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Distinctive TLR7 Signaling, Type I IFN Production, and Attenuated Innate and Adaptive Immune Responses to Yellow Fever Virus in a Primate Reservoir Host

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Why cross-species transmissions of zoonotic viral infections to humans are frequently associated with severe disease when viruses responsible for many zoonotic diseases appear to cause only benign infections in their reservoir hosts is unclear. Sooty mangabeys (SMs), a reservoir host for SIV, do not develop disease following SIV infection, unlike nonnatural HIV-infected human or SIV-infected rhesus macaque (RM) hosts. SIV infections of SMs are characterized by an absence of chronic immune activation, in association with significantly reduced IFN-α production by plasmacytoid dendritic cells (pDCs) following exposure to SIV or other defined TLR7 or TLR9 ligands. In this study, we demonstrate that SM pDCs produce significantly less IFN-α following exposure to live attenuated yellow fever virus 17D strain vaccine, a virus that we show is also recognized by TLR7, than do RM or human pDCs. Furthermore, in contrast to RM, SMs mount limited activation of innate immune responses and adaptive T cell proliferative responses, along with only transient antimicrobial Ab responses, following infection with yellow fever vaccine 17D strain. However, SMs do raise significant and durable cellular and humoral immune responses comparable to those seen in RMs when infected with modified vaccinia Ankara, a virus whose immunogenicity does not require TLR7/9 recognition. Hence, differences in the pattern of TLR7 signaling and type I IFN production by pDCs between primate species play an important role in determining their ability to mount and maintain innate and adaptive immune responses to specific viruses, and they may also contribute to determining whether disease follows infection. The Journal of Immunology, 2011, 186: 6406–6416.

The majority of emerging viruses, including HIV, avian influenza, severe acute respiratory syndrome coronavirus, and Ebola virus, have zoonotic origins (1). Although these viruses typically cause benign infections in their natural reservoir hosts, cross-species transmission to humans can result in severe disease (2–6). Unfortunately, our understanding of host-specific attributes that determine whether disease develops following infection with zoonotic viruses is very limited. To date, mechanisms enabling resistance to infection have generally been better studied than those that allow hosts to tolerate particular infections without disease (7). However, elucidation of the mechanisms by which reservoir hosts remain healthy despite harboring viruses that cause severe disease following zoonotic transmissions will be essential to better understand how host immune responses can either ameliorate or contribute to infection-associated damage. Insights into healthy host–virus equilibria in natural host species will also provide valuable information to guide development of effective therapeutic interventions for treating zoonotic infections. Furthermore, improved understanding of the biology and evolution of natural reservoir hosts for viruses responsible for zoonotic diseases will illuminate whether the association of specific host species (such as bats and African primates) with multiple emerging viruses reflects common or distinctive biological features of the host (8, 9).

Numerous viruses have crossed into humans from African primate species with devastating consequences, such as SIV, yellow fever virus (YFV), and dengue fever virus (8, 10). More than 30 African primate species harboring closely related SIVs in the wild have been described so far (11). SIV from chimpanzees has given rise to HIV-1 in humans, and SIV from a second primate reservoir host, the sooty mangabey (SM), is the zoonotic origin of SIVmac in rhesus macaques (RMs) and HIV-2 in humans (10). Similar to SIV, there is evidence that YFV also originates in Africa, infecting many of the same primate species, including SMs, which do not develop disease upon YFV infection and are important in the ecology of the sylvatic cycle of YFV transmission (12, 13). In contrast, YFV infections of RMs (Asian primates that are not natural hosts for YFV) are severe and often lethal (14, 15).

Among emerging viruses, the immune responses of reservoir hosts for SIV have been studied in the greatest detail (16). Infections of nonnatural RM hosts with SIV, as for HIV infections in humans, lead to the development of immunodeficiency in association with persistent virus replication, chronic aberrant acti-
vation of multiple arms of the immune system, active but ultimately ineffective host antiviral cellular and humoral immune responses, and progressive CD4+ T cell depletion (17). In marked contrast, SIV-infected SMs and other natural hosts do not progress to AIDS despite high levels of virus replication (18). Importantly, a key feature distinguishing nonpathogenic SIV infections of natural hosts is the absence of chronic generalized immune activation (18). Whether this is a result of the active suppression of immune activation or the absence of signals that instigate or maintain ongoing immune activation, or both, is still unclear (19). SIV and HIV have been shown to potently activate human and RM plasmacytoid dendritic cells (pDCs) to produce IFN-α and other cytokines (e.g., IL-12 and TNF-α) via TLR7 and TLR9 (20). We have previously shown that SM pDCs produce significantly less IFN-α, but similar levels of other proinflammatory cytokines, after engagement of TLR7 and TLR9 by SIV or other TLR7/9 ligands, than do pDCs from humans or RMs. SM pDCs produce equivalent levels of IFN-α as do those from RMs and humans following exposure to innate immune response agonists (e.g., live influenza virus) that activate pDCs via TLRs other than TLR7 and TLR9 or via cytosolic pattern recognition receptors (e.g., RIG-I–like helicases), demonstrating that they do not have an intrinsic inability to produce IFN-α (21). This pattern of cytokine production suggests impaired signaling along the IFN regulatory factor (IRF)-7 pathway, but preserved NF-kB–dependent signaling following TLR7 or TLR9 activation in SMs. Currently, it is unknown whether the qualitatively different immune response to SIV in SMs compared with RMs is unique or whether it may also affect immune responses mounted to other viruses that, similar to SIV, are recognized by TLR7 or TLR9.

To investigate this question, we studied both the ex vivo responses of pDCs and the in vivo immune responses of RMs and SMs to two distinct viruses: YFV vaccine 17D strain (YF-17D), a flavivirus, and modified vaccinia virus Ankara (MVA), an orthopoxvirus. YF-17D has an ssRNA genome and in mice has been shown to be recognized by TLRs 2, 7, 8, and 9 (22). In contrast, MVA has a dsDNA genome and its innate recognition, as studied in murine models, has been shown not to involve TLR7 or TLR9 (23, 24). Both of these viruses, the attenuated live YF-17D vaccine strain, and the host range-restricted MVA have been shown to elicit significant levels of antiviral cellular and humoral immune responses in humans after a single inoculation and have proven to be valuable tools in studying primary immune responses to virus infection in humans (25–29). Indeed, contemporary systems biology approaches have recently been applied to study the human immune response to YF-17D in great detail (28, 29). These studies have highlighted the importance of a number of “masterswitch genes” involving several pathways of the innate immune response, including type I IFNs, which are significantly upregulated following infection and are associated with the induction of a substantial and broad Th1/Th2 profile of immune responses elicited by YF-17D immunization (28, 29). Of the identified nodes of immune response gene activation by YF-17D, IRF-7 was found to feature prominently as a key regulatory pathway following YF-17D infection of humans, consistent with its important role in generation of innate and adaptive immune responses in mice (30–32). Additionally, TLR7 and a range of type I IFN response genes were observed to be significantly induced following YF-17D infection (28, 29). Given these studies and our earlier observations of the distinctive patterns of TLR7/9 signaling and type I IFN production in association with nonpathogenic SIV infection of SMs, the present study sought to explore not only the specific biology of the SM immune system in response to virus infections that they harbor as reservoir hosts but also the role that TLR7 signaling and IFN-α production play in the generation of innate and adaptive immune responses to viral infection in primates.

Materials and Methods

Human and animal subjects

Blood samples were obtained from HIV− human volunteers after obtaining written informed consent. Protocols were approved by the Emory University Institutional Review Board. Animal housing, care, and research were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). All animal studies were approved by the Emory University Institutional Animal Use and Care Committee.

Virus

MVA was grown on DF-1 cells (American Type Culture Collection, Manassas, VA) and cell lysates were collected. YF-17D was grown on Vero cells (American Type Culture Collection) and infected cell media collected. Viruses were purified by pelleting through 36% sucrose. Titers were determined by plaque assays. Sucrose-purified MVA was used for all experiments, whereas sucrose-purified YF-17D was used only for in vitro experiments, not for animal infections.

Infection with YF-17D

Humans and animals were s.c. given 1 × 10⁷ PFU of the commercially available YF-17D vaccine (YF-VAX; Aventis Pasteur). Animals were infected in three separate groups: group 1 (RM1, RM2, RM3, RM4), group 2 (SM1, SM2, SM5, SM6), and group 3 (SM3, SM4, SM7, SM8, RM5, RM6). SM5–SM8 were SIV+. Additionally, TLR7 and a range of type I IFN response genes

"...including type I IFNs, which are significantly upregulated following TLR7 or TLR9 activation..."

were determined by plaque assays. Sucrose-purified MVA was used for all experiments, whereas sucrose-purified YF-17D was used only for in vitro experiments, not for animal infections.

Infection with MVA

A subgroup of animals vaccinated with YF-17D (RM1–RM4, SM1, SM2 and SM5, SM6) was given 1 × 10⁷ PFU sucrose-purified MVA both intramuscularly and intradermally.

Determination of YF-17D plasma RNA

YF-17D plasma viremia was quantified by ultrasensitive quantitative real-time RT-PCR as described (25). Limit of detection was 12 copies/ml.

Immunophenotyping and flow cytometry

Whole blood staining was performed with the following mAbs as described (21): anti-CD3 (SP34), anti-CD4 (L200), anti-CD8 (SK1), anti-CD20 (L27 or 2H7), anti-CD16 (3G8), anti-CD11c (S-HCL-3), anti-CD123 (7G3), anti-CD14 (M5E2), and anti-HLA-DR (L243) from BD Pharmingen (San Jose, CA) and anti-CCR7 (150503; R&D Systems, Minneapolis, MN). Samples used for anti-Ki67 or anti-Bcl2 staining were permeabilized (Perm Solution 2; BD Biosciences). Flow cytometric acquisition of at least 100,000 lymphocytes was done on a FACSCalibur or LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR).

YF-17D Ab titres

Plasma was assayed for anti-YF neutralizing Ab by measuring protection conferred on Vero cells against the YF-17D cytopathic effect as described (29).

MVA Ab titres

Microtiter wells were coated with 2 μg/ml sucrose gradient-purified MVA. After blocking with PBS/10% FCS, animal plasma samples were added in sixteen 1:2 serial dilutions. A polyclonal HRP-conjugated goat anti-rhesus Ig was used as secondary Ab (SouthernBiotech, Birmingham, AL) that was cross-reactive with SM IgG. Plates were developed using SureBlue TMB substrate (KPL, Gaithersburg, MD), and absorbance was read at 450 nm, after the reaction was stopped using 1 M phosphoric acid.

Detection of MVA-specific T cells

PBMCs were isolated from blood using sodium citrate Vacutainer CPT tubes (BD Biosciences, Franklin Lakes, NJ) centrifuged at 2500 rpm for 25 min. RBCs were lysed with ACK lysing buffer (BioSource, Camarillo, CA). PBMCs were washed, and 2 × 10⁶ cells incubated in 10% FBS RPMI 1640 at 37 °C for 16 h in 200 μl media with MVA (multiplicity of infection of 2), 5 μg/ml Con A (Sigma-Aldrich, St. Louis, MO), 2 μg/ml staphylococcal enterotoxin B (Sigma-Aldrich), or media alone. Brefeldin A (Sigma-Aldrich) was then added at final concentration of 10 μg/ml and cells were incubated for an additional 5 h. Cells were surface stained for CD3...
and CD8, permeabilized (Perm Solution 2; BD Biosciences), and stained intracellularly for IFN-γ (4S.B3; BD Pharmingen).

**DC stimulations**

PBMCs were isolated from HIV/SIV-negative humans, RMs, or SMs. Cells (4 × 10^5) were stimulated with a multiplicity of infection of 0.4 MVA or YF-17D in 10% FBS RPMI 1640 in the presence or absence of oligodeoxynucleotide (ODN) antagonists IRS661, IRS869, or IRS954 (Dynavax Technologies, Berkeley, CA), or chloroquine diphosphate salt (Sigma-Aldrich) dissolved in PBS, for 17 h in 96-well plates. IFN-α and IL-12 production was measured in the supernatants by ELISA (human IFN-αAldrich) dissolved in PBS, for 17 h in 96-well plates. IFN-α and IL-12 production was measured in the supernatants by ELISA (human IFN-αAldrich) dissolved in PBS, for 17 h in 96-well plates.

**Statistical analyses**

We analyzed data using t tests, Mann–Whitney U tests, or, in cases where more than one group was compared, by Kruskal–Wallis ANOVA (GraphPad Prism). A p value < 0.05 was considered significant.

**Results**

**Reduced IFN-α production of SM PBMCs upon stimulation with YF-17D**

Persistently increased levels of type I IFN have been reported to be associated with increased levels of chronic immune activation during pathogenic SIV infections in nonhuman primates and in HIV infections in humans (21, 33). However, it remains unclear whether this association between IFN-α production and chronic immune activation is causal. Similarly, the extent to which host-specific differences in IFN-α production response to TLR7 and TLR9 stimulation may influence the nature and magnitude of antiviral immune responses generated following infection with viruses whose innate immune recognitions are mediated via TLR7/9 pathways has not been determined previously in humans and nonhuman primates. To investigate the effect of reduced type I IFN production on the generation of immune responses in more detail in primates, we examined the host-specific responses of RMs and SMs to two distinct viruses: YF-17D and MVA.

We first sought to determine whether TLR7 or TLR9 is required for eliciting an IFN-α response to YF-17D or MVA in humans and primates. YF-17D has been reported to activate DCs in mice to produce IL-6 and IL-12 via TLRs 2, 7, and 9, and to activate NF-κB in human HEK293 cells transfected with human TLR8 (22). To date, TLR pathways responsible for activation of type I IFN production by YF-17D in mice have not been reported. Furthermore, although initial host responses to YF-17D immunization manifest a prominent signature of type I IFN production (28, 29), the specific TLR signaling pathways responsible for activation of IFN-α production by pDCs have not been determined in humans or primates. To investigate this, we stimulated human PBMCs with YF-17D or MVA in the presence or absence of inhibitors of TLR7 and/or TLR9 and measured IFN-α production (Fig. 1A).

Controls for these studies included chloroquine, an endosomal acidification inhibitor that blocks activity of endosomal TLRs, including TLR7 and TLR9; three ODN antagonists of TLR signaling that are specific for TLR7 (IRS661), TLR9 (IRS869), or both TLRs (IRS954); as well as a nonspecific ODN, or chloroquine. Histograms represent mean ± SEM of four to six humans. Comparisons are made to without inhibitor, and data are rescaled relative to the level of IFN-α production in the absence of inhibitors. B and C, HIV− human and SIV− RM or SM PBMCs were stimulated with YF-17D or MVA and IFN-α (B) or IL-12 production above background (IL-12 production with media alone is subtracted) (C), measured by ELISA. Mean cytokine production is denoted by a line. *p < 0.05, **p < 0.001, ***p < 0.0001.

**TLR9.** Although these data do not exclude potential contributions of other TLR pathways in the pDC response to YFV, they are consistent with what is known about the nature of TLR7 agonists (35–37) and the key role played by TLR7-mediated activation of IFN-α production by other ssRNA viruses, including dengue virus (38) and coronaviruses (39). None of the TLR inhibitors significantly reduced IFN-α production upon stimulation with MVA (Fig. 1A), suggesting that TLR7 and TLR9 signaling are largely dispensable for generation of an IFN-α response to this virus.

We next examined the type I IFN responses of human, RM, and SM PBMCs to YF-17D or MVA. We found that SM PBMCs produced significantly lower levels of IFN-α upon stimulation with YF-17D than did human or RM PBMCs, consistent with the altered TLR7/9 signaling pathway previously described (21). Conversely, MVA-induced IFN-α production by SM PBMCs is similar to humans and significantly higher than RMs (Fig. 1B). As we have previously shown that the TLR7/9 signaling pathway leading to the production of proinflammatory cytokines in an NF-κB–dependent manner is intact in SMs (21), we investigated whether YF-17D stimulation of SM PBMCs resulted in IL-12 production. Interestingly, SM IL-12 production in response to YF-17D stimulation was not significantly different from that of humans and RMs (Fig. 1C), confirming that although the TLR7/9 signal

**FIGURE 1.** Reduced IFN-α production in SMs compared with RMs and humans upon stimulation with YF-17D, but not MVA. **A**, HIV− human PBMCs were stimulated with YF-17D or MVA for 17 h in the presence of no inhibitor, 5.6 μM ODN of TLR7 (IRS661), TLR9 (IRS869), both TLR7 and TLR9 (IRS954), a nonspecific ODN, or chloroquine. Histograms represent mean ± SEM of four to six humans. Comparisons are made to without inhibitor, and data are rescaled relative to the level of IFN-α production in the absence of inhibitors. B and C, HIV− human and SIV− RM or SM PBMCs were stimulated with YF-17D or MVA and IFN-α (B) or IL-12 production above background (IL-12 production with media alone is subtracted) (C), measured by ELISA. Mean cytokine production is denoted by a line. *p < 0.05, **p < 0.001, ***p < 0.0001.
bifurcation leading to IFN-α production via the IRF-7–dependent pathway is altered in SMs, the NF-kB–dependent signaling pathway leading to production of other proinflammatory cytokines is similarly intact and functional as in RM and human pDCs.

No detectable DC activation in SMs upon YF-17D infection in vivo, despite detectable YF-17D viremia

We sought to determine whether species-specific differences in pDC-derived IFN-α production upon YF-17D exposure are associated with differences in innate and adaptive immune antiviral responses in vivo. A total of eight SMs, of which four were SIV+, and six SIV− RMs were infected with the YF-17D vaccine (YF-VAX). The kinetics of YF-17D viremia was measured by quantifying levels of YFV viral RNA in plasma following infection (Fig. 2A). YF-17D was able to replicate in both primate species, although to a lesser extent in SMs. All RMs and five of eight SMs exhibited detectable YF-17D viremia, with viral replication peaking around day 4 postinfection (Fig. 2A). By way of reference, in recent studies of YF-17D immunization of humans using the same assay for quantification of YFV RNA levels, between 58 and 75% of immunized individuals had peak detected levels of YFV of <1000 copies/ml and 17.5–30% of individuals had peak levels of <100 copies/ml (27, 28). To determine whether YF-17D was recognized in vivo, we assessed whether DCs were activated by YF-17D in SMs and RMs postinfection. As DCs mature, they up-regulate CCR7, a chemokine receptor that binds CCL19 and CCL21 and directs migration to lymphoid organs, facilitating Ag presentation and cross-talk between DCs and T or B cells (40). In RMs there was a rapid upregulation of CCR7 on pDCs in the blood peaking at day 4 postinfection (Fig. 2B). In contrast, there was little increase in pDC CCR7 expression in SMs after YF-17D vaccination, regardless of the extent of YF-17D viremia detected. In the two RMs with the greatest upregulation of CCR7 on pDCs, an increase in CCR7+ myeloid DCs (mDCs) was also observed, whereas CCR7 expression on mDCs remained unchanged in all SMs (Fig. 2B). Hence, our data suggest that differences in the TLR-dependent ability of pDCs to recognize YF-17D may affect early in vivo innate responses following virus infection.

Attenuated immune responses in SMs upon infection with YF-17D

To investigate whether the lack of detectable DC activation in SMs leads to differences in downstream innate responses elicited, we followed the magnitude and kinetics of the NK cell proliferative response following YF-17D immunization in the blood by staining for nuclear expression of Ki67 to identify recently divided cells. Although high levels of NK cell proliferation were seen in RMs by day 7 after YF-17D infection, there was little increase in NK cell proliferation in YF-17D-immunized SMs (Fig. 3A).

Similarly, we observed substantial differences between SMs and RMs in the magnitude of T cell proliferative responses following YF-17D challenge. To identify effector T cell responses, we measured Ki67 expression and expression of the anti-apoptotic protein Bcl-2, which is downregulated upon activation. The combination of these markers has been shown to identify Ag-specific T cell responses upon YF-17D and vaccinia virus immunization in humans, with effector T cells being Ki67+Bcl-2− (25, 31). As in humans, there was an expansion of Ki67+Bcl-2− CD4+ and CD8+ T cells in RMs, whereas in SMs the percentage Ki67+Bcl-2− CD4+ and CD8+ T cells remained unchanged following YF-17D inoculation (Fig. 3B). Because not all Ki67+ T cells were Bcl-2− in RMs, we also examined the kinetics of total proliferating T cells. There were no detectable increases in the percentage Ki67+expressing CD4+ or CD8+ T cells after YF-17D infection in either SIV+ or SIV− SMs (Fig. 3C), nor did we observe increases in CD69 or HLA-DR expression on T cells in SMs following YF-17D inoculation (data not shown). Given the substantially higher baseline percentage of Ki67+ T cells in RMs than in SMs, as is commonly observed (M.B. Feinberg, unpublished data and Refs. 21, 41), we assessed the fold increase in proliferation of CD4 and CD8 T cells from baseline postinfection in both species. Peak fold changes in proliferation of T cells were significantly higher in RMs than in SMs (Fig. 3D). This was also true for CD4+ and CD8+ T cells in lymph nodes of these animals (data not shown). Thus, even in SMs with peak YF-17D viremia at 200–900 RNA copies/ml, there were no detectable NK or T cell proliferative responses following vaccination. Our efforts, using the limited cryopreserved cellular samples available from these studies, to quantify levels of YFV-specific CD4 and CD8 cells in YF-17D–vaccinated RMs (using the same intracellular cytokine assay system we have successfully employed, using freshly prepared PBMCs, to measure YFV-specific T cell responses in YF-17D–vaccinated human volunteers; see Refs. 22, 25, 27) were unsuccessful (data not shown). Without a reliable positive control, we could neither quantify nor exclude a YFV-specific T cell response in SMs.

To determine whether the absence of detectable effector T cell responses in SMs, as assessed by levels of Ki67+Bcl-2− CD8+ T cells quantified by flow cytometric analyses, was due to the reduced level of YF-17D viremia in these animals, we specifically investigated the extent of CD8+ T proliferation in humans vaccinated with YF-17D that had levels of plasma YF-17D viremia <500 RNA copies/ml (Fig. 4). From previous studies of YF-17D–infected humans, it is clear that low YF-17D viremia after immunization is frequently seen, but that robust Ab responses are

![FIGURE 2.](http://www.jimmunol.org/) Increases in CCR7 expression on RM, but not SM, DCs following YF-17D infection, despite detectable YF-17D viremia. A, Plasma YF-17D RNA copies/ml in eight SMs and six RMs. Symbols used for individual animals are as follows: SM1 ( ), SM2 ( ), SM3 ( ), SM4 ( ), SM5 ( ), SM6 ( ), SM7 ( ), SM8 ( ), RM1 ( ), RM2 ( ), RM3 ( ), RM4 ( ), RM5 ( ), RM6 ( ). B, Percentage CCR7+ DCs in RMs and SMs prior to (day 0) and 4 d after YF-17D infection. pDCs are gated on CD123+ Lineage− HLA-DR+ cells, and mDCs are gated on CD11c+ Lineage− HLA-DR+ cells. CCR7 expression was determined by comparison with a fluorescence minus one control. Mean ± SEM is shown. *p < 0.05.
nonetheless elicited (27, 28). Depending on the days where plasma was sampled, 36 (27) to 60% (28) of YF-17D–vaccinated humans had a peak viremia of \(500\) RNA copies/ml. In four representative humans with such low YF-17D replication (peak viremia of \(52–435\) RNA copies/ml), there was nevertheless a substantial increase in percentage Ki67+Bcl-2lo CD8+ T cells by day 14 post-vaccination (Fig. 4). From this comparison with vaccinated humans, we would expect to see detectable increases in T cell proliferation in SMs with measurable, albeit lower, YF-17D viremia. Therefore, it is unlikely that the absence of any increases in T cell proliferation following YF-17D infection in SMs are due only to lower levels of YF-17D replication.

Ab responses play a critical role in protective immune responses induced by YF-17D and persist for decades following a single inoculation in humans (42). In four representative humans with such low YF-17D replication (peak viremia of \(52–435\) RNA copies/ml), there was nevertheless a substantial increase in percentage Ki67+Bcl-2lo CD8+ T cells by day 14 post-vaccination (Fig. 4). From this comparison with vaccinated humans, we would expect to see detectable increases in T cell proliferation in SMs with measurable, albeit lower, YF-17D viremia. Therefore, it is unlikely that the absence of any increases in T cell proliferation following YF-17D infection in SMs are due only to lower levels of YF-17D replication.

Ab responses play a critical role in protective immune responses induced by YF-17D and persist for decades following a single inoculation in humans (42). Thus, we next assessed the magnitude and duration of neutralizing anti–YF-17D Ab titers in both primate species (Fig. 3E). In RMs and SMs the timing of appearance of neutralizing Abs coincided with the diminution of detectable YF-17D viremia. However, peak anti–YF-17D Ab titers were significantly lower in SMs than in RMs (\(p = 0.0013\), Mann–Whitney U test). Furthermore, YF-17D–specific Ab levels declined to levels below detection by 20 wk postvaccination in all but two SMs (Fig. 3E). In contrast, in RMs Ab levels were stably maintained at high levels (Fig. 3E). The lower levels of YF-17D replication in SMs are unlikely to be responsible for the lower and only transient anti–YFV Ab responses seen in SMs since humans who display low-level YF-17D viremia following immunization mount significant and durable anti–YFV Ab responses (Fig. 4). The induction and persistence of significant anti–YFV Ab responses in humans who manifest low peak levels of viremia following YF-17D immunization, including those with undetectable YFV viremia postvaccination, has also been reported by others (28). Interestingly, although transient, anti–YF-17D Abs were nevertheless measured in all animals, even in SMs where YF-17D viremia was undetectable. No differences in Ab titers were observed between SIV+ and SIV– SMs, further attesting to preserved immune system integrity of SIV-infected SMs, unlike HIV-infected humans (43).

HIV viremia in infected humans has been reported to transiently increase following inoculation with specific vaccines (44, 45). This is likely due to the activation and preferential infection of Ag-specific CD4+ T cells, as well as the potential reactivation of latently infected cells, upon exposure to the vaccine (46). Hence, perturbations from the steady-state viremia during chronic SIV infection is a potentially sensitive indicator of CD4+ T cell activation. We therefore determined whether there were increases in
levels of plasma SIV RNA in SIV+ SMs following YF-17D vaccination, which might identify activation of Ag-specific CD4+ T cell responses in these animals that we may not have detected by monitoring levels of Ki67+ CD4+ T cells in the peripheral bloodstream alone. Surprisingly, rather than seeing increased levels of SIV production in SMs following inoculation of YF-17D, there was a transient diminution of SIV replication, with a decline of, on average, a half log in SIV viral loads during the first 14 d after vaccination (Supplemental Fig. 1A). Although, due to the small sample size, this decline is not statistically significant, it nevertheless represents a greater change than is generally observed in such a short time frame during chronic SIV infection of SMs (S.I. Staprans and M.B. Feinberg, unpublished observation) and is striking in that it occurs in all animals during the period of active YF-17D replication. The decline in SIV viral load was not attributable to measurable changes in overall CD4+ T cell levels in the blood or lymph nodes (Supplemental Fig. 1B and data not shown) or in levels of proliferating (Ki67+) CD4+ T cells in these compartments (Fig. 3C and data not shown).

**Equivalent immune responses in RMs and SMs following MVA infection**

To determine whether the reduced adaptive immune responses of SMs raised to YF-17D is due to how this virus is recognized by the innate immune system, we next infected SMs and RMs with MVA, a virus recognized by non-TLR7/9 innate pathways. To rule out animal-specific differences, we used a subset of animals previously infected with YF-17D. A total of four SMs, of which two were chronically SIV+ and four SIV− RMs were infected with MVA. As before, we measured Ki67+Bcl-2lo T cells and total proliferating T and NK cells (Fig. 5). In contrast to YF-17D infection, there was an expansion of Ki67+Bcl-2lo CD4+ and CD8+ T cells in both species after MVA infection. Fold increases in Ki67+ T and NK cells were not significantly different between RMs and SMs (Fig. 5). An intracellular cytokine assay for MVA-specific CD8+ T cell responses confirmed that by day 29 postinfection there were increases in MVA-specific IFN-γ+CD8+ T cells in all infected animals (Fig. 5D). Unlike the lower magnitude and impaired persistence of Ab responses seen in SMs following YF-17D infection, high anti-MVA Ab titers were measured in both species and were sustained up to 16 wk postinfection (Fig. 5E). Thus, in stark contrast to what we observed with YF-17D, SMs raised a detectable T cell response to MVA and maintained stable MVA-specific Ab responses following virus inoculation.

In contrast to the transient decreased levels of SIV viremia following YF-17D infection, we observed increased SIV replication in association with increased CD4+ T cell activation in SMs following MVA infection, a result consistent with reports of vaccine-elicted increases in viremia in HIV-infected humans. There was a 1.4 log increase in the SIV viral load at day 11 postinfection compared with day 0 in the SM (SM5) that had a detectable CD4+ T cell proliferative response following MVA infection (Supplemental Fig. 1C). This increase in SIV replication occurred concomitantly with increased CD4+ T cell counts (Supplemental Fig. 1D). In comparison, the other SIV+ SM that showed little increase in Ki67+CD4+ T cells following MVA infection showed little change in SIV viremia.

**Discussion**

The African primates that are natural hosts for AIDS viruses have, to date, been the most intensively studied only with respect to the nature of the nonpathogenic host–SIV equilibrium. Interestingly, these primates also harbor, without evidence of disease, other viruses that are responsible for serious zoonotic diseases in humans, among them YFV. Until now, little was known about immune responses to these and other viruses in natural reservoir hosts. In particular, although we have previously shown that SM pDCs have a reduced ability to produce large amounts of IFN-α following stimulation with SIV and other TLR7/9 ligands (21), it has been unclear how the altered TLR7/9 signaling pathway affects their ability to mount and maintain innate and adaptive immune responses to other viruses that are recognized by TLR7/9 innate pathways. In this study, to address these questions, we investigated the immune responses of SMs to two viruses, YFV and MVA.

Studies in YF-17D–immunized humans have shown that the activation of type I IFN-response genes represents an early and prominent component of anti-YFV immune responses (28, 29). In this study, we provide evidence elucidating the underlying mechanism of activation of this early innate response to YF-17D infection by showing that YFV requires TLR7 to induce a type I IFN response in pDCs from humans and RMs. Conversely, we show that, as has been previously described in mice (23, 24), in humans and primates MVA elicits IFN-α by non-TLR7/9 innate pathways. As for SIV and other TLR7/9 ligands, we observed that YF-17D stimulation of SM PBMCs ex vivo results in significantly reduced IFN-α production compared with RMs and humans, whereas MVA-induced IFN-α production by PBMCs is similar. Interestingly, SM PBMCs are nevertheless able to produce IL-12 in response to YF-17D stimulation, suggesting that proximal TLR7/9 signaling events are intact. This is consistent with previous studies indicating that NF-kB–dependent signaling pathways induced by TLR7/9 ligand binding are unaltered, whereas activation of the IRF-7–dependent signaling pathway following...
TLR7/9 ligand engagement is markedly attenuated in SMs pDCs, as compared with pDCs from RMs and humans (21). The precise changes in the IRF-7–mediated signaling pathway in SMs, as well as the potential role for additional variations in post-TLR signaling molecules, that result in lower IFN-α production following TLR7/9 stimulation remain to be determined.

Differences in ex vivo SM pDC type I IFN responses to YF-17D and MVA were mirrored by striking differences between SM in vivo immune responses to YF-17D and MVA immunization. In SMs, there was no detectable DC activation or proliferative NK and T cell responses following YF-17D infection, in contrast to increased pDC activation and the significant increases in proliferation of NK and T cells observed in RMs. Furthermore, although all SMs mounted detectable neutralizing Ab responses to YF-17D, this response was significantly lower than in RMs and, in notable distinction to humans and RMs immunized with YF-17D, it was generally not maintained (47). Although YF-17D replication was lower in SMs than in RMs, we show that humans with similarly low peak YF-17D viremia nonetheless have detectable CD8+ T cell proliferation and robust Ab responses, suggesting that the lower YF-17D viremia in SMs is not sufficient to explain the transient Ab responses or absence of detectable T cell responses in this primate species. Furthermore, both SMs and RMs raised innate and adaptive cellular and humoral immune responses to MVA.

**FIGURE 5.** Kinetics and magnitude of T cell, NK cell, and Ab responses in MVA-vaccinated animals. A, Ki67 and Bcl-2 expression on CD8 T cells in a representative RM (RM1) and SM (SM5) 0, 7, 11, 14, and 39 d following MVA infection. FACS plots were gated on CD3+CD8+ cells (upper panel) or CD3+CD4+ cells (lower panel), and numbers on plots indicate percentage Ki67+Bcl-2lo cells. B, Kinetics of percentage Ki67+CD4+ T cells, CD8+ T cells, and NK cells in SMs and RMs. C, Maximum fold increases in proliferation were obtained by comparing day 0 to peak (day 7) Ki67 expression. Mean fold increases are denoted by a line. D, PBMCs from SMs and RMs were stimulated with MVA and stained for IFN-γ on days 0 and 29 following MVA infection. Numbers on FACS plots indicate the percentage IFN-γ+CD8+ T cells (gated on live CD3+CD8+ cells). E, MVA Abs (IgG) were measured by ELISA and endpoint titers calculated. Symbols used for individual animals in B and E are as follows: SM1 (●), SM2 (○), SM5 (◆), SM6 (▲), RM1 (■), RM2 (♦), RM3 (●), RM4 (▲).
that were of similar magnitude and persistence. Hence, the absence of any detectable effector T cell increases even in SMs with substantial YF-17D replication is a notable and significant result.

It is still unclear why YF-17D replicates to lower levels in SMs than in RMs. It has been shown that DCs play a role in flavivirus replication by disseminating virus from the site of infection (48, 49). Hence, the reduced activation and trafficking of DCs observed in SMs might also contribute to the lower viral replication in this species. Alternatively, because YF-17D is able to replicate in activated T cells (Ref. 51 and data not shown), the substantial T cell activation in RMs and humans following immunization may amplify YF-17D replication. If so, reduced T cell activation in SMs might contribute to the more limited YF-17D viremia observed. However, further experiments will be required to specifically address this question.

Importantly, the substantially reduced innate and adaptive immune responses of SMs to YF-17D observed is consistent with the key role of type I IFNs in linking innate and adaptive immunity (28, 29, 51). Studies conducted in mice have shown that IFN-α has numerous effects on innate immune cells that indirectly affect adaptive immune responses, such as stimulating the maturation of DCs, inducing the upregulation of MHC class I, promoting NK cell activation, as well as resulting in production of IL-15 and IFN-γ, which themselves enhance adaptive immune responses (51). Moreover, type I IFN also has direct effects on the induction of T cell responses. Clonal expansion and survival of CD4+ and CD8+ T cells in response to specific viruses are severely diminished upon infection in mice lacking the type I IFN receptor or when IFN-α production is blocked (30, 52, 53). Interestingly, the dependency of T cells on IFN-α- and IFN-γ-mediated survival signals is determined by the pathogen (52, 54). In IRF7−/− mice, which do not produce IFN-α/β upon TLR7/9 stimulation, impairment of T cell responses is seen only when TLR7/9 signaling is required for DC activation (31). More recent support documenting the impact of depressed pDC number or function on the induction and maintenance of CD8+ T cell numbers and function have been obtained from studies of YF-17D infection in rapamycin-treated mice (which results in inhibition of IRF-7-dependent IFN-α production) and VSV-infected mice that had been conditionally depleted of pDCs (30, 55). Although less is known about the role of TLRs, DCs, and IFN-α in the generation of long-lived memory B cell responses (56), IFN-α has been shown to enhance primary Ab responses, promote isotype switching, influence relative polarization of the Ab isotype profile, and, together with IL-6, stimulate plasma cell differentiation in mice (57–60). However, detailed mechanistic studies to better understand the role and mechanisms of potentially important effects of TLR7/9 signaling and IFN-α production on the levels, isotype composition, generation of CD4+ T cell and B cell memory, and in the overall persistence of Ab production following specific infections and immunizations in both humans and nonhuman primates (as well as in other vertebrate species that represent natural hosts for zoonotic infections) are clearly needed.

Together with these data, our results suggest that the ability of pDCs to produce IFN-α following TLR7/9 activation is important in generating multilineage innate and adaptive immune responses to YFV in humans and primates (21, 28, 29). It is likely that the diminished NK and T cell responses to YF-17D in SMs are related to, and perhaps explained by, the reduced IFN-α response to this virus. Additionally, lower magnitude and limited persistence of anti–YF-17D Ab levels in vaccinated SMs may result from the reduced production of IFN-α by their pDCs and the limited CD4+ T cell activation they exhibit following YF-17D infection. However, further studies will be required to directly ascertain that, as the ex vivo PBMC stimulation data suggest, there is a reduced type I IFN response in vivo in SMs following YF-17D immunization.

A recent study of experimental SIV infection of SMs observed a gene expression profile consistent with a transient type I IFN response during the acute infection period (61). Given the exquisite sensitivity of expression levels of IFN response genes to even very low levels of IFN-α and IFN-β (62), these data are not inconsistent with lower level, but still readily detectable, levels of IFN-α produced by SM pDCs following exposure to SIV in ex vivo assays (21). Of note, these studies did not investigate the source of the in vivo type I IFN or ascertain the innate pathways by which its production was being stimulated. The observed absence of a type I IFN response pattern during chronic infection when levels of SIV viremia are very high (61) is consistent with our earlier report (21) and reinforces that important differences exist in the innate immune responses to SIV in SMs and RMs. Induction of immunosuppressive mediators could, as has been hypothesized following SIV infection (19), also play a role in the active inhibition of damaging immune responses to YFV in SMs, for instance, by induction of IDO following exposure to HIV and other TLR7 and TLR9 agonists (63), an NF-kB–mediated response that we have found to be preserved in SMs (data not shown). In light of evidence indicating that potentially immunosuppressive mediators, such as IDO, are produced by pDCs in response to activation of the TLR7 signaling pathway, it is tempting to speculate that such mediators might contribute to the transient diminution of SIV viremia we observed in SMs following vaccination with YF-17D (but not the non-TLR7/9-active virus, MVA) (64, 65). Conceivably, both the limited CD4+ T cell activation and induction of immunosuppressive pathways might be involved in protecting natural hosts from disease. An attenuated innate or compromised early CD4+ T cell response may result in induction of nonresponsive or readily exhausted T cell responses (66), the promotion of a state of active tolerance, and result in impaired Ab responses. Importantly, both following YF-17D and SIV infection of SMs, little change in CD4+ T cell proliferation is observed (data from this study and Refs. 21, 41, 67–69). The extent of early CD8+ T cell proliferation has been found to vary between different animals and in different published reports (ranging from low to modest levels as compared with acute pathogenic SIV and HIV infections), but in all cases when seen, increased levels of CD8+ T cell proliferation in SIV-infected SMs following SIV infection have been only transient (in contrast to pathogenic SIV infections in RMs and HIV infections of humans) (21, 41, 67–69). Notably, in the present study, we could detect little or no increase in effector CD8+ T cell proliferative responses after YF-17D infection. Clearly, pursuit of understanding the specific determinants of the differences as well as the similarities in SM host responses to YF-17D and SIV in much greater detail, and how these differ from those of RMs and humans, represent important topics for future studies.

Of note, in contrast to SIV, YF-17D does not establish persistent infection in SMs despite their dampened immune responses to the virus. Ab responses to YF-17D in SMs, albeit transient, are likely responsible for virus clearance. In contrast to HIV or SIV, YF-17D is extremely sensitive to neutralizing Ab (42). In primates studied herein, as in immunized humans (25), clearance of YF-17D viremia coincides with the appearance of Ab responses. The ability of SMs to produce Ab responses even to viruses whose recognition by the innate immune system involves TLR7/9 may enable them to resist the range of potentially pathogenic virus infections they encounter in the wild. Indeed, experimental infection of SMs with pathogenic YFV has been reported to result in transient viremia that is cleared coincident with the production of YFV-
neutralizing Abs (13). This feature, in concert with the discrete nature of the alteration in TLR7/9 signaling in SMs, as well as the apparent redundancy in innate immune activation inferred from studies of individuals with specific inherited polymorphisms (70), may explain why SMs are nevertheless able to control most infections. Additional immunization studies in SMs and RMs may provide a valuable experimental model to understand how divergent TLR signaling patterns can influence host responses to specific infectious agents, thereby complementing findings from genetically defined mouse models, which are increasingly recognized as being imperfect models of human TLR biology (70–72).

The interaction of the innate immune system with a virus greatly affects the balance between an immune response capable of clearing infection or suppressing virus replication and a response that contributes to disease. For most emerging zoonotic infections, the severe consequences of acute infection are responsible for the greatest morbidity and mortality (2–4, 6). Although acute consequences of these infections can be lethal, hosts surviving the primary infection clear the virus and develop specific immunity. In contrast, in HIV infection host immune responses are fundamentally unable to mediate clearance, and it is the long-term consequences of chronic virus replication and generalized immune activation that lead to disease progression (17). In each instance, however, unintended immunopathologic consequences of the virus–host immune system interactions likely influence disease severity following infection. For instance, higher levels of proinflammatory mediators are seen in people with fatal hemorrhagic YFV infections compared with people experiencing less severe infections (15). Indeed, a more exuberant early immune response is often associated with more severe disease following zoonotic infections, such as SARS coronavirus, dengue virus, hantavirus, chikungunya virus, and the 1918 influenza A virus strain (73–77).

The precise mediators of immunopathologic responses seen in nonnatural hosts following zoonotic viral infections may differ in different hosts and different infections, and many cytokine pathways (e.g., type I IFN) may well play both beneficial and detrimental roles in the host response to infection. However, explication of the role that TLR-mediated signaling of the innate immune response plays in determining the absence of disease in natural reservoir hosts, as well as the pathogenesis of zoonotic diseases in nonnatural hosts, represents an important and fascinating topic for future investigation.

Collectively, our results obtained from the study of two distinct viruses, SIV and YFV, whose primary similarities rest in their possession of ssRNA genomes and their recognition by host innate immune effector cells via TLR7 and/or TLR9, provide complementary evidence demonstrating that in SMs that represent natural hosts for both infections, reduced IFN-α production by pDCs, attenuated pDC and NK responses, and muted adaptive immune responses are observed following infection, regardless of whether the virus in question can deplete CD4+ T cells. In fact, for both YFV and SIV, the altered TLR7/9 signaling in SM pDCs may prevent immunopathology following infection and hence may be beneficial for the host. Additionally, this study gives us a glimpse into the wealth of information that can be gained about host-pathogen relationships from studying the immune system of different species and species harboring zoonotic viruses may, similar to SMs, possess specifically altered innate signaling patterns that enable them to avoid aberrant immune activation, immunopathology, and disease. Furthermore, given that ssRNA viruses have been frequently implicated in crossing species barriers, infecting novel hosts (5), the TLR7/9 pathway likely represents an important candidate for possible host adaptations to tolerate these zoonotic viruses without disease. Interestingly, adaptations to particular viruses by their natural hosts may enable them to harbor additional viruses recognized by the same innate immune pathways, such that some species act as reservoir hosts for multiple zoonotic agents. If so, the observation that specific animal species, including bats and African primates, serve as reservoir hosts for multiple zoonotic viruses, along with the fact that so many emerging infectious diseases are caused by RNA viruses, may not be coincidental (5, 78).

It will be important for future studies to elucidate the immunologic mechanisms that allow reservoir hosts to tolerate infection with single or multiple emerging viruses and to determine whether the underlying mechanisms of apathogenic infection are similar for distinct viruses and reservoir species. Such studies promise to provide novel insights into evolutionary solutions to host–pathogen struggles that have allowed natural hosts to avoid disease. These insights should, in turn, inform efforts to better detect potential reservoir hosts, anticipate the potential emergence of zoonotic infections, and to specifically design therapies to ameliorate the immunopathologic consequences of emerging virus infections.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary Figure 1. Changes in SIV viral load in SIV+ SMs following YF-17D or MVA infection. (a) and (c) SIV RNA copies per ml plasma following YF-17D or MVA infection, respectively. (b) and (d) CD4+ T cell counts per μl blood in SIV+ SMs after YF-17D or MVA inoculation, respectively. Symbols used for individual animals are as follows: SM5 (◆), SM6 (▲), SM7 (△), SM8 (◇).