Enhanced Fc Receptor expression and distinct immunomodulatory properties of neutrophils during HIV-1 infection and therapy

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Keywords

HIV-1, neutrophils, immunomodulation, immune complexes, broadly neutralizing antibodies.

Abstract

Neutrophils are innate immune cells that display multiple immunomodulatory properties. Using a mouse model of retroviral infection, we previously uncovered their key immunomodulatory function in promoting protective immunity during antiviral antibody therapy, which occurs through Fc-FcγR interactions. Here, we investigated the immunomodulatory properties of neutrophils in the context of HIV-1 infection and therapy. We conducted a thorough examination of the functional activation and modulation of Fcγ receptors (FcγRs) expression on neutrophils isolated from healthy donors (HD) and people living with HIV-1 (PLWH). Upon stimulation with virions, either free or in the form of immune complexes (ICs) formed with broadly neutralizing antibodies (bNAbs), neutrophils from HD secreted cytokines and chemokines in modest quantities. Interestingly, priming HD neutrophils with pro-inflammatory cytokines augmented their chemokine production upon exposure to HIV-1 and ICs, underscoring the pivotal role of the inflammatory milieu in eliciting a functional response from neutrophils to these stimuli. Noteworthy, under inflammatory conditions, IC-activated neutrophils showed higher secretion of CXCL1 and CCL4 than HIV-1-stimulated neutrophils. Highlighting the critical influence of the inflammatory environment on their functional properties, neutrophils from PLWH displayed increased release of cytokines (IFNγ) and chemokines (CXCL1, CCL2, CCL3 and CCL4) when stimulated with danger signals associated with HIV-1 infection. In addition, PLWH's neutrophils exhibited higher expression of two activating FcγRs (FcγRI and FcγRIII) than neutrophils from HD. Our study provides new insights into the immunomodulatory properties of neutrophils during HIV-1 infection and therapy. These findings hold potential for optimizing antiviral antibody therapies by harnessing the immunomodulatory properties of neutrophils.

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.

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

Using neutrophils isolated from healthy donors (HD), we report here that their activation by HIV-1 virions, free or in the form of ICs, induced the secretion of very limited amounts of cytokines and chemokines, both in terms of diversity and quantity. However, priming of HD's neutrophils with TNF-α or IFN-γ enhanced their chemokine production upon activation by either free HIV-1 or ICs. Noteworthy, the inflammatory environment increased more the chemokines secretion in IC-activated neutrophils than in HIV-stimulated neutrophils. Furthermore, our findings reveal that neutrophils isolated from PLWH exhibited heightened release of cytokines and chemokines compared to neutrophils from healthy HD when exposed to danger signals associated with HIV-1 infection. We also report that PLWH's neutrophils display higher expression several immune receptors. Our study provides new insights into the immunomodulatory properties of neutrophils during HIV-1 infection and antibody treatment. It also highlights that the inflammatory environment is a key factor for neutrophils to show a functional response to HIV-1/ICs. These findings could have implications in improving the treatment of HIV-1 infection with bNAbs alone or in combinatorial therapeutic approaches.

120 **Results**

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

123 First, we assessed the effect of TLR agonists and inflammatory cytokines (TNF-α and IFN-γ)
124 on the phenotypic and functional activation of human neutrophils isolated from HD. The
125 rationale behind this was to determine whether and how the microenvironment resulting from
126 infection might be determinant for modulating the immune response, both in terms of
127 chemokine/cytokine secretion profile and of FcγR expression pattern, as observed in our mouse
128 studies (20). In this regard, virus-driven inflammation (including TNF-α and IFN-γ signature)
129 has been associated with the development of bNAbs in spontaneous controllers of HIV (22).
130 As for TLR agonists, we mainly focused on TLR4 and TLR7/8 agonists (lipopolysaccharide
131 (LPS) and Resiquimod (R848), respectively), due to their role in immune activation associated
132 with viral sensing (TLR7/8) and to the bacterial translocation observed in PLWH (i.e. LPS
133 binding to TLR4). To assess the phenotypic and functional activation of neutrophils we used
134 the following read-out: (i) the upregulation of the cell surface markers CD11b and CD66b, (ii)
135 the modulation of the expression of the main FcγRs constitutively expressed on neutrophils:
136 FcγRIIa (CD32a) and FcγRIIIb (CD16b), and (iii) the cytokine and chemokine secretion profile
137 (**Figure 1**). Flow cytometry analysis was performed on the viable fraction of $CD15^{+}/CD11b^{+}$
138 cells (**Figure 1A**). While life span of neutrophils has been reported to be short (only few hours),
139 specific culture conditions allow extending it. Upon TLR4 and TLR7/8 agonist stimulation
140 during 16-20h, neutrophil survival ranged between 70-80% in average (**Figure 1B**). TLR4 and
141 TLR7/8 agonists led to significant upregulation of CD11b and CD66b as compared to
142 unstimulated neutrophils, with no significant differences between TLR agonists (**Figure 1C)**.
143 As for FcγR expression, neutrophil stimulation by either TLR agonist induced a strong shedding
144 of the CD16b receptor, in particular TLR4, as well as a significant decrease in CD32a
145 expression as compared to unstimulated neutrophils (**Figure 1C)**. The assessment of neutrophil
146 secretome revealed that both stimuli triggered the secretion of IL-8 and several chemokines (i.e.
147 CCL3, CCL4, CXCL1, CXCL10 and CCL2) albeit to a different extent (**Figure 1D)**. Due to
148 high donor-dependent variability of chemokine secretion, no statistical differences were
149 observed between TLR4 and TLR7/8 activation (**Figure 1E**). However, TLR4 stimulation led
150 to the secretion of IFN-λ2/3 in 3 out of 7 samples, while TLR7/8 stimulation did not.
151

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

185 upregulation of CD11b and CD66b as compared to unstimulated neutrophils (**Supplementary**
186 **Figure 2**) or neutrophils cultured in the presence of free bNAb, notably for CD11b (**Figure**
187 **3B**). No significant differences in the expression of these two cell surface receptors were
188 observed between HIV-1- and IC-activated neutrophils (**Figure 3B**). Despite being similarly
189 activated by both stimuli (as observed by the upregulation of activation markers), we observed
190 a mild but significant reduced expression of CD16b in HIV-1-stimulated neutrophils but not in
191 IC-stimulated cells (**Supplementary Figure 2)**. However, a significant decrease in CD32a
192 expression was observed in both HIV-1- and IC-activated neutrophils (**Supplementary Figure**
193 **2)**, that was more marked in IC-activated cells (**Figure 3B**). Together, these results show a very
194 mild effect of the activation of neutrophils by HIV-1/IC on FcγR expression. Worthy of note,
195 the decrease in FcγRs expression was much less pronounced than that observed upon activation
196 with the TLR7/8 agonist mimicking a viral stimulus (**Figure 1**). This is important to highlight
197 as TLR7/8 is commonly used as surrogate of virus activation, instead of *bona fide* virions.
198
199 ***Neutrophils activated by HIV-1 and ICs secrete low levels of cytokines and chemokines***
200 In parallel, we assessed the activation of neutrophils isolated from HD by HIV-1 virions, free
201 or in the form of ICs. Free 10-1074 bNAb was used as control and showed no effect on either
202 the expression of cell surface markers or the secretome of neutrophils. The study of the
203 secretome of neutrophils activated by these stimuli revealed a weak secretion of several
204 cytokines and chemokines (**Figure 3C**). Among them, IL-8, CXCL1 and CCL4 were
205 significantly increased upon HIV-1 and IC-activation as compared to unstimulated neutrophils
206 (**Figure 3D**). In addition, other cytokines and chemokines were detected in about half of
207 samples tested (i.e. IL-1ß, IFN-λ1, IFN-λ2/3, CCL2 and CCL3). However, no significant
208 differences in the secretome were observed between HIV-1- and IC-activated neutrophils
209 (**Figure 3D**). Collectively, these results show a low activation of neutrophils by these stimuli.
210 This contrasted to the strong secretion of multiple cytokines and chemokines by monocytes
211 activated by HIV-1- and ICs (**Supplementary Figure 3**). This showed that these stimuli are
212 able to induce the secretion of these soluble immune mediators by different myeloid cells but
213 at different extents depending on the cell type.
214
215 ***Inflammatory conditions potentiate the activation of HD's neutrophils by HIV-1 and ICs***
216 As the inflammatory microenvironment resulting from the viral infection and bNAbs therapy
217 might affect the antiviral immune response, we next examined the phenotypic and functional
218 activation of HD's neutrophils by HIV-1 free or in the form of ICs in an inflammatory

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

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

285 maintained their capacity to secrete cytokines and chemokines in response to danger signals
286 associated with HIV-1 infection, but also showed increased release of specific soluble
287 mediators (i.e. CXCL1, CCL2, CCL3 and IFN-γ) compared to neutrophils isolated from healthy
288 donors (HD), particularly under certain stimulatory conditions and/or in specific donor samples.
289

290 ***Neutrophils from PLWH show stronger CD16 shedding in response to danger signals than***
291 ***neutrophils from HD***

292 We analyzed in parallel the expression of FcγRs in neutrophils from PLWH upon activation by
293 different danger signals associated with HIV-1 infection, as this is an important issue in the
294 context of bNAb-based therapy. Upon TLR4 activation, CD16b and CD32a cell surface levels
295 on neutrophils from PLWH were significantly lower than those observed in HD (**Figure 8A**).
296 In contrast, TLR7/8 activation resulted in no significant difference in the expression of either
297 FcγR as compared to HD (**Figure 8A**). Similar to TLR4 activation, TNF-α stimulation led to a
298 significant lower expression of CD16b and CD32a on neutrophils from PLWH, as compared to
299 HD (**Figure 8B**). In contrast, IFN-γ activation only led to significantly lower levels of CD16b
300 (**Figure 8B**). No difference in expression was observed for CD16b or CD32a upon activation
301 by free HIV-1 virions (**Figure 8C**) as with TLR7/8 activation. These results highlight that
302 CD16b shedding can be affected by HIV-1 infection as neutrophils from PLWH showed a
303 stronger CD16 shedding as compared to HD upon activation by TLR4 and inflammatory
304 cytokines. However, such enhanced CD16 shedding was not observed upon activation by viral
305 stimuli (i.e. TLR7/8, HIV-1, free or in the form of ICs).
306

307 ***Neutrophils from PLWH display enhanced expression of FcγRs, MHC-II molecules,***
308 ***CXCR4, and PD-L1***

309 We then performed a phenotypic study of neutrophils isolated from PLWH and HD to further
310 assess to what extent HIV-1 infection affected neutrophil properties. To this end, we measured
311 the expression of 18 cell surface markers in the absence of stimulation. The panel was chosen
312 to follow the expression of markers of interest in HIV-1 infection (CXCR4), neutrophil
313 activation (CD11b, CD66b, CD63, CD62L), neutrophil maturity (CD10), neutrophil
314 immunosuppressive properties (PD-L1) and Fc receptors (CD16, CD32, CD64 and CD89),
315 among others. Within the 18 markers, we found a significantly higher expression on PLWH's
316 neutrophils of two activating FcγRs (CD16b and CD64) and 3 other cell surface molecules,
317 namely CXCR4, PD-L1 and HLA-DR. We also detected a decreased expression of the adhesion

318 molecule CD172α in neutrophils from PLWH (**Figure 9**). Thus, despite similar secretion
319 profiles when activated by HIV-1 virions and/or ICs, neutrophils from PLWH show phenotypic
320 differences as compared to HD. These differences might reflect different functional properties.

321 **Discussion**

322 We have previously shown in a mouse model of retroviral infection that neutrophils have a key
323 immunomodulatory role in the induction of a protective immunity (19,20). Here, we provide
324 new insights into the immunomodulatory properties of neutrophils in the context of HIV-1
325 infection and antiviral mAb-therapy. Our study dissects the secretome and expression of
326 neutrophil immune receptors in a stimulus-specific manner both in HD and PLWH.
327 Noteworthy, most studies on immunomodulatory functions of neutrophils are based on bacterial
328 activation (i.e. TLR4 activation), while the effect of virus-dependent activation is understudied.
329 The latter has been mostly investigated using TLRs as surrogates, and there are few data on
330 whether and how HIV-1 and/or HIV-ICs affect their immunomodulatory properties. Our work
331 highlights that neutrophil from ART-treated PLWH retain the ability to secrete
332 cytokines/chemokines in response to danger signals and even display a stronger secretion of
333 some of these soluble mediators compared to neutrophils isolated from HD. In addition, notable
334 differences in phenotypic features distinguish them from neutrophils of HD origin. Our research
335 also shows that inflammatory environment shapes neutrophils immunomodulatory properties
336 and allows them to better respond to HIV-1 viral determinants, 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 as well as CCL3/CXCL1 and IFN-γ upon TLR activation.

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

421 effects that can be counteracted by blocking the PD-1/PD-L1 axis (46,50). Thus, the
422 upregulation of PD-L1 we found in PLWH suggests a potential immunosuppressive role of
423 PLWH neutrophils.
424
425 Another important observation of our study is the effect of the inflammatory environment on
426 the modulation of neutrophil properties. Thus, the stimulation with inflammatory cytokines
427 potentiated the release of several chemokines by HIV-1 and IC-activated neutrophils.
428 Interestingly, TNF-α and IFN-γ priming led to a significant increased secretion of CXCL1 and
429 CCL4 by IC-activated neutrophils as compared to virus-activated cells. This is in agreement
430 with our previous results using mouse neutrophils activated with IC-made with a murine
431 retrovirus (20). Our work also allowed us to identify a different effect of TNF-α and IFN-γ on
432 the enhancement of the functional properties of IC-activated human neutrophils. Notably, IFN-
433 γ but not TNF-α, led to enhanced secretion of CCL4 and CCL2 although to a lesser extent for
434 the latter. It is worth mentioning that the enhanced secretion of CCL2 by HIV-1 and HIV-IC-
435 activated neutrophils by IFN-γ priming is in agreement with the IFN-γ-mediated upregulation
436 of CCL2 production previously described in a different experimental setting (51). This finding
437 suggests that in a Th1 microenvironment, neutrophil might be involved in the recruitment of
438 monocytes through the secretion of CCL2. Worthy of note, IFN-γ priming led to the
439 upregulation of FcγRI and TLR8 (potentially involved in IC-mediated signaling and HIV-1
440 sensing, respectively). Interestingly, this observation is in agreement with the recent study by
441 Kumola *et al*, showing an upregulation of CD64 and TLR8 upon IFN-γ stimulation as well as
442 an enhanced expression of PD-L1 (52). This suggests that the enhanced expression of CD64
443 and PD-L1 we observed on the neutrophils from PLWH could be due to IFN-γ-priming *in vivo*.
444 However, our results showing that neutrophils from PLWH respond to HIV-1 or ICs as weakly
445 as those from HD, also raise the possibility that they may not be adequately primed *in vivo*, or
446 not to an efficient degree. Thus, the question of whether PLWH's neutrophils are primed or not
447 remains open and deserves further investigation.
448
449 In conclusion, our work dissects the phenotypic and functional properties of PLWH neutrophils.
450 It highlights that they maintain their cytokine and chemokine secretion capacity, while
451 displaying some immunosuppressive features. It also provides new insights on the modulation
452 of FcγR expression in the context of danger signals associated with HIV-1 infection which need
453 to be taken into account in the context of antibody therapy. These findings might be exploited

454 to improve HIV-1 antiviral therapies in PLWH. This could be achieved by tailoring therapeutic
455 interventions aiming at harnessing the immunomodulatory properties of these cells. Strategies
456 could involve leveraging the immunomodulatory properties of neutrophils (through the use of
457 cell-type specific, immunostimulatory compounds) and/or counteracting their
458 immunosuppressive functions (through the design of combinatorial approaches involving the
459 blocking of the PD-1/PD-L1 axis) (53,54).
460

461 **Material & Methods**

462

463 *Ethics*

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

466

467 *Human blood samples*

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

479

480 *Primary cells purification*

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

488

489 Monocytes and neutrophils were then purified from PBMC or granulocytes, respectively, using
490 magnetic-based CD14- or CD15-positive selection kits (STEMCELL TECHNOLOGIES,
491 $CD15^+$ positive selection REF#18651, $CD14^+$ positive selection REF#117858) according to the
492 manufacturer's protocol. Purity assessed by flow cytometry was > 98% for both cell type.

493

494

495 *Cell culture*

496 Purified neutrophils were plated at a concentration of 2 million cells/mL in 96 U-bottom wells
497 plate and cultured in RPMI 1640 supplemented with GlutaMAX^{TM} medium (Gibco, cat
498 #61870-010), 10% fetal bovine serum (FBS, Eurobio, REF#CVFSVF00-01) and 1%
499 penicillin/streptomycin (Gibco, cat #15140-122) with 10 µg/mL of G-CSF (R&D, cat #214-
500 CS-025).

501 Purified monocytes were plated at a concentration of 1 million cells/mL in 96 U-bottom wells
502 and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1%
503 penicillin/streptomycin with 10 µg/mL of GM-CSF (Peprotech, cat #300-03).
504 Cells were then cultured for 16-20h with or without stimulation.

505

506 *In vitro cell stimulation*

507 For TLR or cytokine stimulations, cells were stimulated overnight with TLR4 agonist (LPS ;
508 Sigma-Aldrich, cat #L4524), TLR7/8 agonist (R848 ; Invivogen, cat #tlrl-r848) at 0,5 µg/mL
509 each, with IFN-γ (R&D, cat #285-IF-100) at 1000 IU/mL or with TNF-α (Peprotech, cat #AF-
510 300-01A) at 1ng/mL. Otherwise, cells were stimulated with either free HIV-1 virions (Ad8
511 strain, MOI of 3.10^4), immune complexes made with HIV-1 Ad8 and the 10-1074 bNAb or the
512 bNAb alone.

513

514 *Neutrophils priming*

515 For primed conditions, neutrophils were resuspended in culture medium after purification and
516 stimulated at 37°C 5%CO_2 with IFN-γ at 2000 IU/mL for 30 minutes or with TNF-α at 5ng/mL
517 for 10 minutes. Cells were washed then cultured overnight with either free virus, immune
518 complex or bNAb alone.

519

520 *Virus production, purification and titration:*

521 2.5 million human embryonic kidney cells (293THEK cell) were seeded in 10 ml of growth
522 media 1 day before transfection. At 50-70% confluence, cells were transfected with calcium
523 phosphate precipitate method, with 10 µg total quantity of HIV-1 molecular clone expressing
524 plasmids, as in (55). At 12 hours post transfection, cells were washed and fresh media
525 supplemented with 20mM Hepes was added on the cells. Cell culture supernatant containing
526 viral particles was collected 36 hours post-transfection, pooled and filtrated through 0.45 µm
527 and then purified by ultracentrifugation on a cushion of 25% sucrose in TNE buffer (10 mM
528 Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA) at 100000xg, for 1 hour 30 minutes at 4°C,

529 in a SW32Ti Beckman Coulter rotor. Dry pellet was resuspended with RPMI without serum, at
530 4°C overnight. Viral titer was quantified by alphaLISA immunoassay (Perkin Elmer) using
531 anti-HIV-1 p24. Recombinant HIV-1 p24 protein is used for titer standard range. Viral particles
532 were quantified using Videodrop system (Myriade France).
533
534 *Recombinant* HIV-1 *bNAb production*
535 Recombinant human HIV-1 IgG1 antibodies, anti-CD4bs 3BNC117 (56) and anti-V3-glycan
536 10-1074 (57), were produced by co-transfection of Freestyle 293-F cells (Thermo Fisher
537 Scientific) using the polyethyleneimine (PEI)-based precipitation method as previously
538 described (58), and purified by affinity chromatography using protein G Sepharose 4 fast flow
539 beads according to the manufacturer's instructions (Cytiva). Purified IgG antibodies were
540 dialyzed against PBS using Slide-A-Lyzer Cassettes (10K MWCO; Thermo Fisher Scientific),
541 and quantified using NanoDrop™ ONE instrument (Thermo Fisher Scientific). Both bNAbs
542 are of IgG1 isotype and were used at a concentration of 1µg/mL to form ICs.
543
544 *Immune complexes formation*
545 Viruses (3.10^4 viral particles) and bNAb (1µg/mL) were diluted in culture medium and mixed
546 together on a 1,5mL Eppendorf, then mixed on a rotative agitator for 20 minutes. ICs were
547 gently plated with cells in 96 U-bottom wells plate.
548
549 *Flow cytometry immunophenotyping of neutrophils*
550 Monitoring of phenotypic activation was done by flow cytometry using FACSCantoII, BD
551 LSRFortessa (BD Bioscience) or Novocyte (Agilent Technologies) flow cytometers. Cells were
552 stained at room temperature with a viability dye (Zombie NIR™, Biolegend #423105 or
553 ZombieRed™, Biolegend #423109) for 15 minutes then washed and stained for 20 minutes at
554 room temperature with fluorochrome-conjugated antibodies. Antibodies for flow cytometry
555 were PerCPCy5-5-CD15 (HI98, Biolegend #301922), AF488-CD11b (ICRF44, Biolegend
556 #301317), AF647-CD66b (G10F5, Biolegend #305110), PC7-CD16a/b (3G8, Biolegend
557 #302016), PE-CD32a/b (FUN-2, Biolegend #303206), PE-CD64 (10.1, Biolegend #305008),
558 FITC-CD14 (MoP9, STEMCELL Technologies #60124FI).
559 For immunophenotyping of HD's and PLWH's neutrophils the following antibodies were used:
560 PerCPCy5-5-CD15 (HI98, Biolegend #301922), PE-LOX-1 (15C4, Biolegend #358604),
561 BV510-CD11b (ICRF44, Biolegend, #301334), AF647-CD66b (G10F5, Biolegend, #305110),
562 BV605-CD62L (DREG-52, Biolegend, #304834), BV650-HLA-DR (L243, Biolegend,

563 #307650), BV711-CD63 (H5C6, Biolegend, #353042), PC7-CD16a/b (3G8, Biolegend,
564 #302016), PE-CF594-CD64 (10.1, Biolegend, #565389), APC-Fire750-CD32a/b (FUN-2,
565 Biolegend, #303220), PE-PD-1 (3.13, Miltenyi, #130-177-384), APC-CD177 (MEM-166,
566 Biolegend, #315808), BV605-CD49d (9F10, Biolegend, #304324), BV711-CD31 (WM59,
567 Biolegend, #303136), PC7-PD-L1 (MIH1, BD, #558017), PE-CF594-CD10 (HI10a,
568 Biolegend, #312227), PE-CD89 (Biolegend, #555686), APC- CD172*α* (15-414, eBiosciences
569 #17-1729-42), PC7-CXCR4 (12G5, Biolegend, #306514).
570 Forward scatter area and forward scatter height as well as side scatter area and height were used
571 to remove doublets from flow cytometry analyses. Gating is done on live cells as assessed by
572 viability dye and on CD15-positive cells. Data were analyzed using FlowJo software version
573 10.5.3 (TreeStar).
574
575 *Cytokines and chemokines secretion quantification*
576 Secretion of soluble cytokines and chemokines were assayed from cell-free culture supernatants
577 of *in vitro* stimulated neutrophils and monocytes using bead-based immunoassays (LegendPlex,
578 Biolegend). Two assays were used allowing the quantification of IL-1β, Il-6, TNF-α, CXCL10,
579 IFN-λ1, IL-8, IL-12p70, IFN-α2, IFN-λ2/3, GM-CSF, IFN-β, IL-10, IFN-γ (LegendPlex
580 Human anti-virus response panel, Biolegend #740390) and IL-8, CXCL10, CCL11, CCL17,
581 CCL2, CCL5, CCL3, CXCL9, CXCL5, CCL20, CXCL1, CXCL11, CCL4 (LegendPlex
582 Human proinflammatory chemokines panel, Biolegend #740985). Immunoassays plates were
583 read on a Novocyte (Agilent Technologies) flow cytometer and data were analyzed with the
584 appropriate Biolegend LEGENDplex™ software (https://legendplex.qognit.com).
585
586 *Transcriptomic studies*
587 RNA was extracted from neutrophils obtained from 4 healthy blood donors. Total RNA was
588 then purified from 500,000 to 1 million cells using the miRNeasy Micro kit (Qiagen GmbH)
589 following the manufacturer's instructions. The quantification of all RNA samples was
590 performed using the Nanodrop One (Thermo Fisher Scientific), and RNA integrity was
591 evaluated using the Agilent 2100 Bioanalyzer system. RNA expression profiling was performed
592 using Myeloid Innate Immunity Panel 2.0 (770 genes in 19 different pathways). Gene
593 expression analysis was conducted with the NanoString technology. Briefly, 5 µL/sample
594 containing of 50 ng of total RNA was combined with the nCounter® reporter CodeSet (3 µL)
595 and nCounter® capture ProbeSet (2 µL) along with hybridization buffer (5 µL) for an overnight
596 hybridization reaction at 65 °C. The reaction was then cooled to 4 °C, and the samples were

purified, immobilized on a cartridge, and the data were assessed using the nCounter SPRINT. All expression data was reviewed using NanoString® nSolver™ Analysis software 4.0. To identify main biological processes modulated by cytokine priming, genes upregulated and downregulated were analyzed using Metascape (59).

Surface Plasmon resonance (SPR)

Affinity of bNAbs for Fcγ Receptors was assessed by SPR experiments performed on a Biacore T200 (GE Healthcare). SPR experiments were performed on a T200 apparatus at 25°C in PBS containing 0,05 % P20 surfactant (Cytiva). Anti-histidine antibody (R&D Systems) was covalently immobilized on a CM5-S sensor chip flowcell (Fc2) by amine coupling according to the manufacturer's instructions (Cytiva). A control reference surface (flowcell Fc1) was prepared using the same chemical treatment but without anti-His antibody. All kinetic measurements in Fc1 and Fc2 were performed by single-cycle titration at 100µl/min. Each human FcγR (R&D Systems) was captured on immobilized anti-His antibodies at 100-200 RU level. Five increasing concentrations (3,6, 11, 33, 100, 300 nM) of antibody were injected (injection time = 60s) at 100µl/min on captured receptors. After a dissociation step of 600s in running buffer, sensor surfaces were regenerated using 10µl of glycine-HCl pH1.5. All the sensorgrams were corrected by subtracting the low signal from the control reference surface and buffer blank injections. Kinetic parameters were evaluated from the sensorgrams using a two-states or a steady-state models from the T200 evaluation software.

Statistics

Statistical analyses were performed using Prism software version 9.5.1 (GraphPad). Simple group comparisons were performed using the Wilcoxon signed rank test for paired data or the Mann–Whitney test for unpaired data. Multiple group comparisons were performed using Friedmann test for paired data or using Kruskal-Wallis test for unpaired data with additional multiple comparisons Dunn test. Significance was assigned as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Acknowledgements

This work was supported by grants from Sidaction (A014-2-AEQ-08-01; BI25-1-02278, 20-1-AEQ-12652), ANRS (France Recherche Nord&sud Sida-hiv Hépatites; ECTZ46143; ECTZ47079, ECTZ205786), and INSERM state funding granted to M. Naranjo-Gomez (U1183NAR). This work was also supported by the consortium ACT4COVIDCellnex (220110FF, RAK200019FFA). R.Dibsy was a recipient of a Sidaction PhD fellowship. S. Marsile-Medun, M. Naranjo-Gomez, Manon Souchard, Daouda Abba Moussa, Martine Pugnière and M. Pelegrin are members of the "MabImprove Labex", a public grant overseen by the French National Research Agency (ANR) as part of the "Investments for the future" program (reference: ANR-10-LABX -53-01) that also supported this work. We thank the imaging facility MRI, which is part of the UAR BioCampus Montpellier and a member of the national infrastructure France-BioImaging, supported by the French National Research Agency (ANR-10-INBS-04, "Investments for the future"). We thank the BSL3 research laboratory CEMIPAI (CNRS UAR3725) for providing help and advice, and the Centre de Ressources Biologiques (CRB) for providing blood samples from PLWH. We thank the Transcriptomic Core Facility of the Institute in Regenerative Medicine and Biotherapy, CHU-INSERM-UM Montpellier where the applied Biosystem-Affymetrix microarrays were processed. We thank Cecile Monzo and Carole Crozet that are in charge of the Nanostring Sprint Profiler platform in the Institute for Neurosciences Montpellier (INM), for providing assistance in the transcriptomic studies. We thank the Montpellier Proteomics Platform (PPM-PP2I, BioCampus Montpellier) facilities where the SPR experiments were carried out. We are grateful to Dr. Paris for critical reading of the manuscript.

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.

Conflict of interests

None

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810
811

Figure Legends

Figure 1. Activation of human neutrophils stimulated *in vitro* by Toll-like receptors (TLRs) agonists.
Human neutrophils from healthy donors were activated by TLRs agonists, TLR4 (LPS), TLR7/8 (R848). (A). Gating strategy used for all experiments. (B-C) Viability (B) and phenotypic activation (C) assessed by monitoring cell surface markers. Fold change is measured versus unstimulated cells (dashed lines). It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. (D-E) Cytokines and chemokines secretion profile, represented as heatmap (D) and histograms for each cytokine/chemokine assessed (E). Values are mean +/- SD of at least 25 (B-C) and 8 (D-E) independent measurements. Significance was assigned as follows: $**p < 0{,}01$ and $***p < 0.001$. NS: non-stimulated.

Figure 2. Activation of human neutrophils stimulated *in vitro* by TNF-α or IFN-γ.
Human neutrophils from healthy donors were activated overnight by TNF-α at 1 ng/mL or IFN-γ at 1000 IU/mL. (A-B) Viability (A) and phenotypic (B) activation assessed by monitoring cell surface markers. Fold change is measured versus unstimulated cells (dashed lines). It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. (C-D) Cytokines and chemokines secretion profile, represented as a heatmap (C) and histograms (D) for each cytokine/chemokine assessed. Values are mean +/- SD of at least 19 (A) and 8 (B-D) independent measurements. Significance was assigned as follows: $*p < 0.05$, and $**p < 0.01$. NS: non-stimulated.

Figure 3. Activation of human neutrophils stimulated *in vitro* by HIV-1 virions, ICs or free bNAb.
Human neutrophils from healthy donors were activated by HIV-1 virions, ICs or free bNAb. (A-B) Viability (A) and phenotypic activation (B) assessed by monitoring cell surface markers. Fold change is measured versus unstimulated cells (dashed lines). It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. (C-D) Cytokines and chemokines secretion profile, represented as a heatmap (C) and histograms (D) for each cytokine/chemokine assessed. Values are mean +/- SD of at least 21 (A-B) and 17 (C-D) independent measurements. Significance was assigned as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. NS: non-stimulated.

Figure 4. Secretion profile of human neutrophils primed with TNF-α (B) or IFN-γ (C) and then stimulated *in vitro* by HIV-1 virions, ICs or free bNAb.
(A) Protocol for priming of neutrophils. Dashed lines indicate the level of secretion induced by either TNF-α alone (B) or IFN-γ alone (C). Values are of 10 (B) and 6 (C) independent measurements. Significance was assigned as follows: $*p < 0.05$ and $**p < 0.01$

Figure 5. Transcriptomic analysis of neutrophils primed with TNF-α or IFN-γ.
Heatmap (A) and Volcano plot (B) representation of upregulated and downregulated genes by TNF-α or IFN-γ. (C) Venn diagram of regulated genes after cytokine stimulation. (D) Genes upregulated and downregulated were analyzed using Metascape (https://metascape.org/gp/index.html#/main/step1) to identify main biological processes modulated by cytokine priming. Data from 4 independent samples.

Figure 6. Activation of human neutrophils isolated from HD or PLWH stimulated *in vitro* by TLR agonists LPS (TLR4) and R848 (TLR7/8) or inflammatory cytokines (TNF-α and IFN-γ).
(A-B) Cytokines and chemokines secretion profile upon activation by TLR agonists, represented as a heatmap (A) and histograms (B) for each cytokine/chemokine assessed. (C-D) Cytokines and chemokines secretion profile upon activation by inflammatory cytokines, represented as a heatmap (C) and histograms (D) for each cytokine/chemokine assessed. Values are mean +/- SD of at least 9 (A-B) and 4 (C-D) independent measurements.

861
862 **Figure 7. Activation of human neutrophils isolated from HD or PLWH stimulated *in vitro* by HIV-**
863 **1 virions, ICs or free bNAb.**
864 (A-B) Cytokines and chemokines secretion profile, represented as a heatmap (A) and histograms (B) for
865 each cytokine/chemokine assessed. Values are mean +/- SD of 6 independent measurements.
866
867 **Figure 8. Modulation of the expression of FcγR on human neutrophils isolated from HD or PLWH**
868 **stimulated *in vitro* by TLR agonist, inflammatory cytokines, HIV-1 virions, ICs or free bNAb.**
869 (A-C) Modulation of the cell surface levels of CD16 and CD32 upon activation by TLR agonists (A),
870 cytokines (B), and HIV-1 virions, ICs or free bNAb (C). Fold change is measured versus unstimulated
871 cells (dashed lines). It is represented as the ratio MFI stimulated cells/MFI unstimulated cells. Values
872 are mean +/- SD of at least 14 (A), 8 (B) and 6 (C) independent measurements. Significance was assigned
873 as follows: $*p < 0.05$ and $**p < 0.01$
874
875 **Figure 9. Phenotypic characterization of neutrophils isolated from PLWH and HD blood samples.**
876 Purified neutrophils were immunophenotyped by assessing the expression of 18 cell surface markers.
877 Data include at least 19 PLWH blood samples and 4 HD samples. Statistical analyses are unpaired
878 Mann-Whitney test. Significance was assessed as follows: $*p < 0.05$, $***p < 0.001$, and $****p < 0.0001$.

Figure 1

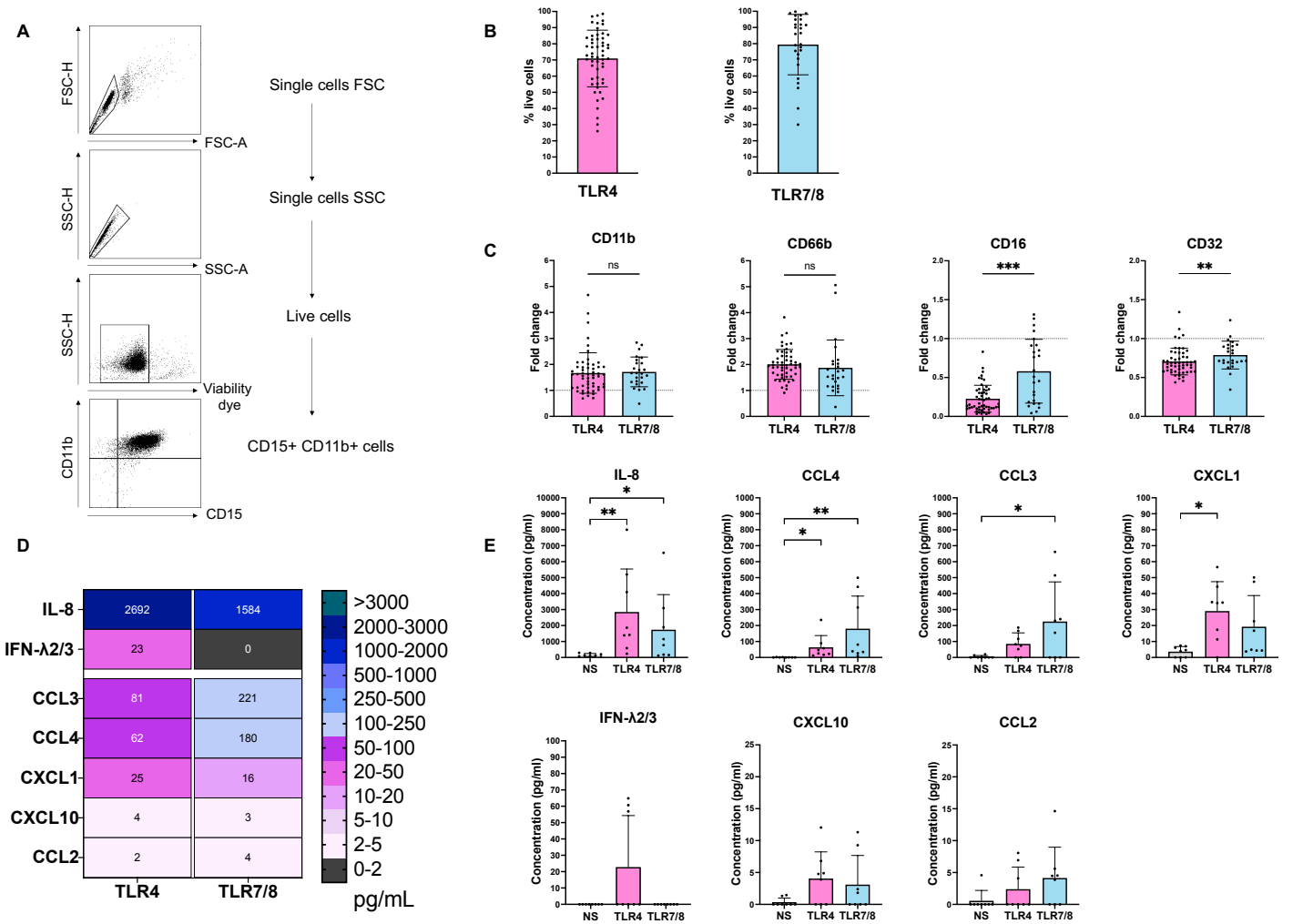

A
FSC-H
FSC-A
SSC-H
SSC-A
SSC-H
Viability dye
CD11b
CD15
Single cells FSC
Single cells SSC
Live cells
CD15+ CD11b+ cells
B
% live cells
TLR4
TLR7/8
C
CD11b
CD66b
CD16
CD32
Fold change
ns
***
**
TLR4
TLR7/8
D
IL-8
IFN-λ2/3
CCL3
CCL4
CXCL1
CXCL10
CCL2
2692
1584
23
0
81
221
62
180
25
16
4
3
2
4
TLR4
TLR7/8
>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

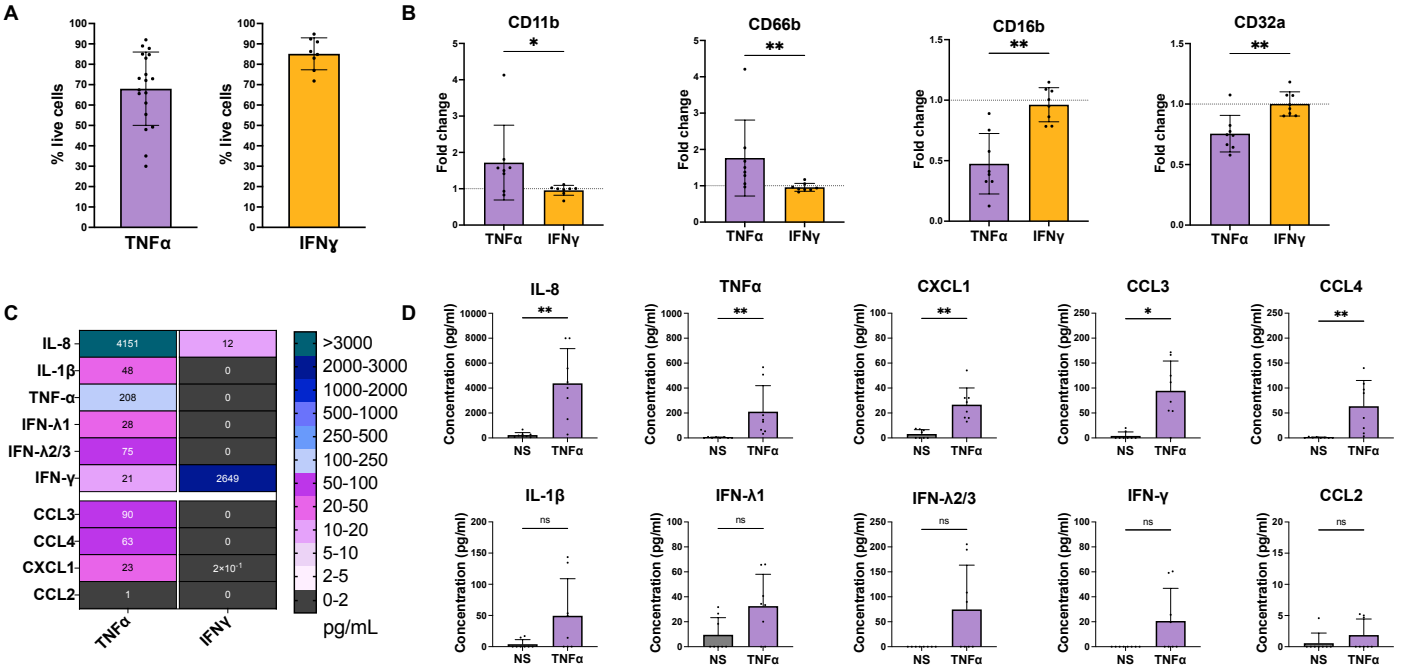

A
% live cells
TNFα
IFNγ
B
CD11b
CD66b
CD16b
CD32a
Fold change
C
IL-8
IL-1β
TNF-α
IFN-λ1
IFN-λ2/3
IFN-γ
CCL3
CCL4
CXCL1
CCL2
4151
12
48
0
208
0
28
0
75
0
21
2649
90
0
63
0
23
2×10-1
1
0
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
D
Concentration (pg/ml)
NS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

Figure 3

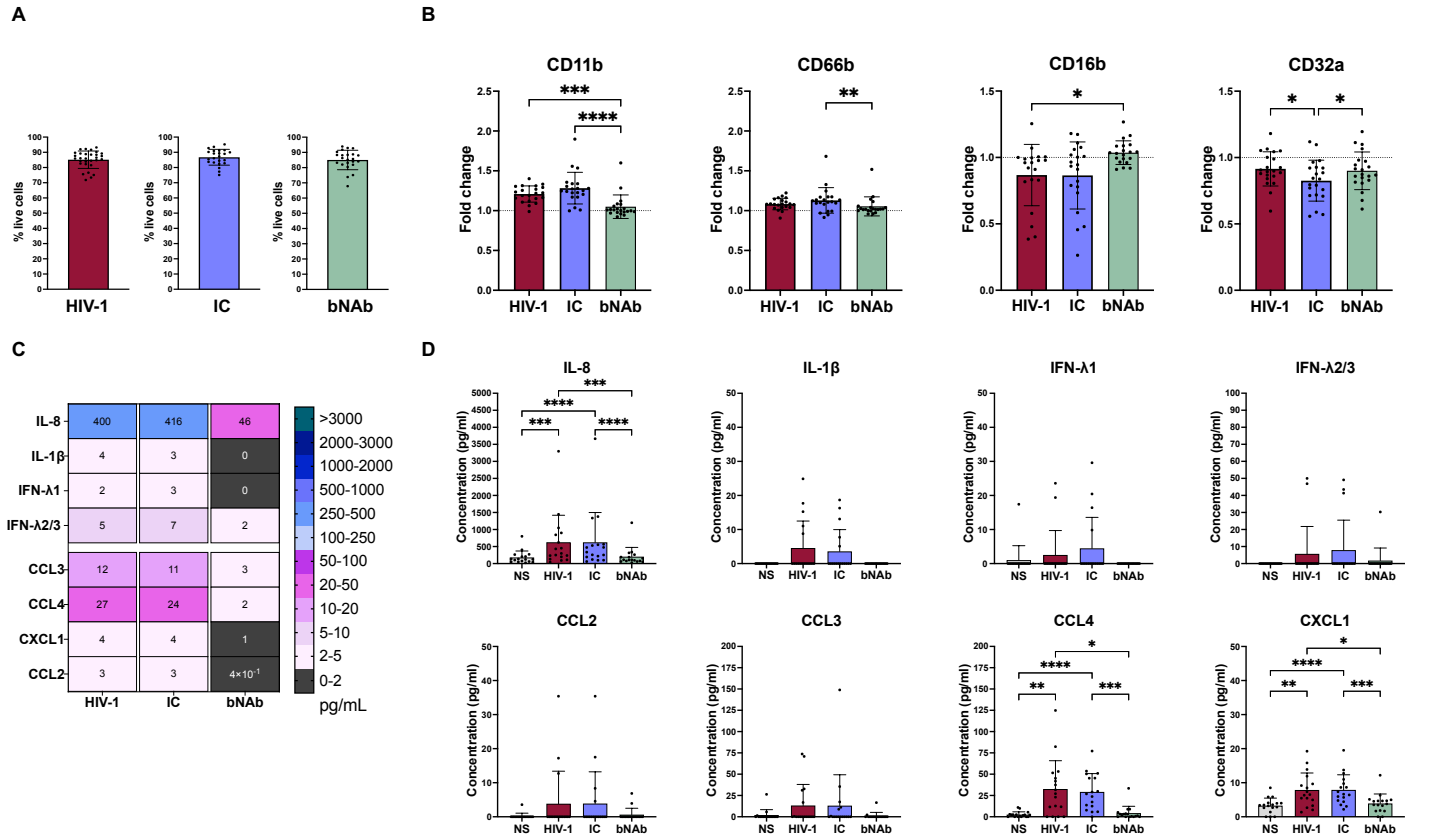

A
% live cells
HIV-1
IC
bNAb
B
CD11b
CD66b
CD16b
CD32a
Fold change
HIV-1 IC bNAb
C
IL-8 400 416 46
IL-1β 4 3 0
IFN-λ1 2 3 0
IFN-λ2/3 5 7 2
CCL3 12 11 3
CCL4 27 24 2
CXCL1 4 4 1
CCL2 3 3 4×10-1
HIV-1 IC bNAb
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
0-2
pg/mL
D
IL-8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

Figure 4

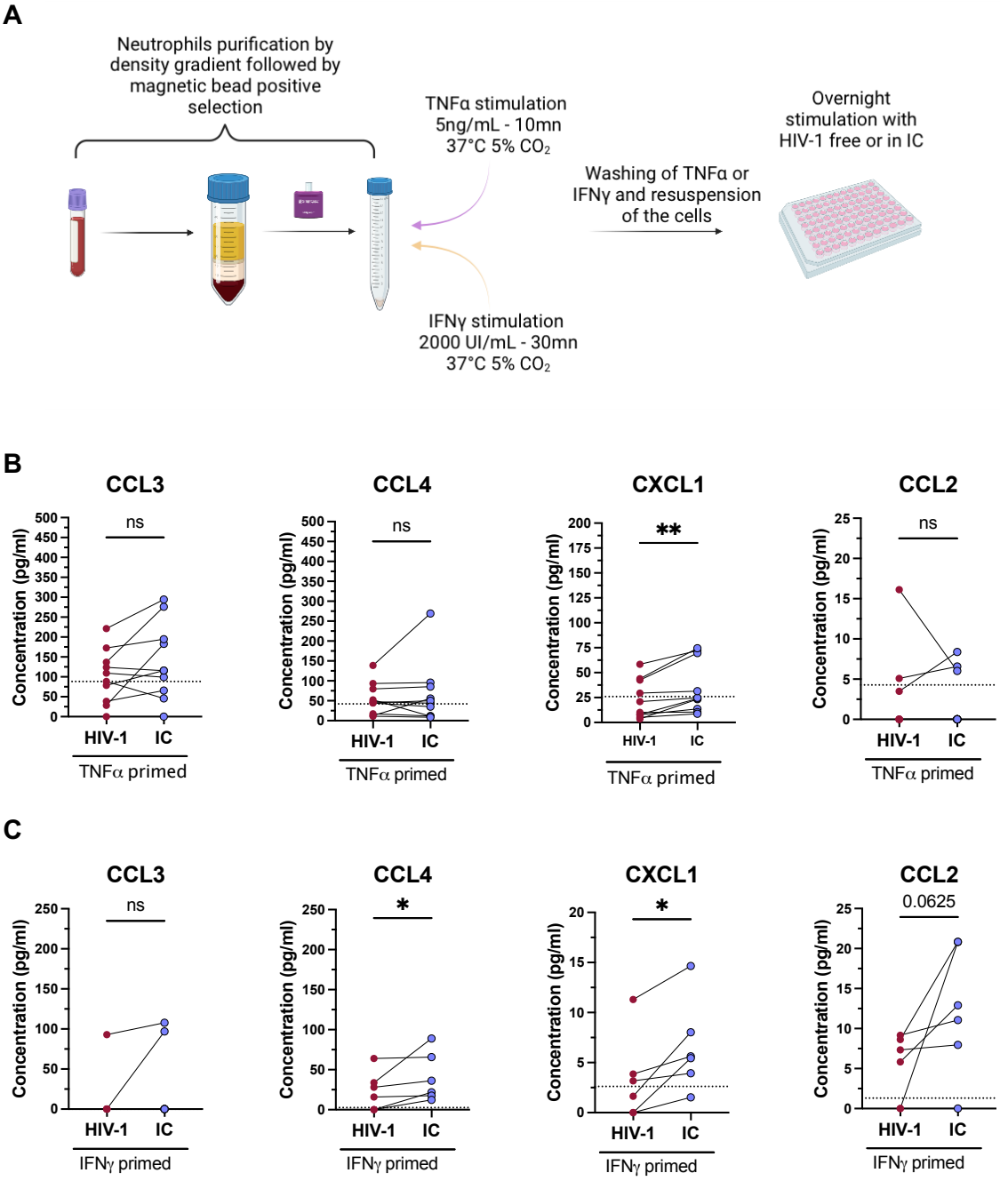
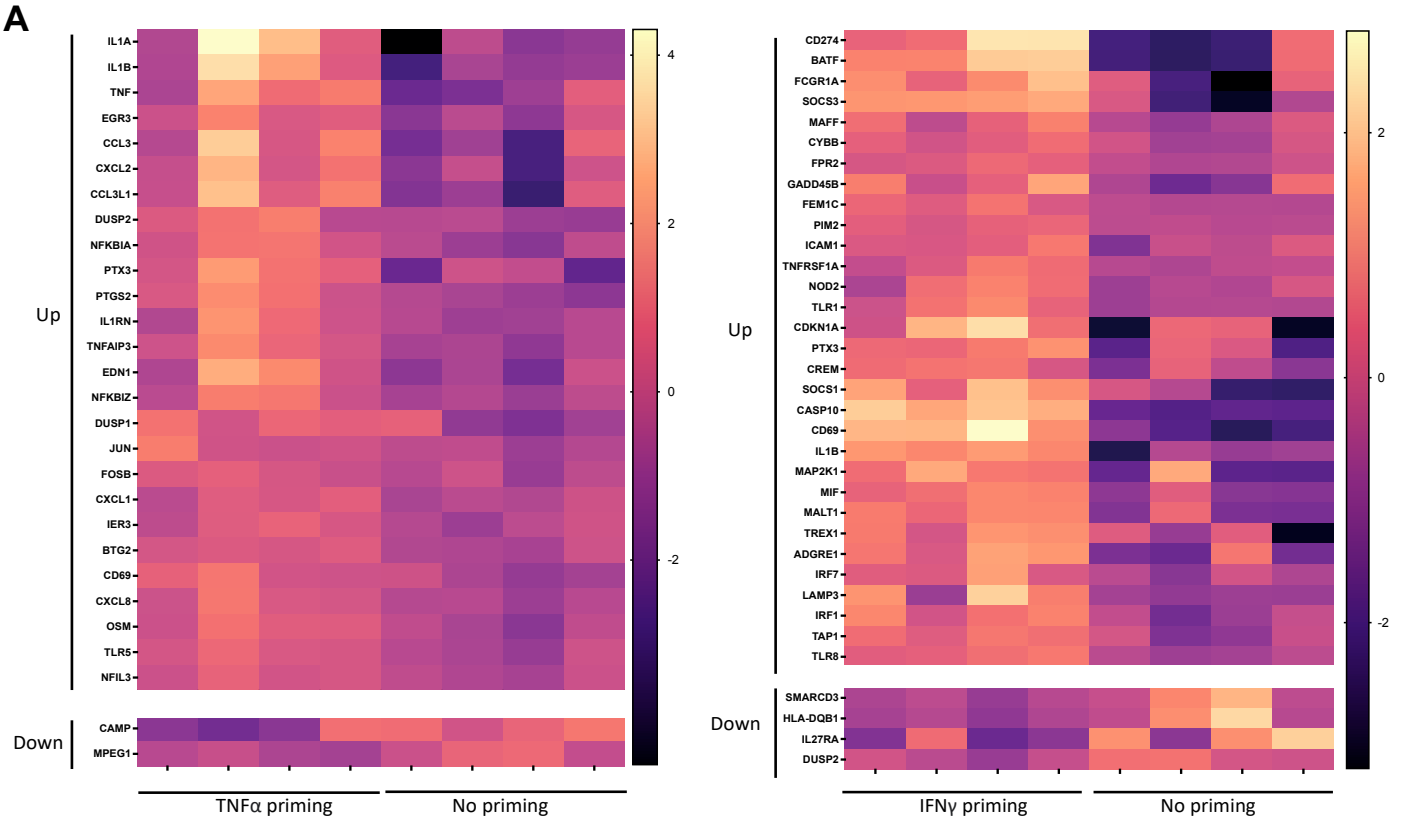
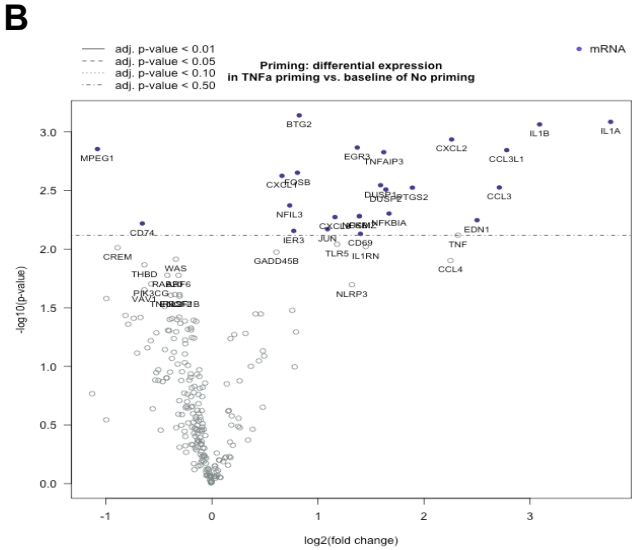
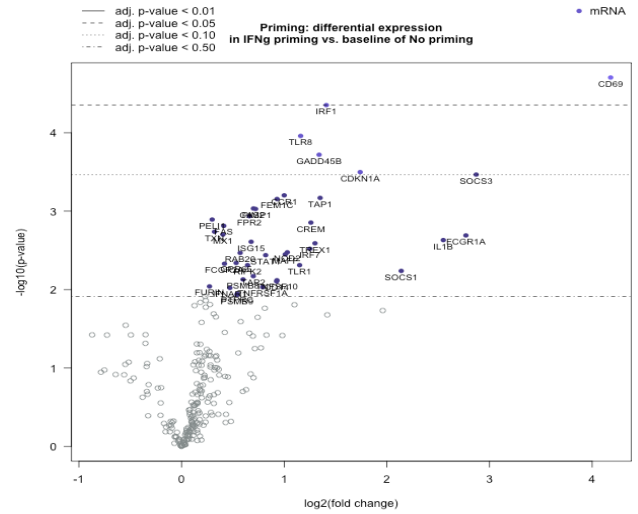
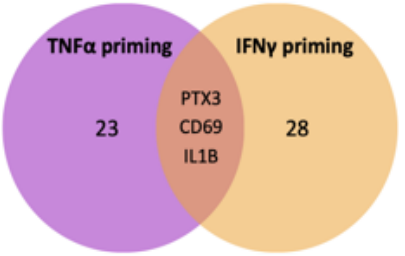

A
Neutrophils purification by density gradient followed by magnetic bead positive selection
TNFα stimulation 5ng/mL - 10mn 37°C 5% CO2
IFNγ stimulation 2000 UI/mL - 30mn 37°C 5% CO2
Washing of TNFα or IFNγ and resuspension of the cells
Overnight stimulation with HIV-1 free or in IC
B
CCL3
CCL4
CXCL1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

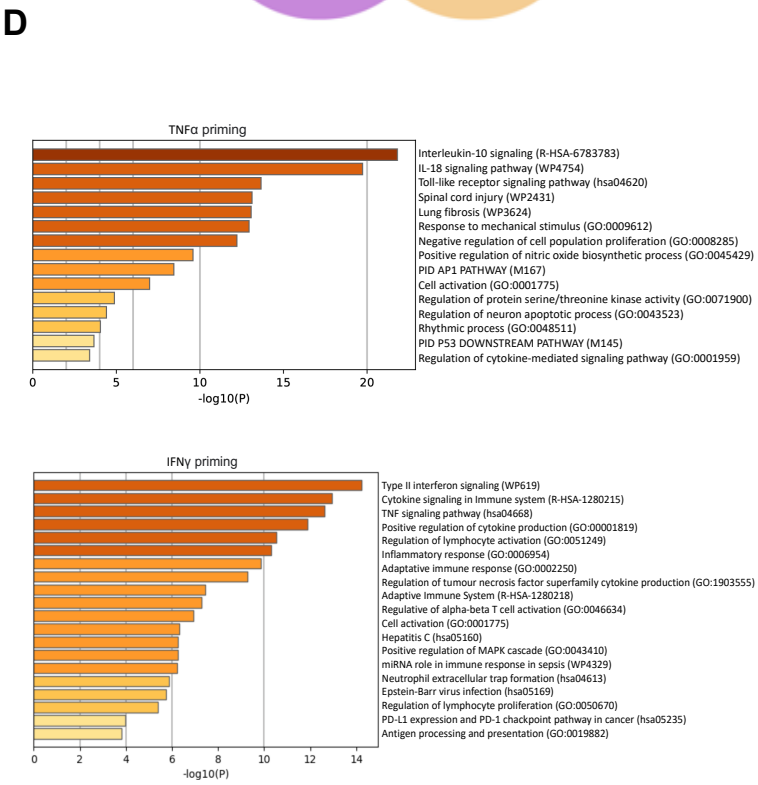

D
TNFα priming
Interleukin-10 signaling (R-HSA-6783783)
IL-18 signaling pathway (WP4754)
Toll-like receptor signaling pathway (hsa04620)
Spinal cord injury (WP2431)
Lung fibrosis (WP3624)
Response to mechanical stimulus (GO:0009612)
Negative regulation of cell population proliferation (GO:0008285)
Positive 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

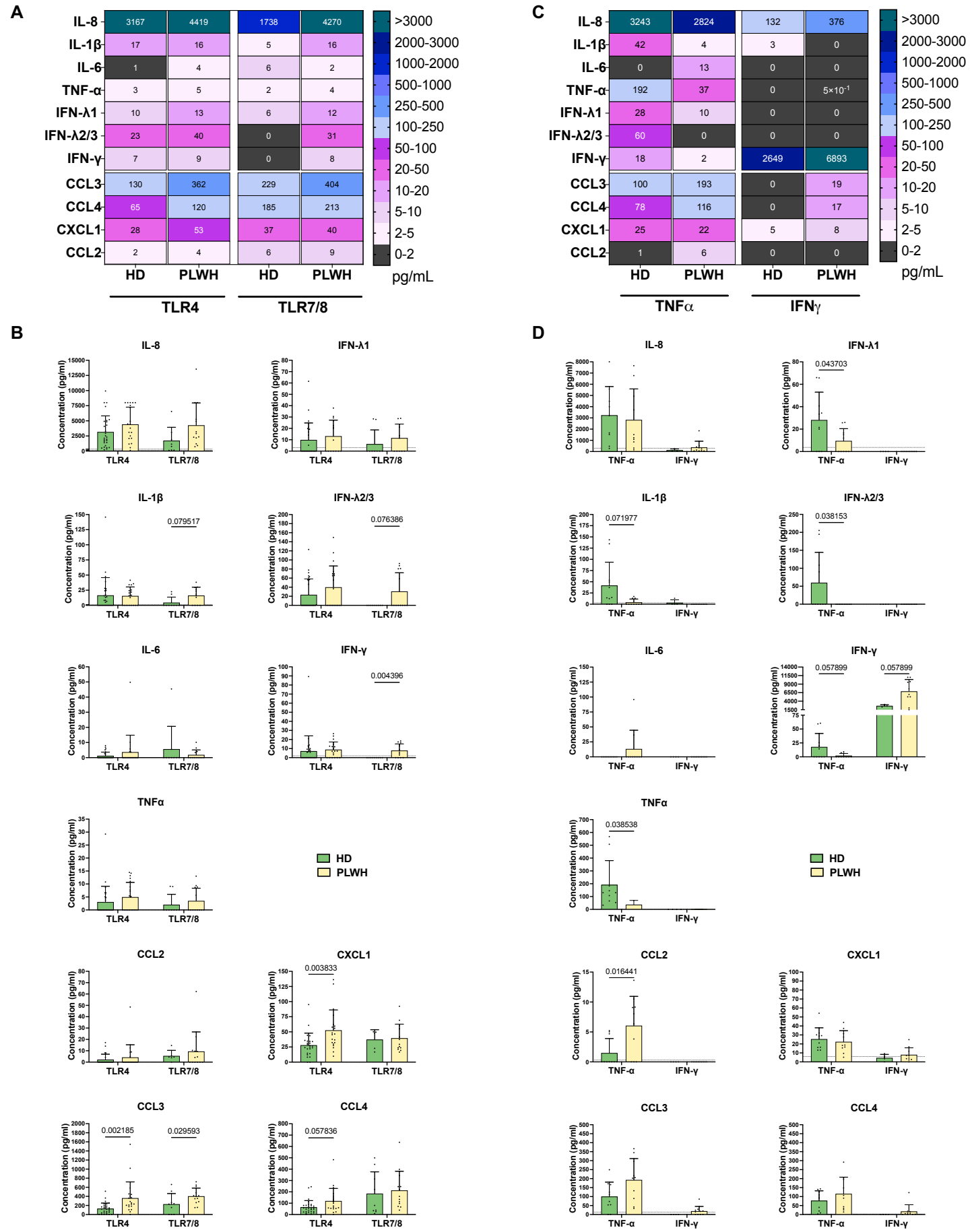
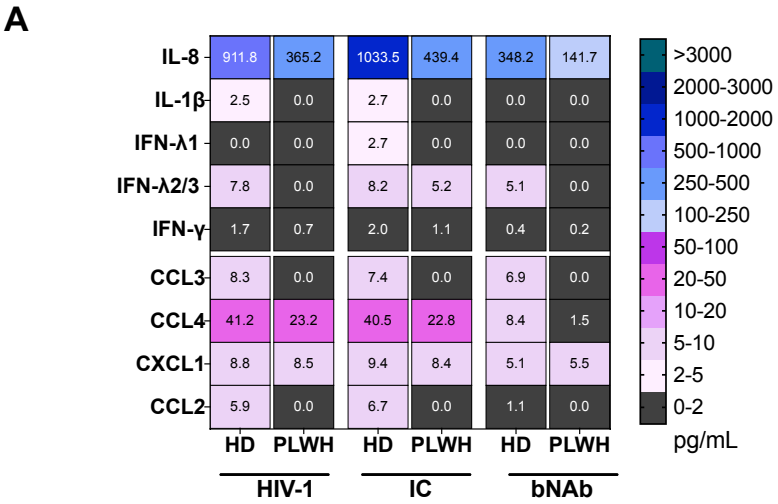

A
IL-8 3167 4419 1738 4270
IL-1β 17 16 5 16
IL-6 1 4 6 2
TNF-α 3 5 2 4
IFN-λ1 10 13 6 12
IFN-λ2/3 23 40 0 31
IFN-γ 7 9 0 8
CCL3 130 362 229 404
CCL4 65 120 185 213
CXCL1 28 53 37 40
CCL2 2 4 6 9
HD PLWH HD PLWH
TLR4
TLR7/8
>3000
2000-3000
1000-2000
500-1000
250-500
100-250
50-100
20-50
10-20
5-10
2-5
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

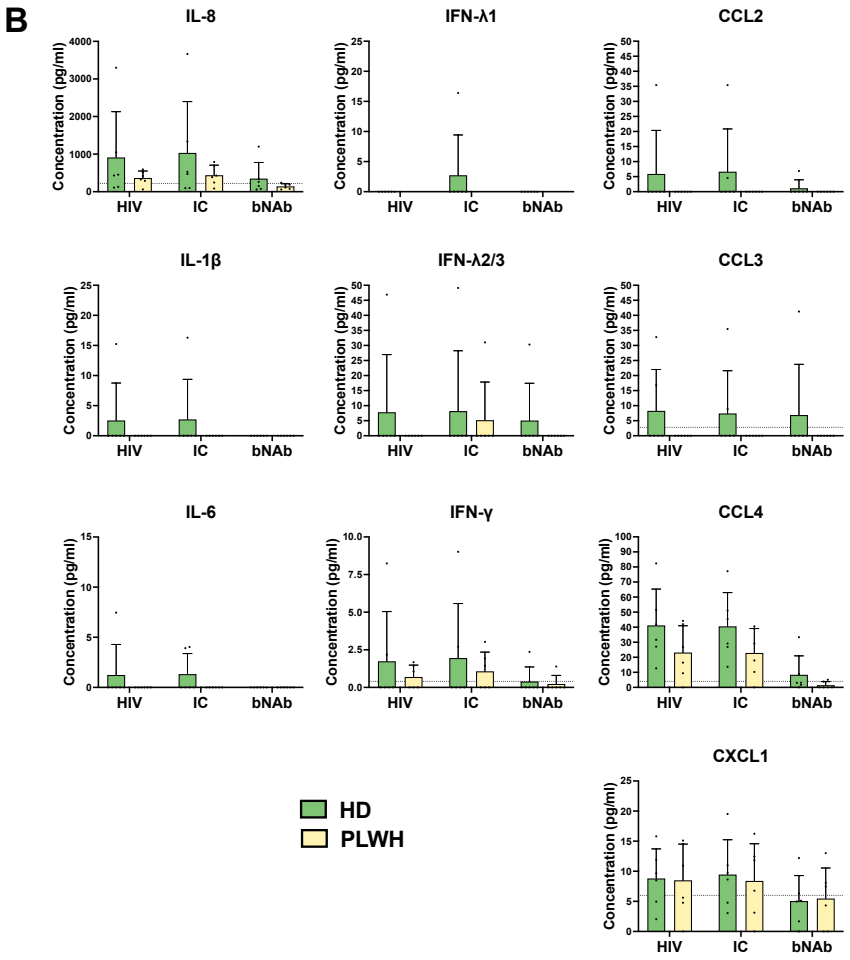

B
IL-8
IFN-λ1
CCL2
IL-1β
IFN-λ2/3
CCL3
IL-6
IFN-γ
CCL4
CXCL1
Concentration (pg/ml)
HIV
IC
bNAb
HD
PLWH

Figure 8

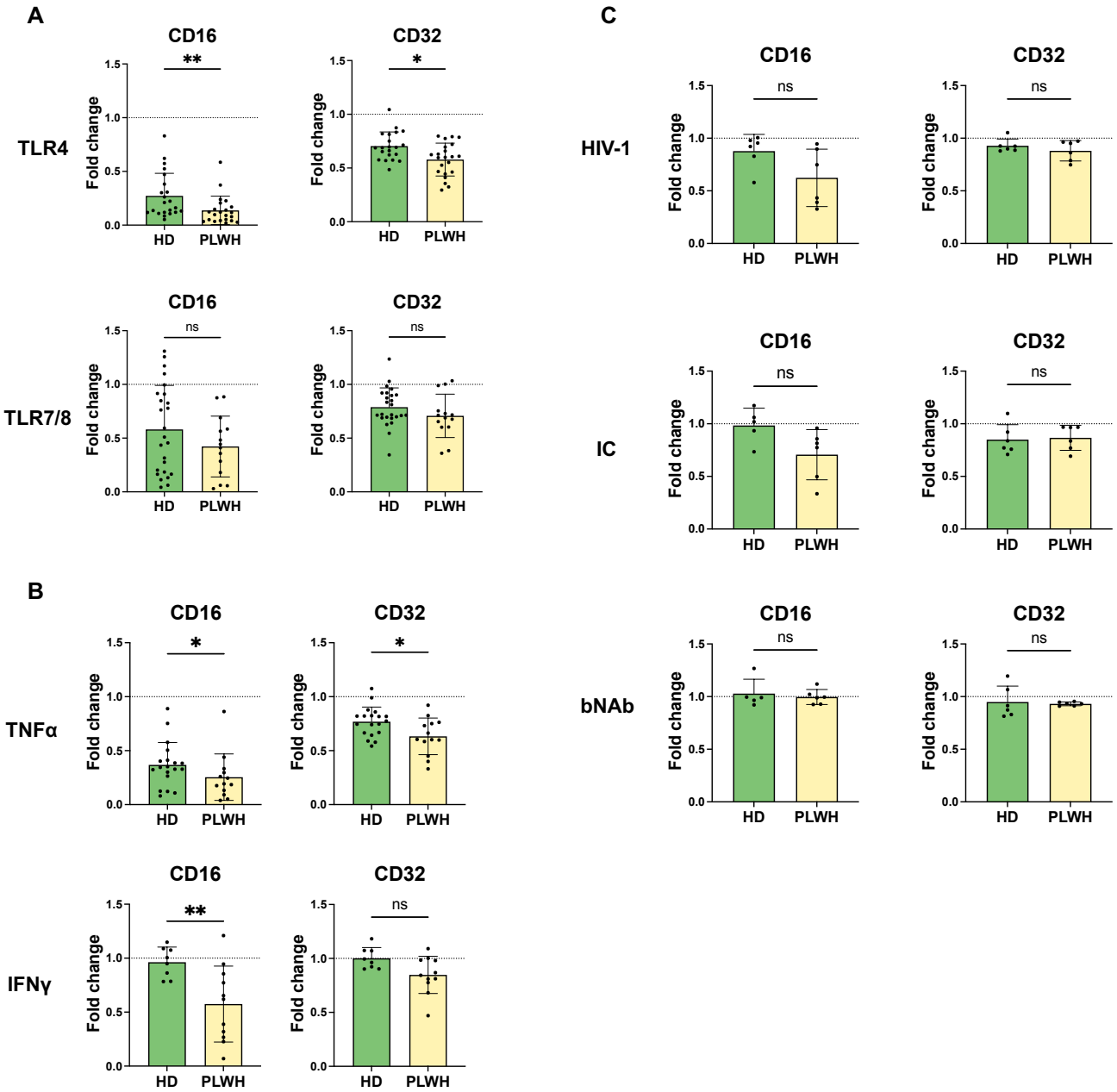
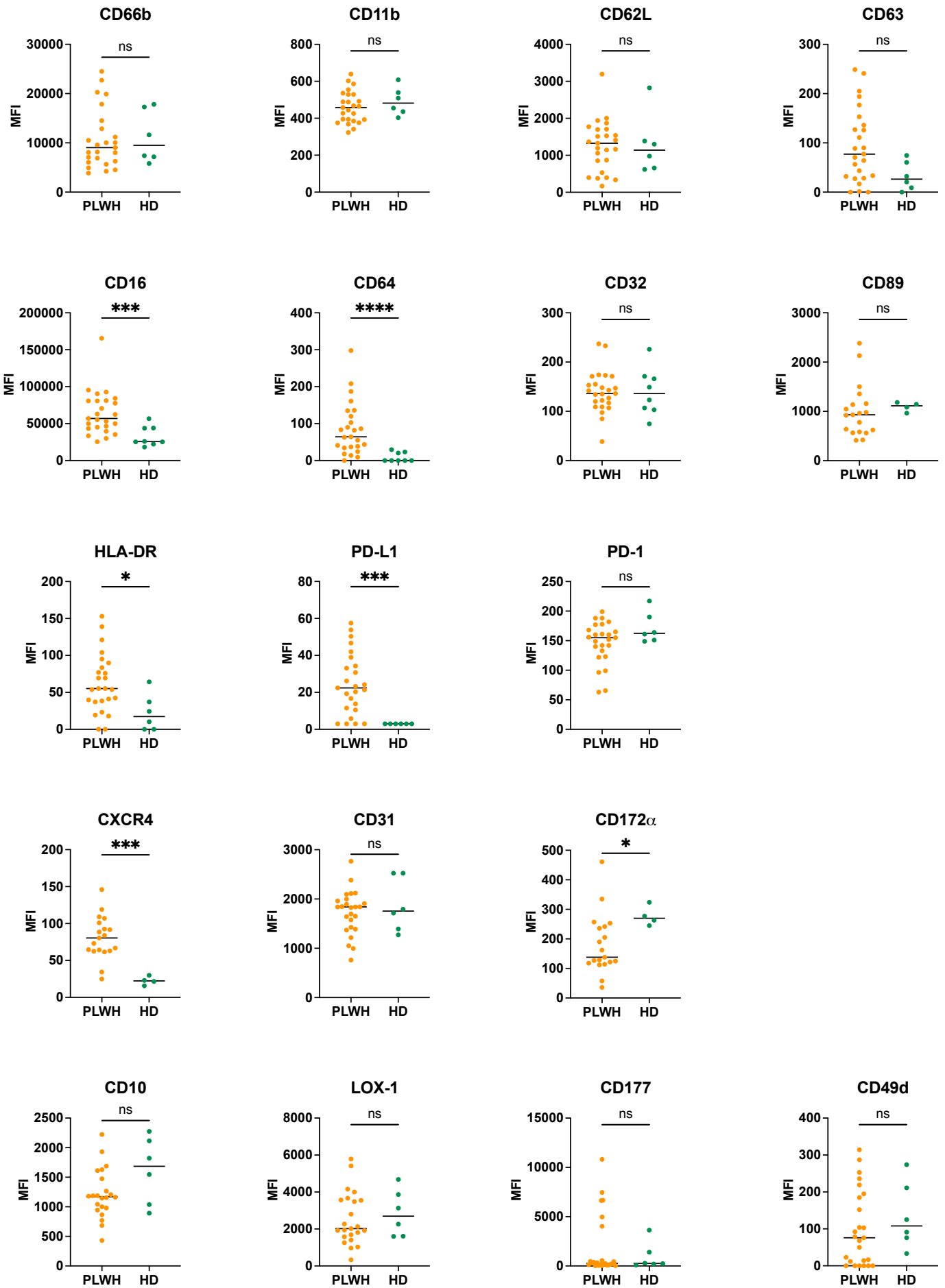

A
CD16
CD32
TLR4
TLR7/8
B
TNFα
IFNγ
C
HIV-1
IC
bNAb
Fold change
HD
PLWH
ns
*
**

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