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Shifting Dynamics of Intestinal Macrophages During SIV Infection in Adult Rhesus Macaques

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Abstract

The intestinal tract is a primary barrier to invading pathogens and contains immune cells including lymphocytes and macrophages. We previously reported that CD163+CD206- (single-positive; SP) interstitial macrophages of the lung are short-lived and succumb early to SIV infection. Conversely, CD163+CD206+ (double-positive; DP) alveolar macrophages are long-lived, survive after SIV infection, and may contribute to the virus reservoir. This report characterizes analogous populations of macrophages in the intestinal tract of rhesus macaques (*Macaca mulatta*) with SIV/AIDS. By flow cytometry analysis, immunofluorescence staining, and confocal microscopy, CD163+CD206+ DP macrophages predominated in the lamina propria of uninfected animals compared to CD163+CD206- SP macrophages that predominated in the lamina propria in animals with SIV infection that were exhibiting AIDS. In submucosal areas, CD163+CD206+ DP macrophages predominated in both SIV-infected and uninfected macaques. Furthermore, BrdU-labeled CD163+CD206+ DP and CD163+CD206- SP macrophages recently arriving in the colon that are both presumed to be shorter-lived were observed to localize only in the lamina propria. Conversely, longer-lived CD163+CD206+ DP macrophages that retained dextran at least two months after in vivo administration localized exclusively in the submucosa. This suggests that CD163+CD206+ DP intestinal macrophages of the lamina propria were destroyed after SIV

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Author contributions

N.T., C.S., and M.J.K. designed the experiments, N.T. and C.S. conducted the flow cytometry studies, N.T. and X.A. performed confocal microscopy studies and imaging, E.S.D, W.-K.K., and M.J.K. provided access to animals and specimens, all authors participated in discussion about data, interpretation of results, and editing the manuscript, N.T., C.A., E.S.D. and M.J.K. helped write the manuscript.

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Disclosures

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infection and replaced by immature CD163+CD206- SP macrophages while longer-lived CD163+CD206+ DP macrophages remained in the submucosa supporting their potential role as an SIV/HIV tissue reservoir. Moreover, the DP macrophages in the submucosa that differ from lamina propria DP macrophages may be missed from pinch biopsy sampling which may preclude detecting virus reservoirs for monitoring HIV cure.

Introduction

Anti-retroviral therapy (ART) prolongs survival by effectively controlling viral replication to undetectable levels in most HIV-infected patients. With ART interruption, however, virus rapidly rebounds due to incomplete clearance of virus from host cell reservoirs. Yet after long-term ART, individuals often exhibit increasing risk for HIV-associated non-AIDS conditions that may include earlier onset of cardiovascular disease, cognitive decline, metabolic syndrome, renal dysfunction, liver fibrosis, and fragility fractures among others (1, 2). Thus, there is a need to eradicate HIV viral reservoirs in organs that become established during latent or productive HIV/SIV infection so that ART may be safely discontinued to avoid viral rebound and to forestall onset of chronic inflammatory diseases associated with the HIV-associated non-AIDS conditions (3).

Relatively more research has focused on memory CD4+ T cells as major sites of virus replication and reservoirs during acute HIV/SIV infection as well as after implementation of ART (4). Follicular helper T cells also support continuous productive virus replication in whole blood and lymph nodes, even during ART administration (5, 6). Tissue-resident macrophages likewise are considered targets of HIV/SIV infection, host cells for the latent viral reservoir, and contributors to HIV/SIV pathogenesis (7). We reported previously that increasing monocyte turnover (MTO) in blood is an indicator of disease progression to terminal AIDS in SIV-infected rhesus macaques (8, 9) and correlates with damage in lung tissue, including destruction of virus-infected shorter-lived tissue macrophages (10, 11). Shorter-lived CD163+CD206- SP macrophages in the interstitial lung tissues, i.e. interstitial macrophages (IM), were readily infected and destroyed by SIV, whereas longer-lived CD163+CD206+ DP macrophages of the alveolar spaces, i.e. alveolar macrophages (AM), primarily survived infection and were considered potential sites for the virus reservoir (12).

The intestinal tract, including gut-associated lymphoid tissue (GALT), is a primary site of HIV/SIV infection during the acute phase that is associated with massive depletion of intestinal CD4+ T-cells and a high rate of viral replication. Administration of ART leads to controlled but long-term persistence of virus which rebounds after disruption of ART (13–16). The intestine also houses among the largest population of macrophages (17), and an increased accumulation of intestinal macrophages has been reported in HIV-positive patients (18) and SIV-infected rhesus macaques (19). This raises questions about virus infections targeting the more available macrophages that could also become sites of viral reservoirs. Therefore, goals of this study were to examine macrophages of the intestinal tract that may be analogous to those of the lung, as well as to examine their contributions to disease progression and possible sites of viral reservoirs. Results from characterizing the phenotype, migration, and localization of intestinal macrophages in SIV-infected and uninfected rhesus

macaques as a model for HIV infection in humans demonstrated an accumulation of shorter-lived CD163+CD206- SP macrophages that replaced infected CD163+CD206+ DP macrophages in the lamina propria of SIV-infected macaques during disease progression. Results also revealed the existence of longer-lived CD163+CD206+ DP macrophages in the intestinal submucosa that may be contribute to an SIV reservoir.

Materials and Methods

Animals and SIV inoculations

A total of 34 adult Indian rhesus macaques (*Macaca mulatta*) of both sexes (31 males, 3 females shown in Table 1) from the Tulane National Primate Research Center were used in these studies. The animals ranged between the ages of 3.4 and 20.9 yrs old (median age = 6.38 yrs), and were specific pathogen-free for SIV, Type D Simian Retrovirus, and Simian T-cell Leukemia Virus type 1 at the time of assignment. Of these, eight animals served as uninfected controls. The remaining animals were inoculated by intravenous (i.v.) or intravaginal routes with virus strains SIVmac251, SIVmac239, SIVmac239ΔGY, or SIVmac239ΔNef (10, 12). All animal procedures were performed according to the “NIH Guide for the Care and Use of Laboratory Animals” (20) and were approved by the Tulane University Institutional Animal Care and Use Committee.

In vivo macrophage labeling and animal specimen collections

The thymidine analog, 5-bromo-2′-deoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO) was prepared at 30 mg/ml in PBS (pH 7.2, Ca/Mg-free; Mediatech, Inc., Manassas, VA) and filter sterilized prior to i.v. inoculation at 60 mg/kg. In some studies, another thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU; Molecular Probes, Eugene, OR), was prepared at 25 mg/ml in PBS and inoculated i.v. at 50 mg/kg. EDTA-preserved blood specimens were obtained 24 hrs after BrdU/EdU injection for evaluation of MTO rates. Some animals were intravenously injected with dextran (75 mg/kg of a 50 mg/ml in saline stock suspension; Thermo Fisher Scientific, Waltham, MA) from those administered 75 mg/kg (50 mg/ml in saline) at least 69 days earlier to detect the longer-lived macrophages that retained dextran. Intestinal tissues were obtained at necropsy and 1-4 days after BrdU/EdU injection for flow cytometry analysis and immunofluorescent antibody staining for confocal microscopy imaging.

Isolation of macrophages and lymphocytes from intestinal tissue

Single-cell suspensions were prepared from jejunum and colon tissue by enzymatic digestion. Intestinal-tissue sections were removed of fat, cut into 1-cm^3 pieces, and resuspended in 45 ml of HBSS (Mediatech, Inc.) supplemented with 5% fetal bovine serum (FBS; Thermo Fisher Scientific), 100 IU/ml penicillin/streptomycin (MP Biomedicals, LLC, Santa Ana, CA), 2 mM L-glutamine (MP Biomedicals, LLC), 25 mM HEPES (Thermo Fisher Scientific) and 5 mM EDTA (Millipore Sigma, St. Louis, MO). The suspensions were incubated at 37˚C for 30 min, followed by mincing the tissues into < 1 mm^3 fragments. Tissue fragment suspensions were resuspended in RPMI 1640 (Lonza, Cohasset, MN) supplemented with 5% FBS, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 25 mM HEPES (Thermo Fisher Scientific), 200 U/ml type II collagenase (Worthington

Biochemical) and 0.05 mg/ml DNase I (Roche, Indianapolis, IN). The tissue digest suspensions were then incubated at 37°C for 30 min, followed by pipetting and incubation for an additional 10 min at 37°C. After discontinuous density centrifugation over layers of 24% and 50% Percoll (GE Healthcare, Chicago, IL) at 2000 rpm for 20 min (Allegra X-12R; Beckman Coulter, Brea, CA), the cells were recovered from the 24-50% Percoll interface, washed with PBS containing 2% FBS, and used for flow cytometry analyses or resuspended in Bambanker freezing media (Wako Chemicals USA, Inc., Richmond, VA) for storage in liquid nitrogen until further analyses.

Cells staining and flow cytometry

Antibodies used for cell surface staining of intestinal cells are shown in Supplemental Table 1. BD Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA) and DNase or Click-iT EdU Pacific Blue Flow Cytometry Assay kit (ThermoFisher Scientific) were used to label intracellular BrdU or EdU, respectively. Stained cells were acquired with FACSAria (BD Biosciences) and the results were analyzed by use of FlowJo software (FLOWJO, LLC, Ashland, OR).

Tissue staining and confocal microscopy imaging

Sections from paraffin-embedded colon or jejunum were incubated with antibodies listed in Supplemental Table II. Imaging was performed with a Leica TCS SP8 confocal microscope, equipped with three lasers (Leica Microsystems, Wetzlar, Germany) at 400X magnification. Adobe Photoshop software (Adobe Systems, San Jose, CA) was used to process and assemble the images. Quantification of macrophage subsets was performed by manually counting 20 fields of each slide using Image J software (https://imagej.net/).

Statistical analysis

Comparisons between two groups were measured by non-parametric Mann Whitney test. For more than two groups, one-way ANOVA (Kruskall Wallis) was performed followed by pairwise comparisons using Dunn's post test. Non-parametric Spearman's test was performed for correlation analyses. Data were analyzed and graphed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA; www.graphpad.com). $P < 0.05$ was considered statistically significant.

Results

CD4 T-cell depletion in the intestine did not correlate with MTO rate in SIV-infected rhesus macaques

Since CD4 T cells of the intestine are among the earliest cells targeted by SIV/HIV, much research has focused on CD4 T cell depletion in the intestine as a major mechanism of AIDS pathogenesis. We previously demonstrated a direct correlation between increasing MTO in blood and disease progression based on clinical signs, lung tissue damage, virus levels, and time until death using the SIV/AIDS rhesus macaque model (8). Therefore, we analyzed the shifts in CD4-to-CD8 T-cell ratios in jejunum and colon prior to and during SIV infection, as well as after progression to simian AIDS (SAIDS), and then compared the results in the intestine of SIV-infected animals with lower MTO (< 20% or low MTO) versus intermediate

(20-29.9%)-to-higher (≥ 30 %) MTO using flow cytometry analyses (Fig 1). Indeed, SIV-infected animals showed significantly lower CD4 T-cell-to-CD8 T-cell ratios in jejunum and colon compared to uninfected animals but there were no statistically significant differences in CD4 T-cell to CD8 T-cell ratios between animals with lower versus intermediate-to-higher MTO (Fig. 1). This suggested that while CD4+ T cells may serve as targets of SIV infection in intestinal tissues, their decline relative to CD8 T cells did not predict or indicate SAIDS progression in relation to increased MTO.

Intestinal macrophage populations shift from DP to SP during progression to SAIDS

To investigate the dynamics in intestinal macrophages associated with increasing MTO during SIV infection, we applied flow cytometry to identify the intestinal macrophages expressing CD163 and/or CD206 during different stages of SIV infection using the gating strategy described in Supplemental Fig. 1. We identified two subsets of macrophages that comprised CD163+CD206+ DP macrophages and CD163+CD206- SP macrophages in the intestines (Fig. 2A). In uninfected animals, DP macrophages dominated over the SP macrophages (Fig. 2A). During SIV infection however, the SP macrophages became more frequent among total macrophages (Fig. 2A). In other words, the flow cytometry shown in Figure 2A examined fewer “events” from the jejunum compared to colon and was applied to compare subsets within the total macrophage population. In both jejunum and colon, there also were statistically significant correlations between decreasing DP-to-SP macrophage ratios and increasing MTO rates (Fig. 2B) as also reported in lung (10). This suggested that as SIV infection disease progressed to AIDS, as indicated by increasing MTO, the DP subset of macrophages became depleted and/or SP macrophages increased in the intestinal tissues.

DP-to-SP macrophage ratios declined in the lamina propria but not in the submucosa of the colon during SIV infection

We next applied immunofluorescence staining and confocal microscopy to assess the localization of the macrophage subsets in the lamina propria and submucosa regions of the colon in uninfected and SIV-infected animals. In the lamina propria, DP macrophages predominated over the SP macrophages of uninfected animals (Fig. 3A), but SP macrophages were more commonly observed in the lamina propria of infected animals with higher MTO at 20.3% (Fig. 3B), consistent with flow cytometry results (Fig. 2). Interestingly, in the submucosal region including the muscular mucosa, DP macrophages were observed more frequently than SP macrophages in both infected and uninfected animals (Fig. 3A and B). After counting macrophage subpopulations in tissue sections, we observed a statistically significantly higher mean DP-to-SP macrophage ratio in the lamina propria of uninfected macaques compared to infected animals exhibiting higher MTO (Fig. 3C). In contrast, there was no significant difference in the DP-to-SP macrophage ratios observed in the submucosa of uninfected versus SIV-infected macaques with intermediate-to-higher MTO (Fig. 3C). Similar trends were observed for the DP-to-SP macrophage ratios in the jejunum of uninfected compared to infected animals with higher MTO (data not shown). These results suggested that during terminal stages of SIV infection in animals with higher MTO, there was a loss in DP macrophages with concurrent increases in SP macrophages in the lamina propria, whereas submucosal DP and SP macrophages were retained even after SIV infection and disease progession.

Blood monocytes traffic and differentiate into SP macrophages in the intestine

To assess the distribution of recently-differentiated macrophages from monocytes that appear to be recruited from blood to renew or restore DP intestinal macrophages during homeostasis in uninfected animals or that may account for the increase in SP macrophages after SIV infection, animals were inoculated i.v. with thymidine analogues, BrdU or EdU. Tissues of the intestine then were examined 48 hrs later by flow cytometry. As shown in Fig. 4A, a majority of recently-dividing BrdU- or EdU-labeled cells were CD163+ macrophages in the jejunum and colon of either uninfected or SIV-infected animals. In addition, a greater number of BrdU/EdU-labeled CD163+ macrophages were observed to accumulate in the intestinal tissues of SIV-infected animals than in those of uninfected animals. Furthermore, a statistically significant correlation was observed between increasing percentages of BrdU/EdU-labeled CD163+ macrophages in jejunum with increasing blood MTO rates and this approached significance in the colon (Fig. 4B). This suggested that with progression to simian AIDS, more monocytes migrated from blood to intestinal tissues.

Submucosal DP macrophages in the colon are longer-lived

Since some DP macrophages appeared to localize and remain primarily in the submucosa regardless of SIV infection status, we hypothesized that these may comprise a longer-lived macrophage population. This is relevant because longer-lived rather than shorter-lived macrophages would be more likely to participate in the SIV/HIV reservoir and contribute to pathogenesis. To explore this, we analyzed the distribution of colon macrophages that incorporated and retained the polysaccharide dextran (i.e. to identify longer-lived macrophages) relative to those that were labeled with thymidine analogues, BrdU or EdU, for identifying recently-dividing shorter-lived macrophages. We observed BrdU+CD163+ macrophages only in the lamina propria but not in the submucosa (Fig. 5A). Conversely, two months after dextran injection, dextran+CD163+ macrophages exclusively localized in the submucosa where there were approximately 10 times more DP macrophages than SP macrophages (Fig. 5B), suggesting that the DP macrophages in the submucosa are longer-lived cells. The results also suggested that shorter-lived macrophages migrate from the blood to the lamina propria where they remain for short periods of time whereas longer-lived macrophages remain localized in the submucosa.

Discussion

We previously reported that increased MTO was associated with terminal disease progression to AIDS in SIV-infected rhesus macaques (8). The increasing MTO was accompanied by lung tissue macrophage destruction and terminal disease progression in the rhesus macaque SIV/AIDS model of HIV/AIDS (10–12). In addition, a higher physiological baseline MTO rate in very young rhesus macaques was associated with greater susceptibility and accelerated disease progression observed in pediatric compared to adult HIV/SIV infections (21–23). The purpose of this study was to build on the earlier results and now relate the effects of SIV infection and MTO shifts on macrophages and T cell populations in the intestine of adult macaques. This is important because historically, disease progression has been linked to depletion of CD4 T cells, especially in the intestine (13, 14). We observed previously (10, 12) and in this study that while increased MTO affected changes in intestinal

and lung macrophage subpopulations, there was no difference in the intestinal CD4-to-CD8 T-cell ratios between SIV-infected animals with lower versus intermediate-to-higher MTO rates. This suggested that while there was a lower proportion of CD4 T cells observed in the intestinal tissues from both groups of animals with higher and lower MTO, loss of CD4 T cells alone may not be the primary driver toward terminal disease progression. Since the increasing MTO was associated with interstitial lung macrophage destruction, and the loss of CD4 T cells may redirect virus to infect new host cells such as macrophages, we focused on intestinal macrophages as another possible contributor to disease progression during SIV infection.

Intestinal macrophage populations express various biomarkers such as CD14, CD11c, CD68 and CD206 during homeostasis that shift during injury, infection, or disease. These changes affect cytokine secretion induced by such insults (27). In mice, Ly6C^{Hi} and Ly6C^{Low} monocytes are observed in tissues sites of injury suggesting that classical and non-classical monocytes infiltrate sequentially to produce pro-inflammatory and anti-inflammatory functions (28). Especially in mice, CX3CR1 is known as one of the biomarkers for tissue-resident macrophages not only in intestinal tissues but also some other organs (25, 29). Although, we did not use CX3CR1 to identify resident r longer-lived omacrophages, we did use dextran and BrdU to identify long-lived and short-lived macrophages, respectively. We recently reported that the half-life of monocytes is approximately 1 day (30) and thus expect that the short lived-macrophages that differentiated from circulating monocytes maintain a similar half-life in tissues. Future studies are required to determine if CX3CR1 macrophages are of mice are analogous to the dextran-positive long-lived macrophages in macaques. However, the impact of SIV infection on changes in macrophage subsets in the intestines in relation to disease progression and pathogenesis have only recently been investigated.

From previous studies, we identified two major populations of macrophages in lung tissues. The shorter-lived CD163+CD206- SP interstitial macrophages (IM) were primarily located in the lung parenchyma and were readily infected and destroyed during SIV infection. Loss of these macrophages appeared to contribute to increased trafficking or accumulation of IM into lung tissues and increased MTO to replace and restore these macrophages that was indicative of disease progression. In contrast, the longer-lived CD163+CD206+ DP alveolar macrophages (AM) found mainly in the alveolar spaces could become infected with virus but survived, and thus were considered to possibly serve as a viral reservoir. By flow cytometric analysis of jejunum and colon, we similarly identified two main populations of CD163+CD206+ DP and CD163+CD206- SP macrophages that appeared phenotypically equivalent to AMs and IMs of the lung, respectively. Uninfected animals and SIV-infected animals with lower baseline levels of MTO, also exhibited higher DP to SP macrophage ratios. With increasing MTO after SIV infection, the SP macrophages accumulated in the intestinal tissues and predominated over the DP macrophages, similar to our previous reports on lung tissue macrophages in SIV-infected rhesus macaques (10, 12).

Confocal microscopy enabled further characterization about the localization and shifts in macrophage populations during SIV infections and in relation to MTO rates. In uninfected animals, DP macrophages predominated in the lamina propria of the colon. After SIV infection, SP macrophages predominated suggesting that there occurred a rapid migration of

recently dividing and differentiating blood monocytes into the intestinal tissues. Intestinal macrophages have been considered differentiated resident myeloid cells that do not proliferate or do so at very low rates (24–26). Using in vivo BrdU labeling, however, we demonstrated the presence of recently-dividing CD163+CD206- SP macrophages in jejunum and colon further suggesting these cells were recruited from circulating monocytes to replenish the damaged tissue macrophages. This was also supported by the observation that incorporation of BrdU into CD163+macrophages in jejunum correlated with increasing MTO in association with disease progression.

In contrast, DP macrophages were predominant in the submucosa of the colon in both the SIV-infected and uninfected animals. Similar to what we observed in the lung AM, it appeared that the submucosal DP macrophages were not massively depleted after SIV infection but were retained. This suggests that there exist subpopulations within the CD163+CD206+ DP macrophages since DP macrophages remained longer in the submucosa than in the lamina propria, similar to the AM of the lung after SIV infection. While we detected DP macrophages that underwent cell division within the previous 48 hrs based on incorporation of thymidine analogues in the lamina propria, no thymidine analogue-labeled DP macrophages were detected in the submucosa/muscular mucosa. Conversely, dextran, a polysaccharide that is incorporated and retained in long-lived macrophages, was detected in DP macrophages of the submucosa but not in the lamina propria. Thus it appeared that recently-dividing and differentiating monocytes expressing CD163+CD206- biomarkers traffic to repopulate the damaged DP CD163+CD206+ macrophages in the lamina propria after SIV infection and then may subsequently differentiate from SP to DP phenotype.

Relevant to these studies is an apparent difference in functions between DP CD163+CD206+ macrophages that are shorter-lived in the lamina propria compared to the dextran-retaining longer-lived DP macrophages in the submucosa. This is important since pinch biopsies would be comprised of the shorter-lived macrophages whereas wedge biopsies or more in-depth sampling (e.g. intestinal resection or harvested tissues at necropsy) would be required to recover the longer-lived macrophages. These findings are reminiscent of limited macrophage sampling observed from our earlier studies on lung macrophages whereby bronchoalveolar lavage sampling captured the longer-lived CD163+CD206+ macrophages but failed to retrieve the short-lived CD163+CD206- SP interstitial macrophages. Interestingly in this study, the DP CD163+CD206+ intestinal macrophages exhibiting longer-lived and shorter-lived characteristics (based on dextran retention versus thymidine analogue labeling, respectively) differed principally by location rather than primarily by surface phenotype biomarkers, as reported for lung tissue macrophages (10, 12). Some studies have suggested possible key roles of intestinal macrophages in the pathogenesis of disease, such as contributing to the viral reservoir during HIV/SIV infection (31, 32). Further studies, however, may identify differential surface biomarkers to distinguish among these macrophages, particularly relating to specific roles in disease, and conversely, in immunity. Studies also are needed to better understand the further differentiation of these DP macrophages in relation to their localization and whether these longer-lived macrophages truly serve as an SIV/HIV reservoir.

African green monkeys (AGMs) are known to be the natural host of SIVagm and have been widely used as a non-pathogenic SIV model, in contrast to rhesus macaques that serve as a pathogenic SIV model. While rhesus macaques exhibit increased monocyte turnover during disease progression in association with damage of short-lived tissue macrophages (12), SIV-infected AGMs consistently maintained low baseline levels of monotyte turnover throughout the infection and in the absence of overt disease progression (unpublished data). This is consistent with our working hypothesis that AIDS disease progression is dependent upon tissue macrophage destruction that induces increased monocyte turnover.

In summary this report on intestinal macrophages built upon results from studies about macrophages of the lung in rhesus macaques infected with SIV. These findings demonstrated that; 1) CD163+CD206+ DP macrophages were dominant in the lamina propria of healthy uninfected rhesus macaques; 2) the loss of DP macrophages in lamina propria was followed by accumulation of CD163+CD206- SP macrophages in the intestinal lamina propria of SIV-infected animals exhibiting increased and higher MTO $\geq$ 30%; 3) there occurred a rapid migration of recently-dividing thymidine-analogue-labeled SP macrophages to the lamina propria but not submucosa in SIV-infected animals exhibiting higher MTO and; 4) there exist relatively shorter-lived and longer-lived macrophages in the intestinal tissues. Taken together, these results contribute to a working model shown in Fig. 6 that blood monocytes (CD163+CD206-) migrate to the intestinal lamina propria and differentiate into mature DP macrophages which can be depleted by SIV infection, similar to the loss of short-lived lung IMs (CD163+CD206-). After SIV infection, the depletion of DP macrophages in the lamina propria is followed by accumulation of newly-migrated immature or differentiating SP macrophages in concert with the increasing and higher MTO indicative of rapid terminal disease progression to SAIDS. In contrast, DP macrophages in the submucosa were not depleted during SIV infection and remained for more than two months based on dextran label retention, suggesting that these longer-lived macrophages, like the lung AM (CD163+CD206+) could serve as an SIV tissue reservoir. Thus, this work forms a basis for future studies to define intestinal virus reservoirs that may include longer-lived macrophages as possible target candidates for eradication of HIV/SIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AM	alveolar macrophage
ART	Antiretroviral therapy
BrdU	5-Bromo-2′-deoxyuridine
DP	CD163+CD206+ double positive
EdU	5-ethynyl-2′-deoxyuridine
IM	interstitial macrophage
MTO	monocyte/macrophage turnover
SAIDS	simian AIDS
SP	CD163+CD206- single positive

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Key points

- Lamina propria macrophages are short-lived and destroyed after SIV infection.
- Intestinal submucosa macrophages are long-lived and may become SIV/HIV reservoirs.
- Gut biopsies are insufficient for monitoring submucosal long-lived macrophages.

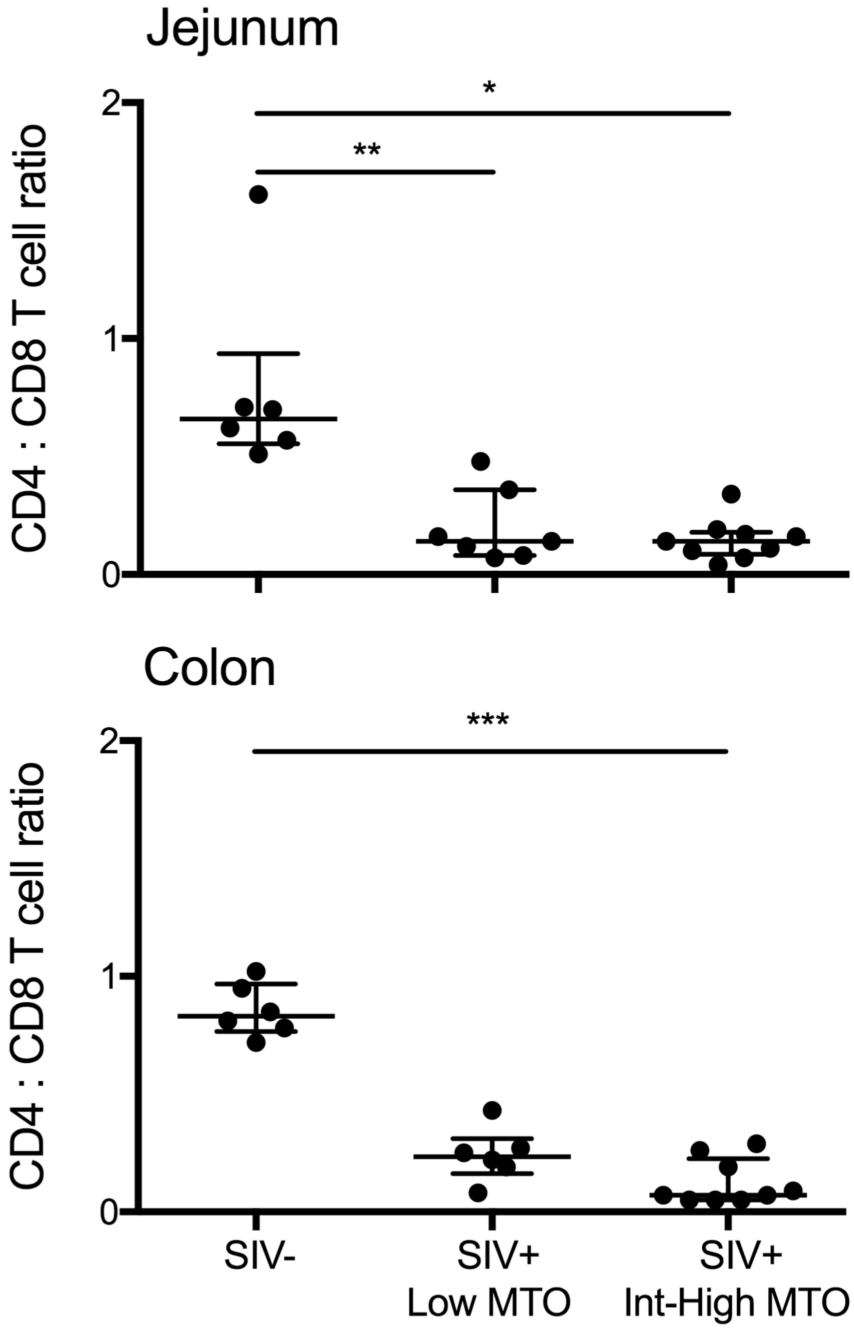

FIG 1.

Reduction of intestinal CD4-to-CD8 T-cell ratio is not associated with increased MTO rate that predicts terminal disease progression to AIDS in SIV-infected rhesus macaques. The ratios of CD4-to-CD8 T cells in jejunum (n = 22) and colon (n =21) representing depletion of CD4 T cells were calculated from flow cytometric data. The SIV-infected animals were divided into groups exhibiting BrdU-labeled MTO rates that were relatively lower (< 20%) or intermediate (Int; 20-29.9%)-to-high (≥ 30%). Analyses were performed by nonparametric one-way ANOVA (jejunum, $P = 0.0015$; colon, $P = 0.001$) and Dunn's post

test for pairwise comparisons. $P < 0.05$ was considered significant. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

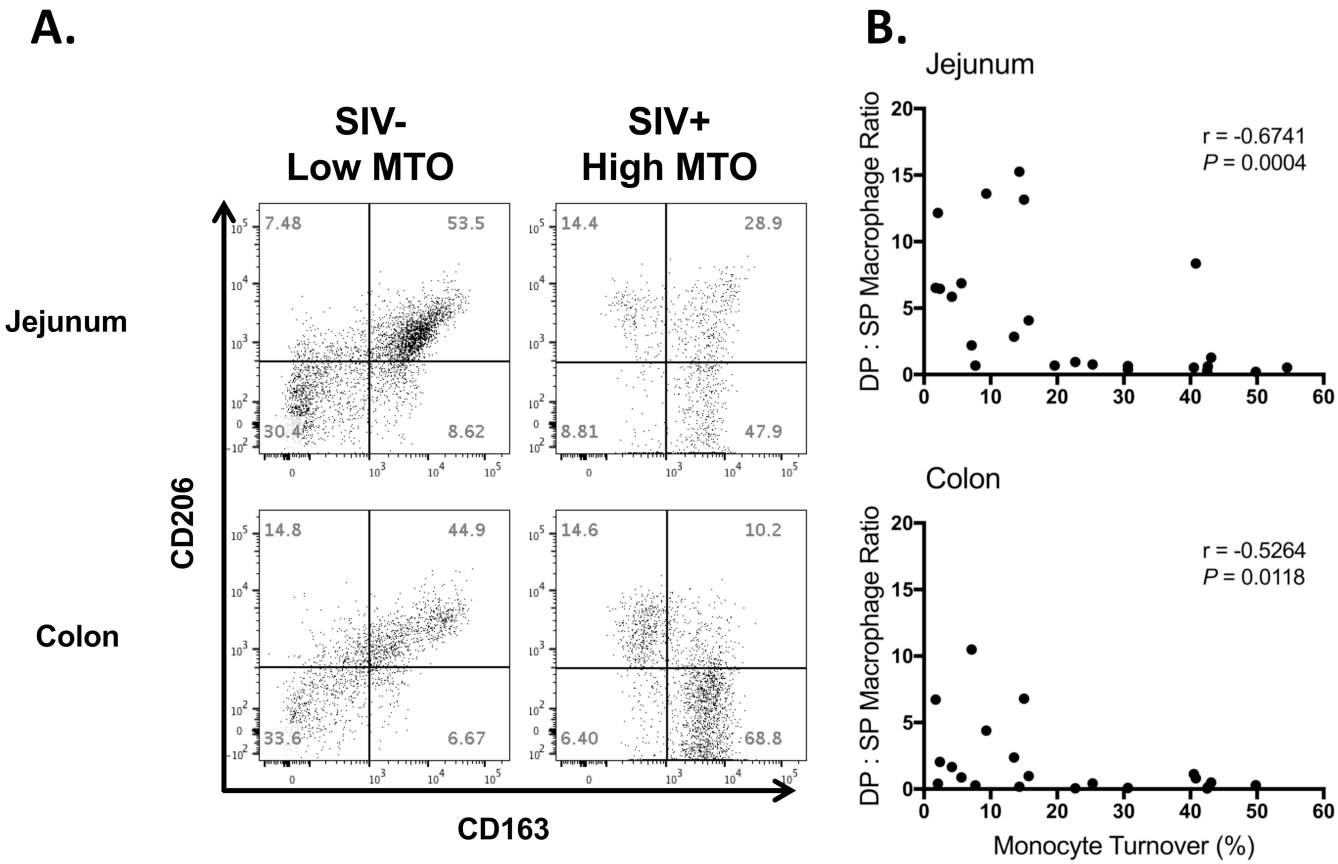

FIG 2.

Identification of macrophage subsets in the intestinal tissues of SIV-infected and uninfected rhesus macaques. (A) Representative flow cytometric analysis of intestinal macrophages expressing CD163 and/or CD206 surface markers are shown for an uninfected macaque (SIV-) that exhibited physiologically normal lower MTO and an SIV-infected macaque (SIV +) with high MTO (≥ 30%). (B) Spearman nonparametric correlation was performed to relate blood MTO rate with DP-to-SP macrophage ratios in jejunum (n = 24) and colon (n = 22).

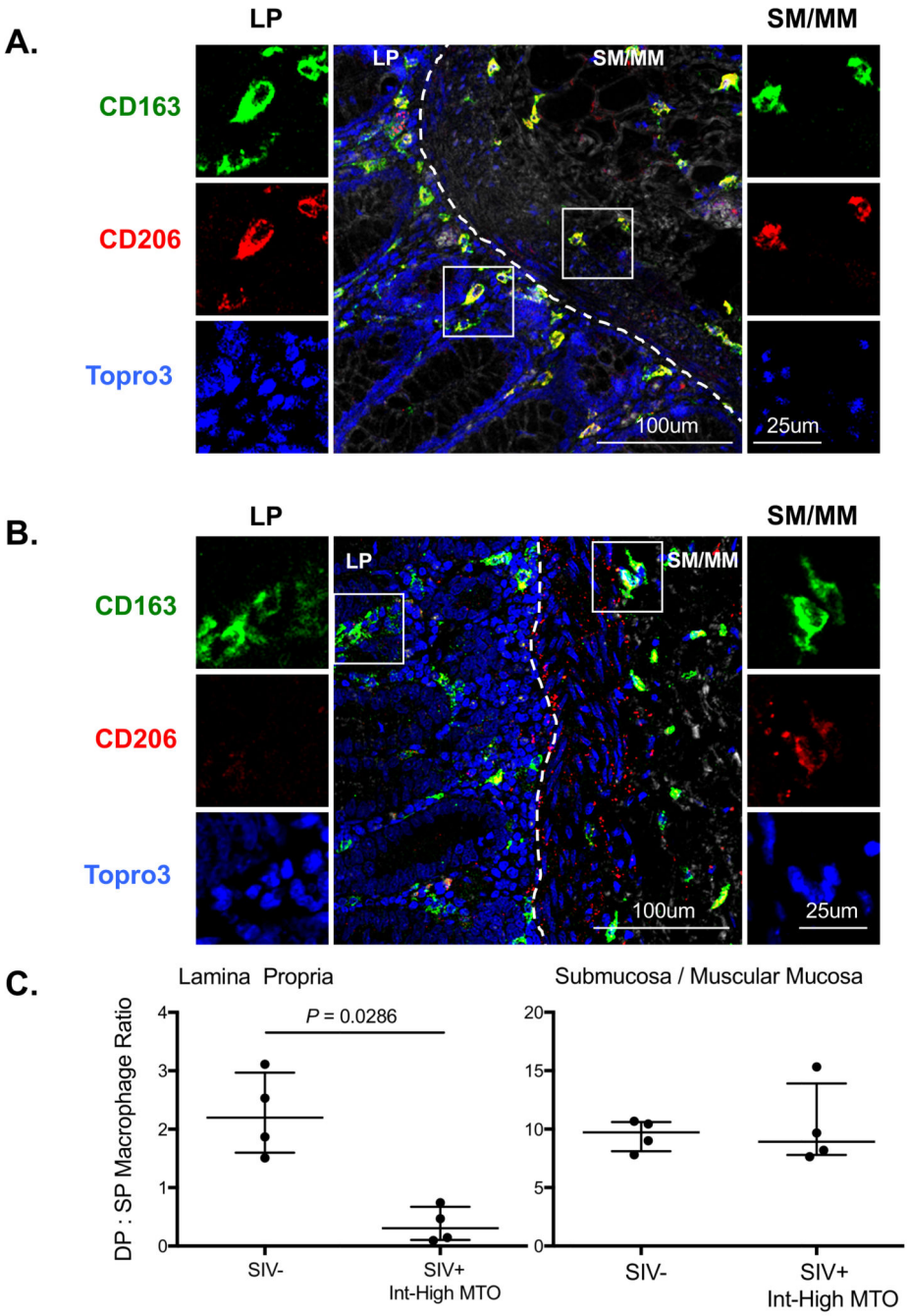

FIG 3.

Localization and phenotypes of macrophages in the colon. Immunostaining and confocal microscopy were used to detect expression of CD163 and CD206 on macrophages, and Topro3 was used to detect cell nuclei in tissue sections of lamina propria (LP) and submucosa/muscular mucosa (SM/MM) of the colon. Representative images are shown of the colon from an uninfected SIV-animal (A) and an SIV-infected animal exhibiting increasing intermediate MTO rate at 20.3 % (B). Images were captured at 400× magnification. Dashed lines indicate the border between lamina propria (left) and

submucosa / muscular mucosa (right). Inserts of LP (left) and SM/MM (right) demonstrate magnified unmerged individual staining characteristics for each biomarker. (C) Comparisons of the DP : SP macrophage ratio in lamina propria and submucosa / muscular mucosa (n = 4 in each group) were measured by nonparametric *t* test (Mann Whitney) and $P < 0.05$ was considered statistically significant.

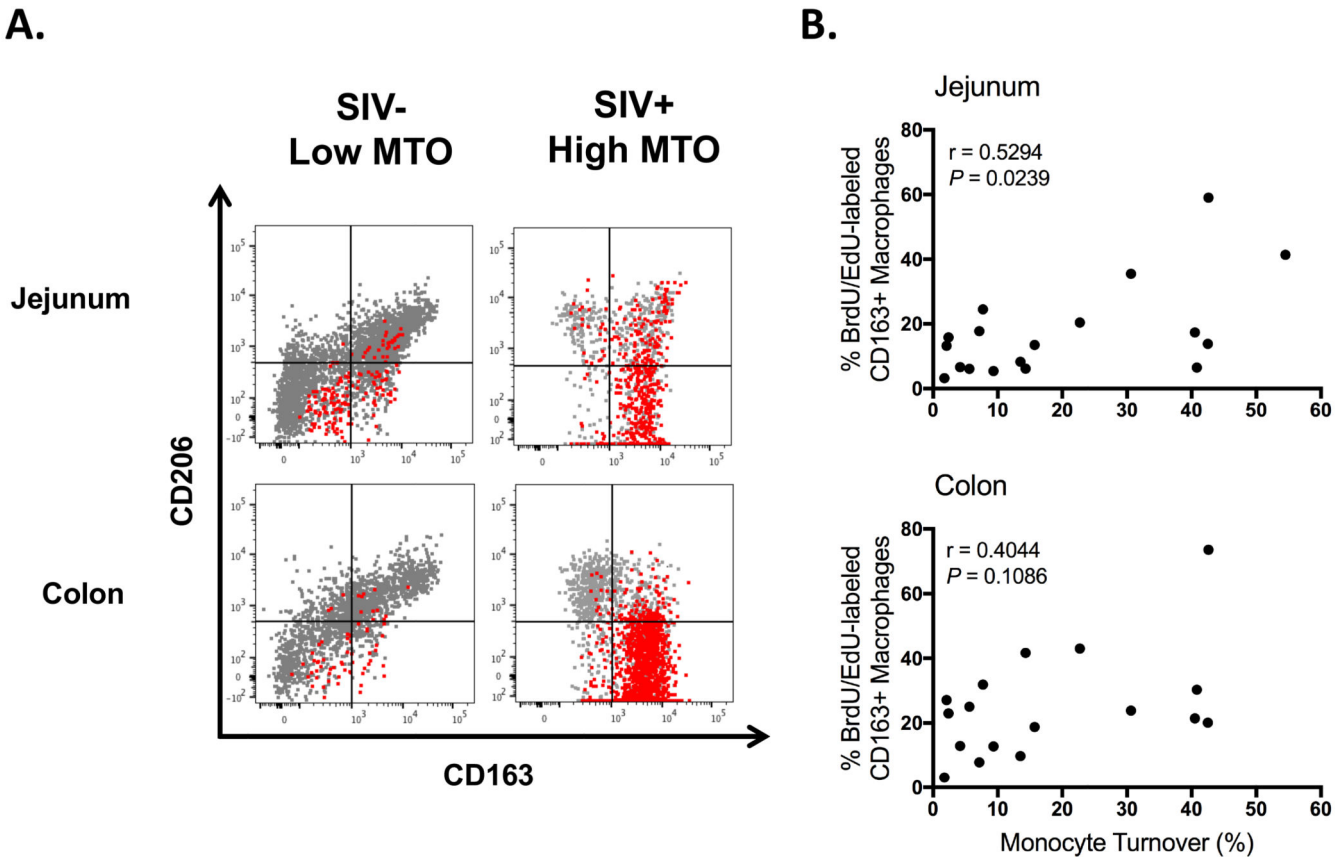

FIG 4.

Identification of recently-replicating intestinal macrophages in rhesus macaques. BrdU or EdU incorporation in recently-dividing macrophages was detected by immunostaining tissues 48 hr after in vivo inoculation of rhesus macaques with the thymidine analogues. (A) Representative flow cytometry plots are shown for CD163+ macrophages in jejunum and colon of an uninfected macaque (SIV−) and an SIV-infected animal (SIV+) exhibiting high MTO. BrdU/EdU-labeled macrophages are shown as red cells. (B) Spearman correlation analyses were performed to compare MTO rates to the percent of BrdU/EdU-labeled CD163+ macrophages in the jejunum (n =18) and colon (n =17). $P < 0.05$ was considered statistically significant.

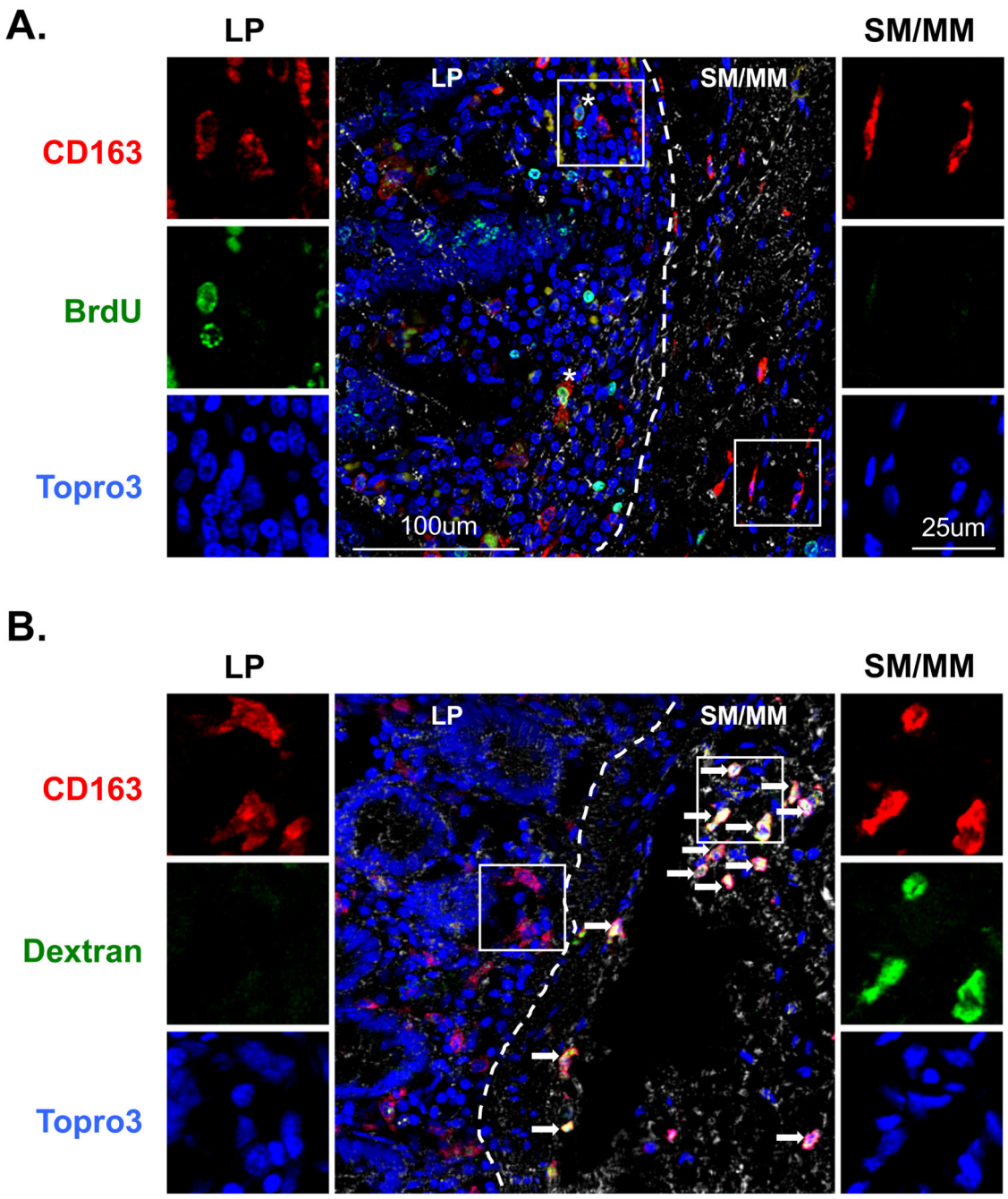

FIG 5.

Immunostaining and confocal microscopy imaging of colon from an SIV-infected animal exhibiting high MTO. (A) A representative image demonstrates the presence of recently-dividing CD163+ macrophages that incorporated BrdU 48 hrs after inoculation as indicated by * symbols. (B) A representative image detecting longer-lived macrophages that retained dextran 69 days after inoculation is indicated by arrows. Dashed lines indicate the border between the lamina propria (LP) and submucosa/muscular mucosa (SM/MM). Topro 3 was

used to identify host cell nuclei. Inserts of LP (left) and SM/MM (right) demonstrate magnified unmerged individual staining characteristics for each biomarker.

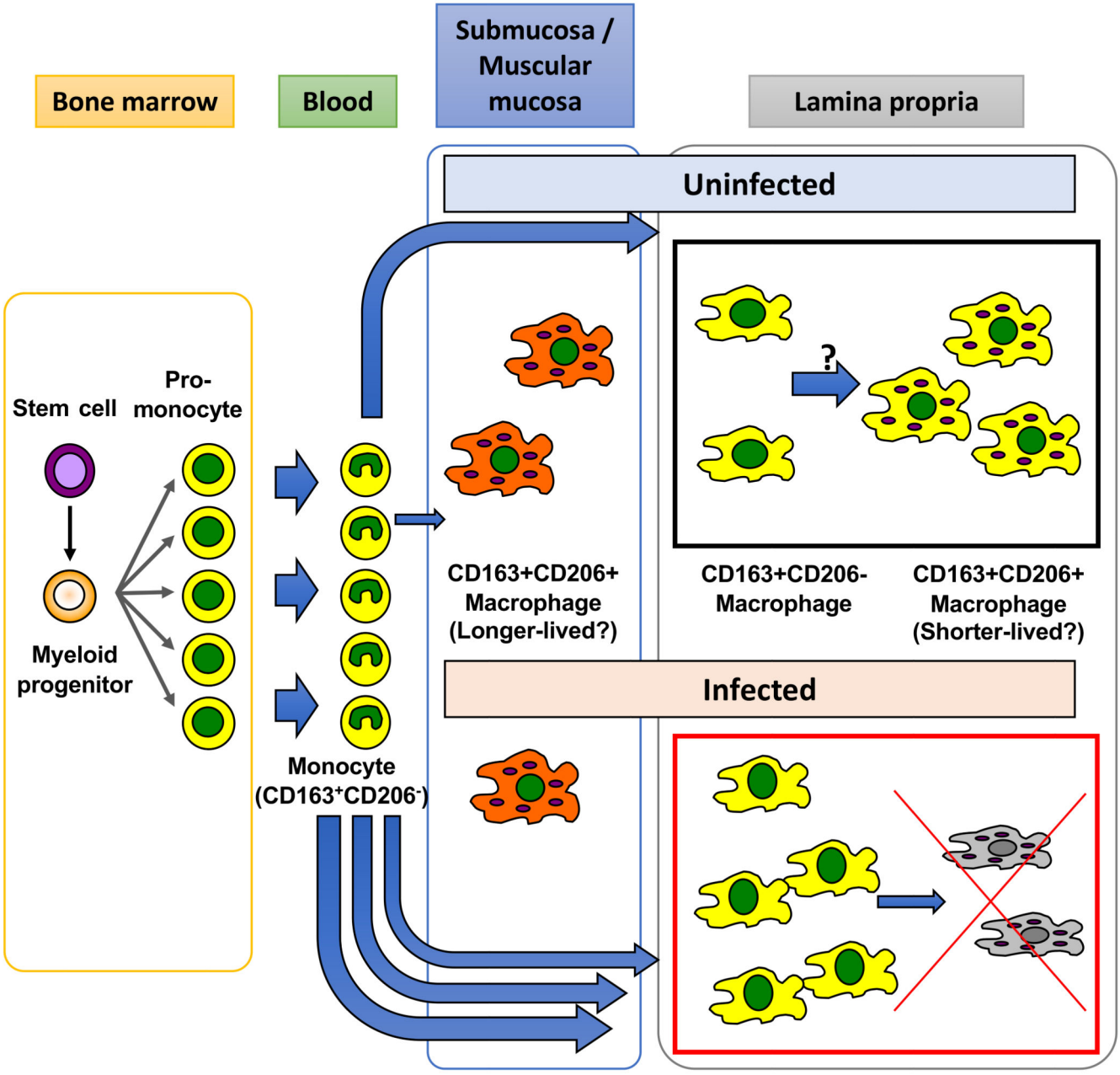

FIG 6.

Proposed mechanism of macrophage shifts in the intestinal tissue after SIV-infection in macaques with high MTO in comparison to uninfected animals. In uninfected healthy animals, monocyte precursors are produced in bone marrow and migrate to the peripheral tissues via blood circulation. CD163+CD206- monocytes then differentiate from CD163+CD206- SP macrophages into CD163+CD206+ DP macrophages as they traffic to the lamina propria. After SIV infection, these DP macrophages in the lamina propria are killed more rapidly than the submucosa DP macrophages and are replaced by trafficking monocytes. Over time, increasing pressure is placed on monocytes to replicate, traffic and replace the lamina propria DP macrophages that were destroyed by SIV infection. This

results in accumulation of SP macrophages in the lamina propria and leads to the increased MTO that predicts disease progression to terminal AIDS. Concurrently, SIV infects DP macrophages in the submucosa/muscular mucosa that remain longer-lived and have the potential to serve as virus reservoirs.

Table 1.

Animals used for the study.

	Animal ID	Viral Load	CD4 T cell count
SIV-negative	EC61		N/A[1]
	GI53		N/A
	GI84		952
	IT02		N/A
	IT24		1218
	FM06		966
	IP62		N/A
	IM47		722
SIV-positive, Low MTO[2]	GN17	1.30E+05	221
	DT18	4.40E+04	270
	BK48	8.58E+06	N/A
	DD87	3.03E+04	254
	FC32	1.40E+07	262
	R945	1.26E+04	374
	CR37	7.40E+06	301
	DR67	1.42E+07	284
	GK40	1.38E+05	243
	FG54	1.49E+07	235
	HA52	9.13E+06	226
	JB71	1.01E+03	N/A
SIV-positive, Int[3]-High MTO	GN24	5.82E+07	667
	GL96	5.86E+07	1502
	FI38	1.54E+08	273
	GP56	2.03E+08	34
	GH64	5.17E+07	762
	GM77	1.42E+07	326
	BA34	1.91E+05	401
	ER17	5.32E+06	185
	DR28	1.48E+07	N/A
	IT27	5.17E+07	237
	IR99	6.88E+05	117
	EM89	4.59E+06	197
	CV39	5.43E+07	221
	CN66	6.43E+08	902

[1] N/A, not available;

[2] MTO, monocyte turnover;

[3] Int, intermediate