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KNJIŽNICA MEDICiNSKOG FAKULTETA

Braće Branchetta 20 | HR - 51000 Rijeka
e-mail:knjiznica_medri@medri.uniri.hr
www.medri.uniri.hr
Tel:+385 (0)51 651199 I +385 (0)51 651123

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'Activated' STAT proteins – a paradoxical consequence of inhibited JAK-STAT signalling in cytomegalovirus-infected cells

Running title: Cytomegaloviral inhibition of STAT3 signalling

Mirko Trilling[†,‡,*], Vu Thuy Khanh Le[‡,†], Jassin Rashidi-Alavijeh[†,‡,§§], Benjamin Katschinski[†‡], Jürgen Scheller[§], Stefan Rose-John[¶], Gabriela Elena Androsiac[‡], Stipan Jonjić[‡‡], Valeria Poli[‖], Klaus Pfeffer[#] and Hartmut Hengel[‡,††,*]

[†]Institute for Virology, Robert-Koch-Haus, University Hospital Essen, University Duisburg-Essen, D-45147, Essen, Germany

[‡]Institute for Virology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, D-40225, Düsseldorf, Germany

[§]Institute for Biochemistry und Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Düsseldorf, D-40225, Düsseldorf, Germany

[¶]Institute of Biochemistry, Christian-Albrechts-University of Kiel, D-24118, Kiel, Germany

[‡‡]Department for Histology and Embryology, School of Medicine, University of Rijeka, 51000 Rijeka, Croatia

[‖]Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin, 10126, Turin, Italy

[#]Institute for Medical Microbiology and Hospital Hygiene, Medical Faculty, Heinrich-Heine-University, Düsseldorf, D-40225, Düsseldorf, Germany

[††] Institute for Virology, University Medical Center, Albert-Ludwigs-University, D-79104 Freiburg, Germany

[§§]Current address: Department of Gastroenterology and Hepatology, University Hospital Essen, University Duisburg-Essen, D-45147, Essen, Germany

[*]Corresponding author

Phone (HH):	+49 (0) 761 203 6534
Phone (MT):	+49 (0) 201 723 83830
Fax:	+49 (0) 761 203 6626
E-mail:	Mirko.Trilling@uk-essen.de
	Hartmut.Hengel@uniklinik-freiburg.de

Abstract:

We have previously characterized mouse cytomegalovirus (MCMV)-encoded immune evasive interferon (IFN) signalling inhibition and identified the viral protein pM27 as inducer of proteasomal degradation of STAT2. Extending our analysis to STAT1 and STAT3, we found that MCMV infection neither destabilizes STAT1 protein nor prevents STAT1 tyrosine Y701 phosphorylation, nuclear translocation or the capability to bind GAS DNA-enhancer elements. Unexpectedly, the analysis of STAT3 revealed an induction of STAT3 Y705 phosphorylation by MCMV. In parallel, we found decreasing STAT3 protein amounts upon MCMV infection although STAT3 expression normally is positive autoregulative. STAT3 phosphorylation depended on the duration of MCMV infection, the infectious dose and MCMV gene expression but was independent of IFNAR1, IL-10, IL-6 and JAK2. Although STAT3 phosphorylation did not require MCMV *immediate early* (IE)1, pM27 and *late* gene expression, it was restricted to MCMV-infected cells and not transmitted to bystander cells.

Despite intact STAT1 Y701 phosphorylation, IFNγ-induced target gene transcription (e.g. *IRF1* and *SOCS1*) was strongly impaired. Likewise, the induction of STAT3 target genes (e.g. *SOCS3*) by IL-6 was also abolished, indicating that MCMV antagonizes STAT1 and STAT3 despite the occurrence of tyrosine phosphorylation. Consistent with the lack of SOCS1 induction, STAT1 phosphorylation was prolonged upon IFNγ treatment. We conclude that the inhibition of canonical STAT1 and STAT3 target gene expression abrogates their intrinsic negative feedback loops, leading to accumulation of phospho-Y-STAT3 and prolonged STAT1 phosphorylation.

These findings challenge the generalization of Tyr-phosphorylated STATs necessarily being transcriptional active and document antagonistic effects of MCMV on STAT1/3-dependent target gene expression.

(250 words)

Introduction

Cytomegaloviruses (CMVs) are prototypical β-herpesviruses with an enveloped virion coating a large double stranded DNA genome of ~230 kbp which encodes numerous viral proteins. Upon infection, CMVs initiate a sequential and highly coordinated gene expression profile, starting with *immediate early* (*IE*) transcripts, followed by *early* and *late* gene products - the latter being simultaneously expressed with and depending on genome amplification by rolling circle replication.

Although the infection in healthy individuals is mostly asymptomatic due to virus control by a concerted response of the innate and adaptive immune system (1), primary as well as recurrent CMV infections cause symptomatic or even fatal pathologies in immuno-compromised or immature individuals. Irrespective of their robust immune stimulatory capacity, CMVs circumvent sterile immunity and instead establish a lifelong latency. Reactivation occurs upon immuno-suppressing and/or stressful conditions due to (re-) initiation of the lytic viral replication program. Human CMV (HCMV, HHV-5, taxonomy ID: 10359) frequently causes congenital infections via a vertical transmission from the mother to the developing foetus and constitutes the most frequent non-genetic congenital complication in western countries (2).

CMVs have undergone an intimate and long lasting co-adaptation with their respective host species and are thus highly species-specific, which restricts efficient viral replication to cells of the native host species and thereby excludes experimentation with HCMV in animal models. The related mouse cytomegalovirus [MCMV, Murid herpesvirus 1, taxonomy ID: 10366]) has a co-linear and partially homologous genome and infects mice (*Mus musculus,* taxonomy ID: 10090) allowing to study cytomegaloviral pathogenesis. Additionally, MCMV represents one of the few DNA viruses naturally infecting *Mus musculus* as its native host and has therefore become a widely used model to explore virus-host interactions and to assess their consequences *in vivo*.

Among the earliest immune responses raised against viruses is the secretion of interferons (IFNs). IFNs are pleiotropic cytokines expressed upon encounter of pathogens or their pathogen-associated molecular patterns (PAMPs). IFNs bind to specific cell surface resident IFN receptors and subsequently induce a rapid janus kinase (JAK) mediated phosphorylation of signal transducer and activator of transcription (STAT) which than instruct specific transcriptional programs to (re-)enforce intrinsic resistance, induce innate immunity and stimulate and recruit adaptive immune responses. Thereby, IFNs orchestrate the induction of an antiviral state that efficiently restricts viral replication (3).

Type I IFNs (IFNα/β) mainly induce STAT1 and STAT2 Tyr (Y) phosphorylation (residues Y701 and Y689, respectively) upon binding of the IFN to the heterodimeric IFNAR1-IFNAR2 receptor complex and the accompanying activation of the receptor-associated kinases JAK1 and tyrosine kinase 2 (Tyk2). STAT3 becomes also Y-phosphorylated (at residue Y705) by type I IFNs. These STAT Y-phosphorylation events are essential for transcriptional activation of STAT molecules (4-7) and are thus widely considered to constitute a hallmark and faithful surrogate marker for STAT activation. Phosphorylated STAT1 and STAT2 recruit IRF9 to form the IFN-stimulated gene factor 3 (ISGF3), which binds to IFN-stimulated response elements (ISRE) to recruit the transcriptional machinery and stimulate the expression of adjacent genes. In contrast, IFNγ mainly signals via Y-phosphorylated STAT1 homodimers which are induced upon binding of IFNγ to the heterodimeric IFNGR1-IFNGR2 receptor and the activation of JAK1 and JAK2.

Besides IFNs, STAT3 Y705 phosphorylation is also induced by a broad variety of growth factors and cytokines (e.g. IL-6, IL-10, EGF, LIF, OSM and Leptin). It is noteworthy that STAT3-activating stimuli play an important role in CMV biology. For example, *IL-6* transcripts are strongly induced in HCMV-infected cells (8, 9) stimulating IL-6 secretion (10). Interestingly, recent results document an important regulation of HCMV reactivation by IL-6 (11-13). Additionally, MCMV induces the expression of IL-10 to suppress MHC class II presentation (14). HCMV encodes a STAT3-activating IL-10 homolog acquired by molecular piracy (15).

Viruses counteract the antiviral activity of the IFN system by expressing IFN antagonists, targeting e.g. JAK-STAT signal transduction (16, 17). We have identified and characterized the MCMV-encoded IFN inhibitor pM27. Replication of a *M27* deletion virus mutant (ΔM27-MCMV) is almost not affected in cell culture (18-21) but is highly attenuated *in vivo* (18, 21). Using a forward genetic screening approach, we identified pM27 to be an IFN antagonist targeting STAT2 (21) and thereby abrogating the induction of IFN target genes (21, 22). Consistently, replication of ΔM27-MCMV is highly susceptible to IFNs in cell culture (21, 23). pM27 acts by recruiting cellular DDB1-containing ubiquitin ligase complexes and to proteasomally degrade STAT2 (23). Like MCMV, HCMV also encodes a yet unknown protein inducing proteasomal degradation of STAT2 (24).

Here, we extend our analysis to the interplay between MCMV and further STAT transcription factors present in CMV target cells. Unexpectedly, we found prolonged STAT1 Y-phosphorylation and seemingly autonomous STAT3 phosphorylation as consequence of viral JAK-STAT inhibition instead of being a hallmark of STAT activation.

Material and Methods:

Cells and cytokines

NIH3T3 (ATCC CRL-1658), RAW 264.7 (ATCC TIB-71), M2-10B4 (kindly provided by Brendan Marshall [Medical College of Georgia, USA]), mHTC-K2 (25), STAT3$^{flox/flox}$, crisis immortalized IFNAR1- (26), respective C57BL/6 control fibroblasts, STAT3- (27), JAK2- (28), IL-6- and primary IL-10-deficient (prepared as described (29)) fibroblasts were grown in Dulbecco's modified eagle medium (D-MEM) with 10% (vol/vol) foetal bovine serum (Gibco [Invitrogen]), streptomycin, penicillin and 2 mM glutamine.

IFNs (mouse IFNα (#12100-1) and mouse IFNγ (#12500-1)) were purchased from PBL Biomedical Laboratories, New Jersey, USA. If not stated otherwise, cells were treated with 500 U/ml IFN. Hyper-IL-6 has been described previously (30).

Viruses, infection conditions and virus titration

Preparation, purification and titration of MCMV stocks was done as described previously (21, 26, 31). wt-MCMV and ΔM27-MCMV have been described previously (21). For the construction of Δm157-MCMV:*mCherry*, a *frt*-site-flanked fragment encompassing the HCMV-derived major IE promoter/enhancer (MIEP) in front of the *mCherry* gene was introduced into a recombinant MCMV bacterial artificial chromosome (which already harbored a *frt* site instead of the *m157* CDS) by flp-mediated recombination. UV inactivation was conducted with indicated J/m^2 doses of UV irradiation in a CL-1000 UV cross-linker (UVP).

Electromobility shift assay (EMSA)

Extraction of fractionated EMSA lysates and the EMSA assay was performed as described (26). Briefly, cells were lysed in cytosolic extraction buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 0.2% [vol/vol] NP-40, 0.1 mM EDTA, 10% [vol/vol] glycerol, 0.1 mM Na-vanadate, 0.1 mM PMSF, 1 mM dithiothreitol (DTT), Complete® protease inhibitors [Roche, Mannheim, Germany]). The extracts were centrifuged at 16,000 g for 16 s at 4°C, the supernatants were collected, centrifuged for 10 min and used as cytosolic extracts for EMSA. The pellets were washed in PBS and suspended in nuclear extraction buffer (20 mM HEPES, pH 7.6, 420 mM KCl, 0.1 mM vanadate, 20% [vol/vol] glycerol, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, Complete® protease inhibitors [Roche, Mannheim, Germany]). After incubation on ice for 25 min the extracts were centrifuged at 16,000 g for 25 min at 4°C, and the supernatants were used as nuclear extracts. Both extracts were frozen immediately in liquid nitrogen until final use. Nuclear or cytosolic lysates were incubated with 1 ng (~50 000 cpm) of (^{32}P)-labelled M67 GAS (32) probe for 20 min at room temperature. The DNA-protein complexes were separated on 4.7% (vol/vol) polyacrylamide, 22.5 mM TrisHCl, 22.5 mM borate and 50 µM EDTA gels, fixed and finally visualized by autoradiography. Supershifts were performed with a STAT1 antibody (Santa Cruz).

Immunoblotting

Cells were lysed in RIPA$^+$-buffer (50 mM Tris-HCl, 150 mM NaCl, 1% [vol/vol] IGEPAL, 1% Na-Deoxycholate [weight/vol], 0.1% [weight/vol] SDS, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml pepstatin, 50 mM NaF, 0.1 mM Na-vanadate with Complete® protease inhibitors [Roche] pH 7.5). Samples were normalized according to Bradford protein staining and equal amounts were subjected to denaturing SDS-PAGE. Gels were blotted on nitrocellulose membranes (GE Healthcare) and probed with indicated antibodies. The same membrane was used and consecutively stripped with reblot solution (Millipore). The following antibodies were used: α-β-actin from Sigma-Aldrich; α-STAT3 (K-15), α-STAT3 (C-20), α-STAT3 (H-190), α-STAT3 (F-2), α-phospho-Y705 STAT3 (B-7), α-STAT1(E-23), α-IκBα (C-21), and α-IRF-1 (M-20) from Santa Cruz; α-phospho-Y701 STAT1, α-phospho-S727 STAT1, α-phospho-S727 STAT3 and α-Lamin A/C from Cell Signaling, α-SOCS3 (ab16030) from Abcam. α-pp89-IE1 (Croma101) and α-pM45 were provided by Stipan Jonjić, University of Rijeka, Croatia. The actual MCMV antigen of the α-pM45 was defined by co-immuno-precipitation and mass-spectrometry by Gabriela E. Androsiac, HHU Düsseldorf, Germany. Quantification of immunoblot signals was conducted using a Fusion FX7 (Vilber Lourmat).

Luciferase Assay

For reporter gene assays, a NIH3T3-derived cell line harbouring a GAS luciferase vector was selected. To do so, a fragment comprising the promoter/enhancer sequence and the fire fly (*Photinus pyralis*) luciferase gene from the pTA-GAS construct (Clontech) was sub-cloned into a pGene vector backbone conferring Zeocine resistance (Invitrogen). Cells were induced with 100 U/ml of the indicated recombinant mouse IFN. Luciferase activity was measured using the *luciferase reporter gene assay, high sensitivity* according to the manufacturer's instructions (Roche) using a microplate luminometer (model LB 96V; Berthold).

Northern blot analysis of specific transcripts

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Total RNA was subjected to MOPS gel electrophoresis and transferred to nylon membranes using the TurboBlotter (Schleicher and Schuell). Probes were prepared by PCR with gene-specific primers (Table 1) and digoxigenin-labelled dUTP (Roche) for detection of indicated transcripts. Hybridization and detection were performed as described in Roche manuals.

Analysis of nuclear translocation of STAT1

For visualization of IFN-γ-induced nuclear translocation of STAT1, we constructed a STAT1-EGFP fusion protein. Based on the expression vector pIRES2EGFP-mSTAT1HA (STAT1HA amplified using primers mSTAT1-1 CGGCTAGCATGTCACAGTGGTTCGAGCTTC and mSTAT1-<u>HA</u>2 CGCTCGAGTTA<u>AGCGTAATCTGGAACATCGTATGGGTA</u>TACTGTGCTCATCATACTGTCAAATTC with total RNA of BALB/c MEF as template), we generated a PCR product using the primers mSTAT1-1 and HA-EGFP-2 CGGAATTCGAGCGTAATCTGGAACATCGTATGGG. This fragment was digested with *Nhe*I and *Eco*RI to clone it in two steps into pEGFP-N1, resulting in STAT1-EGFP. NIH3T3 fibroblasts were transfected (Superfect, Qiagen) with a STAT1-EGFP expression vector. 24 h post transfection, cells were mock-treated or infected with Δm157-MCMV:*mCherry* (2 PFU/cell). 16 h post infection, cells were incubated with 200 U/ml IFN-γ for 45 min. After IFN-treatment, cells were fixed using 4% (weight/vol) paraformaldehyde-PBS for 20 min. IFNγ-induced nuclear translocation of STAT1-EGFP was visualized by fluorescence microscopy using a Leica DM IL LED Fluo and LAS V4.0.

Chromatin immuno-precipitation (ChIP)

Crosslinking was achieved by adding formaldehyde (1% [vol/vol] final concentration) to cells. After 10 min, crosslinking was stopped by addition of glycine (125 mM final concentration). Cells were washed twice with ice cold PBS and subsequently detached from the cell culture plates by scraping in ice cold Na-butyrate containing PBS. Cells were washed in buffer 1 (0.5% [vol/vol] Triton-X-100, 20 mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.5 and 20 mM Na-butyrate) and buffer 2 (400 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.5 and 20 mM Na-butyrate). Cells were lysed in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA, 1% [vol/vol] Triton-X-100, 0.1% [weight/vol] SDS, 0.5% [weight/vol] Na-deoxycholate, 1 mM PMSF, 10 mM Na-butyrate and Complete protease inhibitors (Roche). Lysates were sonicated (5 cycles [2 min each]; amplitude 30; cycle 0.5 on ice in a Sartorius Labsonic P) and subsequently cleared by centrifugation. Supernatants were pre-cleared by addition of protein G sepharose (PGS) in presence of 100 µg/l salmon sperm DNA and 500 µg/l BSA. After the pre-clearing procedure, an aliquot was stored ('input'). Precipitation was performed over night at 4°C using a STAT1-specific antibody (E-23; Santa Cruz). Immune complexes were precipitated using PGS (1-2 h at 4°C). Afterwards, the sepharose was washed twice with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% [weight/vol] SDS, 0.5% Na-deoxycholate and 1% [vol/vol] NP-40), twice with high salt buffer (500 mM NaCl, 50 mM Tris pH 8.0, 0.1% [weight/vol] SDS and 1% [vol/vol] NP-40), twice with LiCl buffer (25 mM LiCl, 50 mM Tris pH 8.0, 0.5% [weight/vol] Na-deoxycholate and 1% [vol/vol] NP-40) and twice with TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA). Immune complexes were eluted using elution buffer (2% [weight/vol] SDS, 0.1 M $NaHCO_3$ and 10 mM DTT). Crosslinking was reversed by addition of 0.05 volume of 4 M NaCl and subsequent incubation for 4 h at 65°C. Proteins were digested using Proteinase K. After standard phenol/chloroform extraction, ethanol precipitation and washing with 70% (vol/vol) ethanol, immune-precipitated DNA was analysed by PCR using previously described primers (33) specific for the mouse *irf-1* promoter (5'- AGCACAGCTGCCTTGTACTTCC-3' and 5'-CTTAGACTGTGAAAGCACGTCC-3') yielding a 229 nucleotide long product.

Results:

MCMV abrogates STAT1 signalling downstream of intact STAT1 Y701 phosphorylation, nuclear translocation and DNA-binding

Intrigued by the central role of STAT1 for type I and type II IFN signalling, we investigated the interplay between MCMV and STAT-activation by IFNs. In clear contrast to STAT2, which is rapidly degraded upon MCMV infection (21, 23), STAT1 protein amounts remained stable in MCMV-infected cells (Fig. 1A). STAT1 Y701 phosphorylation was virtually absent in untreated cells but became strongly induced upon exposure to IFNγ (Fig. 1A). A comparable IFNγ-induced STAT1 phosphorylation was also evident in MCMV-infected cells (Fig. 1A) indicating that IFNGR proximal events of JAK-STAT1 signalling are not affected by MCMV-encoded IFN antagonists. Nuclear translocation of STAT1 was also not inhibited by MCMV since equal amounts of phosphorylated STAT1 were detected in nuclear lysates of IFNγ-incubated MCMV-infected cells and mock control cells (Fig. 1A, right panel). Consistently, the nuclear translocation of transiently transfected GFP-tagged STAT1 was not compromised upon MCMV infection (Fig. 1B). The analysis of the DNA-binding capacity of STAT1 by electro-mobility shift assay (EMSA) revealed that STAT1:STAT1 homodimers retained their capacity to bind to gamma activated sequence (GAS) DNA elements after MCMV infection - irrespective of *M27* coding capacity (Fig. 1C). Chromatin immunoprecipitation (ChIP) experiments confirmed the intact ability of STAT1 to bind to endogenous GAS DNA elements (here the *irf-1* promoter) in MCMV-infected cells upon IFNγ exposure (Fig. 1D). To test the ability of MCMV to antagonize GAS-dependent gene expression, we constructed a stable MCMV-permissive NIH3T3 cell line harbouring the firefly (*Photinus pyralis*) *luciferase* reporter gene under the control of a minimal promoter and a GAS enhancer element. In mock-infected cells, IFNγ strongly induced luciferase activity, but the response was significantly inhibited upon MCMV infection - again irrespective of *M27* coding capacity (Fig. 1E). To rule out that this effect is influenced by previously observed effects of CMV infections on gene expression derived from reporter plasmids (34), we studied the ability of MCMV to counteract the IFNγ-dependent induction of endogenous *IRF-1* mRNA by northern blotting. 4 h of MCMV infection already sufficed to antagonize IFNγ-induced *IRF-1* transcription (Fig. 1F). These results demonstrate that MCMV abrogates IFNγ-induced gene expression without compromising overall STAT1 protein amounts, receptor-proximal Y701 STAT1 phosphorylation, nuclear translocation of STAT1 or the capability to bind to DNA - largely confirming previous reports (35). Additionally, our results reveal that this inhibition is not influenced by pM27, documenting the existence of at least one additional MCMV-encoded antagonist of JAK-STAT signal transduction.

MCMV gene expression induces IFNAR1-independent STAT3 phosphorylation

Given that STAT1 and STAT3 are highly homologous proteins (53% identical and 72% similar) and that STAT3 transduces signals of pro- as well as anti-inflammatory cytokines that play a crucial role in CMV immune control and pathogenesis (11-14), we extended our analysis to STAT3. In contrast to STAT1, a basal STAT3 Y705 phosphorylation was frequently (depending on exposure time of the films) observed in untreated, mock-infected cells. Nevertheless, we observed a substantial increase of STAT3 Y705 phosphorylation upon MCMV infection (Fig. 2A-C). Surprisingly, global STAT3 protein amounts simultaneously declined in MCMV-infected cells as revealed by two different STAT3-specific antibodies recognizing different parts of STAT3 (Fig. 2A-C). Y705 phosphorylation by MCMV was invariably evident in a variety of cells including NIH3T3, M2-10B4, mHTC-K2 and others (see Fig. 2A-F and data not shown). The extent of the virus-induced Y705 phosphorylation was comparable to the effect elicited by treatment with 20 ng/ml Hyper-IL-6 - a STAT3-activating designer cytokine generated by fusion of the coding sequence of IL-6 to the extracellular domain of the gp80 subunit of the IL-6 receptor. Conversely, the MCMV-induced decline in overall STAT3 amounts was not observed in Hyper-IL-6 treated cells suggesting that the phosphorylation event does not preclude STAT3 recognition by the used antibodies (C-20 and K-15). To confirm the detected protein band to represent truly STAT3, we performed an experiment in STAT3-deficient cells. We observed STAT3 phosphorylation upon MCMV infection in STAT3-positive cells, but no signal was detectable in STAT3-deficient cells (Fig. 2B). Since the respective membranes were probed in a sequential manner, STAT3 was also detected on separate membranes by different antibodies in parallel to ensure that such procedure does not mask secondary STAT3 detection. We reproduced increased levels of Y705 phosphorylation despite an overall reduction of STAT3 protein amounts in MCMV-infected cells (Fig. 2C). The decrease of STAT3 and the parallel increase of phosphorylated STAT3 was quantified (Fig. 2D). An increasing MCMV infection dose was directly positively correlated with STAT3 Y705 phosphorylation as well as reduction of the overall STAT3 protein amount (Fig. 2E).

Among other cytokines and growth factors, IFNα is known to induce STAT3 phosphorylation (36). This effect was also observed in MCMV-permissive NIH3T3 cells (Fig. 2F). Since MCMV infection initially induces type I IFN production (31), we used IFNAR1-deficient cells to test if the observed STAT3 phosphorylation is caused by type I IFN. STAT3 phosphorylation and reduction of STAT3 protein amounts were observed with almost congruent kinetics in IFNAR1-deficient and identically immortalized C57BL/6 fibroblasts (Fig. 2G), indicating that both effects on STAT3 are IFNAR1-independent.

To analyse if viral gene expression is required for STAT3 modulation, NIH3T3 cells were infected with MCMV virions which had been inactivated with grading UV doses (250 - 10,000 J/m^2). Subsequently, STAT3 phosphorylation and global STAT3 protein amounts were assessed by immunoblot. UV-inactivation of viral gene expression, as documented by pp89-IE1 detection, abrogated STAT3 phosphorylation and reduction (Fig. 2H), indicating that cytomegaloviral gene expression is essential for the observed modulation of STAT3.

We also detected STAT3 phosphorylation upon reversible application of protein synthesis inhibitor Cycloheximide (CHX) followed by Actinomycin D (ActD) inhibition of transcription leading to MCMV gene expression restricted to *immediate early* (IE) gene products (37). However, this result cannot be interpreted as a proof for the responsibility of viral *IE* genes due to similar drug-induced changes in STAT3 phosphorylation levels in UV-MCMV as well as mock-infected cells (data not shown). A previously described fibroblast cell line stably transfected with the *IE* gene region encompassing the respective *Hin*dIII fragment of MCMV and thus being able to complement the growth of MCMV mutants lacking the essential gene *ie3* (38), did not exhibit overt STAT3 phosphorylation or reduced STAT3 protein amounts in comparison to parental NIH3T3 cells (data not shown), suggesting that *ie* gene expression is not sufficient for the observed changes of STAT3.

To evaluate whether MCMV *late* gene expression is required for the effects on STAT3, we infected cells in the presence of ganciclovir (GCV) - a drug which interferes with MCMV DNA replication and thereby strongly reduces the accompanying viral *late* gene expression. *Late* gene expression was neither essential for the phosphorylation of STAT3 nor for the reduction of STAT3 overall amounts (Fig. 2I). Taken together, these results reveal that (a) MCMV *IE*- and/or *early*-expressed gene product(s) induce(s) a sustained STAT3 phosphorylation and simultaneously reduce(s) STAT3 overall amounts.

The MCMV-induced STAT3 Y705 phosphorylation occurs independent of IL-6, IL-10, gp130 and JAK2 and is restricted to infected cells

As mentioned above, MCMV induces cytokines like IL-6 and IL-10, which mainly signal via STAT3. Therefore, we tested if these cytokines are involved in the MCMV-induced STAT3 Y705 phosphorylation. Fibroblasts deficient for IL-6 and IL-10, respectively, both exhibited

STAT3 phosphorylation upon MCMV infection (Fig. 3A), indicating that these interleukins are not essential for the observed phosphorylation. Since JAK2 is known to be crucial for the signalling of a variety of cytokine receptors (28, 39), we assessed JAK2-deficient fibroblasts, but again we observed STAT3 phosphorylation upon MCMV infection (Fig. 3A). However, we could not exclude the possibility that STAT3 phosphorylation results from redundant signalling via several receptors. Paralleling STAT3 phosphorylation, the reduction of the overall STAT3 protein amount was also evident in the three gene-deficient cells and the control fibroblasts (Fig. 3A).

STAT3 can be phosphorylated by cytokines via receptor bound janus kinases but also by growth factors via receptor tyrosine kinases (RTKs) and by cytoplasmic kinases like c-Src (40) and BCR-ABL (41, 42). Several STAT3-activating cytokines signal via a heterodimeric receptor complex composed of a cytokine-specific subunit and the gp130 signalling module. Soluble gp130-Fc blocks gp130-dependent IL-6 signalling and to a lesser extent LIF and OSM signalling (43). Therefore, we used commercially available soluble gp130-Fc, to test the involvement of gp130 in MCMV-induced STAT3 phosphorylation. As expected, soluble gp130 blocked Hyper-IL-6-dependent STAT3 activation, but the STAT3 phosphorylation by MCMV was not impaired (data not shown).

Next, we transferred conditioned medium (sterile-filtered or UV-inactivated) from MCMV-infected NIH3T3 cells (which exhibited substantial STAT3 phosphorylation) to uninfected fibroblasts and RAW 264.7 macrophages, respectively. Conditioned medium did not induce STAT3 phosphorylation above background in fibroblasts or RAW cells (Fig. 3B). Subsequently, we conducted transwell experiments in which MCMV-infected and uninfected cells continuously share the same medium. Again, STAT3 phosphorylation was only observed in infected cells but not in uninfected bystander cells (Fig. 3C), suggesting that the STAT3 Y705 phosphorylating principle is restricted to infected cells and not transferable to neighbouring cells when MCMV transmission is prevented. From these data we assume that STAT3 phosphorylation does not require a secreted factor released from infected cells, although we cannot formally rule out the implication of membrane bound cytokines (like for example the tumour necrosis factor (TNF) superfamily member LIGHT which has been shown to induce STAT3 phosphorylation (44)).

Under standard (non-serum-starved) culture conditions, cells are constantly exposed to growth factors present in the foetal bovine serum contained in the cell-culture media. Several growth factors (e.g. basic fibroblast growth factor (45) and insulin-like growth factor I (46)) have been demonstrated to induce STAT3 phosphorylation. We therefore tested if and how such growth factors influence the MCMV-induced STAT3 phosphorylation. Even though STAT3 phosphorylation was restricted to cells being MCMV-infected and was not transmitted to bystander cells (see above), we found that increasing foetal bovine serum (FBS) present in the cell culture medium concentration-dependently enhanced STAT3 phosphorylation in MCMV-infected cells (Fig. 3D - for a discussion of this finding see below).

Taken together, MCMV induces STAT3 phosphorylation in infected cells by an IFNAR1-, IL-6-, IL-10-, gp130- and JAK2-independent principle which is restricted to infected cells. In addition, growth factors present in the cell culture media (e.g. FBS) have the potency to further enhance STAT3 phosphorylation in MCMV-infected cells.

MCMV interferes with STAT1 and STAT3-dependent gene expression

Based on our finding that tyrosine phosphorylated STAT1 molecules are transcriptionally inert in MCMV-infected cells (Fig. 1), we raised the question if MCMV-induced Y705 phosphorylated STAT3 is actually active in terms of target gene expression. To this end we chose the canonical target gene SOCS3 encoding an immediately responsive and highly sensitive surrogate marker protein of STAT3 function. Cells were infected with MCMV and 24 h post infection treated with Hyper-IL-6 and IFNγ, respectively. Interestingly, despite efficient induction of Y705 phosphorylated STAT3 in MCMV-infected cells, we did not observe the expected increase of SOCS3 amounts. On top of that, an additional stimulation with Hyper-IL-6 or IFNγ, which strongly induces SOCS3 in mock cells, failed to induce SOCS3 in MCMV-infected cells (Fig. 4A). Adequate MCMV infection was documented by detection of pp89-IE1 and equal protein loading by detection of β-actin. As expected, MCMV infection resulted in reduced STAT3 protein amounts (Fig. 4A).

To test if the blockade of the SOCS3 response is specific or part of a general inhibition and whether it is executed on transcriptional level or acts post-transcriptionally, we performed northern blot experiments. We found that MCMV infection drastically inhibited the induction of *SOCS3*, *SOCS1*, *IRF-1* and *cEBPδ* transcripts by IFNγ and the induction of multiple STAT3 target genes including *SOCS3*, *IRF-1*, *cEBPδ*, *JunB* and *c-Myc* by Hyper-IL-6 (Fig. 4B).

In this context, it is noteworthy that it is well documented that an auto-regulative STAT3 circuit exists since STAT3 gene transcription is STAT3-dependent (47-49). Thus, the decline of STAT3 protein amount might represent a consequence of the cytomegaloviral disruption of canonical STAT3-dependent signal transduction and gene expression. Therefore, we tested the expression of *STAT3* mRNA and found it to be significantly reduced upon MCMV infection (Fig. 4B). In summary, MCMV interferes with STAT1 and STAT3 signal transduction, leading to impaired target gene induction.

To reach the maximal rate of transcriptional activity, certain STAT molecules (including STAT1 and STAT3) require additional phosphorylation of serine residue 727 (50). Since our data uncover a MCMV-encoded inhibition of STAT1 and STAT3 target gene expression at a step beyond tyrosine phosphorylation, we assessed Ser727 phosphorylation of STAT1 and STAT3 in MCMV-infected cells upon treatment with IFNγ or Hyper-IL-6. We found that the IFNγ-induced Ser727 phosphorylation was not inhibited by MCMV (Fig. 5). MCMV infection did also not significantly change the level of constitutive Ser727 STAT3 phosphorylation in unconditioned cells (Fig. 5). Nevertheless, the Hyper-IL-6-induced increase in Ser727 phosphorylation of STAT3 was to a certain extent reduced in MCMV-infected cells (irrespective of *M27* coding capacity) compared to mock-infected cells or cells infected with UV-irradiated MCMV (Fig. 5). We interpret this minor change in Ser727 phosphorylated STAT3 in MCMV-infected cells as indirect consequence of the reduced overall STAT3 amounts.

MCMV infection prolongs IFNγ-induced STAT1 Y701 phosphorylation

JAK-STAT signalling pathways are highly auto-regulative. Upon activation, negative feed-back regulation is initiated by *de novo* expression of proteins like SOCS1, SOCS3, PIAS or Usp18 which terminate the signalling process. Having demonstrated that MCMV infection antagonizes SOCS1 and SOCS3 induction upon IFNγ and Hyper-IL-6 stimulation, we raised the question whether the MCMV-encoded inhibitor, which acts on the level of transcriptional activation after effected phosphorylation, would prevent the expression of negative feed-back regulators and thereby counterintuitively prolong IFNγ-induced STAT1 phosphorylation. To test this hypothesis, we pulse stimulated infected or non-infected cells for a short period (30 min) with IFNγ. Afterwards, we vigorously washed the cells to remove the IFNγ and then followed the slope of declining STAT1 phosphorylation levels over a period of 8 h. As shown in Fig. 6, STAT1 phosphorylation was virtually undetectable in untreated cells but readily observed after adding IFNγ. In mock infected cells, STAT1 phosphorylation declined within 4 h. Conversely, in MCMV-infected cells a substantial amount of phosphorylated STAT1 was still apparent after 8 h. *M27* coding capacity did not affect this prolongation of STAT1 phosphorylation. We concluded that MCMV interferes with IFNγ-induced gene expression on the transcriptional level downstream of STAT1 Y701 phosphorylation resulting in abrogation of the induction of the negative feedback loop (e.g. SOCS1 expression) leading to prolonged STAT1 phosphorylation upon incubation with IFNγ.

Neither pIE1pp89 nor pM27 are essential for the cytomegaloviral STAT3 regulation

HCMV IE1-pp72 has been described as inhibitor of IFN-JAK-STAT signalling which antagonizes ISRE signal transduction and simultaneously induces GAF-like responses (51, 52). Despite the limited primary sequence conservation between HCMV IE1-pp72 and MCMV IE1-pp89 (~22% identity and ~42% similarity within a ~190 amino acid stretch [residues 23-197 in HCMV IE1-pp72 and 33-210 in MCMV pp89-IE1, respectively]), the MCMV homolog IE1-pp89 was found to co-precipitate with human STAT2 (53) defining MCMV-pIE1 as potential candidate for a STAT-specific IFN antagonist. Therefore, we tested if the STAT3 phosphorylation, reduction of the overall STAT3 amount and the inhibition of target gene expression are preserved in cells infected with an MCMV mutant lacking the *ie1* coding capacity. All three effects were found to be independent of *ie1* (Fig. 7A).

Besides pIE1-pp72, the only other known cytomegaloviral antagonist of JAK-STAT signal transduction is pM27 (54). Consequently, we tested if *M27* is required for STAT3 modulation. But ΔM27-MCMV was also fully capable to stimulate STAT3 phosphorylation, reduced overall STAT3 amounts and antagonized SOCS3 expression (Fig. 7B). These results rule out an essential contribution of *M27* to the herein described regulation of STAT3 signal transduction and the accompanying inhibition of target gene induction.

MCMV reveals a dynamic equilibrium of STAT3 activation and deactivation

MCMV interferes with STAT1- and STAT3-dependent gene expression in a strikingly similar manner. In both cases, the expression of target genes is antagonized on the transcriptional level despite the nuclear presence of Tyr-phosphorylated STATs. The lack of target gene expression includes the well-known negative feedback regulators SOCS1 and SOCS3, explaining the exaggerated tyrosine phosphorylation. Nevertheless, STAT1 phosphorylation is prolonged but requires the external stimulus IFNγ, whereas STAT3 phosphorylation is seemingly 'autonomous'. How can this apparent difference be reconciled? We hypothesize that cell culture media including FBS contains stimuli like growth factors which induce low-level of `constitutive` STAT3 Y705 phosphorylation. These growth factors induce STAT3 phosphorylation which stimulates gene expression including the STAT3-dependent mediators of the negative feedback (e.g. SOCS3) constantly balancing phosphorylated STAT3 at low level. Consistently, cre-recombinase induced SOCS3 excision has been found to lead to increased levels of tyrosine-activated STAT3 (55). To test if such a low level STAT3 activation exists in MCMV-permissive cells, we used the broad spectrum phosphatase inhibitor sodium vanadate to interfere with STAT3 de-phosphorylation. A 15 min treatment leading to a blockade of cellular phosphatases already induced accumulation of phosphorylated STAT3 eventually resulting in SOCS3 protein induction (Fig. 8A). This finding indicates that a fine-tuned dynamic equilibrium of STAT3 phosphorylation, gene expression of negative feed-back regulators and subsequent dephosphorylation of STAT3 exists in MCMV-permissive cells and that uncoupling of activation and subsequent negative feed-back inhibition results in accumulating phospho-STAT3. We made further use of this sodium-vanadate-mediated uncoupling to induce a long-term 'tyrosine phosphorylation state' and the accompanied SOCS3 induction in cells. Consistent with general inhibition of STAT3 target gene expression, MCMV-infection also precluded SOCS3 and IRF-1 protein expression upon long-term sodium-vanadate incubation (Fig. 8B).

Taken together, the herein described MCMV-encoded STAT3 inhibition reveals the existence of a dynamic equilibrium of STAT3 by uncoupling phosphorylation and induction of mediators of the negative feed-back loop.

Discussion:

We found that MCMV induces seemingly cytokine-autonomous STAT3 phosphorylation. STAT3 phosphorylation is essential for STAT3 activation and thus considered to constitute a faithful surrogate marker for transcriptional STAT3 activity. Although STAT3 is known to stimulate its own transcription (47-49), STAT3 phosphorylation in MCMV-infected cells was accompanied by a reduction of overall STAT3 amounts. This contradiction led to the finding that even a strong external stimulation like Hyper-IL-6 failed to induce all tested STAT3 target genes in MCMV-infected cells, documenting the existence of a potent MCMV-encoded antagonist of STAT3 signal transduction. A comprehensive molecular analysis revealed that MCMV interferes with STAT1- and STAT3-dependent signalling after effected tyrosine phosphorylation, thereby also compromising the intrinsic negative feedback loop otherwise executed by proteins like SOCS1 and SOCS3. Due to the lack of these negative regulators, the dynamic equilibrium of STAT phosphorylation and subsequent inactivation is uncoupled upon MCMV infection, leading to the accumulation of transcriptional inactive phospho-STAT3. The on-rate of the intrinsic equilibrium is influenced by growth factors present in the cell culture medium and especially the FBS. Therefore, increasing FBS concentrations dose-dependently increase STAT3 phosphorylation in MCMV-infected cells.

In contrast to STAT3, which is activated by a broad variety of cytokines, interleukins and growth factors, only very few cytokines e.g. IFNs and IL-35 (56) critically rely on STAT1. Therefore, STAT1 phosphorylation is not induced by constituents of serum and untreated cells virtually do not phosphorylate STAT1 and therefore no accumulation can be observed upon MCMV infection alone. Nevertheless, once the cell encounters STAT1-activating cytokines like IFNγ, STAT1 phosphorylation is also prolonged in MCMV-infected cells, yet it remains transcriptionally inert.

STAT tyrosine phosphorylation - a true hallmark for transcriptional activity?

Under normal conditions, phosphorylation of STAT1 and STAT3 at Y701 and Y705, respectively, is undoubtedly crucial for STAT activation. Since highly specific antibodies are available, the determination of the phosphorylation status represents a widely applied way to assess activity of STATs. Our data uncover a biological condition (i.e. virus infection) which challenges the view that tyrosine phosphorylated STATs are necessarily transcriptional active. This example of stalled STAT transcription complexes demonstrates the necessity to conduct further experiments to unequivocally prove the transcriptional activity of tyrosine phosphorylated STAT molecule species. Textbook schemes usually simplify JAK-STAT signal transduction by only depicting STAT factors binding to respective DNA enhancer elements directly resulting in target gene expression. Apparently, the process of transcriptional initiation and transcription by STATs is a highly regulated process requiring precisely choreographed activity of multiple proteins like co-activators, the mediator complex and the RNA polymerase II complex (see for example (57, 58)). Our data indicate that MCMV interferes with at least one factor involved in this step of regulation.

Implications for ΔM27-MCMV pathogenesis

We have previously described the MCMV-encoded protein pM27 as potent STAT2-specific IFN antagonist (21-24) which interferes with type I IFN signal transduction by inducing rapid proteasomal degradation of STAT2 via recruiting STAT2 to DDB1-containing ubiquitin ligase complexes (23). In absence of *M27*, MCMV hardly interferes with IFNα-dependent gene expression (21), strongly suggesting that pM27 is essential and sufficient to antagonize formation of functional ISGF3 (STAT2:STAT1:IRF9) complexes. Here, we have characterized a second *M27*-independent MCMV-encoded antagonism targeting both STAT1 and STAT3 signalling. Together both inhibitors preclude type I and type II IFN responses.

The presence of IFNα-dependent gene expression observed in ΔM27-MCMV-infected cells together with the pronounced IFN susceptibility of ΔM27-MCMV *in vitro* and *in vivo* (21-23) indicate that the herein described second MCMV-encoded IFN antagonist does not (or only to a limited degree) affects ISGF3 complexes and that both MCMV-encoded inhibitors possess non-redundant functions.

ΔM27-MCMV replication is over-proportionally susceptible to IFNα, but replication is almost completely abrogated in IFNγ-conditioned cells (21). This might be in part explained by IFNγ-dependent STAT2 phosphorylation (21, 59, 60) but IFNγ-induced Y689 STAT2 phosphorylation is less pronounced compared to stimulation by type I IFNs, yet the antiviral effect of IFNγ is more potent (21). Our data reveal that phosphorylated STAT3 and STAT1 (in presence of IFNγ) accumulate in MCMV-infected cells but remain transcriptionally inert. In the wt-MCMV infection scenario, STAT2-dependent gene expression is abrogated by pM27-dependent STAT2 degradation. Upon ΔM27-MCMV infection, increased and prolonged tyrosine phosphorylation of STAT3 and STAT1, respectively, join STAT2 which seems to bypass the second inhibitor of STAT1 signalling. Together these STATs induce an 'overshooting' antiviral state via trans-signalling from IFNγ to ISRE-driven ISGs.

MCMV effects on ISGF3 vs. GAF

As outlined above, the biological phenotype of ΔM27-MCMV and the results of our molecular analysis of the JAK-STAT signalling events in MCMV-infected cells indicate that ISGF3 complexes are rather resistant against the herein described cytomegaloviral inhibitor, whereas STAT1 and STAT3 homodimers are sensitive. Such a differential inhibition can only be explained if we infer that the (co-) transcription factor complexes (e.g. p300, CBP, the mediator complex and others) recruited by ISGF3 significantly differ from the complexes utilized by STAT1 and/or STAT3 homodimers.

STAT1 is absolutely essential for the generation of functional ISGF3 complexes and thus type I IFNs fail to induce gene expression in STAT1-deficient cells. Nevertheless, a full length transactivation domain (TAD) of STAT1 seems not to be required for ISGF3 signalling: The short splice isoform of STAT1 (STAT1β) lacks large parts of the C-terminal STAT1 TAD and is insufficient to generate transcriptionally active STAT1 homodimers. However, functional ISGF3 complexes can be formed by this STAT1β splice isoform (61). Thus, the transcriptional activation of ISGF3 relies on the TAD of STAT2 whereas the transcriptional activation of STAT1 homodimers depends on the TAD of STAT1. Consistently, specific co-regulatory proteins have been described. For example, *N-myc and STAT interactor* (Nmi) potentiates STAT-dependent gene expression. Interestingly, Nmi binds all STATs except STAT2 (62). Additionally, ISGF3 signalling has been shown to require an interaction with the mediator complex. In this context, STAT2 physically interacts with the mediator components DRIP77 and DRIP150, but none of the tested mediator complex components co-precipitates with STAT1 or STAT3 (57).

We infer that the suspected cytomegaloviral inhibitor of JAK-STAT signalling targets a specific (co-) transactivator involved in STAT1 and STAT3 signalling, which is dispensable (or redundant) in terms of ISGF3 signal transduction.

Potential biological implications for the viral inhibition of STAT3 signalling

Given the wealth of knowledge concerning IFN-induced effector mechanisms directly or indirectly interfering with viral replication, the existence of viral antagonists targeting STAT1 can easily be understood (54). Less obvious is the 'rationale' behind the herewith described viral modulation of STAT3 signal transduction, especially because several viruses (e.g. Rous Sarcoma Virus, Hepatitis C Virus, Herpesvirus Saimiri, Epstein-Barr Virus and Kaposi's Sarcoma-associated Herpesvirus) encode proteins which induce genuine STAT3 activation (63-67). Nevertheless, other viruses besides MCMV also inactivate STAT3, e.g. mumps virus induces proteasomal degradation of STAT3 (68). This divergence might be based on differences in the viral life styles and authentic STAT3 activation may be correlated with cell transforming potential.

A simple explanation for STAT3 inactivation would be that MCMV targets STAT1 and that STAT3 inhibition represents merely a 'collateral damage' resulting from overlapping transcriptional co-activators used by both STATs. We favour the interpretation that STAT3 modulation constitutes a selective advantage for MCMV on its own. Recently, it was shown that IL-6 induces PML expression via STAT3 (69). PML is well known to restrict herpesvirus replication (70) which drove HCMV to evolve specific antagonists (71). Therefore, one explanation for the blockade of STAT3 signal transduction might be interference with the induction of antiviral proteins like PML.

But why should MCMV retain STAT3 phosphorylation? MCMV infection leads to dramatically increased IL-6 secretion (72) and it has been shown that in absence of STAT3, IL-6 induces STAT1 activation and initiates IFNγ-like responses (73). Thus, it is tempting to speculate that MCMV invented this elaborated interference with STAT3 signalling (instead of e.g. STAT3 degradation) to avoid that IL-6 in turn induces an IFNγ-like antiviral program.

Even though phosphorylated STAT3 is transcriptionally inert in terms of canonical STAT3 signal transduction and fails to induce classic target genes (e.g. SOCS3), it might nevertheless fulfil other pro-viral functions in MCMV-infected cells. In this respect, it is noteworthy that the HCMV-encoded IE1-pp72 utilizes phosphorylated STAT1 to elicit an IFN-like host cell response (51) and that the HCMV major IE promoter (MIEP) contains IFN-responsive promoter elements (called VRS1) which resemble STAT-binding sites (or GAS elements) (74). It remains to be elucidated whether phosphorylated STAT3 binds to the MCMV genome to modulate viral gene expression.

Implications for the interpretation of CMV-induced and CMV-expressed cytokines

CMVs induce cellular interleukins like IL-6 and IL-10 whereas HCMV even encodes different splice isoforms of a viral IL-10 homolog. IL-6 and IL-10 signal via STAT3, therefore an inhibition of STAT3-dependent signal transduction has considerable implications for the interpretation of viral cytokine induction. Our results show that cells (e.g. fibroblasts) which are productively infected by MCMV do not respond with a canonical STAT1/3 signalling. Therefore, secretion of STAT3-activating cytokines induced by CMV either have to bypass the described inhibition or the cytokines exclusively act in a paracrine manner to manipulate uninfected bystander cells. This interpretation is consistent with findings showing that conditioned media derived from MCMV-infected cells induced MHC class II down regulation upon transfer to uninfected cells in an IL-10-dependent manner (14). Additionally, the expression of the viral STAT3 antagonists will most likely occur delayed in less permissive cells (e.g. macrophages) so that STAT3-dependent signalling of cytokines like IL-10 is preserved longer to execute the observed autocrine MHC II down modulation.

An additional MCMV-encoded IFN antagonist

Besides the implications for the understanding of phosphorylated STAT molecules and their feedback regulation, our data reveal the existence of a yet unknown IFN antagonist encoded by MCMV in addition to pM27. This inhibitor targets STAT1/3-dependent gene expression on the transcriptional level without disturbing STAT1/3 phosphorylation. Our future work will be focused on the elucidation of the identity of this particular viral inhibitor.

Since STAT1 and STAT3 fail to execute their transcriptional program despite their tyrosine phosphorylation, the identity of the cellular interaction partner(s) of such an inhibitor might be relevant for an understanding of STAT-induced transcription.

Taken together, our findings reveal the existence of a novel MCMV-encoded inhibitor of JAK-STAT signalling which acts downstream of effected phosphorylation, but precludes respective gene expression. This inhibition has the paradoxical consequence that phosphorylation (normally a hallmark of activation) of STAT1 is prolonged and STAT3 phosphorylation is even ‘autonomously’ induced, due to blockade of the negative feedback regulation.

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MT, VTKL and HH designed research. MT, VTKL, JRA, and BK performed research. VP, GEA, SJ, JS, SRJ and KP provided crucial reagents. MT, VTKL and HH analyzed the data. MT, VTKL and HH wrote the paper.

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Abbreviations used:

Foetal bovine serum, FBS; Gamma activated factor, GAF; gamma activated sequence, GAS; human CMV, HCMV; IFN-stimulated gene factor 3, ISGF3; IFN-stimulated response elements, ISRE; immediate early, *IE*; mouse cytomegalovirus, MCMV; suppressor of cytokine signalling, SOCS; transactivation domain, TAD, tyrosine kinase 2, Tyk2

Figure legends:

Fig. 1: MCMV abrogates STAT1 signalling after effected STAT1 Y701 phosphorylation, nuclear localization and DNA-binding

(**A**) Cells were infected with MCMV or left uninfected. 1 d post infection (d p. i.) cells were incubated for 30 min with mouse IFNγ (500 U/ml) and fractionated protein lysates (cytoplasm versus nucleoplasm) were prepared, normalized and subjected to SDS polyacrylamid gel electrophoresis (PAGE). The gel was blotted and the indicated proteins were detected by immunoblotting with indicated antibodies. (**B**) Nuclear translocation of STAT1 was tested as described in the M&M section (**C**) Native protein lysates of uninfected, wt-MCMV- or ΔM27-infected cells treated with IFNα, IFNγ or left untreated were subjected to EMSA analysis using a GAS probe as described in the M&M section. The identity of STAT1-containing complexes (indicated as 'GAF') was ensured by a super-shift upon addition of a STAT1-specific antibody to a lysate of IFNγ-conditioned cells (compare lanes 1 and 4). (**D**) Cells were either mock- or MCMV-infected (for 24 h with 10 PFU/cell) and subsequently exposed to 100 U/ml IFNγ for 30 min. Cells were lysed and subjected to ChIP analysis as described in the M&M section. (**E**) A clonal NIH3T3-based cell line harbouring a luciferase reporter gene under the control of a GAS promoter/enhancer element was generated. Cells were infected (10 PFU/cell) with the indicated viruses for 24 h and then IFN stimulated (100 U/ml) for 5 h. Afterwards, luciferase activity was quantified as described in the M&M section. Arithmetic mean and standard deviation are depicted. Statistical significance was tested using *t*-test (unpaired, two-sided) compared to the respective mock samples. (**F**) IFNγ-dependent (500 U/ml; 2 h) induction of *IRF-1* mRNA in uninfected and MCMV-infected (4+2 h p. i.; 10 PFU/ml) cells was tested by northern blotting.

Fig. 2: MCMV gene expression is required to induce IFNAR1-independent STAT3 phosphorylation

(**A**) Lysates of M2-10B4 cells infected with wt-MCMV (24 h p. i.; 10 PFU/cell) or left uninfected were subjected to immunoblot analysis detecting the indicated proteins. For a comparison, cells were incubated for 30 min with 20 ng/ml Hyper-IL-6. (**B**) As in (A) but M2-10B4 cells were compared with STAT3-deficient cells. (**C**) mHTC-K2 were infected with wt-MCMV or left uninfected. Protein lysates were analyzed by immunoblotting. In contras to previous experiments, separate membranes were probed individually with the indicated antibodies and not sequentially using the same membrane. (**D**) NIH3T3 cells were infected with MCMV (10 PFU/cell). At indicated time points post infection, cells were lysed and analysed by immuno blotting using STAT3- (left panel) and phospho-STAT3-specific antibodies (right panel). Four independent lysates were quantified. Shown is the relative abundance compared to mock cells. The mean values are depicted as dotted line. (**E**) As indicated, NIH3T3 cells were infected with grading infectious doses of wt-MCMV and analyzed by immunoblotting. (**F**) NIH3T3 and IFNAR1-deficient cells were stimulated for 30 min with 500 U/ml IFNα and STAT3 phosphorylation was tested by immunoblotting. (**G**) To describe the time course of viral STAT3 modulation, uninfected and wt-MCMV infected (10 PFU/cell) cells (IFNAR1-deficient and identically immortalized C57BL/6 fibroblasts) were lysed at indicated times [h] p. i. and probed for phospho-Y705-STAT3 and overall STAT3 amounts. (**H**) The necessity of viral gene expression for STAT3 modulation was tested by irradiating MCMV with grading UV doses (in J/m^2) prior to 'infection'. 24 h p. i. cells were lysed and subjected to immunoblotting. (**I**) The dispensability of cytomegaloviral *late* gene expression was analyzed by ganciclovir (GCV) treatment which inhibits genome replication and thereby largely reduces accompanying *late* gene expression.

Fig. 3: The MCMV-induced STAT3 phosphorylation is restricted to infected cells and not influenced by IL-6, IL-10 or JAK2

(**A**) NIH3T3, IL-6-, IL-10- and JAK2-deficient cells were tested for their capacity to support the MCMV-induced STAT3 modulation. Cells were infected (10 PFU/cell, 24 h p. i.) and subjected to immunoblotting using indicated antibodies. (**B**) In a medium transfer experiment, conditioned media of MCMV infected cells (which exhibit pronounced STAT3 Y705 phosphorylation) was inactivated (either by sterile filtration ['sterile'] or by UV irradiation ['UV']) and subsequently transferred to uninfected NIH3T3 or RAW cells. STAT3 phosphorylation was assessed by immunoblotting. (**C**) In a transwell experiment (0.4 µm membrane pore size), MCMV-infected and mock-infected cells were co-incubated sharing the same media. Cells were lysed separately and analyzed by immunoblotting. (**D**) wt-MCMV infected cells (10 PFU/ml; 24 h p. i.) and uninfected cells were cultured during the infection cycle with grading concentrations of foetal bovine serum (0 – 20% [v/v]). Cells were lysed and assessed by immunoblotting using the indicated antibodies. MCMV infection was visualized using a polyclonal anti-MCMV mouse immune serum ('α-MCMV'). One virus-specific band is depicted. Please note, that the viral gene expression was to a certain extend increased in cells being treated with higher FCS concentrations (data not shown).

Fig. 4: MCMV interferes with STAT3-dependent gene expression

(**A**) Fractionated protein lysates (cytoplasmic versus nucleoplasmic) of uninfected or wt-MCMV infected cells (10 PFU/ml, 24 h p. i.) treated for 1 h with or without 20 ng/ml Hyper-IL-6 were subjected to immunoblot analysis using the indicated antibodies. STAT3 target gene expression was analyzed by assessing SOCS3 amounts. (**B**) Northern blot analysis reveals the inhibition of Hyper-IL-6- (1 h; 20 ng/ml) and/or IFNγ (1 h; 500U/ml) -dependent mRNA induction of *SOCS3*, *SOCS1*, *IRF-1*, *JunB*, *cEBPDδ*, *STAT3* and *c-Myc* in cell infected with wt-MCMV (24+1 h p. i.). Appropriate RNA loading of the blotted gel was ensured by ethidium bromide staining of ribosomal 18S and 28S RNAs ('rRNA') and MCMV infection was documented by using an *ie1*-specific DNA-probe for hybridization.

Fig. 5: MCMV infection impairs Ser 727 phosphorylation of STAT3

NIH3T3 cells were infected with 10 PFU/cell wt-MCMV, ΔM27-MCMV, UV-irradiated (10,000 J/m^2) wt-MCMV or left uninfected. 24 h p. i. cells were either treated with 20 ng/ml Hyper-IL-6, 500 U/ml IFNγ or left untreated for 15 min. Cells were lysed and subjected to immunoblot using the indicated antibodies.

Fig. 6: MCMV infection prolongs STAT1 phosphorylation

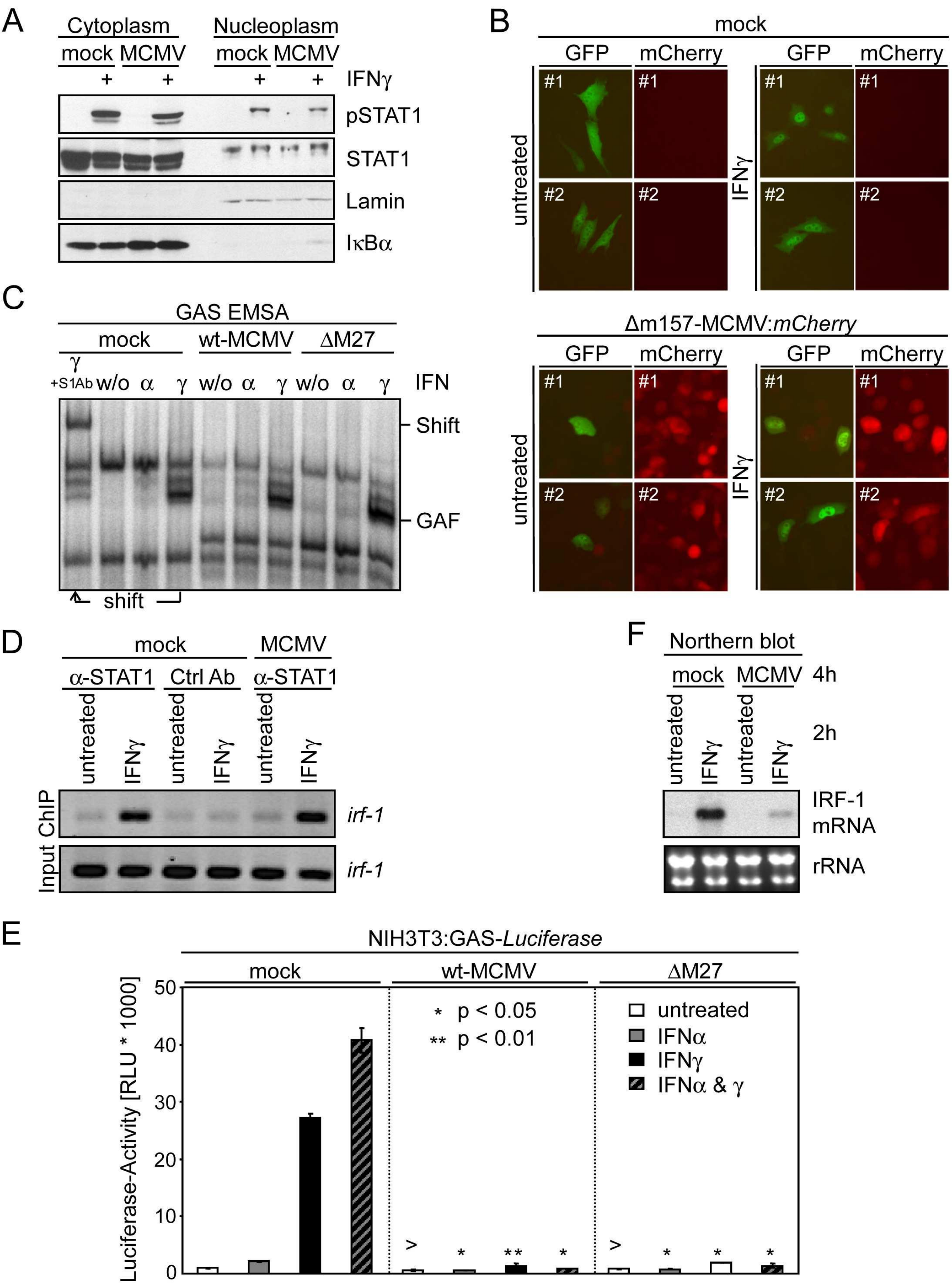
NIH3T3 cells were infected with wt-MCMV or ΔM27-MCMV or left uninfected (24 h p. i.; 5 PFU/ml) and subsequently pulsed with IFNγ (500 U/ml; 30 min). Afterwards, IFNγ was removed by vigorously washing the cells and protein lysates were prepared at the indicated time points post washing procedure ('chase' time in min) to follow the kinetic of STAT1 phosphorylation and dephosphorylation. The indicated proteins were detected by immunoblotting.

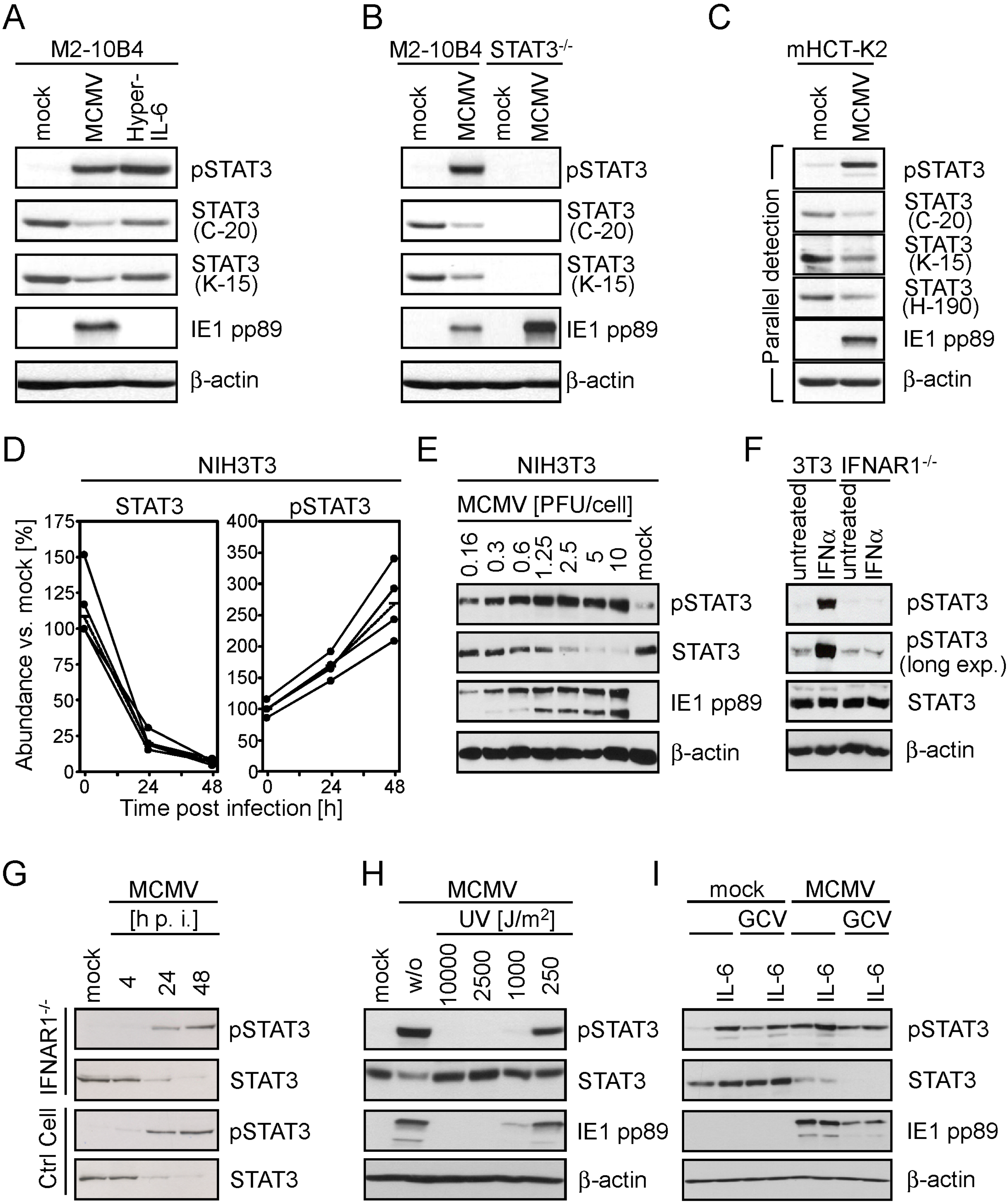
Fig. 7: Neither pIE1 nor pM27 are essential for the cytomegaloviral STAT3 regulation.

(**A**) NIH3T3 cells were infected (10 PFU/ml; 24 h p. i.) with an *IE1*-deletion MCMV, the respective parental virus ('wt') or left uninfected. Cells treated by Hyper-IL-6 (1 h; 20 ng/ml) or left untreated. Mixtures of nuclear and cytoplasmatic fractions (1/1 ratio) were subjected to immunoblot analysis. The deficiency for *IE1* was documented by probing pIE1-pp89 and comparable infection was ensured by probing the viral protein pM45. (**B**) As in (A), but wt-MCMV and ΔM27-MCMV were compared.

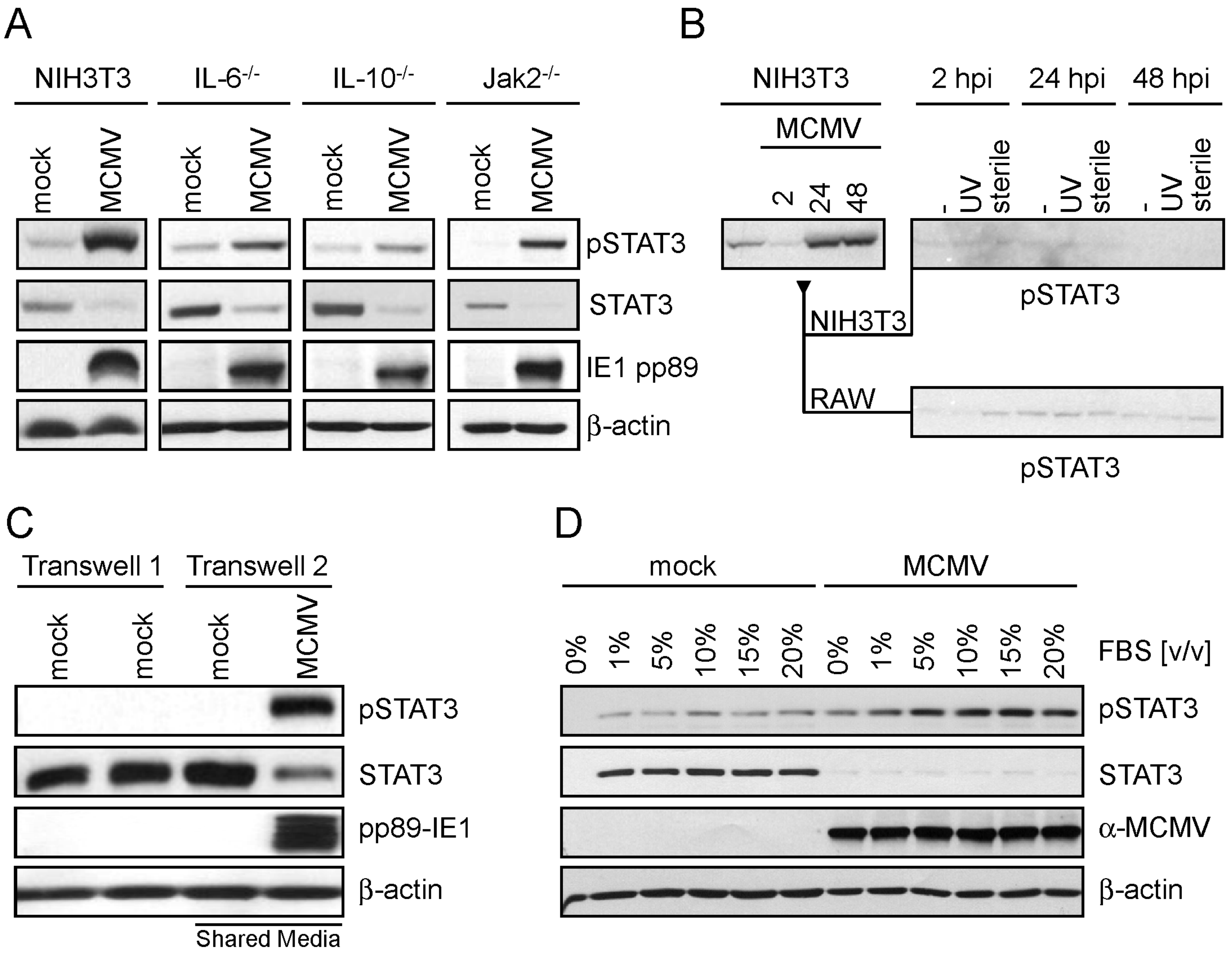
Fig. 8: MCMV reveals a dynamic equilibrium of STAT3 activation and inactivation.

(**A**) The supposed dynamic equilibrium of STAT3 phosphorylation, expression of mediators of the negative feed-back loop (e.g. SCOS3) and STAT3 dephosphorylation in MCMV permissive cells (NIH3T3) was tested by incubation of cells with the broad spectrum phosphatase inhibitor sodium vanadate (Na_3VO_4; 100 µM) for the indicated time periods. Cells were lysed and subjected to immunblot analysis. (**B**) as in (A) but MCMV-infected and uninfected cells were treated with 10 and 100 µM sodium vanadate, respectively, starting at the time of infection or one day post infection.


A
Cytoplasm
Nucleoplasm
mock MCMV mock MCMV
+ + + + IFNγ
pSTAT1
STAT1
Lamin
IκBα
B
mock
GFP mCherry GFP mCherry
untreated
IFNγ
#1
#2
Δm157-MCMV:mCherry
C
GAS EMSA
mock wt-MCMV ΔM27
γ +S1Ab w/o α γ w/o α γ w/o α γ IFN
Shift
GAF
shift
D
mock MCMV
α-STAT1 Ctrl Ab α-STAT1
untreated IFNγ untreated IFNγ untreated IFNγ
ChIP
Input
irf-1
irf-1
F
Northern blot
mock MCMV 4h
2h
untreated IFNγ untreated IFNγ
IRF-1 mRNA
rRNA
E
NIH3T3:GAS-Luciferase
mock wt-MCMV ΔM27
Luciferase-Activity [RLU * 1000]
0 10 20 30 40 50
* p < 0.05
** p < 0.01
untreated
IFNα
IFNγ
IFNα & γ



A
M2-10B4
mock
MCMV
Hyper-IL-6
pSTAT3
STAT3 (C-20)
STAT3 (K-15)
IE1 pp89
β-actin
B
M2-10B4
STAT3-/-
mock
MCMV
mock
MCMV
pSTAT3
STAT3 (C-20)
STAT3 (K-15)
IE1 pp89
β-actin
C
mHCT-K2
mock
MCMV
Parallel detection
pSTAT3
STAT3 (C-20)
STAT3 (K-15)
STAT3 (H-190)
IE1 pp89
β-actin
D
NIH3T3
STAT3
pSTAT3
Abundance vs. mock [%]
Time post infection [h]
E
NIH3T3
MCMV [PFU/cell]
0.16
0.3
0.6
1.25
2.5
5
10
mock
pSTAT3
STAT3
IE1 pp89
β-actin
F
3T3
IFNAR1-/-
untreated
IFNα
untreated
IFNα
pSTAT3
pSTAT3 (long exp.)
STAT3
β-actin
G
MCMV
[h p. i.]
mock
4
24
48
IFNAR1-/-
Ctrl Cell
pSTAT3
STAT3
pSTAT3
STAT3
H
MCMV
UV [J/m2]
mock
w/o
10000
2500
1000
250
pSTAT3
STAT3
IE1 pp89
β-actin
I
mock
MCMV
GCV
GCV
IL-6
IL-6
IL-6
IL-6
pSTAT3
STAT3
IE1 pp89
β-actin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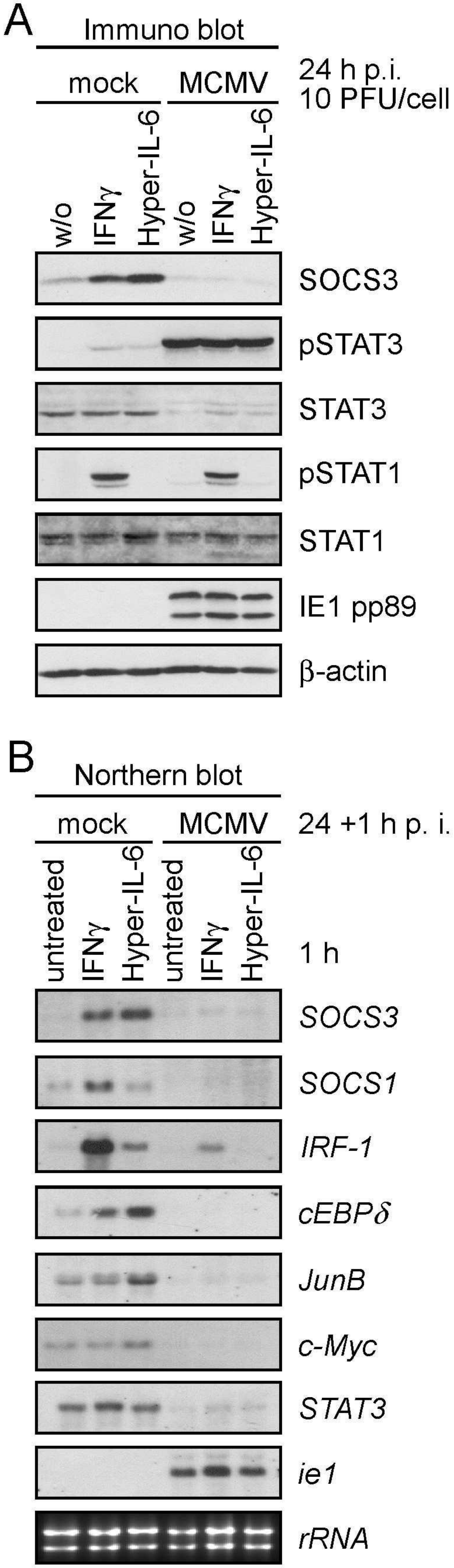
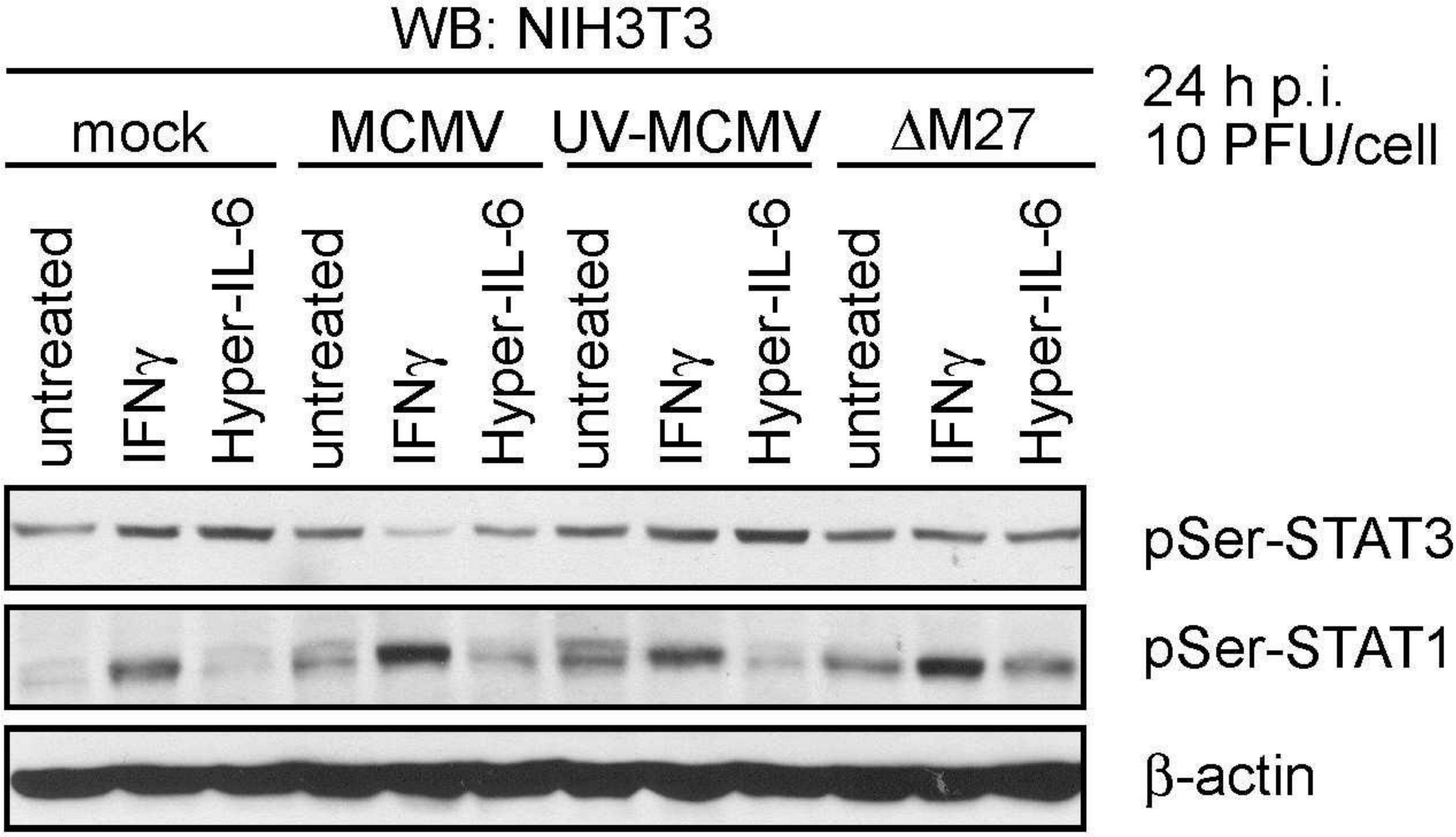
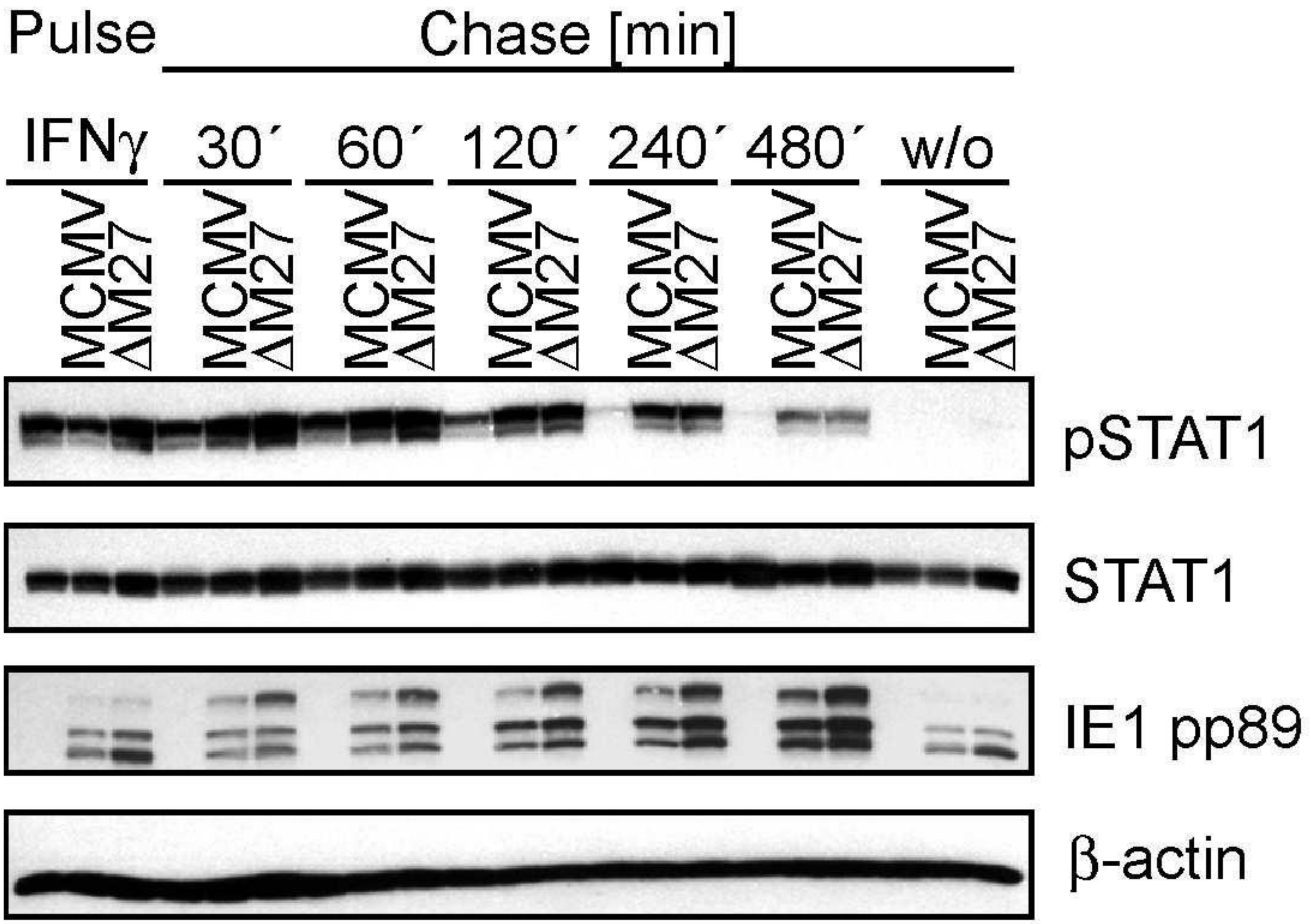
NIH3T3
IL-6-/-
IL-10-/-
Jak2-/-
mock
MCMV
pSTAT3
STAT3
IE1 pp89
β-actin
B
NIH3T3
MCMV
2
24
48
2 hpi
24 hpi
48 hpi
-
UV
sterile
NIH3T3
RAW
pSTAT3
C
Transwell 1
Transwell 2
mock
MCMV
pSTAT3
STAT3
pp89-IE1
β-actin
Shared Media
D
mock
MCMV
0%
1%
5%
10%
15%
20%
FBS [v/v]
pSTAT3
STAT3
α-MCMV
β-actin

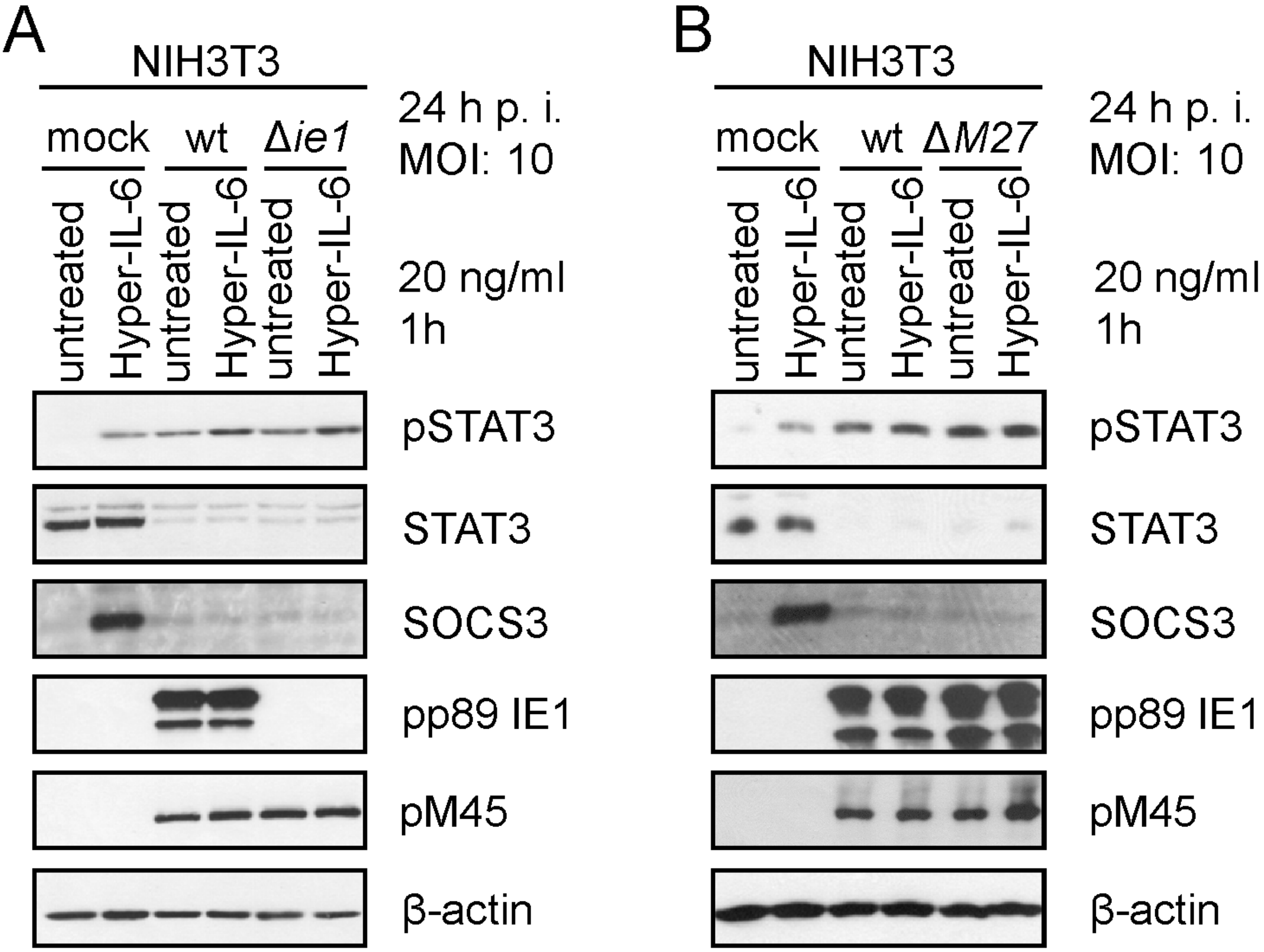


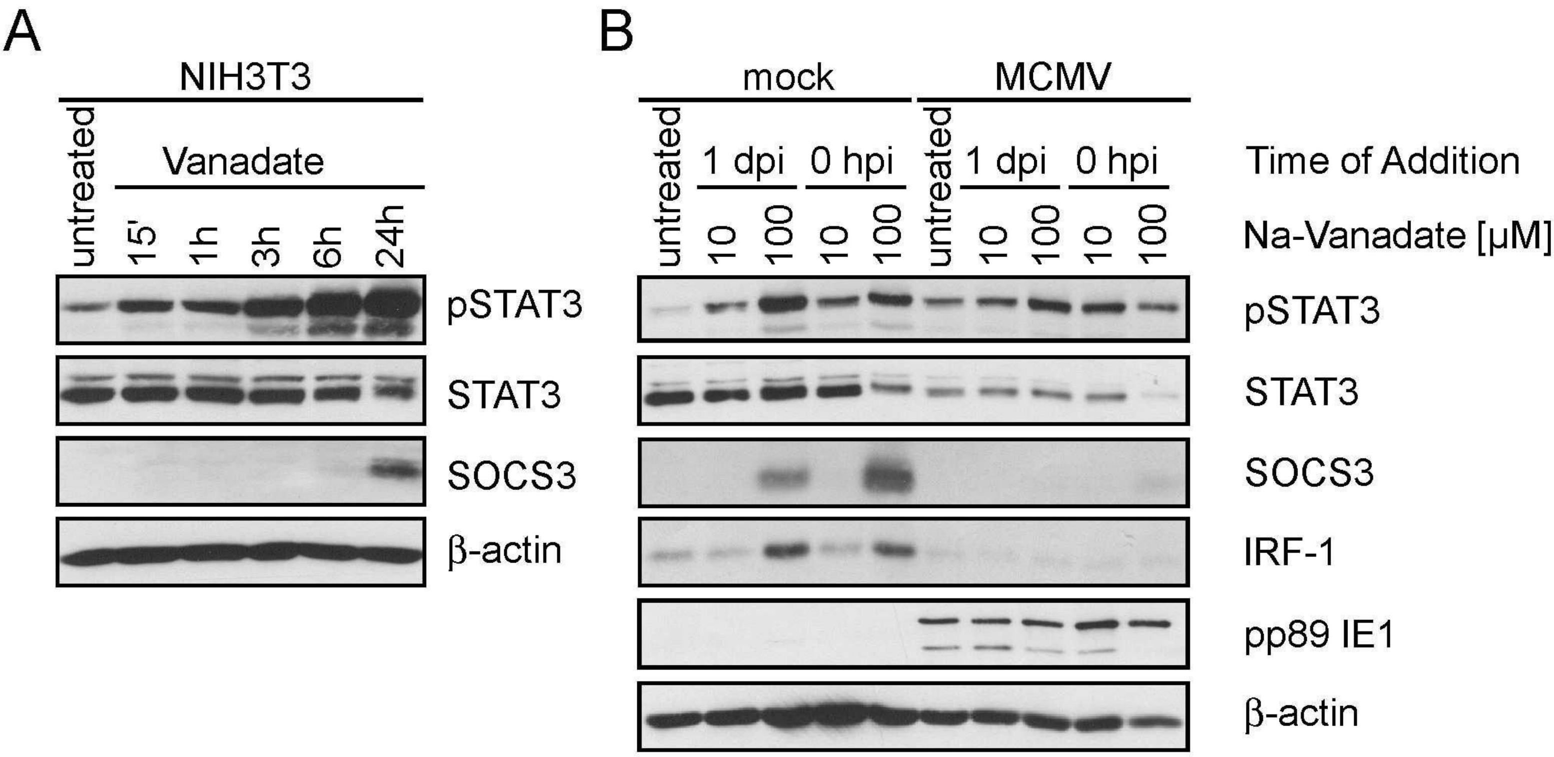
A
Immuno blot
mock
MCMV
24 h p.i.
10 PFU/cell
w/o
IFNγ
Hyper-IL-6
w/o
IFNγ
Hyper-IL-6
SOCS3
pSTAT3
STAT3
pSTAT1
STAT1
IE1 pp89
β-actin
B
Northern blot
mock
MCMV
24 +1 h p. i.
untreated
IFNγ
Hyper-IL-6
untreated
IFNγ
Hyper-IL-6
1 h
SOCS3
SOCS1
IRF-1
cEBPδ
JunB
c-Myc
STAT3
ie1
rRNA


WB: NIH3T3
24 h p.i.
10 PFU/cell
mock
MCMV
UV-MCMV
ΔM27
untreated
IFNγ
Hyper-IL-6
pSer-STAT3
pSer-STAT1
β-actin



Pulse
Chase [min]
IFNγ
30´
60´
120´
240´
480´
w/o
MCMV
ΔM27
pSTAT1
STAT1
IE1 pp89
β-actin


A
NIH3T3
mock
wt
Δie1
24 h p. i.
MOI: 10
untreated
Hyper-IL-6
untreated
Hyper-IL-6
untreated
Hyper-IL-6
20 ng/ml
1h
pSTAT3
STAT3
SOCS3
pp89 IE1
pM45
β-actin
B
NIH3T3
mock
wt
ΔM27
24 h p. i.
MOI: 10
untreated
Hyper-IL-6
untreated
Hyper-IL-6
untreated
Hyper-IL-6
20 ng/ml
1h
pSTAT3
STAT3
SOCS3
pp89 IE1
pM45
β-actin


A
NIH3T3
untreated
Vanadate
15'
1h
3h
6h
24h
pSTAT3
STAT3
SOCS3
β-actin
B
mock
MCMV
untreated
1 dpi
0 hpi
Time of Addition
10
100
Na-Vanadate [µM]
pSTAT3
STAT3
SOCS3
IRF-1
pp89 IE1
β-actin