



HIV Controllers Have Low Inflammation Associated with a Strong HIV-Specific Immune Response in Blood

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**HIV controllers have low inflammation associated with a strong HIV-specific
immune response in blood**

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24 Running Head: Immune response signature of HIV controllers.

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Abstract

HIV-Controllers (HIC) maintain control of HIV replication without combined antiretroviral treatment (cART). The mechanisms leading to virus control are not fully known. We used gene expression and cellular analyses to compare HIC and HIV-1 infected individuals under cART. In the blood, HIC are characterized by a low inflammation, a down modulation of NK inhibitory cell signaling and an up regulation of T-cell activation gene expression. This balance that persists following stimulation of cells with HIV antigens, was consistent with functional analyses showing a bias towards a Th1 and cytotoxic T cell response and a lower production of inflammatory cytokines. Taking advantage of the characterization of HIC based upon their CD8⁺ T lymphocyte capacity to suppress HIV-infection, we show that unsupervised analysis of differentially expressed genes fits clearly with this cytotoxic activity allowing the characterization of a specific signature of HIC. These results reveal significant features of HIC making the bridge between cellular function, gene signatures and the regulation of inflammation and killing capacity of HIV-specific CD8⁺T cells. Moreover, these genetic profiles are consistent through analyses performed from blood to PBMC and T-cells. HIV controllers maintain strong HIV-specific immune responses with low levels of inflammation. Our findings may pave the way for new immunotherapeutic approaches leading to strong HIV-1-specific immune responses while minimizing inflammation.

50 **Importance**

51 A small minority of HIV infected patients, called “HIV Controllers” (HIC) maintains
52 spontaneous control of HIV replication. It is therefore important to identify mechanisms
53 that contribute to the control of HIV replication that may have implications for vaccine
54 design. We observed a low inflammation, a down modulation of natural killer inhibitory
55 cell signaling and an up regulation of T-cell activation gene expression in blood of HIC
56 compared to patients under combined antiretroviral treatment. This profile persists
57 following in vitro stimulation of peripheral blood mononuclear cells with HIV antigens,
58 and was consistent with functional analyses showing a Th1 and cytotoxic T cell
59 response and a lower production of inflammatory cytokines. These results reveal
60 significant features of HIV controllers that maintain strong HIV-specific immune
61 responses with low levels of inflammation. These findings define the immune status of
62 HIC that is probably associated with the control of viral load.

Introduction

If left untreated, HIV-1 infection is characterized by a detectable HIV replication and a rapid decline in CD4⁺ T lymphocytes leading to AIDS, whereas a small minority of patients, called “HIV Controllers” (HIC) maintains spontaneous control of HIV replication (1-3). Although, this population is heterogeneous and several mechanisms leading to the control of HIV replication contribute to this outcome (4, 5), an efficient HIV-specific CD8⁺ T cell response appears to be a key factors associated with the control of viremia. HIC maintain polyfunctional CD8⁺ T cell responses to HIV-1 antigens (6, 7) in particular to gag polypeptide (8). A population of HIC exhibiting strong functional HIV-specific cytotoxic CD8⁺ T cell responses (2) has been characterized (9). Indeed, primary CD8⁺ T cells from many HIC are able to suppress HIV-1 replication *ex vivo* by efficient granzyme B and perforin mediated killing of infected T cells (10). In previous reports (9, 11), we have defined two sub-groups of HIC in function of the capacity of their CD8⁺ T cells to *ex vivo* suppress HIV-1 infection in autologous CD4⁺ T lymphocytes (12). Strong responders HIC (SRHIC) exhibit a higher CD8⁺ T cell HIV-suppressive capacity than weak responders HIC (WRHIC). It was also observed that WRHIC maintain a large pool of HIV Gag-specific central memory T cells that are highly functional and readily expandable upon antigen stimulation, able to reach functions and high frequency similar to those observed in SRHIC (13). A negative correlation between expandable Gag-specific memory T cell responses and residual viremia suggest that these cells actively contribute to the sustained suppression of virus replication (14).

In order to identify mechanisms that may contribute to the spontaneous control of HIV replication in HIC, we hypothesized that comparison of blood gene expression profiles

87 of HIC and chronically HIV-infected patients, with high CD4⁺ T cells counts and
88 suppressed plasma HIV viral load while on cART, might help to identify features of
89 spontaneous HIV control. In a second approach, cellular and genetic analyses of PBMC
90 of these patients stimulated in vitro with HIV antigens were performed. Finally, in order
91 to further characterize the SRHIC and WRHIC, we compared gene profiles of purified
92 CD4⁺ and CD8⁺ T lymphocytes. Globally, our results identified key profiles of
93 immune control of viral replication delineating implications for the design of strategies
94 aimed to a sustained remission of HIV infection.

Results

Characteristics of the study population

The blood samples of the cohort comprised 53 HIC subjects and 27 cART treated patients. Clinical characteristics of the two groups are shown in Table 1. No statistically differences were observed between the two groups in terms of age (median of 47 vs 52 years old), viral load (1.6 vs 1.3 RNA log₁₀ copies/ml), CD4⁺ T (689 vs 588 cells/mm³) and CD8⁺ T (829 vs 725 cells/mm³) cell counts. No statistically differences were also observed for these parameters between the 10 SRHIC and 9 WRHIC subjects used for purification of CD4 and CD8 T cells, and between HIC and cART used for PBMC purification.

HIC are characterized by an increase in T cell activation and a down modulation of inflammatory genes in the blood.

Gene expression profile analysis of whole blood of HIC (n=53) and cART patients (n=27) showed that 1244 genes differentially expressed. Globally, these genes belong to pathways involved in innate immunity and NK signalling, T-cell activation and inflammation. HIC were clearly characterized by a down modulation of genes related to inflammation response with a down regulation of TLRs and TREM1 pathways (TLR1 (-1.73), *TLR4* (-1.91), *TLR6* (-1.87), *TLR8* (-2.61), *CD14* (-1.66), *TREM1* (-2.12) and *TYROBP* (-1.62) and of many pro-inflammatory genes including neutrophils chemotactic factor *IL-8/CXCL8* (-8.14) and its receptors *CXCR1* (-3.12) and *CXCR2* (-4.09) (Fig 1A). More precise analysis revealed also a down modulation in HIC of receptors for the Fc portion of immunoglobulin (*FCGR3A/FCGR3B* (-7.65), *FCER1G* (-1.94) including the *CD32A* gene (*FCGR2A*) (-2.77) as well as killer cell

immunoglobulin-like receptors (*KIR2DL1/KIR2DL3* (-1.85), *KIR3DL1* (-1.82), *KIR2DL4* (-1.7), *KIR2DL5A* (-1.25)) and killer cell lectin like receptors *KLRD1* (-1.76), *KLRC3* (-1.96) and *KLRC2* (-1.69) (Fig. 1A). This result contrasts with an upregulation in HIC of the expression of Src family kinases, *FYN* (+1.66) and *ZAP70* tyrosine kinase (+1.73), *IFNG* (+1.51) and *STAT1* genes (+1.54) (Fig. 1B). Interestingly, the low inflammatory profile in HIC is consistent with the down modulation of inflammation regulatory pathways mitogen-activated protein kinase 1 (*MAPK1*) (-1.59) and PI3-kinase *PIK3CG* (-1.52) and *PIK3CB* (-1.61) (Fig. 1B), a critical regulatory factor that connect immune stimulation and suppression during inflammation (15, 16). Globally, as illustrated in Fig. 1B, analysis revealed significant direct interactions between these pathways linking the down modulation of *PIK3CG* with an increase of T-cell activation (*ZAP70/FYN*) and a decrease of innate cell inhibitory signaling of NK cells (*KIRs*).

We have also looked for immunometabolism pathways that play important role in the modulation of the immune system. In whole blood, we have observed an enrichment of gluconeogenesis and lipid metabolism pathways. In that respect, we observed a down regulation in HIC compared to cART, of *ALDOA* (aldolase, fructose-bisphosphate A) (-1.57), *BPGM* (bisphosphoglycerate mutase) (-2.31), *ME2* (malic enzyme 2) (-1.61), *PGAM1* (phosphoglycerate mutase 1) (-1.54) and *PGAM4* (phosphoglycerate mutase 4) (-1.51) and an up-regulation of *ENO3* (enolase 3) (+1.64). In lipid metabolism, there was a down modulation of *PTGS2* (prostaglandin-endoperoxide synthase 2) (-2.88), *CD36* (-2.24), *ACSL1* and 4 (acyl-CoA synthetase long chain family members) (-2.97 and -1.56), *S1PR1* and *S1PR3* (sphingosine-1-phosphate receptors) (-1.62 and -1.70), *PCTP* (phosphatidylcholine transfer protein) (-2.13) and *PTGS2* (prostaglandin-endoperoxide synthase 2) (-2.88). In contrast, *PTGR2* (prostaglandin reductase 2)

(+2.15), *PLA2G2D* (phospholipase A2 group IID) (+2.18), *SREBF1* (sterol regulatory element binding transcription factor 1) (+1.55) were upregulated in HIC compared to cART. However, the modulated genes did not allow to predict an activation or inhibition of these pathways in HIC compared to cART.

HIC cellular responses to HIV peptides are associated with a low inflammatory gene expression associated with Th1 and cytotoxic profiles

We analyzed differences in gene expression of PBMC isolated from HIC (n=25) and cART (n=15) patients, before and after in vitro stimulation with pools of HIV peptides.

Gene expression analysis before HIV peptides stimulation revealed that 113 genes were differentially expressed. Analysis on the Ingenuity Pathway software showed that these genes are significantly involved in inflammation with a down regulations of many genes such as *IL1A* (-2.28) and *IL1B* (-7.02), *IL6* (-5.71), *CXCL5* (-6.89), *CXCL13* (-1.97), *CCL23* (-1.68), *CXCL1* (-4.19), *TREM1* (-1.66) and *CD14* (-2.86) (Fig. 2A). Some of these genes are also related to granulocytes adhesion and diapedesis (*IL1A*, *IL1B*, *CXCL5*, *CXCL1*, *FPR1*, *FPR2*, *CCL22*, *CXCL13*, *CCL19*, *CCL23*) and to IL6, HMGB and TREM1 signaling (*IL1B*, *IL6*, *CD14*, *IL1A*, *FOS*, *LAT2*, *RHOA*, *TREM1*). We also observed a down regulation of genes involved in iron homeostasis pathway such as *HBA1/HBA2* (-12.93), *HBB* (-12.3), *HBG1* (-8.14), *HBG2* (-7.58), *IL6* (-5.71), *ALAS2* (-4.52), *SLC11A1* (-2.24) and *SLC25A37* (-1.88). Likewise, gene expression analysis of HIV peptides stimulated PBMC between HIC and cART revealed that 144 annotated genes were differentially expressed. Pathway analyses showed, as for unstimulated cells, a down regulation of genes belonging to inflammatory immune response, including *CD14* (-5.12), *CXCL8* (-1.84), *TREM1* (-1.71) and *IL6* (-7.78), as well as *CXCL5* (-7.87), *IL1B* (-5.45), *IL1A* (-4.74), *CCL3L1* (-4.17), *CXCL1* (-3.82) and *CCL24*

(-3.55) (Fig 2B). We further observed a significant up regulation of genes related to the interferon pathway such as *IFIT1* (+3.54), *IFI44L* (+2.50), *IFI44* (+1.94), *MX1* (+2.02), *OAS3* (+1.91) (Fig 2C).

These genetic characteristics were found to be consistent with the profile of cytokine production of in vitro stimulated PBMC from HIC (20 samples) and cART (15 samples), as shown in Fig. 3. We observed a lower production of IL1 β and a higher production of IP10, TNF α and MIP-1 β in HIC compared to cART as measured by Luminex (Fig. 3A). This result was confirmed by ICS analysis after PBMC stimulation with HIV peptides showing a higher frequency of CD8 $^{+}$ T cell producing TNF α , MIP-1 β and IFN γ in HIC patients compared to cART (p=0.0127, Mann-Whitney test) (Fig. 3B). In contrast, no difference was observed in the profile of cytokine production for CD4 T cells of HIC and cART (Fig. 3C).

Genetic and functional analyses of CD8 $^{+}$ T cells from SRHIC and WRHIC reveal specific signatures.

We analysed cytokine patterns of in vitro stimulated PBMC and gene expression profiles of purified CD4 and CD8 $^{+}$ lymphocytes from SRHIC and WRHIC. PBMC stimulation with HIV peptides led to a significant higher production of IL-2, IP-10, Granzyme A, Perforin and MIP-1 β in SRHIC as compared to WRHIC (Fig. 4A), which is consistent with a stronger Th1- and T effector-cytokines response in SRHIC subjects (Fig. 4B). Phenotypic analyses in ICS assay confirmed a higher frequency of CD8 $^{+}$ T cells producing cytokines in SRHIC compared to WRHIC group (p=0.031, Mann-Whitney test), specially MIP-1 β (p=0.024) (Fig. 5A). CD8 $^{+}$ T cells from SRHIC and WRHIC were highly polyfunctional (55 to 60% of cells exhibit 2 or 3 cytokines) in both groups. Although CD4 $^{+}$ T cells from both SRHIC and WRHIC patients were highly

polyfunctional (60 to 75% of the cells exhibit 2 or 3 cytokines), no differences were observed between groups in terms of cytokine production following HIV peptide stimulation (Fig. 5B).

We then compared gene expression profiles of *ex vivo* CD8⁺ and CD4⁺ T lymphocytes purified from SRHIC and WRHIC. In contrast to CD4⁺ T lymphocytes (Fig. 6A), unsupervised hierarchical clustering analysis of CD8⁺ T lymphocytes showed a perfect clustering of SRHIC and WRHIC groups (Fig. 6B). We found 804 annotated differentially expressed genes between SRHIC and WRHIC CD8 cells. Analysis of gene expression profiles of CD8⁺ T lymphocytes showed an up regulation in SRHIC of genes involved in the IFN γ pathway (Fig. 7), while proinflammatory genes such as *CXCL8* (-3.53), *IL1B* (-2.28), *IRAK3* (-1.61), *TYROBP* (-3.13) and *FCER1G* (-3.37) were down regulated. CD8⁺ T lymphocytes from SRHIC exhibited also a significant upregulation of *CX3CR1* (+2.21) gene expression, a marker of CD8 effector memory cells (17).

Among 804 genes differentially expressed between CD8⁺ T cells from SRHIC and WRHIC, 133 were also part of those identified in blood gene expression differences between HIC and cART (Fig. 8A). These genes are mainly associated with a down modulation of inflammation. Among the 671 genes differentially expressed specifically between CD8⁺ T cells from WRHIC and SRHIC (excluding the 133 genes differentiating blood gene expression of HIC from cART), four main functions were identified: three were predicted as activated (T cell response, cytotoxicity of leukocytes and killing Natural Killer cells) and one was predicted as inhibited (activation of leukocytes). The down regulation of genes such as *NFKB1* was consistent with the decrease of leukocyte activation and increase of leukocyte toxicity (Fig. 8B).

215 These data reveal significant features of HIC making the bridge between HIV-specific
216 cellular function; i.e polyfunctionality, low proinflammatory responses, cytotoxic
217 activity and gene signatures. Interestingly enough, these genetic profiles are consistent
218 through the analyses of *ex vivo* whole blood and PBMC to analyses performed at the
219 cellular population levels.

Discussion

We report here results of extensive functional and gene expression analyses performed in whole blood and at cellular level through PBMC and purified CD4 and CD8 T cells in a cohort of HIC. Globally, these analyses performed through the different compartments were consistent. They show that HIC individuals, as compared to chronically HIV-1 infected individuals under cART have a low inflammatory background which contrasts with activation of adaptive immune response pathways. Interestingly this balance persists following *in vitro* stimulation of cells with HIV antigens. This genetic profile was also consistent with functional analyses as assessed by the production and cellular expression of cytokines. Finally, taking advantage of the characterization of HIC based upon their *in vitro* CD8⁺ T lymphocyte capacity of killing HIV-infected cells, we show clearly that unsupervised genetic analysis of differentially expressed genes fits clearly with this cytotoxic activity. Here again we found a balance between low activation and the commitment of genes associated with cytotoxicity and T cell response.

Although cART has significantly improved the prognosis of HIV infected individuals, they remain at increased risk of morbidity and mortality (18, 19). These clinical events are supposed to be related to residual immune activation and inflammation in cART-treated patients. The immune activation is also associated to the poor HIV-specific response in chronically infected patients (20). Several studies have shown that HIC exhibited cellular and serological markers of immune activation and inflammation despite a spontaneous control of HIV replication (21-24). However, no evidence of persistent inflammation was observed when HIC were defined using stringent criteria in relation to the cutoff level of viremia (≤ 50 copies/mL) and a minimum follow-up time

244 of >5 years, compared to HIV uninfected subjects (25). We found here, as compared to
245 cART patients, that the level of inflammatory gene expression remains still dramatically
246 reduced in HIC with a significant down regulation of TLRs, TRIM1 and CXCL8/IL8.
247 This result extends several observations showing that HIC have significantly lower
248 levels of IL-8 mRNA when PBMCs were exposed to exogenous HIV-1 compared to
249 HIV-progressors, cART treated or not, and HIV uninfected control (26). It was also
250 observed a higher expression of CXCL8 in untreated HIV-1 infected progressors and
251 cART nonresponders when compared to LTNPs (long term non progressors) and cART
252 responders, respectively. Furthermore, a negative correlation of plasma levels of
253 CXCL8 with CD4 counts was found in HIV-1 infected cART naïve subjects, while the
254 CXCL8 levels positively correlated with viral load in the cART treated children (27).
255 These observations suggest a strong link between CXCL8 through its proinflammatory
256 action, to viral replication and disease progression. On the other hand, El-Far M et al.
257 (28) underlined the role of proinflammatory IL-32 cytokine in the failure of virus
258 replication control in HIC. We did not find any differences between HIC and cART
259 patients in the expression of IL-32 gene in our study where there was no failure to
260 control viral replication, neither in HIC nor in cART patients. Beside the down
261 regulation of inflammatory genes, HIC down regulated many genes belonging to the
262 natural killer cell signaling pathway such as receptors for the Fc portion of
263 immunoglobulin, inhibitory killer cell immunoglobulin-like receptors and killer cell
264 lectin like receptors. Interestingly, studies on HIV slow progressors linked the
265 protective effect of NK cells with certain killer immunoglobulin-like receptors and their
266 ligands the human leukocyte antigen-class I molecules (HLA) on the target cells (29,
267 30). The responsiveness of NK cells varies depending on the number of inhibitory

receptors (iKIR) expressed in particular KIR2DL1/KIR2DL3 (29, 31, 32). Interestingly, expansion of the activating KIR3DS1+ and the inhibitory KIR3DL1+ NK cells are increased in patients with acute HIV-1 infection in the presence of HLA-B Bw480I. However, it was not associated with reduction in HIV levels in the blood. Engagement of the inhibitory KIR3DL1 receptor on these NK cells with its ligand on the target HIV infected cells could lead to the inhibition of NK cell cytotoxicity. Similarly, studies have shown that CD56[−] CD16⁺ NK cells, which are expanded in HIV-viremic individuals, have impaired function and high expression of inhibitory KIR2DL2 and KIR2DL3 receptors, which would explain their defective lytic capability toward HIV-infected cells (33). Although we did not evaluate the functional capacity of NK cells in HIC, one can hypothesis that the down regulation of iKIR, observed in HIC may result in strong NK cell activation leading to viral load control.

We also observed a down regulation in HIC of receptors for the Fc portion of immunoglobulin (*FCGR3A/FCGR3B, FCGR2A and FCER1G*). Many studies indicate that antibody-induced effectors responses mediated through FCGR signaling contribute to the control and prevention of HIV-1 infection (34-36). FCGR2A (CD32A) receptor has also been reported as a marker of the CD4⁺ T cell HIV reservoir in HIV-infected patients (37), but more recently contradictory works have shown that CD32 is not a marker of HIV-1 reservoir but of CD4⁺ T cell activation in HIV⁺ individuals (38, 39). Despite that the role of the FC receptors in virus control remains to be thoroughly explored, one can speculate that the down regulation of these receptors could be associated with both the lower activation/inflammation and HIV reservoir observed in HIC compared to cART (40). It was also reported that the quality rather than the number of the FCGR signaling, could be responsible of the wider poly-functional Fc-

mediated responses observed in HIC (36, 41). In parallel, there is a down regulation in HIC of mitogen-activated protein kinase 1 (*MAPK1*) and PI 3-kinase (*PIK3CG* and *PIK3CB*), both are critical regulatory factor of immune stimulation and suppression during inflammation (15, 16, 42). In mice, an inhibition of PI3K promotes adaptive immunity and CTL activities (16, 43). Here, we observed a down regulation of PI3K associated to a down regulation of many inflammatory genes including IL-4 especially in HIC presenting a strong viral inhibition capacity. Globally, the observation in HIC of a link between the low expression of *PIK3CG* and both an activation of T-cell signaling and a down modulation of inflammatory pathways is reminiscent to the action of this “switcher” in the balance between immune suppression and inflammation (16). HIC seem to develop an efficient adaptive immune response through a modulation of expression of regulatory molecules of cytoplasmic signal transduction pathways *FYN*, *ZAP70*, *MAPK1*. Indeed, increase in expression of Src family kinases, *FYN* and *ZAP70* tyrosine kinase in HIC are in favor of activation of T cells through the TCR, which allows a specific immune response (44, 45). This specific response was associated to a drastic down regulation of chemoattractive molecules such as *CXCL5*, *IL1B*, *IL1A*, *CCL3L1*, *CXCL1* and *CCL24* in HIV-peptides stimulated PBMC of HIC compared to cART. The same profile was observed with CD8 T lymphocytes of SRHIC compared to WRHIC, that also have less proinflammatory response, through down regulation of mRNA of *CXCL8*, *S100A8*, *S100A9* and *IL1B*, while the *IFNG* response was activated. Immunological and virological aspects in the blood, gut associated lymphoid tissues (GALT) and lymph nodes of HIC and cART showed the crucial role in the virus control of both HIV specific responses and immune activation (44, 45). Our observations highlight only mechanisms involved in the blood of HIC compared to cART patients.

Though, HIV infection induces also the expression of different components of the inflammasomes in GALT (46) and both the immune regulation and delayed progression to AIDS were associated with a particular activation phenotype of T cells in GALT from HIV-controllers (47). Furthermore, in HIV infection the immune activation and inflammation were also associated with immunometabolism reprogramming through the use of glucose and fatty-acid (48). In whole blood, we observed an enrichment of gluconeogenesis and lipid metabolism pathways in differentially expressed genes between HIC and cART, but it was not possible to determine if there was activation or inhibition of these pathways.

Altogether, we show that HIC associate an anti-inflammatory state and strong adaptive immune response to virus that probably allows for the control of viral loads below the limits of detection. Efficient HIV therapeutic vaccine would mimic such response profiles by inducing strong HIV-specific immune response whereas minimizing inflammation.

Materials and Methods

Patients and samples

Whole blood samples were collected from 53 HIV HIC subjects of the ANRS CO21 CODEX cohort and 27 HIV-cART treated patients followed in Henri Mondor Hospital (Créteil, France). HIC individuals were never treated with cART, HIV-infected for at least 5 years and with last five consecutive plasma HIV RNA < 400 HIV-RNA copies/ml (49). Control cART patients exhibited plasma HIV RNA <50 copies HIV-RNA/ml for at least 2 years and CD4 lymphocytes ≥ 500 cells/mm³. CD4 and CD8 T lymphocytes were purified from SRHIC and WRHIC subjects, and Peripheral blood mononuclear cell (PBMC) from HIC and cART patients. The study protocol was approved by the regional investigational review board (Comité de Protection des Personnes Ile-de-France VII and IX) with approval reference 05–22 and 10-023. The study protocol was performed in compliance with the tenets of the Declaration of Helsinki.

RNA isolation and microarray sample preparation

Whole blood RNA was purified using Tempus™ Spin RNA Isolation Kit (ThermoFisher scientific). PBMC, CD4- and CD8-lymphocytes RNA were purified on Qiagen RNeasy Micro Kit. RNA was quantified using a ND-8000 spectrophotometer (NanoDrop Technologies, Fisher Scientific, Illkirch Cedex, France) before being checked for integrity on a 2100 BioAnalyzer (Agilent Technologies, Massy Cedex, France). cDNA was synthesized and biotin-labelled cRNA was generated by an in vitro transcription reaction using Ambion Illumina TotalPrep RNA Amplification Kits

(Applied Biosystem/Ambion, Saint-Aubin, France). Labeled cRNA were hybridized on Illumina Human HT-12V4 BeadChips.

CD4 and CD8 T lymphocytes isolation

CD4- and CD8-lymphocytes were isolated only from SRHIC and WRHIC subjects (9). T cells were isolated with an automated Robosep cell separator (STEMCELL) by indirect magnetic cell sorting with a T cell enrichment kit (STEMCELL) customized to also eliminate gamma/delta T cells. CD4⁺ T cells were subsequently separated by positive selection using anti-CD4 coated beads (STEMCELL) and CD8⁺ T were recovered in the resulting negative fraction. The purities of CD4 and CD8-T cells were > 95%.

In vitro stimulation of purified PBMC with HIV peptides for gene expression and cytokines profile analyses

After resting, 8.10^5 of thawed cells were stimulated for 24 hours in 48-well plates with a HIV peptide pool of 36 peptides (15-mers overlapping by 11 amino acids peptides) covering 5 regions of HIV Gag, Pol and Nef (50). Cells were then pelleted for transcriptomic analysis. In parallel, 5.10^5 cells were cultured in triplicate in 96 deep well plates and stimulated with the same antigens. At day 2, supernatants were collected for Luminex assay. 100 Units/ml IL2 (Miltenyi Biotec) was added in the culture medium at days 2 and 5 for longer stimulation. At day 8, all wells were split in 2, and cells were re-stimulated with the same antigens either for 6 hours in the presence of brefeldin A for ICS assay or for 24h for Luminex assay.

For ICS analyses, cells were first stained with surface monoclonal antibodies: anti-CD3 Alexa 700, anti-CD4 BV421 (BD Biosciences, Le Pont de Claix, France), anti-CD8

eFluor780 (affymetrix/eBioscience, Paris, France) and a viability marker (Live dead fixable Aqua Dead cell stain kit from Life Technologies, Saint Aubin, France), permeabilized and fixed with Cyto fix/Cytoperm Buffer (BD Biosciences). Cells were then stained with intracellular antibodies: anti-IFNG PerCP Cy5.5, anti-TNF α PE-Cy7 and anti-MIP1B PE (BD Biosciences). Data were acquired with a LSRII flow cytometer (BD, Le Pont de Claix, France), with a minimum of 100000 events collected in CD3+ alive cells, analyzed using FlowJo software, and the specific response has been expressed as the percentage of CD4 or CD8 T cells.

For Luminex assay, 14 cytokines have been measured in the supernatants of cell cultures at days 2 and 9 using Millipore reagents (MILLIPLEX Human CD8 T-Cell Panel with IL-2, IL-5, IL-10, IL-13, IFN γ , TNF α , MIP-1 β , Perforin, Granzyme A and Granzyme B; Magnetic beads and antibodies for human IP10, IL-21, IL-17A and IL-1 β , Millipore, Chicago, USA). Data were acquired with the Bio-Plex 200 system™ (Bio-Rad, Marnes-la-Coquette, France).

Statistics

Microarray data analyses were performed using R software version 3.2.2 (The R foundation for Statistical Computing, Vienna, Austria). Gene transcription data were pre-processed (51, 52) and corrected for potential batch effect (53). Statistical comparisons between groups were based on empirical Bayes moderated t-statistics (54). An adaptive FDR procedure was used to control for test multiplicity. Unsupervised hierarchical clustering heatmap analysis was performed on raw scaling expression using Euclidean distance matrix and Ward's linkage (55). Canonical pathway and biological function analyses were then carried out using genes differentially expressed between

groups with adaptive FDR-adjusted $P \leq 0.05$ and fold-change $|FC| \geq 1.5$. Ingenuity Pathway Analysis software (IPA®, Qiagen, Redwood City, www.qiagen.com/ingenuity) was used for gene pathway and function analyses. Mann Whitney tests have been used to compare cytokine production by T cells and PBMC.

Data availability

All microarray data are MIAME compliant, and the raw and normalized data have been deposited in the MIAME-compliant database Gene Expression Omnibus (GEO) under accession number GSE108297.

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H.H., C.La., J-D.L., AS-C., O.L., Y.L. and R.T contributed to study conception and design. J-D.L. and O.L. enrolled patients from the participating hospitals and provided the clinical information. AS-C., H.B., E.F., H.H., C.La., O.L., C.Le. P.V. and P.T. contributed to acquisition of data. H.B., H.H., C.La., J-D.L., Y.L. and R.T participated in the statistical analysis and the interpretation of data. H.H., C.La., Y.L. and R.T. contributed to the drafting of the manuscript. AS-C., H.B., H.H., O.L., C.La., J-D.L., Y.L. and R.T. contributed to critical revisions.

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Figure legends

Fig. 1. Gene expression in HIC and cART in whole blood. (A) Heatmap of genes belonging to the main pathways associated to the differentially expressed genes in whole blood of HIC and cART patients, including NK cells, TLRs, TREM1 and CXCL8 pathways. (B) Relationships between genes differentially expressed in whole blood of HIC compared to cART patients. Red symbols are overexpressed genes in HIC compared to cART patients, green symbols are underexpressed genes. Solid lines represent direct links between genes and dashed lines represent indirect links (with no more than one gene between the two genes).

Fig. 2. Gene expression in HIV peptides unstimulated and stimulated peripheral blood mononuclear cell (PBMC) of HIC and cART. (A) Main differentially expressed genes between HIC and cART patients associated with inflammation before HIV peptides stimulation. (B and C) Differentially expressed genes between HIC and cART patients associated to inflammation and IFN signaling after HIV peptides stimulation of PBMC.

Fig. 3. Cytokines profiles of peripheral blood mononuclear cell (PBMC) from HIC and cART patients stimulated *in vitro* with HIV peptides covering Gag, Pol and Nef antigens. (A) Cytokine measurements (pg/ml) in supernatants of stimulated PBMC from HIC (n=20) and cART (n=15). Cytokine secretion was measured in supernatants after HIV peptide stimulation of PBMC using Bio-Plex 200 system™ (Bio-Rad) at day 2 of stimulation for IP-10 and IL-1 β , and at day 9 after a re-stimulation for 24 hours for TNF α and MIP-1 β . (B, C) CD8 and CD4 T cell

producing cytokines after PBMC stimulation with HIV peptides for 9 days as measured by ICS. **(B)** Frequency of CD8 T cells producing TNF α , IFN α and MIP-1 β (sum of the cytokines or individual cytokines) in 18 HIC and 14 cART patients. **(C)** Frequency of CD4 T cells producing TNF α , IFN γ and MIP-1 β (sum of the cytokines or individual cytokines) in 18 HIC and 14 c-ART patients. Horizontal lines represent the median $\pm$ IQR, and Mann-Whitney test has been used.

Fig. 4. Cytokines profiles of peripheral blood mononuclear cell (PBMC) from strong (SRHIC) and weak (WRHIC) responders HIC, stimulated in vitro with HIV peptides covering Gag, Pol and Nef antigens. (A) Cytokine measurements (pg/ml) in supernatants of stimulated PBMC from 10 SRHIC and 10 WRHIC. Horizontal lines represent median $\pm$ IQR and Mann-Whitney test has been used to compare cytokines secretion among groups of patients. **(B)** Heatmap of 14 cytokine profiles of SRHIC and WRHIC. Cytokine secretion was measured in supernatants after HIV peptide stimulation of PBMC using Bio-Plex 200 system™ (Bio-Rad) at day 2 of stimulation for IL-2, IL-1 β and IP10 or at day 9 after a re-stimulation for 24 hours for all other cytokines. The white color indicates a very low cytokine concentration (or no detection), and the dark red color indicates a high cytokine concentration.

Fig. 5. CD4 and CD8 T cell producing cytokines after peripheral blood mononuclear cell (PBMC) stimulation with HIV peptides for 9 days measured by ICS assay. (A) Frequency of CD8 T cells producing TNF α , IFN γ and MIP-1 β (sum of the cytokines or individual cytokines) in 9 SRHIC and 9 WRHIC. **(B)**

Frequency of CD4 T cells producing $\text{TNF}\alpha$, $\text{IFN}\gamma$ and MIP-1 β (sum of the cytokines or individual cytokines) in 9 SRHIC and 9 WRHIC. Horizontal lines represent the median $\pm$ IQR, and Mann-Whitney test has been used. Pie charts represent the cell poly functionality, ie the relative proportion of CD8 and CD4 T cells producing 1 (grey), 2 (dark grey) or 3 (black) cytokines.

Fig. 6. Unsupervised hierarchical clustering of differentially expressed genes between SRHIC and WRHIC subjects in purified T cell populations. (A) Unsupervised hierarchical clustering of CD4⁺ T lymphocytes samples between 8 SRHIC and 9 WRHIC subjects. **(B)** Unsupervised clustering of CD8⁺ T lymphocytes samples between 10 SRHIC and 10 WRHIC subjects.

Fig. 7. $\text{IFN}\gamma$ pathway associated with differentially expressed genes between SRHIC and WRHIC purified CD8 T cells. Overexpressed genes with $\text{FC} \geq 1.5$ are represented in red, underexpressed genes in green and genes with a $\text{FC} < 1.5$ and > 1.2 in gray.

Fig. 8. Summary of genes differentially expressed in the various experiments performed in the study. (A) Commonly differentially expressed genes between HIC and cART PBMC at 6 and 24 hours of stimulation, between SRHIC and WRHIC CD4, between SRHIC and WRHIC CD8 and between HIC and cART in the whole blood (WB). Commonly differentially expressed genes between HIC and cART in WB and between SRHIC and WRHIC CD8⁺T cells, are indicated by stars. **(B)** Predicted functions committed based on the 671 genes differentially expressed

699 specifically between SRHIC and WRHIC CD8⁺ T lymphocytes, using Ingenuity
700 software. Green symbols are underexpressed genes in SRHIC compared to WRHIC,
701 red symbols are overexpressed genes. Supplementary legends are depicted in the
702 figure.

Table 1. Characteristics of the HIC and cART subjects.

	HIC	cART patients
Number of subjects	53	27
Age in years (Q1/Q3)	47 (20/79)	52 (40/64)
Genders (F; M))	F 24; M 29	F 12; M 15
HIV-1 plasma viral load (RNA copies/ml)		
Mean (sd)	1.6 (0.46)	1.3 (0.16)
Median (Q1/ Q3)	1.4 (1.3/1.9)	1.3 (1.3/1.3)
CD4+ lymphocytes		
Number of subjects	53	27
Count (cells/mm ³)		
Mean (sd)	713 (249)	606 (186)
Median (Q1/ Q3)	689 (502/859)	588 (498/698)
CD8+ lymphocytes		
Number of subjects	50	27
Count (cells/mm ³)		
Mean (sd)	829 (398)	725 (330)
Median (Q1/ Q3)	794 (593/920)	681 (526/852)

Fig. 1A

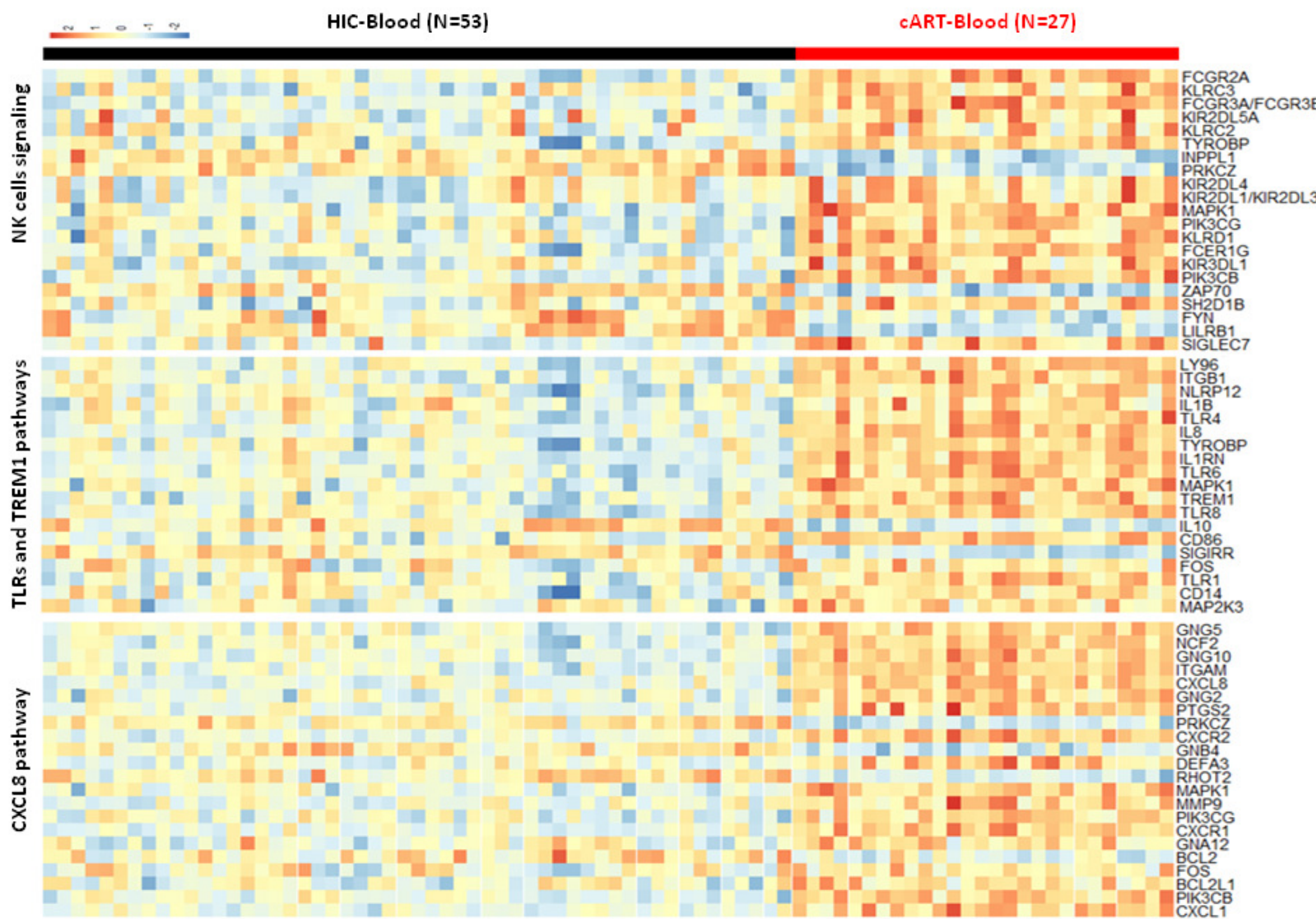


Fig. 1B

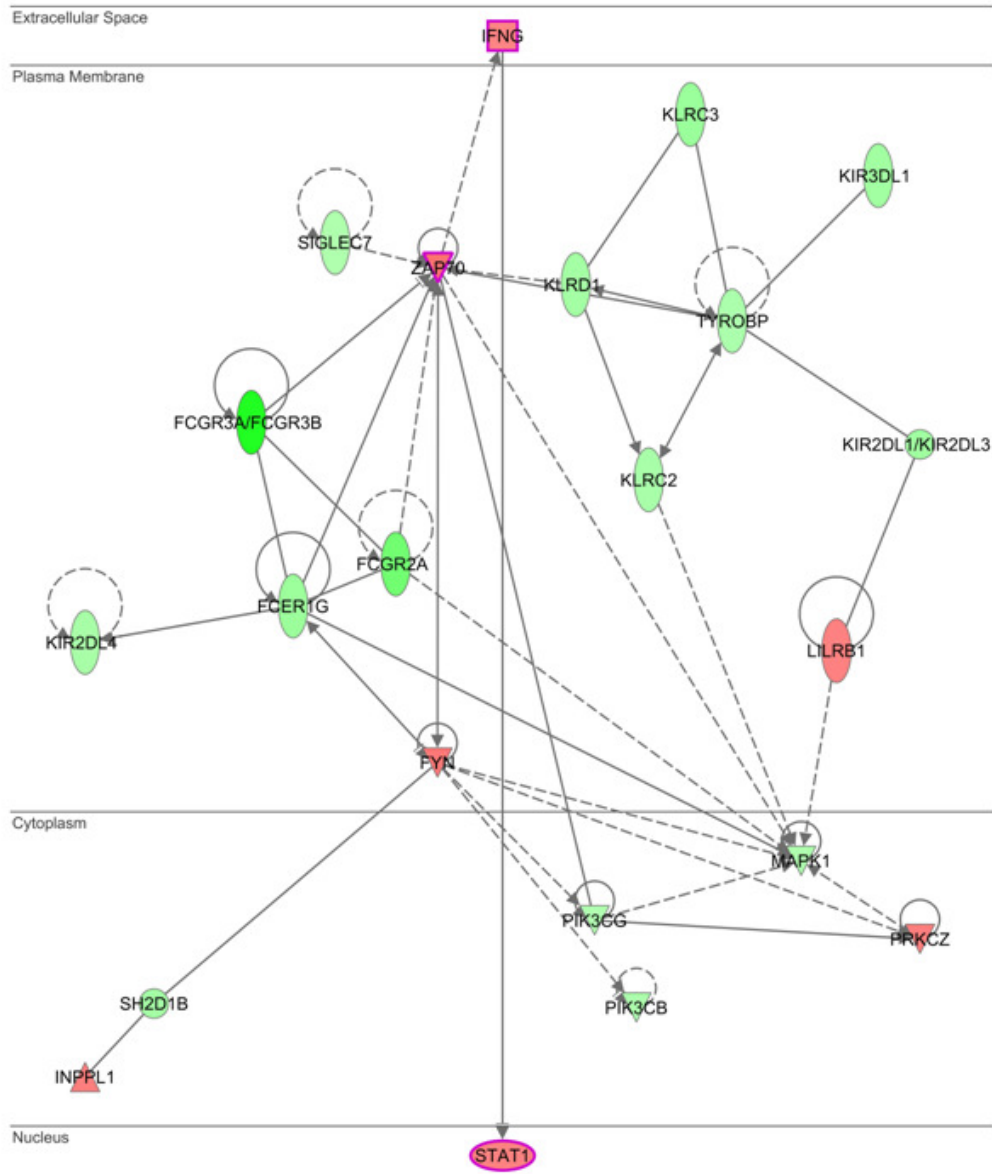


Fig. 2

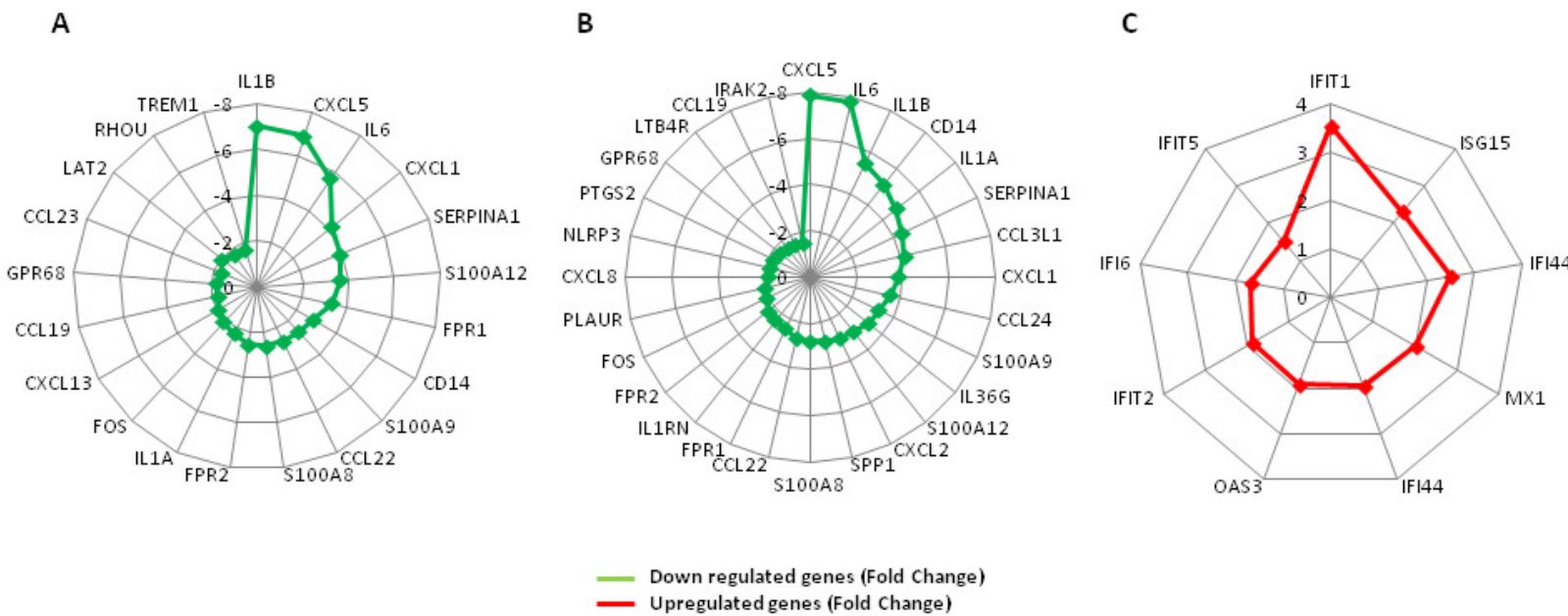


Fig. 3

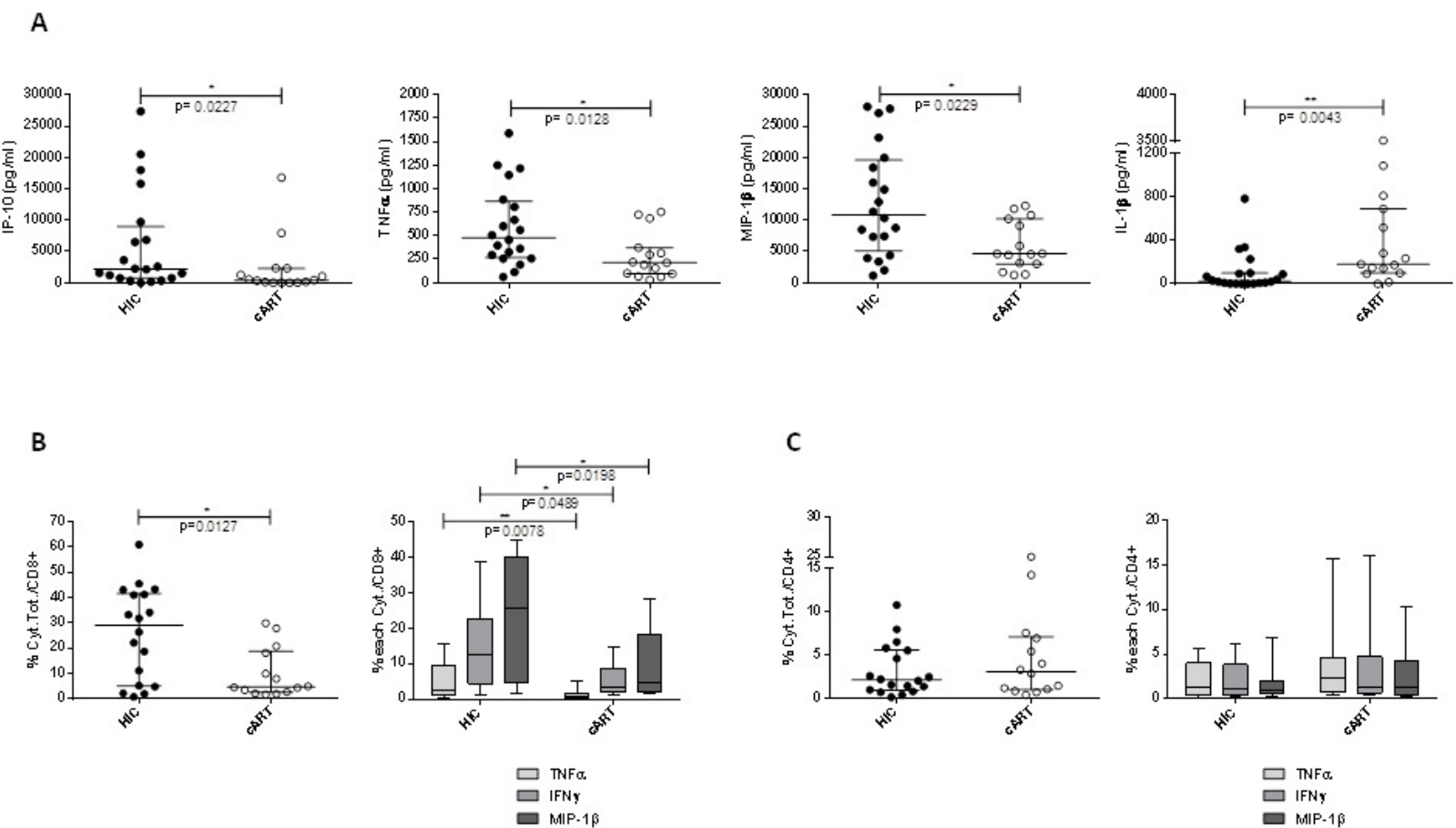


Fig. 4

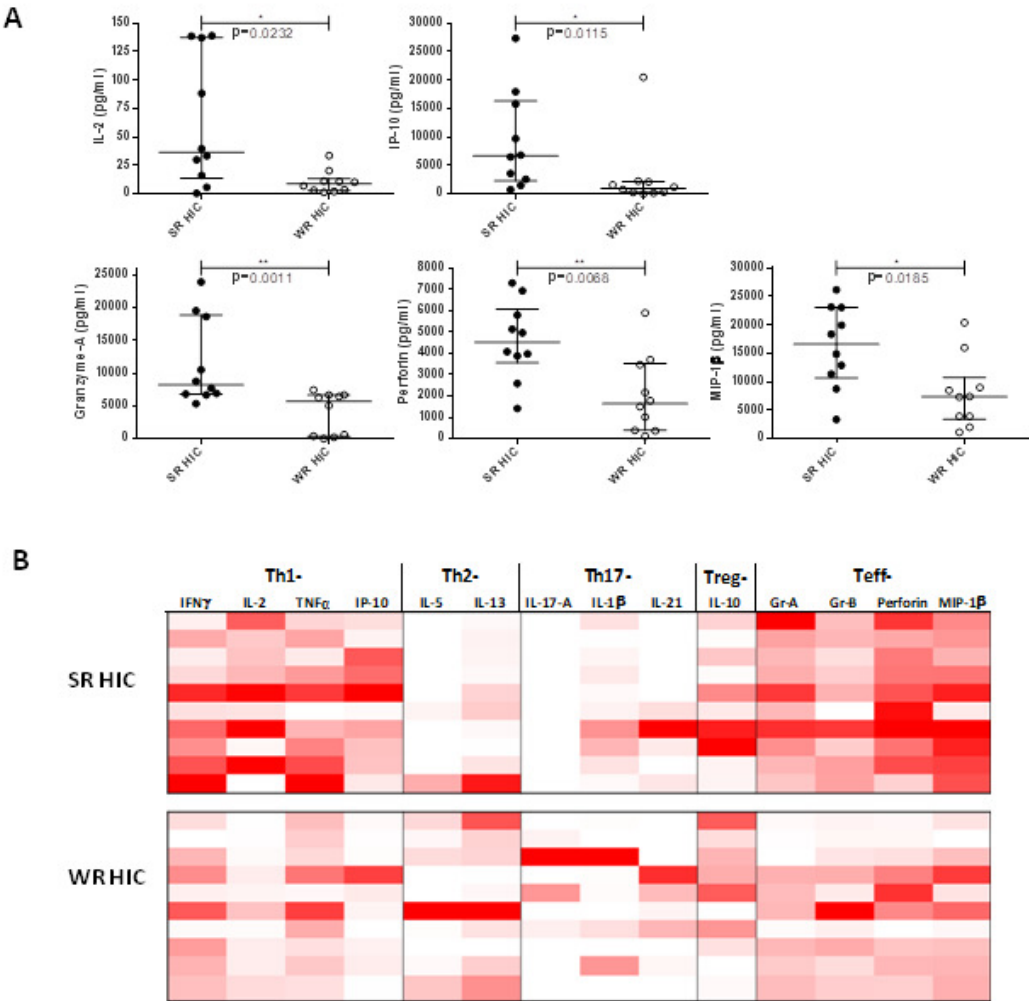


Fig. 5

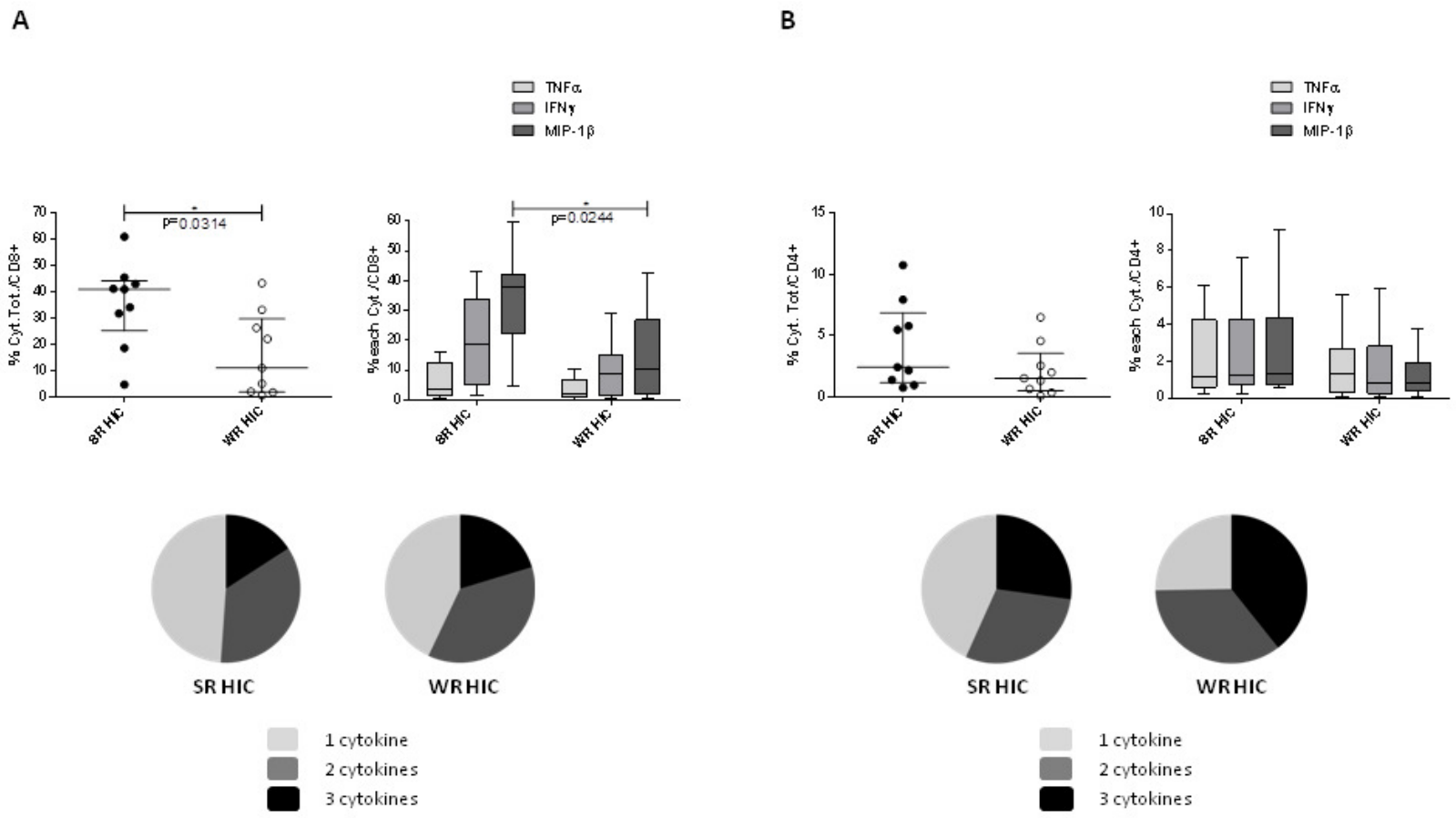


Fig. 6

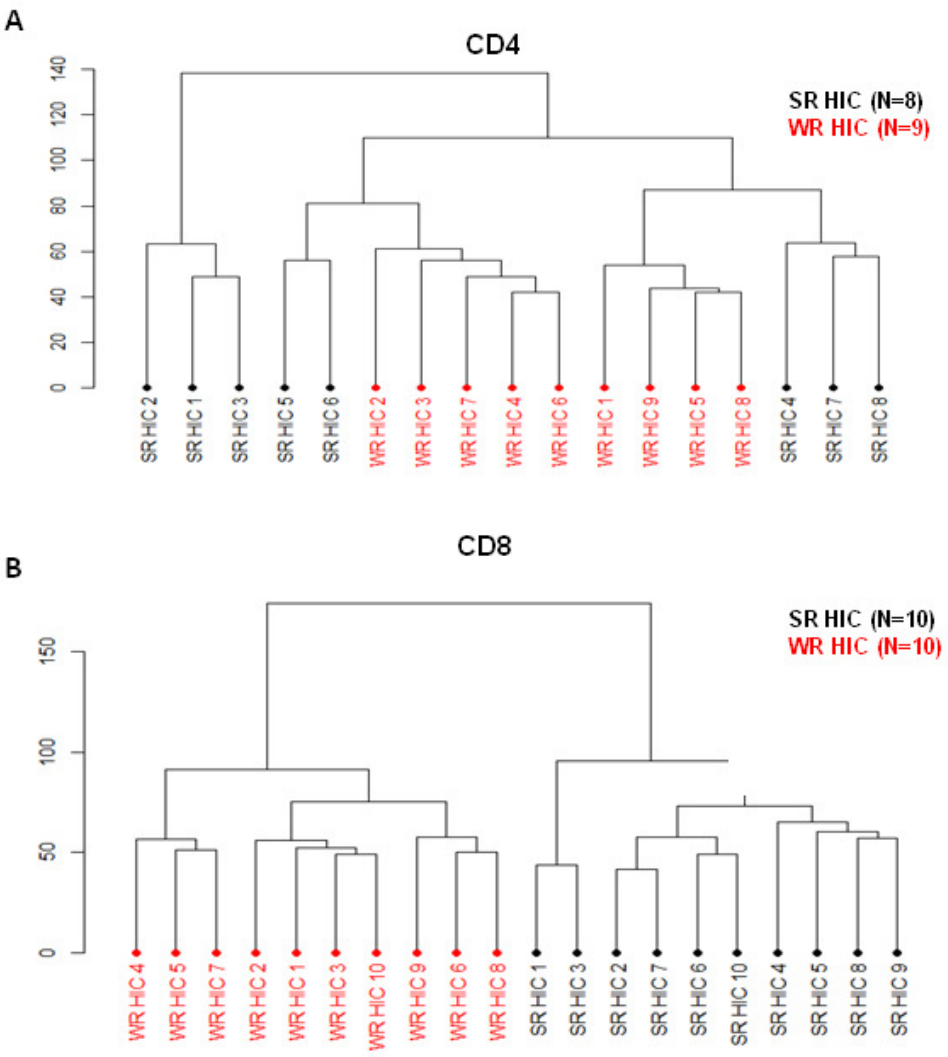


Fig. 7

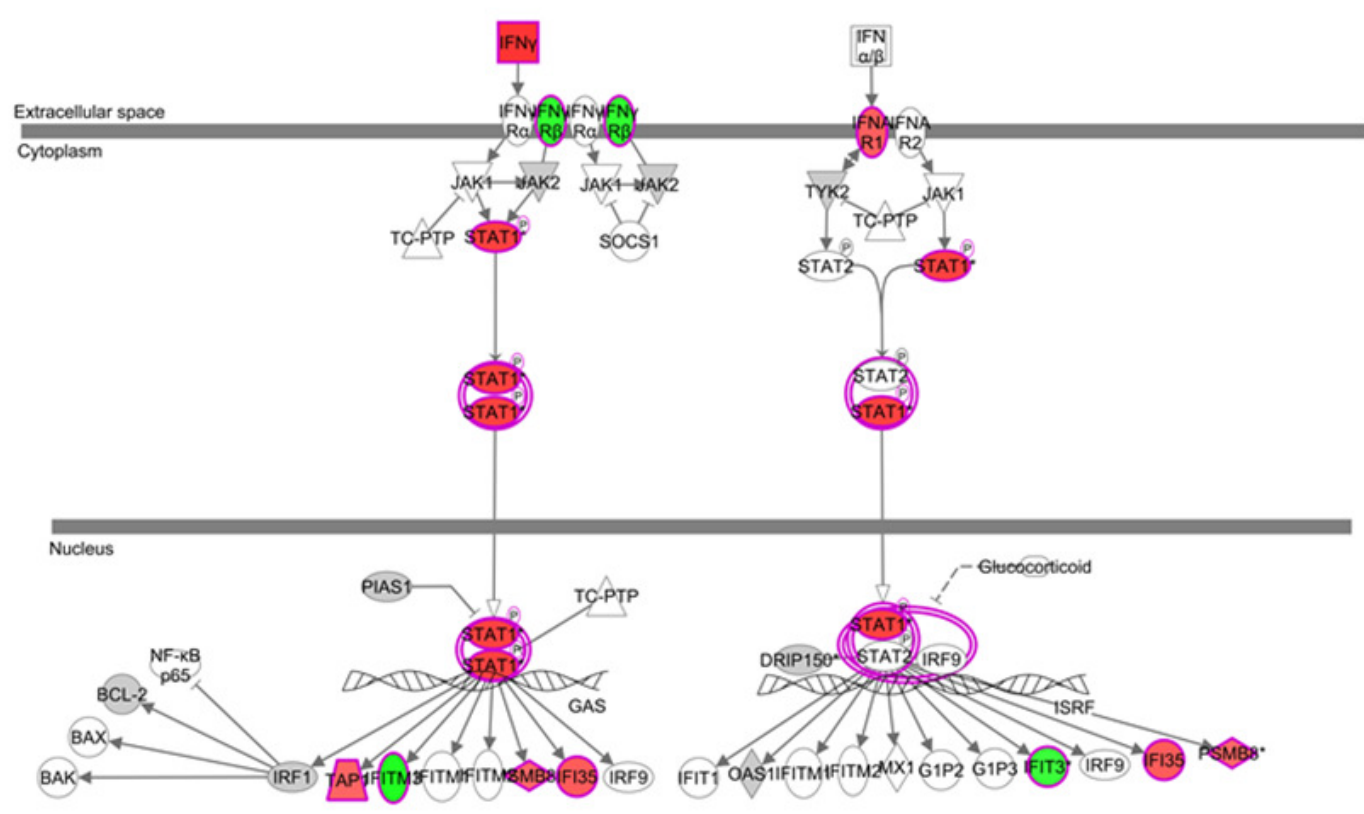


Fig. 8 A

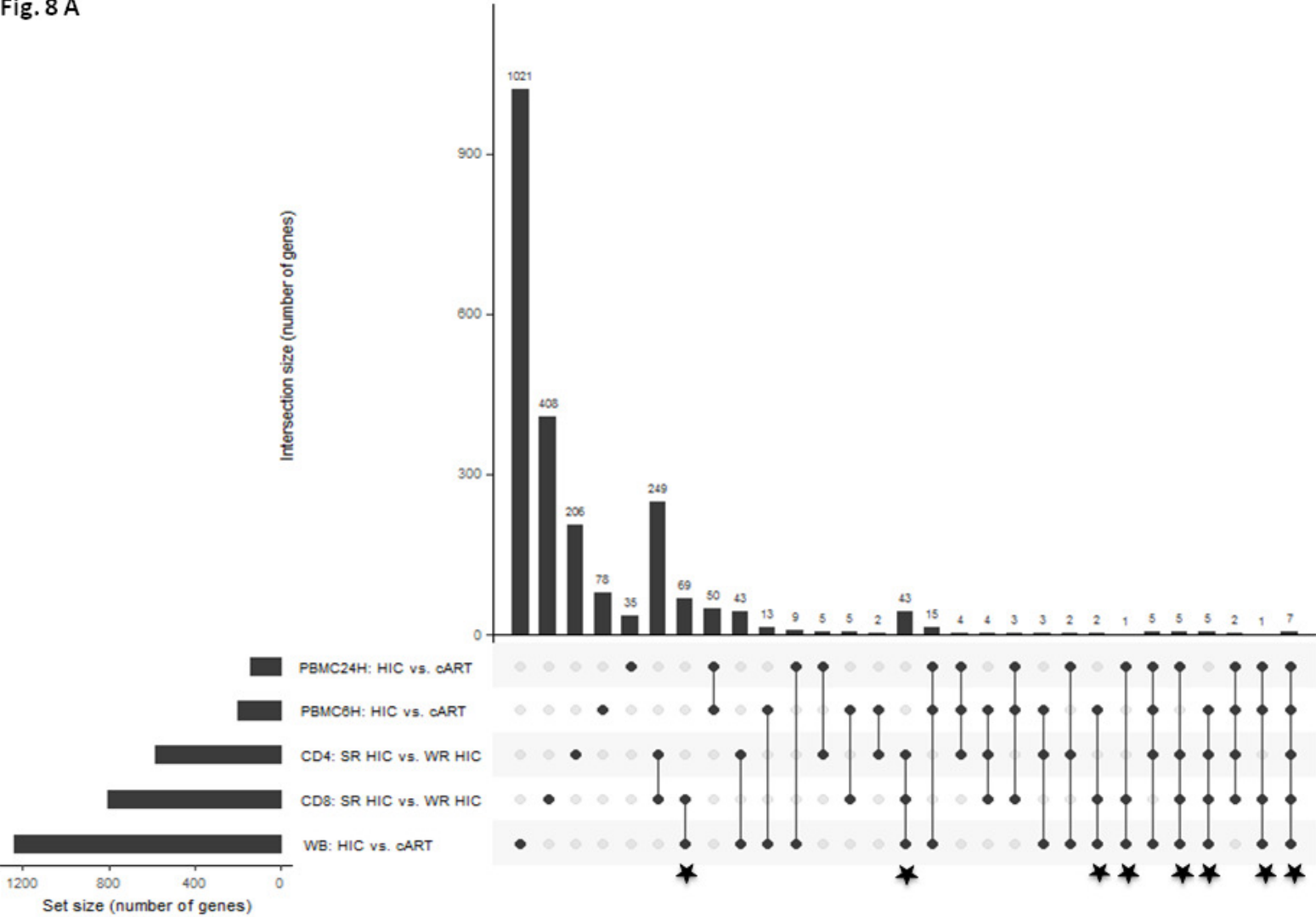


Fig. 8 B

