



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.,

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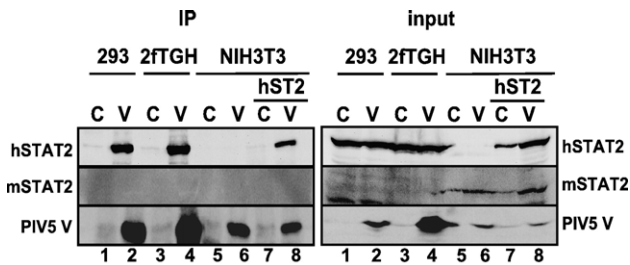


Fig. 1. PIV5 V protein binds human but not mouse STAT2. Mouse NIH3T3 cells or human 2fTGH and HEK293T cells were transfected with either FLAG-tagged PIV5 V protein (V) or FLAG vector control (C). Parallel samples of NIH3T3 were co-transfected with human STAT2 expression vector (hST2). Whole cell extracts were immunoprecipitated with FLAG-M2 agarose beads, eluted with SDS-PAGE buffer and processed for immunoblot with specific antibodies indicated.

1998; Ulane and Horvath, 2002; Andrejeva et al., 2002). 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 ubiquitinylation 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.

Results

PIV5 V preferentially binds to STAT2 from human and not mouse

It has been previously shown that PIV5 V protein can target STAT1 for degradation in human cell lines, and that this degradation is dependent on the expression of STAT2 (Parisien et al., 2002a). In mouse cell lines, expression of human STAT2 is required for PIV5 to target the degradation of STAT1 (Parisien et al., 2002b). To better understand the mechanisms underlying the species specificity, we performed co-immunoprecipitations to examine V:STAT2 interactions. The mouse cell line NIH-3T3 was transfected with FLAG-tagged PIV5 V protein with and without co-expression of human STAT2. Immunoprecipitations show that human STAT2 can readily associate with the PIV5 V protein while mouse STAT2 cannot (Fig. 1). These results verified the earlier assumption that PIV5 V protein fails to recognize murine STAT2 and suggested it may be possible to use a transgene approach to engineer PIV5 susceptible mice.

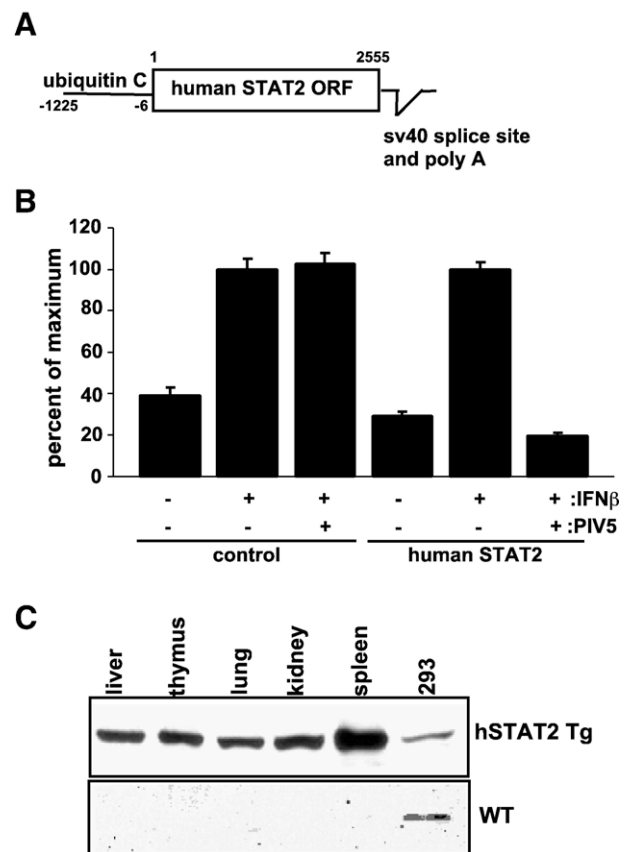


Fig. 2. Expression of human STAT2 in transgenic mice. (A) The 2555-bp open reading frame encoding human STAT2 was flanked by the human ubiquitin C promoter on the 5' end and an SV40 splice site and poly A tail on the 3' end. (B) NIH3T3 cells were transfected with the 5xISRE-luciferase plasmid with or without the hSTAT2 transgene construct, infected with PIV5 for 24 h prior to luciferase assay. Normalized to co-transfected Renilla luciferase. Bars indicate the mean normalized to percent of maximum ($n=3$) and error bars indicate standard deviation. (C) Ubiquitous STAT2 expression in transgenic mice. Protein extracts (20 μ g) from isolated transgenic mice were subject to immunoblot to detect human STAT2. Ubiquitous expression of the transgene throughout the mouse was observed.

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

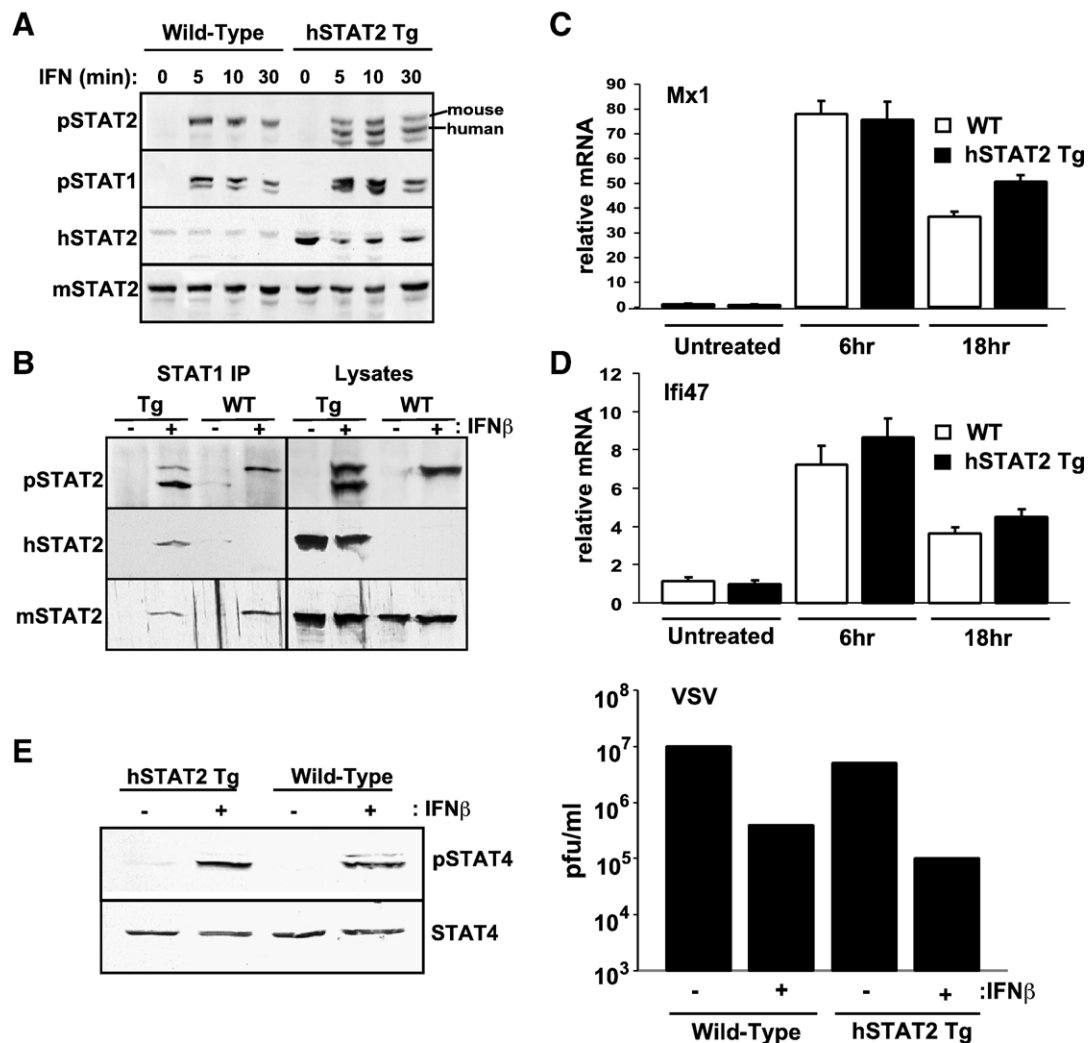


Fig. 3. Human STAT2 activity in mouse does not alter IFN signaling. (A) Splenocytes from transgenic and wild-type mice were treated with murine IFN β for 5–30 min. Cells were immediately lysed and processed for immunoblot with antibodies that recognize human and mouse tyrosine phosphorylated STAT2, human STAT2, mouse STAT2 and STAT1 α/β (recognizes human and mouse). (B) Splenocytes from transgenic and wild-type mice were treated with IFN β for 10 min prior to lysate 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 mIFN β 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 IFN β , 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 IFN β for 20' prior to lysis. Whole cell extracts were separated by SDS–PAGE and immunoblotted with antibodies to detect STAT4 and tyrosine phosphorylated STAT4. IFN β induces STAT4 activation in both wild-type and transgenic splenocytes.

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 IFN β 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 IFN β 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 IFN β 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

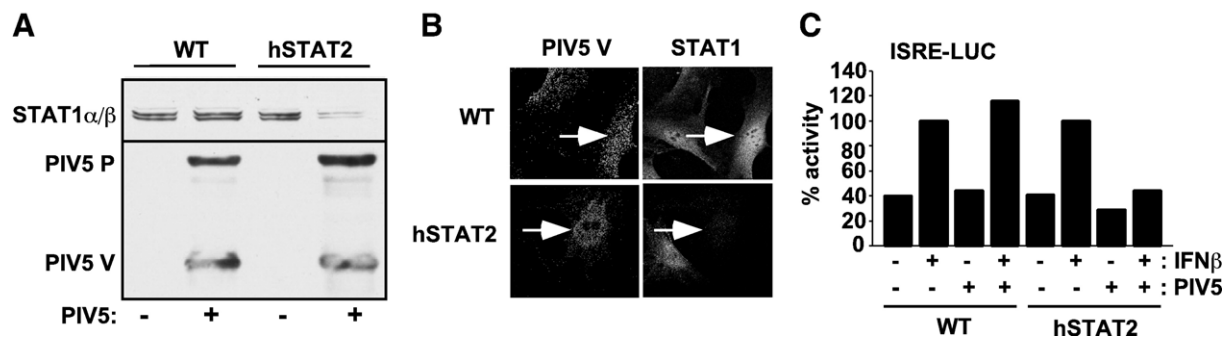


Fig. 4. PIV5 targets STAT1 for degradation and blocks IFN signaling only in transgenic mice. (A) MEFs from wild-type or transgenic mice were infected with PIV5 (50 pfu/cell) for 24 h prior to lysis and immunoblot with STAT1 or P/V antibody. (B) MEFs were infected with PIV5 (1 pfu/cell) and prepared for 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.

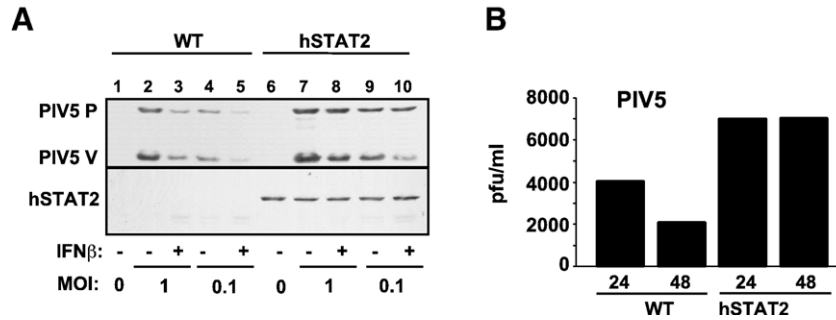


Fig. 5. Transgenic mouse cells support enhanced PIV5 replication. (A) MEFs were infected with PIV5 for 24 h before additional (1000 U/ml) exogenous IFN β for another 24 h. Cells were lysed and processed for immunoblot with human STAT2 and P/V antibodies. (B) PIV5 titer from MEFs infected at low MOI (1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

MEFs were subjected to an IFN β -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).

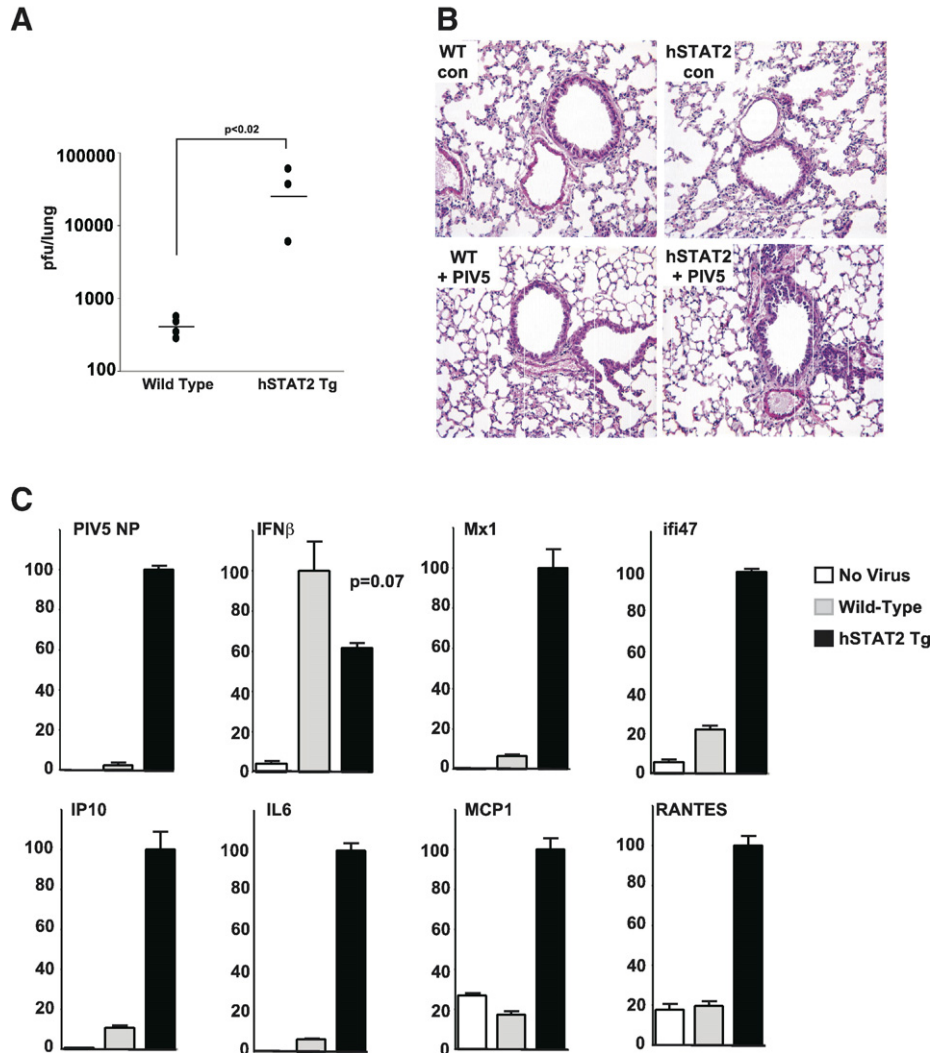


Fig. 6. Enhanced replication in vivo. Groups of transgenic mice were intranasally inoculated with PIV5 (4×10^5 pfu/mouse). Forty-eight to 72 h post-infection, the mice were euthanized and lungs were processed for infection virus, RNA and histological analysis. (A) Concentration of infectious virus in 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, IFN β , Mx1, Ifi47, IP10, IL6, MCP1 and RANTES.

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. IFN β 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 repli-

cation 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 2fTGH and U6A cells were maintained in DMEM supplemented with 10% cosmic calf serum (Hyclone). 2fTGH 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 littermates.

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 IFN β (PBL, Piscataway, NJ) was used at a concentration of 10^3 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 nM 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 background staining. Images were obtained using a Leica TCSSP confocal microscope.

RNA analysis of splenocytes

Total RNA was prepared by using Trizol reagent (GIBCO BRL), digested with DNase I (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'-gactaccactgagatgacc-3', Mx1-AS 5'-ctctattctctcccaaat-3', Ifi47-S 5'-ttgctgaacaagaacagt-3', Ifi47-AS 5'-aagttcccccttgatgtctg-3', GAPDH-S 5' ggcatggactgtggtcatga-3', GAPDH-AS 5'-caccaccatggagaaggca-3'. Mx1 and Ifi47 products were normalized against GAPDH. Untreated RNA was used as the calibrator.

Antiviral assays

Antiviral assays titrating 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 (10³ u/ml) for 12 h. In experiments where PIV5 infection is used, transfected cells were cultured overnight 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 × 10⁵ 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 L-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 titrating on CV-1 cells or for RNA analysis. For RNA analysis for homogenates, 100 µl of

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'-tatgctgatgcaggcaccatg-3', AS 5'-tctgtagtc
caagccacttg-3'
IFN β S 5'-acctacaggccgactcaag-3', AS 5'-gatggcaaagg
cagtgaactctt-3'
IP10 S 5'-tccttgctcctcctagctca-3', AS 5'-ataacccttgggaa
gatgg-3'
IL6 S 5'-agttgctctctgggactga-3', AS 5'-cagaattgccattgca
caac-3'
MCP1 S 5'-cccaatgagtaggctggaga-3', AS 5'-tctggaccattctt
cttg-3'
RANTES S 5'-gccacgtcaaggagtattcta-3', AS 5'-acacattgg
cggctcctc-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

- Andrejeva, J., Poole, E., Young, D.F., Goodbourn, S., Randall, R.E., 2002. The p127 subunit (DDB1) of the UV-DNA damage repair binding protein is essential for the targeted degradation of STAT1 by the V protein of the paramyxovirus simian virus 5. *J. Virol.* 76, 11379–11386.
- Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., Randall, R.E., 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc. Natl. Acad. Sci. U. S. A.* 101 (49), 17264–17269.
- Appel, M., Bemis, D.A., 1978. The canine contagious respiratory disease complex (kennel cough). *Cornell Vet.* 68 (Suppl 7), 70–75.
- Brankin, B., Wisdom, G.B., Allen, I.V., Hawkins, S.A., Cosby, S.L., 1989. Antibodies to simian virus 5 in patients with multiple sclerosis and other neurological disorders. *J. Neurol. Sci.* 89, 181–187.
- Chatziandreou, N., Stock, N., Young, D., Andrejeva, J., Hagmaier, K., McGeoch, D.J., Randall, R.E., 2004. Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5). *J. Gen. Virol.* 85, 3007–3016.
- Cosby, S.L., McQuaid, S., Taylor, M.J., Bailey, M., Rima, B.K., Martin, S.J., Allen, I.V., 1989. Examination of eight cases of multiple sclerosis and 56 neurological and non-neurological controls for genomic sequences of measles virus, canine distemper virus, simian virus 5 and rubella virus. *J. Gen. Virol.* 70, 2027–2036.
- Didcock, L., Young, D.F., Goodbourn, S., Randall, R.E., 1999a. Sendai virus and simian virus 5 block activation of interferon-responsive genes: importance for virus pathogenesis. *J. Virol.* 73, 3125–3133.
- Didcock, L., Young, D.F., Goodbourn, S., Randall, R.E., 1999b. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J. Virol.* 73, 9928–9933.
- Evermann, J.F., Krakowka, S., McKeirnan, A.J., Baumgartner, W., 1981. Properties of an encephalitogenic canine parainfluenza virus. *Arch. Virol.* 68, 165–172.
- Farrar, J.D., Smith, J.D., Murphy, T.L., Murphy, K.M., 2000a. Recruitment of Stat4 to the human interferon-alpha/beta receptor requires activated Stat2. *J. Biol. Chem.* 275, 2693–2697.
- Farrar, J.D., Smith, J.D., Murphy, T.L., Leung, S., Stark, G.R., Murphy, K.M., 2000b. Selective loss of type I interferon-induced STAT4 activation caused by a minisatellite insertion in mouse Stat2. *Nat. Immunol.* 1, 65–69.
- Goswami, K.K., Lange, L.S., Mitchell, D.N., Cameron, K.R., Russell, W.C., 1984a. Does simian virus 5 infect humans? *J. Gen. Virol.* 65, 1295–1303.
- Goswami, K.K., Cameron, K.R., Russell, W.C., Lange, L.S., Mitchell, D.N., 1984b. Evidence for the persistence of paramyxoviruses in human bone marrows. *J. Gen. Virol.* 65, 1881–1888.
- Goswami, K.K., Randall, R.E., Lange, L.S., Russell, W.C., 1987. Antibodies against the paramyxovirus SV5 in the cerebrospinal fluids of some multiple sclerosis patients. *Nature* 327 (6119), 244–247.
- He, B., Paterson, R.G., Ward, C.D., Lamb, R.A., 1997. Recovery of infectious PIV5 from cloned DNA and expression of a foreign gene. *Virology* 237, 249–260.
- He, B., Paterson, R.G., Stock, N., Durbin, J.E., Durbin, R.K., Goodbourn, S., Randall, R.E., Lamb, R.A., 2002. Recovery of paramyxovirus simian virus 5 with a V protein lacking the conserved cysteine-rich domain: the multi-functional V protein blocks both interferon-beta induction and interferon signaling. *Virology* 303, 15–32.
- Horvath, C.M., 2004. Silencing STATs: lessons from paramyxovirus interferon evasion. *Cytokine Growth Factor Rev.* 15, 117–127.
- Keller, M.A., Murphy, S.K., Parks, G.D., 2001. RNA replication from the simian virus 5 antigenomic promoter requires three sequence-dependent elements separated by sequence-independent spacer regions. *J. Virol.* 75, 3993–3998.
- Levy, D.E., Garcia-Sastre, A., 2001. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine Growth Factor Rev.* 12, 143–156.
- Lin, G.Y., Paterson, R.G., Richardson, C.D., Lamb, R.A., 1998. The V protein of the paramyxovirus PIV5 interacts with damage-specific DNA binding protein. *Virology* 249, 189–200.
- McLean, B.N., Thompson, E.J., 1989. Antibodies against the paramyxovirus SV5 are not specific for cerebrospinal fluid from multiple sclerosis patients. *J. Neurol. Sci.* 92, 261–266.
- Nguyen, K.B., Watford, W.T., Salomon, R., Hofmann, S.R., Pien, G.C., Morinobu, A., Gadina, M., O'Shea, J.J., Biron, C.A., 2002. Critical role for STAT4 activation by type I interferons in the interferon-gamma response to viral infection. *Science* 297, 2063–2066.
- Park, C., Lecomte, M.J., Schindler, C., 1999. Murine Stat2 is uncharacteristically divergent. *Nucleic Acids Res.* 27, 4191–4199.
- Paulson, M., Pisharody, S., Pan, L., Guadagno, S., Mui, A.L., Levy, D.E., 1999. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. *J. Biol. Chem.* 274, 25343–25349.
- Parisien, J.P., Lau, J.F., Rodriguez, J.J., Ulane, C.M., Horvath, C.M., 2002a. Selective STAT protein degradation induced by paramyxoviruses requires both STAT1 and STAT2 but is independent of alpha/beta interferon signal transduction. *J. Virol.* 76, 4190–4198.
- Parisien, J.P., Lau, J.F., Horvath, C.M., 2002b. STAT2 acts as a host range determinant for species-specific paramyxovirus interferon antagonism and simian virus 5 replication. *J. Virol.* 76, 6435–6441.
- Precious, B., Childs, K., Fitzpatrick-Swallow, V., Goodbourn, S., Randall, R.E., 2005. Simian virus 5 V protein acts as an adaptor, linking DDB1 to STAT2, to facilitate the ubiquitination of STAT1. *J. Virol.* 79, 13434–13441.
- Ulane, C.M., Horvath, C.M., 2002. Paramyxoviruses PIV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components. *Virology* 304, 160–611.
- Ulane, C.M., Kentsis, A., Cruz, C.D., Parisien, J.P., Schneider, K.L., Horvath, C.M., 2005. Composition and assembly of STAT-targeting ubiquitin ligase

- complexes: paramyxovirus V protein carboxyl terminus is an oligomerization domain. *J. Virol.* 79 (16), 10180–10189 (Aug).
- Young, D.F., Randall, R.E., Hoyle, J.A., Souberbielle, B.E., 1990. Clearance of a persistent paramyxovirus infection is mediated by cellular immune responses but not by serum-neutralizing antibody. *J. Virol.* 64, 5403–5411.
- Young, D.F., Didcock, L., Randall, R.E., 1997. Isolation of highly fusogenic variants of simian virus 5 from persistently infected cells that produce and respond to interferon. *J. Virol.* 71, 9333–9342.
- Young, D.F., Chatziandreou, N., He, B., Goodbourn, S., Lamb, R.A., Randall, R.E., 2001. Single amino acid substitution in the V protein of simian virus 5 differentiates its ability to block interferon signaling in human and murine cells. *J. Virol.* 75, 3363–3370.
- Young, D.F., Carlos, T.S., Hagmaier, K., Fan, L., Randall, R.E., 2007. AGS and other tissue culture cells can unknowingly be persistently infected with PIV5; A virus that blocks interferon signalling by degrading STAT1. *Virology* 365, 238–240.