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Cell associated HIV cross-presentation by pDC is potentiated by non-cognate CD8 T cell preactivation

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- HIV-infected H9 cells induce type I IFN production by pDC
- Type I IFN induces IFN- γ intracellular production in CD8 T cells
- pDC cross-present MHC-I-restricted HIV-Gag antigen to specific CD8⁺ T cells
- IFN- γ secretion by CD8⁺ T cells is potentiated by former type I IFN stimulation, but occurs only in the case of cognate interaction with pDC

Cell-associated HIV cross-presentation by pDC is potentiated by non-cognate CD8⁺ T cell pre-activation¹

Running title: HIV sensing by pDCs potentiates their crosspresentation

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Abstract

Human plasmacytoid dendritic cells (pDC) can cross-present antigens from apoptotic HIV-infected cells or tumor cells to CD8⁺ T cells. As pDC respond to HIV virions by maturing and secreting cytokines, we wondered whether these innate properties would affect cross-presentation from HIV-infected cells. We incubated purified blood DC with apoptotic HIV-infected H9 cells and then explored the activation process of HIV-specific cloned CD8⁺ T cells, in the presence of saquinavir. We studied IFN- γ secretion by HIV-specific T cells, which is known to be tightly regulated by engagement of the T cell receptor. We found that HIV-specific secretion by cloned HIV-specific CD8⁺ T cells was stimulated by pDC and cDC1 more than by cDC2, and was strictly MHC-Class I (MHC-I)-restricted. Surprisingly, intracellular production of IFN- γ was only partly MHC-I restricted for pDC, indicating a non-cognate CD8⁺ T cell activation. Plasmacytoid DC, but not cDC, matured and secreted IFN- α in the presence of apoptotic H9HIV cells. A cocktail of IFN- α , IFN- β and TNF- α induced intracellular production of IFN- γ but not Granzyme-B, mimicked the non-cognate mechanism, and neutralization of type I IFN signaling blocked non-cognate intracellular production of IFN- γ in the co-culture model. Moreover, cognate stimulation was required to induce IFN- γ secretion in addition to the cytokine cocktail. Thus, IFN- γ secretion is tightly regulated by engagement of the TCR as expected, but in the context of virus-infected cells, pDC can trigger intracellular IFN- γ accumulation in CD8⁺ T cells, potentializing IFN- γ secretion once CD8⁺ T cells make cognate interactions. These findings may help manipulate type I IFN signaling to enhance specifically antigen-specific CD8⁺ T cell activation against chronic infections or tumors.

- HIV-infected H9 cells induce type I IFN production by pDC
- Type I IFN induces IFN- γ but not Granzyme intracellular production in CD8⁺ T cells
- pDC cross-present MHC-I-restricted HIV-Gag antigen to specific CD8⁺ T cells
- IFN- γ secretion is potentiated by type I IFN, but only upon cognate interaction

38 **Introduction**

39 HIV-specific CD8⁺ T cells activity is essential to achieve HIV control during acute and chronic
40 infection (1-8). To activate CD8⁺ T cell receptors (TCR) and induce antiviral and cytotoxic
41 responses, antigens must be presented as epitopes bound to class I major histocompatibility
42 complex (MHC-I). In general, antigens that are synthesized in the cytosol of virus-infected
43 cells are digested by the proteasome, transported into the endoplasmic reticulum, where they
44 associate with nascent MHC-I molecules. MHC-I-peptide complexes are then exported to the
45 cell surface: this is the direct antigen presentation pathway, available in all cell types (9). The
46 direct antigen presentation pathway allows the detection and elimination of infected cells by
47 antigen-specific CD8⁺ T-cells.

48 Dendritic cells (DC) are specialized in antigen presentation and activation of antigen-specific
49 T cells. They have specific antigen cross-presentation functions, allowing them to present
50 antigens which are not produced in their cytosol, but captured from other tissues, tumor cells
51 or infected cells (10, 11). They are poorly infected by HIV compared to CD4⁺ T cells,
52 especially because of their high expression of restriction factors (12). Hence, they probably
53 stimulate HIV-specific CD8⁺ T-cells mainly by cross-presentation of exogenous antigens
54 produced in infected CD4⁺ T cells. During cross-presentation, antigens are engulfed into
55 endosomal/phagosomal compartments, and reach MHC-I molecules by either of two pathways:
56 endosome-to-cytosol translocation, then proteasomal digestion and transport into the ER or
57 phagosome; or endosomal pathway, where MHC-I molecules are fed by fusion with the
58 endoplasmic reticulum, and digestion is performed by local enzymes (13-15).

59 Among DC, CD8α⁺ cDC1 appeared to be specialized in cross-presentation in mice (16). Their
60 human homologs, XCR1⁺CD141⁺ cDC1, express a collection of receptors for dead or dying
61 cells, chemokine receptors and adhesion molecules to interact with CD8⁺ T cells, and TLR for
62 microbial recognition, all converging toward a specialization for cross-presentation (17-20).

63 However, the two other populations of human DC, CD1c⁺ cDC2, and plasmacytoid DC (pDC),
64 have also been shown to be able to cross-present antigens (18, 21-23). Human immature pDC
65 have lower endocytic capacities than cDC, and pDC were believed to be poor cross-presenting
66 cells. However, we have shown that pDC can cross-present HIV antigens (18, 21). Other
67 laboratories have also shown that pDC cross-presented antigens from Influenza A Virus (24-
68 27), tumors (22, 23, 28, 29) or Cytomegalovirus (28) either as cell-associated antigens, long
69 peptides, lipopeptides or particulate antigens. Similarly, murine pDC were also shown to be
70 able to perform cross-presentation (30, 31). In addition, pDC have exacerbated sensing
71 properties and strong inflammatory responses, particularly the production of interferon (IFN)-
72 α , which has been shown to activate and increase CD8⁺ T cell function (32-34). Thus, some
73 laboratories have set up clinical protocols to induce antitumor responses using pDC lines or
74 pDC isolated from patient's blood, or combinations of pDC and cDC, loaded with tumor
75 antigens (28, 35).

76 Our laboratory has shown that pDC cross-present HIV antigens to CD8⁺ T cell lines (18, 21).
77 As pDC can respond to HIV by maturation and secretion of different cytokines (32, 33), we
78 wondered how these innate responses to HIV-infected cells would affect cross-presentation.
79 To explore the activation process of CD8⁺ T cells by pDC compared to other DC, we isolated
80 and expanded CD8⁺ T cell clones specific for an HIV-1_{lai}-Gag, HLA-A2-restricted epitope. We
81 tested the capacity of pDC to cross-present HIV-infected cell antigens to these CD8⁺ T cell
82 clones, which secreted IFN- γ in response to cognate recognition. IFN- γ secretion by specific T
83 cells is known to be tightly regulated, being triggered as very short ON/OFF cycles by
84 engagement of the TCR (36, 37). By comparing CD8 T cell clone activation by HLA-A2⁺ or
85 HLA-A2⁻ DC, we uncovered a non-cognate intracellular pre-activation of these CD8⁺ T cells
86 by type I IFN produced by pDC upon stimulation by HIV-infected antigen-donor cells.

Materials and Methods

Cell isolation and culture

Cytapheresis residues were obtained from healthy blood donors from the Etablissement Français du sang (EFS) Crozatier-Saint-Antoine within a convention (C CPSL UST 18/EFS/030) between INSERM and EFS. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Human pancoll, Dutscher, Issy les Moulineaux, France). DC were isolated as previously described (38). Briefly, enriched DC were obtained by depleting CD19⁺ or CD9⁺ B cells, CD3⁺ T cells, CD14⁺ and or CD16⁺ monocytes, CD56⁺ NK cells, CD9⁺ platelets, CD66b⁺ granulocytes and Glycophorin-A⁺ erythrocytes from PBMC (Stemcell, Grenoble, France). Enriched DC were then sorted by FACS to isolate the different populations of blood DC according to the strategy depicted in the Supplemental Fig. 1A. All the cells were maintained in "complete medium" (RPMI 1640-glutamax containing 100 U/mL penicillin; 100 mg/mL streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer; Life Technologies, Saint-Aubin, France) supplemented with 2% human albumin (Vialebex, LF Biomédicaments, Les Ulis, France). In all experiments but those of Sup Fig. 2B, to improve DC survival, we cultured pDC with 10 ng/ml IL-3 (Invivogen, Toulouse, France) and cDC with 1000 U/ml GM-CSF (Peprotech, Neuilly sur Seine, France). When indicated, we stimulated cDC1 with 30 µg/ml Poly I:C (Invivogen) and cDC2 with 100 ng/ml LPS (Sigma). All cells were incubated in a humid incubator at 37°C, 5% CO₂. For each condition, DC were cultured at 10.10³ for ELISA or 25.10³ for flow cytometry in 200 µL, in 96 well plates, with "complete medium" and 2% human albumin.

H9 and H9HIV-1_{IIIB} cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID from Dr. Robert Gallo (39, 40) and maintained in culture in "complete medium" + 10% Fetal Calf Serum (FCS, Life Technologies France). They were irradiated for 1 to 5 min

by UV-C (240 mJ/cm²) for apoptosis induction, and cultured overnight before culture with DC. All experiments involving H9 and H9HIV cells were performed in the presence of Saquinavir (1 μ M), a protease inhibitor used as an antiretroviral drug, to prevent direct presentation of HIV, kindly provided by the NIH AIDS Reagent Program, division of AIDS, NIAID, NIH.

T cell clones were obtained from PBMC of an HLA-A2⁺ HIV-infected donor (Prevac Cohort, IRB 00003835, authorized by the CPP Ile de France IV, June 25, 2010). Briefly, PBMC were pulsed with HIV-1-Gag₇₇₋₈₅ (41) short peptide for 1 h at 4°C, then incubated at 37°C, 5% CO₂ for 4 h, and IFN- γ producing cells were magnetically purified using the IFN- γ secretion Assay (Miltenyi, Paris, France). Cells were then cloned by limiting dilution on irradiated feeders (PBMC and LAZ cells; kind gift from Dr. Fonteneau, Nantes, France) and amplified in "complete medium", with 8% AB-negative heat inactivated human serum, 150 U IL-2/mL (Chiron or Miltenyi) and PHA-L (Millipore, Molsheim, France) as in (42). After amplification, cell specificity was confirmed by incubating T cell clones overnight with HIV-1_{lai}-Gag₇₇₋₈₅ peptide-pulsed HLA-A2⁺ B cell blasts (Supplemental Fig. 2A). Supernatants were collected and IFN- γ measured by ELISA (Mabtech, Courtaboeuf, France). Background IFN- γ secretion with a control Flu peptide (H1N1-A/PR8₅₈₋₆₆) was usually less than 1% of the signal obtained with HIV-1-Gag₇₇₋₈₅ peptide. Percentage of HIV-1-Gag specific cells was analyzed by flow cytometry as described below. More than 95% of CD8⁺ T cell clones produced IFN- γ in response to their cognate antigen. For experiments involving CD8⁺ T cell clones only, 25.10³ cells were cultured in 200 μ L in "complete medium" with 8% AB-negative heat-inactivated human serum.

Flow cytometry

For DC isolation from DC-enriched PBMC, as described in (38), Live/Dead Yellow (Thermofisher) was used to exclude dead or dying cells. The following antibodies were diluted

in labeling buffer (PBS, 2 mM EDTA, 0.5% BSA) and incubated with the cells for 20 min on ice; anti human CD1c (BDCA-1) Pacific Blue (Biolegend, L161 clone, diluted at 1/400), CD141 (BDCA-3) APC-Vio770 (Miltenyi, AD5-14H12 1/50), CD304 (BDCA-4) PE-Vio770 (Miltenyi, AD5-17F6 1/20) and CD3/14/16/19/20/56 (Lin1) FITC (BD, SK7, 3G8, SJ25C1, L27, MphiP9, NCAM16.2 1/50), as in (38). Cells were then washed and purified by FACS (FACS ARIA, Beckton Dickinson or ASTRIOS, Beckman Coulter) with >95% purity (Supplemental Fig. 1A). From 1 billion PBMC per donor, we usually sort 1 million and more of pDC and cDC2, compared to 0.15 million cDC1 only (38). For DC maturation assays, anti-human CD1c Pacific Blue (Biolegend, L161 1/400), CD141 PE (BD, 1A4), CD123 APC (Miltenyi, AC145 1/50), CD3 APC-H7 (BD, SK7 1/50), CD83 PE-Cy5 (BD, HB15e 1/30) and CCR7 FITC (R&D, FAB1971 1/30) were used.

For assessment of CD8⁺ T-cell function (38), 2 PBS washes were performed before adding Live/Dead Blue (Life Technologies), then Fc Block (5% Human Serum (Dutscher) on ice. Anti-human CD1c Pacific Blue (Biolegend, L161 1/400); CD141 PE-Cy7 (Miltenyi, AD5-14H12 1/100); CD123 PE-Cy5 (Biolegend, 6H6 1/50); CD3 APC-H7 (BD, SK7 1/50); CD8 FITC (BD, SK1 1/50); and HLA-A2 PE (BD, BB7.2 1/50) diluted in labelling buffer were incubated with the cells for 20 min on ice for surface staining. Cells were then washed twice in labeling buffer, fixed for 15 min in PBS PFA 4%, washed again and incubated with anti IFN- γ APC (Sony, B27 1/20) in PBS, 0.1% BSA, 0.1% saponin for 45 min on ice for intracellular staining. Cells were washed twice in PBS containing BSA and saponin, and resuspended in labeling buffer before acquisition on a LSR II flow cytometer (BD Biosciences).

Microscopy

After incubation with cytokines for 4 h on poly-D-lysine (Millipore)-coated coverslips, cells were washed once with PBS and fixed using 4% PFA in PBS for 15 min at room temperature.

Permeabilization and blocking were performed using PBS 0.1% Saponine 0.1% BSA 5% Human Serum for 30 min at room temperature. Labeling was performed using anti-CD3 rabbit polyclonal antibodies (Dako, 1/100), anti-human Granzyme–FITC (Sony, GB11 1/20) and anti-human IFN- γ -APC (Sony, B27 1/5) for 1h at room temperature. After washing, Goat anti-Rabbit IgG (H⁺L) Alexa Fluor 546 (Invitrogen, 1/200) were added for 45 min at room temperature. DAPI was used to stain the nucleus. Fluoromount-G (Invitrogen) was used to mount the slides. Images were taken using a confocal microscope (Leica DMI6000, spinning disk Yokogawa CSU-X1M1) and analyzed with ImageJ software.

Antigen cross-presentation assay

We improved the model established in the laboratory (21) by using CD8⁺ T cell clones (38). We incubated UV-C irradiated HIV-1 infected H9 cells (which do not express HLA-A2) with blood DC isolated from HLA-A2⁺ or HLA-A2⁻ donors (5 H9 cells per DC), stimulated or not as indicated, in the presence of saquinavir. After overnight co-culture and washes, we added the HIV-1 Gag₇₇₋₈₅ specific CD8⁺ T cell clone (1 T cell per DC) overnight in the presence of survival factors (IL-3 for pDC, GM-CSF for cDC) and measured IFN- γ in the supernatant by ELISA (Mabtech, France). Alternatively, we added the specific CD8⁺ T cell clone only for 4 h in the presence of Brefeldin A (BFA, Sigma, St Quentin Fallavier, France) and in the absence of survival factors, and analyzed IFN- γ intracellular production by flow cytometry. To assess the role of type I IFN or TNF- α , neutralizing antibodies specific for IFN- α , IFNAR (PBL) or TNF- α (Peprotech) were added at 10 μ g/ml for 30 min before incubation with the CD8⁺ T cell clone, and IFN- γ intracellular production was analysed by flow cytometry.

Cytokine secretion and dendritic cell maturation assays

25.10³ Dendritic cells were separately incubated overnight in 200 µl of complete medium + 2% human albumin with either H9 or H9HIV cells at a 1 DC to 5 H9/H9HIV ratio in the presence of saquinavir and IL-3 (pDC) or GM-CSF (cDC1 and cDC2). Cells were then labeled and analyzed by flow cytometry. IFN-α was quantified in the supernatants by ELISA (Mabtech).

CD8⁺ T cell clone stimulation by cytokines or anti-CD3/CD28 beads

Recombinant PEGylated IFN-α (Roche), IFN-β (Roche) or TNF-α (Peprotech) were added at 200, 100 and 50 ng/ml respectively, on CD8⁺ T cell clones for 4 h in the presence or absence of BFA. To mimic cognate interaction with DC, 2 anti-CD3/CD28 beads (Life Technologies) per CD8⁺ T cell were added for 4 h before supernatant collection. IFN-γ was analysed by intracellular labeling or by ELISA as above.

Statistics

Results are given as medians (95% confidence intervals). All tests were realized with GraphPad Prism v6.10 for Mac OS X. The Wilcoxon test was used to compare IFN productions in response to DC incubated with H9 vs H9HIV. Otherwise, Mann-Whitney tests were applied to compare two different groups, and Kruskal-Wallis test with Dunn's post-test corrections were applied to compare more than two groups.

Results

IFN- γ secretion by CD8⁺ T cells in the presence of pDC incubated with HIV-infected apoptotic cells was MHC-I-restricted, whereas IFN- γ intracellular production was only partly MHC-I-restricted

To understand how different types of human blood DC interact with apoptotic HIV-infected antigen-donor cells and CD8⁺ T cells during cross-presentation, we set up a culture system as previously published, in the presence of saquinavir to eliminate HIV replication and direct presentation (18, 21, 38). Dendritic cells were purified from buffy coats by immunomagnetic sorting, then by flow cytometric cell sorting, yielding typically 150000 cDC1, 1,3 x 10⁶ cDC2 and 1,2 x 10⁶ pDC. They were incubated overnight with apoptotic H9 or H9HIV cells in the presence of survival factors (IL-3 for pDC, or GM-CSF for cDC). To make sure of the origin of IFN- γ , we measured in parallel the secretion of IFN- γ by the CD8⁺ T cell clone in the supernatants (Fig. 1A), and the intracellular production of IFN- γ (Fig. 1B). **IFN- γ secretion** was specific for HIV, as it was only detected with apoptotic H9HIV cells and not with apoptotic uninfected H9 cells (Fig. 1A; for pDC: median 104 (95% confidence interval 57-256) vs 0 (0-5) pg/ml, Wilcoxon $p < 0.0001$; for cDC1: 66 (35-153) vs 11 (2-18) pg/ml, $p = 0.008$; for cDC2: 30 (17-57) vs 0 (0-4) pg/ml, $p = 0.0005$). As expected, IFN- γ secretion by the CD8⁺ T cell clone was MHC-I restricted, as it was detected only in the presence of HLA-A2⁺, but not HLA-A2⁻ DC (Fig. 1A). Comparing HLA-A2-restricted cross-presentation by the different DC populations, no statistical difference was found between the levels of IFN- γ secretion induced in the presence of pDC and cDC1, whereas the levels induced by cDC2 were lower (Mann Whitney $p = 0.0008$ for pDC vs cDC2 and $p = 0.04$ for cDC1 vs cDC2). Conversely to pDC, cDC1 and cDC2 required TLR activation (TLR3 for cDC1 and TLR4 for cDC2) to perform cross-presentation (Fig. 1 A). **IFN- γ intracellular production** was also specific for HIV, as it

was only detected when DC were cultured with infected H9HIV cells, and not with uninfected H9 cells (Fig. 1B; for pDC: median 16.8% (11.3-20.8) vs 0.7% (0.5-1.6) , Wilcoxon $p=0.0005$; for cDC1: 13.5% (1.3-31.0) vs 2% (0.0-5.2), $p=0.03$; for cDC2: 4 (1.6-5.2) vs 0.5% (0.3-1.4), $p=0.01$). Again, pDC and cDC1 induced similar levels of intracellular IFN- γ production by the CD8⁺ T cell clone, and higher levels than cDC2 (Mann-Whitney $p < 0.0001$ for cDC2 vs pDC and $p=0.01$ for cDC2 vs cDC1). Surprisingly, CD8⁺ T cell intracellular production of IFN- γ was also stimulated in a non-cognate manner by HLA-A2⁻ pDC incubated with H9HIV cells, and not with uninfected H9 cells (8.9% (6.4-11.1), vs 0.3% (0.1-1) Wilcoxon $p=0.002$), although weaker than by HLA-A2⁺ pDC (Mann-Whitney $p=0.01$). HLA-A2⁻ cDC1 from one/4 donors induced 76 pg/mL IFN- γ production, and from another 3.4% IFN- γ -positive CD8⁺ T cells upon stimulation with HIV-1 infected cells and poly(I:C), indicating an occasional, but not significant, potential of these cells for inducing non-cognate responses. HLA-A2⁻ cDC2 from the 14 donors did not induce any significant non-cognate response upon stimulation with H9HIV cells and LPS. IL-3 was added to improve pDC viability but otherwise had no effect on CD8⁺ T cell responses (Supplemental Fig. 2B). Therefore, HIV-specific secretion of IFN- γ by the antigen-specific CD8⁺ T cells was stimulated by pDC and both cDC populations and was strictly MHC-I restricted. In contrast, HIV-specific intracellular production of IFN- γ by CD8⁺ T cells was only partly MHC-I restricted for pDC, indicating a non-cognate CD8⁺ T cell activation.

pDC, and not cDC, matured and produced IFN- α in the presence of HIV-infected apoptotic cells

We investigated the mechanisms of the non-cognate CD8⁺ T cell activation found in culture with pDC and H9HIV cells. HIV virions are known to induce maturation and IFN- α secretion

by pDC, but not cDC, thus we tested these two parameters in the different populations of purified DC cultured in the presence of apoptotic HIV-infected H9 cells (Fig. 2). Expression of the chemokine receptor CCR7 and the costimulatory molecule CD83, which are known to be induced on pDC by HIV-1 virions (43), were analyzed by flow cytometry (Fig. 2A). pDC cultured with apoptotic H9HIV cells expressed CCR7 and CD83, but cDC did not. The three populations of purified DC were however able to mature by upregulating CCR7 and CD83 expression, when mixed again and cultured all together and not separately with apoptotic H9HIV cells (Fig.2A), as in (43) with HIV virions. Apoptotic uninfected H9 cells did not induce significant changes in CCR7 or CD83 expression by DC. In the supernatants from purified DC populations, we detected high amounts of IFN- α when purified pDC were cultured with apoptotic H9HIV cells, but not H9 cells (median 21 (3-86) vs 0.1 (0.1-0.3) ng/ml, Wilcoxon p=0.004) (Fig. 2B). In contrast, no IFN- α secretion was detected in cDC1 or cDC2 co-culture. Thus, only purified pDC, and not purified cDC, matured and secreted IFN- α when cultured with HIV- infected H9 cells.

Type I IFN and TNF- α induced IFN- γ intracellular production by CD8⁺ T cells, and increased IFN- γ secretion only upon cognate interaction.

As IFN- α , IFN- β and TNF- α are produced by pDC and not by cDC1 or cDC2 in response to HIV virions (32, 33), and as IFN- α was produced by pDC and not by cDC1 or cDC2 in response to H9HIV cells, we tested whether these cytokines would have a direct activity on CD8⁺ T cells and could explain the non-cognate T cell activation. We incubated cloned CD8⁺ T cells alone for 4h with recombinant cytokines, at concentrations similar to those secreted by pDC in the presence of HIV (32, 33) or H9HIV cells. By flow cytometry, 2.9% ((1-6.2) to 3.9% (0.8-5.4) of CD8⁺ T cells produced **intracellular IFN- γ** after incubation with IFN- α or IFN- β , alone or

277 in combination (Fig 3A, median 2.9%, 2.9% and 3.9% respectively; Dunn's post-test $p=0.3$,
 278 0.1 and 0.1 respectively), but not after incubation with TNF- α alone (median 0.1%). However,
 279 a combination of IFN- α , IFN- β and TNF- α strongly stimulated IFN- γ production by a median
 280 of 10% (5.7-13.0) of the CD8⁺ T cells compared to medium only (0% (0.0-0.1), Fig. 3A,
 281 Dunn's post-test $p=0.009$). The percentage of CD8⁺ T cells expressing IFN- γ was 2.6 times
 282 higher after addition of TNF- α than after IFN- α and IFN- β alone, indicating a potential synergy,
 283 but the results were not statistically different (median 10% (5.7-13.0) vs 3.9% (0.4-7.9) of IFN-
 284 γ^+ CD8⁺ T cells, Wilcoxon $p=0.25$). This stimulation by the three cytokines induced
 285 approximately the same percentage of IFN- γ^+ CD8⁺ T cells as observed after stimulation with
 286 HLA-A2⁻ pDC incubated with apoptotic H9HIV cells (8.9% (6.4-11.0) Fig. 1B). Indeed,
 287 **intracellular IFN- γ** production by the CD3⁺ CD8⁺ T cell clone was detected using confocal
 288 microscopy after stimulation with IFN- α , IFN- β and TNF- α (28 IFN- γ positive cells out of 60
 289 cells) compared to medium alone (NS) (0 IFN- γ positive cells out of 18 cells) (Fig. 3B).
 290 Interestingly, intracellular Granzyme B was expressed in different vesicles from IFN- γ in 100%
 291 of the cells and did not depend on stimulation by the cytokine cocktail. By contrast to
 292 intracellular production, **IFN- γ secretion** was not induced by these three cytokines added in
 293 combination (Fig 3C). We have seen that IFN- γ secretion by the CD8⁺ T cell clone required
 294 cognate interaction with HLA-A2⁺ DC. To mimic cognate stimulation by MHC-peptide
 295 complexes (signal 1) and co-stimulation (signal 2) (44), without adding HIV and its pathogen-
 296 associated molecular patterns (PAMPs), we used anti-CD3 and -CD28 antibody-coated beads,
 297 which induced a weak but consistent IFN- γ secretion from the CD8⁺ T cells (median 93 pg/mL,
 298 min 22, max 286 pg/mL, $n=6$, Fig. 3C). This secretion was normalized to 1 to compare the
 299 effect of the cytokines in multiple experiments. Adding beads to IFN- α , IFN- β or TNF- α -
 300 stimulated cells increased IFN- γ secretion without reaching statistical significance (median 2,
 301 1.9 and 1.2 vs 1, $p=0.4$, $p>0.99$ and $p=0.6$ respectively, Dunn's post-tests). Addition of IFN- α

and IFN- β induced an increase of IFN- γ secretion compared to beads only (median 2.8 (2.0-4.9) vs 1, $p=0.049$), and addition of the three cytokines induced a very significant increase (median 4.7 (1.7-12.1) vs 1, $p=0.002$, Dunn's post-test, Fig. 3C).

Therefore, intracellular IFN- γ production by the CD8⁺ T cell clone was induced by cytokines alone (i.e. IFN- α and IFN- β , perhaps potentiated by TNF- α), at similar levels as those observed with non-cognate stimulation by HLA-A2⁻ pDC incubated with apoptotic H9HIV cells, while intracellular GzB was expressed constitutively. Secretion of IFN- γ by CD8⁺ T cells occurred only upon cognate interaction and was increased by cytokine stimulation.

Blocking type I IFN pathway abolished non-cognate CD8⁺ T cell preactivation

To know whether these cytokines were entirely responsible for the non-cognate T cell activation elicited by pDC, we added a cocktail of three antibodies to neutralize IFN- α , TNF- α and the IFN- α and β Receptor (IFNAR) in the coculture of pDC with apoptotic H9HIV cells, 30 min before adding the CD8⁺ T cell clone for 4 h (Fig. 4). Compared to isotypic controls, these antibodies abolished partly the cognate stimulation by HLA-A2⁺ pDC (median 2.5% (- 3-11) vs 12.6% (5.7-18.2), $n=7$, Wilcoxon $p=0.016$), and seemed to abolish completely the non-cognate stimulation of the CD8⁺ T cell clone by HLA-A2⁻ pDC, when present (median 0.9% (-0.1-2.5) vs 1.4% (-0.3-6.5) IFN- γ ⁺ CD8⁺ T cells, respectively, $n= 6$, Wilcoxon $p=0.13$, not significant; Fig. 4). Blocking TNF- α only ($n=4$) did not have an effect on IFN- γ intracellular production in CD8⁺ T cell clones, while blocking type I IFN receptor and IFN- α appeared to replicate the effects of using all three blocking antibodies compared to isotype controls (Mann-Whitney $p=0.04$, $n=5$). Therefore, IFN- α and IFN- β secreted by pDC in the presence of apoptotic H9HIV cells were indeed responsible for the non-cognate T cell activation elicited by pDC.

Discussion

We previously showed that human blood pDC induced HIV-Gag specific, HLA-A2 restricted, CD8⁺ T-cell activation and IFN- γ secretion through cross-presentation of apoptotic HIV-1-infected cell antigens (18, 21). Here we found that although IFN- γ secretion was MHC-I dependent, intracellular IFN- γ production was only partly MHC-I dependent. By dissociating cognate signals, mimicked by beads without virus, and non-cognate signals, we observed that the non-cognate part of the response was due to IFN- α and IFN- β , possibly potentialized by TNF- α , secreted by pDC in response to HIV contained in antigen-donor cells. This non-cognate signal also enhanced the cognate response. Thus, CD8⁺ T-cells activation induced by cross-presentation of HIV-1 gag from infected cells by pDC was potentiated by non-cognate pre-activation (Fig. 5). This finding questions the dogma that while cytolytic effector molecules such as Granzyme B are expressed continuously in effector and memory T cells, poising them for rapid killing of target cells immediately upon T Cell Receptor (TCR) triggering, IFN- γ production is tightly regulated, being triggered as very short ON/OFF cycles by engagement of the TCR (36, 37). Here, IFN- γ secretion was tightly regulated by engagement of the TCR, but intracellular IFN- γ production was pre-activated upon type I-IFN-signaling.

Conversely to pDC, cDC1 and cDC2 required the addition of TLR ligands for their specific TLRs (either poly-IC for TLR-3 or LPS for TLR4, respectively) to perform cross-presentation. Here, we showed that pDC were already stimulated through detection of HIV contained in antigen-donor cells, and did not require additional stimulation. Comparison between the three DC populations showed that pDC and cDC1 were equivalent at inducing CD8⁺ T cell activation. cDC2 were less effective, even when stimulated by LPS. In our model, MHC-I-independent CD8⁺ T cell activation was not demonstrated for cDC. Indeed, cDC did not upregulate CD83 or CCR7 or secrete IFN- α in response to cell-associated HIV, as they lack

350 both TLR-7 and 9 (45). This is in agreement with other results on pDC and cDC stimulated
 351 with free HIV virions (33)(43).
 352 We then analyzed the mechanism responsible for the MHC-I independent activation of CD8⁺
 353 T cells in the presence of pDC and apoptotic HIV infected cells. IFN- α as a signal 3 cytokine
 354 can activate IFN- γ expression by human CD8⁺ T cells (34, 46). IFN- α is also known to be
 355 secreted at high levels by human pDC in response to HIV virions, among other cytokines such
 356 as IFN- β , TNF- α or IL-6 (32, 33, 43). We indeed showed here that cell-associated HIV led to
 357 pDC maturation and IFN- α secretion by pDC, but not by cDC. HIV might still potentially
 358 stimulate TLR-8 in cDC1 and cDC2 and induce not IFN- α , but IFN- β , IL-12 or TNF- α in
 359 cDC1 or cDC2 through TLR-8 (47), which might explain the occasional non-cognate
 360 stimulation of IFN- γ production by the CD8⁺ T cell clone by cDC1 in one donor in our results
 361 (Fig. 1A). Furthermore, we showed that a combination of IFN- α , IFN- β and TNF- α induced a
 362 higher IFN- γ intracellular production than medium alone in CD8⁺ T cell clones. The level
 363 detected (10% of IFN- γ ⁺ among CD8⁺ T cells) was in the same range as the MHC-I independent
 364 signal observed with HLA-A2⁻ pDC in the presence of apoptotic HIV infected cells (8.9 to
 365 10%). This, in addition to the absence of IFN- γ production observed with HLA-A2⁻ cDC in the
 366 presence of H9HIV cells, ruled out a potential additional direct effect of cell-associated HIV-
 367 1 on the CD8⁺ T cells, which might have been activated by HIV-1 through TLR-8 (48).
 368 Moreover, when these cytokines were neutralized by blocking IFN- α and IFNAR, the
 369 percentage of IFN- γ ⁺ cells among CD8⁺ T cells was either reduced in the case of HLA-A2⁺
 370 pDC or abolished in the case of HLA-A2⁻ pDC. This total abolition showed that other soluble
 371 or cell-associated factors did not seem to contribute to the non-cognate T cell stimulation by
 372 HLA-A2⁻ pDC. The same trend was followed when only blocking IFN- α and IFNAR, but not
 373 when blocking TNF- α only. Thus, IFN- α and - β stimulation of CD8⁺ T cells recapitulated the

stimulation of IFN- γ intracellular production induced by pDC independently of MHC-I presentation.

In our model, this MHC-I independent CD8⁺ T cell activation mechanism found with pDC was not demonstrated with cDC. Indeed, cDC did not secrete type I IFN in response to cell-associated HIV, consistently with their lack of or low expression of TLR-7 and 9 (45). While cDC2 express only low levels of TLR7 and no TLR9 (49), they still express TLR8, which might recognize HIV-1 RNA (50). However, in our experiments, sensing of cell-associated HIV by cDC failed to induce their maturation or secretion of IFN- α , consistently with previous work showing no activation, IFN- α nor TNF- α production of conventional DC in the presence of HIV virions (32, 43). Even though cDC were not activated in response to free or cell-associated virus, and did not induce non-cognate CD8⁺ T cell activation, they are activated by type I IFN, which is secreted by pDC after stimulation with either free HIV (43), or cell-associated HIV (Isnard, in preparation). IFN-DC generated in vitro from monocytes stimulated by GM-CSF and IFN- α induce in vivo cross-priming of protein-specific CD8⁺ T cells against inactivated HIV-1 or HCV NS3 protein more efficiently than DC generated from monocytes stimulated by GM-CSF and IL-4 (51) and protection against HIV-1 infection in SCID-hu mice (52), and IFN- α enhances the capacity of cDC to cross-present HIV-1 and tumor antigens (53). Therefore, in vivo, cDC may act in collaboration with pDC for the cross-presentation of HIV antigens and antiviral activity of CD8⁺ T cell.

Interestingly, this effect was specific for IFN- γ production while Granzyme B production, known to be continuously expressed by cytotoxic and memory CD8⁺ T cells, was indeed independent on type I IFN production. IFN- γ has strong antiviral effects, but during chronic HIV infection, it may also participate in CD8⁺ T cell hyperactivation and exhaustion which correlate with disease progression (54). Hyperactivation has been linked to the persistent type I IFN signature found in this infection (55-57). In line with our results, in vitro IFN- α

stimulation of CD8⁺ T cells from chronic HIV-1 infected patients with progressive disease enhanced their production of IFN- γ , their degranulation, and their HIV-1 suppressive activity (58). In vivo, the effects of IFN- α treatment, induction or inhibition depend on the time and dosage. Before infection of Rhesus macaques, IFN- α treatment prevents infection, but if treatment is extended during SIV infection, it contributes to CD8⁺ T cell activation, type I IFN-desensitization and progression to AIDS (59). During chronic infection, IFN- α treatment decreases viral loads transiently (60) and potentially proviral loads (61), and increases CD8⁺ T cell activation (62). However, chronic IFN- α administration in vivo can induce monocyte-derived suppressor cells and limit CD8⁺ T-cell antiviral functions (63). Antagonization of IFN- α signaling at the onset of infection reduces T cell activation and exhaustion, but accelerates progression to AIDS (59, 64), whereas long-term antagonization during chronic SIV infection, either treated by cART or not, reduces ISG expression without increasing viral replication (65). Hence our results are in line with such findings, showing that type I IFN participate in immune activation by increasing CD8⁺ T-cell activation during HIV/SIV infection.

As a conclusion, in the context of virus-infected cells, pDC can trigger intracellular IFN- γ protein production and accumulation in CD8⁺ T cells, even when they do not present the cognate peptide (Fig. 5A). This potentializes the secretion of IFN- γ once CD8⁺ T cells encounter antigen-presenting cells with their cognate MHC-viral epitope complex (Fig. 5B). This finding may help to understand how to modulate and channel type I IFN production so that it leads to IFN- γ release only toward infected cells, improving elimination of infected or tumor cells.

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Author contributions

SI and EXH performed, analyzed and interpreted most experiments and wrote the manuscript. MI and JBG participated in experiment setup, design analyses and interpretation and critically reviewed the manuscript. AH designed the study, oversaw and participated in experiments and analyses, interpreted the data and wrote the manuscript. All authors approved the final version of this manuscript.

Competing interest

The authors declare no conflict of interest.

Footnotes

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Fig. legends

Fig. 1: IFN- γ secretion by CD8⁺ T cells in the presence of pDC incubated with HIV-infected apoptotic cells was MHC-I restricted, whereas IFN- γ intracellular production was only partly MHC-I restricted

Purified HLA-A2⁺ or HLA-A2⁻ pDC, cDC1 or cDC2 were cultured overnight with apoptotic H9 cells infected (H9HIV, magenta) or not (H9, green) by HIV-1_{lai}, in the presence of saquinavir and survival factors (IL-3 for pDC, GM-CSF for cDC). Conventional DC₁ were stimulated with Poly:IC and cDC2 with LPS. Anti-Gag₇₇₋₈₅ cloned CD8⁺ T cell were then added and IFN- γ secretion or intracellular production analyzed. **A. IFN- γ secretion** was measured by ELISA after 16h incubation. **B. IFN- γ intracellular production** was tested after 4h incubation in the presence of BFA, cells were then stained with live/dead and labeled for CD3, CD8, CD1c, CD141, CD123 and HLA-A2, and intracellularly for IFN- γ , then the percentages of IFN- γ ⁺ cells among CD8⁺ T cells were analyzed by flow cytometry (38). Wilcoxon test between H9 and H9HIV cells, Mann-Whitney test (*italics*) between HLA-A2⁺ (pDC A: n=15, B: n=12; cDC1 A: n=8 with PIC, n=2 without PIC, B: n=6; cDC2 A: n=14 with LPS, n=5 without LPS, B: n=11) and HLA-A2⁻ donors (pDC A: n=9, B: n=10; cDC1 A: n=4, B: n=4; cDC2 A: n=6, B: n=9), Mann-Whitney tests (*italics*) between DC populations.

Fig. 2: pDC, not cDC, matured and produced IFN- α in the presence of apoptotic HIV-infected cells

FACS-purified pDC, cDC1 and cDC2 were incubated overnight with apoptotic H9 or H9HIV cells in the presence of survival factors. **A. Maturation.** Purified DC, either alone (left) or combined (right), were labelled with anti-CCR7 and -CD83 antibodies and analyzed by flow

cytometry. One experiment representative out of 3 for pDC and cDC2, and of 2 for cDC1 is shown. **B. IFN- α secretion.** pDC, cDC1 stimulated with Poly:IC and cDC2 stimulated with LPS were incubated for 16h in the presence of H9 (green) or H9HIV (magenta) cells. IFN- α secretion was measured in the supernatants by ELISA. pDC n =10, cDC1 n=4, cDC2 n=10. Data are shown with medians. Wilcoxon test for comparison of pDC + H9HIV and pDC + H9.

Fig. 3: IFN- α and IFN- β , potentiated by TNF- α , induced by themselves IFN- γ intracellular production in CD8⁺ T cells and IFN- γ secretion upon cognate interaction.

A. Intracellular IFN- γ production in response to cytokines alone. Cloned CD8⁺ T cells were stimulated or not for 4h with IFN- α_2 , IFN- β or TNF- α alone or in combination as indicated. Intracellular IFN- γ production was analyzed by flow cytometry (n=3). Data are shown as medians with interquartile ranges. Statistical analysis by Kruskal-Wallis followed by Dunn's multiple comparison post-test. **B. Intracellular IFN- γ or Granzyme B production.** Cloned CD8⁺ T cells were stimulated with IFN- α , IFN- β and TNF- α (Cytokines), or not (NS), for 4h. The cells were then fixed and permeabilized, then labeled with anti-CD3, anti-Granzyme B and anti-IFN- γ antibodies, and with DAPI. They were analyzed by confocal microscopy. IFN- γ is shown in red, Granzyme B in green, CD3 in grey and DAPI-stained DNA in blue. NS: image representative of 18/18 cells, Cytokines: image representative of 28/60 cells positive for IFN- γ . All cells expressed Granzyme B regardless of stimulation. **C. IFN- γ secretion in response to cognate interaction is potentiated by cytokines** Cloned CD8⁺ T cells were stimulated for 4h with IFN- α_2 , IFN- β or TNF- α , alone or in combination as described in the figure. When indicated, anti-CD3 anti-CD28 coated beads were added for 4h. IFN- γ secretion was measured in the supernatant by ELISA. Results were normalized to the beads-

only condition (n=5 or 6 depending on stimulation conditions). Data are shown as medians with interquartile ranges. Medium-only and cytokines-only conditions did not induce IFN- γ secretion and were not taken into account for the statistical tests. Statistical analysis by Kruskal-Wallis test followed by Dunn's multiple comparison post-test.

Fig. 4: Antibodies blocking IFN- α and IFNAR abolished INF- γ intracellular production by CD8⁺ T cells

HLA-A2⁺ (n=7) or HLA-A2⁻ (n=6) purified pDC were cultured overnight with apoptotic H9 (green) or H9HIV (magenta) cells in the presence of IL-3, then incubated with either neutralizing antibodies targeting IFN- α and IFN- α receptor only, TNF- α only or all three (10 μ g/mL each) or isotype controls at the same concentrations (Isotypes), before cloned anti-Gag₇₇₋₈₅ CD8⁺ T cells were added. After 4h in the presence of BFA, cells were labelled as in Fig. 1B and analyzed by flow cytometry.

Fig. 5: Model. Cell associated HIV cross-presentation by pDC is potentiated by non-cognate CD8⁺ T cell preactivation

H9 cells infected with HIV induce type I IFN production by pDC
Type I IFN induces IFN- γ intracellular production in CD8⁺ T cells
pDC cross-present MHC-restricted HIV-gag antigen to specific CD8⁺ T cells
IFN- γ secretion by CD8⁺ T cells is potentiated by former stimulation by type I IFN, but occurs only in the case of cognate interaction with pDC

Fig. 1

Fig. 1: IFN- γ secretion by CD8⁺ T cells in the presence of pDC incubated with HIV-infected apoptotic cells was MHC-I-restricted, whereas IFN- γ intracellular production was only partly MHC-I-restricted

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In the context of the
other purified DC <avec la
meme présentation qu'à gauche et sans
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Fig. 4

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- pDC cross-present MHC-I-restricted HIV-Gag antigen to specific CD8⁺ T cells
- IFN- γ secretion by CD8⁺ T cells is potentiated by former type I IFN stimulation, but occurs only in the case of cognate interaction with pDC

Supplemental Fig. 1: Gating strategies.

- A. DC sorting and purity assessment from Pan-DC.** SSC then FSC were used to remove doublets. FSC/SSC combination was used to eliminate debris, and dead cells were excluded using Live/Dead dye. Lineage (CD3, CD19, CD20, CD14, CD16 and CD56) negative live cells were then selected and DC were sorted according to CD1c (BDCA-1), CD141 (BDCA-3) or CD304 (BDCA-4) expression. Purity of each DC subtype was immediately checked after sorting.
- B. IFN- γ intracellular production.** IFN- γ expression was tested by flow cytometry in cloned CD8⁺ T cells specific for HIV-1 Gag 77-85 after incubation with HLA-A2⁺ (left) DC or HLA-A2⁻ (right) pDC preincubated with H9HIV cells. CD8⁺ cells were gated among CD3⁺ T cells, which expressed HLA-A2 and not the surface proteins characterizing pDC (CD123) nor other DC (CD141 or CDc1), among live cells not forming doublets. The gating strategy is described in detail in ref (38).

Supplemental Fig. 2: Properties of the anti-HIV CD8⁺ T cell clone. A. Anti HIV-1 Gag₇₇₋₈₅-specific CD8⁺ T cell clone IIB7 specificity test on peptide-pulsed HLA-A2⁺ EBV-B cell line. sP Gag: HIV-1_{lai}-Gag₇₇₋₈₅; sP Flu: IAV (H1N1-A/PR8₅₈₋₆₆).

B. IL-3 did not alter IFN- γ intracellular production by the CD8⁺ T cell clone in response to pDC incubated with apoptotic H9HIV cells. Purified HLA-A2⁺ or HLA-A2⁻ pDC were cultured overnight with apoptotic H9 or H9HIV cells in the presence or absence of IL-3. Cells from the CD8⁺ T cell clone were then added for 4h in the presence of BFA. IFN- γ intracellular production was assessed by labeling cells as in Fig. 1, and cells were analyzed by flow cytometry. Wilcoxon text between IL-3 and no IL-3 (HLA-A2⁺ pDC n=10 for both IL-3 and no IL-3, HLA-A2⁻ pDC n=5 for both IL-3 and no IL-3).