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Abstract

Macrophages from the *decidua basalis* (dM), the main uterine mucosa during pregnancy, are weakly permissive to HIV-1 infection. Here we investigated the mechanisms underlying this natural control. We show, by using freshly purified decidual macrophages and *ex vivo* human decidual explants, that the local decidual environment influences dM differentiation and naturally protects these cells from HIV-1 infection. IFN-γ, present in the decidual tissue, contributes to maintenance of the dM phenotype and restricts HIV-1 infection by mechanisms involving the cyclin-dependent kinase inhibitor p21Cip1/Waf1. We also found that activation of Toll-like receptors 7 and 8 expressed by dM reinforces the low permissivity of dM to HIV-1 by restricting viral replication and inducing secretion of cytokines in the decidual environment, including IFN-γ, that shape dM plasticity. A major challenge for HIV-1 eradication is to control infection of tissue-resident macrophages in the female reproductive tract. Our findings provide clues to the development of novel strategies to prevent HIV-1 macrophage infection.

Introduction

The *decidua basalis*, the main uterine mucosa during pregnancy, is a model for studying natural protection against HIV-1 mucosal infection. Indeed, maternofetal HIV-1 transmission is rare during the first trimester, and HIV-1 dissemination appears to be tightly controlled at the maternofetal interface [1]. Macrophages that reside in decidual tissue (dM) are fully differentiated. They are the main targets of CCR5-tropic HIV-1 but are less permissive to HIV-1 infection than their peripheral blood counterparts [2].

The *decidua basalis* is characterized by a specific pro/anti-inflammatory cytokine balance required to support fetal development. These cytokines maintain a state of local immune tolerance to the semi-allogeneic fetus and probably ensure host defenses against pathogens [3].

Pro- and anti-inflammatory cytokines trigger peripheral monocyte derived macrophage (MDM) differentiation into opposing programs. This polarization gives rise to two major macrophage phenotypes, designated M1 (classic) and M2 (alternative). M1 macrophages generated in response to cytokines such as IFN-γ and TNF-α, produce pro-inflammatory cytokines such as IL-12 and TNF-α and mediate resistance to pathogens. They express the transcription factor IRF5[4], the costimulatory molecules CD80 and CD86, and the FCγ receptor I CD64 [5,6]. M2 macrophages, generated in response to cytokines such as IL-10, produce anti-inflammatory cytokines (especially IL-10 and IL1-RA) and promote tissue remodeling. The surface-marker expression profile of M2 macrophages includes the scavenger receptor CD163 and the mannose receptor CD206 [5,6]. This MDM plasticity is linked to HIV-1 susceptibility [7].

62 Decidual macrophages have an M2-like phenotype and show features of pro-
63 inflammatory and tolerogenic macrophages, simultaneously expressing M1 (CD64,
64 CD80, CD86) and M2 markers (CD163, CD206) [2,8].

65 Another hallmark of the *decidua basalis* is the abundance of decidual natural killer
66 cells (dNK). The dNK cells secrete large amounts of IFN-γ and TNF-α and are
67 involved in the control of fetal cell invasion, initiation of endometrial vasculature.
68 These cells may limit also the spreading of viral infection to fetal tissues by
69 controlling intrauterine infection by hCMV [9] and HIV-1 (Quillay et al., manuscript
70 under revision).

71 dM and dNK cells express innate receptors, such as toll-like receptors 7 and 8
72 (TLR7/8), that sense HIV-1 sequences and are involved in mediating immune
73 activation following HIV-1 infection [3,10]. We have shown that, upon TLR7/8 activation,
74 dM and dNK cells secrete large amounts of cytokines [3]. TLR7/8 activation inhibits
75 HIV-1 replication in monocytes and MDM [11-14]. In addition, TLR7/8 may influence
76 macrophage polarization, either by activating signaling pathways or by inducing the
77 secretion of cytokines associated with macrophage polarization in the
78 microenvironment [3,14-16].

79 The aim of this work was to determine why dM are weakly permissive to HIV-1
80 infection during the first trimester of pregnancy, with a view to the development of
81 novel strategies for eradicating macrophage infection. We postulated that the
82 decidual environment must contain factors that shape dM plasticity and susceptibility
83 to HIV-1 infection, and that TLR7/8, if activated, can reinforce this low dM
84 permissivity by modulating the local environment and blocking HIV-1 replication.

Results

dM in culture undergo dynamic changes toward a more M2 profile and become more permissive to HIV-1 infection

To address whether the local decidual environment influences the susceptibility of dM to HIV-1 infection, dM were isolated and challenged with VSV-G pseudotyped HIV-1 (HIV-1/VSV-G), immediately after purification (day 0) or after 3 or 7 days of culture in cytokine-free medium. HIV-1 replication was monitored by measuring luciferase activity. Surprisingly, we found that freshly purified dM had very low permissivity to HIV-1/VSV-G but became more susceptible with time in culture (Figure 1a). Similar results were obtained when freshly purified dM and cultured dM were challenged with CCR5-using macrophage-tropic HIV-1$_{BaL}$, as shown by p24 Ag measurement (Figure 1b).

To explain these results, we monitored the surface expression of several macrophage markers by flow cytometry. Expression of M2 markers (CD163, CD206) increased in culture (Figures 2a and 2b), whereas expression of M1 markers (CD64, CD80, CD86) remained unchanged (Supplementary figure S1). We then analyzed HIV-1 receptor and co-receptor expression (CD4, CCR5 and DC-SIGN) and found that only CCR5 expression increased significantly over time (Figure 2c). Early after purification, these cells consisted of approximately equal numbers of spindle-shaped fibroblastoid cells and large flat-round cells (Supplementary figure S2a, left panel), whereas round-shaped cells gradually became predominant in culture (Supplementary figure S2a, right panel). This suggested that dM polarization switched towards a more M2 profile in culture. To confirm this, we used western blot to analyze the expression of the M1 transcription factor (IRF5) involved in the M2 to M1 switch. In keeping with our previous observations, IRF5 expression was

111 decreased gradually in culture (Figure 2d). Analysis of cytokine secretion showed

112 that dM maintained their secretion of M2 cytokines (IL-1RA and IL-10) over time

113 (Figure 2e).

114 Together, these results indicate that dM isolated from their local environment

115 acquire more M2 features and that this switch is accompanied by an increase in

116 susceptibility to HIV-1 infection.

117

118 **IFN-γ participates in dM differentiation and in resistance to HIV-1 infection**

119 The next step was to identify the decidual environmental factor(s) that,when

120 lacking in culture, trigger the dM polarization switch and enhance susceptibility to

121 HIV-1 infection. IFN-γ, present in the decidua, is one of the most potent stimuli of

122 endogenous M1 macrophage activation and play a crucial role in immune responses

123 against pathogens and in tumour immunosurveillance [17]. We thus investigated

124 whether IFN-γ affect dM plasticity and susceptibility to HIV-1 infection.

125 IFN-γ treatment immediately after dM purification attenuated the increase in M2

126 marker expression on day 7, as illustrated by CD163 and CD206 expression in

127 (Figures 3a and 3b). In addition, no morphological change occurred when IFN-γ was

128 added to the culture medium (Supplementary figure S2b). Seven days of IFN-γ

129 treatment restored IRF5 expression to the level found in freshly purified dM (Figure

130 3c). In parallel, M1 markers (CD64, CD80, CD86) were strongly upregulated by IFN-γ

131 (Figure 3d). However, CD4, CCR5 and DC-SIGN expression on IFN-γ-treated dM

132 varied greatly among donors (Figure 3e). Significantly lower IL-10 and IL-1RA

133 secretion (Figure 3f) and higher chemokine secretion (CCL3, CCL4 and CCL5)

134 (Figure 3g) were detected on day 7 of IFN-γ treatment. Thus, 7 days of IFN-γ

135 treatment restored certain features of freshly purified dM (cell morphology, M2

markers and IRF5 expression) and induced certain features of M1 macrophages (M1 markers and low IL-10 and IL-1RA secretion).

To determine whether IFN-γ treatment affected HIV-1 permissivity, purified dM were challenged with HIV-1/VSV-G on day 7 of culture with and without IFN-γ (Figure 4a). IFN-γ impaired HIV-1 replication by more than 80%. This effect was not due to cytotoxicity (Supplementary figure S3a). IFN-γ treatment also impaired dM infection by HIV-1$_{BaL}$, as shown by p24 Ag quantification in the culture supernatant (Figure 4b). By comparison with freshly purified dM, HIV-1 replication was also strongly inhibited in dM (IFN-γ) (Figures 4a and 4c).

Together, these results suggest that IFN-γ participates in dM differentiation and contributes to the low permissivity of freshly purified dM to HIV-1 infection.

HIV-1 infection of dM (IFN-γ) is inhibited at the reverse-transcription and integration steps

As freshly purified dM rapidly switch to an M2 profile and become more susceptible to infection in cytokine-free culture, we used dM (IFN-γ), which recover characteristics of freshly purified dM, to investigate the mechanisms underlying the low permissivity of decidual macrophages.

Late reverse transcripts (U5GAG) and integrated proviral DNA that accumulated during the first 48 h of infection by HIV-1/VSV-G in dM (IFN-γ) were quantified and compared to those found in infected dM (US). IFN-γ treatment led to markedly lower levels of U5GAG (Figure 5a) and integrated HIV-1 (Figure 5b). This strongly suggests that HIV-1 infection in dM (IFN-γ) is restricted at early post-entry steps of the replicative cycle.

Expression of the cyclin-dependent kinase inhibitor p21Cip1/Waf1 (p21) correlates with IRF5 expression and restricts HIV-1 replication in dM (IFN-γ)

We then sought to identify the cellular factor(s) responsible for inhibiting the reverse transcription and integration steps. IFN-γ and IFN-γ-induced factors such as IRF5 are known to modulate the expression of genes involved in cell cycling and apoptosis. We focused on p21, because it has been reported to inhibit HIV-1 replication in macrophages [18-20].

First, we determined p21 expression by western blot in freshly isolated dM and dM (US) at day 7. Freshly purified dM expressed higher levels of p21 (Figure 6a). Next, we analyzed p21 expression in dM (US) and dM (IFN-γ) at day 7 (Figure 6b). p21 was expressed significantly more strongly in dM (IFN-γ) than in dM (US), and correlated with strong IRF5 expression (Figure 6c). p21 expression was then knocked down by short interfering RNA (siRNA) in dM (IFN-γ) prior to challenge with HIV-1/VSV-G (Figure 6d). As a control, we used dM (US) and dM (IFN-γ) treated with random siRNA. p21 silencing in dM (IFN-γ) significantly increased U5GAG levels (Figure 6e) and also the numbers of integrated viral copies (Figure 6f) by comparison with dM (IFN-γ) treated with control siRNA. The resulting levels were not significantly different from those of control dM (US) (Figures 6e and 6f). p21 siRNA had no significant cytotoxic effect, and viability was similar in the three conditions (data not shown). These results show that p21 restricts HIV-1 reverse transcription and integration in dM (IFN-γ).

Interestingly, freshly purified dM and dM (IFN-γ) expressed higher levels of p21, than dM (US) (Figure 6a and 6b), suggesting that the increased dM susceptibility to HIV-1 infection in culture is probably associated with a decrease in p21 expression and that IFN-γ addition to the culture medium of freshly purified dM restores both p21

186 expression and low permissivity to infection. Together, these findings suggest that

187 p21 is involved in the control of HIV-1 replication in decidual tissue.

188

189 **TLR7/8 triggering does not alone induce dM polarization towards an M1 or M2**

190 **profile but transiently blocks HIV-1 replication**

191   We have previously shown that dM express functional TLR7/8. We wondered

192 whether these receptors, if activated after HIV-1 sensing, could influence dM

193 polarization and reduce dM susceptibility to infection, as shown for monocytes and

194 MDM [11-14].

195   We first checked whether dM stimulation by a specific agonist (R848) that mimics

196 HIV-1 recognition by TLR7/8 induced a dM polarization switch. dM were treated with

197 R848 immediately after purification or left untreated. TLR7/8 triggering transiently

198 increased CD163 expression on dM after 3 days of R848 treatment, while CD206

199 and M1 expression fell gradually (Figures 7a and 7b). TLR7/8 engagement also

200 down-regulated CD4, CCR5 and DC-SIGN expression (Figure 7c). TLR7/8 triggering

201 by R848 did not induce morphological changes in culture, by comparison to dM (US)

202 (data not shown). Moreover, IRF5 expression was down-regulated in R848-treated

203 dM (Figure 7d). These changes were accompanied by higher IL-10 release by R848-

204 stimulated dM compared to unstimulated dM, whereas IL-1RA secretion was not

205 modified (Figure 7e). Together, these findings show that TLR7/8 activation by itself is

206 not sufficient to fully polarize dM towards an M1 or M2 profile.

207   We then assessed the capacity of R848-stimulated dM to support HIV-1 infection.

208 dM were treated with R848 directly after purification or left untreated, then challenged

209 with HIV-1/VSV-G pseudotyped HIV-1 after 3 days (Figure 8a) or 7 days (Figure 8b)

210 of culture. TLR7/8 triggering impaired HIV-1 replication after 3 days of stimulation,

but this antiviral effect had been lost by day 7 (Figure 8c). The inhibitory effect of R848 on HIV-1 replication was not due to cytotoxicity, as viability was unaffected (Supplementary figure S3b). TLR7/8-treated dM were also able to control HIV-1$_{BaL}$ infection, as shown by p24 Ag quantification in the culture supernatant (Figure 8d). We also found that TLR7/8 activation led to accumulation of late RT products (Figure 8e) and to a marked decrease in integrated HIV-1 (Figure 8f). These results were consistent with the unmodified p21 expression upon TLR7/8 activation, by comparison to unstimulated dM (data not shown).

Together, these results show that TLR7/8 stimulation of dM leads to significant but transient inhibition of HIV-1 replication, after the reverse transcription step and before or at the integration step, potentially reinforcing the low permissivity of dM.

TLR7/8 triggering in decidual explants induces M1 polarization of dM and restricts HIV-1 replication

We have previously shown that TLR7/8 triggering in dNK cells induces IFN-γ and TNF-α secretion, which could potentially influence dM polarization and HIV-1 permissivity [3].

Therefore, to determine whether TLR7/8 stimulation can indirectly promote dM polarization, we stimulated cultured decidual explants with R848. Cytokine secretion by R848-treated and untreated explants was measured. Secretion of IFN-γ and of IFN-γ-induced cytokines such as CXCL9 and TNF-α was increased in R848-treated explants (Figure 9a), as was the secretion of both M1 (IL-1β and IL-12) (Figure 9b) and M2 cytokines (IL-1RA and IL-10) (Figure 9c). Secretion of chemokines (CCL3, CCL4 and CCL5) was also increased in R848-treated explants (Figure 9d). To further investigate the effect of TLR7/8 stimulation on dM polarization, decidual explants

236   were digested and the resulting cell suspensions were analyzed by flow cytometry.

237   Untreated and IFN-γ-treated explants were used as negative and positive controls,

238   respectively. TLR7/8 triggering down-regulated the M2 marker CD206 (Figure 9e) as

239   well as HIV-1 receptors and co-receptors (Figure 9f) on the dM surface, and

240   enhanced M1 marker expression (CD64, CD80 and CD86) (Figure 9g). This

241   phenotype modulation was similar to but less marked than that observed with IFN-γ-

242   treated explants.

243   Finally, we checked whether R848-treated explants controlled HIV-1 replication, by

244   adding a TLR7/8 agonist to the explants prior to challenge with HIV-1$_{BaL}$. HIV-1$_{BaL}$

245   infection was consistently inhibited in TLR7/8-stimulated explants, as shown by p24

246   Ag measurement (Figure 9h). The viability of R848-treated explants was similar to

247   that of untreated controls (data not shown).

248   TLR7/8 stimulation of decidual explants thus induced dM polarization toward an

249   M1 profile and markedly inhibited HIV-1$_{BaL}$ infection.

Discussion

We report for the first time that mucosal macrophages from the *decidua basalis* are characterized by considerable plasticity in their local environment that determine their permissivity to HIV-1 infection.

dM have an M2-like phenotype but share features of both M1 (CD64, CD80, CD86 and IRF5 expression) and M2 macrophages (CD163 and CD206 expression, IL-1RA and IL-10 secretion). We found that, in culture without exogenous cytokines, dM switched towards a more M2 profile. Thus, dM are likely predestined to adopt an M2 program, which is ideally suited to homeostatic remodeling, angiogenesis and tolerogenesis during pregnancy. This is supported here by the observed IL-1RA and IL-10 secretion by dM cultured without exogenous stimuli. IL-10 may play an autocrine/paracrine role in the M2 polarity switch during dM culture [16]. IFN-γ treatment attenuated the increase in M2 marker expression and restored IRF5 expression to the level observed in freshly purified dM, suggesting that the presence of IFN-γ in decidual tissue participates in dM differentiation. However, IFN-γ treatment also induced other features of M1 macrophages, such as strong expression of CD64, CD80 and CD86 and weak secretion of IL-10 and IL-1RA. In contrast, it failed to increase IL-12 secretion, in keeping with reports that additional stimuli are needed to induce IL-12 secretion [21,22]. The combined M1 and M2 signatures of freshly purified dM are probably ensured by several decidual factors in addition to IFN-γ. In particular, contacts with decidual stromal and mesenchymal cells can induce some M2 features [23]. Phagocytosis of trophoblastic debris generated during pregnancy can also deviate dM toward an M2 phenotype, reducing CD80 and CD86 surface expression and inducing IL-10 and IL-1RA secretion [24]. The balance

275 between these different environmental signals likely accounts for the unique

276 phenotype of freshly purified dM.

277 In parallel to the polarization changes observed in cytokine-free culture, we found

278 that freshly purified dM became more permissive to HIV-1 infection over time. Only

279 CCR5 expression increased significantly over time, possibly playing a role in the

280 increased susceptibility to HIV-1$_{BaL}$ infection, but this would not explain why dM

281 became more sensitive to infection by HIV-1/VSV-G, which does not require the

282 presence of HIV-1 receptors.

283 IFN-γ treatment of dM in culture restored the low permissivity of freshly purified

284 dM, strongly inhibiting the HIV-1 reverse transcription and integration steps. R5 HIV-1

285 entry might also be blocked, as CCR5-binding chemokines (CCL3, CCL4, and CCL5)

286 are strongly induced by IFN-γ [25].

287 Thus, our data demonstrate that dM polarization status is responsible for the low

288 permissivity to HIV-1 infection.

289 As freshly purified dM switch rapidly to an M2 profile in cytokine-free culture, and

290 as IFN-γ prevents this switch (in terms of the phenotype and resistance to infection),

291 we used IFN-γ-treated dM to examine the mechanisms underlying the low

292 permissivity of freshly purified dM, and found that p21 inhibited the reverse

293 transcription and integration steps. This is consistent with a previous study showing

294 that IRF5 modulates the expression of growth-regulating and pro-apoptotic genes,

295 including p21 [18]. However, we cannot exclude the possibility that other IFN-γ-induced

296 genes may modulate p21 expression [20]. We also found that IFN-γ treatment restored

297 p21 expression in culture to a level similar to that of freshly purified dM, suggesting

298 that p21 is involved in HIV-1 restriction in decidual tissue. p21 is known to restrict

299 HIV-1 replication in macrophages either by inhibiting dNTP biosynthesis through a

300 RNR2-dependent pathway [26] or by modulating the phosphorylation of SAMHD1, a

301 host restriction factor [27]. We have previously shown that the SAMHD1 pathway is

302 involved in controlling HIV-1 infection of freshly purified dM [8]. RNR2 was

303 undetectable in dM (IFN-γ), and SAMHD1 phosphorylation was slightly lower in dM

304 (IFN-γ) than in controls, whereas the total SAMHD-1 level remained unchanged

305 (preliminary data not shown). Thus, in decidual tissue, p21 might act through the

306 SAMHD1 pathway.

307   Contrary to IFN-γ, TLR7/8 triggering alone did not induce an M1 or M2 switch.

308 However, TLR7/8 triggering impaired HIV-1/VSV-G infection and resulted in

309 accumulation of late RT products, together with a marked reduction in the amount of

310 integrated proviral DNA. These findings corroborate reports that TLR7/8 ligation

311 impairs HIV-1 infection of monocytes and MDM [12-14,28]. However, as in previous

312 studies [11,29], we found no correlation between the TLR7/8-induced antiviral effect and

313 IFN-α secretion, which was undetectable (data not shown). TLR7/8 engagement

314 down-modulated CD4, CCR5 and DC-SIGN expression and induced secretion of

315 CCR5-binding chemokines [3], which are reported elsewhere to inhibit HIV-1 entry [30].

316   The observed antiviral effects peaked 3 days after TLR7/8 activation and then

317 declined, while the IFN-γ-induced antiviral effect was more profound. This difference

318 was probably due to differential IL-10 secretion by R848- and IFN-γ-treated dM.

319 Indeed, TLR activation induces factors such as IL-10 that restrain TLR-induced

320 inflammatory activation and tissue damage. This inhibitory feedback also seems to

321 control the TLR7/8-induced antiviral effect described above. By contrast, IFN-γ

322 suppresses IL-10 secretion and the IL-10-dependent gene expression that regulate

323 IFN-γ responses. IFN-γ signaling also induces proteins of the IRF family, which in

324 turn generate a second wave of IFN-γ target gene transcription [31].

325   TLR7/8 triggering in purified dM failed to promote an M1 switch. In contrast,

326   TLR7/8 triggering in decidual explants drove dM towards an M1 profile and

327   consistently inhibited HIV-1$_{BaL}$ infection. This M1 switch can be attributed, at least in

328   part, to the increased production of IFN-γ and TNF-α by dNK cells upon TLR7/8

329   activation. Cellular contacts with other decidual cells and other factors induced by

330   TLR7/8 stimulation could also be involved in this switch. Indeed, IL-12 secretion,

331   another hallmark of M1 polarization [4], was also increased in R848-treated explants.

332   This increase could reinforce the dM polarity switch by enhancing dNK secretion of

333   IFN-γ [32]. IL-12-related mechanisms and contacts between dM and dNK cells can also

334   favor the emergence or selection of a macrophage population less sensitive to HIV-1

335   infection [33]. dM switching towards an M1 phenotype could also induce TLR7/8

336   overexpression and thereby further reduce the permissivity of dM to HIV-1 [29]. The

337   durable anti-HIV-1 effect observed in decidual explants is probably due to a

338   synergistic action of TLR7/8 activation and IFN-γ secretion.

339   Mucosal inflammation in the FRT promotes HIV-1 transmission and chronic HIV-1

340   disease [34]. TLR stimulation of other FRT mucosae such as the vaginal mucosa

341   induces proinflammatory cytokine and type I interferon secretion by plasmacytoid

342   dendritic cells (pDC), and enhances viral replication [35]. We found that TLR7/8-

343   stimulated decidual explants increased the secretion of both anti-inflammatory (IL-

344   1RA and IL-10) and pro-inflammatory cytokines (TNF-α and IL-1β). The cytokine

345   profile upon TLR7/8 activation was compatible with the pro/anti-inflammatory

346   cytokine balance required to maintain pregnancy during initiation of an immune

347   response to HIV-1 [3]. This cytokine balance, together with the small number of pDCs

348   and the lack of interferon type I expression in the decidua, may explain these

349   discrepancies.

350   To conclude, our data support a model in which several inter-dependent factors

351   mediate the weak permissivity of dM to HIV-1 infection during the first trimester of

352   pregnancy (Figure 10). In fact, the decidual environment necessary for successful

353   pregnancy is unfavorable to HIV-1 replication. At the maternofetal interface,

354   macrophages acquire distinct phenotypic and functional properties directed by the

355   pro/anti-inflammatory balance (IFN-γ/TNF-α versus IL-10/IL1-RA) and by physical

356   contact with decidual cells. dM therefore have low permissivity to HIV-1 infection.

357   Shortly after infection, p21 blocks HIV-1 reverse transcription and integration. In

358   parallel, TLR7/8, which recognize HIV-1 sequences, are activated and further reduce

359   dM permissivity by adding an extra brake after the reverse transcription step and at

360   or before the integration step. TLR7/8 triggering also induces β-chemokine secretion

361   and down-regulates HIV-1 receptor and co-receptor expression, which may limit viral

362   entry. In addition, TLR7/8 triggering induces IFN-γ and TNF-α release from dNK. This

363   favors an M1 switch of neighboring dM and increases p21 expression, thereby

364   limiting infection of new cells. In parallel, TLR7/8 induce IL-1RA and IL-10 secretion,

365   thereby damping inflammation and preserving the pregnancy (Figure 10).

366   In addition to underlining the role of dM differentiation in HIV-1 control, our findings

367   open new avenues of research into the role of dM differentiation in reproductive

368   immunology and implantation failure.

Materials and Methods

Ethics statement

All the donors in this study provided their written informed consent. The French Biomedicine Agency (n° PF508-013), Assistance Publique des Hôpitaux de Paris (n° VAL/2011/06-41/02) and the Biomedical Research Committee of Institut Pasteur, Paris, France (n° 2005.024) approved the study. The blood used for viral amplification on PBMC were obtained from adult healthy donors (Établissement Français du Sang (n°12/EFS/134 / n° HS2013-24916)). All blood donors signed informed consent allowing the use of their blood for research purpose.

Human decidual tissue collection, dM isolation, and reagents

Decidual tissues were obtained from healthy women undergoing voluntary termination of pregnancy during the first trimester (8 - 12 weeks of gestation). dM were purified by positive selection with anti-CD14 magnetic beads. dM purity was checked by flow cytometry and was 93% ± 3.8% (mean + SEM). dM were stimulated 3 or 7 days prior to various assays with R848 at 5µg/ml for TLR7/8 activation and IFN-γ at 100ng/ml for dM polarization switch. Detailed procedures are described in the supplementary materials and methods.

HIV-1 isolates and infection

For single-round infectious challenge, we used HIV-1 particles (600 ng of p24 Ag/10^6 cells) containing the luc reporter gene and pseudotyped with the vesicular stomatitis virus G protein (HIV-1/VSV-G). The efficiency of infection was determined after 72 h by measuring luciferase activity in cell lysates with the Luciferase reagent. For

394   productive infection, HIV-1$_{BaL}$ was used at 10^{-3} MOI. HIV-1$_{BaL}$ replication was

395   measured by p24 Ag ELISA in culture supernatants. Detailed procedures are

396   described in the supplementary materials and methods.

397

Decidual explants

399   Decidual tissues were cut into 0.3-cm^2 pieces. For flow cytometry, explants were

400   placed on collagen sponge gels in 1.5 ml of medium/well/sponge/piece prior to

401   stimulation with R848 or IFN-γ. Decidual tissue and the sponge were minced,

402   digested and filtered. The total cell population was analyzed by flow cytometry. For

403   experiments with HIV-1$_{BaL}$, explants were stimulated overnight with R848, then

404   challenged with HIV-1$_{BaL}$ for 12 h. After several washes the explants were placed on

405   collagen sponge gels. All experiments were performed in triplicate. Details are

406   provided in the supplementary materials and methods.

407

Flow cytometry of cell surface markers

409   Detailed procedures are described in the supplementary materials and methods. In

410   brief, adherent dM were detached from plastic plates by pipetting without scraping.

411   Cells were then incubated with an FcR blocking reagent and stained with conjugated

412   antibodies. Cell surface marker expression was determined with an LSRII 2-Blue 2-

413   Violet 3-Red 5-Yelgr-laser configuration. The results were analyzed with FlowJo

414   9.1.3. software. MFI (%) was calculated as the ratio of treated dM (IFN-γ) or R848-

415   treated dM to the dM (US) control.

416

Western blotting

418   Details are provided in the supplementary materials and methods. Protein bands

419 intensity were quantified with Image J software 1.47V and normalized to the actin

420 band. The protein band intensity was quantified with the Image J software 1,47V and

421 normalized to the actin band. Ratios (protein / actin) were then compared between

422 the different conditions. Fold changes of IRF5 and p21 expression were calculated by

423 comparing the normalized value of IRF5 and p21 from the IFN-γ-treated samples to

424 the normalized value of IRF5 and p21 from the dM (US) control from the same donor.

425

426 **Cytokine quantification**

427 dM culture supernatants were harvested after 1, 3 or 7 days and stored at -80°C.

428 Soluble factors were measured in a Luminex assay with the Human Cytokine 25-plex

429 antibody bead kit as recommended by the manufacturer (Invitrogen, Saint Aubin,

430 France).

431

432 **siRNA transfection**

433 Small interfering RNAs (siRNAs) were purchased from GE Healthcare Dharmacon

434 (Buckinghamshire, UK). The siRNA against the p21 gene was on-target plus siRNA

435 n.12, and had the following sequence: 5′ AGA CCA GCA UGA CAG AUU U 3′.

436 Control siRNAs (Ctrl) were a pool of four on-target plus nontargeting siRNAs. siRNA

437 transfection was performed using INTERFERin kits (Polyplus Transfection, Illkirch-

438 Graffenstaden, France). 50 nM sip21 or siCtlr was prediluted in 1 mL of Opti-MEM, to

439 which 20 µL of INTERFERin was added. The transfection mix was left at room

440 temperature for 10 min and then incubated with dM at 37°C for 16 hours. Cell culture

441 medium was next replaced by siRNA-free culture medium during 8 hours. The

442 transfection mix was then added for additional 16 hours prior to Western Blot and

443 infection by HIV-1/VSV-G. Ctrl siRNA was also added to dM (US).

HIV-1 DNA quantitative PCR (qPCR)

Total DNA in HIV-1/VSV-G-infected dM was extracted 48 h postinfection. Quantitative real-time PCR analysis of late (U5GAG) forms of viral DNA was carried out as previously described [19]. Integrated HIV-1 DNA was quantified by real-time Alu-LTR nested PCR using primers and probes described elsewhere [19]. The amount of viral DNA was normalized to the endogenous reference albumin gene. Detailed procedures are described in supplementary materials and methods.

Viability test

The fixable viability dye 329 eFluor 780 (eBiosciences, Paris, France) was used to determine the viability of freshly purified dM and unstimulated dM on day 7 of culture, as recommended by the manufacturer. On days 3 and 7 of culture, the viability of stimulated dM (IFN-γ and R848) and unstimulated dM was also compared. No significant differences were found.

Statistical analyses

Statistical analyses were done with GraphPad Prism software version 5.0f. When several groups (>=3) were compared, a Kruskal–Wallis test was used. When this test was significant, two by two comparisons were conducted and a Bonferroni correction was applied. Otherwise the Mann-Whitney test and the Wilcoxon matched pairs test were used. Lines or bars represent the mean, and error bars indicate SEM. The figure legends show in parentheses the number of independent donors used in the experiments. P values <0.05 were considered significant (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$). (#) Represents the statistical comparison of MFI values

469   between stimulated and unstimulated dM at a given time point. ($^{#}$p < 0.05, $^{##}$p <

470   0.005, $^{###}$p < 0.0005, $^{####}$p < 0.0001).

471

472   **DISCLOSURES**

473   The authors declared no conflict of interest.

474

475   **Acknowledgments**

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605

Figure legends

Figure 1. dM become more permissive to HIV-1 infection in cytokine-free culture.

(a) Freshly purified dM were challenged with HIV-1/VSV-G at day 0, 3 and 7 of culture. HIV-1 replication was monitored by measuring luciferase activity (n=7).

(b) dM were challenged with HIV-1$_{BaL}$ immediately after isolation (day 0) or after 7 days of cytokine-free culture. Viral production was followed by quantifying p24 Ag in the supernatant. p24 Ag production on day 21 post-infection (n=7).

Mean ± SEM of *n* independent donors.

Figure 2. dM switch towards a more M2 profile in cytokine-free culture.

(a) Representative histograms of CD163 and CD206 expression on dM, as measured by flow cytometry.

(b) CD163 and CD206 mean fluorescent intensity (MFI) over time (n=6).

(c) CD4, CCR5 and DC-SIGN mean fluorescent intensity (MFI) on day 0 and on day 7 of culture (n=6).

(d) Representative western blot of IRF5 expression by dM over time (left panel). The protein band intensity was quantified and normalized to the actin band. Ratio (IRF5 / actin) was then compared over time (right panel) (n=6).

(e) dM cytokine release after 1, 3 and 7 days of culture (n=6).

Mean ± SEM of *n* independent donors.

631 **Figure 3. IFN-γ addition to cultured dM restores partially the phenotype of**

632 **freshly purified dM.**

633 (a) Representative histograms of IFN-γ-induced modulation of CD163 and CD206

634 expression on day 7, as measured by flow cytometry.

635 (b) IFN-γ-induced change (%) in CD163 and CD206 expression (MFI) on dM

636 compared to time-matched unstimulated dM (n=6).

637 (c) Representative western blot of IRF5 expression by IFN-γ-stimulated and

638 unstimulated dM on day 7 of culture (left panel). The protein band intensity was

639 quantified and normalized to the actin band. Ratio (IRF5 / actin) was then compared

640 between IFN-γ-stimulated and unstimulated dM on day 7 of culture (right panel)

641 (n=19).

642 (d) IFN-γ-induced change (%) in CD64, CD80 and CD86 expression (MFI) on dM, as

643 compared to time-matched unstimulated dM (n=6).

644 (e) IFN-γ-induced changes (%) in CD4, DC-SIGN and CCR5 expression (MFI) on dM

645 on day 7 of culture, by comparison with unstimulated dM (n=17).

646 (f) dM cytokine and (G) β-chemokine release after 7 days of culture (n=7).

647 Mean ± SEM of *n* independent donors. # = statistical comparison of MFI values

648 between IFN-γ-stimulated dM versus unstimulated (US) dM at the same time.

649

650 **Figure 4. IFN-γ addition to cultured dM restores the resistance to HIV-1.**

651 (a) Freshly purified dM and 7-day IFN-γ-stimulated and unstimulated (US) dM were

652 challenged with HIV-1/VSV-G. HIV-1 replication was monitored by measuring

653 luciferase activity in cell lysates (n=13).

654 (b) IFN-γ stimulated and US dM were challenged with HIV-1$_{BaL}$ on day 7 of culture.

655 HIV-1 p24 Ag was measured by ELISA in the supernatants (n=6).

(c) p24 Ag production on day 21 after infection by HIV-1$_{BaL}$ was compared between freshly purified dM and 7-day IFN-γ-stimulated or unstimulated dM (n=6). Mean ± SEM of *n* independent donors.

Figure 5. IFN-γ restricts HIV-1 infection of dM at the reverse transcription and integration steps.

7-day IFN-γ-stimulated and unstimulated (US) dM were challenged with HIV-1/VSV-G. Late reverse-transcription products (U5GAG) (n=6) (a) and integrated forms (n=8) (b) were quantified by qPCR. Mean ± SEM of *n* independent donors.

Figure 6. p21 is involved in low dM permissivity to HIV-1.

(a) Representative western blot of p21 expression by freshly purified dM (day 0) and by unstimulated dM at day 7 of culture (left panel). The protein band intensity was quantified and normalized to the actin band. Ratio (p21 / actin) was then compared between dM at day 0 and day 7 (right panel) (n=6).

(b) Representative western blot of p21 expression by IFN-γ-stimulated and unstimulated (US) dM on day 7 of culture (left panel). Ratio (p21 / actin) was then compared between the 7-days IFN-γ-stimulated and unstimulated (US) (right panel) (n=20).

(c) Fold changes of IRF5 and p21 expression were calculated by comparing the normalized value of IRF5 and p21 from the IFN-γ-treated samples to the normalized value of IRF5 and p21 from the dM (US) control.

Correlation between fold changes in IRF5 and p21 expression on day 7 of IFN-γ treatment compared to time-matched unstimulated dM (n=19). Spearman's R value is

681 shown.

682 (d) Representative western blot of p21 expression in 7-day IFN-γ-stimulated and

683 unstimulated dM transfected with p21-specific siRNA or with an irrelevant control

684 siRNA (Ctrl) (n=9).

685 (e) Number of late reverse transcription products (U5GAG) and (F) integrated forms

686 in dM transfected with p21-specific or control siRNA (n=9).

687 Mean ± SEM of *n* independent donors.

688

689 **Figure 7. TLR7/8 triggering is not sufficient to induce dM polarization towards**

690 **an M1 or M2 profile.**

691 (a) % change in dM CD163 and CD206, (b) CD64, CD80 and CD86 expression (MFI)

692 induced by R848 as compared to time-matched unstimulated dM.

693 (c) % change in dM CD4, DC-SIGN and CCR5 expression (MFI) induced by R848 as

694 compared to time-matched unstimulated dM.

695 (d) Ratio of IRF5 to actin expression by R848-stimulated and unstimulated dM on

696 days 3 and 7 of culture.

697 (e) dM cytokine release after 3 days of culture.

698 Mean ± SEM of 6 independent donors. # = statistical comparison of MFI values

699 between R848-stimulated versus unstimulated dM at the same time.

700

701 **Figure 8. TLR7/8 triggering restricts HIV-1 replication after the reverse**

702 **transcription step and at or before the integration step.**

703 R848-stimulated and unstimulated (US) dM were challenged with HIV-1/VSV-G on

704 day 3 (n=13) (a) or day 7 (n=6) (b) of culture. HIV-1 replication was monitored by

705 measuring luciferase activity. The % of luciferase activity inhibition was determined

by comparison with time-matched unstimulated dM (c).

(d) R848-stimulated and US dM were challenged with HIV-1$_{BaL}$ on day 3 of culture.

HIV-1 p24 Ag was measured by ELISA in the culture supernatants (n=9)

Late reverse transcription products (U5GAG) (n=8) (e) and integrated forms (n=8) (f)

were quantified by qPCR in dM infected with HIV-1/VSV-G on day 3. Mean ± SEM of

n independent donors.

Figure 9. TLR7/8 triggering in decidual explants induces M1 polarization of dM and restricts HIV-1 replication

Decidual explants were stimulated with R848 or left unstimulated (US).

(a) (b) (c) (d) cytokine and β-chemokine concentrations in explant supernatants after 3 days of culture.

(e) (f) (g) Marker expression on CD45$^+$ CD14$^+$ dM after explant digestion on day 7 of culture, as measured by flow cytometry of total decidual cells. The % change in MFI induced by R848 and IFN-γ was determined by comparison with time-matched unstimulated explants.

(h) Explants were stimulated with R848 or left untreated prior to challenge with HIV-1$_{BaL}$. HIV-1 p24 Ag was measured by ELISA in explant supernatants.

Mean ± SEM of 7 independent donors. # = statistical comparison of MFI values between R848-stimulated versus unstimulated dM.

Figure 10. Integrated model of the inter-dependent factors involved in dM differentiation and natural resistance to HIV-1 infection.

In case of HIV-1 infection, p21 blocks HIV-1 replication at the reverse transcription

731 and integration steps (1). In parallel, TLR7/8 activation by HIV-1 sequences (2), add

732 an extra brake after the reverse transcription step and at or before the integration

733 step (3). TLR7/8 triggering may also limit viral entry by inducing β-chemokine

734 secretion (4) and by down-regulating HIV-1 receptor and co-receptor expression (5).

735 In addition, TLR7/8 triggering induces IFN-γ and TNF-α release from dNK cells (6),

736 which favors an M1 switch of neighboring dM (7) and increases p21 expression (8).

737 In parallel, TLR7/8 induce IL-1RA and IL-10 secretion that restrain TLR-induced

738 inflammatory activation and tissue damage (9). In conclusion, the decidual pro/anti-

739 inflammatory cytokine balance shape the phenotype of dM and their permissivity to

740 HIV-1 infection (10).

Figure 1

a
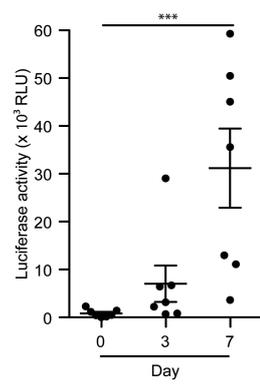
b
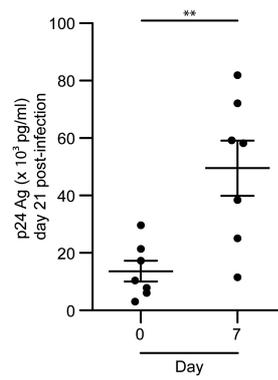
Figure 2

Figure 3

Figure 4

a

Luciferase activity (x 10³) RLU

***
**

US   US   IFN-γ
0   7   7
Day

b

p24 Ag (x 10³ pg/ml)

US
IFN-γ

*   *   *

0   4   8   12   16   20
Days post-infection

c

p24 Ag (x 10³ pg/ml) day 21 post-infection

**
*

US   US   IFN-γ
0   7   7
Day

Figure 5

a

b

Figure 6

a

b

c

R=0,622
P=0,004

d

e


f
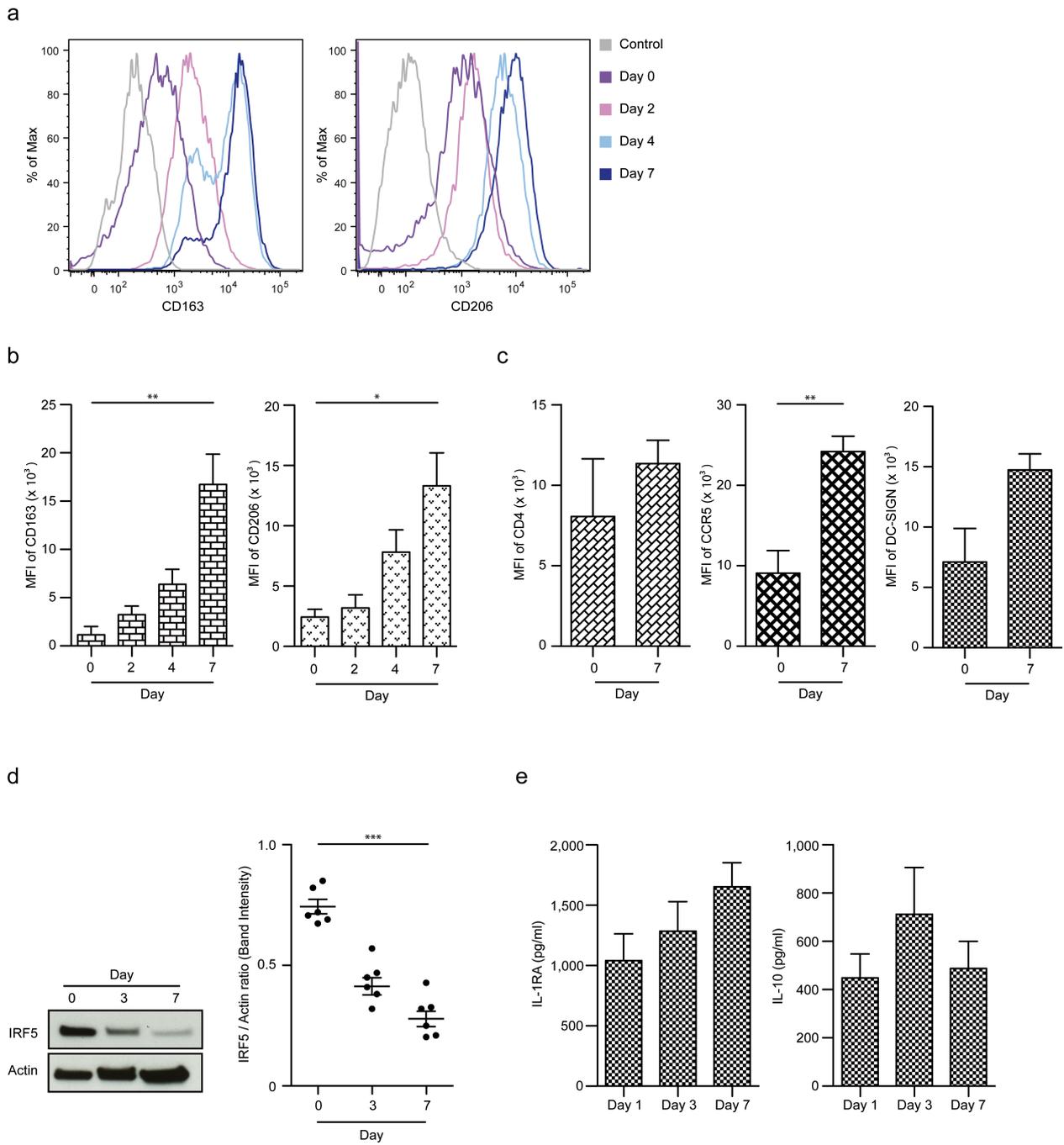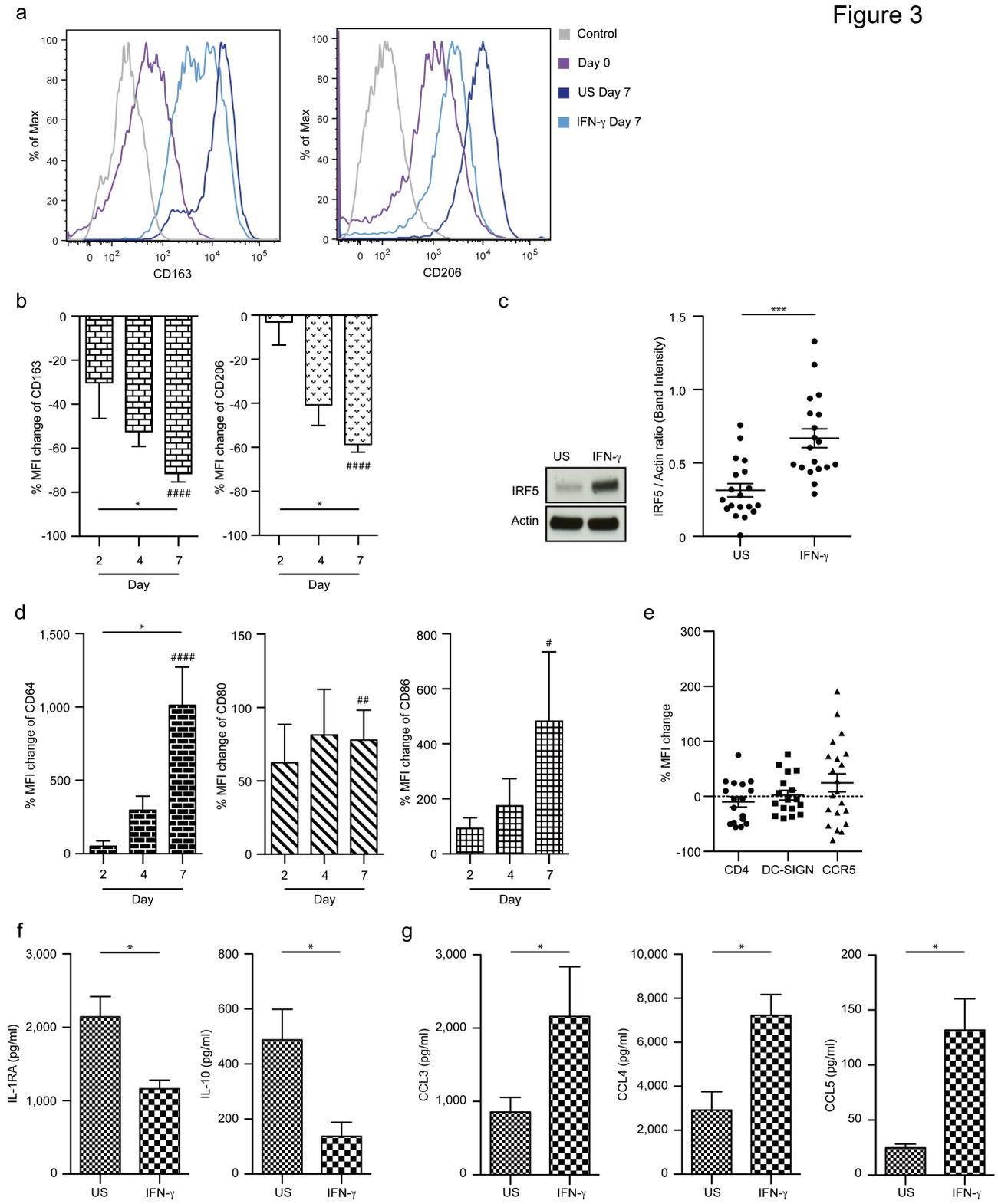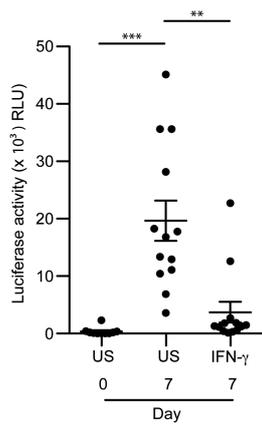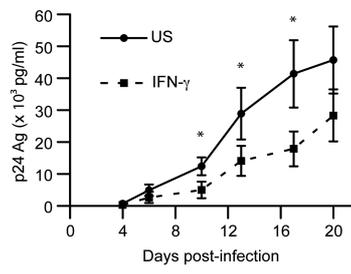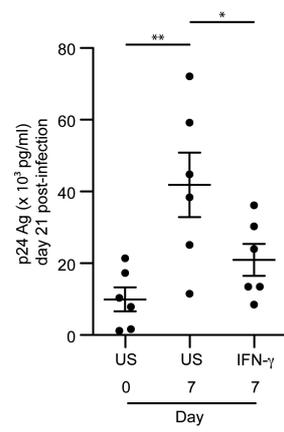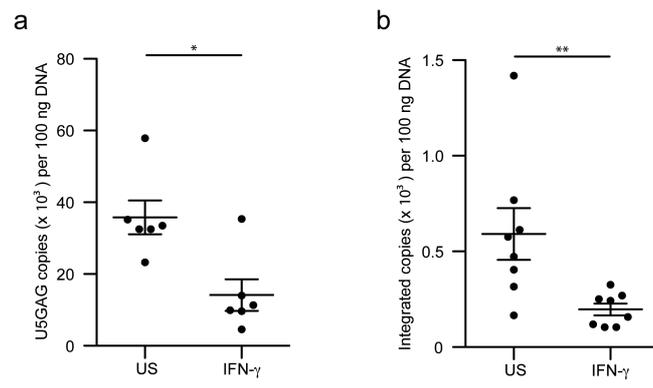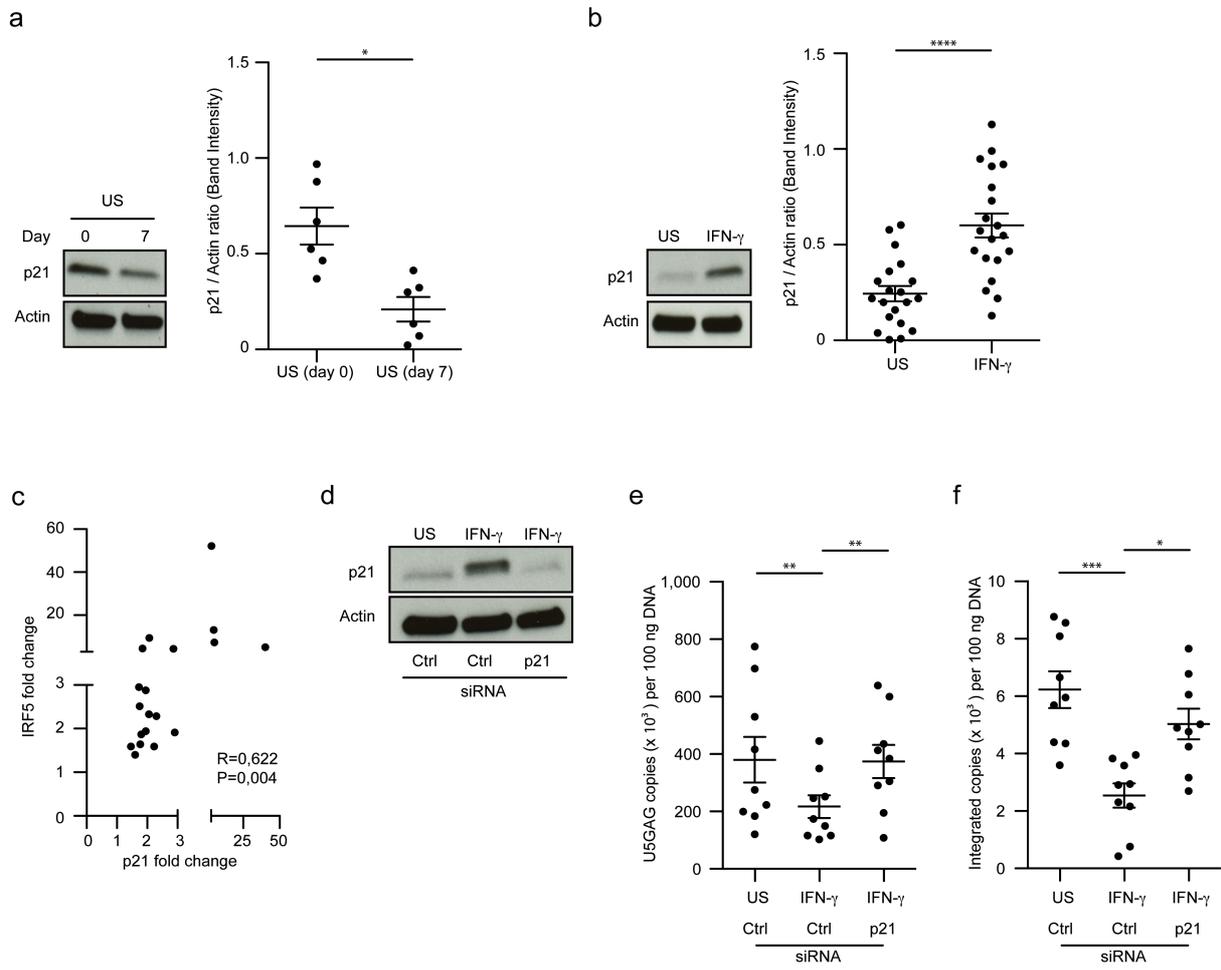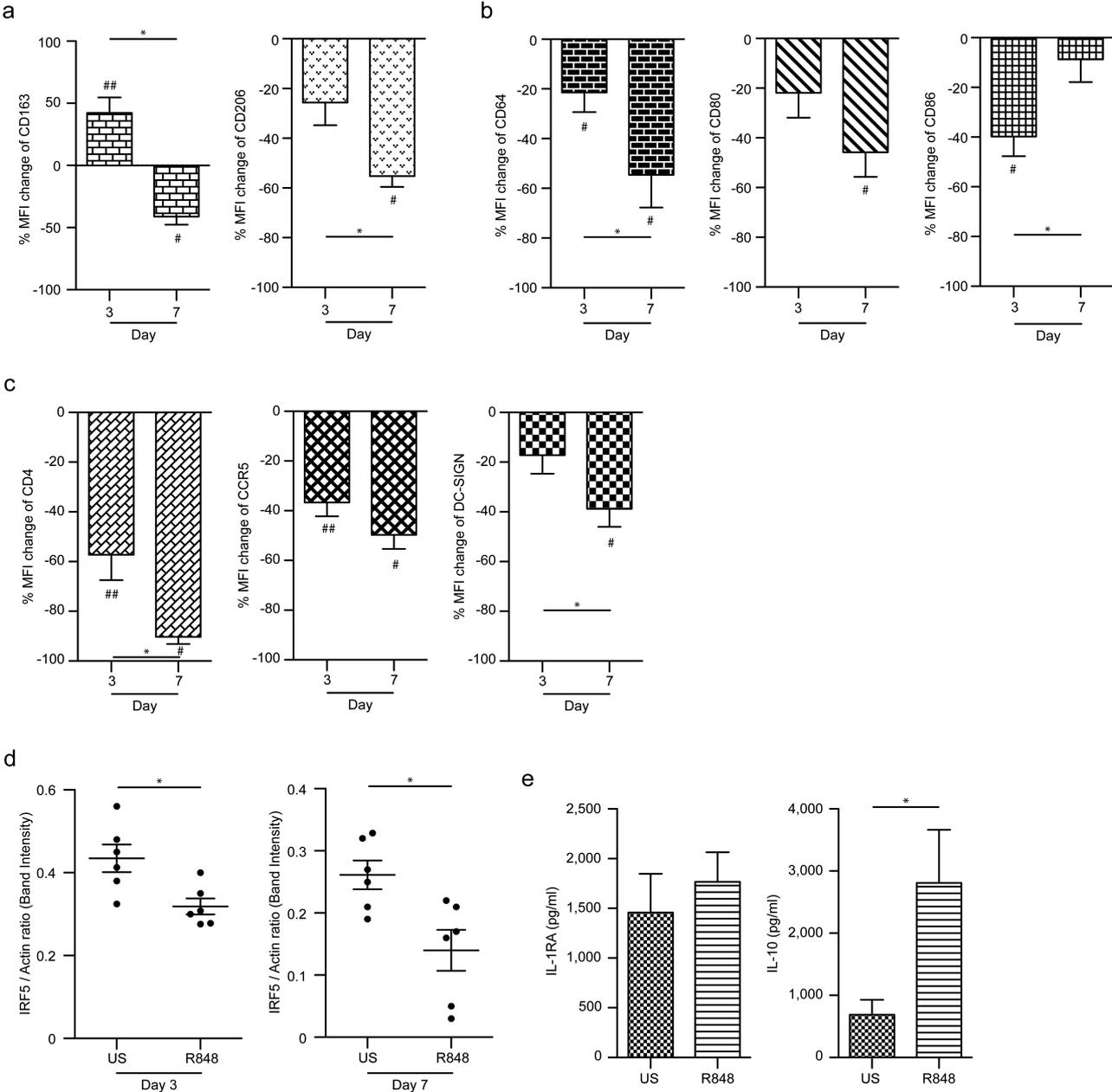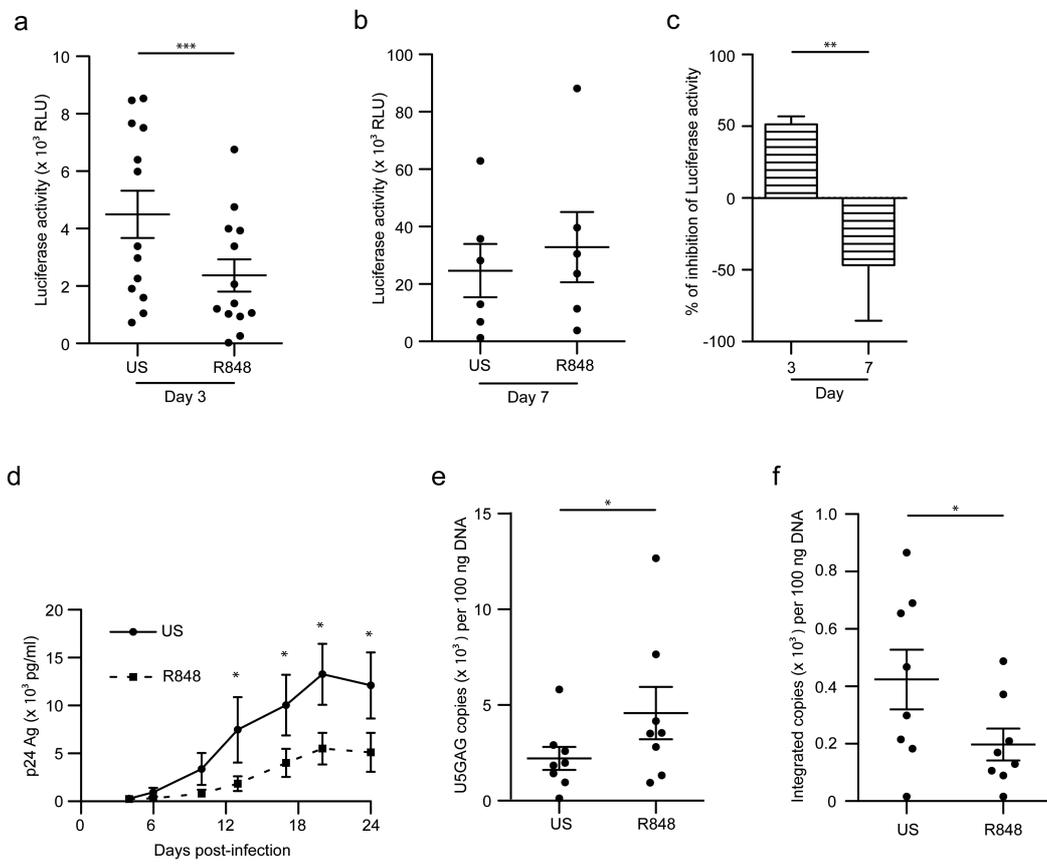
Figure 7

Figure 8

Figure 9
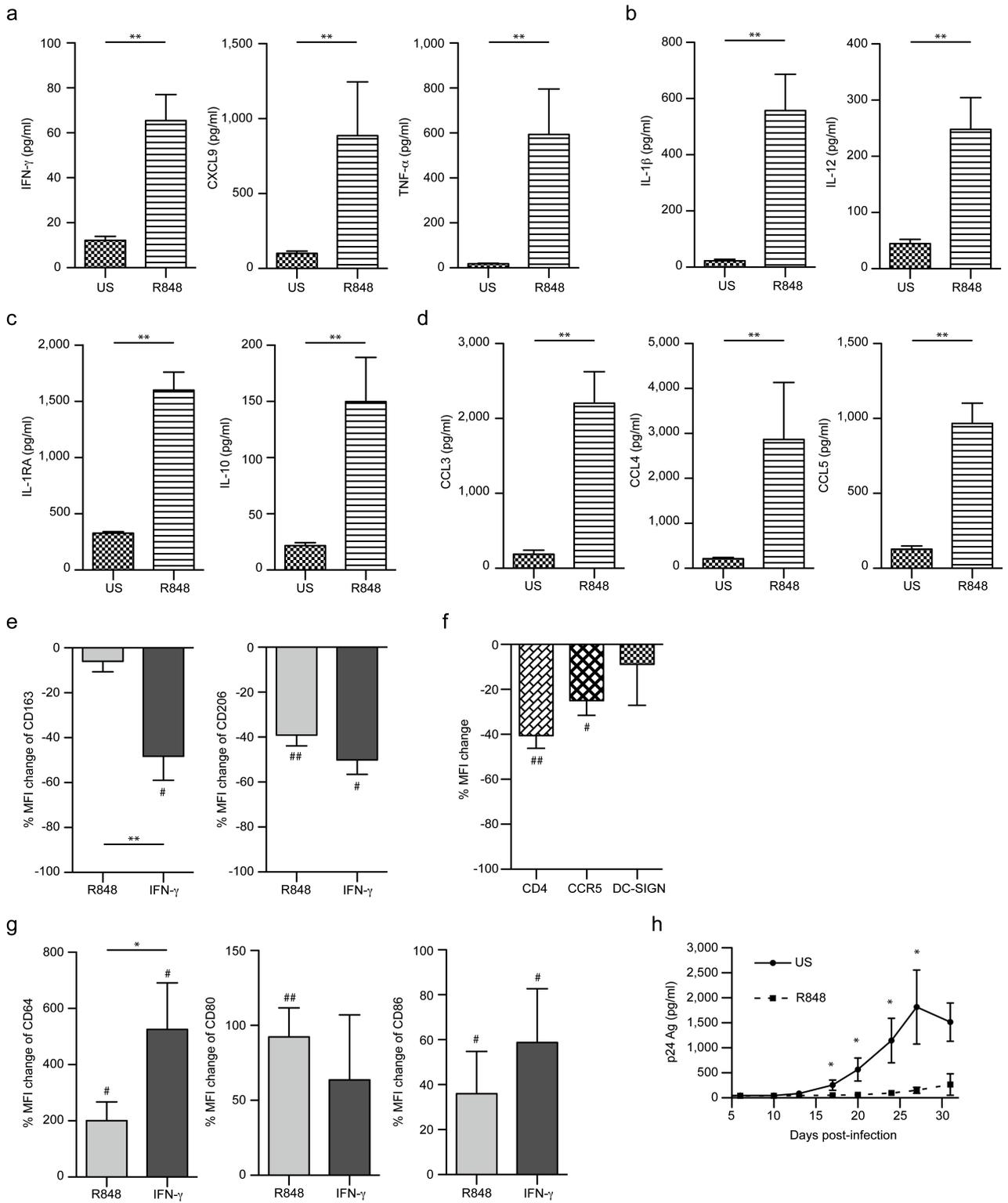
Figure 10
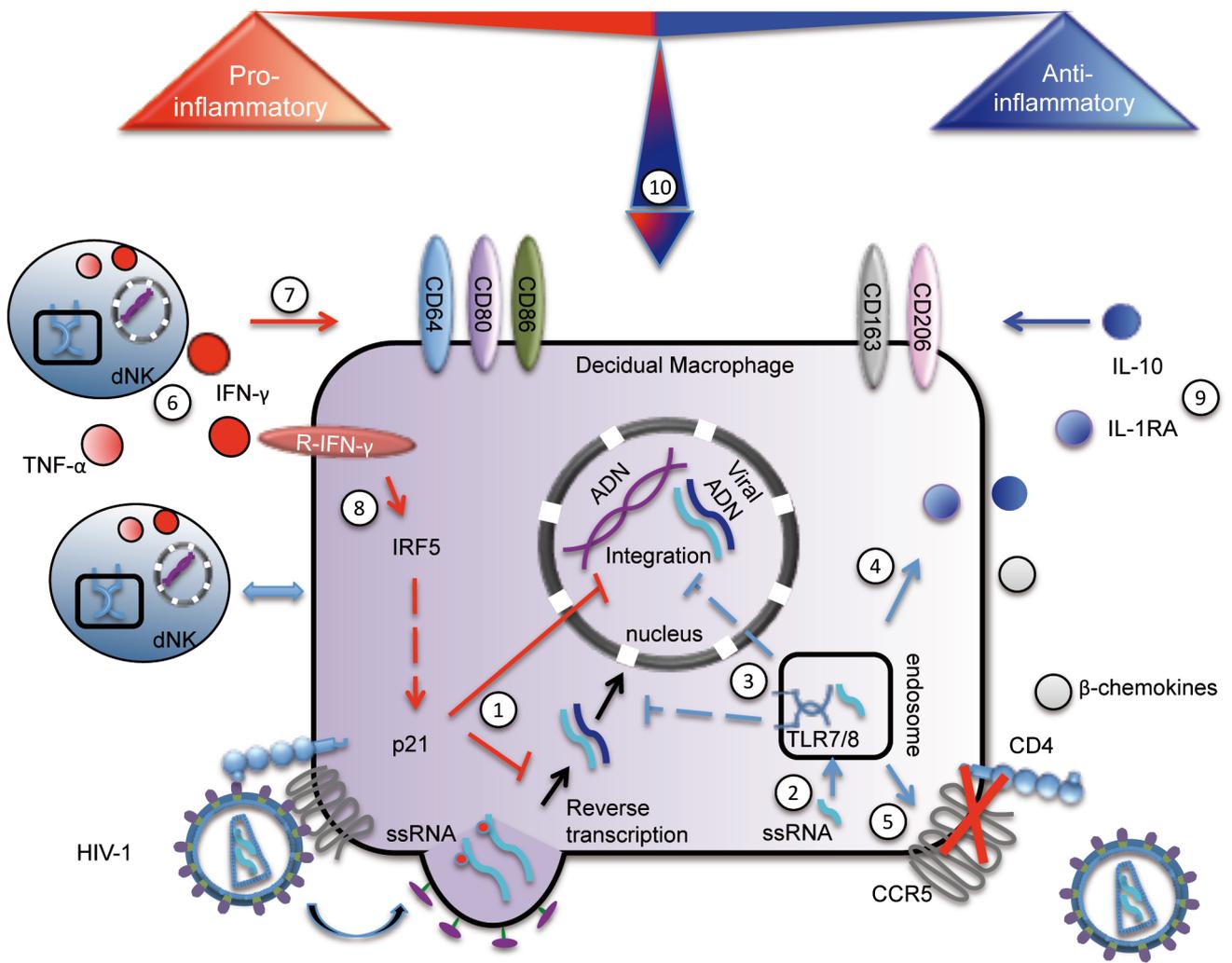
Supplementary figure legends

Supplementary Figure S1. M1 marker expression on dM in cytokine-free culture.

(a) (b) (c) CD64, CD80 and CD86 mean fluorescent intensity (MFI) over time. Mean ± SEM of 6 independent donors.

Supplementary Figure S2. dM morphology in culture.

Morphology of unstimulated (US) (a) and IFN-γ-stimulated (b) dM on days 2 and 7 of culture.

Bars = 100 μm. Original magnification X40.

Supplementary Figure S3. Viability of IFN-γ- and R848-stimulated dM.

 (a) Viability of IFN-γ−stimulated and unstimulated (US) dM after 7 days of culture, as measured by flow cytometry after labeling dead cells. A representative FACS dot plot gated on $CD45^+$ $CD14^+$ dM is shown.

(b) Viability of R848-stimulated and US dM after 3 days of culture, as measured by flow cytometry. A representative FACS dot plot gated on $CD45^+$ $CD14^+$ dM is shown.

Supplementary Materials and Methods

Human decidual tissue collection, dM isolation, and reagents

Decidual tissues were obtained from healthy women undergoing voluntary termination of pregnancy during the first trimester (8 - 12 weeks of gestation) at Antoine Béclère Hospital (Clamart, France) or Pitié-Salpêtrière Hospital (Paris,

France). After isolation, the decidua was minced and digested with 1 mg/mL collagenase IV (Sigma, St Quentin Fallavier, France) and 250 U/mL recombinant DNase I (Roche, Meylan, France) for 45 minutes at 37°C under agitation. The cell suspension was filtered successively through 100-, 70- and 40-µm sterile nylon cell strainers (BD Biosciences, Le pont de Claix, France). Mononuclear cells were isolated from the cell suspension by Ficoll gradient centrifugation using Lymphocyte Separation Medium (PAA, Les Mureaux, France). dM were purified by positive selection using anti-CD14 magnetic beads (Miltenyi, Paris, France). Their purity was checked by flow cytometry and was 93% ± 3.8% (mean + SEM). Contaminating cells were mainly CD45$^-$ cells. dM were cultured at 2x106 cells/mL in HamF12 and DMEM Glutamax (Gibco, Cergy Pontoise, France) supplemented with 15% fetal calf serum (PAA), penicillin (0.1 U/mL) and streptomycin (10-8 g/L). dM were stimulated for 3 or 7 days prior to various assays with R848 at 5 µg/ml for TLR7/8 activation (Invivogen, Toulouse, France) and with IFN-γ at 100 ng/ml for polarization switch studies (R&D, Lille, France).

HIV-1 isolates and infection

For single-round infection, we used HIV-1 particles containing the luc reporter gene and pseudotyped with the vesicular stomatitis virus G protein (HIV-1/VSV-G). HIV1/ VSV-G was produced by transient cotransfection of HEK293T cells with proviral pNL4-3 Nef $^-$ Env $^-$ Luc $^+$ DNA and the pMD2 VSV-G expression vector. Supernatants containing pseudotyped viruses were harvested 72 h after transfection, passed through 0.45-nm pore-size filters and stored at - 80°C. The viral stocks were titrated on HeLa P4P cells. dM were spinoculated with the HIV-1/VSV-G pseudotype (600 ng of p24 Ag/10^6 cells) for 30 minutes at 1200 g then incubated at 37°C for 30

minutes. The efficiency of infection by the viral pseudotype was determined 72h later by luciferase assay in cell lysates, using the Luciferase reagent (Promega, Lyon, France) on a Glomax luminometer, according to the manufacturer's instructions. For productive infection, HIV-1$_{BaL}$ was used. HIV-1$_{BaL}$ was amplified for 11 days in PHA-stimulated PBMC depleted of CD8^{+} cells, from three blood donors. The virus was concentrated by centrifugation on Vivaspin 100 000-Kda columns (Sartorius, Palaiseau, France) at 2000 g for 30 minutes, and was titrated on PBMC. dM were incubated for 1 hour with HIV-1$_{BaL}$ at 10^{-3} MOI at 37°C. Culture supernatants were collected every 3 or 4 days and stored at -80°C. HIV-1BaL production was measured by ELISA (Zeptometrix, Franklin, Massachusetts, USA) measurement of the viral core antigen p24 (p24 Ag) in culture supernatants, according to the manufacturer's instructions.

Decidual explants

Decidual tissues were cut into 0.3 cm^{2} pieces. For flow cytometry, explants were placed on collagen sponge gels (Pfizer, Paris, France) in 1.5 ml of medium/well/sponge/piece prior to stimulation with R848 or IFN-γ. The decidual tissue and sponge were then minced and digested with 1 mg/mL collagenase IV (Sigma, St Quentin Fallavier, France) and 250 U/mL recombinant DNase I (Roche, Meylan, France) for 20 minutes at 37°C under agitation. The cell suspension was filtered through 40-μm sterile nylon cell strainers (BD Biosciences, Le pont de Claix, France). The total cell population was used for flow cytometry. Explants were stimulated overnight with R848 then infected with HIV-1$_{BaL}$ for 12h. After several washes, explants were placed on collagen sponge gels. Experiments were all performed in triplicate and the mean values are reported in the figures.

Flow cytometry of cell surface markers

Adherent dM were washed with cold EDTA/PBS (2 mM) and detached from the plastic plates by pipetting without scraping. Cells were labeled with anti-CCR5 (APC-Cy7,clone 2D7, BD), anti-CD14 (Pacific Blue, clone 3.9, BD), anti-CD163 (PE, clone GHI/61, BD), anti-CD206 (APC, clone 19.2, BD), anti-CD4 (PE-Cy7, clone SK3, BD), anti-CD45 (Amcyan, clone 2D1, BD), anti-CD64 (FITC, clone 10.1, BD), anti-CD80 (PE, clone 307/4, BD), anti-CD86 (PE, clone 2331, BD), and anti-DC-SIGN (PE, clone 120507, R&D) (R&D, Lille, France). dM were incubated with an FcR blocking reagent (Miltenyi, Paris, France) before staining with the conjugated antibodies for 20 minutes at 4°C. After two EDTA/PBS washes, cells were fixed in 1% paraformaldehyde, and surface marker expression was determined with an LSRII 2-Blue 2-Violet 3-Red 5-Yelgr-laser configuration (BD Biosciences, Le pont de Claix, France). The results were analyzed with FlowJo 9.1.3. software (Tristar, Ashland, USA). MFI (%) was calculated as the ratio of treated dM (IFN-γ) or R848-treated dM to the unstimulated dM control.

Western blotting

Freshly isolated dM or dM cultured in 48-well plates were washed with PBS then lysed in 120 μL of lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% TritonX-100) containing a protease and phosphatase inhibitor mixture (Roche, Meylan, France). Cell extracts (10–25 μg) were hybridized with the primary antibodies, followed by secondary horseradish peroxidase anti-mouse (Cell Signaling, Leiden, Netherlands) or anti-rabbit (Sigma-Aldrich, Saint Louis, USA) antibodies. Proteins were revealed on Hyperfilms (Amersham, Velizy-Villacoublay,

France) by using the Pierce ECL western blotting substrate (Thermo Scientific, Waltham, USA). The following primary antibodies were used: anti-p21 (clone CP74, Merck Millipore, Fontenay sous Bois, France), anti-IRF5 (ab140593, abcam, Paris, France) and anti-β-actin (clone AC-74, Sigma-Aldrich, Saint Louis, USA). Primary antibodies were all used at 1 :2000 dilution in 1% skimmed milk in PBS-0.05% Tween, except for anti-b-actin (1 :10 000).

The protein band intensity was quantified with the Image J software 1,47V and normalized to the actin band. Ratios (protein / actin) were then compared between the different conditions. Fold changes of IRF5 and p21 expression were calculated by comparing the normalized value of IRF5 and p21 from the IFN-γ-treated samples to the normalized value of IRF5 and p21 from the dM (US) control from the same donor.

**HIV-1 DNA quantitative PCR (qPCR)**

Total DNA in HIV-1/VSV-G-infected dM was extracted 72 h postinfection (p.i.) by using the DNeasy kit as recommended by the manufacturer (Qiagen, California, USA). Quantitative real-time PCR analysis of late (U5GAG) forms of viral DNA was carried on an ABI Prism 7500 sequence detection system as previously described [19]. Standards for U5GAG amplification products were generated by serial dilution of DNA extracted from HIV-1 8E5 cells containing one integrated copy of HIV-1 per cell. Integrated HIV-1 DNA was quantified by real-time Alu-LTR nested PCR using primers and probes described elsewhere, with some modifications [19]. Briefly, the first round of amplification was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Saint Aubin, France). Integrated HIV-1 sequences were amplified by using the Expand High Fidelity kit (Roche, Meylan, France), two Alu primers (Alu F and Alu R)

and an LTR primer extended with an artificial tag sequence at the 5' end of the oligonucleotide (NY1R). Real-time nested PCR was run on the ABI Prism 7500 system using a 1/10 dilution of the first-round PCR product as template (primers NY2F and NY2R; probe NY2Alu). The integrated HIV-1 DNA copy number was determined with reference to a standard curve generated by concurrent amplification of HeLa R7 Neo cell DNA. A nested PCR conducted in parallel without the Alu primers in the first round gave a very weak background signal. The number of integrated HIV-1 DNA copies was obtained by subtracting the copy number measured without the Alu primers in the first round from the copy number measured in the full reaction. The amount of viral DNA was normalized to the endogenous reference albumin gene (for total DNA extracts). Standard curves were generated by serial dilution of a commercial human genomic DNA (Roche, Meylan, France).

Supplementary Figure S1

Supplementary Figure S2

Day 2   Day 7

a

US

b

IFN-γ

100μm   100μm

100μm   100μm

Supplementary Figure S3

a

b