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Control of the HIV-1 DNA Reservoir Is Associated *In Vivo* and *In Vitro* with NKp46/NKp30 (CD335 CD337) Inducibility and Interferon Gamma Production by Transcriptionally Unique NK Cells

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Abstract

The size of lentiviral DNA reservoirs reflects effectiveness of immune responses against lentiviruses. So far, abundant information has been gathered on the control of HIV-1 replication, Understanding of innate mechanisms contributing to containment of HIV-DNA reservoir, however, are only partly clarified and are relevant to guide interventions for reservoir containment or eradication

We studied the contribution of Natural Killer (NK) cell functional features in HIV patients either controlling replication either spontaneously (HIC) or after progression and antiretroviral treatment (PP). An inverse correlation between HIV-DNA copy numbers (either total or integrated) in circulating $CD4^+$ cells and NK cell function were observed. Induced IFN-γ production and NKp46/NKp30 activating receptor-induced expression correlated inversely with reservoir size. The correlation was present not only when considering a homogeneous cohort of HIC patients, but also when PP patients were included in the analysis. Adaptive ($NKG2C^+CD57^+$) NK cell features were not associated with reservoir size. However a distinct set of 370 differentially expressed transcripts was found to underlie functional differences in NK cells controlling HIV-DNA reservoir size. In proof-of-principle in vitro experiments of $CD4^+$ cell infection with HIV-1, purified NK cells with the above functional/transcriptional features displayed a 10- and 30-fold higher ability to control HIV replication and DNA burden in vitro, respectively, compared to other NK cells.

Thus, NK cells with a specific functional and transcriptional signature contribute to control of HIV reservoir in $CD4^+$ cells. Their selection, expansion and/or adoptive transfer may support strategies to eradicate HIV-1 infection or to safely deescalate antiretroviral treatment.

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52 **Importance**

53 The most relevant feature of HIV-1 infection is represented by its DNA reservoir size in the body
54 that guarantees lifelong infection and resumption of virus replication after antiretroviral treatment
55 interruption. So far, there has been little success in the identification of factors contributing to HIV-1
56 reservoir containment.

57 Here, by studying quantitative total and integrated HIV-1 DNA and NK cells in HIV-1 patients with
58 both progressive and non-progressive disease, we observed that inducible IFN-γ and Natural
59 Cytotoxicity Receptor (NCR) expression in a specific subset of NK cells with characteristic
60 transcriptional signature represents a correlate for HIV-1 reservoir control. This represents an
61 advance in our understanding of mechanism(s) that control lentivirus reservoir. Monitoring,
62 selection, expansion and adoptive transfer of these NK cells could allow monitoring treatment
63 efficacy, likelihood of reservoir control and could support protocols for HIV-1 eradication.

64

Introduction

Since their original description of cells endowed with cytotoxic antitumor activity and CD16 and CD56 antigen expression (1-4), our view of Natural Killer (NK) cells has been upgraded with a number of additional functions, including a finely tuned balance between activating Natural Cytotoxicity Receptors (NKp46, NKp30, NKp44; NCRs) and inhibitory Killer Ig-like Inhibitory Receptors (KIR) function, cytokine production and responsiveness, characterization of TLR and chemokine receptor expression and identification of NK cell developmental stages from $CD34^+$ cell precursors(5-12). In addition, a quite remarkable heterogeneity of circulating NK cell phenotypes has been described by mass cytometry (13) and by computer-assisted flow-cytometric analysis(14)

Similarly, the original view of prevalent NK cell antitumor activity has evolved to encompass their fundamental role in the control of virus infections. Indeed, NK cell absence in humans, or their removal in mice, leads to disseminated Herpes, Influenza and Ebola virus infections (15-18). Their relevance in control of virus infections is reflected by the quantity of mechanisms that were developed to evade NK cell activity including skewed cytokine production(19, 20), reduced NCRs expression with activated NK cell phenotype(21-23) and NCRs-ligand shedding or down-modulation on target cells(22, 24, 25) (26, 27). A distinct pattern of NCRs expression and induction associates with a benign course of HIV infection in a minority of HIV-infected patients with low or undetectable viremia (HIV-controller, HIC; Long-term non progressors; LTNP; Elite Controller EC)(28-30) and in chronic HCV infected patients who respond to treatment(31, 32). In addition, epidemiological DNA carriage studies showed an association between specific KIR:HLA carriage and control of HIV-1 replication (33-35). The impact of coordinate KIR:HLA genotype carriage on disease progression so far partly eludes understanding, due to the variability of molecule transcription and surface expression on T and NK cells. Accordingly, the in vitro effect of HLA:KIR carriage on NK cell control of HIV replication (HIV_{p24}) in patients with delayed disease progression, remains controversial (36, 37) During the infection with different viruses including HCMV(38, 39),

MCMV(40), and possibly Chikungunya Dengue and Hantavirus(41, 42) persistent or transient expansions of a specific NK cell subset bearing NKG2C$^+$ in humans and its homolog in mice, have been described. These cell expansions are interpreted as possible memory-like features of NK cells with resemblance to adaptive immune T cell responses(11, 12).

With regard to NK cells in HIV-1 infection, scientific focus has so far concentrated on their control of HIV-1 replication and of plasma viral load (RNA), leading among other achievements to the identification of particular KIR:HLA haplotypes or NCRs expression regulation that may control virus replication (30, 37, 43) and to their cooperation with adaptive CD8$^+$CTL responses. The hallmark of lentivirus infection is however represented by the persistence of integrated or partly episomal DNA in long-lived cells - referred to as reservoir - which guarantees lifelong infection(44).

Focus on different reservoir sites and cells is being currently actively pursued including CXCR5$^+$PD1$^+$ Tfh cells(45, 46), CD32 CD4$^+$ T cells(47) and tissue monocytes/macrophages, Peripheral blood HIV-1 DNA (HIV-DNA) in circulating CD4$^+$ T cells represents an acknowledged site for estimating the total HIV reservoir in HIV infected patients(44) (48-50). The reservoir is still detected even after 5-14 years of successful (i.e.: with undetectable plasma viral RNA) antiretroviral treatment (ART)(51, 52). Persistence of HIV-DNA is maintained in a relatively constant non-replicating pool of central memory CD4$^+$ T cells in peripheral blood, lymph nodes and gut-associated lymphoid tissue (GALT)(50) and in monocytes and follicular dendritic CD4$^+$ T cells(44). Quantitative determination of HIV-DNA in PBMC contributes to define the risk of disease progression in infected patients(53) in whom low levels of HIV-DNA in CD4$^+$ PBMC are associated with non-progressive disease (HIC) (54) with post-treatment control of viremia(55). Accordingly, one of the major therapeutic objectives for lentiviruses in general and HIV-1 in particular is represented by the containment of HIV-DNA reservoir size, and its targeting with novel therapeutic strategies(56).

Limited information is so far available on the mechanism(s) that contribute to the containment of HIV-1 reservoir *in vivo*. To address this issue with specific focus on innate immune cells, we

studied the relationship between NK cell function and total and integrated HIV-DNA copy numbers in circulating $CD4^+$ PBMC in two widely diverging clinical cohorts (HIV and progressor patients). We here report that the degree of inducibility of IFN-γ production and of NCR have a linear relationship to quantitative control of HIV-1 reservoir in infected patients over a wide spectrum of disease activity and are associated to a specific NK cell transcriptional signature.

123 ## Results

124

125 **IFN-γ production by NK cell associates with the control of HIV-DNA.**

126 We first studied the quantitative HIV-1 reservoir in circulating PBMC of progressor patients (PP)
127 who had been treated successfully and were persistently suppressed for >18 months on ART and
128 in a cohort of HIV-1 controller patients (HIC) with spontaneously stable $CD4^+$ counts >500/μl and
129 low or undetectable plasma viremia As expected, a higher viral reservoir was detected in PP
130 compared to HIC (Fig.1 panel A-C) both in terms of total, integrated and unintegrated (i.e. linear, 1-
131 and 2-LTR circles) viral DNA ($p<0.001$). On the contrary, the evaluation of 2-LTR was not different
132 when comparing the two patient groups (Fig.1, panel D). A relatively wide range of HIV-1 DNA
133 copies/10^5 $CD4^+$ PBMC was detected in PP but also in HIC patients, suggesting that possibly
134 ample variations may exist in the mechanism(s) underlying control of HIV reservoir *in vivo* both
135 across patient groups (i.e. HIV vs. PP) and also within relatively selected groups such as HIC. We
136 therefore next sought to study possible parameters that could associate and explain the wide
137 dispersion in HIV-1 reservoir observed in HIC. In view of the reported association of innate NK cell
138 control of virus replication in HIV (30, 37) and of absent association of adaptive CD8CTL function
139 with post-treatment control or HIC(57) we next studied whether circulating HIV-DNA levels could
140 be associated with specific differences in NK cell function.

141 To this end, HIV-DNA copy numbers in PBMC (expressed as n°/$CD4^+$ PBMC) were first compared
142 to NK cell IFN-γ□□□□□□□□□□□□ by Spearman's rho. NK cells were gated as shown in Fig.2.
143 Lower HIV-DNA loads were detected in patients with higher proportions of IFN-γ producing NK
144 cells. This significant inverse association was detected using all the three NK cell-triggering
145 strategies, and was not restricted to a single activating stimulus (Table 1, Fig.3A-C). A
146 representative example of two HIC patients with different proportions of NK cells producing IFN-γ
147 and highly diverging HIV-DNA reservoir (8cp vs.122 cp/10^5CD4), are shown in Fig. 3D and 3E.

148 Data relative to these two divergent patients are outlined in Fig.3A-C by an open diamond and
149 open triangle, respectively. Nonparametric correlation analysis showed a significant correlation for
150 total and integrated DNA, not for unintegrated DNA (Table 1)

151 Taken together these data indicate that the size of HIV-DNA reservoir is closely associated to the
152 ability of NK cells to produce IFN-γ in response to multiple triggering signals mimicking signaling
153 of NCR:ligand recognition on target cells (e.g.: B7-H6 through NKp30 or PVR/Nectin-2 via DNAM-
154 1), or signaling by mature DCs (e.g.:DNAM-NKp30, rhIL-12/rhIL-15). The overall ability to produce
155 IFN-γ □□□□□□□□□□□ □□□□□□□□□□□t□□□, contributes to up to 10-fold differences (as
156 indicated by the HIV-DNA range, Fig.1A-B) in the control of lentivirus reservoir even among a
157 homogeneous cohort of HIC patients with excellent control of viral replication.

158

159 **Inducible expression of NKp46 and NKp30 upon NK cell activation correlates with**
160 **HIV-DNA reservoir and with IFN-γ production**

161

162 We next studied whether also inducible NCR expression by NK cells, which has been associated
163 with benign disease courses in different chronic infections (TB(58),HCV(31); HIV (59)) (30), could
164 contribute to the control of the size of virus reservoir. To this end, flow cytometric analysis of NCRs
165 expression was assessed on highly purified NK cells before and after rhIL-2 activation (Fig.2, for
166 gating strategy). The inducibility of NCRs (shown in Fig.4E), expressed as fold-increase over
167 baseline (see Materials), was then correlated to HIV-DNA copies. As shown in Figure 4A and C
168 and Table 1, the inducibility of both NKp46 and NKp30 expressed as proportion of expressing cells
169 and also as molecule density (MFI) on NK cells, is inversely correlated with the amount of HIV-
170 DNA (cp/10^5 $CD4^+$ PBMC) detected in PBMC from HIC patients ($p<0.01$ and 0.001, respectively).
171 Similar correlations were detected for total HIV-DNA but not nor for unintegrated DNA (Tab1). No
172 correlation was observed between NKp44 inducibility and reservoir size (Fig.4A).

Analysis of IFN-γ production and of NCR induction upon NK cell activation revealed that these two NK cell functions are directly correlated. Indeed, by inducing *in vitro* activation with rhIL-12+rhIL-15 – i.e. a condition independent on NCRs triggering and a proxy for NK cell activation by mature DCs - we detected a direct correlation between IFN-γ secretion and *de novo* expression of NKp46 and NKp30 ($p<0.01$ and $p<0.05$, respectively) (Fig. 4panel B and D).

Thus, these findings indicate that the size of lentivirus reservoir in a subset of patients with efficient control of virus replication (HIC) correlates inversely with the functional efficiency of NK cells in terms not only of IFN-γ production but also of inducible expression of NKp46 and NKp30.

NK cell function is associated with control of HIV-DNA in PBMC in divergent disease courses

The inverse association between two distinct NK cell functional capabilities and the size of HIV-DNA reservoir in HIC raised the question of whether this could be applied only to the minority (<0,5-2%) of patients with exceptionally conserved NK cell function (HIC) or rather whether this could represent a general mechanism applying also to patients who do not control HIV-1 replication with progressive disease, $CD4^+$T cell loss and who needed ART (PP).

To address this question, we analyzed NK cell function (IFN-γ production and induction of NCRs expression) in a group of stably virologically suppressed PP on cART. HIV-DNA in PBMC from these patients was significantly higher compared to HIV-DNA levels in the PBMC of HIC patients (17 vs. 10 copies/ 10^5 $CD4^+$PBMC) HIC vs. PP, respectively; $p<0.0001$, U-test) (Figure 1).

Analyses of pooled (PP+HIC) patient samples confirmed the inverse relationship between NK cell function and HIV-DNA copies (Table 1). This correlation was detected for both IFN-γ production (Fig.5A) and for induction of NKp46 and NKp30 expression vs. HIV-DNA copies in PBMC (Fig.5B).

Following pooled analysis, PP samples were also evaluated alone. As with HIC (Fig.4), an inverse relationship was observed between virus reservoir and IFN-γ production (Fig 5, panel C), while this was not the case for NCR inducibility (Fig.5D). This was not unexpected, as lack of NKp46 and NKp30 NCR inducibility (but not NKp44) is a hallmark of previously progressive disease even after successful cART(30).

Since expression of NCRs is clearly inducible only in HIC but not in PP patients, we verified whether differences in cytotoxic activity would actually reflect differences in activating surface receptor expression after *in vitro* activation of purified NK cells. Indeed, NCRs-mediated redirected killing of target cells was similar for HIC and PP at baseline (freshly drawn purifiec NK cells, T0) while it was significantly increased in HIC vs. PP after 2 and 4 days of *in vitro* activation in the presence of IL-2 ($p<0.01$) with a 2-10 fold increase over baseline values in different patients (Fig.5E). Interestingly, NK cell cytotoxicity after activation *in vitro* was directly correlated to the fold-increase in NCRs expression (Fig.5F). It should be noted that the differences observed in functional induction of NCRs did not correspond to differences in baseline expression of NKp30, NKp46 or DNAM-1 activating receptors on NK cells (Fig.6A, Fig.2 for gating strategy) and the frequency of NKp30/NKp46+ NK cells were not correlated to IFN-y production. In addition, IFN-y production inversely correlated with reservoir not only in the condition with NK cell triggering via NKp46/30, but also via IL-15/IL-12 and NKp30/DNAM-1 triggering. These findings indicate that the key functional feature with regard to IFN-y production is not represented by basal receptor expression, but rather by intrinsic production upon polyfunctional triggering.

With regard to NKG2D, its expression was increased in HIC compared to HD (Figure 5A), with comparable IFN-γ production upon its triggering in combination with DNAM-1 to reproduce the interaction with DCs *in vitro* (Fig.6B). No defect emerged in the general potential of NK cells from PP to produce IFN-γ when compared to HD, as determined following maximal stimulation with PMA+ionomycin stimulation (Fig.6C), and HIC had increased IFN-γ producing potential compared both to PP and HD (Fig.6C).

223 Thus, these results show that inducibility of NCRs expression parallels acknowledged important
224 NK cell functions such as IFN-γ production and contributes to edit downstream NK cell NCRs-
225 mediated cytotoxicity. The relationship of these functional activities with quantitative HIV-DNA
226 assessment independent of the ability to control HIV-1 replication and $CD4^+$ cell loss, suggests that
227 different set points for virus reservoir (HIV-DNA) are associated to a continuum of quantitative NK
228 cell function. The satisfactory control of HIV-DNA copies observed not only in HIC, but also in
229 some PP after successful ART, suggests a preferential role for *in vivo* NK cell function in the
230 control of lentivirus burden (DNA) even when failing to control viral load/replication (RNA).

231

232 **Distinct NK cell transcriptional signatures underlie the control of reservoir size.**

233

234 Upon the identification of IFN-γ production and of the extent of NCR (NKp46, NKp30) modulation
235 as inverse correlates of HIV reservoir in $CD4^+$ PBMC, we decided to address some open questions
236 including whether the strong correlations observed could indeed be based on substantial
237 differences in transcriptional programs. We wanted to test the possibility that increased transcripts
238 for NKp46 (NCR1) and NKp30 (NCR3) could be detected, or rather whether the increased *de novo*
239 expression of NCR in NK cells from HIC patients would depend predominantly on
240 posttranscriptional regulation. In addition we also wanted to explore additional differences in
241 transcriptional signatures that could help identify specific surface markers for future selection
242 strategies.

243 To this end, we performed a full transcriptional characterization of peripheral purified NK cells.
244 Multiple samples from different patients (4HIC, 4PP) of purified NK cells, either resting or activated
245 *in vitro* with rhIL-2, were evaluated by microarray analysis. Comparative transcript analysis
246 (Student's t Test cut-off $p<0.05$) identified 370 genes, which were differentially expressed by
247 purified resting NK cells derived from HIC and PP patients (Fig.7A). Interestingly a set of 370

transcripts were also found to be differentially expressed in NK cells of HIC vs. PP patients following rhIL-2 stimulation (Student's t Test cut-off $p<0.05$) (Fig.7B). When assessing the identity of the transcripts that were differentially expressed in resting and activated conditions, however, only 5 genes were found to be overlapping between the two sets of 370 transcripts (Fig.7C). In NK cells derived from HIC patients, of the 370 genes differentially expressed upon rh-IL2 stimulation, 306 (Suppl.Tab.1) and 64 (Suppl.Tab.2) were up-regulated and down-regulated, respectively. Interestingly, genes encoding for activating receptors of NK cells such as NCR3 (NKp30) and NCR1 (NKp46) were found to be up-regulated in NK cells derived from HIC patients upon rhIL2 stimulation (Suppl.Tab.1), thus indicating that in NK cells from these patients NCR induction is transcriptionally regulated and differs from PP. Under the same conditions also other transcripts coding for activating NK cell receptors were up-regulated including NKG2D (KLRK1), NKG2F (KLRC4), NKp80 (KLRF1), while transcripts coding for an inhibitory receptor (CTLA4) was down-regulated. Transcripts for NKG2C (KLRC2) showed only a trend towards increased transcription (p=0,17, fold increase 2.5). In addition, a significantly increased expression of inhibitory receptor transcripts KIR2DL4 and KIR3DL3 were detected at baseline in HIC, as compared to PP. At the same time, induction of KIR3DS1 confirms previous reports(37) and may contribute to HIV DNA control only in the fraction of HIC carrying the appropriate HLA class I molecules.

We then explored the functional pathways along the differentially expressed transcripts by Funrich analysis. The entire cohort of 370 transcripts that were differentially expressed between HIV vs. PP following rhIL-2 stimulation showed an involvement of IFN-γ, rhIL-2/rhIL-12, and TNF-receptor signaling pathways (Fig.7D). Furthermore, functional analysis performed by the David tool showed that genes that are upregulated in HIC upon rhIL2 stimulation (n=306) are mostly involved in the NF-kB pathway, in lysosome signaling and in mechanisms shared with lymphocyte cytotoxicity (Suppl.Tab.3).

273 The different transcriptional signatures of purified NK cells in HIC could reflect an inherent, and
274 possibly genetically encoded, regulation of NK cell function, or rather derive from a memory-like
275 expansion of one or few NK cell subsets following persistent lentiviral infection. Both NKG2C
276 activating receptor and CD57 are expressed on $CD56^{dim}$ memory-like NK cell that undergo
277 expansion after HCMV and other virus infections(39, 40). Therefore, we studied the expression of
278 these markers on NK cells from our patient cohorts. In line with the KLRC2 findings by microarray
279 analysis, flow cytometric analysis of peripheral NK cells in HIC or PP patients showed a similar
280 proportion of memory-like NK cells (HIC vs. PP) both in terms of $CD56^{dim}NKG2C^{+}$ and of CD56dim
281 $CD57^{+}NKG2C^{+}$ NK cells, while they were virtually absent in HD as expected, due to lower CMV
282 seroprevalence (Fig.8). Taken together, these results show that differences in major NK cell
283 functional activity in patients who control lentiviral reservoir (HIV-DNA) rely on inherent NK cell
284 transcriptional regulation, with a distinctive signature involving primarily several activating NK cell
285 receptors. Thus, a sufficiently high NK cell enrichment is present, sufficient to be clearly detected
286 by macroarray analysis. This was unrelated to differential involvement in HIV-1 reservoir control of
287 the whole subset of memory-like ($NKG2C^{+}CD57^{+}$) NK cells. However, the possibility cannot be
288 discarded that a particular subset of cells in this memory-like population may be involved and may
289 encompass adaptive NK cells with inducible NCR/NKG2D/NKp80, as indicated by *in vitro* and
290 microarray analysis.

291

292

293 **Transcriptionally unique NK cells from HIC patients control HIV integration in $CD4^{+}$**
294 **T cells in vitro.**

295

296 In order to directly verify that differences in NK cell function and significant differences in
297 transcriptional signature, indeed determine different DNA integration and contribute to sizing the

lentivirus reservoir size, we then studied the effect of coculturing NK cells in an *in vitro* infection experimental model.

Purified activated HD $CD4^+$ blasts were cultured *in vitro* and were infected with $HIV\text{-}1_{Bal}$ at high MOI (0.1). After removal of excess virus, highly purified (>98%) patient activated NK cells were added to the cultures at a 0.5:1 (NK:CD4) ratio. This experimental design aimed at studying *in vitro* control of DNA reservoir by NK cells while monitoring successful infection as represented by HIV RNA/p24. To explore the effect of predominant IFN-γ production and NCR expression on infected $CD4^+$ cells and to minimize the physiological inhibitory effect of KIR:HLA engagement, preactivation of purified NK cells was performed before co-culturing in an allogeneic system.

Virus replication, as measured by supernatant RNA copies and by HIVp24 expression by infected $CD4^+$ T cells, peaked at day 7 after infection. NK cells from HIC patients efficiently controlled virus replication as compared to PP (Figure 9, panel A, B). An 88% reduction of $p24^+CD4^+$ T cells was detected in culture by flow cytometry, and a 93% reduction in HIV-RNA was detected by qRT-PCR. Quantitative evaluation of HIV-DNA was performed by RT PCR and showed that HIC NK cells sharply controlled total and integrated HIV-1 DNA accumulation during peak virus replication, with a 98% and 96% reduction in total and integrated HIV-DNA, respectively, when compared to cultures supplemented with purified NK cells from PP (Fig.9, panel C, D). When comparing the effect of NK cells from different donors (HIC vs. PP) in controlling HIV-DNA accumulation (Fig.9E), purified NK cells from HIC with specific transcriptional signatures had a 35-fold higher effect compared to NK cells from PP (Fig.9F). A more contained comparative potency in containing HIV-1 replication was observed (14- and 8- fold for HIV-RNA and $HIVp24^+$, respectively) (Fig.9F). When compared to NK cells from PP, lacking NCR and IFN-γ inducibility, the presently characterized NK cells with a characteristic function and transcriptional signature, have comparatively an approximately 3-fold higher efficacy in containing HIV-DNA accumulation over HIV replication.

322 Taken together, these experiments show that NK cells with a specific transcriptional signature and
323 function provide a remarkable contribution limit HIV-DNA *in vitro*, thus confirming the *in vivo*
324 observations.

325

Discussion

The control of DNA reservoir, rather than only RNA, represents the main goal for the treatment of lentiviruses and ultimately for retrovirus eradication and clearance(44, 59-61). However, immune mechanism(s) contributing to lentivirus DNA reservoir size shaping and control still elude our full understanding. NK cells are involved in the control of RNA and DNA viruses belonging to different virus families including retrovirus replication (11, 16, 32, 38, 62).

In the present work we explored for the first time the impact of NK cell function and NK cell characterization on the containment of lentivirus reservoir in *vivo* and *in vitro* using HIV-1 as a model. The results provide evidence that transcriptionally characterized NK cells substantially contribute to HIV reservoir containment. The induction of IFN-γ and NCR (NKp46, NKp30) expression represents a quantitative functional correlate of lentiviral reservoir size containment. Previous work showed that NK cells with a specific HLA:KIR carriage may control viral replication (RNA production) without information on HIV reservoir(37) and also that non-progressing disease may be unrelated to carriage of KIR3DS1 carriage(36). In the present *in vivo* and *in vitro* setting, no HLA:KIR carriage was associated with HIC status, and the *in vitro* experimental strategy was chosen to minimize the interference of HLA:KIR interaction on NCR expression/inducibility and IFN-γ production on HIV-DNA accumulation. A doubtless need emerges for future work to include combined analysis of activating and inhibitory NK cell receptors and on the actual expression of KIR3DL1/3DS1 and of HLA-Bw4 on the role of other soluble factors produced by NK cells in addition to IFN-γ.

The present finding of higher levels of HIV-DNA in $CD4^+$PBMC in cART-treated PP is in line with previous reports(54), and with the association of more rapid disease progression in patients with higher $CD4^+$T-cell associated circulating HIV-DNA(53). In fact, the reservoir size decays with shallower slopes after the first year(s) of cART, remains constant and fails to decrease below detectability in most patients(51, 52), and is independent of $CD8^+$CTL activity(52). Virus replication

invariably resumes upon cART interruption(48, 49, 51), with a remarkable exception to this concept being a very small group of early cART-treated patients (post-treatment controller patients, PTCP)[4,5]. The present findings on NK cell-associated control of HIV reservoir size provide a novel reading frame for the above observations, suggesting that NK cell functional potential in terms of NCR and IFN-γ inducibility represent two pivotal elements for proviral DNA containment. Indeed, cART has a remarkable impact on HIV replication in $CD4^+$ cells, however it has a limited effect on the recovery of NK cell function(63, 64) and on DNA size reduction(52). An adaptive CTL-mediated control of DNA reservoir size is unlikely, as suggested by the heterogeneity of $CD8^+$ CTL function in PTCP(65) and by the lack of correlation between $CD8^+$ CTL activation and DNA reservoir(52). Thus, according to the present data, the failure to fully reconstitute NK cell function upon cART in PP is likely to contribute to the limited decrease over time in HIV reservoir (51, 52) and ultimately to the renewed HIV replication observed upon treatment interruption in most patients(48, 49). In view of the reported lack of $CD8^+$CTL control in PTCP, it could be hypothesized that in these patients NK cells may represent an important immune mechanism leading to HIV reservoir and spontaneous post-treatment control.

cART has been shown to reconstitute $CD4^+$cell numbers, to leave NK cell phenotype and function largely unaffected and to provide minimal contribution to HIV reservoir decrease. For this reason, it appears unlikely that the effect on HIV reservoir and NK cell function in treated patients is due to cART. It remains to be determined whether some patients with active replication and progressing to low CD4 numbers may still limit the reservoir size in the presence of robust innate immune defenses (e.g. NK cell function), or rather whether in some PP cART may be effective also on NK cell function and contribute to HIV reservoir. Further focus is needed to understand whether NK cell function/transcriptional profile is preset and innate, or may be rather affected by virus replication or by cART, and to which extent poor control of $CD4^+$ cell HIV reservoir by poor IFN-γ producers may be improved.

If confirmed, the present characterization of transcriptionally and functionally distinctive NK cells could be exploited in the prospective identification of HIV-patients with good chances of success when undergoing cART simplification to monotherapy(66), or cART interruption (e.g. PTCP)(59, 67, 68).

In the present analysis, NK cells controlling HIV-1 reservoir size displayed a set of 370 differentially expressed NK cell genes/transcripts that include, among others, the principal functional NK cell pathways (i.e. cytokine production, cytokine response, NCRs induction) that were assessed by phenotypic and functional assays. This indicates that an inherent individual regulation of NK cell gene expression underlies functional differences, and affects the control of the HIV-1 reservoir set point and disease course(53). This finding is in line with previous observations showing that the NK cell transcriptional signature underlies specific disease courses in chronically infected HCV patients who will respond to treatment(59, 69). In view of the considerable size of the NK cell phenotype spectrum with up to 30.000/100.000 phenotypes(13), the detection of a specialized NK cell population of sufficient size to identify a distinct transcriptional signature may reflect enrichment of circulating NK cells, thus raising the question on their origin. NK cell-mediated memory-like reactions with large expansions of $NKG2C^{+}CD57^{+}$ NK cells have been detected during infections with different viruses in humans(38, 42, 70), and in animal models(71). Enrichment of memory-like NK cells of known phenotype ($NKG2C^{+}CD57^{+}$) was not involved here, since both patient groups (HIC and PP) had a similarly large circulating pool of putative memory-like ($NKG2C^{+}CD57^{+}$) NK cells, while functional and transcriptional differences were detected only in patients with the smaller lentiviral reservoir size (HIC). Support to the view that control of lentiviral reservoir cannot be ascribed to imbalances of memory-like NK subset as a whole, is further provided by the efficient IFN-γ production by NK cells upon rhIL-2 or rhIL-12/rhIL-15 stimulation. This is in contrast to the diminished response to rhIL-12 and rhIL-18 in $NKG2C^{+}$ NK cells from HCMV donors(72),and to reduced IFN-γ production upon H1N1 influenza or antigen or pertussis toxin stimulation(73). Moreover, memory-like NK cell expansion in HIV patients is due to

HCMV rather than to HIV-1 persistent infection (74). Therefore, two possible hypotheses may be proposed to explain our present findings. First, the enrichment in NK cells with such a distinct transcriptional signature in patients with a small HIV-1 reservoir size may represent a novel type of "trained" or adaptive NK cells, either independent of, or rather representing a specialized subset of "memory-like" NKG2C^{+}NK cells that is expanded and functional in HIC but not in PP. These cells could be identified for their ability to induce transcription of NKP46, NKp30, NKG2D, and NKp80 while retaining CD57 and NKG2C expression. Alternatively, in agreement with the high environmental influence of NK cell activating receptor expression(13), they may represent an inherent rather than an acquired inborn feature, therefore preexisting HIV infection and contributing to lentiviral containment following virus entry. In this case their expansion may be detected in the normal population with a low frequency (about 1-2%) superimposable to HIC prevalence among HIV-infected patients. Further work is needed to fully identify the origin and the presence at low frequency of these phenotypes in the overall population.

Finally, current programs and protocols for HIV eradication including histone deacetylase inhibitor (HDACi) administration and immunotherapy reportedly need improved immunotherapeutic strategies to clear virus in reactivated cells and limit new virus integration(44, 56, 60, 75, 76). Selection and expansion of NK cells with high NCR and IFN-γ inducibility could represent a useful tool to efficiently clear HIV-1 when combined within the frame of virus eradication strategies exploiting latency-exit induction, and immuno/antiretroviral therapy(75-77).

In conclusion, HIV reservoir size is dependent on the presence of a defined NK cell population with a specific transcriptional signature, high NCRs (NKp46, NKp30) and IFN-γ inducibility upon NCRs and cytokine receptor engagement. These cells may represent a novel tool offering relevant promise for translational applications including patient monitoring, treatment interruption and virus eradication.

427 ## Materials and Methods

428

429 **Patients.** Five Infectious Diseases Units caring for HIV patients enrolled 20 HIV-1 controllers
430 infected patients (10 ECs and 10 LTNPs), 10 HIV-1 infected patients with progressive disease
431 (Tab.2). Patients were being followed up within a program for surveillance and treatment of HIV-1
432 infection. EC patients had positive HIV-1 serology, ART-naïve, and had $CD4^+$ counts ≥450 cells/μL
433 at all visits for ≥7 y of follow-up with no clinical evidence of disease progression. LTNP met the
434 same definitions as EC, with the exception of HIV-RNA being detectable during the years of
435 observation and <2,000 cp/mL. A group of HIV-infected progressor patients on ART with HIV-RNA
436 < 50 cp/mL for at least 24 months and $CD4^+$T cells > 350/μL was considered as aviremic
437 progressor controls (Progressor Patients; PP) For these patients, evidence from clinical records at
438 cART initiation showed that they had viral replication and low $CD4^+$ cell numbers. HDs (n = 10)
439 were recruited locally among blood bank donors. Carriage of KIR:HLA alleles was analyzed as
440 described previously in HIC and PP(30). No significant association for HLA-Bw4:KR3DL1/S1 was
441 detected in HIC vs. PP.

442 Exclusion criteria were current or previous ART; age<18 years, pregnancy, cancer, and treatment
443 for HCV infection during the previous 6 mos.

444 The study was authorized by the local ethical committee. Patients were enrolled after providing
445 informed consensus. Peripheral blood mononuclear cells (PBMCs) were obtained by density
446 gradient centrifugation (Ficoll–Hypaque) and processed or cryopreserved at -80°C until processed.

447

448 **Abs.** The following panel of mouse anti-human monoclonal antibodies (mAbs) was used: PC7-
449 conjugated anti-CD56 (Immunotech-Coulter, clone N901); FITC- and APC-conjugated anti-CD3
450 (BioLegend, clone HIT3A); APC-conjugated anti-CD19 and anti-CD14 (BioLegend, clone HIB19

and M5E2 respectively); PE- and FITC-conjugated anti-CD16 (BD PharMingen, clone 3G8); 7AAD (BD PharMingen); anti-NKp46 (BAB281 and IgG1), anti-NKp30 (7A6 and IgG1), anti-NKp44 (ZIN231 and IgG1), anti-NKG2D (BAT221 and IgG1), anti-DNAM-1 (F22 and IgG1). FITC-conjugated (Southern Biotechnology) and PE-conjugated goat anti-mouse anti-isotype Abs (BD PharMingen) was used for controls.

Immunofluorescence Analysis. Cells were analyzed by eight-color flow cytometry. Briefly, cells were incubated with primary mAbs followed by PE- or FITC-conjugated anti-isotype-specific goat anti-mouse secondary reagents. Direct staining was performed by fluorochrome-conjugated mAbs as a third step. For cytofluorimetric analysis, cells were gated using forward and side light scatter parameters (FACSCantoII; BD), 10,000 events were always acquired on $CD3/14/19^{-}CD56^{+}$ gating. MFI was used to quantify molecule density. To reduce interassay variability, MFIsample/MFIcontrol (MFIratio) was used to compare sample/groups. Data were analyzed using FlowJo (Tree Star, Inc). Gating strategies are shown in Figure 2.

NK Cell-Activating Receptor Induction Assay. NK cells were isolated from PBMCs using negative immunomagnetic separation using NK Cell Isolation Kit II; Miltenyi Biotec as previously described(78). NK cell purity was ≥95%.

NKp46, NKp30, and NKp44. expression by flow cytometry were analyzed on highly purified NK cells before, 2 and 4 days after *in vitro* culture with rhIL-2 (200 U/mL; Proleukin; Chiron Corp.). Receptor expression changes were determined comparing MFIr to fresh NK cells MFIr: (MFIratioACT_MFIratio baseline)/MFIratio ACT_100 (ACT; Activated).

Cytotoxicity Assay. NK cell cytotoxic activity was determined using PKH26/TO-PRO3 (Sigma-Aldrich and Invitrogen, respectively) assay as previously described(79). FcγR$^+$ P815 mouse mastocytoma cell lines were used as target cells and were PKH26 labeled(80) P815-PKH26$^+$ were incubated with rhIL2-activated NK cells at 1:1 E:T ratios. Cultures were incubated for 4 h at 37 °C in 5% CO_2 in RPMI 1620 (BioWhittaker, Lonza) supplemented with 10% FCS (Complete Medium, CM), in the presence or absence of mAbs and then placed on ice until flow cytometric analysis. Spontaneous and maximal target cell deaths were respectively determined by PKH-26 labeling of cells cultured alone or permeabilized with BD Citofix/Citoperm reagent (BD Pharmigen). To identify dead cells, 5µL of 10-µM stock solutions TO-PRO3 was added to each tube immediately before analysis. Cells were analyzed by FACSCanto II (BD), and 10,000 events were collected. Specific lysis was calculated by the formula (Sample−spontaneous dye/total dye –spontaneous dye) x 100.

IFN-γ production assay. PBMCs were stimulated using FcγR$^+$ P815 target cells at 10:1 E/T ratio in complete medium in the presence or absence of an anti-NKp30 and/or anti-NKp46 and/or anti-NKp30 and/or anti-DNAM-1 mAb mixture (0.1 µg mL^{-1}), rhIL-12 (20 ng mL^{-1}), and rhIL-15 (40 ng mL^{-1}; PeproTech) as previously described(78).

HIV infection

PBMC from healthy HIV-1 seronegative donors were activated with purified PHA (0,25% vol.) and rhIL-2 (50 U/ml) for five days in complete medium RPMI 1620 (BioWhittaker, Lonza) supplemented with 10% FCS, L-glutamine (2 mM) and 1% antibiotic mixture (Penicillin–Streptomycin 5 mg/ml). Activated CD4$^+$ cells were positively purified with antibody-coated magnetic beads (Miltenyi Biotec) and then cultured in rhIL-2 (50 U/ml) for 1 week. The cells were infected with HIV-1 (HIV_{Bal}, MOI=0,01)(kind gift from Dr. G. Poli, Milan, Italy) for 2h at 37°C in a humidified 5% CO_2 atmosphere, washed twice to remove excess virus, cultured (5x10^{5}cells/ml) in CM rhIL-2 (50U/ml)

alone or with purified/activated NK cells in triplicate at 1:2 ratio. Supernatants and cells were collected on day7 and cryopreserved.

Total, unintegrated and integrated HIV-DNA quantification. **Total, unintegrated and integrated HIV-DNA quantification**. DNA was isolated from 1.0-2.0x10^6 PBMC of HIC and PP patients and from 3.0x10^5 *in vitro* infected $CD4^+$ blasts. A constant HIV-1 negative donor background of 1x10^7 uninfected and unstimulated PBMC was mixed to the cell pellets to ensure high DNA extraction efficiencies. Briefly(81) total and unintegrated HIV DNA (the ensemble of linear and circular 1 and 2-LTR), were selectively separated by an optimized chromatographic procedure (a method utilized to separate the high molecular weight DNA containing the proviral HIV-DNA from the low molecular weight DNA consisting of unintegrated HIV-DNA) and analyzed by quantitative SYBR Green I real-time based PCR (qPCR) using a single set of specific primers selected in the LTR-Gag highly conserved region of HIV-1 genome(82) and able to detect all HIV-1 subtypes in the M group. The qPCR measurements of 2-LTR circles were performed using primers flanking the dual-repeat cassette within the circular form. PCR reactions were carried out in a 7500 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc) in a final volume of 50 µL using the HIV-1 DNA qPCR kit (Diatheva s.r.l. Fano, Italy) and according to the above-developed assay. Each sample was analyzed in triplicate in a first PCR (1^{st} qPCR) consisted of two wells containing 0.5 µg each plus one well containing 1.0 µg of DNA (to ensure the detection of the target even in the low copy number, i.e. near the QL 2 copies/PCR) or the equivalent quantity of elution fraction from chromatographic separation to quantify unintegarated forms. For samples with HIV DNA datum quantified below 30 copies (coefficient of variation of 21%), a 2^{nd} PCR experiment was performed. Six 0.5 µg replicates were assayed for samples which in the 1^{st} qPCR had been quantified in the range between 30 to 2 copies; three 0.5 µg replicates and three 1.0 µg replicates were tested for samples which in the 1^{st} qPCR had been quantified near or detected below the QL. In case of negative amplification, a PCR spike-test was performed adding 2 or 10 copies of our plasmid (pPBS) standard to the samples to exclude the presence of inhibitors. HIV-1 DNA copy number was estimated by interpolation of the experimentally determined Ct (threshold cycle) on the standard curves generated using half-log serial dilutions from 10^3 to 2 copies. Integrated HIV DNA was obtained by subtracting unintegrated from total HIV-DNA. The agreement between this indirect and direct measurement of integrated HIV-DNA was demonstrated (A.C, manuscript in preparation). Total, unintegrated, integrated and 2-LTR copy numbers were given adding up the copy number from the 0.5/1.0 µg replicates tested and were

531 expressed to 1 µg of DNA and then normalized to copies/10^5 $CD4^+$ T cells. Values <2 copies/µg
532 were considered 1 for statistical analyses.

533 Serum and supernatant HIV-RNA was quantified by a commercial kit according to manufacturer
534 instructions RNA (Nuclisens EasyQ HIV-1 2.0, BioMérieux SA).

535

536 **Microarray analysis**. Total RNA was purified from freshly isolated, purified (>98%) NK cells
537 after flow cytometric sorting from 4 HIC patients and from 4 PP patients by using miRNeasy minikit
538 (Qiagen, Germantown, MD, USA) according to the manufacture's protocol. RNA quality and
539 quantity was estimated using Nanodrop (Thermo Scientific, Waltham, MA, USA) and Agilent 2100
540 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). First- and second-strands cDNA were
541 synthesized from 10 ng of total RNA by using Nugen Ovation Pico WTA System V2 (Nugen
542 technologies, San Carlos, CA, USA) and following manufacturer's instructions. cDNAs were
543 fragmented and biotinylated by using Nugen Encore Biotin Module (Nugen technologies, San
544 Carlos, CA, USA) and hybridized to the GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa
545 Clara, CA, USA). The arrays were washed and stained on a GeneChip Fluidics Station 450 and
546 scanned by GeneChip Scanner 3000 (Affymetrix, Santa Clara,CA, USA). The global gene
547 expression profiling of NK cells was analyzed using Partek Genomics Suite (St Louis, MO).
548 Functional analysis was performed using the David analysis (https://david.ncifcrf.gov/) and
549 FunRich analysis (Functional Enrichment analysis tool).

550 Analysis of genetic carriage (HLA and KIR) was performed on PBMCs as previously described
551 (30)and failed to show significant gene carriage enrichments.

552

553 **Statistical Analysis.** Statistical analysis was performed using the Mann-Whitney U tests for
554 comparisons between unpaired datasets for comparisons. Correlation analysis was performed by

Spearman's test, and by two-way direct correlation for graphical representation. Analysis was performed using JMP 10.0 (SAS).

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886 ## Legend to figures

887

888 **Figure 1: HIV-DNA in PBMC from patients with different disease courses.** Comparison of total
889 (panel A), integrated (Panel B), unintegrated (Panel C) and 2-LTR (Panel D) HIV-DNA
890 (cp/10^5CD4$^+$) in HIC (EC and LTNP, empty box) compared with PP (greyed box). Box-plot
891 analysis: bars represent range, lines represent median values, and box lines represent 75% and
892 25% percentiles. (***p<0.001, ****p<0.0001, Mann–Whitney u test). HIC: HIV Controller Patients
893 n=20; PP:Progressor Patients. Previously progressing pts who are successfully treated with
894 persistently undetectable HIV-RNA (<50cp/ml), n=10.

895 **Fig.2: Gating strategy for the selection of NK cells.** Panel A: Gating strategy for NK cell
896 identification, analysis and sorting in PBMC. Panel B: Gating strategy for the identification and
897 characterization of PBMC-derived, purified NK cells in vitro after activation in vitro

898 **Fig. 3.: HIV-DNA is inversely correlated with induced IFN-γ production in NK cells from HICs.**
899 Panel A: Linear Correlation analysis of quantitative integrated HIV-DNA (cp/10^5CD4$^+$) with purified
900 NK cell IFN-γ production upon triggering with activating receptors or cytokines. Panel A: anti-
901 NKp46+NKp30 Representative of 17 patients. Panel B: anti-NKp30+DNAM-1. Representative of
902 16 patients. Panel C: rhIL-12 + hIL-15. Representative of 12 patients. Panel D, E Flow cytometric
903 analysis of peripheral NK cells producing IFN-γ in two patients with widely diverging HIV DNA copy
904 numbers (marked with open diamond and open triangle in panel 2A, 2B, 2C) and high (panel D
905 and low (Panel E) HIV-DNA copies: CD3$^-$ cells were gated by flow cytometric analysis and
906 analyzed for CD56 and CD16 expression and for intracellular IFN-g production.

907 **Fig. 4: Induced NCR expression by NK cells inversely associate with HIV-DNA reservoir and**
908 **is correlated to IFN-g production.**

Panel A: Integrated HIV-DNA copy n° are inversely correlated with fold-increase NKp46 or NKp30 expression on purified NK cells after *in vitro* activation with rh-IL2 in HIV controller patients.. Panel B: Peripheral NK cell IFN-γ production is directly correlated with fold-increase of NCRs (NKp46, NKp30) expression on purified activated NK cells in HIV controller patients. Correlation analysis of NK cells producing IFN-γ in response to cytokine stimulation (rhIL12+rh-IL15) and fold increase of NKp46 and NKp30 expression on purified NK cells after *in vitro* activation with rh-IL2 (n=12). Panel C: HIV-DNA reservoir in HIC (n=12) is inversely correlated with fold increase of NCR molecule density, expressed as MFI, on purified NK cells after *in vitro* activation with rh-IL2. Molecule densities are expressed as the ratio of the sample MFI to the control MFI to account for intersample variability. ($p<0.01$). Panel D: Peripheral NK cell IFN-γ production in response to cytokine stimulation (rhIL12/rh-IL15) is directly correlated with fold increase of NKp46 and NKp30 molecule density expressed as MFI on purified NK cells after *in vitro* activation with rh-IL2 ($p<0.01$). Representative of 12 different patients. Panel E: representative shift in NKp46 and in NKp30 molecule expression and density after stimulation with rIL-2 *in vitro* of highly purified NK cells in former progressor patients after 24 month of successful cART (PP) and in HIV controller patients (HIC). Flow cytometric analysis and overlay histogram representation. Grey histogram = negative control, Dark Histogram= resting NCR (NKp46, NKp30) expression; Red Histogram = NCR expression after 48h of culture in the presence of rIL-2 200UI/ml.

Fig. 5: NK cell function inversely correlates to HIV-DNA reservoir size across widely divergent disease courses.

Panel A: Linear correlation analysis of integrated HIV-DNA with IFN-γ production by NK cells triggered by activating receptors (NKp46+NKp30) (n=27) or by cytokines (rhIL-12 plus rhIL-15) (n=27) in HIC (dots) and PP (x). Panel B: Correlation analysis of integrated HIV-DNA with fold-increase of NCR (NKp46, n=21 and NKp30, n=19) expression on purified NK cells after *in vitro* activation with rh-IL2 in HIC (dots) and PP (x). Representative of 21 experiments. Panel C:

935 Correlation analysis in PP alone of integrated HIV-DNA with IFN-γ production by NK cells triggered
936 by activating receptors (NKp46+NKp30) (*n*=10) or by cytokines (rhIL-12 plus rhIL-15) (*n*=10) in PP
937 (x).Panel D: Correlation analysis in PP alone of integrated HIV-DNA with fold-increase of NCR
938 (NKp46, *n*=9 and NKp30, *n*=7) expression on purified NK cells after *in vitro* activation with rh-IL2 in
939 PP (x) (*p*=n.s.).Panel E: Purified NK cell cyototoxicity before and after 2 and 4 days of *in vitro*
940 activation in the presence of rhIL-2 (200 U/mL). PKH-26/TO-PRO3 flow cytometric cytotoxicity
941 assay. Box plots indicate percentage of target cell lysis in the presence of NKp30-specific mAbs in
942 HIC (*n*=14) and PP (*n*=7). In the box plots, bars represent range, lines represent median values,
943 and box lines represent 75% and 25% percentiles. ***p*<0.01, Mann–Whitney *u* test. Panel F:
944 Correlation analysis of NK cell cytotoxicity and NKp30 induction upon *in vitro* rh-IL2 activation in
945 HIC (*n*=6) and PP (*n*=5). HIC=HIV-controller patients; PP=Progressor patients, aviremic, who
946 needed cART to control viral replication.

947 **Figure 6: Baseline expression of activating receptors on NK cells and IFN-γ producing**
948 **potential under NKG2D-DNAM-1 triggering and under maximal stimulation.** Panel A: flow
949 cytometric analysis of peripheral NK cells in HD (empty box), HIC (light gray), PP (dark gray).
950 Panel B: IFN-γ production by NK cells upon NKG2D- and DNAM-1 triggering (Panel B) or via PMA
951 and ionomycin (Panel C). Box-plot analysis: bars represent range, lines represent median values,
952 and box lines represent 75% and 25% percentiles. (**p<0.*05, ****p<0.*001, Mann–Whitney *u* test).
953 HD: Healthy Donors, *n*=10; HIC: HIV Controller Patients, *n*=20; PP: Progressor Patients, *n*=10.

954

955 **Figure 7: Whole genome microarray analysis demonstrates the existence of differential**
956 **gene expression patterns of NK cells between HIC and PP patients.**

957 Panel A: Heat map based on genes differentially expressed HIC vs. PP; Student t test p< 0.05 and
958 Fold change > 1.5 TIME T0 = 370 genes.Panel B: Heat map based genes differentially expressed
959 HIC vs. PP; Student t test p< 0.05 and Fold change > 1.5 TIME T4 = 370 genes. Panel C: Venn

diagram showing genes in common between T0 vs T4. Panel D: Gene Ontology enrichment analysis of purified NK cells by FunRich. Bar graph of biological pathway overrepresented in genes differentially expressed HIC vs. PP is shown. T0= resting NK cells, T4= NK cells after 4 days of IL-2 culture; HIC=HIV-controller patients, *n*=4; PP= Progressor Patients, *n*=4.

Fig 8: NK cells from HIC and PP show similar proportion of memory-like NK cells.

$CD3^{-}CD14^{-}CD19^{-}$ cells were gated by flow cytometric analysis and analyzed for CD56 and NKG2C/CD57 expression in HD, HIC (greyed) and PP (black). In the box plots, bars represent range, lines represent median values, and box lines represent 75% and 25% percentiles. (**p<0.*05, ***p*<0.01, Mann–Whitney *u* test). Representative of 30 experiments. HD= Healthy Donors, *n*=10; HIC= HIV-controller patients, *n*=10; PP= Progressor Patients, *n*=10.

Fig.9: Purified NK cells from HIC patients control HIV integration *in vitro*. IL2/PHA lymphocyte T $CD4^{+}$ were infected with $HIV\text{-}1_{Bal}$ at an MOI of 0.01, in the presence or absence of activated NK cells form PP or HIC patients. After 7 days of culture virus replication was monitored by HIV-RNA (cp/ml) in the supernatant (panel A), p24 levels in intracellular staining of $CD3^{+}56^{-}$ T cells by flow cytometry (panel B) and viral reservoir accumulation was assayed by quantitative assay of total HIV-DNA ($cp/10^{5}CD4^{+}$), (panel C) and of integrated HIV-DNA (panel D). Panel E: Fold-decrease in virus accumulation in the presence of given purified NK cells, over control cultures without NK cells Panel F: Relative potency of purified NK cells from HIC vs. NK cells from PP in inhibiting virus replication or DNA integration/accumulation. HIC= HIV-controller patients, *n*=6; PP= Progressor Patients, *n*=6.

Table 1: Nonparametric correlation analysis of quantitative HIV DNA (total, integrated or unintegrated) with IFN-γ production and with activating receptor induced expression on NK cells after activation *in vitro*. Group composition refers to the group comparison in the respective figure (indicated in column 1), unless otherwise specified.

985 **Table 2:** Demographic and clinical patient data. With the exception of Gender, numbers represent
986 Mean (±S.D.); Age: yrs;

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Table 1: nonparametric correlation analysis (Spearman's test) of quantitative HIV-1 DNAcopy numbers with peripheral NK cell IFN-g production and with basal and induced e of activating

Graphic Representation and Dataset	Variable 1	Variable 2	Spearman ρ	Prob>|ρ|
Fig 2	IFN-γ+(a)	I -HIV DNA	-0,4858	0,0088*
	IFN-γ+(b)	I -HIV DNA	-0,5101	0,0013*
	IFN-γ+(c)	I -HIV DNA	-0,5831	0,0002*
Fig 2, ns	IFN-γ+(a)	T- HIV DNA	-0,5976	0,0013*
Fig 2, ns	IFN-γ+(b)	T- HIV DNA	-0,4329	0,0190*
Fig 2, ns	IFN-γ+(c)	T- HIV DNA	-0,4148	0,0486*
Fig 2, ns	IFN-γ+(a)	U - HIV DNA)	-0,2274	0,2851
Fig 2, ns	IFN-γ+(b)	U - HIV DNA)	0,0446	0,8286
Fig 2, ns	IFN-γ+(c)	U - HIV DNA)	-0,0887	0,7098
3A	F.I. % NKp46 e	I -HIV DNA	-0,5536	0,0075*
	F.I. % NKp30 e	I -HIV DNA	-0,6873	0,0033*
3B	IFN-γ+(a)	F.I. % NKp46 e	0,7169	0,0130*
	F.I. % NKp30 e	IFN-γ+(a)	0,7945	0,0035*
3C	I -HIV DNA	F.I. MFI NKp46 e	-0,6561	0,0205*
	I -HIV DNA	F.I. MFI NKp30 e	-0,6333	0,0365*
	IFN-γ+(a)	F.I. MFI NKp46 e	0,8389	0,0024*
	IFN-γ+(a)	F.I. MFI NKp30 e	0,7939	0,0061*
Fig.3, ns	F.I. % NKp46 e	T- HIV DNA	-0,6423	0,0032*
Fig.3, ns	F.I. % NKp30 e	T- HIV DNA	-0,6527	0,0039*
Fig.3, ns	F.I. % NKp46 e	U - HIV DNA)	0,056	0,7117
Fig.3, ns	F.I. % NKp30 e	U - HIV DNA)	-0,1989	0,1956
Fig.3, ns	T- HIV DNA	F.I. MFI NKp46 e	-0,6555	0,0207*
Fig.3, ns	U - HIV DNA)	F.I. MFI NKp46 e	-0,487	0,0657
Fig.3, ns	T- HIV DNA	F.I. MFI NKp30 e	-0,6872	0,0195*
Fig.3, ns	U - HIV DNA)	F.I. MFI NKp30 e	-0,5612	0,0724
4A	IFN-γ+(a)	I -HIV DNA	-0,6048	0,0003*
	IFN-γ+(b)	I -HIV DNA	-0,4375	0,0053*
4B	F.I. % NKp46 e	I -HIV DNA	-0,5954	0,0056*
	F.I. % NKp30 e	I -HIV DNA	-0,831	<,0001*
4C	IFN-γ+(a)	I -HIV DNA	-0,7185	0,0127*
	IFN-γ+(b)	I -HIV DNA	-0,7219	0,0121*
4D	F.I. % NKp46 e	I -HIV DNA	-0,0251	0,9489
	F.I. % NKp30 e	I -HIV DNA	-0,2523	0,5852
4F	F.I. % NKp30 e	% cytolysis	0,7107	0,0142*
Fig. 4, ns	IFN-γ+(a)	T- HIV DNA	-0,5976	0,0013*
Fig. 4, ns	IFN-γ+(b)	T- HIV DNA	-0,4329	0,0190*
Fig. 4, ns	F.I. % NKp46 e	T- HIV DNA	-0,5051	0,0231*
Fig. 4, ns	F.I. % NKp30 e	T- HIV DNA	-0,5258	0,0250*
Fig. 4, ns	IFN-γ+(a)	T- HIV DNA (only PP)	-0,7165	0,0059*
Fig. 4, ns	IFN-γ+(b)	T- HIV DNA) (only PP)	-0,8988	<0,0001*
Fig. 4, ns	F.I. % NKp46 e	T- HIV DNA (only PP)	0,0418	0,9149

Fig. 4, ns	F.I. % NKp30 e	T- HIV DNA (only PP)	-0,1441	0,7578
Fig. 4, ns	IFN-γ+(a)	U - HIV DNA)	-0,2274	0,2851
Fig. 4, ns	IFN-γ+(b)	U - HIV DNA)	0,0446	0,8286
Fig. 4, ns	F.I. % NKp46 e	U - HIV DNA)	-0,2325	0,324
Fig. 4, ns	F.I. % NKp30 e	U - HIV DNA)	-0,3976	0,114
Fig. 4, ns	IFN-γ+(a)	U - HIV DNA) (only PP)	0,1215	0,7219
Fig. 4, ns	IFN-γ+(b)	U - HIV DNA)) (only PP)	0,4014	0,221
Fig. 4, ns	F.I. % NKp46 e	U - HIV DNA)) (only PP)	0,1187	7611
Fig. 4, ns	F.I. % NKp30 e	U - HIV DNA)) (only PP)	0,3964	0,3786
Fig. 4, ns	NKG2D e on NK resting	I -HIV DNA	-0,1258	0,4518
Fig. 4, ns	NKG2D e on NK resting	T- HIV DNA	-0,1832	0,2516
Fig. 4, ns	NKG2D e on NK resting	U - HIV DNA)	0,0519	0,7603

ns: not shown; HIV DNA= (copies/10e4 CD4); I=Integrated; T=total; U=unintegrated; IFN–γ+ NK : %CD56+ IFN-g+ NK cells; F.I: = fold-increase; (a) after rhIL-12+rhIL15 activation; (b): NK cells after a-NKp46+a-NKp30 mAb activation c): after antiNKG2D+DNAM-1 activation; e=expression

Table 2: Demographic and clinical data of the patients

	Age	Male	Female	$CD4^+$% baseline	$CD4^+$ no. baseline	CD4% last	$CD4^+$ count last	HIV^+ years
EC	52 (±4.41)	3	7	35 (±6.59)	1,033 (±479.8)	35.8 (±5.07)	739.5 (±347.6)	20 (±4.4)
LTNP	49 (±12.2)	4	6	36 (±7.83)	876 (±378)	34.6 (±8.33)	776 (±362)	18 (±8.3)
PP	51 (±5.9)	4	6	9.9 (±5.56)	85 (±75.5)	24.6 (±8.33)	325 (±182)	17 (±4.9)

Fig. 1

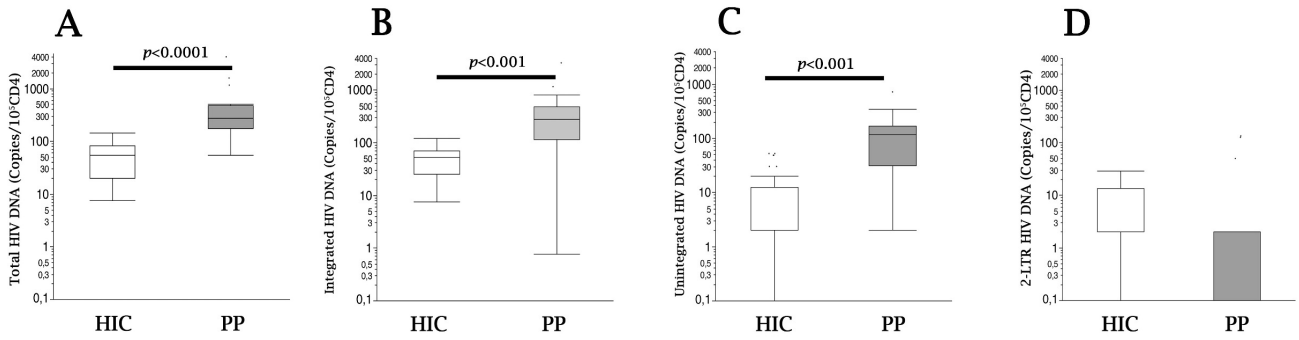
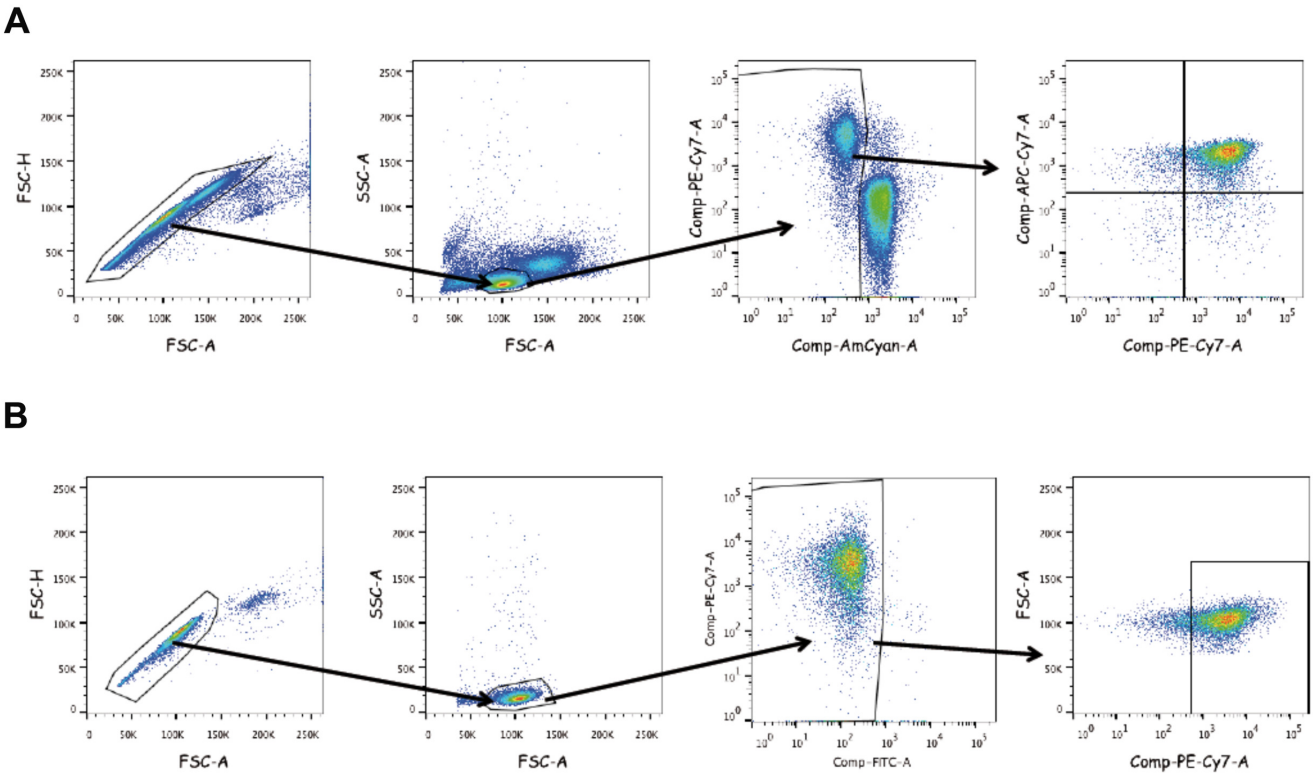
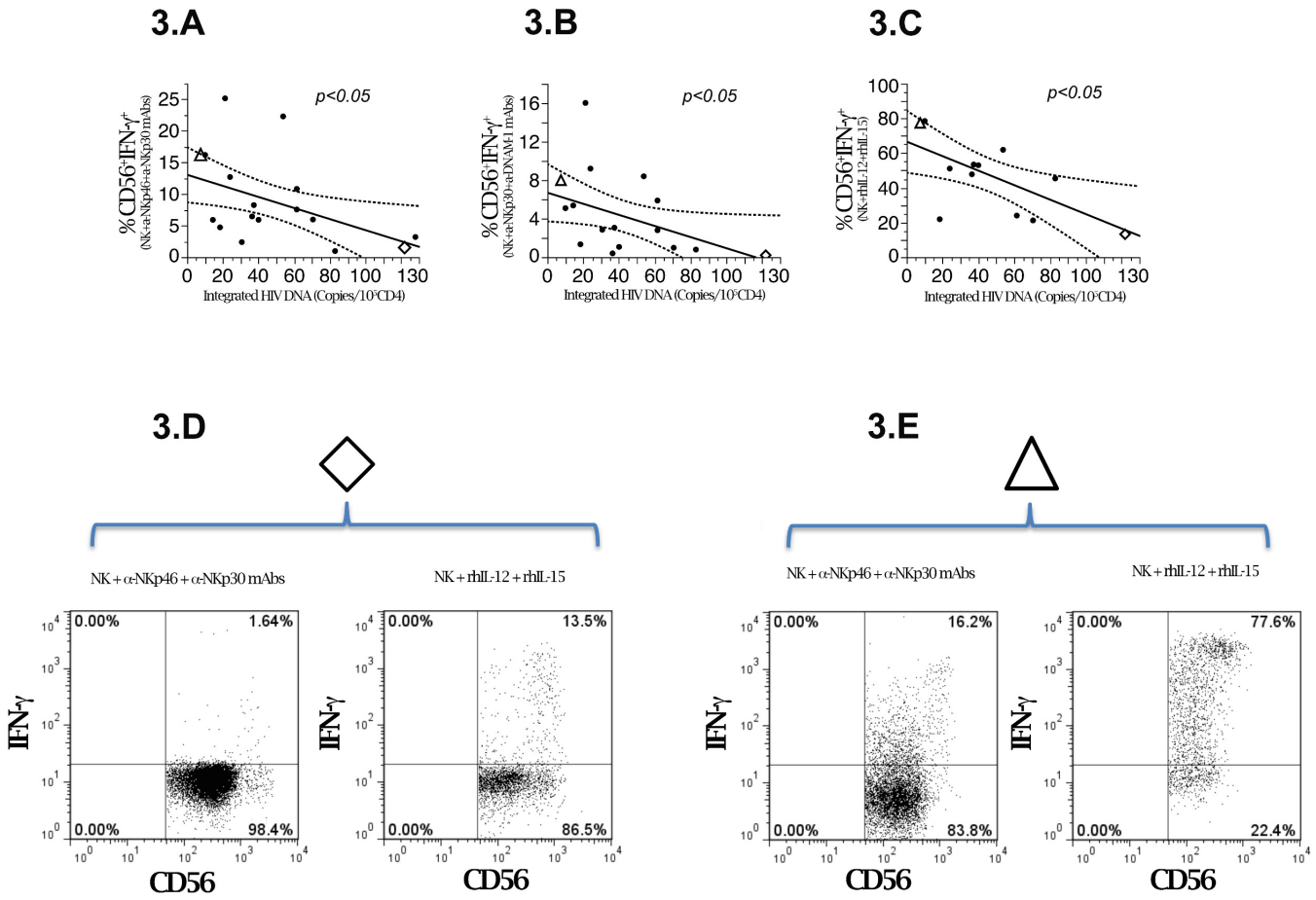
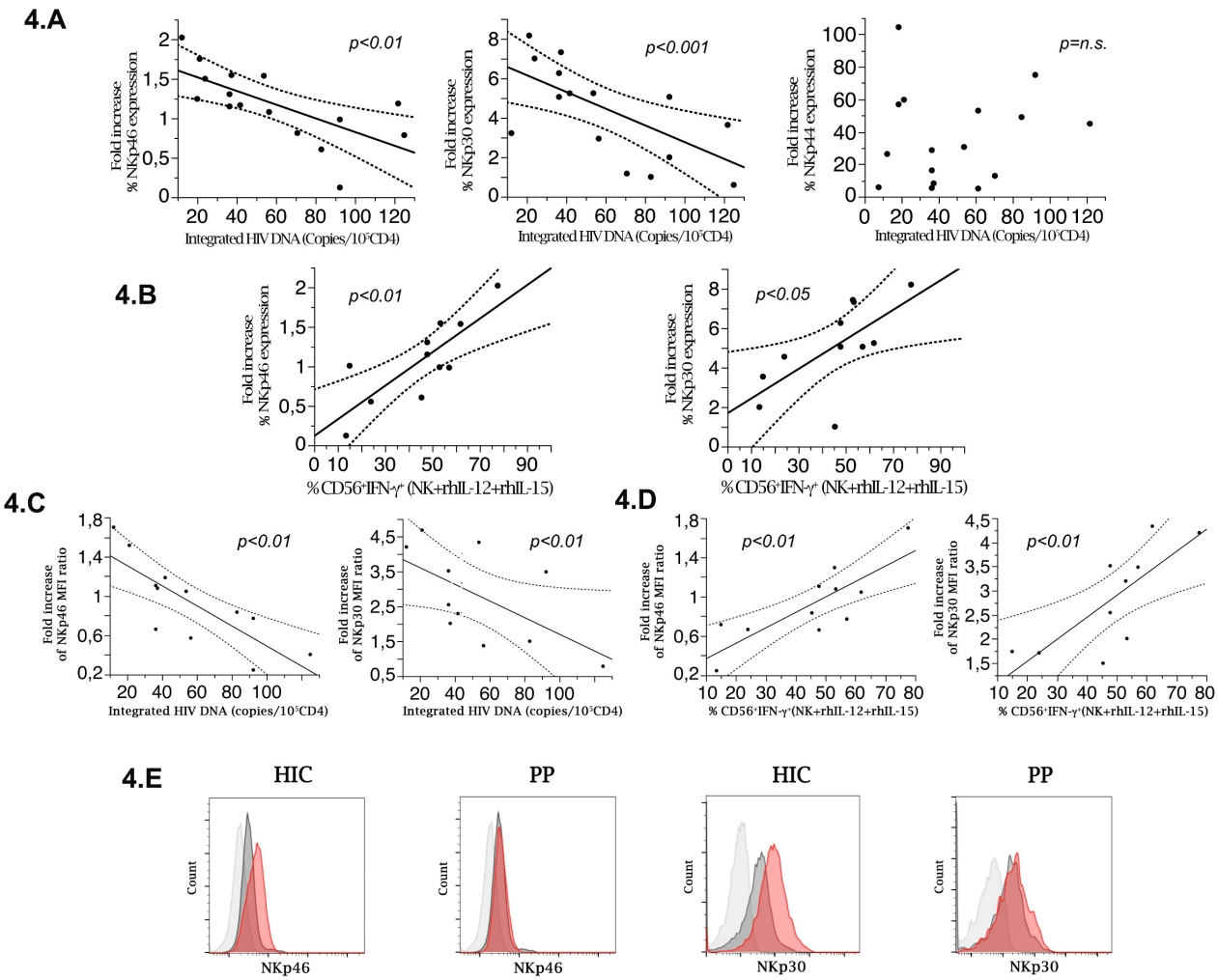


Figure 2







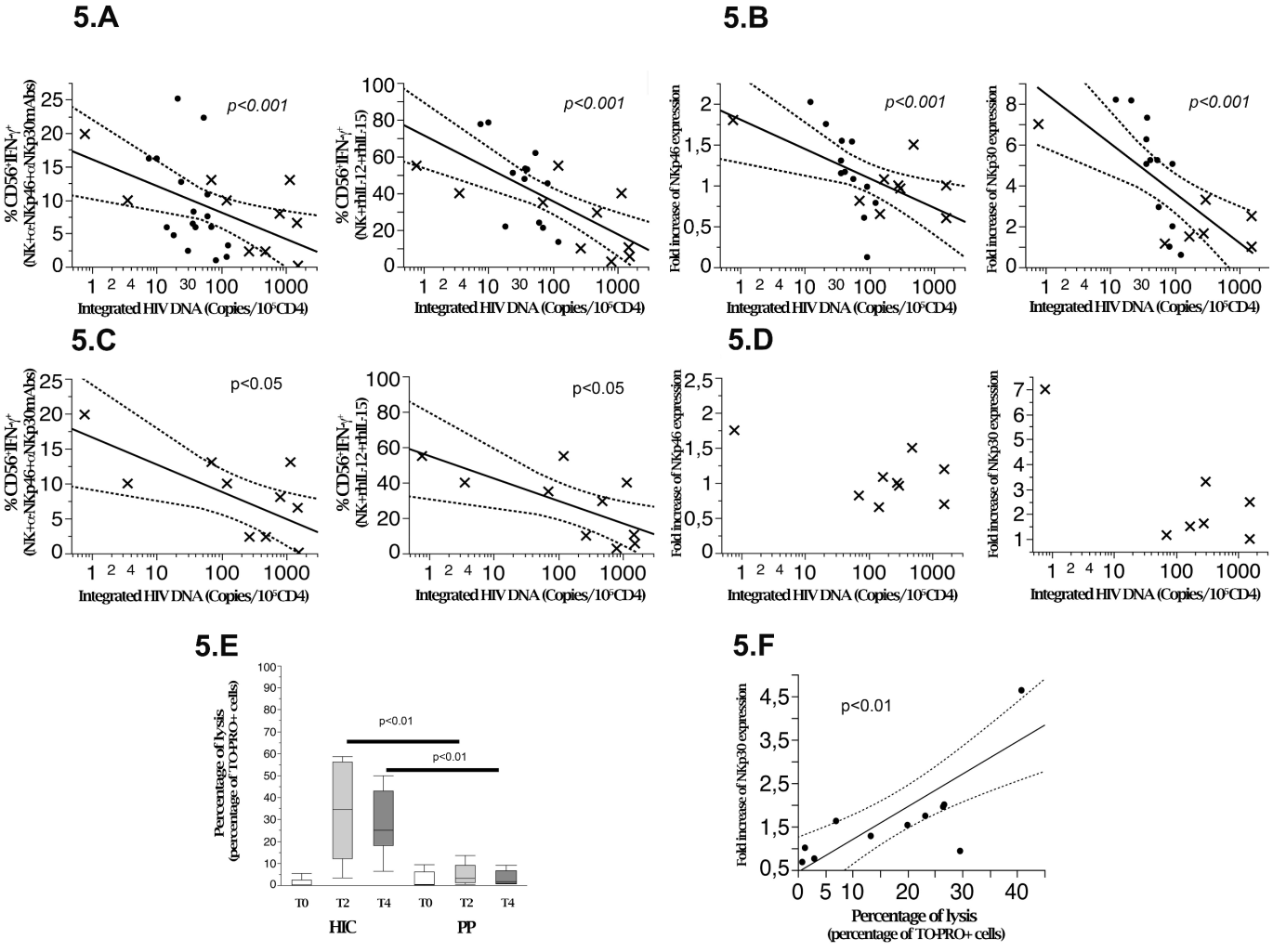


Figure 7

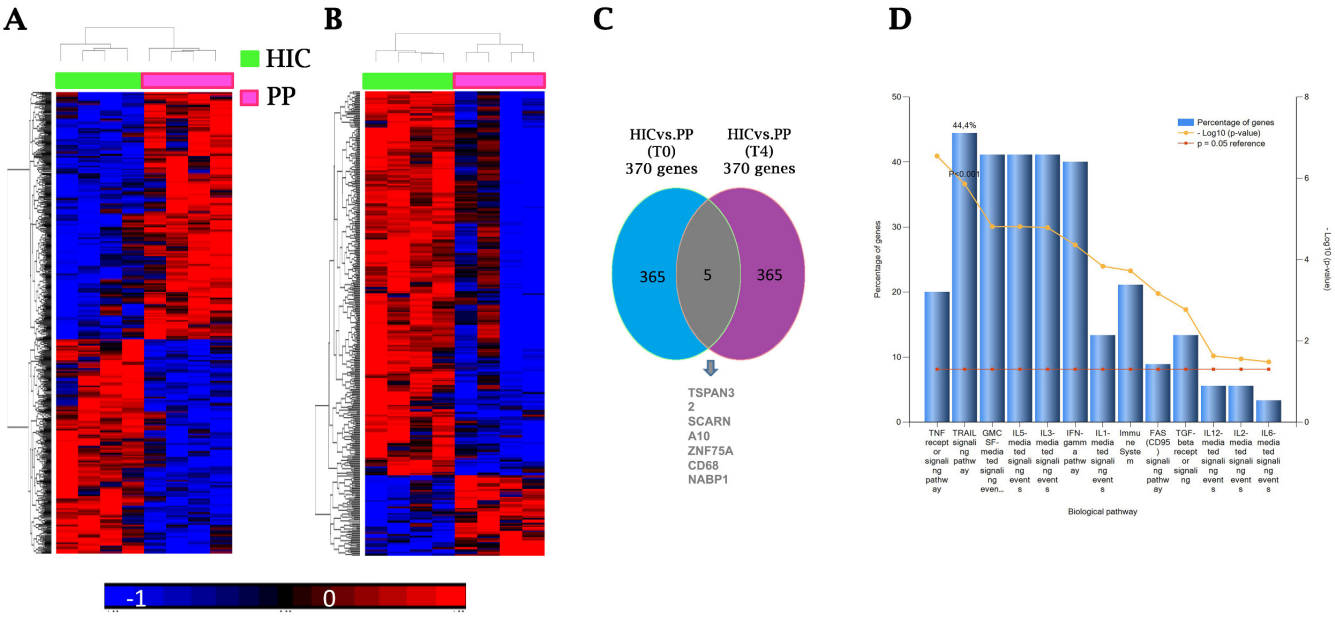


Figure 8

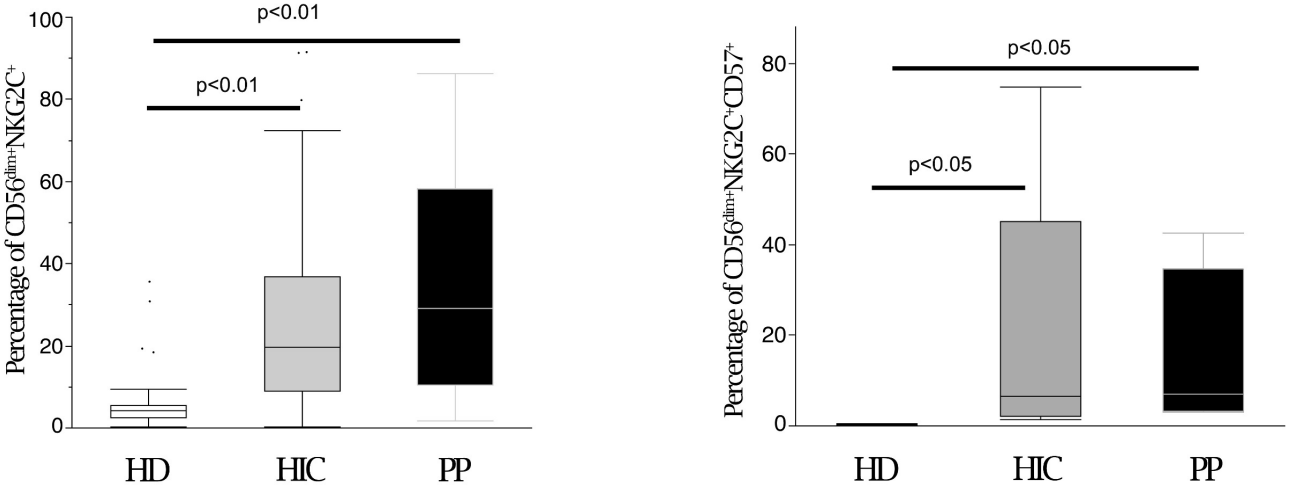


Figure 9