Reverse Translation and Extension of Human Tyk2 Biology to a Murine Model

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Reverse Translation and Extension of Human Tyk2 Biology to a Murine Model

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A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Autoimmune disease is a broad term that encompasses over 80 different syndromes, and collectively affects greater than 23.5 million Americans (Rosenblum et al., 2012). Since cytokines play a pivotal role in inflammation and autoimmunity, more defined treatment regiments targeting selective cytokines and/or their signaling pathways continue to be explored. Type I and type II cytokine receptors lack ability to produce kinase activity relying on the JAK family proteins for internal signaling in response to receptor binding. Tyk2 is a JAK family member that is downstream of multiple cytokines that modulate inflammation and Tyk2 variants show strong GWAS correlations to protection from multiple autoimmune diseases. P1104A is a homozygous SNP which protects from various autoimmune diseases without the adverse effects associated with pan-JAK inhibition. Characterization of immune cells from patients with this SNP indicates loss of Type 1 IFN, IL-12 and IL-23 STAT activity but no change in the IL-6, IL-10 and IL-13 signaling pathways (Dendrou et al., 2016). In an effort to develop a translational model of the P1104A SNP, we have generated the genetically modified mice (GEM) carrying the correlative of the P1104A mutation (P1124A GEM). In proximal assays P1124A GEM responded to IFNα, IL-12, IL-6 and IL-10 STAT phosphorylation in a similar manner as P1104A PBMCs. A significant variation to the human variant was seen with IL-23 stimulation where STAT activity was unaffected. Cytokine driven proximal activity was consistent with functional cytokine stimulated mediator production in mice. Published data indicates that when PBMCs from P1104A patients are stimulated
with CD3ε/CD28 they shift towards a Th2 GATA3+, IL-4 producing phenotype (Couturier et al., 2011; Shimoda et al., 2000). Our data indicates that CD4 T cells loss of IFNγ, IL-10 and IL-21 cytokine production when cells were polarized to Th1 or Th17 phenotypes, respectively, contributes to the shift in P1124A mice. When Th1 differentiated, CD4 cells trend towards a Th2 profile expressing low levels of GATA3. Bone Marrow cell CD11b and CD11c expression was reduced in Tyk2 KO and P1124A GEMs. To further delineate the effect of P1124A, they were evaluated in a delayed-type hypersensitivity (DTH) model, in which they showed an attenuated response. Animal modeling suggest that GEM P1124A is protected from Th1 CD4 dependent DTH inflammation. We also measured the activity of the P1124A variant and compared it to other Tyk2 GEMs including: Tyk2 KO, the K950A kinase-dead knock-in (KDKI) and the Y1074F/Y1075F trans-phosphorylation mutant and have determined that the P1124A, and by extension possibly the P1104A SNP does not function by deficiency of Tyk2 activity, loss of auto-phosphorylative ability nor by loss of TYK2 trans-phosphorylative activity.
Dedication

To: Anthony (Tony) Dorvilien
Acknowledgments

First and foremost, I would like to acknowledge my thesis advisor Denise Manfra Ph.D. who through tremendous influence, immense and indispensable knowledge was instrumental in all phases of this massive endeavor. Only with her invaluable guidance and unmeasurable assistance was I able to perform the tasks required for a project of this magnitude. I would also like to thank Laxminarayan G. Hedge and Janice Woodhouse for their support and assistance, especially with current and future animal modeling. I must thank my family for their unwavering support, not only during the research and writing of this thesis but during the entirety of my graduate degree. So thank you Wilkens Dorvilien, MaryAnn Adams and Larry Adams for enduring the day to day stress that comes along with undertaking a graduate thesis. I would also like to thank Joseph Tumang, Ph.D who was a mentor throughout most of my professional career and degree candidacy, without whom my career would not be what it is today.
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Chapter I.

Introduction

Autoimmune diseases affect both men and women affecting about 23.4 million Americans which are approximately 5-8% of the population (Fenu & Caturegli, 2018; National Institute of Health, 2012). In general, autoimmune diseases are a broad group of conditions containing over 80 distinct syndromes defined by dysregulation of immune cells that result in the recognition and attacking of one’s own body (self) (Rosenblum, Gratz, Paw & Abbas, 2012). A leading theory for this self-destructive activity is the disruption of the sensitive balance between cellular immunity, humoral immunity and immunological inhibition. This theory proposes that cytokine dis-regulation leading to either abhorrent pro-inflammatory signatures or lack of inflammatory resolution contributes to disease development and is supported by genetic associations (Rosenblum et al., 2012). Human patient samples and animal models of rheumatoid arthritis have shown an aberrant type 1 inflammatory signature, the multiple sclerosis mouse model experimental autoimmune encephalomyelitis (EAE) is thought to be type 3/17 dependent, and atopic dermatitis and asthma are type 2 phenotype driven (Damsker, Hansen & Caspi, 2010; Kidd, 2003; Patel & Kuchroo, 2015). Further understanding of cytokine regulation and how it impacts inflammatory states is a popular field that could be instrumental in developing new treatment options to combat autoimmune diseases.
Cytokines

Due to the intricate yet complex nature of immune regulation there are infinite possibilities for mutations that could alter or halt necessary cell signaling, disrupting immune homeostasis and possibly causing immune dis-regulation. Cellular communication via secreted proteins, a major facet of cellular regulation and subsequent signaling pathways, could be implicated in disease progression and resolution. For all cells, a major form of communication is via cytokines; which can act in either an auto- or paracrine manner to form a biological response (Fiji, 2007). The definition of a cytokine is very broad and flexible, due to the ever expanding knowledge surrounding cytokine activity, but the working definition for the purpose of this research is polypeptides secreted from multiple nucleated cell types that function locally to influence immune cell responses (O’Shea, Gadina & Siegel, 2019). Of this group, cytokines secreted solely by lymphocytes have the designation interleukin (IL) (O’Shea, Gadina & Siegel, 2019). The importance of cytokines is highlighted by their integral nature in both innate and adaptive immune cell function. Direct cytokine function is important in polarization of myeloid cells (Eager and Miller, 2019) and contributes the 3rd signal required for T cell activation, subsequent to T cell receptor – MHC engagement and costimulatory receptor signaling (Eager and Miller, 2019). There are multiple ways that cytokines can be grouped but the most widely used is grouping by their receptors, protein structure, or subunit interactions since many members of cytokine receptor families use the same unit for multiple members (Fiji, 2007; Hunter, 2005). The cytokine categories based on their receptors are as follows (O’Shea, Gadina & Siegel, 2019):
1. Hematopoietic Cytokine Receptor Family (Type I Cytokines), which have homologous structures and can be sub-grouped by the receptor subunits that they share.

2. Interferons and Interleukin (IL), which encompass 10 related cytokines (Type II Cytokines) whose members function in both pro-inflammatory and anti-inflammatory responses.

3. Tumor Necrosis Factor (TNF) Receptor Family

4. IL-1 and Toll-Like Receptor Family

5. IL-17 Receptors

Independent of the cytokine receptor subgroup that a particular cytokine belongs to, cytokines are integral to the formation, control and resolution of inflammatory response.

Figure 1. Cytokine Dependent Cellular Functions

*Diagram of how Type I and II cytokines signal through JAK families to influence immunological response (Clark, Flanagan & Telliez, 2014).*
Cytokines and Inflammation

Defense against disease and infection is a complex system that has multiple components, all functioning together in order to protect self. The first line of defense against infection is physical barriers which include skin, mucosa and antimicrobials that function to keep invading organism out of the body. The second line of defense is the innate immune system, which recognizes invading organisms by their conserved pathogen-associated molecular patterns (PAMPs) (McDonald & Levy, 2019). All known innate immune cells produce and respond to cytokines in response to biological assault to protect self. The innate immune system effectively forms a defensive clearing response via 2 main mechanisms; cell mediated clearing of the assaulting pathogen and activation/shaping of the adaptive immune system. The former employs multiple mechanisms including phagocytosis, cytolytic activities and production of cytokines and chemokines leading to alterations in the surrounding tissue and recruitment of lymphocytes to ward off the infect or insult (Joffre, Nolte, Sporri & Reis e Sousa, 2009). Adaptive mechanisms depend on myeloid derived antigen presenting cells (APCs) and innate immune cell cytokine production to elicit T cell activation.

Mononuclear phagocytes, a group of cells that include various tissue specific populations of monocytes and macrophages, primarily function by engulfing invading pathogens but can differentiate into 2 pro-inflammatory groups depending on the cytokine(s) that these cells encounter. Interferon-γ (IFNγ) and granulocyte macrophage – colony stimulating factor (GM-CSF) are classic pro-inflammatory cytokines that induce the macrophage-1 (M1) polarization, enhancing their cytolytic activity and type 1 cytokine production (O'Shea, Gadina, & Siegel, 2019). The second group is the
macrophage-2 (M2), which can be further grouped depending on the cytokines they produce. Ubiquitous and consistent low levels of macrophage – colony stimulating factor (M-CSF) M2 cells function in homeostatic maintenance, in the presence of IL-4 and IL-13 cells polarize to the M2a phenotype which function in type 2 mediated pro-inflammatory function, and in the presence of IL-10 cells polarize to M2c cells which are anti-inflammatory using cytokines such as transforming growth factor-β (TGFβ) and M2c produced IL-10 to inhibit T cell activation (McDonald & Levy, 2019; Martinez & Gordan, 2014; Ushach & Zlotnok, 2016).

Natural killer (NK) cells are polarized by expression of IL-15, IL-12 and IL-18, the former produced by dendritic cells (DCs) and the latter two by macrophages (McDonald & Levy, 2019). NK cells are also divided into subsets. The first subset expresses high levels of cluster of differentiation (CD)-56 and is negative for CD16 (McDonald & Levy, 2019). This subset is not cytotoxic via direct mechanisms such as cell to cell mediated killing, but kills indirectly via production of large concentrations of IFNγ leading to M1 macrophage activation and type 1 polarization of adaptive T cells (McDonald & Levy, 2019). NK cells that express low levels of CD56 and high levels CD16 are important for antibody-dependent, cell mediated cytotoxicity (McDonald & Levy, 2019).

NK T cells are classified into four groups, depending on their surface receptor expression (McDonald & Levy, 2019). They produce IFNγ, TNFα, IL-4, IL-13 and IL-10 and therefore are influence in both adaptive and innate immune responses. With NKT cells as with many other cellular populations, location of the cell is important. NK T
cells located in the blood produce large concentrations of cytokines compared to those located in the thymus (McDonald & Levy, 2019).

Eliciting an adaptive immune response requires a significant interplay between multiple cell types. DCs, by their function as the pre-eminent antigen presenting cell (APC), truly links the innate immune system to the adaptive by traveling to primary lymphoid organs to activate or inhibit T cell activity via interactions between the APC cell (MHC) and the T cell receptor (TCR) (McDonald & Levy, 2019). How DCs influence of lymphocytes is dependent on the DC’s activating stimuli. DCs activated by PAMPs have high MHC expression and are effective T cell activators, but when DCs are stimulated in the absence of PAMPs by tissue derived cytokines DCs activity is determined by the location the signal is generated (Joffre et al., 2009). Both, PAMP activated DCs and T cells, respond to and secrete cytokines to influence the adaptive immune system. The adaptive immune system consists mostly of B cells, CD8+ T cells and CD4+ T cells. CD8+ T cells function via direct cell killing using perforin and granzyme, perforin and/or by Fas ligand/TNF-related apoptosis-inducing ligand (TRAIL) mediated cell death (Nutt & Huntington, 2019). These cells also have the ability to influence pro-inflammatory responses by their ability to produce cytokines like IFNα, TNFα, GM-CSF, IL-5, IL-10 and IL-13 (Nutt & Huntington, 2019). CD4+ T cells function by providing “help” to CD8+ cells by secreting cytokines that are pro-survival and/or proliferative or by modulating the immunological response by assisting with B cell antibody class switching, activating macrophages increasing phagocytic activity and activating cytotoxic T cells (Nutt & Huntington, 2019). There are 8 known subtypes of CD4+ T cells; they include Th1, Th2, Th9, Th17, Th22, T follicular helper (fh), induced
T regulatory (iTreg) and T memory (mem). IL-12 is primarily responsible, along with IFNγ, type I IFNs and IL-18 in Th1 differentiation. Th1 cells secrete TNFβ, IFNγ, GM-CSF and IL-2 to promote direct cell mediated cytotoxicity (Eagar & Miller, 2019; O'Shea, Gadina, & Siegel, 2019). IL-4 polarizes CD4+ T cells to a Th2 phenotype, which are associated with IL-4, IL-5, IL-9, IL-10 and IL-13 cytokine production which function to active eosinophils and mast cells, cause B cell immunoglobulin (Ig) class switching from IgG to IgE and IgA (Eagar & Miller, 2019). Th9 cells are polarized by IL-4, IL-21 and transforming growth factor – β (TGFβ-1) and secrete IL-9 and IL-10. These cells function to prevent apoptosis. Th17 CD4 T cells are pro-inflammatory secreting IL-17A/F, IL-21, IL-22, IL-26 and GM-CSF when polarized with IL-6, IL-21, IL-23, TGFβ-1 and IL-1β and play a role in IL-17 driven diseases (Eagar & Miller, 2019). IL-6 and TNFα stimulate Th22, a skin associated CD4 cell type, which regulates homeostasis in the skin and dermal protection from inflammation (also in the gut) (Eagar & Miller, 2018). Tfh cells functions in a pro-survival, pro-proliferative, Ig class switching affinity maturation activity by direct interaction and IL-21 secretion (Eagar & Miller, 2019). IL-12, IL-6 and IL-21 are necessary for the development of the Tfh CD4 phenotype (Eager & Miller, 2019). The resolution of inflammation occurs by two major mechanisms, removal of cytokines from the local microenvironment and direct cellular inhibition. Treg cells, both natural thymic derived and peripheral tissue induced Treg cells, are instrumental in the cellular inhibition of inflammation.. Induced Tregs (iTregs) are polarized by prolonged antigen exposure or exposure to antigen by immature DCs and produce the anti-inflammatory cytokines IL-10 and TGF-β (Eagar & Miller, 2019).
Table 1. Type 1 and Type II Cytokines.

<table>
<thead>
<tr>
<th>Cytokine Receptor γ chain</th>
<th>Cytokine Receptor β chain</th>
<th>Gp130</th>
<th>Homodimeric Receptors</th>
<th>Tyrosine Kinase Receptors</th>
<th>Type I &amp; Type II IFNs</th>
<th>IL-10 family</th>
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<tr>
<td>IL-2</td>
<td>IL-3</td>
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<td>GH</td>
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<td>IL-5</td>
<td>IL-11</td>
<td>Prolactin</td>
<td>SCF</td>
<td>IFN-β</td>
<td>IL-19</td>
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<tr>
<td>IL-7</td>
<td>GM-CSF</td>
<td>Oncostatin M</td>
<td>EPO</td>
<td>IFN-ω</td>
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<td>IL-9</td>
<td>LIF</td>
<td>TPO</td>
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<td>IFN-τ</td>
<td>IL-22</td>
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<tr>
<td>IL-13</td>
<td>CNF</td>
<td>G-CSF</td>
<td></td>
<td>IFN-γ</td>
<td>IL-24</td>
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<tr>
<td>IL-15</td>
<td>BSF-3</td>
<td>IL-12</td>
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<td>TSLP</td>
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<td>IL-29</td>
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Type 1 and Type 2 cytokines listed group by which receptor chains they share (Fujii, 2007; Hunter, 2005).

Type I and Type II cytokine receptors lack the inherent ability to initiate the activity needed for down-stream cellular processes (Watfield & O'Shea, 2006). Therefore their signaling capabilities are due to their cytoplasmic domains binding to Janus kinase family (JAK) and recruitment of signal transducer and activator of transcriptions (STATs) (O'Shea, Gadina, & Siegel, 2019). In the mammalian JAK-STAT pathway, there a four JAK (Jak1, Jak2, Jak3 and Tyk2) and seven STAT (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) proteins that work in concert to exert cytokine/receptor functional regulation (Fujii, 2007).
Figure 2. JAK/STAT Pathway and Structures.

A schematic of JAK/STAT activity. Type I or type II cytokine binding to their receptors activate JAKs by phosphorylation. JAKs phosphorylate STATs causing dimerization and translocation from the cytoplasm into the nucleus, where they function as transcription factors to increase transcription of immediate response genes (Aaronson & Horvath, 2002). b) The structure of the conserved regions of the JAKs. JAKs are bound to type I and type II cytokine receptors by their JH6 and JH7 domains, and activated at the JH1 kinase domain (Shuai & Liu, 2003).

JAK protein family structure is conserved and contains seven JAK homology (JH) domains. The catalytically active JH1 kinase domain and the inactive regulatory JH2 pseudo kinase domain are at the carboxyl end of the protein (Schindler, Levy, & Decker, 2007). Moving from the carboxyl end towards the amino end, JH3 and half of JH4 contain a src-homology domain with unknown function (Strobl, Stoiber, Sexl, & Mueller, 2011). The other half of the JH4 domain up to the JH7 domain is the four point one, ezrin, radixin, moesin (FERM) domains which undergo a confirmation change when the
cytokine binds causing the receptor to oligomerize bringing the JAKs closer in proximity for trans phosphorylation. This in turn activates both JAKs, phosphorylating the cytokine receptors and causes the recruitment of STATs. The STATs oligomerize and are transported to the nucleus where they function as transcription factors influencing cellular lineages and pro-inflammatory states (Fujii, 2007).

Figure 3. JAK Activation and Signaling.

*A schematic of JAK phosphorylation patterns. Type I or type II cytokines binding to their receptors causes JAKs to auto-phosphorylate and then trans-phosphorylate leading to receptor phosphorylation of tyrosine residues, causing STAT binding (Fujii, 2007).*

Tyk2 Cytokine RegulationTyk2 is one of the four members of the JAK family that can function during cytokine receptor association to alter biological function. Multiple pro-inflammatory cytokines establish the inflammatory response; some of the most researched include IL-1, IL-6, TNFα, IFNγ, IL-12, IL-18, IL-23 and GMCSF (Hanada & Yoshimura, 2002). Resolving inflammation is triggered by anti-inflammatory cytokines
such as IL-10 and TGF-β (Hanada & Yoshimura, 2002). The balance between these two arms of inflammation provide protection against internal (i.e. cancer, autoimmune disease) and external (i.e. infection) mediated pathologies, but when mis-regulated deleterious effects like sepsis or autoimmune diseases occur. Of these pro-inflammatory/anti-inflammatory cytokines IL-6, IL-12, IL-23, IL-10, IL-13 and type I IFNs can signal through Tyk2. This combination of cytokines puts Tyk2 in the interesting position of being influential in the initiation, duration and resolution of the inflammatory response.

Much of Tyk2’s activation has been elucidated in human tissue cell lines. JAKs can be both auto-phosphorylated and trans-phosphorylated. Phenylalanine substitution at tyrosines 1054 and 1055, Y1054F/Y1055F, in human cell line 11, 1 resulted in Tyk2’s inability to be phosphorylated by other JAKs with retention of its auto-phosphorylative ability when stimulated by IFNα (Gauzzi et al., 1996). A substitution of lysine 930, a conserved lysine in the ATP binding site of the kinase domain, with arginine results in a loss of Tyk2’s auto-phosphorylative ability but retention the ability to be trans-phosphorylated (Gauzzi et al., 1996). These experiments highlight that conserved K930 and Y1054/Y1055 are important for Tyk2 function. Although Tyk2 functions in cytokine signaling, it always partners with either Jak1 or Jak2 in the types I and II cytokine signaling cascades. Function through other JAKs may allow for signaling compensation if a particular JAK is inactive.

The majority of Tyk2’s activity has been discovered using engineered human cell lines or some limited data from naturally occurring human variants. However, research with animal models allows for more controlled manipulation of whole biological systems.
B10.Q/J mice have a natural missense mutation in the pseudo kinase domain resulting in a single nucleotide polymorphism (SNP) resulting in an E557K point mutation (Shaw et al., 2003). Phenotypically, these mice display a Tyk2 knockout (KO) like cytokine response, which included impaired signaling after IL-12, IL-23 and type I IFN stimulation. Due to these defects in cytokine responsiveness, B10.Q/J mice do not respond to extracellular infectious agents, like protozoan parasite infection, nor intracellular infectious agents and are resistant to collagen-induced arthritis (Shaw et al., 2003). The loss of activity seen with the E775K SNP is similar to the responses seen with complete loss of Tyk activity.

Loss of Tyk2 activity in Tyk2 KO mice dampened but did not ablate Tyk2 influenced cytokine responses measured by immunoprecipitation and western blotting. This is contrary to the research done with Tyk2 KO human cell lines which showed abrogated type I IFN and IL-12 responses including a loss of IFNγ production (Karaghiosoff et al., 2000). Data also indicated that while IL-10 is a Tyk2 signaling cytokine, Tyk2’s role in IL-10 activity can be compensated for by Jak1, the other IL-10 associated JAK, since the IL-10 response was not impacted (Karaghiosoff et al., 2000; Corey, Tan & Uleet, 2012). Multiple mouse models support the proof of concept that Tyk2 modulates multiple autoimmune diseases. Studies with Tyk2 KO mice backcrossed on a Balb/c background show Tyk2 deficiency can be protective in Th1 and Th17 driven animal models, but exacerbates Th2 driven diseases. In the psoriasis-like mouse animal model stimulated by imiquimod (IMQ), a Th17 focused disease, knocking-out Tyk2 resulted in a reduction of ear swelling and depletion in the total amount of immune cells that infiltrated the stimulated ear (Ishizaki et al., 2013).
Using multiple Th1 and Th17 driven animal models including methylated-BSA in a 1:1 emulsion of CFA stimulated DTH model, the same IMQ model mentioned above, a 3% DSS induced colitis model and a hapten TNBS-induced colitis model it was shown that Tyk2 is important for the development of Th1 and Th17 dependent autoimmune diseases (Ishizaki et al., 2011). Knocking-out Tyk2 inhibited DTH footpad swelling; reduced IMQ ear swelling; reduced DSS induced colitis DAI scores (body weight and colon length); and increased TNBS-induced colitis mouse survival (Ishizaki et al., 2011). With a Th2 polarization dependent autoimmune disease manifestation use of Ova for inflammation in a mouse model of asthma, disease is exacerbated in Tyk2 KO (Seto et al., 2003). Tyk2 is necessary for regulation of the Th1, Th17 and Th2 pro-inflammatory responses and manipulation of its pathway could be groundbreaking in the ability to modulate autoimmune diseases.

Tyk2 and Human Disease

Tyk2’s relevance to human disease is undeniable. As Tyk2 is down-stream of Type I and Type II cytokines IL-12, IL-23, IL-13, IL-6, IL-10 and Type I IFNs, it functions predominantly in type 1 and type 3/17 inflammatory responses but also has a role in Th2 related activities through it IL-13 regulation and a role in the resolution of inflammation based on influence in IL-10 signaling. Tyk2 is also downstream of IL-22, IL-26, IFNλ1, IFNλ2, and IFNλ3, but its function and importance in these cytokine pathways is more elusive (Strobl, Stoiber, Sexl, & Mueller, 2011). Human Tyk2 loss leads to human Primary Immunodeficiency Syndrome PID (Watfield & O'Shea, 2006). Two patients have been identified that lacks Tyk2 due to a homozygous mutation resulting in a premature stop codon. Phenotypically one of the patients was highly...
susceptible to viral infections, suffered from mycobacterial infections, had impaired Th1 cell differentiation, impaired IFNγ production and signaling, developed skin abscesses and atopic dermatitis with high levels of IgE, and had more Th2 polarized cells than healthy patients. These symptoms were consistent with the loss of Type I IFN which can lead to heightened risk of infection; loss of IL-12 which could cause deficient Th1 polarization via loss of IFNγ production and response, and loss of IL-23 which can lead to skin abscesses (Watfield & O'Shea, 2006; Kilic et al., 2012).

Tyk2 is not only important for signaling but also for scaffolding. Tyk2’s physical importance is highlighted by IFNα receptor 1 (IFNAR1). IFNAR1 is a component of the type 1 interferon receptor that is required for type I IFN signaling. IFNAR1’s surface expression, internalization and degradation is relative to the amount of Tyk2 expression measured using human cell lines either lacking Tyk2 or expressing various amounts (Ragimbeau et al., 2003). In the absence of adequate Tyk2 expression IFNAR1 is internalized and degraded at a faster rate than if sufficient Tyk2 is present. While Tyk2 is responsible for stabilizing IFNAR1 on the surface of human cells it is not the case for murine cells, highlighting one of the major species differences in Tyk2 activity (Karaghiosoff et al., 2000).

Tyk2 Confers Protection against Autoimmune Disease

Multiple Tyk2 SNPs have been associated with various autoimmune diseases. In a family association study in the United Kingdom (UK) with 380 trios, which consisted of both parents and the patient, double stranded (ds) SNP rs12720270 individually associated with SLE and dsSNP rs280519 had a trend of association (Graham, Akil, &
Of the multiple Tyk2 SNPs related with autoimmune diseases, ds SNP rs34536443 has the strongest links. Rs34536443 is a SNP that causes a substitution of proline 1104 with an alanine residue (P1104A variant), and has been established as a protective allele in multiple sclerosis (MS) in 4 independent GWAS studies spanning multiple case-control cohorts with differing countries of origin and differing ethnic backgrounds (Couturier et al., 2011). As recently as 2016, the P1104A SNP was shown in GWAS studies to be protective from 10 autoimmune diseases including multiple sclerosis, Crohn’s disease, ulcerative colitis, psoriasis, ankylosing spondylitis, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, juvenile idiopathic arthritis and primary biliary cirrhosis (Dendrou et al., 2016). Although this polymorphism is established as being protective, there is controversy surrounding how its protective phenotype is established. Analysis of activated peripheral blood mononuclear cell (PBMC) samples from heterozygous Tyk2 variant patients indicated a decrease of Tyk2 dependent Type I IFN activity, based on Tyk2 phosphorylation or STAT phosphorylation after IFNα and IFNβ treatment (Couturier et al., 2011; Dendrou, et al., 2016). Dendrou, et al (2016) located a population, previously thought to be nonexistent, of homozygous P1104A variant patients who showed an almost complete loss of STAT phosphorylation in response to both IFNα and IFNβ stimulation.

However, the contribution of the Tyk2 P1104A variation IL-6 and IL-10 signaling remains unclear. Couturier et al (2011) measured IL-6 and IL-10 activity using quantitative real-time polymerase chain reaction (RT qPCR) with a SOCS3 read-out. In their studies the P1104A substitution significantly reduced SOCS3 upregulation when PMBCs were incubated with respective cytokines for 2 hours. The Dendrou et al (2016)
group measured IL-6 and IL-10 activity with a more proximal cell STAT phosphorylation assessed using FLOW cytometry after 15 minutes of cytokine stimulation. In Dendrou’s (2016) studies IL-6 and IL-10 activity is unaffected by heterozygous and homozygous Tyk2 P1104A substitutions. While the Couturier (2011) group focused on IFNβ, IL-10 and IL-6 activity the Dendrou (2016) group also evaluated Tyk2 influenced cytokines IL-13, which showed no proximal changes, and IL-12 and IL-23, both showing loss of pSTAT phosphorylation in homozygous patients. Although some of these discrepancies can be attributed to the assays used and the time-frame measured, questions remain about the exact role of the Tyk2 allele’s with respect to their protection from disease. Further, studies would be necessary to determine assess IL-6 and IL-10 activity relative to kinetics, JAK compensation, or other potential regulating factor led to these discrepancies.

With respect to further downstream effects of Tyk2, T lymphocyte polarization initially measured by RT qPCR with T-bet (Th1), GATA3 (Th2), RORγt (Th17) and Foxp3 (Treg) and then measured by cytokine secretion were used to determine if the P1104A variant altered T cell polarization (Couturier et al., 2011). Interestingly, the results showed no change in Th1 or Th17 responses but an upregulation of TH2 associated readouts. These results are unexpected since Tyk2 functions down-stream of cytokines mostly associated with Th1 (IL-12) and Th17 (IL-22 and IL-23) dominate phenotypes but not down-stream of a Th2 dominate cytokine.

Translational Modeling

Efforts to alter cytokine signaling using therapeutics in autoimmune disease have included direct cytokine modulation typically with kinase inhibitors, sequestering molecules, antibodies and memetic therapeutics (Bryan & Rajapaksa, 2018). Historically
high doses of recombinant cytokines, due to short half-lives, were tested in oncologic clinical trials without much success due to the exceeding high doses required to achieve effects and to severe side effects (Klatzmann & Abbas, 2015). Another way of treating autoimmune diseases is via the cytokine signaling pathway via development of jakinibs, ATP competitive JAK kinase family small compound inhibitors (Schwartz, Bonelli, Gadina, & O'Shea, 2016). While multiple jakinibs (Tofacitinib, Ruxolitinib and Baricitinib) have been studied for the treatment of various autoimmune diseases they are all also plagued with multiple side effects (Schwartz, Bonelli, Gadina, & O'Shea, 2016). These side effects cannot necessarily be categorized as off target effects since due to their very nature, they modulate multiple cytokine signaling pathways resulting. Treatment with the jakinibs increases risk of infection alters immune cell populations by decreasing lymphocytes, neutrophils, NK cells and platelets, increases levels of liver lipids including cholesterol, causes gastrointestinal perforation and increases risk of malignancy (Winthrop, 2017). However, as previously mentioned, multiple GWAS studies link SNP rs34536443 to protection against various autoimmune diseases; Dendrou’s publication (2016) linking proximal signaling of the major Tyk2 related cytokines with STAT phosphorylation in these patients could lead to a model phenotype for a Tyk2 inhibitor with a better therapeutic window than other jakinibs and or Tyk2 depletion.

Unfortunately, the rs34536443 SNP is rare making extensive research on its modulation of Tyk2 activity difficult. Discovering a translational animal model with the analogous variation would contribute to furthering the Tyk2 biology especially with respect to cytokine signaling. It would also create a translational model that could aid in the understanding of the functional consequence of Tyk2 modulation in more complex
situations and disease settings. These GEMs were chosen based on research surrounding either patient samples or genetic alteration of human cell lines. The GEMs include the previously described Tyk2 KO, K950A KDKI, Y1074F/Y1075F, and the P1124A murine version of the rs34536443 SNP.

With the GEMs, research could establish an rs34536443 translational model by determining if the mouse SNP analog is comparable to the human P1104A variant, and how it compares with other Tyk2 GEMs. If the murine variant’s activity is similar to the human variant, the additional GEMs could elicit if the variant activity is due to changes in Tyk2 auto- or trans-phosphorylative activity, or has a completely independent mode of action. The GEMs would also allow for the further evaluation of the functional and downstream consequences of the altered STAT activation seen in the human and mouse. By also determining Tyk2 cytokine dependent mediator production, changes in cellular polarization and alterations in response to animal models of human disease we can help elucidate how rs3456443 alters Tyk2 regulation of the immune response and understand the phenotype necessary to treat autoimmune diseases with a Tyk2 specific jakinib without side effects.
Chapter II.
Research Methods

The materials and methods used for the Tyk2 studies are outlined in this section. All laboratory work for this project was completed in a BSL-2 facility. For the in vitro experiments spleen and lymph node (LN) tissues were harvested from one female and one male mouse per experiment LN cells were pooled from brachial, inguinal, lumbar and popliteal LNs. Spleen and bone marrow derived cells were used to test GM-CSF activity specifically. There were 2 experiments per assay.

Genetically Engineered Mice (GEMs)

The GEMs used in these studies were generated at Taconic. All of the mouse strains are on the C57Bl/6 background.

Figure 4. Schematic of the genotype of strain 12684: A constitutive Tyk2 knockout.

*The knockout was engineered by insertions of Ioxp sites downstream of Tyk2’s 4th and 6th exons, resulting in exon deletions. These deletions are though to result in loss of*
Tyk2 function. Due to the creation of a premature stop codon introduced with the exon 3 to 7 frame-shift in the FERM domain (Taconic, 2018).

Figure 5. A schematic of the genotype of strain 13560.

K950A point mutation in exon 20 generated the kinase dead knock-in GEM (Taconic, 2018).

Figure 6. A schematic of the genotype of strain 13762.

Y1074A and Y1075 two point mutations in exon 22 to generate the trans-phosphorylation mutated GEM (Taconic, 2018).
**Figure 7.** A schematic of the genotype of strain 13763.

*P1124A point mutation on exon 23 to generate the GEM with a single point variation and as will be discussed is the equivalent of humans carrying the Tyk2 P1104A mutation. Heterozygous and homozygous versions of these GEMS were included in this research (Taconic, 2018).*

**Tyk2 Signaling Proximal Studies**

Proximal activity was measured by percent of cells with STAT phosphorylation after cytokine stimulation. Tissues were ground through a 70μc filter in RPMI media containing 10% fetal bovine serum (FBS) and 55μM 2-mercaptoethanol, into a single cell suspension. 100,000 cells/well in 200μLs were stimulated in NUNC 96 well u-bottom plate with various concentrations of cytokine for 20 minutes at 37°C. The cytokines used for stimulation where IFNα (PBL assay science, 12100-1), IL-6 (RnD, 406-ML-025/CF), IL-10 (RnD, 417-ML-025/CF), IL-12 (RnD, 419-ML-025/CF), IL-23 (RnD, 1887-ML-010/CF), GM-CSF (RnD, 415-ML-o5o/CF) and IL-2 (RnD, 402-ML-020/CF). The cells were then fixed for 20 minutes at room temperature with BD Cytofix fixation buffer according to the manufacturer’s recommendations. The cells were washed once with
phosphate buffered saline (PBS) permeabilized on ice for 30 minutes with BD Phosflow perm buffer III, and stained for both surface antigens and phosphorylated STAT proteins overnight at 4°C. The STAT staining antibodies include: anti (α)-pSTAT1 (BD, 560190), αpSTAT5 (BD, 612567), αpSTAT3 (BD, 562072) and αpSTAT4 (BD, 558137).

Table 2. Flow Staining Panels

<table>
<thead>
<tr>
<th>Panel 1</th>
<th>Panel 2</th>
<th>Panel 3</th>
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<tbody>
<tr>
<td>CD3 Percp</td>
<td>B220 BV421</td>
<td>CD8a AF700</td>
</tr>
<tr>
<td>CD4 Fitc</td>
<td>NK1.1 Fitc</td>
<td>B220 BV421</td>
</tr>
<tr>
<td>CD8a AF700</td>
<td>CD335 PE Cy7</td>
<td>CD11b APC Cy7</td>
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<tr>
<td>CD62L APC Cy7</td>
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<td>CD11c BV711</td>
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<td>CD44 PE Cy7</td>
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<td>CD14 PE Cy7</td>
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<tr>
<td></td>
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<td>CD103 Fitc</td>
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Table 3. Cytokine Induced STAT Phosphorylation

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>STATs Activated</th>
<th>STATs Measured</th>
</tr>
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<tbody>
<tr>
<td>IFNα</td>
<td>STAT1/STAT2/STAT3</td>
<td>STAT3/STAT4</td>
</tr>
<tr>
<td>IL-6</td>
<td>STAT3/STAT4</td>
<td>STAT3/STAT4</td>
</tr>
<tr>
<td>IL-10</td>
<td>STAT3/STAT4</td>
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<td>IL-12</td>
<td>STAT3/STAT4</td>
<td>STAT3/STAT4</td>
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<tr>
<td>IL-23</td>
<td>STAT4</td>
<td>STAT3/STAT4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>STAT3/STAT5</td>
<td>STAT1/STAT5</td>
</tr>
<tr>
<td>IL-2</td>
<td>STAT3/STAT5</td>
<td>STAT1/STAT5</td>
</tr>
</tbody>
</table>

Flow cytometry was used as the read-out to measure STAT phosphorylation. The samples were acquired on a BD Fortessa using DIVA software and the flow data was analyzed with FLOWjo software. Percent of cells that were positive for fluorescence compared to unstimulated negative controls are considered positive for phosphorylation. Data was graphed using Prism software and curves were fit using nonlinear regression (curve fit) analyses with log (agonist) vs response (three-parameter) analysis.
Tyk2 Signaling functional (distal) Studies

Cytokine stimulated mediator (cytokine or chemokine) production was used for functional activity measurements. Cells were stimulated with various concentrations of IFNα, IL-6, IL-10, IL-12, IL-23, GM-CSF, and IL-2 for 16 hours at 37°C. Supernatant was collected and analyzed at a 1:2 dilution on U-plex MSD panels that measured IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-21, IP-10 and TNFα. Concentrations of the mediators produced were calculated using linear regression. Data was graphed in Prism software and curves were fit using nonlinear regression (curve fit) analyses with log(agonist) vs response (three-parameter) analysis.

Cellular Polarization

Naïve CD4+ T and naïve CD8+ T cells were isolated using miltenyi biotech magnetic bead based negative selection kits (130-096-543) and (130-104-453), respectively. The cells were either unstimulated or differentiated (100,000 cells/well) using kits from RnD Systems to one of the following phenotypes: Th1 (CDK018), Th2 (CDK019), Th17 (CDK019) or Treg (CDK007). Supernatant from day 6 post stimulation were analyzed at a 1:2 dilution on a custom U-plex MSD panel that measured IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-21, IP-10 and TNFα. MSD panels were run as per the manufacturer’s suggested U-plex protocol. The total mediator concentrations were calculated using linear regression. Data was graphed in Prism software and curves were fit using nonlinear regression (curve fit) analyses with log (agonist) vs response (three-parameter) analysis. The cell pellets were fixed, permeabilized and stained for Tbet, GATA3, RORγT or Foxp3 transcription factors. Flow data was collected on a BD Fortessa using Diva software and the transcription factor expression was analyzed using
FlowJo software. Percent of positive cells was relative to unstimulated negative controls were reported.

M1/M2 polarized cells were generated by incubating 1x10^6 bone marrow derived cells (BMDCs) for 6 days with either 500ng/mL of GM-CSF (415-ML-050/CF) or 500ng M-CSF (416-ML-050/CF) from R and D Systems. Supernatant from day 6 post stimulation were analyzed at a 1:2 dilution on a custom U-plex MSD panel that measured IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-21, IP-10 and TNFα. MSD panels were run as per the manufacturer’s suggested U-plex protocol. Mediator production was analyzed using linear regression. Data was graphed in Prism software and curves were fit using nonlinear regression (curve fit) analyses with log(agonist) vs response (three-parameter) analysis.

Delayed Type Hypersensitivity (DTH) in Vivo Model

Female 8-10 week old GEMs were sensitized to KLH subcutaneously between the shoulder blades with 6μg/mouse in 100μls/site with a suspension of 1:1:1 Keyhole Limpet Hemocyanin (KLH) from Calbiochem (374807), Complete Freund’s Adjuvant (CFA) from Sigma (F5881) and Incomplete Freund’s Adjuvant (IFA) from Sigma (F5506) emulsions.. Mice were challenged intradermally in one ear with 10μg/mouse in 10μls/site seven days post sensitization. Ear thickness was measured pre-sensitization, and 24 and 48hrs post challenge using a precision engineering micrometer (Mitutoyo Absolute). The read-out for this assay is ear-swelling, plotted as change in ear thickness. Ears were harvested from each group and snap frozen for future analysis.
Chapter III.

Results

The primary focus of this research was to determine if the murine Tyk2 P1124A variant could be used as a translational model of the P1104A human Tyk2 SNP. To confirm or negate similarities we measured STAT phosphorylation (pSTAT) stimulated by cytokines signaling through Tyk2 containing pathways. Secondarily, if P1124A proximal activity was similar to its human homolog, determining down-stream functional consequences of the murine SNP in is important in understanding how it confers its protective abilities. To this end cytokine induced mediator production, T cell and monocyte polarization and a DTH animal model of inflammation was run. Lastly, we wanted to determine if we could track the physical alteration that occurs with Tyk2 variant was consistent or divergent from other Tyk2 alterations. Therefore the heterozygous Tyk2 P1124A, Tyk2 knockout, Tyk2 K950A and Tyk2 Y1074F/Y1075F GEMs were used for comparison. Due to the scope of the study, ancillary insights into basic Tyk2 and JAK function can be elucidated including accompanying JAK compensatory activity when there are alterations in Tyk2 function and the influence of different phosphorylation activities of Tyk2.

Proximal Activity: STAT Phosphorylation

Tyk2 influenced STAT phosphorylation was measured in spleen, LN and bone marrow derived cells (GM-CSF only) stimulated with various cytokines containing Tyk2 in its signaling pathway and counter non-Tyk2 related cytokines. Tyk2 homozygous P1104A PBMCs had reduced STAT activity when stimulated with type 1 IFNs, IL-12
and IL-23, and retained IL-13 STAT activity, but IL-10 and IL-6 stimulated STAT phosphorylation remains controversial possibly due to different methods of analysis or the use of PBMC vs expanded T cells (Couturier et al., 2011; Dendrou et al., 2016). Therefore, we hypothesized that analyzing multiple cell subsets from multiple tissues using phospho-Flow may resolve this discrepancy as the cells would be in different physiological states depending on tissue.

IFNα was used for type I IFN stimulation. Data on human JAK activity attributes type I IFN activity to STAT1 and STAT2 or STAT3, controlled by SOCS3. Dendrou’s group (2016) utilized STAT1 and STAT3 to measure type I IFN activity in P1104A PBMCs in response to both IFNα and IFNβ stimulation. In our studies STAT4, but not STAT3 was phosphorylated in wildtype cells at all concentrations of IFNα tested. Similar to the STAT3 phosphorylation pattern published, splenic CD3+CD4+ T cells (figure 8A), CD335+NK1.1+ NKT cells (figure 8C) and B200+ B cells (figure 8D) from the P1124A homozygous variant loss STAT4 phosphorylation in response to IFNα stimulation. CD3+CD8+ T P1124A cells (figure 8B) showed an attenuated response, which is in contrast to the human PBMC data.

Similarly, the GEM Tyk2 KO CD8 T cells showed an attenuated pSTAT response. This activity was not replicated in either the K950A or the Y1074F/Y1075F GEMs. Cells from both spleen (figure 8) and LN (figure 9) showed similar patterns of IFNα stimulated STAT4 phosphorylation. The GWAS allelic dosage affect additive model, where gene dosages is thought to function in a linear format, was not consistent with Tyk2 activity where heterozygous P1124A did not consistently respond in a dose dependent manner, response to reduced allele frequency was cell type dependent.
Figure 8. IFNα Stimulated STAT4 Phosphorylation of Splenic Cells.

CD4+ (A), CD8+ (B), NK T (C) and B cells (D) were plotted as percent of cells that were pSTAT4 positive after IFNα stimulation, n=2. P1124A variant CD4+, NK T and B cells do not respond to type I IFN stimulation. P1124A and KO CD8+ T cells have a delayed onset of type I IFN stimulated pSTAT4.

Figure 9. IFNα Stimulated STAT4 Phosphorylation in cells from LNs.
IFNα Stimulated STAT4 Phosphorylation in cells from LNs. CD4+ (A), CD8+ (B), NK T (C) and B cells (D) were plotted as percent of cells that were pSTAT4 positive after IFNα stimulation, n=2. P1124A variant CD4+, NK T and B cells do not respond to type I IFN stimulation. P1124A and KO CD8+ T cells also have a delayed onset of type I IFN stimulated pSTAT4.

IL-12 stimulated both pSTAT3 (figure 10) and pSTAT4 (figure 11) in splenic cells but not in any of cell populations tested from pooled LNs. This may reflect difference in IL-12 receptor levels as the murine cells were not TCR stimulated prior to IL-12 treatment. CD335+/NK1.1+ NK T and B220+ B cells from the spleen did not phosphorylate STAT3 in response to IL-12 stimulation. P1124A CD4+ (figure 10A) and CD8+ (figure 10B) T cells failed to activate STAT3 in response to IL-12 stimulation. Similarly, cells from the K950A and Y1074F/Y1075F GEMs had an attenuated pSTAT4 response. The heterozygous GEM’s STAT3 and STAT4 phosphorylation was not upregulated in response to IL-12 stimulation. Surprisingly, the Tyk2 KO GEMs retained IL-12 stimulated STAT3 phosphorylation and heightened activity in CD8+ T cells (Figure 10B), an activity not replicated in any of the other GEMs. This data would indicate that when Tyk2 is completely removed that another JAK, likely JAK2, can compensate for Tyk2 absence in these particular cells.

IL-12 stimulated CD4+, CD8+ and NK T cells pSTAT4 but not in B cells. Similar to pSTAT3 data, P1124A GEMs failed to induce pSTAT4 in response to IL-12 stimulation. This observation is consistent with published human P1104A variant PBMC pSTAT3 activity (Dendrou et al., 2016). Again the Tyk2 KO, but not the other GEMs, retained their pSTAT3 strengthening the argument for JAK compensation in response to Tyk2 protein loss.
Figure 10. IL-12 Stimulated STAT3 Phosphorylation.

CD4 (A) and CD8 (B) T cells induced pSTAT3 in response to IL-12 stimulation. n =2. P1124A variant did not induce pSTAT3 in response to IL-12 stimulation. pSTAT3 was induced n Tyk2 KO GEMs.

Figure 11. IL-12 Stimulated STAT4 Phosphorylation.

CD4 (A), CD8 (B) T cells and NK T (C) phosphorylated STAT4 in response to IL-12 stimulation. n =2. P1124A variant did not phosphorylate STAT4 in response to IL-12 stimulation. pSTAT4 was induced n Tyk2 KO GEMs pSTAT4 was attenuated in cells form the heterozygous Tyk2 P1124A, K950A and Y1074F/Y1075F GEMs.
For IL-23 evaluation, a direct activation with IL-23 in the absence of pre-activation via the TCR was performed. CD3+CD8+ T cells and CD335+NK1.1 NK T cells responded to IL-23 stimulation (figure 12). Wildtype C57Bl/6 cells stimulated with IL-23 showed a modest pSTAT4 response. Tyk2 heterozygous P1124A variant and Y1074F/Y1075F cells showed an elevated pSTAT4 response in CD3+CD8+ T cells. CD335+NK1.1 NKT cells from all GEM lines except Tyk2 KO retained pSTAT4 in response to IL-23. IL-23 did not stimulate pSTAT3 in cells from either spleen or LNs, nor did it induce pSTAT4 in any of the cell populations from the LNs.

Figure 12. IL-23 Stimulated STAT4 Phosphorylation.

CD8 (A) T cells and NK T (B) cells pSTAT4 in response to IL-23 stimulation. n =2. CD8+ T cells from Tyk2 heterozygous P1124A and Y1074F/Y1075F GEMs had a heighten response to IL-23 stimulation. None of the other GEM’s CD8+ T cells, except Tyk2 KO, lost IL-23 pSTAT4.

Although published data on IL-6 stimulated P1104A PBMC STAT activation focused on STAT3. IL-6 induces murine pSTAT3 and pSTAT4 therefore we evaluated
the activity of both transcription factors. Splenic P1124A CD4+ and CD8+ T cells lost STAT3 activity (figure 13) in response to IL-6 stimulation. The Tyk2 KO and Tyk2 KDKI showed a complete and partial loss of activity, respectively, in CD8+ T cells. CD4+ T cells from the lymph node, replicated the splenic CD4+ T cell pSTAT3 response, but the other cell population’s retained their activity. Cell type based variation is also observed with splenic cells from the Tyk2 KO GEMs, which show a heightened pSTAT3 response in CD4+ T cells but loss in CD8+ T cells. LN cells from the Tyk2 KO showed either an unaffected or an increased pSTAT3 in CD4+ (14A), CD8+ T (14B) and B cells (14D) but a lessened NKT (14C) response. In contrast to the pSTAT3 data, P1124A GEM IL-6 induced pSTAT4 was unaffected by P1124A variant. With the exception of B cells, both splenic (figure 15) and lymphatic (figure 16) cells had similar trends.

Figure 13. IL-6 Stimulated STAT3 Phosphorylation in Splenic cells.

CD4+ (A) and CD8+ (B) T cells phosphorylated STAT3 in response to IL-6 stimulation. n =2. Tyk2 P1124A lost Tyk2 dependent IL-6 STAT3 activation.
Figure 14. IL-6 Stimulated STAT3 Phosphorylation in cells from LN.

CD4+ (A), CD8+ (B) T cells, NK T (C) and B cells (D) phosphorylated STAT3 in response to IL-6 stimulation. n =2. CD4+ Tyk2 P1124A lost Tyk2 dependent IL-6 STAT3 activation and NKT Tyk2 P1124A had a reduced response. The pSTAT3 response in both CD8 T cells and B cells from the P1124A GEM remained unchanged.
Figure 15. IL-6 Stimulated STAT4 Phosphorylation.

All of the GEMs CD4+ (A), CD8+ (B) T cells and NK T cells (C) upregulated STAT4 activity in response to IL-6 stimulation. n = 2.

Figure 16. IL-6 Stimulated STAT4 Phosphorylation.

All of the GEMs CD4+ (A), CD8+ (B) T cells and NK T cells (C) upregulated STAT4 activity in response to IL-6 stimulation. Homozygous and heterozygous P1124A and Tyk2 K950A GEMS lost B cell (D) specific STAT4 activity. n = 2.

IL-10 stimulates Tyk2 and Jak1 dependent STAT3 and STAT4 activation.

pSTAT3 of P1104 PBMCs was used as the primary readout for our comparative study in IL-10 proximal assays (Dendrou et al., 2016). Splenic CD4+ (figure 17A) and CD8+ (figure 17B) T cells modestly activated STAT3 in response to IL-10 stimulation. Contrary to P1104A, P1124A variant CD4+ and CD8+ T cells had a decreased pSTAT3 response. Similarly, decreased responses were observed in cells from the K950A and Y1074F/Y1075F GEMs. Interestingly, the pSTAT3 response was not affected in CD8+ T
cells from the spleen of Tyk2 P1124A variant which is different than the response of LN cells where homozygous and heterozygous P1124A CD8+ T cells (figure 18A) and B220+ B cells (figure 18B) had heighten STAT3 activity in response to IL-10 stimulation; while NK T activity was unchanged (figure 18B).

Although pSTAT3 was the dominate readout for IL-10 stimulated human PBMCs, STAT4 produced a more vigorous response in our murine assays. Similar to human pSTAT3 data, pSTAT4 was not attenuated in spleen or (figure 19) LN cells (figure 20) from P1124A GEMs. In many cases cells from P1124A had a heightened response to IL-10 stimulation, cells with a heightened response where splenic CD8+ T and NK T cells and CD4+ and CD8+ T cells from the LN.

Figure 17. IL-10 Stimulated STAT3 Phosphorylation of Splenic Cells.

Tyk2 P1124A variant and Y1074F/Y1075F but not Tyk2 KO CD4+ T cells (A) lost IL-10 stimulated pSTAT3. CD8+ T cells (B) pSTAT3 was unaltered when Tyk2 is modified. n = 2.
Figure 18. IL-10 Stimulated STAT3 Phosphorylation of cells from the LN.

*Heterozygous and Homozygous P1124A variant CD8+ T cells (A) and B220+ B cells (C) had a heightened STAT3 response to IL-10 stimulation. K950A and Y1074F/Y1075F also had a higher IL-10 stimulated STAT3 activation compared to wildtype and Tyk2 KO B cells but that response was not as dramatic as either P1124A GEM. NK T cell STAT3 phosphorylation was unaltered. n = 2*

Figure 19. IL-10 Stimulated STAT4 Phosphorylation.

*P1124A GEMS show and unaltered CD4+ T cells (A) or heightened CD8+ T cells (B) and NK T cells (C) IL-10 stimulated pSTAT4. n = 2*
Figure 20. IL-10 Stimulated STAT4 Phosphorylation in cells from LNs.

*P1124A GEMS show an unaltered B cell (D) or heightened CD4+ T cell (A), CD8+ T cell (B) and NK T cell (C) IL-10 stimulated pSTAT4. n = 2*

As a counter screen, we evaluated IL-2 and GM-CSF, which are proposed to be nonTyk2 containing cytokines. IL-2 functions through Jak1, Jak2 and Jak3 to stimulate STAT3 and STAT5. IL-2 stimulated pSTAT5 expression in splenic CD4+ (figure 21A) and CD8+ (figure 21B) T cells and B220+ B cells (figure 21C) was unaltered by Tyk2 modulation.
GM-CSF JAK specific activity functions via Jak2 stimulation of STATs 3 and 5. Tyk2 related GM-CSF pSTAT5 activity was measured in CD11b\textsuperscript{Hi} (figure 22A), CD11b\textsuperscript{Int} (figure 22B) and CD11c (figure 22C) cells isolated from bone marrow. Surprisingly although Tyk2 is not known to function in the GM-CSF pathway, Tyk2 associated pSTAT5 modulation was observed. Tyk2 KO CD11b\textsuperscript{Hi} and CD11b\textsuperscript{Int} myeloid cells had a higher percentage of cells phosphorylated STAT5 in response to GM-CSF. Decreases in CD11b\textsuperscript{Hi} pSTAT5 expression were not observed in any of the other GEMS; Tyk2 KO even showed an elevated response. Conversely, the percentage of Tyk2 KO CD11b\textsuperscript{Int} myeloid cells that pSTAT5 in response GM-CSF stimulation was greatly reduced. No other GEMS showed an altered response in CD11b\textsuperscript{Int} cells. Tyk2 alterations had a much more pronounced effect on CD11c\textsuperscript{+} myeloid cells where K950A had a heightened pSTAT5 response, homozygous and heterozygous P1124A variants as well as Tyk2 KO cells pSTAT5 activity was ablated and Y1074/Y1075F pSTAT5 response was to GM-CSF matched the wildtype. In addition, investigations into Tyk2’s influence on myeloid cell populations led to discovering that Tyk2 modulated CD11b and CD11c cell
densities (figure 23). Tyk2 KO GEMS had a lower percentage of CD11b\textsuperscript{Hi}, CD11b\textsuperscript{Int} and CD11c cells than those of C57Bl/6 wildtype mice. The percentage of CD11b\textsuperscript{Hi} cells was unaltered in the other GEMs. Homozygous P1124A cells had a higher amount of CD11b\textsuperscript{Int} cells than wildtype cells. CD11c cell densities were reduced in all of the GEMs, a 60% reduction was observed in hetero- and homozygous P1124A GEMs.

Figure 22. GM-CSF Stimulated pSTAT5 Bone Marrow Cells.

GM-CSF stimulated bone marrow CD11b\textsuperscript{Hi} (A) cells from Tyk2 KO GEMs had a heightened pSTAT5 response, while CD11b\textsuperscript{Int} (B) cells from the same GEM had reduced activity. CD11c cells (C) GM-CSF stimulated pSTAT5 activity was higher in K950A, lower in KO and P1224A (homo- and heterozygous) and unaltered in Y1074F/Y1075F GEMs.
Tyk2 Signaling functional (distal) Studies

In order to correlate the effects of Tyk2 mutations on the pSTAT response to a downstream longer term activity, we evaluated the effect of the Tyk2 P1124A variant in cytokine induced mediator production. Although most data is consistent with respect to effects of Tyk2 deficiency or mutations on IL-12 and Type I interferon signaling, there are discrepancies with respect to the effect of these mutations on IL-6 and IL-10 activity (Couturier et al., 2011; Dendrou et al., 2016). Published data evaluating proximal STAT activity showed that Tyk2 P1104A had no effect on IL-6 and IL-10 activity, while another article using IL-6 and IL-10 stimulated suppressor of cytokine signalling-3

Fluctuations in the percentage of \(CD11b^{Hi}\), \(CD11b^{Int}\) and \(CD11c\) cells in the bone marrow of GEMS
(SOCS3) transcription showed reduced activity (Couturier et al., 2011; Dendrou et al., 2016). One hypothesis is that this discrepancy could be due differences in the read-outs since STAT phosphorylation is a very proximal and short term event occurring within 20 minutes and SOCS3 transcription stimulation is a significantly more distal function measured at 2 hours post cytokine stimulation in activated cells. The disconnect could be due to differences in the cell types analyzed and/or secondary effects from other immune regulators induced by IL-6 and IL-10 that have a Tyk2 component, causing a more pronounced effect on IL-6 and/or IL-10 cytokine reaction due to modulation of the downstream signals (i.e. SOCS3). Or P1104A could cause an abhorrent and more pronounced IL-6 and/or IL-10 cytokine reaction caused by loss of SOCS3 production.

To further the biology associated with the role of Tyk2 in IL-6 and IL-10 as well as determine the effect of Tyk2 mutations on IL-12, Type I interferon and other Tyk2 influenced cytokines, assays were developed to explore the biological effects of Tyk2 modulation downstream of both STAT phosphorylation and SOCS transcription in the GEMS. Cytokine dependent mediator production was measured in resting and cytokine stimulated cells after 16 hours. IFNα and IL-12 induced IFNγ Induced Protein -10 (IP-10) (figure 25A and 26) and IFNγ (figure 25B), respectively. Cytokines produced in response to both IFNα and IL-12 tightly paralleled their respective STAT activation. IFNα dependent IP-10 production was attenuated and IL-12 dependent IFNγ production was ablated in all GEMs from both spleen (Figure 25) and LN (Figure 26). CD8+ T cells from P1124A and KO GEM cells had a more pronounced effect on IP-10 production in cells from the spleen than their lymphatic counter parts producing a lessened but substantial
concentration of cytokine.

Figure 24. IFNα and IL-12 Dependent IP-10 and IFNγ Production.

*IFNα dependent IP-10 production was attenuated and IL-12 dependent IFNγ production was ablated in all GEMs. n = 2*

Figure 25. IFNα Dependent IP-10 Production in LN Cells.

*IFNα. IFNα dependent IP-10 production was attenuated in all GEMs. n = 2*

The most prominent difference between human P1104A PBMC and murine P1124A spleen and LN proximal STAT activity identified in these studies was with IL-
23 stimulation. While this can reasonably be explained by assay discrepancies, differences in tissue and cell types analyzed or species related differences, the data remains that although STAT activation was significantly reduced in P1104A studies, this response was not replicated in the P1124A studies (Dendrou et al., 2016). Further, there was no decreased IL-23 induced IL-17α production in spleens cells from the variant, but was ablated in the Tyk2 KO and K950A and Y1075F/Y1075F mutants. In contrast, the variant data indicated an apparent upregulation of IL-17α.

Figure 26. IL-23 Stimulated IL-17α Production of Spleen Cells.

*Variable IL-23 responses in the GEM mice. n = 2*

P1124A cells stimulated with IL-6 from both spleen (figure 28) and LN (figure 29) had unaltered IL-21 and TNFα production. Similar observations were made with the other GEMs. Consistent with the IL-10 pSTAT response in the GEMs, no decrease was observed in the production of IL-10 induced mediators from spleen (figure 28) or LN (figure 29). Despite the modest production of IL-2 by IL-10, the Tyk2 P1124A variant
and the Tyk2 KO appeared to have an increased production (figure 28). No decrease in
the production of any IL-10 induced mediator, and even an apparent increase in IL-1β
production from the LN cells from the P1124A variant. Tyk2 KO showed a decreased
production of IL-6 induced IL-21 and TNFα, but no effect on IL-10 induced IL-4 or IL-
1β production (figure 29) in cells from LN.

Figure 27. IL-6 and IL-10 induced mediator production from spleen cells.

*IL-6 stimulated IL-21 (A) and TNFα (B) and IL-10 stimulated IL-1β (C), IL-2 (D), IL-4
(E) and TNFα (F) production. n =2*
IL-6 and IL-10 induced mediators in LN cells.

*IL-6 stimulated IL-21 (A) and TNFα (B) and IL-10 stimulated IL-1β (C), IL-2 (D), IL-4 (E) and TNFα (F) production. n = 2*

IL-2 and GM-CSF stimulated mediator production was used as a measure to counter screen nonTyk2 influenced cytokine activity. IL-2 stimulated IFNγ, IL-6 and IL-10 (figure 29) production had minimal Tyk2 related differences. IL-6, IL-10 and TNFα production in response to GM-SCF stimulation was unaltered in the Tyk2 GEMs. Interestingly, GM-CSF stimulated IFNγ and IP-10 cytokine production was inhibited in all of the GEMs.
Figure 29. IL-2 induced mediator production from spleen cells.

*IL-2 stimulated IFNγ (A) and IL-6 (B) and IP-10 (C), production. n = 2*

Figure 30. GM-CSF induced mediator production from spleen cells.

*GM-CSF stimulated IL-6 (A), IL-10 (B) TNFα, (C), IFNγ (D) and IP-10 (E) production. n = 2*
Cellular Polarization

Cytokine signaling via Tyk2 contributes to all stages and types of immune responses. Type 1 IFNs and IL-12 drive Th1, IL13 supports Th2 polarization and IL-6, IL-23 supports Th17 cell polarization and survival and IL-10 is necessary for the resolution of inflammation. Therefore, understanding how Tyk2 modulation affects these intricate processes of inflammation is of great interest. We utilized GEM mice carrying various Tyk2 mutations to assess how Tyk2 modulates cell polarization, which could contribute to modulation of the overall immune response. Up to this point, the research has focused on how individual cell populations respond to genetic manipulation of Tyk2 in simple proximal experiments. But understanding how the P1124A variant as well as other Tyk2 mutations modulate complex biological functions, such as cell polarization is necessary to determine the net effect of Tyk2 on immune responses. Naïve CD4+ Tyk2 KO 129/Ola T cells polarized to Th1 using immobilized αCD3/CD28 in combination with αIL-4 and recombinant IL-12 showed a reduction in IFNγ production (Shimoda et al., 2000). Stimulation of the Th1 cells under Th2 conditions led to cells with a dual phenotype: reduced IFNγ production and heightened IL-4 secretion, indicating that altered Tyk2 in CD4 T cells could hamper Th1 differentiation and cause preferential Th2 polarization (Shimoda et al., 2000).

Homozygous variants are both protected from various autoimmune diseases and exempt from Tyk2 deficiency associated PID, therefore determining if and how alteration in Tyk2 changes inherent inflammatory phenotype responses to cellular activation is necessary to understand how P1104A elicits protection against disease (Dendrou et al., 2016; Watfield & O’Shea, 2006). Transcription factor messenger RNA (mRNA) levels
from heterozygous P1104A Tyk2 T lymphocytes stimulated with CD3ε/CD28 beads expressed significantly more GATA3, the Th2 associated transcription factor, but no alteration of T-bet (Th1), RORγt (Th17) or Foxp3 (Treg) expression (Couturier et al., 2011). When cultured with IL-2 these cells also produced significantly higher concentration of IL-5 and IL-13, although IL-4 production was unchanged (Couturier et al., 2011). This data indicates that like Tyk2 KO, P1104A T cells maybe pre-wired towards a Th2 inflammatory response. The effect of the P1124A mutation on cell differentiation in both the heterozygous and homozygous mice is presented in this section.

Th1 differentiated CD4+ T cells isolated from homozygous P1224A and K950A KDKI spleens lost IFNγ, IL-10 and IL-21 production; and had a reduced concentration of TNFα (figure 32A). Reduction of IL-21 cytokine production was gene dosage dependent. No significant alteration to splenic CD4+ T cell Th2 (figure 32B) or Treg (figure 32D) polarizations was observed. While there were no differences in the amount of IL-1β, IL-6 nor IL-17α; IL-21 and IL-10 cytokine production is reduced in homozygous Th17 differentiated P1124A and K950A CD4+ T cell (figure 32C). Due to Tyk2 GEM IL-17α concentration remaining consistent, Th17 polarization is seemingly intact in P1224A GEMs, but how their lack of IL-21 production would affect other immune cells in an in vivo system remains unknown. Inhibition of immunosuppressive cytokine IL-10 production in P1124A GEMs indicate a loss in anti-inflammatory signaling which could contribute to sustained inflammatory responses.

Variation in levels of IFNγ, IL-10 and TNFα production in Th1 polarized cells from naïve P1124A CD4+ T LN cells was seen in GEMs other than just homozygous
P1124A and K950A (figure 32E). Polarized Th1 cells derived from Tyk2 KO and heterozygous P1124A from the LN also had decreased IFNγ, IL-10 and TNFα concentrations. All Th2 polarized GEMs, except Y1074F/Y1075F, CD4+ T LN cells, had increased levels of IL-2 production (figure 32F). LN CD4+ T cells differentiated to Tregs had no change in cytokine signaling (figure 32H). Th17 polarized CD4+ T cells from the LN had unchanged IL-1β production but heightened IL-6 production in all of the GEMs.

CD4+ T cells differentiated to either Th1 or Th2 phenotypes where stained for GATA3 expression. GATA3 was upregulated in Th2 polarized cells from all of the GEMs. Th1 and Th2 reciprocally inhibit the other. Since Tbet is the transcription factor associated with Th1 polarization, and functions downstream of Tbet would inhibit GATA3 activity, Th1 polarized cells should not express GATA3. Both K950A and homozygous P1124A splenic CD4+ T cells that were Th1 polarized expressed low levels of GATA3 (figure 33). GATA3 expression is also seen in Tyk2 KO and homozygous P1124A CD4+ T cells from the LN (figure 34).
Figure 31. CD4+ T cell Polarization.

Naïve CD4 T cells from the spleen (A-D) and LN (E-H) were polarized using a combination of CD3ε/CD28 activation and cytokine stimulation to 1 of 4 phenotypes Th1 (A and E), Th2 (B and F), Th17 (C and G) and Treg (D and H). Cytokine concentrations are in pg/mL. n = 2
Figure 32. Splenic CD4+ T cell GATA3 Expression.

*Th2 (red) and Th1 (blue) polarized CD4+ T cells were stained for GATA3 expression. n = 2*

Figure 33. CD4+ T cell GATA3 and Tbet expression in cells from LNs.

*Th2 (red) and Th1 (blue) polarized CD4+ T cells were stained for GATA3 and Tbet expression.*
Naïve CD8+ T cells were differentiated to Tc1, Tc2, Tc17 or Treg phenotypes with in intent to understand the role of Tyk2 in CD8 T cell polarization. Unlike the results seen with Th1 differentiated CD4+ T cells from homozygous P1124A and K950A GEMs, Tc1 polarized CD8+ T cells from the spleen retained IFNγ production (figure 35A). Similar to Th1 polarized CD4+ T cells, IL-10 and TNFα concentrations were reduced in supernatants from Tc1 CD8+ T cells from homozygous P1124A GEMS. Further, Tc1 CD8+ T cell IL-10 production was decreased in all homozygous GEMs, indicating a potential role for Tyk2 in CD8 T cell driven anti-inflammatory responses. However, splenic CD8+ T cell TNFα production of Tyk2 KO, K950A KDKI and homozygous P1124A GEMs was also reduced, which would lead to less inflammation. Homozygous P1124A GEMs had unaltered Tc2 (figure 35B) and Treg (figure 35D) differentiation. CD8+ T cell from homozygous P1124A GEMs polarized towards a Tc17 phenotype produced lower concentrations of IL-21 and TNFα. Homozygous P1124A CD8+ T cell from the LN had no significant cytokine driven mediator alterations in any of the Tc phenotypes (figure 35E-H). K950A CD8+ T cells polarized towards a Tc1 phenotype had reduced concentrations of IL-10 and TNFα (figure 35E). K950A CD8+ T cells singularly expressed low levels of GATA3 when Tc1 stimulated (figure 36).
Figure 34. CD8 Polarization.

Naïve CD8 T cells from the spleen (A-D) and LN (E-H) were polarized using a combination of CD3ε/CD28 activation and cytokine stimulation to 1 of 4 phenotypes Tc1 (A and E), Tc2 (B and F), Tc17 (C and G) and Treg (D and H). n = 2.
Defective Th1 polarization leads to questions about how Tyk2 modulates macrophage polarization. Macrophages are integral in the establishment of an inflammatory response and produces IFNγ to induce Th1 polarization, which can subsequently produce cytokines that could influence macrophage polarization. Therefore, we explored the effect of the various Tyk2 mutations on macrophage polarization using bone marrow derived macrophages (BMDMs). BMDMs polarized to either M1 or homeostatic M2 phenotypes with GM-CSF and M-CSF, respectively, had minimal differences in cytokine production due to polarization (figure 37A). M1 differentiated BMDMs from the GEMs showed a decreased IL-6 production from Tyk2 KO, Tyk2
KDKI, and Y1074F/Y1075F GEMs. M2 polarized BMDCs had the same trend in IL-6 and a reduction of IP-10 production in all homozygous GEMs.

Figure 36. M1/M2 Polarization.

Bone marrow derived cells were polarized towards either M1 via GM-CSF stimulation (A) or M2 by M-CSF stimulation (B). n = 2

Delayed Type Hypersensitivity

Tyk2’s influence in inflammation has been established in various Th1 and Th17 dependent animal models simulating human diseases. Tyk2 depletion attenuated DSS and TNBS models of colitis, as well as IMQ, IL-23, and mBSA-DTH induced models of skin inflammation (Ishizaki et al., 2011; Ishizaki et al., 2013). P1124A has also been shown to be protective in a Th17 driven EAE model of multiple sclerosis (Dendrou et al., 2016). To explore the role of the various Tyk2 mutations, including the P1124A variant on in-vivo immune responses, our goal was to run acute CD4+ T cell dependent Th1 and
Th17, and a CD8+ T cell dependent animal model. The proposed models included CD4+ Th1 dependent KLH DTH, CD4+ Th17 dependent IMQ and a CD8+ T cell dependent 2,4-dinitrofluorobenzene (DNFB) induced contact hypersensitivity. Of the three animal models proposed only the KLH DTH was completed in enough time for this thesis due to GEM availability. The results from the other two models are pending.

Difference in the ear swelling of sensitized and challenged wildtype mice (Fig 38 labeled C57Bl6) compared to unchallenged mice were of the magnitude expected for the model (fig 38). All of the Tyk2 GEMs had a reduced ear swelling by 24 hours and almost complete ablation by 48 hours (figure 38). Area under the curve (AUC) changes in ear swelling showed similar trends in loss of inflammatory activity. Due to this assay having too few animals, no hard conclusions can be extrapolated from this data. The repeat experiment is pending. Although, this experiment is not high powered enough to measure significance both the homozygous and heterozygous P1124A GEMS, as well as the other GEMs, showed a trend in protection against KLH induced ear swelling.

Figure 37. Delayed Type Hypersensitivity.
Ear swelling data at 24 hours (A) and 48hrs (B and C) post KLH challenge. Mouse numbers are too small to determine statistical significance or see any variation between the GEMs but there is a trend in Tyk2 mediated reduction of ear swelling. n =1, repeat pending.
Chapter IV.

Discussion

The more we learn about the mechanisms that coordinate the immune systems and how it governs the inflammatory response the more we appreciate the sophistication and complexity of it. The elicitation of an immune response depends on an appropriate stimulus, receptors, signaling pathways and/or mediators. It encompasses tissue specific signatures, cytokine and chemokine expression, cellular interactions, transcription factor regulation and epigenetic modifications; amongst other systems. The complexity of these systems is compounded greatly during times when there is a defective immune response. Cellular communication via cytokines is only one piece of the very complicated inflammatory response and influences multiple facets of immunology from stem cell differentiation to cellular maturation (Lewis & Blutt, 2019). Here we studied a small, yet complex, snapshot of cellular communication elicited by the JAK protein, Tyk2, and how it modulates inflammation. Compelling genetic data links the P1104A Tyk2 variation with a phenotype that is protective against development of multiple autoimmune diseases (Dendrou et al., 2016; Couturier et al., 2011; Diogo et al., 2015; Li et al., 2013). Due to Tyk2’s function in type 1 and 2 cytokine signaling pathway it is contributes in various manners to the development, maintenance and resolution of inflammation. Using GEM mice we explored pivotal steps in the understanding of Tyk2’s contribution to the immune system.
P1124A is a murine model of the human P1104A variant

P1124A is the murine homologue of the P1104A SNP, which is protected from autoimmune diseases. This GEM was created to understand the functional consequences of the A variant in cell signaling, cell differentiation, and overall pathology. The potential use of the P1124A GEM as an animal model to study how this variant alters the inflammatory process would be instrumental in understanding how the human P1104A variant may confer its protective phenotype. Therefore, our goal was to back-translate the known human variant molecular phenotype and to extend this characterization with the murine P1124A. The first step in that process was determining if the P1124A GEM replicates human P1104A activity. Using cytokine stimulation and pSTAT detection methods similar to that previously reported (Dendrou et al., 2016; Couturier et al., 2011, our data suggest that the P1124A replicates the human biology with a few exceptions, discussed below.

Type 1 IFNs are antiviral inflammatory cytokines with high affinity to IFN alpha receptor - 2 (IFNAR2) subunit of the IFN receptor (IFNR) (Lopez de Padilla et al., 2016; Ivashkiv et al., 2014). Type I IFN activity signals through Tyk2 and Jak2 signaling to activate multiple STAT transcription factors. STAT1 and STAT2 can be tyrosine phosphorylated, causing binding to IRF9 and the formation of the transcription factor complex ISGFB (Lopez de Padilla et al., 2016; Ivashkiv et al., 2014). STAT3 is activated, which stimulates it to dimerize an action which functions to inhibit STAT1 dependent gene activation and subsequent down regulation of IFNα induced inflammatory responses by competitively binding to type I IFN promoters (Lopez de Padilla et al., 2016; Ivashkiv et al., 2014). Loss of only pSTAT1 could be indicative of
defective type 1 IFN pro-inflammatory responses, while loss of only pSTAT3 would show a flawed IFNα/β anti-inflammatory response. 80% reductions in both pSTAT1 and pSTAT3 seen in CD4+ and CD8+ T cells, B cells and monocytes from P1104A PBMCs indicate an overall loss in responsiveness to type 1 IFNs (Dendrou et al., 2016). In our studies STAT4, but not STAT3, was activated in response to IFNα stimulation. There is a greater than 90% reduction in pSTAT4 in CD4+ T cells, B cells and NKT cells from P1124A spleens and LN. Data from both splenic and LN P1124A CD8+ T cells was the only cellular type to have a dissimilar IFNα response; IFNα stimulated pSTAT4 was delayed and only moderately reduced. Whether the CD8 response is truly different than what would occur in P1104A PBMCs if tested in a dose response instead of a single concentration remains to be seen, but our data suggest that the level of disruption to type 1 IFN signaling is cell type dependent and more dramatically effects CD4 vs CD8 T cells.

The type 1 immunological phenotype is dependent on IL-12 stimulation, is regulated by the Tbet transcription factor and produces IFNγ from multiple cell lineages including Th1 CD4+, Tc1 CD8+, M1 and ILC1 cells. The IL-12 protein (IL-12p70) is a homodimer that consists of a 35Kda α-chain subunit (p35) and a 40Kdaβ-chained subunit (p40) (Zundler et al., 2015). β-chain binding to the IL-12 receptor β-2 (IL12Rβ2) recruits Jak2, while α-chain binding to the IL-12 receptor β-1 (IL12Rβ1) recruits Tyk2 and predominately leads to STAT4 activation and homo-dimerization (Teng et al., 2015; and Zundler et al., 2015). Although IL-12 also signals through a transcription factor complex formed by STAT1, STAT3 and STAT5, its dominate signaling mechanism is via STAT4 (Teng et al., 2015). The P1104A SNP caused a 70% reduction in IL-12 induced pSTAT4 in stimulated PBMCs (Dendrou et al., 2016). Mimicking P1104A’s defective pSTAT4
activity, IL-12 stimulated cells from P1124A spleen had a >90% reduction in pSTAT4 (CD4+, CD8+ and NK T cells) and pSTAT3 (CD4+ and CD8+ T cells) activity. Although Dendrou, et al’s article does not address the fate of the STAT1/STAT3/STAT5 transcription factor complex, P1124A indicates that both IL-12 induced pSTAT3 and pSTAT4 are impacted.

IL-23, an IL-12 family member, uses IL12Rβ1-p40 along with IL-23 receptor β p19 (IL23R-p19) to signal through Tyk2 and Jak2 predominately activating STAT3 (Teng et al., 2015). IL-23 singularly cannot stimulate the Th17 immunological phenotype but does support the IL-17α production, RORC transcription (the gene responsible for RORγT) and Th17 cell survival (Gaffin et al., 2014). Due to IL23R-p19 not being expressed at on naïve human and mouse T cells, P1104A PBMCs were pre-activated via the TCR system to elevate the receptor level in order to determine loss of IL-23 activity (Dendrou et al., 2016). However, since a moderate mediator response was detected in the murine cells without pre-activation, the P1124A cells were evaluated in the presence of IL-23 direct stimulation to eliminate any contribution from a pre-activation step. An IL-23 pSTAT4 response was detected in a small percentage of CD8+ and NK T spleen cells. IL-23 stimulated P1124A CD8+ and NK T cell’s pSTAT4 activation was at a similar, or in the case of NK T cells higher, then what was seen from wildtype spleens. This marks one observed difference between murine P1224A and human P1104A mutations. While this alteration could be due to assay related differences, the further retention of IL-23 stimulated IL-17α production from spleen cells indicates that P1124A cells retain partial IL-23 response and Th17 cell activity. GEM P1124A preservation of STAT4 phosphorylation could indicate a compensatory mechanism that is either not existent or is
delayed in P1104A PBMCs, or is modulated differently in cells of different activation or maturation states. The difference seen could also be due to cellular localization, where cells from the spleen do not respond to IL-23 in the same manner as PBMCs. The later hypothesis would require experimentation of murine PBMCs or human spleen cells since the state of cell differentiation or activation in blood vs spleen cells are different.

IL-6 is pleiotropic cytokine that can be either pro-inflammatory or anti-inflammatory depending on the conditions at the time of signaling. IL-6 functions not only as a cytokine but also in a manner similar to that of a hormone (Hunter & Jones, 2015). Systemic IL-6 activity includes its role in vascular disease, lipid metabolism, insulin resistance, mitochondrial energy production and activity in various neurological processes including neuroendocrine regulation and behavior (Hunter & Jones, 2015). IL-6 cytokine pro-inflammatory functions include type 1 pro-inflammatory cell activity, assistance in Th17 lineage commitment, humoral immunity promotion via B cell differentiation, inhibition of Treg function and induced Treg commitment (Hunter, 2005). IL-6 binds to the IL-6 receptor and GP130 forming a complex that signals through multiple kinase family members for its function including the GTPase Ras and its effector Ras, the mitogen-activated protein kinase (MAPK) signaling cascade and the JAK family (Hunter & Jones, 2015). JAK activity is proposed to be via TYK2, Jak1 and Jak2, which activate STAT1 and STAT3, predominately, and also STAT5 (Rose-John, Winthrop & Calabrese, 2017).

As mentioned previously, two articles had conflicting results when measuring alterations in IL-6 activity in P1104A cells. Lack of reduction in the percent of IL-6 stimulated pSTAT3 in homozygous P1104A PBMCs indicated that the P1104A variant
does not cause alteration of IL-6 activity (Dendrou et al., 2016). Significant reduction of CD3ε/CD28 activated IL-6 stimulated heterozygous P1104A T cells induced SOCS3 transcription was interpreted as a variant dependent reduction in IL-6 activity (Couturier et al., 2011). IL-6 does stimulate SOCS3 translation, a negative regulator of IL-6, forming a negative feedback loop to control the timing of inflammation. SOCS3 upregulation is not only important for controlling pro-inflammatory IL-6 activity but also in determining IL-6 function, where the absence of SOCS3 causes IL-6 to act in an anti-inflammatory manner (Yasukawa et al., 2003). In the studies presented here, IL-6 stimulated pSTAT3 and pSTAT4 in P1124A cells from both spleen and LN led to opposing results. In spleen T cell populations, IL-6 induced pSTAT3 activity was abrogated while IL-6 induced pSTAT4 activity was minimally altered.

Due to STAT4 having a much more robust response to IL-6 stimulation than STAT3 in these murine studies, this polarized activity could be due to species related differences in STAT activity where STAT4 is the more dominate and least effected by Tyk2 variations in mice. If these two STATs initiate differing transcriptional programs with one having unaltered activity and another with activity that is deficient, namely the transcription factor responsible for SOCS3 upregulation, IL-6 could take cause a more anti-inflammatory role in P1124A GEMs and possibly in P1104A PBMCs. These murine results could potentially explain the discrepancies in the human P1104A PBMC activity, where some parts of the IL-6 signaling pathway is unchanged but other aspects of its activity are causing alterations in traditional IL-6 functions. This information leads to questions about conserved STAT activity and whether the signaling pathways are regulated similarly across cells and species. It is unclear which measured STAT is more
correlative to pro-inflammatory or anti-inflammatory activity and which would be the
most reflective of human biology; unfortunately without more information in the distal
activity of the P1104A patients it will be impossible to make a definitive conclusion.

IL-10 is an anti-inflammatory cytokine that binds to a receptor with two chains,
IL-10 receptor-1 (IL-10R1) and IL-10 receptor-2 (IL-10R2) (Ouyang, Rutz, Crellin,
Valdez & Hymowitz, 2011). IL-10R2 and IL-10R1 bind Tyk2 and JAK1, respectively,
predominantly activating STAT3 but also STAT1 and STAT5 (Ouyang et al., 2011).
STAT3 activity and upregulation of SOCS3 are necessary for IL-10 stimulated immune
cell anti-inflammatory functions (Ouyang et al., 2011). Similar to IL-6, there is some
controversy surrounding Tyk2’s influence on IL-10 stimulated activity in human P1104A
PBMC samples. Dendrou, et al. (2016) concluded that there was no Tyk2 P1104A related
difference in pSTAT3 activation, whereas Couturier, et al. (2011) observed a significant
reduction of IL-10 function based on SOCS3 transcription. IL-10 induced pSTAT3 and
pSTAT4 activity from both LN and spleen in the P1124A cells showed variable results
based on cell type. P1124A lost pSTAT3 in CD4+ T cells from the spleen, was unaltered
in splenic CD8 and LN NK T cells, and was heightened in CD8 T cells and B cells from
the LN. This collection of non-linear cell responses was also seen in IL-10 stimulated
pSTAT4 activity. CD4 T cells from both spleen and LN, and CD8 T cells and B220 B
cells from the LN had minimal changes in pSTAT4 in homozygous P1124A cells, while
NK T cells from the spleen and CD8 T cells from the LN showed an elevated pSTAT4
response to IL-10 stimulation. Similar to the IL-6 data, these results exemplify the
complicated role of Tyk2 in various cells from various tissues.
Unlike the similarities between the murine and human IFNα and IL-12 data, the combined IL-6 and IL-10 data highlight the very specific and cell type dependent manner by which JAK signaling influences cell activity. The data suggest that different cell types rely on cytokine induced Tyk2 signaling to varying degrees. Due to the differences in STAT activation patterns, we unable to conclude whether IL-6 or IL-10 activity is lost or retained in P1124A with this data alone, and therefore we cannot easily extrapolate the findings to P1104A. It is interesting to observe that murine STAT4 activity trended much more similarly to the human STAT3 activity of P1104A patients leading to the question of the translatability of STAT activity between human and mouse, and whether or not they control the transcription of the same gene signatures. Interestingly, functional IL-6 and IL-10 data exemplified by mediator production (IL-6 induced IL-21 and TNFα; IL-10 induced IL-1β, IL-4 and TNFα) indicates no effect Tyk2 on P1224A variation despite differences in pSTAT activity between cells or tissues.

While no animal model can perfectly reproduce human disease or model human behavior, the use of animal models in translational efforts is absolutely essential in the progression of our understanding of the inflammatory process. Taken together all of the data generated suggest that the P1124A GEM is an accurate model for most of the Tyk2 P1104A SNP activity modulations, except of IL-23 driven functions, especially at the distal functional level. Our data suggest that with the reduction of the pSTAT and mediator production of activity which pushes the type 1 inflammatory phenotype due to decreased responsivness toType I IFNs and IL-12 along with retention of most pSTAT and cytokine production needed for IL-6 pro- or anti-inflammatory processes and IL-10
inflammatory resolution in P1124A GEM and can be used to study and potentially predict P1104A responses.

Mechanism of the Variant’s Altered Phenotype

Published data suggest that the Tyk2 P1104A SNP does not remove all Tyk2 function, indicated by the various GWAS studies that link this SNP to protection from autoimmune diseases and the lack of PID similar to what has seen in the one of the two known Tyk2 deficient patients (Dendrou et al., 2016; Couturier et al., 2011; Diogo et al., 2015; Li et al., 2013; Watford & O’Shea, 2006). A major consensus amongst P1104A studies is that these patients have a partially defective vs deficient Tyk2 activity suggesting that to the observation from the P1124A vs the KO would be divergent. How it compares to other SNP is also of interest. Therefore, one major question that is still of interest is how the P1104A SNP alters Tyk2 activity. Using P1124A as a model to answer questions around the physical consequence of the point mutation, the KO, K950A KDKI and Y1074F/Y1075F trans-phos mutant GEMS were included in the presented studies. P1124A activity did not consistently trend with the stimulated responses from either K950A KDKI, KO or Y1074F/Y1075F in any of the studies indicating that P1124A function is wholly different than interference in auto- or trans- Tyk2 phosphorylation as well as from the deficiency of Tyk2.

Tyk2 and Inflammation

Understanding how alteration in the Tyk2 protein effects immune responses requires addressing its role in 1) internal changes to individual cells including modifications in their signaling and transcriptional regulation due to Tyk2 mutations and
2) on how these changes affect the inflammatory microenvironment, autoimmune diseases, and responses to infection. As previously mentioned this human variant represents 0.5% of the population and has been linked to protections from autoimmune disease (Dendrou et al., 2016; Gauzzi et al, 1996). All of the GEMs were evaluated in proximal and distal studies to elucidate the impact of different mutations in Tyk2 on cellular responses. We have already established that if activity is measured by STAT phosphorylation and more so, the functional/distal levels alone, then the P1124A GEM closely models the type 1, but not the type 17 immunomodulatory responses of the human P1104A SNP. In this section we will look the data presented and how Tyk2 alterations affect cell polarizations and overall inflammatory responses.

Myeloid cells have a high degree of plasticity which allows them to go from their homeostatic M2/cDC2 subtype to a pro-inflammatory phenotype when in a pro-inflammatory environment (Usbach et al, 2016; others). During periods of infection the immune system re-orients itself to tailor its response depending on the type of assault that is presented (Annuensiato et al, 2015; others). Contingent on the infectious agent, microenvironment and location, the immune system mobilizes to mount a type 1, type 2 or type 17 responses. While there is a lot of variability that can occur in the buildup of inflammation, these basic principles apply: innate immune cells while surveying tissues come across a foreign body, antigen presenting cells (APCs) phagocytose and process the invader and presents antigens (Ag) via the major histocompatibility complex (MHC). These cells then travel to secondary lymphoid organs to present the Ag to T cells and B cells to initiate the adaptive immune response. APCs provide the first two steps of initiating an immune response, MHC – T cell interaction and co-receptor binding. But it
is the pattern of cytokines produced by the APCs, activated T cell, and maturated B cells, ILCs and surrounding tissue that determines the type of inflammatory response that takes place. All of these cells contribute in concert to from the proper immune response to the specific invader.

M-CSF is produced ubiquitously and consistently at a low level as a signal to innate immune cells, specifically monocytes, macrophages and dendritic cells, to maintain homeostasis during periods without external offense (Usbach et al., 2016). When bone marrow derived cells were polarized towards a homeostatic M2 phenotype. IL-6 and IP-10 production was Tyk2 modification dependent but IL-10 and TNFα production was unaffected. Tyk2 KO, K950A and Y1074F/Y1075F had less IL-6 and IP-10 production while P1124A cells had slightly more IL-6 activity. While IL-6 is not a traditionally thought of M-CSF stimulated cytokine, alterations in its production could be due to alterations in homeostatic/pro-inflammatory states.

A type 1 response is triggered when the invading pathogen infects the cell; like bacteria, protozoa and viruses (Annunziato et al, 2015). In classical activation APCs express IL-12, type I IFNs, IL-1β, TNFα, and IL-6 to facilitate the type 1 phenotype (Annunziato et al, 2015). Of these cytokines IL-12 is the most important for the type 1 polarization of T cells. Th1/Tc1 polarized cells produce IFNγ, GM-CSF, IL-2, TNFα and LTα causing more T cell and APCs to adopt a type 1 phenotype, increasing their cytolytic activity, pushing IgG, IgM and IgA antibody production from B cells, and generating further type 1 cytokine production culminating in a cyclic growth of type 1 immune cells in the microenvironment until inflammatory resolution occurs (Annunziato et al, 2015). GM-CSF is pivotal in reprogramming myeloid cells from a homeostatic state to
M1/cDC1 inflammatory type driving the production of type 1 T cell polarization as well as further macrophage differentiation. In this pathway three key cytokines are known to be regulated by Tyk2: IL-12, type I IFNs and IL-6. IL-12 stimulated activity is most often attributed to being regulated through IL12Rβ1-JAK2 association, where Jak2 has been reported as the main JAK family member responsible for STAT4 phosphorylation (Teng et al., 2015; Zundler et al., 2015). Our research indicates that IL-12Rβ1, which associates with JAK2, is a critical player in IL-12 signaling in the absence of Tyk2, but not when the intact or mutated protein is present. CD4 and CD8 T cells from the Tyk2 KO but not the GEMS carrying the variant or other mutations, retains signaling via pSTAT3 and pSTAT4. The percent of pSTAT3 and pSTAT4 decreases in CD4+, CD8+ and NK T cells by >75% in all of the GEMs except in the Tyk2 KO. Minimal modulation of pSTAT activity in the Tyk2 KO indicates that when the Tyk2 protein is absent, JAK2 can compensate during the proximal response, not during longer term functional responses. However, IL-12 produced IFNγ is absent in all GEMS including the KO suggesting a major defect in the ability of Tyk2 GEMs to initiate a type 1 immune response. This data indicates that surprisingly, in functional responses, but not all proximal responses, Tyk2 activity is seemingly dominate.

Type I IFNs are antimicrobial and antiviral cytokines produced by innate immune cells when PAMP receptors are activated (Ng et al., 2016). While these cytokines have often not been associated as pivotal players for development of the type 1 phenotype as IL-12, they are still important in driving the immune cell differentiation and chemotaxis, cytolytic, epithelial barrier protection, pro-inflammatory responses, amongst other functions (Kovarik et al., 2016). Homozygous alterations in Tyk2 activity causes a loss of
IFNα stimulated STAT phosphorylation in CD4+ T cells, NK T cells and B cells. In contrast, the homozygous variant and Tyk2 KO CD8 cells had an attenuated but not ablated response to IFNα. These results indicate that IFNα induced pSTAT4 activity in CD8+ T cells, are less sensitive to alterations of Tyk2 activity than the other cells. Gene dosage based on Tyk2 allele frequency had varying results based on cell type and location, but Tyk2 activity trended towards dominance. However, despite these observes differences in cell subsets at the pSTAT level, at the functional level splenocytes from all GEMs showed an attenuated IFNα induced IP-10 response. At the proximal and functional levels, both auto-phosphorylation and trans-phosphorylation activity are required for IFNα signaling. This indicates that both auto – and trans-phosphorylation may be necessary for full Tyk2 activity and that retention of one cannot compensate for the activity of the other.

Depending on the proteins synthesized during rapid response gene translation upregulated during infection and inflammatory stimulation, IL-6 can be either pro- or anti-inflammatory. IL-6 is also important in type 1 and type 17 inflammatory responses. While not necessary for type 1 cell polarization, IL-6 activity stimulates Th1 CD4+ T cell IL-1β and TNFα production (Opal & DePalo, 2000). In these studies, IL-6 STAT4 activity was predominately unaltered, except in LN B cells where both the homozygous and, heterozygous P1124A and K950A had reduced pSTAT4. In contrast, IL-6 induced pSTAT3 varied significantly by cell type and location and would require additional studies to further understand the importance of murine IL-6 induced pSTAT3. At the functional level, of the mediators analyzed in response to IL-6, only TNFα, and IL-21 showed an attenuated response in the LN cells from the Tyk2 KO. Possibly, TNFα is an
important IL-6 pSTAT4 driven mediator downstream of STAT4, due to the similar pSTAT and mediator profiles in all GEMs.

To further assess how Tyk2 modulation of pSTAT and mediators effected overall inflammatory responses, various cell polarization studies were performed. Th1 polarized CD4+ T cells from the spleens of P1124A variant and K950A, and LN of Tyk2 KO completely lost their ability to produce IFNγ. These same cells expressed low concentrations of GATA3. Since Tbet and GATA3 reciprocally downregulate the each other’s transcription, the Tyk2 GEMs are actively polarizing towards a Th2 cell despite being generated under Th1 polarizing cytokines. Although the loss of IFNγ production was not duplicated in polarized CD8 T cells, the K950A cells did have a low level of GATA3 expression. This altered inflammatory response is similar to published data linking Tyk2 KO mice and P1104A PBMCs to a reduction in IFNγ production and an increase in IL-4 production when cells were polarized or bead stimulated, respectively (Couturier et al., 2011; Seto et al., 2003). In addition, pro-inflammatory cytokines IL-21 and TNFα were also reduced in type 1 polarized CD4 and CD8 T cells. This data indicates that the absence of IL-12 responses and subsequently IFNγ production in the GEMS drives CD4 T cells towards a non-optimal pro-inflammatory response. Counter screens are important to assure that the effects observed are Tyk2 dependent and not random and therefore, non-Tyk2 signaling pathways were evaluated. IL-2 signals via JAK families members Jak1 and Jak2, as well as through Ras-MAP kinase and phosphoinositol 3-kinase (PI 3K) (Liao, Lin & Leonard, 2011). Importantly, no effects of the GEMs were observed on IL-2 induced pSTAT3 and pSTAT5 or the functional mediator response.
GM-CSF reportedly signals via Jak2 homo-dimerization in addition to extracellular signal-regulated kinase (ERK), V-Akt murine thymoma viral oncogene homolog 1 (AKT), NF-κB with IRF5 and Jak2/STAT3 for its activity (Martinez & Gordon, 2014). GM-CSF, produced in response to multiple cytokines including IL-2, TNFα and IFNγ (Bhattacharya et al., 2015) and is important in polarizing myeloid cells the type I phenotype and supporting granulocyte and macrophage cell survival and proliferation (Bhattacharya et al., 2015). Surprisingly, bone marrow CD11b^{Hi}, CD11b^{Int} had altered GM-CSF stimulated pSTAT5 activity in Tyk2 KO cells. Both Tyk2 KO and P1124A (homo- and heterozygous) cells had less CD11c cells than wildtype mice. Classical GM-CSF mediated cytokines, IL-6, TNFα, and IL-10, where not affected by Tyk2 alterations. Interestingly, GM-CSF stimulated IFNγ and IP-10 was lost in all GEMs except the heterozygous P1124A variant. This data may indicate a possible novel inclusion of Tyk2 in the GM-CSF pathway where its activity could influence some GM-CSF stimulated myeloid cell activity including IFNγ and IP-10. Although GM-CSF stimulated IL-6 production was heightened or unchanged in GEMs during short-term assays, less IL-6 was detected in bone marrow derived cells cultured with GM-CSF to stimulate M1 polarization indicating either that alteration of activity is dependent on location or that it dependent on time. In support of Tyk2’s influence on the myeloid cell compartment of the immune systems, Tyk2 KO mice had drastically less CD11b^{Hi}, CD11b^{Int} and CD11c in their bone marrow. While the CD11b^{Hi} population was unaltered in the other GEMs, homozygous P1124A mice had more CD11b^{Int} and both P1124A had much less CD11c cells indicating that the loss of Tyk2 function attributed to P1124A variant causing this reduction is a dominate trait. Two plausible explanation of thise
results are valid and require further explanation. The first explanation is that these changes in cell density could be caused by Tyk2 dependant alteration in CD11b and CD11c receptor expression. The second is that this data is consistent with known JAK function during embryogenesis where Jak2 and Jak3 have roles in lymphoid development, innate lymphoid cell development and myeloid cell hematopoiesis (Robinette et al., 2018; Zhu, Liu, Guan, Zeng, Yin & Zhang, 2017; Nosaka et al., 1995).

The type 2 inflammatory phenotype is preferential when the invading organism is extracellular, and it requires a heightened antibody response to remove the harmful substance appropriately (Kidd, 2016). Th2 and Tc2 polarized cells are characterized by expression of GATA3 and the production of IL-4 and IL-13 (Wynn, 2015). IL-4 is indispensable in type 2 phenotype polarization but IL-13 and IL-5 are necessary for cellular activity (Annuenziato et al, 2015). IL-4 and IL-13 are Tyk2 independent and dependent, respectively (Annuenziato et al, 2015). Contrary to published data measuring Tyk2 KO or P1104A PBMC cytokine production from Th2 polarized cells, our Th2 and Tc2 polarization studies showed no significant upregulation in IL-4 production and no significant alterations in GATA3 expression in the GEMs (Shimoda et al., 2000; Couturier et al, 2011). Our results indicate that type 2 polarized cells are not becoming more productive type 2 cells, but that defects Tyk2 lead to type 1 cells defaulting to a type 2 response.

The type 17 immune response is initiated during extracellular bacterial or fungal infection (Annuenziato et al, 2015). The type 17 phenotype is stimulated by IL-21, IL-1β, IL-6, TGFβ, and stabilized by IL-23 cytokines which are responsible for the promoting expression STAT3 and RORγT transcriptions factors inhibiting that of Foxp3,
a Treg promoting transcription factor (Gaffen, Jain, Garg & Cua, 2014; Miossec, Korn & Kuchroo, 2009). Th17 cell function can be divided into two types of activity, protective/homeostatic function characterized by the expression of IL-17A and IL-10 and pro-inflammatory activity characterized by the expression of IL-17A, IL-22, IFNγ and GM-CSF (Gaffen, Jain, Garg & Cua, 2014). We have addressed IL-6 proximal STAT activity and its role in type 1 inflammation in prior sections, IL-6 is also required for type 17 polarizations. Tyk2’s influence on IL-6 stimulated production of IL-21 varied depending on the cell’s tissue of origin. IL-6 stimulated spleen cells produce IL-21 is Tyk2 independent, conversely IL-6 stimulated IL-21 from LN was dependent on Tyk2 function. As discussed earlier, pSTAT3 activity was significantly altered in a cell type and location dependent manner. This could indicate a tissue specific regulation of IL-6 activity and since STAT3 is required for epithelial cell differentiation and homeostatic activity this could signal a change in the maintenance arm of type 17 activities (Annuenziato et al, 2015).

IL-23 is another Tyk2 influential cytokine required for type 17 cell survival and expansion (Gaffen, Jain, Garg & Cua, 2014). Tyk2 KO was the only GEM that lost all of its IL-23 pSTAT4 proximal activity, while cells from Y1074F/Y1075F and heterozygous P1124A mice has a heighten response to IL-23 stimulation and cells from all other GEMs were unaffected by changes in Tyk2 sequence. These alterations in STAT signaling manifested as a loss in IL-23 stimulated IL-17α production in Tyk2 KO, K950A and Y1074F/Y1074F and heighten IL-17α production from homozygous and heterozygous P1124A cells. This discrepancy between proximal and distal cellular activity requires more experimentation before a definitive conclusion can be drawn, but may influence
Th17 and Tc17 cell polarization and expansion. CD4 T and CD8 cells polarized towards a Th17 and Tc17 cells, respectively, showed a reduction in IL-10 and IL-21 production and CD8 Tc17 cells also produced less TNFα. Surprisingly, IL-17α and IL-6 production remained consistent in all of the GEMs polarized towards a Th17/Tc17 response. This data indicates that global type 17 polarization might be hampered but that polarization is still intact. To help further understand the influence of Tyk2 on the type 17 response, a Th17 driven Imiquimod model is planned.

The termination of inflammation is characterized by anti-inflammatory co-receptors like CTLA4 and PD-1, the absence of pro-inflammatory cytokines and production of IL-10, an anti-inflammatory cytokine. Proximal IL-10 stimulated Tyk2 pSTAT3 and pSTAT4 activity was largely unchanged in the GEMs and was paralleled by the IL-10 functional response, where the only cytokine that has a meaningful change in production was IL-2 was highly upregulated in Tyk2 KO and modestly upregulated homozygous P1124A cells. Since IL-2 is not traditionally downstream of IL-10 the cause of this upregulation is unknown. Possible RNAseq or proteomic data could assist in identifying additional IL-10 regulated genes and proteins. Taking all data into consideration the resolution of inflammation mediated by IL-10 is unaffected by Tyk2 modulation and probably contributes to the overall protective response.

A new wave of Jakinibs: Understanding the Molecular Profile of Protective Alleles

Pharmacological intervention of autoimmune diseases has occurred for years with both biologics, large antibodies or antibody like structures that typically bind at the surface of the cell, and chemical matter, small chemical molecules that can bind to targets both on the surface and inside cells (Bryan & Rajapaksa, 2018). Of these two modalities,
biologics have been more successful in autoimmune disease treatment with more approvals from Food and Drug Administration (FDA), European Medical Association (EMA) and other governmental agencies, but kinase inhibitors are of great interest due to the associations of kinases alleles with disease associations and ease in targeting with small molecules (Schwartz et al, 2016). Frequently, these small molecules bind in the adenosine triphosphate (ATP) binding pocket (Bryan & Rajapaksa, 2018). High homology between Jak family kinases, specifically the conserved sequences in their ATP binding pocket, has made development of JAK-specific jakinibs challenging, despite their critical role in autoimmune diseases due their modulation of pathways important in inflammation (Winthrop, 2017). High homology between JAK family kinases, specifically the conserved sequences in the JAKs phosphate binding pocket, has made development of single JAK specific jakinibs that a specific elusive (Winthrop, 2017).

Currently, Xeljanz (tofacitinib), a pan JAK inhibitor with some selectivity towards JAK1 and JAK3, is approved for autoimmune diseases including rheumatoid arthritis (RA) (Winthrop, 2017; Pfizer, 2018), ulcerative colitis and active psoriatic arthritis. Similarly, Olumiant (baracitinib), a JAK1, JAK2, Tyk2 inhibitor, is also approved for RA, and Ruxolitinib (Jakafi) is a Jak1/Jak2 inhibitor approved for the treatment of myeloproliferative disease (Schwartz et al, 2016; Lily & Incyte, 2018). Unfortunately, these drugs being less selective towards a specific JAK protein, is often associated with adverse events (AEs) and/or dose limited (Schwartz et al., 2016). For example, tofacitinib is dose limited due to neutropenia and lymphopenia, possibly due to Jak2 inhibition, amongst other AEs (Bryan & Rajapaksa, 2018). Hence, identifying new jakinibs with more JAK selectivity is a desirable goal (Bryan & Rajapaksa, 2018).
GWAS studies have linked the human Tyk2 P1104A variant with protection from multiple autoimmune diseases, making this an attractive JAK family member to target (Dendrou et al., 2016; Couturier et al., 2011; Diogo et al., 2015; Li et al., 2013). Further, as presented in multiple articles and confirmed and extended in these studies, Tyk2 plays a pivotal role in regulating cytokine pathways important for the development of type 1 and type 17 inflammatory phenotypes. Current Tyk2 jakinibs are focused on potency and specificity to obtain 10-100fold higher Tyk2 selective compounds vs other JAK family members (Nimbus, 2018; Nimbus, 2018; Bristol-Myers Squibb, 2018).

Critical data supporting and facilitating the development of Tyk2 selective compounds was recently presented by Dendrou and colleagues (2016). Those studies which measured the effect of a P1104A Tyk2 mutation on STAT phosphorylation gave the beginnings of a roadmap towards this much desired activity (Dendrou et al., 2016). This research confirmed and back-translated much of the human proximal pSTAT data to a mouse GEM model, and further extended the studies by addressing the selectivity of Tyk2 vs the of JAKs on distal functions. The data will facilitate determining a blueprint to understanding the necessary phenotype to confer protection without the AEs of traditional jakinibs. The Tyk2 P1124A variant, and by correlation the P1104A variant, functions by prewiring CD4 T cells towards a type 2 phenotype during infection but retains CD8 T cell type 1 polarization capabilities. Although Tyk2’s influence on proximal activity is cell type dependent, its influence in alteration in function and cellular differentiation leads to attenuated type 1 and 17, in human cells, inflammation associated cytokine pathways and trends to modulate a Th1 driven DTH response. Consistent with this data, the P1124A GEM, as well as all other GEMs showed a trend towards and
attenuated type 1 CD4+ T cell driven pro-inflammatory DTH response supporting recent literature that the P1120A mutation is sufficient to protect from autoimmune diseases. These important studies are being repeated with increase mouse numbers to obtain significance.

Yet, there appears to be retention of CD8 type 1 induced polarization responses to retain abilities for fighting intra and extracellular small microbe infection. Hence, a proposed phenotype for protection from disease without AEs may be inhibition of the type I IFNs, IL-12 and IL-23 but retention of IL-6 and IL-10 cytokine pathways including both pSTAT activity and subsequent functional signaling. Additionally, Tyk2 modulation causes functional mediator alterations which makes CD4 T cells more inclined to polarize to a Th2 phenotype when stimulated when in an inflammatory environment and hence contribute to the suppression of autoimmune diseases such as RA, MS, inflammatory bowel disease, amongst others. Although, Th17 responses in the P1124A were not consistent with human P1104A variant activity additional studies are necessary to further parse the role of Tyk2 in Th17 biology and protection from a disease such as MS, which has a Th17/IL-23/IL-17 component. Preliminary data suggest that Tyk2 could function in modulating myeloid cell populations or CD11b and CD11c surface expression not only in KO GEMs but also in P1124A variants, if this data holds it could mean that the protective phenotype seen in P112A4 mice is not only due to changes in cytokine signaling but introduce possible novel roles for Tyk2 in myeloid cells independent of its influence on polarization. With this research we have successfully validated a novel animal model that translates the type 1 inflammatory phenotype of the protective Tyk2 P1104A variant, elucidated alterations in Tyk2 influenced cytokines that confer this protection,
determined that P1124A and by extension P1104A’s activity is not due to loss of Tyk2 function or auto-/trans-phosphorylative abilities, and possibly found the beginnings of a cytokine signaling independent Tyk2 function.
References


Annunziato, Francesco; Romagnani, Chiara & Romagnani, Sergio (2014). The 3 major types of innate and adaptive cell-mediated effector immunity. American Academy of Allergy, Asthma & Immunology 135(3), 626-635

Bhattacharya, Palash; Budnick, Isadore; Singh, Medha; Thiruppathi, Muthusamy; Alharshawi, Khaled; Eishabrawy, Hatem, Holterman, Mark J & Prabhakar, Bellur S (2015). Dual Role of GM-CSF as a Pro-Inflammatory and Regulatory Cytokine: Implications for Immune Therapy. Journal of Interferon & Cytokine Research 35(8), 585-599


Carey, Alison J; Tan, Chee K & Ulett, Glen C (2012). Infection-induced IL-10 and JAK-STAT: A review of the molecular circuitry controlling immune hyperactivity in response to pathogenic microbes. JAK-STAT 1(3), 159-167

Couturier, Nicolas; Buccioni, Florence; Nurtdinov, Ramil N; Debouverie, Marc; Lebrun-Frenay, Christine; Defer, Gilles; Moreau, Thibault; Confavreux, Christian; Vukusic, Sandra; Cournu-Rebeix, Isabelle; Goertsches, Robert H; Zettl, Uwe K; Comabella, Manuel; Montalban, Xavier; Rieckmann, Peter; Weber, Frank; Muller-Myhsok, Bertram; Edan, Gilles; Fontaine, Bertrand; Mars, Lennart T; Saoudi, Abdelhadi; Oksenberg, Jorge R; Clanet, Michel; Liblau, Roland S & Brassat, David (2011). Tyrosine kinase 2 variant influences T lymphocyte polarization and multiple sclerosis susceptibility. Brain 134, 693-703


Dendrou, Calliope A; Cortes, Adrian; Shipman, Lydia; Evans, Hayley G; Attfield, Kathrine E; Jostins, Luke; Barber, Thomas; Kaur, Gurman; Kuttikkatte, Subita B;
Leach, Oliver A; Desel, Christiane; Faergeman, Soren L; Cheeseman, Jane; Neville, Matt J; Sawcer, Stephen; Compston, Alastair; Johnson, Adam R; Everett, Christine; Bell, John I; Karpe, Fredrik; Ultsch, Mark; Eigenbrot, Charles; McVean, Gil & Fugger, Lars (2016). Resolving TYK2 locus genotype-to-phenotype differences in autoimmunity. Science Translational Medicine 8, 363ra149

Diogo, Dorothée; Bastarache, Lisa; Liao, Katherine P; Graham, Robert R; Fulton, Robert S; Greenberg, Jeffrey D; Eyre, Steve; Bowes, John; Cui, Jing; Lee, Annette; Pappas, Dimitrios A; Kremer, Joel M; Barton, Anne; Coenen, Marieke JH; Franke, Barbara; Kiemeney, Lambetus; Marriette, Xavier; Richard-Miceli, Corrine; Canhao, Helena; Fonseca, Joao E; de Vries, Neik; Tak, Paul P; Crusius, J. Bart A; Nurmohamed, Michael T; Kurreeman, Fina; Mikuls, Ted R; Okada, Yukinori; Stahl, Eli A; Larson, David E.; Deluca, Tracie L; O’Laughlin, Michelle; Fronick, Catrina C; Fulton, Lucinda L; Kosoy, Roman; Ranson, Michael; Bhangale, Tushar R; Ortmann, Ward; Cagan, Andrew; Gainer, Vivian; Karlson, Elizabeth W; Kohane, Isaac; Murphy, Shawn N; Martin, Javier; Zhernakova, Alexandra; Klareskog, Lars; Padyukov, Leonid; Worthington, Jane; Mardis, Elaine R; Seldin, Michael F; Gregersen, Peter K; Behrens, Timothy; Raychaudhuro, Soumya; Denny, Joshua C & Plenge, Robert M (2015). TYK2 Protein-Coding Variants Protect against Rheumatoid Arthritis and Autoimmunity, with No Evidence of Major Pleiotropic Effects on Non-Autoimmune Complex Traits. PLOS One 10(4)


Gaffen, Sarah L; Jain, Renu; Garg Abhishek V & Cua, Daniel J (2014). The IL-23 – IL-17 immune axis: from mechanisms to therapeutic testing. Nature Reviews Immunology 14, 585-600

Gauzzi, M Cristina; Velazquez, Laura; McKendry, Roslyn; Mogensen, Knud E, Fellous, Marc & Pellegrini, Sandra (1996). Interferon-α-dependent Activation of Tyk2 Requires Phosphorylation of Positive Regulatory Tyrosines by Another Kinase. The Journal of Biological Chemistry 271, 20494-20500

Hanada, Toshitkatsu & Yoshimura (2002). Regulation of cytokine signaling and inflammation. Cytokine & Growth Factor Reviews 13, 413-421

Hunter, Christopher A (2005). New IL-12-Family Members: IL-23 and IL-27, Cytokines with Divergent Functions. Nature Reviews Immunology 5, 521-531


Ishizaki, Masayuki; Akimoto, Toshihiko; Muromoto, Ryuta; Yokoyama, Mika; Ohshiro, Yuya; Sekine, Yuichi; Maeda, Hiroaki; Shimoda, Kazuya; Oritani, Kenji & Matsuda, Tadashi (2011). Involvement of Tyrosine Kinase-2 in Both the IL-12/Th1 and IL-23/Th17 Axes In Vivo. Journal Immunology 187, 181-189

Ishizaki, Masayuki; Muromoto, Ryuta; Akimoto, Toshihiko; Sekine, Yuichi; Kon, Shigeyuki; Diwan, Manish; Maeda, Hiroaki; Togi, Sumihito; Shimoda, Kazuya; Oritani, Shigeyuki & Matsuda, Tadashi (2013) Tyk2is a therapeutic target for psoriasis-like skin inflammation. International Immunology


Joffre, Olivier; Nolte, Martijn A; Sporri, Roman & Reis e Sousa, Caetano (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunological Reviews 227, 234-247

Karaghiosoff, Marina; Neubauer, Hans; Lassnig, Caroline; Kovarik, Pavel; Schindler, Heike; Pircher, Hanspeter; McCoy, Barbara; Bogdan, Christian; Decker, Thomas; Brem, Gottfried; Pfeffer, Klaus & Muller, Mathias (2000). Partial Impairment of Cytokine Responses in Tyk2-Deficient Mice. Immunity 13, 549-560


Kilic, Sara S; Hacimustafaoğlu, Mustafa; Boisson-Dupuis, Stephanie; Kreins, Alexandra Y; Grant, Audrey V; Abel, Laurent & Casanova, Jean-Laurent (2012). A Patient with Tyrosine Kinase 2 Deficiency without Hyper-IgE Syndrome. The Journal of Pediatrics 160(6), 1055-1057


Kovarik, Pavel; Castiglia, Virginia; Ivin, Masa & Ebner, Florian (2016). Type I Interferons in Bacterial Infections: A Balancing Act. Frontiers in Immunology 7(652)

Li, Zhi; Gakovic, Milica; Ragimbeau, Josiane; Eloranta, Maija-Leena; Ronnblom, Lars; Michel, Frederique & Pellegrini, Sandra (2013). Two Rare Disease-Associated Tyk2 Variante Are Catalytically Impaired but Signaling Competent. Journal of Immunology 190, 2335-2344


Martinez, Fernando O & Gordon, Siamon (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000 Prime Reports 6(13)


Ng, Cherie T; Mendoza, Juan L; Garcia, K Christopher & Oldstone, Michael BA (2016). Alpha and Beta Type 1 Interferon Signaling: Passage for Diverse Biologic Outcomes. Cell 164, 349-352

Nosaka, Tetsuya; van Deursen, Jan M; Tripp, Ralph A; Thierfelder, William E; Witthuhn, Bruce A; McMickle, Anthony P; Doherty, Peter C; Grosveld, Gerald C & Ihle, James N (1995). Defective Lymphoid development in Mice Lacking Jak3. Science 270, 800-802


Ragimbeau, Josiane; Dondi, Elisabetta; Alcover, Andres; Eid, Pierre; Uze, Gilles & Pellegrini, Sandra (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. The EMBO Journal 22(3), 537-547

Robinette, MI; Cella, M; Telliez, JB; Barrow, AD; Capuder, K; Gilfillan, S; Lin, L-L; Nortarangelo, LD & Colonna, M (2018). Jak3 deficiency blocks innate lymphod cell development. Nature 11(1), 50-60

Rose-John, Stefan; Winthrop, Kevin & Calabrese, Leonard (2017). The role of IL-6 in host defence against infection: immunobiology and clinial implications. Nature Reviews Rheumatology 13, 399-409

Rosenblum, Michael D; Gratz, Iris K; Paw, Johnathan S & Abbas, Abul K (2012). Treating Human Autoimmunity: Current Practice and Future Prospects 4(125), 125sr1

Schwartz, Daniella M; Bonelli, Michael; Gadina, Massimo & O’Shea, John J (2016) Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. Nature Reviews Rheumatology 12, 25-36

Seto, Yohei; Nakajima, Hiroshi; Suto, Akira; Shimoda, Kazuya; Saito, Yasushi; Nakayama, Keiichi I & Iwamoto, Itsuo (2003) Enhanced Th2 Cell-Mediated Allergic Inflammation in Tyk2-Deficient Mice. Journal of Immunology 170, 1077-1083

Shaw, Michael H; Boyartchuk, Victor; Wong, Sandy; Karaghlosoff, Marina; Ragimbeau, Josiane; Pellegrini, Sandra; Muller, Mathias; Dietrich, William F & Yap, George S (2003). A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. PNAS 100(20), 11594-11599

Shimoda, Kazuya; Kato, Kouji; Aoki, Kenichi; Matsuda, Tadashi; Miyamoto, Akitomo; Shibamori, Masafumi; Yamashita, Masakatsu; Numata, Akihiko; Takase, Ken; Kobayashi, Shinji; Shibata, Shouichirou; Asano, Yoshinori; Gondo, Hisashi; Sekiguchi, Kazuo; Nakayama, Keiko; Nakayama, Toshinori; Okamura, Takashi; Okamura, Seichi; Niho, Yoshiyuki & Nakayama, Kei-ichi (2000). Tyk2 Plays a Restricted Role in IFNα Signaling, Although It Is Required for IL-12-Mediated T Cell Function. Immunity 13, 561-571


Strobl, Birgit; Stoiber, Dagmar; Sexl, Veronika & Mueller, Mathias (2011). Tyrosine kinase 2 (TYK2) in cytokine signalling and host immunity. Frontiers in Bioscience 16, 3224-3232


Teng, Michele W; Bowman, Edward P; McElwee, Joshua J; Smyth, Mark J; Casanova, Jean-Laurent; Cooper, Andrea M & Cua, Daniel J (2015). IL-12 and IL-23 cytokines: from discovery to targets therapies for immune-mediated inflammatory diseases. Nature Medicine 21(7), 719-729


Yasukawa, Hideo; Ohishi, Masanobu; Mori, Hiroyuki; Murakami, Masaaki; Chinen, Takatoshi; Aki, Daisuke; Hanada, Toshikatsu; Takeda, Kiyoshi; Akira, Shizuo; Hoshijima, Masahiko; Hirano, Toshio; Chien, Kenneth R & Yoshimura, Akihiko (2003). Il-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nature Immunology 4(6), 551-556

Zhu, Xianmin; Liu, Rui; Guan, Jun; Zeng, Wen; Yin, Jin & Zhang, Yicheng (2017). Jak2a regulated erythroid and myeloid hematopoiesis during zebrafish embryogenesis. Internation Journal of Medical Science 14(8), 758-763