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Candidate TLR agonist restores DC-NK cross-talk and improves immunization outcomes in nicotine-exposed hosts

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Purpose: One of the priorities of vaccine program agencies (WHO, NIH, CDC) today is to put a global halt to infectious diseases by developing new and improved vaccines that are safe and effective in all target populations. However, the reduced protection seen in smokers compared to nonsmokers after vaccination has been one of the major hurdles to this effort (*1-4*). We reported that exposure to nicotine significantly diminishes host response to an otherwise protective vaccine due to defects in immunobiology of dendritic cells (DCs) (*5-9*). While these studies from our lab and others have identified the existing problem, none has provided a solution. In this study, we further investigated whether the addition of an appropriate immunological adjuvant to vaccine formulation can overcome the degrading effects of nicotine on vaccination outcome. Methods**:** Osmotic pump delivery of nicotine. Ovalbumin(OVA) transgenic and EG7-OVA mouse model to evaluate host responses to vaccines. Findings: Here we show that among TLR agonists tested, a candidate agonist overcame the impact of nicotine and restored DC-NK cross-talk in vitro. Furthermore, the addition of this agonist to vaccine formulation significantly improved vaccination outcome in nicotine-exposed mice as evidenced by clonal expansion and differentiation of adoptively transferred OVA-specific T cells into IFN-g-secreting effector Th1 cells in the spleen and lymph nodes of both control and nicotine-exposed hosts. Discussion: Our research has revealed the physiological changes triggered by nicotine on host immunity and opened the possibility of developing a vaccine formulation that works optimally in all target populations.

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Candidate TLR agonist restores DC-NK cross-talk and improves immunization outcomes in nicotine-exposed hosts

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Abstract

Background: Vaccines aid in saving lives from infectious diseases and biological warfare attacks. Unless they are effective in all target populations, the likelihood that an unprotected person will transmit disease to a vulnerable individual is greatly increased. There is compelling evidence that smokers, consisting of over one billion people worldwide, are less responsive to vaccines. Yet, there are no scientific organizations or commercial entities offering an enhanced vaccine or working on such optimization. We have made progress in our understanding of mechanisms underlying the impairment of vaccine-induced immunity and reported that prophylactic and therapeutic vaccines fail to protect and cure animals from disease due to nicotine-induced defects in the biological activities of Dendritic Cells (DC). We also reported that the defects observed in DCs are reversible and IFN-γ known to be produced by Natural Killer (NK) cells, is an absolute requirement in this process. *Hypothesis:* We tested whether an adjustment of vaccine formulation with immunological adjuvant(s) capable of restoring interactions between DC and NK cells will improve vaccine efficacy. *Methods:* Osmotic pump delivery of nicotine, Ovalbumin transgenic mice and Flow cytometry were used to evaluate DC:NK cross-talk and immune responses to a protein-based vaccine formulated with TLR agonists. *Results:* We show that among TLR agonists tested, nicotine had limited adverse effects on DC and NK cell responses to the candidate agonist in vitro. Furthermore, addition of this agonist to the vaccine formulation significantly improved vaccination outcomes in nicotine-exposed mice as evidenced by clonal expansion and differentiation of adoptively transferred ovalbumin-specific T cells into IFN-γ-secreting effector Th1 cells in lymphoid tissues of both control and nicotine-exposed hosts. *Conclusion:* Our research has revealed the physiological changes triggered by nicotine on host immunity and opened the possibility of developing a formulation that optimizes the efficacy of vaccines in all target populations

Introduction

One of the most important contributions for public health has been the creation of vaccines. The effectiveness of a vaccine, however, depends on the competency of the individual immune system to adequately respond to that vaccine (*1, 2*). There is compelling evidence that smokers are less responsive to vaccines. Finklea and colleagues showed that smokers have lower titers and a decreased half-life of antibodies to influenza virus after natural disease and immunization (*3*). Holt's group also reported that the longevity of the immune response to subunit vaccine was severely depressed 50 weeks post-vaccination in smokers (*4*). Reduced protection seen in smokers compared to non-smokers after vaccination is further supported by a study where it was found that smokers who received hepatitis B vaccines at 0, 1 and 6 months (standard schedule) had lower antibody levels than non-smokers after 3, 7 and 13 months (*5*). The observed immunosuppression in smokers combined with the importance of DCs in vaccine outcome (*6, 7*) led us to study whether the biological activities of DCs are severely affected by exposure to cigarette smoke.

Several approaches have been used (e.g., smoking machine, smoke extract, nicotine) to evaluate the *in vivo* effects of cigarette smoke on the immune system. All of these approaches are regarded as model systems that imperfectly reproduce some aspect of human smoking. However, studies using these techniques in animal models have provided great insight and are likely to continue to do so (*8*). Cigarette smoke contains more than 4,500 chemicals in its gaseous and particulate phases (*9*) including nicotine, ammonia, carbon monoxide, carbon dioxide, formaldehyde, acrolein, acetone, benzopyrenes, hydroxyquinone, nitrogen oxides and cadmium. Many of these agents are known to be carcinogenic and toxic to the cells (*8*). However, nicotine is the only one considered synonymous with smoking in modulating the immune system (*10*).

Research identified a family of APCs called dendritic cells (DCs) as being the body's key immune cells that are pivotal in the initiation of immune responses against invading pathogens and neoplastic cells. DCs express various receptors including a wide repertoire of the Toll-like receptors (TLRs) which allow for their recognition of pathogen-associated molecular patterns (PAMPs) (*11*) that are the basis of many adjuvants. The recognition of PAMPs by TLRs triggers DC maturation and chemokine receptor CCR7 expression resulting in their migration from tissues to secondary lymphoid organs, upregulation of major histocompatibility complex (MHC) and costimulatory molecules such as CD80 and CD86 that are essential for T-cell priming (*6, 12*). In addition, maturing DCs release cytokines, especially interleukin (IL)-12, which drives the differentiation of naïve T cells into effector memory Th1 cells. DCs, however, do not act in isolation but potentiate their efficiency by interacting with NK cells, resulting in regulation of the adaptive immune response. DC subsets regulate different aspects of NK-cell activation, with type I IFN of plasmacytoid DCs increasing NK-cell cytotoxicity, and IL-12/IL-18 by myeloid DCs primarily triggering IFN-γ cytokine production by NK cells (*13-15*). In turn, NK cells provide immunoregulatory "helper" functions by causing further DC maturation and IL-12 production for efficient Th1 polarization (*14, 16-21*).

Our pioneering in vitro and in vivo work revealed that nicotine-induced defects in the differentiation and the biological activities of DCs (henceforth called nicDCs) diminishes the development of antigen-specific effector memory Th1 cells and antibody production, leading to poor host response to an otherwise protective and therapeutic vaccine (*22-26*). We also reported that the nicotine-induced defects observed in DCs are reversible, and IFN-γ is an absolute requirement in this process (*24*). In this study, we further investigated remedial strategies to overcome the degrading effects of nicotine on DC-NK cross-talk through the use of immunological adjuvant(s).

Results

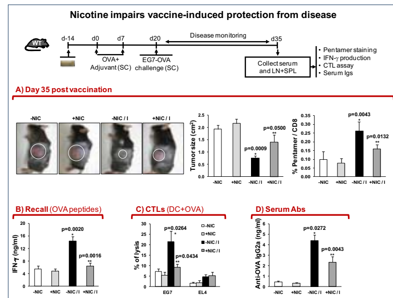


FIGURE 1. Vaccine efficacy in mice exposed to nicotine. **(A)** Unvaccinated (-NIC or +NIC) or vaccinated (-NIC/I or +NIC/I) mice implanted with an osmotic pump containing saline (-NIC) or nicotine (+NIC) were challenged with OVA-EG.7 tumor cells one week after the last vaccination. Photographs of mice two weeks after tumor challenge. Circles and bars indicate the tumor site and size. Bars show the tumor sizes and frequency of endogenous OVA-specific CD8+ (Pentamer/CD8) T cells within CD19-negative lymphoid and splenic cells. **(B, C)** Cells from these mice were also re-stimulated in vitro with OVA peptides (B) or OVA-pulsed DCs (C) for 5 days. (B) Bars display the amount of IFN-γ released in the supernatant of cultures. (C) Bars display the killing activity of cytotoxic effector cells assessed against target tumor cells and expressed as a percentage of specific lysis. **(D)** Bars depict the titers of OVA-specific IgG2a in the serum of mice. Bars are expressed as means ± SEM. * -NIC vs. –NIC/I and ** -NIC/I vs. +NIC/I, p < 0.05.

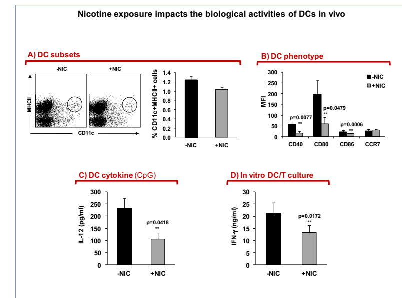
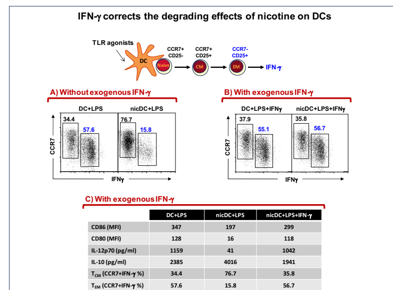
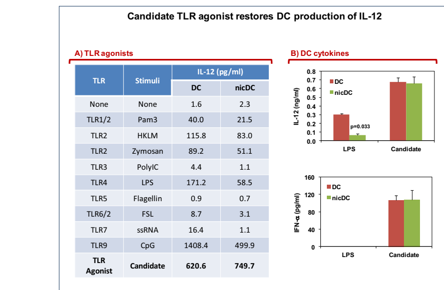


FIGURE 2. Impact of nicotine exposure on DCs in vivo. WT mice were left intact (-NIC) or exposed to nicotine (+NIC) for up to two weeks. **(A)** Gates and bars identify the total CD11c/MHCII DCs and their frequency within splenic cells. **(B, C, D)** Total DCs (CD11c+ fraction) were enriched from spleen tissue. (B) Bars show the mean fluorescence intensity of indicated cell surface markers with isotype control subtracted. (C) Bars represent the amount of cytokine IL-12 produced by DCs from control and nicotine-exposed mice after 24 hrs in culture with the TLR agonist, CpG. (D) DCs were loaded with OVA peptide (323-339) and co-cultured with OVA-specific CD4+ T cells isolated from OVA-transgenic mice. Bars depict the amount of Th1-cytokine IFN-γ released in culture supernatant after 3-4 days of co-culture. Bars are expressed as means ± SEM. ** -NIC vs. +NIC, p < 0.05.



	DC+LPS	nicDC+LPS	nicDC+LPS+IFN-γ
CD86 (MFI)	347	197	299
CD80 (MFI)	128	16	118
IL-12p70 (pg/ml)	1159	41	1042
IL-10 (pg/ml)	2385	4016	1941
T_{CM} (CCR7+IFN-γ %)	34.4	76.7	35.8
T_{EM} (CCR7+IFN-γ %)	57.6	15.8	56.7

FIGURE 3. Effect of NK-derived IFN-γ on nicDC properties. Monocytes-derived DCs generated in the absence (DC) or the presence of nicotine (nicDC) were exposed to the TRL agonist LPS in the absence **(A)** or presence **(B)** of exogenous IFN-γ. Then they were co-cultured with purified allogeneic naïve T cells. After 5 days, these T cells were collected and rested in IL-2 for an additional 7 days. (A, B) Flow cytometry plots shows the percentages of central (CCR7+IFN-γ-) and effector (CCR7-IFN-γ+) memory cells within expanded T cells. **(C)** Table shows 1) the mean fluorescence intensity (MFI) of indicated cell surface markers with the isotypes control MFI values subtracted, 2) the mean value of IL-12p70 and IL-10 measured in the supernatant, and 3) the percentages of central (CCR7+IFN-γ-) and effector (CCR7-IFN-γ+) memory cells within expanded T cells.



TLR	Stimuli	IL-12 (pg/ml)	
		DC	nicDC
None	None	1.6	2.3
TLR1/2	Pam3	40.0	21.5
TLR2	HKLM	115.8	83.0
TLR2	Zymosan	89.2	51.1
TLR3	PolyIC	4.4	1.1
TLR4	LPS	171.2	58.5
TLR5	Flagellin	0.9	0.7
TLR6/2	FSL	8.7	3.1
TLR7	ssRNA	16.4	1.1
TLR9	CpG	1408.4	499.9
TLR Agonist	**Candidate**	**620.6**	**749.7**

FIGURE 4. IL-12 production by nicDC in response to different TLR agonists. Bone marrow-derived DCs were generated with Flt3-ligand in the absence (DC) or presence of nicotine (nicDC). **(A)** Table shows the amount of IL-12 cytokine produced by DCs and nicDCs after 24hr in culture with the indicated TLR agonists. **(B)** Bars display the amount of IL-12 (top) and IFN-α (bottom) cytokines released in the supernatant following 24hr culture with indicated TLR agonists. Bars are expressed as means ± SEM, p < 0.05.

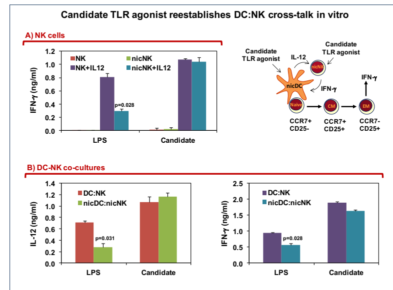


FIGURE 5. Impact of nicotine on TLR agonist-mediated DC:NK cross-talk in vitro. **(A)** NK cells freshly isolated from WT mice were stimulated for 24 hrs with TLR agonists supplemented with or without exogenous IL-12 in the absence (NK) or presence of nicotine (nicNK). Bars display the amount of IFN-γ released in the supernatant of cultures. **(B)** Bone marrow-derived DCs generated with Flt3-ligand in the absence (DC) or presence of nicotine (nicDC) were co-cultured with purified NK or nicotine-exposed NK cells at a 1:2 ratio and stimulated with indicated TLR agonists. After 24hr, culture supernatants were harvested and the production of IL-12 (left panel) and IFN-γ (right panel) measured by ELISA. Bars are expressed as means ± SEM, p < 0.05.

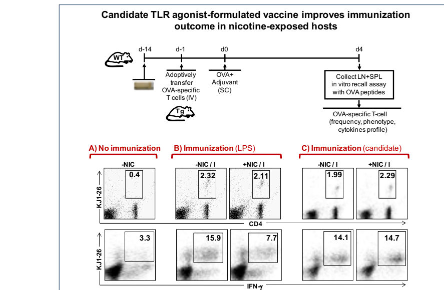


FIGURE 6. Impact of TLR agonists on immunization outcome in mice exposed to nicotine. WT BALB/c mice implanted with an osmotic pump containing saline (-NIC) or nicotine (+NIC) and adoptively transferred with OVA-specific T cells were left intact (-NIC) **(A)** or immunized (-NIC/I, +NIC/I) with OVA protein antigen and TLR agonist, LPS **(B)** or Candidate agonist **(C)**. After 4 days, spleen and lymph nodes were isolated and analyzed by flow cytometry (top panels) or re-stimulated in vitro with OVA peptide (323-339) for 72 hrs (lower panels). (A, B, C, Top panels) Gates indicate the percentage of in vivo expanded transgenic OVA-specific CD4+ (KJ1-26/CD4) T cells. (A, B, C, Lower panels) Gates show the frequency of OVA-specific KJ1-26/CD4 T cells producing IFN-γ.

Materials and Methods

Mice: Wild type (WT) C57BL/6J, BALB/cJ, and ovalbumin (OVA)-transgenic DO11.10 mice (The Jackson Laboratory) are housed in a specific animal facility. Systemic administration of nicotine is performed using miniature osmotic pumps (Durect) filled with nicotine bitartrate salt (Sigma) and implanted s.c. during surgery. Subsequently, mice are injected s.c. with OVA antigen (2mg/100μl) plus TLR agonists (LPS, CpG or Candidate) as adjuvants (50μg). For tumor inoculation (vaccination protocol), age- and sex-matched C57BL/6 recipient mice are given $1x10^6$ EG7 tumor cells s.c. For adoptive transfer (immunization protocol), age- and sex-matched BALB/c recipient mice are given $2x10^6$ DO11.10 TCR transgenic CD4+ T cells i.v. The research procedures outlined in this project is conformed to the guidelines of NIH OLAW and USDA.

T-cell phenotype: Spleen and Lymph nodes (LNs) cells are removed aseptically and kept on ice. Single cell suspensions are obtained by rubbing the tissue between two frosted glass slides and suspended in PBS. Then, cells are suspended in staining media (PBS, 2%FCS, 2mM EDTA). For vaccination protocol, spleen and LNs are first stained with MHC Class I + OVA (257-264) peptide pentamer (Proimmune) followed by staining with anti-CD8 (53-6.7) mAbs. For immunization protocol, cells are stained with anti-CD4 (L3T4), anti-IFN-γ (XMG1.2) (eBioscience), and DO11.10 clonotypic TCR (KJ1-26) mAbs. The labeled cells are then analyzed by flow cytometry.

Recall assay: For vaccination protocol, spleen and LNs are stimulated with either OVA peptides (257-264, 323-339) or OVA-loaded DCs in 6-well plates (25:1 ratio). After incubation at 37ºC for several days, the culture supernatants from these co-cultures are collected and amounts of IFN-γ measured using ELISA kit according to manufacturer's protocols. Expanded cells are also collected and the killing activity of tumor-specific immune effector cells analyzed by cytotoxic assay. For immunization protocol, spleen and LNs are re-stimulated in vitro with 1μM OVA (323-339) peptide. After 4 days, lymphoid cells are harvested; re-stimulated for 4hrs with a leukocyte activation cocktail containing Brefeldin A (BD Biosciences) and the frequency of OVA-specific T cells producing IFN-γ is measured by flow cytometry.

Cytotoxicity Assay: Briefly, targets cells, EL4 (OVA negative), and EG7 (OVA positive), are labeled with calcein AM (15μM, Invitrogen) for 1hr, then washed three times with CM (RPMI1640, 1% L-Glutamine, 1% penicillin/Streptomycin, 50μM 2-β-Mercaptoethanol, 1% Sodium-pyruvate, 1% Essential amino-acids and heat-inactivated 10% FCS). Effectors and targets are co-cultured at 37ºC for 4hr in 96-well round-bottomed tissue culture plates. Supernatants are recovered, and calcein AM release measured using a fluorescent microplate reader (Ex=485nm and Em=530nm). The percentage-specific lysis is calculated using the formula: % lysis = 100 x [(experimental – spontaneous)/(maximal – spontaneous)]. Maximal lysis is achieved with 3% Triton X-100.

Dendritic cell (DC) generation: Femurs are removed from 6-8 weeks age WT mice and the marrow flushed with PBS using a syringe with 0.45μm needle. Cluster within the marrow suspension are dissociated by vigorous pipetting and filtrated through a 70-μm cell strainer. Bone marrow cells are suspended in CM plated at $5x10^6$/ml in 6-well culture plate. They are cultured for up to 6 days in the presence of 200ng/ml of *fms*-like tyrosine kinase-3 ligand (FL) (PeproTech) at 37ºC, 5% CO_2. Nicotine (200μg/ml) is added at the onset of culture and every other day thereafter. Cells are then harvested, stained with CD11c (N418) and positively selected (StemCell Technologies). DC activation is achieved by culturing $1x10^6$ cells in 48-well plates with indicated TLR ligands (1-2.5μg/ml). Supernatants are collected 24hr later and IL-12 and IFN-α cytokines measured by ELISA according to manufacturer's protocol.

DC-NK cross-talk: $1x10^5$ DC are co-cultured in 48-well plates with $2x10^5$ purified NK cells (EasySep positive selection enrichment kit, StemCell Technology) and stimulated with indicated TLR agonists (1-2.5μg/ml, InvivoGen). In parallel, $2x10^5$ purified NK cells are cultured alone with the same TLR agonists in the absence or the presence of recombinant IL-12 (1ng/ml). After 24hr, supernatants are collected and the amount of IL-12, IFN-α, and IFN-γ measured by ELISA according to manufacturer's protocol.

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Conclusion

Despite substantial progress in tobacco control, there are still more than one billion smokers and almost half of the world's children breathe air polluted by tobacco smoke (WHO). Effective vaccination of this large population that may not be receiving the entire benefit from current vaccine programs is critical to overall public health. Our studies have opened the possibility of developing a vaccine formulation that works optimally in all target populations. However, integration of this knowledge for development of current and future vaccines necessitates further investigation into the mechanisms by which the candidate adjuvant but not others exerts its unique adjuvant effects in nicotine-exposed hosts.