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Simian Immunodeficiency Virus Infection in the Brain and Lung Leads to Differential Type I IFN Signaling during Acute Infection

Luna Alammar,* Lucio Gama,* and Janice E. Clements*,†

Using an accelerated and consistent SIV pigtailed macaque model of HIV-associated neurologic disorders, we have demonstrated that virus enters the brain during acute infection. However, neurologic symptoms do not manifest until late stages of infection, suggesting that immunological mechanisms exist within the CNS that control viral replication and associated inflammation. We have shown that IFN-α, a type I IFN central to viral innate immunity, is a major cytokine present in the brain during acute infection and is responsible for limiting virus infection and inflammatory cytokine expression. However, the induction and role of IFN-α in the CNS during acute SIV infection has never been examined in this model. In the classical model of IFN signaling, IFN-β signals through the IFN-α/β receptor, leading to expression of IFN-α. Surprisingly, although IFN-β is upregulated during acute SIV infection, we found that IFN-α is downregulated. We demonstrate that this downregulation is coupled with a suppression of signaling molecules downstream of the IFN receptor, namely tyrosine kinase 2, STAT1, and IFN regulatory factor 7, as indicated by either lack of protein phosphorylation, lack of nuclear accumulation, or transcriptional and/or translational repression. In contrast to brain, IFN-α is upregulated in lung and accompanied by activation of tyrosine kinase 2 and STAT1. These data provide a novel observation that during acute SIV infection in the brain, there is differential signaling through the IFN-α/β receptor that fails to activate expression of IFN-α in the brain. The Journal of Immunology, 2011, 186: 4008–4018.

H uman immunodeficiency virus-associated neurologic diseases are a mounting problem in HIV treatment despite the introduction of highly active antiretroviral therapy (HAART). HAART has greatly decreased the prevalence of people with HIV-associated neurologic diseases; however, the incidence has increased as more HIV-infected individuals are living longer (1–3). HIV is thought to enter the CNS through a “Trojan horse” mechanism, where infected monocytes in the peripheral blood traffic to the brain and mature into macrophages where they produce virus and infect neighboring macrophages, resident microglia, and astrocytes (4). Because of the blood–brain barrier, antiretrovirals that are administered to HIV-infected individuals have variable CNS penetration and do not consistently control replication (5–7). Thus, the brain represents a significant viral reservoir that can be reactivated during infection and lead to neurologic damage. Therefore, it is critical to understand how the innate immune response during acute infection initially controls virus replication and inflammation and how this control fails, leading to increased virus expression, inflammation, and neurologic disease. If therapeutics could be identified that maintain immunological control mechanisms that limit inflammation in brain, they would be candidates for adjunctive therapy with HAART.

We have developed and characterized an accelerated, consistent SIV model of HIV AIDS and neurologic disease (8). Like HIV infection in humans, SIV infection is characterized by stages of disease. In our accelerated SIV model, acute infection occurs between 4 and 21 d postinoculation (p.i.). Viral load in plasma peaks at 7 d p.i. paralleled by a decrease in CD4⁺ T cell counts. CD4⁺ T cell counts rebound, and the asymptomatic phase occurs from 21 to 42 d p.i. (9–11). SIV-infected macaques then start to develop signs of disease and develop AIDS by 84 d p.i. (9, 10). In addition, 90% of animals develop SIV-associated neurologic disease, as indicated by neuropathological lesions and inflammation. Analyses of the brain from these SIV-infected macaques at various stages of infection have made it possible to examine both the viral and host factors throughout the course of disease.

Using this model, we have shown that although virus enters the brain and actively replicates in macrophages as early as 4 d p.i., inflammatory changes that accompany acute infection are transient, and clinical signs of neurologic disease do not manifest themselves until late stages of infection (9, 10). SIV replication in the brain is downregulated during the acute infection, but this does not occur in the peripheral blood (11). This suggests that virus replication is regulated differently in brain compared with that in the periphery.

Two of the major cytokines involved in the innate immune response to viral infections are the type I IFNs IFN-α and IFN-β. Whereas there is only a single gene encoding the IFN-β protein in both humans and macaques, there are 13 different IFN-α genes encoding 12 different proteins located on chromosome 9 (in humans) and chromosome 15 (in macaques) (12). In the classical IFN signaling pathway, pattern recognition receptors such as TLRs, nucleotide-binding oligomerization domain-like receptors, and cytosolic receptors such as RIG-I and MDA5 are stimulated, and these receptors trigger activation of numerous kinases such as...
TANK binding kinase 1 and the inhibitor of NF-κB kinases. These kinases are thought to phosphorylate the transcription factor IFN regulatory factor (IRF)3, which leads to its dimerization, nuclear translocation, and the transcription of IFN-β (13, 14). IFN-β is then secreted and engages one of the two subunits of the heterodimeric IFN receptor (IFNAR1 and IFNAR2) in an autocrine and paracrine manner, which leads to receptor dimerization. IFNAR1 is associated with the Janus kinase tyrosine kinase 2 (Tyk2), whereas IFNAR2 is associated with Jak1. Jak1 and Tyk2 phosphorylate themselves, each other, and the IFN receptor subunits. Phosphorylation of the receptor creates a docking site for STAT1 and STAT2 to bind through Src homology 2 domains. Phosphorylated STAT1, STAT2, and IRF9 form the ISGF3 complex and translocate into the nucleus, where it upregulates transcription of IRF7. IRF7 then undergoes phosphorylation, dimerization, and translocation into the nucleus where it stimulates IFN-α transcription. IFN-α is then secreted and continues to signal in an autocrine and paracrine manner through the IFN receptor, thus amplifying IFN production and in turn the antiviral response through a positive feedback loop (15, 16).

We have demonstrated in our SIV macaque model that IFN-β is critical in controlling virus replication and establishing virus latency during acute infection in the brain (17, 18). IFN-β production in the brain follows the same pattern as virus replication in the brain, with peak mRNA levels at 7 d.p.i. (18). IFN-β in turn leads to the translation of a truncated dominant negative isoform of C/EBP beta, which binds to the SIV long-term repeat through its DNA binding domain and represses transcription (18, 19). It is thought that this mechanism is responsible for the downregulation of both viral and cytokine gene expression (9). Although the production of IFN-β usually leads to the production of IFN-α, it has been reported that HIV-infected monocytes have a reduced ability to produce IFN-α whereas IFN-β levels remain the same (20). This implies that differential IFN signaling patterns may exist, which may not be surprising because IFN-α and IFN-β have been shown to have different activities, especially in the CNS (21). Whereas IFN-β has been reported to be protective against autoimmune encephalitis in mice, studies have shown IFN-α to be associated with CNS disease (22, 23). Therefore, we examined the regulation of IFN-α in the brain in our SIV model.

Surprisingly, we found that in the brain, IFN-α mRNA is downregulated during acute infection when IFN-β mRNA is upregulated. Furthermore, we determined that the signaling molecules Tyk2, STAT1, and IRF7 are all suppressed transcriptionally, translationally, and/or posttranslationally. This is consistent with our previous studies that demonstrated that IL-12 mRNA is downregulated during acute infection in the brain, as Tyk2 is a component of the IL-12 receptor as well (9). In contrast to the brain, IFN-α mRNA is downregulated during acute infection in the brain, as Tyk2 is a component of the IL-12 receptor as well (9). In contrast to the brain, IFN-α mRNA is downregulated during acute infection in the brain, as Tyk2 is a component of the IL-12 receptor as well (9).

Materials and Methods

Animal studies

Pigtailed macaques (Macaca nemestrina) were dually inoculated with an immunosuppressive swarm of SIV (SIV/Deltab670) and a neurovirulent clone (SIV/17E-Fr) as previously described (8, 10). Macaques were euthanized without infection (six animals) or at days 4 (six animals), 7 (six animals), 10 (six animals), 14 (six animals), 21 (six animals), 42 (eight animals), and 56 (eight animals) p.i. in accordance with federal guidelines and with approval of the institutional review board. Tissues were snap frozen at necropsy and were later used for RNA and protein isolation.

Quantitative RT-PCR

RNA isolation. RNA was isolated from 50 mg of snap-frozen tissue from the parietal cortex, basal ganglia, and lung using STAT60 reagent (IsoTec Diagnostics, Friendswood, TX). RNA was treated with turbo DNase (Ambion, Austin, TX) for 30 min at 37°C and then purified with the RNaseasy kit (Qiagen, Valencia, CA).

IFN-α quantitative RT-PCR primer development. Forward and reverse primers for IFN-α were made off two conserved regions between an alignment of all subtypes. However, for the forward primer, three α subtypes (alphas 6, 8, and 13) had 1–2 nucleotide differences within the middle of the conserved region, so a mixture of four primers was made that contained exact sequences for alphas 6, 8, 13 and the 10 subtypes that had the same sequence. Similarly, for the reverse primer, alphas 13, 4, and 2 had 1–2 nucleotide differences within the middle of the conserved region, so a mixture of primers was made using the same approach. Although a third conserved region exists between the two primers, the sequence would not yield a functional probe, so SYBR Green chemistry was used for all quantitative RT-PCR reactions. To verify that these primers would amplify all subtypes, RNA was isolated from 6 animals (3 uninfected and 3 SIV infected) and then reverse transcribed to cDNA using SSSH reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was done on each animal using the IFN-α primers using High Fidelity PCR Supermix (Invitrogen), and then the amplification product was cloned into the vector pcr 2.1 using TOPO TA cloning kit (Invitrogen). Between 26 and 96 colonies were sequenced from each animal, making a total of 467 colonies for sequence analysis. Twelve of thirteen IFN-α subtypes were identified using these primers, with the exception being IFN-α 21 (data not shown).

Because IFN-α real-time PCR results indicate levels of expression for all subtypes except IFN-α 21. IFN-α quantitative RT-PCR. One microgram of total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen). cDNA was then incubated with RNaseH (Invitrogen) for 20 min at 37°C. Two to four microliters of the cDNA stock was used for real-time PCR using the SYBR Green mix (Qiagen) in a Chromo4 thermocycler (Bio-Rad, Hercules, CA). Reactions were run in triplicate with appropriate controls, including reactions that lacked DNA template or reverse transcriptase enzyme, and then normalized to GAPDH run in parallel wells. Several samples were also normalized to 18S rRNA to ensure the same results using two different housekeeping genes. All quantitative RT-PCR samples were analyzed using the ∆∆Ct method (27) and expressed as a fold induction over the average of uninfected controls.

Quantitative RT-PCR for all other genes. Two hundred nanograms of RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and then analyzed by real-time PCR using Multiplex NoRox mix (Qiagen) in a Chromo4 Thermocycler (Bio-Rad). Cellular RNA levels were normalized using 18S rRNA. Reactions were run in triplicate with controls that lacked either DNA template or reverse transcriptase enzyme. All quantitative RT-PCR samples were analyzed using the ∆∆Ct method (27) and expressed as a fold induction over the average of uninfected controls.

Primer and probe sequences

Sequences for all primers were based on the rhesus genome (Macaca mulatta) obtained from GenBank. IFN-α: forward 5′-TCTCCATGAGGTGATCCGACGACCT-3′ (α 6), 5′-TCTCCATGAGATTACCCAACGACGACCT-3′ (α 8), 5′-TCTCCATGAGGTGATCCGACGACCT-3′ (α 13), 5′-TCTCCATGAGGACTGCTTCCAGCT-3′ reverse 5′-GACTCTAAATTCTGCTGCTAACCC-3′ (α 2), 5′-GATTCCATGATTCTTCCTGAGACACC-3′ (α 4), 5′-GATTCCATGATTCTTCTGAGACACC-3′ (α 13), 5′-GATTCCATGATTCTTCTGAGACACC-3′ (all other alphas). GAPDH primers and probe sequences: forward 5′-AGGCTCTAAGATCATCGAAATGT-3′, probe 5′-CCAACTGTCTTAGCACCCCTGACCC-3′, reverse 5′-ATGAGCTGCTGATCGACTGCTTCT-3′. IL-12 and 185 primers and probes were described elsewhere (7). Tyk2 primers were as follows: forward 5′-TCTAACTTCTCGCTCCCTTCT-3′, probe 5′-CCCATACACCATCTTCGATCCAC-3′, reverse 5′-GGAGCTTGTGATCCAC-3′, reverse 5′-GATCTCCGACGACCT-3′.
Western blots and Abs

Protein lysates were made by using 100-mg punches of parietal cortex and lung snap-frozen tissues homogenized in RIPA buffer (0.25% Na deoxycholate, 0.1% SDS, 25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 in water) with protease and phosphatase inhibitors (Calbiochem, Darmstadt, Germany), 1 mM DTT, and 0.1 nM PMSF. Protein lysates were sonicated for 10 s every 10 min for 30 min on ice and then spun down to pellet cellular debris. Protein was quantitated using Bio-Rad Protein Assay kit. Protein lysates were run on 10% Tris-HCl criterion gels (Bio-Rad) and then transferred onto polyvinylidene difluoride membranes using the iBlot apparatus (Invitrogen). For all Western blots except p-Tyk2, membranes were blocked in 5% milk for 1 h and then probed with primary Ab overnight in 5% milk. Blots were washed three times for 15 min with TBST and then incubated with HRP-conjugated secondary Abs of the correct isotype that were diluted in 5% milk. Blots were then washed three times with TBST for 20 min and were developed using either ECL Advance (Amersham, Piscataway, NJ) substrate (Tyk2, p-Tyk2, p-STAT3, p-STAT1), Supersignal West Pico (Pierce, Rockford, IL) substrate (GAPDH, STAT1, IRF7), or Supersignal West Dura (Pierce) substrate (TFIID). For p-Tyk2, blots were washed three times for 15 min with TBST after blocking in 5% milk, and primary Ab was diluted in 5% BSA. All further steps were carried out in the same way as for other blots, with the exception that all-washes from that point on were done for 5 min. Abs and concentrations were as follows (primary and secondary, respectively): Tyk2 (Abcam, Cambridge, MA) 1:200, 1:5000; GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) 1:5000, 1:5000; p-STAT3 S727 (Santa Cruz Biotechnology) 1:500, 1:5000; p-Tyk2 Y1054/1055 (Cell Signaling, Danvers, MA) 1:500, 1:2000; STAT1 (Santa Cruz Biotechnology) 1:1000, 1:5000; p-STAT1 Y701 (Cell Signaling) 1:1000, 1:5000; TFIID (TBP, SI-1) (Santa Cruz Biotechnology) 1:1000, 1:5000; IRF7 (Abcam) 1:4000, 1:10,000. Western blot quantitations were carried out by scanning the developed film using a CanoScan 8400F scanner, and then the images were quantitated using ImageQuant TL 7.0 software.

Cells and reagents

Primary macaque macrophages were isolated from whole blood as previously reported (19). Cells were allowed to differentiate for 7 d in culture. On day 7, cells were stimulated with 100 U/ml IFN-α (PBL, Piscataway, NJ) for 5, 10, and 15 min. Nuclear and cytoplasmic cellular lysates were prepared using the Nuclear Isolation kit (Marligen, Ijamsville, MD).

Nuclear and cytoplasmic lysate preparation from tissue

Nuclear and cytoplasmic lysates were prepared from the parietal cortex of two uninfected and eight SIV-infected macaques (four from 7 d p.i. and four from 10 d p.i.). One hundred milligrams of snap-frozen parietal cortex was homogenized in 800 μl of buffer A (400 μl) and Buffer B (400 μl) from the Nuclei Enrichment kit for Tissue (Pierce) with protease and phosphatase inhibitors (Calbiochem), 1 mM DTT, and 0.1 nM PMSF. Samples sat on ice for 10 min, and nonhomogenized debris was pelleted. Twenty-five microliters of detergent solution (Marligen Nuclear Isolation kit) was added to the supernatant, and nuclear lysates were prepared via the manufacturer’s protocol (Marligen). Protein was quantitated using the Bio-Rad Protein Assay kit (Bio-Rad).

Statistical analysis

The data from the quantitative RT-PCR and Western blot analysis were analyzed using the nonparametric Mann–Whitney U test. Correlations were determined using the Spearman nonparametric test.

Results

IFN-α is downregulated in the brain but not the lung during acute SIV infection

We have previously reported that during acute SIV infection in the brain, IFN-β mRNA and protein are significantly upregulated (Fig. 1A, data from Ref. 9). However, the expression of IFN-α had not been examined. To examine the pattern of expression of IFN-α in the brain during SIV infection, RNA was isolated from the parietal cortex of pigtailed macaques that were sacrificed at different times postinfection. The parietal cortex (hereon referred to as brain) was chosen because previous reports from the laboratory have looked at this region in the context of SIV infection (11). Expression of IFN-α mRNA was quantitated by quantitative RT-PCR using primers that spanned regions that were conserved among all IFN-α subtypes. We validated that these primers detected all subtypes as described in Materials and Methods. RNA levels were expressed as a ratio of the infected brain RNA over the average of uninfected controls using the ΔΔCt method (27). Notably, during acute infection, there is a significant downregulation (p = 0.0101) of IFN-α mRNA expression, with its nadir level at 7 d p.i. (Fig. 1B). There is on average a 65% fold decrease in IFN-α mRNA expression compared with that of uninfected controls. Levels of IFN-α mRNA expression do not peak above uninfected controls until 56 d p.i. This is in contrast to levels of IFN-β mRNA, which shows a reciprocal expression pattern (Fig. 1C). Viral RNA in the brain during these same time points has been previously reported (9). In addition, we previously demonstrated that IL-12 mRNA is also downregulated in the brain (Fig. 1D, data from Ref. 9), which is in contrast to many other cytokines that are upregulated during acute infection.

To determine whether the pattern of IFN signaling was specific to the brain, we examined IFN-α expression in the periphery. We first examined IFN-α expression in PBMCs obtained at necropsy from the same animals used in the brain studies, however no induction was detected (data not shown). This is not surprising because plasmacytoid dendritic cells, the major source of IFN-α in PBMCs, rapidly migrate to lymph nodes during acute infection (28, 29). We next examined IFN-α expression in the lung as a peripheral comparison, because, like the brain, macrophages are the major cell infected by SIV and HIV (24–26). Furthermore, it has been reported in our SIV model that viral infection in the lung is followed by induction of IFN-β during acute infection (30). RNA was isolated from lung of the same animals used in the brain studies, and quantitative RT-PCR was performed. We measured IFN-α mRNA levels in the lung and found that unlike the brain, levels are increased during acute infection (p = 0.0519) (Fig. 1F). In addition, we examined the level of IL-12 mRNA because it is the only other cytokine that we have measured that is downregulated in the brain during acute infection. Similar to IFN-α, IL-12 mRNA expression increased in the lung during acute infection. (Fig. 1F). These results indicate that SIV infection leads to a differential IFN signaling pattern during acute infection in the brain and lung.

Transcriptional and translational expression of Tyk2 is impaired in the brain

Because IFN-α and IL-12 are the only cytokines in the brain that we have examined that are downregulated during acute infection, the signaling pathways of the two cytokines were compared to identify signaling molecules that are involved in the regulation of both cytokines. Like type I IFNs, it has been reported that IL-12 can signal in a positive feedback loop with IFN-γ as the main mediator (31, 32). Both IFN-α and IL-12 signal through receptors that are associated with specific tyrosine kinases that are then activated by phosphorylation and signal through the JAK–STAT pathway. Tyk2 is associated with both the IL-12 and IFN-α/β receptors. Because IFN-α production is maintained and amplified in a positive feedback loop through the IFN receptor, we examined Tyk2 mRNA expression levels by quantitative RT-PCR in the brains of the same SIV-infected macaques in Fig. 1. At 4 d p.i., Tyk2 mRNA was significantly (p = 0.0022) downregulated by roughly 70% (Fig. 2A) and by 10 d p.i., Tyk2 mRNA levels returned to uninfected control levels. To determine if this transcriptional downregulation correlated with protein levels, protein homogenates were made from the brain, and Tyk2 protein levels were assayed by Western blot. We found that levels of Tyk2 protein were significantly downregulated at days 4 (p = 0.0087), 7...
FIGURE 1. IFN-α and IL-12 mRNA are downregulated in the brain but upregulated in the lung during acute SIV infection. RNA was isolated from the brain and lung from SIV-infected pigtailed macaques that were sacrificed at days 4, 7, 10, 14, 21, 42, and 56 and from 6 uninfected controls and analyzed by quantitative RT-PCR. Each animal is represented by a black dot, and dashes connect medians. Values are normalized to GAPDH (for IFN-α) and 18S rRNA (for IL-12) and are expressed as a ratio over the average of the uninfected controls (fold induction) using the ΔΔCt method (14). A, IFN-β mRNA expression in the brain. B and E, IFN-α mRNA expression in brain and lung (respectively). *p = 0.0101, †p = 0.0519. Primers were made against conserved regions between all subtypes. C, Overlay of the medians for mRNA expression of IFN-β and IFN-α during SIV infection in the brain. D and F, IL-12 RNA expression in brain and lung, respectively.

(p = 0.015), and 10 (p = 0.0022) p.i. (Fig. 2B). In parallel, we examined Tyk2 levels in the lung during acute infection when both IFN-α and IL-12 are induced. We found that both Tyk2 mRNA and protein are upregulated (Fig. 2C, 2D, respectively), thus illustrating that IFN-α signaling is specifically suppressed in the brain.

Although these data indicate that Tyk2 is suppressed at both the transcriptional and translational levels in SIV-infected brain, residual protein was still present, and it was possible that this residual protein could be activated. Therefore, we assessed the phosphorylation status at Y1054/1055 of Tyk2 at 4, 7, and 10 d p.i. by Western blot. We detected no p-Tyk2 protein (Fig. 2E) in brain at these times p.i. To ensure that the lack of p-Tyk2 signal was not due to an insufficient amount of protein analyzed, Western blots were performed with up to 100 μg protein lysate from brain, and we consistently found that no p-Tyk2 could be detected (data not shown). Protein lysates made from IFN-β–stimulated macrophages isolated from pigtailed macaques were used as a positive control for Tyk2 phosphorylation (Fig. 2E).

In addition to Tyk2 protein phosphorylation, we also examined the level of p-STAT3 (S727) to ensure that there were no technical difficulties in the detection of phosphorylated proteins in the brain lysates. STAT3 is involved in IL-10 signaling, and this cytokine is known to be upregulated in the brain during acute infection (9). We detected p-STAT3 in brain of all of the animals during this time, thus controlling for the detection of phospho protein (Fig. 2E). These data indicate that Tyk2 is suppressed at both the transcriptional and translational levels and that the lack of Tyk2 protein phosphorylation further suggests that the residual Tyk2 protein was not in an active state to signal.

STAT1 is transcriptionally and translationally upregulated

In the classical IFN signaling pathway, Tyk2-mediated IFN receptor phosphorylation creates a docking site for STAT1. Here, STAT1 is phosphorylated and forms the ISGF3 complex with STAT2 and IRF9, where it translocates into the nucleus and leads to the transcription of genes necessary for the antiviral response, including IFN-α. We first measured STAT1 mRNA by quantitative RT-PCR and protein by Western blot analysis to determine whether this transcription factor is expressed in the brain during acute infection. We found that STAT1 mRNA and protein are significantly (p = 0.0079, p = 0.002, respectively) upregulated by 7 d p.i. (Fig. 3A, 3B), thus indicating that this protein is indeed present in the brain.

**STAT1 phosphorylation and nuclear accumulation do not occur in SIV-infected brain despite the increase in mRNA and protein**

Because STAT1 RNA and protein are upregulated during acute infection in brain, we determined whether the protein was in an active state, as this would indicate the activation of signaling downstream of Tyk2. Therefore, we examined phosphorylation at Y701, which is required for IFN signaling, at 4, 7, and 10 d p.i. by Western blot. We were unable to detect tyrosine phosphorylation of STAT1, confirming a lack of STAT1 activation and upstream kinase activity (Fig. 4A). Western blots were also performed using up to 75 μg protein lysates from macaque brain to ensure that sufficient amounts of protein were analyzed (data not shown). Further, as a control, Western blot analyses were done for p-STAT3, which we were readily able to detect in the brain lysates (Fig. 2E). IFN-β–stimulated macrophages isolated from pigtailed macaques were used as a positive control to ensure species cross-reactivity of the Ab (Fig. 4A). In contrast to the brain, p-STAT1 was detected in the lung at 4 d p.i. (Fig. 4B).

We then analyzed the cellular localization of STAT1 in nuclear and cytoplasmic protein extracts made from brain of SIV-infected macaques. Because activation of STAT1 by phosphorylation results in nuclear translocation, we would expect to see little or no STAT1
FIGURE 2. Tyk2 mRNA and protein expression is downregulated during acute SIV infection in brain. A, Quantitative RT-PCR analysis for Tyk2 on RNA isolated from the brain of pigtailed macaques sacrificed between days 4 and 56 p.i. including uninfected controls. Five to six animals used per group. Values are normalized to 18S rRNA and expressed as fold induction. Medians are connected by red dashes. $^* p = 0.0649$, $^{**} p = 0.0022$. B, Western blot analysis of Tyk2 protein in lysates from brain of pigtailed macaques sacrificed between days 4 and 10 p.i. including uninfected controls. Six animals were used per group. All are normalized to GAPDH and expressed as fold induction over uninfected. Medians are connected by dashed lines. $^* p = 0.0087$, $^{**} p = 0.0152$, $^{***} p = 0.0022$. C, Quantitative RT-PCR analysis for Tyk2 on RNA isolated from the lungs of the same pigtailed macaques sacrificed between days 4 and 14 p.i. including uninfected controls. Values normalized to 18S rRNA. Medians are connected by dashed lines. $^* p = 0.0317$, $^# p = 0.0635$. D, Western blot analysis of Tyk2 protein on lysates made from lung of pigtailed macaques sacrificed on day 4 p.i. including uninfected controls. Values normalized to GAPDH and expressed as fold induction. Medians are connected by dashed lines. $^* p = 0.0571$. E, Western blot analysis of p-Tyk2 (Y1054/1055) and p-STAT3 (S727) on the same protein lysates, as well as a lysate made from macrophages of pigtailed macaques stimulated with IFN-β (100 U/ml) for 10 min (designated “+”).
in nucleated extracts. Consistent with our results that STAT1 is not phosphorylated, Western blot analysis revealed no STAT1 nuclear accumulation at days 7 and 10 p.i. (Fig. 4C). GAPDH, a cytoplasmic protein, and TFHID, a nuclear protein, were used to assess the purity of the nuclear and cytoplasmic lysates of brain. As a positive control, nuclear and cytoplasmic lysates were prepared from pigtailed macaque macrophages stimulated with IFN-α for 5, 10, and 15 min, and STAT1 nuclear accumulation was detected (Fig. 4D). Therefore, although an antiviral response is initiated that results in transcriptional and translational upregulation of STAT1, activation of STAT1, as indicated by phosphorylation and nuclear accumulation, could not be detected. This suggests that signaling downstream of the IFN receptor is impaired in the brain.

FIGURE 3. STAT1 mRNA and protein expression is upregulated during acute SIV infection. A, Quantitative RT-PCR analysis for STAT1 was done on RNA isolated from brain of pigtailed macaques sacrificed on days 4–21 p.i. including uninfected controls. Six animals were used for each group. Values are expressed as a fold induction normalized to 18S rRNA. Medians are connected by dashed lines. **p = 0.0079. B, Western blot analysis for STAT1 done on protein lysates made from brain of macaques sacrificed on days 4–10 p.i. including uninfected controls (six animals per group). Values are expressed as fold induction normalized to GAPDH. Medians are indicated by red dashes. **p = 0.0022.

Tyk2-mediated gene transcription is suppressed

To assess the effect of Tyk2 suppression on the regulation of downstream gene expression, we next examined another gene that is regulated by Tyk2, TRAIL. It has previously been reported that TRAIL is one of the few IFN-stimulated genes (ISGs) whose transcriptional induction by IFN-β is ablated in the absence of Tyk2 (33). Therefore, we measured TRAIL mRNA expression levels in brain from the same SIV-infected macaques (Fig. 5A). TRAIL mRNA was not induced during acute infection and did not significantly increase until 56 d p.i. (p = 0.017), the time at which Tyk2 and IFN-α mRNA is induced (Fig. 5A). Furthermore, using Spearman correlation analysis, there is a significant correlation (R = 0.442, p = 0.0043) between Tyk2 and TRAIL mRNA expression, providing further support for the relationship between Tyk2 and TRAIL regulation. This lack of induction of TRAIL in brain is in contrast to the lung. Quantitation of TRAIL mRNA in lung demonstrated that it is upregulated at 4 d p.i. (Fig. 5B). These data provide strong evidence that the Tyk2-mediated IFN signaling pathway is suppressed during acute SIV infection in brain compared with that in a peripheral tissue such as lung, suggesting that there is differential regulation of this pathway in vivo in a tissue-specific manner.

IRF7 expression is blocked at the level of translation

In addition to Tyk2 and STAT1, IRF7 is another major protein in the pathway that regulates IFN-α expression. IRF7 mRNA transcription is most notably induced by the ISGF3 complex, composed of STAT1, STAT2, and IRF9. Additionally, it has also been reported to be induced in a STAT1-independent manner through NF-κB (34, 35). IRF7 serves as the transcription factor mediating IFN-α expression. Therefore, we measured expression of IRF7 mRNA in the brain during acute infection of the same animals. Surprisingly, IRF7 mRNA was significantly upregulated (p = 0.01) 20-fold by days 4, 7, and 10 p.i. and more than 200-fold at days 14 and 21 p.i. (Fig. 6A). However, the regulation of mRNA during acute infection does not correlate with IRF7 protein expression. Western blot analysis revealed that IRF7 protein levels do not increase relative to uninfected controls between 4 and 21 d p.i. (Fig. 6B). These results suggest that there is an additional block in the IFN signaling pathway that occurs after IRF7 mRNA transcription preventing translation of the protein.

Discussion

In this study, we report a novel observation that SIV infection in the brain leads to repression of a branch in the IFN signaling pathway, where IFN-α mRNA expression is significantly downregulated during acute infection. IFN-α mRNA levels rebound during late stage of infection, when inflammatory cytokines increase and there is infiltration of lymphocytes (8). This expression pattern rivals that of IFN-β, which is upregulated during acute infection at the same time as the nadir of IFN-α levels (9). We show that this transcriptional downregulation of IFN-α is associated with suppression of signaling elements downstream of the IFN-α/β receptor. Tyk2, a Janus tyrosine kinase that is associated with the IFN receptor, is suppressed at both the transcriptional and translational levels by 4 d p.i. Despite residual Tyk2 protein present in the brain, it is not phosphorylated and thus in an inactive state. We also demonstrate that the lack of Tyk2 activation affects the regulation of downstream genes such as TRAIL. Moreover, STAT1, which is a protein phosphorylated by the IFN receptor-associated tyrosine kinases, although upregulated at the mRNA and protein levels during SIV acute infection, is neither phosphorylated nor does it accumulate in the nucleus, signifying a lack of activation. Another important finding is that IRF7, a transcription factor mediating IFN-α production, is upregulated at the transcriptional level, but protein levels remain comparable with those of uninfected controls throughout acute infection. This indicates that in addition to lack of activation of Tyk2, there is yet another block in the IFN signaling pathway that occurs after IRF7 transcription. We demonstrate that suppression of IFN signaling was characteristic of SIV infection in the brain, as the same signaling mediators (Tyk2 and STAT1) in the lung were activated, and IFN-α as well as TRAIL were produced. This indicates that the type I IFN response to acute SIV infection occurs in a tissue-specific manner. Taken together, these data demonstrate that innate antiviral pathways are differentially regulated during acute SIV in-
The IFN signaling pathway can be divided into an “early” phase where IFN-β is induced via the transcription factor IRF3, and a “late” phase where IFN-α is produced after activation of signaling factors downstream of the IFN receptor (38). Our data demonstrate that the late phase of this pathway is altered in the SIV-infected brain during the acute infection. This pattern has been previously observed in HIV-infected monocytes. While they have an impaired ability to produce IFN-α, production of IFN-β remains normal (20).

We focused on Tyk2 as a candidate for mediating the block in IFN-α transcription because it is a common component of the IL-12 receptor, and IL-12 is the only other cytokine examined that is downregulated during acute SIV infection in brain. Tyk2 is a 135-kDa Janus kinase associated with IFNAR1 and is activated upon receptor engagement by type I IFNs, where it not only phosphorylates itself (Y1054/1055) but also the IFN receptor, jump starting the IFN-mediated JAK–STAT pathway (39). Notably, there are published reports that implicate Tyk2 specifically in IFN-α, as opposed to IFN-β, signaling (40, 41). Several studies have reported that Tyk2-deficient cells are completely unresponsive to IFN-α, whereas binding of IFN-β is not only intact but still results in signaling (33, 40–43). It has also been suggested that Tyk2 plays a structural role particularly in receptor engagement of IFN-α (44). This was further supported by the observation that whereas Tyk2 deficiency abrogated IFN-α receptor engagement, cells that contained the inactive kinase restored partial IFN-α, whereas binding of IFN-β remains normal (20).

A, Western blot analysis of p-STAT1 (Y701) on the brain of pigtailed macaques and of a lysate made from IFN-β-stimulated macrophages harvested after 10 min (last sample designated “+”). B, Western blot analysis of STAT1 and p-STAT1 (Y701) in lung protein lysates made from pigtailed macaques (five animals) sacrificed at 4 d p.i. and from one uninfected control. C, Nuclear (N) and cytoplasmic (C) lysates were made from the brain from animals sacrificed at 7 and 10 d p.i. (four each) and from two uninfected controls. Western blot analysis for STAT1, GAPDH, and TFIID were done on lysates. D, Nuclear (N) and cytoplasmic (C) lysates were made from pigtailed macaque macrophages that were either untreated or treated with IFN-β for 5, 10, and 15 min. Western blot analyses for STAT1, GAPDH, and TFIID were done on lysates.
downregulated at the levels of transcription, translation, and phosphorylation as early as 4 d p.i. in brain, which is when virus replication is first detected in the CNS. This suggests that either viral infection or pathways activated by the virus are triggering an inhibitory mechanism that prevents IFN signaling through the type I IFN receptor specifically in the brain. This is further supported by the lack of TRAIL induction, which is a gene dependent upon Tyk2 activation. Because IFN-α production is regulated in a positive feedback loop, the direct implication that Tyk2 has on IFN-α signaling suggests that the downregulation and inactivation of Tyk2 during acute SIV infection is likely to be responsible for the selective downregulation of IFN-α in brain.

One question that arises from our studies is that if the JAK–STAT signaling pathway downstream of the IFN receptor is indeed suppressed, how is it possible that ISGs such as IRF7 and MxA (9) are upregulated? Previously published work in the field has described similar results when ISG expression is activated despite disruption in JAK–STAT signaling. STAT1-deficient mice show an increase in IRF7 RNA expression in response to lymphocytic choriomeningitis virus infection (35), and this was attributed to signaling through TNF-α, which activates NF-κB. This in turn binds NF-κB binding site sequences in the IRF7 promoter region, activating transcription (34). In our macaque model, TNF-α mRNA expression is upregulated during acute infection (9), so it is possible that IRF7 transcription is mediated through an NF-κB–dependant pathway. Furthermore, in either cells deficient in Tyk2 or cells expressing an inactive form of Tyk2, stimulation with IFN-β resulted in upregulation of almost all of the same ISGs as control cells with one of the exceptions being TRAIL (49). In addition, fibroblasts and splenocytes from Tyk2-deficient mice were still able to respond to high concentrations of IFN-α as indicated by upregulation of the ISGs IRF1 and MHC class I, as well as protection against vesicular stomatitis virus (50). These data can be explained by the fact that alternative pathways stimulated by type I IFN that do not signal through the JAK–STAT pathway exist and can mount an antiviral response. These pathways include p38...
cytokine in the CNS in terms of its antiviral and antiproliferative signaling pattern is an inherent characteristic of the primate brain. SIV enters the brain at 4 d p.i. It is possible that this differential type I IFN signaling is a hallmark of the CNS. In our studies, IFN-β mediates antiviral effects against encaphalomyocarditis virus which is reduced in cells treated with a p38 pharmacological inhibitor. It has been shown in our macaque model that both phosphorylated ERK and phosphorylated p38 expression are upregulated during acute infection in the brain (55). Taken together, these reports strongly suggest that in the brain of SIV-infected macaques, the upregulation of IFN-β during acute infection may be activating alternative pathways that do not involve JAK or STAT activation leading to the production of ISGs such as MxA.

The regulation of STAT1 and IRF7 in our SIV model is very striking. What is surprising is that although we did not detect phosphorylation of STAT1 (Y701) during acute infection in the brain, there was a significant increase in transcription and translation of this protein. However, it has been reported that unphosphorylated STAT1 does have several biological functions (reviewed in Ref. 56). It associates with IRF1 to activate transcription of LMP2, as well as being involved in TNF-α-mediated apoptosis (57–59). Furthermore, microarray analysis showed similar expression patterns in cells expressing STAT1 and a mutant STAT1 that is unable to become tyrosine phosphorylated (58). Moreover, serine phosphorylation of STAT1 (S727) also regulates gene transcription that is independent of Y701 signaling (59–61). Therefore, it is possible that the transcriptional and translation upregulation of STAT1 serves an alternative function from the IFN response. Furthermore, although IRF7 transcription is induced 20-fold by day 7 and 200-fold by day 14, protein level remains constant. This suggests that there is not only a posttranscriptional block in IRF7 production but also a compensatory mechanism that drives transcription of IRF7 mRNA when no protein is produced.

The specificity in determining which parts of the IFN response are activated (such as IFN-β and MxA) and which are not (such as IFN-α, Tyk2, and TRAIL) most likely results from the particular microenvironment where infection occurs. For example, our results comparing IFN signaling in the brain and the lung suggest that down-modulation of the pathway is specific to the brain and not more generally to SIV infection. The brain, unlike many other tissues, contains neurons that are not renewed and have been shown to be vulnerable to inflammation and inflammatory cytokines (reviewed in Ref. 62). However, the CNS must mount innate immune responses to pathogenic insult to reduce both infections and the inflammatory processes that accompany them, which damage or kill neurons (63, 64), thus illustrating that there must be an immunological balance. In our studies, IFN-β is upregulated at the peak of viral infection, and this is accompanied by a striking downregulation of both IFN-α and Tyk2 mRNA as soon as virus enters the brain at 4 d p.i. It is possible that this differential type I IFN signaling pattern is an inherent characteristic of the primate CNS, as it has been reported that while IFN-β is a beneficial cytokine in the CNS in terms of its antiviral and antiproliferative effects, IFN-α and Tyk2 are both harmful. For example, transgenic mice that chronically express IFN-α in the brain developed a marked inflammatory response resulting in neurodegeneration and encephalitis (22). Two human genetic neurologic disorders, Aicardi–Goutiéres syndrome and Cree encephalitis, which are characterized by neurodegeneration, brain lesions, and ongoing inflammatory processes, are associated with elevated and persistent levels of IFN-α in the CNS (65, 66). Furthermore, hepatitis C virus-infected patients receiving IFN-α treatment have been reported to suffer from neuropsychiatric side effects including depression, seizures, and EEG changes (67–70). Tyk2 has been found by genome-wide association studies to be associated with the CNS inflammatory disease multiple sclerosis (71, 72). Mutations in the Tyk2 gene that result in reduced kinase activity are reported to be protective against multiple sclerosis (71). Additionally, studies in mice report that attenuation of the Tyk2 protein results in increased CNS repair in response to Theiler’s murine encephalomyelitis virus (73).

In contrast, IFN-β has been reported to be neuroprotective through the secretion of nerve growth factors, as well as anti-inflammatory by limiting T cell differentiation and increasing production of anti-inflammatory cytokines (23, 74–76). This is further supported by reports showing that IFN-β knockout mice have an increased chance of developing experimental autoimmune encephalitis (23). Therefore, it is possible that the primate brain has evolved a mechanism to elicit an antiviral response through the production of IFN-β but prevents inflammatory damage by downregulating IFN-α and Tyk2. This is most likely characteristic of the early stages of CNS infection, before infiltration of peripheral cells that may serve to overcome the block of IFN-α and Tyk2 activation.

Our studies indicate that the IFN pathway consists of a complex network of branches that are each regulated in a tissue-specific way. We found that viral infection does not result in a collective upregulation of all known IFNs and ISGs. Rather, a fine-tuned specificity exists that results in the differential regulation of antiviral genes that appear to be dependent upon cell and tissue type. Thus, it may not be surprising that TRAIL, a gene whose primary function involves apoptosis, is not activated in the brain, a tissue consisting of nonrenewable cells, but is activated in the lung where there is ongoing cellular turnover. Because astrocytes are the immunomodulatory cells of the CNS and are only present in the brain, it is possible that these cells are mediating the specific IFN response to acute SIV infection. The multiple signaling pathways that can be activated in response to IFN-β and viral infections, such as JAK–STAT, MAPK, and PI3K, provide subtle levels of regulation that can tailor the antiviral response in a tissue and cell type specific way.

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References


