The Journal of Immunology

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J Immunol 2014; 192:447-458; Prepublished online 6 December 2013;
doi: 10.4049/jimmunol.1203516
http://www.jimmunol.org/content/192/1/447

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

"Activated" STAT Proteins: A Paradoxical Consequence of Inhibited JAK-STAT Signaling in Cytomegalovirus-Infected Cells

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We have previously characterized mouse CMV (MCMV)–encoded immune-evasive IFN signaling 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 γ-activated sequence 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* 1, pM27, and *late* gene expression, it was restricted to MCMV-infected cells and not transmitted to bystander cells. Despite intact STAT1 Y701 phosphorylation, IFN-γ–induced target gene transcription (e.g., *IRF1* and *suppressor of cytokine signaling* [SOCS] *1*) 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 IFN-γ 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–tyrosine-STAT3 and prolonged STAT1 phosphorylation. These findings challenge the generalization of tyrosine-phosphorylated STATs necessarily being transcriptional active and document antagonistic effects of MCMV on STAT1/3-dependent target gene expression. *The Journal of Immunology*, **2014, 192: 447–458.**

Cytomegaloviruses (CMVs) are prototypical β-herpesviruses with an enveloped virion coating a large, double-stranded DNA genome of ∼230 kbp that 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 because of virus control by a concerted response of the innate and adaptive immune system (1), primary and recurrent CMV infections cause symptomatic or even fatal pathologies in immunocompromised or immature individuals. Irrespective of their robust immune-stimulatory capacity, CMVs circumvent sterile immunity and instead establish a lifelong latency. Reactivation occurs upon immunosuppressing and/or stressful conditions because of (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 fetus and constitutes the most frequent nongenetic congenital complication in western countries (2).

CMVs have undergone an intimate and long-lasting coadaptation with their respective host species and are thus highly species specific, which restricts efficient viral replication to cells of the native host

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Received for publication December 26, 2012. Accepted for publication October 30, 2013.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 974 Project A09 and Grant GRK1045 to H.H.), the Helmholtz Society (Viral Strategies of Immune Evasion [VISTRIE] grant to H.H.), the Medical Faculty of the University Duisburg-Essen (as support for the TRR60 to J.R.-A. [as holder of a Gerok position]), and the Medical Faculty of the Heinrich-Heine-University Düsseldorf (Grant 9772473 to M.T.).

M.T., V.T.K.L., and H.H. designed research; M.T., V.T.K.L., J.R.-A., and B.K. performed research; V.P., G.E.A., S.J., J.S., S.R.-J., and K.P. provided crucial reagents; M.T., V.T.K.L., and H.H. analyzed the data; and M.T., V.T.K.L., and H.H. wrote the paper.

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Abbreviations used in this article: GAF, γ-activated factor; GAS, γ-activated sequence; HCMV, human CMV; *IE*, *immediate early*; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response elements; MCMV, mouse cytomegalovirus; NP-40, Nonidet P-40; p.i., postinfection; SOCS, suppressor of cytokine signaling; TAD, transactivation domain; wt, wild type.

species and thereby excludes experimentation with HCMV in animal models. The related mouse CMV (MCMV; Murid herpesvirus 1, taxonomy ID: 10366) has a colinear and partially homologous genome, and infects mice (*Mus musculus*, taxonomy ID: 10090), allowing to study cytomegaloviral pathogenesis. In addition, 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 IFNs. IFNs are pleiotropic cytokines expressed upon encounter of pathogens or their pathogen-associated molecular patterns. IFNs bind to specific cell-surface resident IFN receptors and subsequently induce a rapid JAK-mediated phosphorylation of STAT, which then 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 (IFN-α/β) mainly induce STAT1 and STAT2 tyrosine 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. STAT3 becomes also tyrosine phosphorylated (at residue Y705) by type I IFNs. These STAT tyrosine-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. Phospho-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, IFN-γ mainly signals via tyrosine-phospho-STAT1 homodimers, which are induced upon binding of IFN-γ 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). In addition, 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, for example, 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).

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

Materials 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]), mHTC-K2 (25), $STAT3^{flox/flox}$, crisis immortalized IFNAR1-deficient (26), respective C57BL/6 control fibroblasts, STAT3- (27), JAK2- (28), IL-6–, and primary IL-10–deficient (prepared as described previously [29]) fibroblasts were grown in Dulbecco's MEM with 10% (v/v) FBS (Life Technologies [Invitrogen]), streptomycin, penicillin, and 2 mM glutamine.

IFNs (mouse IFN-α [#12100-1] and mouse IFN-γ [#12500-1]) were purchased from PBL Biomedical Laboratories (Piscataway, NJ). 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 were done as described previously (21, 26, 31). Wild type (wt)-MCMV and ΔM27-MCMV have been described previously (21). For the construction of Δm157-MCMV: *mCherry*, an *frt*-site-flanked fragment encompassing the HCMV-derived major IE promoter/enhancer in front of the *mCherry* gene, was introduced into a recombinant MCMV bacterial artificial chromosome (which already harbored an *frt* site instead of the *m157* CDS) by flp-mediated recombination. UV inactivation was conducted with indicated doses (J/m^2) of UV irradiation in a CL-1000 UV cross-linker (UVP).

EMSA

Extraction of fractionated EMSA lysates and the EMSA assay was performed as described previously (26). In brief, cells were lysed in cytosolic extraction buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 0.2% [v/v] Nonidet P-40 [NP-40], 0.1 mM EDTA, 10% [v/v] glycerol, 0.1 mM Na-vanadate, 0.1 mM PMSF, 1 mM 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% [v/v] glycerol, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, Complete protease inhibitors [Roche]). 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) [^{32}P]-labeled M67 γ-activated sequence (GAS) (32) probe for 20 min at room temperature. The DNA–protein complexes were separated on 4.7% (v/v) polyacrylamide, 22.5 mM Tris HCl, 22.5 mM borate, and 50 μM EDTA gels, fixed, and finally visualized by autoradiography. Supershifts were performed with a STAT1 Ab (Santa Cruz).

Immunoblotting

Cells were lysed in radio-immunoprecipitation assay$^+$ buffer (50 mM Tris-HCl, 150 mM NaCl, 1% [v/v] IGEPAL, 1% Na-Deoxycholate [w/v], 0.1% [w/v] SDS, 1 mM DTT, 0.2 mM 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 Abs. The same membrane was used and consecutively stripped with reblot solution (Millipore). The following Abs were used: anti–β-actin (Sigma-Aldrich); anti-STAT3 (K-15), anti-STAT3 (C-20), anti-STAT3 (H-190), anti-STAT3 (F-2), anti–phospho-Y705 STAT3 (B-7), anti-STAT1 (E-23), anti-IκBα (C-21), and anti-IRF1 (M-20; Santa Cruz); anti–phospho-Y701 STAT1, anti–phospho-S727 STAT1, anti–phospho-S727 STAT3, and anti-Lamin A/C (Cell Signaling), and anti-SOCS3 (ab16030; Abcam). Anti–pp89-IE1 (Croma101) and anti-pM45 were provided by Stipan Jonjić (University of Rijeka, Rijeka, Croatia). The actual MCMV Ag of the anti-pM45 was defined by coimmunoprecipitation and mass spectrometry by Gabriela E. Androsiac (Heinrich-Heine-University, Düsseldorf, Germany). Quantification of immunoblot signals was conducted using a Fusion FX7 (Vilber Lourmat).

Luciferase assay

For reporter gene assays, an NIH3T3-derived cell line harboring a GAS luciferase vector was selected. To do so, we subcloned a fragment comprising the promoter/enhancer sequence and the firefly (*Photinus pyralis*) luciferase

gene from the pTA-GAS construct (Clontech) 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 I) and digoxigenin-labeled 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 5′-CGGCTAGCATGTCACAGTGGTTCGAGCTTC-3′ and mSTAT1-HA2 5′-CGCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTATACTGTGCTCATCATACTGTCAAATTC-3′ with total RNA of BALB/c MEF as template), we generated a PCR product using the primers mSTAT1-1 and HA-EGFP-2 5′-CGGAATTCGAGCGTAATCTGGAACATCGTATGGG-3′. This fragment was digested with NheI and EcoRI 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. Twenty-four hours posttransfection, cells were mock treated or infected with Δm157-MCMV: *mCherry* (2 PFU/cell). Sixteen hours postinfection (p.i.), cells were incubated with 200 U/ml IFN-γ for 45 min. After IFN-treatment, cells were fixed using 4% (w/v) paraformaldehyde-PBS for 20 min. IFN-γ–induced nuclear translocation of STAT1-EGFP was visualized by fluorescence microscopy using a Leica DM IL LED Fluo and LAS V4.0.

Chromatin immunoprecipitation

Cross-linking was achieved by adding formaldehyde (1% [v/v] final concentration) to cells. After 10 min, cross-linking 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% [v/v] 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% [v/v] Triton X-100, 0.1% [w/v] SDS, 0.5% [w/v] 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 precleared by addition of protein G-Sepharose in the presence of 100 μg/l salmon sperm DNA and 500 μg/l BSA. After the preclearing procedure, an aliquot was stored ("input"). Precipitation was performed overnight at 4°C using a STAT1-specific Ab (E-23; Santa Cruz). Immune complexes were precipitated using protein G-Sepharose (1–2 h at 4°C). Afterward, the Sepharose was washed twice with radio-immunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% [w/v] SDS, 0.5% Na-deoxycholate, and 1% [v/v] NP-40), twice with high-salt buffer (500 mM NaCl, 50 mM Tris pH 8.0, 0.1% [w/v] SDS, and 1% [v/v] NP-40), twice with LiCl buffer (25 mM LiCl, 50 mM Tris pH 8.0, 0.5% [w/v] Na-deoxycholate, and 1% [v/v] 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% [w/v] SDS, 0.1 M $NaHCO_3$, and 10 mM DTT). Cross-linking was reversed by addition of 0.05 vol 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% (v/v) ethanol, immune-precipitated DNA was analyzed by PCR using previously described primers (33) specific for the mouse *irf-1* promoter (5′-AGCACAGCTGCCTTGTACTTCC-3′ and 5′-CTTAGACTGTGAAAGCACGTCC-3′) yielding a 229-nt-long product.

Results

MCMV abrogates STAT1 signaling 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 signaling, we investigated the interplay between MCMV and STAT activation by IFNs. In clear contrast with 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 IFN-γ (Fig. 1A). A comparable IFN-γ–induced STAT1 phosphorylation was also evident in MCMV-infected cells (Fig. 1A), indicating that IFNGR proximal events of JAK-STAT1 signaling are not affected by MCMV-encoded IFN antagonists. Nuclear translocation of STAT1 was also not inhibited by MCMV because equal amounts of phospho-STAT1 were detected in nuclear lysates of IFN-γ–incubated MCMV-infected cells and mock control cells (Fig. 1A, *right*). 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 EMSA revealed that STAT1:STAT1 homodimers retained their capacity to bind to GAS DNA elements after MCMV infection, irrespective of *M27* coding capacity (Fig. 1C). Chromatin immunoprecipitation experiments confirmed the intact ability of STAT1 to bind to endogenous GAS DNA elements (in this study, the *irf-1* promoter) in MCMV-infected cells upon IFN-γ exposure (Fig. 1D). To test the ability of MCMV to antagonize GAS-dependent gene expression, we constructed a stable MCMV-permissive NIH3T3 cell line harboring the firefly (*P. pyralis*) *luciferase* reporter gene under the control of a minimal promoter and a GAS enhancer element. In mock-infected cells, IFN-γ 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 IFN-γ–dependent induction of endogenous *IRF1* mRNA by Northern blotting. Four hours of MCMV infection already sufficed to antagonize IFN-γ–induced *IRF1* transcription (Fig. 1F). These results demonstrate that MCMV abrogates IFN-γ–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). In addition, 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 proinflammatory and anti-inflammatory cytokines that play a crucial role in CMV immune control and pathogenesis (11–14), we extended our analysis to STAT3. In contrast with 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 Abs 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 (Fig. 2A–F and data not shown). The extent of the virus-induced Y705 phosphorylation was comparable with 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-6R. Conversely, the MCMV-induced decline in overall

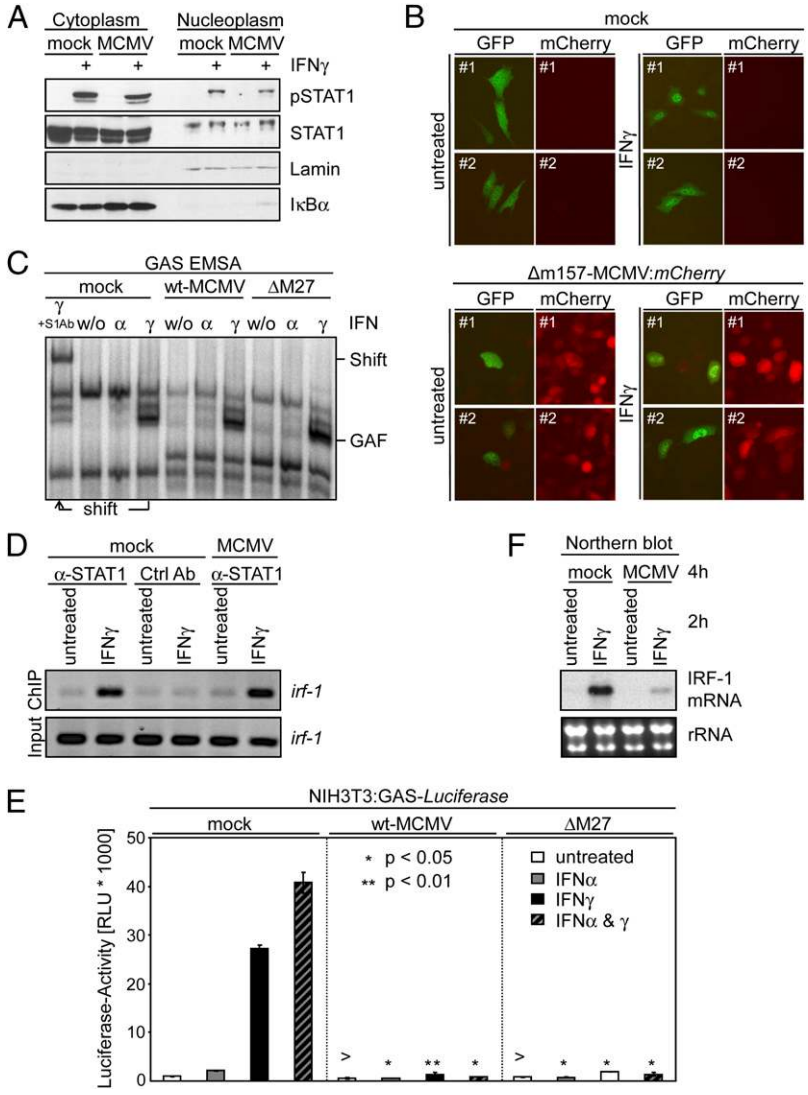

FIGURE 1. MCMV abrogates STAT1 signaling after effected STAT1 Y701 phosphorylation, nuclear localization, and DNA binding. (**A**) Cells were infected with MCMV or left uninfected. One day p.i., cells were incubated for 30 min with mouse IFN-γ (500 U/ml) and fractionated protein lysates (cytoplasm versus nucleoplasm) were prepared, normalized, and subjected to SDS-PAGE. The gel was blotted and the indicated proteins were detected by immunoblotting with indicated Abs. (**B**) Nuclear translocation of eGFP-tagged STAT1 was tested as described in *Materials and Methods*. Original magnification ×55. (**C**) Native protein lysates of uninfected, wt-MCMV– or ΔM27-infected cells treated with IFN-α, IFN-γ, or left untreated were subjected to EMSA analysis using a GAS probe as described in *Materials and Methods*. The identity of STAT1-containing complexes (indicated as GAF) was ensured by a supershift upon addition of a STAT1-specific Ab to a lysate of IFN-γ–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 IFN-γ for 30 min. Cells were lysed and subjected to chromatin immunoprecipitation analysis as described in *Materials and Methods*. (**E**) A clonal NIH3T3-based cell line harboring 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. Afterward, luciferase activity was quantified as described in *Materials and Methods*. Arithmetic mean and SD are depicted. Statistical significance was tested using *t* test (unpaired, two-sided) compared with the respective mock samples. (**F**) IFN-γ–dependent (500 U/ml; 2 h) induction of *IRF1* mRNA in uninfected and MCMV-infected (4+2 h p.i.; 10 PFU/cell) cells was tested by Northern blotting.

STAT3 amounts was not observed in Hyper–IL-6–treated cells, suggesting that the phosphorylation event does not preclude STAT3 recognition by the used Abs (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 $STAT3^+$ cells, but no signal was detectable in STAT3-deficient cells (Fig. 2B). Because the respective membranes were probed in a sequential manner, STAT3 was also detected on separate membranes by different Abs 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 phospho-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, IFN-α is known to induce STAT3 phosphorylation (36). This effect was also observed in MCMV-permissive NIH3T3 cells (Fig. 2F). Because MCMV infection initially induces type I IFN production (31), we used IFNAR1-deficient cells to test whether 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 analyze whether viral gene expression is required for STAT3 modulation, we infected NIH3T3 cells with MCMV virions that 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 followed by actinomycin D inhibition of transcription leading to MCMV gene expression restricted to *IE* gene products (37). However, this result cannot be interpreted as a proof for the responsibility of viral *IE* genes because of 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 HindIII 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 with parental NIH3T3 cells (data not shown), suggesting that *IE* gene expression is not sufficient for the observed changes of STAT3.

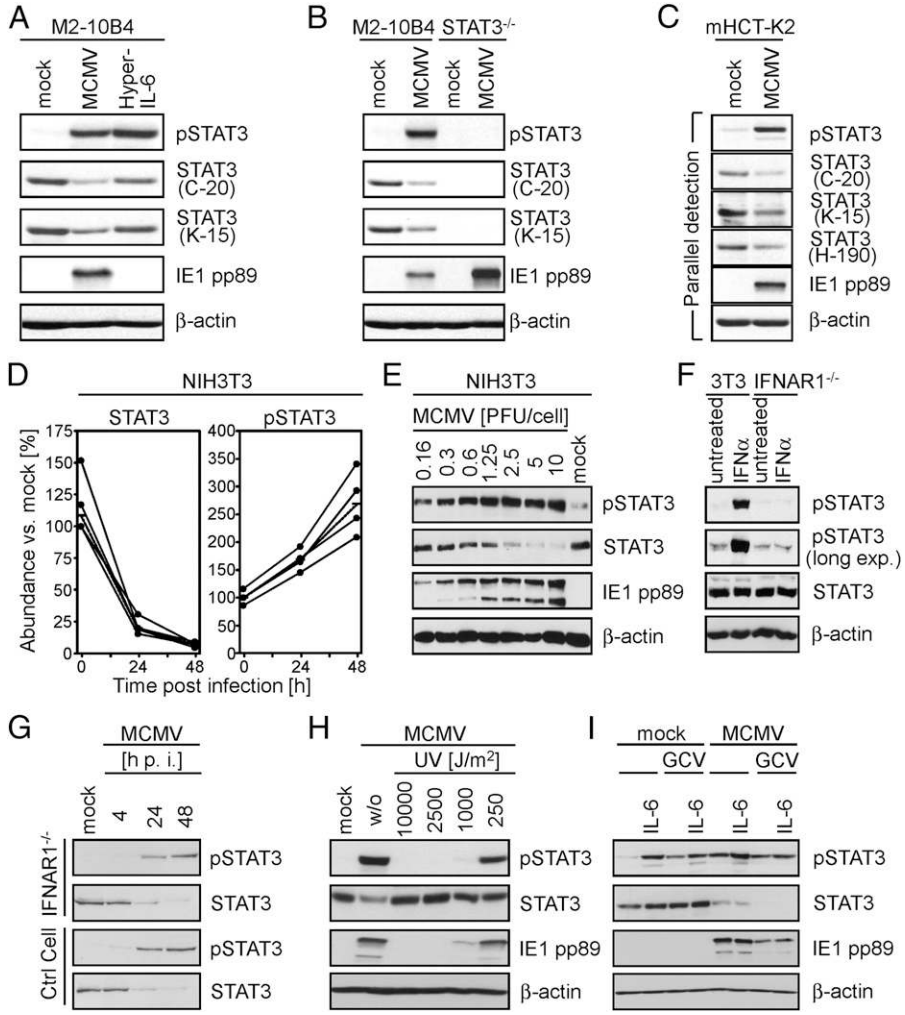

FIGURE 2. MCMV gene expression is required to induce IFNAR1-independent STAT3 phosphorylation. (**A**) Lysates of M2-10B4 cells infected with wt-MCMV (24 h p.i.; 10 PFU/cell) or left uninfected were subjected to immunoblot analysis detecting the indicated proteins. For a comparison, cells were incubated 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 contrast with previous experiments, separate membranes were probed individually with the indicated Abs and not sequentially using the same membrane. (**D**) NIH3T3 cells were infected with MCMV (10 PFU/cell). At indicated time points p.i., cells were lysed and analyzed by immunoblotting using STAT3- (*left panel*) and phospho-STAT3–specific Abs (*right panel*). Four independent lysates were quantified. Shown is the relative abundance compared with mock cells. The mean values are depicted as the 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 IFN-α, and STAT3 phosphorylation was tested by immunoblotting. (**G**) To describe the time course of viral STAT3 modulation, we lysed uninfected and wt-MCMV–infected (10 PFU/cell) cells (IFNAR1-deficient and identically immortalized C57BL/6 fibroblasts) 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) before "infection." Twenty-four hours 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.

To evaluate whether MCMV *late* gene expression is required for the effects on STAT3, we infected cells in the presence of ganciclovir, a drug that interferes with MCMV DNA replication, and thereby strongly reduces the accompanying viral *late* gene expression. *Late* gene expression was not essential for the phosphorylation of STAT3 or for the reduction of STAT3 overall amounts (Fig. 2I). Taken together, these results reveal that an MCMV *IE*- and/or *early*-expressed gene product or products induce a sustained STAT3 phosphorylation and simultaneously reduce STAT3 overall amounts.

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

As mentioned earlier, MCMV induces cytokines such as IL-6 and IL-10, which mainly signal via STAT3. Therefore, we tested whether 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 ILs are not essential for the observed phosphorylation. Because JAK2 is known to be crucial for the signaling 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 signaling 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 JAKs, but also by growth factors via receptor tyrosine kinases and by cytoplasmic kinases such as c-Src (40) and BCR-ABL (41, 42).

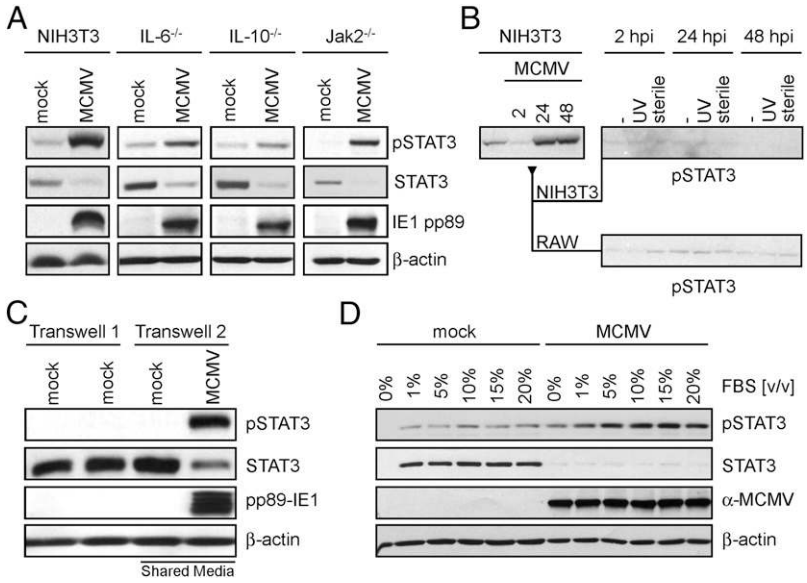

FIGURE 3. The MCMV-induced STAT3 phosphorylation is restricted to infected cells and not influenced by IL-6, IL-10, or JAK2. (**A**) NIH3T3, IL-6-, IL-10-, and JAK2-deficient cells were tested for their capacity to support the MCMV-induced STAT3 modulation. Cells were infected (10 PFU/cell, 24 h p.i.) and subjected to immunoblotting using indicated Abs. (**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 [ultraviolet]) 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 coincubated sharing the same media. Cells were lysed separately and analyzed by immunoblotting. (**D**) wt-MCMV–infected cells (10 PFU/cell; 24 h p.i.) and uninfected cells were cultured during the infection cycle with grading concentrations of FBS (0–20% [v/v]). Cells were lysed and assessed by immunoblotting using the indicated Abs. MCMV infection was visualized using a polyclonal anti-MCMV mouse immune serum (α-MCMV). One virus-specific band is shown. Please note that the viral gene expression was to a certain extent increased in cells being treated with higher FCS concentrations (data not shown).

Several STAT3-activating cytokines signal via a heterodimeric receptor complex composed of a cytokine-specific subunit and the gp130 signaling module. Soluble gp130-Fc blocks gp130-dependent IL-6 signaling and, to a lesser extent, LIF and oncostatin M signaling (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 observed only in infected cells and not in uninfected bystander cells (Fig. 3C), suggesting that the STAT3 Y705 phosphorylating principle is restricted to infected cells and not transferable to neighboring 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 (e.g., the 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 FBS 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 whether 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 earlier), we found that increasing FBS present in the cell culture medium concentration-dependently enhanced STAT3 phosphorylation in MCMV-infected cells (Fig. 3D, see later for a discussion of this finding).

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-phospho-STAT1 molecules are transcriptionally inert in MCMV-infected cells (Fig. 1), we raised the question whether MCMV-induced Y705 phospho-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 p.i. treated with Hyper–IL-6 and IFN-γ, respectively. Interestingly, despite efficient induction of Y705 phospho-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 IFN-γ, 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 whether the blockade of the SOCS3 response is specific or part of a general inhibition and whether it is executed on

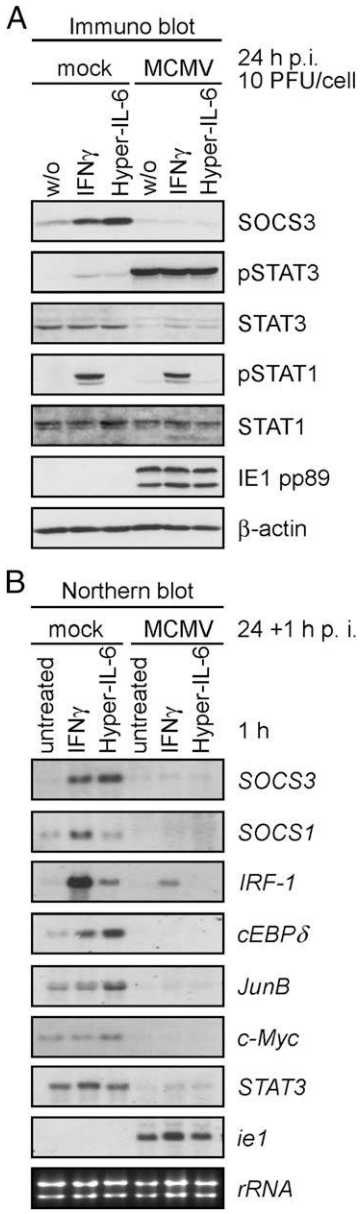

FIGURE 4. MCMV interferes with STAT3-dependent gene expression. (**A**) Fractionated protein lysates (cytoplasmic versus nucleoplasmic) of uninfected or wt-MCMV–infected cells (10 PFU/cell, 24 h p.i.) treated for 1 h with or without 20 ng/ml Hyper–IL-6 were subjected to immunoblot analysis using the indicated Abs. 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 IFN-γ (1 h; 500U/ml)–dependent mRNA induction of *SOCS3*, *SOCS1*, *IRF1*, *JunB*, *cEBPDδ*, *STAT3*, and *c-Myc* in cells 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.

transcriptional level or acts posttranscriptionally, we performed Northern blot experiments using gene-specific probes (Table 1). We found that MCMV infection drastically inhibited the induction of *SOCS3*, *SOCS1*, *IRF1*, and *cEBPδ* transcripts by IFN-γ and the induction of multiple STAT3 target genes including *SOCS3*, *IRF1*, *cEBPδ*, *JunB*, and *c-Myc* by Hyper-IL-6 (Fig. 4B).

In this context, it is noteworthy that it is well documented that an autoregulative STAT3 circuit exists because 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.

Table I. Primers used to generate probes for Northern blotting

Primer	Sequence
KL-cEBPd-1	CGACCTCTTCAACAGCAACC
KL-cEBPd-2	TCGCAGGTCCCAAAGAAAC
KL-cmyc-1	ACGACGAGACCTTCATCAAGA
KL-cmyc-2	TTGCTCTTCTTCAGAGTCGCT
KL-mIRF1-1	CAGAGGAAAGAGAGAAAGTCC
KL-mIRF1-2	CACACGGTGACAGTGCTGG
KL-JunB-1	CCGGATGTGCACGAAAATG
KL-JunB-2	TCTTTAAAGGCGGAAGCGC
KL-Bcl2-1	GGGGGACTTCGTAGCAGTCA
KL-Bcl2-2	AATCGGGAGTTGGGGTCTG
KL-Bcl6-1	CGGCTGACAGCTGTATCCA
KL-Bcl6-2	CACGGGGAGGTTTAAGTGC
KL-SOCS1-1	GCCCCTCGAGTAGGATGGTA
KL-SOCS1-2	CAGCCGGTCAGATCTGGAA
KL-SOCS3-1	CCATGGTCACCCACAGCAA
KL-SOCS3-2	TCCAGGAACTCCCGAATGG

To reach the maximal rate of transcriptional activity, certain STAT molecules (including STAT1 and STAT3) require additional phosphorylation of serine residue 727 (50). Because our data uncover an MCMV-encoded inhibition of STAT1 and STAT3 target gene expression at a step beyond tyrosine phosphorylation, we assessed S727 phosphorylation of STAT1 and STAT3 in MCMV-infected cells upon treatment with IFN-γ or Hyper–IL-6. We found that the IFN-γ–induced S727 phosphorylation was not inhibited by MCMV (Fig. 5). MCMV infection also did not significantly change the level of constitutive S727 STAT3 phosphorylation in unconditioned cells (Fig. 5). Nevertheless, the Hyper–IL-6–induced increase in S727 phosphorylation of STAT3 was to a certain extent reduced in MCMV-infected cells (irrespective of *M27* coding capacity) compared with mock-infected cells or cells infected with UV-irradiated MCMV (Fig. 5). We interpret this minor change in S727 phospho-STAT3 in MCMV-infected cells as an indirect consequence of the reduced overall STAT3 amounts.

MCMV infection prolongs IFN-γ–induced STAT1 Y701 phosphorylation

JAK-STAT signaling pathways are highly autoregulative. Upon activation, negative feedback regulation is initiated by de novo expression of proteins such as SOCS1, SOCS3, PIAS, or Usp18, which terminate the signaling process. Having demonstrated that MCMV infection antagonizes SOCS1 and SOCS3 induction upon IFN-γ 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 feedback regulators and thereby counterintuitively prolong IFN-γ–induced STAT1 phosphorylation. To test this hypothesis, we pulse stimulated infected or noninfected cells for a short period (30 min) with IFN-γ. Afterward, we vigorously washed the cells to remove the IFN-γ 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 IFN-γ. In mock-infected cells, STAT1 phosphorylation declined within 4 h. Conversely, in MCMV-infected cells, a substantial

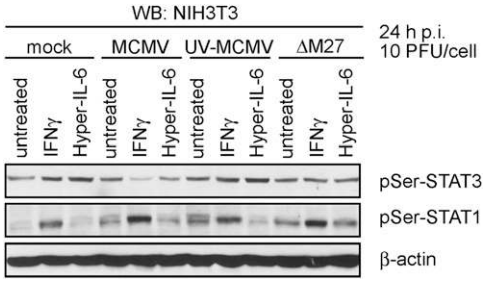

FIGURE 5. MCMV infection impairs Y727 phosphorylation of STAT3. NIH3T3 cells were infected with 10 PFU/cell wt-MCMV, ΔM27-MCMV, UV-irradiated (10,000 J/m^2) wt-MCMV, or left uninfected. Twenty-four hours p.i. cells were either treated with 20 ng/ml Hyper–IL-6, 500 U/ml IFN-γ for 15 min, or left untreated. Cells were lysed and subjected to immunoblot using the indicated Abs.

amount of phospho-STAT1 was still apparent after 8 h. *M27* coding capacity did not affect this prolongation of STAT1 phosphorylation. We concluded that MCMV interferes with IFN-γ–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 IFN-γ.

Neither pIE1pp89 nor pM27 are essential for the cytomegaloviral STAT3 regulation

HCMV IE1-pp72 has been described as an inhibitor of IFN-JAK-STAT signaling, which antagonizes ISRE signal transduction and simultaneously induces γ-activated factor (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 an ~190-aa stretch [residues 23–197 in HCMV IE1-pp72 and 33–210 in MCMV pp89-IE1, respectively]), the MCMV homolog IE1-pp89 was found to coprecipitate with human STAT2 (53) defining MCMV-pIE1 as a potential candidate for a STAT-specific IFN antagonist. Therefore, we tested whether the STAT3 phosphorylation, reduction of the overall STAT3 amount, and 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 whether *M27* is required for STAT3 modulation. However, ΔM27-MCMV was also fully capable to stimulate STAT3

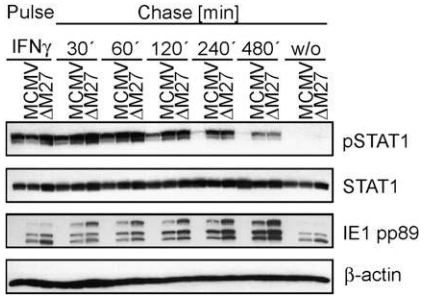

FIGURE 6. MCMV infection prolongs STAT1 phosphorylation. NIH3T3 cells were infected with wt-MCMV or ΔM27-MCMV or left uninfected (24 h p.i.; 5 PFU/cell) and subsequently pulsed with IFN-γ (500 U/ml; 30 min). Afterward, IFN-γ was removed by vigorously washing the cells, and protein lysates were prepared at the indicated time points after washing procedure ("chase" time in min) to follow the kinetic of STAT1 phosphorylation and dephosphorylation. The indicated proteins were detected by immunoblotting.

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 tyrosine-phospho-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 IFN-γ, whereas STAT3 phosphorylation is seemingly "autonomous." How can this apparent difference be reconciled? We hypothesize that cell culture media including FBS contain stimuli like growth factors that induce a 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 phospho-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 whether such a low-level STAT3 activation exists in MCMV-permissive cells, we used the broad-spectrum phosphatase inhibitor sodium vanadate to interfere with STAT3 dephosphorylation. A 15-min treatment leading to a blockade of cellular phosphatases already induced accumulation of phospho-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 feedback regulators, and subsequent dephosphorylation of STAT3 exist in MCMV-permissive cells, and that uncoupling of activation and subsequent negative feedback 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 IRF1 protein expression upon long-term sodium-vanadate incubation (Fig. 8B).

Taken together, the MCMV-encoded STAT3 inhibition described in this article reveals the existence of a dynamic equilibrium of STAT3 by uncoupling phosphorylation and induction of mediators of the negative feedback loop.

Discussion

We found that MCMV induces seemingly cytokine-autonomous STAT3 phosphorylation. STAT3 phosphorylation is essential for STAT3 activation, and thus is 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 signaling after effected tyrosine phosphorylation, thereby also compromising the intrinsic negative feedback loop otherwise executed by proteins like SOCS1 and SOCS3. Because of the lack of these negative regulators, the

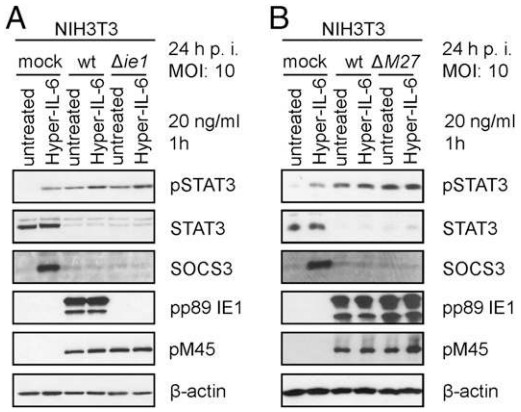

FIGURE 7. Neither pIE1 nor pM27 are essential for the cytomegaloviral STAT3 regulation. (**A**) NIH3T3 cells were infected (10 PFU/cell; 24 h p.i.) with an *IE1*-deletion MCMV, the respective parental virus (wt), or left uninfected. Cells were treated by Hyper–IL-6 (1 h; 20 ng/ml) or left untreated. Mixtures of nuclear and cytoplasmic 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.

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 with STAT3, which is activated by a broad variety of cytokines, ILs, and growth factors, only very few cytokines, for example, 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 IFN-γ, 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. Because highly specific Abs 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) that challenges the view that tyrosine phospho-STATs are necessarily transcriptionally active. This example of stalled STAT transcription complexes demonstrates the necessity to conduct further experiments to unequivocally prove the transcriptional activity of tyrosine phospho-STAT molecule species. Textbook schemes usually simplify JAK-STAT signal transduction by depicting only 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 such as coactivators, the mediator complex, and the RNA polymerase II complex (e.g., see Refs. 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 a potent STAT2-specific IFN antagonist (21–24) that 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 IFN-α–dependent gene expression (21), strongly suggesting that pM27 is essential and sufficient to antagonize formation of functional ISGF3 (STAT2:STAT1:IRF9) complexes. In this study, we have characterized a second *M27*-independent, MCMV-encoded antagonism targeting both STAT1 and STAT3 signaling. Together, both inhibitors preclude type I and type II IFN responses.

The presence of IFN-α–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 second MCMV-encoded IFN antagonist described in this article does not (or only to a limited degree) affect ISGF3 complexes, and that both MCMV-encoded inhibitors possess nonredundant functions.

ΔM27-MCMV replication is overproportionally susceptible to IFN-α, but replication is almost completely abrogated in IFN-γ–conditioned cells (21). This might be explained, in part, by IFN-γ–dependent STAT2 phosphorylation (21, 59, 60), but IFN-γ–induced Y689 STAT2 phosphorylation is less pronounced compared with stimulation by type I IFNs, yet the antiviral effect of IFN-γ is more potent (21). Our data reveal that phospho-STAT3 and -STAT1 (in presence of IFN-γ) 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 signaling. Together, these STATs induce an "overshooting" antiviral state via *trans* signaling from IFN-γ to ISRE-driven ISGs.

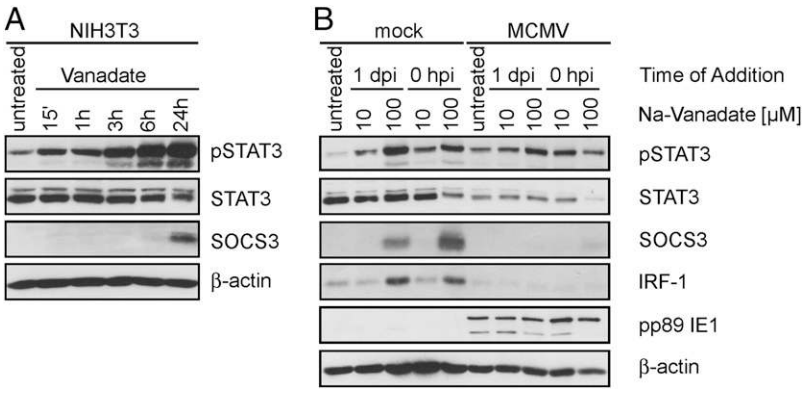

FIGURE 8. MCMV reveals a dynamic equilibrium of STAT3 activation and inactivation. (**A**) The supposed dynamic equilibrium of STAT3 phosphorylation, expression of mediators of the negative feedback loop (e.g., SCOS3), and STAT3 dephosphorylation in MCMV-permissive cells (NIH3T3) was tested by incubation 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 immunoblot 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 1 d p.i.

MCMV effects on ISGF3 versus GAF

As outlined earlier, the biological phenotype of ΔM27-MCMV and the results of our molecular analysis of the JAK-STAT signaling 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 be explained only 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 used by STAT1 and/or STAT3 homodimers.

STAT1 is absolutely essential for the generation of functional ISGF3 complexes; 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 signaling: 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. Specific coregulatory proteins have been consistently described. For example, *N-myc and STAT interactor* potentiates STAT-dependent gene expression. Interestingly, *N-myc and STAT interactor* binds all STATs except STAT2 (62). In addition, ISGF3 signaling 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 coprecipitates with STAT1 or STAT3 (57).

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

Potential biological implications for the viral inhibition of STAT3 signaling

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 viral modulation of STAT3 signal transduction described in this article, 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 that induce genuine STAT3 activation (63–67). Nevertheless, other viruses besides MCMV also inactivate STAT3, for example, mumps virus induces proteasomal degradation of STAT3 (68). This divergence might be based on differences in the viral lifestyles, 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 "collateral damage" resulting from overlapping transcriptional coactivators used by both STATs. We favor 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 IFN-γ–like responses (73). Thus, it is tempting to speculate that MCMV invented this elaborated interference with STAT3 signaling (instead of, e.g., STAT3 degradation) to avoid that IL-6, in turn, induces an IFN-γ–like antiviral program.

Even though phospho-STAT3 is transcriptionally inert in terms of canonical STAT3 signal transduction and fails to induce classic target genes (e.g., SOCS3), it might nevertheless fulfill other proviral functions in MCMV-infected cells. In this respect, it is noteworthy that the HCMV-encoded IE1-pp72 uses phospho-STAT1 to elicit an IFN-like host cell response (51) and that the HCMV major IE promoter contains IFN-responsive promoter elements (called VRS1) that resemble STAT-binding sites (or GAS elements) (74). It remains to be elucidated whether phospho-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 ILs such as 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) that are productively infected by MCMV do not respond with a canonical STAT1/3 signaling. Therefore, secretion of STAT3-activating cytokines induced by CMV either has 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 downregulation upon transfer to uninfected cells in an IL-10–dependent manner (14). In addition, the expression of the viral STAT3 antagonists will most likely occur delayed in less permissive cells (e.g., macrophages) so that STAT3-dependent signaling of cytokines like IL-10 is preserved longer to execute the observed autocrine MHC II downmodulation.

An additional MCMV-encoded IFN antagonist

Besides the implications for the understanding of phospho-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.

Because 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 signaling that 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, because of blockade of the negative feedback regulation.

Disclosures

The authors have no financial conflicts of interest.

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