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SERINC5 Is an Unconventional HIV Restriction Factor That Is Upregulated during Myeloid Cell Differentiation

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Keywords
SERINC5 · Innate restriction factor · Adaptive immunity · HIV · Myeloid cell differentiation

Abstract
Classical antiviral restriction factors promote cellular immunity by their ability to interfere with virus replication and induction of their expression by proinflammatory cytokines such as interferons. The serine incorporator proteins SERINC3 and SERINC5 potently reduce the infectivity of HIV-1 particles when overexpressed, and RNA interference or knockout approaches in T cells have indicated antiviral activity also of the endogenous proteins. Due to lack of reagents for detection of endogenous SERINC proteins, it is still unclear whether SERINC3/5 are expressed to functionally relevant levels in different primary target cells of HIV infection and how the expression levels of these innate immunity factors are regulated. In the current study, analysis of *SERINC3/5* mRNA steady-state levels in primary lymphoid and monocyte-derived cells revealed selective induction of their expression upon differentiation of myeloid cells. Contrary to classical antiviral restriction factors, various antiviral α-interferon subtypes and proinflammatory interleukins had no effect on *SERINC* levels, which were also not dysregulated in CD4+ T cells and monocytes isolated from patients with chronic HIV-1 infection. Notably, HIV-1 particles produced by terminally differentiated monocyte-derived macrophages with high *SERINC5* expression, but not by low-expressing monocytes, showed a Nef-dependent infectivity defect. Overall, these findings suggest endogenous expression of SERINC5 to antivirally active levels in macrophages. Our results classify SERINC5 as an unconventional HIV-1 restriction factor whose expression is specifically induced upon differentiation of cells towards the myeloid lineage.

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Introduction

The serine incorporator proteins SERINC1 to SERINC5 display no sequence homology to other proteins, but are conserved from yeast to mammals and share >30% amino acid identity among themselves. They belong to a family of transmembrane carrier proteins that have been initially anticipated to be responsible for the incorporation of the amino acid serine into lipid membranes. Furthermore, it is believed that they comprise a molecular scaffold for the binding of 3-phosphoglycerate dehydrogenase facilitating also the synthesis of phosphatidylserine and sphingolipids [1]. However, recent studies could neither show an effect of SERINC1 and SERINC5 on cellular lipid composition [2, 3] nor on cell function (SERINC1 [3]) or lipid composition of viral particles (SERINC5 [2]), thus questioning previously accepted functions of SERINC proteins.

In 2015, SERINC3 and SERINC5, but not SERINC1, SERINC2, and SERINC4, were identified as cell-associated innate immunity factors which can potently suppress the particle infectivity of HIV upon overexpression. Thereby, SERINC3/5 become incorporated into newly generated particles and compromise their fusion with new target cells. The antiviral activity of SERINC3/5 has been shown to be counteracted by the Nef proteins encoded by HIV-1, HIV-2, and SIV [4, 5]. Analysis revealed that in the presence of Nef, the amount of SERINC3/5 was markedly reduced in newly produced virus particles, and Nef expression caused redistribution of SERINC into a Rab7-positive cellular compartment [4, 5]. These findings were supported by a surface proteome study of HIV-1 infected CD4+ T cells, which revealed reduction of several plasma membrane-bound proteins upon Nef expression – among them SERINC3/5 [6]. Interestingly, and contrary to SERINC3 and SERINC5, Nef increased the surface abundance of SERINC1 [6], revealing a so far unappreciated level of selectivity of the Nef-SERINC interaction. Notably, downregulation of SERINC5 from the cell surface seems not to be sufficient for enhancement of viral infectivity [7].

While a context-dependent requirement for Nef for HIV-1 particle infectivity is well established and this phenotype correlates well with *SERINC5* mRNA levels [4, 5], detection of endogenous SERINC proteins in cells has been precluded so far by the lack of suitable antibodies. Whether SERINC3/5 proteins are expressed to functionally relevant levels in primary target cells of productive HIV infection and how their expression levels are regulated has remained largely elusive.

Here, we analyzed *SERINC3/5* mRNA steady-state levels in lymphoid and monocyte-derived cells from healthy donors. Our findings disclose significant variances of *SERINC3/5* abundance in these cells and reveal a selective induction of *SERINC* gene expression upon differentiation of myeloid cells. We further assessed the immune modulatory effect of several α-interferons (IFNs) and proinflammatory interleukins (ILs) on *SERINC3/5* mRNA levels and analyzed possible differences in HIV target cells, i.e., CD4+ T cells and monocytes, isolated from a cohort of chronically HIV-1-infected patients and healthy controls. Surprisingly, neither stimulation with various IFNs/ILs nor HIV-1 infection had a measurable effect on *SERINC* mRNA expression levels. Finally, the impact on HIV-1 particle infectivity was studied and demonstrated in the context of the observed differentiation-dependent mRNA induction of *SERINC5* in cells of the myeloid lineage. Overall, our results corroborate the physiological relevance of the antiviral function of SERINC5, but question its categorization as a classical innate restriction factor.

Materials and Methods

Blood Cell Isolation and Differentiation

Blood cones (Terumo BCT leukocyte reduction system) containing red blood cells and enriched leukocytes were received from the Hospital of the University of Munich, Department of Immunohematology, infection screening and blood bank (ATMZH). Blood cells were derived exclusively from anonymized healthy donors in the age range of 20–55 years. Blood cells were diluted with PBS (Gibco) and different cell types were isolated via the EasySep™ Rosette Human CD4+ T Cell ("resting" CD4+ T cells), Human CD8+ T Cell (CD8+ T cells), Human NK Cell (NK cells), and Human B Cell (B cells) enrichment kits (STEMCELL Technologies, Canada) according to the manufacturer's protocols. Monocytes were isolated via the Human Monocyte Isolation Kit II and the autoMACS® Pro Separator (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Monocytes were further differentiated into monocyte-derived macrophages (MDMs) by growing cells in surface-repellent plates (Greiner Bio-One, Austria) at 37 °C and 5% CO_2 for 7–9 days in DMEM Glutamax medium (Gibco) supplemented with 10% heat-inactivated FCS (Sigma Aldrich), antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]), and 10% human AB serum (Sigma Aldrich). Differentiation was confirmed by microscopy and attaching behavior of the cells. Subsequently, cells were detached by incubation with ice-cold detach buffer (5 mM EDTA/PBS). For differentiation into dendritic cells, monocytes were cultivated at 37 °C and 5% CO_2 for 7 days in RPMI Glutamax medium (Gibco) containing 10% heat-inactivated FCS (Sigma Aldrich), antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]), together with IL-4 (580 U/mL; R&D Systems, Wiesbaden, Germany) and GM-CSF (10 ng/mL; R&D Systems). Subsequently,

cells were either directly used for further experiments (immature monocyte-derived dendritic cells [imMDDCs]) or treated with 10 ng/mL lipopolysaccharide (Sigma Aldrich) for another 2 days to obtain mature monocyte-derived dendritic cells (mMDDCs). Resting CD4+ T cells were either directly used or further activated using one of two strategies: For activation procedure I, cells were grown at 37 °C and 5% CO_2 for 4 days in RPMI Glutamax medium (Gibco) containing 10% heat-inactivated FCS (Sigma Aldrich), antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]), IL-2 (100 U/mL; Biomol, Germany), and phytohemagglutinin-P (PHA) (5 µg/mL; Sigma Aldrich). Subsequently, medium was exchanged to RPMI Glutamax medium (Gibco) containing 10% heat-inactivated FCS (Sigma Aldrich), antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]), and IL-2 (100 U/mL; Biomol), and cells were cultivated for another 1–4 days. Activation II was achieved by using Human T-Activator CD3/CD28 Dynabeads™ (Gibco) according to the manufacturer's protocol.

The identity and purity of cell types were validated by flow cytometry using cell type-specific fluorophore-coupled antibodies (see also online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000504888). 293T cells, which were used as reference for mRNA normalization, and HT-1080 cells were cultivated at 37 °C and 5% CO_2 in DMEM Glutamax medium (Gibco) supplemented with 10% heat-inactivated FCS (Sigma Aldrich) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]). Jurkat-TAg cells were cultivated at 37 °C and 5% CO_2 in RPMI Glutamax medium (Gibco) supplemented with 10% heat-inactivated FCS (Sigma Aldrich) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]).

Lineage Marker Validation and Purity of Isolated Cell Types

Cells were stained with cell type-specific fluorophore-coupled antibodies and analyzed on a FACSverse™ flow cytometer (BD Biosciences). The following antibodies were used: B cells (CD19 FITC [Biolegend], CD20 APC [Biolegend]), monocytes (CD14 PE [BD Biosciences], CD3 APC [BD Biosciences]), NK cells (CD56 PE [BD Biosciences], CD3 APC [BD Biosciences]), CD8+ T cells (CD8 PE [BD Biosciences], CD3 APC [BD Biosciences]), CD4+ T cells (CD3 APC [BD Biosciences], CD4 PerCP-Cy5.5 [BD Biosciences]), MDMs (CD14 APC [BD Biosciences], CD1a PE [Biolegend]), MDDCs (CD86 APC [Biolegend], CD1a PE [Biolegend]), and activated CD4+ T cells (CD25 PE [BD Biosciences], CD69 PE [BD Biosciences]). The purity and/or activation state were determined based on the flow cytometric data. All data sets were processed with the FlowJo software package (FlowJo LLC, USA).

Fluorescence-Activated Cell Sorting of Samples from HIV-Infected Donors

PBMC samples were separated from EDTA blood by Ficoll density gradient centrifugation (Biocoll Separation Solution; Biochrom, Germany) from healthy donors (Healthy) or from chronically HIV-1-infected patients either without antiretroviral therapy (ART) (Chronic) or under suppressive ART (see also Fig. 3b). Study participants were recruited from the outpatient clinic at the Department of Infectious Diseases of the University Hospital Munich. Cells were stained for CD3 (FITC; BD Biosciences), CD4 (PerCP; BD Biosciences), CD8 (BV510; Biolegend), CD14 (PE; BD Biosciences), CD16 (BV421; Biolegend), CD56 (PE-Cy7; Biolegend), and HLA-DR (APC; BD Biosciences) and subsequently sorted using a Beckman Coulter MoFlo Astrios fluorescence-activated cell sorting (FACS) sorter. Sorted cells were immediately transferred into TRI reagent (Sigma Aldrich) and stored at –80 °C until further use.

Cytokine Treatment

For cytokine treatment, MDMs or activated CD4+ T cells were cultured at 37 °C and 5% CO_2 for 4 or 24 h in RPMI Glutamax medium (Gibco) containing 10% heat-inactivated FCS (Sigma Aldrich) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Merck KGaA]) supplemented with the respective cytokine(s) at the indicated low or high concentration. The following cytokines were used (brackets indicate low and high dose and supplier): IFN-α1 (100 U/mL; 1,000 U/mL; provided by U.D. and K.S.), IFN-α2 (100 U/mL; 1,000 U/mL; Miltenyi Biotech), IFN-α6 (100 U/mL; 1,000 U/mL; provided by U.D. and K.S.), IFN-α7 (100 U/mL; 1,000 U/mL; provided by U.D. and K.S.), IFN-α14 (100 U/mL; 1,000 U/mL; provided by U.D. and K.S.), IFN-γ (100 U/mL; 1,000 U/mL; Miltenyi Biotech), TNF-α (100 U/mL; 1,000 U/mL; Miltenyi Biotech), IL-1β (100 U/mL; 1,000 U/mL; PeproTech, Germany), IL-4 (100 U/mL; 1,000 U/mL; PeproTech), IL-6 (100 U/mL; 1,000 U/mL; Miltenyi Biotech), IL-10 (10 ng/mL; 100 ng/mL; PeproTech), IL-12 (10 ng/mL; 100 ng/mL; PeproTech), IL-18 (10 ng/mL; 100 ng/mL; InvivoGen, USA), and IL-27 (10 ng/mL; 100 ng/mL; PeproTech).

RNA Preparation

Cells were washed with PBS and lysed in TRI reagent (Sigma Aldrich). The lysate was cleared from debris by centrifugation (12,000 *g* for 10 min at 4 °C), and subsequently the supernatant was transferred to a fresh reaction tube, mixed with chloroform (1× $CHCl_3$: 5× lysate) and vortexed vigorously for 15 s. Samples were incubated for 15 min at room temperature and the resulting mixture was centrifuged at 12,000 *g* for 15 min at 4 °C. The upper, RNA-containing phase was transferred to fresh reaction tubes and 0.5 mL 2-propanol per mL TRI reagent used during sample preparation were added. Samples were incubated for 5–10 min at room temperature and afterwards centrifuged at 12,000 *g* at 4 °C. Precipitated RNA was washed with 75% ethanol, air-dried and dissolved in RNase-free water. For preparation of RNA from cytokine-treated cells, either the TRI-based method (as described above) or the NucleoSpin® 96 RNA Core Kit (Macherey-Nagel, Germany) in combination with the NucleoVac 96 Vacuum Manifold (Macherey-Nagel) was used. The concentration of purified RNA was determined with a Nanodrop One© spectrometer (Thermo Fisher Scientific) and RNA was stored at –80 °C.

Quantitative RT-PCR

RNA was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA without enzyme mix served as an internal control for RT-PCR. The subsequent qPCR reaction was performed using TaqMan probes and kits (Thermo Fisher Scientific). The following TaqMan probes (Thermo Fisher Scientific) were used: *SERINC3* (01566572_m1), *SERINC5* (Hs00968169_m1), *HsCCL8* (Hs04187715_m1), *CXCL10* (Hs00171042_m1), *IFI16* (Hs00986757_m1), *BST2* (Hs00171632_m1), and TaqMan *RNase P* Control Reagent Kit. *RNase P* mRNA served as an internal control and reference for the normalization of all samples. For overall normalization, the $2^{-\Delta\Delta Ct}$ method [8] was employed using

either 293T cells, untreated controls, or timepoint 0 h as calibrator. Samples were analyzed in 384-well plates using a 7900HT real-time cycler (Applied Biosystems) or in 96-well plates using a Quantstudio 3 real-time cycler (Thermo Fisher Scientific).

Production of VSV-G Pseudotyped HIV-1

293T cells were seeded at a density of 5×10^6 cells/15-cm dish and cultivated at 37 °C and 5% CO_2 in DMEM Glutamax medium (Gibco) supplemented with 10% heat-inactivated FCS (Invitrogen) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Invitrogen]). After 24 h, cells were cotransfected with 25 µg/15-cm dish pHIV-1 NL4-3 WT (SF2 Nef wt) and pHIV-1_{NL4-3} Nef stop (ΔNef), respectively, and 2.8 µg pVSV-G/15-cm dish using PEI (Sigma-Aldrich) (3 µL of a 1 µg/µL stock solution/µg of DNA) in 2 mL OptiMEM (Gibco). After 48 h, the supernatant was harvested and virus was purified via sucrose cushion centrifugation. The relative infectious titer was determined for concentrated virus stocks as reported [9].

HIV-1 Infection Assay

Monocytes were isolated from healthy donors and differentiated into MDMs as described before. Monocytes were infected with VSV-G pseudotyped HIV-1 Nef wt or HIV-1 ΔNef at a multiplicity of infection of 1 using spinoculation (300 rpm, 90 min, RT). MDMs from the same donor were infected by direct addition of virus-containing medium. Four hours after inoculation, cells were washed and fresh medium was added. Five days after inoculation, virus-containing supernatants were harvested and analyzed for reverse transcriptase activity using the SG-PERT assay [10] and for infectious titer using the TZM-bl luciferase assay [11]. Relative infectivity data for each donor were calculated as the arbitrary ratio of infectivity values and units of reverse transcriptase activity. Uninfected cells were used as mock control.

Data Analysis and Figure Preparation

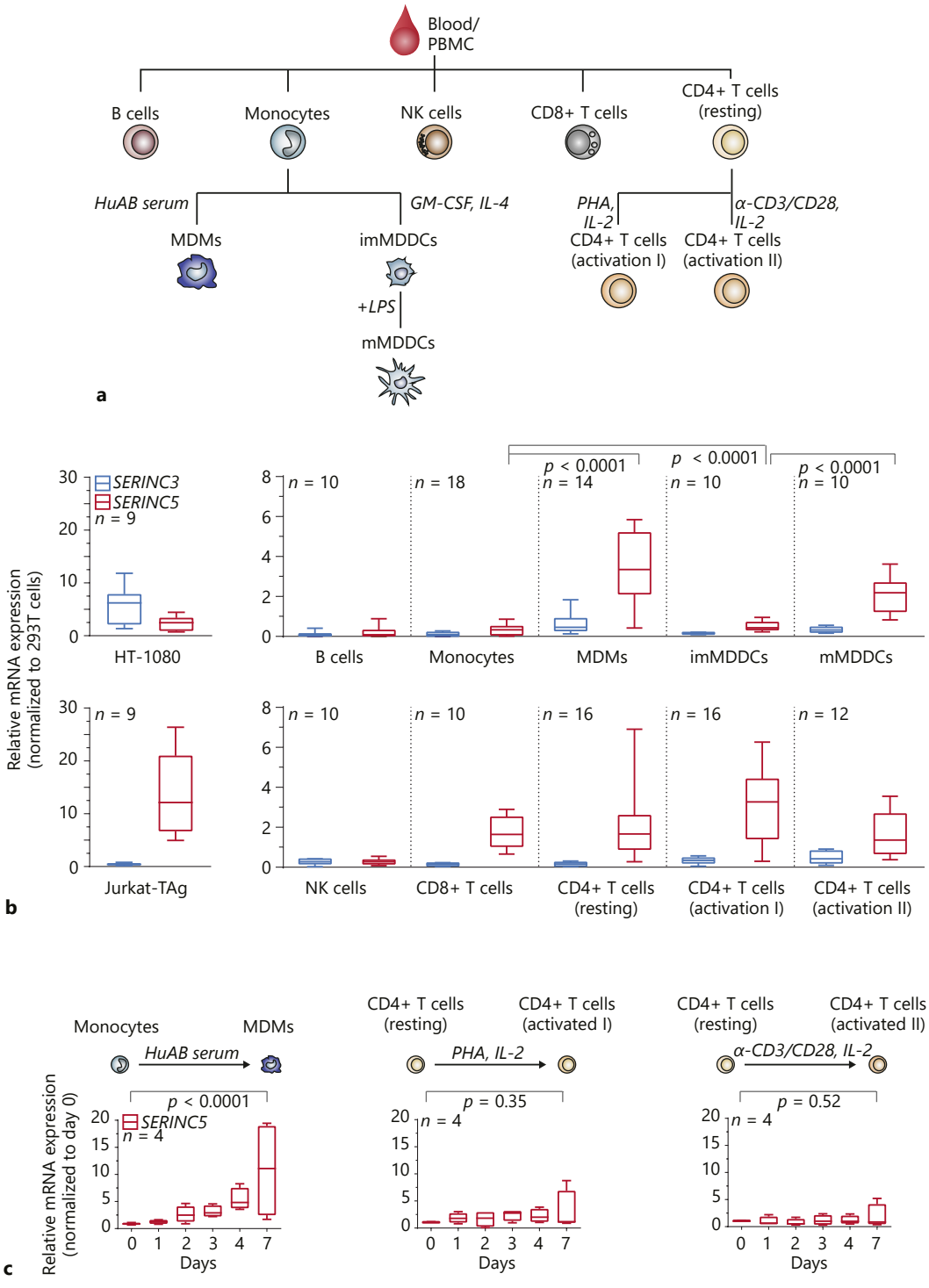
FACS data were processed with the FlowJo software package. Real-time PCR data analysis was performed using the ABI 7900HT and Quantstudio 3 real-time PCR software. Data were further processed and statistically analyzed using GraphPad Prism version 7. For tissue level comparison, data were taken from the EMBL-EBI expression atlas/The Human Protein Atlas (www.ebi.ac.uk/gxa/experiments/E-MTAB-2836/Results) and processed graphically via GraphPad Prism version 7. Figures were created in Adobe Illustrator CS6.

Results

Taking into account the lack of appropriate antibodies for the detection of endogenous SERINC proteins, we made use of real-time quantitative PCR to systematically investigate abundance levels of *SERINC3* and *SERINC5* mRNA in PBMC-derived primary cell populations, covering the major immune cells relevant for HIV-1 biology in vivo (Fig. 1a; online suppl. Fig. S1). The investigated cell types included B cells, NK cells, resting CD4+ T cells, CD8+ T cells, and monocytes. Furthermore, we analyzed MDMs, imMDDCs and mMDDCs, as well as activated CD4+ T cells, the latter having been activated either by stimulation with mitogen (activation method I: PHA/IL-2) or through the T cell receptor (activation method II: α-CD3/CD28 monoclonal antibodies/IL-2). All analyzed *SERINC* mRNAs were normalized to the mRNA of the ubiquitous single-copy gene *RNase P* and compared to those from Jurkat-TAg and HT-1080 cells, which were reported to display high and low levels of *SERINC5* mRNA as well as corresponding restriction capabilities on HIV infectivity, respectively [4] (Fig. 1b). To allow for a direct comparison between the different cell lines and

Fig. 1. Quantification of endogenous *SERINC3* and *SERINC5* mRNA levels in human hematopoietic cells. **a** Schematic representation of isolation and differentiation of cell types used in this study. B cells, monocytes, NK cells, CD8+ T cells, and resting CD4+ T cells were directly isolated by MACS or immunodensity cell isolation. MDMs or MDDCs were differentiated from monocytes using either human serum or GM-CSF together with IL-4, respectively. Maturation from imMDDCs to mMDDCs was induced by lipopolysaccharide treatment. CD4+ T cells were activated either by PHA/IL-2 (activation method I) or α-CD3/CD28 mAbs/IL-2 (activation method II). For validation of cell purity see also online supplementary Figure S1. **b** Relative mRNA levels of *SERINC3* and *SERINC5* normalized to 293T cells in either HT-1080 cells (known for low *SERINC5* levels; upper left panel), Jurkat-TAg cells (known for high *SERINC5* levels; lower left panel), or different primary cell populations (right panels). CD4+ T cell activation method I is based on PHA/IL-2 stimulation, activation II is based on α-CD3/CD28 mAbs/IL-2 stimulation (see also **a**). Box plots represent the relative mRNA levels of SERINC3/5 as determined by qPCR. *RNase P* mRNA levels in each cell type and in 293T cells ($n = 29$) were used for normalization. Relative levels in 293T cells served as an overall experimental calibrator and values in these cells were set to 1. **c** Time-resolved analysis of relative mRNA expression levels of *SERINC5* during differentiation and activation, respectively. Left panel: mRNA levels of *SERINC5* during MDM differentiation. Middle and right panel: mRNA levels of *SERINC5* during CD4+ T cell activation by method I (middle panel) or method II (right panel). Box plots show the relative mRNA levels normalized to levels in undifferentiated cells (monocytes) or resting CD4+ T cells, respectively, values for which were set to 1. Box plot elements: centerline, median; box limits, 25th to 75th percentiles; whiskers from minimum to maximum. Relevant statistical information based on unpaired *t* test analysis is provided where applicable (*p* values). HuAB, human AB; IL, interleukin; imMDDCs, immature monocyte-derived dendritic cells; mAbs, monoclonal antibodies; MDDCs, monocyte-derived dendritic cells; MDMs, monocyte-derived macrophages; mMDDCs, mature monocyte-derived dendritic cells; *n*, number of donors tested; PHA, phytohemagglutinin-P.

(For figure see next page.)


Blood/
PBMC
B cells
Monocytes
NK cells
CD8+ T cells
CD4+ T cells
(resting)
HuAB serum
GM-CSF, IL-4
PHA,
IL-2
α-CD3/CD28,
IL-2
MDMs
imMDDCs
CD4+ T cells
(activation I)
CD4+ T cells
(activation II)
+LPS
mMDDCs
a
SERINC3
SERINC5
n = 9
HT-1080
n = 10
n = 18
p < 0.0001
n = 14
p < 0.0001
n = 10
p < 0.0001
n = 10
B cells
Monocytes
MDMs
imMDDCs
mMDDCs
Relative mRNA expression
(normalized to 293T cells)
n = 9
Jurkat-TAg
n = 10
n = 10
n = 16
n = 16
n = 12
NK cells
CD8+ T cells
CD4+ T cells
(resting)
CD4+ T cells
(activation I)
CD4+ T cells
(activation II)
b
Monocytes
MDMs
HuAB serum
p < 0.0001
SERINC5
n = 4
Relative mRNA expression
(normalized to day 0)
Days
CD4+ T cells
(resting)
CD4+ T cells
(activated I)
PHA, IL-2
p = 0.35
n = 4
CD4+ T cells
(resting)
CD4+ T cells
(activated II)
α-CD3/CD28, IL-2
p = 0.52
n = 4
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1

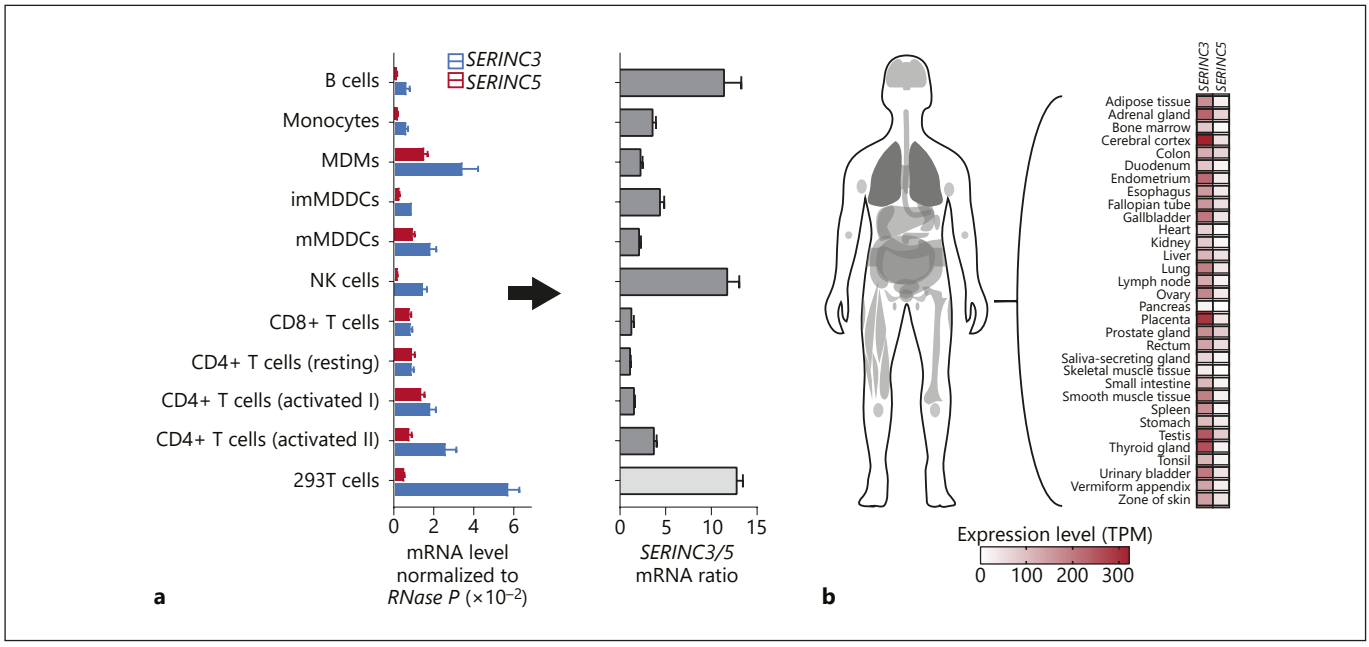

Fig. 2. *SERINC3* and *SERINC5* mRNA expression profiles in primary hematopoietic cells and body tissues. **a** The left panel shows mRNA levels of *SERINC3* and *SERINC5* normalized to *RNase P*. Levels in 293T cells are displayed as an informative reference. The right panel depicts the resulting mRNA ratios of *SERINC3* and *SERINC5*. The arithmetic means and standard errors of the mean are shown. **b** RNA-seq data of coding RNA of 122 human individuals. The heatmap represents the mRNA expression levels of *SERINC3* and *SERINC5* in 32 different tissues. Data are given in transcripts per million (TPM) and are taken from the EMBL-EBI expression atlas/The Human Protein Atlas (www.ebi.ac.uk/gxa/experiments/E-MTAB-2836/Results). imMDDCs, immature monocyte-derived dendritic cells; MDMs, monocyte-derived macrophages; mMDDCs, mature monocyte-derived dendritic cells.

primary cell sets, we first depicted relative quantifications in Figure 1b by normalizing all datasets to the relative mRNA expression levels of *SERINC* in 293T cells, which do not exert a significant restriction of HIV-1 particle infectivity and endogenously express very low and intermediate levels of *SERINC5* and *SERINC3* mRNA, respectively [4]. While none of the primary cell types analyzed showed marked differences in relative *SERINC3* mRNA expression levels compared to 293T cells (Fig. 1b), significantly higher *SERINC5* mRNA levels were found in MDMs, mMDDCs, CD8+ T cells, and CD4+ T cells. Levels of elevation of *SERINC5* in these cell types relative to 293T cells ranged from 1.7- to 3.5-fold and were characterized by a high donor-dependent variance (Fig. 1b). Remarkably, we observed a strong increase in *SERINC5* mRNA expression within the myeloid lineage during terminal differentiation (monocytes to MDMs [$p < 0.0001$] or to mMDDCs [$p < 0.0001$]) as well as activation (imMDDCs to mMDDCs [$p < 0.0001$]). In contrast, *SERINC5* mRNA levels in primary CD4+ T cells were not significantly affected by mitogen (activation method I) or T cell receptor (activation method II) stimulation and cell proliferation (Fig. 1b, c). Notably, time-resolved analysis of *SERINC5* levels during differentiation of monocytes into MDMs revealed a time- and differentiation-dependent modulation of mRNA expression (Fig. 1c, left panel).

To also allow a direct comparison of cell type-intrinsic differences in *SERINC* mRNA levels, the *SERINC3* and *SERINC5* expression levels were depicted normalized to *RNase P* mRNA (Fig. 2a, left panel) or as a ratio of *SERINC3/SERINC5* (Fig. 2a, right panel). Highest *SERINC3/5* mRNA ratios were found for primary CD19+CD20+ B cells and CD56+CD3– NK cells (which were comparable to the ratios in 293T cells), displaying more than 10 times higher levels of *SERINC3* than *SERINC5* (Fig. 2a). For all other cell types the *SERINC3/5* ratios ranged from 1.1 (resting CD4+ T cells) to 4.4 (imMDDCs) (Fig. 2a, right panel). Overall, mRNA of *SERINC3* was generally more prevalent in hematopoietic cells than that of *SERINC5*. This observation is further supported by extended data mining of RNA-seq analyses from 32 different tissues from 122 individuals showing higher levels of *SERINC3* compared to *SERINC5* mRNA across all tissues with highest abundancies in the cerebral

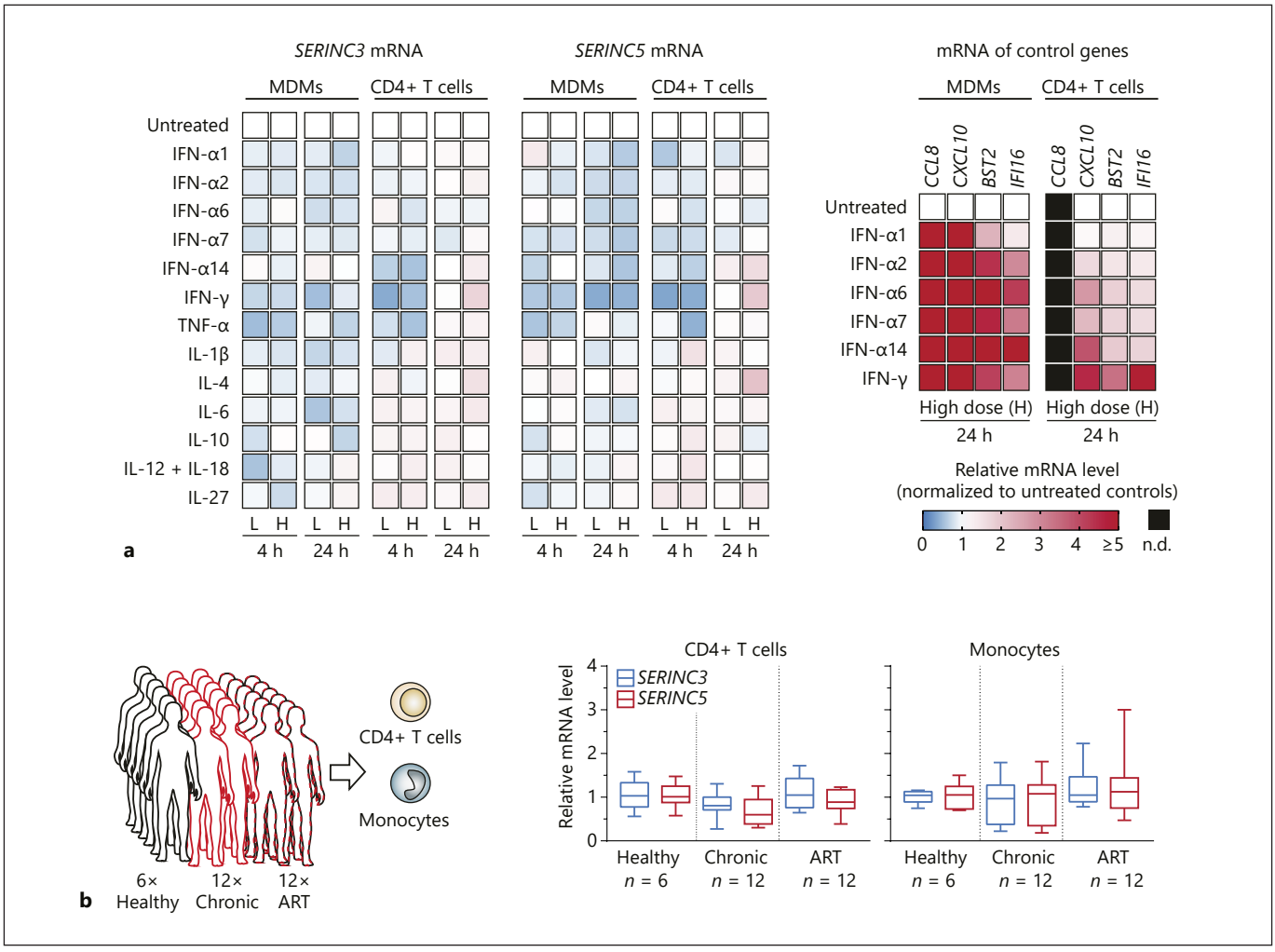

Fig. 3. *SERINC3* and *SERINC5* mRNAs are not modulated by major proinflammatory cytokines or by chronic HIV-1 infection. **a** Effect of the indicated cytokines on *SERINC3/5* mRNA expression in MDMs and PHA/IL-2-activated CD4+ T cells. Cells were treated with two different concentrations – low dose (100 U/mL or 10 ng/μL, indicated by L) or high dose (1,000 U/mL or 100 ng/μL; indicated by H] (see also Materials and Methods) – of the indicated cytokines for either 4 or 24 h. Subsequently, relative *SERINC3/5* mRNA levels were determined by real-time PCR. The activity of certain cytokines was confirmed by mRNA analysis of established marker genes (*CCL8*, *CXCL10*, *BST2*, or *IFI16*) after 24 h of treatment (right panel). All mRNA levels were normalized to the respective untreated sample at the indicated time points, which were set to 1. The heatmap represents the arithmetic mean of at least three independent measurements. For further information see also online supplementary Figures S2–S5. **b** *SERINC3/5* mRNA levels in CD4+ T cells and monocytes were not affected by chronic HIV-1 infection. Monocytes and CD4+ T cells were isolated via FACS from HIV-negative donors (Healthy) and chronically infected patients, either with viremia (Chronic) or under effective antiretroviral therapy (ART). Box plots show relative *SERINC3* or *SERINC5* mRNA levels normalized to *RNase P*. Relative mRNA levels in healthy donor samples were used as calibrator and were set to 1. Box plot elements: centerline, median; box limits, 25th to 75th percentiles; whiskers, from minimum to maximum. FACS, fluorescence-activated cell sorting; IL, interleukin; MDMs, monocyte-derived macrophages; *n*, number of donors tested; n.d., not detectable; PHA, phytohemagglutinin-P.

cortex and placenta and lowest levels in pancreas and skeletal muscle cells (EMBL-EBI expression atlas/The Human Protein Atlas (E-MTAB-2836) [12–15]) (Fig. 2b).

Innate immunity factors can directly or indirectly mediate resistance to retroviral infection. A defining feature of these factors is their induced expression by IFNs [16, 17]. The mRNA levels in primary target cells under steady-state conditions might therefore not reflect SERINC3/5 abundancies in the context of antiviral immune reactions in vivo. We therefore explored the

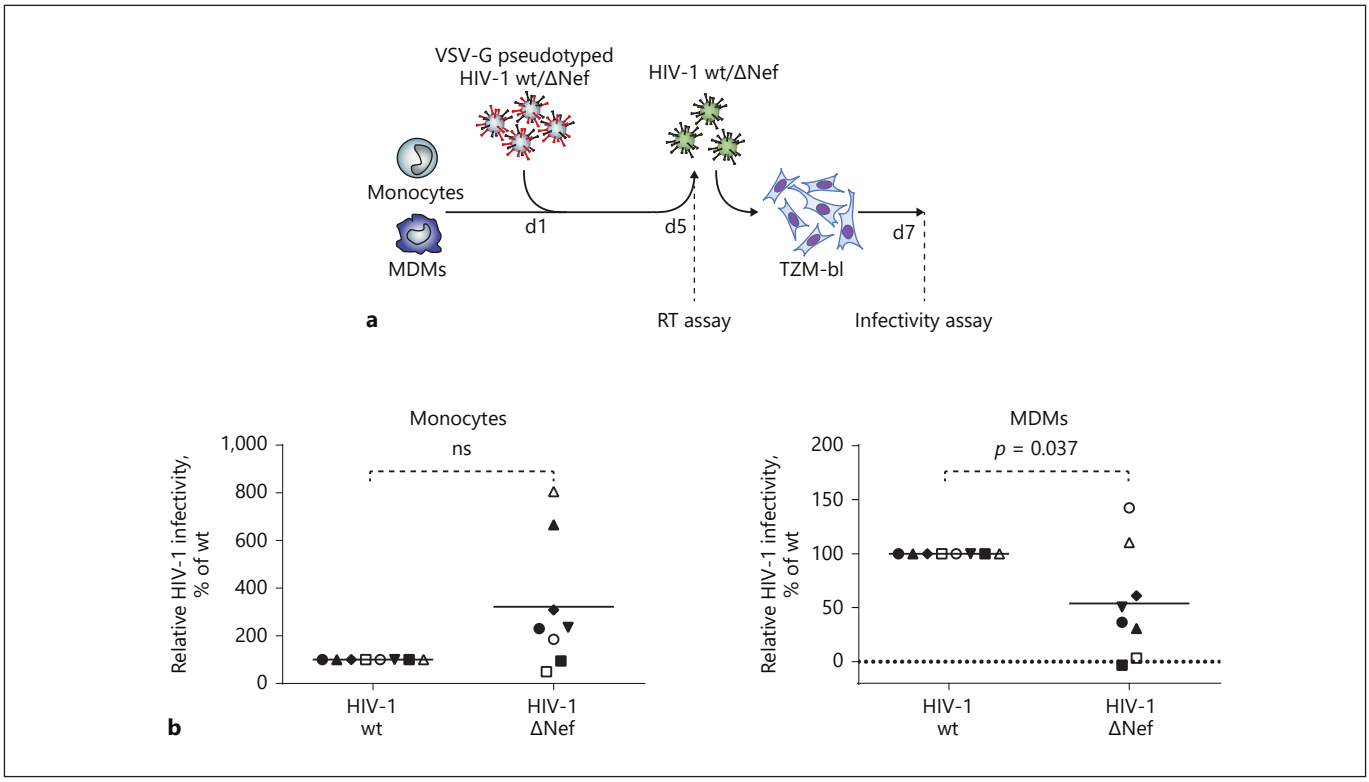

Fig. 4. Evidence that MDMs physiologically express SERINC5 protein to biologically relevant levels that can be antagonized by Nef. **a** Schematic representation of the experimental setup for infectivity assessment of HIV-1 particles derived from either monocytes or MDMs. Both cell types were infected with an identical multiplicity of infection of VSV-G pseudotyped HIV-1 wt or ΔNef virus. Four days later, supernatants containing newly produced HIV-1 wt or ΔNef particles equipped with HIV-1 Env were quantified for reverse transcriptase units by SG-PERT assay (see also online suppl. Fig. S9) and infectivity by TZM-bl luciferase assay (infectivity readout), respectively. **b** Relative infectivity (infectivity per reverse transcriptase units) of HIV-1 wt and HIV-1 ΔNef particles released from either monocytes (left panel) or MDMs (right panel) from 8 different donors (individual symbols), respectively. Values for HIV-1 wt were set to 100%. Center lines indicate the mean of each group. Paired *t* test was used for statistical analyses and the respective *p* value is given on top of the graph. MDMs, monocyte-derived macrophages; ns, not significant; RT, reverse transcriptase.

regulation of *SERINC3/5* mRNA in the two major HIV target cell populations (activated CD4+ T cells and MDMs) isolated or differentiated from PBMCs of healthy, HIV-negative donors upon exposure to a panel of cytokines, which exert antiviral activity or are upregulated within the course of HIV-1 infection [18–23] (Fig. 3a; online suppl. Fig. S2, S3). Cytokines included a set of IFN subtypes with known anti-HIV activity [18]. The strong induction of mRNAs from *CCL8*, *CXCL10*, *BST2* (Tetherin, CD317), or *IFI16* by various IFNs served as positive controls (Fig. 3a, right panel; online suppl. Fig. S4, S5) [24–26]. While in MDMs most cytokine stimuli resulted in a modest reduction of *SERINC3* and *SERINC5* mRNA levels compared to untreated control cells, *SERINC3/5* expression was slightly induced 24 h after cytokine stimulation in activated CD4+ T cells. For example, exposure to IFNα-14, IFN-γ, or IL-4 resulted in a 1.5-, 1.7-, or 2.1-fold upregulation of *SERINC5* mRNA levels, respectively) (Fig. 3a; online suppl. Fig. S3). However, compared to classical cytokine-regulated innate immunity factors, e.g., *CXCL10* or *CCL8*, with inductions of >100-fold in MDMs (Fig. 3a; online suppl. Fig. S4) and nearly 5-fold in CD4+ T cells (Fig. 3a; online suppl. Fig. S5), the *SERINC* changes observed were rather modest or absent and did not support a strong transcriptional modulation of *SERINC3/5* by IFNs and ILs. To exclude transient inductions, we also quantified *SERINC3/5* levels following exposure to selected cytokines, i.e., TNF-α, IFN-γ, and IFN-α14, during the first 4 h of stimulation (online suppl. Fig. S6A, B, S7, S8). Also here, no relevant *SERINC* mRNA changes were observed, while for instance in MDMs *CCL8* and/or *CXCL10* responded strongly (online suppl. Fig. 6C, D, S8). Thus, overall our data do not indicate a modula-

tion of SERINC3/5 levels by cytokines in primary cells relevant for replication of HIV.

Next, we explored whether mRNA levels of *SERINC3/5* might be altered during chronic HIV-1 infection and/or by the patient's treatment status. To this end, CD4+ T cells and monocytes were isolated by FACS from untreated, chronically HIV-infected patients with viremia ("chronic," $n = 12$; see also online suppl. Table S1) or with long-term ART-mediated suppression of viremia ("ART," $n = 12$), or from HIV-negative controls ($n = 6$) and analyzed by real-time PCR. Remarkably, mRNA levels of *SERINC3* and *SERINC5* were found to be indistinguishable between the three patient groups (Fig. 3b).

Based on our analysis of *SERINC3* and *SERINC5* abundancies in various PBMC-derived cell subpopulations, we surmised that increased levels of SERINC5 protein in MDMs may negatively affect the infectivity of Nef-deficient viral particles produced in these cells compared to monocytes. The latter harbor only low levels of *SERINC5*, on average more than 10-fold less compared to MDMs (Fig. 1b, c). To address this hypothesis, we investigated whether a Nef-dependent impact on the relative infectivity of HIV-1 particles released from MDMs compared to monocytes could be observed. Therefore, both cell types were infected with an identical multiplicity of infection of HIV-1 wt or ΔNef virus, which had been pseudotyped for a single round with VSV-G. Newly produced HIV-1 particles were harvested 4 days after infection and culture supernatants analyzed by measuring reverse transcriptase units (SG-PERT assay) as well as reporter cell infection (TZM-bl luciferase assay), respectively (Fig. 4a). Using this experimental approach, the amount of released particles from either monocytes or MDMs, determined by the activity of reverse transcriptase in supernatants, was comparable for HIV-1 wt and ΔNef viruses within and between the two cell types (online suppl. Fig. S9).

Nef did not significantly enhance the relative infectivity of particles released from monocytes and even appeared to impair HIV-1 infectivity in some donors (Fig. 4b, left panel). In contrast to monocyte-derived viral particles, HIV-1 produced in MDMs showed significantly less infectivity when produced in the absence of Nef ($p = 0.037$) (Fig. 4b, right panel). Also here, a high donor variability was noted, with some donors showing no Nef-dependent effect while others showed a complete loss of the infectivity of Nef-deficient HIV-1 particles. Together, these results support the notion that HIV-susceptible MDMs physiologically express SERINC5 protein to biologically relevant levels that can be antagonized by Nef.

Discussion and Conclusion

The recent discovery of antiviral activities of SERINC3 and SERINC5 has fueled research into this unique protein family, but has also raised questions regarding the physiological relevance of SERINC in the context of HIV infection. Here, we systematically analyzed the abundance levels of *SERINC3/5* mRNA in primary immune cells of the lymphoid and myeloid lineage. *SERINC3/5* abundance or dynamics during differentiation only for the myeloid lineage mirrored the natural increase in HIV susceptibility from monocytes to macrophages that would be expected for an innate immunity factor. In contrast, the transition of largely nonsusceptible quiescent to activated proliferating CD4+ T cells did not go along with a boost in *SERINC3/5* mRNA expression. Of note, our observation of increased *SERINC5* mRNA levels in macrophages was made in human AB serum-differentiated MDMs. Since macrophages are known to be significant drivers of HIV pathogenesis in vivo [27], it cannot be excluded that certain subsets of macrophages harbor lower amounts of SERINC3 and 5 and thus are more likely to release infectious HIV-1. Preliminary observations in cells from two donors indicate that M2 macrophages may express higher levels of *SERINC5* mRNA than M1 macrophages (data not shown). Together, our current findings and the established role of tissue-resident macrophages for HIV production in vivo [27] warrant future studies in this direction.

As the physiological expression levels of SERINC proteins under steady-state conditions might not reflect their abundancies during inflammation or viral encounter, we exposed major target cells of HIV-1, namely macrophages and activated CD4+ T cells, to different cytokines and IFNs, which have been described to be relevant within the course of HIV infection. As reported previously for IFN-β and lipopolysaccharide by Rosa et al. [4] and expanded in the current study to a larger and more diverse set of stimuli, the mRNA expression of *SERINC5* and *SERINC3* displayed no marked modulation by TNF-α, IFNs, or proinflammatory ILs, whereas classical HIV innate immunity factors like *Tetherin* (*BST2*, *CD317*) or *IFI16* were upregulated. While the absent or modest mRNA changes observed for *SERINC* by multiple cytokine stimuli call their biological relevance into question, one cannot exclude that also a two-fold upregulation, e.g., as observed for *SERINC5* mRNA in CD4+ T cells upon IL-4 treatment, could in principle affect the infectivity of Nef-deficient virions released from this cell type. Overall, this may not be likely, since the >10-fold upregulation of *SERINC5* mRNA in MDMs rela-

tive to monocytes resulted in an only two-fold, Nef-dependent impairment of HIV infectivity (Fig. 4). Moreover, no significant changes in *SERINC3/5* mRNA expression were observed in cells during chronic HIV-1 infection compared to healthy controls, thus apparently not reflecting a common anti-HIV response by the infected host.

Nevertheless, the analyzed *SERINC5* mRNA levels upregulated during monocyte to MDM differentiation go in line with the observed Nef-sensitive defect in particle infectivity. Similarly, no positive effect of Nef on HIV infectivity was observed in *SERINC5* mRNA-low-expressing monocytes. Since monocytes, in contrast to terminally differentiated macrophages, are largely refractory to productive HIV-1 infection in vivo [28], the expression of antiviral factors that act late in the replication cycle may be dispensable for the host's intrinsic defense in this undifferentiated cell type. However, it cannot be excluded that during myeloid differentiation also other, yet to be identified Nef-sensitive antiviral factors might be upregulated, as recently suggested for T cells [29].

Overall, our data allow us to classify SERINC proteins as unconventional innate HIV-1 restriction factors. This notion is supported by a recent bioinformatics study, which indicated that the evolution of SERINC3 and SERINC5 proteins in primates did not follow characteristics of an arms race between hosts and lentiviruses documented for other antiviral restriction factors, including APOBEC3 F/G, Trim5α, SAMHD1, and Tetherin [30]. Nevertheless, based on previous SERINC3/5 overexpression studies in cell lines, which demonstrated a strong, Nef-dependent impact on HIV particle infectivity, and our current results with a more moderate, yet significant Nef-dependent phenotype in primary macrophages, it seems justified to conclude that these SERINC family members are capable of impeding HIV infection in the human host. Future studies will need to address at which stage and to what degree SERINC proteins shape the dynamics of HIV transmission and infection in vivo.

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Statement of Ethics

Usage of blood cones was approved by the ethics committee of the LMU München, Munich, Germany (project No. 17-202-UE). HIV-1-infected individuals were recruited from the outpatient clinic at the Department of Infectious Diseases of the University Hospital Munich. The study was approved by the Institutional Review Board of the LMU München, Munich, Germany (project No. 274-03). All participating donors provided written informed consent.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A. Zutz, C. Schölz, S. Schneider, M. Münchhoff, V. Pierini, O.T. Fackler, and O.T. Keppler designed the study and/or performed experiments. K. Sutter and U. Dittmer provided IFN-α subtypes, R. Draenert and J.R. Bogner provided PBMCs from HIV patients, and G. Wittmann provided blood cones. A. Zutz, C. Schölz, and V. Pierini performed statistical analyses. A. Zutz, C. Schölz, O.T. Fackler, and O.T. Keppler wrote the manuscript. All authors reviewed the manuscript.

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