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HIV-1 with gag processing defects activates cGAS sensing

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Abstract

Background Detection of viruses by host pattern recognition receptors induces the expression of type I interferon (IFN) and IFN-stimulated genes (ISGs), which suppress viral replication. Numerous studies have described HIV-1 as a poor activator of innate immunity *in vitro*. The exact role that the viral capsid plays in this immune evasion is not fully understood.

Results To better understand the role of the HIV-1 capsid in sensing we tested the effect of making HIV-1 by co-expressing a truncated Gag that encodes the first 107 amino acids of capsid fused with luciferase or GFP, alongside wild type Gag-pol. We found that unlike wild type HIV-1, viral particles produced with a mixture of wild type and truncated Gag fused to luciferase or GFP induced a potent IFN response in THP-1 cells and macrophages. Innate immune activation by Gag-fusion HIV-1 was dependent on reverse transcription and DNA sensor cGAS, suggesting activation of an IFN response by viral DNA. Further investigation revealed incorporation of the Gag-luciferase/GFP fusion proteins into viral particles that correlated with subtle defects in wild type Gag cleavage and a diminished capacity to saturate restriction factor TRIM5α, likely due to aberrant particle formation. We propose that expression of the Gag fusion protein disturbs the correct cleavage and maturation of wild type Gag, yielding viral particles that are unable to effectively shield viral DNA from detection by innate sensors including cGAS.

Conclusions These data highlight the crucial role of capsid in innate evasion and support growing literature that disruption of Gag cleavage and capsid formation induces a viral DNA- and cGAS-dependent innate immune response. Together these data demonstrate a protective role for capsid and suggest that antiviral activity of capsid-targeting antivirals may benefit from enhanced innate and adaptive immunity in vivo.

Keywords HIV-1, DNA sensing, Capsid, Interferon, cGAS

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Background

Viral infection can be sensed by host pattern recognition receptors (PRRs) that detect viral nucleic acids and/or proteins. PRR engagement activates transcription factors belonging to the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon (IFN) regulatory factor (IRF) families, to induce expression of type I IFNs and inflammatory cytokines and chemokines [1]. IFNs activate signalling cascades dependent on Janus kinase (JAK) and signal transducer and activator of transcription (STAT) and the expression of IFN-stimulated genes (ISGs), including viral restriction factors [2]. A series of studies have demonstrated sensing of HIV-1 by RNA and DNA sensors. For example, the RNA genome has been reported to be sensed by DDX3 [3] and MDA5 [4] and viral DNA reverse transcripts by cyclic GMP-AMP synthase (cGAS) [5–7], IFI16 [8, 9], PQBP1 [10, 11] and NONO [12]. Further, DDX41 may sense RNA/DNA hybrids formed during reverse transcription [13]. Importantly, the central HIV DNA sensor appears to be cGAS, as it is required for HIV detection by other DNA sensors. cGAS is DNA sequence independent and when activated catalyses synthesis of cyclic GMP-AMP (2′,3′-cGAMP) [14–16] which induces STING phosphorylation and translocation to perinuclear regions [17]. STING recruitment of TBK1 and IRF3 results in IRF3 phosphorylation by TBK1 and IRF3 nuclear translocation [18, 19]. Activated STING also activates IKK and the NF-κB family of transcription factors [20], which with IRF3, activate expression of type I IFN and subsequently ISGs. ISGs include an array of anti-HIV restriction factors including APOBEC3G, SAMHD1, tetherin, TRIM5α, MxB and the IFITMs [21]. Despite all these examples of HIV-1 sensing, other studies demonstrate HIV replication in permissive primary cells without IFN induction. We hypothesise that sensing is context and particularly viral dose dependent. Thus whilst high dose infection can be sensed, particularly in cells that do not support HIV replication, e.g. dendritic cells [6, 22], in permissive macrophages and T-cells, HIV-1 replication is a poor stimulator of IFN [23, 24] and the virus can replicate without triggering innate immune sensing through hiding nucleic acid PAMPs inside intact capsids [7, 25, 26], which uncoat and release genome inside the nucleus immediately prior to integration [27–30].

Growing evidence supports a crucial role for cellular cofactors in HIV-1 avoiding host immunity. Recruitment of cleavage and polyadenylation specificity factor 6 (CPSF6) and cyclophilin A (cypA) promote evasion of sensing, with cypA being particularly important for escaping HIV-1 capsid sensing by TRIM5α [7, 31]. Conversely, other cellular proteins that target the HIV-1 capsid, including NONO [12] and PQBP1 [11], have been described to promote sensing by cGAS. In order to better understand the role of the HIV-1 capsid in sensing, and establish whether it promotes evasion, or is responsible for HIV-1 detection in infected cells, we tested the effect of making HIV-1 by co-expressing a truncated Gag encoding the first 107 amino acids of capsid fused with either luciferase or GFP with wild type Gag-pol. We found that truncated Gag fused to luciferase or GFP was incorporated into viral particles, yielding virions with subtle defects in wild type Gag cleavage, a reduced capacity to saturate TRIM5α and a failure to shield viral DNA from cGAS detection. These findings further evidence a role for the HIV-1 capsid in protecting HIV-1 genome from being sensed and support a model in which the principle function of capsid is to protect viral genomes from sensors to promote replication in sensing-competent target cells.

Results

HIV-1 gag-fusion viruses trigger a robust type I IFN-dependent innate immune response in THP-1 cells

Whilst seeking to design an HIV-1 Gag fusion virus expressing firefly luciferase (LUC) in frame with capsid (CA) (Gag-LUC), we found that mixed viral particles made by co-transfecting Gag-LUC (Suppl Fig. 1, Fig. 1A) with wild type Gag-pol, and a VSV-G envelope, triggered sensing in THP-1 cells. Gag-LUC was based on HIV-1 LAI strain [32] and also encodes GFP in the place of Nef. It activated a dose-dependent innate immune response whilst the WT VSV-G pseudotyped ΔEnv LAI-GFP did not, as previously observed [26] (LAI, Suppl Fig. 1, Fig. 1A). Innate induction was assessed by measuring luciferase activity in the supernatants of infected monocytic THP-1 cells that had been modified to express Gaussia luciferase under the control of the *IFIT-1* (also known as *ISG56*) promoter, which is both IRF-3- and IFN-sensitive [33]. Virus dose in these experiments was normalised according to RT activity, as measured by SG-PERT (see Methods). Mixed particles were equally infectious in cell lines as the number of infectious units per unit of RT (Suppl Fig. 2A), or per genome copy (Suppl Fig. 2B), was equivalent between WT and Gag-fusion viruses. Innate induction was not unique to the Gag-luciferase fusion as a second HIV-1 LAI virus carrying a similar Gag-GFP fusion also resulted in dose-dependent ISG induction (Gag-GFP, Suppl Fig. 1, Fig. 1A), ruling out an immunostimulatory feature in the luciferase sequence. Fusion of Gag to either GFP or luciferase makes it non-functional, therefore co-transfection with a WT Gag-pol packaging construct (e.g. p8.91, Suppl. Figure 1) is required to produce infectious particles. To rule out differences in 8.91 and LAI Gag sequences/proteins that could potentially explain the observed differences in innate immune activation, we also co-transfected WT LAI with 8.91 Gag-pol by co-transfecting the ΔEnv

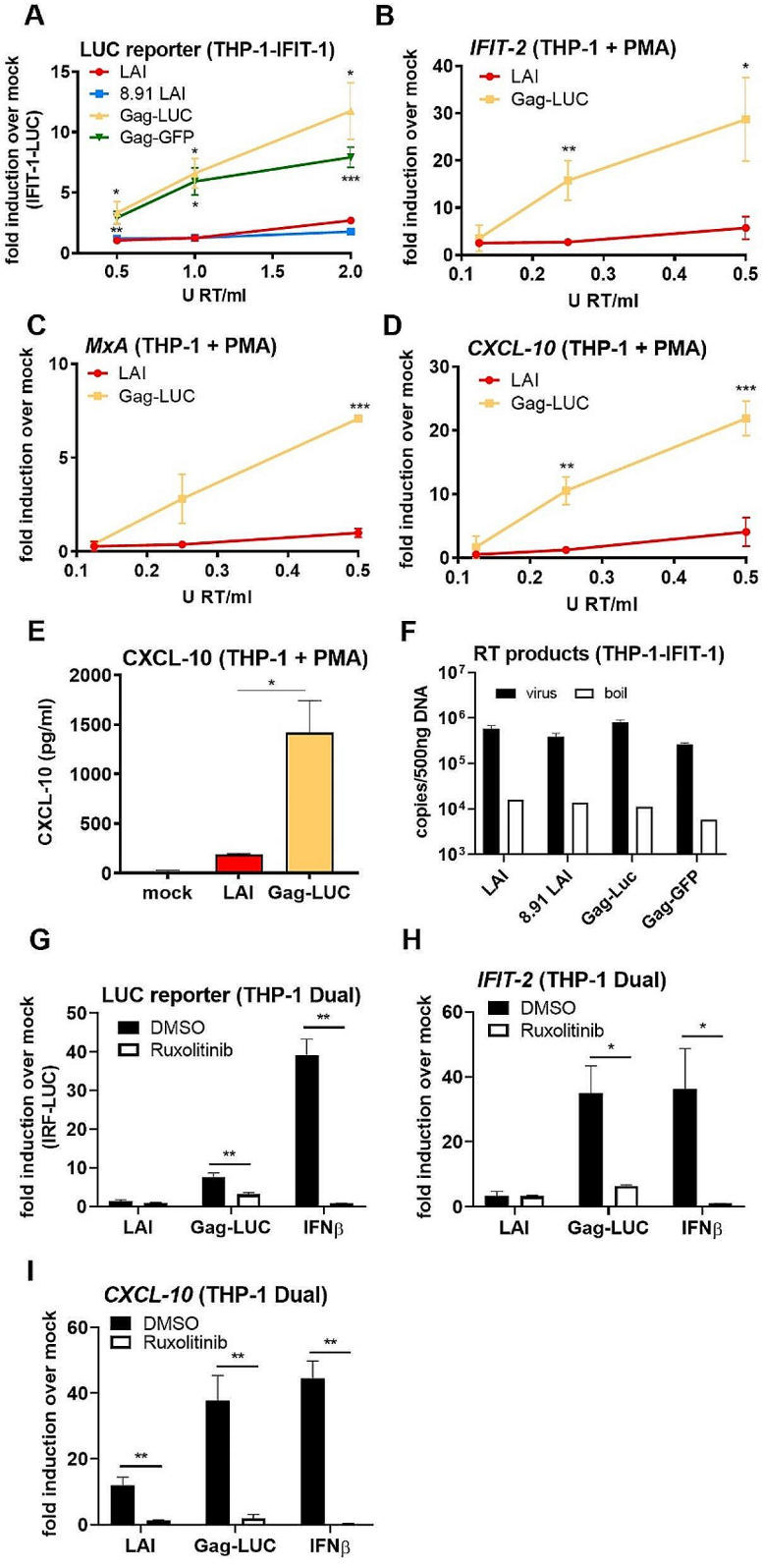

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Fig. 1 HIV-1 expressing a Gag-fusion protein triggers a type I IFN response in THP-1 cells. **A**: IFIT-1 reporter activity from monocytic THP-1-IFIT-1 cells transduced for 24 h with WT LAI (LAI), LAI packaged with 8.91 Gag (8.91 LAI), LAI expressing gag fused to luciferase and packaged with 8.91 Gag (Gag-LUC) or LAI expressing Gag fused to GFP and packaged with 8.91 Gag (Gag-GFP) (See Suppl. Figure 1) at 0.5, 1 or 2 U RT/ml. **B-D**: ISG qRT-PCR from PMA-treated THP-1 shSAMHD1 cells transduced for 24 h with LAI or Gag-LUC viruses at 0.125, 0.25 and 0.5 U RT/ml. **E**: CXCL-10 protein in supernatants from B-D (0.5 U RT/ml, ELISA). **F**: RT products from THP-1-IFIT-1 cells transduced for 24 h with 1 U RT/ml of the indicated viruses or transduced with boiled virus as a control. **G**: IRF reporter activity from monocytic THP-1 Dual cells transduced for 24 h with 1.5 U RT/ml LAI or Gag-LUC viruses, or stimulated with 1 ng/ml IFNβ as a control, in the presence of DMSO vehicle or 2 μM ruxolitinib. **H, I**: ISG qRT-PCR from monocytic THP-1 Dual cells transduced for 24 h with 1.5 U RT/ml LAI or Gag-LUC viruses, or stimulated with 1 ng/ml IFNβ as a control, in the presence of DMSO vehicle or 2 μM ruxolitinib. Data are mean ± SD from biological triplicates of a single experiment, representative of at least 3 repeats. Statistical analyses were performed using Student's t-test, with Welch's correction where appropriate, comparing each virus with WT LAI at the same dose (A-E) or pairs of samples -/+ ruxolitinib (G-I). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

LAI genome and p8.91 packaging construct (8.91 LAI, Suppl Fig. 1). This virus behaved the same as WT ΔEnv LAI alone and failed to induce ISG reporter activity at the doses tested, thus ruling out differences in Gag as an explanation for ISG induction in the Gag fusion viruses (Fig. 1A).

To confirm the findings above from monocytic THP-1 cells, we also infected PMA differentiated THP-1 cells stably depleted for restriction factor SAMHD1. SAMHD1 was depleted to permit HIV transduction [26, 34]. The Gag-LUC virus, but not WT LAI, again induced high levels of endogenous ISGs *IFIT-2* (Fig. 1B), *MxA* (Fig. 1C) and *CXCL-10* (Fig. 1D) measured by qPCR, as well as CXCL-10 protein (Fig. 1E), measured by ELISA. Levels of viral reverse transcripts were equivalent in WT- and Gag fusion virus-infected cells, as assessed by qPCR (Fig. 1F).

To assess whether Gag-fusion viruses induced type I IFN production we infected THP-1 Dual reporter cells (Invivogen) that also express luciferase under the control of an IRF- and ISG-sensitive promoter, in the presence of JAK1/2 inhibitor ruxolitinib [35]. Signal transduction downstream of the type I IFN receptor is dependent on JAK and thus ruxolitinib efficiently blocks IFNβ-induced ISG induction (Fig. 1G-I). Expression of luciferase (Fig. 1G), as well as endogenous *IFIT-2* (Fig. 1H) and *CXCL-10* (Fig. 1I) was significantly reduced following ruxolitinib treatment of Gag-LUC-infected cells indicating that infection with this Gag-LUC fusion virus induces type I IFN production, to induce endogenous ISG and IFN reporter expression.

HIV-1 gag-fusion viruses activate a restrictive type I IFN response in primary macrophages

To determine whether HIV-1 Gag-fusion viruses also induced a type I IFN response in primary human cells we infected primary monocyte-derived macrophages (MDM) with the Gag-LUC virus and the corresponding pseudotyped WT LAI strain and measured ISG induction by qPCR and ELISA. As in THP-1 cells, infection of MDM with Gag-LUC induced a robust type I IFN response leading to significantly higher expression of *CXCL-10* (Fig. 2A), *IFIT-2* (Fig. 2B) and *MxA* (Fig. 2C), as well as CXCL-10 protein (Fig. 2D) compared to VSV-G pseudotyped LAI infection, all of which was reduced by ruxolitinib treatment. Gag-LUC virus infection levels were lower in MDM than WT LAI at the same input dose, assessed by measuring GFP-positive cells by flow cytometry, and this was partially rescued by blocking IFN signalling with ruxolitinib indicating an IFN-dependent suppression of infection (Fig. 2E). Taken together, Gag-fusion viruses, unlike their WT counterparts, induce a robust type I IFN response, which is restrictive even in a single round infection in primary macrophages.

IFN induction by HIV-1 gag-fusion viruses is dependent on viral DNA synthesis

To establish whether the source of immune stimulation during Gag-fusion virus infection was the viral genome, reverse transcripts, or a later stage of infection we generated Gag-LUC viruses that were defective for reverse transcription (Gag-LUC RT D185E) or integration (Gag-LUC INT D116N) by co-transfecting p8.91 Gag-pol carrying the RT D185E and INT D116N mutations. Luciferase IFN reporter in monocytic THP-1 IFIT-1 reporter cells (Fig. 3A) and endogenous ISG induction in PMA-differentiated THP-1 shSAMHD1 cells (Fig. 3B-D) was entirely RT-dependent (RT mutant did not trigger sensing) and did not require integration (Integrase mutant triggered normally, or even slightly higher). Concordantly, reporter activity (Fig. 3E) and ISG expression (Fig. 3F, G) was also significantly reduced in monocytic THP-1 Dual reporter cells following treatment with RT inhibitor nevirapine, but not with integrase inhibitor raltegravir.

As expected, no GFP positive cells were observed following Gag-LUC RT D185E infection (Suppl Fig. 3A, B) and levels of infectivity were also significantly reduced following nevirapine treatment (Suppl Fig. 3C). Whilst GFP positivity was minimal in integrase defective Gag-LUC infection in differentiated THP-1 cells (Suppl Fig. 3B), GFP positive cells were still detected with Gag-LUC INT D116N infection (Suppl Fig. 3A) or following raltegravir treatment (Suppl Fig. 3C) in monocytic THP-1 cells. This is in agreement with our previous findings [26] and likely due to GFP expression from unintegrated 2'-LTR circles that have been observed in other cell types [36, 37]. Together, these data rule out the viral

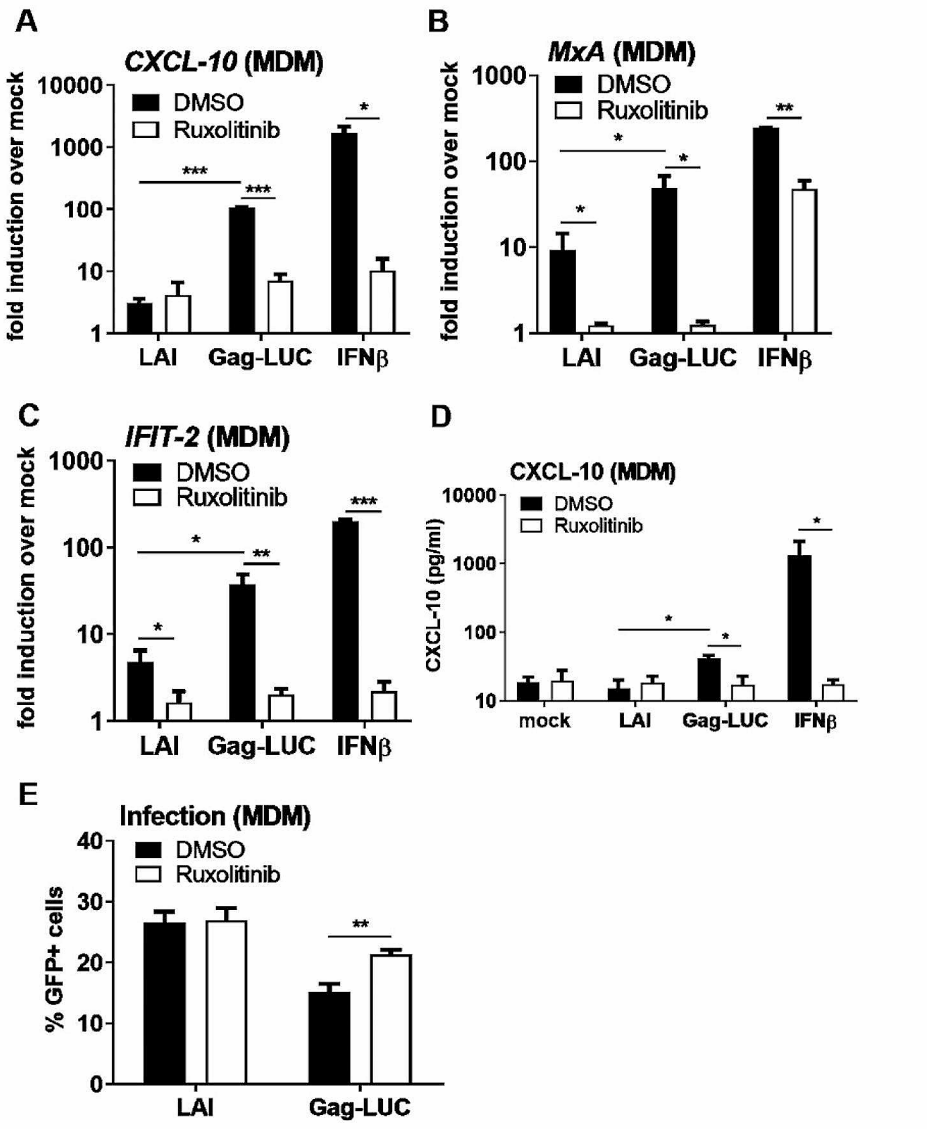

Fig. 2 HIV-1 Gag-fusion viruses activate a restrictive type I IFN response in primary macrophages. **A-C**: ISG qRT-PCR from primary MDM transduced for 24 h with 0.5 U RT/ml LAI or Gag-LUC viruses, or stimulated with 1 ng/ml IFNβ as a control, in the presence of DMSO vehicle or 2 μM ruxolitinib. **D**: CXCL-10 protein in supernatants from A-C (ELISA). **E**: Infection data from A-D measured by flow cytometry at 48 h. Data are mean ± SD from biological triplicates of a single experiment, representative of at least 3 repeats. Statistical analyses were performed using Student's t-test, with Welch's correction where appropriate, comparing pairs of samples -/+ ruxolitinib as indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

RNA genome as the immunostimulatory feature of the Gag-fusion viruses and instead point to innate immune detection of viral DNA.

ISG induction by HIV-1 gag-fusion virus is dependent on cGAS and STING

To further investigate the source for immune stimulation in the Gag-fusion viruses we sought to determine which host innate sensors were required for innate immune detection. As expected, THP-1 IFIT-1 reporter cells lacking STING failed to respond to herring testis DNA (HT-DNA) stimulation, but did respond to transfected RNA mimic poly I: C and TLR4 agonist lipopolysaccharide (LPS). MAVS -/- cells responded to HT-DNA and LPS, but not transfected poly I: C (Suppl. Figure 4A). Luciferase reporter activity (Fig. 4A) and endogenous ISG expression (Fig. 4B, C) of Gag-LUC infection was entirely dependent on STING. Levels of infection were equivalent between WT and STING- or MAVS-null cells (Suppl Fig. 4B). Furthermore, THP-1 Dual cells lacking cGAS failed to respond to HT-DNA (Suppl Fig. 4C) and Gag-LUC infection (Fig. 4D-F), consistent with a cGAS/STING-dependent DNA sensing response. Again, levels of infection were equivalent in WT and cGAS-/- cells

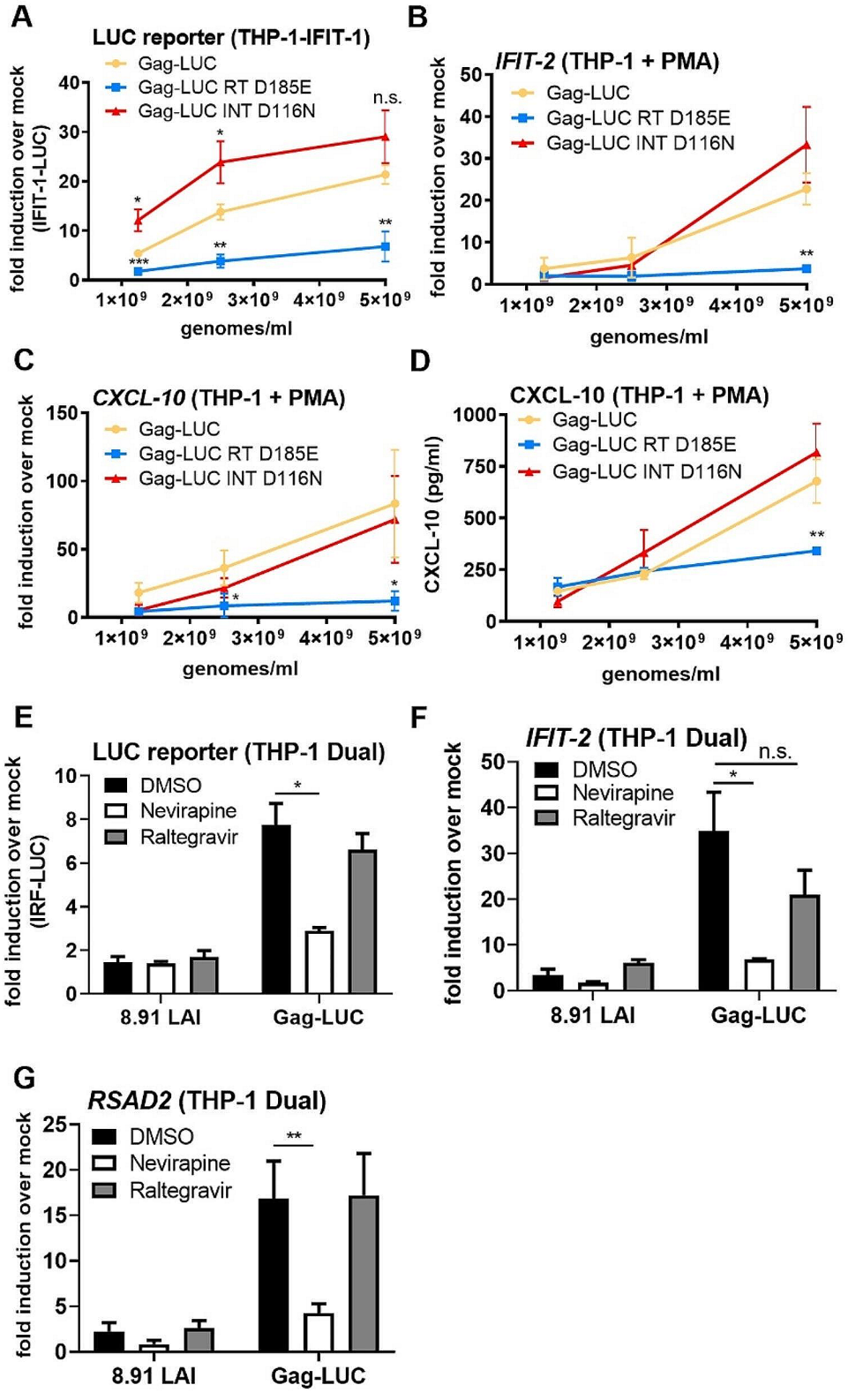

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Fig. 3 ISG induction by HIV-1 Gag-fusion virus is RT-dependent. **A**: IFIT-1 reporter activity from monocytic THP-1-IFIT-1 cells transduced for 24 h with Gag-LUC, RT-defective Gag-LUC (Gag-LUC RT D185E) or integrase-defective Gag-LUC (Gag-LUC INT D116N) at 1.25×10^9, 2.5×10^9 and 5×10^9 genomes/ml. **B, C**: ISG qRT-PCR from PMA-treated THP-1 shSAMHD1 cells transduced for 24 h with Gag-LUC, Gag-LUC RT D185E or Gag-LUC INT D116N at 1.25×10^9, 2.5×10^9 and 5×10^9 genomes/ml. **D**: CXCL-10 protein in supernatants from B, C (ELISA). **E**: IRF reporter activity from THP-1 Dual cells transduced for 24 h with 8.91 LAI or Gag-Luc (1.5 U RT/ml) in the presence of DMSO vehicle, 5 μM neviripine or 10 μM raltegravir. **F, G**: ISG qRT-PCR from THP-1 Dual cells transduced for 24 h with 8.91 LAI or Gag-Luc (1.5 U RT/ml) in the presence of DMSO vehicle, 5 μM neviripine or 10 μM raltegravir. Data are mean ± SD from biological triplicates of a single experiment, representative of at least 3 repeats. Statistical analyses were performed using Student's t-test, with Welch's correction where appropriate, comparing mutant viruses with WT Gag-LUC at the same dose (A-D) or to the DMSO control as indicated (E-G). *$P < 0.05$, **$P < 0.01$, n.s. non-significant

(Suppl Fig. 4D). Finally luciferase reporter activity in Gag-LUC infected THP-1 Dual cells was significantly reduced in the presence of STING inhibitor H151 [38] and cGAS inhibitor RU.521 [39] (Fig. 4G, Suppl Fig. 4E), confirming cGAS/STING-dependent sensing of viral reverse transcripts during Gag-fusion virus infection.

Gag-fusion viruses display subtle defects in maturation and are less able to saturate TRIM5α

Given that the genome sequences of the LAI and Gag-LUC/Gag-GFP viruses only differ by the inclusion of the chimeric Gag-LUC/GFP reporter gene, and encode for all the same accessory proteins, we hypothesised that rather than specific features of the genome enhancing sensing, the Gag-fusion viruses may have defects in particle maturation and instead fail to efficiently shield RT products from cGAS. Indeed, immunoblotting of extracted viral particles (purified through sucrose cushion ultracentrifugation) with anti-luciferase or anti-GFP antibodies showed evidence of incorporation of Gag-LUC/GFP fusion proteins, or their cleavage products, in mixed particles (Fig. 5A). Importantly, incorporation of these fusions correlated with additional p24 capsid-positive bands not observed with WT HIV-1 (LAI, 8.91+LAI, Fig. 5A). These additional bands resulted from 8.91-derived WT Gag and not the Gag-GFP or Gag-LUC plasmids as transfection of HEK293T cells with Gag-GFP or Gag-LUC alone in the absence of the 8.91 Gag-pol plasmid did not yield any bands recognised by the p24 capsid antibody in either the whole cell lysate or released into the cell supernatant (Suppl. Figure 5). This was in contrast to transfection with the 8.91 plasmid alone which yielded p24-positive bands in both the cell lysate and supernatant as expected (Suppl. Figure 5). Transfection of the Gag-GFP and Gag-LUC plasmids was confirmed by blotting for either the Gag-GFP fusion protein or free GFP respectively. Taken together these data suggest that the presence of Gag-LUC/GFP causes subtle defects in WT Gag processing (Fig. 5A).

To assess HIV-1 core integrity in the Gag-fusion viruses we measured their ability to saturate rhesus monkey TRIM5α in an abrogation-of-restriction assay. Rhesus monkey TRIM5α binds and forms hexameric cage-like structures around intact HIV capsid lattices [40, 41], leading to proteasome-dependent viral disassembly and subsequent innate immune activation [42–44]. Restriction by TRIM5α can be overcome by co-infection with high doses of a saturating virus, dependent on the stability of the incoming viral capsid [45, 46]. The Gag-LUC fusion protein was cloned into the p8.91 Gag-pol packaging plasmid and HEK 293T cells were transfected with varying proportions of WT or Gag-LUC p8.91, thus producing VSV-G psuedotyped mixed particles with increasing amounts of Gag-fusion protein. In all cases the same genome expressing luciferase (CSLW) was packaged. Rhesus FRhK cells were then co-infected with a fixed dose of HIV-1 LAI bearing GFP and increasing doses of the WT/Gag-LUC chimeric viruses. Flow cytometry was used to assess rescue of HIV-1 LAI infectivity from TRIM5α restriction measuring GFP positive cells. As expected, the virus with 100% WT Gag (0% Gag-LUC) efficiently saturated TRIM5α restriction and rescued HIV-1 LAI GFP expression (Fig. 5B, Suppl. Figure 6). Increasing the proportion of luciferase-fused Gag in the saturating virus, reduced rescue of GFP expression, which reached statistical significance at the highest proportion of Gag-LUC (90% Gag-LUC, Fig. 5B).

We conclude that expression of this Gag-LUC fusion protein during viral production interferes with the maturation process of co-transfected WT Gag, yielding particles with reduced stability and a diminished ability to saturate TRIM5α, which fail to shield their RT products from DNA sensor cGAS. This finding adds to growing literature that intact capsid plays a crucial role in HIV-1 evasion of cGAS and that antiviral activity of capsid-targeting antivirals may benefit from triggering innate immune detection and subsequent antiviral gene expression in vivo.

Discussion

Numerous studies have described HIV-1 as a poor activator of innate immunity in vitro [6, 7, 23, 26] unless infection is high dose or target cells are not usually permissive to HIV replication e.g. dendritic cells [6, 22]. This suggests that, like many other viruses, HIV-1 has evolved strategies to evade the host response. In addition to encoding accessory proteins that block innate signalling cascades and activation of transcription factors such as NF-κB and IRF3 [47–52], growing evidence points to a critical role for capsid in innate immune evasion.

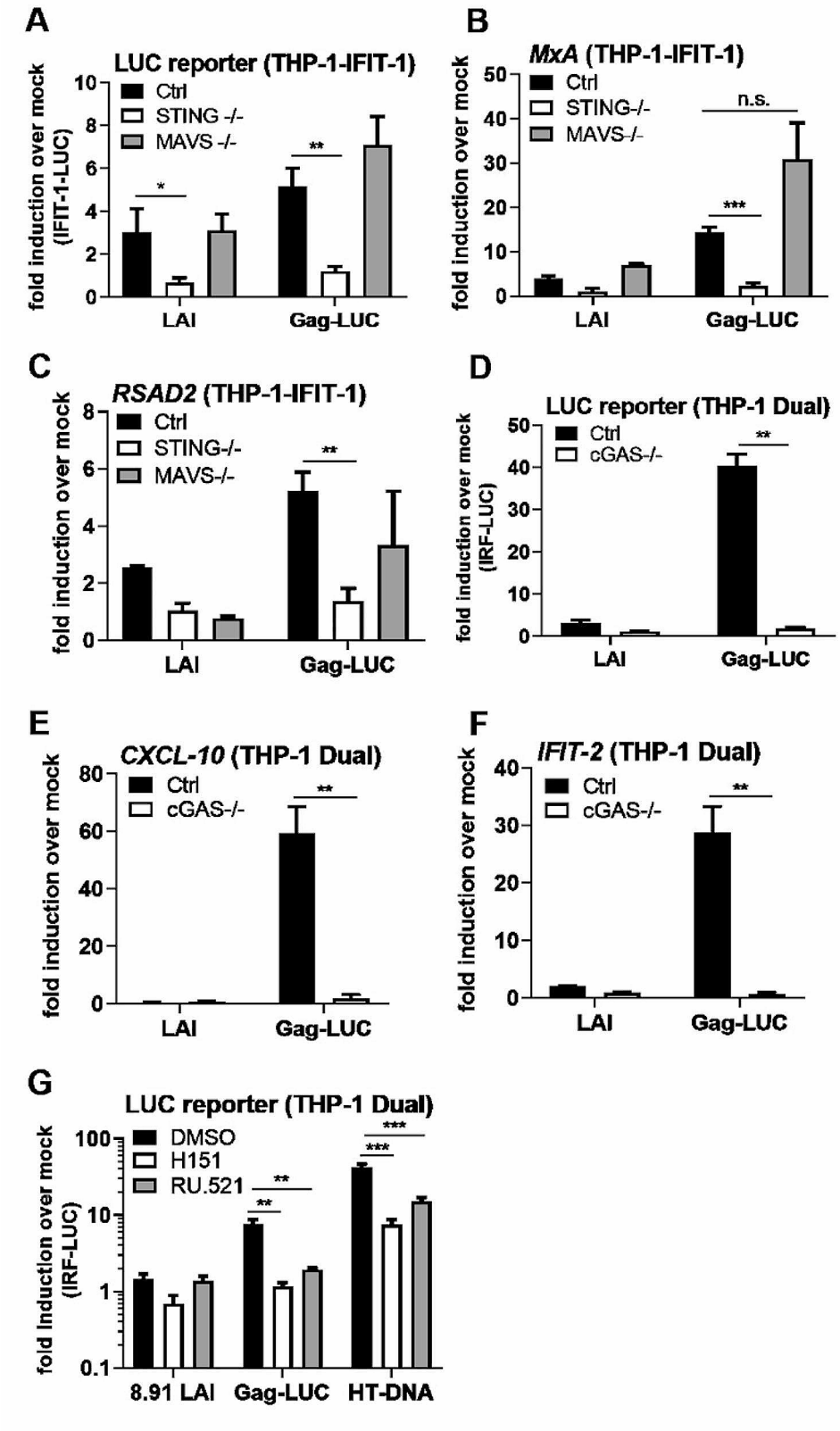

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Fig. 4 ISG induction by HIV-1 Gag-fusion virus is dependent on cGAS and STING. **A**: IFIT-1 reporter activity from monocytic THP-1-IFIT-1 cells lacking STING or MAVS, or a gRNA control (Ctrl) cell line transduced for 24 h with WT LAI or Gag-LUC (1.5 U RT/ml). **B, C**: ISG qPCR from monocytic THP-1-IFIT-1 cells lacking STING or MAVS, or a gRNA control (Ctrl) cell line transduced for 24 h with WT LAI or Gag-LUC (1.5 U RT/ml). **D**: IRF reporter activity from monocytic THP-1 Dual cells lacking cGAS, or a gRNA control (Ctrl) cell line transduced for 24 h with WT LAI or Gag-LUC (1.5 U RT/ml). **E, F**: ISG qPCR from monocytic THP-1 Dual cells lacking cGAS, or a gRNA control (Ctrl) cell line transduced for 24 h with WT LAI or Gag-LUC (1.5 U RT/ml). **G**: IRF reporter activity from monocytic THP-1 Dual cells lacking cGAS, or a gRNA control (Ctrl) cell line transduced for 24 h with WT LAI or Gag-LUC (1.5 U RT/ml), or stimulated by transfection with 0.05 µg/ml HT-DNA in the presence of DMSO vehicle, 0.5 µg/ml STING inhibitor H151 or 10 µg/ml cGAS inhibitor RU.521. Data are mean ± SD from biological triplicates of a single experiment, representative of at least 3 repeats. Statistical analyses were performed using Student's t-test, with Welch's correction where appropriate, comparing to Ctrl cells (A-F), or to DMSO vehicle treated cells (G) as indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. non-significant

Cellular cofactors CPSF6 and cyclophilin A are recruited by capsid and are critical for evasion of sensing, the latter being important for avoiding TRIM5α restriction [7, 31]. Encapsidated DNA synthesis is expected to protect viral RT products from DNA sensors such as cGAS and from degradation by cellular nucleases such as TREX-1 [7, 45, 53]. Supporting this, recent studies have linked capsid stability to activation of cGAS sensing [54], including our own work demonstrating that disrupting capsid maturation using protease inhibitors, or by mutating cleavage sites in Gag, yields aberrant viral particles that fail to protect RT products from cGAS [26]. Furthermore, differences in the ability of HIV-1, and HIV-2 and other non-pandemic lentiviruses to evade innate immunity has been mapped to the viral capsid, with the ability to evade cGAS activation and TRIM5α correlating with pandemicity [6, 25]. In this study we report the unexpected finding that unlike WT HIV-1, HIV-1 viruses carrying a truncated Gag fusion protein trigger a robust type I IFN response in macrophages (Figs. 1 and 2), dependent on reverse transcription (Fig. 3) and host DNA sensing machinery cGAS and STING (Fig. 4). Importantly, virus made with Gag fusions showed evidence of maturation defects and had a reduced capacity to saturate restriction factor TRIM5α in an abrogation-of-restriction assay, indicative of defective capsids (Fig. 5). This work adds to a growing body of evidence that the HIV-1 capsid plays a crucial role in shielding RT products from cGAS.

Exactly how the expression of Gag fused to a reporter gene such as luciferase or GFP inhibits Gag cleavage and functional capsid formation is not known. Immunoblotting analysis of virions revealed luciferase or GFP positive bands in the Gag-LUC/Gag-GFP viruses (Fig. 5A) suggesting that Gag fusion proteins can be incorporated into nascent virions, subtly interfering with WT Gag processing and reducing their capacity to saturate TRIM5α in an abrogation of restriction assay (Fig. 5B). Although subtle, these cleavage defects had profound consequences in sensing competent cells, such as macrophages, where they induced a cGAS-dependent IFN response (Figs. 1 and 2). Similar results were observed with viruses that were treated with low doses of protease inhibitors (lopinavir, darunavir) or mixed particles containing a mutant of Gag that cannot be cleaved between CA and spacer peptide 1 (CA-SP1), where again even small defects in Gag processing led to cGAS sensing in macrophages [26]. Of note, high proportions of Gag-LUC in mixed particles were required to observe significant defects in TRIM5α saturation in the abrogation of restriction assay, despite lower proportions of Gag-LUC triggering sensing. This is likely a reflection on the sensitivity of the abrogation of restriction assay whereby relatively large defects in capsid integrity/stability are necessary to observe appreciable reduction in the ability of these viruses to saturate TRIM5α. Again similar effects were observed with HIV-1 produced in the presence of very low doses lopanivir or with a small proportion of CA-SP1 that triggered sensing but were not significantly defective for saturating TRIM5α [26]. The Gag fusion viruses used in this study may have altered stability, may prematurely uncoat and subsequently activate a potent host innate response that is not observed for similar doses of WT virus. Furthermore, interactions with host proteins may also differ, and whether the Gag-LUC and Gag-GFP viruses still interact appropriately with cofactors such as CPSF6 and cypA, or incorporate the capsid stabilising cellular metabolite inositol hexakisphosphate (IP6) that is dependent on the immature lattice [55], remains to be determined.

Thus far cGAS has been described to sense DNA in a sequence-independent manner [15, 56], but whether there are particular features of viruses or their genomes that enhance recognition is unclear. Additional proteins may be involved in fine-tuning the cGAS response or breaking capsid open to expose viral DNA within. For example, PQBP1 has recently been described to directly bind and decorate the HIV-1 capsid, 'licensing' it for subsequent cGAS recruitment and sensing of viral DNA [11]. Furthermore nuclear protein NONO has been shown to promote cGAS sensing of HIV-2 [12]. Whether HIV-1 can be sensed at the point of integration has not been fully explored. Of note we did observed an increase in innate sensing with Gag-LUC carrying the D116N integration mutation in some assays (Fig. 3A), which may hint that defects in integration, particularly those associated with D116N, may influence sensing.

As we have previously observed [26], activation of an IFN response by maturation defective viruses during single round infection of THP-1 cells was not sufficient

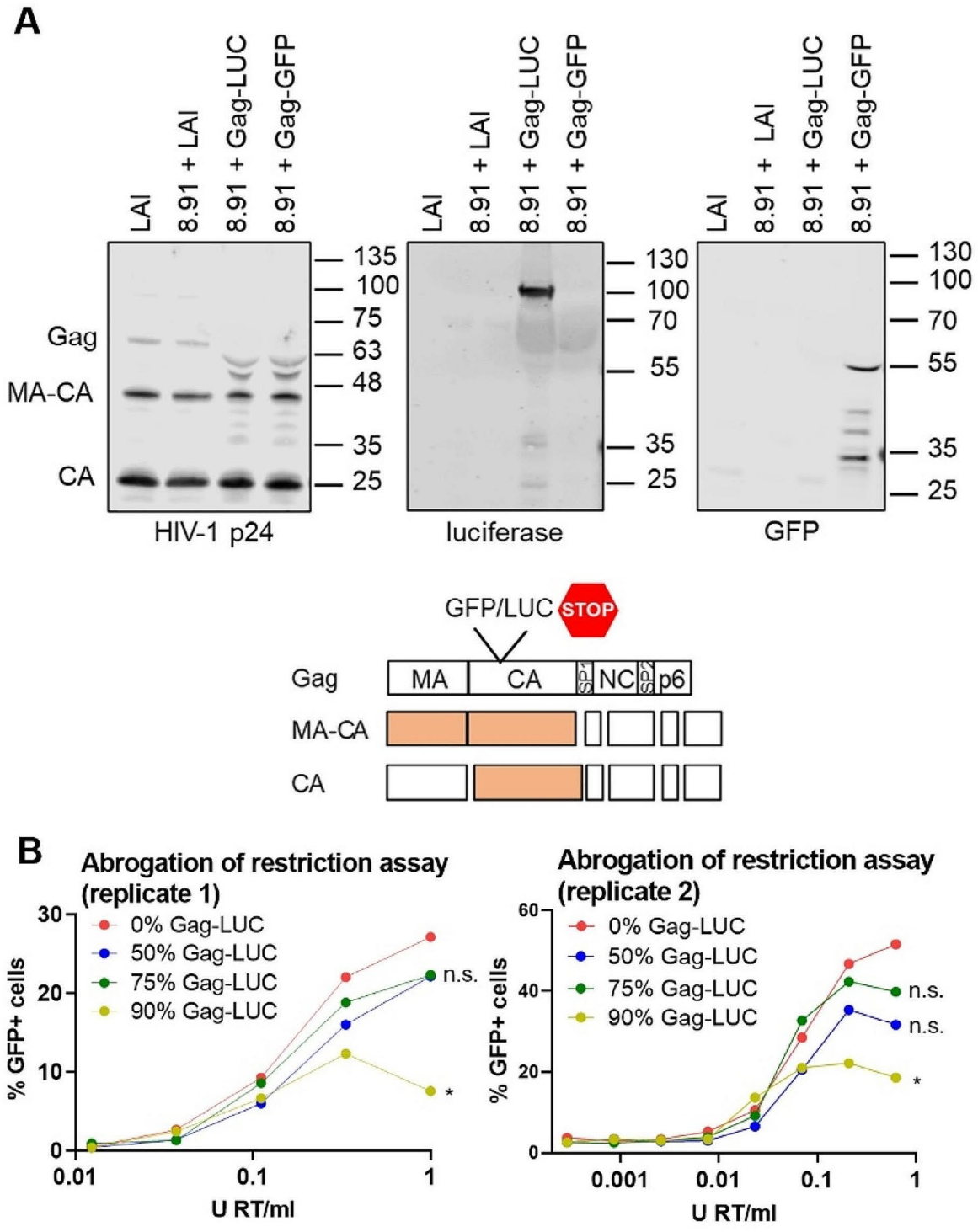

Fig. 5 Gag-fusion viruses display defects in Gag processing and are less able to saturate TRIM5α. **A**: Immunoblot of WT LAI, 8.91 LAI, Gag-LUC and Gag-GFP virus particles (2×10^{11} genomes) detecting HIV-1 p24, firefly luciferase or GFP and a schematic of Gag cleavage products. MA: matrix, CA: capsid, SP1: spacer peptide 1, NC: nucleocapsid, SP2: spacer peptide 2 and location of the GFP/luciferase gene insertion. **B**: Abrogation-of-restriction assay in FRhK4 cells expressing restrictive rhesus TRIM5. FRhK4 cells were co-transduced with a fixed dose of WT LAI.GFP (5×10^7 genomes/ml) and increasing doses of the WT/Gag-LUC chimeric viruses carrying a luciferase-expressing genome (0.0005–1 U RT/ml). Rescue of GFP infectivity was assessed by flow cytometry at 48 h. Data are singlet % GFP values and two repeats of the experiment are shown. Statistical analyses were performed using 2-way ANOVA with multiple comparisons. * $P < 0.05$, n.s. non-significant

to block infection, with WT and Gag-LUC/GFP viruses being equally infectious in THP-1 and U87 cells (Suppl Fig. 2). Infectivity of the Gag-LUC virus was however reduced compared to WT in primary macrophages, and this was partially rescued by blocking IFN signalling (Suppl Fig. 2E). Primary cells may express higher levels of IFN, be more sensitive to IFN, or may express a wider range of restrictive ISGs than cell lines such as THP-1 that could explain these differences. Unprotected RT products during Gag-LUC infection may also be subject to degradation by TREX1, which could also account for some of the remaining restriction in MDM.

In summary we have discovered an unanticipated effect on the maturation of WT Gag by coexpression of a truncated Gag fusion protein, yielding viral particles that fail to shield their DNA from cGAS and induce a restrictive type I IFN response in macrophages. This finding supports the crucial role of capsid in innate immune evasion and highlights this viral protein as an important target for novel therapeutics. Indeed, it will be interesting to test whether recently described capsid-targeting inhibitors, such as those from Gilead [57], also induce sensing of HIV-1 RT products as we recently demonstrated for PF-74 [26], which accelerates capsid opening [58]. Likewise, maturation inhibitors such as bevirimat [59] may also lead to enhanced sensing in a similar manner to that observed with protease inhibitors [26]. It remains to be seen whether capsid or protease inhibitors leverage innate immune responses to improve their efficacy in vivo.

Materials and methods

Cells and reagents

HEK293T, FRhK and U87 cells were maintained in DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS, Labtech) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Pen/Strep; Gibco). THP-1 cells were maintained in RPMI (Gibco) supplemented with 10% FBS and Pen/Strep. THP-1-IFIT-1 cells that had been modified to express Gaussia luciferase under the control of the *IFIT-1* promoter [33] and versions lacking MAVS or STING [34] were described previously. THP-1 cells stably depleted for SAMHD1 were also previously described [26]. THP-1 Dual Control and cGAS-/- cells were obtained from Invivogen. Nevirapine and raltegravir were obtained from AIDS reagents. STING inhibitor H151 and cGAS inhibitor RU.521 were obtained from Invivogen. JAK inhibitor ruxolitinib was obtained from CELL guidance systems. Lipopolysaccharide and IFNβ were obtained from Peprotech. Herring-testis DNA was obtained from Sigma. cGAMP and poly I: C were obtained from Invivogen. For stimulation of cells by transfection, transfection mixes were prepared using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Isolation of primary monocyte-derived macrophages

Primary monocyte-derived macrophages (MDM) were prepared from fresh blood from healthy volunteers as described previously [26]. The study was approved by the joint University College London/University College London Hospitals NHS Trust Human Research Ethics Committee and written informed consent was obtained from all participants. Replicate experiments were performed with cells derived from different donors.

Generation of gag fusion, RT D185E and INT D116N viruses

pLAIΔEnvGFP.Gag-LUC/GFP and p8.91 Gag-LUC were generated by cloning the firefly luciferase gene/GFP into the unique SpeI site of CA (after amino acid 107) followed by a stop codon. pLAIΔEnvGFP.Gag-LUC RT D185E and INT D116N were generated by site-directed mutagenesis using Pfu Turbo DNA polymerase (Agilent) and the following primers:

LAI_ RT D185E fwd: 5' ATAGTTATCTATCAATACATG GAAGATTTGTATG 3'.
LAI_ RT D185E rev: 5' AAGTCAGATCCTACATACAA ATCTTCCATGTATTG 3'.
LAI_ INT D116N fwd: 5' GGCCAGTAAAAACAATACA TACAAACAATGGCAGC 3'.
LAI_ INT D116N rev: 5' ACTGGTGAAATTGCTGCCA TTGTTTGTATGTATTG 3'.

In all cases mutated sequences were confirmed by sequencing, excised by restriction digestion and cloned back into the original plasmid.

Viral production in HEK293T cells

Lentiviral particles were produced by transfection of HEK293T cells in T150 flasks using Fugene 6 transfection reagent (Promega) according to the manufacturer's instructions. For LAI WT each flask was transfected with 2.5 µg of VSV-G glycoprotein expressing plasmid pMDG (Genscript) and 6.25 µg pLAIΔEnvGFP (Suppl. Figure 1). For viruses requiring a packaging plasmid each flask was transfected with 2.5 µg of pMDG (Genscript), 2.5 µg of p8.91 (encoding Gag-Pol, Tat and Rev) [60], and 3.75 µg of genome plasmid (pLAIΔEnvGFP, pLAIΔEnvGFP.Gag-LUC, pLAIΔEnv.Gag-GFP, Suppl. Figure 1). WT/Gag-LUC chimeric viruses were generated by transfecting cells with 2.5 µg of pMDG, 3.75 µg of a firefly luciferase-expressing genome plasmid (CSLW) and varying proportions of p8.91 and p8.91Gag-LUC packaging plasmids, up to 2.5 µg per flask. Virus supernatants were harvested at 48 and 72 h post-transfection, pooled, DNase treated (2 h at 37 °C, DNaseI, Sigma) and subjected to ultracentrifugation over a 20% sucrose cushion. Viral particles

were resuspended in RPMI supplemented with 10% FBS. Viral titres were calculated by infecting PMA-treated THP-1 cells (2×10^5 cells/ml) or U87 cells (10^5 cells/ml) with dilutions of virus in the presence of polybrene (8 μg/ml, Sigma) for 48 h and enumerating GFP-positive cells by flow cytometry using the FACS Calibur (BD). Analysis was performed using FlowJo software.

SG-PERT

Reverse transcriptase activity of virus preparations was quantified by qPCR using a SYBR Green-based product-enhanced RT (SG-PERT) assay as described [61].

Genome copy/RT products measurements

Viral genome copies and RT products were measured by qPCR as previously described using primers specific for GFP [26]:

GFP fwd: 5'- CAACAGCCACAACGTCTATATCAT – 3'.
GFP rev: 5'- ATGTTGTGGCGGATCTTGAAG – 3'.
GFP probe: 5'- FAM-CCGACAAGCAGAAGAACGGCA TCAA-TAMRA – 3'.

As a control for the presence of contaminating plasmid DNA, cells were also transduced in parallel with the same virus preparations that had been boiled for 10 min.

Infection assays

THP-1 cells were infected at a density of 2×10^5 cells/ml in 24 well plates for luciferase reporter assays or 12 well plates for qPCR and ELISA. For differentiation, THP-1 cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Peprotech) for 48 h. Infections in THP-1 cells were performed in the presence of polybrene (8 μg/ml, Sigma). Input dose of virus was normalised either by RT activity (measured by SG-PERT) or genome copies (measured by qPCR) as indicated. Infection levels were assessed at 48 h post-infection through enumeration of GFP positive cells by flow cytometry.

Luciferase reporter assays

Gaussia/Lucia luciferase activity in supernatants was measured by transferring 10 μl to a white 96 well assay plate, injecting 50 μl per well of coelenterazine substrate (Nanolight Technologies, 2 μg/ml) and analysing luminescence on a FLUOstar OPTIMA luminometer (Promega). Fold inductions were calculated by normalising to a mock-treated control.

ISG qPCR

ISG induction in infected THP-1 cells and primary MDM was assessed by qPCR as previously described [26]. Expression of each gene was normalised to an internal control (*GAPDH*) and these values were then normalised to mock-treated control cells to yield a fold induction. The following primers were used:

GAPDH Fwd: 5'-GGGAAACTGTGGCGTGAT-3',
GAPDH Rev: 5'-GGAGGAGTGGGTGTCGCTGTT-3'.
CXCL-10 Fwd: 5'-TGGCATTCAAGGAGTACCTC-3'.
CXCL-10 Rev: 5'-TTGTAGCAATGATCTCAACACG-3'.
IFIT-2 Fwd: 5'-CAGCTGAGAATTGCACTGCAA-3'.
IFIT-2 Rev: 5'-CGTAGGCTGCTCTCCAAGGA-3'.
MxA Fwd: 5'-ATCCTGGGATTTTGGGGCTT-3'.
MxA Rev: 5'-CCGCTTGTCGCTGGTGTCG-3'.
RSAD2 Fwd: 5'-CTGTCCGCTGGAAAGTG-3'.
RSAD2 Rev: 5'-GCTTCTTCTACACCAACATCC-3'.

ELISA

Cell supernatants were harvested for ELISA at 24 h post-infection/stimulation and stored at -80 ºC. CXCL-10 protein was measured using Duoset ELISA reagents (R&D Biosystems) according to the manufacturer's instructions.

Immunoblotting

For immunoblotting of viral particles, 2×10^{11} genome copies of virus were boiled for 10 min in 6X Laemmli buffer (50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 100 mM β-mercaptoethanol) before separating on 4–12% Bis-Tris polyacrylamide gradient gel (Invitrogen). For immunoblotting from HEK293T cells, whole cell lysates were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 0.1% (w/v) SDS, supplemented with protease inhibitors (Roche)), supplemented with 6X Laemmli buffer and boiled for 5 min. HEK293T cell supernatants were filtered through a 0.45 μM filter, supplemented with 6X Laemmli buffer and boiled for 5 min. After PAGE, proteins were transferred to a Hybond ECL membrane (Amersham biosciences) using a semi-dry transfer system (Biorad). Mouse-anti-HIV-1capsid p24 was from AIDS reagents (183-H12-5 C), mouse-anti-firefly luciferase was from Santa Cruz Biotechnology (sc-57,604), mouse-anti-GFP was from Proteintech (66002-1-Ig), rabbit-anti-β-actin was from Proteintech (20536-1-AP) and were detected with goat-anti-mouse/rabbit IRdye 680/800CW infrared dye secondary antibody and membranes imaged using an Odyssey Infrared Imager (LI-COR Biosciences).

Abrogation-of-restriction assay

FRhK cells were plated in 48 well plates at 5×10^4 cells/ml. The following day cells were co-transduced in the presence of polybrene (8 μg/ml, Sigma) with a fixed dose of HIV-1 LAI expressing GFP (5×10^7 genome copies/ml) and increasing doses of the WT/Gag-LUC chimeric viruses carrying a luciferase-expressing genome, CSLW (0.0005–1 U RT/ml). Rescue of GFP infectivity was

assessed 48 h later by flow cytometry using the FACS Calibur (BD) and analysing with FlowJo software.

Statistical analyses

Statistical analyses were performed using an unpaired Student's t-test (with Welch's correction where variances were unequal) or a 2-way ANOVA with multiple comparisons, as indicated. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Abbreviations

cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CA	capsid
CPSF6	cleavage and polyadenylation specificity factor 6
cypA	cyclophilin A
env	envelope
GFP	green fluorescent protein
HIV	human immunodeficiency virus
HT-DNA	herring testis DNA
IFN	interferon
IP6	inositol hexakisphosphate 6
IRF	interferon regulatory factor
ISG	interferon stimulated gene
IU	infectious unit
JAK	Janus kinase
LPS	lipopolysaccharide
LTR	long terminal repeat
Luc	luciferase
MA	matrix
MDM	monocyte-derived macrophage
NC	nucleocapsid
NF-κB	nuclear factor kappa B
PAMP	pathogen-associated molecular pattern
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RT	reverse transcriptase
SG-PERT	SYBR Green-based product-enhanced RT
SP	spacer peptide
STAT	signal transducer and activator of transcription
WT	wild-type
VSV-G	vesicular stomatitis virus G protein

Supplementary Information

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Supplementary material

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Author contributions

RPS and GJT conceptualised the study. RPS, HB and ML performed the experiments and analysed the data. RPS, CMM and GJT wrote the manuscript. GJT and CMM obtained funding. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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