each pair, forward and reverse primers were specific to different exons, so that potential DNA contamination could be ruled out. PCR was performed using the RedTaq PCR kit (Sigma, USA), according to the supplied protocol. Primers were always tested with total brain cDNA (positive control), and the optimal annealing temperature (T_m) was determined empirically (with the use of a Temperature gradient thermocycler), while maintaining high stringency conditions. A negative control (no cDNA template), was included in every set of reactions. PCR products for each primer pair from positive controls were sequenced in order to ensure the accuracy of our results.

Overall nAChR Expression Assessed by Radioligand Saturation Binding

Epibatidine, which is selective for β 2- and β 4-containing nAChRs (which can assemble with α 4, α 5, α 6 and β 3 subunits), was used to assess the amount of β 2-containing nAChRs, as done previously [14,15]. In order to maximize assay sensitivity, we used high specific activity [125 I]epibatidine (I-Epi; 2200 Ci mmol $^{-1}$; PE Life Sciences, Waltham, MA). To this end, purified NK cells were used immediately after isolation. Cells were resuspended in isotonic binding buffer (mM: NaCl, 144; KCl, 2; CaCl $_2$, 2; MgSO $_4$, 1; HEPES 20; pH = 7.5), supplemented with bovine serum albumin (0.1% (w/v)). Incubations were performed at 22 °C for 2 h in 96-well plates. Samples were incubated with a range of eight concentrations (6.25 – 800 pM) of I-Epi in a total volume of

30 μ l of supplemented isotonic binding buffer. Total (no peptide added) and non-specific binding (defined using 100 mM carbamylcholine) were measured at each concentration, in triplicate. To differentiate β 2- from β 4-containing nAChRs, 10 nM A85380 was added in conjunction with 200 pM I-Epi [14]. Binding reactions were terminated and washed by filtration using a 96-place manifold. Particulate fractions were collected onto single layers of Inotech 0.75 μ m retention glass fiber filters soaked in 0.5 % polyethyleneimine. Bound radioligand were quantified using a Microbeta plate counter (PerkinElmer Life Sciences, Wellesley, MA).

Nicotine Treatment in vivo and in vitro

For in vivo delivery, a 100 mg/ml solution containing (-)nicotine bitartrate (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) or a solution of PBS alone was freshly prepared 24 h before pump implantation and loaded into Alzet[®] osmotic minipumps (model 2006, Durect Corporation, Cupertino, CA, USA). The pumps were implanted subcutaneously on the right side of the back of the mouse and continuously delivered either PBS or nicotine salt at 3.6 μ l/d for 6 weeks, and then the pumps were removed. This equated to delivery of 0.39 mg of nicotine free base per mouse per day. For an \sim 30 gm mouse, which is at the upper end of weight for animals used in the study, this equates to \sim 13 mg of nicotine free base/kg/d or \sim 0.54 mg of nicotine free base/kg/hr. Plasma nicotine levels in mice are \sim 100–200 ng/ml (\sim 0.6–1.2 mM) after infusion of \sim 2–4 mg/kg/hr of drug and \sim 45 ng/ml (\sim 280 nM) after infusion at \sim 0.5 mg/kg/hr. For comparison, human smokers have peak plasma nicotine levels of 10–50 ng/ml (\sim 60–310 nM). Thus, nicotine levels in plasma (extrapolated to be \sim 49 ng/ml or \sim 300 nM) of mice used in the studies are comparable to those in the plasma of human smokers. Some control mice received PBS via direct injections rather than through minipumps, but either delivery method produced similar results. For the in vitro experiments, 0.1–100 μ M/ml of nicotine was added to the cell cultures.

B16 Melanoma Cell Line and Tumor Challenge

B16-F10-luc2 (B16) is a luciferase-expressing cell line from mouse melanoma (Caliper Life Sciences, Alameda, CA, USA). Mice of the indicated genotypes were challenged i.v. with B16 cells

Table 1. Primers used to detect nAChR subunits.

nAChR	NCBI	Forward Primer	Reverse Primer	Product	Tm
Subunit	Template	Sequence	Sequence	Size (bp)	(°C)
α 3	NM_145129.2	gccaacctcacaagaagctc	atgtggggttagcagcaac	680	58
α 4	NM_015730.5	cagtagccaatatctcagat	gtagaacagtgaggcagtcgg	577	55
α 5	NM_176844.3	gggttcgtcctgtggaacacctga	ggtcctgtaggattatctcg	431	58
α 6	NM_021369.2	tggtccagcagataacatctg	tgaattgaacactctcgatg	987	55
α 7	NM_007390.3	cgtggcctctcagtggtcg	ggccatgaggcacaagcggt	514	58
α 9	NM_001081104.1	ctattccccttgacag	ttttgctagcttcatagc	798	55
β 2	NM_009602.3	ctccaactctatggcgtgct	cgctggcctggcagtgcgat	623	58
β 3	NM_173212.3	acggagagtaaggaaccgt	accagcagccctcagttcta	364	58
β 4	NM_148944.4	tctctgtctgctctgctca	acacagtggtgacgatggaa	913	58

Primer pairs were designed with the use of PubMed's "Primer-blast" tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each pair, forward and reverse primers were specific to different exons, so that potential DNA contamination could be ruled out. The optimal annealing temperature (T_m) was determined empirically with the use of a temperature gradient thermocycler, while maintaining high stringency conditions. PCR products for each primer pair were sequenced in order to ensure the accuracy of our results.

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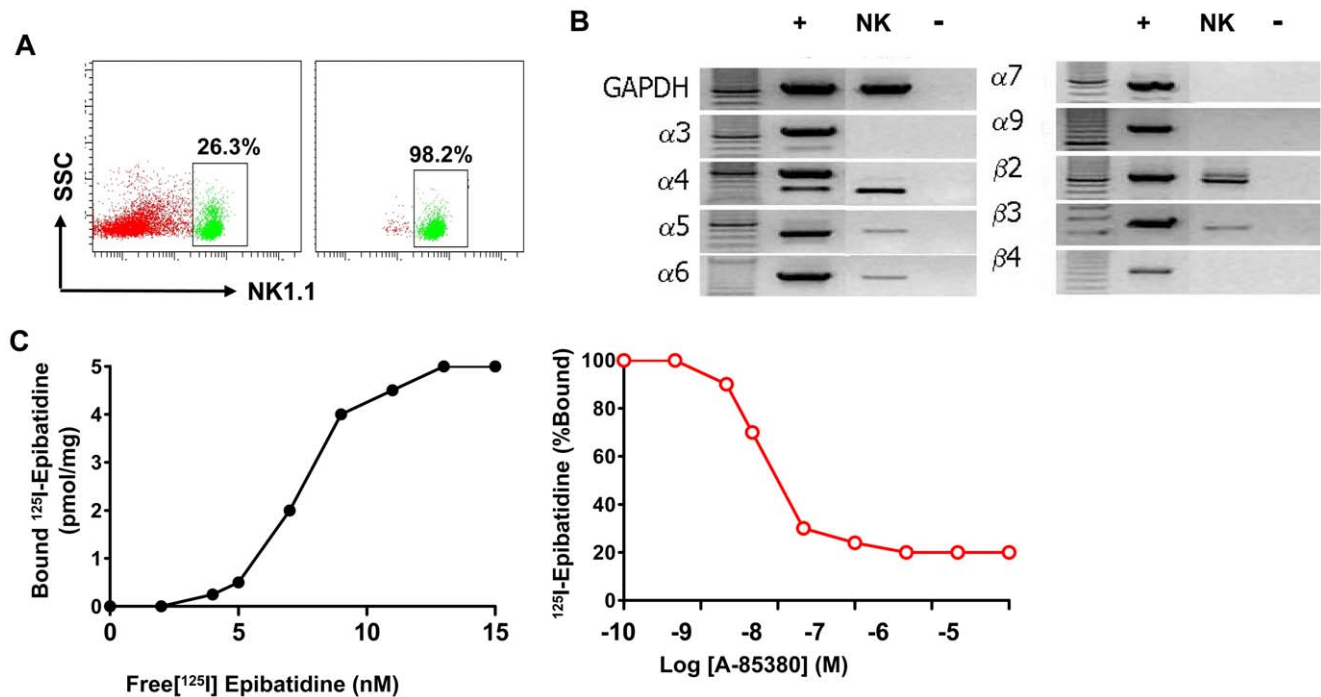


Figure 1. Nicotinic acetylcholine receptor (nAChR) expression on NK cells. Spleen, or lymph node cell suspensions were obtained RAG2^{-/-} mice, and FACS was performed as described in the *Materials and Methods* section. **A.** Representative dot plots of cell populations before and after sorting are shown in the left- and right-hand panels, respectively. The purity of NK cells reached >98% purity after sorting. **B.** mRNA was purified from the sorted NK cells, and cDNA was synthesized by reverse transcription. PCR was then performed using primers specific for each mouse nAChR subunit. A positive control (+), using whole mouse brain cDNA, and a negative control (-), without cDNA, were included in each reaction. For each nAChR subunit, bands from the positive control and at least one sample from each cell type were sequenced and confirmed as the correct PCR product. **C.** nAChR expression assessed by radioligand saturation binding. Data depicted are representative from 2 to 3 separate experiments with similar results.

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in 0.2 ml of PBS via the tail vein. Cell numbers used and times of sacrifice are referred to previously established protocols [9,15,16] and specified in the results section. Animals were anesthetized and euthanized via exsanguinations through abdominal vein puncture. Lungs were removed and placed in PBS. Tumor nodules were counted using a dissecting microscope by a researcher blinded to the treatment groups. In some cases, lungs were immersed in a 67% ethanol, 9% formaldehyde, and 4% glacial acetic acid solution and the number of tumor foci was enumerated 24–48 h later.

Isolation of Lung Mononuclear Cells

Lung mononuclear cells were isolated as described previously [17]. Briefly, lungs were perfused with prewarmed HBSS from the right ventricle. Cell suspensions from excised organs were generated by collagenase digestion and followed by mechanical mincing. Cell debris were removed by passing through nylon mesh. Cells were washed and resuspended in HBSS. Nonparenchymal cells were isolated by density-gradient centrifugation with Lympholyte-M (Cedarlane Laboratories).

NK Cell Cytotoxicity Assays

NK cell cytotoxicity was assessed by the chromium release assay using ⁵¹Cr-labeled YAC-1 murine lymphoma cell line [18] or B16 tumor cells. Freshly purified NK cells were incubated with ⁵¹Cr-labeled target cells (5×10^3) at various effector cell/target cell ratios. After 4 h of incubation, the supernatants were harvested and ⁵¹Cr-release was measured with a γ -counter (PerkinElmer, Waltham, MA). The percentage of specific lysis was calculated

according to the following formula: (experimental release – spontaneous release) / (maximum release – spontaneous release) \times 100. Cytotoxicity assays were done in triplicate [18].

NK Cell Proliferation Assays

NK cells were isolated from the spleen of mice that were treated with nicotine or PBS. Cells were suspended in culture medium containing Dulbecco's modification of Eagle's medium (Gibco, Paisley, UK) supplemented with 1% (v/v) minimum essential medium (Gibco), 2 mM glutamine (Flow Laboratory, Irvine, CA, USA), 50 IU/ml penicillin, 50 mg/ml streptomycin, and with 10% (v/v) FCS (all from Gibco). 4×10^5 cells in 200 μ l of culture medium were placed in each well of 96-well, round-bottom microtiter plates (Nunc, Copenhagen, Denmark). For the in vitro nicotine exposure experiments, nicotine (0.1–100 μ M) was added to the culture medium in addition to LPS (10 μ g/ml). After 3 days of incubation, the cells were pulsed for 18 h with 10 μ l aliquots containing 1 μ Ci of ³H-methylthymidine (specific activity of 42 Ci/mmol; MP Biomedicals, Irvine, CA, USA) per well. Cells were harvested onto glass fiber filters and thymidine incorporation proportional to the degree of cell proliferation was then measured. The results are expressed as counts per minute (cpm).

FACS Analysis

Single cell suspensions (10^6 cells) were prepared from spleen or lymph nodes, and stained with fluorochrome-conjugated antibodies. All antibodies were purchased from BD Biosciences or eBioscience unless otherwise indicated. Antibodies were directly labeled with one of the following fluorescent tags: FITC, PE,

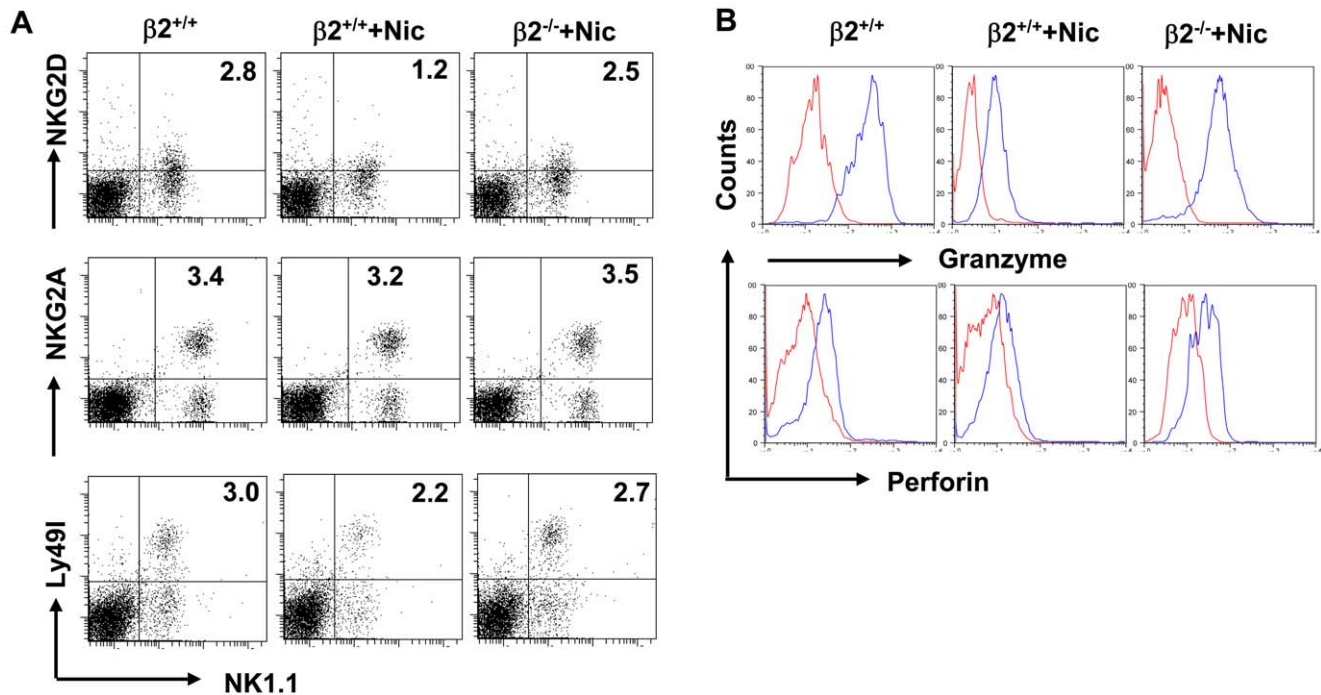


Figure 2. Nicotine alters NK cell phenotype via nAChR $\beta 2$. Wild type ($\beta 2^{+/+}$) or nAChR $\beta 2$ knock-out ($\beta 2^{-/-}$) mice received nicotine (Nic) or PBS for 21 days before they were sacrificed. Single cell suspensions were prepared from spleens. Frequency and phenotypes of NK cells within the mononuclear cells were analyzed by FACS. **A.** Expression of NKG2D, NKG2A and Ly49I on gated NK1.1⁺CD3⁻ cells. **B.** Expression of perforin and granzyme B secreted by gated NK1.1⁺CD3⁻ cells. The plot data represents results from two separate experiments (n=3–4 mice/group). P values, Student's *t*-test, **p*<0.05. doi:10.1371/journal.pone.0057495.g002

PerCP, allophycocyanin (APC), PC5, or PC7. The following anti-mouse antibodies were employed: CD3 (145-2C11), NKG2A (20d5), NKG2D (CX5), Ly49 I (YLI-90), NK1.1 (PK136), perforin (eBioOMAK-D) and granzyme B (16G6). Flow cytometric data were collected on a FACSAria™ flow cytometer (Becton Dickinson, Mountain View, MD) and analyzed with Diva™ software. Isotype-matched negative control mAbs were used for all stains. To determine the percentage of cells producing selected cytokines, values obtained with isotype controls were subtracted from those with specific mAb.

Tumor in vivo Imaging

Bioluminescence images were obtained using an IVIS Imaging System 200 Series (Caliper Life Sciences, Hopkinton, MA). For *in vitro* imaging, bioluminescent cells were serially diluted from 10⁵ cells in complete media into black, clear bottomed, 24-well plates (Costar, Acton, MA, USA). D-luciferin (Caliper Life Sciences, Hopkinton, MA) at 150 $\mu\text{g}/\text{ml}$ in media was added to each well 5–10 min before imaging. Imaging time was 1 min/plate. For *in vivo* imaging, Mice received an intraperitoneal injection of D-Luciferin 150-mg/kg (Caliper Life Sciences, Hopkinton, MA). Mice were then placed onto the warmed stage inside the light-tight camera box with continuous exposure to 1–2% isoflurane. Imaging times ranged from 1 s to 3 mins. Regions of interest from displayed images were identified around the tumor sites and were quantified as total photon counts or photons/s using Living Image® software (Caliper Life Sciences, Hopkinton, MA).

Tumor challenge experiments were conducted with melanoma B16 cells expressing luciferase. For quantitating tumor burden, at 10 minutes after the intraperitoneal injection of 3 mg/mouse D-luciferin (Caliper Life Sciences, Hopkinton, MA), mice were

anesthetized and placed into the light tight chamber of an IVIS 200 bioluminescence imaging system (Caliper Life Sciences, Hopkinton, MA). Data was collected as photons/sec/cm² using the Living Image® software (Caliper Life Sciences, Hopkinton, MA).

MRI was performed using a 7T small animal, 30-cm horizontal-bore magnet and BioSpec Avance III spectrometer (Bruker, Billerica, MA) with a 116 mm high power gradient set (600 mT/m) and a 72 mm whole-body mouse transmit/surface receive coil configuration. Axial and coronal T1-weighted (MSME; TE 10.5 ms, TR 322 ms, 0.5 mm slice thickness, matrix 256×256, field of view (FOV) 2.8 cm, eight averages, 40 coronal slices, scan time 22 minutes, and 20 axial slices, scan time 16 min) and fat-suppressed turbo spin echo T2-weighted (RARE; TE1 14.5 ms, TE2 65.5 ms, TR 4500 ms, 0.5 mm slice thickness, Matrix 256×256, FOV 2.8 cm, eight averages, 40 coronal slices, scan time 28 minutes, and 20 axial slices, scan time 28 minutes) images were acquired, covering the volume of brain from the olfactory bulb/frontal lobe fissure to the cervical spinal cord. MRI data were analyzed using the MEDx3.4.3 software package (Medical Numerics, Virginia, USA) on a LINUX workstation.

Cytokine Quantification

Standard ELISAs for assessment of cytokine release by NK cells (IFN- γ , MIP1 α , and GM-CSF) were performed using BD OptEIA ELISA kit (BD Biosciences Pharmingen, San Diego, CA) as previously described [18]. Results were expressed as mean cytokine concentration (pg/ml) \pm SEM.

For intracellular cytokine staining, single cell suspensions were prepared and incubated at 37°C for four days in round-bottomed plates (2×10⁶ cells/well), and stimulated with a mixture of PMA

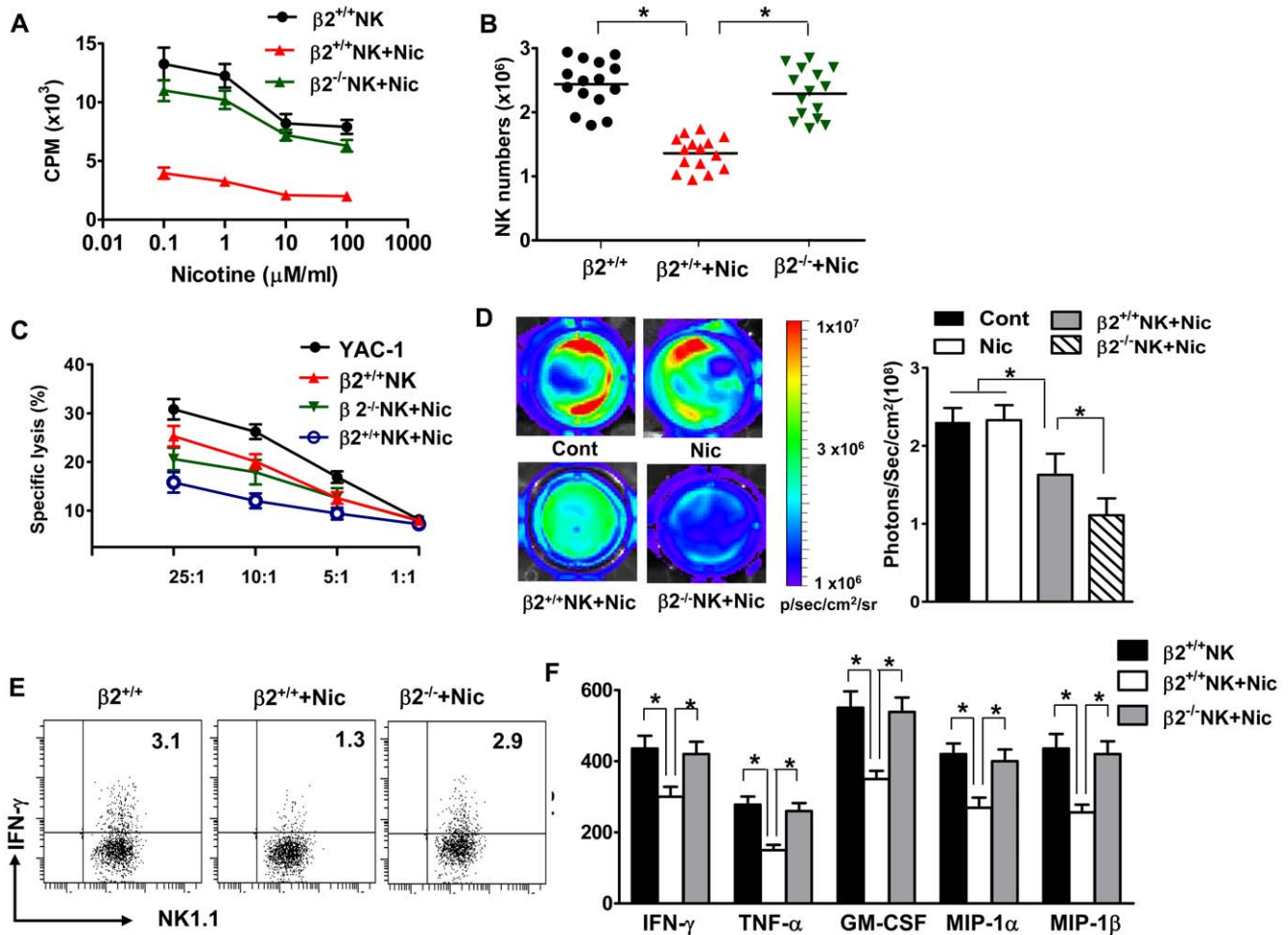


Figure 3. Nicotine impairs NK cell proliferation and killing of target cells by acting on nAChR $\beta 2$. NK cells were sorted from splenocyte single cell suspensions of wild type ($\beta 2^{+/+}$) or nAChR $\beta 2$ knock-out ($\beta 2^{-/-}$). **A.** Cells were then cultured in the presence of various concentrations of nicotine (0.1, 1, 10 or 100 μM), and [^3H]thymidine incorporation was measured (10^3 cpm + SEM; ordinate). **B.** NK cell numbers were assessed from $\beta 2^{+/+}$ or $\beta 2^{-/-}$ mice in the presence or absence of nicotine. ($N=6-8$ per group; Student's t -test, * $p<0.05$). **C.** ^{51}Cr -labelled target cell YAC-1 were incubated with NK cells derived from $\beta 2^{+/+}$ or $\beta 2^{-/-}$ mice in the presence or absence of nicotine, at the indicated effector/target cell ratio. Killing of target cells are measured by ^{51}Cr release assay. **D.** B16 cells were incubated with NK cells derived from $\beta 2^{+/+}$ or $\beta 2^{-/-}$ mice in the presence or absence of nicotine. D-Luciferin was added to each well and the plate was imaged to obtain photons/s per cell. Wells with cells alone (no nicotine) or cells (added nicotine) were included as controls. The killing of B16 cells are measured via bioluminescence imaging. The data of one experiment out of two performed is shown, $n=3-4$ mice/group. P values, Student's t -test, * $p<0.05$. **E.** Production of IFN- γ was measured by intracellular cytokine staining. Dot plots are representative of two separate experiments ($n=6-18$ mice). **F.** Production of IFN- γ , TNF- α , GM-CSF, MIP-1 α and MIP-1 β by sorted NK cells was measured by ELISA. P values, Student's t -test, * $p<0.05$. doi:10.1371/journal.pone.0057495.g003

(20 ng/ml), ionomycin (1 $\mu\text{g}/\text{ml}$), and brefeldin A (5 $\mu\text{g}/\text{ml}$) for another 5 h at 37°C. After harvesting, cells were stained for surface markers, fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences), then stained with anti-IFN- γ mAb (XMG1.2) conjugated with Alexa 647. All samples were analyzed on a FACS AriaTM using DivaTM software.

qRT-PCR

Total RNA was extracted from cell suspensions of spinal cords using TRIzol (Invitrogen). First-strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen). RT-PCR was performed as previously described, using an ABI Prism 7900-HT sequence system (Applied Biosystems) with the QuantiTect SYBR Green PCR kit (QIAGEN), in accordance with the manufacturer's instructions. The following primers were used: NF- κB forward, 5'-ATTTGAAACACTGGAAGCACGG-3'; NF-

kB reverse, 5'-CCGCCTTCTGCTTGTAGATAGG-3'; IKK α forward, 5'-TGCACACCGTGCAGAGTCA-3'; IKK α reverse, 5'-TGCTTGCAGCCCAACAAC-3'; hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward, 5'-AGCCTAAGATGAGCGCAAGT-3'; and HPRT reverse, 5'-TTACTAGGCAGATGGCCACA-3'. The HPRT gene was amplified and served as an endogenous control. 1 μl of first-strand cDNA product was amplified with platinum Taq polymerase (Invitrogen) and gene-specific primer pairs. Each sample was assayed in triplicate and experiments were repeated twice. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), and mean relative expression was determined by the $2^{-\Delta\Delta\text{Ct}}$ comparative method.

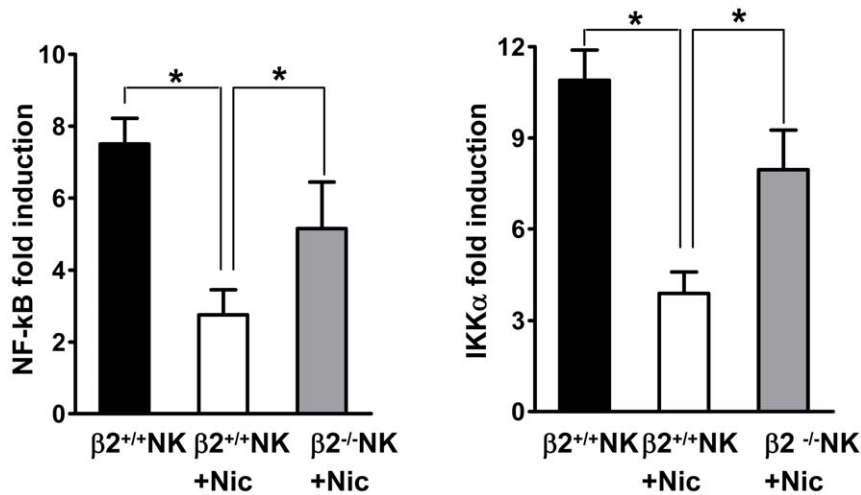


Figure 4. Nicotine inhibits NF- κ B activation in NK cells through nAChR $\beta 2$. NK cells were sorted from splenocyte single cell suspensions of RAG2^{-/-} and RAG2^{-/-} $\beta 2^{-/-}$ mice. NF- κ B and IKK α mRNA of sorted NK cells in $\beta 2^{+/+}$ or $\beta 2^{-/-}$ mice received nicotine (Nic) was measured by qRT-PCR. The data of one experiment out of two performed is shown, n=3–4 mice/group. P values, Student's *t*-test, *p<0.05. doi:10.1371/journal.pone.0057495.g004

Results

nAChR Expression Profile on NK Cells and Confirmation by Ligand Binding Assays

To determine nAChR mRNA expression on NK cells, we sorted NK cells from pooled splenocytes of RAG2^{-/-} C57BL/6 mice that are devoid of T cells, NKT cells and B cells. mRNA was extracted from highly purified NK cells (Figure 1A) and nAChR expression profile was determined by PCR. Our results indicate that NK cells express nAChR $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ and $\beta 3$ but not $\alpha 3$, $\alpha 7$, $\alpha 9$; nor $\beta 4$. Furthermore expression of $\beta 2$ was particularly strong (Figure 1B). We then investigated whether nAChR proteins capable of engaging in nicotinic radioligand binding are assembled in NK cells by using an ¹²⁵I-labeled epibatidine (I-Epi) binding assay (200 pM I-Epi +/- 100 mM carbamylcholine), as described elsewhere [14,19,20,21]. NK cells (~1 million) were purified by FACS, and mouse brain cortex homogenate was used as a positive control. Specific I-Epi binding was 99 fmol/mg protein in NK cells, about equal to specific binding levels in mouse whole brain (Figure 1C). These data demonstrate that NK cells express significant numbers of assembled nAChRs (as well as nAChR subunit mRNAs as shown previously) at levels that can be quantified with this assay. Since I-Epi binds to both $\beta 2$ - and $\beta 4$ -containing nAChRs [14], competitive binding with the nAChR $\beta 2$ -selective compound A85380 was used to discriminate between these two receptor subtypes. We found that A85380 largely abrogated I-Epi binding, demonstrating that the vast majority of nAChRs present on NK cells contain the $\beta 2$ subunit (Figure 1C). Collectively, our results reveal that NK cells express an array of nAChR mRNAs and that most of these subunits assemble into $\beta 2$ -containing nAChRs. The strong expression of nAChR $\beta 2$ in NK cells, both at the mRNA and protein levels suggest that $\beta 2$ -containing nAChRs are candidates for mediation of nicotine's effects on NK cell activity.

Influence of nAChR $\beta 2$ Deficiency on Expression of NK Cell Receptors and Effector Molecules

NK cell functions are dictated by stimulatory and inhibitory receptors on NK cells. Activating signals are generated through NK group 2 member (NKG2D), which recognize the stress-

inducible molecules MHC class I chain-related proteins (MICA and MICB) and the UL-16-binding proteins 1-4 (ULBP1-4), also known as RAET proteins, or through natural cytotoxicity receptors (NCRs) that recognize viral hemagglutinin and as yet undefined tumor-associated ligand. The inhibitory signals are generated by binding of MHC class I molecules to killer cell immunoglobulin-like receptors (KIRs) in humans, or to Ly49 in mice, in addition to the CD94/NKG2A heterodimer in both species [22,23]. We compared the expression of several of these receptors on spleen NK cells isolated from nicotine- or PBS-exposed mice and investigated the influence, if any, of nAChR $\beta 2$ deficiency. Expression of inhibitory receptor NKG2A is not significantly altered on NK cells by nicotine; whereas expression of NKG2D and Ly49I were reduced in mice that received nicotine, and such reductions are largely abrogated in nAChR $\beta 2^{-/-}$ mice (Figure 2A) [13]. Similar findings were obtained regarding expression of NK cell effector molecular Granzyme B and perforin (Figure 2B). We also observed that nicotine has a similar effect on lung-derived NK cells receptor profile and effector molecules (data not shown).

nAChR $\beta 2$ Deficiency Reverses the Inhibitory Effects of Nicotine on NK Cell Functions

To understand the biological consequences of altered NK cell receptor expression induced by nicotine exposure, we investigated the effects of nicotine on NK cell mediated killing of target cells, as well as release of cytokines by NK cells. NK cells normal represent 5–10% of peripheral blood mononuclear cells in human and 1–3% of monocytes in mouse spleen or lymph node. For NK cells to perform their functions, these cells must proliferate under pathological situations. Thus, we first assessed the influence of nicotine on NK cell proliferation and found that nicotine reduced NK cell proliferation and numbers (Figure 3A, B). nAChR $\beta 2$ deficiency largely restored the capacity of NK cells to proliferate and numbers in the presence of nicotine (Figure 3A, B).

Next, we assessed the influence of nicotine on NK cell lytic activity and potential roles for nAChR $\beta 2$. Compared with control mice that received PBS, NK cells from nicotine-treated mice exhibited reduced cytotoxicity against YAC-1, a classic NK target cell with lower MHC class I expression (Figure 3C). Primary

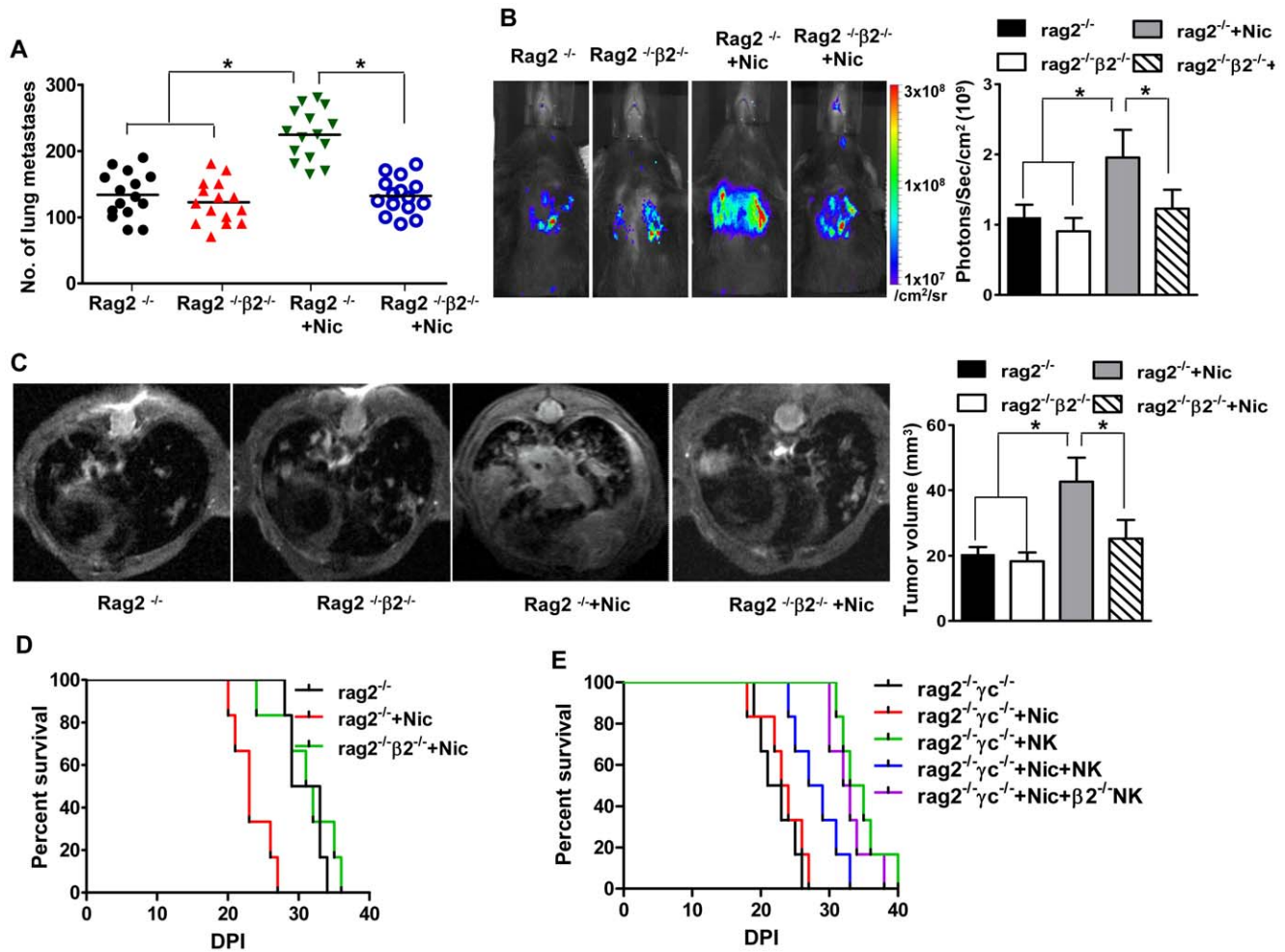


Figure 5. Impairment of NK cell-dependent suppression of B16 tumor cell metastasis by nicotine is mediated by nAChR $\beta 2$. Mice of different genotypes received nicotine (Nic) or PBS for 21 days and engrafted with the B16 melanoma cell line (1×10^6 cells/mouse). **A**. Seven or 14 days later, a portion of mice were euthanized and the lung dissected. Total numbers of melanoma nodules counted in these organs are shown ($n = 12$ mice/group). **B**, **C**. Quantification of tumor growth in these animals during the period are achieved via bioluminescence imaging (**B**) and high field MRI (**C** for T1 images; Figure S2 denotes Th2 images). $N = 15-18$ /group. P values, ANOVA, $*p < 0.05$. **D**. Survival for the remaining animals are monitored for 60 days ($n = 15-18$ mice/group). **E**. Mice were engrafted with melanoma cells lines (1×10^6 /mouse) in the presence or absence of nicotine, and NK cells (5×10^5 NK cells/mouse) from wild type or nAChR $\beta 2^{-/-}$ mice. Mice were then monitored for survival up to 60 days. Results are pooled from three experiments with similar results. $N = 6-8$ mice/group. doi:10.1371/journal.pone.0057495.g005

human and mouse melanoma, or cell lines, shared expression of ligands for natural cytotoxicity receptors (NCRs) and DNAX accessory molecule-1 (DNAM-1), the two emerging NK cell receptors key for cancer recognition [15]. Further, these cells have low MHC class I expression [15]. NK cells are thus able to recognize and lyse human and mouse melanoma cells in vitro and in vivo, and to prevent melanoma metastasis in adoptive transfer experiments [24]. We therefore used the murine B16 cell line, derived from spontaneous murine melanoma, and demonstrated that NK cells readily kill B16 tumor cells, and that this effect was significantly reduced when exposed to nicotine (Figure 3C, D). To investigate the contributions of nAChR $\beta 2$ receptor to mediate the effects of nicotine, we perform these experiments using nAChR $\beta 2^{-/-}$ NK cells (Figure 3C, D). In all experiments, nAChR $\beta 2$ deficiency significantly abrogated the effects of nicotine.

Last, we examine the impact of nicotine on cytokine production by NK cells. Compared with control mice, IFN- γ , GM-CSF, MIP-1 α , MIP-1 β and TNF- α secretion were reduced in mice received nicotine (Figure 3E, F). Again, these parameters were reversed to a

great extent in nAChR $\beta 2^{-/-}$ mice. Collectively, our results demonstrate that nicotine impairs NK cell functions including proliferation, cytotoxicity toward YAC-1 cell and B16 tumor cells, and production of cytokines. Our findings also show that despite expression of multiple nAChRs, nAChR $\beta 2$ emerged as a key mediator for nicotinic effects on NK cells, as deficiency of nAChR $\alpha 7$ fails to significantly alter the influence of nicotine on NK cells (data not shown).

Nicotine Inhibits NF- κ B Transactivation in NK Cells through nAChR $\beta 2$

Mobilization of the transcription factor NF- κ B is an essential step for NK cell activation and proceeds its cytotoxic activity and production of cytokines. To evaluate the impact of nicotine on NF- κ B activity in NK cells, we sorted NK cells from the spleen of RAG2 $^{-/-}$ mice, and cultured the isolated NK cells alone or with nicotine for 24 h. We performed a series of real-time PCR experiments looking at changes in transcript abundance, and saw

that addition of nicotine suppressed gene transcription of NF- κ B, and IKK α , an alternative regulator of NF- κ B in NK cells (Figure 4). To determine if nAChR $\beta 2$ is involved, we cross-bred RAG2 $^{-/-}$ with nAChR $\beta 2^{-/-}$ mice to generate double knockout mice. When NK cells derived from these mice were used, the effect of nicotine on NF- κ B and IKK α transcription becomes minimum (Figure 4). Thus, nAChR $\beta 2$ mediates the effects of nicotine on NK cell transcription genes that are critical for their functions.

Nicotine Exposure Promotes Lung Metastasis, a Process Dependent on nAChR $\beta 2$

Cigarette smoke has been experimentally demonstrated to increase lung metastatic tumor burden [11]. As multiple chemicals contained in cigarette smoke can have this effect, we sought to specifically address the role of nicotine exposure in this process. To this end, we adopted the murine B16 cell line derived from spontaneous murine melanoma. Because B16 tumor cells have high lung metastasis potential and such metastasis can be inhibited by NK cells, this model is ideal to address the role of nicotine and its receptors in this pathological process. For this purpose, C57BL/6 RAG2 $^{-/-}$ mice were treated with nicotine or PBS via osmotic pump implantation for 14 days and beyond. The dosage selection is based on smokers and justified in detail in the methods section and in our previous publications [7,8]. These mice were then challenged i.v. with 1×10^6 B16-melanoma cells. Based on the literature [11,15,16], we did preparation experiments in which tumor burden and survival rates were compared in mice receiving 1×10^5 , 2.5×10^5 , 5×10^5 , 1×10^6 and 2.5×10^6 B16 cells. We found that transplantation of 1×10^6 provided the most efficient comparison of tumor burden, and thus this concentration was used for all subsequent experiments (Table S1). 14 days of nicotine or PBS exposure at the onset of tumor challenge lead to no significant difference in tumor burden between the two groups (data not shown). Nicotine was thus continuously released in these mice until 21 days post tumor challenge. Compared with control mice that received PBS, mice treated with nicotine had significantly greater lung tumor burden (Figure S1; Figure 5A), increased bioluminescence imaging (Figure 5B), tumor volume when assessed by high field MRI of both T1 (Figure 5C) and T2 imaging (Figure S2), as well as decreased survival rates (Figure 5D).

To determine the contributions of nicotinic receptors, we cross-bred RAG2 $^{-/-}$ with mice lacking nAChR $\alpha 7$ or $\beta 2$, two nAChRs prominently expressed by NK cells, and performed similar experiments as described above. Lack of $\alpha 7$ receptors exerted little influence on B16 cell lung metastasis promoted by nicotine (data not shown). In contrast, promotion of metastasis by nicotine is largely diminished in RAG2 $^{-/-}$ $\beta 2^{-/-}$ mice, as the tumor burden was indistinguishable between RAG2 $^{-/-}$ and RAG2 $^{-/-}$ $\beta 2^{-/-}$ mice (Figure 5A–C). Thus, $\beta 2$ receptor plays a critical role in mediating the effects of nicotine's facilitation of lung tumor metastasis.

nAChR $\beta 2$ Determines NK Cell-dependent Tumor Metastasis

The effects of nicotine in promoting B16 cell line lung metastasis can be achieved via a direct effect on tumor cells, lung microenvironment, or immune cells. We focused our investigations on NK cells because of their known effects in constraining B16 cell metastasis, as well as strong expression of several nAChRs in these cells. In addition, as RAG2 $^{-/-}$ mice have no intrinsic T and B cells, the role for nAChR $\beta 2$ in determining the effect of nicotine in tumor metastasis is likely via NK cells. We therefore

first compared tumor burden and survival rates in RAG2 $^{-/-}$ and RAG2 $^{-/-}$ $\gamma c^{-/-}$ mice. Clearly, the lungs of RAG2 $^{-/-}$ $\gamma c^{-/-}$ mice had larger tumor burden and majority of these mice quickly succumb to death after B16 cell challenge (Figure S1; Figure 5E). Our results thus confirm the role of endogenous NK cells in restricting B16 lung metastasis.

Next, we determined the role of nAChR $\beta 2$ on NK cells in curbing tumor metastasis. For this purpose, we co-transferred B16 tumor cells with NK cells isolated from wild type and nAChR $\beta 2^{-/-}$ mice, into the recipient RAG2 $^{-/-}$ $\gamma c^{-/-}$ mice. Lung tumor number and survival rates were indistinguishable between B16-recipient RAG2 $^{-/-}$ $\gamma c^{-/-}$ treated with or without nicotine (Figure 5E). Thus significant roles for macrophage, dendritic cells and other cells in B16 lung metastasis are largely ruled out. Further, co-transfer of NK cells isolated from PBS-treated control mice improve the survival rate of recipient mice. However, such improvement is reduced markedly when NK cells derived from nicotine-treated mice are used for transfer experiments. Importantly, when transfer of NK cells isolated from $\beta 2^{-/-}$ mice, irrespective nicotine treatment, improved the survival rate (Figure 5E), whereas nicotine maintains its impact on B16 tumor cell metastasis when $\alpha 7^{-/-}$ NK cells are co-transferred (data not shown). Therefore, our results suggest that nAChR $\beta 2$ determines NK cell-dependent tumor metastasis.

Discussion

Despite ongoing efforts to reduce smoking prevalence, over 1.1 billion people continue to smoke, and smokers in countries like China comprise a large portion of the overall smoking population, which is estimated about one-sixth of the world's population [2]. Due to the prominent association of smoking with lung tumor and lung metastasis, we addressed several critical issues regarding cigarette smoke, immunity and tumor metastasis in a well established murine lung metastasis model following B16 melanoma challenge. The combined effects of enormous carcinogens, as well as multiple immune suppressive chemicals contained in cigarette smoke contribute to tumorigenesis. Until now, how nicotine, the psychoactive component of tobacco products, affects NK cells and their ability to perform immune surveillance against lung tumors, and which nAChRs mediate such events, were not known.

Several findings revealed by the current study are significant. First, we defined the influence of nicotine on the phenotype and functions of NK cells. Overall, we found that nicotine exposure reduces expression of NK cell-related activating signals as well as NK cell-associated immune effector molecules. This was associated with reduced NK cell proliferation, production of cytokines, cytolytic activity against target cells, and transactivation of NF- κ B. Second, we are able to identify that among several nAChRs, the $\beta 2$ subunit emerged as a principle mediator for these inhibitory effects of nicotine on NK cells. Third, we demonstrated the impact of nicotine on NK cells, as well as the involvement of nAChR $\beta 2$, on the ability of these cells to perform immunosurveillance against murine B16 melanoma, a counterpart to a highly prevalent and malignant cancer in humans.

Effective immune responses against lung tumor growth and metastasis rely on a strong activation of NK cells, CTLs as well as type 1 immunity. Weak antigenic stimuli by tumor antigens coupled with active suppression by regulatory cells preclude the difficulty in achieving effective anti-tumor immunity *in vivo*. Tumor intrinsic factors including reduced expression of class I molecule triggers NK cell cytotoxic activity. Tumor over growth, tissue damage and inflammation mobilize the cytokine synthesis

machinery in NK cells. Early and burst release of IFN- γ in turn boosts type 1 immunity and CTLs and amplifies anti-tumor immunity. Suppression of both cytotoxic activity and cytokine production by NK cells essentially blunt two arms of NK cell – mediated anti-tumor immunity. Indeed, we found a significant increase in lung tumor burden in mice that received nicotine, and such an increase can be linked to compromised NK cell functions. In terms of melanoma, recognition of tumor cells by NK cells appears important to prevent metastasis. Expression of NK receptors NCR and DNAM-1 by both human and murine melanoma cells, as well as reduced expression of MHC-class I molecule, lead to recognition by NK cells and trigger the loss of NK cell tolerance. The importance of NCR is highlighted by evidence that NCR-expressing NK cell clones were required for the elimination of lung carcinoma and, conversely, loss of NCR expression on lung-resident NK cells led to tumor progression [15]. Whether nicotine directly affects expression of NCR or the accessory molecule DNAM-1 awaits further investigation. The lung metastasis model recapitulates the homogenous spread of melanoma. In adoptive transfer models, NK cells are sufficient to reduce tumor burden in the lung as well as other organs (data not shown).

Mammalian nAChR subunits are derived from a family of sixteen different genes ($\alpha 1$ – $\alpha 7$, $\alpha 9$ – $\alpha 10$, $\beta 1$ – $\beta 4$, γ , δ and ϵ) and each have distinct distributions. The combinations and order of nAChR subunits within functional pentamers dictate nAChR subtype properties [25,26]. Despite that nAChR expression has been examined in macrophages, dendritic cells, monocytes, T cells and B cells [27], expression profile of nAChRs and their functions on NK cells have not been previously reported. Here we demonstrated that NK cells express nAChR $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ and $\beta 3$. In addition, positive ligand binding assay results imply nAChR $\beta 2$ expression on NK cells may have biological functions. Indeed, influence of nicotine on NK cells, both at cellular and molecular levels, was largely abrogated in nAChR $\beta 2$ deficient mice. The in vivo effects of nicotine in augmenting B16 metastasis become minimum in nAChR $\beta 2$ /RAG1 double knock-out mice, implying a crucial role for nAChR $\beta 2$ in NK cells, which can not be compensated by other nAChRs. The role for $\alpha 7$ expression on macrophages in the peripheral immune system and microglia within the central nervous system in mediating the cholinergic anti-inflammatory effects are highlighted by recent studies [7,8]. Despite the prominent role for nAChR $\alpha 7$, expression of other nAChRs appears to also influence the functions of macrophages

and microglia [3,4]. However, we found that only nAChR $\beta 2$ appears as a prominent mediator for nicotine's effects on NK cells. The overlapping expression pattern and their functional discrepancy of nAChR on macrophage and NK cells are interesting and deserve in depth investigation.

Taken together, our studies have revealed that NK cells express nAChR $\beta 2$ which determine their function in controlling lung tumor metastasis in a murine model. These data reveal new insight in smoking-induced pathologies. Restoration of altered NK cell function induced by nicotine and smoking may possibly boost their capacity to prevent dissemination of early stage melanoma.

Supporting Information

Figure S1 Gross appearance of B16 tumor cell metastasis to lung in mice received PBS or nicotine. Mice of different genotypes received nicotine (Nic) or PBS for 21 days and engrafted with B16 melanoma cells lines (1×10^6 cells/mouse). Seven of 14 days later, a portion of mice were euthanized and the lung dissected. Representative pictures of lungs are shown. (TIF)

Figure S2 B16 melanoma cells dissemination in lung visualized by MRI T2 images. Mice of different genotypes received nicotine (Nic) or PBS for 21 days and engrafted with B16 melanoma cells lines (1×10^6 cells/mouse). T2 7MRI image denote B16 melanoma tumor cell dissemination in lung. (TIF)

Table S1 Comparison of numbers of lung metastasis in mice engrafted with different numbers of tumor cells. Mice of different genotypes received nicotine (Nic) or PBS for 21 days and engrafted with B16 different numbers of melanoma cells lines as indicated. 14 of 21 days later, mice were euthanized and the lung dissected. Total numbers of melanoma nodules counted in these lungs are compared (n = 6 mice/group). (DOC)

Author Contributions

Supervised nAChR typing, binding and nicotine dosing experiments: PW. Conceived and designed the experiments: JH QZ FS. Performed the experiments: JH MA AS SXS. Analyzed the data: JH QZ FS RL. Contributed reagents/materials/analysis tools: JH QZ FS. Wrote the paper: JH QZ FS.

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