Cotrimoxazole reduces systemic inflammation in HIV infection by altering the gut microbiome and immune activation

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Overline: HIV

One Sentence Summary: Cotrimoxazole reduces systemic and intestinal inflammation in HIV infection by suppressing gut-resident Streptococci and immune cell activation.

ABSTRACT

Long-term cotrimoxazole prophylaxis reduces mortality and morbidity in HIV infection but the mechanisms underlying these sustained clinical benefits are unclear. Here we investigate the impact of cotrimoxazole on systemic inflammation, an independent driver of HIV mortality. We show that plasma inflammatory markers were lower in HIV-positive Ugandan and Zimbabwean children receiving antiretroviral therapy after randomization to continue (n=144) versus stop (n=149) cotrimoxazole. This was not explained by clinical illness, HIV progression or nutritional status. Since sub-clinical enteropathogen carriage and enteropathy can drive systemic inflammation, we explored the impact of cotrimoxazole on the gut microbiome and biomarkers of intestinal inflammation. Although global microbiome community composition was unchanged, viridans group Streptococci and streptococcal mevalonate pathway enzymes were lower among children who continued (n=36) versus stopped (n=36) cotrimoxazole. These changes were associated with lower fecal myeloperoxidase. To isolate direct effects of cotrimoxazole on immune activation from its antibiotic properties, we established *in vitro* models of systemic and intestinal inflammation. *In vitro* cotrimoxazole treatment had modest but consistent inhibitory effects on pro-inflammatory cytokine production by blood leukocytes from HIV-positive (n=16) and HIV-negative (n=8) U.K. adults. It also reduced IL-8 production by inflamed gut epithelial cell lines. Together, these data demonstrate that cotrimoxazole reduces systemic and intestinal inflammation both indirectly via antibiotic effects on the microbiome, and directly by blunting immune and epithelial cell activation. Synergy between these pathways may explain the clinical benefits of cotrimoxazole despite high antimicrobial resistance, providing further rationale for extending coverage among people living with HIV in sub-Saharan

Africa.

INTRODUCTION

In 2017, 36.9 million people were living with HIV and 940,000 died from AIDS-related illnesses(*1*). To reduce mortality and morbidity(*2, 3*), World Health Organization (WHO) guidelines recommend long-term cotrimoxazole prophylaxis for all people living with HIV in areas with a high prevalence of malaria and/or severe bacterial infections(*4*). However, it is unclear how cotrimoxazole reduces mortality and morbidity, given the high rates of antimicrobial resistance and selection for resistant pathogens with long-term use(*2*). There is therefore a need to better understand the effect of cotrimoxazole on HIV pathogenesis.

Systemic inflammation is independently associated with mortality in HIV infection(*5-7*). Cotrimoxazole might plausibly confer benefits by reducing inflammation, either indirectly by targeting pathogens, or directly by modulating cells that produce pro-inflammatory mediators. Animal models suggest that antibiotics confer anti-inflammatory benefits(*8*), and observational studies of HIV-positive adults suggest that cotrimoxazole can reduce plasma inflammatory biomarkers(*9, 10*). Data from randomized trials and low-income settings are lacking and no studies have evaluated the effects of cotrimoxazole on pro-inflammatory pathways in HIV-positive individuals.

HIV drives a chronic enteropathy, characterized by loss of villous architecture, increased gut permeability, mucosal CD4+ T cell depletion(*11*), leukocyte infiltration(*12-14*), and microbial translocation(*15, 16*), accompanied by increased pathogen carriage and an altered microbiome(*17, 18*); together, these changes contribute to systemic inflammation.

Cotrimoxazole prophylaxis could influence intestinal inflammation through antibiotic effects on enteropathogens and/or the microbiome, or via direct effects on mucosal leukocytes and gut epithelial cells(*19, 20*). Among HIV-positive Ugandan adults cotrimoxazole had limited effects on the gut microbiome(*21*), however the effects of cotrimoxazole have not been assessed in a randomized trial or in children.

Cotrimoxazole comprises two folate pathway inhibitors, trimethoprim and sulfamethoxazole. The hypothesis that cotrimoxazole can directly inhibit pro-inflammatory immune cell activation was first posited in 1970, following the observation that intramuscular trimethoprim prolonged skin graft retention in mice(*22*). However, subsequent *in vitro* studies of the direct effects of cotrimoxazole on immune cells have yielded conflicting results(*23-26*) and none have assessed its anti-inflammatory effects in HIV-positive individuals. Cotrimoxazole treatment of rats impacts absorption across the gut epithelium(*19*), suggesting cotrimoxazole may influence gut barrier function, a critical regulator of cross-talk between the circulation and gut-resident microorganisms.

Thus, cotrimoxazole prophylaxis confers long-term clinical benefits in HIV infection, which are not entirely explained by its antibiotic effects(*2, 3*). Inconsistent evidence suggests that cotrimoxazole may have anti-inflammatory properties, but conclusive data are lacking. We therefore capitalized on a randomized trial of continuing versus stopping cotrimoxazole in HIV-positive children in sub-Saharan Africa, to test the hypothesis that cotrimoxazole reduces systemic inflammation. We then explored mechanistic pathways through which

this may occur, using clinical data, stored specimens and *in vitro* models.

RESULTS

Cotrimoxazole reduces systemic inflammation in HIV-positive children

We have previously shown that randomization to continue versus stop cotrimoxazole prophylaxis reduced hospitalization or death among HIV-positive children on long-term antiretroviral therapy (ART) in the ARROW trial in Uganda and Zimbabwe(*27*). Since the inflammatory biomarkers C-reactive protein (CRP) and interleukin (IL-) 6 were independently associated with mortality in ARROW(*5*), we hypothesized that the benefits of cotrimoxazole might be partly mediated through reductions in systemic inflammation. CRP, IL-6, soluble (s)CD14, and tumor necrosis factor (TNF)α) were quantified in longitudinal plasma samples from children randomized to continue (n=144) versus stop (n=149) cotrimoxazole (**Fig. 1**).

Biomarkers were similar between groups at baseline (**Fig. 1A-D**), but subsequent CRP concentrations from week-24 until the end of follow-up were lower in children randomized to continue cotrimoxazole (global p: 0.006; **Fig. 1A**). IL-6 was also significantly lower among children continuing cotrimoxazole, particularly at early timepoints (Global p: 0.010; week-12 p: 0.014, week-24 p: 0.003; **Fig. 1B**). There was no evidence of global differences between groups in sCD14 (**Fig. 1C**) or TNFα (**Fig. 1D**). Serum albumin was significantly higher (median: 42 versus 41g/L, p=0.041) and total protein significantly lower (76 versus 78g/L, p=0.038) in children continuing cotrimoxazole at week-48 (**Fig. 1E**), consistent with less systemic inflammation. Collectively these results show that cotrimoxazole reduces systemic inflammation in HIV-positive children.

To estimate the clinical implications of these findings, we used relative risk estimates of adverse outcomes (death, new or recurrent WHO clinical stage 4 events, or poor immunological response to ART) associated with baseline (i.e. pre-ART) concentrations of CRP and IL-6 in ARROW(*5*). Stopping cotrimoxazole led to 1.65-fold higher CRP (stop, 2.71mg/L versus continue, 1.64mg/L; **Fig. 1A**) and 1.18-fold higher IL-6 (stop, 5.36pg/mL versus continue, 4.54pg/mL; **Fig. 1B**) at week-24, corresponding to an increased relative risk of adverse clinical outcomes among children stopping cotrimoxazole of 13% (95% CI: 4-24%) and 11% (95% CI: 4-18%), respectively, within 24 weeks(*5*). Relative differences in CRP peaked at week-48 (1.92-fold increase; stop, 2.86mg/L versus continue, 1.49mg/L; **Fig. 1A**), corresponding to an 18% (95% CI: 6-32%) increased risk of adverse clinical outcomes. Thus, differences in CRP and IL-6 with continued cotrimoxazole are important for long-term survival, health and immune restoration among HIV-positive children.

Reduced systemic inflammation is not solely due to less clinical disease

Lower systemic inflammation with long-term cotrimoxazole could be due to reductions in HIV disease progression or clinical illness(*2*, *27*). However, there was no evidence of global differences in the proportion of children with viral suppression (<80 HIV RNA copies/mL; **Fig. 2A**) or in CD4+ T cell percentages (%CD4; **Fig. 2B**) between randomized groups. There was also no evidence for global differences in caregiver-reported cough (**Fig. 2C**), fever (**Fig. 2D**), nausea/vomiting (**Fig. 2E**) or abdominal pain (**Fig. 2F**). Too few children had persistent, bloody or moderate-to-severe diarrhea, difficult/fast breathing and/or weight loss for comparison between groups. HIV-positive children frequently have malnutrition; antibiotics (including cotrimoxazole) have been shown to improve growth(*28*) and slow weight-loss(*29*).

We therefore compared anthropometry between randomized groups, reasoning that differences in systemic inflammation might be explained by underlying wasting or stunting. We found no evidence of differences in weight-for-age (**Fig. 2G**) or height-for-age Z-scores (**Fig. 2H**). Thus, effects of cotrimoxazole on systemic inflammation were not explained by differences in HIV disease progression, symptomatic infections or malnutrition between groups.

Cotrimoxazole alters circulating CD4+ T cell phenotypes in HIV-positive children

Although cotrimoxazole continuation had no impact on total CD4+ T cell counts, we hypothesized that CD4+ T cell phenotypes would differ between randomized groups because systemic inflammation is associated with T cell activation and proliferation(*30, 31*) (*5*). T cell immunophenotyping in a subset of Ugandan ARROW participants (stop n=48, continue n=47; **fig. S1A**) revealed no evidence of differences between randomized groups in the proportions of total CD4+ T cells expressing the activation marker HLA-DR or the proliferation marker Ki67 (**fig. S1B** and **C**). Children continuing cotrimoxazole had higher percentages of recent thymic emigrant-like cells (RTE, CD4+CD45RA+CD31+ T cells; an indicator of thymic output(*32*)) than children stopping prophylaxis (**fig. S1B**). There was no evidence of differences in proportions of naïve (CD4+CD45RA+CD31-) or effector-memory (CD4+CD45RA-CD31-) T cells or in the expression of HLA-DR on any CD4+ T cell sub-populations (**fig. S1C**). However, children continuing cotrimoxazole had lower percentages of proliferating (Ki67+) RTE and naïve T cells, particularly at later timepoints post-randomization (**fig. S1D**). Thus, cotrimoxazole continuation led to some changes in circulating T cells consistent with reduced systemic inflammation(*5*).

Cotrimoxazole suppresses abundance and function of gut-resident Streptococci

The gut microbiome is disrupted by HIV infection, which contributes to local and systemic inflammation(*17, 33*). We hypothesized that continuing cotrimoxazole would drive sustained sub-clinical differences in gut pathogens and commensals. We conducted whole metagenome shotgun sequencing of total fecal DNA from children randomized to continue (n=36 at week-84; n=33 at week-96) versus stop cotrimoxazole (n=36 at week-84; n=35 at week-96). Randomized groups did not differ in species-level diversity at week-84 or week-96 (Shannon indices: 13.1 continue versus 14.3 stop, p=0.27; and 13.5 continue versus 14.8 stop, p=0.72) or evenness (Pileou's index: 0.59 continue versus 0.60 stop, p=0.605; and 0.60 continue versus 0.61 stop, p=0.883). Bacterial community composition was also similar between groups (**Fig. 3A** and **B**) . However, false discovery rate (FDR)-adjusted zero-inflated beta regression analysis of individual microbiome characteristics identified 7 bacterial species (*Alistipes onderdonkii, Eggerthella lenta, Clostridium bartlettii, Haemophilus parainfluenzae, Streptococcus mutans, Streptococcus parasanguinis* and *Streptococcus vestibularis*; **fig. S2**) and 11 protein families (Pfam; **fig. S3**) mapping to *Streptococcus parasanguinis, Streptococcus salivarius* and *Haemophilus parainfluenzae*, that were consistently less abundant at both timepoints in the continue versus stop group (relative abundance ratio <1). The differentially abundant Streptococci are all within the viridans group (VGS), and largely fell in the quadrant of the NMDS ordination plot where the extremes of the treatment groups lay (**Fig. 3A** and **B**). The relative abundance of Enterobacteriaceae, which includes gastrointestinal pathogens (e.g. *Salmonella*, *Escherichia coli*, and *Shigella*) that are frequently resistant to cotrimoxazole(*34, 35*), was not affected by cotrimoxazole at week-84 (relative abundance ratio: 0.65, adjusted p=0.108) and was

increased in those continuing versus stopping cotrimoxazole at week-96 (4.48, adjusted p<0.001).

To understand the effect of cotrimoxazole on microbiome function, we quantified the abundance of full sets of genes involved in metabolic pathways across bacterial taxa. Only mevalonate pathway I, which influences neutrophil and monocyte recruitment and function, was consistently different between groups at both timepoints. Mevalonate pathway-associated genes were significantly less abundant in stool samples from children continuing cotrimoxazole (week-84 adjusted p=0.042, and week-96 adjusted p=0.019; **Fig. 3C**). Of the enzyme-encoding genes within mevalonate pathway I, those with identity to *Streptococcus parasanguinis* (5 enzymes, KEGG EC: 1.1.1.88 (hydroxymethylglutaryl-CoA (HMG-CoA) reductase), 2.3.3.10 (HMG-CoA synthase), 2.7.1.36 (mevalonate kinase) and 4.1.1.33 (diphosphomevalonate decarboxylase) and 5.3.3.2 (isopentenyl-diphosphate Delta-isomerase); adjusted p<0.05 at both timepoints) and *Streptococcus salivarius* (2 enzymes, KEGG EC: 1.1.1.88 and 5.3.3.2; adjusted p<0.05 at both timepoints) were significantly less abundant in the continue group (**Fig. 3C**), suggesting that continuation of cotrimoxazole reduces VGS metabolic function in the gut.

To confirm this metagenomic signature of VGS suppression by cotrimoxazole, we conducted high-resolution mapping of metagenome sequencing reads to Streptococci pangenome datasets using *PanPhlAn* software, which has a lower false-positive rate for species identification and better discrimination between samples containing the same versus different

bacterial genomes than *MetaPhlAn*(*36*). Of the 140 stool samples sequenced (both groups at week-84 and week-96), *PanPhlAn* identified 29 samples positive for any Streptococci (9 species present: *S. salivarius, S. parasanguinis, S. mutans, S. vestibularis, S. australis, S. infantarius, S. oligofermentans, S. pasteurianus,* and *S. sanguinis*) and, of these, 20 samples positive for at least one of the 4 VGS species identified using *MetaPhlAn* (7 at week-84 and 13 at week-96). *PanPhlAn* identified a lower percentage of VGS-positive samples on account of its higher species-level resolution (**Fig. 3D** and **E**). Six samples from children continuing and 14 samples from children stopping cotrimoxazole were confirmed VGS-positive across both timepoints, corroborating VGS suppression by cotrimoxazole. Individual VGS species were present less often in children continuing cotrimoxazole (**Fig. 3E**). Together, these findings show that continuing compared to stopping long-term cotrimoxazole does not affect global microbiome community composition, but does drive specific alterations in gut microbiome structure and function, with suppression of VGS and associated reductions in VGS mevalonate pathway genes.

Cotrimoxazole-induced changes in Streptococci reduce intestinal inflammation

We next tested whether these microbiome changes influenced HIV enteropathy. We compared levels of fecal inflammatory markers at week-84 and week-96 post-randomization to continue (n=37) or stop (n=38) cotrimoxazole. At week-84, fecal myeloperoxidase was significantly lower in children continuing versus stopping cotrimoxazole (median: 1694ng/mL versus 3178ng/mL, p=0.022; **Fig. 4A**), but there was no evidence of differences in neopterin, alpha-1-antitrypsin, or REG1β between groups (p>0.15, **fig. S4A**). At week-96, myeloperoxidase did not significantly differ between randomized groups (1262 versus

1473ng/mL, p=0.093; **Fig. 4B**) and there was no evidence of differences in neopterin, alpha-1-antitrypsin or REG1β (p>0.15, **fig. S4B**). Since myeloperoxidase is an abundant peroxidase enzyme in monocytes and neutrophils that perpetuates granulocyte activation(*37*) and both cell types home to the gut mucosa during HIV infection(*13*, *14*), these observations suggest that cotrimoxazole reduces innate immune cell activity in the gut.

Of the bacterial species suppressed by cotrimoxazole, *Streptococcus mutans*, *Streptococcus vestibularis*, *Streptococcus parasanguinis*, and *Haemophilus parainfluenzae* were positively associated with myeloperoxidase levels at week-96 (*Streptococcus* spp. summarized in **Fig. 4C**; analysis of all species in **fig. S5**), after adjustment for age, sex, and cotrimoxazole group. Myeloperoxidase was also positively associated with Pfam that were differentially abundant according to cotrimoxazole treatment: 5 with identity to *Streptococcus parasanguinis*, 2 to *Streptococcus salivarius*, 2 to *Haemophilus parainfluenzae*, and 1 to *Eubacterium bioforme* at week-96 (Pfam with identify to *Streptococcus* spp. summarized in **Fig. 4C**; analysis of all Pfam in **fig. S6**). Overall mevalonate pathway I abundance was significantly associated with higher myeloperoxidase at week-96 (adjusted p<0.001, **Fig. 4C**). Of the mevalonate pathway I enzymes that differed between randomized groups, only those with identity to *Streptococcus parasanguinis* (5 enzymes, adjusted p<0.001) and *Streptococcus salivarius* (2 enzymes, adjusted p<0.01) had a significant positive association with myeloperoxidase (**Fig. 4C**). We therefore show that all VGS components suppressed by cotrimoxazole (**Fig. 3C**) were positively associated with myeloperoxidase (**Fig. 4C**), suggesting that reduced VGS abundance and function contribute to lower intestinal inflammation among children continuing cotrimoxazole.

Cotrimoxazole blunts pro-inflammatory cytokine responses in vitro

Having established that cotrimoxazole reduces both systemic and intestinal inflammation, we next investigated whether cotrimoxazole has direct immunomodulatory properties. To isolate any direct effects of cotrimoxazole on immune cells from its impact on enteropathy and the microbiome, we optimized an *in vitro* model of whole blood cytokine responses to bacterial and fungal antigens: heat-killed *Salmonella typhimurium* (HKST), which activates immune cells via Toll-like receptor (TLR) 2, 4 and 5; purified *Escherichia coli* lipopolysaccharide (LPS), which engages TLR4; and the *Saccharomyces cerevisiae* cell-wall component zymosan, which engages TLR2 and dectin-1. Antigens engaging pattern recognition receptors were chosen to reflect microbial translocation, which drives systemic inflammation and immune activation in HIV infection(*7*, *15*, *18*, *33*). The cotrimoxazole dose was chosen to reflect maximum (high-dose; 8μg/mL trimethoprim and 200μg/mL sulfamethoxazole) and minimum (low-dose; 2μg/mL trimethoprim and 50μg/mL sulfamethoxazole) serum concentrations in HIV-positive patients taking cotrimoxazole(*38*). Laboratory cotrimoxazole preparations were confirmed to have antibiotic activity (**fig. S7A**) and doses did not reduce leukocyte viability in culture (**fig. S7B-D**).

Since the inflammatory milieu can affect immune cell responses, we obtained blood samples from three groups of U.K. adults (HIV-positive ART-treated (n=6), HIV-positive ART-naïve (n=10) and HIV-negative (n=8), **table S1**), with distinct baseline inflammatory profiles (**fig. S8**). There was no difference between groups in spontaneous cytokine production in 24h unstimulated cultures (**Fig. 5**).

High-dose cotrimoxazole significantly reduced HKST-, LPS- and zymosan-induced TNFα (**Fig 5A**) and IL-6 (**Fig 5B**) production relative to control treatment with drug diluent alone (dimethyl sulfoxide, DMSO) in ≥1 group. This was particularly evident for HKST- and LPS-induced TNFα and LPS- and zymosan-induced IL-6, which were significantly lower across all three clinical groups. LPS- and zymosan-induced TNFα and zymosan-induced IL-6 were also significantly reduced by low-dose cotrimoxazole in the HIV-positive ART-naïve group (**Fig 5A** and **B**). These observations confirm our hypothesis that cotrimoxazole directly modulates pro-inflammatory immune cell activation by pathogen antigens, both in HIV-positive and in HIV-negative individuals, independently of its effects on the microbiome or intestinal inflammation.

To determine the immune cell types modulated by cotrimoxazole, we evaluated intracellular TNFα production and surface expression of HLA-DR by monocytes and T cells during 6h PBMC culture with or without high-dose cotrimoxazole (gating strategy shown in **fig. S9**; antibodies in **table S2**). Cotrimoxazole reduced the proportion of TNFα+ monocytes after HKST stimulation relative to control-treated cultures in the HIV-negative group but not in the HIV-positive groups (**Fig. 5C**). Cotrimoxazole did not alter HKST-induced up-regulation of HLA-DR by monocytes (**Fig. 5D**). Cotrimoxazole also had no effect on the proportion of TNFα+ or HLA-DR+ CD4+ or CD8+ T cells after polyclonal stimulation with staphylococcal enterotoxin B (SEB; **Fig. 5C** and **D**). Thus, although cotrimoxazole reduces pro-inflammatory cytokine production by blood leukocytes and TNFα production by monocytes specifically, it did not directly reduce monocyte maturation or T cell activation.

Cotrimoxazole reduces IL-8 production by gut epithelial cells

The gut epithelium provides a barrier between the microbiota and mucosal immune cells, responds to TLR ligands, and produces leukocyte chemoattractants under inflammatory conditions; direct effects of cotrimoxazole on epithelial cell function could contribute to its anti-inflammatory effects. To isolate direct effects of cotrimoxazole on the epithelial barrier from its impact on leukocytes or the microbiome, we used transwell cultures of the Caco-2 human colonic epithelial cell-line as a well-established model of gut epithelium. We induced epithelial inflammation with IL-1β and evaluated the effect of cotrimoxazole on four epithelial functions: epithelial integrity (trans-epithelial resistance, TEER), epithelial cell death (%Lactose dehydrogenase (LDH) activity), apical-to-basal translocation of a fluorescent dye (%Lucifer Yellow passage, a proxy for gut-to-circulation microbial translocation), and production of the neutrophil chemoattractant IL-8 (**Fig. 6A**). We used high cotrimoxazole concentrations for these experiments to reflect the concentration found in the gut lumen following oral dosing, after first titrating cotrimoxazole in Caco-2 cultures to identify a dose that did not differ in cytotoxicity from DMSO controls (1mg/mL; **Fig. 6B**).

Cotrimoxazole treatment throughout Caco-2 growth did not significantly alter the rate of monolayer confluence (mean TEER/plate >800Ω; **Fig. 6C**), ΔTEER, %LDH activity or %Lucifer yellow passage under inflammatory conditions (1, 10, or 100μg/mL IL-1β for 24h; **Fig. 6D**). However, cotrimoxazole-treated monolayers produced significantly less IL-8 than control-treated cultures when the inflammatory stimulus was highest (100μg/mL IL-1β, p=0.003, **Fig. 6D**). Taken together, these experiments suggest that cotrimoxazole directly

inhibits IL-8 production by gut epithelial cells, which may contribute to reduced neutrophil recruitment to the intestinal mucosa under inflammatory conditions.

DISCUSSION

Inflammation drives morbidity and mortality in HIV infection. There is therefore interest in using anti-inflammatory agents with ART to improve clinical outcomes(*39-42*). Long-term cotrimoxazole prophylaxis is recommended for children and adults living with HIV in settings with high prevalence of malaria or invasive bacterial infections, although global coverage remains poor(*4, 43*). We show here that cotrimoxazole reduces systemic inflammation in ART-treated children in sub-Saharan Africa, and demonstrate several underlying mechanisms, including antibiotic effects on the gut microbiome and direct anti-inflammatory effects on leukocytes and gut epithelial cells. Synergy between antibiotic and anti-inflammatory pathways may explain the sustained clinical benefits of cotrimoxazole(*3, 27*) and provides an additional rationale for increasing cotrimoxazole coverage in sub-Saharan Africa.

Using samples from the ARROW trial, we show definitively, using the randomized stop-versus-continue design, that systemic inflammatory biomarkers (CRP and IL-6) are reduced by cotrimoxazole. . Pre-ART levels of CRP and IL-6, but not TNFα or sCD14, predicted mortality, WHO stage 4 clinical events, and poor CD4 reconstitution in ARROW; a 2-fold increase in CRP or IL-6 was independently associated with 19% and 54% increased risk, respectively(*5*). Based on these predictions, the reductions in CRP and IL-6 among children continuing cotrimoxazole would reduce the relative risk of adverse outcomes by 13% and 11%, respectively. HIV-positive children have lower absolute mortality risk after starting ART; however, our estimates highlight that the additive anti-inflammatory benefits of

continuing cotrimoxazole are clinically meaningful. Previous studies suggest that Systemic inflammatory mediators are better predictors of poor clinical outcomes than T cell activation among HIV-positive people in resource-limited settings(*44*). Our assessment of circulating immune cell activation was limited to HLA-DR expression on CD4+ T cells, which did not differ between randomized groups. However, we observed lower percentages of proliferating naïve CD4+ T cells among children continuing cotrimoxazole, which we interpret as beneficial, since elevated CD4+ T cell proliferation without a corresponding increase in total counts leads to depletion of the naïve T-cell pool(*45*).

We went on to explore potential explanatory mechanisms. Systemic inflammation in HIV infection is partly driven by enteropathogen carriage and chronic enteropathy(*11-13, 15*). Using stool samples from a subset of ARROW children, we demonstrated that VGS were less abundant at week-84 and week-96 post-randomization in those continuing cotrimoxazole. Since speciation of VGS is challenging, we confirmed these differences using high-resolution mapping of metagenome sequencing reads to Streptococcal pangenomes databases(*36*). Cotrimoxazole effects on VGS are particularly striking because global microbiome community composition did not differ between randomized groups, likely because all children had received cotrimoxazole for median 2 years pre-randomization(*27*). VGS are a heterogeneous group of bacteria, which can be both commensal and pathogenic(*46*). They are found throughout the healthy human gut(*47, 48*) and are enriched in stool samples from children with stunting(*49*), a form of chronic malnutrition associated with systemic inflammation(*50*). VGS express several immune-stimulatory antigens that may drive intestinal inflammation, and potently trigger innate immune cell cytokine production *in*

vitro(*51*). In contrast to changes in VGS, we found no evidence for suppression of Enterobacteriaceae, which include pathogens causing severe bacterial infections in sub-Saharan Africa(*34*, *35*). Our microbiome analyses focused on later timepoints post-randomization, due to stool sample availability; there may plausibly be additional cotrimoxazole-driven changes at earlier timepoints and at other anatomic sites.

Children randomized to continue cotrimoxazole had lower fecal myeloperoxidase, an antimicrobial peroxidase enzyme abundant in neutrophils, and a biomarker of enteropathy(*52*). Of the cotrimoxazole-affected VGS, *S. mutans*, *S. parasanguinis* and *S. vestibularis* were positively associated with myeloperoxidase, suggesting that sub-clinical antibiotic effects of cotrimoxazole on VGS reduce intestinal inflammation. This does not appear to be a universal characteristic of antibiotic treatment since suppression of gut-resident gram-positive bacteria with vancomycin in rhesus macaques subsequently infected with SIV did not reduce IL-6 or CD4+ T cell activation in mesenteric lymph nodes(*53*). It is likely that timing of treatment, baseline microbiome, ART history, intercurrent infections, and antibiotic specificity influence the relationship between antibiotic prophylaxis, gut microbiome and enteropathy.

Functional analysis of ARROW stool samples identified a metagenomic signature of mevalonate metabolism, predominantly mapping to VGS, which was positively associated with fecal myeloperoxidase and suppressed by cotrimoxazole. The mevalonate pathway is one of two metabolic processes that produce isoprenoids, naturally-occurring organic

precursors in eukaryote cholesterol and prokaryote cell wall peptidoglycan (a TLR2 ligand) synthesis (*54*). Several *in vitro* studies indicate that inhibition of mevalonate pathway enzymes impairs innate leukocyte recruitment and pro-inflammatory cytokine responses, providing a precedent for how inhibiting VGS mevalonate metabolism might influence HIV enteropathy. For example, inhibiting farnesyl pyrophosphate synthesis reduces neutrophil priming by IL-8(*55*), and inhibiting HMG-CoA reductase reduces monocyte IL-6 and IL-8(*56*) and neutrophil trans-epithelial migration(*39, 42*). HMG-CoA reductase with identity to *Streptococcus parasanguinis* and *Streptococcus salivarius* was among the cotrimoxazole-suppressed mevalonate pathway enzymes identified.

Leukocytes are an abundant source of pro-inflammatory cytokines. Levels of circulating microbial products that could trigger these pathways are elevated during HIV infection, including the TLR4 ligand LPS(*10, 15*). We developed an *in vitro* model of leukocyte activation by TLR ligands to isolate direct anti-inflammatory effects of cotrimoxazole from its antibiotic effects, using blood samples from HIV-negative and HIV-positive U.K. adults not receiving cotrimoxazole. Although this cohort differed in age, geographic location, likely HIV clade and co-morbidities compared to children in ARROW, these *in vitro* experiments provide proof-of-concept that physiologically-relevant cotrimoxazole doses consistently inhibited whole blood TNFα and IL-6 production elicited via TLR2, 4 and 5. Collectively, these findings suggest that modulation of innate pro-inflammatory cytokine production is a property of cotrimoxazole *per se*, affects multiple innate signaling pathways, and occurs independently of its antibiotic effects, HIV-driven inflammation or ART exposure. Intracellular cytokine staining suggested that monocyte rather than T cell cytokine production

was most affected by cotrimoxazole. Our demonstration of direct modulation of pro-inflammatory cytokine production by human leukocytes clarifies a longstanding theory that cotrimoxazole modulates immune responses in mice via an undefined mode-of-action(*22*), for which subsequent *in vitro* models have yielded opposing conclusions for innate and adaptive immune cells (*23-26*). Although these immunomodulatory effects were quantitatively subtle, our relative risk estimates in ARROW indicate that even small reductions in inflammatory markers may improve clinical outcomes(*5*). The pharmacology of cotrimoxazole-mediated immunosuppression, its interaction with TLR signaling and potential therapeutic value in other inflammatory disorders are yet to be established.

Cotrimoxazole reduced production of the neutrophil chemoattractant IL-8 by gut epithelial cells *in vitro*. This is a putative pathway through which cotrimoxazole could directly contribute to reduced neutrophil recruitment and myeloperoxidase production in the gut mucosa. Cotrimoxazole did not alter epithelial characteristics associated with barrier function *in vitro*; however, it remains possible that cotrimoxazole alters these pathways *in vivo* by affecting gut barrier components such as mucus(*19*) and tight junction proteins(*53*), which we did not model. Primary epithelial cells and biopsies, which would better mimic trans-epithelial transport *in vivo*, were not available from ARROW. Since VGS express abundant TLR2 ligands and Caco-2 have limited TLR2 expression(*57*), alternative epithelial models are required to explore inter-relationships between cotrimoxazole, VGS metabolism and epithelial barrier function.

Our study raises the possibility that antibiotics other than cotrimoxazole may confer anti-inflammatory benefits that contribute to their impact at scale, including the recent finding of reduced child mortality following mass administration of azithromycin in sub-Saharan Africa(*58*). Accessory benefits from antibiotics are important considerations in the debate around antimicrobial stewardship, particularly in settings where antimicrobial resistance is already high and in conditions such as HIV, where chronic inflammation combines with intercurrent infection to exacerbate clinical outcomes. Whether cotrimoxazole has clinical benefits for HIV-positive people in high-income settings, where long-term cotrimoxazole prophylaxis is not currently recommended and ART alone does not fully prevent pathology, warrants further study. Recognition of its anti-inflammatory benefits should drive renewed efforts for universal cotrimoxazole coverage to improve clinical outcomes for all people living with HIV in sub-Saharan Africa.

MATERIALS & METHODS

Study design

The study objective was to determine whether cotrimoxazole has anti-inflammatory effects, and to elucidate underlying mechanisms. Experimental work comprised: 1) analysis of longitudinal blood samples (using ELISA and flow cytometry) and stool samples (using ELISA and whole metagenome sequencing) collected from HIV-positive Ugandan and Zimbabwean children randomized to continue versus stop open-label cotrimoxazole in the ARROW trial(*27*), until 16th March 2012; and, 2) *in vitro* cotrimoxazole treatment using blood samples from U.K. adults (ELISA and flow cytometry) and epithelial cell-line (Caco-2) cultures. Full details are in **Supplementary Materials and Methods**.

Within ARROW, children/adolescents (median age: 7.9 years, IQR: 4.6, 11.1) who had been receiving ART and once-daily cotrimoxazole prophylaxis (200mg of sulfamethoxazole and 40mg of trimethoprim, 400mg sulfamethoxazole/80mg trimethoprim, or 800mg sulfamethoxazole/160mg trimethoprim for body weight 5-15, 15-30, or >30 kg, respectively) for >96 weeks at four sites in Uganda and Zimbabwe, were randomized to stop (n=382) or continue (n=386) cotrimoxazole(*27, 59*). Children with a history of *Pneumocystis jirovecii* pneumonia were excluded(*27*). 98% of children enrolled into ARROW during the last 6 months of recruitment were also included in an immunology sub-study; additional assays were conducted for these children and for a random 23% sample of all remaining non-immunology sub-study children (*5*). The current analysis included children with available baseline plasma of sufficient volume to measure inflammatory biomarkers (stop n=149,

continue n=144). Stool samples were collected at week-84 and week-96 post-randomization from a subgroup of children in Zimbabwe to assay intestinal inflammation. Total DNA was extracted from 150mg stool for whole metagenome sequencing (stop n=36, continue n=36).

Blood was collected from 8 HIV-uninfected adults, 6 HIV-positive adults on ART for ≥2 years, and 10 HIV-positive ART-naïve adults (**table S1**) who were not taking cotrimoxazole, for 24h whole blood culture and 6h PBMC culture with bacterial and fungal antigens. Pro-inflammatory cytokine responses were compared between parallel cultures treated with cotrimoxazole and volume-matched diluent without drug (DMSO).

Caco-2 monolayers were grown in transwell cultures as a gut epithelium model. Epithelial functions (integrity, cell death, translocation across the epithelium and chemokine production) were quantified after 24h stimulation with IL-1β and compared between cultures treated with cotrimoxazole or DMSO throughout growth, run in triplicate. Transwell cultures were repeated 3 times using separate Caco-2 passages. Data from individual transwells were excluded if monolayers were sub-confluent.

Ethics

ARROW (ISRCTN Registry# ISRCTN24791884) was approved by Research Ethics Committees in Uganda, Zimbabwe, and the U.K. Written informed consent from all caregivers and assent from participants (where appropriate) was obtained (*27, 59*). Approval for U.K. donor recruitment was provided by the National Health Service Research Authority

(IRAS project ID: 209553; Research Ethics Council reference: 17/WM/0018) and the Research Ethics Committee of Queen Mary University of London. All participants provided written informed consent.

Statistical analysis

For ARROW data, fold-change in geometric means between randomized groups were compared for continuous variables at each timepoint using standard regression models and globally across all timepoints using generalized estimating equations (GEE; normal distribution for log-transformed values), both with adjustment for recruitment center and baseline values, and assuming variation in treatment effect by timepoint. Proportions of children with HIV viral load <80 copies/mL were compared between randomized groups at each timepoint using Exact tests and globally across all timepoints using GEE (binomial distribution) with adjustment for recruitment center and assuming variation in treatment effect by timepoint. Relative risk projections for CRP and IL-6 differences between randomized groups were calculated from the output of models based on enrolment (i.e. pre-ART and pre-cotrimoxazole) biomarker levels in the ARROW immunology sub-cohort (*5*). GEE and Exact tests were conducted in *STATA* version 15.1 (StataCorp LLC). Concentrations of fecal inflammatory markers and serum protein (Shapiro-Wilk test for normality, $p<0.05$) were compared between randomized groups using Mann-Whitney U test in *Prism* version 7.02 (GraphPad).

For microbiome sequencing data, differences in species relative abundance and diversity

between randomized groups were evaluated at each timepoint by intention-to-treat analysis using linear regression models fitted against natural log-transformed inverse Shannon species-level alpha-diversity indices. Species-level beta diversity was evaluated using the Bray–Curtis dissimilarity index, and visualized using NMDS. Differences in relative abundance of species, Pfam, metabolic pathways, and enzymes (microbiome characteristics) were evaluated at each time point by intention-to-treat analysis using separate zero-inflated beta regression models fitted against relative abundances for each microbiome characteristic. Cotrimoxazole treatment effect was the ratio of relative microbiome characteristic abundance in continue versus stop groups. P-values were adjusted for multiple comparisons to maintain the FDR significance level (α=0.05)(*60*). Only differentially abundant microbiome characteristics with consistent significant differences between groups at both week-84 and week-96 were interpreted as causally related to cotrimoxazole continuation. Rank-based regression models were fitted against fecal myeloperoxidase concentration adjusted for age, sex, and randomized group, with FDR adjustment for multiple comparisons. Microbiome analyses were conducted in *R* version 3.3.2. *VEGAN*(*61*) was used to calculate Shannon diversity, Bray-Curtis dissimilarity and NMDS. *Gamlss* was used for zero-inflated beta regression(*62*). *Rfit* was used for rank-based regression(*63*).

For U.K. adults, continuous variables were compared between groups using unpaired Kruskall-Wallis tests. Comparisons between drug treatments were only conducted for responses that were significantly up-regulated in antigen-stimulated cultures without drug treatment versus un-stimulated cultures without drug treatment (paired Wilcoxon test, $p<0.05$). Comparisons between drug treatments used Freidman tests with post-hoc pair-wise

comparisons via uncorrected Dunn's test; post-hoc tests were only conducted where the global test was statistically significant. Caco-2 read-outs (TEER, ΔTEER, % LDH activity, % Lucifer Yellow passage, and IL-8; Shapiro-Wilk test for normality, $p>0.05$) were compared between cotrimoxazole-treated and DMSO-treated cultures using paired two-tailed t-tests. All analyses were conducted using *Prism*.

SUPPLEMENTAL MATERIALS

Materials and Methods

Figure S1. Cotrimoxazole alters circulating CD4+ T cell phenotype in HIV infection.

Figure S2. Fecal bacterial species that differ between HIV-positive ART-treated Zimbabwean children randomized to continue versus stop cotrimoxazole prophylaxis.

Figure S3. Protein families that differ between stool samples from HIV-positive ART-treated Zimbabwean children randomized to continue versus stop cotrimoxazole prophylaxis.

Figure S4. Fecal biomarkers of enteropathy that were unaffected by continuing versus stopping cotrimoxazole prophylaxis.

Figure S5. Associations between all fecal bacterial species that differed between HIV-positive children randomized to continue versus stop cotrimoxazole prophylaxis and fecal myeloperoxidase.

Figure S6. Associations between all fecal Pfam that differed between HIV-positive children randomized to continue versus stop cotrimoxazole prophylaxis and fecal myeloperoxidase.

Figure S7. Optimization of in vitro blood leukocyte activation and cotrimoxazole treatment conditions.

Figure S8. HIV-positive adults have greater systemic inflammation, monocyte and T cell activation than HIV-negative adults.

Figure S9. Flow cytometry gating strategy for analysis of monocyte and T cell intracellular cytokine responses.

Table S1. Characteristics of HIV-negative and HIV-positive U.K. adult volunteers

Table S2. Details of fluorophore-conjugated antibody combinations used for flow cytometry analysis of PBMC from HIV-negative and HIV-positive adults.

Data file S1. Primary data for *in vitro* cotrimoxazole treatment assays.

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ACKNOWLEDGEMENTS

We thank participants, caregivers, and staff from all study centers; ViiV Healthcare/GlaxoSmithKline who donated ART and funded ARROW viral loads; Ministries for Health in Uganda and Zimbabwe who provided cotrimoxazole for ARROW; Margaret Govha, Sandra Rukobo, Macklyn Kihembo, Lydia Nakiire and Hannah Poulsom for laboratory work in ARROW; Anele Waters, Sarah Murphy and James Hand for U.K. participant recruitment; and Aine McKnight who hosted U.K. laboratory work.

FUNDING

Supported by Wellcome Trust (grant# 093768/Z/10/Z and 108065/Z/15/Z to A.J.P. with sub-contract to A.R.M; and 206225/Z/17/Z to C.D.B, co-funded by The Royal Society); Canadian Institutes of Health Research (grant to E.K.G.); Medical Research Council (MRC; grant# G0300400 to A.J.P., V.M., M.B-D., A.K., N.K., D.M.G., A.S.W. and G1001190 to A.J.P., V.M., M.B-D., A.K., N.K., D.M.G., A.S.W.); Department for International Development under MRC/DFID Concordat agreement and EDCTP2 programme supported by the European Union; MRC Clinical Trials Unit at UCL (A.J.S., M.J.S., D.M.G., A.S.W.; grant# MC_UU_12023/26).

AUTHOR CONTRIBUTIONS

C.D.B. conceptualized and implemented *in vitro* work, analyzed data, prepared figures, recruited donors and led manuscript preparation; E.K.G. conceptualized and implemented microbiome analysis, prepared figures and led manuscript preparation; G.P., A.S. and

C.B. implemented ARROW biomarker assays; L.T. recruited U.K. donors with oversight from J.R.D. and C.D.B.; L.B. and Y.K. assisted C.D.B. with whole blood culture and Caco-2 assays; N.C. supported Caco-2 experiments; D.M.G. and A.S.W. conceptualized and managed ARROW; N.K., A.J.P., A.S.W., D.M.G., M.J.S. and A.J.S. conceptualized and managed ARROW immunology work; M.B-D., V.M., J.L. and A.K. undertook ARROW clinical management; K.M. managed ARROW assays in Zimbabwe; M.G. and H.G. assisted E.K.G. with microbiome assays; C.P. supported U.K.-based experiments; T.J.E. and A.R.M. conducted *PanPhlAn* analysis; A.S.W. and A.J.S. conducted ARROW statistical analysis and prepared figures; A.J.P. and A.R.M. conceptualized the study and had primary responsibility for the manuscript. All authors read and contributed to the manuscript and approved submission. C.D.B. and E.K.G. contributed equally. A.R.M. and A.J.P. contributed equally.

COMPETING INTERESTS

The authors declare no competing interests.

DATA AND MATERIALS AVAILABILITY

All data associated with this study are presented in the paper or Supplementary Materials.

FIGURES

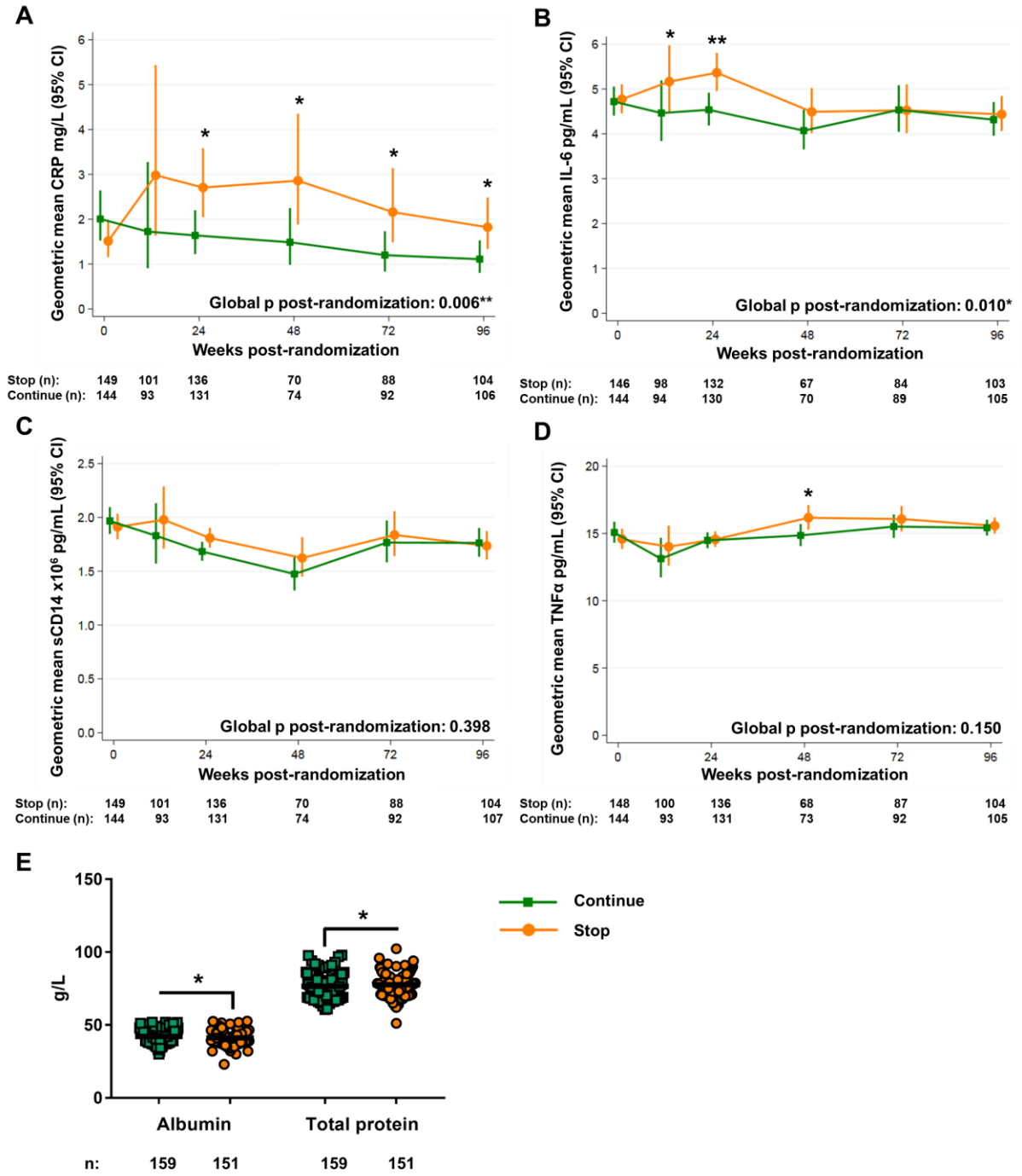
A
Geometric mean CRP mg/L (95% CI)
Global p post-randomization: 0.006**
Weeks post-randomization
Stop (n): 149 101 136 70 88 104
Continue (n): 144 93 131 74 92 106
B
Geometric mean IL-6 pg/mL (95% CI)
Global p post-randomization: 0.010*
Weeks post-randomization
Stop (n): 146 98 132 67 84 103
Continue (n): 144 94 130 70 89 105
C
Geometric mean sCD14 x10⁶ pg/mL (95% CI)
Global p post-randomization: 0.398
Weeks post-randomization
Stop (n): 149 101 136 70 88 104
Continue (n): 144 93 131 74 92 107
D
Geometric mean TNFα pg/mL (95% CI)
Global p post-randomization: 0.150
Weeks post-randomization
Stop (n): 148 100 136 68 87 104
Continue (n): 144 93 131 73 92 105
E
g/L
Albumin
Total protein
n: 159 151 159 151
Continue
Stop

Figure 1. Systemic inflammation is lower among HIV-positive children randomized to continue daily oral cotrimoxazole prophylaxis. Geometric mean concentrations of (**A**) CRP, (**B**) IL-6, (**C**) TNFα, and (**D**) sCD14 in plasma of HIV-positive children who had been receiving ART and cotrimoxazole for ≥96 weeks and were then randomized to stop (orange circles) or continue (green squares) cotrimoxazole. Randomized groups were compared across timepoints using generalized estimating equations and at individual timepoints using standard regression models (normal distribution for log-transformed values), adjusted for center and baseline concentrations (global p; **A-D**); *p<0.05, **p<0.01 ***p<0.001. (**E**) Serum protein concentrations at week-48 post-randomization; horizontal bars indicate means. Comparisons between groups by Mann-Whitney U test; *p<0.05, **p<0.01 ***p<0.001.

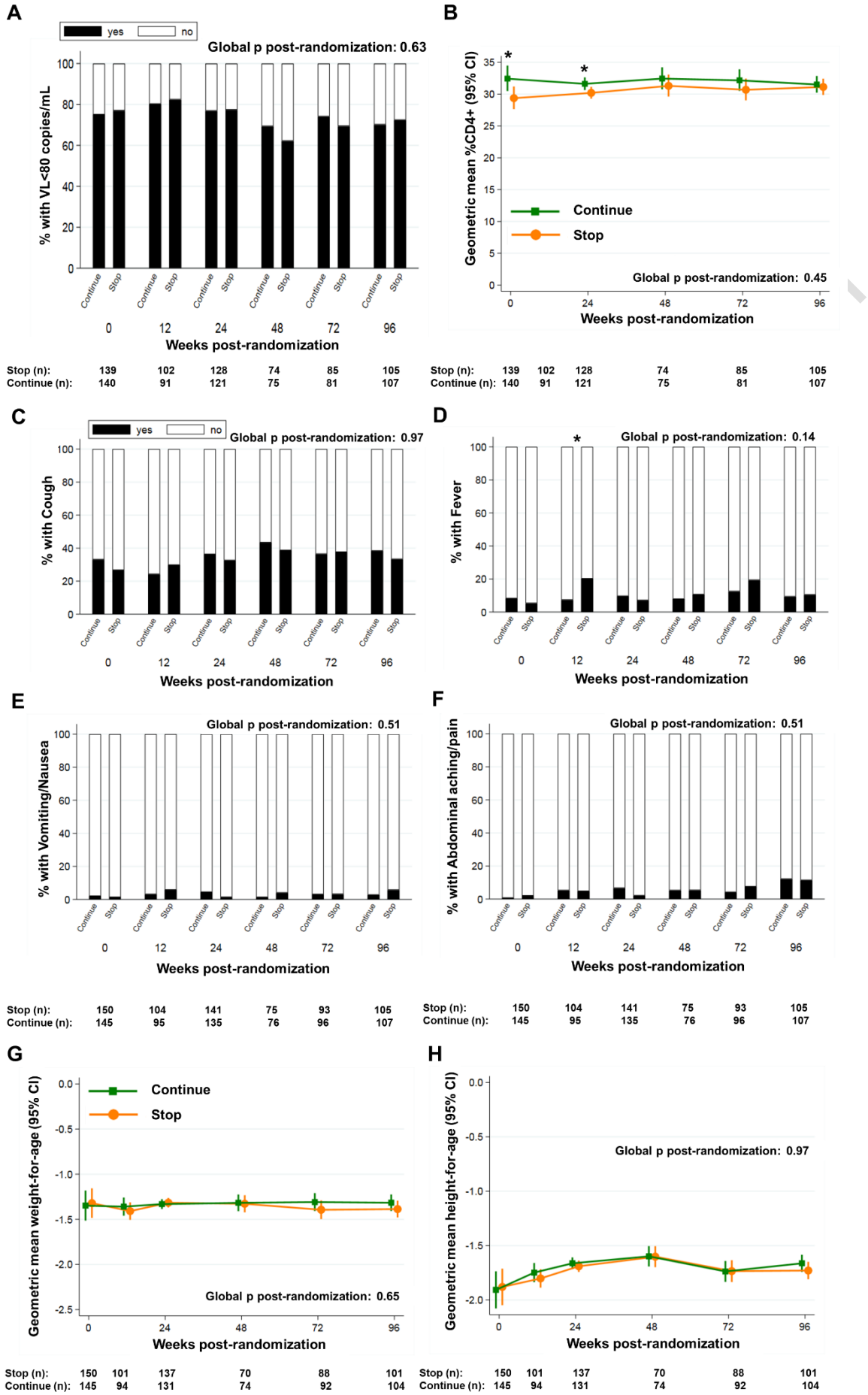
A
yes
no
Global p post-randomization: 0.63
% with VL<80 copies/mL
Continue
Stop
0 12 24 48 72 96
Weeks post-randomization
Stop (n): 139 102 128 74 85 105
Continue (n): 140 91 121 75 81 107
B
Geometric mean %CD4+ (95% CI)
Continue
Stop
Global p post-randomization: 0.45
Weeks post-randomization
Stop (n): 139 102 128 74 85 105
Continue (n): 140 91 121 75 81 107
C
yes
no
Global p post-randomization: 0.97
% with Cough
Weeks post-randomization
D
Global p post-randomization: 0.14
% with Fever
Weeks post-randomization
E
Global p post-randomization: 0.51
% with Vomiting/Nausea
Weeks post-randomization
Stop (n): 150 104 141 75 93 105
Continue (n): 145 95 135 76 96 107
F
Global p post-randomization: 0.51
% with Abdominal aching/pain
Weeks post-randomization
Stop (n): 150 104 141 75 93 105
Continue (n): 145 95 135 76 96 107
G
Geometric mean weight-for-age (95% CI)
Continue
Stop
Global p post-randomization: 0.65
Weeks post-randomization
Stop (n): 150 101 137 70 88 101
Continue (n): 145 94 131 74 92 104
H
Geometric mean height-for-age (95% CI)
Global p post-randomization: 0.97
Weeks post-randomization
Stop (n): 150 101 137 70 88 101
Continue (n): 145 94 131 74 92 104

Figure 2. Cotrimoxazole effects on systemic inflammation are not solely due to differences in HIV disease progression, symptomatic infections, or nutritional status.

(**A**) Percentage of children with viral load <80 copies/mL; (**B**) geometric mean percentage CD4+ T cells; mean proportions of children with caregiver-reported (**C**) cough, (**D**) fever, (**E**) vomiting/nausea and (**F**) abdominal pain; geometric mean (**G**) weight-for-age and (**H**) height-for-age Z-scores in children randomized to continue versus stop cotrimoxazole prophylaxis (n per group shown under each graph). Randomized groups were compared by generalized estimating equations across timepoints (global p) and at individual timepoints using standard regression models (binomial distribution for viral load; normal distribution for log-transformed values) adjusted for recruitment center; *p<0.05, **p<0.01 ***p<0.001.

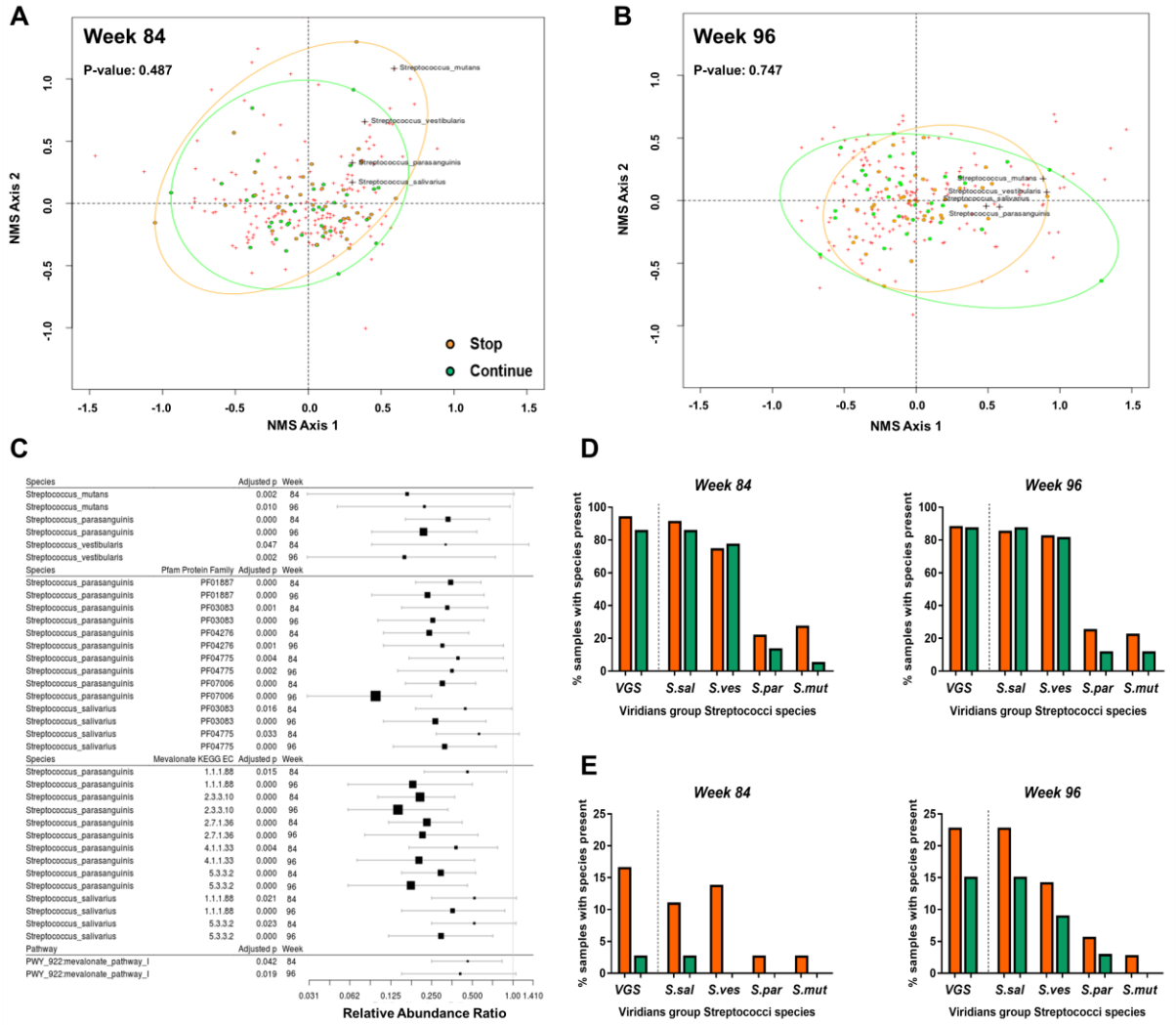

Figure 3. Continuation of cotrimoxazole suppresses the abundance and function of viridans group Streptococci in stool samples from HIV-positive children. Non-metric multidimensional scaling plots of the Bray–Curtis dissimilarity index for stool samples from 72 HIV-positive Zimbabwean children randomized to stop (orange) versus continue (green) cotrimoxazole at (**A**) week-84 and (**B**) week-96 post-randomization. Red crosses indicate individual bacterial species irrespective of randomized group; VGS species that consistently differed between randomized groups are labelled. Randomized groups were compared by

permutation tests. (**C**) Effect size plots of relative abundance ratios (±95% confidence interval) for all *Streptococcus* spp. and their protein families (Pfam) and mevalonate pathway-associated genes (KEGG EC), and metabolic pathways (all bacterial species) that significantly differed between randomized groups at both week-84 and week-96 in FDR-adjusted zero-inflated beta regression. Identities for Pfam and KEGG EC were established using *HUMANn2* against the UniRef90 database. Relative abundance ratio <1.0 indicates lower relative abundance in children who continued versus stopped cotrimoxazole. Vertical line indicates null value. Size of square is inversely proportional to p-value. Percentage of samples positive for any of the four VGS or individual species according to (**D**) *MetaPhlAn* and (**E**) *PanPhlAn* analysis at week-84 (continue n=36, stop n=36) and week-96 (continue n=33, stop n=35)

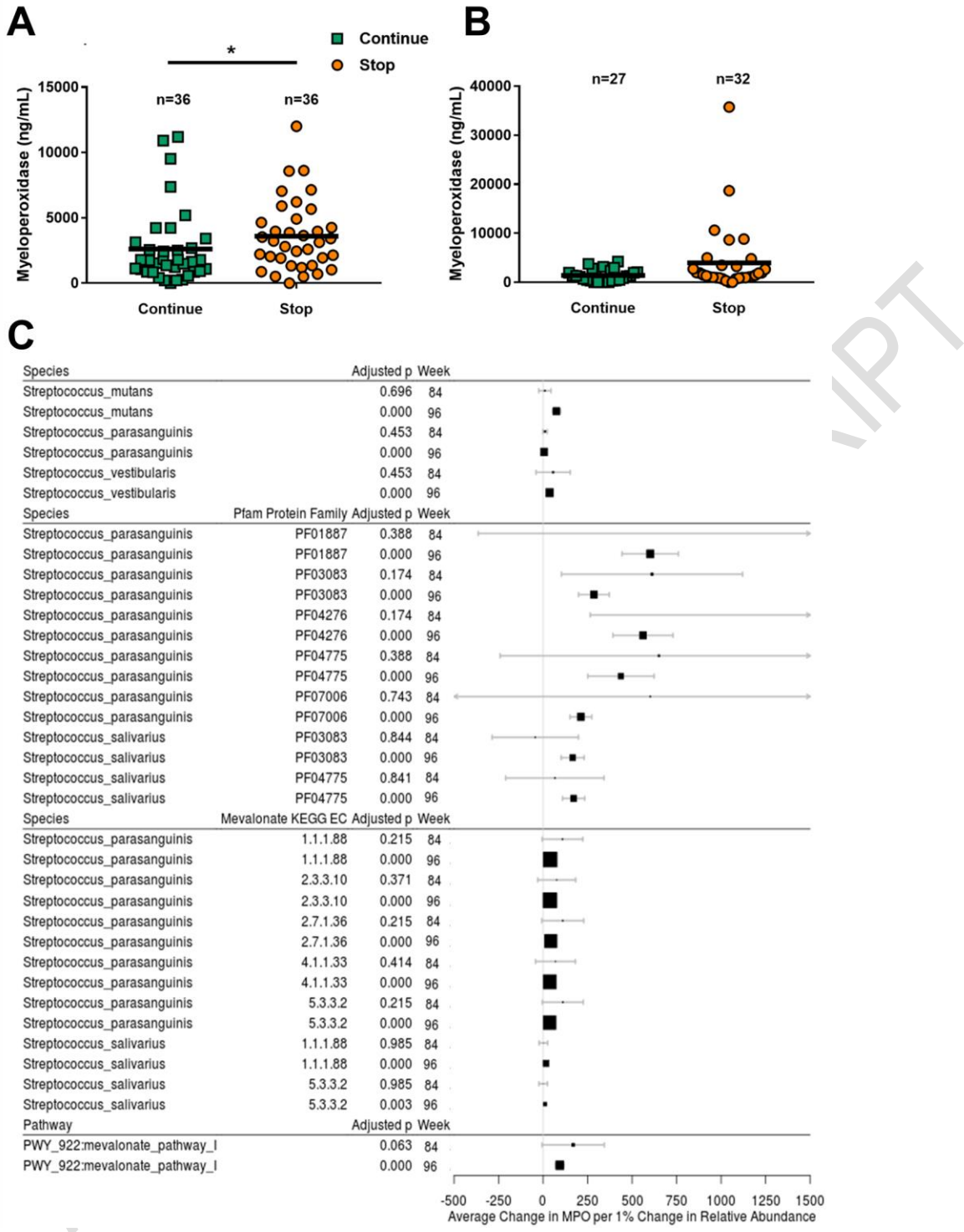

Figure 4. Intestinal inflammation in HIV-positive children is associated with gut-resident viridans group Streptococci that are suppressed by continuation of cotrimoxazole. Myeloperoxidase at (**A**) week-84 and (**B**) week-96 in stool samples from HIV-positive Zimbabwean children randomized to stop versus continue cotrimoxazole.

Randomized groups compared by Mann-Whitney U test; *p<0.05, horizontal lines indicate median. (**C**) Effect size plots showing average change in myeloperoxidase per 1% change in relative abundance (±95% confidence interval) for all *Streptococcus* spp. and their protein families (Pfam) and mevalonate pathway-associated genes (KEGG EC), and metabolic pathways (all bacterial species) that significantly differed between randomized groups at both week-84 and week-96 in FDR-adjusted zero-inflated beta regression (**Fig. 3C**). Identities for Pfam and KEGG EC were established using *HUMANn2* against the UniRef90 database. Average change >1.0 indicates increase in myeloperoxidase with increased abundance. Vertical line indicates null value. Size of square inversely proportional to p-value.

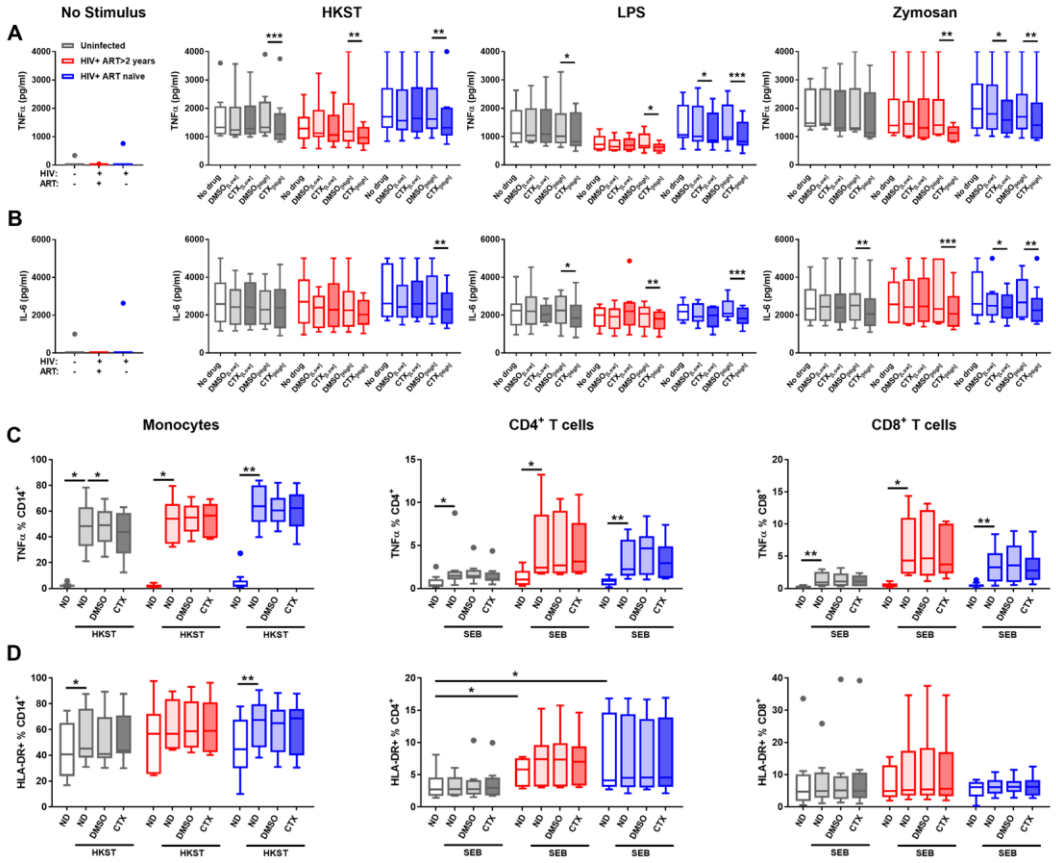

Figure 5. Cotrimoxazole inhibits *in vitro* pro-inflammatory cytokine responses to bacterial and fungal antigens. Tukey boxplots of (**A**) TNFα and (**B**) IL-6 concentrations in supernatants from whole blood cultures without antigen (No Stimulus), with heat-killed *Salmonella typhimurium* (HKST), lipopolysaccharide (LPS); or zymosan. Cultures were treated with low-dose cotrimoxazole ($CTX_{[Low]}$: 2 μg/mL trimethoprim, 50 μg/mL sulfamethoxazole), high-dose cotrimoxazole ($CTX_{[High]}$: 8 μg/mL trimethoprim, 200 μg/mL sulfamethoxazole) or volume-matched controls ($DMSO_{[Low]}$, $DMSO_{[High]}$). Proportions of monocytes (left), CD4+ (center) and CD8+ T-cells (**C**) producing TNFα and (**D**) expressing HLA-DR after 6h PBMC culture with HKST or staphylococcal enterotoxin B (SEB). Grey bars indicate HIV-negative (n=8); red indicate HIV-positive ART-treated (n=6); and blue

indicate HIV-positive ART-naïve group (n=10). Cytokine concentrations in cotrimoxazole-treated cultures are indicated by darker shading. Drug treatments compared within groups by Freidman tests with post-hoc uncorrected Dunn's tests; *p<0.05, **p<0.01, ***p<0.001.

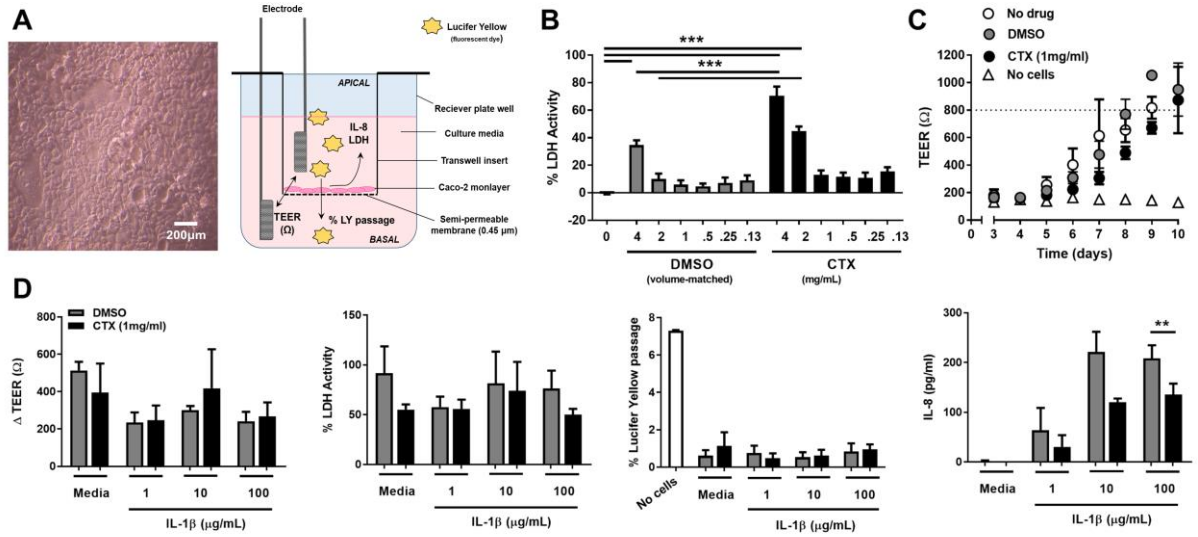

Figure 6. Cotrimoxazole reduces *in vitro* IL-8 production by gut epithelial cells under inflammatory conditions. (**A**) Light microscopy of confluent Caco-2 monolayer (200 µm scale bar) and diagram showing transwell culture model. (**B**) Percentage lactose dehydrogenase activity relative to lysed cells (%LDH) of Caco-2 cultured for 24h with titrated concentrations of cotrimoxazole (CTX; black bars) or volume-matched DMSO control (grey bars); %LDH compared to untreated controls and between volume-matched pairs of cotrimoxazole and DMSO by adjusted Tukey's test; ***p<0.001. (**C**) Daily trans-epithelial resistance (TEER) in transwell Caco-2 cultures without drug (white circles), 1 mg/mL cotrimoxazole (black circles) or DMSO (grey circles) relative to transwells without Caco-2 (no cells; white triangles); mean ±SEM, n=3 separate experiments. Dotted line indicates culture confluence (TEER≥800Ω) . (**D**) Epithelial cell functions (Δ TEER, % LDH, % apical-to-basal passage of Lucifer Yellow dye relative to transwells without Caco-2 cells, and IL-8 concentration in apical supernatants) of confluent Caco-2 monolayers treated with 1 mg/mL CTX or DMSO since seeding, then incubated with media alone (no stimulus) or IL-1β for 24h; mean ±SEM, n=3 separate experiments. Cotrimoxazole and DMSO-treatment compared by 2-tailed t-tests; *p<0.05, **p<0.01