Nicotinic Receptor β2 Determines NK Cell-Dependent Metastasis in a Murine Model of Metastatic Lung Cancer

Junwei Hao2, Fu-Dong Shi2,3, Mohammed Abdelwahab3, Samuel X. Shi3, Alain Simard4, Paul Whiteaker3, Ronald Lukas3, Qinghua Zhou1*

1 Tianjin Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin, China, 2 Department of Neurology, Tianjin Neurological Institute, Tianjin Medical University General Hospital, Tianjin, China, 3 Departments of Neurology, Neurobiology, Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, Arizona, United States of America, 4 Department of Chemistry and Biochemistry, Université de Moncton, Moncton, New Brunswick, Canada

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 β2. Nicotine exposure impairs the ability of NK cells to kill target cells and release cytokines, a process that is largely abrogated by nAChR β2 deficiency. Further, nicotinic suppression of NF-κB-induced transcriptional activity in NK cells is dependent on nAChR β2. This nAChR subtype also plays a large role in the NK cell-mediated control of melanoma metastasis, in a murine lung metastasis model exposed to nicotine. Our findings suggest nAChR β2 as a prominent pathway for nicotine induced impairment of NK cell functions which contributes to the occurrence of smoking-related pathologies.

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 α7 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 -specific autoimmune responses in an nAChR α7-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 β2 as a key determinant for nicotine-mediated impairment of NK cell functions. Further, we demonstrate that nicotinic inhibition of NK cell functions via nAChR β2 significantly increases melanoma metastasis in a xenogenic model.

Materials and Methods

Animals

Female C57BL/6 mice (6–8 wk old), RAG2−/−, Rag2−/−γc−/−, all on a C57BL/6 background, were purchased from Taconic Farms. α7 and β2 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 (∼1.5×10⁶ cells per sample) by using the mAGS mRNA isolation kit (Milenyi 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).

Table 1. Primers used to detect nAChR subunits.

| Subunit | NCBI   | Forward Primer   | Reverse Primer   | Product     | Tm  |
|---------|--------|------------------|------------------|-------------|-----|
| σ3      | NM_145129.2 | gccaacctcacaagaagcc | atgtgggttttagcagaca | 680 | 58  |
| σ4      | NM_015730.5 | cagtgccatcactccgat | tgtagaacagtgcgactg | 577 | 55  |
| σ5      | NM_176844.3 | ggggtctgcttggaacacgt | ggctctgtgattatatcg | 431 | 58  |
| σ6      | NM_021369.2 | tgttcagcagataactctg | tgaattgaacactctcg | 987 | 55  |
| σ7      | NM_007390.2 | cgtgctggcctcagt | ggcctagggcactctc | 514 | 58  |
| σ9      | NM_001081104.1 | ctatttcctctgtgccagc | ttgtatcgctgctatg | 798 | 55  |
| β2      | NM_009602.3 | cttcaacttcatgctgct | cttgctgcagcttcgct | 623 | 58  |
| β3      | NM_173212.3 | acggagatgaaggagacgct | accacgacccctagctca | 364 | 58  |
| β4      | NM_148944.4 | tctctgctgcgctccta | accacgaggtgcatagga | 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 (Tm) 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.

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 μl/hr 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 ~30 gm mouse, which is at the upper end of weight for animals used in the study, this equates to ~15 mg of nicotine free base/kg/hr or ~0.54 mg of nicotine free base/kg/hr. Plasma nicotine levels in mice are ~100–200 ng/ml (~0.6–1.2 mM) after infusion of ~2–4 mg/kg/hr of drug and ~45 ng/ml (~290 mM) after infusion at ~0.5 mg/kg/hr. For comparison, human smokers have peak plasma nicotine levels of 10–50 ng/ml (~60–310 mM). Thus, nicotine levels in plasma (extrapolated to be ~49 ng/ml or ~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 μM/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...
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 × 10^5) 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) × 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 μl 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 μM) was added to the culture medium in addition to LPS (10 μg/ml). After 3 days of incubation, the cells were pulsed for 18 h with 10 μl aliquots containing 1 μCi 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,
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 FACSAriaTM flow cytometer (Becton Dickinson, Mountain View, MD) and analyzed with DivaTM 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 vivo imaging, bioluminescent cells were serially diluted from $10^5$ cells in complete media into black, clear bottomed, 24-well plates (Costar, Acton, MA, USA). D-luciferin (Caliper Life Sciences, Hopkinton, MA) at 150 mg/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).

Cytokine Quantification

Standard ELISAs for assessment of cytokine release by NK cells (IFN-γ, MIP1α, 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) ± SEM.

For intracellular cytokine staining, single cell suspensions were prepared and incubated at 37°C for four days in round-bottomed plates (2 x 10^6 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 β2. NK cells were sorted from splenocyte single cell suspensions of wild type (β2+/-) or nAChR β2 knock-out (β2-/-). 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 β2+ or β2- mice in the presence or absence of nicotine. (N=6–8 per group; Student's t-test, * p<0.05). C. 51Cr-labeled target cell YAC-1 were incubated with NK cells derived from β2+ or β2- mice in the presence or absence of nicotine, at the indicated effector/target cell ratio. Killing of target cells are measured by 51Cr release assay. D. B16 cells were incubated with NK cells derived from β2+ or β2- 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-1α and MIP-1β by sorted NK cells was measured by ELISA. P values, Student's t-test, *p<0.05.

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(20 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/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 FACSAria™ using Diva™ 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 7900HT 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-kB forward, 5'-ATTTGAAACACTGGAAGCGG-3'; NF-kB reverse, 5'-CCGGCTTCTGTCTGATAGATGC-3'; IKKα forward, 5'-TGCACACCGTGCTGAGTCA-3'; IKKα reverse, 5'-TGCTTGAGCCCAAGAACTC-3'; hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward, 5'-AGCCACATGACTGCAAAGT-3'; and HPRT reverse, 5'-TTACTAGAGCATGGCCCACTG-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^-ΔΔCt comparative method.
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 α1, α5, α6, β2 and β3 but not α3, α7, α9; nor β4. Furthermore expression of β2 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 β2- and β4-containing nAChRs [14], competitive binding with the nAChR β2-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 β2 subunit (Figure 1C). Collectively, our results reveal that NK cells express an array of nAChR mRNAs and that most of these subunits assemble into β2-containing nAChRs. The strong expression of nAChR β2 in NK cells, both at the mRNA and protein levels suggest that β2-containing nAChRs are candidates for mediation of nicotine’s effects on NK cell activity.

Influence of nAChR β2 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 β2 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 β2−/− mice (Figure 2A) [13]. Similar findings were obtained regarding expression of NKG2D and Ly49I in human NK cells [12].

nAChR β2 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 β2 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 β2. 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
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 β2 receptor to mediate the effects of nicotine, we performed these experiments using nAChR β2−/− NK cells (Figure 3C, D). In all experiments, nAChR β2 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-1α, MIP-1β and TNF-α secretion were reduced in mice received nicotine (Figure 3E, F). Again, these parameters were reversed to a great extent in nAChR β2−/− 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 β2 emerged as a key mediator for nicotinic effects on NK cells, as deficiency of nAChR α7 fails to significantly alter the influence of nicotine on NK cells (data not shown).

**Nicotine Inhibits NF-κB Transactivation in NK Cells through nAChR β2**

Mobilization of the transcription factor NF-κB 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-κB 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-κB, and IKKα, an alternative regulator of NF-κB in NK cells (Figure 4). To determine if nAChR β2 is involved, we cross-bred RAG2−/− with nAChR β2−/− mice to generate double knockout mice. When NK cells derived from these mice were used, the effect of nicotine on NF-κB and IKKα transcription becomes minimum(Figure 4). Thus, nAChR β2 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 β2

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 1×10⁵ 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 1×10⁵, 2.5×10⁵, 5×10⁵, 1×10⁶ and 2.5×10⁵ B16 cells. We found that transplantation of 1×10⁶ 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 and B16 cell metastasis when transferred to recipient RAG2−/− mice (Figure 5A–C). Thus, nicotine treatment, improved the survival rate (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 β2−/− mice, irrespective nicotine treatment, improved the survival rate (Figure 5E), whereas nicotine maintains its impact on B16 tumor cell metastasis when a7−/− NK cells are co-transferred (data not shown) Therefore, our results suggest that nAChR β2 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-κB. Second, we are able to identify that among several nAChRs, the β2 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 β2, 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 an 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 NRC 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 (α1–α7, β2–β4, γ1, 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 β2, α4, α5, α6, β2 and β3. In addition, positive ligand binding assay results imply nAChR β2 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 β2 deficient mice. The in vivo effects of nicotine in augmenting B16 metastasis become minimum in nAChR β2/RAG1 double knock-out mice, implying a crucial role for nAChR β2 in NK cells, which can not be compensated by other nAChRs. The role for α7 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 α7, expression of other nAChRs appears to also influence the functions of macrophages and microglia [3,4]. However, we found that only nAChR β2 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 β2 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 MRI 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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