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SIV infection of rhesus macaques differentially impacts mononuclear phagocyte responses to virus-derived TLR agonists

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Abstract

Background—During progressive SIV infection, the ability of innate mononuclear phagocytes to function when responding to the invading pathogen has yet to be determined.

Methods—We generated single-stranded RNA (ssRNA) oligonucleotides from the infecting strain of virus and utilized them to stimulate mononuclear phagocytes from blood and lymph nodes of naïve and SIVmac251 infected rhesus macaques.

Results—Soon after infection and continuing through to chronic disease, plasmacytoid dendritic cells (pDC), monocytes, and macrophages from SIV infected macaques were less able to produce pro-inflammatory cytokines after exposure to virus-derived TLR agonists. In contrast, myeloid dendritic cells (mDC) became hyper-responsive during acute and stable chronic infection.

Conclusions—pDC, monocytes, and macrophages may not instigate continued immune activation by recognizing the ssRNA from SIV as they are left dysfunctional after infection. Conversely, mDC functionality may be beneficial as their hyper-responsiveness is related to slowed disease progression.

Keywords

Innate immunity; HIV/AIDS; Nonhuman primate

Introduction

Simian immunodeficiency virus (SIV) infection in non-natural hosts is an important model system that parallels the disease course of human immunodeficiency virus (HIV) infection in humans [1]. HIV and SIV disease progression has been linked with chronic immune activation and in particular continued stimulation of the innate immune system has been suggested to be a contributing cause [2-6]. Exploring the roles of mononuclear phagocytes in HIV and SIV infection is crucial as their functions span the gap between the innate and adaptive immune responses [7]. The mononuclear phagocyte family consists of monocytes, macrophages and dendritic cells (DC), specifically plasmacytoid DC (pDC) and myeloid (mDC). Each of these cell types is able to sense invading pathogens through pathogen associated molecular pattern (PAMP) recognition receptors, including toll-like receptors

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(TLRs) [8]. Plasmacytoid DC recognition of viral single-stranded RNA (ssRNA) by TLR7 induces copious amounts of IFN-α production [9-13]. Additionally, TLR8 activation in monocytes and mDC through ssRNA exposure leads to production of multiple pro-inflammatory cytokines including TNF-α, IL-12 and IL-6 [9].

It has been suggested that persistent recognition of viral genomes by TLR7 in pDC results in chronic production of IFN-α and leads to chronic immune activation [2, 14, 15], but these cells are significantly depleted after HIV or SIV infection [16-20]. The functionality of the remaining pDC has been explored but the results remain conflicting [21-27]. In progressive disease, circulating mDC are also depleted, while elevated levels have been shown to be related to stable disease [16, 17, 28-30]. The remaining mDC have been shown to retain their ability to respond to TLR8 activation [24, 27, 30], but thus far a link between mDC responsiveness to the invading pathogen and disease progression has yet to be found. After pathogenic SIV infection, monocytes have been shown to experience increased turnover rates that can be positively correlated with disease progression [31, 32]. Published evidence exploring monocyte and macrophage ability to respond to TLR activation remains conflicting, as dysfunction and hyper-activation have both been reported [24, 33].

Previously, the innate function of pDC has been explored by using synthetic TLR agonists [21, 24, 27], which may not accurately reflect activation by pathogens. This highlights the need for more relevant TLR agonists that directly test the ability for mononuclear phagocytes to respond to the invading pathogen itself. Because it has been shown that poly-uridine rich sequences from the HIV-1 genome are able to specifically stimulate TLR7 and TLR8 in pDC and mDC [9, 14], we identified and generated SIVmac251-derived single-stranded RNA (ssRNA) oligonucleotides and used them to stimulate either circulating or lymphatic mononuclear phagocytes *ex vivo*. We characterized the functionality of each mononuclear phagocyte population during acute, post-acute, and chronic infection when responding to our virus-derived TLR agonist. We found that soon after infection, pDC, monocytes, and macrophages all became hyporesponsive to virus-derived TLR ligands, whereas mDC appear more sensitive to stimulation. We were able to link mDC function with a positive prognosis of disease as animals that had hyper-responsive mDC at viral set point remained disease free one year after infection [34].

Materials and methods

Animals, virus, anti-retroviral therapy (ART), and samples

All experiments involving animals were performed with appropriate oversight and approval by the Institutional Animal Care and Use Committee of the University of Pittsburgh. For acutely infected samples, ten adult male Indian origin rhesus macaques (*Macaca mulatta*) were infected through intravenous inoculation of 100 $TCID_{50}$ of SIVmac251 (generously provided by Preston Marx, Tulane University, LA). Five of these animals were administered anti-retroviral therapy (ART) consisting of 9-R-2-phosphonomethoxypropyl adenine (PMPA), emtricitabine (FTC; both PMPA and FTC provided by Michael Miller, Gilead Science, Foster City, CA), and L-000870812 (provided by Daria J. Hazuda, Merck Research Laboratories Rahway, NJ) beginning at 7 days post-infection as described [34]. Periodic samplings of peripheral blood leukocytes, peripheral blood mononuclear cells, and lymph nodes were processed into single cell suspensions as previously described [19] and necropsies were performed after 5 weeks of infection. Chronically infected samples that had been previously characterized and archived [30] were analyzed for responsiveness at viral set-point (10 weeks post-infection).

SIVmac251-derived ssRNA oligonucleotides

HIV-1-derived RNA40 and RNA41 have been described elsewhere [9]. The SIVmac251 consensus sequence was scanned for regions that were 20 nucleotides long, >50% uridine-rich and were not predicted to make hairpin loops. The oligonucleotides were named according to their location in the genome. The corresponding control sequences were generated to replace uridines with adenines and all oligonucleotides were ordered from Invitrogen with desalting purification. For *ex vivo* stimulations, 10 μg of each ssRNA oligonucleotide was coupled to DOTAP and incubated in low-volume mixed cultures for two hours. The culture volumes were then increased and brefeldin A was added for the remaining five hours. The appropriate control stimulation using the A-variant oligonucleotide was included for gating purposes [34].

Characterization of mononuclear phagocyte functionality

Flow cytometric analysis was utilized to characterize the functionality of each cell type after stimulation. Monocytes were identified in the blood as HLA-DR$^+$ CD3$^-$ CD20$^-$ CD14$^+$, while macrophages were defined as HLA-DR$^+$ CD3$^-$ CD20$^-$ CD163$^+$ in lymphatic tissue. pDC were identified as HLA-DR$^+$ CD3$^-$ CD20$^-$ CD14/163$^-$ CD11c$^-$ CD123$^+$. mDC were HLA-DR$^+$ CD3$^-$ CD20$^-$ CD14/163$^-$ CD11c$^+$ CD123$^-$. Intracellular TNF-α and IFN-α were identified through flow cytometry as described elsewhere [34].

Results

Virus-derived ssRNA oligonucleotides are potent stimulators of mononuclear phagocytes

As depicted in Figure 1, we identified >50% poly-uridine rich sequences that were 20 nucleotides long and named them according to their location in the SIVmac251 genome. Eleven locations in the SIVmac251 genome were identified to fit these criteria. Four of the sequences were predicted to create hairpin loops and were therefore deemed inappropriate for our application as they would likely become double-stranded RNA. Of the remaining seven, we selected three sequences (Env35, Env687, and Env976) and their corresponding adenine-containing variants (Env35A, Env687A, and Env976A) to synthesize and be evaluated as our TLR7 and TLR8 stimulants. We then tested the stimulatory capacity of each sequence to cause circulating pDC taken from naïve rhesus macaques to produce TNF-α and IFN-α. As seen in Figure 2, each uridine-rich oligonucleotide was specifically able to stimulate pDC to produce IFN-α and TNF-α as compared to the adenine-containing variant. The HIV-1 version RNA40 caused 9% of pDC to produce IFN-α and 43% to produce TNF-α. Of our SIVmac251-derived agonists, Env976 yielded the most robust stimulation of pDC given 14% produced IFN-α and 52% produced TNF-α. We further quantified the stimulatory capacity of Env976 to activate pDC, monocytes, and mDC in blood and pDC, macrophages, and mDC in lymph nodes taken from naïve rhesus macaques [34]. We determined that blood pDC were able to produce IFN-α and TNF-α, whereas circulating monocytes and mDC were only able to produce TNF-α [34]. In contrast in lymphatic tissue, pDC and, to a larger extent, macrophages were both able to produce IFN-α and TNF-α, whereas mDC only made TNF-α [34].

Mononuclear phagocytes experience divergent changes in functionality upon SIV infection when responding to virus-derived TLR agonists

We next wanted to determine how mononuclear phagocytes taken from SIVmac251 infected rhesus macaques function in response to *ex vivo* exposure to TLR agonists derived from the infecting virus. Very soon after infection, pDC, monocytes, and macrophages experienced a rapid decrease in their ability to produce pro-inflammatory cytokines upon exposure to Env976 as published elsewhere [34] and summarized in Figure 3. The dysfunction of

circulating pDC and monocytes was evident seven days post-infection, and pDC and macrophages from biopsied lymph nodes were rendered less functional at 14 days post-infection, effects that were largely reversed with 4 weeks of ART. This dysfunction was maintained at viral set point (ten weeks post-infection) in a separate cohort of animals that were followed long term for disease progression. In stark contrast, mDC exhibited a transient surge in production of TNF-α after exposure to Env976 during acute infection that was diminished by five weeks post-infection. Furthermore, in a separate cohort of animals that were monitored for progressive or stable disease, hyper-responsive mDC were found at viral set point only in the stably infected animals, suggesting that mDC hyper-responsiveness early on may be beneficial to control disease [34].

Discussion

In order to determine how SIV infection impacts innate immune cells and overall immune activation, we generated relevant TLR agonists that would likely be encountered, as they are derived from the invading pathogen. In *ex vivo* mixed cultures, each ssRNA oligonucleotide was able to stimulate circulating and lymphatic mononuclear phagocytes to produce pro-inflammatory cytokines. Using these oligonucleotides to stimulate TLR7 and TLR8, we were able to conduct a direct comparison of functionality between pDC, monocytes, macrophages, and mDC after pathogenic SIV infection. We were also able to use these physiologically relevant stimuli to explore *ex vivo* how each innate immune cell may function at fighting chronic SIV infection *in vivo*.

IFN-α presence has been shown to be beneficial to the host by inhibiting HIV replication, decreasing viral load in patients, and by being elevated in infected patients experiencing elite control without therapy [35-38]. In contrast, IFN-α has also been argued to be detrimental by being implicated in chronic up-regulation of interferon stimulated genes (ISGs) leading to persistent immune activation [2, 15, 39-41]; thus, it is important to determine the ability for IFN-α producing cells to function in response to the invading pathogen *in vivo*. By seven days post-infection, pDC experience up to a seven-fold mobilization into the blood [20] and by 14 days after infection, macrophages experience a significant accumulation in lymph nodes [34, 42]. Already at these time points and continuing through to chronic infection, these cells are less able to produce IFN-α when exposed to virus-derived TLR agonists [34]. Likewise, there is a peak of type I IFN by two weeks post-infection [43] and our data suggests this peak may not be coming from the traditional IFN-α producers as they are rendered hyporesponsive [34]. This suggests that there are other cell types creating type I IFN that may be inducing chronic immune activation, and data from our lab and others shows that mDC from infected animals may be gaining the ability to produce IFN-α [44, 45].

In stark contrast to pDC and macrophage impairment, mDC become hyper-responsive to virus-derived TLR agonists during acute infection and this phenotype may be beneficial as it is related to disease control [34]. Previous work from our lab has shown that circulating mDC from SIV infected rhesus macaques express more CCR7 which causes them to home to lymphatic tissues [30]. The mDC found in the lymph node of SIV infected macaques express less co-stimulatory molecules suggesting that these cells are semi-mature leaving them readily activated by TLR stimulation [30, 34, 46-48]. This beneficial, hyper-responsive phenotype appears only in the chronic stages of stable disease [34], suggesting that some function of semi-mature, hyper-responsive mDC may be important for initiating an appropriate anti-SIV immune response or controlling continued immune activation. Understanding the beneficial and detrimental roles mononuclear phagocytes play on chronic immune activation may be key in understanding disease control and progression. The use of SIV infection in nonhuman primates as a model for AIDS remains crucial to improve our

understanding of early immunologic events that may ultimately set the environment for chronic inflammation.

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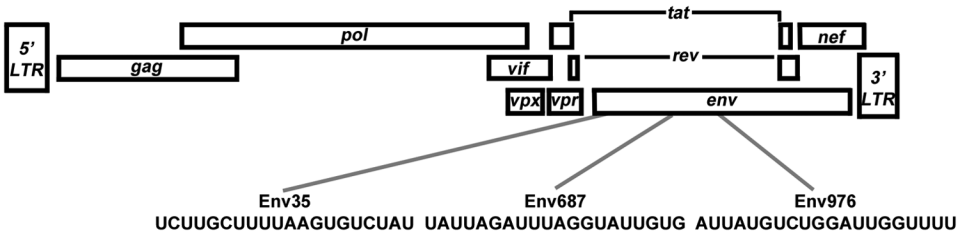

Figure 1. Location of virus-encoded TLR agonists in the SIVmac251 genome

The poly-uridine rich sequences found in SIVmac251 are given and named for their location.

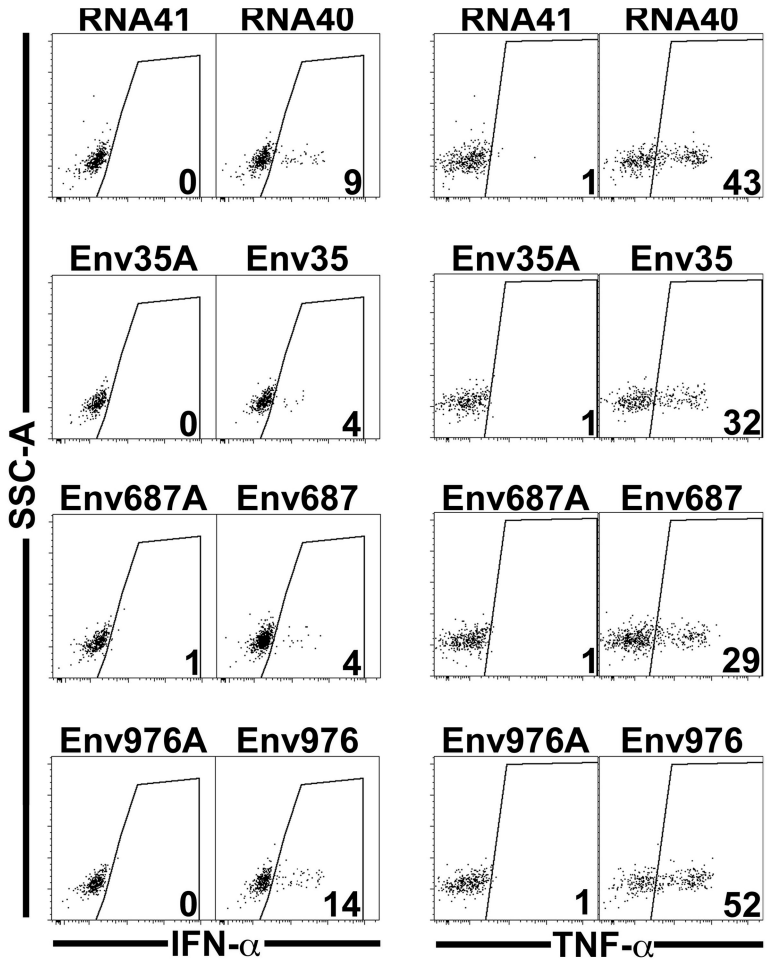

Figure 2. SIVmac251-derived ssRNA oligonucleotides stimulate pDC from SIV-naïve rhesus macaques to produce cytokines at varying levels

PBMC were exposed to TLR7 and TLR8 agonists derived from either HIV-1 (RNA40) or SIVmac251 (Env35, Env687, Env976) or the respective control oligonucleotides (RNA41, Env35A, Env687A, Env976A). Plasmacytoid DC were then identified through flow cytometry as MHC-II^{+} CD3^{-} CD20^{-} CD14/163^{-} CD11c^{-} and CD123^{+}. Each representative dot plot shows the percentage of pDC staining positively for each cytokine.

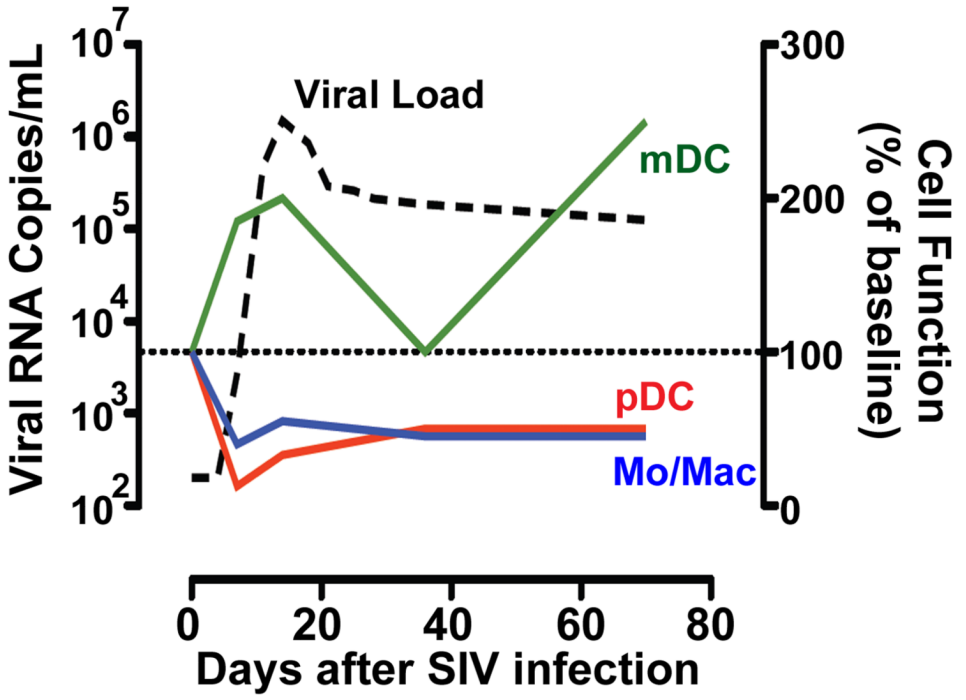

Figure 3. SIV infection of rhesus macaques renders pDC, monocytes, and macrophages less responsive, whereas mDC become hyper-responsive, upon exposure to virus-derived TLR ligands

As the plasma viral load (dashed line) becomes detectable, changes in the functional responses of each cell type (right axis) are evident when compared to pre-infection (dotted line). As early as 7 days after infection, plasmacytoid DC (red), monocytes, and macrophages (blue) are left hypo-responsive. In contrast, mDC (green) become hyper-responsive to stimulation as viral load peaks during acute infection as well as during chronic stable infection.