Synthetic Abortive HIV-1 RNAs Induce Potent Antiviral Immunity

Melissa Stunnenberg, Joris K. Sprokholt, John L. van Hamme, Tanja M. Kaptein, Esther M. Zijlstra-Willems, Sonja I. Gringhuis and Teunis B. H. Geijtenbeek*

Department of Experimental Immunology, Amsterdam Infection and Immunity Institute, Amsterdam UMC, University of Amsterdam, Amsterdam, Netherlands

Strong innate and adaptive immune responses are paramount in combating viral infections. Dendritic cells (DCs) detect viral infections via cytosolic RIG-I like receptors (RLRs) RIG-I and MDA5 leading to MAVS-induced immunity. The DEAD-box RNA helicase DDX3 senses abortive human immunodeficiency virus 1 (HIV-1) transcripts and induces MAVS-dependent type I interferon (IFN) responses, suggesting that abortive HIV-1 RNA transcripts induce antiviral immunity. Little is known about the induction of antiviral immunity by DDX3-ligand abortive HIV-1 RNA. Here we synthesized a 58 nucleotide-long capped RNA (HIV-1 Cap-RNA_{58}) that mimics abortive HIV-1 RNA transcripts. HIV-1 Cap-RNA_{58} induced potent type I IFN responses in monocyte-derived DCs, monocytes, macrophages and primary CD1c{\textsuperscript{+}} DCs. Compared with RLR agonist poly-I:C, HIV-1 Cap-RNA_{58} induced comparable levels of type I IFN responses, identifying HIV-1 Cap-RNA_{58} as a potent trigger of antiviral immunity. In monocyte-derived DCs, HIV-1 Cap-RNA_{58} activated the transcription factors IRF3 and NF-κB. Moreover, HIV-1 Cap-RNA_{58} induced DC maturation and the expression of pro-inflammatory cytokines. HIV-1 Cap-RNA_{58}-stimulated DCs induced proliferation of CD4{\textsuperscript{+}} and CD8{\textsuperscript{+}} T cells and differentiated naïve T helper (T{\textsubscript{H}1}) cells toward a T{\textsubscript{H}2} phenotype. Importantly, treatment of DCs with HIV-1 Cap-RNA_{58} resulted in an efficient antiviral innate immune response that reduced ongoing HIV-1 replication in DCs. Our data strongly suggest that HIV-1 Cap-RNA_{58} induces potent innate and adaptive immune responses, making it an interesting addition in vaccine design strategies.

Keywords: abortive HIV-1 RNA, type I IFN, viral sensing, DDX3, pattern recognition receptor, dendritic cells, antiviral immunity

INTRODUCTION

Evoking potent and tailored antiviral responses by the host is paramount in combating viral infections (1). Dendritic cells (DCs) induce antiviral immune responses by recognizing invading viruses via pattern recognition receptors (PRRs). PRR triggering by viral pathogen-associated molecular patterns (PAMPs) induces DC maturation and activation as well as differentiation of naïve T cells (2–4). Certain PRRs such as the RIG-I-like receptors (RLRs) induce strong antiviral innate immune responses initiated by expression of type I interferon (IFN) responses. RLRs are cytosolic PRRs and sense virus infection by detection of specific viral RNA structures (5, 6). RIG-I (DDX58) and MDA5 are two well-described RLRs and important in antiviral immunity to e.g., Influenza viruses, Dengue virus, and West Nile virus (7–9). RIG-I recognizes both uncapped 5′ppp
single stranded (ss) RNA and short double stranded (ds) RNA, whereas MDA5 senses long dsRNA (7, 10, 11). Upon activation, RIG-I and MDA5 engage with mitochondrial antiviral protein MAVS, leading to MAVS multimerization and subsequent recruitment of adaptor molecule TRAF3. TRAF3 mediates recruitment of serine/threonine-protein TANK-binding kinase 1 (TBK1) and IκB kinase ε (IKKε), resulting in activation of transcription factors IRF3 and NF-κB, leading to type I IFN and cytokine transcription (12, 13). Autocrine and paracrine ligation of IFNβ on the cell surface ultimately results in transcription of a broad spectrum of interferon-stimulated genes (ISGs), of which many exhibit strong antiviral activity (14–18). NF-κB activation regulates transcriptional activation of a plethora of cytokine genes, including IL-1β and TNF-α that are important in innate immunity (19). In addition, IFNβ is crucial in driving IL-27 synthesis and thereby CD8+ T cell-dependent adaptive immune responses (20). Moreover, both IRF3 and NF-κB are involved in the induction of cytokines important in T helper (T_H1) 1 differentiation (21–23).

Recently, the cytosolic DEAD-box RNA helicase 3 (DDX3) that resembles cytosolic DEAD box helicase RIG-I and MDA5, was shown to function as a PRR for human immunodeficiency virus 1 (HIV-1) (24–27). DDX3 acts as a host factor for various viruses including hepatitis B and C virus and West Nile virus, facilitating virus replication (8, 28, 29). Thus, DDX3 is an important host factor for viruses and its function as a PRR might prevent escape of viruses from DDX3.

DDX3 is a well-known host factor required for HIV-1 propagation due to its role in transport of viral Tat mRNA and subsequent formation of translation initiation complexes (30–32). Upon initiation of HIV-1 infection, deficient transcription elongation leads to formation of prematurely aborted RNAs (33, 34). Interestingly, DDX3 senses these abortive HIV-1 RNA transcripts, leading to MAVS-dependent type I IFN responses, indicating that DDX3 is a viral PRR for HIV-1 (27).

Abortive HIV-1 RNAs are generated during the early steps of HIV-1 transcription, consisting of an HIV-1-specific complex secondary RNA hairpin-like structure (TAR loop) and a 5′cap, but lacking a poly A tail (33, 34). The complex structure of the TAR loop, together with the 5′cap is required for binding to DDX3, while the absence of the poly A tail prevents engagement of DDX3 with the cellular translational machinery (30). Gringhuis et al. have shown that DDX3 is an important PRR that senses abortive HIV-1 RNA transcripts upon HIV-1 infection. However, during infection of DCs, HIV-1 hijacks DC-SIGN function to block MAVS signaling, thereby preventing type I IFN and cytokine responses, and subsequently preventing the induction of antiviral innate and adaptive immune responses (27, 35). Due to this viral inhibition mechanism, the breadth and potency of abortive HIV-1 RNAs in inducing antiviral immunity remains elusive.

To characterize the role of abortive HIV-1 RNA in establishing innate and adaptive immune responses without interference due to DC-SIGN inhibition, we developed a synthetic 5′capped HIV-1 RNA of 58 nucleotides that mimics the naturally occurring abortive HIV-1 RNA (HIV-1 Cap-RNA58). Our data strongly suggest that HIV-1 Cap-RNA58 induces potent type I IFN responses in monocyte-derived DCs, macrophages as well as primary CD1c+ DCs. Furthermore, HIV-1 Cap-RNA58 is a potent stimulus that induces both innate and adaptive immune responses in monocyte-derived DCs. HIV-1 Cap-RNA58-dependent induction of DC maturation and cytokine secretion leads to Th1 differentiation. Notably, HIV-1 Cap-RNA58 responses inhibited ongoing HIV-1 infection. Our data further define the importance of sensing abortive HIV-1 transcripts to evoke strong antiviral immunity and provide a rationale for using HIV-1 Cap-RNA58 in vaccine design strategies.

MATERIALS AND METHODS

RNA Constructs

The synthetic abortive HIV-1 RNAs were designed based on the HIV-1 genome. HIV-1 Cap-RNA58 consists of nucleotides 1–58 from the HIV-1 genome, including a 5′m7G cap but lacking the poly A tail. HIV-1 Cap-RNA630 consists of nucleotides 1–630 and also contains the 5′m7G cap and is lacking the poly A tail. The 5′m7G cap was incorporated using co-capping of 5′m7GTP during in vitro transcription (IVT) (Biosynthesis, Table S1) as previously described (27). As a control RNA, 1–58 nucleotides were synthesized lacking the 5′cap (HIV-1 control RNA58). HIV-1 Cap-RNA58 and HIV-1 control RNA58 structures were predicted with the MFOLD program.

Myeloid Cell Stimulations

This study was performed according to the Amsterdam University Medical Centers, location AMC Medical Ethics Committee guidelines and all donors gave written informed consent in accordance with the Declaration of Helsinki. CD14+ monocyte isolation and subsequent generation of monocyte-derived DCs was performed as previously described (36). CD14+ monocytes were cultured in IMDM supplemented with 10% FCS, 10 U/mL penicillin and 10 mg/mL streptomycin (IMDM complete, Invitrogen) O/N at 37°C. 5% CO2 for monocyte stimulations or cultured for 6 days in IMDM complete supplemented with GM-CSF (800 U/mL, Invitrogen) to obtain monocyte-derived macrophages. Stimulations were performed on day 1 (monocytes) or day 6 (monocyte-derived macrophages and DCs). CD1c+ DCs were isolated using human CD1c+ dendritic cell isolation kit (Milenyi Biotec) according to manufacturer’s instructions. Cells were stimulated with synthetic HIV-1 RNAs (1 nM, Biosynthesis) complexed with transfection reagent lyevec (Invitrogen), polyinosinic:polycytidylic acid complexed with lyevec (poly-I:C, 4 µg/mL, Invitrogen) and lipopolysaccharide (LPS) Salmonella enterica serotype typhimurium (10 µg/mL, Sigma). Poly-I:C concentration series were performed with molecular weight ranges from 223801.1 to 895204.4 g/mol. Cells were pre-incubated with blocking IFNα/βR antibodies (clone MMHar2, 20 µg/mL) and BAY 11–7082 (2 µM) for 30 min and 2 h, respectively.
RNA Interference

RNA interference was performed using the Neon Transfection System according to manufacturer’s protocol (Thermo Fisher). On day 4 of monocyte-derived DC cultures, cells were washed with PBS, resuspended in buffer R (Thermo Fisher) and divided according to the different short interfering (si) SMARTpool RNAs (all from Dharmacon), siDDX3 (M-006874-01), siMAVS (M-024237-02) or siNon-Target as a control (D-001206-13) were added to DC-buffer R mixtures and transfection of DCs with the siRNAs was achieved by subjecting them to 1,500V for 20 ms. Transfected cells were seeded in 24-wells plates in RPMI 1640 with 10% FCS (Invitrogen) and 2 mM L-glutamine (Lonza), without antibiotics. After 48 h, viable cells were harvested, washed and seeded in a 96-wells round bottom plate and incubated overnight at 37°C, 5% CO2. Seventy-two hours after transfection, silencing of expression of target proteins in DCs was confirmed by quantitative real-time PCR and flow cytometry (Figure S1) and cells were stimulated as previously described.

Quantitative Real-Time PCR

mRNA was extracted using an mRNA capture kit (Roche) and was reverse transcribed to cDNA using a reverse transcriptase kit (Promega). Quantitative real-time PCR was performed on an ABI 7500 Fast Real-Time PCR detection system (Applied Biosystems) using SYBR Green (Thermo Fisher), with primers that were designed using Primer Express 2.0 (Applied Biosystems, Table S2). Expression of genes of interest was normalized to expression of household gene GAPDH, according to the formula Nt = 2Ct(GAPDH)−Ct(target). For each donor, expression levels induced upon stimulation with HIV-1 Cap-RNAs58 were set as 1.

Flow Cytometry

DCs were stimulated for 24 or 48 h, fixed with 4% paraformaldehyde (pFA) and stained with PE-conjugated anti-CD80 (1:12.5, 557227, BD pharbounding), allopurinocyanin-conjugated CD83 (1:25, 551073, BD Pharmingen), FITC-conjugated anti-CD86 (1:25, 555657, BD Pharmingen), PE-conjugated anti-HLA-DR (1:25, 555812, BD Pharmingen), or PE-Cy7-conjugated anti-CD40 (1:100, 2165055, Sony Biotechnology). Expression levels after RNA interference were determined using anti-DDX3 or anti-MAVS (1:50, 26355 or 3993S, Cell Signaling) followed by PE-conjugated donkey anti-rabbit (1:200, Jackson Immuno Research). HIV-1 infection levels were assessed using anti-p24 (1:200, KC57-RD1, Beckman Coulter). Flow cytometric analysis was performed using the FACS Canto II (BD Biosciences) and FlowJo software v10.

p65 and IRF3 Translocation

DCs were stimulated for 4 h, fixed with 4% pFA and permeabilized with 0.2% Triton X-100 in PBS, p65 was stained with anti-p65 (1:50, 8242S, Cell Signaling) and IRF3 with anti-IRF3 (1:50, 4302S, Cell Signaling), followed by a secondary donkey anti-rabbit labeled with Alexa-546 (1:400, Invitrogen) and 1 μg/mL Hoechst (Invitrogen) and cellular localization was visualized with a 100x magnification, using Leica DM6 B upright microscope. Analysis was performed with LAS X Navigator software. Nuclear extracts (NE) were prepared 4 h after DC stimulation, using the NucBuster protein extraction kit (Novagen). Twenty micrograms of NE per sample was used to detect nuclear p65 or IRF3 using the TransAM NF-kB-p65 and IRF3 kits (Active Motif). OD450 nm values were measured using BioTek Synergy HT.

Elisa

DC supernatants were harvest 24 or 48 h after stimulation and secretion of TNF, IL-6, and IL-12p70 protein (eBiosciences) was measured by ELISA as described by manufacturer. OD450 nm values were measured using BioTek Synergy HT.

T Cell Proliferation and Differentiation

To assess T cell proliferation levels, DCs were primed with stimuli and cocultured in a 1:4 ratio with allogeneic peripheral blood lymphocytes (PBLs) isolated from buffy coats of healthy donors (Sanquin) labeled with CellTrace Violet (Thermo Fisher). On day 3, IL-2 was added (10 U/mL, Chiron). After 5 days, cells were fixed with 4% pFA and stained with FITC-conjugated anti-CD3 (1:100, 11003642, eBioscience), PerCP-Cy5.5-conjugated anti-CD4 (1:20, 332772, BD Biosciences), PE-Cy7-conjugated anti-CD8 (1:100, 25008742, eBioscience) and fixable viability dye eFluor780 (1:4000, 65-0865-14, eBioscience). Proliferation was assessed in viable CD4+ and CD8+, CD3+ T cells. To examine Th cell differentiation, DCs were primed for 48 h with stimuli or LPS (10 ng/mL) in combination with IFNγ (1,000 U/mL, uCyTech) or PGE2 (1 μM, Sigma), as positive controls for Th1 and 2 skewing, respectively. DCs were cocultured in the presence of Staphylococcus aureus enterotoxin B (10 pg/mL, Sigma) in a 1:4 ratio with allogeneic naïve T cells that were isolated from PBMCs or PBLs from buffy coats with a human CD4+ T cell isolation kit II (Miltenyi Biotec), with PE-conjugated anti-CD45RO (Dako) and anti-PE beads (Miltenyi Biotec) according to manufacturer’s instructions. On day 5, IL-2 was added (10 U/mL, Chiron). After 11–13 days, resting T cells were restimulated with PMA (10 ng/mL, Sigma) and ionomycin (1 mg/mL, Sigma) for 6 h and treated with brefeldin A (10 μg/mL, Sigma) for the final 4 h. Cells were fixed for flow cytometry analysis with 4% pFA, permeabilized using 0.1% saponin in PBS and stained with FITC-conjugated anti-IFNγ (1:5, 340449, BD Biosciences) and allopurinocyanin-conjugated anti-IL-4 (1:25, 554486, BD Biosciences) to determine Th1 and 2 skewing, respectively. Cells were analyzed using the FACS Canto II (BD Biosciences) and FlowJo software v10.

Virus and Infection

DCs were infected with R5 HIV-1 strain NL4.3 BaL. NL4.3 BaL was produced as described previously (34, 37). For DC infection, a multiplicity of infection (MOI) of 0.1–0.2 was used, depending on the virus batch. DCs were infected for 24 h, washed extensively and left in the presence of HIV-1 control RNA or HIV-1 Cap-RNAs8 for 5 days, after which intracellular p24 levels were measured using flow cytometry to determine infection.
**Statistical Analysis**

Statistics were performed using Student's t-test for paired (BAY 11-7082 inhibitor, IL-6 and TNF ELISAs, proliferation assay) and unpaired observations (all other experiments) using GraphPad version 8. Statistical significance was set at \( P < 0.05 \).

**RESULTS**

**HIV-1 Cap-RNA\textsubscript{58} Induces Type I IFN Responses in Various Myeloid Cells**

We used a synthetic RNA that mimics abortive HIV-1 RNAs: this synthetic HIV-1 Cap-RNA\textsubscript{58} consists of the first 58 nucleotides common to all HIV-1 transcripts and contains a 5' cap while lacking a poly A tail (**Table S1, Figure 1A**). We investigated whether HIV-1 Cap-RNA\textsubscript{58} induced type I IFN responses in monocyte-derived DCs (DCs) by treating DCs with HIV-1 Cap-RNA\textsubscript{58} complexed with transfection reagent lipofectamine (vehicle control) to facilitate cytoplasmic delivery (27). HIV-1 Cap-RNA\textsubscript{58} induced strong IFNB transcription in DCs after 10 h of stimulation (**Figure 1B**). Expression levels of interferon-stimulated gene (ISG) Myxovirus resistance protein 1 (Mxα) transcripts were significantly induced by HIV-1 Cap-RNA\textsubscript{58} in DCs as well when compared to untreated DCs (**Figure 1B**). HIV-1 Cap-RNA\textsubscript{58}-induced type I IFN levels were compared to those observed with RLR agonist poly-I:C. After equalizing the amount of molecules of both HIV-1 Cap-RNA\textsubscript{58} and poly-I:C (**Figure S1**), HIV-1 Cap-RNA\textsubscript{58}-induced type I IFN were significantly higher than poly-I:C-induced levels after 10 h of stimulation (**Figure 1B**). We next investigated whether HIV-1 Cap-RNA\textsubscript{58} activated responses in primary myeloid cells. Notably, treatment of CD14\textsuperscript{+} monocytes and monocyte-derived macrophages with HIV-1 Cap-RNA\textsubscript{58} induced type I IFN responses 10 h after stimulation, whereas primary blood CD1c\textsuperscript{+} DCs showed type I IFN responses 8 h after stimulation. HIV-1 Cap-RNA\textsubscript{58}-induced type I IFN levels were higher than or comparable to poly-I:C-induced type I IFN responses (**Figure 1C**). To assess the specificity of HIV-1 Cap-RNA\textsubscript{58} for DC activation and MAVS-dependent signaling, both DDX3 and MAVS were silenced in DCs by RNA interference (RNAi) (**Figure S2**). Silencing of either DDX3 or MAVS expression completely abrogated IFNB induced by HIV-1 Cap-RNA\textsubscript{58}, 2 h after stimulation (**Figure 1D**). Both vehicle control and HIV-1 control RNA did not induce IFNB expression (**Figures 1A, D**). These data indicate that HIV-1 Cap-RNA\textsubscript{58} triggers type I IFN in a variety of myeloid cells via DDX3 and MAVS, and that the 5’cap is required for sensing by DDX3.

**HIV-1 Cap-RNA\textsubscript{58} Induces DC Maturation**

To determine whether HIV-1 Cap-RNA\textsubscript{58} induces adaptive immunity, we first examined the expression levels of costimulatory molecules CD80, CD83, and CD86 after stimulation with HIV-1 Cap-RNA\textsubscript{58}, by flow cytometry. HIV-1 Cap-RNA\textsubscript{58} induced expression of CD80, CD83, and CD86 compared to unstimulated DCs, albeit to a lesser extent compared to LPS (**Figure 2A**). HIV-1 Cap-RNA\textsubscript{58} induced expression levels of activation marker HLA-DR to a similar extent as LPS, whereas the expression levels of costimulatory molecule CD40 remained unaffected in contrast to LPS stimulation (**Figure 2A**). To investigate the role of IFNβ signaling in DC maturation, we neutralized IFNβ signaling by treatment with blocking IFNα/βR antibodies. CD86 induction by HIV-1 Cap-RNA\textsubscript{58} was partially but significantly blocked by blocking IFNα/βR antibodies (**Figures 2B, C**), suggesting that type I IFN induction increases CD86. Besides type I IFN-dependent CD86 expression, expression of costimulatory molecules can also be induced by the transcription factor NF-κB (38, 39). To test whether CD86 expression is also NF-κB dependent, we blocked NF-κB activation using BAY 11-7082, a small molecule inhibitor for IkBα, as release of the inhibitory protein IkBα from the NF-κB dimer within the cytoplasm is mandatory for NF-κB activation (40). BAY 11-7082 treatment significantly reduced HIV-1 Cap-RNA\textsubscript{58}-induced CD86 expression (**Figure 2C**). Our results imply that HIV-1 Cap-RNA\textsubscript{58} treatment leads to IFNβ- and NF-κB-dependent DC maturation.

**HIV-1 Cap-RNA\textsubscript{58} Activates IRF3 and NF-κB p65**

We next investigated whether HIV-1 Cap-RNA\textsubscript{58} activated transcription factors IRF3 and NF-κB, known to be involved in transcriptional regulation of a plethora of cytokine and other genes required for the orchestration of innate and adaptive immune responses by DCs (14, 15, 19). DCs were treated with HIV-1 Cap-RNA\textsubscript{58} or LPS and translocation of p65, one of the primary active subunits within the dimeric NF-κB transcription factor family, was analyzed by immunofluorescence microscopy. Similarly, as was observed for IRF3, HIV-1 Cap-RNA\textsubscript{58} induced p65 translocation to the nucleus, albeit to a lesser extent than observed for LPS-treated DCs (**Figures 3A, B**). We next quantified nuclear translocation of p65 and IRF3 using a transcription factor binding assay using nuclear extracts from HIV-1 Cap-RNA\textsubscript{58}-activated DCs. HIV-1 Cap-RNA\textsubscript{58} induced significant translocation of the p65 unit, 4 h after stimulation, in contrast to HIV-1 control RNA (**Figure 3C**). Similarly, we quantified the nuclear translocation of IRF3, which was also detected in HIV-1 Cap-RNA\textsubscript{58}-activated DCs. HIV-1 Cap-RNA\textsubscript{58} induced significant translocation of the p65 unit, 4 h after stimulation, in contrast to HIV-1 control RNA (**Figure 3C**). Thus, these data strongly indicate that HIV-1 Cap-RNA\textsubscript{58} activates both IRF3 and NF-κB, implying that it can play a significant role in establishing innate and adaptive immune responses.

**HIV-1 Cap-RNA\textsubscript{58} Induces Expression of Pro-Inflammatory Cytokines**

We next assessed whether treatment of HIV-1 Cap-RNA\textsubscript{58} triggered cytokine expression in DCs. HIV-1 Cap-RNA\textsubscript{58} significantly induced expression of the pro-inflammatory cytokines IL-6 and TNF at both mRNA and protein level, compared to vehicle control and HIV-1 control RNA-treated DCs (**Figures 4A–C**). Interestingly, HIV-1 Cap-RNA\textsubscript{58} treatment of DCs did not induce mRNA expression of IL1B, IL8, IL10, and IL23A (data not shown). mRNA expression levels of IL6 and TNF peaked at 10 h, while the ISG IL27A, which encodes IL-27p28, a subunit of IL-27, peaked at 8 h (**Figure 4A**). At peak level, the
expression levels of HIV-1 Cap-RNA$_{58}$-induced IL6, TNF, IL27A, and IL12A mRNA were significantly increased compared to both vehicle control and HIV-1 control RNA (Figure 4B). Whereas, IL12A transcription was induced upon treatment with HIV-1 Cap-RNA$_{58}$, IL12B mRNA could not be detected (Figure 4A). In line with the lack of expression of the IL12B subunit, no bioactive
IL-12p70 protein could be detected in the supernatant of DCs after HIV-1 Cap-RNA₅₈ treatment (Figure 4C). Thus, HIV-1 Cap-RNA₅₈ induces a specific cytokine program primarily directed at pro-inflammatory conditions.

**Different Viral HIV-1 RNAs Induce Similar Levels of Type I IFN and Pro-Inflammatory Cytokines**

To assess whether the ability of HIV-1 Cap-RNA₅₈ to induce type I IFN and cytokine responses is due to the short RNA construct length, we examined the ability of a longer viral HIV-1 RNA in inducing immune activation. DCs treated with a 5′capped 630 nucleotides long RNA corresponding to the Tat transcript, which includes the same 1–58 sequence as the HIV-1 Cap-RNA₅₈ at its start, but lacking a poly A tail (HIV-1 Cap-RNA₆₃₀) induced antiviral responses comparable to those induced by HIV-1 Cap-RNA₅₈. Similar to HIV-1 Cap-RNA₅₈, HIV-1 Cap-RNA₆₃₀ induced IFNB 2 h after stimulation reaching a peak level after 10 h (Figure 5A). Similarly, HIV-1 Cap-RNA₆₃₀ induced MXA and also another ISG A3G (encoding for APOBEC3G protein).
transcription at similar levels as HIV-1 Cap-RNA58, peaking again at 10 h after stimulation (Figure 5B). At peak levels, HIV-1 Cap-RNA58 significantly induced IFNB, MxA, and A3G gene expression compared to DCs treated with vehicle control or HIV-1 control RNA (Figure 5C). We also assessed HIV-1 Cap-RNA58- and HIV-1 Cap-RNA630-induced IL6, TNF, IL12A, and IL27A transcription at time points that we had previously shown to have significantly enhanced expression after HIV-1 Cap-RNA58 treatment compared to untreated DCs and DCs treated with vehicle control and HIV-1 control RNA (Figures 4A,B). At 6,
FIGURE 4 | HIV-1 Cap-RNA \textsubscript{58} induces expression of pro-inflammatory cytokines. (A) DCs were not treated or stimulated with 1 nM HIV-1 Cap-RNA \textsubscript{58} and mRNA expression levels of \textit{IL6}, \textit{TNF}, \textit{IL27A}, \textit{IL12A}, and \textit{IL12B} were measured every 2 h with quantitative real-time PCR, relative to GAPDH. HIV-1 Cap-RNA \textsubscript{58}-induced responses at 8 h were set as 1 for \textit{IL6} and at 10 h for all other cytokines genes. (B) DCs were left untreated, treated with vehicle control or 1 nM HIV-1 control RNA or stimulated with 1 nM HIV-1 Cap-RNA \textsubscript{58} and mRNA expression levels of \textit{IL6}, \textit{TNF}, \textit{IL27A}, and \textit{IL12A} were measured at peak level with quantitative real-time PCR, relative to GAPDH. HIV-1 Cap-RNA \textsubscript{58}-induced responses at 8 h were set as 1 for \textit{IL6} and \textit{TNF}, at 10 h for \textit{IL27A} and at 24 h for \textit{IL12A} gene expression. (C) DCs were left untreated, treated with vehicle control or 1 nM HIV-1 control RNA or stimulated with 1 nM HIV-1 Cap-RNA \textsubscript{58} and supernatant was harvested after 24 or 48 h, to determine expression levels of IL-6 (48 h), TNF (24 h), and IL-12p70 (48 h) with ELISA. Data are representative of collated data of three donors (A,B) or representative of three donors of different experiments (C) (mean ± s.d.). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test. NS, not significant.
**FIGURE 5** Different viral HIV-1 RNAs induce similar levels of type I IFN and pro-inflammatory cytokines. (A) DCs were not treated or treated with 1 nM HIV-1 Cap-RNA<sub>58</sub> or 1 nM HIV-1 Cap-RNA<sub>630</sub>. mRNA was extracted every 2 h and analyzed for IFNB by quantitative real-time PCR and mRNA expression was analyzed relative to GAPDH. HIV-1 Cap-RNA<sub>58</sub>-induced responses at 10 h after stimulation were set as 1. Data point at 8 h after stimulation was also shown in Figure 1A. (B) MXA or APOBEC3G mRNA expression was assessed similarly. Data point at 8 h after stimulation was also shown in Figure 1B. (C) DCs were left untreated, treated with either vehicle control, 1 nM HIV-1 control RNA or 1 nM HIV-1 Cap-RNA<sub>58</sub> and mRNA expression levels of IFNB, MXA, and A3G were measured at peak level with quantitative real-time PCR, relative to GAPDH. HIV-1 Cap-RNA<sub>58</sub>-induced responses at 10 h were set as 1. (D) IL6, TNF, IL12A, and IL27A mRNA expression was assessed similarly. Data are representative of collated data of three (A,B,D) or four (C) donors (mean ± s.d.). Statistical significance was determined between both HIV-1 RNA constructs and between unstimulated DCs and DCs treated with HIV-1 Cap-RNA<sub>58</sub> or HIV-1 Cap-RNA<sub>630</sub>. **(D)** *P < 0.05, ****P < 0.0001, Student’s t-test. NS, not significant.
8 and 10 h after stimulation with the two HIV-1 Cap-RNAs, we observed that both constructs induced similar levels of IL6, TNF, IL12A, and IL27A mRNA (Figure 5D). Although not significant, stimulation with HIV-1 Cap-RNA630 showed a trend toward increased IL12A expression compared to HIV-1 Cap-RNA58 after 6, 8, and 10 h of stimulation, and to a trend of increased IL27A expression after 10 h (Figure 5D). These data suggest that the length of viral RNA constructs does not affect the strength of the type I IFN and pro-inflammatory cytokine responses as long as it contains a 5′cap and the first 58 nucleotides of the HIV-1 genome that form the TAR loop. Thus, synthetic HIV-1 RNAs induce antiviral innate immune responses independent of length.

**HIV-1 Cap-RNA58-Activated DCs Induce T Cell Proliferation and Differentiation**

We next investigated the ability of HIV-1 Cap-RNA58-treated DCs to activate T cells. First we analyzed proliferation of CellTrace Violet-labeled peripheral blood cells (PBLs) induced by coculture for 5 days with DCs that were treated with vehicle control, HIV-1 control RNA, HIV-1 Cap-RNA58 or LPS. Flow cytometry analysis showed that HIV-1 Cap-RNA58-activated DCs enhanced proliferation of both CD4+ and CD8+ T cells compared to untreated DCs and DCs treated with vehicle control or HIV-1 control RNA (Figures 6A,B and Figure S3). We next analyzed TIL1 and TIL2 differentiation after DC-naive CD4+ T cell cocultures by intracellular IFNγ and IL-4 expression, respectively. HIV-1 Cap-RNA58-activated DCs showed significant skewing toward TIL1 differentiation compared to the DCs treated with vehicle control or HIV-1 control RNA (Figures 6C,D). As expected, positive controls LPS and PGE2 or LPS and IFNγ induced TIL2 and TIL1 responses, respectively, whereas LPS gave a mixed TIL1/TIL2 response (Figures 6C,D). Thus, our data show that HIV-1 Cap-RNA58-treated DCs induce CD4+ and CD8+ T cell activation and skew adaptive immune response toward a TIL2 phenotype.

**HIV-1 Cap-RNA58 Inhibits HIV-1 Infection of DCs**

To investigate whether treatment with HIV-1 Cap-RNA58 would block HIV-1 replication in DCs after infection, DCs were infected by R5-tropic HIV-1 NL4.3 BaL. After 24 h of ongoing HIV-1 infection the DCs were treated with HIV-1 Cap-RNA58 or LPS After 5 days, infection levels were assessed by measuring intracellular p24+ cells by flow cytometry. Although infection levels differed per donor, HIV-1 Cap-RNA58 decreased the percentage of HIV-1 p24+ DCs compared to HIV-1 control RNA-treated DCs in four different donors, albeit to a lesser extent than LPS (Figures 7A,B). These results imply that HIV-1 Cap-RNA58 induces a functional antiviral response that limits HIV-1 infection.

**DISCUSSION**

RNA helicase DDX3 is important for the transport of HIV-1 Tat mRNA as well as the formation of translation initiation complexes required for HIV-1 translation (30–32). Besides its role as a host factor for HIV-1, DDX3 also functions as a viral sensor (27). Here we investigated the potency and breadth of DD3X ligand HIV-1 Cap-RNA58, a synthetic mimic of the naturally occurring abortive HIV-1 RNAs. We observed that HIV-1 Cap-RNA58 resulted in type I IFN responses in various myeloid cells in a DDX3- and MAVS-dependent manner. Our data further showed that HIV-1 Cap-RNA58 inhibited ongoing HIV-1 replication in DCs, most likely via the induced innate antiviral type I IFN responses. Moreover, HIV-1 Cap-RNA58 induced DC maturation and cytokine responses that led to adaptive T cell activation as well as differentiation. Thus, our data suggest that DDX3 is a PRR and shows that synthetic abortive HIV-1 RNA58 is immunostimulatory.

The potency of abortive HIV-1 RNA to exert an antiviral role in response to viral sensing by its natural ligand DDX3 is not well-understood. Therefore, we aimed to assess the role of abortive HIV-1 RNA in induction of innate and adaptive immune responses by using a synthetic mimic of abortive HIV-1 RNA (HIV-1 Cap-RNA58). Our data strongly suggest that synthetic HIV-1 Cap-RNA58 encapsulated in polyamine triggered DD3X and MAVS-mediated IFNβ responses. It has been described that DD3X has been found in complexes with RIG-I and MDA5 and might therefore induce IFNβ responses via a RIG-I or MDA5-MAVS-dependent way (41). We have previously shown in 293T cells treated with CRISPR-cas9 that depletion of RIG-I and MDA5 did not affect HIV-1 Cap-RNA58-induced type I IFN responses, indicating that the HIV-1 Cap-RNA58-induced type I IFN responses described here are generated in a DD3X-MAVS-TBK1-IRF3-dependent manner (27). Previous studies have shown that knockdown of DD3X or the generation of phosphorylation-deficient DD3X mutants in cell lines resulted in TBK1/IKKe-dependent decrease of the IFNB promoter activity, providing evidence that DD3X is involved in the induction of IFNB transcription (24, 25). In line with the previous reported data obtained in cell lines, we have shown that DD3X induces type I IFN responses in monocyte-derived DCs as well as primary monocytes, monocyte-derived macrophages and primary human CD1c+ DCs.

Synthetic abortive HIV-1 Cap-RNA58 contains a 5′cap and a secondary TAR loop structure and is a ligand for DD3X, which leads to the induction of type I IFN responses (27). The HIV-1 Cap-RNA58-induced type I IFN responses were similar if not stronger than those observed by poly-L:C, which triggers RIG-I and MDA5. RIG-I and MDA5 distinguish the recognition of their viral ligands based on RNA structure and length. RIG-I recognizes ssRNAs and short dsRNAs, whereas MDA5 recognizes longer dsRNAs (42). Soto-Rifo et al. have described that DD3X recognizes viral RNA constructs due to the presence of a 5′cap in close proximity to a complex secondary structure (30). Whether DD3X is able to distinguish between different viral RNA lengths and adapts subsequent immune activation is unknown. Here we aimed to assess whether differences in HIV-1 Cap-RNA construct length would lead to varying immune activation levels. We observed that the length of the synthetic viral ligand did not affect the strength of the antiviral type I IFN responses as both HIV-1 Cap-RNA58 and HIV-1 Cap-RNA630...
induced similar levels of IFNB and ISG mRNA expression. Similar levels of pro-inflammatory cytokines were also observed. Whereas, HIV-1 Cap-TAR58 induced type I IFN responses, HIV-1 control RNA did not result in IFNB mRNA expression, indicating that the 5’cap is required for recognition by DDX3 and subsequent induction of antiviral responses. Thus, HIV-1 Cap-RNAs induces potent type I IFN in DCS via DDX3 and MAVS-dependent signaling, due to the presence of a 5’cap and complex secondary TAR loop structure.

Besides type I IFN responses, HIV-1 Cap-RNAs8 resulted in increased upregulation of costimulatory molecules CD80, CD83, and CD86. Furthermore, HIV-1 Cap-RNA58 enhanced expression of HLA-DR but not CD40. Our data further indicate that DC maturation is dependent on both type I IFN and NF-kB activation. It remains to be established whether the effect of NF-kB is mediated via IFNβ or that other cytokines activated by NF-kB further affect DC maturation in combination with IFNβ. It has previously been described for various cell lines.
that DDX3 expression knockdown results in decreased NF-κB p65 phosphorylation and cytokine responses suggesting that DDX3 plays a stimulatory role in NF-κB signaling (43). In addition, Ku et al. (44) described that in THP-1-differentiated macrophages DDX3 is important for TNF, IL-1β, CCL2, and CCL5 expression as knockdown of DDX3 expression impaired cytokine and chemokine expression in response to LPS and poly-I:C stimulations. Besides affecting pro-inflammatory cytokine responses, DDX3 knockdown also led to impaired migration and phagocytic capacities of THP-1-differentiated macrophages (44). Although it is unclear whether these functions can be induced by viral ligands, these data potentially imply that DDX3 could be involved in orchestrating various important functions in DCs. Our data underscore the importance of DDX3 as a viral sensor important for the induction of antiviral immunity.

We observed that HIV-1 Cap-RNA58 induced expression of pro-inflammatory cytokines IL-6 and TNF, which are important for both innate and adaptive immune responses. Furthermore, HIV-1 Cap-RNA58 induced *IL12A* but not *IL12B* mRNA expression which resulted in the absence of heterodimeric IL-12p70 protein that is crucial for the induction of *T*H1 differentiation. The observed lack of IL-12p70 upon stimulation with HIV-1 Cap-RNA58 might explain skewing of T helper differentiation toward a *T*H2 phenotype by HIV-1 Cap-RNA58-treated DCs. Furthermore, we observed HIV-1 Cap-RNA58-dependent induction of *IL27A*, encoding for one of the subunits of heterodimeric IL-27 protein, important in the induction of follicular T helper (*T*FH) cells (45). Both *T*H2 and *T*FH cells are important for the induction of antibody responses against invading pathogens including viruses (46). *T*FH cells are important for the formation and maintenance of germinal centers (GCs) and subsequent differentiation of B cells in GCs (46). Once a B cell exits the GC, *T*H2-induced IL-4 production can direct class switching from immunoglobulin G (IgG) to IgE antibodies (47, 48). Recent studies show that *T*FH responses are required to induce broadly neutralizing antibodies against HIV-1 (49–51). Although it is unclear yet whether HIV-1 Cap-RNA58 induces a *T*FH phenotype, the cytokine responses and

---

**FIGURE 7** | HIV-1 Cap-RNA58 reduces ongoing HIV-1 infection in DCs. (A,B) DCs were infected with NL4.3 BaL for 24 h, washed and incubated with 1 nM HIV-1 control RNA, 1 nM HIV-1 Cap-RNA58 or LPS. After 5 days, intracellular p24 levels were measured by flow cytometry. Data are representative of four donors (A,B). (mean ± s.d.). *P < 0.05, ***P < 0.001, Student’s t-test. NS, not significant.
T<sub>H</sub>2 differentiation suggest that HIV-1 Cap-RNA<sub>58</sub> can be useful in vaccines to induce neutralizing antibodies against HIV-1 or other viruses.

Microbial LPS is increased in serum of HIV-1 infected individuals due to intestinal damage upon CD4<sup>+</sup> T cell depletion. LPS as a potent immunostimulatory compound could be involved in inflammatory responses during chronic phase of infection (52, 53). Interestingly, several studies suggest that HIV-1 replication in latent infected cells produces short abortive RNAs such as the DDX3 ligand HIV-1 Cap-RNA<sub>58</sub> (54, 55). Our study suggest that these abortive RNAs can induce inflammatory responses. Thus, besides increased translocation of microbial LPS, the production of HIV-1 Cap-RNA<sub>58</sub> in latent infected cells can also contribute to immune activation observed in HIV-1 infected individuals during chronic phase of infection.

In conclusion, DDX3 is a highly versatile protein involved in a multitude of cellular processes. During HIV-1 infection, the dual role of DDX3 in exerting both proviral and antiviral capacities provides insight into its complexity and to the various roles DDX3 might play in establishing immunity. Here we have identified the antiviral role of DDX3 upon sensing of a viral-derived RNA and how its ligands can be used as adjuvants. Our data strongly indicate that HIV-1 Cap-RNA<sub>58</sub> induces potent antiviral innate and adaptive immune responses in human DCs or directed by human DCs. Our data shows that DDX3 is a pattern recognition receptor and its synthetic ligands can be used as adjuvants to induce potent immune responses.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**REFERENCES**

1. Alcami A, Koszinowski UH. Viral mechanisms of immune evasion. *Trends Microbiol.* (2000) 8:410–8. doi: 10.1016/S0966-842X(00)01830-8
2. Hou B, Reizis B, DeFranco AL. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinscic and -extrinsic mechanisms. *Immunity.* (2008) 29:272–82. doi: 10.1016/j.immuni.2008.05.016
3. Spörri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD<sup>+</sup> T cell populations lacking helper function. *Nat Immunol.* (2005) 6:163–70. doi: 10.1038/nili162
4. Kratky W, Reis e Sousa C, Oxenius A, Spörri R. Direct activation of antigen-presenting cells is required for CD8<sup>+</sup> T-cell priming and tumor vaccination. *Proc Natl Acad Sci USA.* (2011) 108:17414–9. doi: 10.1073/pnas.1108945108
5. Loo YM, Gale M. Immune signaling by RIG-I-like receptors. *Immunity.* (2011) 34:680–92. doi: 10.1016/j.immuni.2011.05.003
6. Chen N, Xia P, Li S, Zhang T, Wang TT, Zhu J. RNA sensors of the innate immune system and their detection of pathogens. *IUBMB Life.* (2017) 69:297–304. doi: 10.1002/iub.1625
7. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-1 helicases in the recognition of RNA viruses. *Nature.* (2006) 441:101–5. doi: 10.1038/nature04734
8. Frederiksen BL, Keller BC, Formek J, Katze MG, Gale M. Establishment and maintenance of the innate antiviral response to west nile virus infection involves both RIG-I and MDA5 signaling through IPS-1. *J Virol.* (2008) 82:609–16. doi: 10.1128/JVI.01305-07
9. Sprokholt JK, Kaptein TM, van Hamme JL, Overmars RJ, Gringhuis SI, Geijtenbeek TBH. RIG-I-like receptor triggering by dengue virus drives dendritic cell immune activation and T<sub>H</sub>1 differentiation. *J Immunol.* (2017) 198:4764–71. doi: 10.4049/jimmunol.1602121
10. Pichlmair A, Schulz O, Tan CP, Naslund TJ, Liljestrom P, Weber F, et al. RIG-I-Mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science.* (2006) 314:997–1001. doi: 10.1126/science.1132998
11. Hornung V, Kato H, Poec H, Akira S, Conzelmann K, Schlee M. 5'-Triphosphate RNA is the ligand for RIG-I. *Science.* (2006) 314:994–7. doi: 10.1126/science.1132505
12. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol.* (2005) 6:981–8. doi: 10.1038/nili1243
13. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF3. *Cell.* (2005) 122:669–82. doi: 10.1016/j.cell.2005.08.012
14. Larner AC, Jonakt G, Chengt YSE, Korant B, Knightt E, Darnell JE. Transcriptional induction of two genes in human cells by beta interferon. *Proc Natl Acad Sci USA.* (1984) 81:6733–7. doi: 10.1073/pnas.81.21.6733
15. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol.* (2011) 1:519–25. doi: 10.1016/j.covirol.2011.10.008
16. Goujon C, Moncorgé O, Bauby H, Doyle T, Ward CC, Schaller T, et al. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature.* (2013) 502:559–62. doi: 10.1038/nature12542
17. Kane M, Yadav SS, Bitzegio J, Kuthiyu SB, Zang T, Wilson SJ, et al. Human MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature.* (2013) 502:563–6. doi: 10.1038/nature12653

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Amsterdam University Medical Centers, location AMC Medical Ethics Committee according to the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

MS designed, performed, and interpreted most experiments and prepared the manuscript. JS helped with study design. JH performed HIV-1 infections and subsequent FACS analyses. TK and EZ-W performed nuclear extract isolations. SG helped with study design and interpretation of data. TG supervised all aspects of this study.

**FUNDING**

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

**ACKNOWLEDGMENTS**

We thank the Host Defense group members for their discussions and input on the data.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00008/full#supplementary-material
18. Yan N, Chen ZJ. Intrinsic antiviral immunity. Nat Immunol. (2012) 13:214–22.
doi: 10.1038/ni.2229

19. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. (2017) 2:17023.
doi: 10.1038/sigtrans.2017.23

20. de Groot R, van Beelen AJ, Bakdash G, van der Drift M, Kapsenberg ML. Viral dsRNA-activated human dendritic cells produce IL-27, which selectively promotes cytotoxicity in naive CD8+ T cells. J Leukoc Biol. (2012) 92:605–10.
doi: 10.1189/jlb.1120145

21. Goriely S, Nguyen M, Albarani V, Haddou NO, Lin R, De Wit D, et al. Interferon regulatory factor 3 is involved in toll-like receptor 4 (TLR4)– and TLR3-induced IL-12p35 gene activation. Blood. (2006) 107:1078–84.
doi: 10.1182/blood-2005-06-2416

22. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-κB-containing half-site. Mol Cell Biol. (1995) 15:5258–67.
doi: 10.1128/MCB.15.10.5258

23. Hayden MS, West AP, Ghosh S. NF-κB and the immune response. Oncogene. (2006) 25:6758–80.
doi: 10.1038/sj.onc.1209943

24. Soulat D, Bürckstümmer T, Westermayer S, Goncalves A, Bauch A, Stefanovic A, et al. The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. EMBO J. (2008) 27:2135–46.
doi: 10.1038/emboj.2008.126

25. Schröder M, Baran M, Bowie AG. Viral targeting of DEAD box protein 1 couples ID2 kinase e to interferon regulatory factor 3 activation. Mol Biol Cell. (2013) 23:2004–15.
doi: 10.1091/mbc.e12-06-0482

26. Gu L, Fullam A, Brennan R, Schroder M, Human DEAD box helicase 3 couples IκB kinase to interferon regulatory factor 3 activation. Mol Biol Cell. (2008) 19:2147–57.
doi: 10.1091/mbc.e08-04-0405

27. Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, et al. DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol. (2007) 81:13922–6.
doi: 10.1128/JVI.01517-07

28. Warr J, Ryu WS. Hepatitis B virus polymerase blocks pattern recognition receptor signaling via interaction with DDX3: implications for immune evasion. PLoS Pathog. (2010) 6:1–11.
doi: 10.1371/journal.ppat.1000986

29. Liu L, Fullam A, Brennan R, Schroder M, Human DEAD box helicase 3 couples IκB kinase e to interferon regulatory factor 3 activation. Mol Biol Cell. (2013) 23:2004–15.
doi: 10.1091/mbc.e12-06-0482

30. Stunnenberg M, Geijtenbeek TBH. HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. Nat Immunol. (2007) 8:13922–6.
doi: 10.1128/JVI.01517-07

31. Stunnenberg and Geijtenbeek. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC-BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.