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# Novel Functions of Tyrosine Kinase 2 in the Antiviral Defense against Murine Cytomegalovirus<sup>1</sup>

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We have recently reported that tyrosine kinase 2 (Tyk2)-deficient mice have a selective defect in the in vivo defense against certain viruses. In our current study we show that Tyk2 is essential for the defense against murine CMV (MCMV). In vivo challenges with MCMV revealed impaired clearance of virus from organs and decreased survival of mice in the absence of Tyk2. Our in vitro studies demonstrate that MCMV replicates to dramatically higher titers in Tyk2-deficient macrophages compared with wild-type cells. We show an essential role of type I IFN (IFN- $\alpha\beta$ ) in the control of MCMV replication, with a prominent role of IFN- $\beta$ . MCMV infection leads to the activation of STAT1 and STAT2 in an IFN- $\alpha\beta$  receptor 1-dependent manner. Consistent with the role of Tyk2 in IFN- $\alpha\beta$  signaling, activation of STAT1 and STAT2 is reduced in Tyk2-deficient cells. However, lack of Tyk2 results in impaired MCMV-mediated gene induction of only a subset of MCMV-induced IFN- $\alpha\beta$ -responsive genes. Taken together, our data demonstrate a requirement for Tyk2 in the in vitro and in vivo antiviral defense against MCMV infection. In addition to the established role of Tyk2 as an amplifier of Jak/Stat signaling upon IFN- $\alpha\beta$  stimulation, we provide evidence for a novel role of Tyk2 as a modifier of host responses. *The Journal of Immunology*, 2005, 175: 4000–4008.

**T**yrosine kinase 2 (Tyk2)<sup>3</sup> belongs to the Jak family of nonreceptor tyrosine kinases. To date, four mammalian members, Jak1 to -3 and Tyk2, have been identified. Jaks play a pivotal role in signaling via a number of cytokines and growth hormones. Jaks associate with the intracellular domains of cytokine receptors and become phosphorylated upon ligand binding. Activated Jaks phosphorylate tyrosine residues of the respective receptor chains, which, in turn, leads to the recruitment and subsequent phosphorylation of STATs. Phosphorylated STAT proteins form homo- and/or heterodimers, translocate to the nucleus, and regulate cytokine-responsive genes (1, 2). Tyk2 was initially identified by its essential role in type I IFN (IFN- $\alpha\beta$ ) signaling (3, 4) and was subsequently shown to be involved in signaling via a number of other cytokines (1). Studies of human cell lines also demonstrated structural functions of Tyk2 for transport and stabilization of the IFN- $\alpha\beta$  receptor 1 chain (IFNAR1) (5). Tyk2-deficient mice show a partial requirement for Tyk2 in IFN- $\alpha\beta$  sig-

naling, arguing for an amplifying function of Tyk2 in IFN- $\alpha\beta$  signaling. Tyk2-deficient splenocytes show defective STAT3 and STAT4 activation upon IL-12 stimulation and, as a consequence, fail to produce IFN- $\gamma$  (6, 7). Surprisingly, a partial defect in IFN- $\gamma$  signaling was also observed (6). We have recently reported that Tyk2 is not essential for the in vivo defense against vesicular stomatitis virus infections. In contrast, Tyk2 is required for harboring efficient CTL responses upon lymphocytic choriomeningitis virus infection and Tyk2<sup>-/-</sup> mice fail to clear vaccinia virus from spleen (6). The selective requirement of Tyk2 in the defense against certain virus infections suggested that different Tyk2 functions are required for the defense against specific viruses. In this study we investigate the role of Tyk2 in the antiviral defense using murine CMV (MCMV) as a model system. MCMV is a  $\beta$ -herpesvirus widely used as an experimental model for human CMV based on their considerable resemblance at both molecular and biological levels (8). CMV infections are normally asymptomatic, with the establishment of life-long latency and the risk of reoccurrence, but infection causes serious disease and high mortality in immunocompromised hosts (9). The importance of IFN- $\gamma$  in the defense against MCMV infection is well established both in vivo (10–13) and in vitro (14). Although the essential role of IFN- $\alpha\beta$  has been extensively studied during in vivo infections (15–18), little is known about the function of IFN- $\alpha\beta$  in cellular immunity against MCMV. We show in this study that Tyk2 is essential for the defense against MCMV in vivo and in vitro. We demonstrate that increased intrinsic sensitivity of Tyk2-deficient macrophages to MCMV infection is not solely due to decreased IFN signaling and suggest a novel function for Tyk2 as a modifier of host responses.

## Materials and Methods

### Mice, viruses, and infections

All mice were on the C57BL/6 background. STAT1<sup>-/-</sup> (19) and inducible NO synthase (iNOS)<sup>-/-</sup> (20) mice were provided by D. E. Levy (New York University School of Medicine, New York, NY) and C. Bogdan (University of Freiburg, Freiburg, Germany) respectively. IFNAR1<sup>-/-</sup> mice (21) were backcrossed for 10 generations into C57BL/6. IFN- $\beta$ <sup>-/-</sup> (22) and Tyk2<sup>-/-</sup> (6) mice were described previously (23). All animals

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<sup>3</sup> Abbreviations used in this paper: Tyk2, tyrosine kinase 2; C<sub>T</sub>, threshold cycle; IFNAR1, IFN- $\alpha\beta$  receptor 1; iNOS, inducible NO synthase; ISGF3, IFN-stimulated gene factor 3; MCMV, murine CMV; MEF, mouse embryo fibroblast; MOI, multiplicity of infection; p.i., postinfection; SG-, salivary gland derived; SOCS, suppressor of cytokine signaling; TC-, tissue culture derived; WT, wild type.

were housed under specific pathogen-free conditions. MCMV Smith strain VR-194 (American Type Culture Collection) was used. Isolation of salivary gland-derived MCMV (SG-MCMV) was described previously (24). Tissue culture-derived MCMV (TC-MCMV) was grown in mouse embryo fibroblasts (MEFs), purified through a sucrose cushion, and passed through a 0.45- $\mu$ m pore size filter (25). All in vitro infections were made with TC-MCMV. In vivo infection experiments were conducted with weight- and age-matched animals. SG-MCMV was injected i.p. with the indicated number of PFU in PBS, and survival was monitored for 7 days. TC-MCMV ( $2 \times 10^5$  PFU) was injected i.p., and virus titer was determined in various organs after indicated times postinfection (p.i.) using standard plaque forming assays (25). All animal experiments were conducted in accordance with protocols approved by European directives.

### Cells and reagents

Bone marrow-derived macrophages were isolated and grown in the presence of CSF-1 derived from L929 cells as described previously (26) and were cultivated for 6 days before infection. BALB/c MEFs were isolated and grown as previously described (27). Immortalized STAT1<sup>-/-</sup> MEF were grown in DMEM supplemented with 10% FCS, 100  $\mu$ g/ml penicillin/100 U/ml streptomycin, and L-glutamine (1 mM). Anti-phospho-STAT1, anti-STAT1, anti-phospho-STAT3, and anti-STAT3 Abs were purchased from Cell Signaling Technologies; anti-pan-ERK and anti-iNOS Ab were obtained from BD Transduction Laboratories; anti-phospho-STAT2 and anti-STAT2 Abs were purchased from Upstate Biotechnology. The CROMA 101 cell line producing anti-IE1 Abs was a gift from H. Hengel (Heinrich Heine University, Dusseldorf, Germany). Purified recombinant murine IFN- $\beta$  was obtained from Calbiochem.

### Virus growth curves

Cells ( $2 \times 10^5$ /well) were plated into 24-well plates. Cells were infected at a multiplicity of infection (MOI) of 1 for 2 h, washed three times with PBS, and cultured in 0.5 ml of medium. Three days p.i., 0.5 ml of fresh medium was added. On the indicated days p.i., plates were frozen at  $-80^\circ\text{C}$ . Lysates were scraped into a 1-ml total volume, and virus was titrated using standard plaque forming assays (25), except that titrations were performed on STAT1<sup>-/-</sup> MEF to exclude potential effects of IFN present in cell lysates/supernatants.

### Nitrite assay and TNF- $\alpha$ assay

Accumulation of nitrite in cell culture supernatants was determined by Griess reaction as described previously (28). TNF- $\alpha$  secretion was measured with ELISA (R&D Systems).

### Cell lysis, Western blot analysis, and EMSA

Cells ( $10^6$ ) were lysed in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40 (v/v), 10% glycerol (v/v), 1 mM DTT, 0.1 mM EDTA, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 3  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1 mM PMSF. Cell debris was removed by centrifugation, and whole-cell lysates were used for EMSA or Western blot analysis. Ten to 20  $\mu$ g of extract was separated on 6.5 or 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed using the indicated Abs and the ECL detection system (Amersham Biosciences). EMSAs were performed using 15  $\mu$ g of whole-cell lysate as described previously with oligonucleotides containing either an IFN- $\gamma$  activation site (29) or an IFN-stimulated regulatory element consensus site (30), respectively.

### Quantitative RT-PCR

RNA was isolated from  $10^6$  cells using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA was treated with DNase I (RQ1 RNase-free DNase; Promega) before cDNA synthesis. One microgram of total RNA was reverse transcribed using random hexamer priming and the First Strand cDNA Synthesis Kit (Fermentas). RT-minus samples served as a control to exclude the amplification from contaminating undigested genomic DNA. cDNA corresponding to 250 ng of input RNA was amplified in duplicate with the TaqMan Universal PCR Master Mix on a custom TaqMan Low Density Array (assay specifications are available upon request). Real-time PCR was conducted with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Differential target gene expression was calculated according to the  $2^{-\Delta\Delta C_T}$  method (31), using *UBE2D2* and *18S rRNA* as endogenous controls. The SD values calculated for the threshold cycle ( $C_T$ ) values of *UBE2D2* and *18S rRNA* were  $\pm 0.48$  and  $\pm 0.55$ , respectively ( $n = 48$ ). Interarray reproducibility was proven by repeated measurements of eight cDNA samples on two different arrays (Pearson correlation coefficient = 0.993;  $p <$

0.001;  $r^2 = 0.985$ ;  $n = 186$ ). Quantitative RT-PCR for *IFN- $\beta$*  was performed as previously described (23), except that *UBE2D2* was used as an endogenous control (32). The quantitative RT-PCR assays for *UBE2D2* and *IFN $\beta$*  were submitted to the Real-Time PCR Primer and Probe Database (RTPrimerDB identification nos. 3377 and 3378) (33).

### Statistical analysis

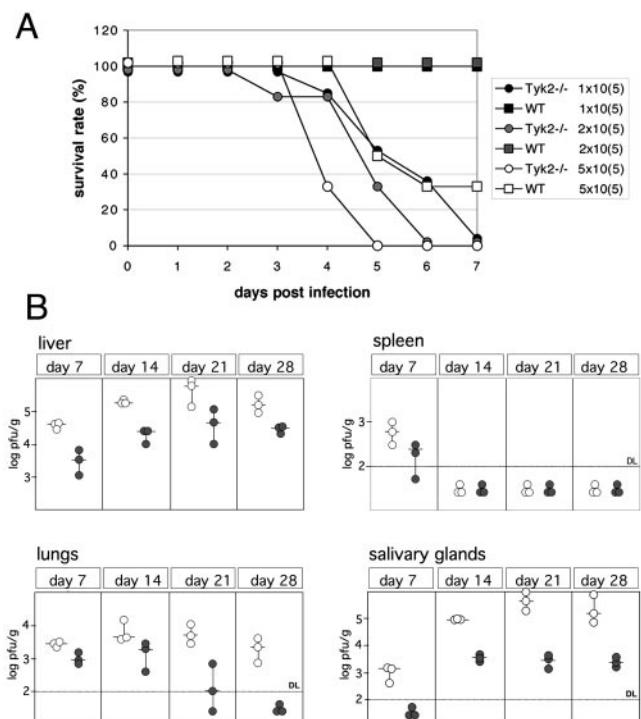
TaqMan array gene expression data were investigated for differences among genotypes and time after challenge.  $C_T$  values were approximately normally distributed. Target gene  $C_T$  values were normalized to the mean of *18S rRNA* and *UBE2D2* values. Univariate ANOVA was calculated for genotypes on  $C_T$  value differences (control at 0 h and time-course samples for the respective genotype), treating the experiment as the random factor. In the case of *IFN- $\beta$* , log-transformed  $n$ -fold changes in gene expression were used. Tukey's B post-hoc comparisons were applied. Calculations were made using SPSS 8 for Windows (SPSS).

## Results

### Tyk2-deficient mice are highly susceptible to MCMV infection

To assess the importance of Tyk2 to the defense against MCMV, we inoculated Tyk2-deficient and wild-type (WT) mice with different concentrations of SG-MCMV and monitored survival for 7 days. Using  $1 \times 10^5$  and  $2 \times 10^5$  PFU of SG-MCMV, all WT mice survived, whereas all Tyk2<sup>-/-</sup> mice succumbed to infection within 7 days (Fig. 1A). At the highest virus dose used ( $5 \times 10^5$  PFU), 50% of WT mice died by day 5, and 70% died by day 7, whereas all Tyk2-deficient mice died by day 5.

MCMV pathogenesis is significantly different dependent on the source of the virus. SG-MCMV is more virulent than TC-MCMV and induces high in vivo levels of circulating proinflammatory cytokines and a shock-like syndrome (34). In contrast, TC-MCMV infections are usually asymptomatic and are characterized by a



**FIGURE 1.** Tyk2<sup>-/-</sup> mice are hypersensitive to in vivo MCMV infections. Mice of the indicated genotype were infected i.p. with the indicated dose of SG-MCMV, and survival was monitored for a period of 7 days (A). Mice were infected i.p. with  $2 \times 10^5$  PFU of TC-MCMV, and virus titer was determined in various organs at 7, 14, 21, and 28 days p.i. using standard plaque forming assays. Virus titers in organs from WT mice (●) and from Tyk2<sup>-/-</sup> mice (○) are shown (B).

relatively low virus load in tissues of infected mice, except in salivary glands. To determine whether the defect of the *Tyk2*<sup>-/-</sup> mice in the antiviral defense against MCMV is particular to the SG-MCMV infection model, we infected *Tyk2*<sup>-/-</sup> and WT mice with TC-MCMV and monitored virus load in various organs up to 28 days p.i. As shown in Fig. 1B, increased virus load was observed in *Tyk2*<sup>-/-</sup> compared with WT mice in all organs tested from 7 to 28 days p.i., except in spleen, where MCMV replicated very poorly.

These data demonstrate that *Tyk2* is essential for the in vivo antiviral defense against MCMV infection regardless of the source of the virus. *Tyk2* is required for both the initial control of TC-MCMV replication and virus clearance from organs at later stages of infection.

#### *Tyk2* is required for control of MCMV replication in macrophages

Next we assessed the role of *Tyk2* in the intrinsic defense against MCMV at the single-cell level. We chose to use macrophages for the in vitro studies for two reasons: firstly, macrophages play an important role during MCMV infections (35–38), and secondly, the ability of MCMV to replicate in macrophages has been correlated with its in vivo virulence (39–41). *IFN-β* pretreatment can inhibit MCMV replication in vitro in fibroblasts and macrophages (42, 43), although the effect is relatively modest. Because the role of endogenous *IFN-αβ* has not been studied, we also assessed the importance of *IFNAR1* in innate cellular immunity against MCMV.

MCMV replicated poorly in WT macrophages, whereas *IFNAR1* deficiency led to a >10<sup>4</sup>-fold increase in virus yield. A 10<sup>3</sup>-fold increase in MCMV replication was found in *Tyk2*-deficient macrophages (Fig. 2A). The defect of *Tyk2*- and *IFNAR1*-deficient macrophages in their ability to control MCMV growth was not restricted to the MOI used in this particular set of experiments, but could be observed over a range of MOI from 0.1 to 10 (data not shown). To assess the relative contributions of *IFN-α* and *IFN-β* to the phenotype observed in *IFNAR1*-deficient macrophages, we included *IFN-β*-deficient cells in our studies. As shown in Fig. 2B, *IFN-β*-deficient macrophages were also highly susceptible to

MCMV replication. MCMV replicates to slightly higher titers in *IFN-β*-deficient macrophages compared with *Tyk2*<sup>-/-</sup> cells and to slightly lower titers than in *IFNAR1*<sup>-/-</sup> cells. These data show that the autocrine/paracrine action of type I IFN is essential for the inhibition of MCMV replication in macrophages. Among the type I IFNs, *IFN-β* plays a dominant role, but other type I IFNs also contribute. Importantly, we can rule out differences in virus binding and entry, because the expression of the MCMV early gene product IE1 was similar in WT, *Tyk2*<sup>-/-</sup>, and *IFNAR1*<sup>-/-</sup> macrophages (Fig. 2C).

#### *IFN-β* can inhibit MCMV replication in *Tyk2*<sup>-/-</sup> macrophages

Given the only partial requirement of *Tyk2* for *IFN-αβ* signaling, the replication efficiency of MCMV in *Tyk2*<sup>-/-</sup> macrophages is surprisingly close to the phenotype obtained with *IFN-β*<sup>-/-</sup> macrophages. We therefore analyzed the ability of *IFN-β* to inhibit MCMV replication in *Tyk2*<sup>-/-</sup> macrophages. We infected macrophages, which were either pretreated with *IFN-β* for 18 h or left untreated, and determined virus yield for up to 6 days. *IFN-β* pretreatment efficiently inhibited MCMV replication in *Tyk2*-deficient cells (Fig. 3A). Thus, *IFN-β*-mediated inhibition of MCMV replication is not impaired in *Tyk2*-deficient macrophages.

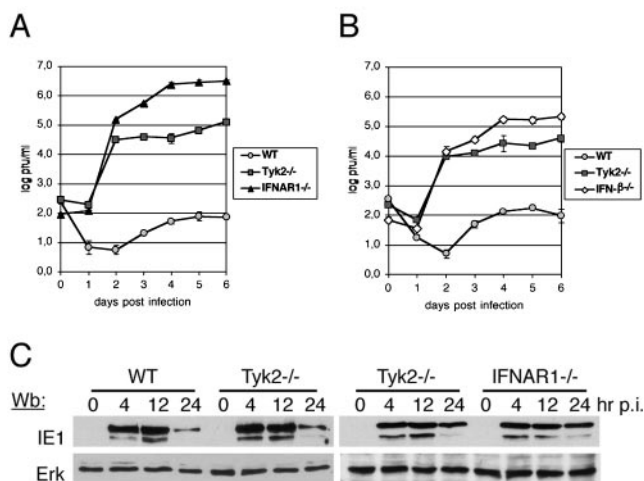
#### *IFN-β* and *TNF-α* production are not impaired in *Tyk2*-deficient macrophages

The fact that exogenous *IFN-β*-mediated anti-MCMV activity is not impaired in *Tyk2*-deficient cells raises the question of whether the increased sensitivity of *Tyk2*<sup>-/-</sup> cells is due to impaired endogenous production of *IFN-β*. MCMV induces the expression of *IFN-αβ* via activation of TLR9 and TLR3 (43–45), and engagement of both TLRs leads to the induction of proinflammatory cytokines (e.g., *TNF-α* and IL-6) (46). As shown in Fig. 3B, MCMV infection induced *IFN-β* expression >100-fold at 2 h p.i., remained constant until 8 h p.i., and declined modestly at 24 h p.i. No significant difference could be observed between WT and *Tyk2*<sup>-/-</sup> macrophages at any of the time points analyzed. Maximal levels of *TNF-α* were observed in the supernatants of infected macrophages within 24 h p.i., and no difference between *Tyk2*<sup>-/-</sup> and WT cells was observed for up to 6 days p.i. (Fig. 3C). Similar *TNF-α* production was confirmed at MOIs ranging from 0.1 to 10 (data not shown).

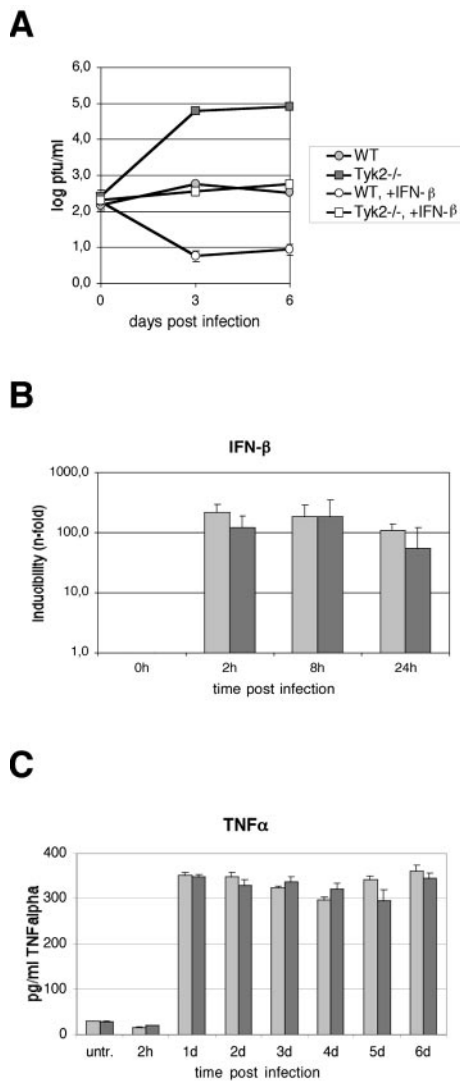
#### *STAT* activation upon MCMV is dependent on *IFNAR1* and partially dependent on *Tyk2*

Next, we addressed the question of whether MCMV-induced autocrine/paracrine IFN signaling is affected by the lack of *Tyk2*. In WT macrophages, MCMV-mediated *STAT1* and *STAT2* phosphorylation was first detected at 2 h p.i., started to decline at 6 h p.i., and was very low, but still detectable, at 24 h p.i. (Fig. 4, A and B). *STAT1* and *STAT2* were phosphorylated only in the presence of *IFNAR1*; hence, phosphorylation is completely dependent on autocrine/paracrine action of *IFN-αβ*. *STAT1* and *STAT2* phosphorylation was decreased, but was still induced significantly in *Tyk2*<sup>-/-</sup> macrophages.

In agreement with previous observations, the *STAT1* protein level was decreased in *Tyk2*- and *IFNAR1*-deficient cells (6, 47) (Fig. 4A). Notably, we report a similar phenomenon concerning *STAT2*; protein expression was reduced in *Tyk2*<sup>-/-</sup> and *IFNAR1*<sup>-/-</sup> macrophages (Fig. 4B). Both *STAT1* and *STAT2* are IFN-inducible proteins, and an increase in protein expression could be seen in WT and *Tyk2*<sup>-/-</sup> cells at 8–24 h p.i., indicating intact IFN-induced gene induction in *Tyk2*<sup>-/-</sup> cells (Fig. 4, A and B). As expected, neither *STAT1* nor *STAT2* protein expression was increased in *IFNAR1*<sup>-/-</sup> cells (data not shown).



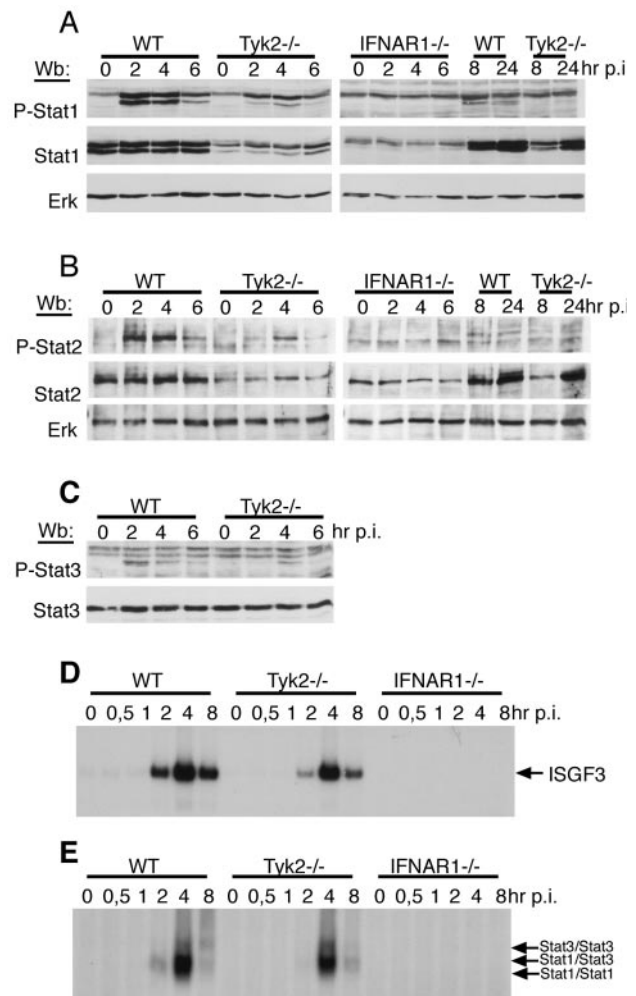
**FIGURE 2.** *Tyk2*, *IFNAR1*, and *IFN-β* are required for the control of MCMV replication in vitro. Macrophages derived from mice of the indicated genotype were infected with MCMV with an MOI of 1 (A–C). At the indicated times p.i., virus titer was determined in whole lysates (cells and supernatants) using plaque forming assays on *STAT1*<sup>-/-</sup> MEF (A and B). Whole-cell extracts were prepared at the indicated times p.i., and the expression of MCMV IE1 protein was determined by Western blot analysis (C). Protein loading was controlled by reprobing with anti-ERK Ab (D).



**FIGURE 3.** IFN- $\beta$ -induced anti-MCMV activity, induction of IFN- $\beta$ , and TNF- $\alpha$  production are not dependent on Tyk2. Macrophages were treated with 100 U/ml IFN- $\beta$  (+IFN- $\beta$ ) for 18 h or were left untreated before infection with MCMV (MOI, 1). At the indicated times p.i., virus titer was determined as described in Fig. 2 (A). Macrophages were infected without prior treatment at an MOI of 1 (B and C), and at the times indicated, IFN- $\beta$  expression was determined by quantitative RT-PCR. UBE2D2 was used for normalization, and expression levels were calculated relative to the uninfected state of the respective genotype; data are derived from three independent experiments (B). At the indicated times, TNF- $\alpha$  accumulation was measured in the supernatants by ELISA (C).  $\square$ , WT macrophages;  $\blacksquare$ , Tyk2<sup>-/-</sup> macrophages (B and C).

MCMV also induced phosphorylation of STAT3 in an IFNAR1-dependent manner, although the induction was very low and could only be detected early after infection. Similar to STAT1 and STAT2, STAT3 phosphorylation was reduced in Tyk2<sup>-/-</sup> cells. Neither differences in basal expression nor an increase in STAT3 protein expression were observed in WT, Tyk2<sup>-/-</sup>, and IFNAR1<sup>-/-</sup> macrophages (Fig. 4C and data not shown).

We next monitored the DNA binding activity of STAT1/2 upon MCMV infection. In WT macrophages, formation of IFN-stimulated gene factor 3 (ISGF3) was first observed at 2 h p.i., peaked at ~4 h p.i., and declined thereafter (Fig. 4D). A low level of STAT1/2 activation was observed up to 48 h p.i. (data not shown). As expected from the phosphorylation data, ISGF3 formation was



**FIGURE 4.** MCMV-induced STAT phosphorylation is completely dependent on the presence of IFNAR1 and is partially dependent on Tyk2. Macrophages were infected with MCMV at an MOI of 1 (A–F). At the indicated times p.i., total cell lysates were prepared and subjected to Western blot analysis (A–C). Membranes were probed with the indicated Abs specific for either phosphorylated STAT (P-STAT) or STAT protein (STAT). Protein loading was controlled by reprobing with anti-ERK Ab (A–C). Whole-cell lysates were subjected to EMSA analysis using oligonucleotides containing an ISRE (D) or a IFN- $\gamma$  activation site (E) consensus motif, respectively.

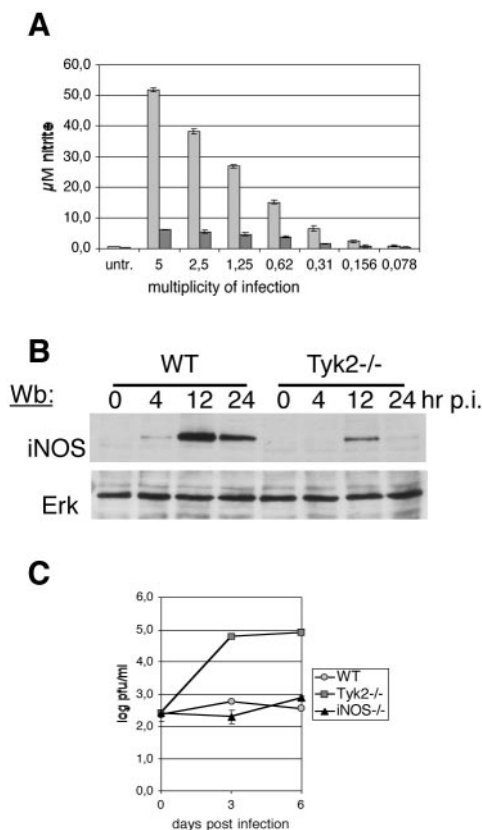
not observed in IFNAR1<sup>-/-</sup> cells. Similar to STAT1/2 phosphorylation, STAT1/2 DNA binding activity was reduced in Tyk2<sup>-/-</sup> macrophages. However, ISGF3 formation was still induced significantly in the absence of Tyk2 and occurred with similar kinetics as in WT cells for at least up to 48 h p.i. (Fig. 4D and data not shown).

As expected for IFN- $\alpha\beta$  signaling, MCMV infection induced low level of STAT1 homodimer DNA binding activity (Fig. 4E). We also observed a low level of STAT1/3 heterodimer formation and hardly detectable STAT3/3 homodimer DNA binding activity in an IFNAR1-dependent manner (Fig. 4E and data not shown).

Taken together, the data show that autocrine/paracrine IFN- $\alpha\beta$  signaling is reduced, but not absent, in Tyk2<sup>-/-</sup> cells. Reduced signaling in Tyk2<sup>-/-</sup> cells is still sufficient for efficient induction of STAT1 and STAT2 protein expression, suggesting that neither IFN- $\alpha\beta$  production nor IFN- $\alpha\beta$  signaling shows a gross defect in the absence of Tyk2.

### Tyk2 is required for MCMV-induced production of NO

Tyk2-deficient peritoneal macrophages fail to produce NO in response to LPS (6). It has been previously reported that NO plays an important role in MCMV infections in vivo (13, 48). We therefore determined NO production in the supernatants of MCMV-infected macrophages derived from Tyk2<sup>-/-</sup> and WT mice. Fig. 5A shows that NO production was strongly impaired in Tyk2-deficient cells infected with a wide range of MOIs, although very low residual NO production was consistently seen. Reduced production of NO was due to reduced induction of iNOS in Tyk2<sup>-/-</sup> cells (Fig. 5B). It has been reported that iNOS deficiency leads to increased replication of MCMV after long infection periods with low MOI in peritoneal exudate macrophages (48). We determined whether the defective NO production in Tyk2-deficient cells contributes to the increased replication of MCMV in our experimental system. We compared growth of MCMV in WT, iNOS<sup>-/-</sup> and Tyk2<sup>-/-</sup> macrophages. As shown in Fig. 5C, iNOS deficiency did not influence MCMV replication for up to 6 days p.i. Taken together, the data show that Tyk2<sup>-/-</sup> cells fail to efficiently produce NO upon MCMV infection, but this defect is not the cause of the dramatic increase in MCMV replication in vitro.



**FIGURE 5.** Lack of Tyk2 results in impaired NO production. WT (□) and Tyk2<sup>-/-</sup> macrophages (■) were infected with the indicated MOIs of MCMV. Three days p.i., cell culture supernatants were collected, and accumulation of nitrite was determined with the Griess reaction (A). Whole-cell extracts were prepared at the indicated times p.i., and the expression of iNOS was analyzed by Western blot; protein loading was controlled by reprobing with anti-ERK Ab (B). Macrophages of the indicated genotype were infected with MCMV at an MOI of 1, and at the indicated times p.i., virus titer was determined as described in Fig. 2 (C).

### Tyk2 is differentially required for MCMV-mediated gene induction

We next assessed MCMV-induced gene expression in Tyk2<sup>-/-</sup> cells over a broader range of target genes. To comparatively determine the dependence of MCMV-mediated gene induction on functional IFN- $\alpha\beta$  signaling, we again included IFNAR1<sup>-/-</sup> cells in these studies.

Using quantitative real-time PCR array technology, we assessed changes in the expression of a number of genes upon MCMV infection at 2, 8, and 24 h p.i. As shown in Fig. 6, Tyk2 deficiency had a differential effect on the expression of certain genes (see Table I for detailed statistical analysis). Induction of Mx1 (IFN-induced GTP-binding protein Mx1, influenza resistance protein), ISG56K (IFN-induced protein with tetratricopeptide repeats 1, Ifit1), STAT1, PKR (IFN-induced, dsRNA-activated protein kinase), and TNF- $\alpha$  were similar in Tyk2<sup>-/-</sup> and WT cells, whereas maximal induction of suppressor of cytokine signaling 1 (SOCS1), SOCS3, IFN regulatory factor 1, TLR3, TLR9, MIP-1 $\alpha$  (CCL3), RNase L (2-5A-dependent RNase), and caspase 8 and repression of Bcl2 (apoptosis regulator Bcl-2) required the presence of Tyk2. In the case of 2',5'-oligoadenylate synthase 1B, Tyk2 was not essential for induction up to 8 h p.i., but was required for the subsequent increase in expression. The induction of most IFN-responsive genes was dramatically reduced in IFNAR1-deficient cells, although a significant induction was still observed with the majority of genes analyzed. TLR9 and MIP-1 $\alpha$  expression were significantly reduced in the absence of functional IFN- $\alpha\beta$  signaling. In contrast, ISG56K was induced to an even greater extent in the absence of IFNAR1 at 2 h p.i., whereas at 8 and 24 h p.i., no significant difference among the three genotypes was observed. IFN- $\alpha\beta$ -independent induction of ISG56K has been reported in human cells in response to polyinosinic-polycytidylic acid and virus infections (49–51). Importantly, Tyk2 dependency did not parallel IFNAR1 dependency. For example, STAT1 induction was completely dependent on IFNAR1, but was induced normally in Tyk2<sup>-/-</sup> cells. In contrast, SOCS1 and SOCS3 inductions were similarly affected by the lack of either Tyk2 or IFNAR1.

No significant difference was observed for the relatively low induction of cyclin-dependent kinase inhibitor 1B (p27, Kip1) among the three genotypes (data not shown).

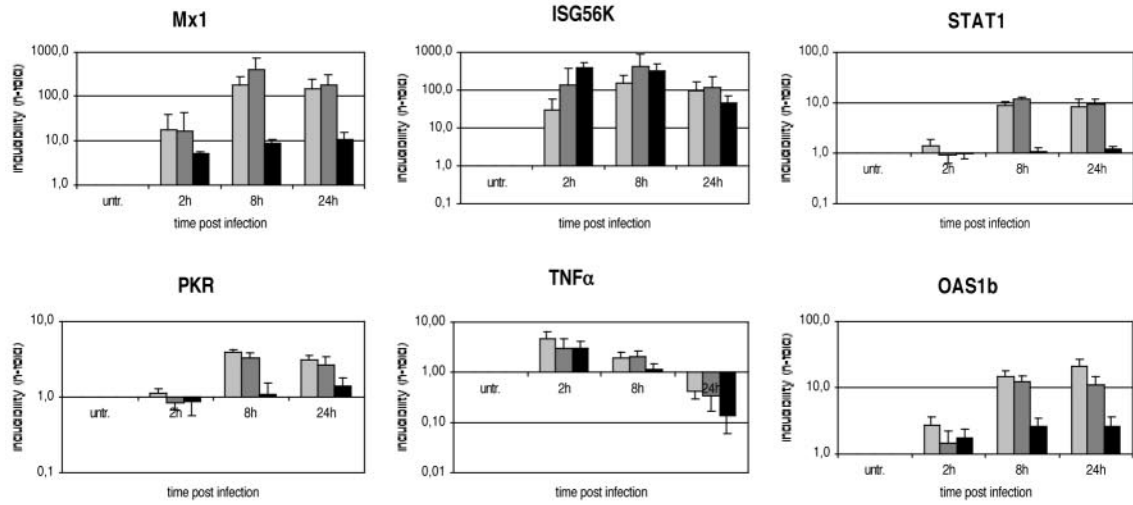
No significant change in gene expression upon MCMV infection in any of the genotypes tested was observed for Bax (apoptosis regulatory protein Bax) or caspase 9 (Fig. 6C).

In summary, these data demonstrate that MCMV-mediated gene induction occurs via both Tyk2-dependent and -independent pathways, and Tyk2 dependency does not parallel the dependence on functional autocrine/paracrine IFN- $\alpha\beta$  signaling.

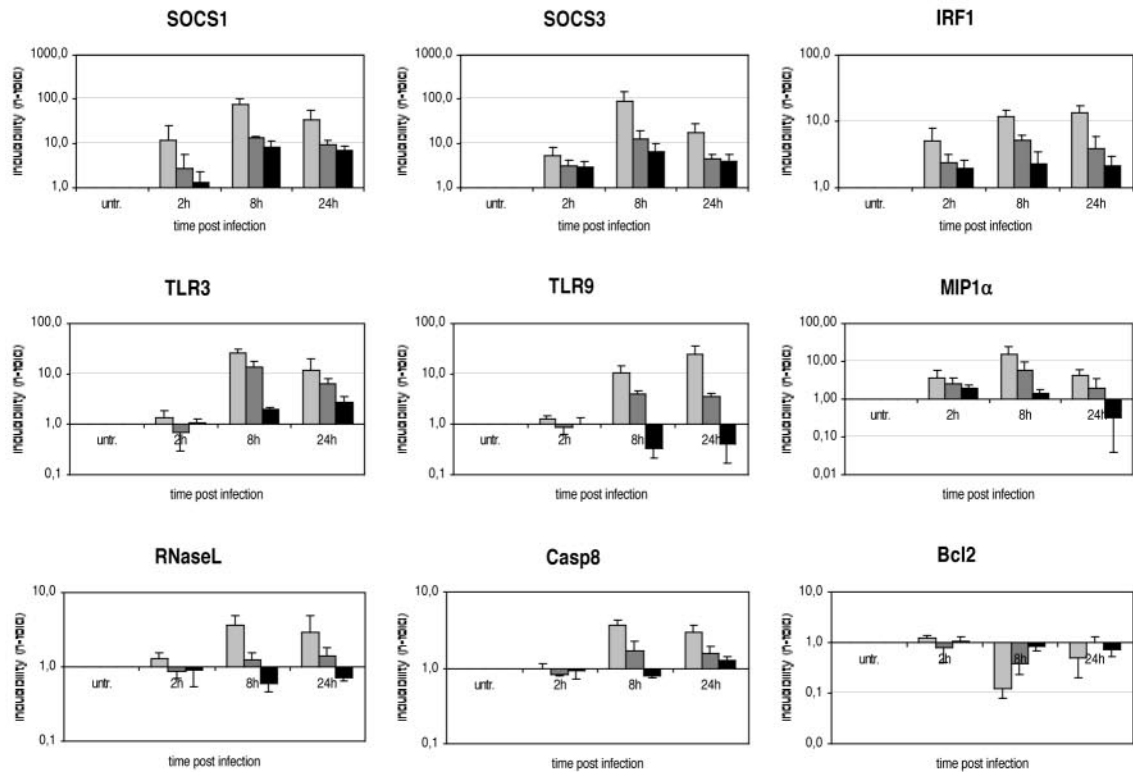
### Discussion

In this work we demonstrate that Tyk2 plays a pivotal role in the defense against MCMV infection both in vivo and in vitro. We report that Tyk2<sup>-/-</sup> mice show increased virus load upon TC-MCMV infection and are highly susceptible to SG-MCMV-induced death. Resistance against MCMV infection depends on functional IFN- $\alpha\beta$  as well as IFN- $\gamma$  signaling. IFN- $\gamma$  derived from NK, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells is essential for the antiviral effect of CD4<sup>+</sup> T cells and CTL responses (34). IL-12-induced IFN- $\gamma$  from NK cells has been shown to be crucial for resistance against MCMV infection (52). IFN- $\alpha\beta$  is required for the limitation of early virus replication; the recruitment/activation of CD8<sup>+</sup> T cells, NK cells, and macrophages (16, 17); and lymphocyte survival (53). Either IFNAR1 or IFN- $\gamma$ R1 deficiency leads to increased susceptibility to MCMV infection; the most prominent phenotype

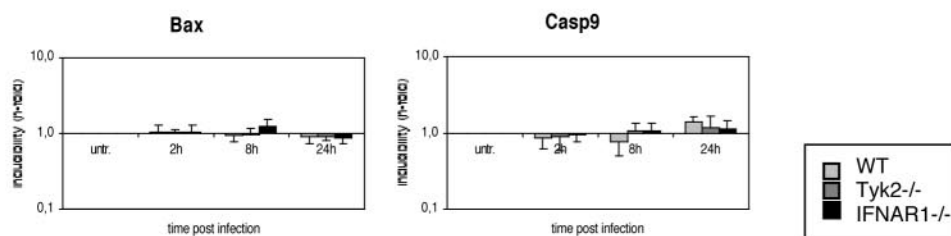
**A** Tyk2-independent



**B** Tyk2-dependent



**C** no change in gene expression



**FIGURE 6.** Tyk2 is differentially required for the induction of IFN-responsive genes. Macrophages were infected with MCMV as described in Fig. 2. At the indicated times p.i., total RNA was extracted, and quantitative RT-PCR was performed using TaqMan array technology. 18S rRNA and UBE2D2 were used for normalization; expression levels were calculated relative to the uninfected state of the respective genotype. The data are derived from four independent experiments. Casp, caspase; OAS1b, 2',5'-oligoadenylate synthase 1B.

Table I. Detailed statistical analysis of differences in gene expression<sup>a</sup>

Gene	2 h p.i.		8 h p.i.		24 h p.i.	
	Groups	<i>p</i>	Groups	<i>p</i>	Groups	<i>p</i>
Tyk2 independent						
Stat1	NS		W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	<0.001	W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	<0.001
Mx1	NS		W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.001	W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.002
OAS1b <sup>b</sup>	NS		W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	<0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>cb</sup>	<0.001
ISG56K	W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.002	NS		NS	
PKR	NS		W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.001	W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.007
TNF- $\alpha$	W <sup>a</sup> , T <sup>b</sup> , I <sup>b</sup>	=0.036	NS		W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.007
Tyk2 dependent						
SOCS1	W <sup>a</sup> , T <sup>a,b</sup> , I <sup>b</sup>	=0.026	W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>a,b</sup> , I <sup>b</sup>	=0.025
SOCS3	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>b</sup>	=0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>b</sup>	=0.008
TLR3	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>a,b</sup> , I <sup>b</sup>	=0.024
TLR9	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001
IRF1	W <sup>a</sup> , T <sup>a,b</sup> , I <sup>b</sup>	=0.036	W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	=0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>b</sup>	=0.001
Bc12	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>a,b</sup>	=0.034
RNaseL	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	=0.001	W <sup>a</sup> , T <sup>a</sup> , I <sup>a,b</sup>	=0.008
MIP1 $\alpha$	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>a</sup> , I <sup>b</sup>	=0.003
Casp8	NS		W <sup>a</sup> , T <sup>b</sup> , I <sup>c</sup>	<0.001	W <sup>a</sup> , T <sup>b</sup> , I <sup>b</sup>	=0.004
No differences						
Bax	NS		NS		NS	
Casp9	NS		NS		NS	
Cdk1b	NS		NS		NS	

<sup>a</sup> Detailed statistical analysis of differences in gene expression in WT (W), Tyk2- (T), and IFNAR1- (I) deficient macrophages upon MCMV infection. a, b, c = statistically different groups (Tukey-B post hoc comparison  $p < 0.05$ ); analysis was done with univariate ANOVA (see Materials and Methods).

<sup>b</sup> OAS1b is Tyk2 independently induced (8 h p.i.), but further increase is dependent on Tyk2 (24 h p.i.).

is observed with IFNAR1/IFN- $\gamma$ R1 double-knockout mice (18). The sensitivity of Tyk2-deficient mice might be due to defects in IL-12-mediated IFN- $\gamma$  production, a partial defect in IFN- $\alpha\beta$ -mediated activation/recruitment/survival of leukocytes, or a combination thereof. We report in this work that susceptibility of Tyk2-deficient mice might at least partly be caused by increased sensitivity of Tyk2<sup>-/-</sup> macrophages to MCMV replication. The role of exogenous IFN- $\gamma$  in the antiviral response has been studied in vitro in fibroblasts and macrophages (14, 54), whereas the effect of IFN- $\alpha\beta$  is less well characterized. In this study we identify an essential role for autocrine/paracrine IFN- $\alpha\beta$  in the antiviral defense of macrophages against MCMV infection, with a main role for IFN- $\beta$  (Fig. 2B). Lack of Tyk2 results in sensitivity to MCMV replication nearly as pronounced as in the absence of IFN- $\beta$ . However, we demonstrate that increased replication of MCMV in Tyk2<sup>-/-</sup> macrophages is not due to a defective IFN- $\beta$  response, because IFN- $\beta$  is able to inhibit MCMV in the absence of Tyk2 (Fig. 3).

Recently, it has been reported that MCMV infection leads to the production of IFN- $\alpha\beta$  via activation of TLR9 in dendritic cells, IFN-producing cells, and in vivo in a MyD88-dependent manner (43–45). Activation of the IFN- $\alpha\beta$  gene locus via the TLR3-Toll-IL-1 receptor domain-containing adaptor-inducing IFN-dependent pathway has been shown to contribute to the anti-MCMV response (45). We show in this study that neither IFN- $\beta$  nor TNF- $\alpha$  production is impaired in the absence of Tyk2, arguing that TLR3/9 signaling is not defective in Tyk2-deficient macrophages. In contrast, Tyk2 is required for TLR4-mediated IFN- $\beta$  production (6, 23), and as a consequence, Tyk2 deficiency results in a dramatic delay and reduction of STAT1 activation. In this study we show that STAT1 and STAT2 activation after MCMV infection occurs with similar kinetics in Tyk2<sup>-/-</sup> and WT cells, although the activation is reduced. Similar kinetics, but reduced STAT1/2 activation is in accordance with the normal IFN- $\beta$  production reported above and the previously described amplifying role of Tyk2 in IFN- $\alpha\beta$  signaling (6, 7).

Similar to what we have reported after TLR4 stimulation (6, 23), Tyk2<sup>-/-</sup> macrophages show strongly impaired induction of iNOS and, as a consequence, impaired production of NO. Although iNOS is required for the defense against MCMV in vivo (13, 48), we show in this study that iNOS is not required for cellular immunity against MCMV. Impaired NO production can therefore not be the cause of the increased replication of MCMV in Tyk2-deficient cells, but might contribute to the in vivo sensitivity of Tyk2<sup>-/-</sup> mice. In contrast to the induction of iNOS protein, two IFN-responsive proteins, STAT1 and STAT2, are similarly induced in Tyk2<sup>-/-</sup> and WT macrophages. A differential requirement for Tyk2 for MCMV-mediated gene induction is confirmed by the analysis of a larger number of target genes. We demonstrate that MCMV-mediated gene induction occurs via Tyk2-dependent and -independent pathways. As expected, comparative analysis of IFNAR1-deficient cells showed that most of the genes are induced in an IFNAR1-dependent manner, although a residual low level of induction was observed for most genes analyzed. Direct activation of IFN-responsive genes by viral infections has been described previously (51, 55, 56), although it has not been demonstrated for MCMV. Importantly, Tyk2 dependency was differential and did not parallel IFNAR1 dependency. Thus, in the context of MCMV infection, the function of Tyk2 is more complex than to solely amplify IFN- $\alpha\beta$ -mediated responses. Macrophages have been reported to secrete IFN- $\gamma$  in response to IL-12. Because IL-12 is itself a product of macrophages, the existence of an autocrine activation loop has been suggested (57, 58). Controversially, it has recently been reported that minor contamination with NK and T cells is the source of IFN- $\gamma$  in macrophage populations (59). We show in this study that STAT1 activation upon MCMV infection is completely dependent on functional IFN- $\alpha\beta$  signaling and thereby exclude the contribution of an autocrine/paracrine action of IFN- $\gamma$ . Furthermore, we could not detect any IFN- $\gamma$  in the supernatants of MCMV-infected macrophages (data not shown).

The essential role of Tyk2 in the defense against MCMV can therefore not be explained by either generally reduced IFN- $\alpha\beta$  or

impaired IL-12/IFN- $\gamma$  signaling. Thus, our results demonstrate a novel function of Tyk2 in intrinsic antiviral responses. The data presented in this study suggest a modifying, rather than an initiating, role of Tyk2 in host responses. Tyk2 might exert this function via modification of IFN- $\alpha$ -derived signals or by IFN- $\alpha$ -independent pathways. Generation of IFNAR1/Tyk2 double-knockout mice is underway and will help to clarify this question. The molecular mechanisms responsible for anti-MCMV action have not yet been identified. Tyk2<sup>-/-</sup> macrophages show reduced induction of genes with well-described antiviral activity (60–62), but their importance in the defense against MCMV is not known yet. The dramatically enhanced replication of MCMV in Tyk2-deficient cells might be a combination of reduced activation of genes with antiviral activity or, alternatively, might be caused by defects in an as yet unknown anti-MCMV mechanism. In macrophages, novel antiviral pathways have been postulated (14); their mechanisms and a possible involvement of Tyk2 remain to be identified.

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## Disclosures

The authors have no financial conflict of interest.

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