

# SIV-induced Translocation of Bacterial Products in the Liver Mobilizes Myeloid Dendritic and Natural Killer Cells Associated With Liver Damage

Tristan I. Evans,<sup>1,a</sup> Haiying Li,<sup>2,a</sup> Jamie L. Schafer,<sup>2</sup> Nichole R. Klatt,<sup>3</sup> Xing-Pei Hao,<sup>4</sup> Ryan P. Traslavina,<sup>4</sup> Jacob D. Estes,<sup>4</sup> Jason M. Brenchley,<sup>5</sup> and R. Keith Reeves<sup>1,2</sup>

<sup>1</sup>Division of Immunology, New England Primate Research Center, Harvard Medical School, Southborough, and <sup>2</sup>Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Boston, Massachusetts; <sup>3</sup>Department of Pharmaceutics, Washington National Primate Research Center, University of Washington, Seattle; <sup>4</sup>AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory, and <sup>5</sup>Program in Barrier Immunity and Repair, Immunopathogenesis Section, LMM, NIAID NIH, Bethesda, Maryland

**Disruption of the mucosal epithelium during lentivirus infections permits translocation of microbial products into circulation, causing immune activation and driving disease. Although the liver directly filters blood from the intestine and is the first line of defense against gut-derived antigens, the effects of microbial products on the liver are unclear. In livers of normal macaques, minute levels of bacterial products were detectable, but increased 20-fold in simian immunodeficiency virus (SIV)-infected animals. Increased microbial products in the liver induced production of the chemoattractant CXCL16 by myeloid dendritic cells (mDCs), causing subsequent recruitment of hypercytotoxic natural killer (NK) cells expressing the CXCL16 receptor, CXCR6. Microbial accumulation, mDC activation, and cytotoxic NK cell frequencies were significantly correlated with markers of liver damage, and SIV-infected animals consistently had evidence of hepatitis and fibrosis. Collectively, these data indicate that SIV-associated accumulation of microbial products in the liver initiates a cascade of innate immune activation, resulting in liver damage.**

**Keywords.** HIV; liver disease; microbial translocation; myeloid dendritic cells; natural killer cells; SIV.

In human immunodeficiency virus (HIV) infection, disease progression is more strongly associated with immune activation than with viral load. During acute infection, the rampant viral replication that occurs in the gut causes severe CD4<sup>+</sup> T-cell depletion and results in decreased integrity of the intestinal epithelium [1, 2]. This damaged barrier leads to translocation of microbial products out of the lumen of the gastrointestinal tract into the circulation [3, 4], and in addition replicating virus can be a source of systemic immune activation

observed in chronic HIV disease [5]. Indeed, even pre-existing gastrointestinal dysfunction at the time of infection is associated with more rapid simian immunodeficiency virus (SIV) disease progression [6]. Not surprisingly, recent reports also suggest that microbial translocatants accumulate in blood-filtering organs such as the liver [7, 8], but the immunological consequences for this organ and associated disease pathologies in HIV/SIV infection are poorly understood.

The liver plays a significant role in the maintenance of immune responses to immunodeficiency viruses. Large numbers of HIV/SIV-specific CD8<sup>+</sup> T cells are found in livers of SIV-infected macaques [9, 10], and this organ is largely responsible for the clearance of virus from the bloodstream [11]. Although often underappreciated, a major source of morbidity and mortality in HIV patients is liver disease, contributing to approximately 15% of non-AIDS-related deaths, even in highly active antiretroviral therapy (HAART)-treated patients

Received 8 April 2015; accepted 24 July 2015.

<sup>a</sup>T. I. E. and H. L. contributed equally to this work.

Correspondence: R. Keith Reeves, PhD, Center for Virology and Vaccine Research, 3 Blackfan Circle, CLS1024, Boston, MA 02215 (rreeves@bidmc.harvard.edu).

The Journal of Infectious Diseases®

© The Author 2015. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.

DOI: 10.1093/infdis/jiv404

[12]. Tissue fibrosis, steatosis, and nodular regenerative hyperplasia are all found at higher frequencies in HIV patients, as are more advanced conditions such as nonalcoholic fatty liver disease and liver cancer [12–14]. In addition, coinfection of HIV with hepatitis C virus (HCV) affects 5–7 million people [15], which decreases viral clearance during acute HCV infection [16, 17] and accelerates the development of hepatocellular carcinoma [18]. Progression of HCV is also exacerbated by microbial translocation, and circulating lipopolysaccharide (LPS) is associated with the development of cirrhosis [19].

Natural killer (NK) cells are found at high frequencies in the liver, some 30%–50% of human hepatic lymphocytes [20]. NK cells also play critical roles in defense against a number of viral infections, including HIV and HCV [21–23]. Numerous studies have reported that robust NK cytotoxicity, as well as NK cell modulation of adaptive responses, is associated with control of HIV disease. Long-term nonprogressors (LTNPs) have increased NK cell cytotoxicity compared to viremic individuals [24], whereas an early NK cell response during acute SIV infection is associated with viral control [25]. Furthermore, expression of certain killer-cell immunoglobulin-like receptor (KIR) in concert with their major histocompatibility complex class I ligands is associated with delayed disease progression in HIV infection [26], and immune pressure exerted by NK cells is evidenced by KIR-associated polymorphisms observed in HIV [27]. Conversely, a subset of macaque inhibitory *KIR3DL* alleles is associated with higher viral loads in SIV infection [28]. The importance of NK cells for control of HCV infection has also been demonstrated by epidemiologic studies that show that individuals who express a specific subset of inhibitory KIRs in combination with the appropriate human leukocyte antigen (HLA) ligands are more likely to clear HCV infection [29]. Moreover, HCV has evolved a mechanism for evading NK responses by expression of a viral peptide that stabilizes HLA-E expression, thereby inhibiting NK-mediated cytotoxicity through interaction with the inhibitory receptor NKG2A [30].

NK cells express CXCR6 and can be recruited to the liver via its ligand CXCL16 [31]. Myeloid dendritic cells (mDCs) are one of the main producers of CXCL16 [32]. Therefore, in this study we set out to investigate how microbial translocation during SIV infection affects hepatic mDCs and, in turn, hepatic NK cells. These findings have implications for the liver pathology associated with HIV, especially in instances of coinfection with HCV.

## METHODS

### Animals and SIV Infections

Animals were housed at the New England Primate Research Center or National Institutes of Health and cared for according to guidelines of the American Association for Accreditation of Laboratory Animal Care, in Association for Assessment and Accreditation of Laboratory Animal Care–accredited facilities,

and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the National Institutes of Health (NIH) or Harvard Medical School. Animals were monitored daily by veterinary staff, and those showing signs of significant weight loss, disease, or distress were provided dietary supplementation and medication as necessary. Euthanization with an overdose of barbiturates was carried out in accordance with the guidelines of the American Veterinary Medical Association.

Tissue samples from Indian rhesus macaques were analyzed in this study, including 11 SIV-naïve animals and 17 chronically infected with SIVmac239. Animals were infected intravenously. Chronically SIV-infected macaques were infected between 112 and 756 days, with a median duration of 308 days. Chronic viral loads at time of necropsy were between 1.7 and 6.9 log<sub>10</sub> copies of viral RNA/mL, plasma, with a median of 5.3 log<sub>10</sub> copies of viral RNA/mL, plasma, determined as previously described [33]. All animals were free of simian retrovirus type D or simian T-lymphotropic virus type 1.

### Tissue Collection and Processing

Peripheral blood mononuclear cells were isolated by density gradient centrifugation of ethylenediaminetetraacetic acid-treated blood over lymphocyte separation media (MP Biomedicals, Solon, Ohio), followed by lysis of red blood cells using a hypotonic ammonium chloride solution. Single-cell suspensions of liver tissue were prepared as described [34].

### Antibodies and Flow Cytometry

Antibodies to the following antigens were used in this study and were obtained from BD Biosciences unless otherwise specified: active-caspase-3-Alexa647 (clone C92–605), CD3-APC-Cy7 (clone SP34.2), CD4-PE (clone L-200), CD8 $\alpha$ -Qdot605 (clone T8/7Pt-3F9, NIH Nonhuman Primate Reagent Resource Program), CD8 $\alpha$ -APC-Cy7 (clone SK1), CD11c-PE or -APC (clone S-HCL-3), CD14-Alexa700 (clone Tuk4, Invitrogen), CD16-Alexa-700 (clone 3G8), CD20-PerCP-Cy5.5 (clone L27), CD45-FITC (clone D058–1283), CD45-PerCP-Cy5.5 (clone Tu116), CD56-PE-Cy7 (clone NCAM16.2), CXCR6-PE (clone 56811, R&D Systems), NKG2A-PE (clone Z199, Beckman-Coulter), NKG2A-Pacific Blue (clone Z199, in-house custom conjugate, Beckman-Coulter), Ki67-FITC (clone B56), and Perforin-Pacific Blue (in-house custom conjugate, clone Pf-344, Mabtech). Samples were acquired on an LSR II (BD Biosciences, La Jolla, California) and analysis was conducted with FlowJo (version 9.6.4, Tree Star Inc, Ashland, Oregon).

### mDC-stimulation Assay

Mononuclear cells were incubated with 1  $\mu$ g/mL LPS or cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) (R10) alone. Golgi-Plug (brefeldin A) was included at 6  $\mu$ g/mL. Samples were

incubated for 12 hours at 37°C in 5% CO<sub>2</sub>, then permeabilized using FIX & PERM reagents (Invitrogen) and stained intracellularly with anti-CXCL16 (APC conjugate, clone 256213, R&D Systems).

### NK-stimulation Assay

Mononuclear cells were incubated with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (1 µg/mL) or cultured in RPMI 1640 medium containing 10% FBS (R10) alone. For all samples, anti-CD107a (PE-Cy5, clone H4A3) was included at a concentration of 20 µL/mL, and GolgiPlug (brefeldin A) and GolgiStop (monensin) were included at 6 µg/mL. Samples were incubated for 12 hours at 37°C in 5% CO<sub>2</sub>, then permeabilized using FIX & PERM reagents (Invitrogen) and stained intracellularly with anti-interferon-γ (anti-IFN-γ) (PE-Cy7 conjugate, clone B27; Invitrogen), and anti-tumor necrosis factor-α (anti-TNF-α) (Alexa700 conjugate, clone Mab11).

### Immunohistochemistry

Immunohistochemical staining for LPS and *Escherichia coli* were performed as previously described [4]. Briefly, liver tissues were obtained at necropsy, fixed in 4% paraformaldehyde, and paraffin embedded. Immunohistochemistry was performed using a biotin-free polymer approach (Golden Bridge International, Inc) on 5-µm tissue sections mounted on glass slides.

The slides were dewaxed in xylenes and rehydrated through graded ethanols into double-distilled water. Antigen retrieval was performed using either 1X Diva (Biocare Medical) or 0.1 M Tris-HCl (pH 8.6) antigen-retrieval buffer in a pressure cooker (Biocare Medical) set at 122°C for 30 seconds. Slides were stained with optimal conditions determined empirically on an IntelliPATH autostainer (Biocare Medical) that consisted of a blocking step using blocking buffer (Tris buffered saline [TBS] with 0.05% Tween-20 and 0.5% casein) for 10 minutes and an endogenous peroxidase block using 1.5% (v/v) H<sub>2</sub>O<sub>2</sub> in TBS (pH 7.4) for 10 minutes. Slides were incubated with primary antibodies mouse monoclonal anti-LPS-core (clone WN1 222-5; 0.4 µg/mL; Hycult) or rabbit polyclonal anti-*E. coli* (Cat. # B0357; 1:5000; Dako) for 60 minutes. Tissue sections were washed, and incubated with either the Rabbit Polink-1 or Mouse Polink-2 staining systems (Golden Bridge International, Inc) for 30 minutes at room temperature for each step. Sections were developed with Impact 3,3'-diaminobenzidine (Vector Laboratories), counterstained with CAT hematoxylin (Biocare Medical) and mounted in Permount (Fisher Scientific). All stained slides were scanned at high magnification (400×) using the Aperio AT2 System (Leica Biosystems) yielding high-resolution data for the entire tissue section. These methods enumerate both live and dead as well as intracellular and extracellular bacteria/bacterial products.

**Table 1. Liver Pathology in SIV-infected Rhesus Macaques**

Animal	Infection Status	Morphology (H&E stain)	Morphology (Masson's Trichrome stain)
RHDBV1	SIV-	Minimal, multifocal, random lymphocytic hepatitis.	NSF
RHDB9Z	SIV-	NSF	NSF
RH4016	SIV-	NSF	NSF
RHDBXG	SIV-	Mild, multifocal, centrilobular hemosiderosis and fibrosis.	NSF
RHDBM6	SIV-	NSF	NSF
RHDB4E	SIV+	Minimal, multifocal, random lymphocytic hepatitis with single-cell necrosis.	Mild, multifocal, sinusoidal fibrosis and fibrosis surrounding lymphocytic infiltrate/hepatitis.
PSP1010	SIV+	Minimal, multifocal, periportal, lymphocytic and neutrophilic hepatitis, hepatocellular karyolysis.	Mild, multifocal, sinusoidal fibrosis surrounding lymphocytic infiltrate/hepatitis.
RHDB17	SIV+	Mild, multifocal, centrilobular fibrosis. Mild multifocal random Ito cell hyperplasia, hepatocellular disarray, hepatocellular vacuolation, and rare single-cell necrosis. Rare multifocal sinusoidal and hepatocellular pigment (bile and hemosiderin). Focal minimal centrilobular lymphocytic and neutrophilic hepatitis.	Mild, multifocal, centrilobular fibrosis.
RHCF4J	SIV+	Moderate, multifocal, lymphoplasmacytic and eosinophilic periportal hepatitis with bridging fibrosis, lobular collapse, and oval cell hyperplasia. Moderate multifocal hepatocellular anisocytosis and anisokaryosis, hepatocellular disarray, and Ito cell hyperplasia.	Severe, multifocal, bridging fibrosis and fibrosis surrounding lymphocytic infiltrate/hepatitis and throughout sinusoids.
RHCF5T	SIV+	Severe, multifocal, random necrotizing neutrophilic hepatitis with Cowdry type A intranuclear herpetic inclusion bodies. Mild multifocal chronic periportal lymphoplasmacytic hepatitis and Ito cell hyperplasia, hepatocellular anisocytosis.	Moderate, multifocal, significant fibrosis and surrounding necrotizing regions, lymphocytic infiltrate/hepatitis and throughout sinusoids.

Abbreviations: H&E, hematoxylin and eosin; NSF, no significant findings; SIV, simian immunodeficiency virus.

## Histopathology

High-magnification images from scanned liver stained by hematoxylin and eosin (H&E) stain or Masson's Trichrome stain from SIV-negative and SIV-infected animals were obtained with an Aperio Scanscope (Aperio, Vista, California) with the aim to evaluate lymphocyte infiltrates and collagen deposition as previously described [35].

## Statistical Analyses

Prism 6.0 software (GraphPad Software Inc, La Jolla, California) was used for all graphical and statistical analyses. Two-tailed Mann-Whitney *U*, Wilcoxon matched pairs, and Spearman's correlation tests were used where indicated, and a *P* value <.05 was considered statistically significant.

## RESULTS

### Hepatitis and Accumulation of Microbial Products in the Liver During Chronic SIV Infection

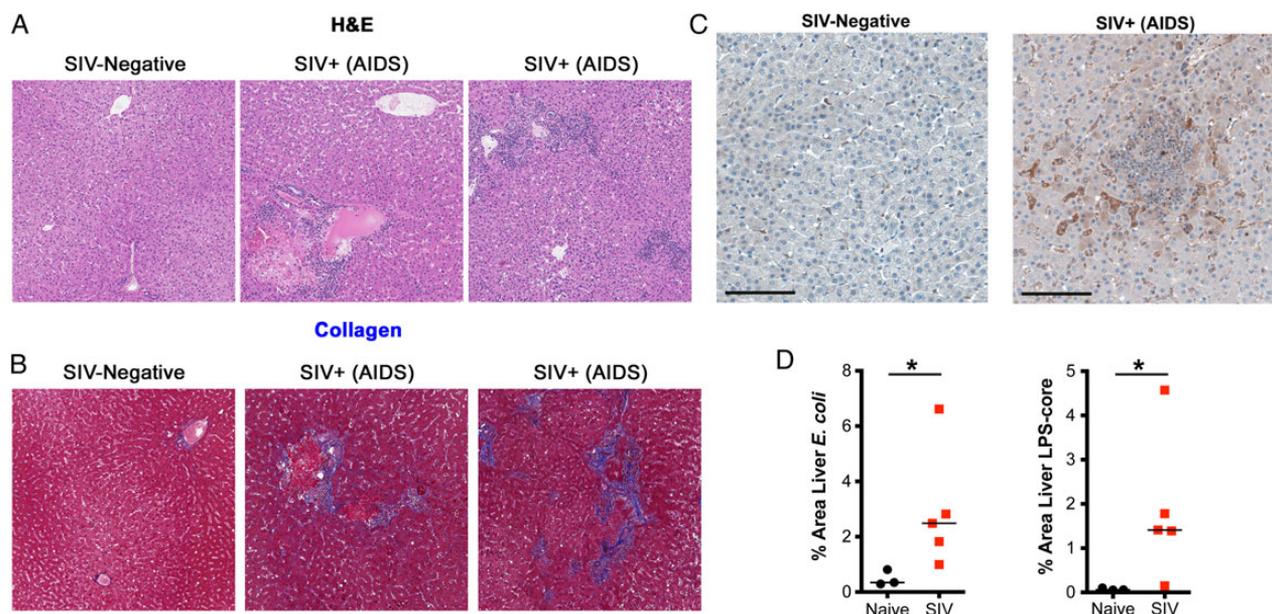
Even in the post-HAART era, comorbidities associated with HIV infection are common. Among the least understood are liver pathologies that occur even in the absence of coinfection with HCV and include tissue fibrosis, steatosis, and liver cancer [9–14]. Indeed, even in a cohort of chronically SIV-infected rhesus macaques, we found significant hepatitis, lymphocyte infiltration, and collagen deposition in the liver (Table 1; Figure 1A

and 1B). All of these pathologic findings are indicative of a higher degree of hepatitis associated with chronic SIV infection, but the underlying mechanisms are unclear.

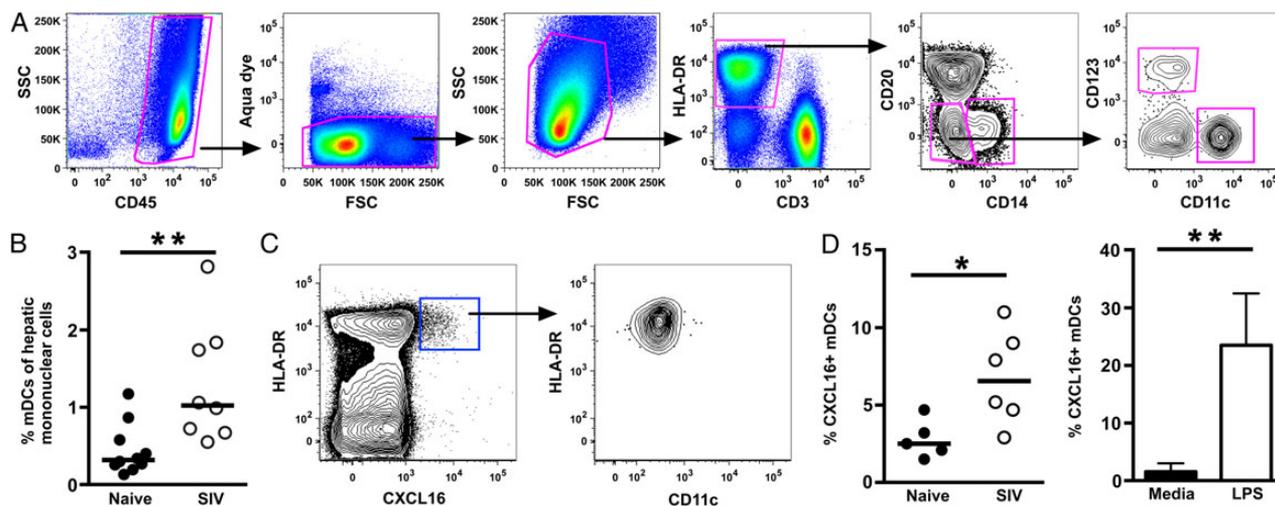
Although multiple groups have now shown that bacteria and bacterial products translocate out of the disrupted gastrointestinal tract during chronic HIV and SIV infection [4, 5, 36], it is less clear how such products are cleared from the blood. Because blood circulates and is filtered through the liver (hepatic portal system), we investigated whether bacterial products might be present in liver tissues during SIV infection via enteric bacterial translocation. In normal livers, *E. coli* and total LPS were detectable at only very low levels by quantitative immunohistochemistry (Figure 1C), but by comparison were increased 10- and 20-fold, respectively, in chronically SIV-infected macaques (Figure 1D). These data suggested that the large quantities of microbial products that translocate into the systemic circulation during HIV and SIV infection are filtered by and can accumulate in the liver and hepatic portal circulation.

### Accumulation of Microbial Products in the SIV-infected Liver is Associated With Activation of CXCL16-producing mDCs

Persistent immune activation due to translocated microbial products in HIV and SIV infection is well documented, and most analyses have focused on activation of T cells. However, in this same cohort of naive and SIV-infected macaques, we found that frequencies of liver CD11c<sup>+</sup> myeloid dendritic cells



**Figure 1.** Accumulation of microbial products in the liver during chronic SIV infection. Representative histopathology shown by H&E (A) and Masson's trichrome (collagen) (B) of liver sections from SIV-naive and chronically SIV-infected macaques. Notice focal necrosis and diffuse inflammatory cell infiltrations in triads on H&E and blue-stained fibrosis on trichrome sections in chronically SIV-infected but not SIV-naive RMs. C, Representative immunohistochemistry demonstrating staining for LPS in liver (DAB, brown). Sections are representative of 3 naive and 5 chronically SIV-infected animals. D, Quantification of *E. coli* and LPS in liver from naive and SIV-infected macaques. Horizontal bars indicate median values, and asterisks indicate significant differences by Mann-Whitney *U* test (\**P* < .05). Abbreviations: DAB, diaminobenzidine; H&E, hematoxylin and eosin; LPS, lipopolysaccharide; RMs, rhesus macaques; SIV, simian immunodeficiency virus.



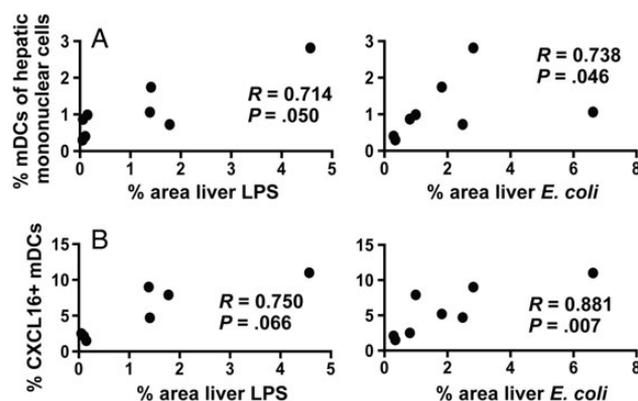
**Figure 2.** CXCL16-producing mDCs infiltrate the liver during SIV infection. *A*, Gating strategy identifying mDCs in macaque liver representative of 8 naive and 10 chronically SIV-infected macaques. *B*, Frequencies of hepatic mDCs among mononuclear cells from naive and chronically SIV-infected animals. *C*, Representative intracellular cytokine staining flow cytometry plot demonstrating mDCs as the primary producers of CXCL16 in the liver. *D*, CXCL16 production ex vivo by hepatic mDCs (left panel) and following in vitro stimulation with LPS (right panel) was analyzed in 5 naive and 6 chronically SIV-infected macaques. Horizontal bars indicate median values, and error bars represent medians  $\pm$  IQR. Asterisks indicate significant differences by Mann–Whitney *U* test, or in vitro evaluations from matched samples were determined by Wilcoxon matched pairs test (\* $P < .05$ ; \*\* $P < .01$ ). Abbreviations: FSC, forward scatter; IQR, interquartile range; HLA, human leukocyte antigen; LPS, lipopolysaccharide; mDCs, myeloid dendritic cells; SIV, simian immunodeficiency virus.

were significantly elevated during infection (medians 0.32% and 1.1%, normal and SIV-infected, respectively) (Figure 2*A* and 2*B*). By comparison, no significant differences in pDC frequencies were observed (data not shown). Liver mDCs were also found to be the primary producers of CXCL16 (Figure 2*C*), a chemokine associated with lymphocyte homing to the liver [31, 32, 37]. In infected animals, the frequency of mDCs producing CXCL16 ex vivo was 3-fold greater than in naive animals, and mDCs specifically produced CXCL16 in response to in vitro stimulation with LPS (Figure 2*D*). Collectively, these data suggested that mDC activation and CXCL16 production might be directly associated with the accumulation of bacterial products in the liver, likely due in part to the high expression of Toll-like receptor 4, the LPS receptor, in macaque mDCs [38]. Indeed, both the total frequencies of mDCs and frequencies of CXCL16-producing mDCs strongly correlated with both LPS and *E. coli* in the liver (Figure 3*A* and 3*B*), but did not correlate with contemporaneous viral loads (data not shown).

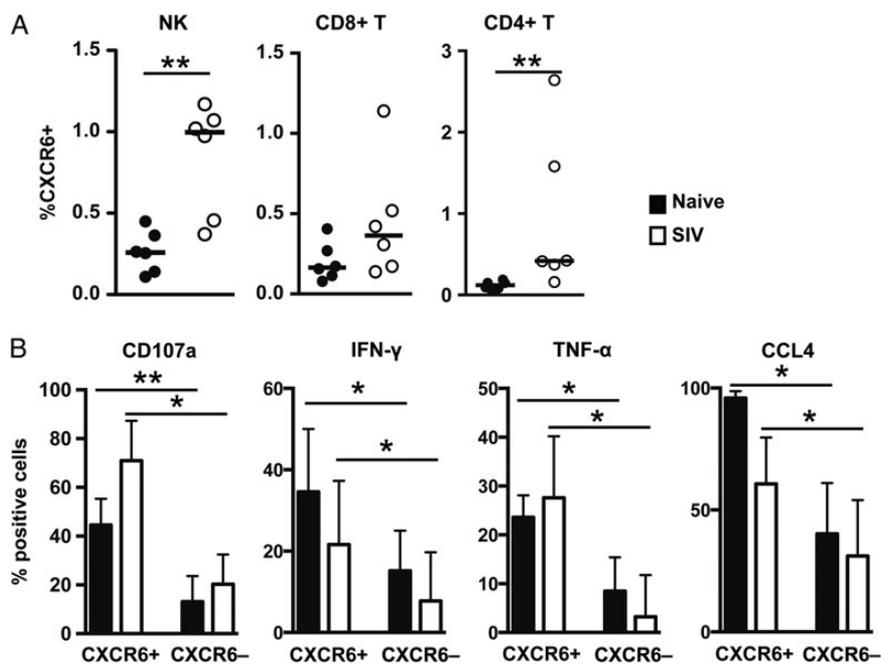
### CXCR6<sup>+</sup> Hyperfunctional NK Cells Are Increased in Circulation During SIV Infection

Previous research has indicated that CXCR6 is associated with lymphocyte trafficking to the liver [39]. Coupled with our observation that production of the CXCR6 ligand, CXCL16, increases in the liver during SIV infection, we next investigated whether CXCR6<sup>+</sup> lymphocytes might be mobilized in infected animals. On NK cells and CD8<sup>+</sup> and CD4<sup>+</sup> T cells, CXCR6 expression was generally very low in naive animals, representing only 0.26%,

0.16%, and 0.12% (medians) of the total cell populations, respectively (Figure 4*A*). However, expression of CXCR6 increased some 2- to 4-fold on lymphocyte populations in chronically infected animals, albeit only significantly on NK cells and CD4<sup>+</sup> T cells. Together, these data suggested that in addition to enhanced production of CXCL16 in the liver during SIV infection, overall liver-homing of circulating lymphocytes may also increase.



**Figure 3.** CXCL16-producing mDCs correlate with accumulation of microbial products in the liver. *A*, Correlations of liver LPS and *E. coli* (as in Figure 1) with frequencies of hepatic mDCs. *B*, Correlations of liver LPS and *E. coli* (as in Figure 1) with frequencies of ex vivo CXCL16<sup>+</sup> hepatic mDCs. All correlations were determined by nonparametric Spearman test for 8 animals (naive or chronically SIV-infected). Abbreviations: LPS, lipopolysaccharide; mDCs, myeloid dendritic cells; SIV, simian immunodeficiency virus.



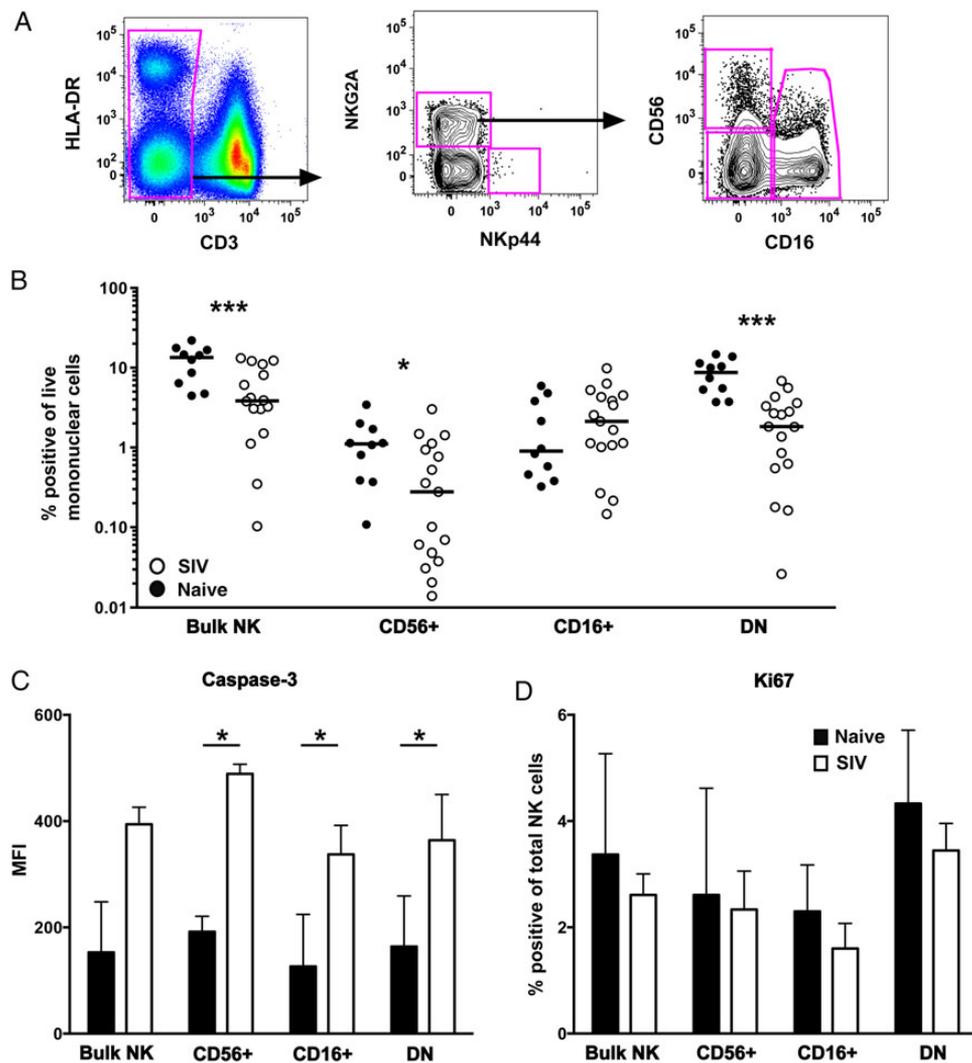
**Figure 4.** Expression of liver-trafficking CXCR6 on circulating lymphocytes. *A*, Frequency of CXCR6<sup>+</sup> circulating lymphocytes in naive and SIV-infected macaques. *B*, Expression of degranulation marker, CD107a, and IFN- $\gamma$ , TNF- $\alpha$ , and CCL4 (MIP-1 $\beta$ ) in CXCR6<sup>+</sup> and CXCR6<sup>-</sup> NK cells following mitogen stimulation. Horizontal bars indicate median values, and asterisks indicate significant differences by Mann–Whitney *U* test (*A*). Error bars represent medians  $\pm$  IQR for in vitro evaluations from matched samples and were evaluated by Wilcoxon matched pairs test (*B*) (\**P* < .05; \*\**P* < .01). Only significant values are shown. Abbreviations: IFN, interferon; IQR, interquartile range; NK, natural killer; SIV, simian immunodeficiency virus; TNF, tumor necrosis factor.

Because the most dramatic gain in liver-homing lymphocytes was observed in circulating NK cells and because NK cells have a complex interplay with mDCs, we next evaluated the functionality of CXCR6<sup>+</sup> liver-homing NK cells. These cells had similar functional repertoires to CXCR6<sup>-</sup> NK cells, but were hyperfunctional with regard to mobilization of CD107a, as well as production of IFN- $\gamma$ , TNF- $\alpha$ , and CCL4 in response to mitogen stimulation (Figure 4*B*). These data could suggest that liver-homing NK cells are functionally poised for rapid cytotoxicity or production of cytokines. Interestingly, in SIV-infected animals, these functional differences became even more disparate. While little change was observed in the functionality of CXCR6<sup>-</sup> NK cells when comparing naive and infected animals, CXCR6<sup>+</sup> NK mobilized CD107a and produced TNF- $\alpha$ , while simultaneously downregulating production of IFN- $\gamma$  and CCL4. This may suggest that liver-homing NK cells are skewed toward cytotoxicity and inflammatory functions compared with other subpopulations of NK cells. Also, of note, virtually all CXCR6<sup>+</sup> NK cells in circulation expressed CD16, a hallmark of cytotoxic NK cells (Supplementary Figure 1).

#### Liver NK Cells Demonstrate Increased Apoptosis in SIV-infected Macaques

NK cells in the liver, like in most tissues in macaques, are readily identifiable by high expression of NKG2A [40] and can be

divided into subpopulations on the basis of CD56 and CD16 expression (Figure 5*A* and 5*B*). Compared with most tissues, we found NK cells to be relatively abundant in the liver, representing a median of 15% of total liver lymphocytes in normal macaques. Phenotypically, liver NK cells were dominated by CD16<sup>+</sup> and double-negative (CD16<sup>-</sup>CD56<sup>-</sup>) NK cell subpopulations. Interestingly, NKp44<sup>+</sup> innate lymphoid cells (ILCs) were found at only low frequencies in the liver. In SIV-infected macaques, the frequencies of bulk, CD56<sup>+</sup>, and double-negative NK cells were reduced by approximately 50% (Figure 5*B*). Associated with this overall loss of NK cells in the livers of infected animals were increases in expression of the apoptosis marker, active-caspase-3 (Figure 5*C*), but a lack of compensatory proliferation, as no difference in intracellular Ki67 was observed (Figure 5*D*). Together, these data could explain, at least partially, the overall loss of NK cells in the liver during SIV infection. However, CD16<sup>+</sup> NK cells, despite demonstrating increased apoptosis and no proliferation in the liver, remained unchanged in number. It is tempting to speculate that the maintenance of this cytotoxic subpopulation in the liver is driven by recruitment of CXCR6<sup>+</sup>CD16<sup>+</sup> NK cells from other tissues, as demonstrated in Figure 4. Not surprisingly, neither bulk nor subset frequencies of NK cells correlated with viral load, likely due to the turnover of NK cells found in the liver.



**Figure 5.** Hepatic natural killer cells in naive and SIV-infected macaques. *A*, Representative gating strategy to identify NK cells among liver mononuclear cells. *B*, Absolute frequencies of total and subset NK cells in liver from naive ( $n = 11$ ) and SIV-infected macaques ( $n = 17$ ). Horizontal bars represent medians. *C* and *D*, Intracellular expression of active-caspase-3 and Ki67 in total and subset NK cells. Horizontal bars indicate median values, error bars represent medians  $\pm$  IQR, and asterisks indicate significant differences by Mann–Whitney  $U$  test ( $*P < .05$ ;  $**P < .01$ ;  $***P < .001$ ). Only significant values are shown. Abbreviations: HLA, human leukocyte antigen; IQR, interquartile range; NK, natural killer; SIV, simian immunodeficiency virus.

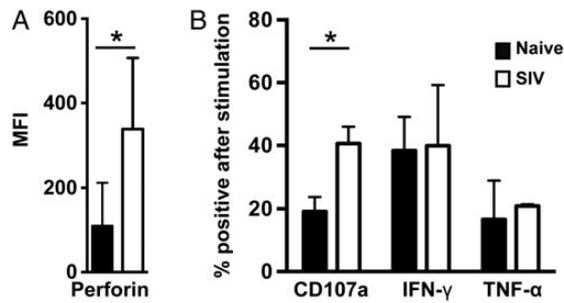
### Hepatic NK Cells Are Functionally Skewed Toward a More Cytotoxic Phenotype During SIV Infection

To further corroborate the finding that CD16<sup>+</sup> cytotoxic NK cells are maintained in the liver during SIV infection, we next directly investigated the functionality of liver NK cells. Intracellular perforin expression measured in NK cells ex vivo was 2-fold higher in samples from infected compared to naive animals (Figure 6A). Similarly, there was a 2-fold increase in CD107a mobilization following mitogen stimulation, but little change in production of IFN- $\gamma$  or TNF- $\alpha$  (Figure 6B). These findings are well in line with overall increased cytotoxicity of NK cells during SIV infection [40] and also are in agreement with the finding that CXCR6<sup>+</sup> liver-homing NK cells are more cytotoxic (Figure 4B). However, due to the fact that NK cells can also

become dysfunctional in chronic infection, the overall impact of hypercytotoxicity could be somewhat overestimated by these surrogate markers [21, 41].

### Microbial Accumulation in the Liver Is Associated With Liver Damage

Liver damage is common in HIV disease, and generally includes cirrhosis, collagen deposition, and nonalcoholic fatty liver disease, among other pathologies. Changes in serum liver enzymes are often used as surrogate clinical indicators to measure damage. Hence, we evaluated changes in serum liver enzymes in naive and SIV-infected macaques to evaluate whether microbial accumulation and immune activation might also be associated with liver damage. In naive animals, aspartate aminotransferase



**Figure 6.** Functional repertoire of hepatic NK cells in naive and SIV-infected macaques. *A*, Intracellular perforin expression in total NK cells ex vivo from animals in Figure 5. *B*, Expression of degranulation marker, CD107a, and IFN- $\gamma$  and TNF- $\alpha$  following mitogen stimulation. Error bars represent medians  $\pm$  IQR, and asterisks indicate significant differences by Mann-Whitney *U* test ( $*P < .05$ ). Only significant values are shown. Abbreviations: IFN, interferon; IQR, interquartile range; NK, natural killer; SIV, simian immunodeficiency virus; TNF, tumor necrosis factor.

levels were between 23 and 31 U/L (median 29 U/L), but were significantly increased to between 35 and 56 U/L (median 47 U/L) in SIV-infected animals ( $P = .049$ , Mann-Whitney *U* test). In naive animals, alkaline phosphatase levels were between 119 and 250 U/L (median 134 U/L), but increased to between 123 and 479 U/L (median 341 U/L) in chronically SIV-infected animals, albeit not significantly ( $P = .23$ , Mann-Whitney *U* test). Interestingly, while neither viral load nor duration of infection correlated with these indicators of liver damage (data not shown), *E. coli*, LPS, mDC, and CD16<sup>+</sup> NK cell frequencies all correlated (Supplementary Figure 2). Collectively, these data indicate that accumulation of microbial products in the liver and the associated mDC activation and NK cell recruitment are a likely source of SIV-induced liver damage.

## DISCUSSION

Microbial translocation in addition to the virus itself is now considered a cause of inflammation and ongoing disease in HIV, even in the context of HAART. Furthermore, a common pathology in advanced HIV infection is liver disease, including hepatocellular carcinoma, hepatitis, and steatosis, among others [12–18]. Although the underlying mechanisms are not entirely clear, we present here new evidence that accumulation of microbial products in the liver activates local innate immune cascades that can result in liver damage.

Even under homeostatic conditions the liver is constantly exposed to pathogen-associated molecular patterns, including LPS and bacterial DNA, through bacterial translocation via the portal vein system connecting it to the intestine [42]. Normal degradation and clearance limit activation and inflammation, but overt signaling activates inflammasomes and has been previously associated with incidence of liver fibrosis [43–45]. Even in the

absence of HIV or HCV, microbial translocation to the liver is a predictor of poor prognosis of cirrhosis [19]. Similar to studies of SIV-induced disease [8], probiotics have been shown to be useful at treating cirrhosis and fatty liver disease [46].

Typically, most NK cell research focuses on cells in circulation, but we and others have demonstrated broad activation of NK cells by HIV and SIV, most notably in the mucosa. Similarly, we show here that liver NK cells become hypercytotoxic and recruited to the liver, but that this NK cell activity is highly related to mDC activation by microbial products. Increases in NK cell cytotoxicity are common in both HIV and SIV infections, but most studies focus on how NK cells can lyse infected cells [21, 23, 47]. However, the data we present here suggest NK cell cytotoxicity could also damage otherwise healthy tissue. Although most evidence indicates NK cell activation is driven by ongoing virus replication, including decreases in activation during HAART [48–50], these data agree with previous observations that it is primarily indirect.

In summary, these data suggest a complex mechanism by which microbial products accumulate in the liver, resulting in mDC production of CXCL16 that in turn recruits hypercytotoxic NK cells to the liver. Although the net effects include inflammation and liver damage, the full spectrum of pathologies remain to be elucidated. Regardless, these data make it tempting to speculate that, in addition to combination antiretroviral therapy, therapies aimed at restoring gastrointestinal function and alleviation of microbial translocation could help to control chronic liver complications found in HIV disease.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** The authors thank Michelle Connole, Fay E. Wong, and Yi Yu for expert technical assistance; Angela Carville and Elaine Roberts for dedicated animal care; and Jeff Lifson and Michael Piatak, Jr, of the Quantitative Molecular Diagnostics Core of the AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc, Frederick National Laboratory, for plasma SIV RNA determinations. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US government.

**Financial support.** This work was supported by Center for HIV/AIDS Vaccine Immunology (CHAVI)/HIV Vaccine Trials Network (HVTN) Early Career Investigator (National Institutes of Health [NIH] grant U19 AI067854), American Foundation for AIDS Research (amFAR) (108547-53-RGRL), Center for Aids Research (CFAR) Developmental (P30 AI060354), and NIH (R21 AI118468) grants to R. K. R. Funding for this study was provided in part by the Division of Intramural Research/National Institute of Allergy and Infectious Diseases/NIH and with federal funds from the National Cancer Institute, NIH, under Contract No. HHSN261200800001E.

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

1. Epple HJ, Allers K, Troger H, et al. Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. *Gastroenterology* **2010**; 139:1289–300.
2. Klatt NR, Estes JD, Sun X, et al. Loss of mucosal CD103+ DCs and IL-17+ and IL-22+ lymphocytes is associated with mucosal damage in SIV infection. *Mucosal Immunol* **2012**; 5:646–57.
3. Klatt NR, Harris LD, Vinton CL, et al. Compromised gastrointestinal integrity in pigtail macaques is associated with increased microbial translocation, immune activation, and IL-17 production in the absence of SIV infection. *Mucosal Immunol* **2010**; 3:387–98.
4. Estes JD, Harris LD, Klatt NR, et al. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. *PLOS Pathog* **2010**; 6:e1001052.
5. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* **2006**; 12:1365–71.
6. Canary LA, Vinton CL, Morcock DR, et al. Rate of AIDS progression is associated with gastrointestinal dysfunction in simian immunodeficiency virus-infected pigtail macaques. *J Immunol* **2013**; 190:2959–65.
7. Compare D, Coccoli P, Rocco A, et al. Gut–liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* **2012**; 22:471–6.
8. Klatt NR, Canary LA, Sun X, et al. Probiotic/prebiotic supplementation of antiretrovirals improves gastrointestinal immunity in SIV-infected macaques. *J Clin Invest* **2013**; 123:903–7.
9. Schmitz JE, Kuroda MJ, Veazey RS, et al. Simian immunodeficiency virus (SIV)-specific CTL are present in large numbers in livers of SIV-infected rhesus monkeys. *J Immunol* **2000**; 164:6015–9.
10. Ahsan MH, Gill AF, Lackner AA, Veazey RS. Acute and chronic T cell dynamics in the livers of simian immunodeficiency virus-infected macaques. *J Virol* **2012**; 86:5244–52.
11. Zhang L, Dailey PJ, Gettie A, Blanchard J, Ho DD. The liver is a major organ for clearing simian immunodeficiency virus in rhesus monkeys. *J Virol* **2002**; 76:5271–3.
12. Puoti M, Moioli MC, Travi G, Rossotti R. The burden of liver disease in human immunodeficiency virus-infected patients. *Semin Liver Dis* **2012**; 32:103–13.
13. Kim AY, Chung RT. Coinfection with HIV-1 and HCV—a one-two punch. *Gastroenterology* **2009**; 137:795–814.
14. Vallet-Pichard A, Mallet V, Pol S. Nonalcoholic fatty liver disease and HIV infection. *Semin Liver Dis* **2012**; 32:158–66.
15. Chen JY, Feeney ER, Chung RT. HCV and HIV co-infection: mechanisms and management. *Nat Rev Gastroenterol Hepatol* **2014**; 11:362–71.
16. Alter MJ, Margolis HS, Krawczynski K, et al. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. *N Engl J Med* **1992**; 327:1899–905.
17. Thomas DL, Astemborski J, Rai RM, et al. The natural history of hepatitis C virus infection: host, viral, and environmental factors. *JAMA* **2000**; 284:450–6.
18. Brau N, Fox RK, Xiao P, et al. Presentation and outcome of hepatocellular carcinoma in HIV-infected patients: a U.S.-Canadian multicenter study. *J Hepatol* **2007**; 47:527–37.
19. Bellot P, Garcia-Pagan JC, Frances R, et al. Bacterial DNA translocation is associated with systemic circulatory abnormalities and intrahepatic endothelial dysfunction in patients with cirrhosis. *Hepatology* **2010**; 52:2044–52.
20. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. *Nat Rev Immunol* **2011**; 11:658–71.
21. Alter G, Teigen N, Davis BT, et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. *Blood* **2005**; 106:3366–9.
22. O'Connor GM, Holmes A, Mulcahy F, Gardiner CM. Natural killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. *Clin Immunol* **2007**; 124:277–83.
23. Giavedoni LD, Velasquillo MC, Parodi LM, Hubbard GB, Hodara VL. Cytokine expression, natural killer cell activation, and phenotypic changes in lymphoid cells from rhesus macaques during acute infection with pathogenic simian immunodeficiency virus. *J Virol* **2000**; 74:1648–57.
24. O'Connor GM, Holmes A, Mulcahy F, Gardiner CM. Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. *Clin Immunol* **2007**; 124:277–83.
25. Pereira LE, Johnson RP, Ansari AA. Sooty mangabey and rhesus macaques exhibit significant divergent natural killer cell responses during both acute and chronic phases of SIV infection. *Cell Immunol* **2008**; 254:10–9.
26. Martin MP, Qi Y, Gao X, et al. Innate partnership of *HLA-B* and *KIR3DL1* subtypes against HIV-1. *Nat Genet* **2007**; 39:733–40.
27. Alter G, Heckerman D, Schneidewind A, et al. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* **2011**; 476:96–100.
28. Chaichompoo P, Bostik P, Stephenson S, et al. Multiple KIR gene polymorphisms are associated with plasma viral loads in SIV-infected rhesus macaques. *Cell Immunol* **2010**; 263:176–87.
29. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **2004**; 305:872–4.
30. Nattermann J, Nischalke HD, Hofmeister V, et al. The HLA-A2 restricted T cell epitope HCV core 35–44 stabilizes HLA-E expression and inhibits cytotoxicity mediated by natural killer cells. *Am J Pathol* **2005**; 166:443–53.
31. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. *J Immunol* **2006**; 177:7833–40.
32. Tabata S, Kadowaki N, Kitawaki T, et al. Distribution and kinetics of SR-PSOX/CXCL16 and CXCR6 expression on human dendritic cell subsets and CD4+ T cells. *J Leukoc Biol* **2005**; 77:777–86.
33. Li H, Richert-Spuhler LE, Evans TI, et al. Hypercytotoxicity and rapid loss of NKp44+ innate lymphoid cells during acute SIV infection. *PLOS Pathog* **2014**; 10:e1004551.
34. Ortiz AM, DiNapoli SR, Brenchley JM. Macrophages are phenotypically and functionally diverse across tissues in SIV-infected and uninfected Asian macaques. *J Virol* **2015**; 89:5833–94.
35. Estes JD, Reilly C, Trubey CM, et al. Antifibrotic therapy in simian immunodeficiency virus infection preserves CD4+ T-cell populations and improves immune reconstitution with antiretroviral therapy. *J Infect Dis* **2015**; 211:744–54.
36. Klatt NR, Funderburg NT, Brenchley JM. Microbial translocation, immune activation, and HIV disease. *Trends Microbiol* **2013**; 21:6–13.
37. Borchers AT, Shimoda S, Bowlus C, Keen CL, Gershwin ME. Lymphocyte recruitment and homing to the liver in primary biliary cirrhosis and primary sclerosing cholangitis. *Semin Immunopathol* **2009**; 31:309–22.
38. Trott KA, Richardson A, Hudgens MA, Abel K. Immune activation and regulation in simian immunodeficiency virus-*Plasmodium fragile*-coinfected rhesus macaques. *J Virol* **2013**; 87:9523–37.
39. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. *J Immunol* **2006**; 177:7833–40.
40. Reeves RK, Gillis J, Wong FE, Yu Y, Connole M, Johnson RP. CD16-natural killer cells: enrichment in mucosal and secondary lymphoid tissues and altered function during chronic SIV infection. *Blood* **2010**; 115:4439–46.
41. Schafer JL, Li H, Evans TI, Estes JD, Reeves RK. Accumulation of cytotoxic CD16+ NK cells in simian immunodeficiency virus-infected lymph nodes associated with in situ differentiation and functional anergy. *J Virol* **2015**; 89:6887–94.

42. Aoyama T, Paik YH, Seki E. Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract* **2010**; doi:10.1155/2010/192543.
43. Seki E, De Minicis S, Osterreicher CH, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* **2007**; 13:1324–32.
44. Isayama F, Hines IN, Kremer M, et al. LPS signaling enhances hepatic fibrogenesis caused by experimental cholestasis in mice. *Am J Physiol Gastrointest Liver Physiol* **2006**; 290:G1318–28.
45. Schroder K, Tschopp J. The inflammasomes. *Cell* **2010**; 140:821–32.
46. Cesaro C, Tiso A, Del Prete A, et al. Gut microbiota and probiotics in chronic liver diseases. *Dig Liver Dis* **2011**; 43:431–8.
47. Bostik P, Kobkitjaroen J, Tang W, et al. Decreased NK cell frequency and function is associated with increased risk of KIR3DL allele polymorphism in simian immunodeficiency virus-infected rhesus macaques with high viral loads. *J Immunol* **2009**; 182:3638–49.
48. Gonzalez VD, Falconer K, Michaelsson J, et al. Expansion of CD56- NK cells in chronic HCV/HIV-1 co-infection: reversion by antiviral treatment with pegylated IFNalpha and ribavirin. *Clin Immunol* **2008**; 128:46–56.
49. Marras F, Nicco E, Bozzano F, et al. Natural killer cells in HIV controller patients express an activated effector phenotype and do not up-regulate NKp44 on IL-2 stimulation. *Proc Natl Acad Sci USA* **2013**; 110:11970–5.
50. Michaelsson J, Long BR, Loo CP, et al. Immune reconstitution of CD56(dim) NK cells in individuals with primary HIV-1 infection treated with interleukin-2. *J Infect Dis* **2008**; 197:117–25.