HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2013 May 01.

Published in final edited form as:

Mucosal Immunol. 2012 November ; 5(6): 646–657. doi:10.1038/mi.2012.38.

Loss of CD103+ DCs and Mucosal IL-17+ and IL-22+ Lymphocytes is Associated with Mucosal Damage in SIV Infection

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Abstract

HIV/SIV disease progression is associated with multifocal damage to the GI tract epithelial barrier that correlates with microbial translocation and persistent pathological immune activation but the underlying mechanisms remain unclear. Investigating alterations in mucosal immunity during SIV infection, we found that damage to the colonic epithelial barrier was associated with loss of multiple lineages of IL-17-producing lymphocytes, cells that microarray analysis showed express genes important for enterocyte homeostasis, including IL-22. IL-22-producing lymphocytes were also lost after SIV infection. Potentially explaining coordinate loss of these distinct populations, we also observed loss of CD103+ DCs after SIV infection which associated with loss of IL-17 and IL-22-producing lymphocytes. CD103+ DCs expressed genes associated with promotion of IL-17/IL-22+ cells, and co-culture of CD103+ DCs and naïve T-cells led to increased *IL17A* and *RORc*

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Disclosure The authors declare no conflicts of interest.

expression in differentiating T-cells. These results reveal complex interactions between mucosal immune cell subsets providing potential mechanistic insights into mechanisms of mucosal immune dysregulation during HIV/SIV infection, and offer hints for development of novel therapeutic strategies to address this aspect of AIDS virus pathogenesis.

Introduction

During progressive HIV/SIV infection there is a significant and progressive insult to the intestinal mucosa, beginning with massive infection of and subsequent depletion of CD4+ T cells and eventual compromise of the structural epithelial barrier of the gastrointestinal (GI) tract[1,2]. These changes lead to translocation of GI tract microbial products[1,3,4] which contributes to persistent pathological immune activation and exacerbation of disease progression[3,5-8]. However, the mechanisms responsible for the damage to the tight epithelial barrier of the GI tract during HIV/SIV infection, which may initiate these processes, remain unclear.

Another manifestation of mucosal immune dysfunction during pathogenic HIV/SIV infections is the preferential loss of IL-17-producing CD4+ and CD8+ T cells from mucosal tissues[9-16]. These IL-17-producing T cells are essential for mucosal immunity as they respond to extracellular bacteria and fungi, and are involved in maintenance of the structural barrier of the GI tract, driven by production of cytokines such as IL-22, as well as induction of claudin, anti-bacterial defensin, and/or mucin expression[10,17-21]. While T lymphocytes are clearly important sources of IL-17 and IL-22, recent studies have demonstrated that T cells are not the only lymphocytic source of IL-17-associated cytokines *in vivo*, nor are they the only subset important for maintenance of the structural barrier of the GI tract and mucosal immunity. Indeed, specialized subsets of innate lymphocytes in mucosal tissues can produce IL-22 and/or IL-17 and contribute to homeostasis of epithelial cells along mucosal surfaces[22-27]. Furthermore, it was recently demonstrated that NKp44+ natural killer (NK) cells produce IL-17 and are lost from mucosal sites in progressive SIV infection[16].

With continuous exposure to dietary antigens and commensal microbes, the gut represents a unique immunological environment, requiring a balance between prompt, effective responses to pathogens and tolerance of frequent exposures to non-infectious antigens. Appropriate mucosal immune responses depend on specialized dendritic cells (DCs) to sample GI tract antigens, migrate to lymphoid tissues and present antigens to the adaptive immune system to convey signals critical for directing lymphocytes to either become activated or to maintain tolerance against specific antigens[28-31]. One such specialized subset of antigen presenting cells in the GI tract is CD103+ DCs which can metabolize vitamin A into retinoic acid (RA)[31-33]. This is of particular importance as RA production is essential for inducing expression of RORγt, a transcription factor required for development and maintenance of IL-17 and IL-22-producing cells[34-40]**.** Thus, interactions between different DC and lymphocyte populations in the mucosal immune system define the unique local immune microenvironment, and disruption of these cells and their interactions may contribute to the mucosal immune dysfunction seen during pathogenic HIV/SIV infections.

We studied the frequency, phenotypes and functionality of mucosal IL17− and IL-22-producing lymphocytes, comparing results for uninfected and SIV-infected rhesus macaques to assess the potential contribution of loss or dysfunction of these cells to loss of GI tract structural integrity and associated aspects of disease progression. Given the close and critical interactions between mucosal APCs and lymphocytes in the mucosal immune system, we also studied the frequency, phenotype and functionality of mucosal APCs as a potential factor contributing to loss of IL-17-producing lymphocytes. Our results reveal that SIV infection results in multiple disruptions in these cell populations and their interactions and provide novel insights and a plausible mechanistic basis for the mucosal immune system dysfunction and the GI epithelial damage observed in progressive HIV/SIV infections.

Results

Loss of IL-17-producing lymphocytes is associated with damage to the mucosal barrier of the colon

As recent studies have demonstrated the importance of IL-17 in mucosal immunity[41], we performed flow cytometric analysis of IL-17-producing lymphocytes, including CD4+ T cells (Th17), CD8+ T cells (Tc17) and CD20-CD3-CD8+ natural killer (NK)-like lymphocytes from mucosal tissues of uninfected and chronically SIV-infected rhesus macaque (RM), after mitogenic stimulation (Supplementary Figures 1, 2a). Of note, while CD20-CD3-CD8+ lymphocytes in peripheral blood are classically defined as NK cells[42], in mucosal tissues these cells do not homogenously express any given NK cell marker, including NKp44, NKp46, NKp80 or NKG2A (data not shown), and thus are referred to as “CD3-CD8+ lymphocytes”. This *ex vivo* analysis showed all subsets of IL-17-producing lymphocytes were decreased in mesenteric lymph node (MLNs) and colon tissues of chronically SIV-infected RMs compared to uninfected RMs (Supplementary Figure 1a-1b). As the flow cytometry analysis of tissue derived cells addresses only the relative frequencies of different populations, to discriminate between relative loss of IL-17-producing lymphocytes and potential influx of non-IL-17+ cells in explaining this observation, we performed immunohistochemical (IHC) analysis and quantitative analysis of IL-17+ cells in paraffin-embedded colonic tissue from SIV-infected and uninfected RMs to assess absolute numbers of IL-17+ cells per unit area of lamina propria (Supplementary Figure 1c-d). These data confirmed that IL-17-producing cells were in fact depleted from the GI tract of SIV-infected RMs with a significant loss of IL17+ cells/mm^2 in the lamina propria of the colon (P=0.0012, Supplementary Figure 1e). Preservation of IL-17-producing lymphocyte subsets in tissues of SIV-infected natural host sooty mangabeys (Supplementary Figure 1f), which replicate SIV to high levels after SIV infection but do not develop GI mucosal damage and immunological dysfunction or progress to simian AIDS, is consistent with a potential role for these cells in maintenance of mucosal integrity in non-progressive SIV infection.

A hallmark of progressive HIV/SIV infection is focal damage to the epithelial barrier of the colon, leading to microbial translocation[1] but the mechanism(s) underlying this damage and associated changes in intestinal permeability are unknown. Given data implicating IL-17-producing lymphocytes in GI tract immunity and maintenance of GI epithelial integrity, we investigated the relationship between loss of different IL-17-producing lymphocyte

populations and structural, multifocal, damage to the GI tract using IHC with antibodies against the tight junction protein claudin-3 to assess the continuity of the structural epithelial barrier of the colon (breach/intact ratio) as previously described[1,43]. We found a significant, inverse correlation between the frequency of colonic Th17 cells and damage to the epithelial barrier of the colon (P=0.0219, r=−0.6643, Figure 1a), and a trend toward a negative relationship between MLNs Th17 cell frequency and structural damage (P=0.1351, r=−0.4198, Figure 1b). For Tc17 cells we found a significant, inverse correlation between the extent of damage to the epithelial barrier of the colon and the frequency of Tc17 cells in both the colon (P=0.0033, r=−0.7902, Figure 1c) and MLNs (P=0.0389, r=−0.5560, Figure 1d**).** Intriguingly, we observed the most robust inverse correlations for the extent of colonic epithelial damage and the frequency of IL-17-producing CD3-CD8+ lymphocytes in the colon (P=0.0011, r=−0.7758, Figure 1e), and MLNs (P=0.0009, r=−0.7846, Figure 1f). These significant correlations were maintained when SIV-uninfected RM were excluded from the analysis (data not shown). Furthermore, we also found inverse correlations between the frequency of mucosal IL-17-producing lymphocytes and systemic immune activation, as measured by Ki67 expression by peripheral blood CD4+ and CD8+ T cells, suggesting that loss of mucosal IL-17 producing lymphocytes is not only associated with epithelial damage, but also with the persistent, systemic immune activation that characterizes pathogenic HIV/SIV infection (data not shown). Given the role of IL-17-producing lymphocytes in contributing to the maintenance of the epithelial barrier of the GI tract, these data suggest that loss of these cells may provide an explanation for the observed damage to the tight epithelial barrier of the GI tract and the ensuing microbial translocation and pathological systemic immune activation characteristic of progressive SIV infection of RMs.

IL-17-producing lymphocytes express genes essential for enterocyte homeostasis

While IL-17 production is associated with preservation of epithelial cell homeostasis, the actual mechanism by which IL-17-producing lymphocytes exert this protective function is unclear. To further characterize these cells, we performed gene array analysis on mRNA isolated from MLN lymphocyte subpopulations separated based upon production of IL-17 following mitogenic stimulation. Supervised cluster analysis of results for cells that did or did not produce IL-17 demonstrated distinct gene expression profiles, apparent in heatmap cluster analysis of the top 200 differentially expressed genes (Figure 2a). Further analysis of genes differentially expressed between the two subsets revealed that the gene most significantly up-regulated in the IL-17+ lymphocytes was IL-17F, validating our approach for separating IL-17-producing and non-producing lymphocytes (Figure 2b). Bioinformatic analysis identified more than 1269 genes which were significantly differentially regulated between the two subsets (fold change +/−1.5 and $p<0.05$). Furthermore, analysis of specific genes demonstrated several key functional distinctions between IL-17-producing and non-IL-17-producing lymphocytes based on FDR adjusted P values for the IL-17− vs IL-17+ microarray specific genes calculated using the R package Limma). Several genes that are essential for GI tract function were up-regulated in IL-17+ lymphocytes, including IL-22, which is an essential growth factor for epithelial cells[27,44] (Figure 2b). In addition, the chemokines CCL1 and CCL20, both of which are immune chemoattractants important for mucosal immunity, were up-regulated in IL-17+ lymphocytes[45,46]. Furthermore, IL-17+ lymphocytes also expressed the integrin β7 which is essential for mucosal homing and

retention of lymphocytes[47], and TNFRSF4 (OX40), a co-stimulatory molecule of the TNF super family implicated in regulation and survival of mucosal lymphocytes[48,49], as well as RORc, which encodes RORγt, and is essential for IL-17 production[50,51].

In comparison, in the IL-17− lymphocytes, genes essential for Th1 and Th2 responses were notably up-regulated, including genes associated with interferon signaling and production, by such as IFNGR1, STAT3, and STAT6[52]. Furthermore, genes associated with TGF-β signaling, including TGIF1[53], were also expressed by the IL-17-lymphocytes (Figure 2b). A complete list of these genes is presented (Supplementary Table 1).

Network analysis of the expression profile of the IL-17+ lymphocytes (compared to IL-17-) demonstrated the association of genes essential for promotion of IL-17 and IL-22 in these mucosal lymphocytes (Figure 2c). T-helper cell pathway analysis further demonstrates the skewing of the expression profile of the IL-17+ cells away from a “Th1” phenotype, and towards a “Th17” phenotype (Figure 2d). Thus, the transcriptional analysis of IL-17-producing lymphocytes demonstrated expression of genes implicated in mucosal immunity and maintenance of the GI tract epithelial barrier.

IL-22-producing lymphocytes are lost after SIV infection in association with damage to the GI tract epithelial structural barrier

Recent studies have identified IL-22 as a cytokine important for enterocyte homeostasis[23,25,27,44]. Notably, our transcriptional analysis demonstrated that upon mucosal lymphocytes that express IL-17 upon stimulation express significantly higher levels of IL-22 transcript than IL-17− lymphocytes (4.53 fold change and $p<0.0001$) (Figure 2). To identify other cell types capable of producing IL-22 upon *ex vivo* stimulation we used flow cytometry (Supplementary Figure 2b). High frequencies of all subsets of IL-17+ lymphocytes from colon and MLNs expressed IL-22 after mitogenic stimulation (Figure 3a-b), findings consistent with our gene expression array data. Given this evidence of production of IL-22 by IL-17+ lymphocytes and the observation that IL-17+ lymphocytes are lost from mucosal tissues in SIV-infected RMs, we compared IL-22 production by mucosal CD4+ and CD8+ T cells and CD3-CD8+ lymphocytes from uninfected and SIV-infected RMs. In SIV-infected RMs we found significantly decreased IL-22 production by CD4+ T cells from both colon and MLNs (*P*=0.0159, *P*=0.0048, Figure 3c-d**, left**), as well as from CD3-CD8+ lymphocytes in colon and MLNs (*P*=0.0491, *P*=0.0360, Figure 3c-d**, right**). We also observed a trend for decreased IL-22 production by CD8+ T cells from colon and MLNs of SIV-infected RMs (Figure 3cd**, center**). Of note, production of IL-22 and IL-17 are not mutually exclusive; IL-22+IL-17+ as well as IL-22+IL-17− and IL-22-IL-17+ lymphocyte populations can all be demonstrated and are all decreased after SIV infection (data not shown). Since IL-22 is known to be essential for enterocyte proliferation and homeostasis[44], we hypothesized that loss of IL-22 after SIV infection might also be associated with damage to the tight epithelial barrier of the GI tract. Indeed, examining different IL-22 producing lymphocyte subpopulations, we found significant inverse correlations between the frequency of colonic and MLNs CD4+IL-22+ T cells and damage to the structural barrier of the colon (*P*=0.0108, r=−0.8167; *P*=0.0068, r=−0.6760, Figure 3e-f**, left**) and inverse trends between the frequency of colon and MLNs CD8+IL-22+ T

cells and damage to the epithelial barrier of the colon (Figure 3e-f**, center**). We also observed an inverse trend for the relationship between IL-22+CD3-CD8+ lymphocytes in the colon and damage to the barrier (*P*=0.0968, r=−0.6025, Figure 3e**, right**) and a significant inverse correlation in MLNs (*P*=0.0042, r=−0.6929, Figure 3f**, right**). Taken together, these data demonstrate that loss of IL-22-producing lymphocytes after SIV infection is associated with damage to the tight epithelial barrier of the GI tract.

CD103+ DCs are lost after SIV infection, and are associated with loss of IL-17 and IL-22-producing lymphocytes

Given the critical importance for local APCs in priming lymphocytes towards specific functionalities and in maintaining cells with such functions, we investigated the relationship between the frequency of IL-17 and IL-22-producing lymphocytes and individual subsets of dendritic cells (DCs) within the GI tract and MLNs. We measured the frequencies of CD103+ DCs, identified as live, lineage-, HLA-DR+CD103+ cells, during SIV infection (Supplementary Figure 2c) and found significant decreases in CD103+ DCs in both colon (*P*=0.0260, Figure 4a) and MLNs (*P*=0.0040, Figure 4b) in SIV-infected animals. To determine whether loss of these CD103+ DCs might contribute to the loss of IL-17 and IL-22-producing lymphocytes, we investigated the relationship between these cell subsets in colon and MLNs. We found a significant, positive correlation between the frequency of CD103+ DCs and Th17 cells (*P*<0.0001, r=0.7158, Figure 4c), Tc17 cells (*P*=0.0011, r=0.6249, Figure 4d) and IL-17+CD3-CD8+ lymphocytes (*P*=0.0053, r=0.5401, Figure 4e) in these mucosal tissues. Similarly, we found a significant, positive correlation when we compared the frequency of CD103+ DCs and CD4+IL-22+ T cells (*P*=0.0032, r=0.5876, Figure 4f), and IL-22+CD3-CD8+ lymphocytes (*P*=0.0317, r=0.4488, Figure 4h) and a trend towards an association between CD8+IL-22+ T cells and CD103+ DCs (Figure 4g). Of note, we found no significant changes in the frequencies of either CD11c+ myeloid DCs (mDCs, Supplementary Figure 4a) or CD123+ plasmacytoid DCs (pDCs, Supplementary Figure 4b) in the colon after SIV infection. Furthermore, we found no similar correlations between frequencies of either mDCs (Supplementary Figure 4c) or pDCs (Supplementary Figure 4d) and IL-17+ lymphocytes in mucosal tissues. In addition, maintenance of CD103+ DCs during non-progressive SIV infection of natural host sooty mangabeys suggests conservation of CD103+ DCs in mucosal tissues of SMs may contribute to maintenance of healthy mucosal immunity during non-progressive SIV infection (Supplementary Figure 4e).

CD103+ DCs promote IL-17 and IL-22-producing lymphocytes

To characterize further CD103+ DCs and to understand whether these APCs can directly support development or maintenance of IL-17 and IL-22-producing lymphocytes, we performed transcriptional analysis of CD103+ and CD11c+CD103− DCs isolated from MLNs of uninfected macaques. Striking differences between the transcriptional profiles of these DCs were evident by heatmap unsupervised cluster analysis of the top 200 differentially regulated genes (Figure 5a). In fact, we observed 1177 genes that are significantly and differentially expressed (fold change +/−1.5 and p value <0.05) when we compared CD103+ to CD103− DCs (a complete list of genes available in Supplementary Table 2). Furthermore, analysis of key differentially regulated genes demonstrated the induction of genes that strongly associated with the described functions of these DC subsets

(Figure 5b). For example, CD103+ DCs expressed genes which are essential for the promotion of IL-17+ and IL-22+ lymphocytes, including the transcriptional regulator IL-6, along with MAP3K7, APOE, and RXRB (retinoid X receptor B) (Figure 5b). Furthermore, other immunomodulatory genes, such as CCL22, IL-15 and IRF3 were expressed by CD103+ DCs, further demonstrating the important role for CD103+ DCs in mucosal immune regulation (Figure 5b). In contrast, CD103-CD11c+ DCs expressed numerous genes essential for induction and regulation of interferon responses, including JAK1, IFI6, IRF8 and CXCL10 (Figure 5b), as well as immunomodulatory genes such as CCR4 and TGFB1 (Figure 5b). We performed gene network analysis to investigate potential associations of genes within the IL-17 pathways in CD103+ and CD103− DCs (Figure 5c). Increased expression of IL-6 represented a significant node within the network connecting IL-17, IL-22 and RXRA genes, all known to be important in the regulation and promotion of IL-17 (Figure 5c).

CD103+ DCs can directly promote a Th17 phenotype *in vitro*

While transcriptional profiling demonstrated gene expression associated with IL-17 and IL-22 production, to address whether mucosal CD103+ DCs can directly prime naïve CD4+ T cells towards a Th17 phenotype, we performed co-cultures with flow cytometrically sorted, naïve CD4+ T cells and DCs isolated from the MLNs of two SIV-uninfected RMs. Naïve CD4+ T cells were cultured with either CD103+ DCs, CD103-DCs, or no DCs and anti-CD3/anti-CD28 beads (control) under Th17 conditions, with or without IL-6. We found that in the presence of IL-6, CD103+ DCs promoted *IL17A* expression that was 1.88-fold higher than expression by CD4+ T cells cultured with CD103− DCs (figure 6a). Furthermore, the importance of IL-6 in promoting IL-17 production was demonstrated, as co-cultures with CD103+ DCs in the absence of IL-6 expressed 0.84 fold less *IL17A* (figure 6b), though CD4+ T cells co-cultured with CD103+ DCs maintained higher expression of *IL17A* (figure 6b), suggesting that other functions of CD103+ DCs, such as RA production, are also essential for promotion of IL17 production. In addition to *IL17A*, we also measured expression of *RORc* , which encodes RORγt, the transcription factor essential for IL-17 and IL-22 production[49,53], (figure 6c-d). We found that CD4+ T cells co-cultured with CD103+ DCs expressed, on average, 6.31-fold higher levels of *RORc* compared to CD4+ T cells co-cultured with CD103− DCs (figure 6c). CD103+ DC co-cultures without IL-6 expressed an average of 3.27 fold less *RORc* than cultures with IL-6, however still expressed levels of *RORc* higher than co-cultures with CD103− DCs (figure 6d). Of note, due to the very low numbers of CD103+ APCs obtainable from MLNs and the very large numbers of cells required for these experiments, we could only isolate a sufficient number of cells from two sacrificed RMs (Rh1 and Rh2), thus statistical analysis was not possible, though the same trends were present in all animals in all conditions. Consistent with this implied role for RA in inducing Th17 cells in vivo, in a separate pilot experiment we found moderate increases in the frequencies of Th17 cells in a chronically SIV-infected animal therapeutically dosed with 50 mg/kg all trans retinoic acid sub-cutaneously (data not shown).

Taken together, our data demonstrate that CD103+ DCs can directly induce naïve T cells to express *RORc*. In contrast, other DCs can produce indoleamine 2,3-dioxygenase (IDO), which has been shown to be inversely associated with frequencies of Th17 cells during

pathogenic SIV and HIV infections[13,16,55]. Indeed, we found that increased IDO expression in mucosal tissues (Supplementary Figure 5a-f) significantly, negatively correlates with the frequencies of CD103+ DCs (Supplementary Figure 5gh). Thus, alterations in the mucosal APC landscape after SIV infection likely contribute to the decreased CD103+ DCs and IL-17+ and IL-22+ lymphocytes we observe here and provide a plausible mechanism whereby maintenance of mucosal CD103+ DCs promoting the development and survival of IL-17+ and IL-22+ lymphocytes is important for maintenance of the tight epithelial barrier of the GI tract and mucosal immunity.

Discussion

To understand potential mechanisms by which SIV infection leads to focal damage to the intestinal epithelial barrier, resulting in microbial translocation which contributes to persistent immune activation, we studied subpopulations of resident immune cells in the GI tract and draining MLNs, comparing uninfected and chronically SIV-infected RMs, as well as nonprogressively SIV-infected and uninfected SMs. We have shown that CD4+ T cells, CD8+ T cells and CD3-CD8+ lymphocytes isolated from mucosal tissues can produce IL-17 and IL-22 after mitogenic stimulation, and were significantly decreased after pathogenic SIV infection. Furthermore, transcriptional analysis revealed multiple genes expressed by IL-17-producing lymphocytes that are essential for enterocyte homeostasis and mucosal immune function. Indeed, IL-22, which is essential for enterocyte proliferation, was up-regulated by IL-17+ cells, and we show that IL-22 is produced by IL-17+ cells and these lymphocyte populations are lost in pathogenic SIV infection. Importantly, loss of these IL-17 and IL-22-producing lymphocytes was associated with damage to the tight epithelial barrier of the GI tract. This suggests that IL-17 and IL-22-producing lymphocytes may play an essential role in maintenance of the GI tract epithelial barrier, and that loss of these cells during HIV/SIV infections may contribute to the damage observed.

As we found no correlations between levels of plasma viral load and frequencies of IL-17 or IL-22-producing lymphocytes (data not shown), viral replication, itself, is unlikely leading to direct loss of these cells. Indeed, previous studies found no evidence for preferential infection of Th17 cells, and neither CD3-CD8+ lymphocytes nor CD8+ T cells capable of expressing IL-17 are targets for the virus *in vivo*[9]. Furthermore, preservation of IL-17-producing lymphocytes in natural hosts, which maintain GI epithelial integrity and avoid microbial translocation, persistent systemic immune activation and progressive disease despite high viral loads during infection, indicates that virus replication is not sufficient for the dysfunction of the mucosal immune system observed in progressively SIV-infected RM.

Our results suggest that modulation of different mucosal APC populations represents a plausible mechanism underlying loss of mucosal IL-17 and IL-22-producing cells. Increased activation and inflammation in mucosal tissues after SIV infection may lead to increased levels of proinflammatory immune cells that may inhibit CD103+ DCs through mechanisms such as tryptophan metabolism manifested by up-regulated IDO. Furthermore, the general activated state in the mucosa may promote the influx of extrinsic DC populations which do not, generally, reside within mucosal tissues, thus overwhelming the numbers and/or effects of mucosal resident CD103+ DCs. In turn, lack of CD103+ DCs in the mucosal tissues

likely results in decreased IL-6 production and metabolism of vitamin A to retinoic acid (RA), and the combination of decreased IL-6 and RA and increased IDO could easily lead to loss of IL-17-producing lymphocytes. Indeed, we have demonstrated here that CD103+ DCs have increased expression of IL-6 genes compared to CD103− DCs, and that removing IL-6 from co-cultures results in decreased *IL17A* and *RORc* expression. This loss of the IL-17− and IL-22-producing cells may contribute to damage to the tight epithelial barrier of the GI tract with subsequent microbial translocation and immune activation; all of which contribute to disease progression during pathogenic SIV and HIV infection. Of note, previous studies in mice have shown that CD103+ DCs specialize in promoting T regulatory cells[32,33]; however, recent murine studies have demonstrated that, consistent with our primate results, retinoic acid and IL-6 produced by CD103+ DCs are also essential for IL-17 production[36]. Furthermore, recent studies have demonstrated that the functional specializations of intestinal DCs in mice is dependent on many factors, including source of mice and APC:T cell ratios[56], demonstrating the diverse functionality and importance of CD103+ DCs.

In conclusion, here we have demonstrated that multiple IL-17 and IL-22-producing lymphocyte subsets are preferentially lost from mucosal tissues of chronically SIV-infected RM, and that this loss may adversely affect GI tract epithelial homeostasis. Furthermore, loss of IL-17-producing cells is associated with loss of CD103+ DCs, which we show can directly promote *IL17A* as well as *RORc*, essential for induction of IL-17 and IL-22 production by lymphocytes. Furthermore, transcriptional analysis demonstrates that CD103+ DCs express genes essential for survival of mucosal IL-17+ and IL-22+ lymphocytes. These data provide mechanistic insight underlying dysregulation of mucosal immunity during HIV/SIV infections, and may provide clues for developing therapeutic strategies intended to preserve or restore these populations, potentially improving the epithelial integrity of the GI tract and limiting microbial translocation and the associated immune activation.

Methods

Animals and Sample Collection

For this study, 12 chronically (day 90+) SIV-infected rhesus macaques (RM, *Macaca mulatta*) and 6 SIV-uninfected RMs were euthanized and blood, mesenteric LN, and colon tissues were collected. Of the 12 chronically SIV-infected RMs, 3 animals were infected i.v. with 1 TCID50, 3 with 10 TCID50, and 3 intrarectally with 3000 TCID50 of SIVsmE543, and 3 animals were infected i.v. with 3000 TCID50 of SIVmac239. RMs were infected with multiple pathogenic viruses and via different inoculation routes and animals were sacrificed at different time points post SIV infection in order to establish a diverse cohort of progressively SIV-infected animals, the goal being to ensure our observations were reproducible in multiple infection scenarios and to maximize our chances of observing a spectrum of immunological abnormalities (Supplementary Table 3). None of the animals had clinical signs of simian AIDS. 10 rectal pinch biopsies and blood samples were also collected from 19 naturally SIV-infected sooty mangabeys (SM, *Cercocebus atys*) during chronic infection and from 15 SIV-uninfected SMs. Lymphocytes were isolated as previously described[42]. Animals were housed and cared in accordance with American Association for Accreditation of Laboratory Animal Care standards in AAALAC accredited

facilities, and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (RMs), or of Emory University (SMs).

Flow Cytometry

Multicolor flow cytometric analysis was performed on samples stimulated and stained as previously described[42] using cross-reactive anti-human Abs: CD3 (clone SP34-2, BDPharmigen), CD8 (RPA-T8, BDPharmigen), CD8 (3B5, Invitrogen), CD4 (OKT4, eBioscience), Ki67 (B26, BDPharmigen), HLA-DR (L243, BDPharmigen), CD45 (MB46D6, Miltenyi), CD14 (M5E2, BDPharmigen or TUK4, Invitrogen), CD20 (2H7, eBioscience), IFNγ (4S.B3, BDPharmigen), IL-17 (eBio64CAP17, eBioscience), CD103 (B-Ly7, eBioscience), IL-22 (IL22JOP, eBioscience), CD123 (6H6, eBioscience), CD11c (3.9, eBiocience) and Aqua Live/Dead amine dye (Invitrogen). Flow cytometric acquisition was performed on a BD LSRFortessa driven by the FACS DiVa software (v6.0; BD). Analysis of the acquired data was performed using FlowJo software (v9.0.2; TreeStar). We used a minimum threshold of 200 collected events for all analysis. We performed Mann-Whitney *U* test for all t-test *P* values, horizontal bars in figures reflect median. Spearman rank calculation and linear regression were calculated for comparative correlations, performed using Prism 5.0 software (Prism).

Immunohistochemistry and Quantative Image Analysis

Immunohistochemical staining was performed as previously described[1,42] on specimens of tissues fixed in Prefer fixative (Anatech) or neutral buffered 4% paraformaldahyde (Electron Microscopy Sciences). Primary antibodies used were polyclonal rabbit anti-Claudin-3 (Labvision), polyclonal rabbit anti-IDO (Millipore) and monoclonal human anti-IL-17 (clone eBio64CAP17, eBioscience). We performed Mann-Whitney *U* test for all t-test *P* values, horizontal bars in figures reflect median. Spearman rank calculation and linear regression were calculated for comparative correlations, performed using Prism 5.0 software (Prism).

DC Co-Cultures

Sorted naïve (live, singlet, CD3+CD28+CD95-CCR7+CD27+) CD4+ T cells were co-cultured with sorted CD103+ DCs (live, lineage-CD14-HLA-DR+CD11c+) (100:1), sorted CD103− DCs (lineage-CD14-HLA-DR+CD11c+CD103-) (100:1), or anti-CD3/anti-CD28 beads (control, 4:1) in X-Vivo15 media (Lonza) under Th17 conditions (10 U/mL recombinant human IL-2, 12.5 ng/mL rhIL-1β, 25 ng/mL rhIL-21, 25 ng/ml rhIL-23, 10 μg/mL anti-IFNγ, 10 μg/mL anti-IL-12 and 2 ng/mL TGF-β, with or without 25 ng/mL rhIL-6). All cytokines were from (R&D), except IL-2, IL-6 and IL-21 (Peprotech). DC co-cultures were stimulated with SEB (1 μg/mL, Sigma Aldrich). All cultures were fed on day 3 with rhIL-2 (2 U/mL), anti-IFNγ (10 μg/mL) and anti-IL-12 (10 μg/mL). After 7 days of co-culture, qRT-PCR was performed for *IL17A* and *RORc* gene expression (Applied Biosystems). Gene expression was quantified using ΔΔCT analysis in excel (version 12.3.0).

Microarray analysis

For microarray analysis, 4 subsets of live cells were sorted from SIV- RM MLNs: CD45+ IL-17+ lymphocytes, as measured using an IL-17 secretion/enrichment and detection assay (Miltenyi) after 4 hours of PMA and ionomycin stimulation, and CD45+ lymphocytes that were IL-17−, as well as live, lineage-, HLA-DR+ CD103+ DCs, and CD103-CD11c+ DCs. Eleven RNA samples (four groups: CD103+ CD11c− DCs, CD103-CD11c+ DCs, IL17+ MLNs lymphocytes, IL17-MLNs lymphocytes) were hybridized to the Illumina HumanHT-12 version 4 Expression BeadChip according to the manufacturer's instructions, and quantified using an Illumina iScan System. The data were collected with Illumina GenomeStudio software, normalized with the quantile normalization method of the Bioconductor package limma[57], and then log transformed. Missing values were imputed with R package impute (http://cran.r-project.org/web/packages/impute/index.html). Two contrasts were analyzed from MLNs of SIV-uninfected animals; CD103+CD11c− DCs versus CD103-CD11c+ DCs, and IL17+ versus IL17− lymphocytes. Genes with significant differential expression levels were identified using Bioconductor limma package with > 1.5 fold change (up or down), and the raw *P* value <0.05. *P* values are corrected for multiple comparisons. We used the R package Limma (http://bioconductor.org/packages/release/bioc/html/limma.html) to estimate the FDR adjusted *P* values, and since a parametric t test is inappropriate for our small sample size with normal distribution, we used the linear model with empirical Bayes method[57]; these analyses are stable even for experiments with small number of arrays (http://www.statsci.org/smyth/pubs/limma-biocbook-reprint.pdf, page 397).

Ingenuity pathway analysis

All network analysis was done with Ingenuity Pathway Analysis (IPA: Ingenuity Systems, Redwood City, CA). The input data includes genes whose expression levels meet the following criteria: fold change >= 1.5 (up or down) and raw P-value < 0.05. Genes were mapped to the Ingenuity pathway knowledge base with different colors (red: up-regulated; green: down-regulated) based on Entreze Gene IDs. The canonical pathways were ranked with −log p values. The network for a few selected genes, including IL17F, IL22, IRF3, was further explored based on the Ingenuity knowledge base.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to acknowledge Heather Cronise-Santis, JoAnne Swerczek, Richard Herbert, and all the veterinary staff at the NIH animal center. We would like to thank Tracy Meeker and Stephanie Ehnert and the veterinary staff at YNPRC. We would like to thank the Cleveland Immunopathogenesis Consortium (BBC/CLIC) for advice and helpful discussions. We would like to thank Mark Cameron and Peter Wilkinson from the Genomics core at VGTI-FL for gene array consultation. Histology support was provided by the Pathology/Histotechnology Laboratory (PHL) core service located at the National Cancer Institute-Frederick, Frederick MD. These studies were supported by the Intramural National Institute of Allergy and Infectious Diseases, US National Institutes of Health program and in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E, and National Institutes of Health R01 AI-084836 under M. Paiardini. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S.

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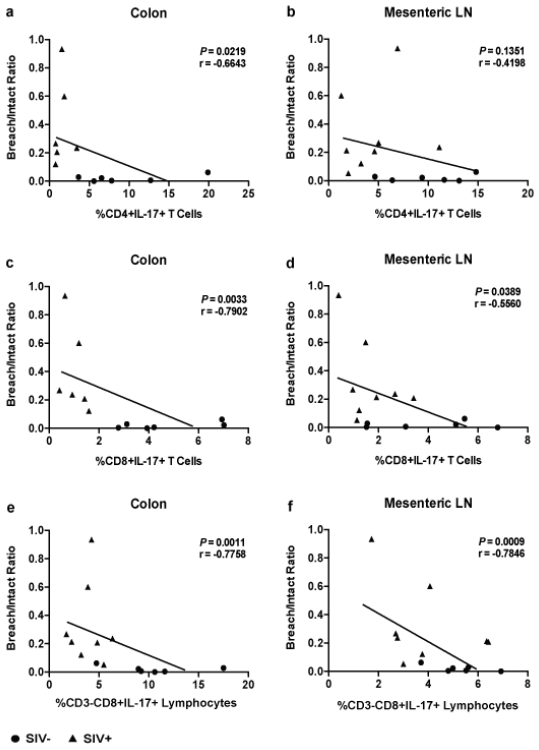

Figure 1. Loss of IL-17-producing lymphocytes correlates with damage to the tight epithelial barrier of the colon

The frequency of IL-17-producing CD4+ T cells in (**a**) colon, and (**b**) MLNs, IL-17-producing CD8+ T cells in (**c**) colon, and (**d**) MLNs, and IL-17-producing CD3-CD8+ lymphocytes in (**e**) colon and (**f**) MLNs were compared to the extent of damage to the structural barrier of the colonic epithelium as measured by the breach (no claudin) to intact (claudin) ratio. Circles, SIV-; Triangles, SIV+. Diagonal lines represent linear regression.

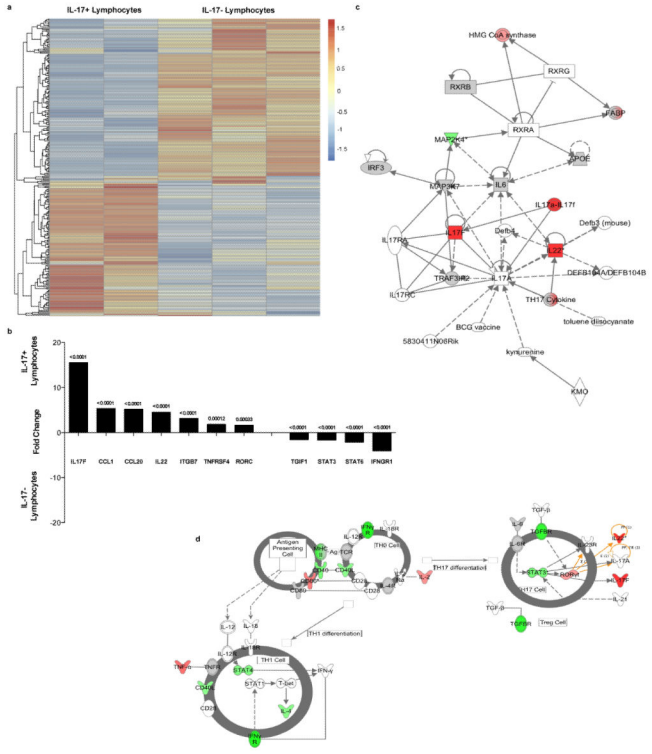

Figure 2. IL-17-producing lymphocytes upregulate genes essential for mucosal homeostasis

(**a**) The top 200 genes based on *P* value for the greatest contrast between IL17+ and IL17− lymphocytes from MLNs of SIV-uninfected RM. The complete linkage method was used for hierarchical cluster analysis. Both rows and columns are clustered. Each row represents normalized expression value for a single gene, and each column represents a sample (data are based upon RNA extractions from IL17+ lymphocytes from 2 SIV-uninfected RM, left, and IL17− lymphocytes from 3 SIV-uninfected RM, right). (**b**) Fold change of selected genes that were up-regulated by IL-17+ lymphocytes (top) or IL-17− lymphocytes (bottom). *P* values donated above each gene fold change. (**c-d**) The network (**c**) and pathway (**d**) from IPA analysis for selected genes is shown. Red indicates up-regulation (fold change >= 1.5); green indicates down-regulation (fold change <= −1.5); grey indicates genes whose expression values are between −1.5 and 1.5. (**c**) Shape: triangle represents kinase; square represents cytokine; rectangle represents ligand-dependent nuclear receptor; diamond represents enzyme; trapezoid represents transporter; ellipse represents transcription regulator; circle represents others.

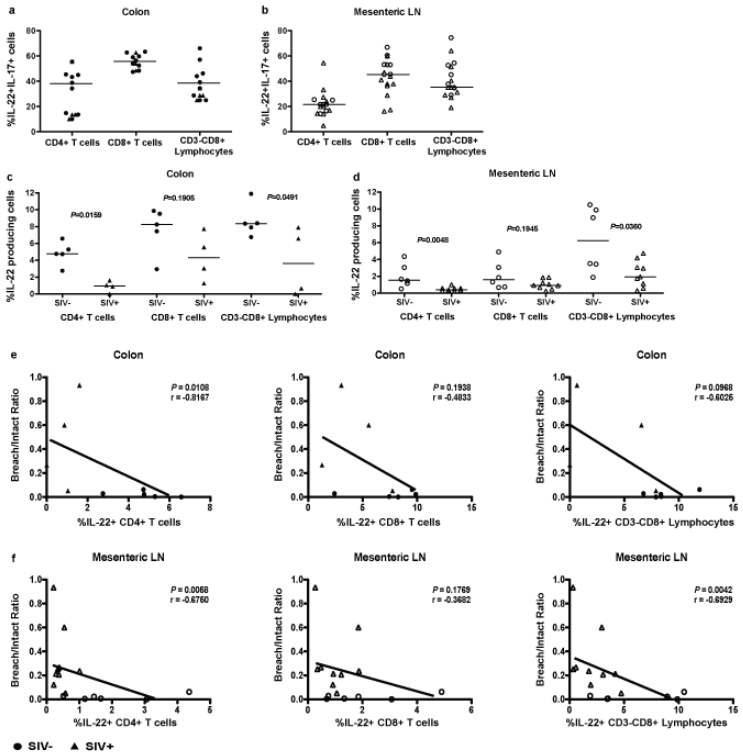

Figure 3. IL-17-producing lymphocytes produce IL-22, and IL-22-producing lymphocytes are lost after SIV infection and associated with mucosal barrier integrity

(**a**-**b**) The frequency of IL-22+ cells within IL-17-producing CD4+ T cells (left), CD8+ T cells (center) and CD3-CD8+ lymphocytes (right) in (**a**) colon, and (**b**) MLNs. (**c**-**d**) The frequency of IL-22-producing CD4+ T cells (left), CD8+ T cells (center) and CD3-CD8+ lymphocytes (right) in uninfected (circles) or SIV-infected (triangles) RMs in (**c**) colon and (**d**) MLNs. (**e**-**f**) The frequency of IL-22-producing CD4+ T cells (left), CD8+ T cells (center) and CD3-CD8+ lymphocytes (right) in (**e**) colon and (**f**) MLNs were compared to damage to the structural barrier of the colon as measured by the breach (no claudin) to intact (claudin) ratio. Closed circles, SIV− colon; closed triangles, SIV+ colon; open circles, SIV− MLNs; open triangles, SIV+ MLNs. Horizontal bars represent median, diagonal lines represent linear regression.

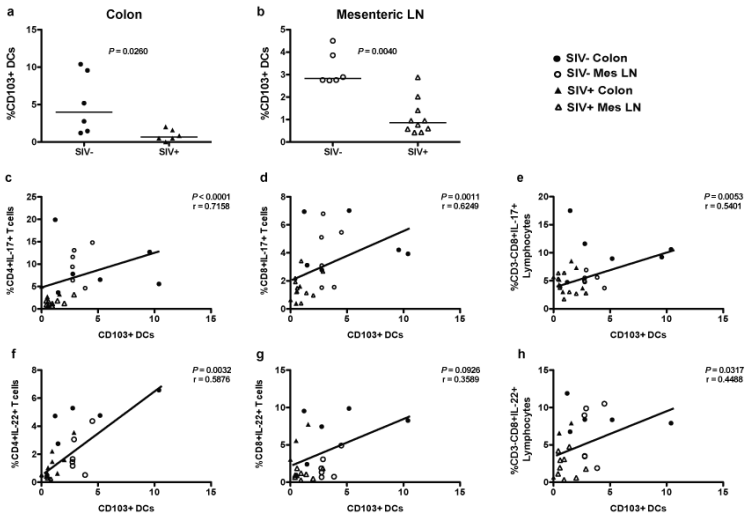

Figure 4. CD103+ DCs are lost after SIV infection, and associated with the frequency of IL-17 and IL-22-producing lymphocytes

(**a-b**) The frequency of live, lineage-, HLA-DR+ CD103+ DCs in the colon (**a**) or MLNs (**b**). (**c-e**) The frequency of CD103+ DCs is compared to the frequency of IL-17-producing CD4+ T cells (**c**), CD8+ T cells (**d**) or CD3-CD8+ lymphocytes (**e**). The frequency of CD103+ DCs is compared to the frequency of IL-22-producing CD4+ T cells (**c**), CD8+ T cells (**d**) or CD3-CD8+ lymphocytes (**e**). Closed circles, SIV− colon; closed triangles, SIV+ colon; open circles, SIV− MLNs; open triangles, SIV+ MLNs. Horizontal bars represent median, diagonal lines represent linear regression.

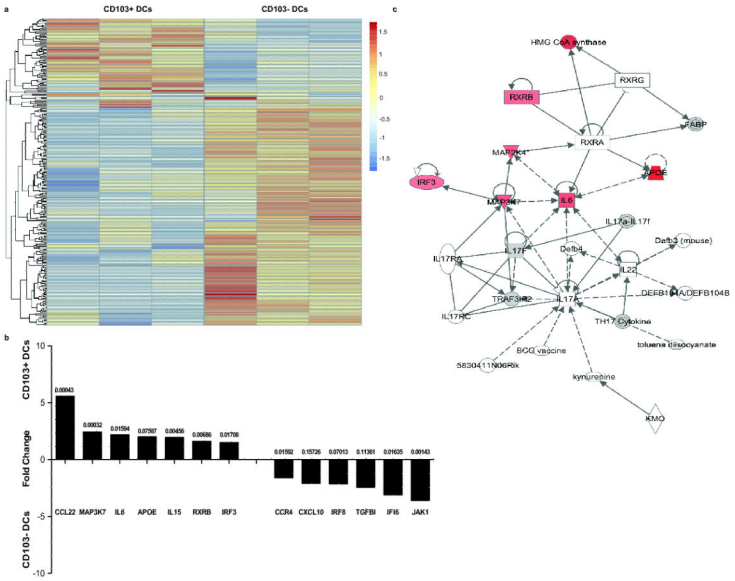

Figure 5. CD103+ DCs upregulate IL-17 promoting genes

(**a**) The top 200 genes based on *P* value for the contrast between CD103+CD11c DCs and CD103-CD11c+ DCs from MLNs of SIV-uninfected RM. The complete linkage method was used for hierarchical cluster analysis. Both rows and columns are clustered. Each row represents normalized expression value for a single gene, and each column represents a sample (data are from MLS of 3 SIV-uninfected animals and CD103+CD11c− DCs are left and CD103-CD11c+ DCs are right). (**b**) Fold change of selected genes that were upregulated in CD103+ DCs (top) or CD103-CD11c+ DCs (bottom). *P* values donated above each gene fold change. (**c**) The network from IPA analysis for selected genes is shown. Color: red indicates up-regulation (fold change >= 1.5); green indicates down-regulation (fold change <= −1.5); grey indicates the genes whose expression values are between −1.5 and 1.5. Shape: triangle represents kinase; square represents cytokine; rectangle represents ligand-dependent nuclear receptor; diamond represents enzyme; trapezoid represents transporter; ellipse represents transcription regulator; circle represents others.

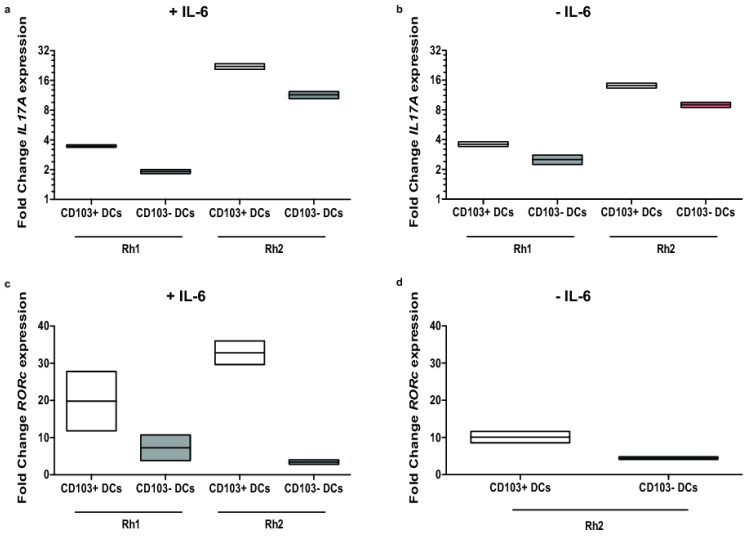

Figure 6. CD103+ DCs directly promote *IL17A* and *RORc* expression by naïve CD4+ T cells

Naïve CD4+ T cells were co-cultured under Th17 conditions with stimulatory anti-CD3 beads alone (control), with CD103+ DCs, or with CD103− DCs in the presence of IL-6 (**a, c**) or absence of IL-6 (**b, d**). Gene expression for *IL17A* (**a-b**) and *RORc* (**c-d**) was measured by ΔΔCt compared to controls. Cells were sorted from live, SIV− rhesus macaque mesenteric lymph nodes. Rh1 and Rh2 designate the two rhesus macaques used for this experiment.