ARTICLE

Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection

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The pathway causing CD4 T-cell death in HIV-infected hosts remains poorly understood although apoptosis has been proposed as a key mechanism. We now show that caspase-3-mediated apoptosis accounts for the death of only a small fraction of CD4 T cells corresponding to those that are both activated and productively infected. The remaining over 95% of quiescent lymphoid CD4 T cells die by caspase-1-mediated pyroptosis triggered by abortive viral infection. Pyroptosis corresponds to an intensely inflammatory form of programmed cell death in which cytoplasmic contents and pro-inflammatory cytokines, including IL-1β, are released. This death pathway thus links the two signature events in HIV infection—CD4 T-cell depletion and chronic inflammation—and creates a pathogenic vicious cycle in which dying CD4 T cells release inflammatory signals that attract more cells to die. This cycle can be broken by caspase 1 inhibitors shown to be safe in humans, raising the possibility of a new class of 'anti-AIDS' therapeutics targeting the host rather than the virus.

The progressive loss of CD4 T cells in HIV-infected individuals lies at the root of AIDS. Despite more than three decades of study, the precise mechanism(s) underlying the demise of CD4 T cells during HIV infection remains poorly understood and has been highlighted as one of the key questions in HIV research[1]. In almost all cases, loss of CD4 T cells has been linked to apoptosis, both in *in vivo*[2–6] and *ex vivo*[5,7,8] studies. However, various features of apoptotic cell death including maturation of executioner caspase 3, DNA fragmentation and plasma membrane permabilization are commonly shared with other programmed cell death pathways[9]. Importantly, most studies have focused on the death of productively infected cells circulating in peripheral blood[10]. Very little is known about the death of 'bystander' CD4 T cells in tissues that are refractory to productive HIV infection. However, these resting CD4 T lymphocytes represent the main cellular targets encountered by HIV in lymphoid tissues[11–13].

To investigate how CD4 T cells die during HIV infection, we took advantage of an *ex vivo* human lymphoid aggregate culture (HLAC) system formed with fresh human tonsil or spleen tissues[13]. HLACs can be infected with a small number of viral particles in the absence of artificial mitogens, allowing analysis of HIV cytopathicity in a natural and preserved lymphoid microenvironment[12]. Infection of these cultures with HIV-1 produces extensive loss of CD4 T cells, but over 95% of the dying cells are abortively infected with HIV, reflecting their non-permissive, quiescent state. The HIV life cycle is attenuated during the chain elongation phase of reverse transcription, giving rise to incomplete cytosolic viral DNA transcripts. Cell death is ultimately caused by a cellular innate immune response elicited by these cytosolic DNA intermediates[11]. This response is associated with production of type I interferon and activation of both caspase 3 and caspase 1. Caspase 3 activation leads to apoptosis without inflammation[14], whereas caspase 1 activation can trigger pyroptosis, a highly inflammatory form of programmed cell death in which dying cells release their cytoplasmic contents, including inflammatory cytokines, into the extracellular space[9,15]. The consequences of apoptosis versus pyroptosis may affect HIV pathogenesis by influencing the state of inflammation and immune activation, but their relative contribution to CD4 T-cell death in lymphoid tissues had remained unexplored.
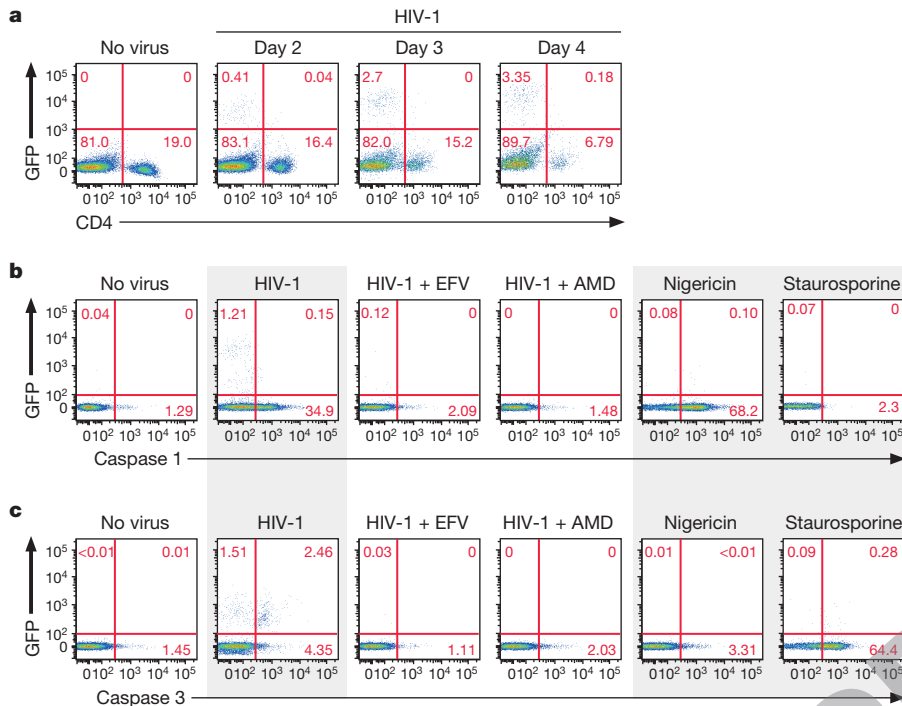
Host permissivity determines the form of cell death

Previous reports have implicated caspase 3 activation and apoptosis in most instances of cell death caused by HIV-1 (refs 3, 7, 8). To explore the role of caspase 1 in dying HIV-infected CD4 T cells, HLACs formed with freshly dissected human tonsillar tissues were infected with a GFP reporter virus (NLENG1), prepared from the X4-tropic NL4-3 strain of HIV-1. This reporter produces fully replication-competent viruses. An internal ribosome entry site (IRES) upstream of the *nef* gene preserves Nef expression and supports long terminal repeat (LTR)-driven GFP expression[16], allowing simultaneous quantification of HIV-1 infection and caspase activation in CD4 T cells. NL4-3 was selected because tonsillar tissue contains a high percentage of CD4 T cells that express CXCR4 (90–100%). Consistent with our previous report[11], infection with HIV-1 produced extensive depletion of 'bystander' non-productively infected CD4 T cells. No more than 4% of the CD4 T cells were productively infected with HIV-1, but most of the remaining CD4 T cells underwent abortive infection and ultimately died after four days in culture (Fig. 1a).

To determine the distribution of active caspase 1 and caspase 3 in the dying CD4 T cells, we used fluorescently labelled inhibitor of caspases (FLICA) probes with sequences targeted by specific activated caspases[17]. Interestingly, a large fraction of non-productively infected CD4 T cells exhibited activation of caspase 1. Conversely, essentially no caspase 1 activity was detected in the productively infected cells (Fig. 1b). Caspase 3 activity was markedly less abundant, and mainly confined to the productively infected subset of cells (Fig. 1c). Treatment, with efavirenz (a non-nucleoside reverse transcriptase inhibitor, NNRTI) or AMD3100 (an inhibitor of CXCR4-dependent HIV entry) prevented activation of both caspases. Infection with the primary, dual-tropic 89.6 HIV isolate[18] produced similar results (Extended Data Fig. 1). The two FLICA probes appeared to bind their respective caspases with reasonable specificity based on exclusive caspase 3 staining in cells treated with staurosporine, a protein kinase inhibitor known to induce apoptosis versus robust

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Figure 1 | Host permissivity determines the CD4 T-cell death pathway employed following HIV infection. a, Kinetics of spreading viral infection versus depletion of CD4 T cells after infection of HLACs with a replication-competent HIV reporter virus encoding GFP. The relative proportion of CD8 T cells was not altered (not shown). Consistent with our previous report, HIV-infected HLACs contain a small number of productively infected cells, whereas almost all of the dying cells are abortively infected[11]. **b**, Abortively infected CD4 T cells exclusively activate caspase 1. Nigericin induces abundant caspase 1 activation in uninfected cells. **c**, Productively infected CD4 T cells activate caspase 3 but not caspase 1. The samples (**b**, **c**) represent cells from the same infected tonsil culture. Efavirenz (EFV) and AMD3100 were added to the indicated cultures before HIV infection. These data are representative of four independent experiments performed with tonsil cells isolated from four different donors.

caspase 1 staining in cells treated with the cationic ionophore nigericin which promotes NLRP3 inflammasome assembly, caspase 1 activation and pyroptosis[19].

Healthy lymphoid CD4 T cells express pro-IL-1β

IL-1β activity is controlled at several levels including pro-IL-1β expression, processing and secretion. Pro-inflammatory stimuli induce expression of pro-IL-1β whereas processing and release are regulated by caspase 1 activation in inflammasomes[20]. The signals required for caspase 1 activation and release of IL-1β differ between immune cells. In circulating human blood monocytes, caspase 1 is constitutively active[21]. Stimulation of these cells with lipopolysaccharide (LPS) promotes pro-IL-1β expression leading to the rapid release of bioactive IL-1β. In contrast, macrophages and dendritic cells require a second signal to activate caspase 1 (ref. 22). Nigericin can function as this second signal activating caspase 1 in LPS-primed macrophages[19,23]. Surprisingly, nigericin alone proved sufficient to activate caspase 1 in uninfected lymphoid CD4 T cells (Fig. 1b) and to promote the release of the 17-kDa bioactive form of IL-1β (Fig. 2a). Treatment with monensin, a different monovalent cationic ionophore, or A23187, a calcium ionophore, did not promote mature IL-1β release[23,24]. Maturation and secretion of the bioactive form of IL-1β was inhibited by Z-VAD-FMK (a pan-caspase inhibitor), Z-WEHD-FMK or Z-YVAD-FMK (two independent caspase 1 inhibitors, which also block other inflammatory caspases—caspase 4 and caspase 5), but not by Z-FA-FMK (a negative control for caspase inhibitors) indicating that caspase 1 activation was required.

Pro-IL-1β expression in human tonsil and spleen HLACs was examined next. Western blotting analysis revealed large amounts of intracellular pro-IL-1β in both untreated tonsil and spleen HLACs (Fig. 2b). Removal of dead cells by Ficoll-Hypaque density centrifugation resulted in an even higher intracellular pro-IL-1β signal, indicating that these normal lymphoid tissues constitutively express high levels of pro-IL-1β. The presence of pro-IL-1β in spleen indicated that expression in tonsil is not solely caused by infection (tonsillitis). Fractionation of the lymphocytes present in these HLACs revealed high levels of intracellular pro-IL-1β in isolated CD4 T cells, but not in CD8 T-cell or B-cell populations.

Most tonsillar CD4 T cells express CXCR4, but only around 5% of these cells also express CCR5 (refs 12, 25). When CCR5-positive and CCR5-negative lymphoid CD4 T-cell subsets were isolated and studied,

the CCR5-expressing cells displayed much higher levels of intracellular pro-IL-1β (Fig. 2b). The CCR5-expressing CD4 T cells also released notably more 17 kDa IL-1β into the supernatant after infection with HIV-1 (Fig. 2c). These results suggest that most of the mature form of IL-1β is released by the small population of CCR5-expressing CD4 T cells. The resident CCR5-expressing cells in lymphoid tissues are primarily memory CD4 T cells, which might be more permissive for productive HIV infection[26]. However, the activation status of these cells varied (Fig. 2d). Two-thirds exhibited a memory phenotype as determined by surface expression of CD45RO, but only a small fraction of these cells were permissive to productive infection with either X4-tropic or R5-tropic HIV-1 strains (Extended Data Fig. 2). Notably, lymphoid CCR5-expressing CD4 T cells also express CXCR4 and thus can be targeted by either X4 or R5-tropic HIV-1 strains[12,27,28]. Memory T cells continually recirculate within lymphoid tissues scanning for presentation of their cognate antigen[29–31]. It seems likely that many of these cells have returned to a sufficient state of quiescence that they are susceptible to abortive HIV infection and thus could contribute importantly to chronic inflammation through the release of bioactive IL-1β .

CD4 T-cell death by HIV-1 is mediated by pyroptosis

Caspase 1 is a pro-inflammatory caspase whose catalytic activity is tightly regulated by signal-dependent auto-activation within inflammasomes[20]. Inflammasome-dependent caspase 1 activity results in a highly inflammatory form of cell death known as pyroptosis, primarily described in myeloid cells infected with intracellular bacterial pathogens[9,15,32]. Pyroptosis is caspase 1 dependent by definition and occurs independently of other pro-apoptotic caspases[9,32]. Based on our finding that caspase 1 is activated in lymphoid CD4 T cells following abortive HIV infection, we investigated whether pyroptosis is triggered within these cells.

Fresh HLACs were infected with HIV-1 and cultured for 12 h to initiate viral spread and then treated with various caspase inhibitors or controls. Extensive and selective depletion of CD4 T cells occurred in untreated, HIV-infected cultures after 3 days. However, treatment with either pan-caspase or caspase 1 inhibitors prevented the depletion of CD4 T cells as efficiently as the viral inhibitors efavirenz and AMD3100 (Fig. 3a). Inhibitors of caspase 3 or caspase 6 and the control compound did not prevent CD4 T-cell depletion. Necrostatin-1, a RIP1 inhibitor,
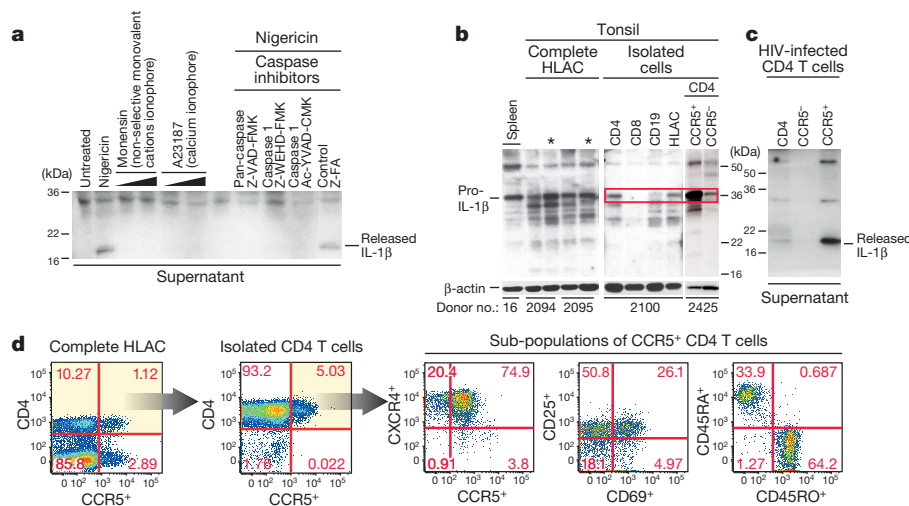
Figure 2 | Lymphoid CD4 T cells are primed to mount an inflammatory response and constitutively express high levels of pro-IL-1β. **a**, A secondary inflammatory stimulus by nigericin induces lymphoid CD4 T cells to process and release bioactive IL-1β. Supernatants from cell cultures were filtered to remove all remaining cells and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) immunoblotting analyses for bioactive 17-kDa IL-1β. **b**, High levels of constitutive pro-IL-1β are selectively expressed in lymphoid CD4 T cells. Levels of intracellular pro-IL-1β were assessed in HLACs from fresh tonsils or spleen tissue from different donors. Asterisks indicate samples in which dead cells were removed. CD4 T cells were positively isolated from HLAC. Cells were lysed and analysed for pro-IL-1β expression. **c**, Nearly all bioactive IL-1β produced by HIV-infected lymphoid CD4 T cells is released from CCR5-expressing cells. Indicated CD4 T-cell populations were isolated from HLAC and infected with HIV-1. Supernatants of cultures were filtered and analysed for bioactive 17-kDa IL-1β. **d**, HLACs were characterized for expression of memory and activation markers by flow cytometry. The majority of CCR5-expressing CD4 T lymphocytes exhibit a memory phenotype. All CCR5-expressing CD4 T cells co-express the CXCR4 receptor.

did not inhibit CD4 T-cell depletion (Extended Data Fig. 3a, b), indicating that cell death does not reflect necroptosis. Analysis of spleen cells yielded similar results (Extended Data Fig. 3c). Inhibiting type-I interferon signalling with neutralizing antibodies directed against IFNα/β receptor did not prevent CD4 T-cell death (Extended Data Fig. 4), indicating that this antiviral response is not critical for the innate-immune-mediated onset of programmed cell death. Distinct from apoptosis, pyroptosis features cellular swelling, plasma membrane rupture and release of intracellular content into the extracellular milieu[15], including cytosolic enzymes like lactate dehydrogenase (LDH)[33]. LDH release was readily detected after HIV infection (Fig. 3b), and was blocked by two antiviral inhibitors, efavirenz and AMD3100 and by a caspase 1 inhibitor, but not by a caspase 3 inhibitor. Thus, the form of cell death associated with abortive HIV infection appears to involve caspase 1 activation and the release of cytoplasmic contents. Caspase 1 inhibitors also prevented death of CCR5-expressing CD4 T cells in HLACs infected with a CCR5-dependent strain of HIV-1 (Fig. 3c). Inhibition of cell death by the caspase 1 inhibitor was as effective as the CCR5 receptor antagonist TAK779, suggesting that most CCR5-expressing CD4 T cells in the culture are dying by caspase-1-mediated pyroptosis. These findings are consistent with the large amounts of bioactive IL-1β released by these cells after HIV-1 infection.

Because caspase inhibitors are not exquisitely specific, we designed short hairpin (shRNA) vectors to silence the expression of caspase 1, the ASC (PYCARD) adaptor, which recruits pro-caspase 1 to inflammasome complexes[20], caspase 3 and NLRP3 (Extended Data Fig. 5). For these experiments, a third generation shRNA-encoding lentiviral vector (shRNA LV) pSico[34], bearing an EF1α:mCherry reporter expression cassette was used. To relieve the resistance of lymphoid CD4 T cells to shRNA LV infection, target cells were initially challenged with lentiviral particles harbouring Vpx (Vpx-VLPs), which induce proteasomal degradation of SAMHD1 in non-permissive human resting CD4 T cells[35]. Infections with Vpx-VLPs did not lead to activation of resting CD4 T cells, as measured by surface expression of the CD69 and CD25 activation markers (not shown). The shRNA LV particles and Vpx-VLPs were pseudotyped with a CXCR4-tropic Env of HIV-1, which supports efficient fusion to quiescent CD4 T lymphocytes[36]. Under these conditions, infection with shRNA LVs markedly suppressed expression of a variety of targeted genes whereas the scrambled shRNA LV control

did not (Fig. 3d). We next investigated whether any of these shRNA LVs inhibited pyroptosis induced by nigericin. Nigericin induced massive pyroptosis in mCherry positive CD4 T cells infected with scrambled and caspase 3 shRNA LV particles, but this response was blocked by the caspase 1, ASC or NLRP3 shRNA LV particles (Fig. 3e). Next, the effect of these shRNAs on CD4 T-cell death elicited by HIV-1 was examined. HIV-1 infection caused extensive death of mCherry-positive CD4 T cells expressing shRNAs against scramble, caspase 3 and NLRP3, but not caspase 1 or ASC. Thus, cell death occurring during abortive HIV infection appears to be mediated through caspase 1 dependent pyroptosis involving an inflammasome that contains ASC but lacks NLRP3.

HIV-1 stimulates caspase 1 to secrete IL-1β

To independently confirm that abortive HIV-1 infection leads to the activation of caspase 1, we investigated the appearance of the active p10 subunit of caspase 1. As controls for pyroptosis and apoptosis, uninfected cells were treated with either nigericin or staurosporine, respectively. An active 10 kDa subunit of caspase 1 (p10) was detected in the lysates of HIV-infected cultures as well as in nigericin-treated cells, and in blood monocytes in which caspase 1 is constitutively active[21]. Treatments with viral or caspase 1, but not caspase 3, inhibitors prevented caspase 1 cleavage (Fig. 3f). These findings confirm the induction of caspase 1 in quiescent CD4 T cells following abortive infection with HIV-1. Caspase 3 activation in these infected cultures was markedly less abundant (Extended Data Fig. 6). To test whether caspase 1 activation leads to proteolytic maturation of pro-IL-1β, we used various caspase inhibitors and analysed the culture media for the mature 17-kDa form of IL-1β. Interestingly, release of mature IL-1β was completely inhibited by a pan-caspase inhibitor and by two different caspase 1 inhibitors (Fig. 3g). Inhibitors of apoptotic caspases, caspase 3, caspase 6 or caspase 8, or necrostatin did not interrupt this release. Similar findings were observed using a quantitative IL-1β enzyme-linked immunosorbent assay (ELISA) (Extended Data Fig. 7a). Thus, caspase 1 activation is specifically required for the release of bioactive IL-1β in lymphoid CD4 T cells infected with HIV-1. In accord with shRNA analyses, treatment with four separate NLRP3 inhibitors did not prevent release of bioactive IL-1β by HIV-1 (Fig. 3g), nor CD4 T-cell death by HIV-1 (Extended Data Fig. 7b, c).
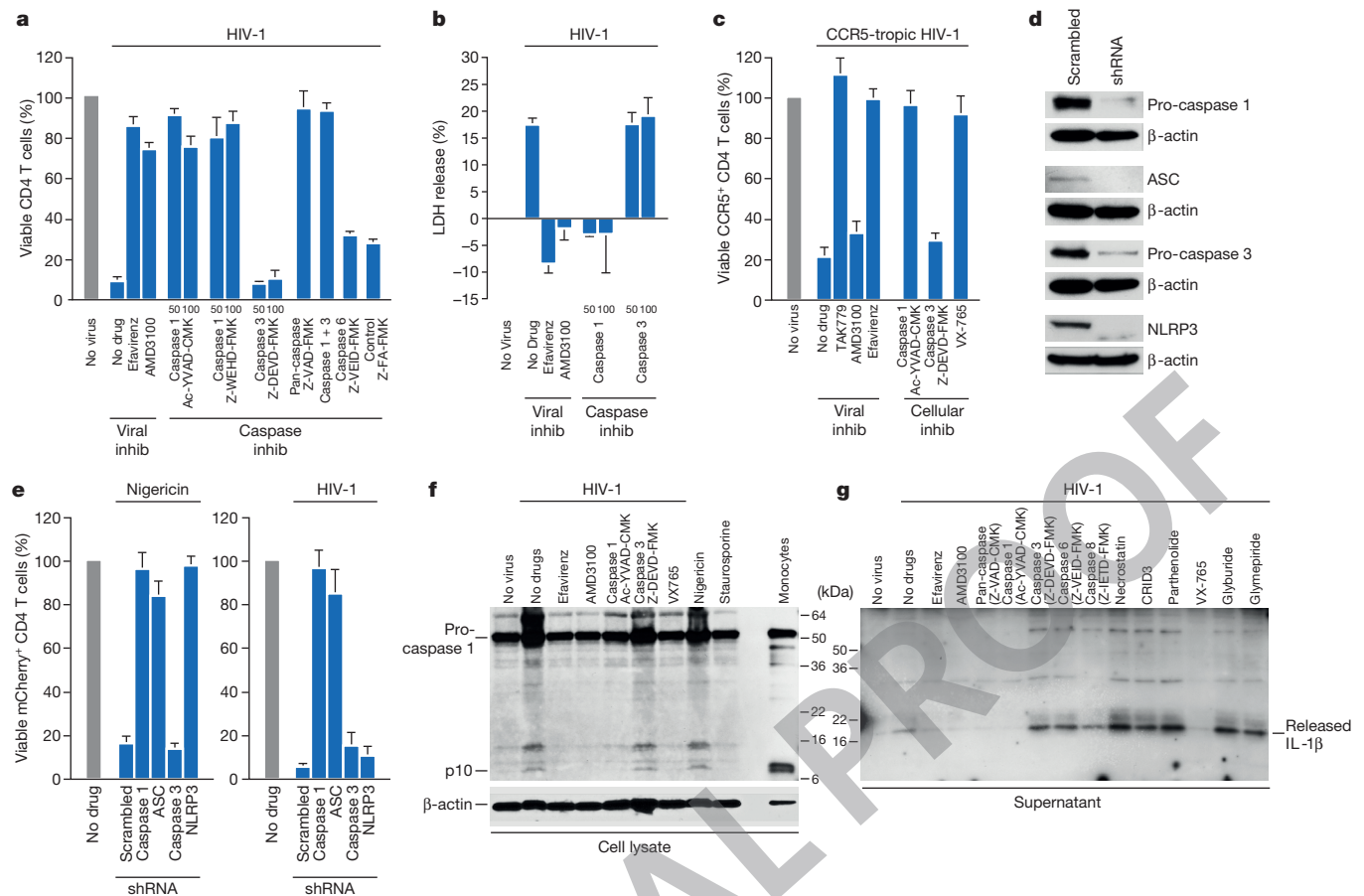
Figure 3 | Death of HIV-infected lymphoid CD4 T cells and release of bioactive IL-1β are controlled by caspase 1. a, b, Caspase 1 inhibitors are sufficient to prevent CD4 T-cell death in HIV-infected HLACs. Viable CD4 T cells were counted by flow cytometry and supernatants were analysed for levels of cytoplasmic LDH enzyme release[33]. **c**, Infection with CCR5-dependent HIV-1 induces pyroptosis of lymphoid CD4 T cells. Death of CCR5-expressing CD4 T cells is prevented by caspase 1 inhibitors and TAK779, but not by the CXCR4 antagonist, AMD3100. Due to the small number of target CCR5-expressing cells, this experiment was performed by overlaying tonsil cells on a monolayer of 293T cells that had been transfected with an R5-tropic proviral HIV-1 clone, as previously described[11]. The co-culture conditions for the R5 virus experiment induced no activation of the overlaid cells. **d**, Efficient

repression of target genes by shRNA-coding lentiviral vectors. **e**, shRNA LV designed to silence either caspase 1 or ASC, key components of the pyroptotic pathway, protect lymphoid CD4 T cells from death by nigericin treatment or HIV-1 infection. To specifically assess non-productively infected cells, cultures were treated with 3′-azido-3′-deoxythymidine (AZT) before infections with HIV-1. **f**, Caspase 1 cleavage in HIV-infected CD4 T cells is blocked by specific caspase 1 inhibitors. **g**, Inhibitors of caspase 1, but not NLRP3, prevent release of bioactive IL-1β from HIV-infected lymphoid CD4 T cells. Error bars represent s.e.m from at least three independent experiments using tonsil cells from at least three different donors. Protein analyses represent results from three independent experiments using tonsillar CD4 T cells from three different donors.

## In vivo evidence for HIV–mediated pyroptosis

To extend our *ex vivo* HLAC findings, we next examined fresh lymph node tissue obtained from a consenting untreated subject infected with R5-tropic HIV and displaying a high viral load and a low CD4 T-cell count. *In situ* immunostaining revealed a distinct zone of HIV p24 Gag expression between the mantle zone and germinal centres, where activated CD4 T and B cells proliferate (Ki-67) and interact in the follicles (Fig. 4). Conversely, staining for caspase 1 revealed abundant activity in the surrounding paracortical zone (CD3) comprised primarily of resting CD4 T cells. Staining of uninfected tonsil or spleen (not shown) tissues revealed no such positive signals (Extended Data Fig. 8). Because this antibody reacts with both the active p20 component of caspase 1 and pro-caspase 1, we cannot completely exclude the possibility that abortive HIV-1 infection produced localized increase in pro-caspase 1 expression. However, large amounts of IL-1β were also detected in the paracortical zone, particularly in the extracellular space between the T cells, as well as the cell death marker annexin V. In sharp contrast, active caspase 3 staining was limited to the areas in the germinal centre where HIV-1 p24 Gag expression was detected. These findings strongly agree with the HLAC results (Fig. 1b) indicating that caspase 3 activity occurs in a set of productively infected cells, anatomically separated from most of

the resting CD4 T cells undergoing abortive infection, caspase 1 activation, IL-1β processing and pyroptosis.

A clinically safe drug blocks pyroptosis by HIV–1

Identifying pyroptosis as the predominant mechanism mediating CD4 T-cell depletion during HIV infection provides novel targets, such as caspase 1, for potential therapeutic intervention. The role of caspase 1 in the chronic inflammatory response has attracted therapeutic interest[37]. VX-765 is a caspase 1 inhibitor that has been tested in chronic epilepsy and psoriasis (Extended Data Fig. 9a)[38–41], and found in a phase IIa trial (http://clinicaltrials.gov/ct2/show/NCT01048255) to be safe and well tolerated over the six weeks length of the trial[42]. In our studies, VX-765 inhibited IL-1β secretion by nigericin-induced lymphoid CD4 T cells (Extended Data Fig. 7b), indicating it efficiently blocks caspase 1 activity in these cells. VX-765 also blocked caspase 1 cleavage (Fig. 3f), IL-1β secretion (Fig. 3g) and CD4 T-cell death in HIV-infected tonsillar and splenic HLACs (Figs 3c and 5a, b and Extended Data Fig. 9b). Cell death was not markedly inhibited by VRT-043198 (the active form of the VX765 pro-drug), probably because of reduced cellular permeability[38]. HIV-1 infection was not restored to productive infection when caspase 1 was blocked (Extended Data Fig. 10). These findings demonstrate that a
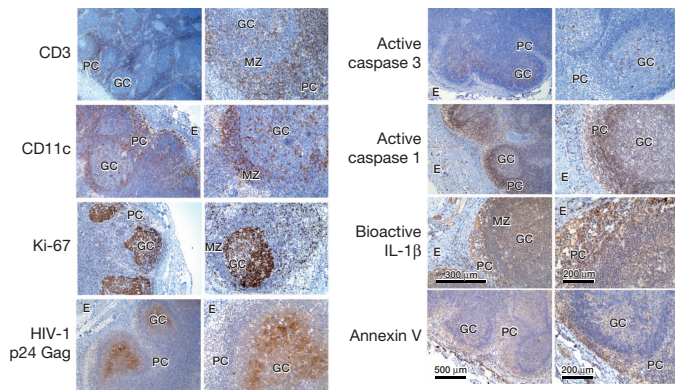
Figure 4 | Distinct regions of caspase 1 and caspase 3 activity in lymph node of a patient chronically infected with R5-tropic HIV. Inguinal lymph node was collected from a 50-year-old immunosuppressed HIV-1 infected subject during the chronic phase of disease. The patient was first identified with HIV in 1985, has not been on anti-retroviral therapy and displayed a CD4 count of 156 cells per μl and viral load of 85,756 copies per ml at the time of lymph node resection (see also Extended Data Fig. 8). E, epithelium; GC, germinal centre; MZ, mantle zone; PC, paracortical zone.

small-molecule inhibitor of caspase 1, shown to be safe in humans, suppresses CD4 T-cell death and inflammation elicited in lymphoid tissues by HIV-1.

Discussion

HIV's lethal attack on its principal cellular target, the CD4 T cell, has been generally attributed to apoptosis[2-8,43]. We now demonstrate that the permissivity status of the host cell dictates the pathway through which lymphoid CD4 T cells die following HIV infection. Specifically, when HIV infects permissive, activated CD4 T cells, cell death occurs silently through caspase-3-dependent apoptosis. Conversely, when either R5- or X4-tropic HIV abortively infects non-permissive, quiescent CD4 T cells from lymphoid tissue, these cells die by caspase-1-dependent pyroptosis, an intensely inflammatory form of programmed cell death. Our recent studies have identified IFI16 as the host DNA sensor that recognizes the incomplete HIV reverse transcripts thereby initiating activation of caspase 1 (ref. 44). In most human lymphoid tissues including tonsil, lymph node and spleen, the activated and permissive subset of cells represents 5% or less of the total CD4 T cells, whereas the non-permissive quiescent cells represent 95% or more of the targets encountered by HIV[12,25]. Thus, in sharp contrast to previous studies[2-8,10], caspase-1-mediated pyroptosis, not caspase-3-mediated apoptosis, appears predominantly responsible for driving CD4 T-cell death following HIV infection of these lymphoid tissues. These findings are further supported by analysis of fresh lymph nodes from subjects infected with R5-tropic HIV, in which caspase 1 and IL-1β are detected in the paracortical zone that is rich in resting CD4 T cells, whereas caspase 3 activity is detected in the anatomically distinct germinal centres where productively infected cells are found.

Our studies also highlight how lymphoid CD4 T cells are selectively primed to mount inflammatory responses as evidenced by constitutive expression of cytoplasmic pro-IL-1β. This is particularly prominent within the CCR5-expressing subset of lymphoid CD4 T cells. The pyroptotic death of these cells would lead to high level release of IL-1β potentially further fuelling chronic inflammation.

Pyroptosis probably promotes the rapid clearance of various bacterial infections by removing intracellular replication niches and enhancing the host's defensive responses through the release of pro-inflammatory cytokines and endogenous danger signals. However, in pathogenic chronic inflammation, such as in HIV infection, pyroptosis is not a protective response and does not lead to clearance of the primary infection. In fact, pyroptosis appears to create a pathogenic vicious cycle in which dying CD4 T cells release inflammatory signals that attract more cells into the
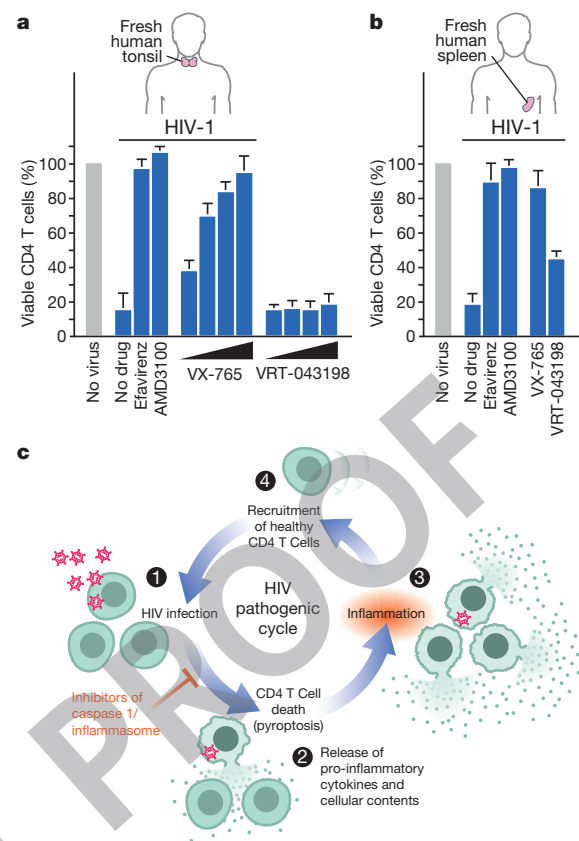
Figure 5 | Targeting caspase 1 via an orally bioavailable and safe drug prevents lymphoid CD4 T-cell death by HIV-1. a, b, VX-765 efficiently blocks CD4 T-cell death in HIV-infected tonsillar and splenic lymphoid tissues. No toxicity was observed at any of these drug concentrations. Error bars represent s.e.m. from three independent experiments using tonsil or spleen cells from three different donors. **c,** Pyroptosis in HIV-infected lymphoid tissues may establish a chronic cycle of CD4 T-cell death and inflammation, which attracts new CD4 T cells and ultimately contributes to disease progression and tissue damage. Inhibitors of caspase 1 such as VX-765 may inhibit pyroptosis in a manner that both preserves CD4 T cells and reduces inflammation.

infected lymphoid tissue to die and to produce more inflammation[45] (Fig. 5c). These events establish a chronic state of inflammation that probably fuels disease progression and tissue injury[46]. Chronic inflammation might also promote maintenance of the latent HIV reservoir through the dysregulated action of the IL-7 or IL-15 cytokines stimulating homeostatic proliferation of memory CD4 T cells. In this regard, it will be interesting to assess to what extent pyroptosis persists in lymphoid tissues of HIV-infected subjects on effective anti-retroviral therapy.

The depletion of CD4 T cells and the development of chronic inflammation are signature processes in HIV pathogenesis that propel disease progression[47]. Our studies now reveal how pyroptosis provides an unexpected link between these two disease-promoting processes. In non-pathogenic infections in which simian immunodeficiency virus (SIV) infects its natural non-human primate hosts, caspase 3 apoptosis in productively infected cells may signal for most of the cell death rather than caspase 1, thus reducing inflammation. The pathogenic cycle of cell death and inflammation created by pyroptosis obligately requires the activation of caspase 1. As such, it may be possible to break this pathogenic cycle with safe and effective caspase 1 inhibitors. These agents could form a new and exciting 'anti-AIDS' therapy for HIV-infected subjects in which the treatment targets the host instead of the virus.

METHODS SUMMARY

Human tonsil or splenic tissues were obtained from the National Disease Research Interchange and the Cooperative Human Tissue Network and processed as previously described[11]. Dead cells within the complete HLACs were first removed by

Ficoll-Hypaque gradient centrifugation. CD4 T cells (CD3^{+}) were isolated from HLACs by positive selection using CD4 microbeads (Miltenyi) as described[11]. CCR5-expressing CD4 T cells were positively separated (PlusCellect R&D Systems), from CD4 T cells negatively isolated from complete HLACs (STEMCELL Technologies, EasySep Human CD4^{+} T-cell Enrichment Kit). In shRNA experiments, infections with R5-tropic HIV-1, and when splenic cells that are extremely refractory to HIV-1 infection were used, we modified the infection system by overlaying HLAC cells on a monolayer of 293T cells that had been transfected with HIV-1 proviral clones, as previously described[11]. Flow cytometry data were collected on a FACS Calibur (BD Biosciences) and analysed with FlowJo software (Treestar). HIV-1 viruses were generated by transfection of proviral DNA into 293T cells using calcium phosphate.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions G.D. identified the involvement of caspase 1 and pyroptosis in lymphoid CD4 T-cell death by HIV-1, developed and designed most of the studies, collected the data and wrote the manuscript; N.L.K.G. performed IL-1β protein assays and examined VX-765 in HIV-infected tonsils; X.G. performed FLICA and shRNA analyses in HLACs; Z.Y. analysed caspase cleavage in HIV-infected cultures; K.M.M. examined caspase inhibitors and LDH release assays; O.Z. tested caspase inhibitors,

METHODS

Preparation of HIV-1 virions. To generate replication-competent viruses, pNL4-3, pNLENG1 or 89.6, proviral expression DNAs were transfected into 293T cells by the calcium phosphate method. The medium was replaced after 16 h. After 48 h, the supernatants were collected and clarified by sedimentation, the virions were concentrated by ultracentrifugation, and were stored at −80 °C in 100% fetal bovine serum. All viral stocks were quantitated by measuring p24 Gag levels by ELISA (1 ng of p24[gag] equals approximately 2×10^6 viral particles). The R5-tropic GFP reporter virus (pBRNL43_005pf135(R5)nef+_IRES_eGFP) was derived from the pNLENG1 clone replaced with gp120 V3 loop sequence of R5-tropic HIV primary isolates as previously described[48].

Culture and infection of HLACs. Human tonsil or splenic tissues were obtained from the National Disease Research Interchange and the Cooperative Human Tissue Network and processed as previously described[11]. HLACs were infected with HIV-1 in 96-well V-bottomed polystyrene plates by spinoculation of 80 ng p24 Gag of HIV particles with 1×10^6 cells in a total of 100 μl per well. Cells were chilled on ice for 15 min and HIV-1 was then added to each well and mixed with cold cells. Virions and cells were subjected to high-speed centrifugation (1200g) for 2 h at 4 °C. This step promotes high-level attachment of virions to target cell membranes. Immediately after centrifugation, cells were cultured at 37 °C as a pellet to facilitate synchronized fusion of the attached viruses. After 10 h of incubation and establishment of productive infection, the indicated drugs were added. Because splenic cells are extremely refractory to HIV infection, we modified the infection system by overlaying splenic HLAC cells on a monolayer of 293T cells that had been transfected with HIV-1 proviral clones. Analysis of CCR5-expressing CD4 T-cell death was similarly performed using 293T transfected with the R5-tropic 81A strain of HIV-1. We also used this method for assays using shRNA-infected HLACs. The 293T cells were transfected with 50 μg of HIV-1 DNA in a 24-well plate. After 12 h, 293T cells were overlayed with 4×10^6 HLACs per well in RPMI media in the presence of the indicated drugs. Virus-producing 293T cells directly interact with overlaid target HLACs. After 24–72 h, the HLAC suspensions were collected from wells and analysed by flow cytometry. Unless otherwise stated, drugs were used at the following concentrations: AMD3100 (250 nM); efavirenz (100 nM); nigericin (8–10 μM); staurosporine (50 nM); Ac-YVAD-CMK, Z-WEHD-FMK, Z-DEVD-FMK, Z-VAD FMK, Z-VEID-FMK, Z-VAD FMK, Z-IETD-FMK or Z-FA-FMK (all 50 μM) (100 μM was determined to be the maximal concentration of these caspase inhibitors that is not associated with toxicity); VX-765 (10 μM); VRT-043198 (10 μM); necrostatin (5 μM); CRID3 (50 μM); parthenolide (10 μM); glyburide (20 μM); glimepiride (20 μM) (20 μM of glyburide and glimepiride was determined to be the maximal drug concentration that does not induce toxicity).

FACS analysis and gating strategy. HLACs were washed in FACS buffer (PBS supplemented with 2 mM EDTA and 2% fetal bovine serum), stained with PE-conjugated anti-CD4, PerCP-conjugated anti-CD19, and APC-conjugated anti-CD8 (all from BD Pharmingen) and fixed in 2% paraformaldehyde. For analysis of CCR5-expressing CD4 T cells, HLACs were stained with 1:3 dilutions of mouse anti-human CCR5 (BD Pharmingen, clone 2D7/CCR5) on ice for 3 h. In isolated CD4 T-cell cultures a standard number of fluorescent beads (Flow-Count Fluorospheres, Beckman Coulter) were added to each cell-suspension sample before data acquisition. Data were collected on a FACSCalibur (BD Biosciences) and analysed with FlowJo software (Treestar). The percentages of viable CD4 T cells were defined by sequential gating beginning with forward scatter versus side scatter to select live lymphocytes, then calculating the number of CD4 T cells divided by the number of CD8 T cells, or by normalization based on the number of fluorescent beads acquired by volume.

Protein analysis, LDH assay, IFN inhibition and intracellular caspase stainings. In order to stimulate the processing and secretion of IL-1β, CD4 T cells were isolated from HLACs by positive selection and treated with 8–10 μM nigericin (Sigma) for 12 h at 37 °C. The potassium ionophore nigericin mediates an electroneutral exchange of intracellular K$^+$ ions for extracellular protons, providing a second inflammatory stimulus, which results in the NLRP3-mediated activation of caspase 1 (ref. 19). In order to assess the processing and secretion of IL-1β in infected CD4 T cells, CD4 T cells were isolated from HLACs as described above, spinoculated with or without NL4-3 (80 ng p24 Gag per 1×10^6 cells) and the indicated drugs as described in the figures. For cytoplasmic pro-IL-1β (Fig. 2b) and other intracellular protein analyses, cells were washed in PBS and immediately lysed in cell extraction buffer (Life Technologies) containing a protease inhibitor cocktail (Roche). For NLRP3 detection cells were lysed using digitonin lysis buffer (digitonin 0.5%, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl) with the addition of a protease inhibitor cocktail (Roche). Lysates were subjected to SDS–PAGE protein analysis using mouse anti human IL-1β antibody (R&D systems clone 8516, catalogue number MAB201), which recognizes the pro- as well as the cleaved form of IL-1β (Fig. 2a, b). For analysis of secreted IL-1β (Figs 2a, c, and 3g), cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Supernatants were collected 3–5 days after infection with HIV-1 or 12 h after treatment with nigericin, filtered through 0.22-μm filter plates (Millipore) and subjected to SDS–PAGE protein analysis using rabbit polyclonal anti-human IL-1β (Abcam, catalogue number ab2105), which primarily recognizes the cleaved form of IL-1β, or assessed for release of cytoplasmic lactate dehydrogenase (LDH) as previously described[33]. For SDS–PAGE immunoblotting analysis, Bio-Rad Criterion 15% pre-cast Tris-HCL gels were used. Gels were wet transferred onto PVDF membranes (Bio-Rad) at maximum current for 3 h at 4 °C and then blocked in 5% non-fat milk for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 1 h at room temperature. Additional primary antibodies used for SDS–PAGE analysis were 1:1,000 rabbit anti-caspase 1 p10 (clone c-20, Santa Cruz, catalogue number SC-515), 1:1,000 rabbit anti-caspase 3 (clone 8G10, Cell Signaling, catalogue number 9665S), 1:1,000 mouse anti NLRP3 (Abcam, catalogue number ab17267), 1:1,000 rabbit polyclonal anti-human ASC (Imgenex, catalogue number IMG-5662), 1:100 Phospho-Stat1 (ser727, Cell Signaling, catalogue number 9177), and 1:10,000 of the mouse monoclonal anti-β-actin (Sigma, catalogue number A5316). The secondary antibody used was 1:5,000 anti-rabbit secondary (Thermo Scientific, catalogue number 32460) or 1:5,000 anti-mouse secondary (Thermo Scientific, catalogue number 32430) developed using a 1:4 dilution of SuperSignal West Femto substrate (Thermo Scientific). To neutralize interferon receptors in HLACs, cultures were added with 1–5 μg of anti-interferon-α/β receptor chain 2 antibody, clone MMHAR-2 (Millipore). To determine intracellular activation of specific caspases, fluorescent labelled inhibitors of caspases (FLICA) probe assays (ImmunoChemistry Technologies) were performed. Each FLICA probe contains a 3 or 4 amino acid sequence targeted by a specific activated caspase. There is no interference from pro-caspases or the inactive form of the enzymes[17]. FLICA probes were added directly to the cell culture media, incubated for 15 min at 37 °C, and washed five times with PBS supplemented with 2 mM EDTA and 2% fetal bovine serum. FLICA probes are cell-permeable and covalently bind to the active forms of specific caspases. After washing, FLICA fluorescent signal is specifically retained within cells containing the appropriate active form of the caspase while the reagent is washed away in cells lacking the appropriate active caspase.

Production and infection of Vpx-VLPs and shRNA-coding HIV LV particles. SIVmac 251 virus-like particles for Vpx delivery (Vpx-VLPs) were produced using the pSIV3$^+$ plasmid, provided by A. Cimarelli[49]. These Vpx-VLP particles are non-infectious as they do not contain any viral genetic material, but they are used to transiently deliver Vpx into target cells where it promotes degradation of SAMHD1 thereby rendering the cells permissive to HIV LV infection[50]. Rather than using the typical pseudotyping method with VSV-G glycoprotein, we pseudotyped the Vpx-VLPs with the CXCR4-tropic Env of HIV-1, which supports efficient fusion of viral particles to quiescent CD4 T lymphocytes[36]. For production of Vpx-VLPs 293 T-cells were co-transfected with 8 μg pSIV3$^+$ and 2 μg CXCR4-tropic Env (gp160)-encoding plasmid. The amount of lentiviral particles was determined by SIV p27 Gag ELISA assay. shRNA-coding vectors were cloned using a modified version of the pSicoR (plasmid for stable RNA interference, conditional) lentiviral vector[34], which encodes an mCherry reporter driven by an EF-1α promoter (pSicoR-MS1)[51]. To generate shRNA lentiviral particles, 293T cells were co-transfected with 10 μg pSicoR-mCherry shRNA constructs, 9 μg HIV-based packaging construct NL4-3 8.91 (ref. 52), and 2 μg CXCR4-tropic Env (gp160)-encoding plasmids. Cells were transfected using the standard phosphate calcium transfection protocol[53]. The lentiviral particle stocks were quantitated by HIV p24 Gag ELISA assay (1 ng of p24 Gag equals approximately 2×10^6 viral particles).

To achieve productive infection of shRNA-encoding LV particles, complete HLACs or isolated lymphoid CD4 T cells were initially challenged with Vpx-VLPs, followed by a second infection with an shRNA-coding LV of interest after 24 h. This sequential infection strategy allowed Vpx to establish an optimal permissive state within the target cells at the time when the shRNA LV infection was performed. To facilitate a synchronized delivery of Vpx and fusion of shRNA LV particles, cells and particles were subjected to high-speed spinoculation at each step. To assess the efficiency of gene silencing by the shRNA-coding vectors (Fig. 3d), highly infectious SupT1 were infected with shRNA LV (without prior Vpx-VLP infection), and were subjected to protein analysis after 48 h.

For cloning of caspase 1 coding shRNA vector the following oligonucleotides were used: sense: 5′-TACACGTCTTGCTCTCATTATTCAAGAGATAATGA GAGCAAGACGTGTTTTTTTC-3′; antisense: 5′-TCGAGAAAAAAACACGT CTTGCTCTCATTATCTCTTGAATAATGAGAGCAAGACGTGTA-3′. For cloning of caspase 3 coding shRNA vector the following oligonucleotides were used: sense: 5′-TAAAGGTGGCAACAGAATTTTTCAAGAGAAAAATTCTGTTGCC ACCTTTTTTTTTC-3′; antisense: 5′-TCGAGAAAAAAAAAGGTGGCAACAG AATTTTCTCTTGAAAAATTCTGTTGCCACCTTTA-3′. For cloning of ASC-coding shRNA vector the following oligonucelotides were used: sense: 5′-TGAA GCTCTTCAGTTTCACATTCAAGAGATGTGAAACTGAAGAGCTTCTTTT

TTC-3′; antisense: 5′-TCGAGAAAAAAGAAGCTCTTCAGTTTCACATCTC TTGAATGTGAAACTGAAGAGCTTCA-3′. For cloning of NLRP3-coding shRNA vector the following oligonucelotides were used: Sense: 5′-TGAAATGGATTGAA GTGAAATTCAAGAGATTTCACTTCAATCCATTTCTTTTTTC-3′; antisense: 5′-TCGAGAAAAAAGAAATGGATTGAAGTGAAATCTCTTGAATTTCACT TCAATCCATTTCA-3′.

Tissue samples. HIV-infected lymph node tissues were obtained from patients participating in the SCOPE cohort at HIV/AIDS clinic of the San Francisco General Hospital (SFGH) Positive Health Program. All tissues were obtained with full consent from the patients and under a protocol fully approved by the Committee on Human Research at UCSF. For the results presented, an inguinal lymph node was harvested from two different HIV-infected patients: A 50-year-old immunosuppressed, untreated R5-tropic HIV-1-infected subject in the chronic phase of disease. This individual exhibited a viral load of 87,756 RNA copies per ml, and CD4 T-cell count of 227 cells per μl. A 41-year-old African-American male, infected with an R5-tropic strain of HIV-1, had been on intermittent anti-retroviral therapy between 2004–2009 and stopped anti-retroviral therapy in 2009. This individual exhibited a viral load of 30,173 RNA copies per ml, and a CD4 T-cell count of 259 cells per μl. The fresh specimens were immediately fixed with 4% PFA and subjected to immunostaining analysis. Sections of the HIV-infected lymph node and of a fresh human tonsil were processed in parallel and analysed for the indicated markers. IRB approval number 10-03606 with study title: the use of lymph node biopsies to support HIV pathogenesis studies.

Tissue preparation and immunohistochemistry. Five-micron sections were cut from formalin-fixed paraffin-embedded tissue blocks and mounted on X-tra microscope slides (Leica Microsystems). Specimens were stepwise deparaffinized in xylene and rehydrated in descending alcohols to water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide (Sigma Chemicals) in PBS for 15 min. Antigen retrieval was performed by microwaving the sections in 10 mM citrate buffer, pH 6.0. Sections were then blocked in the secondary antibody host's normal serum (Vector Labs; horse S-2000, goat S-1000, catalogue number S5000). The following primary antibodies were diluted in PBS with 0.1% bovine serum albumin (BSA) and applied to the slides overnight at 4°C: monoclonal mouse anti-human CD3 (1:100, Clone F7.2.38 Dako, catalogue number M725429-2), monoclonal rabbit anti-human CD11c (1:100, clone EP1347Y, Abcam catalogue number ab52632), monoclonal mouse anti-human Ki-67 (1:100, clone MIB1, Dako catalogue number M724029-2), monoclonal mouse anti-HIV p24 Gag (1:50, clone KaI-1, Dako Cytomation catalogue number M0857), rabbit anti-human cleaved caspase 3 (1:300, Cell Signalling Technology catalogue number 9661), goat anti-p20 subunit of active human caspase 1 (1:200, clone c15, Santa Cruz Biotechnology catalogue number sc-1780), rabbit anti-human against bioactive 17-kDa IL-1β (1:100, Abcam catalogue number ab2105), and annexin V (1:50, Abcam catalogue number EPR3979). The following day sections were washed in 0.05% Tween-20 in PBS followed by incubation with Vector laboratories biotinylated secondary IgG antibodies diluted 1:200 in PBS for 30 min at room temperature (donkey anti-mouse BA-2000, goat anti-rabbit BA-1000, rabbit anti-goat BA-5000). Slides were then rinsed in 0.05% Tween-PBS, and incubated in streptavidin horseradish peroxidase complex at a 1:200 dilution in PBS for 30 min at room temperature (Vector Laboratories catalogue number SA-5004). Specimens were rinsed in 0.05% Tween-20 in PBS then incubated with 3,3-diaminobenzadine (DAB) chromogenic substrate (Sigma Chemicals) using hydrogen peroxide as a substrate (Sigma Chemicals) for 10 min. Sections were counterstained in haematoxylin dehydrated through graded alcohols, cleared in xylene and mounted in depex.

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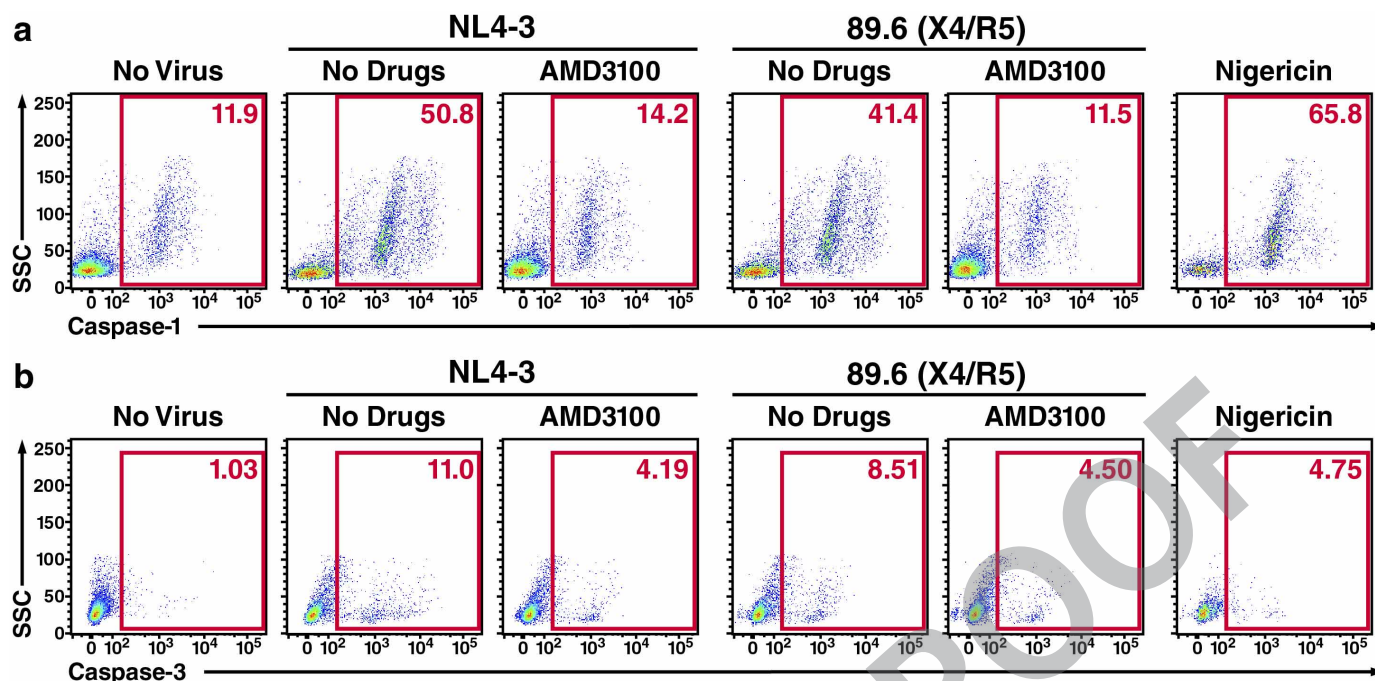
Extended Data Figure 1 | Extensive caspase 1 activation in dying lymphoid CD4 T cells infected with either NL4-3 or a primary HIV-1 isolate. a, Dying CD4 T cells activate caspase 1. HLACs were infected with NL4-3 or with a primary HIV-1 isolate 89.6 obtained from a mixed PBMC culture from an AIDS patient. The 89.6 viral isolate replicates to high titres in primary human cells such as macrophages and lymphocytes. It is highly cytopathic and utilizes both CCR5 and CXCR4 as co-receptors (dual-tropic)[18,54]. Infected cells were treated either with no drugs or with AMD3100 (250 nM) entry inhibitor, as indicated. Caspase 1 activity was determined by flow cytometry using FLICA 12 h after treatment with nigericin (10 μM) or 3 days after infection with HIV. Notably, equivalent levels of caspase 1 activation were observed in CD4 T cells infected with NL4-3 or 89.6 HIV-1 isolate. AMD3100 prevented caspase 1 activity with both viruses, indicating the abundant presence of CXCR4-expressing target CD4 T cells in these cultures. **b**, Low levels of caspase 3 activity in dying CD4 T cells. The same cultures as in (**a**) were tested for caspase 3 activity using FLICA. Compared to caspase 1, infections with NL4-3 and 89.6 HIV-1 isolate induced low levels of caspase 3 activation in dying CD4 T cells. No caspase 3 activation was observed in cells treated with nigericin, which signals the NLRP3 inflammasome to activate caspase 1 (ref. 19), indicating a specific recognition of caspase 1 and caspase 3 activity by the FLICA probes. These data are the representative results of four independent experiments performed in tonsil cells isolated from four different donors.
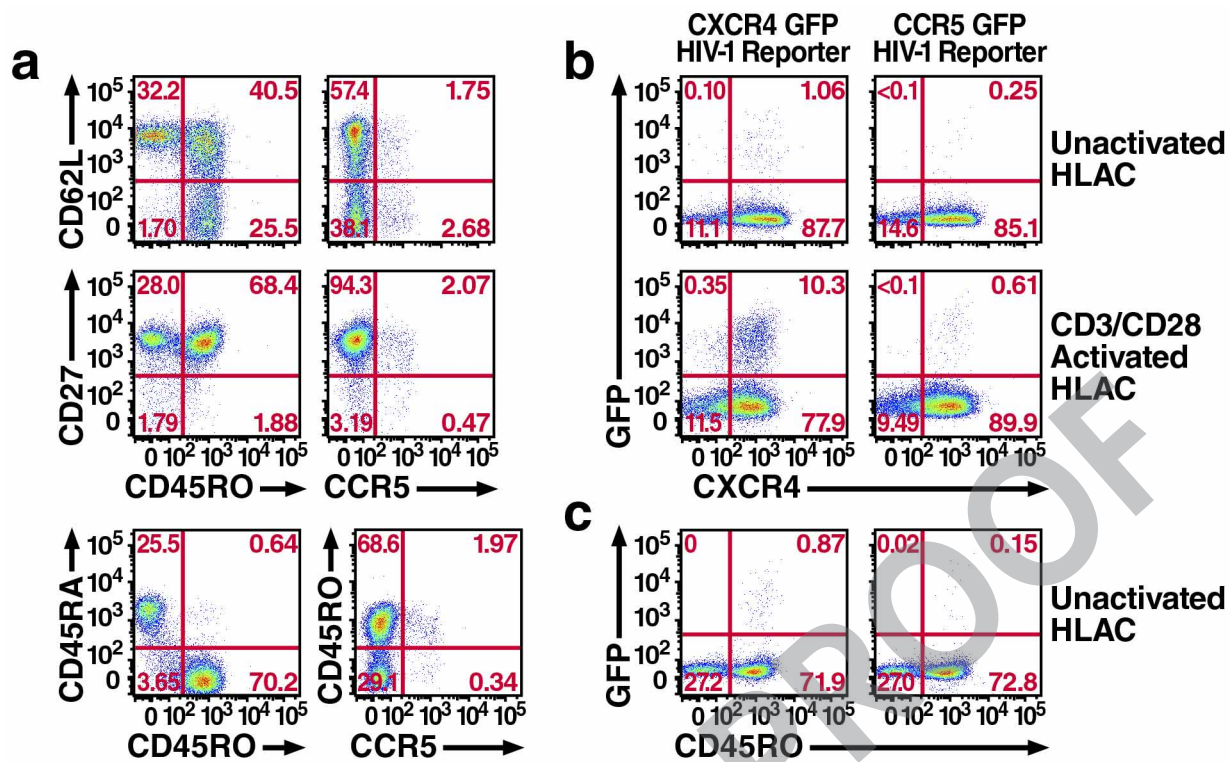
Extended Data Figure 2 | Resting CD4 T cells from tonsil include both naive and memory subsets. a, CD4 T lymphocytes in lymphoid tissues contain a large population of central memory cells. To identify the sub-populations of CD4 T cells in human tonsil histocultures, we examined the expression pattern of CCR5, CD45RA, CD45RO, CD62L and CD27. Central memory CD4 T cells (T_{CM}) are characterized by expression of CD45RO$^+$/CD62L$^+$ or CD45RO$^+$/CD27$^+$ [29,31,55,56]. T_{CM} lack effector function and constantly travel through the lymph nodes in large quantities for antigen sampling, whereas effector memory cell (T_{EM}) mainly migrate to peripheral tissues [29–31]. Analysis of these surface markers revealed at least three distinct maturation phenotypes. The majority of CD4 T lymphocytes exhibit a memory phenotype as determined by surface expression of CD45RO, among them more than two-thirds were found to be central memory cells (CD45RO$^+$/CD62L$^+$ and CD45RO$^+$/CD27$^+$). Similarly, a large population of CCR5-expressing CD4 T cells was found to have central memory phenotype (CCR5$^+$/CD62L$^+$ and CCR5$^+$/CD27$^+$). These findings are in accordance with previous studies in primary human lymphoid cultures [12,57,58]. **b, c**, Memory lymphoid CD4 T cells represent preferential targets for productive infection by both the R5- and X4-tropic strains of HIV-1. To determine whether cell maturation influences susceptibility for productive infection, we measured the levels of productive infection using GFP reporter viruses harbouring either an X4-tropic or R5-tropic Env of HIV-1. Except for their select V3 loop envelope determinants, both reporters were derived from the same bicistronic Nef-IRES-GFP clone which produces fully replication-competent viruses [16]. Interestingly, productive infection of both X4-tropic or R5-tropic viral strains was detected in CXCR4-expressing cells, indicating that the CXCR4 co-receptor is equally present on CCR5-expressing cells, as was previously shown [12,57–59]. Memory CD4 T cells (CD45RO$^+$) were selectively productively infected in cultures infected with either X4-tropic or R5-tropic reporter virus. Similar findings were found in infected cultures activated with CD3/CD38 beads to achieve higher rates of infection. Among the memory CD4 T cells, T_{EM} cells became productively infected in higher quantities than T_{CM} (not shown). These data are the representative results of six independent analyses performed in tonsil cells isolated from six different donors.
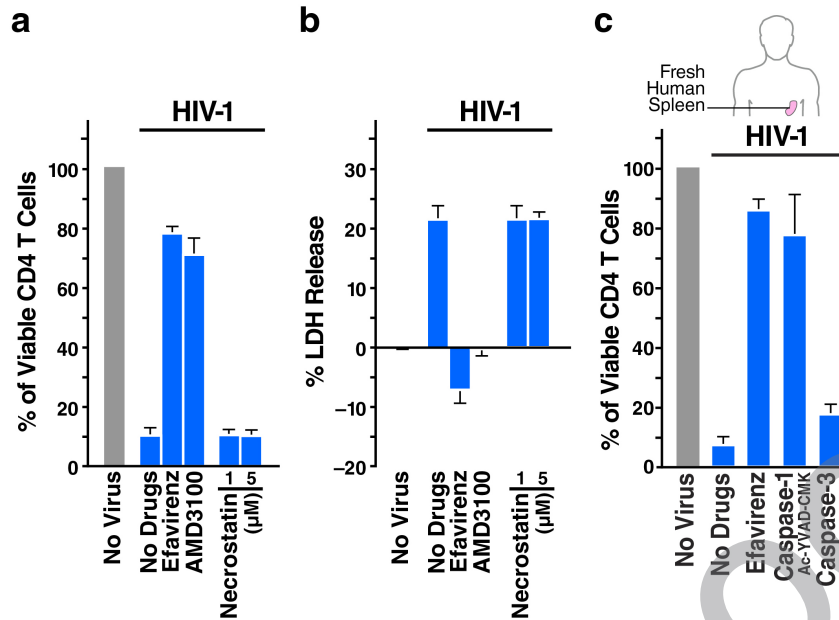
Extended Data Figure 3 | Necrostatin-1 does not prevent lymphoid CD4 T-cell death and cell lysis in HIV-infected cultures. a, b, Necrostatin was tested at 1 or 5 μM, a concentration that yields maximal inhibition without inducing toxicity (not shown). Pyroptosis shares cell death features with necroptosis which similarly leads to the release of intracellular contents into the extracellular space[9]. To test whether cell death involves necrotic signalling, we treated HIV-infected CD4 T cells with necrostatin, a specific inhibitor of RIP1, whose kinase activity is essential for programmed necroptosis to occur[60]. Concentrations of necrostatin that block necroptotic signalling (not shown) did not inhibit CD4 T-cell depletion in HIV-infected cultures (**a**), and did not prevent the release of intracellular contents into the culture medium, as indicated by LDH activity in the supernatants (**b**). Thus, although pyroptosis shares features with necroptosis, these data demonstrate that the signalling pathways linking caspase 1 activation to CD4 T-cell death are specific.

Together, these findings indicate that the CD4 T-cell depletion and release of cytoplasmic contents in HIV-infected lymphoid cultures reflects pyroptosis rather than apoptosis or necroptosis. Error bars represent s.e.m. of at least three independent experiments using tonsil cells from at least three different donors. **c,** Caspase 1 inhibitors prevent CD4 T-cell death in HIV-infected splenic tissues. Splenic HLACs were cultured with no virus or were infected with HIV-1. The HIV-infected cultures were treated as indicated, either with no drugs, efavirenz (100 nM), the caspase 1 inhibitor Ac-YVAD-CMK (50 μM), or the caspases-3 inhibitor Z-DEVD-FMK (50 μM). After 4 days, viable CD4 T cells were counted by flow cytometry. Viable CD4 T cells are presented as the percentage remaining live T cells CD4 using CD8 T cells to normalize each HIV-infected or uninfected culture. Error bars represent s.e.m. from four independent experiments using tonsil cells isolated from four different donors.
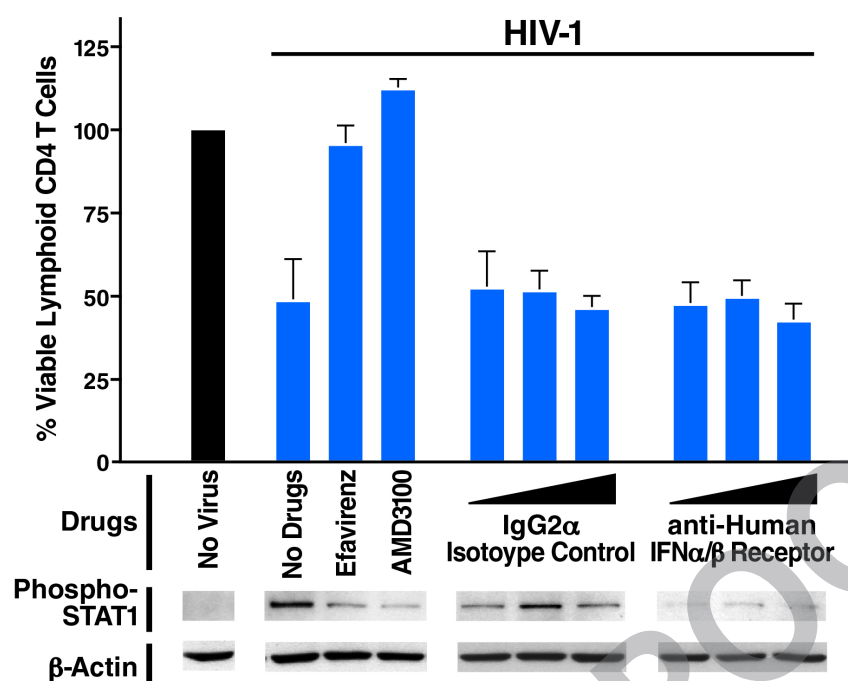
Extended Data Figure 4 | Induction of type-I interferon is not required to trigger a death response in HIV-infected lymphoid CD4 T cells.
HIV-1 infections induce type-I interferon *in vitro* and *in vivo*[61]. To test the involvement of this antiviral response in modulating CD4 T-cell death, isolated CD4 T cells were infected with HIV-1 in the presence of neutralizing antibodies against the human interferon alpha receptor (IFNAR2), which blocks biological action of type I interferons. To determine the state of interferon signalling, cells were analysed in parallel for the presence of tyrosine-phosphorylated STAT1, which plays a central role in mediating type-I IFN-dependent biological responses, including induction of an antiviral state[62]. Phosphorylated STAT1 readily appeared in HIV-infected CD4 T cells, but not in HIV-infected cells treated with efavirenz (100 nM), AMD3100 (250 nM) or anti-IFNAR2 neutralizing antibodies (1–5 μg ml^{-1}). Notably, blocking interferon signalling with anti-IFNAR2 neutralizing antibodies did not prevent the death of CD4 T cells by HIV-1, although tyrosine phosphorylation of STAT1 was inhibited indicating effectiveness of the antibody blockade. The data suggest that this antiviral IFN induction is not critical to the onset of the innate immune death response leading to caspase 1 activation and pyroptosis. Error bars represent s.e.m. from three independent experiments using tonsil cells from three different donors.
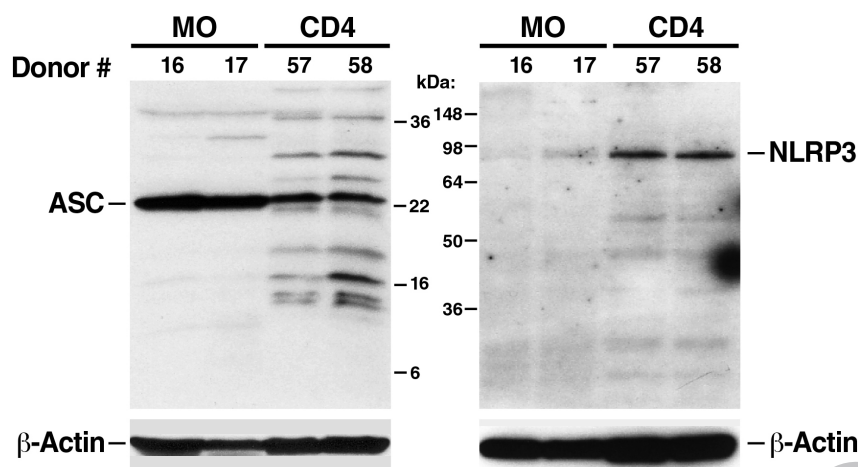
Extended Data Figure 5 | Lymphoid CD4 T cells express detectable levels of ASC and NLRP3 relative to blood-derived monocytes. The bipartite adaptor protein ASC (PYCARD) plays a central role in the interaction between (NOD)-like receptor and caspase 1 in inflammasome complexes[63]. Lymphoid CD4 T cells are primed to mount such inflammatory responses, and constitutively express high levels of cytoplasmic pro-IL-1β, but also ASC and NLRP3, compared to blood-derived monocytes. CD4 T lymphocytes express constitutive levels of NLRP3. In contrast to lymphocytes, monocytes require stimulation with TLR ligands such as LPS to induce NLRP3 expression[21]. Thus, the release of intracellular 5′-ATP by pyroptotic CD4 T cells may provide a second inflammatory stimulus to induce activation of caspase 1 by the NLRP3 inflammasome in nearby CD4 T cells that are already primed as reflected by their high levels of ASC, NLRP3 and pro-IL-1β expression. Thus, pyroptosis activated initially by HIV may result in cascade of new rounds of pyroptosis in primed CD4 T cells by the repeated release of intracellular ATP in a virus-independent manner. Such an 'auto-inflammation' scenario could result in persistent rounds of pyroptosis, chronic inflammation and loss of CD4 T cells even when viral loads are reduced by antiretroviral therapy (ART).
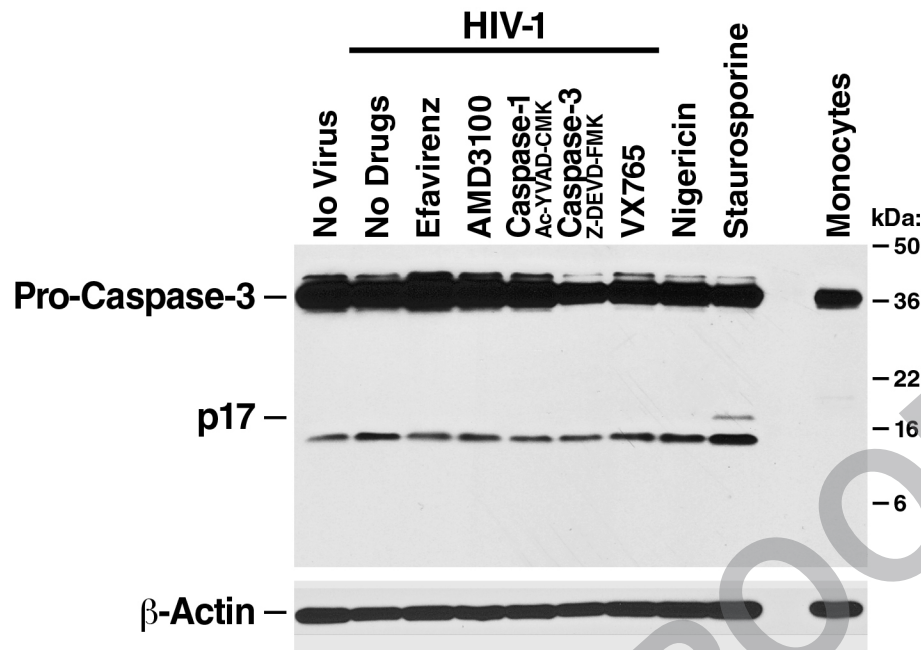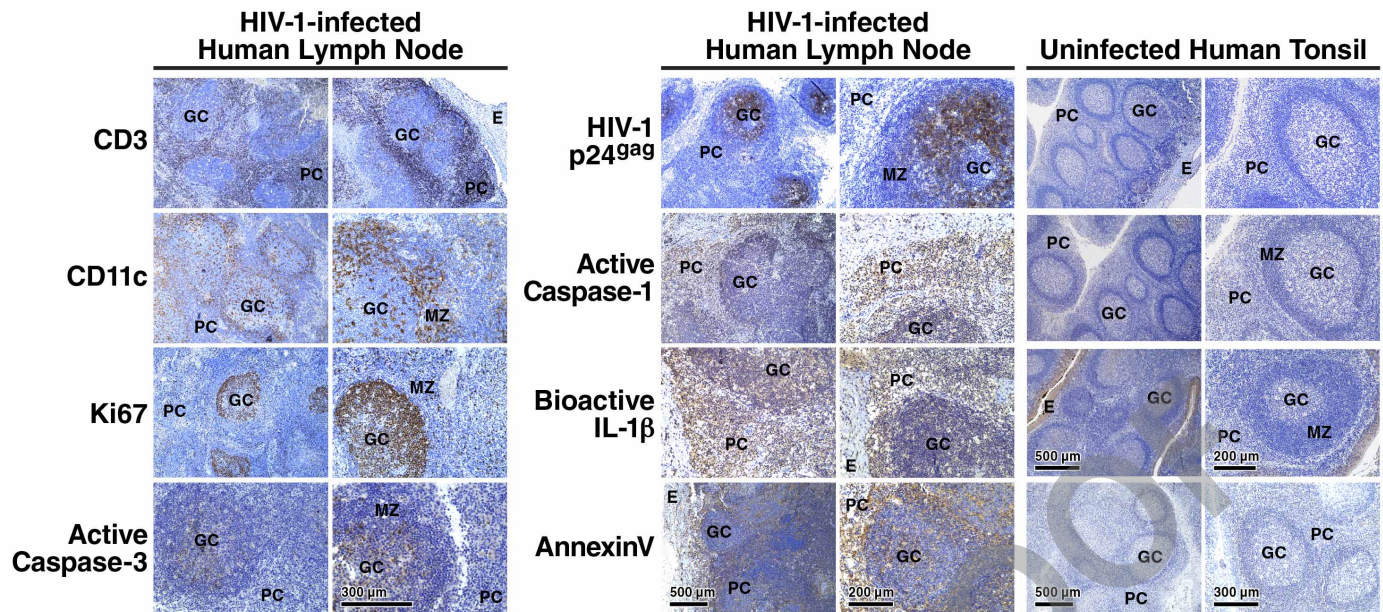
Extended Data Figure 6 | Low levels of caspase 3 activation in HIV-infected lymphoid CD4 T-cell cultures. Although the endogenous levels of pro-caspase 3 and pro-caspase 1 expression are similar in lymphoid CD4 T cells, caspase 3 activation in these cells was markedly less abundant after infection with HIV-1, compared to caspase 1. These data are in accord with our findings using fluorescently labelled inhibitor of caspases (FLICA) probes in cultures infected with a GFP reporter HIV-1. In these cultures, the majority of CD4 T cells were abortively infected and showed activation of intracellular caspase 1. No caspase 1 activity was observed in productively infected cells (Fig. 1b). In sharp contrast, caspase 3 activity in these cultures was markedly less abundant, and specifically occurred in productively infected, but not in non-productively infected cells (Fig. 1c). These data are the representative results of three independent experiments performed in tonsillar CD4 T cells isolated from three different donors.
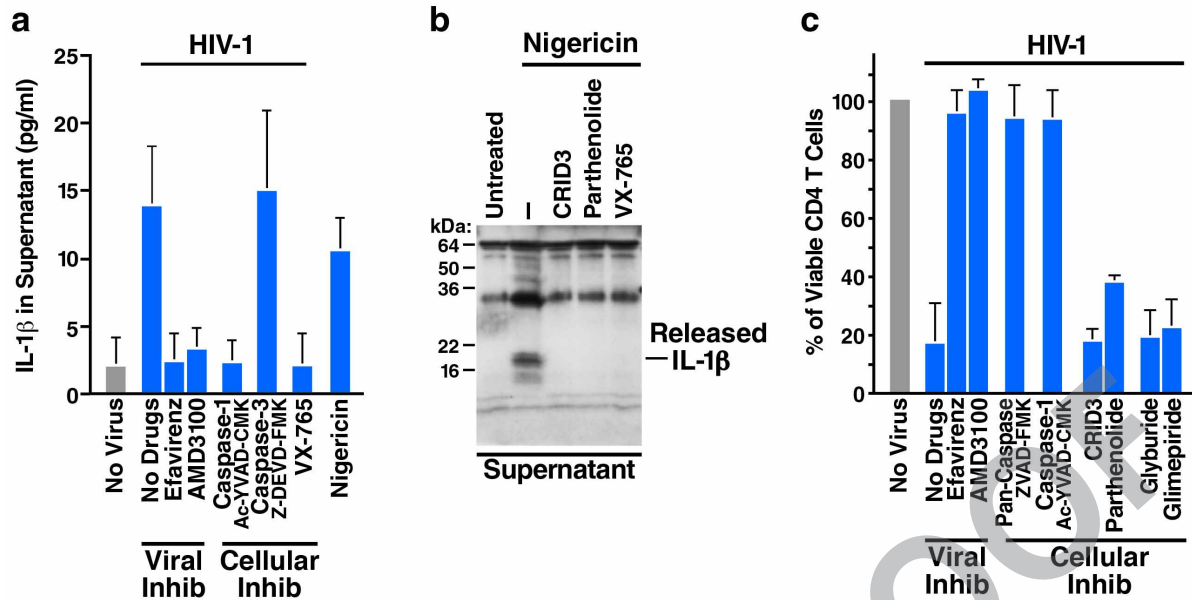
Extended Data Figure 7 | Inhibitors of caspase 1, but not of NLRP3, prevent CD4 T-cell death by HIV-1. **a**, Quantitative evaluation of bioactive IL-1β secreted in HIV-infected CD4 T-cell cultures using ELISA. Isolated tonsillar CD4 T cells were left uninfected or infected with HIV in the presence of the indicated drugs. Four days after infection, supernatants were filtered through 0.22-μm filter plates and subjected to IL-1β ELISA analysis. A total of 200 μl of supernatant from 2 million isolated CD4 T cells was used for each condition. The assay was performed as described by the manufacturer's instructions (R&D Systems). Bioactive IL-1β was detected in supernatants of HIV-infected cultures, at levels comparable to those in uninfected cells treated with nigericin. Treatments of HIV-infected cultures with viral or caspase 1 inhibitors, but not caspase 3 inhibitor, reduced accumulation of IL-1β in the supernatants to levels comparable to those detected in uninfected cultures. These finding demonstrate that caspase 1 activation is specifically required for the release of bioactive IL-1β in lymphoid CD4 T cells infected with HIV-1. Error bars represent s.e.m. of three independent experiments using tonsil cells from at least three different donors. **b**, Inhibitors of caspase 1 and the NLRP3

inflammasome prevent release of mature IL-1β induced by nigericin, but not CD4 T-cell death by HIV-1. Because nigericin engages the NLRP3 inflammasome to activate caspase 1 in lymphoid CD4 T cells, we sought to determine if NLRP3 also similarly controls caspase 1 activity in response to HIV-1 infection. Cell cultures were treated with four separate NLRP3 inhibitors including CRID3[64], parthenolide[65], and the sulfonylureas glyburide[66] and glimepiride. Treatments with CRID3, parthenolide or sulfonylureas (not shown) completely inhibited NLRP3-dependent release of mature IL-1β by nigericin, but had no effect on IL-1β release triggered by HIV infection of lymphoid CD4 T-cell cultures (Fig. 3f). **c**, Treatments with CRID3, parthenolide or sulfonylureas did not prevent HIV-1-mediated CD4 T-cell death. These results suggest that the NLRP3 inflammasome does not control the caspase-1-mediated death responses in lymphoid CD4 T cells abortively infected with HIV-1. Cell death results are represented as ratios of viable CD4 versus CD8 T cells in each HIV-infected or uninfected culture. Error bars represent s.e.m. of four independent experiments using tonsil cells from four different donors.

Extended Data Figure 8 | Distinct regions of caspase 1 and caspase 3 activity in lymph node of a chronically infected HIV patient. Inguinal lymph node was collected from a 41-year-old African-American male, infected with an R5-tropic strain of HIV-1. The patient had been on intermittent anti-retroviral therapy between 2004–2009, and stopped anti-retroviral therapy in 2009. This individual exhibited a viral load of 30,173 RNA copies per ml, and CD4 T-cell count of 259 cells per μl. The fresh specimen was immediately subjected to immunostaining in parallel with fresh uninfected human tonsil. Note the immunostain against CD3 highlights the paracortical region, which is almost entirely composed of resting T cells. Note also the sparse presence of CD3-positive T cells in the mantle zones and germinal centres, where lymphocytes become activated (Ki-67) and differentiate into memory and plasma cells. These CD4 T cells are responsible for antigen-dependent activation of B cells in the follicle. Staining for CD11c reveals scattered dendritic cells[67,68] in the germinal centre and largely in the mantle zone. HIV p24 Gag expression is located between the mantle zone and germinal centres, where activated CD4 T cells reside. Remarkably, caspase 3 activity also occurs in this anatomical region, which is separated from the majority of non-productively infected T cells in the paracortical zone and exhibit caspase 1 activation, IL-1β processing and pyroptosis. The anti-caspase 1 antibody was raised against a peptide mapping to the C terminus of caspase 1 p20 of human origin and detects both the cleaved p20 subunit and the precursor of caspase 1. Therefore, in the absence of an equivalent uninfected lymph node it is hard to absolutely determine whether abortive HIV-1 infection affects pro-caspase expression. However, staining of uninfected tonsil or spleen (not shown) tissue revealed no positive HIV p24 Gag, active or pro-caspase 1, bioactive IL-1β or annexin V signals. These data closely correlate with the findings in HIV-infected HLACs where the 95% of the CD4 T cells are non-productively infected CD4 T cells and show activation of intracellular caspase 1, whereas caspase 3 activity is markedly less abundant and specifically occurs in productively infected CD4 T cells. GC, germinal centre; MZ, mantle zone; PC, paracortical zone.

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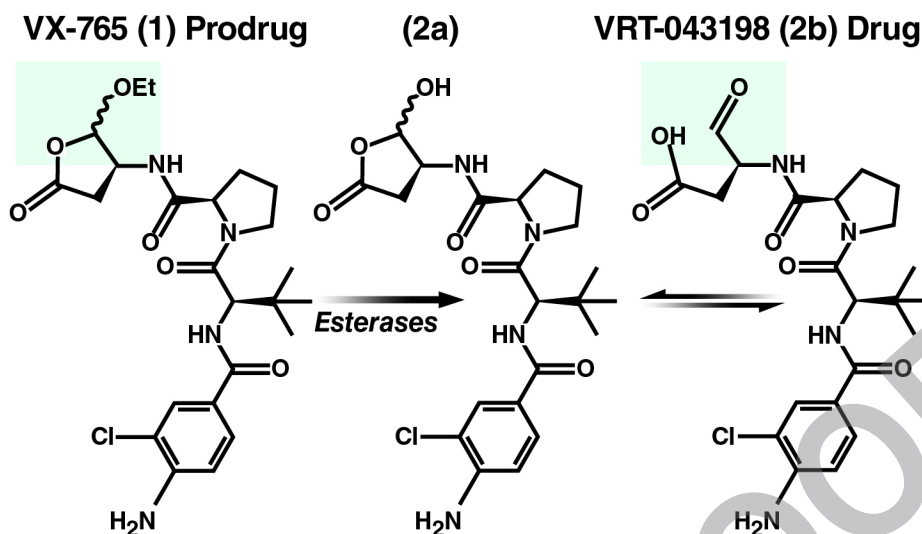
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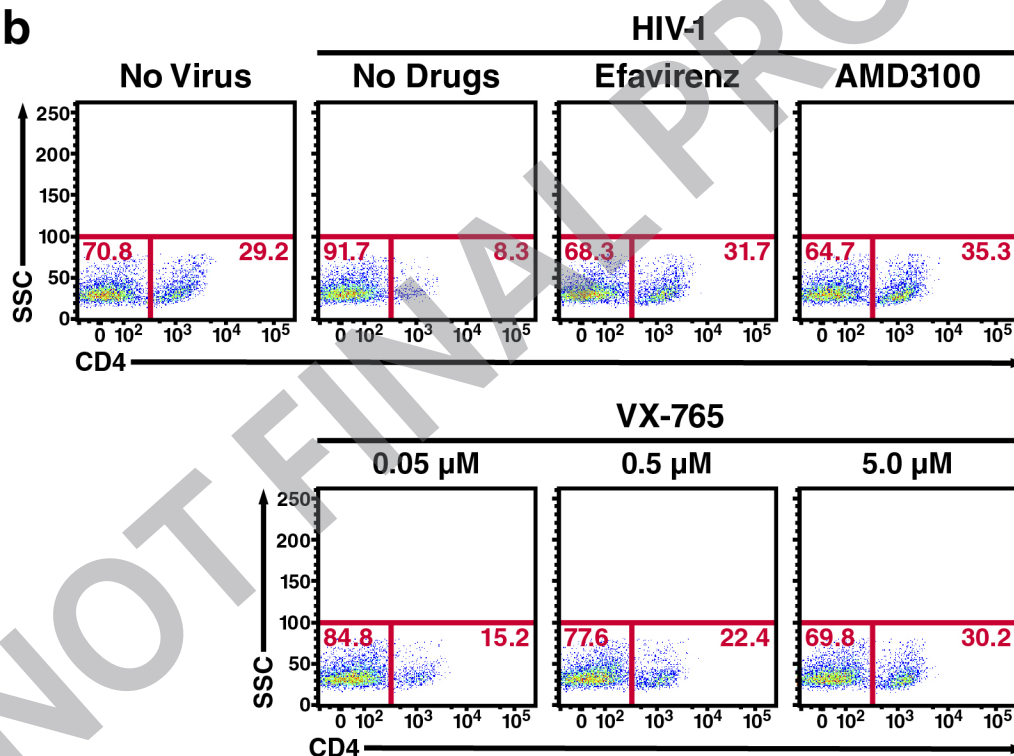
Extended Data Figure 9 | Targeting caspase 1 via an orally bioavailable small molecule inhibitor prevents lymphoid CD4 T-cell death by HIV-1. a, VX-765 is a cell permeable pro-drug (**1**) that requires intracellular esterase cleavage in the cell to yield the aldehyde functionality (green) of the drug VRT-043298 (**2b**), which acts as a potent caspase 1 inhibitor. Adapted from ref. 38 with permission. **b,** VX-765 prevents CD4 T-cell death in a dose-dependent manner in HIV-infected lymphoid tissues. HLACs were either not infected or infected with HIV-1 in the absence of drugs or in the presence of efavirenz (100 nM), AMD3100 (250 nM) or VX-765 (0.05, 0.5 or 5 μM) as indicated. Flow cytometry plots depict gating on live cells based on the forward-scatter versus side-scatter profile of the complete culture. These results are representative of three independent experiments performed using tonsil cells from three different donors.
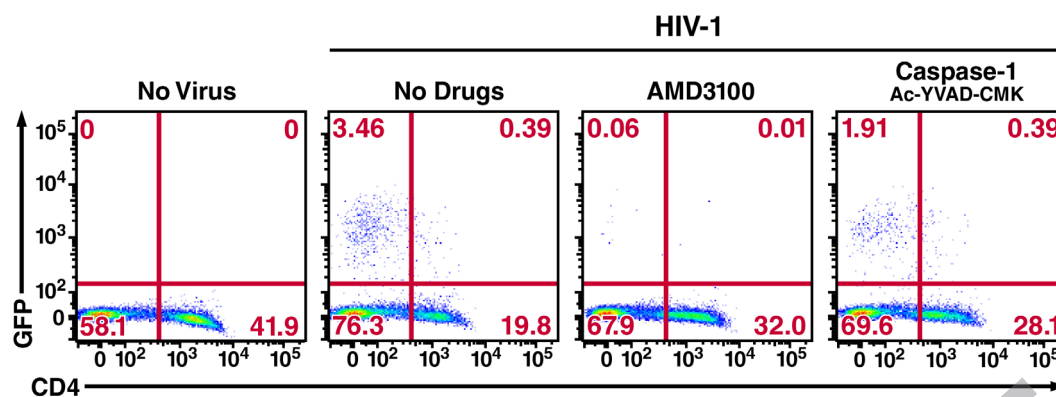
Extended Data Figure 10 | Treatment with a caspase1 inhibitor does not increase productive HIV-1 infection. To determine whether inhibition of caspase-1-mediated pyroptosis would result in higher levels of productive HIV-1 infection, tonsillar HLACs were treated with AMD3100 or with the caspase 1 inhibitor Ac-YVAD-CMK before infection with a GFP reporter virus (NLENG1). After 5 days, flow cytometry analysis of the infected cultures revealed no increase in GFP-positive cells in the infected cultures treated with the caspase 1 inhibitor Ac-YVAD-CMK. This result likely reflects the continued function of the host restriction factor SAMHD1 (refs 35, 69). These findings argue against the possibility that pyroptosis functions as a defence against productive infection. Instead, pyroptosis appears to represent an overall harmful response that centrally contributes to HIV pathogenesis. These results also argue that interdiction of the pyroptosis pathway with caspase 1 inhibitors would produce beneficial rather than harmful therapeutic effects.