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Indirect activation of rhesus macaque (*Macaca mulatta*) NK cells in oral and mucosal-draining lymph nodes

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Abstract

The oral mucosae and draining lymph nodes are primary entry points for invading pathogens, particularly during immunosuppressive HIV/SIV infections. Innate immunity against oral stimuli, including natural killer (NK) cells, is understudied. Herein, we demonstrate functional NK cell responses to pathogen-associated molecular patterns (PAMPs) of potential oral pathogens in rhesus macaques.

Introduction

Gastrointestinal and oral mucosae are two of the most heavily immune-surveyed areas in the body. However, compared to gut mucosal studies, the network of lymph nodes and immune cells in the oral mucosae monitoring and maintaining homeostasis with the resident commensal microbiome while also responding to pathogenic microorganisms remains poorly understood [1, 2]. Innate immune cells contribute significantly to the initial response towards invading pathogens, and specifically natural killer (NK) cells play crucial roles in preventing oral cancers [3–7] and oral thrush/candidiasis[8]. Interestingly, while systemic NK cell function diminishes with immunodeficiency resulting from HIV or SIV infections[9–11], previous work from our laboratory showed little functional differences between oral NK cells from SIV-infected and uninfected animals when stimulated with mitogens[12]. Unfortunately artificial stimulation does not recapitulate toll-like receptors (TLR) activation resulting from pathogen-associated molecular patterns (PAMPs). TLR expression on NK cells has been a controversial topic, but a consensus in the field suggests that while NK cells may express some TLR they do not seem to rely directly on TLR engagement in order to respond to an infection or abnormal cell, but more on their environment [8, 9]. Rather antigen-presenting cells such as dendritic cells (DC), macrophages (MØ) or B cells respond to these signals and indirectly activate the NK cell response.

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Materials and Methods

Animals

Indian rhesus macaques (*Macaca mulatta*) were analyzed in this study. All animals were experimentally naïve and euthanized as normal animals for an unrelated research project. Matched oral and mesenteric lymph nodes were isolated from all animals following euthanasia. All samplings were reviewed and approved by the local Institutional Animal Care and Use Committee and were carried out in accordance with recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with recommendations of the Weatherall report; "The use of non-human primates in research".

Indirect NK cell stimulation using TLR ligands

Mononuclear cells from OLN or MLN were rested in R5 media (RPMI + 5%FBS + 1% penstrep) at 37°C for 6h, then stimulated in the presence of Golgi Stop (BD Biosciences, concentrations as recommended by manufacturer) with either 1μg/mL CpG ODN 2395 (InvivoGen, CA) in DOTAP (ROCHE, Germany), 1μg/mL LPS-PG (InvivoGen, CA), 10μg/mL Zymosan (InvivoGen, CA) in R5, or were cultured in R5 only for 12h at 37°C.

Flow cytometry

All antibodies used were purchased from BD Biosciences unless specified otherwise. For surface staining antibodies against the following antigens were used: CD3 (SP34.2), CD8α (SK1), CD11c (3.9, Biolegend), CD14 (MφP9), CD20 (L27), CD16 (3G8), CD45 (D058-1283), CD56 (NCAM16.2), CD80 (L307.4), CD86 (2311), CD159 (Z199, Beckman Coulter). Intracellular staining antibodies included TNF-α (MAb11), IFN-γ (B27), CD107a (H4A3) and MIP1β (24006, R&D Systems). Flow cytometry data was acquired on a LSRII (BD Biosciences, La Jolla, CA) and analyzed with FlowJo software (version 10.2, Tree Star, Ashland, OR).

Statistical analyses

Statistical and graphing analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA). Wilcoxon Matched pairs test and Student's t-test were used where indicated, and p-values of $p < 0.05$ were considered to be statistically significant.

Results

Strengths of using non-human primate models include investigation of the earliest responses at tissue sites of infection. Despite this, the initial responses to infection by NK cells, and the preceding mechanisms leading to their activation are still incompletely understood. Our group has previously shown that there are little differences in oral NK cell responses in normal and SIV-infected macaques [12] following mitogen stimulation, but it is unclear whether NK cells in mucosal-draining lymph nodes might respond differently against more typical pathogen agonists. In order to address this deficit we established a rapid TLR stimulation assay mimicking the earliest responses to infection in OLN and matched MLN, while decreasing feedback-signaling events from long term-stimulation. Total NK cells were

identified as CD3-CD14-CD20-NKG2A/C+ (Fig. 1A) among total mononuclear cells using standard NK cell phenotyping for rhesus macaques optimized in our laboratory [12]. Frequencies of NK cells in MLN were not statistically different compared to those found OLN, though there was a trend towards a greater NK Cell frequency in the MLN (Fig. 1B). Interestingly all three agonists tested elicited measurable responses from NK cells in both OLN and MLN (Fig. 1C) with Zymosan eliciting the most robust IFN-γ response in MLN ($p = 0.015$, Student's *t*-test), and LPS-PG eliciting the strongest CD80 response in MLN over OLN ($p = 0.012$, Student's *t*-test).

Discussion

Many works attributing NK responses following TLR stimulation have long incubation times (≥18h), and in some cases do not use pure NK populations [16–18]. As a result, our indirect assay maybe a better approximation of *in vivo* NK cell stimulation during infection. Here we show that NK cells are stimulated using TLR and Dectin-1 agonists, but due to the abbreviated time of our experiment, we posit that the observed NK cell activation is indirect, rather than a direct activation via NK-cell specific TLR. These agonists include the TLR2/Dectin-1 agonist Zymosan – a proxy for fungal infections such as candidiasis [19], TLR2/4 agonist LPS-PG from *Porphyromonas gingivalis* to mimic common infections in the mouth, and TLR9 agonist CpG ODN to mimic pathogenic bacteria as well as KSHV infection which may lead to Kaposi sarcoma[20, 21]. We hypothesized that stimulation with these agonists will allow us to better elucidate the role of NK cells in microbial infections in the oral mucosae, especially in the context of immunocompromised diseases/conditions, and indeed show here that OLN NK cells are activated by these agonists. We propose that using rhesus macaques will provide a valuable model system with which to examine the NK response during the earliest activation events where antigen-presenting cells initiate a response via TLR activation.

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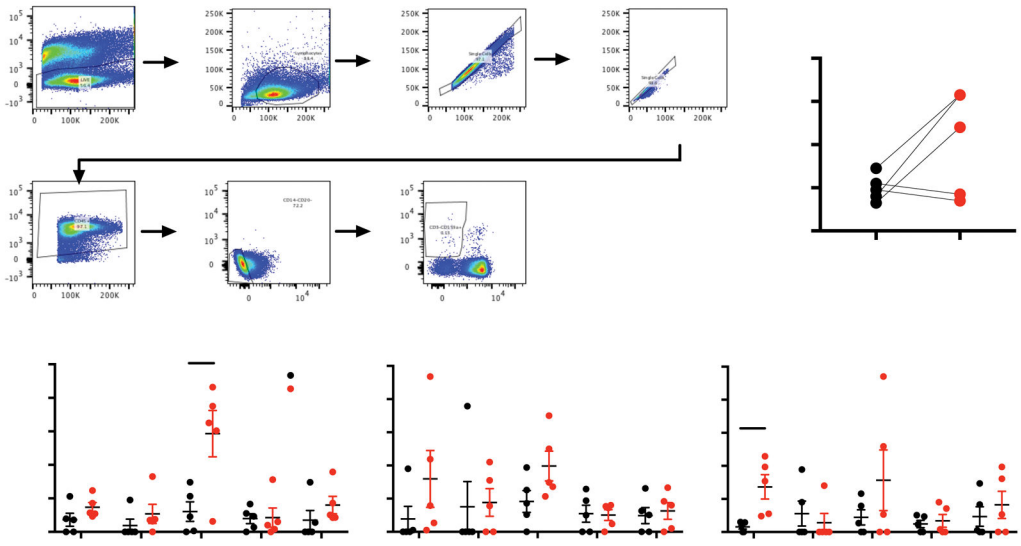

Figure 1. Activation of mucosal draining lymph node NK cells by TLR ligands

(A) Representative gating strategy used to identify NK cells in lymph nodes. (**B**) Frequencies of NK cells found in OLN and MLN samples. **(C)** Background subtracted NK cell responses to ligands for TLR2/Dectin-1 (Zymosan), TLR9 (CpG) and TLR4/2 (LPS-PG) using CD107a, IFNγ, MIP1β, TNFα, CD80 as indicators of NK cell activation. Values from unstimulated controls were subtracted from stimulated values and any negative values were zeroed for readability. Wilcoxon Matched pairs test was used to compare samples between OLN and MLN groups, and Student's *t* test was used for in vitro analyses; *, $P < 0.05$.