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MyD88-Dependent and -Independent Murine Cytomegalovirus Sensing for IFN-α Release and Initiation of Immune Responses In Vivo[1]

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Antiviral immunity requires early and late mechanisms in which IFN-α and IL-12 play major roles. However, the initial events leading to their production remain largely unclear. Given the crucial role of TLR in innate recognition, we investigated their role in antiviral immunity in vivo. Upon murine CMV (MCMV) infection, both MyD88$^{-/-}$ and TLR9$^{-/-}$ mice were more susceptible and presented increased viral loads compared with C57BL/6, TLR2$^{-/-}$, TLR3$^{-/-}$, or TLR4$^{-/-}$ mice. However, in terms of resistance to infection, IFN-α production and in many other parameters of early inflammatory responses, the MyD88$^{-/-}$ mice showed a more defective response than TLR9$^{-/-}$ mice. In the absence of the TLR9/MyD88 signaling pathway, cytokine production was dramatically impaired with a complete abolition of bioactive IL-12p70 serum release contrasting with a high flexibility for IFN-α release, which is initially (36 h) plasmacytoid dendritic cell- and MyD88-dependent, and subsequently (44 h) PDC-, MyD88-independent and, most likely, TLR-independent. NK cells from MCMV-infected MyD88$^{-/-}$ and TLR9$^{-/-}$ mice displayed a severely impaired IFN-γ production, yet retained enhanced cytotoxic activity. In addition, dendritic cell activation and critical inflammatory cell trafficking toward the liver were still effective. In the long term, except for isotype switching to MCMV-specific IgG1, the establishment of Ab responses was not significantly altered. Thus, our results demonstrate a critical requirement of TLR9 in the process of MCMV sensing to assure rapid antiviral responses, coordinated with other TLR-dependent and -independent events that are sufficient to establish adaptive immunity. *The Journal of Immunology,* **2005, 175: 6723–6732.**

Viral infection presents a significant challenge to host survival in which the earliest events of the immune response are decisive in counteracting viral spread. Through cytokine release, sentinel cells orchestrate the initiation and development of both innate and acquired immunity, including NK cells whose activation is critical for a wide range of viruses and other pathogens (1, 2).

Murine CMV (MCMV)[3] is a hepatotropic herpesvirus that induces early dendritic cell (DC)-dependent type I IFN and IL-12 responses essential for mouse resistance (3–11). Through their unique capacity to secrete IFN-α, and to a lesser extent IL-12 and other innate cytokines, the plasmacytoid DC (PDC) are a cornerstone in the initiation of both innate and adaptive immune responses to MCMV (8, 10, 11). In addition to directly interfering with viral replication through ubiquitous cellular mechanisms (12), IFN-α controls NK cell cytotoxic activity (11, 13) and critical CCL3 (MIP-1α)-dependent trafficking to the liver (9). In addition, IFN-α regulates T cell functions by activating classical DC to more efficiently present Ags (11, 14). IL-12 secretion is required to prime a strong NK cell-dependent IFN-γ response (4–6). This process is essential to counteract MCMV infection in the liver, in contrast to a perforin-dependent mechanism in the spleen (15).

NK cell antiviral functions also depend on direct virus sensing through the NK cell activating receptor Ly49H (16–18) that interacts with the MCMV-encoded protein m157 expressed on the surface of infected cells (19, 20). Consistent with deficiencies for type I IFN, IL-12, IFN-γ, CCL3, or CXCL9 (6, 7, 9, 21, 22), a deficiency for Ly49H is sufficient to highly increase mouse susceptibility to MCMV infection (16, 18). DC-derived cytokines initiate both nonspecific NK cell activation and Ly49H$^+$-specific subset expansion that in turn contributes to the maintenance of the DC population (23, 24). Thus, both direct pathogen recognition and DC-derived cytokines are vital for NK cell functions and resistance to MCMV appears to be strongly dependent on NK/DC interactions.

The TLR family is a major class of pattern-recognition receptors that detect host invasion by a wide variety of pathogens. The detection of particular pathogen-associated molecular patterns by TLR leads to the release of specific cytokine profiles (25, 26). The common TLR signaling pathway triggers the activation of NF-κB and MAPK through the MyD88 adaptor, but some other alternative

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[3] Abbreviations used in this paper: MCMV, murine CMV; DC, dendritic cell; gB, glycoprotein B; Ie-1, immediate-early gene 1; PDC, plasmacytoid DC; p.i., postinfection; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; MFI, mean fluorescence intensity.

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pathways also induce type I IFN synthesis (25–27). Although TLR7 and TLR9 mediate type I IFN production by a MyD88-dependent mechanism (28, 29), TLR3 and TLR4 use a MyD88-independent pathway (30, 31). Given the crucial role of TLR in innate recognition of pathogens and their unique capacity to induce IFN synthesis, several groups have recently described a major role for TLR in virus sensing. Although TLR2 and TLR4 are involved in viral envelope or protein detection to induce inflammatory cytokines (32–34), production of IFN-α and IL-12 have been predominantly related to the recognition of nucleic acid structures by PDC either through TLR9 for DNA viruses (35, 36) or TLR7 for ssRNA viruses (37–39). Lastly, the role of TLR3 in dsRNA viral structure recognition has been suggested for Lang reovirus (40), yet this remains controversial (41). Thus, depending on virus type or pattern, various sensors could be involved during early phases of infection to induce specific antiviral responses.

In the present study, we define the in vivo roles for TLR in the detection of MCMV to induce early cytokine release and initiate coordinated NK cell functions. In agreement with Krug et al. (42) and Tabeta et al. (43), we demonstrate the critical role of the TLR9/MyD88 signaling pathway for a rapid MCMV clearance. However, in terms of resistance to infection, IFN-α production and in many other parameters of early inflammatory responses, the MyD88$^{-/-}$ mice showed a more defective response than TLR9$^{-/-}$ mice. In addition, although other DNA herpesviruses such as human CMV or HSV-1 trigger TLR2 (33, 34), we exclude a major involvement of TLR2, TLR3, or TLR4 in MCMV sensing. Furthermore, our results describe TLR-independent processes that provide a high flexibility for IFN-α release contrasting with a strong TLR9 and MyD88 dependency for IL-12. This argues for a multimodal recognition of MCMV in which TLR are essential to rapidly counteract virus propagation, but not for the set up of efficient immunity.

Materials and Methods

Mice

Specific pathogen-free female C57BL/6 mice were purchased from Charles River Breeding Laboratories. MyD88$^{-/-}$, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF)$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ were obtained from Osaka University (Suita, Osaka, Japan) (44–47) and TLR3$^{-/-}$ from Yale University School of Medicine (New Haven, CT) (40). The Ikaros$^{L/L}$ mouse line was previously described (48). They were backcrossed to C57BL/6 for 6, 2, 5, 5, 3, 2, and over 7 generations, respectively, and we consistently confirmed the expression of NK1.1 and/or Ly49H on TLR9$^{-/-}$ NK cells by flow cytometry. All mice were bred under pathogen-free conditions at the Charles River Breeding Laboratories animal facility and were used between 6 and 12 wk of age. Experiments were performed following protocols approved by the institutional animal committee and in accordance with European Economic Community Council Directive 86/609 as well as institutional animal care and use guidelines. The Ikaros$^{L/L}$ mice were maintained under specific pathogen-free conditions at the Institut de Génétique et de Biologie Moléculaire et Cellulaire mouse facility, and the mice were used between 5 and 7 wk of age.

Virus, infections, and in vivo treatments

Stocks of Smith strain MCMV salivary gland extracts were prepared from CD1 mice (4) and titered in a standard plaque assay on BALB/c MEF cells (4). Infections were initiated on day 0 by i.p. injection of 2×10^5 PFU either of the wild-type MCMV (Smith strain or, in a few indicated experiments, the K181-Perth strain) or the clone RVG-102 (MCMV-GFP) (49), which is recombinant for the enhanced GFP under the promoter of the immediate-early gene 1 (Ie-1). For PDC depletion experiments, mice were i.p. injected with an optimal dose of anti-Ly6G/C (RB6-8C5; DNAX), 120G8 mAb, or control ascite (GL113) once a day beginning 2 days before infection as described (8). The anti-Ly6G/C treatment eliminated >85% of 120G8$^+$ PDC in total CD11c$^+$ spleen DC. For survival experiments, mice were infected with 2 or 5×10^5 PFU and assessed for mortality and weight loss once daily for >15 days.

Sample collection and leukocyte preparation

At indicated times postinfection (p.i.), mice were sacrificed by CO_2 inhalation. Serum and organs were collected and prepared as described (8). Splenocyte suspensions were prepared as described (8, 50) and NK cells were enriched by successive negative and positive selections. Splenocytes were first incubated for 30 min at 4°C with a mixture of anti-CD3 molecular complex (17A2), anti-CD8β (53-5.8), anti-CD19 (1D3), and anti-erythrocyte (TER119). Unless otherwise specified, Abs were from BD Pharmingen. Ab-coated cells were then removed with Dynabeads (Dynal Biotech) on a magnet. Second, depleted cells were enriched for NK cells using anti-DX5 magnetic beads and positive selection columns MS$^+$ according to the manufacturer's instructions (Miltenyi Biotec). Cell viability was determined by trypan blue exclusion and NK cell purity was evaluated at 75–85% by flow cytometry for NK1.1$^+$CD3ϵ^- staining.

Flow cytometry analyses

For all FACS analyses, isolated total spleen cells were first incubated with anti-CD16/32 (2.4G2) to ensure blocking of FcRs. Cell surface stainings were then performed with the following Abs: CD3ϵ (145-2C11), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD69 (H1.2F3), CD86 (GL1), NK1.1 (PK136), 120G8 (50), and Ly49H generously provided by S. Vidal (University of Ottawa, Ottawa, Ontario, Canada). Negative controls were performed with the corresponding isotypes. Conventional DC were discriminated on CD11c^{high} expression and were distinctly composed of CD8α^+ DC and CD11b$^+$ DC. PDC were defined as CD11c^{low}, 120G8$^+$, CD8$\alpha^{+/-}$ cells (50). Lastly, NK cells were defined as NK1.1$^+$CD3ϵ^- cells. Stained cells were acquired using a FACSCalibur flow cytometer (BD Biosciences).

Real-time RT-PCR for quantification of MCMV mRNA

Total RNA was extracted from 5×10^6 isolated splenocytes using RNeasy kit (Qiagen) digested with DNase I (RNase-free DNase set; Qiagen) and reverse transcribed into cDNA by using Superscript II (Invitrogen Life Technologies). Quantification of cDNA was performed using the ICycler IQ Real-Time PCR Detection System (Bio-Rad) and the SYBR Green PCR kit (Qiagen). To quantify MCMV gene expression, we cloned the Ie-1 and the glycoprotein B (gB) gene of MCMV into the plasmid pTOPO 2.1 (Invitrogen Life Technologies). Standards consisted of 10-fold dilutions of gB and Ie-1 plasmids from 10^7 to 10^2 copies. The primers were designed to amplify a single specific fragment of 137 and 160 bp, respectively, for Ie-1 forward (GAGTCTGGAACCGAAACCGT) and reverse GTCGCT GTTATCATTCCCCAC) and gB forward (GTCGGCCATCTAC GAGAGAC) and reverse (GACCAGCGGTCTCGAATAAC) primers. The amounts of cellular 18 S rRNA used as an internal standard were determined with the primers forward GCTGGAATTACCGCGGCTGCT and reverse CGGCTACCACATCCAAGGAAG.

Cytokine measurements

For the determination of cytokine production, weighted and homogenized organs (21), sera, or culture supernatants were tested by ELISA at indicated times according to manufacturer's instructions. IFN-α levels were determined by using a specific ELISA kit (PBL Biomedical Laboratories) detecting IFN-αA, α1, α4, α5, α6, and α9. IL-12p40, IFN-γ, IL-6, TNF, and CXCL10 (IP-10) production were assayed using Duoset ELISA kits (R&D Systems), IL-12p70 and CCL3 levels using Quantikine kits (R&D Systems).

NK cell culture and cytotoxic activity

Enriched NK cells were purified from spleens of uninfected or 36 h infected mice and cultured at 10^6 cells/ml for 24 h in complete medium: RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES (all from Invitrogen Life Technologies) at 37°C in 5% CO_2. Supernatants were harvested and stored at −20°C for further analysis by ELISA. NK cell cytotoxic activity was assessed by ^{51}Cr release, in a 4-h assay at 37°C, from 5×10^3 labeled YAC-1 target cells (TIB-160; American Type Culture Collection), as described (4).

Histology

Liver samples were isolated at 60 h p.i., fixed in 10% buffered formalin and paraffin embedded for histological analysis. Tissue sections (5 μm) were affixed to slides, deparaffinized and stained with hematoxylin (Vector Laboratories) and eosin Y alcoholic solution (Sigma-Aldrich) to determine morphologic changes. As described (4), inflammatory foci were defined as discrete clusters of 6–60 individual, small nucleated cells visible throughout the liver. Numbers of inflammatory foci were determined by counting,

at a magnification of ×40, clusters of cells in 20 relative areas of representative tissue. Councilman bodies (dying hepatocytes morphologically identified as large, eosinophilic staining cells) and cytomegalic inclusion bodies (characteristic of CMV-infected cells) were enumerated from >100 representative inflammatory foci.

MCMV-specific IgG Ab titration

The presence of specific anti-MCMV Abs in the serum was investigated at days 15, 40, and 50 p.i. by ELISA using MCMV Ag-coated plates (PL-018; Charles River Breeding Laboratories). Each sample was tested on partially purified MCMV Ag or tissue control coated wells at a 60-fold dilution. Secondary Abs used for MCMV Ag detection recognized either total IgG (Charles River Breeding Laboratories) or the IgG1 subtype (X56; BD Pharmingen).

Statistical analyses

Statistical analyses were performed using Student's two-tailed *t* tests. Significant differences were done with respect to the C57BL/6 control values. Unless otherwise indicated, data are mean ± SEM.

Results

Inefficient control of MCMV propagation in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice

Using the recombinant RVG-102 virus expressing GFP (49), we assessed the percentage of MCMV-infected cells in C57BL/6 mice compared with the TLR-deficient mice (Fig. 1*A*). As already reported (Ref. 11 and data not shown), at 36 h and more obviously at day 4 p.i., a small proportion of splenocytes were positive for GFP in C57BL/6 mice with 3.3 ± 0.9% of GFP$^+$ conventional CD11c^{high} DC. Significant increases in GFP$^+$ splenocytes and CD11c^{high} DC were observed in MCMV-infected MyD88$^{-/-}$ mice and, to a lesser extent, in MCMV-infected TLR9$^{-/-}$ mice. Compared with C57BL/6 mice, similar percentages of GFP$^+$ splenocytes and DC were detected in MCMV-infected TLR2$^{-/-}$, TLR3$^{-/-}$, and TLR4$^{-/-}$ mice. Quantitative RT-PCR analysis for MCMV transcripts such as gB and Ie-1 in splenocytes from infected mice at day 3 allowed us to observe a significant increase in viral load in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice compared with C57BL/6 animals. This increase was not observed in splenocytes from mice infected with TLR2$^{-/-}$, TLR3$^{-/-}$, and TLR4$^{-/-}$ (Fig. 1, *B* and *C*, *left panels*). Kinetic experiments indicated that although the viral load peaked at day 2 in C57BL/6 splenocytes, a further increase of 2 and 3 logs of gB and Ie-1 transcripts, respectively, was measured at days 3–4 both in splenocytes from MyD88$^{-/-}$- and TLR9$^{-/-}$-infected mice (Fig. 1, *B* and *C*, *right panels*). However, at day 7, no significant differences were observed between MyD88$^{-/-}$, TLR9$^{-/-}$, or C57BL/6 mice (data not shown). Thus, control of MCMV infection and replication in the spleen involved MyD88 and TLR9, whereas TLR2, TLR3, and TLR4 did not seem to play a significant role.

Increased susceptibility to MCMV infection in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice

To assess the protective role of TLR in acute infection, mice deficient for TLR signaling were challenged with MCMV and followed for mortality and weight loss. Because sharp differences in mortality are observed with small changes of viral dose (7), we used inoculums of either 2 or 5 × 10^5 PFU that did not induce mortality in C57BL/6 mice. In accordance with the viral load data, TLR9$^{-/-}$ and, particularly, MyD88$^{-/-}$ mice were more susceptible to MCMV than C57BL/6 mice with a LD$_{50}$ ranging between 2 and 5 × 10^5 PFU (Fig. 2, *A* and *B*). Of note, deficiency for TLR2, TLR3, and TLR4 did not affect mouse resistance (data not shown). Unlike that which was observed with C57BL/6 and all other TLR-deficient mice during the first 2 days p.i., the behavior and general appearance of MyD88$^{-/-}$ animals was unaffected in comparison

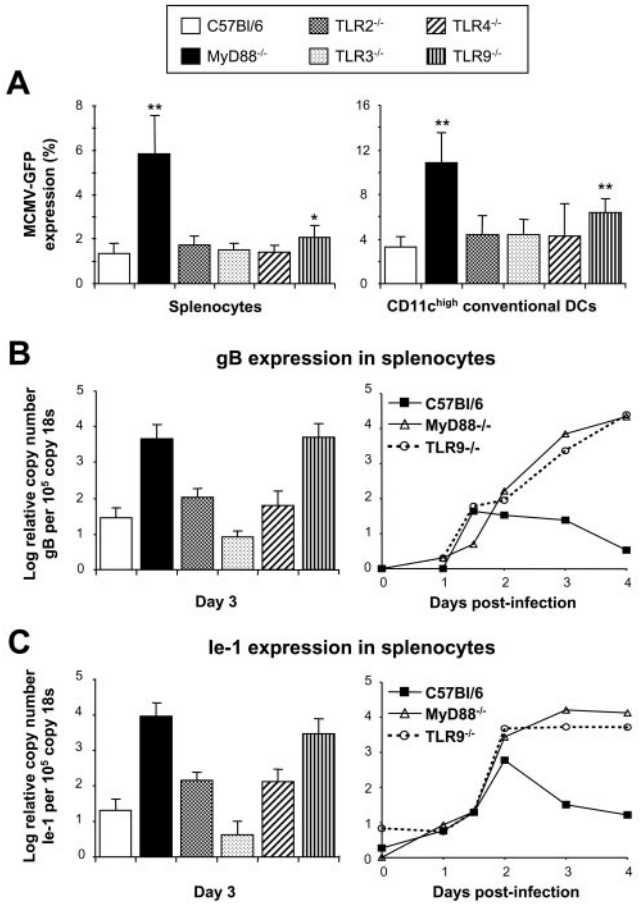

FIGURE 1. Infection and viral load of MyD88$^{-/-}$, TLR2$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ mice upon MCMV infection. *A*, Infection of splenocyte and CD11c^{high} conventional DC was investigated at day 4 p.i. with the MCMV clone RVG-102 expressing the GFP under the Ie-1 promoter. Cells were analyzed by flow cytometry for GFP and CD11c expression. Number of events analyzed was >150,000 for total and 2500 for GFP$^+$ splenocytes. The data are from one representative experiment ($n = 4$ mice) of two (**, $p < 0.01$; *, $p < 0.05$). *B* and *C*, Viral load was investigated through MCMV transcripts expression in splenocytes. RNA was extracted from total splenocytes isolated from mice at indicated times p.i. and transcripts for 18 S, the gB (*B*) and Ie-1 (*C*) were quantified by real-time RT-PCR. Results are expressed in relative copy number of gB (*B*) or Ie-1 (*C*) per 10^5 copy of 18 S. *Left panels* are focused on day 3 ($n = 3$ mice) and *right panels* display evolution of gene expression from days 0 to 4 ($n = 2$ mice per time point).

with noninfected animals. This observation correlated with a significant 2-day delay in weight loss in infected MyD88$^{-/-}$ animals compared with C57BL/6 mice ($p < 0.002$ from days 2 to 4) for both doses of MCMV tested (Fig. 2*C*). At later times, obvious signs of increased morbidity were observed for both MyD88$^{-/-}$- and TLR9$^{-/-}$-infected mice, for which the onset of weight recovery was delayed by 2 days ($p < 0.03$ from days 6 to 8). Thus, based on the follow-up of mortality and morbidity, both MyD88$^{-/-}$ and TLR9$^{-/-}$ mice were more susceptible to MCMV infection than C57BL/6 mice.

Deficient early serum cytokine secretion in response to MCMV infection in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice

Because MCMV clearance is clearly cytokine-dependent (2, 3, 5), we monitored the levels of cytokines in serum from C57BL/6 and TLR-deficient mice from day 0 to 4 p.i. (Fig. 3). We observed that in vitro IFN-α and IL-12 secretion by DC in response to CpG or

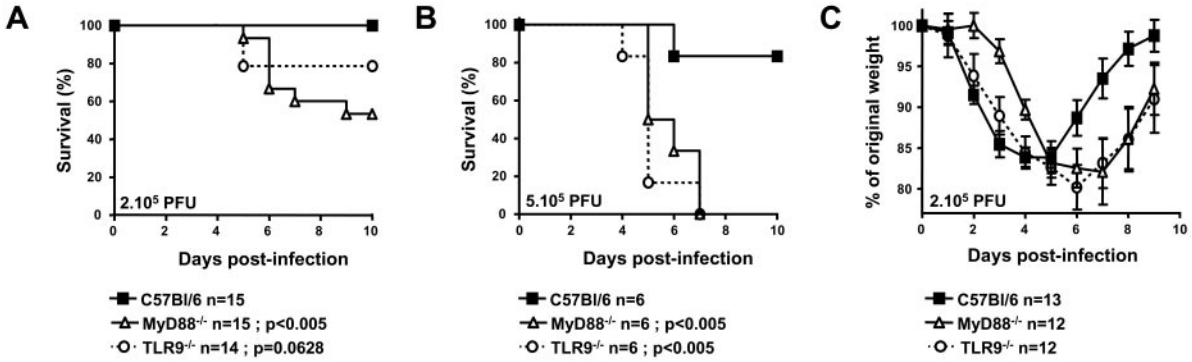

FIGURE 2. Susceptibility to MCMV infection of MyD88$^{-/-}$ and TLR9$^{-/-}$ mice. Animals were infected i.p. either with 2×10^5 PFU (*A* and *C*) or 5×10^5 PFU (*B*) of MCMV at day 0. Animals were then monitored at least once daily for 15 days after infection to assess mortality (*A* and *B*) and morbidity by means of weight loss (*C*). Mortality was presented as the percentage of survival mice and weight loss as indicated by the drop in the percentage of original weight. As indicated, the results were from 6 to 15 mice per group. Differences in survival between TLR9$^{-/-}$, MyD88$^{-/-}$, and control mice for an infection with 5×10^5 PFU were statistically significant ($p < 0.005$).

MCMV were MyD88- and TLR9-dependent (data not shown). In vivo, as the cytokine production was optimal at 36 h, we focused on this time point. We noted a complete abolition of bioactive IL-12p70 production (Fig. 3*A*) as well as a dramatic decrease of IL-12p40 (Fig. 3*B*) in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice, whereas an impairment of IFN-α release was only observed in MyD88$^{-/-}$ mice (Fig. 3*C*). IFN-γ production was also dramatically impaired in MyD88$^{-/-}$ mice and to a lower extent in TLR9$^{-/-}$ mice (Fig. 3*D*). Although IFN-α levels were low to undetectable at 36 h in MyD88$^{-/-}$ mice, we observed a delayed wave of secretion between 40 and 60 h p.i. that reached a maximum of 590 ± 195 pg/ml at 44 h (Fig. 3*A*, *right*). TRIF$^{-/-}$ mice produced similar or increased levels of IFN-α compared with wild-type mice both at 36 and 44 h (data not shown). Consistent levels of type I IFN were measured in a bioassay (data not shown). Concurrently, in MyD88$^{-/-}$ mice, a short delayed peak of IFN-γ secretion was observed that reached 664 ± 194 pg/ml (Fig. 3*D*, *right*). In addition, we observed in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice a severely deficient secretion of chemokines that are responsible for inflammatory cell trafficking toward sites of infection. Production of IFN-dependent chemokines CCL3 (MIP-1α) and CXCL10 (IP-10) were severely disturbed in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice at 36 h p.i., but appeared at day 3–4 p.i. (Fig. 3, *E* and *F*). Finally, we observed a dramatic decrease of proinflammatory cytokine secretion in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice compared with C57BL/6, particularly for TNF levels (Fig. 3*G*), but also to a lesser extent for IL-6 (Fig. 3*H*). Except for a notable and reproducible increase in IFN-α levels in TLR4$^{-/-}$ mice at 36 h p.i. (3321 ± 716 pg/ml), we did not observe significant differences for any cytokine tested as described in TLR2$^{-/-}$, TLR3$^{-/-}$, or TLR4$^{-/-}$ mice compared with C57BL/6 (data not shown).

High flexibility of IFN-α release during MCMV infection

By orchestrating both innate and acquired immune responses, IFN-α plays a central role in MCMV immunity. As presented in Fig. 3*C*, the deficiency in MyD88 resulted in a complete loss of IFN-α secretion at 36 h p.i. However, starting from 42 h, delayed levels were detected, suggesting successive MyD88-dependent and -independent mechanisms of IFN-α synthesis in normal mice. Because early IFN-α production (36 h p.i.) was previously demonstrated to be PDC-dependent (8, 10), we investigated the role of PDC in the MyD88-independent IFN-α production. PDC depletion using anti-Ly6G/C Ab (Fig. 4*A*) resulted in a dramatic reduction of IFN-α production at 36 h p.i. in C57BL/6 mice, as well as in TLR9$^{-/-}$ mice (Fig. 4*B*). The treatment also induced a substantial increase in splenocyte infection both in C57BL/6 and MyD88$^{-/-}$ mice with a boost at 44 h p.i. from 0.18 ± 0.03% to 0.65 ± 0.05% ($p < 0.001$) and from 0.29 ± 0.06% to 0.64 ± 0.10% ($p < 0.025$) of GFP-MCMV positive cells, respectively (data not shown). Of interest, at 44 h p.i., although anti-Ly6G/C Ab treatment depleted PDC by >90%, as detected by analysis of the number of CD11c^{int} 120G8$^+$ cells in the spleen (Fig. 4*A*), it did not alter IFN-α levels in C57BL/6, MyD88$^{-/-}$, TLR9$^{-/-}$, or TLR3$^{-/-}$ mice (Fig. 4*B*). Similarly, depletion experiments performed in 129Sv mice, using either anti-Ly6G/C Ab (data not shown) or the PDC-specific mAb 120G8 (Fig. 4*C*), decreased IFN-α production by ~90% at 36 h, but did not affect production at 44 h p.i. IFN-α production at either 36 or 44 h p.i. was not decreased in TRIF$^{-/-}$ mice (Fig. 4*D*). As a further test for the role of PDC in IFN-α production in MCMV infection, we used a strain of Ikaros-deficient mice that lacks PDC while having relatively normal numbers of other hemopoietic cells and conventional DC (D. Allman, M. Dalod, C. Asselin-Paturel, T. Delale, G. Trinchieri, C. Biron, P. Kastner, and S. Chan, submitted for publication and Ref. 48). Although at 36 h p.i. the Ikaros-deficient mice produced only minimal levels of IFN-α, at 42 h they produced significant levels (Fig. 4*E*). Thus, whereas early IFN-α release was strictly MyD88- and PDC-dependent, IFN-producing cells other than PDC released at later times IFN-α in a MyD88-, TRIF-, TLR3-, and TLR9-independent manner.

DC maturation in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection

In the course of MCMV infection, proportions of DC subsets were comparably affected in C57BL/6, MyD88$^{-/-}$, and TLR9$^{-/-}$ mice (data not shown). However, at 36 h p.i., DC from MyD88$^{-/-}$ mice were more infected than those from C57BL/6 with a more than 3-fold increase in GFP-MCMV expression in CD8α^+ DC (data not shown). To consider the impact of TLR signaling deficiency on DC, we monitored their maturation through CD86 costimulatory molecule expression (Fig. 5). A significant increase in CD86 expression was observed at day 1–3 peaking at 36–48 h p.i. in all mice. PDC, CD8α^+ DC, and to a lesser extent CD11b$^+$ DC from MyD88$^{-/-}$ mice at 36 h p.i. expressed significantly less CD86 than DC from C57BL/6, TLR2$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$, or TLR9$^{-/-}$ mice. However, compared with noninfected mice, CD86 up-regulation in DC from infected MyD88$^{-/-}$ mice at 36 h p.i. was still largely significant, with an increase in mean fluorescence intensity (MFI) from 24 to 125 MFI on PDC, from 78 to 408 MFI

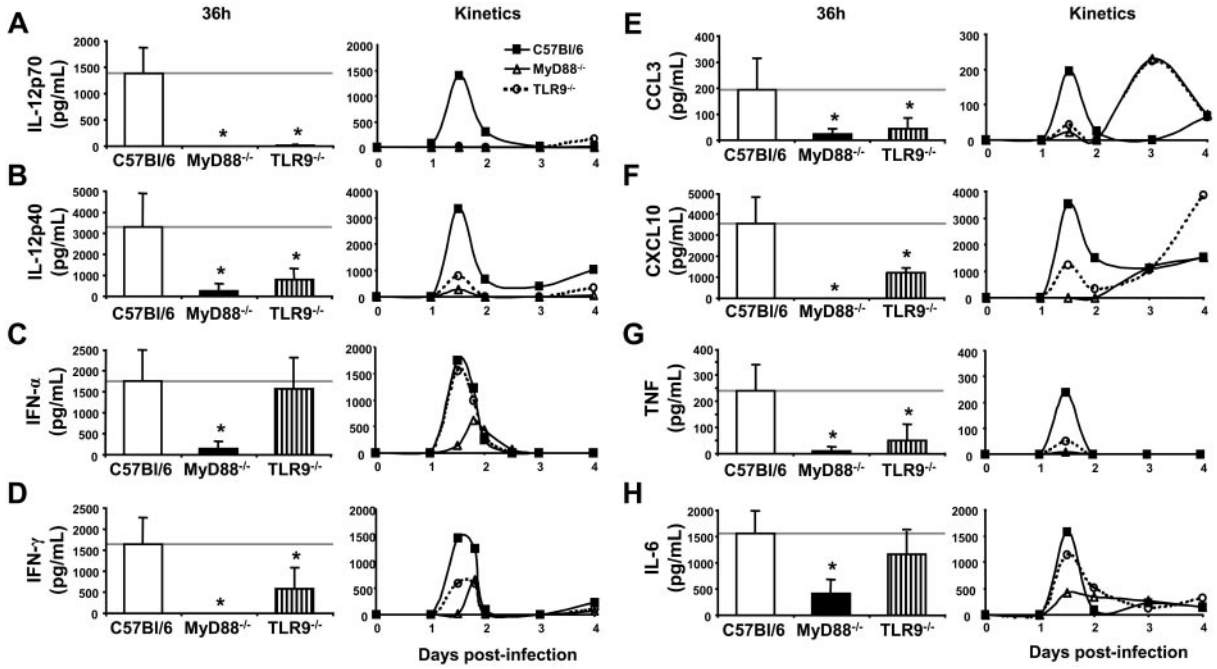

FIGURE 3. Serum cytokines and chemokines in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection. Bioactive IL-12p70 (*A*), IL-12p40 (*B*), IFN-α (*C*), IFN-γ (*D*), CCL3/MIP-1α (*E*), CXCL10/IP-10 (*F*), TNF (*G*), and IL-6 (*H*) were measured by ELISA in serum of MCMV-infected mice at 36 h (*left*) and at the indicated times up to day 4 p.i. (*right*). Values are expressed in picograms per milliliter $\pm$ SEM and were performed on over nine mice in three to five independent experiments (*left*), on six mice for the time point 44 h in *A–D* and on two mice (*right*) at each other indicated times (*, $p < 0.005$).

on CD8α^+ DC and from 42 to 342 MFI on CD11b$^+$ DC. In addition, CD86 expression on DC from MyD88$^{-/-}$ mice reached at 48 h p.i. the levels observed on DC from C57BL/6. Thus, unlike the early cytokine defect, the absence of the TLR9/MyD88 signaling pathway only marginally altered DC activation.

Failure of IFN-γ secretion, but efficient up-regulation of cytotoxic potential in NK cells from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection

Due to the critical role of NK cells in the control of MCMV propagation either through a perforin- and Ly49H-dependent mechanism in the spleen or an IFN-γ secretion-dependent role in the liver (15), we investigated NK cell activities in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice upon MCMV challenge. NK cell maturation was evaluated by analyzing CD69 expression, which peaked at 36 h p.i. in C57BL/6 mice. Similarly to CD69 expression on B and T cells (data not shown), NK cells from MyD88$^{-/-}$ mice expressed lower levels of CD69 at 36 h p.i. than C57BL/6, TLR2$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$, or TLR9$^{-/-}$ mice (Fig. 6*A* and data not shown). In addition, we did not observe a recovery of CD69 expression at later times of infection (Fig. 6*B*). However, CD69 expression was still significantly increased compared with noninfected animals (120 vs 15 MFI). NK cell functions (IFN-γ secretion and cytotoxic activity) were investigated in ex vivo-purified cells from 36 h infected mice. In agreement with IFN-γ impairment in serum, NK cells from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice upon in vitro culture displayed profound defects in IFN-γ secretion. Although NK cells from C57BL/6 produced ~3500 pg/ml IFN-γ, a 10-fold decrease and virtually undetectable levels of IFN-γ were observed with NK cells from TLR9$^{-/-}$ and MyD88$^{-/-}$ mice, respectively (Fig. 6*C*). As for cytolytic functions against YAC-1 cells, we observed comparable activities among NK cells from C57BL/6, MyD88$^{-/-}$, and TLR9$^{-/-}$ mice (Fig. 6*D*). Thus, MyD88 and TLR9 deficiencies partially altered the activation of NK cells that could still acquire a cytotoxic potential but failed to produce IFN-γ.

Normal inflammatory cell recruitment to the liver, but altered hepatotoxicity in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection

Critical inflammatory cell trafficking, and particularly NK cell recruitment toward the liver in response to MCMV has been shown to be highly cytokine- and chemokine-dependent (9, 21, 22). At day 4, MCMV infection of C57BL/6 mice was associated with high levels of virus replication in the liver exceeded by >100-fold levels of gB expression measured in splenocytes (data not shown). In addition, gB and Ie-1 expression in both MyD88$^{-/-}$ and TLR9$^{-/-}$ liver homogenates was 10-fold higher than in C57BL/6 mice (data not shown). Inflammatory foci, represented by clusters of leukocyte infiltrates in infected livers, are present early p.i. (51). Their number peaks at days 2 to 3 when they colocalize with Councilman bodies (dying hepatocytes with eosinophilic staining) (51). From day 3 to 7, infiltrates and Councilman bodies progressively disappear whereas cytomegalic inclusion bodies (characteristic of infected hepatocytes) appear (51). To compare MCMV-induced liver pathology in C57BL/6, MyD88$^{-/-}$ and TLR9$^{-/-}$ mice, we analyzed H&E stained liver sections of 60 h infected mice. As previously described (51), we observed large numbers of inflammatory foci in C57BL/6 mice in which Councilman bodies became readily detectable (Fig. 7*A*). MyD88$^{-/-}$ (Fig. 7*B*) and TLR9$^{-/-}$ liver sections displayed a number of leukocyte infiltrates comparable to those of C57BL/6 mice (Fig. 7*C*). However, the number of Councilman bodies was significantly reduced in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice (Fig. 7*D*). Furthermore, we observed a high number of hepatocytes from MyD88$^{-/-}$ mice with cytomegalic inclusion bodies, whereas only a few of these lesions were detectable either in C57BL/6 or TLR9$^{-/-}$ mice (Fig. 7, *B* and *E*). Because the outcome of liver pathology during early MCMV infection mainly depends on the recruitment of NK cells and their unique capacity to produce IFN-γ (4, 6), we monitored the cytokine secretion in liver homogenates (Fig. 7*F*). In C57BL/6 mice,

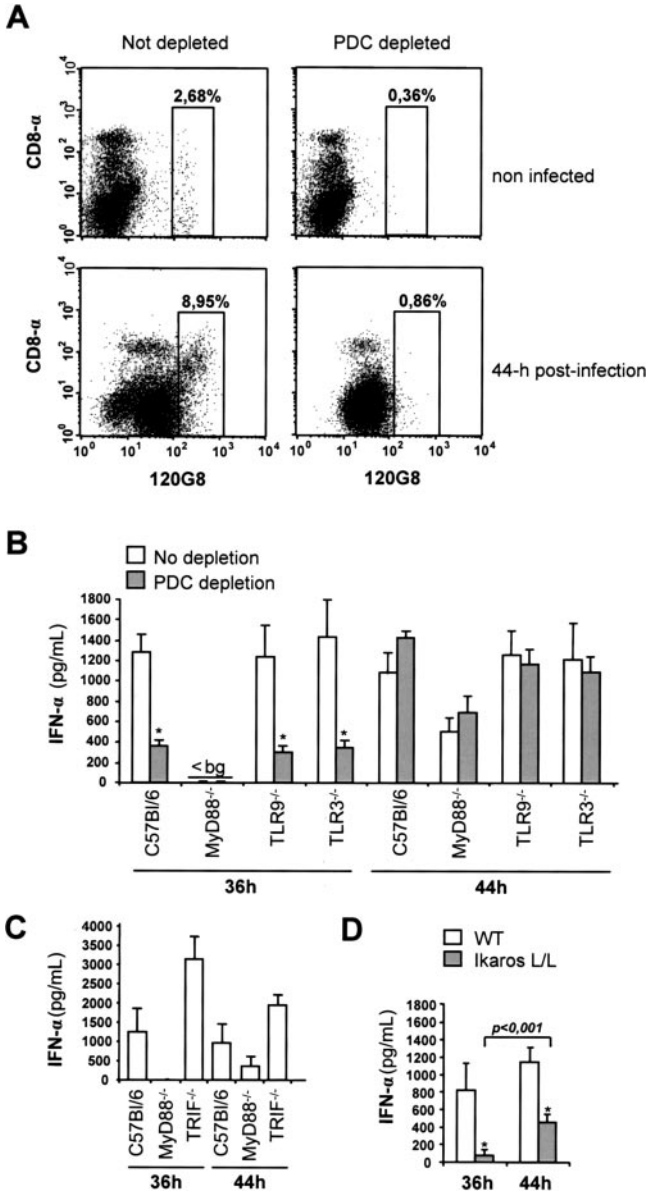

FIGURE 4. Serum IFN-α secretion during MCMV infection in the presence or absence of PDC. *A* and *B*, Anti-Ly6G/C Ab-mediated depletion of PDC in C57BL/6, MyD88$^{-/-}$, TLR9$^{-/-}$, and TLR3$^{-/-}$ mice. Mice were i.p. injected four times either with anti-Ly6G/C ($n = 3$) or control anti-GL113 ($n = 3$) Abs 48, 24, and 4 h before, and also 24 h after infection. *A*, Depletion of PDC (as detected by staining with 120G8 mAb) was verified in the spleen of anti-Ly6G/C-treated C57BL/6 mice 44 h p.i. with MCMV (Smith strain). *B*, Blood sera were collected from same mice at 36 and 44 h p.i., and IFN-α levels were measured by ELISA. Data are representative of three independent experiments (*, $p < 0.001$). *C*, 120G8 Ab-mediated depletion of PDC in 129Sv mice. Mice were i.p. injected four times either with 120G8 ($n = 3$) or control anti-GL113 ($n = 3$) Abs 48, 24, and 4 h before, and also 24 h after infection. Blood sera were collected from the same mice at 36 and 44 h p.i., and IFN-α levels were measured by ELISA. Data are representative of three independent experiments (*, $p < 0.001$). *D*, IFN-α production in MCMV (K181-Perth)-infected C57BL/6, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice. Blood sera were collected from same mice ($n = 3$) at 36 and 44 h p.i., and IFN-α levels were measured by ELISA. *E*, Use of PDC-deficient Ikaros$^{L/L}$ mice. Infections were initiated in wild-type ($n = 6$) or Ikaros$^{L/L}$ ($n = 6$) mice on day 0 by i.p. injection of 2×10^4 PFU of a salivary gland-extracted GFP-recombinant MCMV Smith strain (RVG-102). Blood sera were collected from same mice at 36 and 44 h p.i., and IFN-α levels were measured by ELISA (*, $p < 0.001$).

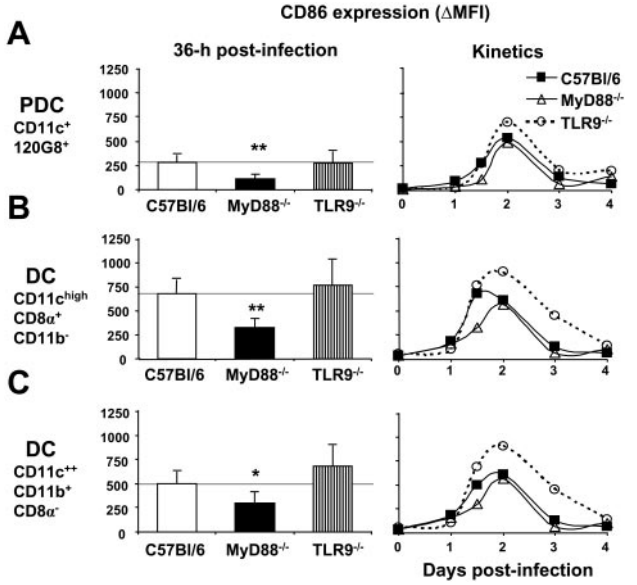

FIGURE 5. CD86 expression on DC subsets from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection. Splenocytes were isolated from mice at the indicated times up to day 4 p.i. and were analyzed by flow cytometry of CD86 expression on PDC CD11c^{low}120G8^{+}CD8$\alpha^{+/-}$ (*A*), DC CD11c^{high}CD8α^{+}CD11b^{-} (*B*), and DC CD11c^{high}CD8α^{-}CD11b^{+} (*C*) cells. Results are shown as ΔMFI between CD86 expressions at indicated times p.i. and the steady state at day 0. Data were performed on over 11 mice in three independent experiments at time point of 36 h and on 2 mice at each other indicated times (**, $p < 0.001$; *, $p < 0.02$).

IFN-γ was produced in two steps with peaks at 36 h and day 4. In contrast, it was first detected only at day 3 in MyD88$^{-/-}$ mice and at day 2 in TLR9$^{-/-}$ mice and the secretion continuously increased thereafter. Thus, unlike the dramatic default in the serum, a delayed but significantly high level of IFN-γ is produced in livers from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice.

Normal MCMV-specific Ab production, except isotype switching to IgG1 in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection

Acquired immunity is closely related to innate responses that orchestrate the orientation of B and T cell differentiation through cytokine-dependent mechanisms. Because we observed major alterations of Th1 cytokines such as IL-12 in MCMV infected MyD88$^{-/-}$ and TLR9$^{-/-}$ mice, we investigated the profile of MCMV-specific Ab production. Assay of anti-MCMV IgG subtypes in the serum were performed by ELISA at 15, 40, or 50 days p.i. Except for a slightly lower level of specific total IgG at 15 days p.i. in MyD88$^{-/-}$ serum, we did not measure significant differences between total specific IgG amounts in MyD88$^{-/-}$, TLR9$^{-/-}$, and C57BL/6 sera (Fig. 8*A*). Of interest, production of specific IgG1 Abs was measured in the serum of infected MyD88$^{-/-}$ and TLR9$^{-/-}$ mice yet were undetectable in the serum of C57BL/6 (Fig. 8*B*). Serum of the different deficient mice analyzed contained comparable high levels of specific-IgG2a and IgG2b, but no specific-IgE could be measured in the serum of MyD88$^{-/-}$ and TLR9$^{-/-}$ mice (Fig. 8, *C* and *D*). In addition, IgG profiles of TLR2$^{-/-}$ and TLR4$^{-/-}$ mice matched with those of C57BL/6. Thus, both innate and acquired immune responses are affected in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice, but high levels of potentially neutralizing antiviral Abs are still produced.

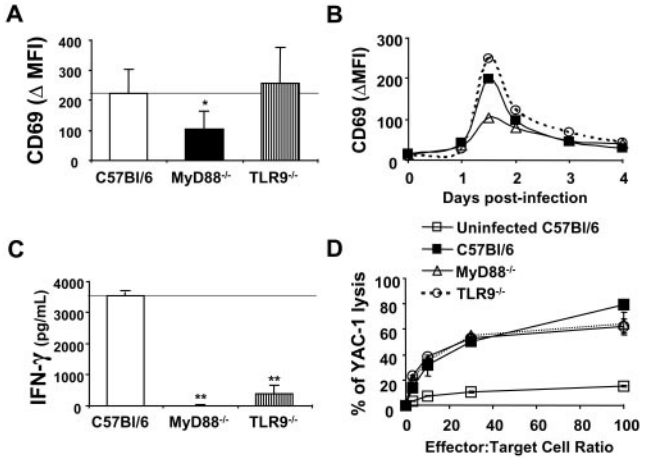

FIGURE 6. NK cell functions in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection. NK cell activation was assessed by flow cytometry of CD69 expression on NK1.1$^+$ CD3ϵ^- splenocytes at 36 h (*A*) or in kinetics up to day 4 (*B*). Results are shown as ΔMFI between CD69 expressions at indicated times p.i. and the steady state at day 0. Data were performed on over 11 mice in three independent experiments (*, $p < 0.002$) (*A*) and on 2 mice at each indicated time (*B*). NK cell activity was assessed in vitro after isolation and purification from spleens by each lineage at 36 h p.i. as well as of uninfected C57BL/6 mice ($n = 4$). *C*, IFN-γ production was measured in a culture at 24 h of 10^6 cells/ml (**, $p < 0.001$) and cytotoxicity was investigated against YAC-1 cells in 4-h ^{51}Cr release assays at varying E:T ratios as indicated (*D*). Percentage of lysis was calculated as 100 × [(mean cpm of samples − mean cpm of spontaneous release)/(mean cpm of maximum release − mean cpm of spontaneous release)].

Discussion

In this study we demonstrate the critical role of the TLR9/MyD88 signaling pathway in MCMV sensing. This pathway acts to rapidly counteract viral spread by initiating early cytokine and chemokine release and by coordinating the priming of effective NK cell activity. We also emphasize a high flexibility of the signal necessary for IFN-α release as opposed to a strict TLR9 requirement for IL-12 production in serum. Indeed, our results argue for a multimodal detection of MCMV with coordinated and partially redundant TLR-dependent and -independent responses highlighted by the relative resistance of MyD88$^{-/-}$ and TLR9$^{-/-}$ mice as can be compared with the severe susceptibility of mice deficient for certain critical cytokines such as IL-12, IFN-γ, CCL3, or CXCL9 (6, 7, 21).

In the absence of the TLR9/MyD88 pathway, bioactive IL-12 release is abolished and as a consequence IFN-γ secretion by NK cells in serum and spleen is dramatically impaired. Contrasting with IL-12 and consistent with the observed delayed DC activation, IFN-α secretion is relayed by TLR-independent mechanisms and as a result many type I IFN-dependent effects are not altered. Indeed, contrary to what has been observed in IFN-αβR$^{-/-}$ MCMV infected mice (13), spleen NK cells from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice retained their cytolytic potential activity against YAC-1 cells, but according to Krug et al. (42), not against RMA-S lymphoma cells. Because NKG2D ligands are expressed on YAC-1 but not on RMA-S cells (52), it is possible that NKG2D plays a major role in the killing of YAC-1 cells in vitro. However, the costimulatory function of NKG2D may not have a major role in vivo, as some of its cell surface expressed ligands are specifically down-regulated by MCMV gene products (53, 54). In agreement with our results, deficiencies for the TLR recruited IL-1R-associated kinase or for IL-12p35 also disrupt NK cell IFN-γ release yet do not affect cytotoxic activity against YAC-1 cells (6, 55). Thus, the TLR9/MyD88/IL-1R-associated kinase axis is critical to induce nonspecific NK cell-dependent IFN-γ secretion as well as particular mechanisms of lysis.

In the liver, defenses against MCMV require a critical cascade of cytokines and chemokines to ensure NK cell trafficking and effective responses that are IFN-γ-dependent but perforin- and Ly49H-independent (15, 21, 22). Initially, liver responses require type I IFN release for CCL3-dependent NK cell recruitment (9, 21, 22). Then, NK cells secrete IFN-γ (4–6), which has a direct antiviral action and recruits T cells for long-term responses through induction of CXCL9 and CXCL10 (21). Despite the severe alteration of CCL3 and IFN-α release at early times p.i. in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice, hepatic accumulation of leukocyte infiltrates was not impaired. However IFN-γ secretion was severely delayed and Councilman bodies did not appear normally suggesting a decreased killing of infected hepatocytes. This lack of infected-hepatocyte killing could be attributed to deficient IFN-γ secretion because IFN-γ, unlike IL-1β, TNF, IL-6, or IFN-α, induces hepatocyte apoptosis in vitro (56) as well as in vivo as observed during adenovirus infection (57). Thus, although inflammatory cells from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice are normally recruited to the liver during MCMV infection, antiviral activity of infiltrating NK cells, assessed through IFN-γ release, is delayed and probably contributing to viral spread and augmented mouse pathology.

Although our study mostly focuses on early times of infection, adaptive immunity is also required for the termination of the productive infection and the establishment of latency (3). Innate-derived cytokines also drive adaptive immunity by polarizing naive CD4$^+$ T cells toward either a Th1 or Th2 response and consequently B cells toward IgG2a or IgG1 and IgE isotype switching (25, 26). Despite critical deficiencies in Th1 cytokine production, i.e., IL-12 and IFN-α, and in contrast to responses against some other pathogens (25), Th1 immune responses were not abolished in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice as MCMV-specific IgG2a Abs were still produced. Thus, the observed isotype switching toward IgG1 could not be considered as reflecting a Th2 response, but was possibly related to a removal of IgG1 inhibition mediated in B cells by the TLR9-dependent induction of T-bet as described by Liu et al. (58). Thus, in addition to the delayed expansion of specific CD8$^+$ T cells (42), TLR9 and MyD88 deficiency only partially delays and modifies acquired immune responses against MCMV.

Although the TLR9/MyD88 signaling pathway is obviously critical in vivo for resistance to MCMV infection by the induction of early cytokine secretion, a number of alternative responses were still effective despite the absence of TLR9. The residual and/or delayed cytokine release could be attributed to partially redundant receptors that were sufficient to induce: 1) DC maturation; 2) IFN-α release; 3) inflammatory cell trafficking; and 4) establishment of adaptive immunity. MyD88 deficiency disrupted additional responses to those induced by TLR9. In this case, IFN-γ secretion was completely abolished and IFN-α secretion was reduced and delayed, yet the resulting viral loads were only slightly higher. Because the MyD88 adaptor is also required for the signal transduction of all other TLR, except TLR3 (25, 26), as well as receptors for IL-18 and IL-1 (44), these pathways could also play a role in either MCMV sensing or subsequent responses. Although IL-18 signaling has been reported to be nonessential for the survival of MCMV-infected mice, it is required for the release of IFN-γ in serum (6), suggesting a contribution of IL-18 at least for the residual IFN-γ production in TLR9$^{-/-}$ mice. The role of IL-1 signaling in MCMV infection remains to be determined. Although other viruses belonging to the same family are detected by TLR2, i.e., human CMV or HSV-1 (33, 34), this was not the case for

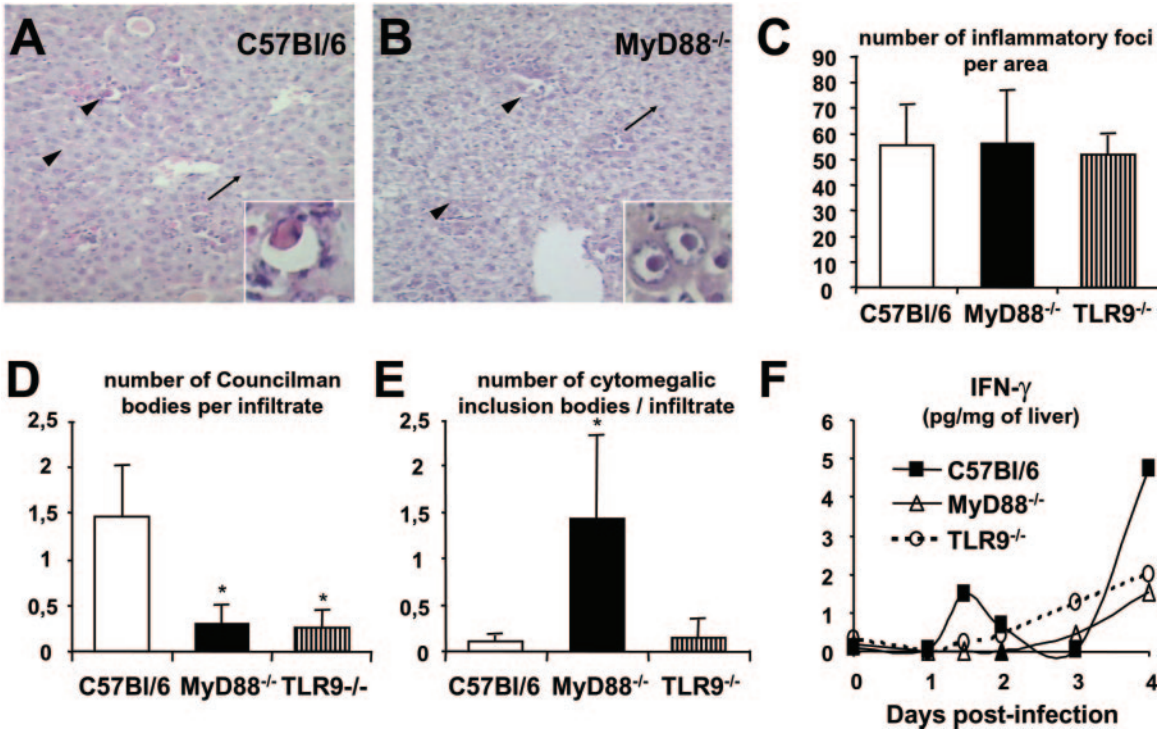

FIGURE 7. Leukocyte accumulation and IFN-γ secretion in MyD88$^{-/-}$ and TLR9$^{-/-}$ livers during MCMV infection. Livers were harvested and H&E-stained tissue sections were prepared from C57BL/6 (*A*), MyD88$^{-/-}$ (*B*), and TLR9$^{-/-}$ (*C*) mice at 60 h p.i. Arrows denoted inflammatory foci with leukocyte infiltrates, and arrowheads denoted Councilman bodies (*A*) or cytomegalic inclusion bodies (*B*), which were presented at a higher magnification (*inset*). Images were digitally captured at the original magnifications of ×100 and ×400. *C*, Numbers of inflammatory foci per relative areas of representative liver sections were determined as well as the mean numbers of Councilman bodies (*D*) and cytomegalic inclusion bodies (*E*) per inflammatory focus (*, $p < 0.002$). *F*, IFN-γ levels were assessed by ELISA on liver homogenates prepared at indicated times p.i. Values are expressed in picograms per milligram of liver on two mice per time point.

MCMV. MCMV also initiated immune responses independently of TLR4 and in agreement with Edelmann et al. (41), but in contrast to the report by Tabeta et al. (43), independently of TLR3 and TRIF. The contribution of TLR7, which is highly expressed on PDC and has the capacity to induce high levels of IFN-α in response to ssRNA viruses (37–39), was not determined. Our observation that, unlike in MyD88$^{-/-}$ mice, PDC-dependent IFN-α production in vivo is largely maintained in TLR9$^{-/-}$ mice, contrast with data reported by other investigators using BALB/c TLR9$^{-/-}$ mice (42) and TLR9^{CpG1} mutant mice (43) that display a striking deficiency in IFN-α production similar to that of MyD88$^{-/-}$ mice. Using the highly virulent K181-Perth MCMV strain, we also observed that TLR9$^{-/-}$ mice produced somewhat reduced but significant levels of IFN-α at 36 h and identical levels at 44 h compared with wild-type mice (61). It is possible that, according to the genetic background, PDC would have alternative pathways allowing IFN-α secretion in vivo. We also show in this study that non-PDC have the capacity, with somewhat slower kinetics compared with PDC, to release IFN-α in response to MCMV infection as shown by the finding that depletion of PDC with either the anti-Ly6G/C or the 120G8 Ab largely decreased IFN-α production at 36 h p.i. but not at 42–44 h p.i. This PDC-independent production of IFN-α, already described in vivo in response to lymphocytic choriomeningitis virus (10), was also confirmed in MCMV-infected Ikaros$^{-/-}$ mice, which are naturally deficient of PDC (61) and which failed to produce IFN-α at 36 h p.i., but produced significant levels at 42 h p.i. The PDC-independent production of IFN-α at 42–44 h p.i. was TLR3-, TLR9-, and MyD88-independent. The normal or slightly enhanced production of IFN-α at both 36 and 42 h in TRIF$^{-/-}$ mice suggests that it is also TRIF-independent, although this was not formally proven by PDC-depletion experiments in TRIF$^{-/-}$ mice. The identification of in vivo IFN-α producing cells other than PDC has proven challenging and conventional DC as well as many other hemopoietic and nonhemopoietic cells are able to produce IFN-α in response to virus infection and could all participate in the in vivo production. We have observed that unlike bone marrow-derived PDC in FLT3L that are strictly dependent on MyD88 and TLR9 for in vitro IFN-α production in response to infectious MCMV (42 and data not shown), bone marrow-derived DC in GM-CSF produce IFN-α through a TLR3-, TLR9-, MyD88-, and TRIF-independent pathway (data not shown). Thus, conventional DC can produce IFN-α in response to MCMV infection in a TLR-independent fashion, similar to what was previously shown for lymphocytic choriomeningitis virus and NS1-deficient influenza virus (59). The dsRNA-binding enzyme protein kinase R and the recently described retinoic acid inducible gene 1 (RIG-1) provide precedents for PDC- and TLR-independent IFN-α releases in response to viruses (59, 60). Thus, although PDC are responsible for most in vivo IFN-α production during MCMV infection through a MyD88-dependent mechanism, other cells can produce lower levels of IFN-α with a slightly delayed kinetics most likely using TLR-independent mechanisms.

In conclusion, the initiation of effective antiviral immunity against MCMV would engage coordinated and partially redundant MyD88-dependent and -independent processes. MyD88-mediated signaling offers an early innate induction of cytokine release and is critical for a rapid pathology remission, whereas MyD88-independent events are delayed but sufficient to maintain a relative resistance by offering liver defenses and the establishment of adaptive immunity.

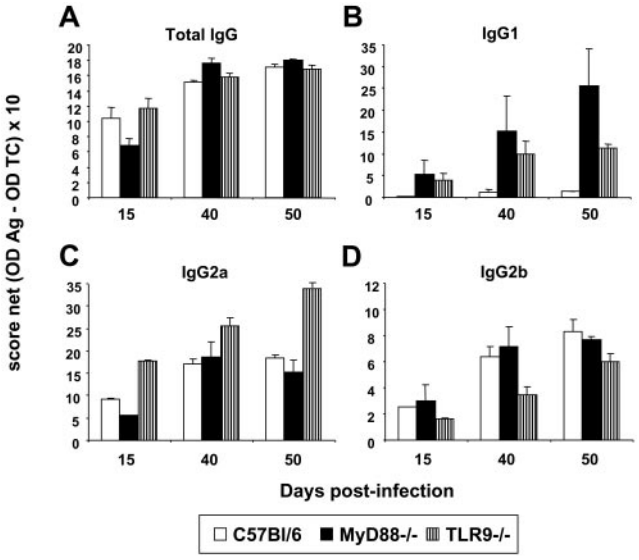

FIGURE 8. MCMV-specific Ab response in MyD88$^{-/-}$ and TLR9$^{-/-}$ mice during MCMV infection. MCMV-specific total IgG (*A*), IgG1 (*B*), IgG2a (*C*), and IgG2b (*D*) were measured by ELISA in serum of MCMV-infected mice at 15, 40, and 50 days p.i. Values are presented as relative score net of OD corresponding to formula: [(OD measured in MCMV Ag-coated well) − (OD measured in tissue control (TC)-coated well) × 10] and were performed on two mice per time point.

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Disclosures

The authors have no financial conflict of interest.

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