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RNA-DNA hybrids and ssDNA differ in intracellular half-life and toll-like receptor 9 activation

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ABSTRACT

The innate immune system senses viral and bacterial RNA or DNA via different cytoplasmic or endosomal localized pattern recognition receptors. In general, the preference of these receptors for single-stranded (ss), double-stranded (ds) RNA or DNA has been thoroughly characterized. Recently, RNA-DNA hybrids have also been identified as ligands for pattern recognition receptors such as Toll-like receptor 9 (TLR9). However, a comparison of RNA-DNA hybrids and ssDNA in terms of TLR9 stimulation potential and intracellular stability has not been addressed. RNA-DNA hybrids are formed transiently during normal cellular processes (e.g. replication), consist as part of some viral genomes (e.g. cytomegalovirus (CMV) or hepatitis B virus (HBV)) and exist during retroviral infection. Here we report that virus-derived synthetic RNA-DNA hybrids stimulate human peripheral blood mononuclear cells (PBMCs) as well as murine FMS-like tyrosine kinase 3 ligand (FLT3L) induced dendritic cells to secrete interferon alpha (IFN-α) in a TLR9-dependent manner. Furthermore, we could show that RNA-DNA hybrids exhibit increased intracellular stability, which correlates with enhanced activation of TLR9 in comparison to corresponding ssDNA. Overall, these data suggest a prominent role for TLR9 in the immune recognition of RNA-DNA hybrids in retroviral and CMV infection.

1. Introduction

The innate immune system relies on germline-encoded pattern recognition receptors (PRRs) to sense pathogen-derived molecules and to initiate an appropriate immune response. Bacterial and viral RNA or DNA are prominent target structures that are recognized by cytoplasmic and endosomal/lysosomal PRRs leading to cytokine production and cellular activation (Barbalat et al., 2011; Schlee and Hartmann, 2016). In the cytoplasm, receptors such as the RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) recognize viral and bacterial RNA (Kato et al., 2006; Monroe et al., 2009). Of note, 5'-triphosphate-RNA and high molecular weight viral RNA are very potent ligands for RIG-I or MDA5, respectively (Hornung et al., 2006; Pichlmair et al., 2006, 2009). The receptor absent in melanoma 2 (AIM2) is activated by double-stranded DNA leading to IL-1 production (Hornung et al., 2009; Ishikawa et al., 2009). Cytoplasmic DNA driven IFN-α production is mediated by the nucleotidyl transferase cGAMP synthase (cGAS) that upon interaction with DNA synthesizes a 2'-5'-linked cyclic dinucleotide second messenger leading to the activation of stimulator of interferon genes (STING) (Ablasser et al., 2013; Gao et al., 2013; Ishikawa et al., 2009; Sun et al., 2013; Wu et al., 2013).

In the endosome/lysosome a subgroup of the Toll-like receptor (TLR) family that consists of 13 members recognizes DNA and RNA (Barbalat et al., 2011). Single-stranded RNA is recognized by TLR7, TLR8 and TLR13 (Diebold et al., 2004; Heil et al., 2004; Oldenburg et al., 2012), whereas double-stranded RNA is sensed by TLR3 and TLR7 (Alexopoulou et al., 2001; Hornung et al., 2005). In contrast, TLR9 is activated by single-stranded synthetic oligodeoxynucleotides (ODNs) and bacterial genomic DNA containing a non-methylated sequence motif termed CpG (Bauer et al., 2001; Dalpke et al., 2006; Hemmi et al., 2000). However, under circumstances of enhanced DNA uptake and subsequent ligand availability also non CpG-motif containing oligodeoxnucleotides and self-DNA are immunostimulatory (Boule et al., 2004; Yasuda et al., 2005). Furthermore, RNA-DNA hybrids containing a 30mer GU-repetitive RNA-strand and a 30mer CA-repetitive DNA-strand as well as RNA-DNA hybrids containing a sequence of the HIV-1 group-associated antigen (gag) have been described to activate immune cells via TLR9 (Rigby et al., 2014). Additionally cytoplasmic RNA-DNA hybrids consisting of poly(rA) and

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poly(dT) activate the cGAS-STING axis (Mankan et al., 2014) and bacterial RNA-DNA hybrids stimulate the NLRP3 inflammasome complex (Kailasan Vanaja et al., 2014). In addition, also self-RNA when complexed to antimicrobial peptides or bound in antibody immune complexes triggers TLR7 and TLR8 (Ganguly et al., 2009; Roers et al., 2016; Vollmer et al., 2005). Under these conditions type I interferon is induced by dendritic cells and therefore possibly sustains autoimmune diseases such as systemic lupus erythematosus (SLE) (Christensen et al., 2006; Savarese et al., 2006). RNA-DNA hybrids usually are formed transiently during DNA replication, telomere elongation, transcription and reverse transcription of endogenous retroelements as well as during reverse transcription in the infection cycle of retroviruses such as HIV or murine retroviruses (Varmus, 1988). RNA-DNA hybrids are also part of the genomic structure of some viruses such as human cytomegalovirus (HCMV) (Prichard et al., 1998) and hepatitis B virus (HBV) (Miller et al., 1984).

To investigate the immunostimulatory capacity of RNA-DNA hybrids, we selected RNA-DNA hybrid sequences from the origin of replication of HCMV (Prichard et al., 1998) and the polypurine tract from HIV-1 (Powell and Levin, 1996), which naturally obtain RNA-DNA hybrids. Importantly, RNA-DNA hybrids stimulate human PBMCs and murine FLT3L-induced dendritic cells in a TLR9-dependent manner to secrete IFN-α. Interestingly, IFN-α secretion in response to RNA-DNA hybrids is more robust than IFN-α secretion induced by the corresponding ssDNA and this effect is mediated by an enhanced intracellular stability of RNA-DNA hybrids in comparison to ssDNA. These observations support an important role of TLR9 in sensing RNA-DNA hybrids in retroviral and CMV infection.

2. Materials and methods

2.1. Ethics statement

The local ethics committees of Justus-Liebig-University Gießen and Philipps-University Marburg approved the use of human blood samples for this study. For experiments with murine immune cells, mice were sacrificed and tissue/organs removed. These experiments were performed in accordance with the National German welfare law §4 (3) TierSchG and §2 and Annex 2 (TierSchVerV) of the National Order for the use of animals in research and do not need the approval by a local ethics committee. According to the regulations, the number of mice used was reported to the animal welfare officer of the Philipps-University Marburg.

2.2. Reagents

The sequence of CpG-ODN 2216 (5'-GsGsGGGACGATC GTCsGsGsGsGsGsG-3') where 's' depicts a phosphorothioate linkage was synthesized by TIB MOLBIOL (Berlin, Germany). RNA40 (5'-GCC CGUCUGUUGUGUGACUC), RNA- and DNA- oligonucleotides were synthesized by IBA (Göttingen, Germany). Phosphodiester RNA- and DNA-oligonucleotides used in this study for RNA-DNA hybrid generation are depicted in Table 1. DOTAP was purchased from Carl Roth (Karlsruhe, Germany) for murine cells or Roche (Darmstadt, Germany) for human cells.

FLT3-ligand (FLT3L) was prepared from an FLT3L-secreting cell line (H. Hochrein, Bavarian Nordic GmbH, Martinsried, Germany)

2.3. Annealing of RNA-DNA hybrids

Equal molar amounts of complementary oligonucleotides were combined in annealing buffer (20 mM HEPES, 150 mM NaCl) resulting in a final concentration of 20 μM of double-stranded nucleic acids. The solution was heated up to 95 °C for ten minutes and cooled down to room temperature. Hybrid formation was verified by 15% non-denaturing polyacrylamide gel-electrophoresis or via detection by the RNA-DNA hybrid-specific antibody S9.6(HB-8730, ATCC).

2.4. Melting curve analysis

Double-stranded nucleic acids were stained with SybrGold (Thermo Fisher Scientific, Waltham, USA) after dilution in buffer containing physiological pH 7.4 or acidic pH 5.5. Melting curve analysis were performed with the MiniOpticon System (Bio-Rad, Hercules, California, USA).

2.5. Cells

FLT3L-induced mixed cultures of murine myeloid and plasmacytoid dendritic cells were grown as described (Spies et al., 2003). Shortly, murine bone marrow cells were seeded at 1.5×10^6 cells/ml in Opti-MEM (Thermo Fisher Scientific, Waltham, USA) supplemented with 0.05 mM β-mercaptoethanol (Thermo Fisher Scientific, Waltham, USA), 100 U/ml penicillin (PAA, Cölbe, Germany), 100 μg/ml streptomycin (PAA, Cölbe, Germany), 1% FCS (Biochrom AG, Berlin, Germany) and cultured with the FLT3L containing supernatant in a 1:250 dilution for 7 days.

Human PBMCs were isolated by Ficoll density gradient centrifugation. Therefore lymphocyte separation medium LSM1077 (PAA, Cölbe, Germany) was used according to the manufacturer's recommendation. PBMCs were cultured in RPMI1640 (PAA, Cölbe, Germany) supplemented with 2 mM L-glutamine (PAA, Cölbe, Germany), 100 U/ml penicillin (PAA, Cölbe, Germany), 100 μg/ml streptomycin (PAA, Cölbe, Germany), 1 mM sodium pyruvat solution (PAA, Cölbe, Germany), 1% non-essential amino acids (PAA, Cöbe, Germany), 2% human AB-serum.

2.6. Mice

TLR7- and TLR9-deficient mice were established as described and have been backcrossed to C57BL/6 mice for at least 12 generations (Hemmi et al., 2000). C57BL/6 wild type mice were from Harlan (Borchen, Germany). Mice were kept under specific pathogen free conditions in the animal facility of the Philipps-University Marburg at the biomedical research center.

2.7. Retroviral infection of FLT3L-induced DCs

FLT3L-induced DCs were spin-infected with MuLV pZAP (GFP-tagged Moloney-MuLV) (Logg et al., 2001) on day 5 of differentiation. 2×10^6 cells were incubated with 0.175×10^6 IU/ml of MuLV pZAP and polybrene in a final concentration of 6.6 μg/ml and centrifuged at 32 °C for 90 min at 400 g. After 3 additional days of differentiation cells were analyzed for RNA-DNA hybrid formation by intracellular staining with the RNA-DNA hybrid-specific antibody S9.6.

2.8. Cell stimulation

For cell stimulation human PBMCs were seeded at 3×10^5 cells/well and murine FLT3L-induced DCs at 2×10^5 cells/well. Cells were incubated with 1 μM CpG-ODN 2216, 0.75 μM RNA40, RNA- and DNA-oligonucleotides as well as RNA-DNA hybrids at concentrations as indicated for 20 h. All oligonucleotides except CpG-ODN 2216 were complexed to DOTAP. Therefore, oligonucleotides in 50 μl Opti-MEM were mixed with 50 μl DOTAP-solution (final concentration 12.5 μg/ml) and incubated for 10 min at room temperature. An equal volume of complete medium was added to this mixture. 100 μl of primary cells were incubated with 100 μl of stimuli in 96-well microplates. Supernatants were analyzed for IFN-α secretion with reagents from PBL (Piscataway, USA).

2.9. Intracellular staining

Mouse FLT3L-induced DCs were infected with MuLV pZAP or incubated for 1 h with 0.5 μM of appropriate ssRNA, ssDNA and RNA-DNA hybrid complexed to DOTAP as described for cell stimulation. Cells were fixed with 2% paraformaldehyde (Carl Roth, Karlsruhe, Germany), permeabilized with 0.5% saponin (Sigma, München, Germany), blocked with 1% BSA (Carl Roth, Karlsruhe, Germany) and RNA-DNA-hybrids were detected by staining with antibody S9.6 (final concentration 2 μg/ml) and Alexa568-labeled secondary antibody (final concentration 6.7 μg/ml) (Thermo Fisher Scientific, Carlsbad, USA). Nuclei were stained with 0.1 μg/ml DAPI (Merck, Darmstadt, Germany). Pictures were taken with a TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany).

2.10. Analysis of intracellular DNA and RNA-DNA hybrid stability

Murine FLT3L-DCs were incubated with 0.5 μM of Alexa488-labeled ssDNA or Alexa488-labeled RNA-DNA hybrids (whereby the DNA strand was fluorescent-labeled at the 3' end) complexed to DOTAP. After 1 h of incubation, cells were extensively washed to remove free nucleic acids. Cells were analyzed for presence of ssDNA and RNA-DNA hybrids by flow cytometry and PAGE. For flow cytometry, cells were fixed at indicated time points with paraformaldehyde and analyzed with a BD FACSCalibur. For PAGE, cytoplasmic cell extracts were prepared using cytoplasmic extract buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT and 0.25% NP-40). Lysates were analyzed by 15% non-denaturing PAGE and fluorescence labeling was visualized by the ChemiDoc MP System (Bio-Rad, Hercules, California, USA).

3. Results

3.1. Synthetic RNA-DNA hybrids derived from HCMV and HIV stimulate human PBMCs

Since Prichard et al. have described the presence of stable as well as persistent RNA-DNA hybrids, that may serve as primer for lytic-phase DNA replication within a cis-acting origin of replication (oriLyt) of human cytomegalovirus (HCMV) (Prichard et al., 1998), we selected two sequences from this oriLyt (hybrid CMV27 and CMV63) to investigate immune stimulation by RNA-DNA hybrids (Fig. 1A, Table 1). In addition, we selected a sequence from the polypurine tract (PPT) of HIV-1 (hybrid HIVPPT) (Fig. 1A, Table 1). The existence of an RNA-DNA hybrid within this PPT, whose RNA-part acts as the primer for plus-strand DNA synthesis during the reverse transcription process, has been reported (Powell and Levin, 1996). In general, the RNA sequences in these corresponding regions were selected in such a manner that complementary ssDNA containend no classical CpG motif. Corresponding single-stranded (ss) RNA and ssDNA phosphodiester oligonucleotides were annealed at an equal molar ratio and hybrid formation was analyzed by non-denaturing polyacrylamide gel electrophoresis (Fig. 1B). All hybrids showed a uniform banding pattern supporting an efficient hybridization with minor contamination of ssRNA or ssDNA within the preparation (Fig. 1B). We observed a larger band especially for ssRNA HIVPPT in non-denaturing polyacrylamide gel electrophoresis (Fig. 1B). This band is most likely the result of secondary structure formation since it disappeared after hybridization. Hybrid CMV27 formation was also validated with an ELISA utilizing the RNA-DNA hybrid-specific antibody S9.6 (Hu et al., 2006). The antibody only recognized the RNA-DNA hybrid but not the corresponding ssRNA or ssDNA (Fig. 1C). A murine isotype control IgG2a antibody showed no reactivity (Fig. 1C). The additional two hybrids CMV63 and HIVPPT were equally recognized by antibody S9.6 (Fig. 1D). To proof the existence of RNA-DNA hybrids during viral infection, we infected murine FLT3L-DCs with the Moloney murine leukemia virus (MoMuLV) (Logg et al., 2001) and verified the presence of intracellular RNA-DNA hybrids using the RNA-DNA hybrid-specific antibody S9.6 (Fig. 1E). Mock-infected as well as cells stained with the secondary Alexa568-labeled antibody solely served as controls and showed no staining at all. However, we could not detect any type I interferon production by MoMuLV infected murine FLT3L-DCs (data not shown).

For immune stimulation RNA-DNA hybrids and corresponding ssRNA or ssDNA were complexed to the cationic transfection reagent DOTAP that protects RNA from nucleases and facilitates nucleic acid uptake (Capaccioli et al., 1993). Since nucleic acid transfection with DOTAP leads predominantly to endosomal uptake, we determined the stability of the hybrid at acidic pH found in endosomes and lysosomes. Of note, analysis of thermal melting curves at physiological pH 7.4 and pH 5.5 showed similar melting points for RNA-DNA hybrids CMV27, CMV63 and HIVPPT suggesting a stable hybrid composition at acidic pH (Fig. 2A). Stimulation of human PBMCs with ssRNAs, ssDNAs and the corresponding hybrids showed that ssRNA did not or only weakly induce IFN-α. The ssDNA and the RNA-DNA hybrids led to increased IFN-α secretion and interestingly, RNA-DNA hybrids CMV27 and CMV63 induced significantly higher amounts of IFN-α in comparison to the corresponding ssDNAs (Fig. 2B). Hybrid HIVPPT lead to higher amounts of IFN-α production in comparison to ssDNA, but not at significant levels.

In summary, RNA-DNA hybrids are stable at acidic pH and specifically stimulate human PBMCs to secrete IFN-α at higher amounts than the corresponding ssDNAs.

3.2. RNA-DNA hybrids are recognized by TLR9

We further screened RNA-DNA hybrid-driven cytokine production in FLT3L-induced dendritic cells (DCs) from various PRR-deficient mice to identify the corresponding receptor. Importantly, TLR9-deficient DCs did not produce IFN-α upon stimulation with RNA-DNA hybrids CMV27, CMV63 and HIVPPT, whereas WT DCs as well as TLR7-deficient DCs responded strongly (Fig. 3A). The corresponding ssRNA CMV27 and CMV63 were rarely immunostimulatory and surprisingly, ssRNA HIVPPT seems to be detected by TLR9 and not as expected by TLR7. The ssDNA from CMV27 and CMV63 produced IFN-α, albeit at reduced levels, whereas the ssDNA from HIV did not induce any IFN-α secretion (Fig. 3A). The immunostimulatory potential of RNA-DNA hybrids CMV27, CMV63 and HIVPPT to induce IFN-α secretion was significantly higher than that of corresponding ssDNA (Fig. 3A). Hybrids could also be detected in CMV27/DOTAP-transfected FLT3L-induced dendritic cell cultures with the RNA-DNA hybrid-specific antibody S9.6 (Fig. 3B). In contrast, transfection with corresponding ssRNA or ssDNA showed no staining. These findings demonstrate that neither transfection of ssRNA nor transfection of ssDNA leads to intracellular formation of RNA-DNA hybrids with endogenous nucleic acids. Overall, these results demonstrate that RNA-DNA hybrids are detectable within immune cells after transfection and induce IFN-α production in a TLR9-dependent manner.

3.3. Direct effect of ssRNA on hybrid-mediated TLR9 activation

To exclude the possibility that ssRNA somehow affects the mechanism of TLR9 activation by ssDNA without being part of an RNA-DNA hybrid, we stimulated mouse FLT3L-DCs with ssRNA and ssDNA in one DOTAP liposome without prior hybridization or with individually complexed and then combined ssRNA and ssDNA. Stimulation with individually complexed ssRNA and ssDNA lead to similar IFN-α levels compared to ssDNA complexed to DOTAP. Interestingly, ssRNA and ssDNA complexed in one DOTAP liposome stimulated the cells to produce significantly higher amounts of IFN-α compared to individually packaged ssDNA- and ssRNA-liposomes and furthermore this IFN-α secretion was similar to IFN-α production induced by RNA-DNA hybrids (Fig. 4A). These observations gave rise to the assumption that RNA-DNA hybrids were formed when delivered within one DOTAP liposome.

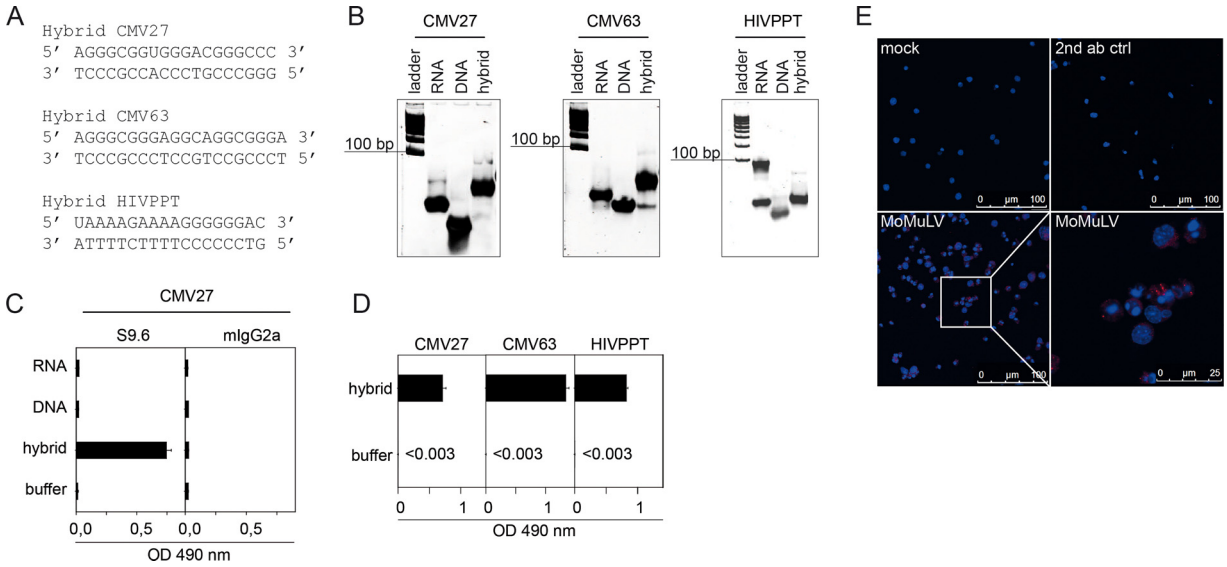

Fig. 1. Formation and detection of RNA-DNA hybrids. (**A**) RNA-DNA hybrid containing sequences of the origin of replication of HCMV and of the polypurine tract of HIV-1. (**B**) Hybrid formation was analyzed by 15% non-denaturing polyacrylamide gel electrophoresis for every individual hybrid preparation. Nucleic acids were detected with SybrGold. The ladder is a 100 bp DNA ladder. One representative gel out of at least five is shown. (**C, D**) Formation of RNA-DNA hybrids was verified using the RNA-DNA hybrid-specific antibody S9.6 in an ELISA based assay. One representative experiment out of at least five is shown. (**E**) RNA-DNA hybrids were detectable within MoMuLV infected FLT3L-induced DCs by the RNA-DNA hybrid-specific antibody S9.6. Nuclei were stained with DAPI (blue), RNA-DNA hybrids were visualized by an Alexa568-labeled secondary antibody against the RNA-DNA hybrid-specific antibody S9.6 (red). Scale bar 100 µm. Scale bar in higher magnification image 25 µm.

Therefore, we incubated murine FLT3L-DCs with the different liposomes and analyzed RNA-DNA hybrid formation by S9.6 staining. Indeed, we found RNA-DNA hybrids within cells incubated with ssRNA and ssDNA in one liposome, whereas we could hardly detect RNA-DNA hybrids within cells stimulated with individually complexed and combined ssRNA and ssDNA (Fig. 4B). In summary, TLR9 activation is mediated by the RNA-DNA hybrid directly.

3.4. RNA-DNA hybrids exhibit enhanced intracellular stability compared to ssDNA within FLT3L-DCs

To address the intracellular stability of RNA-DNA hybrids in comparison to corresponding ssDNA, we transfected murine FLT3L-DCs with Alexa488-labeled RNA-DNA hybrid CMV27 (the ssDNA-strand was labeled at the 3' end) or the corresponding labeled ssDNA. After one hour of incubation, cells were extensively washed to remove free nucleic acids and cells were analyzed for presence of Alexa488-labeled nucleic acids after different time points by flow cytometry (Fig. 5A, B). In general, we detected a transfection efficiency greater than 95% for both ssDNA and the RNA-DNA hybrid and MFIs of 519 for ssDNA and 267 for the RNA-DNA hybrid after one hour of incubation. However, we found that the reduction of fluorescence intensity with time was more dramatic for ssDNA compared to RNA-DNA hybrids and this effect was most prominent after two to three hours of incubation (Fig. 5A, B).

To exclude the possibility that the detected fluorescent-signal is emitted by remaining Alexa488-labeled ssDNA after RNA-degradation within the RNA-DNA hybrid, we prepared cytoplasmic cell extracts of Alexa488-CMV27/DOTAP-transfected FLT3L-DCs, loaded the cytoplasmic extracts onto a non-denaturing polyacrylamide gel and examined intracellular stability of RNA-DNA hybrids and ssDNA. Again, we found an increased half-life for RNA-DNA hybrid CMV27 in comparison to ssDNA CMV27 after three hours of incubation (Fig. 5C). In both preparations, we observed a non-specific band that does not match the ssDNA or RNA-DNA hybrid.

In summary, RNA-DNA hybrid CMV27 exhibits enhanced intracellular stability within FLT3L-DCs in comparison to corresponding ssDNA.

4. Discussion

The innate immune system senses single or double stranded RNA and DNA and RNA-DNA hybrids via different pattern recognition receptors such as RIG-I like helicases, AIM2, nucleotidyl transferase cGAMP synthase (cGAS), STING, Toll-like receptors (TLR) or NOD-like receptors (NLR) (Gurtler and Bowie, 2013; Roers et al., 2016). RNA-DNA hybrids are part of the genome of human cytomegalovirus (HCMV) (Prichard et al., 1998) and hepatitis B virus (HBV) (Miller et al., 1984) and exist temporarily during infection with retroviruses such as human immunodeficiency virus (HIV) or murine leukemia virus (MuLV) (Varmus, 1988). Therefore, RNA-DNA hybrids are bona fide pathogen derived structures with the potential to alert and stimulate the innate immune system. Bacterial and synthetic RNA-DNA hybrids (virus-derived or artificial sequence) have been described to activate TLR9, the cGAS-STING axis or the NLRP3 inflammasome (Kailasan

Table 1
Phosphodiester RNA- and DNA-oligonucleotides used in this study for RNA-DNA hybrid generation.

Name	Sequence	Origin
CMV27_DNA	5'GGGCCCGTCCCACCGCCCT	nt 94182 to 94200, gb_HQ380895.1
CMV27_RNA	5'AGGGCGGUGGGACGGGCCC	complementary to CMV27_DNA
CMV63_DNA	5'TCCCGCCTGCCTCCCGCCCT	nt 94118 to 94137, gb_HQ380895.1
CMV63_RNA	5'AGGGCGGGAGGCAGGCGGGA	complementary to CMV63_DNA
HIVPPT_DNA	5'GTCCCCCCTTTTCTTTTA	complementary to HIVPPT_RNA
HIVPPT_RNA	5'UAAAAGAAAAGGGGGGAC	nt 9098 to 9115, dbj_AB641837.2

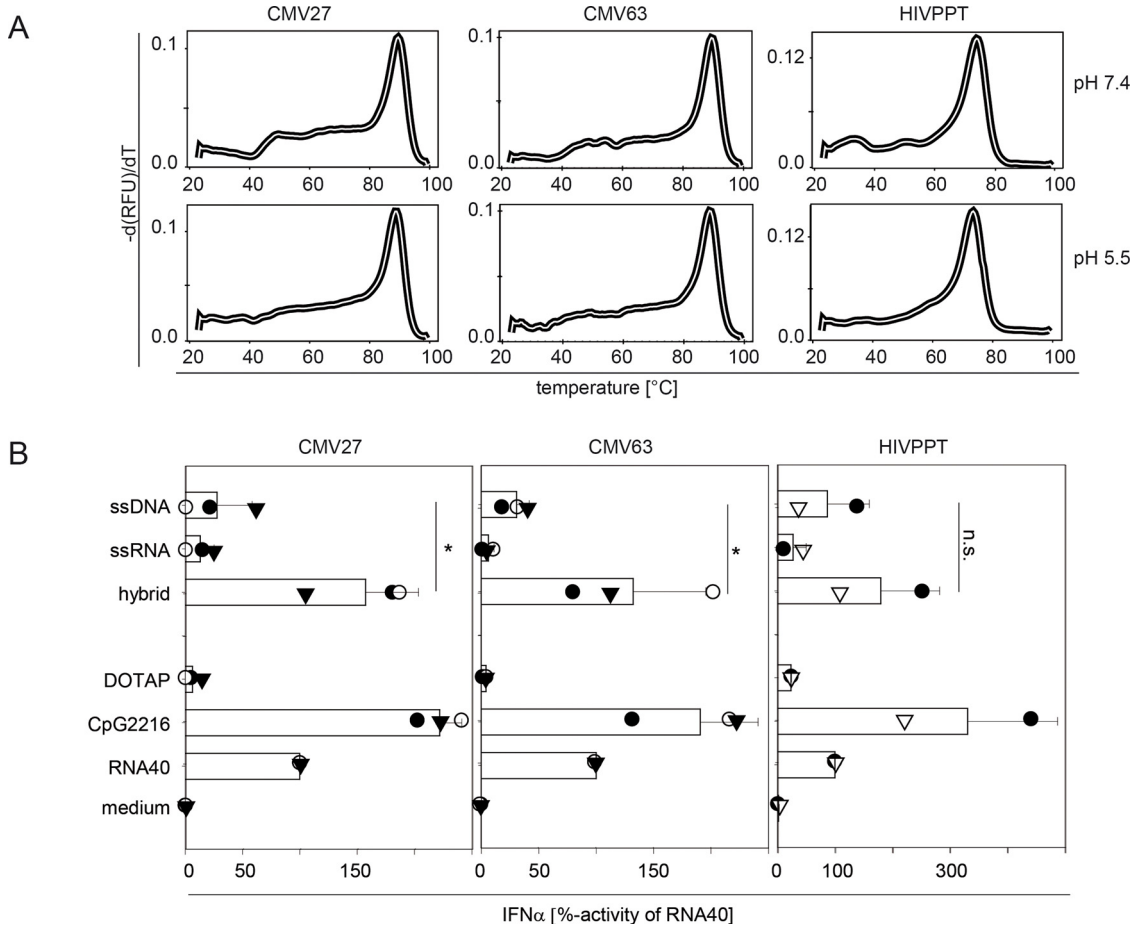

Fig. 2. Synthetic RNA-DNA hybrids derived from HCMV and HIV-1 induce IFN-α production in human PBMCs. (**A**) RNA-DNA hybrids were either diluted in buffer at physiological pH 7.4 or acidic pH 5.5 and stained with SybrGold before melting curve analyses were performed. Two representative melting curves out of ten are shown for RNA-DNA hybrid CMV27, RNA-DNA hybrid CMV63 and RNA-DNA hybrid HIVPPT. (**B**) Human PBMCs were stimulated with 0.125 μM (CMV27 and HIVPPT) or 0.5 μM (CMV63) of RNA-DNA hybrids and corresponding ssRNAs and ssDNAs. Medium, RNA40 (0.75 μM), CpG2216 (1 μM) and DOTAP served as controls. Hybrids and single stranded oligonucleotides (except CpG2216) were complexed to DOTAP and PBMCs were incubated with these stimuli. Supernatants were harvested after 20 h of stimulation and IFN-α was detected by ELISA. IFN-α secretion was normalized to the IFN-α production induced by stimulation with RNA40 (100%). Each symbol represents an individual donor (n = 3 (CMV27), n = 3 (CMV63), n = 2 (HIVPPT)). Data shown are means ± SD. *p ≤ 0.05, n.s. not significant, unpaired t-test.

Vanaja et al., 2014; Mankan et al., 2014; Rigby et al., 2014). Since RNA-DNA hybrids also form during normal cellular processes such as replication, telomere elongation and transcription, aberrant self-RNA or self-DNA clearance may lead to accumulation of endogenous RNA-DNA hybrids that induce cytokines and promote autoimmune diseases such as Aicardi-Goutières syndrome (AGS) (Crow et al., 2006).

Here we describe that TLR9 recognizes synthetic RNA-DNA hybrids derived from HCMV (Prichard et al., 1998) or the polypurine tract of HIV (Fitzgerald and Drohat, 2008) and this recognition leads to enhanced IFN-α secretion in comparison to IFN-α induction mediated by corresponding ssDNA. Additionally, we could show that this IFN-α production is indeed mediated by RNA-DNA hybrids and not due to an influence of ssRNA on ssDNA-inuced TLR9-activation. Furthermore, this enhanced IFN-α secretion correlates with increased intracellular stability of RNA-DNA hybrids. In HCMV a cis-acting lytic origin of DNA replication (oriLyt) contains a bidirectional promoter element and a RNA-DNA hybrid region that are substrate for the protein UL84 that belongs to core replication proteins for initiating lytic replication (Pari, 2008). Importantly, TLR9 has been described as pattern recognition receptor of CMV since mice with non-functional TLR9 are more susceptible to mouse CMV infection and show impaired production of type I interferon or NK cell activity (Krug et al., 2004; Tabeta et al., 2004). Our data therefore support the hypothesis that the conserved and for replication essential RNA-DNA hybrid could serve as a specific pattern to sense CMV infection. Interestingly, two polymorphisms in TLR9 (-1486 T/C and 2848C/T SNPs) are associated with CMV infections in infants (Paradowska et al., 2016).

During the life cycle of retroviruses, single stranded viral RNA is converted into double stranded DNA by reverse transcriptase (RT). The minus-strand DNA synthesis is primed by host-derived tRNA, whereas the plus-strand synthesis is initiated from a purine-rich region termed the polypurine tract (PPT) (Fitzgerald and Drohat, 2008). Accordingly, this region resists digestion by RNase H and could serve as a unique structure for immune sensing. Of note, we could demonstrate a TLR9 dependent type I interferon production by a synthetic RNA-DNA hybrid mimicking the PPT. Furthermore, RNA-DNA hybrids are formed within murine FLT3L-DCs upon infection with MoMuLV. However, the immunostimulatory potential of these RNA-DNA hybrids within the cell could not be further addressed since MoMuLV and MoMuLV-based retroviral replicating vectors do not induce type I interferon and may also act as IFN antagonist (data not shown and (Lin et al., 2014)). Of note, in case of the synthetic HIVPPT RNA-DNA hybrid, we observed that also the corresponding ssRNA itself induced IFN-α in a TLR9-dependent manner. This observation is somewhat surprising since TLR7 would be the prime candidate to recognize ssRNA (Heil et al., 2004). We could rule out that the HIVPPT RNA binds small complementary

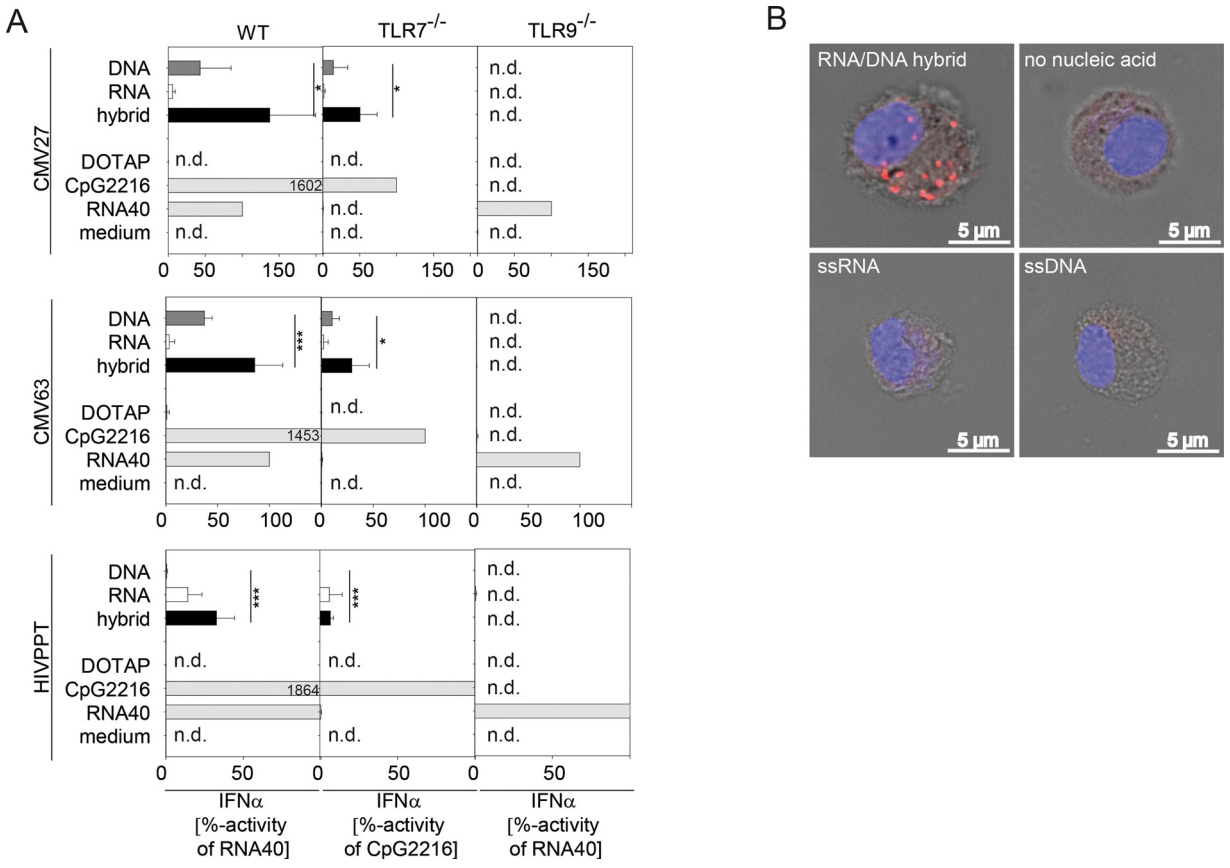

Fig. 3. RNA-DNA hybrids induce IFN-α production in a TLR9-dependent manner and can be detected intracellularly by fluorescence microscopy. (**A**) WT, TLR7- and TLR9-deficient FLT3L-induced DCs were stimulated with 0.5 µM RNA-DNA hybrids (black bars), ssRNA (white bars) or ssDNA (grey bars). Medium, RNA40 (0.75 µM), CpG2216 (1 µM) and DOTAP served as controls (light grey bars). Stimuli (except CpG2216) were complexed to DOTAP and IFN-α production was detected by ELISA after 20 h of incubation. IFN-α secretion was normalized to the IFN-α production induced by stimulation with RNA40 (100%) for wt and TLR9-deficient FLT3L-induced DCs or CpG2216 (100%) for TLR7-deficient FLT3L-induced DCs. n.d.: not detectable, < 8 U/ml. Data shown are means from six (CMV27, wt and TLR9-deficient), five (CMV27, TLR7-deficient), seven (CMV63, wt), five (CMV63, TLR7-deficient), six (CMV63, TLR9-deficient), five (HIVPPT, wt) or four (HIVPPT, TLR7- and TLR9-deficient) individual experiments ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-test. (**B**) FLT3L-induced DCs were transfected with 0.5 µM RNA-DNA hybrid CMV27, its corresponding ssRNA or ssDNA complexed to DOTAP. After paraformaldehyde fixation, cells were permeabilized and RNA-DNA hybrids were stained with antibody S9.6 followed by a secondary Alexa568-labeled antibody (red). Nuclei were stained with DAPI (blue). Cells were examined by confocal laser scanning microscopy. Scale bar 5 µm.

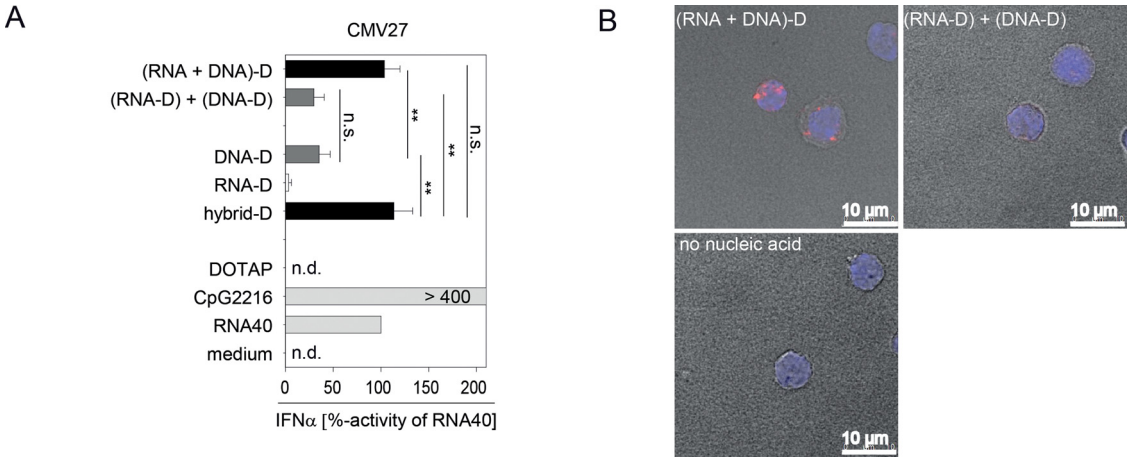

Fig. 4. Direct effect of ssRNA on hybrid-mediated TLR9 activation. (**A**) Murine FLT3L-DC of WT mice were stimulated with 0.5 µM of nucleic acids (CMV27) complexed to DOTAP (D) (hybrid-D, RNA-D, DNA-D). ssRNA and ssDNA were mixed without initial temperature-induced annealing ((RNA + DNA)-D) or ssRNA and ssDNA were complexed to DOTAP separately ((RNA-D) + (DNA-D)). Medium, RNA40 (0.75 µM), CpG2216 (1 µM) and DOTAP served as controls (light grey bars). IFN-α production was detected by ELISA after 20 h of incubation. IFN-α secretion was normalized to the IFN-α production induced by stimulation with RNA40 (100%). n.d.: not detectable, < 8 U/ml. Data shown are means from three individual experiments ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-test. (**B**) Mouse FLT3L-induced DCs were transfected as described in (A). After 30 min of incubation, cells were fixed and permeabilized to stain RNA-DNA hybrids with antibody S9.6 followed by a secondary Alexa568-labeled antibody (red). Nuclei were stained with DAPI (blue). Cells were examined by confocal laser scanning microscopy. Scale bar 10 µm.

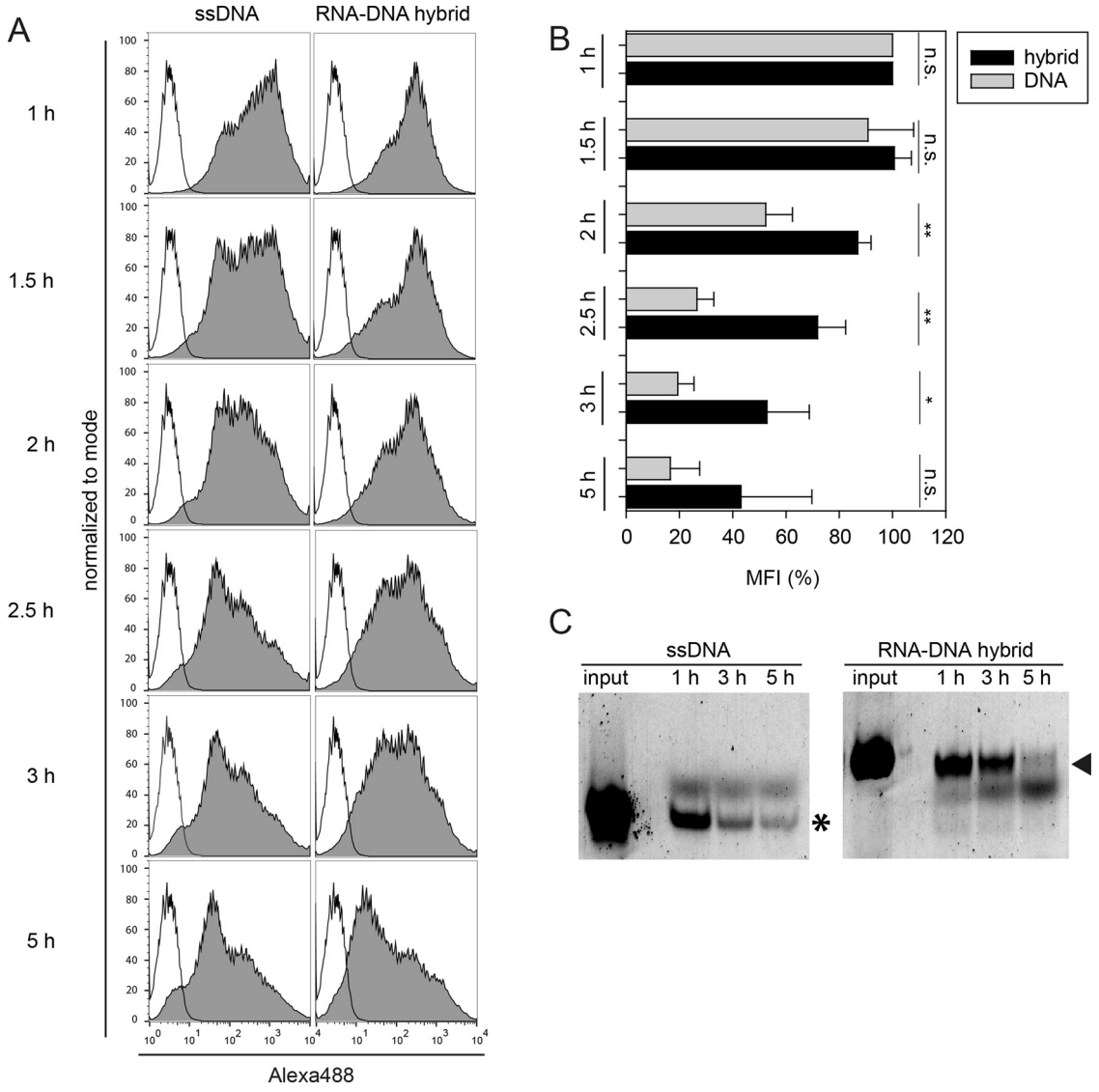

Fig. 5. RNA-DNA hybrids exhibit enhanced intracellular stability compared to ssDNA within FLT3L-DCs. (**A**) FLT3L-DCs were incubated with 0.5 μM Alexa488-labeled ssDNA or RNA-DNA hybrids (filled histograms) from the origin of replication of HCMV (CMV27) complexed to DOTAP. Cells were fixed after indicated time points and analyzed by flow cytometry. Untreated cells served as controls (unfilled histograms). Representative histograms out of three independent experiments are shown. (**B**) Half-life of ssDNA and RNA-DNA hybrids is shown as percent MFI normalized to the average MFI after 1 h of incubation (100%) (n = 3 ± SD). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, n.s. not significant, unpaired t-test. **(C)** FLT3L-DCs were treated as described above. After preparation of cytoplasmic cell extracts, presence of Alexa488-labeled ssDNA or Alexa488-labeled RNA-DNA hybrids was visualized by non-denaturing PAGE. Input represents the amount of ssDNA or RNA-DNA hybrid, which was initially transfected. Asterisk indicates ssDNA and the arrowhead indicates RNA-DNA hybrids. One representative experiment out of three independent experiments is shown.

DNA fragments and forms immunostimulatory RNA-DNA hybrids, since no RNA-DNA hybrids could be detected following ssRNA transfection (data not shown). Interestingly, the HIVPPT RNA contains a poly(G) stretch, which forms quadruplex-like structures (Curtis and Liu, 2013) that may influence TLR9 activity. In case of CpG-containing ODNs with a phosphodiester backbone G-quadruplex formation can be used to increase their immunostimulatory activity due to increased ODN uptake and nuclease resistance (Hoshi et al., 2019). However, this does not explain why a RNA with G-quadruplex activates TLR9. Therefore, it will be important to investigate the potential of ssRNA as TLR9 agonist and its dependency on sequence and G-quadruplex in future studies.

In general, a role for TLR9 in HIV-1 mediated immune recognition or pathology is supported by genetic studies. For example, two single nucleotide polymorphisms (SNPs) in TLR9 (1635A/G and +1174 G/A) that are in linkage disequilibrium might have a role in HIV-1 clinical disease progression and risk of mother to child transmission (Bochud et al., 2007; Freguja et al., 2010; Ricci et al., 2012; Soriano-Sarabia et al., 2008). Also in sheep, the TLR9 polymorphism G520R is associated with seropositivity for small ruminant lentivirus (Sarafidou et al., 2013).

Overall, we have demonstrated that RNA-DNA hybrids induce IFN-α production in a TLR9 dependent manner, which is more robust in comparison to IFN-α secretion in response to corresponding ssDNA. This enhanced IFN-α production is most likely a result of increased intracellular stability of RNA-DNA hybrids compared to corresponding ssDNA. Thus, RNA-DNA hybrids serve as a pathogen-derived structure for sensing infection.

Author contributions

HLO and IE performed experiments. HLO, PY and AK conceived or designed the experiments. HLO, AK and SB analyzed the data and HLO,

AK and SB wrote the manuscript. SB and HLO conceived and supervised the study.

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Declaration of Competing Interest

None.

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References

Ablasser, A., Goldeck, M., Cavlar, T., Deimling, T., Witte, G., Rohl, I., Hopfner, K.P., Ludwig, J., Hornung, V., 2013. cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING. Nature 498, 380.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732.

Barbalat, R., Ewald, S.E., Mouchess, M.L., Barton, G.M., 2011. Nucleic acid recognition by the innate immune system. Annu. Rev. Immunol. 29, 185.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A. 98, 9237.

Bochud, P.Y., Hersberger, M., Taffe, P., Bochud, M., Stein, C.M., Rodrigues, S.D., Calandra, T., Francioli, P., Telenti, A., Speck, R.F., Aderem, A., 2007. Polymorphisms in Toll-like receptor 9 influence the clinical course of HIV-1 infection. Aids 21, 441.

Boule, M.W., Broughton, C., Mackay, F., Akira, S., Marshak-Rothstein, A., Rifkin, I.R., 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. J. Exp. Med. 199, 1631.

Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T., Quattrone, A., 1993. Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. Biochem. Biophys. Res. Commun. 197, 818.

Christensen, S.R., Shupe, J., Nickerson, K., Kashgarian, M., Flavell, R.A., Shlomchik, M.J., 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 25, 417.

Crow, Y.J., Leitch, A., Hayward, B.E., Garner, A., Parmar, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., Baumann, C., Baxter, P., Bertini, E., Chandler, K.E., Chitayat, D., Cau, D., Dery, C., Fazzi, E., Goizet, C., King, M.D., Klepper, J., Lacombe, D., Lanzi, G., Lyall, H., Martinez-Frias, M.L., Mathieu, M., McKeown, C., Monier, A., Oade, Y., Quarrell, O.W., Rittey, C.D., Rogers, R.C., Sanchis, A., Stephenson, J.B., Tacke, U., Till, M., Tolmie, J.L., Tomlin, P., Voit, T., Weschke, B., Woods, C.G., Lebon, P., Bonthron, D.T., Ponting, C.P., Jackson, A.P., 2006. Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection. Nat. Genet. 38, 910.

Curtis, E.A., Liu, D.R., 2013. Discovery of widespread GTP-binding motifs in genomic DNA and RNA. Chem. Biol. 20, 521.

Dalpke, A., Frank, J., Peter, M., Heeg, K., 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. Infect. Immun. 74, 940.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529.

Fitzgerald, M.E., Drohat, A.C., 2008. Structural studies of RNA/DNA polypurine tracts. Chem. Biol. 15, 203.

Freguja, R., Gianesin, K., Del Bianco, P., Malacrida, S., Rampon, O., Zanchetta, M., Giaquinto, C., De Rossi, A., 2010. Polymorphisms of innate immunity genes influence disease progression in HIV-1-infected children. Aids 26, 765.

Ganguly, D., Chamilos, G., Lande, R., Gregorio, J., Meller, S., Facchinetti, V., Homey, B., Barrat, F.J., Zal, T., Gilliet, M., 2009. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. J. Exp. Med. 206, 1983.

Gao, P., Ascano, M., Wu, Y., Barchet, W., Gaffney, B.L., Zillinger, T., Serganov, A.A., Liu, Y., Jones, R.A., Hartmann, G., Tuschl, T., Patel, D.J., 2013. Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. Cell 153, 1094.

Gurtler, C., Bowie, A.G., 2013. Innate immune detection of microbial nucleic acids. Trends Microbiol.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A toll-like receptor recognizes bacterial DNA. Nature 408, 740.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., Fitzgerald, K.A., 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., Hartmann, G., 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314, 994.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A., Endres, S., Hartmann, G., 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat. Med. 11, 263.

Hoshi, K., Yamazaki, T., Sugiyama, Y., Tsukakoshi, K., Tsugawa, W., Sode, K., Ikebukuro, K., 2019. G-quadruplex structure improves the immunostimulatory effects of cytosine-phosphate-Guanine oligonucleotides. Nucleic Acid Ther.

Hu, Z., Zhang, A., Storz, G., Gottesman, S., Leppla, S.H., 2006. An antibody-based microarray assay for small RNA detection. Nucleic Acids Res. 34, e52.

Ishikawa, H., Ma, Z., Barber, G.N., 2009. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461, 788.

Kailasan Vanaja, S., Rathinam, V.A., Atianand, M.K., Kalantari, P., Skehan, B., Fitzgerald, K.A., Leong, J.M., 2014. Bacterial RNA:DNA hybrids are activators of the NLRP3 inflammasome. Proc. Natl. Acad. Sci. U. S. A. 111, 7765.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101.

Krug, A., French, A.R., Barchet, W., Fischer, J.A., Dzionek, A., Pingel, J.T., Orihuela, M.M., Akira, S., Yokoyama, W.M., Colonna, M., 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity 21, 107.

Lin, A.H., Burrascano, C., Pettersson, P.L., Ibanez, C.E., Gruber, H.E., Jolly, D.J., 2014. Blockade of type I interferon (IFN) production by retroviral replicating vectors and reduced tumor cell responses to IFN likely contribute to tumor selectivity. J. Virol. 88, 10066.

Logg, C.R., Logg, A., Tai, C.K., Cannon, P.M., Kasahara, N., 2001. Genomic stability of murine leukemia viruses containing insertions at the Env-3' untranslated region boundary. J. Virol. 75, 6989.

Mankan, A.K., Schmidt, T., Chauhan, D., Goldeck, M., Honing, K., Gaidt, M., Kubarenko, A.V., Andreeva, L., Hopfner, K.P., Hornung, V., 2014. Cytosolic RNA:DNA hybrids activate the cGAS-STING axis. EMBO J. 33, 2937.

Miller, R.H., Marion, P.L., Robinson, W.S., 1984. Hepatitis B viral DNA-RNA hybrid molecules in particles from infected liver are converted to viral DNA molecules during an endogenous DNA polymerase reaction. Virology 139, 64.

Monroe, K.M., McWhirter, S.M., Vance, R.E., 2009. Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila. PLoS Pathog. 5, e1000665.

Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S., Koedel, U., Akira, S., Kawai, T., Buer, J., Wagner, H., Bauer, S., Hochrein, H., Kirschning, C.J., 2012. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science 337, 1111.

Paradowska, E., Jablonska, A., Studzinska, M., Skowronska, K., Suski, P., Wisniewska-Ligier, M., Wozniakowska-Gesicka, T., Nowakowska, D., Gaj, Z., Wilczynski, J., Lesnikowski, Z.J., 2016. TLR9 -1486T/C and 2848C/T SNPs are associated with human cytomegalovirus infection in infants. PLoS One 11, e0154100.

Pari, G.S., 2008. Nuts and bolts of human cytomegalovirus lytic DNA replication. Curr. Top. Microbiol. Immunol. 325, 153.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., Reis e Sousa, C., 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science 314, 997.

Pichlmair, A., Schulz, O., Tan, C.P., Rehwinkel, J., Kato, H., Takeuchi, O., Akira, S., Way, M., Schiavo, G., Reis e Sousa, C., 2009. Activation of MDA5 requires higher-order RNA structures generated during virus infection. J. Virol. 83, 10761.

Powell, M.D., Levin, J.G., 1996. Sequence and structural determinants required for priming of plus-strand DNA synthesis by the human immunodeficiency virus type 1 polypurine tract. J. Virol. 70, 5288.

Prichard, M.N., Jairath, S., Penfold, M.E., St Jeor, S., Bohlman, M.C., Pari, G.S., 1998. Identification of persistent RNA-DNA hybrid structures within the origin of replication of human cytomegalovirus. J. Virol. 72, 6997.

Ricci, E., Malacrida, S., Zanchetta, M., Mosconi, I., Montagna, M., Giaquinto, C., De Rossi, A., 2012. Toll-like receptor 9 polymorphisms influence mother-to-child transmission of human immunodeficiency virus type 1. J. Transl. Med. 8, 49.

Rigby, R.E., Webb, L.M., Mackenzie, K.J., Li, Y., Leitch, A., Reijns, M.A., Lundie, R.J., Revuelta, A., Davidson, D.J., Diebold, S., Modis, Y., MacDonald, A.S., Jackson, A.P., 2014. RNA:DNA hybrids are a novel molecular pattern sensed by TLR9. EMBO J. 33, 542.

Roers, A., Hiller, B., Hornung, V., 2016. Recognition of endogenous nucleic acids by the innate immune system. Immunity 44, 739.

Sarafidou, T., Stamatis, C., Kalozoumi, G., Spyrou, V., Fthenakis, G.C., Billinis, C.,

Mamuris, Z., 2013. Toll like receptor 9 (TLR9) polymorphism G520R in sheep is associated with seropositivity for Small Ruminant Lentivirus. PLoS One 8, e63901.
Savarese, E., Chae, O.W., Trowitzsch, S., Weber, G., Kastner, B., Akira, S., Wagner, H., Schmid, R.M., Bauer, S., Krug, A., 2006. U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7. Blood 107, 3229.
Schlee, M., Hartmann, G., 2016. Discriminating self from non-self in nucleic acid sensing. Nat. Rev. Immunol. 16, 566.
Soriano-Sarabia, N., Vallejo, A., Ramirez-Lorca, R., Rodriguez Mdel, M., Salinas, A., Pulido, I., Saez, M.E., Leal, M., 2008. Influence of the Toll-like receptor 9 1635A/G polymorphism on the CD4 count, HIV viral load, and clinical progression. J. Acquir. Immune Defic. Syndr. 49, 128.
Spies, B., Hochrein, H., Vabulas, M., Huster, K., Busch, D.H., Schmitz, F., Heit, A., Wagner, H., 2003. Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J. Immunol. 171, 5908.
Sun, L., Wu, J., Du, F., Chen, X., Chen, Z.J., 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339, 786.
Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L., Flavell, R.A., Beutler, B., 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl. Acad. Sci. U. S. A. 101, 3516.
Varmus, H., 1988. Retroviruses. Science 240, 1427.
Vollmer, J., Tluk, S., Schmitz, C., Hamm, S., Jurk, M., Forsbach, A., Akira, S., Kelly, K.M., Reeves, W.H., Bauer, S., Krieg, A.M., 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J. Exp. Med. 202, 1575.
Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., Chen, Z.J., 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 339, 826.
Yasuda, K., Yu, P., Kirschning, C.J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., Wagner, H., 2005. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J. Immunol. 174, 6129.