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IL-21 and Probiotic Therapy Improve T_H17 Frequencies, Microbial Translocation, and Microbiome in ARV-Treated, SIV-Infected Macaques

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Abstract

Increased mortality in antiretroviral (ARV)-treated, HIV-infected individuals has been attributed to persistent immune dysfunction, in part due to abnormalities at the gastrointestinal barrier. In particular, the poor reconstitution of gastrointestinal T_H17 cells correlates with residual translocation of dysbiotic, immunostimulatory microflora across a compromised intestinal epithelial barrier. We have previously demonstrated that oral probiotics promote increased intestinal CD4⁺ T-cell reconstitution during ARV treatment in a non-human primate model of HIV infection; however, essential mucosal T-cell subsets, such as T_H17 cells, had limited recovery. Here, we sought to promote T_H17 cell recovery by administering IL-21 to a limited number of ARV-treated, probiotic-supplemented, SIV-infected pigtailed macaques. We demonstrate that probiotic and IL-21 supplementation of ARVs is associated with enhanced polyfunctional T_H17

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DISCLOSURE

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expansion and reduced markers of microbial translocation and dysbiosis as compared to infected controls receiving ARVs alone. Importantly, treatment resulted in fewer morbidities compared to controls, and was independent of increased immune activation or loss of viral suppression. We propose that combining ARVs with therapeutics aimed at restoring intestinal stasis may significantly improve disease prognosis of ARV-treated, HIV-infected, individuals.

Introduction

Progressive HIV infection is characterized by persistent innate and adaptive immune activation that is predictive of disease progression and that is not resolved with effective ARV therapy^{1, 2}. Although mediators of immune activation are multi-faceted, it is evident that even with effective viral suppression, persistent immune dysfunction at the intestinal epithelial barrier permits the translocation of immunostimulatory luminal contents into intestinal tissues and systemic circulation³. In non-human primate (NHP) models of HIV infection, microbial translocation distinguishes progressive from non-progressive models of SIV infection, with progressive infection models such as rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemistrina*) macaques displaying evidence of microbial translocation and immune activation in stark contrast to non-progressive hosts such as sooty mangabeys (*Cercocebus atys*)⁴⁻⁶. Importantly, although both progressive and non-progressive infected hosts exhibit some loss of intestinal CD4+ T-cells, progressive models exhibit a preferential loss of T_H17 cells, implicating T_H17 cell loss as a primary contributor to microbial translocation and disease progression⁶⁻⁸.

T_H17 cells are a subset of differentiated, IL-17-expressing memory CD4+ T-cells important for control of the microbiota at mucosal barrier sites⁹. T_H17 cells develop from naïve CD4+ T-cells in response to TGF- β and pro-inflammatory cytokine signaling induced by microbial antigens¹⁰⁻¹². Although T_H17 differentiation can be calibrated by the microbiome^{13, 14}, the effects of T_H17 cells on gastrointestinal immunity extend beyond interplay with commensal species. Thus, while commensal species are sufficient for the generation of T_H17 cells^{13, 14}, the ability of T_H17 cells to induce production of antimicrobial peptides and chemotactic cytokines from the intestinal epithelium contributes to resistance against colonization with *Salmonella typhimurium*, *Streptococcus pneumoniae*, and *Candida albicans*^{8, 9}. Implicit in this counter-regulation, microbial dysbiosis – an imbalance in the relative frequencies of commensal and pathogenic species – is associated with a dysregulation of T_H17 frequencies and function¹⁵. This, in turn, is implicated in inflammation in a multitude of chronic autoimmune and infectious diseases. In HIV-infected individuals and SIV-infected macaques, microbial dysbiosis has been described as a contributor to T_H17 loss and microbial translocation, with some dysbiotic species shown to be enriched in both treated and untreated subjects, and to be associated with markers of disease progression^{16, 17}. Given the contributions of dysbiotic and translocating bacterial species to disease progression in treated, HIV-infected individuals, we previously considered that augmenting ARV-therapy in chronically SIV-infected macaques with probiotics might promote a restoration of the commensal microbiota, initiating a sequence of immune restorative processes. Although probiotic supplementation was associated with intestinal CD4+ T-cell restoration and enhanced T_H17 functionality, we did not observe a significant improvement in the frequency

of T_H17 cells nor was there any discernible impact on the relative frequencies of fecal microbial communities¹⁸.

Subsequent to priming by APCs, T_H17 development is augmented by autocrine production of the common γ -chain cytokine, IL-21^{19, 20}. Though not essential for the differentiation of T_H17 cells^{14, 21}, blockade of IL-21 during T_H17 development limits the expansion and functional capacity of T_H17 cells^{19, 20}. In progressive SIV infection, a paucity of IL-21-producing CD4+ T-cells correlates with T_H17 loss²². Moreover, while IL-21 treatment during acute SIV infection has been shown to delay T_H17 loss independent of ARV therapy, cessation of IL-21 administration results in a rapid decline in T_H17 frequencies²³.

Herein, we considered that augmenting ARV therapy with combination probiotic and IL-21 therapy might enhance the recovery and maintenance of T_H17 cells in chronic SIV infection. As described, we observed that this regimen is associated with increased frequencies of intestinal polyfunctional T_H17 cells as compared to ARV-only controls, and that supplementation is further associated with improvements in microbial translocation, microbial dysbiosis and clinical status. Importantly, as our therapy did not promote immune activation or negate viral suppression, we believe that our findings suggest that probiotic and IL-21 supplementation of ARVs may translate into a safe and effective therapeutic for the reduction of co-morbidities suffered by ARV-treated, HIV-infected individuals.

Results

Experimental Design

We previously demonstrated that treatment of SIV-infected, virus-suppressed pigtailed macaques (PTM) with probiotics improved gastrointestinal integrity and immunity; however, probiotic supplementation alone did not promote a restoration of T_H17 cells nor was there evidence for a shift towards a significantly less inflammatory microbiome¹⁸. Here, we sought to determine if treatment with IL-21^{19, 20} enhances probiotic treatment in SIV-infected, ARV- and probiotic-treated PTMs by promoting T_H17 recovery and reducing microbial translocation and dysbiosis. As depicted in Fig. 1, we infected 11 PTM with SIV_{mac239} and, at day 98 post-infection (p.i.), all animals began daily treatment with the reverse transcriptase inhibitors, PMPA and FTC, and the integrase inhibitor, L'812. Of these animals, 6 were simultaneously treated with the daily probiotic, VLS#3[®] (consisting of 10¹¹ live *Bifidobacterium*, *Lactobacillus* and *Streptococcus* species) and with early and late administration courses of IL-21 to assess refractoriness to continued IL-21 therapy. We routinely sampled the blood and stool of all animals, sampled the pulmonary airways through bronchoalveolar lavage (BAL) and obtained jejunal (Jej), lymph node (LN), and rectal (RB) biopsies.

IL-21 and Probiotic Supplementation Does Not Alter Viral Suppression in ARV-Treated Macaques

IL-21 is a pleiotropic cytokine known to promote the development of T_H17 cells from naïve CD4+ T-cell precursors^{19, 20}. Since mature CD4+ T-cells are the preferential targets for HIV and SIV infection, we assessed the impact of the IL-21 and probiotic regimen on viral

suppression. With the initiation of therapy, all animals exhibited a rapid decline in plasma viral RNA (vRNA) with all but one probiotic, IL-21-supplemented animal (PTA2P031) achieving suppression to below 100 copies/mL within 30 days of treatment initiation (Fig. 2A). In each group, all but one animal exhibited at least one instance of breakthrough viremia; however, measurable virus in these animals did not exceed 100 copies/mL. Importantly, breakthrough viremia in supplemented animals was not associated with increased activation of systemic CD4+ or CD8+ memory T-cells (TM), as assessed by HLA-DR and Ki67 expression, or with increases in plasma soluble CD14 (sCD14) or LPS-binding protein (LBP) (Figure S1 and data not shown).

IL-21 has been shown to promote the expansion of antiviral CD8+ T-cells²³⁻²⁵; however, we considered that CD4+ T-cell reconstitution might counteract ARV- and CD8+ T cell-mediated antiviral effects by expanding the viral reservoir. Indeed, the addition of IL-21 to probiotic and ARV therapy was associated with enhanced intestinal CD4+ T-cell recovery as compared ARV-only controls (Figure S2), similar to what we previously reported using probiotic-supplementation alone¹⁸. To quantify the viral reservoir within intestinal tissues – which exhibit the greatest loss of CD4+ T-cells after infection – we sorted and measured cell-associated viral DNA (vDNA) within colonic, CD4+ memory T-cells (TM; CD95+) at necropsy. Despite a trend for higher cell-associated vDNA in IL-21- and probiotic-supplemented animals, no significant differences were noted in residual vDNA between ARV-only (0.0188 ±0.00566 copies/100 cells; mean±SEM) and IL-21- and probiotic-supplemented (0.0364 ±0.0219) animals (Fig. 2B). Thus, probiotic- and IL-21-supplementation does not appear to dramatically alter virus production or infectivity within intestinal tissues in SIV-infected, ARV-treated PTMs.

Improved Clinical Status in ARV-Treated Macaques Supplemented with IL-21 and Probiotics

Effective ARV-therapy significantly improves disease progression and clinical prognosis in chronically HIV-infected individuals; however, prolonged therapy remains fraught with non-AIDS related co-morbidities^{1, 2}, especially in situations in which dysbiosis is likely to be present²⁶. To determine if probiotic and IL-21 supplementation might improve the prognosis of our animals, we compared the incidence of clinical complications between our experimental groups, as diagnosed by positive bacterial culture or altered blood chemistries (Fig. 3). Interestingly, although no overt complications were noted in the probiotic and IL-21 supplemented group, opportunistic infection (respiratory *Enterobacter cloacae*) and renal injury (azotemia as determined by elevated plasma levels of urea nitrogen or creatinine) were evident among 3 of the 5 ARV-only animals^{27, 28}. While low platelet counts have been shown to be associated with disease progression in SIV-infected PTM²⁹, we found similar counts between experimental groups throughout the study period (data not shown). Of animals that exhibited complications, all displayed subsequent instances of severe diarrhea with one animal requiring unplanned euthanasia, per protocol-specified humane clinical endpoints. To quantitatively assess disease progression, we compared the time to the first diagnosed clinical complication in ARV-only (median day 128 p.i.) and probiotic, IL-21-supplemented (no complication through study termination) animals and determined that supplementation was associated with a significant difference in the time to first complication

($p=0.0316$, Mantel-Cox). These results indicate that probiotic and IL-21 supplementation of ARV-therapy decreased the incidence of non-AIDS morbidities.

IL-21 and Probiotic Supplementation Promote Polyfunctional T_H17 Recovery In ARV-Treated Macaques

In ARV-treated individuals and macaques, persistent microbial translocation and dysbiosis are associated with continued immune activation, thereby possibly exacerbating lentiviral-mediated T_H17 losses^{16, 17}. As our previous findings indicated that probiotic supplementation of ARV-therapy alone was unable to promote significant restoration of T_H17 cells, we considered that additional supplementation with IL-21 might promote sustained restoration of these cells. We assessed the frequency of IL-17 expressing memory CD4+ T-cells (T_H17) longitudinally in the PBMCs, BAL, Jej, LN, and RB. Among BAL, LN and PBMC CD4+ T-cells, probiotic and IL-21 supplemented animals exhibited no significant differences in T_H17 frequencies as compared to ARV-only controls or as compared to the pre-treatment timepoint of day 84 p.i. (data not shown). Indeed, preferential loss of T_H17 within chronically HIV/SIV-infected individuals is not observed within these specific anatomic sites^{7, 30}. However, modest to significant improvements in the frequencies of intestinal T_H17 cells were observed in supplemented animals as compared to the pre-treatment baseline timepoint (Fig. 4A–B and S3A). Coupled with increased intestinal CD4+ T-cell frequencies (Fig. S2), the improved relative frequencies of intestinal T_H17 cells led to significantly improved absolute frequencies of intestinal T_H17 cells (% IL-17-expressing CD4+TM of CD3+ T-cells). We observed increases in the absolute frequency of jejunal T_H17 cells from day 84 p.i. to days 274 and 365 p.i. ($p=0.016, 0.031$) and in rectal T_H17 cells from day 84 p.i. to days 126, 274, and 365 p.i. ($p=0.031, 0.031, 0.016$; Fig. 4A–B) within the probiotic and IL-21 supplemented animals. No significant differences were evident in ARV-only controls (Table S1). Furthermore, at day 182 p.i. (98 days post-intervention), ARV-only controls exhibited a significantly lower absolute frequency of jejunal T_H17 cells as compared to probiotic and IL-21 supplemented animals ($p=0.0286$).

Superior control of disease progression in HIV-infected individuals has been attributed to improved functionality (multiple cytokine expression, or polyfunctionality) among T-cells^{31–33}. As such, we further investigated the frequency of intestinal, polyfunctional T_H17 cells – as assessed here by the additional expression of IL-2, IL-22, or TNF α – in our animals. Although no significant differences were observed between groups in the expression of individual cytokines (data not shown), significant differences in the relative frequency of polyfunctional jejunal T_H17 cells were noted exclusively among probiotic and IL-21 supplemented animals at necropsy as compared to days 84 and 274 p.i. ($p=0.031, 0.031$; Fig. S3B, Table S1). As compared to ARV-only controls, the relative frequencies of polyfunctional T_H17 cells were lower in supplemented animals at day 274 p.i. within the Jej ($p=0.033$) and RB ($p=0.032$) but comparable at all other timepoints. However, an examination of absolute intestinal polyfunctional T_H17 frequencies revealed significant increases specific to probiotic- and IL-21-supplemented animals after the initiation of therapy (Fig. 4C). Within jejunal tissues, significant differences in the absolute frequencies of polyfunctional T_H17 cells were observed from day 84 p.i. to day 274 and 365 p.i. ($p=0.031, 0.031$) in supplemented animals, in whom a significantly higher absolute

frequency of polyfunctional T_H17 cells was observed as compared to ARV-only controls at day 182 p.i. ($p=0.029$). As compared to day 84 p.i., differences were additionally observed among probiotic and IL-21 supplemented animals in the absolute frequency of RB polyfunctional T_H17 cells at days 126, 274, and 365 p.i. ($p=0.031$, all). No significant differences as compared to baseline were observed among the ARV-only controls at any timepoint (Table S1). Thus, probiotic and IL-21 supplementation of SIV-infected, ARV-treated macaques led to a significant improvement in the absolute frequencies of intestinal T_H17 cells and in turn, the absolute frequencies of polyfunctional T_H17 cells.

Since IL-21 similarly promotes the differentiation and maintenance of IL-17-expressing memory CD8⁺ T-cells (T_C17), we additionally examined the longitudinal relative frequencies of T_C17 cells. No significant differences between experimental groups were noted at any timepoint in any tissue examined (Fig. S3C and data not shown). However, a significant improvement in jejunal T_C17 cells was noted in the probiotic- and IL-21-supplemented animals at day 274 p.i. as compared to the pre-treatment timepoint ($p=0.0469$) – no differences were noted in relative RB T_C17 frequencies between or among groups. We similarly examined the relative frequencies of polyfunctional T_C17 cells. No differences were noted between experimental groups and, again, significant changes were limited to jejunal T_C17 cells in the probiotic and IL-21 supplemented animals (Fig. S3D). Significantly higher relative frequencies of polyfunctional, jejunal T_C17 cells were noted in these animals at days 274 and 365 p.i. as compared to day 84 p.i. ($p=0.031$, 0.031). These data indicate that probiotic- and IL-21-supplementation of ARV therapy promotes a recovery of T_H17 and T_C17 cells during chronic SIV infection in macaques.

IL-21 and Probiotic Supplementation Reduce Microbial Translocation and Dysbiosis in ARV-Treated Macaques

Persistent immune activation, T_H17 depletion, residual microbial translocation, and dysbiosis form a synergistic axis in ARV-treated individuals that likely results in the increased morbidities and mortality observed in ARV-treated subjects²⁶. As our results indicate that probiotic and IL-21 supplementation of ARVs promotes the recovery of intestinal T_H17 cells (Fig. 4), tempers some markers of immune activation (Fig. S1), and results in better clinical outcomes (Fig. 3), we next considered the possibility that such treatment might further be associated with a reduction in microbial translocation and dysbiosis. We assessed levels of microbial translocation in our animals by immunohistochemical (IHC) staining for *Escherichia coli* antigens in the colonic lamina propria, MLN paracortex, and liver tissue from samples obtained at necropsy. *E. coli* antigens were evident in all tissues and only within the liver of probiotic- and IL-21-supplemented animals was there a trend for lower levels of *E. coli* staining ($p=0.0556$; Fig. S4 A+B).

To assess whether probiotic and IL-21 supplementation influences the composition of microbial communities, we measured relative levels of commensal *Bifidobacterium* and *Lactobacillus* 16S DNA in the stool and rectal tissues of our experimental animals. No significant differences were observed in the ARV-only animals as compared to baseline with regards to *Bifidobacterium* levels ($p=0.0625$, all timepoints), but these animals exhibited

significantly lower frequencies of *Bifidobacterium* as compared to probiotic and IL-21 supplemented animals at day 154 p.i. ($p=0.0333$; Fig. 5A). Importantly, probiotic- and IL-21-treated animals exhibited significant increases in the relative frequency of *Bifidobacterium* species as compared to the pre-treatment timepoint at days 154 and 365 p.i. ($p=0.0156, 0.0313$). Supplemented animals additionally exhibited significant increases in *Lactobacillus* species at day 154 p.i. as compared to the pre-treatment timepoint ($p=0.0156$; Figure 5B) – no significant differences were noted as compared to or among ARV-only animals ($p=0.0625$, all timepoints). Thus, probiotic and IL-21 supplementation of ARV-therapy during chronic SIV infection promoted a reduction in distal site microbial translocation as well as an increase in the level of probiotic species within the GI tract, reflecting a significant improvement in disease progression.

Reduced IDO Activity in IL-21-, Probiotic- and ARV-treated Macaques

The persistent depletion of T_H17 cells in ARV-treated, HIV-infected individuals correlates with elevated expression and activity of indoleamine-2,3-dioxygenase (IDO), a cytokine-inducible, tryptophan (Trp) catabolizing enzyme^{16, 34}. Trp metabolites are known to inhibit the generation and maintenance of T_H17 cells³⁴, and persistently elevated IDO activity has been postulated to contribute to poor T_H17 recovery in HIV-infected individuals^{16, 34} while also predicting poor CD4+ T-cell recovery and increased mortality. To determine if probiotic and IL-21 supplementation reduced IDO expression and activity, we first quantified IDO expression by IHC in colon and mesenteric lymph node (MLN) tissue samples obtained at necropsy. Tissue staining revealed no significant differences in MLN IDO between the two groups and only a trend for reduced colonic IDO in probiotic and IL-21 supplemented animals as compared to ARV-controls ($p=0.0857$) (Fig S4C+D). We next considered whether T_H17 recovery might correspond with a reduction in circulating levels of kynurenine (Kyn), the first immunoreactive Trp catabolite generated in the IDO pathway. When we measured the concentration of plasma Kyn, we observed that whereas ARV-only animals exhibited a steady increase in Kyn throughout the study period, probiotic and IL-21 supplemented animals displayed reduced Kyn from day 154 to 365 p.i. (Fig. 5C). Although no significant differences were noted between experimental groups at the timepoints examined, significantly lower Kyn was apparent between these later timepoints within the supplemented group ($p=0.0469$). To ascertain whether the observed Kyn levels might result from reduced IDO expression and/or activity, we additionally measured the ratio of plasma Kyn:Trp. Similar to the pattern observed for Kyn itself, while ARV-only animals exhibited a steady increase in plasma Kyn:Trp throughout the experimental period, probiotic and IL-21 supplemented animals exhibited an increase only through the final examined pre-necropsy timepoint, day 154 p.i. (Fig. 5D). There was no significant difference between the final two timepoints, however, although supplemented animals exhibited a plateau in Kyn:Trp levels. Thus, probiotic and IL-21 supplementation of ARVs during chronic SIV infection is associated with a trend towards reduction in intestinal IDO and an apparent reduction in circulating levels of the Trp catabolite, Kyn.

Discussion

ARV-treatment significantly prolongs the length and quality of life of HIV-infected individuals; however, disease progression in these individuals remains evident even with decades of effective viral suppression^{1, 2}. In treated HIV-infected individuals, morbidities and mortality have been attributed to a self-reinforcing cycle of microbial translocation and dysbiosis, immune activation, and a persistent, loss of antimicrobial T_H17 cells^{3, 35}. While we have previously demonstrated that probiotic supplementation of ARVs in SIV-infected macaques promotes a restoration of intestinal CD4+ T-cells¹⁸, in our prior study we did not observe significant T_H17 expansion nor was there a measurable impact on microbial translocation or on the composition of the GI tract microbiome. Herein, we demonstrate that the supplementation of ARVs with probiotics and IL-21 promotes intestinal T_H17 recovery, a reduction in measures of microbial translocation, and an enhanced microbiome. Importantly, our treatment regimen did not promote immune activation or a significant expansion of the viral reservoir. Although limited in size, these findings suggest that probiotic and IL-21 supplementation of ARV therapy should be further evaluated as a therapeutic in the treatment of non AIDS-related comorbidities.

The development of T_H17 cells from naïve CD4+ T-cell precursors or T_{regs} is augmented by the autocrine production of IL-21¹⁹⁻²¹. In this capacity, IL-21 enhances ROR γ T transcription, FOXP3 suppression, and the downstream transcription of IL-17 and additional T_H17-associated cytokines such as IL-22^{19, 20}. Importantly, IL-21 alone is insufficient to induce T_H17 development²¹, requiring additional signaling originating from the commensal microbiome. Thus, in murine models, although the presence of the commensal microbiome – specifically, segmented filamentous bacteria – is sufficient to drive the development of T_H17 cells *in vivo*^{13, 14}, an absence or blockade of IL-21 signaling diminishes T_H17 differentiation^{19, 20}. That the inclusion of IL-21 in our regimen promotes T_H17 reconstitution (Fig. 4) where probiotic supplementation of ARVs alone does not¹⁸ indicates that therapeutically provided probiotic species alone are either insufficient or incorrectly parlayed into the development of T_H17 cells during chronic SIV infection. We believe that the prolonged regeneration of T_H17 cells in our animals subsequent to IL-21 withdrawal (approximately 7 months between early and late administration; Fig. 1) suggests that IL-21 functioned directly at the level of CD4+ T-cells to establish a basal threshold of T_H17 cells necessary for self-propagation. Although a direct assessment of circulating IL-21 was not performed, attainment of a theoretical threshold is further supported by the observation that late IL-21 administration did not augment already enhanced T_H17 frequencies (Figure 4). To this end, it would be of significant interest to determine whether increasing the concentration of administered IL-21 or lengthening early IL-21 therapy might not augment or accelerate T_H17 recovery beyond what we describe here. Although we cannot discount alternative roles for IL-21 in improving disease progression in our supplemented animals – such as altered functionality among IL-21-responsive B-cells and NK-cells³⁶ – the administration of IL-21 during acute SIV infection in macaques leads to a selective maintenance of T_H17 cells that is strictly dependent on the continued administration of IL-21²³, arguing against an ancillary role for IL-21 in sustaining T_H17 frequencies. An assessment of the effects of IL-21 supplementation to ARV therapy independent of probiotic administration will significantly

further our understanding of the conditions required for the generation and maintenance of T_H17 cells in treated chronic lentiviral infections.

Although *de novo* T_H17 development from naïve CD4+ T-cell precursors is largely reliant on the ability of APCs to integrate microbial sensing into pro-T_H17 cytokine signaling^{10, 11}, T_H17 and T_{reg} cells exist as a transcriptional dichotomy^{19, 20, 37, 38} which may further be reciprocally programmed by aryl hydrocarbon receptor (AHR) ligand signaling^{34, 39}. As recent findings have demonstrated a role for the IDO-derived AHR-agonist Kyn in reducing the T_H17:T_{reg} during lentiviral infection³⁴, reduced plasma Kyn (Fig. 5C) in our supplemented animals suggests that T_H17 recovery may have included mechanisms adjunct to the direct influence of therapeutic IL-21. Given that species of the dysbiotic microbiota encode enzymatic homologues to primate Trp-catabolizing enzymes¹⁶, we cannot discount the possibility that an IL-21-mediated recuperation of T_H17 cells preempted a reduction of dysbiotic species and hence, Kyn. Indeed, the prolonged recovery of commensal *Bifidobacteria* in our supplemented animals (Fig. 5B) in contrast to animals receiving probiotic supplementation alone¹⁸ suggests a role for IL-21 in promoting the colonization of probiotic species. However, as a replete commensal microbiome is itself necessary for T_H17 maintenance¹⁴, prolonged T_H17 recovery after IL-21 cessation may thus be dependent upon a reduction in the frequency of dysbiotic species.

Microbial translocation has been extensively described as a significant contributor to immune activation and disease progression in chronic lentiviral infections³. In HIV-infected humans and SIV-infected NHPs the influence of microbial translocation on immune activation has been causally associated by the correlation of disseminated LPS with systemic IFN α ^{4, 5}, and formally demonstrated by the prevention of immune activation with acute use of the LPS-sequestering drug sevelamer⁴⁰. Intriguingly, T_H17 recovery (Figure 4) and improved disease progression (Figure 3) occurred independent of systemically reduced *E. coli* antigen (albeit a trend for reduced *E. coli* in the liver; Figure S4A+B) or persistent immune activation (Figure S1). In this regard, immune activation in our supplemented animals may reflect improved immune function rather than a manifestation of innate cell refractoriness or lymphocyte exhaustion^{4, 41, 42}. Indeed, as compared to ARV-only controls, supplemented animals exhibited control over rectal CD4+ T-cell proliferation (Fig S1A) concomitant with improved CD4+ T-cell reconstitution (Fig. S2), which is suggestive of homeostatic restoration. A more detailed assessment of immune function, particularly alterations in inflammatory biomarkers, will be necessary to address these hypotheses.

Increased mortality among HIV-infected individuals remains evident even with decades of effective viral suppression², especially in conditions when the IDO-mediated kynurenine pathway is induced and microbial dysbiosis may be present²⁶. Consistent with these observations in humans, co-morbidities were evident in our ARV-only animals where, despite continued viral suppression (Fig. 2), 3 of 5 animals displayed repeated complications. That none of our supplemented animals displayed complications despite co-housing with the ARV-only controls is particularly notable given the propensity of PTMs toward microbial translocation and systemic immune activation, even in the absence of SIV infection⁴³. Although the recovery of T_H17 cells in our supplemented animals (Figure 4) likely contributed to improved resistance to co-infection^{8, 9}, an association between T_H17

recovery and ARV tolerance is more tenuous. Indeed, ARV complications were reduced in animals supplemented with probiotics alone, where T_H17 recovery was absent¹⁸. While the presence of commensal bacterial species has been demonstrated to improve the efficacy of anticancer therapeutics such as cyclophosphamide^{44, 45}, the influence of probiotics and commensal species in mediating pharmaceutical toxicities has not been formally explored. A thorough characterization of the contributions of our individual therapeutic constituents – including individual probiotic species – to immune function in the presence and absence of ARVs will be necessary to fully uncover the mechanisms which govern disease progression in treated chronic lentiviral infections.

In summary, we demonstrate that probiotic and IL-21 supplementation of ARV therapy in chronically SIV-infected macaques improves clinical outcomes, as manifested by a reduction in non-AIDS associated morbidities. Importantly, improved clinical outcomes were accompanied with an increase in the absolute frequency of intestinal, polyfunctional T_H17 cells, and reduced measures of intestinal microbial dysbiosis. A larger, mechanistic dissection of the immune processes associated with improved disease progression in our supplemented animals will significantly inform the design of future therapeutics for the treatment of HIV-infected individuals.

Methods

Animal Infections and Interventions

Eleven, healthy pigtail macaques (*Macaca nemestrina*) were accessed for this study and infected with 3,000 TCID₅₀ SIV_{mac239}. At day 98 p.i., all animals began a daily ARV regimen consisting of PMPA and FTC (30 mg/kg at 120 mg/mL in PBS s.c. once-daily) and L'812 (120 mg oral, twice-daily) – PMPA was reduced to 20 mg/kg after 30 days. Of these animals, 6 additionally received daily probiotic VSL#3[®] (oral, two capsules daily) for the entirety of the study and two courses of rMamu IL-21-IgFc (50 ug/kg at 1 mg/mL in H₂O s.c., 5 once-weekly doses) initiated at days 98 and 333 p.i. Animals were assigned to treatment groups to evenly distribute animals by weight and MHC genotype (Mane-A10) and were sampled as a mixed population. The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health (NIH) Intramural Research Program, approved all of the experimental procedures (protocol LMM 12). Animals were sacrificed according to the recommendations set forth by the American Veterinary Medical Association Guidelines on Euthanasia.

Sample Collection

Blood, BAL, and biopsies from colon, liver, lymph nodes, jejunum, and rectum were collected longitudinally or at necropsy. Biopsies were maintained in RPMI prior to processing. For longitudinal LN biopsies, axillary or inguinal LNs were locally exposed and excised by blunt dissection. For longitudinal RBs, fecal material was removed from the rectum and biopsies obtained with biopsy forceps. Jejunal biopsies were obtained by video-guided endoscopy. For BAL collection, silicone tubing was directed into the trachea with the assistance of a laryngoscope, whereupon warmed normal PBS or saline was instilled and

subsequently aspirated for collection. Biopsies collected at necropsy were directly accessed immediately post-mortem. Stool and luminal swabs were obtained as previously described¹⁷. Plasma was isolated from blood by centrifugation. Mononuclear cells were isolated from blood by Ficoll gradient centrifugation and from BAL and tissue biopsies by straining/grinding samples through a 0.22 μ m cell strainer.

Determination of vRNA and vDNA

Plasma vRNA levels were determined by qRT-PCR (ABI Prism 7900 sequence detection system; Applied Biosystems). vRNA was reverse transcribed and cDNA amplified (45 cycles/default setting) with Ampli Taq Gold DNA polymerase (PCR core reagents kit; Perkin-Elmer/Roche) utilizing primer pairs corresponding to SIV_{mac239} gag gene sequences (forward, nucleotides 1181 to 1208, and reverse, nucleotides 1338 to 1317). Cell-associated viral DNA was measured as previously⁴⁶. Briefly, qPCR was performed on sorted, lysed cells using the Taq DNA polymerase kit (Invitrogen) and the SIV_{mac239} forward primer GTCTGCGTCATYTTGGTGCATTC, reverse primer CACTAGYTGCTCTGCACTATRTGTTTTG, and probe sequence CTTCTCAGTYTGTTTCACTTTCTCTTCTGCG. Results assessed below the limit of detection were normalized to the threshold cutoff.

Flow Cytometry and Immunophenotypic Analysis

Polychromatic flow cytometry and cell sorting were performed on stained mononuclear cells utilizing the BD LSRFortessa and FACS Aria, respectively, equipped with FACS DiVA software. The following Abs were used for staining at predetermined concentrations: CD3 (clone SP34-2) AL700, HLADR (G46-6) APC-H7, IL-2 (MQ1-17H12) FITC, and Ki67 (B56) FITC from BD; CD4 (OKT4) eFluor450, CD8 (SK1) PerCP-e710, IFN γ (4S.B3) eFluor450, IL17A (eBio64DEC17) PE, IL22 (IL22JOP) APC, and TNF α (MAb11) PE-Cy7 from eBioscience; CD28 (CD28.2) ECD from Beckman Coulter; and CD95 (DX2) PE-Cy5 from Biolegend. Cell viability was assessed using the Live/Dead Aqua Fixable Dead Cell Stain (Invitrogen). Cells were permeabilized with Cytotfix/Cytoperm (BD) prior to intracellular staining. Acquired data were analyzed using FlowJo software. To quantify cell subset frequencies, we used a threshold cutoff of 100 cells of the parent population. For T_H17 functional analyses, cytokine gates were established on a threshold cutoff of 100 CD4⁺ or CD8⁺ TM and Boolean gates utilized to ascertain frequencies – populations not meeting this threshold were not considered for analysis.

IHC and quantitative image analysis

IHC for rabbit polyclonal anti-*E. coli* (Cat. # B0357; Dako) and rabbit monoclonal anti-IDO (Millipore; clone EPR1230Y) was performed using a biotin-free polymer approach (Rabbit Polink-1 for *E. coli* and Mouse Polink-2 for IDO, Golden Bridge International, Inc.) on 5-mm tissue sections mounted on glass slides, which were dewaxed and rehydrated with double-distilled H₂O. Antigen retrieval was performed by heating slides in 1 \times DIVA Decloacker reagent (Biocare Medical) or 0.01% citraconic anhydride containing 0.05% Tween-20 in a pressure cooker (122°C for 30sec). Slides were stained with optimal conditions determined empirically on an IntelliPATH autostainer (Biocare Medical) that consisted of a blocking step using blocking buffer (TBS with 0.05% Tween-20 and 0.5%

casein) for 10min and an endogenous peroxidase block using 1.5% (v/v) H₂O₂ in TBS (pH 7.4) for 10min. Primary Abs were diluted 1:5,000 in blocking buffer and incubated for 1h at room temperature. Tissue sections were washed, and either Rabbit Polink-1 or Polink-2 staining systems (Golden Bridge International, Inc) were applied for 30min at room temperature. Sections were developed with Impact™ 3,3'-diaminobenzidine (Vector Laboratories), counterstained with hematoxylin and mounted in Permount (Fisher Scientific). All stained slides were scanned at high magnification (400×) using the Aperio® AT2 System (Leica Biosystems) yielding high-resolution data for the entire tissue section. Representative high magnification (400×) images were acquired from these whole tissue scans.

Quantification of systemic inflammatory markers

Concentrations of Kyn and Trp were quantified from plasma by liquid-chromatography-tandem mass spectrometry, as previously described³⁴. Levels of LBP and sCD14 were assessed from plasma using commercially available ELISA kits (Antibodies Online and R&D, respectively), according to the manufacturers' protocols.

Assessment of bacterial 16S DNA

16S DNA was isolated and quantified from stool or from luminal swabs as previously described¹⁷.

Statistics

Statistical analyses were performed using Prism (v6.0; GraphPad Software Inc.). The MannWhitney *U* test was used for comparisons between groups; the Wilcoxon Signed-Rank test for within groups. The Mantel-Cox test was used to assess disease progression as measured by time to the first presentation of health complication. Averaged data are presented as arithmetic mean ± SEM. *p* values less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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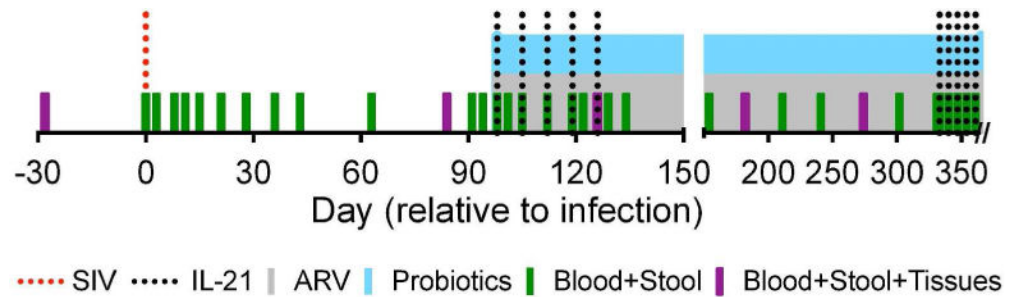


Figure 1. Experimental Design

Eleven PTM were infected with SIV_{mac239} at day 0 (dotted red line) and daily ARV therapy (grey shaded region) was initiated at day 98 p.i. Of these animals, 6 PTM were additionally treated with daily probiotic therapy (blue shaded region) as well as two rounds of IL-21 therapy, administered once weekly for five weeks (dotted black lines). One ARV-only PTM was euthanized at day 126 p.i. per protocol specified clinical endpoint. Blood, stool and tissues (BAL, Jej, LN, and RB) were collected at pre-determined intervals, as indicated (green and purple bars).

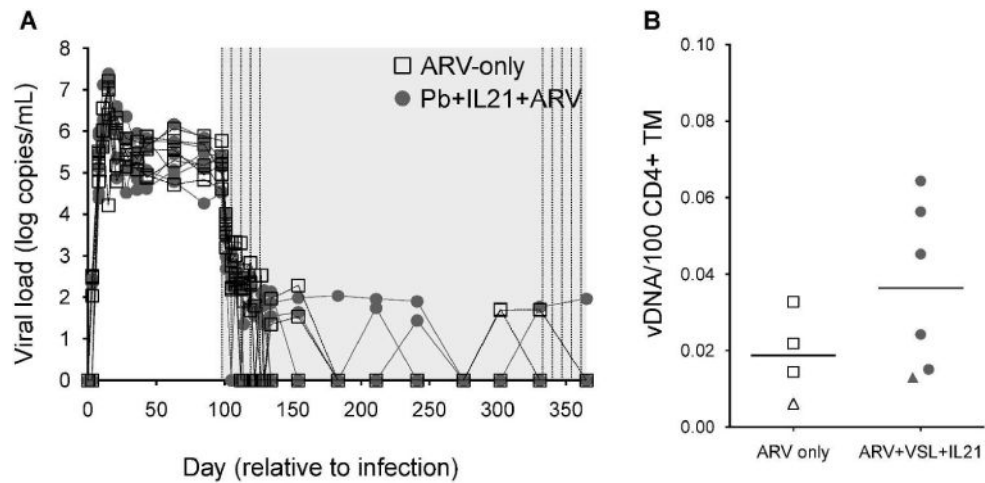


Figure 2. IL-21 and Probiotic Therapy does not Alter Viral Load or Cellular Distribution in ARV-Treated PTMs

a. Longitudinal, log copies/mL of plasma vRNA from animals treated with ARVs only (open squares) or with ARVs, probiotic VSL#3, and IL-21 (closed circles). Grey shading indicates treatment initiation and duration. Dotted lines denote IL-21 administration timepoints. Symbols and shading are consistent throughout the manuscript unless otherwise noted. **b.** Copies of vDNA per 100 colonic CD4+ TM at the time of necropsy. Triangles indicate samples normalized to the limit of detection

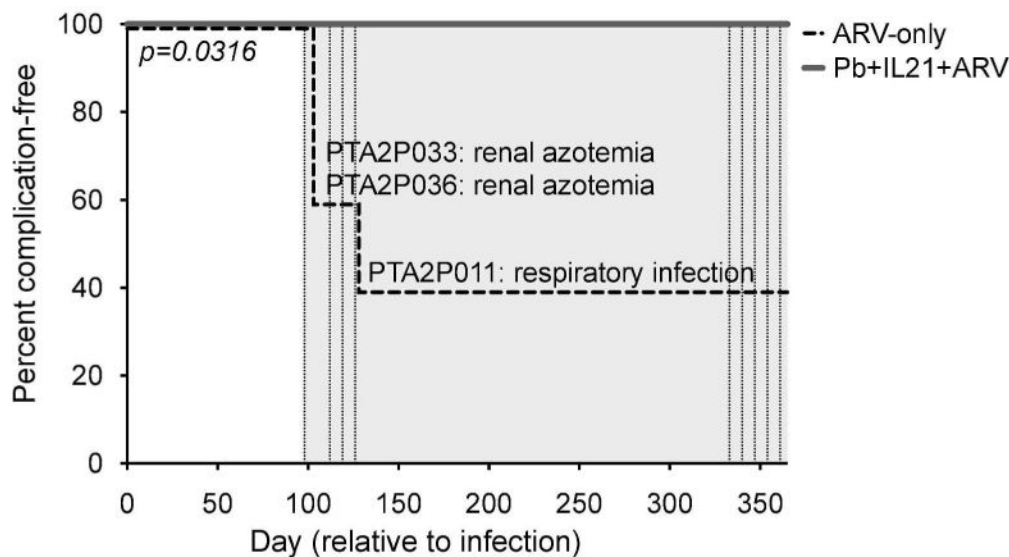


Figure 3. IL-21 and Probiotic Therapy Improves Clinical Status in ARV-Treated PTMs
 Percent of animals remaining free from clinical complications over time. Initial complications per animal are noted alongside changes in the incidence-free curve, with ARV-only animals represented by the dashed line and supplemented animals by the solid line. Significance in the time to the first reported clinical complication between groups was assessed by the Mantel-Cox Log-Rank test.

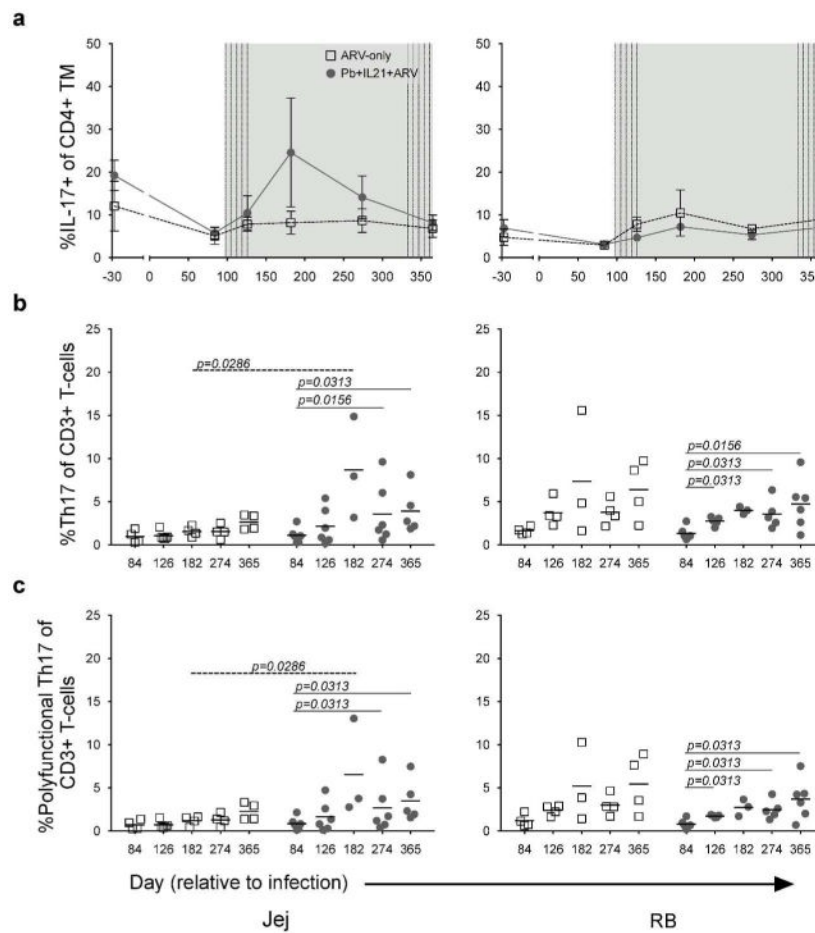


Figure 4. IL-21 and Probiotic Therapy Promotes the Absolute Expansion of Polyfunctional T_H17 Cells in ARV-Treated PTMs

a. Longitudinal mean (\pm SEM) percent IL-17+ of CD4+ TM (T_H17) from the Jej (left) and RB (right). **b.** Absolute percent T_H17 (i.e., the percent of IL-17+ CD4+ TM found within CD3+ T cells) from the Jej and RB at the indicated days p.i. **c.** Absolute percent polyfunctional T_H17 from the Jej and RB at the indicated days p.i. Line through samples represents mean; lines above samples span timepoints of significance. Significance within groups was assessed by Wilcoxon Signed-Rank test. Significance across groups was assessed by Mann-Whitney U test.

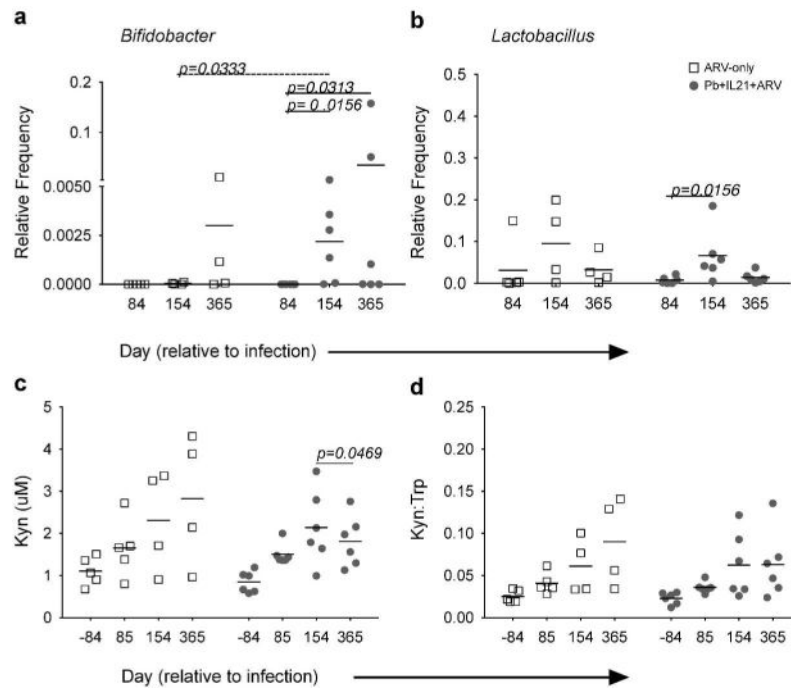


Figure 5. IL-21 and Probiotic Therapy Improve Commensal Colonization and Reduce Circulating Kyn

a–b. Relative frequency of *Bifidobacter* (**a**) and *Lactobacillus* (**b**) 16S DNA of total 16S DNA in rectal tissues at the indicated timepoints. **c.** Concentration of plasma Kyn (μM) at the indicated days p.i. **d.** Ratio of plasma Kyn to Trp concentrations at the indicated days p.i. Line through samples represents mean; lines above samples span timepoints of significance. Significance within groups was assessed by Wilcoxon Signed-Rank test. Significance across groups was assessed by Mann-Whitney U test.