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Alternative splicing takes control of cytokine signaling

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Abstract

Alternative splicing takes control of cytokine signaling

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Immune responses must be tightly controlled for dose, location, strength and duration using genetic, epigenetic or biochemical regulation. Among these, the generation of alternatively-spliced transcripts is an efficient and dynamic way to increase transcriptional and proteomic diversity. Specifically, this thesis explains how splice variation dictates the biological functions of interleukin-22 (IL-22) binding protein (IL-22BP) and interferon lambda 4 (IFNλ4), two proteins that participate in key cytokine responses to infection and inflammation.

IL-22BP is a soluble receptor for IL-22 that is expressed as three isoforms in humans, IL-22BPi1, IL-22BPi2 and IL-22BPi3. The murine homolog of IL-22BPi2 has been characterized as an antagonist of IL-22 while the physiological relevance of IL-22BPi1 and IL-22BPi3 are unknown. Here, we present findings demonstrating that alternative splicing tailors IL-22BP activity to specific spatiotemporal conditions. Inclusion of a unique third exon renders IL-22BPi1 inactive by preventing its secretion, while exclusion of exons 5 and 6 makes IL-22BPi3 is a weaker antagonist than IL-22BPi2. While IL-22BPi3 is the most dominant isoform under homeostatic conditions, stimulation by Toll-like receptor 2 (TLR2) or retinoic acid induces only IL-22BPi2. Response to environmental cues therefore generates a gradient of IL-22BPi2 and IL-22BPi3 as a rheostat for IL-22 activity.

In the case of IFNλ4, alternative splicing suppresses protein expression and secretion to prevent its antiviral activity. Unlike the tightly linked *IFNL3*, *IFNL4* is genetically associated with hepatitis C virus persistence. Thus, we examined whether any of its three natural protein-coding isoforms IFNλ4p107, IFNλ4p131 or IFNλ4p179 may account for unexpected pro-viral activity. Using overexpression systems and recombinant proteins, we found that only full-length IFNλ4p179 is bioactive and, like IFNλ3, exhibits antiviral activity without blocking other IFN signaling. Even so, little of this active cytokine is made as alternative splicing favors the expression of the inactive isoforms or unproductive transcripts instead . Thus, alternative splicing is a major gene regulatory mechanism that limits IFNλ4 bioactivity during infection, causing the genetically linked IFNλ3 to be the dominant antiviral effector instead.

Overall, our data show that alternative splicing is an important response to pathogen sensing and infection that efficiently fine-tunes or controls expression of immune receptors and effectors. In these ways, it dictates the outcome of many cytokine signaling pathways.

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Chapter 1. INTRODUCTION

Generating alternative isoforms of a single protein or gene is a major mechanism by which the complexity of the proteome is magnified in multicellular eukaryotes. Isoforms can be created at the transcript level via alternative splicing of pre-messenger RNA, the use of alternative transcription start sites, alternative polyadenylation or truncation by proteolytic cleavage. These processes alter a genetic program's passage to change protein localization and function, with potentially profound effects in all central physiological processes, including but not limited to development, homeostasis, immunity and regeneration. Therefore, alternative splicing is extensively used by organisms to regulate gene expression or protein function in tissue-specific manners. While exciting new technologies now enable us to profile splicing events on a more global, unbiased scale, it is still often impossible to predict the biological consequences of alternative protein isoforms without experimental testing. Given the variety and magnitude of changes alternative isoforms can confer on gene function, their differing effects are an important consideration for the function of many immune genes, but often remain neglected.

To understand how an immune response is initiated and resolved, it is essential to comprehend the control and function of cytokines, small protein messengers in the immune system that, among other things, can coordinate immune responses against infection. In particular, cytokines are often heavily regulated by transcriptional and post-transcriptional mechanisms as directed cellular responses to specific immune stimuli. In this thesis, I present our findings on how alternative splicing of the cytokine interferon (IFN) lambda 4 (IFNλ4) and the soluble cytokine receptor interleukin-22 (IL-22) binding protein (IL-22BP) generates multiple isoforms of each protein that alter antiviral activity and barrier immunity effected by IFNs and IL-22 respectively.

The findings presented in this thesis shed light on novel methods of regulating cytokine activity either by fine-tuning cytokine responses or controlling the production of functional cytokines in response to activation of pathogen sensing.

1.1 REGULATION OF PROTEIN EXPRESSION AND ACTIVITY BY ALTERNATIVE SPLICING

1.1.1 Basic mechanism of pre-mRNA splicing

Genes in eukaryotic, metazoan organisms contain sections of nucleotide sequences that contain information for making a protein sequence (exons) interspersed with non-protein-coding sequences (introns). Splicing is the essential process that removes the introns and joins the adjacent exons into mature mRNA that contain contiguous open reading frames (ORFs) for direct translation into polypeptide sequences. However, most genes have more than three exons that can be included or excluded in different permutations for the final mRNA transcript. Alternative splicing is the use of different exon combinations to generate multiple transcripts from a single gene, and it often produces multiple protein isoforms of different lengths. It is estimated that 95% of multi-exon genes in humans undergo alternative splicing, and that 100,000 intermediate-to-high abundance alternative splicing events occur in major human tissues[1]. The core process of alternative splicing has been long established and details well covered in numerous reviews[2-5]; a brief summary of the process is presented below.

The process of pre-mRNA splicing process depends on a hierarchy of multiple elements. At its foundation, the spliceosomal machinery contains several RNA-binding proteins such as branchpoint binding protein (BBP), U2AF65 and U2AF35 along with five small nuclear ribonuclear proteins (snRNP)s: U1, U2, U4, U5 and U6. Together, the spliceosome identifies consensus sequences required for splicing near the intron-exon boundaries: the 5′ “donor” GU site

in the upstream exon; the 3′ "acceptor" site at the AG in the downstream exon, usually guided by an adjacent polypyrimidine tract; and a branch point sequence in the intron containing a crucial adenosine residue. After activation by RNA-dependent helicases and ATPases, the spliceosome catalyzes the two transesterification reactions to excise introns and ligate neighboring exons (**Fig. 1.1A**). First, the branch point is identified by BBP, while the U1 snRNP recognizes the 5′ splice site and U2AF factors bind the 3′ splice site and polypyrimidine tract. U2 snRNP then displaces BBP at the branch point, which it binds with high affinity, and recruits the pre-formed U4-U5-U6 snRNP complex to initiate the first transesterification process. In this reaction, the 2′-OH of the branch site adenosine attacks the 5′ splice site to form an intermediate branched-loop structure called the intron lariat. U1 and U4 are displaced by rearrangements in RNA-RNA and RNA-protein interactions, leaving U2, U5 and U6 to co-ordinate the second transesterification process in which the -OH overhang of the upstream exon attacks the 3′ splice site. This causes release of the intron lariat structure and ligation of the two neighboring exons. The spliceosome then dissociates and reassembles on new splice sites to begin the cycle anew.

An exon is included only if the spliceosome is able to recognize and bind its flanking splice sites. Splice sites vary in strength based on this selection: "strong" sites are fully used and lead to constitutive splicing, while choice of "weak" sites leads to alternative splicing, i.e., the generation of multiple, distinct mature mRNAs from a single pre-mRNA sequence. This is partially dependent on how closely the splice sites match consensus sequences. The likelihood of exon usage is adjusted further by cis-acting factors: sequences on the pre-mRNA including exonic splice enhancer (ESE), intronic splice suppressor (ESS), exonic splice suppressor (ESS) and intronic splice suppressor (ISS) sequences lie close to the splice junctions and recruit proteins and ribonucleoproteins that in turn interact with the spliceosomal complexes on the splice sites (**Fig.

1.1B). The most common trans-acting factors associated with this alternative splicing process are the Ser/Arg-rich protein families (SR) and heterogeneous nuclear ribonucleoproteisn (hnRNPs), although tissue-specific factors such as the FOX and neuro-oncological ventral antigen (NOVA) proteins may also participate. Together, these elements guide the spliceosomal protein machinery to the appropriate splice junctions required, preventing random binding while preserving bias towards certain combinations of exons.

1.1.2 Factors that influence alternative splicing

Changes in splice site, branch point or other cis-regulating elements affect the binding of spliceosomal machinery and inclusion of a neighboring exon. As of July 2016, about 9.1% of characterized human genomic mutations have known effects on splicing (http://www.hgmd.cf.ac.uk/), although this is likely an underestimate due to scant characterization of changes in splicing caused by novel genetic variants. Much of our knowledge about how cis-regulatory elements change splicing comes from genomic variants of a small handful of well-studied genes: cystic fibrosis transmembrane conductance regulator (*CFTR*), neurofibromin-1 (*NF1*), breast cancer 1 (*BRCA1*) and ataxia-telangiectasia mutated (*ATM*). Examples in this subsection will draw from this short list.

Firstly, some mutations directly change the consensus sequences required for spliceosome binding[6]. In *CFTR*, a G>A transition at intron 9 destroys a weak acceptor site in some cystic fibrosis patients and results in use of alternative sites within exon 10 or skipping of exon 10 altogether[7,8]. Mutations in the branch point and polypyrimidine tract are very rare but also influence splicing. Again in *CFTR*, a dinucleotide TG repeat lies in the polypyrimidine tract upstream of the exon 9 acceptor site; a low repeat number (≤5) seen in some patients enhances

exon skipping due to changes in RNA structure[9,10]. Naturally, mutations in these sites provide the most direct way of absolutely targeting a given exon.

About 80% of splicing variability in the genome falls within ORFs, while the remaining variability occurs in untranslated regions[4]. Splicing changes are the most common result of pathological mutations in neurofibromatosis type I, causing half of all modifications to *NF1* mRNA[11]. Exonic mutations on the open reading frame are usually expected to alter protein function solely by nonsense or missense effects that directly change amino acid sequence or polypeptide length. However, translationally-silent synonymous mutations have been associated time and again with alterations in splicing. *CFTR* have been shown to change splicing efficiency of exons 9-12 as they alter ESE sequences to promote exon skipping[8,12,13]. Similarly, other translationally silent mutations of *BRCA1* alter the inclusion of its exon 11 or its use of an alternative splice site[14]. Mutations within exon 12 of *CFTR*, away from the splice sites, also reduce exon inclusion to 15%[15]. In addition, intronic variants can generate new splice sites and therefore, pseudoexons; for instance, a variant in intron 20 of *ATM* more than 0.5kb from the nearest exons disrupts an intron-splicing processing element and creates an aberrant transcript with a cryptic exon[16]. Given that we are still a long way from solving the splicing code, it is likely that many genomic variants that affect splicing in other genes remain unaccounted for due to poor predictive methods.

Alternative splicing can also be altered by changes in trans-acting elements required for normal splicing, which have broader effects on multiple genes. More than 150 proteins have been identified to be trans-acting factors in splicing, including kinases, phosphatases, helicases, mRNA transport factors and transcription factors[4]. While some of these factors are constitutively active in all cells , such as the SR proteins, many have heterogeneous tissue expression that

changes exon usage at different tissue sites for the same gene. For example, in activated murine macrophages, splicing during activation by cytokine stimulation or infection is highly correlated with activation of additional myeloid factors including members of the Pit-Oct-Unc (POU), cAMP response element binding protein (CREB) and CCAAT-enhancer-binding protein (C/EBP) families[17]. At the same time, many exon configurations are unique to the placental tissues and highly correlated with the expression of splicing factors such as epithelial splicing regulatory protein (ESRP) and muscleblind-like protein 3 (MBNL3) [18]. Thanks to the co-occurrence of transcription, there is interaction between the spliceosome and transcription factors, initiation factors, elongation factors, RNA polymerase II and modified nucleosomes that determine exon inclusion or exclusion[3]. More recently, it has been recognized that non-coding RNAs including microRNAs and long non-coding RNAs can regulate binding of spliceosome machinery. In addition, secondary structure of RNA can also affect splicing by steric hindrance that alters accessibility to the spliceosomal complex[19].

A final caveat remains in the use of animal models to study alternative splicing. Changes in alternative exon usage evolve quickly, with much larger and quicker transitions between species than gene expression largely due to changes in cis-acting elements[20]. In support of this, 6-8% of exons are spliced differently in human and chimpanzee frontal cortex and heart tissue, despite the two species having 99% genomic identity[21]. This presents a major challenge to endogenous expression and regulation *in vivo* that also applies to our study of IL-22 binding protein and interferon lambda 4, as the isoforms of interest are both absent in popular animal models.

1.1.3 Consequences of alternative splicing

Alternative splicing of a transcript can occur in several modes including (1) inclusion of alternative cassette exons; (2) inclusion of mutually exclusive exons; (3) retention of introns

leading to nonsense-mediated decay (NMD) and (4) use of alternative 5′ or 3′ splice sites **(Fig. 1.2)**. Hence this process can create a large variety of insertions, deletions, truncations and avenues for transcript suppression.

The choice of splice site affects transcriptional regulation, translational efficiency and the inclusion of functional domains in the resultant protein. For example, many soluble cytokine and growth factor receptors are generated from the same genes as membrane-bound receptors simply by exclusion of exons encoding the transmembrane domains. Often, this generates a soluble receptor that competitively blocks signaling by the membrane bound receptor from the same gene, as in the case of interleukin-1 (IL-1) receptor accessory protein (IL-1RacP) for IL-1 and TLR4[22,23]. Immediately downstream of these innate immune receptors, the proximal adaptor protein myeloid differentiation primary response gene 88 (MyD88) may also be spliced to generate a shorter isoform without exon 2 that prevents activation of IL-1 receptor-associated kinase 1 (IRAK1), but preserves activation of the distinct pathway driven by c-Jun N-terminal kinase (JNK) and activator protein (AP-1)[24-26]. In turn, IRAK-1 naturally undergoes alternative splicing in humans to disrupt kinase activity of two isoforms and NF-κB-dependent cytokine production without changing their association with signaling co-factors [27,28]. Alternative splicing in MyD88, IRAK-1 and other links of the chain including the TLRs, tumor necrosis factor receptor associated (TRAF) proteins and inhibitor of κB kinase (IKK) subtly offer the immune system many opportunities to modulate the magnitude of activation of the inflammatory transcription factor nuclear factor κB (NFκB) – all through the selective disruption of functional domains through alternative splice site and exon usage.

Excluding intron retention, about 1/3 of all alternative splicing events lead to premature stop codons that cause mRNA degradation by nonsense mediated decay[29]. While NMD can

function as a surveillance process that detects and eliminates the misfolded products of aberrant splicing, it is increasingly recognized as a targeted, efficient process coupled with alternative splicing to quickly and tightly regulate gene expression in many developmental and physiological pathways[30,31]. NMD is used to reduce expression of specific transcripts in adult tissue, while during development the exons containing premature stop codons are skipped to increase expression[32]. Identifying splice variation that leads to intron retention or premature stop codons is important for getting the full picture of regulation of gene networks.

Naturally, alteration of splicing events often has impacts on disease and the establishment of immunity. It is estimated that 60% of deleterious mutations cause diseases by changing splicing patterns[33]. Mutations in snRNP assembly complexes give rise to neural diseases such as spinal muscular atrophy (SMA) and retinitis pigmentosa, caused by mutations in survival of motor neuron-1 (SMN-1) and pre-mRNA-processing factor gene homologues PRPF31, PRPF8 and HPRP3 respectively[4]. Changes in alternative splicing also have a major hand in alterations of important survival, antiapoptotic, metastatic and metabolic pathways that drive cancer progression, such as the tumor suppressor genes *BRCA1* and adenomatous polyposis coli (*APC*)[34,35]. In innate immunity, extensive alternative splicing of TLR mRNAs generates new variants that change signaling strength or act as antagonists to the full-length protein, keeping runaway inflammatory signals in check[23]. Alternative splicing is also multiple methods to generate diversity in adaptive immune receptors required to detect an ever growing list of pathogens. Notably, alternative splicing of major histocompatibility complex (MHC) molecules and other relevant proteins such as tapasin and transporter associated with antigen processing 1 and 2 (TAP1/2), which are required for peptide loading, greatly increase the complexity of antigen presentation[36]. Alternative splicing between two exons, Cμ1 and Cδ1, enables production of both

immunoglobulin M (IgM) and IgD) from the same B lymphocyte, controls relative IgM/IgD proportions and controls reduction of IgD expression during B cell maturation. Furthermore, splicing of the immunoglobulin pre mRNAs generates soluble and transmembrane receptors with separate, specialized jobs in antimicrobial defense[37]. Further downstream, the strong genetic association of *Irf5* with systemic erythematosus lupus (SLE) implicates haplotypes of specific exon usage and splice sites that are likely to alter conveyance of interferon signaling[38-40]. Despite the continuing challenges of parsing out a splicing code to accurately predict exon usage and splicing patterns, it remains a paramount consideration as a potential effect of RNA modifications and genomic variation.

1.2 ALTERNATIVE SPLICING IN CYTOKINES AND THEIR RECEPTORS

Many of the interleukins have known alternative isoforms that, when expressed, exhibit altered biological activity[41]. In addition, their receptors also undergo extensive alternative splicing to yield multiple forms that either compete for the ligand to suppress functional signaling, or otherwise synergize with the membrane-bound receptor to enhance downstream signaling[41]. Of this extensive list, I will expand on two specific examples below that demonstrate the profound and complex effects of splicing of cytokines and their receptors on the nature and strength of signaling. They are summarized in **Fig. 1.3-1.5**.

1.2.1 Splicing of IL-6 and its receptor diversifies signaling and regulatory modalities

The signaling pathway of IL-6 is subject to extensive regulation by alternative isoform usage. The inflammatory cytokine IL-6 is expressed by many cells including activated monocytes, T cells and fibroblasts. IL-6 binds to its specific receptor IL-6R and the common family receptor chain gp130, to activating principally STAT3 but also NFκB and PI3K pathways[42]. IL-6 itself has

several splice variants in both human and mouse. Isoforms of human IL-6 lacking exons 2 and 4 have been identified in lung tissue; exclusion of exon 2 completely prevents translation of the protein, while IL-6 lacking exon 4 competitively inhibits full-length IL-6 binding to IL-6R and thus suppresses functional IL-6 signaling[43]. The decision to include or exclude these exons enables compartmentalization of IL-6 function.

In addition, both chains of the IL-6 receptor have classical membrane-bound forms as well as soluble forms (sIL-6R and sgp130) that can either be generated by alternative splicing or limited proteolysis of the membrane bound receptors by the proteases a disintegrin and metalloproteinase (ADAM)10 and ADAM17[44-47]. Soluble IL-6R is able to bind IL-6 with high affinity and signal cells expressing only gp130 in a process known as trans-signaling, in contrast to cis- signaling that is mediated by IL-6 binding to mIL-6R and gp130 on the same cell (**Fig. 1.3**). This process has several important implications that diversifies the biological functions and regulatory mechanisms of IL-6 signaling. Firstly, while mIL-6R expression is limited to hepatocytes, megakaryocytes, neutrophils and specific subsets of activated T cells, gp130 is expressed on all cells; therefore, IL-6 trans-signaling can occur in cells without mIL-6R, including human smooth muscle cells, neural cells, the endothelium and embryonic stem cells. Furthermore, the gene expression signatures of cis- and trans-signaling differ greatly; while cis-signaling is required for antimicrobial defenses, many of the pathological effects of IL-6-driven inflammation can be attributed to trans-signaling[48]. For example, it is mostly trans-signaling that drives gliosis, vasculopathy and neurodegeneration in IL-6-overexpressing mice[49]. Trans-signaling by IL-6 is also largely responsible for pathological immune cell infiltrate in murine arthritic models as well as osteoarthritis and rheumatoid arthritis patients[50]; indeed, sIL-6R expression is normally elevated in rheumatoid arthritis and cancers[51]. It is certain that splicing to generate membrane-bound or soluble IL-6R has far-reaching

consequences for IL-6 signaling that do not only alter the strength of the signal but also drastically change its effector program.

Herein sgp130 presents an interesting layer to regulation. Notably, trans-signaling can be selectively blocked by sgp130, which acts as a sink for free sIL-6R but does not affect membrane-bound IL-6R. While free IL-6 expression is low in the steady state, sIL-6R occurs at about a 10-fold excess while, in turn, sgp130 is often present in 5-10-fold excess. Since sgp130 also has a high affinity for the IL-6:sIL-6R complex with a K_D of 1pM, it is able to tightly bind the complex and thus tightly control trans-signaling. Since sgp130 interacts only with the IL-6:sIL-6R without any effect on classical signaling by mIL-6R, it is an excellent experimental tool to determine the dominant signaling pathway in IL-6-dependent disease phenotypes and is currently under clinical trial for selective blockade of IL-6-driven inflammation[49,50]. Several splice forms of sgp130 exist that are generated by alternative splicing or alternative polyadenylation that all bind IL-6:sIL-6R, albeit at different affinities and with different dimerization properties[46,52,53]. It is possible that these splice forms have evolved to enable graded control of trans-signaling.

1.2.2 Splicing of IL-15 and its receptor controls stability and secretion

IL-15 and its specific receptor chain, IL-15Rα, also exhibit interesting splicing patterns that alter IL-15 activity. IL-15 signals on many cells, though its main biological function is the homeostasis, function and activation of $CD8^+$ lymphocytes and natural killer cells[54]. Two splice variants exist that encode either a long signal peptide (LSP) or short signal peptide (SSP) upstream of the same full-length IL-15 (LSP-IL-15 and SSP-IL-15) and have very different tissue expression patterns[55-58] (**Fig. 1.4**). In addition, while the isoform with the SSP is efficiently translated, it remains in the cytoplasm and nucleus; the isoform encoded with the LSP is poorly translated, but it is transported through the Golgi apparatus, undergoes post-translational modifications and is

efficiently secreted. Furthermore, SSP-IL-15 competes with LSP-IL-15 for binding to IL-15Rα, when all three are expressed in the same cell, and destabilizes the IL-15:IL-15Rα complex[59]. Alternative exon configurations of IL-15 isoforms in mice are generated in activated immune cells that change expression and activity, including an internally spliced exon 5 that improves translational efficiency, a variant lacking exon 6 that competitively inhibits IL-15 receptor binding, and an isoform lacking 5′ end of exon 7 that ameliorates dermal infiltrates of immune cells and acanthosis normally induced by full-length IL-15[60-62]. These highlight a major role for splicing in the regulation of IL-15 translation, secretion and activity.

The cytokine IL-15 is normally not secreted in a monomeric form but rather held tightly by its unique receptor IL-15Rα, which is expressed on many cell types[63]. This complex further requires binding to the IL-2/15βR and γC subunits (hereafter referred to as the β/γC complex) as to signal (**Fig. 1.5**). Early intracellular association mutually increases stability of both variants of IL-15 as well as IL-15Rα, allowing their secretion as a complex[59,64]. Interestingly, membrane-bound complexes of IL-15 and IL-15Rα on monocytes are recycled in endosomes and able to trans-activate neighboring T cells expressing only the complementary the β/γC complex[65].

Like IL-6R, IL-15Rα is expressed in both transmembrane and soluble forms, and shedding of the soluble form from IL-15Rα-expressing cells can occur in proteolysis-dependent and -independent mechanisms[66]. The heterodimeric complex of soluble IL-15 and soluble IL-15Rα comprises the majority of bioactive IL-15 in serum, as the soluble IL-15 is comparatively more unstable and less agonistic in its monomeric form[67,68] (**Fig. 1.5**). Indeed, addition of soluble IL-15Rα is able to enhance IL-15 agonism of lymphocyte proliferation, unlike the closely related soluble IL-2Rα which antagonizes IL-2 signaling[69].

Both soluble and membrane-bound IL-15Rα have multiple isoforms resulting from extensive splicing variation of their pre-mRNAs that may exclude exon 2, exon 3 or both in addition to usage of two alternative exon 7 sequences (**Fig. 1.6**). All these isoforms are well-expressed in various tissues and, therefore, functionally relevant to IL-15 signaling[70]. Exon 2 encodes the sushi domain, which is essential for post-translational modification and cytokine binding[70,71], so isoforms lacking this exon are binding-deficient and cannot stabilize or activate IL-15 signaling. The linker domain encoded by exon 3 contributes to the strong association between IL-15:IL-15Rα complex[72-74]. Importantly, the N-terminus sequence of exon 3 determines whether the soluble IL-15Rα is able to antagonize IL-15:IL-15Rα signaling. Meanwhile, how alternative exon 7 usage alters IL-15Rα function remains unknown. Eight additional splice variants have been more recently identified specifically in human monocytes and dendritic cells, with a novel insertion between exons 1 and 2 and further alternative exon 6 and 7 sequences that alter glycosylation, maturation, secretion and trans-presentation of the receptor[75]. Thus, the unusually large variety of splicing patterns of both IL-15 and IL-15Rα provide many ways to tightly regulate IL-15 signaling through changes in affinity, stability and trafficking.

1.2.3 Splicing of IFN genes and their receptors

Interferons are a family of cytokines that have essential, particular antiviral activities. They are categorized as type I (IFNα1-14, β, ε, κ, ω), type II (IFNγ) and type III (IFNλ). As one of the genes in interest of this thesis is the most recently discovered type III IFN, IFNλ4, a brief discussion of known splice variation in the cytokine and receptor genes follows to provide context for the unique splicing properties of IFNλ4.

Compared to other cytokine families, splicing and alternative isoform generation of IFN genes is more limited. Generally, splicing diversity and the biological effects of splice variation

have not been explored in IFNs, although it has been sporadically examined in their receptors. Importantly, the question is moot in type I IFNs because nearly all transcripts arise from a single exon; thus, splice variation does not occur. The contrasting multi-exon configuration of type III IFNs hint at functional differences between the families despite highly similar downstream effector pathways. Of these, IFNλ1 and IFNλ2 only have one transcript annotated, while IFNλ3 has two transcripts, although their functional differences have not been discovered. IFNγ, the sole representative of type II IFNs, is a multi-exon gene for which only one transcript exists. Therefore, IFNλ4 is unique among all the IFN families for having several endogenously expressed splice variants. These are further discussed in Section 3.1.

The IFN family receptors demonstrate more splicing diversity than their ligands, although the true extent of variation and consequences are also poorly understood. In the case of type I IFN receptors, no natural variant of IFNαR1 has been identified; in contrast, there are multiple isoforms of IFNαR2 that are generated by alternative splicing and polyadenylation. Both humans and mice have a soluble IFN receptor lacking the transmembrane domain, and the murine soluble receptor has both agonistic and antagonistic roles in IFN signaling *in vivo*[76-80]. The type III IFN receptor chain, IFNλR1, also has two additional known isoforms: one isoform excludes exon 6 and generates a frame-shifted, truncated soluble receptor that inhibits type III IFN-dependent signaling, while a second isoform uses an alternative splice site to delete part of exon 7. Both bind IFNλ but do not signal[81,82]. Given that these isoform transcripts are expressed in human hepatocytes, keratinocytes, airway epithelium and immune cells, with the soluble receptor expressed at different proportions in different tissue, it is tempting to speculate that the choice of splice variant for IFNλR1 impacts these cells' responsiveness to IFNλ stimulation and, therefore, their propensity for antiviral effector functions. The two receptor chains for type II IFNs, namely IFNγR1 and

IFNγR2, have multiple annotated splice forms that have not been explored in detail. Perhaps the increased splice variation of IFN receptors compensates for their single-isoform ligands in an evolutionary bid to preserve this mechanism for controlling IFN signaling.

1.3 Splicing as an important paradigm of immune regulation

IL-6 and IL-15 teach us the large variety of ways in which splicing can change immune function. These are particularly complex examples, although it is clear from other immune genes and cytokines that splicing causes the removal of important functional domains and generates competitive inhibitors for signaling[23,41,83]. Furthermore, as discussed in section 1.1.3, we still lack a "splicing code" for computational prediction of the strength of each splicing site, much less combinations of exons used under specific environments. Therefore, the expression of specific isoforms in a given context still has to be determined empirically for each specific gene under experimental conditions. While some genes, such as *CFTR*, have been extensively studied and annotated due to well-known genetic disorders, the effects and biological significance of splicing and isoform generation remain mysterious for most. This knowledge is especially lacking for many immune genes which were discovered more recently.

In my thesis, we have chosen to examine two molecules for which alternative splicing is likely to have important functional effects but remains poorly studied. IL-22 binding protein (IL-22BP; gene symbol *IL22RA2*) is a soluble receptor for the IL-10 family cytokine IL-22 that has two special properties: first, it is the only soluble receptor known in the IL-10 family of cytokines, and second, it is encoded on a separate gene from the membrane-bound receptor for IL-22. While IL-6R, gp130 and IL-15Rα are excellent examples for complex roles of soluble receptors, they are generated by alternative splicing (or in the case of the IL-6 receptor, limited proteolysis) from the same gene as the membrane-bound receptors. While genetic associations have implicated a role

for *IL22RA2* in autoimmune disease, these do not distinguish between the three known splice variants of the receptor. It is likely that the alternative splicing of IL-22BP changes its function, localization or secretion, and these would be important to determine in the context of other alternatively spliced cytokine receptors.

Our second gene of interest, *IFNL4*, is a novel member of the IFN pathway that uniquely has multiple naturally-expressed transcripts; apart from the full-length protein, the functions of the isoforms are not known. The endogenous expression of isoforms contrasts other type III IFN genes that do not appear to undergo functional splicing. Since genetic associations already implicate a pro-viral function for IFNλ4 atypical of type III IFNs, it is likely that alternative splicing is an important mechanism that controls its gene expression, and that exon inclusion or exclusion dictates viral clearance or persistence as an outcome of IFNλ4 activity.

In our investigations of IL-22BP and IFNλ4 described in the following chapters, we examined the function and regulation of isoforms generated by alternative splicing. We found that choice of splice variant is crucial to for regulating expression of functional isoforms of both proteins. Alternative splicing in IL-22BP is needed to fine-tune the response both for IL-22 alone as well as its cooperative action with a co-expressed cytokine IL-17. Additionally, functional study of IFNλ4 isoform expression allowed us to determine the relative contributions of two tightly linked genetic variants in *IFNL3* and *IFNL4* to antiviral defense that were previously difficult to discern. Our findings cement the paramount importance of addressing the function and regulation of alternative transcripts from cytokine and receptor genes, as we show that they diversify and often determine the contribution of each signaling pathway to immune homeostasis and defense. Therefore, we encourage this approach to the study of many other multi-exon cytokine and

receptor genes, for which the biological effects of alternative splicing still remain poorly understood.

Figures

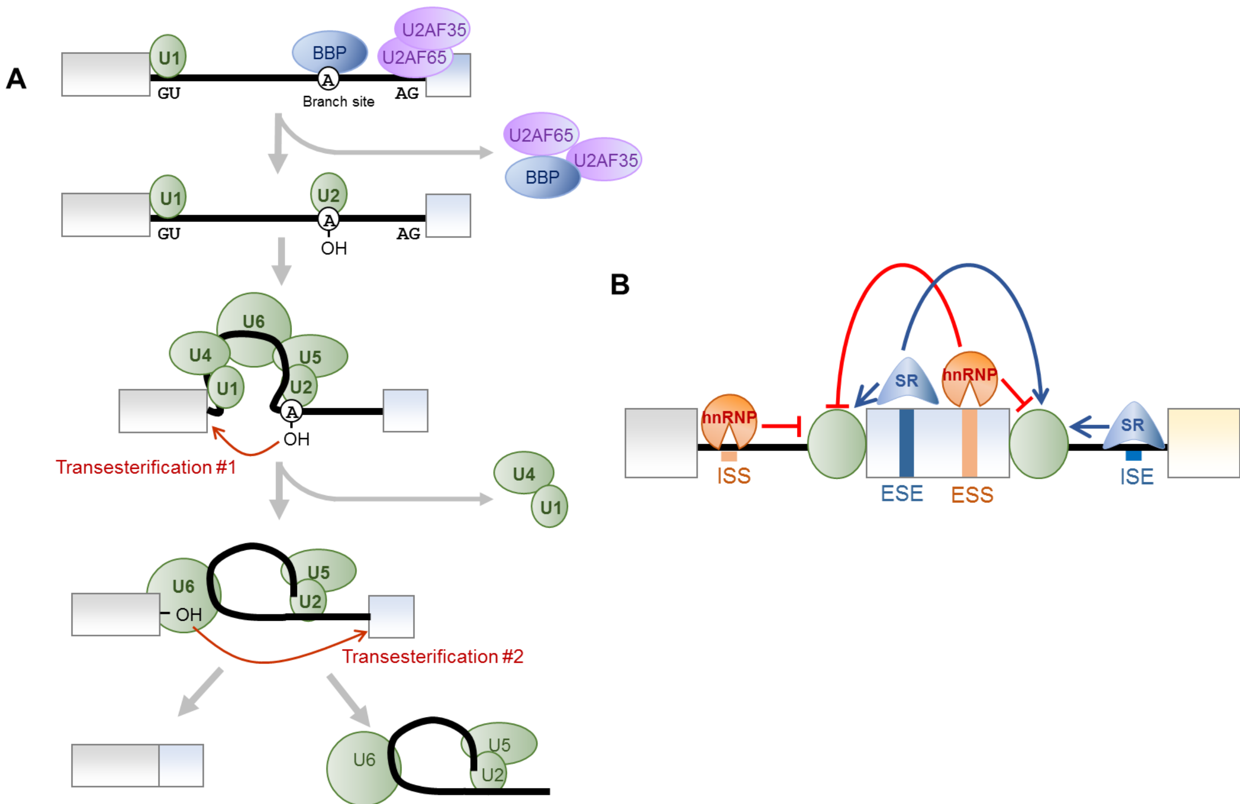

Figure 1.1. The processes of constitutive and alternative splicing.

(**A**) General mechanism of constitutive splicing. The core spliceosomal complex comprises the U1, U2, U4, U5 and U6 snRNPs and associated proteins that identify several important sequences: the 5′ splice site (GU), the 3′ splice site (AG) and an intronic branch site proximal to the 3' splice site. Upon recognition of the splice sites and branch site, U2 is recruited to the branch site where it and hydrolyzes the branch point adenosine residue. This initiates a cascade that recruits the U4-U5-U6 tri-snRNP complex and catalyzes the first transesterification reaction to yield the intron lariat. The U2, U5 and U6 snRNPs then guide the second transesterification process that ligates the two exons and releases the intron lariat.

(**B**) Mechanism of decision-making during alternative splicing. Not all splice sites are fully used. Choice of "weaker" splice sites depends on the association of trans-acting factors, such as hnRNPs and SR proteins, with cis-regulatory elements: intronic and exonic splice enhancers (ISE/ESE) and suppressors (ISS/ESS). The positive and negative signals are processed by the spliceosome to define whether the exon is included or excluded.

Figure panels adapted from Kornblihtt A.R. et al., (2013) *Nat Rev Mol Cell Biol* 14: 153 – 165.

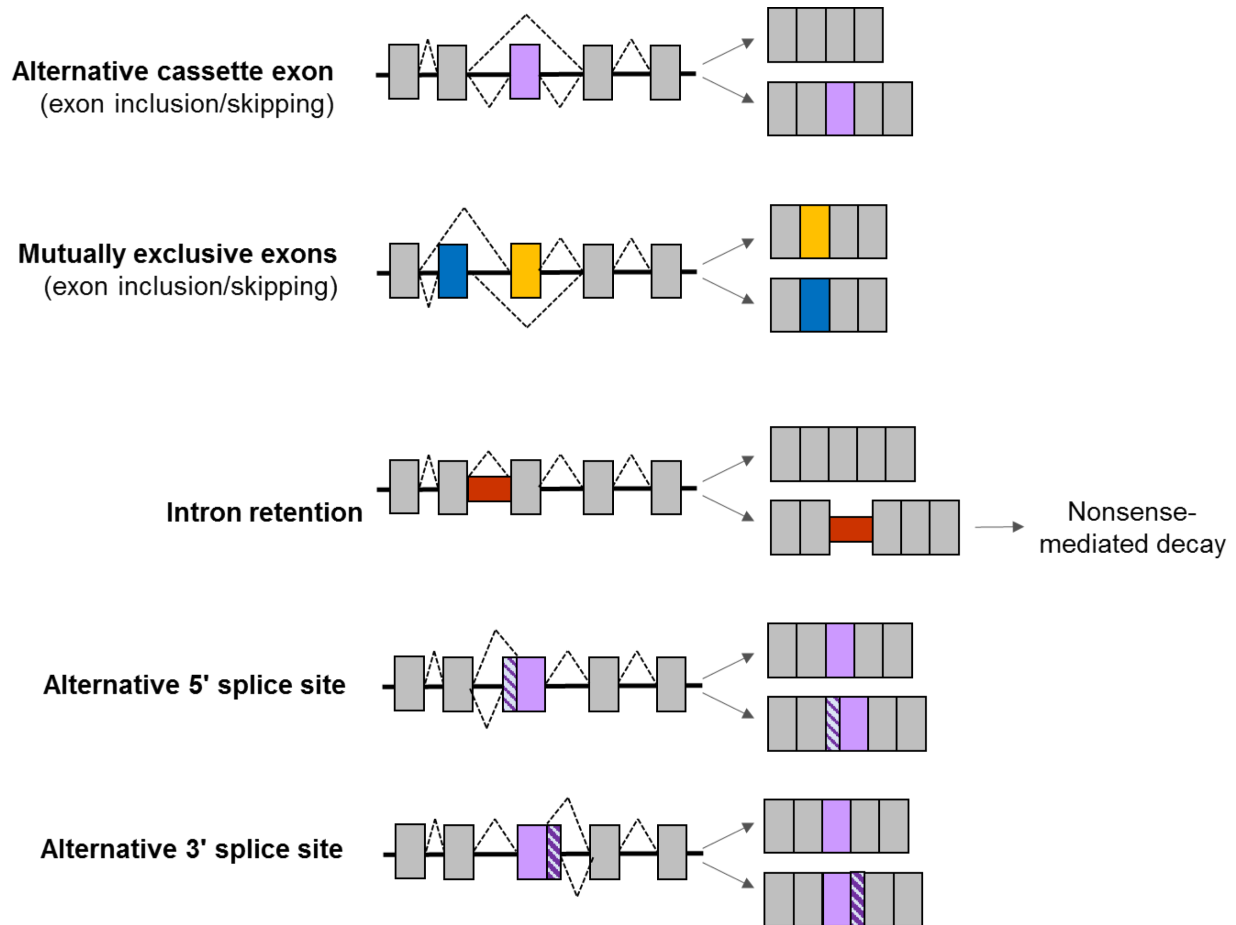

Figure 1.2. Outcomes of alternative splicing.

Schematics showing multiple ways in which alternative splicing can occur and their effects on the resulting transcript. Alternative exon usage: allows exon inclusion or exclusion; mutually exclusive exons generate different combinations; intron retention targets the transcript for nonsense-mediated decay; and alternative 5′ or 3′ splice site usage generates insertions or deletions that may keep the transcript in-frame or create a frame-shift that sometimes truncates the protein. Multiple mechanisms can be used the same gene individually or in combination. Grey boxes represent constitutive exons; purple, blue, gold and shaded boxes represent alternative exons; shorter red boxes represent retained introns.

Figure panels adapted from Blencowe, B.J. (2006) *Cell* 126: 37 – 47.

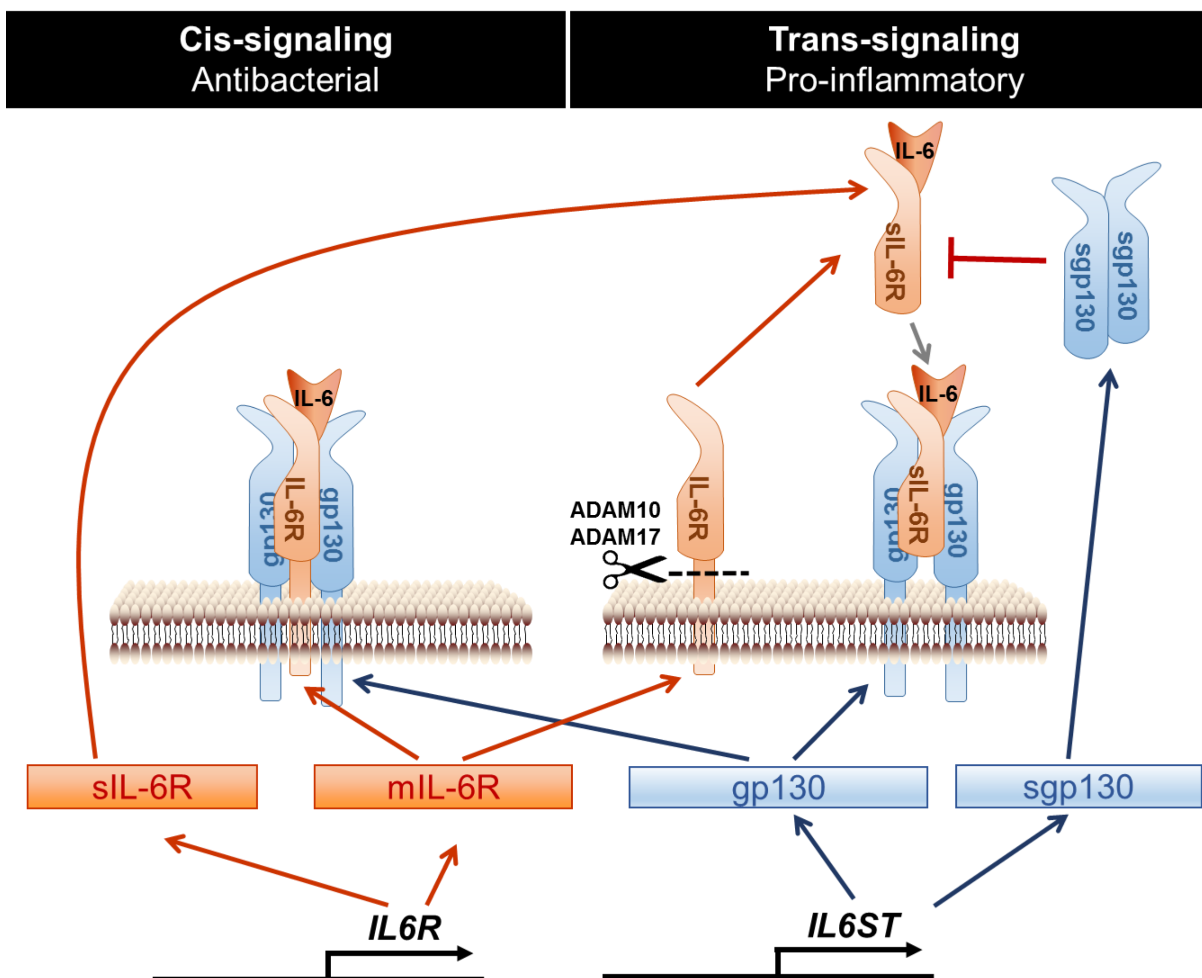

Figure 1.3. Alternative splicing of the IL-6 receptor increases diversity in IL-6 signaling.

Schematic of IL-6 membrane-bound receptors, soluble receptors and how they regulate one another to cause either cis- or trans-signaling. Both IL-6R and gp130 (gene symbol *IL6ST*) have membrane-bound and soluble isoforms that can be generated by alternative splicing or, in the case of IL-6R, proteolysis by ADAM10 or ADAM17. Cis-signaling through both membrane-bound receptors is limited to hepatocytes, megakaryocytes, neutrophils and specific subsets of activated T cells that express full-length IL-6R (mIL-6R). In contrast, trans-signaling occurs when soluble IL-6R (sIL-6R) binds IL-6 and then interacts with gp130 on cells lacking mIL-6R. Due to the ubiquitous expression of gp130, trans-signaling can occur on many cell types. Importantly, each mode of signaling has divergent effector functions: while classical signaling is required for the beneficial antimicrobial activites of IL-6, pathogenic pro-inflammatory signaling associated with autoimmunity and neoplasia seems to be solely dependent on trans-signaling. Soluble gp130 (sgp130) specifically inhibits IL-6 trans-signaling but leaves classical IL-6 signaling intact; its high concentration provides a physiological buffer to limit IL-6 trans-signaling in the steady state.

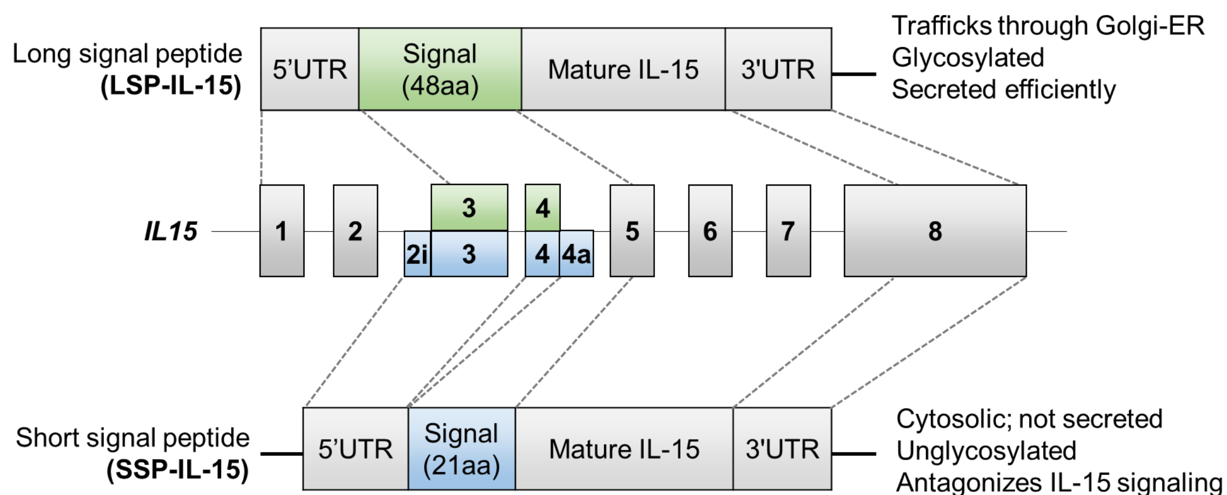

Figure 1.4. Alternative splicing of IL-15 changes its localization and post-translational modification.

Schematic of the *IL15* transcript and generation of two major isoforms by alternative splicing. Use of different transcription start sites generates two transcripts of *IL15* encoding the same mature peptide with two different lengths of signal peptide (long and short, for LSP-IL-15 and SSP-IL-15 respectively). The long signal peptide is able to target the protein for transport through the endoplasmic reticulum and Golgi apparatus, but not the short peptide. As a result, LSP-IL-15 is glycosylated and efficiently secreted, while SSP-IL-15 remains unglycosylated and intracellular. Relative expression of LSP-IL-15 and SSP-IL-15 regulates the export of bioactive IL-15. In addition, SSP-IL-15 competes with LSP-IL-15 for binding to IL-15Rα intracellularly and destabilizes the receptor. Therefore, alternative splicing to generate SSP-IL-15 instead of LSP-IL-15 creates a natural antagonist of IL-15 signaling.

Figure adapted from Bugadian, V. et al (2006) *Cytokine Growth Factor Rev* 17: 259-280.

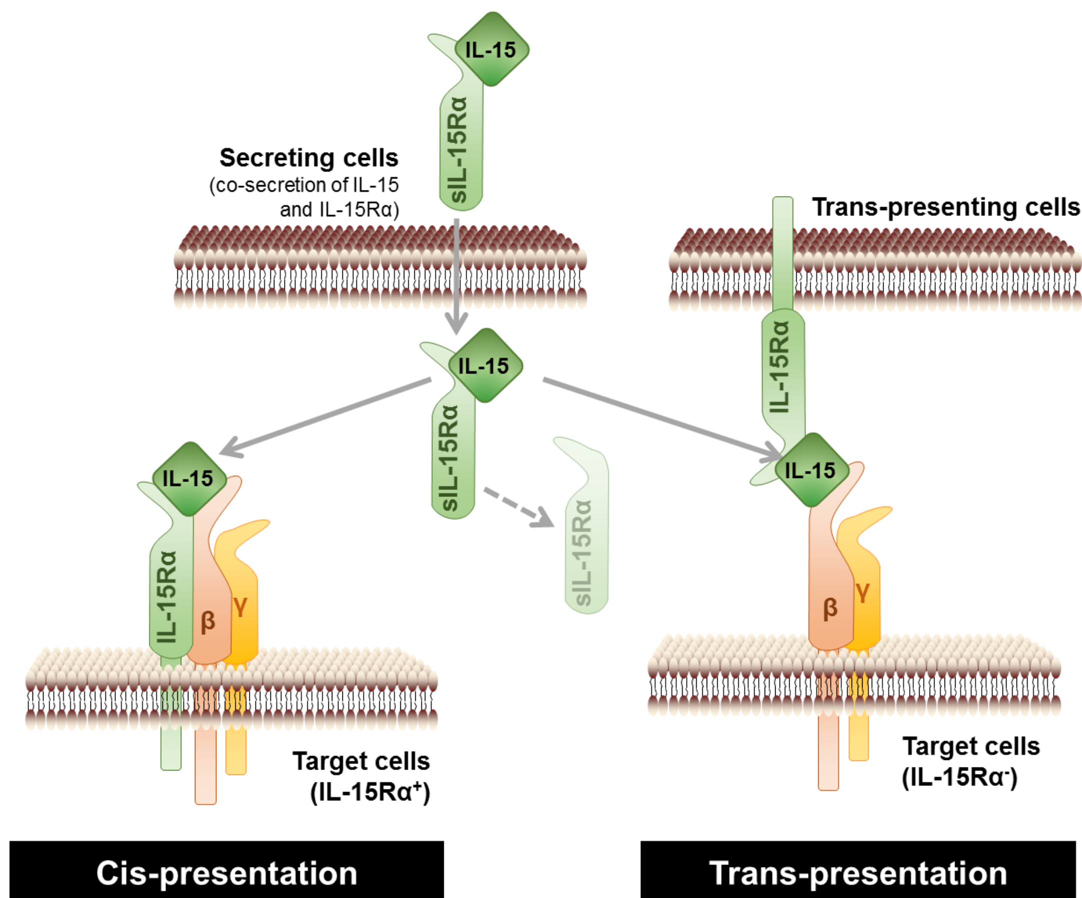

Figure 1.5. Alternative splicing of IL-15Rα generates a soluble receptor that stabilizes IL-15.

Schematic of signaling modes through IL-15/IL-15Rα, and the role of soluble IL-15Rα. This complex associates intracellularly to increase stability of both proteins and remains bound in serum. IL-15 binds membrane-bound IL-15Rα, IL-2/15Rβ and common γ chain (the βγ complex) on the target cell to signal. In cis-presentation, IL-15Rα is expressed on the same cell as the βγ complex. In trans-presentation. IL-15 is able to bind the βγ complex on a target cell due to stabilization and presentation by an adjacent cell. Trans-presentation is an important mechanism for IL-15 signaling in lymphocytes, which require IL-15 activity for homeostasis, activation and memory formation despite a lack of cell-intrinsic IL-15Rα expression.

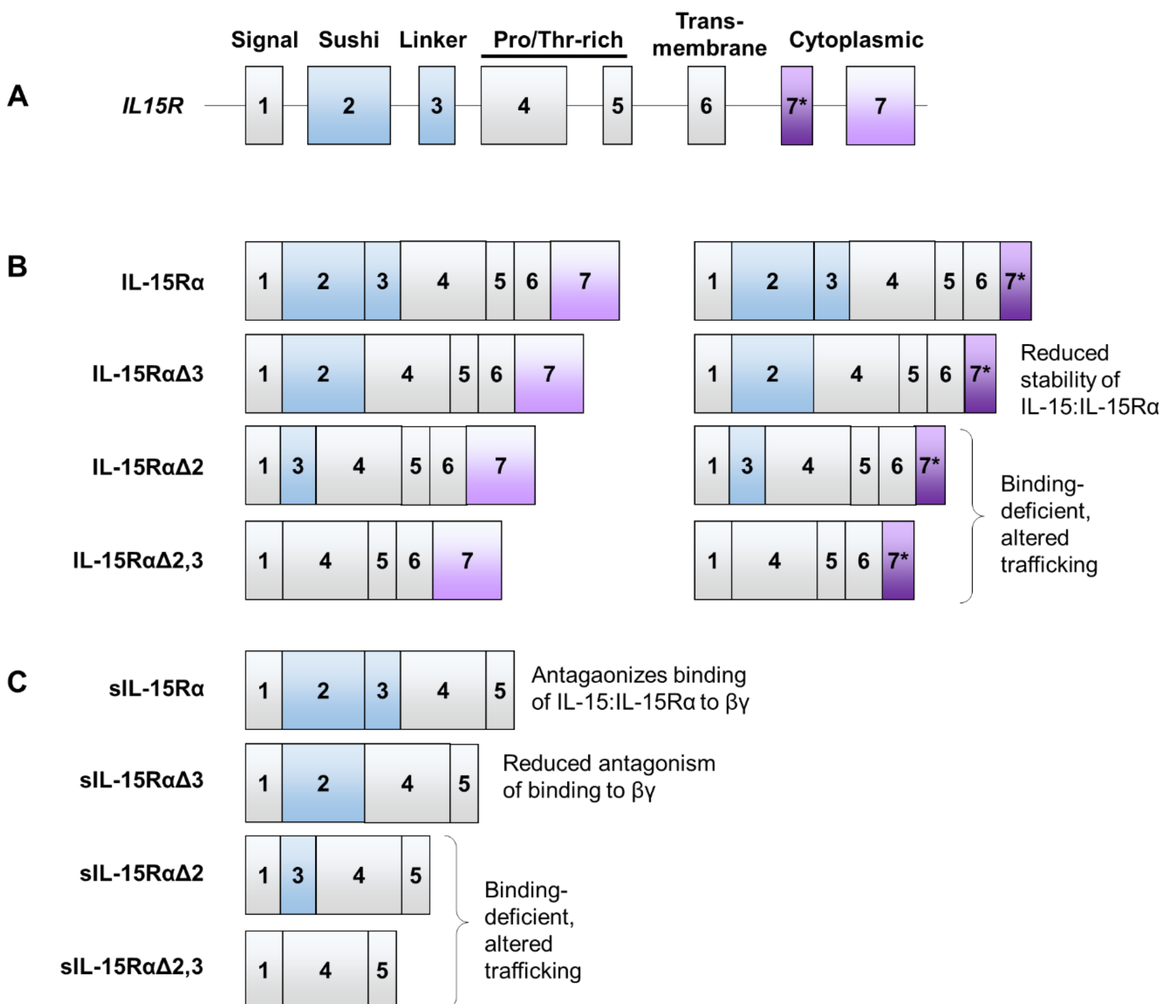

Figure 1.6. Alternative exon usage in IL-15Rα isoforms diversifies its activities.

(**A**) Major domains encoded by exons of IL-15Rα used in splice variants.

(**B**) 8 splice variants of IL-15Rα exist; apart from the full-length protein, variants may lack exon 2, exon 3 or both. In addition, they may use an alternative exon 7 (labelled 7*). The sushi domain encoded by exon 2 is essential for binding IL-15, while exon 3 contributes to stability of binding with IL-15. The effect of differential exon 7 usage is not known.

(**C**) Similar to full-length IL-15Rα, soluble IL-15Rα may exist lacking either exon 2, exon 3 or both. Again, exon 2 is essential for cytokine binding. An N-terminus 13 amino acid sequence in exon 3 is essential for antagonizing the binding and active signaling of IL-15:IL-15Rα.

Figure partially adapted from Dubois, S. et al (1999) *J Biol Chem* 274 (38): 26978–26984.

Chapter 2. THE HUMAN IL-22 BINDING PROTEIN ISOFORMS ARE A RHEOSTAT FOR IL-22 SIGNALING

Parts of the following work has been accepted for publication in Science Signaling *as a scientific article by authors Chrissie Lim, MeeAe Hong and Ram Savan. Additionally, sections of the introduction have been published in* Cytokines and Growth Factor Reviews *as a review by authors Chrissie Lim and Ram Savan.*
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The cytokine interleukin-22 (IL-22) activates signal transducer and activator of transcription 3 (STAT3) to drive various processes central to tissue homeostasis and immune surveillance; its dysregulation causes inflammatory diseases. IL-22 has a soluble receptor, IL-22 binding protein (IL-22BP; *IL22RA2*), which antagonizes IL-22 activity and has genetic associations with autoimmune diseases. Humans express three IL-22BP isoforms, whereas mice only express an IL-22BPi2 homolog. Given the lack of IL-22BPi1 and IL-22BPi3 expression in mice, there has not been a thorough characterization of their activities to date. In this study, we show that IL-22BPi2 and IL-22BPi3 differentially modulate IL-22 activity: IL-22BPi3 has lower inhibitory activity than IL-22BPi2, but is expressed more abundantly throughout the body during homeostasis. IL-22BPi2 and IL-22BPi3 also differentially alter the cooperative actions between IL-22 and IL-17, which are commonly co-expressed in autoimmune settings. In addition, we demonstrate that IL-22BPi1 is inactive because it is not secreted. Importantly, while IL-22BPi3 is the dominant isoform expressed constitutively, only IL-22BPi2 is modulated when myeloid cells are activated by TLR2 or retinoic acid. These data suggest that the IL-22BP isoforms have distinct spatial and temporal roles and coordinately fine-tune IL-22-dependent STAT3 responses in tissues as a rheostat. This work is the first to date to perform a thorough biochemical and molecular characterization of the human IL-22BP isoforms.

2.1 INTRODUCTION

IL-22 is an α-helical class II cytokine of the IL-10 family cytokine secreted mainly by $CD4^+$ helper T cells, γδ T cells, innate lymphoid cells and NKT cells[84-86]. IL-22 signaling through its unique receptor IL-22R1 and the common IL-10 family receptor chain IL-10R2 activates principally STAT3 to induce transcription of genes with context-specific pro- or anti-inflammatory, pro-survival, regenerative and anti-fibrotic effects[87-94]. IL-22 also activates STAT1 in specific contexts, particularly in the presence of interferons, to mediate antiviral activity and inflammation in the gut epithelium[90,95-97]. Therefore, IL-22 plays important roles in tissue repair, mucosal immunity and metabolic activities[91,94,98-109]. Dysregulated expression of IL-22 drives inflammation in psoriasis and Crohn's disease[88,90,92]. IL-22 also has complex, stage-specific pro- and anti-tumor effects[110]. Consequently, IL-22 inhibitors are of significant clinical interest and currently under trial in the treatments of psoriasis, rheumatoid arthritis and atopic dermatitis (www.clinicaltrials.gov).

IL-22 also has a soluble receptor, IL-22 binding protein (IL-22BP; gene symbol *IL22RA2*). Studies in *Il22ra2*$^{-/-}$ mice have revealed striking effects in colitis and experimental autoimmune encephalitis (EAE)[94,111-113], pointing to a crucial role for IL-22BP in the control of pathological IL-22 signaling. However, data from mouse models have not translated into understanding the mechanisms behind the associations with human disease. This is likely because *IL22RA2* encodes three alternatively-spliced isoforms in humans named as *IL22RA2v1* (IL-22BPi1), *IL22RA2v2* (IL-22BPi2) and *IL22RA2v3* (IL-22BPi3) but only one isoform in mice, *Il22ra2* (Il-22bp), which is homologous to human *IL22RA2v2*/IL-22BPi2 (**Fig. 2.1; Suppl. Fig. 2.1**). Knowledge about IL-22BP is largely limited to the shared murine and human homolog, IL-22BPi2, which inhibits IL-22 signaling *in vitro*[114,115]. However, the control of IL-22BP expression remains poorly

understood. The only known stimulus of IL-22BP production thus far is retinoic acid in myeloid cells *in vitro* and, even so, it is unclear which specific isoforms are induced. Meanwhile, expression of IL-22BPi1 and IL-22BPi2 has been examined in the steady state in limited contexts, but not the expression of IL-22BPi3. Furthermore, the biological functions of IL-22BPi1 and IL-22BPi3 remain uncharacterized.

In this chapter, we use molecular and biochemical approaches to define the functions of IL-22BPi1 and IL-22BPi3 in comparison to IL-22BPi2, and go on to profile the distinct expression of the isoforms in homeostasis and inflammation. We expect that our findings will add invaluable insight to how these human-specific isoforms contribute to disease progression in patients and begin to provide possible functional explanations for genetic associations for disease.

2.1.1 IL-22 performs pleiotropic, context-dependent functions

The IL-22 gene (gene symbol *IL22*) is located on chromosome 12 in humans, in close proximity to the loci encoding interferon-gamma (IFN-γ; gene symbol *IFNG*) and IL-26[116]. IL-22 has also been cloned from non-mammals such as fish, where its chromosomal location between the *IFNG* and *IL26* loci remains conserved[117]. Thus we can infer that its importance to homeostasis and immunity has persisted for a long time in evolutionary history.

Principal sources of IL-22 include $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, γδ T cells, innate lymphoid cells (ILCs) encompassing natural killer (NK) cells and lymphoid tissue inducer (LTi)-like cells [84,85], and NKT cells which express both NK cell markers and T cell receptor [86]. It is important to bear in mind that the principal cellular sources of IL-22 varies widely between tissues, so regulation of IL-22 production and signaling may involve targeting different immune cell populations or niches. For example, the expression of CCR10 on Th22 cells but not Th17 cells causes preferential recruitment of Th22 cells to the skin where they are the primary source of IL-

22[118-120], whereas they are overshadowed by γδT cells and ILCs in the gut[121-123]. In immune challenge, kinetics also dictate the main cellular sources of IL-22: during early response, IL-22 production primarily comes from ILCs and tissue-resident γδT cells which are poised for rapid activation, while later on the adaptive immune system provides a second wave of IL-22 from conventional αβT cells [124,125]. This is likely why different IL-22-producing subsets are major players for different models of colitis, particularly with respect to the innate/adaptive immune divide[126]. The local milieu and disease kinetics will be major factors to account for when dissecting IL-22-dependent mechanisms of disease. A summary of principal sources of IL-22 and its downstream signaling effects is presented in **Fig. 2.2**.

Although IL-22 is secreted exclusively by immune cells, it signals solely on non-immune cells including hepatocytes, keratinocytes, lung epithelial cells, colonic epithelial cells and pancreatic acinar cells, to which IL-22R1 expression is restricted[87,91-93,101,127-133]. IL-22R1 is also expressed in some other tissue cells such as hepatic stellate cells[134], colonic subepithelial myofibroblasts[135], smooth muscle cells[136] and thymic epithelial cells[137], but their sensitivity to IL-22 stimulation appears to vary. IL-22 signals through a heterodimeric receptor comprising its unique receptor IL-22R1 (gene symbol *IL22RA1*) and IL-10R2[130,138] to activate Signal Transducers and Activators of Transcription (STATs) 1, 3 and 5, nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and phosphatidylinositide 3-kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) pathways[125,139]. The consequences of these signaling pathways are detailed below. Their variety demonstrate the profound effects of IL-22 signaling throughout the body as well as the great need to learn how it can be controlled in specific tissues and situations.

2.1.1.1 Signaling effects downstream of IL-22/IL-22R1

IL-22 stimulation induces expression of a medley of genes principally downstream of STAT3, with pro-inflammatory, anti-inflammatory, mitogenic, pro-survival, anti-apoptotic or anti-fibrotic effects depending on specific tissue and disease context[124,125,140,141] (detailed in section 2.1.1.2). Constitutive association of STAT3 molecules with the intracellular domain of IL-22R1 poises the cell for rapid activation, though activation also requires association of Src Homology Phosphatase 2 (SHP2) with activated IL-22R1 at phosphorylated residues Tyr-251 and Tyr-301[142,143]. IL-22 signaling enacts negative feedback through induction of suppressors of cytokine signaling 1 and 3 (SOCS1/3), which inhibiting STAT3 activity[139]. On the other hand, since STAT3 enhances IL-22 production by Th17 cells, enrichment of IL-6, IL-21, IL-23 and IL-1β in a STAT3-driven niche sustains local differentiation of these cells and resultant maintenance of IL-22 signaling[118,144,145].

To a smaller extent, STAT1 activation by IL-22 is observed in some cells including hepatocytes, colonic epithelial cells and renal carcinoma cells[90,91,97,130,131,141,146-148] that can be enhanced by IFNα costimulation[97]. Rare STAT5 activation has also been observed in hepatocytes and kidney cells, but downstream effects, if any, are yet unknown[129,130,139,141]. Multiple mitogen-activated protein kinase (MAPK) pathways are activated downstream of IL-22R1 including the p38, extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK) cascades, though sensitivity to induction, exact pathways activated and subsequent downstream events are varied and cell-type specific[135,136,141,143,146,147,149-152]. In addition, IL-22 activates the phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway in some cells. Akt activation via IL-22 is necessary for proliferation in normal human epithelial keratinocytes and fibroblast-like synoviocytes, while in renal tubular epithelial cells it inhibits

mitochondrial apoptosis[149,153]. PI3K activation is also required for migration of colonic epithelial cells and hepatocytes [90,139].

Lastly, IL-22 is able to functionally activate NF-κB[135,136,154,155]. Notably, NF-κB can promote expression of genes involved in cell cycle, proliferation and prevention of apoptosis, particularly in synergy with STAT3[154,156,157]. So far, inflammatory cytokine induction and proliferation have been observed downstream of IL-22 signaling via NF-κB[135,136,154,155]; the intersection of these pathways with STAT3 signaling may yet prove interesting especially for the role of IL-22 in transformation and cancer progression.

Because immune cells are not responsive to IL-22, it has generated immense interest as a therapeutic target with likely fewer side effects than IL-6, IL-17, IL-21 or other principal STAT3-activating cytokines[125]. However, its pleiotropic functions throughout the body still make it a challenging cytokine to manipulate for treatment of disease. For example, the IL-22/IL-22R1 has complex effects on carcinogenesis and tumor progression that are origin- and stage-specific; while anti-inflammatory effects of IL-22 can reduce initial tumorigenesis, prolonged dysregulation and activation can act as a proliferative driver of established cancers[110]. Furthermore, IL-22 can either be protective or pathogenic in different murine colitis models[126]. Therefore, care must be taken in interpreting the contribution of IL-22 to disease progression.

2.1.1.2 Physiological roles of IL-22 signaling

Since many IL-22-responsive cell populations are epithelial cells lining mucosal barriers, it is no surprise that IL-22 has many pro-inflammatory effects to rapidly defend against invading pathogens. In hepatocytes and colonic epithelial cells, IL-22 potently induces an acute phase response including the induction of serum amyloid protein, α-chymotrypsin and haptoglobin [93,116]. IL-22 also induces the production of ELR^+ chemokines CXCL1, CXCL2 and CXCL5 which

recruit neutrophils to the site of invasion[101,123,134,158,159]. IL-22 instigates the production of inflammatory cytokines such as IL-6, IL-8, TNFα in various cells including hepatic stellate cells, myofibroblasts and keratinocytes; enhanced processing of the NLRP3 inflammasome also yields IL-1β[134,135,160]. This further hints at oxidative signaling induced by IL-22, which employs NADPH oxidase to activate STAT3 and may synergize with IFN-γ to produce nitric oxide species[148,161]. Furthermore, IL-22 stimulates the production of an impressive repertoire of antimicrobial peptides, including several members of the S100A family, the regenerating protein (RegI, RegII, RegIII) families, lipocalin, defensins and the cathelicidin LL-37 in the skin and colon[87-89,100,101,162,163]. IL-22 suppresses differentiation-related tissue factors including loricrin, involucrin and calmodulin in keratinocytes, which together with its inflammatory activities drives progression of psoriasis[88,92,99,159,164,165]. While these actions are transiently pro-inflammatory, clearance of pathogens decreases the duration and extent of inflammatory signals[125]. IL-22 also suppresses IFN-γ-dependent induction of pro-inflammatory chemokines *in vitro*[166] with an overall anti-inflammatory effect.

In particular, IL-22 has specialized roles the maintenance of barrier immunity in the gut, where large amounts of the cytokine are produced by ILCs in the lamina propria. IL-22 stimulates mucin production in colonic epithelium cells[94] and enhances lymphotoxin-α production to increase barrier protection. Commensal microbiota further enhance IL-22 activity to increase fucosoylation of glycans on epithelial cells, a biochemical modification important for preventing dysbiosis, colonization by invasive bacteria and systemic septicaemia from spread of *E.faecalis*[105,167]. Serum amyloid protein induction by IL-22 from innate lymphoid cells is also essential for the maintenance of Th17 barrier immunity[168].

Metabolic functions of IL-22 have been more recently characterized. IL-22 induces hepcidin in hepatocytes, an enzyme which decreases iron bioavailability in blood[169,170]. IL-22 also decreases expression of lipogenetic transcription factors and genes in the liver in a hepatic steatosis model[171]. Since IL-22 is already so important to maintenance in the gastrointestinal tract, it may have other metabolic functions that have not yet been discovered.

While IL-22 itself has pleiotropic pro- and anti-inflammatory roles in different sites and environments, interactions and cooperation with other cytokines in the environment can alter its function. The balance of pathogenic and protective functions of IL-22 in a bleomycin-induced lung inflammation model depended on the presence of IL-17 in the microenvironment: while IL-22 enhanced inflammation in the presence of IL-17, IL-22 was required for tissue protection in the absence of IL-17[172]. Thus, IL-22 is able to exert both pro- and anti-inflammatory actions in synergy with the local cytokine milieu. IL-22 is also able to synergize with type III IFNs to enhance antiviral immunity against rotaviral infection[96]. Since interactions between IL-22 and other cytokines are a major factor in determining the outcomes of its signaling, cooperative activity of these cytokines should be taken into consideration when studying regulators of IL-22 activity.

2.1.2 The soluble receptor IL-22 binding protein (IL-22BP)

IL-22 is unique among the IL-10 family of cytokines to have a soluble receptor, IL-22 binding protein (IL-22BP; gene symbol *IL22RA2*)[114,173-176]. IL-22BP is highly expressed in the placenta and mammary gland; found at intermediate levels in the stomach, lung, skin, spleen, thymus and lymph nodes; and modestly expressed in peripheral blood leucocytes, prostate, heart and brain tissues[114,173-176]. Only recently have myeloid cells been confirmed as specific sources of IL-22BP. IL-22BP is expressed in the human gut by eosinophils and conventional dendritic cells, and can be further induced from dendritic cells by all-trans retinoic acid or suppressed by IL-18

through the NLRP3/NLRC4 inflammasomes[111,112,177]. While genetic associations between *IL22RA2* and autoimmune disease, inflammatory disease or cancer have been identified[147,178-182], the role of IL-22BP *in vivo* remains an enigma and other mechanisms of its regulation are still unknown. IL-22 and IL-22BP expression is inversely regulated in dextran sodium sulfate (DSS) colitis models, although they are both elevated in patients with Crohn's disease and ulcerative colitis[94,111,112]. IL-22BP protein levels were elevated in the cerebrospinal fluid of patients with active multiple sclerosis, while there was a trend of increased transcript expression in their monocytes alongside increased IL-22 levels in their PBMCs[183]. Patients with active lupus nephritis in a small study also had increased levels of IL-22BP in urine to accompany increased IL-22 in serum, although IL-22BP levels in serum did not differ from controls [184]. Given that IL-22BP was protective in a colitis-associated colon carcinoma model yet detrimental to the progression of EAE[112,113] despite a pathological role for IL-22 in both, it is likely an active member of the IL-22/IL-22R1 axis in health and disease and its role is likely dependent on tissue and inflammatory context, as is that of IL-22.

Notably, IL-22BP exists as three splice variants in humans (**Fig. 2.1**; **Suppl. Fig. 2.1**). Human isoforms of IL-22BP have demonstrated different binding affinities to IL-22[185], which will be referred to from here as IL-22BP isoforms 1, 2 and 3 (IL-22BPi1, 2 and 3). Murine IL-22BP is homologous to IL-22BPi2, which is the isoform that has been studied almost exclusively. Compared to IL-22BPi2, IL-22BPi1 contains a unique exon 3 insertion while IL-22BPi3 lacks exon 5 and has a truncated exon 6 which comprises 5 frame-shifted amino acids with a premature stop codon. *IL22RA2* sequences have been annotated for many vertebrates, but splice variation appears to be relatively recent. Indeed, exon 3 of IL-22BPi1 is encoded in a long terminal repeat retrotransposon introduced in rhesus macaques, which was later exonized in orangutans through

the generation of a cryptic splice site by an A>G transition[186]. This source could explain why the isoform is absent in rodent models, which completely lack the genomic sequence containing exon 3[175,186].

Studies to date have nearly exclusively studied IL-22BPi2, likely due to its homology with the murine protein for which the crystal structure has been solved. Despite poor amino acid sequence identity and location on a different chromosome from both *IL22* and *IL22RA1*, it folds in a highly similar structure to the extracellular domain of IL-22R1, sharing crucial amino acid interactions for IL-22 binding but with much higher affinity and specificity[115,185,187] (**Fig. 2.1**). IL-22BPi2 has therefore been employed extensively *in vitro* as a competitive inhibitor of IL-22 signaling. The sole biochemical characterization of the other isoforms remains a surface plasmon resonance study which found that IL-22BPi3 binds to IL-22 with a similar affinity to the membrane bound receptor IL-22R1, both a thousand fold lower than that for IL-22BPi2[185].

2.1.3 Research objectives on IL-22BP and its isoforms

Much of current knowledge about IL-22BP and *IL22RA2* is restricted to genetic associations and biochemical assays, with little information of how it functions in biological systems. Even so, the small number of studies performed *in vitro* and *in vivo* study only exclusively IL-22BPi2; while its antagonism of IL-22 is consistent and reproducible *in vitro*, the divergent effects of IL-22BP on IL-22-driven murine colitis and EAE suggest a more complicated role *in vivo*. Clearly, several questions remain on the functional relevance of IL-22BP and its isoforms.

Furthermore, the presence of multiple isoforms in humans adds an important layer to the study of IL-22BP and how it interacts with IL-22. The roles of IL-22BP isoforms have largely been ignored due to the lack of isoform-specific reagents in humans and splice variants in the mouse; indeed, many present studies employ immunostaining or quantitative PCR approaches that

do not distinguish between the isoforms. Unfortunately, neither genomic variants nor *Il22ra2*$^{-/-}$ mice effects or dominance of these human-specific isoforms. Functionally, pharmacological binding affinity also does not determine biological relevance due to localization of the protein or more complicated receptor-ligand interactions that can cause trans-presentation, as in IL-6 where cis- and trans-activation results in different gene transcription programs. Determining the separate functions of IL-22BP isoforms will be essential for mechanistic insights into the genetic associations linking *IL22RA2* and human disease. Lastly, whether the expression of each isoform is distinctly expressed needs to be determined so that it is clear where the signaling effect of each isoform is dominant.

In this chapter, we identify distinct roles for the IL-22BP isoforms by investigating their molecular and biochemical activities. We demonstrate that IL-22BPi3 is able to functionally block IL-22 signaling, but is more dominant in weak homeostatic control of IL-22 while expression of the stronger antagonist IL-22BPi2 is modulated in inflammatory conditions. By combining function and expression to determine spatio-temporal effects of IL-22BP isoforms on IL-22 signaling, we propose a model in which IL-22BPi2 and IL-22BPi3 collaborate to set the threshold for activation of IL-22 signaling and cooperation with other cytokines in human health and disease.

2.2 RESULTS

2.2.1 IL-22BP isoforms exhibit distinct activities against IL-22 signaling.

The functions of IL-22BPi1 and IL-22BPi3 and their effects on IL-22 signaling remain unknown. As alternative splicing of *IL22RA2* generates structural differences between IL-22BP isoform proteins (**Fig. 2.1, 2.3A**; **Suppl. Fig. 2.1**), we compared their biochemical properties and functions. We over-expressed IL-22BPi1, IL-22BPi2 and IL-22BPi3 and observed expression of all the isoforms in cell lysates, but only IL-22BPi2 and IL-22BPi3 in the supernatants, even after

concentrating supernatant proteins by trichloroacetic acid (TCA) (**Fig. 2.3B**). Treatment of lysates with PNGase F also revealed that all three isoforms are N-linked glycosylated, with double bands for IL-22BPi1 and IL-22BPi2 (**Fig. 2.3C**).

To assess how the IL-22BP isoforms affect IL-22 signaling, we tested the activity of IL-22 in the presence of IL-22BP isoforms. Briefly, we harvested the supernatants from cell cultures overexpressing IL-22BP isoforms and pre-incubated IL-22 with the supernatants to allow association. This mixture was transferred onto Huh7 hepatocytes. To measure downstream signaling, we performed immunoblots for the phosphorylation of STAT3, the main proximal signaling protein activated by IL-22 signaling[88,90,91]. We also measured expression of the IL-22-inducible gene *SOCS3* by real-time quantitative PCR (qPCR). IL-22BPi2 was able to block phosphorylation of STAT3 (**Fig. 2.3D**; **Suppl. Fig. 2.2A**) and IL-22-dependent *SOCS3* induction (**Fig. 2.3E**) in Huh7 cells. Thus, overexpressed IL-22BPi2 effectively blocked IL-22 signaling *in vitro*, confirming prior studies[114,115]. IL-22BPi1 did not significantly alter IL-22 signaling (**Fig. 2.3, D-E**), which was expected given its absence in supernatants. On the other hand,IL-22BPi3, which is found in supernatants, functionally antagonized IL-22 signaling.

2.2.2 Inactive IL-22BPi1 is retained intracellularly due to its third exon.

We were intrigued by the intracellular retention of IL-22BPi1 and its inactivity against IL-22. We observed that the defect was at the stage of secretion as all the isoforms were detected in the cell lysates, but only IL-22BPi1 was absent from the supernatant (**Fig. 2.3B**). Since the coding sequences of IL-22BPi1 and IL-22BPi2 differ only by exon 3, we hypothesized that this sequence could be responsible for IL-22BPi1 retention within the cell. To test this, we sub-cloned exon 3 from IL-22BPi1 into IL-22BPi3-HA to generate an artificial 162 aa open reading frame, termed IL-22BPi3-ex3 (**Fig. 2.4A**). We then transfected this vector, along with those encoding IL-22BPi1

and IL-22BPi3, into cells to determine if this protein is secreted in the supernatant. We found that the insertion of exon 3 was sufficient to retain IL-22BPi3 intracellularly, even though it is normally secreted (**Fig. 2.4B**). We also found that insertion of exon 3 into IL-22BPi3 abolished the suppressive effect of IL-22BPi3 on IL-22 signaling as read out by a STAT3-dependent luciferase reporter (**Fig. 2.4C**; **Suppl. Fig. 2.2B**).

2.2.3 Functional IL-22BP isoforms differentially modulate IL-22 activity.

Overexpression systems do not allow us to control the molarity of IL-22BP isoform proteins provided in each experiment or to measure dose-dependent responses. To accurately compare the dose-dependent effects of human IL-22BP isoforms on IL-22 signaling, we purified recombinant human IL-22BPi3, using a eukaryotic Schneider 2 (S2) cell Drosophila expression system to retain glycosylation. We expressed IL-22BPi3 with a Histidine (6x) tag in the C-terminus under control of a copper (II) ion-inducible metallothionein promoter so that recombinant protein could be harvested and extracted from cell supernatant by affinity purification (**Fig. 2.5, A-C**). We then stimulated Caco-2 intestinal epithelial cells and Huh7 hepatocytes with IL-22 in the presence of recombinant human (rh)IL-22BPi3 in varying molar ratios to IL-22 as a dose curve of its effects on IL-22 activity. The blocking effect of rhIL-22BPi3 was compared against rhIL-22BPi2 applied in the same dose curve. We found that recombinant IL-22BPi3 is able to block IL-22 signaling in a dose-dependent fashion as assayed by phospho-STAT3 immunoblot in Huh7 and Caco-2 cells (**Fig. 2.5D, Table 2.1**). To precisely compare the antagonistic efficacy of the two isoforms, we measured the IC_{50} values of IL-22BPi2 and IL-22BPi3 acting on IL-22-dependent induction of STAT3-dependent luciferase reporter activity (**Fig. 2.5E**); for this constant dose of 10 ng/ml (0.606 nM), IL-22BPi2 had an IC_{50} value of 1.47 nM (2.426-fold molar excess of IL-22, 75.42 ng/ml),

while IL-22BPi3 had an IC_{50} of 40.22 nM (66.37-fold molar excess of IL-22, 58.32 ng/ml,), giving an IC_{50} shift in 27.36 between the two isoforms.

2.2.4 IL-22BPi2 and IL-22BPi3 differentially skew the IL-17/IL-22 axis.

In addition to proximal signaling via STAT3, we investigated the induction of IL-22-dependent effector genes in the presence of IL-22BPi2 and IL-22BPi3. IL-22BPi3 suppressed the induction of IL-22-dependent induction of *SOCS3* in Huh7 cells (**Fig. 2.6A**) as well as *CXCL5*, *DMBT1* and *MUC1* (**Fig. 2.6B**) in Caco-2 cells. As expected from the IC_{50} values, IL-22BPi3 consistently showed weaker antagonistic efficacy than IL-22BPi2 supplied at the same molar ratio against IL-22. Based on the IC_{50} ratio of 27.36 calculated for IL-22BPi2 and IL-22BPi3, we compared the induction of *CXCL5* and *DMBT1* in Caco-2 cells when stimulated in the presence of either 97.2 ng/ml (1 unit, 3.13-fold molar excess of IL-22) of rhIL-22BPi2, 878.9 ng/ml (32 units) of IL-22BPi3 or 1757.9 ng/ml (64 units) of IL-22BPi3 (**Table 2.1**). We found that IL-22BPi3 exhibited similar blockade of *CXCL5* and *DMBT1* induction to IL-22BPi2 when supplied at 32 to 64-fold excess of IL-22BPi2 (**Fig. 2.6C**), corroborating with the ratio of IC_{50} values obtained from the STAT3-dependent luciferase assay.

Many IL-22-producing lymphocytes co-express IL-17, which has overlapping and co-operative functions with IL-22 but is a more potent inducer of pro-inflammatory genes[89,159,188]. Therefore, we assessed if the presence of IL-17 altered the effects of IL-22BP isoforms on IL-22 signaling. We incubated IL-17 and IL-22, individually and in combination, with either IL-22BPi2 or IL-22BPi3 100-fold in excess of IL-22, then added the pre-incubated mixtures to Caco-2 cells to model co-stimulation of epithelial cells in physiological settings. We used *DMBT1* as a readout for IL-22 activity, while β2-defensin (gene symbols *DEFB4A/B*) indicated IL-17-specific activity. In turn, *CXCL5* could be induced by either cytokine. While β2-defensin induction has been shown

in IL-22-stimulated keratinocytes[87,89], we did not observe this induction in Caco-2 (**Fig. 2.6D**). *DMBT1*, *DEFB4A/B* and *CXCL5* were synergistically induced upon stimulations with IL-17 and IL-22. IL-22BPi2 and IL-22BPi3 acted specifically on IL-22 and did not affect IL-17 activity. IL-22BPi2 was able to completely eliminate IL-22 activity while IL-17 signaling was retained. Incubation with IL-22BPi3 resulted again in a partial blockade, which dampened some of the synergy between IL-22 and IL-17 but did not abolish it (**Fig. 2.6D**).

2.2.5 *IL22RA2v3* is the widely expressed isoform during homeostasis.

Given that IL-22BPi2 and IL-22BPi3 differentially modulate IL-22 activity, we surveyed the expression of all IL-22BP isoforms to determine where each signaling mode was dominant. To quantify the expression of *IL22RA2* isoforms in humans, we designed qPCR probes that were specific for each isoform based on their unique exon usage (**Fig. 2.7A**). We obtained similar probe efficiencies and specificity using expression vectors for each isoform and established standard curves for each probe to quantify copy numbers (**Suppl. Fig. 2.3**). We then performed quantitative PCR on a human tissue RNA panel to determine the expression of *IL22RA2* isoforms (**Fig. 2.7B**). We found that *IL22RA2v1* was expressed solely in the placenta. *IL22RA2v2* expression was also highest in the placenta, but it was also detected at low levels in the spleen, stomach, thymus and thyroid gland. In contrast, *IL22RA2v3* expression was widespread across many tissues and at higher copy numbers than the other isoforms even where co-expressed.

To examine if there was any correlation between the expression of any *IL22RA2* isoform and that of local tissue responsiveness to IL-22, we amplified the same tissue RNA for expression of the two membrane-bound receptor chains for IL-22, *IL22RA1* and *IL10RB*. Surprisingly, we found no correlation between the expression levels of any of the IL-22BP isoforms and the two membrane-bound receptor chains (**Suppl. Fig. 2.4**).

2.2.6 *IL22RA2v2* is the only isoform induced by the TLR2-retinoic acid pathway in myeloid cells.

In addition to steady state expression, we sought to identify dominant IL-22BP functions in other immune environments. Myeloid cells were recently shown to be the main sources of IL-22BP in the gut. Huber et al. (2012) showed that expression of *Il22ra2* transcripts in the murine gut was correlated with that of CD11c, a commonly-used dendritic cell marker. Martin et al. (2014) then found IL-22BP was induced during *in vitro* differentiation of primary human monocytes into dendritic cells (DCs), and that expression was enhanced by retinoic acid treatment during differentiation[177]. The same group also identified intestinal eosinophils as an additional source of human IL-22BP[111].

Based on these reports, we used myeloid cells to determine if *IL22RA2* isoforms were differentially regulated. We used undifferentiated THP-1 cells and THP-1 cells differentiated with GM-CSF and IL-4, which have been previously used as a model of DCs *in vitro*[189,190]. We confirmed induction of *IL22RA2* in THP-1 cells upon retinoic acid stimulation, matching prior observations in primary monocyte-derived DCs[177]. Previously, *IL22RA2v1* and *IL22RA2v2* were observed in primary monocyte-derived DCs by reverse-transcriptase PCR[177]. While we detected *IL22RA2v1* and *IL22RA2v3* constitutively at low levels in all treatments, *IL22RA2v2* was the predominant isoform expressed in myeloid cells (**Fig. 2.7C**). Furthermore, when treated with retinoic acid during differentiation, *IL22RA2v2* was also strongly induced by stimulation, followed by marginal increases of *IL22RA2v1*, with minimal effect on *IL22RA2v3* expression.

Retinal dehydrogenase 2 (RALDH2, gene symbol *ALDH1A2*) is an essential component to retinoic acid biosynthesis that is induced by stimulation of the pathogen sensor Toll-like receptor 2 (TLR2)[191,192]. Since retinoic acid enhances *IL22RA2v2* expression, we hypothesized that TLR2 stimulation would induce *IL22RA2v2* through RALDH2 activity (**Fig. 2.8A**). Indeed, stimulation

of THP-1 cells with TLR2 agonist Pam3CSK4 induced the expression of *IL22RA2v2* alongside *ALDH1A2*, with no change in *IL22RA2v1* or *IL22RA2v3* (**Fig. 2.8, B-C**). THP-1-derived DCs, which basally express much higher levels of *ALDH1A2* than their undifferentiated counterparts, also further increased *ALDH1A2* and specifically *IL22RA2v2* expression in response to Pam3CSK4 and lipopolysaccharide derived from *E.coli* 0111:B4 (LPS-EB) that activates both TLR2 and TLR4 (**Fig. 2.8, D-E**; **Suppl. Fig. 2.5A**). Since retinoic acid metabolism is important for multiple immune cell processes, particularly myeloid cell maturation[193-195], we investigated whether *IL22RA2v2* was also induced by TLR2 stimulation of other myeloid cell models. We differentiated HL-60 myeloid cells using dimethyl sulfoxide (DMSO) into a neutrophil model[196-198] and stimulated them with Pam3CSK4 and LPS-EB. Again, while we detected all three isoforms before stimulation, only *IL22RA2v2* was induced by treatment with Pam3CSK4 and LPS-EB (**Suppl. Fig. 2.5, B-C**). Importantly, stimulation of THP-1 monocytes and THP-1 derived DCs in the presence of a pan-retinoic acid receptor (RAR) inverse agonist greatly reduced *IL22RA2v2* induction by Pam3CSK4 stimulation, revealing dependence of this induction on the retinoic acid biosynthesis pathway (**Fig. 2.3, F-G**).

We examined whether induction patterns of *IL22RA2* observed in our myeloid cell models were consistent with expression in primary cells by stimulating $CD14^+$ monocytes and monocyte-derived dendritic cells from healthy donors with Pam3CSK4 over 48 hours (**Fig. 2.3H**). Monocytes did not express *IL22RA2* at 12 hours after stimulation, while *IL22RA2v1* and *IL22RA2v2* were detected at 24-48 hours. On the other hand, monocyte-derived dendritic cells expressed high basal levels of *IL22RA2v1* and *IL22RA2v2* that decreased initially upon Pam3CSK4 stimulation, although recovery was achieved by 48 hours after stimulation. *IL22RA2v3* was not detected in both cell types.

2.3 DISCUSSION

In this study, we identify the distinct properties of the IL-22BP isoforms and their effects on IL-22 signaling, alone and in concert with IL-17. While IL-22BPi2 and IL-22BPi3 differentially modulate IL-22 activity, IL-22BPi1 is retained intracellularly and inactive. We show that IL-22BPi3 is the most abundantly expressed isoform in homeostatic conditions, while IL-22BPi2 alone is induced upon stimulation of myeloid cells. This study sheds light on the properties of human IL-22BP that are not reflected by murine models expressing only the IL-22BPi2 homolog.

We began by defining the biochemical characteristics and cellular localization of the IL-22BP isoforms. Bioinformatics sequence analysis strongly predicts two N-linked glycosylation sites each for IL-22BPi1 and IL-22BPi2 and one site for IL-22BPi3 (**Suppl. Fig. 2.1**), suggesting that the double bands in IL-22BPi1 and IL-22BPi2 lysates arise from alternative glycosylation. Many cytokines and inteferons undergo glycosylation, which enhances their activity and stability. Indeed, IL-22 requires N-linked glycosylation for optimal binding to IL-10R2[199]. We expect that the glycosylation of IL-22BP isoforms is similarly important to their function. Therefore, we used a eukaryotic *Drosophila* expression system to generate glycosylated recombinant IL-22BPi3 and accurately assess signaling effects.

We found that IL-22BPi1 is retained intracellularly by exon 3, which is unique to this isoform. Since insertion of exon 3 into IL-22BPi3 is sufficient to prevent its secretion and ablate its suppressive capacity, lack of secretion is likely the principal reason why IL-22BPi1 fails to significantly alter IL-22 signaling. Previous evolutionary analysis found that exon 3 is a long terminal repeat retrotransposon introduced in the ape lineage [186], which also explains the absence of IL-22BPi1 in rodents. Our data have now identified that a sequence in exon 3 changes the localization of IL-22BPi1 from that of its splice variants and prevents it from modulating IL-22

activity. Indeed, inclusion of exon 3 by alternative splicing may be a regulatory mechanism that diverts functional IL-22BPi2 or IL-22BPi3 expression to favor production of the inactive, intracellular isoform IL-22BPi1 instead. Also interesting is the location of exon 3 immediately upstream of Tyr-67 of IL-22BPi2 (Tyr99 of IL-22BPi1; **Suppl. Fig. 2.1**), which is alone responsible for four of six vital interactions between IL-22 and IL-22BPi2 [115]. Thus, exon 3 has the potential to alter conformation and affinity of the IL-22:IL-22BP bond. However, as IL-22BPi1 did not significantly alter IL-22 activity, any effects of exon 3 on the IL-22:IL-22BP interaction must be overridden by prevention of IL-22BPi1 release and hence opportunity to bind IL-22.

Our data from overexpression systems and recombinant proteins both present compelling evidence that IL-22BPi2 and IL-22BPi3 have differential antagonistic activity against IL-22 signaling. IL-22BPi2 comprises two fibronectin III domains [115], while IL-22BPi3 lacks the C-terminus domain save for 5 frame-shifted residues (**Suppl. Fig. 2.1**). Prior structural analysis has shown that IL-22BPi2 interacts with IL-22 through 7 residues in its N-terminus fibronectin III domain and 3 residues in its C-terminus fibronectin III domain [115,200]. Retention of interactions in the N-terminus domain may explain the ability of IL-22BPi3 to antagonize IL-22 signaling, while the absence of the C-terminus interactions likely causes its weaker strength of blockade compared to IL-22BPi2. Together, our signaling data show that IL-22 signaling can be inhibited by both IL-22BPi2 and IL-22BPi3. However, the relative ability of the isoforms to inhibit IL-22 is different . Thus, alternative splicing of *IL22RA2* to generate IL-22BPi2 and IL-22BPi3 enables graded fine-tuning of the extent of IL-22 antagonism based on exon inclusion under different conditions. Our results provide functional support for the different binding kinetics shown by a previous surface plasmon resonance (SPR) study, which found the dissociation constant for IL-22BPi2 with IL-22 a thousand fold lower than that for IL-22BPi3[185]. Notably, we found an IC_{50} shift of 27.36 between

IL-22BPi2 and IL-22BPi3 using a STAT3-luciferase reporter for biological IL-22-dependent activation. The different ratios found between SPR and our luciferase assay may be due to different sources of recombinant protein; unlike proteins from the *Drosophila* expression system we used, recombinant protein from bacterial sources are not glycosylated and may therefore have different binding properties. They may also reflect the different temperatures at which measurements were performed or competition with the membrane-bound receptor IL-22R1 in a biological setting. While SPR provides insight into the bimolecular interaction, our readout of downstream signaling quantifies the biological function of IL-22BPi2 and IL-22BPi3 and therefore more closely reflects physiological effects. Differential blockade of IL-22 signaling extends to its synergy with IL-17, such that IL-22BPi2 and IL-22BPi3 skew the IL-17/IL-22 axis to different degrees in Th17 environments: complete blockade achieved by IL-22BPi2 favors expression of IL-17-dependent genes, while partial blockade by IL-22BPi3 retains co-operative actions between IL-22 and IL-17. Therefore, the composition of IL-22BP isoforms in an immune microenvironment may have a regulatory effect on the expression profile of inflammatory genes associated with barrier immunity. Existing clinical studies examining IL-22BP in Th17-driven disease have yet to distinguish between the isoforms; it is imperative for future ones to do so, as the relative abundance of each isoform may also reflect an imbalance between the contributions of IL-17 and IL-22 to inflammatory signaling. During these investigations, we also documented a loss of detection of IL-22 protein by enzyme-linked immunosorbent assay (ELISA) in the presence of IL-22BPi2 but not IL-22BPi3 (**Suppl. Fig. 2.6**). Less sensitive detection of IL-22 in the presence of IL-22BPi2 might be due to the blockade of epitopes at the interface. Poor binding of detection or capture antibodies to IL-22 due to interference by IL-22BPi2 may be one explanation why IL-22 has proven difficult to detect in clinical samples by ELISA.

While quantifying expression of *IL22RA2* isoforms in healthy human tissues, we found the first clues that *IL22RA2* isoforms are independently regulated. Previous studies have carried out semi-quantitative expression analyses of *IL22RA2v1* and *IL22RA2v2*, but not *IL22RA2v3*, in human tissues[114,173-176]. Our expression data for *IL22RA2v1* and *IL22RA2v2* concur with previous observations in the placenta, stomach, spleen and thymus[114,173,201]; while significant *IL22RA2v2* expression has also been reported in mammary gland and lung, we did not see this in our samples. We found that *IL22RA2v3* was the most widely expressed isoform among the three, being found in many tissues at high copy numbers compared to *IL22RA2v1* and *IL22RA2v2*. This was surprising as we expected *IL22RA2v2* to be the major isoform based on previous studies, which likely missed these differences by failing to examine *IL22RA2v3* gene expression. Distinct *IL22RA2* isoform expression is likely caused by differences in promoter usage or the modulation of cell type-specific trans-acting factors that affect exon inclusion. Thus, splicing to generate *IL22RA2v3* may be favored by expression of trans-acting factors in secretory cells, which are abundant in tissues where *IL22RA2v3* expression was shown to be highest. Based on the differential biological activity of IL-22BPi2 and IL-22BPi3 against IL-22, we speculate that these isoforms provide spatial and temporal control of IL-22 activity during homeostasis and inflammation. We were surprised that expression levels of *IL22RA2* isoforms did not correlate with those of the membrane-bound receptors for IL-22, since we expected IL-22BP isoforms to be expressed in IL-22-responsive tissues where they could respond quickly to IL-22 activity. This finding suggests that IL-22BP may have few local paracrine signaling effects and instead play a more complex role modulating IL-22 signaling at a distal site. Additional studies should be performed on human tissue samples to examine whether protein expression of the isoforms

correlate well with mRNA expression, which would strongly support the importance of IL-22BPi3-mediated antagonism in homeostatic conditions.

Other groups have shown that myeloid cells are an important source of *IL22RA2* transcripts[111,112,177]. We found that stimulation with retinoic acid, either directly or indirectly through upstream activation of TLR2, induced only *IL22RA2v2* in three different models representing different major populations: THP-1 monocytes, dendritic cells derived from THP-1 and granulocytic neutrophils from HL-60. *IL22RA2v1* and *IL22RA2v3* expression were much lower and largely unaltered in these models. Similarly, *IL22RA2v2* was the principal isoform modulated by TLR2 stimulation in primary monocytes and monocyte-derived DCs, while *IL22RA2v3* was not detected. In primary monocytes, *IL22RA2v1* and *IL22RA2v2* were only induced late at 24 – 48 hours after Pam3CSK4 stimulation, but undetectable at 12 hours after activation; on the other hand, monocyte-derived DCs had high basal expression levels of these isoforms that decreased by 12 hours after stimulation, but recovered over the course of 48 hours. Our results in monocyte-derived DCs concur with previous findings that TLR4 and TLR9 stimulation of these cells results in loss of *IL22RA2* expression[177], while we are the first to observe that expression is increased in primary monocytes stimulated *via* TLR2. The differing kinetics of *IL22RA2* expression in monocytes (late) and monocyte-derived DCs (early) may reflect different physiological niches they occupy. Since IL-18 can be induced downstream of TLR stimulation and contributes to the suppression of IL-22BP in mice[112], differences in its maturation particularly by the NLRC4 inflammasome may contribute to discrepancies between our immortalized and primary monocyte-derived DCs. It is intriguing that *IL22RA2v1* is coexpressed with *IL22RA2v2* in primary cells despite its inactivity; its expression may be one way in which levels of active IL-22BPi2 are controlled.

Combining our data on the functions of exogenous IL-22BP and the regulation of endogenous IL-22BP, we conclude that IL-22BPi2 and IL-22BPi3 are the dominant isoforms actively controlling IL-22 signaling in humans, while IL-22BPi1 is inactive as it is intracellular. Therefore, we propose a model where IL-22BPi2 and IL-22BPi3 collaborate to form an IL-22BP rheostat that tunes IL-22 activity (**Fig. 2.9**). During homeostasis, the rheostat is set low with IL-22BPi3 as the principal isoform regulating IL-22 activity, allowing low level IL-22 signaling and synergy with IL-17. Several tissue sites, such as the placenta, are more resistant to these small, aberrant changes in IL-22 activity in the steady state due to higher expression of IL-22BPi3. Increase of IL-22 levels during bacterial challenge or inflammatory disease is able to overcome this weaker blockade by steady-state IL-22BPi3. However, simultaneous TLR2 stimulation or retinoic acid biosynthesis in response to infection or inflammation would modulate expression levels of the much stronger antagonist IL-22BPi2 in various myeloid populations, causing much larger changes to IL-22 signaling that determine IL-17 dominance in Th17 settings. This rheostat presents a novel method by which IL-22 signaling is stringently controlled along a gradient specifically in humans, with likely impact on systemic IL-22 activity. Our characterization of the context-specific expression of *IL22RA2* isoforms and their differential modulation of IL-22 activity also adds crucial understanding of genetic associations between *IL22RA2* and various inflammatory conditions, which could not otherwise have been identified by genome-wide associations which do not distinguish the contributions of splice variants. Among these, one single nucleotide polymorphism has been identified in the *IL22RA2* 3′UTR, although the authors failed to identify a regulatory element that affected *IL22RA2* expression[202]. Other polymorphisms identified in these genetic association studies are not in the open reading frame. Based on our data,

it would be interesting to identify additional polymorphisms in linkage disequilibrium that could affect the splicing of *IL22RA2* isoforms.

Altogether, our results open avenues to understanding how IL-22BP acts in concert with IL-22 to modulate inflammation, and begin to parse the complex picture of how the genetic association of *IL22RA2* with various inflammatory diseases translates into functions of IL-22BP in patients.

2.4 MATERIALS AND METHODS

Cell culture conditions.

Mammalian cell lines for signaling studies. Caco-2 cells were grown in complete Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 20 % (v/v) heat-inactivated fetal bovine serum (HI-FBS, Atlanta Biologicals, Norcross, GA) and 1X penicillin-streptomycin-glutamine (P/S/G, Mediatech, Manassas, VA), 1X non-essential amino acids (NEAA) and 1mM sodium pyruvate (Gibco, Carlsbad, CA). Huh7 hepatocytes were cultured in DMEM containing 10 % HI-FBS and 1X P/S/G.

Myeloid cells for expression studies. THP-1 monocytes were cultured in complete RPMI containing 10 % (v/v) HI-FBS, 1X P/S/G, 10 mM HEPES, 1X NEAA, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol (Sigma). To obtain THP-1-derived myeloid dendritic cells, THP-1 cells were seeded at 0.25 x 10^6 cells/ml in tissue culture (TC)-treated T25 or T75 tissue culture flasks (Thermo Scientific) and stimulated with 100 ng/ml GM-CSF and 100 ng/ml IL-4 (Shenandoah Biotechnology, Warwick, PA) over 6 days, with half the medium replaced on day 3. HL-60 cells were grown in Iscove's modified Eagle's Medium (Sigma) containing 20 % (v/v) HI-FBS. To differentiate them into a neutrophil model, HL-60 cells were seeded at 0.25 x 10^6 cells/ml in tissue culture-treated T25 or T75 tissue culture flasks in the presence of 1.25 % (v/v) DMSO (Sigma) for

5 days. Medium was replaced on day 3 and cells were diluted to 0.5 x 10^6 cells/ml to maintain their capacity for differentiation. Stimulations were performed with 100 ng/ml Pam3CSK4, 1 µg/ml LPS-EB from E.coli 0111:B4 (Invivogen, San Diego, CA) and 100 mM all-trans retinoic acid (ACROS Organics, NJ) in 12-well TC-treated plates.

Primary monocytes and DCs. Primary cells were thawed and cultured in RPMI 1640 containing 10% (v/v) HI-FBS, 1X P/S/G, 25 mM HEPES and additional 2 mM glutamine. Positively-isolated primary $CD14^+$ monocytes were purchased frozen from a vendor. Monocytes were thawed and seeded at 1 x 10^6/ml in T25 flasks for stimulations. To obtain monocyte-derived DCs, monocytes were seeded at 3 x 10^6/ml in 6-well plates and treated with 178 ng/ml GM-CSF, 30 ng/ml IL-4 and 50 µM 2-mercaptoethanol for 7 days, with half the medium replaced on days 2 and 4. On day 5, monocyte-derived DCs were re-seeded at 1 x 10^6/ml in 6-well plates for stimulations.

Drosophila S2 cells for recombinant IL-22BPi3 expression. S2 cells (Life Technologies, Carlsbad, CA) were cultured in complete S2 medium containing Schneider's Drosophila Medium (Life Technologies), 10 % (v/v) HI-FBS, 1X P/S/G and 20 µg/mL gentamycin (Amresco, Solon, OH). Complete medium was replaced one day after transfection. Cells were selected with 25 µg/mL blasticidin (Invivogen) in complete medium for one passage, and then permanently transferred into ExpressFive Serum-Free medium (Life Technologies) supplemented with 1X P/S/G, 20 µg/ml gentamycin and 25 µg/ml blasticidin. Cultures were grown at 28 °C in a humidified incubator without additional CO_2.

RNA tissue samples. RNA tissue samples were obtained from the Human Total RNA Panel II and Human Mammary Gland Total RNA (Clontech, Mountain View, CA).

RNA extraction and quantitative PCR. RNA was extracted using either the RNEasy Mini kit (QIAgen, Valencia, CA) or the Nucleospin RNA kit (Macherey-Nagel, Bethlehem, PA) and cDNA synthesized using the Quantitect Reverse Transcriptase Kit (QIAgen) according to manufacturers' instructions. Samples were then amplified by qPCR using *Taq*man reagents on the ViiA7 qPCR system (Life Technologies). Gene expression levels for cell lines were normalized to *HPRT* or *GAPDH*, and those for human primary tissue or cellular RNA were normalized to *ACTB* (β-actin). Gene expression was measured by qPCR using *CXCL5*, *DEFB4A, DMBT1, MUC1* and *SOCS3* *Taq*man probes (Life Technologies). Specific qPCR probes for *IL22RA2* isoforms were custom-made (Integrated DNA Technologies) with the sequences listed in Supplementary Table S2.

Transfections and stimulations. The IL-22BP isoforms were cloned on a pUNO backbone (Invivogen) under control of EF1-HTLV promoters and transfected using Xtremegene 9 (Roche, San Francisco, CA) or calcium phosphate transfection (Life Technologies) according to manufacturers' instructions. Additionally, IL-22BPi1, IL-22BPi3 and IL-22BPi3-ex3 were cloned into pC4-FV1E (Clontech) backbones under control of CMV promoters. Supernatants were harvested 24 hours after transfection and incubated for 1 hour in the presence of recombinant cytokines prior to stimulation.

Untransfected cells were serum-starved for 4-6 hours before medium was replaced with supernatants containing cytokines pre-incubated with overexpressed or recombinant IL-22BP. Cells were stimulated with 10 ng/ml IL-22 (Peprotech, Rocky Hill, NJ), 10 ng/ml IL-17 (Shenandoah Biotechnology) or both. Whole cell lysates were harvested after 15 min of stimulation for phospho-STAT3 immunoblotting and after 24 hours for gene induction. Luciferase

reporter cells were transfected in 48-well plates using Xtremegene with a constitutively active eGFP vector and a luciferase reporter with a STAT3-responsive promoter (pGL4.47, Promega, Madison, WI) 24 hours before serum starvation.

Purification of recombinant IL-22BPi3. S2 cells were stably co-transfected with plasmids encoding IL-22BPi3-Histag and a blasticidin resistance gene in a ratio of 19:1 using calcium phosphate. Cell cultures were expanded in serum-free medium under constant blasticidin selection to a 500 ml culture suspended at 125 r.p.m. When density exceeded 5.0 x 10^6 cells/ml, we induced IL-22BPi3 expression with 0.8 mM $CuSO_4$ over 8 days. Recombinant IL-22BPi3 was isolated from the filtered supernatant by affinity chromatography onto Ni^{2+}-NTA resin (Thermo Pierce, Rockford, IL) and eluted in an imidazole (Sigma) gradient. Enriched eluate fractions were identified by SimplyBlue Safestain (Life Technologies), concentrated by ultracentrifugation and desalted by dialysis in PBS. Single-use aliquots were frozen in 20 % glycerol and 0.1 % BSA.

Coomassie staining and immunoblotting. 15-30 µg of cell lysates, 20 µL of untreated supernatant, or 1 ml of supernatant concentrated by TCA (Sigma) was subjected to SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, Waltham, MA). After blocking in 5 % (w/v) non-fat milk dissolved in Tris buffer saline containing Tween-20 (Sigma), staining was performed in either 5 % milk or 5 % bovine serum albumin (BSA, Sigma) for phospho-STAT3 (Tyr705), total STAT3, β-actin (13E5) (Cell Signaling, Danvers, MA) or IL-22BP (R&D Systems, Minneapolis, MN). Coomassie staining was performed on SDS-PAGE gels with SimplyBlue SafeStain and destained overnight in 20 % (w/v) NaCl.

ELISA. ELISA was performed using the IL-22 Quantikine kit (R&D Systems) and the IL-22 LEGEND MAX kit (Biolegend, San Diego, CA) according to the manufacturers' instructions. Samples containing IL-22 only or IL-22 in the presence of rhIL-22BP were preincubated for 1 hour in PBS before application to assay plates.

Statistics. Statistics were performed for quantitative assays in GraphPad Prism using one-way analysis of variance (ANOVA) with multiple comparisons against IL-22 stimulated samples, except in Fig. 2.2H where comparisons are made against IL-17 and IL-22 co-stimulated samples.

2.5 FIGURES

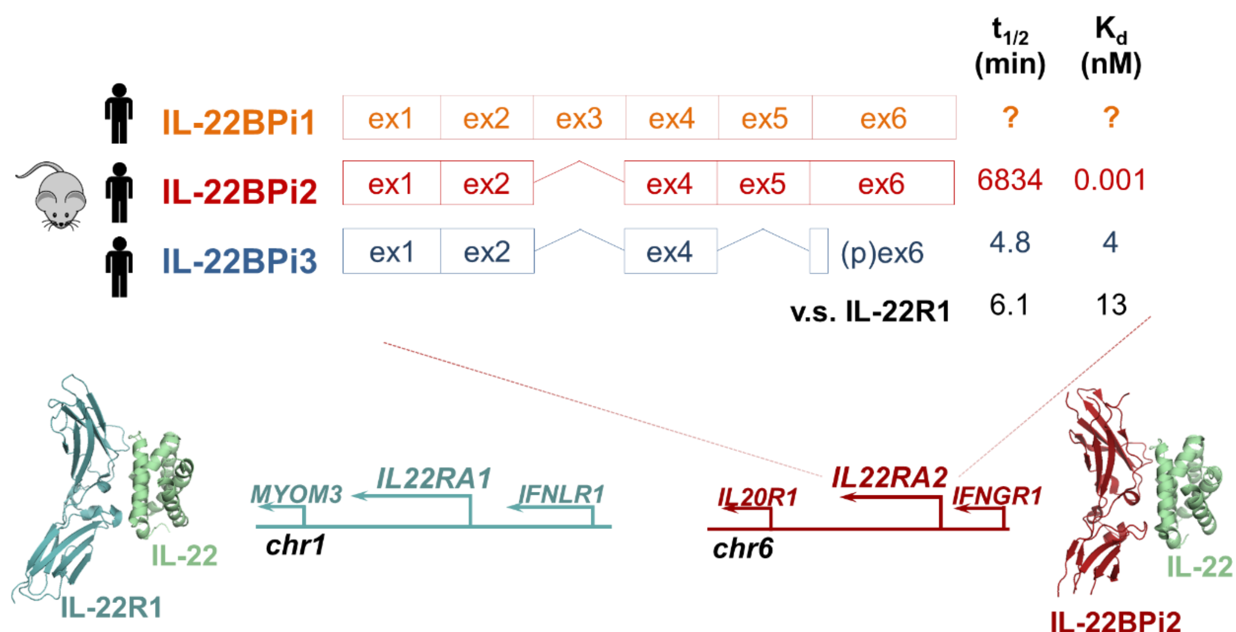


Figure 2.1. Known characteristics of IL-22 binding protein (IL-22BP) isoforms.

IL-22BP, encoded by *IL22RA2*, is a soluble receptor for IL-22 expressed as three alternatively spliced transcripts in humans, of which only IL-22BPi2 is found in mice. IL-22BPi2 and IL-22BPi3 have different binding affinities for IL-22 determined by surface plasmon resonance. IL-22BPi2 has a similar three-dimensional structure to the membrane bound receptor IL-22R1, which is encoded on a different gene and chromosome. The locations of *IL22RA1* and *IL22RA2* on different chromosomes contrast many cytokine receptor soluble/transmembrane pairs, which are usually encoded on the same gene and generated by alternative splicing or enzymatic cleavage.

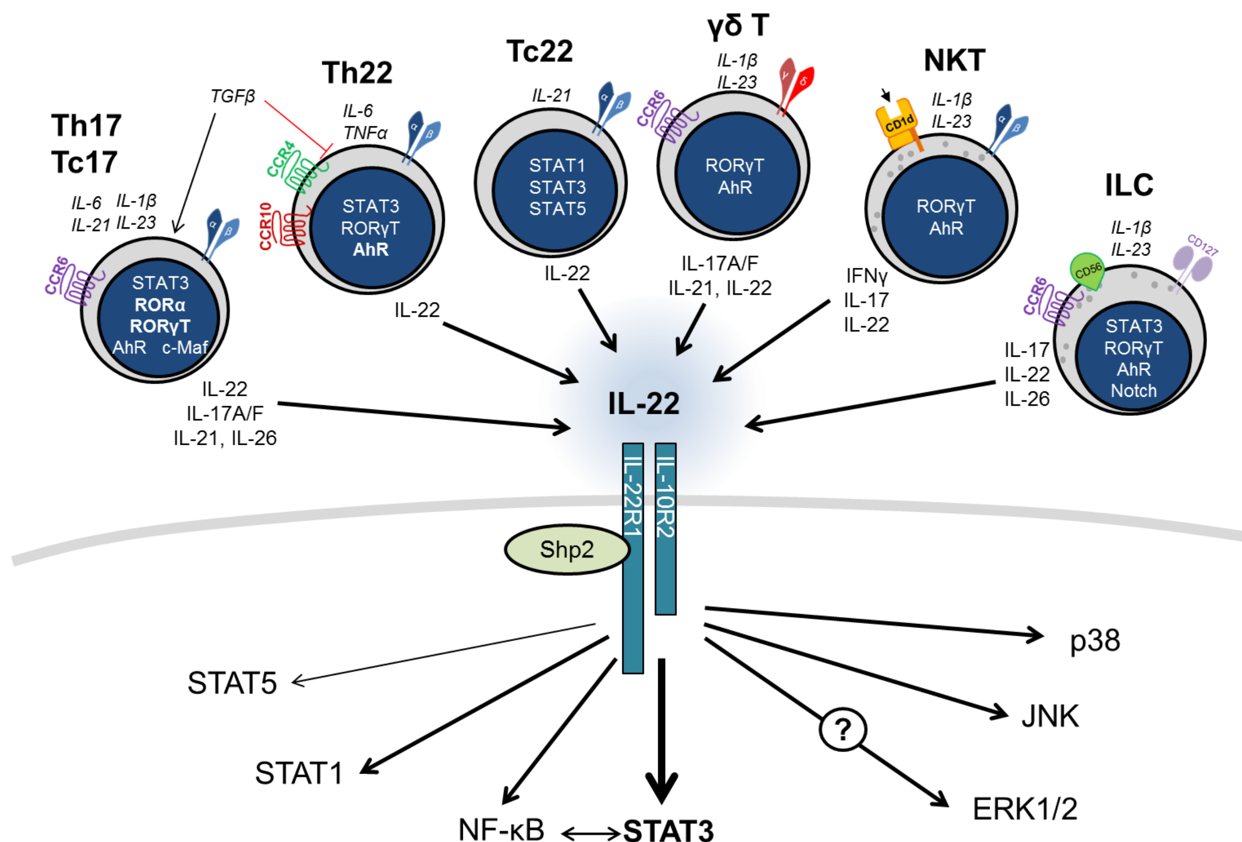


Figure 2.2. Sources of IL-22 and its principal downstream signaling pathways.

IL-22 is produced by several populations of lymphoid cells, largely under control of STAT3, RORα, RORγT and aryl hydrocarbon receptor (AhR). These activate signaling via the receptor comprising IL-22R1 and IL-10R2 that is expressed exclusively on non-immune cells under normal conditions, including hepatocytes, keratinocytes and epithelial cells of the colon and lung. Principal functions of IL-22 signaling are mediated through STAT3 activation, although activation of STATs 1 and 5, MAP kinases, NFκB, p38 and JNK have also been reported. STAT3 constitutively associates with IL-22R1 and rapidly responds to phosphorylation of IL-22R1 by Shp2 following IL-22 stimulation.

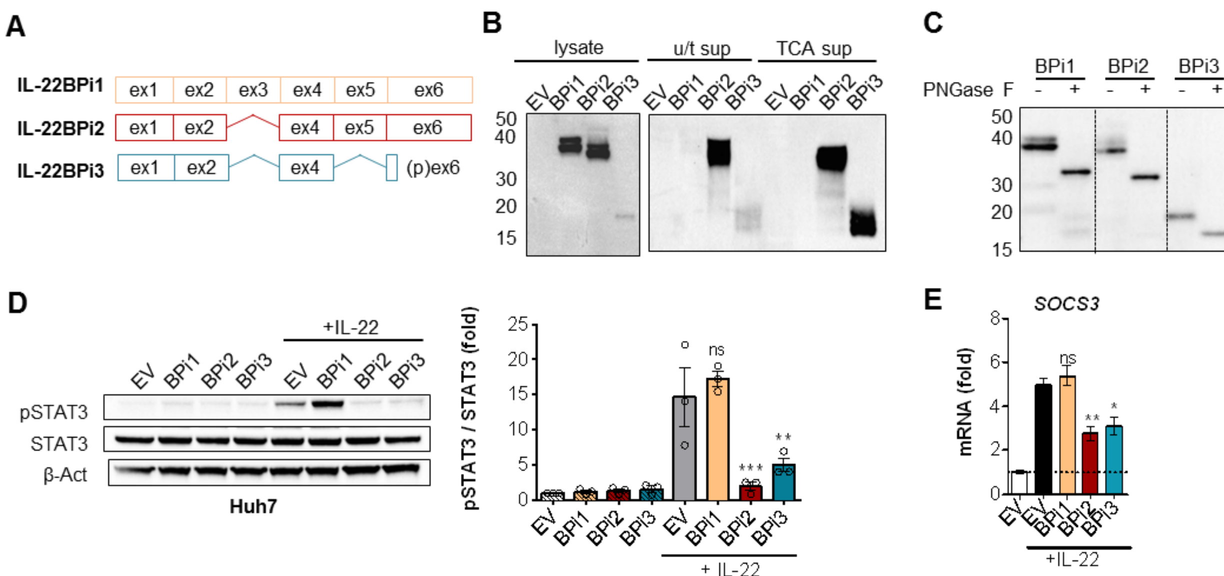


Figure 2.3. IL-22BP isoforms differentially modulate IL-22 activity when overexpressed.

(**A**) Schematic showing exon usage in the coding sequences of IL-22BPi1, IL-22BPi2 and IL-22BPi3 generated by alternative splicing. (**B**) Immunoblot of IL-22BPi1, IL-22BPi2 and IL-22BPi3 in transfected Huh7 cell lysate, untreated cell culture supernatant and supernatant concentrated by trichloroacetic acid (TCA). (**C**) Immunoblot of lysates from IL-22BP isoform-overexpressing cells treated with Peptide-N-Glycosidase F (PNGase F) to determine their N-linked glycosylation. (**D**) Effects of overexpressed IL-22BP isoforms on IL-22 signaling measured by immunoblot of STAT3 phosphorylation 15 min post-incubation in hepatocyte cell line Huh7, with total STAT3 and β-actin used as loading controls for normalization for densitometry. (**E**) Effects of overexpressed IL-22BP isoforms on IL-22 signaling measured by qPCR of *SOCS3* in Huh7 cells 24 hours post-incubation, normalized to endogenous control *HPRT*. (**B,D,E**) Data are representative of 2-3 independent experiments and presented as mean ± s.e.m. ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

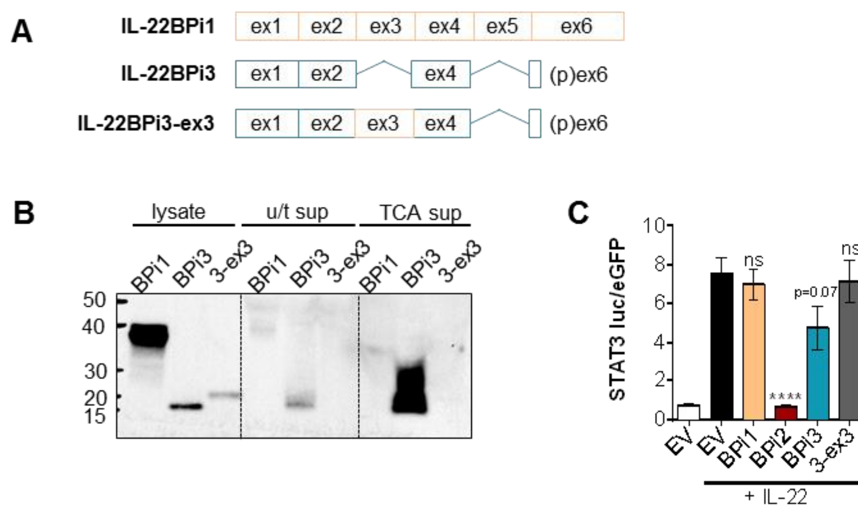


Figure 2.4. IL-22BPi1 is inactive due to intracellular retention by inclusion of exon 3.

(**A**) Schematic showing IL-22BPi3-ex3 protein created by sub-cloning exon 3 from IL-22BPi1 into the open reading frame of IL-22BPi3. (**B**) Immunoblot of IL-22BPi1, IL-22BPi3 and IL-22BPi3-ex3 in transfected Huh7 lysate, untreated supernatant and TCA-treated supernatant. (**C**) Effect of overexpressed IL-22BPi1, IL-22BPi3 and IL-22BPi3-ex3 in supernatants on IL-22 activity 6 hours post-incubation in Huh7 cells, measured by luciferase reporter activity downstream of a STAT3-responsive promoter. (**B**,**C**) Data are representative of 2-3 independent experiments and presented as mean ± s.e.m. ns = not significant.

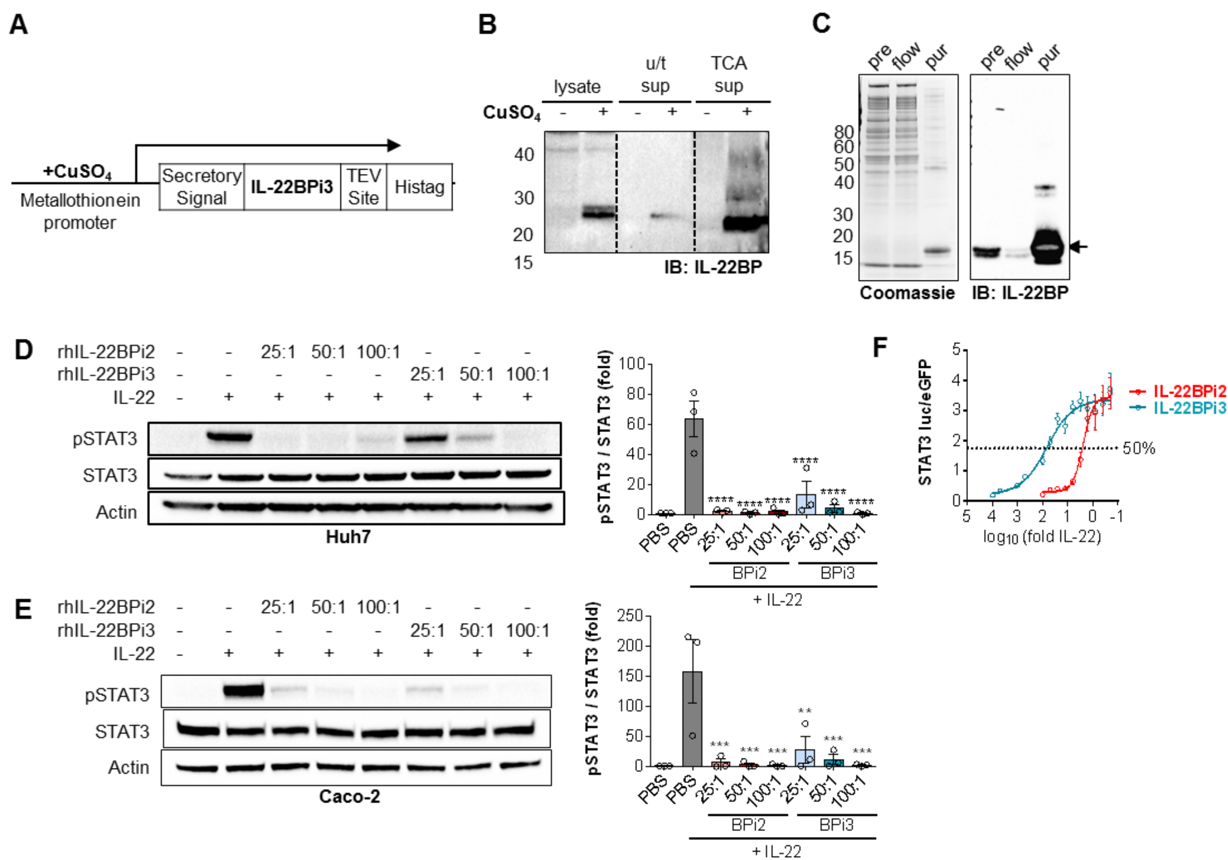


Figure 2.5. Recombinant human IL-22BPi2 and IL-22BPi3 proteins modulate IL-22 activity.

(**A**) Schematic showing IL-22BPi3-encoding construct with a 6xHis tag driven by a metallothionein promoter, which was stably transfected into an S2 Drosophila expression system for secretion. (**B**) Immunoblot of IL-22BPi3 in lysate and supernatant of S2 cells induced by copper (II) sulfate. (**C**) Detection of IL-22BPi3 before and after isolation via Ni^{2+}-NTA affinity chromatography by Coomassie stain and immunoblot. (**D,E**) Effects of increasing doses of recombinant IL-22BPi2 or IL-22BPi3 on IL-22 signaling in Huh7 hepatocytes and Caco-2 colonic epithelial cells measured by activation of STAT3 15 min post-incubation. Concentrations are expressed as the molar ratios of IL-22BP:IL-22. Total STAT3 and β-actin were used as loading controls for normalization in densitometry. (**F**) IC_{50} curves of IL-22BPi2 and IL-22BPi3 for their blockade of IL-22 activity 6 hours post-co-incubation in Huh7 cells, measured by luciferase reporter activity downstream of a STAT3-responsive promoter. (**D-F**) Data are representative of 2-4 independent experiments and are presented as mean ± s.e.m. ns = not significant, ** p<0.01, *** p<0.001, **** p<0.0001.

Figure 2.6. Recombinant human IL-22BPi2 and IL-22BPi3 proteins modulate the Th17 effector response.

(**A,B**) Effects of increasing doses of recombinant IL-22BPi2 or IL-22BPi3 on induction of *SOCS3* in Huh7 cells (**A**) and *CXCL5*, *DMBT1* and *MUC1* in Caco-2 cells (**B**) 24 hours after IL-22 stimulation. (**C**) Effects of recombinant IL-22BPi2 and IL-22BPi3 on induction of *CXCL5* and *DMBT1* in Caco-2 cells 24 hours after IL-22 stimulation, when IL-22BPi3 is supplied at 32 or 64-fold molar excess to IL-22BPi2. (**D**) Effect of 100-fold molar excess of IL-22BPi2 and IL-22BPi3 on synergistic induction of IL-22-dependent *DMBT1,* IL-17-dependent *DEFB4A/B* and IL-17 or IL-22-inducible *CXCL5* in Caco-2 cells. Each IL-22BP isoform was applied to IL-17-only and IL-22-only stimulations as well as costimulations of IL-17 and IL-22. (**A-D**) *HPRT* used as endogenous control. Data are representative of 2-4 independent experiments and are presented as mean ± s.e.m. ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

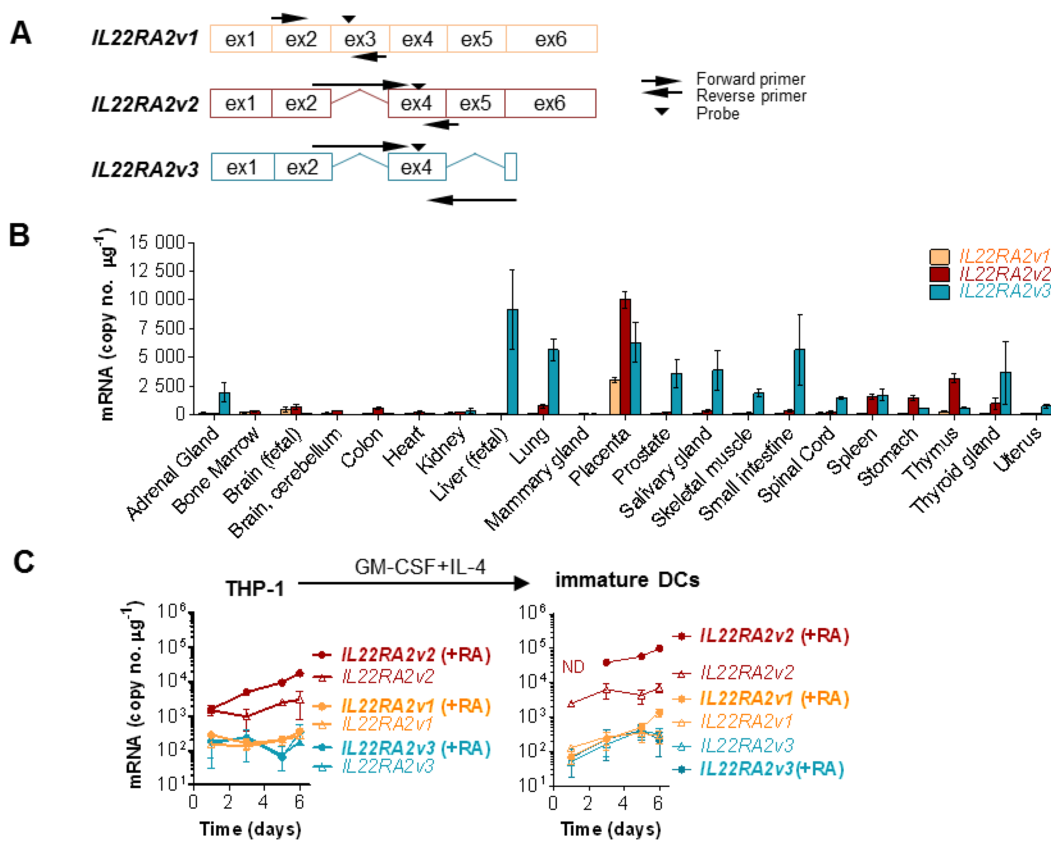


Figure 2.7. ***IL22RA2v3*** **is the most widely expressed isoform, while** ***IL22RA2v2*** **is the principal isoform induced by retinoic acid.**

(**A**) Schematic showing the gene structure encoding the three IL-22BP protein isoforms (*IL22RA2v1*, *IL22RA2v2* and *IL22RA2v3*). Specific Taqman primers and probes that exploit the unique exon/intron configurations in each isoform are indicated by arrows.

(**B**) Expression of *IL22RA2v1*, *IL22RA2v2* and *IL22RA2v3* were measured and quantified by qPCR in 21 human tissues. Copy numbers of *IL22RA2* isoforms were obtained from standard curves in Suppl. Fig. 2.3A.

(**C**) THP-1 cells were used as undifferentiated monocytes or differentiated into immature DCs using GM-CSF and IL-4 over 6 days. Expression of each *IL22RA2* isoform was quantified by qPCR in the presence or absence of retinoic acid stimulation. Data are representative of 2 experiments and presented as mean ± s.e.m.

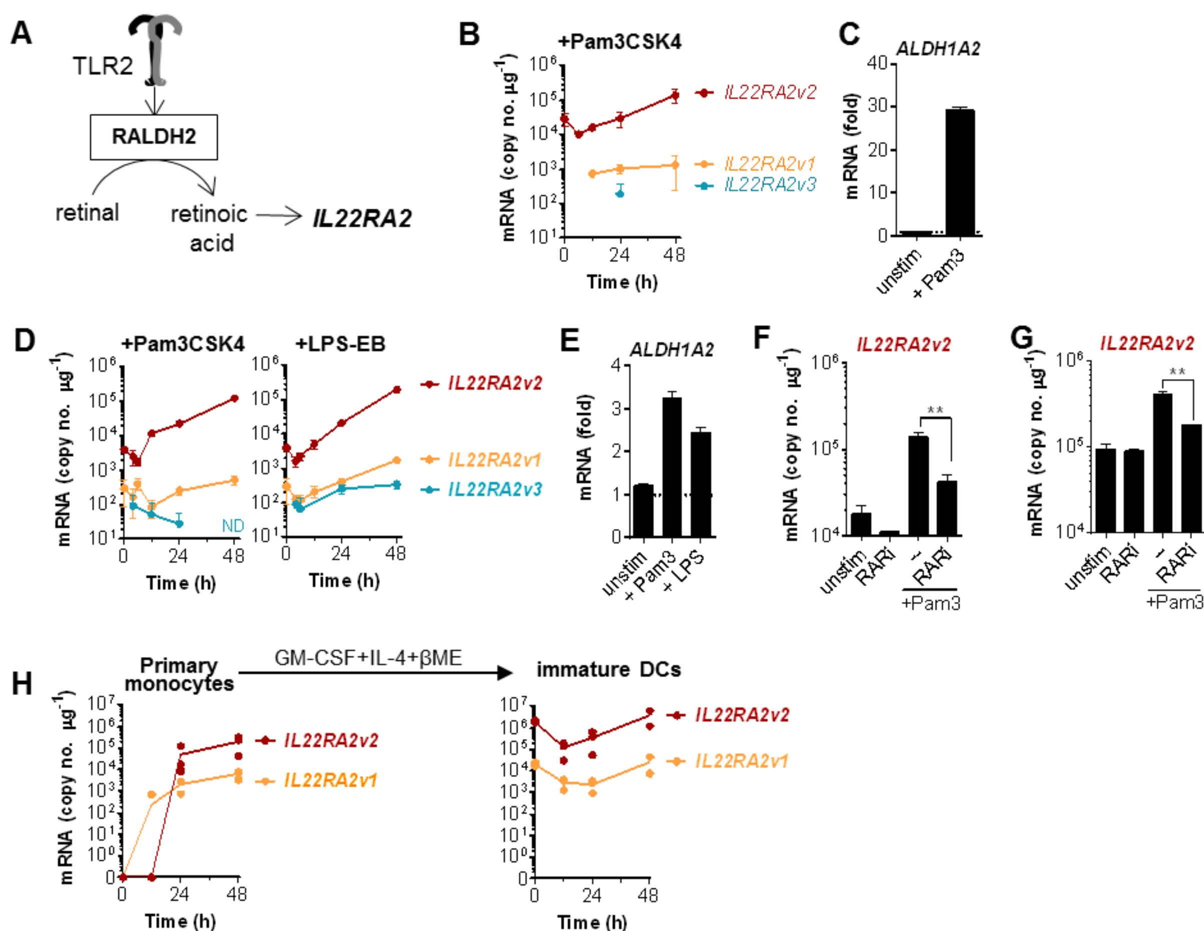


Figure 2.8. ***IL22RA2v2*** **is the principal isoform modulated by TLR2** ***via*** **retinoic acid biosynthesis.**

(**A**) Schematic of pathway linking IL-22BP induction with TLR2 stimulation. TLR2 induces RALDH2 (*ALDH1A2*) expression and activity, increasing retinoic acid biosynthesis and in turn inducing *IL22RA2* expression. (**B**,**C**) Undifferentiated THP-1 were stimulated with TLR2 ligand Pam3CSK4. (B) Expression of *IL22RA2* isoforms was measured over 48 hours post-stimulation. (**C**) Expression of *ALDH1A2* by qPCR 12 hours post-stimulation. (**D**,**E**) THP-1-derived DCs were stimulated via TLR2 with Pam3CSK4 and LPS-EB. (D) Expression of *IL22RA2* isoforms measured over 48 hours post-stimulation. (**E**) Expression of *ALDH1A2* 12 hours post-stimulation. (**F**, **G**) Undifferentiated THP-1 cells (**F**) and THP-1-derived DCs (**G**) were stimulated with Pam3CSK4 in the presence or absence of pan-RAR inverse agonist BMS 493 for 48 hours and *IL22RA2v2* expression measured. (**H**) Primary $CD14^{+}$ monocytes and monocyte-derived DCs were stimulated over 48 hours with Pam3CSK4 and expression of each IL22RA2 isoform was quantified. *IL22RA2v3* was not detected. (**C**,**E**) *HPRT* was used as an endogenous control. (B-G) Data are representative of 2 experiments and presented as mean ± s.e.m. (**H**) Data from three donors are displayed as individual symbols with connecting lines representing means. ** p<0.01. ND, not detected.

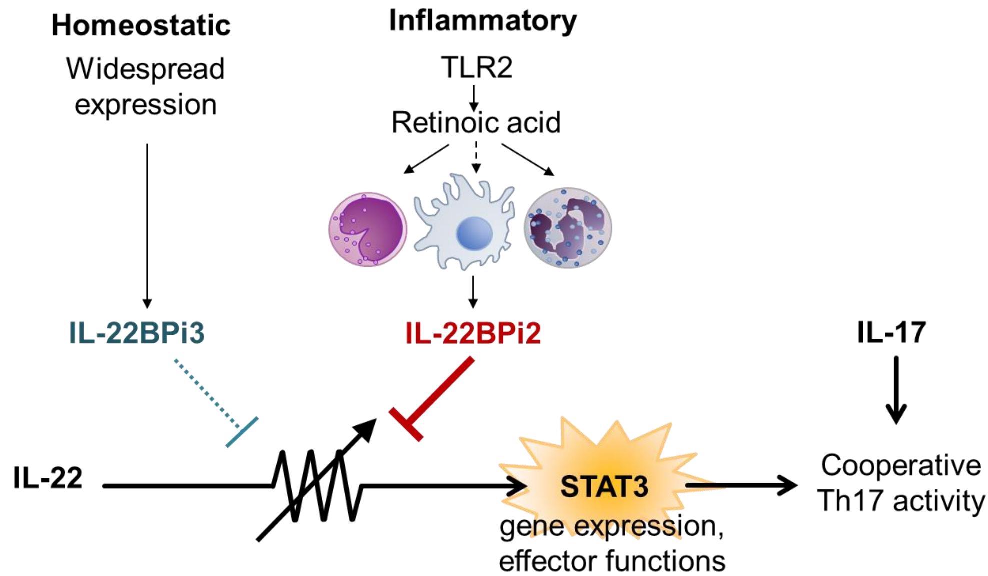
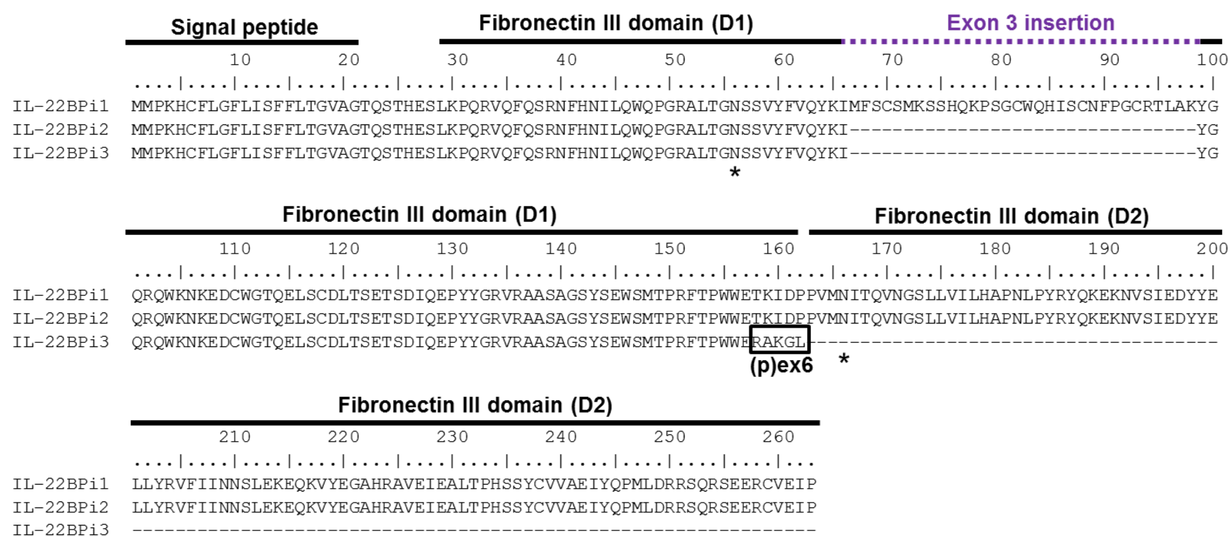


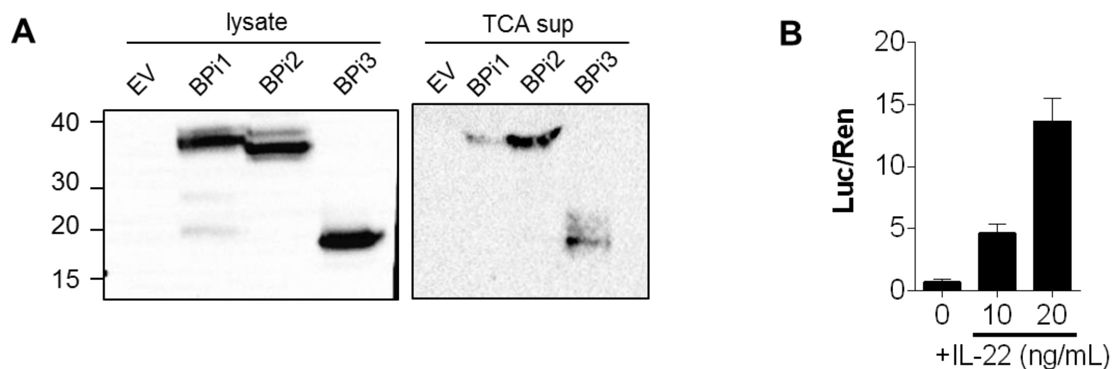
Figure 2.9. IL-22BPi2 and IL-22BPi3 collaborate to form a rheostat that tunes IL-22 activity.

During homeostasis, IL-22BPi3 is widely expressed, preventing aberrant IL-22 signaling while allowing low level activation and synergy with IL-17 essential to important barrier immunity and regenerative functions. When inflammation activates the TLR2-retinoic acid pathway, only IL-22BPi2 is modulated for its strong antagonism of IL-22 activity. The balance of isoform expression forms a gradient which controls STAT3 activation downstream of IL-22 activation and subsequent gene expression, effector functions and Th17 activity.



Suppl. Figure 2.1. Alignment of IL-22BPi1, IL-22BPi2 and IL-22BPi3 protein sequences.

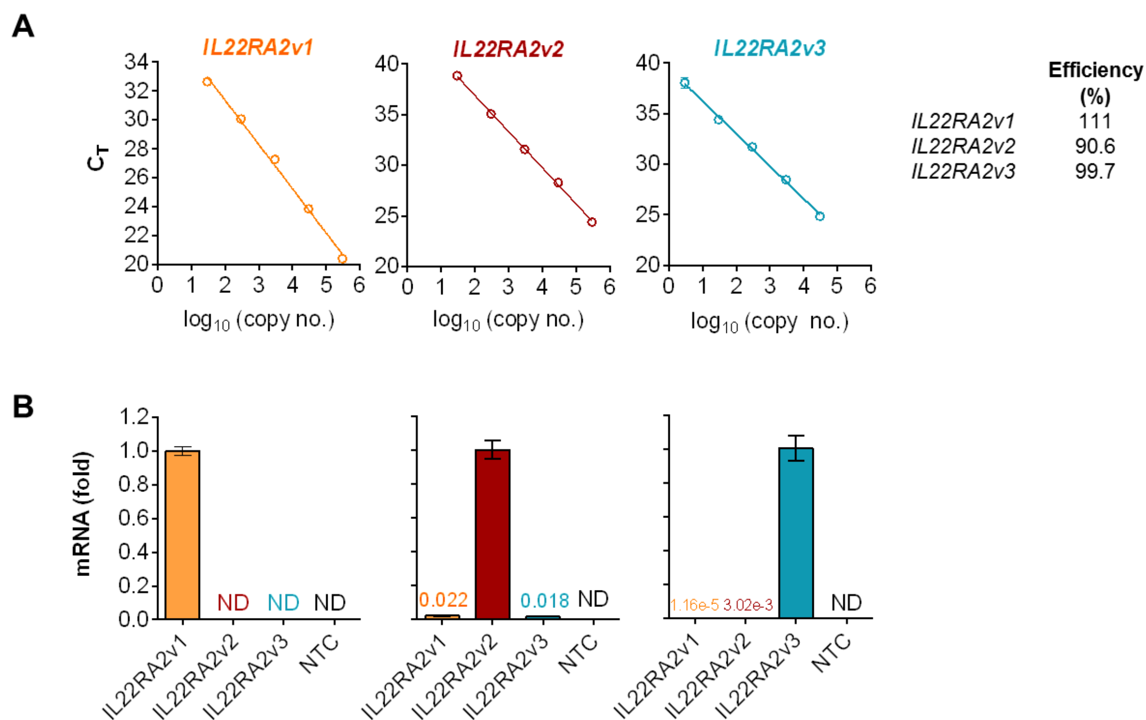
Alignment was performed using CLUSTALW in BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences are provided from NCBI (accessions NP_443194, NP_851827, NP_851826 respectively) and Uniprot (accession Q969J5). Major protein domains are annotated as in Uniprot and based on crystallography by de Moura et al. [115]. Asterisks indicate N-glycosylation sites strongly predicted by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).



Suppl. Figure 2.1. Expression of IL-22BP isoforms in phospho-STAT3 immunoblot assays and optimization of dose for STAT3 luciferase reporter activity.

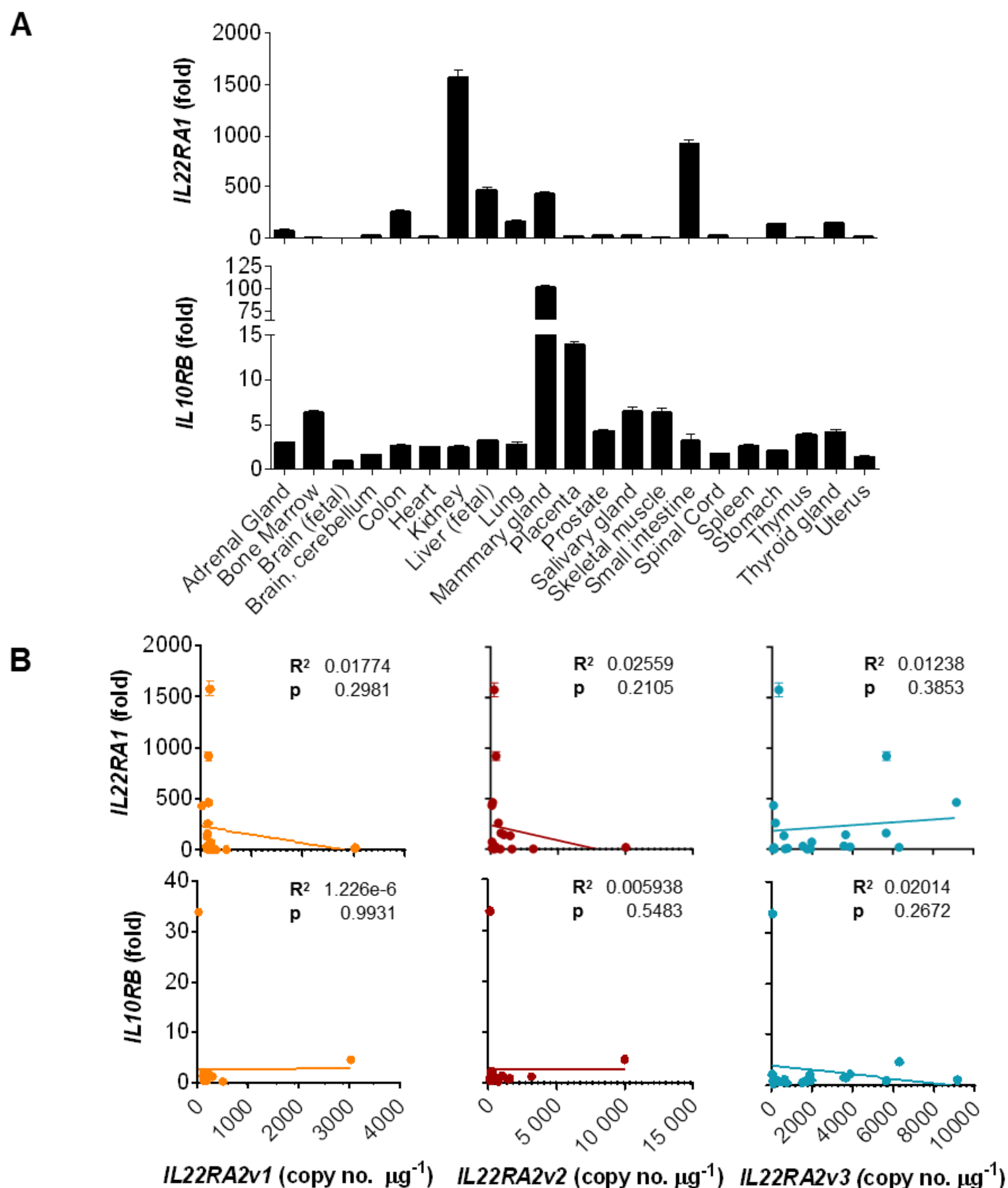
(**A**) Lysate and supernatant overexpression of IL-22BPi1, IL-22BPi2 and IL-22BPi3 in Huh7 cells 24h after transfection, alongside empty vector (EV) control. These correspond to the representative experiment in Fig. 2.3D.

(**B**) Optimization of dose for STAT3 luciferase reporter activity 6 hours after IL-22 stimulation in HepG2 hepatocytes. Firefly luciferase activity was normalized against co-transfected *Renilla* luciferase activity.



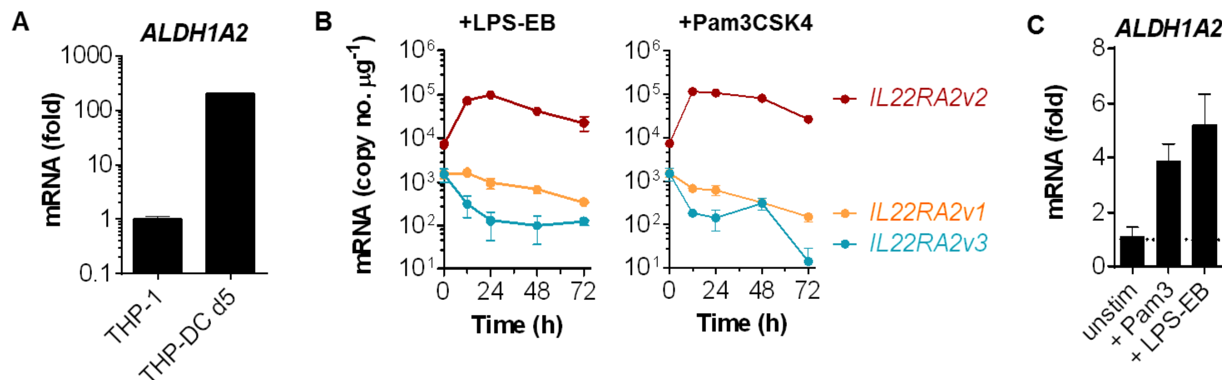
Suppl. Figure 2.2. Efficiency and specificity of the *IL22RA2* Taqman probes.

(**A**) Standard curves were obtained by amplifying known copy numbers of vectors expressing each isoform of *IL22RA2*. Efficiency of each probe is provided in accompanying table. (**B**) 3×10^5 copies of expression vectors for *IL22RA2v1, IL22RA2v2 and IL22RA2v3* were each amplified with all three sets of qPCR probes to verify their specificity for each isoform.



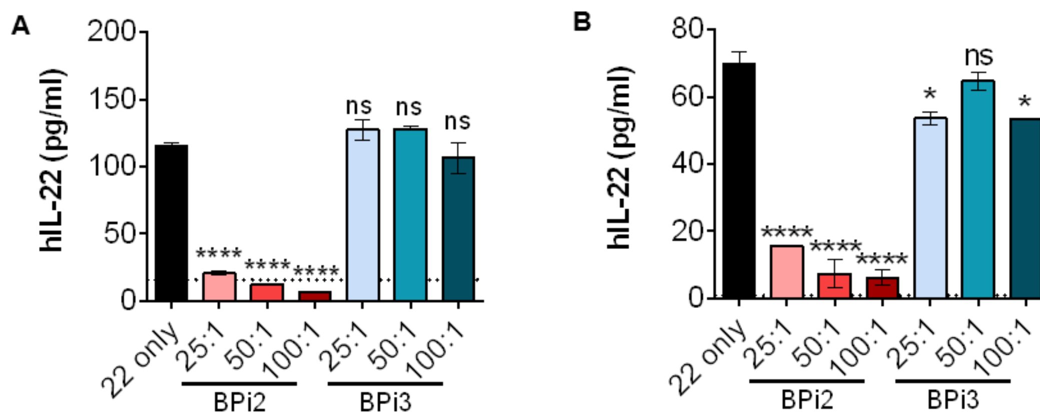
Supplementary Figure 2.3. Correlation between expression levels of IL-22 receptor and *IL22RA2* isoforms.

(**A**) Expression of *IL22RA1* and *IL10RB* were measured by qPCR in 21 human tissues and plotted as fold difference from expression in fetal brain. *ACTB* was used as an endogenous control. (**B**) Linear regression analysis was performed to assess correlation between *IL22RA2* isoform copy number and expression of *IL22RA1* or *IL10RB*. R^2 and p (slope ≠ 0) values are inset for each graph. ND, not detected. NTC, no template control. Data are represented as mean ± s.e.m.



Suppl. Figure 2.4. *IL22RA2* and *ALDH1A2* expression in HL-60-derived neutrophils, THP-1 and derivative DCs

(**A**) Basal expression of *ALDH1A2* in undifferentiated THP-1 cells and THP-1-derived DCs after 5 days of differentiation. (**B**,**C**) HL-60 cells were differentiated over 5 days with DMSO into a neutrophil model and stimulated with Pam3CSK4 and LPS-EB. (**B**) Expression of *IL22RA2* isoforms measured over 48h post-stimulation. (**C**) Expression of *ALDH1A2* 12h post-stimulation.



Suppl. Figure 2.5. Effects of IL-22BPi2 and IL-22BPi3 on IL-22 ELISA.

Detection of human IL-22 by ELISA after incubation with or without rhIL-22BPi2 or rhIL-22BPi3 in varying molar ratios of IL-22BP:IL-22. Assays were performed using (**A**) R&D's Quantikine ELISA kit and (**B**) Biolegend's LEGEND MAX ELISA kit for human IL-22. **** $p < 0.0001$, * $p < 0.05$, ns = not significant.

2.6 TABLES

Table 2.1 Concentrations of rhIL-22BP used as 25:1, 50:1 and 100:1 excess of IL-22.

IL-22BP:IL-22 ratio	IL-22 / ng ml^{-1} (16.5 kDa monomer)	rhIL-22BPi2-Fc / ng ml^{-1} (51.3 kDa)	rhIL-22BPi3-Histag / ng ml^{-1} (14.5 kDa)
3.12:1	10.0	97.2	27.5
25:1	10.0	777.3	219.7
50:1	10.0	1554.6	439.5
100:1	10.0	3109.1	878.9
25:1	20.0	1554.6	439.5
50:1	20.0	3109.1	878.9
100:1	20.0	6218.2	1757.9

Table 2.2. Sequences of the qPCR probes and primers used to quantify *IL22RA2* isoforms.

Transcript	Primer	5′ - Sequence - 3′
IL22RA2v1	Forward	ATTTTGCAATGGCAGCCTG
	Probe	TCTCACCAGAAGCCAAGTGGATGC
	Reverse	GCCTGGGAAGTTACAAGAAATG
IL22RA2v2	Forward	GTGCAGTACAAAATATATGGACAGA
	Probe	CTCGGCTGGGAGCTACTCAGAAT
	Reverse	GAGGATCTATTTTTGTTTCCCACC
IL22RA2v3	Forward	GTGCAGTACAAAATATATGGACAGA
	Probe	CTCGGCTGGGAGCTACTCAGAAT
	Reverse	CTTTTGCTCTTTCCCACCAG

Chapter 3. EVOLUTIONARY ADAPTATIONS SUPPRESS IFNλ4 EXPRESSION DURING VIRAL INFECTION.

Parts of the following work has been submitted for publication by the authors MeeAe Hong, Johannes Schwerk, Chrissie Lim, Alison Kell, Abigail Jarret, Joseph Pangallo, Yueh-Ming Loo, Shuanghu Liu, Curt Hagedorn, Michael Gale Jr. and Ram Savan.
MeeAe Hong, Johannes Schwerk and Chrissie Lim contributed equally as co-first authors.

Interferon lambdas are critical antiviral effectors in hepatic and mucosal infections. While IFNλ1, IFNλ2 and IFNλ3 act antiviral, genetic association studies have shown that expression of the recently discovered *IFNL4* is detrimental to hepatitis C virus (HCV) infection through a yet unknown mechanism. Intriguingly, human *IFNL4* harbors a genetic variant that introduces a premature stop codon. We performed a molecular and biochemical characterization of IFNλ4 to determine its role and regulation of expression. We found that IFNλ4 exhibits similar antiviral activity to IFNλ3 without negatively affecting antiviral IFN activity or cell survival. We show that humans deploy several mechanisms to limit expression of functional IFNλ4 through non-coding splice variants and non-functional protein isoforms. Furthermore, protein-coding *IFNL4* mRNA are not loaded onto polyribosomes and lack a strong polyadenylation signal, resulting in poor translation efficiency. This study provides mechanistic evidence that humans have sustained adaptations to suppress IFNλ4 expression, suggesting that immune function is dependent on other *IFNL* family members.

3.1 INTRODUCTION

3.1.1 Type III IFNs (IFNλs) in antiviral immunity

Interferons (IFNs) are cytokines central to innate immune responses. They have been categorized as type I (e.g. IFN-α, β, ω), type II (IFN-γ) and type III (IFN-λ). Each category occupies a different niche in the immune response as they interact with distinct families of receptors on different cells, tissues and organs. The most recently discovered family, the type III IFNs, are encoded on in close proximity on the human *IFN lambda* (*IFNL*, IFNλ) locus on chromosome 19, composed of *IFNL1 (IL29)*, *IFNL2 (IL28A), IFNL3 (IL28B)* and *IFNL4* genes located on chromosome 19[203]. Of these members, IFNλ4 has been most recently discovered and most studies to date characterize IFNλ1, IFNλ2 and IFNλ3. The latter IFNs signal through a heterodimeric receptor composed of IFNλR1 and IL-10R2 chains that activates the Jak/STAT pathway to induce IFN stimulated genes (ISGs) and antiviral activity. Among them, IFNλ3 has the greatest antiviral activity, at 2-fold higher than IFNλ1 and 16-fold higher than IFNλ2[204,205].

While type I and III IFNs share many antiviral functions, including activation of signal transducer and activator of transcription (STAT)1, STAT2, IRF3, IRF7 and IRF9, the type I IFN receptor chains (IFNαR1 and IFNαR2) are nearly ubiquitously expressed while IFNλR1 expression is limited to hepatocytes, epithelial cells of the lung, intestine, skin and cells of myeloid lineage so that the type III IFN response is far more tissue-restricted[203,206,207]. Other distinctions between the type I and III IFN pathways are slowly coming to light, including the strength of signaling, kinetics of activation and mediators of upstream induction. To begin, initial type III IFN activity is generally of lower strength and slightly delayed compared to type I IFN, but expression and activity are far more stable, making them the principal mediators of long-term antiviral activity[208-211]. Also, while the STAT, ISG and antiproliferative signatures activated by type I and

III IFNs are highly similar [205,212-215], IFNλ3 uniquely activates Janus kinase 2 (JAK2) for phosphorylation of STAT1 or 2 [216]. Furthermore, type I and III IFN induction via viral sensing by MAVS may occur through distinct pathways depending on where MAVS is localized, although opposing results have been found by different groups[216,217]. Thus, type III IFNs have non-redundant functions from type I IFNs in antiviral immunity.

IFNλ-mediated immunity is essential to fight viral infections in the liver and at epithelial surfaces. *Ifnlr1*$^{-/-}$ mice show that IFNλ activity is required for antiviral protection against respiratory viruses including the influenza virus and the severe acute respiratory syndrome (SARS) coronavirus[218,219]. In turn, human IFNλ is the dominant IFN secreted by respiratory epithelial cells in response to influenza virus infection[220,221] and is also produced by myeloid and lung epithelial cells during rhinovirus infection[222]. Similar to the respiratory tract, epithelial cells of the gastrointestinal tract are predominantly responsive to IFNλ[219,223], which initiates antiviral signaling critical for control of pathogenic enteric viruses[223-225], rather than type I IFN. Uniquely among the IFNλs, IFNλ1 is also expressed by T helper 17 (Th17) cells and is the only IFN associated with antiviral gene expression in psoriatic lesions[226] Although these studies underscore the importance of IFNλs in antiviral immunity, the expression, regulation and activities of the individual members of the *IFNL* family during viral infection remain poorly understood.

Genome-wide association studies identified *IFNL* as a strong susceptibility locus for both natural and treatment-induced clearance of hepatitis C virus (HCV)[227-233]. Of multiple SNPs with high predictive strength, two major variants are known to be functional. The first, rs4803217, is a 3′ UTR variant in *IFNL3* that dictates the stability and expression of the *IFNL3* mRNA[233,234] (**Fig. 3.1**). The second is a dinucleotide variant that controls the expression of functional *IFNL4* and will be further discussed in the following sections.

3.1.2 IFNλ4: Serendipitous discovery of a genetic paradox

IFNL4 is a novel gene that was discovered, ironically, first by its genetic variant before its open reading frame. Prokunina-Olsson et al. performed an RNA screen by deep sequencing to search for differential expression of transcripts in response to HCV therapy in primary human hepatocytes and found strong association between a dinucleotide polymorphism rs368234815 (TT/ΔG) and clearance of hepatitis C virus (HCV) infection[232]. Intriguingly, the variant occurred in the first exon of a novel open reading frame (ORF), named *IFNL4* for its location within the *IFNL* locus, and determined whether a productive transcript was made. While the "TT" dinucleotide variant of *IFNL4* creates a premature stop codon due to a frame shift that renders it a pseudogene, the ancestral "ΔG" single nucleotide variant encoded functional IFNλ4 proteins of predicted sizes 179aa (full-length), 170aa, 131aa and 107aa (hereafter termed "IFNλ4p179", "IFNλ4p170", "IFNλ4p131" and "IFNλ4p107" respectively). Surprisingly, the "TT" variant that prevents expression of functional *IFNL4* associated with improved HCV clearance, while the "ΔG" variant encoding the full-length protein associated with HCV persistence (**Fig. 3.1**). The conclusion that IFNλ4 ORF expression is pro-viral starkly contrasts the highly established, classical antiviral activity of all other known IFNs in many infections, especially for HCV which is treated traditionally with pegylated IFN-α and for which IFNλ3 expression increases viral clearance. Many studies have since replicated the strong *IFNL4* association with HCV clearance/persistence[234-239]. Naturally, the robust genetic association and high odds ratios has prompted great academic interest in the potential mechanisms by which IFNλ4 acts in a pro-viral manner, a major paradox for the IFN field which remains unexplained. Furthermore, the *IFNL4* "ΔG" (risk) allele is in strong linkage with the risk allele for the functional *IFNL3* variant at rs4803217[234], making the role of each variant particularly hard to distinguish.

3.1.3 Known characteristics and function of IFNλ4

Of the type II cytokines and interferons, IFNλ4 shares the highest amino acid similarity and identity with the IFN lambdas, and highest with IFNλ3 (similarity 40.8%, identity 29.1%)[232]. Homology modelling shows that general folding of the cytokines and specific helices interacting with IFNλR1 are expected to be conserved between IFNλ3 and IFNλ4, though amino acid differences enable and support formation of a disulfide bridge in IFNλ4 that is absent in IFNλ3[240].

Of the isoforms predicted in the original discovery, IFNλ4p107, IFNλ4p131 and IFNλ4p179 were verified and detectable by antibodies. Prokunina-Olsson et al. showed that overexpressed IFNλ4p179 is able to activate a co-transfected interferon stimulatory response element (ISRE) luciferase reporter, while IFNλ4p107 and IFNλ4p131 did not induce any response[232]. Several groups have gone on to examine gene induction by IFNλ4p179 and generally found it to be similar to IFNλ3. RNA sequencing of HepG2 cells overexpressing IFNλ4p179 revealed an enrichment mainly of pathways associated with standard antiviral interferon responses and pattern recognition of bacteria or viruses, with some hits for the *BRCA*-mediated DNA damage response and the IL-17 response. A side-by-side comparison later showed that both overexpressed and recombinant IFNλ4p179 activated an ISRE luciferase reporter, induced canonical interferon-stimulated genes and increased clearance of HCV and coronavirus infection *in vitro* with equal efficacy as the same dose of IFNλ3[240]. A gene array also revealed similar classical antiviral gene induction in primary human hepatocytes and airway epithelial cells stimulated with recombinant IFNλ3 or IFNλ4p179[82]. A recent study suggested that IFNλ4p179 but not p131 overexpression may be able to induce cell death and reduce proliferation of stably transfected HepG2 hepatocytes, although the effect on mortality was comparatively modest and not compared alongside other IFNs[241]. Thus, the numerous similarities in signaling downstream of IFNλ4p179 and IFNλ3

generally fail to explain their opposite genetic associations with viral clearance. While IFNλ4p107 and IFNλ4p131 remain poorly characterized, they have already demonstrated differential activity from IFNλ4p179 and could be responsible for pro-viral functions of IFNλ4.

Meanwhile, poor detection of endogenous IFNλ4 in primary samples poses obstacles to study of its activity and regulation. Consequently, some studies stratify patient cohorts and discern the effect of IFNλ4 by whether it is "detectable" or "undetectable", being unable to perform two-dimensional quantitative analyses[242,243]. This contrasts the ready detection of other type III IFNs in these studies and raises questions on the biological relevance of IFNλ4 activity. Nonetheless, the expression of IFNλ4p107, p131 and p179 isoform transcripts has been recapitulated in liver samples, primary human hepatocytes and A549 lung carcinoma cells. Surprisingly, the majority of the transcripts cloned from primary samples were of the TT genotype that encodes a premature stop codon[235]. Analyses have also been performed examining correlations between genotype and markers of the antiviral state that alas extend the paradox: although carrying the ΔG allele associates with poorer sustained virological response as expected, it still correlates with increased expression of important antiviral effector ISG15, supporting the transcriptomic profiles of overexpressed IFNλ4[242,244].

An additional genetic polymorphism, rs117648444, encodes a P70S substitution in IFNλ4 which attenuates its ability to induce antiviral ISGs. The allele encoding the less active S70 variant associates independently with improved both spontaneous clearance of HCV and response to pegylated IFN/ribavirin therapy[244]. Thus, it appears that IFNλ4 may have already undergone other forms of intrinsic selection in its short evolutionary history.

3.1.4 Research objectives on IFNλ4 and its isoforms

While genetic association between *IFNL4* and response to treatment of HCV is clear, its mechanism of action remains unclear. Similarities between overexpressed full-length IFNλ4p179 and IFNλ3 fail to explain the paradoxical association between IFNλ4 expression and poor viral clearance. Furthermore, several studies have failed to detect secretion of IFNλ4 protein, prompting speculation on unique activities of IFNλ4, including an intracellular role[232,235,245-247] (**Fig. 3.2**). The observation that IFNλ4 has multiple naturally expressed isoforms adds an interesting angle regarding non-canonical function or regulation of this IFN, as all other type I, II and III IFNs do not undergo alternative splicing. Again, genetic variation does not clearly implicate any particular isoform with the association; strong linkage disequilibrium with a functional *IFNL3* genotype at rs12979860 and rs4803217 further complicates efforts to distinguish the contributions of IFNλ3 and IFNλ4 to the striking disease phenotype[234,248]. Much remains to be elucidated of the function of these isoforms and the regulation of their expression, which are likely to provide important clues to the non-canonical biological activities of IFNλ4 in viral infection.

In this study, we use molecular and biochemical approaches to show that IFNλ4 has similar antiviral activities as IFNλ3 but is weakly induced and poorly translated during viral infection. Our investigation revealed that the lower expression of *IFNL4* is due to host adaptation suppressing the functional full-length isoform (179aa) of *IFNL4* through induction of alternative, non-functional, intron-retaining splice forms and a weak polyadenylation signal. This study provides clear mechanistic evidence that humans have sustained adaptations to suppress IFNλ4 expression suggesting that antiviral function is determined by the activity of other *IFNL* family members, whose effects were previously difficult to distinguish from those of *IFNL4* due to high linkage disequilibrium between their genetic variants.

3.2 RESULTS

3.2.1 Bioactivity of IFNλ4 in comparison to IFNλ3.

Differential mRNA splicing of the *IFNL4* gene produces three protein-coding isoforms termed *IFNL4P107*, *IFNL4P131* and *IFNL4P179* based on the number of amino acids encoded (**Fig. 3.3A**). To test their individual activities, we overexpressed IFNλ4p107, IFNλ4p131, IFNλ4p179 isoforms and IFNλ3, all tagged with C-terminal hemagglutinin (HA) in Huh7 cells. Overexpression was verified by immunoblot using α-HA and α-IFNλ4 antibodies (**Fig. 3.3B**). As the α-IFNλ4 antibody was raised against a peptide encoded in exon 2, this antibody only detects IFNλ4p131 and IFNλ4p179 isoforms. However, α-HA detected equal expression of all IFNλ4 isoforms and IFNλ3 in the whole cell lysate.

By immunoblotting, we detected two bands in the lysates for IFNλ4p131, IFNλ4p179 and IFNλ3 (**Fig. 3.3B**). These bands usually arise from differential glycosylation, a post-translational modification that is coded for, and which a majority of IFNs and cytokines require for efficient secretion and stability. To test if the higher molecular weight bands reflect glycosylated forms of IFNλ4p131, IFNλ4p179 and IFNλ3, we treated the overexpression cell lysates with PNGase F and immunoblotted with α-IFNλ4 and α-HA. We observed that the higher molecular weight band was reduced, indicating that IFNλ4p131 and IFNλ4p179 were glycosylated (**Fig. 3.3C**). While two bands were detected for IFNλ3, PNGase F failed to reduce the higher molecular weight band, suggesting a non-N-glycosyl modification for IFNλ3.

Interferons require secretion from the cell to engage with their cognate receptors at the cell surface and activate Jak/STAT signaling. To test if the IFNλ4 isoforms were secreted into the supernatant, we performed immunoblots on supernatants, both before (neat) and after

concentration by tricholoroacetic acid (TCA) precipitation (**Fig. 3.3D**). When *IFNL* isoforms were overexpressed, we documented secreted IFNλ4p179 and IFNλ3 in both neat and TCA-treated supernatants. However, we did not detect IFNλ4p107 or IFNλ4p131 (**Suppl. Fig. 3.1A**). These data suggest that IFNλ4p179 and IFNλ3 are released extracellularly, while IFNλ4p107 and IFNλ4p131 are retained intracellularly. The supernatants containing IFNλ4p179 or IFNλ3 proteins were then subjected to PNGase F treatment. The higher molecular weight band of IFNλ4p179 was reduced to a lower molecular weight suggesting that the secreted proteins are also glycosylated (**Fig. 3.3E**).

Another nonsynonymous variant of *IFNL4* (Pro70Ser; rs117648444) exists that changes Proline to Serine at position 70 of the IFNλ4 protein [232,244]. This SNP lowers induction of antiviral genes by IFNλ4, presumably due to changes in the protein structure, and has been associated with improved spontaneous HCV clearance and better treatment response in patients with "ΔG" at rs368234815 [244]. We also tested the secretion of IFNλ4p179 S70 (P70S) in comparison to IFNλ4p179 (P70) after expression of both variants in Huh7 cells and found that the S70 variant is secreted less efficiently (**Suppl. Fig. 3.1A**). Furthermore, expression of *IFNL4P179* S70 (P70S) results in a lower interferon-stimulated gene (ISG) response compared to the *IFNL4P179* P70 variant, as measured by *MX1* qPCR (**Suppl. Fig. 3.1B**). Overall, in our overexpression system, the glycosylated form of IFNλ4p179 is efficiently secreted out of the cell, the P70 variant more than the P70S variant, while IFNλ4p107 and IFNλ4p131 are predominantly intracellular.

3.2.2 IFNλ4 signals exclusively through the extracellular IFNλR1:IL-10R2 receptor complex.

Similar to other type III IFNs, IFNλ4 is thought to signal through its cognate heterodimeric receptor composed of IFNλR1 and IL-10R2 subunits [240]. Type III IFNs evolved from a common lineage with IL-10 family cytokines, many of which feature alternative receptor usage. Since

IFNL4 shares this lineage and has low sequence identity with other type III IFNs [232], we examined if IFNλ4 could also signal through a different IL-10 family receptor. We co-expressed the *IFNL4* isoforms and *IFNL3* together with a luciferase reporter downstream of an interferon stimulated response element (ISRE) in Huh7 wild-type and *IFNLR1*$^{-/-}$ cells. We found that expression of IFNλ4p179, the only secreted isoform, and IFNλ3 strongly induced ISRE luciferase reporter activity in wild-type Huh7 cells, whereas the non-secreted IFNλ4p107 and IFNλ4p131 were inactive (**Fig. 3.4A**). ISRE luciferase reporter activity was completely abrogated in *IFNLR1*$^{-/-}$ Huh7 cells, suggesting that IFNλR1 was necessary for ISG induction by IFNλ4p179. To further test if IFNλ4 signals through extracellular IL-10R2, we blocked the receptor using a neutralizing αIL-10R2 antibody and co-expressed IFNλ4p179 or IFNλ3 together with the ISRE luciferase reporter. IL-10R2 blockade decreased ISRE luciferase reporter activity for both IFNλ4p179 and IFNλ3 (**Fig. 3.4B**). Lastly, downstream signaling via *MX1* was completely abrogated in *IFNLR1*$^{-/-}$ cells upon stimulation with IFNλ4p179 (**Fig. 3.4C**). These data not only confirm that IFNλ4 requires both IFNλR1 and IL-10R2 chains, but also shows that it signals through this extracellular heterodimeric receptor complex.

3.2.3 Intracellular IFNλ4 isoforms do not affect type I and III IFN signaling.

As the majority of IFNλ4 remain in the cytoplasm, including both functional and inactive isoforms, it has been proposed that intracellular IFNλ4 regulates cell surface IFNλR1 by binding and sequestering IFNλR1 or IL-10R2 in the cytoplasm [232,240]. To quantify the effects of IFNλ4 on receptor surface expression and consequent downstream signaling of other type III IFNs, we treated Huh7 cells overexpressing IFNλ4 isoforms or an empty vector (EV) with recombinant human (rh)IFNλ3 for 6 h and quantified ISG induction represented by *MX1*. We found that induction of *MX1* by rhIFNλ3 was unaltered in the presence of overexpressed IFNλ4p107 or

IFNλ4p131 compared to EV transfection (**Fig. 3.4D**). Again, IFNλ4p179 alone was able to induce *MX1*, and in this overexpression system it does so at similar levels compared with EV-transfected cells treated with 100 ng/ml rhIFNλ3. To exclude that observed *MX1* expression is due to the non-transfected fraction of cells not expressing *IFNL4* isoforms, we co-transfected a GFP plasmid together with the IFNλ4 overexpression constructs and sorted for GFP^+ cells. The sorted cells were immediately treated with IFNλ3 for 9 h, after which their RNA was harvested for qPCR analysis. Again, overexpression of IFNλ4p107 and IFNλ4p131 isoforms did not interfere with the cell's ability to respond to exogenous IFNλ3 (**Fig. 3.4E**). We performed the same stimulation with IFNβ and found that overexpression of intracellular IFNλ4 isoforms does not affect type I IFN signaling (**Fig. 3.4F**). These data show that IFNλ4p179 has a similar ability to induce ISGs as rhIFNλ3 and that IFNλ4 isoforms do not interfere with type I or type III IFN induced ISG responses intracellularly or extracellularly.

3.2.4 Transfected IFNλ4p179 and IFNλ3 have comparable antiviral activity on HCV.

The IFNλ4 polymorphism (rs368234815) is presumed functional because full-length IFNλ4 protein coding potential encoded by the "ΔG" genotype correlates with HCV persistence [232]. This paradoxical association has led to the hypothesis that IFNλ4 may have non-canonical functions such as blockade of antiviral activity. To test this hypothesis, we overexpressed IFNλ4 isoforms and IFNλ3 in Huh7 cells and infected them with a *Renilla*-luciferase tagged HCV reporter virus [249]. We observed that IFNλ4p179 and IFNλ3 mediated similar antiviral activity and comparably suppressed HCV replication, while the intracellular IFNλ4p107 and IFNλ4p131 isoforms were unable to block HCV replication (**Fig. 3.4G**). These data suggest that IFNλ4p179 has antiviral activities on HCV comparable to IFNλ3 when overexpressed and indeed appears to perform similar to type III IFNs in the context of antiviral defense.

3.2.5 Recombinant human IFNλ4p179 has canonical antiviral activity

To confirm the observations made with our plasmid-based overexpression system, we purified recombinant human (rh) IFNλ4p179 (rhIFNλ4) protein using a *Drosophila* Schneider 2 (S2) cell expression system. We cloned the *IFNL4P179* open reading frame with a C-terminal 6xHistidine tag into a construct under control of a copper (II) ion-inducible metallothionein promoter and transfected the expression plasmid into S2 cells. Upon induction by copper (II) sulfate, rhIFNλ4 was secreted into the supernatant, collected for affinity purification on a nickel column, and further isolated by size exclusion chromatography (**Fig. 3.5, A-C**). We compared the activities of rhIFNλ3 and rhIFNλ4 by quantifying *MX1* induction as a functional read out in PH5CH8 hepatocytes. The induction pattern of *MX1* over several logs of IFNλ concentration yielded an EC_{50} of 189.1 pM (3.801 ng/ml) for rhIFNλ3 and an EC_{50} of 577.0 pM (11.01 ng/ml) for rhIFNλ4, leading to an EC_{50} ratio of 3.051 (**Fig. 3.5D**). Intriguingly, the largest differences in activity were seen at lower concentrations of IFNλ. These data suggest that IFNλ3 exhibits activity that is marginally higher than that of IFNλ4, although these differences are minimal compared to differences in activities between IFNλ3 and IFNλ2[204]. We further tested the specificity for downstream STAT signaling in wild-type or *IFNLR1*$^{-/-}$ PH5CH8 cells (**Suppl. Fig. 3.2**) stimulated with rhIFNβ, rhIFNλ3, or rhIFNλ4 for 15 min (**Fig. 3.5E**). Treatment with rhIFNβ, rhIFNλ3, or rhIFNλ4 induced phosphorylation of STAT1 (pSTAT1) which was completely abrogated in *IFNLR1*$^{-/-}$ cells, when stimulated with rhIFNλ3 or rhIFNλ4. To confirm downstream gene expression, we treated wild-type or *IFNLR1*$^{-/-}$ PH5CH8 cells with rhIFNβ, rhIFNλ3 or rhIFNλ4 for 6 h and quantified the induction of ISGs by qPCR. *MX1*, *OAS1* and *ISG15* were induced by rhIFNλ4 treatment at comparable levels to rhIFNλ3 treatment. This induction was again abrogated in *IFNLR1*$^{-/-}$ cells

(**Fig. 3.5, F-H**). Control treatment with rhIFNβ resulted in stronger activation of pSTAT1 and induction of ISGs regardless of the *IFNLR1*$^{-/-}$ status.

Since genetic association data show that individuals carrying the in-frame "ΔG" variant have increased risk of viral persistence, we examined whether IFNλ4 administration had unique detrimental effects on the host that were independent of virus infection *per se*, for example causing increased cell death. To test if IFNλ4 causes cell death, as another potential mechanism rendering IFNλ4 expression detrimental to the host, PH5CH8 cells were treated with either rhIFNλ4 (100 ng/ml), rhIFNλ3 (100 ng/ml), or actinomycin D (ActD, 10 μg/ml). Cell death and confluence were assessed over 70 h using an IncuCyte imaging system. The cells were treated in the presence of Sytox green, a dye which enters dying cells as they lose membrane integrity. Neither IFNλ3 nor IFNλ4 induced cell death, and cell viability was comparable to mock-treated cells (**Fig. 3.5I**). ActD treated cells served as a positive control for cell death in these assays and *MX1* induction was assessed as a control to show similar IFNλ activities (**Fig. 3.5J**). When measured for cell confluence over time as a readout for proliferation, we found that IFNλ3 and IFNλ4 treatment shows a similar antiproliferative effect on cells compared to control cells (**Fig. 3.5I**).

To test if the induction of antiviral ISGs by rhIFNλ4 translates into functional suppression of viral propagation, as we had observed with the use of our plasmid-based overexpression system, we measured replication of HCV (**Fig. 3.6A**) and West Nile virus (WNV) (**Fig. 3.6B**) in the presence of rhIFNλ3 or rhIFNλ4. We documented robust antiviral activity against these viruses with rhIFNλ4 treatment, which was comparable to that exerted by rhIFNλ3 (**Fig. 3.6, A-B**). Comparable gene induction of 37 ISGs was observed in WNV infection post rhIFNλ3 or rhIFNλ4 treatments (**Fig. 3.6C**). These data confirm observations from our overexpression studies and conclusively show that rhIFNλ4 displays antiviral activity comparable to that of IFNλ3.

3.2.6 *IFNL4* is expressed at basal levels during viral infection.

Previous studies have identified transcripts of the protein-coding isoforms *IFNL4P107*, *IFNL4P131* and *IFNL4P179* harboring the "ΔG" allele [232]. As the induction pattern of *IFNL4* isoforms during infection is not well documented, we cloned and generated a cDNA library from HepG2 hepatocytes (heterozygotes at rs368234815) stimulated with a RIG-I ligand (HCV 5'ppp RNA) or poly(I:C). Using primers flanking the coding region of *IFNL4P179*, we identified three additional *IFNL4* transcripts with intron retention, which have a similar exon configuration to *IFNL4P107*, *IFNL4P131* and *IFNL4P179* (**Fig. 3.7, A-B**; **Suppl. Fig. 3.3**). We also identified pseudogenes with premature stop codons carrying the "TT" allele (**Suppl. Fig. 3.3**). Genes with intron retentions are not exported to the cytoplasm from the nucleus, or are subjected to nonsense-mediated decay in the rare event export occurs.

To evaluate the induction patterns of these *IFNL4* splice forms by qPCR, we designed primers specific to each protein-coding transcript as well as a specific primer pair that detects all *IFNL4* isoforms with intron retention (*IFNL4* IR). We established standard curves for these probes and for *IFNL3* to measure absolute copy numbers for each isoform allowing to directly compare abundance across the transcripts (**Table 3.1**). Analysis of Huh7 cells (TT/ΔG), HepG2 cells (TT/ΔG), HeLa cells (ΔG/ΔG) and HEK293 cells (ΔG/ΔG) stimulated with the RIG-I ligand HCV 5'ppp RNA (HCV PAMP) and primary human hepatocytes (PHH; TT/ΔG or ΔG/ΔG) stimulated with poly(I:C) revealed that *IFNL3* was highly induced compared to all the *IFNL4* transcripts **(Fig. 3.7, C-F, I-J)**. Surprisingly, the next most abundant transcripts were *IFNL4* with retained introns, rather than any of the protein-coding isoforms. These were followed by *IFNL4P131*, *IFNL4P179* and *IFNL4P107* isoforms. As dendritic cells have been reported to express *IFNL1-3* during viral infection [250,251], we also stimulated myeloid DCs differentiated from MUTZ-3 cells (TT/ΔG) and

THP-1 cells (TT/ΔG) with poly(I:C) and examined for *IFNL* expression patterns (**Fig. 3.7, G-H**). While poly(I:C) induced high *IFNL3* expression in these DCs, the protein-coding *IFNL4* isoforms were not induced, while the intron-retaining *IFNL4* transcripts were observed only in THP-1 cells differentiated to acquire DC-like characteristics. We found that similar expression patterns, including low level *IFNL4P179* expression, were seen in hepatoma cells during infections with WNV, Dengue virus (DenV), and Sendai virus (SeV) **(Fig. 3.7, K-M)**. Our data reveal that *IFNL4* is poorly induced compared to *IFNL3* during viral PAMP stimulation and viral infection, and that non-functional, intron-retaining *IFNL4* isoforms are preferentially transcribed instead of the protein-coding isoforms.

3.2.7 Endogenous *IFNL4* isoforms are not translated efficiently during infection

The poor induction of functional *IFNL4P179* compared to *IFNL3* during viral PAMP stimulation and viral infections would reflect low secretory output of the IFNλ4p179 protein. To test this, we infected Huh7 and HepG2 cells with HCV, WNV and SeV, and the cell lysates and supernatants were immunoblotted for endogenous IFNλ4 protein. In conformity with previous reports, cell lysates, direct cell culture supernatants and TCA-concentrated supernatants did not yield detectable IFNλ4 protein. To ensure that this was not due to poor antibody sensitivity, we performed polysome fractionation to determine efficiency of active translation of *IFNL3* and *IFNL4* isoforms. Cell lysates from HepG2 cells stimulated with poly(I:C) or mock treated for 12 h were separated using a sucrose gradient to separate non-translating monosome fractions of low centrifugal weight and polysome fractions of high centrifugal weight containing actively translated mRNA (**Fig. 3.8, A-D**). Polysome fractions were then pooled and *IFNL3* and *IFNL4* isoforms were quantified by qPCR. Intriguingly, *IFNL4* isoforms were poorly detected (less than 40 copies) in the heavy polysome and non-translated monosome fractions, despite detection of high levels of

IFNL3 in these fractions (**Fig. 3.8, C-D**), suggesting that *IFNL4* isoforms have only low association with actively translating ribosomal fractions compared to *IFNL3*. We propose two main reasons for inefficient translation of *IFNL4* mRNA. First, multiple isoforms of *IFNL4*, including intron-retaining variants, are induced upon viral infection or stimulation with viral PAMP. Expression of multiple splice variants, especially non-protein-coding transcripts, reduces transcriptional and translational resources for expression of the only functionally active *IFNL4P179* isoform. Second, the 3' UTR of *IFNL4*, unlike other *IFNL* genes, does not harbor a canonical polyadenylation (polyA) signal (**Fig. 3.8E**). For mRNA termination, a polyA signal (canonical motif: AAUAAA) is essential for downstream cleavage and polyadenylation of mRNA. Polyadenylation signal sequences are not only critical for mRNA termination, but also for recruitment of RNA binding proteins essential for stability and subsequent translation. *In silico* analysis of the *IFNL4* 3' UTR did not yield a strong canonical polyA signal compared to the other *IFNL* genes (**Suppl. Fig. 3.4**). To test the polyA signal usage and to identify the downstream cleavage site (CS) essential for *IFNL4* mRNA termination, we employed 3' Rapid Amplification of cDNA Ends (3' RACE). Cloning and analysis of the 3' UTRs documented three distinct cleavage sites used by *IFNL4* mRNA for termination (**Fig. 3.8E**). More importantly, 70.6% of the analyzed sequences used the second cleavage site (CS#2) followed by CS#3 (23.5%) and CS#1 (5.9%). Intriguingly, the 5' ends of CS#2 (major cleavage site) and CS#1 (minor cleavage site) do not encode for a canonical or non-canonical polyA signal. Only a small percentage of *IFNL4* mRNA sequences use CS#3, where a weak non-canonical polyA signal (AUUAAA) was detected, suggesting that this is not the major termination site. Previous studies have shown that eukaryotic mRNA do not tolerate changes in these nucleotide motifs and the efficiency of mRNA termination, polyadenylation and translation are severely hampered [252]. Therefore, we propose that the majority

of *IFNL4* mRNA that is not efficiently terminated, is rapidly degraded and weakly translated. Overall, basal low-levels of in-frame *IFNL4* mRNA expression and poor translation lead to lack of IFNλ4 protein expression.

3.2.8 Non-functional *IFNL4* splice variants arose before the ΔG>TT frame-shift variant in humans.

Our studies suggest that IFNλ4 mediates comparable antiviral activity to IFNλ3, but its action is limited through mechanisms including poor endogenous expression, expression of non-functional alternative splice variants, isoforms with intron retention, a frame-shift mutation that begets a premature stop codon (ΔG>TT), and the absence of a canonical polyA signal. As non-human primates do not carry the ΔG>TT frame-shift mutation and therefore have the potential to express full-length *IFNL4*, it is possible that humans have evolved multiple strategies for limiting the production of IFNλ4 for yet unknown reasons. We hypothesized that if IFNλ4 was detrimental to the host, we would observe such selection in non-human primates. Therefore, we stimulated *Gorilla gorilla* fibroblasts with poly(I:C) and found that they also expressed non-functional *IFNL4P107* and *IFNL4P131* isoforms, as well as unstable splice variants with intron retention (**Fig. 3.8F**). Like in humans, expression levels of functional *IFNL4P179* were low. These data suggest that the expression of functional *IFNL4P179* isoform has been selected against in non-human primates even before the dinucleotide frame-shift mutation (ΔG>TT) evolved in humans. Based on our evidence, it is likely that non-human primates evolved strategies to limit expression of functional IFNλ4, and consequently, neither human nor non-human primates express IFNλ4 protein to sufficient levels for biological consequences.

3.3 DISCUSSION

IFNL4 is a member of the type III IFNs that was most recently identified through a genetic association study [232]. Genetic studies postulate a cell-autonomous, intracellular role for IFNλ4 in dampening the antiviral response, but have failed to provide functional support for this hypothesis. Therefore, we carried out a comprehensive biochemical and molecular study to investigate the functional role of IFNλ4 during viral infections.

Our IFNλ4 overexpression studies show that the IFNλ4p179 variant can be secreted and has comparable antiviral activities to IFNλ3, confirming previous data [235,240]. We extended these observations by producing recombinant full-length IFNλ4p179 protein in *Drosophila* S2 cells, further demonstrating that antiviral activity of IFNλ4 is preserved and its potency against viruses such as HCV and WNV is comparable to that of IFNλ3. In contrast, IFNλ4p107 and IFNλ4p131 are both intracellular when overexpressed in hepatocytes. We further observed that neither intracellular nor secreted IFNλ4 isoforms interfered with type I or III IFN signaling, as they did not affect induction of ISGs *via* either type I or type III IFN receptors. Lastly, we found that hepatocytes treated with IFNλ4p179 did not exhibit a different rate of cell death compared to untreated cells or cells treated with IFNλ3. These data contradict a previous report that found IFNλ4 could induce cell death[241]. The authors of that study observed differences in the endoplasmic reticulum stress response and cell death in HepG2 cells using a plasmid-based overexpression system; this is in contrast to our use of titrated, recombinant IFNλ proteins, which are likely a cleaner measure of cell death responses that would occur *in vivo*. This may also be a result of variation between cell lines as our experiments were done not in HepG2 cells but in PH5CH8 cells, which are non-neoplastic and more closely resemble primary hepatocytes due to similar permissivity of infection and fully intact viral-sensing pathways[253,254]. All these

observations are paradoxical to the findings from genetic association studies in HCV patients, where the ΔG allele that codes for full-length *IFNL4P179* associates with a worse clinical outcome [232,238,248]. While it is possible that the intracellular retention and inactivity of IFNλ4p107 are specific to overexpression in hepatocytes, our findings that endogenous *IFNL4* is hardly expressed in multiple cell types during different viral infections or stimulations make it difficult to assess the trafficking of these isoforms in more physiological conditions.

We proceeded to examine whether endogenous IFNλ4 acts similarly during PAMP stimulations or viral infection. By cloning endogenous *IFNL4* isoforms from stimulated hepatocytes, we found additional mRNA splice variants with intron retention in the cDNA, which would not be translated as the introns prevent their export to the cytoplasm. This discovery warranted a comprehensive expression profiling analysis of *IFNL4* isoforms induced in multiple cell lines and primary human hepatocytes containing both variants at rs368234815 (ΔG and TT) during stimulation with PAMP and upon viral infections. Although previous studies documented induction of *IFNL4* [243], they have used primer/probes that do not differentiate the isoforms and in most cases amplify functional and non-functional isoforms indiscriminately. Using qPCR probes specific for each transcript, we were surprised to find that the intron-retaining and *IFNLP107* isoforms were the most abundant among all *IFNL4* isoforms regardless of their rs368234815 genotype. Intron-retaining transcripts are usually targeted for nonsense-mediated decay, and in recent years this process has been recognized as an efficient way to control expression of particular transcripts under different developmental phases or environmental contexts [31]. Therefore, we hypothesize that preferential expression of the intron-retaining isoforms suppresses expression of functional *IFNL4P179.* More intriguingly, *IFNL3* was induced several (2-3) logs-fold higher than any *IFNL4* splice forms. This induction pattern was consistent irrespective of PAMP stimulations

or viral infections. We propose two possibilities for inefficient translation of *IFNL4* mRNA. First, multiple isoforms of *IFNL4*, including intron-retaining variants, are induced upon viral infection or stimulation with viral PAMP. Expression of multiple splice variants, especially non-protein-coding transcripts, may reduce transcriptional resources for expression of the only functionally active *IFNL4P179* isoform. Second, we determined that endogenous *IFNL4* isoforms are not efficiently translated into proteins compared to *IFNL3* as they are poorly loaded onto polyribosomes upon PAMP stimulation. Notably, *IFNL4* is the only *IFNL* gene that does not encode a strong canonical polyA signal, which recruits proteins that cleave and polyadenylate the primary mRNA. Polyadenylation sequences then lead to the recruitment of several RNA-binding proteins essential for mRNA stability and subsequent translation. Longer polyA tails provide higher mRNA stability and higher translation potential [252]. Indeed, we found poor usage of its non-canonical polyA signal. Based on these data, we hypothesize that the *IFNL4* 3' UTR could be playing additional roles in reducing the expression levels and lowering the translation potential of *IFNL4*.

Altogether, we show that multiple mechanisms combine to minimize IFNλ4 protein expression in the host, including alternative splicing of *IFNL4* for significant production of non-functional proteins, preferential expression of unstable intron-retaining mRNA variants, poor loading onto polyribosomes for protein production and a weak polyA signal that further lowers the stability and translation potential of all *IFNL4* splice forms (**Fig. 3.9**). In addition to these regulatory controls, humans have evolved a frame-shift "ΔG>TT" mutation that further disrupts the coding potential of the *IFNL4* gene. While non-human primates bear only the ancestral ΔG allele and thus do not have this recent adaptation, we documented favored induction of non-

functional *IFNL4* isoforms compared to the functional *IFNL4P179* isoform in gorilla fibroblasts, as we observed in human cells.

Our mechanistic evidence shows that humans have sustained adaptations suppressing IFNλ4 activity even when the genotype at rs368234815 allows production of in-frame IFNλ4. Our data suggest that the expression of functional *IFNL4P179* isoform has been selected against in non-human primates even before the dinucleotide frame-shift mutation (ΔG>TT) evolved in humans. We propose that splicing and translational control mechanisms to suppress expression of functional IFNλ4 protein appeared prior to the frame shift mutation that evolved in humans to further silence its expression. Taken together, this study highlights differential activities of the *IFNL* genes during viral infection and the relatively low contribution of *IFNL4* compared to *IFNL3* in physiological immune contexts. A question of why bioactive *IFNL4* expression is so greatly suppressed, but not *IFNL3* can only be speculated. Similar to other interferon/cytokine genes, *IFNL4* may have arisen from gene duplication but failed to subfunctionalize or neofunctionalize leading to high redundancy with *IFNL3*.

Our findings still do not fully explain why genetic association implicates a pro-viral role for IFNλ4. It is possible that *IFNL4* may have more complex pathological roles detrimental to the host which led to its suppression – for example, it may increase viral permissivity dependent on communication between different cell types or in three-dimensional organoid structures that cannot be replicated *in vitro*. These are just speculations that can be tested only if the functional *IFNL4* is expressed at physiological levels during infection.

We and others have previously found the *IFNL3* variant to have functional effects on antiviral immunity against HCV infection and unfortunately, high linkage disequilibrium makes it difficult to distinguish the contributing effects of individual polymorphism. Our comparison of

endogenous *IFNL3* and *IFNL4* expression in this study showed that *IFNL3* is far more efficiently transcribed and translated upon stimulation with PAMPs or viral infection than *IFNL4*. Therefore, we propose that the clinical association between the genetic variant encoding in-frame IFNλ4 andpoor HCV clearance is not a direct biological effect of *IFNL4*, because hardly any functional IFNλ4 protein is produced regardless of genotype. Rather, viral persistence or clearance depends on the tightly linked functional variant in *IFNL3*, which is far more efficiently transcribed and translated to protein. Thus, our extensive characterization of the different antiviral effects of IFNλ4 isoforms and comparison of endogenous *IFNL3* and *IFNL4* expression clearly distinguish the contribution of two genetic variants and identified *IFNL3* as the true functional variant between the two.

3.4 MATERIALS AND METHODS

Cell culture conditions. HepG2, PH5CH8, Huh7, HeLa, HEK293 cells and *Gorilla gorilla* fibroblasts were cultured in complete DMEM (Sigma) (cDMEM) media containing 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals) and 1% penicillin-streptomycin-glutamine (PSG; Mediatech). The cells were incubated at 37 °C with 5 % CO_2. Primary human hepatocytes (PHH) were purchased from Life Technologies and cultured according to the vendor's instructions. THP-1 cells were grown in complete RPMI 1640 containing 10% FBS, PSG, 10mM HEPES, 1mM sodium pyruvate, 1X non-essential amino acids (Mediatech) and 50 μM 2-mecaptoethanol (Sigma). MUTZ-3 cells were grown in MEMα containing nucleotides (Gibco), 20% FBS and 10% conditioned medium from 5637 renal carcinoma cells. To differentiate myeloid cell lines into dendritic cells, THP-1 and MUTZ-3 cells were seeded at 0.2 x 10^6 cells/ml and cultured for 7 days in the presence of cytokines (for THP-1, GM-CSF 100 ng/ml and IL-4 100

ng/ml in complete RPMI 1640; for MUTZ-3, GM-CSF 100 ng/ml, IL-4 10 ng/ml and TNFα 2.5 ng/ml in MEMα containing nucleotides, 20% FBS), with half the medium replaced every 3 days.

Generation of *IFNLR1$^{-/-}$* hepatocytes. *IFNLR1* targeting guide RNA (gRNA, 5′-GCTCTCCCACCCGTAGACGG-3′) was cloned downstream of the U6 promoter in the pRRLU6-empty-gRNA-MND-cas9-t2A-Puro vector using In-Fusion enzyme mix (Clontech). Hepatocytes were transfected with either cas9-expressing or *IFNLR1* gRNA-cas9-expressing plasmids. For transfection of Huh7 cells, $3x10^6$ cells were seeded onto a 10 cm dish and 10 µg of plasmid was transfected using the $CaPO_4$ transfection kit (Invitrogen) according to the manufacturer's instructions. After 48 h cells were pre-selected by addition of 2 µg/ml puromycin to the media for 2 days. To confirm successful gene targeting in pre-selected cells, genomic DNA was extracted from wt/cas9 control and *IFNLR1$^{-/-}$* cells (NucleoSpin Tissue; Clontech) and subjected to T7 endonuclease I assay. Pre-selected cells were then single-cell sub-cloned and analyzed for *IFNLR1$^{-/-}$* knockout efficiency by checking for downstream activation of STAT1 and *MX1* induction upon stimulation with IFNλ3.

Stimulations. IFNβ was purchased from PBL and used at 100 IU/ml each. IFNλ3 was purchased from R&D Systems and used at 100 ng/ml. Neutralizing IL-10R2 antibody (MAB874, R&D) was pre-incubated with cells at 2-6 µg/ml for one hour prior to cytokine stimulation. Poly(I:C) (InvivoGen) was used at 1 µg/ml. The RIG-I ligand HCV 5'ppp RNA (HCV PAMP) was transcribed *in vitro* and used at 1 µg/ml. Both were transfected into cell lines using XtremeGene HP (Roche) or Mirus TransIT-X2 reagent (Mirus). Primary human hepatocytes were stimulated by adding 1 µg/ml poly(I:C) directly to the culture media.

Cloning and sequencing of *IFNL4* isoforms. We amplified *IFNL4* from HepG2 cells stimulated with poly(I:C) for 12 h and the mRNA was amplified with IFNL4-cDNA fwd and IFNL4-cDNA rev primers (**Suppl. Table 1**). The amplified products were cloned into a pCR2.1 TA-cloning vector (Invitrogen) and the inserts were sequenced. *IFNL4P107*, *IFNL4P131* and *IFNL4P179* were cloned into a C-terminal HA vector [pCMV-HA(c) pHOM-Mem1]. The three isoforms were amplified using the primers IFNL4-FL-HA-fwd and IFNL4-FL-HA-rev (**Table 3.1**) and cloned into the vector using *EcoRI* and *SpeI* restriction sites. To identify 3' UTR lengths and sequences of endogenous *IFNL4*, 3' Rapid Amplification of cDNA Ends (3' RACE) was carried out based on the manufacturer's instruction (Invitrogen). IFNL4-RACE-nest1 and IFNL4-RACE-nest2 forward primers (**Table 3.1**) were used for 3' RACE to amplify the 3' UTR of *IFNL4*. The 3' RACE products were cloned into a pCR2.1 TA-cloning vector and sequenced as above.

RNA isolation, reverse transcription and quantification of gene expression. Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. cDNA was synthesized from 1 μg total RNA using the QuantiTect RT kit (Qiagen) according to manufacturer's instructions. qPCR was carried out using the ViiA7 qPCR system with *Taq*Man reagents (Life Technologies) using custom-made isoform-specific *IFNL4 Taq*Man probes (**Table 3.1**, IDT), *IFNL3*, *IFNB*, *ACTB, HPRT* and interferon stimulated genes *MX1*, *OAS1* and *ISG15* (Life Technologies). Gene expression levels were normalized to either *ACTB* or *HPRT*.

Immunoblot analysis. 30 μg of cell lysates, 10 μl supernatant or 1 ml TCA precipitated supernatant from cell culture were subjected to SDS-PAGE and transferred to PVDF membranes

(Thermo Scientific). The membranes were then probed in 5 % BSA in TBST (Tris-Buffered Saline and Tween 20) or 5 % non-fat milk in TBST for IFNλ4 (4G1, Millipore), phospho-STAT1 (Y701) (58D6, Cell Signaling), STAT1 (42H3, Cell Signaling), HA (6E2, Cell Signaling) or β-actin (13E5, Cell Signaling).

Interferon bioactivity reporter assay. An interferon-stimulated response element (ISRE)-luciferase reporter construct along with eGFP control and overexpression constructs for IFNλ3 or IFNλ4 isoforms were co-transfected using XtremeGene HP (Roche) into Huh7 cells plated in a 96-well plate. 24 h post transfection, the cells were lysed with 1x Passive Lysis Buffer (Promega) and luciferase and eGFP values were measured using a multi-mode microplate reader (Synergy HT, BioTek).

Culture conditions of S2 cells. *Drosophila* Schneider 2 cells (S2) were grown in complete Schneider's *Drosophila* Medium (Gibco, Invitrogen), 10% heat-inactivated fetal bovine serum, penicillin-streptomycin (Gibco) and 20 µg/ml gentamycin (Amresco).

Production of recombinant IFNλ4 protein. We expressed IFNλ4 in a *Drosophila* S2 cell expression system (Invitrogen). The cells were stably co-transfected with plasmids encoding *IFNL4*-Histag and a blasticidin resistance gene in a ratio of 19:1 using calcium phosphate (Invitrogen). Cells were then passed into ExpressFive serum-free medium (Invitrogen) containing 25 µg/ml blasticidin and scaled up under constant selection to 1L suspension cultures at 125 rpm in spinner flasks. At a density of 5.0 x 10^6 cells/ml, cells were induced by 0.8 mM $CuSO_4$ to produce IFNλ4 for eight days. Recombinant IFNλ4 protein was isolated from the supernatant by

affinity chromatography, eluted in an imidazole gradient on a $Ni2^{+}$-IDA-based His60 resin (Clontech). The eluate was analyzed by a Coomassie gel to identify enriched fractions, which were subsequently concentrated by ultracentrifugation columns and desalted in PBS using PD-10 columns (GE Healthcare). To remove non-specifically-bound proteins, we performed size exclusion chromatography using an ÄKTA 9 high pressure liquid chromatography system with a Superdex 200 analytical column (Amersham Biosciences). Finally, we added 0.1 % BSA as a carrier protein and froze single-use aliquots in 20 % glycerol.

Cell death assays. PH5CH8 cells were treated with either IFNλ4 (100 ng/ml), IFNλ3 (100 ng/ml) or actinomycin D (ActD, 10 μg/ml) (Thermo Fisher). Cell death and confluence were assessed over time using an IncuCyte (Essen Bioscience) imaging system for 70 h with 100 nM Sytox Green (Thermo Fisher), a cell-impermeable DNA-binding fluorescent dye which stains only dead cells.

Genotyping assays. Genotyping assays were performed using *Taq*Man primers and probes as previously described[232] (**Table 3.1**).

Virus infections. HCV-*Renilla* infections were carried out as previously described [249]. Briefly, Huh7 cells (1.5×10^5 cells/ml) were plated on 96-well plates in cDMEM and incubated overnight at 37 °C to ensure 60-70 % confluency. We infected the cells with HCV-*Renilla* (MOI=0.3) diluted in serum-free DMEM in a total volume of 35 µl for 4 h. The media was replaced with 100 µl cDMEM and the cell lysate was harvested for luciferase assay 48 h after infection. WNV isolate TX 2002-HC (WNV-TX) was described previously [255]. Dengue virus type 2 (DV2) stocks were generated from seed stocks provided by A. Hirsch and J. A. Nelson (Oregon Health and Sciences

University). Virus stocks were titered with a standard plaque assay on Vero cells. Huh7 cells were infected at MOI=1-2 with either WNV-TX or DV2, or 25 HAU/ml SeV Cantell strain (Charles River) diluted in serum-free DMEM, or mock infected. The virus inoculum was removed 2 h post infection and replaced with cDMEM supplemented with 10% FBS. Total RNA was extracted using the NucleoSpin RNA kit, treated with DNase I (Ambion) and evaluated by real-time qPCR for relative gene expression and intracellular viral RNA levels using SYBR Green (Applied Biosystems). Real-time qPCR methods for quantifying intracellular WNV viral RNA is described previously [256].

Polysome analyses. $10x10^6$ HepG2 (*IFNL4* ΔG/TT) cells were stimulated with poly(I:C) for 6 h. Cells were then treated with 100 µg/ml of cycloheximide (MP Biomedicals) for 5 min, then washed twice with ice-cold PBS and harvested. The cell pellet was resuspended in polysome lysis buffer and cells were left to lyse on ice for 20 min, then centrifuged at 8,000 x g for 10 min at 4 °C. Supernatants were layered above 10 %-50 % sucrose gradient and centrifuged at 36,000 rpm for 2 h 30 min at 4 °C. Gradients were fractionated while continuously monitoring absorbance at 254 nm.

Statistical analyses. Statistics were performed using one-way ANOVA in GraphPad Prism 6.0. For Figure 1L, multiple comparisons were made against EV-transfected cells; for Figure 3A-B, multiple comparisons were made against mock-treated cells.

3.5 Figures

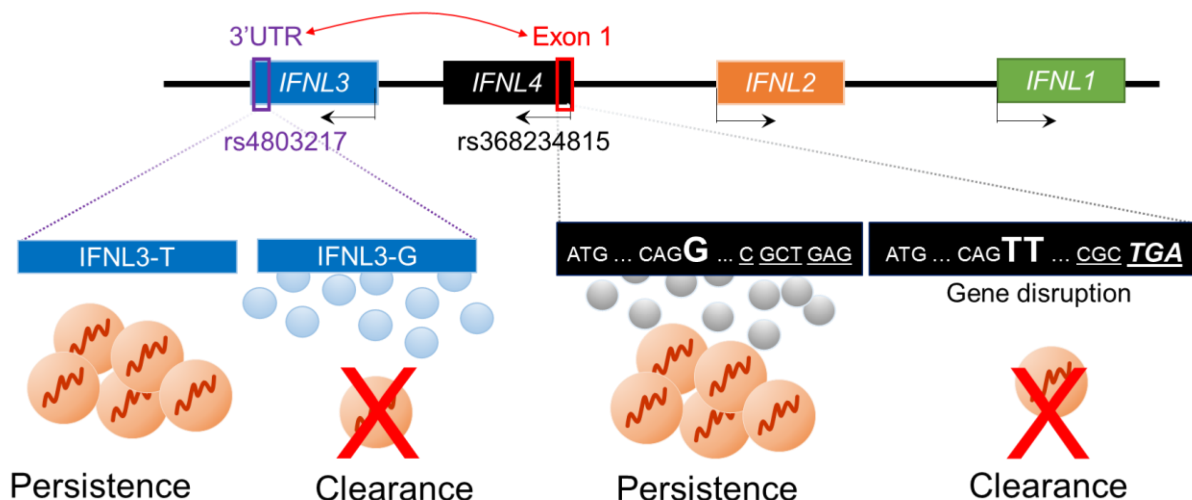


Figure 3.1. IFNλ4 expression paradoxically associates with viral persistence.

Schematic showing the *IFNL* locus that encodes the four members of the type III IFN family. IFNλ1, IFNλ2 and IFNλ3 have classical antiviral activity and are antiviral; indeed the functional variant rs4803217 in *IFNL3* strongly determines clearance of hepatitis C virus by controlling post-transcriptional decay of the IFNL3 transcript. A separate dinucleotide genetic variant, rs368234815 (TT/ΔG), lies in the first exon of *IFNL4* that results either in protein-coding transcripts (ΔG) or termination from a premature stop codon (TT). While the rs4803217 variant allowing higher *IFNL3* expression associates with viral clearance, the rs368234815 variant which enables *IFNL4* expression is instead associated with viral persistence. This suggests that IFNλ4 performs non-canonical signaling. The high linkage disequilibrium between rs4803217 and rs368234815 pose a challenge to discerning the relative functional contribution of each polymorphism.

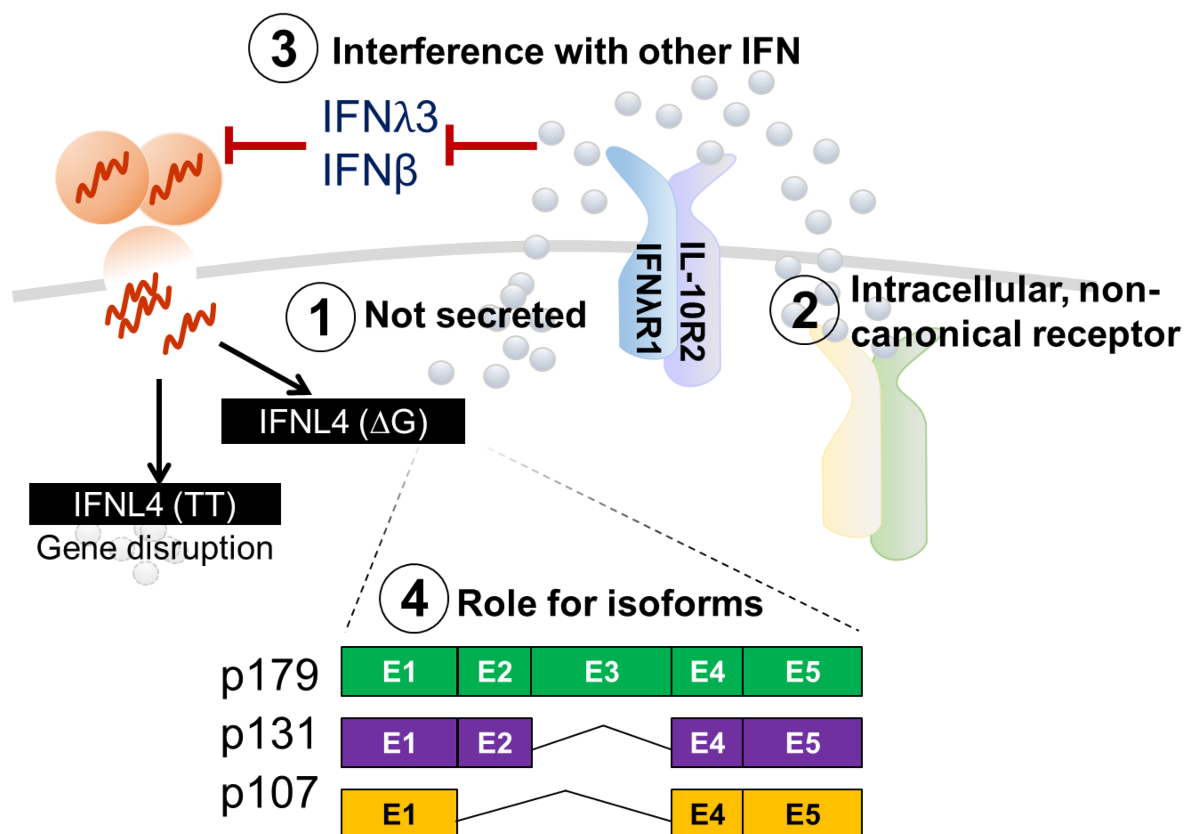


Figure 3.2. Suggested non-canonical functions of IFNλ4 and its isoforms.

Due to the paradoxical genetic association which suggests pro-viral functions for IFNλ4, several mechanisms have been proposed in the field for how IFNλ4 may reduce viral clearance. The possibilities are numbered as such in the above figure: (1) IFNλ4 is not secreted, so that it performs (2) intracellular functions, such as binding to a non-canonical receptor and activating signaling pathways distinct from those of other IFNs. (3) IFNλ4 may interfere with antiviral signaling by other type I or type III IFNs, either due to suppressive signaling *via* the non-canonical receptor or competing for IFNλR binding. (4) Lastly, as IFNλ4 is expressed as several alternatively spliced isoforms, it may be only specific isoforms that exhibit pro-viral activities. These possibilities are each addressed in our work.

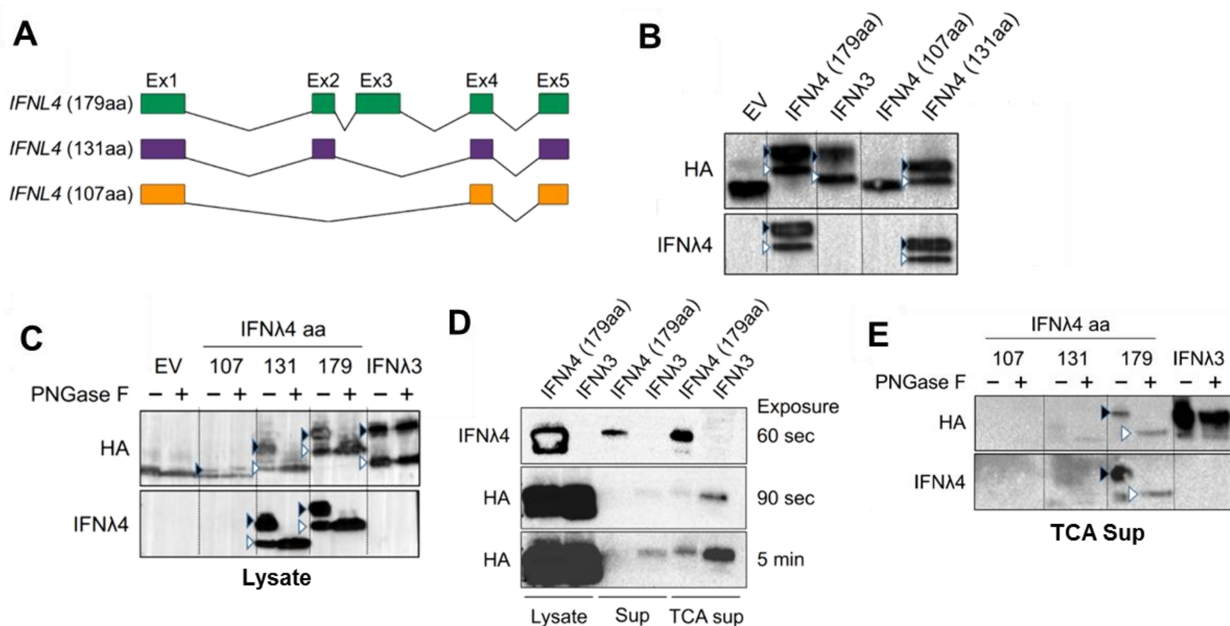


Figure 3.3. IFNλ4 isoforms differ in secretory efficiency and glycosylation.

(**A**) Schematic of the gene structure of protein-coding splice variants of *IFNL4* generated by alternative splicing. (**B**) Immunoblot of HA-tagged IFNλ3 and IFNλ4 isoforms overexpressed in Huh7 cells. (**C**) Immunoblot of PNGase F-treated lysates from cells overexpressing HA-IFNλ4 isoforms or HA-IFNλ3. (**D**) Immunoblot of cell lysates, neat supernatants and supernatants concentrated by trichloroacetic acid (TCA) treatment. (**E**) Immunoblot of TCA-treated supernatants of Huh7 cells overexpressing IFNλ4 isoforms. Above experiments are representative of at least 2-3 biological replicates. (**B, C&E**) Solid arrows: glycosylated forms; clear arrows: deglycosylated forms.

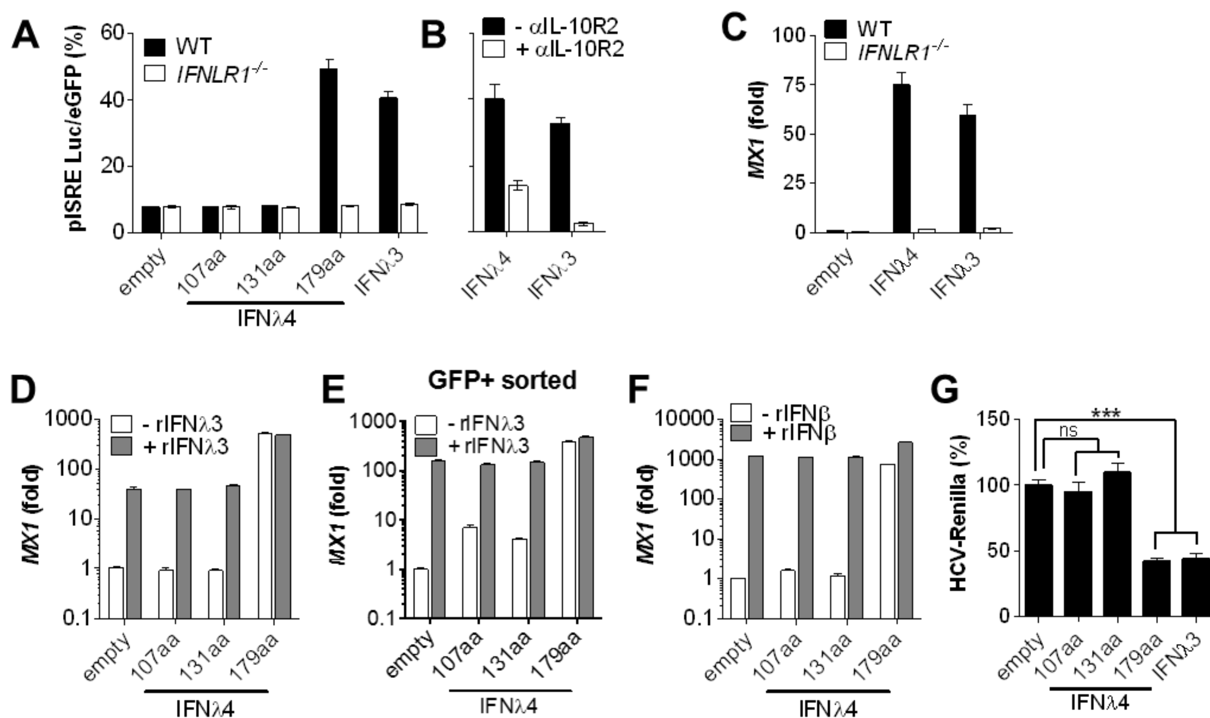


Figure 3.4. IFNλ4p179 has similar biological activity to IFNλ3, while other isoforms are inactive.

(**A**) Luciferase reporter assay measuring ISRE activity in wild-type and *IFNLR1*$^{-/-}$ Huh7 cells overexpressing IFNλ3 or IFNλ4 isoforms. (**B**) Luciferase reporter assay measuring ISRE activity in Huh7 cells treated with IL-10R2 neutralizing antibody and overexpressing IFNλ4p179 and IFNλ3. (**C**) *MX1* expression in wild-type and *IFNLR1*$^{-/-}$ Huh7 cells overexpressing IFNλ4p179 and IFNλ3. (**D-F**) *MX1* expression in Huh7 cells overexpressing IFNλ4 isoforms and treated with either IFNλ3 (**D,E**) or IFNβ (**F**). (**E**) *MX1* expression in GFP^{+} cells after 9h of IFNλ3 stimulation immediately after sorting from cells co-transfected with a GFP plasmid and either the *IFNL4* or *IFNL3* overexpression construct. (**G**) Luciferase reporter activity measured in Huh7 cells overexpressing IFNλ3 or IFNλ4 isoforms infected with with hepatitis C virus (HCV) tagged with *Renilla* luciferase. Above experiments are representative of at least 2-3 biological replicates. *** $p<0.001$, ns = not significant.

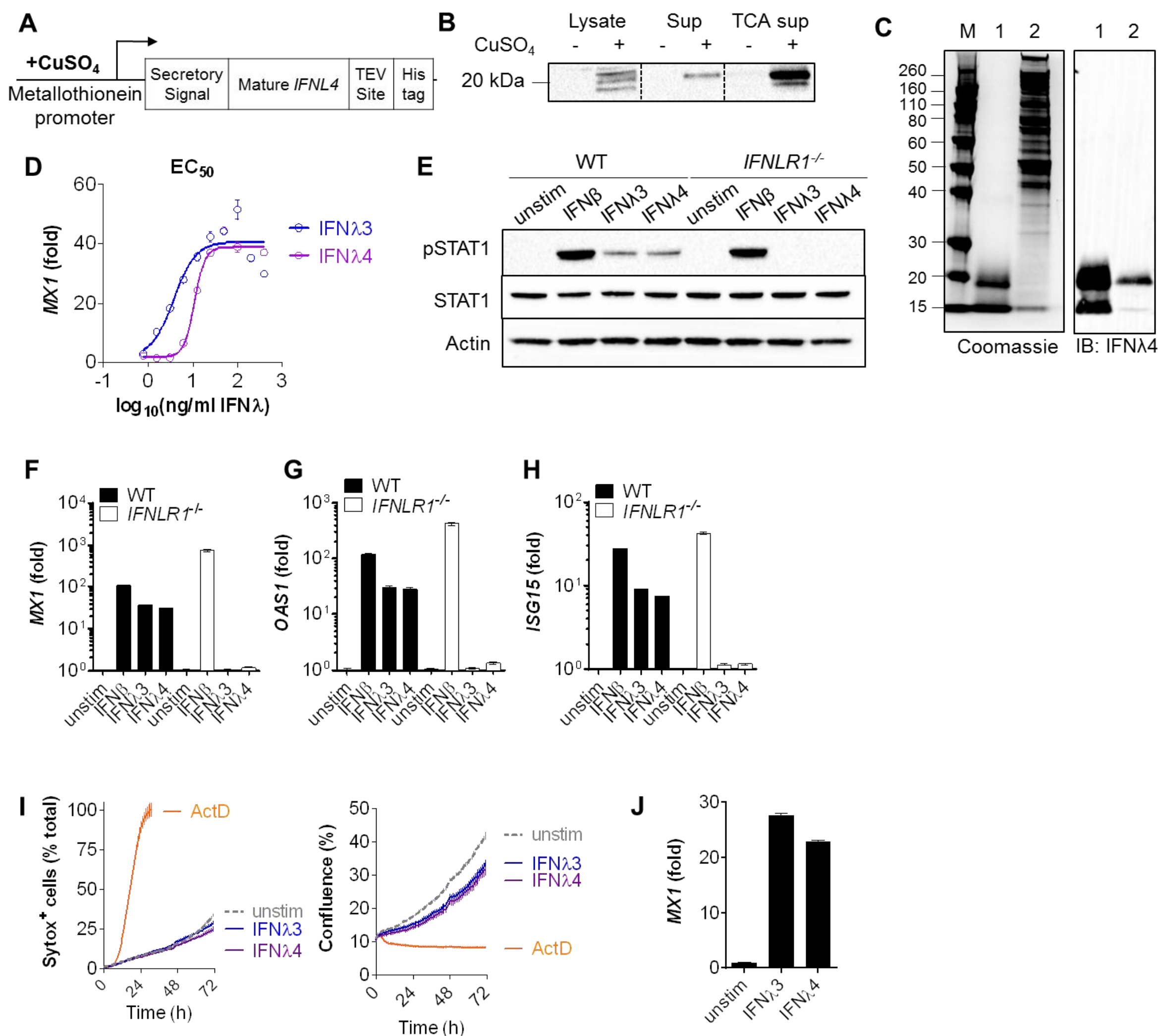


Figure 3.5. Recombinant IFNλ4 induces ISGs but not cell death.

(**A**) Schematic the expression vector for recombinant human IFNλ4p179 (rhIFNλ4) in a *Drosophila* S2 Schneider cell expression system. (**B**) Immunoblot showing expression of rhIFNλ4 in lysates and supernatants upon treatment with copper (II) sulfate for 8 days. (**C**) Coomassie stain and immunoblot of rhIFNλ4 purified by affinity chromatography and gel filtration. Lane: M, molecular weight marker; 1, purified rhIFNλ4; 2, pre-purified supernatant from S2 cells. (**D**) EC_{50} curve of IFNλ3 and IFNλ4 activity assayed by *MX1* gene expression. (**E**) Immunoblot of pSTAT1 in wild-type and *IFNLR1*$^{-/-}$ PH5CH8 cells, treated with recombinant IFNβ, IFNλ3 and IFNλ4. (**F-H**) Gene expression of *MX1* (**F**), *OAS1* (**G**) and *ISG15* (**H**) in wild-type and *IFNLR1*$^{-/-}$ PH5CH8 cells treated with recombinant IFNβ, IFNλ3 or IFNλ4 for 6 h. (**I**) Proliferation and cell death (object counts) in wild-type PH5CH8 hepatocytes stimulated with IFNλ3, IFNλ4 and actinomycin D (positive control), measured using Incucyte time-lapse live fluorescent microscopy of a quantifiable cell viability dye Sytox green, which marks dead cells. (**J**) *MX1* gene expression measured at 70 h to confirm stimulation of the hepatocytes by IFNλ3 and IFNλ4 in cell death assays. Above experiments are representative of at least 2-3 biological replicates.

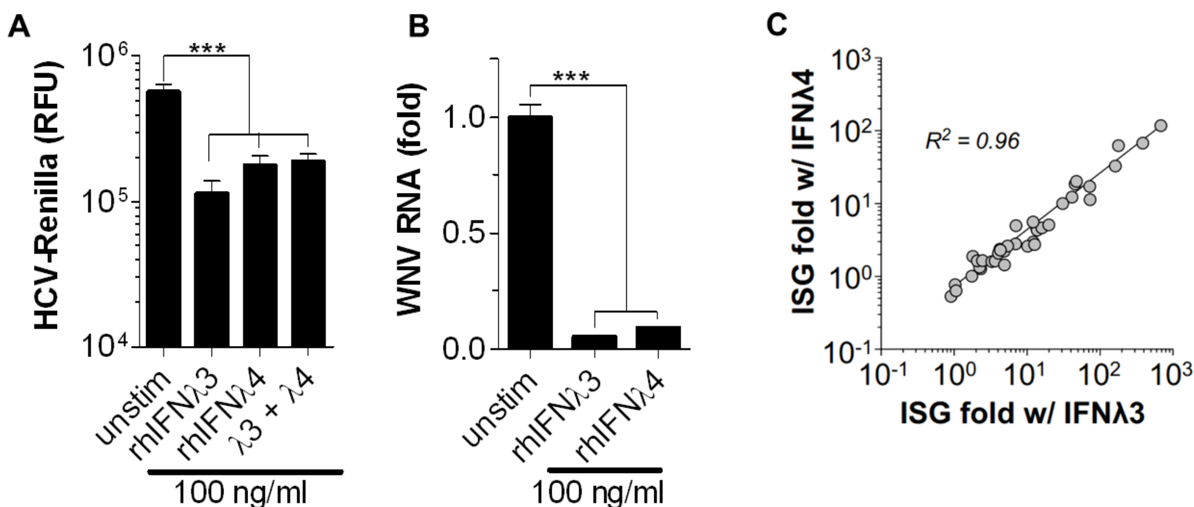


Figure 3.6. Recombinant human IFNλ4 shows similar antiviral activity to IFNλ3.

(**A**) Luciferase reporter activity measuring HCV replication in Huh7 cells infected with *Renilla* luciferase-tagged HCV and treated with rhIFNλ3 and rhIFNλ4. (**B**) West Nile virus (WNV) RNA load upon treatment of infected Huh7 cells with equal doses of rhIFNλ3 and rhIFNλ4. (**C**) Correlation of ISG induction in WNV-infected Huh7 cells (from Fig. 3B) stimulated with 100 ng/ml rhIFNλ3 and rhIFNλ4, based on a *Taq*Man qPCR array of 37 ISGs. Above experiments are representative of at least 2-3 biological replicates. *** $p<0.001$.

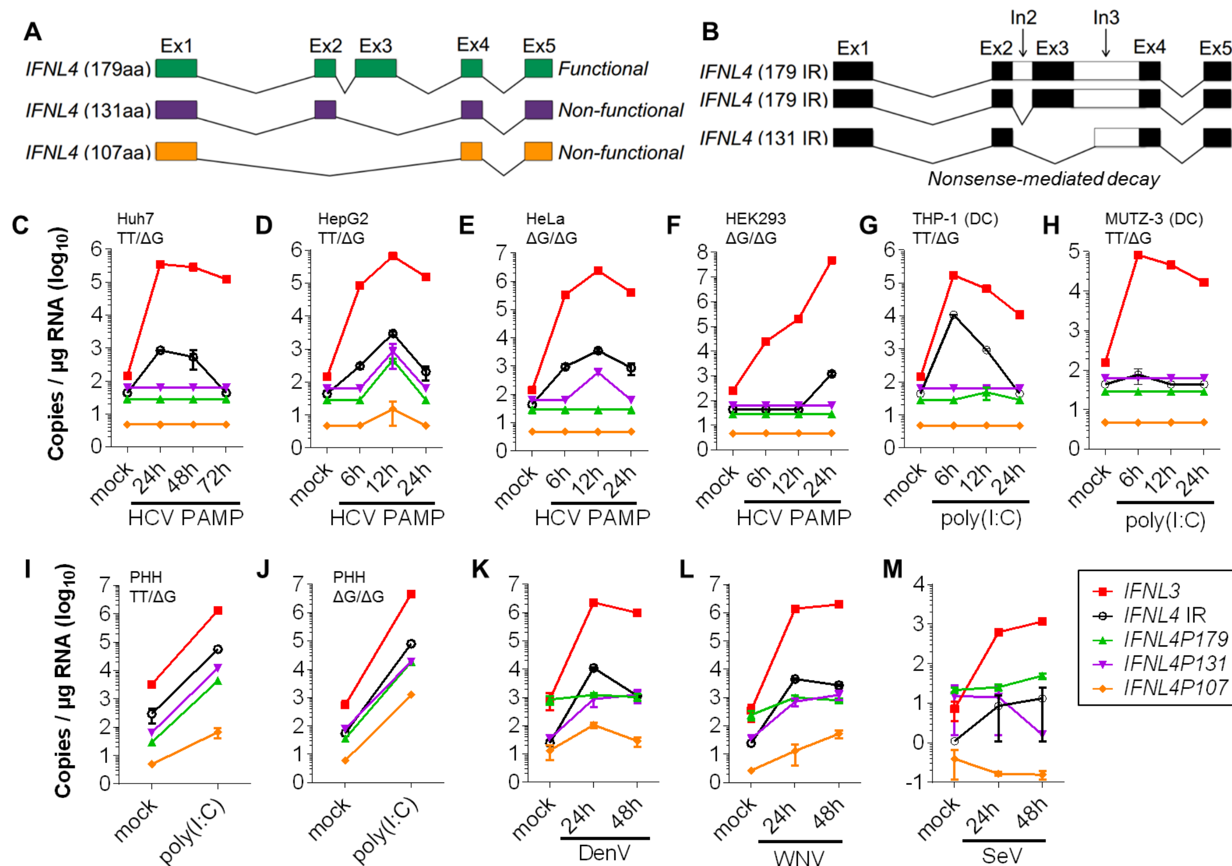


Figure 3.7. Low *IFNL4* induction during PAMP stimulation and viral infection.

(**A-B**) Schematic of *IFNL4* mRNA exhibiting intron retention (IR), compared to the known protein-coding isoforms of *IFNL4*. (**C-H**) Gene expression kinetics of *IFNL*3 and *IFNL4* isoforms measured by real-time PCR using custom made *Taq*Man qPCR probes in Huh7 (**C**), HepG2 (**D**), HeLa (**E**), HEK293 (**F**) cells upon stimulation with HCV PAMP, and DCs derived from THP1 (**G**) and MUTZ-3 (**H**) cell lines stimulated with poly(I:C). (**I-J**) Gene expression of *IFNL3* and *IFNL4* isoforms in primary human hepatocytes, heterozygous (**I**) or homozygous (**J**) for the ΔG variant, stimulated with poly(I:C). (**K-M**) Gene expression of *IFNL*3 and *IFNL4* isoforms in HepG2 cells infected with Dengue virus (**K**), West Nile virus (**L**) and Sendai virus (**M**). All experiments are representative of at least 2-3 biological replicates.

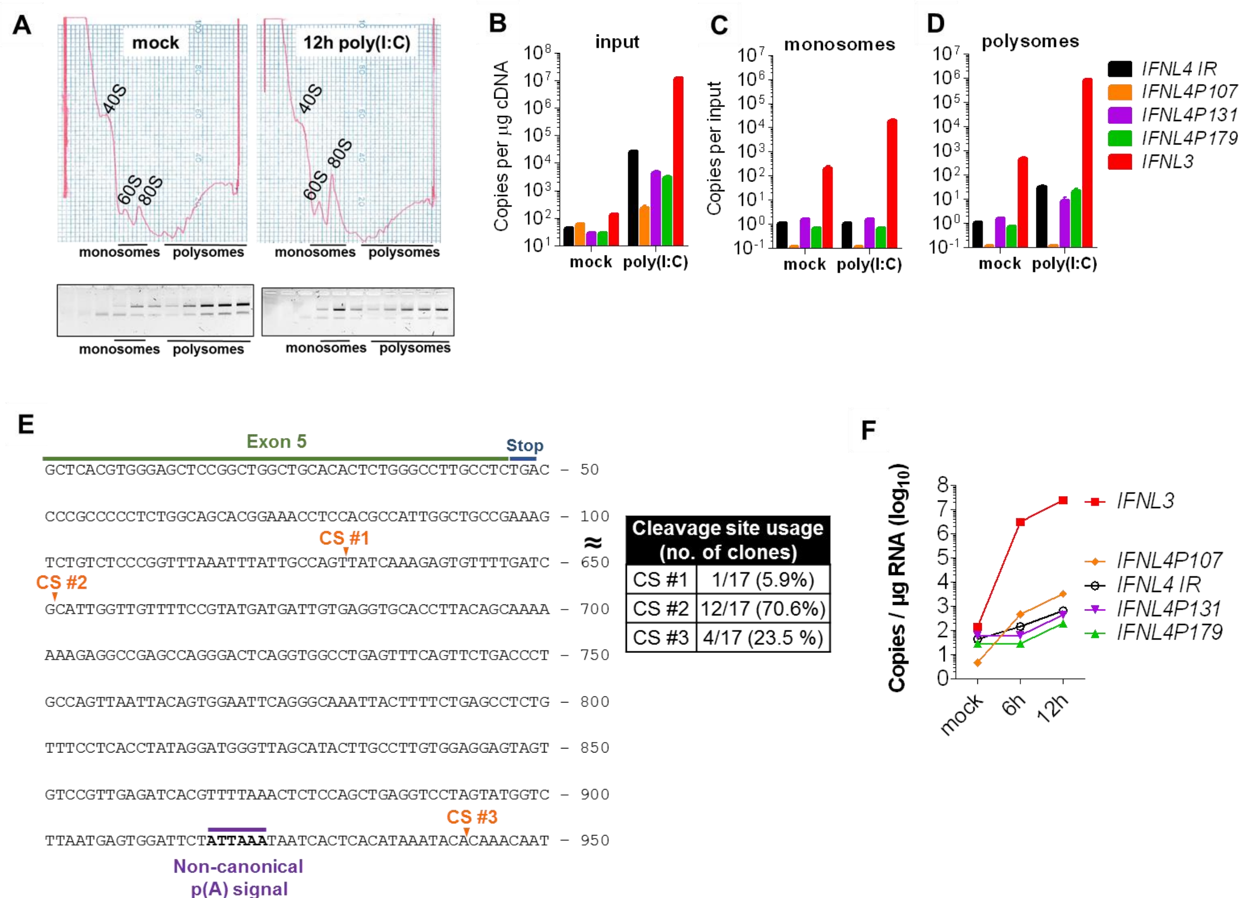


Figure 3.8. ***IFNL4*** **mRNA are poorly loaded onto polyribosomes for translation and suppressed in evolution.**

(**A**) Whole-cell extracts from mock or poly(I:C) treated HepG2 were resolved by density sedimentation in 10-50 % sucrose gradients. The UV absorbance trace (254 nm) obtained during fractionation is shown with the positions of the 40S, 60S, 80S, and polyribosomes. The lower panel shows an agarose gel of polysome fractions to check for 28S and 18S ribosomal RNA in the fractions. Copy number expression of *IFNL*3 and *IFNL4* isoforms in input **(B)**, monosome **(C)**, and polysome **(D)** fractions measured by qPCR. (**E**) Cleavage sites (CS) and frequency of CS usage in the human *IFNL4* 3' UTR determined by 3' RACE. (**F**) Gene expression of *IFNL*3 and *IFNL4* isoforms in gorilla fibroblasts upon stimulation with poly(I:C). All experiments are representative of at least 2-3 biological replicates.

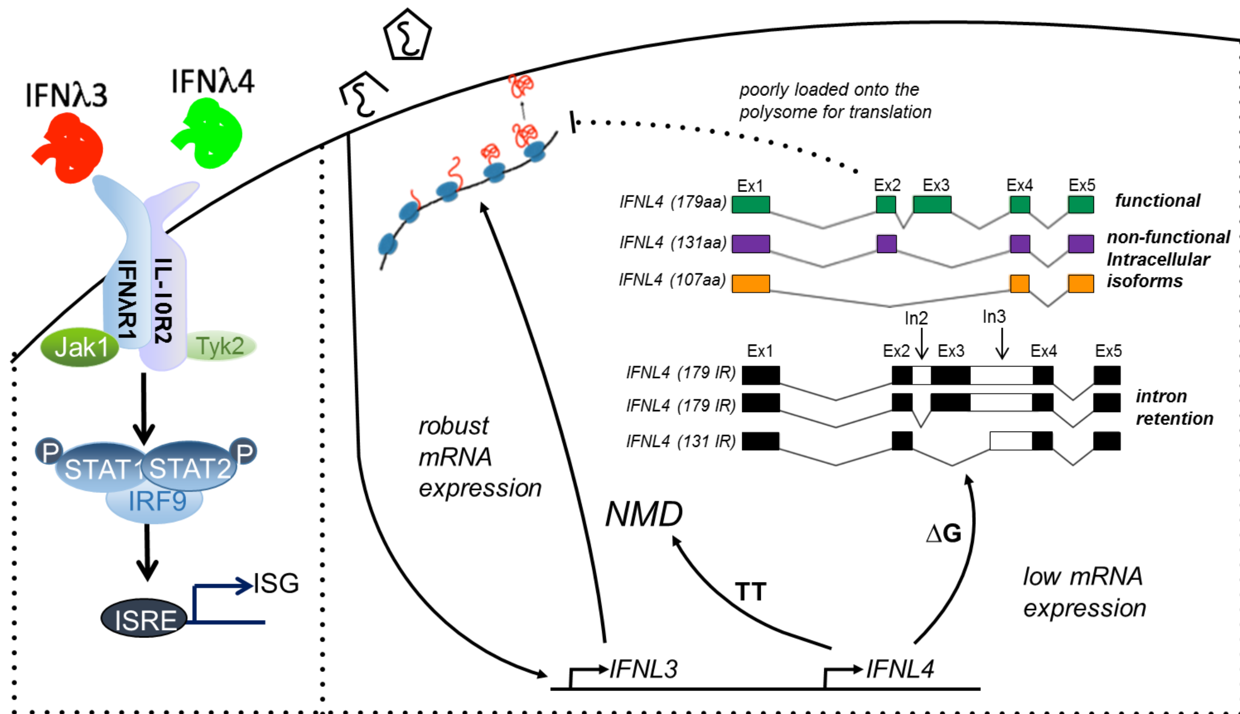
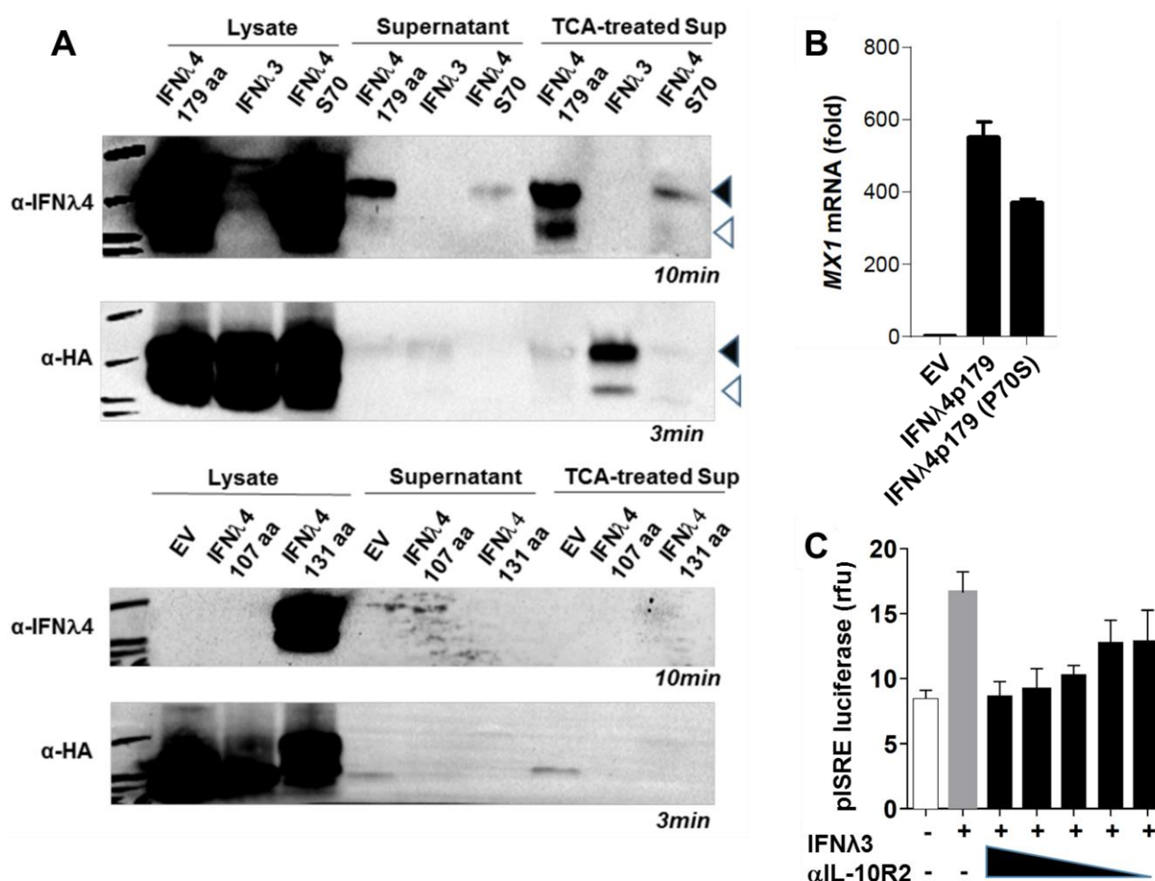


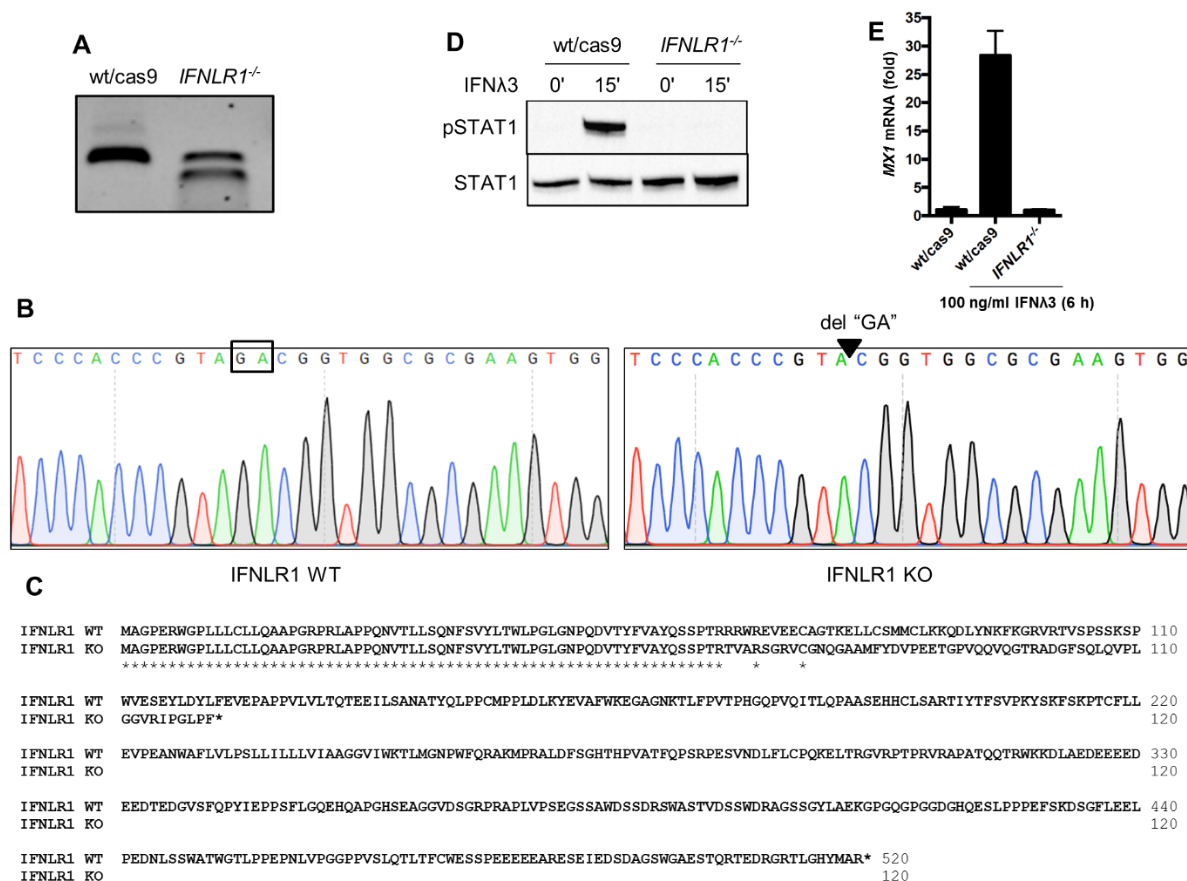
Figure 3.9. IFNλ4 expression is suppressed during viral infections.

IFNλ4p179 has similar antiviral properties as IFNλ3, signaling exclusively through the extracellular IFNλR1 and IL-10R2 heterodimer to activate STAT1 and induce ISGs. During viral infections, several mechanisms are in place which suppress the expression of the functional *IFNL4* isoform compared to robust *IFNL3* expression.



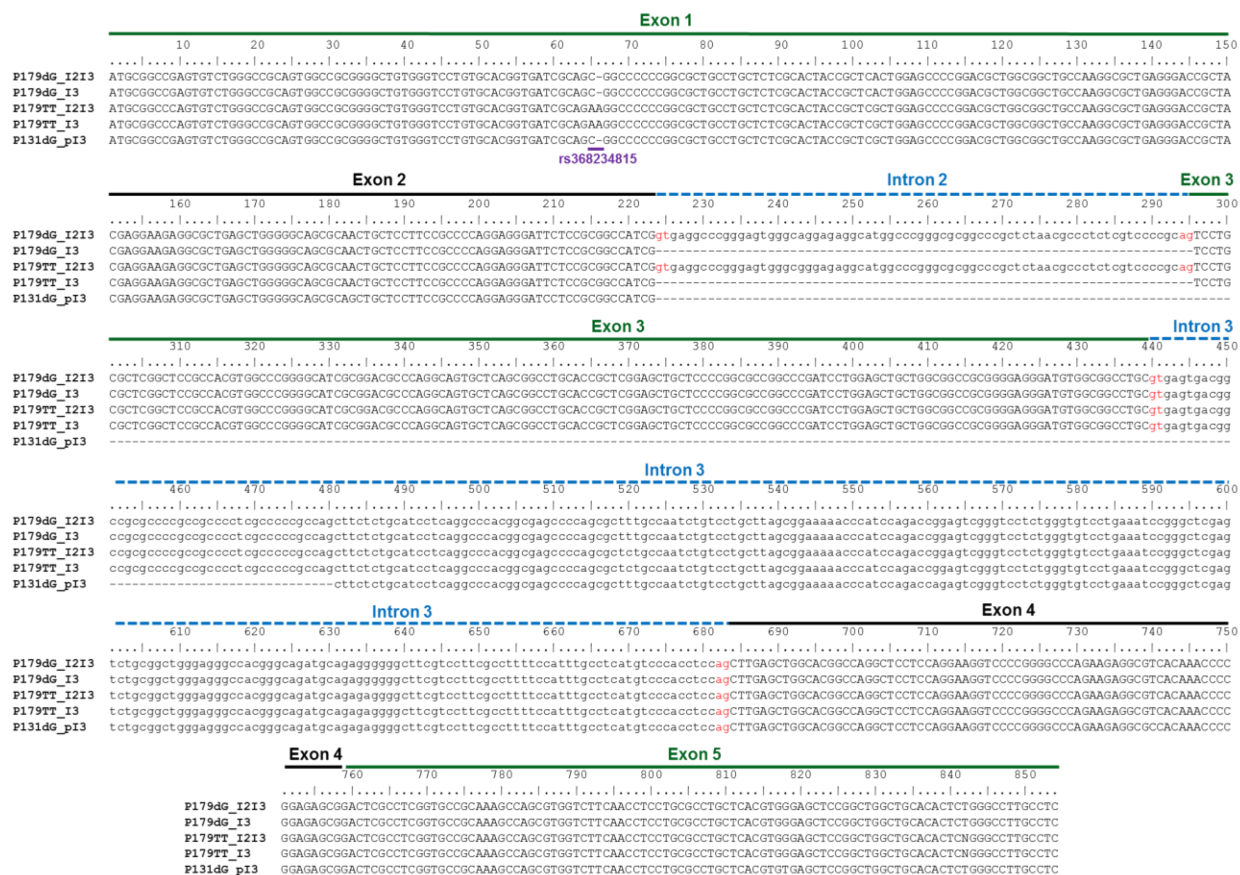
Suppl. Figure 3.1. Titration of IL-10R2 antibody.

(**A**) Immunoblot of lysates, neat supernatants and supernatants concentrated by trichloroacetic acid (TCA) of HA-tagged IFNλ4p179 (P70), IFNλ4p179 P70S variant, IFNλ4p131, IFNλ4p107, and IFNλ3 overexpressed in Huh7 hepatocytes. Arrows indicate glycosylated (solid) and non-glycosylated (clear) forms, respectively. (**B**) *MX1* induction upon overexpression of IFNλ4p179 P70 and IFNλ4p179 P70S variants in Huh7 cells. (**C**) pISRE luciferase reporter activity in transfected Huh7 hepatocytes stimulated with rhIFNλ3 in the presence of varying doses of αIL-10R2 antibody (2-6 µg/ml).



Suppl. Figure 3.2. Generation and characterization of *IFNLR1*$^{-/-}$ hepatocytes.

(**A**) CRISPR/cas9-mediated *IFNLR1* targeting confirmed by T7 endonuclease I cleavage assay; two bands in the *IFNLR1*$^{-/-}$ lane indicates targeting of genomic DNA by CRISPR. (**B**) Sequencing chromatogram of the CRISPR target region in the human *IFNLR1* gene. Clonal cell line derived from CRISPR targeted *IFNLR1*$^{-/-}$ cells have a two base pair deletion (ΔGA) compared to wild type non-targeted cells. (**C**) Alignment of wild type *IFNLR1* amino-acid sequence with truncated CRISPR targeted *IFNLR1*$^{-/-}$ sequence due to a two base pair deletion resulting in a pre-mature stop codon. (**D**) Immunoblot of pSTAT1, total STAT1 and (**E**) *MX1* gene induction in wild type and *IFNLR1*$^{-/-}$ PH5CH8 cells stimulated with rhIFNλ3 for 6 h.



Suppl. Figure 3.3. Sequence alignment of intron-retaining *IFNL4* isoforms.

The above sequences were identified from a cDNA library from HepG2 cells treated with poly(I:C). Alternating exons are labelled with solid lines and capital letters, introns with dotted lines and small letters, position of rs368234815 in purple, and intronic splice donor and acceptor sites in red. Multiple sequence alignment was performed using CLUSTALW in BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

```
          10        20        30        40        50        60        70        80        90
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hIFNL3    TGACCCTTCCGCCAGTCATGCAACCTGAGATTTTATTTATAAATTAGCCACT-----TGGCTTAATTTATTGTCACCCAGTCGCTATTTA
hIFNL2    .......C..A.........................................-----..T...........C..................
hIFNL1    ------------------..AC...CC.C.CC........GCGC.GAG.C..ACTCC.TC...........TC.T.T..CC.TT......

                   100       110       120       130       140       150       160
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
hIFNL3        TGTATTTGTGTATGTAAATCCAACTCACCTCCAGGAAAATGTTTATTTTTCTACTTTTTGAAATCCTTGTTGAAATAAA
hIFNL2        ...........G...............................................AT..C...............
hIFNL1        ..A.GC..C------.GCC.TG...G.GACAT...GCTGA.......G..T.......-AT.CATTA..CAC.......
```

Suppl. Figure 3.4. Alignment of 3' UTR sequences of human *IFNL1*, *IFNL2* and *IFNL3* genes.

The canonical polyadenylation signal (AATAAA) is labeled in red. Dots in *IFNL2* and *IFNL1* sequences indicate sequence homology to *IFNL3*. The stop codon (TGA) is underlined. Multiple sequence alignment was performed using CLUSTALW in BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

3.6 TABLES

Table 3.1. Sequences of primers and probes used to clone, detect and genotype *IFNL4*.

Primer/probe name	Sequence (5'>3')
IFNL4-cDNA fwd	ATGCGGCCGAGTGTCTGG
IFNL4-cDNA rev	GAGGCAAGGCCCAGAGTGT
IFNL4-FL-HA-fwd	GAATTCGCGCGTATGCGGCCGAGTGTCTGG
IFNL4-FL-HA-rev	ACTAGTGAGGCAAGGCCCAGAGTGT
IFNL4-RACE-nest1	GTGGTCTTCAACCTCCTGCG
IFNL4-RACE-nest2	CTGCTCACGTGGGAGCTCCG
IFNL4-Genot-fwd	ATGCGGCCGAGTGTCTGG
IFNL4-Genot-rev	GCTCCAGCGAGCGGTAGTG
IFNL4-ΔG probe	GGCCGCTGCGAT
IFNL4-TT probe	GGCCTTCTGCGAT
IFNL4P179-fwd	CGATCCTGGAGCTGCTG
IFNL4P179-rev	TTTGTGACGCCTCTTCTGG
IFNL4P179 probe	CAAGGCAGGCCGCCACATC
IFNL4P131-fwd	CAACTGCTCCTTCCGCC
IFNL4P131-rev	GGGTTTGTGACGCCTCTTC
IFNL4P131 probe	CGGCCATCGCTTGAGCTGG
IFNL4P107-fwd	CTCTCGCACTACCGCTC
IFNL4P107-rev	TTTGTGACGCCTCTTCTGG
IFNL4P107 probe	CGCTGAGGGACCGCTACCTTG
IFNL4 IR-fwd	GTCCTTCGCCTTTTCCATTTG
IFNL4 IR-rev	AGACCACGCTGGCTTTG
IFNL4 IR probe	CGTCACAAACCCCGGAGAGCG

Chapter 4. CONCLUDING REMARKS

Alternative splicing is a powerful tool used by eukaryotes to increase diversity in their proteome from a finite genome. Exon skipping, use of alternative splice sites and selection of different transcription start or polyadenylation sites generate proteins containing different sets of functional domains, allowing organisms to fine-tune their biological activities in different tissues or physiological contexts. In addition, intron retention to target transcripts for nonsense-mediated decay provides an opportunity to suppress particular transcripts but not others from the same gene. However, it is still difficult to accurately predict how splice variation changes protein function without methodical experimental validation. Furthermore, alternative splicing in many immune genes remains poorly studied, yet it may hold important clues for their activity.

We have investigated how the isoforms of IL-22BP and IFNλ4 differ in their functions and examined their differential expression under various stimuli. Both are generated by splice variation of their parent genes in patterns seemingly specific to humans and higher primates. While these cytokine families appear unrelated at first glance, activating different STAT pathways and occupying quite different physiological niches, the combined results from both studies add fascinating diversity to how protein isoforms alter immune function. These splice variants are important determinants of cytokine function that escape detection in genetic association studies and are also poorly modelled in animals due to high species specificity of splicing decisions. In approaching the question of how isoforms differ in both genes, we first examined their biological activities using overexpression systems and purification of glycosylated recombinant protein from an eukaryotic expression system. With the knowledge of their differential activities, we could then examine the relative endogenous expression of each isoform in different human cells or tissues, both in the steady state and in response to different stimuli. This would allow us to put the role of

alternative splicing in the appropriate immune contexts and relate our findings to putative roles in diseases associated with *IL22RA2* or *IFNL4*.

Alternative splicing of human IL-22BP results in three variants of the soluble receptor that perform different functions in IL-22 signaling. The inclusion of exon 3 in IL-22BPi1 renders it inactive, while the inclusion or exclusion of exon 5 and most of exon 6 generates two variants of the protein with two different levels of antagonism, IL-22BPi2 and IL-22BPi3. This splicing flexibility allows it to fine-tune IL-22 signaling in a gradient generated between homeostatic and inflammatory conditions. The different biological activities of IL-22BP isoforms may ascribe new roles to *IL22RA2* genetic polymorphisms that have been associated with inflammatory disease. Like variants both distant and proximal to splice junctions in *CFTR* (described in section 1.1.2), we expect that polymorphisms in *IL22RA2* lie in or tag cis-regulatory factors skew alternative splicing towards particular isoforms under specific inflammatory stimuli, adjusting the rheostat to a pathological setting that fails to correctly control IL-22 activity.

The hand of the spliceosome is heavy in changing the activity of IL-22BP. IL-22BPi3 is generated by skipping of exon 5 and an alternative splice site in exon 6 that encodes a frame shift and truncates the protein. This results in the absence of the second fibronectin domain of IL-22BPi2 that is known to contain one important stabilizing interaction with IL-22. While this alone causes major functional changes to the antagonistic activity of IL-22BP, different expression patterns of IL-22BPi2 and IL-22BPi3 in homeostasis and inflammation make it clear that tissue-specific and myeloid-specific factors further alter the biological relevance of IL-22BPi2 and IL-22BPi3 by transforming splice site usage. Meanwhile, the inactivation of IL-22BPi1 by exonization of a retrotransposon recalls previous observations in IL-15, which uses alternative transcription start sites and alternative exon 4 usage results to generate different signal peptides

that control the intracellular trafficking, secretion and consequent activity of the cytokine. In both these cases, alternative splicing enables expression of a transcript, yet limit its bioactivity; at least for IL-22BPi1, this mechanism emerged in higher primates and appears to have been evolutionarily selected for since. Since the dose and location of IL-22 signaling dictates its highly context-dependent roles, perhaps separating the parent genes of the membrane-bound and soluble receptors allows more flexible diversification of isoforms that have provided greater dynamic range and sensitivity for tweaking IL-22 signaling. The gradient of resistance created by the balance of IL-22BPi2 and IL-22BPi3 parallels the consequences of alternative splicing in *IL6R*; exclusion of specific exons creates receptor isoforms with different binding affinities for IL-6 and, therefore, different potencies for activating IL-6 signaling.Now that we have shown the IL-22BP isoforms have different effects on IL-22 signaling, the next important step would be to determine whether the expression of specific isoforms are being dysregulated in human disease: that is, whether it is IL-22BPi2 or IL-22BPi3 that shows aberrant expression, or whether alternative splicing favors the generation of IL-22BPi1 over the other two isoforms to limit IL-22BP activity. In addition to using the custom-designed qPCR probes generated during the course of my thesis, advanced technologies such as next-generation sequencing may be able to provide a comprehensive, unbiased answer even from limited amounts of clinical samples.

In contrast, we found that the generation of alternative splice forms of IFNλ4 serves mostly as a mechanism to limit expression of a functional cytokine. In addition to three known protein-coding isoforms, we identified a high abundance of transcripts generated with retained introns, which would target the *IFNL4* mRNA for NMD and prevent expression of a productive cytokine. Yet these transcripts are expressed in preference to the protein-coding isoforms, and among these only one is truly secreted from the cell and active in signaling. As discussed in section 1.1.3,

splicing of transcripts to achieve NMD is being increasingly recognized as a directed process that efficiently suppresses the expression of an active, functional protein, so this mechanism is not without precedent. For IFNλ4, splicing is exploited to suppress a transcript whose expression is genetically associated with detrimental effects in infection. This property is truly unique to IFNλ4 as type I IFNs are single-exon transcripts that cannot undergo alternative splicing, while alternative transcripts have not been found for type II and III IFNs. Thus, our findings present a fresh perspective on how it is regulated differently from other IFNs. Along with the paradoxical pro-viral association, for which an explanation still has not been satisfactorily presented, splicing as a regulatory mechanism adds to IFNλ4's status as the oddball of the IFN family. It would be interesting to investigate whether similar regulation by alternative splicing occurs in the other type III IFNs. Although only one transcript has been found for IFNλ1 and IFNλ2 each, and only two transcripts for IFNλ3, there has not been an unbiased profiling as we have performed by generating a cDNA library. Perhaps alternative splicing could be an important regulatory mechanism specific to the resolution of type III IFN responses, which would be crucial for preventing the onset of autoimmunity from excessive IFN signaling. In addition, while we have studied the role of IFNλ4 in virally-infected hepatocytes, it remains to be seen whether any of the IFNλ4 isoforms can perform additional roles in other immune responses independent of ISRE activation or ISG induction. In turn, regulation of *IFNL4* by alternative splicing may be more dominant in viral infection than in response to other immune stimulations.

It bears repeating that our ability to obtain these insights required a combination approach in both studies: first to determine the biological function of overexpressed or purified proteins and then to examine their endogenous expression. These aspects were often examined separately in previous IL-22BP and IFNλ4 studies, which made it difficult to determine overall where and how

they would act. Being able to clarify both how IL-22BPi3 functioned and where it was expressed in comparison to IL-22BPi2, for example, allowed us to propose the rheostat model of regulation, whereas such a consideration could not be confidently made with either finding in isolation. Similarly, while the discovery of intron retention revealed a new, important regulatory mechanism for IFNλ4, verifying the biological inactivity of the additional protein-coding isoforms IFNλ4p107 and IFNλ4p131 against hypotheses in the field for pro-viral functions of these proteins provided a larger context that even “productive” transcripts contributed to suppression of IFNλ4 activity. Therefore, both remain important questions that should be approached simultaneously when studying novel regulators of cytokine signaling.

Overall, our studies on the human IL-22BP and IFNλ4 isoforms have united studies of function and regulation to demonstrate the central role alternative splicing has to controlling signaling in cytokine responses. The mechanisms we have discovered for the regulation of IL-22BP and IFNλ4 function have precedent in other genes and transcriptomic networks, so it is with delight that we add mucosal immunity and antiviral responses among their number. Furthermore, our findings on the function of alternative splicing in these two gene families are exciting additions to the lush regulatory landscape of the immune system, a network that especially requires tight control of timing, dose and strength of its responses.

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VITA

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