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CD4 T cell subsets in the Mucosa are CD28⁺Ki-67⁺HLA-DR⁺CD69⁺ but show differential infection based on $\alpha 4\beta 7$ receptor expression during acute SIV infection

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Abstract

Background—CD4 T cell depletion in the mucosa has been well documented during acute HIV and SIV infections. The demonstration the HIV/SIV can use the $\alpha 4\beta 7$ receptor for viral entry suggests that these viruses selectively target CD4 T cells in the mucosa that express high levels of $\alpha 4\beta 7$ receptor.

Methods—Mucosal samples obtained from SIV infected rhesus macaques during the early phase of infection were used for immunophenotypic analysis. CD4 T cell subsets were sorted based on the expression of $\beta 7$ and CD95 to quantify the level of SIV infection in different subsets of CD4 T cells. Changes in IL-17, IL-21, IL-23 and TGF β mRNA expression was determined using Taqman PCR.

Results—CD4 T cells in the mucosa were found to harbor two major population of cells; ~25% of CD4 T cells expressed the $\alpha 4^+\beta 7^{\text{hi}}$ phenotype, whereas the rest of the 75% expressed an $\alpha 4^+\beta 7^{\text{int}}$ phenotype. Both the subsets were predominantly CD28⁺Ki-67⁺HLA-DR⁺ but CD69⁺, and expressed detectable levels of CCR5 on their surface. Interestingly, however, $\alpha 4^+\beta 7^{\text{hi}}$ CD4 T cells were found to harbor more SIV than the $\alpha 4^+\beta 7^{\text{int}}$ subsets at day 10 pi. Early infection was associated with a dramatic increase in the expression of IL-17, and IL-17 promoting cytokines IL-21, IL-23, and TGF β that stayed high even after the loss of mucosal CD4 T cells.

Conclusions—Our results suggest that the differential expression of the $\alpha 4\beta 7$ receptor contributes to the differences in the extent of infection in CD4 T cell subsets in the mucosa. Early infection associated dysregulation of the IL-17 network in mucosal tissues involves other non-Th-17 cells that likely contributes to the pro-inflammatory environment in the mucosa during acute stages of SIV infection.

Keywords

HIV; SIV; simian; acute; immunodeficiency; Mucosa; CD4; Gut; Intestine; IL-17; IL-21; IL-23; TGF β

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Introduction

Mucosal tissues are a major site for viral entry, replication and dissemination. Both Human immunodeficiency virus (HIV) and Simian immunodeficiency virus (SIV) infections are associated with a significant loss of CD4 T cells in the mucosa(3,10,14,17,20,24-26,30). This massive loss occurs within the first 2-3 weeks after infection.

The recent demonstration that HIV can use high levels of the $\alpha 4\beta 7$ receptor to infect CD4 T cells suggests that mucosal CD4 T cells likely serve as preferential targets for viral infection (1). Lending support to this argument is the recent observation that mucosal homing CD4 T cells in peripheral blood that express high levels of the $\alpha 4\beta 7$ receptor were preferentially infected and depleted during acute SIV infection when compared to the non-mucosal homing CD4 T cell subsets(15).

Though most mucosal CD4 T cells express the $\alpha 4\beta 7$ receptor, it is not known if the $\alpha 4\beta 7$ receptor is differentially expressed on mucosal CD4 T cells, and if this variable expression contributes to the differences in susceptibility to viral infection. We hypothesized that, if, $\alpha 4\beta 7$ was differentially expressed, then mucosal CD4 T cells that express high levels of $\alpha 4\beta 7$, like their peripheral blood counterparts(15), may harbor more SIV than mucosal CD4 T cells that express lower levels of the $\alpha 4\beta 7$ receptor. We used the SIV infection model to address this question.

The overall objectives of these study was to determine if the expression level of $\alpha 4\beta 7$ receptor varied on mucosal CD4 T cells, and if this variation in expression was associated with differential infection in these subsets. Further, as the loss of mucosal CD4 T cells during acute viral infection has been associated with a dysregulation of the IL-17 network(2,4,9,15,22,29), we evaluated the expression of IL-17, IL-21, IL-23 and TGF β mRNA prior to, and after the acute depletion of mucosal CD4 T cells. Our results demonstrated that mucosal CD4 T cells harbor two major subsets of cells, with ~25% expressing the $\alpha 4^+\beta 7^{\text{hi}}$ phenotype, and the rest expressing an $\alpha 4^+\beta 7^{\text{int}}$ phenotype. $\alpha 4^+\beta 7^{\text{hi}}$ CD4 T cells were found to harbor higher levels of SIV as compared to $\alpha 4^+\beta 7^{\text{int}}$ CD4 T cells. Expression of IL-17 increased prior to the loss of mucosal CD4 T cells, and stayed high even after the depletion of CD4 T cells suggesting a role for non-Th-17 cells in the dysregulation of IL-17 network. Increased IL-17 expression was accompanied by an increase in the expression levels of IL-17 promoting cytokines, namely IL-21, IL-23, and TGF β .

Materials and Methods

Animals, infection and tissue samples

Archival samples collected from SIV infected Rhesus macaques (*Macaca mulatta*) were used in this study. Animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines and were sero-negative for SIV, simian retrovirus and simian T-cell leukemia virus type-1. All the animals were infected with 100 animal-infectious doses of uncloned pathogenic SIVmac251 intravenously. Jejunal samples were obtained at necropsy from uninfected, and infected animals during the first 2-3 weeks after infection. After processing of samples by enzymatic digestion with collagenase (Sigma-Aldrich, Inc. St. Louis, MO), cells were isolated using a percoll gradient as previously described(24) and cryopreserved for later use.

Antibodies and flow cytometry

All antibodies used in this study were obtained from BD Biosciences (San Diego, CA), and titrated using rhesus macaque PBMC. For phenotypic analysis cryopreserved cells were labeled with CD3-Cy7APC, CD8-Alexa-700 and CD4-APC, $\beta 7$ -Cy5-PE, CCR5-PE, CD28-Cy7-PE

and CD95-FITC. Labeled cells were fixed in 0.5% paraformaldehyde, and analyzed using a Becton Dickinson LSR-II instrument.

qPCR assay for SIV infection—Infection in sorted CD4 T cells subsets was determined using a qPCR assay for SIV *gag* on a Perkin-Elmer ABI 7500 instrument as previously described(7,24) using SIV *gag* primers and probe as described by Lifson et al(21). Cell numbers were quantified simultaneously using rhesus macaque Albumin specific primers and probe as described previously(24).

qPCR assay for IL-17, IL-21, IL-23 and TGF β —Relative expression of IL-17, IL-21, IL-23 and TGF β mRNA were determined in total jejunal cells from uninfected (n = 2) and SIV infected (day 7-10 pi, n = 3; day 14-17 pi, n = 4) animals using the dCt method with previously described primers/probes(12). β 2M(7,24) was used as an endogenous control using a Perkin-Elmer ABI 7500 instrument. Data are shown as fold change relative to uninfected animals.

Data analysis

Flow cytometric data was analyzed using FlowJo version 8.6 (Tree Star, Inc., Ashland, OR). Statistical analysis was performed with GraphPad Prism Version 4.0 software (GraphPad Prism Software, Inc. San Diego, CA).

Results

CD4 T cells in the mucosa can be delineated into $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets

To determine if mucosal CD4 T cells differentially express the $\alpha 4\beta 7$ receptor, we evaluated the expression of $\alpha 4\beta 7$ receptor on CD4 T cells from uninfected animals using flow cytometry. We used an antibody against the $\beta 7$ integrin to identify the $\alpha 4\beta 7$ subsets. We have previously shown that most $\beta 7^+$ CD4 T cells coexpresses the $\alpha 4$ integrin(15), and costains with ACT-1, an antibody that recognizes the $\alpha 4\beta 7$ heterodimeric epitope(19).

Our results showed that, as expected, all mucosal CD4 T cells expressed the $\alpha 4\beta 7$ receptor. However, based on the intensity of $\alpha 4\beta 7$ expression, mucosal CD4 T cells could be delineated into two major subsets, namely $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets (Fig. 1 a). Approximately 25% of the mucosal CD4 T cells expressed high levels of $\alpha 4\beta 7$ ($\alpha 4\beta 7^{\text{hi}}$) receptor, and the remaining 75% were found to express $\alpha 4\beta 7$ ($\alpha 4\beta 7^{\text{int}}$) at intermediate levels (Fig. 1a-b). Both $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets expressed CD95 indicative of their memory phenotype. Previous studies have shown that CD95 $^+$ T cells were memory cells(27).

Following infection, most of the mucosal CD4 T cells were depleted by 2-3 weeks after challenge (Fig. 1c-d) as shown previously, suggesting that both $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets of mucosal CD4 T cells were equally susceptible to loss during the acute phase of viral infection.

To determine if the high levels of $\alpha 4\beta 7$ expression rendered $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells in the mucosa more susceptible to infection, we quantified the SIV loads in sorted subsets of $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells from the mucosa at day 10 pi and compared them to SIV loads in $\alpha 4\beta 7^{\text{int}}$ subsets from the same animals.

Our results demonstrated that $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells harbored higher levels of SIV DNA than the $\alpha 4\beta 7^{\text{int}}$ subsets (Fig. 1e). Due to the small sample size we could not perform statistical analysis. However, in each of the 3 animals examined, $\alpha 4\beta 7^{\text{hi}}$ subsets had consistently higher levels of SIV DNA than $\alpha 4\beta 7^{\text{int}}$ subsets. Interestingly, most of the $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets expressed detectable levels of CCR5 on their surface (Fig. 1f) suggesting that both subsets had sufficient levels of CCR5 to be infected by SIV. More than 80% of both the

$\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets expressed CD28 (Fig. 1g) indicating that most of the mucosal CD4 T cells had a central memory phenotype. Previous studies have shown that rhesus macaque central memory T cells were CD95⁺CD28⁺(27).

Next we evaluated if the higher level of infection in $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells was due to the differences in the state of activation of $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets. To address this question, we determined the expression of Ki-67, HLA-DR, and CD69 on both these subsets in uninfected and infected animals (Fig. 2a). Our results demonstrated that few $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets expressed either Ki-67 or HLA-DR prior to or after infection. On the other hand, both the $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ subsets were found to express CD69 suggesting that both subsets had an activated phenotype.

IL-17, IL-21, IL-23 and TGF β levels are upregulated very early during SIV infection

Previous studies have shown that Th-17 cells in the mucosa of SIV and HIV infected subjects were rapidly destroyed(2,4,9,15,22,29). To determine if early viral replication was associated with changes in the endogenous expression of IL-17 in the mucosa, we evaluated the levels of IL-17 mRNA in total mucosal cells prior to (day 7-10 pi), and after the depletion (day 13-17 pi) of CD4 T cells (Fig. 2b). We found an ~15 fold increase in IL-17 mRNA levels in the mucosa very early during infection prior to the depletion of CD4 T cells. Interestingly, the level of IL-17 mRNA expression increased ~35 fold following depletion of CD4 T cells by 2 weeks pi. However, this increase did not approach statistical significance due to the variation between animals.

Next we evaluated if the increased expression of IL-17 mRNA was due to alterations in cytokines that have been shown to promote the production of IL-17, namely, IL-21, IL-23 and TGF β . Our results demonstrated that the increased level of IL-17 mRNA expression was accompanied by a ~10 – 25 fold increase in the expression of IL-21, IL-23 and TGF β genes suggesting that these cytokines likely play a role in upregulating the expression of IL-17 in mucosal tissues..

Discussion

Recent studies have shown that HIV/SIV can use high levels of the $\alpha 4\beta 7$ receptor to infect CD4 T cells in the mucosa suggesting that mucosal CD4 T cells are selectively targeted by HIV/SIV upon viral entry. Interestingly, not all mucosal CD4 T cells expressed high levels of the $\alpha 4\beta 7$ receptor; a minor population of mucosal CD4 T cells was $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells, whereas a majority of them expressed intermediate levels of $\alpha 4\beta 7$ receptor suggesting that $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells were likely the early targets for viral infection. In fact, we found higher levels of SIV DNA in $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells at peak viral infection as compared to the $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets.

Most of the mucosal CD4 T cells expressed CD28, indicating that they were central memory T cells. Previous studies have shown that central memory CD4 T cells were infected during acute SIV infection(24). However, unlike previous reports by Li et al(20), nearly all $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4 T cells in the mucosa were CD69⁺ suggesting that they were activated T cells. The absence of any major difference in either the phenotypic or activated nature between the two subsets would suggest that the high level of $\alpha 4\beta 7$ receptor expression likely plays a role in the increased infection of $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells in the mucosa. Lending support to this argument is our recent observation that $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells in peripheral blood that displayed a predominantly central memory (CD45RA⁻CD28⁺CCR7⁺) and resting (Ki-67⁻HLA-DR⁻CD69⁻CD25⁻) phenotype were preferentially infected very early during the course of infection(15). It is difficult to determine the exact reasons for the differences in CD69 expression observed in our study, and the lack of CD69 expression by SIV infected CD4 T

cells reported by Li et al. It is possible that the different techniques used to obtain tissues/cells may have contributed to these apparent differences; Li et al(20) used in situ hybridization along immunohistochemistry to identify SIV infected and CD69+CD4+ T cells, whereas we used purified cells from tissues that were subjected to rigorous enzymatic digestion followed by percoll gradient centrifugation.

Interestingly, early viral infection was associated with ~15 fold increase in the endogenous expression of IL-17 in the mucosa. This increase was observed prior to the loss of mucosal CD4 T cells. Studies(2,4,9,15,22,29) have shown that mucosal CD4 T cells were a primary source of Th-17 cells, and it is likely that these cells significantly upregulate the expression of IL-17 in response to viral infection. We have previously shown that mucosal homing CD4 T cells harbored most Th-17 cells as compared to non-mucosal CD4 T cell subsets(15).

The loss of mucosal CD4 T cells by 2 weeks pi was accompanied by an increase in the expression of IL-17 in the mucosa suggesting that non-Th-17 cells, most likely CD8 T cells, were upregulating the expression of IL-17 in the mucosa following the depletion of CD4 T cells in the mucosa. Previous studies(13) have shown that loss of Th-17 cells was accompanied by an increase in IL-17 production by mucosal homing CD8 T cells. Others have shown that CD8⁺ T cells were capable of producing IL-17(11).

IL-17 is a pro-inflammatory cytokine that has been shown to play a role in the recruitment of neutrophils to mucosal tissues. It is likely that the high levels of mucosal inflammation observed during HIV infection is associated with the dysregulation of the IL-17 network in mucosal tissues.

High level of IL-17 expression was associated with an increase in the expression of IL-17 promoting cytokines, namely IL-21, IL-23 and TGF β , suggesting that the dysregulation of the mucosal IL-17 network is likely aided by these cytokines. Though TGF β is an anti-inflammatory cytokine, and plays a critical role in maintaining homeostasis in the normal mucosa, increased levels of TGF β following infection likely contributes to the pro-inflammatory environment in the mucosa by promoting IL-17 production by non-Th-17 cells such as CD8 T cells. TGF β is produced by a variety of cells(18), and increased levels of TGF β production has been reported in acute SIV infected rhesus macaques(6). Kekow et al (16) showed that TGF β is upregulated in plasma during acute HIV infection, and that it remains elevated throughout chronic infection. Likewise, Estes et al(8) reported high levels of TGF β in mucosal tissues very early during SIV infection. Despite elevated expression of this anti-inflammatory cytokine, high levels of immune activation, commonly seen in HIV and SIV infection, still persisted(28).

Likewise, other studies have shown that the dysregulated IL-23/IL-17 axis plays a role in chronic intestinal inflammation(23). Production of IL-23 by antigen presenting cells, including dendritic cells and macrophages, is not required for initial differentiation of Th-17 cells, but is believed to be necessary for terminal differentiation and maintenance of the Th-17 phenotype. Other studies have shown that IL-23 drives pathogenic IL-17 producing CD8 T cells.(5)

Taken together, our data suggests that mucosal CD4 T cells that express high levels of the α 4 β 7 receptor harbor greater levels of viral DNA than other subsets. The loss of these cells along with other mucosal CD4 T cells is associated with a significant dysregulation of the IL-17 network and IL-17 promoting cytokines that likely contributes to the pro-inflammatory environment in the mucosa of SIV infected rhesus macaques.

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M.K and S.B performed the experiments and helped in the analysis and interpretation of data, and preparation of the manuscript. M.R and R.V helped provided some of the samples. J.J M designed, helped with preparation of the manuscript, and supervised the study.

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Figure 1. Mucosal tissues harbor $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets

(a) Representative dotplots showing that two major subsets can be delineated in the mucosa based on $\alpha 4\beta 7$ expression. (b) Proportions of $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4⁺ T cell subsets in the mucosa. (c) Representative dotplots and (d) dynamics of CD4 T cells showing that most mucosal CD4 T cells are destroyed by day 14-17 pi. (e) $\alpha 4\beta 7^{\text{hi}}$ CD4 T cells harbor more SIV DNA as compared to $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets. (f) Most $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets express detectable levels of CCR5 on their surface. (g) Majority of the both $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets are CD28⁺.

Figure 2. Mucosal CD4 T cells are Ki-67⁻HLA-DR⁻CD69⁺

(a) Representative dotplots showing that most of the $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{int}}$ CD4 T cell subsets are Ki-67⁻HLA-DR⁻CD69⁺ prior to and after SIV infection. (b) Relative expression of IL-17, IL-21, IL-23, and TGFβ in total mucosal cells at day 7-10 and 14-17 pi.