HIV-1 infection in macrophages and genes involved throughout: Big eaters versus small invaders
Cobos Jiménez, Viviana

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CHAPTER FOUR
SINGLE-STRAND RNA ENDONUCLEASE G3BP1 RESTRICTS HIV-1 REPLICATION IN MACROPHAGES AND T CELLS

Viviana Cobos Jiménez
Fernando O. Martinez
Karel A. van Dort
Siamon Gordon
Teunis B.H. Geijtenbeek and
Neeltje A. Kootstra

1 Department of Experimental Immunology, Sanquin Research, Landsteiner Laboratory, and Center for Infectious Diseases and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands
2 Kennedy Rheumatology Institute, University of Oxford, Oxford, United Kingdom
3 Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

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SUMMARY PARAGRAPH

Antiretroviral therapy has dramatically improved the clinical outcome of HIV-1 infection. However, total eradication of HIV-1 is not achieved due to persistence of the latent viral reservoir\(^1\)\(^-\)\(^3\). HIV-1 exploits the cellular machinery of the target cells for successful infection; however innate defense mechanisms that prevent production of viral particles in these cells may in turn induce latent infection. Hence there is an urgent need to understand what cellular factors and mechanisms contribute to viral latency and establishment of the viral reservoir in macrophages and T cells. Here we identified the GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) as a novel HIV-1 restriction factor expressed in macrophages and T cells. G3BP1 was found by analyzing gene expression profiles of macrophages upon stimulation with a combination of IFN\(\gamma\) and TNF\(\alpha\), which restricts HIV-1 replication after proviral integration\(^4\)\(^,\)\(^5\). Expression of G3BP1 increased upon macrophage stimulation with IFN\(\gamma\) in combination with TNF\(\alpha\), but not with other cytokines. Silencing of G3BP1 by RNA interference resulted in increased HIV-1 replication and HIV-1 LTR-mediated gene expression. We demonstrated that G3BP1 was able to bind HIV-1 genomic RNA as well as single or multiple-spliced mRNAs. G3BP1 was also highly expressed in resting naïve or memory T cells from healthy donors and HIV-1 infected patients; however expression decreased significantly upon T cell activation by IL-2. G3BP1 was able to restrict post-integration steps of HIV-1 replication in primary macrophages, indicating that it may contribute to the establishment of the viral reservoir. Identification of such cellular restriction factors contributes to our understanding of HIV-1 latency and provides novel therapeutic alternatives that could help eliminate the HIV-1 reservoir.
Previously, we reported that HIV-1 infection is restricted in macrophages stimulated with IFNγ and TNFα, IL-4 and IL-10 as well as type I interferon. Restriction of viral replication occurred at different steps in the viral life cycle and was dependent on the cytokine used to stimulate the macrophages. Recently, several new cellular factors have been identified to restrict HIV-1 replication upon type 1 interferon stimulation of macrophages, such as MX2, IFI16, and SAMHD1, or in activated CD4+ T cells (MCPIP1). These newly identified restriction factors mediate HIV-1 inhibition predominantly at early steps of the replication cycle. To identify novel HIV-1 restriction factors that block post-integration and thereby contribute to the establishment of the viral reservoir, we evaluated genome-wide transcriptional profiles in human macrophages, in comparison to IFNγ and TNFα-stimulated macrophages (figure 1a), where HIV-1 infection is restricted at a post-integration level. Analysis of gene expression profiles resulted in the identification of 120 genes that were regulated in IFNγ+TNFα-stimulated macrophages, but not during maturation of monocytes into macrophages (Figure 1b). Expression of most of the 120 selected genes was exclusively regulated by IFNγ+TNFα stimulation, and changes in expression were not observed in IL-4, IL-10 or M-CSF-stimulated macrophages (figure 1c). From the initial selection, only 4 genes were previously identified to be required for HIV-1 replication, in siRNA/shRNA genomic screens. Another 19 genes either possessed antiviral characteristics or could be involved in viral infections, according to the Ingenuity Knowledge Database (Extended Data, table 1). One of these genes was MX1, recently reported to be an IFN-α induced gene, capable of inhibiting influenza virus but not HIV-1 replication. We identified G3BP1 as an important gene upregulated after IFNγ and TNFα treatment. G3BP1 has been shown to be involved in infection by viruses such as Poliovirus, Hepatitis C Virus, Sindbis Virus, Dengue virus and members of the Arenavirus genus, among others, and also in formation of stress granules. During maturation of monocytes into macrophages G3BP1 levels increased only on the first day of culture, but remained constant during maturation throughout day 5 (figure 1d). G3BP1 mRNA levels increased strongly upon stimulation of macrophages with IFNγ+TNFα, but not with IFNγ alone or with other cytokines (Figure 1d).

To determine the effect of G3BP1 on HIV-1 infection, we silenced G3BP1 expression in HEK293T cell with shRNA (Extended data, figure 1a, b) and measured replication of a VSV-g-pseudotyped HIV-1-luciferase reporter virus. Notably, upon silencing of G3BP1, HIV-1 replication was significantly increased in a dose dependent manner (Figure 2a). Additionally, silencing of G3BP1 also led to a dose dependent increase of luciferase expression mediated by the HIV-1 LTR (Figure 2b), which indicates that G3BP1 regulated HIV-1 replication at a transcriptional or post-transcriptional step. G3BP1 down-regulation also slightly enhanced luciferase expression driven by Hepatitis B Virus S1 promoter; however it neither affected expression driven by the Simian Immunodeficiency Virus (SIV).
Figure 1. IFNγ+TNFα stimulation of macrophages induces expression G3BP1. A) Hierarchical clustering of genes significantly induced during maturation of monocytes (Mo) into macrophages (MΦ) and stimulation with IFNγ+TNFα, using median-centred values; B) Genes that were regulated only by stimulation with IFNγ+TNFα were selected by a Pavlidis Template Matching (PMT) analysis, using median-centred values; C) Hierarchical clustering of expression of the PMT selected genes in monocytes (Mo), unstimulated macrophages (MΦ) stimulated with M-CSF, IL-4 or IL-10, using median centred values. The location of G3BP1 is indicated on the side; D) Expression of G3BP1 relative to β-actin mRNA, in monocytes and macrophages cultured for 1 to 7 days, and isolated from 5 donors; E) Expression of G3BP1 relative to β-actin mRNA, in monocytes, and unstimulated macrophages (MΦ) or stimulated with IFNγ, IFNγ+TNFα, IL-4 and IL10. Significant differences in the expression levels are indicated by asterisks (One-way ANOVA and Bonferroni's Multiple Comparison Test, p<0.05*, p<0.01**, ***p<0.001).
promoter, HBV core promoter, Cytomegalovirus (CMV) promoter nor the human elongation Factor 1α (eF1α) promoter (figure 2c-h).

G3BP1 is a single-strand-specific endonuclease capable of binding mRNA transcripts at the 3'UTR inside stress granules\(^\text{(18)}\) that control their translation during cellular stress\(^\text{(19,20)}\). Therefore, we hypothesized that G3BP1 could bind and sequester HIV-1 transcripts inside cytosolic organelles, thus delaying translation and synthesis of viral proteins. To test this, we performed RNA-immunoprecipitation assays in macrophages cultured with or without IFN\(_\gamma\)+TNF\(\alpha\), after HIV-1 infection. The different HIV-1 RNA transcripts (unspliced, single spliced or multiple spliced) were measured. Levels of all viral transcripts in total RNA were decreased upon IFN\(_\gamma\)+TNF\(\alpha\) stimulation of macrophages (Figures 3), which is evidence of the strong inhibition of HIV-1 replication in these cells. Viral RNA transcripts were bound by G3BP1 at higher levels in IFN\(_\gamma\)+TNF\(\alpha\) stimulated macrophages, compared to unstimulated cells (figure 3). As a negative control, cellular Trim5\(\alpha\) mRNA was not detected after immunoprecipitation (data not shown). Therefore, higher expression of G3BP1 in IFN\(_\gamma\)+TNF\(\alpha\) stimulated macrophages resulted in increased binding of G3BP1 to viral transcripts, which may prevent of RNA translation and thereby production of viral particles. This could also lower translation of luciferase viral transcripts expressed by the VSV-g pseudotyped virus as well as with the HIV-1 LTR-driven reporter constructs (figure 2). This could indicate that G3BP1 recognizes viral elements in the 5'UTR of the mRNA, and thereby inhibits translation.

CD4\(^+\) T cells are also major targets for HIV-1 infection and can become latently infected by the virus. G3BP1 was highly expressed in resting CD4\(^+\) naïve T cells (CD27\(^-\)CD45RO\(^-\)) and memory T cells (CD27\(^+\)CD45RO\(^+\)) obtained from healthy blood donors (figure 4a) and no differences in the expression levels were observed between the two subpopulations. In addition, G3BP1 was also highly expressed in resting T cells isolated from HIV-1 infected patients (participants of the Amsterdam Cohort Studies), and again no differences were observed between memory or naïve cells (Figure 4b). These results indicate that high levels of G3BP1 could play a role to the formation of the viral reservoir in resting T cells. Since productive HIV-1 infection can only be established upon T cell activation\(^\text{(21)}\), we measured G3BP1 mRNA in IL-2 stimulated T cells and observed a significant decrease upon T cell activation (figure 4c). To confirm that G3BP1 is able to control HIV-1 replication, we silenced mRNA expression in macrophages (extended data, figure 1c), and measured replication of the VSV-g pseudotyped reporter virus. Silencing of G3BP1 resulted in increased HIV-1 replication in macrophages (figure 4d). These observations indicate that G3BP1 restricts viral translation in both macrophages and T cells.

In this study, we have identified human G3BP1 as a novel HIV-1 restriction factor that is highly expressed in T cells and is induced by IFN\(_\gamma\)+TNF\(\alpha\) in macrophages. Our results demonstrated that G3BP1 is able to bind HIV-1 RNA transcripts, which may sequester them in the cytosolic organelles, leading to a delay in translation,
**Figure 2.** G3BP1 restricts HIV-1 replication at a post-transcriptional level. A) 293T cells transfected with shRNA-3 targeting G3BP1 or shRNA control (50 ng), subsequently inoculated with VSV-g/NL4-3Luc and luciferase levels were measured 48 hours later. shRNA-G3BP1 concentrations are 50 ng, 25 ng, 12.5 ng and 6.25 ng. Bars represent the average luciferase activity of triplicate measurements and the error bars represent the standard error of the mean (SEM); B) 293T cells were transfected with shRNA targeting G3BP1 or shRNA control (50 ng), together with the HIV-1 LTR Luciferase reporter construct (10 ng); luciferase levels were measured 48 hours after transfection. shRNA concentrations are 50 ng, 25 ng, 12.5 ng and 6.25 ng. Bars represent
The average luciferase activity of triplicate measurements and the error bars represent the Standards Error of the Mean (SEM). 293T cells were transfected with shRNA targeting G3BP1 or an shRNA control (50ng) and luciferase reporter constructs (10ng) containing the different promoter regions: C) HIV-1 LTR; D) SIV; E) HBV-S1; F) HBV Core; G) CMV; H) eF1α. Luciferase levels were measured 48 hours after transfection. Luciferase levels of cells treated with shRNA for G3BP1 were compared with cells treated with the shRNA control. Bars represent the average values of two separate experiments and the error bars represent the standard error of the mean (SEM). Significant differences are indicated by asterisks (A,B: one-way ANOVA test and subsequent Bonferroni multiple comparison test; C: Student’s T test; p<0.05*, p<0.01**, ***p<0.001).

Figure 3. G3BP1 binds to HIV-1 RNA transcripts. Detection of HIV-1 RNA transcripts after cDNA synthesis was performed by agarose-gel electrophoresis and the intensity of the PCR product band was quantified. PCR products from viral RNA transcripts in total RNA isolated from macrophages stimulated with IFNγ+TNFα, compared to total RNA isolated from unstimulated macrophages are represented by the light grey bars; RNA-immunoprecipitation assays were performed in samples from unstimulated macrophages or macrophages stimulated with IFNγ+TNFα, and infected with HIV-1. G3BP1-RNA complexes were precipitated and RNA was subsequently isolated. PCR products from viral RNA transcripts in RNA isolated from macrophages stimulated with IFNγ+TNFα, compared to RNA isolated from unstimulated macrophages, after immunoprecipitation are represented by the dark grey bars. Values represented here correspond to the log2 transformed ratio of the intensity of the PCR products.

Low viral protein levels and therefore low viral replication. Our study is the first to report the interaction between G3BP1 and HIV-1, yet the mechanism of G3BP1 binding to viral RNA and inhibition of translational events still needs to be elucidated in detail. It is known that G3BP1 plays an important role in immune mechanisms that detect viral RNA and trigger IFN-β production, during influenza virus infection. G3BP1-mediated reduction of mRNA translation and viral protein production could however prevent recognition of the infected cell by the immune system, facilitating latent infection. Since latent HIV-1 reservoirs are a major hurdle in HIV-1 eradication, it is of great importance to define mechanisms that contribute
to the establishment of viral latency. Cellular factors, like G3BP1, that restrict replication after proviral integration, could induce viral latency. Identification of these antiviral factors contributes to our understanding of the establishment of the viral reservoir in macrophages and T cells. This knowledge will contribute to the design of new therapeutic strategies towards the elimination of the latent reservoir as part of a sterilizing cure for HIV-1 infection23.

METHODS SUMMARY

Cells and Cell Culture: Primary human monocytes were isolated from buffy coats from healthy blood donors and stimulated with cytokines for 5 days, as described in the Methods.
Gene expression array: Microarrays were performed in the Illumina Hg12v4 platform using RNA isolated from monocyte-derived macrophages (MDM) derived from 2 donors and stimulated with cytokines for 5 days. All datasets have been deposited in Geoprofiles: SubSeries GSE49240 (samples GSM1195728-39), part of the SuperSeries GSE3549524.

G3BP1 silencing and virus infection: Down-regulation of G3BP1 expression in HEK293T cells was achieved by transient transfection of pLKO.1-puro constructs expressing shRNAs against G3BP1 or shRNA control from the MISSION™ TRC-Hs 1.0 library, using the calcium chloride method. Down-regulation of G3BP1 expression in primary MDMs was performed by transient transfection of siGENOME siRNA SMART pool libraries using DharmaFECT 4 transfection Reagent (ThermoFisher Scientific). To assess the effect of G3BP1 down-regulation on HIV-1 infection, transfected cells were inoculated with VSV-g pseudotyped HIV-1 luciferase reporter virus and luciferase was measured 48 or 72 hours post inoculation.

RNA-Immuno Precipitation Assays: RIP assays were performed with the EZ-Magna RIP™-RNA Binding Immunoprecipitation Kit (Millipore, Billerica, MA, USA) together with the RIPAb™ G3BP1 – RIP Antibody and Primer set (Millipore), according to the manufacturer’s protocol, using samples from MDM and M1 polarized macrophages infected with HIV-1 NL4-3 Ba-L. RNA was isolated after immunoprecipitation and HIV-1 cDNA synthesized using random primers. HIV-1 sequences were detected using the following primers: Primer set 1: R-U5-Fw-1 5'-GAGCTCTCTGCTAAGCTAGG-3', GAG-1-Rv 5'-TTTGGCGTACTCACCAGTCG-3'. Primer set 2: POL-1-FW 5'-GACAACATCTGAGTGTTG-3', POL-3-Rv 5'-GCCAGTCTAGCTCTGCTTC-3'. Primer set 3: POL-2-Fw 5'-GATGGACCACACAACTATTGC-3', POL-2-Rv 5'-CTATGGACCACACAACTATTGC-3'. Primer set 4: Nef-2-Fw 5'-GTAGTGTATTGGATGTCCTTG-3', LTR-3U-Rv-1 5'-GGAAGTAGCCTTGTGTGG-3'.


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MATERIALS AND METHODS

Isolation of monocytes and cell culture

Monocytes and peripheral blood lymphocytes (PBL) were obtained from buffy coats from healthy blood donors. Written informed consents were obtained from all donors in accordance with the ethical principles set out in the declaration of Helsinki. This study was approved by the Medical Ethics Committee of the Academic Medical Center and the Ethics Advisory Body of the Sanquin Blood Supply Foundation in Amsterdam, The Netherlands. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation. Monocytes were isolated by adherence to plastic and cultured in Iscove’s modified Dulbecco’s medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% [v/v] heat-inactivated human pooled serum (HPS), penicillin (100 U/ml; Invitrogen, Carlsbad, CA), streptomycin (100 µg/ml; Invitrogen) and ciproxin (5 µg/ml; Bayer, Leverkusen, Germany) for 5 days in the presence of different cytokines: M-CSF (50ng/ml; Sigma-Aldrich, St. Louis, MO, USA), IFN-γ (50 U/ml; Sigma-Aldrich) in combination with TNF-α (12.5 ng/ml; Peprotech, Rocky Hill, NJ, USA), IL-4 (50 ng/ml; Peprotech), IL-10 (50 ng/ml; Peprotech), or medium alone at 37°C in a humidified atmosphere supplemented with 5% CO₂.

PBMC were also obtained from HIV-1 seropositive participants of the Amsterdam Cohort Studies. All individuals were infected with HIV-1 subtype B. The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set put in the declaration of Helsinki and written consent was obtained prior to data collection. PBMC from healthy blood donors and HIV-1 positive individuals were sorted into CD4+ naïve T cells (CD27+CD45RO−) and memory T cells (CD27−CD45RO+) by flow cytometry with the FACS Canto II (BD Biosciences, San Jose, CA, USA). Additionally, PBMC isolated from healthy blood donors were cultured for 3 days with IMDM supplemented with 10% [v/v] heat-inactivated fetal bovine serum, penicillin, streptomycin, ciproxin and IL-2 (20U/ml; Chirion Benelux, Amsterdam, The Netherlands).
HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) without Hepes (Lonza), supplemented with 10% [v/v] inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in a humidified atmosphere supplemented with 10% CO₂.

**Transcriptional profiles study and data mining**
At day 5 the medium was removed and MDM were harvested with TriPure Isolation reagent (Roche, Basel, Switzerland). RNA was isolated according to the manufacturer’s protocol. RNA samples were cleaned with RNeasy Mini Spin columns (Qiagen RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to the clean-up protocol from the manufacturer. The concentration was analysed with the Nanodrop ND-1000 spectrophotometer and A260/280 values were above 2.0 for all samples. Microarrays were performed in the Illumina Hg12v4 platform. Background-subtracted data were normalised by Quantiles in R/Bioconductor. All datasets have been deposited in Geoprofiles: SubSeries GSE49240 (samples GSM1195728-39), part of the SuperSeries GSE35495.24. Genes with fold change ≥ 2 were considered differentially expressed. Statistical differences and data processing was performed in TMev v4.8.1.25.

**qPCR**
For mRNA quantification, cDNA from TriPure-isolated RNA was prepared using the M-MLV Reverse Transcriptase kit (Promega, Madison, WI) using an oligo-dT primer. The qPCR was performed with a Lightcycler 480 with SYBR Green I Master (Roche) using the primers: G3BP1-Fw1: 5’-GCTCATGTGCTCGTGTCCGCA-3’ and G3BP1-Rv1: 5’-CACCCACTGCTGCCCATC-3’ to determine G3BP1 expression levels and β-actin-Fw: 5’- GGCCCAAGTCCTCTCCCAGTCCAC-3’ and β-actin-Rv: 5’- GGTAAAGCCCTGCTGCTGCCACC to determine the β-actin expression levels. The following cycling conditions were used in the LightCycler 480 Real-Time PCR System (Roche): denaturation for 95°C for 10 min; amplification: 40 cycles of 95°C for 10 sec, 58°C for 20 sec and 72°C for 30 sec. Specificity of the primer pairs was confirmed by melting curve analysis of the PCR products. Values for G3BP1 copies relative to β-actin copies were calculated by the “Advanced relative quantification” method using LightCycler 480 software, version 1.5.

**Viruses and Expression vectors**
HIV-1 NL4-3 Ba-L and single-round luciferase virus pseudotyped with the vesicular stomatitis glycoprotein (VSV-G) were produced by transient transfection of HEK293T cells with pNL4-3 Ba-L or pNL4-3.Luc.R-E-construct in combination with pCMV-VSV-G using a calcium phosphate method.26 Infectious virus was harvested at 48 and 72 h after transfection and filtered through a 0.22 μm filter. NL4-3 Ba-L titers were determined on PHA-stimulated PBMC as described previously.27 Titers of the VSV-G pseudotyped single-round luciferase virus were determined on HEK293T cells.
Myc-His-tagged G3BP1 expression construct was generated by PCR amplification of G3BP1 from cDNA obtained from monocyte-derived macrophages using primers containing restriction sites for XbaI and HindIII restriction enzymes: G3BP1-Fw1-XbaI: 5’-GGCCCTCTAGAATGGTGATGG-3’ and G3BP1-Rv1-HindIII: 5’-GCCCAA GCTTGCCTGCAGCTG-3’. The PCR product was digested with XbaI and HindIII and cloned into the multiple cloning site of pcDNA3.1A(-) containing a Myc-His tag.

pLKO.1-puro constructs expressing a shRNA against G3BP1 or shRNA control from the MISSION™ TRC-Hs 1.0 library were used (1: G3BP1 TRCN0000008719, 2: G3BP1 TRCN0000008720, 3: G3BP1 TRCN0000008721, 4: G3BP1 TRCN0000008722, 5. G3BP1 TRCN0000008723 and empty vector SHC001; Sigma-Aldrich St. Louis, MO, USA).

The LTR-Luciferase reporter construct pBlue3′ LTR-luc was a kind gift from Dr. R. Jeeninga and Dr. B. Berkhout, Academic Medical Center, Amsterdam, The Netherlands. The pGL 4.51 Luc2/CMV/Neo vector (Promega) was used for evaluating luciferase expression mediated by the CMV promoter region. To generate the hEF1α-Luc construct, expressing luciferase under control of the human elongation factor 1 promoter, the luciferase gene was amplified using the primers FW_Luciferase_BamHI (5’-GATAGGATCCATGGAAGACGCCAAAAAC-3’) and REV-Luciferase-Kpn1 (5’-CTATGGTACCTTACACGGCGATCTTTCC-3’). The amplification product was digested with BamH1 and Kpn1 and ligated after the hEF1α promoter in a lentiviral vector digested with the same enzymes. The luciferase reporters under control of the different HBV promoters were generated as described by Du et al., with minor modifications to the primers to make them suitable to the HBV adw subtype. The different promoter sequences were amplified by PCR from the R9 vector, which contains a 1.2 x overlength HBV DNA genome (subtype adw) in a pGEM 7zf+ backbone (kindly provided by Dr. Baumert). All constructs were validated by BDT sequencing.

**G3BP1 Silencing**

To determine the effect of G3BP1 down-regulation on HIV-1 infection, HEK293T cells seeded on 6-well plates and transfected with the shRNA-3 construct targeting G3BP1 using a calcium phosphate method. After 24 hours, the medium was replaced and the cells were harvested with trypsin-EDTA (PAA), and plated in white-bottom 96-well plates. After overnight culture, cells were inoculated with 1000 TCID50 units of VSV-G/NL4-3Luc. Luciferase activity was analysed 48 hours after inoculation by the addition of 50 µl of luciferase substrate (0.83 mM ATP, 0.83 mM d-luciferin (Duchefa Biochemie B.V., Haarlem, The Netherlands), 18.7 mM MgCl2, 0.78 mM Na2HPO4, 38.9 mM Tris [pH 7.8], 0.39% [v/v] glycerol, 0.03% [v/v] Triton X-100, and 2.6 µM dithiothreitol) per well. Luminescence was measured for 1 sec per well, using a luminometer (Berthold Technologies, Bad Wildbad, Germany).
To determine the effect of G3BP1 down-regulation on HIV-1 infection of in primary MDMs, siGENOME siRNA pool libraries targeting Human G3BP1 (ThermoFisher Scientific, Waltham, MA, USA) were used. Monocytes were obtained from buffy coats from healthy blood donors, as described above, and seeded on white-bottom 96-well plates, at a density of 50,000 cells per well. MDM were transfected at day 4 with 50ng of siRNA pool library with DarmaFECT 4 Transfection reagent (ThermoFisher Scientific) in antibiotic-free medium containing 5% HPS, according to manufacturer’s protocol. After 48 hours, the transfection medium was replaced by culture medium containing antibiotics and 10% HPA. Cells were inoculated with 15,000 TCID50 units of VSV-g/NL4-3Luc 72 hours after transfection. Luciferase activity was analysed 72 hours after inoculation by the addition of 50 µl of luciferase substrate, as described above.

To determine the effect of G3BP1 down-regulation on transcription under HIV-1 or other types of promoters, HEK293T cells were transfected with shRNA-3 construct targeting G3BP1, together with the different LTR-Luc constructs, in 96-well plates using a calcium phosphate method26. After 24 hours, the medium was replaced and after overnight culture, cells were inoculated with 1000 TCID50 units of VSV-G/NL4-3Luc. Luciferase activity was analysed 48 hours after inoculation by the addition of 50 µl of luciferase substrate, as described above.

Western Blotting
HEK 293T cells were cultured in 6 well plates and transfected with 1 ug of pcDNA3.1-G3BP1 and 1ug of constructs expressing shRNAs targeting G3BP1 (1-5) or shRNA control using the calcium phosphate method26. After 24 hours, the cell medium was replaced and cultures were continued for another 24 hours. The cells were harvested with trypsin-EDTA (PAA, Pasching Austria), collected and washed with PBS and centrifuged at 400 g for 10 minutes. The cell pellet was lysed in 200 µl of RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with Complete® EDTA free protease inhibitor (Roche). The lysate was denatured at 70°C for 10 minutes in 1× NuPAGE LDS sample buffer (Invitrogen) and 0.1 M DTT. Proteins were separated by electrophoresis on a 10% Bis-Tris gel (NuPAGE 10% Bis–Tris precast gel) together with the Odyssey Protein Weight Marker (LI-COR, Lincoln, NE, USA) using MES SDS running buffer (Invitrogen). Subsequently, proteins were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany; 2 hours 150V) using NuPAGE transfer buffer. Blots were stained overnight at 4°C in PBS (Gibco) supplemented with 0.01% Tween 20 (Merck) and 1% Protifar (Nutricia, Schiphol, The Netherlands) with a monoclonal mouse anti-c-Myc antibody (1:5,000; Calbiochem, San Diego, CA, USA) or goat anti-Actin antibody (1:200; Novagen). IRDye 800CW conjugated goat anti-mouse IgG (1:15,000) and IRDye 680RD donkey anti-goat IgG (1:15,000; LI-COR, Lincoln, NE, USA) were...
used as secondary antibodies to visualize the proteins using the Odyssey infrared image system (LI-COR).

**RNA-Immuno Precipitation Assays (RIP)**

To determine if HIV-1 RNA species could be bound to G3BP1, we obtained samples from MDM and M1 macrophages and infected them at day 5 with HIV-1 NL4-3 Ba-L. Samples isolated 72 hours after inoculation were used for RIP assays with the EZ-Magna RIP™-RNA Binding Immunoprecipitation Kit (Millipore, Billerica, MA, USA) together with the RIPAb™ G3BP1 – RIP Antibody and Primer set (Millipore), according to the manufacturer’s protocol. Briefly, monocytes were obtained from buffy coats from healthy blood donors, as described above, and incubated with IFNγ and TNFα or medium alone, in T25 flasks at a density of 5 x 10⁶ cells. After 5 days, cells were inoculated with 375.000 TCID50 units of NL4-3BaL. Cells were harvested 72 hours after inoculation with the Complete RIP Lysis buffer, and stored at -80°C. 10µl of lysate were stored for subsequent RNA isolation. Cell lysates were incubated with magnetic beads coated with anti-G3BP1 and control antibodies, at 4°C over night and immunoprecipitation was carried out according to the manufacturer’s instructions. Subsequently, RNA from the lysate and immunoprecipitated samples was isolated using a phenol:chloroform:isoamyl alcohol isolation procedure, as described by the manufacturer. cDNA was synthesized form the RNA isolated after RIP, using Random Primers and the M-MLV reverse transcriptase (Promega). The primers used to detect HIV-1 cDNA were: Primer set 1: R-U5-Fw-1 5’-GAGCTCTCTGGCTAACTAGG-3’, GAG-1-Rv 5’-TTTGGCGTACTCACCAGTCTCG-3’. Primer set 2: POL-1-FW 5’-GAC AACATCTGTAGGTGGG-3’, POL-3-Rv 5’-GCCAGTTCTAGCTCTGCTTC-3’. Primer set 3: POL-2-Fw 5’-GCTCATCAGAACAGTCAGAC-3’, POL-2-Rv 5’-GTTAGTCTACGGATGGGGTC-3’. Primer set 4: Nef-2-Fw 5’-GTAGTCTACGGATGGGGTC-3’, LTR-3U_Rv-1 5’-GGAAGTAGCCTTGTTGCGG-3’. PCR products were visualized after 2% agarose gel electrophoresis containing Ethidium Bromide (Promega), with a UV-light transilluminator.

**Statistical analysis**

Differences G3BP1 expression levels and luciferase levels were analysed with a one-way ANOVA test and subsequent Bonferroni multiple comparison test or a student T-test, using GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA). Luciferase levels in silencing experiments on MDM were analysed with a mixed linear model in SPSS 10 (Chicago, IL, USA).
Extended Data - Figure 1. Confirmation of G3BP1 down-regulation upon silencing. A) HEK293T cells were transfected with constructs encoding for 5 shRNAs targeting G3BP1 or an shRNA control construct, together with a non-functional, Myc-His-tagged G3BP1shRNA construct. Sample from cells transfected only the G3BP1 expression construct (-). Actin was detected as a loading control. B) Quantification of G3BP1 in shRNA treated cells, compared to the shRNA-control treated cells. C) MDM and M1 macrophages from 3 donors were transfected with siRNA libraries targeting G3BP1 or non-targeting siRNAs as control, and inoculated with the VSV-g/NL4-3Luc virus. G3BP1 mRNA levels relative to β-actin levels were measured. Significant differences in the expression levels are indicated by asterisks (student’s T-Test, p<0.05*, p<0.01**, ***p<0.001).
Extended Data - Table 1. Genes with antiviral characteristics, expressed in macrophages stimulated with IFNγ and TNFα

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