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Minocycline attenuates HIV-1 infection and suppresses chronic immune activation in humanized NOD/LtsZ-scidIL-2Rγ^{null} mice

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Summary

More than a quarter of a century of research has established chronic immune activation and dysfunctional T cells as central features of chronic HIV infection and subsequent immunodeficiency. Consequently, the search for a new immunomodulatory therapy that could reduce immune activation and improve T-cell function has been increased. However, the lack of small animal models for *in vivo* HIV study has hampered progress. In the current study, we have investigated a model of cord blood haematopoietic progenitor cells (CB-HPCs) -transplanted humanized NOD/LtsZ-scidIL-2Rγ^{null} mice in which progression of HIV infection is associated with widespread chronic immune activation and inflammation. Indeed, HIV infection in humanized NSG mice caused up-regulation of several T-cell immune activation markers such as CD38, HLA-DR, CD69 and co-receptor CCR5. T-cell exhaustion markers PD-1 and CTLA-4 were found to be significantly up-regulated on T cells. Moreover, increased plasmatic levels of lipopolysaccharide, sCD14 and interleukin-10 were also observed in infected mice. Treatment with minocycline resulted in a significant decrease of expression of cellular and plasma immune activation markers, inhibition of HIV replication and improved T-cell counts in HIV-infected humanized NSG mice. The study demonstrates that minocycline could be an effective, low-cost adjunctive treatment to regulate chronic immune activation and replication of HIV.

Keywords: HIV; humanized NOD/LtsZ-scidIL-2Rγ^{null} mice; immune activation; minocycline.

Introduction

Chronic immune activation is a hallmark of HIV pathophysiology.[1] Current understanding suggests that persistent antigenic stimulation, microbial translocation, disruption of T-cell homeostasis and various co-infections initiate and perpetuate chronic immune activation and dysfunction of T cells.[2,3] Highly active antiretroviral therapy (HAART) has been successful in suppressing virus replication and improving overall immune function in HIV-infected individuals. However, even after several years on HAART, markers of immune cell activation, proliferation, apoptosis and soluble markers of inflammation do not reach baseline levels in HIV-infected individuals.[4–6] Elevated immune activation is associated with progression towards AIDS in non-treated patients and to non-opportunistic infections, cardiovascular disease, lipodystrophy, renal disease, alteration in bone metabolism and an increased risk of non-AIDS-defining malignancies in individuals on HAART.[1] Persistent immune activation, which disrupts the functional organization of the immune system and reduces its regenerative capacity, has emerged as a potential determinant of HIV-associated morbidity and mortality.[7]

Development of new immunomodulatory therapies to curb HIV-associated immune activation and T-cell dysfunction has been hampered by lack of *in vivo* experimental models. The inherent differences between SIV and HIV pathogenesis,[8,9] along with the prohibitive cost for using non-human primates, have slowed the search for novel therapies to combat chronic immune activation induced by HIV. The new generation of humanized NOD/LtsZ-scidIL-2R$^{\gamma null}$ (NSG) or NSG-bone marrow-liver-thymus (BLT) mouse models sustain a long-term HIV replication

and mimic the immune activation phase of HIV pathogenesis.[10–12] Hence, they provide a new opportunity for *in vivo* trials to predict the efficacy of new therapies.

Recently, an antibiotic from the tetracycline family – minocycline – has been proposed as anti-HIV adjuvant therapy.[13,14] It fits into a new unique class of drug in possessing both immunomodulatory and anti-viral properties against SIV and HIV. In the experimental SIV model of HIV central nervous system disease, minocycline has a strong influence in reducing the severity of encephalitis and neurodegeneration through the reduction of monocyte activation and the suppression of p38 and c-jun-n-terminal kinase (jnk) activation pathways, which are essential for the generation of inflammatory mediators.[15,16] An *in vitro* study has shown that minocycline affects activation-induced markers (CD69, HLA-DR, CD25, CCR5 and Ki-67) expression and secretion of cytokines [interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)] from T cells.[14] Previous studies have also shown that minocycline has strong anti-viral properties. Minocycline treatment of SIV-infected macaques significantly lowers their viral RNA in cerebrospinal fluid and plasma.[17,18] Zink *et al.* observed 92 and 99% reduction in HIV replication (HIV-1Bal and HIV-1III) at a 40 μg/ml dose of minocycline in lymphocytes and macrophages.[17] In another *in vitro* study using a 20 μg/ml dose, minocycline-treated T cells showed a twofold decrease in HIV-1 NL4-3 infectivity.[14] Computational docking and molecular dynamics simulation studies have also proposed high binding affinity of minocycline for HIV-1 integrase.[19]

In a clinical trial for the treatment of HIV-associated cognitive impairment, minocycline was found to be safe and tolerable in HIV-infected individuals.[20] In another recent pilot study, minocycline nevertheless failed to modulate cerebrospinal fluid HIV infection and immune activation.[21] However, this study had several inherent design limitations, including its size, short duration of study and more importantly, absence of an untreated control group for comparison. Hence, potential role and efficacy of minocycline in the management of HIV-induced chronic immune activation still needs to be investigated.

In the present study, we have used humanized NSG mice transplanted with cord blood-haematopoietic progenitor cells (CB-HPCs),[11] to evaluate the therapeutic potential of minocycline for the treatment of chronic immune activation induced by HIV-1 infection. HIV infection in humanized mice caused a chronic activation of the immune system, and treatment of minocycline at a dose of 100 mg/kg/day for 60 days led to significantly lower viral load and improved T-cell count. Expression of cellular activation (CD38, HLA-DR, CD69 and CCR5) and exhaustion markers (PD-1 and CTLA-4) was significantly lower in the treated group of mice. Level of sCD14, lipopolysaccharide (LPS) and IL-10 was also lower in this group of humanized mice.

In summary, we demonstrate that the CB-HPC-transplanted humanized NSG mouse model can recapitulate the HIV-induced chronic immune activation observed in infected humans and minocycline can be proposed as adjuvant therapy with HAART to treat the chronic immune activation and inflammation.

Materials and methods

Mouse conditioning

NSG mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were kept in microisolator cages and were fed with autoclaved food and water. Experiments on animals were performed according to institutional animal care and research committee guidelines, ethical application number-670. Busulfan (Sigma Aldrich, Munich, Germany) was dissolved in DMSO, diluted with RPMI-1640 and injected intraperitoneally in 3- to 4-week-old mice. Mice received 50 mg/kg of busulfan as two doses of 25 mg/kg with a 12-hr interval.

Transplantation of CB-HPCs and HIV inoculation in NSG mice

Human cord blood (CB) was obtained from the Cord Blood Bank Central Hospital University (Liège, Belgium) after obtaining informed consent. CB mononuclear cells were obtained after Ficoll–Hypaque density gradient centrifugation and $CD34^+$ haematopoietic stem cells were isolated from CB mononuclear cells using a magnetic-activated cell sorting $CD34^+$ progenitor cell isolation kit (Stem Cell Technologies, Grenoble, France) according to the manufacturer's instructions. After three-time enrichment, 96% of $CD34^+$ cells were positively selected. We transplanted 2×10^5 freshly isolated $CD34^+$ cells into mice by intravenous tail injection within 16–24 hr of a second busulfan injection. Infection and maintenance of NSG mice were performed in GIGA-R-Biosafety Level 3 facilities under standard caging conditions. After 130–150 days of CB-HPC transplantation, nine groups of mice ($n = 70$) (Table 1) were inoculated with HIV-1JRCSF (5000 $TCID_{50}$) by intraperitoneal route. Group 5, 6, 7 and 8 mice were treated with minocycline at a dose of 100 mg/kg/day by intraperitoneal injection. Six mice of group 9 were used to measure the basal level of cytokines and LPS in the plasma samples.

Flow cytometry of activation markers

Peripheral blood was collected from the tail vein of mice at 10-day intervals. Red blood lysis was performed before labelling with antibodies and cells were phenotyped by

Table 1. Groups of humanized mice and experimental analysis performed

Group number	Number of mice	Minocycline-treated	HIV inoculation	Analysis performed
1	10	No	Yes	CD38, T cells/μl, viral load
2	10	No	Yes	PD-1, T cells/μl, viral load
3	6	No	Yes	HL-DR, CD69, sCD14
4	6	No	Yes	CCR5, CTLA-4, cytokines, lipopolysaccharide (LPS)
5	10	Yes	Yes	CD38, T cells/μl, viral load
6	10	Yes	Yes	PD-1, T cells/μl, viral load
7	6	Yes	Yes	HL-DR, CD69, sCD14
8	6	Yes	Yes	CCR5, CTLA-4, cytokines, LPS
9	6	No	No	Cytokines, LPS

using BD FACSCanto II Flow Cytometer (BD Biosciences, Erembodegem, Belgium). Antibodies used for flow cytometric analysis were allophycocyanin-conjugated anti-human CD45, FITC-conjugated anti-human CD4, phycoerythrin-conjugated anti-human CD38, CD69, PD-1, CTLA-4, HLA-DR and peridinin-chlorophyll protein-conjugated anti-human CD8 from BD Biosciences. We also used phycoerythrin-conjugated anti-human CCR5 from R&D Systems (Abingdon, UK). Human leucocyte reconstitution of CD4 and CD8 cells was enumerated using a BD Trucount tube (BD Biosciences, Erembodegem, Belgium). The absolute numbers of cells were calculated using the BD trucount equation.

Viral load determination

The viral load was determined using Cobas ampliPrep/cobas TaqMan HIV-1 Test, version 2.0 (v2.0) (Roche, Vilvoorde, Belgium). This is an automated *in vitro* nucleic acid amplification test for the quantification of HIV-1 RNA in plasma using the Cobas ampliPrep instrument. We processed 20 mouse samples for each time-point. Fifty microlitres of murine plasma samples was diluted with 950 μl of assay diluent and processed with control seropositive and seronegative human plasma samples.

Measurement of sCD14 and LPS in plasma

Sixty days after HIV inoculation, 100 μl of plasma samples was used to determine LPS levels with the EndoLISA LPS measurement kit (Hyglos GmbH, Bernried, Germany). Reagents were prepared in LPS-free glass tubes and LPS-free tips and disposables were used. Levels of sCD14 were measured by quantikine human sCD14 immunoassay (R&D Systems).

Cytokine levels

Levels of cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α were quantified with 50 μl of plasma using a BD CBA human enhanced sensitivity kit and the master buffer kit or CBA human Th1/Th2 Cytokine Kit II (BD Biosciences, Erembodegem, Belgium) according to the manufacturer's instructions. Data were acquired with FACS Canto and analysed using FCAP Array software.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software. Data were expressed as the mean value ± standard deviation (SD). Significant differences between data groups were determined by two-sample Student's *t*-test analysis. A P-value $< 0{\cdot}05$ was considered to be significant. Two-tailed Spearman correlation coefficient (r) was calculated, with resulting P-values at 95% confidence.

Results

Effect of minocycline on viral load and T-cell count

HIV replicated efficiently in the humanized mouse model as early as 10 days after HIV inoculation and viral load was around 5 log copies/ml in both groups (untreated and minocycline-treated) of humanized mice ($n = 20$, each group) (Table 1) (Fig. 1a). At 10 days after HIV inoculation, the untreated group of mice had a viral load of $4{\cdot}95 \pm 0{\cdot}34$ log copies/ml that steadily increased to $6{\cdot}20 \pm 0{\cdot}60$ log copies/ml at day 60. Twenty days after HIV inoculation, viral load remained constant in the minocycline-treated group. It was around $5{\cdot}17 \pm 0{\cdot}55$ at day 30 after HIV inoculation and remained similar $5{\cdot}20 \pm 0{\cdot}55$ at day 60. Compared with the untreated infected group of humanized mice, viral load in the minocycline-treated group of mice was significantly lower at day 30 ($P = 0{\cdot}020$), 40 ($P = 0{\cdot}0054$), 50 ($P = 0{\cdot}0001$) and 60 ($P < 0{\cdot}0001$).

Number of $CD4^+$ T cells decreased after HIV inoculation in both groups of humanized mice (Fig. 1b). Before HIV inoculation, the concentration of $CD4^+$ T cells in untreated mice was $528{\cdot}4 \pm 33{\cdot}08$ cells/μl and it rapidly decreased by roughly 50% to $254{\cdot}6 \pm 66{\cdot}71$ cells/μl after 20 days of infection. At day 0 in HIV-infected,

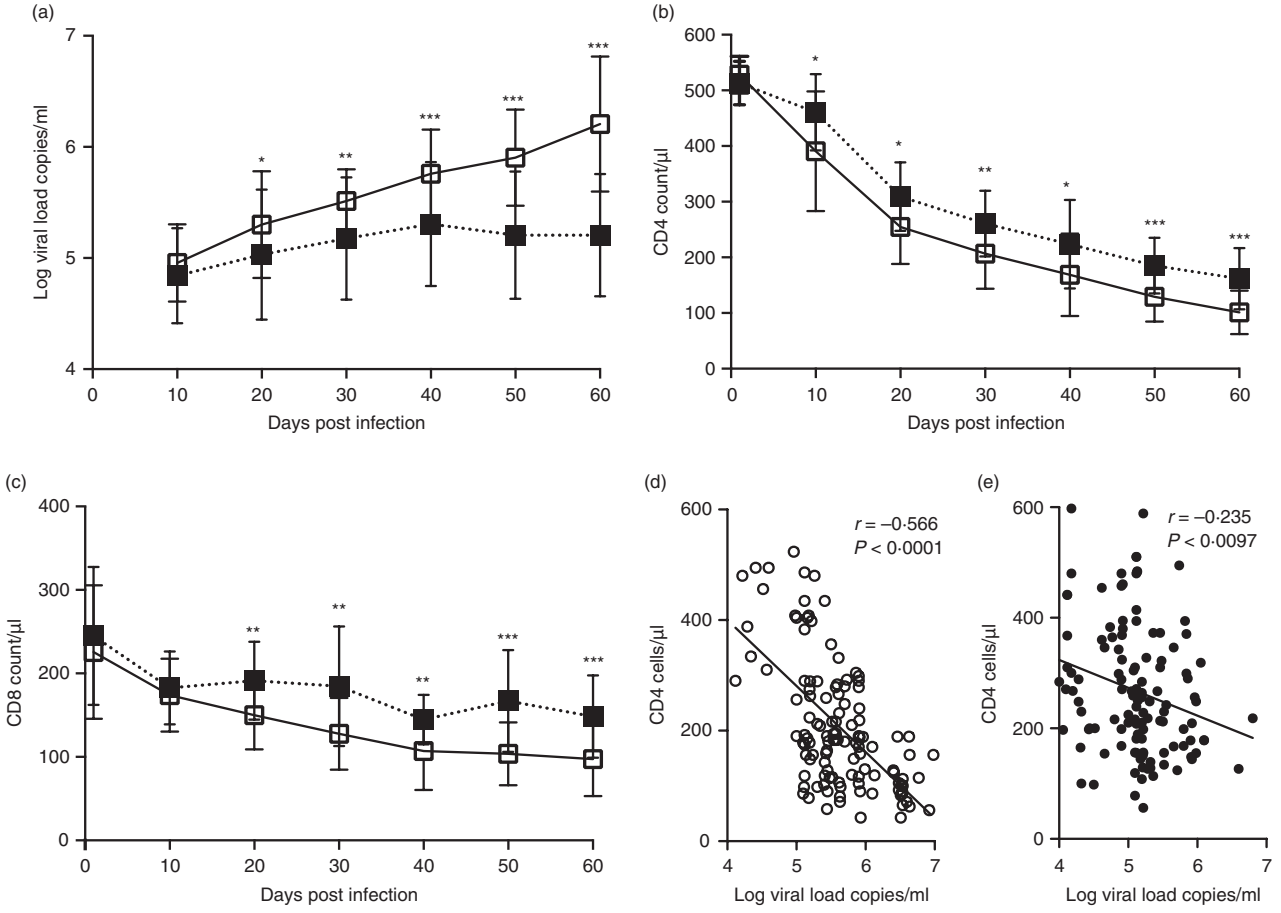

Figure 1. Viral load and T-cell count in HIV-infected non-treated and minocycline-treated mice. (a) HIV viral load during 60 days of HIV infection in non-treated (open boxes) and minocycline-treated (solid black boxes) humanized mice. (b, c) CD4 and CD8 T cells/μl during 60 days of HIV infection in non-treated (open boxes) and minocycline-treated (solid black boxes) humanized mice. (d) Strong inverse correlations of human CD4 cell count with the plasma viral load during 60 days of infection in HIV-infected non-treated humanized mice (open circle: non-treated). (e) Weaker inverse correlations between human CD4 count and plasma viral load in infected minocycline-treated humanized mice (solid circle: non-treated). Error bars indicate standard deviation. Results are presented as mean ± SD. *P* values were determined by unpaired Student's *t*-tests. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$. Linear regression along with trend lines from analysis are shown, and the two-tailed Spearman correlation coefficient (*r*) was calculated, with resulting *P* values at 95% confidence.

minocycline-treated mice the $CD4^+$ T-cell number was 513·1 ± 39·04 cells/μl and it decreased to 309·3 ± 61·37 cells/μl at 20 days after HIV inoculation. $CD4^+$ T-cell decline was much slower during the remaining period of the study. Fifty days after HIV inoculation, the minocycline-treated group had more $CD4^+$ T cells, 185·5 ± 50·13 cells/μl, compared with the untreated group, 129·2 ± 44·63 cells/μl ($P = 0{\cdot}0006$), of humanized mice. Similarly, at day 60, the minocycline-treated group had more $CD4^+$ T-cells, 161·6 ± 55·08 cells/μl, compared with the untreated group of mice, 101·3 ± 38·89 cells/μl ($P = 0{\cdot}0003$).

HIV infection also caused a slow but steady decrease in the number of $CD8^+$ T cells in the untreated group of mice. Compared with $CD4^+$ T cells, the decline of the CD8 cell count was less drastic. The most significant decline of $CD8^+$ T cells occurred during the first 10 days of infection (Fig. 1c). During this period, $CD8^+$ T-cell number declined from 225·6 ± 79·77 to 174·2 ± 43·69 cells/μl in the untreated humanized mice, whereas the drop was less pronounced in the minocycline-treated group (from 245·0 ± 82·70 to 182·8 ± 43·81 cells/μl). In the remaining 50 days of the study, there was a small decline of $CD8^+$ T-cell number in the minocycline-treated group (182·8 ± 43·81 to 148·5 ± 49·32 cells/μl). However, during the same period, there was a sharp decline of $CD8^+$ T cells in the untreated group and the CD8 count was only 97·5 ± 44·28 cells/μl 60 days after HIV inoculation. Interestingly, we observed a strong inverse correlation ($r = -0{\cdot}602$; $P < 0{\cdot}0001$) between viral load and CD4 count in HIV-infected untreated humanized mice (Fig. 1d). In the minocycline-treated group of humanized mice, this correlation was weak ($r = -0{\cdot}238$; $P < 0{\cdot}0088$) (Fig. 1e).

Effect of minocycline on CD38 mean florescence intensity on T cells

CD38 expression on $CD8^+$ cells is a surrogate marker for HIV disease progression.[22,23] The expression level of CD38 on $CD8^+$ T cells strongly correlates directly with the viral load and indirectly with CD4 cell count.[24] Peripheral blood leucocytes from HIV-infected untreated ($n = 10$) and minocycline-treated humanized mice ($n = 10$) were assessed for CD38 mean fluorescence intensity (MFI) on $CD4^+$ and $CD8^+$ T cells. We performed CD38 expression analysis seven times, at 10-day intervals (Fig. 2a, c). The MFI of CD38 expression increased soon after HIV inoculation. Minocycline-treated mice had lower levels of CD38 expression on $CD4^+$ T cells compared with the untreated humanized mice. The more pronounced difference of CD38 MFI on $CD4^+$ T cells was observed at day 50 (5477 ± 1518 versus 7697 ± 1130, $P = 0{\cdot}0016$) and day 60 (5719 ± 1486 versus 8251 ± 1916, $P = 0{\cdot}0040$) after HIV inoculation between the minocycline-treated and untreated groups (Fig. 2a). MFI of CD38 on $CD8^+$ T cells remained significantly different between two groups of mice starting from 20 days after HIV inoculation. At the last three time-points of analysis, MFI was 8984 ± 1067, 9638 ± 1419 and $10\,096 \pm 1737$ in minocycline-treated group of humanized mice and much higher in the untreated group at $10\,756 \pm 850$ ($P = 0{\cdot}0007$), $12\,192 \pm 809{\cdot}9$ ($P = 0{\cdot}0001$) and $12\,806 \pm 1090$ ($P = 0{\cdot}0006$), respectively (Fig. 2b).

Several former studies have shown that CD38 expression on $CD8^+$ T cells is well correlated with plasma viral load.[24] Hence we verified the correlation between viral load and the frequency of CD38 expression on $CD8^+$ T cells in the HIV-infected humanized mice. In the untreated group of infected humanized mice the MFI of CD38 on $CD8^+$ T cells was moderately correlated with HIV viral load ($r = 0{\cdot}365$; $P = 0{\cdot}0041$) (Fig. 2c). In the minocycline-treated group, the MFI of CD38 on $CD8^+$ T cells was weakly correlated with the viral load ($r = 0{\cdot}261$; $P = 0{\cdot}0439$) (Fig. 2d). We also observed a strong inverse correlation between CD38 MFI on $CD8^+$ and $CD4^+$ T-cell counts in untreated ($r = -0{\cdot}637$; $P < 0{\cdot}0001$) (Fig. 2e) and minocycline-treated ($r = -0{\cdot}705$; $P < 0{\cdot}001$) (Fig. 2f) humanized mice groups. Levels of CD69, CCR5 and HLA-DR also differed between infected non-treated and minocycline-treated groups (see Supporting information, Fig. S1).

Minocycline limits the expression of immune exhaustion markers PD-1 and CTLA-4

In HIV-infected individuals, PD-1 and CTLA-4 expression on T cells is driven by viral replication and associated with T-cell exhaustion and immune cell dysfunction.[25,26]

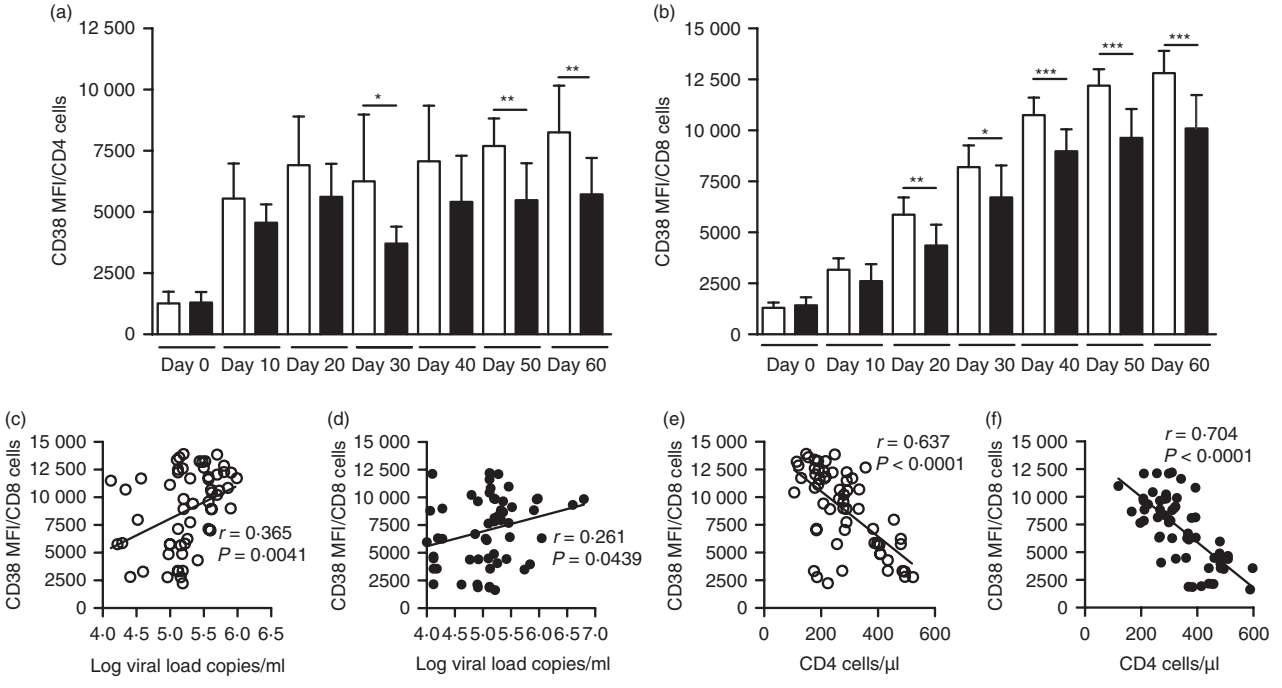

Figure 2. Expression of cellular immune activation marker CD38 on T cells. (a, b) Change in mean florescence intensity of $CD38^+$ on CD4 and CD8 T cells in the peripheral blood on HIV-infected non-treated (open bars) and HIV-infected minocycline-treated (solid black bars) humanized NSG mice during 60 days of infection. Results are presented as mean ± SD. *P*-values were determined by unpaired Student's *t*-tests. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$. (c–f) Mean florescence intensity of CD38 on CD8 T cells correlates with viral load and inversely to CD4 count in HIV-infected non-treated mice (open circle: non-treated and solid circle: minocycline-treated). Linear regression along with trend lines from analysis are shown, and the two-tailed Spearman correlation coefficient (r) was calculated, with resulting P values at 95% confidence.

Peripheral blood leucocytes from HIV-infected non-treated ($n = 10$) and minocycline-treated ($n = 10$) humanized mice were analysed seven times at 10-day intervals to determine the frequency of PD-1-expressing cells. This frequency increased after HIV inoculation in both groups of mice. Minocycline-treated humanized mice had a significantly lower level of PD-1-expressing CD4 cells at day 20 after HIV inoculation. At day 50, it was 47·4 ± 11·5% in the minocycline-treated group and 61·1 ± 8·0% in non-treated mice ($P = 0·0071$). On day 60, it was 52·4 ± 8·6% in the minocycline-treated mice and 66·86 ± 8·2% in non-treated mice (Fig. 3a). The frequency of CD8 T cells expressing PD-1 also differed between infected non-treated and minocycline-treated mice as soon as 30 days after HIV inoculation. At day 50, the frequency of $CD8^+$ $PD\text{-}1^+$ T cells was 51·2 ± 6·8% in the minocycline-treated group and 63·8 ± 5·8% ($P = 0·0003$) in non-treated mice. Sixty days after HIV inoculation, this frequency was 71·5 ± 5·8% in the non-treated and 60·8 ± 8·0% ($P = 0·0030$) in the minocycline-treated group (Fig. 3b, g).

PD-1 expression on $CD4^+$ T cells has been shown to correlate positively with viral load and inversely with $CD4^+$ T-cell count.[25] Accordingly, we observed a strong correlation between the frequency of $CD4^+$ $PD\text{-}1^+$ T cells and viral load in non-treated mice ($r = 0·588$; $P < 0·0001$) (Fig. 3c) whereas this correlation was weak in the minocycline-treated mice ($r = 0·138$; $P = 0·2911$) (Fig. 3d). However, there was a strong inverse correlation with CD4 cells/μl in both minocycline-treated ($r = -0·750$; $P < 0·0001$) and non-treated ($r = -0·744$; $P < 0·0001$) groups (Fig. 3e, f).

It has also been shown that up-regulation of CTLA-4 on $CD4^+$ T cells correlates with HIV disease progression and defines a reversible immune dysfunction in infected individuals.[27] We measured CTLA-4 frequency at days 0, 30 and 60 after HIV inoculation in non-treated ($n = 6$) and minocycline-treated ($n = 6$) humanized mice and observed a significant increase in CTLA-4 expression on $CD4^+$ T cells. Compared with non-treated mice, the frequency of CD4 T cells expressing CTLA-4 was significantly lower in the minocycline-treated group. Thirty days after HIV inoculation, untreated mice had a $CD4^+$ $CTLA\text{-}4^+$ frequency of 41·9 ± 6·8%, which was significantly higher ($P = 0·0258$) than what was observed in minocycline-treated mice (31·8 ± 8·0%). This difference was even more significant ($P = 0·0035$) at day 60. The frequency of $CD8^+$ $CTLA\text{-}4^+$ T cells was much lower compared with $CD4^+$ T cells in both groups of mice and there was no difference between non-treated and minocycline-treated mice (Fig. 3h, i).

Effect of minocycline on LPS, sCD14 and IL-10

Increased microbial translocation from the gastrointestinal tract has been implicated in driving the chronic immune activation observed in HIV-infected individuals.[28] We measured LPS levels in plasma samples of non-infected, infected untreated and infected minocycline-treated humanized mice. LPS level was below the detection limit in non-infected humanized mice ($n = 6$). Infected untreated mice ($n = 6$) had significantly higher levels of LPS (10·04 ± 3·30 EU/ml), compared with minocycline-treated mice (0·50 ± 0·20 EU/ml) ($P < 0·001$) ($n = 6$) (Fig. 4a). An increased level of sCD14 is a marker of monocyte response to LPS in HIV-infected individuals.[29] Non-infected mice ($n = 6$) had 124·5 ± 7·10 ng/ml of sCD14. The levels of sCD14 significantly increased (156·3 ± 13·70 ng/ml) in infected untreated mice ($n = 6$). Minocycline-treated mice ($n = 6$) had significantly lower levels of sCD14 (135·1 ± 5·48 ng/ml) ($P = 0·0025$) compared with untreated infected mice (Fig. 4b).

HIV infection resulted in disruption of homoeostasis of cytokine profiles. Levels of cytokines increased significantly in infected mice ($n = 6$ each group) compared with non-infected mice ($n = 6$) (see Supporting information, Fig. S2). However, only the IL-10 level differed significantly (325·7 ± 35·90 versus 36·87 ± 20·62 pg/ml, $P = 0·0033$) between infected untreated and infected minocycline-treated mice (Fig. 4c).

Discussion

The results presented here demonstrate that NSG mice humanized by transplantation of CB-HPCs and infected with HIV emulate the chronic immune activation state observed during HIV infection in humans. They furthermore show that a chronic treatment with minocycline has a significant inhibitory effect on HIV replication and on expression levels of cellular/plasma immune activation markers in humanized mice.

Because of the inherent complexity of HIV-associated immune activation and the existence of controversies regarding its mechanism, it is difficult to draw parallels for all aspects of HIV-associated immune activation observed in humans with those observed in this murine model in a single study. However, features such as the increased expression of activation (CD38, HLA-DR, CD69 and CCR5) and exhaustion markers (PD-1 and CTLA-4) are precisely described in humans. It is also the case for the increased levels of circulating LPS and sCD14. Moreover, the strong correlation between $CD38^+$ MFI and PD-1 expression with the viral load and CD4 count are also similarly present in human infection and in our model. Early establishment of T-cell activation highlights the relevance of this phenomenon for the pathogenesis of HIV disease in our murine model. Studies performed on HIV-infected individuals have also suggested that the immune activation set point is established during the early acute phase of infection and is responsible for determining the rate of disease progression.[30]

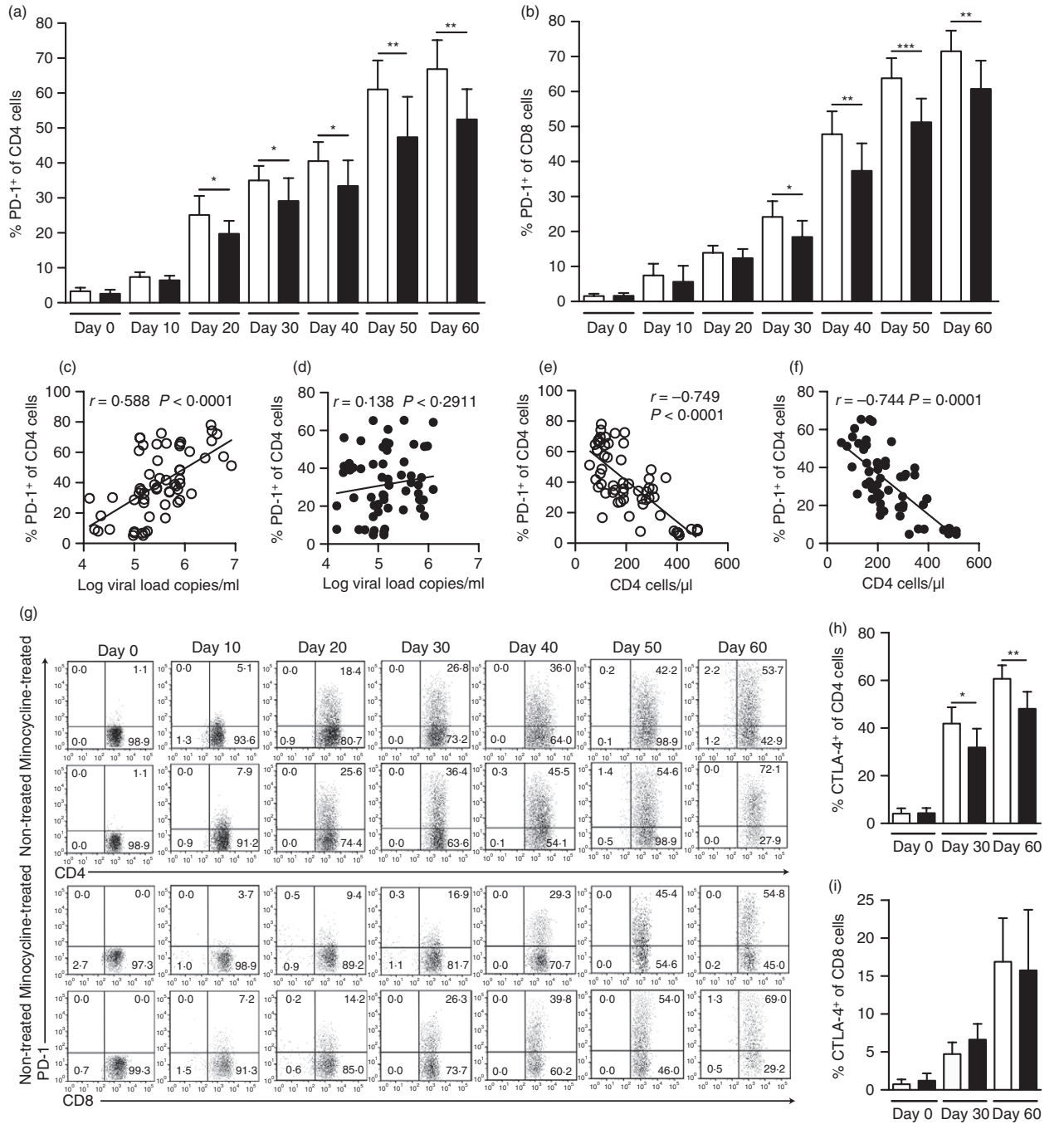

Figure 3. Frequencies of T cells expressing immune exhaustion markers PD-1 and CTLA-4. (a, b) Change in frequencies of CD4 PD-1$^+$ and CD8 PD-1$^+$ T cells in the peripheral blood in HIV-infected non-treated (open bars) and HIV-infected minocycline-treated (solid black bars) humanized NSG mice during 60 days of infection. Frequency of CD4 PD-1$^+$ T cells strongly positively correlated with viral load (c) and CD4 cells/µl (e) in HIV-infected non-treated mice (open circle: non-treated). Weaker correlation existed between the frequency of CD4 PD-1$^+$ T cells and viral load in minocycline-treated groups of mice (d, solid circle: minocycline) but a stronger correlation was found with CD4 cell/µl (f). (g) Dot plot for PD-1 frequency on CD4 and CD8 cells. Acquisition gate was set on human CD45 cells and further gatings were performed on CD4 and CD8 cells. (h, i) Frequency of CTLA-4 on CD4 and CD8 T cells of peripheral blood, at days 0, 30 and 60 after HIV infection. Results are presented as mean ± SD. *P* values were determined by unpaired Student's *t*-tests. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ and ***$P < 0{\cdot}001$. Linear regression along with trend lines from analysis are shown, and the two-tailed Spearman correlation coefficient (*r*) was calculated, with resulting *P* values at 95% confidence.

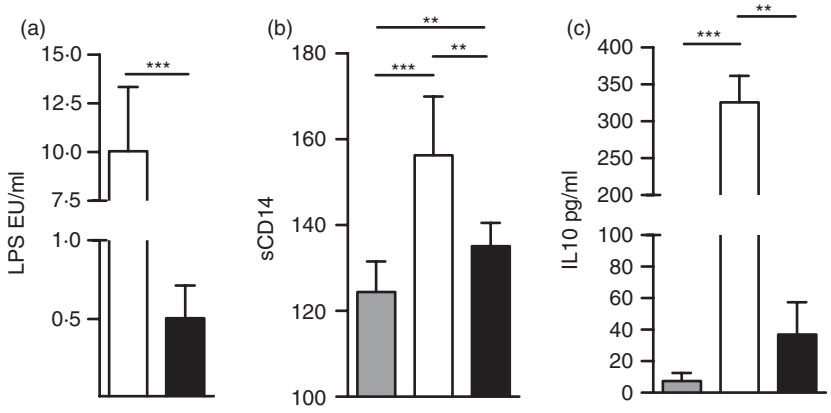

Figure 4. Levels of lipopolysaccharide (LPS), sCD14 and interleukin-10 (IL-10) in plasma of non-infected, infected non-treated and infected minocycline-treated mice. (a) Significant difference in plasma LPS levels in between infected non-treated and infected minocycline-treated mice. LPS levels were below detection limits in non-infected age-matched mice (b, c). Higher plasma sC14 and IL-10 levels in HIV-infected mice compared with the other two groups (open bars: HIV-infected non-treated, solid black bars: HIV-infected minocycline-treated, solid grey bars: non-infected non-treated mice). Results are presented as mean ± SD. *P* values were determined by unpaired Student's *t*-tests. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$.

Unlike what has been described in the humanized BLT-NSG model,[10,31] we have also observed a strong and inverse correlation between viral load and CD4 count in the course of infection. This allowed us to investigate and to evaluate the potential of minocycline as a way to prevent the establishment of HIV-associated immune activation during the acute phase of infection.

Our study in humanized NSG mice suggests that minocycline inhibits both HIV replication and the expression of chronic immune activation markers. HIV infection is a self-perpetuating cycle in which HIV replication drives chronic immune activation, which further promotes replication of the virus. Based on previous observations, we can speculate that minocycline can interrupt this vicious cycle at various steps as its immunomodulatory effects are well recognized.[32–35] Szeto *et al.*, showed that minocycline has indeed a strong influence on the expression of activation markers and cytokines after *in vitro* stimulation of non-infected T cells.[14] Recently, it was also shown that minocycline administered in a vaginal microbicidal preparation, down-regulates the activation of $CD4^+$ T cells and the recruitment of immune cells into vaginal tissues of mice challenged with HIV-1.[36] Another report suggests that minocycline acts by suppressing activation of the nuclear factor of activated T cells 1 (NFAT-1) in $CD4^+$ T cells.[37] Hence, our observations of lower levels of HIV-associated activation markers (CD38, HLA-DR, CCR5) are consistent with previous reports and possibly reflect the effect of minocycline on NFAT-1 function.

One of the direct consequences of immune activation is the facilitation of virus replication. In SIV-infected macaques and in HIV-infected individuals, immune activation was correlated with the induction of viral replication *in vivo*.[38] We can therefore make a link between the blunted immune activation and the lower levels of viral load in minocycline-treated mice. However, it is difficult to formally establish the direction of the causality between immune activation and virus replication because a direct role of minocycline in inhibiting HIV replication cannot be ruled out. Previous studies have indeed predicted high binding affinity of minocycline to HIV-1 integrase.[19] Moreover, blocking of nuclear factor-κB induction by minocycline could also interfere with HIV-1 transcription and infection in T cells.[39] Significantly lower viral loads have also been observed in plasma samples of SIV-infected rhesus macaques treated with minocycline.[18]

It is known that substantial decline of $CD4^+$ T cells in untreated HIV infection is the result of apoptosis of T cells.[18] Increased caspase-3 activity is observed in individuals with progressive HIV infection and caspases have been associated with HIV-mediated apoptosis.[40,41] Minocycline has been shown to interfere with both caspase-dependent and independent cell death processes.[42–44] Hence higher T-cell numbers in HIV-infected minocycline-treated mice might be the result of direct anti-apoptotic effects.

Microbial translocation of commensal microbial products from the gut into the systemic circulation plays a key role in driving chronic persistent immune activation in HIV-infected individuals.[45] A strong correlation between plasma LPS level and the frequency of circulating activated $CD38^+$ $HLA\text{-}DR^+$ CD8 T cells is reported in HIV-infected individuals.[46,47] We have also observed increased levels of LPS in the plasma of HIV-infected humanized mice, as previously reported in other studies.[47,48] Increased secretion of TNF-α has been proposed as a cause for increased intestinal permeability by disrupting tight junctions and inducing apoptosis of intestinal

epithelial cells in HIV-infected humanized RAG$^{-/-}$ $\gamma^{-/-}$ mice.[48] Interestingly, we also found increased TNF-α levels in our model of HIV-infected mice; however, it is noteworthy that, in our hands, minocycline had no significant effect on plasma levels of TNF-α (Fig. S2) despite the associated strong reduction of microbial translocation. We postulate that the observed difference in LPS levels might be a direct antibiotic effect of minocycline leading to an overall reduction of intestinal bacterial load and therefore, to reduced microbial translocation. This assumption is supported by the fact that minocycline has a 38-fold higher concentration in bile than in serum[49] and has been shown to decrease the bacterial load of colon and caecum.[50] Furthermore, minocycline might also play a role in preserving gut integrity as it attenuates nitric oxide synthase and matrix metalloproteinase synthesis and preserves gut integrity in an experimental model of colitis.[51] We also found significant differences in sCD14 levels between minocycline-treated and non-treated HIV-infected humanized mice. To our knowledge, it is the first study to measure levels sCD14 in HIV-infected humanized mice.

HIV infection leads to a significant increase in cytokine levels in infected humanized mice. Interestingly, we did not observe any difference in levels of cytokines between HIV-infected non-treated and minocycline-treated groups except for IL-10, which was significantly higher in the infected non-treated group. This observation does not correspond with previous findings, which suggested that minocycline can preclude nuclear factor-κB translocation and impede the mitogen-activated protein kinase pathways and therefore, block the secretion of pro-inflammatory cytokines.[52–54] The reason for this discordance is not clear. It is conceivable that the strong inhibition of IL-10 (a cytokine with anti-inflammatory properties) by minocycline has irrevocably antagonized its direct inhibitory effects on the other cytokines. Additional experiments are needed to clarify this point.

Persistent antigenic stimulation during HIV infection leads to immune exhaustion with subsequent loss and proliferative capacity of effector T cells. It is also characterized by up-regulation of PD-1 and CTLA-4 expression on T cells. We observed a significant difference in the levels of expression of these markers between infected non-treated and minocycline-treated groups. Minocycline had been earlier shown to regulate levels of NFAT-1 in T cells.[37] The observed difference in the levels of PD-1 expression on T cells might be correlated to this finding because NFAT-1 regulates NFATc1 levels, on the one hand,[55] and NFATc1 regulates levels of PD-1 expression on T cells on the other.[56,57] Therefore, minocycline regulation of PD-1 expression on T cells via NFAT-1/NFATc1 cannot be ruled out, although further experiments might be needed to verify this correlation. Moreover, it is also known that NFAT-1 directly regulates levels of CTLA-4 expression,[57] which is suppressed by minocycline in T cells. It would be interesting to find out if the blunting of PD-1 expression by minocycline is also associated with improved CD8$^+$ T-cell function, especially directed against HIV antigens. This could pave the way for the use of minocycline as an adjunctive treatment during therapeutic vaccine trials aimed at restoring an efficient anti-HIV immune function in chronically infected patients.

In summary, our data demonstrate that immune activation observed in HIV-infected individuals can be modelled in HIV-infected humanized NSG mice. This model is therefore, an effective tool to dissect the mechanisms involved. Moreover, the potential of therapeutic interventions to reduce persistent T-cell activation in the setting of HIV infection can be screened more rapidly in this humanized NSG mouse model. The encouraging results observed in this study confirm that minocycline has potential as an anti-HIV therapy, specifically in the context of immune activation and inflammation.

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Disclosure

The authors declare that no conflict of interest exists.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Frequency of T cells expressing immune activation markers CD69, HLA-DR A and co-receptor CCR5 (a, b) Frequency of early immune activation marker CD69 on CD4 and CD8 T cells of peripheral blood at day 0, 7 and 14 after HIV infection. (c, d) CCR5 frequency on CD4 and CD8 T cells at days 0, 30 and 60 after HIV infection in peripheral blood of humanized mice. (e, f) Frequency of HLA-DR on T cells from peripheral blood of humanized mice. Open bars: HIV-infected non-treated; solid bars: HIV-infected minocycline-treated. Error bars indicate standard deviation. Results are presented as mean ± SD. *P* values were determined by unpaired Student's *t*-tests. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$.

Figure S2. Levels of cytokines in plasma samples. (a, b, c, d, e) Levels of interleukin-2 (IL-2), IL-4, IL-6, interferon-γ (IFN-γ)and tumour necrosis factor-α (TNF-α) cytokines in humanized mice. Open bars: HIV-infected non-treated; solid black bars: HIV-infected minocycline-treated; and solid grey bars: non-infected and minocycline-treated. Error bars indicate standard deviation. Results are presented as mean ± SD. *P*-values were determined by unpaired Student's *t*-tests. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$.

