

The local environment orchestrates mucosal decidual macrophage differentiation and substantially inhibits HIV-1 replication

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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. Interferon (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 p21^{Cip1/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 interferon (IFN)- γ and tumor necrosis factor (TNF)- α , produce pro-inflammatory cytokines such as interleukin (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 FC γ 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

Decidual macrophages have an M2-like phenotype and show features of pro-inflammatory and tolerogenic macrophages,

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simultaneously expressing M1 (CD64, CD80, CD86) and M2 markers (CD163, CD206).^{2,8}

Another hallmark of the *decidua basalis* is the abundance of decidual natural killer cells (dNK). The dNK cells secrete large amounts of IFN- γ and TNF- α and are involved in the control of fetal cell invasion and initiation of endometrial vasculature. These cells may also limit the spreading of viral infection to fetal tissues by controlling intrauterine infection by hCMV⁹ and HIV-1 (Quillay *et al.*, manuscript under revision).

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

The aim of this work was to determine why dM are weakly permissive to HIV-1 infection during the first trimester of pregnancy, with a view of developing of novel strategies for eradicating macrophage infection. We postulated that the decidual environment must contain factors that shape dM plasticity and susceptibility to HIV-1 infection, and that TLR7/8, if activated, can reinforce this low dM 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 vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 (HIV-1/VSV-G), immediately after purification (day 0) or after 3 or 7 days of culture in a 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 (**Figure 2a** and **b**), whereas expression of M1 markers (CD64, CD80, CD86) remained unchanged (**Supplementary Figure S1** online). 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),

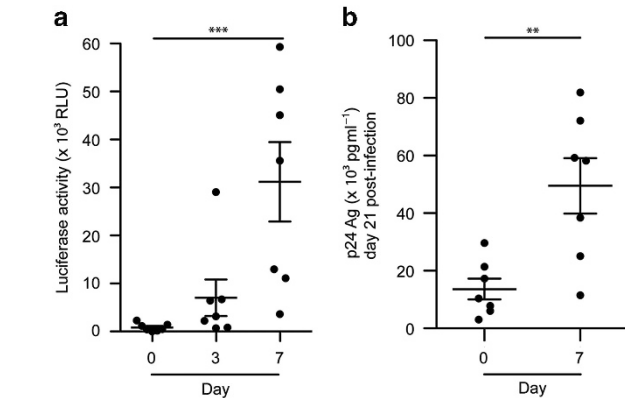


Figure 1 dM become more permissive to HIV-1 infection in cytokine-free culture. **(a)** Freshly purified dM were challenged with HIV-1/VSV-G at day 0, 3, and 7 of culture. HIV-1 replication was monitored by measuring luciferase activity ($n=7$). **(b)** dM were challenged with HIV-1_{BaL} immediately 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 \pm s.e.m. of n independent donors. dM, decidual macrophages.

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 decreased gradually in culture (**Figure 2d**). Analysis of cytokine secretion showed that dM maintained their secretion of M2 cytokines (IL-1RA and IL-10) over time (**Figure 2e**).

Together, these results indicate that dM isolated from their local environment acquire more M2 features and that this switch is accompanied by an increase in susceptibility to HIV-1 infection.

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

The next step was to identify the decidual environmental factor(s) that, when lacking in culture, trigger the dM polarization switch and enhance susceptibility to HIV-1 infection. IFN- γ , present in the decidua, is one of the most potent stimuli of endogenous M1 macrophage activation and have a crucial role in immune responses against pathogens and in tumor immunosurveillance.¹⁷ We thus investigated whether IFN- γ affect dM plasticity and susceptibility to HIV-1 infection.

IFN- γ treatment immediately after dM purification attenuated the increase in M2 marker expression on day 7, as illustrated by CD163 and CD206 expression in **Figure 3a** and **b**. In addition, no morphological change occurred when IFN- γ was added to the culture medium (**Supplementary Figure S2b**). Seven days of IFN- γ treatment restored IRF5 expression to the level found in freshly purified dM (**Figure 3c**). In parallel, M1 markers (CD64, CD80, and CD86) were strongly upregulated by IFN- γ (**Figure 3d**). However, CD4, CCR5, and DC-SIGN expression

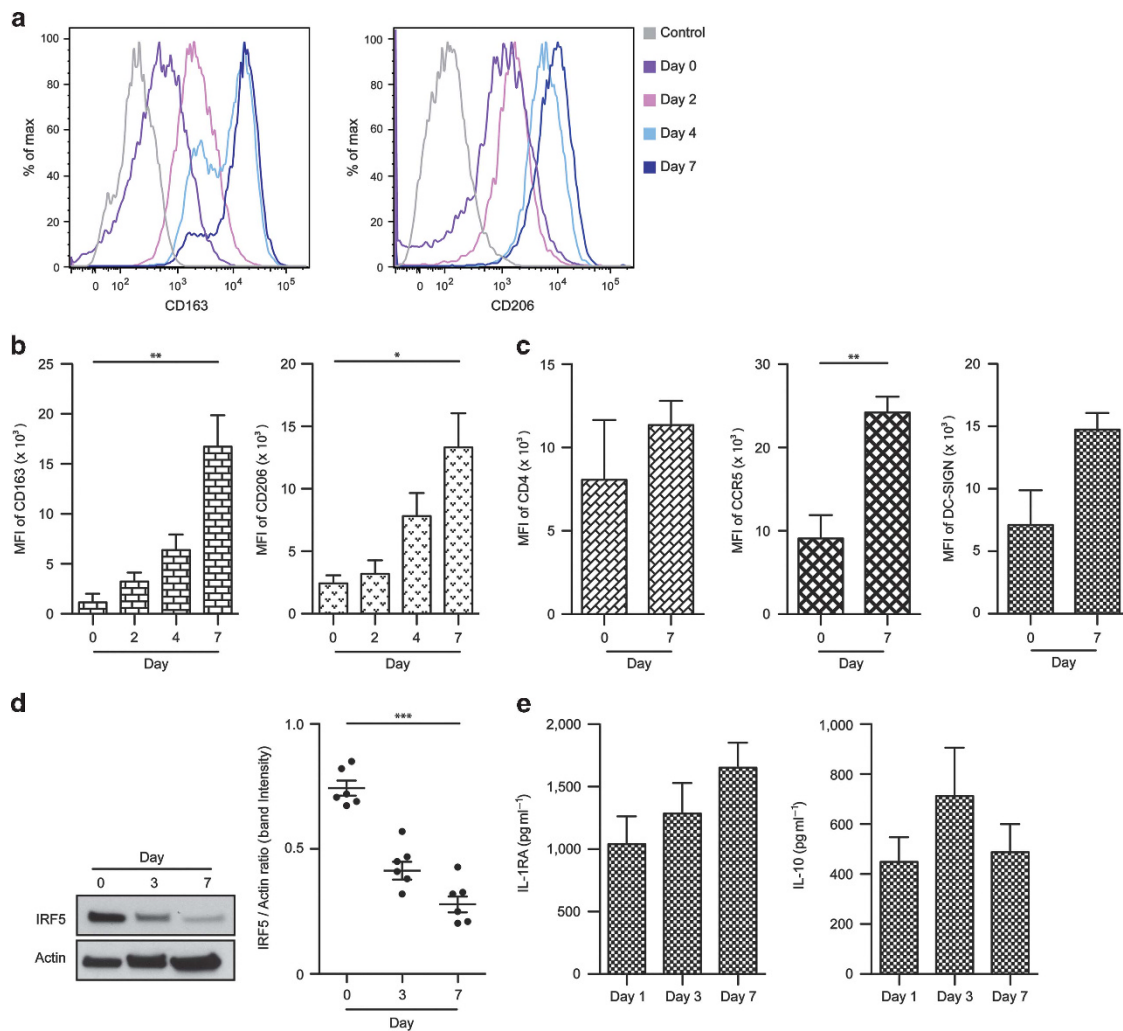


Figure 2 dM switch towards a more M2 profile in cytokine-free culture. **(a)** Representative histograms of CD163 and CD206 expression on dM, as measured by flow cytometry. **(b)** CD163 and CD206 mean fluorescent intensity (MFI) over time ($n = 6$). **(c)** CD4, CCR5 and DC-SIGN MFI on day 0 and on 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 \pm s.e.m. of n independent donors. dM, decidual macrophages; MFI, mean fluorescent intensity.

on IFN- γ -treated dM varied greatly among donors (**Figure 3e**). Significantly lower IL-10 and IL-1RA secretion (**Figure 3f**) and higher chemokine secretion (CCL3, CCL4, and CCL5) (**Figure 3g**) were detected on day 7 of IFN- γ treatment. Thus, 7 days of IFN- γ 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) (**Figure 4a** and **c**).

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 a 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

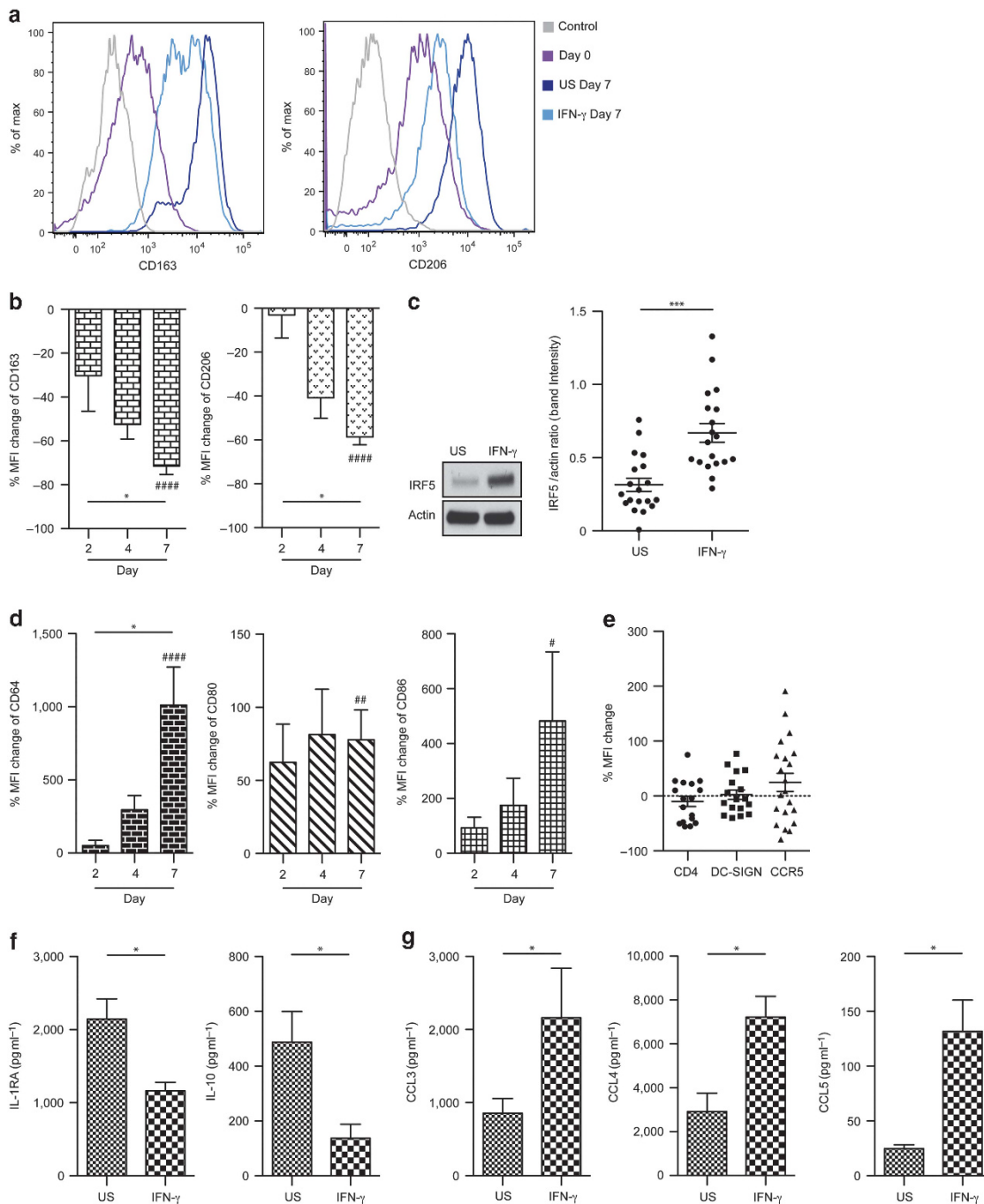


Figure 3 IFN- γ addition to cultured dM restores partially the phenotype of freshly purified dM. **(a)** Representative histograms of IFN- γ -induced modulation of CD163 and CD206 expression on day 7, as measured by flow cytometry. **(b)** IFN- γ -induced change (%) in CD163 and CD206 expression (MFI) on dM compared with time-matched US dM ($n=6$). **(c)** Representative western blot of IRF5 expression by IFN- γ -stimulated and US dM on day 7 of culture (left panel). The protein band intensity was quantified and normalized to the actin band. Ratio (IRF5/actin) was then compared between IFN- γ -stimulated and US dM on day 7 of culture (right panel) ($n=19$). **(d)** IFN- γ -induced change (%) in CD64, CD80, and CD86 expression (MFI) on dM, as compared with time-matched US dM ($n=6$). **(e)** IFN- γ -induced changes (%) in CD4, DC-SIGN, and CCR5 expression (MFI) on dM on day 7 of culture, by comparison with US dM ($n=17$). **(f)** dM cytokine and **(g)** β -chemokine release after 7 days of culture ($n=7$). Mean \pm s.e.m. of n independent donors. # = statistical comparison of MFI values between IFN- γ -stimulated dM vs. US dM at the same time. dM, decidual macrophages; IFN, interferon; MFI, mean fluorescent intensity; US, unstimulated.

with those found in infected dM (unstimulated, 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.

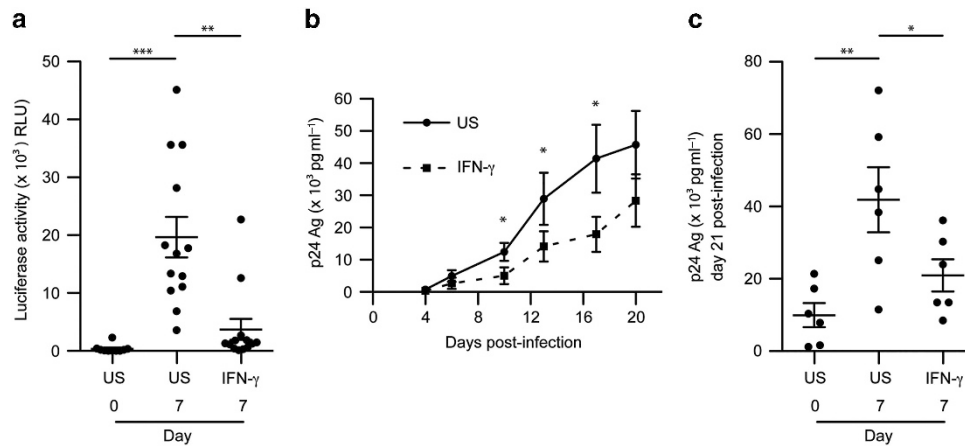


Figure 4 IFN- γ addition to cultured dM restores the resistance to HIV-1. (a) Freshly purified dM and 7-day IFN- γ -stimulated and US dM were challenged with HIV-1/VSV-G. HIV-1 replication was monitored by measuring luciferase activity in cell lysates ($n=13$). (b) IFN- γ stimulated and US dM were challenged with HIV-1_{BaL} on day 7 of culture. 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 US dM ($n=6$). Mean \pm s.e.m. of n independent donors. dM, decidual macrophages; IFN, interferon; US, unstimulated; VSV-G, vesicular stomatitis virus G protein.

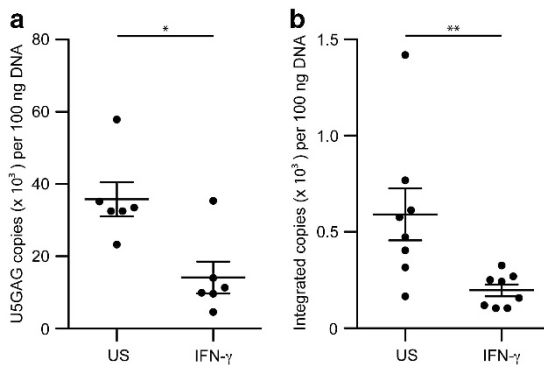


Figure 5 IFN- γ restricts HIV-1 infection of dM at the reverse transcription and integration steps. Seven-day IFN- γ -stimulated and US dM were challenged with HIV-1/VSV-G. Late reverse-transcription products (U5GAG) ($n=6$) (a) and integrated forms ($n=8$) (b) were quantified by qPCR. Mean \pm s.e.m. of n independent donors. dM, decidual macrophages; IFN, interferon; qPCR, quantitative PCR; US, unstimulated; VSV-G, vesicular stomatitis virus G protein.

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) (Figure 6e and f). 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 b), 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 expression and low permissivity to infection. Together, these findings suggest that p21 is involved in the control of HIV-1 replication in decidual tissue.

TLR7/8 triggering does not alone induce dM polarization towards an M1 or M2 profile but transiently blocks HIV-1 replication

We have previously shown that dM express functional TLR7/8. We wondered whether these receptors, if activated after HIV-1 sensing, could influence dM polarization and reduce dM susceptibility to infection, as shown for monocytes and MDM.^{11–14}

We first checked whether dM stimulation by a specific agonist (R848) that mimics HIV-1 recognition by TLR7/8 induced a dM polarization switch. dM were treated with R848 immediately after purification or left untreated. TLR7/8 triggering transiently increased CD163 expression on dM after 3 days of R848 treatment, whereas CD206 and M1 expression fell gradually (Figure 7a and b). TLR7/8 engagement also downregulated CD4, CCR5, and DC-SIGN expression (Figure 7c). TLR7/8 triggering by R848 did not induce morphological changes in culture, by comparison with dM (US) (data not shown). Moreover, IRF5 expression was

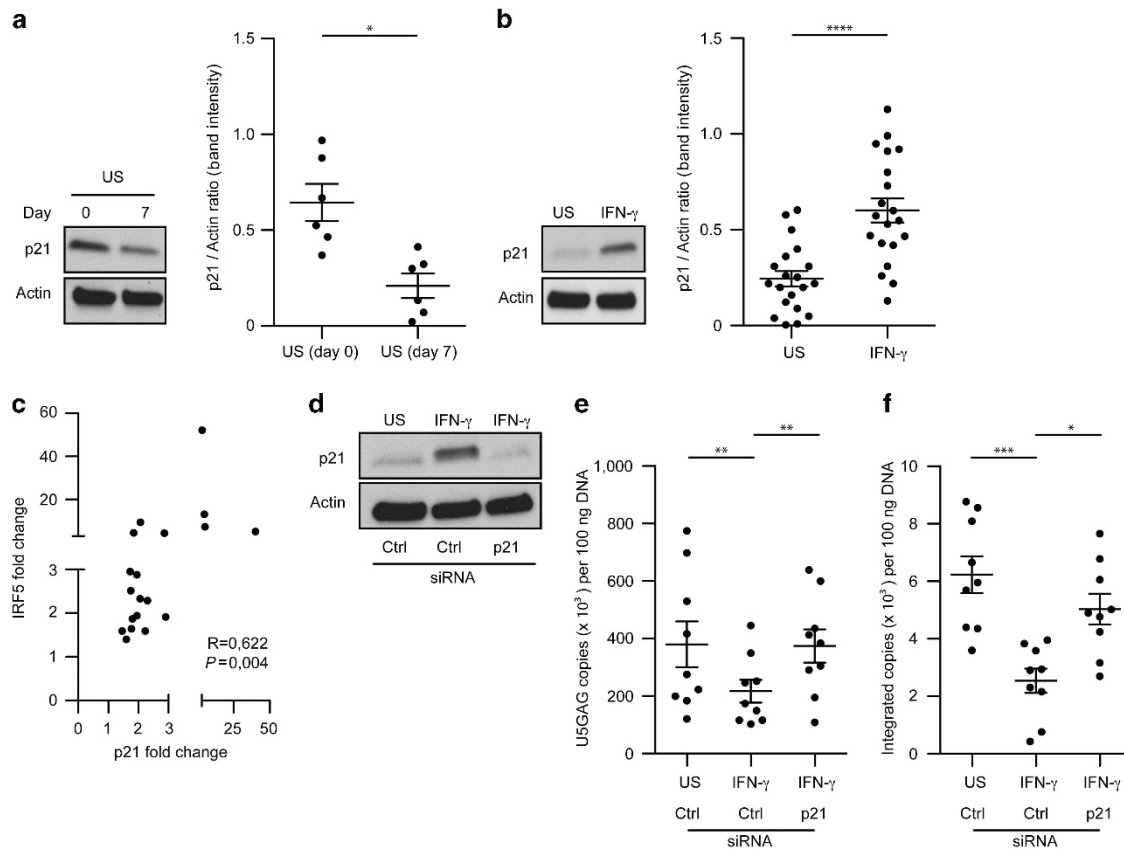


Figure 6 p21 is involved in low dM permissivity to HIV-1. **(a)** Representative western blot of p21 expression by freshly purified dM (day 0) and by US dM at day 7 of culture (left panel). The protein band intensity was quantified and normalized to the actin band. Ratio (p21/actin) was then compared between dM at day 0 and day 7 (right panel) ($n=6$). **(b)** Representative western blot of p21 expression by IFN- γ -stimulated and US dM on day 7 of culture (left panel). Ratio (p21/actin) was then compared between the 7-day IFN- γ -stimulated and 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 with time-matched US dM ($n=19$). Spearman's R value is shown. **(d)** Representative western blot of p21 expression in 7-day IFN- γ -stimulated and US dM transfected with p21-specific siRNA or with an irrelevant control siRNA (Ctrl) ($n=9$). **(e)** Number of late reverse transcription products (U5GAG) and **(f)** integrated forms in dM transfected with p21-specific or control siRNA ($n=9$). Mean \pm s.e.m. of n independent donors. dM, decidual macrophages; IFN, interferon; siRNA, short interfering RNA; US, unstimulated.

downregulated in R848-treated dM (**Figure 7d**). These changes were accompanied by higher IL-10 release by R848-stimulated dM compared with US dM, whereas IL-1RA secretion was not modified (**Figure 7e**). Together, these findings show that TLR7/8 activation by itself is not sufficient to fully polarize dM towards an M1 or M2 profile.

We then assessed the capacity of R848-stimulated dM to support HIV-1 infection. dM were treated with R848 directly after purification or left untreated, then challenged with HIV-1/VSV-G after 3 days (**Figure 8a**) or 7 days (**Figure 8b**) 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 the accumulation of late reverse transcription 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 with US 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

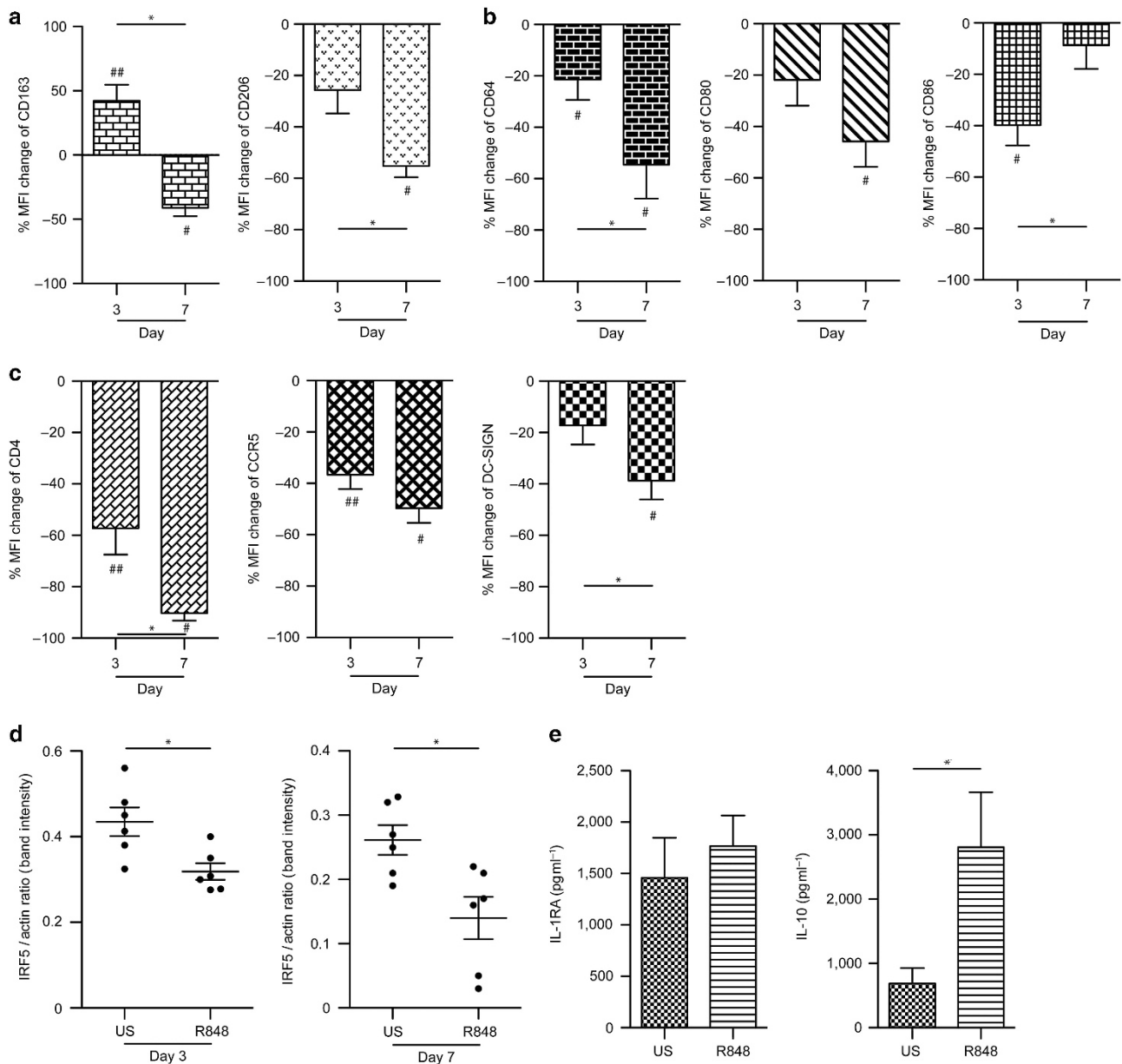


Figure 7 TLR7/8 triggering is not sufficient to induce dM polarization towards an M1 or M2 profile. **(a)** % change in dM CD163 and CD206, **(b)** CD64, CD80, and CD86 expression (MFI) induced by R848 as compared with time-matched unstimulated dM. **(c)** % change in dM CD4, DC-SIGN, and CCR5 expression (MFI) induced by R848 as compared to time-matched unstimulated dM. **(d)** Ratio of IRF5 to actin expression by R848-stimulated and unstimulated dM on days 3 and 7 of culture. **(e)** dM cytokine release after 3 days of culture. Mean \pm s.e.m. of 6 independent donors. # = statistical comparison of MFI values between R848-stimulated vs. unstimulated dM at the same time. dM, decidual macrophages; MFI, mean fluorescent intensity; TLR, Toll-like receptor.

TNF- α was increased in R848-treated explants (**Figure 9a**), as was the secretion of both M1 (IL-1 β 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 were digested and the resulting cell suspensions were analyzed by flow cytometry. Untreated and IFN- γ -treated explants were used as negative and positive controls, respectively. TLR7/8 triggering downregulated the M2 marker CD206 (**Figure 9e**) as well as HIV-1 receptors and

co-receptors (**Figure 9f**) on the dM surface, and enhanced M1 marker expression (CD64, CD80, and CD86) (**Figure 9g**). This phenotype modulation was similar to but less marked than that observed with IFN- γ -treated explants.

Finally, we checked whether R848-treated explants controlled HIV-1 replication, by adding a TLR7/8 agonist to the explants prior to challenge with HIV-1_{BaL}. HIV-1_{BaL} infection was consistently inhibited in TLR7/8-stimulated explants, as shown by p24 Ag measurement (**Figure 9h**). The viability of R848-treated explants was similar to that of untreated controls (data not shown).

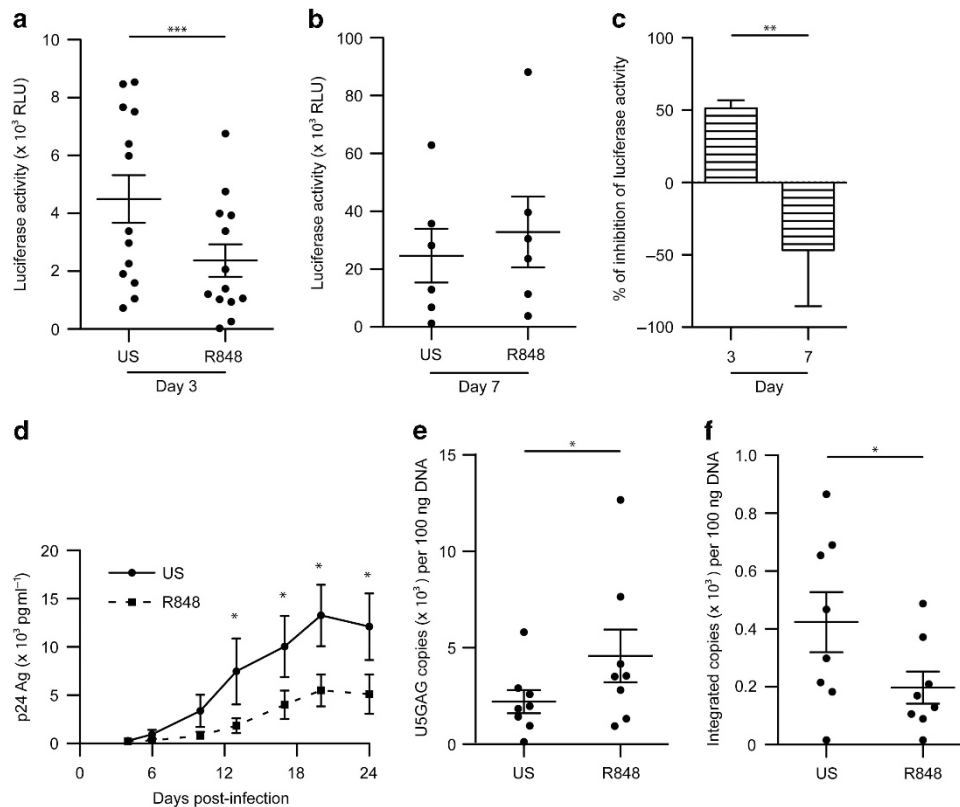


Figure 8 TLR7/8 triggering restricts HIV-1 replication after the reverse transcription step and at or before the integration step. R848-stimulated and US dM were challenged with HIV-1/VSV-G on day 3 ($n=13$) (a) or day 7 ($n=6$) (b) of culture. HIV-1 replication was monitored by measuring luciferase activity. The % of luciferase activity inhibition was determined by comparison with time-matched US 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 \pm s.e.m. of n independent donors. dM, decidual macrophages; qPCR, quantitative PCR; TLR, Toll-like receptor; US, unstimulated; VSV-G, vesicular stomatitis virus G protein.

TLR7/8 stimulation of decidual explants thus induced dM polarization toward an 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 determines 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 have 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 between these different environmental signals likely accounts for the unique phenotype of freshly purified dM.

In parallel to the polarization changes observed in the cytokine-free culture, we found that freshly purified dM became more permissive to HIV-1 infection over time. Only CCR5 expression increased significantly over time, possibly playing a role in the increased susceptibility to HIV-1_{BaL} infection, but this would not explain why dM became more sensitive to infection by HIV-1/VSV-G, which does not require the presence of HIV-1 receptors.

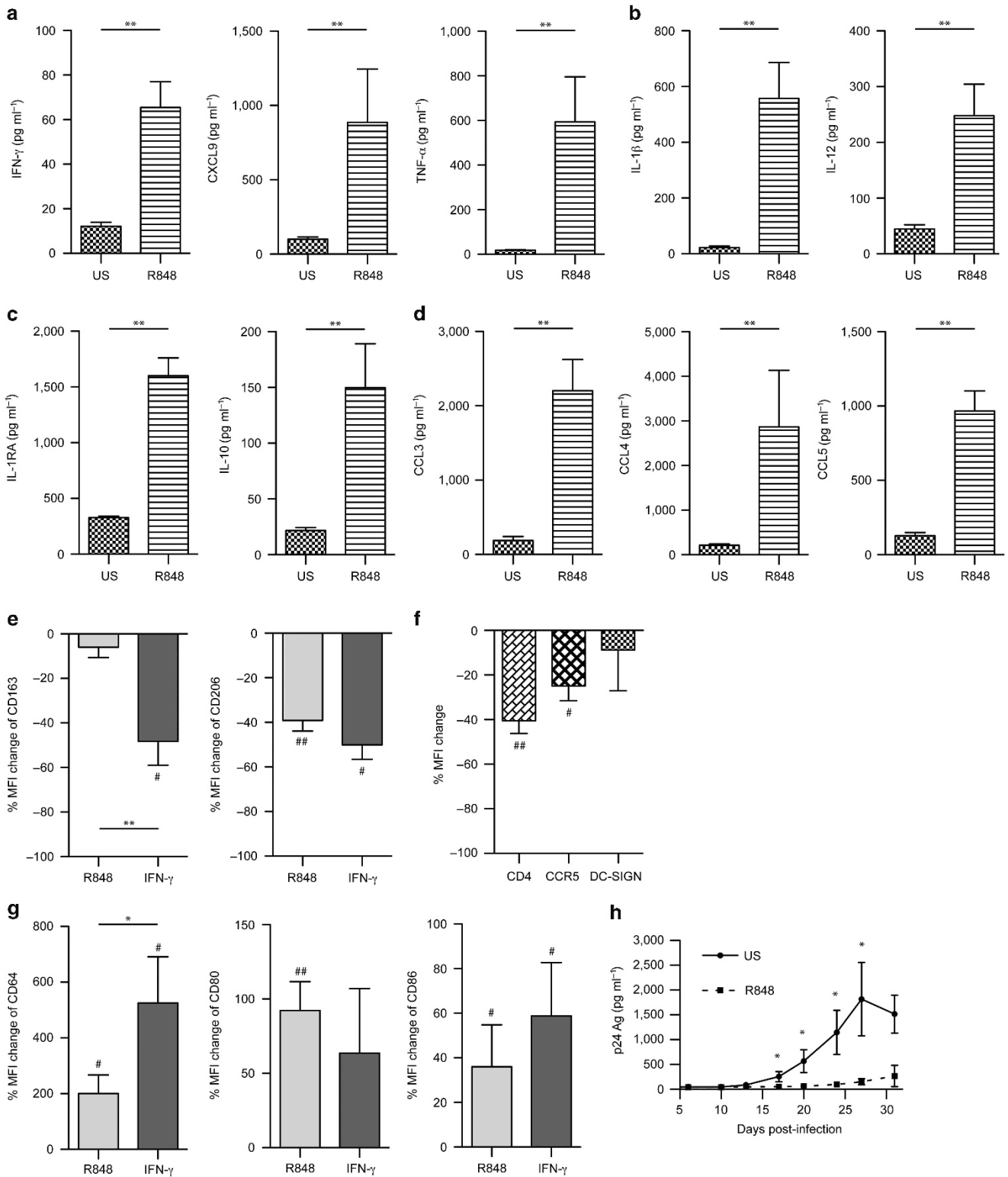


Figure 9 TLR7/8 triggering in decidual explants induces M1 polarization of dM and restricts HIV-1 replication. Decidual explants were stimulated with R848 or left US. **(a–d)** Cytokine and β -chemokine concentrations in explant supernatants after 3 days of culture. **(e–g)** Marker expression on CD45⁺ 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 US 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 \pm s.e.m. of 7 independent donors. # = statistical comparison of MFI values between R848-stimulated vs. US dM. dM, decidual macrophages; MFI, mean fluorescent intensity; TLR, Toll-like receptor; US, unstimulated.

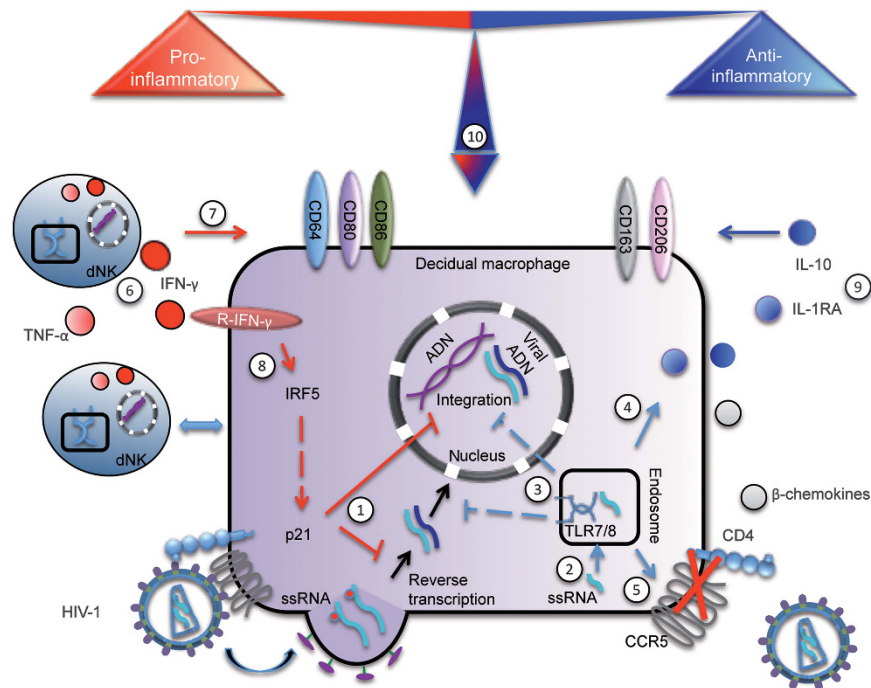


Figure 10 Integrated model of the inter-dependent factors involved in dM differentiation and natural resistance to HIV-1 infection. In case of HIV-1 infection, p21 blocks HIV-1 replication at the reverse transcription and integration steps (1). In parallel, TLR7/8 activation by HIV-1 sequences (2) add an extra brake after the reverse transcription step and at or before the integration step (3). TLR7/8 triggering may also limit viral entry by inducing β -chemokine secretion (4) and by downregulating HIV-1 receptor and co-receptor expression (5). In addition, TLR7/8 triggering induces IFN- γ and TNF- α release from dNK cells (6), which favors an M1 switch of neighboring dM (7) and increases p21 expression (8). In parallel, TLR7/8 induce IL-1RA and IL-10 secretion that restrain TLR-induced inflammatory activation and tissue damage (9). In conclusion, the decidual pro/anti-inflammatory cytokine balance shape the phenotype of dM and their permissivity to HIV-1 infection (10). dM, decidual macrophages; dNK, decidual natural killer cells; IFN, interferon; TLR, Toll-like receptor; TNF, tumor necrosis factor.

IFN- γ treatment of dM in culture restored the low permissivity of freshly purified dM, strongly inhibiting the HIV-1 reverse transcription and integration steps. R5 HIV-1 entry might also be blocked, as CCR5-binding chemokines (CCL3, CCL4, and CCL5) are strongly induced by IFN- γ .²⁵

Thus, our data demonstrate that dM polarization status is responsible for the low permissivity to HIV-1 infection.

As freshly purified dM switch rapidly to an M2 profile in a cytokine-free culture, and as IFN- γ prevents this switch (in terms of the phenotype and resistance to infection), we used IFN- γ -treated dM to examine the mechanisms underlying the low permissivity of freshly purified dM, and found that p21 inhibited the reverse transcription and integration steps. This is consistent with a previous study showing that IRF5 modulates the expression of growth-regulating and pro-apoptotic genes, including p21.¹⁸ However, we cannot exclude the possibility that other IFN- γ -induced genes may modulate p21 expression.²⁰ We also found that IFN- γ treatment restored p21 expression in culture to a level similar to that of freshly purified dM, suggesting that p21 is involved in HIV-1 restriction in decidual tissue. p21 is known to restrict HIV-1 replication in macrophages either by inhibiting dNTP biosynthesis through an RNR2-dependent pathway²⁶ or by modulating the phosphorylation of SAMHD1, a host restriction factor.²⁷ We have previously shown that the SAMHD1 pathway is involved in controlling HIV-1 infection of freshly purified dM.⁸ RNR2 was

undetectable in dM (IFN- γ), and SAMHD1 phosphorylation was slightly lower in dM (IFN- γ) than in controls, whereas the total SAMHD-1 level remained unchanged (preliminary data not shown). Thus, in decidual tissue, p21 might act through the SAMHD1 pathway.

Contrary to IFN- γ , TLR7/8 triggering alone did not induce an M1 or M2 switch. However, TLR7/8 triggering impaired HIV-1/VSV-G infection and resulted in the accumulation of late reverse transcription products, together with a marked reduction in the amount of integrated proviral DNA. These findings corroborate reports that TLR7/8 ligation impairs HIV-1 infection of monocytes and MDM.^{12–14,28} However, as in previous studies,^{11,29} we found no correlation between the TLR7/8-induced antiviral effect and IFN- α secretion, which was undetectable (data not shown). TLR7/8 engagement down-modulated CD4, CCR5, and DC-SIGN expression and induced the secretion of CCR5-binding chemokines,³ which are reported elsewhere to inhibit HIV-1 entry.³⁰

The observed antiviral effects peaked 3 days after TLR7/8 activation and then declined, whereas the IFN- γ -induced antiviral effect was more profound. This difference was probably due to differential IL-10 secretion by R848- and IFN- γ -treated dM. Indeed, TLR activation induces factors such as IL-10 that restrain TLR-induced inflammatory activation and tissue damage. This inhibitory feedback also seems to control the TLR7/8-induced antiviral effect described above.

By contrast, IFN- γ suppresses IL-10 secretion and the IL-10-dependent gene expression that regulate IFN- γ responses. IFN- γ signaling also induces proteins of the IRF family, which in turn generate a second wave of IFN- γ target gene transcription.³¹

TLR7/8 triggering in purified dM failed to promote an M1 switch. In contrast, TLR7/8 triggering in decidual explants drove dM towards an M1 profile and consistently inhibited HIV-1_{BaL} infection. This M1 switch can be attributed, at least in part, to the increased production of IFN- γ and TNF- α by dNK cells upon TLR7/8 activation. Cellular contacts with other decidual cells and other factors induced by TLR7/8 stimulation could also be involved in this switch. Indeed, IL-12 secretion, another hallmark of M1 polarization,⁴ was also increased in R848-treated explants. This increase could reinforce the dM polarity switch by enhancing dNK secretion of IFN- γ .³² IL-12-related mechanisms and contacts between dM and dNK cells can also favor the emergence or selection of a macrophage population less sensitive to HIV-1 infection.³³ dM switching towards an M1 phenotype could also induce TLR7/8 over-expression and thereby further reduce the permissivity of dM to HIV-1.²⁹ The durable anti-HIV-1 effect observed in decidual explants is probably due to a synergistic action of TLR7/8 activation and IFN- γ secretion.

Mucosal inflammation in the female reproductive tract promotes HIV-1 transmission and chronic HIV-1 disease.³⁴ TLR stimulation of other female reproductive tract mucosae such as the vaginal mucosa induces proinflammatory cytokine and type I IFN secretion by plasmacytoid dendritic cells, and enhances viral replication.³⁵ We found that TLR7/8-stimulated decidual explants increased the secretion of both anti-inflammatory (IL-1RA and IL-10) and pro-inflammatory cytokines (TNF- α and IL-1 β). The cytokine profile upon TLR7/8 activation was compatible with the pro/anti-inflammatory cytokine balance required to maintain pregnancy during initiation of an immune response to HIV-1.³ This cytokine balance, together with the small number of plasmacytoid dendritic cells and the lack of IFN type I expression in the decidua, may explain these discrepancies.

To conclude, our data support a model in which several inter-dependent factors mediate the weak permissivity of dM to HIV-1 infection during the first trimester of pregnancy (Figure 10). In fact, the decidual environment necessary for successful pregnancy is unfavorable to HIV-1 replication. At the maternofetal interface, macrophages acquire distinct phenotypic and functional properties directed by the pro/anti-inflammatory balance (IFN- γ /TNF- α vs. IL-10/IL1-RA) and by physical contact with decidual cells. dM therefore have low permissivity to HIV-1 infection. Shortly after infection, p21 blocks HIV-1 reverse transcription and integration. In parallel, TLR7/8, which recognize HIV-1 sequences, are activated and further reduce dM permissivity by adding an extra brake after the reverse transcription step and at or before the integration step. TLR7/8 triggering also induces β -chemokine secretion and downregulates HIV-1 receptor and co-receptor expression, which may limit viral entry. In addition, TLR7/8 triggering

induces IFN- γ and TNF- α release from dNK. This favors an M1 switch of neighboring dM and increases p21 expression, thereby limiting infection of new cells. In parallel, TLR7/8 induce IL-1RA and IL-10 secretion, thereby damping inflammation and preserving the pregnancy (Figure 10).

In addition to underlining the role of dM differentiation in HIV-1 control, our findings open new avenues of research into the role of dM differentiation in reproductive 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 peripheral blood mononuclear cells 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% \pm 3.8% (mean + s.e.m.). dM were stimulated 3 or 7 days prior to various assays with R848 at 5 μ g ml⁻¹ for TLR7/8 activation and IFN- γ at 100 ng ml⁻¹ 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/10⁶ 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 productive infection, HIV-1_{BaL} was used at 10⁻³ multiplicity of infection. HIV-1_{BaL} replication was measured by p24 Ag ELISA in culture supernatants. Detailed procedures are described in the **Supplementary Materials and Methods**.

Decidual explants. Decidual tissues were cut into 0.3-cm² pieces. For flow cytometry, explants were placed on collagen sponge gels in 1.5 ml of medium/well/sponge/piece prior to stimulation with R848 or IFN- γ . Decidual tissue and the sponge were minced, digested, and filtered. The total cell population was analyzed by flow cytometry. For experiments with HIV-1_{BaL}, explants were stimulated overnight with R848, and then challenged with HIV-1_{BaL} for 12 h. After several washes, the explants were placed on collagen sponge gels. All experiments were performed in triplicate. Details are provided in the **Supplementary Materials and Methods**.

Flow cytometry of cell surface markers. Detailed procedures are described in the **Supplementary Materials and Methods**. In brief, adherent dM were detached from plastic plates by pipetting without scraping. Cells were then incubated with an FcR blocking reagent and stained with conjugated antibodies. Cell surface marker expression was determined with an LSRII 2-Blue 2-Violet 3-Red 5-Yelgr-laser configuration. The results were analyzed with FlowJo 9.1.3. software. Mean fluorescent intensity (%) was calculated as the ratio of treated dM (IFN- γ) or R848-treated dM to the dM (US) control.

Western blotting. Details are provided in the **Supplementary Materials and Methods**. 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.

Cytokine quantification. dM culture supernatants were harvested after 1, 3, or 7 days and stored at -80°C . Soluble factors were measured in a Luminex assay with the Human Cytokine 25-plex antibody bead kit as recommended by the manufacturer (Invitrogen, Saint Aubin, France).

siRNA transfection. siRNAs were purchased from GE Healthcare Dharmacon (Buckinghamshire, UK). The siRNA against the p21 gene was on-target plus siRNA n.12, and had the following sequence: 5' AGA CCA GCA UGA CAG AUU U 3'. Control siRNAs (Ctrl) were a pool of four on-target plus nontargeting siRNAs. siRNA transfection was performed using INTERFERin kits (Polyplus Transfection, Illkirch-Graffenstaden, France). Fifty nanomolar of sip21 or siCtrl was prediluted in 1 ml of Opti-MEM, to which 20 μl of INTERFERin was added. The transfection mix was left at room temperature for 10 min and then incubated with dM at 37°C for 16 h. Cell culture medium was next replaced by siRNA-free culture medium during 8 h. The transfection mix was then added for additional 16 h prior to western blot and infection by HIV-1/VSV-G. Ctrl siRNA was also added to dM (US).

HIV-1 DNA quantitative PCR. Total DNA in HIV-1/VSV-G-infected dM was extracted 48 h post infection. 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 US 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 US dM was also compared. No significant differences were found.

Statistical analyses. Statistical analyses were carried out 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 s.e.m. 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 mean fluorescent intensity values between stimulated and US dM at a given time point. ($\#P < 0.05$, $\##P < 0.005$, $\###P < 0.0005$, $\####P < 0.0001$).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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