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Rate of AIDS Progression Is Associated with Gastrointestinal Dysfunction in Simian Immunodeficiency Virus–Infected Pigtail Macaques

Lauren A. Canary,* Carol L. Vinton,* David R. Morcock,‡ Jordan B. Pierce,* Jacob D. Estes,‡ Jason M. Brenchley,* and Nichole R. Klatt*,‡

During HIV/SIV infection, mucosal immune system dysfunction and systemic immune activation are associated with progression to AIDS; however, it is unclear to what extent pre-existing gastrointestinal damage relates to disease progression postinfection. Pigtail macaques (PTM) are an excellent model in which to assess mucosal dysfunction in relation to HIV/SIV pathogenesis, as the majority of these animals have high levels of gastrointestinal damage, immune activation, and microbial translocation prior to infection, and rapidly progress to AIDS upon SIV infection. In this study, we characterized the mucosal immune environment prior to and throughout SIV infection in 13 uninfected PTM and 9 SIV-infected PTM, of which 3 were slow progressors. This small subset of slow progressors had limited innate immune activation in mucosal tissues in the periphery, which was associated with a more intact colonic epithelial barrier. Furthermore, we found that preinfection levels of microbial translocation, as measured by LPS-binding protein, in PTM correlated with the rate of progression to AIDS. These data suggest that pre-existing levels of microbial translocation and gastrointestinal tract dysfunction may influence the rate of HIV disease progression. The Journal of Immunology, 2013, 190: 2959–2965.

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Address correspondence and reprint requests to Dr. Jason M. Brenchley or Dr. Nichole R. Klatt, Laboratory of Molecular Microbiology, National Institutes of Health/National Institute of Allergy and Infectious Diseases, 9000 Rockville Pike, Building 4, Room 301, Bethesda, MD 20892 (J.M.B.) or Department of Pharmaceutics, Washington National Primate Research Center, University of Washington, Seattle, WA 98121 (N.R.K.). E-mail addresses: jbrenchl@mail.nih.gov (J.M.B.) or klartrr@uw.edu (N.R.K.)

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Abbreviations used in this article: ALN, axillary lymph node; GI, gastrointestinal; HIC, immune chemokine; LBP, LPS-binding protein; MLN, mesenteric lymph node; MxA, myxovirus protein A; PTM, pigtail macaque; RM, rhesus macaque; SP, slow progressor.
Materials and Methods

Study animals and sample collection

Nine PTM were infected i.v. with 3000 50% tissue culture-infective dose of SIVmac239. PTM were monitored prior to SIV infection to progression to AIDS (6 of 9 animals; progressors) or scheduled euthanasia after lack of AIDS progression after >700 d (3 of 9 animals; SP). Thirteen SIV-uninfected animals were also analyzed for comparison (of note, in SIV-PTM, the number of animals analyzed is variable depending on tissues collected). Upon necropsy, blood, axillary lymph nodes (ALN), mesenteric lymph nodes (MLN), jejunum, and colon were collected. Lymphocytes were isolated and cryopreserved from lymph nodes and GI tract tissues, as previously described (15). Of note, one animal (SIV+ progressor) was not available for necropsy claudin analysis and another animal (SIV+ progressor) was not available for LBP analysis. All animals were housed and cared for in accordance with the American Association for Accreditation of Laboratory Animal Care standards in American Association for Accreditation of Laboratory Animal Care–accredited facilities, and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Viral loads

Viral RNA levels in plasma were determined by real-time RT-PCR (ABI Prism 7700 sequence detection system; Applied Biosystems, Carlsbad, CA) using reverse-transcribed viral RNA in plasma samples from SIVmac239-infected PTM, as previously described (20).

Immunohistochemistry and quantitative image analysis

Staining of tissue sections and quantitative image analysis were performed, as previously described (15, 21). Epithelial barrier integrity in the colon was determined by immunohistochemistry (IHC) for the tight junction protein claudin-3 (rabbit polyclonal; Labvision, Fremont, CA), and immune activation in the MLN, colon, jejunum, and ALN by IHC for the type I IFN-responsive gene product, myxovirus protein A (MxA; mouse monoclonal, clone M143; from O. Haller and G. Kochs, Department of Virology, University of Freiburg, Freiburg, Germany). High-power (>400x) whole-tissue scans were obtained using a ScanScope CS System (Aperio Technologies, Vista, CA) and imported into Photoshop CS3 (Adobe Systems, Mountain View, CA). The percent area of tissue stained for MxA was determined in the entire cellular area of the MLN and the lamina propria of colon and jejunum, as previously described (15), using Photoshop CS3 tools with FoveaPro plugins (version 3.0; Reindeer Graphics, Asheville, NC), and the ratio of damaged to intact colonic epithelium was measured using the Aperio pen tool to outline and calculate the length of colonic breaches (claudin-3–negative regions) divided by the length of intact colonic epithelium (claudin-3–positive regions).

Cellular staining and flow cytometry

Multicolor flow cytometric analysis was performed on thawed samples, as previously described (15), using predetermined optimal concentrations of Abs that cross-react with PTM, which included the following: CD3 (clone SP34-2; BD Biosciences, San Jose, CA), CD4 (OKT4; eBioscience, San Diego, CA), CD8 (RPA-T8; eBioscience or 3B5; Invitrogen, Grand Island, NY), CD14 (M5E2; BioLegend, San Diego, CA or TuK4; Invitrogen), CD45 (MB46/4; Miltenyi Biotec, Cambridge, MA), CD20 (2H7; eBioscience), CD27 (O323; eBioscience), CD38 (OKT10; National Institutes of Health Nonhuman Primate Reagent Resource), CD28 (28.2; Beckman Coulter, Brea, CA), CD95 (DX2; BD Biosciences), CD103 (B-Ly7; eBioscience), IL-17 (eBio8CAP17; eBioscience), IL-22 (IL22OP; eBioscience), and Aqua Live/Dead dye (Invitrogen). Flow cytometric acquisition and analysis were performed on at least 100,000 cells on a BD LSRSort II flow cytometer driven by the FACS DiVa software (version 6.0; BD Biosciences). Analysis of the acquired data was performed using FlowJo software (version 9.4.11; Tree Star, Ashland, OR). We used a threshold of 200 collected events for all analysis of specific cell subsets.

Plasma LPS-binding protein levels

Plasma LPS-binding protein (LBP) was quantified with a commercially available ELISA (Biometec, Greifswald, Germany), according to the manufacturer’s protocol. We ran samples in duplicate and subtracted the background.

Statistical analysis

We performed Mann–Whitney U test for all t test p values and performed Bonferroni adjustment for multiple comparisons; horizontal bars in figures reflect mean. Spearman rank calculation and linear regression were calculated for comparative correlations, performed using Prism software (version 5.0; GraphPad, La Jolla, CA).

Results

Slow progression in PTM

GI immune dysfunction is a key factor in driving immune activation and HIV/SIV disease pathogenesis (1, 3, 6). However, it is unclear to what extent mucosal determinants relate to the rate of AIDS progression. Thus, to better understand the mucosal immunology of pathogenic SIV infection relative to the rate of AIDS progression, we studied mucosal tissues in a cohort of 9 SIVmac239-infected PTM with differential disease progression, in which SIV disease kinetics have been previously described in peripheral blood (16), and 13 uninfected PTM (15). Six of the 9 SIV-infected PTM rapidly progressed to AIDS, whereas 3 maintained a SP phenotype, evident by a lack of AIDS progression after >700 d postinoculation, at which point the animals were euthanized (16). Of note, because these animals were scheduled for euthanasia and may have otherwise eventually progressed, we refer to them as SP rather than long-term nonprogressors. The 6 progressing animals exhibited AIDS-defining illnesses at an average of 296 d postinfection, which was significantly more rapid than the 3 SP, who were euthanized on days 727, 729, and 733 (p = 0.02, Fig. 1A), a comparison that is most likely understated, because the SPs had not yet progressed to AIDS at the time of necropsy. Of note, all 3 of the SP, along with 2 progressors and 2 SIV-PTM, possessed the potentially protective Mane- AI084 allele. Consistent with this SP phenotype, despite similar peak viremia between the groups, the SP were better able to suppress viremia during the chronic stage of SIV infection (Fig. 1B). The set point viral load during the chronic stage of SIV infection was, on average, 2.78 log10 SIV copies/ml plasma in SP, significantly lower than that of the progressors who had an average of 5.54 log10 SIV copies/ml plasma (p = 0.02, Fig. 1B).

HIV preferentially infects CD4+ T cells, and a characteristic of disease progression is rapid loss of this crucial subset in mucosal tissues, followed by chronic depletion of CD4+ T cells in peripheral tissues (7, 22–24). Thus, to assess whether differences in viral load were reflected in CD4+ T cell maintenance, we measured the percentage of lymphocyte populations in tissues from necropsy by flow cytometry (Supplemental Fig. 1A). In PBMCs, SP maintained a slightly higher percentage of CD4+ T cells than progressors, although not significantly so (Fig. 1C, left). However, when progressors were compared with SIV+ PTM, there is, as expected, a significant decline of CD4+ T cells in peripheral blood (Fig. 1C, p = 0.02).

Because depletion of CD4+ T cells is most prevalent in the GI tract (7, 23), we quantified the CD4+ T cell populations in mucosal sites, including MLN, colon, and jejunum, to represent mucosal secondary lymphoid tissues, large intestine, and small intestine, respectively. In the MLN, SP had an average of 45.97% CD4+ T cells, whereas progressors had an average of 36.47% (p = 0.33; Fig. 1C, left center). Similar to PBMCs, when compared with uninfected PTM, the loss of CD4+ T cells in the MLN was more pronounced in progressors than SP (p < 0.01 and p = 0.02, respectively). Furthermore, the loss of CD4+ T cells in the colon was more pronounced in progressors than SP (p < 0.01 versus p = 0.10; Fig. 1C, right center) when compared with uninfected PTM. However, in the jejunum, low pre-SIV levels of CD4+ T cells persisted after SIV infection, with only a slight indication of CD4+ T cell decline in progressors versus SIV+ PTM (p = 0.17; Fig. 1C, right). This depletion of CD4+ T cells from the GI tract and limited peripheral CD4+ T cell loss of SP is consistent with models of nonprogressive SIV infection of natural hosts, which...
also have moderate depletion of mucosal CD4+ T cells, but do not progress to AIDS (25–27).

Recent data have shown that specific mucosal CD4+ T cell subsets that produce IL-17 and IL-22, cytokines associated with mucosal immune homeostasis, are lost during pathogenic SIV infection of RM (6, 28–30). Similarly, in our cohort of SIV-infected progressor PTM in this study, we found significant loss of IL-17– and IL-22–producing CD4+ T cells in mucosal tissues (p, 0.01, Supplemental Fig. 1B, 1C). Furthermore, loss of essential homeostatic lymphocytes in mucosal tissues during SIV infection has been associated with loss of CD103+ dendritic cells, which are crucial for directing homeostatic T cell responses, including Th17 (6, 31). Indeed, these CD103+ dendritic cells are also significantly lost in SIV-infected progressor PTM (p < 0.01, Supplemental Fig. 1B, 1C). Furthermore, loss of essential homeostatic lymphocytes in mucosal tissues during SIV infection has been associated with loss of CD103+ dendritic cells, which are crucial for directing homeostatic T cell responses, including Th17 (6, 31). Indeed, these CD103+ dendritic cells are also significantly lost in SIV-infected progressor PTM (p < 0.01, Supplemental Fig. 1B, 1C). Notably, slow progressors suffered no significant loss of CD103+ dendritic cells (p = 1.00, Supplemental Fig. 1D).

**Immune activation is decreased in PTM SP**

Given that systemic immune activation is a principal predictor of CD4+ T cell loss and AIDS progression (12–14), we next characterized immune activation in mucosal and systemic tissues from PTM. It has been previously established that PTM exhibit higher levels of immune activation in the absence of SIV infection compared with RM, and that, after SIV infection, PTM have high levels of T cell turnover and activation in peripheral blood (16). We quantified immune activation in tissue sections stained by IHC with a mAb against MxA, a protein produced in response to type-1 IFN (indicating local innate immune activation) (32) (Fig. 2A).

Comparing MxA expression in the SIV-negative, SP, and progressor PTM revealed that SP exhibited markedly less innate immune activation than progressors in all three of the GI tract sites we evaluated (Fig. 2B–D). In the MLN, SP had an average of 4.74% of the tissue occupied by MxA as compared with progressors, who had an average of 15.08% of the tissue occupied by MxA (p = 0.05; Fig. 2B). However, the SP had similar levels of activation compared with uninfected PTM, who had an average of 9.36% MxA (p = 0.69; Fig. 2B) in the MLN. In the lamina propria of the colon, SP demonstrated noticeably less activation than both progressors (average of 6.89% MxA; p = 0.10) or uninfected PTM (average of 8.23% MxA; p = 0.06) with SP having only an average of 1.07% MxA (Fig. 2C). However, perhaps surprisingly, the amount of activation in the colon was comparable between progressors and uninfected animals (p = 1.00). Furthermore, in the lamina propria of the jejunum, SP showed significantly less innate immune activation compared with progressors, with SP having an average of 0.39% of the lamina propria occupied by MxA compared with 3.85% MxA in progressors (p = 0.02; Fig. 2D) (uninfected PTM data not available). These data are consistent with previous studies demonstrating high levels of underlying mucosal damage and immune activation in the GI tract of uninfected PTM (15), but indicate that SP may have additional mechanisms to dampen immune activation in the GI tract, independent of SIV infection.

Although SP were able to limit activation locally in the GI tract, we also sought to investigate innate immune activation in peripheral lymphoid tissue. Of interest, whereas adaptive immune cell activation in T and B cells tended to be increased in progressors compared with SPs, it was not significantly different as measured by Ki67, CD38, and HLA-DR in CD4+ T cells (Supplemental Fig. 2A, 2B) and CD8+ T cells (Supplemental Fig. 2C, 2D), as well as CD27 and Ki67 in B cells (Supplemental Fig. 3), although this could be due to the limited numbers of animals. However, we
hypothesized that innate immune activation may be increased, and, thus, we quantified levels of MxA in the ALN at necropsy (Fig. 2E). Of interest, SP, with an average of 8.03% MxA in the ALN, had levels of peripheral innate immune activation comparable to that of uninfected PTM who had an average of 9.39% MxA (p = 1.00; Fig. 2E). Activation in the ALNs of progressors, in contrast, was markedly higher, with an average of 17.52% MxA, when compared with uninfected PTM (p = 0.03), and slightly higher than SP (p = 0.10). Thus, a key feature of progressive SIV infection in PTM is robust innate immune activation in systemic lymphoid sites.

Systemic immune activation is associated with damage to the colon barrier
Pathogenic HIV/SIV infections are strongly associated with immune dysfunction and destruction of the GI epithelial barrier (1–4, 9). Thus, we quantified the continuity of the colonic barrier by IHC using Abs against claudin-3, a tight junction protein, and quantified the ratio of the length of breached epithelium (not stained for claudin) to intact epithelium (stained for claudin) (Fig. 3A). Of interest, we found the underlying level of breaches in uninfected PTM was comparable to that of SIV+ progressors (p = 1.00; Fig. 2E). Activation in the ALNs of progressors, in contrast, was markedly higher, with an average of 17.52% MxA, when compared with uninfected PTM (p = 0.03), and slightly higher than SP (p = 0.10). Thus, a key feature of progressive SIV infection in PTM is robust innate immune activation in systemic lymphoid sites.

Microbial translocation predicts AIDS progression
Intestinal damage in SIV infection has been shown to result in microbial translocation, a cause of systemic immune activation (3, 9, 15). Given that we observed greater intestinal damage as well as local and peripheral innate immune activation in progressors, we sought to determine whether a potential relationship existed between microbial translocation and progression to AIDS. To measure microbial translocation, we quantified the concentration of LBP throughout infection in the plasma of PTM by ELISA. LBP is produced in response to bacterial LPS, and thus indicates the presence of microbial products in the bloodstream (33), and LBP may be the most reliable measurement of systemic microbial translocation in plasma of nonhuman primates (34). Prior to SIV infection, the PTM that went on to become progressors tended to have higher levels of plasma LBP (Fig. 4A). Furthermore, at necropsy, whereas all animals had increases in LBP, the PTM with AIDS had slightly higher levels of LBP compared with the SP (p = 0.14, Fig. 4A). Importantly, levels of LBP prior to SIV infection significantly correlated with the rate to AIDS progression after SIV infection (p < 0.01, Spearman r = −0.84, Fig. 4B). Thus, pre-existing levels of microbial translocation may dictate the rate of disease progression once infected. Furthermore, we also found a significant correlation between microbial translocation as measured by LBP at necropsy and progression to AIDS (p = 0.04, Fig. 3C). Furthermore, we found the correlation between barrier integrity and activation was also significant locally in the colon (p = 0.04, Spearman r = 0.76, Fig. 3D). There was also a trend toward an association between barrier integrity and MLN activation (p = 0.11, data not shown). These data suggest a relationship between colonic barrier integrity and local and systemic activation.

FIGURE 2. Immune activation in the GI tract and periphery is reduced in SP. The amount of immune activation in necropsy tissues was measured by immunohistochemistry staining for MxA, an IFN-responsive protein. (A) Representative MxA stain (brown) in lymph node samples from SIV− (left), SIV+ SP (center), and SIV+ progressors (right). Original magnification ×200. The percent area of tissue quantified as positively stained for MxA in (B) MLN, (C) colon, (D) jejunum, and (E) ALN. Horizontal lines represent mean, and p values were calculated using Mann–Whitney nonparametric U test. For multiple comparisons, p value was adjusted with Bonferroni correction. SIV-uninfected, circles; SIV-uninfected Mane-A1*084+, open circles; SIV+ Mane-A1*084+ SP, open triangles; SIV+ progressors, squares; SIV+ Mane-A1*084+ progressors, open squares.
Spearman r = −0.76, Fig. 4C). Notably, this relationship was statistically stronger than the relationships we observed between time to AIDS progression and typical disease indicators, such as set point viral load and absolute CD4+ T cell count at necropsy (Supplemental Fig. 4A, 4B). Furthermore, it presented a more extreme disparity than the comparison of CD4 count at necropsy between groups, as slow progressors had only slightly higher absolute CD4 counts than progressors, and the relationship was not statistically significant (p = 0.26, Supplemental Fig. 4C). Although our findings are limited to a small subset of PTM, our data indicate that plasma LBP might serve as a potential identifier of susceptibility to AIDS progression both prior to and during SIV infection.
infection. Furthermore, our data support the role of microbial translocation in predicting AIDS progression.

Discussion
To better understand how mucosal immune dysfunction relates to disease progression after HIV/SIV infection, we studied immune activation, GI barrier function, mucosal immunity, and microbial translocation in uninfected PTM, SIV-infected PTM who progressed to AIDS, and SIV-infected SP PTM. We found that, despite moderate depletion of CD4+ T cells in SP, these animals had dampened innate immune activation compared with either uninfected PTM or progressors, as measured by the IFN-α response gene Mxα. Furthermore, we found that this decreased innate immune activation was associated with a more intact tight epithelial barrier of the colon compared with progressors, who had significantly more damage to the GI tract. Finally, we found a correlation between microbial translocation preinfection and at necropsy with the time to AIDS progression. These data suggest the importance of limitation of peripheral immune activation, maintenance of mucosal homeostatic immune subsets, and suppression of microbial translocation in limiting, and possibly predicting, progression to AIDS.

The decreased innate immune activation observed in all tissues in the SP is of interest considering the incomplete understanding of cause versus effect in HIV-infected SP and long-term non-progressors. Although it is intuitive to suggest that lower virus replication results in decreased immune activation and disease progression in SP, in this study the finding that SP had lower immune activation than uninfected animals suggests that dampened immune activation preinfection may play a role in the lack of AIDS progression, and potentially could be itself why viremia is lower in SP. Indeed, HIV preferentially infects activated CD4+ T cells (35); thus, pre-existing lower levels of immune activation may limit the pool of targets for HIV infection, and thus decrease viremia in SP. This is also consistent with the moderate CD4+ T cell depletion we observed in mucosal tissues in SP, which also occurs in natural host nonprogressive SIV infection (25, 26, 36), without inducing local activation or microbial translocation. SP also had decreased damage to the tight epithelial barrier in the colon compared with progressors and uninfected PTM, further supporting that preinfection mucosal factors may, in part, underlie lack of progression, and that these observations are not the result of decreased virus alone.

The role of the somewhat protective ManeA1*084 allele in this study is unclear. Whereas all of the SP possessed this allele, several SIV− and SIV+ progressors were also ManeA1*084−, with no protective effects. Thus, the importance of this allele in slow progression, and whether ManeA1*084 plays a role in mucosal immunity, needs to be further investigated. Furthermore, another caveat of these data is the comparison of healthy animals to those that progressed to AIDS, with opportunistic infections and poor health. Whereas comparing animals that have progressed to AIDS with uninfected animals or with SP has the potential to confound the conclusions, the clear trend for increased GI dysfunction, even prior to SIV infection, indicates that the results are not compromised by the presence of opportunistic infections.

Interestingly, uninfected and progressively infected animals demonstrated similarly high amounts of immune activation in the GI tract; however, elevated innate immune activation in the ALN was only observed in progressively infected animals. Systemic immune activation in HIV/SIV infections is multifaceted, although it has been demonstrated that one factor that contributes to systemic activation is microbial translocation (9, 15). Indeed, our finding that damage to the colonic epithelium in infected animals closely correlated with the amount of both local and peripheral innate immune activation suggests that local damage to the GI tract can result in systemic microbial translocation in the context of SIV infection. Furthermore, this indicates that damage to the GI tract and local microbial translocation in the lamina propria may be the first breakdown of this chain of events, and that dysfunctional GI immunity postinfection results in reduced microbial immune responses and microbial clearance, leading to systemic microbial translocation and immune activation during SIV infection.

Our finding that levels of microbial translocation at death correlated with AIDS progression supports the role of microbial translocation in contributing to disease progression and mortality in HIV infection (37). However, we extend on this premise with our evidence showing a direct relationship between preinfection levels of microbial translocation and AIDS progression. Indeed, it has been demonstrated that PTM have greater levels of microbial translocation in the absence of SIV infection and that they progress faster than models such as RM, who have less inherent microbial translocation (15). This direct association may point to an important role for monitoring plasma bacterial products and/or serum proteins expressed in response to microbial products (i.e., LBP) in uninfected animals to ascertain the susceptibility and rate to disease progression in SIV infection. Given our small sample size, future studies are warranted to assess the strength of preinfection microbial translocation as a predictor of AIDS progression in larger cohorts. Indeed, ascertaining pre-existing levels of microbial products in nonhuman primate studies may be an ideal and informative opportunity in vaccination and pre-exposure prophylaxis experiments to further determine the role of pre-existing GI damage and disease progression in addition to its potential role in acquisition given that microbial translocation increases T cell activation at mucosal sites.

In this study, we have characterized SIV infection in mucosal tissues of PTM and have found that GI tract immune subsets may have a role in limiting microbial translocation and systemic activation, and that preinfection microbial translocation is associated with disease progression upon SIV infection. The observations in this study point to key factors to be explored in the mucosal immunology of SIV infection. Importantly, our study highlights the benefit of using PTM to elucidate key factors in progression, as we have seen in this study that factors causing pathology in uninfected PTM, such as local immune activation and damage to the barrier of the colon, are not sufficient in and of themselves to cause the levels of systemic activation observed in SIV-infected PTM. Rather, the underlying innate immune activation and GI damage may be potentiated by mucosal immune dysfunction caused by infection, which predicts progression. Indeed, the pre-existing GI immune activation may make PTM a better model for mucosal immunology studies in the context of heightened immune activation and/or GI enteropathies that exist in areas with high prevalence of HIV such as sub-Saharan Africa (38, 39). Understanding how SIV-infected SP PTM prevent systemic immune activation may allow for better treatment regimens, especially considering that in HIV-infected, highly active antiretroviral therapy–treated individuals, morbidity and mortality are associated with systemic immune activation and microbial translocation (37, 40–42). Furthermore, these data point to the GI tract as an essential target for therapies, such as probiotics, aimed to support GI tract physiology and suppress microbial translocation.

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