DOI: 10.1002/JLB.4A0721-365R

JLB JOURNAL OF LEUKOCYTE BIOLOGY

ARTICLE

HOST DEFENSE AND PATHOPHYSIOLOGY

Crosstalk between R848 and abortive HIV-1 RNA-induced signaling enhances antiviral immunity

Melissa Stunnenberg | **John L. van Hamme** | **Esther M. Zijlstra-Willems** | **Sonja I. Gringhuis** | **Teunis B.H. Geijtenbeek**

Department of Experimental Immunology, Amsterdam institute for Infection & Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

Correspondence
Teunis B.H. Geijtenbeek, Meibergdreef 9, 1105 AZ, Amsterdam, the Netherlands.
Email: t.b.geijtenbeek@amsterdamumc.nl

Abstract

Pathogens trigger multiple pattern recognition receptors (PRRs) that together dictate innate and adaptive immune responses. Understanding the crosstalk between PRRs is important to enhance vaccine efficacy. Abortive HIV-1 RNA transcripts are produced during acute and chronic HIV-1 infection and are known ligands for different PRRs, leading to antiviral and proinflammatory responses. Here, we have investigated the crosstalk between responses induced by these 58 nucleotide-long HIV-1 RNA transcripts and different TLR ligands. Costimulation of dendritic cells (DCs) with abortive HIV-1 RNA and TLR7/8 agonist R848, but not other TLR agonists, resulted in enhanced antiviral type I IFN responses as well as adaptive immune responses via the induction of DC-mediated T helper 1 (T_H1) responses and IFNγ^+CD8$^+$ T cells. Our data underscore the importance of crosstalk between abortive HIV-1 RNA and R848-induced signaling for the induction of effective antiviral immunity.

KEYWORDS
adaptive immunity, immunomodulation, innate immunity, pathogen-associated molecular pattern, pattern recognition receptor, TLR8

1 | INTRODUCTION

The ability of dendritic cells (DCs) to induce potent innate and adaptive immune responses is paramount to combating invading pathogens.[1] Host pattern recognition receptors (PRRs) present on the cell surface, in the cytoplasm or in endosomes, detect pathogen-associated molecular patterns (PAMPs), resulting in responses directed against the encountered pathogen.[2–5]

TLRs are a well-described family of PRRs that induce DC activation and specific cytokine production, leading to innate and adaptive immune responses.[6,7] Triggering of multiple TLRs together can result in synergistic immune responses that are more potent than the responses induced by triggering of one single TLR, but more importantly also lead to tailored T cell responses.[8–10] Besides crosstalk between TLRs, crosstalk between TLRs and members of other PRR families, such as C-type lectin receptors, NOD-like receptors, and RIG-I-like receptors (RLRs), has also been reported, which dictates cytokine responses based on the specific crosstalk induced by these receptors.[11–13] However, the effect of crosstalk between TLRs and other PRRs on antiviral immunity is less well established.

Human immunodeficiency virus 1 (HIV-1) triggers a variety of PRRs, including cGAS,[14,15] TLR7/8,[16–18] RIG-I,[19] DEAD-box RNA helicase 3 (DDX3),[20–22] and protein kinase RNA-activated (PKR).[23,24] During HIV-1 infection, endosomal TLR7 and TLR8 recognize single-stranded

Abbreviations: DCs, dendritic cells; DDX3, DEAD-box RNA helicase 3; ISGs, IFN-stimulated genes; LTA, lipoteichoic acid; PAM3CSK4, Pam3CysSerLys4; PAMPs, pathogen-associated molecular patterns; PKR, protein kinase RNA-activated; Poly-I:C, polyinosinic:polycytidylic acid; PRRs, pattern recognition receptors; RLRs, RIG-I-like receptors; ss RNA, single-stranded ribonucleic acid; TAR, trans-activation response element; T_H, T helper

(ss) HIV-1 RNA and induce immune responses upon infection.[16–18,25] Furthermore, the cytosolic PRRs DDX3 and PKR recognize abortive HIV-1 transcripts that are generated during both acute and chronic HIV-1 infection as well as in latently infected cells.[22,23,26,27] These abortive HIV-1 transcripts are formed after transcription initiation, when transcription elongation is blocked or not induced.[27] Abortive HIV-1 transcripts consist of the first 58 nucleotides common to all HIV-1 transcripts and contain the trans-activation response (TAR) element that adopts a hairpin structure.[18,27] DDX3 detects abortive HIV-1 RNAs and induces antiviral type I IFN responses and adaptive responses via DC-mediated T cell polarization,[22,28] while detection by PKR induces proinflammatory responses.[37] Interestingly, abortive HIV-1 transcripts are secreted from cells since they are detected in exosomes, both in vitro as well as in vivo.[29–33] Stimulation of macrophages with isolated short TAR RNA-containing exosomes results in proinflammatory responses,[34] confirming their stimulatory properties and suggesting that TAR RNA affects bystander cells.

TLR7 and 8 have been shown to sense HIV-1 ssRNA[16,17] and are therefore triggered during HIV-1 infection.[18] Thus, during HIV-1 infection, both TLR7/8 ligands and abortive HIV-1 RNA are present.[17,18] Triggering TLR7/8 by R848 induces antiviral immune responses.[25,35,36] Similarly, abortive HIV-1 RNA also induces antiviral and proinflammatory responses by triggering DDX3 and PKR.[22,28,37] However, it is unknown whether there is crosstalk between abortive HIV-1 RNA and HIV-1 ssRNA-specific TLR7/8-induced responses and how this affects antiviral immunity.

Here, we demonstrate that crosstalk between signaling by abortive HIV-1 RNA and TLR7/8 agonist R848 enhanced antiviral innate responses, which was not observed with other TLR agonists. Moreover, costimulation of DCs with both R848 and abortive HIV-1 RNA enhanced specific adaptive responses by inducing T_H1 polarization and IFNγ^+CD8$^+$ T cell responses. Our data strongly suggest that crosstalk between R848 and abortive HIV-1 RNA-induced signaling is paramount for an effective antiviral immune response. Considering that HIV-1 triggers TLR7/8 signaling and the production of abortive HIV-1 RNAs, these effective antiviral immune responses could be especially important to limit HIV-1 infection.

2 | MATERIALS AND METHODS

2.1 | Cells and stimulations

This study was performed in accordance with the Declaration of Helsinki and the Amsterdam University Medical Centers (location AMC) Medical Ethics Committee guidelines. Human buffy coats (Sanquin) were processed using a lymphoprep density separation step to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were used to isolate naïve CD4$^+$ T cells or a percoll gradient was obtained to isolate monocytes or peripheral blood lymphocytes (PBLs). Naïve CD4$^+$ T cells were isolated using a CD4$^+$ T cell isolation kit II (Miltenyi Biotec) and subsequent depletion of memory CD4$^+$ T cells (CD45RO) was performed using PE-conjugated anti-CD45RO (Dako) and anti PE-beads (Miltenyi Biotec), according to manufacturer's instructions. Monocytes were cultured in RPMI medium supplemented with 10% FCS (Biological Industries), L-glutamine (2 mM; Lonza), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; Thermo Fisher) and either stimulated directly or cultured in the presence of GM-CSF and IL-4 (800 and 500 U/ml, respectively; Invitrogen) at 37°C 5% CO_2 for 6 days to generate monocyte-derived DCs. On day 6, DCs were stimulated with optimal concentrations of TLR 1/2 agonist Pam3CysSerLys4 (PAM3CSK4, 10 μg/ml; Invivogen), TLR2/6 agonist lipoteichoic acid (LTA, 10 μg/ml; Invivogen), TLR3 agonist polyinosinic:polycytidylic acid (poly-I:C, 10 μg/ml; Invivogen), TLR4 agonist LPS *Salmonella enterica* serotype typhimurium (10 ng/ml; Sigma), TLR5 agonist flagellin from *Bacillus subtilis* (10 μg/ml; Invivogen), TLR7/8 agonist R848 (10 μg/ml; Invivogen), and/or stimulated with synthetic abortive HIV-1 RNA (HIV-1 Cap-RNA_{58} Biosynthesis) or a similar RNA sequence without the 5′cap (HIV-1 Cap-RNA_{58}) complexed with transfection reagent lyovec (Invivogen). Both HIV-1 RNA constructs were used at a concentration of 1 nM as this induced a potent IFN response in DCs. HIV-1 Cap-RNA_{58} mimics the first 58 nucleotides of the HIV-1 genome and contains a 5′m^{7}GTP cap, while lacking a poly A tail. Synthetic abortive HIV-1 RNA was synthesized using an in vitro transcription (IVT) method including cocapping techniques, as previously described.[22,28]

2.2 | Quantitative real-time PCR

mRNA extraction of DCs was performed using lysis buffer obtained from the mRNA capture kit (Roche) and mRNA was reversely transcribed to cDNA using the reverse transcriptase kit (Promega). Quantification of gene expression levels was performed with SYBR Green (Thermo Fisher) and primer sets (Sigma) using an ABI 7500 Fast Real-Time PCR detection system (Applied Biosystems). Primer sets were designed with Primer Express 2.0 (Applied Biosystems; Table S1). Expression levels of target genes were normalized to expression levels of household gene *GAPDH*, with the formula: $N_t = 2^{Ct(GAPDH)-Ct(target)}$.

2.3 | ELISA

The secretion of TNF, IL-6, IL-10, IL-12p70, IL-23, and IL-27 in DC supernatants was analyzed 24 h after cell stimulation and quantified using ELISA according to manufacturer's instructions (eBiosciences). Secretion of IFNβ in the DC supernatant was analyzed 6 h after cell stimulations (R&D systems). OD450 nm values were read using BioTek Synergy HT.

2.4 | T cell differentiation

DCs were stimulated with HIV-1 Cap-RNA_{58}, R848, or a combination. As a positive control for T helper (T_H) 1 skewing, DCs were stimulated with LPS (10 ng/ml; Sigma) and IFNγ (1000 U/ml; u-CyTech) and with LPS and PGE_2 (1 μM; Sigma) to induce T_H2 skewing. After 48 h, DCs

were collected, washed, and cocultured with isolated naïve $CD4^+$ T cells from an allogeneic donor in a 1:4 ratio. DC-T cell cocultures were performed in IMDM medium (Invitrogen) supplemented with 10% FCS, penicillin, and streptomycin (100 U/ml and 100 μg/ml, respectively; Thermo Fisher, IMDM complete) and in the presence of *Staphylococcus aureus* enterotoxin B (10 pg/ml; Sigma). On day 5, IL-2 (10 U/ml; Chiron) was added and on day 11–13, resting T cells were restimulated using PMA (10 ng/ml; Sigma) and ionomycin (1 mg/ml; Sigma) for 6 h and brefeldin A (10 μg/ml; Sigma) for the final 4 h. Cells were fixed with 4% PFA, permeabilized with 0.1% saponin in PBS with 0.5% BSA, and stained with FITC-conjugated anti-IFNγ (1:5, 340449; BD Biosciences) and APC-conjugated anti-IL-4 (1:25, 554486; BD Biosciences), indicative of T_H1 and T_H2 skewing, respectively. Flow cytometry was performed using FACSCanto II (BD Biosciences) and analyzed with FlowJo software v10.7.

2.5 | Cytotoxic T lymphocyte responses

DCs were stimulated with HIV-1 Cap-RNA_{58}, R848 or a combination, for 48 h and subsequently cocultured with allogeneic PBLs in IMDM complete, using a 1:8 ratio. As a positive control, T cells were stimulated with anti-CD3 (1:10,000; Sanquin) and anti-CD28 (1 μg/ml; Sanquin). After 3 days, IL-2 was added to the cocultures. After 6 days, cells were restimulated using PMA (10 ng/ml; Sigma) and ionomycin (1 mg/ml; Sigma) for 6 h, and brefeldin A (10 μg/ml; Sigma) for the final 4 h, followed by fixation and permeabilization using the fixation/permeabilization solution kit (BD Biosciences) according to manufacturer's instructions. Cells were then stained using APC-Cy7-conjugated anti-CD3 (1:100, 300317; Biolegend), PE-Cy7-conjugated anti-CD8 (1:100, 344711; Biolegend), FITC-conjugated anti-IFNγ (1:5, 340449; BD Biosciences), PE-conjugated anti-Perforin (1:10, 12-9994-42; Thermo Fisher) and AF700-conjugated anti-Granzyme B (1:200, 560213; BD Biosciences) and flow cytometry was performed using FACS Canto II (BD Biosciences) and analyzed with FlowJo software v10.7.

2.6 | Data analysis

Statistical analysis was performed using Student's *t*-test for paired observations using GraphPad version 8. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | R848 enhances *IFNB* transcription induced by abortive HIV-1 RNA

To assess the crosstalk between HIV-1-specific TLR- and abortive HIV-1 RNA-induced signaling, we stimulated monocyte-derived DCs with TLR7/8 agonist R848 together with HIV-1 Cap-RNA_{58}, a synthetic abortive HIV-1 RNA that triggers DDX3 and PKR.[22,28,37] Stimulation with HIV-1 Cap-RNA_{58} alone induced antiviral *IFNB* transcription[22,28] (Figure 1(A)). Strikingly, costimulation of HIV-1 Cap-RNA_{58} with R848 significantly enhanced *IFNB* transcription (Figure 1(A)). In contrast, costimulation with control HIV-1 RNA_{58}, lacking the 5′ cap structure, did not enhance *IFNB* transcription, suggesting that the crosstalk is mediated by DDX3 as PKR recognizes both uncapped control HIV-1 RNA_{58} and HIV-1 Cap-RNA_{58}[38]. To address whether costimulation with HIV-1 Cap-RNA_{58} and other TLR ligands resulted in a similar enhancement of *IFNB*, DCs were stimulated with HIV-1 Cap-RNA_{58} in combination with TLR agonists PAM3CSK4 (TLR1/2), LTA (TLR2/6), poly-I:C (TLR3), LPS (TLR4), or flagellin (TLR5). Similar to R848, stimulations with PAM3CSK4 and flagellin induced low levels of *IFNB* transcription, while LTA, poly-I:C, and LPS induced high levels of *IFNB* transcription similar to HIV-1 Cap-RNA_{58} (Figure 1(A)). However, none of these TLR agonists enhanced HIV-1 Cap-RNA_{58}-induced *IFNB* transcription (Figure 1(A)), suggesting that crosstalk between abortive HIV-1 RNA- and specifically TLR7/8-induced responses leads to enhanced *IFNB* transcription. In addition to enhanced *IFNB* transcription levels, costimulation with R848 and HIV-1 Cap-RNA_{58} resulted in enhanced IFNβ protein secretion (Figures 1(B) and S1). Furthermore, we have stimulated monocytes with HIV-1 Cap-RNA_{58}, R848, or a combination (Figure 1(C)). Interestingly, a similar enhancement of *IFNB* transcription was observed (Figure 1(C)), suggesting that R848 enhances abortive HIV-1 RNA-induced *IFNB* transcription in primary myeloid subsets.

3.2 | Costimulation with R848 and abortive HIV-1 RNA modulates cytokine gene expression

Next, we examined whether crosstalk between R848 and HIV-1 Cap-RNA_{58} modulates expression of cytokine genes. Treatment of DCs with R848 resulted in transcription of all measured cytokine genes. Treatment with HIV-1 Cap-RNA_{58} alone induced low levels of *TNF* and *IL6* transcription and higher levels of *IL12A* and *IL27A* (*IL27p28*) transcription,[28] without inducing *IL23A*, *IL10*, *IL12B*, or *IL27B* (*IL27EBI3*) transcription (Figure 2). Strikingly, costimulations with R848 and HIV-1 Cap-RNA_{58}, but not the uncapped HIV-1 RNA_{58}, synergistically enhanced transcription of *IL6*, *IL10*, *IL12A*, *IL12B*, *IL27A*, and *IL27B* compared with either stimulus alone, while R848-induced *TNF* and *IL23A* transcription was unaffected (Figure 2). Thus, crosstalk between HIV-1 Cap-RNA_{58} and R848 modulates the expression of cytokine genes and enhanced antiviral type I IFN responses via enhanced transcription of *IFNB* and IFN-stimulated gene (ISG) *IL27A*.

3.3 | Crosstalk between abortive HIV-1 RNA and R848 enhances IL-12p70 and IL-27 expression

We next examined the protein expression levels upon costimulation with R848 and HIV-1 Cap-RNA_{58}. Of note, due to high levels of donor variation and to perform statistical analysis, data are shown as fold

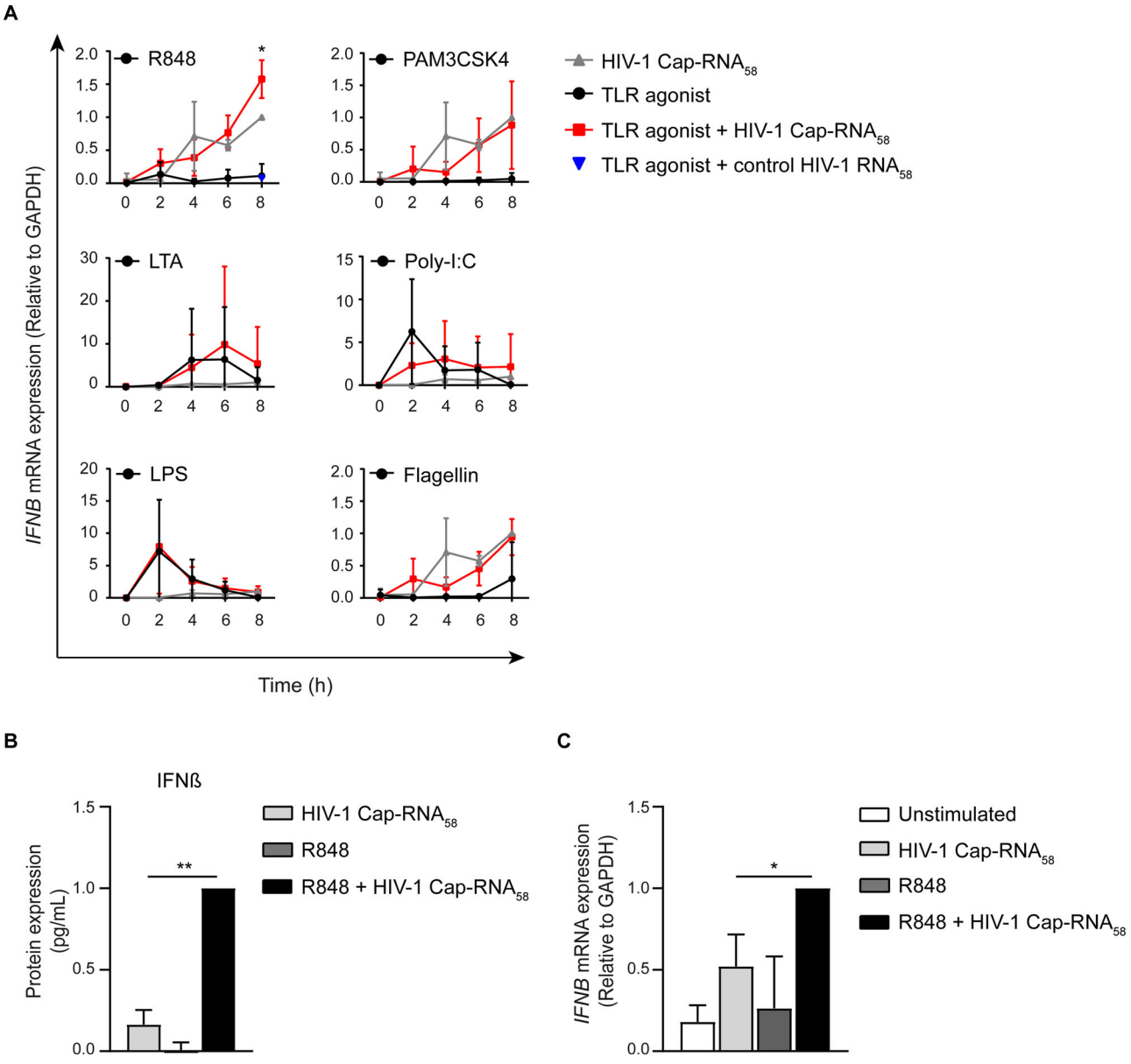

FIGURE 1 **R848 enhances *IFNB* transcription induced by abortive HIV-1 RNA.** Monocyte-derived dendritic cells (DCs) were (co)stimulated with HIV-1 Cap-RNA_{58} or control HIV-1 RNA_{58} and different TLR agonists (TLR7/8 agonist R848, TLR1/2 agonist PAM3CSK4, TLR2/6 agonist LTA, TLR3 agonist poly-I:C, TLR4 agonist LPS, and TLR5 agonist flagellin) (A–C). Every 2 h (and only 8 h after costimulation with control HIV-1 RNA_{58}), mRNA was extracted to analyze *IFNB* transcription by quantitative real-time PCR. mRNA expression was relative to *GAPDH*. Responses induced by HIV-1 Cap-RNA_{58} at 8 h were set at 1 (A). IFNβ protein secretion was detected in the supernatant of DCs 6 h after stimulation and quantified using ELISA (B). *IFNB* transcription in primary monocytes was assessed 8 h after stimulations with R848 and HIV-1 Cap-RNA_{58}. mRNA expression was relative to *GAPDH* and responses induced by HIV-1 Cap-RNA_{58} and R848 were set at 1 (B and C). Data are representative of collated data of 4 (A) or 3 (B and C) donors (mean ± SD). Significance was determined between responses induced by cotreatment with HIV-1 Cap-RNA_{58} and R848, and HIV-1 Cap-RNA_{58}-induced responses (A). $^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t*-test

change in protein expression (Figures 3(A)–3(C)) and as individual concentrations in pg/ml in Figure S2 (Figures S2(A)–S2(C)). TNF and IL-23 were secreted upon stimulation with R848 and costimulation with HIV-1 Cap-RNA_{58} did not enhance TNF and IL-23 levels (Figures 3(A) and S2(A)), similar to our mRNA results (Figure 2). Although mRNA transcripts of *IL6* and *IL10* were enhanced by costimulation (Figure 2), this was not observed for IL-6 and IL-10 protein expression (Figures 3(A) and S2(A)). In contrast, costimulation with R848 and HIV-1 Cap-RNA_{58} significantly enhanced IL-12p70 and IL-27 (Figures 3(A) and S2(A)), following transcriptional patterns (Figure 2). Both IL-12p70 and IL-27 are important cytokines in directing adaptive responses.[38–41] Next, we further assessed whether the enhanced IL-12p70 and IL-27 levels upon costimulation with HIV-1 Cap-RNA_{58} and R848 are unique for TLR7/8 triggering, or that other TLRs lead

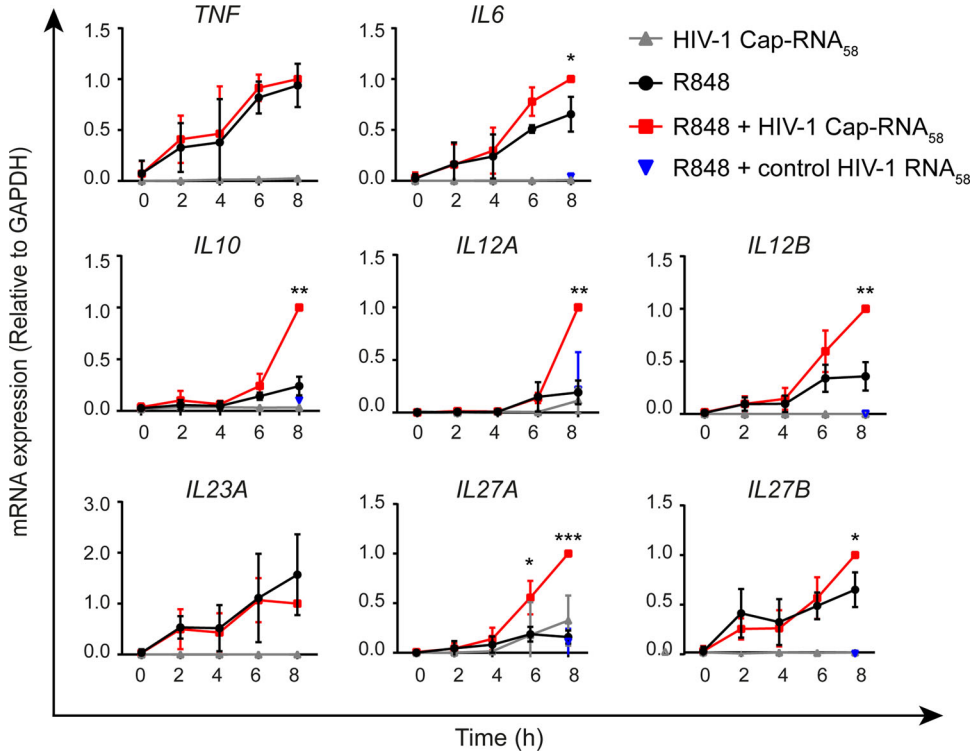

FIGURE 2 **Costimulation with R848 and abortive HIV-1 RNA modulates cytokine gene expression.** DCs were (co)stimulated with R848 and HIV-1 Cap-RNA_{58} or control HIV-1 RNA_{58} Every 2 h, (and only 8 h after costimulation with control HIV-1 RNA_{58}) mRNA was extracted to analyze *TNF*, *IL6*, *IL10*, *IL12A*, *IL12B*, *IL23A*, *IL27A*, and *IL27B* transcription by quantitative real-time PCR. mRNA expression was relative to *GAPDH*. Responses induced by HIV-1 Cap-RNA_{58} with R848 at 8 h were set at 1. Data are representative of collated data of 3 (*IL10*, *IL12A*) or 4 donors (mean ± SD). Significance was determined between responses induced by cotreatment with HIV-1 Cap-RNA_{58} and R848, and R848-induced responses. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test

to a similar enhancement of cytokine production. We observed that costimulation with HIV-1 Cap-RNA_{58} and PAM3CSK4 or LTA significantly enhanced IL-12p70 secretion, while poly-I:C-, LPS-, or flagellin-induced IL-12p70 levels were only marginally enhanced by costimulation with HIV-1 Cap-RNA_{58} (Figures 3(B) and S2(B)). Notably, although the different TLR agonists induced IL-27 expression, these levels were not significantly affected by costimulation with HIV-1 Cap-RNA_{58} (Figures 3(C) and S2(C)). These data suggest that crosstalk between TLR- and abortive HIV-1 RNA-induced signaling synergistically enhanced IL-12p70 expression, whereas only signaling induced by abortive HIV-1 RNA and R848 enhanced IL-27 expression.

3.4 | Costimulation with R848 and abortive HIV-1 RNA leads to T_H1 differentiation

Next, we set out to assess the effect of crosstalk between R848- and abortive HIV-1 RNA-induced signaling on DC-mediated T helper (T_H) differentiation, which was assessed by production of intracellular cytokines IFNγ and IL-4, indicative of a T_H1 or T_H2 response, respectively. Treatment with HIV-1 Cap-RNA_{58} alone resulted in DC-mediated T helper 2 (T_H2) differentiation[28] (Figure 4(A)). R848-primed DCs also induced IL-4$^+$ CD4$^+$ T cells, while decreasing IFNγ^+CD4$^+$ T cells, indicative of T_H2 skewing (Figure 4(A)). Remarkably, costimulation of R848-primed DCs with HIV-1 Cap-RNA_{58} redirected T_H differentiation towards T_H1 polarization, as shown by high levels of intracellular IFNγ and low levels of IL-4, comparable to the T_H1 priming induced by the LPS + IFNγ control (Figures 4(A) and 4(B)). These results imply that crosstalk between abortive HIV-1 RNA- and TLR7/8-induced signaling specifically redirects abortive HIV-1 RNA-induced T_H2 differentiation, resulting in strong T_H1 polarization.

3.5 | Abortive HIV-1 RNA and R848-induced crosstalk enhances CD8$^+$ T cell IFNγ responses

An important aspect of antiviral adaptive immunity is the induction of cytotoxic CD8$^+$ T cells.[42,43] Thus, we next investigated the effect of abortive HIV-1 RNA- and R848-induced crosstalk on induction of DC-mediated antiviral CD8$^+$ T cells. DCs (co)stimulated with R848 and/or HIV-1 Cap-RNA_{58} were cocultured with PBLs, and the percentages of cells positive for intracellular expression of cytolytic molecules granzyme B and perforin, and IFNγ in CD8$^+$ T cells were assessed (Figure S3). DCs treated with R848 but not HIV-1 Cap-RNA_{58} induced IFNγ^+CD3$^+$CD8$^+$ T cells, while, strikingly, crosstalk between R848 and HIV-1 Cap-RNA_{58} significantly enhanced the IFNγ^+CD3$^+$CD8$^+$ T cell responses (Figures 5(A) and 5(B)). Crosstalk did not affect the levels of granzyme B$^+$ and perforin$^+$CD3$^+$CD8$^+$ T cells compared with the

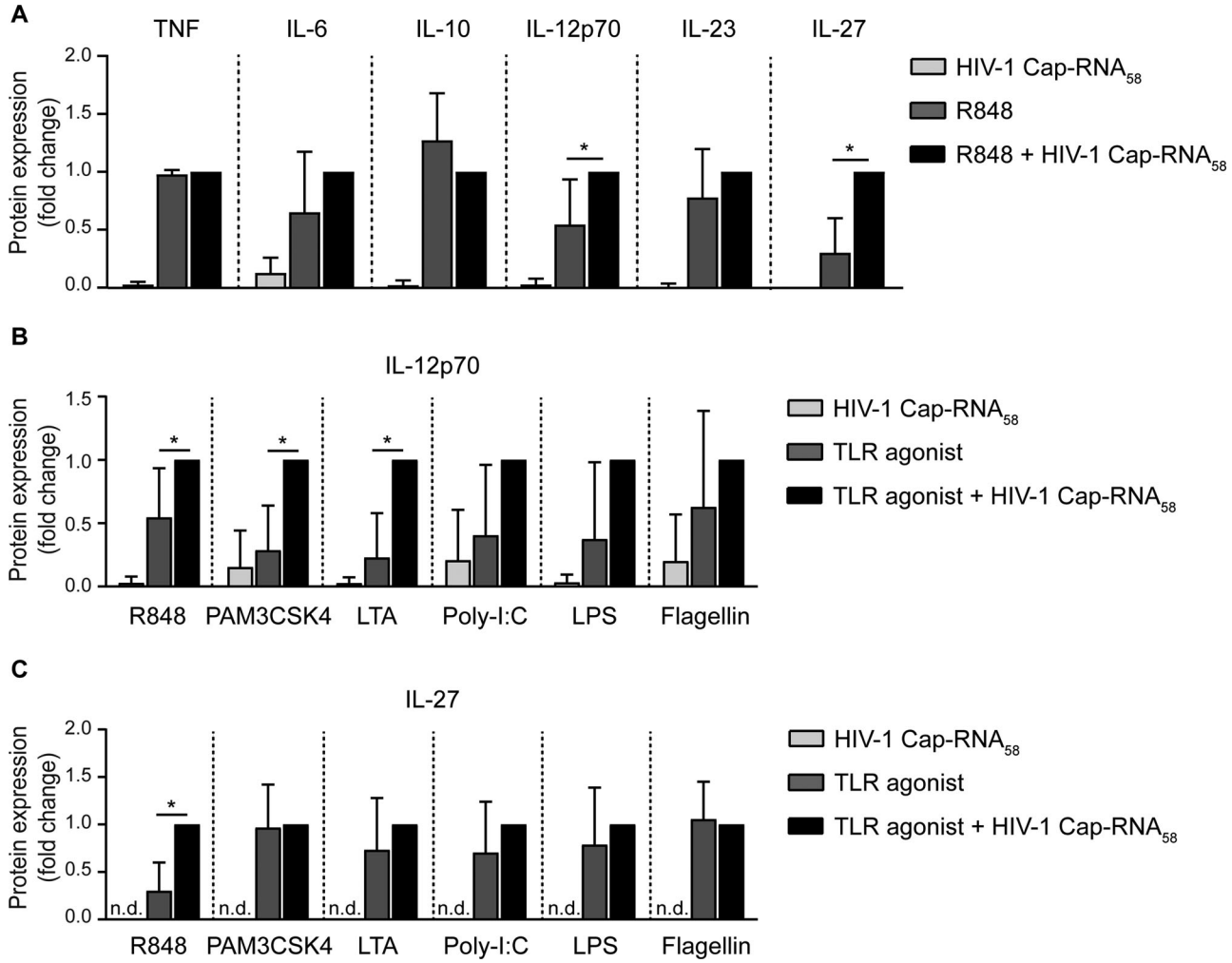

FIGURE 3 **Crosstalk between abortive HIV-1 RNA and R848 enhances IL-12p70 and IL-27 expression.** (A–C) DCs were (co)stimulated with R848 (A–C) or TLR agonists (TLR1/2 agonist PAM3CSK4, TLR2/6 agonist LTA, TLR3 agonist poly-I:C, TLR4 agonist LPS, TLR5 agonist flagellin) (B and C) and HIV-1 Cap-RNA$_{58}$. TNF, IL-6, IL-10, and IL-23 (A), IL-12p70 (A, B) or IL-27 (A, C) expression levels in the DC supernatant were measured after 24 h by ELISA (A–C). Responses induced by HIV-1 Cap-RNA$_{58}$ with TLR agonists were set at 1. Data are shown as fold change in protein expression due to donor variation in cytokine expression levels. Data are representative of collated data of 4 donors and R848 stimulations are used in A–C. HIV-1 Cap-RNA$_{58}$-induced IL-27 expression was not detected (n.d.) (C). (mean ± SD). $^*p < 0.05$, Student's *t*-test

levels induced by R848 and HIV-1 Cap-RNA$_{58}$ stimulations alone (Figures 5(A) and 5(B)). Our data indicate that crosstalk between R848 and abortive HIV-1 RNA enhances antiviral CD8$^+$ T cells, albeit without affecting the expression of cytolytic molecules.

4 | DISCUSSION

Triggering of specific PRRs induces innate and adaptive responses.[5] However, when innate immune cells encounter pathogens, distinct PAMPs are exposed, resulting in the triggering of multiple PRRs and leading to a tailored immune response to that pathogen.[2–5,13] Here, we show that crosstalk between abortive HIV-1 RNA and TLR7/8 agonist R848 leads to antiviral innate and adaptive immunity.

Abortive HIV-1 RNA is a potent inducer of antiviral type I IFNs as it induces high levels of *IFNB* and ISG transcription.[22,28] Most TLR agonists did not modulate the abortive HIV-1 RNA-induced *IFNB* transcription; however, crosstalk with TLR7/8 agonist R848 enhanced transcription of *IFNB* and the ISG *IL27A*, and also enhanced IFNβ protein secretion. Moreover, *IFNB* transcription was also observed in primary blood monocytes in response to R848 and abortive HIV-1 RNA, suggesting that the effects described apply to primary myeloid subsets. Furthermore, synergy between R848- and abortive HIV-1 RNA-induced signaling enhanced the transcription of *IL6*, *IL10*, *IL12A*, *IL12B*, *IL27A*, and *IL27B*. Interestingly, this synergy was detected for bioactive IL-12p70 and IL-27 protein as well, which relies on transcription of *IL12A*/*IL12B* and *IL27A*/*IL27B*, respectively. In contrast to the enhanced transcription of *IL6* and *IL10* by abortive HIV-1 RNA and R848

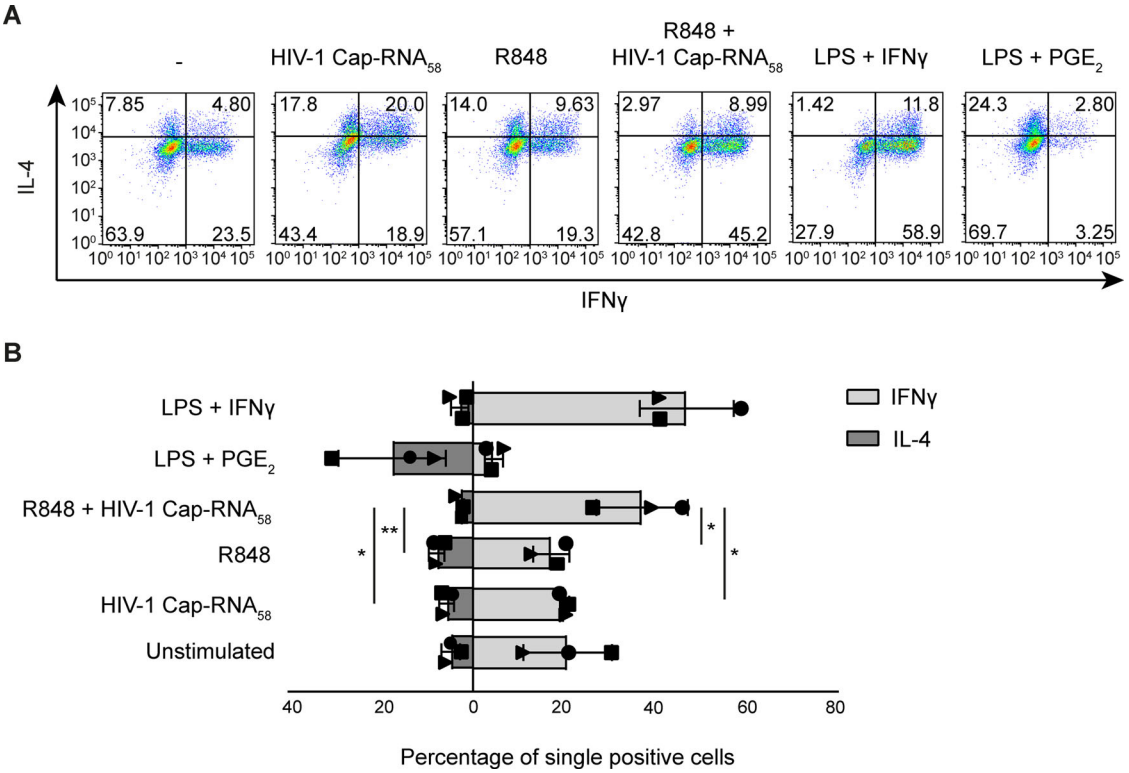

FIGURE 4 **Costimulation with abortive HIV-1 RNA and R848 leads to T_H1 differentiation.** (A and B) DCs were (co)stimulated with R848 and HIV-1 Cap-RNA_{58}. DCs were treated with either LPS + IFNγ or LPS + PGE_2 to induce T_H1 and T_H2 skewing, respectively (A and B). After 48 h, DCs were cocultured with naïve $CD4^+$ T cells for 11–13 days. Resting T cells were re-stimulated and intracellular expression levels of IFNγ and IL-4 were assessed by flow cytometry to indicate T_H1 and T_H2 differentiation, respectively (A and B). The percentage of cells in each quadrant is indicated by numbers in the plots (A) and only the percentage of single IL-4^+ or IFNγ^+ cells were plotted in B. Data are representative of 3 donors of different experiments (A) or are collated data of 3 donors with every symbol representing a different donor (B), (mean ± SD). $^*p < 0.05$, $^{**}p < 0.01$, Student's *t*-test

costimulation, no difference in IL-6 and IL-10 protein levels was observed, possibly due to a crosstalk-specific negative control of translation. These data indicate that crosstalk between abortive HIV-1 RNA and R848 modulates cytokine gene transcription and protein secretion.

We have previously reported that abortive HIV-1 RNA is sensed by the cytosolic PRR DDX3 and result in antiviral type I IFN responses.[22] Additionally, abortive HIV-1 RNA is also recognized by PKR, which upon triggering induces inflammasome activation and IL-1β secretion.[37] In addition to type I IFN, we have shown that sensing of abortive HIV-1 RNA by DDX3 also induced the transcription of various cytokine genes such as *TNF*, *IL6*, and *IL12A*, to protein production and to T_H2 responses.[28] Here, similar responses were detected and enhanced by the presence of R848 and were not observed when we used a capless HIV-1 RNA_{58} control, which is also sensed by PKR,[37] suggesting that the responses induced by R848 and abortive HIV-1 RNA-induced crosstalk are mediated by DDX3-dependent sensing of abortive HIV-1 RNA.

Abortive HIV-1 RNA induces DC-mediated T_H2 differentiation,[28] a response predominantly important for B cell help and immunity against extracellular pathogens.[44,45] In contrast, T_H1 responses are crucial against intracellular pathogens such as viruses.[46] Our data show that crosstalk between abortive HIV-1 RNA and R848, re-directed T_H polarization towards a T_H1 response, possibly due to a synergistic effect between enhanced IL-12 and IL-27 production, leading to the enhanced IFNγ-producing T_H1 responses.[47] Synergy between IL-12 and IL-27 as a result of crosstalk between abortive HIV-1 RNA and R848 might also be the contributing factor leading to the enhanced percentage of IFNγ^+CD8^+ T cells, which also play a crucial role in the cellular control of viruses.[43,48–52] IFNγ is important for enhanced $CD8^+$ T cell function and killing.[53–56] Therefore, our data suggest that abortive HIV-1 RNAs contribute to antiviral immunity by promoting the propagation of IFNγ^+CD8^+ T cells. Of note, granzyme B and perforin play an important role in killing of HIV-1 infected cells.[57–59] Although crosstalk does not further enhance granzyme B^+ and perforin$^+CD8^+$ T cell numbers, granzyme B and perforin were abundantly expressed in the $CD8^+$ T cells. Their presence, in combination with the observed propagation of IFNγ^+CD8^+ T cells, could potentially lead to higher antiviral responses during viral infection.

As genomic HIV-1 RNA is a ligand for TLR7/8,[16–18] our data reveal that crosstalk between R848 and abortive HIV-1 RNA-induced signaling leads to potent antiviral immune responses that might shape responses against HIV-1. We have previously shown that the triggering of TLR8 plays a role in HIV-1 transcription initiation steps,

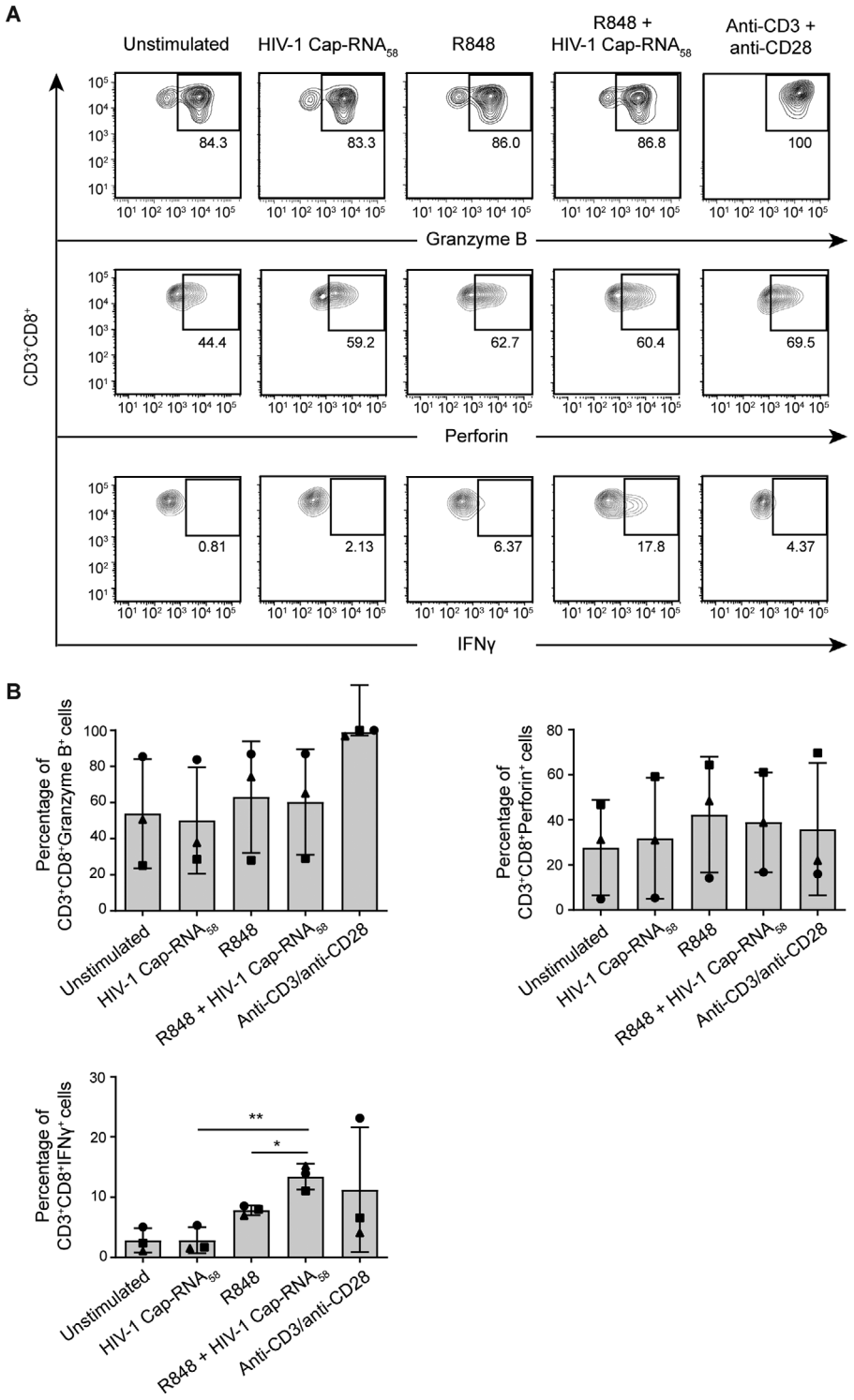

FIGURE 5 **Synergy between abortive HIV-1 RNA and R848-induced signaling enhances CD8$^+$ T cell IFNγ responses. (A and B)** DCs were (co)stimulated with R848 and HIV-1 Cap-RNA$_{58}$ for 48 h and cocultured with PBLs. PBLs were directly treated with anti-CD3 and anti-CD28 as a positive control. After 6 days, cells were restimulated and the percentages of CD3$^+$CD8$^+$ cells with intracellular expression levels of granzyme B, perforin, and IFNγ were assessed by flow cytometry. The percentage of cells in each gate is indicated by numbers in the plots (A). Data are representative of 3 donors of different experiments (A) or are collated data of 3 donors with each symbol representing a different donor (B), (mean ± SD). $^*p < 0.05$, $^{**}p < 0.01$, Student's *t*-test

thus enabling for the production of abortive HIV-1 RNA transcripts.[18] Therefore, these linked events demonstrate that multiple different PRRs are triggered during HIV-1 infection, together determining the final immune response and highlighting the importance of investigating crosstalk between these receptors. Here, we show that both HIV-1-specific events have the potency to induce crosstalk, leading to synergistic antiviral responses. Further studies on the effects of crosstalk between different PRRs, specifically those triggered by HIV-1 products, will provide insight into the immune responses elicited or blocked by this crosstalk and thus would be valuable for vaccination strategies.

Defining the effects of crosstalk between PRRs provides valuable targets needed to enhance vaccine efficacy. TLR8 agonists are currently used as adjuvants to treat viral infections and as antitumor treatment.[35,36,60,61] Here, our data imply that abortive HIV-1 RNAs have a strong capacity to modulate R848-induced signaling, leading to enhanced antiviral immunity. Our findings provide a rationale for the use of abortive HIV-1 RNA as an adjuvant in current TLR8-based antiviral and possibly antitumor strategies. Overall, our results underscore the importance of crosstalk between different PRRs for the final direction and potency of innate and adaptive immune responses.

AUTHORSHIP

M. S. contributed to study conceptualization, investigation, data analysis and wrote the original draft. J. L. V. H. and E. Z. W. contributed to study investigation. S. I. G. and T. B. H. G. contributed to study conceptualization, and, together with M. S. assisted with reviewing and editing the final version of this manuscript.

DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by Aidsfonds (P-9906) and the European Research Council (Advanced grant 670424).

REFERENCES

1. Reis e Sousa C. Dendritic cells as sensors of infection. *Immunity*. 2001;14:495-498.
2. Akira S, Takeda K, Kaisho T, Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2, 675–680.
3. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783-801.
4. Hou B, Reizis B, DeFranco AL. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. *Immunity*. 2008;29:272-282.
5. Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.
6. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol*. 2003;3:984-993.
7. Spörri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol*. 2005;6:163-170.
8. Alam MM, Yang D, Trivett A, et al. HMGN1 and R848 synergistically activate dendritic cells using multiple signaling pathways. *Front Immunol*. 2018;9:2982.
9. Gautier G, Humbert M, Deauvieau F, et al. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med*. 2005;201:1435-1446.
10. Napolitani G, Rinaldi A, Bertoni F, et al. Selected TLR agonist combinations synergistically trigger a TH1 polarizing program in dendritic cells. *Nat Immunol*. 2005;6:769-776.
11. Gerosa F, Baldani-Guerra B, Lyakh LA, et al. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J Exp Med*. 2008;205:1447-1461.
12. Gringhuis SI, den Dunnen J, Litjens M, et al. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB. *Immunity*. 2007;26:605-616.
13. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34:637-650.
14. Lahaye X, Satoh T, Gentili M, et al. The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells. *Immunity*. 2013;39:1132-1142.
15. Sun L, Wu J, Du F, et al. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the Type I interferon pathway. *Science (80-)*. 2013;339:786-791.
16. Beignon A, Mckenna K, Skoberne M, et al. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor – viral RNA interactions. *J Clin Invest*. 2005;115:3265-75.
17. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science (80-)*. 2004;303:1526-1529.
18. Gringhuis SI, van der Vlist M, van den Berg LM, et al. HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. *Nat Immunol*. 2010;11:419-426.
19. Berg RK, Melchjorsen J, Rintahaka J, et al. Genomic HIV RNA induces innate immune responses through RIG-I-dependent sensing of secondary-structured RNA. *PLoS One*. 2012;7:1-10.
20. Yedavalli VSRK, Neuveut C, Chi YH, et al. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. *Cell*. 2004;119:381-392.
21. Soto-Rifo R, Rubilar PS, Limousin T, et al. DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. *EMBO J*. 2012;31:3745-3756.
22. Gringhuis SI, Hertoghs N, Kaptein TM, et al. HIV-1 blocks the signaling adaptor MAVS to evade antiviral host defense after sensing of abortive HIV-1 RNA by the host helicase DDX3. *Nat Immunol*. 2017;18:225-235.
23. Maitra R, McMillan NA, Desai S, et al. HIV-1 TAR RNA has an intrinsic ability to activate interferon-inducible enzmes. *Virology*. 1994;204:823-827.
24. Kim I, Liu CW, Puglisi JD. Specific recognition of HIV TAR RNA by the dsRNA binding domains (dsRBD1-dsRBD2) of PKR. *J Mol Biol*. 2006;358:430-442.
25. de Marcken M, Dhaliwal K, Danielsen AC, et al. TLR7 and TLR8 activate distinct pathways in monocytes during RNA virus infection. *Sci Signal*. 2019;12:1-19.
26. Clemens MJ, Elia A. The double-stranded RNA-dependent protein kinase PKR: structure and function. *J Interf Cytokine Res*. 1997;17:503-524.
27. Kao S, Calman AF, Luciw PA, et al. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature*. 1987;330:489-493.
28. Stunnenberg M, Sprokholt JK, van Hamme JL, et al. Synthetic abortive HIV-1 RNAs induce potent antiviral immunity. *Front Immunol*. 2020;11:1-14.

29. Adams M, Sharmeen L, Kimpton J, et al. Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. *Proc Natl Acad Sci USA*. 1994;91:3862-3866.
30. Greene WC, Peterlin BM. Charting HIV's remarkable voyage through the cell: basic science as a passport to future therapy. *Nat Med*. 2002;8:673-680.
31. Hladnik A, Ferdin J, Goričar K, et al. Trans-activation response element RNA is detectable in the plasma of a subset of aviremic HIV-1–infected patients. *Acta Chim Slov*. 2017;64:530-536.
32. Lassen KG, Bailey JR, Siliciano RF. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo. *J Virol*. 2004;78:9105-9114.
33. Lin X, Irwin D, Kanazawa S, et al. Transcriptional profiles of latent human immunodeficiency virus in infected individuals: effects of tat on the host and reservoir. *J Virol*. 2003;77:8227-8236.
34. Sampey GC, Saifuddin M, Schwab A, et al. Exosomes from HIV-1-infected cells stimulate production of pro-inflammatory cytokines through trans-activating response (TAR) RNA. *J Biol Chem*. 2016;291:1251-1266.
35. Vanwalscappel B, Tada T, Landau NR. Toll-like receptor agonist R848 blocks Zika virus replication by inducing the antiviral protein viperin. *Virology*. 2018;522:199-208.
36. Mark KE, Corey L, Meng TC, et al. Topical resiquimod 0.01% gel decreases herpes simplex virus type 2 genital shedding: a randomized, controlled trial. *J Infect Dis*. 2007;195:1324-1331.
37. Stunnenberg M, van Hamme JL, Trimp M, et al. Abortive HIV-1 RNA induces pro-IL-1β maturation via protein kinase PKR and inflammasome activation in humans. *Eur J Cell Biol*. 2021. Epub ahead of print.
38. Carreno BM, Becker-Hapak M, Huang A, et al. IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity. *J Clin Invest*. 2013;123:3383-3394.
39. de Groot R, van Beelen AJ, Bakdash G, et al. Viral dsRNA-activated human dendritic cells produce IL-27, which selectively promotes cytotoxicity in naive CD8+ T cells. *J Leukoc Biol*. 2012;92:605-610.
40. Heufler C, Koch F, Stanzl U, et al. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-γ production by T helper 1 cells. *Eur J Immunol*. 1996;26:659-668.
41. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nat Immunol*. 2000;1:199-205.
42. Collins DR, Gaiha GD, Walker BD. CD8+ T cells in HIV control, cure and prevention. *Nat Rev Immunol*. 2020;20:471-482.
43. Goonetilleke N, Liu MKP, Salazar-Gonzalez JF, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med*. 2009;206:1253-1272.
44. Allen JE, Sutherland TE. Host protective roles of type 2 immunity: parasite killing and tissue repair, flip sides of the same coin. *Semin Immunol*. 2014;26:329-340.
45. Crotty S. Follicular helper CD4 T cells (T FH). *Annu Rev Immunol*. 2011;29:621-663.
46. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4 + T cells in immunity to viruses. *Nat Rev Immunol*. 2012;12:136-148.
47. Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+T cells. *Immunity*. 2002;16:779-790.
48. Klavinskis LS, Geckeler R, Oldstone MBA. Cytotoxic T lymphocyte control of acute lymphocytic choriomeningitis virus infection: interferon γ, but not tumour necrosis factor α, displays antiviral activity in vivo. *J Gen Virol*. 1989;70:3317-3325.
49. Kuwano K, Scott M, Young JF, et al. HA2 subunit of influenza A H1 and H2 subtype viruses induces a protective cross-reactive cytotoxic T lymphocyte response. *J Immunol*. 1988;140:1264-128.
50. Lukacher AE, Braciale VL, Braciale TJ. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J Exp Med*. 1984;160:814-826.
51. Sridhar S, Begom S, Bermingham A, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med*. 2013;19:1305-1312.
52. Tzannou I, Nicholas SK, Lulla P, et al. Immunologic profiling of human metapneumovirus for the development of targeted immunotherapy. *J Infect Dis*. 2017;216:678-687.
53. Bhat P, Leggatt G, Waterhouse N, et al. Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis*. 2017;8:e2836.
54. Schroder K, Hertzog PJ, Ravasi T, et al. Interferon-γ: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004;75:163-189.
55. Whitmire JK, Tan JT, Whitton JL. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. *J Exp Med*. 2005;201:1053-1059.
56. Zhou F. Molecular mechanisms of IFN-γ to up-regulate MHC class i antigen processing and presentation. *Int Rev Immunol*. 2009;28:239-260.
57. Addo MM, Yu XG, Rathod A, et al. Comprehensive epitope analysis of human immunodeficiency virus Type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol*. 2003;77:2081-2092.
58. Hersperger AR, Pereyra F, Nason M, et al. Perforin expression directly ex vivo by HIV-specific CD8+ T-cells is a correlate of HIV elite control. *PLoS Pathog*. 2010;6:1-13.
59. Migueles SA, Osborne CM, Royce C, et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity*. 2008;29:1009-1021.
60. Mullins SR, Vasilakos JP, Deschler K, et al. Intratumoral immunotherapy with TLR7/8 agonist MEDI9197 modulates the tumor microenvironment leading to enhanced activity when combined with other immunotherapies. *J Immunother Cancer*. 2019;7:1-18.
61. Bourquin C, Pommier A, Hotz C. Harnessing the immune system to fight cancer with Toll-like receptor and RIG-I-like receptor agonists. *Pharmacol Res*. 2020;154:104192.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Stunnenberg M, van Hamme JL, Zijlstra-Willems EM, Gringhuis SI, Geijtenbeek TBH Crosstalk between R848 and abortive HIV-1 RNA-induced signaling enhances antiviral immunity. *J Leukoc Biol*. 2022;112:289–298. https://doi.org/10.1002/JLB.4A0721-365R