Enabled interferon signaling evasion in an immune-competent transgenic mouse model of parainfluenza virus 5 infection

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Abstract

Parainfluenza virus 5 (PIV5 or SV5) infects several mammalian species but is restricted from efficient replication in mice. In humans, PIV5 evades IFN signaling by targeting STAT1 for proteasomal degradation in a STAT2-dependent reaction. In contrast, cell culture experiments have demonstrated that the divergent murine STAT2 protein fails to support STAT1 targeting. Expression of human STAT2 in mouse cells can overcome the species restriction to enable PIV5-induced STAT1 degradation and subsequent IFN antagonism. Here, we describe a transgenic mouse that ubiquitously expresses human STAT2. PIV5 infection induces STAT1 degradation leading to enhanced virus replication and protein expression in the cells from the transgenic mouse but not from the non-transgenic littermates. Importantly, intranasal inoculation with PIV5 results in increased viral load in the lungs of the transgenic mice compared to wild-type littermates. These transgenic mice provide a small animal model to study the role of innate immune evasion in paramyxovirus pathogenesis.

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Introduction

The co-evolution of viruses with their hosts has resulted in diverse mechanisms to subvert the anti-viral immune system (Levy and Garcia-Sastre, 2001). Understanding how these virulence factors affect viral pathogenesis is a critical first step in identifying targets for pharmaceutical intervention or vaccine development. The paramyxovirus PIV5 (previously known as simian virus 5, SV5) has been isolated from human, canine, porcine and non-human primate populations (Chatziandreou et al., 2004). In canines, PIV5 has been implicated in upper respiratory tract disease (kennel cough) and has been isolated from the cerebral spinal fluid of a dog with posterior paralysis (Appel and Bemis, 1978; Evermann et al., 1981). In humans, PIV5 has been linked to both multiple sclerosis and chronic fatigue and immune dysfunction syndrome (CFIDS), although direct causative associations are poorly understood (Goswami et al., 1984a,b, 1987; Brankin et al., 1989; Cosby et al., 1989; McLean and Thompson, 1989) and (http://www.ncf-net.org/library/PIV5HostChallenge-0606.htm). PIV5 can persistently infect cells and has been isolated from several cultured cell lines (Young et al., 2007). The observation that humans are one of the favored hosts for PIV5 replication is linked to the virus’s efficacy in blocking type I and type II interferon signaling as a result of the specific degradation of the interferon signaling molecule, STAT1 (Didcock et al., 1999a,b).

PIV5-induced STAT1 degradation requires the actions of a single viral protein known as V. The V protein assembles de novo STAT1-targeting ubiquitin ligases from cellular components. This V-dependent degradation complex, known as the VDC, is composed of a number of cellular proteins that directly or indirectly bind to the V protein, including DDB1, the Cullin family member Cul4A, and both STAT1 and STAT2 (Lin et al., 2003). The VDC degrades both STAT1 and STAT2, resulting in enhanced virus replication. To overcome this innate immune restriction, humans express a divergent STAT2 protein that does not support STAT1 targeting. Expression of human STAT2 in mouse cells can overcome the species restriction to enable PIV5-induced STAT1 degradation and subsequent IFN antagonism. Here, we describe a transgenic mouse that ubiquitously expresses human STAT2. PIV5 infection induces STAT1 degradation leading to enhanced virus replication and protein expression in the cells from the transgenic mouse but not from the non-transgenic littermates. Importantly, intranasal inoculation with PIV5 results in increased viral load in the lungs of the transgenic mice compared to wild-type littermates. These transgenic mice provide a small animal model to study the role of innate immune evasion in paramyxovirus pathogenesis.
Available evidence indicates that the combined actions of these proteins produce an E3 ubiquitin ligase capable of targeting STAT1 for proteasome-mediated degradation (Ulane and Horvath, 2002). PIV5 V associates with STAT2, which serves as an interface for recruitment of the target, STAT1. Evidence suggests that the V protein forms a subcomplex with cellular ubiquitin ligase machinery, including DDB1, Cul4A and Roc1. The V protein assembles the two subcomplexes, forcing the interaction of the hijacked ubiquitylation machinery with the STAT1 protein target, enabling the modification of STAT1 to signal its destruction (Ulane et al., 2005; Precious et al., 2005). V proteins also impinge upon IFN biosynthesis mediated by the MDA5 RNA helicase (Andrejeva et al., 2004), but this reaction is not dependent on STAT2.

PIV5-dependent STAT1 degradation in human cells is contingent upon the expression of human STAT2, and the failure to induce STAT1 degradation in infected mouse cells has been attributed to the significant divergence in STAT2 between mice and humans (Park et al., 1999). Indeed, PIV5 V protein cannot inhibit IFN signaling in mouse cells (Didcock et al., 1999a). Apparently mouse STAT2 cannot efficiently substitute for human STAT2 in the VDC. Expression of human STAT2 in mouse cell lines enables the V protein to target STAT1 for proteasome-mediated degradation and block IFN signaling, allowing PIV5 to replicate more efficiently, even in the presence of exogenous IFN (Parisien et al., 2002a).

To validate these observations in an intact animal, and as a means to establish a model system to better understand how the ability to block IFN signaling affects viral pathogenesis in vivo, a transgenic mouse ubiquitously expressing human STAT2 (hSTAT2) was created. These mice are normal by all criteria, but unlike WT mice, infection with PIV5 induces loss of STAT1 and inhibition of IFN signaling, recapitulating the phenotype observed in cultured cells. Furthermore, results indicate that the enabled IFN signaling inhibition is advantageous to virus replication in vivo, as the lungs of PIV5 infected transgenic mice contain more virus than wild-type mice. The increased viral load resulted in a coordinate increase in the expression of inflammatory signaling proteins.
Mice ubiquitously expressing human STAT2

A transgenic mouse expressing human STAT2 broadly in vivo was desired. A plasmid vector was constructed by engineering the human STAT2 ORF downstream of the murine ubiquitin C gene promoter (Fig. 2A). The ability of this human STAT2 vector to enable PIV5 V protein-mediated IFN signaling interference was tested using reporter gene assays in mouse cells. In murine NIH-3T3 cells, PIV5 infection does not inhibit IFN-induced luciferase activity, but expression from the ubiquitin C-human STAT2 construct resulted in PIV5-dependent inhibition of IFN responsive transcriptional activity (Fig. 2B).

This vector was linearized and used for microinjection into fertilized eggs that were implanted into pseudo-pregnant surrogate mothers for generation of transgenic mice.

Primary screening of live born pups was carried out by PCR analysis of total DNA to detect the human STAT2 sequences. Screening was followed by examining STAT2 protein expression by immunoblotting. To test for transgenic protein expression levels, an antibody recognizing the C-terminal region specific to human STAT2 was used. The transgenic mice were found to express human STAT2 in all tissues analyzed, including liver, thymus, kidney, lung and spleen (Fig. 2C), as well as bone marrow and brain (not shown). Two males exhibiting moderate

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Fig. 3. Human STAT2 activity in mouse does not alter IFN signaling. (A) Splenocytes from transgenic and wild-type mice were treated with murine IFNβ for 5–30 min. Cells were immediately lysed and processed for immunoblot with antibodies that recognize human and mouse tyrosine phosphorylated STAT2, human STAT2, mouse STAT2 and STAT1α/β (recognizes human and mouse). (B) Splenocytes from transgenic and wild-type mice were treated with IFNβ for 10 min prior to lysis preparation and anti-STAT1 immunoprecipitation. Precipitated proteins were separated by SDS-PAGE and processed for immunoblot with antibodies for human STAT2, mouse STAT2 and tyrosine phosphorylated STAT2. (C) Splenocytes from hSTAT2 transgenic and wild-type mice were isolated and treated with mIFNβ for 6 or 18 h prior to RNA isolation and reverse transcription. Real-time PCR with primers specific for Mx1 and Ifi47 were performed and normalized to GAPDH. Graphs indicate average values for n = 3, with error bars to represent standard deviation. (D) Transgenic and wild-type MEFs were pretreated 2 h with murine IFNβ, then infected with VSV (1 pfu/cell) for 16 h. Infectious virus released into the supernatant was estimated by titration on CV1 cells. IFN treatment provides a similar level of protection in transgenic and wild-type cells. Graph shows data from an individual VSV titration experiment. (E) Splenocytes were isolated from transgenic and wild-type mice and stimulated with IFNβ for 20’ prior to lysis. Whole cell extracts were separated by SDS-PAGE and immunoblotted with antibodies to detect STAT4 and tyrosine phosphorylated STAT4. IFNβ induces STAT4 activation in both wild-type and transgenic splenocytes.

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to high expression of human STAT2 protein in PBMCs were chosen to generate two independent colonies that behaved similarly in all experiments.

**Human STAT2 is activated by mouse IFN stimulation**

Although it has been shown that murine STAT2 can complement IFN signaling defects in STAT2-deficient human cells (Paulson et al., 1999), the ability of human STAT2 to participate in the murine IFN response complex has not been characterized. To address this question, the activity of human STAT2 in the transgenic mice was characterized. Activation of STAT2 can be evaluated using antiserum specific for the tyrosine-phosphorylated form. This reagent reacts well with both phosphorylated human STAT2 (Y690) and phosphorylated mouse STAT2 (Y689). Splenocytes from transgenic and wild-type mice were treated with IFNβ for 5 to 30 min, and cell extracts were subjected to immunoblot with phosphorylated STAT2 antiserum. Both the endogenous murine STAT2 and the ectopic human STAT2 were found to be activated in response to IFN stimulation (Fig. 3A). To test the downstream consequences of hSTAT2 activation, the ability to form heterodimers with murine STAT1 was tested by Co-IP assay.

The tyrosine phosphorylated hSTAT2 was also found to heterodimerize with STAT1 in IFN-treated splenocytes (Fig. 3B). This result led us to consider the possibility that if it were rate limiting, addition of human STAT2 might influence the quality of IFN responses of the cell. To analyze IFN signaling in the transgenic cells, we performed RT-PCR analysis of IFN-stimulated target genes (ISGs). Treatment of splenocytes with IFNβ resulted in increased mRNA accumulation for all ISGs tested, including Mx1 and Ifi47 (Fig. 3C). No significant differences were found between WT and transgenic IFN responsive gene expression. To test the biological activity of IFN antiviral responses in the mice, transgenic and wild-type MEFs were infected with vesicular stomatitis virus (VSV), a virus known to be sensitive to the IFN-induced antiviral state. The sensitivity of VSV to the IFN response did not differ between the hSTAT2 transgenic and wild-type MEFs (Fig. 3D illustrates representative results). Therefore, we conclude that although human STAT2 can get activated by the murine IFN system and participate in the ISGF3 complex formation, the general biological consequences for IFN signaling and anti-viral responses in the human STAT2 transgenic mice are minimal.

**Activation of STAT4**

One other controversial aspect of IFN responses attributed to sequence diversity between human and mouse STAT2 is the ability of IFN to induce STAT4 tyrosine phosphorylation (Farrar et al., 2000a,b). As differential STAT4 activation might contribute to immune responses in our transgenic mice we tested the hSTAT2 transgenic mice for differences in IFN-induced STAT4 tyrosine phosphorylation. Splenocytes from transgenic and wild-type siblings were treated with IFNβ for 20 min before processing for immunoblot with antisera that recognizes tyrosine phosphorylated STAT4 (Fig. 3E). STAT4 tyrosine 693 phosphorylation was detected in both WT mice and transgenic mice at similar levels. These data support the conclusion that activation of STAT4 by IFN occurs equally well in murine splenocytes regardless of the presence or absence of human STAT2 expression, consistent with the results of (Nguyen et al., 2002). Moreover, this result confirms that the IFN signaling pathway is intact in the transgenic mice and does not differ significantly from WT littermates.

**Human STAT2 transgene supports PIV5-mediated STAT1 degradation**

To characterize the ability of the transgenic mice to support PIV5-mediated STAT1 targeting and inhibit IFN signaling, transgenic and wild-type MEFs were infected with PIV5 for 24 h. While STAT1 levels were unaffected in the wild-type MEFs, STAT1 was greatly reduced in the transgenics (Fig. 4A). To verify STAT1 targeting on a single cell level, indirect immunofluorescence was performed. In the wild-type cells, STAT1 was readily detected at a level similar to adjacent uninfected cells (Fig. 4B). In cells from the transgenic mice, STAT1 was absent from infected cells. Therefore, the basic properties of PIV5-induced STAT1 destruction were recapitulated in the transgenic mouse cells. The ability of PIV5 to alter the IFN response was tested using a reporter gene assay. Transgenic and wild-type

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**Fig. 4.** PIV5 targets STAT1 for degradation and blocks IFN signaling only in transgenic mice. (A) MEFs from wild-type or transgenic mice were infected with PIV5 (50 pfu/cell) for 24 h prior to lysis and immunoblot with STAT1 or P/V antibody. (B) MEFs were infected with PIV5 (1 pfu/cell) and prepared for indirect immunofluorescence microscopy. Viral proteins are detected in infected cells by Texas Red. STAT1 is detected by FITC. (C) Luciferase assay in MEFs carried out as in Fig. 2. IFN signaling is inhibited only in the infected transgenic mice.
MEFs were subjected to an IFNβ-responsive ISRE-luciferase reporter gene prior to PIV5 infection. Twenty-four hours post-infection, cells were treated with IFN for 12 h. The results show that while IFN signaling can occur in both the transgenic and wild-type cells in the absence of PIV5 infection, only the wild-type can retain this activity after PIV5 infection (Fig. 4C).

Fig. 5. Transgenic mouse cells support enhanced PIV5 replication. (A) MEFs were infected with PIV5 for 24 h before additional (1000 U/ml) exogenous IFNβ for another 24 h. Cells were lysed and processed for immunoblot with human STAT2 and P/V antibodies. (B) PIV5 titer from MEFs infected at low MOI (1 pfu/cell) with PIV5 after 24 and 48 h. Viral supernatant was titrated by serial dilution on CV-1 cells. Results show greater viral replication in transgenic MEFs.

Fig. 6. Enhanced replication in vivo. Groups of transgenic mice were intranasally inoculated with PIV5 (4 × 10^5 pfu/mouse). Forty-eight to 72 h post-infection, the mice were euthanized and lungs were processed for infection virus, RNA and histological analysis. (A) Concentration of infectious virus in lung homogenates was determined by plaque assays on CV1 cells. (B) Histological evaluation. At 48 hpi, lungs were fixed and sectioned for H&E staining. Micrographs illustrate typical sections of WT and hSTAT2 mice with and without PIV5 infection. (C) RNA was isolated from lung homogenates 72 hpi and real-time RT-PCR was performed with specific primers for PIV5 NP, IFNβ, Mx1, Ifi47, IP10, IL6, MCP1 and RANTES.

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Human STAT2 provides a replication advantage for PIV5 in primary cells

This acquired ability to block IFN activity should result in a replication advantage for the virus. To test the biological outcome of PIV5-mediated IFN evasion, wild-type and transgenic MEFs were infected with PIV5 at a low MOI (0.1 and 1) for 24 h, then treated with or without IFN for 24 h prior to lysis and immunoblot for viral protein expression. Greater accumulation of viral protein was detected in the infected transgenic cells compared to wild-type (Fig. 5A, compare PIV5 P protein expression, lanes 9 to 4 and lanes 7 to 2). Furthermore, in the wild-type cells, IFN treatment caused a marked reduction in viral protein synthesis (compare PIV5 P protein expression in lanes 2 to 3, or lanes 4 to 5). In contrast, the transgenic cells were less sensitive to IFN stimulation (compare PIV5 P expression in lanes 7 to 8, lanes 9 to 10).

Furthermore, PIV5 gained a replication advantage in the transgenic MEFs, achieving a higher titer within 24 h compared to wild-type (Fig. 5B). The viral load in the transgenic cells remained high at 48 hpi, a time where WT MEFs exhibit a decline in virus load. This confirms that the ability to limit PIV5 replication is impaired in the transgenic mice.

Transgenic mice support greater PIV5 replication in vivo

The analysis of transgenic mouse cells indicates that human STAT2 enables PIV5-mediated STAT1 destruction, IFN signaling evasion and more efficient virus replication. To test the theory that this enabled IFN evasion increases PIV5 replication in vivo, transgenic and wild-type mice were infected intranasally with PIV5 and analyzed 72 h later. While PIV5 was detected in both wild-type and transgenic mouse lungs, the hSTAT2 transgene resulted in an increase in lung viral titers by an average of one hundred fold (Fig. 6A). This result supports the conclusion that PIV5-mediated STAT1 degradation in the lungs of the transgenic mice can dampen the innate antiviral response and allow for unrestricted viral replication.

Despite the greater virus replication at 72 hpi, histological assessment of infected lungs did not reveal substantial differences in pathology at 48 hpi. However, areas of greater inflammatory cell infiltration were observed at greater frequency in infected transgenic mouse lungs (Fig. 6B), suggesting a response to the increased viral load. To measure inflammation in the lungs of these mice, we isolated RNA from the lung homogenates and performed real-time PCR for inflammatory cytokines and interferon stimulated gene expression. Fig. 6C shows that there is higher expression of the cytokines IL6, MCP1 and Rantes, and higher expression of the IFN stimulated genes Mx1 and Ifi47 in the lungs of the infected transgenic mice. IFNβ itself is induced in infected lungs, but the transgenic animals exhibited a lower maximum level at this time point. It is interesting to note that when the virus has the ability to inhibit IFN signaling, the resulting increased viral load stimulates a greater anti-viral response. Taken together, these experiments show that viral IFN evasion of PIV5 results in an immediate increase in viral replication at the site of primary infection followed by an increase in a local inflammatory response.

Discussion

PIV5 is a zoonotic virus found in humans whose pathogenesis is uncertain. In immuno-competent mice, PIV5 appears to be non-pathogenic. However, because its normal IFN evasion strategies are compromised, efficient replication of the virus is restricted from mice. Viral attachment and entry often appears to be a factor for species specificity as well as cell tropism for many viruses including certain paramyxoviruses. However, for PIV5, restriction of mice from the host range is in large part dictated by the ability to block IFN signaling in a species specific fashion. For this virus, restriction from mice is conferred in part by the inability to use the intracellular signal transducer and activator of transcription, STAT2, as a cofactor to degrade murine STAT1.

In the human STAT2-expressing transgenic mice described here, PIV5 is better able to recapitulate the human infection due to the enabled block in IFN signaling, a critical parameter for viral pathogenesis.

In this unique animal infection model, IFN signaling and antiviral responses remain intact, providing the ability to examine virus replication in a more natural context of a fully immune-competent host. This situation differs greatly from strategies used previously to investigate PIV5 immune responses and pathogenesis. In previous reports, immune compromised mice were used to study the immune response to and pathogenesis of PIV5 infections. When SCID mice were used, PIV5 infection resulted in a short-term weight loss and efficient recovery from infection, leading to the conclusion that the adaptive immune system is not vital to the antiviral immune response to PIV5 (Young et al., 1990). Indeed these studies were the first to demonstrate the importance of IFN responses in controlling PIV5 infection. Later studies took advantage of mice harboring a deficiency in the STAT1 gene. In this situation, the host is systemically deficient in responses to both type I and type II IFNs, causing dramatic consequences on innate and adaptive immunity. Infection of STAT1 deficient mice with PIV5 results in 100% mortality (He et al., 2002), dramatically different from the outcome of any natural PIV5 infection reported. These data prove that the virus can replicate efficiently in mice, and that the innate immune response is critical. However, a drawback to these immune-compromised mouse experiments is that it is not possible to determine the natural progression of pathogenesis during infection or evaluate the contributions of host responses to viral pathogenesis. Based on our analysis of SV5-dependent STAT2 degradation in the cultured transgenic mouse cells, during a natural PIV5 infection, we expect that STAT1 would be degraded in the infected cells, leaving the STAT1-dependent immune response of non-infected cells intact.

Although PIV5 does not robustly infect murine cells for reasons that may include differences in receptor binding and membrane fusion as well as immune effects, low level persistent infections can be established in cultured mouse cells. It has been
reported that prolonged serial passage of PIV5 in mouse cells can result in the selection of mouse adapted variants (Young et al., 1997). One isolate, termed mci-1, was found to be highly fusogenic and had acquired the ability to spread more rapidly than wild-type virus via cell to cell fusion. The second isolate, mci-2, contained a mutation in the P/V gene that allowed for more efficient IFN evasion in the mouse cells (Young et al., 2001). When a recombinant PIV5 was constructed harboring the P/V gene mutation (N100D), greater virus replication in murine cells was observed. It has been proposed that the N100D mutation may enable greater interaction with murine STAT2 to facilitate destruction of STAT1, but the ability of this mouse adapted virus to replicate in animals has not been reported. This result reinforces the importance of STAT2 in STAT1 destruction, IFN evasion and virus replication.

It is quite interesting to note that in infected transgenic mice, where IFN evasion has led to an early increase in viral load, there is a parallel increase in inflammatory response. Based on these findings, it is tempting to speculate that PIV5-mediated IFN evasion offers only a short-lived advantage to the virus, and that clearance by the host immune system occurs efficiently with or without IFN evasion. In other words, the consequence of IFN evasion for PIV5 is an increase in the viral load at the primary site of infection, providing a short-term increase in viral load that also may heighten the chance of horizontal transmission. Further experimentation is required to test this concept.

PIV5 has long served as a prototypic member of the larger Paramyxovirus family. This family of viruses includes re-emerging viruses like measles and mumps, as well as newly emerging deadly viruses Hendra and Nipah virus. All of these pathogenic paramyxoviruses express V proteins which inhibit IFN responses (Horvath, 2004). In all cases, the consequence of IFN evasion has only been examined in vitro. The human STAT2 transgenic model described here provides an excellent experimental system to probe the consequences of innate immune evasion during infection of an intact host organism. The data reported here lay the foundation to study the role that IFN evasion has on adaptive immune responses, viral pathogenesis, disease progression, virus clearance and virus transmission.

Materials and methods

Cell lines, transfections and viruses

PIV5 (strain W3A, derived from a genetically defined recombinant virus system; He et al., 1997; Keller et al., 2001) was propagated and titered in CV-1 cells (African green monkey kidney cells). NIH 3T3 cells (gift from Stuart Aaronson, Mount Sinai School of Medicine, New York), 293T cells and 2fTG and U6A cells were maintained in DMEM supplemented with 10% cosmic calf serum (Hyclone). 2fTG and U6A cells were transfected by Superfect (Qiagen), 293T were transfected by the calcium phosphate method and 3T3 cells were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Generation of transgenic mice

The construct containing the 5′ flanking region (−1225 to −6) of the human ubiquitin C gene was a kind gift of Dr. Peter Angel (Heidelberg, Germany). The full-length human STAT2 cDNA was subcloned into the multiple cloning site upstream of an 850-bp SV40 splice site and poly A region (Fig. 1A). The construct was tested for protein expression and for PIV5-dependent STAT1 degradation in U6A cells (STAT2−/−) before microinjection of the purified DNA into both B6C3 hybrids and pure Balb/c backgrounds by the Mount Sinai mouse genetics facility (Mount Sinai Medical Center, New York, NY). Two different transgenic lines were constructed, one with B6C3 background and the other with Balb/c background. The in vivo experiments were carried out on the Balb/c background, while in vitro experiments were carried out in B6C3. In both backgrounds, the human STAT2 transgene enabled efficient PIV5 V-mediated IFN signaling evasion. Of the 65 pups born in B6C3 hybrids, 23 had integration of the transgene as assayed by tail tip genomic PCR. Of the 16 pups born in the Balb/c background, 8 had integration. Most had moderate to high human STAT2 protein expression in peripheral blood cells. Independent lines were generated by mating a male founder with wild-type C57BL/6 or Balb/c females (Charles River Laboratories). Experiments were carried out with first generation transgenic and wild-type sibling littersmates.

Generation of MEFs

Male transgenics were mated to C57BL/6 or Balb/c females, depending on the background of the transgenics. MEFs were generated by harvesting day 14 embryos from matings between male transgenics and wild-type females by removing the head and liver and running them through a 5-cc syringe fitted with an 18-gauge needle. The resulting tissue was pipetted up and down in 5 ml Trypsin/EDTA solution (Gibco), and incubated for 15 min at 37 °C. Tissue was pipetted again, then 15 ml DMEM with 10% Cosmic calf serum (Hyclone) was added and the cells were cultured undisturbed for 48 h. Media were changed and the cultures of primary fibroblasts were allowed to expand. Each embryo was cultured separately and genotyped to confirm expression of the transgene.

Primary cell cultures

For splenocytes, spleens were teased into single cell suspensions and depleted of RBCs with 0.75% NaHPO4. Cells were cultured in DMEM supplemented with 10% CCS, 1% penicillin–1% streptomycin (Gibco), L-glutamine (Gibco), non-essential amino acids (Gibco) and 2-mercapto-ethanol (5×10⁻⁵ M). For IFN activation, mouse IFNβ (PBL, Piscataway, NJ) was used at a concentration of 10⁴ U/ml.

Protein immunoblot assays

Phosphospecific antibodies against STAT2 and STAT4 as well as antibodies against murine STAT4 and the unique C-term
region of human STAT2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used according to the manufacturer’s instructions. DDB1 antibody was purchased from Pharmingen (San Diego, CA). Anti-PIV5 P/V protein antibody was purchased from Serotec (Raleigh, NC). Polyclonal antisera against murine STAT2 was a kind gift of Dr. Christian Schindler (Columbia University). Total protein extracts were prepared in whole cell extract buffer (50 mM Tris pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM DTT) supplemented with a protease inhibitor cocktail (Complete, Roche), 1 mM DTT and 10 mM Na3VO4. Proteins were separated by SDS–PAGE and transferred to nitrocellulose filters, immunoblotted by standard procedures, and prepared for chemiluminescent detection according to manufacturer’s protocol (NEN Renaissance).

For STAT1 immunoprecipitations, cell extracts were incubated with 1 μg antibody overnight followed by 30 μl protein A-agarose beads (Roche) in 50% slurry for 1 h and washed five times with 1 ml of whole-cell extract buffer. Samples were boiled in 30 μl of SDS gel loading buffer, and 15 μl was loaded directly onto an SDS polyacrylamide gel for analysis. For FLAG immunoprecipitations, lysates were precleared and incubated with (anti-FLAG) M2 beads (Sigma) for 4 h, washed 5 times followed by addition of SDS gel loading buffer.

Indirect immunofluorescence

For indirect immunofluorescence experiments, MEFs from transgenic and non-transgenic embryos were grown to 60 to 80% confluence on Permanox chamber slides (Nalgene Nunc) and infected with PIV5 at an MOI of 1. At 24 h post-infection, cells were fixed in 1% formaldehyde in PBS for 15 min and permeabilized in an ice-cold methanol–acetone solution (1:1) for 10 min at −20°C. After five washes with PBS, samples were blocked with 1% bovine serum albumin in PBS for 15 min at 37°C. After every subsequent antibody exposure, samples were washed and blocked. Antibody staining was performed sequentially, with the PK antibody at a 1:100 dilution first, followed by an Texas red-conjugated mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, Pa) to visualize PIV5 P/V protein. The second stain for STAT1 was detected with fluorescein isothiocyanate-conjugated rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific immunofluorescence were precleared on fixed and permeabilized rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). STAT1 polyclonal antisera used for immunofluorescence were precleared on fixed and permeabilized STAT1-deficient U3A cells to reduce non-specific imm

RNA analysis of splenocytes

Total RNA was prepared by using Trizol reagent (GIBCO BRL), digested with DNase 1 (Promega) and subjected to reverse transcriptase with SuperScript III Reverse Transcriptase (Invitrogen) as per manufacturer’s recommendations. Mock reactions were carried out with no reverse transcriptase added to confirm the absence of genomic DNA. Real-time PCR was performed on the MX3000 (Stratagene) by a three step protocol with a denaturation temp of 94 °C, an annealing temp of 61 °C and a 72 °C extension. The primer sets were as follows: Mx1-S 5′-gactacaactgagatgcc-3′, Mx1-AS 5′-ctetattctcecccaatg-3′, Ifi47-S 5′-tgctgcaagaaacagt-3′, Ifi47-AS 5′-aagttccccctgatgctg-3′, GAPDH-S 5′ ggcatgacagtgctag-3′, GAPDH-AS 5′-caacactagaggaac-3′. Mx1 and Ifi47 products were normalized against GAPDH. Untreated RNA was used as the calibrator.

Antiviral assays

Antiviral assays titering PIV5 and vesicular stomatitis virus (VSV) were performed as follows: Cells were washed with serum free media (SFM), infected with virus at the indicated multiplicity of infection (MOI) for 2 h, washed with SFM, and cultured in DMEM with 2% CCS. Supernatants were then titered in plaque assays using simian CV-1 cells with an overlay containing 0.5% agar with DMEM, 10 mM HEPES (pH 7.2), and 1% pen/strep (Gibco). After plaque formation, the monolayer was fixed in 3.7% formaldehyde and stained with 0.1% crystal violet (Sigma) dissolved in 20% ETOH.

Reporter gene assays

MEFs were plated for 80–90% confluency the following day. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 5× ISRE-luciferase and Renilla (Promega, Madison, WI) constructs. Cells were cultured for 24 h before the addition of IFNβ (10 μg/ml) for 12 h. In experiments where PIV5 infection is used, transfected cells were cultured under infection before infection with PIV5 (10 pfu/cell) in serum free media. Twenty-four hours post-infection, all cells were washed and cultured with DMEM with 2% Cosmic calf serum, +/−IFNβ.

Infection of mice

While anesthetized with an intraperitoneal administration of 2,2,2-Tribromoethanol (Avertin), mice were infected with an intranasal inoculation of 4 × 10^5 plaque forming units of PIV5 in 10 μl DMEM. Control mice were subjected to anesthesia and intranasal administration of sterile PBS. At 48–72 h post-infection, mice were euthanized and their lungs were removed for analysis.

Analysis of infected mouse lung

Whole lung was removed, rinsed in PBS, blotted dry and transferred to a 14-ml round bottom tube (Fisher) with 1 ml ice-cold homogenate buffer (DMEM supplemented with 0.75% BSA, 2 mM t-glutamine, 1% pen/strep, 10 mM HEPES, pH 7.2). Tissue was homogenized using a Powermax 250 (VWR) for two 10-s bursts, followed by incubations on ice. Tissue homogenate was then used for virus titering on CV-1 cells or for RNA analysis. For RNA analysis for homogenates, 100 μl of

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homogenated tissue was added to 1 ml Trizol. RNA isolation and cDNA synthesis was performed as above. Real-time PCR was performed using specific primers listed below:

PIV5NP S 5′-tatcgctcgacggaacccatg-3′, AS 5′-ctcgagatgccgacctg-3′

IFNβ S 5′-acctacagggggaacctcaag-3′, AS 5′-gatggcaaggaactgtaacctt-3′

IP10 S 5′-ctctgctctgctctagca-3′, AS 5′-ataaaccctggttaggg-3′

IL6 S 5′-agtgctctctgtgaga-3′, AS 5′-cagaagttgccgactcaac-3′

MCP1 S 5′-cccacacgtagctgctgaga-3′, AS 5′-ctctgacccacctccctttg-3′

RANTES S 5′-gccccacgtcgaagatttgtctca-3′, AS 5′-acaacactggcagttcctc-3′

Histology of lungs

Mice were infected as above. At 48 h post-infection, mice were anesthetized and lungs were perfused with formalin before removal for fixation. 10 mm longitudinal cross-sections were prepared for hematoxylin and eosin (H&E) staining.

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References


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