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Combination of TLR8 and TLR4 agonists reduces the degrading effects of nicotine on DC-NK mediated effector T cell generation

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Abstract

The magnitude of immune responses to vaccination is a critical factor in determining protection from disease. It is known that cigarette smoke dampens the immune system and increases the risk of vaccine-preventable diseases. We reported that nicotine, the immunosuppressive component of cigarette smoke, disrupts the differentiation and functional properties of DC, which are pivotal in the initiation of immune response to vaccines. We also reported that TLR agonists act in synergy and boost DC maturation, DC-NK crosstalk and ultimately naïve T cell polarization into effector Th1 and Tc1 cells. Here, we investigated whether the combination of TLR agonists could diminish the degrading effects of nicotine on DC-NK mediated effector T cell generation.

We found that none of TLR agonists, single or combined, were able to diminish completely the adverse effects of nicotine on DC. However, TLR3, TLR4, and TLR8 agonists acted as the most effective adjuvants to increase the expression levels of antigen-presenting, costimulatory molecules and production of cytokines by nicotine-exposed DC (nicDC). When combined, TLR3+8 and TLR4+8 synergistically optimized nicDC maturation and IFN- γ secretion from nicotine-exposed NK (nicNK) during co-cultures. Interestingly, in contrast to DC-NK-T, co-cultures of nicDC-nicNK-T treated with TLR3+8 or TLR4+8 agonists produced a similar frequency of effector memory Th1 and Tc1 cells. However, the effector cells from TLR4+8 followed by TLR3+8 treated nicDC-nicNK-T co-cultures produced significantly more IFN- γ when compared with aluminum salt treated co-culture. Our data suggest that addition of appropriate TLR agonists to vaccine formulation could potentially augment the immune response to vaccination in smokers.

Keywords

DC-NK-T crosstalk; TLR agonists; vaccine; nicotine

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Conflict of interest

The authors have no financial conflicts of interest.

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1. Introduction

Vaccines are one of the most successful and cost-effective tools for preventing communicable diseases. They have aided public health by directly protecting vaccinated individuals and by indirectly preventing circulation of infectious agents to individuals who are not eligible for vaccines through their herd effect [1, 2].

Today, one of the priorities of vaccine program agencies (WHO, NIH, CDC) 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 [3–5] has been one of the major hurdles to this effort. Indeed, there is compelling evidence that smokers are less responsive to vaccines. Finklea and colleagues [4] showed that smokers had lower titers and a decreased $t_{1/2}$ of antibodies to influenza virus after natural disease and immunization. In addition, Holt's group [6, 7] reported that the longevity of the immune response to subunit vaccine was severely depressed 50 weeks post-vaccination in smokers. Reduced protection seen in smokers compared with nonsmokers after vaccination is further supported by a study from Winter et al. [5] in which it was found that smokers who received hepatitis B vaccines at 0, 1, and 6 months (standard booster vaccine) had lower Ab levels than nonsmokers after 3, 7, and 13 months. The reduced immune response seen in smokers compared to nonsmokers necessitates refining vaccines with adjuvant formulations that induce protection in all individuals.

Toll-like receptors (TLRs) are pathogen associated molecular pattern (PAMP) recognition receptors that are an important link between innate and adaptive immunity [8]. They are expressed on most innate immune cells including dendritic cells (DC), natural killer (NK) cells, monocytes and granulocytes [9]. PAMPs or TLR agonists mediate the interaction between DC and NK cells, which is a critical step in initiating adaptive immunity. Indeed, a number of published clinical trials utilizing TLRs 2, 3, 4, 7/8 and 9 as adjuvants concluded that TLR ligands are safe, well-tolerated, and effective vaccine adjuvants [9–13].

In response to vaccines, DC sense and deliver the antigen and shape the adaptive immune response [14]. However, DC do not act in isolation but potentiate their efficiency by interacting with NK cells, resulting in regulation of the adaptive immune response [15]. Indeed, secretion of IL-12 by DC activated through TLRs regulates the function of NK cells. In turn, NK cells, which are activated by IL-12 and through their own TLRs, provide an early source of IFN- γ necessary for enhancing stable IL-12 production by DC and favoring the generation of effector memory T cells [16, 17].

We previously reported that nicotine, the known immunosuppressive component of cigarette smoke, induces alterations in the biological activities of DC (henceforth called nicDC), particularly the ability to prime effector memory Th1 cells [18–20]. Importantly, we found the defects seen in nicDC are reversible and IFN- γ is a requirement in this process [21]. Indeed, IFN- γ exposure restored the ability of nicDC to drive the progressive differentiation of naïve T cells towards central-(T_{CM}) and effector-(T_{EM}) memory Th1 cells with T_{CM} cells capable of generating a rapid recall response to secondary antigen challenge [18, 19, 21]. We

also recently showed that TLR agonists act in synergy and promote human DC maturation, DC-NK crosstalk and ultimately naïve T cell polarization into effector Th1 and Tc1 cells.

Therefore, in this study we investigated whether the use of TLR agonists improve nicDC +nicNK crosstalk and their Th1/Tc1 promoting capacity.

2. Materials and methods

1.1. Materials

Recombinant human Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), Interleukin (IL)-4, and IL-2 were purchased from Peprotech (Rocky Hill, NJ). TLR agonists were purchased from Invivogen (San Diego, CA). (–) Nicotine was purchased from Sigma (St Louis, MO). The concentration of nicotine used in this work was chosen according to previous studies [18–28] and correlates with nicotine levels achievable in blood [28, 29] and local tissues, [30] where immune cells encounter antigens.

1.2. Media

Complete culture media (CM) contained RPMI 1640, 1% L -glutamine, 1% penicillin/streptomycin, 50 μ M 2 β -Mercaptoethanol, 1% sodium-pyruvate, 1% non-essential amino acids and heat-inactivated 10% Fetal Calf Serum (FCS). Media for staining cells contained PBS 1X without Ca²⁺/Mg²⁺, heat-inactivated 2% FCS and 2 mM EDTA.

1.3. Generation and immunophenotyping of DC

Immature monocyte-derived DC and nicDC were generated from peripheral blood adhering monocytes cultured in CM with GM-CSF (100 ng/mL) and IL-4 (10 ng/mL) in the absence or presence of nicotine (200 μ g/ml), respectively. Cultures were fed every 2 days with medium containing cytokines and nicotine (200 μ g/ml). Day 6 immature DC (2.5×10^5 /ml) were suspended in CM and activated for 48 hrs with aluminum hydroxide gel 2% (Alum, 2 μ l/ml) or TLR agonists TLR2 (Pam3CSK4, 1 μ g/ml), TLR3 (Polyinosinic I:C, 5 μ g/ml), TLR4 (Monophosphoryl Lipid A, 2.5 μ g/ml), TLR5 (Flagellin, 1 μ g/ml), TLR7 (Adenine analog CL264, 2.5 μ g/ml), TLR8 (Thiazoloquinoline CL075, 2.5 μ g/ml), and TLR9 (Type C CpG oligonucleotide ODN 2395, 5 μ g/ml). Immature and mature DC were stained with fluorochrome-labeled monoclonal Antibodies (mAbs) CD1a (HI149), CD14 (61D3), HLA-DR (LN3), CD80 (L307.4), CD83 (HB15a), CD86 (IT2.2) and CD40 (5C3) (BD Biosciences, San Jose, CA and eBiosciences, San Diego, CA) and analyzed by multi-color Flow Cytometry. The supernatants of mature DC cultures were analyzed by ELISA for production of cytokines, TNF- α , IL-1 β , IL-10 and IL-12 (eBioscience and BD Bioscience, San Diego, CA).

1.4. NK cell isolation

NK cells were isolated from peripheral blood mononuclear cells (PBMC) using the EasySep Human NK cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). The purity of the enriched NK cell population was >90% based upon prevalence of CD56+CD3– phenotypes.

1.5. DC-NK crosstalk

Immature DC and NK cells or nicDC and nicotine-exposed NK (nicNK) cells, (1×10^5 DC: 2×10^5 NK/well) were exposed to Alum or TLR agonists. Supernatants collected from day 2 co-cultures were analyzed by ELISA for IL-12 and IFN- γ production by DC and NK cells, respectively. NK cells were also stained with corresponding fluorochrome-labeled mAbs CD3 (UCHT1), CD56 (MEM188), and CD69 (L78) and analyzed by Flow Cytometry for activation status of NK cells.

1.6. DC-NK-T co-culture

Syngeneic immature DC and NK cells or nicDC and nicNK cells (1×10^5 DC: 2×10^5 NK/well) exposed to Alum or TLR agonists for 8 hrs were subsequently co-cultured in 48-well plates (Falcon, Franklin Lakes, NJ) at a 1:10 ratio with CFSE labeled allogeneic naïve T cells isolated from PBMC using T cell isolation kit (StemCell Technologies, Vancouver, Canada). On day 5, the proliferating cells were transferred into new plates and rested in IL-2-containing medium (5 ng/ml) up to 10 days. The cells were subsequently collected and stained with CD4 (L200), CD8 (SK1), and CCR7 (3D12) (primary co-cultures). The remaining T cells were transferred to plates pre-coated with 10 μ g/ml mAbs CD3 (UCHT1) and 2 μ g/ml soluble CD28 (CD28.2) (BD Biosciences, San Diego, CA) for 72 hrs. The T cells were then stimulated for 4–6 hrs with leukocyte activation cocktail (BD Biosciences) containing Brefeldin A before staining with CD4 (L200), CD8 (SK1), CCR7 (3D12), and intracellular IFN- γ (4S.B3) (BD Biosciences, San Diego, CA) (secondary co-cultures). The frequency and amount of IFN- γ were further analyzed using Flow Cytometry and ELISA.

1.7. Statistical analysis

Statistical significance of differences between Immature or Alum and TLR agonists were calculated using unpaired Mann-Whitney test. A p value <0.05 was considered statistically significant and denoted with an asterisk (*). Analysis was performed using the Prism program (GraphPad, San Diego, CA). Results are expressed as mean $\pm$ SEM. Synergistic effect is considered as 3-fold increase in the sum of individual TLR agonist effects on the expression or production of indicated parameters.

2. Results

2.1. Maturation and cytokine production of human nicDC in response to single TLR agonists

In response to “danger signals” such as microbial products or tissue damage [31], maturing DC not only increase the expression of antigen-presenting and co-stimulatory molecules, but also produce pro-inflammatory cytokines [32, 33] that are critical for optimal activation, proliferation and final differentiation of naïve T cells to effector memory T cells [14, 34, 35]. We have previously shown that nicotine exposure adversely affects human monocyte-derived DC generation (nicDC) and maturation in response to bacterial antigen, lipopolysaccharide [18, 21]. Here, we further evaluated the changes in expression of antigen-presenting and co-stimulatory molecules as well as the production of pro-inflammatory cytokines by maturing

nicDC in response to other TLR agonists, TLR2 (Pam3CSK4), TLR3 (Poly I:C), TLR4 (MPLA), TLR5 (Flagellin), TLR7 (CL264), TLR8 (CL075), and TLR9 (CpG).

Our data revealed that TLR3, TLR5, TLR7, and TLR9 agonists significantly increased the expression level of antigen-presenting molecule, HLA-DR, on maturing nicDC when compared with immature nicDC (Figure 1). Interestingly, further assessment of treated nicDC showed that TLR3, TLR4, and TLR8 agonists significantly increased the expression levels of co-stimulatory molecules CD40, CD80, and CD86 molecules involved in T-cell priming, as well as the maturational marker CD83. TLR2 agonist significantly increased the expression levels of CD40, CD80, and CD86 while TLR5, TLR7 and TLR9 agonists significantly increased the expression levels of CD86 (Figure 1). Maturing nicDC also responded to the TLR agonists by producing different levels of pro-inflammatory and anti-inflammatory cytokines. TLR3, TLR5, TLR7 and TLR9 treated nicDC produced minimal amounts of TNF- α , IL-1 β , IL-10, and IL-12, a key cytokine for Th1 polarization. nicDC treated with TLR2, TLR4 and TLR8 secreted significantly more TNF- α , IL-1 β , and IL-10; and nicDC treated with TLR4 and TLR8 secreted more IL-12 (Figure 2). These data suggests that TLR3, TLR4, and TLR8 agonists were the most effective adjuvants to increase the expression levels of antigen-presenting, co-stimulatory molecules, and maturational marker while TLR4 and TLR8 were the most effective adjuvants to increase the production of cytokines by maturing nicDC (Figures 1 & 2). Hence, we next tested whether various combinations of these selected TLR agonists could act additively or synergistically to further optimize the nicDC maturation.

2.2. Maturation and cytokine production of human nicDC in response to combined TLR agonists

Live, attenuated vaccines are more effective than subunit vaccines [10] due to the fact that upon exposure to the pathogens the immune cells recognize multiple PAMPs through their TLRs and each TLR is coupled with a specific signal transduction pathway [36, 37]. Therefore, we next evaluated the combined effects of TLR3, TLR4, and TLR8 agonists that showed to be most effective in inducing nicDC expression of co-stimulatory molecules and production of inflammatory cytokines (Figures 1 & 2). We found that the TLR3+4 combination demonstrated significant effects on nicDC expression of antigen presenting and costimulatory molecules and maturation marker. Alternately, the TLR3+8 combination increased nicDC expression of CD40, CD80, CD86, and CD83 and the TLR4+8 combination increased nicDC expression of HLA-DR, CD40, CD80, and CD83 when compared with immature nicDC (Figure 3). In addition, we observed no additive or synergistic effects on nicDC expression of HLA-DR, CD40, CD80, CD86 and CD83 with any indicated TLR combinations (Figure 3). We also assessed the amount of cytokines (TNF- α , IL-1 β , IL-10 and IL-12) secreted by nicDC treated with the combined TLR agonists. Interestingly, our data show that in contrast to having almost no additive or synergistic impact on nicDC maturational markers (Figure 3), selected TLR combinations influenced the amount of the cytokines produced by treated nicDC (Figure 4). The TLR3+4 combination altered additively IL-1 β , IL-10, and IL-12 and synergistically TNF- α production. In contrast, TLR3+8 and TLR4+8 combinations altered additively TNF- α and synergistically IL-1 β , IL-10, and IL-12 production (Figure 4). Overall, our data show that

TLR3+8 and TLR4+8 combinations were able to enhance the production of TNF- α (1.7-fold and 2.3-fold respectively), IL-1 β (3.5-fold and 11-fold respectively), IL-10 (3.2-fold and 5-fold respectively), and IL-12 (3-fold and 16-fold respectively) when compared with the highest single producer, TLR8 agonist (Figures 2 & 4). We next examined whether the TLR3+8 or TLR4+8 combinations, the highest producers of IL-12, could act in synergy and effectively program nicDC for further DC-NK crosstalk and Th1/Tc1 polarization.

2.3. Selected TLRs promote nicDC-nicNK crosstalk and lead to activation of more functional IFN- γ producing NK cells

DC and NK cells interaction during the innate immune responses to pathogen is thought to provide an early source of IFN- γ necessary for enhancing the generation of effector memory T cells [38]. We found that exposure to IFN- γ during maturation can alter the differential expression of co-stimulatory molecules on nicDC to the mature DC level and induce IL-12 production by nicDC which leads to an improved Th1/Tc1 development [21]. Therefore, we measured the extent to which selected TLR agonists promote nicDC mediated activation of nicNK cells. When compared with Alum, an adjuvant commonly used in human vaccines, the combination of selected TLR agonists significantly increased both the percentage of the NK cells expressing the activation marker CD69 (Figure 5A) and their production of IFN- γ during DC-NK co-cultures (Figure 5B). The combination of selected TLR agonists also significantly, but less effectively, increased nicDC mediated activation of nicNK cells (Figure 5A). Interestingly, TLR3+8 and TLR4+8 combinations induced the lower amounts of IL-12 production by nicDC (Figure 5C), but to a sufficient extent to promote the significant production of Th1/Tc1 polarizing cytokine IFN- γ by nicNK cells when compared with Alum (Figure 5B). We next compared how effectively TLR3+8 or TLR4+8 combinations prime the development of IFN- γ producing effector T cells.

2.4. Selected TLR agonists improve nicDC-nicNK mediated Th1 polarization

After exposure to pathogens, the innate immune cells initiate the differentiation of naïve T cells to distinct effector T cells [39, 40]. Therefore, we next compared the frequency of effector memory CD4 and CD8 T cells generated when naïve T cells co-cultured with the DC+NK or nicDC+nicNK cells were exposed to Alum or selected TLR combinations. We observed minimal differences in percentages of central memory CFSE-CCR7+ CD4 and CD8 T cells developed in the primary co-cultures (Figure 6A). However, Alum treated co-culture produced the highest percentage (49%) of effector memory CFSE-CCR7- CD4 T cells while TLR4+8 treated co-culture produced the highest percentage (68%) of effector memory CFSE-CCR7- CD8 T cells (Figure 6A). In addition, when central and effector memory T cells from the primary co-cultures were restimulated, significantly higher percentages of IFN- γ -producing effector CD8 T cells developed in DC+NK co-cultures that were originally treated with TLR3+8 or TLR4+8 combinations when compared with Alum co-culture (Figure 6B–C). Interestingly, although both TLR3+8 or TLR4+8 treated nicDC +nicNK co-cultures generated similar frequencies of Th1 and Tc1 effector cells, these effector cells produced significantly higher amount of IFN- γ , 3-fold and 4.2-fold, respectively when compared with Alum co-culture (Figure 6C). Overall, our data suggest that the TLR4 and TLR8 act in synergy and effectively optimize nicDC+nicNK mediated

polarization of naïve T cells into effector Th1 and Tc1 cells producing quantitatively higher amounts of IFN- γ than Alum treated co-cultures.

3. Discussion

In the present study, we have evaluated and sequentially selected the TLR agonists that displayed the highest adjuvant effect for the induction of nicDC maturation, nicDC+nicNK crosstalk, and Th1/Tc1 effector cells. We observed that none of TLR agonists, single or combined, were able to diminish completely the adverse effects of nicotine on DC. However, we found that TLR3, TLR4, and TLR8 were the most effective agonists to induce nicDC maturation (Figure 1). TLR8 followed by TLR4 were also the most effective agonists to induce the production of cytokines by nicDC, especially IL-12 (Figure 2). In addition, TLR3+8 and TLR4+8 agonists were the combinations that had the highest synergistic effects on IL-12 secretion by nicDC and promoted IFN- γ production during nicDC and nicNK interaction. Finally, we observed that the TLR4+8 combination optimally triggered nicDC +nicNK mediated polarization of naïve T cells into IFN- γ producing Th1/Tc1 effector cells.

TLRs signal through pathways that involve distinct adaptor molecules, leading to the activation of different transcription factors. These transcription factors (NF- κ B, IRF3, IRF7, AP-1) induce the expression of genes coding for antigen presenting molecules, co-stimulatory molecules, cytokines and chemokines that play a key role in the priming, expansion and polarization of immune cells. The molecular mechanisms by which the single or combinations of TLR agonists differentially regulate the corresponding genes in DC could be dependent on expression of TLRs, adaptor molecules, signaling pathways and duration of signaling [41]. Similar to what we reported for DC [42], TLR5, TLR7, and TLR9 compared with other TLR agonists were less effective in inducing the expression of antigen presenting and co-stimulatory molecules on nicDC and their production of cytokines (Figures 1 & 2). One possible explanation is that DC express relatively lower levels of TLR5, TLR7, and TLR9 when compared to TLR1-4 [43]. Another possible explanation is that TLR5, TLR7, and TLR9 use the myeloid differentiation primary-response gene 88-dependent (MyD88) to activate NF- κ B while TLR2 and TLR4 recruit two adaptor proteins, the Toll/IL-R (TIR)-domain-containing adaptor protein (TIRAP) and MyD88 to activate NF- κ B and AP-1 for induction of inflammatory cytokines. Indeed, reports indicate that DC stimulated with ligands for TLR that signal through MyD88/TIRAP produce higher amounts of IL-12 [44]. TLR3 as well as TLR4 uses TRIF to trigger an alternative pathway leading to activation of NF- κ B and AP-1 for induction of inflammatory cytokines [41], suggesting that the concomitant activation of NF- κ B and AP-1 transcription factors also contribute to differential expression of the co-stimulatory and cytokines genes in DC.

TLR8 signaling depends on the recruitment of a single adaptor protein MyD88 that culminates in NF- κ B activation [41]. We observed that TLR8 agonist is the most effective inducer of IL-12 production by nicDC (Figure 2) and DC [42]. The difference in IL-12 induction by TLR8 could be explained by the previous observation that IL-12p35 mRNA was up-regulated only by TLR8 [45]. Since high levels of proinflammatory cytokines require NF- κ B activation, it needs to be determined whether TLR8 compared to other TLRs is a more effective inducer of NF- κ B in innate immune cells. Using HEK cells transfected

with either TLR8 or TLR7, Gorden et al., observed that TLR8 agonist rather than the TLR7 agonist is more efficient inducer of NF- κ B [46].

Interestingly, we observed variable degrading effects of nicotine on expression levels of maturational markers and cytokines production by nicDC in response to TLR3, TLR4, and TLR8 agonists. Indeed, the TLR3 agonist induced the expression of HLA-DR and CD86 to the levels observed on maturing DC (Table 1). TLR4 agonist induced the production of IL-10 while the TLR8 agonist was able to induce the production of TNF- α , IL-1 β , and IL-10 to the levels observed on maturing DC (Table 1). It is shown that nicotine exposure inhibits the nuclear binding of NF- κ B and expression of inflammatory cytokines induced by LPS [47]. Our data suggest a broader suppressive role for nicotine on signaling pathways downstream of various TLRs.

We and others [42, 48, 49] have reported that TLR combinations do not increase the expression of antigen-presenting and co-stimulatory molecules over the expression induced by single TLR agonists (Figures 3 & 4) but certain combinations of TLR ligands act in synergy to enhance the release of cytokines, in particular IL-12 by MoDC and PBMC [42, 48–52]. Similar to DC [42], we observed that combinations of TLR3, TLR4, and TLR8 had noticeable synergy for induction of cytokines by nicDC (Figure 4), probably due to sustained signaling provided by dual TLR engagement [48]. Specifically, nicDC produced the highest level of IL-12 when TLR4+8 followed by TLR3+8 agonists were combined (Figure 4). This may be because TLR3 activates the Toll/IL-R (TIR)-domain-containing adaptor protein inducing interferon- β (TRIF) pathway and the stimulation of TLR8 activates MyD88 pathway. TLR4 acts through both pathways. The combination of TRIF-associated TLR ligands with MyD88-associated TLR-ligands induces a cooperation between the two signal-transduction pathways downstream of MyD88 and TRIF that may results in synergistic effects [53]. It has been shown that TLR7, which is less represented on DC under normal conditions, is dramatically upregulated after stimulation by TLR4 agonist LPS [52, 54]. Whether TLR3 (and to higher extent TLR4) agonist induces the higher expression of TLR8 on DC and makes them more responsive to TLR8 agonist is yet to be determined. Interestingly, we also observed various degrading effects of nicotine on expression levels of maturational markers and cytokines production by nicDC in response to selected combinations of TLR agonists. The TLR3+8 agonists only induced the production of TNF- α , IL-1 β , and IL-10 to the levels observed on maturing DC (Table 1). The TLR4+8 agonists quantitatively produced more of these cytokines by nicDC but they were unable to induce the production of TNF- α , IL-1 β , and IL-10 to the levels observed on maturing DC (Table 1).

DC and NK cells interact with each other through soluble factors and cell-cell contact [55]. Our data shows that compared to Alum, TLR3+8 and TLR4+8 combinations significantly increased the percentage of activated NK cells and the production of IFN- γ by activated NK cells during nicDC and nicNK crosstalk (Figures 5A–B). However, the amounts of IFN- γ produced by nicNK cells were lower than the amounts of IFN- γ produced by NK cells during DC and NK crosstalk (Figure 5B). Our data suggest that this is due to 1) less than optimal production of IL-12, a central soluble factor for induction of IFN- γ by NK cells [56, 57], by nicDC as compared to DC (Figure 5C) and 2) the degrading effect of nicotine on NK cells responsiveness to TLR agonists. Indeed, we have observed that NK cells exposed to

nicotine produce lower amounts of IFN- γ in the presence of exogenous IL-12 (data not shown). Other interactions that promote NK cell production of IFN- γ include CXCL10 expressed on DC with its receptor on NK cells and triggering of activation receptor NKG2D [58, 59]. Whether nicotine further compromises the DC-NK interaction by modulating expression of CXCL10 and other receptor-ligands and/or other soluble factors merits further investigation.

Increased protection from reinfection in smokers requires the selection of adjuvants that increase the number of memory T cells. Our data demonstrate that Alum was an effective adjuvant to increase the frequency of effector memory CD4 T cells whereas the TLR3+8 and TLR4+8 combinations were more effective to promote effector memory CD8 T cells (Figure 6). Overall, however, the TLR4+8 combination showed superior adjuvant activity to polarize the nicDC+nicNK mediated differentiation of naïve T cells into both effector memory CD4 and in particular CD8 cells producing IFN- γ (Figure 6). Indeed, while treatment with Alum or TLR3+8 and TLR4+8 combinations resulted in almost the same frequency of total effector memory cells, the effector cells produced in response to TLR4+8 secreted significantly much more IFN- γ in the supernatant (Figure 6C). Our findings are consistent with previous studies, which demonstrated that the combination of TLR4 and TLR8 improves the expansion of peptide-specific human CD8 T cells *in vitro* [60]. This adjuvant effect of the TLR4+8 combination on effector CD4 and CD8 polarization may be directly correlated to high levels of IL-12, IL-1 β , and TNF- α produced by DC and nicDC during co-cultures. Indeed, there is considerable evidence indicating that IL-1 β and IL-12 provide a third signal to support clonal expansion and development of CD4 and CD8 T effector memory function in response to antigen, respectively [61].

Using an animal model, we have previously reported that both prophylactic and therapeutic vaccines formulated with LPS alone fail to protect and cure the nicotine-exposed mice from disease [20]. This is consistent with the *in vitro* human results presented here, which shows that some TLR agonists are not optimal adjuvants to induce maturation of nicDC. Using the same animal model, we have recently observed that the addition of TLR8 agonist to vaccine formulation not only recruits more DC and IFN- γ -producing NK cells into draining LNs but it also increases both cellular and humoral immune responses in mice exposed to nicotine when compared to Alum or LPS. We have also observed that DC isolated from nicotine-exposed mice and treated with LPS express lower levels of transcription factors IRF3, IRF7, and NF- κ B involved in the regulation of pro-inflammatory cytokines. However, DC isolated from WT and nicotine-exposed mice express similar levels of IRF3 and NF- κ B in response to TLR8 agonist (manuscript in preparation).

In conclusion, our data suggest that the addition of appropriate TLR agonists to vaccine formulation could potentially reduce the degrading effects of nicotine and increase the vaccination outcome in smokers.

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Highlights

- Combination of selected TLR agonists reduces the adverse effects of nicotine
- TLR3, TLR4, and TLR8 agonists are the most effective adjuvants to increase antigen-presenting and co-stimulatory molecules expression and to induce production of cytokines by nicotine-exposed DC.
- TLR3+8 and TLR4+8 synergistically optimize nicDC maturation and IFN- γ secretion by nicotine-exposed NK (nicNK).
- Effector cells from nicDC-nicNK-T co-culture treated with TLR4+8 agonists produced significantly more IFN- γ when compared with aluminum salt treated co-culture.

Fig. 1.

Exposure of immature nicDC to indicated TLR agonists. DC were generated in the presence of nicotine (nicDC) and exposed to the indicated TLR agonists. Bar graphs (left panels) show the geometric mean fluorescence intensity (GMFI $\pm$ SEM) of indicated surface makers expressed on nicDC. The GMFI values are shown with the control GMFI subtracted. Plots (right panels) display overlaid histograms of indicated cell surface maturational markers (filled black) and control (filled white) on nicDC after 48 hrs stimulation. One representative flow cytometry data is shown. (n=3–10, n denotes number of individual donor-derived nicDC). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, relative to immature nicDC.

Fig. 2.

Exposure of immature nicDC to indicated TLR agonists. DC were generated in the presence of nicotine (nicDC) and exposed to the indicated TLR agonists. **A–D)** Bar graphs show the amount of indicated cytokines released by nicDC in the supernatants after 48 hrs stimulation. Data are expressed as mean $\pm$ SEM (n=3–7, n denotes number of individual donors).

Fig. 3.

Exposure of immature nicDC to indicated combinations of TLR agonists. DC were generated in the presence of nicotine (nicDC) and exposed to the indicated TLR agonists. Bar graphs (left panels) show the geometric mean fluorescence intensity (GMFI $\pm$ SEM) of indicated surface markers expressed on nicDC. The GMFI values are shown with the control GMFI subtracted. Plots (right panels) display overlaid histograms of indicated cell surface maturational markers (filled black) and control (filled white) on nicDC after 48 hrs stimulation. One representative flow cytometry data is shown. (n=3–10, n denotes number of individual donor-derived nicDC). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, relative to immature nicDC.

Fig. 4.

Immature nicDC exposure to indicated TLR agonists. DC were generated in the presence of nicotine (nicDC) and exposed to the indicated TLR agonists. **A–D**) Bar graphs show the amount of indicated cytokines released by nicDC in the supernatants after 48 hrs stimulation. Data are expressed as mean $\pm$ SEM ($n=3-7$, n denotes number of individual donors).

Fig. 5. nicDC and nicNK cells exposure to aluminum salt or indicated combinations of TLR agonists. DC were generated, co-cultured with NK cells in the absence (DC+NK) or presence of nicotine (nicDC+nicNK) and exposed to the indicated TLR agonists. **A)** Dot plots and bar graphs display percentages of CD56⁺CD69⁺ on pre-gated CD56⁺CD3⁺ NK and nicNK cells after 48 hrs culture with DC and nicDC, respectively. One representative flow cytometry data is shown. **B)** Bar graphs shows the amount of IFN- γ (pg/ml) secreted by activated NK cells in DC+NK (white bar) and nicDC+nicNK (black-striped bar) co-cultures. **C)** Bar graphs show IL-12 production by DC and nicDC in the same co-cultures. B–C) Data are expressed as mean $\pm$ SEM (n=3–4, n denotes number of individual donors). * p<0.05, ** p<0.01, relative to Alum.

Fig. 6.

DC, NK, and naïve T cells exposure to aluminum salt or indicated combinations of TLR agonists. DC+NK (white bar) and nicDC+nicNK (black striped bar) were exposed to the indicated TLR agonists and then co-cultured with T cells. **A)** Bar graphs show the frequency of central (CCR7+CFSE-) and effector (CCR7-CFSE-) memory CD4+ and CD8+ T cells generated in the primary co-cultures. **B)** Flow plots and bar graphs show the frequency of IFN- γ producing effector CD4 (top panels) and CD8 (lower panels) T cells detected within CFSE negative expanded T cells in secondary cultures. One representative flow cytometry data is shown. **C)** Bar graph shows IFN- γ released in the supernatants of secondary cultures. A–C) Data are expressed as mean $\pm$ SEM ($n=3$, n denotes number of individual donors). * $p<0.05$, ** $p<0.01$, relative to Alum.

Table 1

Phenotype of and cytokine production by DC and nicDC

		Im	TLR3	TLR4	TLR8	TLR3+4	TLR3+8	TLR4+8
DR	DC	79.49	119	148	117.5	138.1	105.5	74.96
	nieDC	59.53	116.8	88.01	62.65	115.5	81.43	39.15
	Fold reduction	1.3	1.0	1.7	1.9	1.2	1.3	1.9
CD40	DC	1.51	5.06	7.22	5.56	4.62	5.33	4.86
	nieDC	1.45	4.48	3.53	3.98	4.10	3.98	4.30
	Fold reduction	1.0	1.1	2.0	1.4	1.1	1.3	1.1
CD80	DC	4.72	17.79	35.00	28.07	24.24	26.35	21.98
	nieDC	3.13	13.25	21.37	16.13	22.35	15.60	16.80
	Fold reduction	1.5	1.3	1.6	1.7	1.1	1.7	1.3
CD86	DC	7.295	50.52	90.76	55.36	67.54	58.33	28.82
	nieDC	11.2	54.2	48.53	23.77	60.25	41.38	19.65
	Fold reduction	none	none	1.9	2.3	1.1	1.4	1.5
CD83	DC	1.06	2.75	5.32	4.18	3.92	4.30	2.40
	nieDC	0.61	1.71	3.55	2.02	3.40	1.83	1.88
	Fold reduction	1.7	1.6	1.5	2.1	1.2	2.4	1.3
IL-12	DC		16.06	85.51	610.40	100.40	5187.00	27444.00
	nieDC	ND	0.72	9.23	237.00	19.47	700.90	3791.00
	Fold reduction		22.2	9.3	2.6	5.2	7.4	7.2
TNF-α	DC		142.40	1983.00	8390.00	2068.00	26936.00	62839.00
	nieDC	ND	102.40	1523.00	19251.00	5641.00	34198.00	45345.00
	Fold reduction		1.4	1.3	none	none	none	1.4
IL-10	DC		11.32	234.60	852.60	311.00	1983.00	5467.00
	nieDC	ND	8.11	254.00	843.40	425.00	2767.00	4195.00
	Fold reduction		1.4	none	1.0	none	none	1.3

	Im	TLR3	TLR4	TLR8	TLR3+4	TLR3+8	TLR4+8
DC		28.01	22.38	40.20	26.94	201.70	2104.00
nicDC	ND	0.93	9.99	71.14	30.47	258.30	817.20
IL-1β							
Fold reduction		30.2	2.2	none	none	none	2.6

Immature DC and nicDC were exposed to indicated TLR agonists for up to 48 hr. The values of geometric mean fluorescence intensity (GMFI) of indicated surface markers are shown with control GMFI values subtracted Mean values of indicated cytokines produced by matured nicDC (pg/ml). Fold change is calculated relative to DC. ND, not done.