Neutrophils in People Living With HIV-1 on antiretroviral treatment conserve their cytokine and chemokine secretion capacity and display enhanced expression of Fc receptors

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Abstract

Neutrophils are innate immune cells that display immunomodulatory properties by secreting cytokines and chemokines. By using a mouse model of retroviral infection, we previously uncovered the key immunomodulatory function of neutrophils in promoting protective immunity during antiviral antibody therapy which occurs in a Fc-dependent manner. Here, we investigated the immunomodulatory properties of neutrophils in the context of HIV-1 infection and therapy. We characterized the functional activation and the modulation of Fcγ receptors (FcγRs) expression on neutrophils isolated from healthy donors (HD) or people living with HIV-1 (PLWH) upon stimulation with virions, free or in the form of immune complexes (ICs) made with broadly neutralizing antibodies (bNAbs). HD's neutrophils secreted cytokines/chemokines in small amount upon activation by either stimulus. Interestingly, conditioning of HD's neutrophils with pro-inflammatory cytokines enhanced their cytokine/chemokine production upon activation by HIV-1 and ICs, highlighting that the inflammatory environment is key for neutrophils to show a functional response to these stimuli. Noteworthy, under inflammatory conditions, IC-activated neutrophils showed higher secretion of CXCL1 and CCL4 than HIV-1-stimulated neutrophils. Importantly, neutrophils from PLWH on antiretroviral treatment (ART) maintained their cytokine/chemokine secretion abilities when stimulated by TLR agonists, TNFα or HIV-1/ICs. However, they displayed different phenotypic features as compared with HD's neutrophils, notably higher expression of FcγRs. Our study provides new insights into the immunomodulatory properties of neutrophils during HIV-1 infection and therapy. This might contribute to the improvement of antiviral antibody therapies through the use of therapeutic approaches exploiting the immunomodulatory properties of these cells.

57 **Introduction**

58 Neutrophils are the most abundant population of circulating white blood cells and are massively
59 and rapidly recruited following infection. Through diverse mechanisms such as phagocytosis,
60 releasing of reactive-oxygen species (ROS), trapping of the pathogenic agent by neutrophil
61 extracellular traps (NET) formation (known as NETosis) (1), neutrophils can limit and
62 counteract invading pathogens. Moreover, immunomodulatory properties of neutrophils have
63 been described in recent years, showing their ability to shape the adaptive immune response
64 (2,3). Neutrophils migrate from the site of infection to draining lymph nodes where they interact
65 with multiple immune cells through the production of several cytokines and chemokines (3),
66 making neutrophils key cells in the orchestration of innate and adaptive immunity (3,4).
67 However, the immunomodulatory role of neutrophils in antiviral immunity is still ill-
68 understood, including in the context of persistent viral infections such as HIV-1, as well as in
69 the frame of antiviral monoclonal antibody (mAb)-based immunotherapies. Noteworthy, it has
70 been reported that the functional activation of neutrophils is compromised upon HIV-1
71 infection which leads to impaired effector functions (i.e. phagocytosis, ROS release, …) and
72 increased apoptosis (5,6). However, much less is known on the effect of HIV-1 infection on
73 their cytokine and chemokine secretion profile as well as on the modulation of Fcγ receptors
74 (FcγRs) expressed at their surface, which is a key point in the context of antibody therapy.
75
76 The discovery of potent broadly neutralizing antibodies (bNAbs) against HIV-1 has made
77 passive immunotherapy a potential strategy for the prevention and treatment of HIV-1 infection
78 (7). Importantly, recent clinical data have demonstrated the efficacy of several anti-HIV-1
79 bNAbs to control viremia when administered to people living with HIV-1 (PLWH) as well as
80 to induce adaptive immune responses (so-called "vaccinal effect") (reviewed in 8,9). This has
81 given strong support to the idea that bNAbs could broaden the therapeutic arsenal against HIV-
82 1 infection (10–14). Beyond their neutralization capacity through the binding of their Fab
83 fragment to viral antigens, the biological activity of mAbs is also mediated by the Fc moiety
84 upon interaction with the complement system and FcγRs expressed by many cells of the
85 immune system (15,16). This allows to clear free virions from the blood as well as to guide host
86 immune effector cells to kill infected cells by several Fc-mediated mechanisms (i.e. antibody-
87 dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity
88 (ADCC), …). Furthermore, mAbs can form immune complexes (ICs) *via* binding with different
89 viral determinants (virions or infected cells) and the subsequent engagement of FcγRs by ICs
90 has immunomodulatory effects leading to the induction of protective immunity.

91

92 We have previously identified different FcγR-expressing immune cells involved in the vaccinal
93 response during mAb-based therapies (8,9). By using a mouse model of retroviral infection, we
94 have shown a key role for ICs in enhancing antiviral immune responses by multiple innate
95 immune cells such as dendritic cells (DC) (17,18), neutrophils (19,20), monocytes (20) and NK
96 cells (21) in a FcγR-dependent manner. Notably, our findings revealed a key
97 immunomodulatory role for murine neutrophils in the induction of protective immunity upon
98 mAb-therapy (19,20). This occurs through the secretion of multiple cytokines and chemokines
99 upon activation by free virions and ICs. With the aim of extending our observations to the
100 context of HIV-1 infection, here we addressed the immunomodulatory properties of human
101 neutrophils upon activation by different stimuli/danger signals associated with HIV-1 infection
102 and mAb-based therapy.
103

104 Using neutrophils isolated from healthy donors (HD), we report here that their activation
105 by HIV-1 virions, free or in the form of ICs, induced the secretion of very limited amounts of
106 cytokines and chemokines, both in terms of diversity and quantity. However, priming of HD's
107 neutrophils with TNF-α or IFN-γ enhanced their chemokine production upon activation by
108 either free HIV-1 or ICs. Noteworthy, the inflammatory environment increased more the
109 chemokines secretion in IC-activated neutrophils than in HIV-stimulated neutrophils. We also
110 report that neutrophils isolated from PLWH showed a similar secretome as compared to those
111 isolated from HD upon stimulation with TLRs agonists, cytokines or HIV/ICs but displayed
112 different phenotypic features. Our study provides new insights into the immunomodulatory
113 properties of neutrophils during HIV-1 infection and antibody treatment. It also highlights that
114 the inflammatory environment is a key factor for neutrophils to show a functional response to
115 HIV-1/ICs. These findings could have implications in improving the treatment of HIV-1
116 infection with bNAbs alone or in combinatorial therapeutic approaches.
117
118

119 **Results**

120 ***Different cytokine/chemokine secretion profile and FcγR cell surface levels in neutrophils***
121 ***stimulated by TLR agonists or pro-inflammatory cytokines***

122 First, we assessed the effect of TLR agonists and inflammatory cytokines (TNF-α and IFN-γ)
123 on the phenotypic and functional activation of human neutrophils isolated from HD. The
124 rationale behind this was to determine whether and how the microenvironment resulting from
125 infection might be determinant for modulating the immune response, both in terms of
126 chemokine/cytokine secretion profile and of FcγR expression pattern, as observed in our mouse
127 studies (20). In this regard, virus-driven inflammation (including TNF-α and IFN-γ signature)
128 has been associated with the development of bNAbs in spontaneous controllers of HIV (22).
129 As for TLR agonists, we mainly focused on TLR4 and TLR7/8 agonists (lipopolysaccharide
130 (LPS) and Resiquimod (R848), respectively), due to their role in immune activation associated
131 with viral sensing (TLR7/8) and to the bacterial translocation observed in PLWH (i.e. LPS
132 binding to TLR4). To assess the phenotypic and functional activation of neutrophils we used
133 the following read-out: (i) the upregulation of the cell surface markers CD11b and CD66b, (ii)
134 the modulation of the expression of the main FcγRs constitutively expressed on neutrophils:
135 FcγRIIa (CD32a) and FcγRIIIb (CD16b), and (iii) the cytokine and chemokine secretion profile
136 (**Figure 1**). Flow cytometry analysis was performed on the viable fraction of $CD15^{+}/CD11b^{+}$
137 cells (**Figure 1A**). While life span of neutrophils has been reported to be short (only few hours),
138 specific culture conditions allow extending it. Upon TLR4 and TLR7/8 agonist stimulation
139 during 16-20h, neutrophil survival ranged between 70-80% in average (**Figure 1B**). TLR4 and
140 TLR7/8 agonists led to significant upregulation of CD11b and CD66b as compared to
141 unstimulated neutrophils, with no significant differences between TLR agonists (**Figure 1C)**.
142 As for FcγR expression, neutrophil stimulation by either TLR agonist induced a strong shedding
143 of the CD16b receptor, in particular TLR4, as well as a significant decrease in CD32a
144 expression as compared to unstimulated neutrophils (**Figure 1C)**. The assessment of neutrophil
145 secretome revealed that both stimuli triggered the secretion of IL-8 and several chemokines (i.e.
146 CCL3, CCL4, CXCL1, CXCL10 and CCL2) albeit to a different extent (**Figure 1D)**. Due to
147 high donor-dependent variability of chemokine secretion, no statistical differences were
148 observed between TLR4 and TLR7/8 activation (**Figure 1E**). However, TLR4 stimulation led
149 to the secretion of IFN-λ2/3 in 3 out of 7 samples, while TLR7/8 stimulation did not.

150 We also assessed the viability as well as the phenotypic and functional activation of
151 human neutrophils stimulated with TNF-α and IFN-γ (**Figure 2**). At the concentrations used,

152 TNF-α led to a lower neutrophil survival than IFN-γ (68% versus 85%, respectively) (**Figure**
153 **2A**). TNF-α led to a significant upregulation of CD11b and CD66b, a strong shedding of CD16b
154 receptor as well as a significant decrease in CD32a expression while IFN-γ did not significantly
155 affect the phenotypic activation of neutrophils (**Figure 2B**). Thus, significant differences
156 between IFN-γ and TNF-α activation were observed for all the cell surface markers evaluated.
157 The assessment of neutrophil's secretome revealed that TNF-α activation induced the secretion
158 of several cytokines (IL-8, IL-1ß, IFN-λ2/3, IFN-λ1 and IFN-γ) and chemokines (CCL3, CCL4
159 and CXCL1) (**Figure 2C**). The secretion of IL-8, CCL3, CCL4 and CXCL1 was significantly
160 higher than for unstimulated neutrophils, while increased secretion of IL-1ß, IFN-λ2/3 and IFN-
161 γ was observed for half of the samples tested (**Figure 2D**). In contrast, IFN-γ activation only
162 resulted in a very low secretion of IL-8 (**Figure 2C**). Overall, these results highlight a stimulus-
163 specific effect on neutrophil activation, both phenotypically (FcγR and activation markers
164 levels) and functionally (secretome). Furthermore, by studying multiple parameters, these
165 results enabled to establish a comprehensive and comparative landscape of neutrophil activation
166 by these stimuli (some of them used as a surrogate of viral activation) as a basis for comparison
167 with neutrophil activation by HIV-1 and ICs. This is important to highlight as neutrophil
168 activation by TLR agonists and cytokines has been partially addressed in previous studies (23–
169 27), but the data are scattered and based on few analysed parameters.
170
171
172 ***Mild effect of neutrophil activation by HIV-1 virions, free or as ICs, on the expression of***
173 ***FcγRs***
174 Considering that neutrophil activation by TLR agonists and TNF-α led to decreased levels of
175 FcγRs, we next assessed the modulation of the expression of FcγRs in presence of HIV-1
176 virions, free or in the form of ICs. This is important to assess, as in the context of HIV-1
177 infection and antibody therapy, the expression of FcγRs might affect the activation of
178 neutrophils by therapeutic antibodies. ICs were made using the anti-HIV-1 bNAb 10-1074. This
179 bNAb is currently being tested in clinical trials and has been shown to induce vaccinal effects
180 (28,29). The 10-1074 bNAb was chosen due to its higher affinity to the FcγRs CD16b and
181 CD32a as compared to other bNAbs (**Supplementary Figure 1**). Activation by HIV-1 virions,
182 ICs and free bNAb had a slight effect on neutrophil survival, which was maintained at around
183 85%. (**Figure 3A**). The stimulation with HIV-1 virions and ICs led to mild but significant
184 upregulation of CD11b and CD66b as compared to unstimulated neutrophils (**Supplementary**

185 **Figure 2**) or neutrophils cultured in the presence of free bNAb, notably for CD11b (**Figure**
186 **3B**). No significant differences in the expression of these two cell surface receptors were
187 observed between HIV-1- and IC-activated neutrophils (**Figure 3B**). Despite being similarly
188 activated by both stimuli (as observed by the upregulation of activation markers), we observed
189 a mild but significant reduced expression of CD16b in HIV-1-stimulated neutrophils but not in
190 IC-stimulated cells (**Supplementary Figure 2)**. However, a significant decrease in CD32a
191 expression was observed in both HIV-1- and IC-activated neutrophils (**Supplementary Figure**
192 **2)**, that was more marked in IC-activated cells (**Figure 3B**). Together, these results show a very
193 mild effect of the activation of neutrophils by HIV-1/IC on FcγR expression. Worthy of note,
194 the decrease in FcγRs expression was much less pronounced than that observed upon activation
195 with the TLR7/8 agonist mimicking a viral stimulus (**Figure 1**). This is important to highlight
196 as TLR7/8 is commonly used as surrogate of virus activation, instead of *bona fide* virions.
197
198 ***Neutrophils activated by HIV-1 and ICs secrete low levels of cytokines and chemokines***
199 In parallel, we assessed the activation of neutrophils isolated from HD by HIV-1 virions, free
200 or in the form of ICs. Free 10-1074 bNAb was used as control and showed no effect on either
201 the expression of cell surface markers or the secretome of neutrophils. The study of the
202 secretome of neutrophils activated by these stimuli revealed a weak secretion of several
203 cytokines and chemokines (**Figure 3C**). Among them, IL-8, CXCL1 and CCL4 were
204 significantly increased upon HIV-1 and IC-activation as compared to unstimulated neutrophils
205 (**Figure 3D**). In addition, other cytokines and chemokines were detected in about half of
206 samples tested (i.e. IL-1ß, IFN-λ1, IFN-λ2/3, CCL2 and CCL3). However, no significant
207 differences in the secretome were observed between HIV-1- and IC-activated neutrophils
208 (**Figure 3D**). Collectively, these results show a low activation of neutrophils by these stimuli.
209 This contrasted to the strong secretion of multiple cytokines and chemokines by monocytes
210 activated by HIV-1- and ICs (**Supplementary Figure 3**). This showed that these stimuli are
211 able to induce the secretion of these soluble immune mediators by different myeloid cells but
212 at different extents depending on the cell type.
213
214 ***Inflammatory conditions potentiate the activation of HD's neutrophils by HIV-1 and ICs***
215 As the inflammatory microenvironment resulting from the viral infection and bNAbs therapy
216 might affect the antiviral immune response, we next examined the phenotypic and functional
217 activation of HD's neutrophils by HIV-1 free or in the form of ICs in an inflammatory
218 environment (i.e. priming with TNF-α and IFN-γ). Neutrophil priming with TNF-α or IFN-γ

219 (for 10 or 30 min, respectively, **Figure 4A**) enhanced chemokine production (i.e. CXCL1,
220 CCL2, CCL3 and CCL4) upon activation of neutrophils by either free HIV-1 or ICs.
221 Interestingly, under these conditions, IC-activated neutrophils showed a significantly greater
222 secretion of several chemokines than free HIV-stimulated neutrophils. Notably, TNF-α
223 enhanced the secretion of CXCL1 in IC-stimulated neutrophils as compared to free virions
224 stimulation (**Figure 4B**), while IFN-γ led to an enhanced secretion of CXCL1 and CCL4
225 (**Figure 4C**) as well as a trend to an increased secretion of CCL2. The enhanced secretion of
226 chemokines by neutrophils upon cytokine priming indicates that the inflammatory environment
227 (i) is key for the functional response of neutrophils to HIV-1 virions, and (ii) potentiates
228 neutrophil chemokine secretion upon activation by ICs.
229
230 ***Neutrophil priming with TNF-α or IFN-γ leads to a dramatically different transcriptomic***
231 ***profile***
232 To identify the potential molecular mechanisms involved in the enhancement of chemokine
233 secretion by IC-activated neutrophils upon pro-inflammatory cytokine priming, we next studied
234 the modulation of gene expression in neutrophils briefly conditioned by TNF-α or IFN-γ using
235 the NanoString technology. This transcriptomic analysis allowed us to identify differentially
236 expressed genes upon TNF-α or IFN-γ priming (**Figure 5**). Heatmap representation of
237 upregulated and downregulated genes (**Figure 5A**) shows that IFN-γ and TNF-α activation
238 resulted in changes in the expression pattern of a different set of genes. Volcano plots provided
239 in **Figure 5B** show the differential gene expression by the stimulation by either cytokine. Only
240 three genes (CD69, PTX3 and IL-1β) were upregulated by both cytokines (**Figure 5C**). IFN-γ
241 induced a set of genes related to innate immune responses, viral infections, cytokine signaling,
242 regulation of lymphocyte activation, antigen processing/presentation, MAPK cascade and
243 PD1/PD-L1 axis. In contrast, TNF-α induced a set of genes related to IL-10/IL-18 signaling,
244 AP1/NF-κB signaling, regulation of cell proliferation and apoptosis responses as well as nitric
245 oxide biosynthesis (**Figure 5D**). The list of genes upregulated or downregulated and their fold
246 increase is shown in **Supplementary Tables 1 and 2**. Worthy of note, IFN-γ led to the
247 upregulation of the inducible FcγRI (CD64) (confirmed at the protein level, **Supplementary**
248 **Figure 4**), while no effect of TNF-α activation on FcγR expression was observed. In addition,
249 IFN-γ induced the expression of the TLR1 and TLR8 (involved in bacterial lipoproteins and
250 viral sensing, respectively), in contrast to TNF-α that induced the expression of TLR5 (involved
251 in flagellin sensing) (**Figure 5A**). These results show a very distinct transcriptomic regulation

252 of neutrophils by TNF-α and IFN-γ, with IFN-γ priming leading to the upregulation of FcγRI
253 and TLR8 genes that could potentiate Fc-mediated antibody signaling and viral sensing,
254 respectively.
255
256 ***Neutrophils from PLWH maintain their cytokine and chemokine secretion capacity in***
257 ***response to danger signals***
258 Neutrophil functions have been shown to be compromised upon HIV-1 infection. Notably, it
259 has been reported that HIV-1 infection impaired the effector functions of neutrophils as well as
260 increased their apoptosis (5,6). However, the effect of HIV-1 infection on their cytokine and
261 chemokine secretion profiles is much less studied and mostly assessed in the AIDS stage (30).
262 Thus, it is necessary to investigate how HIV-1 infection and ART treatment affect the functional
263 activation of neutrophils, particularly their immunomodulatory potential. To this end, we
264 assessed the activation of neutrophils isolated from the blood of ART-treated PLWH
265 (**Supplementary 5A-D**) activated by TLR agonists, pro-inflammatory cytokines, HIV-1 or ICs
266 as compared to HD. Importantly, no significant differences in the frequency of neutrophils in
267 the blood of PLWH were observed as compared to HD (**Supplementary Figure 5E**).
268 Furthermore, no significant differences in neutrophil frequencies were neither observed in
269 PLWH regardless of the time since diagnosis (**Supplementary Figure 5F**). Neutrophils from
270 PLWH had similar secretion profiles than HD upon activation with either TLR4 or TLR7/8,
271 both qualitatively (type of cytokine/chemokine) and quantitatively (amount secreted) (**Figure**
272 **6A**), with few exceptions. In particular, neutrophils from PLWH showed an enhanced secretion
273 of CCL3 and CXCL1 upon TLR4 stimulation and CCL3 and IFN-γ upon TLR7/8 activation
274 (**Figure 6B**). The secretion of other chemokines (CCL4) and cytokines (IFN-λ2/3, IL-1ß)
275 showed a trend of increase in neutrophils isolated from PLWH, but the difference did not reach
276 statistical significance (**Figure 6B**). We assessed in parallel the response of PLWH's
277 neutrophils to inflammatory cytokines (TNF-α and IFN-γ) (**Figure 6C**). Few significant
278 differences were observed in the secretome of neutrophils from PLWH versus HD upon
279 activation by either cytokine. Upon TNF-α activation we observed a decreased secretion of
280 IFN-λ1, IFN-λ2/3 and TNF-α (as well as a trend to a decrease in IL-1ß) and on the contrary,
281 an increased secretion of CCL2 (**Figure 6D**). Neutrophils from PLWH also had similar
282 secretion profiles than those from HD upon stimulation with free HIV-1 virions or in the form
283 of ICs (bNAb alone, was used as a control) (**Figure 7)**. Overall, these results show that
284 neutrophils from PLWH under ART maintain the ability to secrete cytokines/chemokines in

285 response to danger signals, and for some analytes (i.e. CXCL1, CCL2, CCL3 and IFN-γ) they
286 can even display a stronger secretion than neutrophils isolated from HD, at least for certain
287 stimulatory conditions and/or donor samples. These findings also show that neutrophils from
288 PLWH respond to HIV-1 and ICs as weakly as those from HD, suggesting no preceding priming
289 induced by the pathology-related inflammation.
290
291 ***Neutrophils from PLWH show stronger CD16 shedding in response to danger signals than***
292 ***neutrophils from HD***
293 We analyzed in parallel the expression of FcγRs in neutrophils from PLWH upon activation by
294 different danger signals associated with HIV-1 infection, as this is an important issue in the
295 context of bNAb-based therapy. Upon TLR4 activation, CD16b and CD32a cell surface levels
296 on neutrophils from PLWH were significantly lower than those observed in HD (**Figure 8A**).
297 In contrast, TLR7/8 activation resulted in no significant difference in the expression of either
298 FcγR as compared to HD (**Figure 8A**). Similar to TLR4 activation, TNF-α stimulation led to a
299 significant lower expression of CD16b and CD32a on neutrophils from PLWH, as compared to
300 HD (**Figure 8B**). In contrast, IFN-γ activation only led to significantly lower levels of CD16b
301 (**Figure 8B**). No difference in expression was observed for CD16b or CD32a upon activation
302 by free HIV-1 virions (**Figure 8C**) as with TLR7/8 activation. These results highlight that
303 CD16b shedding can be affected by HIV-1 infection as neutrophils from PLWH showed a
304 stronger CD16 shedding as compared to HD upon activation by TLR4 and inflammatory
305 cytokines. However, such enhanced CD16 shedding was not observed upon activation by viral
306 stimuli (i.e. TLR7/8, HIV-1, free or in the form of ICs).
307
308 ***Neutrophils from PLWH display enhanced expression of FcγRs, MHC-II molecules,***
309 ***CXCR4, and PD-L1***
310 We then performed a phenotypic study of neutrophils isolated from PLWH and HD to further
311 assess to what extent HIV-1 infection affected neutrophil properties. To this end, we measured
312 the expression of 18 cell surface markers in the absence of stimulation. The panel was chosen
313 to follow the expression of markers of interest in HIV-1 infection (CXCR4), neutrophil
314 activation (CD11b, CD66b, CD63, CD62L), neutrophil maturity (CD10), neutrophil
315 immunosuppressive properties (PD-L1) and Fc receptors (CD16, CD32, CD64 and CD89),
316 among others. Within the 18 markers, we found a significantly higher expression on PLWH's
317 neutrophils of two FcγRs (CD16b and CD64) and 3 other cell surface molecules, namely

318 CXCR4, PD-L1 and HLA-DR. We also detected a decreased expression of the adhesion
319 molecule CD172α in neutrophils from PLWH (**Figure 9**). Thus, despite similar secretion
320 profiles when activated by HIV-1 virions and/or ICs, neutrophils from PLWH show phenotypic
321 differences as compared to HD. These differences might reflect different functional properties.

322 **Discussion**

323 We have previously shown in a mouse model of retroviral infection that neutrophils have a key
324 immunomodulatory role in the induction of a protective immunity (19,20). Here, we provide
325 new insights into the immunomodulatory properties of neutrophils in the context of HIV-1
326 infection and antiviral mAb-therapy. Our study dissects the secretome and expression of
327 neutrophil immune receptors in a stimulus-specific manner both in HD and PLWH.
328 Noteworthy, most studies on immunomodulatory functions of neutrophils are based on bacterial
329 activation (i.e. TLR4 activation), while the effect of virus-dependent activation is understudied.
330 The latter has been mostly investigated using TLRs as surrogates, and there are few data on
331 whether and how HIV-1 and/or HIV-ICs affect their immunomodulatory properties. Our work
332 highlights that neutrophil from ART-treated PLWH conserve the ability to secrete
333 cytokines/chemokines in response to danger signals but display different phenotypic features
334 than HD's neutrophils. It also shows that inflammatory environment shapes neutrophils
335 immunomodulatory properties and allows them to better respond to HIV-1 viral determinants,
336 free or in the form of ICs.

337

338 Based on the observed upregulation of CD11b and CD66b cell surface activation markers, our
339 data show that both TLR4 and TLR7/8 agonists similarly activate neutrophils. However, TLR4
340 agonist led to a significantly decrease expression of two FcγRs (CD16b and CD32a) as
341 compared to the TLR7/8 agonist. This suggests a stronger effect of bacterial-associated danger
342 signals on FcγRs modulation than virus-associated danger signals, notably on the shedding of
343 CD16b. Similarly, our data also show that free HIV-1 and ICs had either a mild effect or no
344 effect, respectively, on the modulation of CD16b cell surface levels, despite that both stimuli
345 significantly increased the expression of CD11b and CD66b. However, TNF-α, but not IFN-γ,
346 led to a strong CD16b shedding as well as a decrease in the CD32a cell surface levels (although
347 the latter to a much lesser extent). This shows a clear effect of inflammatory conditions in
348 promoting CD16b shedding that might have biological consequences. On one hand, soluble
349 CD16b might have immunomodulatory functions as it has been shown to play a dominant role
350 in clearing ICs (31) and to bind complement receptors (32) leading to an increased cell
351 activation. On the other hand, it has been suggested that CD16 shedding might grant access to
352 cell-activating CD32a (33). In addition, CD16b shedding can be affected by pathological
353 conditions as observed for neutrophils from PLWH that showed stronger CD16 shedding upon
354 activation than HD's neutrophils. This stimulus-dependent and pathology-dependent
355 modulation of FcγRs might have to be taken into account in the context of HIV-1 infection and

356 bNAb-based immunotherapies alone or in combinatorial therapeutic approaches involving TLR
357 agonists (14,34–38) as it might affect their therapeutic efficacy. This might also be relevant to
358 consider it in the context of bacterial co-infections in PLWH (39).
359
360 Our work also allowed us to dissect, at the protein level, the chemokine/cytokine secretion
361 profiles of neutrophils in a stimulus-dependent manner under both physiological (HD) and
362 pathological (PLWH) conditions. This is important to point out as multiple studies assessing
363 the secretion profile of neutrophils upon different stimuli were based on transcriptomic
364 approaches instead of *bona fide* cytokine/chemokine secretion. In agreement with previous
365 studies, we have shown that neutrophils isolated from HD and activated by LPS, R848 or TNF-
366 α secreted IL-8, CXCL1, CCL3 and CCL4 (23–25,27), while IFN-γ only induced the secretion
367 of IL-8. We also observed the secretion of IFN-λ2/3 by LPS-activated neutrophils in half of the
368 samples tested, which has not been described thus far. Finally, our work finely describes the
369 secretome of HIV-1-and IC-activated neutrophils, thus far unreported. Importantly, the
370 cytokines/chemokines secreted upon activation with HIV-1 and ICs are involved in neutrophil
371 activation (IL-8) and recruitment (CXCL1, CCL3) and/or act as chemoattractant for NK cells,
372 monocytes and various other immune cells in the site of inflamed or damaged tissue (CCL4)
373 (40). Interestingly, although it is only detected in a proportion of samples, the secretion of IFN-
374 λ by neutrophils observed upon HIV-1 stimulation has not been described to date. This family
375 of cytokines have been shown to modulate antiviral immune response (41), but its role in HIV-
376 1 infection is still unclear. Further investigations are necessary to ascertain the secretion of IFN-
377 λ by neutrophils activated by the different stimuli and to determine the biological consequences
378 of its production in the context on neutrophil biology and HIV-1 infection. In this regard, it has
379 been shown that IFN-λ (i) diminishes the production of reactive oxygen species and
380 degranulation in neutrophils (42) and (ii) IFN-λ3 inhibits HIV-1 infection of macrophages (43).
381
382 In the context of HIV-1 infection, a key question not yet fully addressed is the capacity of
383 neutrophils to respond, in terms of cytokines/chemokines secretion, to pathogen-associated
384 danger signals and cytokine stimulation. Our data show that neutrophils isolated from ART-
385 treated PLWH respond to TLR agonists, pro-inflammatory cytokines, HIV-1 and ICs in a
386 similar way than neutrophils from HD, if not better. This is the case for the enhanced secretion
387 of CCL2 upon TNFα activation and CCL3/CXCL1 upon TLR activation. These observations
388 indicate that (i) neutrophils retain their capacity to secrete cytokines/chemokines in the immune

389 context associated with HIV-1 infection, and (ii) such immune context may even potentiate the
390 secretion of some specific chemokines. Our results contrast with previous studies reporting
391 impaired cytokine secretion by LPS-activated neutrophils isolated from PLWH on ART for less
392 than 2 years and having developed AIDS (30). Thus, in advanced stages of disease, the ability
393 of neutrophils to respond to danger signals is apparently altered, which can contribute to the
394 disease evolution. This does not seem to be the case for neutrophils isolated from aviremic,
395 ART-treated PLWH as shown here, highlighting the importance of ART in maintaining not
396 only their effector functions (6,44), but also their cytokine/chemokine secretion capacity.
397
398 Our work also sheds new light on the phenotypic characteristics of neutrophils in PLWH. We
399 found that several cell surface molecules were significantly increased in PLWH. First, we
400 observed an increased expression of CD16b and CD64, while CD32a levels were not affected.
401 However, the upregulation of these FcγRs in PLWH did not result in an increased secretion of
402 cytokines and chemokines upon IC-activation of neutrophils as compared to HD. Indeed, while
403 increased FcγR-mediated phagocytic activity may directly influence immune activation, the
404 effect of specific FcγRs in modulating cytokines/chemokines secretion in neutrophils is still ill-
405 understood. Further investigations are required to determine the potential FcγR-dependent
406 enhancement of cytokines/chemokine by ICs as well as the FcγRs involved. Second, our results
407 show the upregulation of HLA-DR in PLWH neutrophils. This observation is also in agreement
408 with the increased frequency of HLA-DR^{high} neutrophils in SIV-infected macaques as described
409 by Lemaitre et al (45). On the contrary, Bowers *et al* (46) reported that the expression of HLA-
410 DR was unmodified in PLWH neutrophils. However, experiments were performed using whole
411 blood and not purified neutrophils as we did. These controversial observations could therefore
412 result from the presence of several neutrophil subsets in whole blood as previously described
413 (47–49). Third, our results also show an enhanced expression of CXCR4 and PD-L1.
414 Interestingly, the upregulation of these two cell surface molecules has recently been associated
415 with neutrophil aging in PLWH (50). However, in contrast to our study, the assessment of
416 CXCR4 and PD-L1 expression was made using samples from ART-naive PLWH. Finally, our
417 findings provide further evidence of the upregulation of PD-L1 in PLWH neutrophils. PD-L1
418 expression has been previously described in different populations of neutrophils (46,47,50) and
419 irrespective of ART (46,50) in PLWH. It has been associated with T-cell immunosuppressive
420 effects that can be counteracted by blocking the PD-1/PD-L1 axis (46,50). Thus, the

421 upregulation of PD-L1 we found in PLWH suggests a potential immunosuppressive role of
422 PLWH neutrophils.
423
424 Another important observation of our study is the effect of the inflammatory environment on
425 the modulation of neutrophil properties. Thus, the stimulation with inflammatory cytokines
426 potentiated the release of several chemokines by HIV-1 and IC-activated neutrophils.
427 Interestingly, TNF-α and IFN-γ priming led to a significant increased secretion of CXCL1 and
428 CCL4 by IC-activated neutrophils as compared to virus-activated cells. This is in agreement
429 with our previous results using mouse neutrophils activated with IC-made with a murine
430 retrovirus (20). Our work also allowed us to identify a different effect of TNF-α and IFN-γ on
431 the enhancement of the functional properties of IC-activated human neutrophils. Notably, IFN-
432 γ but not TNF-α, led to enhanced secretion of CCL4 and CCL2 although to a lesser extent for
433 the latter. It is worth mentioning that the enhanced secretion of CCL2 by HIV-1 and HIV-IC-
434 activated neutrophils by IFN-γ priming is in agreement with the IFN-γ-mediated upregulation
435 of CCL2 production previously described in a different experimental setting (51). This finding
436 suggests that in a Th1 microenvironment, neutrophil might be involved in the recruitment of
437 monocytes through the secretion of CCL2. Worthy of note, IFN-γ priming led to the
438 upregulation of FcγRI and TLR8 (potentially involved in IC-mediated signaling and HIV-1
439 sensing, respectively). Interestingly, this observation is in agreement with the recent study by
440 Kumola *et al*, showing an upregulation of CD64 and TLR8 upon IFN-γ stimulation as well as
441 an enhanced expression of PD-L1 (52). This suggests that the enhanced expression of CD64
442 and PD-L1 we observed on the neutrophils from PLWH could be due to IFN-γ-priming *in vivo*.
443 However, our results showing that neutrophils from PLWH respond to HIV-1 or ICs as weakly
444 as those from HD, also raise the possibility that they may not be adequately primed *in vivo*, or
445 not to an efficient degree. Thus, the question of whether PLWH's neutrophils are primed or not
446 remains open and deserves further investigation.
447
448 In conclusion, our work dissects the phenotypic and functional properties of PLWH neutrophils.
449 It highlights that they maintain their cytokine and chemokine secretion capacity, while
450 displaying some immunosuppressive features. It also provides new insights on the modulation
451 of FcγR expression in the context of danger signals associated with HIV-1 infection which need
452 to be taken into account in the context of antibody therapy. These findings might be exploited
453 to improve HIV-1 antiviral therapies in PLWH. This could be achieved by tailoring therapeutic

454 interventions aiming at either harnessing the immunomodulatory properties of these cells
455 (through the use of cell-type specific, appropriate immunostimulatory host-directed therapies)
456 or at counteracting their immunosuppressive function (through the design of combinatorial
457 approaches involving the blocking of the PD-1/PD-L1 axis) (53,54).
458

459 **Material & Methods**

460

461 *Ethics*

462 All human blood samples were fully anonymized and given with previous written consent of
463 the donor. Samples were given under ethical approval of appropriate institutions.

464

465 *Human blood samples*

466 Blood samples from anonymized healthy donors (HD) were provided by the French
467 establishment for blood donations (Etablissement Français du Sang, EFS, Montpellier, France)
468 according to the agreement between this establishment and INSERM (21PLER2018-0069).
469 EFS provided 7 to 8mL of fresh whole blood in EDTA for each healthy donor.
470 The use of blood samples from anonymized people living with HIV-1 (PLWH) under
471 antiretroviral therapies (ART) was approved by the Centre for Biological Resources (DRI#305-
472 2019-NC and amendment DRI_2022-69-SR). The samples were collected and provided by the
473 Virology laboratory of the Centre Hospitalier Universitaire (CHU) Lapeyronie of Montpellier
474 (France). Median age was 57 years old ([22;76]) for 31 PLWH donors (19M; 13F). All donors
475 were under successful ART with no detectable viral load. 2 to 3mL of whole blood in EDTA
476 were provided for each PLWH donors.

477

478 *Primary cells purification*

479 Peripheral blood mononuclear cells (PBMC) and granulocytes were isolated using a
480 Histopaque-1119 density gradient. Briefly, whole blood sample was diluted to three times its
481 volume with a solution of PBS 1X (Gibco, cat #10010-015) supplemented with EDTA at 0,2
482 mM (Invitrogen, cat #15575-038) and loaded on Histopaque-1119 (Sigma-Aldrich, cat
483 #11191). Density gradient was performed by 800g centrifugation with slow acceleration and
484 no brake (acceleration 4, brake 0) for 20 minutes. Then, PBMCs ring was collected as well as
485 the granulocytes- containing fraction underneath.

486

487 Monocytes and neutrophils were then purified from PBMC or granulocytes, respectively, using
488 magnetic-based CD14- or CD15-positive selection kits (STEMCELL TECHNOLOGIES,
489 $CD15^+$ positive selection REF#18651, $CD14^+$ positive selection REF#117858) according to the
490 manufacturer's protocol. Purity assessed by flow cytometry was > 98% for both cell type.

491

492

493 *Cell culture*
494 Purified neutrophils were plated at a concentration of 2 million cells/mL in 96 U-bottom wells
495 plate and cultured in RPMI 1640 supplemented with GlutaMAX^{TM} medium (Gibco, cat
496 #61870-010), 10% fetal bovine serum (FBS, Eurobio, REF#CVFSVF00-01) and 1%
497 penicillin/streptomycin (Gibco, cat #15140-122) with 10 µg/mL of G-CSF (R&D, cat #214-
498 CS-025).
499 Purified monocytes were plated at a concentration of 1 million cells/mL in 96 U-bottom wells
500 and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1%
501 penicillin/streptomycin with 10 µg/mL of GM-CSF (Peprotech, cat #300-03).
502 Cells were then cultured for 16-20h with or without stimulation.
503
504 Human embryonic kidney cells (293T HEK cells) (HEK 293T-ATCC-CRL-1575TM) were
505 used for the production of HIV-1 virions. 293T HEK cells were cultured in Dulbecco's
506 Modified Eagle's Medium (DMEM, Gibco, cat #31966) supplemented with 10% fetal bovine
507 serum, sodium pyruvate and antibiotics (penicillin-streptomycin). Cells were grown at 37˚C in
508 a 5% CO_2 atmosphere.
509
510 *In vitro cell stimulation*
511 For TLR or cytokine stimulations, cells were stimulated overnight with TLR4 agonist (LPS ;
512 Sigma-Aldrich, cat #L4524), TLR7/8 agonist (R848 ; Invivogen, cat #tlrl-r848) at 0,5 µg/mL
513 each, with IFN-γ (R&D, cat #285-IF-100) at 1000 IU/mL or with TNF-α (Peprotech, cat #AF-
514 300-01A) at 1ng/mL. Otherwise, cells were stimulated with either free HIV-1 virions (Ad8
515 strain, MOI of 3.10^4), immune complexes made with HIV-1 Ad8 and the 10-1074 bNAb or the
516 bNAb alone.
517
518 *Neutrophils priming*
519 For primed conditions, neutrophils were resuspended in culture medium after purification and
520 stimulated at 37°C 5%CO_2 with IFN-γ at 2000 IU/mL for 30 minutes or with TNF-α at 5ng/mL
521 for 10 minutes. Cells were washed then cultured overnight with either free virus, immune
522 complex or bNAb alone.
523
524 *Virus production, purification and titration:*
525 2.5 million human embryonic kidney cells (293THEK cell) were seeded in 10 ml of growth
526 media 1 day before transfection. At 50-70% confluence, cells were transfected with calcium

527 phosphate precipitate method, with 10 µg total quantity of HIV-1 molecular clone expressing
528 plasmids, as in (55). At 12 hours post transfection, cells were washed and fresh media
529 supplemented with 20mM Hepes was added on the cells. Cell culture supernatant containing
530 viral particles was collected 36 hours post-transfection, pooled and filtrated through 0.45 µm
531 and then purified by ultracentrifugation on a cushion of 25% sucrose in TNE buffer (10 mM
532 Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA) at 100000xg, for 1 hour 30 minutes at 4°C,
533 in a SW32Ti Beckman Coulter rotor. Dry pellet was resuspended with RPMI without serum, at
534 4°C overnight. Viral titer was quantified by alphaLISA immunoassay (Perkin Elmer) using
535 anti-HIV-1 p24. Recombinant HIV-1 p24 protein is used for titer standard range. Viral particles
536 were quantified using Videodrop system (Myriade France).
537
538 *Recombinant* HIV-1 *bNAb production*
539 Recombinant human HIV-1 IgG1 antibodies, anti-CD4bs 3BNC117 (56) and anti-V3-glycan
540 10-1074 (57), were produced by co-transfection of Freestyle 293-F cells (Thermo Fisher
541 Scientific) using the polyethyleneimine (PEI)-based precipitation method as previously
542 described (58), and purified by affinity chromatography using protein G Sepharose 4 fast flow
543 beads according to the manufacturer's instructions (Cytiva). Purified IgG antibodies were
544 dialyzed against PBS using Slide-A-Lyzer Cassettes (10K MWCO; Thermo Fisher Scientific),
545 and quantified using NanoDrop™ ONE instrument (Thermo Fisher Scientific). Both bNAbs
546 are of IgG1 isotype and were used at a concentration of 1µg/mL to form ICs.
547
548 *Immune complexes formation*
549 Viruses (3.10^4 viral particles) and bNAb (1µg/mL) were diluted in culture medium and mixed
550 together on a 1,5mL Eppendorf, then mixed on a rotative agitator for 20 minutes. ICs were
551 gently plated with cells in 96 U-bottom wells plate.
552
553 *Flow cytometry immunophenotyping of neutrophils*
554 Monitoring of phenotypic activation was done by flow cytometry using FACSCantoII, BD
555 LSRFortessa (BD Bioscience) or Novocyte (Agilent Technologies) flow cytometers. Cells were
556 stained at room temperature with a viability dye (Zombie NIR™, Biolegend #423105 or
557 ZombieRed™, Biolegend #423109) for 15 minutes then washed and stained for 20 minutes at
558 room temperature with fluorochrome-conjugated antibodies. Antibodies for flow cytometry
559 were PerCPCy5-5-CD15 (HI98, Biolegend #301922), AF488-CD11b (ICRF44, Biolegend
560 #301317), AF647-CD66b (G10F5, Biolegend #305110), PC7-CD16a/b (3G8, Biolegend

561 #302016), PE-CD32a/b (FUN-2, Biolegend #303206), PE-CD64 (10.1, Biolegend #305008),
562 FITC-CD14 (MoP9, STEMCELL Technologies #60124FI).
563 For immunophenotyping of HD's and PLWH's neutrophils the following antibodies were used:
564 PerCPCy5-5-CD15 (HI98, Biolegend #301922), PE-LOX-1 (15C4, Biolegend #358604),
565 BV510-CD11b (ICRF44, Biolegend, #301334), AF647-CD66b (G10F5, Biolegend, #305110),
566 BV605-CD62L (DREG-52, Biolegend, #304834), BV650-HLA-DR (L243, Biolegend,
567 #307650), BV711-CD63 (H5C6, Biolegend, #353042), PC7-CD16a/b (3G8, Biolegend,
568 #302016), PE-CF594-CD64 (10.1, Biolegend, #565389), APC-Fire750-CD32a/b (FUN-2,
569 Biolegend, #303220), PE-PD-1 (3.13, Miltenyi, #130-177-384), APC-CD177 (MEM-166,
570 Biolegend, #315808), BV605-CD49d (9F10, Biolegend, #304324), BV711-CD31 (WM59,
571 Biolegend, #303136), PC7-PD-L1 (MIH1, BD, #558017), PE-CF594-CD10 (HI10a,
572 Biolegend, #312227), PE-CD89 (Biolegend, #555686), APC- CD172*α* (15-414, eBiosciences
573 #17-1729-42), PC7-CXCR4 (12G5, Biolegend, #306514).
574 Forward scatter area and forward scatter height as well as side scatter area and height were used
575 to remove doublets from flow cytometry analyses. Gating is done on live cells as assessed by
576 viability dye and on CD15-positive cells. Data were analyzed using FlowJo software version
577 10.5.3 (TreeStar).
578
579 *Cytokines and chemokines secretion quantification*
580 Secretion of soluble cytokines and chemokines were assayed from cell-free culture supernatants
581 of *in vitro* stimulated neutrophils and monocytes using bead-based immunoassays (LegendPlex,
582 Biolegend). Two assays were used allowing the quantification of IL-1β, Il-6, TNF-α, CXCL10,
583 IFN-λ1, IL-8, IL-12p70, IFN-α2, IFN-λ2/3, GM-CSF, IFN-β, IL-10, IFN-γ (LegendPlex
584 Human anti-virus response panel, Biolegend #740390) and IL-8, CXCL10, CCL11, CCL17,
585 CCL2, CCL5, CCL3, CXCL9, CXCL5, CCL20, CXCL1, CXCL11, CCL4 (LegendPlex
586 Human proinflammatory chemokines panel, Biolegend #740985). Immunoassays plates were
587 read on a Novocyte (Agilent Technologies) flow cytometer and data were analyzed with the
588 appropriate Biolegend LEGENDplex^{TM} software (https://legendplex.qognit.com).
589
590 *Transcriptomic studies*
591 RNA was extracted from neutrophils obtained from 4 healthy blood donors. Total RNA was
592 then purified from 500,000 to 1 million cells using the miRNeasy Micro kit (Qiagen GmbH)
593 following the manufacturer's instructions. The quantification of all RNA samples was
594 performed using the Nanodrop One (Thermo Fisher Scientific), and RNA integrity was

595 evaluated using the Agilent 2100 Bioanalyzer system. RNA expression profiling was performed
596 using Myeloid Innate Immunity Panel 2.0 (770 genes in 19 different pathways). Gene
597 expression analysis was conducted with the NanoString technology. Briefly, 5 µL/sample
598 containing of 50 ng of total RNA was combined with the nCounter® reporter CodeSet (3 µL)
599 and nCounter® capture ProbeSet (2 µL) along with hybridization buffer (5 µL) for an overnight
600 hybridization reaction at 65 °C. The reaction was then cooled to 4 °C, and the samples were
601 purified, immobilized on a cartridge, and the data were assessed using the nCounter SPRINT.
602 All expression data was reviewed using NanoString® nSolver™ Analysis software 4.0. To
603 identify main biological processes modulated by cytokine priming, genes upregulated and
604 downregulated were analyzed using Metascape (59).
605
606 *Surface Plasmon resonance (SPR)*
607 Affinity of bNAbs for Fcγ Receptors was assessed by SPR experiments performed on a Biacore
608 T200 (GE Healthcare). SPR experiments were performed on a T200 apparatus at 25°C in PBS
609 containing 0,05 % P20 surfactant (Cytiva). Anti-histidine antibody (R&D Systems) was
610 covalently immobilized on a CM5-S sensor chip flowcell (Fc2) by amine coupling according
611 to the manufacturer's instructions (Cytiva). A control reference surface (flowcell Fc1) was
612 prepared using the same chemical treatment but without anti-His antibody. All kinetic
613 measurements in Fc1 and Fc2 were performed by single-cycle titration at 100µl/min. Each
614 human FcγR (R&D Systems) was captured on immobilized anti-His antibodies at 100-200 RU
615 level. Five increasing concentrations (3,6, 11, 33, 100, 300 nM) of antibody were injected
616 (injection time = 60s) at 100µl/min on captured receptors. After a dissociation step of 600s in
617 running buffer, sensor surfaces were regenerated using 10µl of glycine-HCl pH1.5. All the
618 sensorgrams were corrected by subtracting the low signal from the control reference surface
619 and buffer blank injections. Kinetic parameters were evaluated from the sensorgrams using a
620 two-states or a steady-state models from the T200 evaluation software.
621
622 *Statistics*
623 Statistical analyses were performed using Prism software version 9.5.1 (GraphPad). Simple
624 group comparisons were performed using the Wilcoxon signed rank test for paired data or the
625 Mann–Whitney test for unpaired data. Multiple group comparisons were performed using
626 Friedmann test for paired data or using Kruskal-Wallis test for unpaired data with additional
627 multiple comparisons Dunn test. Significance was assigned as follows: $*p < 0.05$, $**p < 0.01$,
628 $***p < 0.001$, and $****p < 0.0001$.

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Authors contribution

Soledad Marsile-Medun (SMM), Mar Naranjo-Gomez (MN-G) and Mireia Pelegrin (MP) defined the research program; SMM, Manon Souchard (MS), Daouda Abba Mousssa (DAM), Giang Ngo (GN), Martine Pugnière (MPu,) and MN-G, performed the experiments; Valerie Lorin (VL) and Hugo Mouquet (HM) provided advice on and generated bNAbs; Elisa Reynaud (ER) and Edouard Tuaillon (ET) managed and provided PLWH blood samples. Rayane Dibsy (RD) and Delphine Muriaux DM) provided advice on and performed HIV-1 production, purification and quantification. SMM, MS, MN-G and MP carried out the data analyses. SMM, MN-G and MP wrote the manuscript. Grants to SMM, MN-G and MP funded the study. All authors discussed, commented on and approved the manuscript in its final form.

662

663 **Conflict of interests**

664 None

665

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819
820

821 **Figure Legends**
822
823 **Figure 1 Activation of human neutrophils stimulated *in vitro* by Toll-like receptors (TLRs)**
824 **agonists.**
825 Human neutrophils from healthy donors were activated by TLRs agonists, TLR4 (LPS), TLR7/8 (R848).
826 (A). Gating strategy used for all experiments. (B-C) Viability (B) and phenotypic activation (C) assessed
827 by monitoring cell surface markers. Fold change is measured versus unstimulated cells (dashed lines).
828 It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. (D-E) Cytokines and
829 chemokines secretion profile, represented as heatmap (D) and histograms for each cytokine/chemokine
830 assessed (E). Values are mean +/- SD of at least 25 (B-C) and 8 (D-E) independent measurements.
831 Significance was assigned as follows: $**p < 0{,}01$ and $***p < 0.001$. NS: non-stimulated.
832
833 **Figure 2. Activation of human neutrophils stimulated *in vitro* by TNF-α or IFN-γ.**
834 Human neutrophils from healthy donors were activated overnight by TNF-α at 1 ng/mL or IFN-γ at
835 1000 IU/mL. (A-B) Viability (A) and phenotypic (B) activation assessed by monitoring cell surface
836 markers. Fold change is measured versus unstimulated cells (dashed lines). It is represented as the ratio
837 MFI stimulated cells/MFI unstimulated cells. (C-D) Cytokines and chemokines secretion profile,
838 represented as a heatmap (C) and histograms (D) for each cytokine/chemokine assessed. Values are
839 mean +/- SD of at least 19 (A) and 8 (B-D) independent measurements. Significance was assigned as
840 follows: $*p < 0.05$, and $**p < 0.01$. NS: non-stimulated.
841
842 **Figure 3. Activation of human neutrophils stimulated *in vitro* by HIV-1 virions, ICs or free bNAb.**
843 Human neutrophils from healthy donors were activated by HIV-1 virions, ICs or free bNAb. (A-B)
844 Viability (A) and phenotypic activation (B) assessed by monitoring cell surface markers. Fold change
845 is measured versus unstimulated cells (dashed lines). It is represented as the ratio MFI stimulated
846 cells/MFI unstimulated cells. (C-D) Cytokines and chemokines secretion profile, represented as a
847 heatmap (C) and histograms (D) for each cytokine/chemokine assessed. Values are mean +/- SD of at
848 least 21 (A-B) and 17 (C-D) independent measurements. Significance was assigned as follows: $*p <$
849 0.05, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. NS: non-stimulated.
850
851 **Figure 4. Secretion profile of human neutrophils primed with TNF-α (B) or IFN-γ (C) and then**
852 **stimulated *in vitro* by HIV-1 virions, ICs or free bNAb.**
853 (A) Protocol for priming of neutrophils. Dashed lines indicate the level of secretion induced by either
854 TNF-α alone (B) or IFN-γ alone (C). Values are of 10 (B) and 6 (C) independent measurements.
855 Significance was assigned as follows: $*p < 0.05$ and $**p < 0.01$
856
857 **Figure 5. Transcriptomic analysis of neutrophils primed with TNF-α or IFN-γ.**
858 Heatmap (A) and Volcano plot (B) representation of upregulated and downregulated genes by TNF-α
859 or IFN-γ. (C) Venn diagram of regulated genes after cytokine stimulation. (D) Genes upregulated and
860 downregulated were analyzed using Metascape (https://metascape.org/gp/index.html#/main/step1) to
861 identify main biological processes modulated by cytokine priming. Data from 4 independent samples.
862
863 **Figure 6. Activation of human neutrophils isolated from HD or PLWH stimulated *in vitro* by TLR**
864 **agonists LPS (TLR4) and R848 (TLR7/8) or inflammatory cytokines (TNF-α and IFN-γ).**
865 (A-B) Cytokines and chemokines secretion profile upon activation by TLR agonists, represented as a
866 heatmap (A) and histograms (B) for each cytokine/chemokine assessed. (C-D) Cytokines and
867 chemokines secretion profile upon activation by inflammatory cytokines, represented as a heatmap (C)
868 and histograms (D) for each cytokine/chemokine assessed. Values are mean +/- SD of at least 9 (A-B)
869 and 4 (C-D) independent measurements.

870
871 **Figure 7. Activation of human neutrophils isolated from HD or PLWH stimulated *in vitro* by HIV-**
872 **1 virions, ICs or free bNAb.**
873 (A-B) Cytokines and chemokines secretion profile, represented as a heatmap (A) and histograms (B) for
874 each cytokine/chemokine assessed. Values are mean +/- SD of 6 independent measurements.
875
876 **Figure 8. Modulation of the expression of FcγR on human neutrophils isolated from HD or PLWH**
877 **stimulated *in vitro* by TLR agonist, inflammatory cytokines, HIV-1 virions, ICs or free bNAb.**
878 (A-C) Modulation of the cell surface levels of CD16 and CD32 upon activation by TLR agonists (A),
879 cytokines (B), and HIV-1 virions, ICs or free bNAb (C). Fold change is measured versus unstimulated
880 cells (dashed lines). It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. Values
881 are mean +/- SD of at least 14 (A), 8 (B) and 6 (C) independent measurements. Significance was assigned
882 as follows: $*p < 0.05$ and $**p < 0.01$
883
884 **Figure 9: Phenotypic characterization of neutrophils isolated from PLWH and HD blood samples.**
885 Purified neutrophils were immunophenotyped by assessing the expression of 18 cell surface markers.
886 Data include at least 19 PLWH blood samples and 4 HD samples. Statistical analyses are unpaired
887 Mann-Whitney test. Significance was assessed as follows: $*p < 0.05$, $***p < 0.001$, and $****p < 0.0001$.

Figure 1

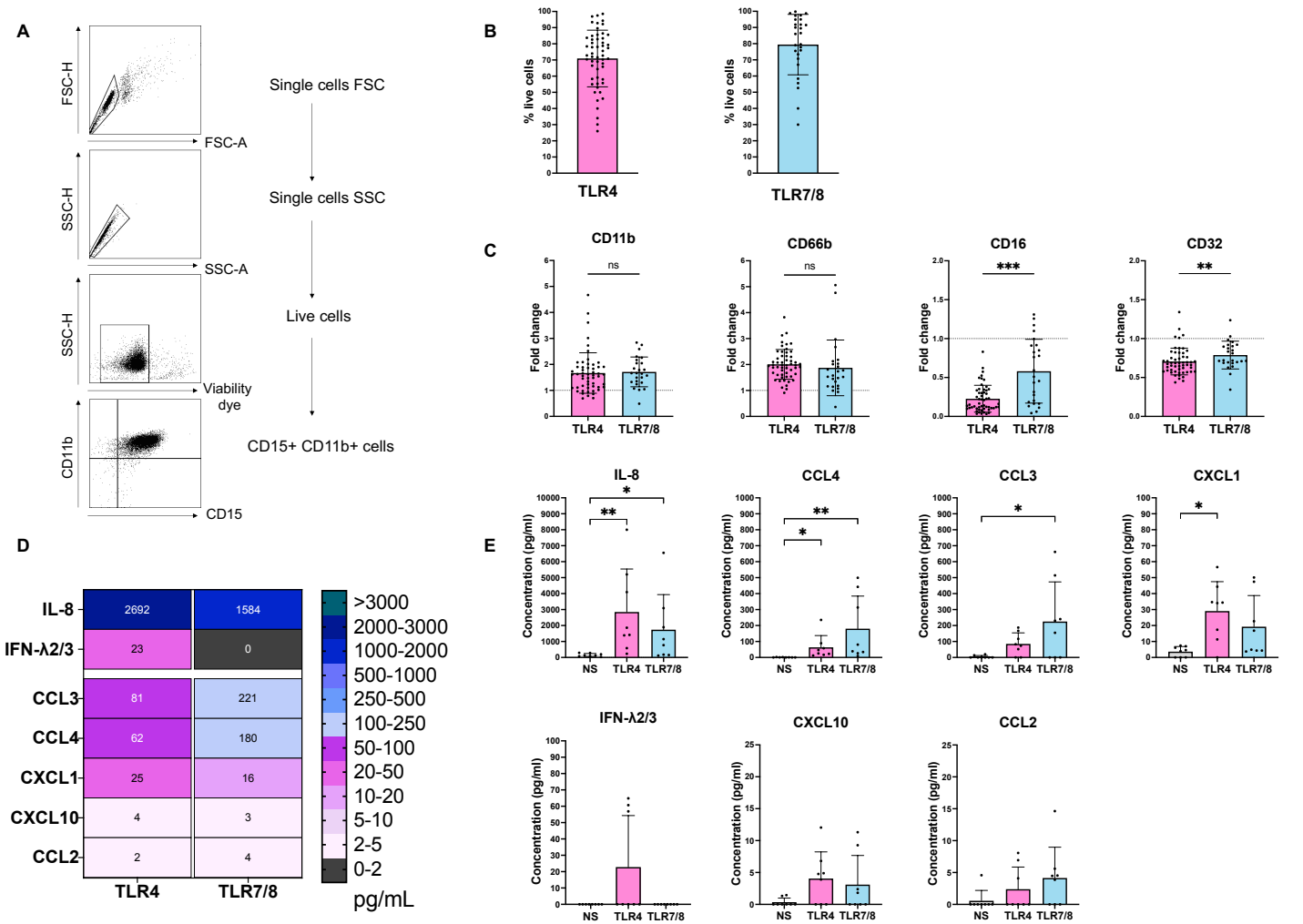

A
FSC-H
FSC-A
SSC-H
SSC-A
SSC-H
Viability dye
CD11b
CD15
Single cells FSC
Single cells SSC
Live cells
CD15+ CD11b+ cells
B
% live cells
TLR4
TLR7/8
C
CD11b
CD66b
CD16
CD32
Fold change
ns
***
**
TLR4
TLR7/8
D
IL-8
IFN-λ2/3
CCL3
CCL4
CXCL1
CXCL10
CCL2
2692
1584
23
0
81
221
62
180
25
16
4
3
2
4
TLR4
TLR7/8
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
E
IL-8
CCL4
CCL3
CXCL1
IFN-λ2/3
CXCL10
CCL2
Concentration (pg/ml)
NS
TLR4
TLR7/8

Figure 2

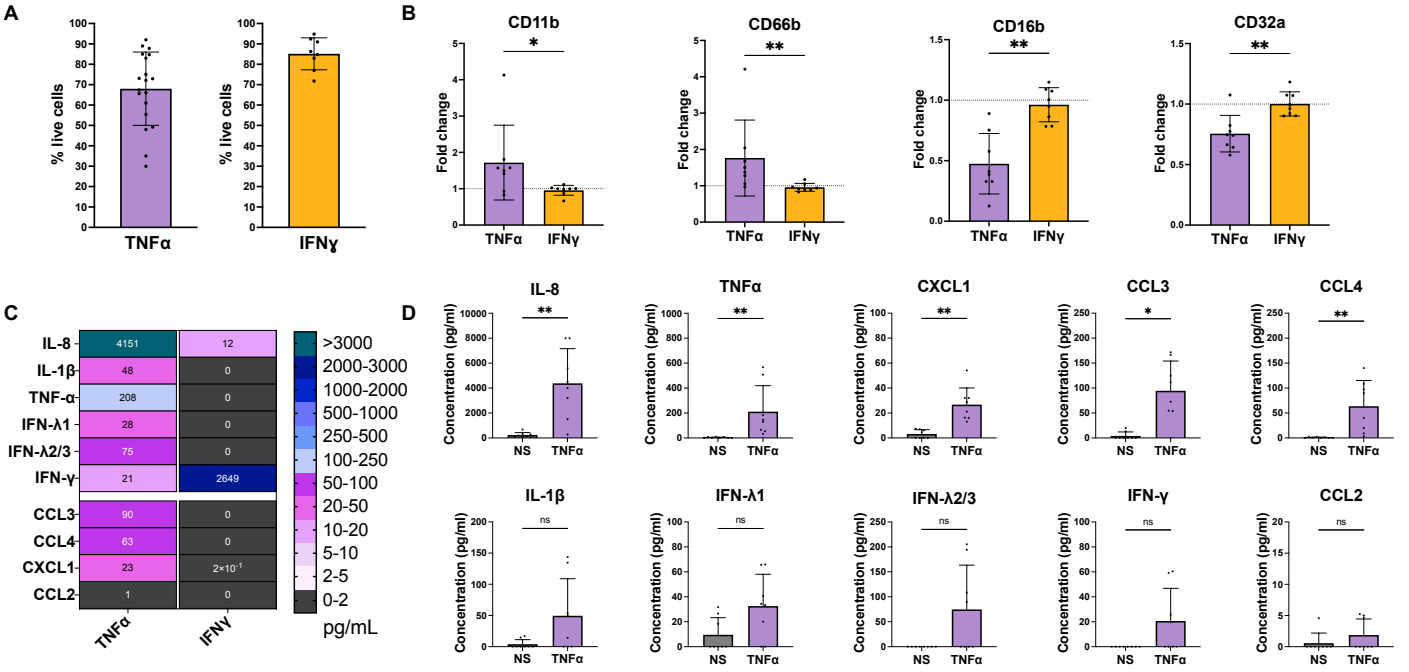

A
% live cells
TNFα
IFNγ
B
CD11b
CD66b
CD16b
CD32a
Fold change
C
IL-8
IL-1β
TNF-α
IFN-λ1
IFN-λ2/3
IFN-γ
CCL3
CCL4
CXCL1
CCL2
4151
12
48
0
208
0
28
0
75
0
21
2649
90
0
63
0
23
2×10-1
1
0
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
D
Concentration (pg/ml)
NS
ns

Figure 3

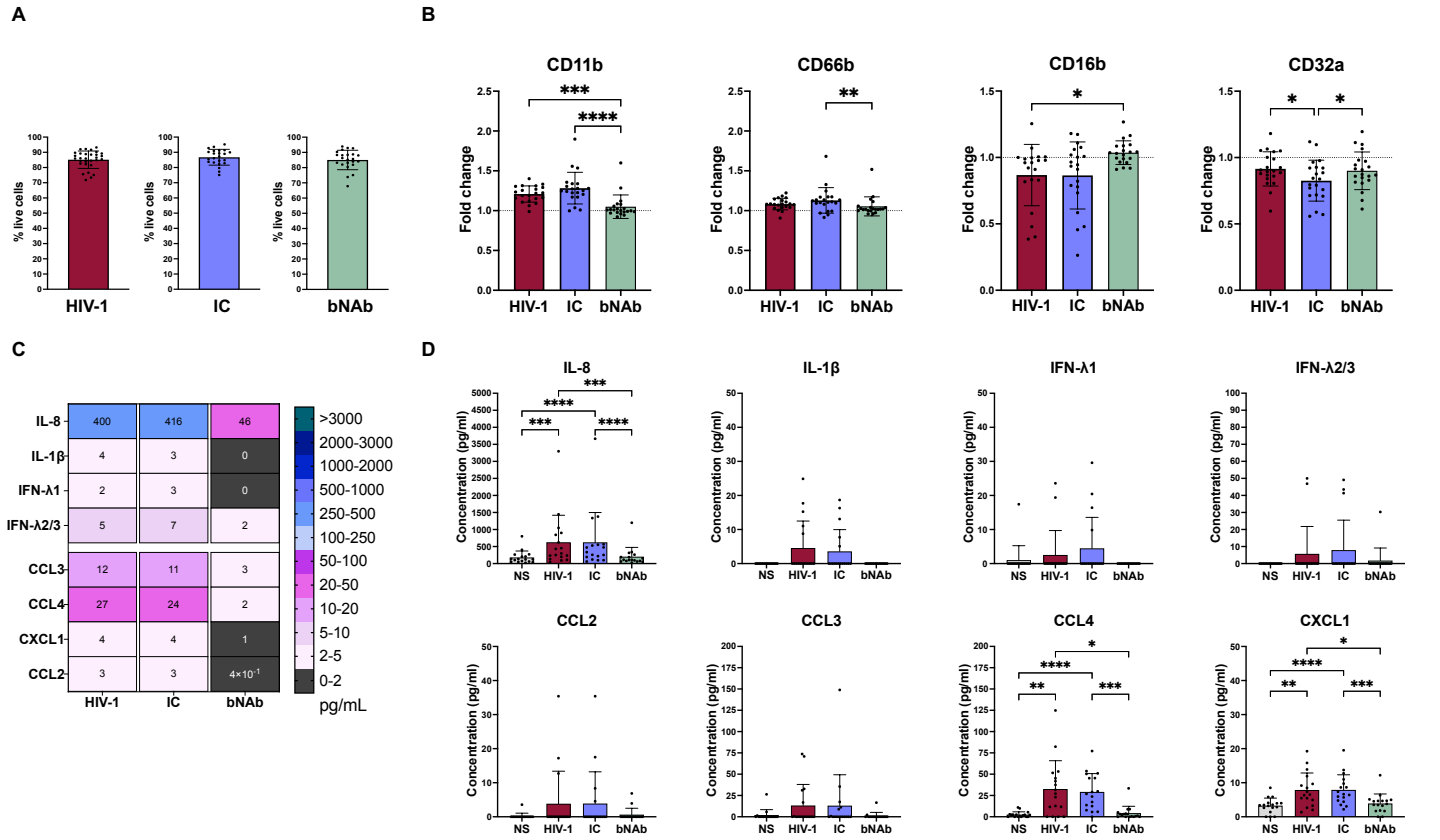

A
% live cells
HIV-1
IC
bNAb
B
CD11b
CD66b
CD16b
CD32a
Fold change
HIV-1 IC bNAb
C
IL-8 400 416 46
IL-1β 4 3 0
IFN-λ1 2 3 0
IFN-λ2/3 5 7 2
CCL3 12 11 3
CCL4 27 24 2
CXCL1 4 4 1
CCL2 3 3 4×10-1
HIV-1 IC bNAb
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
D
IL-8
IL-1β
IFN-λ1
IFN-λ2/3
CCL2
CCL3
CCL4
CXCL1
Concentration (pg/ml)
NS HIV-1 IC bNAb

Figure 4

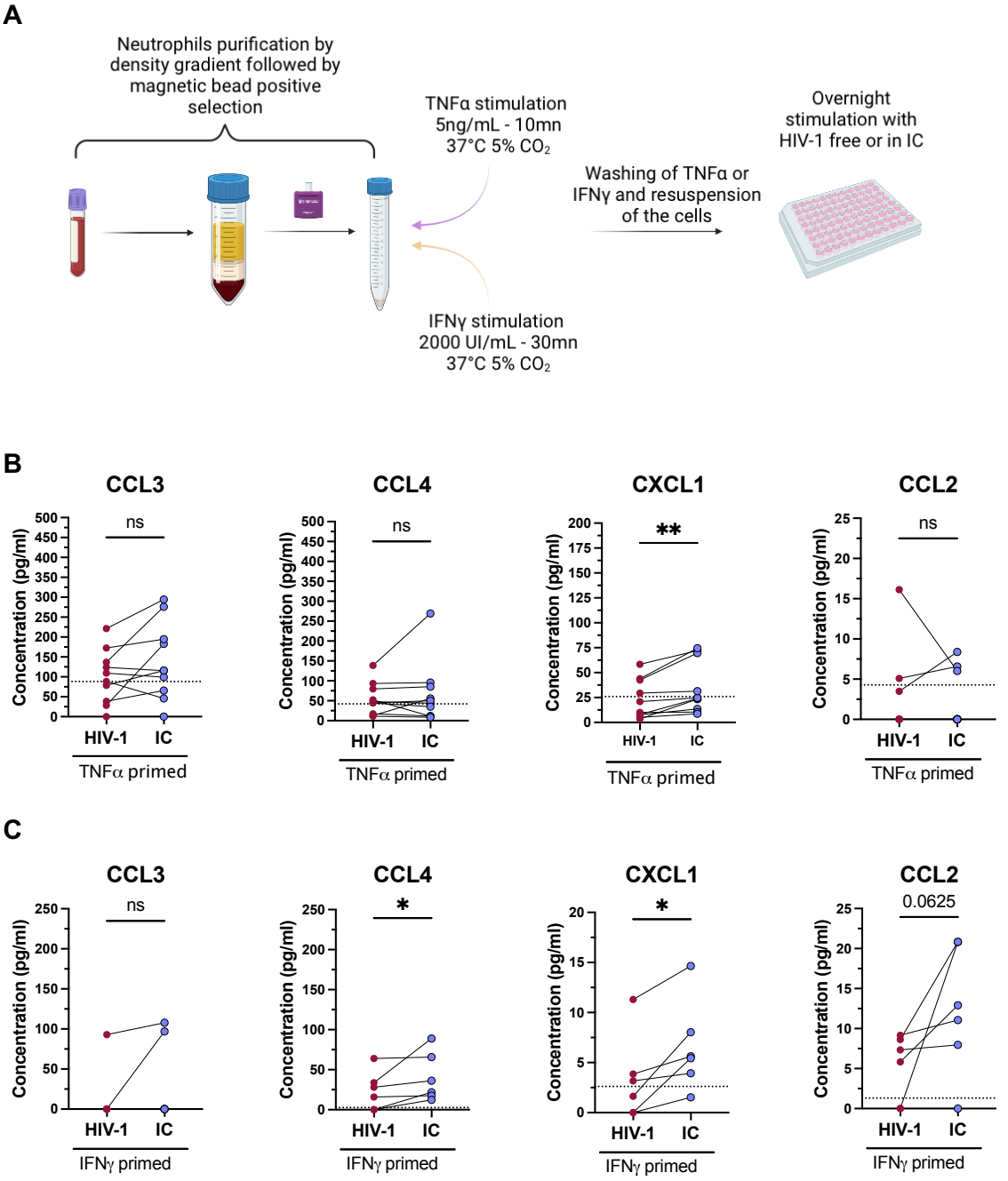
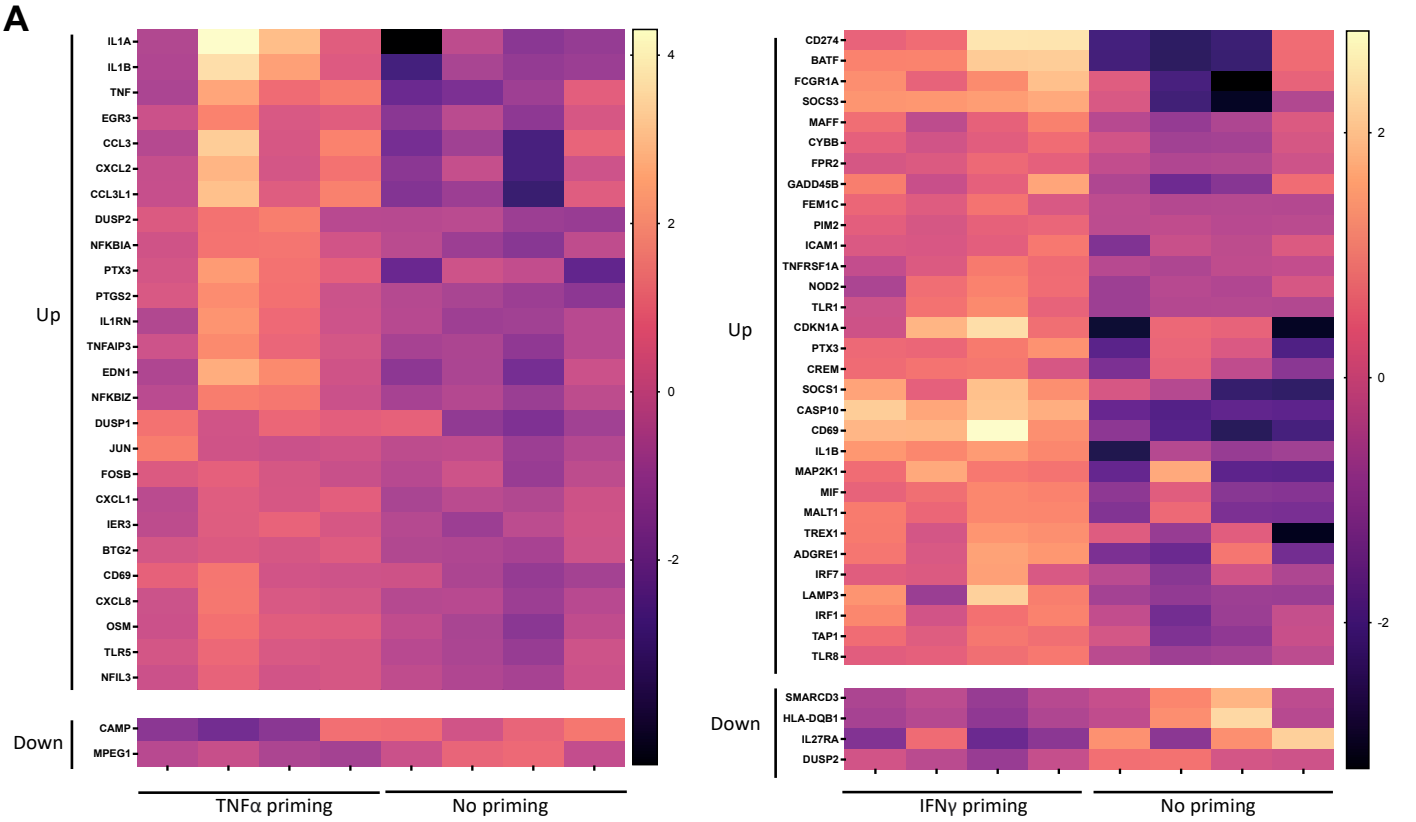
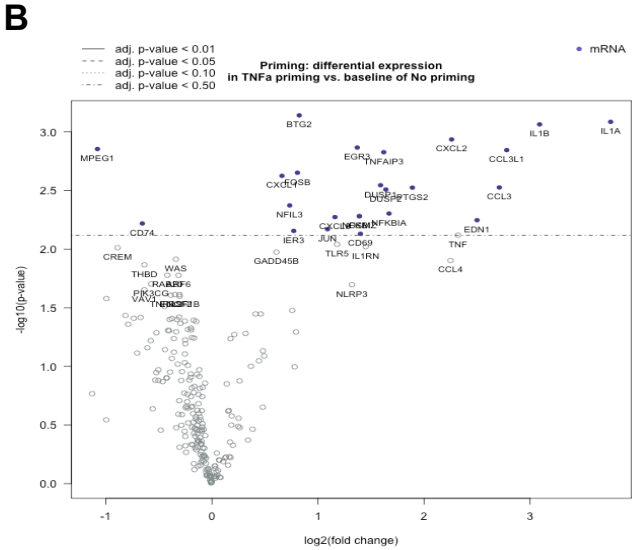
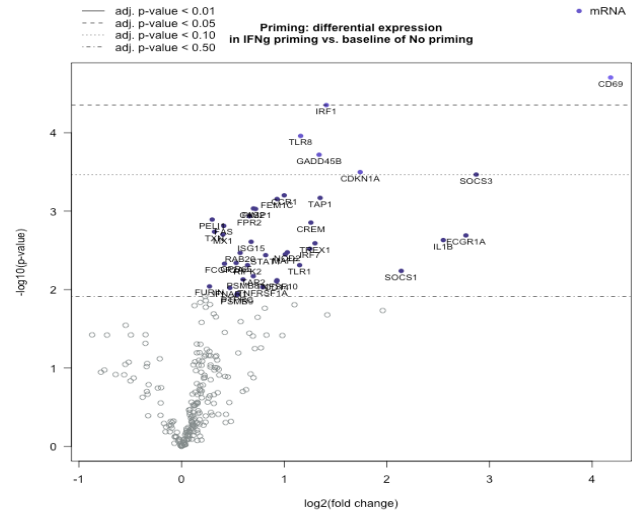
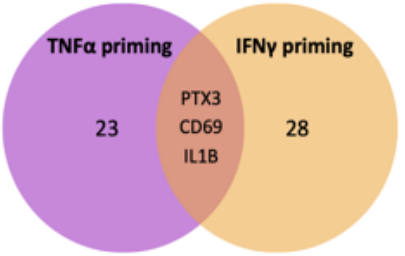

A
Neutrophils purification by density gradient followed by magnetic bead positive selection
TNFα stimulation 5ng/mL - 10mn 37°C 5% CO2
IFNγ stimulation 2000 UI/mL - 30mn 37°C 5% CO2
Washing of TNFα or IFNγ and resuspension of the cells
Overnight stimulation with HIV-1 free or in IC
B
CCL3
CCL4
CXCL1
CCL2
Concentration (pg/ml)
ns
**
HIV-1
IC
TNFα primed
C
*
0.0625
IFNγ primed

Figure 5


A
Up
IL1A
IL1B
TNF
EGR3
CCL3
CXCL2
CCL3L1
DUSP2
NFKBIA
PTX3
PTGS2
IL1RN
TNFAIP3
EDN1
NFKBIZ
DUSP1
JUN
FOSB
CXCL1
IER3
BTG2
CD69
CXCL8
OSM
TLR5
NFIL3
Down
CAMP
MPEG1
TNFα priming
No priming
CD274
BATF
FCGR1A
SOCS3
MAFF
CYBB
FPR2
GADD45B
FEM1C
PIM2
ICAM1
TNFRSF1A
NOD2
TLR1
CDKN1A
PTX3
CREM
SOCS1
CASP10
CD69
IL1B
MAP2K1
MIF
MALT1
TREX1
ADGRE1
IRF7
LAMP3
IRF1
TAP1
TLR8
Up
Down
SMARCD3
HLA-DQB1
IL27RA
DUSP2
IFNγ priming
No priming


B
adj. p-value < 0.01
adj. p-value < 0.05
adj. p-value < 0.10
adj. p-value < 0.50
mRNA
Priming: differential expression
in TNFa priming vs. baseline of No priming
-log10(p-value)
log2(fold change)


adj. p-value < 0.01
adj. p-value < 0.05
adj. p-value < 0.10
adj. p-value < 0.50
mRNA
Priming: differential expression
in IFNg priming vs. baseline of No priming
-log10(p-value)
log2(fold change)


C
TNFα priming
IFNγ priming
23
PTX3
CD69
IL1B
28

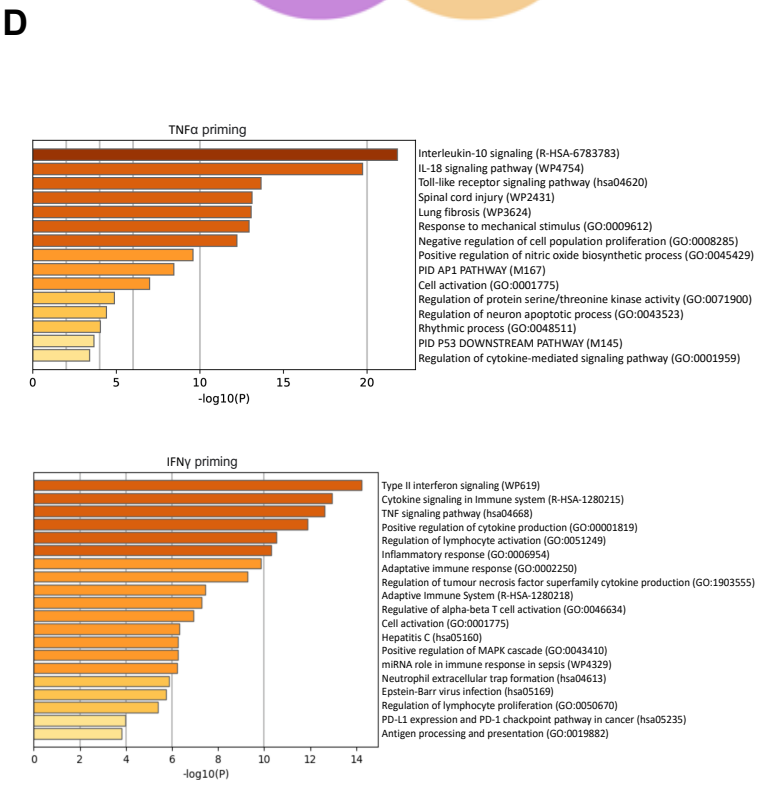

D
TNFα priming
Interleukin-10 signaling (R-HSA-6783783)
IL-18 signaling pathway (WP4754)
Toll-like receptor signaling pathway (hsa04620)
Spinal cord injury (WP2431)
Lung fibrosis (WP3624)
Response to mechanical stimulus (GO:0009612)
Negative regulation of cell population proliferation (GO:0008285)
Positive regulation of nitric oxide biosynthetic process (GO:0045429)
PID AP1 PATHWAY (M167)
Cell activation (GO:0001775)
Regulation of protein serine/threonine kinase activity (GO:0071900)
Regulation of neuron apoptotic process (GO:0043523)
Rhythmic process (GO:0048511)
PID P53 DOWNSTREAM PATHWAY (M145)
Regulation of cytokine-mediated signaling pathway (GO:0001959)
-log10(P)
IFNγ priming
Type II interferon signaling (WP619)
Cytokine signaling in Immune system (R-HSA-1280215)
TNF signaling pathway (hsa04668)
Positive regulation of cytokine production (GO:00001819)
Regulation of lymphocyte activation (GO:0051249)
Inflammatory response (GO:0006954)
Adaptative immune response (GO:0002250)
Regulation of tumour necrosis factor superfamily cytokine production (GO:1903555)
Adaptive Immune System (R-HSA-1280218)
Regulative of alpha-beta T cell activation (GO:0046634)
Cell activation (GO:0001775)
Hepatitis C (hsa05160)
Positive regulation of MAPK cascade (GO:0043410)
miRNA role in immune response in sepsis (WP4329)
Neutrophil extracellular trap formation (hsa04613)
Epstein-Barr virus infection (hsa05169)
Regulation of lymphocyte proliferation (GO:0050670)
PD-L1 expression and PD-1 chackpoint pathway in cancer (hsa05235)
Antigen processing and presentation (GO:0019882)
-log10(P)

Figure 6

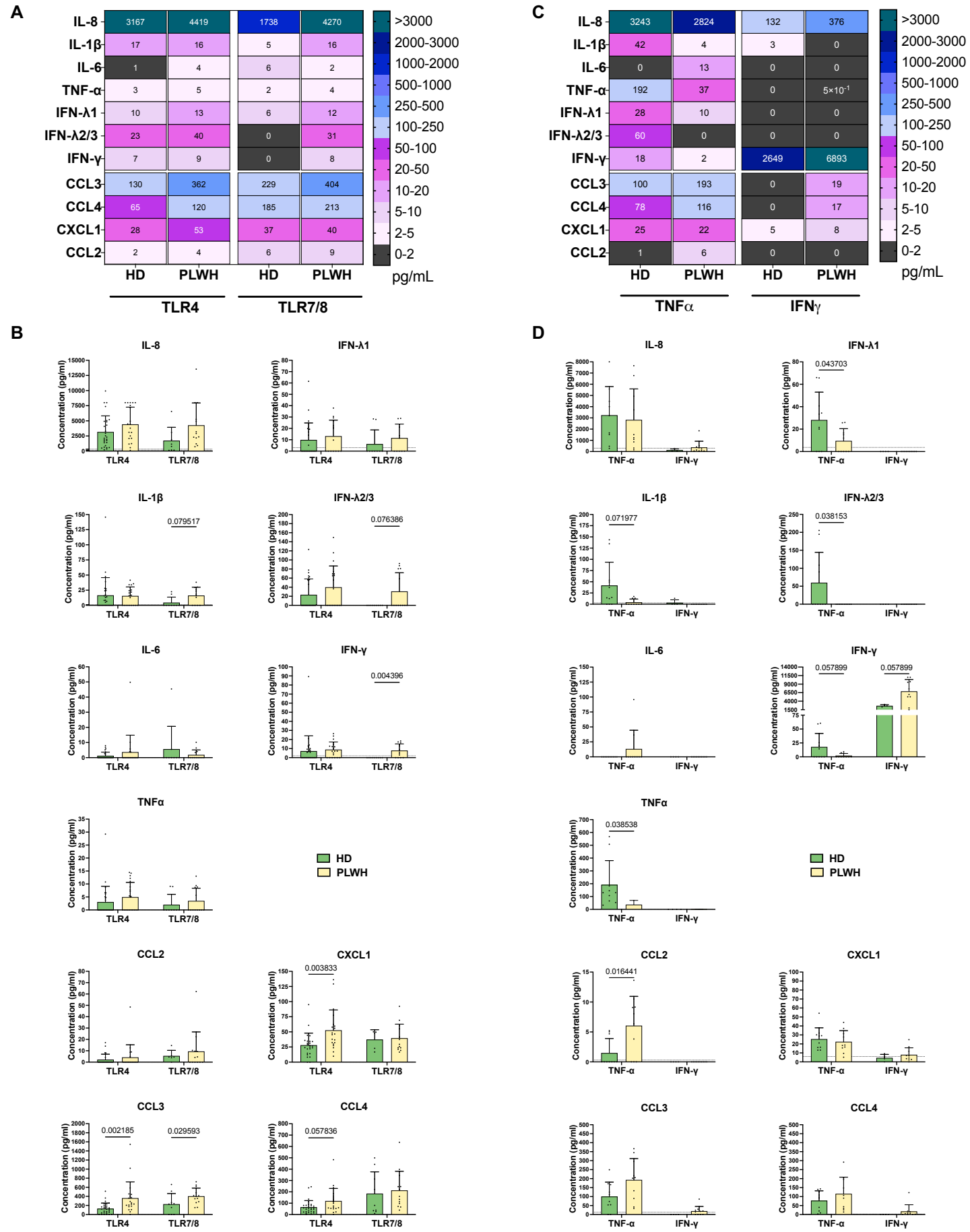
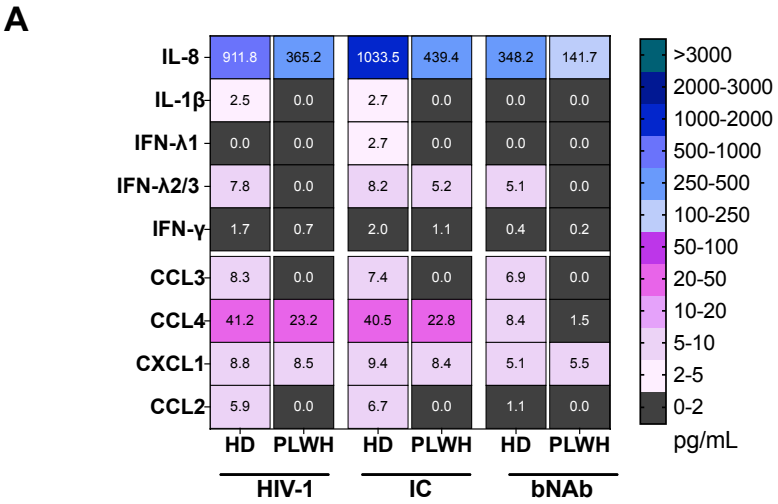

A
IL-8 3167 4419 1738 4270
IL-1β 17 16 5 16
IL-6 1 4 6 2
TNF-α 3 5 2 4
IFN-λ1 10 13 6 12
IFN-λ2/3 23 40 0 31
IFN-γ 7 9 0 8
CCL3 130 362 229 404
CCL4 65 120 185 213
CXCL1 28 53 37 40
CCL2 2 4 6 9
HD PLWH HD PLWH
TLR4
TLR7/8
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
C
IL-8 3243 2824 132 376
IL-1β 42 4 3 0
IL-6 0 13 0 0
TNF-α 192 37 0 5×10-1
IFN-λ1 28 10 0 0
IFN-λ2/3 60 0 0 0
IFN-γ 18 2 2649 6893
CCL3 100 193 0 19
CCL4 78 116 0 17
CXCL1 25 22 5 8
CCL2 1 6 0 0
HD PLWH HD PLWH
TNFα
IFNγ
B
IL-8
IFN-λ1
IL-1β 0.079517
IFN-λ2/3 0.076386
IL-6
IFN-γ 0.004396
TNFα
CCL2
CXCL1 0.003833
CCL3 0.002185 0.029593
CCL4 0.057836
Concentration (pg/ml)
TLR4
TLR7/8
HD
PLWH
D
IL-8
IFN-λ1 0.043703
IL-1β 0.071977
IFN-λ2/3 0.038153
IL-6
IFN-γ 0.057899 0.057899
TNFα 0.038538
CCL2 0.016441
CXCL1
CCL3
CCL4
TNF-α
IFN-γ

Figure 7


A
IL-8
IL-1β
IFN-λ1
IFN-λ2/3
IFN-γ
CCL3
CCL4
CXCL1
CCL2
911.8 365.2 1033.5 439.4 348.2 141.7
2.5 0.0 2.7 0.0 0.0 0.0
0.0 0.0 2.7 0.0 0.0 0.0
7.8 0.0 8.2 5.2 5.1 0.0
1.7 0.7 2.0 1.1 0.4 0.2
8.3 0.0 7.4 0.0 6.9 0.0
41.2 23.2 40.5 22.8 8.4 1.5
8.8 8.5 9.4 8.4 5.1 5.5
5.9 0.0 6.7 0.0 1.1 0.0
HD PLWH HD PLWH HD PLWH
HIV-1
IC
bNAb
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL

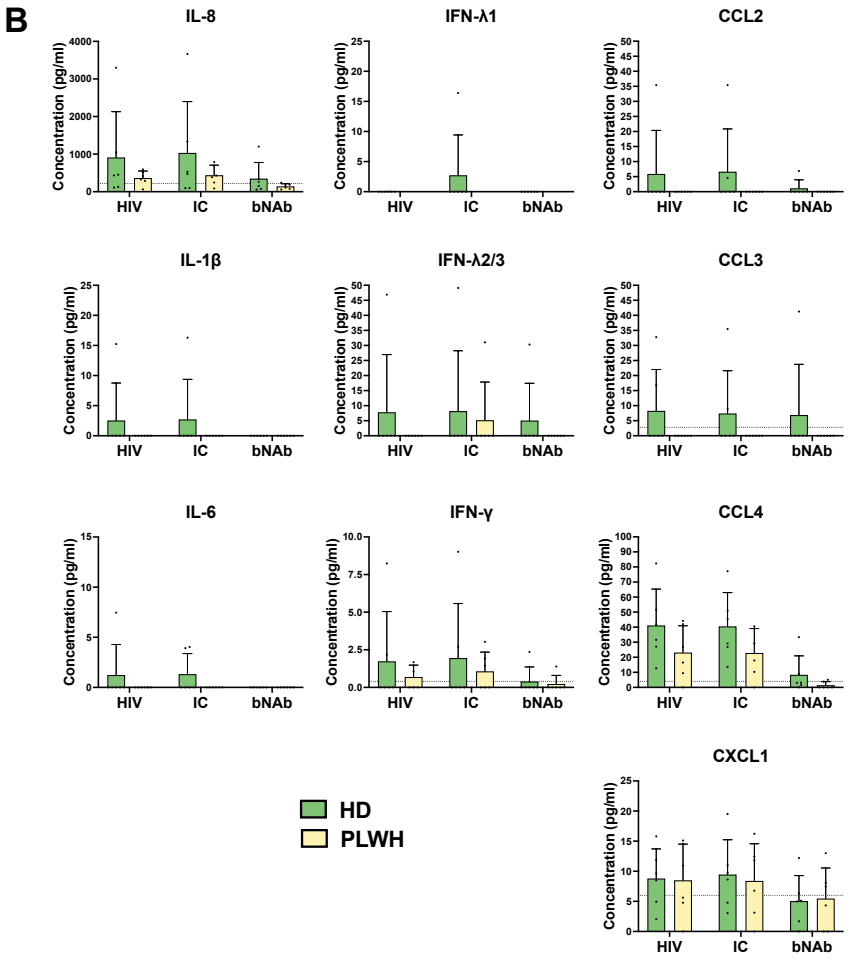

B
IL-8
IFN-λ1
CCL2
IL-1β
IFN-λ2/3
CCL3
IL-6
IFN-γ
CCL4
CXCL1
Concentration (pg/ml)
HIV
IC
bNAb
HD
PLWH

Figure 8

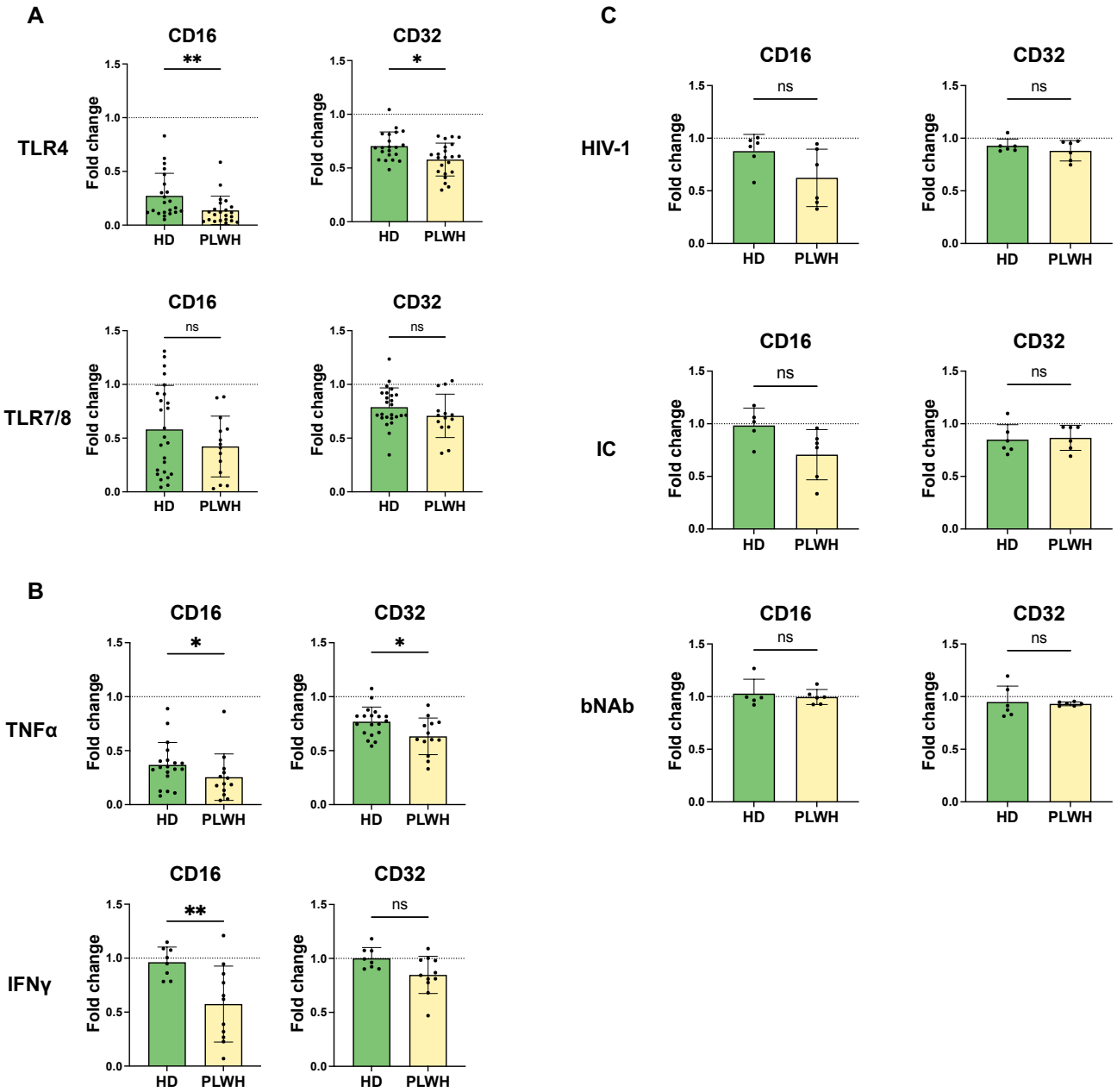
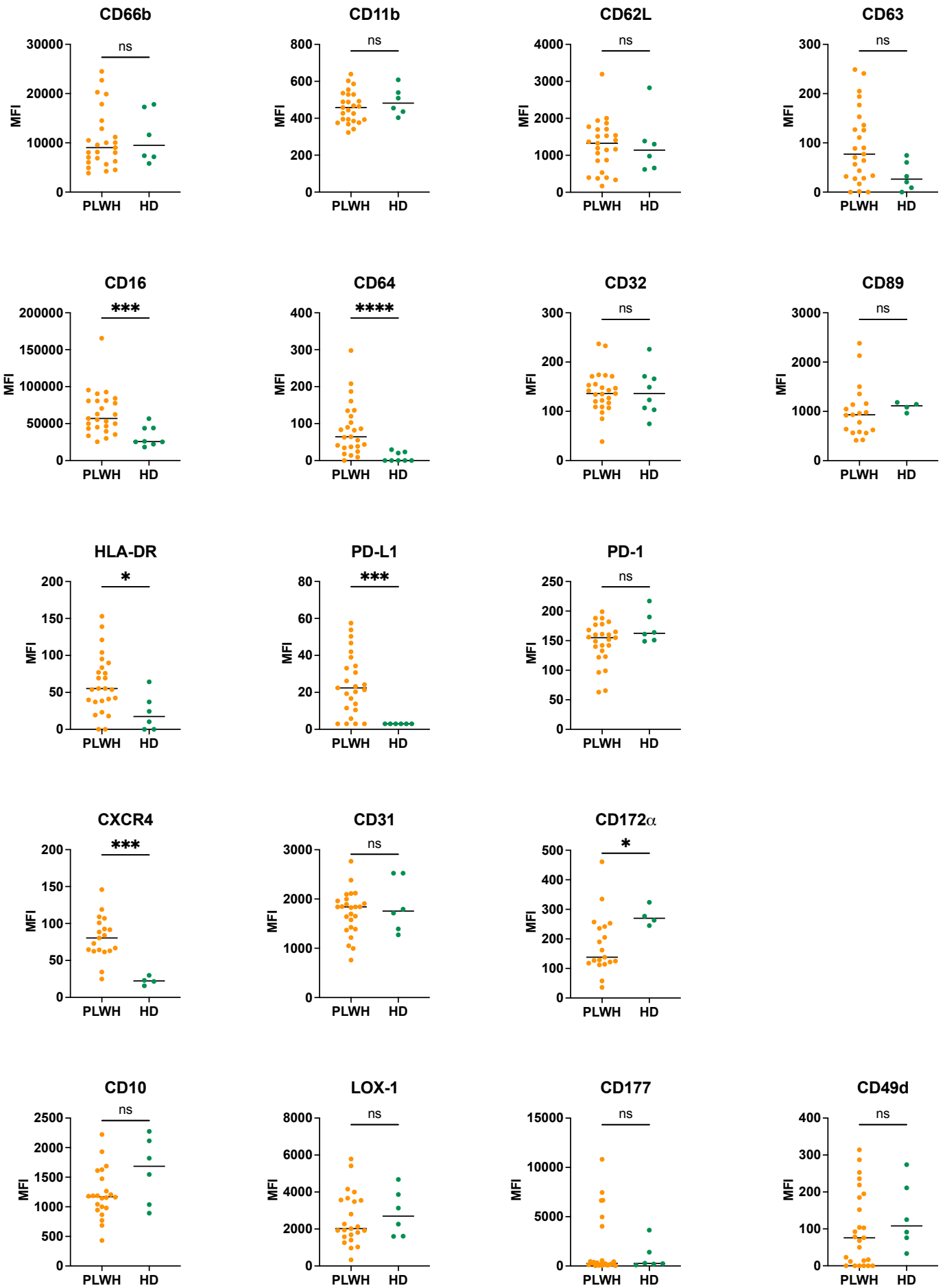

A
CD16
CD32
TLR4
TLR7/8
B
TNFα
IFNγ
C
HIV-1
IC
bNAb
Fold change
HD
PLWH
ns
*
**

Figure 9


CD66b
ns
MFI
30000
20000
10000
0
PLWH
HD
CD11b
ns
MFI
800
600
400
200
0
PLWH
HD
CD62L
ns
MFI
4000
3000
2000
1000
0
PLWH
HD
CD63
ns
MFI
300
200
100
0
PLWH
HD
CD16
***
MFI
200000
150000
100000
50000
0
PLWH
HD
CD64
****
MFI
400
300
200
100
0
PLWH
HD
CD32
ns
MFI
300
200
100
0
PLWH
HD
CD89
ns
MFI
3000
2000
1000
0
PLWH
HD
HLA-DR
*
MFI
200
150
100
50
0
PLWH
HD
PD-L1
***
MFI
80
60
40
20
0
PLWH
HD
PD-1
ns
MFI
250
200
150
100
50
0
PLWH
HD
CXCR4
***
MFI
200
150
100
50
0
PLWH
HD
CD31
ns
MFI
3000
2000
1000
0
PLWH
HD
CD172α
*
MFI
500
400
300
200
100
0
PLWH
HD
CD10
ns
MFI
2500
2000
1500
1000
500
0
PLWH
HD
LOX-1
ns
MFI
8000
6000
4000
2000
0
PLWH
HD
CD177
ns
MFI
15000
10000
5000
0
PLWH
HD
CD49d
ns
MFI
400
300
200
100
0
PLWH
HD