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ABSTRACT

A variety of signals influence the capacity of dendritic cells (DCs) to mount potent antiviral cytotoxic T-cell (CTL) responses. In particular, innate immune sensing by pathogen recognition receptors (PRRs), such as TLR and C-type lectines, influences DC biology and affects their susceptibility to HIV infection. Yet, whether the combined effects of PPRs triggering and HIV infection influence HIV-specific CTL responses remain enigmatic. Here, we dissect the impact of innate immune sensing by PRRs on DC maturation, HIV infection and on the quality of HIV-specific CTL activation. Remarkably, ligand-driven triggering of TLR-3, -4, NOD2 and DC-SIGN, despite reducing viral replication, markedly increased the capacity of infected DCs to stimulate HIV-specific CTLs. This was exemplified by the diversity and the quantity of cytokines produced by HIV-specific CTLs primed by these DCs. Infecting DCs with viruses harboring members of the APOBEC family of antiviral factors enhanced the antigen-presenting skills of infected DCs. Our results highlight the tight interplay between innate and adaptive immunity and may help develop innovative immunotherapies against viral infections.

INTRODUCTION

In humans, several DC subsets have been identified, including BDCA-1$^+$ and BDCA-3$^+$ conventional DC (cDC) and inflammatory monocyte-derived DC (MDDC) [1]. DCs share common features such as the capacity to capture antigen (Ag), migrate and form privileged interactions with effector T cells in lymphoid tissues. While migrating, DCs process captured antigens such as proteins, virions or infected cells leading to the loading of major histocompatibility class I (MHC-I) or class II (MHC-II) molecules and activation of CD8$^+$ or CD4$^+$ T cells, respectively [2]. Alternatively, DCs can be directly infected and present newly synthetized antigens (so called endogenous antigens) to T cells [3]. The sensing of microbes by pathogen recognition receptors (PRRs) initiate the maturation of DCs that enhances their capacities to interact and present antigen to T cells [4]. DC maturation is characterized by a higher cell surface expression of MHC-I and MHC-II molecules, of co-stimulatory molecules, but also changes in vesicular trafficking or composition of proteases involved in antigen processing [5]. PRRs include transmembrane receptors such as TLR- and C-type lectins as well as cytosolic sensors including NOD2 [6]. PRRs bind distinct pathogen-associated molecular patterns (PAMPs) and trigger different cascades of intracellular signalings leading to the expression of lymphokines that strongly influence the capacity of DC to cross-present infected cells and soluble antigens to cytotoxic CD8$^+$ T cells (CTLs) [2]. In addition, PRR-triggering initiates the expression of antiviral factors and the secretion of antiviral cytokines/chemokines [6].

cDCs contribute to HIV-1 infection (hereafter referred as HIV) and spread while initiating innate and adaptive anti-HIV immune responses [7]. cDCs and MDDCs, that are located or attracted at HIV entry sites, are among the targets of HIV infection [8] and contribute to chronic infection [9]. In the absence of treatment, infected cDCs and monocytes are found in the blood of HIV$^+$ donors [8, 10]. Ex vivo, sorted BDCA1$^+$ cDCs support productive infection of HIV strains [11, 12]. MDDCs are equipped with HIV receptors and express molecules involved in HIV capture (e.g. DC-SIGN) that facilitate infection and viral transfer [7]. Nonetheless, HIV replicates poorly in DCs as compared to activated CD4$^+$ T cells [13, 14]. This is due to the expression of viral restriction factors blocking HIV replication at different stages of DC infection, e.g. SAMHD1 depletes intracellular dNTPs and degrades viral RNA, and APOBEC-3G (A3G) and -3F (A3F) interfere with reverse transcription and introduce point mutations in HIV DNA [15, 16]. DC maturation further reduces

susceptibility to infection and is associated with an increase in A3G and A3F expressions [17]. HIV also exploits innate immune signaling pathways to facilitate productive infection of DCs [18]. Hence, depending on the PRRs involved, triggering of innate antiviral responses in DCs has contrasting roles on viral replication [18-20].

HIV specific (HS)-CTLs play a critical role in controlling HIV replication. During acute infection, expansion of HS-CTLs is associated with decreased viremia and determines viral set point during chronic infection [21]. Resistance to disease progression correlates with detection of HIV Gag-specific CTLs and with expression of particular HLA alleles, such as HLA-B*27. HIV rapidly mutates to evade virus-specific CTL responses, underlying the selection pressure exerted by CTLs [22]. However, our understanding of T-cell efficacy in HIV infection is still limited. The quality, defined as the secretion of multiple antiviral cytokines/chemokines, and not the magnitude of T-cell responses determines HIV disease outcome [23]. The quality of T-cell activation is linked to various parameters such as the avidity of the TcR/MHC interactions, the cytokine environment but also the kinetics and quantity of antigen presented on APCs. These factors are influenced by PRR-activation [4]. Previous reports have shown, in vivo and *in vitro,* that treatments with TLR-3 and TLR-7 ligands improve the capacity of DCs to present HIV protein antigens or HIV peptides to HS-CTLs [24, 25].

In the present work, we examined the consequences of PRR-triggering of DCs on immunological and virological parameters: maturation, HIV replication and quality of CTL stimulation by HIV-infected DCs. We show that HIV infection induces an intermediate maturation of DCs. However, PRR activation fully restores DC maturation. Only a limited set of PRR agonists (TLR-3, TLR-4, NOD2 and DC-SIGN ligands) influence HIV replication, highlighting that DC maturation is not systematically associated with lower viral replication. Notably, the agonists that reduce viral replication promoted the expression of antiviral factors, such as APOBECs, but also enhanced the capacity of infected DC to stimulate HS-CTLs. This is exemplified by the magnitude and the quality of HS-CTL activation. Finally, we demonstrate that the antiviral factors A3G and A3F enhance the ability of DCs to activate HS-CTL responses, thus linking innate and adaptive immunity.

RESULTS

Human MDDCs express NOD2 and various levels of TLR-1 to TLR-9

Using RT-qPCR, we first analyzed the relative expression levels of PRRs in sorted DC-SIGN$^+$ MDDCs. As expected [26, 27], transcripts encoding TLR-1 to -8 and NOD2 were detected, though to variable rates (not shown). In contrast to Li et al. [26] but in accordance with the work of Tada et al. [27], we also detected TLR9 mRNA. TLR-2 and TLR-4 mRNA were the most abundant. We thus selected a library of ligands binding to TLR-1 to -9, NOD2 and DC-SIGN, a lectin also involved in HIV antigen presentation [28]. As described in Supporting Information Fig. 1A, MDDCs were infected 24 h or 3 days, with the R5-tropic HIV_{Yu2b} strain, in the presence or absence of reverse transcription inhibitors, respectively, and treated at the time of infection with the agonists (or untreated as negative control). At each time point, the maturation and the capacity of PRR-ligand treated MDDCs to present HIV antigens to HIV-specific CTLs was compared. 3-day post infection (p.i.), HIV replication in MDDC cultures was also monitored. To certify that the concentrations of PRR agonists used in our study were sufficient to induce MDDC activation, we also analysed, 3 day post-treatment and/or infection the cytokine/chemokine secretion patterns (Supporting Information Fig. 1B).

HIV infection does not interfere with PRR-induced maturation of human MDDCs

We monitored the consequences of PRR-triggering on the cell surface expressions of the classical DC maturation markers: CD86, CD83, HLA-I (class I), HLA-DR and DC-SIGN (Fig. 1). A representative staining using MDDCs from one individual using TLR-4 ligand (LPS) is shown in Fig. 1A. This experiment was repeated using MDDCs from 8 healthy individuals and the results compiled in Fig. 1B.

At early time points (24h), LPS induced a strong up-regulation of CD86, CD83, MHC-I and HLA-DR (2.5 to 4.1 fold increase). LPS-activated DCs also down-regulate DC-SIGN expression (average fold change < 0.7) (Fig. 1B and Supporting Information Fig. 2A). We interpreted the decrease of DC-SIGN expression is a hallmark of DC maturation. With the exception of TLR-2, all PRR-ligands induced to various extend the maturation of MDDCs 24 h after treatment (Fig. 1B). CD86, CD83 and HLA-DR were highly up-regulated following TLR-1/2, TLR-4 and -SIGN triggering (fold change ranging 1.7 to 4.1), while other PRR ligands moderately changed their expression levels (average fold change ranging

1.5 to 2). At 24h, the loading of MDDCs with viral particles induced a marked increase of CD86 and HLA-DR, (fold change expression of 1.6 and 2.8, respectively) and a slight increase of other markers. However, loading MDDCs simultaneously with HIV and PRR agonists restored a maturation profile similar to PRR-agonists alone.

At later time points (72 h after treatment), TLR-2 and TLR-9 induced a modest maturation of MDDCs (Fold change > 1.5). All other agonists induced a significant increase of 2 (e.g. TLR-8) to 5 (e.g. TLR-4) maturation markers (average fold increases ranging from 1.5 to 5.1). TLR-4 and DC-SIGN agonists induced a significant DC-SIGN down-regulation both at 24h or 72h post-treatment. Remarkably, HIV-infection of MDDCs (72h), induced a strong and significant increase of CD86 and a slight, non-significant, increase of all other maturation markers. This induction of maturation was also exemplified by the cytokine secretion patterns induced by HIV infection of MDDCs (Supporting Information Fig. 1B). PRR-activation of infected MDDCs established a matured phenotype, exacerbating the maturation profile observed for uninfected DCs (Fig. 1B). We then compared the maturation profile of productively infected (Gag-p24 positive) and "by-stander" (Gag-p24 negative) cells (Supporting Information Fig. 2B). Among PRR-treated but also untreated cells, Gag-p24 positive cells showed a higher expression of CD83 and HLA-DR than Gag-p24 negative cells. The expression profiles of CD86 and HLA-I were only slightly up-regulated in Gag-p24 positive cells as compared with Gag-p24 negative cells.

In summary, PRR agonists induced a moderate (TLR-2) to strong (TLR-1/2, TLR-4, NOD2 and DC-SIGN) MDDC maturation. HIV provoked a slight maturation of MDDCs with productively infected MDDCs (Gag-p24$^+$ cells) being more matured than Gag-24 negative cells. HIV infection did not interfere with the maturation induced by each of the ligands.

Triggering of TLR-3, -4, NOD2 and DC-SIGN diminishes HIV replication in MDDCs

We then analyzed the capacity of HIV to replicate in MDDCs treated at the time if infection with the panel of PRR agonists (Fig. 2 and Supporting Information Fig. 1A). A representative experiment using MDDCs from a single donor is presented in Fig. 2A. The results from 8 independent experiments using MDDCs derived from 8 individuals are presented as percent of Gag-p24$^+$ cells (Fig. 2B) and as relative levels of infection (Fig. 2C). The infection rate of untreated MDDCs using HIV_{Yu2b} was on average 12.4 % (ranging from 4.3 to 24.7 %). None of the PRR agonists significantly increased HIV-infection of MDDCs. In contrast, DC treatment with TLR-3, TLR-4, NOD2 and DC-SIGN ligands was associated with a significant ($p<0.05$) reduction in HIV replication (as exemplified by the decrease in

the frequency of Gag-p24$^+$ cells). TLR-5, TLR-8 and TLR-9 agonists also lead to a slight reduction of Gag-p24$^+$ cells (average fold decrease of 0.8). The most dramatic decrease was observed using the agonists of TLR-4 (LPS) and TLR-3 (PolyI:C), for which viral replication was on average 80 % and 55 % reduced as compared with untreated cells, respectively (Fig. 2). We obtained similar results when collecting cell-culture supernatant and performed a p24-ELISA (not shown).

Innate antiviral factors are upregulated in human MDDCs upon PRR activation

We measured by RT-qPCR the expression of viral restriction factors (A3G, A3A, A3F, SAMHD1, Tetherin, CyPA and ADAR1) and viral sensors or enzymes that interact with PRR signaling (RIG-I, MDA5, A20 and TREX1) [29]. We used the innate immune activation marker, MxA, as control (Fig. 3). We focused our analysis on PRR-ligands that induced an inhibition of HIV replication in DCs (TLR-3, TLR-4, NOD2, and DC-SIGN) and as a control TLR-6/2 agonist, which did not impact HIV infection (Fig. 2). MDDCs were treated with PRR-agonists in the presence or absence of replicative competent HIV_{YU2b} and the relative mRNA expression levels were compared to the untreated uninfected controls. With the exception of SAMHD1 and CypA, TLR-3 and -4 agonists induced modest (1.2 and 3.3 fold increases in Tetherin expression, respectively) to very strong (31 and 563 fold increases in A3A expression) up-regulation of all mRNA analyzed. HIV infection further enhanced the mRNA expression levels without changing the overall profiles. TLR-6/2 ligand only slightly increased the expression of A3F, ADAR-1 and A20 mRNA. NOD2 and DC-SIGN agonists induced an intermediate expression profile with a slight increase of A3G, A3F, ADAR1, RIG-I, MDA5, A20 and MxA (folds changes between 1.9 and 6.9). In contrast, mRNA of A3A, SAMHD1 and CypA were downregulated by NOD2 and DC-SIGN ligands. These down-modulations were even more pronounced in HIV-infected MDDCs. Overall, we observed an increase of transcripts encoding for the antiviral restriction factors A3G, A3A, A3F, ADAR-1 and Tetherin upon treatment with TLR-3, -4, NOD2, DC-SIGN but not TLR-6/2 ligands (Fig. 3).

Triggering of TLR-3, TLR-4, NOD2 and DC-SIGN at the time of infection enhances HS-specific CTL activation by infected MDDC.

We then analyzed the capacity of PRR-agonist-treated DCs to activate HS-CTLs. 24 h and 3 d p.i., MDDCs loaded or infected with HIV_{Yu2b}, respectively, and treated with the panel of PRR ligands were co-cultured with an HS-CTL clone restricted by HLA-A*0201 and

specific for SL9 peptide from HIV Gag-p17 (Fig. 4). Note that for DCs loaded with HIV for 24h in the presence of RT inhibitors (AZT+NVP), HIV antigens are derived solely from incoming viral particles (so-called exogenous presentation) [28]; in contrast, upon 3-day of infection, the source of HIV antigens corresponds mainly to newly synthetized HIV proteins (so-called endogenous presentation) [30]. A representative IFN-γ ELISpot, using MDDCs from a single donor is presented in Supporting Information Fig. 3 and the data combined with three additional independent experiments in Fig. 4. PRR activation had no significant impact on the capacity of HIV- or peptide-loaded MDDCs to activate the SL9-specific CTL clone (Fig. 4A). LPS (TLR-4)-treatment of MDDCs induced a slight, but not significant, decrease of T cell activation by HIV-loaded MDDCs (Fig. 4A). Interestingly, with the exception of LPS that decreases T cells activation levels, PRR triggering had, at first sight, also a minor influence on the capacity of HIV-infected cells (3 d p.i.) to stimulate the SL9-specific CTL clone (Fig. 4B). However, 3-day post-infection the main source of antigens is derived from newly synthetized Gag antigens [30]. We thus examined T cell activation relative to the infection rates (Fig. 4B, right panel). Strikingly, relative to their capacity to reduce viral replication (Fig. 2), TLR-3, TLR-4, NOD2 and DC-SIGN agonists enhanced (3 to 6 fold) the activation of the SL9-specific CTL clone. Note that, 3-day post treatment, PRR-triggering increased modestly, if any, peptide-mediated activation of the SL9-specific CTL (Fig. 4B, left panel). Overall, our results strongly suggest that TLR-3, TLR-4, NOD2 and DC-SIGN triggering improve the capacity of infected MDDCs to stimulate HS-CTLs, and this is not uniquely due to increased MHC-I expression.

We then sought in extending these findings to primary blood derived DCs. To this end, BDCA1$^+$ DCs were sorted from PBMCs of HLA-A*02$^+$ donors, infected with HIV$_{Yu2b}$ (+/- RT inhibitors) and simultaneously treated with TLR-3 ligand (Supporting Information Fig. 4A). Due to the limited amount of DCs sorted (1.3 +/- 0.6 million cells), we could not envisage performing multiple PRR-agonist treatments. The infection and maturation levels were assessed using intracellular Gagp24 or cell-surface CD86 stainings, respectively. BDCA1$^+$ DCs were then co-cultured with SL9-specific CTLs and T cell activation monitored. 2-day pi, BDCA1$^+$ DCs exhibited a marked maturation phenotype induced by HIV particles and further enhanced by TLR3-trigerring (Supporting Information Fig. 4B). As compared to uninfected cells, regardless of the presence of RT inhibitors, BDCA1$^+$ DCs stained positive with the anti-Gagp24 Ab, suggesting that 2 days pi, the anti-Gagp24 Ab staining allowed the detection of the viral input and not exclusively of HIV-infected cells. Consequently, BDCA1$^+$ DCs loaded with HIV +/- RT inhibitors induced the same levels of

SL9-specific CTL activation (Supporting Information Fig. 4C). As observed in Fig. 4, TLR-3 triggering of BDCA1$^+$ DCs, did not significantly influence this exogenous HIV antigen presentation (Supporting Information Fig. 4C). Overall, these experiments showed that the viral input might be used as a source of antigen by BDCA1$^+$ DC to activate Gag-specific CTLs. However, it did not allow drawing conclusions on the influence of PRR-triggering on the presentation of newly synthetized viral antigens.

Triggering of TLR-3, TLR-4, NOD2 and DC-SIGN at the time of infection improves the quality of HS-specific CTL activation by infected MDDCs.

We then examined the capacity of HIV-loaded or -infected PRR-treated MDDCs to induce T cell polyfunctional responses using two HLA-B*27 restricted CTL clones (E2C and H8B) specific for Gag-p24 KK10 epitope and whose polyfunctional profiles have been previously characterized [31]. As illustrated in Supporting Information Fig. 5, polyfunctional activation was analyzed using IFN-γ, IFN-α, IL-2, TNF-α, MIP-1β and CD107a mobilization [31]. As for the SL9-specific CTL clones, 24 h post PRR-stimulation, MDDCs loaded with KK10 peptide or with HIV, induced similar levels of activation (not shown). 72 h post PRR-treatments, peptide-loaded MDDCs also brought comparable levels of HS-CTL activation (Fig. 5, left panels). 72 h post infection, most PRR ligands provoked a modest increase of KK10-specific CTL activation (Fig. 5). To highlight the quality of T cell activation, the results were also expressed as a polyfunctional index that allows a quantitative assessment of T cell poly functionality [32]. The polyfunctional indexes induced by KK10-peptide loaded- or HIV-infected PRR-treated MDDCs followed a similar trend than the global HS-CTL activation levels (Fig. 5). However, relatively to their capacity to reduce viral replication (Fig. 2), TLR-3, TLR-4, NOD2 and DC-SIGN agonists increased the activation and the polyfunctionality of the KK10-specific CTL clones from 3-16 fold (Fig. 5, right panels).

Thereafter using distinct HS-CTL clones and analyzing both the magnitude and quality of T cell activation, we observed that TLR-3, TLR-4, NOD2 and DC-SIGN pathways have a dual role: they limit HIV replication in MDDCs while inducing highly functional HS-CTL responses.

APOBEC-3G (A3G) and -3F (A3F) enhance HIV antigen presentation by MDDCs.

We have previously shown that A3G-mediated viral restriction contributes to the immunogenicity of HIV-infected cells [33]. Since A3G but also A3F expression are induced

upon TLR-3, TLR-4, NOD2 and DC-SIGN MDDC activation, we decided to define their role in the enhancement of CTL activation by MDDCs. We first designed shRNA targeting A3G and A3F expression to stably abolish A3G and A3F expression in MDDCs using lentiviral vectors. However, although the shRNA strongly reduced the expression levels of A3G and A3F in untreated uninfected MDDCs, shRNA-mediated A3G and A3F inhibitions were saturated by TLR-3-, TLR-4-, NOD2- or DC-SIGN-triggering that induced a strong up-regulation of A3G and A3F (not shown and Fig. 3). We thus used an alternative approach by introducing A3G and A3F in HIV_{SF2} particles prior to infecting MDDCs (Fig. 6A) [33]. In this approach, A3G and A3F are packaged into newly formed HIV particles and subsequently edit the nascent viral DNA leading to G- to -A hypermutations in the proviral genome [34]. As expected, infecting a T cell line with A3G- or A3F-containing HIV virions lead to reduced viral replication, as compared to wild-type HIV, but increase activation of the SL9-specific CTL clone than HIV alone (Fig. 6B). These results confirmed, and further extend to A3F, our previous demonstration that A3G editing enhances the ability of HIV-infected $CD4^+$ T cells to activate HS-CTLs [33].

Using a similar approach, MDDCs were infected with HIV, $A3G^+$ and $A3F^+$ containing HIV, viral replication monitored and cells co-cultured with the SL9-specific CTL clone (Fig. 6C). As previously, the antiviral activity of A3G and A3F reduced MDDC infection as compared to HIV alone but enhanced the capacity of MDDCs to activate the HS-CTL clone. MDDCs treated with RT-inhibitors did not induce a significant CTL activation. As in $CD4^+$ T cells, these results strongly suggest that in DCs, the editing activity of A3G and A3F favors the generation of endogenous MHC-I restricted antigens that improve HS-CTL activation.

DISCUSSION

DCs express membrane-bound, endosomal or cytosolic PRRs that are involved in the sensing of microbes and viruses. In pDCs, previous studies have shown that the ssRNA of HIV is sensed by TLR-7/8 [35]. In MDDCs, the uptake of HIV leads to NF-KB activation, also through the triggering of TLR-8, and results in abortive transcription of HIV genome [18]. However, whether HIV-mediated TLR-8-activation leads to MDDC maturation is less clear [18]. In fact, the effect of HIV infection on MDDC maturation is controversial. Using single cycle virus or AZT to block productive infection, some reports have shown that the viral inoculum does not induce maturation [14, 36]. In contrast, using highly purified chemically inactivated virus, others have shown that the viral input induces, in a dose dependent manner, a partial maturation of MDDCs [37, 38]. In our work, we also observed that abortive HIV infection (HIV+RT inhibitors) induces an intermediate maturation of DCs. The impact of productive infection of MDDCs is also a matter of debate as some authors described that HIV replication induces MDDC maturation [14, 37-39] while others did not [36, 40, 41]. Using 8 MDDC preparations, we show here that HIV replication induces MDDC maturation with productively infected cells (HIV Gag-p24^{+}) exhibiting a more mature phenotype. The treatment of MDDCs with TLR-1 to -9, DC-SIGN and NOD-2 ligands further enhanced the maturation of DCs loaded or infected with the virus.

PRR triggering initiates an antiviral state involving the secretion of antiviral cytokines and the expression of Interferon-stimulated genes (ISGs). Some ISGs including A3G, A3A, A3F, SAMHD1, Tetherin, CyPA and ADAR1 inhibit HIV replication [29]. HIV replication in MDDCs also induces the expression of ISG [14]. However in HIV-infected cells, ISGs upregulation is delayed as compared to PRR-agonist treated MDDCs [14], potentially due to hijacking of the TBK-1/IRF3 transduction pathway [37]. Productive infection of MDDCs might also, at least partially, block TLR-induced phenotypic maturation of MDDCs [40]. On the other hand, TLR-3 and -4 ligands increase A3G and A3F expression limiting HIV replication in macrophages and DCs [16, 19, 42]. Triggering of NOD2 also reduces HIV replication in MDDCs [43]. The kinetics of PRR-triggering differentially influence viral replication: for instance, a 24 h- or a short 2 h-pulse with TLR-2 ligand, prior to MDDC HIV infection, has been shown to decrease or enhance HIV replication, respectively [19, 43].

In our study, we decided to focus on TLR-, NOD2 and DC-SIGN ligands because i) these PRRs are expressed by MDDCs and other cDCs; ii) primary HIV infection is often associated with co-infections; iii) TLR- ligands are already included in vaccine

formulations; iv) TLR- ligands are currently considered as potential adjuvants for therapeutic vaccination against HIV. With the exception of the work by Thibault et al. [19] who focused on TLR-2 and TLR-4, most authors studied the impact of PRR ligands on HIV replication using fully matured MDDCs (e.g. infecting 24h post PRR-treatment). In our work, MDDCs were treated with the agonists during HIV loading to mimic potential HIV co-infections and more importantly vaccine administration. We show that none of the PRR agonists significantly increased HIV-infection of MDDCs. In contrast, treatment with TLR-3, TLR-4, NOD2 and DC-SIGN ligands reduced HIV replication in DCs. TLR-5, TLR-8 and TLR-9 agonists only slightly diminished MDDC infection. We observed an increase of transcripts encoding for A3G, A3A, A3F, ADAR-1 and Tetherin upon treatment with TLR-3, -4, NOD2, DC-SIGN but not TLR-6/2 ligands that does not influence replication, suggesting a potential link between the expression of these antiviral factors and the inhibition of HIV replication in MDDCs. PRR-triggering induces the secretion of various cytokines such as IFNs, IL-2, -7, -15 and -27 that might increase A3G/A3F expression [17]. In particular, TLR3- and TLR4-mediated increase of A3G expression relies on type-I IFNs [42]. However, the pathways influencing A3G/A3F expression probably rely on various transduction signals and/or cytokine environments depending on the PRR engaged. Indeed, in contrast to NOD2 that also favors IFN production [4], DC-SIGN activation represses the expression of IFN-related genes [6]; and TLR6/2 that mediates IFN secretions does not influence A3G/A3F expression in our experimental settings [4]. Note that other ISGs, such as TRIM5 or Mx2 that influence HIV replication at different steps of the viral cycle might also account for the inhibition of HIV replication [29]. Although we did not investigate this point, PRR activation might influence replication at the level of entry by reducing CCR5 expression [19, 43]. However, reduction of CRR5 expression does not necessary correlate with reduced viral replication e.g. TLR2-activation of MDDCs although reducing CCR5 expression, enhanced viral replication [19]. In DCs, to bypass the entry steps, Pion et al [16] used VSV-G pseudotyped HIV to highlight the role of A3G and A3F in viral restriction.

PRR triggering also regulates antigen presentation by DCs [4]. TLR-signals redirect recycling MHC-I molecules to phagosomes allowing cross-presentation of antigens to CTLs [2] and upregulate the expression of rapidly degraded proteins, a major source of MHC-I restricted antigens [44]. TLR-activation increases the expression of factors involved in the MHC-I restricted processing pathways such as TAP, Tapasin, and favors a switch between standard to classical immunoproteasomes in DCs, potentially impacting the nature of peptide loaded on MHC-I molecules [2]. The cytokines secreted by activated MDDCs might

also influence CTL activation. We analyzed the capacity of PRR-activated DCs to present the cognate peptide, antigens derived from incoming viral particles or from productive viral infection, thus including newly-synthetized viral products. PRR-triggering did not influence the presentation of the control peptide nor of exogenous HIV antigens to HS-CTLs. Suggesting that the cytokines secreted by activated-MDDCs (Supporting Information Fig. 1B) did not directly influence CTL stimulation. Other studies have shown that TLR-triggering differentially impact cross-presentation of cellular antigens [2]. However, the kinetics of PRR activation might have different outcome depending on the routes of antigen entry, e.g. the presentation of MHC-I-restricted HIVGag-p24 antigens derived from viral particles relies on fusion of HIV and host membranes [28]. Although we used CTLs specific for different viral proteins (Gag-p17 and Gag-p24) and exhibiting various functional avidities, our work, with T cell clones, might also underestimate the impact of PRR-triggering of CTL activation.

Nonetheless, using HS-CTL clones, we demonstrate that TLR-3, -4, NOD2 and DC-SIGN ligands enhanced the capacity of infected MDDCs to stimulate HS-CTLs. This was exemplified by the magnitude and the quality of HS-CTL activations. At first sight, our results reveal a potential paradox as triggering of these PRRs decreased viral replication, reducing the quantity of Gag-p24 antigens, but enhanced HS-CTL responses. However, the source of MHC-I-antigens is not limited to full length proteins as misfolded or truncated proteins provide peptides for the loading of MHC-I molecules [45]. In T cells, we have shown that A3G enhances the recognition of HIV-infected cells by HS-CTLs. This phenomenon requires the enzymatic activity of A3G that introduces hypermutations in HIV genome, leading to the expression of truncated viral peptides [17, 33]. In this study, we demonstrate that A3F also enhances the immunogenicity of infected T cells. In MDDCs, confirming previous findings [16, 19, 42, 43], we show that the expression of A3G and A3F is strongly induced upon TLR-3, -4, NOD2 activation. We observed that DC-SIGN signalling also increases their expression. In addition, we demonstrate that in MDDCs as in T cells, A3G and A3F when present in viral particles strongly favour CTL activation, thus providing a mechanism explaining PRR-mediated enhancement of CTL activation.

We intended to extend our observations to primary blood derived DCs. We showed that under our experimental conditions, BDCA1$^+$ DC had a remarkable ability to present antigens derived from incoming viral particles. However, this exogenous presentation impeded the detection and monitoring of endogenous HIV antigen presentation by productively infected BDCA1$^+$ DCs. We could not perform CTL activation experiments at

more distant time points from the infection since BDCA1$^+$ DC did not survive prolonged in vitro culture. Thereafter, although *in vitro* derived MDDCs share common features with inflammatory DCs, it will be important to extend our results on newly endogenous viral antigen presentation to primary blood derived or tissue resident DC subsets. Remarkably in the context of vaccinations, A3G expression in mucosal DCs and monocytes correlates with the activation of polyfunctional CTLs and upon challenge, to lower viral loads [46].

Overall, we demonstrate that triggering of TLR-3, -4, NOD2 and DC-SIGN decreases HIV replication but in contrast enhances the quality of CTL activation mediated by HIV-infected DCs (Fig. 7). In DCs, we highlight the role APOBEC family members in enhancing CTL activation.

MATERIALS AND METHODS

Cells

PBMCs from the blood of HIV-seronegative donors (Etablissement Français du Sang, Paris, France) were screened by FACS and/or using Luminex xMAP for the expression of HLA-A*02- (BB7.2, Biolegends) or HLA-B27-positive donors. Monocytes were isolated with $CD14^+$ magnetic beads (Miltenyi Biotec) and cultured with RPMI 1640 containing 10 % FBS, GM-CSF (20 ng/mL) and IL-4 (2 ng/mL, Miltenyi Biotec). On day 5, MDDCs were infected with HIV and simultaneously treated with PRR ligands (see Supporting Information Fig. 1A). The SL9c2 and0 $CD8^+$ T cell clones, specific for HIV Gag-p17 (SLYNVATL, aa 77–85, SL9 peptide KK1; restricted by HLA-A*0201) and for HIV Gag-p24 (KRWIILGLNK, aa 263-272, KK10 peptide, restricted by HLA-B*2705) respectively, were restimulated and expanded, as previously described [28, 31]. CEM-$HLA*02^+$ (CEM-$A2^+$) cell were cultured in RPMI 10 % FBS.

$BDCA1^+$ DCs were isolated from the PBMCs of HLA-A*02-positive donors using a first step of enrichment (EasyStep Human pan-DC enrichment kit, Stemcell Technologies) and sorting by flow cytometry (FACS Aria flow cytometer, BD Biosciences) using CD1c, CD11c, CD45-, HLA-DR, CD14 and CD123 expressions. $BDCA1^+$ DCs were maintained in culture in GM-CSF containing medium (3ng/ml).

Antibodies

On day 5, the purity of immature MDDCs was controlled by flow cytometry (FACScanto Flow Cytometer; BD Biosciences) using CD14-PE (M5E2; BD Pharmingen), and DC-SIGN-APC antibodies (DCN46; BD Pharmingen). DC maturation was analyzed 24 h or 72 h post PRR-treatment using antibodies to HLA class I-FITC (W6/32; Sigma-Aldrich), HLA-DR–PE (L243; BD Biosciences), CD86-FITC (2331; BD Pharmingen), CD83-PE (HB15e; BD Pharmingen), DC-SIGN-APC, and fluorochrome matched isotype controls. $BDCA1^+$ DCs were sorted using the following antibodies: CD1c-PE-Cy7 (Biolend), CD11c-PE-CF594, CD45-APC-H7, HLA-DR-AF700, CD14-V450 (BD Pharmingen) and CD123-APC (Miltenyi Biotec).

PRR ligands

TLR-1 to -9 and NOD2 ligands (Invivogen) were used at the following concentrations: Pam3CSK4 (TLR-1/2; 0.2 µg/ml); HKLM (TLR-2; 0.2 10^8 cells/ml); Poly(I:C) LMW

(TLR-3; 2 µg/ml); *E. coli K12* LPS (TLR-4; 200 ng/ml); *S. Typhimurium* Flagellin (TLR-5; 200 ng/ml); FSL1 (TLR-6/2; 200 ng/ml); Imiquimod (TLR-7; 0.2 µg/ml); ssRNA40 (TLR-8; 0.2 µg/ml); ODN2006 (TLR-9; 1 µM) and Muramyl dipeptide (MDP) (NOD2; 20 µg/ml). DC-SIGN ligand (ManLam; 2 µg/ml) was a kind gift from O. Neyrolles (Toulouse, France).

Virus and infection

HIV_{YU2b} or $HIV_{SF2\Delta nef}$ were produced as previously described [33]. For incorporation of A3G or A3F into viral particles, A3G and A3F encoding vectors were added to the transfection DNA mix, respectively [33]. The Gag-p24 content of all viral supernatants was measured using ELISA (PerkinElmer). MDDCs and BDCA1+ DCs were exposed to the indicated HIV strains at 200 ng/mL of p24 (4 h, 37 °C), washed, and cultured in medium with IL-4+GM-CSF or GM-CSF, respectively. In the HIV+ART conditions (24 h treatment of PRR-ligands), DCs were loaded as before with HIV but in the presence of 5 µM AZT and 1.2 µM NVP. Three-day post-treatment, infection of MDDCs was analyzed by Gag-p24 ICS (KC57-RD1, Beckman Coulter).

Cytokine/chemokine secretions

Cytokine/chemokine release in the cell culture supernatants of MDDCs was quantified according the manufacturer's instructions using the Luminex technology (Cytokine 25-Plex Human Panel, Biorad).

T-cell activation assay

For IFN-γ ELISpot, MDDCs (100,000 cells per well) were co-cultured (16 h) with HS-CTLs (ranging from 2,500 to 10,000 $CD8^+$ T cells per well) and IFN-γ production measured as previously described [28]. As positive controls, MDDCs were loaded (1 h) with cognate peptides (1 µg/ml). For ICCS, MDDCs and T cells were co-cultured (6 h) at a (1:1) ratio. Brefeldin A (5 µg/mL) and Monensin (2.5 µg/mL, Sigma) were added after 1 h. CD8-A405 (Invitrogen MHCD0826), CD4-APC-Cy7 (BD 7871), MIP1-β-FITC (RD system IC271F), TNFα -PECy7 (BD 557647), IL-2-APC (BD 341116), IFN-γ-A700 (BD 557995) and CD107a-PE-Cy5 (BD 555802) antibodies and Flow cytometry (BD Fortessa) were used. Data were analyzed with FlowJo software (Tree Star). Multifunctional data were analyzed with PESTLE v1.3.2 and SPICE v3.1 (Mario Roederer, VRC/NIAID/NIH). Polyfunctionality of HS-CTLs was evaluated by calculating a polyfunctional index (P_index, 1) using FunkyCells ToolBox V.0.1.2 software (www.FunkyCells.com) [32]. Briefly,

$$\text{Polyfunctionality index} = \sum_{i=0}^{n} F_i \cdot \left(\frac{i}{n}\right)^q \qquad (1)$$

where Fi is the frequency of cells performing *i* simultaneous functions. The polyfunctionality parameter *q* was set conservatively to 1.

Real time RT-qPCR

Four µg total RNA was reverse transcribed with oligo(dT)15 primers (Promega) using SuperScript III Reverse Transcriptase (Invitrogen). SYBR Green PCR was performed with 50 ng of cDNA templates using commercial kit (Applied Biosystems) and GeneAmp 7300 Sequence Detection System (Applied Biosystems). Each sample was analyzed in duplicates, and the amounts of templates normalized to internal controls (β-actin). Primer sequences are listed in Supplemental Table 1. PCR were confirmed on agarose gel (data not shown).

Statistical analysis

For statistical analyses of fold change variations, two-tailed paired t tests were used. Prism 6.0 (GraphPad) was used to process all the statistical analyses.

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Conflict of Interest Disclosure

M.L. is inventor of the polyfunctionality index (patent number: WO2013127904) and proprietary owner of the Funky Cells ToolBox software (www.FunkyCells.com). All other authors declare no financial or commercial conflict of interest.

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FIGURE LEGENDS

Figure 1. HIV infection does not interfere with PRR-induced maturation of MDDCs. MDDCs were infected with HIV_{YU2b} and maturation at 24 h and 72 h p.i. was analyzed using antibodies to the indicated markers and flow cytometry. **(A)** Representative staining using MDDCs from one individual and using TLR-4 ligand (LPS). Values correspond to the fold changes to the untreated (untx) or uninfected (NI) conditions induced by HIV, LPS or HIV+LPS (f= fold change). **(B)** MDDCs from 8 healthy individuals were submitted to infection and the indicated PRR treatments (as in Supporting Information Fig. 1A) and the results for each maturation marker combined in a radar chart. For each marker, MFIs were normalized to untreated non-infected samples and the data expressed as fold increase. In red and green are depicted the results from HIV-loaded/infected (red) or uninfected (green) MDDCs, respectively. Standard deviations are not depicted for clarity. Raw data are presented in Supporting Information Fig. 2.

Figure 2. Triggering of TLR-3, -4, NOD2 and DC-SIGN at the time of infection diminishes HIV replication in MDDCs. MDDCs were treated with PRR agonists and simultaneously infected with HIV_{YU2b}. 72 h p.i., viral replication was analyzed by intracellular staining for Gag-p24. **(A)** Representative stainings using MDDCs from one individual. MDDCs were co-stained with anti-HLA-I Abs to allow a better discrimination of infected cells. PRR agonists are indicated on the top of each plot. MDDCs were gated on SSC and FSC (not shown). Values in each quadrant indicate the % of Gag-$p24^+$ cells. This quadrant was set based on the staining of uninfected cells (not shown). **(B)** MDDCs from 8 healthy individuals were submitted to infection and PRR-treatments (as in Supporting Information Fig. 1A) and the percentage of Gag-$p24^+$ cells is shown. **(C)** For each donor, the infection rate was normalized to untreated infected samples and the data expressed as fold change. Data are expressed as mean ± SD of the 8 donors. two-tailed paired t tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Innate antiviral factors are upregulated upon PRR activation.
Heat map of antiviral and innate factor mRNA expression. MDDCs were infected with HIV_{YU2b} in the presence of the indicated PRR agonists. As a control, MDDCs were untreated (untx), uninfected (ni) or untreated and uninfected. The mRNA encoding for the indicated antiviral and innate factors were quantified by RT-qPCR and normalized to a

house-keeping gene. The fold increase of each transcript is compared to untx ni sample. Data shown correspond to the mean fold increase of two independent experiments.

Figure 4. Triggering of TLR-3, TLR-4, NOD2 and DC-SIGN at the time of infection enhances HIV Gag-p17 SL9-specific CTL activation by infected MDDCs. MDDCs from four HLA-A*02$^+$ donors were loaded with HIV_{YU2b} in the presence of AZT/NVP (ART, 24 h) **(A,** right) or productively infected (72 h pi) (**B**, middle and right) in the presence of the indicated PRR-agonists and co-cultured with the HIV Gag-p17 SL9-specific CTL clone. (**A and B,** left) as a control, uninfected cells were treated with the PRR ligands and loaded with SL9 peptide. T-cell activation was monitored using IFN-γ ELISpot. For each donor and condition (infected or uninfected), activations were normalized to untreated (untx) samples and the data expressed as fold change. **(B,** right) Data expressed as a ratio of percent of activated cells to percent of infected Gag-p24$^+$ cells (Fig. 2). The results from 4 independent experiments performed with cells from different donors are presented as mean ± SD. *p<0.05, ***p<0.001, two-tailed paired t tests.

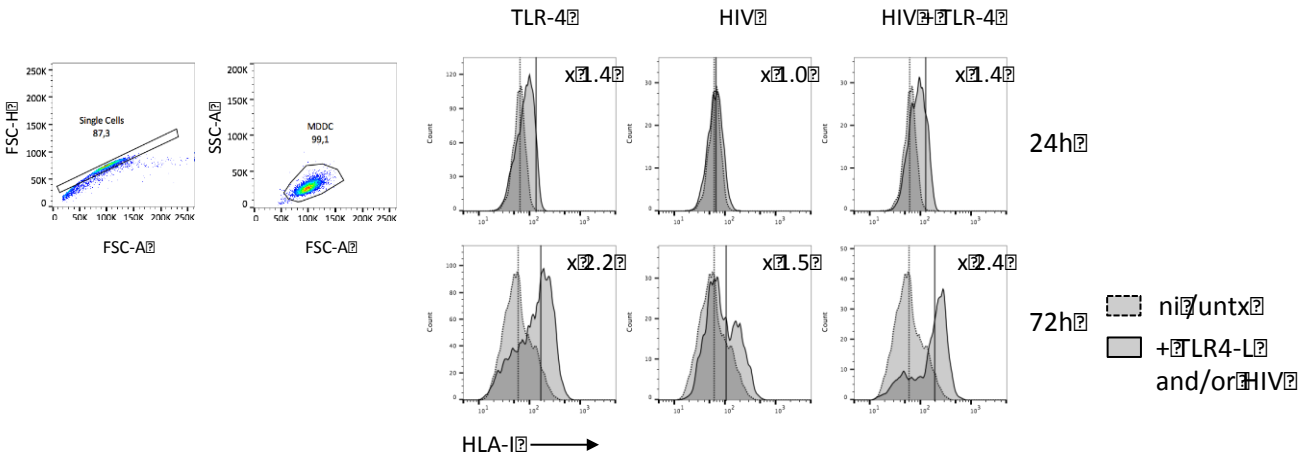
Figure 5. Triggering of TLR-3, TLR-4, NOD2 and DC-SIGN at the time of infection improves the quality of HIV Gag-p24 KK10-specific CTL activation by infected MDDCs. HLA-B*27$^+$ MDDCs were infected with HIV_{YU2b} in the presence of the indicated PRR-agonists (as in Supporting Information Fig. 1A) and co-cultured with the HIV Gag-p24 KK10-specific CTL clones (DCs + HIV, 72 h, middle). As a control, uninfected cells were treated with the PRR ligands and loaded with KK10 peptide prior co-culture with the clone (DCs + KK10 peptide, left). T-cell activation was monitored by intracellular cytokine staining for IFN-γ, IFN-α, IL-2, TNF-α, MIP-1β and CD107a mobilization and flow cytometry. For each donor and conditions (infected or uninfected), activation levels were normalized to untreated (untx) samples and the data expressed as fold change. Polyfunctional activations were also analyzed and expressed as an index allowing a quantitative assessment of T-cell polyfunctionality (bottom panels). Data are expressed as a ratio of percent of activated cells or polyfunctional index to percent of infected Gag-p24$^+$ cells (right). Results shown as mean ± SD of data pooled from 3 independent experiments, performed with cells from two different donors. *p<0.05, ***p<0.001, two-tailed paired t tests.

Figure 6. APOBEC-3G (A3G) and -3F (A3F) enhance HIV antigen presentation by MDDCs. (A) Representation of the experimental procedure. Viruses were produced upon cotransfection of 293T cells with HIV genome and plasmids encoding for A3G or A3F. CEM-A2$^+$ cells or MDDCs were then infected. A Nef-deficient isolate was used since Nef interferes with HLA-A2 expression. CEM-A2$^+$ cells do not express A3G, thus A3G and A3F exert their editing activity exclusively during the first cycle of replication. **(B)** CEM-A2$^+$ cells were incubated with $HIV_{SF2\Delta nef}$, or $HIV_{SF2\Delta nef}$ + A3G or $HIV_{SF2\Delta nef}$ + A3F (5 to 20 ng/mL of Gag-p24) and the kinetics of viral infection were analyzed by Gag-p24 FACS staining (topl). 24 h p.i., infected cells were collected and used to stimulate the SL9-specific CTL clone in an IFN-γ Elispot (1200 CTLs/well) (bottom). Background IFN-γ production induced by uninfected cells and CEM-A2$^+$ cells alone were subtracted. Activation levels with SL9 peptide–loaded cells were around 500 IFN-γ$^+$ spots/well (not depicted). The percentages of infected (top) and IFN-γ-producing (bottom) cells were normalized to CEM-A2$^+$ cells infected with WT HIV (middle). Data are presented as a ratio of IFN-γ$^+$ spots to percentage infection (right). Each symbol represents an independent experiment. Data are shown as mean ±SD of 5 experiments. **(C)** As in (B) using HLA-A*02$^+$ MDDCs as target cells. From 24 to 72 h p.i., infected DCs were collected, stained for Gag-p24$^+$ cells (top) and used to stimulate HIV Gag-p17 SL9-specific CTL clone in ICCS (bottom). As a negative control DCs were also infected in the presence of ART (AZT/NVP) and co-cultured with the clones. Activation levels induced by SL9 peptide–loaded DCs were ranging from 25 to 60 % (not depicted). Data are normalized to the results using HIV-infected DCs (middle). Data are presented as a ratio of % activation to % infection. Each symbol represents an independent experiment using cells from different donors and data are shown as mean ±SD of 4 experiments. ***p<0.001, **p < 0.01; *p < 0.05, two-tailed paired t tests.

Figure 7. Radar chart summarizing the differential impact of PRR triggering by its ligands on DC maturation, infection and HIV Ag-presentation. All calculated indices (maturation, infection, IFN-γ production in ELISpot and polyfunctional index (P-Index) were assembled for each PRR ligand.

Figure-1

A.


TLR-4
HIV
HIV+TLR-4
Single Cells
87,3
MDDC
99,1
FSC-H
FSC-A
SSC-A
FSC-A
x1.4
x1.0
x1.4
x2.2
x1.5
x2.4
24h
72h
ni/untx
+TLR4-L
and/or HIV
HLA-I

B.

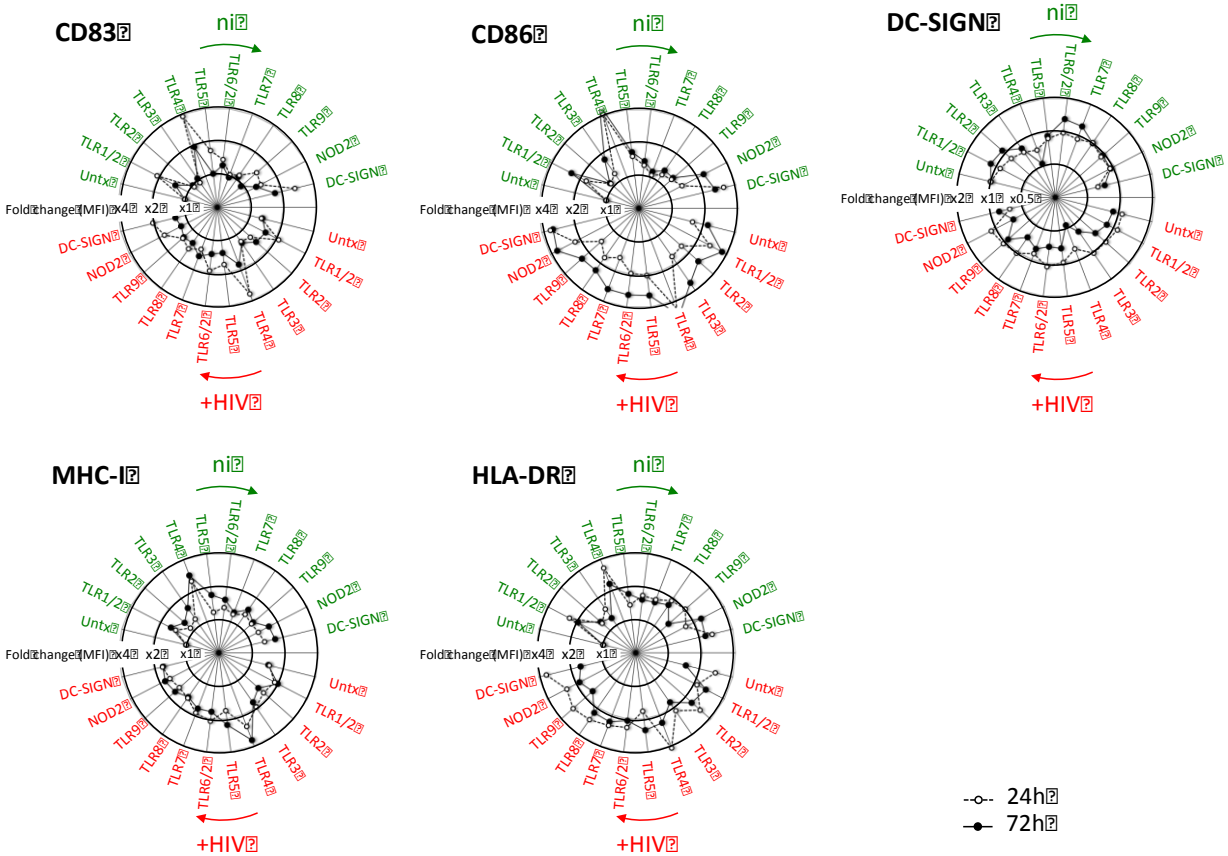

CD83
CD86
DC-SIGN
MHC-I
HLA-DR
ni
+HIV
Fold change (MFI)
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
24h
72h

Figure-2

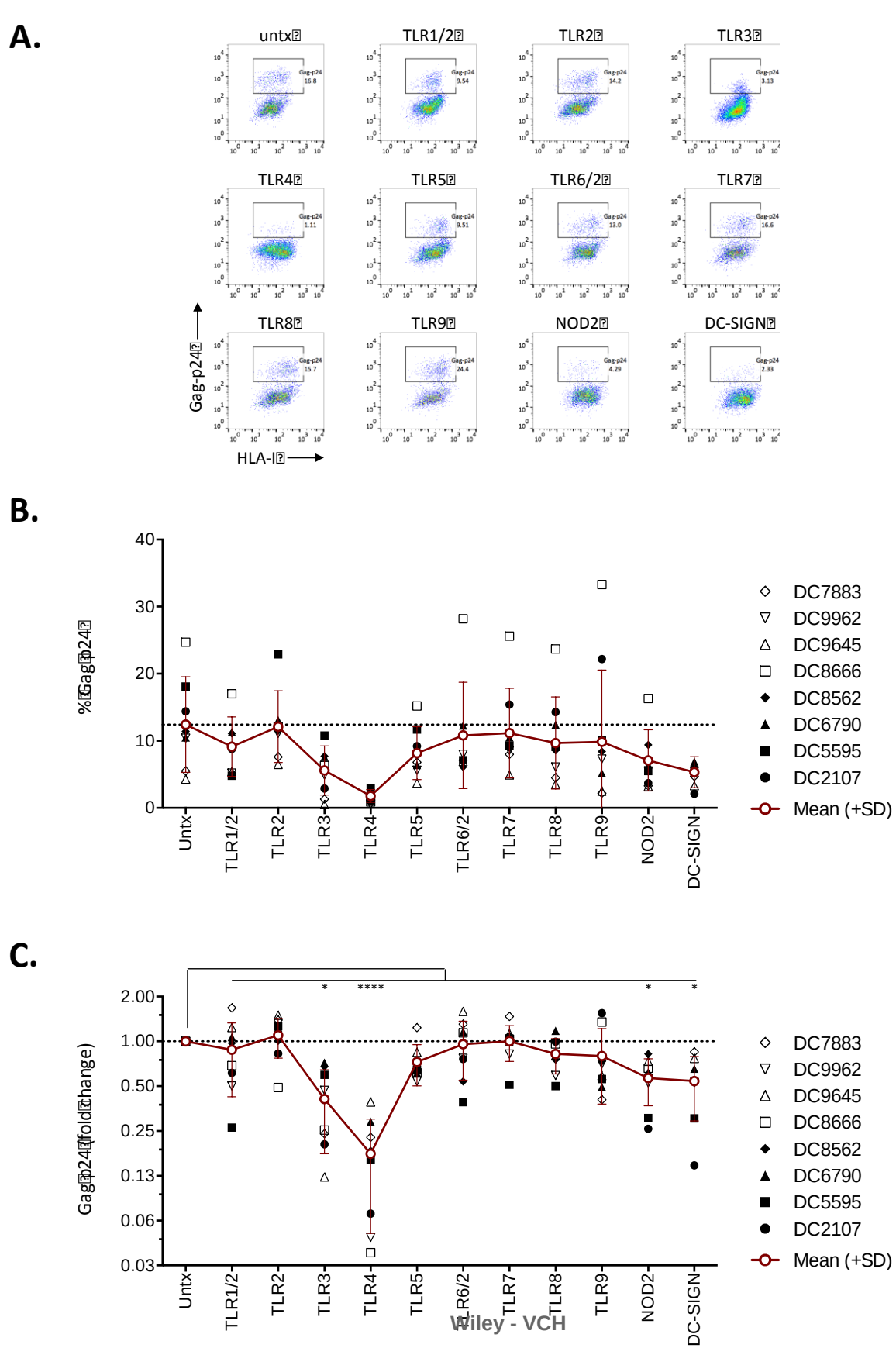

A.
untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
Gag-p24
HLA-I
B.
%Gag-p24
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
DC7883
DC9962
DC9645
DC8666
DC8562
DC6790
DC5595
DC2107
Mean (+SD)
C.
Gag-p24 (fold change)
****
Wiley - VCH

Figure-3

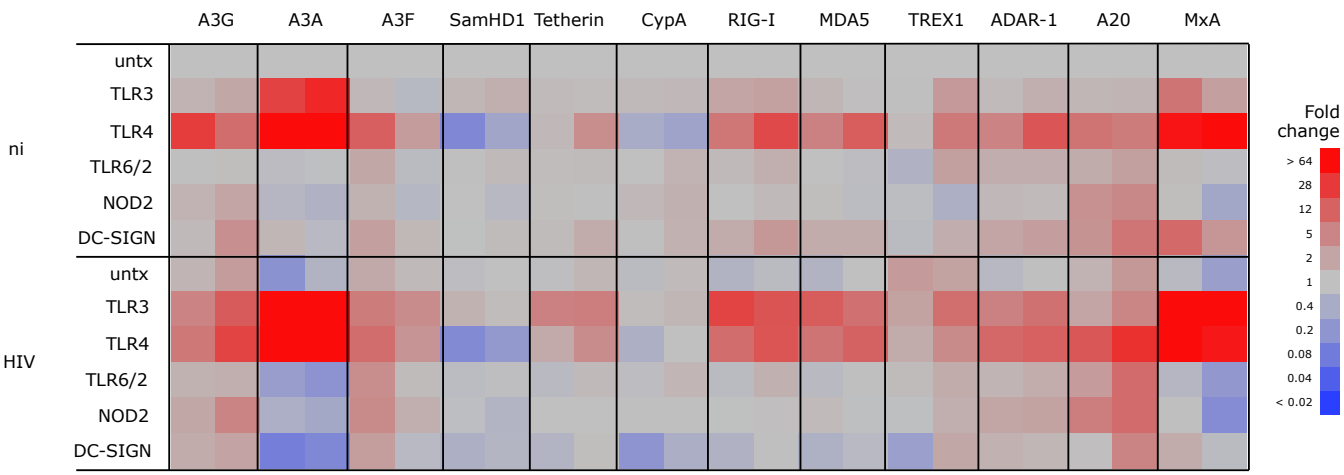
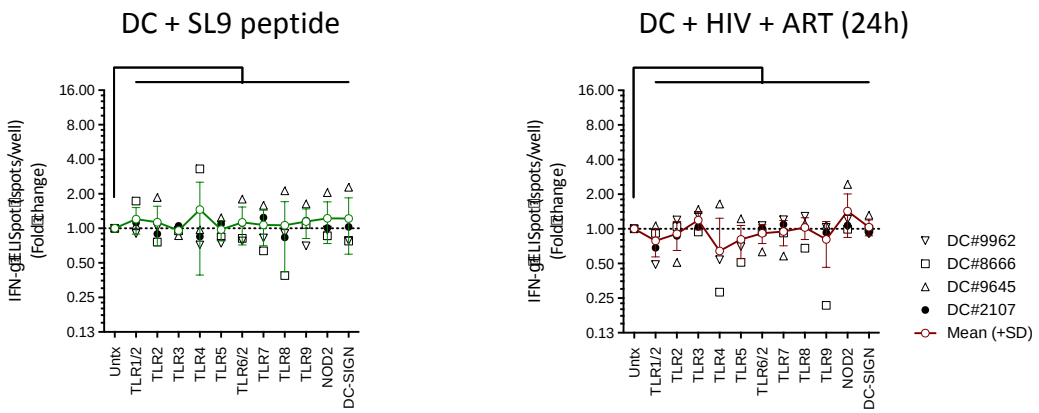

A3G
A3A
A3F
SamHD1
Tetherin
CypA
RIG-I
MDA5
TREX1
ADAR-1
A20
MxA
ni
HIV
untx
TLR3
TLR4
TLR6/2
NOD2
DC-SIGN
Fold change
> 64
28
12
5
2
1
0.4
0.2
0.08
0.04
< 0.02

Figure-4

A.


DC + SL9 peptide
DC + HIV + ART (24h)
IFN-γ ELISpot (spots/well)
(Fold change)
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
DC#9962
DC#8666
DC#9645
DC#2107
Mean (+SD)

B.

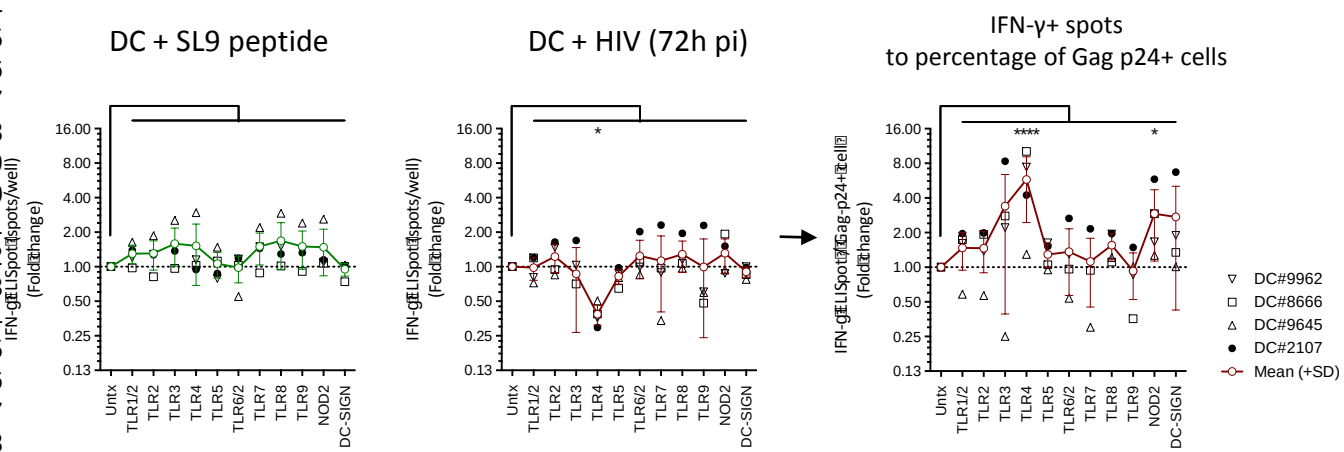

DC + SL9 peptide
DC + HIV (72h pi)
IFN-γ+ spots
to percentage of Gag p24+ cells
IFN-γ ELISpot (spots/well)
IFN-γ ELISpot (Gag-p24+ cell)
(Fold change)
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
*
****
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
DC#9962
DC#8666
DC#9645
DC#2107
Mean (+SD)

Figure-5

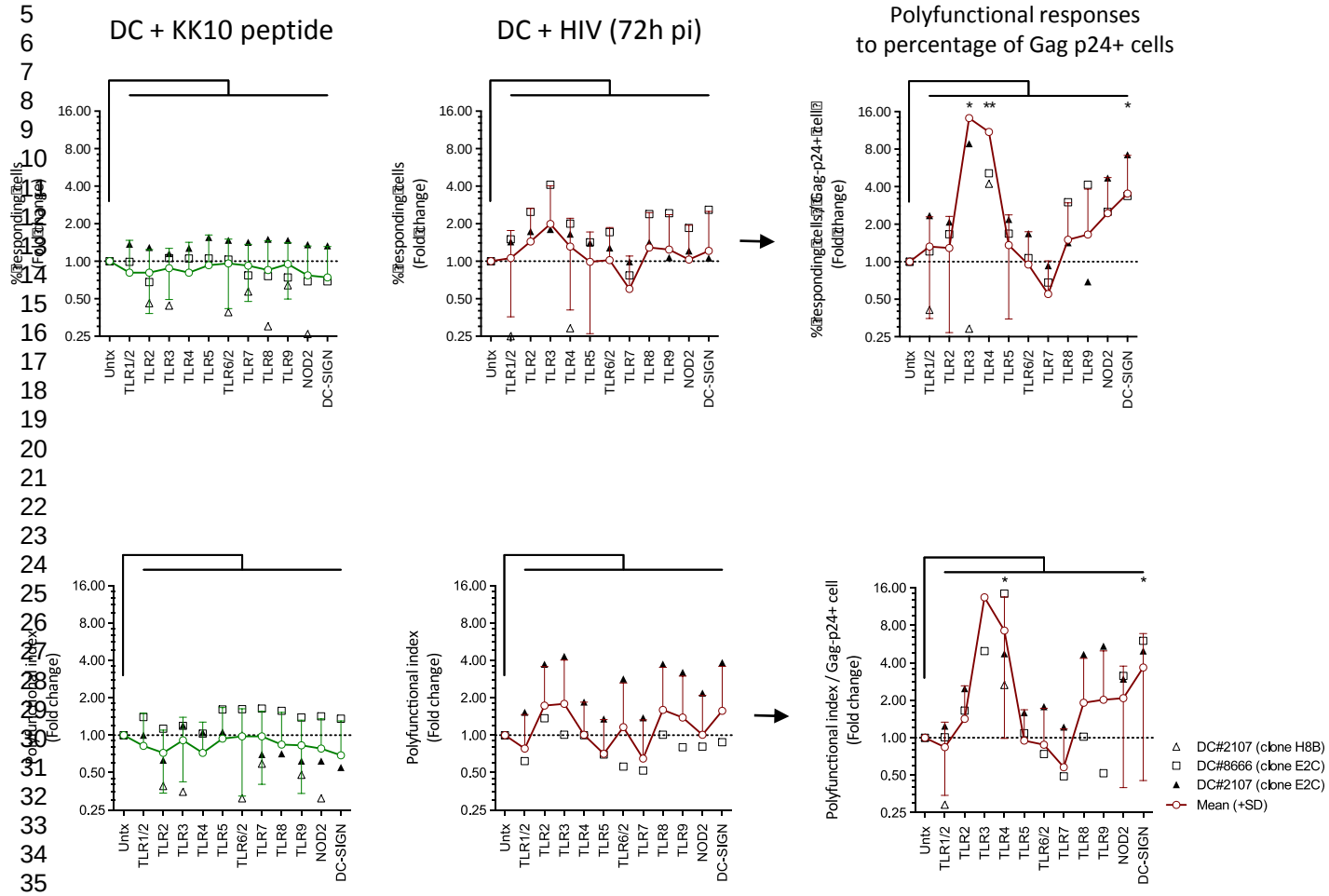

DC + KK10 peptide
DC + HIV (72h pi)
Polyfunctional responses
to percentage of Gag p24+ cells
%Responding cells (Fold change)
Polyfunctional index (Fold change)
Polyfunctional index / Gag-p24+ cell (Fold change)
16.00
8.00
4.00
2.00
1.00
0.50
0.25
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
DC#2107 (clone H8B)
DC#8666 (clone E2C)
DC#2107 (clone E2C)
Mean (+SD)

Figure 6

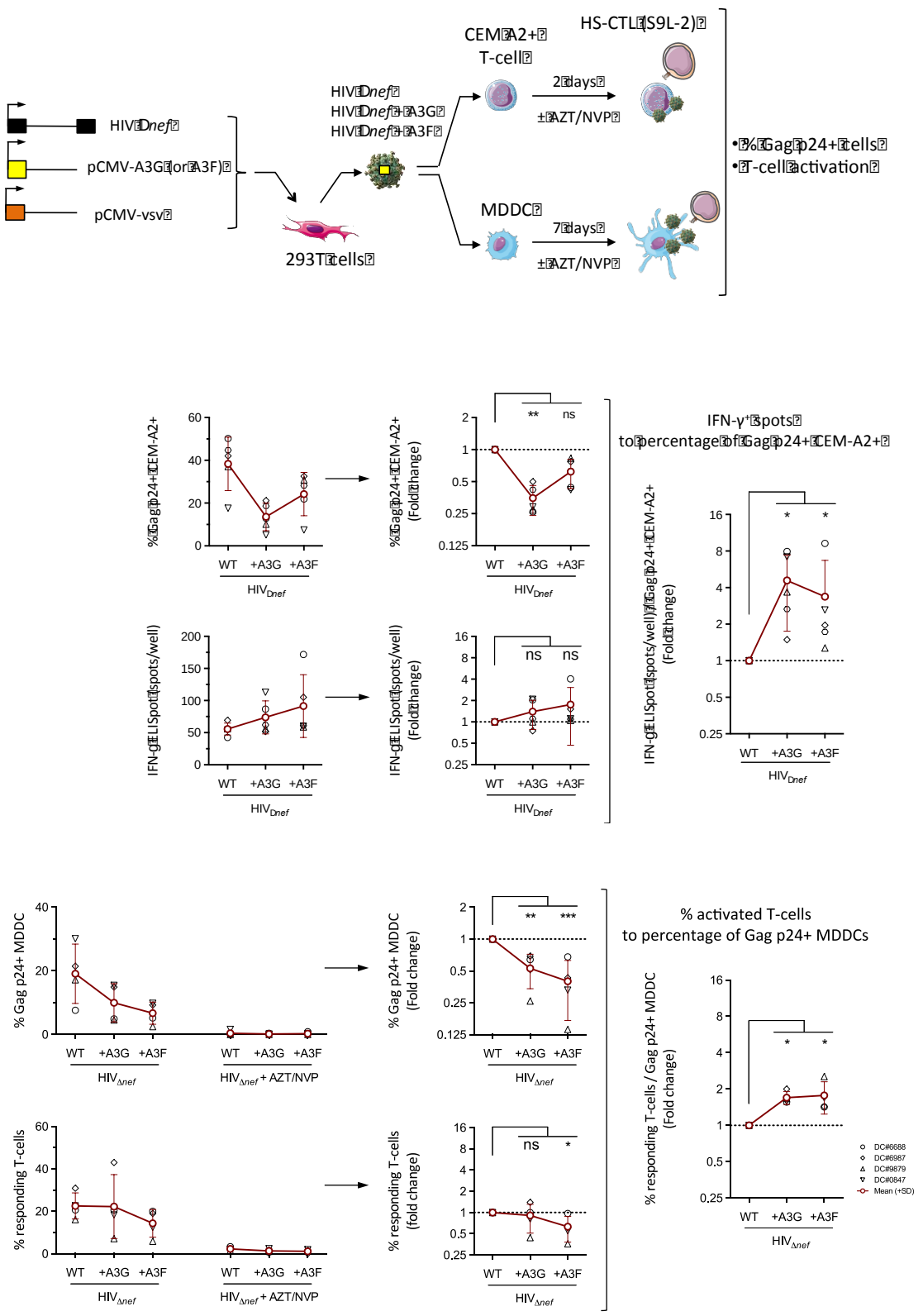
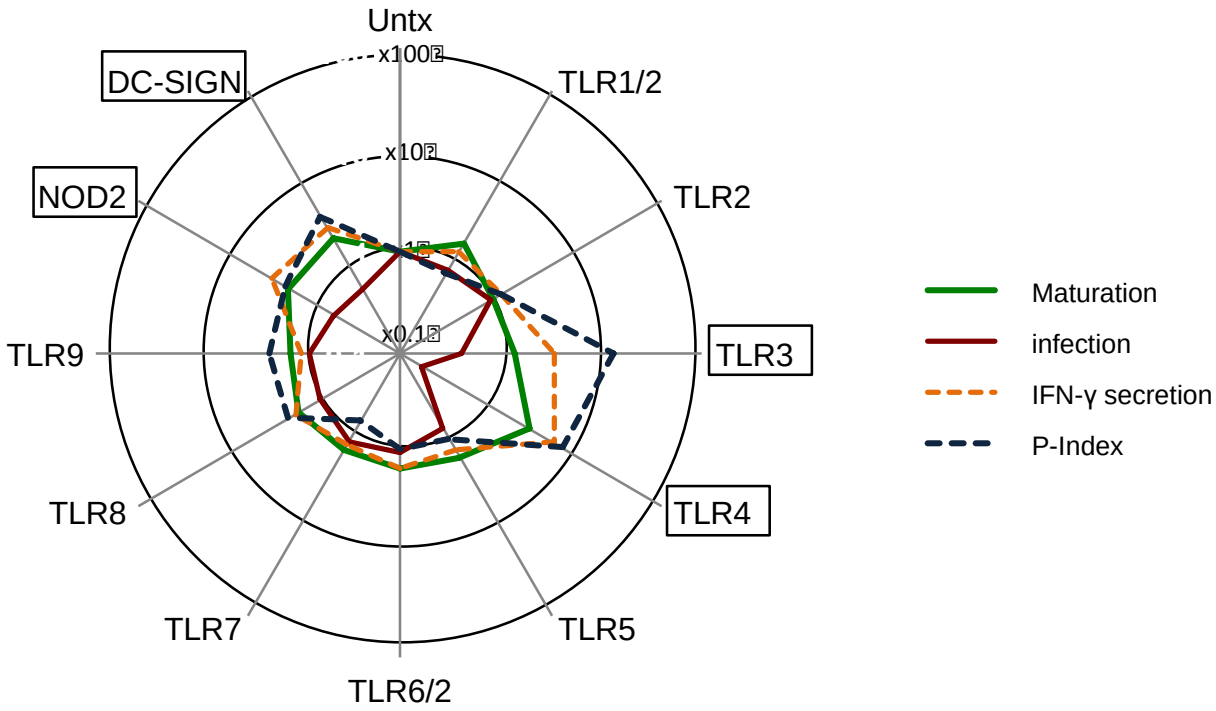

A.
HIV Δnef
pCMV-A3G (or A3F)
pCMV-vsv
293T cells
HIV Δnef
HIV Δnef+A3G
HIV Δnef+A3F
CEM A2+ T-cell
2 days
±AZT/NVP
HS-CTL (S9L-2)
MDDC
7 days
±AZT/NVP
• % Gag p24+ cells
• T-cell activation
B.
% Gag p24+ CEM-A2+
% Gag p24+ CEM-A2+ (Fold change)
IFN-γ ELISpot (spots/well)
IFN-γ ELISpot (spots/well) (Fold change)
WT +A3G +A3F
HIV Δnef
** ns
ns ns
IFN-γ+ spots to percentage of Gag p24+ CEM-A2+
IFN-γ ELISpot (spots/well)/% Gag p24+ CEM-A2+ (Fold change)
* *
C.
% Gag p24+ MDDC
% Gag p24+ MDDC (Fold change)
% responding T-cells
% responding T-cells (fold change)
WT +A3G +A3F
HIV Δnef
HIV Δnef + AZT/NVP
** ***
ns *
% activated T-cells to percentage of Gag p24+ MDDCs
% responding T-cells / Gag p24+ MDDC (Fold change)
* *
DC#6688
DC#6987
DC#9879
DC#0847
Mean (+SD)

Figure 7


Untx
x100
x10
x0.1
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN
Maturation
infection
IFN-γ secretion
P-Index

Supporting Information Fig. 1:

Experimental procedure and cytokine production by PRR-agonist treated MDDCs. **(A)** Schematic representation of the experimental procedure. MDDC were generated from CD14+ monocytes using IL4 and GM-CSF. MDDC were then loaded (AZT/NVP) or infected with HIV_{Yu2b} and simultaneously treated with PRR agonists. HIV-loaded (24h) or infected (72h) MDDC were analyzed using flow cytometry for the expression of DC-maturation markers and co-cultured with HS-CTL clones. CTL activation was monitored using IFN-γ ELISpot assay or ICCS. 72h p.i. replication was also analyzed using anti-Gag-p24 antibody or ELISA (not shown). **(B)** Cytokine/chemokine expressions by PRR-agonist treated MDDCs. 72h p.i. and/or treatment with the agonists, the release of cytokines/chemokines in the cell culture supernatants was evaluated using the luminex technology (25plex). The results from two independent experiments using MDDCs from two different donors (DC9645 and DC2107) are presented. The cytokines/chemokines that could be detected are showed. The numbers correspond to the concentrations in pg/ml. The red color code highlights higher expression levels compared to the untreated controls for each analyte and for donors (the darker the highest). Ni: not infected

Supporting Information Fig. 2 (related to Fig. 1):

Productive HIV infection enhances PRR-induced MDDC maturation. **(A)** Raw data from Fig. 1B presented as fold change of expression for each maturation markers for the 8 independent experiments. Data were normalized to uninfected untreated MDDC. For each maturation marker, mean expression (±SD) of 8 independent experiments using 8 donors are indicated and statistical difference to the untx ni samples were determined as in Fig 1. **(B)** Increased expression of maturation markers on productively infected MDDC analyzed and presented as in Fig. 1. In bold and light red are depicted the results from productively infected (Gag p24+) and "by-standard" uninfected (Gag p24-) MDDC from the same co-cultures, respectively. Individual percentages of infection are presented in Fig 2B (ranging from 4.3 to 24.7%). For each PRR treatment, fold change of maturation marker were compared between the Gag-24- versus Gag-24+ cells using a two-way Holm-Sidak ANOVA multiple comparison test. Adjusted significant p values are presented (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, *** $p<0.0001$).

Supporting Information Fig. 3 (related to Fig. 4):

TLR-3, TLR-4, NOD2 and DC-SIGN agonists enhance HIV Gag-p17 SL9- specific CTL responses. Representative experiment using productively infected MDDC. MDDC from donor DC#2107 were productively infected in the presence of the indicated PRR-agonists (as in Fig. S1B). Three days p.i., viral replication was evaluated using HIV-Gag p24 intracellular staining (right panels) and cells co-cultured with an HIV Gag-p17 SL9-specific CTL clone (1000 CTL/well). T cell activation was then monitored using IFNγ-ELISpot (left panels). T cell activation and percentage infection were normalized to untreated (untx) samples and the data expressed as fold change (left and right middle panels, respectively). Relative to their capacity to reduce viral replication (bottom panel), TLR-3, TLR-4, NOD2 and DC-SIGN agonists enhanced (4 to 8 fold) the activation of the SL9-specific CTL clone. Background IFN-γ production induced by uninfected cells and treated MDDC alone were subtracted; both were at least 10 times lower than with SL9-specific CTL. Activation levels with SL9 peptide–loaded cells were around 500 IFN-γ+ spots/well (not depicted). Data are the mean (±SD) of triplicates using cells from the DC#2107 donor.

Supporting Information Fig. 4:

HIV infection of primary BDCA1+ DC and activation of HIV Gag-p17 S9L-specific CTL clones. (A) Schematic representation of the experimental procedure. BDCA1$^+$ DCs were FACS-sorted from PBMCs of HLA-A2+ donors based CD45, HLA-DR, CD14, CD123, CD11c and CD1c (BDCA1) expressions. Depending on the donors, we obtained 1.3 +/- 0.6 million cells. 200,000 BDCA1$^+$ DC were then loaded (AZT/NVP) or infected with HIV_{Yu2b} (200 ng/ml of p24 / million cells) and simultaneously treated with TLR-3 agonist. Two to five days p.i., the infection and maturation levels were assessed using intracellular Gagp24 or cell-surface CD86 stainings, respectively. BDCA1$^+$ DCs were then co-cultured with SL9-specific CTLs and T cell activation monitored using ICCS**. (B)** Results from of 1 out of 6 independent experiments are presented. BDCA1+ DC infection and maturation was evaluated 48h p.i.. **(C)** Infected or HIV loaded BDCA1+ DC were co-cultured with HIV-Gagp17 SL9-specific CT clones and CTL activation monitored using ICCS.

Supporting Information Fig. 5 (related to Fig. 5):

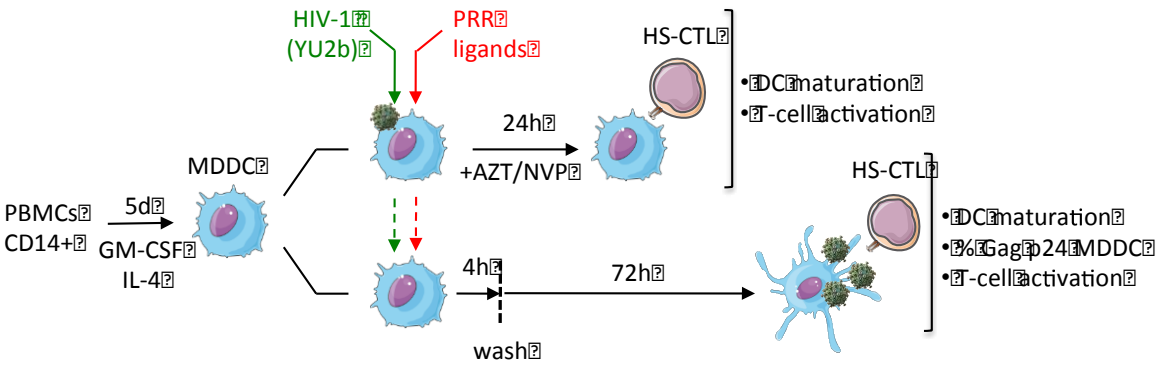
TLR-3, TLR-4, NOD2 and DC-SIGN agonists improve the quality of HIV Gag-p24 KK10- specific CTL responses. Representative experiment using productively infected MDDC from donor DC#2107. MDDC were productively infected in the presence of the

indicated PRR-agonists (as in Fig. S1B). Three days p.i., viral replication was evaluated using HIV Gag-p24 intracellular staining (not shown) and cells co-cultured with an HIV Gag-p24 KK10-specific CTL. T cell activation was monitored by ICCS and flow cytometry using IFN-γ, IL-2, TNF-α, MIP-1β and CD107a mobilization. **(A)** Representative ICCS experiment using TLR-4 agonist (LPS). The percentage of activated KK10 HS-CTL clones induced by KK10-peptide loaded or infected DC are indicated for non-treated cells (untx) and LPS-treated cells. Background secretions induced by uninfected cells were close to zero (not shown). Marked boxes indicated whether the specific cytokines/chemokines or CD107a were positive for each condition. **(B)** The same data are also presented as pies showing the proportion of cells producing one or multiple cytokine/chemokine or marked for their cytolytic activity (CD107a mobilization). The total % of activated cells (mono or polyfunctional) and the polyfunctional indexes are also indicated for the 4 conditions tested. **(C)** Raw data from donor DC#2107 presented as percentage of responding KK10 HS-CTL clones and polyfunctional index (top panel) and as fold change to untreated cells (bottom left panel) for each PRR agonist. Taking into account to their capacity to reduce viral replication (bottom right panel), TLR-3, TLR-4, NOD2 and DC-SIGN agonists enhanced (3 to 17 fold) the activation of the KK10-specific CTL clone. The polyfunctional index follows the exact trend as the percentage of activation.

Supplementary Table 1 related to Fig. 3: Primers used for qRT-PCR

Gene targeted	Primer		Gene targeted	Primer	
A3G	Forward 5'-	CCGAGGACCCGAAGGTTAC	RIG-I	Forward 5'-	GACCCTCCCGGCACAGA
	Reverse 5'-	TCCAACAGTGCTGAAATTCG		Reverse 5'-	TCAGCAACTGAGGTGGCAATC
A3F	Forward 5'-	CCGTTTGGACGCAAAGAT	A20	Forward 5'-	TGCCCAGGAATGCTACAGAT
	Reverse 5'-	CCAGGTGATCTGGAAACACTT		Reverse 5'-	ACAAGTGGAACAGCTCGGATT
A3A	Forward 5'-	GAGAAGGGACAAGCACATGG	ADAR-1	Forward 5'-	CTTCCAGTGCGGAGTAGCG
	Reverse 5'-	TGGATCCATCAAGTGTCTGG		Reverse 5'-	ATTCATTGCGCCCGCGAG
SamHD1	Forward 5'-	AAAACCAGGTTTCACAACTTCTGC	CypA	Forward 5'-	GTCTCCTTTGAGCTGTTTGC
	Reverse 5'-	TGCGGCATACAAACTCTTTCTGT		Reverse 5'-	CGTATGCTTTAGGATGAAGTTCTC
Tetherin	Forward 5'-	AAGAAAGTGGAGGAGCTTGAGG	TREX1	Forward 5'-	GCATCTGTCAGTGGAGACCA
	Reverse 5'-	CCTGGTTTTCTCTTCTCAGTCG		Reverse 5'-	AGATCCTTGGTACCCCTGCT
MDA5	Forward 5'-	GGTCTGGATATTAAAGAATGTAACATTGTTATC	MxA	Forward 5'-	GCCGGCTGTGGATATGCTA
	Reverse 5'-	CCAGGACGTAGGTGCTCTCATC		Reverse 5'-	TTTATCGAAACATCTGTGAAAGCAA
Actin-B	Forward 5'-	TCCTTCCTGGGCATGGAGT			
	Reverse 5'-	AGCACTGTGTTGGCGTACAG			

A.

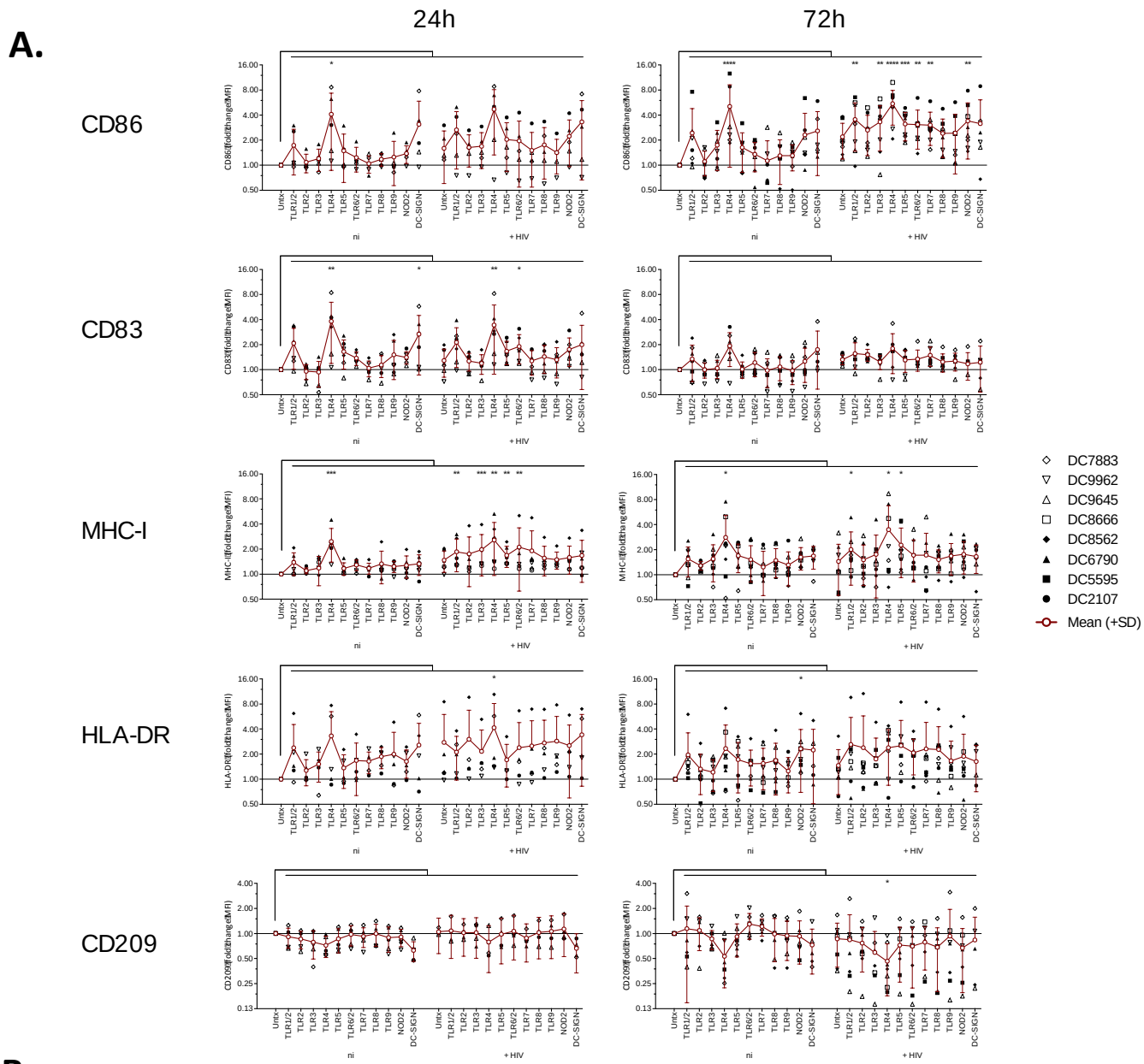


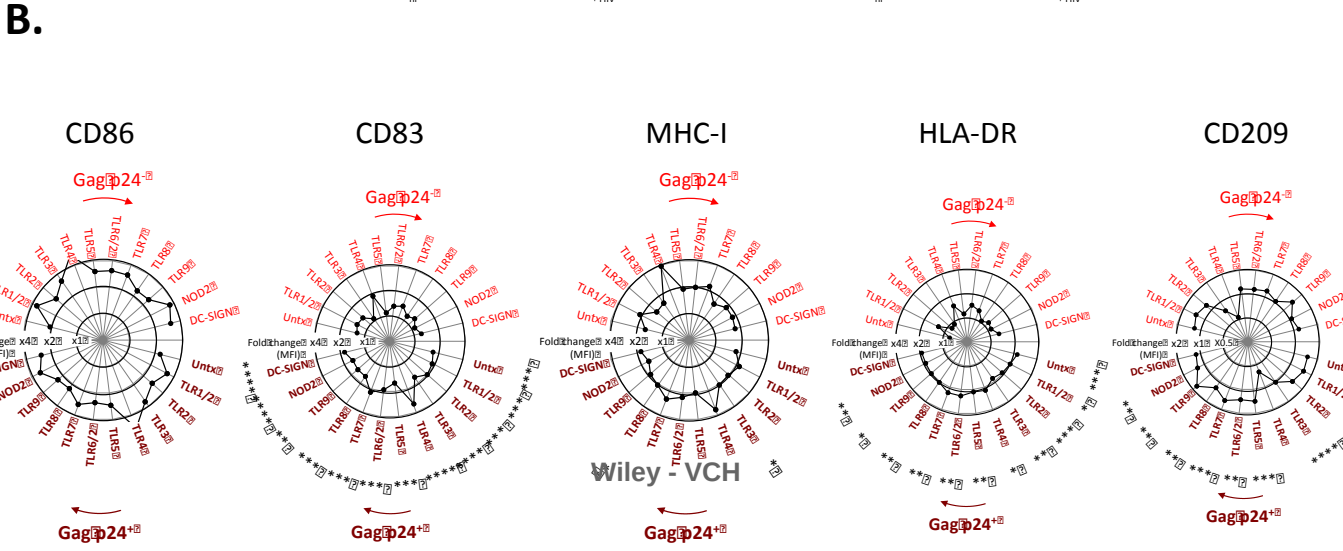
B.

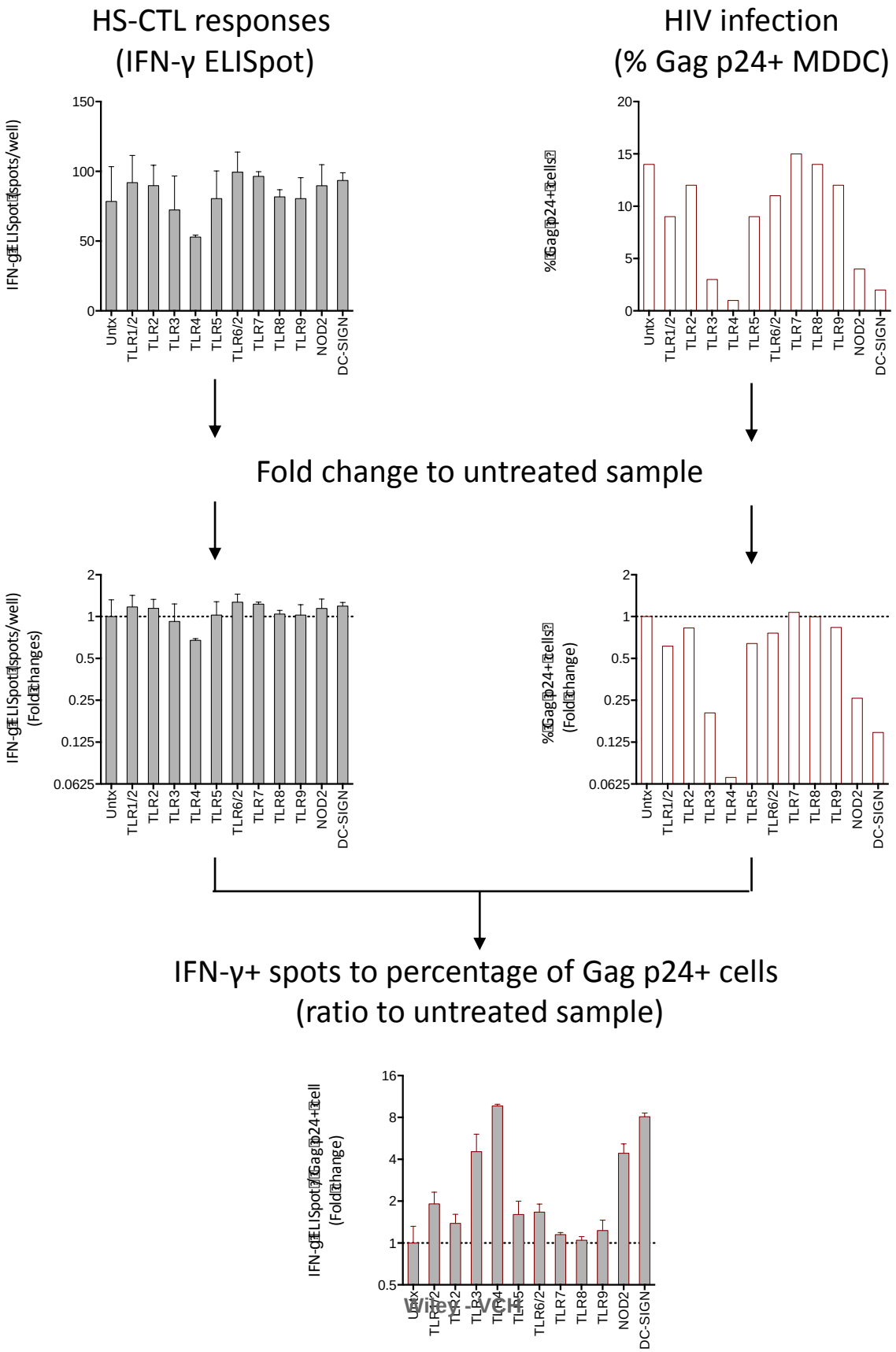
		IL-8		IL1RA		MCP-1		IP10		IL-6		IL-12		MIP1a	
		DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107
Ni	untx	1680	1400	650	340	490	150	20	0	50	20	0	110	50	30
	TLR1/2	3300	16270	600	1240	520	880	0	10	240	4040	140	3990	20	60
	TLR2	1490	810	4100	450	1350	150	30	0	100	10	20	110	140	20
	TLR3	1580	1410	2350	960	1400	200	90	450	140	30	20	110	100	20
	TLR4	7840	16480	670	1190	700	540	100	80	300	5000	460	5330	100	2660
	TLR5	3180	5000	1150	710	720	470	10	70	350	470	60	280	40	40
	TLR6/2	2520	3330	1810	2380	1030	3780	10	140	300	70	20	220	50	490
	TLR7	2030	340	1120	290	720	70	10	30	90	20	20	30	130	0
	TLR8	5360	1920	2070	410	680	310	20	20	190	30	50	80	120	40
	TLR9	5530	930	970	400	620	170	10	0	110	20	20	120	90	20
	NOD2	6690	4320	920	720	570	300	10	10	80	30	20	90	60	20
	DC-SIGN	5450	14500	810	910	610	600	10	140	230	580	20	810	50	110
HIV	untx	12660	4780	8910	2750	730	5540	340	430	700	100	150	160	290	710
	TLR1/2	14440	13120	8070	1790	420	4430	60	140	1960	1420	450	1510	130	110
	TLR2	8830	3220	11500	2340	700	3380	670	230	400	120	30	80	140	330
	TLR3	4420	2900	9540	1580	560	4390	10000	10000	410	70	20	50	90	40
	TLR4	14260	15620	8590	1070	1450	670	10000	170	5000	2500	1860	730	330	270
	TLR5	10450	3300	9330	1530	350	1960	120	130	990	130	210	270	90	130
	TLR6/2	14040	3180	11230	2340	590	3990	250	130	1110	70	180	210	170	470
	TLR7	12470	2300	6730	3040	7350	4900	240	390	470	70	80	80	3610	540
	TLR8	4050	830	11580	2750	410	3350	460	190	280	30	10	70	100	450
	TLR9	5340	1010	12490	2890	350	4900	270	310	270	40	20	110	100	1090
	NOD2	17440	9300	15770	2840	490	2840	160	190	1400	90	260	220	320	400
	DC-SIGN	16940	12050	14520	2170	560	3670	290	360	5000	210	280	430	230	280

		MIP1b		IL-15		IL-2R		IFN-a		Rantes		TNF-a		IL-10	
		DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107	DC9645	DC2107
Ni	untx	90	30	170	180	0	0	20	20	0	0	0	0	0	0
	TLR1/2	40	80	170	250	0	30	20	40	0	10	0	10	0	10
	TLR2	140	20	180	160	10	0	20	20	0	0	0	0	0	0
	TLR3	120	20	160	250	20	0	30	10	0	0	0	0	0	0
	TLR4	170	2830	200	290	20	300	30	40	1	670	0	20	0	210
	TLR5	70	60	190	190	0	0	20	30	0	0	0	0	0	0
	TLR6/2	70	540	160	210	0	50	20	40	0	0	0	20	0	0
	TLR7	130	10	160	170	10	0	30	20	0	0	0	0	0	0
	TLR8	100	50	180	160	10	0	30	20	0	0	0	10	0	0
	TLR9	180	20	200	160	10	0	30	20	0	0	0	0	0	0
	NOD2	140	40	160	160	0	0	20	30	0	0	0	0	0	0
	DC-SIGN	80	170	170	230	0	20	20	30	0	0	0	20	0	0
HIV	untx	210	680	250	230	50	70	30	50	10	0	20	20	0	0
	TLR1/2	90	160	220	250	30	40	30	50	0	0	10	10	0	0
	TLR2	90	390	270	200	30	30	20	40	0	0	10	20	0	0
	TLR3	50	60	370	360	10	0	20	50	0	0	10	0	0	0
	TLR4	380	560	400	210	200	40	30	30	170	40	10	0	10	10
	TLR5	60	190	210	190	30	10	20	30	0	0	10	10	0	0
	TLR6/2	130	530	240	200	40	60	20	50	0	0	10	10	0	0
	TLR7	1900	620	250	260	170	70	60	40	30	0	30	0	0	0
	TLR8	40	570	230	200	20	60	20	40	0	0	0	0	0	0
	TLR9	30	1030	230	240	10	100	20	50	0	0	10	0	0	0
	NOD2	170	540	210	210	70	50	30	40	30	0	20	10	0	0
	DC-SIGN	110	430	240	260	60	50	30	40	10	0	20	20	0	0

Supporting Information Fig. 2

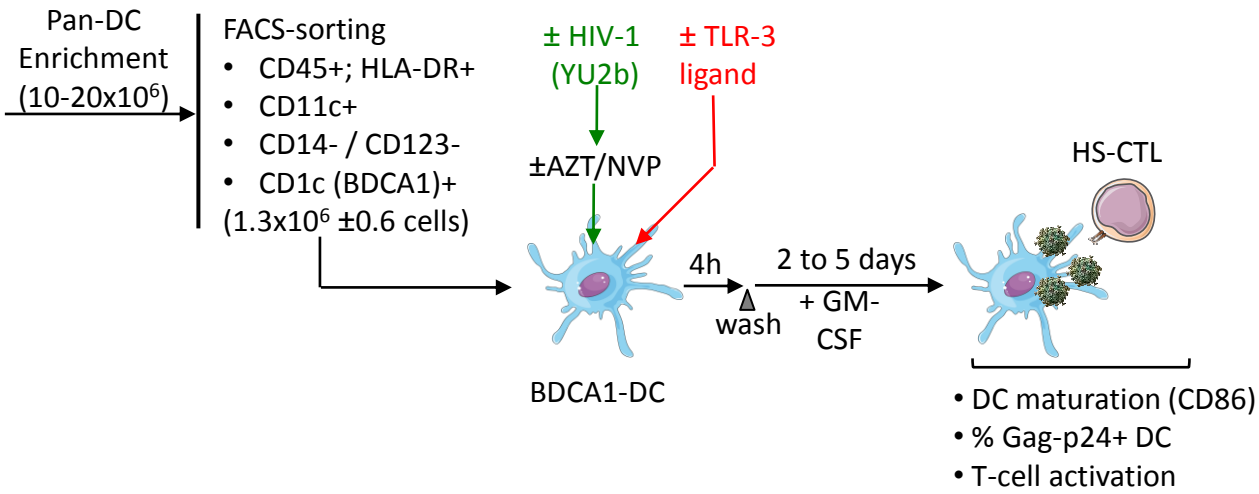




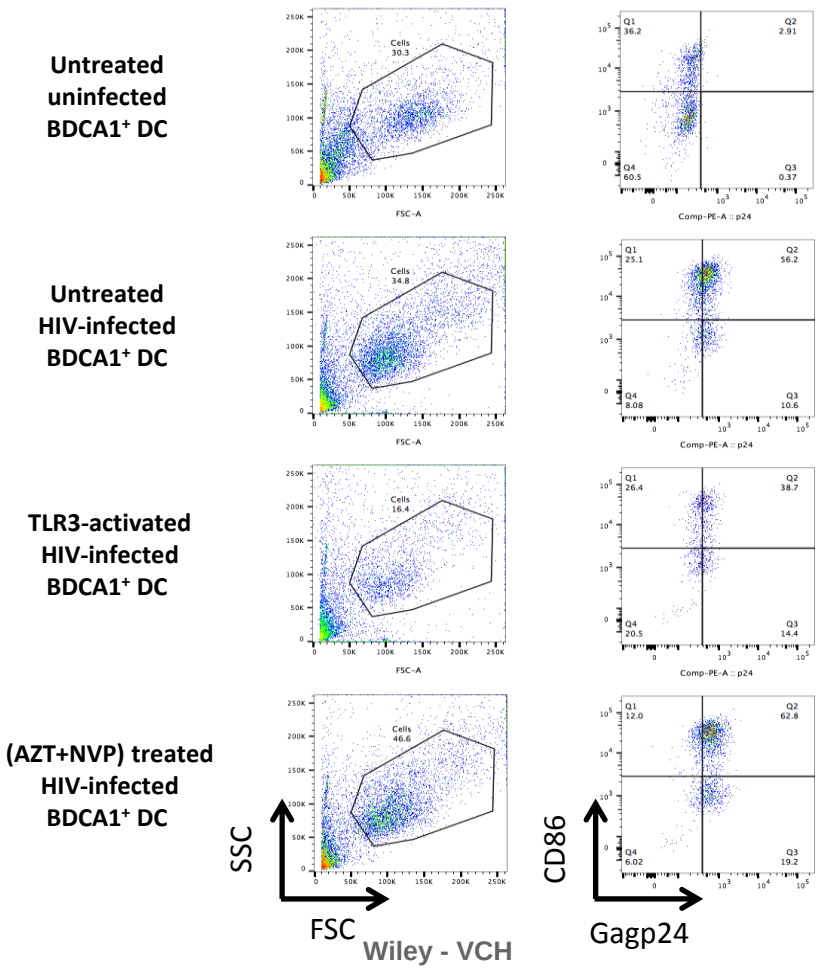

HS-CTL responses
(IFN-γ ELISpot)
HIV infection
(% Gag p24+ MDDC)
Fold change to untreated sample
IFN-γ+ spots to percentage of Gag p24+ cells
(ratio to untreated sample)
Untx
TLR1/2
TLR2
TLR3
TLR4
TLR5
TLR6/2
TLR7
TLR8
TLR9
NOD2
DC-SIGN

Supporting Information Fig. 4

A. Experimental procedure

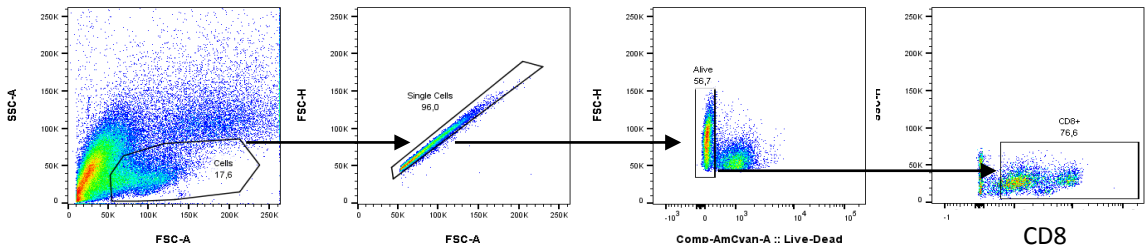


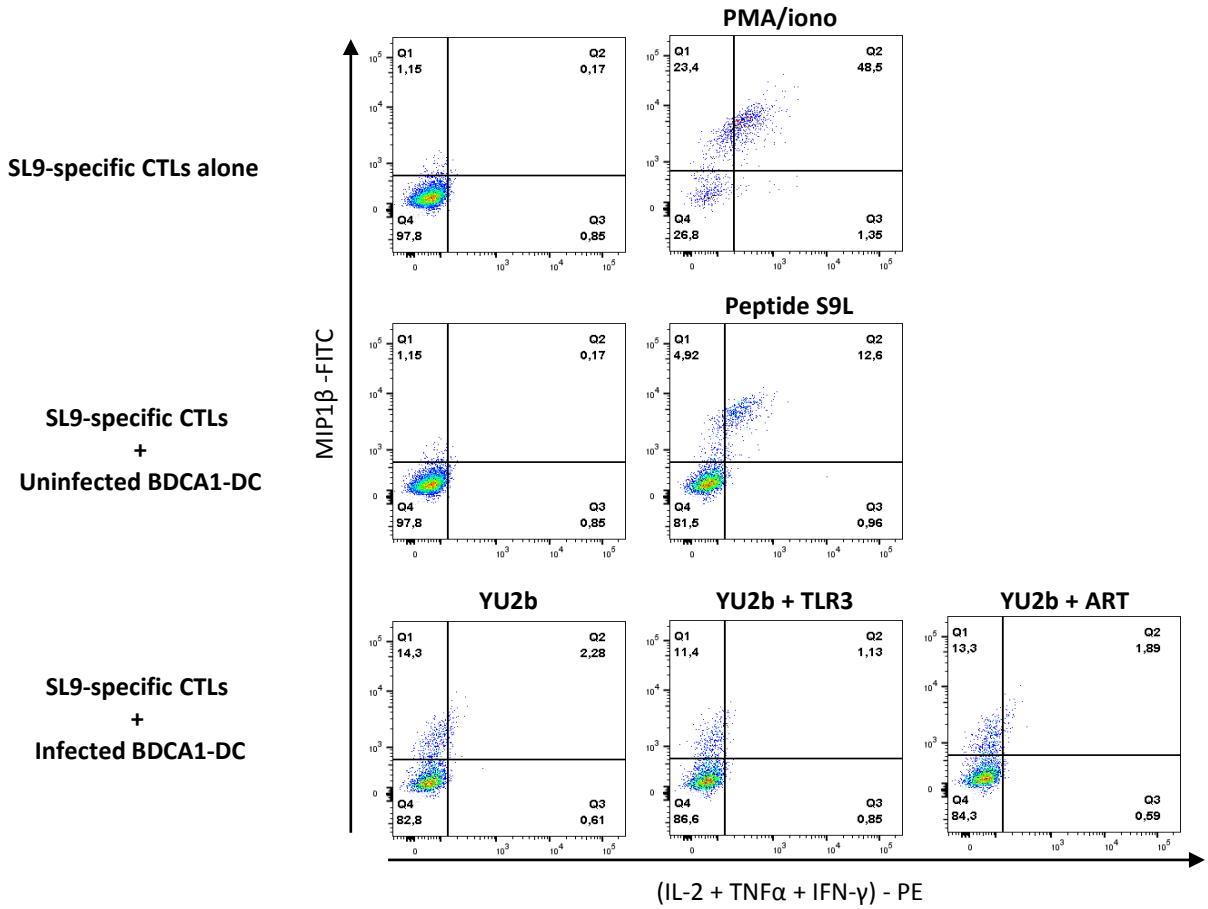
B. Evaluation of BDCA1⁺ DC infection and maturation



C. Activation of HIV-Gagp17 SL9-specific CTL clones (ICCS)

Gatings





Supporting Information Fig. 5