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The Immunosuppressive Role of IL-32 in Lymphatic Tissue during HIV-1 Infection

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One pathological hallmark of HIV-1 infection is chronic activation of the immune system, driven, in part, by increased expression of proinflammatory cytokines. The host attempts to counterbalance this prolonged immune activation through compensatory mediators of immune suppression. We recently identified a gene encoding the proinflammatory cytokine IL-32 in microarray studies of HIV-1 infection in lymphatic tissue (LT) and show in this study that increased expression of IL-32 in both gut and LT of HIV-1–infected individuals may have a heretofore unappreciated role as a mediator of immune suppression. We show that: 1) IL-32 expression is increased in CD4$^+$ T cells, B cells, macrophages, dendritic cells, and epithelial cells in vivo; 2) IL-32 induces the expression of immunosuppressive molecules IDO and Ig-like transcript 4 in immune cells in vitro; and 3) in vivo, IL-32-associated IDO/Ig-like transcript 4 expression in LT macrophages and gut epithelial cells decreases immune activation but also may impair host defenses, supporting productive viral replication, thereby accounting for the correlation between IL-32 levels and HIV-1 replication in LT. Thus, during HIV-1 infection, we propose that IL-32 moderates chronic immune activation to avert associated immunopathology but at the same time dampens the antiviral immune response and thus paradoxically supports HIV-1 replication and viral persistence. *The Journal of Immunology*, **2011, 186: 6576–6584.**

Chronic activation of the immune system is a pathological hallmark of HIV-1 infection (1) and now widely accepted as an important negative prognosticator of disease progression in infected individuals (2). This chronic immune activation is thought to be due to the persistent nature of HIV-1 replication and the host's inability to clear the virus (3). As such, the immune response does not contract and fully return to a quiescent-like state, instead remaining in a state of sustained interplay between mediators of immune activation and immune suppression, which ultimately determines the magnitude, pace, and duration of an immune response to the invading pathogen (4). In HIV-1 infection, this indelicate balance between mediators of immune activation and immune suppression is skewed in a way that supports viral persistence, CD4$^+$ T cell depletion, and other pathologies that result in disease progression (5–7).

Immune activation during HIV-1 infection is the result of a robust host response in lymphatic tissue (LT) (6), the primary anatomical site of viral replication, CD4$^+$ T cell depletion, and pathology. This response is characterized by the upregulation of a vast array of genes controlling immune activation and antiviral molecules. Although ongoing immune activation is one important feature throughout HIV-1 infection in LT, there is a compensatory upregulation in expression of genes promoting immune suppression during early HIV-1 infection, likely serving as a counterbalance to moderate the immunopathological consequences of sustained immune activation (6). Maintaining this equilibrium between immune activation and immune suppression is essential, as imbalances can benefit HIV-1 replication—too much immune activation provides permissive target cells for the virus, whereas too much immune suppression can dampen innate and cell-mediated immune responses needed to contain the virus.

We have examined the complex relationship between immune activation and suppression during HIV-1 infection by transcriptionally profiling the global host response to HIV-1 infection in LT (6, 7). Using this experimental approach, we have identified global, stage-specific transcriptional signatures during HIV-1 infection (6), transcriptional correlates of viral load (7), and particular genes that provide tantalizing clues to factors and mechanisms that may be critically affecting HIV-1 replication and the host response in LT.

One such gene, the proinflammatory cytokine IL-32, was originally identified in 1992 as an unknown transcript for which expression increased in activated NK and T cells (8). IL-32 is a multi-isoform cytokine that has received growing attention recently as an important component in numerous autoimmune and inflammatory disorders (9). Thus, when we identified IL-32 in our microarray analysis as a gene increased in expression in LT during HIV-1 infection (6), we investigated its potential functional role in this anatomical niche. In this report, we show that there is a significant increase in IL-32 expression in both lymph node and gut during HIV-1 infection, that this cytokine is a potent inducer of immunosuppressive molecules IDO and Ig-like transcript 4

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A.J.S. designed the experiments; A.J.S., C.M.T., S.W.W., and L.D. performed the experiments; T.W.S. recruited subjects and procured biopsy samples; C.S.R. performed statistical analyses; and A.J.S. and A.T.H. analyzed the data and wrote the manuscript.

The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE16363.

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Abbreviations used in this article: ILT4, Ig-like transcript 4; LT, lymphatic tissue; NA, numerical aperture; RWB, riboprobe wash buffer; T_{Reg}, T regulatory.

(ILT4), and that IL-32 expression is associated with a dampening of the antiviral immune response by reducing cell-mediated cytotoxicity, potentially accounting for the correlation between increased IL-32 levels and higher HIV-1 replication in vivo. We thus propose that the nominally proinflammatory cytokine IL-32 actually functions as a double-edged sword during HIV-1 infection, suppressing both immune activation and the antiviral immune response, thereby supporting HIV-1 replication and viral persistence.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Minnesota. All patients provided written informed consent for the collection of samples and subsequent analysis.

Gut and lymph node biopsy specimens

Ileal, rectal, and inguinal lymph node biopsies from 4 HIV-negative individuals and 26 untreated HIV-1–infected individuals at different clinical stages were obtained for this University of Minnesota Institutional Review Board-approved study. Viral load measurements were obtained the same day as biopsies. Each biopsy was immediately placed in fixative (4% neutral buffered paraformaldehyde or Streck's tissue fixative) before embedding in paraffin.

Immunohistochemistry/immunofluorescence

Immunohistochemistry and immunofluorescence were performed as previously described (10) using a biotin-free detection system on 5-μm tissue sections mounted on glass slides. Tissues were deparaffinized and rehydrated in deionized water. Heat-induced epitope retrieval was performed using either a water-bath (95–98°C for 10–20 min) or high-pressure cooker (120°C for 30 s) in one of the following buffers: DiVA Decloaker (Biocare Medical, Concord, CA), 10 mM sodium citrate (pH 6), or 1 mM EDTA (pH 8), followed by cooling to room temperature. Tissues sections were blocked with SNIPER Blocking Reagent (Biocare Medical) for 15 min at room temperature. Endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in methanol. Primary Abs were diluted in Tris-NaCl–blocking buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.05% Tween 20 with Dupont blocking buffer) and incubated overnight at 4°C. After the primary Ab incubation, sections were washed with PBS. Sections for immunofluorescence were then incubated with rabbit or mouse fluorophore-conjugated secondary Abs (Invitrogen, Carlsbad, CA) in 5% nonfat milk for 2 h at room temperature. These sections were washed, nuclei counterstained blue with TOTO-3, and mounted using Aqua Poly/Mount (Polysciences, Warrington, PA). Sections for immunohistochemistry were incubated with mouse or rabbit polymer system reagents conjugated with HRP (DakoCytomation, Carpinteria, CA) according to the manufacturer's instructions, developed with 3,3′-diaminobenzidine (Vector Laboratories), counterstained with Harris hematoxylin (Surgipath Medical, Richmond, IL), and mounted using Permount (Fisher Scientific, Pittsburgh, PA). Stained sections were examined either by light microscopy or immunofluorescent confocal microscopy at ambient temperatures. Light micrographs were taken using an Olympus BX60 upright microscope (Olympus) with the following objectives: ×10 (0.3 numerical aperture [NA]), ×20 (0.5 NA), and ×40 (0.75 NA); images were acquired using a Spot color mosaic camera (model 11.2) and Spot acquisition software (version 4.5.9; Diagnostic Instruments). Immunofluorescent micrographs were taken using an Olympus BX61 Fluoview confocal microscope (Olympus) with the following objectives: ×20 (0.75 NA), ×40 (0.75 NA), and ×60 (1.42 NA); images were acquired using Olympus Fluoview software (version 1.7a; Olympus). Isotype-matched IgG- or IgM-negative control Abs in all instances yielded negative staining results (Supplemental Table I, which lists the primary Abs and Ag-retrieval methodologies).

To quantify levels of IL-32, IDO, or ILT4 expression in the gut and lymph node, 15 randomly stained images from each specimen were captured and positive cells enumerated using Photoshop (CS2, version 9.0; Adobe Systems) with plug-ins from Reindeer Graphics. Specifically, this program utilizes a threshold tool to set a gray level that discriminates positively stained cells from background and marks the positive signal with a red overlay. The program then measures the area of the signal above the threshold, expressed as a percentage of the total area. Data were expressed as the percentage tissue area positive for IL-32, IDO, or ILT4.

In situ hybridization of HIV-1 RNA$^+$ cells

HIV-1 RNA was detected in cells in formalin-fixed and paraffin-embedded tissues as previously described (11). Briefly, 5-μm sections were cut, adhered to silanized glass slides, and deparaffinized. Sections were first permeabilized by treating with HCl, digitonin, and proteinase K, then acetylated, and finally hybridized to [^{35}S]-labeled HIV-1–specific riboprobes. After hybridization, the slides were washed in 5× SSC/10 mM DTT at 42°C, 2× SSC/10 mM DTT/50% formamide at 60°C, and a 2× riboprobe wash buffer (RWB) (0.1 M Tris-HC1 [pH 7.5], 0.4 M NaCl, and 50 mM EDTA] before digestion at 37°C with RNase A (25 μg/ml) in 1× RWB. After washing in RWB, 2× SSC, and 0.1× SSC, sections were dehydrated in graded ethanol solutions containing 0.3 M ammonium acetate, dried, and then coated with nuclear track emulsion, exposed at 4°C, developed, and counterstained with Harris hematoxylin (Surgipath Medical). Light micrographs were taken using an Olympus BX60 upright microscope (Olympus).

PBMCs treated with IL-32

PBMCs from healthy donors were isolated by density centrifugation using Ficoll/Hypaque (Sigma-Aldrich, St. Louis, MO) and maintained in RPMI 1640 medium supplemented with 10% FBS (heat-inactivated) and 50 μg/ml gentamicin (Invitrogen) at 37°C/5% CO_2. After overnight culture, 50 ng IL-32γ (R&D Systems, Minneapolis, MN) or 5 μg PHA was added to the culture and incubated for 24–48 h. After incubation, cells were washed in PBS, spotted onto silanized glass slides, fixed using SAFEFIX II (Fisher Scientific), dried, and stained for cellular markers using specific Abs according to the above protocol. To quantify the fold-change in IDO or ILT4 production, 10 randomly stained images from each donor's PBMCs (3 donors in total) in each experimental condition were captured using an Olympus BX60 upright microscope (Olympus) and positive cells enumerated by manually counting positively stained cells in each image. The number of positively stained cells was determined as a percentage of total cells and then compared with the control percentage in untreated samples, yielding a fold-change in protein expression (fold-change is set at 1 for control samples).

Statistical analysis

To test for the effect of stage on IL-32 expression, a mixed model was fit with the logarithm of IL-32 expression as the response variable, stage as a four-level factor explanatory variable, and anatomical location as a dichotomous variable to distinguish lymph node from gut tissue (we included additive random effects for subjects to model the within-subject correlation). This model found significant differences between all HIV$^+$ stages and the uninfected subjects (all $p < 0.0001$) as well as a significant difference between lymph node and gut; however, no significant differences were detected between the HIV$^+$ stages. To test for an effect of day and dose in the in vitro experiments, a two-way ANOVA was conducted using the logarithm of the expression levels. To test for associations between IL-32 and IDO or ILT4, linear models were fit for which the log of IL-32 was the outcome and infection status plus the log of IDO or ILT4 were covariates in addition to the interaction between the two variables. This model found significant positive associations between IL-32 and IDO ($p = 0.003$), IL-32 and ILT4 ($p < 0.0001$), and IDO and ILT4 ($p < 0.0001$) among HIV-1–infected individuals. Associations between continuous variables (IL-32 and immune activation markers, cell proliferation markers, cytotoxic mediators, or CD4$^+$ T cell counts) were estimated using Pearson's correlation coefficient and tests conducted using the usual t test for a correlation. All calculations were conducted using the statistical software R version 2.10.1.

Microarray data accession number

All microarray results have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE16363).

Results

IL-32 expression is increased in lymph node and gut during HIV-1 infection

To examine changes in IL-32 expression during HIV-1 infection, we used Abs recognizing IL-32 to stain gut (ileum and rectum) and lymph node (inguinal) biopsies from uninfected and HIV-1–infected individuals in each clinical stage of disease: acute (defined as individuals sampled within 4 mo of documented seroconversion), asymptomatic (defined as individuals infected for at

least 4 mo with a $CD4^+$ T cell count >200 cells/μl), and AIDS (defined as infected individuals with a $CD4^+$ T cell count <200 cells/μl) (Table I). Compared with uninfected individuals, levels of IL-32 were significantly increased in both gut and LT during all stages of HIV-1 infection, with the highest IL-32 levels in the acute stage of disease for gut (4.8-fold increase) and AIDS stage of disease for lymph node (5.8-fold increase) (Fig. 1).

To determine the cellular source(s) of IL-32 within gut and LT, we used Abs to CD4 or CD8 (T cells), CD163 (macrophages), CD20 (B cells), CD11c (dendritic cells), killer cell lectin-like receptor subfamily C, member 1 (NK cells), and cytokeratin (epithelial cells) to colocalize IL-32 with the cell types producing this cytokine. As shown in representative images in Fig. 2, IL-32 was expressed during HIV-1 infection in $CD4^+$ T cells, B cells, macrophages, and dendritic cells; minimal expression was detected in $CD8^+$ T cells or NK cells (data not shown). Additionally, IL-32 was highly expressed in the mucosal epithelium of HIV-1–infected gut. Finally, the majority of $IL\text{-}32^+$ cells were also expressing IL-18, a proinflammatory cytokine implicated in initiating IL-32 expression (12) (Supplemental Fig. 1). Thus, IL-32 is significantly increased during HIV-1 infection and broadly expressed in many cell types in both gut and LT.

IL-32 induces IDO expression

IL-32 has been categorized as a proinflammatory cytokine due to its elevated levels in various inflammatory diseases (13–15) as well as its ability in vitro to induce other proinflammatory mediators, such as TNF-α, IL-1β, and IL-8 (12, 13, 16). Despite IL-32's association with inflammation and induction of proinflammatory cytokines, recent work has also suggested a role for IL-32 in immune suppression through its ability to stimulate IL-10 production (17). To better understand the role of IL-32 during HIV-1 infection, we first examined its effects in vitro. We treated human leukocytes (both polymorphonuclear cells and PBMCs) with rIL-32γ, the most biologically active isoform (18) and then examined expression of markers of immune activation or suppression. We found IL-32γ to be a potent inducer of IDO, a tryptophan-degrading enzyme with far-reaching immunosuppressive effects (19). IL-32γ increased IDO expression ~6-fold ($p < 0.001$) in both polymorphonuclear cells and PBMCs (Fig. 3). IL-32γ induced IDO production in $CD163^+$ macrophages, $CD4^+$ T cells, $Foxp3^+$ T regulatory (T_{Reg}) cells, and $CD11c^+$ dendritic cells (Supplemental Fig. 2). In contrast, IL-32γ had minimal effects on immune activation and proliferation, with no detectable increases in cell activation/proliferation markers such as Ki-67, CD38, or CD69 (data not shown), in agreement with a previous report (14).

We then showed that IL-32 expression correlated with IDO production in vivo. We stained for IDO in a subset of study individuals and found a similar pattern of protein expression as for IL-32 (Fig. 4*A*), resulting in a significant positive association between $IL\text{-}32^+$ cells and IDO expression ($r = 0.6850$, $p = 0.003$) (Fig. 4*B*). Moreover, IDO often was coexpressed with IL-32 (Fig. 4*C*), predominantly in LT macrophages (Fig. 4*D*) and gut epithelial cells (Fig. 4*A*). Thus, increased expression of IL-32 during HIV-1 infection coincides with induction of the tryptophan-degrading enzyme IDO in diverse anatomical compartments such as the gut and lymph node, thereby lowering environmental tryptophan (20), which can inhibit immune cell proliferation/activation (21, 22), promote the generation of T_{Reg} cells (23, 24), and stimulate expression of other immunosuppressive molecules such as the ILT receptors (25).

IL-32 induces ILT4 expression

ILT receptors are cell-surface inhibitory molecules that bind MHC class I and render target cells anergic (26). Because we had

Table I. Clinical characteristics of study subjects

Patient No.	Disease Stage	Gender	Age (y)	Race	Peripheral Blood $CD4^+$ T Cell Count (Cells/μl)	Plasma HIV-1 RNA Levels (Copies/ml)
AP1476	Uninfected	Female	28	White	704	Undetectable
DB1472	Uninfected	Female	52	White	837	Undetectable
GG1425	Uninfected	Male	43	White	1,351	Undetectable
MS1452	Uninfected	Male	40	White	742	Undetectable
MH1458	Acute	Male	51	White	400	439,000
PH1329	Acute	Male	59	White	370	484,694
PR1389	Acute	Male	32	White	824	32,173
SQ1484	Acute	Male	49	White	301	23,721
SR1469	Acute	Male	44	White	180	>100,000
TH1449	Acute	Male	30	White	333	>100,000
TS1435	Acute	Male	42	White	663	>100,000
WB1391	Acute	Male	37	African American	234	24,718
AE1419	Asymptomatic	Male	37	White	245	61,432
CF1463	Asymptomatic	Male	23	African American	259	27,200
DB1468	Asymptomatic	Male	30	White	875	2,150
DS1335	Asymptomatic	Male	32	White	400	15,284
EO1429	Asymptomatic	Male	27	African American	1,058	2,620
JF1086	Asymptomatic	Male	30	White	512	20,562
RC1293	Asymptomatic	Male	36	White	905	14,225
TS1317	Asymptomatic	Male	31	White	399	120,469
WU1459	Asymptomatic	Male	36	White	286	>100,000
DD1446	AIDS	Female	45	White	200	150,500
EL1474	AIDS	Male	40	African American	98	10,000
GL1438	AIDS	Male	49	White	147	4,960
MO1263	AIDS	Male	44	White	3	>100,000
RB1413	AIDS	Male	50	African American	42	59,401
TK1462	AIDS	Male	43	White	81	35,000
VM1327	AIDS	Female	40	African American	112	12,046
95-6082	AIDS	Female	42	White	110	>100,000
93-1547	AIDS	Male	46	White	75	>100,000

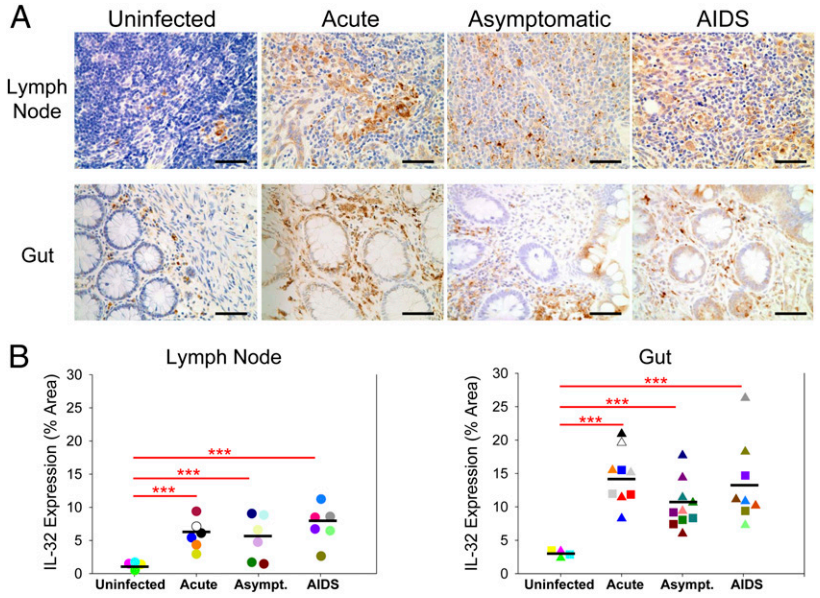

FIGURE 1. IL-32 expression is significantly increased in both gut and lymph node during HIV-1 infection. *A*, Representative immunohistochemical images reveal increased IL-32 expression in the gut and inguinal lymph node during HIV-1 infection (IL-32–positive cells appear brown, whereas cell nuclei appear blue). Original magnification ×400. Scale bars, 50 μm. *B*, IL-32 expression was quantified in each biopsy and reported as percent tissue area positive for IL-32. The results are shown with significance where applicable. Symbols: circles, triangles, and squares represent inguinal lymph node, ileal, and rectal biopsies, respectively, whereas the black bars denote the mean expression level of IL-32 in each stage of disease. ***$p < 0.0001$. Asympt., asymptomatic.

previously identified ILT4 from our microarray analysis as a gene increased in expression in LT during HIV-1 infection (6), we examined the possibility that IL-32–induced IDO might be creating a local environment in LT that favors ILT4 expression.

We first tested in vitro whether IL-32γ could induce ILT4 expression in PBMCs. As we had shown for IDO induction, IL-32γ also increased ILT4 expression in PBMCs (~2.6-fold; $p < 0.001$) (Fig. 5). In vivo, we also found a similar pattern of protein expression for IL-32 and ILT4 (Fig. 6*A*), resulting in a significant positive association between IL-32$^+$ cells and ILT4 expression ($r = 0.9246$, $p < 0.0001$) (Fig. 6*B*). Moreover, ILT4 often was coexpressed with IL-32 (Fig. 6*C*), predominantly in LT macrophages (Fig. 6*D*) and gut epithelial cells (Fig. 6*A*). Not surprisingly, ILT4 and IDO were also significantly associated with one another ($r = 0.8790$, $p < 0.0001$) and expressed within the same cell (Supplemental Fig. 3*A*, 3*B*). Thus, increased expression of IL-32

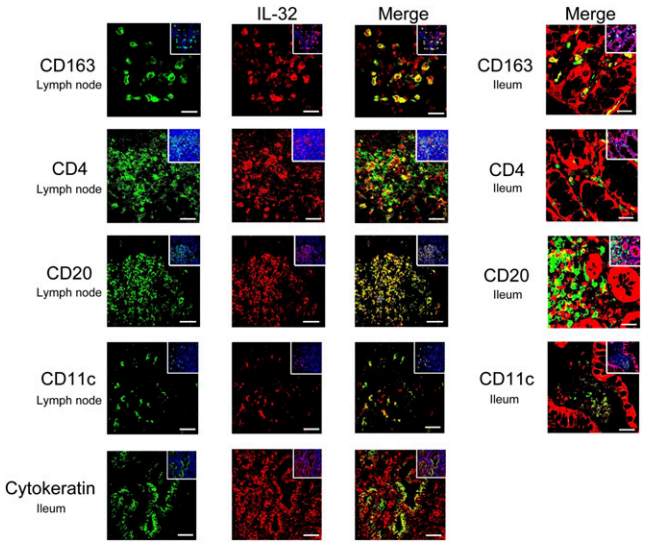

FIGURE 2. IL-32 is expressed in CD4$^+$ T cells, B cells, macrophages, dendritic cells, and epithelial cells during HIV-1 infection. Representative immunofluorescent images of IL-32 (red staining) and various cell-surface markers (green staining) in the inguinal lymph node and gut from HIV-1–infected individuals, showing colocalization between IL-32 and CD163$^+$ macrophages, CD4$^+$ T cells, CD20$^+$ B cells, CD11c$^+$ dendritic cells, and cytokeratin$^+$ epithelial cells. The *insets* show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification ×600. Scale bars, 10 μm.

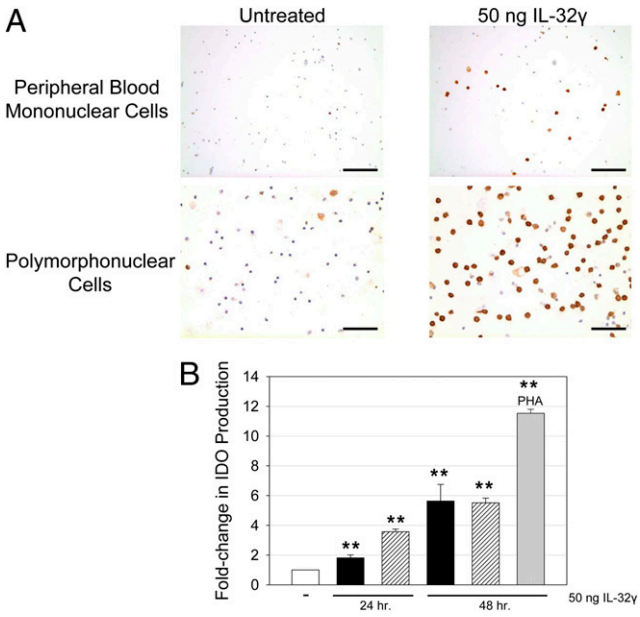

FIGURE 3. IL-32γ stimulates the production of IDO by both polymorphonuclear cells and PBMCs. *A*, Representative immunohistochemical images reveal increased IDO expression in polymorphonuclear cells and PBMCs treated with 50 ng IL-32γ (IDO-positive cells appear brown, whereas cell nuclei appear blue). A dose-response curve was initially used (5, 50, and 500 ng IL-32γ) in designing these in vitro experiments. There was no appreciable change in cell markers at 5 ng compared with untreated controls. However, 50 and 500 ng yielded significant changes in IDO expression compared with untreated controls, with little difference between the two dosages. Thus, we report a time-dependent effect of 50 ng IL-32γ on IDO expression. Original magnification ×400. Scale bars, 50 μm. *B*, IDO-positive cells were enumerated for each condition and reported as fold-change in IDO production. Data are expressed as the mean ± SEM for which three independent experiments were performed in triplicate. Black bars represent PBMCs, whereas hashed bars represent polymorphonuclear cells. PBMCs were treated with 5 μg PHA as a positive control. The results are shown with significance where applicable. **$p < 0.001$.

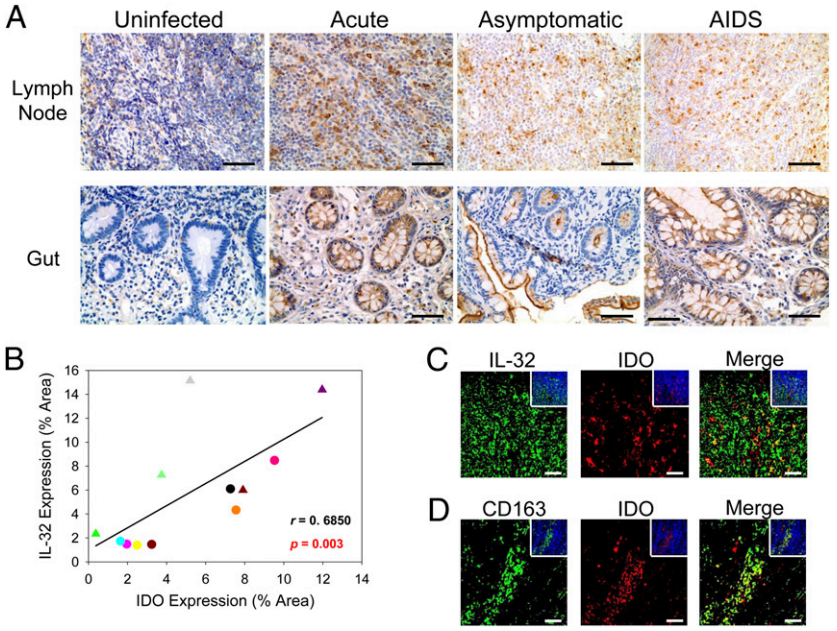

FIGURE 4. Increased IDO expression is significantly associated with IL-32 production in both gut and lymph node during HIV-1 infection. *A*, Representative immunohistochemical images reveal increased IDO expression in the gut and inguinal lymph node during HIV-1 infection (IDO-positive cells appear brown, whereas cell nuclei appear blue). *B*, IL-32 expression was significantly correlated with IDO expression in both gut and inguinal lymph node during HIV-1 infection. Symbols: circles and triangles represent inguinal lymph node and ileal biopsies, respectively. *C* and *D*, Representative immunofluorescent images of IDO (red staining) and IL-32 or CD163 (green staining) in the inguinal lymph node from HIV-1–infected individuals, showing colocalization between IDO and IL-32 and between IDO and CD163$^+$ macrophages. The *insets* show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification ×400. Scale bars, 50 μm.

during HIV-1 infection likely initiates a cascade of events to moderate immune activation by inducing two potent immunosuppressors, IDO and ILT4, in both gut and lymph node.

IL-32 expression is associated with reduced immune activation, cell proliferation, and cytotoxic factors in vivo

Immunosuppressive molecules such as IDO and ILT4 could play an important role in moderating the immunopathological consequences of chronic immune activation during HIV-1 infection, but these molecules also may be detrimental to the host by dampening the antiviral immune response through inhibition of innate and adaptive cytotoxic functions. Consistent with this concept of a balance between moderating effects on immune activation/proliferation and inhibition of host defenses, we found significant inverse correlations between IL-32 expression and mRNA levels of cell proliferation markers (Ki-67, proliferating cell nuclear Ag), cell activation markers (CD38, HLA-DR), and cytotoxic mediators (perforin, granzyme, NK cell group 7 sequence, and killer-specific secretory protein of 37 kDa) in lymph node (Fig. 7).

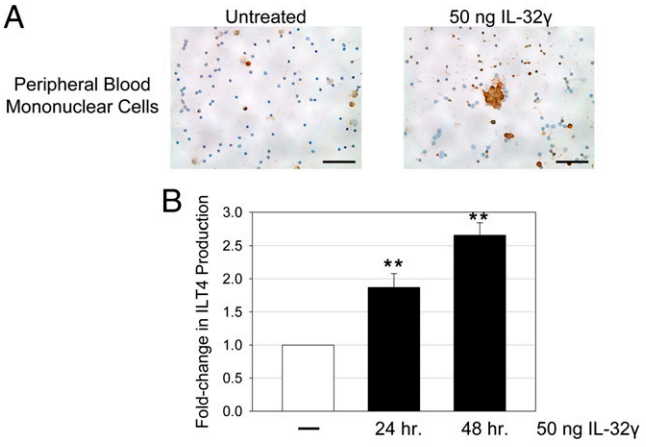

FIGURE 5. IL-32γ stimulates the production of ILT4 by PBMCs. *A*, Representative immunohistochemical images reveal increased ILT4 expression in PBMCs treated with 50 ng IL-32γ (ILT4-positive cells appear brown, whereas cell nuclei appear blue). A dose-response curve was initially used (5, 50, and 500 ng IL-32γ) in designing these in vitro experiments. There was no appreciable change in ILT4 at 5 ng compared with untreated controls. However, 50 and 500 ng yielded significant changes in ILT4 expression compared with untreated controls, with little difference between the two dosages. Thus, we report a time-dependent effect of 50 ng IL-32γ on ILT4 expression in PBMCs. Original magnification ×400. Scale bars, 50 μm. *B*, ILT4-positive cells were enumerated for each condition and reported as fold-change in ILT4 production. Data are expressed as the mean ± SEM for which three independent experiments were performed in triplicate. The results are shown with significance where applicable. $^{**}p < 0.001$.

IL-32 expression is associated with HIV-1 replication in vivo and inversely correlates with CD4$^+$ T cell survival

The immunosuppressive effects of IDO, other immunoregulatory cytokines, and T_{Reg} cells have been implicated in facilitating SIV and HIV-1 replication (22, 27, 28). Because IL-32 can promote IDO and ILT4 expression, we looked at the relationship between HIV-1 replication and IL-32 expression in a small subset of lymph node biopsies and found a qualitative relationship between the number of HIV-1–infected cells and IL-32 expression (Fig. 8). Consistent with a role in immune suppression, we also found an inverse relationship between IL-32 expression and granzyme-producing cells in the lymph node (Fig. 8). Finally, IL-32 expression and augmented HIV-1 replication was inversely correlated with CD4$^+$ T cell numbers in both peripheral blood ($r = -0.451$, $p = 0.035$) (Supplemental Fig. 3*C*) and lymph node (Fig. 8). These data suggest that IL-32–induced IDO and ILT4 expression may suppress cell proliferation/activation and downmodulate cytotoxic mediators required for effective viral clearance, creating an environment more conducive for productive HIV-1 replication, which also contributes to CD4$^+$ T cell depletion.

Discussion

IL-32 is thought to play an important role in various inflammatory disorders due to its purported role in fueling inflammation by inducing expression of other proinflammatory mediators (9). However, until recently (29), IL-32 has received little attention as a moderator of chronic immune activation. In this study, we provide evidence that IL-32 may have such a role in countering immune activation and inflammation during HIV-1 infection by promoting immune suppression via the induction of immunosuppressive molecules

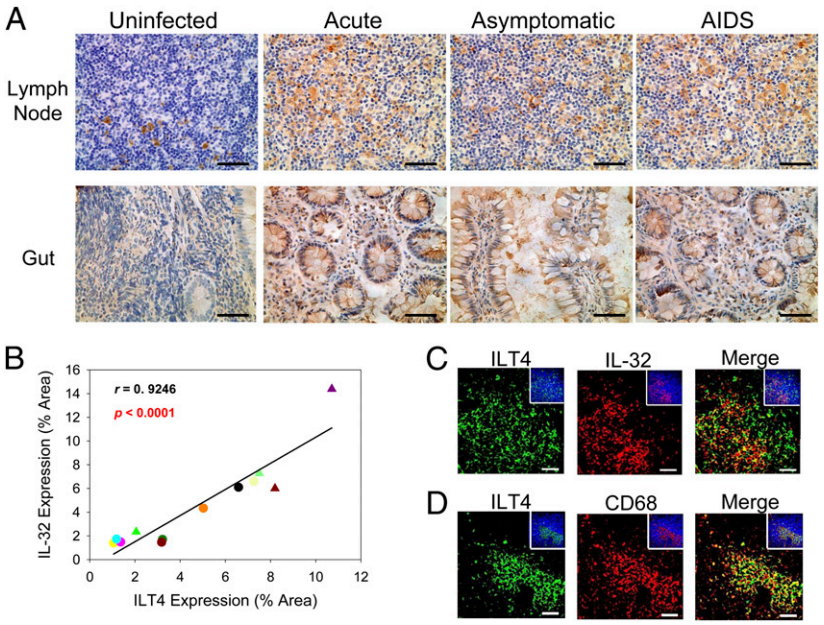

FIGURE 6. Increased ILT4 expression is significantly associated with IL-32 production in both gut and lymph node during HIV-1 infection. *A*, Representative immunohistochemical images reveal increased ILT4 expression in the gut and inguinal lymph node during HIV-1 infection (ILT4-positive cells appear brown, whereas cell nuclei appear blue). *B*, IL-32 expression was significantly correlated with ILT4 expression in both gut and inguinal lymph node during HIV-1 infection. Symbols: circles and triangles represent inguinal lymph node and ileal biopsies, respectively. *C* and *D*, Representative immunofluorescent images of ILT4 (green staining) and IL-32 or CD68 (red staining) in the inguinal lymph node from HIV-1–infected individuals, showing colocalization between ILT4 and IL-32 and between ILT4 and $CD68^+$ macrophages. The *insets* show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification ×400. Scale bars, 50 μm.

IDO and ILT4. This observation complements a previous study in which IL-32 was shown to induce the immunosuppressive cytokine IL-10 (17).

The balance between immune activation and immune suppression is critical throughout the immune system, whereby perturbation of this balance can have deleterious, immunopathological consequences for the host (30). Additionally, this balance is crucial in determining the effectiveness and ability of the immune system to initially clear acute infections or partially control persistent infections. Because HIV-1 is usually not cleared in the acute stage of infection (31), the immune system, without antiviral therapy, is confronted over a period of years with the sustained challenge of managing this balance between chronic immune activation, needed to maintain host defenses to partially control persistent

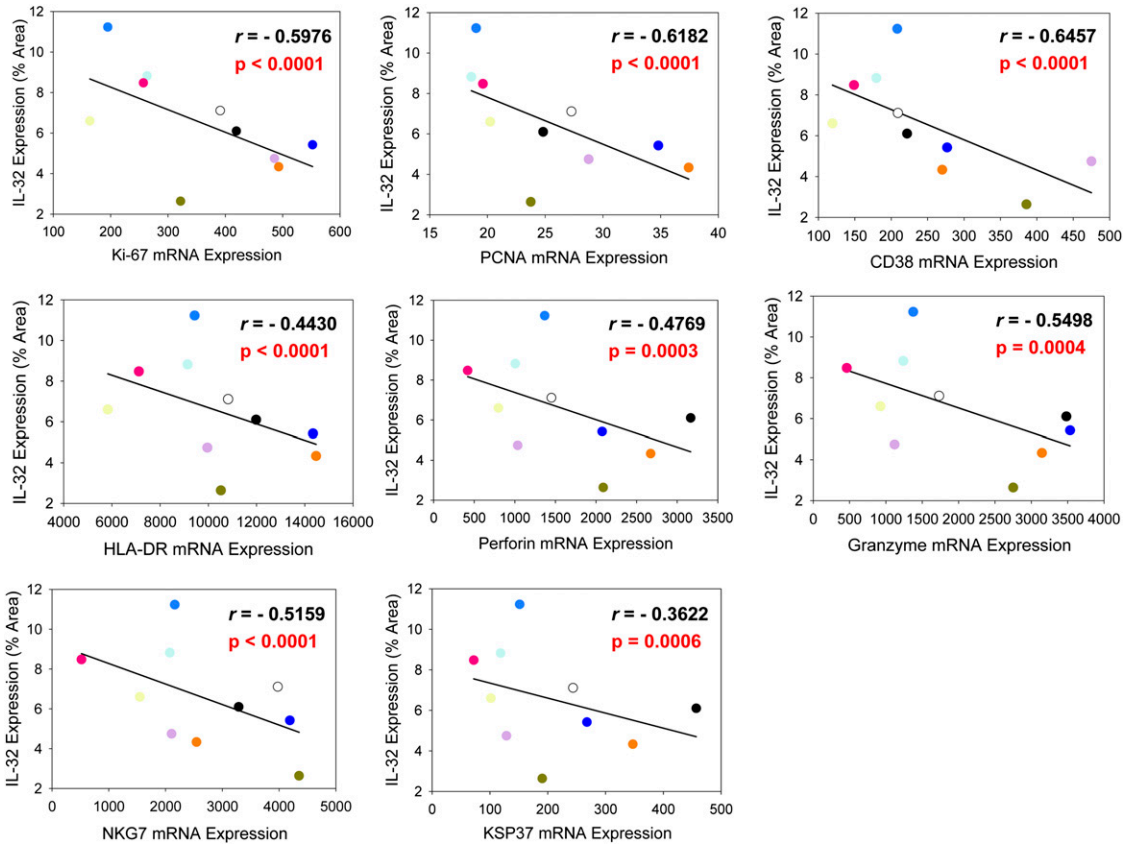

FIGURE 7. IL-32 expression in the lymph node of HIV-1–infected individuals is associated with reduced cell proliferation, cell activation, and cytotoxic mediators. IL-32 expression within the inguinal lymph node was inversely correlated with mRNA levels of cell proliferation markers Ki-67 and proliferating cell nuclear Ag, immune activation markers CD38 and HLA-DR, and cytotoxic mediators perforin, granzyme, NK cell group 7 sequence (NKG7), and killer-specific secretory protein of 37 kDa (KSP37). mRNA levels are taken from Li et al. (6).

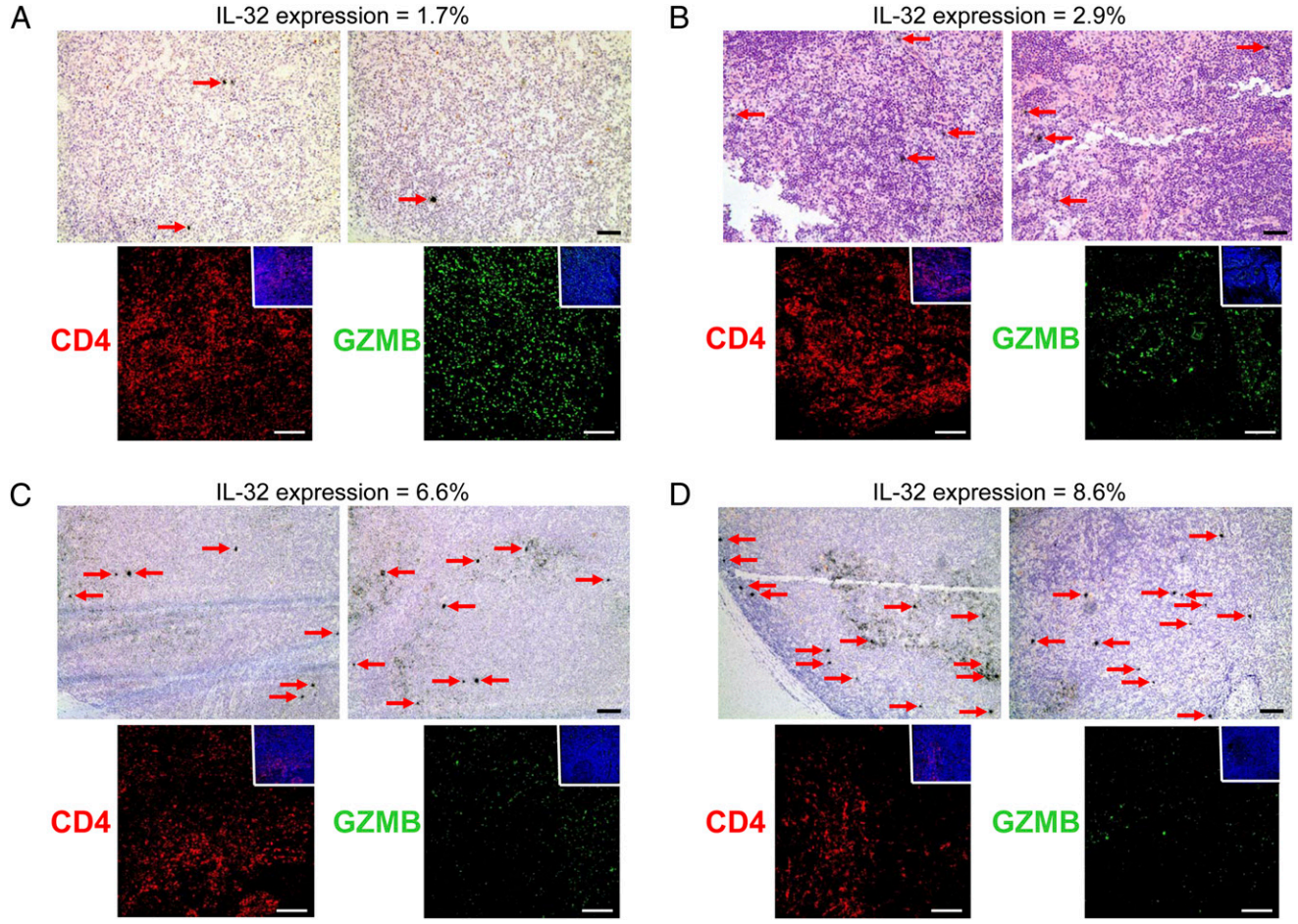

FIGURE 8. IL-32 expression in the lymph node of HIV-1–infected individuals is associated with productive viral replication, lower granzyme expression, and decreased $CD4^+$ T cell viability. *A–D*, Representative light micrographs reveal a qualitative association between HIV-1 replication (HIV-1 RNA^+ cells appear as collections of black silver grains overlying a single cell, whereby each red arrow denotes a single HIV-1 RNA^+ cell; cell nuclei appear blue) and IL-32 expression in the inguinal lymph node of HIV-1–infected individuals. Immunofluorescent images reveal decreased granzyme B expression (green) and lower $CD4^+$ T cell numbers (red) with increasing IL-32 expression in the same lymph nodes. Original magnification ×100 (light micrographs) and ×200 (immunofluorescent micrographs). Scale bars, 100 μm.

viral replication, and moderating the immunopathological consequences of this chronic immune activation.

The transcriptional profiles of the acute, asymptomatic, and AIDS stages of HIV-1 infection (6) have revealed the complexity of managing chronic immune activation in persistent infection. Initially, the host's immune system responds by upregulating expression of large numbers of genes that mediate immune activation and innate and adaptive defenses. With the general failure of these defenses in clearing infection, there is an abrupt decrease in expression of most of these genes to levels indistinguishable from HIV-1–uninfected individuals in the asymptomatic stage of infection, which we have interpreted as immunoregulatory mechanisms mounted by the host to strike a balance between moderating chronic immune activation and maintaining host defenses to partially contain infection.

The mediators of this immunoregulatory transition, however, were not immediately obvious in the lists of genes with altered expression, with the exception of IL-32, IDO, and ILT4 (6), and hence the focus on these genes in the work we now report. From the evidence we present, IL-32 certainly could be one of these early immunoregulatory mediators, with increased expression at the right place and right time—in many immune cells during acute HIV-1 infection in both the gut and lymph node as well as in intestinal epithelial cells (Figs. 1, 2). The induction of IL-32 itself is likely due to other proinflammatory cytokines increased during HIV-1 infection (32), particularly IL-18 (Supplemental Fig. 1), which has been shown to be a potent inducer of IL-32 in vitro (12). The antigenicity of HIV-1 itself is unlikely to stimulate IL-32 production, as Nold et al. (33) demonstrated that infection of PBMCs with various strains of HIV-1 actually inhibited production of this cytokine rather than enhancing it.

We had also observed increased expression of ILT4 and IDO in early HIV-1 infection (6) and conjectured that these immunosuppressors were also partly responsible for the immunoregulatory transition. In this study, we show that IL-32, IDO, and ILT4 are coregulated and associated with decreased immune activation, proliferation, and cytotoxic host factors. In vitro, IL-32 induced both IDO and ILT4 expression in PBMCs, whereas in vivo, the levels of IL-32 in LT and gut correlated with both IDO and ILT4 expression levels and were, in turn, inversely correlated with markers of cell proliferation and cytotoxic NK and T cell markers.

The role of IDO in HIV-1 infection has been well documented (20, 34) in suppressing various arms of the immune system by depleting locally available stocks of the essential amino acid tryptophan. A tryptophan-depleted environment has been repeatedly described in HIV-1 infection (35–38) and thought to be responsible for inhibiting essential cellular functions through its potent antiproliferative and immunosuppressive effects (21, 22,

39). Additionally, high tryptophan catabolism within the environment can promote the local generation of T_{Reg} cells (23, 24) and other immunosuppressive molecules (e.g., ILT4) (25), a process that can lead to further IDO induction (34), resulting in a continuous cycle of immunosuppressive amplification.

Like IDO, ILT4 can also serve to further amplify an immunosuppressive environment; ILT4 expression on APCs such as macrophages can inhibit proliferation of immune cells (26, 40), render immune cells anergic and unresponsive to extracellular stimuli (26, 40, 41), and promote the local generation of T_{Reg} cells (25, 41, 42). The localization of ILT4 and IDO in vivo, mainly in macrophages, is consistent with these functions.

We think that IL-32, IDO, ILT4, and T_{Reg} cells comprise important components of an immunoregulatory axis designed to counter the pathological effects of chronic immune activation in persistent HIV-1 infections and the primate counterparts in pathogenic SIV infections. The price the host pays for these moderating effects are impaired host defenses, ranging from smaller numbers of virus-specific CTLs (27, 43) to the decreased expression of cytotoxic effectors (44–46) associated with increased IL-32 expression. In support of this, a recent study showed that splenocytes isolated from mice injected with IL-32–expressing epithelial cells displayed dampened cell-mediated cytotoxicity compared with splenocytes isolated from control mice (47). Although IL-32 has been shown in vitro to have IFN-mediated antiviral activity (33), we think in vivo that IL-32's immunosuppressive effects contribute, at best, to partial control of untreated HIV-1 infection. In sum, although it remains difficult to quantify the suppressive contributions from IL-32 itself in terms of overall cell-mediated cytotoxicity compromised during HIV-1 infection (48), these data, nevertheless, suggest that IL-32 may be a contributing factor in an immunoregulatory axis that collectively acts as a double-edged sword in lentiviral immunodeficiency infections.

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Disclosures

The authors have no financial conflicts of interest.

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