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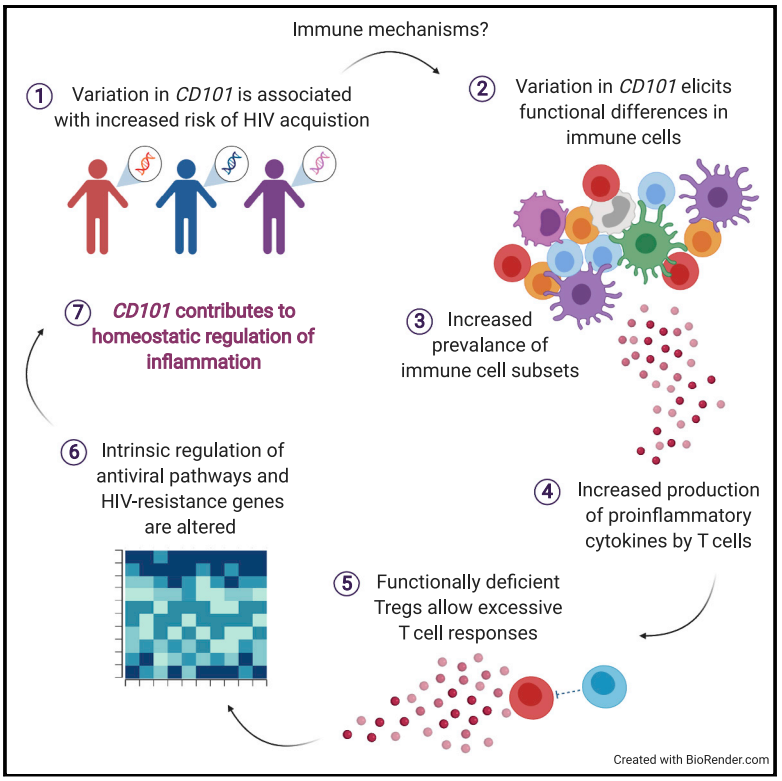
CD101 genetic variants modify regulatory and conventional T cell phenotypes and functions

Graphical abstract



Highlights

- Circulating immune cell frequencies are modified in individuals with *CD101* variants
- *CD101* variants are associated with increased proinflammatory T cell function
- Individuals with a particular *CD101* variant have reduced Treg cell suppression capacity
- *CD101* contributes to regulation of inflammation at steady state

Authors

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In brief

Genetic variation in *CD101* is associated with increased risk of HIV acquisition, but the mechanism linking the genotype and observed phenotype has not been established. Richert-Spuhler et al. demonstrate an immunologic link by uncovering an association between these *CD101* variants and homeostatic regulation of inflammation.

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CD101 genetic variants modify regulatory and conventional T cell phenotypes and functions

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SUMMARY

We recently reported that the risk of sexually acquired HIV-1 infection is increased significantly by variants in the gene encoding CD101, a protein thought to modify inflammatory responses. Using blood samples from individuals with and without these variants, we demonstrate that *CD101* variants modify the prevalence of circulating inflammatory cell types and show that *CD101* variants are associated with increased proinflammatory cytokine production by circulating T cells. One category of *CD101* variants is associated with a reduced capacity of regulatory T cells to suppress T cell cytokine production, resulting in a reduction in the baseline level of immune quiescence. These data are supported by transcriptomics data revealing alterations in the intrinsic regulation of antiviral pathways and HIV resistance genes in individuals with *CD101* variants. Our data support the hypothesis that CD101 contributes to homeostatic regulation of bystander inflammation, with *CD101* variants altering heterosexual HIV-1 acquisition by facilitating increased prevalence and altered function of T cell subsets.

INTRODUCTION

Inflammation is a double-edged sword in the host response to pathogens; some inflammatory responses enhance critical defense mechanisms to eliminate an invading pathogen, but others may provide unintended pathogen entry mechanisms by increasing the concentration of target cells for the pathogen or mediating harmful immunopathology. Clearly, efforts to develop an HIV-1 vaccine have been focused on the former effect: to guide the inflammatory response toward protection against HIV-1 infection. However, prior studies of natural host resistance to HIV-1 infection have suggested that host resistance to HIV-1 acquisition may be rooted in intrinsic differences in levels of bystander inflammation.[1] To identify host inflammatory pathways that affect HIV-1 acquisition risk, we previously applied whole-genome sequencing to African heterosexual individuals with epidemiologically quantified exposure to HIV-1, some of whom became HIV-1 infected and others who remained HIV-1 uninfected over follow-up. We identified functional variation in the *CD101* gene (GenBank: Gene ID 9398) as having the strongest genome-wide association with HIV-1 acquisition risk.[2] We identified three different single-nucleotide variants (SNV) that alter distinct amino acids in separate extracellular immunoglobulin (Ig)-like domains in the *CD101* coding region and are collectively associated with a significant increase in HIV-1 acquisition.[2] We also identified four missense variants that alter amino acids in the cytoplasmic domain of the protein (Figure S1) and are associated with an increase in HIV-1 acquisition risk that did not reach statistical significance after adjustment for multiple comparisons.[2]

Previous studies have shown that the *CD101* gene encodes a transmembrane protein highly expressed on T cells, monocytes, and dendritic cells.[3] Early studies have demonstrated that T cell receptor (TCR) cross-linking induces expression of CD101 on human T cells[4,5] and that proliferation of T cells in response to stimulation with anti-CD3 is inhibited by treatment with an anti-CD101 antibody,[3,6,7] suggesting that CD101 plays a role in TCR-dependent T cell activation. Specifically, it has been shown that treatment of $CD4^+CD101^+$ T cells with a presumed agonistic anti-CD101 antibody leads to inhibition of interleukin-2 (IL-2) production induced by CD3 stimulation.[7] This immunoregulatory

role of CD101 is further supported by a study demonstrating that a higher level of wild-type CD101 expression on murine regulatory T (Treg) cells is associated with an increased capacity to suppress effector T cells in a model of graft versus host disease;[8] however, expression of CD101 was not found to discriminate more suppressive Treg cells in individuals with rheumatoid arthritis.[6] CD101 is also highly expressed on activated mucosal tissue-resident memory T cells[9] and may regulate a balance between anti-inflammatory Treg cells and proinflammatory Th17 cells in mucosal tissue.[10] Recent work has also demonstrated that, during chronic viral infection, CD101 is highly expressed on terminally differentiated, exhausted, and highly dysfunctional circulating $CD8^+$ T cells,[11] contributing to the notion that CD101 is an immunoregulatory protein that may play a role in restraining T cells in various tissues and contexts, including inflammatory processes such as autoimmunity[12,13] and infectious diseases.[2]

Given the evidence that CD101 plays an immunoregulatory role, we sought to better understand mechanisms by which *CD101* missense variation modifies host responses that may be relevant to HIV-1 acquisition. Specifically, we hypothesized that candidate *CD101* Ig-like or cytoplasmic missense variants facilitate increased HIV-1 infection risk by mediating a heightened homeostatic inflammation set point through altered Treg cell function and activation of effector $CD4^+$ and $CD8^+$ T cells. To address this hypothesis, we used peripheral blood mononuclear cells (PBMCs) from individuals with and without these missense variants in *CD101* to assess the association of *CD101* variants with differences in phenotype, function, and transcriptomics profile of circulating conventional and Treg cells.

RESULTS

CD101 variants affect the phenotypes of circulating PBMCs

To test the effect of *CD101* genetic variation on immune cell frequency and phenotype, we identified cryopreserved PBMCs from 118 HIV-1-uninfected individuals (cases) with a single missense variant located in the *CD101* Ig-like domains (n = 85) or cytoplasmic domains (n = 33) and 117 HIV-1-uninfected individuals (controls) with no *CD101* functional variants (Figure S1). The epidemiologic characteristics of the sampled individuals were similar by variant (Table S1). We hypothesized that variants in different regions of *CD101* could differentially alter immune cell function. Thus, we compared immune cell phenotypes for PBMCs from cases with Ig-like-variants with controls and, separately, cases with cytoplasmic variants with controls. We used a broad panel of antibodies (Table S2) to characterize lymphocyte (T and B cell), monocyte, and dendritic cell (DC) subsets by high-parameter flow cytometry and analyzed the data by conventional manual gating (Figures S2–S4) and via the recently described full annotation using shape-constrained trees (FAUST) method,[14] which combines new algorithms for unbiased clustering, variable selection, and feature selection (Figure S6).

Phenotypic effect of Ig-like CD101 variants

These studies demonstrated multiple significant phenotypic differences across all cell type categories evaluated in individuals with Ig-like variants compared with those without functional *CD101* variants (Table 1; Figure S5; Tables S3 and S4). However, most differences were evident in the proportion of activated immune cell subsets expressing CD101, which were generally higher for T cell subsets from individuals with Ig-like variants (Figures 1A and 1B; Table 1; Tables S3 and S4). For example, *CD101* Ig-like variants were associated with an increased proportion of circulating $CD8^+$ T cells expressing CD101 (Figure 1B). FAUST analysis also identified an increased frequency of activated $CD101^+CD8^+$ $CD45RA^+CCR7^-CD38^+$ T effector memory (T_{EMRA}) cells as well as $CD101^+$HLA-DR^+-expressing CD4+ T cells among individuals with Ig-like variants (Figure S6A). Similarly, Ig-like variants were associated with elevated proportions of CD101-expressing subsets of Treg cells, including $CD25^+CD127^{lo}Foxp3^+$ cells with and without Helios expression (Figure 1C; Figures S5N and S5P). Moreover, $CD101^+$ Treg cells expressing the activation and proliferation markers CTLA-4, CD39, or Ki-67 were also increased in frequency among individuals with Ig-like variants (Figure 1C; Figures S5N and S5P; Table 1), suggesting a complex role of altered immunoregulatory capacity in the context of genetic *CD101* variation.

In contrast, proportions of $CD101^+CD141^+$ DCs, $CD101^+$ intermediate ($CD14^{++}CD16^+$) and non-classical monocytes ($CD14^+$ $CD16^{++}$), and $CD101^+$ B cells were reduced in Ig-like-cases compared with controls (Figures 1D–1F). Notably, the proportion of classical and intermediate monocytes expressing the HIV-1 co-receptor CCR5 was elevated for Ig-like variants compared with controls, irrespective of CD101 expression (Figure S5E). Additionally, expression of the chemokine receptor and HIV-1 co-receptor CXCR4 was increased on $CD1c^+$ DCs from individuals with Ig-like variants compared with controls (Figure 1D).

Interestingly, across all our investigated cell phenotypes, individual Ig-like variants contributed to the observed immune alterations to a greater or lesser degree. Specifically, the phenotypic effects of *rs12093834* were generally stronger than those of *rs17235773*, and no significant effects of rs3754112 were identified (Table S3).

Phenotypic effect of cytoplasmic CD101 variants

We also compared immune cell phenotypes using PBMCs from individuals without *CD101* functional variants with those from individuals with *CD101* cytoplasmic domain variants, either *rs34248572* or *rs150494742* (Tables S3 and S4). Cytoplasmic *CD101* variation was associated with few differences in the frequencies or activation of immune cell subsets in general (Figures S5G–S5L). However, when specifically considering frequencies of $CD101^+$ cells by manual gating, cases with *CD101* cytoplasmic variants had similar changes in Treg cell and DC subset phenotypes compared with those with Ig-like variants (Figure 2; Table S3). Additionally, FAUST identified a significant reduction in the frequency of $Helios^+Foxp3^+$ Treg cells among total Treg cells in individuals with cytoplasmic variants compared with no functional variants (Figure S6B), suggesting a potential deficit in immunoregulation among cases.[15] Moreover, we found a marked reduction in plasmacytoid DCs that co-expressed CCR5 and CD40 (Figure S6C), suggesting the possibility of altered plasmacytoid DC (pDC)-mediated antiviral immunity, including type I interferon responses—a mechanism relevant to host:pathogen interactions with HIV-1. Overall, cytoplasmic variants were associated with a smaller change in T cell and monocyte subsets than the alteration

Table 1. Phenotypic effects of the presence of Ig-like versus no Ig-like *CD101* variants

Cell type characteristics	No *CD101* functional variants (N = 117) (median ± σ)	≥ 1 *CD101* Ig-like variant (N = 92) (median ± σ)	p value[a]
CD4$^+$ T cells			
CD45RA$^-$CCR7$^-$CD101$^+$ effector mem of CD4$^+$ T cells	1.2 ± 1	2 ± 1.5	**<0.0001**
CD8$^+$ T cells			
%CD45RA$^+$CCR7$^-$CD101$^+$ effector of CD8$^+$ T cells	2.2 ± 2.9	5.4 ± 5.2	**<0.0001**
%CD45RA$^-$CCR7$^-$CD101$^+$ effector mem of CD8$^+$ T cells	9.7 ± 8.7	17.9 ± 10.7	**<0.0001**
%CD101$^+$ of CD8$^+$ T cells	37.9 ± 16.9	51 ± 14.8	**<0.0001**
%CXCR4$^+$CD101$^+$ of CD8$^+$ T cells	33.6 ± 14.2	45.9 ± 14.3	**<0.0001**
%CCR5$^+$CD101$^+$ of CD8$^+$ T cells	7.9 ± 6.4	13.7 ± 9.4	**<0.0001**
Regulatory T (Treg) cells			
%CD101$^+$ of Treg cells	13.7 ± 11.1	20.1 ± 9	**<0.0001**
%CTLA-4$^+$CD101$^+$ of Treg cells	8.3 ± 6.7	13.6 ± 6.7	**<0.0001**
%CD39$^+$CD101$^+$ of Treg cells	9.1 ± 8	13.4 ± 8	**0.0001**
%Ki-67$^+$CD101$^+$ of Treg cells	0.8 ± 0.8	1.3 ± 0.8	**<0.0001**
Dendritic cell (DC) characteristics			
%CXCR4$^+$ of CD1c$^+$ DCs	47.4 ± 32.7	58.8 ± 26.6	0.0063
%CXCR4$^+$CD101$^+$ of CD1c$^+$ DCs	55.6 ± 29.8	68.9 ± 18.1	**<0.0001**
%CD101$^+$ of CD141$^+$ DCs	29.9 ± 13.1	18.9 ± 10.2	**<0.0001**
%CD40$^+$CD101$^+$ of CD141$^+$ DCs	21 ± 11.1	12.4 ± 8.4	**<0.0001**
%CD80$^+$CD101$^+$ of CD141$^+$ DCs	1.7 ± 1.5	1 ± 0.9	**0.0002**
Monocyte characteristics			
%CCR5$^+$ of classic monocytes	27.1 ± 17.4	37.3 ± 18	**<0.0001**
%CCR5$^+$CD101$^+$ of classic monocytes	26.8 ± 17.3	36 ± 17.4	**0.0002**
%CCR5$^+$ of intermediate monocytes	43 ± 19.8	53.6 ± 16.9	**<0.0001**
%CD101$^+$ of intermediate monocytes	85.6 ± 10.2	79.2 ± 13.3	**0.0002**
%CCR5$^+$CD101$^+$ of intermediate monocytes	38.4 ± 18.1	45.4 ± 15.6	0.0034
%CD101$^+$ of nonclassic monocytes	25.2 ± 19.4	16.5 ± 14.6	**0.0002**
%CD40$^+$CD101$^+$ of nonclassic monocytes	16.9 ± 13.7	11 ± 10	**0.0004**
B cell characteristics			
%CD101$^+$ of B cells	6.6 ± 3.4	4.9 ± 2.4	**<0.0001**
%CD80$^+$CD101$^+$ of B cells	1.7 ± 1.4	1.2 ± 1	0.0094
%CD40$^+$CD101$^+$ of B cells	6.2 ± 3.1	4.7 ± 2.2	**<0.0001**

[a]Uncorrected p values are shown; p values in bold are significant after Bonferroni correction for 100 comparisons.

in these subsets associated with Ig-like variants. Individual portions of the CD101 receptor certainly serve functionally distinct purposes, and genetic alterations therein could be surmised to result in distinct immunologic consequences. However, variation at several distinct genetic locations in *CD101* altered immune outcomes, and further analysis of the CD101 protein structure will be useful in elucidating associated mechanisms.

CD101 variants are associated with conventional T cells that manifest an elevated proinflammatory response to stimuli

To examine changes in the functional potential of T cells associated with specific *CD101* genetic variants, we performed *ex vivo* stimulation assays in combination with intracellular cytokine staining (ICS; Figure S7). PBMCs were stimulated with an Epstein-Barr virus (EBV) lysate or with a combination of anti-CD3 and anti-CD28 to separately examine T cell responses to a viral versus a polyclonal stimulus. EBV was selected as the antigenic stimulus instead of HIV-1 because we previously showed that, in HIV-1-exposed, uninfected individuals, the prevalence of circulating T cells responding to HIV-1 antigens is low.[16] Further, EBV seroprevalence is high in African adults, and therefore most individuals were expected to have EBV-specific T cells present in the blood as a result of prior EBV exposure. This allowed us to quantify an individual's T cell response to viral antigen challenge through ICS, which we then stratified based on *CD101* variant status.

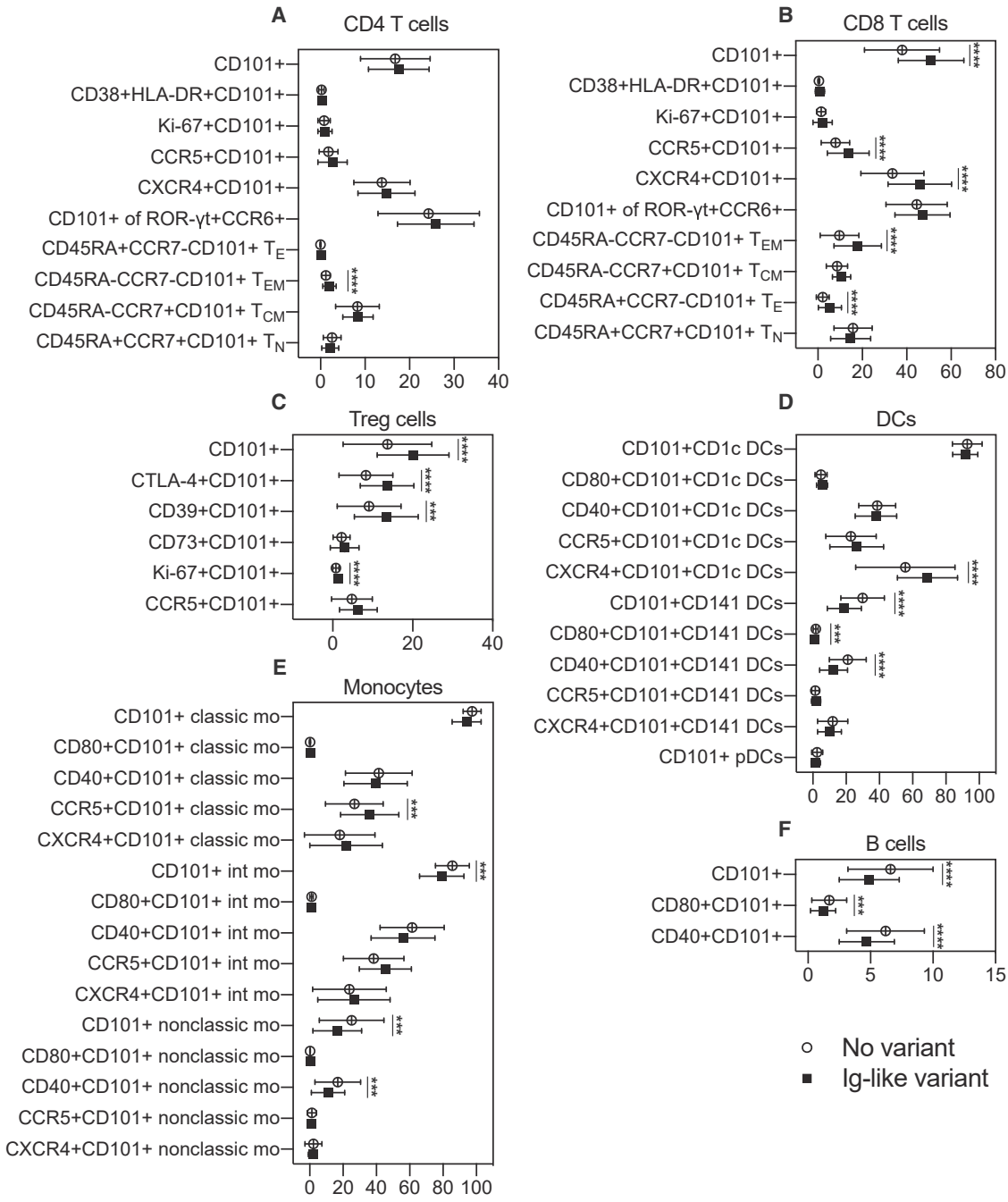


Figure 1. CD101$^+$ immune cell frequency and phenotype vary based on the presence of genetic variants in the Ig-like region of *CD101*

Circulating PMBCs from study participants possessing Ig-like variants in *CD101*, including *rs12093834*, *rs17235773*, and rs3754112 (N = 85), versus no functional variant (N = 117) were assessed by high-parameter flow cytometry for expression of various subset-specific and activation markers on CD101$^+$ cells.

(A–C) The mean and SD of the frequency of activation and subset-specific markers expressed on CD101$^+$CD4 T cells (A), CD101$^+$CD8 T cells (B), and CD101$^+$ Treg cells (C) for Ig-like variants versus controls.

(D) CD101$^+$ DCs are broken into CD1c$^+$ DCs and CD141$^+$ DCs alongside various activation markers within those subsets (D).

(E) The total frequencies of classic (CD14^{++}CD16$^-$), nonclassic (CD14$^+$CD16^{++}), and intermediate (CD14^{++}CD16$^+$) monocytes expressing CD101 alongside the frequencies of various activation markers within each indicated subset.

(F) CD101$^+$ B cells and expression of CD80 and CD40 among CD101$^+$ B cells.

Data points represent the mean and SD of biologic replicates as indicated by N per group. Two-sample t tests were performed with Bonferroni correction for 100 comparisons to determine significance. ***p < 0.001; ****p < 0.0001.

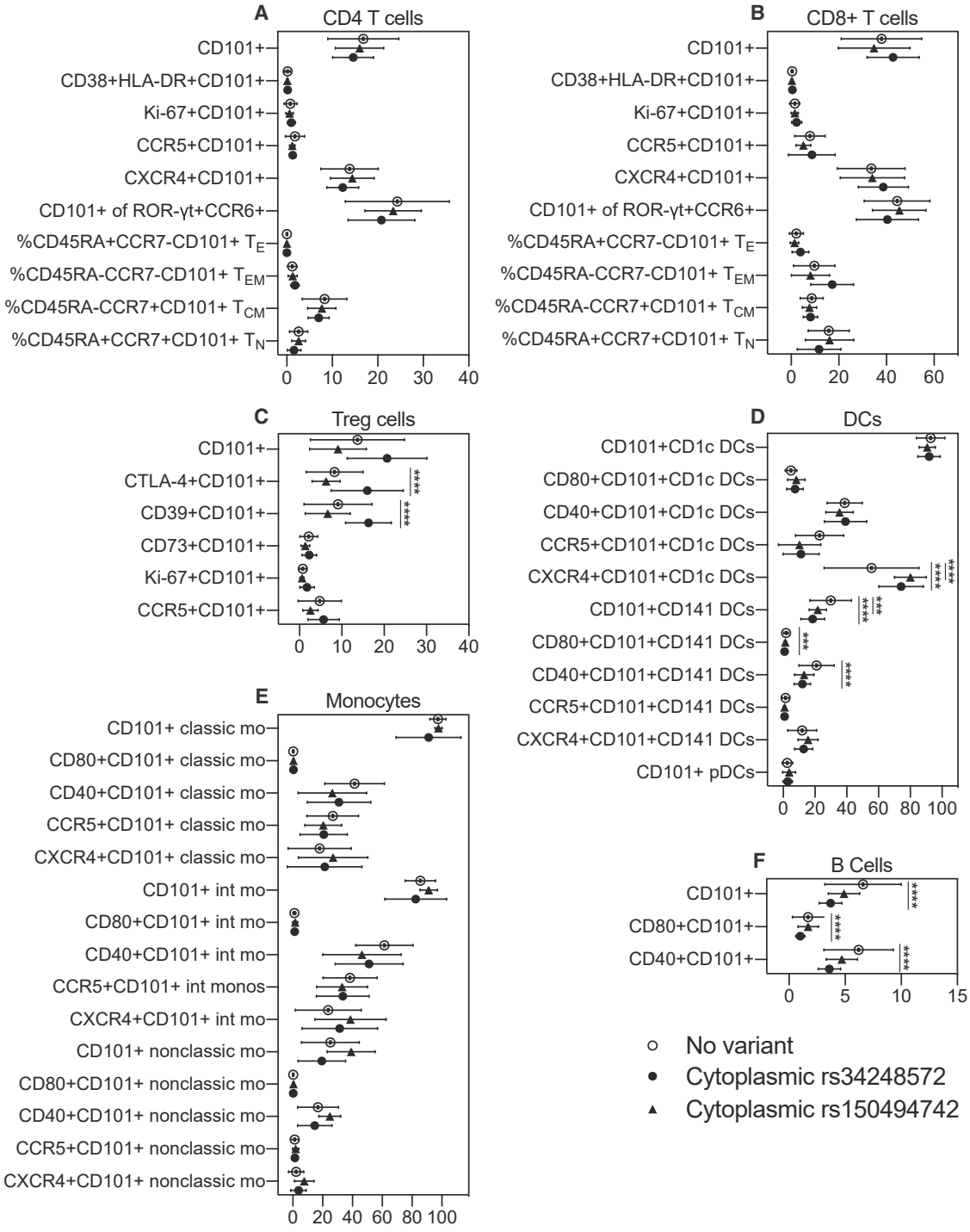


Figure 2. Circulating CD101$^+$ immune cell frequency and phenotype vary minimally based on the presence of genetic variants in the cytoplasmic domain of *CD101*

Circulating PMBCs from study participants possessing cytoplasmic variants in *CD101*, including *rs34248572* (N = 20) and *rs150494742* (N = 13), versus no functional variant (N = 117) were assessed by high-parameter flow cytometry for expression of various subset-specific and activation markers on CD101$^+$ cells. (A–C) The mean and SD of the frequency of activation and subset-specific markers expressed on CD101$^+$CD4 T cells (A), CD101$^+$CD8 T cells (B), and CD101$^+$ Treg cells (C) for cytoplasmic variants versus controls.

(D) CD101$^+$ DCs are broken into CD1c$^+$ DCs and CD141$^+$ DCs, alongside various activation markers within those subsets.

(legend continued on next page)

We found that, compared with individuals with no functional *CD101* variants, those with the Ig-like variant *rs12093834* or cytoplasmic variant *rs34248572* had an increased frequency of CD101$^+$ interferon (IFN)-γ^+CD8$^+$ T cells after EBV or CD3/CD28 stimulation (Figures 3A and 3B; Table 2; Table S5). Comparisons were done using a nonparametric rank regression using an unadjusted analysis (only variant as the predictor) and an adjusted analysis (controlling for batch, gender, and age). Notably, four individuals homozygous for *rs12093834* had increases in the frequency of CD101$^+$IFN-γ^+ CD8$^+$ T cells that were at or above the median of all *rs12093834*$^+$ individuals, suggesting a possible allele dose-response relationship (Figures 3A and 3B). Similarly, individuals with the Ig-like variant *rs12093834* had an increased frequency of CD101$^+$IFN-γ^+ CD4$^+$ T cells after EBV lysate stimulation (Figure 3C). Consistent with our phenotypic results, individuals with the cytoplasmic variant *rs34248572* demonstrated more limited functional differences; however, we observed a trend ($p = 0.057$) toward increased frequency of IFN-γ^+tumor necrosis factor alpha (TNF-α)$^+$ CD8$^+$ T cells after CD3/CD28 stimulation, aligned with the suggestion that *CD101* variants may result in increased proinflammatory cytokine production by T cells (Figure 3D).

Our phenotypic analysis revealed that CD101-expressing T cells (and subsets thereof) were particularly sensitive to functional alterations in *CD101*. We therefore additionally assessed the effect of *CD101* variants to alter the ability of CD101$^+$ T cells to produce proinflammatory cytokines upon *ex vivo* stimulation by gating on only CD101$^+$ T cells prior to quantifying cytokine expression. Interestingly, stimulation with EBV resulted in an augmented frequency of CD4$^+$ and CD8$^+$ CD101$^+$ T cells dually expressing IFN-γ and TNF-α in individuals with the cytoplasmic variant *rs34248572* but not the Ig-like variant *rs12093834* (Figures 3E and 3G; Table S5). However, when cells were stimulated polyclonally with anti-CD3/CD28, individuals carrying the Ig-like or cytoplasmic *CD101* variant demonstrated an increased frequency of CD101$^+$ CD4$^+$ and CD8$^+$ T cells co-expressing IFN-γ and TNF-α compared with individuals with no functional variants (Figures 3F and 3H). Moreover, we found that individuals with the Ig-like variant *rs12093834* or cytoplasmic variant *rs34248572* have an increased frequency of CD101$^+$ CD8$^+$ T cells that are TNF-α^+IFN-γ^- after polyclonal stimulation of sorted CD3$^+$ cells (Figure S8; Table S5). Our data suggest that these variants may be associated with increased proinflammatory potential of CD101$^+$CD8$^+$ and CD4$^+$ T cells in the circulation.

CD101 variation diminishes Treg cell-mediated restraint of effector T cells

Given that we observed that variants in *CD101* are associated with increased proinflammatory cytokine expression by circulating CD101$^+$ T cells (Figure 3), we next wanted to determine whether this may be due to increased pro-inflammatory responses from effector T cells and/or reduced Treg cell suppression capacity in individuals with variants in *CD101*. As shown in Figure 4A, we performed an indirect Treg cell suppression assay to examine the effect of *CD101* variants on the ability of Treg cells to suppress T cell effector function. This involved comparing cytokine production of antigen-stimulated whole PBMCs with Treg cells present at a natural frequency ("whole PBMCs") with cytokine production of antigen-stimulated PBMC made deficient in Treg cells (through fluorescence-activated cell sorting [FACS]; "Treg depleted"). We first evaluated the overall effect of Treg cell depletion by comparing the ability of T cells to produce cytokines in the absence of Treg cells across individuals with Ig-like or cytoplasmic *CD101* variants compared with no functional variants. In the absence of Treg cells, CD4$^+$ T cells and CD8$^+$ T cells exhibited no functional differences in the context of *CD101* genetic variation (Figure 4B; see gating strategy including without stimulation controls in Figure S7). These results suggest that the effector T cell responses are not intrinsically more pro-inflammatory in individuals with *CD101* variants. This finding, combined with our demonstration that the frequencies of CD4$^+$ and CD8$^+$ T cells producing proinflammatory cytokines were increased in individuals with *CD101* variants (Figure 3), led us to compare cytokine production in Treg cell-depleted cultures with whole PBMCs. We performed this comparison separately for individuals with no variants, those with the Ig-like variant *rs12093834*, and individuals with the cytoplasmic variant *rs34248572* (Table S5). We reasoned that depletion of functionally suppressive Treg cells would be associated with a relative increase in proinflammatory cytokines compared with whole PBMCs, whereas depletion of impaired Treg cells would result in a smaller increase in proinflammatory cytokines. Indeed, we found that depletion of Treg cells from individuals with the Ig-like variant *rs12093834* resulted in a smaller change in EBV-induced IL-2 production by CD4$^+$ T cells, but there were not other differences in T cell cytokine production in the absence of Treg cells (Table 2; Table S5). Figure 4C shows a trajectory plot of the change in the frequency of IL-2-^{producing} CD4$^+$ T cells in whole PBMCs and Treg cell-depleted PMBCs for each individual, and Figure 4D shows boxplots by variant, indicating the difference in percent of IL-2$^{-producing}$ CD4$^+$ T cells in Treg cell-depleted minus whole PBMCs. These data suggest that individuals with the Ig-like *CD101* variant have Treg cells that are less able to suppress EBV-specific CD4$^+$ T cell production of IL-2, in line with lower levels of Treg cell-mediated immune quiescence in individuals with *CD101* Ig-like variants.

CD101 variants are associated with distinct inflammatory transcriptional signatures

Given our observations that the phenotype and function of multiple T cell subsets are affected by *CD101* variants, we next sought to determine whether there were corresponding transcriptional differences that could explain the altered functional

(E) The total frequencies of classic (CD14^{++}CD16$^-$), nonclassic (CD14$^+$CD16^{++}), and intermediate (CD14^{++}CD16$^+$) monocytes expressing CD101 alongside the frequencies of various activation markers within each indicated subset.

(F) CD101$^+$ B cells and expression of CD80 and CD40 among CD101$^+$ B cells.

Data points represent the mean and SD of biological replicates as indicated by N per group. Two-sample t tests were performed, comparing each variant to samples with no functional variants, with Bonferroni correction for 100 comparisons to determine significance. ****$p < 0.0001$, ***$p < 0.001$.

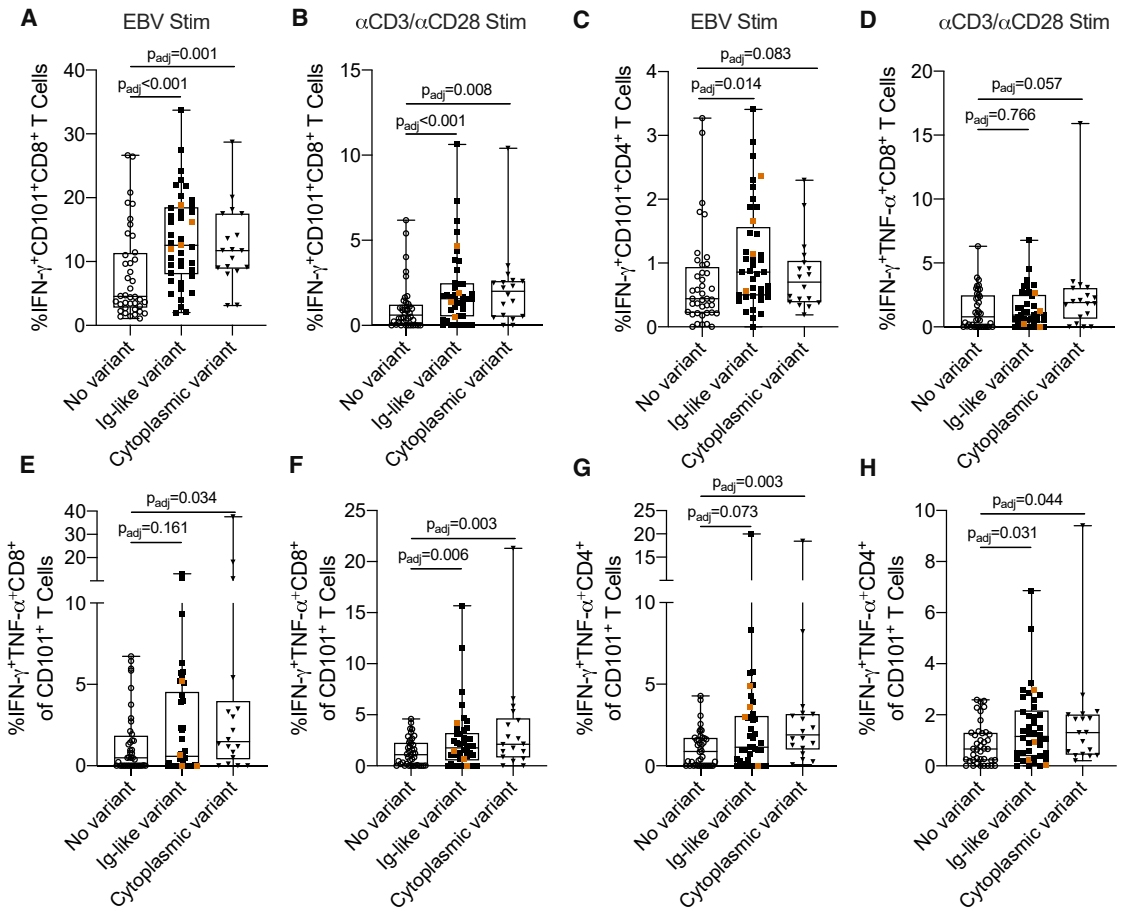


Figure 3. T cells from individuals with variants in *CD101* have increased potential to express cytokines

(A–D) Cytokine-producing $CD8^+$ or $CD4^+$ T cells from EBV-stimulated (A and C) or αCD3/αCD28-stimulated (B and D) whole PBMCs for *CD101* Ig-like (N = 42) and cytoplasmic variants (N = 18) compared with no functional *CD101* variants (missense, 3′ or 5′ untranslated region, splice site) (N = 40). Live-sorted PBMCs, including lymphocytes and APCs, were recovered and stimulated with EBV lysate or glycine control (A and C) or αCD3/αCD28 or medium control (B and D) for 6 h prior to staining for intracellular cytokine production. The frequencies of $CD101^+CD8^+$ T cells producing IFN-γ after subtracting background values were affected by the presence of Ig-like and cytoplasmic-*CD101* variants (A and B). Similarly, production of IFN-γ by $CD101^+CD4^+$ T cells after EBV stimulation was increased significantly for individuals carrying an Ig-like variant, whereas possessing a cytoplasmic variant did not have a significant effect (C). $CD8^+$ T cells co-producing IFN-γ and TNF-α after αCD3/αCD28 stimulation also trended toward an increased frequency in individuals with the cytoplasmic variant (D). Four participants were homozygous for Ig-like variant *rs12093834*; they are denoted by orange symbols.

(E–H) Live-sorted PBMCs, including lymphocytes and APCs, were recovered and stimulated with EBV lysate or glycine control (E and G) or αCD3/αCD28 or medium control (F and H) for 6 h prior to staining for intracellular cytokine production. Total $CD4^+CD3^+$ or $CD8^+CD3^+$ T cells were gated by CD101 positivity and then assessed for their ability to co-produce IFN-γ and TNF-α. EBV lysate stimulation of PBMCs isolated from individuals with cytoplasmic variants resulted in an increased frequency of IFN-γ^+TNF-α^+CD8^+ (E) and $CD4^+$ (G) T cells, whereas having an Ig-like variant did not produce a significant effect. Stimulation with αCD3/αCD28 antibodies elicited an increased frequency of IFN-γ^+TNF-α^+ $CD8^+$ (F) and $CD4^+$ (H) T cells in individuals with both Ig-like and cytoplasmic-variants. Matched background control values were subtracted for all participants. Four participants were homozygous for Ig-like variant *rs12093834*; they are denoted by orange symbols.

Each data point represents one individual. Adjusted p values were calculated as described in STAR Methods.

patterns in circulating T cells from individuals with *CD101* variants. To address this question, we used FACS to sort $CD8^+$ T cells or conventional, non-Treg $CD4^+$ T cells from PBMCs collected from individuals with no functional variants in *CD101* and compared them with cells sorted from individuals with an Ig-like variant or cytoplasmic variant in *CD101* (Figure 5A). Low RNA recovery from the limited number of sorted Treg cells from available cryopreserved PBMCs prevented us from performing RNA sequencing (RNA-seq) on this population.

Comparison of $CD4^+$ T cells from individuals with an Ig-like variant or a cytoplasmic-variant in *CD101* with $CD4^+$ T cells from individuals with no functional variants yielded many differentially expressed genes (Table S6A). Genes that were downregulated in $CD4^+$ T cells from individuals with Ig-like variants included many IFN-stimulated genes (ISGs), including *IFIT3*, *IFI44L*, *IFIT1*, *IFI27*, *ISG15*, and *IRF7*, as well as the HIV resistance genes *BST2* and *MX1*. Similarly, many ISGs were downregulated in $CD4^+$ T cells from individuals with cytoplasmic

Table 2. Effect of *CD101* variation on T cell activation and Treg cell functional characteristics

Measure	Stimulation condition	Whole PBMCs: Median difference in response for stimulation minus control		Unadjusted		Adjusted	
		No functional variants (N = 40)	Ig-like variant *rs12093834* (N = 41)	Coefficient	p value	Coefficient	p value
%CD101^{+}IFNγ^{+} of CD8^{+} T cells	EBV lysate	4.553	12.550	5.900	<0.001	5.690	<0.001
%CD101^{+}IFNγ^{+} of CD8^{+} T cells	anti-CD3/CD28	0.606	1.570	0.762	0.003	0.819	<0.001
%CD101^{+}IFNγ^{+} of CD4^{+} T cells	EBV lysate	0.442	0.835	0.268	0.016	0.252	0.007
Measure	Stimulation condition	No functional variants (N = 40)	Cytoplasmic variant *rs34248572* (N = 18)	Coefficient	p value	Coefficient	p value
%CD101+ IFNγ+ of CD8+ T cells	EBV Lysate	4.553	11.730	5.726	0.000	4.941	0.001
%CD101+ IFNγ+ of CD8+ T cells	anti-CD3/CD28	0.606	2.000	0.289	0.003	0.73	0.008
%IFNγ+ TNFα+ of CD8+ T cells	anti-CD3/CD28	0.790	1.905	0.684	0.045	0.5	0.057
Measure	Stimulation condition	Treg depleted minus whole PBMC: Median difference in response for stimulation minus control		Unadjusted		Adjusted	
		No functional variants (N = 40)	Ig-like variant rs12093834 (N = 41)	Coefficient	p value	Coefficient	p value
%IL-2^{+} of CD4^{+} T cells	EBV lysate	0.506	0.080	−0.353	0.014	−0.327	0.002

variants, including IFI44L, IFIT3, and IFIT1 (Figures 5B and 5C; Table S6A). Overall, this decreased expression of ISGs in CD4^{+} T cells from individuals with *CD101* variants is consistent with the finding that HIV-1 infection risk is increased in individuals with these variants because it is possible that reduced ISG expression renders CD4^{+} T cells more susceptible to infection with HIV, in part through a less potent anti-viral response. Many genes were also increased in expression in CD4^{+} T cells with *CD101 rs12093834* or *rs34248572* compared with no variants. Notably, expression of *CREM*, which encodes a transcriptional activator of T cells that has been shown to contribute to T cell dysregulation in autoimmune conditions,[17–20] was increased in CD4^{+} T cells from individuals with an Ig-like variant or a cytoplasmic variant (Figures 5B and 5C). Thus, increased expression of CREM could lead to a dysregulated and inflammatory T cell profile, but additional experiments to investigate the effects of ectopic expression of this gene are required to validate this possibility.

When we compared CD8^{+} T cells from individuals with an Ig-like variant or a cytoplasmic variant in *CD101* with individuals with no functional variants, we also found many genes to be differentially expressed (Table S6A). Among genes upregulated in CD8^{+} T cells from individuals with an Ig-like variant in *CD101* were *RGS1*, *TNF*, *CTLA4*, *CD28*, and *IFNGR1*, and those from individuals with a cytoplasmic variant in *CD101* were *CREM*, *RGS1*, *TNF*, *CTLA4*, and *GZMK* (Figures 5B and 5C; Table S6A). Increased expression of *TNF* is consistent with our finding that CD8^{+} T cells from individuals with variants in *CD101* express increased levels of TNF-α following stimulation (Figure 3), again pointing to their increased inflammatory potential. However, CD8^{+} T cells from individuals with an Ig-like or cytoplasmic variant in *CD101* have reduced expression of *GZMB* and, therefore, may have reduced cytolytic potential, which could reduce the efficacy of HIV protection in the face of HIV-1 exposure.

Finally, functional characterization of the differentially expressed genes was performed using Gene Ontology (GO) term enrichment analysis to determine whether particular biological processes were significantly over- or underrepresented in the differentially expressed genes. Notably, for CD4^{+} T cells from individuals with either type of variant in *CD101*, downregulated pathways were dominated by terms associated with "response to virus" (GO:0009615) and "defense response to virus" (GO:0051607) and for CD4+ T cells from individuals with an Ig-like variant in *CD101* "response to interferon beta" (GO:0035456) (Table S6B). For CD8^{+} T cells from individuals with an Ig-like variant in *CD101*, the GO term "regulation of immune response" (GO:0050776) was enriched significantly among downregulated pathways (Table S6B). In addition, in CD8^{+} T cells and from individuals with an Ig-like variant, the GO term "inflammatory response" (GO:0006954) was enriched significantly among upregulated pathways (Table S6B). These results suggest that individuals with variants in *CD101* may have CD4^{+} T cell subsets with reduced anti-viral activity and, thus, increased susceptibility to HIV infection and CD8^{+} T cell subsets with increased pro-inflammatory potential.

DISCUSSION

Our data support the concept that variation in *CD101* modifies the homeostatic set point toward a proinflammatory environment. Specifically, our results are consistent with three principal conclusions: (1) candidate *CD101* variants are associated with increased prevalence of proinflammatory phenotypes for a wide range of circulating immune cell types (Table 1), with the strongest associations revealed among CD101^{+} immune cells (Figures 1 and 2); (2) conventional CD4^{+} and CD8^{+} CD101^{+} T cells from individuals with these candidate *CD101* variants appear to be more proinflammatory, as assessed by cytokine expression (Figure 3) and overall transcriptional profile (Figure 5); and (3) although bulk effector T cells did not differ in cytokine responses by *CD101* variant status, Treg cells from individuals with Ig-like variants are less able to suppress IL-2^{+}CD4^{+} T cells

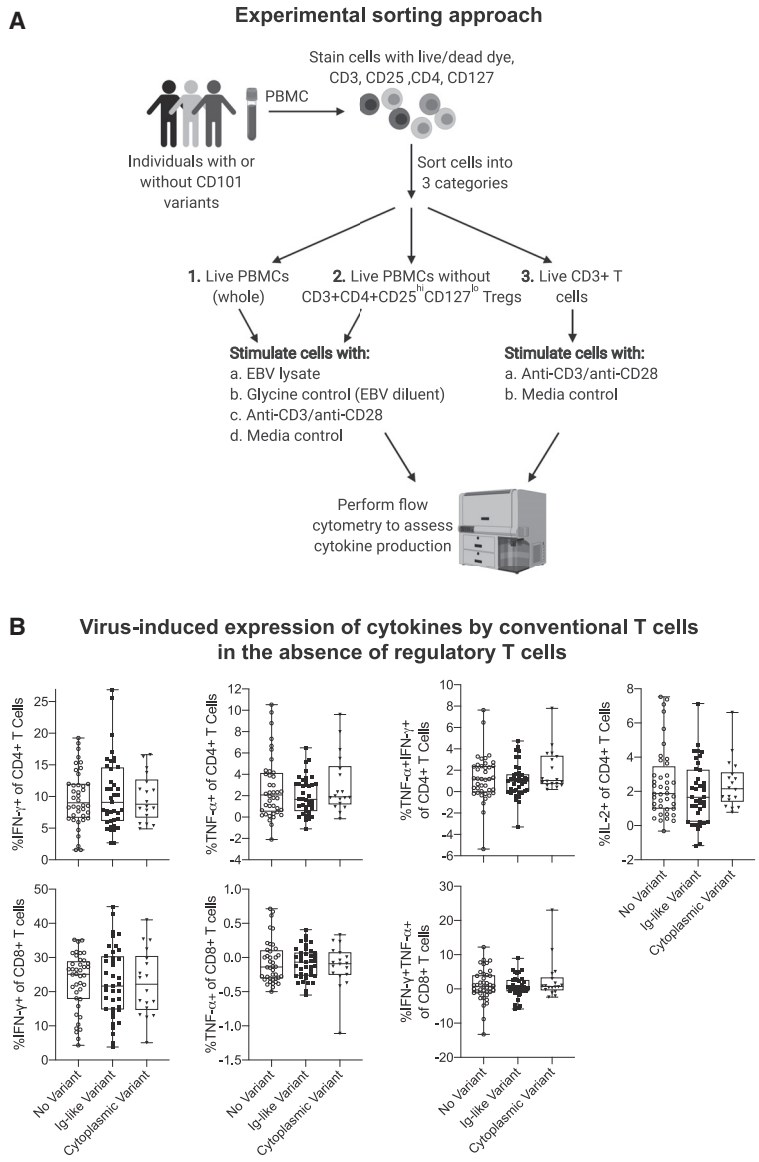


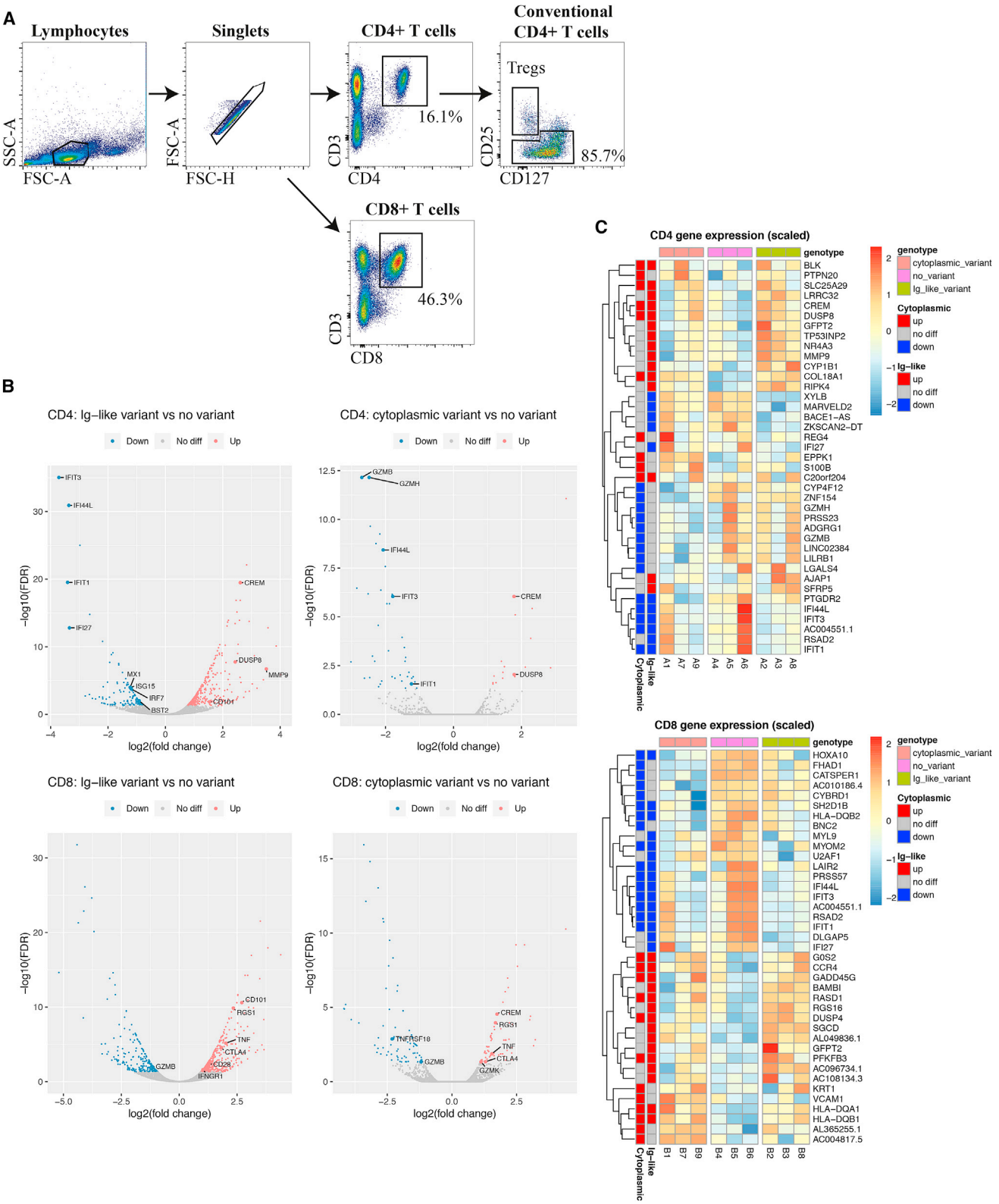
Figure 4. CD101 variation diminishes Treg cell-mediated restraint of effector T cells

(A) Schematic of the PBMC sorting and stimulation protocol (created with BioRender). PBMCs (N = 100) were sorted into (1) whole live PBMCs and (2) Treg cell-depleted or (3) purified $CD3^+$ T cell fractions. Cells were stimulated with EBV lysate or control or αCD3/αCD28 or control for 6 h. Cells were then analyzed for their expression of cytokines by intracellular cytokine staining (ICS).

(B) The frequency of $CD4^+$ and CD8 T cells producing proinflammatory cytokines in response to EBV among Treg-cell-depleted PBMCs was analyzed per case or control.

(C and D) Individual trajectory plots (C) and summary results (D) of Δ% $IL\text{-}2^+CD4^+$ T cells from Treg cell-depleted and whole PBMCs for CD101 cytoplasmic (N = 18) and Ig-like variants (N = 42) compared with no functional variants (N = 40). Live-sorted "whole" PBMCs, including lymphocytes and APCs, or PBMCs sorted to deplete Treg cells were recovered and stimulated with EBV lysate or glycine control for 6 h prior to staining for intracellular cytokine production. In (C), the frequencies of IL-2-producing $CD4^+$ T cells recovered from the "whole" PBMC fraction and the Treg cell-depleted fraction are plotted as a trajectory plot, and in (D), the difference between the percentage of $IL\text{-}2^+$ $CD4^+$ T cells in Treg cell-depleted compared with whole PBMCs is plotted.

Results are stratified by the presence of an Ig-like or cytoplasmic variant. Each data point represents one individual. Adjusted p values were calculated as described in STAR Methods.



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compared with Tregs from individuals with no functional *CD101* variants (Figure 4). We hypothesize that these inflammatory phenotypes contribute mechanistically to why these variants are associated with increased risk of HIV-1 acquisition.

Rather than investigating the effects of genetic variation as we did here, prior studies investigating the role of CD101 as an immunoregulatory marker have probed differences in the function of cells with or without wild-type CD101 protein expression. For example, a previous study of human cells reported that $CD8^+$ T cells lacking CD101 expression have a significantly greater cytotoxicity potential compared with $CD101^+$ $CD8^+$ T cells.[6] This is in line with the more recent finding that CD101 expression can be used to identify a population of dysfunctional, terminally exhausted $CD8^+$ T cells that lack proliferation potential in the context of chronic virus infection.[11] Those studies concluded that $CD101^+$ $CD8^+$ T cells have reduced cytotoxicity and proliferation potential. The key findings from our study are that (1) individuals with specific variants in the *CD101* Ig-like domain compared with those with the reference *CD101* sequence have a higher frequency of $CD101^+$ $CD8^+$ T cells producing proinflammatory cytokines, and (2) based on a Treg cell depletion assay, individuals with these variants may have Treg cells with a reduced capacity to suppress bystander proinflammatory $CD4^+$ T cells. These changes may arise from the effect of these gene variants on CD101 function, such as its ability to bind its natural ligand and/or transmit an intracellular signal. Given that we did not formally test the cytotoxic function of CD8 T cells, we cannot comment on how these gene variants affected T cell cytotoxicity or proliferation potential. Notably, the increased frequency of cells expressing variant CD101 may also represent a feedback response to the decreased functionality of this molecule. Other studies found that ligation of CD101 blocks TCR-induced proliferation by inhibiting calcium flux and activation of tyrosine kinase, resulting in suppression of IL-2 transcription.[7,21] However, because the natural ligand for CD101 has not yet been identified, we were not able to assess how genetic variation affects binding of CD101 to its ligand or downstream intracellular signaling cascades and therefore assessed the downstream effects of gene variants on T cell proliferation. Additional studies of T cell proliferation potential in antigen-presenting cell (APC)-free assays in the context of wild-type (WT) *CD101* or *CD101* variants will be required to determine how variants may directly affect T cell proliferation and other functional properties. Nevertheless, given that we identified increases in cytokine expression by $CD101^+$ T cells in individuals with *CD101* variants (Figures 3 and 4), it is possible that this increased proinflammatory environment, at least in part, supports an immune-mediated increase in risk of HIV-1 acquisition. In earlier studies, we assessed the peripheral cytokine milieu of individuals with or without functional *CD101* variants and found a reduced concentration of serum IL-1RN as well as a tendency toward a reduction in sCD40L in cases, although we found no difference among the remaining 25 cytokines assessed.[2] Given that the described cohort is comprised of healthy individuals, perhaps it is not surprising that systemic cytokine concentrations are comparable. Further, given our data showing a similar proinflammatory capacity of effector T cells by *CD101* variant status, our indirect evidence (through Treg cell depletion) supports the hypothesis that Treg cells from individuals with *CD101* variants are less able to suppress viral antigen-driven production of IL-2 by $CD4^+$ T cells (Figure 4), and this may contribute to the association between *CD101* variants and increased risk of HIV-1 infection. In phenotypic assays, we specifically evaluated bulk Treg cells ($CD25^+CD127^{lo}Foxp3^+$) as well as those that do or do not additionally express Helios. Interestingly, FAUST revealed a deficit in the frequency of $Helios^+$ Treg cells among individuals with *CD101* cytoplasmic variants. This may be particularly important, given that Helios-deficient mice exhibit increased frequencies of activated effector T cells (as well as other autoimmune-related issues).[15]

In addition to the noted differences in T cell phenotypes and function based on *CD101* variants, we also identified several aspects of APC phenotypes that differed according to the type of variant. It is possible that variants modify the ability of APCs to activate T cells, thus affecting HIV-1 acquisition risk via an indirect DC-mediated mechanism, and our assay results cannot rule out this possibility. Indeed, we did observe differential phenotypic effects of *CD101* variation on DCs, including activated pDCs expressing the HIV co-receptor CCR5. These data suggest a possible role of differential type I IFN signaling among individuals with variants—a potentially important anti-HIV mechanism. Moreover, a previous study of cutaneous DCs found that ligation of CD101 on DCs via a monoclonal antibody led to IL-10-mediated inhibition of T cell proliferation.[22] Because we found that the presence of *CD101* variants was associated with a reduced frequency of DCs expressing CD101 protein (Table 1), it is possible that this reduced expression of CD101, in turn, leads to reduced potential to produce IL-10 and, thus, restrain T cell activation and proliferation. Indeed, because our *ex vivo* stimulation assays used whole PBMCs and, thus, included DCs and other APC subsets that also express CD101, it is possible that our findings of increased production of cytokines by $CD4^+$ and $CD8^+$ T cells in individuals with CD101 variants (Figures 3 and 4) were due to alterations in DC function alone or working in concert with modifications in T cell activation because of *CD101* variants. In either case, this more proinflammatory

Figure 5. CD101 variation is associated with transcriptional changes in circulating $CD4^+$ and $CD8^+$ T cells

$CD4^+$ conventional or $CD8^+$ T cells were sorted from PBMCs sampled from 3 individuals with no functional variants in *CD101* or from 3 individuals with an Ig-like variant (*rs12093834*) or 3 with a cytoplasmic variant (*rs34248572*) in *CD101*.

(A) The gating strategy for sorting included gates for lymphocytes and singlets, and $CD8^+$ T cells were sorted as $CD3^+CD8^+$, whereas conventional $CD4^+$ T cells were sorted as $CD3^+CD4^+$ and, to exclude Treg cells, were further gated as $CD25^-$.

(B) Volcano plots showing genes that are differentially expressed between the indicated groups. Genes were considered differentially expressed when false discovery rate (FDR) values were less than 0.05.

(C) Heatmaps showing the top 10 differentially upregulated genes and top 10 differentially downregulated genes for each genotype category (Ig-like or cytoplasmic) versus no variant, with samples ordered by genotype.

environment supported by elevations in bystander inflammation may contribute to increased HIV-1 infection risk as a counterpoint to the previously noted association of natural resistance to HIV-1 infection with immune quiescence.[1] However, additional testing of the immunosuppressive potential of DCs from individuals with and without *CD101* variants is required to formally test this prediction.

The importance of studying *CD101* variation as a risk factor for bystander inflammation is underscored by the collective population prevalence of these variants. Although *rs12093834* itself is present in less than 10% of Kenyans, we previously identified at least three and possibly five or more variants in Kenyans in the seven *CD101* Ig-like domains that were associated with risk for HIV-1 acquisition.[2] In total, 20%–25% of East Africans may retain one or more of these variants. Although we grouped variants by structural location, the inflammatory effect of each variant was distinct; this, along with the fact that many of the specific variants we analyzed here are not present in other ancestral populations (e.g., *rs12093834* is absent in Europeans), suggests that the specific *CD101* variants associated with proinflammatory signatures may differ by population. Although our data suggest a proinflammatory risk signature that could be helpful in identifying variants that play a similar role in other populations, rapid, high-throughput methods are needed to more readily identify such variants. However, identification of an immunogenetic mechanism that directly connects bystander inflammation with sexually transmitted HIV-1 also underscores a link between HIV-1 acquisition and host inflammation that has been appreciated since early in the HIV-1 pandemic.

In conclusion, our data support the hypothesis that host genetic variants in *CD101* confer increased T cell activation and may mediate Treg cell dysfunction. Although there are currently no known drugs that directly modify CD101 function, our data raise the prospect that development of drugs that modulate specific CD101 functions could reduce inflammation and the risk of infection by modifying the host rather than directly targeting the pathogen. Furthermore, given the potential relationship of CD101 function to autoimmunity, we speculate that an intervention targeting CD101 function that is designed to increase immune quiescence could benefit other diseases where excess immune activation or inflammation can be detrimental, such as coronavirus disease 2019 (COVID-19), autoimmunity, or tissue rejection. Although additional studies are required to identify how gene variants in *CD101* affect binding of this receptor to its natural ligand as well as downstream signaling events, our study provides insight into the potential mechanism whereby *CD101* variation may increase the risk of HIV-1 acquisition.

Limitations of the study

Our Treg cell depletion data suggest that Treg cells from individuals with the identified functional *CD101* variants exhibit more limited suppressive activity of virally exposed $CD4^+$ T cells. However, we were limited in our ability to directly demonstrate these results using a more conventional suppression assay because of finite numbers of available PBMCs. Additional studies should be undertaken to directly assess Treg cell suppression capacity in the setting of *CD101* variants. Other approaches to corroborate our observations, including identification of the CD101 ligand and dissection of potentially distinct functions of CD101 on APCs versus T cells, will provide further clarity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Study population
  - Study approval
  - Ethics committees (local and national African study sites):
  - Ethics committees (site-affiliated international institutions):
- METHOD DETAILS
  - Phenotype analysis by flow cytometry
  - FAUST analysis
  - Sorting and stimulation assays
  - RNA-seq Expression Analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistics

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

L.E.R.-S. and P.S. performed experiments, acquired data, and performed data analyses. E.G. and R.G. conceived the FAUST analysis method and performed FAUST data analysis. C.M.M., F.W., T.H., S.H., and K.T. performed data curation/management and/or statistical analysis. N.M., G.d.B., C.C., J.M.B., and J.R.L. conducted the clinical trial from which samples for this study were provided and/or provided study supervision. L.E.R.-S., J.R.L., and J.M.L. designed the research study and supervised completion of the experiments. L.E.R.-S., J.R.L., and J.M.L. wrote the first draft of the manuscript, and all authors provided editorial contribution and approved the final draft.

DECLARATION OF INTERESTS

All data analysis conducted by E.G. and R.G. were completed while E.G. was a full-time employee of the Fred Hutchinson Cancer Research Center. E.G. declares ownership interest in Ozette Technologies. R.G. has received consulting income from Takeda Vaccines, speaker fees from Illumina and Fluidigm, and research support from Janssen Pharmaceuticals and declares ownership in Ozette Technologies.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD39 FITC	eBioscience	eBioA1; RRID:AB_837099
CD127 BB700	BD	HIL-7R-M21; RRID:AB_2744279
CD101 APC	Biolegend	BB27; RRID:AB_2121761
CD4 APC-Cy7	Biolegend	OKT4; RRID:AB_571947
Foxp3 PE	eBioscience	PCH101; RRID:AB_1518782
CTLA-4 Pe-Dazzle594	Biolegend	L3D10; RRID:AB_2566198
CCR5 PeCy7	BD	2D7/CCR5; RRID:AB_396858
Helios eF450	eBioscience	22F6; RRID:AB_2574136
CD25 BV605	Biolegend	BC96; RRID:AB_11218989
CD3 BV650	BD	SK7; RRID:AB_2738535
Ki-67 BV786	BD	B56; RRID:AB_2732007
CD73 BUV737	BD	AD2; RRID:AB_2739217
CD8 PerCP eF710	eBioscience	SK1; RRID:AB_1834411
CD38 AF700	Biolegend	HB-7; RRID:AB_2566424
ROR-gt PE	eBioscience	AFKJS-9; RRIDAB_1834470
CD45RA ECD	Beckman Coulter	2H4LDH11LDB9; RRID:AB_10640553
CCR7 BV421	Biolegend	G043H7; RRID:AB_11203894
CCR6 BV605	Biolegend	G034E3; RRID:AB_2561449
HLA-DR BV711	Biolegend	L243; RRID:AB_2562913
CXCR4 BUV395	BD	12G5; RRID:AB_2738490
CD3 BB515	BD	HIT3a; RRID:AB_2744379
CD25 APC-R700	BD	2A3; RRID:AB_2870475
CD127 BV570	Biolegend	A019D5; RRID:AB_2832685
IL-2 PE	BD	MQ1-17H12; RRID:AB_1727541
IFN-γ V450	BD	B27; RRID:AB_1645594
CD69 BV605	Biolegend	FN50; RRID:AB_2562307
TNF-α BV711	Biolegend	Mab11; RRID:AB_2562740
CD8a BUV395	BD	RPA-T8; RRID:AB_2722501
CD14 BUV737	BD	M5E2; RRID:AB_2870095)
CD3	eBioscience	HIT3a; RRID:AB_468859
CD28	BD	CD28.2; RRID:AB_396068
HLA-DR FITC	BD	TU36; RRID:AB_395942
CD1c BB700	BD	F10/21A3; RRID:AB_2743468
CD16 APC-Cy7	Biolegend	3G8; RRID:AB_314217
CD11c PE	Biolegend	S-HCL-3; RRID:AB_2616899
CXCR4 PeCF594	BD	12G5; RRID:AB_11153132
CD123 PeCy5	BD	9F5; RRID:AB_394029
CD14 BV421	Biolegend	HCD14; RRID:AB_2563296
CD3 BV605	Biolegend	OKT3; RRID:AB_2561911
CD141 BV711	BD	1A4; RRID:AB_2738033
CD80 BV786	BD	L307.4; RRID:AB_2738631
CD40 BUV395	BD	5C3; RRID:AB_2739110
CD20 BUV737	BD	2H7; RRID:AB_2687489

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Cryopreserved PBMC samples African participants enrolled in the Partners in Prevention HSV/HIV Transmission Study and the Partners PrEP Study	University of Washington repository; [23] and [24]	Partners in Prevention HSV/HIV Transmission Study (ClinicalTrials.gov number, NCT00194519), the Couples Observational Study, and the Partners PrEP study (ClinicalTrials.gov number, NCT00557245)
CD101 genomic data from African participants enrolled in the Partners in Prevention HSV/HIV Transmission Study and the Partners PrEP Study	University of Washington repository; [2], [23], and [24]	Partners in Prevention HSV/HIV Transmission Study (ClinicalTrials.gov number, NCT00194519), the Couples Observational Study, and the Partners PrEP study (ClinicalTrials.gov number, NCT00557245)
Chemicals, peptides, and recombinant proteins		
Live/Dead Aqua	Invitrogen	L34957
Live/Dead Blue	Invitrogen	L34962
EBV Lysate	East Coast Bio	EV012
Critical commercial assays		
SMART-Seq v4 Ultra Low Input RNA Kit	Clontech Laboratories	635026
Nextera XT DNA Library Preparation Kit	Illumina	FC-131-1096
Deposited data		
RNA sequencing data have been deposited in the GEO repository	GEO Repository	GSE152381
Software and algorithms		
FlowJo Software	BD	V9.9.6
FAUST Algorithm	[14]	https://www.biorxiv.org/content/10.1101/702118v2
Real Time Analysis v3.4.4 Software	Illumina	v3.4.4
bcl2fastq2 Conversion Software	Illumina	v2.20
R package Rfit	[25–28]	N/A
Bioconductor package GOseq v1.36 was used to perform enrichment analysis on differentially expressed genes against GO Biological Processes	[29] and GO_BP_DIRECT from DAVID database; https://david.ncifcrf.gov/home.jsp	N/A
The filtered expression matrix was normalized by TMM method and subject to significance testing using GLM LRT method.	[30]	N/A
Bioconductor package edgeR 3.26.8 was used to detect differential gene expression between genotypes	[31]	N/A
FeatureCounts in Subread 1.6.5 was used to quantify gene-level expression by counting unstranded paired-end reads	[32]	N/A
FastQC 0.11.8 and RSeQC 3.0.0 were used for QC including insert fragment size, read quality, read duplication rates, gene body coverage and read distribution over different genomic regions	[33]	N/A
STAR v2.7.1 with 2-pass mapping was used to align paired-end reads to human genome build hg38 and GENCODE gene annotation V31	[34]	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by the lead contact, Jennifer Lund (jlund@fredhutch.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA sequencing data have been deposited in the GEO repository (GSE152381).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study population

The studies reported here utilized cryopreserved peripheral blood mononuclear cell (PBMC) samples, and CD101 genomic data from African participants enrolled in the Partners in Prevention HSV/HIV Transmission Study[23] and the Partners PrEP Study.[24] Demographic details are provided in Table S1. *CD101* sequence data was generated through our prior study[2] in which either whole genome sequencing, or targeted re-sequencing of the *CD101* exons, splice sites, and 3′- and 5′ untranslated regions was applied to samples collected from N = 1329 individuals with quantified levels of HIV-1 exposure and known HIV-1 infection outcomes. Among these individuals we defined a case of *CD101* Ig-like variation as a participant who was either homozygous or heterozygous for only one of the three candidate *CD101* Ig-like SNVs (*rs12093834*, *rs17235773*, or rs3754112). Cases of *CD101* cytoplasmic variation were defined as individuals homozygous or heterozygous for only one of two *CD101* cytoplasmic SNVs (*rs150494742* or *rs34248572*). Cases were restricted to those individuals who had only the identified candidate variant with no other functional (i.e., missense, splice site, or untranslated region) *CD101* SNVs. Controls for this analysis were identified as individuals whose *CD101* sequence lacked any functional SNVs.

Study approval

We identified individuals for this study from HIV-1 serodiscordant couples recruited into three cohorts of African heterosexual HIV-1 serodiscordant couples: the Partners in Prevention HSV/HIV Transmission Study (ClinicalTrials.gov number, NCT00194519), the Couples Observational Study, and the Partners PrEP study (ClinicalTrials.gov number, NCT00557245). Detailed procedures have been reported elsewhere for each of these studies.[23,24,35] All participants provided written informed consent for participation in the clinical study, and samples for this study were drawn from those participants who provided additional consent for future research on HIV including genotyping. Relevant study documents went through ethical review and approval by the following committees:

Ethics committees (local and national African study sites):

Kenya Medical Research Institute Ethics Committee;
Kenyatta National Hospital Ethics Committee;
Kilimanjaro Christian Medical College;
Moi University Ethics Committee;
Republic of Botswana Ministry of Health;
South Africa Medicines Control Council;
Uganda National Council for Science & Technology;
Uganda National AIDS Research Committee;
Uganda Virus Research Institute;
University of Witwatersrand Ethics Committee;
University of Cape Town Institutional Review Board

Ethics committees (site-affiliated international institutions):

Harvard School of Public Health;
Indiana University Institutional Review Board;
London School of Hygiene and Tropical Medicine;
United States Centers for Disease Control and Prevention;
University of California, San Francisco Institutional Review Board;
University of Washington Institutional Review Board
The University of Washington Institutional Review Board also was the institutional review board for the UW coordinating center applications for all three studies.

METHOD DETAILS

Phenotype analysis by flow cytometry

Cryopreserved PBMCs (N = 268) were maintained at −150°C according to guidelines established by the University of Washington Repository, and transported to the laboratory on LN_2 on the day of thaw. PBMCs were quickly thawed in complete media with 50 U/ml benzonase (Millipore, Burlington, MA). Thirty-three had viability less than 30% as determined by trypan blue staining and were excluded from analysis leaving aliquots from N = 235 participants (Table 1). Cells were counted and stained for flow cytometry (including panels designed to assess T cell activation and Tregs, monocytes, dendritic cells, and B cells) and collected on a BD FACSymphony X50 for analysis. Antibodies and clones are described in Table S2. Data files were analyzed using FlowJo v9.9.6 (BD, Franklin Lakes, NJ), and gating trees are available in Figures S2–S4. Researchers were blinded to *CD101* variant identities throughout.

FAUST analysis

We additionally analyzed our flow cytometry data using the recently-described unbiased clustering strategy, Full Annotation Using Shaped-constrained Trees (FAUST) to reveal potentially unexplored cell phenotypes of importance.[14] Briefly, FAUST was applied to the three staining panels as follows: 1) live lymphocytes (identified by manual gating) within the T cell panel; 2) live CD3-CD20- cells (identified by manual gating) within the DC/monocyte panel; and 3) live lymphocytes with a CD3+CD4+CD25+$CD127^{lo}$ phenotype (identified by manual gating) within the Treg panel. After tuning, FAUST selected multiple markers within each panel for the discovery and annotation of phenotypes (Table S4). FAUST phenotypes were tested for association to compare phenotypes among individuals with *CD101* variants versus no functional variants using a binomial generalized linear mixed-effects model with a subject level random effect. In each panel, the set of hypotheses generated by this procedure were jointly adjusted for multiple comparisons using Bonferroni correction at p = 0.05.

Sorting and stimulation assays

For functional assays, a second cryopreserved aliquot of PBMCs was retained from 100 individuals (Table S1) selected as a subset of those analyzed for cellular phenotypes. These were used to specifically assess the impact of *CD101* variation on conventional T cell and Treg functional responses. Since only ~10% of HIV-1 exposed individuals maintain peripheral blood T cells that are responsive to HIV-1 peptides,[36] we sought to evaluate Treg function by measuring a more prevalent antiviral response, namely CD4+ and CD8+ T cell response to Epstein-Barr Virus (EBV) lysate, which we expected to be present in most individuals included in our cohort. Cryopreserved PBMCs were thawed as above with individual aliquots sorted via BD FacsAria II into three groups: whole live PBMCs (including APCs and all lymphocytes); whole live PBMCs depleted of Tregs (as defined by $CD3^{+}CD4^{+}CD25^{+}CD127^{lo}$); and isolated live CD3+ cells (T cells). Recovered cells were washed, resuspended in complete media with brefeldin A (eBioscience, San Diego, CA), and plated for stimulation. Average cell counts were as follows i) sorted whole live PBMCs: 3.35 X 10^5; ii) Treg depleted PBMCs: 4.95 X 10^5, and iii) CD3+ T cells: 2.15 X 10^5. Sorted whole live PBMCs and PBMCs depleted of Tregs were stimulated with i) 60 μg/ml EBV lysate (East Coast Bio, North Berwick, ME); ii) 100 mM glycine (EBV diluent) control; iii) anti-CD3 and CD28; or iv) media control. Sorted live CD3+ T cells were stimulated with i) anti-CD3 and CD28 or ii) media. All cells were incubated in stimulation cocktails for 6 hours at 37°C. Following stimulation, cells were washed and stained for cytokine production. Samples were acquired on a BD FACSymphony X50 flow cytometer and analyzed using FlowJo v9.9.6 (BD, Franklin Lakes, NJ).

RNA-seq Expression Analysis

For RNA-sequencing studies, PBMC aliquots were selected from 3 individuals with no functional variants in *CD101*, from 3 individuals homozygous for *CD101* Ig-like variant rs12093834, and from 3 individuals heterozygous for *CD101* cytoplasmic variant rs34248572. Cryopreserved PBMCs were thawed as above, stained with CD3, CD4, CD25, CD127, and CD8, and sorted via BD FacsAria II into two groups: CD8+ T cells (CD3+CD8+) and conventional CD4+ T cells (CD3+CD4+CD25-). Recovered cells were used for RNA isolation by QIAGEN kit.

RNA-seq libraries were prepared from total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA). Library size distribution was validated using an Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Additional library QC, blending of pooled indexed libraries, and cluster optimization was performed using Life Technologies' Invitrogen Qubit® 2.0 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, USA). RNA-seq libraries were pooled (18-plex) and clustered onto one SP flow cell. Sequencing was performed using an Illumina NovaSeq 6000 employing a paired-end, 50 base read length (PE50) sequencing strategy. Image analysis and base calling was performed using Illumina's Real Time Analysis v3.4.4 software, followed by 'demultiplexing' of indexed reads and generation of FASTQ files, using Illumina's bcl2fastq2 Conversion Software v2.20 (https://support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html).

STAR v2.7.1[34] with 2-pass mapping was used to align paired-end reads to human genome build hg38 and GENCODE gene annotation V31. FastQC 0.11.8 and RSeQC 3.0.0[33] were used for QC including insert fragment size, read quality, read duplication rates, gene body coverage and read distribution over different genomic regions. FeatureCounts[32] in Subread 1.6.5 was used to quantify gene-level expression by counting unstranded paired-end reads. Bioconductor package edgeR 3.26.8[31] was used to detect

differential gene expression between genotypes. Genes with low expression were excluded by requiring at least one count per million in at least N samples (N is equal to the number of samples in the smallest genotype group). The filtered expression matrix was normalized by TMM method[30] and subject to significance testing using GLM LRT method. Genes were deemed differentially expressed if FDRs were less than 0.05. Bioconductor package GOseq v1.36[29] was used to perform enrichment analysis on differentially expressed genes against GO Biological Processes (GO_BP_DIRECT from DAVID database; https://david.ncifcrf.gov/home.jsp).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

Phenotype analysis

We used a two-sample t test to compare prevalence of specific PBMCs phenotypes. Individuals were sampled based previously defined *CD101* genotype categories: a) those with no functional *CD101* variants [N = 117], b) individuals having one of the three more prevalent *CD101* Ig-like domain variants (*rs12093834*, or *rs17235773*, or rs3754112) [N = 85], or c) individuals having one of the two more prevalent *CD101* cytoplasmic domain variants (*rs150494742* or *rs34248572*) [N = 33] (Table S1).

Functional analysis

After stimulation, we characterized the intracellular T cell cytokine responses in the presence or absence of Tregs using archived PBMCs from individuals with *rs12093834* (Ig-like variant, N = 42), with *rs34248572* (cytoplasmic variant, N = 18), or with no variant (N = 40). In the Treg depletion analyses we dropped data from individual assays for batches that showed no variation across individuals tested with that assay. We used a nonparametric rank regression test for this data as the data had outliers and the median appeared to be a better measure of the central tendency than the mean (R package Rfit).[25–28] The ''unadjusted'' analyses had only one predictor variable: variant (1 or 0, ig-like or none, cytoplasmic or none). The ''adjusted'' analyses were a multivariate analysis the included batch, gender, and age as control variables.

We used a nominal p = 0.05 as the threshold for statistical significance. Those markers in the phenotypic analysis that satisfied a Bonferroni correction for 100 comparisons are indicated in the text. We treated the functional analysis as a hypothesis generating effort, and as such did not correct for multiple comparisons. We do indicate functional analysis values that pass Bonferroni correction for 250 comparisons.