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A New Mouse Model Reveals a Critical Role for Host Innate Immunity in Resistance to Rift Valley Fever

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Rift Valley fever (RVF) is an arthropod-borne viral disease repeatedly reported in many African countries and, more recently, in Saudi Arabia and Yemen. RVF virus (RVFV) primarily infects domesticated ruminants, resulting in miscarriage in pregnant females and death for newborns and young animals. It also has the ability to infect humans, causing a feverish syndrome, meningoencephalitis, or hemorrhagic fever. The various outcomes of RVFV infection in animals and humans argue for the existence of host genetic determinants controlling the disease. We investigated the susceptibility of inbred mouse strains to infection with the virulent RVFV ZH548 strain. Compared with classical BALB/cByJ mice, wild-derived Mus m. musculus MBT/Pas mice exhibited earlier and greater viremia and died sooner, a result in sharp contrast with their resistance to infection with West Nile virus and influenza A. Infection of mouse embryonic fibroblasts (MEFs) from MBT/Pas mice with RVFV also resulted in higher viral production. Microarray and quantitative RT-PCR experiments showed that BALB/cByJ MEFs displayed a significant activation of the type I IFN pathway. In contrast, MBT/Pas MEFs elicited a delayed and partial type I IFN response to RVFV infection. RNA interference-mediated inhibition of genes that were not induced by RVFV in MBT/Pas MEFs increased viral production in BALB/cByJ MEFs, thus demonstrating their functional importance in limiting viral replication. We conclude that the failure of MBT/Pas murine strain to induce, in due course, a complete innate immune response is instrumental in the selective susceptibility to RVF. The Journal of Immunology, 2010, 185: 6146–6156.

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moderate febrile reactions to high fevers, severe prostration, and death in the most susceptible animals (2). Experiments with the rat model confirmed the existence of genetic determinants in RVF (10–13). However, surprisingly, an influence of genetic factors could not be demonstrated in a large survey of 34 classical inbred laboratory mouse strains (11). To further decipher the host–pathogen interaction in mice, we tested the susceptibility of additional inbred strains of mice by including mouse strains derived from mice of various Mus subspecies recently trapped in the wild. This collection of wild-derived inbred strains encompasses genetic variation accumulated over ∼1 million years (14), offering a much larger genetic variation than classical laboratory strains. The latter originated from only a small number of founder animals and shows a remarkably high level of shared ancestry, largely contributed by the M. m. domesticus subspecies (15, 16). The MBT/Pas inbred strain was derived from M. m. musculus animals trapped in Bulgaria in 1980; the mouse colony was later propagated by sib-mating at the Institut Pasteur (17).

In this article, we report that MBT/Pas mice exhibit an extreme susceptibility to experimental infection with the virulent RVFV ZH548 and Kenya 98 strains compared with BALB/cByJ mice, demonstrating for the first time, to the best of our knowledge, natural variation in the severity of susceptibility of the host to RVF among inbred strains of mice. To investigate the underlying mechanism, we compared gene expression profiles of BALB/cByJ and MBT/Pas cells following infection with RVFV. Our data show that MBT/Pas cells exhibit an impaired induction of type I IFN response compared with BALB/cByJ cells, although MBT/Pas cells were capable of producing type I IFNs. Interestingly, this poor response results from the inability of MBT/Pas cells to induce a complete set of IFN-stimulated genes (ISGs) p.i. with RVFV.

Materials and Methods

Ethics statement

All studies on animals followed the guidelines on the ethical use of animals from the European Communities Council Directive of November 24, 1986 (86/609/EEC). All animal experiments were approved and conducted in accordance with the Institut Pasteur Biosafety Committee.

Mice, cells, and virus

BALB/cByJ and C57BL/6J inbred mice were purchased from Charles River Laboratories (L’Arbresle, France). 129/Sv/Pas and MBT/Pas mice were bred in the Institut Pasteur facilities. Mouse embryonic fibroblasts (MEFs) were generated from embryos at day 13.5 of gestation. Cultures were genotyped by PCR for sex determination using the Smcx and Smcy genes to identify cells from male embryos (18). Only MEFs from male embryos were used for further experiments. RVFV strain ZH548 was isolated from a human case in Egypt (19). Strain rec-ZHΔNSs was produced by reverse genetics from the ZH548 genome by deleting the complete NSs gene (6). RVFV strain Kenya 98 (0523) was isolated from a human case in Kenya (20). West Nile virus strain IS-98-ST1 was isolated from a white stork in Israel; it is highly neuroinvasive in adult mice (21). A/Puerto Rico/8/34 (H1N1) PR8 FR 0807 influenza virus is a mouse-adapted strain (22, 23). Viral stocks were produced under biosafety level 3 conditions.

Mice infection and monitoring

Groups of age-matched 9–12-wk-old males were inoculated with 10^2 PFU RVFV, 10^3 PFU West Nile virus i.p., or 2 × 10^3 PFU influenza A virus intranasally. Infected mice were monitored for mortality daily. Experiments were performed in biosafety level 3 isolators. For analysis of viremia, blood samples were collected by retro-orbital puncture from 10 mice at days 1 and 3 p.i. Virus was titrated using plaque assays on Vero cells.

IFN dosage

IFN-α and IFN-β ELISAs were performed on BALB/cByJ and MBT/Pas sera, 24 and 48 h p.i., using the Mouse IFN-α and -β ELISA kits, according to the manufacturer’s instructions (PBL Biomedical Laboratories,
Piscataway, NJ). The kits’ lower limits of quantification were 12.5 and 15.6 pg/ml for IFN-α and IFN-β, respectively.

Cell infection

MEFs from BALB/cByJ and MBT/Pas male embryos were plated at identical densities in culture dishes 24 h prior to infection. To determine the efficacy of virus replication in MEFs, cells were infected using a multiplicity of infection (MOI) of 1, 5, or 10 for the ZH548 strain. Experiments were carried in triplicates. After 1 h, cells were washed in PBS twice and grown in DMEM supplemented with 2% FCS. Supernatants were collected at 15 and 20 h p.i. Titration was performed by plaque assays in Vero cells. For the microarray experiments, MEFs were infected using an MOI of 5. Cell monolayers were harvested 9 h later, and total RNAs were extracted. For the quantitative RT-PCR (qRT-PCR) experiments, MEFs from three BALB/cByJ and MBT/Pas male embryos were plated at identical cell densities. Twenty-four hours later, they were infected using an MOI of 5 with RVFV strain ZH548 or rec-ZHΔNSs or with sterile medium (mock infected). Cell monolayers were harvested 3, 6, and 9 h later, and total RNAs were extracted.

Immunocytology

For the measurement of the infectivity efficiency, BALB/cByJ and MBT/Pas MEFs were infected with ZHS48 RVFV at an MOI of 0.1, 1, and 5, as determined by plaque assays on Vero cells. Nine hours p.i., cells were fixed and labeled with specific Abs against the nucleoprotein N and the non-structural protein NSs of RVFV, followed by fluorescent secondary Abs. Infectivity efficiency was calculated by counting the number of infected cells against the total number of Hoechst-stained nuclei.

RNA extraction

Total RNAs from infected and mock-infected MEF monolayers were extracted using TRIzol reagent (Roche, Basel Switzerland), according to the manufacturer’s instructions. DNA was digested by DNase treatment using a DNA-free kit (Ambion, Austin, TX). RNA quality was assessed by electrophoresis and OD.

Expression microarrays and data analysis

Gene-expression profiling was performed using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA). The 430 2.0...
chip contains >27,000 unique transcripts. Experiments were performed according to the manufacturer’s protocol. Raw data files were background corrected, quantile normalized, and summarized using the robust multi-array averaging method (24) and transformed in log2 values. Principal-component analysis of all regulated genes confirmed that the expression changes were true biological variations and were not caused by variations in experimental conditions. Differentially expressed genes were identified using dChip software (25). A false discovery rate <0.05 using 100 permutations was applied. Genes were further analyzed using the Functions and Disease tool from Ingenuity Pathways Analysis (IngenuitySystems; http://www.ingenuity.com/).

qRT-PCR
Equal amounts of total RNAs from infected and mock-infected MEFs 0, 3, 6, and 9 h p.i. were used in a two-step qRT-PCR. RT-PCR was performed using random primers (p(dN)6, Roche Diagnostic, Mannheim, Germany) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Then, quantitative PCR was done using SYBR Green Master Mix (Applied Biosystems, Warrington, U.K.) and previously described specific primers (Primer bank, http://pga.mgh.harvard.edu/primerbank/). To avoid interference due to polymorphisms between the sequences of the BALB/cByJ and MBT/Pas genomes, the hybridization site of each primer was sequenced in the BALB/cByJ and MBT/Pas genomic DNA. When polymorphisms were identified, the corresponding primers were substituted with a new pair. Supplemental Table I shows the list of the primers used. Data were analyzed by the 2^{-ΔΔCt} method, whereby changes in expression of target genes are calculated relative to an internal control gene (26). Five internal control genes, Gapdh, B2m, Tbp, Rrm2, and Tubb5, were tested in mock- and RVFV-infected MEFs from both backgrounds at each time point p.i. Expression of the Tbp gene was similar in BALB/cByJ and MBT/Pas MEFs and was unaffected by infection with RVFV until 9 h p.i. Hence, Tbp was selected as internal control gene for the quantitative PCR experiments.

RNA interference experiments
The sequences of the three individual stealth RNAi small interfering RNA (siRNA; Invitrogen, Carlsbad, CA) used to target Irf7, Ifi15, Oasl2, and Rig-1 are presented in Supplemental Table II. Briefly, BALB/cByJ MEFs (2 × 10^6 cells) were plated onto 35-mm plates. Transfections were carried out according to the manufacturer’s instructions using a final concentration of 10 nM siRNA and 1.7 μl/ml Lipofectamine RNAiMAX (Invitrogen). Twenty-four hours after siRNA transfection, the MEFs were infected with RVFV strain rec-ZH548 at an MOI of 5 or were treated with 100 IU/ml recombinant mouse IFN-α/IFN-α (PBL Biomedical Laboratories, Piscataway, NJ). The RNAs were extracted 6 h p.i. or 4 h post–IFN-α addition. The levels of mRNA specific for each target gene were monitored by qRT-PCR. Experiments were done in triplicates. The induction of Irf7 gene by siRNA was tested in MEFs transfected with the specific or the scrambled siRNA compared with nontransfected cells. The effect of transient expression of siRNA on viral production was measured on transfected BALB/cByJ MEFs. Twenty-four hours posttransfection, the MEFs were infected with the ZH548 RVFV strain or mock infected. At 20 h p.i., supernatants were harvested from the culture, and virus titers were determined using plaque assay on Vero cells.

Statistical analysis and microarray data
The survival curves were compared using Kaplan–Meier tests (27). For viral burden in mice, the nonparametric Mann–Whitney U test was used. For qRT-PCR data and viral production in cells, Student t tests were performed on log_{10} transformed data. All data were analyzed using StatView software (SAS Institute, Cary, NC). Data are given as mean ± SEM. The complete microarray data have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (28) and are available for download (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18064) through GEO Series accession number GSE18064.

Results
Increased susceptibility of MBT/Pas mice to RVFV infection
To identify genetic factors that may influence susceptibility to RVFV among inbred strains of mice, we used the virulent strain ZH548, a human isolate from the Egyptian outbreak in 1977–1978 (19). Groups of 9–12-wk-old, age-matched male mice of various genetic backgrounds, including classical laboratory inbred strains (BALB/cByJ, C57BL/6J, and 129/Sv/Pas) and the wild-derived MBT/Pas inbred strain were infected i.p. with 10^6 PFU (= 10 LD₅₀) of RVFV ZH548 strain. Mortality was monitored daily for 2 wk. In agreement with an earlier report (11), classical inbred strains showed little phenotypic variation in susceptibility to RVF, with a mean time to death of 7.19 ± 0.21, 6.06 ± 0.37, and 6.06 ± 0.45 d for BALB/cByJ, C57BL/6J, and 129/Sv/Pas strains, respectively. C57BL/6J mice were only slightly more susceptible than BALB/cByJ mice. 

FIGURE 4. Expression profiles of RVFV-responsive genes 9 h p.i. A, Heat maps showing 29 genes related to the IFN innate-immune response that were upregulated i.p. in BALB/cByJ MEFs. B, Expression of the same genes in MBT/Pas MEFs. Green and red squares indicate reduced and increased levels of expression, respectively. Black squares indicate no change in expression level. The color scale at the bottom indicates the magnitude of changes. Values are in log2. Data from three biological replicates are shown for each experimental condition.
and MBT/Pas mice with 10^2 PFU RVFV Kenya 98 strain. Survival curves obtained with ZH548 or Kenya 98 strains were similar, indicating that the susceptible phenotype of MBT/Pas mice is not specific to the ZH548 viral strain. To test whether these mice may exhibit a generalized immunodeficiency, MBT/Pas and Ifnar1^−/− mice were infected i.p. with 10^3 PFU RVFV rec-ZHANSs, which lacks a functional NSs gene and, therefore, is avirulent in immunocompetent mice (6). Rec-ZHANSs–infected MBT/Pas mice remained alive, whereas Ifnar1^−/− mice, which are nonresponsive to type I IFN, died within 2 d p.i. (data not shown). We assessed the ability of MBT/Pas mice to express type I IFN proteins. The levels of IFN-α in sera were significantly higher 48 h after ZH548 infection in MBT/Pas mice (151–883 pg/ml) than in BALB/cByJ mice (<12.5 pg/ml). IFN-β remained below ELISA’s detection levels in sera from ZH548-infected MBT/Pas and BALB/cByJ mice. This may be due to the fact that, at low concentrations, IFN-β rapidly binds to its receptor, and serum concentrations of the free IFN-β decrease rapidly (29). In addition, we tested the susceptibility of MBT/Pas mice to other viruses. BALB/cByJ and MBT/Pas mice were infected i.p. with 10^3 PFU (= 100 LD50) West Nile virus strain IS-98-ST1 (21). West Nile virus–infected BALB/cByJ mice died, whereas MBT/Pas mice survived (Fig. 1C) (30). Similar results were obtained p.i. with the mouse-adapted influenza virus PR8 FR0708. Influenza A–infected MBT/Pas mice had a higher survival than did BALB/cByJ mice (p = 0.015; Fig. 1D). Together, these results suggest that MBT/Pas mice are able to produce and respond to type I IFN and that they carry a selective defect that does not enable this strain to successfully combat an RVFV infection.

We further investigated this difference in susceptibility by in vitro infection of MEFs. BALB/cByJ and MBT/Pas MEFs were infected at an MOI of 1, 5, and 10. Supernatants were analyzed by plaque assay for the production of infectious RVFV at 15 and 20 h p.i. Notably, RVFV titers were significantly higher in MBT/Pas MEF supernatants than in BALB/cByJ MEF supernatants at different MOIs and times p.i. (Fig. 2). To test whether the higher viral production in MBT/Pas MEFs resulted from increased infection rate, BALB/cByJ and MBT/Pas MEFs were infected with RVFV ZH548 at an MOI of 0.1, 1, and 5. The percentage of nucleocapsid (N) protein^+ and NSs^+ (i.e., infected) cells was 1.5, 17.6, and 30.4 for MOIs of 0.1, 1, and 5, respectively, in BALB/cByJ MEFs. The infection efficiency was similar in MBT/Pas MEFs (p > 0.5 for each MOI). Thus, increased cell infectivity is unlikely to explain

Table 1. Upregulation of the innate-immune response genes in RVFV-infected BALB/cByJ and MBT/Pas MEFs, as determined by microarray experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Transcript Accession Number</th>
<th>Fold Change in BALB/cByJ Cells^a</th>
<th>p Value for BALB/cByJ Cells^a</th>
<th>Fold Change in MBT/Pas Cells^a</th>
<th>p Value for MBT/Pas Cells^a</th>
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<tr>
<td>Dai/Zbp1</td>
<td>Z-DNA–binding protein 1</td>
<td>NM_021394</td>
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<td>0.02*</td>
<td>0.01</td>
<td>0.9</td>
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<td>Gvi1</td>
<td>GTase, very large IFN inducible 1</td>
<td>NM_029000</td>
<td>2.72</td>
<td>0.0003***</td>
<td>–0.09</td>
<td>0.3</td>
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<td>Ifi202b</td>
<td>IFN-activated gene 202B</td>
<td>NM_011940</td>
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<td>Ifi203</td>
<td>IFN-activated gene 203</td>
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<td>0.003**</td>
<td>0.94</td>
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<tr>
<td>Ifi35</td>
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<td>0.59</td>
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<td>Ifi44</td>
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<td>Ifi10/Ifx56</td>
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<td>3.95</td>
<td>0.0009**</td>
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<td>Ifi3/Ifx49</td>
<td>IFN-induced protein with tetratricopeptide repeats 1</td>
<td>NM_010501</td>
<td>1.99</td>
<td>0.005**</td>
<td>0.88</td>
<td>0.01*</td>
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<td>Ifisp1</td>
<td>IFN-induced protein with tetratricopeptide repeats 3</td>
<td>AF194871</td>
<td>2.41</td>
<td>0.00002**</td>
<td>0.39</td>
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<tr>
<td>Ifisp2/Ifg2</td>
<td>IFN-induced GTase 2</td>
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<td>0.23</td>
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<td>I6</td>
<td>IL 6</td>
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<td>1.53</td>
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<td>Ip10/Cxcl10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
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<td>0.00002**</td>
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<td>If27I2a</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>NM_015783</td>
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<td>0.001**</td>
<td>1.54</td>
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<td>If20</td>
<td>IFN-stimulated protein</td>
<td>NM_020583</td>
<td>2.11</td>
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<td>0.0002**</td>
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<td>If9/Ifg3g</td>
<td>IFN-dependent positive-acting transcription factor 3γ</td>
<td>NM_008394</td>
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<td>0.0001**</td>
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<td>Lpg2/Dh58a</td>
<td>DEXH (Asp-Glu-X-His) box</td>
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<td>0.0004**</td>
<td>1.02</td>
<td>0.003**</td>
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<td>Mda5/Ifb1</td>
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<td>Oas1a</td>
<td>2′-5′ oligoadenylate synthetase 1A</td>
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<td>0.001**</td>
<td>0.26</td>
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<td>NM_145209</td>
<td>1.65</td>
<td>0.002**</td>
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<td>Oas2l</td>
<td>2′-5′ oligoadenylate synthetase-like 2</td>
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<td>4.63</td>
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<td>Pkr/Eif2ak2</td>
<td>Eukaryotic translation initiation factor 2-α kinase 2</td>
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<td>0.04*</td>
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<td>Rig-I/Dd58</td>
<td>DEAD (Asp-Glu-Ala-Asp) box</td>
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<td>1.38</td>
<td>0.002**</td>
<td>0.35</td>
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<td>Stat1</td>
<td>Signal transducer and activator of transcription 1</td>
<td>NM_009283</td>
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<td>0.005**</td>
<td>0.36</td>
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<td>Stat2</td>
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<td>Trim25</td>
<td>Tripartite motif protein 25</td>
<td>NM_009546</td>
<td>1.06</td>
<td>0.005**</td>
<td>0.71</td>
<td>0.1</td>
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^aThe fold changes in mRNA levels in RVFV-infected cells relative to mock-infected cells are displayed in log2 values.

^bThe microarray probe for Isg15 also hybridizes with the hypothetical Gm9708 (see Results).

*p < 0.05; **p < 0.01; ***p < 0.001.
the higher production of RVFV by MBT/Pas MEFs. Altogether, these data further confirm the importance of the host genotype in controlling RVFV spreading and strongly suggest that one of the mechanisms could be the efficiency of RVFV replication in infected cells.

MBT/Pas MEFs exhibit a weak IFN-dependent response against RVFV ZH548 infection compared with BALB/cByJ MEFs

To examine the global effects of RVFV infection and the associated host response, we compared the gene-expression profiles of RVFV-infected BALB/cByJ and MBT/Pas MEFs using microarrays. An MOI of 5 was used to ensure that a significant percentage of cells would be infected. Total RNA from three culture dishes of mock- or ZH548-infected MEFs from BALB/cByJ and MBT/Pas embryos were extracted at 9 h p.i., a time at which NSs-induced inhibition of transcription is still low (9). Total RNAs were hybridized to Affymetrix Mouse Genome 430 2.0 chips. Data were normalized and transformed into log2 values, and the fold changes between ZH548- and mock-infected MEFs were calculated. A gene was considered regulated by RVFV if its expression in infected cells was ≥2-fold higher or lower than its expression in mock-infected cells of the same genetic background. Quantitative analysis of the data obtained with BALB/cByJ MEFs showed that 229 unique genes (0.82% of cellular transcripts) fulfilled these criteria. Of these, 152 were upregulated, with a maximal 29.2-fold increase; 77 were downregulated, with a maximal 24.1-fold decrease (Fig. 3A, 3B, Supplemental Table III). In MBT/Pas MEFs, 819 genes were regulated by RVFV. Of these, 205 were upregulated, whereas 614 were downregulated (Fig. 3A, 3B). Downregulation of this high number of genes is likely a reflection of the high amount of NSs that is able to inhibit the transcriptional activity of constitutive promoters (31).

To identify the main biological functions that were regulated by RVFV, we used the Ingenuity Pathway Analysis database. In BALB/cByJ MEFs, the upregulated genes were primarily related to “viral functions” and “immune response” categories, whereas the downregulated genes were mostly related to the “cell death” category (Fig. 3C). “Viral functions” describes functions associated with the normal activity of viruses after they have infected the host cell. Examples include activation, replication, and transcription of viruses. “Immune response” includes functions such as Ab response, engulfment, and proliferation of immune cells and tissues, as well as functions specific to an immune response, such as phagocytosis, fever, and Th1 immune response. “Cell death” describes functions associated with cellular death and survival. Examples of functions included in this category are cytolysis, necrosis, survival, and recovery of cells. In MBT/Pas MEFs, the upregulated genes also showed an overrepresentation of the immune response and viral functions (Fig. 3D), suggesting that MBT/Pas cells were able to elicit an innate immune response. However, these categories are less significantly represented in MBT/Pas than in BALB/cByJ response (compare Fig. 3C, 3D). Clear differences between strains became apparent when the differentially regulated genes in BALB/cByJ and MBT/Pas MEFs were compared. Seventy-eight percent of the genes that were downregulated in BALB/cByJ MEFs were also downregulated in MBT/Pas MEFs. In contrast, only 35% of the genes upregulated in

**FIGURE 5.** Genes induced by RVFV infection in BALB/cByJ and MBT/Pas cells 9 h p.i. The IFN-α/β gene induction occurs in two steps. Early signaling events following virus infection (left panel). Viral components are sensed by cytoplasmic pathogen recognition receptors, which trigger cascades that activate NF-κB and IRF3. These proteins enter the nucleus and stimulate the transcription of Ifnb1 and a first set of ISGs, such as Isg15 and Ifit1. The produced IFN-β binds the type I IFNAR and activates the JAK/STAT pathway (late signaling events; right panel). Phosphorylated STAT1 and STAT2 bind IRF9 to form ISGF3. ISGF3 enters the nucleus and stimulates the transcription of ISGs, including Irf7. IRF7, together with IRF3, activates Ifna and Ifnb1 genes, thus creating a positive-feedback loop.
BALB/cByJ MEFs were also upregulated in MBT/Pas MEFs (Fig. 3B). These observations suggest that MBT/Pas MEFs were able to elicit only a restricted range of antiviral responses compared with BALB/cByJ MEFs.

Thus, among the genes that were upregulated in BALB/cByJ MEFs, we considered those that are typically induced after type I IFN stimulation (Fig. 4A). Table I shows a list of 29 of these genes and their fold changes in BALB/cByJ and MBT/Pas MEFs. Many of these genes were not upregulated by RVFV in MBT/Pas cells (also compare Fig. 4A, 4B). Indeed, only seven genes encoding RNA helicases MDA5 (Mda5/Ifih1) and LGP2 (Lgp2/Ddx58), IFN-stimulated exonuclease ISG20 (32), ubiquitin-like modifier protein ISG15 (Isg15), IFN-induced protein with tetra-tricopeptide repeats 1 (Ifi44), and chemokine CXCL10 (Cxc10/IP10) were upregulated in MBT/Pas MEFs. Of note, the genes for RNA helicase RIG-I (Rig-I/Ddx58), dsRNA-dependent protein kinase PKR (Eif2ak2), the three ISGF3 subunits (i.e., IRF9, STAT1, STAT2), IRF7, and three 2’-5’-oligoadenylate synthetases (OAS1a, OASL1, OASL2) (34) were stimulated at significantly lower levels in MBT/Pas MEFs than in BALB/cByJ MEFs. Fig. 5 provides an illustration of the two successive phases of the induction of ISG expression, in which genes upregulated in BALB/c MEFs and/or MBT/Pas MEFs are indicated. A number of genes in the early and late phases were stimulated by RVFV in BALB/cByJ MEFs but not in MBT/Pas MEFs. Altogether, these data suggest that the lack of an appropriate type I IFN response most likely accounts for the higher replication of RVF viral particles in MBT/Pas MEFs.

Infection of BALB/cByJ and MBT/Pas MEFs with virulent RVFV ZH548 stimulates IFN-β and IFN-α4 expression

The representative virulent isolate ZH548 expresses the NSs protein, which was shown previously to block IFN-β production (6, 31). To our surprise, we observed an upregulation of many ISGs in response to ZH548 infection. Therefore, we decided to assess the effect of NSs on type I IFN expression in MEFs. We compared the expression of Ifnb1 and Ifna4 genes p.i. with ZH548 or rec-ZHΔNSs, a strain derived from ZH548 in which the NSs gene is totally deleted (6). BALB/cByJ MEFs were infected with ZH548 and rec-ZHΔNSs strains using an MOI of 5, and the expression of Ifnb1 and Ifna4 genes was examined 0, 3, 6, and 9 h later by qRT-PCR. Because NSs viral protein inhibits RNA polymerase II TFIIH factor beyond ∼8–9 h p.i (9), gene expression was not analyzed at later times. Markedly higher Ifnb1 and Ifna4 mRNA levels (76- and 1,034-fold, respectively) were observed at 6 h p.i. when BALB/cByJ MEFs were infected with rec-ZHΔNSs compared with ZH548 (Fig. 6). Similar increases in Ifnb1 and Ifna4 mRNA levels were obtained when MBT/Pas MEFs were infected with rec-ZHΔNSs compared with ZH548 (data not shown). These experiments confirmed the role of NSs as an inhibitor of Ifnb1 gene transcription. They revealed that Ifna4 expression was also strongly inhibited when the RVFV genome carried NSs. Finally, they showed that the expression of Ifnb1 and Ifna4 genes was still significantly stimulated by the virulent ZH548 strain in MEFs, thus allowing a possible response to IFN-α or IFN-β.

In general, the upregulation of ISGs was weak in MBT/Pas cells compared with BALB/cByJ cells. To test whether this was due to a disparity in IFN production, we studied the induction of Ifnb1 and Ifna4 in MEFs of both genotypes. Ifnb1 and Ifna4 genes showed a congruent and significant difference in transcript levels (Fig. 7).

**FIGURE 6.** Effect of NSs viral protein on the expression of Ifnb1 and Ifna4 genes. Quantification of Ifnb1 (A) and Ifna4 (B) mRNA in BALB/cByJ MEFs infected with the virulent ZH548 or attenuated rec-ZHΔNSs strain of RVFV. Expression levels are displayed relative to the Tbp gene. Statistical analysis was performed using the Student t test on log10-transformed data. **p < 0.01; ***p < 0.001.

**FIGURE 7.** Induction kinetics of Ifnb1 and Ifna4 genes in BALB/cByJ and MBT/Pas fibroblasts infected with RVFV. Quantification of Ifnb1 (A) and Ifna4 (B) mRNA in BALB/cByJ and MBT/Pas MEFs infected with the virulent ZH548 strain of RVFV. Expression levels are displayed relative to the Tbp gene. Statistical analysis was performed using the Student t test on log10-transformed data. *p < 0.05.
At 6 h p.i., Ifnb1 and Ifna4 mRNA levels were similar in BALB/cByJ and MBT/Pas MEFs. At 9 h p.i., the mRNA levels for Ifnb1 and Ifna4 were higher in the MBT/Pas MEFs than in the BALB/cByJ MEFs. Therefore, the weak upregulation of ISGs and higher viral production in MBT/Pas MEFs cannot simply be explained by stronger inhibition of Ifnb1 by NSs nor by the defective induction of type I IFNs.

Incomplete and delayed innate immune response to RVFV ZH548 infection in MBT/Pas MEFs

To further examine the response of MBT/Pas cells to virus infection, we selected seven key genes from the IFN-a/b gene-induction pathways for kinetics analysis: Rig-I, Stat2, Ifit3 (Ifi49), Ifit1 (Isg56), Irf7, Oasl2, and Isg15. RNAs were extracted at 0, 3, 6, and 9 h p.i. from mock- and ZH548-infected BALB/cByJ and MBT/Pas MEFs. Most selected genes showed congruent and significant differences in transcript levels (Fig. 8). Overall, the results obtained by qRT-PCR were largely consistent with the signal intensities of DNA microarray hybridization, except for Isg15, which was highly upregulated by ZH548 in BALB/cByJ MEFs but not in MBT/Pas MEFs, whereas the results obtained by microarrays indicated that Isg15 was upregulated in BALB/cByJ and MBT/Pas MEFs. We found that the microarray probes for Isg15 are not specific for this gene because they also hybridize with the hypothetical Gm9706 gene (Affymetrix database; data not shown). Sequencing of the amplicon confirmed the specificity of Isg15 primers used in qRT-PCR and validated data of the quantitative PCR (data not shown).

Comparison of the levels and kinetics of expression for BALB/cByJ and MBT/Pas MEFs allowed classification of the seven genes into three groups, according to their induction profile. The first profile was observed for Ifit3. This gene was expressed at higher levels in MBT/Pas MEFs than in BALB/cByJ MEFs early in infection (Fig. 8A). The second profile was observed for Ifit1, Stat2, and Rig-I. These genes had delayed kinetics of induction in MBT/Pas MEFs; their expression was higher in BALB/cByJ MEFs early in infection, but they reached similar levels in both cultures at 9 h p.i. (Fig. 8B–D). The last profile consisted of genes that were induced weakly or not at all in MBT/Pas MEFs. The latter profile was observed for the Irf7, Oasl2, and Isg15 genes (Fig. 8E–G).

Thus, the MBT/Pas cells elicited a delayed and only partial type I IFN response to RVFV infection.

Knockdown of Isg15 and Oasl2 leads to increased virus production

The functional importance of genes that were not fully induced in RVFV-infected MBT/Pas MEFs was evaluated further. We used siRNAs to inhibit their expression in BALB/cByJ MEFs and measured the effect of this reduced expression on the viral production.

We determined the ability of different stealth RNAi siRNAs to downregulate Irf7, Isg15, Oasl2, and Rig-I gene expression. RVFV rec-ZHΔNSs strain was shown to be an efficient trigger for the expression of type I IFN (Fig. 6), which, in turn, induces ISG transcription. Therefore, rec-ZHΔNSs was first used to stimulate the expression of type I IFN, which eventually induces Irf7, Isg15, Oasl2, and Rig-I gene expression. Three siRNAs for Irf7, Isg15, Oasl2, and Rig-I were independently transfected in BALB/cByJ MEFs; 24 h later, the transfected MEFs were infected with RVFV rec-ZHΔNSs. At 6 h p.i., total RNAs were extracted, and

![FIGURE 8. Induction kinetics of immune-response genes in BALB/cByJ and MBT/Pas fibroblasts infected with RVFV. Expression of Ifit3 (A), Ifit1 (B), Rig-I (C), Stat2 (D), Irf7 (E), Isg15 (F), and Oasl2 (G) was measured by qRT-PCR. Expression levels are displayed relative to the Tbp gene. Statistical analysis was performed with the Student t test on log_{10} transformed data. *p < 0.05; **p < 0.01; ***p < 0.001.](http://www.jimmunol.org/DownloadedFrom/pg106153fig8.jpg)
**Figure 9.** Knockdown effects of *Irf7*, *Isg15*, *Oasl2*, and *Rig-I* genes on their expression and viral production. qRT-PCR analysis showed the inhibition of *Irf7* (A), *Isg15* (B), *Oasl2* (C), and *Rig-I* (D) gene expression after transfection with specific siRNA (siRNA-1, -2 or -3) or scrambled siRNA (scrambled). mRNA levels are calculated relative to the gene expression in cells transfected with scrambled siRNA. BALB/cByJ MEFs were transfected with the most efficient siRNAs (scrambled). mRNA levels are calculated relative to the gene expression in MEFs that were infected with an RVFV or treated with type I IFN. siRNAs can also trigger the IFN response under certain conditions. Therefore, it was important to exclude this possibility. BALB/cByJ MEFs were transfected with the most efficient siRNAs for *Irf7*, *Isg15*, *Oasl2*, and *Rig-I*. Thirty hours after transfection, total RNAs were extracted, and *Ifnb1* mRNA levels were measured by qRT-PCR. The specific siRNA-treated MEFs did not express higher *Ifnb1* mRNA levels than MEFs treated with scrambled siRNA or control (no siRNA), indicating that the siRNAs did not stimulate *Ifnb1* expression (data not shown).

Finally, to determine whether siRNAs targeting *Irf7*, *Isg15*, *Oasl2*, or *Rig-I* are able to increase RVFV production, specific and scrambled siRNAs were transfected into BALB/cByJ MEFs. Twenty-four hours later, the transfected cells were infected with RVFV strain ZH548 at an MOI of 5. The supernatants were harvested 20 h p.i. and assayed for virus titers. Fig. 9E shows that virus titers were significantly increased in the supernatants of the MEFs transfected with specific siRNAs for *Isg15* and *Oasl2* compared with MEFs receiving scrambled siRNA (p = 0.0007 and p = 0.0262, respectively). Thus, these findings demonstrate the functional importance of both genes in controlling infection.

**Discussion**

RVFV infection causes symptoms of various severities in several mammalian species. In contrast to European breeds, indigenous African sheep, goats, and cattle may show no clinical signs of illness, despite exhibiting a brief period of viremia (2). Such observations suggest that genetic factors are crucial for an effective host defense against RVFV infection. Previous investigations in the mouse did not describe reproducible differences in the susceptibility to RVF among various inbred mouse strains (11). Because this failure may be due to the limited genetic diversity that exists among common mouse laboratory strains, we tested mouse strains that were derived from mice trapped in the wild. The wild-derived MBT/Pas inbred strain belongs to the *Mus m. musculus* subspecies, whereas most laboratory strains carry a genetic make-up that is mainly derived from the subspecies *Mus m. domesticus* (16). The results presented in this article showed that MBT/Pas mice, in contrast to many common laboratory mouse strains, exhibited high susceptibility to RVF, which makes them a useful model for further study of RVFV pathology.

After inoculation with the virulent RVFV ZH548 strain, mice developed similar clinical and histological manifestations in MBT/Pas and BALB/cByJ genetic backgrounds. In MBT/Pas mice, the virus was detected in the liver at day 2 p.i. High levels of liver enzymes were observed (15- and 27-fold increase versus noninfected controls for aspartate aminotransferase and alanine transaminase, respectively), reflecting hepatocytes' injury and death. A few hours before death, MBT/Pas mice were prostrated and adopted a hunched posture. At necropsy, the liver exhibited extensive damage. Histopathologic examination confirmed acute hepatic disease as the fatal lesion. The same features were observed in ZHS48-infected BALB/cByJ mice but with a delay of 4 d.
The rapid death of MBT/Pas mice within 4 d p.i. and the 3000-fold higher production of infectious viral particles in the serum at day 3 p.i. suggested that a less efficient innate intracellular antiviral response contributes to the high susceptibility of MBT/Pas mice to the RVFV ZH548 strain. The higher virus production in MBT/Pas mice compared with BALB/cByJ mice in vivo could be reproduced in cultured MEFs. Therefore, we compared the response to RVFV infection in MBT/Pas and BALB/cByJ MEFs. At a high MOI, ISGs were among the main upregulated genes in BALB/cByJ cells, thus implicating that the IFN-signaling pathway was significantly induced by RVFV. This was unexpected, given that the Ifnb1 gene transcription was reported to be blocked by the RVFV NSs protein (6). Our data show that, although the infection of MEFs with wild-type ZH548 virus induced a 70-fold lower Ifnb1 expression compared with an NSs-null virus, Ifnb1 and Ifna4 genes were still induced by ZH548 infection. Thus, despite the strategies developed by RVFV to escape host-defense mechanisms, the virus was still able to activate the host innate-immune system and induced ISGs. The essential role for type I IFN in RVFV pathogenesis is corroborated by the fact that mice deficient for IFN-α/β receptor subunit 1 (Ifnar1<sup>−/−</sup>) exhibited enhanced viremia and earlier lethality than did wild-type mice p.i. with ZH548 (35) (data not shown). Moreover, treatment with an IFN inducer is efficacious in preventing the disease in mice (36).

Our analyses further revealed that MBT/Pas MEFs elicited a weak and only partial innate-immunity response to viral infection compared with BALB/cByJ MEFs. Ifn7 mRNA was only weakly induced by infection in MBT/Pas cells compared with BALB/cByJ cells. Ifn7 plays a critical role within the IFN pathway. It is required for Ifna4 gene induction, and its absence is associated with increased susceptibility to various pathogens, such as encephalomyocarditis virus and vesicular stomatitis virus (37). However, we could not confirm a functional role for Ifn7 down-regulation in RVFV production because it was not possible to abolish its expression by siRNA transfection. Therefore, we tested the functional role of other genes that were also induced in BALB/cByJ MEFs but not induced in susceptible MBT/Pas MEFs. Isg15 was differentially regulated by RVFV infection in both MEFs. Isg15 encodes an ubiquitin-like protein that modifies >150 proteins through ISGylation (38). Isg15 inhibits the degradation of IRF3, thus establishing a direct positive feedback loop to enhance IFN-β expression (39). Furthermore, the increased susceptibility of Isg15-deficient mice to infection with Sindbis virus, influenza virus, and HSV-1 suggests that Isg15 is critical for the host response to viral infections (40). The antiviral effect of Isg15 seems to be specific, because Isg15-deficient mice exhibited no increase susceptibility to infection with vesicular stomatitis virus or lymphocytic choriomeningitis virus compared with wild-type mice (41). Our data point to a significant role for the ISG15 ubiquitin-like protein in the antiviral host defense against RVFV infection. Similarly, the Oas12 gene was upregulated 24-fold p.i. in BALB/cByJ MEFs, whereas its expression remained low in MBT/Pas MEFs. The OasL2 protein is active as a 2′-5′-oligoadenylate synthetase (42), and the OAS family members represent potent antiviral proteins. Indeed, Oas1b is involved in the innate response of mice to West Nile virus infection (30, 43), and OasJ3 is a genetic determinant of West Nile virus susceptibility in humans (44). OAS3 also exerts antiviral effects against Chikungunya alphavirus (45). The RNA interference-mediated inhibition of Oas12 significantly increased viral replication, suggesting that OASL2 is a potent anti-RVFV effector.

However, one should keep in mind that the weak activation of ISGs contributes to the susceptibility of MBT/Pas mice to RVFV, but it does not render these mice susceptible to all viral infections. MBT/Pas mice survived an infection with 10<sup>2</sup> PFU of an NSs-deficient RVFV, whereas the same viral dose killed mice deficient in type I IFN signaling (35) (data not shown). Accordingly, MBT/Pas MEFs were able to produce type I IFNs after viral infection and respond to them. Moreover, MBT/Pas mice are resistant to infection with West Nile virus and influenza A (30). Interestingly, infection of BALB/cByJ or MBT/Pas MEFs induced a weak stimulation of type I IFN genes compared with the 23- and 41-fold induction of genes for IFN-β and IFN-α4 seen in West Nile virus-infected MEFs (46). This weak stimulation in virus-infected cells was probably due to the synthesis of viral inhibitors of Ifnb1 gene, such as NSs protein. In this study, we focused on the immune response and investigated the role of ISGs differentially activated in the two types of MEFs and we found that at least, some of them contribute to the antiviral defense.

In summary, our study shows that the inability of the MBT/Pas cells to limit virus production is the result of several defects in the early and late phases of the IFN response, although MBT/Pas mice are capable of producing IFNs and responding to them. These defects result in a failure to control the spread of the fast-growing RVFV in cultured cells and are likely to contribute to the early death of RVFV-infected MBT/Pas mice. According to our current model, increased viremia leads to earlier infection and higher viral burden in the liver, as well as to rapid acute hepatitis and death of infected MBT/Pas mice. This hypothesis is supported by seminal experiments by Cedric Mims (47), who showed that histological changes in the mouse liver during RVFV infection correlated with viral load in the blood. Interestingly, the defects seen in MBT/Pas mice affect specifically the response against RVFV, because these mice were resistant to other viral infections. Finally, the MBT/Pas inbred strain seems an interesting model to study the selective susceptibility to RVF. This strain would also be appropriate to test RVFV vaccine candidates.

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