Innate Lymphoid Cells Are Depleted Irreversibly during Acute HIV-1 Infection in the Absence of Viral Suppression

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="https://doi.org/10.1016/j.immuni.2016.01.006">https://doi.org/10.1016/j.immuni.2016.01.006</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Version</td>
<td>Original manuscript</td>
</tr>
<tr>
<td>Accessed</td>
<td>Sat Apr 28 13:44:20 EDT 2018</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/114947">http://hdl.handle.net/1721.1/114947</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Creative Commons Attribution-NonCommercial-NoDerivs License</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td><a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a></td>
</tr>
</tbody>
</table>
IRREVERSIBLE DEPLETION OF INNATE LYMPHOID CELLS IN EARLY ACUTE HIV-1 INFECTION IN THE ABSENCE OF VIRAL SUPPRESSION


1KwaZulu-Natal Research Institute for Tuberculosis & HIV, K-RITH, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.
2Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark.
3Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, USA.
4Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.
5Institute for Medical Engineering & Science, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.
6Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA.
7Center for Infectious Medicine, Karolinska Institute, Stockholm, Sweden.
8HIV Pathogenesis Programme, Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa.
9Department of Paediatrics, University of Oxford, Oxford, United Kingdom.
10Agency for Science, Technology and Research (A*STAR), Singapore Immunology Network (SIgN), Singapore.
11Department of Surgery, Inkosi Albert Luthuli Hospital, KwaZulu-Natal, Durban, South Africa.
12ENT department Stanger Hospital, University of KwaZulu-Natal, Durban, South Africa.
13Department of Infectious Diseases, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.
14Center for the AIDS Programme of Research in South Africa – CAPRISA, Durban, South Africa.
15Division of Medical Virology and Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa
16Department of Epidemiology, Columbia University, New York City, USA.
17Division of Health Sciences and Technology, Harvard Medical School, Boston, MA, USA.
18Max Planck Institute for Infection Biology, Berlin, Germany.
19Howard Hughes Medical Institute, Chevy Chase, MD, USA.

Corresponding author: henrik.kloverpris@k-rith.org and hkloverpris@sund.ku.dk

Keywords: Innate Lymphoid Cells, HIV-1 Infection, Acute Infection, Chronic Infection

Running title: Innate Lymphoid Cells and HIV-1 Infection
SUMMARY

Innate Lymphoid Cells (ILCs) play a central role in the response to infection by secreting cytokines critical for immune-regulation, tissue homeostasis and repair. Although dysregulation of these systems is central to pathology, the impact of HIV-1 on ILCs remains unknown. Here we show that human blood ILCs are severely depleted in viremic HIV-1 infection in correlation with disease stage. Depletion occurs during acute infection and ILCs do not reconstitute following resolution of peak-viremia or antiretroviral therapy (ART) begun in chronic disease. However, ILC levels are preserved by treatment in acute infection. Accordingly, transcriptional profiling of ILCs from untreated patients during acute infection reveals significant up-regulation of genes associated with apoptosis and cell-death that is curbed by early ART. Remaining circulating ILCs in chronic infection are activated but not apoptotic. These data comprehensively describe the impact of HIV-1 on ILCs and introduce a novel aspect of HIV-1 pathology.
INTRODUCTION

Hallmarks of HIV-1 pathology include immunodeficiency, lymphoid tissue destruction, impaired integrity of the gut barrier and systemic immune activation (Veazey et al., 1998). These features are only partially reversed by fully suppressive long-term antiretroviral therapy (ART) (Sanchez et al., 2014; Zeng et al., 2012). The underlying mechanisms of this pathology remain unclear, presenting a serious barrier to the development of novel interventions to improve immune reconstitution in HIV-1 infected individuals. Recent studies have suggested that the rapid depletion of IL-17 and IL-22 producing CD4+ T cells within gut associated lymphocyte tissue (GALT) during acute infection (Schuetz et al., 2014) may lead to gut epithelial break down, as IL-17 and IL-22 are the key cytokines involved in mucosal homeostasis. In fact, the most rapid and prolific producers of IL-17 and IL-22 in tissue are Innate Lymphoid Cells (ILCs) rather than conventional Th17 and Th22 cells (Cella et al., 2008; Cupedo et al., 2009; Satoh-Takayama et al., 2008; Zheng et al., 2008), prompting the question: what happens to this important immune subset after HIV-1 infection?

Although ILCs are not antigen specific – they lack rearranged B- and T-cell receptors – they are grouped into three heterogeneous subsets, ILC1, ILC2 and ILC3, that share functional characteristics with Th1, Th2 and Th17 cells, respectively (Walker et al., 2013). ILCs respond rapidly to damage, prior to antigen specific B- and T-cell expansion, and are therefore crucial for tissue homeostasis and repair during acute and chronic disease (McKenzie et al., 2014). In particular, ILCs play important roles in mucosal barrier maintenance through tissue repair, wound healing, and regulation of the immune response to the commensal microbiota (Kurashima et al., 2013; Tait Wojno and Artis, 2012). As a result, ILCs are emerging as key players in many infectious and non-infectious diseases, where they may aid or impair proper immune response. Illustratively, group 2 ILCs (ILC2s) accumulate in the
lung in response to influenza infection and restore epithelial integrity (Chang et al., 2011; Gorski et al., 2013; Monticelli et al., 2011), and loss of ILC3s in the gut precipitates inflammatory bowel conditions through the IL-22 axis (Sonnenberg et al., 2012a; Tait Wojno and Artis, 2012). In contrast, untreated Multiple Sclerosis, Allergic Asthma and Psoriasis are all associated with expansions of specific ILC subsets in peripheral blood that are thought to drive disease pathology (Bartemes et al., 2014; Perry et al., 2012; Teunissen et al., 2014).

The impact of HIV-1 infection on ILC populations in circulation or at mucosal barrier sites remains unclear. Given the central role of ILCs in gut epithelial integrity, immune regulation and other hallmarks dysregulated in HIV-1 disease, this represents a significant gap in our understanding of HIV-1 pathology. We hypothesized that HIV-1 infection depletes ILC levels, which exacerbates disease progression and limits immune reconstitution observed either after resolution of primary viremia or following successful ART mediated viral suppression.

In this study, we use blood and tissue samples collected from individuals in both chronic and very early acute HIV-1 infection to demonstrate for the first time that ILCs are indeed depleted from circulation within 7-14 days of infection, do not reconstitute, and remain depleted from the blood even with long term fully suppressive ART. In contrast, using a unique set of samples, we find that ILC populations are maintained at normal levels if ART therapy is started in early acute infection before peak viremia. We show that levels of ILCs in circulation from chronic disease display an altered and activated phenotype. However, we do not find direct evidence of apoptosis during chronic disease or enrichment of tissue resident ILCs identified in lymphoid and gut mucosal barrier sites that explain the observed depletion of circulating ILCs. RNA sequencing of ILCs during early acute infection, however, reveals
significant up-regulation of genes involved in apoptosis and cell death and down-regulation of genes involved in cell proliferation and cell survival. These gene signatures are diminished or lost when ART is started before peak viremia, and suggest that depletion of circulating ILCs is mediated by cell death driven by high viral load and immune activation during acute HIV-1 infection, associated with markers of acute viral response. Together these data comprehensively describe the impact of HIV-1 infection in humans on this novel subset of innate helper cells that have recently been shown to be vital for lymphoid and mucosal barrier tissue homeostasis, and are likely to be important in the pathology of this disease.
RESULTS
ILCs are severely depleted in chronic HIV-1 infection with inverse correlation to viral load setpoint
ILCs are defined as lymphocytes negative for B- and T-cell lineage markers and conventional NK cell markers (CD16 and CD94), but positive for CD127 and CD161. Using flow cytometry of peripheral blood samples, we adopted a traditional gating strategy and identified three phenotypically distinct ILC populations as described in the literature (Spits et al., 2013): CRTH2^{-}CD117^{-}CD56^{-}CD25^{-/+} (ILC1), CRTH2^{+}CD117^{-/+}CD56^{-}CD25^{-/+} (ILC2) and CRTH2^{-}CD117^{-/+}CD56^{-/+}CD25^{-/+} (ILC3) (Figure 1A and Figure S1A). To verify these 3 ILC populations, we turned to an unbiased data analysis tool, t-distributed stochastic neighbour embedding (tSNE), which simultaneously analyses all flow-measured parameters, rather than sequentially gating on two markers at a time. tSNE, like a principal component analysis (PCA), clusters cells that share similar expression patterns together while accounting for potential non-linear relationships between markers (Amir et al., 2013; Becher et al., 2014; van der Maaten and Hinton, 2008). Using this approach, we identified 5 clusters within the Lin^{-}/CD127^{+} population, ILC1, ILC2, ILC3, NK and ‘non ILCs’ that correspond to the phenotypes of the recently described nomenclature of human ILCs (Figure 1B) (Spits et al., 2013). To confirm these ILC subsets, we performed intracellular cytokine staining, controlled by CD4^{+} T cells, and, as expected, found mutually exclusive IFNγ and IL13 production from ILC1 and ILC2 subsets, respectively (Figure 1C). The ILC3 subset in blood produced IL2 and TNFα, but did not express NKp44 (not shown) and therefore do not secrete IL22 (Teunissen et al., 2014). However, NKp44^{+} ILC3 cells isolated from tonsil tissue produce high levels of this cytokine (Figure 1C). In addition, ILC subsets display the T-bet, GATA-3 and RORγt transcription factor expression patterns of ILC1, ILC2 and ILC3 subsets, respectively and in relation to conventional NK (T-bet) and CD4-Th1 (T-bet) and Th2 (GATA-3) cells (Figure
ID) (Spits et al., 2013). Finally, we performed RNA sequencing of sorted ILC and CD4+ T cells from blood in 9 healthy donors and find that the ILC populations display a highly significant ILC transcriptional signature compared to sample matched CD4+ T-cell populations (Figure 1E) (Table S1-S3). The ILC subsets themselves are closely related but transcriptionally distinct (Table S1-3), typified by expression levels of canonical ILC lineage genes, such as CD117 (cKit; ILC3), IL1R (ILC3) and KLRG1 (ILC2, Figure S1C). Together, these data confirm precise identification of the main human ILC subsets described.

Next, we compared the absolute frequencies of blood ILC counts in a total of 223 subjects from HIV-1 uninfected individuals to those of ART-naive viremic (HIV-1 RNA>50 copies/ml plasma) and aviremic (<50 copies/ml) individuals with chronic HIV-1 infection (Figure 1D). In viremic subjects, we observed depletion of all three ILC populations (P < 0.0001). However, ILC1 and ILC2, but not ILC3s (P = 0.009), were preserved in aviremic subjects (Figure 1D). ILC frequencies expressed as percentage of total CD45+ lymphocytes confirmed chronic HIV-1 depletion (Figure 1G), and show that changes in frequency of other hematopoietic subsets do not significantly impact ILC measurement in chronic infection. In addition, we observed a significant negative correlation between HIV-1 RNA viral load setpoint and ILC frequencies (P = 0.07 to 0.007, R = -0.18 to -0.32) (Figure S2A,B), similar to the well described correlation between viral load and absolute CD4+ T-cell count (P < 0.001, R = -0.47) (Figure S2C). Thus, ILCs are severely depleted in chronic viremic infection with a direct negative correlation to viral load setpoint.

To examine shifts in ILC subsets driven by chronic HIV-1 infection, we used the tSNE algorithm (Becher et al., 2014; van der Maaten and Hinton, 2008) to obtain an unbiased analysis of ILC distribution. By gating on Lin’CD127+, we identified 38 distinct clusters with shared surface
marker expression characteristics (Figure 1H). Ten of these clusters were significantly enriched or depleted in HIV-1 infected subjects (Figure 1I), predominantly from within the ILC2 and ILC3 populations. Of these, cluster 9 (ILC2) and cluster 20 (ILC3) remained significant after controlling for multiple comparisons ($P = 4.5 \times 10^{-5}$ and $P = 1.7 \times 10^{-11}$), suggesting that the circulating ILCs that remain in chronic infection are phenotypically altered. Taken together, these data suggests that circulating ILCs in chronic HIV-1 infection, are not only severely depleted, but also display skewed phenotypic properties.

**ILCs are depleted during early acute HIV-1 infection**

To further investigate the dynamics of ILC depletion, we turned to a unique acute HIV-1 infection cohort (Ndhlovu et al., 2015). These females are tested for the presence of HIV-1 nucleic acid in plasma twice a week, and therefore identified within a maximum of 4 days from their last negative test and approximately 5-14 days after the transmission event; corresponding to Feibig stage I (McMichael et al., 2010). We tracked ILC1, ILC2 and ILC3s in seven individuals throughout the course of peak viremia and up to 250 days into chronic infection (one representative example is shown in Figure 2A). We found normal ILC levels at the first time-points, sampled before peak viremia, but observed a rapid ILC depletion that coincided with the peak viral load (day 7-14) (Figure 2B,C) and persisted without rebound into chronic infection ($P < 0.016$) (Figure 2D). This observation remained significant when we analyzed the absolute ILC counts (Figure 2E) (Figure S3A-C), demonstrating ILC depletion is not a result of the drastic changes in the frequency of other immune subsets during this disease phase. In contrast, and as expected, the characteristic early nadir of absolute CD4$^+$ T-cell count, temporally associated with peak viremia, rebounded rapidly, although to suboptimal levels (Figure 2F). Thus, these data show that the ILC depletion observed during chronic viremic infection occurs very early in the acute phase of infection.
and that, unlike CD4 T cells, ILCs fail to recover following the resolution of acute viremia to setpoint viral load.

Early depletion of ILCs coincide with spikes in epithelial gut breakdown

ILCs are required to maintain an effective gut barrier and to regulate the immune response to commensal microbiota (Sonnenberg et al., 2012b). We therefore next sought to define the kinetics of ILC decline in early acute HIV-1 infection in relation to the well-documented damage to gut associated lymphoid tissue that occurs during primary infection in non-human primates (Brenchley et al., 2004; Veazey et al., 1998). Changes to gut integrity during acute HIV-1 infection were assessed indirectly by measuring the levels of intestinal fatty acid binding protein 1 (I-FABP), a plasma marker previously associated with gut barrier breakdown (Hunt et al., 2014). In one subject (PID 0398-271), we found a peak in I-FABP levels one week after ILC depletion and 2 weeks after HIV-1 plasma RNA detection (Figure S4A). When we compared the relative levels of I-FABP for the entire cohort, we consistently found maximum levels occurring 2 weeks after HIV-1 RNA detection that coincided with ILC depletion (Figure S4B). Although I-FABP levels return to baseline once viral load reaches setpoint, the association between ILC levels and I-FABP was significant at 2 and 3 weeks post HIV-1 detection ($P < 0.008$) (Figure S4C), corresponding to one week after peak viral replication (Figure S4D). To our knowledge, this is the first report to measure I-FABP levels longitudinally through acute infection in humans and show a spike in gut epithelial breakdown that corresponds with peak viremia. This data is consistent with the hypothesis that massive viral replication during acute infection leads to profound damage to the gut epithelial barrier and precipitates the well described association between microbial translocation, immune activation and disease progression. Since ILCs are central to gut epithelial repair, the 4-fold increase in I-FABP levels and coincident loss of ILC from
circulation suggests a possible link, although this may be circumstantial and additional data is required to test this correlation.

**ILC depletion is irreversible despite successful suppression of viremia by antiretroviral treatment in chronic infection**

We next sought to investigate the relationship between ILC depletion and immune reconstitution following ART initiation. In a longitudinal treatment cohort (Abdool Karim et al., 2010), we measured ILC levels in chronically infected individuals at the last time-point before ART initiation and 2 years into successful treatment (Figure 3A,B) (Figure S5), as indicated by partial CD4 reconstitution and reduced immune activation (Figure 3C). Unexpectedly, all three ILC populations failed to restore to normal levels, and remained significantly lower than in uninfected individuals despite undetectable viral loads ($P < 0.02$) (Figure 3D-G). Only blood ILC3s significantly rebounded with ART ($P < 0.0001$), but never to the levels observed in uninfected individuals (median 70 vs 430 ILC3 cells). There was no correlation between the recovery of ILCs and CD4$^+$ T cells in the same subjects ($P > 0.7$) (data not shown). Thus, the ILC depletion observed during both acute and chronic HIV-1 infection is irreversible when treatment is initiated in the chronic phase.

**ILC depletion is prevented by antiretroviral treatment initiated during early acute HIV-1 infection**

To investigate the potential capacity of ART initiated in early infection to reverse the negative impact of HIV-1 on circulating ILCs, we analyzed a unique subset of individuals in whom ART was initiated at the earliest possible time-point: on the first day of HIV-1 RNA detection and within 5-14 days after HIV-1 transmission (McMichael et al., 2010). From one subject (PID 0444-312), we observed reduced peak viremia, preserved CD4$^+$ T cells (871 before vs...
745 cells/ul 4 weeks after infection) and found no depletion of blood ILCs (Figure 4A). The preservation of ILCs relative to untreated subjects was consistent in all seven individuals receiving ART during early acute infection (Figure 4B) in striking contrast to acute infected individuals not receiving treatment (Figure 4C). ILC levels in these subjects fluctuate to some extent during the course of acute infection but no clear patterns emerge. Whether this represents heterogeneity in the response of ILCs in these individuals to their ART, or natural variation is unclear. However, at >6 weeks into infection for all three ILC subsets are significantly higher than in untreated subjects ($P < 0.018$) (Figure 4D). Thus, ILC depletion during HIV-1 infection can be prevented by early ART.

**ILC depletion associated with signatures of activation and Fas up-regulation**

ILCs are unlikely to be directly infected by HIV-1 as they do not express CD4 co-receptor and, *in vitro*, we were unable to infect ILCs with HIV-1 using high titers of X4 and R5 virus (data not shown). In order to investigate alternative mechanisms of ILC depletion, we first measured levels of the anti-apoptotic factor Bcl-2 and activated caspase-3, involved in apoptosis. Although we were able to induce high levels of caspase-3 in all ILC subsets by incubation with the pro-apoptotic molecule camptothecin, we did not detect any significant changes in absolute (Figure 5A) or relative levels (Figure 5B) of Bcl-2 or caspase-3 expression when comparing ILCs from HIV-1 uninfected and chronic infected subjects. As expected (Petrovas et al., 2004), we did detect elevated signatures of apoptosis in CD8$^+$ T cells from chronically infected individuals using this assay (data not shown).

We next examined the activation status of the remaining circulating ILCs after HIV-1 infection, since HIV-1 induced immune activation is implicated in CD4 T-cell depletion (Brenchley et al., 2004). We found a significant increase in the lymphocyte activation
marker CD69 on ILC2 and ILC3 (P < 0.0001) (Figure 5C) and on T cells (data not shown) in HIV-1 positive subjects, demonstrating activation of ILCs in response to infection. However, when we measured CD38, a marker of general immune activation on T cells, we found low expression levels on ILC1, ILC2 and ILC3s compared to CD4+ and CD8+ T cells with no correlation to ILC frequencies in HIV-1 infected individuals (data not shown). In addition, we found no difference in CD38 expression on ILC1, ILC2 and ILC3s comparing infected and uninfected individuals in contrast to significant differences observed for both CD4+ and CD8+ T cells (P < 0.01) (data not shown) suggesting that different activation markers exist for ILCs and T cells in HIV-1 infection. We observed significant up-regulation of the mucosal tissue homing receptors α4β7, but only in the ILC3 subset (Figure S6) and not in their T-cell counterparts or in ILC1 and ILC2 subsets (data not shown).

Plasma IL-7 is known to be elevated in chronic HIV-1 infection (Hodge et al., 2011) and is the ligand for CD127, a receptor that is expressed on all ILC subsets and is critical for their generation and maintenance (Spencer et al., 2014). Therefore, we next measured plasma levels of IL-7 by ELISA in matched samples and surprisingly find a weak positive associations with frequency of ILC1 and ILC2 and plasma IL-7 levels in HIV-1 infected individuals, but no association with ILC3s (Figure S7). These data do not suggest a role for IL7 in persistent ILC depletion in chronic HIV-1 infection.

A recent study suggests Fas-FasL interactions are involved in ILC3 apoptosis in a humanized mouse model of HIV-1 infection (Zhang et al., 2015). Therefore, we measured the levels of CD95 (Fas) expression in chronic HIV-1 and found significant up-regulation on ILC2 and ILC3 subsets measured by % CD95 expression (Figure 5D), which also were significant for the ILC1 subset by relative MFI expression levels (P = 0.004) (data not shown). The Fas mediated apoptosis reported by Zhang et al. was driven by IFN-α, and we therefore sought to correlate the ILC depletion
observed in our acute HIV-1 cohort with levels of this cytokine, reported to be induced in this early phase of infection (Stacey et al., 2009). Plasma levels of IFN-α and the IFN-induced protein IP-10 were measured in samples from 2-12 weeks before infection (plotted as day -7) followed by 3, 7, 14 and 30 days after first HIV-1 RNA detection. Large amounts of IP-10 were induced by primary viremia, confirming immune activation and the existence of a strong IFN signature associated with acute infection ($P = 0.038$) (Figure 5E). In addition, we observed a modest increase in IFN-α over baseline at the earliest time-point and prior to peak viremia ($P = 0.077$) Figure 5F.). In the limited samples available (n=4), we find that IP-10 levels are greatly reduced by immediate ART, consistent with a blunted IFN response, but the impact on IFN-α is not apparent (Figure 5E,F). These data are consistent with the hypothesis that ILC depletion is driven by immune activation during acute viremia, and that is prevented by early treatment, although the role of IFN-α specifically is not clear.

RNA-Seq analysis reveals down-regulation of genes associated with cell survival and proliferation in ILCs immediately following HIV-1 infection

In order to gain a deeper understanding of the depletion of circulating ILCs in HIV-1 infection, we performed bulk RNA-Seq measurements on patient samples from early acute infection. RNA-Seq is a sensitive and powerful method for determining changes in cell populations and behaviors (Hu et al., 2010; Rapaport et al., 2013). mRNA from CD4$^+$ T-cell, ILC2, and ILC3 populations isolated at various time points in early acute infection were sequenced from two untreated patients and two patients who started ART immediately after viral RNA detection. Transcriptional comparisons were made between both HIV detection and peak viremia, and peak viremia and 6 weeks after detection for each patient in order to generate lists of significantly up- or down-regulated genes (Figure 6A, Figure S8A).

2009
First, we compared initial viral detection and peak viremia in an attempt to understand the transcriptional changes undergone by ILCs in response to the antigenemia and cytokine response associated with this disease phase (McMichael et al., 2010). Both ILC2s and ILC3s in untreated patients showed statistically significant ($P < 0.01$) down-regulation of genes associated with cell proliferation and survival, and up-regulation of those linked to apoptosis and cell death (Figure 6B, Figure S8B, Table S4). ILCs in an early acute ART treated patient (PID 0629-453) exhibited less down- and up-regulation respectively, demonstrating a mitigated response to HIV infection. CD4$^+$ T cells, on the other hand, only show significant differential gene expression in one patient and, in this case, opposite trends were observed (data not shown). In addition, several key upstream immune regulators were found to change in ILC2s and ILC3s immediately following infection (Figure S8D). Although ILCs completely lack T-cell receptor expression measured at the protein level, there were significant changes in genes associated with CD3, also recently reported in transcriptional profiling of ILCs in murine models (Robinette et al., 2015). However, we found no evidence of TCR gene modules and therefore the role of these genes in ILCs is unclear. ILCs have previously been shown to regulate CD4$^+$ T-cell responses to intestinal commensal bacteria and have been implicated in ensemble responses to SIV (Hepworth et al., 2015; Hepworth et al., 2013; Li et al., 2014). Down-regulation of SYVN1 and Leukocyte Extravasation Signaling pathways suggest cellular shutdown in ILCs: SYVN1 coordinates removal of unfolded proteins and down-regulation leads to measured de-activation of surface receptor genes like IL7R, ITGB1, and CD44 (full gene lists can be found in Table S4); decreased Leukocyte Extravasation Signaling implies that ILCs could be unable to properly traffic towards tissues. Combined with measured immune transcriptional change, we propose that ILCs are initially activated in the blood, but quickly lose function and undergo apoptosis during the onset of peak viremia.
We also compared transcriptional profiles of ILCs at peak viremia and approximately 6 weeks after infection to probe the state of the ILCs that survive acute infection. Counter to early changes, we found that ILC2s and ILC3s from untreated patients displayed up-regulated cell proliferation and cell survival, and down-regulated cell death and apoptosis (Figure 6C, Figure S8C, Table S5). This is consistent with our flow cytometry data showing that ILCs persisting in chronic infection show no evidence of increased apoptosis or cell death. Additionally, although phenotypically skewed – (see Figure 1H,I), the remaining ILCs, show signs of typical cellular function and immune response (Figure S8E). The early ART treated patients, on the other hand, show mixed responses after peak viremia. Since these patients were treated very early in infection, one cannot accurately compare time points between patients using the typical standard of viral load; thus, it is not surprising that these patients have varied responses in comparison to the coordinated response of the untreated patients. Further longitudinal profiling of patient specific changes in function, upstream regulators, and signaling pathways could reveal distinct regulatory networks influencing ILC mediated homeostasis in chronic HIV-1 or ART mediated viral suppression.

**Tonsil and gut resident ILCs are not enriched or depleted in chronic HIV-1 infection**

Current studies of the involvement of ILCs in infectious and non-infectious disease have focused primarily on the effector function of these cells in lymphoid tissue (McKenzie et al., 2014). Therefore, to investigate how changes in frequency and phenotype of circulating ILCs relate to lymphoid tissue resident cells, we next turned to tissue samples from HIV-1 infected and uninfected subjects. We first examined surgically removed tonsils, lymphoid organs that are known to support high levels of HIV-1 production and to undergo profound tissue remodeling during progressive disease (Doitsh et al., 2014; Sanchez et al., 2014). Using the accepted ILC definitions (Spits 2013) we identified tissue resident ILCs and confirmed these subsets by T-bet, GATA-3, Eomes, Helios, AHR and RORγt transcription factor staining.
controlled by NK and CD4+Th2 cells (Figure 7A). In this lymphoid tissue, we found higher frequencies of ILC1s and ILC3s, but not ILC2s, compared to the blood (Figure S9), with a distinct CD69+ and CD62L− phenotype (Figure 7B) that is characteristic of tissue resident T-lymphocytes (Schenkel and Masopust, 2014). No significant depletion of CD4+ T cells from the HIV-1 infected tonsils, known to be directly infected by the virus, was observed (Figure 7E) (Doitsh et al., 2014). Interestingly, no significant effect of HIV-1 on ILC frequency, subtype or phenotype distribution in tonsil tissues was observed (Figure 7D,E), nor any differences in apoptotic markers such as active caspase-3 and anti-apoptotic Bcl-2 (data not shown), that might explain their loss from circulation. We next examined ILC frequencies in the gut mucosa, which is known to be a major site of HIV-1 replication and CD4+ T-cell depletion (Mattapallil et al., 2005), and where ILC3s are known to play a crucial role in homeostasis and barrier function. Using human gut biopsies from subjects undergoing colonoscopy, we identified a distinct lineage negative CD127+ ILC population that was dominated by cKit positive ILC3 cells expressing CD56, NKp44, CCR6 and CD69 (Figure 7F). Again RORγt, Helios and AHR transcription factor staining to confirmed that these are ILC3s (Figure 7F). In 46 subjects undergoing colonoscopy we found increased levels of ILC1 and ILC3, but not ILC2, compared to tonsil and blood (P < 0.0001) (Figure 7G). However, we observed no differences in gut resident ILC frequencies between HIV-1 infected (n=9) and uninfected (n=37) individuals, nor do we detect any difference in NKp44 and CD56 ILC3 phenotype distribution (Figure 7G). Together these data provide no evidence that ILC are either recruited to or depleted from lymph nodes or the gut mucosal barrier.

Finally, to assess the impact of HIV-1 infection on ILC function, we measured cytokine production in tonsil resident ILCs from HIV-1 infected and uninfected individuals, stimulated non-specifically with PMA/ionomycin. Cytokine production in these cells is almost
exclusively restricted to NKp44$^+$ILC3s (Figure 7H, Figure S10; $P < 0.0001$), and dominated by IL-2, TNFα and GM-CSF and, to a lesser extent IL-22. **No IFN-γ or IL-13 production was detected** from NKp44$^+$ RORγt$^+$ ILC3 cells (data not shown)(Glatzer et al., 2013). Overall, we observe a consistent trend for decreased cytokine production in tonsil resident ILC3s from HIV-1 infected individuals, but these differences were not significant in the sample size obtained here.
The role of ILCs during chronic viral infection in humans remains incomplete (Diefenbach, 2013), despite compelling evidence highlighting the importance of ILCs in immune regulation and mucosal barrier maintenance (Sonnenberg et al., 2012a; Tait Wojno and Artis, 2012). Here, we report a rapid and irreversible depletion of blood ILCs during acute HIV-1 infection that persists in chronic infection in proportion to viral load. During the chronic phase, ILC depletion is associated with altered subset composition and increased expression of activation (CD69), tissue homing (α4β7) markers, and the Fas death receptor (CD95). Up-regulation of FAS was found to make ILC3s more susceptible to anti-CD95 antibody induced apoptosis in vitro (Zhang et al., 2015), however, we detect no apoptotic ILCs ex-vivo in chronic human HIV-1 infection measured either by increased expression of activated caspase 3 or down-regulation of bcl-2. In contrast, in early acute infection we find ILCs up-regulate genes associated with cell death and apoptosis, potentially explaining their disappearance in the absence of early ART. This coincides with a strong IFN response induced by viral up-ramp, demonstrated by the rapid elevation in plasma levels of the IFN induced protein IP10 and to a lesser extent IFNα. We find no evidence of enrichment of tissue resident ILCs in either tonsil and gut samples, suggesting that ILC depletion from circulation is explained by apoptosis rather than tissue redistribution.

Importantly, despite the correlation between viral load and ILC levels in chronic infection, removal of viral burden and reduction of immune activation during this phase of the disease with fully suppressive ART is unable to restore circulating ILC populations. There is a partial rebound of the circulating ILC3 subset, but they remain well below the levels found in healthy donors. This suggests that sustained viremia may lead to a fundamental impairment of the ILC arm of the immune system, which could have far reaching immunological
The role of acute viremia in ILC depletion is supported by data from our unique subset of patients in whom ART was initiated prior to peak viremia. In these individuals we observe no sustained depletion of ILCs and, in stark contrast to untreated individual or those treated later, ILCs remain at the levels observed in uninfected individuals. This is associated with an absence of the transcriptional signature of apoptosis, and the strong plasma IP-10 response observed in the untreated individuals. What role this early IFN response has in ILC depletion is not clear from our data, although IFNa in particular has been implicated in ILC3 depletion in the humanized mouse model(Zhang et al., 2015). Importantly, depletion of ILCs from blood is not a general acute phase response to infection, as filarial infection in humans is associated with expansion of blood ILCs(Boyd et al., 2014), suggesting the specific relevance of this phenomena to HIV-1. Indeed, more than 90% of individuals sampled from the same populations investigated in this study were infected with CMV and EBV(Kloverpris H et al, unpublished data), yet displayed normal blood ILC levels compared to individuals from areas without endemic viral infections(Munneke et al., 2014). In addition, direct infection of ILCs by HIV-1 is highly unlikely as they lack viral entry receptors and we are completely unable to infect them in vitro with high titres of either X5 or R4 virus (data not shown).

Our RNA-Seq data suggests that at some point in early acute infection ILCs may lose their ability to effectively traffic throughout the body. However, those ILCs that do survive peak viremia and persist in chronic infection show no evidence of apoptosis by protein expression (Bcl-2 and active caspase-3) and exhibit comparatively down-regulation of apoptosis and cell death transcription signatures. In fact, genes associated with viral infection and immune response are up-regulated in these populations, consistent with the measured increase in CD69 expression. Whether a certain subset of ILCs never initiates apoptosis in early acute
infection or a population is consistently renewed at lower levels in the blood during chronic infection remains unclear. Single-cell RNA-Seq analysis of ILCs during acute infection could reveal deeper insights into the fate of this important group of cells both in blood and primary tissues like tonsils and gut.

In addition to apoptosis induced by acute infection, a potential mechanism for the loss of ILCs from circulation is that they home to major sites of HIV-1 replication and tissue damage. The fact that we do not detect a significant increase in any ILC subset within tonsils and gut tissue may be limited to an issue of sensitivity. There are approximately 3-6 fold more T-lymphocytes residing in lymphoid organs compared to the blood (Farber et al., 2014), and this, in combination with the fact that certain ILC subsets are enriched within these compartments, may mask the redistribution of circulating ILCs to these sites. In addition, as we observe no apparent impact of HIV-1 infection on tonsil resident CD4+ T cells, it is possible that insufficient numbers and patient heterogeneity limit our ability to detect such changes. Significant increases in ILC subsets are detectable in the livers of fibrotic mice (Mchedlidze et al., 2013) and skin of human psoriasis suffers (Teunissen et al., 2014), possibly as a result of sustained recruitment from a blood pool that is itself presumably continually replenished from ILC precursors in the bone marrow or other lymphoid structures (Montaldo et al., 2014; Yu et al., 2014). Furthermore, significant enrichment of NKp44+ lineage negative cells are observed in the tonsils and oral lymph nodes of SIV infected macaques (Reeves et al., 2011). Although, not precisely defined, these cells are most likely analogous to the ILC3 NKp44+ subset we observed enriched in the tonsils compared to blood. Subsequent studies observed depletion of the ILC3 NKp44+ subset from the GALT and mesenteric lymph nodes of acutely and chronically SIV infected Macaques (Li et al., 2014; Xu et al., 2015). Why an enrichment of ILC NKp44+ cells occurs in the tonsils of SIV challenged...
monkeys and not in naturally infected human subjects remains unclear, but these data imply that either enrichment or depletion of ILCs in the context of retroviral infection would be possible to detect. Finally, the ILC response to HIV-1 infection is likely to be different in different tissue compartments. The ILC2 subset for example is severely depleted in blood and yet our data suggest that oral lymph nodes and gut are not a major reservoir of this subset. Tissue compartments known to be major sites of ILC2 activity, such as the liver (Mchedlidze et al., 2013) and lung (Monticelli et al., 2011), were not sampled in this study but would be interesting to follow in future experiments.

The fact that Li et al observed a depletion of an ILC3-like subset from the GALT of acutely SIV infected Macaques (Li et al., 2014) is also consistent with the depletion of circulating ILC3s we observe in acute HIV-1 infection and concomitant spike in I-FABP levels. The limited recovery of blood ILC3s observed after successful drug treatment suggests that this subset remains impaired, and that continued immune activation might relate to the functional inability of gut resident ILC3s in HIV-1 infected individuals (Zhang et al., 2015) to restore gut barrier integrity and prevent microbial translocation (Brenchley et al., 2008; Brenchley et al., 2004). Whether ILCs play a direct role in the HIV-1 pathology is difficult to study in human samples and therefore animal studies are warranted to elucidate the mechanistic details relating to the direct consequence of ILC depletion in HIV/SIV pathology (Klatt et al., 2012; Li and Reeves, 2012; Reeves et al., 2011; Xu et al., 2012; Zhang et al., 2015). However, we believe that ILCs are likely to play an important role in HIV-1 pathology given the existence of an IL-22 producing NKp44+ ILC3 subset (Glatzer et al., 2013) that is required to both maintain the gut mucosa and limit the response to gut microbial contents (Cella et al., 2008; Sonnenberg et al., 2012b). Irreversibly depleted ILC3s by HIV-1 infection would suggest a clear mechanism behind the continued immune activation observed even in individuals with
successful long term viral suppression by ART (Sanchez et al., 2014; Zeng et al., 2012), which is the strongest predictor for the onset of AIDS (Hunt, 2012; Hunt et al., 2014).

In summary, we demonstrate persistent and irreversible ILC depletion that occurs immediately after HIV-1 acquisition, correlates with disease stage and is not restored by long term fully suppressive ART, but can be blocked by early treatment. This provides a potential novel mechanistic link between HIV-1 infection, lymphoid tissue breakdown and persistent immune dysfunction that merits further exploration, and suggests the importance of early ART administration in maintaining normal immune system composition and functionality.
MATERIALS AND METHODS

Subjects

We used samples from a total of 122 HIV-1 uninfected subjects and 137 HIV-1 infected subjects. All participants were women with sub-Saharan Zulu/Xhosa ancestry from four independently collected cohorts within or in the greater area of Durban, KwaZulu-Natal, South Africa. All subjects were treatment naïve for antiretroviral therapy unless otherwise indicated. The cohorts were followed from 4 independent studies; i) ‘iThimba’ Cohort from McCord Hospital, Durban, ii) the ‘CAPRISA002’ cohort iii) the ‘GATEWAY’ cohort (n=48) at Prince Mshiyeni Memorial Hospital, Umlazi. Durban and iv) the Females Rising through Empowerment, Support, and Health ‘FRESH’ from Umlazi. Durban(Ndhlovu et al., 2015).

The median viral load was 14,000 copies/ml (interquartile range 640 - 79,302 copies/ml). Absolute CD4+ T-cell counts were 397 per μL of blood (interquartile range 297 - 548 cells per μL). Samples from ‘iThimba’ and ‘CAPRISA002’ were processed from frozen PBMCs, whereas samples from the ‘GATEWAY’ and ‘FRESH’ cohorts were processed from fresh blood samples. All PBMCs were purified using standard ficoll separation.

Tissue samples were obtained from surgical resection following tonsillectomy at King Edward Hospital, Durban, South Africa and from Stanger Hospital, KwaDukuza, KwaZulu-Natal, South Africa with a total of 12 HIV-1 infected and 12 HIV-1 uninfected individuals enrolled.

All subjects provided informed consent and each study was approved by the respective institutional review boards including the Biomedical Research Ethics Committee of the University of KwaZulu-Natal for all the studies.

Clinical parameters
Viral loads were obtained using the Roche Amplicor 1.5 assay (iThimba and CAPRISA002 cohorts) or the BioMerieux Nuclisens v2.0 (FRESH and GATEWAY cohorts) at Global Clinical and Viral Laboratories, Durban, South Africa. CD4+ T-cell counts and total lymphocyte counts were determined as previously described (Abdool Karim et al., 2010).

**Flow cytometry**

Two different multicolor flow cytometry panels were used. The transcription factor panel, shown in Fig. S1b and Fig 4e, was surface stained with near-infrared live/dead cell viability staining kit (Invitrogen) and monoclonal antibodies: αCD45-V500 Horizon clone HI30 (BD Biosciences), αCD56 Brilliant Violet 711 clone HCD56 (BioLegend), αCD94 PerCP-Cy5.5 clone HP-3D9 (BD Biosciences), αCRTH2 PE-CF594 clone BM16, αCD127 PE-Cy7 clone R34.34 (Beckman Coulter), CD161 Brilliant Violet 605 clone HP-3G10 (BioLegend) and lineage markers conjugated to FITC or AlexaFlour488: αCD4 clone OKT4 (BioLegend), αCD11c clone 3.9 (BioLegend), αCD14 clone HCD14 (BD Biosciences), αCD19 clone 6D5 (BD Biosciences), αCD34 clone 561 (BioLegend), αFcER1 clone AER-37 (eBioscience), αBDCA2 clone 201A (BioLegend), αTCRαβ clone IP26 (BioLegend), αTCRγδ clone B1 (BioLegend) and intracellularly stained after Fix/Perm kit by (eBioscience) with αCD3 Brilliant Violet 785 OKT3 (BioLegend) and αCD3-FITC clone HIT3A (BD Biosciences), αGATA3-eFlour660 clone TWAJ (eBioscience), αT-bet Brilliant Violet 421 clone 4B10 (BioLegend), αEomes-PerCP-eFlour710 clone WD1928 (eBiosciences), αRORγt clone Q21-559 (BD Biosciences), α-aryl hydrocarbon receptor (AHR) clone F3399 (eBioscience) and αHelios clone 22F6 (BioLegend). The eBioscience Fixation/Permeabilization kit from eBioscience were used for intracellular staining of transcription factors and blocked with 20% goat serum for 20 mins prior to antibody staining.
For phenotype identification, cells were surface stained with near-infrared live/dead cell viability staining kit (Invitrogen) and monoclonal antibodies: αCD45-V500 Horizon clone HI30 (BD Biosciences), αCD56 Brilliant Violet 711 clone HCD56 (BioLegend), αCD94 PerCP-Cy5.5 clone HP-3D9 (BD Biosciences), αCRTH2 Alexa-Flour647 clone BM16 (BD Biosciences), αCD127 PE-Cy7 clone R34.34 (Beckman Coulter), αCD161 Brilliant Violet 605 clone HP-3G10 (BioLegend), αCD117 Brilliant Violet 650 clone 3G8 (BioLegend), αCD25 Brilliant Violet 785 clone BC96 (BioLegend), αCD69 Brilliant Ultra Violet 395 clone FN50 (Brilliant Horizon), αCCR6 Brilliant Ultra Violet 496 clone 11A9 (Brilliant Horizon), αNKp44 PE and PECy5 clone Z231 (Beckman Coulter), α4-β7 monoclonal antibody (cat#11718) (NIH) conjugated in house to PacificBlue (Life Technologies) and lineage markers conjugated to FITC or AlexaFlour488: αCD4 clone OKT4 (BioLegend), αCD11c clone 3.9 (BioLegend), αCD14 clone HCD14 (BD Biosciences), αCD19 clone 6D5 (BD Biosciences), αCD34 clone 561 (BioLegend), αFcER1 clone AER-37 (eBioscience), αBDCA2 clone 201A (BioLegend), αTCRαβ clone IP26 (BioLegend), αTCRγδ clone B1 (BioLegend), and intracellularly stained after Fix/Perm kit by BD Biosciences αCD3-PE-CF594 clone UCHT1 (BD biosciences) and αCD3-FITC clone HIT3A (BD Biosciences). Intracellular cytokine production from all ILC subsets was measured by PBMC stimulation for 6 hours with PMA/ionomycin or medium, with Golgiplug present. The following antibodies were used for intracellular cytokine stimulation (ICS); αTNFα Alexa700 Mab11 (BD Biosciences), αIL13 V450 JES10-5A2 (BD Biosciences), IFNg Brilliant Violet 785 4S.B3 (BioLegend), αIL22 PerCP-eFlour710 22URTI (eBioscience), αIL17A Brilliant Violet 605 clone BL168 (BioLegend), αIL2 PE clone MQ1-7H12 (BD Bioscience). All samples were surface stained at room temperature for minimum 20 mins and intracellularly stained at room temperature for
minimum 20 mins. All samples were fixed in 2% Paraformaldehyde before acquisition on a 4 laser, 17 parameter BD Fortessa flow cytometer within 24 hours of staining. Data were analysed using FlowJo v. 9.7.2 (TreeStar).

**ELISA**

Intestinal Fatty Acid Binding Protein (I-FABP) was measured using the ELISA kit human FABP2 DuoSet, R&D systems and plasma IL-7 levels were measured using the recombinant human IL-7 kit from R&D systems (cat#207-iL). IFNα and IP-10 were measured using the Milliplex kit (Millipore) and completed according to the manufacturers protocol.

**tSNE analysis of flow cytometry data**

Unbiased representations of multi-parameter flow cytometry data were generated using the t-distributed stochastic neighbor embedding (tSNE) algorithm (van der Maaten and Hinton, 2008). tSNE is a non-linear dimensionality reduction method that optimally places cells with similar expression levels near to each other and cells with dissimilar expression levels further apart. The R package ‘Rtsne’ available on CRAN (provided by Jesse Krijthe, github.com/jkrijthe/Rtsne) was used to perform the Barnes Hut implementation of tSNE on flow cytometry data. FlowJo software was used to export events of interest (in fcs format) for each sample analyzed in a manner similar to that described (Becher et al., 2014). After using the Bioconductor ‘flowCore’ R package to import .fcs file data and the Logicle transform (Parks et al., 2006) to scale the data similarly to that displayed in FlowJo (Top of scale = 500,000, width = 10), a similar number of events from each sample analyzed in parallel were merged and the relevant fluorescent parameters were used as input for tSNE. The results were unmerged into and appended to the corresponding input .fcs files for subsequent analysis in FlowJo. Clusters of cells identified by tSNE plots were gated manually.
and then further evaluated for median fluorescence intensities of each phenotypic marker using heatplots.

**RNA-Seq**

CD4+ T cells, ILC2s, and ILC3s (100,000 - 50 cells) were sorted from PBMCs as described above into 300 μL of RLT Lysis Buffer (Qiagen) supplemented with 1% v/v 2-mercaptoethanol, briefly vortexed, spun down, and snap-frozen on dry ice. Cellular mRNA was then isolated and processed for RNA-Seq as described previously (Trombetta et al., 2014). Briefly, 10 μL of mixed lysate from each sample was transferred to a skirted 96 well plate. Genetic material was pulled down and purified by mixing the lysate in each well with 2.2x volumes of Agencourt RNAClean XP SPRI beads (Beckman Coulter) and washing thrice with 100 μL of 80% ethanol. After drying, the SPRI beads were re-suspended in 4 μL of pre-reverse transcription (RT) mix, incubated for 3 min at 72°C, and placed on ice. Next, Smart-Seq2 Whole Transcriptome Amplification (WTA) was performed: 7 μL of RT mix was added to each well and RT was carried out; then, 14 μL of PCR mix was added to each well and PCR was performed. After WTA, 0.8x volumes of Agencourt AMPure XP SPRI beads (Beckman Coulter) were used to clean up the cDNA product, which was then quantified using a Qubit dsDNA HS Assay Kit (Life Technologies). Library size and quality, meanwhile, were measured by Bioanalyzer using a High Sensitivity DNA Analysis Kit (Agilent Technologies).

Sequencing libraries were then prepared from WTA product using Nextera XT (Illumina). After library construction, one final AMPure XP SPRI clean-up (0.8 volumes) was conducted. Library concentration and size were measured with the KAPA Library Quantification kit (KAPA Biosystems) and a TapeStation (Agilent Technologies), respectively. Finally, samples
were sequenced on a NextSeq500 (30 bp paired-end reads) to an average depth of 350,000 reads.

**Gene Expression Data Analysis**

Sequencing data from the NextSeq500 was demultiplexed and aligned against hg19 using TopHat (Li and Dewey, 2011; Trapnell et al., 2014). Expression values, in counts, were generated in RSEM for every sample. Genes expressed in less than one third of the samples were discarded from future analyses as were samples with fewer than 2500 total counts or 1000 detected genes (genes with counts>0). A simple PCA of all samples was also conducted in order to identify any samples of low technical quality for removal.

EdgeR was used to calculate differential expression between CD4⁺ T cells, ILC2 cells, and ILC3 cells isolated from 9 HIV negative FRESH cohort patients. First, expression counts were normalized and gene-specific dispersions were estimated. Global fitting was used to conduct likelihood ratio tests between pairs of cell types. The top differentially expressed genes (P < 0.01, FDR < 0.01) between each of the following comparisons can be found in Table S1-S3: CD4 vs. ILC2, CD4 vs. ILC3, ILC2 vs. ILC3.

Additionally, to succinctly demonstrate differences between cell types over subsets of genes previously linked to ILCs, we adapted a previously described scoring system (Shalek et al, Nature, 2014). Here, we used human orthologs of genes found to be differentially expressed between various ILC populations in mice (Robinette et al., 2015). Orthologs were identified biomaRt (http://bioconductor.org/packages/release/bioc/html/biomaRt.html). Briefly, to generate each cell’s Innate Lymphoid Cell Score, we transformed normalized expression counts for each gene into “induction values” by dividing by average expression and summing...
the induction values for each gene in our list for each sample. Finally, scores across all
samples were linearly scaled to fit between (0, 1).

Differential expression analysis was conducted using edgeR, available on Bioconductor, via
generalized linear modeling (Robinson and Oshlack, 2010). Lacking biological replicates in
patient samples, dispersion estimates were calculated from 9 HIV uninfected patient samples.
Tag-wise dispersion estimates were generated following trended dispersion estimates
calculated using a log-linear trend. For each patient, Likelihood Ratio Tests (LRTs) were
conducted for each cell type between both peak viremia and detection, and peak viremia and
the time point closest to 6 weeks after infection (Figure 6 Figure S8A). These LRTs generated
a log fold change (logFC) and P-value for each gene. All genes with P<0.01 were then
uploaded into Ingenuity Pathway Analysis (IPA, Qiagen) with their corresponding logFCs.
Core analyses were conducted on each cell type for each patient for each LRT test; the
significant functionally enriched gene sets, upstream regulators, and identified signaling
pathways were then compared across patients and cell types. A list of all gene sets identified
by IPA for each core analysis for the detection vs. peak viremia and peak viremia vs. 6 weeks
comparisons is provided in Table S1 and S2, respectively.

Statistical analyses
We used the Mann-Whitney U-test for comparison of median values between two groups only
and the Dunn’s multi comparisons test to compare median values of more than 2 groups. The
Wilcoxon matched-pairs signed rank test was used for paired testing of median values before
and after antiretroviral treatment for matched samples. We used the Spearman rank
correlation test to compare correlation between two parameters and reported r-values and P-
values. Statistical analyses were performed using GraphPad Prism version 6.0c (GraphPad software, Inc).
ACKNOWLEDGEMENTS

The authors have declared that no competing interests exist.

H.N.K is funded by the Danish Agency for Science, Technology and Innovation (grant #12-132295), The Lundbeck Foundation (grant #R151-2013-14624) and the MAERSK Foundation for Medical Improvement. The study was also supported by the Collaboration for AIDS Vaccine Discovery of the Bill and Melinda Gates Foundation, and NIH grant AI067073 (BDW). Partial support for this work was received from the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative (IAVI) (UKZNRSA1001), and the NIAID (R37AI067073). T.N. received additional support from the South African Research Chairs Initiative, an International Early Career Scientist Award from the Howard Hughes Medical Institute and the Victor Daitz Foundation. S.W.K is funded by the National Science Foundation Graduate Research Fellowship Program (NSF GRFP). A.K.S. is supported by the Searle Scholars Program.

We thank Dr Hollis Shen for extensive support and help during cell sorting and flow cytometry acquisition. We thank all the participants in ‘iThimba’, ‘Gateway’, ‘CAPRISA002’ and ‘FRESH’ Acute Infection Study participants who are continuing to make an important personal contribution to HIV research through their support and participation in our studies. The scientific and supportive role of the whole CAPRISA004 and CAPRISA002 study and protocol teams are gratefully acknowledged. We would also like to thank Carly G. K. Ziegler, Travis K. Hughes, and the other members of the Shalek Lab for insightful conversations concerning the analysis of the RNA-Seq data.

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: α4-β7 monoclonal antibody (cat#11718) from Dr. A. A. Ansari.


FIGURE LEGENDS

Figure 1. Identification of human ILCs from blood shows HIV specific depletion of circulating ILCs. (a) Representative conventional flow cytometry plots from an HIV-1 uninfected donor showing the hierarchical phenotype gating strategy from singlet lymphocytes to the ILC1 (orange), ILC2 (red) and ILC3 (blue) populations indicated by arrows and color-coded gates. Lineage (Lin) gate contains anti-CD3, CD4, CD11c, CD14, CD19, CD34, BDCA2, FcER1, TCR\(\alpha\)\(\beta\), TCR\(\gamma\)\(\delta\) antibodies. (b) tSNE clustering of human PBMCs pre-gated for lymphocytes/singlets/live/CD45\(^+\)/CD3\(^-\)/Lin\(^-\)/CD127\(^+\) that shows two-dimensional representation of high dimensional space based on phenotype markers CD161, CD117, CD56, CRTH2, CD4, NKp44, CD25, CD62L, CD69, CCR6, CD94 with gates indicating 5 identified clusters with heat-map showing the relative expression intensity for each marker within the 5 identified clusters (NK, ILC3, ILC1, ‘non-ILC’, ILC2). (c) Cytokine production after media or PMA/Ionomycin stimulation from blood CD4\(^+\) T cells, ILC1s, ILC2s and ILC3s for 6 cytokines IL2, IL13, IFN\(\gamma\), TNF\(\alpha\), IL17A and IL22 with tonsil derived ILC3 shown as NKp44 vs IL22. (d) Transcription factor expression within CD4\(^+\) Th2 cells (CRTH2 gated), CD4\(^+\) Th1 cells (CD56 gated), NK cells (CD3\(^-\)CD94\(^+\)CD56\(^-\)CD16\(^-\)) ILC1, ILC2 and ILC3 for T-bet, GATA-3 and ROR\(\gamma\)\(t\). (e) Innate lymphoid cell score based on RNAseq generated gene expression within CD4\(^+\) T-cell, ILC2 and ILC3 sorted populations from blood of n=9 HIV uninfected individuals based on recent published gene transcripts of ILCs(Robinette et al., 2015). (f) Absolute ILC counts for HIV-1 uninfected (n=136), HIV-1 infected with undetectable plasma virus (VL<50) (n=16) and HIV-1 infected individuals with detectable viremia (VL>50) (n=91). (g) ILC frequency expressed as % of CD45\(^+\) lymphocytes for HIV-1 uninfected (n=122), HIV-1 infected with undetectable plasma virus (VL<50) (n=14) and HIV-1 infected individuals with detectable viremia (VL>50) (n=115). P - values by Dunn’s test for multiple comparisons. (h) Grouping of cells gated from
lymphs/singlets/live/CD45^+/CD3^-/Lin^-/CD127^+ according to automatic (unbiased) cluster
designation for accumulated data from 18 HIV-1 uninfected (left) and 21 HIV-1 infected
subjects with each distinct cluster named by its unique number inside gates. (i) Bar graph
showing the mean percentage contribution from each cluster (x-axis), corresponding to the
tSNE plots in (h), to the overall Lin^-CD127^+ population with P<0.02 indicated by * and
calculated by t-test comparing HIV-1 uninfected (n=18) and HIV-1 infected individuals
(n=21). P-values by student t-test and Sidak-Bonferroni method for multiple comparisons.
Figure 2. All ILC populations are depleted during early acute HIV-1 infection. (a) Data from the acutely HIV-1 infected PID 0398-271 subject followed longitudinally over 10 time-points from day 1 to day 249 from the day of first HIV+ RNA test, with ILC1, ILC2 and ILC3s shown as a percentage of the total CD45+ lymphocytes (left y-axis, coloured line) and the plasma viral loads shown as HIV+ RNA copies/ml plasma (right y-axis, black line). (b) Data as shown in (a), but for the entire cohort (n=7). (c) Data as shown in (b), but cumulative data presented as mean values for the entire cohort (n=7) with error bars showing s.e.m. (d) Percentage ILC1, ILC2 and ILC3 cells of total CD45+ lymphocytes shown for week 0 and week 6 after day of first HIV-1 positive test and compared to the HIV uninfected subjects (116). (e) ILC frequencies expressed as absolute ILC counts shown for week 0 and week 6 after day of first HIV-1 positive test and compared to the HIV uninfected subjects (n=116). (f) Data from the acutely HIV-1 infected PID 0398-271 subject as shown in (a) but for absolute CD4+ T-cell counts (pink line, right y-axis).

P-values by Dunn’s test for multiple comparisons. All samples are from ‘FRESH’ cohort(Ndhlovu et al., 2015).
Figure 3. ILCs are not reconstituted after successful treatment initiated in chronic infection. Twenty-four individuals were tested 3-monthly for presence of early detectable HIV-1 specific p24 antibodies and followed over 9 years with average treatment initiation starting at median values of 213 weeks after infection and sampled again 2 years later. (a) Median HIV-1 RNA copies/mL plasma before and after treatment with the absolute CD4$^+$ T-cell counts shown in (b). (c) Percentage of HLA-DR$^+$CD38$^+$ expression gated on CD4$^+$ T cells and CD8$^+$ T cells before and after ART treatment. (d) Absolute ILC counts comparing HIV-1 uninfected individuals (n=81) to unmatched HIV-1 infected individuals (n=22) with successful viral suppression after 2 yrs of treatment. (e) Absolute ILC1, ILC2 and ILC3 counts from matched HIV-1 infected individuals before and after ART start (n=22) with horizontal dotted line representing median absolute ILC counts for HIV-1 uninfected individuals. (f) ILC frequencies expressed as % of CD45$^+$ lymphocytes comparing HIV-1 uninfected individuals (n=84) to unmatched HIV-1 infected individuals with successful viral suppression after 2 yrs of treatment (n=22). (g) Data as in (e) but expressed as % ILCs of total CD45$^+$ lymphocytes. $P$ – values by the Wilcoxon matched-paired signed rank test, Mann-Whitney U test and with correlation coefficients shown as spearman rank $r$ – and $P$ – values.
Figure 4. ILCs are preserved by treatment initiation during early acute HIV-1 infection.

(a) Data from one acute infected subject (FRESH cohort) that was treated one day after detection of HIV-1 RNA in plasma (VL=2,900) as indicated by the black arrow and with ILC frequency shown as % of CD45 lymphocytes tracked throughout 400 days after HIV-1 infection (left y-axis) and with HIV-1 RNA copies/mL plasma (right y-axis). (b) Longitudinal data from 7 acutely HIV-1 infected subjects initiated on ART within one day of plasma HIV-1 detection and shown as % ILC of CD45 lymphocytes throughout acute infection. (c) Longitudinal mean values comparing 7 acutely infected individuals initiated on ART one day after HIV RNA detection (colored lines) to 7 acutely infected individuals not receiving ART (grey lines) throughout 11 weeks after HIV detection with error bars representing s.e.m. (d) ILC1, ILC2 and ILC3 frequencies for six acutely infected individuals receiving ART by day 1 of HIV detection ‘ART+’ individuals at >6 weeks after HIV detection compared to 9 treatment naïve acutely infected individuals from the same cohort 6 weeks into infection ‘no ART (6w)’. P - values by Dunn’s test for multiple comparisons.
Figure 5. ILCs from peripheral blood of chronic HIV-1 infected individuals are not apoptotic but display an activated phenotype and upregulation of Fas (CD95). (a) Activated caspase-3 measured by median fluorescence intensity (MFI) after log fold titration of camptothecin at 0, 8, 80 and 800 μM shown for ILC1, ILC2 and ILC3 with cumulative data for MFI values for caspase-3 comparing 20 HIV negative and 18 chronic HIV positive individuals for ILC1, ILC2 and ILC3 subsets. (b) One representative example of percentage Bcl-2 and caspase-3 negative ILC1, ILC2, ILC3 and NK (CD3-CD56-CD94+CD16+) cell subsets with cumulative data shown on the right as the percentage of Bcl-2-/casp-3- cells of ILC1, ILC2 and ILC3 subsets. (c) MFI expression for CD69 on ILC1, ILC2 and ILC3 cells comparing HIV negative (18) and chronic HIV positive subjects (n=21). (d) Fas (CD95) expression on ILC1, ILC2 and ILC3 gated cells overlayed by HIV-1 uninfected (grey) and HIV-1 infected (color) comparing 20 uninfected and 20 infected subjects (right). (e) Plasma IP-10 mean levels (left y-axis) shown for 14 acutely infected treatment naïve subjects sampled before infection (7-60 days prior to HIV detection plotted as day -7) followed 30 days into infection (left) and with 4 subjects treated 1 day after HIV-1 detection (right) and with plasma HIV RNA copies/ml levels shown on the right y-axis. (f) Same as for (e) but with data for IFNα2. Error bars represent s.e.m. values. P - values by Mann-Whitney U test and paired t-test.
Figure 6. ILCs show up-regulation of genes associated with apoptosis and cell death in early acute HIV-1 infection. RNA-Seq was performed on samples from two untreated and two early ART treated subjects (FRESH cohort) during the course of early acute HIV infection. (a) Sampling points and associated viral loads for each patient. NB Patient 0444-312 did not have a sample collected prior to peak viral load. (b) Heat map of activation z-scores for functionally enriched gene sets differentially expressed between initial viral detection and peak viremia. (c) Similar plot comparing enrichments between peak viremia and approximately 6 weeks after detection. Z-score was calculated using log fold change in expression values (see Methods).
Figure 7. Magnitude and phenotype of tissue resident ILCs identified using transcription factors within tonsil and gut tissue (a) Gating of live CD45+CD3- ILC1 (Lin-CD127+CD161+CRTH2+CD117-CD56+), ILC2 (Lin-CD127-CD161+CRTH2+), ILC3 (Lin-CD127+CD161+CRTH2+), NK (CD3-CD94-CD56+) and CD4+Th2 (CD3+CD4+CRTH2+) cells and overlayed for CD69, CD56 and NKp44 expression and stained for T-bet, Eomes, GATA-3, Helios, AHR and RORγt transcription factors. (b) Median fluorescence intensity (MFI) of CD69 and CD62L expression on ILC1, ILC2 and ILC3 subsets in tonsil and blood resident T cells with NKp44+/− ILC3 subsets in tonsils (NKp44 not expressed in blood cells). (c) Frequency of CD4+ T cells shown as % of CD45+ cells within tonsil cells obtained from 12 HIV uninfected and 10 HIV infected subjects. (d) Comparing ILC1, ILC2 and ILC3 subsets expressed as % of CD45+ lymphocytes in HIV infected (n=12) and uninfected individuals (n=12). (e) ILC3 phenotype distribution of CD56 and NKp44 positive and negative subsets with FACS plot showing one representative example and pie charts showing data for a total of 21 subjects. (f) Gut tissue resident live CD45+CD3-CD4+ lymphocytes gated as in (a) with ILC3 cells overlayed on Lin+ (CD45+CD3-Lin+) shown for CCR6 and CD69 and shown for RORγt, Helios and AHR transcription factors for Gut Lin+, CD4+ T, NK and ILC3 cells. (g) Cumulative data for ILC1, ILC2 and ILC3 frequencies expressed as % of CD45+ lymphocytes from gut, tonsil and blood (left) with ILC1, ILC2 and ILC3 frequencies shown for n=39 HIV-1 uninfected and n=9 HIV infected subjects (middle) and comparing ILC3 NKp44/CD56 phenotype expression from HIV-1 uninfected and infected subjects (right). (h) Intracellular cytokine staining after media or PMA/Ionomycin stimulation for 5 hrs shown for IL2, TNFα, IL22 and GM-CSF production in NKp44+ ILC3 gated cells and with cumulative data shown in (i) obtained from HIV-1 infected (n=9) and uninfected individuals (n=14). P-values by the Wilcoxon matched-paired signed rank test and Mann-Whitney U test with horizontal bars representing median values.
Figure 1

a) Flow cytometry plots showing sorting of different cell populations.

b) Heatmap of expression levels of various proteins.

c) Heatmaps showing changes in gene expression before and after stimulation.

d) Heatmaps illustrating changes in gene expression across different cell types.

e) Box plot showing variation in expression levels.

f) Heatmaps showing changes in gene expression before and after stimulation.

g) Heatmaps illustrating changes in gene expression across different cell types.

h) Heatmaps showing expression levels of different proteins.

i) Bar graph showing changes in expression levels of specific proteins.
Figure 2

(a) % ILC1 of CD45

(b) % ILC2 of CD45

(c) % ILC3 of CD45

(d) HIV+ RNA test

(e) HIV+ RNA test

(f) HIV+ RNA test
Figure 3

(a) Viral load (RNA copies/mL plasma) with ART - ART+ comparison, showing a significant decrease in viral load with ART treatment. 

(b) CD4 count (cells/μL) with ART - ART+ comparison, indicating an increase in CD4 count with ART treatment. 

(c) % HLA-DR+CD38+ of CD4 cells with ART - ART+ comparison, showing an increase in activated CD4 cells with ART treatment. 

(d) ILC count (cells/mL) with HIV - HIV+ and HIV ART - HIV ART+ comparison, showing a significant increase in ILC counts with ART treatment. 

(e) ILC1 count and ILC2 count (cells/mL) with ART - ART+ comparison, showing a significant increase in ILC1 and ILC2 counts with ART treatment. 

(f) % ILC of CD4+ cells with HIV - HIV+ and HIV ART - HIV ART+ comparison, showing a significant increase in ILC of CD4+ cells with ART treatment. 

(g) % ILC cells of CD45+ with ART - ART+ comparison, showing a significant increase in ILC cells of CD45+ with ART treatment.
Figure 4

(a) % ILC1 of CD45
(b) % ILC2 of CD45
(c) % ILC3 of CD45
(d) Viral load (RNA copies/ml plasma)
Figure 5

a) 

- ILC1, ILC2, ILC3
- Cells vs. Casp-3
- MFI CD69
- P < 0.0001

b) 

- ILC1, ILC2, ILC3, NK
- Bcl-2 vs. Casp-3
- % Casp-3/Bcl-2
- % CD69 of ILCs

P < 0.0001

P = 0.002


c) 

- ILC1, ILC2, ILC3
- CD69
- MFI CD69

P < 0.0001

P = 0.002


d) 

- ILC1, ILC2, ILC3
- CD95
- % CD95 of ILCs

P < 0.0001

P = 0.002


e) 

- Days post first HIV+ RNA test
- IP-10 (pg/ml)
- Viral load (RNA copies/ml plasma)
- n=14 (no ART)

- Days post first HIV+ RNA test
- IP-10 (pg/ml)
- Viral load (RNA copies/ml plasma)
- n=4 (early ART)

f) 

- Days post first HIV+ RNA test
- IFNα (pg/ml)
- Viral load (RNA copies/ml plasma)
- n=12 (no ART)

- Days post first HIV+ RNA test
- IFNα (pg/ml)
- Viral load (RNA copies/ml plasma)
- n=4 (early ART)
Figure 6

<table>
<thead>
<tr>
<th>Patient</th>
<th>VL Detection</th>
<th>VL Peak Viremia</th>
<th>6 Weeks</th>
<th>VL 6 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection (Days)</td>
<td>Peak Viremia (Days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0450-318</td>
<td>1</td>
<td>7</td>
<td>38</td>
<td>790,000</td>
</tr>
<tr>
<td>0387-272</td>
<td>1</td>
<td>7</td>
<td>49</td>
<td>2,300</td>
</tr>
<tr>
<td>0629-453 (ART)</td>
<td>1</td>
<td>6</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>0444-312 (ART)</td>
<td>--</td>
<td>2</td>
<td>41</td>
<td>20</td>
</tr>
</tbody>
</table>

Patient Detection (Days)

VL Detection

Viremia Peak (Days)

VL Peak Viremia (Days)

6 Weeks (Days)

VL 6 Weeks

---

**Figure 6**

**a**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Detection (Days)</th>
<th>VL Detection</th>
<th>VL Peak Viremia</th>
<th>6 Weeks</th>
<th>VL 6 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0450-318</td>
<td>1</td>
<td>160,000</td>
<td>1,400,000</td>
<td>38</td>
<td>790,000</td>
</tr>
<tr>
<td>0387-272</td>
<td>1</td>
<td>87,000</td>
<td>100,000,000</td>
<td>49</td>
<td>2,300</td>
</tr>
<tr>
<td>0629-453 (ART)</td>
<td>1</td>
<td>2,900</td>
<td>3,400</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>0444-312 (ART)</td>
<td>--</td>
<td>--</td>
<td>24,000</td>
<td>41</td>
<td>20</td>
</tr>
</tbody>
</table>

**b**

**c**
Figure S1. Phenotype and selected transcriptional gene expression of ILC1, ILC2 and ILC3 subsets in blood. Pie charts showing distributions of 8 different ILC1, ILC2 and ILC3 subpopulations based on differential expression of CD25, CD56, CD117 and color-coded as indicated below x-axis and with y-axis showing the relative frequency of ILC1, ILC2 and ILC3 gated cells (see Fig 1a). Data from n=43 HIV uninfected individuals. Selected canonical ILC gene expression (CD127, cKit/CD117, KLRG1 and IL1R) from 9 HIV-1 uninfected individuals obtained by RNAseq after ILC2, ILC3 and CD4+ T cell sorted cells from blood.
Viral load (RNA copies/ml plasma)

- **ILC1** count (cells/ml)
  - **Viral load**
  - **CD4 count**
  - \( P = 0.02 \)
  - \( r = -0.27 \)

- **ILC2** count (cells/ml)
  - **Viral load**
  - **CD4 count**
  - \( P = 0.02 \)
  - \( r = -0.23 \)

- **ILC3** count (cells/ml)
  - **Viral load**
  - **CD4 count**
  - \( P = 0.07 \)
  - \( r = -0.18 \)

- **ILC1 % of CD45**
  - **Viral load**
  - **CD4 count**
  - \( P = 0.007 \)
  - \( r = -0.32 \)

- **ILC2 % of CD45**
  - **Viral load**
  - **CD4 count**
  - \( P = 0.002 \)
  - \( r = -0.30 \)

- **ILC3 % of CD45**
  - **Viral load**
  - **CD4 count**
  - \( P = 0.007 \)
  - \( r = -0.26 \)
Figure S2. ILCs are depleted in HIV-1 infection and correlates negatively with HIV RNA copies/mL plasma. (a) Correlation of ILC counts to the viral load set-points for HIV-1 infected individuals. (b) Correlation of ILC frequencies expressed as % of total CD45+ lymphocytes to the viral load set-points. (c) Correlation of absolute CD4+ T cell count and HIV-1 viral load setpoints. P and R values by correlation coefficients and shown as spearman rank r – and P – values.
Fig S3

Days post first HIV RNA test

ILC1 count (cells/ml)

Viral load (RNA copies/ml plasma)

n = 7

Days post first HIV RNA test

ILC2 count (cells/ml)

Viral load (RNA copies/ml plasma)

n = 7

Days post first HIV RNA test

ILC3 count (cells/ml)

Viral load (RNA copies/ml plasma)

n = 7
Figure S3. ILCs are depleted during acute HIV-1 infection. (a) Data from the acutely HIV-1 infected PID 0398-271 subject followed longitudinally over 10 time-points from day 1 to day 249 from the day of first HIV⁺ RNA test showing the absolute ILC1, ILC2 and ILC3 counts (left y-axis) with the plasma viral load shown as HIV⁺ RNA copies/mL plasma (right y-axis, black line). (b) Data as in (a), but for the entire cohort (n=7). (c) Absolute ILC counts (cells/ml of blood) presented as cumulative data with median values shown for the entire cohort (n=7).
Fig S4

(a) Percentage ILC1 of CD45+ I-FABP Plasma (pg/mL) with respect to days post first HIV+ RNA test.

(b) Percentage ILC2 of CD45+ I-FABP Plasma (pg/mL) with respect to weeks post first HIV+ RNA test.

(c) Percentage ILC3 of CD45+ I-FABP Plasma (pg/mL) with respect to weeks post first HIV+ RNA test.

(d) Viral load (RNA cps/ml plasma) with respect to weeks post first HIV+ RNA test.
Figure S4. Depletion of ILCs in early acute HIV-1 infection is associated with elevated levels of gut epithelial break down. (a) Absolute I-FABP levels for the acutely HIV-1 infected PID 0398-271 subject shown longitudinally over 8 time-points from day 1 to day 77 from the day of first HIV+ RNA test with percentages of ILC1, ILC2 and ILC3 of total CD45+ lymphocytes (left y-axis) and with absolute plasma I-FABP levels (right y-axis, purple. (b) Cumulative median Plasma I-FABP levels (% of the maximum value) for all acutely infected subjects, measured at the last time-point before HIV-1 infection and 7 subsequent time-points over acute infection into chronic infection (week 24) (left y-axis), plotted against the median value for ILCs over the same time period, plotted as % of week 0 ILC frequency for each ILC subset (right y-axis). (c) Plasma Intestinal Fatty Acid Binding protein (I-FABP) levels for each subject expressed as percentage of maximum value detected during acute infection compared for week 0, 2 and 3 for matched acutely infected subjects (n=14). (d) Data as in (a) but showing the median values of the maximum I-FABP levels from n=14 acutely infected subjects in comparison to HIV-1 RNA copies/ml plasma. P - values by paired t-test.
Fig S5

(a) HIV infection, ART start, ART+ weeks post infection

(b) % change CD4 absolute count (cells/µL) over median 2 yrs ART
Figure S5. Characterization of the CAPRISA002 longitudinal treatment cohort.  
(a) Twenty-four individuals were tested 3-monthly for presence of early detectable HIV-1 specific p24 antibodies and followed over 9 years with average treatment initiation starting at median values (vertical bars) of 213 weeks after infection and sampled again at a median of 2 years into effective ART treatment at week 308 (top). Sampling for each subject is shown for the last sample available before ART initiation (median week 213 after HIV-1 infection) and again 2 years into treatment (median week 308 after HIV-1 infection) (bottom). (b) Shows the percentage change in absolute CD4⁺ T cell counts comparing sampling at week 213 and week 308. Thus, the median absolute reconstitution after 2 years of ART is a doubling of CD4⁺ T cell counts compared to week 213 at ART initiation.
Fig S6

- ILC3 MFI α4β7

HIV- vs HIV+

P = 0.001

- P value indicating significant difference between HIV- and HIV+ groups.
Figure S6. Relative expression levels of α4β7 expression on ILC3 gated blood cells from HIV-1 uninfected and infected subjects. *P* value by Mann-Whitney U-test.
Fig S7

IL-7 pg/ml plasma

ILC1 count (cells/ml)

$P = 0.08$

$r = 0.31$

ILC2 count (cells/ml)

$P = 0.06$

$r = 0.30$

ILC3 count (cells/ml)

$P = 0.66$

$r = 0.07$
Figure S7. Correlation of plasma IL7 levels (pg/ml plasma) and absolute ILC counts from chronic HIV-1 infected subjects. $P$ and $R$ values by spearman rank correlation.
Figure S8. RNA-Seq of ILCs in early acute HIV infection reveals dynamic changes in gene expression. (a) Viral load (black), ILC2 (red), and ILC3 (blue) population data for each patient. Horizontal lines denote time points used for detection, peak viremia, and 6 weeks after detection, respectively. (b) Heat map of $P$-values for functionally enriched gene sets differentially expressed between initial viral detection and peak viremia (Fig. 6b) and (c) between peak viremia and approximately 6 weeks after detection (Fig. 6c). (d) Heat map of activation z-scores for putative upstream regulators and signaling pathways whose targets or constituents, respectively, are differentially expressed between initial viral detection and peak viremia and (e) between peak viremia and approximately 6 weeks after detection.
Fig S9

% ILC1 cells of CD45+

10^4 10^3 10^2 10^1

ILC1 Tonsil   ILC1 Blood

P = 0.002

% ILC2 cells of CD45+

10^4 10^3 10^2 10^1

ILC2 Tonsil   ILC2 Blood

P = 0.003

% ILC3 cells of CD45+

10^4 10^3 10^2 10^1

ILC3 Tonsil   ILC3 Blood

P < 0.0001
Figure S9. Comparison of ILC1, ILC2 and ILC3 frequencies obtained from matched tonsil and blood gated cells. $P$ values by paired t-test.
Fig S10
Figure S10. Intracellular cytokine production of IL2, TNFα, IL22 and GM-CSF comparing NKp44- and NKp44+ ILC3 gated tonsil cells. P values by paired t-test.
Click here to access/download

Supplemental Movies & Spreadsheets
Table S1 ILC2vsCD4_DE_table.xlsx
Click here to access/download
Supplemental Movies & Spreadsheets
Table S3 ILC3vsCD4_DE_table.xlsx
Click here to access/download
Supplemental Movies & Spreadsheets
Table S4 HIV Detection vs Peak VL.xls
Click here to access/download

**Supplemental Movies & Spreadsheets**

Table S5 - Peak VL Vs Chronic VL.xls