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Multiple Homozygous Variants in the STING-Encoding *TMEM173* Gene in HIV Long-Term Nonprogressors

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Among HIV-infected individuals, long-term nonprogressor (LTNP) patients experience slow CD4 T cell decline and almost undetectable viral load for several years after primary acquisition of HIV. Type I IFN has been suggested to play a pathogenic role in HIV pathogenesis, and therefore diminished IFN responses may underlie the LTNP phenotype. In this study, we examined the presence and possible immunological role of multiple homozygous single-nucleotide polymorphisms in the stimulator of IFN genes (STING) encoding gene *TMEM173* involved in IFN induction and T cell proliferation in HIV LTNP patients. We identified LTNPs through the Danish HIV Cohort and performed genetic analysis by Sanger sequencing, covering the R71H-G230A-R293Q (HAQ) single-nucleotide polymorphisms in *TMEM173*. This was followed by investigation of STING mRNA and protein accumulation as well as innate immune responses and proliferation following STING stimulation and infection with replication-competent HIV in human blood–derived cells. We identified G230A-R293Q/G230A-R293Q and HAQ/HAQ homozygous *TMEM173* variants in 2 out of 11 LTNP patients. None of the 11 noncontrollers on antiretroviral treatment were homozygous for these variants. We found decreased innate immune responses to DNA and HIV as well as reduced STING-dependent inhibition of CD4 T cell proliferation, particularly in the HAQ/HAQ HIV LTNP patients, compared with the age- and gender-matched noncontrollers on antiretroviral treatment. These findings suggest that homozygous HAQ STING variants contribute to reduced inhibition of CD4 T cell proliferation and a reduced immune response toward DNA and HIV, which might result in reduced levels of constitutive IFN production. Consequently, the HAQ/HAQ *TMEM173* genotype may contribute to the slower disease progression characteristic of LTNPs. ***The Journal of Immunology*****, 2018, 200: 3372–3382.**

Infection with HIV remains a major health problem, with ∼18.5 million untreated patients globally in 2016 reported by the World Health Organization. HIV-1 is responsible for the majority of these infections, which leads to rapid CD4 T cell decline, elevated viral load (VL), and almost inevitably to the development of AIDS if not treated. The mean antiretroviral treatment (ART)–free survival is 7.4–11.5 y, depending on HIV-1 strain and country (1). In contrast, around 1% of HIV-1–infected individuals experience a slower disease progression. These long-term nonprogressor (LTNP) patients maintain a high CD4 T cell count and low VL despite many years of untreated infection. A subgroup of LTNP patients are elite controllers (ECs), defined by having a VL below the limit of detection. Common genetic variants, such as the Δ32-*CCR5* mutation and certain protecting HLA alleles, are estimated to account for 25% of the LTNP/EC phenotype (2, 3). Additionally, the ability to establish a high transient IFN response early during infection and the ability to prevent overactivation of the immune system during chronic infection have been suggested as contributing factors to the LTNP/EC phenotype (4).

The main sources of type I IFN during HIV infection originate from recognition of genomic HIV RNA or RNA replication intermediates by TLR7/8 or alternatively by RIG-I in plasmacytoid dendritic cells (5, 6). Moreover, translocation of intestinal bacteria over the gut barrier induces immune activation mainly through TLR4 (7, 8). In

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Abbreviations used in this article: AQ, G230A-R293Q; ART, antiretroviral treatment; CDN, cyclic dinucleotide; cGAMP, cyclic GMP-AMP; cGAS, cGAMP synthase; dbSNP, Single Nucleotide Polymorphism database; EC, elite controller; HAQ, R71H-G230A-R293Q; htDNA, herring testes DNA; ID, identification number; IFI16, IFN-γ–inducible protein 16; ISG, IFN-stimulated gene; LTNP, long-term nonprogressor; LV, lentiviral; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; NCART, noncontroller on ART; Q, R293Q; qPCR, quantitative PCR; SeV, Sendai virus; SNP, single-nucleotide polymorphism; STING, stimulator of IFN genes; TBK1, TANK-binding kinase 1; UT, untreated; VL, viral load; VLP, virus-like particle; WT, wild type.

addition, bacterial DNA as well as HIV-derived DNA can be recognized by cytosolic DNA sensors, such as IFN-γ–inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthase (cGAS), and signal through the adaptor molecule stimulator of IFN genes (STING) (also known as *TMEM173*, MITA, *MYPS*, and ERIS) by cGAMP formation (9). Cyclic dinucleotides (CDNs) from some bacteria can be sensed directly by STING (10, 11). STING subsequently activates TANK-binding kinase 1 (TBK1), leading to phosphorylation of IFN regulatory factor 3 (IRF3) and transcription of type I IFN and IFN-stimulated genes (ISGs) (12, 13). Furthermore, recent papers have described a novel apoptotic and antiproliferative role of STING in mouse and human T cells (14–16). Importantly, several studies have also reported a combined effect of three single-nucleotide polymorphisms (SNPs) in *TMEM173*, resulting in STING protein variants that can affect type I IFN production (14, 17–20) and rescue proliferation in cells with constitutive active STING (14). The haplotype is termed HAQ because of the altered amino acids: R71H-G230A-R293Q (HAQ). R71H (the Single Nucleotide Polymorphism database [dbSNP] identification number [ID] rs11554776) is located in the ectodomain, whereas G230A (dbSNP ID rs78233829) and R293Q (Q) (dbSNP ID rs738082) are both located in the cytoplasmic tail of the STING molecule. R293Q may affect the disulfide bond formation at the neighboring amino acid C292 (17). G230A is in close proximity to the amino acid 232, both covering (but not forming) the CDN binding pocket (21). The amino acid 232 is either histidine or arginine; both are considered wild type (WT) in different studies (19, 22) and do not affect the DNA-sensing pathway (20). The proportion of HAQ homozygosity varies depending on ethnicity: 3% homozygosity exists in Europe and America and 15% of East Asian descendants are homozygous for HAQ, whereas homozygosity is nonexistent in African populations (17–19). In vitro, two independent studies showed ∼90% reduction of constitutive IFN-β production in the presence of the HAQ/HAQ genotype compared with WT STING (17, 18). Specifically, macrophages expressing homozygous HAQ STING were found to be deficient in their ability to respond to *Listeria monocytogenes* (17) and to specific CDNs (18). Some studies even suggest that the three SNPs might also affect IFN-β production independently or in pairs of two. Finally, heterozygous STING variants are also suggested to affect functionality (7, 17–19).

The consequence of multiple homozygous STING variants in HIV patients remains poorly understood. In this study, we investigated the presence of multiple homozygous SNPs in the *TMEM173* gene in a cohort of 11 LTNPs (of whom 4 were also ECs) and a control group of 11 normally progressing HIV patients on treatment, termed noncontrollers on ART (NCARTs). We demonstrate that an HIV LTNP with the HAQ/HAQ STING variant has decreased STING protein expression, reduced innate responses to DNA and HIV, reduced HIV replication, and reduced inhibition of CD4 T cell proliferation compared with WT STING–carrying HIV NCARTs. Another LTNP homozygous for the G230A-R293Q (AQ) STING variant had similar characteristics regarding STING-induced type I IFN response, although not as pronounced. Reduced STING-dependent innate immune sensing may contribute to reduced chronic immune activation and reduced inhibition of CD4 T cell proliferation during chronic HIV infection. Hence, we suggest that the homozygous HAQ/HAQ STING variant represents a contributing factor in the slow disease progressing phenotype in certain HIV-infected individuals with this genetic constitution.

Materials and Methods

Study aim and design

We conducted a cross-sectional study enrolling 11 HIV ECs and LTNPs (of whom 4 were ECs and 7 were LTNPs) for examination of SNPs in the STING-encoding gene *TMEM173* and investigation of innate immune response to DNA, HIV, and alterations in STING-dependent proliferation. After inclusion, a priori to stimulation experiments, HIV ECs and LTNPs were age- and gender-matched to HIV NCARTs.

The Danish National Committee on Health Research Ethics (1-10-72-369-14) and the Danish Data Protection Agency (journal number 2007-58-0010) approved the project. All patients provided written consent prior to study inclusion.

Study population

Study participants were identified based on screening of the entire Danish HIV Cohort (23) for patients who met the definitions of HIV EC or LTNP. Inclusion criteria for ECs were as follows: age >18 y, HIV diagnosis for a minimum of 5 y, at least three VL measurements with >1 y between the first and last sampling, and a maximum of one CD4 T cell count <500 cells/μl. Exclusion criteria were as follows: ART, AIDS-defining events, VL measurement ≥1000 copies/ml, two consecutive VL measurements >400 copies/ml, or >20% of VL measurements above the detection limit. Inclusion criteria for LTNPs were as follows: age >18 y, HIV diagnosis for a minimum of 5 y, and at least three VL measurements with at least 1 y between the first and last sampling. Exclusion criteria were as follows: CD4 count <350 cells/μl by two consecutive measurements, ART, AIDS-defining events, two consecutive VL measurements ≥2000 copies/ml, or >20% of VL measurements ≥2000 copies/ml. Inclusion criteria for the control group with normal HIV progression (NCARTs): age >18 y, HIV diagnosis for a minimum of 5 y, nadir CD4 count below 350 cells/μl leading to initiation of ART within the first 5 y of HIV diagnosis, and ART for ≥1 y.

PBMC purification

PBMCs were purified by a Ficoll (GE Healthcare) gradient. Frozen PBMCs were resuspended in RPMI 1640 medium with L-glutamine supplemented with 10% heat inactivated FBS, penicillin (100 IU/ml), and streptomycin 100 μg/ml), all from Biowest. PBMCs were incubated overnight at 37°C in 5% CO_2 before stimulations.

Monocyte-derived macrophage differentiation

Freshly thawed PBMCs were seeded as 0.75×10^6 cells in cRPMI supplemented with 10% human serum albumin (Sigma-Aldrich) and 15 ng/ml M-CSF (PeproTech) in a 48-well plate. At day 4, media were changed to monocyte-derived macrophage (MDM) media: DMEM with L-glutamine (Biowest), penicillin (100 IU/ml), and streptomycin (100 μg/ml) supplemented with 10% human AB serum (Sigma-Aldrich) and 15 ng/ml M-CSF (PeproTech). At day 7, MDM media were replaced again. At day 9, MDMs were stimulated in DMEM with L-glutamine supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Biowest).

CD4 T cell isolation

$CD4^+$ T cells were negatively isolated from PBMCs using the MACSxpress CD4 T Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany).

Stimulations

Two micrograms per milliliter herring testes DNA (htDNA) (Sigma-Aldrich) was transfected into PBMCs and MDMs using Lipofectamine 3000 (Thermo Fisher Scientific), with a Lipofectamine:DNA ratio of 1:1 diluted in Opti-Mem (Life Technologies) following manufacturer's instructions. Lipofectamine 3000 alone was used as a mock control. 1:500 volume Sendai virus (SeV) Cantell strain (VR-907; American Type Culture Collection, Mannassas, VA) was used as a positive control. cRPMI was used as an untreated (UT) control. Cells were lysed after 6 h using a lysis buffer from High Pure RNA Isolation Kit (Roche). Supernatants were harvested after 24 h.

Healthy donor cells were stimulated with 1 or 10 μl of 2′3′cGAMP (InvivoGen) containing virus-like particles (VLP) or empty VLPs or were left UT for 6 h. Polybrene (Santa Cruz Biotechnology) (6 μg/ml) was added to the culture medium to enhance infection. After 6 h, supernatant was harvested for bioassay, and cells were lysed for quantitative PCR (qPCR) or Western blot.

Viral plasmid propagation and purification

Plasmids of NL4.3 X4 eGFP and AD8 were produced according to manufacturer's protocol for chemical transformation of One Shot TOP10 Chemically Competent *E. coli* (Invitrogen), with the exception of a change in overnight incubation temperatures from 37 to 30°C. One colony was propagated in Lysogeny broth medium (Sigma-Aldrich) supplemented with 100 μl carbenicillin (100 μg/ml) (Novagen) at 34°C with vigorous shaking for 18 h.

After incubation, plasmid DNA purification was performed according to manufacturer's protocol (Plasmid Plus Midi Kit; Qiagen), and concentrations were determined using NanoDrop technology (Thermo Fisher Scientific).

Virus production

HIV was produced in HEK293T cells: 10 μg NL4.3 X4 eGFP plasmid was transfected using 3 μg/ml Polyethylenimine HCl MAX (PEI MAX) (PolyScience). Five micrograms of AD8 plasmid was transfected using FuGENE 6 (Promega) in a 3:1 relationship to DNA. Virus was harvested 48 h posttransfection and stored at −80°C.

Vpx production

Lentiviral (LV) particles carrying Vpx were produced in HEK293T cells transfected with PEI Max and 8 μg DNA in a 1:3 ratio. The DNA consisted of LV-SIV3$^+$ and pMD2.G in a 2:5 ratio. Sixteen hours after transfection, the media were exchanged to fresh cDMEM and harvested after 48 h. LV-Vpx was concentrated using 5% sucrose gradient by centrifugation at 25,000 × *g* for 2 h at 4°C and finally resuspended in PBS (Sigma-Aldrich).

Production of carrier particles

VLPs carrying cGAMP or nothing (empty) were produced in HEK293T cells. HEK293T cells were transfected with Lipofectamine 2000 (#11669-019; Thermo Fisher Scientific) and 14 μg DNA in a 3:1 ratio. The DNA consisted of two packaging plasmids (pCSGW-rev-nef-GFP and 8.91-gag-pol), an envelope plasmid (pMD2.G-VSV-G), and either pCDNA3-Flag-mcGAS WT or pCDNA3-Flag-mcGAS AA in a 2:2:1:2 ratio, respectively. pCDNA3-Flag-mcGAS WT was used to produce VLPs containing cGAMP, whereas the catalytically dead mutant of cGAS (pCDNA3-Flag-mcGAS GS > AA) was used to produce empty control VLPs. Sixteen hours posttransfection, the media were exchanged to fresh cDMEM and harvested after 48 h. VLPs were concentrated using 20% sucrose gradient by centrifugation at 25,000 × *g* for 2 h at 4°C and finally resuspended in pure DMEM (Lonza).

HIV infection

PBMCs were preactivated for 3 d using 1 μg/ml anti-human CD3 (Functional Grade Purified clone OKT3 lot 4284902; eBioscience), anti-human CD28 (Functional Grade Purified clone CD28.2 lot 4274199; eBioscience), and 40 U/ml IL-2 (PeproTech) in cRPMI. Next, cells were infected with NL4.3 eGFP or AD8 at multiplicity of infection (MOI) 0.1 or left untreated. After 6 h, cells were washed three times in cRPMI to remove excess virus. After 72 h, supernatants were harvested and inactivated using 1:1 2% EMPIGEN BB detergent (Fluka) and used for p24 ELISA.

MDMs were used for infection with AD8 at MOI ∼0.5. One hour prior to HIV infection, MDMs received 250 pg VLP-Vpx with 4 pg/ml Polybrene. Cells were washed three times in cDMEM 6 h postinfection. After a total of 18 h, cells were lysed for mRNA measurements.

CD4 T cell proliferation assay

CD4 T cells were stained with eBioscience CFSE (Thermo Fisher Scientific) and cultured in cRPMI with 40 U/ml IL-2 (PeproTech) with or without αCD3 (no. MAB100; Bio-Techne)/αCD28 (CD28.2, no. 555725; BD Biosciences) activation and 40 μg/ml 2′3′cGAMP (InvivoGen) stimulation for 3 d. Next, cells were stained with LIVE/DEAD fixable Far-Red Dead Cell Stain (molecular probes by Life Technologies, Thermo Fisher Scientific), monoclonal mouse IgG1 anti–human-CD4-PC7 (SFCI12T4D11; Beckman Coulter), and mouse BALB/c IgG_1 anti–human-CD3-BV421 (SK7; BD Horizon) and were analyzed by flow cytometry on NovoCyte (ACEA Biosciences) using FlowJo_V10.1. The gating strategy applied was as follows: single cells gated on forward scatter height versus forward scatter area, followed by gating on live cells, nondebris (forward and side scatter), and CD3$^+$/CD4$^+$ double-positive cells. Lastly, the fraction of proliferated cells was gated according to dilution of the CFSE stain. The number of proliferation cycles were not taken in to account.

Viability assay

Cells were left untreated or stimulated with 40 μg/ml 2′3′cGAMP (InvivoGen) or 500 nM staurosporine (ApexBio) for 20 h with or without 48 h preactivation with αCD3 (OKT3; eBioscience)/αCD28 (CD28.2; eBioscience) and IL-2. Next, viability was measured by CellTiter-Glo (Promega) following manufacturer's instructions.

RNA purification, cDNA, and qPCR

RNA was purified by High Pure RNA Isolation Kit (Roche). cDNA was synthesized from RNA using QuantiTect Reverse Transcription Kit (Qiagen). mRNA levels were determined by TaqMan qPCR using PerfeCTa qPCR FastMix II and by ROX (Quantabio) using the following primers and probes, all from Thermo Fisher Scientific: TBP (Hs00427620_m1), CXCL10 (Hs01124251_g1), TNF-α (Hs01113624_g1), IFNB1 (Hs01077958_s1), IFIT1 (Hs03027069_s1), and STING (TMEM173) (Hs00736955_g1). Manufacturer's instructions were used for all methods in this section.

PCR and Sanger sequencing

STING/*TMEM173* full-length mRNA were produced using forward primer 5′-TTGGCTGAGTGTGTGGAGTC-3′ and reverse primer 5′-CAGTC-CAGAGGCTTGGAGAC-3′ (Eurofins) (19). The PCR products were prepared for DNA sequencing using the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific). HAQ SNPs and H/R232 alleles were identified by Sanger sequencing with GATC Biotech using two different primers: 5′-GTTCTGCTGAGTGCCTG-3′ and 5′-TTCCTGGTAGG-CAATGAG-3′. Sequences were analyzed using Geneious R9 version 9.1.8.

ELISA and functional type I IFN bioassay on culture supernatants

CXCL10 protein expression was measured using Human CXCL10/IP-10 DuoSet ELISA (R&D Systems) following manufacturer's instructions. HIV-1 p24 Ag was measured by a homemade ELISA as previously described (24).

Functional type I IFN was quantified using the reporter cell line HEK-Blue IFN-α/β (no. hkb-ifnab; InvivoGen). After 24 h of incubation with 50 μl culture supernatant, secreted embryonic alkaline phosphatase activity was measured according to manufacturer's instructions. Standard range was made with IFN-α (A2) (PBL Assay Science). OD was measured in a microplate reader (ELx808; BioTek).

Western blotting

Cells were lysed in a Triton-based lysis buffer (Cell Signaling Technology) with cOmplete Mini Protease Inhibitor Cocktail (Roche). Lysates were spun for 5 min at 2000 g, and protein concentrations were determined by DC Protein Assay (Bio-Rad). Samples were mixed with XT Reducing Agent (Bio-Rad) and XT Sample Buffer (Bio-Rad) and heated for 5 min at 95°C. Proteins were separated on 10% precast gel (Criterion TGX; Bio-Rad) and transferred to Trans-Blot Turbo polyvinylidene difluoride and nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were washed with TBS supplemented with 0.05% Tween20 (TBS–Tween20) and blocked in 5% BSA (Roche). The following primary Abs were used in TBS–Tween20 1% BSA solution: mouse anti-human IFI-16 IG/SC-8023 (Santa Cruz Biotechnology), vinculin (hVIN-1) monoclonal IgG1 mouse (Sigma-Aldrich), STING (D2P2F) no. 13647 rabbit mAb IgG (Cell Signaling Technology), and rabbit anti-human p-TBK1 (D52C2 no. 5483s; Cell Signaling Technology). The following HRP-conjugated secondary Abs were used in 1% BSA solution: Donkey anti-rabbit (Jackson ImmunoResearch) and donkey anti-mouse (Jackson ImmunoResearch). Membranes were exposed using Clarity ECL Western Blot Substrate (Bio-Rad) and visualized using a ImageQuant LAS 4000 Mini (GE Healthcare) luminescent image analyzer.

Statistics

GraphPad Prism version 6 was used for graphic illustrations. Comparison of NCARTs to LTNP responses was performed using multiple *t* tests. Significance level was set at 0.05.

Results

Study population and genotyping of TMEM173 and CCR5

HIV ECs (n = 4) and LTNPs (n = 7) and 11 NCARTs were included in the study. After inclusion, LTNPs and ECs were age- and gender-matched to the control group (Supplemental Table I). HIV disease characteristics for the individual study participants are shown in Supplemental Table II. Using PCR, gel separation, and Sanger sequencing, we determined the *CCR5* and *TMEM173* genotype for all patients and controls. STING protein architecture is shown in Fig. 1A. Based on ethnicity, we would expect 3.4% in each group to be HAQ or AQ homozygous in the *TMEM173* gene (19). We found that LTNP 011 was HAQ homozygous and LTNP 010 was AQ homozygous, whereas no NCARTs were homozygous for the three HAQ or the two AQ SNPs in *TMEM173* (see Table I). Thus, ∼18% of the ECs and LTNPs were homozygous,

whereas 0% of the NCARTs were homozygous. The allele distribution of STING amino acid 232 (R or H) (19) is shown in Supplemental Table I. Furthermore, LTNP 011 and the matched control NCART 011 were both found to be heterozygous for the Δ32 deletion in *CCR5* (see Table I). A striking observation was that 2 out of 11 LTNPs were homozygous for multiple SNPs in STING, and thus we chose to investigate these patients further. LTNP 011 is a 67-y-old Caucasian male with diagnosed HIV infection for at least 25 y without any treatment. After 12 y with infection, he experienced a decline in CD4 count to around 250 cells/μl; however, he continuously maintained a very low VL in plasma until study inclusion, thus for at least 14 more years. At inclusion, he had a CD4 count of 295 cells/μl and a VL of 22 copies/ml. LTNP 010 is a 57-y-old African female with verified HIV infection for at least 13 y without treatment. At inclusion, she had a CD4 count of 1070 cells/μl and a VL of 43 copies/ml. We found that both patients had HLA subtypes predicted to contribute to both fast and slow progression, which was therefore unlikely to fully explain the LTNP phenotypes of these two patients (2, 25–27) (Supplemental Table III).

STING mRNA accumulation is decreased in LTNPs carrying STING variants

It has previously been shown that the HAQ variant may affect the cellular mRNA accumulation from *TMEM173* (19). Therefore, we examined the accumulation level of STING in the two patients and their matched controls, plus two additional controls with homozygous WT *TMEM173* alleles. In PBMCs, a modest, but significant, reduction in STING mRNA accumulation was observed for LTNP 010 compared with NCARTs (Fig. 1B). However, the STING mRNA accumulation level in PBMCs was generally low in both patients and controls. In contrast, STING mRNA accumulation in MDMs was generally higher for the controls, and a significant reduction was observed for LTNP 011 (Fig. 1C). Interestingly, STING protein expression levels in both PBMCs and MDMs were diminished in both the HAQ/HAQ LTNP 011 and the AQ/AQ LTNP 010 compared with the controls (Fig. 1D, 1E).

The HAQ/HAQ TMEM173 genotype confers reduced innate immune responses toward transfected DNA

In healthy controls, we next investigated induction of the STING-dependent type I IFN pathway in PBMCs and nonactivated as well as activated CD4 T cells after stimulation of STING. We observed phosphorylation of TBK1, IFN-β mRNA accumulation, and functional type I IFN protein secretion in PBMCs after cGAMP delivery by VLPs but no response in any of the T cell populations (Supplemental Fig. 1). Further studies on patient material were therefore carried out in PBMCs and in MDMs, which are known to sense HIV (e.g., by engulfing infected CD4 T cells). Next, we investigated antiviral and proinflammatory immune responses in the patients with multiple homozygous *TMEM173* SNPs and their matched controls. PBMCs were transfected with htDNA, which is a classic way to activate the DNA sensors cGAS and IFI16 upstream of STING (12). SeV infection was used as a control for immune activation through non–DNA-sensing pathways. Cells were harvested 6 and 24 h after stimulation or infection to measure cytokine mRNA and protein levels, respectively. For both LTNPs, mRNA accumulation of IFN-β, CXCL10, and TNF-α after DNA transfection were significantly reduced (except for CXCL10 in LTNP 011), compared with the matched controls. In contrast, immune responses to SeV were largely similar between the LTNPs and their controls (Fig. 2A–C). However, the CXCL10 protein levels from AQ LTNP 010 in response to SeV were slightly reduced compared with HIV NCARTs; this might be due to cross-talk between DNA and RNA sensor signaling mediated through STING and MAVS, respectively (28) (Fig. 2D). Of note, after mock transfection with lipofectamine, a slightly significant IFN-β, CXCL10, and TNF-α mRNA induction was seen in the controls. This signal may result from membrane fusion–dependent activation of STING (29). Moreover, CXCL10 protein expression in supernatants in response to DNA transfection measured by ELISA was also significantly lower in both patients compared with controls (Fig. 2D). Taken together, these results confirm previous findings of reduced activation of the STING pathway in patients carrying multiple homozygous *TMEM173* SNPs.

In order to confirm these results, we repeated the DNA transfection experiment in MDMs, which are specialized innate-sensing cells and represent a more homogeneous cell population. In this experiment, we included additional *TMEM173* WT/WT controls in each experiment. For the HAQ/HAQ LTNP 011, we once again observed a significant reduction in IFN-β and TNF-α responses toward DNA compared with the pooled controls (Fig. 3A, 3C), whereas CXCL10 was more evenly expressed in response to DNA transfection (Fig. 3B). The AQ/AQ LTNP 010 did not, however, display the same phenotype in MDMs with reduced inflammatory responses as observed in PBMCs.

HIV replication and sensing are reduced in the HAQ/HAQ carrying LTNP

Next, we evaluated the impact of the *TMEM173* SNPs on HIV replication. It has previously been shown that LTNPs have reduced HIV replication and/or higher HIV inhibition (30). The STING variant–carrying LTNPs had lower IFN production after DNA transfection compared to controls. Low IFN response to DNA sensing might, in theory, have a negative effect on control of acute HIV infection in vivo. In contrast, weak IFN responses to DNA sensing may result in less exhausted immune cells because of reduced chronic inflammation, hence less NF-κB–induced HIV replication (31) and thus an overall improved control of HIV replication in vivo. To investigate these different possibilities, we infected PBMCs preactivated by IL-2 and αCD3/αCD28 with different HIV strains and evaluated viral replication measured by p24 levels in supernatants after 72 h (Fig. 4). We first evaluated the CCR5 tropic AD8 HIV strain, which is known to adopt a macrophage-tropic phenotype. Our data demonstrated that HIV-AD8 showed a trend ($p = 0.055$) toward reduced replication in the HAQ/HAQ LTNP 011 compared with the group of controls (Fig. 4A). To ensure that the reduced replication observed was not solely due to the heterozygous *CCR5*-Δ32 allele present in this patient, we also challenged patient PBMCs with the CXCR4 tropic NL4.3 HIV strain. Indeed, LTNP 011 had significantly lower HIV replication than the pooled NCARTs (Fig. 4A). However, this phenomenon was not observed in the patient with the AQ variants. We suspected that the minor degree of replication in LTNP 011 might be due to limited innate immune sensing of the virus. We finally investigated type I IFN and ISG induction in response to HIV infection. For this, we infected MDMs because CD4 T cells have previously been shown to be almost devoid of IFN responses to HIV (32). Prior to infection, we treated cells with the SAMHD1 antagonist Vpx to thereby increase the magnitude of potential innate immune sensing. We found that the HAQ/HAQ STING variant LTNP 011 had significantly reduced IFN-β and IFIT1 induction compared with controls after Vpx-AD8 infection (Fig. 4B, 4C). In contrast, the innate immune response toward HIV was only slightly and not significantly reduced in the AQ/AQ LTNP 010 (Fig. 4B, 4C).

The HAQ STING variant results in impaired inhibition of CD4 T cell proliferation

Recent papers suggest an apoptotic or antiproliferative role of STING activation in T cells, especially in mice (14–16). STING

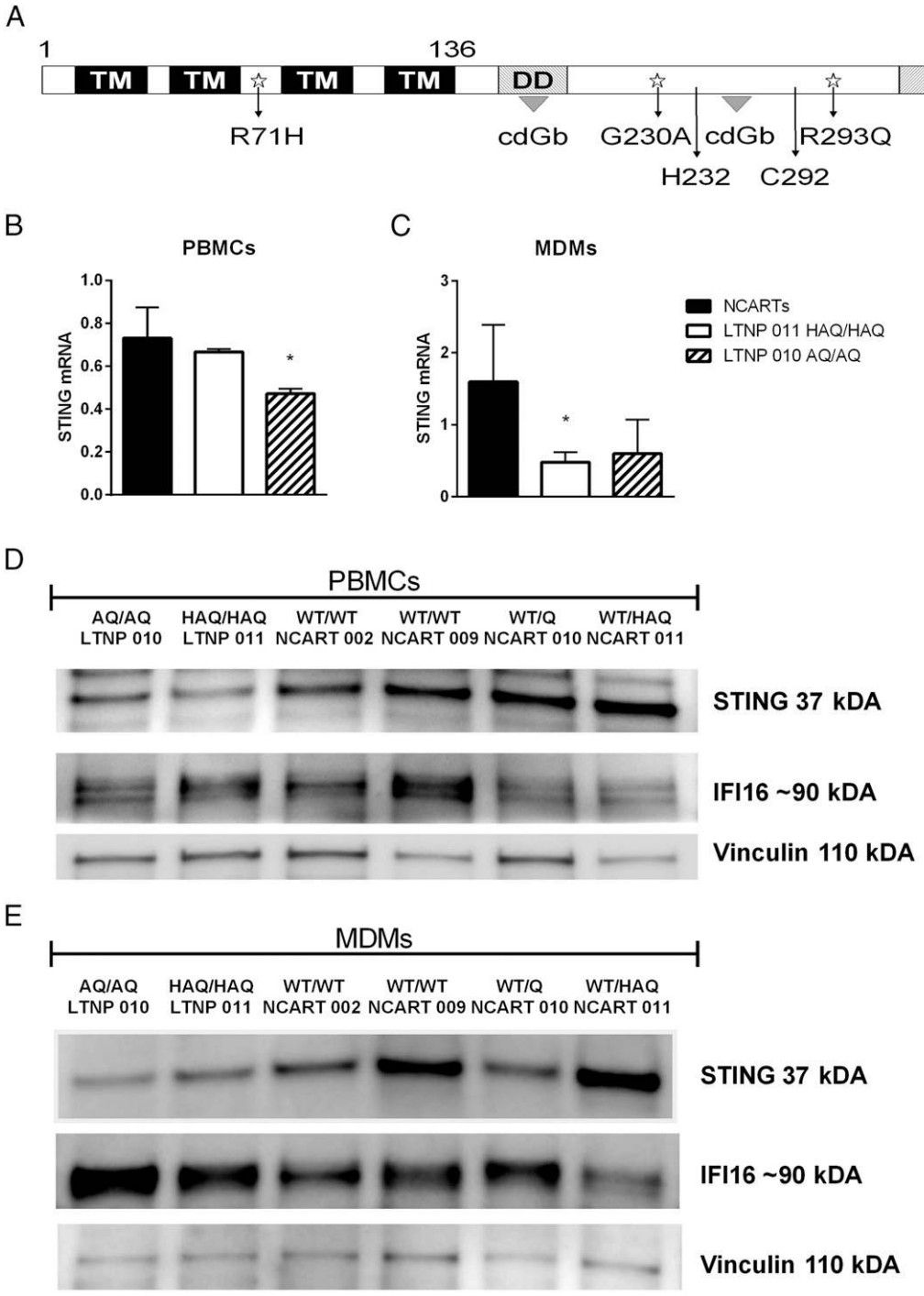

FIGURE 1. STING accumulation is reduced in HIV LTNP cells carrying the HAQ *TMEM173*/STING variant compared with controls. (**A**) Schematic illustration of STING protein architecture: the 379 aa cover four transmembrane (TM) regions and a cytoplasmic region (137–379), including the dimerization domain (DD) and carboxy-terminal tail (CTT), which is phosphorylated by TBK1. Cyclic-di-GMP binding (cdGb) sites are marked with gray triangles. R71H, G230A, and R293Q variants are marked with stars and black arrows. Important amino acids in close proximity of variants are marked with black arrows: R232 (considered WT allele, but frequently substituted with H232) and C292 (disulfide bond formation [17]). STING mRNA accumulation was measured in technical triplicates in PBMCs (**B**) and MDMs (**C**) from the two LTNPs and four pooled controls: NCART 002 (WT/WT), NCART 009 (WT/WT), NCART 010 (WT/Q), and NCART 011 (WT/HAQ). mRNA accumulation levels are relative to TBP; means with SDs are shown. Protein expression levels in PBMCs (**D**) and MDMs (**E**) for STING, the upstream DNA sensor IFI16, and the housekeeping protein vinculin detected by Western blotting. $*p \leq 0.05$, multiple *t* test comparison.

activation has also been shown to induce type I IFN in murine CD4 T cells (15, 16). Therefore, we investigated if STING activation by cGAMP might affect PBMC and CD4 T cell viability and type I IFN production in preactivated and nonactivated cells. Contradictory to previous mouse studies (15, 16), cGAMP stimulation did not affect cell viability significantly in either human PBMCs or blood-derived CD4 T cells, regardless of activation status (Fig. 5A, 5B). Type I IFN was also not detected in CD4 T cells following cGAMP stimulation (Supplemental Fig. 1B, 1C). The difference between our results

Table I. STING and CCR5 genotypes

Patient	*TMEM173* Genotype	*CCR5* Genotype
EC 001	WT/WT	WT/WT
EC 002	WT/WT	Δ32/WT
EC 003	WT/WT	Δ32/WT
EC 004	WT/WT	WT/WT
LTNP 005	WT/WT	WT/WT
LTNP 006	HAQ/WT	Δ32/WT
LTNP 007	AQ/WT	WT/WT
LTNP 008	WT/WT	WT/WT
LTNP 009	WT/WT	Δ32/WT
LTNP 010	**AQ/AQ**	WT/WT
LTNP 011	**HAQ/HAQ**	Δ32/WT
NCART 001	HAQ/WT	WT/WT
NCART 002	WT/WT	WT/WT
NCART 003	WT/WT	Δ32/WT
NCART 004	WT/WT	WT/WT
NCART 005	HAQ/WT	WT/WT
NCART 006	WT/WT	WT/WT
NCART 007	AQ/WT	WT/WT
NCART 008	AQ/WT	WT/WT
NCART 009	WT/WT	WT/WT
NCART 010	Q/WT	WT/WT
NCART 011	HAQ/WT	Δ32/WT

TMEM173 and *CCR5* genotypes were determined by Sanger sequencing and agarose gel separation, respectively. Patients with multiple homozygous SNPs are highlighted with bold type. WT allele (WT), non-WT alleles: R71H-G230A-R293Q (HAQ), G230A-R293Q (AQ), R293Q (Q), and 32 aa deletion (Δ32).

and previous type I IFN measurements in mice (15, 16) may be due to only 68% similarity between the human and mouse *TMEM173* gene as well as binding of different types of agonists (33). Therefore, we focused on cell proliferation and found that CD4 T cell proliferation after αCD3/αCD28 preactivation was strongly inhibited by cGAMP stimulation in STING WT cells (Fig. 5C, 5D). We then examined if the homozygous HAQ/HAQ and AQ/AQ variants in LTNP 011 and 010, respectively, could counteract the cGAMP-induced, STING-mediated block of cell proliferation observed in HIV NCARTs with WT STING. Remarkably, we demonstrated that the HAQ/HAQ constitution in LTNP 011, but not the AQ/AQ constitution in LTNP 010, exhibited a lower degree of inhibition of CD4 T cell proliferation upon cGAMP stimulation compared with HIV NCARTs (Fig. 5E).

Discussion

In the current study, we identified SNPs in *TMEM173* encoding the DNA pathway signaling adaptor STING in two patients with slow HIV disease progression. We demonstrated that PBMCs and MDMs from the HAQ/HAQ LTNP 011 had reduced type I IFN and ISG responses to DNA stimulation and HIV infection compared with controls. Furthermore, STING protein expression in both PBMCs and MDMs were reduced in this patient and in AQ/AQ LTNP 010 compared with controls. Similarly, the AQ/AQ LTNP 010 also showed some degree of reduced IFN and ISG production in response to DNA, although this was less clear than for the HAQ/HAQ genotype in LTNP 011. Accordingly, we found reduced inhibition of proliferation after cGAMP activation of STING in HAQ/HAQ LTNP 011 but not in AQ/AQ LTNP 010. Nevertheless, none of the patients showed a complete lack of IFN

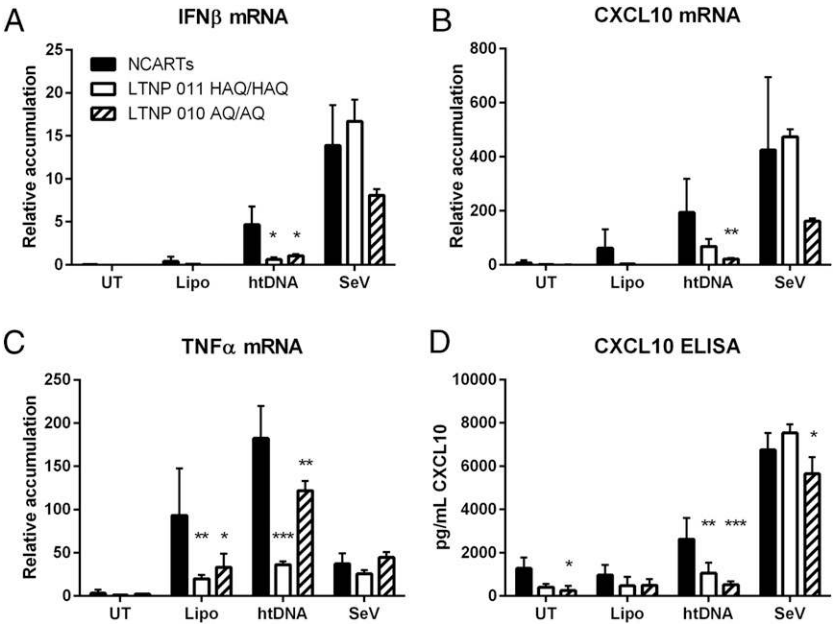

FIGURE 2. Reduced innate immune responses to DNA in PBMCs from STING variant-carrying LTNPs compared with controls. mRNA accumulation levels of IFN-β (**A**), TNF-α (**B**), and CXCL10 (**C**) in PBMCs after 6 h stimulation. Protein expression levels of CXCL10 in supernatants from PBMCs 24 h poststimulation (**D**). Technical triplicates from LTNP 011 and LTNP 010 are compared with pooled age- and gender-matched NCART 011 and NCART 010. Simulations and mRNA measurements in LTNP 010 are representative results from two independent experiments; for LTNP 011, the experiment was only performed once because of limited sample material. Means with SDs are shown. Statistics were performed by multiple *t* test comparison: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. htDNA, herring testes dsDNA; Lipo, Lipofectamine 3000; NCARTs, noncontrollers on ART; SeV, Sendai virus; UT, untreated.

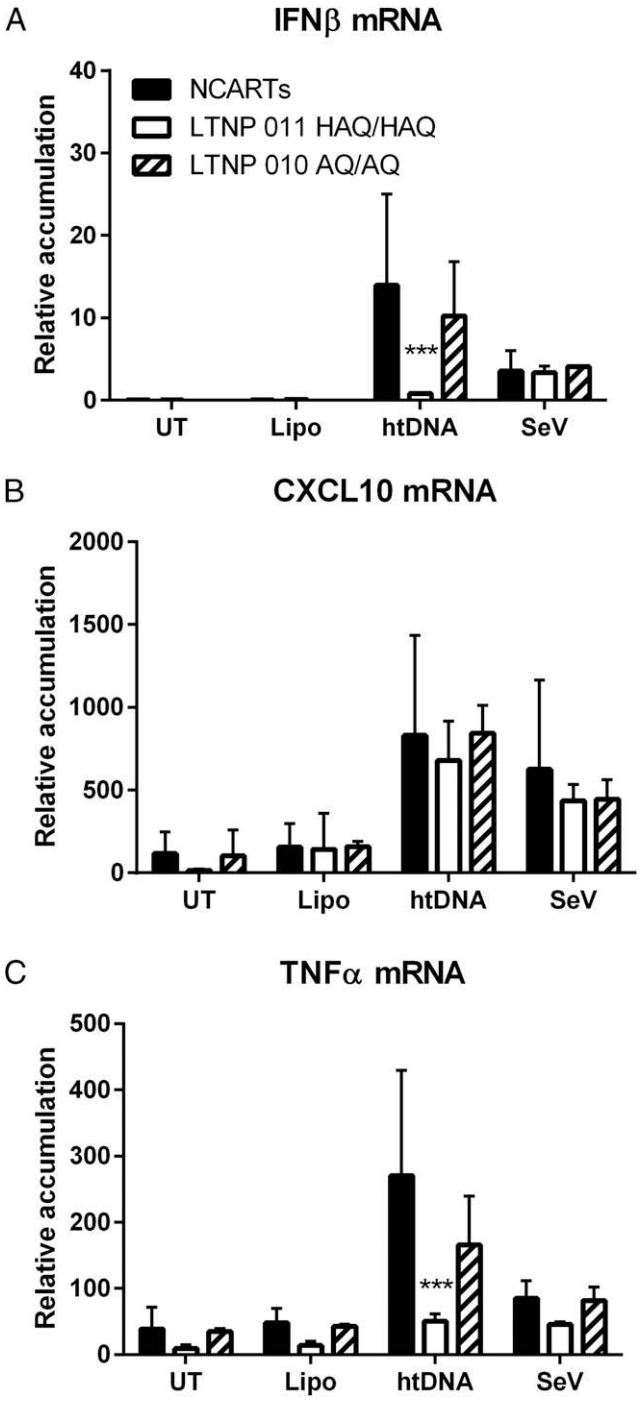

FIGURE 3. Reduced innate immune responses to DNA in MDMs from HAQ/HAQ LTNP 011 compared with controls. mRNA accumulation levels of IFN-β (**A**), CXCL10 (**B**), and TNF-α (**C**) in MDMs after 6 h stimulation. Technical triplicates from LTNP 011 and LTNP 010 are compared with pooled controls: NCART 011 (WT/HAQ), NCART 010 (WT/Q), NCART 009 (WT/WT), and NCART 002 (WT/WT). Means with SDs are shown. Statistics were performed by multiple *t* test comparison: $***p \leq 0.001$. htDNA, herring testes dsDNA; Lipo, Lipofectamine 3000; LTNPs, long term nonprogressors; NCARTS, noncontrollers on ART; SeV, Sendai virus; UT, untreated.

responses or proliferation, indicating that both the homozygous HAQ and particularly the AQ STING variant retain some functionality.

Reduced activity of STING variants might be overcome by high expression levels (7, 19). It might therefore be speculated that patients with the homozygous HAQ/HAQ genotype have a lower cellular level of functional STING protein and therefore a reduced level of constitutive IFN production. However, under certain conditions (e.g., during an acute viral infection), STING protein expression reaches a certain threshold to produce sufficient IFNs for the patient not to succumb to infection. Furthermore, it is important to note that HIV-induced IFNs during acute in vivo infection in the LTNPs harboring STING variants is likely enhanced through other innate sensing pathways, such as RIG-I and TLR7 (5, 7), and might therefore not be markedly reduced. This hypothesis could also explain how STING variants are relatively common in some populations (19).

Besides contributing to a lower level of constitutive inflammation, the hypomorphic HAQ STING variant may also prevent depletion of CD4 T cells in the HAQ/HAQ LTNP 011 during HIV disease progression. It is likely that the less pronounced inhibitory effect of STING activation upon T cell proliferation (possibly driven by ligands derived from HIV or from bacterial translocation over the damaged gut) may have partly contributed to a preserved CD4 T cell count in the patient for more than 25 y without ART.

The literature disagrees on the precise consequences of the individual SNPs in *TMEM173* and whether different STING agonists can induce an IFN response through the HAQ STING variant (17–19, 34–36). These differences are most likely due to differences between human and mouse innate sensing, whether results have been normalized to background IFN expression (which according to some articles may be lower in HAQ STING donors), as well as different STING (over)expression levels in the cells or cell lines used (19, 33, 37, 38). The accumulation level of STING and its signaling threshold also varies in different primary cells; thus, it is well documented that monocytes and macrophages exhibit high levels of STING as well as several innate HIV sensors (5, 9, 39) and contribute to chronic inflammation and cytokine production after sensing microbial translocation over the gut barrier (40, 41). Monocytes from peripheral blood are thought to sense HIV after engulfing T cells. Additionally, macrophages are able to maintain HIV replication in vitro in humans (42) and in vivo in humanized myeloid-only mice (42). In contrast, blood-derived CD4 T cells from the PBMC pool display only a weak, if any, innate immune response to DNA and HIV (32, 43) despite expression of both cGAS, STING, and IFI16 (32).

In the current study, both the homozygous HAQ and AQ STING alleles when present in homozygous form exhibited a hypomorphic phenotype in PBMCs regarding induction of the IFN pathway. Similar results were observed for the HAQ LTNP 011 in MDMs; however, the phenotype was not significantly different from controls for AQ LTNP. To support these observations, we also found that the IFN-β and IFIT1 responses to HIV in the HAQ/HAQ LTNP 011 were significantly reduced compared with controls, whereas only a trend toward reduction was observed for AQ/AQ LTNP 010. Contrary to others' reports, we did not observe any STING-dependent $CD4^+$ T cell death in healthy controls. Our only explanation for this might be differences between using mouse versus human experimental models. Nevertheless, we did observe less inhibition of T cell proliferation for the HAQ/HAQ genotype (but not for the AQ/AQ genotype) compared with controls, indicating a more severe STING defect conferred by the HAQ variant. This finding is supported by a recent study in which transduction with lentivectors carrying HAQ STING rescued proliferation in CD4 T cells from patients carrying the constitutively active V155M STING variant (14). We acknowledge that the precise functional differences between HAQ and AQ variants cannot be concluded without examining a larger cohort of patients or by quantifying the HAQ/HAQ and AQ/AQ genotype frequencies in

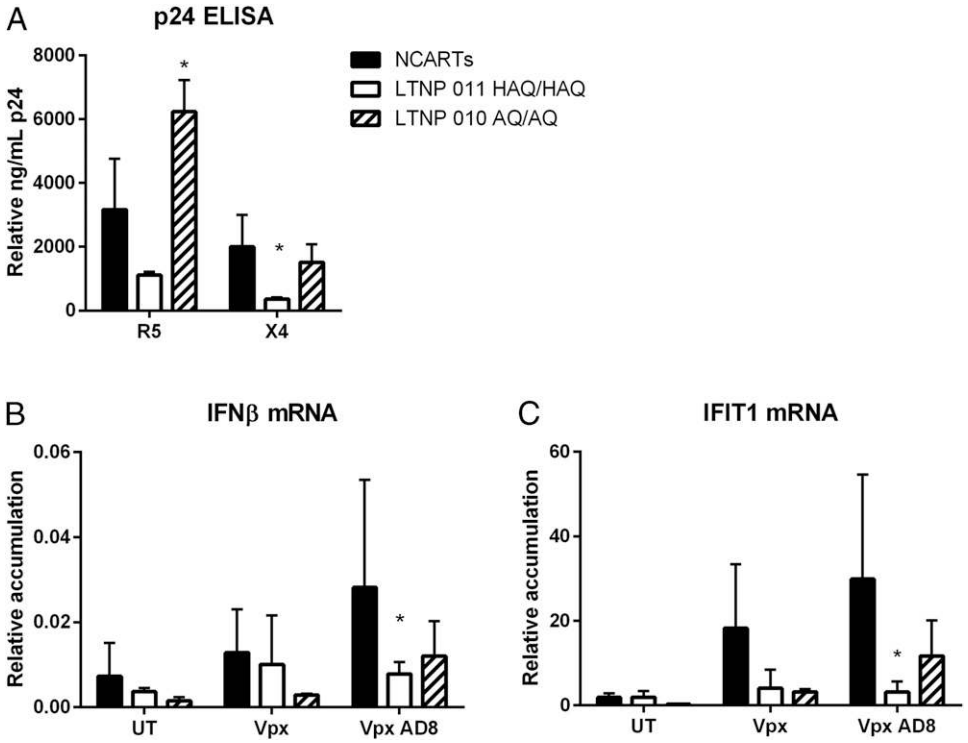

FIGURE 4. Reduced HIV replication and reduced ISG response to HIV in HAQ variant-carrying LTNPs compared with controls. (**A**) In technical triplicates, HIV replication in the two LTNPs is compared with pooled controls: NCART 002 (WT/WT), NCART 009 (WT/WT), and NCART 010 (WT/Q). The amount of p24 protein was measured by ELISA on supernatants from IL-2 and αCD3/αCD28 preactivated PBMCs after 72 h AD8 HIV infection or NL4.3 HIV infection. MOI 0.1 was used for both viruses with tropism for CCR5 (R5) and CXCR4 (×4), respectively. p24 levels were normalized to background after washing. (**B** and **C**) In technical triplicates, HIV-sensing in the two LTNPs was compared with pooled controls: NCART 002 (WT/WT), NCART 004 (WT/WT), NCART 009 (WT/WT), and NCART 010 (WT/Q). mRNA accumulation for IFN-β (B) and the ISG IFIT1 (C) 18 h postinfection with AD8 MOI 0.5 in MDMs pretreated with LV-Vpx and polybrene (Vpx). mRNA accumulation is relative to TBP; means with SDs are shown. Statistics were performed by multiple *t* test comparison: $*p \leq 0.05$. LTNPs, long term nonprogressors; NCARTs, noncontrollers on ART; UT, untreated.

HIV LTNPs compared with noncontrolling HIV patients. Also, we cannot exclude that minor degrees of tolerance in MDMs from patients because of persistent HIV load and/or chronic inflammation might potentially affect our results. However, both HIV LTNPs and HIV NCART patients have very limited VL because of the nature of their phenotype or because of ART, respectively. Therefore, it is our conviction than any tolerance effect in patients would be most probably minimal or absent.

A strong IFN response during the acute infection is extremely important to restrict HIV (44–48). In contrast, persistent chronic immune activation is associated with cellular and humoral immune dysfunction and failure to control viral replication (49, 50). Chronically elevated plasma levels of different types of IFN are a major driving force in CD4 T cell depletion and progression toward AIDS (51–54). Hence, type I IFN levels correlate positively with HIV-1 VL and negatively with CD4 T cell count (55). These clinical observations are supported by studies in natural primate SIV hosts with slowly progressing infection, which have been found to downregulate type I IFN responses in early chronic infection. In contrast, persistently elevated type I IFN levels are observed during pathogenic SIV infection (46, 56). In several studies, vaccinating against IFNs or directly inhibiting IFN-α has resulted in decreased immune activation and lower rates of HIV-1 related events (57–60).

The beneficial versus detrimental effects of IFN are clearly a fine balance between timing, dose, and duration. The results from the current study indicate that a HAQ/HAQ genotype might contribute to the complex HIV LTNP phenotype through decreased chronic type I IFN production and increased CD4 T cell homeostatic proliferation. It could be hypothesized that reducing IFN responses through treatment with a JAK1/2 inhibitor (targeting the hyperactive IFN response in STING-associated vasculopathy with onset in infancy patients with *TMEM173* gain of function mutation; see Ref. 61) or a direct STING inhibitor might be beneficial for HIV patients who fail to regain a normal CD4 T cell count despite ART. A STING inhibitor may act to prevent chronically elevated type I IFN levels, to avoid exhaustion of immune cells, and to minimize the inhibitory effect of STING activation on CD4 T cell proliferation, thereby limiting progressive CD4 T cell depletion during chronic HIV infection. Understanding the HIV LTNP phenotype might therefore theoretically enable us to modulate the clinical phenotype in those for whom the current treatment options with ART are insufficient.

In conclusion, the present study makes several noteworthy contributions to understanding the impact of different STING variants on the pathogenesis in HIV LTNPs. Despite previous findings of elevated IFN in HIV ECs and HIV LTNPs, in this study, we demonstrate a reduced IFN and ISG response toward DNA sensing in an HIV LTNP with the HAQ/HAQ *TMEM173* genotype. Despite low IFN-β and IFIT1 mRNA accumulation after HIV infection, the HAQ/HAQ LTNP 011 exhibited reduced HIV replication. This indicates that reduced chronic IFN production due to a defective STING molecule may be an advantage for HIV patients: without chronic inflammation, immune cells are less exhausted, whereas other sensing pathways may compensate during an acute infection with other pathogens. Interestingly, we found that the HAQ/HAQ STING variant

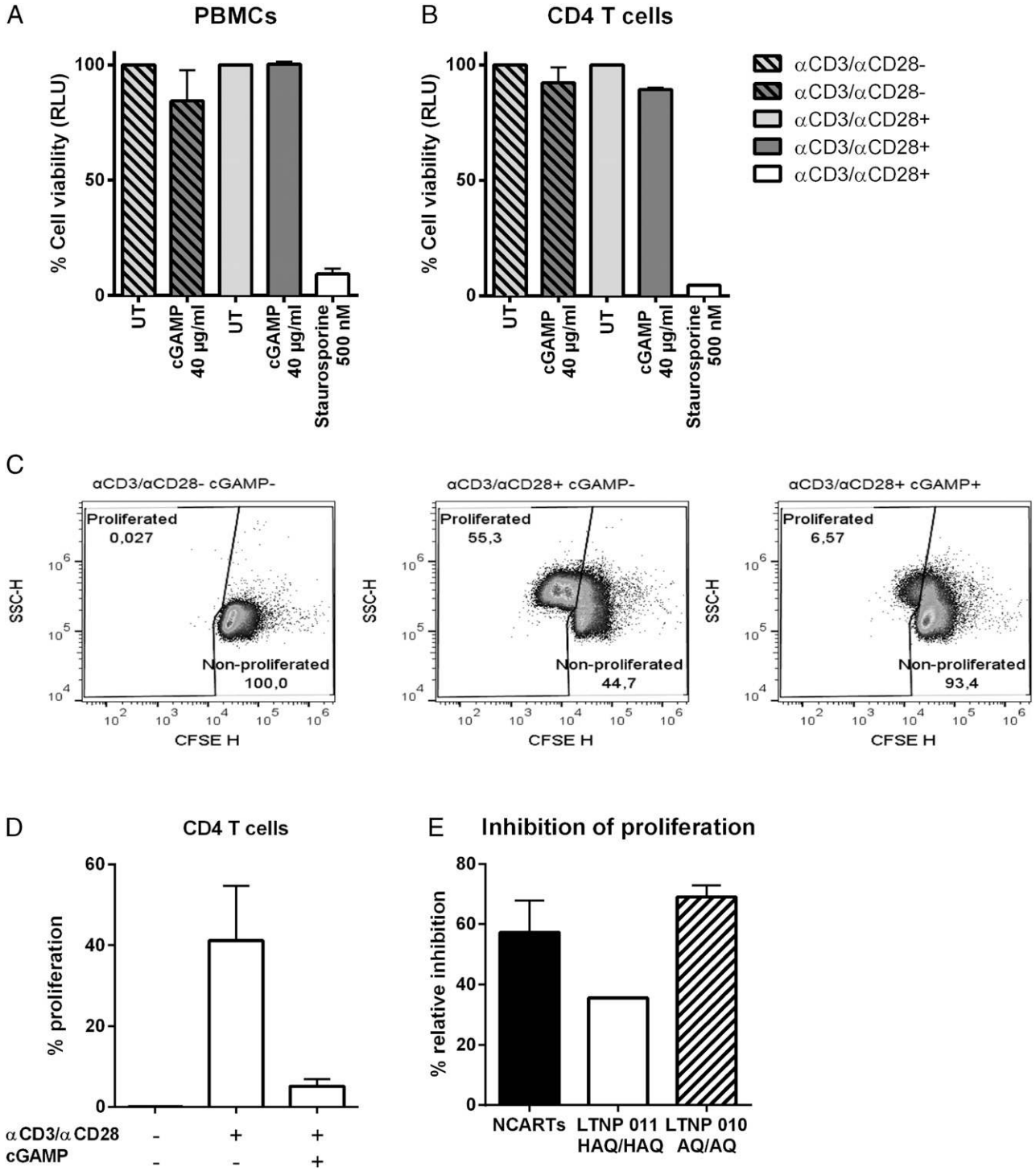

FIGURE 5. The inhibition of T cell proliferation is less pronounced in cells harboring the HAQ STING variant than in controls with WT STING. Viability of preactivated and nonactivated (**A**) PBMCs and (**B**) CD4 T cells from two healthy donors after cGAMP or staurosporine treatment normalized to UT. (**C** and **D**) Preactivation (αCD3/αCD28) and inhibition (40 μg/ml cGAMP) of CD4 T cell proliferation measured by flow cytometry in two healthy donors. (C) Representative flow panel from one donor. (**E**) Inhibition of proliferation after 40 μg/ml cGAMP stimulation normalized to αCD3/αCD28 preactivation induced proliferation alone in LTNP 010 and 011 compared with NCART 002 (WT/WT), NCART 004 (WT/WT), NCART 009 (WT/WT), and NCART 010 (WT/Q). Means with SDs are shown. All experiments were performed in duplicates, with the exception of LTNP 011, which was only measured in singlets due to lack of patient material. LTNP, long term nonprogessor; NCARTs, noncontrollers on ART; UT, untreated.

demonstrated an impaired ability to inhibit T cell proliferation after activation of the STING-dependent DNA-sensing pathway, suggesting a possible direct contribution of the HAQ STING variant to the HIV LTNP phenotype, i.e., preserved CD4 T cell count during chronic HIV. Finally, knowledge on the *TMEM173* genotype and STING variants of individual HIV patients might be essential in future clinical trials, such as HIV DNA vaccine trials. Further studies on genetic variants involving STING or any of the other molecules of the innate DNA-sensing machinery could therefore provide a better understanding of HIV pathogenesis and also support selection of optimal treatment options for each individual HIV patient.

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Disclosures

The authors have no financial conflicts of interest.

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