AGONISTS AND ANTAGONISTS OF IFN-GAMMA SIGNALING

By

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To the mice

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Abstract of Thesis Presented to the Graduate School
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We have designed a series of peptide agonists and antagonists of IFN-γ and JAK/STAT signal transduction based upon an alternative understanding of the classical model for this pathway. We have demonstrated previously that the C-terminal gamma interferon (IFN-γ) mimetic peptide consisting of residues 95 to 132 [IFN-γ(95-132)] and contains the requisite IFN-γ nuclear localization sequence (NLS), has antiviral activity in tissue culture. IFN-γ(95-132) prevented EMC virus-induced lethality in mice in a dose-dependent manner compared to controls. IFN-γ(95-132) also protected mice against lethal vaccinia virus infection with the virulence factor B8R, capable of rescuing 100% of animals two days into infection. B8R protein is a homologue for the extracellular interferon gamma receptor (IFNGR) encoded by poxviruses like vaccinia virus as a defense mechanism to neutralize host IFN-γ. IFN-γ(95-32) bypasses B8R because its mode of action is intracellular, and synthesized with a lipophilic attachment, penetrates the cell plasma membrane in lieu of interacting with IFNGR extracellularly. The mimetic also possessed adjuvant effects which boosted humoral and cellular immunity to vaccinia virus. Suppressor of cytokine signaling (SOCS)-1 protein modulates signaling by IFN-γ by binding to the autophosphorylation site of JAK2 and by targeting bound

JAK2 to the proteosome for degradation. We have developed two small tyrosine kinase inhibitor peptides, Tkip and SOCS1-KIR, that are SOCS-mimetics. Both are based on the kinase inhibitory region (KIR) of SOCS-1 and bind the autophosphorylation site of JAK2, JAK2(1001–1013) though not in precisely the same way. Tkip and SOCS1-KIR inhibited STAT1α phosphorylation, antagonizing IFN-γ-induced biological activity, including its impact as an adjuvant, demonstrated here by the Tkip/SOCS1-KIR suppression of APC activation and Ag-specific splenocyte proliferation. The fact that SOCS1-KIR binds to pJAK2(1001–1013) suggests that the JAK2 peptide could function as an antagonist of SOCS-1. Thus it was found that pJAK2(1001–1013) enhanced suboptimal IFN-γ activity, blocked SOCS-1-induced inhibition of APCs, and enhanced IFN-γ activation site promoter activity. Additionally, pJAK2(1001–1013) protected mice against lethal vaccinia and EMC virus infection. pJAK2(1001–1013) increased the intracellular level of the constitutive IFN-β, which may play a role in the IFN-γ agonist effect at the cellular level, and also synergizes with IFNs as per IFN-γ mimetic to exert a multiplicative antiviral effect at the level of transcription. pJAK2(1001-1013) also exhibits adjuvant effects on humoral and cellular immunity in several direct and indirect ways including through the toll-like receptors on APCs. These peptides present novel, effective approaches to either promote, as with the IFN-γ agonist peptides, or inhibit, via the IFN-γ antagonist peptides, innate and adaptive host defenses.

CHAPTER 1
INTRODUCTION

IFN-γ signaling is one of the major systems coordinating the development, strength, and character of innate and adaptive immune responses. Its many biological effects include, but are not limited to, the induction of a number of antiviral proteins, up-regulation of major histocompatibility complex antigen expression, and involvement in B-cell maturation and immunoglobulin isotype switching (1, 2, 3). IFN-γ is also strongly correlated with the promotion of a T-helper (Th)1 response, for which it has roles in the suppression of Th2 and Th17 specializations, activation and homing of natural killer cells, CD8+ T cells, and mononuclear macrophages, and the secretion of specific cytokine profiles, especially inflammatory ones marked by TNF-α and itself (4, 5, 6).

The classical model of signaling for IFN-γ contends that its effects are exerted solely through interactions with the extracellular domain of its receptor, consisting of an α-subunit, IFNGR-1, and a β-subunit, IFNGR-2 (7). Receptor crosslinking results in the activation of receptor–associated tyrosine kinases of the Janus kinase family, JAK1 and JAK2, leading to phosphorylation and dimerization of the STAT1α transcription factors, which then dissociate from the receptor cytoplasmic domain and translocate to the nucleus. (8) This view ascribes no further role to the ligand or the receptor in the signaling process. (9) Further, there is the implicit assumption that the phosphorylated STAT1α homodimer possesses an intrinsic nuclear localization sequence (NLS) that is responsible for its nuclear translocation. (8) However, based on current knowledge there is a potential paradox, as STATs like STAT1α do not contain a polycationic or functional NLS (10).

We have proposed an alternative model for JAK/STAT signal transduction in which the ligand, receptor and auxiliary proteins are more involved. It has been known for some time that IFN-γ translocates to the nucleus of receptor-expressing cells with kinetics as rapid as those for the activation and nuclear translocation of the transcription factor STAT1α that it activates (11, 12). More recently, nuclear translocation of IFN-γ has been shown to be driven by an NLS in its C terminus (13, 14) Mutations of the IFN-γ NLS destroy its biological activity, which can be restored by reconstitution with the NLS from T Ag of SV40 virus (14, 15) The T Ag NLS is known to localize to the nucleus in a IMPα/β1/Ran-dependent fashion. Excess T Ag NLS peptide inhibits IFN-γ NLS-dependent nuclear import, suggesting that IFN-γ NLS mediates nuclear import through the same pathway (13). Results from immunoprecipitation experiments, which detected endocytosed IFN-γ bound to IMPα5 (NPI-1) in cells actively transporting IFN-γ to the nucleus, are consistent with this conclusion (16) Subsequent experiments showed that the receptor IFNGR1, of the hetero-oligomeric receptor also translocates to the nucleus in IFN-γ-treated cells, while the IFNGR2 is not translocated (14, 12, 17). IFN-γ binds to a soluble form of its receptor in which its N terminus interacts with the extracellular domain of the receptor, while the C terminus from residues 95 through 132, binds instead to the membrane-proximal region of the cytoplasmic domain of the receptor (15, 18, 19, 20) This binding, which requires the NLS, also increases the affinity of the JAK2 for IFNGR1 (7, 21)

Insights from this alternative model led to the design of a short peptide mimetic of IFN-γ which could demonstrate the intracellular role we suspected IFN-γ fulfilled. The mimetic corresponds to a section of the C-terminus [IFN-γ(95-132)] that interacts with

the intracellular domain of IFNGR1 and includes the polycationic NLS motif, ^{126}RKRKRSK (11, 22). A lipophilic attachment, palmitate, was added to enable cell penetration (15, 23). This mimetic proved capable of forming a complex with STAT1α along with IFNGR1 in the cytoplasm and provided the NLS signaling for nuclear transport (22). Significantly, IFN-γ(95-132) possessed agonist activity including upregulation of MHC class II in macrophages and protection against viral infection, without toxicity, on macrophages similar to that of full length IFN-γ. (14, 19, 20, 23). Like IFN-γ, initial findings indicated the mimetic had a broad spectrum of effectiveness against viruses including large dsDNA viruses like HSV-1, a herpes family virus that replicates in the nucleus, and vaccinia virus (VV), a pox family virus that replicates in the cytoplasm, and encephalomyocarditis, a small ssRNA member of picornavaridae (24, 25, 26).

Viruses have developed a variety of mechanisms to antagonize the antiviral apparatus of IFN-γ. Poxviruses are particularly astute at thwarting the IFN system. These are large double-stranded viruses that replicate in the cytoplasm of the cell. The variola strain of the poxviruses is responsible for smallpox, which historically has been the causative agent of pandemics that have resulted in considerable loss of human life until the unprecedented campaign of global immunization (27). Eradication of smallpox was not, however the permanent exit of pox viridae from the global stage, as evidenced by recurrent outbreaks of monkeypox in Africa and recently in the United States (28) The assembly of poxviruses in the cytoplasm of infected cells is complex, involving the generation of four types of infectious virus particles (27). Attachment, internalization, and disassembling of poxviruses is followed by initiation of three waves of mRNA

synthesis. The first or early wave codes for virus growth factors and decoy cytokine receptors. Decoy receptors for both type I and type II IFNs are produced during early protein synthesis in poxvirus-infected cells. An important virulence factor of poxviruses is the B8R protein, which is a homolog of the extracellular domain of the IFN-γ receptor and can therefore bind to intact IFN-γ and prevent its interaction with the receptor (29). The rodent picornovirus, encephalomyocarditis virus (EMCV), has an extremely wide range of hosts including humans (30, 31, 32). Instances of human infection with EMCV have manifested as generalized febrile illness, but the virus has also been isolated from patients with more-severe illnesses, such as encephalitis, meningitis, and cardiomyopathy (31, 33). In mice, EMCV infection is lethal (32, 34, 35).

We characterized the antiviral effects of the IFN-γ(95-132), with and without the NLS region, and evaluated the therapeutic activity of the IFN-γ mimetic peptide in vivo in the presence and absence of B8R protein in models of lethal EMCV and VV infection in mice (25, 26). The peptide mimetics act intracellularly to activate the JAK/STAT signaling apparatus and do not recognize the IFN-γ receptor extracellular domain and therefore bypass interactions with virulence factors like B8R (23).

A family of proteins called suppressors of cytokine signaling (SOCS) negatively regulates JAK/STAT signaling (36, 37, 38). These inducible proteins share domains of homology that characterize the SOCS family. SOCS proteins are also negative regulators of signaling by other cytokines, growth factors, and hormones (39, 40, 41). There are currently eight identified members of the SOCS family, SOCS-1 to SOCS-7 and cytokine-inducible SH2 protein. SOCS-1 is of particular interest, because it is a

negative regulator of JAK2 as well as several other cytokines and hormone receptor systems, including epidermal growth factor receptor (EGFR) (42).

We designed a short 12-mer peptide, WLVFFVIFYFFR, which antagonizes IFN-γ signal transduction by binding to the autophosphorylation site of JAK2, resulting in inhibition of its autophosphorylation as well as its phosphorylation of IFNGR subunit IFNGR-1 (43). Tkip specifically recognizes the autophosphorylation sequence 1001–1013 containing the phosphotyrosine residue (pY1007) in the activation loop of JAK2 similar to SOCS-1 KIR. From the SOCS1 kinase inhibitory region (KIR) we also designed another peptide corresponding to residues ^{53}DTHFRTFRSHSDYRRI near its N-terminus, which we called SOCS1-KIR and showed that it has properties similar to Tkip (44). Tkip and SOCS1-KIR inhibit JAK2 phosphorylation activity and activation of STAT1α. IL-6 activity is similarly suppressed by Tkip and SOCS1-KIR via inhibition of JAK2 phosphorylation of STAT3 (41, 45). The proliferation of prostate cancer cells, which depend on activation of the oncogene STAT3, were similarly blocked by Tkip inhibition of JAK2. Hence, these peptide antagonist of IFN-γ signaling appear to have anti-inflammatory and antitumor properties. We have shown that the kinase inhibitor region (KIR) of SOCS-1 interacts with the autophosphorylation site of JAK2 (44, 46)

We have tested Tkip in a murine model of multiple sclerosis (MS) called experimental allergic encephalomyelitis (EAE). (46) In the EAE model, immunization of mice with CNS antigens such as myelin basic protein (MBP), proteolysis protein, and myelin oligondendrocyte glycoprotein results in tail and limb paralysis due to lymphocytic infiltration and demyelination in the CNS (47, 48). It was widely felt that Th1 cells driven by the cytokine interleukin-12 (IL-12) were primarily responsible for the CNS

pathology of MS (49). Inflammatory cytokines that employ the JAK/STAT pathway such as IFN-γ, TNF-α and IL-2 were under this model implicated in the pathogenesis of both EAE and MS. (50-54). More recently, IL-17 producing CD4+ T (Th17) cells have supplanted Th1 cells as the primary cause of MS (49, 55-58). During our investigation into the binding specificity of SOCS-1 KIR and Tkip we created another peptide, pJAK2(1001-1013). pJAK2(1001–1013) corresponded to the activation loop of JAK2, and we demonstrated that it blocked SOCS-1 activity in cells (44). The ability of pJAK2(1001-1013) to function as an antagonist of SOCS1 was reflected by its ability to function as an agonist of IFN type I and type II signaling through the JAK/STAT pathway. Specifically, pJAK2(1001–1013) enhances suboptimal IFN activity, blocks SOCS-1 induced inhibition of STAT3 activation, enhances IFN-γ activation site (GAS) promoter activity, and enhances Ag-specific proliferation.

It would be a great benefit for patients to receive the bonus of an adjuvant effect, and therefore long lasting immunity, as a side effect from a therapeutic already being used. The adjuvant effects of IFN-γ are well known, and have been shown in various animals (59-64). Its upregulation of MHC, and modulations of APCs, as well as stimulation of B and CD8+T cells and effect on toll-like receptors (TLR)s have long since been identified with its performance as an adjuvant in both cellular and humoral arms. The ability to enhance immune memory is desirable as it plays an important role in effective vaccines. This is especially true in scenarios where causative agents, or their components, present as weak antigens to the immune system. Such is often the case with tumors, which, in the absence of an adjuvant, are very difficult to mount any response against. Recently, tuberculosis patients that have resistance to standard

chemotherapy, have experienced a beneficial effect of IFN-γ when it is used as adjuvant in treatment (64). As IFN-γ(95-132) has previously shown similarity to the effects expressed by native IFN-γ, it followed that its mimetic and its agonist, pJAK2, could also boost adjuvancy and were examined in a model with strong antigens, as with VV, and with weak ones, as with bovine serum albumin (BSA). These were quantified both by splenocyte proliferation and antibody production after a delayed second exposure. Also, because JAK2 is a link in the Toll like receptor 3 (TLR3) signaling cascade, which serves both innate and adaptive arms, we tested to see whether this peptide had an adjuvant effect independent of Tcells. Contrastingly, we show that the JAK2 antagonism of SOCS1-KIR, as well as Tkip, inhibit IFN-γ-induced macrophage activation, and some of the underlining mechanisms associated with the formation of immune memory.

CHAPTER 2
MATERIALS AND METHODS

Cell culture, virus, B8R protein, and interferons. Murine L929 fibroblasts were from the American Type Culture Collection (Manassas, VA) and maintained in Eagle's minimal essential medium (JHR Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium) at 37°C in a 5% CO2 atmosphere incubator. Raw 264.7 were maintained in RPMI 1640 medium (SAFC Biosciences) supplemented with 10% FBS (HyClone), 100 U/ml penicillin and 100 U/ml streptomycin (complete medium). EMCV was obtained from the American Type Culture Collection and stored at -70°C until use. Vaccinia virus Western Reserve was a gift from Dr. Richard Condit (University of Florida, Gainesville, FL). The B8R protein was a kind gift from Tilahun Yilma (University of California, Davis). Rat and mouse IFN-gamma (both at 10^7 U/mg) were purchased from PBL Biomedical Laboratories (Piscataway, NJ) and kept at -70°C until use.

Peptide synthesis. All peptides [IFN-γ(95-132), IFN-γ(95-125), IFN-γ(95-106), IFNGR1(253-287), Tkip, SOCS1-KIR, pJAK2(1001-1013)] were synthesized with an Applied Biosystems 9050 automated peptide synthesizer (Foster City, CA) using conventional fluorenylmethyloxycarbonyl chemistry as described previously (18, 19, 79). IFN-γ(95-132) peptide was synthesized without the cysteine residue at the C-terminal end (residue no. 133). The addition of a lipophilic group (palmitoyl-lysine) to the N termini of the synthetic peptides was performed as the last step by using a semi-automated protocol. Peptides were characterized by mass spectrometry and purified by high-performance liquid chromatography. Peptides were dissolved in deionized water

and used for experimentation. All peptide solutions were negative for endotoxins as determined by a Limulus amebocyte lysate test (E-toxate kit; Sigma, St. Louis, MO).

Antiviral assays. Antiviral assays were performed by using a cytopathic effect reduction assay with EMCV. Murine L929 cells (6 x 10^4cell/well) were plated in a 96-well plate and grown overnight to confluence for optimal growth. Various concentrations of IFN-γ, IFN-γ(95-132), pJAK2(1001-1013), derivatives, and control peptides were incubated for various times (7 to 24 h) at 37°C. In other experiments, B8R protein (33 μg/ml) was preincubated for 2 h with rat IFN-γ and IFN-γ mimetic and control peptides. EMC virus (200 PFU/well) was added to the plate and incubated for 1 h, after which plates were washed and media added. Cells were incubated for at least 24 h and then stained with 0.1% crystal violet. Unbound crystal violet was aspirated, and the plates were thoroughly rinsed with deionized water, blotted, and allowed to air dry. Plates were then scanned using an Astra 2100U flatbed computer scanner (UMAX Technologies, Dallas, TX) and analyzed using Image J 1.29 software (National Institutes of Health, Bethesda, MD) to assess cell survival. Percentages of cell survival were determined by comparing survival for the experimental treatment groups with that for the virus-only control group.

Macrophage activity. Murine macrophage cells, Raw 264.7, were seeded on 24-well plates at a concentration of 3 x 10^5 cells/well (300 μl/well) and allowed to adhere. Varying concentrations of lipo peptides, Tkip, SOCS1-KIR, or muIFN-γ, IFN-γ(95–106), were then added to the wells and the cells incubated for2hat 37°C in a 5% CO2 incubator. Varying concentrations of IFN-γ were then added and the cells were incubated for an additional 72 h at 37°C in a 5% CO2 incubator, after which

supernatants were transferred to fresh tubes and assayed for nitrite levels as a measure of NO production using Griess reagent, according to the manufacturer's instructions (Alexis Biochemicals). To test for synergy between Tkip and SOCS1-KIR, the cells were incubated in the presence of IFN-γ and varying concentrations of peptides as described above and also in the presence of both lipo-Tkip and lipo-SOCS1-KIR or lipo-Tkip and lipo-MuIFN-γ(95–106) and were collected after 48 h and tested for NO production as described above.

MBP-specific proliferation assay. SJL/J mice were immunized with bovine myelin basic protein (MBP) as previously described (46), and spleens were extracted and homogenized into a single-cell suspension. Splenocytes (1 x 10^5 cells/well) were incubated with medium, MBP (50 μg/ml), lipo-Tkip, lipo-SOCS1-KIR, or lipo-MuIFNGR (253–287) for 48 h at 37°C in 5% CO2. The cultures were pulsed with [^{3}H]thymidine (1.0 μCi/well; Amersham Biosciences) 18 h before harvesting onto filter paper discs using a cell harvester. Cell-associated radioactivity was quantified using a β-scintillation counter, and data are reported as counts per minute.

In vivo studies of mice. One-year-old or younger (C57BL/6) or (SJL/J) mice were purchased from Jackson Laboratories and cared for at the Animal Resource Center of the University of Florida (Gainesville, FL). The Institutional Animal Care and Use Committee (IACUC) of the University of Florida approved all protocols prior to any study initiation. For EMCV experiments, female C57BL/6 mice were pretreated by intraperitoneal injection using a tuberculin syringe for 3 or 6 days with rat or mouse IFN-γ and IFN-γ(95-132) and control peptides at various concentrations (100 to 200 μg/mouse) every day. In some studies, B8R protein (250 μg/ml, diluted from crude stock

preparation) was preincubated with the rat IFN-γ (200 U/mouse) and peptide (100 μg/mouse) injection cocktails prior to intraperitoneal administration. On the last day of treatment, mice were challenged with 50 PFU of EMC virus. The numbers of surviving mice were recorded starting on the day of EMC virus challenge (day 0) and are presented as percent survival. Ten mice per treatment group were used in all mouse studies.

For Vaccinia experiments, female C57BL/6 mice (6–8 wk old) were used. Peptides dissolved in PBS in a volume of 100 μL were administered i.p. Vaccinia was administered i.p. in a volume of 100 μL. For intranasal administration, vaccinia virus was taken in a volume of 10 μL, and 5 μL were delivered in each of the nostrils of a lightly anesthetized mouse. After infection, mice were observed daily for signs of disease, such as lethargy, ruffled hair, weight loss, and eye secretions. Moribund mice were euthanized and counted as dead.

Measurement of anti-vaccinia, or anti-BSA Ab response by ELISA Microtiter plates were coated with 10^6 PFU of either purified UV-inactivated vaccinia virus (900,000 μJ/cm^2 for 5 min in a DNA cross-linker), or 0.5μg BSA in 100 μL of binding buffer (carbonate-bicarbonate, pH 9.6) overnight at 4°C. Plates were blocked for 2h at room temperature with PBS containing 5% FBS. Mouse sera (n = 5) were serially diluted in PBS containing 0.1% Tween 20 (wash buffer); 0.1 ml of the diluted serum was added to each well. The plate was incubated for 2h at room temperature and washed three times with wash buffer. Peroxidase-conjugated goat anti-mouse IgM (micro-chain specific), or IgG (γ-chain specific), both from Santa Cruz Biotechnology, diluted at 1/2000 in a volume of 0.1 ml was added to each well, incubated for 1 h, and washed

five times with wash buffer. o-Phenylenediamine (Pierce) in a volume of 0.1 ml was added and incubated for 15 min. The reaction was stopped by addition of 50 µl of 3 N HCl. The OD490 was determined using a microtiter plate reader.

Measurement of vaccinia virus-specific or BSA-specific cellular response by proliferation assay. Spleens from naive or pre-inoculated mice, at times indicated, were homogenized to single-cell suspension. Splenocytes (10^5 cells/well) were incubated with medium alone or medium containing UV-inactivated vaccinia virus, or BSA depending on the experiment, at 37˚C for 96 h. The cultures were then pulsed with [^{3}H]thymidine (1 µCi/well; Amersham Biosciences, Piscataway, NJ) for 8 h before harvesting onto filter paper discs using a cell harvester. Cell-associated radioactivity was counted using a scintillation counter. Stimulation index refers to the incorporation in splenocytes cultured with test Ag divided by incorporation in splenocytes cultured with medium alone.

CHAPTER 3
RESULTS

The Gamma Interferon (IFN-γ) Mimetic Peptide IFN-γ(95-132) Prevents Encephalomyocarditis Virus Infection both in Tissue Culture and in Mice

We have demonstrated previously that the C-terminal γ interferon (IFN-γ) mimetic peptide consisting of residues 95 to 132 [IFN-γ(95-132)], which contains the crucial IFN-γ nuclear localization sequence (NLS), has antiviral activity in tissue culture. Here we evaluate the efficacy of this peptide and its derivatives first in vitro and then in an animal model of lethal viral infection with the encephalomyocarditis virus (EMCV). Deletion of the NLS region from the IFN-γ mimetic peptide IFN-γ(95-132) resulted in loss of antiviral activity. However, the NLS region does not have antiviral activity in itself. Replacing the NLS region of IFN-γ(95-132) with the NLS region of the simian virus 40 large T antigen retains the antiviral activity in tissue culture. IFN-γ(95-132) prevented EMCV-induced lethality in mice in a dose-dependent manner compared to controls. Mice treated with IFN-γ(95-132) had no or low EMCV titers in their internal organs, whereas control mice had consistently high viral titers, especially in the heart tissues. Injection of B8R protein, which is encoded by poxviruses as a defense mechanism to neutralize host IFN-γ, did not inhibit IFN-γ(95-132) protection against a lethal dose of EMCV, whereas mice treated with rat IFN-γ were not protected. The data presented here show that the IFN-γ mimetic peptide IFN-γ(95-132) prevents EMCV infection in vivo and in vitro and may have potential against other lethal viruses, such as the smallpox virus, which encodes the B8R protein.

Antiviral activity of IFN-γ(95-132) peptide in the presence of the B8R protein in tissue culture and in vivo. Since we have shown previously that IFN-γ 95-132) binds to the cytoplasmic domain of the IFN-γ receptor and thus triggers signal

transduction events associated with IFN-γ, this mimetic peptide was evaluated in the presence of the B8R protein, which is produced by poxviruses for neutralization of IFN-γ activity. The B8R protein is a homolog of the IFN-γ receptor extracellular domain; therefore, IFN-γ 95-132) should retain its antiviral activity in the presence of this virulence factor of poxviruses. To demonstrate this in tissue culture, murine L929 cells were plated to confluence on a 96-well plate, after which rat IFN-γ, IFN-γ 95-132), and IFN-γ 95-125), which were all preincubated for 2 h with B8R protein, were added to the plate. Rat IFN-γ was used here instead of mouse IFN-γ due to the fact that B8R does not bind to mouse IFN-γ but does bind to rat IFN-γ (65). Furthermore, rat IFN-γ has activity on mouse cells, as shown previously (65). After overnight incubation, EMCV was added, and the cells were washed and reincubated in media for 24 h, followed by determination of EMCV cytopathic effects. As shown in Fig. 3-1A, IFN-γ had antiviral activity against EMCV at all concentrations (100 U/ml, 33 U/ml, and 11 U/ml) used, but in the presence of B8R, IFN-γ antiviral activity was lost at 33 U/ml and 11 U/ml. In contrast, the antiviral activities of IFN-γ(95-132) were similar in the presence or absence of B8R protein, as denoted by almost 100% cell viability at the 11 μM peptide concentration used (Fig. 3-1A and B). The control peptide IFN-γ 95-125) did not have any antiviral activity in either case.

Thus, B8R protein neutralized IFN-γ antiviral activity but not IFN-γ (95-132) antiviral activity against EMCV in tissue culture. Based on the above-described tissue culture study, the antiviral activity of IFN-γ(95-132) was assessed in the presence of B8R protein in the mouse model of lethal EMCV infection. C57BL/6 mice were pretreated for 3 days with PBS, IFN-γ(95-132) (100 μg/day), or rat IFN-γ (200 U/day).

The 200-U/day dose of rat IFN-γ, which still had significant antiviral activity, was administered in order to detect the neutralizing effect of the B8R protein. On the last day of treatment, mice were challenged with 50 PFU of EMCV. The numbers of surviving mice were recorded over time. As shown in Fig. 3-2, injection of rat IFN-γ resulted in 20% survival of mice in response to EMCV challenge. In contrast, administration of B8R with rat IFN-γ resulted in 0% of mice surviving, which was similar to results for PBS controls. Administration of IFN-γ(95-132) in the presence or absence of B8R resulted in 40% and 30% survival, respectively, 9 days after the EMCV challenge. Furthermore, there was a delay in the onset of lethality in IFN-γ(95-132)- and rat IFN-γ-treated groups compared to results for PBS- and IFN-γ/B8R-treated groups. Thus, the IFN-γ mimetic was effective against EMCV in the presence of B8R.

I additionally contributed to the characterization of IFN-γ mimetic by assisting with injection, observation, non-survival surgeries and euthanization of C57BL/6 mice which were challenged with EMCV. I conducted and assisted in various cytopathic effect reduction assays, and virus yield reduction and plaque assays all involving EMCV. For the above procedures which involved tissue I maintained murine L929 fibroblast cell lines.

IFN Mimetic as a Therapeutic for Lethal Vaccinia Virus Infection: Possible Effects on Innate and Adaptive Immune Responses

We have developed small peptide mimetics of IFN-γ that can bypass the poxvirus virulence factor B8R protein, which binds to intact IFN-γ and prevents its interaction with receptor extracellular domain. Thus, these peptides inhibit vaccinia virus (VV) replication in cell culture where intact IFN-γ is ineffective. We demonstrate here that the mouse IFN-γ-mimetic peptide, IFN-γ(95–132), protects C57BL/6 mice against

overwhelming lethal VV infection. The mimetic peptide was synthesized with an attached lipophilic group for penetration of cell plasma membrane. Injection of mimetic i.p. before and at the time of intranasal (10^6 PFU) or i.p. (10^7 PFU) challenge with virus resulted in complete protection at 200 μg of mimetic and 40–60% protection at 5 μg of mimetic. Initiation of treatment of mice with IFN-γ mimetic up to 2 days postinfection resulted in complete protection against death, whereas initiation of treatment at 6 days postinfection resulted in 40% protection. Administration of mimetic by the oral route also completely protected mice against the intranasal route of a lethal dose of VV challenge. In addition to its direct antiviral effect, the mimetic also possessed adjuvant effects in boosting humoral and cellular immunity to VV. The combination of antiviral and adjuvant effects by the IFN mimetic probably plays a role in its potent anti-VV properties. These results suggest an effective therapeutic against ongoing, lethal poxvirus infections that taps into innate and adaptive host defenses.

IFN-mimetic IFN-γ(95–132) possesses adjuvant activity. We were interested in determining whether the IFN mimetic possessed adjuvant activity that may have contributed to the anti-VV immune response in protecting mice. Thus, mice were infected with a sub-lethal dose of VV in the presence of mimetic or control peptide, and the cellular and humoral immune response to virus were monitored. Mice were injected i.p. with peptides at days -2, -1, and 0 relative to the intranasal challenge with 10^4 PFU of VV. Proliferation in the presence of VV was determined with splenocytes at 4 wk postinfection. As shown in Fig. 3-3A, both lipo-IFN-γ(95–132) and nonlipo-IFN-γ(95–132) treated murine splenocytes showed greater proliferation in the presence of VV than those of the control peptide-treated mice, with the lipophilic form being more

effective. An unrelated peptide from residues 253 to 287 from IFNGR1 with a lipophilic residue was used as a control peptide in this experiment. Thus, the mimetic in either a lipophilic state for cell penetration or in a nonlipophilic state enhanced the proliferation of splenocytes from mice infected with VV. Uptake of a nonlipophilic peptide suggests uptake required pinocytosis by antigen presenting cells (APC)s. Since proliferation for the nonlipophilic group corresponded closely to the lipophilic group, we can speculate that APCs may account for most of the proliferation effect, and additionally that this may be the route of mimetic action.

VV is a potent Ag and thus may possess intrinsic adjuvant activity. This in turn could mask some of the adjuvancy of the mimetic. We thus determined the adjuvancy of the IFN mimetic against a soluble protein, BSA, focusing on the humoral response. As shown in Fig. 3-3B, lipo-mimetic-treated mice showed significant increases in anti-BSA IgG Ab by ELISA at weeks 2 through 4 when compared with the BSA treatment alone. Mice treated with lipo-control peptide from residues 95 to 106 had Ab responses similar to that of BSA alone. The IFN mimetic thus possesses adjuvant effects against VV and BSA Ags.

Both the Suppressor of Cytokine Signaling 1 (SOCS-1) Kinase Inhibitory Region and SOCS-1 Mimetic Bind to JAK2 Autophosphorylation Site: Implications for the Development of a SOCS-1 Antagonist

Suppressor of cytokine signaling (SOCS)-1 protein modulates signaling by IFN-γ by binding to the autophosphorylation site of JAK2 and by targeting bound JAK2 to the proteosome for degradation. We have developed a small tyrosine kinase inhibitor peptide (Tkip) that is a SOCS-1 mimetic. Tkip is compared in this study with the kinase inhibitory region (KIR) of SOCS-1 for JAK2 recognition, inhibition of kinase activity, and regulation of IFN-γ-induced biological activity. Tkip and a peptide corresponding to the

KIR of SOCS-1, ^{53}DTHFRTFRSHSDYRRI (SOCS1-KIR), both bound similarly to the autophosphorylation site of JAK2, JAK2(1001–1013). The peptides also bound to JAK2 peptide phosphorylated at Tyr1007, pJAK2(1001–1013). Dose-response competitions suggest that Tkip and SOCS1-KIR similarly recognize the autophosphorylation site of JAK2, but probably not precisely the same way. Although Tkip inhibited JAK2 autophosphorylation as well as IFN-γ-induced STAT1α phosphorylation, SOCS1-KIR, like SOCS-1, did not inhibit JAK2 autophosphorylation but inhibited STAT1α activation. Both Tkip and SOCS1-KIR inhibited IFN-γ activation of Raw 264.7 murine macrophages and inhibited Ag-specific splenocyte proliferation. The fact that SOCS1-KIR binds to pJAK2(1001–1013) suggests that the JAK2 peptide could function as an antagonist of SOCS-1. Thus, pJAK2(1001–1013) enhanced suboptimal IFN-γ activity, blocked SOCS-1-induced inhibition of STAT3 phosphorylation in IL-6-treated cells, enhanced IFN-γ activation site promoter activity, and enhanced Ag-specific proliferation. Furthermore, SOCS-1 competed with SOCS1-KIR for pJAK2(1001–1013). Thus, the KIR region of SOCS-1 binds directly to the autophosphorylation site of JAK2 and a peptide corresponding to this site can function as an antagonist of SOCS-1.

Tkip and SOCS-KIR inhibit IFN-γ-induced activation of macrophages. IFN-γ plays an important role in activation of macrophages for innate host defense against intracellular pathogens as well as serving to bridge the link between innate and adaptive immune responses (1). Tkip and SOCS1-KIR were examined for their ability to block IFN-γ activation of the macrophage cell line Raw 264.7 as determined by inhibition of nitric oxide (NO) production using Griess reagent (Alexis Biochemicals). Lipo versions of the peptides were synthesized with palmitic acid for penetration of the cell membrane

(66). Both Tkip and SOCS-KIR, compared with control peptide, inhibited induction of NO by various concentrations of IFN-γ as shown in Fig. 3-4A. Dose response with varying concentrations of the peptides against IFN-γ (6 U/ml) resulted in increased inhibition of NO production by Tkip and SOCS1-KIR with Tkip being the more effective of the inhibitors as shown in Fig. 3-4B. Control peptide was relatively ineffective at inhibition, providing evidence for the specificity of Tkip and SOCS1-KIR inhibition. Tkip and SOCS1-KIR in combination (33 μM each) were the most effective in inhibition of IFN-γ induction of NO in macrophages. This synergy may reflect differences in recognition of the autophosphorylation site of JAK2 by the two peptides. Thus, Tkip and SOCS1-KIR both inhibited IFN-γ induction of NO in macrophages with Tkip being the more effective inhibitor.

Tkip and SOCS1-KIR inhibit Ag-specific lymphocyte proliferation. We have previously shown that Tkip inhibits Ag-specific proliferation of mouse splenocytes in vitro (25). Specifically, Tkip inhibited proliferation of splenocytes from mice immunized with bovine MBP. In this study, we compared Tkip and SOCS1-KIR for their relative ability to inhibit proliferation of MBP-specific splenocytes in cell culture. Splenocytes (3 x 10^5cells/well) were incubated with MBP (50 μg/ml) in the presence of lipo-Tkip, lipo-SOCS1-KIR, or lipo-control peptide for 48 h and proliferation was assessed by testing for [^{3}H] thymidine incorporation. As shown in Fig. 3-5, both Tkip and SOCS1-KIR inhibited MBP-induced proliferation of splenocytes, while the control peptide had a negligible effect on proliferation. Similar to inhibition of NO production by macrophages, Tkip was more effective than SOCS1-KIR in inhibition of MBP-induced splenocyte proliferation with 84, 88, and 97% inhibition at 1.2, 3.7, and 11 μM, respectively,

compared with 61, 67, and 72% for SOCS1-KIR. Thus, both Tkip and SOCS1-KIR inhibited Ag-induced splenocyte proliferation, which is consistent with SOCS-1 protein inhibition of Ag-specific lymphocyte activity (67).

I contributed to the Tkip, SOCS1-KIR experiments by maintaining LNCaP and murine macrophage cells (RAW 267.4). I helped in peptide preparation and dilution, antiviral assays, and immunoblot analysis of STAT1α.

I cooperated with Dr. Mujtaba in the EAE experiments on SJL/J mice in which we found SOCS1-KIR could protect mice from relapsing/remitting EAE. In this experiment I administered injections of the various peptide and control species, as well as MBP immunizations to induce disease. Throughout the experiment I assisted in the supervision of mice, the progression of their disease, and some of the post-mortem surgeries, such as splenectomies.

I also assisted Dr. Mujtaba when we examined SOCS1-KIR in a role of prevention and reversal of lymphocyte infiltration into the CNS during EAE. Here, we harvested the brains from three individual mice, one naïve, and two immunized with MBP. The two MBP mice had been treated every other day for 38 days post-immunization, one with PBS (60µg), and the other with SOCS1-KIR2A (60µg). The three mice were sacrificed, their brains removed to a 4% PFA in PBS and fixed overnight before being transferred to 70% ethanol. Brains were embedded in paraffin, sliced, and stained with H&E for imaging.

In addition to work directly associated with the Tkip model, I helped train other members of our lab in animal handling, injection, anesthetization, blood sampling, organ harvesting, euthanasia and other often difficult, intimidating, and precarious

experimental procedures which revolve around live, willful animals during in vivo experimentation.

Enhancement of Antiviral Immunity by Small Molecule Antagonist of Suppressor of Cytokine Signaling

Suppressors of cytokine signaling are negative regulators of both innate and adaptive immunity via inhibition of signaling by cytokines such as type I and type II interferons (IFN)s. We have developed a small peptide antagonist of SOCS-1 that corresponds to the activation loop of JAK2. SOCS-1 inhibits both type I and type II IFN activities by binding to the kinase activation loop via the kinase inhibitory region of the SOCS. The antagonist, pJAK2(1001–1013), inhibited the replication of VV and EMCV in cell culture, suggesting that it possesses broad antiviral activity. In addition, pJAK2(1001–1013) protected mice against lethal VV and EMCV infection. pJAK2(1001–1013) increased the intracellular level of the constitutive IFN-γ, which may play a role in the antagonist antiviral effect at the cellular level. Ab neutralization suggests that constitutive IFN-γ may act intracellularly, consistent with recent findings (19). pJAK2(1001–1013) also synergizes with IFNs as per IFN-γ mimetic to exert a multiplicative antiviral effect at the level of transcription, the cell, and protection of mice against lethal viral infection. pJAK2(1001–1013) binds to the kinase inhibitory region of both SOCS-1 and SOCS-3 and blocks their inhibitory effects on the IFN-γ activation site promoter. In addition to a direct antiviral effect and synergism with IFN, the SOCS antagonist also exhibits adjuvant effects on humoral and cellular immunity as well as an enhancement of polyinosinic-polycytidylic acid (poly I:C) activation of TLR3. The SOCS antagonist thus presents a novel and effective approach to enhancement of host defense against viruses.

pJAK2(1001–1013) exerts an adjuvant effect on the immune system. In addition to its inhibitory effects on virus replication in cells and related to the potent anti-VV response, we were interested in determining possible adjuvant effects of SOCS-1 antagonist on the immune response. Accordingly, C57BL/6 mice were immunized i.p. with 50 μg BSA, treated i.p. with 200 μg pJAK2(1001–1013) on days -2, -1, and 0, and then assessed for enhancement of cellular and humoral immune responses. BSA is a relatively weak Ag in mice. Four weeks postimmunization, splenocytes from the mice were stimulated in cell culture with 0.5 μg BSA. As shown in Fig. 3-6A, untreated mice or mice given PBS mounted a weak proliferation response. By comparison, mice treated with pJAK2 (1001–1013) had an ~8-fold greater proliferative response to BSA. The humoral immune response as assessed by the serum IgG Ab response to BSA in the mice was also significantly enhanced in the pJAK2(1001–1013)-treated mice at 3 and 4 weeks postimmunization (Fig. 3-6B). The SOCS antagonist can also enhance the Ab response to the T cell-independent Ag LPS. This is shown in Fig. 3-6C, in which the Ab response of mice immunized with LPS was significantly enhanced by i.p. injection of pJAK2(1001–1013). We previously showed that staphylococcal enterotoxin superantigens, staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB), enhanced T cell-dependent Ab production (68). SEA/SEB did not enhance the anti-LPS response. SEA/SEB did enhance the Ab response to BSA, a T cell-dependent Ag. Thus, the SOCS antagonist has a direct effect on B cell function independent of T helper cells. At the level of macrophage TLR function, RAW264.7 cells treated with pJAK2(1001–1013) produced a ~5-fold increase in NO production upon LPS stimulation (via TLR4) compared with a control peptide (Fig. 3-7A). We also examined the effect of

pJAK2 (1001–1013) on TLR3 activation. Poly I:C is a synthetic dsRNA that activates macrophages and dendritic cells via TLR3 (69). TLR3 thus plays an important role in the antiviral responses to HSV-1, influenza virus, CMV, and respiratory syncytial virus, all of which have a dsRNA stage in their replication (32, 33). SOCS-1 negatively regulates TLR signaling at several stages including signaling by type I IFNs and by NF-κB transcription factor (45, 72, 73, 74). Given the importance of TLR3 in the innate immune response against viruses, we treated the macrophage cell line RAW264.7 with poly I:C and determined the enhancing effect of the SOCS-1 antagonist pJAK2(1001–1013) on NO production. As shown in Fig. 3-7B, poly I:C at 0.1 μg/ml had a negligible effect on NO production, which was increased >20-fold by 25 μM pJAK2 (1001–1013). Alanine-substituted JAK2(1001–1013)2A had a negligible effect on NO production. Thus, the SOCS-1 antagonist enhanced the poly I:C activation of TLR3. The SOCS-1 effect would suggest that SOCS-1 induction has a regulatory effect on TLR3 activation and that the SOCS antagonist blocks this effect. These results demonstrate that pJAK2(1001–1013) has an adjuvant effect in terms of the cellular and humoral immune responses as well as in macrophage activation. Thus, in addition to direct inhibition of virus replication, the antagonist also has an adjuvant effect on the immune response.

I additionally contributed to many other levels of the pJAK2 investigations, performing numerous antiviral assays that assessed the strength and toxicity of pJAK2 in L929 cell cultures. Here, cells treated with various doses of peptides, including pJAK2 and IFNγ(95-132), were challenged with EMCV or VV. I also reassessed EMCV and VV viral stocks for their infectivity. I ran other experiments, especially in animal models, to assess peptide utility, including the IFNγ-mimetic and Tkip. The protective

abilities of the peptides were tested in VV in and sepsis mouse models. I investigated and quantified the interference associated with DMSO which we used to dissolve relatively insolvent peptides pJAK2, IFNγ(95-132), Tkip and related controls. DMSO is a known anti-inflammatory, and could compete with the inflammatory or anti-inflammatory natures of our peptides. I revealed the basal 'noise' which consistently arose from supposedly inactive controls which were dissolved in DMSO, suggesting an alternative solution be used. I also ran a number of assays and mouse experiments comparing the therapeutic effects of IFN-γ mimetic, and the superantigens, SEA and SEB, in an EMCV model which may have provided small insights.

CHAPTER 4
DISCUSSION

Interferon-gamma is central to the development and character of the host defense, responsible for targeting a wide scope of immunologically relevant genes that range from the initiation of innate cellular programs, to the local recruitment and activation of leukocytes, to the selection and maturation of adaptive immune effecter and memory cells (97). As a potent cytokine, IFN-γ is subject to intensive positive and negative regulation from the host and also from pathogens. While control of IFN-γ is a necessary dimension to the host's immune system in order for it to avoid the excessive inflammation and pathology associated with IFN-γ dysregulation, control of IFN-γ can similarly be seen as one of the essential attributes of successful pathogens such as poxviruses, *Mycobacterium*, and *Leishmania* which, without virulence factors to antagonize IFN-γ, would otherwise fall prey to its downstream machinations (29, 75, 76).

Poxviruses are highly adept at evading the host innate immune response due to their many evasion genes and their resultant protein products (77). There are, for example, greater than 18 proteins produced by poxviruses that interfere with different aspects of the host defense, including the actions of IFNs, TNF, chemokines, interleukins, and others. Vaccinia virus (VV) produces decoy receptors to deal with type I and type II IFNs. These homologues of IFNGR, and IFNAR extracellular domain tie up the ligand in an effective dead end (77). Owing to its importance, the B8R gene is transcribed in the first wave of viral protein synthesis (29).

We have engineered small, peptide mimetics of IFN-γ corresponding to the C terminus amino acids 95 to 132 which possesses antiviral and agonist activity, and is

nontoxic to animals (23). These mimetics were developed contrary to the classical model of JAK/STAT signaling in which IFN-γ triggers a cascade by extracellular interaction. Rather the design for a C terminal IFN-γ mimetic is in following with the logic of direct intracellular signaling by IFN-γ (10, 78). IFN-γ, its receptor subunit IFNGR1, and transcription factor STAT1α, are delivered to the nucleus of cells as a conglomerate where IFN-γ, acting as a chaperone, provides a classical polycationic NLS for the nuclear importation of the group (14). Partial IFN-γ mimetics demonstrate the importance of the NLS as a component in this complex molecule. An NLS peptide, IFN-γ(126-132), and the truncated IFN-γ mimetic where the NLC has been removed, IFN-γ(95-125), were equally without antiviral activity in viral assays. Replacement of the IFN-γ(95-132) NLS region with the NLS of the SV40 large T antigen maintained the mimetics antiviral activity as assessed by its ability to protect cells from encephalomyocarditis virus (EMCV) in a cytopathic assay (25). This suggests that the NLS in IFN-γ is classic and functions through the components of the Ran/importin pathway utilized by the SV40 T antigen NLS for transportation into the nucleus (15, 16, 18). We have previously shown that IFN-γ(95-132) introduced into the cytoplasm was capable of forming a complex with IFNGR1 and STAT1α and that it provided the NLS signaling for nuclear transport like that of full length IFN-γ (80). Both mouse IFN-γ(95–132) and human IFN-γ(95–134) mimetics induced an antiviral state and up-regulated MHC class I molecules in cell culture (23).

Intracellular signaling begins when IFN-γ or its peptide mimetics bind to the residues 253 to 287 on the cytoplasmic domain of receptor subunit IFNGR1. This binding induces the tyrosine phosphorylation events catalyzed by JAK1 and JAK2

kinases that result in the phosphorylation and binding of STAT1α to the cytoplasmic domain of IFNGR1 (9). It may also be the case that JAK1 and JAK2, which are known to be initially attached to IFNGR1, remain attached and are carried on the IFN-γ/IFNGR/STAT1α vehicle into the nucleus where they could have some further role in the transcription of IFN-γ genes such as the phosphorylation of histones and enhancement of promoter binding (81, 82, C.M. Ahmed, E.N. Noon-Song, and H.M. Johnson, unpublished observations). Inside the nucleus, chromatin immunoprecipitations and reporter gene studies of IFN-γ and IFN-γ mimetic-treated cells find these ligands along with IFNGR1 and STAT1α, bound to the IFN-γ activation site element on the DNA and indicate that they participate in STAT1α-mediated transcription (83).

We have characterized IFN-γ(95–132) fitted with the membrane penetrating lipophilic group palmitate for its ability to counteract lethal viral disease in tissue cultures and C57BL/6 mice. In a dose dependent manner IFN-γ(95-132) protected L929 against EMCV challenge (25). In an animal model where EMCV had 100% lethality in controls, a three day prophylactic regimen of IFN-γ(95-132) resulted in 100% survival of mice, equal to that obtained by IFN-γ treatment. Internal tissues processed for viral titer 6 days postinfection showed little or no EMCV in the hearts, livers, and spleens of IFN-γ(95-132)-treated mice, opposite what we found for control peptide and PBS treatment groups. Major organs assayed for EMCV 13 days after inoculation showed that IFN-γ(95-132)-treated mice had achieved total clearance of the virus (25). The prophylactic effect observed in these experiments is probably the consequence of IFN-γ and the IFN-γ mimetic preparing an antiviral state within cells and generally inducing immune

system activity, for example the enhancement of immune surveillance. An additional experiment was devised to exhibit IFN-γ(95-132) intracellular mode of action wherein B8R virulence factor was combined with EMCV and added to cell cultures treated with IFN-γ or the mimetic. Consistent with our understanding that IFN-γ(95-132) activity was independent of the extracellular domain of IFNGR, it retained its protective effect where native IFN-γ was neutralized.

The strong antiviral activity IFN-γ(95-132) against EMCV like IFN-γ, and against EMCV+B8R in contrast to IFN-γ, encouraged us to apply the mimetic against a poxvirus model in mice. Treating a highly lethal VV infection with IFN-γ(95-132) was completely protective compared with the 100% fatality of controls, even for mice who had therapy withheld two days after viral ingress. Initiation of treatment as late as days 6 resulted in the recovery of 40% of the infected animals (26). By bypassing the virulence factor B8R, the IFN-γ mimetic was able to convert an aggressive infection in mice into a rather benign one.

In addition to the direct antiviral properties, IFN-γ95–132 also had an adjuvant effect on both the humoral and cellular immune response. Mimetic enhanced the IgG antibody (Ab) response to both VV and BSA. Cellular immunity to VV was also enhanced as determined by splenocyte proliferation. The adjuvancy of the mimetic was observed with or without the lipophilic group attached. Thus, it does not depend on mimetic penetration of the plasma membrane of nonphagocytic cells. Given that uptake of the mimetic by APCs does not depend on the lipophilic group, internalization by pinocytosis could play an important role in the enhanced of adaptive immune responses.

We have been particularly interested in regulation by the suppressors of cytokine signaling (SOCS) proteins that modulate interferons, including IFN-γ, among other cytokines and growth factors, as they attempt to signal through the JAK/STAT pathway. SOCS-1 function is requisite for an individual's survival past early infancy. Although SOCS-1 knockout mice appear to be normal at birth, they exhibit stunted growth and die as neonates in their third week (84). These mice suffer a syndrome characterized by severe lymphopenia, activation of T lymphocytes, fatty degradation and necrosis of the liver, hemopoietic infiltration of multiple organs, and high levels of constitutive IFN-γ as well as an abnormal sensitivity to it (41, 84, 85). IFN-γ likely plays a central role in the pathology since SOCS-1 knockout mice deficient in IFN-γ or IFNGR do not die as neonates. Similar pathology and lethality is observed in normal neonates that are injected in excess with IFN-γ.

SOCS-1 is transcribed alongside other IFN-γ targeted genes operating apparently in a negative feedback loop (45, 72, 73, 74, 86). The dynamics of induction of SOCS-1 by IFN-γ in cells and the activation of STAT1α is illustrative of how SOCS-1 attenuates IFN-γ functions under physiological conditions. For example, treatment of monocytes or astrocytes with IFN-γ was followed by activation of the SOCS-1 gene at ~90 min (87, 88). Low doses of IFN-γ resulted in transient increases in SOCS-1 mRNA that returned to baseline after 4 h, whereas high concentrations of IFN-γ resulted in increases of SOCS-1 mRNA up to 24 h. Thus, SOCS-1 suppression appears to be induced by the IFN-γ signal. Treatment of hepatocytes from SOCS-$1^{+/+}$ mice with IFN-γ resulted in STAT1α activation within 15 min, peaking at 2 h before declining (88). Although STAT1α is activated in SOCS-1-deficient livers in a similar fashion, it persists for 8 h.

SOCS-1 thus normalizes IFN-γ signaling, attenuating persistent activation of STAT1α, while still permitting a lower amplitude of signaling more likely to be beneficial.

The power to antagonize cytokines which depend on tyrosine kinases for signal transduction could have much therapeutic usefulness, especially in inflammatory disease where IFN-γ has been implicated as an aggressor. SOCS-1 supplementation used in conjunction with other modulaters may be able to abolish certain proinflammatory activity, or to restore equilibrium, as well as to affect long-term development within the adaptive arm, as within the T-helper subsets that are sensitive to the presence of certain cytokines and interleukins. Given the critical importance of SOCS-1 modulation of IFN-γ and other cytokines employing JAK2, we have developed small peptide mimetics of SOCS-1, Tkip and SOCS1-KIR (43, 44). Tkip and SOCS1-KIR recognized the autophosphorylation site on JAK2, specifically residues 1001–1013 including the critical tyrosine at 1007 which would be phosphorylated (44, 45). We showed that Tkip blocked JAK2 autophosphorylation as well as tyrosine phosphorylation of substrates such as STAT1α and the IFNGR chain, IFNGR1 (43). We subsequently showed that Tkip blocked IL-6-induced activation of the STAT3 oncogene in LNCaP prostate cancer cells, which involved inhibition of JAK2 activation (90). These studies presented a proof-of-concept demonstration of a peptide mimetic of SOCS-1 that regulates JAK2 tyrosine kinase function.

We chose EAE, the rodent model for MS, to test the potential of SOCS1-KIR and Tkip in an T-cell-mediated auto-immune disease considered to involve cytokines which depend on JAK2 (46). SJL/J mice were immunized with MBP for induction of the relapsing/remitting form of EAE. The SOCS-1 mimetic and KIR protected the mice

against relapses compared with control groups in which >70% of the mice relapsed after primary incidence of disease. Protection of mice correlated with lower MBP Ab titers in Tkip- and SOCS1-KIR-treated groups as well as suppression of MBP-induced proliferation of splenocytes taken from EAE-afflicted mice (44). Consistent with its JAK2 inhibitory function, the mimetics also inhibited the activity of inflammatory cytokine TNF-α, which uses the STAT1α transcription factor (44). Thus, Tkip and SOCS1-KIR, as with SOCS-1, possess anti-inflammatory activity that protect mice against ongoing relapsing/remitting EAE. More recent findings have indicated the additional involvement of the Th17 subset after its isolation from MS patients (90). Mice with EAE produce IFN-γ in addition to IL-17A when stimulated with IL-23. Pathogenic Th17 cells can be generated by a combination of IL-6, Il-23, and IL-1β (91). Not only will SOCS1-KIR and Tkip downregulate the IFN-γ production in these cells, but IL-23 also signals through JAK2 and is regulated by SOCS1-KIR/Tkip itself (L. Jager, R. Dabelic, and H.M. Johnson unpublished results). Treg cells, essential to the bodies maintenance of self-tolerance through their inhibitor effects on effecter cells, are also relevant to diseases like MS and EAE defined by self-reactive T-cells (92). It was thought that Treg and Th17 effectors arose in a mutually exclusive fashion, depending on whether they were activated in the presence of TGF-β or TGF-β plus IL-6 (90). Even more recent studies show plasticity between T cell phenotypes involves cytokines that differ from those previously considered the central players (93). IL-6 is still considered to be involved in the current formulas for Th17 generation. Since Tkip and SOCS1-KIR can suppress IL-6 transduction, it suggests cytokine antagonists like SOCS mimetics may have influence over active T cell phenotypes.

Tkip and SOCS1-KIR were also significant in their ability to blunt IFN-γ's adjuvant effect on cellular and humor immunity. Activated macrophages exhibit enhanced microbicidal behavior, as well as improved and expanded antigen presentation (1). IFN-γ has similar effects upon other APCs, and together they experience a highly suppressive effect when exposed to SOCS1-KIR or Tkip. This was determined by the SOCS mimetics ability to inhibit nitric oxide (NO) production, a telltale indicator of activation. NO production was blocked in RAW macrophages, especially so when Tkip and SOCS1-KIR were administered in tandem, an effect which reflects their different means of recognizing JAK2. Another experiment focused on SOCS1-KIR and Tkip hindrance of Ag-specific induction of splenocyte proliferation. The expansion of MBP sensitized splenocytes after stimulation was significantly reduced by the SOCS mimetics, with Tkip being the more effective inhibitor.

Regulation or control of the SOCS-1 modulatory arm of the immune system provides an approach to enhance host responses that would normally be suppressed or reduced. As we showed with the SOCS mimetics Tkip and SOCS1-KIR, the regulatory role of SOCS-1 extends to APCs and Ag presentation. Dendritic cells (DC)s are probably the most efficient cells at capturing, processing, and presentating Ags. In another study, knockdown of DC SOCS-1 by siRNA led to more effective cancer vaccination (94). Specifically, presentation of murine melanocyte differentiation Ag tyrosinerelated Ag 2 by DCs transfected with SOCS-1 siRNA protected mice against the well-established B16 melanoma tumor. The enhanced antitumor immunity was accompanied by enhanced tyrosine-related Ag 2-specific CTLs in protected mice as assessed by IFN-γ ELISPOT and CTL responses. The authors concluded that

regulation of Ag presentation by suppression of DC SOCS-1 showed promise for more effective tumor vaccines. Related to the SOCS-1 siRNA studies is the observation that SOCS-1$^{-/-}$ mice are more resistant to viral infection than their wild-type counterparts due to enhanced type I IFN activity involving the IFNAR1 receptor subunit (95).

The fact that the KIR of SOCS-1 can bind directly to pJAK2(1001–1013) raised the possibility that pJAK2(1001–1013) might function as an antagonist of SOCS-1. Four initial experiments explored the potential of pJAK2(1001-1013) to antagonize SOCS-1 function. First, pJAK2(1001–1013) enhanced suboptimal IFN-γ activity in EMCV infection. Second, prostate cancer cells transfected for constitutive production of SOCS-1 protein had reduced activation of STAT3 by IL-6 treatment. pJAK2(1001–1013) reversed the SOCS-1 effect. Third, pJAK2(1001–1013) enhanced IFN-γ activation of the luciferase reporter gene via the GAS promoter (44). Fourth, pJAK2(1001–1013) enhanced Ag-specific splenocyte proliferation. The fact that JAK2(1001-1013) not only has the ability to neutralize SOCS1, which impedes the pathways that develop adaptive immunity, but also contributes to cellular and humoral immunity directly as an IFN agonist, suggest it is a capable adjuvant This was further underlined by the apparant range of different routes through which pJAK2(1001-1013) could affect adaptive immunity.

Toll like receptors (TLRs) are key players in both the innate and adaptive arms of host defense and represent T-cell independent means of effecting immunity. TLRs signal through transcription factors such as NF-κB and are subject to SOCS1 regulation (73). pJAK2(1001-1013) working as an adjuvant could stimulate Ab production to LPS through TLR4 as observed by the downstream production of IgG by B-cells in a mouse

model where antibodies to LPS were insignificantly produced when LPS was coupled to the typically strong adjuvants, staphylococcal enterotoxins, which require Tcells. TLR3 functions in viral immunity to detect dsRNAs specifically, a signature of the replication of HSV-1, influenza, CMV and others. Treatment of the macrophages with poly I:C, synthesized dsRNA, in the presence of pJAK2(1001–1013) resulted in significant enhancement of NO production suggesting they had been activated through TLR3 which trigger type I IFNs, which upregulate NO, hydrogen peroxide, MHC, etc. The diversity in ways which pJAK2(1001-1013) can amplify a signal directed at specific cells of adaptive immunity, as through T-cells or APCs, not only amplifies an immune response by increasing the number of participating cells, but may also strengthen alternative routes to immune responses. This could be critical in a system that has been compromised in one capacity or another, as for example, cases of T cell depletion.

Another mechanism by which pJAK2(1001–1013) may exert direct antiviral effects has to do with a well-recognized but not fully understood aspect of IFN function in which cells constitutively produce low levels of intracellular IFN-β. Type I and type II interferons overlap in their signaling pathways and their genes (96). The signaling of one type of IFN will increase the frequency that mutual components are in circulation and therefore more readily available for use by other IFNs, as is the case with SOCS1 homodimers, ISGF3, and JAK2 (IFN OVERVIEW HUME) . IFNs may also benefit from the expression of shared genes, and cooperate to synergize or antagonize certain functions, for example the induction of the antiviral state (overview). Low level IFN-β activity is thought to keep the cell's signaling apparatus primed for potential interactions with other IFNs (98). We found that pJAK2(1001–1013) treatment increased intracellular

levels of IFN-β, and also STAT1 activation (99). pJAK2(1001-1013) coupled with IFN-γ(95-132) worked in synergy, and cells that received them in combination required lower doses for complete protection against EMCV and VV. Measurements made at the level of transcription demonstrated that the two IFN-γ agonists working synergistically to boost GAS promoter activation. The cooperative nature of the SOCS-1 antagonist and IFN appears to arise from the reduction of regulatory restraints imposed by SOCS-1 under normal physiological conditions as was indicated by reduced SOCS-1 levels in pJAK2(1001–1013) treated cells (99). The mechanism of this reduction is currently not known, but may be related to proteasomal degradation via the SOCS box of SOCS-1 (100).

IFN-γ adjuvants have shown success against Tuberculosis (TB) which are notorious for immune evasion (64). One of the causitve agents of TB, *M. bovis*, has recently been shown to cultivate immune tolerance by inducing SOCS-1 and SOCS-3 (75). The dual strength of pJAK2(1001-1013) as both an antagonizer of SOCS1 and an agonist of IFN-γ suggest it as a suitable candidate for treatment and vaccine adjuvancy.

The studies presented here further contribute to the development and classification of IFN-γ agonists, SOCS-1 (mimetics) and SOCS antagonist. My focus has primarily involved adjuvant properties of the IFN-γ agonists and SOCS antagonists. I helped to show how the mimetic and the SOCS antagonists could significantly enhance immune memory when they are associated with specific antigens whether they be weak or strong. I also helped to establish the involvement that APCs had in conjunction with our peptides ability to elicit long term adaptive immunity. IFN-γ agonists can activate APCs directly and indirectly by strengthening pathways like TLRs.

Through such pathways in APCs we have shown that our peptides can increase splenocyte proliferation and B cell antibody production. In relation to this, I was also involved in investigations as to how IFN-γ adjuvancy could be suppressed by antagonists, specifically our synthetic SOCS-1 mimetics, Tkip and SOCS1-KIR. The SOCS-1 mimetics inhibit the activation of APCs directly and indirectly, limiting their ability to process antigens and transmit them to T and B lymphocytes as evidenced by their stunted proliferation despite the presence of strong antigens. As we have shown that IFN-γ(95-132) synergizes with pJAK2, and also Tkip to some degree with SOCS1-KIR, we present here an effective, dual approach to adjuvancy with potential for future therapeutic application.

Table 3-1. The amino acid sequences of synthetic peptides used in this study

Peptide	Sequence
IFNγ(95-132)	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSR
IFNγ(95-125	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSL
IFNγ(95-132)SV40	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSLPKKRKV
V40 T antigen	PKKKRKV
IFNgamm(126-132	^{126}RKRKRSR
IFNGR(253-287	^{253}TKKNSFKRKSIMLPKSLLVVKSATLETKPESKYS
SOCS1-KIR	^{53}DTHFRTFRSHSDYRRI
Tkip	WVLVFFVIFYFFR
pJAK2(1001-1013	^{1001}LPQDKEYYKVKEP
JAK2(1001-1013)2A	^{1001}LPQDKEAAKVKEP

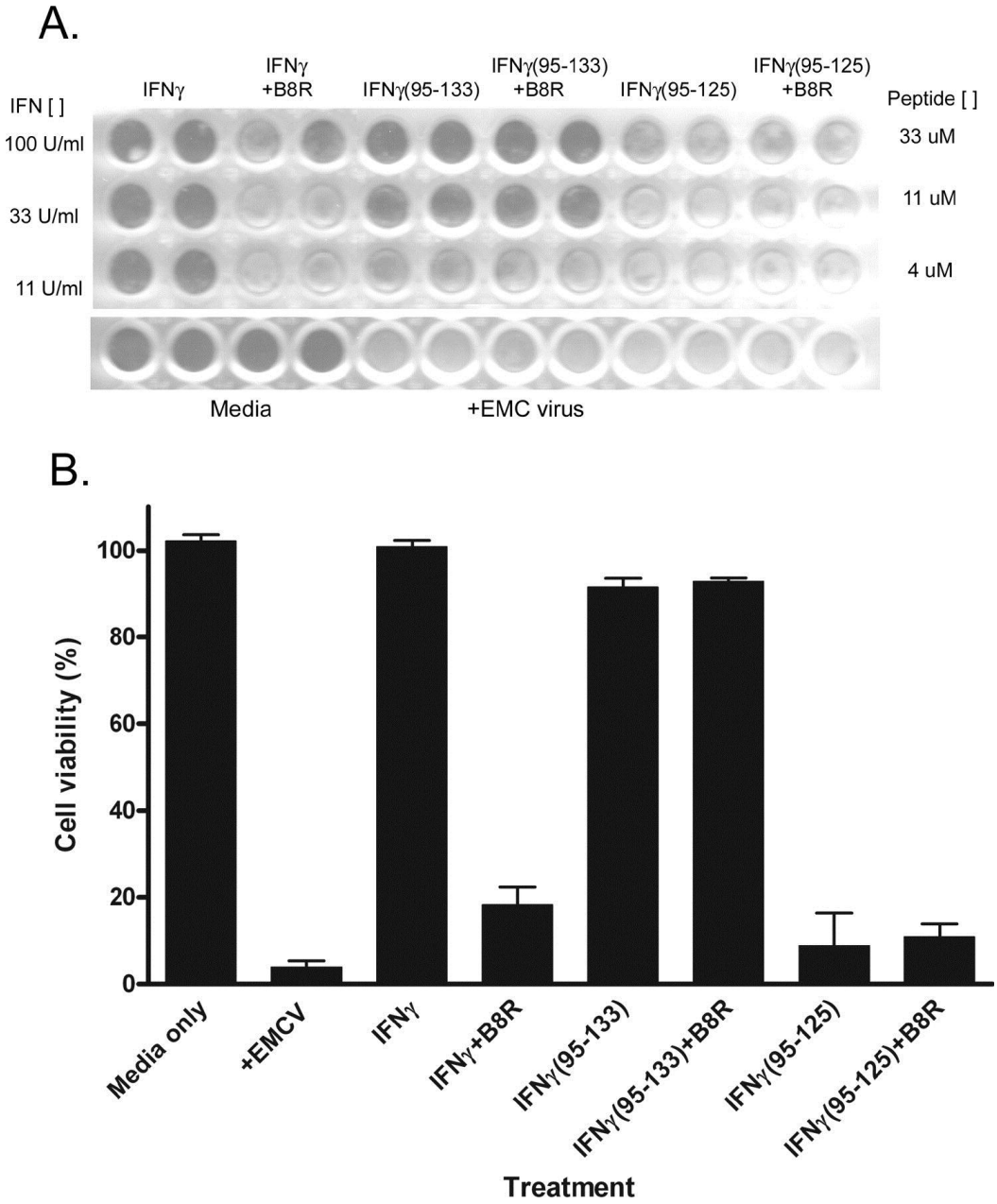

Figure 3-1. B8R neutralizes IFN-γ but not IFN-γ(95-132) antiviral activity. Murine L929 cells were plated to confluence, after which media and various concentrations of IFN-γ, IFN-γ(95-132), and IFN-γ(95-125) that were preincubated for 2 h with or without B8R (33 µg/ml) were added to the plate. After 24 h of incubation, EMCV (EMCV) (200 PFU/ml) was added for1hof incubation and washed with media. Cells were then incubated with media for 24 h, after which wells were stained with crystal violet and washed. A) Digital image of the plate. B) The plate was scanned for cell viability assessment using Image J software (NIH). Percent cell viability is presented for 33 U/ml of IFN-γ and 11 µM of IFN-γ(95-132). Error bars indicate standard errors of the means.

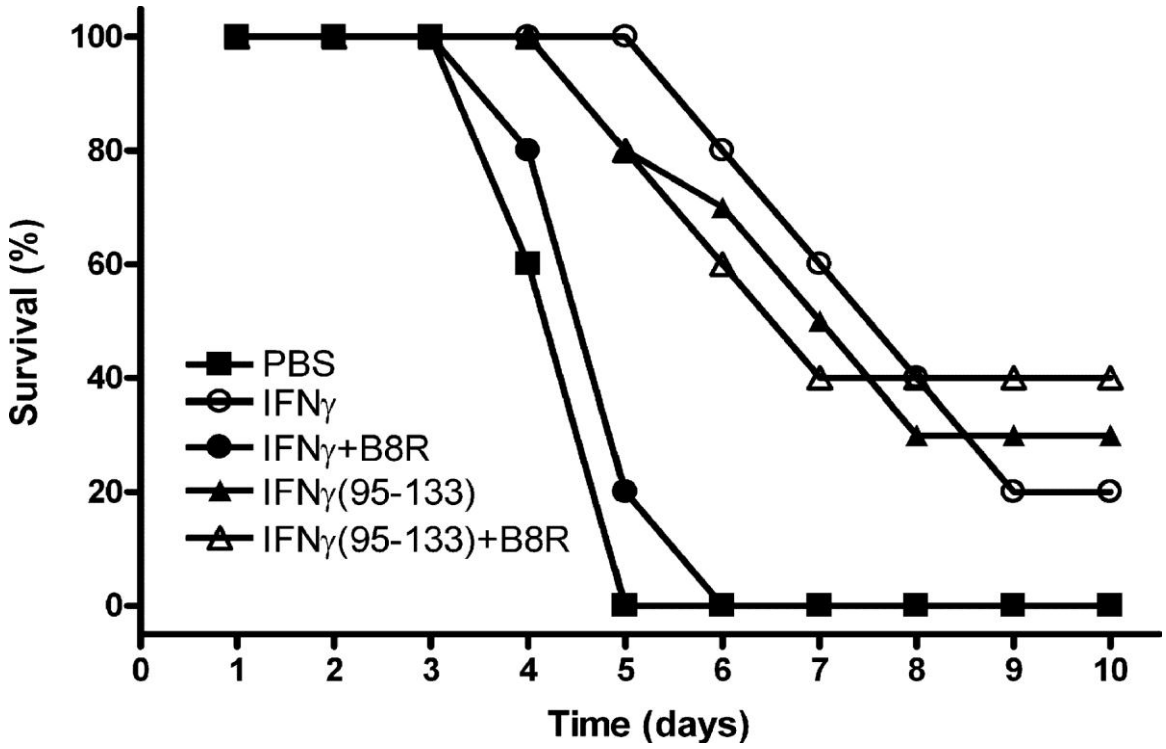

Figure 3-2. Protection of mice from EMC virus challenge by the IFN-γ(95-132) peptide in the presence of B8R protein. C57BL/6 mice were pretreated for 3 days with PBS, IFN-γ(95-132) (100 µg/day), or rat IFN- γ(200 U/day) in the presence or absence of the B8R protein (25 µg). On the last day of treatment, mice were challenged with 50 PFU of EMC virus. The numbers of surviving mice were recorded starting on the day of EMC virus challenge (day 0) and are presented as percent survival. Ten mice per treatment group were used, and representative data from one of two experiments are shown.

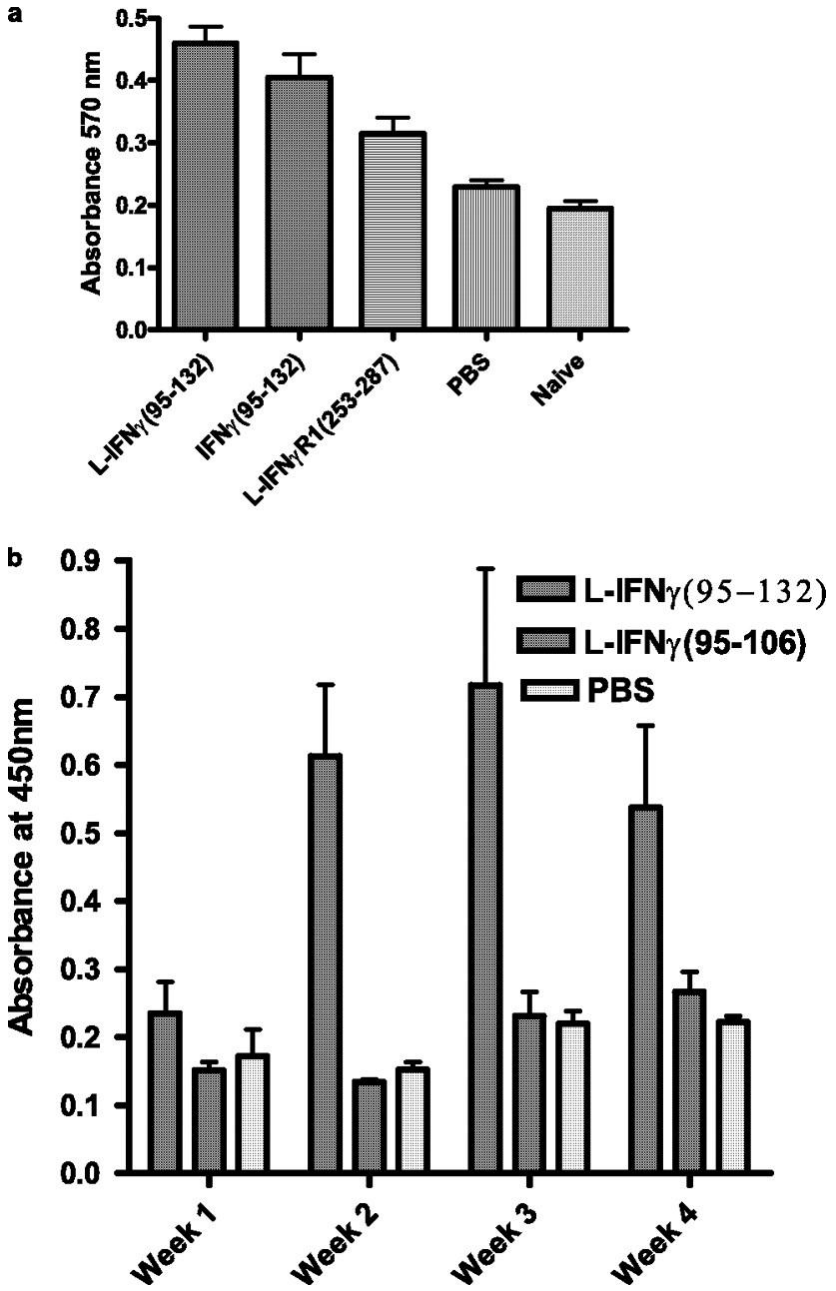

Figure 3-3. Adjuvant effect of IFN-γ mimetic for VV and BSA response. A) Mice (n = 5) were infected intranasally with VV in the presence of lipo (L)-IFN-γ(95–132), IFN-γ(95–132), control peptide, or PBS. Proliferation in the presence of purified inactivated VV was determined 48 h later by using Alamar blue dye (25). Statistical measurements using the Wilcoxon-Mann-Whitney rank sum test indicated $p < 0.05$ for mimetic vs control. B) Mice (C57BL/6, n = 5) were immunized using BSA as an Ag in the presence of lipo-IFN-γ(95–132), control peptide, or PBS. On the weeks indicated, blood was drawn from mice and measured for the presence of BSA-specific Abs in an ELISA format. The values represent the average with SD. $p < 0.05$ was obtained for the lipo-mimetic vs the PBS-treated group.

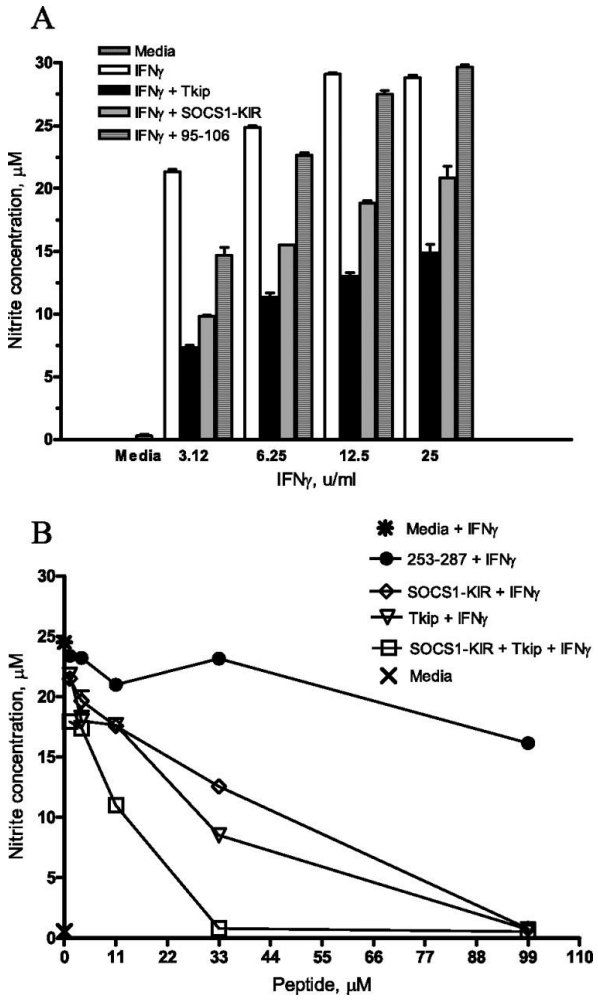

Figure 3-4. SOCS1-KIR and Tkip inhibit IFN-γ-induced macrophage activation. A) Inhibition of IFN-γ-induced NO production in macrophages. Murine macrophage cells, Raw 264.7, were incubated with varying concentrations of IFN-γ alone or in the presence of either lipo-Tkip, lipo-SOCS1-KIR, or lipo-control peptide MuIFN-γ(95–106), all at 15 µM final concentration, for 72 h at 37°C and 5% CO2 atmosphere. Culture supernatants were collected and nitrite concentration determined using Griess reagent. SOCS1-KIR and Tkip, but not the control peptide, significantly inhibited IFN-γ-induced nitrite production. The inhibition of macrophage activation by Tkip and SOCS1-KIR compared with control peptide was statistically significant as determined by two-way ANOVA ($p < 0.0001$). B) Dose-response inhibition of induction of NO and synergy between Tkip and SOCS-KIR. Raw 264.7 cells were treated with IFN-γ (6 U/ml) alone or in the presence of varying concentrations of Tkip, SOCS1-KIR, or control peptide (MuIFN-γ (253–287)) and assayed for NO production as described above. The differences between Tkip and SOCS1-KIR compared with the control peptide are statistically significant as determined by two-way ANOVA ($p < 0.0001$). To show synergy, Raw 264.7 cells were treated with IFN-γ in the presence of varying concentrations of Tkip and SOCS1-KIR and screened for NO production as described above.

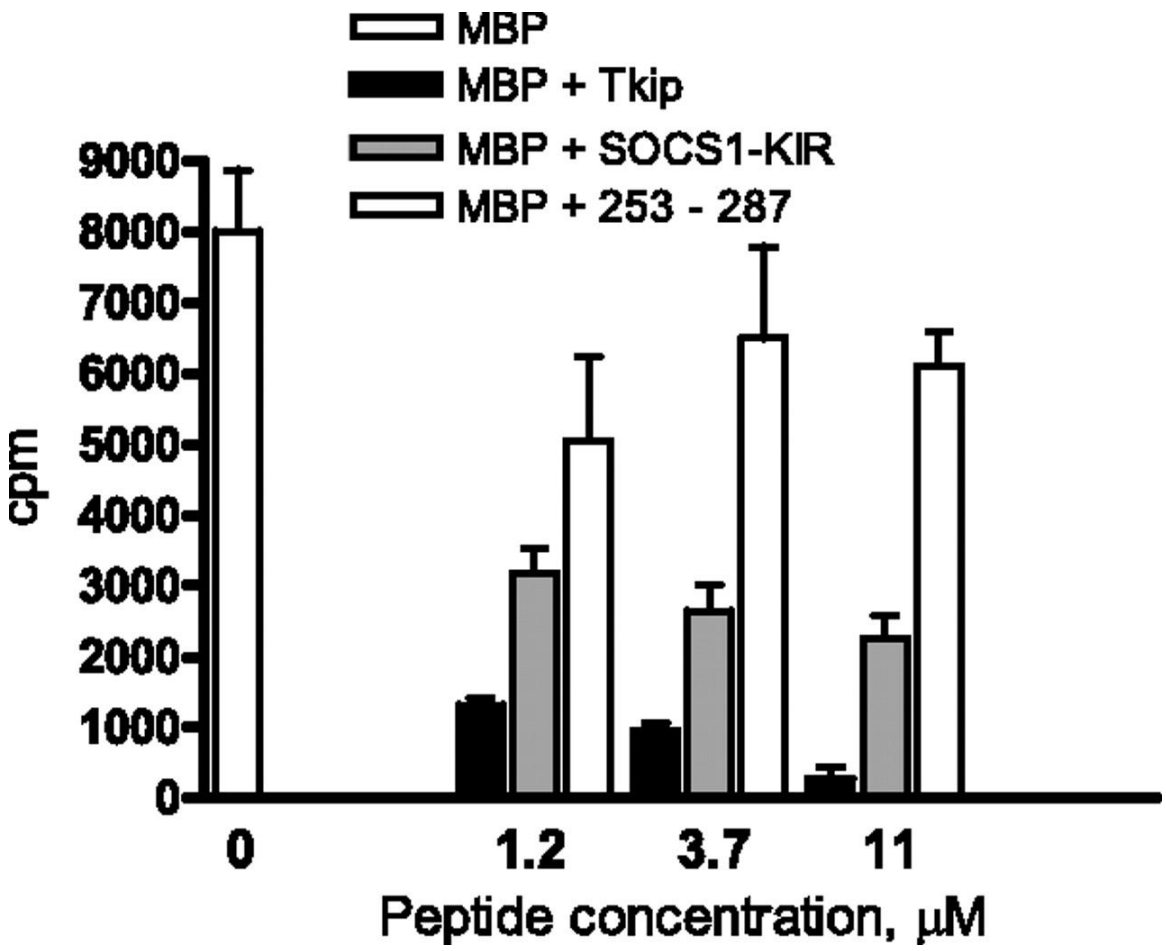

Figure 3-5. Both SOCS1-KIR and Tkip inhibit proliferation of murine splenocytes. Splenocytes (1 x 105cells/well) were obtained from MBP-sensitized SJL/J mice that had developed EAE and were in remission. The splenocytes were incubated with RPMI 1640 medium containing MBP (50μg/ml) and varying concentrations of lipo-SOCS1-KIR, lipo-Tkip, or lipo-control peptide MuIFNGR1 (253–287) for 48 h. Cultures were then incubated with [3H]thymidine for 18 h before harvesting. Radioactivity was counted on a liquid scintillation counter and data reported as counts per minute above background (medium only). Both lipo-SOCS1-KIR and lipo-Tkip, but not the control peptide, inhibited splenocyte proliferation in a dose-dependent manner. The inhibition of proliferation by lipo-SOCS1-KIR and lipo-Tkip, compared with the control peptide, was statistically significant as determined by two-way ANOVA ($p < 0.0003$). The data are representative of two independent experiments, each conducted in triplicate.

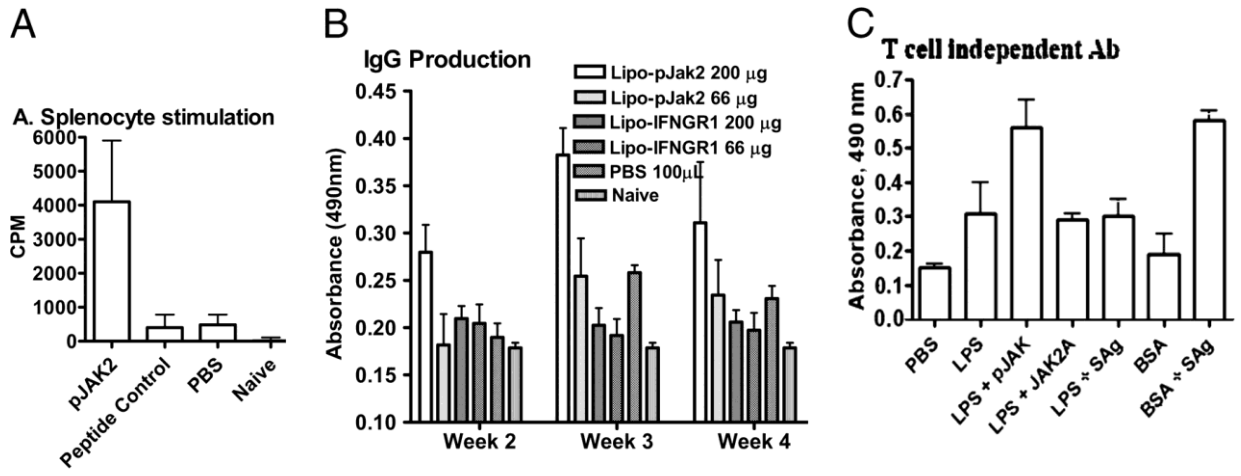

Figure 3-6. pJAK2(1001–1013) exerts an adjuvant effect at both cellular and humoral levels. A) Splenocyte stimulation. Mice (n = 5) were pretreated i.p. on day -2, -1, and 0 with pJAK2(1001–1013), control peptide JAK2(1001–1013)2A, or PBS. On day 0, 50 μg BSA was injected in mice in all groups, except the naive group. Four weeks later, isolated splenocytes (5 x 106/well) were seeded in quadruplicate and incubated with 0.5 μg BSA for 3 d with the addition of 1 μCi/well [3H] thymidine for the last 6 h, and its incorporation was measured. Data are representative of three individual experiments. B) IgG production. Mice (n = 5) were treated as in A. Sera obtained in the weeks indicated were diluted (1:1000) and added to microtiter plates. IgG Abs were measured in an ELISA assay. C) SOCS antagonist enhances T cell-independent Ab production. Mice (C57BL6, n = 3) were injected i.p. with T cell-independent Ag, LPS (50 μg each), or the T cell-dependent Ag BSA (50 μg). Some of the mice received SOCS antagonist (200 μg), the control peptide (JAK2A) (200 μg), or a combination of SEA/SEB (SAg, 25 μg each). A set of mice was also injected with BSA (50 μg) and SAg. Two weeks later, mice were bled. Sera were tested for IgG to LPS or BSA by ELISA. The secondary Ab used was anti-mouse IgG conjugated to HRP. After washing, substrate was added, and color was allowed to develop before reading absorbance at 490 nm. Comparison of LPS versus LPS and SOCS antagonist by Student t test resulted in $p < 0.01$ at 1/100 dilution.

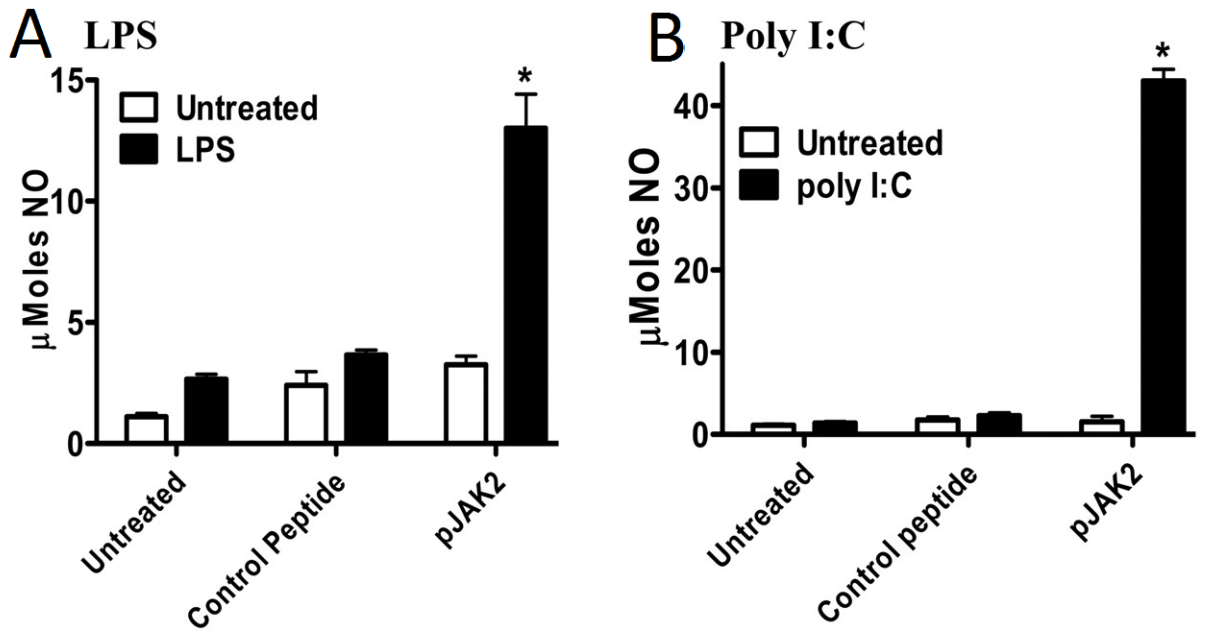

Figure 3-7. pJAK2(1001–1013) enhances macrophage activation via Toll like receptors. A) LPS stimulation. RAW264.7 cells (5 x 106/well) were seeded in triplicate and incubated overnight. The indicated amounts of pJAK2(1001–1013) or control peptide were added to the cells and incubated for 4 h, after which 2 µg/ml LPS was added, and the cells were incubated for 3 d. NO was measured by Griess reagent, and absorbance was read. B) Poly I:C stimulation. Murine macrophages (RAW264.7) were incubated with lipophilic pJAK2(1001–1013), or control peptide for 2 h, followed by stimulation with poly I:C at 0.1 µg/ml for 72 h. Culture supernatants were collected and nitrite concentration determined using Griess reagent. *$p < 0.001$.

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BIOGRAPHICAL SKETCH

James Martin is both a biologist and an artist. He has previously earned a bachelor's degree in microbiology and cell science from the University of Florida College of Liberal Arts. He is also working on a novel and a series of paintings. This thesis marks his departure from the field of immunology, he looks to continue his research in either the fields of plant genetics or aging.