Chronic HIV infection induces transcriptional and functional reprogramming of innate immune cells

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Abstract

Chronic inflammation and immune dysfunction play a key role in the development of non-AIDS related comorbidities. The aim of our study was to characterize the functional phenotype of immune cells in people living with HIV (PLHIV). We enrolled a cross-sectional cohort study of PLHIV on stable antiretroviral therapy and healthy controls. We assessed ex vivo cytokine production capacity and transcriptomics of monocytes and T-cells upon bacterial, fungal and viral stimulation. PLHIV exhibited an exacerbated pro-inflammatory profile in monocyte-derived cytokines, but not in lymphocyte-derived cytokines. Particularly, the production of the IL-1β to imiquimod, E. coli LPS and Mycobacterium tuberculosis was increased, and this production correlated with plasma concentrations of hsCRP and sCD14. This increase in monocyte responsiveness remained stable over time in subsequent blood sampling after >1 year. Transcriptome analyses confirmed priming of the monocyte IL-1β pathway, consistent with a monocyte trained immunity phenotype. Increased plasma concentrations of β-glucan, a well-known inducer of trained immunity, were associated with increased innate cytokine responses. Monocytes of PLHIV exhibit a sustained pro-inflammatory immune phenotype with priming of the IL-1β pathway. Training of the innate immune system in PLHIV likely plays a role in long-term HIV complications and provides a promising therapeutic target for inflammation-related comorbidities.
Introduction

Persistent inflammation and immune dysfunction play an important role in the development of non-AIDS related comorbidities in people living with HIV (PLHIV). Those include cardiovascular disease (CVD), neurocognitive dysfunction and cancer (1-4). Reducing inflammation is considered to be an attractive therapeutic target to reduce the burden of non-AIDS related events. However, immune dysfunction and persistent inflammation in PLHIV are complex processes that are still incompletely understood. These involve changes in the adaptive immune system, including dysfunction and senescence of T-cell lymphocytes (5), as well as changes in the innate immune system. Mediators of the latter, including soluble (s)CD14, sCD163 and high-sensitivity C-reactive protein (hsCRP) are indicative of persistent immune activation and are associated with non-AIDS related events (6-8).

Ongoing exposure to microbial products, for example by cytomegalovirus virus (CMV), continuing HIV replication or microbial translocation, may be one of the drivers of persistent inflammation (2, 9-12). Increased inflammation can also result from a functional adaptation of innate immune cells induced by epigenetic reprogramming; a process termed ‘trained immunity’ (13-16). Trained immunity is recognized to play an important beneficial role in host defense processes, but may also contribute to conditions like atherosclerosis or type 2 diabetes mellitus (17, 18). Whether trained immunity contributes to persistent inflammation in PLHIV is currently unknown.

The Human Functional Genomics Project (HFGP) aims to investigate variation in the immune responses (19). Studies in HFGP cohorts in healthy individuals have yielded different novel insights in the genetic and non-genetic regulation of inflammatory cytokine responses in response to microbial ligands (20-23). We established a HFGP cohort of Dutch PLHIV and in this cohort we describe alterations in the function and phenotype of peripheral blood mononuclear cells (PBMC) by using an integrative approach of functional and transcriptional analyses. We also explored underlying mechanisms of the inflammatory monocyte profile, including the possible contribution of exposure to microbial translocation, CMV seropositivity and HIV reservoir size.
Results

Characteristics
A total of 211 PLHIV on combination antiretroviral therapy (cART) and 56 HIV-uninfected controls were included in the analyses. Baseline characteristics are shown in Table 1. Compared to the healthy controls, PLHIV were more often male (91.5% vs. 60.7%; p<0.001) and of older age (median [IQR] age of 52.5[13.2] vs 30.0 [27.1] years, p<0.0001). The median (IQR) duration of cART use in PLHIV was 6.6yr (4-12yr) and 89% were virally suppressed (HIV viral copies ≤50 copies/mL) for more than one year.

Circulating inflammatory markers and innate cytokine responses
We first measured concentrations of circulating inflammatory markers. Compared to healthy controls PLHIV had significantly higher concentrations of sCD14, hsCRP, IL-18, IL-18 binding protein (IL-18BP) and IL-6 (Figure 1A-G). These differences remained after adjusting for age, sex, body mass index (BMI), or seasonality (Figure 1A). Concentrations of adipokines were similar between groups after correction (Figure 1A).

Next, we analyzed functional changes in the innate and adaptive immune response. PBMCs were incubated with 12 stimuli (4 bacterial, 3 fungal, 1 viral and 6 Toll like receptor (TLR) ligands), followed by measurement of different monocyte-derived (IL-6, IL-1β, TNFα) and lymphocyte-derived (IL-17, IL-22, IFNγ) pro-inflammatory cytokines, as well as anti-inflammatory cytokines (IL-10, IL-1Ra) in the supernatant. We observed markedly increased monocyte-derived cytokine responses in PLHIV, especially IL-1β responses upon stimulation with LPS (TLR4), imiquimod (TLR7) and Mycobacterium tuberculosis (Figure 2A,C,D). Production of TNFα and IL-6 were also increased in PLHIV (Figure 2A,E), except for lower production in response to Staphylococcus aureus (TNF-α) and Candida albicans (IL-6; Figure 1F,G). This increased pro-inflammatory cytokine response in PLHIV was not associated with a concurrent increase in the anti-inflammatory cytokines IL-1Ra or IL-10. However, the release of IL-1Ra by unstimulated cells was higher in PLHIV (Figure 2H), which is consistent with increased basal IL-1β production (Figure 2A). The number of monocytes in the isolated PBMCs (Supplemental Figure 1A-B) may influence cytokine production, but adjusting for the monocyte fraction in PBMCs did not significantly alter the outcome (Supplemental Figure 1C). T-cell-derived cytokine responses were generally lower for most stimuli in the PLHIV cohort (Supplemental Figure 2), but these differences were abrogated following adjustment for age, sex and seasonality (Figure 1A), except for IFNγ responses to M. tuberculosis and C. albicans hyphae (Figure 2A-B). There were no associations between cytokine production capacity and smoking or different HIV-specific traits, including recent CD4 cell count, CD4/8 cell ratio, CD4 nadir, cART-regimen or co-medication (metformin, statins or aspirin) (Supplemental Figure 3).

Next, we assessed associations between innate cytokine responses, circulating inflammatory markers, and monocyte phenotypes (Figure 3A-I). Increased ex vivo cytokine responses were associated with persistent inflammation. For example, we found positive associations between IL-6 responses and circulating hsCRP (Figure 3A,I), and between LPS induced IL-1β responses and plasma sCD14 and IL-18BP (Figure 3A,D). In addition, non-
classical and intermediate monocyte subsets (Supplemental Figure 4A-F), which are considered pro-inflammatory monocyte phenotypes, correlated with higher IL-1β and IL-6 production after LPS or imiquimod, respectively (intermediate monocytes vs LPS-IL-1β R=0.23, p<0.001; Figure 3A-C) (24). Taken together, we show that PLHIV have an altered innate immune profile with markedly elevated monocyte cytokine responses, particularly IL-1β, which correlates with blood biomarkers of persistent inflammation.

**Increased IL-1β production capacity of PLHIV is stable over time**

We then assessed the longevity of the elevated monocyte-derived cytokine responses. We resampled 28 male PLHIV, aged >45yr after >1yr and stratified them on the basis of their initial IL-1β production capacity in a group of low (lowest quartile) and high (highest quartile) IL-1β producers. Fourteen age and sex-matched uninfected controls were enrolled concurrently (Supplemental Table 1). Upon resampling, those assigned to the high IL-1β producer group still exhibited higher IL-1β and IL-6 production compared to participants in the low IL-1β producer group and matched healthy controls, suggesting that the enhanced monocyte responsiveness is stable over time (Figure 4A-B). TNFα followed a similar, but non-significant pattern (P = 0.075; Figure 4C). To examine if the presence of T-lymphocytes affected the outcome, we isolated monocytes using magnetic beads (purity >95%, Supplemental Figure 4G) and repeated the stimulation experiments. We found that IL-1β production remained increased, suggesting that the elevated monocyte-derived cytokine response is due to an enhanced functional state of the monocytes themselves.

**Enhanced pro-inflammatory functional state of monocytes**

Based on the previous findings, we postulated that a trained immunity functional phenotype contributes to the enhanced monocyte responsiveness in PLHIV. Hence, we isolated monocytes from eight PLHIV and four age and sex matched uninfected controls and analyzed their transcriptomes by RNA sequencing. Using principal component analysis (PCA), we observed clustering of the PLHIV and controls (Figure 5A). This clustering was even more pronounced when monocytes were differentiated towards macrophage phenotype by culture for 24hr in serum-free medium (RPMI; Figure 5B) and was also observed using hierarchical clustering (Figure 5C). Gene ontology (GO) analysis of 303 upregulated and 30 downregulated genes (Supplemental Data) showed that pro-inflammatory pathways were upregulated in PLHIV, including the ‘inflammatory response’, ‘regulation of innate immune response’ and ‘IL-1β production’ pathways (Figure 5D-E). Among the upregulated genes in these pathways were intracellular signaling proteins and inflammasome related molecules (e.g. NLRP3, STAT1), cytokines (e.g. IL1B, CCL2, MMP9, IL1RN), and pattern-recognition receptors (e.g. TLR2, TLR4, TLR7 and NOD2), underlining the broad upregulation of inflammatory pathways in PLHIV on long-term cART.

**Enhanced IL-1β gene expression in monocytes of PLHIV**

The release of active IL-1β from blood monocytes involves a priming signal that is mostly transcriptionally driven, which results in the synthesis of the IL-1β precursor (proIL-1β). This precursor is subsequently processed via caspase-1 into active IL-1β (25). Using RT-qPCR on monocytes stimulated with IMQ, we found that the IL1B (gene encoding for proIL-1β) RNA expression was higher in monocytes from PLHIV compared to controls (Figure 6A, P
= 0.021). IL6 mRNA followed a similar trend (Figure 6B, \( P = 0.027 \)), whereas TNFA expression did not (data not shown, \( P = 0.74 \)). Additionally, we measured intracellular proIL-1β and active IL-1β protein by ELISA and both were significantly elevated in PLHIV (Figure 6D-E). The inflammasome promotes the proteolytic cleavage of proIL-1β into active IL-1β, resulting in reduced proIL-1β / active IL-1β ratios in situations with increased caspase-1 activity (25). Instead, we observed a trend towards an increased ratio of intracellular proIL-1β and active IL-1β (Figure 6F) in the high IL-1β producing PLHIV, and no differences in levels of NLRP3 expression (Figure 6C). Together, these results demonstrate that the enhanced IL-1β transcription, rather than increased processing through caspase-1, is the biological process that primarily drives the increased IL-1β production in PLHIV.

**β-glucan exposure induces a pro-inflammatory monocyte phenotype**

We next examined possible pathways underlying the pro-inflammatory monocyte phenotype in PLHIV. Persistent exposure to microbial ligands derived from gut microbes, CMV or HIV itself has been suggested to play a role in the persistent inflammation in PLHIV (2, 9-12). We postulated that these factors also play a role in the pro-inflammatory monocyte phenotype. We, therefore, first assessed whether innate cytokine responses were associated with parameters of the HIV viral reservoir. The HIV reservoir was assessed by analyzing CD4+ cell-associated HIV-1 DNA (CA-DNA) and CA-RNA. In virally suppressed patients, the CA-DNA roughly equals the integrated HIV-1 DNA, being replication competent or not (26), while CA-RNA is associated with recent HIV-1 transcriptional activity and serves as a proxy for the active proviral reservoir (27). While cell associated HIV-1 CA-DNA levels in CD4+ T-cells were associated with plasma IL-6 concentrations (Figure 7A), there was no association between IL-1β cytokine responses (Supplemental Figure 5) or plasma sCD14 concentrations and HIV-1 CA-DNA or CA-RNA levels in our cohort (Figure 7B-H).

Next, we assessed associations between cytokine responses and CMV seropositivity. In total 198/211 (93.8%) of participants were CMV seropositive. There were no differences in IL-1β cytokine responses, sCD14 concentrations or HIV-1 CA-DNA between CMV seropositive vs. seronegative individuals (Figure 7I-L, Supplemental Figure 5).

Another source of chronic microbial ligand exposure in PLHIV is increased microbial translocation (9). Plasma concentrations of Intestinal fatty acid binding protein (iFABP), a marker for microbial translocation, were significantly increased in PLHIV compared to controls (Figure 7M). We found a modest negative correlation of iFABP concentrations with IL-1β cytokine responses (Figure 7N-O), but no correlation with sCD14 concentrations (Figure 7P).

Increased intestinal translocation may also increase circulating concentrations of β-glucan, which is a well-known inducer of trained immunity (28). We therefore determined serum β-glucan concentrations in the subsequent cohort of 28 PLHIV and 12 controls. Compared to controls (4/14; 29%), a significantly higher proportion of PLHIV (16/28; 57%), and especially those in the high IL-1β producer group (11/15; 73%), had detectable β-glucan in serum (Figure 8A-B). Individuals with detectable serum β-glucan had elevated IL-1β production (Figure 8C-D), showed increased IL1β gene expression (Figure 8E) and increased intracellular proIL-1β upon stimulation (Figure 8F). Concurrently, IL1Ra production was increased in PLHIV with detectable β-glucan concentrations (Figure 8G). A similar, non-significant trend was observed for IL-6 and TNFα responses.
(Figure 8H-I). A similar, non-significant trend was observed for IL-6 and TNFα responses (Figure 8I-L). Next, using a well-established in vitro training protocol of trained immunity (29), we confirmed that pre-stimulation of adherent monocytes with β-glucan resulted in a pronounced enhancement of IL-6 release upon re-stimulation with LPS at day 6 (Figure 8M). We also investigated the effects of pre-stimulation with LPS. As previously reported, pre-stimulation with LPS induced immune tolerance (Figure 8D) (15). Taken together, these findings suggest that circulating β-glucan is a possible driver of the pro-inflammatory immune phenotype of monocytes in PLHIV.
Discussion

In this study, we investigated the functional phenotype of circulating immune cells in a cohort of PLHIV on stable cART. We show that PLHIV exhibit a sustained elevation in monocyte cytokine responsiveness, whereas lymphocyte-derived cytokine responses were mostly unaffected. Our immune profiling data, which included transcriptome analysis of circulating monocytes, further showed an increased expression and activation of the IL-1β pathway. HIV infection is associated with increased microbial translocation (9) and we identified a, so far unrecognized, role for elevated circulating β-glucan concentrations in the altered inflammatory monocyte phenotype in PLHIV.

These data corroborate findings from earlier, smaller studies in PLHIV, which reported an increased production of pro-inflammatory cytokines to stimulation with ligands of TLR4 (30, 31) or TLR7 (32, 33), or *M. tuberculosis* (34). The increased monocyte-derived cytokine responsiveness in our PLHIV cohort was associated with increased plasma concentrations of different circulating inflammatory markers, such as hsCRP, sCD14, IL-18 and IL-6, suggesting a functional link between changes in the innate immune profile and systemic inflammation in PLHIV. Remarkably, HIV infection had little effect on lymphocyte-derived cytokine responses, despite the fact that alterations in T-cell immune phenotype have been described in long-term viral suppressed PLHIV (35, 36). Previous research in HFGP cohorts of HIV-uninfected individuals showed that age, environmental, microbial and genetic factors have a clear impact on the function of circulating T-cells (21-23, 37).

The observed intrinsic pro-inflammatory phenotype of monocytes in PLHIV was stable over time and associated with upregulation of pro-inflammatory pathways, most notably the IL-1β pathway. This corroborates a recent study showing a pronounced pro-inflammatory phenotype, including upregulation of *IL1B*, of monocyte derived macrophages in PLHIV (38). Long-term epigenetic reprogramming of monocytes in response to microbes has been termed ‘trained immunity’. This functional adaptation of monocytes enables a greater response when subjected with a secondary stimulus as protective mechanism against a secondary infection (14-16). Nonetheless, a trained immunity phenotype of monocytes has also been related to conditions such as cardiovascular diseases and diabetes (39-42). In PLHIV, altered epigenetic profiles such as DNA methylation have been reported (43, 44, 45). Specific DNA methylation patterns in PLHIV were associated with progressive aging and non-AIDS related co-morbidities, such as insulin resistance, neurocognitive disorders and chronic kidney disease (17, 46-48). Interestingly, HIV/Simian immunodeficiency virus (SIV) DNA vaccination was recently shown to induce a trained immunity phenotype in vivo through upregulation of IL-1β related genes (49). This upregulation correlated with protection against subsequent SIV infection in macaques. Furthermore, HIV-1 itself has also been shown to increase IL-6 production via epigenetic modification of STAT3 in microglial cells (50). Although epigenetic modification has been shown to underlie the trained immunity phenotype, we did not determine epigenetic changes in the current study. Future studies are warranted to investigate the epigenetic processes underlying the trained immunity phenotype reported here.
Another question concerned the identification of the possible mechanisms responsible for the observed changes in innate immune responses. CMV (10), microbial translocation (9), and HIV reservoir (11) have each been related to persistent inflammation in PLHIV. However, our data did not show a positive correlation between monocyte responsiveness and CMV seropositivity, levels of iFABP (microbial translocation marker), or parameters of the HIV reservoir. Although, we do not exclude a possible accessory role for these markers of microbial exposure, we did find an association of circulating β-glucan concentrations and IL-1β responsiveness. β-glucan is a component of the cell wall of fungi and is known to be a strong inducer of trained immunity (16). Furthermore, IL-1β signaling is reported to be important in β-glucan induced trained immunity in vivo (51). Our present data corroborate earlier reports that increased circulating β-glucan concentrations can be found in virally-suppressed PLHIV (28, 52, 53). Acute HIV infection leads to an early and pronounced loss of mucosal Th17 CD4+ T cells (54). This cellular compartment is critical for regulation of mucosal host defense against Candida and regulation of epithelial cell permeability (55). Increased levels of other microbial products, including LPS, have also been reported in PLHIV (28, 56). However, in contrast to β-glucan, LPS generally induces immune tolerance, even at low concentrations (15, 16), a notion we confirmed in vitro. This notion is supported by our observation that microbial integrity, as measured by iFABP, is associated with decreased monocyte responsiveness. An HIV infection is associated with alterations in the bacterial gut microbiome composition and these changes play a role in the incidence of co-morbidities and inflammation (2, 57, 58). While studies on the mycobiome in PLHIV are limited, fungal dysbiosis with a high prevalence of Candida species has been found in stool samples of PLHIV (59). Furthermore, our data showed lower IL-6 and higher IL-10 production by monocytes upon Candida stimulation in PLHIV compared to uninfected controls, suggesting an altered fungal immune response. This defective immunity could contribute to fungal dysbiosis in virally suppressed PLHIV. Hence, our present data support the need for future studies exploring the role of the mycobiome in persistent inflammation in PLHIV, as well as possible strategies to reduce β-glucan exposure.

Reducing inflammation is considered an attractive therapeutic target to reduce the burden of cardiovascular diseases. The CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial that was carried out in HIV uninfected subjects who were at risk for a cardiovascular event. The trial revealed that specific neutralization of IL-1β reduced the incidence of subsequent cardiovascular events and death from lung cancer (60, 61). Similarly, the anti-inflammatory drug colchicine reduced cardiovascular events in patients with coronary disease (62). In contrast, the immunosuppressive drug methotrexate did not reduce CVD, underlining the importance of IL-1β in CVD (63). Recent findings that canakinumab reduced atherosclerotic inflammation in PLHIV at high-risk for CVD are therefore promising (64) and support the importance of IL-1β pathway in HIV. Orally administrated drugs that inhibit the IL-1β pathway, such as inflammasome inhibitors, may form an attractive alternative (65). This also applies to epigenetic modifying drugs, such as histone deacetylase (HDAC) inhibitors. These drugs have been shown to reduce CRP concentrations, IL-1β expression in PBMCs (66) and IL-1β production to LPS stimulation in whole blood from PLHIV (67). Taken together, our present data support the use of interventions targeting the IL-1β pathway or reduce β-glucan exposure as an adjunctive therapy to reduce non-AIDS related co-morbidities.
A particular strength of our study is that in-depth functional analysis of adaptive and innate immune cell function in a relatively large cohort of PLHIV. The same HFGP approach was successfully used to investigate genetic, environmental and microbial factors influencing the immune system in HIV-uninfected individuals (21-23, 37). Limitations of our study include that PLHIV and control cohort differed in age and sex distribution. All analyses were therefore adjusted for these differences using multivariate analyses with sufficient sample size. Moreover, the primary findings were confirmed when we resampled a selection of age and sex matched PLHIV and controls. However, we could not correct for men who have sex with men (MSM), as this information was not available for the control group (68). Second, the nature of our cross-sectional study does not allow to draw strong causal inferences, for example on the role of HIV or β-glucan in the observed immune changes. Third, the heterogeneity and activity of the HIV reservoir is not fully determined by HIV CA-DNA and HIV CA-RNA measurement only, residual viremia with single copy assay could quantifiy recent HIV activity. Fourth, recently monocyte alterations in PLHIV were linked to cART induced oxidative stress (69). The enrolment in our cohort was limited to virally-suppressed PLHIV on cART and, therefore, a possible independent effect of cART on immune responses could not be assessed. However, an effect of different cART regimens appears unlikely, since no differences in cytokine responses were found across the different cART regimens. Finally, due to limited inclusion of women in our cohort, generalizability to women or sex-specific analyses were not feasible.

In conclusion, we found that PLHIV on stable cART exhibit a marked and long-lasting increase in the production of IL-1β with subsequent downstream monocyte-derived cytokines, suggesting a trained immunity phenotype of monocytes. Increased translocation of β-glucan from the gastrointestinal tract was identified as a possible inducer of this innate immune phenotype. Our findings provide mechanistic insights and shed new light on the usefulness of interventions targeting the innate immune system, as well as the gut mycobiome, as an adjunctive therapy to reduce non-AIDS related co-morbidities.

Methods

Study population

A total of 211 PLHIV were recruited from the HIV clinic of the Radboud university medical center between December 2015 and February 2017 with a follow up measurement in 2018. Caucasian individuals age ≥ 18 years, on cART > 6mo with an HIV-RNA load <200 copies/mL and showed no signs of opportunistic infections or active hepatitis B/C were included. The control group consisted of 56 healthy individuals not using any medication at inclusion. Inclusion, sampling and sample processing of both cohorts were conducted simultaneously and uninfected controls were sampled every three months during the inclusion of PLHIV. In the follow-up measurement, we resampled 28 male PLHIV, aged >45yrs after >1yr and stratified them on the basis of their initial IL-1β production capacity in a group of low (lowest quartile) and high (highest quartile) IL-1β producers. A control group of fourteen age-sex matched healthy controls was also included. These groups were used for monocyte-only, RNA expression and β-glucan measurements. All experiments were performed by the same personnel using the HFGP methodologies (http://www.humanfunctionalgenomics.org) (19). General information
from all participants was recorded in an electronic case report form (CastorEDC, Amsterdam, The Netherlands). Clinical data were extracted from the electronic hospital information system and the ‘Stichting HIV Monitoring’ registry (Amsterdam, The Netherlands).

**Ex vivo PBMC and monocyte stimulation**

Venous blood was collected in sterile 10 mL EDTA and 8 mL serum BD Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 1-4h. Isolation of PBMCs was performed freshly collected blood by density centrifugation over Ficoll-Paque (VWR, Amsteram, The Netherlands) as described previously (70). Monocytes were isolated by magnetic activated cell sorting using negative bead selection with the Pan Monocyte Isolation kit according to manufacturer’s instructions (Miltenyi Biotec, Leiden, the Netherlands). Cell counts, cell purity and composition were evaluated by XN-450 hematology analyzer (Sysmex Corporation, Kobe, Japan).

Fresh isolated cells (monocytes 1.10^5 cells per well, PBMCs 5.10^5 cells per well) were incubated with different bacterial, fungal, viral stimuli (Table S2) at 37°C and 5% CO₂ for either 24h or 7d. For the 7d stimulation, 10% human pooled serum was added to the wells. IL-1β, IL-6, IL-1Ra, IL-10 and TNFα were determined in the supernatants of the 24hr PBMC or monocyte stimulation experiments, using enzyme-linked immunosorbent assays (ELISA; Duoset ELISA, R&D Systems, Minneapolis, MN, USA). IL-17, IL-22 and IFNγ were measured after 7d stimulation of PBMCs (PeliKine Compact or R&D Systems). For intracellular cytokine measurements of active IL-1β and pro IL-1β (Quantikine, R&D Systems), cell pellets were lysed using Triton X-100 (Sigma, Zwijndrecht, The Netherlands).

**Measurements of plasma markers and immunophenotyping**

Intestinal fatty acid binding protein (iFABP), a marker of enterocyte damage, resistin, adiponectin, leptin, IL-18 binding protein (IL-18BP), IL-18, hsCRP, sCD14 and sCD163 were measured using ELISA (Duoset or Quantikine, R&D Systems). IL-6, TNFα, IL-10 and IL-1Ra were measured using SimplePlex Cartridges (Protein Simple, San Jose, CA, USA). 1,3-β-D-glucan (β-glucan) levels were measured with Fungitell (Associates of Cape Cod, Liverpool, UK) and CMV IgG by ELISA (Genway Biotech, San Diego, CA, USA). All assays were performed according to manufacturer’s recommendations, and samples of the different cohorts were measured simultaneously in the same plates or SimplePlex Cartridges. Monocyte subsets were measured in whole blood on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) as described elsewhere. Antibodies used are listed in Table S3 (22).

**RNA Extraction and gene expression**

For qPCR analyses, total RNA was extracted from monocyte cell pellets (5.10^5 cells), collected in TRizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Subsequently, cDNA was synthesized using iScript reverse transcriptase according to manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA) with 500ng RNA input in 10ul reaction mix. Relative expression of genes NLRP3, TNF, IL6, IL1B was measured using SYBR Green assays (Invitrogen) on an Applied Biosciences Step-one PLUS qPCR machine (Thermo Fischer Scientific, Carlsbad, CA, USA). Reaction volumes of 10 μL with 2 μL cDNA input. Normalization was performed using ΔΔCq based on two (18S and B2M) reference genes which were stable after stimulation. Cycling
conditions included: 95°C for 10mins, [95°C for 15 seconds, 60°C for 1min seconds] (40 cycles), followed by dissociation curve analysis. Primers were designed to be intron-spanning and are listed in table 54.

For transcriptome analysis by RNaseq, total RNA was extracted from monocytes (5.10^5) using the QIAGEN RNeasy RNA extraction kit (Qiagen, Venlo, The Netherlands), using on-column DNase treatment. Next, ribosomal RNA removal and library preparation for next generation RNA-sequencing was achieved utilizing KAPA RNA HyperPrep Kit with RiboErase (Roche, Basel, Switzerland), following the manufacturer’s protocol. The integrity and quality of prepared libraries were assessed using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). Sequencing was performed using Illumina NextSeq 500 machine (San Diego, CA, USA) in a paired end sequencing fashion. Sequencing reads obtained from RNA-seq measurement were aligned to the hg38 human genome reference using STAR (71). A lower cutoff of average 50 reads among all samples was used to designate a gene as being expressed in our cohort. R software and DESeq2 differential analysis package (72) were used to normalize and assess differentially expressed genes, utilizing FDR 5% and log2(Fold change) > 1 as the significance cutoff. The network of genes connected to their corresponding gene ontology was generated using cytoscape (73). The processed RNA-seq data including normalized read counts can be accessed via GEO (GSE160184)

HIV Reservoir quantification

HIV-1 CA-DNA and CA-RNA were measured in triplicate by droplet digital PCR (ddPCR QX200 – Bio-Rad, Hercules, CA, USA) (Table S3) in CD4+ T cells isolated using EasySep Human CD4+ T Cell Isolation Kit (Stemcell technologies, Vancouver, Canada) as described previously (74). Briefly, genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s protocol with an additional step of adding 75µl elution buffer on the column heated at 56°C for 10 min. CA-RNA was extracted using the Innuprep RNA Kit (Westburg, Leusden, The Netherlands) with 30µl elution buffer. Total RNA was reversely transcribed to cDNA by qScript cDNA SuperMix according to manufacturer's protocol (Quantabio, Beverly, MA, USA). Before PCR amplification, genomic DNA was restricted by EcoRI (Promega, Madison, WI, USA) and cycling conditions were implemented as described previously (74). Total HIV-1 DNA measurements were normalized by measuring the reference gene RPP30 (Table S4) in duplicate by ddPCR and expressed per million PBMCs. CA-RNA was normalized using three reference genes per patient, (B2M, ACTB and GADPH), which were measured with LightCycler 480 SYBR Green I Master mix (Bio-Rad). HIV-1 RNA copies were divided by the geometric mean of the reference genes and expressed per million PBMCs. Droplet classification and absolute quantification was performed using the ddpcRquant analysis tool with standard settings (75).

Statistics

Depending on normality, log10- or inverse rank-based transformation was applied. Values outside the assay quantification limits were imputed at the respective limit. Parameters for which one of the detection limits contained > 50% of the measurements were excluded. Comparisons in baseline characteristics between groups were made using Student’s t test or Mann-Whitney U test depending on data distribution. Differences in non-continuous data were analyzed by the Pearson’s Chi-square test or Fisher’s exact test in case of expected counts less than five. Data were analyzed using a linear regression model. The crude model included an adjustment for
sampling time and the primary analyses included age, sex and seasonality as co-variates. P values less than 0.05 were considered statistically significant and correction for multiple testing was applied using the Benjamini-Hochberg method (FDR-correction). Data were analyzed using R (R Core Team, 2015).

**Study approval**

The study protocol was approved by the Medical Ethical Review Committee region Arnhem-Nijmegen (CMO2012-550) and experiments were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

**Author contributions**

WH, QM, MGN and AV designed the study. WH, LW and MJ recruited and included the participants. WH, JW, LW, MJ, WT, SR and performed the laboratory experiments. WH, RH and LW analyzed the data and interpreted the data together with QM, AV, MGN, CD, LVDK, HK, HS, IJ and JL. WH, LW, AV and QM wrote the manuscript. All authors have read and contributed significantly to the final manuscript.

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Figure 1 Circulating factors in people living with HIV (PLHIV) vs uninfected healthy controls (HC). (A) Circulating factors in PLHIV and uninfected controls. Crude model is linear regression after inverse rank-based transformation. Adjusted model included age, sex, and seasonality as covariates. Red depicts the marker is significantly increased in PLHIV; blue depicts the marker is decreased in PLHIV compared to healthy controls. All P values are FDR corrected (Benjamini-Hochberg method). (B-G) Boxplots depicting circulating factors of inflammation stratified by cohort, PLHIV (blue), uninfected controls (yellow). P values are calculated using Student’s t test. Boxplots are depicted according to Tukey with median (line), interquartile range (edge of boxplot), range (whiskers) and outliers 3 times interquartile range are depicted as dots. All plots include data from PLHIV (n = 211) and HC (n = 56). hsCRP: high-sensitivity C-reactive protein; sC14: soluble CD14; sCD163: soluble CD163; IL-6: interleukin-6; IL-18: interleukin-18; IL-18BP: interleukin 18 binding protein.
Figure 2 Cytokine production capacity in people living with HIV (PLHIV) vs uninfected healthy controls (HC). (A) Ex vivo cytokine production capacity between PLHIV and HC after 24hr (in case of IL-1β, TNFα, IL-6, IL-10 and IL-1Ra) and 7 days stimulation (IL-22, IL-17 and IFNγ). FDR-corrected (Benjamini-Hochberg method) P values are depicted from an adjusted model included age, sex, seasonality as covariates. Red depicts significantly higher in PLHIV, blue depicts lower in PLHIV compared to HC. (I-P) boxplots depicting ex vivo cytokine production capacity stratified by cohort. PLHIV depicted in blue and uninfected controls in yellow. P values, depicted in boxplots were calculated using Student’s t test after log-transformation. All boxplots are depicted according to Tukey with median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. All plots include data from PLHIV (n = 211) and HC (n = 56). LPS: lipopolysaccharide; oxLDL: oxidized low-density lipoprotein; Pam3Cys: synthetic Toll-Like-Receptor (TLR)2 ligand; Poly IC: TLR3 ligand; imiquimod (IMQ): TLR7 ligand. RPMI: Roswell Park Memorial Institute medium.
Figure 3 Cytokine production capacity vs circulating factors. (A) Correlation plot with adjusted p-value shown after correcting for age, sex, seasonality as covariates. Red depicts a significant positive correlation and blue depicts a negative correlation within people living with HIV (PLHIV). All P values were FDR corrected per circulating factor. (B-I) Correlation plot without cofactor adjustment. Pearson’s coefficient ($r$) with $P$ value after log-transformation are shown. All plots include data from PLHIV ($n = 211$) and HC ($n = 56$). LPS: lipopolysaccharide; oxLDL: oxidized lipoprotein density protein; Pam3Cys: synthetic Toll-Like-Receptor (TLR)2 ligand; Poly IC: TLR3 ligand; imiquimod (IMQ): TLR7 ligand; hsCRP: high-sensitivity C-reactive protein; sC14: soluble CD14; sCD163: soluble CD163; IL-6: interleukin-6; IL-18: interleukin-18; IL-18BP: interleukin 18 binding protein. *$P < 0.05$ after FDR-correction.
Figure 4 Ex vivo cytokine production capacity of monocytes in a validation cohort. (A) IL-1β production upon 24h stimulation with imiquimod (1μg/mL), lipopolysaccharide (LPS; 100ng/mL) or M. tuberculosis (1μg/mL) in PBMCs or monocyte only culture (magnetic beads CD14+ isolation). (B) IL-6 production upon imiquimod stimulation. (C) TNFα production upon imiquimod stimulation. All data are stratified by healthy controls (HC; n = 14), people living with HIV (PLHIV) low initial IL-1β producers (n = 13) and PLHIV high producers (n = 15). All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. A Kruskal–Wallis test was performed with a post hoc testing when the P value was below 0.05. Post hoc testing was performed by using a Mann-Whitney U test on the comparison PLHIV high vs HC and PLHIV high vs PLHIV low. A Bonferroni multiple testing correction was used by setting the significance level at P < 0.025 for post hoc analysis. * P <0.025, ** P <0.001, # P value <0.05 (above multiple testing threshold), ns non-significant
Figure 5 Transcriptome analysis of monocytes. (A) Principal component (PC) analysis (PCA) plot of transcriptome of monocytes from people living with HIV (PLHIV) and healthy controls (HC) directly after isolation. (B) PCA plot of transcriptome of monocytes from PLHIV and HC after 24 hours macrophage differentiation in medium only. (C) Hierarchical clustering plot PLHIV vs HC. (D) Gene ontology of differentially expressed genes (PLHIV vs HC) including adjusted P value and gene count. (E) Top pathways of gene ontology plot after macrophage differentiation (GO interaction terms). PLHIV: n = 8, HC: n = 4.
Figure 6 IL1B gene expression and intracellular (pro)IL-1β. (A) IL1B gene expression after imiquimod 1μg/mL stimulation depicted as fold change (FC) from medium (RPMI). (B) IL6 gene expression after imiquimod stimulation depicted as fold change from medium. (C) NLRP3 gene expression (by ΔCT) in RPMI. (D) Intracellular levels of proIL-1β protein after Imiquimod stimulation. (E) Intracellular mature IL-1β protein after imiquimod stimulation. (F) Ratio of intracellular proIL-1β vs IL-1β after imiquimod stimulation protein. All data are stratified by healthy controls (HC; n = 14), people living with HIV (PLHIV) low initial IL-1β producers (n = 13) and PLHIV high producers (n = 15). All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. P-values were calculated by ANOVA and subsequently by pair-wised Student’s t test.
Figure 7 Parameters of HIV reservoir, CMV seropositivity and microbial integrity. (A-D) Log10-transformed cell associated HIV-1 DNA (CA-DNA) in CD4+ cells correlation with: (A) circulating IL-6 levels, (B) IL-1β production after LPS (100ng/mL) stimulation, (C) IL-1β production after imiquimod (IMQ; 1ug/mL) stimulation and (D) soluble CD14 plasma concentration. (E-H) Log10-transformed cell associated HIV-1 RNA (CA-RNA) in CD4+ cells correlation with: (E) circulating IL-6 levels, (F) IL-1β production after LPS stimulation, (G) IL-1β production after imiquimod stimulation and (H) soluble CD14 plasma concentration. (I-L) Stratified by Cytomegalovirus (CMV) seropositivity: (I) Log10-transformed cell associated HIV DNA (CA-DNA) in CD4+ cells, (J) IL-1β production after LPS stimulation, (K) IL-1β production after imiquimod stimulation and (L) soluble CD14 plasma concentration. (M) Intestinal fatty-acid binding protein (iFABP) in plasma, a marker of intestinal integrity, between healthy controls (HC; n = 56) and people living with HIV (PLHIV; n = 211). (N-P) Plasma iFABP concentration correlation with: (N) IL-1β production after LPS stimulation, (O) IL-1β production after imiquimod and (P) soluble CD14 plasma concentration. All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. Pearson’s coefficient ($r$) after log-transformation is shown in correlation plots. Data in boxplots are analyzed using Student’s t test after log10-transformation. All plots depict data from PLHIV only (n = 211) unless otherwise stated.
Figure 8 β-glucan induces a pro-inflammatory phenotype in monocytes. (A) β-glucan (βG) in serum stratified by healthy controls (control), people living with HIV (PLHIV) with low or high initial IL-1β response. (B) Percentage of detectable levels of β-glucan in serum, stratified by control, PLHIV with high and low initial IL-1β response. (C-D) IL-1β production after 24h stimulation with imiquimod (C; 1μg/mL) or Mtb (D; 1μg/mL) in PLHIV stratified by detectable β-glucan levels. (E) IL1B gene expression after imiquimod stimulation depicted as fold change (FC) from medium (RPMI). (F) Intracellular levels of proIL-1β protein after Imiquimod stimulation. (G-H) IL1Ra production after 24h stimulation with imiquimod (G) or Mtb (H) in PLHIV stratified by detectable β-glucan levels. (I-J) IL-6 production after 24h stimulation with imiquimod (I) or Mtb (J) in PLHIV stratified by detectable β-glucan levels. (K-L) TNFα production after 24h stimulation with imiquimod (K) or Mtb (L) in PLHIV stratified by detectable β-glucan levels. (A-L) PLHIV n = 28, healthy controls (HC) n = 14. All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. Data were analyzed using Student’s t test after log10-transformation. (M) Initial training with either LPS (TLR4 ligand), β-glucan (10μg/mL) or medium only (RPMI + 10% serum) was performed for 24h at day 1. Thereafter a 5-day resting period in medium only (supplemented by 10% serum), at day 6 adherent monocytes were restimulated with LPS 10ng/mL. IL-6 was measured in the supernatant and fold change from training with medium only are depicted. Data shown are from three separate experiments (n=9). Data were analyzed using Wilcoxon matched pairs signed-rank test. * P < 0.05 ** P < 0.01.
<table>
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<tr>
<th></th>
<th>PLHIV (n=211)</th>
<th>Healthy controls (n=56)</th>
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</thead>
<tbody>
<tr>
<td><strong>Sex (% Male)</strong></td>
<td>193 (91.5)</td>
<td>34 (60.8)$^a$</td>
</tr>
<tr>
<td><strong>Age (years, mean (SD))</strong></td>
<td>51.4 (10.8)</td>
<td>39.9 (17.3)$^b$</td>
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<tr>
<td><strong>BMI (kg/m$^2$, mean (SD))</strong></td>
<td>24.5 (3.6)</td>
<td>24.1 (3.1)</td>
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<tr>
<td><strong>HIV infection duration (years, median [IQR])</strong></td>
<td>8.5 [4.9, 14.2]</td>
<td></td>
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<tr>
<td><strong>Way of transmission (%)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Heterosexual</td>
<td>9 (4.3)</td>
<td></td>
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<tr>
<td>IDU</td>
<td>3 (1.4)</td>
<td></td>
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<tr>
<td>MSM</td>
<td>159 (75.4)</td>
<td></td>
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<tr>
<td>Other/unknown</td>
<td>40 (19.0)</td>
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<tr>
<td><strong>CD4 nadir (10$^6$ cells/mL; median [IQR])</strong></td>
<td>250.0 [135.0, 360.0]</td>
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<tr>
<td><strong>CD4 count (10$^6$ cells/mL; median [IQR])</strong></td>
<td>660.0 [480.0, 810.0]</td>
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<tr>
<td><strong>HIV load below 200 copies/mL (n (%))</strong></td>
<td>211 (100)</td>
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<tr>
<td><strong>HIV load below 50 copies/mL for &gt;1yr (n (%))</strong></td>
<td>188 (89%)</td>
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<td><strong>CD4/CD8 ratio (median [IQR])</strong></td>
<td>0.8 [0.6, 1.1]</td>
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<tr>
<td><strong>cART duration (years; median [IQR])</strong></td>
<td>6.6 [4.2, 11.8]</td>
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<td><strong>cART regimen</strong></td>
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<tr>
<td>NRTI-use (%)</td>
<td>203 (96.2)</td>
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<td>NT-RTI-use (%)</td>
<td>99 (46.9)</td>
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<tr>
<td>NNRTI-use (%)</td>
<td>63 (29.9)</td>
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<tr>
<td>PI-use (%)</td>
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<td>Maraviroc-use (%)</td>
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<tr>
<td>INSTI-use (%)</td>
<td>141 (66.8)</td>
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<td><strong>Active smoking, n/N (%)</strong></td>
<td>63 (29.9)</td>
<td>2 (3.6)$^b$</td>
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<td><strong>Heavy drinking (%)</strong>$^*$</td>
<td>28 (13.3)</td>
<td>11 (19.6)</td>
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<tr>
<td><strong>Regular substance use, (%)$^{</strong>}$</td>
<td>61 (28.9)</td>
<td>3 (5.4)$^b$</td>
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<tr>
<td><strong>Hypercholesterolemia (%)</strong></td>
<td>58 (27.5)</td>
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<td><strong>Hypertension (%)</strong></td>
<td>41 (19.4)</td>
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<tr>
<td><strong>Diabetes Mellitus (%)</strong></td>
<td>9 (4.3)</td>
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<tr>
<td><strong>No cardiovascular risk factors (%)</strong></td>
<td>50 (23.7)</td>
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<tr>
<td><strong>Statins (%)</strong></td>
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<td>19 (9.0)</td>
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<tr>
<td><strong>Metformin (%)</strong></td>
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**Table 1 Baseline characteristics;** BMI: body mass index. IDU: intravenous drug-use. MSM: men who have sex with men. cART: combination antiretroviral therapy. NRTI: Nucleoside reverse transcriptase inhibitor. NtRTI: nucleotide reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor. INSTI: integrase inhibitor. *Classified according to the CDC definition: for men, ≥15 drinks per week and for women, ≥8 drinks/week. http://www.cdc.gov/alcohol/faqs.htm#heavyDrinking (page accessed April 22, 2020, Page last reviewed: January 15, 2020, Content source: Division of Population Health, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention). **Defined as use of any psychoactive substance (with the exception of alcohol and tobacco) during periods ≥ 1 time per week including ≥ 1 time during the 30 days prior to the study visit. $^a$Significantly different between cohorts using Students T-test. $^b$Significantly different between cohorts using Chi-square test.