

# Critical Roles for Both STAT1-Dependent and STAT1-Independent Pathways in the Control of Primary Dengue Virus Infection in Mice<sup>1</sup>

Sujan Shrestha,<sup>2</sup> Kristin L. Sharar,<sup>2</sup> Daniil M. Prigozhin,<sup>2</sup> Heidi M. Snider, P. Robert Beatty, and Eva Harris<sup>3</sup>

Dengue virus (DEN), a flavivirus, causes dengue fever and dengue hemorrhagic fever/dengue shock syndrome, the most common mosquito-borne viral illnesses in humans worldwide. In this study, using STAT1<sup>-/-</sup> mice bearing two different mutant *stat1* alleles in the 129/Sv/Ev background, we demonstrate that IFN $\alpha$ -dependent control of primary DEN infection involves both STAT1-dependent and STAT1-independent mechanisms. The STAT1 pathway is necessary for clearing the initial viral load, whereas the STAT1-independent pathway controls later viral burden and prevents DEN disease in mice. The STAT1-independent responses in mice with primary DEN infection included the early activation of B and NK cells as well as the up-regulation of MHC class I molecules on macrophages and dendritic cells. Infection of bone marrow-derived dendritic cell cultures with either DEN or Sindbis virus, another positive-strand RNA virus, confirmed the early vs late natures of the STAT1-dependent and STAT1-independent pathways. Collectively, these data begin to define the nature of the STAT1-dependent vs the STAT1-independent pathway in vivo. *The Journal of Immunology*, 2005, 175: 3946–3954.

Dengue virus (DEN)<sup>4</sup> causes dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), the most prevalent mosquito-borne viral diseases in humans worldwide, with an estimated 2.5 billion people at risk for infection (1). DEN is a positive-sense ssRNA virus that belongs to the family *Flaviviridae* and the genus *Flavivirus*, which includes yellow fever and the West Nile, Japanese, and St. Louis encephalitis viruses. Primary infection with any one of the four DEN serotypes typically leads to DF, a debilitating, but self-limited, acute febrile illness. However, some primary infections and a larger percentage of secondary infections with a different serotype result in the severe, life-threatening DHF/DSS, characterized by increased vascular permeability, thrombocytopenia, and hemorrhagic manifestations (1). Despite the global morbidity and mortality, DEN-specific therapies and vaccines are currently unavailable, and DEN pathogenesis is not yet fully understood.

Epidemiologic and in vitro data suggest that DHF/DSS may be an immunopathogenic disease (2–5). However, the precise role of

the immune system in response to DEN infection in vivo is not fully defined, in part due to the lack of an adequate animal model for DEN infection and disease. Therefore, to investigate the immune response to DEN infection in vivo as well as to begin to develop a better animal model for DEN infection and disease, we have optimized a mouse model of primary DEN infection (6). Based on this mouse model, we have recently demonstrated that the combined receptor activities of both IFN- $\alpha\beta$  and IFN- $\gamma$  are essential and are more important than T and B cell-mediated responses in controlling primary DEN infection in vivo (7). Specifically, 129/Sv/Ev (129) mice lacking receptors for both IFN- $\alpha\beta$  and IFN- $\gamma$  (IFN- $\alpha\beta$ R<sup>-/-</sup> × IFN- $\gamma$ R<sup>-/-</sup> 129/Sv/Ev (AG129)) exhibit paralysis and die due to primary DEN infection, whereas T and B cell-deficient RAG1<sup>-/-</sup> C57BL/6J or RAG2<sup>-/-</sup> 129 mice can resolve acute DEN infection. Based on these results, we are now investigating the mechanism of IFN action in mice with primary DEN infection.

A hallmark feature of the IFN system in viral infections is a highly coordinated production of IFN- $\alpha\beta$ , involving several proteins from the IFN regulatory factor (IRF) family (8). In the early phase, constitutively expressed IRF3 induces the production of IFN- $\beta$ , which, in turn, induces IRF7. IRF3 and IRF7 then collectively initiate and amplify IFN- $\alpha\beta$  production. During RNA virus infections, IRF3 may be activated via the TLR-dependent and -independent pathways (9, 10). The TLR-dependent pathway is likely to involve the TLR3-Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  or TLR7/8-MyD88 signaling cascades, whereas the TLR-independent pathway may include the recently identified protein, retinoic acid inducible gene-1 (11). After the production of IFN- $\alpha\beta$ , the transcription of several IFN-stimulated genes (ISGs) is up-regulated through the IFN signaling cascade. However, accumulating evidence indicates that some ISGs may be induced by IRF3 in the absence of IFN production, providing an IFN-independent pathway for activating certain ISGs (12).

In the IFN signaling cascade, the STAT1 pathway is critical for regulating the expression of most ISGs. Mice lacking an intact *STAT1* gene are defective in their ability to mount many classical

Division of Infectious Diseases, School of Public Health, University of California, Berkeley, CA 94720

Received for publication February 1, 2005. Accepted for publication June 30, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by the National Institutes of Health (National Research Scientist Award 1F32AI51070-01 to S.S.) and the Ellison Medical Foundation (Grant 1D-1A-0031 to E.H.).

<sup>2</sup> Current address: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121.

<sup>3</sup> Address correspondence and reprint requests to Dr. Eva Harris, Division of Infectious Diseases, School of Public Health, 140 Warren Hall, University of California, Berkeley, CA 94720-7360. E-mail address: eharris@berkeley.edu

<sup>4</sup> Abbreviations used in this paper: DEN, dengue virus; DC, dendritic cell; DEN1, DEN serotype 1; DEN2, DEN serotype 2; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; Flt3L, Flt3 ligand; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; MCMV, murine CMV; MOI, multiplication of infection; p.i., postinfection; PKR, dsRNA-dependent protein kinase; SV, Sindbis virus; WT, wild type.

responses to IFN- $\alpha\beta$  and IFN- $\gamma$  and are highly susceptible to numerous viral infections (13–16). However, STAT1-deficient 129 (STAT129) mice have been reported to be more resistant than IFN- $\alpha\beta$ R- and IFN- $\gamma$ R-deficient mice to infection with Sindbis virus (SV) or murine CMV (MCMV) (17), indicating that some antiviral responses may be mediated by STAT1-independent mechanisms (18–20). Certain viruses may compromise STAT1 functions (21–23), requiring the STAT1-independent pathway to provide the host with an alternative mechanism for IFN-dependent signaling. Indeed, the DEN nonstructural protein NS4B has recently been demonstrated to inhibit IFN signaling by blocking STAT1 activity *in vitro* (24), suggesting that the IFN system may need to mediate its responses against DEN infection *in vivo* at least partially through the STAT1-independent pathway. Therefore, to determine whether IFNs control DEN infection *in vivo* via the STAT1-dependent or STAT1-independent pathway, we examined the role of STAT1 in resolving primary DEN infection in mice.

Using two different DEN serotypes and STAT1<sup>-/-</sup> 129 mice carrying a deletion mutation in the DNA binding domain of STAT1 (13) or the N-terminal domain of STAT1 (14), we demonstrate in this study that the IFN-mediated responses to primary DEN infection involve both the STAT1-dependent and STAT1-independent pathways. Virologic analyses revealed that the STAT1 pathway controls the initial viral burden, whereas the STAT1-independent response ultimately resolves the infection in mice. Immunologic analyses showed that the STAT1-independent pathway involves some of the major immunoregulatory effects of IFNs, including 1) early activation of B and NK cells and 2) up-regulation of MHC class I molecules on macrophages and dendritic cells (DCs). Infection of bone marrow-derived DC cultures with either DEN or SV, another positive-strand RNA virus, confirmed the role of STAT1 in mediating early viral clearance. Additionally, these studies demonstrated that both DEN and SV can induce IFN- $\alpha$  production in DC cultures via the STAT1-independent pathway, and that STAT1 normally inhibits IFN- $\gamma$  production by these cells. These findings provide new insights on IFN-dependent, STAT1-dependent vs IFN-dependent, STAT1-independent responses during viral infections *in vivo*.

## Materials and Methods

### Mice

Mice were bred and maintained under specific pathogen-free conditions in the animal facility at University of California-Berkeley. 129 Sv/Ev mice lacking receptors for both IFN- $\alpha\beta$  and IFN- $\gamma$  (AG129) and for IFN- $\alpha\beta$  alone (IFN- $\alpha\beta$ R<sup>-/-</sup> 129/Sv/Ev (A129)) were obtained from Dr. S. Virgin (Washington University School of Medicine, St. Louis, MO). Wild-type (WT129) and STAT1<sup>-/-</sup> 129/Sv/Ev mice carrying a deletion of the N-terminal domain of STAT1 (STAT129) were purchased from Taconic Farms. 129/Sv/Ev mice bearing a deletion in the DNA binding domain of the *STAT1* gene were obtained from Dr. J. Durbin (Ohio State University, Columbus, OH). DEN-infected mice were kept in the biosafety level 2 suites of the animal facility, and all procedures involving mice were approved by the animal care and use committee at University of California-Berkeley.

### Viruses

DEN2 PL046 (Taiwanese isolate, *Ae. albopictus* C6/36 mosquito cell-adapted) and DEN1 Mochizuki (mouse brain-adapted) strains were originally obtained from Drs. H.-Y. Lei (National Cheng Kung University, Tainan, Taiwan) and R. Tesh (University of Texas Medical Branch, Galveston, TX), respectively. Viral stocks were amplified in C6/36 cells, titrated by plaque assays using BHK-21 cells, and concentrated via ultracentrifugation as previously described (6). Mice at 5–6 wk of age were infected *i.v.* with 10<sup>7</sup> or 10<sup>8</sup> PFU of DEN2 or 4.4 × 10<sup>4</sup> PFU of DEN1 in 250  $\mu$ l. For infection of bone marrow-derived DCs with SV, a plasmid encoding the full-length Sindbis genome plus GFP (25) was obtained from Dr. I. Frolov (University of Texas Medical Branch, Galveston, TX). The plasmid was purified using the Qiagen Plasmid Midi kit and then linearized with *Xho*I for *in vitro* transcription. RNA was transcribed in the presence

of m<sup>7</sup>G cap analog using RiboMAX Large Scale RNA Production System-SP6 (Promega) according to the manufacturer's instructions. The transcription reaction mixture was electroporated into BHK-21 cells, and virus was harvested 24 h after electroporation, as previously described (25). Virus was titrated by standard plaque assays using BHK-21 cells, with fixing and visualization of plaques on day 2 postinfection (*p.i.*).

### Quantitation of virus in infected mice

Mice were euthanized by isoflurane inhalation just before obtaining whole blood via cardiac puncture. Serum and other tissue samples were harvested, processed, and tested for the presence of infectious virus by both direct and amplified plaque assays, as previously reported (6).

### Flow cytometric analysis of spleen cells from infected mice

After harvest, spleens were disrupted between the frosted ends of two glass slides in complete RPMI 1640 medium, which consists of RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (HyClone), 10 mM HEPES, 200 mM L-glutamine, 10,000 U/ml penicillin and streptomycin, 50 mM 2-ME, 1% nonessential amino acids, and 1% sodium pyruvate. Single-cell suspensions were prepared in FACS buffer (PBS/5% FCS/10 mM HEPES/5 mM EDTA/0.05% NaN<sub>3</sub>), blocked with anti-mouse CD16 (clone 24G2) hybridoma supernatant, and stained with directly conjugated mAbs against mouse cell surface markers as previously reported (6). Combinations of the following mAbs labeled with FITC, PE, or PE-Cy5 were used: anti-CD3, anti-H-2K<sup>b</sup>, anti-I-A/I-E<sup>b</sup>, and anti-DX5 (BD Pharmingen); anti-CD19, anti-CD69, and anti-CD11c (eBioscience); and anti-F4/80 (Caltag Laboratories). For intracellular staining of IFN- $\gamma$ , anti-IFN- $\gamma$ -PE (Caltag Laboratories) and the Fixation and Permeabilization kit (eBioscience) were used, and all procedures were performed according to the manufacturer's instructions. An EPICS XL-MCL (Beckman Coulter) flow cytometer was used to acquire at least 100,000 events/sample. Data were reanalyzed using FlowJo software (TreeStar).

### Bone marrow-derived DCs

To generate CD11c<sup>+</sup> DCs from bone marrow, the femur and tibiae were isolated and processed based on a published protocol (26). Briefly, the marrow was flushed out with complete RPMI 1640 medium injected with a syringe. After lysis of RBC in a buffered ammonium chloride solution, bone marrow cells were washed and then plated at 2 × 10<sup>6</sup> cells/ml in complete RPMI 1640 and 50 ng/ml murine Flt3 ligand (Flt3L; Research Diagnostics). Culture medium was removed and replaced with fresh medium containing Flt3L every 3 days. On day 12, cells were harvested using HBSS/5 mM EDTA solution, washed with complete RPMI 1640, and processed for flow cytometric analysis and infection with DEN2 or SV. For flow cytometry, cells were incubated with anti-CD16 to block FcRs, stained with anti-CD11c-PE-Cy5, anti-B220-PE, and anti-Ly6C-FITC, and analyzed on a Beckman Coulter flow cytometer, as described above. For infection with DEN2 PL046 strain or SV, 10<sup>6</sup> cells/well in a 12-well plate were infected at a multiplication of infection (MOI) of 10 in a total volume of 0.5 ml. After 2-h incubation in a humidified 37°C/5% CO<sub>2</sub> incubator, cells were extensively washed with PBS and then cultured in complete RPMI 1640 for 6 days. Culture supernatants were harvested daily, stored frozen at -80°C, and used later to test for the presence of infectious virus and IFNs. Standard plaque assays and ELISAs were performed to quantify the levels of infectious virus and IFNs, respectively, in the culture supernatant. The mouse IFN- $\gamma$  ELISA Ready-SET-Go (eBioscience) and mouse IFN- $\alpha$  ELISA (PBL Biomedical Laboratories) kits were used as recommended by the manufacturer. The mouse IFN- $\alpha$  ELISA kit detects murine IFN- $\alpha$ A, - $\alpha$ 1, - $\alpha$ 4, - $\alpha$ 5, - $\alpha$ 6, and - $\alpha$ 9. The OD at 450 nm of each sample was measured using an EL808 microplate reader (Bio-Tek Instruments) and KCjunior software (Bio-Tek Instruments). Cytokine levels were expressed as the number of picograms per milliliter. The limits of detection of IFN- $\gamma$  and IFN- $\alpha$  were 15 and 10 pg/ml, respectively.

### Statistical analyses

Kaplan-Meier survival curves were generated using PRISM software, version 3.0cx (GraphPad). The same software was used to assess statistical significance by Student's *t* test and one-way ANOVA with Tukey's multiple comparison test. Comparisons were considered significant at *p* < 0.05.

## Results

### STAT1-deficient 129 mice lacking the N-terminal domain of STAT1 demonstrate resistance to DEN disease

Using the doubly deficient AG129 mice, we have demonstrated that the combined activities of the two IFNRs are essential for

resistance to DEN-induced disease (7). Because both IFNRs signal through STAT1, the present study examined the contribution of STAT1 to controlling primary DEN infection in mice. STAT129 and WT129 mice were infected i.v. with  $4.4 \times 10^4$  PFU of the DEN1 Mochizuki strain or  $10^8$  PFU of the DEN2 PL046 strain, and the development of DEN-induced paralysis was monitored daily until day 30 p.i. After infection with a mouse-adapted strain of DEN1, 28% of STAT129 and 7% of WT129 mice exhibited hind leg paralysis (Fig. 1A). Similarly, 17% of STAT129 and 7% of WT129 mice developed disease after infection with a DEN2 strain that has not been mouse-adapted (Fig. 1B). In contrast, 100% of AG129 mice infected with either DEN1 or DEN2 showed paralysis. As expected, infectious virus was detected in the brain and spinal cord of the paralytic mice by standard plaque assays (data not shown). At  $10^7$  PFU of DEN2, 0% of STAT129 mice, but almost 80% of AG129 animals, succumbed to DEN-induced paralysis (data not shown). These results indicate that STAT1 plays a minimal role in protection against DEN-induced disease in mice. To confirm these findings, STAT1<sup>-/-</sup> mice carrying a different mutant *stat1* allele were tested. As expected, STAT1<sup>-/-</sup> 129 mice lacking a portion of the DNA binding domain of the *STAT1* gene (DBD KO) were resistant to disease after infection with either DEN1 (Fig. 1C) or DEN2 (Fig. 1D). Therefore, we conclude that the IFN system protects against DEN disease in 129 mice via a STAT1-independent pathway.

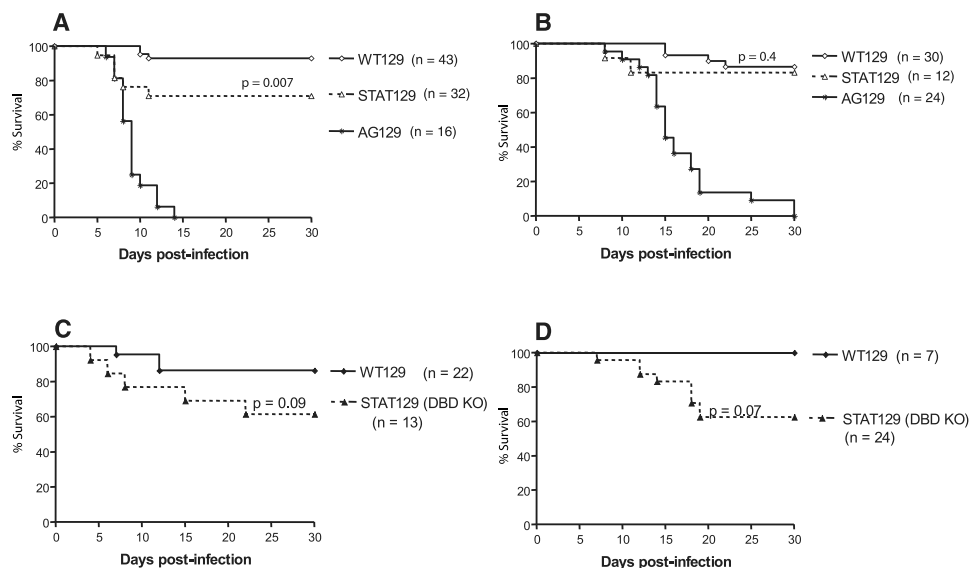
#### STAT1-deficient 129 mice lacking the N-terminal domain of STAT1 display defective early DEN clearance

We have previously shown that 129 mice lacking only the IFN- $\alpha\beta$ R (A129) are unable to control DEN2 titers at early times p.i., but are able to resist DEN-induced disease (7). Therefore, we questioned whether STAT1 would have a similar role in mediating early viral clearance. Amplified plaque assays (7), which represent a sensitive measure for detecting infectious virus, revealed that

STAT129, A129, and AG129, but not WT129, mice harbored infectious DEN2 on day 1 p.i. in several tissues (Table I). By day 3 p.i., STAT129 mice had undetectable amounts of virus in the serum, liver, and spleen, whereas both A129 and AG129 mice still carried infectious virus in these tissues. By day 7 p.i., STAT129 and A129 mice contained undetectable levels of virus in all tissues examined, whereas AG129 mice had virus in the brain and spinal cord. Additionally, in our previous study, G129 mice lacking IFN- $\gamma$ R alone were shown to have no infectious DEN at these early time points p.i. (7). Considered together, these findings demonstrate a role for STAT1 in limiting early viral load, and comparison of the phenotype between STAT129 and A129 mice suggests that the STAT1 activity responds to the IFN- $\alpha\beta$ R engagement. Therefore, the data reported above reveal the contributions of both STAT1-dependent and -independent systems to the control of primary DEN infection in mice.

#### STAT1-independent responses regulate early activation of B and NK cells in 129 mice with primary DEN infection

Consistent with our results, STAT129 mice have been shown to be more resistant than AG129 mice to infection with SV or MCMV (17). However, little is known about the nature of this STAT1-independent signaling in IFN-induced biological responses in vivo. Therefore, we sought to determine which of the major IFN-mediated responses might be regulated by the STAT1-independent mechanism in mice with primary DEN infection. WT A/J, 129, and C57BL/6J mice with primary DEN infection have an increased number of activated B and NK cells in the spleen on day 3 p.i. (6) (S. Shresta and E. Harris, unpublished observations), raising the possibility that IFNs induce certain B and NK cell-mediated responses early during primary DEN infection in mice. In 129 mice, this IFN action may be STAT1 independent, because infectious virus is no longer detectable in the spleen of STAT129 mice on day 3 p.i., as shown in Table I. Therefore, we tested whether the



**FIGURE 1.** Susceptibility of STAT1-deficient 129/Sv/Ev (STAT129) mice to primary DEN infection. *A* and *B*, Inoculation of STAT1-deficient mice lacking the N-terminal domain of *STAT1* with DEN. WT129, STAT129, and AG129 mice were injected via the tail vein with  $4.4 \times 10^4$  PFU of DEN1, Mochizuki strain (*A*) or  $10^8$  PFU of DEN2, PL046 strain (*B*), and observed daily until day 30 p.i. Mice that exhibited paralysis were immediately euthanized, according to the guidelines of the Office of Laboratory Animal Care at University of California-Berkeley. Data from two to four separate experiments were pooled to generate Kaplan-Meier survival curves. The log-rank test yielded  $p < 0.0001$  for STAT129 vs AG129 in both *A* and *B*;  $p$  values for differences between WT129 and STAT129 mice are indicated. *n*, number of mice per group. *C* and *D*, Inoculation of STAT1-deficient mice lacking the DNA binding domain of *STAT1* with DEN. WT129, STAT129, AG129 mice were i.v. inoculated with  $4.4 \times 10^4$  PFU of DEN1, Mochizuki strain (*C*), or  $10^8$  PFU of DEN2, PL046 strain (*D*), and followed for 30 days as described above. Data from two or three independent experiments were combined to plot Kaplan-Meier survival curves. The  $p$  values for differences between WT129 and STAT129 mice are indicated.

Table I. Detection of infectious DEN2 in tissues by amplified plaque assay<sup>a</sup>

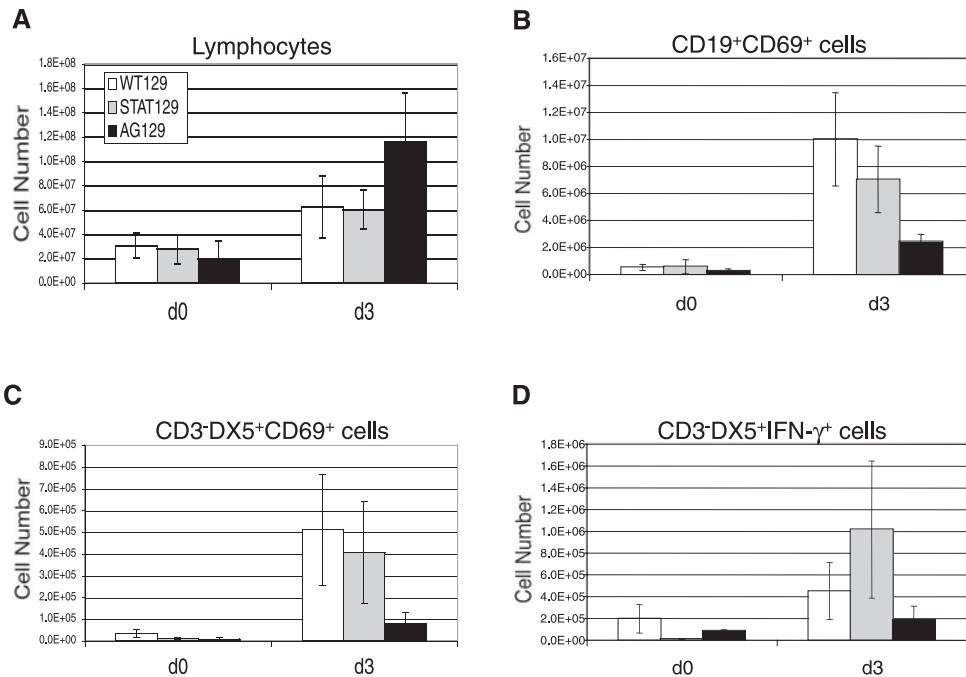
	Serum	Liver	Spleen	Lymph Nodes	Brain	Spinal Cord
Day 1 p.i.						
WT129	-	-	-	-	-	-
A129	+	+	+	+	+/-	+/-
STAT129	+	+	+	+	+/-	+/-
AG129	+	+	+	+	+/-	+/-
Day 3 p.i.						
WT129	-	-	-	-	-	-
A129	+	+	+	+	+/-	+/-
STAT129	-	-	-	+/-	+/-	+/-
AG129	+	+	+	+	+	+
Day 7 p.i.						
WT129	-	-	-	-	-	-
A129	-	-	-	-	-	-
STAT129	-	-	-	-	-	-
AG129	-	-	-	-	+	+

<sup>a</sup> Mice were i.v. inoculated with  $10^8$  PFU of DEN2, PL046 strain. On days 1, 3, and 7 p.i., tissues were harvested and processed for indirect plaque assay. Each group consisted of three to five mice per time point. This experiment was performed three times, and similar results were obtained in all experiments. +, virus was detectable; -, virus was undetectable; +/-, virus was detected in only some mice or some experiments.

early activation of B and NK cells depends on the IFN-mediated, STAT1-independent pathway by performing flow cytometric analysis of spleen cells from WT129, STAT129, and AG129 mice.

The activation status of CD19<sup>+</sup> B cells and CD3<sup>-</sup>DX5<sup>+</sup> NK cells was assessed by their expression of CD69, an early activation marker, and activated NK cells were further phenotyped for intracellular IFN- $\gamma$  expression. As expected, uninfected WT129, STAT129, and AG129 mice had similar numbers of spleen cells

(Fig. 2A), and they contained a low percentage of CD19<sup>+</sup>CD69<sup>+</sup> (activated B), CD3<sup>-</sup>DX5<sup>+</sup>CD69<sup>+</sup> (activated NK), and CD3<sup>-</sup>DX5<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (activated NK) splenocytes (Fig. 2, B–D). Compared with uninfected mice, the number of lymphocytes in spleens from all three types of mice increased after 3 days of infection with DEN2, with approximately twice as many splenocytes in AG129 mice as in WT129 or STAT129 mice. However, both WT129 and STAT129 spleens had higher percentages and numbers of



**FIGURE 2.** DEN-induced activation of B and NK cells in the spleen of 129 mice on day 3 p.i. WT129, STAT129, and AG129 mice were i.v. injected with  $10^8$  PFU of DEN2 (PL046 strain). Spleen cells from uninfected (d0) or DEN2-infected mice on day 3 p.i. (d3) were isolated, counted by hemocytometer, and stained with combinations of fluorescent Abs against mouse cell surface markers CD19, CD3, DX5, and CD69 and intracellular IFN- $\gamma$  for flow cytometric analysis. Lymphocyte gates were set based on forward and side scatter profiles, and the number of positive cells was calculated by multiplying the percentage of fluorescence-positive cells of 100,000 gated events by the total spleen cell number. Data are combined from four independent experiments with three or four mice per group in each experiment. The values shown represent the average number of lymphocytes  $\pm$  SD and were assessed using one-way ANOVA with Tukey's multiple comparison test. A, Total numbers of lymphocytes.  $p < 0.001$  for WT129 vs AG129;  $p < 0.001$  for STAT129 vs AG129 on day 3 p.i. B, Numbers of CD19<sup>+</sup>CD69<sup>+</sup> activated B cells.  $p < 0.001$  for comparisons between WT129 vs AG129 and STAT129 vs AG129;  $p < 0.01$  for WT129 vs STAT129 on day 3 p.i. C, Numbers of CD3<sup>-</sup>DX5<sup>+</sup>CD69<sup>+</sup> activated NK cells.  $p < 0.001$  for comparisons between WT129 vs AG129 and STAT129 vs AG129 on day 3 p.i. D, Numbers of CD3<sup>-</sup>DX5<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NK cells.  $p > 0.05$  for WT129 vs AG129;  $p < 0.001$  for STAT129 vs AG129;  $p < 0.01$  for WT129 vs STAT129 on day 3 p.i.

Table II. Percentages of activated B and NK cells in the spleens of DEN2-infected mice on day 3 p.i.<sup>a</sup>

	CD19 <sup>+</sup> CD69 <sup>+</sup>	CD3 <sup>-</sup> DX5 <sup>+</sup> CD69 <sup>+</sup>	CD3 <sup>-</sup> DX5 <sup>+</sup> IFN- $\gamma$ <sup>+</sup>
WT129	15.4 $\pm$ 6.3	26.5 $\pm$ 10.2	18.5 $\pm$ 3.1
STAT129	9.3 $\pm$ 2.3	11.3 $\pm$ 3.9	25.1 $\pm$ 8.4
AG129	1.7 $\pm$ 0.6 <sup>b,c</sup>	7.4 $\pm$ 2.3 <sup>b,d</sup>	6.1 $\pm$ 1.9 <sup>b,c</sup>

<sup>a</sup> Spleens were harvested on day 1 or 3 p.i., as indicated, from mice that were i.v. infected with 10<sup>8</sup> PFU of DEN2, PL046 strain, and were processed for flow cytometric analysis. Values are shown as the mean percentage  $\pm$  SD. Data were pooled from four independent experiments, with three to four mice per group in each experiment.

<sup>b</sup> AG129 vs WT129.

<sup>c</sup> AG129 vs STAT129.

<sup>d</sup> AG129 vs STAT129.

CD69<sup>+</sup>CD19<sup>+</sup> activated B cells and CD3<sup>-</sup>DX5<sup>+</sup> NK cells expressing either CD69 or IFN- $\gamma$  than spleen cells from AG129 mice (Table II and Fig. 2). This activation of immune cells was not due to LPS contamination, because only live, not UV- or heat-inactivated, DEN preparations induced significant increases in the number of activated B and NK cells (data not shown). These results indicate that the early activation of B and NK cells in 129 mice with primary DEN infection is induced by the IFN-dependent, STAT1-independent response in the absence of STAT1. To confirm the role of the STAT1-independent pathway in mediating NK and B cell responses on day 3 p.i., the activation status of NK and B cells was examined at a time point earlier than day 3. On day 1 p.i., the total numbers of activated NK and B cells in both WT129 and STAT129 mice were lower than the day 3 p.i. values (data not shown), confirming that DEN-infected mice have higher numbers and percentages of activated NK and B cells on day 3 p.i. than on day 1 p.i.

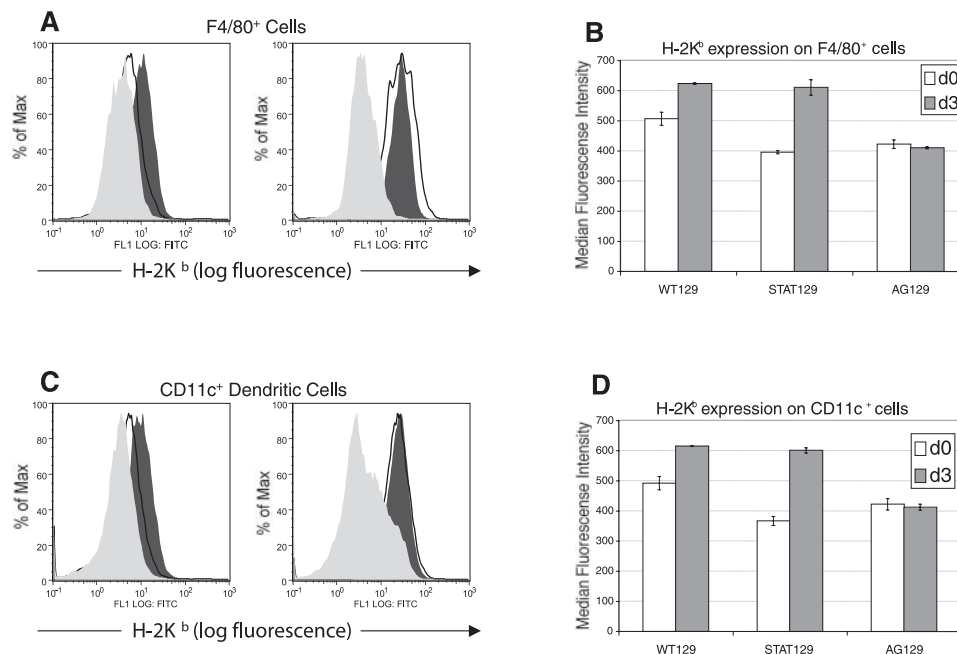
#### STAT1-independent responses control MHC class I up-regulation in 129 mice with primary DEN infection

Besides regulating the activities of B and NK cells, IFNs modulate the function of other immune cells, including professional APCs.

One of the shared functions of IFN- $\alpha\beta$  and IFN- $\gamma$  is the up-regulation of MHC class I molecules on APCs. Therefore, WT129, STAT129, and AG129 mice were infected with DEN2, and spleen cells on day 3 p.i. were analyzed for the expression of MHC class I molecules by flow cytometry. In uninfected mice, F4/80<sup>+</sup> macrophages expressed basal levels of H-2K<sup>b</sup> (Fig. 3A). After 3 days of infection, F4/80<sup>+</sup> cells in spleens of both WT129 and STAT129 mice had higher levels of H-2K<sup>b</sup> expression than uninfected mice, whereas this difference was not observed between uninfected and infected AG129 mice (Fig. 3, A and B). Similarly, CD11c<sup>+</sup> DCs from WT129 and STAT129, but not AG129, mice displayed an up-regulation of H-2K<sup>b</sup> (Fig. 3, C and D). Therefore, in the absence of STAT1, IFNs use a STAT1-independent pathway to increase MHC class I levels on the surface of macrophages and DCs in 129 mice with primary DEN infection.

#### STAT1-dependent and STAT1-independent responses control DEN infection of bone marrow-derived DC cultures from 129 mice

Based on the finding that the IFN system regulates MHC class I levels on DCs from DEN-infected mice, we next focused our studies on DCs as a possible source of IFN production during DEN



**FIGURE 3.** DEN-induced up-regulation of MHC class I expression on macrophages and dendritic cells. Mice were infected with DEN2 as in Fig. 1, and spleen cells from uninfected (d0) or infected (d3) mice were processed for flow cytometric analysis as described in Fig. 2. A and B, Splenocytes bearing the macrophage surface marker F4/80 were examined for the expression for H-2K<sup>b</sup> MHC class I molecules. C and D, CD11c<sup>+</sup> splenocytes were stained for H-2K<sup>b</sup> MHC class I expression. A and C, Representative histograms of individual WT129, STAT129, and AG129 mice. Dark gray, WT129; white with black line, STAT129; light gray, AG129. B and D, Pooled data from four separate experiments with  $n = 12$ –16 mice per group. The median fluorescence intensity values are shown as the mean  $\pm$  SD. White bars, uninfected; dark bars, day 3 p.i.

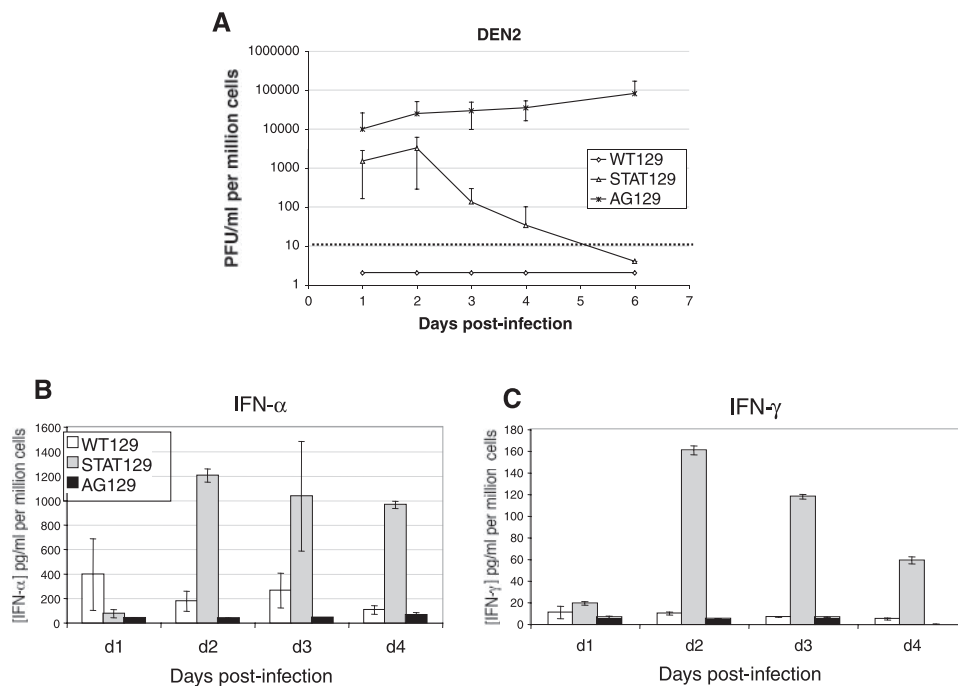
infection. DCs may be the key APCs and initial targets of DEN *in vivo* (27–31), although the exact phenotype of the cell types that support DEN replication *in vivo* is presently unclear. Because small numbers of virus-infected cells in mice at early time points after DEN infection are difficult to quantitate (S. Shresta and E. Harris, unpublished observations), we generated bone marrow-derived DC cultures and examined whether IFNs and STAT1 controlled IFN production as well as viral infection in DEN-infected cultures. Bone marrow cells from WT129, STAT129, and AG129 mice were cultured in the presence of Flt3L for 12 days, and the resulting population of cells was phenotyped by flow cytometric analysis. Approximately 90% of cells from each mouse genotype were CD11c<sup>+</sup> DCs, and the remaining contaminants were Gr1<sup>+</sup> granulocytes and F4/80<sup>+</sup> macrophages, but not B, T, or NK cells, as indicated by the absence of staining for CD19, CD3, and DX5, respectively. Additionally, <2% of the CD11c<sup>+</sup> DC population included plasmacytoid DCs that coexpressed B220 and Ly6C.

To test the susceptibility of these bone marrow-derived DCs to DEN infection, cells were incubated with DEN2 at an MOI of 10 for 2 h, washed to remove unbound virions, and analyzed for infectious virus in the culture supernatant at various times p.i. by standard plaque assay. At all time points after infection, infectious virus was undetectable in cultures from WT129 mice, whereas high levels of infectious DEN were present in AG129 cultures throughout the experimental period (Fig. 4A). In STAT129 cultures, viral titers were almost as high as AG129 levels on days 1 and 2 p.i., but they gradually decreased starting on day 3 p.i. to an undetectable level by day 6 p.i. Microscopically, no cytopathic effects were observed at any time point p.i. in either wild-type or STAT1-deficient cultures, whereas AG129 cultures exhibited increasing levels of cellular cytotoxicity (cell rounding and detach-

ment) starting on day 2 p.i. By day 6 p.i., a vast majority of AG129 cells were dead; in contrast, both WT129 and STAT129 cultures were confluent with live cells. Taken together, these results demonstrate that IFNs are essential for limiting viral growth in bone marrow-derived DC cultures *in vitro* at all time points p.i., and that the STAT1 pathway controls early viral replication, whereas a STAT1-independent pathway reduces later viral titers. These findings support the *in vivo* data shown in Table I, validating the use of this *in vitro* model of DEN infection for additional studies.

To confirm that the STAT1-independent pathway in DEN-infected DC cultures from STAT129 mice is dependent on IFNs, we next examined IFN- $\alpha$  and IFN- $\gamma$  production by these cells. Fig. 4B shows IFN- $\alpha$  levels, as measured by an ELISA that specifically detects murine IFN- $\alpha$ A, - $\alpha$ 1, - $\alpha$ 4, - $\alpha$ 5, - $\alpha$ 6, and - $\alpha$ 9. At all time points p.i., low amounts of IFN- $\alpha$  were detected in WT129 culture supernatants, and the levels of IFN- $\alpha$  in AG129 cultures were even lower than those in WT129 cultures. Similar to AG129 results, on day 1 p.i., the level of IFN- $\alpha$  in STAT129 culture supernatants was lower than that in WT129 cultures; however, on days 2, 3, and 4 p.i., IFN- $\alpha$  levels in STAT129 culture supernatants were dramatically higher than those in WT129 cultures. These results reveal that STAT1-deficient cells produce IFN- $\alpha$  at later times after infection with DEN.

Compared with IFN- $\alpha$  results, IFN- $\gamma$  levels in the same DEN-infected DC cultures were low, as measured by murine IFN- $\gamma$ -specific ELISA (Fig. 4C). Both WT129 and AG129 DC culture supernatants contained IFN- $\gamma$  levels that were low at all time points tested, whereas STAT129 culture supernatants had significantly higher amounts of IFN- $\gamma$  than WT129 and AG129 cultures starting on day 2 p.i. In STAT129 cultures, the level of IFN- $\gamma$  was highest on day 2 p.i. and then progressively decreased at later time



**FIGURE 4.** DEN infection of bone marrow-derived DC cultures. Bone marrow cells from WT129, STAT129, or AG129 mice were cultured in the presence of Flt3L for 12 days to generate DCs. After confirming that at least 90% of cells in these cultures were expressing the DC surface marker CD11c, cells were infected with DEN2 (PL046 strain) at an MOI of 10, and culture supernatants were harvested daily to determine the levels of infectious virus and IFNs. *A*, Infectious DEN virions in culture supernatants. Virus was measured using standard plaque assay in BHK-21 cells. Data are shown as the mean  $\pm$  SD PFU per milliliter per million cells from four independent experiments. The horizontal dotted line represents the limit of detection of the assay. *B*, IFN- $\alpha$  levels in culture supernatants. Levels of IFN- $\alpha$  were measured by an ELISA that specifically detects murine IFN- $\alpha$ A, - $\alpha$ 1, - $\alpha$ 4, - $\alpha$ 5, - $\alpha$ 6, and - $\alpha$ 9. Data are the mean  $\pm$  SD of three different experiments. *C*, IFN- $\gamma$  levels in culture supernatants. IFN- $\gamma$  levels were determined by ELISA. Data represent the mean  $\pm$  SD of three separate experiments.

points. These results show that STAT1-deficient cells also produce IFN- $\gamma$  in response to DEN infection at later time points p.i. Together, the results presented in Fig. 4, B and C, confirm that STAT1-deficient cells use the IFN-dependent, STAT1-independent pathway to control DEN infection at later times p.i. In addition, sera from DEN2-infected STAT129 mice contained IFN- $\alpha$  on day 1 p.i. ( $1615.3 \pm 95.4$  pg/ml) and day 3 p.i. ( $60.0 \pm 121.8$  pg/ml) as well as IFN- $\gamma$  on day 1 p.i. ( $759.5 \pm 215.1$  pg/ml) and day 3 p.i. ( $71.4 \pm 40.8$  pg/ml), and both IFNs were undetectable by day 7 p.i. Therefore, both in vivo and in vitro findings support the conclusion that the STAT1-independent pathway involves both IFN- $\alpha$  and IFN- $\gamma$  production.

#### STAT1-dependent and STAT1-independent responses control SV infection in bone marrow-derived DC cultures from 129 mice

To extend the results described above to another viral infection model, parallel experiments were performed using bone marrow-derived DC cultures infected with SV, a member of the family *Togaviridae* and the genus *Alphavirus*. Similar to DEN, SV is a ssRNA virus, and STAT129 mice are less susceptible than AG129 mice to infection with SV (17). Fig. 5A shows infectious SV titers in WT129, STAT129, and AG129 DC culture supernatants at various time points p.i., as determined by plaque assay. Similar to results obtained from DEN-infected cultures (Fig. 4A), the level of infectious virus was consistently higher in AG129 culture supernatants compared with WT129 and STAT129 cells, confirming an essential role of the IFN system in controlling SV replication. Unlike DEN, SV established productive infection in WT129 DC cultures. In both WT129 and STAT129 culture supernatants, viral titers peaked on day 1 p.i. and progressively decreased at later time points. The levels of infectious SV in STAT129 cultures tended to be higher than those in WT129, but lower than AG129 values, at all time points p.i. By day 6 p.i., a low level of SV was still present in STAT129 cultures, whereas infectious virus was no longer detectable in WT129 cultures, which consisted of normal, viable cells. Similar to findings obtained from DEN infection studies, these results demonstrate that the STAT1-independent pathway controls viral titers at later times p.i.

We next examined IFN- $\alpha$  and IFN- $\gamma$  levels in SV-infected DC culture supernatants by ELISA. Significant amounts of IFN- $\alpha$  were

observed in both WT129 and STAT129 cultures, whereas levels of IFN- $\alpha$  were just above background in AG129 cultures (Fig. 5B). IFN- $\alpha$  levels in both WT129 and STAT129 cultures were similar, and they decreased over time, reflecting the drop in viral titers with time (Fig. 5A). In comparison with IFN- $\alpha$  induction, at all time points p.i., little or no IFN- $\gamma$  was present in the WT129 and AG129 culture supernatants, but significant amounts of IFN- $\gamma$  were detected in STAT129 cultures (Fig. 5C). Fig. 5, B and C, shows that STAT1-deficient cells produce IFN- $\alpha$  and IFN- $\gamma$  in response to SV infection, supporting the conclusion that the STAT1-independent pathway in SV-infected DC cultures is also IFN dependent.

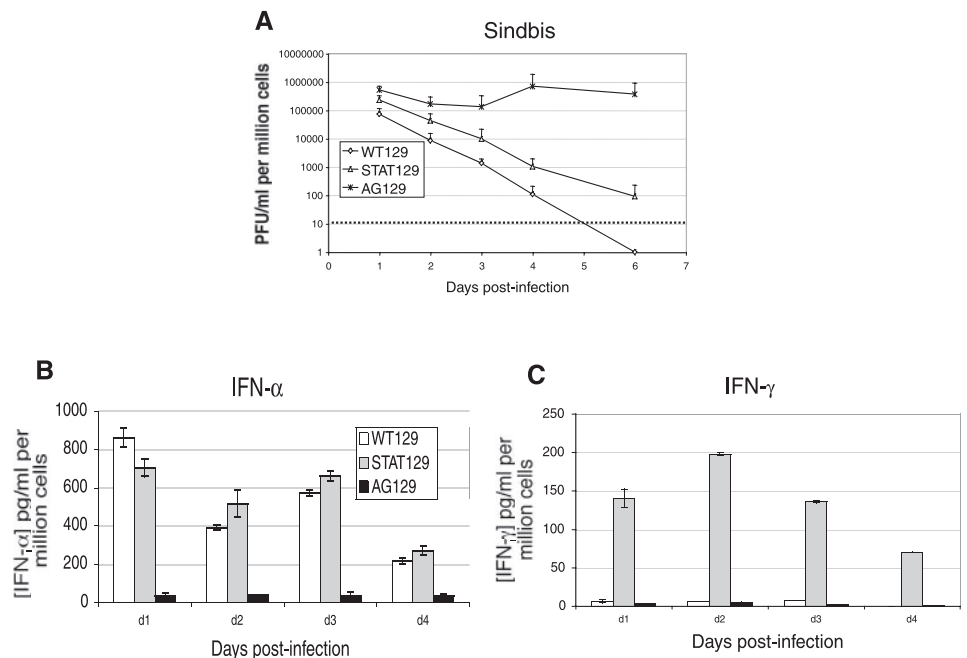
## Discussion

In this study we demonstrated that in 129 mice with primary DEN infection, the IFNRs transduce signals through both STAT1-dependent and STAT1-independent mechanisms. The STAT1 pathway acts early and plays a role in controlling initial viral replication, whereas the STAT1-independent pathway functions later and is responsible for complete viral clearance and prevention of disease. Therefore, using a mouse model of primary DEN infection, this report defines physiologic settings in which the STAT1-independent or STAT1-dependent pathway predominates.

A previous study using MCMV- or SV-infected STAT1<sup>-/-</sup> 129 mice was the first to document the importance of the STAT1-independent pathway in mediating protective responses against viral infections in vivo (17). The present study extends the published observation in several directions. First, we have demonstrated the role of STAT1-dependent and STAT1-independent responses, respectively, as initial and subsequent pathways for resolving a viral infection in vivo. Second, we have identified specific IFN-dependent, STAT1-independent immune responses in vivo. Finally, using DEN- or SV-infected bone marrow cultures in vitro, we have confirmed that the STAT1-independent pathway is IFN dependent.

The early vs late roles of the STAT1-dependent and STAT1-independent pathways in clearing viral infection were defined based on kinetic experiments involving DEN infection in vivo and in vitro as well as SV infection in vitro. The STAT1 pathway limits infectious viral titers on day 1 p.i., whereas the STAT1-independent pathway decreases viral burden by day 3 p.i. On both days 1 and 3 p.i., IFN- $\alpha$ /IFN- $\gamma$  activities control DEN replication, and our

**FIGURE 5.** SV infection of bone marrow-derived DC cultures. Bone marrow-derived DC cultures were established from WT129, STAT129, or AG129 mice as described in Fig. 5. Cells were infected with SV (TE strain) at an MOI of 10, and culture supernatants were collected daily for measuring infectious SV and IFNs. A, Infectious SV in culture supernatants. Virus was measured using standard plaque assay in BHK-21 cells. Data are the mean  $\pm$  SD PFU per milliliter per million cells from three independent experiments. The horizontal dotted line represents the limit of detection of the assay. B, IFN- $\alpha$  levels in culture supernatants. C, IFN- $\gamma$  levels in culture supernatants. Both IFN- $\alpha$  and IFN- $\gamma$  levels were determined by ELISA as described in B and C. Data represent the mean  $\pm$  SD of three separate experiments.



previous study with mice lacking IFN- $\gamma$ R alone revealed that the IFN- $\gamma$ R pathway does not participate in early DEN clearance (7). Based on these findings, the STAT1 pathway involves IFN- $\alpha\beta$ , but not IFN- $\gamma$ , receptor-mediated responses; however, the STAT1-independent pathway mediates both IFN- $\alpha\beta$ R and IFN- $\gamma$ R activities. In support of this conclusion, studies using SV-infected A129 mice have demonstrated that IFN- $\alpha\beta$  receptors alone play a critical role in controlling viral replication during the early stages of infection before a specific Ab response develops (32, 33). Furthermore, studies with SV-infected DC cultures have indicated that the IFN- $\alpha\beta$ R activity uses the dsRNA-dependent protein kinase (PKR) pathway at early time points p.i., whereas IFN- $\alpha\beta$ Rs act independently of PKR at later time points p.i. (34). Moreover, in STAT1<sup>-/-</sup> bone marrow-derived macrophages, the IFN- $\gamma$ -dependent, STAT1-independent signaling does not require PKR (17, 20). Taken together, these observations suggest that in vivo, the early STAT1 pathway may be mediated by PKR, whereas the late STAT1-independent pathway is likely to be PKR independent. A recent study has demonstrated that STAT3 is activated by IFN- $\gamma$  in STAT1-deficient mouse embryo fibroblasts, leading to the transcription of several genes that are normally induced by STAT1 in WT cells (35). Therefore, STAT3 is a plausible candidate for transducing some of the STAT1-independent responses in vivo. Future experiments using 129 mice with primary DEN infection should help determine the roles of both PKR-independent and STAT3-dependent mechanisms in mediating the STAT1-independent pathway in vivo.

Besides identifying the kinetic difference of the STAT1-dependent and STAT1-independent pathways, this study defines specific IFN-dependent, STAT1-independent immune responses in vivo. Specifically, in STAT129 mice with primary DEN infection, the STAT1-independent pathway includes the early activation of splenic B cells. At present, the significance of the early activation of B cells in mice with primary DEN infection is unclear, because B cell-deficient mice resolve primary DEN infection as efficiently as WT mice (7). Similar to DEN, rotavirus, a dsRNA virus of the family *Reoviridae*, also induces a massive increase in the number of activated B cells at early times p.i. (36, 37), and B cell-deficient mice clear primary rotavirus infection (38). However, B cell-deficient mice are not protected against rechallenge with rotavirus, indicating a requirement for B cells in immunity against rotavirus reinfection (38). Similarly, B cells may play a more important role during secondary DEN infection in vivo. According to the Ab-dependent enhancement theory, DEN serotype-cross-reactive Abs at subneutralizing concentrations enhance viral infection of FcR-positive cells, such as macrophages, during sequential DEN infection (39). In 129 mice, the STAT1-dependent and STAT1-independent pathways may shape the memory B cell repertoire during primary DEN infection as well as directly regulate B cell functions during secondary DEN infection.

In addition to the early activation of B cells, another STAT1-independent response in STAT129 mice with primary DEN infection is the early activation of NK cells. Again, the role of NK cells in protection against primary DEN infection is presently unclear, because 129 mice treated with anti-asialo-GM1 Abs, which deplete NK cells in vivo, have a normal ability to clear primary DEN infection (S. Shresta and E. Harris, unpublished observations). However, experiments using better loss-of-function models for murine NK cells may show a role for NK cells in limiting primary and/or secondary DEN infection in mice. In comparison, NK cells are essential for early control of MCMV replication in mice (40–42), suggesting that in STAT1-deficient mice, the STAT1-independent pathway may be clearing MCMV infection by regulating NK cell functions. NK cells use both effector functions, namely,

cell-mediated cytotoxicity and IFN- $\gamma$  production, to limit MCMV infection in mice (43). Similar to results obtained from 129 mice with primary DEN infection, a large number of splenic NK cells in MCMV-infected mice express intracellular IFN- $\gamma$  at early time points p.i. (44). Furthermore, the observation that in 129 mice with primary DEN infection, spleens from STAT1-deficient mice have higher numbers of IFN- $\gamma$ -expressing NK cells than WT spleens agrees with a published finding that the STAT1 response inhibits IFN- $\gamma$  production by NK cells in mice infected with lymphocytic choriomeningitis virus, a negative-stranded RNA virus of the family *Arenaviridae* (45). Therefore, during viral infections in general, the STAT1 pathway may negatively regulate early IFN- $\gamma$  production by NK cells to avoid immunopathology, whereas the STAT1-independent pathway may activate NK cells to control rising viral titers at later times p.i.

Besides NK and B cell activation, the STAT1-independent response induces the up-regulation of MHC class I molecules on professional APCs in mice with primary DEN infection. Several reports have demonstrated that in vitro infection of mammalian cells by a variety of flaviviruses, including DEN, up-regulates cell surface expression of MHC class I molecules (46–48). Although this phenomenon appears to be a by-product of flavivirus assembly, it may allow flaviviruses to evade NK cell attack (49). However, based on the observation that flavivirus-infected cells are highly susceptible to CTL-mediated lysis in vitro, King et al. (48) have hypothesized that flaviviruses, as part of their survival strategy in vivo, may generate massive numbers of low affinity T cells that clear virus poorly. Therefore, the up-regulation of MHC class I molecules on macrophages and DCs may have important implications in vivo, because a major role of MHC molecules is to present viral Ags to specific T cells, and both macrophages and DCs may be crucial targets of DEN infection in vivo.

Bone marrow-derived DC cultures from both STAT129 and AG129, but not WT129, mice are highly susceptible to DEN infection at early times p.i. At later time points p.i., the IFN-dependent, STAT1-independent response leads to a clearing of infectious DEN in DC cultures from STAT129 mice, confirming our in vivo data. This in vitro model of DEN infection using nontransformed, primary cells that are more relevant to human infection should allow us to address the IFN mechanism of action at the cellular level. However, the cellular sources of IFNs in these DEN-infected DC cultures have yet to be identified. Both the conventional DCs as well as the small number of contaminating plasmacytoid DCs and non-DCs may be contributing to the production of IFNs in these bone marrow-derived DC cultures. Future studies using our mouse model of primary DEN infection should uncover the exact mechanisms by which the IFN system mediates antiviral responses.

## Acknowledgments

We thank Dr. Joan Durbin for providing STAT1-deficient mice, Dr. Ilya Frolov for the SV infectious clone, and Dr. Robert Schreiber for his generous gift of reagents and advice. We are grateful to Drs. Sondra Schlesinger and Milt Schlesinger for critical scientific discussions and reading of the manuscript.

## Disclosures

The authors have no financial conflict of interest.

## References

- Burke, D. S., and T. P. Monath. 2001. Flaviviruses. In *Fields Virology*, Vol. 1. D. M. Knipe and P. M. Howley, eds. Lippincott Williams & Wilkins, Philadelphia, pp. 1043–1125.
- Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* 239: 476–481.

3. Rothman, A. L., and F. A. Ennis. 1999. Immunopathogenesis of dengue hemorrhagic fever. *Virology* 257: 1–6.
4. Rothman, A. L. 2003. Immunology and immunopathogenesis of dengue disease. *Adv. Virus Res.* 60: 397–419.
5. Lei, H. Y., T. M. Yeh, H. S. Liu, Y. S. Lin, S. H. Chen, and C. C. Liu. 2001. Immunopathogenesis of dengue virus infection. *J. Biomed. Sci.* 8: 377–388.
6. Shrestha, S., J. L. Kyle, P. Robert Beatty, and E. Harris. 2004. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. *Virology* 319: 262–273.
7. Shrestha, S., J. L. Kyle, H. M. Snider, M. Basavapatna, P. R. Beatty, and E. Harris. 2004. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. *J. Virol.* 78: 2701–2710.
8. Levy, D. E. 2002. Whence interferon: variety in the production of interferon in response to viral infection. *J. Exp. Med.* 195: F15–F18.
9. Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, et al. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17: 251–263.
10. Vaidya, S. A., and G. Cheng. 2003. Toll-like receptors and innate antiviral responses. *Curr. Opin. Immunol.* 15: 402–407.
11. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5: 730–737.
12. Collins, S. E., R. S. Noyce, and K. L. Mossman. 2004. Innate cellular response to virus particle entry requires IRF3 but not virus replication. *J. Virol.* 78: 1706–1717.
13. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84: 443–450.
14. Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, et al. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84: 431–442.
15. Karst, S. M., C. E. Wobus, M. Lay, J. Davidson, and H. W. T. Virgin. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 299: 1575–1578.
16. Hogan, R. J., G. Gao, T. Rowe, P. Bell, D. Flieder, J. Paragas, G. P. Kobinger, N. A. Wivel, R. G. Crystal, J. Boyer, et al. 2004. Resolution of primary severe acute respiratory syndrome-associated coronavirus infection requires Stat1. *J. Virol.* 78: 11416–11421.
17. Gil, M. P., E. Bohn, A. K. O'Guin, C. V. Ramana, B. Levine, G. R. Stark, H. W. Virgin, and R. D. Schreiber. 2001. Biologic consequences of Stat1-independent IFN signaling. *Proc. Natl. Acad. Sci. USA* 98: 6680–6685.
18. Ramana, C. V., M. P. Gil, Y. Han, R. M. Ransohoff, R. D. Schreiber, and G. R. Stark. 2001. Stat1-independent regulation of gene expression in response to IFN- $\gamma$ . *Proc. Natl. Acad. Sci. USA* 98: 6674–6679.
19. Deb, A., S. J. Haque, T. Mogensen, R. H. Silverman, and B. R. Williams. 2001. RNA-dependent protein kinase PKR is required for activation of NF- $\kappa$ B by IFN- $\gamma$  in a STAT1-independent pathway. *J. Immunol.* 166: 6170–6180.
20. Ramana, C. V., M. P. Gil, R. D. Schreiber, and G. R. Stark. 2002. Stat1-dependent and -independent pathways in IFN- $\gamma$ -dependent signaling. *Trends Immunol.* 23: 96–101.
21. Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall. 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J. Virol.* 73: 9928–9933.
22. Garcin, D., J. B. Marq, F. Iseni, S. Martin, and D. Kolakofsky. 2004. A short peptide at the amino terminus of the Sendai virus C protein acts as an independent element that induces STAT1 instability. *J. Virol.* 78: 8799–8811.
23. Shaw, M. L., A. Garcia-Sastre, P. Palese, and C. F. Basler. 2004. Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *J. Virol.* 78: 5633–5641.
24. Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre. 2003. Inhibition of interferon signaling by dengue virus. *Proc. Natl. Acad. Sci. USA* 100: 14333–14338.
25. Frolova, E. I., R. Z. Fayzulin, S. H. Cook, D. E. Griffin, C. M. Rice, and I. Frolov. 2002. Roles of nonstructural protein nsP2 and  $\alpha/\beta$  interferons in determining the outcome of Sindbis virus infection. *J. Virol.* 76: 11254–11264.
26. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X. L. Xu, G. Trinchieri, A. O'Garra, and Y. J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 195: 953–958.
27. Wu, S. J., G. Grouard-Vogel, W. Sun, J. R. Mascola, E. Brachtel, R. Putvatana, M. K. Louder, L. Filgueira, M. A. Marovich, H. K. Wong, et al. 2000. Human skin Langerhans cells are targets of dengue virus infection. *Nat. Med.* 6: 816–820.
28. Ho, L. J., J. J. Wang, M. F. Shaio, C. L. Kao, D. M. Chang, S. W. Han, and J. H. Lai. 2001. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. *J. Immunol.* 166: 1499–1506.
29. Tassaneetrihep, B., T. H. Burgess, A. Granelli-Piperno, C. Trumppfeller, J. Finke, W. Sun, M. A. Eller, K. Pattanapanyasat, S. Sarasombath, D. L. Birx, et al. 2003. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med.* 197: 823–829.
30. Navarro-Sanchez, E., R. Altmeyer, A. Amara, O. Schwartz, F. Fieschi, J. L. Virelizier, F. Arenzana-Seisdedos, and P. Despres. 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Rep.* 4: 723–728.
31. Libraty, D. H., S. Pichyangkul, C. Ajariyakhajorn, T. P. Endy, and F. A. Ennis. 2001. Human dendritic cells are activated by dengue virus infection: enhancement by  $\gamma$  interferon and implications for disease pathogenesis. *J. Virol.* 75: 3501–3508.
32. Ryman, K. D., W. B. Klimstra, K. B. Nguyen, C. A. Biron, and R. E. Johnston. 2000.  $\alpha/\beta$  interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J. Virol.* 74: 3366–3378.
33. Byrnes, A. P., J. E. Durbin, and D. E. Griffin. 2000. Control of Sindbis virus infection by antibody in interferon-deficient mice. *J. Virol.* 74: 3905–3908.
34. Ryman, K. D., L. J. White, R. E. Johnston, and W. B. Klimstra. 2002. Effects of PKR/RNase L-dependent and alternative antiviral pathways on alphavirus replication and pathogenesis. *Viral Immunol.* 15: 53–76.
35. Qing, Y., and G. R. Stark. 2004. Alternative activation of STAT1 and STAT3 in response to interferon- $\gamma$ . *J. Biol. Chem.* 279: 41679–41685.
36. Blutt, S. E., K. L. Warfield, D. E. Lewis, and M. E. Conner. 2002. Early response to rotavirus infection involves massive B cell activation. *J. Immunol.* 168: 5716–5721.
37. Blutt, S. E., S. E. Crawford, K. L. Warfield, D. E. Lewis, M. K. Estes, and M. E. Conner. 2004. The VP7 outer capsid protein of rotavirus induces polyclonal B-cell activation. *J. Virol.* 78: 6974–6981.
38. Franco, M. A., and H. B. Greenberg. 1995. Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to rotavirus infection in mice. *J. Virol.* 69: 7800–7806.
39. Halstead, S. B. 2003. Neutralization and antibody-dependent enhancement of dengue viruses. *Adv. Virus Res.* 60: 421–467.
40. Welsh, R. M., J. O. Brubaker, M. Vargas-Cortes, and C. L. O'Donnell. 1991. Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency: the stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *J. Exp. Med.* 173: 1053–1063.
41. Biron, C. A., and L. Brossay. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr. Opin. Immunol.* 13: 458–464.
42. Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21: 107–119.
43. Tay, C. H., and R. M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J. Virol.* 71: 267–275.
44. Nguyen, K. B., T. P. Salazar-Mather, M. Y. Dalod, J. B. Van Deusen, X. Q. Wei, F. Y. Liew, M. A. Caligiuri, J. E. Durbin, and C. A. Biron. 2002. Coordinated and distinct roles for IFN- $\alpha/\beta$ , IL-12, and IL-15 regulation of NK cell responses to viral infection. *J. Immunol.* 169: 4279–4287.
45. Nguyen, K. B., L. P. Cousens, L. A. Doughty, G. C. Pien, J. E. Durbin, and C. A. Biron. 2000. Interferon  $\alpha/\beta$ -mediated inhibition and promotion of interferon  $\gamma$ : STAT1 resolves a paradox. *Nat. Immunol.* 1: 70–76.
46. Lobigs, M., R. V. Blanden, and A. Mullbacher. 1996. Flavivirus-induced up-regulation of MHC class I antigens; implications for the induction of CD8<sup>+</sup> T-cell-mediated autoimmunity. *Immunol. Rev.* 152: 5–19.
47. Lobigs, M., A. Mullbacher, and M. Regner. 2003. MHC class I up-regulation by flaviviruses: immune interaction with unknown advantage to host or pathogen. *Immunol. Cell Biol.* 81: 217–223.
48. King, N. J., B. Shrestha, and A. M. Kesson. 2003. Immune modulation by flaviviruses. *Adv. Virus Res.* 60: 121–155.
49. Lobigs, M., A. Mullbacher, and E. Lee. 2004. Evidence that a mechanism for efficient flavivirus budding upregulates MHC class I. *Immunol. Cell Biol.* 82: 184–188.