The role of a non-canonical JAK-STAT pathway in IFN therapy of poxvirus infection and multiple sclerosis
An example of Occam’s Broom?

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Keywords: experimental allergic encephalomyelitis, interferon mimetics, multiple sclerosis, poxvirus therapeutics, signal transduction, interferon, JAK-STAT

Abbreviations: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis, EGF, epidermal growth factor; FGF, fibroblast growth factor, PDGF, platelet derived growth factor

Introduction

Interferon (IFN) was discovered in 1957 as an innate host defense response against viral infections. The implication of this discovery was not fully realized for nearly 20 years as IFNs have been shown to be immune regulatory cytokines as well as antiviral proteins. We and others independently made the definitive observations that IFNα/β negatively regulated the in vitro antibody response, thus providing the first evidence that IFNs were not only antivirals but also immunoregulators. IFNα/β was shortly thereafter shown to induce suppressor cells (regulatory cells), which in turn produced a soluble suppressor factor(s) that mediated the immunosuppression. During this time, small scale clinical studies showed that IFNα and IFNβ had some therapeutic efficacy in the treatment of relapsing/remitting multiple sclerosis (MS) as well as some cancers. These early IFN studies served as precursors to the discovery and characterization of other cytokines as well as to studies at the level of signal transduction that provided insight into the mechanism of cytokine signaling.

In the classical model of cytokine signaling, ligand activates both JAKs, or the receptor in the signaling process. Further, there is evidence of a functional non-canonical model of JAK-STAT signaling that bears a striking resemblance to that of steroid hormone (SH)/steroid receptor (SR) signaling. Thus, both types I and II IFN signaling involves nuclear translocation of complexed ligand, receptor, activated JAKs, and activated STATs to the promoters of the genes that are specifically activated by the IFNs, where they are involved in specific gene activation and epigenetic remodeling. Receptor intracellular domains play an important role in binding the C-terminus of the IFNs, which is the basis for our development of IFN mimetics. The IFN mimetics are not recognized by poxvirus decoy receptors, since the decoy receptors compete for extracellular binding and not intracellular binding. Further, the type I IFN mimetics provide therapeutic protection against experimental allergic encephalomyelitis (EAE), a model of multiple sclerosis, without the side effects. Extracellular receptor binding by intact IFN is the primary reason for undesirable side effects of flu-like symptoms, bone-marrow suppression, and weight loss. The non-canonical model of IFN signaling thus provides insight into the specificity of such signaling and a mechanism for development of IFN mimetics. It is our contention that this model applies to other cytokines.

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Submitted: 08/01/2013; Revised: 08/19/2013; Accepted: 08/20/2013
Citation: Ahmed CM, Johnson HM. The role of a non-canonical JAK-STAT pathway in IFN therapy of poxvirus infection and multiple sclerosis: An example of Occam’s Broom? JAK-STAT 2013; 2:e26227; http://dx.doi.org/10.4161/jkst.26227
interaction between different STATs in gene activation/suppression, which provides more insight into STAT mediation of cytokine signaling. It is not clear, however, as to how these STAT interactions at the level of DNA binding translate into specific gene activation by the inducing cytokine. It suggests some sort of intrinsic built-in specificity of STATs beyond response element recognition. With the long interest and focus on STATs, one would have expected that such an intrinsic specificity-determining structure would have been discovered by now if it existed.

There are also studies of differential effects of IFNβ in a small cohort of patients with relapsing/remitting MS, where differential activation of p38, NFκB, and STATs 1, 2, and 3 were observed in the context of apoptosis in monocytes and granulocytes. These studies bear some resemblance to those summarized above concerning IFNγ signaling.

We will focus in this review on a non-canonical model of IFN signaling that has implication for insight into the mechanism of type I IFN therapy of multiple sclerosis (MS), particularly in the context of therapy vs. undesirable side effects associated with IFN treatment. Related to this, we will show that it is possible to dissociate type I IFN therapy in the mouse model of MS, experimental allergic encephalomyelitis (EAE), from the side effects of such therapy. Such dissociation has been made possible as a result of our discovery of a non-canonical pathway of IFN signaling. The model involves the JAK-STAT pathway where STAT is but one of a number of important players in a series of complex events that shed light on specific gene activation as well as the associated epigenetic events. Thus far, the classical model of JAK-STAT signaling has not been helpful in linking activated STATs and activated JAKs in the nucleus for gene activation. The non-canonical model makes such a linkage.

The basics of the classical model of IFN signaling as well as that of other ligands that use the JAK-STAT pathway are quite simple and appealing. For example, see Figure 1A for IFNγ. Ligand interacts with the receptor extracellular domain, which somehow transmits a signal through the receptor hydrophobic transmembrane domains to the cytoplasmic domain. This causes allosteric changes in the cytoplasmic domain that result in JAK auto-activation (phosphorylation) and subsequent activation of the relevant STATs. The activated STATs form dimers, dissociate from the receptor and translocate to the nucleus via intrinsic nuclear localization sequences (NLS) where they determine the specificity of gene activation by the particular ligand. This occurs even in the many cases where functionally different ligands activate the same STATs. The model is assumed to provide insight into important things like specific epigenetics associated with gene activation such as histone phosphorylations, histone methylations, dimethyllations, or acetylations. The obvious question is where is such insight contained within the model?

It has been known for some time that internalized types I and II IFNs possessed anti-viral activity. Further, it has been shown that cells treated with IFNs showed nuclear transport of IFN and receptors. In fact, all of the ligand signaling systems that use the JAK-STAT signaling pathway have, where examined, been shown to possess classic polycationic NLSs in their ligands and/or receptors and to undergo active nuclear translocation. This also applies to well-known tyrosine kinase receptors such as epidermal growth factor (EGF) receptor, fibroblast growth factor (FGF) receptor, and platelet derived growth factor (PDGF) receptor. To date none of these observations have been allowed into the conventional lexicon of ligand/receptor signaling either through signaling by receptor-associated tyrosine kinases or by receptor tyrosine kinases.

The classical model of JAK-STAT signaling by IFNs and other cytokines thus takes an Occam’s Razor approach to signaling events in that it is treated as if it explains all the pertinent aspects of specific gene activation by cytokines in the most concise manner. The flip side of Occam’s Razor is Occam’s Broom, a term coined by Sidney Brenner in reference to the willful overlooking of data that does not fit into a particular model. It is our view that Occam’s Broom is operating in all of its glory in attempts to not modify the classical model of JAK-STAT signaling substantively and in accepting it in present form as explaining the
mechanism of specific gene activation by cytokines such as the IFNs in the essentials. We present here our non-canonical model of IFN signaling to more completely understand and mechanistically explain the complexity of events of specific gene activation including the associated epigenetics.

**Basics of the Non-Canonical Model**

It was previously shown that IFNγ and one of its receptor subunits, IFNGR1, are translocated to the nucleus together with activated STAT1α.14,15,20 Active nuclear transport depended on a polycationic nuclear localization sequence (NLS) in the C-terminus of IFNγ, the nuclear import proteins importins α and β, and ATP/GTP as an energy source.14,21 The nuclear targets of IFNγ and IFNGR1 were also identified.15,22 By chromatin immunoprecipitation (ChIP) followed by PCR, IFNγ, its receptor subunit IFNGR1, and STAT1α were found to be associated with the IFN-activated sequence (GAS) element in the promoter of two genes stimulated by IFNγ. Examination of nuclear extracts from IFNγ treated WISH cells showed that IFNγ, IFNGR1, and STAT1α proteins were associated with the GAS promoter. The same associations were also demonstrated by electrophoretic mobility shift assay (EMSA). Transfection with a GAS-luciferase gene together with IFNGR1 and nonsecreted IFNγ resulted in enhanced promoter activity. Additionally, IFNGR1 fused to the yeast GAL-4 DNA binding domain resulted in enhanced transcription from the GAL-4 response element in IFNγ treated cells, suggesting the presence of a transactivation domain in IFNGR1. These nuclear studies suggest a transcriptional/co-transcriptional role for IFNGR1, which may provide insight into the specificity of IFNγ signaling. A model for these non-canonical IFNγ signaling events is presented in Figure 1B. The movement to nucleus of IFNGR1 occurs in a complex with IFNγ activated STAT1α and activated JAK1 and JAK2.22 The activated JAKs provide a direct link between transcription factors and specific epigenetic events such as pJAK2 (activated) phosphorylation of tyrosine 41 on histone H3. Details concerning JAKs and epigenetics are dealt with below.

Cytokines such as IFNs are assumed to bind solely to the receptor extracellular domain, resulting in allosteric changes on the cytoplasmic domain that initiate signaling events. It was shown, however, that IFNγ bound first to IFNGR1 extracellular domain involving in part its N-terminus and then, during endocytosis, to IFNGR1 cytoplasmic domain via its C-terminus.14 The intracellular binding was blocked by an intracellular excess of a peptide representing the cytoplasmic binding site on IFNGR1 for the C-terminus of IFNγ. Moreover, such cells were also blocked with respect to the tyrosine phosphorylation of STAT1α. Thus, internalized IFNγ appeared to be able to interact with the cytoplasmic domain of IFNGR1 in intact cells as part of the signal transduction events leading to STAT1α tyrosine phosphorylation. Cytosolic injection of antibodies to IFNγ C-terminal amino acids 95–132 blocked STAT1α nuclear translocation in response to extracellular IFNγ,21 consistent with these observations. This further supports the idea that the C-terminus of endocytosed IFNγ access IFNGR1 cytoplasmic domain in the cytosol, although the mechanism is as yet unknown.

The intracellular IFNγ effects can be replicated by internalized C-terminus residues 95–132 of mouse IFNγ or residues 95–134 of human IFNγ.23 Thus, peptides corresponding to these residues, mIFNγ(95–132) and hIFNγ(95–134) respectively, with a palmitate attached for cell penetration, function as IFNγ mimetics. The properties and uses of these IFNγ mimetics as well as type I IFN mimetics are described in detail below in the IFN mimetic section. It is noteworthy that there are no IFN or other cytokine mimetics based on extracellular recognition and cross-linking of receptor chains as per the classical model.

Recently, insight has been gleaned on the intracellular aspects of type I IFN signaling. It was shown by western blotting of nuclear extracts that type I IFN signaling involves activated TYK2 in the nucleus, similar to pJAK2 in the nucleus of IFNγ treated cells.10 The nucleus of WISH cells contained constitutively expressed nonphosphorylated TYK2, but activated TYK2, pTYK2, as well as pJAK1 were found in the nucleus of cells only after treatment with type I IFNs IFNα or IFNβ. Both activated STAT1 and STAT2 were present in the nucleus of cells treated with type I IFNs. With IFNγ, only the receptor subunit IFNGR1 underwent nuclear translocation in IFNγ treated cells, but both receptor subunits IFNAR1 and IFNAR2 underwent nuclear translocation in type I IFN treated cells as determined by western blotting of nuclear extracts and confocal microscopy of GFP-receptor fusion proteins. The GFP-IFNγ fusion protein also underwent nuclear import, thus demonstrating that type I IFNs also translocated to the nucleus.

With all of these components of the type I IFN signaling system in the nucleus, there was interest in determining where they went in terms of promoters and whether they were associated with each other for some coordinate nuclear function. Therefore, ChIP-qPCR assays were performed to determine if the type I IFN players were specifically recruited to the promoter region of a gene activated by IFNα in cells.10 The promoter region of the oligoadenylate synthetase 1 (OAS1) gene, which has an IFN sensitive response element (ISRE) and is involved in IFN antiviral activity was thus examined.10 IFNAR1, IFNAR2, TYK2, pSTAT1, and H3pY41 were found at the OAS1 promoter, but not at the β-actin promoter, a gene that is not directly affected by type I IFNs. Consistent with the ChIP data, immunoprecipitation of IFNAR1 in nuclear extracts of IFNα treated cells followed by western blotting showed TYK2, pSTAT1, and H3pY41 associated with IFNAR1. Thus, the various players in type I IFN signaling were found associated in the nucleus of IFN treated cells specifically at the promoter of a key gene in IFN antiviral activity.

Given the specific epigenetic events that are associated with gene activation, ChIP analysis was used to monitor demethylation/acetylation of lysine 9 on histone H3.10 Type I IFN treated cells showed decreased trimethylated lysine on H3, H3K9me3, in the OAS1 promoter region of cells. Acetylation of H3K9, H3K9ac, occurred concomitantly over the same time span.
Demethylation/acetylation of H3K9 is associated with gene activation.24-25 Related to this, phosphorylation of H3 at Y41, H3pY41, increased as H3K9me3 decreased over the same time period, providing a functional linkage of activated JAKs in the above complex epigenetic event. By comparison, the constitutively activated β-actin gene, which is not affected by IFN, showed constitutive H3K9ac, no H3pY41, and no H3K9me3. The nuclear trafficking and activities at specific genes that are associated with treatment of cells with IFN suggest that the receptor/transcription factor/JAK complex plays a key role in specific gene activation, including the related heterochromatin modifications.

Activated JAKs and STATs in the Nucleus: Evidence for Coordination

Activated JAK2 has recently been reported to be present in the nucleus and was shown to perform the epigenetic function of phosphorylation of tyrosine 41 on histone H3.26 It is highly unlikely that the activated JAK2 is acting randomly in the nucleus, so how and with what are its epigenetic functions coordinated? Of particular interest is how is activated JAK activity coordinated with that of activated STATs in the nucleus? In the study, constitutively activated mutated JAK2 JAK2V617F was found in the nucleus and shown to phosphorylate histone H3 on tyrosine 41 (H3pY41), which led to dissociation of heterochromatin protein 1α (HP1α) from H3.26 The resultant heterochromatin remodeling was associated with exposure of euchromatin for gene activation. Wild-type JAK2 was shown to be constitutively present in the nucleus of cells also, but unlike JAK2V617F, was only activated when K562 cells were treated with the growth factors PDGF or leukemia inhibitory factor, or when BaF3 cells were treated with the cytokine IL-3.26 It would seem obvious that the nuclear H3 phosphorylations are not random, but must be under the control of factors associated with the activating cytokine. It is difficult to address this issue in the context of the classical JAK/STAT model as it says nothing about activated JAKs in the nucleus.

The question of JAK-STAT coordination in the nucleus was addressed in IFN studies by treating cells with IFNγ and tracking activated JAK2 in the nucleus.22 Using ChIP analysis, it was shown that activated JAK2 (pJAK2) and H3pY41 were associated with the GAS promoter element at the IRF-1 gene, a gene that is activated by IFNγ.22 pJAK1 was also associated with the IRF-1 GAS element. None of these factors were associated with the promoter of the β-actin gene, a gene not affected by IFNγ. A similar result was observed with TYK2 in IFNα treated cells where TYK2 and H3pY41 were present at the promoter of the OAS 1 gene, a gene activated by type I IFNs, but were absent from the promoter of the β-actin gene.19 It is important to note that ChIP analysis also showed the presence of STAT1 at the IRF1 and OAS1 promoters of IFNγ and/or IFNα treated cells, but not at the β-actin promoter. This would suggest that the activated JAKs and the STATs track to the same promoters, which would suggest that their nuclear activities are coordinated.

The fact that the hematological disorders associated with JAK2V617F show characteristic phenotypic similarities would suggest that the epigenetic activity of JAK2V617F also occurs in association with the relevant hematological receptor. It is of interest therefore that it has been shown that JAK2V617F activation required the association of the mutant JAK2 with a homodimeric type I cytokine receptor.27-28 Specifically, either erythropoietin receptor, thrombopoetin receptor, or granulocyte colony-stimulating receptor was required for hormone/growth factor-independent activation of JAK2V617F. This raises the question of whether there are receptor/JAK2V617F complexes in the vicinity of promoters of genes that are activated in cancers caused by or associated with JAK2V617F. All of this has implications for how particular tyrosine kinases cause or are associated with specific cancers and provides insight into the phenotypes of such cancers.

IFN Mimetics Development and Use as Therapeutics against Poxvirus and EAE, a Mouse Model of Multiple Sclerosis

As indicated above, we have exploited the non-canonical model of IFN signaling to develop IFNγ mimetics where the internalized mimetic, lipo-IFNγ(95–132), was also expressed via chemical synthesis (95–132), was also expressed via chemical synthesis.29 Using ChIP analysis, it was shown that activated JAK2 (pJAK2) and H3pY41 were associated with the GAS promoter element at the IRF-1 gene, a gene that is activated by IFNγ.22 pJAK1 was also associated with the IRF-1 GAS element. None of these factors were associated with the promoter of the β-actin gene, a gene not affected by IFNγ. A similar result was observed with TYK2 in IFNα treated cells where TYK2 and H3pY41 were present at the promoter of the OAS 1 gene, a gene activated by type I IFNs, but were absent from the promoter of the β-actin gene.19 It is important to note that ChIP analysis also showed the presence of STAT1 at the IRF1 and OAS1 promoters of IFNγ and/or IFNα treated cells, but not at the β-actin promoter. This would suggest that the activated JAKs and the STATs track to the same promoters, which would suggest that their nuclear activities are coordinated.

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for poxviruses and thus are potential candidates for antivirals against smallpox virus.25-32,33

It was further shown that lipo-mIFNγ(95–132) at 2000 units protected C57BL/6 mice against an overwhelming lethal vaccinia virus infection (Table 1).32,33 Control mice injected with a non-cell penetrating IFNγ peptide or PBS died at 6–9 d post infection, but intraperitoneal injection of the mimetic as late as 6 d post infection resulted in 40% protection. Administration of mimetic by the oral route also completely protected mice against the intranasal route of a lethal dose of vaccinia virus challenge. In addition to the direct antiviral effects, the mimetic also possessed adjuvant effects in boosting humoral and cellular immunity. This combination of antiviral and adjuvant effects by the IFN mimetic probably played a role in its potent anti-vaccinia properties. IFNγ is generally not extensively used as a therapeutic, the reason for which is not well understood. It should be noted that the presence of receptors on a large number of cells could serve as a “sink”, thus affecting access of IFNγ to sites and cells for which it was intended. The IFNγ mimetics do not recognize the receptor extracellular domain and thus could possibly have better access to intended targets.

The pattern of nuclear signaling by type I IFNs is similar to that of IFNγ nuclear signaling.10,22,34 Thus, in order to determine if IFNoα and IFNB possessed similar C-terminus function intracellularly while losing extracellular function, truncated IFNs IFNoα(69–189)R9 and IFNB(100–187)R9 with 9 arginines (R9) for cell penetration were expressed in a bacterial system and purified.10 As controls, these truncations were also expressed without R9. Both IFNoα(69–189)R9 and IFNB(100–187)R9 possessed antiviral activity against EMC virus, while the same constructs without R9 for cell penetration lacked antiviral activity.10 This is consistent with previous studies that showed that intracellularly expressed IFNoα possessed antiproliferative and antiviral activity.15

In addition to the B8R IFNγ decoy receptor, poxviruses also produce a type I IFN decoy receptor, B18R.35 The type I IFN mimetics inhibited vaccinia virus growth in the presence of B18R, while the corresponding intact IFNs were ineffective (Ahmed and Johnson, in preparation). We have refined the requirements for IFNoα mimic activity and have shown that a shorter sequence, lipo-IFNoα(152–189), was as effective as IFNoα(69–189)R9 in inhibiting virus replication (Ahmed and Johnson, in preparation). It should be noted that the non-canonical model of IFN signaling provides a conceptual foundation such that the IFN mimetics described here are remarkably easy to produce. The question arises, therefore, as to the applicability of the non-canonical model to other cytokines and the potential benefit of development of mimetics of these cytokines using the IFN mimetic approach. There are over 20 different isoforms of type I IFNs and they all function through the same heterodimeric receptor complex.36 In addition to their similar antiviral activities, these IFNs vary with respect to anticytolytic and cytotoxic (apoptotic) effects. In this regard, IFNB is the treatment of choice for relapsing/remitting multiple sclerosis.57,58 Further, it has been shown that higher doses of IFNB result in better therapeutic efficacy,59 but undesirable toxic side effects of flu-like symptoms, liver damage, and bone marrow suppression limit the dose.60 We showed that type I IFN toxicity (apoptosis) was due to differential extracellular IFN receptor recognition where greater receptor occupancy due to higher binding affinity contributed to the toxic effects.61 This observation has been confirmed by others.62

The IFNoα(69–189)R9 mimetic was tested for its ability to therapeutically treat SJL/J mice in EAE, a mouse model of MS, as well as for its toxicity relative to that of IFNB. Immunization of mice with bovine myelin basic protein (MBP) where cellular infiltration into the CNS has occurred by day 12 was used to test the truncated IFNs.10 SJL/J mice were injected i.p. with saline, IFNoα(69–189)R9 (15 μg/mouse), or the control peptide, IFNoα(69–189) (15 μg/mouse), every other day starting from day 12 post-immunization with MBP. The IFNoα mimetic with the R9 reduced paralysis essentially completely, while the mice treated with saline or the mimetic lacking R9 developed paraplegia (Table 1).

For toxicity studies, mice were injected i.p. on alternate days with IFNB, IFNB(100–187)R9, or IFNoα(69–189)R9, all of the same antiviral activity (2000 U).10 Injection of mice with IFNB resulted in approximately 15% weight loss by day 10, while mice injected with the IFN mimetic gained weight, which is expected under normal growth conditions (Table 1). A similar pattern of bone marrow suppression occurred as reflected by peripheral lymphocyte count. IFNB was also apoptotic, while the mimetics did not induce apoptosis. Thus, under conditions of the same antiviral activity, IFNB was toxic and type I IFN mimetics lacked toxicity of weight loss, lymphopenia, and cellular toxicity. These mimetic results would suggest, as with binding studies mentioned above, that it is the IFN signal at the receptor extracellular domain that is responsible for their toxic effects, while antiviral and anti-EAE (MS) effects are associated with intracellular actions that are retained by the IFN mimetics. It is important to emphasize that the IFN mimetics are products of the non-canonical model of JAK-STAT signaling by IFN presented here.

Table 1. Summary of the effects of IFN mimetics and intact IFN

| Function | IFN mimetics | Intact IFN |
|----------|--------------|------------|
| Protection against vaccinia virus | Yes | No |
| Protection against EAE | Yes | Yes |
| Toxicity by weight loss, apoptosis, and bone marrow suppression | No | Yes |

These effects were observed both in cell culture and in C57BL/6 mice infected intranasally with 106 pfu of vaccinia virus,32 and SJL/J mice immunized with myelin basic protein (MBP).10 IFN mimetics showed dose dependent protection in vaccinia virus-infected mice with 100% protection at 200 μg per mouse, while the PBS and non-cell penetrating peptide injected mice died between days 6 and 9. Mice immunized with MBP and treated with type I IFN mimetic showed low incidence of paralysis in 2/5 at severity of 0.5 and 2 by day 29 and recovered quickly and were healthy for up to 60 d of observation. Mice injected with non-cell penetrating peptide or PBS progressively became more paralyzed with 3 out of 5 and 2 out of 5 mice were dead by day 56, respectively. Most importantly, the weight loss and bone marrow suppression observed with intact type I IFN was not seen with the type I IFN mimetics.
Context and Conclusion

In a search for precedents and templates, we have found that our non-canonical model of IFN signaling bares remarkable similarity to that of steroid receptor (SR) signaling. Steroid hormone (SH) binds to SRs located in the cytoplasm or nucleus of the cell. In the absence of hormone, SR monomers are associated with heat shock proteins (HSPs) and usually possess some basal level of phosphorylation. Upon binding of hormone, SRs dissociate from HSPs, dimerize and translocate to the nucleus where they bind to HREs (hormone-response elements) at genes that are activated by SHs. The complex of SH/SR recruits a series of co-activators to both regulate target gene transcription as well as the associated epigenetic events that accompany such activation. Site-specific phosphorylation of receptors occurs subsequent to hormone binding with varied kinetics, depending on the kinase and the target in the receptor complex.

The kinases, although not the only components of the receptor associated co-activator complexes, are important for their action on members of the complex, as well as for specific epigenetic events of gene activation and thus act on histones as well as on members of the receptor complex. Many of the SH phosphorylation sites contain serine/threonine/proline motifs involving proline-specific kinases, such as the cyclin-dependent kinases or members of the receptor complex. Many of the SH genetic events of gene activation and thus act on histones as action on members of the complex, as well as for specific epigenetic events, are important for their involvement in nuclear aspects of gene activation. We further feel that all of the cytokines, hormones, and growth factors that use the JAK-STAT pathway are likely to also share these similarities. In our view the template for all of this resides in the SH/SR system of specific gene activation.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest.

Acknowledgments

This work was supported by NIH grant R01 AI 056152 to HM Johnson.

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