The immune modulatory role of interferon lambda on human B-cell functions

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Indien

Basel, 2019

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Statement of my thesis

This work was carried out in the group of Dr. Adrian Egli in the Applied Microbiology Research group at the Department of Biomedicine, University Hospital Basel and University of Basel, Switzerland.

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My PhD thesis is written as a cumulative dissertation. It consists out of an executive summary, a general introduction, aims of my PhD thesis, a result section composed out of manuscripts ready for submission and published articles, a discussion and an outlook.

Table of Contents

1 Abbreviations 1

2 Summary 5

3 Introduction 7

3.1 Interferons (IFNs): an overview 7
3.1.1 Type I IFNs 7
3.1.2 Type II IFNs 8
3.1.3 Type III IFNs 9

3.2 IFN-λ expression and signaling pathways 9

3.3 Interferon lambda receptor expression 11

3.4 The impact of IFN-λ SNPs 12
3.4.1 IFN-λ SNPs in innate immunity 12
3.4.2 IFN-λ SNPs in adaptive immunity 13

3.5 Role of IFN in infectious diseases 14

3.6 Role of IFN in other diseases 15
3.6.1 IFN-λ in cancer 15
3.6.2 IFN-λ in autoimmune diseases 15

3.7 Immune modulatory role of IFN-λ 16

3.8 mTOR pathway 17
3.8.1 mTOR and B-cells 19

3.9 B-cell activation and differentiation 20
3.9.1 T-independent B-cell activation and response 21
3.9.2 T-dependent B-cell activation and response 21
3.9.3 Memory B-cell activation and response 22

3.10 B-cell differentiation and transcription factors 23

4 Aims of the thesis 24

5 Results 25

5.1 Interferon-λ enhances the differentiation of naïve B-cells into plasmablasts via mTORC1 pathway 25

5.2 Interferon Lambda: Modulating Immunity in Infectious Diseases 58

5.3 An ELISA Based Binding and Competition Method to Rapidly Determine Ligand-receptor Interactions 72

5.4 An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers 84

5.5 IL-28B is a key regulator of B- and T-cell vaccine responses against influenza 97
5.5.1 Abstract/ Summary 98

5.6 Sequential models in regression of vaccine-induced antibody titers in stem cell transplant recipients 99
5.6.1 Abstract/ Summary 100

5.7 Influenza vaccination of cancer patients during PD-1 blockade induces serological protection but may raise the risk for immune-related adverse events 101
5.7.1 Abstract/ Summary 102
5.8 Additional papers 103
6 Discussion 104
6.1 *In vitro* ELISA assays to study IFN-λ1, -λ2, -λ3 and the IFNLR1 interactions 104
6.2 Responsiveness of immune cell populations to IFN-λ 107
6.3 IFN-λ increases mTORC1 activity in B-cells 107
6.4 IFN-λ enhances cell cycle/proliferation of the BCR-activated B-cells 108
6.5 IFN-λ boosts naïve B-cells into plasmablasts differentiation through mTORC1 109
6.6 Overall conclusion 109
7 Outlook 111
8 References 113
9 Acknowledgement 126

1 Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AA	Amino Acid
AID	Activation-induced cytidine deaminase
APCs	Antigen presenting cells
ASCs	Antibody secreting cells
BCR	B-cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
BV	Brilliant violet
CD	Cluster of differentiation
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocytes
CTV	Cell trace violet
CXCL-10	C-X-C motif chemokine 10
EIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
GAS	Gamma interferon activation site
GC	Germinal center
GDP	Guanosine diphosphate
GO	Gene Ontology
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma

IFN-ε	Interferon-epsilon
IFN-κ	Interferon-kappa
IFN-λ	Interferon-lambda
IFN-ω	Interferon-omega
IFNAR	Interferon alpha-receptor
IFNs	Interferons
IgA	Immunoglobulin A
IGF	Insulin like- growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin-10
IL-21	Interleukin-21
IL-6	Interleukin-6
IL10RB	Interleukin 10 receptor beta
IL28RA	Interleukin 28 receptor alpha
IRF-4	Interferon regulatory factor-4
IRF-9	Interferon regulatory factor-9
ISG	Interferon-stimulated genes
ISGF-3	IFN-stimulated gene factor-3
ISRE	Interferon-stimulated response element
JAK1	Janus Kinase 1
LD	Linkage disequilibrium
LPS	Lipopolysaccharides
MHC	Major histocompatibility complex
mLST8	Mammalian lethal with SEC13 protein 8
mTORC1	Mechanistic/mammalian target of rapamycin
MX1	Interferon-induced GTP-binding protein Mx1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK-cells	Natural killer cells
OAS1	2′-5′-oligoadenylate synthetase.
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell

PBs	Plasmablasts
PCs	Plasma cells
pDCs	Plasmacytoid dendritic cells
PI3K	Phosphoinositide 3-kinase
PKCα	Protein kinase cα
PRDM1	PR domain zinc finger protein 1
PRRs	Pattern-recognition receptors
RLR	RIG-1-like receptor
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
S6K	S6 kinase
SARS	Severe acute respiratory syndrome
SD	Standard deviation
SGK1	Serum- and glucocorticoid-induced protein kinase 1
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TD	T-cell dependent (or) Thymus dependent
Tfh	Follicular T helper cells
TI	T-cell independent (or) Thymus independent
TLR	Toll like receptor
TLR	Toll-like receptors
TSC 1	Tuberous sclerosis 1
TSLP	Thymic stromal lymphopoietin
Tyk2	Tyrosine kinase 2
USP-18	Ubiquitin specific peptidase
VSV	Vesicular stomatitis virus
WNV	West Nile virus
XBP1	X-box binding protein 1

Units:

CPM	Counts per million
h	hour
K_D	dissociation constant
min	minute
ml	milliliter
ng	nanogram
µg	microgram

2 Summary

Interferon lambda (IFN-λ) mediates a crucial antiviral response to protect the host cells during viral infection, as well as functioning as a potential immune modulator. In this thesis, we investigated the immune modulatory role of IFN-λ in B-cells. In the first part, we established an ELISA-based *in vitro* assays to study IFN-λ ligands and its receptor (Interferon lambda receptor 1 - IFNLR1 and IL10R2) interactions. First, we determined the receptor - ligand (IFNLR1 - IFN-λ1-3) dissociation constant (K_D) as a measure of the ligand and receptor binding affinity. We found that IFN-λ1 showed higher binding affinity to IFNLR1 compared to IFN-λ2 and IFN-λ3. Further, we screened antagonistic peptides, which act to interfere in IFNLR1 - IFN-λs interactions. The peptides are designed to compete with IFN-λs at their IFNLR1 binding sites and this experiment thus allowed us to develop a molecular understanding of the interaction. We have also performed the small molecules screen to identify the potential substances targeting for IFN-λ signaling, it will allow us the modulation of IFN-λ signaling which is an interesting target for a broad range of applications. In the second part, we screened the immune cell populations to understand the direct response to IFN-λ, to resolve discrepancies with previously reported data. B-cells and the subpopulations of naïve, class switched and non-class switched memory B-cells were found to directly respond to IFN-λs. On the other hand, T-cells, NK-cells and monocytes did not show any response to IFN-λs. Since B-cells showed a response to IFN-λ, we performed transcriptomic profiling of sorted B-cell, to examine the immune modulatory role of IFN-λ in B-cells. On the basis of B-cell transcriptome analysis and follow up *in vitro* experiments, the IFN-λ increases the mTORC1 (mammalian/mechanistic target of rapamycin complex 1) activity in B-cells, upon B-cell receptor (BCR) cross linking with anti-IgM. The BCR and IFN-λ signaling cascade engage the mTORC1 pathway via phosphoinositide 3-kinase (PI3K). However, it needs further evaluation to see if IFN-λ increase the mTORC1 activity indirectly via ISGs. IFN-λ enhances the BCR-induced cell cycle progress though this mTORC1 and IFN-λ alone did not induce any cell proliferation. Consequently IFN-λ further boosts the differentiation of naïve B-cells into plasmablasts upon BCR-activation, so the cells gain effector functions such as cytokines release (IL-6, IL-10) and antibody production

(IgM). The role of IFN-λ in plasmablast differentiation was previously not known. In this study, we have shown how IFN-λ functionally binds to B-cells and that it systematically boosts the differentiation of naïve B-cells into plasmablasts via mTORC1 and cell cycle progression in BCR-activated cells.

3 Introduction

3.1 Interferons (IFNs): an overview

IFNs are a large group of signaling proteins also known as cytokines, induced by host cells in response to a variety of viruses and other pathogens. Due to their ability to 'interfere' with viral infections, they are termed interferons [1]. IFNs play a crucial role in protecting host cells from many infectious diseases caused by viruses, bacteria, fungi and parasite [2-7]. IFNs are also involved in other tasks such as pro- and anti-inflammatory actions, have regulatory roles in autoimmune diseases, facilitate immune cells maturation, and control of tumor cell proliferation [8-15]. The interferon family is represented by three major classes, designated type I, type II, and type III IFNs [16].

3.1.1 Type I IFNs

The type I IFN family members include 13 subtypes of IFN-α (IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17, and -α21) as well as IFN-β, IFN-ε, IFN-κ and IFN-ω in human [17]. These type I IFNs exclusively bind to the cell surface IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains [18]. The Janus-activated family kinases (JAKs), tyrosine kinase 2 (Tyk2) and JAK1 are associated with the cytoplasmic domain of IFNAR1 [19]. The receptor engagement leads to the phosphorylation of signal transducer and activator of transcription (STAT)1 and 2. STAT1 and STAT2 interact with IFN regulatory factor (IRF)-9, forming a complex called IFN-stimulated gene factor (ISGF)-3, which translocates into the nucleus. This complex binds to specific nucleotide sequences called IFN-stimulated response elements (ISREs) and activates the expression of hundreds of IFN-stimulated genes (ISGs) (**Fig. 1**). Moreover, in specific cell types, STAT homodimers or heterodimers form in different combinations between STAT1, 2, 3, 5, 6 [20-23]. Type I IFNs exhibit a potent antiviral effect and enhance the activity of natural killer (NK)-cells and macrophage functions and increase the expression of major histocompatibility complex (MHC) class I on virus infected cells [24-27]. Type I IFNs modulate T-cells functions, including generation and activation of cytotoxic T lymphocytes (CTL) [28,29]. Nearly every cell type in the body can produce type I IFNs [5].

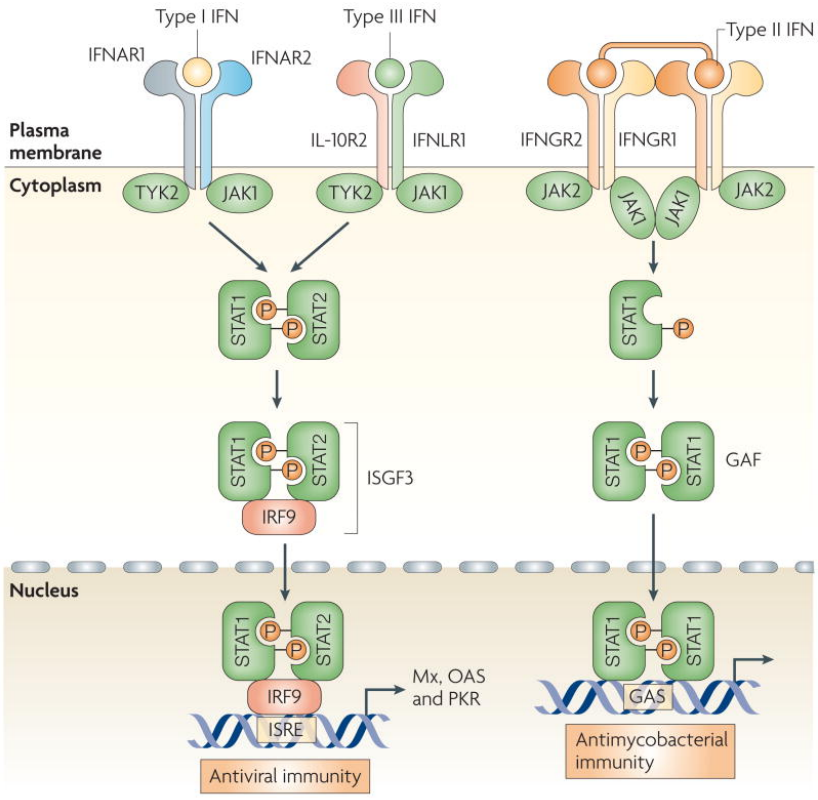

Fig. 1: IFN signaling: an overview

The interaction between Type I IFNs and IFNAR (heterodimer of IFNAR1 and IFNAR2); Type II IFN and IFNGR (IFNGR1 and IFNGR2); Type III interferon and IFNLR (IFNLR1 and IL-10R2). All activate classical JAK-STAT pathways, leading to the translocation of transcription factor complexes ISGF3 or GAF (IFN-γ) into the nucleus, which bind to ISRE or GAS promotor sites and activate ISG expression. From Sadler AJ and Williams BR, Nat Rev Immunol, 2008, 8 (7).

3.1.2 Type II IFNs

IFN-γ is the only representative of the type II IFN family. It binds to the heterodimeric IFN-γ receptor (IFNGR), which consists of two chains: IFNGR1 and IFNGR2 [30-32]. JAK1 and JAK2 tyrosine kinases are associated with the cytoplasmic domain of IFNGR1. The phosphorylation of two STAT1 molecules allows them to form a homodimeric complex, which moves to the nucleus, where it induces the expression of genes with gamma interferon activation site (GAS) elements (**Fig. 1**) [22]. IFN-γ activates NK-cells and macrophages and plays a major role in both innate and adaptive immune responses against viral, fungal and bacterial infections [33]. Further, it induces the expression of MHC II molecules. NK-cells, cytotoxic T-cells and T helper (Th) cells type 1 (Th1) mainly release IFN-γ [34-36].

3.1.3 Type III IFNs

Type III IFNs are a recently discovered group of IFNs [37,38]. Type III IFNs show about 5-18% amino acid sequence identity with type I IFNs [39]. In humans, the Type III IFN family consists of four members: IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4, which are encoded by genes located on chromosome 19 (19q13.13 region). Among these IFN-λ family candidates, IFN-λ1 and IFN-λ2 share 81% amino acid identity, whereas IFN-λ2 and IFN-λ3 share 96% amino acid identity. IFN-λ4 and other IFN-λs share only about 28% amino acid identity [37-41]. Among these IFN-λs only IFN-λ1 is N-linked glycosylated [37]. In mice, only IFN-λ2 and IFN-λ3 are functional and IFN-λ1 and IFN-λ4 are pseudogenes, encoded by genes which all are located on chromosome 7 (7A3 region) [39,42]. Both IFN-λ2 and IFN-λ3 are glycosylated [42,43]. The antiviral properties of IFN-λ have been studied extensively with many viruses [44-48]. The IFN-λ mediated immunity is further extended to other pathogens like bacteria, parasites, and fungi [49-53].

3.2 IFN-λ expression and signaling pathways

IFN-λ is expressed mainly in response to many viruses and bacteria [53]. Sensing of pathogen-associated molecular patterns (PAMPs) by specific pattern-recognition receptors (PRRs) induces IFN expression. PRRs such as membrane-bound Toll like receptors, cytoplasmic receptors like RIG-I, and cytosolic DNA sensor Ku-70 lead to the activation of the NK-kB transcription factors and IRFs, which induce the expression of IFN-λs [54-58]. The following figure describes the expression of IFN-λs through various pathways (**Fig. 2**).

Epithelial cells are the dominant producer of IFN-λ, such as respiratory epithelial cells against influenza virus in the lung and airway; epithelial cells against rhinovirus; gut epithelial cells against enteric virus [59-63]. Likewise, hepatocytes produce type III IFNs during the acute stage of HCV infection [64]. Immune cell populations, such as plasmacytoid dendritic cells (pDCs), monocytes and BDCA3+ myeloid dendritic cells also produce IFN-λ in response to double-stranded RNA (poly I:C) or viral infections [65-68]. A recent study has described that the activation of TLR5 by *Salmonella* might induce the expression of IFN-λ [69].

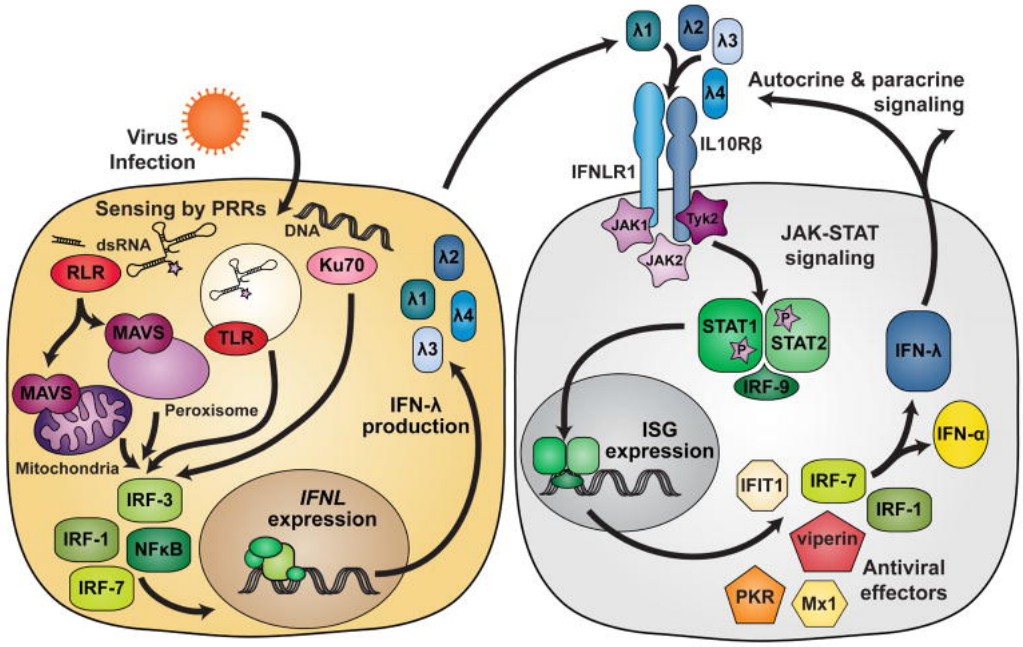

Fig. 2: IFN-λ release and signaling pathways

Pathogens are sensed by pattern recognition receptor (PRRs), including Toll like receptors (TLRs), cytoplasmic RIG-I-like receptor (RLR) and DNA sensor Ku70, which activate multiple signaling pathways and induce IFN-λ expression. On the other side, IFN-λs bind to IFNLR (composed of IFNLR1 and IL10Rβ) and activates JAK-STAT downstream signaling pathway. The expression of IFN stimulated genes (ISGs) lead the effector functions against viruses. From Lazear HM *et al*., Immunity, 2015, 43 (1).

IFN-λ1-4 all bind to a heterodimeric surface receptor, which is composed of the unique IFNLR1 (also known as IL-28RA) chain and the ubiquitously expressed IL10Rβ (IL-10R2) chain. The IL10Rβ chain is also a part of the receptor complexes for IL-10, IL-22 and IL-26 [70-72]. The initial binding of IFN-λs to the IFNLR1 chain causes rapid conformational changes and recruits the second chain, IL10Rβ, to form a receptor complex. The Janus tyrosine kinases JAK1, JAK2, and Tyk2 are associated with the receptor complex and mediate the trans-phosphorylation of IFNLR1 that facilitates transient docking site for cytosolic STAT proteins [39]. The phosphorylation of STAT1 and STAT2 causes the heterodimer to interact with IRF-9 and form a transcription factor complex ISGF-3. This complex then translocates into the nucleus, where it binds to specific ISRE promotor region and activates the transcription of over a hundred ISGs. IFN-λ induced expression of the ISGs promotes the antiviral and other cellular responses (**Fig. 2**).

Although type I and type III signaling pathways seem similar with their cascades, the signaling kinetics differ, mainly due to their own specific feedback mechanisms. The ISG ubiquitin specific peptidase (USP-18) is expressed from both signaling cascades: it acts as a negative regulator for type I, but not type III signaling. Mechanistically, USP18 binds to IFNAR2 and blocks the further interaction between IFNAR2 and JAK1 [73,74].

3.3 Interferon lambda receptor expression

Nearly every cell type expresses the receptor for type I (IFN-α/β) and type II (IFN-γ) interferons [75]. In contrast, the expression of IFN-λ receptor is limited to epithelial cells, especially at mucosal surfaces, gut epithelial cells, hepatocytes and very few immune cell types [46,63,76-78]. The primary hepatocytes initially show baseline response to IFN-λ: treatment with IFN-α significantly increases the mRNA level of IFNLR1 [79]. Likewise, cytomegalovirus (CMV) infection in fibroblasts increases the mRNA level of IFNLR1 about two-fold; however, protein expression levels were found to be unaltered [80]. Furthermore, the endothelial cells in the blood-brain barrier show limited response to IFN-λ during West Nile virus (WNV) infection in mouse [52].

Only specific immune cell types express IFNLR1. In mouse immune cells, only neutrophils directly respond to IFN-λ [81,82]. The NK-cells do not express the IFN-λ receptor, but it indirectly gets activated via macrophages during influenza infection [83]. In human immune cells, many reported data show inconsistencies with the expression of IFNLR1. Human pDCs strongly express IFNLR1 and respond to IFN-λ. NK-cells seem not to express IFNLR1. The IFNLR1 mRNA is measurable in B-cells, but the reports differ on whether B-cells can directly respond to IFN-λ. The expression of functional receptor IFNLR1 in T-cells and monocytes is subject to ongoing debate [44,84-91]. Monocyte-derived macrophages express IFNLR1 and respond to IFN-λ [92,93]. Overall, the cell specific receptor expression and the signaling kinetics make IFN-λ signaling distinct from that of other interferons.

3.4 The impact of IFN-λ SNPs

Genome-wide association studies (GWAS) describe a number of IFN-λ single nucleotide polymorphisms (SNPs), which are strongly correlated with important clinical outcomes. The following figure shows the location of SNPs within the IFN-λ genes (**Fig. 3**).

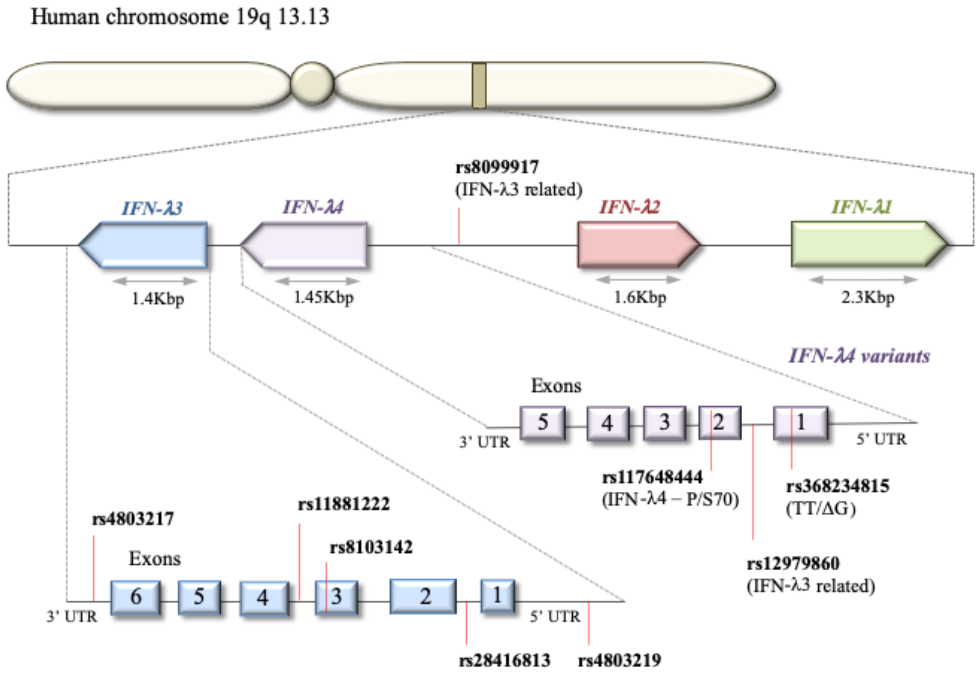

Fig. 3: Location of IFNL genes and the SNPs

The IFN-λ genes are located on human chromosome 19 (19q 13.13). *IFN-λ1, IFN-λ2,* and *IFN-λ3* genes are functional. *IFN-λ4* generally exists as a pseudogene: only a subset of the human population carries the SNP rs368234815 with ΔG frameshift mutation in the first exon of *IFN-λ4*, producing an in-frame protein. Key single-nucleotide polymorphisms (SNPs) in coding and non-coding regions of IFN-λ genes are indicated. Adapted from Syedbasha M et Egli A, 2017, Front Immunol, 8 (119).

3.4.1 IFN-λ SNPs in innate immunity

Many studies have demonstrated the impact of IFN-λ SNPs in innate immunity over the last 10 years. The location of SNPs in IFN-λ genes are described in Figure 3. Several SNPs in the IFN-λ3 locus correlate with the response of IFN-based therapeutics and spontaneous clearance of hepatitis C virus (HCV) [94-97]. The individuals carrying the rs12979860-C allele (CC) respond better to standard HCV treatment (pegylated-IFN with ribavirin) than the individuals carrying the rs12979860-

T allele (CT or TT). The unfavorable rs12979860-T allele is more prevalent in those of African descent compared to those of Asian or European descent. Similarly, those carrying the rs8099917-T allele (TT) respond better than individuals with the TG or GG allele at this locus. This favorable rs8099917-T allele is more prevalent in Asians and Europeans compared to Africans. These two SNPs (rs12979860 and rs8099917) are in linkage disequilibrium (LD) [95,98]. The molecular mechanism behind many IFN-λ SNPs and their associations with treatment outcome is not understood.

A recent study has described the mechanism of the IFN-λ3 SNP rs4803217 where the occurrence of the G allele is associated with HCV clearance, whereas the T allele favors HCV persistence [99]. HCV regulates two microRNAs: miR-208b and miR-499a-5p, which target the 3' UTR of IFN-λ3. The T allele enhances the binding of virus induced microRNAs at the 3' UTR and facilitates AU-rich element mediated decay of IFN-λ3 [100]. The ss469415590 (TT/ΔG) allele causes a frameshift mutation ablating the expression of IFN-λ4. Approximately 40% of Caucasians have this SNP. The ΔG allele in IFN-λ4 is associated with HCV persistence, whereas a TT allele favors viral clearance [27,53].

3.4.2 IFN-λ SNPs in adaptive immunity

The impact of IFN-λ SNPs in the adaptive immune response have been described. Previously reported data indicates that the IFN-λ3 SNP rs10853727 minor alleles (AG or GG) are associated with high post-vaccine antibody titers in measles vaccinated children [101]. The IFN-λ3 SNP rs8099917 minor alleles (TG or GG) correlate with increased seroconversion rate after influenza vaccination. In addition, rs8099917 minor alleles (TG or GG) show low levels of Th1 cytokines (IFN-α, IL-2 and IL-6) secretion in PBMCs with influenza stimulation. On the other hand, the major alleles correlate with low level of Th2 cytokines (IL-4, IL-5 and IL-13) and antibody production [93]. However, the mechanism behind these SNPs are yet to be understood.

3.5 Role of IFN in infectious diseases

IFN-λ plays an important role in controlling a wide variety of viral infections. Many *in vitro* and *in vivo* studies have been explained by IFN-λ-mediated immunity against viruses in the liver, respiratory tract, gastrointestinal mucosa, blood-brain barrier, and immune cells. The miR-122- and CD81-expressing HepG2, primary hepatocytes, and other *in vivo* studies with chimpanzees, all indicate that HCV induces IFN-λ response primarily, rather than IFN-α or IFN-β [64,102-105]. Many *in vitro* studies have shown that IFN-λ can also inhibit the replication of HCV and HBV [106-109]. These studies highlight the fact that IFN-λ might be used as an alternative for HCV patients who are resistant to IFN-α based therapy.

Respiratory epithelial cells predominantly produce IFN-λ during infection with influenza and other respiratory viruses [60,61,110,111]. Many *in vivo* studies have demonstrated that IFNLR1$^{-/-}$ mice are more susceptible to influenza, respiratory syncytial virus (RSV) and SARS coronavirus infections [47,112-114]. Human bronchial epithelial cells produce IFN-λ in response to rhinovirus infection and inhibit the replication of rhinovirus in bronchial epithelial cells [59]. The mouse stomach and intestinal tissues express high level of IFNLR1 [115]. Furthermore, the epithelial cells in the gastrointestinal tract respond to IFN-λ [46,114]. IFN-λ exclusively controls the infection of rotavirus, reovirus and norovirus infection in epithelial cells [46,63,116,117]. The exogenous administration of IFN-λ plays a major role in controlling the entry of West Nile virus (WNV) into the central nervous system (CNS) by restricting the blood-brain barrier [52]. Also, IFN-λ inhibits herpes simplex virus (HSV)-2 and zika virus replication in vaginal mucosa in mice [49,118]. In immune cells, IFN-λ is able to inhibit human immune deficiency virus type 1 (HIV-1) infection of IFN-λ receptor expressing macrophages in blood [119].

Besides antiviral immunity, IFN-λ has also been studied in the context of other microbial infections. Bacteria such as *Mycobacterium tuberculosis*, *Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus* spp., are able to induce IFN-λ expression [53,120-125]. An *in vivo* study with IFNLR1$^{-/-}$ mice exhibited less pathology without changes in cell infiltrates during *Staphylococcus*

and *Pseudomonas* infection [121]. Further investigation is necessary to find the role of IFN-λ in parasitic and fungal diseases.

3.6 Role of IFN in other diseases

IFN-λ not only engages in infectious diseases, but also might play a role in other diseases such as cancer and autoimmune diseases.

3.6.1 IFN-λ in cancer

The type I interferons (IFN-α/β) exhibit anti-tumor activity through the induction of cell apoptosis or immune cell priming [126,127]. Several *in vitro* and *in vivo* studies demonstrated that IFN-λ could alter tumorigenesis directly or indirectly. Like type I IFNs, IFN-λ signaling induces apoptosis in colorectal cancer cells [128]. The virus induced IFN-λ promotes anti-tumor responses. The oncolytic treatment with vesicular stomatitis virus (VSV) strain induces IFN-λ expression in hematopoietic cells, which enhances the anti-tumor responses of NK-cells [129]. The lower level of IFN-λ expression correlates with the progression of cervical cancer triggered by papilloma virus [130].

IFN-λ also play a potential role in the tumor micro-environment. IFN-λ signaling induces the expression of chemokine CXCL-10 in mammary epithelial cells, which promotes the recruitment of CD4 T-cells into the tumor micro-environment [131]. The higher IFN-λ expression controls the tumor growth in a breast cancer mouse model [131]. In addition to that, the role of IFN-λ has been showed in colon cancer, melanoma and fibrosarcoma tumor models, where IFN-λ mainly activates anti-tumor NK and T-cells [42,132,133]. Over all, evidence indicates that IFN-λ might be a potential therapeutic target for some cancers.

3.6.2 IFN-λ in autoimmune diseases

The role of type I IFNs (IFN-α/β) in autoimmunity is well established. The level of type I IFNs are elevated in autoimmune diseases such as Aicardi syndrome Goutières (AGS), Sjogren's syndrome, psoriasis, type I diabetes and systemic lupus erythematosus (SLE) [134,135]. However, in humans, the role of IFN-λ in autoimmune diseases is not established yet. The protective role of IFN-λ in allergic asthma has

been showed in a mouse model. IFN-λ downregulates Th2 cytokines (IL-4, IL-5 and IL-13), which progress the asthma pathogenesis. Mice treated with IFN-λ show reduced production of Th2 cytokines and decreased eosinophil infiltration into the lung [136-138]. Another study with an arthritis mouse model showed that IFN-λ improves the disease outcome by reducing IL-1β production and neutrophil recruitment into the arthritic joints [81].

3.7 Immune modulatory role of IFN-λ

The recent papers describe the immune modulatory role of IFN-λ. Mouse neutrophils express IFN-λ receptor, which is further upregulate after LPS treatment or exposure to *Aspergillus fumigatus.* IFN-λ acts directly on neutrophils and modulates its function via JAK2, which controls the AKT signaling and subsequent reactive oxygen species (ROS) production and degranulation process. IFN-λ suppresses the intestinal inflammation by inhibiting ROS production via a distinct mechanism which is independent of the canonical JAK-STAT signaling [82]. A recent study has described the immunomodulatory effect of IFN-λ during influenza infection in the mouse respiratory tract. IFN-λ enhances the adaptive mucosal immunity after infection of the respiratory tract with live-attenuated influenza. Upon infection, IFN-λ triggers the upper-airway M cells to produce thymic stromal lymphopoietin (TSLP). In turn, TSLP leads the activation of migratory dendritic cells (DCs). In draining lymph nodes, the activated migratory DCs boost the antigen-dependent germinal center (GC) reactions, resulting in increased production of immunoglobulins IgG1 and IgA [139]. Another mouse study showed that using IFN-λ as an adjuvant in HIV vaccination reduces the number of regulatory T-cells and Th2 cytokine (IL-4) release. However, IFN-λ increased the IgG2a response compared to IL-12 adjuvanted vaccine [140].

In humans, IFN-λ modulates the T cell responses indirectly. The stimulation of PBMCs with IFN-λ and concanavalin A increases Th1 cytokine (IFN-γ) and suppresses Th2 (IL-4, IL-5, IL-13) production [84,136,138]. The IFN-λ3 SNP rs8099917 TT allele correlates with high IFN-λ3 expression and reduced seroconversion after influenza vaccination. Further *in vitro* stimulation of PBMCs with inactivated influenza antigen and IFN-λ lowers the release of Th2 cytokines and antibodies release. *In vitro*

blocking of IFN-λ with antagonistic peptides results in increased antibody production [93]. Overall, these observations from mouse and human experiments suggest that the role IFN-λ in adaptive immunity seems to be context dependent and requires further evaluation. Also, the interaction of IFN-λ with other signaling pathways is not yet well studied.

3.8 mTOR pathway

The mechanistic/mammalian target of rapamycin (mTOR) is a protein serine/threonine kinase and a member of the phosphoinositol 3-kinase related kinase protein family, which is encoded by the human mTOR gene [141]. mTOR regulates a variety of important cellular processes such as cell survival, cell growth, cell motility, cell proliferation, cellular metabolism, cytoskeletal organization, autophagy, mitochondrial biogenesis, lipid synthesis, transcription, and protein synthesis [142,143].

mTOR forms two distinct complexes with additional regulatory proteins: mTOR complex 1 and mTOR complex 2. mTORC1 is made up of five components including the catalytic subunit of the complex mTOR, the regulatory associated protein of mTOR (Raptor), the mammalian lethal SEC13 protein 8 (mLST8), and the non-core components proline-rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein Deptor [144]. The impact of proteins interacting with mTOR are often not clear. PRAS40 and Deptor work as negative regulators of mTORC1 [144-146]. mTORC2 complex is made up of six components, including mTOR, the rapamycin insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSiN1), protein observed with Rictor-1 (Protor-1), DEPTOR, and mLST8. Among these components Rictor and mSiN1 contribute to mTORC2 structural organization [147,148]. Deptor is a negative regulator of mTORC2 activity [144]. Protor-1 interacts with Rictor, however the exact roles not clear (**Fig. 4**) [149,150].

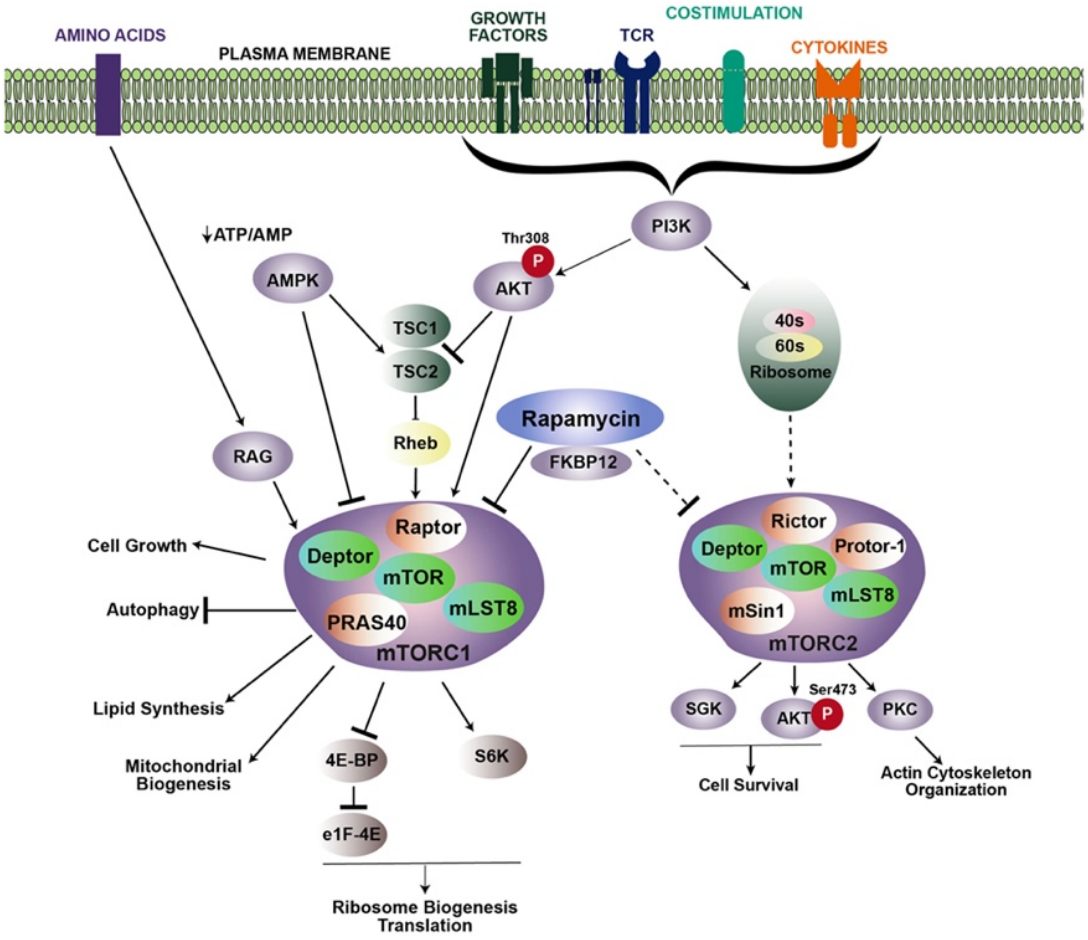

Fig. 4: mTOR signaling pathway

Activation of mammalian target of rapamycin takes place via a complex signaling cascades by external and internal cues as shown. mTORC1 complex comprises five proteins: mTOR, Raptor, mLST8, Deptor and PRAS40, whereas mTORC2 complex contains six proteins. mTOR, mLST8, mSin1, Rictor, Protor-1 and Deptor. Upon activation, mTORC1 phosphorylates S6K and 4E-BP1 downstream targets and enhances protein production. Activation of mTORC2 leads to the phosphorylation of substrates SGK, PKC and AKT, and subsequent activation of biological processes. From Keating R et McGargill MA, 2016, Front Immunol, 7 (180).

Many growth factors and cytokines activate mTORC1 signaling via PI3K, PI3K leads the phosphorylation of AKT; in turn AKT activates mTORC1. The two main mTORC1 effector substrates S6 kinase 1 (S6K1; also known as P70-S6 kinase 1 or ribosomal protein S6 kinase beta 1) and 4E-BP1 (eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1) are involved in downstream signaling [151]. Activated mTORC1 employs numerous downstream biological effects by phosphorylating S6K1 and 4E-BP1, which are associated with mRNA translation initiation and elongation

process [152]. Initially 4E-BP1 blocks mRNA translation: upon 4E-BP1 phosphorylation by mTORC1, 4E-BP1 dissociates from eIF4E, in turn, eIF4E recruits translation initiation factor eIF4G to the 5' end of mRNA. On the other side mTORC1 phosphorylates and activates S6K1, which further phosphorylates S6 ribosomal protein and initiates mRNA translation (**Fig. 4**) [152-154].

Less is known about the upstream signaling pathways that leads to mTORC2 activation and the cellular functions of mTORC2 [155]. mTORC2 has been shown to regulate actin cytoskeletal organization and ion transport by phosphorylating PKCα (protein kinase cα) and SGK1 (serum- and glucocorticoid-induced protein kinase 1) respectively [156-158]. mTORC2 activity is strongly connected to AKT activity, as mTORC2 phosphorylates AKT at Ser 473 [159]. Insulin activated PI3K promotes AKT (Ser 473) phosphorylation. Inhibition of PI3K reduces the mTORC2 kinase activity, as PI3K lies upstream of mTORC2 and promotes the phosphorylation of mTORC2 at Ser 1261. SGK1, PKCα and AKT1 respond to different growth factors through mTORC2 activation [160,161].

3.8.1 mTOR and B-cells

The mTOR serine/threonine kinase is a major regulator of cell growth and lymphocyte proliferation. mTORC1 is involved in metabolic reprogramming of immune cells and has been connected to T-cell differentiation, migration and tolerance as well as B-cell maturation and humoral immunity [162-166]. Recent studies describe the intrinsic role of mTOR in B-cell development and function. Conditional mTOR gene knockout (KO) mice exhibited lower numbers of splenic germinal centers and lower antibody responses than controls [167]. Also, the deletion of Rictor in mTORC2 was found to decrease the survival of mature B-cells and antibody responses [168]. The mTORC1 inhibitor rapamycin significantly impairs the proliferation of B-cells and suppresses antibody responses in both mouse and human [169,170]. The ATP-competitive mTOR kinase inhibitor (PP242) targets the active site of mTOR in both mTORC1 and 2. This inhibitor causes cell cycle arrest in pre-B leukemia cells; interestingly at the concentration, the inhibitor did not block the proliferation and function of normal mature B-cells [170].

3.9 B-cell activation and differentiation

B-cells are part of the adaptive immune response and function in the humoral immunity, secreting antibodies and regulatory cytokines in response to infection. Also, B-cells function as a professional antigen presenting cells (APCs). Naïve B-cells get activated upon encountering a pathogen or extracellular antigen through infection or vaccination. The activated naïve B-cells differentiate into antibody secreting plasma cells and memory B-cells. The activation of B-cells and the follow up humoral response takes place in two ways based on the nature of the antigen. Antigens activate B-cells with or without the help of T-cells, either in T-dependent or T-independent B-cell activation [171,172].

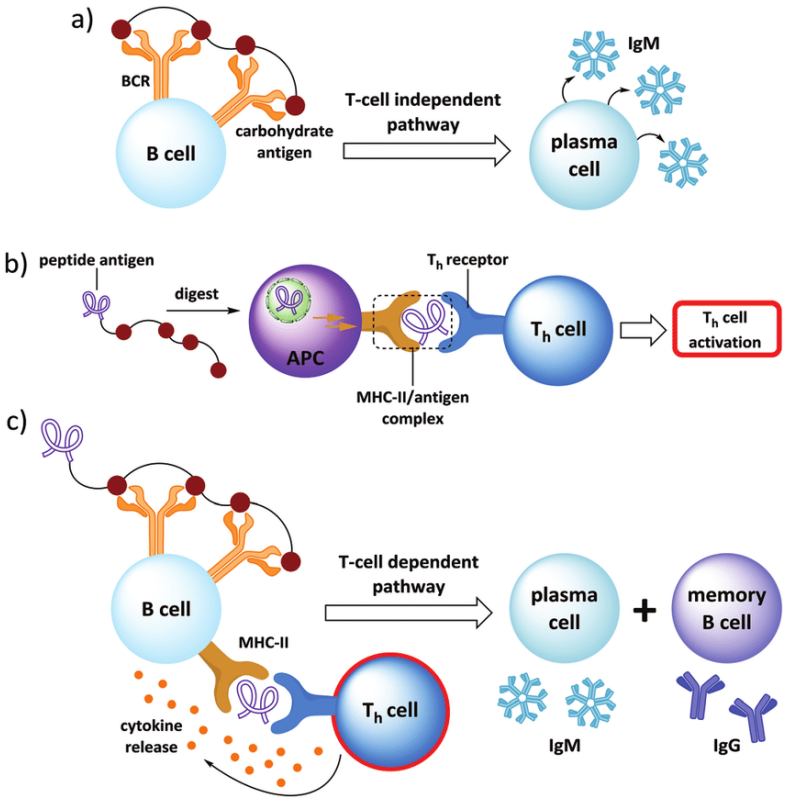

Fig. 5: T-independent and T-dependent B-cell activation and response

a) Activation of B-cells by carbohydrate antigens through BCR and generation of IgM producing plasmablasts via T-cell independent pathway. **b)** Antigen presenting cells (APCs) display processed peptides via MHC class II molecules to T-cells and activation of T-cells. **c)** Activated B-cells presenting peptides to activated T-cells via MHC-II. B- and T-cell interaction takes place, generation of plasma cells and memory B-cells through T-cell dependent pathway. From Pifferi C *et al.,* 2017, Biomater Sci, 5 (5).

3.9.1 T-independent B-cell activation and response

Non-protein antigens like glycoproteins, lipids and nucleic acids, can activate B-cells without T-cell help, and as such are named T-cell independent or thymus independent (TI) antigens. TI antigens are further divided into type I and type II antigens. Type I TI antigens are recognized by toll-like receptors (TLRs). The mitogenic stimuli such as poly IC (TLR 3), LPS (TLR 4), CPG (TLR 9) active B-cells via TLRs [173]. Type I TI antigens can activate both immature and mature B-cells and induce B-cell proliferation and antibody (IgM) production. This type of response is very rapid during the early stage of extracellular infection and it lacks any affinity maturation or isotype class switching.

The type II TI antigens are generally polysaccharides from encapsulated bacteria, which are highly repetitive surface structures [174]. These antigens activate B-cells through cross-linking BCRs, resulting in B-cell differentiation and antibody release. Type II TI antigens only activate mature B-cells, due to the need for extensive cross linking of BCRs for activation; in this condition immature B-cells become energized and do not show any immune response [175]. Type II T1 antigen polysaccharide vaccines such as Pneumovax (against *Streptococcus pneumoniae*) and Menomune (against *Neisseria meningitidis*) elicit long-term humoral response in adults, however Type II TI antigens do not produce a recall response (**Fig. 5**) [176-179].

3.9.2 T-dependent B-cell activation and response

The antigens requiring T-cell help to activate B-cells, are called T-cell dependent or thymus dependent (TD) antigens. Unlike T-independent activation, B-cell takes multiple days to elicit mature and high affinity antibody responses in T-dependent activation. The B-cell activation occurs in two phases. The early phase happens in T-cells and primary follicles outside the lymphoid follicles. In this phase, activated B-cells proliferate and undergo isotype class switching, and initial antibody secretion takes place. In the late phase, activated B-cells enter into the lymphoid follicle. In this GC environment, B-cells undergo isotype class switching, affinity maturation with somatic hypermutation [175].

Naïve $CD4^+$ T-cells get activated through the recognition of antigen by professional APCs such as dendritic cells and macrophages [180]. Meanwhile B-cells also recognize the same antigen. Upon activation, B-cells move from the follicle into the T-cell area, where the activated T and B-cells interact with each other [181]. BCR bound TD antigens are taken up by B-cells via receptor mediated endocytosis, then antigens are degraded, and presented to cognate $CD4^+$ T-cells as peptide fragments via MHC Class II molecules. T-cells recognize the MHC-peptide complex through TCR, during this interaction B-cells also express B7 (CD80/CD86) molecule, which binds to CD28 from T-cells. Followed by T-cells express co-stimulatory molecule CD40L that binds to B-cell CD40 receptor. This cognate interaction and T-cells release cytokines such as IL-2, IL-4 and IL-21. These cytokines promote B-cell proliferation and differentiation, isotype class switching and somatic hypermutation [175,182]. During somatic hypermutation, the enzyme AID (Activation-induced cytidine deaminase) generates random mutations in the variable domains of the BCR, resulting in a BCR with high affinity to the antigen. These whole processes generate both high-affinity memory B-cells and antibody releasing plasma cells. Later these cells can migrate into the bone marrow (**Fig. 5**) [175].

3.9.3 Memory B-cell activation and response

The antigen- or virus-specific memory B-cells get activated upon binding of their target antigen via BCR without T-cell help, whereas other memory B-cells need T-cell help. The BCR bound antigens are taken through receptor mediated endocytosis by memory B-cells, then the antigens are processed and presented in MHC II molecules to follicular T helper (Tfh) cells [183]. The T-cells recognize MHC II-peptide complexes through their TCR, then the same T- and B-cell cognate CD40-CD40L interactions takes place, further T-cell release its cytokines. All these events together promote the activation and proliferation of memory B-cells. The activated memory B-cells differentiate into plasmablasts or plasma cells. It is not clear whether memory B-cells undergo further affinity maturation in GCs or not [183,184].

3.10 B-cell differentiation and transcription factors

The differentiation of B-cells into plasma cells requires coordinated molecular changes so that the cells are able to change phenotype and gain effector functions such as antibody production. The changes happen in many hundreds of genes: a set of transcription factors get activated or silenced during plasma cell generation [185]. Transcription factors such as IRF-4, Blimp-1 (encoded by the PRDM1 gene) and XBP-1 guide this differentiation process. IRF-4 initiates the differentiation process through the activation of PRDM1 [186,187]. The expression of PRDM1 and XBP-1 are critical for plasma cell generation and survival. The activation of B-cells through BCR or TLRs or CD40 results in upregulation of IRF-4 and XBP-1. The cytokines such as IL-21, IL-10, IL-6 upregulate the expression of PRDM1 via STAT3 activation [188,189].

The expression of PRDM1 after B-cell activation is the primary trigger for B-cell differentiation. PRDM1 upregulates chemokine receptor CXCR4 and homing receptor integrin alpha 4, which enables the homing and survival of long-lived plasma cells [190]. The transcription factor XBP1 induces the unfold protein response by switching the surface immunoglobulins to the cytoplasm [191,192]. The other transcription factors such as E2A and Pax5 positively regulate the expression of AID [193,194]. The overall regulation of these transcription factors is very crucial for the B-cell differentiation process.

Several studies showed the important role of IFN-λ in infectious diseases mainly against broad range of virus infections. However, the impact of IFN-λ in immune cell functions are not well studied. So, we wanted to explore IFN-λ signaling in immune cells to understand how it modulates the immune cell functions.

4 Aims of the thesis

Vaccination significantly reduces the burden of infectious diseases. The functions of B-cells are tightly linked to successful vaccination. Interestingly, particular IFN-λ genotypes have been linked to vaccine outcomes in humans. Although IFN-λ modulates immune responses, the underlying mechanisms in B-cells remain largely unknown. Overall, understanding the molecular mechanisms behind the immune modulatory function of IFN-λ signaling in B-cells may help to optimization vaccine efficacies e.g. as adjuvants, but may also be linked to other B-cell associated diseases such as auto-immunity and lymphoproliferative disorders.

My main specific questions were the following:

a. How IFN-$\lambda_{1\text{-}3}$ ligands differ between each other in interacting with the receptor (e.g. binding affinities to receptor)?
b. Which immune cell directly respond to IFN-λ in human?
c. How IFN-λ modulate immune cell function, mainly B-cell functions such as antibody release and in vaccine outcome?

5 Results

5.1 Interferon-λ enhances the differentiation of naïve B-cells into plasmablasts via mTORC1 pathway

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Manuscript is submitted

Contribution of my work:
Design of experiments, Performance of experiments, Analysis of data, Writing the paper.
Figure 1 (Fig. 1a; Fig. 1b; Fig. 1c; Fig. 1d); Figure 2 (Fig. 2b; Fig. 2c; Fig. 2d; Fig. 2e; Fig. 2f); Figure 3 (Fig. 3d; Fig. 3e); Figure 4 (Fig. 4b; Fig. 4c; Fig. 4d); Figure 5

Note: The following part contains the whole manuscript

Interferon-λ enhances the differentiation of naïve B-cells into plasmablasts via mTORC1 pathway

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ABSTRACT

Type III interferon (IFN-λ) is known to be a potential immune modulator, but the mechanisms behind its immune modulatory functions and its impact on plasmablast differentiation in humans, remain unknown. Since human B-cells directly respond to IFN-λ, we performed B-cell transcriptome profiling to investigate the immune modulatory role of IFN-λ in B-cells. We found that IFN-λ enhances the mTORC1 (mammalian/mechanistic target of rapamycin complex 1) pathway in B-cell receptor activated B-cells (BCR/anti-IgM). The engagement of mTORC1 by BCR and IFN-λ induces the cell cycle progress in B-cells. Subsequently IFN-λ boosts the differentiation of naïve B-cells into plasmablast upon activation and the cells gain effector functions such as cytokine release (IL-6, IL-10) and antibody production. Our study shows how IFN-λ systematically boosts the differentiation of naïve B-cells into plasmablasts by enhancing the mTORC1 pathway and cell cycle progression in activated B-cells, which is a previously unknown immune modulatory role of IFN-λ.

INTRODUCTION

IFN-λ is a crucial antiviral effector. IFN-λ mediated immunity is not only limited to viruses such as Hepatitis C virus (HCV), Human immunodeficiency virus (HIV), influenza, norovirus, West Nile virus (WNV), Zika Virus but also extends to other pathogens such as bacteria, parasite and fungi[1-5]. The IFN-λ family (type III) consist of four members: IFN-λ1, IFN-λ2, IFN-λ3, IFN-λ4. They bind to a heterodimeric surface receptor, which is composed of the ubiquitously expressed IL10Rβ chain and the unique IFNLR1 chain. Receptor binding activates the JAK-STAT pathway and induces the expression of hundreds of interferon stimulated genes (ISGs)[6]. Interferon-α/β receptor (type I) and interferon-γ receptor (type II) are expressed in nearly every cell type[7]. In contrast, the expression of interferon-λ receptor is limited to hepatocytes, epithelial cells and a few immune cell types[1,8,9]. The cell specific receptor expression and the signaling kinetics make IFN-λ unique compared to other interferons[10]. In mouse immune cells, only neutrophils have been shown to directly respond to IFN-λ[11]. In human immune cells, however, much contradictory data has been reported on the expression of IFN-λ receptor. This is mainly due to low level of receptor expression, low assay sensitivity and the lack of receptor-specific antibodies to detect the functional IFN-λ receptor. Moreover, the impurities in immune cells isolation and the detection of IFN-λ receptor in mRNA level by quantitative PCR (qPCR) can provide misleading data on the expression of functional IFN-λ receptor in specific immune cell subtype. In brief, plasmacytoid dendritic cells (pDCs) have been shown to strongly express IFN-λ receptor while direct response of other immune cells to IFN-λ is ongoing long-standing debates[12-19]. Understanding of which immune cells respond to IFN-λ is critical for further studying the impact of IFN-λ in the cellular functions.

IFN-λ is secreted by many cell types including dendritic cells following infection or vaccination[20,21]. Triggering of B-cell receptor (BCR) by extracellular antigens or ligands promotes resting naïve/memory B-cells to proliferate and differentiate into antibody-secreting cells (ASCs). Activation of BCR signals instructs B-cells to make crucial cell-fate decisions. The B-cell differentiation process is linked to a certain number of cell divisions that are necessary to allow the expression of transcription factors such as Blimp1 (B lymphocyte-induced maturation protein-1) and IRF4

(Interferon regulatory factor 4)[22]. During this process, the phenotypic changes takes place in naïve/memory B-cells and the cells gain additional functions such as protein secretion[23]. The T-cells release cytokines including IL-5 (in mouse), IL-21 (in human), which are known to enhance plasmablast differentiation[24,25]. In this context of B-cell differentiation, the role of IFN-λ is not known.

In this study, we first show the specific responsiveness of various immune cells populations, including B- and T-cell subtypes, to IFN-λ, using a highly sensitive phospho-flow cytometry assay. Next, we performed B-cell transcriptome profiling and finally follow up *in vitro* assays to investigate the immune modulatory role of IFN-λ in B-cells and their subtypes. Our data systematically indicates that IFN-λ boosts the differentiation of naïve B-cells into plasmablasts by enhancing the mTORC1 signaling pathway and cell cycle progression in BCR-activated B-cells.

RESULTS

Immune cells specific response to IFN-λ

We investigated specific responsiveness of various immune cells subtypes to IFN-λ by phospho-flow cytometry assay. To investigate if IFN-λ signals through a JAK-STAT pathway to stimulate gene expression like type I interferons such as IFN-α or IFN-β[26], IFN-α2 was used as a positive control in the following assays. First, we quantified IFN-α2 (1000 U/mL) or IFN-λ1 (1 µg/mL) induced STAT1 phosphorylation in PBMCs using phospho-flowcytometry. All analysed immune cell subtypes from PBMCs, i.e. CD3, CD4, CD8-T cells, B-cells, NK-cells, monocytes and plasmacytoid dendritic cells (pDCs), responded to IFN-α2. Remarkably, B-cells and pDCs responded to IFN-λ1, but not other cell subtypes **(Fig. 1a, Supplementary Fig. 1a)**. Comparatively, pDCs showed more response to IFN-λ1 than B-cells **(Supplementary Fig. 1b)**. Independent of flow cytometry analysis, we confirmed responsiveness of B-cells to IFN-λ1 by western blot with pSTAT1 measurement **(Supplementary Fig. 1c)**. IFN-λ1 induced STAT1 phosphorylation in a dose-dependent manner with an EC50 of 56 ng/mL **(Fig. 1b)**. Furthermore, IFN-λ2 and IFN-λ3 inducing pSTAT1, a similar manner as IFN-λ1 in B-cells **(Supplementary Fig. 1d)**. We then investigated isolated

B-cells to compare the level of STAT1 phosphorylation induced by IFN-λ1 within B-cell sub populations. Interestingly, IFN-λ1 induced pSTAT1 level is slightly higher in naïve B-cells compared to $CD27^+$ memory B-cells **(Supplementary Fig. 1e)**. A JAK inhibitor assay was used to confirm that IFN-λ signals through JAK/STAT pathway: JAK inhibitor (3 μM ruxolitinib) almost completely blocked IFN-α2 or IFN-λ1 induced STAT1 phosphorylation in isolated B-cells **(Fig. 1c)**. IFN-λ induced gene expression was confirmed with Mx1 (MX dynamin like GTPase 1) measurement at 24 h **(Fig. 1d)**. Mx1 encoded protein is induced by type I and type II interferons against a broad range of viruses[27]. In addition, B-cell gene expression from transcriptome analysis showed that IFN-λ induced expression of interferon stimulated genes (ISGs) increased over 72 h **(Fig. 1e)**. So IFN-λ directly induce the ISG expression in human B-cells via JAK-STAT signaling pathway.

IFN-λ elevates BCR-induced mTORC1 pathway

To understand the immune modulatory effect of IFN-λ with B-cell fate and function, we isolated the B-cell population via FACS sorting (gating strategy outlined in **Supplementary Fig. 2a**) and performed B-cell transcriptional profiling using RNAseq (schematic workflow described in **Supplementary Fig. 2b**). More than thousands of genes were found dysregulated in each stimulation condition, most interestingly 271 genes were further altered by IFN-λ3 over α-IgM stimulation (**Supplementary Fig. 2c)**. We performed a gene set enrichment analysis to identify the pathways enriched by IFN-λ during BCR-activation **(Table 1)**. IFN-λ3 enriched metabolic (mTORC1, MYC) and cell cycle (E2F, G2M) related gene sets following the genes sets (commonly shared) of IFN-α or IFN-γ responses.

Based on this finding, first, we wanted to explore the mTORC1 signaling pathway. The genes involved in mTORC1 signaling were significantly upregulated in α-IgM + IFN-λ3 condition compared to α-IgM alone **(Fig. 2a)**. To verify the effect of IFN-λ on mTORC1 signaling in BCR-activated B-cells, the phosphorylation of well-established mTORC1 targets S6 (S235/p236) and 4EBP1 (T37/46) along with mTORC1 (S2448), was assessed[28]. First, we quantified the phosphorylation of S6 induced by IFN-λ3 or α-IgM or α-IgM and IFN-λ3 in combination over a time course of

16 h in isolated B-cells. We found that IFN-λ3 alone did not increased S6 phosphorylation **(Fig. 2b)**. Without additional induction, only BCR-induced S6 phosphorylation was increased gradually up to 4 h and sustained over 16 h. Interestingly, IFN-λ3 significantly enhanced BCR-induced S6 phosphorylation over 16 h **(Fig. 2b)**. The number of pS6 positive cells were increased from approximately 10% to 25% respectively from α-IgM to α-IgM together with IFN-λ3 condition at 16 h **(Fig. 2c)**. Next, we focused on the phosphorylation of other mTORC1 candidates mTORC1 and 4EBP1 along with S6 at 16h. As expected, IFN-λ3 significantly increased BCR-induced phosphorylation of mTORC1 and 4EBP1 along with S6 as measured at 16 h **(Fig. 2d, e)**.

Finally, we performed checkpoint inhibitor assays to confirm stimuli specific induction of mTORC1 pathway by pS6 quantification at 16 h. Inhibition of mTORC1 by rapamycin completely blocks S6 phosphorylation by α-IgM + IFN-λ3, whereas inhibition of IFN-λ signaling by ruxolitinib (JAK1/2 Iinhibitor) blocks the IFN-λ3 induced boost of S6 phosphorylation. Moreover, inhibition of phosphoinositide 3-kinase (PI3K) by wortmannin completely blocks S6 phosphorylation, which also confirms that the BCR-induced activation of mTORC1 acts via PI3K **(Fig. 2f)**[29].

IFN-λ increases BCR-induced cell cycle progression in B-cells

mTORC1 controls cell proliferation and cell growth by modulating mRNA translation via the phosphorylation of downstream targets like 4E-BP1 to -BP3 and ribosomal protein S6 kinases[30]. Since IFN-λ boosted the phosphorylation of BCR-induced mTORC1 downstream targets S6 and 4EBP1, we sought to identify whether IFN-λ is able to increase BCR-induced cell cycle. The gene set enrichment analysis indicated indeed that E2F targets (FDR = 5.44E-19) and G2M checkpoint (FDR = 1.17E-15) genes involved in the cell cycle process were significantly upregulated in α-IgM together with IFN-λ3 condition compared to α-IgM alone **(Fig. 3a, b)**. In addition, significant up-regulation of genes involved in cell cycle related biological processes were observed when testing the enrichment against Gene Ontology (GO) terms database **(Fig. 3c)**.

To verify the influence of IFN-λ in the cell cycle of BCR-activated cells, we measured the Ki-67 in isolated B-cells. The expression of Ki-67 is associated with cell proliferation and actively increases during the S phase of the cell cycle[31]. As expected, IFN-λ3 significantly increased the expression Ki-67 in α-IgM together with IFN-λ3 condition compared to α-IgM. The number of Ki-67$^+$ cells were remarkably increased from 12 % (α-IgM) to 30 % (α-IgM + IFN-λ3) (p = 0.03) **(Fig. 3d)**. Additionally, we performed proliferation assays with CTV labeled B-cells. IFN-λ3 alone failed to induce the proliferation of B-cells, whereas activation of B-cells with α-IgM induced proliferation. Notably, the proliferative response of BCR-activated B-cells was further significantly increased from 14% to 23% by IFN-λ3 (p = 0.005) **(Fig. 3e)**.

Effect of IFN-λ on naïve B-cells to plasmablast differentiation

Activation of mTORC1 and cell cycle progression can promote the cellular differentiation process[32]. To identify the functional role of IFN-λ in B-cell differentiation, we performed gene set enrichment analysis on the transcriptomic data. The top 10 hits of immunological signature gene sets are shown in **Figure 4a**. Genes involved in naïve B- cell to plasmablast differentiation were strongly upregulated when stimulated with α-IgM + IFN-λ3 compared to α-IgM alone (FDR = 4.38E-163) **(Fig. 4b)**. It is known that the transcription factors IRF4 and Blimp1 are essential for the differentiation of B-cells into ASCs, and that IRF4 initiates the differentiation process by activating PRDM1 gene which encodes Blimp1 protein[33]. The upregulation of PRDM1 and IRF4 was observed under α-IgM + IFN-λ3 stimulation, compared to α-IgM alone, in total B-cells **(Supplementary Fig. 4a, b)**.

To confirm the specific effect of IFN-λ in naïve B-cells to plasmablast differentiation, we performed the following *in vitro* assays with sorted naïve B-cells (gating strategy outlined in **Supplementary Fig. 3**). First, we measured the changes in the phenotypic markers CD27, CD38 and CD71 as described previously[34]. The naïve B-cells were stimulated with IFN-λ3 or α-IgM or with α-IgM + IFN-λ3 in combination. After four days, changes in surface markers were quantified by flow cytometry. The expression of surface markers CD71 and CD38 was found to be significantly increased under α-IgM + IFN-λ3 stimulation compared to α-IgM alone

(Fig. 4c). A similar effect was observed with CD27 expression **(Supplementary Fig. 4c)**. Likewise, $CD38^{+}IgM^{+}$ cells were increase more than 50% after four days of IFN-λ3 with BCR-activation **(Supplementary Fig. 4d)**.

Next, we examined the effector functions of BCR- and IFN-λ activated cells upon B-cell differentiation process. The release of IL-6 and IL-10 was greatly induced by IFN-λ3 in BCR-activated condition (72 h), but IFN-λ3 alone failed to induce any cytokines **(Fig. 4d)**. In contrast, no release of other cytokines IL-4, IFN-γ **(Fig. 4d)**, TNF-α, IL-13, IL-2, TNF-β, IL-17A, IL-12p70, APRIL, BAFF, CD40L was observed in any other stimulation conditions. A similar result was seen in the release of IL-6 or IL-10 when IgM+ memory B-cells were subject to the above stimulation conditions **(Supplementary Fig. 4e)**.

Lastly, we measured the immunoglobulins from the supernatants collected (at day five) from BCR- and IFN-λ3 activated naïve B-cells, cultured with or without mTORC1 checkpoint inhibitors. The analysis was performed using a multi-analyte human immunoglobin isotyping kit. IFN-λ3 was found to boost the release of IgM from BCR-activated cells without any inhibitors **(Fig. 4e)**. At the same time, no release of immunoglobins IgD, IgA or IgG1-4 under any stimulation conditions was noticed **(Supplementary Fig. 4f)**, which indicates that IFN-λ3 in combination with anti-IgM enhances the differentiation of naïve B-cells into IgM releasing plasmablasts. A similar response was seen with IgM+ memory B-cells **(Supplementary Fig. 4g)**. PI3K inhibitor wortmannin or mTORC1 inhibitor rapamycin both blocked the IgM release completely, whereas JAK1/2 inhibitor ruxolitinib only reduced the IFN-λ3 induced boost of IgM release by blocking the IFN-λ signaling independent of BCR response **(Fig. 4d)**. Overall these data suggest that IFN-λ boosts the differentiation of naïve B-cells into IgM releasing plasmablasts by enhancing the mTORC1 pathway.

DISCUSSION

In this study, we clearly demonstrate the direct responsiveness of B-cells to IFN-λ by using different functional techniques. This allowed us to study the immune modulatory role of IFN-λ in B-cell. We have performed transcriptomics on B-cells to study their response to IFN-λ. We have shown the systematic link of how IFN-λ

enhances B-cell differentiation by boosting the mTORC1 signaling and cell cycle process in BCR-activated cells.

We initially performed broad analysis to identify which immune cells express functional IFN-λ receptor to resolve the discrepancies in reported data on IFN-λ receptor expression on immune cell populations. We clearly showed that IFN-λ does not induce STAT1 phosphorylation on NK-cells, monocytes and T-cells (including CD4, CD8+ T-cells). Previously, NK-cells have been shown not to be directly affected by IFN-λ, rather via IFN-λ stimulated alveolar macrophages[35,36]. However, the expression of IFN-λ receptor on T-cells and monocytes has been under debate[13-17,19]. The activation and differentiation state of the immune cells might influence the expression of IFN-λ receptor. In agreement with previously published data[17,18], we observed a strong response of pDCs to IFN-λ. The expression of IFNLR1 mRNA was described on B-cells[14,17]. We evidently showed the direct responsiveness of B-cells to IFN-λ via different functional assays (phospho-flow assay, WB and transcriptome profiling by RNAseq). IFN-λ induces STAT1 phosphorylation in a dose-dependent manner in B-cells. While all B-cell sub subtypes directly respond to IFN-λ, the naïve B-cell response is higher compared to that of memory B-cells. Moreover, IFN-λ induced gene expression increased over 72h. It might suggest that in B-cells, IFN-λ signaling is steady and prolonged like in hepatocytes with specific feedback mechanism[37,38].

The metabolic regulator mTORC1 has a crucial role in B-cell fate decision and immune response[39]. Our B-cell transcriptomics and follow up *in vitro* experimental data indicate that IFN-λ boosts the mTORC1 pathway upon BCR-activation in B-cells: a previously unknown effect of IFN-λ. Though IFN-λ alone did not significantly increase the mTORC1 activity, IFN-λ prolongs the BCR-induced phosphorylation of S6 ribosomal protein over 16 h. S6 is phosphorylated by p70 S6 kinase (S6K) under the regulation of mTORC1. JAK inhibition confirms the IFN-λ specific enhancement of mTORC1 pathway. The engagement of the mTORC1 by BCR and IFN-λ receptor takes place via PI3K which is confirmed by inhibition of PI3K.

mTORC1/S6 plays an important role in cell-cycle progression[40]. As IFN-λ increased mTORC1 activity, we investigated the effect of IFN-λ on cell-cycle progress in B-cells. Our data showed that, IFN-λ increases the expression of Ki-67 and proliferation of BCR-activated B-cells. However, IFN-λ alone did not induce any proliferation. Additionally, hallmark gene set enrichment and Gene Ontology (GO) analysis signified that IFN-λ amplifies the expression of gene sets involved in G2M, E2F and other cell cycle related biological process in BCR-activated cells. Overall, our data suggest that IFN-λ boost the cell cycle progress by enhancing the mTORC1 pathway. Ongoing mTORC1 signaling and cell cycle progression leads to cellular differentiation. The mTORC1 signaling is known to be involved in immune cell differentiation[41,42].

Our B-cell transcriptomics and follow up *in vitro* experimental data suggests that IFN-λ boost the differentiation of naïve B-cells into plasmablast with gained effector functions such as cytokine and antibody release. While mouse B-cells lack IFN-λ receptor, IFN-λ indirectly triggers germinal center reaction and antibody production by a thymic stromal lymphopoietin (TSLP) dependent mechanism[43]. In humans the mechanistic role of IFN-λ in humoral immunity needs to be further evaluated in precise context and properly defined environment, especially how IFN-λ affects B-cells mechanistically during the complex interaction with antigen presenting cells and T-cells. Also, the further role of IFN-λ induced ISGs in mTORC1 activity and follow-up functions can be explored **(Fig. 5)**. The role of IFN-λ in autoimmune diseases is not established yet. However, IFN-λ is shown to be associated with pathogenesis for lupus nephritis (LN)[44]. Further, the level of p-mTORC1 in $CD19^{+}$ B-cells positively correlated with the amount of peripheral plasmablasts and systemic lupus erythematosus (SLE) disease activity score index[45]. To connect our findings with previous clinical observations, the role of IFN-λ signaling might be interesting to evaluate in hyper active B-cell in development of SLE and LN.

In conclusion, our work demonstrates the direct response of B-cells to IFN-λ. Further, it reveals how IFN-λ systematically boost the plasmablast differentiation by enhancing mTORC1 pathway and cell cycle progression in activated B-cells **(Fig. 5).** These findings are particularly important in the context of IFN-λ signaling as a potential

therapeutic target. Our data have provided an insight into the molecular mechanisms behind the immune modulatory function of IFN-λ signaling in B-cells, which might help the optimization of vaccine efficacies and improve strategies to target B-cell associated auto-immune and infectious disease treatment.

METHODS

Purification of human B-cells

Blood samples and buffy coats were collected from healthy blood donors after written informed consent (Blood donor center, University Hospital Basel). PBMCs were isolated from buffy-coats or from whole blood by a ficoll density gradient centrifugation method. B-cells were then purified from the PBMC fraction using negative selection Easysep human B-cell enrichment kit (STEMCELL Technologies). Zombie UV or zombie aqua fixable viability kit was used for live-dead staining according to manufacturer's instructions (BioLegend). Live $CD19^+$ B-cells were sorted using BD FACSARIA III cell sorter (BD Biosciences). Sorted B-cells were cultured in RPMI 1640 medium (Sigma) supplemented with L-glutamine, 10% heat inactivated FBS (Gibco). The cells were incubated at 37ºC with 5% CO_2.

pSTAT1 or Mx1 measurement

5 x 10^5 PBMCs or isolated B-cells were stimulated with IFN-α2 (1000 U/mL, PBL assay Sciences) or IFN-λ1 (1 µg/mL, R&D Systems) for 30 min. The cells were surface stained for 20 min, with following surface markers in different panels 1) For Immune cell sub-populations: CD19 (PE/Cy7), CD3 (FITC), CD14 (PE), CD335 (NKp46) (BV605). 2) For pDCs: CD3 (BV-785), CD19 (APC/Cy7), BDCA-2 (BV-421), CD123 (PE/Cy7). 3) For CD4, CD8 T cells: CD4 (FITC), CD8 (PE/Cy7), CD3 (BV-785), CD19 (APC/Cy7). 4) For Isolated B cells CD19 (PE/Cy7), IgD (BV-605), CD27 (BV-421). The cells were fixed with fix buffer I (BD Biosciences, 557870) for 10 min at 37°C and permeabilized with perm buffer III (BD Biosciences, 558050) for 30 min on ice. After washing, pSTAT1 (AF-647) (BD Biosciences) antibody was added for 1 h, at RT. For Mx1 measurement, the cells were fixed at 24h. After permeabilization mouse anti-human Mx1 primary antibody was added for 45 min, at RT. After washing anti-mouse AF-647 secondary antibody was added for 30 min, at RT. Finally, cells were harvested

for flow cytometry analysis. Data were acquired on LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Ster Inc).

Immunoblotting

PBMCs were stimulated with or without IFN-α2 (1000 U/mL) or IFN-λ1 (1 µg/mL) for 30 min. The cells were fixed with fix buffer I (BD Biosciences). The surface markers CD3, CD19, CD335 (Nkp46), and CD14 were used to sort T-, B-, NK-cells and monocytes respectively by using BD FACSARIA III cell sorter (BD Biosciences). The sorted immune cell subpopulations were directly lysed in 4X laemmli buffer (Bio-Rad). Proteins were separated by 10% SDS gel electrophoresis and then transferred on to nitrocellulose membranes. The membranes were blocked using 5% BSA in TBST buffer for 1 h, at RT. After washing, pSTAT1 (Cell Signaling Technology, CST) or β-actin (Sigma) primary antibodies were added for overnight incubation at 4°C. After washing with TBST buffer, HRP linked anti-rabbit (CST) or anti-mouse (Jakson Immuno Research) secondary antibody was added, followed by detection with ECL substrate (Thermo Fisher Scientific) using ChemiDoc imaging system (Bio-Rad)

mTORC1 inhibitor assay

5 x 10^5 isolated B-cells were pre-incubated with Ruxolitinib (3 µM/mL, JAK1/JAK2 inhibitor) (Selleckchem) or Wortmannin (0.5 µM/mL, PI3K inhibitor) (Selleckchem) or Rapamycin (0.05 µM/mL, mTORC1 inhibitor) (Selleckchem) for 1 h. After washing, the cells were stimulated with IFN-λ3 (100 ng/mL) (R&D Systems) or α-IgM (2 µg/mL) (Jakson Immuno Research) or with a combination of α-IgM and IFN-λ3. The cells were fixed at 16 h with fix buffer I (BD Biosciences) for 10 min at 37°C and permeabilized with perm buffer III (BD Biosciences) for 30 min on ice, after washing anti-human phospho-S6 (Ser235/236, AF-488) (BD Biosciences) or anti-human phospho-mTOR (pS2448, AF-647) (BD Biosciences) antibodies were added for 1 h. For p4EBP1 staining, primary antibody rabbit anti-human phospho-4EBP1 (T37/46) (CST) was added for 45 min. After washing, anti-rabbit (AF-647) secondary antibody (CST) was added for 30 min. Finally, cells were collected for flow cytometry analysis. For immunoglobulin measurements, supernatants were collected after 5 days.

Ki-67 measurement

5 x 10^5 isolated B-cells were stimulated with IFN-λ3 (100 ng/mL) or α-IgM (2 µg/mL) or with combination of α-IgM and IFN-λ3 for 4 days. After surface and live-dead staining, cells were fixed with fix buffer I (BD Biosciences) for 10 min at 37°C and permeabilized with perm buffer III (BD Biosciences) for 30 min on ice. After washing, Ki-67 (BV-421) (BioLegend) antibody was added for 45 min at RT. Finally, cells were collected for flow cytometry analysis. Data were acquired on LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Ster Inc).

B-cell proliferation assay

Isolated $CD19^+$ B-cells were labeled using CTV (Cell Trace Violet) proliferation kit (Thermo Fisher Scientific) according to the manufacturer instructions. CTV labeled B-cells were cultured with IFN-λ3 (100 ng/mL) or α-IgM (5 µg/mL) or with combination of α-IgM (5 µg/mL) and IFN-λ3 (100 ng/mL) for 5 days. The proliferation was measured using LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Ster Inc).

Measurement of surface markers for naïve to plasmablast differentiation

5 x 10^5 sorted naïve B-cells were stimulated with IFN-λ3 (100 ng/mL) or α-IgM (2 µg/mL) or with combination of α-IgM and IFN-λ3 for 4 days. Zombie UV fixable viability kit was used for live-dead staining according to manufacture directions (BioLegend). The expressions of surface markers CD27 (BV421), CD71 (APC/Cy7), CD38 (BV711) (BioLegend) were quantified using LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Ster Inc).

Immunoglobulins and Cytokines measurement

2 x 10^5 sorted naïve or IgM^+ memory B-cells were stimulated with IFN-λ3 (100 ng/mL) or α-IgM (2 µg/mL) or with a combination of α-IgM and IFN-λ3. The supernatants were collected at 72 h for cytokine measurements. The cytokines were measured using multi-analyte flow assay (Legendplex human B-cell cytokines panel, BioLegend). The supernatants were collected at day 5 for immunoglobulin measurements. The immunoglobulins were measured using multi-analyte flow assay (Legendplex human Ig isotyping panel, BioLegend).

Reagents for flow cytometry and immunoblotting

The following antibodies, reactive to human antigens, were used. For FACS: CD3-BV785 (OKT3), CD4-FITC (SK3), CD14-PE (M5E2), CD19-APC (SJ25C1), CD19-PE/Cy7 (H1B19), CD19-APC/Cy7 (H1B19), CD27-BV421 (M-T271), CD27-PE (M-T271), IgD-BV605 (IA6-2), CD38-BV711 (HIT2), IgM-PE/Cy7 (MHM-88), CD8-PE/Cy7 (SK1), CD123-PE/Cy7 (6H6), BDCA-2-BV421 (201A), CD38-APC/Cy7 (HIT2), CD335-BV605 (9E2), CD71-APC/Cy7 (CY1G4), Ki-67-BV421 (Ki-67), Anti-mouse-AF-647 (Poly4053) from BioLegand. pSTAT1 (pY701)-AF647 (4a), phospho-S6 (pS235/p236)-AF488 (N7-548), phospho-mTOR (pS2448)-AF647 (021-404) from BD Biosciences. Phospho-4EBP1 (T37/46) rabbit mAb (236B4), Anti-rabbit-AF-647 from Cell Signaling technology. For immunoblotting analysis: pSTAT1 (Tyr701) rabbit mAb (58D6), HRP-anti-rabbit antibody from Cell Signaling technology. Monoclonal anti-β-Actin antibody (mouse) from Sigma. HRP-goat-anti-mouse antibody from Jakson Immuno Research.

Statistical analysis

Statistical analysis was performed using Graphpad prism version 7 and R. Data are represented as median with interquartile range. Statistical significance was determined by paired two-tailed Student's t-test, Wilcoxon signed-rank test, as specified in the relevant figure legends. P values < 0.05 were considered statistically significant.

B-cell transcriptomics

1 x 10^6 isolated B-cells were used per condition, stimulated with IFN-λ3 (100 ng/mL) or α-IgM (0.5 µg/mL) or with a combination of α-IgM and IFN-λ3. Schematic workflow diagram of B-cell transcriptomics experiment is described in **Supplementary Fig. 2b**. In total 4 donors were used for IFN-λ3 alone conditions and total 6 donors were used for control, α-IgM and α-IgM + IFN-λ3 conditions. The total RNA was isolated using RNeasy micro kit (Qiagen). The quality of the isolated RNA was determined with a Fragment Analyzer (Agilent, Santa Clara, California, USA). The TruSeq Stranded mRNA kit (Illumina, Inc, California, USA) was used in the subsequent steps. Briefly, total RNA samples (60 ng) were polyA enriched and then reverse-transcribed into double-stranded cDNA. The cDNA samples were fragmented, end-repaired and adenylated before ligation of TruSeq adapters containing unique dual indices (UDI) for

multiplexing. Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using the Fragment Analyzer (Agilent, Santa Clara, California, USA). The product is a smear with an average fragment size of approximately 260 bp. The libraries were normalized to 5 nM in Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20. The Novaseq 6000 (Illumina, Inc, California, USA) was used for cluster generation and sequencing according to the standard protocol (single read, 100 bp). RNA quality control, library preparation and sequencing was carried out by Functional Genomics Center Zurich (FGCZ) (https://fgcz.ch).

The raw RNAseq reads were quality assessed using FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and sequencing adaptors trimmed with fastp v0.19.6[46]. STAR v2.6.0c was used to align fastq files to the human primary assembly (GRCh37 release 92), and to produce counts of mapped reads per gene[47]. Count tables were imported in the R statistical environment and normalized via the TMM method implemented in EdgeR[48,49]. Genes that had less than 1 count per million (CPM) in more than four samples were filtered out, together with outlier samples identified in principal component analysis (PCA). Genes differentially expressed (DEGs) were independently assessed by fitting a quasi-likelihood negative binomial model and testing the expression in relation to a minimum required log fold-change threshold (logFC = 1.5)[50]. As cutoff for significant DEGs after multiple testing correction (BH), a false discovery rate (FDR) of 1% was used. Smear plots were produced using ggplot2 (https://ggplot2.tidyverse.org) and heatmaps generated with pheatmap (https://CRAN.R-project.org/package=pheatmap).

To test whether a condition was enriched for relevant up/down-regulated pathways, the Camera approach was used together with the collection of hallmark and immunologic gene-sets and Gene Ontology (GO) terms from Molecular Signatures Database (MSigDB)[51]. Using gene-wise moderated t-statistics, Camera tests whether a gene-set is highly ranked relative to condition signature in terms of differential expression (logFC), accounting for inter-gene correlation[52]. In order to show the enrichment of gene sets amongst logFC ranked genes, barcode plots were produced using the function implemented in the limma package[53].

FIGURE LEGANDS

Figure 1. Immune cells specific response to IFN-λ

(a) Phospho-flow cytometry analysis was performed to quantify the phosphorylation of STAT1 upon IFN-α2 or IFN-λ1 stimulation in PBMCs (for 30 min) from 4 to 5 healthy donors. In the upper row, the representative FACS histogram plot with geometric mean fluorescence intensity of pSTAT1 induction are shown for each sub-population from PBMCs (T-cells: CD3, B-cells: CD19, NK-cells: Nkp46, Monocytes: CD14, pDCs: CD123 & BDCA-2). The collective donors of corresponding sub-populations are shown below. The statistical significance was determined using a paired two-tailed Student's t-test. **(b)** Dose responsive induction of pSTAT1 in B-cells, IFN-λ1 induced STAT1 phosphorylation in B-cells in a dose-dependent manner (n = 3, EC50 = 56.1 ng/mL). **(c)** Ruxolitinib (3 μM/mL) pretreated (for 30 min) isolated B-cells were un-stimulated or stimulated with IFN-α2 or IFN-λ1 for 30 min, induction of STAT1 phosphorylation was measured, statistical significance was calculated using Wilcoxon signed-rank test for IFN-α2 + Ruxolitinib vs IFN-α2, IFN-λ1+ Ruxolitinib vs IFN-λ1, data are shown as median with IQR. **(d)** Expression of Mx1 was measured in isolated B-cells, after 24 h stimulation with IFN-α2 or IFN-λ1 by using flow cytometry (n = 3). **(e)** Heat map of interferon stimulated genes (ISGs) expression in B-cells by IFN-λ3 for 24 h and 72 h. The expression of genes is shown in mRNA copies in counts per million (CPM).

Figure 2. IFN-λ elevates BCR-induced mTORC1 pathway

(a) Hallmark gene set enrichment barcode plot showing mTORC1 signaling genes to be relatively more activated by IFN-λ3 in α-IgM + IFN-λ3 condition compared to α-IgM alone (FDR = 3.50E-06). The log Fold Change (FC) are ranked left to right from smallest to largest. The ranked statistics are represented by shaded bars, and the positions of the specified subsets are marked by vertical bars. The enrichment worm (top) shows the relative enrichment of the vertical bars in each part of the plot. **(b)** Purified B-cells were stimulated with IFN-λ3 or α-IgM or combination of α-IgM with IFN-λ3 over 16 h time course to quantify the mTORC1 target protein, S6 phosphorylation (Ser235/236) (n = 3), the shaded area in the graph indicates the confidence interval. **(c)** The representative FACS plot is shown the phosphorylation of S6 upon stimulations at 16 h. The percentage of pS6 cells from collective donors is

shown at right side (n = 6). **(d,e)** The phosphorylation of different targets of mTORC1 pathway pmTOR1 (S2448) and p4EBP1 (T37/46) were quantified at 16h by flow cytometry. To measure IFN-λ3 induced phosphorylation increase (in geometric mean fluorescence intensity), α-IgM + IFN-λ3 was compared to α-IgM condition by Wilcoxon signed-rank test: ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001. **(f)** The checkpoint inhibitor assay was performed to confirm stimuli specific induction of mTORC1 pathway by quantification of S6 phosphorylation at 16 h. The percentage of pS6 induction are shown in scatter dot plot (n = 5) as median with IQR, Wilcoxon signed-rank test was used for statistical analysis.

Figure 3. IFN-λ increases BCR-induced cell cycle

(a, b) Hallmark gene set enrichment barcode plot showing set of genes from E2F target and G2M check points were upregulated by IFN-λ3, α-IgM + IFN-λ3 was compared to α-IgM alone condition (E2F targets – FDR = 5.44E-19, G2M check points – FDR = 1.17E-15). **(c)** The Gene Ontology (GO) enrichment analysis are presented the upregulation of cell cycle related biological process (top 20 listed) by IFN-λ3 in BCR- primed B-cells. **(d)** The intracellular Ki-67 expression was measured by flow cytometry after 4 days (n = 4) **(e)** CTV – labelled B-cells were cultured in the presence of IFN-λ3 or α-IgM or α-IgM + IFN-λ3 for total of 5 days, the proliferation of B-cells was analyzed by flow cytometry. The percentage of proliferating B-cells are shown in representative FACS plots and collective results in scatter dot plot at right (n = 6). Data are shown as median with IQR, statistical analysis by Wilcoxon signed-rank test.

Figure 4. Effect of IFN-λ on naïve B-cells to plasmablast differentiation

(a) List of top 10 pathways were upregulated by IFN-λ in B-cells over IgM stimulation from hallmark gene set enrichment analysis of Immunological signature categories. **(b)** Hallmark gene set enrichment barcode plot showing the candidate genes involved in differentiation of naïve to plasmablast process were strongly upregulated by IFN-λ3 in BCR-primed B-cells (FDR = 4.38E-163, α-IgM + IFN-λ3 vs α-IgM). **(c)** Sorted naïve B-cells were stimulated with IFN-λ3 or α-IgM or combination of α-IgM and IFN-λ3 for 4 days, flow cytometry analysis was performed to measure the upregulation of surface markers CD38, CD71 (in geometric mean fluorescence intensity). **(d)** For the cytokine release, the sorted naïve B-cells were cultured with indicated stimuli and supernatants

were harvested at 72 h. The cytokines were measured by human B-cell cytokines multi analyte flow assay kit. **(e)** Sorted naïve B-cells were treated with above indicated conditions and supernatants were collected at day 5. The released immunoglobulins were measurement by using human Ig isotyping multi analyte flow assay kit. Data are shown as median with IQR, statistical analysis by Wilcoxon signed-rank test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 5. IFN-λ synergizes with BCR signaling through the mTORC1 pathway

Schematic diagram showed how IFN-λ synergizes with BCR signaling through the mTORC1 pathway to enhance the differentiation of naïve B-cells into plasmablast and further follow up effector functions.

Supplementary Figure 1. Responsiveness of immune cell subpopulations to IFN-λ

(a) PBMCs were stimulated with or without IFN-α2 or IFN-λ1 for 30 min, phosphorylation of STAT1 was measured by flow cytometry (n = 5), responsiveness of CD4 and CD8 T-cells to IFN-α2 or IFN-λ1 are shown. The statistical significance was measured using a paired two-tailed Student's t-test. **(b)** The different responsiveness of B-cell and pDCs to IFN-α2 or IFN-λ1 are shown (n = 4), the responsiveness differences of both populations to IFN-α2 or IFN-λ1 was calculated by Wilcoxon signed-rank test. **(c)** Immunoblot analysis of pSTAT1 and β-actin (loading control). PBMCs were stimulated with or without IFN-α2 or IFN-λ1 for 30 min, after fixation, T-cell, B-cell, NK- and Monocytes were separated via FACS sorting. The sorted immune cell populations were subjected to immunoblot analysis. **(d)** Induction of STAT1 phosphorylation in B-cells by IFN-λ1-3 (n = 4). **(e)** The different responsiveness of B-cell subtypes to IFN-α2 or IFN-λ1 are shown (n = 4). Geometric mean fluorescence intensity of pSTAT1 of each condition are shown in the graph, median with IQR, statistical analysis by Wilcoxon signed-rank test.

Supplementary Figure 2. Modulatory effect of IFN-λ on B-cells upon BCR-activation

(a) The B-cells were sorted via following gating strategy. Partially enriched B-cells were gated based on forward and side scatters, then single cells to live cells were gated and $CD19^+$ live B-cells were collected for B-cell transcriptomics experiment. **(b)**

Schematic plan diagram of B-cell transcriptomics experiment is shown. The B-cells were enriched from buffy-coat by using magnetic based negative selection enrichment kit and further purified via FACS sort. The pure viable B-cells were stimulated with IFN-λ3 or α-IgM or combination of α-IgM with IFN-λ3. **(c)** The number of significantly dis-regulated genes for each condition are shown (X-axis - log fold change, Y-axis – mRNA copies in counts per millions - CPM, red dots – significantly changed genes with FDR ≤ 0.01, blue dots – no significant changes)

Supplementary Figure 3. Gating strategy used to sort naïve B-cells from enriched total B-cells

Partially enriched B-cells were gated based on forward and side scatters, then single cells, live cells were gated. From live $CD19^+$ $CD38^-$ cells naïve and memory B-cells were collected for the further experiment.

Supplementary Figure 4. Enhancement of B-cell differentiation and follow up functions by IFN-λ

(a) The upregulation of PRDM1 (BLIMP1) in number of mRNA copies as CPM by different treatment conditions (at 72 h) **(b)** The isolated B-cells were stimulated with IFN-λ3 or α-IgM or combination of α-IgM and IFN-λ3 for 4 days, the upregulation of intracellular IRF4 was measured by FACS. **(c, d)** Sorted naïve B-cells were stimulated with above stimulation conditions for 4 days, upregulation of surface marker CD27 (in geometric mean fluorescence intensity) and percentage of $CD38^+IgM^+$ cells were quantified by FACS (n = 5). **(e)** The release of cytokines from IgM^+ memory B-cells are shown. For cytokines measurement the cells were treated with IFN-λ3 or α-IgM or combination of α-IgM and IFN-λ3 for 72 h. **(f, g)** The release of immunoglobulins from naïve and IgM^+ memory B-cells are shown, the cells were subjected to above mentioned treatments for 5 days. Data are shown as median with IQR, statistical analysis by Wilcoxon signed-rank test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table. 1 Pathways upregulated by IFN-λ over BCR-primed B-cells

List of top 10 pathways were upregulated by IFN-λ in B-cells over IgM stimulation from hallmark gene set enrichment analysis.

Abbreviations: 4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1; APRIL: A proliferation-inducing ligand; ASCs: Antibody-secreting cells; BAFF: B-cell activating factor; FACS: Fluorescence-activated cell sorting; GO: Gene Ontology; IFNLR1: Interferon lambda receptor 1; IQR: Interquartile range; Ig: Immunoglobulin; IL10Rβ: Interleukin 10 receptor beta; ISGs: Interferon stimulated genes; JAK: Janus Kinase; NK-cells: Natural killer cells; PCA: Principal component analysis; PI3K: Phosphoinositide 3-kinase; STAT: Signal transducer and activator of transcription; TNF-α: Tumor necrosis factor alpha; USP-18: Ubiquitin specific peptidase;

Author contribution:

M.S., F.B., J.L., CS., D.W. and A.E. designed the experiments. M.S. performed the experiments. M.S. and J.L. performed data analysis. F.B. performed bioinformatic analyses. M.S. and A.E. wrote the initial draft of the manuscript with other authors providing editorial comments.

Acknowledgements: We thank Dr. Peter Staeheli, Institute of Virology, Medical Center University of Freiburg, Germany for kindly providing Mx1 antibody. We thank Flow cytometry core facility, Department of Biomedicine, University of Basel. We thank Dominik Vogt for his excellent technical assistance. We thank Dr. Helena Seth-Smith for critical reading of the manuscript. This work was supported by a research grant to AE Swiss National Science Foundation project grant (PZ00P3_154709/1) and the Swiss National Science Foundation SystemsX iPhD program. In addition, grants from the Nachwuchsförderung Universität Basel and Bangerter Rhyner Stiftung, Stiftung für Infektionskrankheiten.

Data availability: The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no competing interests.

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Fig. 1 Immune cells specific response to IFN-λ

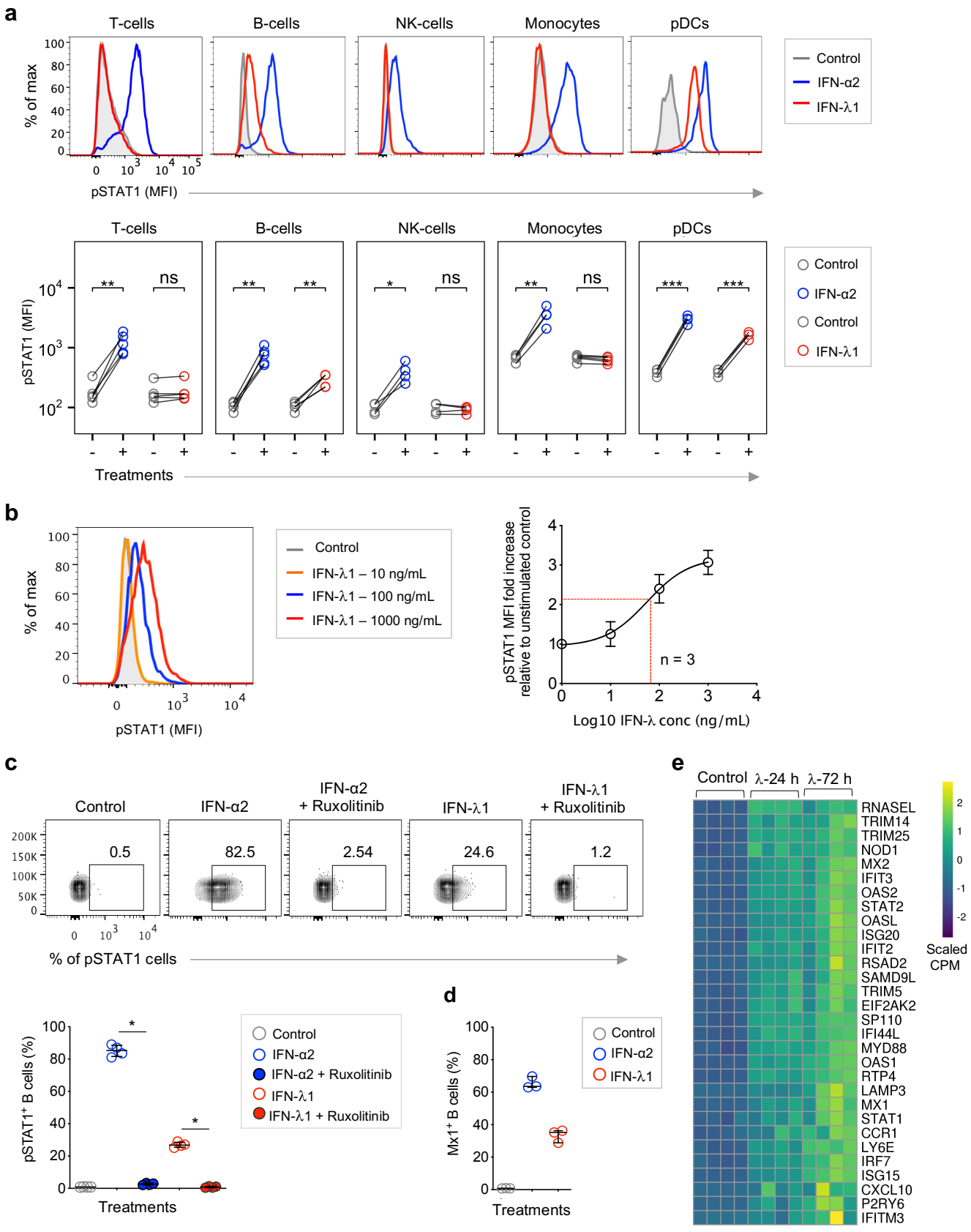


Fig. 2 IFN-λ elevates BCR-induced mTORC1 pathway

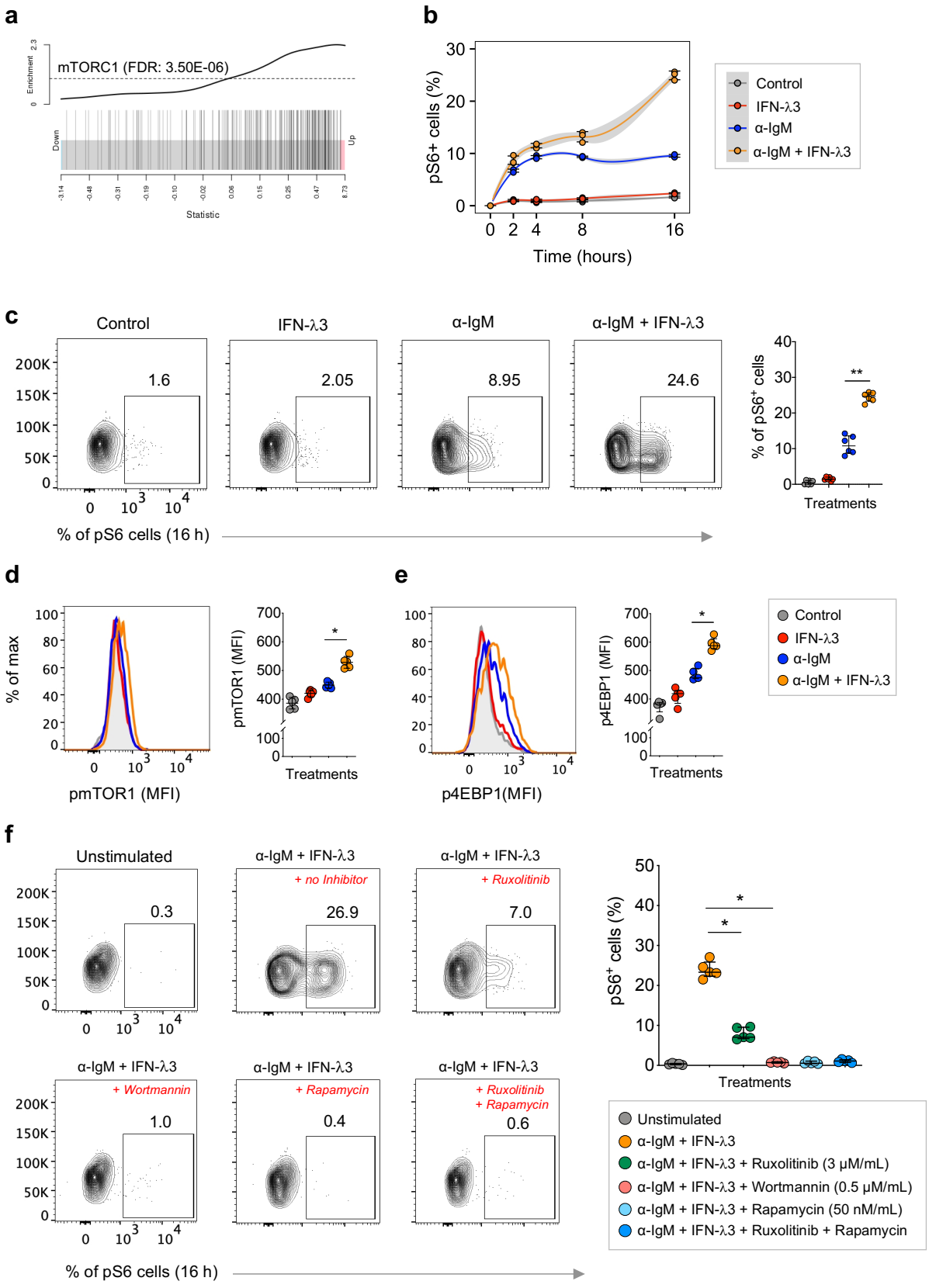


Fig. 3 IFN-λ increases BCR-induced cell cycle progression in B cells

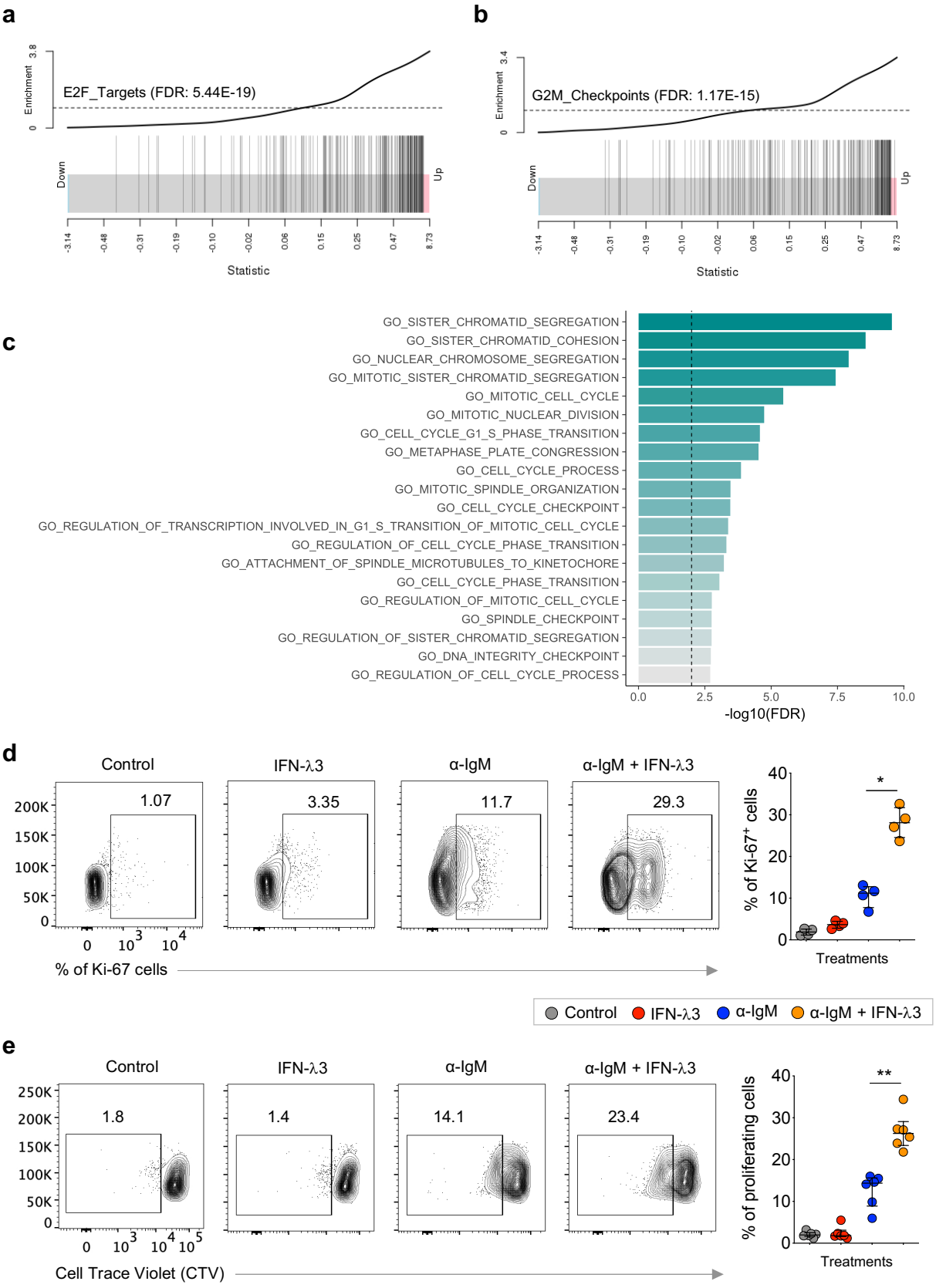


Fig. 4 Effect of IFN-λ on naïve B-cells to plasmablast differentiation

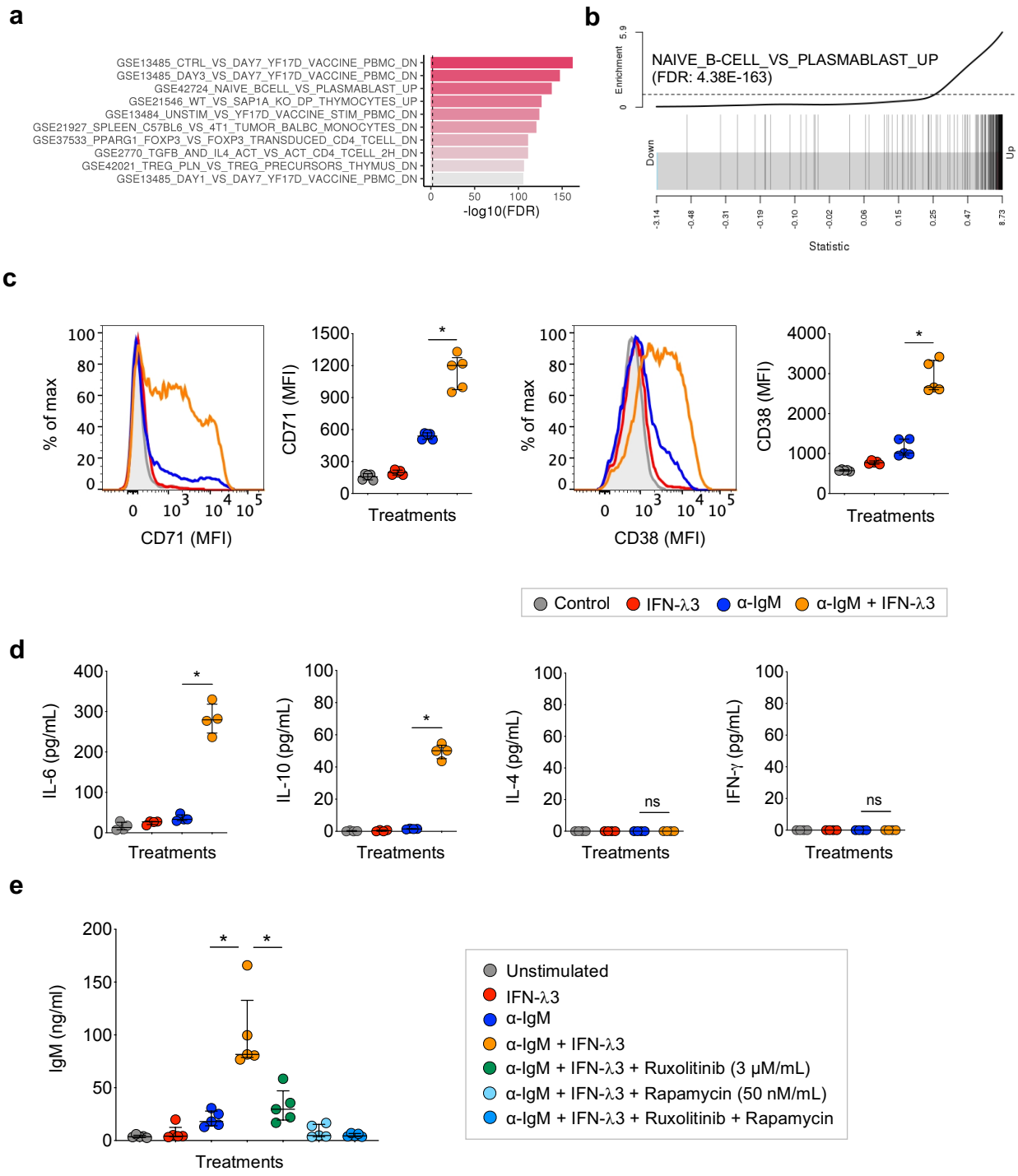
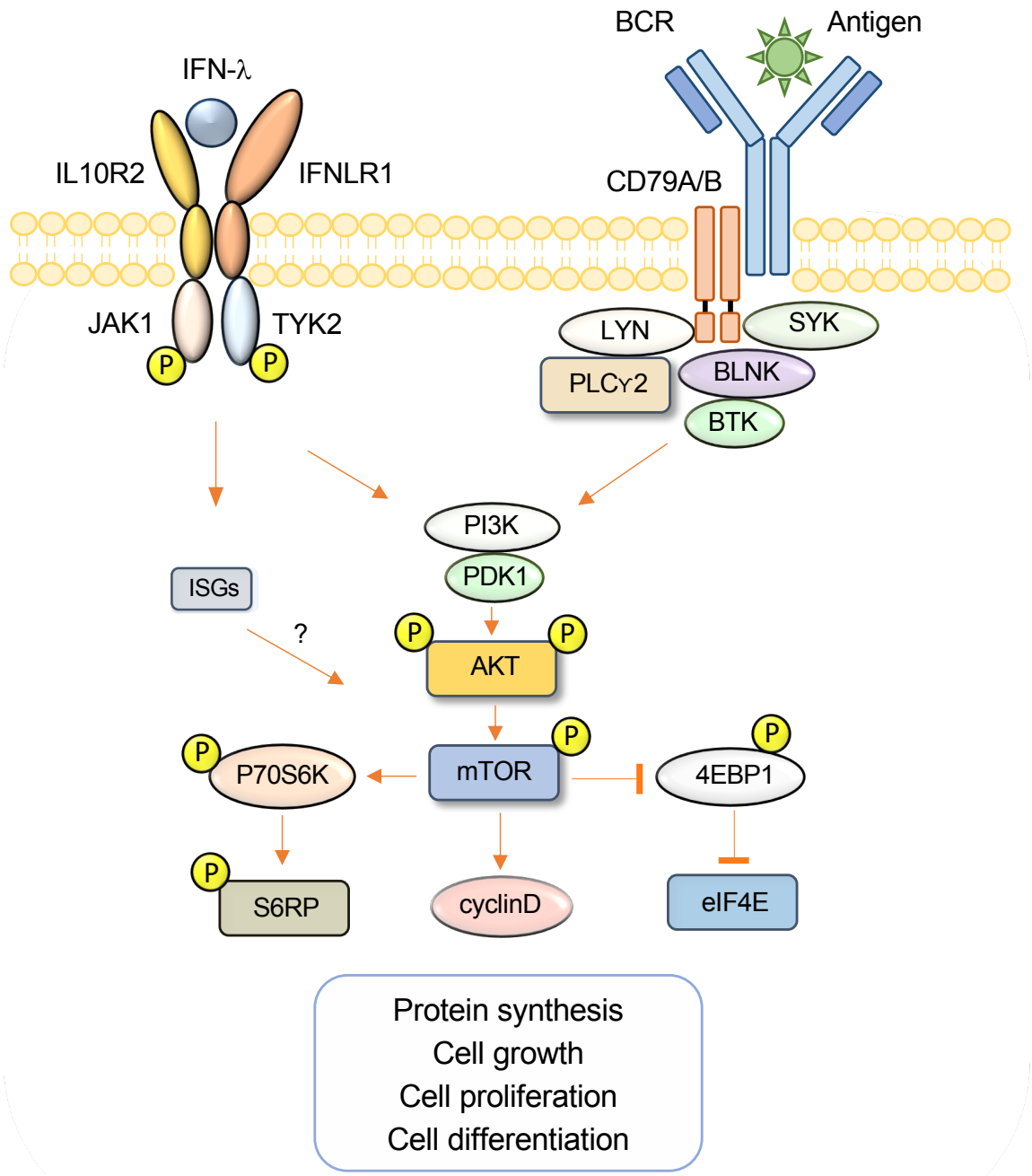


Fig. 5 IFN-λ synergizes with BCR-signaling through the mTORC1 pathway

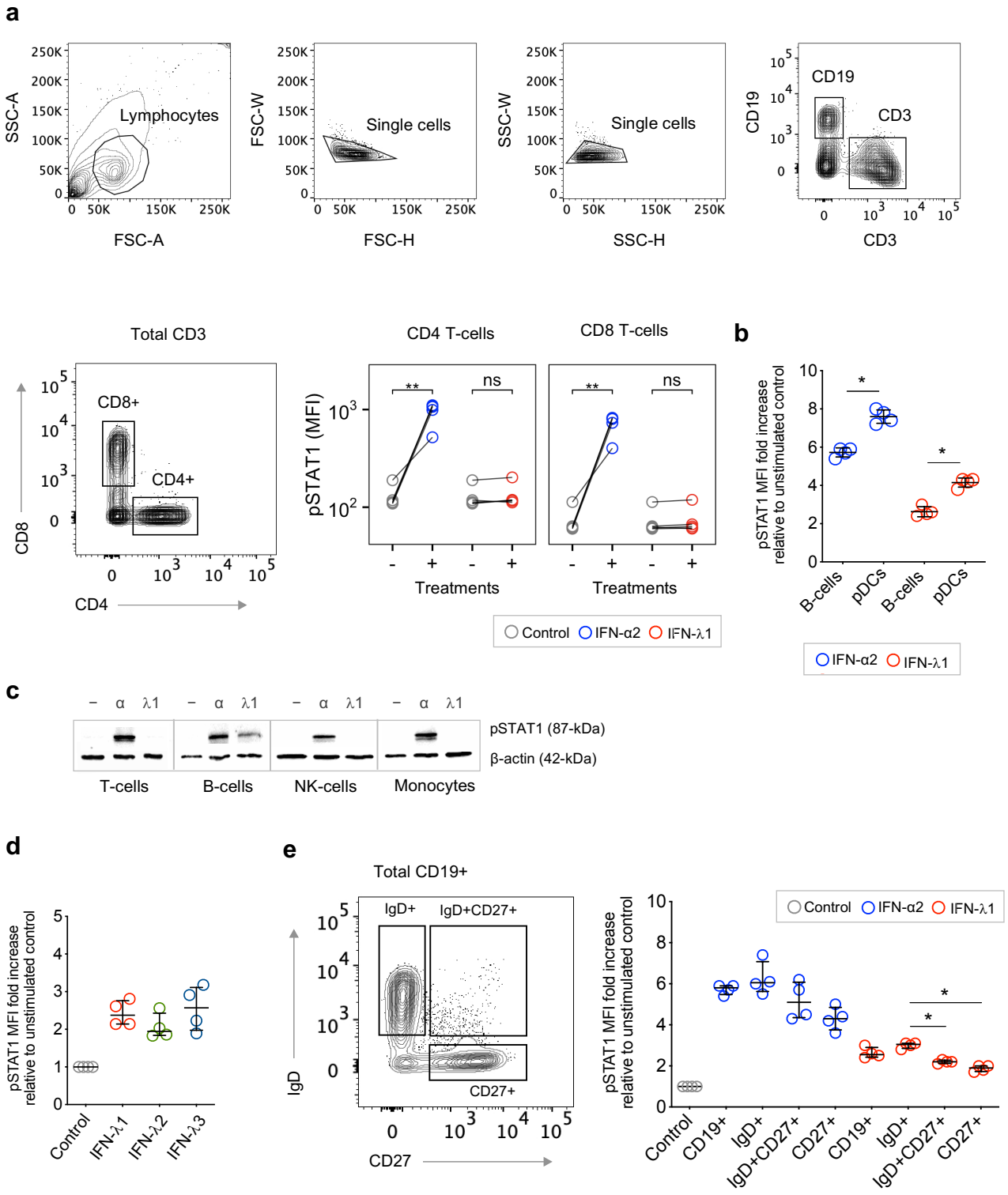


Table

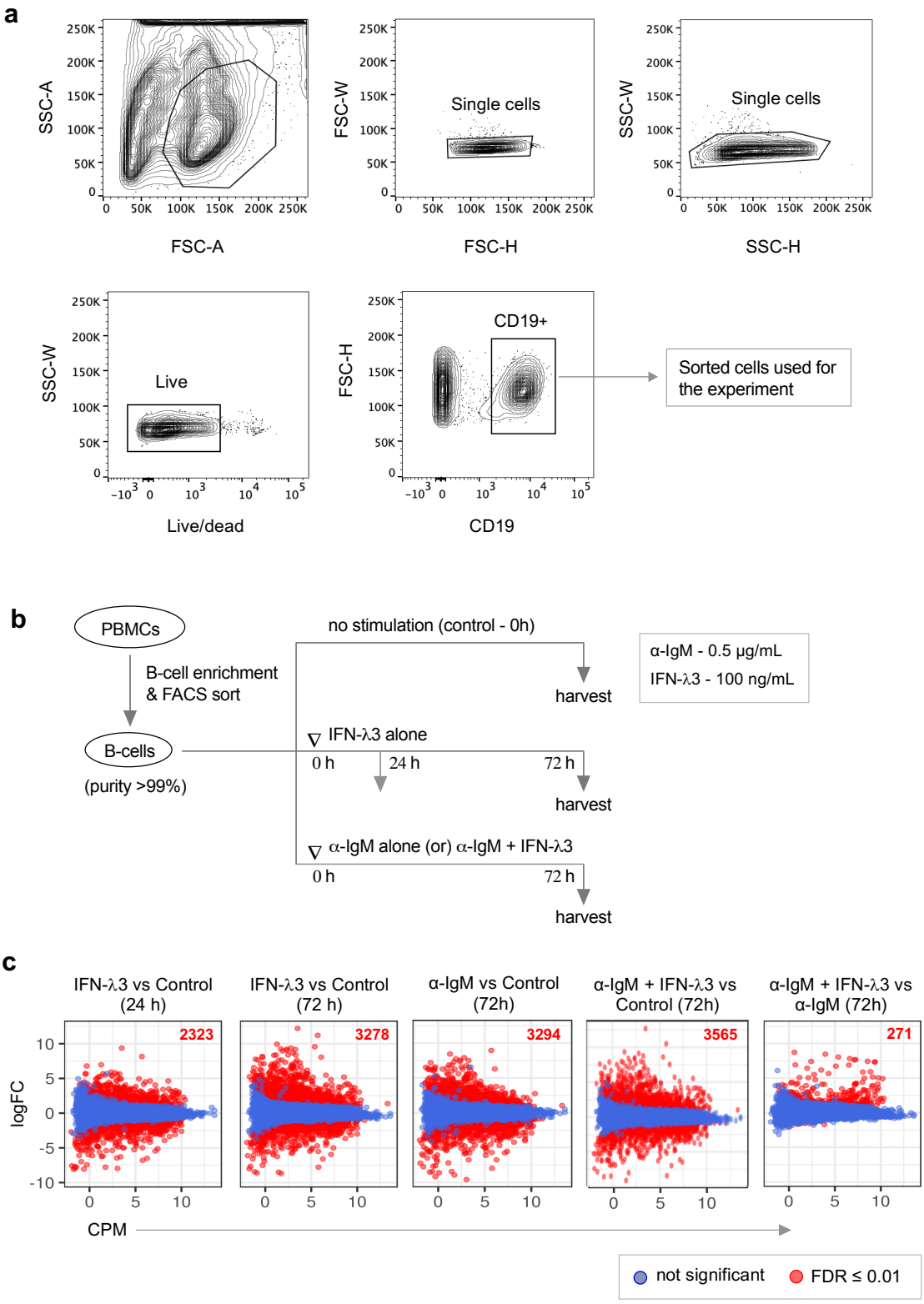
Table. 1 Pathways up-regulated by IFN-λ over IgM stimulation (Hallmark geneset enrichment analysis)

Geneset	pValue	FDR
INTERFERON_ALPHA_RESPONSE	2.18E-147	1.09E-145
INTERFERON_GAMMA_RESPONSE	1.69E-80	4.22E-79
E2F_TARGETS	3.27E-20	5.44E-19
G2M_CHECKPOINT	9.34E-17	1.17E-15
MTORC1_SIGNALING	3.50E-07	3.50E-06
MYC_TARGETS_V1	3.57E-06	2.97E-05
INFLAMMATORY_RESPONSE	1.51E-05	1.08E-04
UNFOLDED_PROTEIN_RESPONSE	8.16E-05	5.10E-04
MYC_TARGETS_V2	1.66E-04	9.23E-04
COMPLEMENT	3.98E-04	1.99E-03

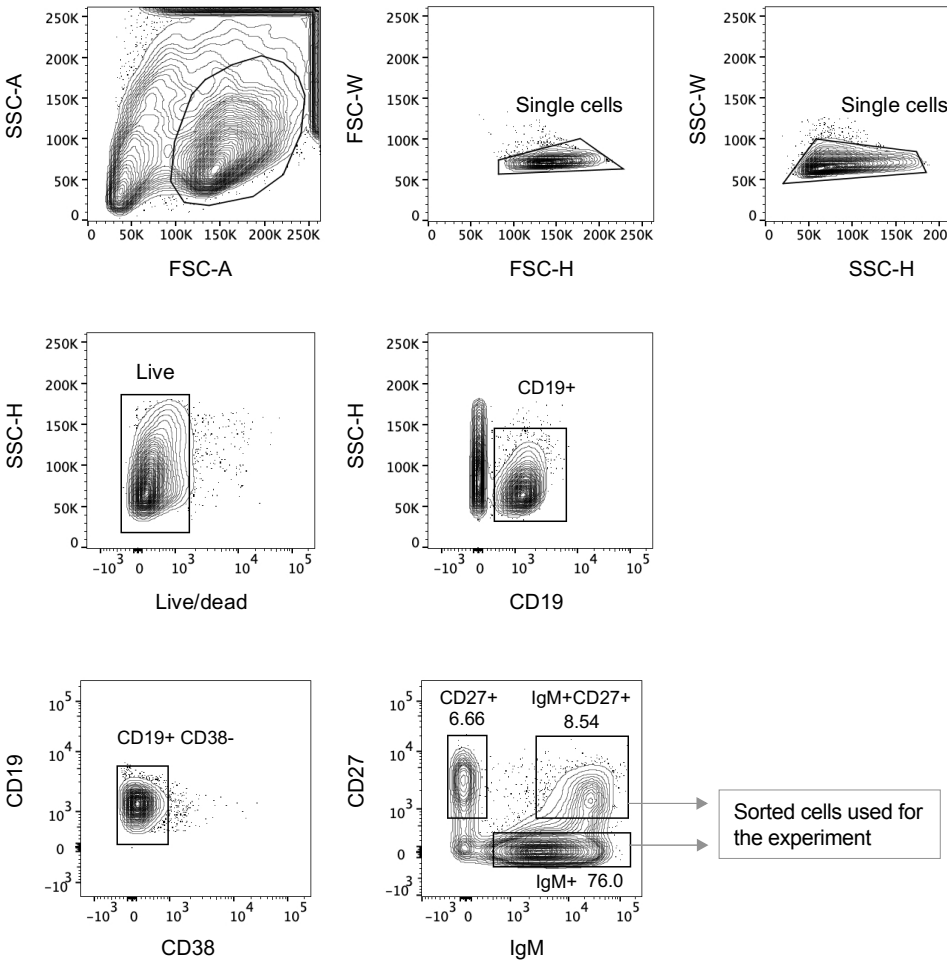
Supplementary Figure. 1 Responsiveness of Immune cell subpopulations to IFN-λ



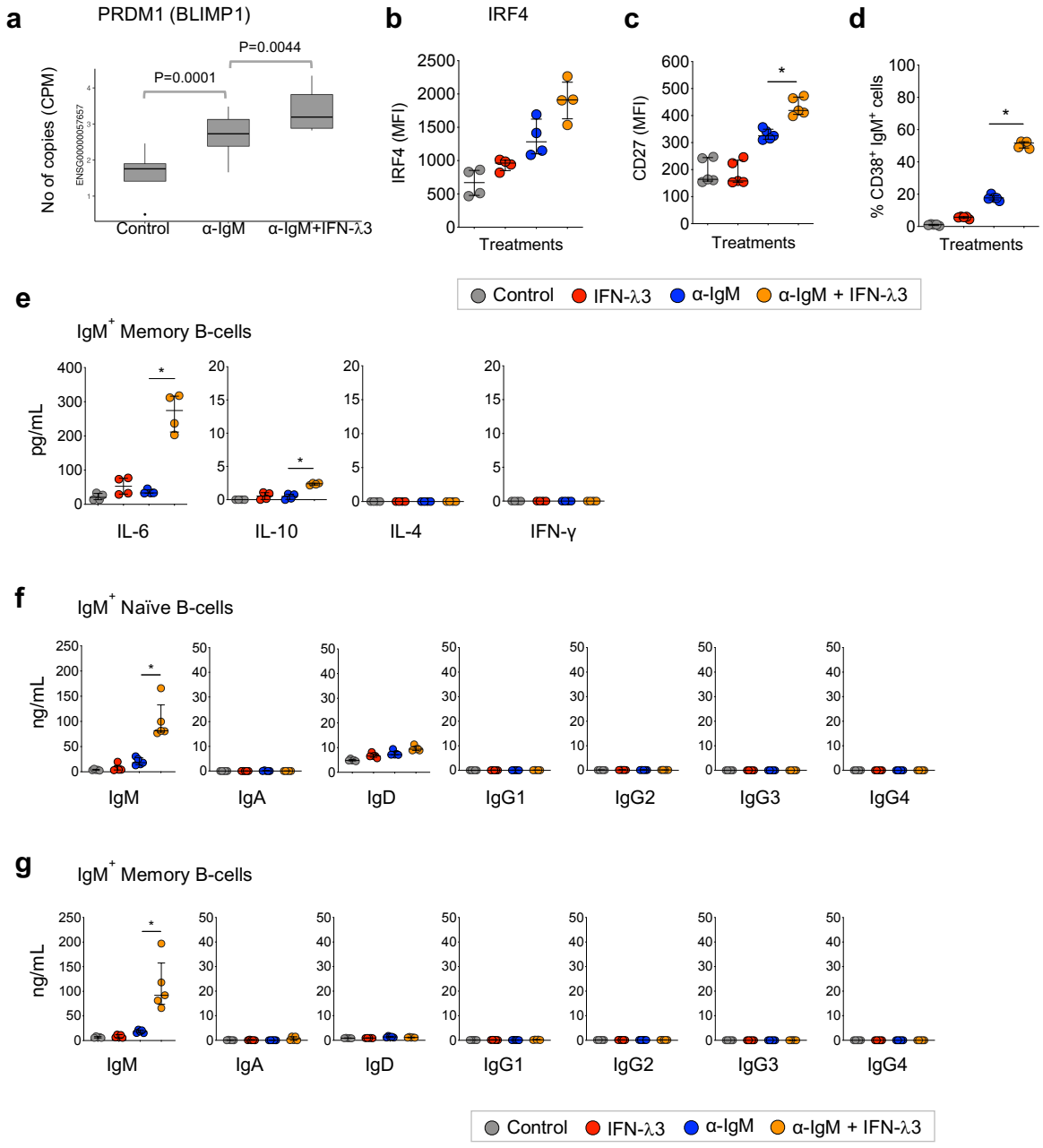
Supplementary Figure. 2 Modulatory effect of IFN-λ on B-cells upon BCR-activation



Supplementary Figure. 3 Gating strategy used to sort naïve B-cells from enriched total B-cells



Supplementary Figure. 4 Enhancement of B-cell differentiation and follow-up functions by IFN-λ



5.2 Interferon Lambda: Modulating Immunity in Infectious Diseases

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Manuscript has been published in Frontiers in Immunology, 2017; 28;8:119.
doi: 10.3389/fimmu.2017.00119. *Review.*

Contribution of my work:
Partly contributed to writing and preparing Figure 1 & 2; Table 1 & 2

Note: The following part contains the whole manuscript

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Contribution of my work:
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Note: The following part contains the whole manuscript

frontiers in Immunology

REVIEW
published: 28 February 2017
doi: 10.3389/fimmu.2017.00119

Interferon Lambda: Modulating Immunity in Infectious Diseases

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Interferon lambdas (IFN-λs; IFNL1-4) modulate immunity in the context of infections and autoimmune diseases, through a network of induced genes. IFN-λs act by binding to the heterodimeric IFN-λ receptor (IFNLR), activating a STAT phosphorylation-dependent signaling cascade. Thereby hundreds of IFN-stimulated genes are induced, which modulate various immune functions *via* complex forward and feedback loops. When compared to the well-characterized IFN-α signaling cascade, three important differences have been discovered. First, the IFNLR is not ubiquitously expressed: in particular, immune cells show significant variation in the expression levels of and susceptibilities to IFN-λs. Second, the binding affinities of individual IFN-λs to the IFNLR varies greatly and are generally lower compared to the binding affinities of IFN-α to its receptor. Finally, genetic variation in the form of a series of single-nucleotide polymorphisms (SNPs) linked to genes involved in the IFN-λ signaling cascade has been described and associated with the clinical course and treatment outcomes of hepatitis B and C virus infection. The clinical impact of IFN-λ signaling and the SNP variations may, however, reach far beyond viral hepatitis. Recent publications show important roles for IFN-λs in a broad range of viral infections such as human T-cell leukemia type-1 virus, rotaviruses, and influenza virus. IFN-λ also potentially modulates the course of bacterial colonization and infections as shown for *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Although the immunological processes involved in controlling viral and bacterial infections are distinct, IFN-λs may interfere at various levels: as an innate immune cytokine with direct antiviral effects; or as a modulator of IFN-α-induced signaling *via* the suppressor of cytokine signaling 1 and the ubiquitin-specific peptidase 18 inhibitory feedback loops. In addition, the modulation of adaptive immune functions *via* macrophage and dendritic cell polarization, and subsequent priming, activation, and proliferation of pathogen-specific T- and B-cells may also be important elements associated with infectious disease outcomes. This review summarizes the emerging details of the IFN-λ immunobiology in the context of the host immune response and viral and bacterial infections.

Keywords: interferon lambda, immunity, immune cells, virus, infectious diseases, bacteria, fungi, parasites

OPEN ACCESS

Edited by:
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Reviewed by:
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Megan Tierney Baldridge, Washington University School of Medicine, USA
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *12 November 2016*
Accepted: *25 January 2017*
Published: *28 February 2017*

Citation:
Syedbasha M and Egli A (2017) Interferon Lambda: Modulating Immunity in Infectious Diseases. Front. Immunol. 8:119. doi: 10.3389/fimmu.2017.00119

IFN-λ EXPRESSION AND SIGNALING PATHWAYS

Patients with infectious diseases often show heterogeneous clinical courses with a range of associated morbidities and variable mortality. This is dependent on a series of factors covering the complex aspects of host–pathogen interactions (1–5). IFNs play a crucial role in these interactions—defining the outcome of many viral, bacterial, fungal, and parasitic infections (6–16) (see **Figure 1**). In addition, IFNs reduce tumor cell proliferation (17, 18) and show important immune regulatory functions in autoimmunity (19, 20). These broad effects are explained through the induction of hundreds of IFN-stimulated genes (ISGs) (21). Three types of IFNs have been described, which can induce ISG expression, and add further complexity: type I with mainly IFN-αs and -βs (22–26), type II with only IFN-γ (27), and type III with IFN-λs (28–31). Although most cells can induce and release various types of IFNs, specialized immune cells are the main producers during an inflammatory process. The effects induced by single or combined IFNs in exposed cells are very heterogeneous and range from differential patterns of ISG expression, regulation of cell proliferation (18), changes in cell surface molecules such as HLA DR (32), to the maturation of monocytes to dendritic cells (33). The effects depend on the plasticity of the various IFNs involved, including the peak concentrations, concentration changes over time, binding affinities of IFNs to the specific receptors, receptor expression, potentially induced feedback mechanisms, and the target cell type itself (34).

Four IFN-λ ligands have been described: IFNL1–4, with each family member having antiviral effects on various viruses within different cell types (28). IFNL1–3 share high amino acid sequence homologies, whereas IFNL4 is more divergent with only 40.8% amino acid similarity to IFNL3 (35). The expression of IFN-λs is induced in a broad range of cell types by pattern recognition receptors including toll-like mediated (36–41), Ku70 (21398614) and RIG-1-like (24952503). Type 2 myeloid dendritic cells have been described as the main producers of IFN-λ (42–48). In mice, commonly used as a model organism for infectious disease and immune function, only IFNL2 and IFNL3 are functional, as IFNL1 and IFNL4 are present as inactive pseudogenes (49).

After release, IFN-λ binds to its heterodimeric IFN-λ receptor (IFNLR). The IFNLR consists of two subunits: α-subunit (IL28RA) and β-subunit (IL10RB) (35, 50–53). Despite high sequence homologies, binding affinities of the different IFN-λs to the IFNLR1 differ greatly. IFNL1 shows the highest binding affinity to IL28RA, and IFNL3 the lowest (54). The dimerization of the receptor subunits leads to activation of Janus Kinase 1 and tyrosine kinase 2 and phosphorylation of STAT-1 and -2, which

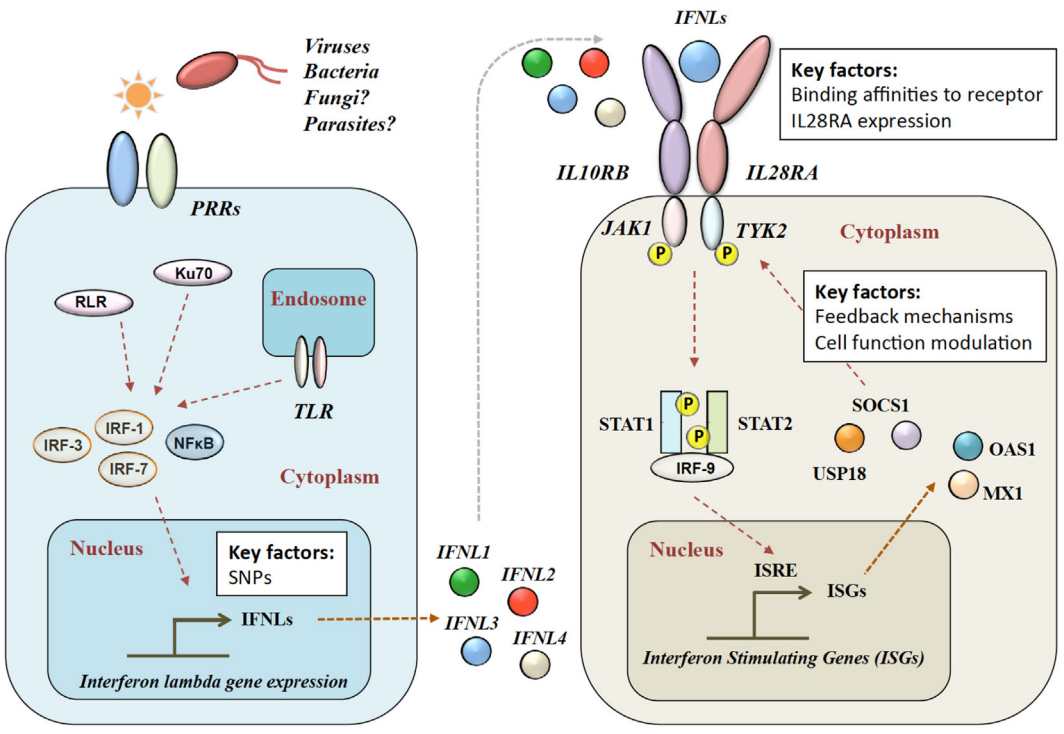

FIGURE 1 | Type III IFN signaling pathway. Viral infection is sensed by pattern recognition receptors (PRRs), which induce IFN-λ production *via* various signaling pathways. IFN-λs bind to the heterodimeric IFN-λ receptor (IFNLR), which consists of IL28RA and IL10RB subunits. Upon binding, a JAK–STAT signaling cascade induces hundreds of IFN-stimulated genes (ISGs). RLR, RIG-1-like receptor; TLR, toll-like receptors; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL28RA, interleukin 28 receptor alpha; IL10RB, interleukin 10 receptor beta; JAK1, Janus Kinase 1; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; MX1, interferon-induced GTP-binding protein Mx1; OAS1, 2′-5′-oligoadenylate synthetase.

induces the subsequent downstream signaling with the induction of hundreds of ISGs (31) (see **Figure 1**). IFN-α and IFN-λ both show a complex mechanism of positive and negative feedback loops, mainly modulated *via* the suppressor of cytokine signaling 1 and the ubiquitin-specific peptidase 18 (31, 55).

IFN-λ RESPONSIVENESS TO COUNTERACT PATHOGENS

Two aspects are crucial to understanding the role of IFN-λs in the context of infectious diseases: (i) IFNLR distribution in infected cells and tissues and (ii) single-nucleotide polymorphisms (SNPs) in and around the genes encoding IFN-λs and IFNLR. Both aspects show important differences between humans and mice, which complicate studies and conclusions drawn from infectious disease models (56).

IFNLR Receptor Expression

The IL10RB subunit is expressed in many cell types (57), whereas the IL28RA subunit expression is much more restricted. Expression of IL28RA mRNA has been detected in the lung, intestine, liver tissues, immune cells such as B cells, neutrophils, macrophages, and plasmacytoid dendritic cells (28, 29, 43, 58–62). Human NK cells seem not to express IFNLR (63), whereas mouse NK cells show deficient function in IL28R knockout animals (25901316). The effects of IFN-λ on cells and tissues are often measured *in vitro via* indirect markers, such as downstream expression of ISGs or changes in specific cellular phenotypes. Data on the induction of STAT phosphorylation, as the most direct measurement of signal induction, are still missing for some cell types and tissues. The IFNLR expression is regulated *via* transcription factors (31) and may show variability during an inflammatory process, which adds an additional level of complexity. Primary hepatocytes show relatively low baseline responsiveness to IFN-λs, yet upon IFN-α treatment a marked increase in IL28RA mRNA levels is observed (64, 65). Similarly, during cytomegalovirus (CMV) infection of fibroblasts, IL28RA mRNA levels increase by about twofold, but protein expression levels remain stable (66). A recent paper by Lazear et al. suggested that endothelial cells in the blood–brain barrier may be sensitive to IFN-λs, reducing permeability to West Nile virus in a mouse model (67).

Understanding which immune cells and subsets are responsive to IFN-λs in humans can be experimentally and technically challenging due to low target cell densities and less accessible cell types such as tissue resident cell types. In contrast, peripheral blood mononuclear cells (PBMCs) are relatively easy to access in order to explore responses to IFN-λs; therefore, most literature focuses on hepatocytes (from liver biopsies) and immune cells from the blood. The direct impact of IFN-λs on T-cells *via* surface expression of the specific IFNLR is subject to ongoing debate (58, 68–71). IFN-λs may also induce FOXP3-expressing regulatory T-cells (72), which may impact a series of immunoregulatory aspects during an infection as part of the inflammatory response. Several research groups confirm that IFN-λs influence the T-helper cell balance, which is shifted toward Th1 (70, 71, 73–76). The Th1/Th2 balance might be important for controlling specific infections such as helminths (6, 77, 78). In addition, the B-cell-driven humoral immune responses are also modulated by the presence of Th2 cytokines, e.g., during vaccination. We have recently shown that IFNL3 is a key regulator of the influenza virus-specific B-cell proliferation and antibody production (76). The exact mechanism of how Th1/Th2 balancing and B-cell activation is modulated by IFN-λs and how this impacts infectious disease outcome has to be explored in more detail in the future.

Impact of SNPs

A series of SNPs in IFN-λ ligand and receptor genes have been described (see **Figure 2**). Most importantly, these SNPs have been associated with a series of important clinical phenotypes in the context of infectious diseases (see **Table 1** for more details).

Modulation of IFNLR expression may have a great impact on the effects of a particular IFN-λ ligand, and thereby influence the subsequent signaling pathway and the outcome of infectious diseases. Multiple SNPs in the gene encoding IL28RA have been described (94–97). The rs10903035 SNP is located within the 3′UTR of the IL28RA mRNA sequence, suggesting a potential microRNA binding site. This particular SNP was identified as an independent risk factor for IFN-α treatment failure against hepatitis C virus (HCV) (44, 98). In addition, this SNP has been associated with insulin resistance in HIV/HCV coinfected patients (94). Another SNP in this gene, rs4649203, has been linked to the risk of psoriasis in four independent populations (96), and to the development of systemic lupus erythematosus (97). These observations suggest an important influence of IL28RA on infectious and autoimmune diseases.

Expression of IFN-λ ligands is modulated by SNPs in both transcription factor binding sites and methylation sites of the promoter region, as well as frameshift mutations (99–102). The IFN-λ gene layout is shown in **Figure 2**. The clinical impact of SNPs in the IFNL3/4 locus was originally observed in the context of IFN-α treatment outcomes in patients with chronic HCV (79, 80, 87, 90, 103). SNPs within this locus are in high linkage disequilibrium, e.g., rs12979860 with ss469415590 (103, 104), which complicates the exploration of the effects of individual SNPs. Therefore, the impact of some SNPs on IFN-λ expression is still debated. Most studies have concluded that the minor alleles of SNPs rs12979860 (CT/TT) and rs8099917 (TG/GG) are associated with reduced IFNL3 expression during chronic HCV infection, observed in liver biopsies (80, 105–107), serum, and PBMCs stimulated with polyI:C-, CMV-, and influenza virus (66, 76, 108, 109). However, it has also been shown that the TT allele of rs12979860 in hepatocytes expresses higher levels of IFNL1 and IFNL3 (110). This minor allele genotype of rs12979860 (TT) has also been associated with a higher and prolonged ISG expression in HCV infection (79, 80, 87, 90, 103, 111). Interestingly, the same SNP of the IFNL3 gene is associated with a higher ISG expression in mothers after childbirth, suggesting that postpartum the normalization of physiological control of IFN signaling depends on the IFNL3 genotype (112). Although the rs12979860 SNPs have been specifically associated with IFNL3/L4 expression, these SNPs might also affect the expression of the other IFN-λ genes (80, 87, 113).

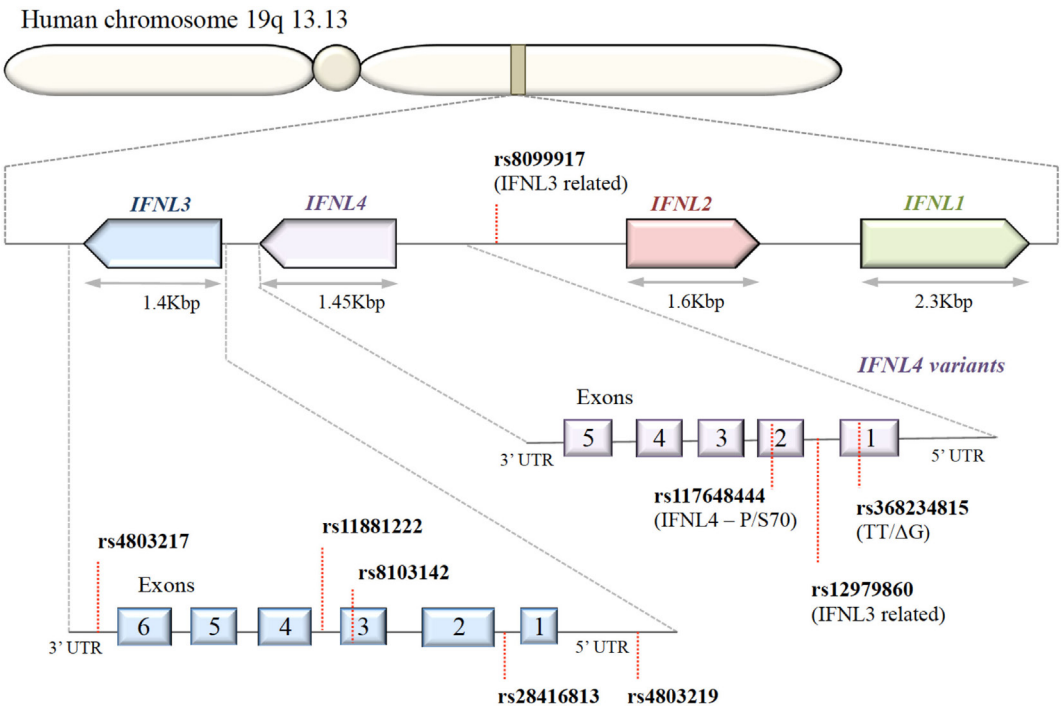

FIGURE 2 | Organization of IFNL genes in the human genome. The IFN-λ genes are located in tandem on chromosome 19. Key single-nucleotide polymorphisms (SNPs) in coding and non-coding regions of IFN-λ genes are shown. IFNL1, IFNL2, and IFNL3 genes are functional; only a subset of the human population possess the SNP rs368234815 with ΔG frameshift mutation in exon 1, producing an in-frame IFNL4.

The impact of the ss469415590 SNP on the expression of IFNL4 is, in contrast, very well described: in the context of a delta-G polymorphism, a frameshift mutation generates a gene containing an alternative reading frame, which causes IFNL4 to be functionally expressed in about 40% of Caucasians (90). An amino acid substitution at residue 70 of IFNL4 (P70S) decreases the antiviral activity *via* a reduction in the ISG expression levels (111).

Beside the impact of SNPs on innate immune signaling *via* differences in ISG expression profiles, an important impact on adaptive immune functions has been noted. We have shown that IFN-λ decreases virus-induced B-cell proliferation and antibody secretion in a dose-dependent manner. In addition, IFN-λ increases influenza-induced Th1 cytokines (IFN-γ, IL6), whereas influenza-induced Th2 cytokines decrease (IL4, IL5, IL9, IL13). These effects can also be reproduced with specific allelic combinations. In particular, the TG/GG allele of rs8099917 shows significantly lower levels of IFN-α, IL2, and IL6 secretion in influenza-stimulated PBMCs. In an influenza vaccine cohort, vaccine recipients with the rs8099917 TG/GG (minor) allele showed significantly higher vaccine-induced humoral immune responses (76). Similarly, in a cohort of children vaccinated against measles, the post-vaccine antibody titers were significantly higher in the group with the rs10853727 SNP AG and GG (minor allele) (89). Both SNPs rs8099917 and rs10853727 lie within the IFNL3 promoter region and have been associated with lower IFNL3 expression (76, 89).

IFN-λ AND INFECTIOUS DISEASES

The dual role of IFN-λs, with direct antiviral effects (innate immunity) and more long-term immunomodulatory effects on T- and B-cell activation and modulation, can result in multiple possible interactions with different types of infectious disease. **Table 2** summarizes the role of IFN-λs in several infectious diseases.

Viral Infections

IFNs protect cells against viral infections. In response, every virus has evolved specific ways to counteract IFN signaling and its effects (139–143). Only a few studies have explored this in the context of IFN-λs. Parainfluenza virus 3 blocks antiviral mediators downstream of the IFNLR signaling by modulation of the STAT1 phosphorylation in BEAS 2B cells, a bronchial epithelial cell line (144). Dengue virus was recently shown to induce IFNL1 *via* its non-structural protein (NS1) in order to facilitate dendritic cell migration (114).

Using cell culture-based *in vitro* models, IFN-λs have been shown to play a role in controlling viral replication. In most studies, cultured cells were treated with IFN-λs and the impact of viral infection was assessed. These studies investigated human (66) and murine CMV (59), dengue virus (114, 145), encephalomyocarditis virus (28, 29, 146), herpes virus type 2 (120), hepatitis B virus (115), HCV (37, 60, 113, 115, 116, 147), HIV (40, 117, 118), human meta pneumovirus (121), influenza virus (122, 148–152),

TABLE 1 | Single-nucleotide polymorphisms (SNPs) within the IFNL3/IFNL4 gene locus and impact on infectious diseases.

Gene	SNP	Allele type	Effects of the allele on infectious diseases	Reference
IFNL3	rs12979860	C/T and T/T (C-major, T-minor)	HCV: decrease of effective treatment for HCV	(79, 80)
		C/T and T/T (C-major, T-minor)	HTLV1: higher proviral load and higher risk of developing HTLV-1-associated myelopathy and tropical spastic paraparesis (TSP)	(81)
		C/C (C-major)	HBV: higher inflammation and liver fibrosis in chronic hepatitis B patients	(82)
		T/T (T-minor)	EBV: observed higher level of EBV DNA in the plasma of EBV viremia patients	(83)
		T/T (T-minor)	CMV: less CMV replication in solid-organ transplant recipients	(66)
		T/T (T-minor)	CMV: lower incidence of active CMV infection and reduced CMV DNAemia in allogeneic stem cell transplant patients	(84)
		C/T and T/T (C-major, T-minor)	HSV: increased rate of HSV-1-related herpes labialis and more clinical severity	(85)
		T/T (T-minor)	ANDV: associated with mild disease progression	(86)
	rs8099917	T/G (T-major, G-minor)	HCV: lower response to PEG-IFN-α/RBV treatment	(87)
			HTLV1: high risk for developing HTLV-1-associated myelopathy and TSP	(88)
			CMV: trend to show less CMV replication in solid-organ transplant recipients	(66)
		G/G (G-minor)	ANDV: associated with mild disease progression	(86)
		T/G and G/G (T-major, G-minor)	Influenza vaccination: increased Th2 cytokine production and higher rate of seroconversion following influenza vaccination	(76)
	rs4803217	C/T (C-major, T-minor)	HCV: decreased response to PEG-IFN-α/RBV treatment	(80)
	rs10853727	A/G and G/G (A-major, G-minor)	Measles vaccination: increased post-vaccine titers against measles vaccination	(89)
	rs12980275	A/G (A-major, G-minor)	HCV: failure to clear infection (null virological response: NVR)	(80, 87)
IFNL4	ss469415590	ΔG/TT and ΔG/ΔG (frameshift variant from TT genotype)	HCV: creates a new IFNL4 gene and poorer response to PEG-IFN-α/RBV treatment	(90)
	(rs368234815)		CMV: increases susceptibility to CMV retinitis among HIV-infected patients	(91)
			CMV: higher susceptibility to CMV infection in solid-organ transplant recipients	(92)
			HIV: higher prevalence of AIDS-defining illness and lower CD4 lymphocytes levels	(93)
IFNLR1	rs10903035	A/G and G/G (A-major, G-Minor)	HIV/HCV: early treatment failure with HIV/HCV coinfected patients	(94)

IFNL3, interferon lambda 3; IFNL4, interferon lambda 4; IFNLR1, interferon lambda receptor 1; HCV, hepatitis C virus; HTLV-1, human T-lymphotrophic virus type 1; HBV, hepatitis B virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HSV, herpes simplex virus; ANDV, Andes virus; HIV, human immunodeficiency virus; PEG-IFN-α/RBV, pegylated-Interferon-α/Ribavirin.

lymphocytic choriomeningitis virus (LCMV) (125), norovirus (124), respiratory syncytial virus (128, 153, 154), sendai virus (155–157), and vesicular stomatitis virus (131, 158, 159).

In vivo, the complexity of the role of IFN-λ within tissues and between various immune cells has been explored using an IL28RA$^{-/-}$ mouse model, leading to the discovery of multiple important aspects of IFN-λ signaling (122, 130, 150).

A recent study by Lin et al. demonstrated that the effects of type III IFNs change with increasing age. Rotavirus was controlled by both type I and III IFN in suckling mice, whereas epithelial cells in particular were responsive. In adult mice, epithelial cells were responsive only to type III and not type I IFNs, suggesting an orchestrated spatial and temporal organization of the IFN-α and IFN-λ responses in the aging murine intestinal tract (160). However, there is some controversy regarding the rotavirus data, as other researchers have shown that rotavirus is specifically controlled by type III and not type I IFN (21518880). Mahlakoiv et al. showed that leukocyte-derived IFN-α/β and epithelial IFN-λ constitute a compartmentalized mucosal defense system to restrict enteric viral infection in mice. The authors concluded that epithelial barriers to IFN-λ may have evolved to reduce frequent triggering of IFN-α/β and thus reduce exacerbated inflammation (161). A study by Baldridge et al. showed that antibiotics could prevent the persistence of enteric murine norovirus infection, but only in the presence of functional IFN-λ signaling. The IL28RA$^{-/-}$ mice showed a high rate of infection, despite the administration of antibiotics. This may suggest cross talk between the gut microbiota and IFN-λ signaling in modulating chronic viral infections (162). Important synergistic effects in the intestine have been described, with IL22-inducing IFN-λ expression in intestinal epithelial cells in a murine rotavirus infection model (163).

The role of IFN-λ during respiratory tract infections has also been explored using the IL28RA$^{-/-}$ mouse model. The studies so far have concentrated on the classical role of IFNs as antiviral cytokines. The IL28RA$^{-/-}$ mouse displayed a significantly higher burden of disease than wild-type mice during infections with influenza virus and SARS coronavirus (122, 130, 150). One study showed the immunoregulatory function of IFN-λ in an LCMV model. The authors noted that in an acute LCMV infection model, the IL28RA$^{-/-}$ mouse showed a greater than normal CD4^{+} and CD8^{+} T-cell response compare to the wild type, whereas in a chronic LCMV infection model, the IL28RA$^{-/-}$ mice showed a greater disease burden and a significantly reduced LCMV-specific T-cell response. The paper showed that germinal center B-cells

TABLE 2 | Described role of IFN-λσ in infectious diseases.

Pathogens	Model	Role of IFN-λ	Reference
Viruses			
Cytomegalovirus (CMV)	*In vitro:* HFF cell line and stimulated peripheral blood mononuclear cells (PBMCs) Clinical study	IFNL3 reduces CMV-induced CD4 T cell proliferation in PBMCs	(66)
Dengue virus	*In vitro*: DC and human lung epithelial cell line A549	IFNL1 induce CCR7 expression and DC migration upon dengue virus infection	(114)
HBV	*In vitro:* murine hepatocyte cell line (HBV-Met)	IFNL induces IFN-α/β-like antiviral response and inhibition of HBV replication in murine heptocyte cell line	(115)
Hepatitis C virus (HCV)	*In vitro: primary* hepatocytes and HUH7 cell lines.	IFNL induces type-1 interferon-like antiviral response and blocks HCV infection in human primary hepatocyte and HUH7 cells	(59, 115, 116)
HIV	*In vitro*: monocyte-derived macrophages *In vitro*: T-cells and clinical study	IFNL3 inhibits HIV infection of macrophage through the JAK-STAT pathway. IFNL induce antiviral state in culture primary T-cells and supress HIV-1 integration and posttranscriptional events	(117, 118)
HSV-1	*In vitro*: human lung epithelial cell line A549 Clinical study	Mediator complex (Med23) interacts with IRF-7 to enhance IFNL production and it inhibits HSV-1 replication	(119)
HSV-2	*In vitro*: human cervical epithelial cells	IFNL contributes to TLR3/RIG-1-mediated HSV-2 inhibition	(120)
Human metapheumovirus (HMPV)	*In vitro*: human lung epithelial cell line A549	Mice treated with IFNL prior to HMPV infection develop lower viral titer and reduced inflammatory responses	(121)
Influenza virus	*In vivo:* mice *In vitro:* cell lines *In vivo:* infected mice	IFNL restricts virus infection in epithelial cells of respiratory and gastrointestinal tracts IFNL reduced Influenza A virus-induced disease, with less inflammatory side effects in comparison to IFN alpha	(122, 123)
Murine CMV	*In vitro:* intestinal epithelial cell lines	IFNL1 mediates antiproliferative and antiviral signals in intestinal epithelial cells	(59)
Norovirus	*In vivo:* infected mice	IFNL cures persistent murine norovirus infection	(124)
Lymphocytic chorimeningitis virus	*In vitro*: human lung epithelial cell line A549	IFNL2 showed more potent antiviral response to lymphocytic choriomeningitis virus than IFNL3	(125)
Rhinovirus	*In vitro:* human bronchial epithelial cell line (BEAS-2B)	Increased IFNL production reduces rhinovirus replication in bronchial epithelial cells	(126)
RSV	*In vitro:* primary human and mouse airway epithelial cells *In vitro:* Hep-2 and Vero cells	TLR-s mediates IFNL production in primary airway epithelial cells and induces the antiviral response IFNL-1 shows prophylactic potential against RSV	(127, 128)
Rotavirus	*In vivo:* infected mice	IFNL reduces viral replication in epithelia cells	(129)
SARS coronavirus	*In vitro*: human lung epithelial cell line A549 *In vivo:* infected mice	Ifnlr1$^{-/-}$ mice exhibit increased susceptibility to SARS corona virus	(122, 130)
VSV	*In vitro:* mouse hepatocyte cell line	IFNL attenuates VSV replication in immortal mouse hepatocytes (MMHD3 cells)	(131)
West Nile virus	*In vitro:* Huh7.5 and HeLa cells *In vivo: infected* mice	IFNL can efficiently prevent West Nile Virus infection in cell line IFNL knockout animals show increased viral load in brain. Treatment with IFNL reduced blood–brain permeability for the virus	(67, 132)
Bacteria			
Staphylococcus aureus and *Pseudomonas aeruginosa*	*In vivo:* infected mice	Ifnlr1$^{-/-}$ mice exhibits less pathology without changes in cell infiltrates	(133)
Mycobacterium tuberculosis	*In vitro:* human lung epithelial cell line A549 Clinical study	Induces IFNL expression on A549 lung epithelial cells Observed increased concentration of IFNL2 in sputum of pulmonary tuberculosis patients	(134, 135)

(*Continued*)

TABLE 2 | **Continued**

Pathogens	Model	Role of IFN-λ	Reference
Listeria monocytogenes	*In vivo:* infected mice	IFNL-mediated immune response may control bacterial colonization	(136)
Salmonella typhimurium	*In vitro:* human monocyte-derived macrophages	The activation of type III interferon by live and heat killed bacteria in phagocytic dentritic cells, but role in pathogenesis is not clear	(137)
Borrelia burgdorferi	*In vitro: stimulated* PBMCs	The ability of IFNL induction correlates with clinical isolates, type III IFN pathway in pathogenesis is yet to be determined	(138)

HSV-1, herpes simplex virus-1; HSV-2, herpes simplex virus-2; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus; murine CMV, murine cytomegalovirus; SARS, severe acute respiratory syndrome.

were more frequent in peripheral blood in the IL28RA$^{-/-}$ mice than wild-type mice. However, the LCMV-induced memory B-cell response, in terms of frequencies and LCMV-specific antibodies, was comparable (164).

The immunoregulatory actions of IFN-λs have been explored in an ovalbumin (OVA)-induced asthma model. The IL28RA$^{-/-}$ mice showed a clear shift to increased Th2 cytokines and a more severe asthma phenotype. Importantly, IgE antibodies were also significantly increased (73). In this model, the IFNL2 (IL28A) immunoregulatory activity was dependent on lung CD11c^{+} dendritic cells to decrease OX40L, increase IL-12p70, and thereby promote Th1 differentiation (73). The potential role in infection-triggered asthma has also been explored in humans (72, 126).

Although these conclusions from mice studies are very important, a series of important differences to human effects have also been noted. In a human chimeric mouse model using human hepatocytes, the response rates of human and mice hepatocytes toward IFN-λs were very different, specifically in that mouse, hepatocytes did not respond to IFN-λ (56). In addition, the expression of IFNLR in immune cells seems to be strikingly different. Whereas B-cells in humans respond to IFN-λs, in B-cells from mice there seems to be no direct effect from IFN-λs (69, 164).

Studies on the impact of IFN-λs in clinical scenarios have been dominated by the strong association of IFNL3/L4 SNPs with spontaneous clearance of HCV and IFN-α treatment response (79, 80, 87, 90, 103, 111). Details on this important association have been reviewed in detail elsewhere (165–167). The association between IFN-λ SNPs and other infectious diseases is far less well explored. Not many studies have linked the genetic associations with mechanistic immunological assay.

Several studies have explored the association between SNPs in the IFNL3/L4 signaling and CMV replication. Transplant recipients with the rs8099917 GG allele demonstrate significantly less CMV primary replication. This SNP has been associated with reduced ISG expression upon infection (66). We postulate that this phenomenon has two reasons: (i) significant primary CMV replication is less likely due to a higher baseline ISG expression and (ii) naïve CMV-specific T cells from seronegative healthy blood donors show reduced proliferation capacity when pretreated with IFNL3 and stimulated with CMV lysate (66). In contrast, the rs368234815 ΔG SNP shows a higher risk for CMV retinitis in HIV-infected patients (91) and has been associated in a transplant cohort with an increased risk of CMV replication and disease, especially in patients receiving grafts from seropositive donors (92). Non-immunosuppressed patients with chronic periodontitis due to herpes virus infection show significant lower IFNL1 levels in gingival fluid compared to a healthy control group without viral replication (168), suggesting a protective effect of IFNL1 on virus replication, or CMV-induced antagonism of IFN-λ expression. These results highlight the different roles of IFN-λs in acute or chronic infection scenarios and viral reactivation.

The impact of IFN-λs on human T-cell leukemia type-1 virus has also been explored in several independent cohorts. The first evidence came from Kamihira et al. showing that the IFNL3 mRNA expression level was significantly higher in HTLV-1 mono-infection than HTLV-1/HCV coinfection. In addition, the high expression level was associated with the rs8099917 TT SNP (169). The impact of the rs8099917 GG SNP on the risk of HTLV-1 associated myelopathy/tropical spastic paraparesis (TSP) has since been confirmed (88). The impact of the rs12979860 SNP is more controversial. One study on the rs12979860 SNP showed that the CT/TT alleles were more frequent in patients with HTLV-1-associated myelopathy/TSP (81), although this finding was not replicated in two additional studies (170, 171). de Sa et al. reported that the major alleles of IFNL3 SNPs (rs12979860 CC and rs8099917 TT) are associated with a shift in the Th1/Th2 immune response toward a Th1 response (172). The Andes virus causes a hantavirus cardiopulmonary syndrome; in a cohort of Andes virus-infected patients, the minor alleles of rs12979860 and rs8099917 (TT and GG) were linked to milder disease compared to CT/CC and TG/TT (86).

The impact of the IFN-λ signaling on humoral immune function has been described in two vaccine cohorts: immunosuppressed patients vaccinated against influenza (76) and healthy children vaccinated against measles (89). These important observations hold promise for personalized vaccine strategies and adjuvant development (4).

Bacterial Infections

The cytokine microenvironment of a tissue may have an impact on the rate at which a particular infectious bacterium can colonize and also influence the rate of infections. Planet et al. showed that IFN-λs might lead to important changes in the local microbiota during influenza infection. In a mouse model of influenza infection, the authors observed that mice with functional IL28 signaling showed more profound changes in their respiratory microbiota and subsequent higher colonization rates with *Staphylococcus aureus* compared to IL28RA$^{-/-}$ mice (173). These

important findings should be confirmed in a human cohort, as *S. aureus* is an important source of bacterial superinfection after an influenza infection. In addition, microbiota changes upon common clinical scenarios such as antibiotic treatment may be modulated by IFN-λs and their genotypes.

Bacteria including *M. tuberculosis* induce IFN-α/β and IFN-γ; however, little is known about the effects of IFN-λs in epithelial immunity. Gram-positive bacteria such as *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Listeria monocytogenes* induce IFN-λs, whereas *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Chlamydia trachomatis* do not substantially induce IFN-λs, in intestinal and placental cell lines (134). Others have reported that *S. enterica* serovar Typhumurium can induce IFN-λs in human DCs (137). IFN-λ gene expression can be increased within DCs upon stimulation with bacterial components such as lipopolysaccharide. In particular, during *M. tuberculosis* infection, IFN-α plays an important regulatory role in the pathogenesis (12, 174). *M. tuberculosis* in A549 lung epithelial cells stimulates expression of IFN-λs. In addition, the IFNL2 concentration in sputum of patients with pulmonary tuberculosis is significantly higher than that in the sputum of healthy controls (135). Although the impact of IFN-λs has not been explored in more detail, the cross talk between IFN-α and IFN-λs may play a crucial role in the pathogenesis of *M. tuberculosis*. The modulation of Th1/Th2 toward Th1 may be of additional importance.

Neutrophil functions are crucial in clearing bacterial infections and wound repair (175, 176). A major target of the effects of IFN-λs may be neutrophils (62, 177). A study by Blazek et al. showed that in a collagen-induced arthritis model, IFNL1 showed anti-inflammatory function by reducing the numbers of IL17-producing Th17 cells and the recruitment of IL-1b expressing neutrophils, which is important to amplify the inflammatory process (62). Similar effects on neutrophil recruitment to the lung have been observed in an OVA-based asthma mouse model (73). Although somewhat speculative, this may suggest an important modulatory function of IFN-λs *via* neutrophil recruitment toward sites of bacterial infection.

So far, only one study has linked SNPs in genes involved in the IFN-λ signaling pathway with an increased risk of bacterial infections. Xiao et al. showed that SNP rs10903035 with G allele in the IL28RA was associated with significantly less frequent urinary tract infection (178).

Parasite and Fungal Infections

The role of IFN-λs in parasitic and fungal disease has not yet been explored. Although somewhat speculative, helminth infections in particular might be regulated by SNPs in the IFN-λ system, considering the profound evidence on the importance of Th1/Th2 balance (6, 77, 78). Furthermore, for parasite infections of the liver such as *Plasmodium* spp. there is important evidence on the importance of the IFN-α signaling (13, 179–182). Due the regulatory interactions of IFN-α and IFN-λ and the clinical importance of relevant SNPs (31), it is not unreasonable to postulate an impact.

SUMMARY

IFN-λs, and their modulation *via* SNPs, are increasingly recognized as important players in a broad range of infectious diseases. Although the literature is still dominated by reports on HCV, work especially in mouse models has pointed out the important role in viral, respiratory, and gastrointestinal infections. Bacterial colonization and bacterial infections may also be modulated by IFN-λs. The important diversity in IFNs and the large number of SNPs adds a difficult-to-address layer of complexity. Therefore, further research on IFN-λs outside the HCV field is required to understand their roles and diagnostic and therapeutic potential. Most importantly, predictions of risks associated with infectious diseases have to be confirmed in independent cohorts to allow personalized medicine strategies.

AUTHOR CONTRIBUTIONS

Both the authors (AE and MS) have significantly contributed by writing the manuscript and designing the graphs.

ACKNOWLEDGMENTS

The authors thank Dr. Helena Seth-Smith (Applied Microbiology Research, Department of Biomedicine, University of Basel) for critically reading the manuscript.

FUNDING

AE was supported by a research grant from the SNSF Ambizione Score PZ00P3_154709.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.3 An ELISA Based Binding and Competition Method to Rapidly Determine Ligand-receptor Interactions

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Manuscript has been published in Journal of Visualized Experiments, 2016; 14;(109). doi: 10.3791/53575.

Contribution of my work:
Design of experiments, Performance of experiments, Partly analysis of data and writing the paper.
Figure 1; Figure 2; Figure 3; Figure 4; Table 1; Table 2

Note: The following part contains the whole manuscript

Video Article

An ELISA Based Binding and Competition Method to Rapidly Determine Ligand-receptor Interactions

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URL: http://www.jove.com/video/53575
DOI: doi:10.3791/53575

Keywords: Chemistry, Issue 109, ELISA, screening, ligand-receptor interaction, dissociation constant, binding affinity, blocking

Date Published: 3/14/2016

Citation: Syedbasha, M., Linnik, J., Santer, D., O'Shea, D., Barakat, K., Joyce, M., Khanna, N., Tyrrell, D.L., Houghton, M., Egli, A. An ELISA Based Binding and Competition Method to Rapidly Determine Ligand-receptor Interactions. *J. Vis. Exp.* (109), e53575, doi:10.3791/53575 (2016).

Abstract

A comprehensive understanding of signaling pathways requires detailed knowledge regarding ligand-receptor interaction. This article describes two fast and reliable point-by-point protocols of enzyme-linked immunosorbent assays (ELISAs) for the investigation of ligand-receptor interactions: the direct ligand-receptor interaction assay (LRA) and the competition LRA. As a case study, the ELISA based analysis of the interaction between different lambda interferons (IFNLs) and the alpha subunit of their receptor (IL28RA) is presented: the direct LRA is used for the determination of dissociation constants (K_D values) between receptor and IFN ligands, and the competition LRA for the determination of the inhibitory capacity of an oligopeptide, which was designed to compete with the IFNLs at their receptor binding site. Analytical steps to estimate K_D and half maximal inhibitory concentration (IC_{50}) values are described. Finally, the discussion highlights advantages and disadvantages of the presented method and how the results enable a better molecular understanding of ligand-receptor interactions.

Video Link

The video component of this article can be found at http://www.jove.com/video/53575/

Introduction

A comprehensive understanding of signaling pathways requires detailed knowledge about the ligand-receptor interaction. Most methods for assessing the interaction of a particular ligand with its specific receptor are expensive, time consuming, labor intensive and require specific equipment and expertise [1].

This article describes two fast and reliable point-by-point protocols to investigate the ligand-receptor interaction based on an enzyme linked immunosorbent assay (ELISA): the direct ligand-receptor interaction assay (LRA) and the competition LRA. ELISA is a highly sensitive, specific and readily available technique, routinely used in almost every laboratory. ELISA can be performed and adapted in various fashions. The presented protocols are optimized for the investigation of the interaction between different lambda interferons (INFLs) and their receptor.

The direct LRA allows for a quantification of ligand-receptor binding with respect to ligand concentration and thus yields a binding curve. Using an appropriate model for the ligand-receptor interaction, the data can be further analyzed to estimate the dissociation constant (K_D).

In the presented protocol, the commonly used Hill equation is applied to model the ligand-receptor binding. Although other methods such as the surface plasmon resonance technology [2,3] allow the determination of the binding affinities between two proteins, this technology is often labor intensive, expensive, and requires special laboratory equipment.

The competition LRA enables the screening of inhibitory peptides: The ligand-receptor binding is quantified with respect to peptide concentration. This yields a dose-response curve describing the inhibitory effect of the peptide. The data can be further analyzed to estimate the half maximal inhibitory concentration (IC_{50}) of the blocking peptide.

Both ELISA protocols are easy to use and can be adapted to a broad range of research questions. Recombinant proteins of any kind can be used to reliably and fast determine the interaction parts. In addition, the competition LRA can be used to determine critical interaction sites of

ligands and receptors by using blocking peptides, which are designed to mimic either the ligand or the receptor. If the blocking peptide shows efficient and specific inhibition, the peptide occupies a critical interaction site of the ligand (if the peptide mimics the receptor) or of the ligand (if the peptide mimics the ligand).

The first protocol describes the K_D value determination of different INFLs and the alpha subunit of their receptor, *i.e.,* the interleukin-28 receptor (IL28RA) using the direct LRA. Next, the second protocol shows how to determine the capability of a 20 amino acid long peptide to inhibit the INFL-IL28RA interactions. The peptide is designed to compete with IFNLs at their receptor binding site and thus enables a molecular understanding of the interaction. Furthermore, this peptide can be used to block IL28RA in *in vitro* experiments to determine the impact on downstream signaling effects[4].

Protocol

1. Reagent Preparation

1. To prepare carbonate coating buffer, dissolve 0.36 g Na_2CO_3 and 0.84 g $NaHCO_3$in 100 ml distilled water; sterile filter the buffer by using a vacuum driven 0.22 µm polyethersulfone (PES) membrane filter and store at RT until usage.
2. Prepare washing solution by adding 0.05% v/v Tween 20 in Phosphate buffered saline (PBS).
3. Prepare a 5% Bovine Serum Albumin (BSA) (blocking solution) in PBS solution by dissolving 5 g BSA (≥98%) in 100 ml PBS and store at 4 °C.
4. Recombinant Receptor, Ligands and Blocking Peptides
    1. Reconstitutethe recombinant human interleukin receptor alpha subunit (IL28RA) and recombinant His-tagged ligands of human IFN (IFNL1-3) according to the manufacturer's instructions and store at -80°C. Synthetize blocking peptides and used as previously described [4]. Use PBS to prepare different concentrations of ligands and peptides for use in the assays.

5. To prepare the primary antibody, dilute 6x His Mouse monoclonal antibody in PBS with 0.1% BSA at 1:1,000 dilution. To prepare the secondary antibody, dilute horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (H+L) in PBS with 0.1% BSA at 1:10,000 dilution.
6. Prepare TMB solution by mixing the reagents A and B according to the manufacturer's instructions.
7. Prepare stop solution by adding 5 N sulphuric acid (H_2SO_4) in distilled water and store at RT.

2. Enzyme-linked Immunosorbent Assays (ELISAs)

NOTE: The direct ligand-receptor interaction ELISA (direct LRA, **Figure 1**) can be used to measure the receptor-ligand dissociation constant (K_D), as a measure of the receptor-ligand binding affinity. The competition ligand-receptor interaction ELISA (competition LRA, **Figure 2**) allows screening of peptides (and other blocking compounds), which act to interfere with the interaction between ligand and receptor. The basic protocol that was previously published [5] was further optimized.
NOTE: In both ELISA methods use multichannel pipette for adding solutions to the wells of 96-well plate in each step. In solution decant or washing steps, throw out the solutions directly into the sink.

1. Direct Ligand-Receptor-Interaction Assay (direct LRA)
   NOTE: For an illustration of the workflow (see **Figure 1**).
    1. Coating Plate with Recombinant Receptor
        1. Dilute the recombinant receptor in carbonate buffer to a final concentration of 100 ng/µl. Coat wells of 96-well microtitre plate with fixed receptor concentration (100 ng/µl) by pipetting 100 µl to each well using a multichannel pipette. Exclude outer walls of the plate to avoid well edge artifact. Cover the plate with a lid and incubate the plate at 4 °C O/N.

    2. Blocking and Addition of Ligands
        1. The next day, remove the coating solution by tilting the plate against the sink and wash the plate 3 times with washing solution (PBS + 0.05% v/v Tween 20).
        2. Block the free receptor-binding sites in the coated plate using 200 µl of 5% BSA solution to each well using a multichannel pipette and incubate the plate for 2 hr at RT.
        3. Discard the blocking solution (see step 2.1.2.1.) and wash the plate 3 times with washing solution.
        4. Prepare the recombinant His-tagged ligands at different concentrations (*e.g.,* 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.063 µg/ml, 0.031 µg/ml, 0.0 µg/ml) in PBS. Add only PBS in the blank wells.
        5. Add 100 µl of each ligand concentration to the wells in duplicate and incubate the plate for 2 hr at RT allowing receptor-ligand interaction.

    3. Incubation with Antibodies
        1. Following incubation with the ligands, wash the plate 3 times with washing solution.
        2. Pipette 100 µl of primary anti-His mouse monoclonal antibody solution (1:1,000) to each well.
        3. Incubate the plate at RT for 2 hr; after incubation, discard the antibody solution (see step 2.1.2.1.) and wash the plate 3 times with washing solution.
        4. Add 100 µl of HRP coupled goat anti-mouse IgG secondary antibody solution (1:10,000) to each well. Incubate the plate for 45 min at RT.
        5. Discard the antibody solution (see step 2.1.2.1.) and wash the plate 3 times with washing solution.

    4. Addition of Substrate and Development
        1. Bring the TMB substrate solutions to RT and prepare TMB substrate solution A and B at 1:1 ratio. Add 100 µl freshly prepared substrate to each well and keep the plate at RT for 15-30 min. After sufficient color development add 50 µl stop solution.

5. Reading the Plate and Data Analysis
   NOTE: The described protocol is based on the assumption that the measured signal rises from specific binding. It might be necessary to estimate the contribution of unspecific binding to the signal but this is out of the scope of this protocol.
   1. Read the absorbance (optical density, OD) directly at 450 nm.
   2. Subtract the background signal from the measured OD values and normalize them. Transform all values of the ligand concentration to logarithmic scale (base 10, $\log_{10}$).
   3. Plot the normalized and background corrected OD values (Y-axis, corresponds to the fraction of occupied receptor binding sites) against the logarithm of the ligand concentration (X-axis, $\log_{10}$ scale).
   4. To estimate the K_D value, fit the data to the following form of the Hill equation:

$$Y = \frac{Y_{max}[L]^n}{(K_D)^n + [L]^n}$$

   NOTE: Here Y denotes the fraction of occupied receptor binding sites and Y_{max} the maximal binding; [L] denotes the concentration of free ligand and the Hill coefficient. If there is only one binding site for the ligand, the Hill coefficient is n = 1. For systems with more than one ligand binding site, the binding exhibits positive cooperativity if n >1, negative cooperativity if n<1 and no cooperativity if n = 1. The microscopic dissociation constant is termed and corresponds to the half maximal effective concentration EC_{50}[6]. The apparent dissociation constant is $K_d = (K_D)^n$. In the simplest case where n = 1, the dissociation constant corresponds to the ligand concentration at which half of the receptor binding sites are occupied and $K_d = K_D$. This model assumes mass action binding under equilibrium conditions, as well as that only a small fraction of the added ligand is bound to the receptor, *i.e.,* [L] >> [RL].

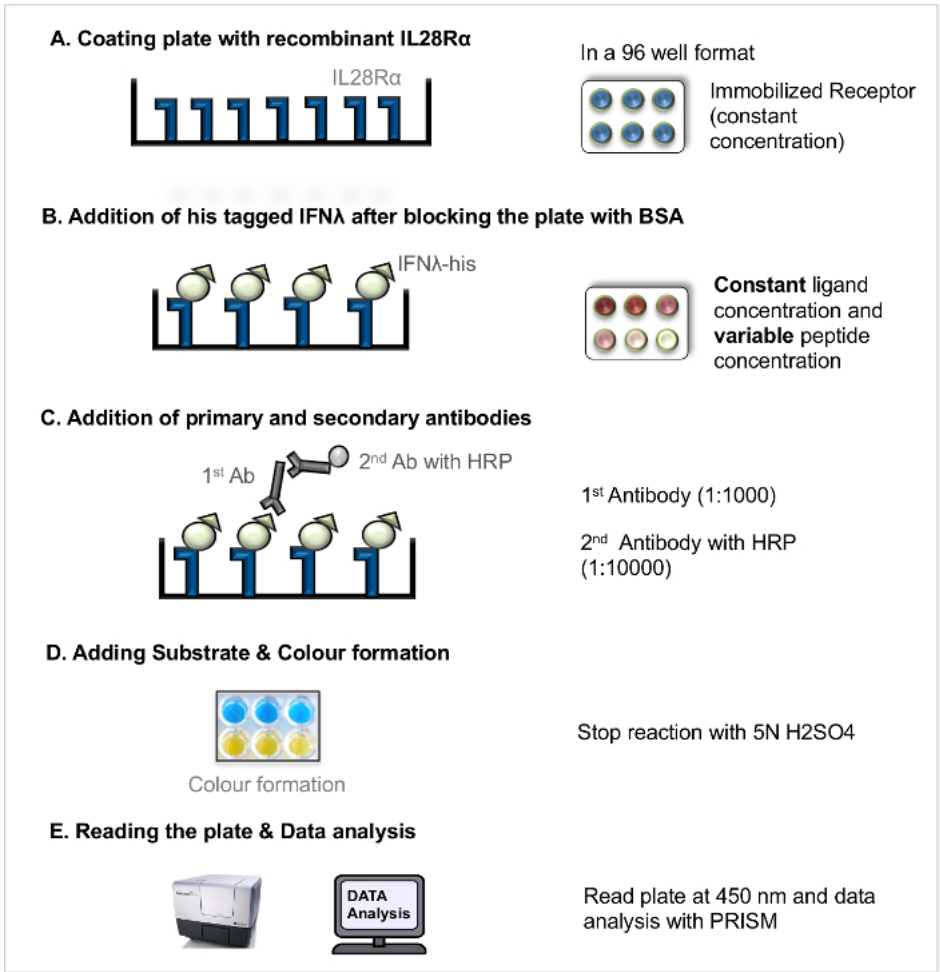

Figure 1. Direct ligand-receptor-interaction assay (direct LRA). Step-by-step protocol for direct LRA. Please click here to view a larger version of this figure.

2. Competition Ligand-Receptor-Interaction Assay (competition LRA)
   NOTE: For an illustration of the workflow see **Figure 2**. The competition LRA procedure follows the same steps as the direct LRA (coating the plate, antibody incubation, plate development) except for important changes in the ligand and peptides addition step. Proper negative controls are essential for this assay. In a previous screening study [4], the scrambled blocking peptide did not show antagonistic effects.
   1. Blocking - Addition of Ligands and Blocking Peptides
      1. The next day, remove the coating solution and wash the plate (see 2.1.2.1).

2. Block the coated plate by adding 200 µl of 5% BSA solution to each well and incubate the plate for 2 hr at RT.
3. Prepare the recombinant His-tagged ligands (IFNL1-3) at a fixed concentration (2x-20 ng/ml) in PBS.
4. Prepare the blocking peptide (cf. **Table 3**) with different concentrations ranging from 10 nM to 100 µM in PBS to guarantee a dose-response curve.
   NOTE: This enables subsequent determination of the IC_{50} value for the blocking peptide. In control wells, add only fixed ligand concentration without peptide to derive the maximum (100%) binding. In the blank, add only PBS without ligand or peptide.
5. Add 50 µl of the ligands (IFNL1-3) and 50 µl of each peptide concentration to the wells in duplicates.
6. Incubate the plate for 2 hr at RT.

2. Reading the Plate and data Analysis
   NOTE: The described protocol is based on the assumption that the measured signal rises from specific binding. It might be necessary to estimate the contribution of unspecific binding to the signal but this is out of the scope of this protocol.
   1. Read the absorbance (optical density, OD) directly at 450 nm.
   2. Subtract the background signal from the measured OD values and normalize them. Transform all values of the peptide concentration to logarithmic scale (base 10, $\log_{10}$).
   3. Plot the normalized and background corrected OD values (Y-axis, corresponds to the fraction of occupied receptor binding sites) against the logarithm of the ligand concentration (X-axis, $\log_{10}$ scale).
   4. To estimate the IC_{50} value, fit the data to the following equation:

$$Y = \frac{100}{1 + 10^{h(\log_{10}(IC_{50}) - \log_{10}[P])}}$$

   NOTE: Here [P] is the peptide concentration and the Hill slope. The Hill slope describes the steepness of the dose-response curve. The IC_{50} corresponds to the inhibitor concentration at which 50% inhibition of binding between ligand and receptor is observed.

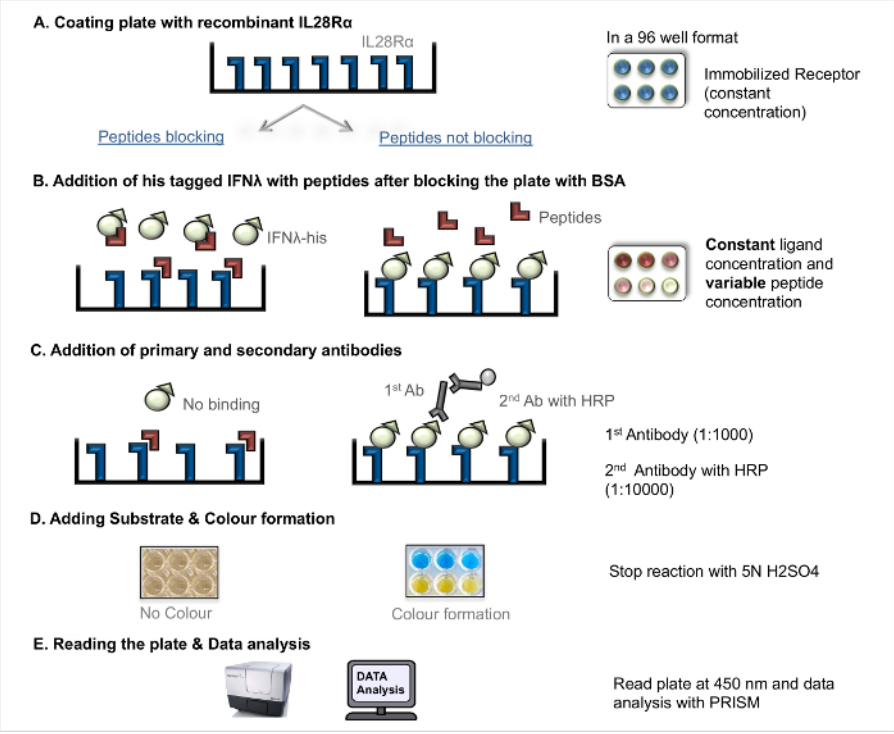

Figure 2. Competition ligand-receptor-interaction assay (competition LRA). Step-by-step protocol for competition LRA. Please click here to view a larger version of this figure.

Representative Results

The dissociation constants between INFL1-3 and their receptor alpha subunit IL28RA were determined using the direct LRA. The results are shown in **Figure 3**: The fraction of occupied binding sites is plotted against the logarithm of the respective IFN concentration. The Scatchard plot of the data is shown in the bottom right corner. The results illustrate that the direct LRA yields a binding curve, which can be further analyzed to estimate the K_D value. The K_D value was determined by fitting the data to the Hill equation (Equation 1).

IFNL1 has the highest binding affinity, followed by IFNL2 and IFNL3. The Hill coefficient n >1 suggests increased affinity for additional ligands after the initial ligand-receptor interaction (see Discussion). The estimated dissociation constants and Hill coefficients are summarized in **Table 1**.

Competitive LRA was used to quantitate the impact of a blocking peptide on the interaction between IFNL1-3 and the IL28RA (**Figure 4**). The fraction of occupied binding sites for a ligand concentration of 10 ng/ml is plotted against the logarithm of the peptide concentration. To estimate the IC_{50} values, the data is fitted to Equation 2.

The blocking peptide inhibited the interaction between IFNL3 and IL28RA (IC_{50} = 0.26 µM) to the greatest extent. The IC_{50} is twice as high for the IFNL2-IL28RA interaction (IC_{50} = 0.50 µM) and one order of magnitude higher for the IFNL1-IL28RA interaction, indicative the peptide was less effective at disrupting IFNL1-IL28RA interactions. The determined IC_{50} values and Hill slopes are summarized in **Table 2**.

Proper data analysis is essential for understanding the ligand-receptor interaction. The shown results were generated using a scientific graphing software such as GraphPad PRISM. For the K_D value determination, the data was fitted to the 'One site - specific binding with Hill slope' (corresponds to Equation 1 in Sec. 2.2.1). For the IC_{50} value determination, the data is fitted to the function 'log(inhibitor) vs. normalized response - variable slope' (see Equation 2 in Sec. 2.2.2). However, any software for non-linear regression analysis can be used.

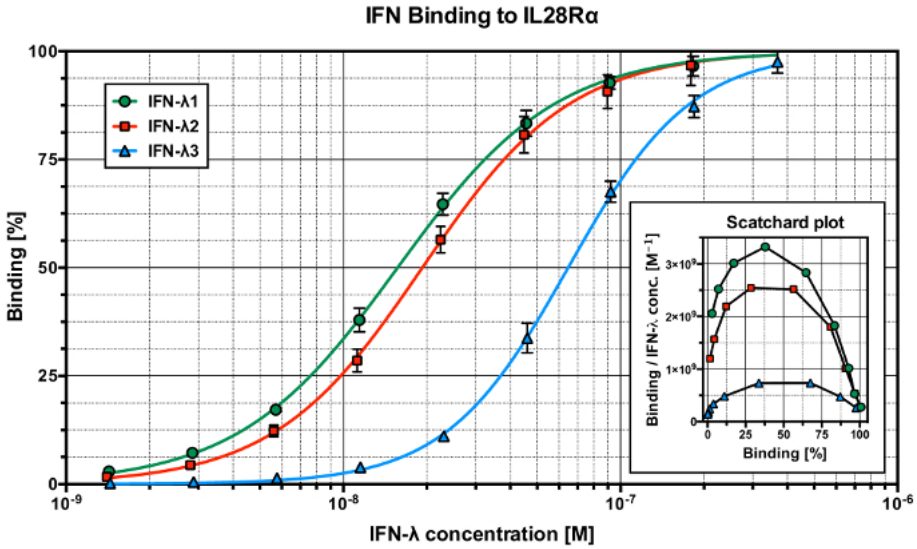

Figure 3. Results of the direct ligand-receptor assay (direct LRA). Binding curves for the binding of IFNL1 (green), IFNL2 (red) and IFNL3 (blue) to IL28RA. The respective Scatchard plot in the bottom right corner suggests positive co-operativity of the binding. Please click here to view a larger version of this figure.

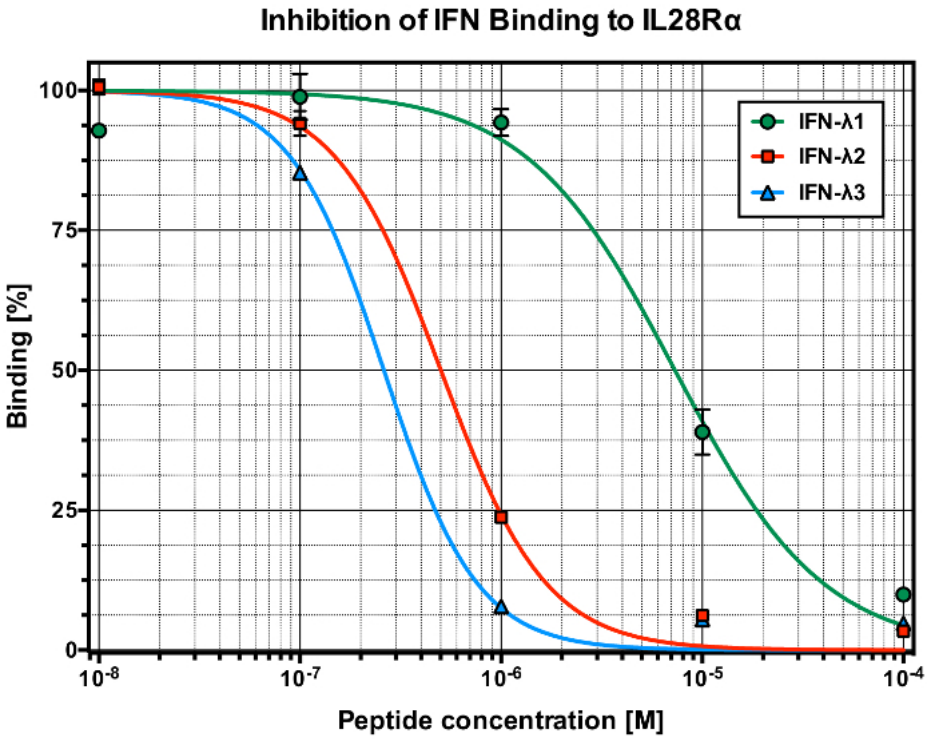

Figure 4. Results of the competitive ligand-receptor assay (competitive LRA). Dose-response curves showing inhibition of the binding of IFNL1 (10 ng/ml, green), IFNL2 (10 ng/ml, red) and IFNL3 (10 ng/ml, blue) to IL28RA by the 20 aa peptide. Please click here to view a larger version of this figure.

IFN	K_D (nM)	SE	Hill coeff.	SE
IFNL1	15.7 ± 0.3	1.90%	1.52 ± 0.04	2.60%
IFNL2	19.3 ± 0.6	3.10%	1.62 ± 0.07	4.30%
IFNL3	64.7 ± 1.3	2.00%	1.96 ± 0.06	3.00%

Table 1: Estimated dissociation constants (K_D) and Hill coefficients of IFNL1-3 binding to IL28RA. The standard error (SE) is given for a sample size of four replicates per data point.

IFN (10 ng/ml)	IC_{50} (uM)	Hill slope
IFNL1	7.31	-1.2
IFNL2	0.5	-1.7
IFNL3	0.26	-1.9

Table 2: Estimated half maximal inhibitory concentrations (IC_{50}) of the blocking peptides and the Hill slope of the dose-response curve for the binding of IFNL1-3 to IL28RA. The IFN concentration is 10 ng/ml. The number of replicates is three.

Discussion

ELISA is a standard and well-established method for many laboratories. We have further modified and improved a previously published method [5,7]. The demonstrated step-by-step protocol shows how it can be used in a simple way to determine the K_D values of ligand-receptor interactions. In addition, the IC50 of a blocking peptide that interferes with the ligand-receptor interaction can be determined.

Major advantages are the rapid setup, easy preparation of reagents and familiar handling, as most researchers have used an ELISA protocol before. The direct LRA protocol is highly flexible and can be adapted to measure many protein-protein interactions. Recombinant proteins with His6- or alternative tag should be used as the binding partner to an immobilized partner. The competition LRA can be exploited as a screening tool to (i) determine the inhibitory potential of blocking compounds (peptides, antibodies, or small molecules) and to (ii) determine the critical interaction sites by using blocking peptides designed to mimic the receptor or the ligand.

Negative controls are essential for a proper interpretation of the presented assays. In a previous screening study [4], the scrambled sequence of the used blocking peptide did not show antagonistic effects. However, other peptides showed a blocking capacity also after scrambling, likely due to unspecific electrostatic interactions.

A potential limitation is that this assay reflects an *in vitro* situation. In particular, heterodimeric receptors often form a more complex structure. It is not possible to distinguish whether the ligand just binds to the receptor or whether the ligand also activates the receptor by triggering a conformational change or a dimerization, which in turn leads to an intracellular signal. In the presented assay, we used a recombinant receptor, which is immobilized to a solid phase. This setup does not work to test the activation or to investigate the interaction of receptors, which require the membrane environment or membrane cholesterol such as G protein coupled receptors (GPCRs). Also the use of recombinant protein raises caveats. For example, the folding and tertiary structure of a recombinant protein may be different compared to an *in vivo* situation. The binding of ligand and receptor usually occurs at RT, however in humans the optimal temperature would be 37 °C. Finally, the use of commercial recombinant ligand and receptor can prove expensive. Despite these limitations, these two ELISA protocols show potential to rapidly explore the ligand-receptor interaction.

The presented results show that IFNL1 has a slightly higher affinity for IL28RA compared to IFNL2, and the affinity of IFNL3 is three-fold lower than IFNL2. This is remarkable considering the similarity between IFNLs. IFNL1 and IFNL2 differ in 33 amino acids while IFNL2 and IFNL3 differ only in seven amino acids [8]. The interaction between IL28RA and IFNLs involves Helix A and the AB-loop of IFNL [9]. Alignment of IFNL sequences reveals four significant differences in Helix A and the AB-loop between IFNL1 and IFNL3 (**Figure 5A**). One affects the salt bridge Arg54-Glu119. The amino acid residues in this section are enumerated according to the UniProt entries Q8IU54 (IFNL1), Q8IZI9 (IFNL3), Q8IZJ0 (IFNL2) and Q8IU57 (IL28RA), which has been found in the crystal structure of the IFNL1-IL28R1 complex (**Figure 5B**). Structural alignment shows that Arg57 in IFNL1 is replaced by Lys57 in INFL3, which is also able to form a salt bridge with Glu118 (**Figure 5C**). Consistent with the decreased affinity of IFNL3 and IL28RA, computational [10,11] and mutational [12] analyses show, that Lys-Glu salt bridges are in general less stable than Arg-Glu salt bridges.

However, the differences in Helix A do not satisfactorily explain the lower affinity of IFNL3, since the amino acid sequence of Helix A is identical for IFNL2 and IFNL3. It is thought that the main difference arises from the mutations in the AB-loop, where Arg74 and His76 in IFNL2 are replaced by Lys70 and Arg72 in IFNL3 [8].

Moreover, differences in stability and solubility between IFNLs may also affect the outcome of the assay. Since the direct LRA assay uses concentrations from nM to M and the physiological concentration of cytokines in serum lies in the pM to nM range, aggregation of IFNL cannot be excluded. We can assume that the observed positive cooperativity of the IFN binding is caused by a specific or non-specific increase in ligand-receptor binding, *e.g.,* a dimerization of the recombinant IL28RA receptor in solution, or a binding of a second ligand or ligand fragment with higher affinity. However, further studies are required to verify this.

The blocking peptide used in the competition LRA mimics the AB-loop of IFNL3 (**Figure 6)**. As previously described, the AB-loop plays an essential role in the interaction between IFNLs and IL28RA [9], particularly IFNL2 and IFNL3. Homology modeling of IFNL3 with the IL28RA/IFNL1 crystal structure shows that the region, which corresponds to the blocking peptide lies in close spatial proximity to the interaction interface with IL28RA (**Figure 6**). Supposed that the peptide blocks the interaction of the AB-loop of IFNL and IL28RA. The results of the competition LRA support this: The peptide has an inhibitory effect on the binding of all IFNLs, however the peptide is a more effective inhibitor of the interaction between IL28RA and either IFNL3 or IFNL2 than of the interaction of IFNL1 and IL28A. As expected, the peptide blocks the binding of IFNL3 most effectively since it occupies exactly the same binding pocket as the AB-loop of IFNL3.

As shown in **Table 3**, the AB-loops of IFNL1 and IFNL2 aligned to the blocking peptide differ. Consistent with the lower IC_{50} value of the peptide for inhibition of the IFNL1/IL28RA interaction, twelve amino acids differ between IFNL1 and the peptide whereas and only two amino acids differ between IFNL2 and the peptide. This indicates that there are slightly different binding modes for interaction between the AB-loops of the IFNLs and IL28RA and predicts that peptides which mimic IFNL1 would be more effective at blocking the interaction between IFNI1 and IL28RA than the interaction between IFNL3 and IL28RA.

Further, the peptide is overall positively charged (four arginine and two lysine residues but only two aspartate residues) and computational analysis shows that the peptide has no defined secondary motifs. Due to its coiled, flexible structure the peptide might also bind to other regions of the receptor. This could potentially block glutamate and aspartate residues such as D118, which forms a salt bridge to stabilize the ligand-receptor complex (**Figure 5B** and **5C**).

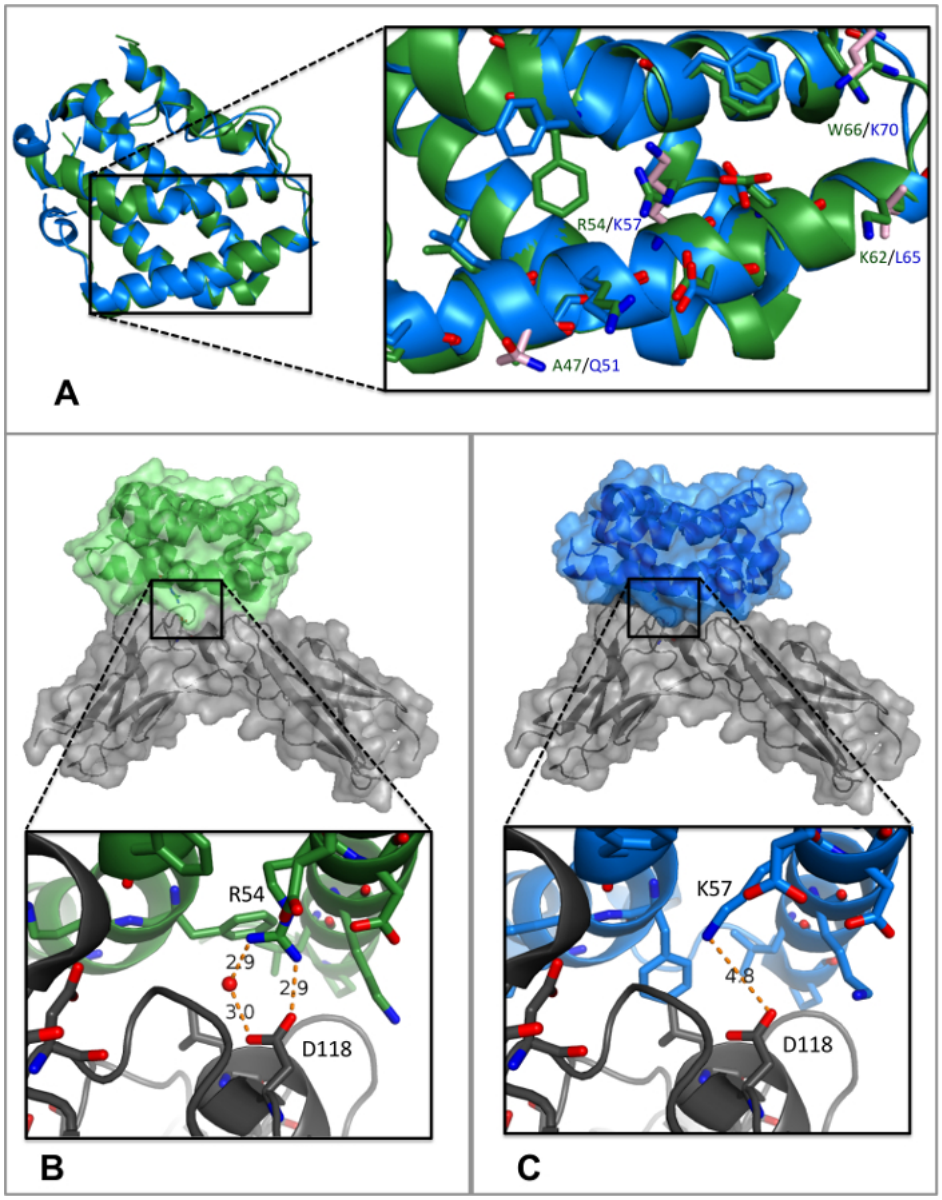

Figure 5. Structural comparison of IFNL1 (green) and IFNL3 (blue). Oxygen atoms are shown in red, nitrogen atoms are shown in blue. (**A**) Superposition of the IL28RA-bound IFNL1 and IFNL3 (IL28RA not shown). The root mean square deviation (RMSD) of all aligned atoms is 0.672 Å. Side chains of IFNL3 that differ from IFNL1 are highlighted in light-pink. (**B**) Salt bridge between Arg54 and D118. (**C**) IFNL3 aligned to IL28RA-bound IFNL1 (IL28RA is shown in grey). The alignment shows that the Arg-Glu salt bridge is probably replaced by a less stable Lys-Glu, a salt-bridge between Lys57 and D118. PyMol was used for the preparation of figures and for the alignment. Please click here to view a larger version of this figure.

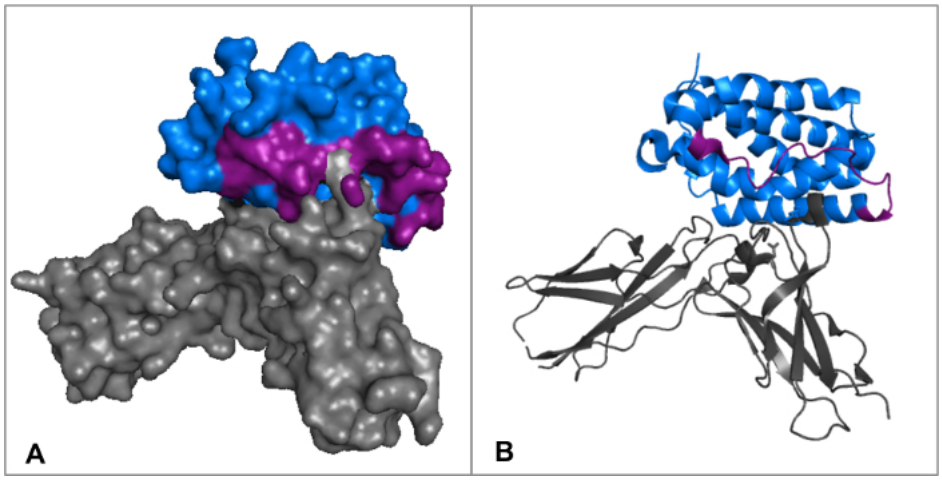

Figure 6. Alignment of IFNL3 and the IFNL1-IL28RA complex (IFNL1 not shown). IFNL3 is shown in blue and the IL28RA in grey. The regions corresponding to the blocking peptide are highlighted in purple. Please click here to view a larger version of this figure.

65 70 85 90	
▮LKLKNWSCSSPVFPGNWDLRLLQ▮	IFNL1
L-LKDCKCRSRLFPRTWDLR--Q	Pep
75 80 85	
▮LLKDCRCHSRLFPRTWDLRQ▮	INFL2
LLKDCKCRSRLFPRTWDLRQ	Pep
65 70 75 80	
▮LLKDCKCRSRLFPRTWDLRQ▮	INFL3
LLKDCKCRSRLFPRTWDLRQ	Pep

Table 3: Sequence alignment of IFNL1-3 and inhibitory peptide. The peptide mimics the AB-loop of INFL3. Amino acid mismatches are highlighted in red.

Disclosures

A.E. was supported by a research grants from the "SNSF Ambizione Score" program (PZ00P3_154709), "Forschungsfond, Förderung strategischer Projekte" University of Basel, Stiftungsinfektionskrankheiten Basel, and Bangeter Rhyner Stiftung. J.L. acknowledges support by an iPhD fellowship of the SystemsX.ch initiative in systems biology program (9th call).

Acknowledgements

We thank Prof. J. Stelling (Department of Biosystems Science and Engineering, ETH Zurich and Swiss Institute for Bioinformatics, Basel, Switzerland) for his critical review of the manuscript.

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Materials List for:

An ELISA Based Binding and Competition Method to Rapidly Determine Ligand-receptor Interactions

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URL: https://www.jove.com/video/53575
DOI: doi:10.3791/53575

Materials

Name	Company	Catalog Number	Comments
Nunc-Immunoplate (F96 Maxi sorp)	Thermo Scientific	442404	ELISA plate
Sodium carbonate (Na2CO3)	Merck	497-19-8	For ELISA plate coating buffer
Sodium hydrogen carbomnate(NaHCO3)	Merck	144-55-8	For ELISA plate coating buffer
Bovine Serum Albumin (BSA)	Sigma	A7030-100G	5% BSA in PBS for Blocking
rhIL-28Rα/IFNλR1	R&D systems	5260-MR	Recombinant human interlukin-28 Receptor alpha
rhIL-29/IFNλ1	R&D systems	1598-IL/CF	Recombinant human interlukin-29/ Carrier free/C-terminal 10-His tag
rhIL-28A/IFNλ2	R&D systems	1587-IL/CF	Recombinant human interlukin-28A/Carrier free/C-terminal 6-His tag
rhIL-28B/IFNλ3	R&D systems	5259-IL/CF	Recombinant human interlukin-28B/Carrier free/C-terminal 6-His tag
6x His Monoclonal antibody (Mouse)	Clontech	631212	Primary antiboy to capture His tagged Ligands
Goat anti-Mouse igG (H+L)	Jackson Immuno Research	115-035-166	Horseradish Peroxidase conjucated secondary antibody
BDoptEIA TMB reagent set	BD Biosciences	555214	ELISA - TMB substrate solution
Sulfuric acid (H2SO4)	Fulka	84720	5 N H_2SO_4 (Enzyme reaction stop solution)
Synergy/H1 - Microplate reader	BioTeK		ELISA plate reader

5.4 An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers

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Manuscript has been published in Journal of Visualized Experiments, 2017; 1;(130). doi: 10.3791/55833.

Contribution of my work:
Design of experiments, Performance of experiments, Partly analysis of data and writing the paper.
Partly contributed to the figure 1 to 6 and figure 8 & 9; Figure 4; Table 1& 2;

Note: The following part contains the whole manuscript

Video Article

An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers

Lukas Kaufmann*[1], Mohammedyaseen Syedbasha*[1], Dominik Vogt[1], Yvonne Hollenstein[1], Julia Hartmann[1], Janina E. Linnik[1,2,3], Adrian Egli[1,4]

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URL: https://www.jove.com/video/55833
DOI: doi:10.3791/55833

Keywords: Medicine, Issue 130, Hemagglutination inhibition assay, screening, titer, antibody, influenza A, influenza B, H1N1, H3N2, cross-reactivity, titration, antigen

Date Published: 12/1/2017

Citation: Kaufmann, L., Syedbasha, M., Vogt, D., Hollenstein, Y., Hartmann, J., Linnik, J.E., Egli, A. An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers. *J. Vis. Exp.* (130), e55833, doi:10.3791/55833 (2017).

Abstract

Antibody titers are commonly used as surrogate markers for serological protection against influenza and other pathogens. Detailed knowledge of antibody production pre- and post-vaccination is required to understand vaccine-induced immunity. This article describes a reliable point-by-point protocol to determine influenza-specific antibody titers. The first protocol describes a method to specify the antigen amounts required for hemagglutination, which standardizes the concentrations for subsequent usage in the second protocol (hemagglutination assay, HA assay). The second protocol describes the quantification of influenza-specific antibody titers against different viral strains by using a serial dilution of human serum or cell culture supernatants (hemagglutination inhibition assay, HI assay).

As an applied example, we show the antibody response of a healthy cohort, which received a trivalent inactivated influenza vaccine. Additionally, the cross-reactivity between the different influenza viruses is shown and methods to minimize cross-reactivity by using different types of animal red blood cells (RBCs) are explained. The discussion highlights advantages and disadvantages of the presented assays and how the determination of influenza-specific antibody titers can improve the understanding of vaccine-related immunity.

Video Link

The video component of this article can be found at https://www.jove.com/video/55833/

Introduction

Infection with influenza virus is associated with considerable morbidity, mortality, and high healthcare costs[1,2,3,4]. In particular, elderly, newborns, pregnant women, and patients with chronic disease are at risk for more severe clinical outcomes. Therefore, vaccination against circulating influenza virus strains is the primary measure to decrease the burden of disease in these high-risk populations. The increase of the individual immune response after vaccination, *e.g.*, influenza-specific antibodies above a protective threshold, reduces the individual risk of infection and in general the likelihood of viral transmission within a population[5]. A detailed understanding of the vaccine-induced humoral immune response in different populations and across various age groups is a key element to answer important clinical questions[6,7,8,9], such as: Why do some elderly patients have infections despite previous vaccination? What is a "good" and "sufficient" vaccine-induced protection? How often should a vaccine be applied to an immunosuppressed patient to reach protective titers? What is the most effective dosage? What is the impact of a novel adjuvant on post-vaccination antibody titers? The measurement of the vaccine-specific antibody production may help answer these important questions and improve vaccination outcomes.

The quantification of virus-specific antibody titers can be performed with various immunological methods. This includes solid-phase[10] or bead-based ELISA[11] assays, the HI assay[12], and neutralizing assays[13]. ELISA-based methods allow the screening of relatively large amounts of serum samples against various antigens. Also, pathogen-specific Immunoglobulin (Ig)M and IgG can be separately explored. Although the characteristics of an antigen, *e.g.*, the linear amino acid sequence or virus-like particle may influence the binding of antibodies, the spectrum of potential epitopes is very broad and does not provide information on whether an antibody response has functional relevance.

In contrast, the neutralization assay determines the potential of antibodies to functionally inhibit the infection of cells and therefore reflects the neutralization potential. However, this method is very labor intensive, requires culturing of specific cell lines and live viruses, and therefore, it is time-consuming, expensive, and requires special equipment.

This article describes a step-by-step the World Health Organization (WHO)-based HI protocol[12] to quantify influenza-specific antibody titers. Hemagglutination is a characteristic effect of some viruses leading to the agglutination of erythrocytes. The inhibition of this effect with patient sera allows the measurement of inhibitory antibody concentrations, which reflects a neutralizing effect.

We have modified the workflow of the WHO-protocol to allow a more efficient handling of multiple samples at the same time and thereby reducing the required time. The first protocol describes the determination of the agglutination potential of a particular influenza antigen. In doing so, the correct influenza antigen concentration is determined for the second protocol. This part should be repeated with every new viral antigen, as well as each batch of blood.

The second protocol describes the determination of influenza-specific antibody titers. The presented protocols are optimized for the investigation of influenza virus and human serum samples however, it can also be applied for mouse serum samples or cell-culture supernatants from stimulated immune cells, *e.g.*, virus-specific B-cells. Results can be determined as absolute measured titers. In many vaccine studies, the geometric mean titers and the 95% confidence interval are shown for each particular population. For interpretation, seroprotection or seroconversion are often used to describe the susceptibility of a population to a certain virus. Seroprotection is defined as a titer of ≥1:40, and seroconversion as a more than 4-fold titer increase with achievement of seroprotective titers between two time points (most commonly pre-vaccination and 30 days post-vaccination are used).

Both protocols are easy to use and they can be adapted to a broad range of research questions. In particular, they can be used to determine reliably and quickly the antibody titers against various other viruses with the capacity for hemagglutination, such as measles, polyomaviruses, mumps, or rubella[14,15,16].

Protocol

The study protocols were approved through the local ethical review board (www.EKNZ.ch) and written informed consent was obtained from all participants.

1. Serum Collection

1. Collect serum samples from humans at time points of interest. For this study, we collected sera at days 0 (time of influenza vaccination), +7, +30, +60, and +180 after vaccination.
2. To obtain the serum, centrifuge the sample tubes at 1,200 x g for 10 min at room temperature (20 - 25 °C).
   NOTE: Non-centrifuged blood samples should be stored at 4 °C, and for no longer than 24 h.
3. Aliquot the serum into different tubes (cryo-vials) and freeze at -80 °C until use.
4. Perform the subsequent assays batch-wise, including all the time-points of one person to reduce variability within a patient.

2. Preparation of Antigens

CAUTION: Five different antigens are used (see **Table of Materials**). Prepare antigens in a Biosafety Level 2 (BSL-2) laboratory.

1. According to the manufacturer's instructions, reconstitute the total contents of one lyophilized influenza antigen ampoule with 1.0 mL of distilled water and allow the dissolved antigen to stand for a minimum of 5 min at room temperature before proceeding.
2. Aliquot the antigen solution to 1.5 mL tubes and freeze at -80 °C until further use.

3. Preparation of Cholera Filtrate

NOTE: Cholera filtrate is used as a receptor destroying enzyme (RDE) according to the WHO protocol[12]. This removes innate inhibitors from the serum which would interfere with the assay[17].

1. Reconstitute the lyophilized RDE according to the manufacturer's instructions.
2. Store the RDE solution in a 15 mL tube at 4 °C until further use.

4. HA Assay

NOTE: To ensure that the HI assays are comparable between several plates, the same amount of virus particles must be used for each plate. The HA assay (also called HA titration) is performed to quantify the virus particles necessary for hemagglutination, and is recorded in HA units. A "unit" of hemagglutination is an operational unit dependent on the method used for HA titration and is not a measurement of an absolute amount of virus. Thus, an HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized RBC suspension. According to the WHO, the standard amount used for the HI assay is 4 HA units per 25 µL. For an illustration of the principle of the HA assay see **Figure 1**.

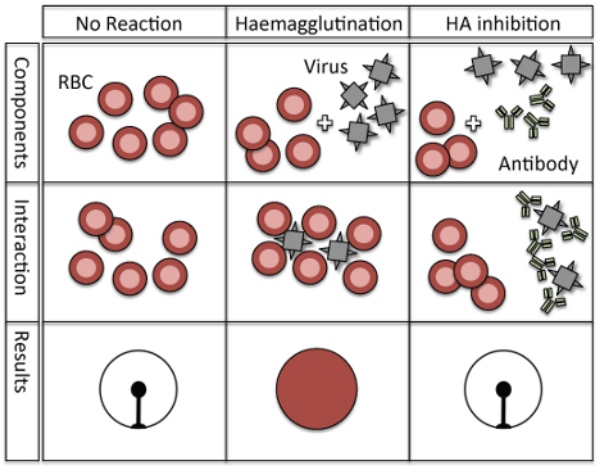

Figure 1: Principle of hemagglutination and hemagglutination inhibition. No hemagglutination occurs in a negative control situation without viruses and antibodies (left column), and erythrocytes hemagglutinate only in the presence of influenza virus (middle column). However, when the hemagglutinin of the influenza virus is blocked by virus-specific antibodies then no hemagglutination can occur (right column). Please click here to view a larger version of this figure.

NOTE: The RBCs used are dependent on the type of influenza virus in the assay (**Table 1**). Further, for various types of 96-well micro titer plates, the incubation time as well as the appearance of the non-agglutinated cells differ (**Table 2**).

Influenza antigen	A/California/7/09 (H1N1)	A/Switzerland/9715293/2013 (H3N2)	A/Texas/50/2012 (H3N2)	B/ Brisbane/60/08	B/Massachusetts/02/2012
RBC species	Chicken	Guinea Pig	Guinea Pig	Turkey	Turkey

Table 1: Influenza antigens and corresponding species of RBCs. According to the manufacturer's instructions (NIBSC).

RBC species	**Chicken**	**Turkey**	**Guinea pig**	**Human type O**
Concentration of RBCs (v/v)	0.75%	0.75%	1%	1%
Type of microtiter plate	V bottom	V bottom	U bottom	U bottom
Incubation time, RT	30 min	30 min	1 hour	1 hour
Appearance of non-agglutinated cells	Button*	Button*	halo	halo

Table 2: Assay conditions with different species of RBCs. According to the WHO protocol. (* flows when tilted).

1. **Preparation of the RBC Suspension**
   1. Dilute the RBC stock suspension (10%, v/v; except human type O) (see **Table of Materials**) with phosphate buffered saline (PBS) to make the proper concentrations for avian and mammalian RBCs of 0.75% and 1%, respectively.

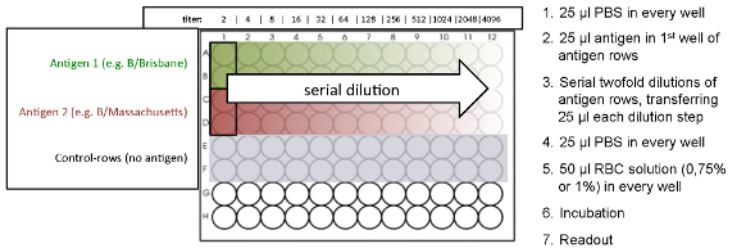

Figure 2: Plate design of the HA assay. The HA titration is performed in duplicates. No antigen was added to the control rows. Also see **Figure 4** for the determination of the best antigen concentration. Please click here to view a larger version of this figure.

2. **Preparation of the 96-well Micro Titer Plate**
   NOTE: See **Figure 2** for an overview of the plate design.
   1. Add 25 µL of PBS to wells 1 to 12 of each used row of a 96-well micro titer plate by using a multichannel pipette (**Figure 2**). Use the V-shaped micro titer plate when working with avian RBCs, like chicken and turkey. Use the U-shaped micro titer plate when working with mammalian RBCs, like guinea pig and human type O (**Table 2**).

2. Add 25 µL of influenza antigen to the first well of the antigen-rows, which are arranged in duplicates. No antigen is added to the control rows. The control rows should not show a hemagglutination effect and serve as negative controls (**Figure 2**).
3. Perform a serial 2-fold dilution by transferring 25 µL from the first well of the antigen-rows to successive wells by using a multichannel pipette. Mix each dilution step by pipetting up and down gently 10 times.
4. Discard the final 25 µL of the last wells.
5. Add 25 µL of PBS to wells 1 to 12 of each used row by using a multichannel pipette, in order to reach a total volume of 50 µL per well.
6. Add 50 µL of the RBC suspension to each used well by using a multichannel pipette.
7. Tap the plate carefully 10 times on all four sides to mix.
8. Cover the plate with a lid and incubate at room temperature for the appropriate amount of time depending on the RBC species used (see **Table 2**). Do not move the plate while incubating.

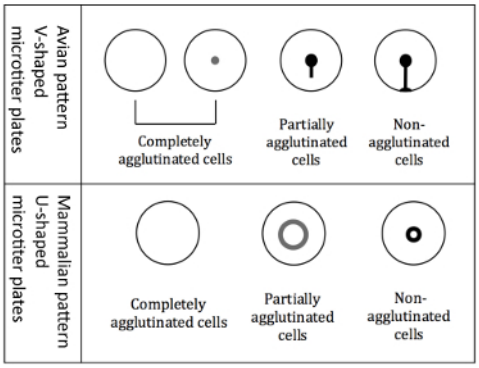

Figure 3: Agglutination patterns of avian and mammalian RBCs. V-shaped micro titer plates are used when working with avian RBCs. The readout is performed in a tilted plate position, and non-agglutinated RBCs start to run down forming a tear-like shape. U-shaped microtiter plates are used when working with mammalian RBCs. The readout is then performed in a non-tilted position, and non-agglutinated RBCs form a small halo. Please click here to view a larger version of this figure.

3. **Reading the plate**
NOTE: The readout is slightly different when using avian RBCs compared to mammalian RBCs, because of the different shaped micro titer wells (**Figure 3**).
    1. Readout of avian RBCs
        1. Tilt the plate 90° for 25 s.
        NOTE: Tilting the plate is crucial for the differentiation of avian patterns, because all three different types of agglutination patterns (completely agglutinated, partially agglutinated, and non-agglutinated) appear as a button when not tilted.
        2. Mark the results immediately, while the plate is still in a tilted position, on a printed scheme of the 96-well plate. The agglutination patterns of the avian RBCs are shown in **Figure 3**.
    2. Readout of mammalian RBCs
        1. Mark the results on a printed scheme of the 96-well plate, without tilting the plate (horizontal position on the bench).
        NOTE: When hemagglutination occurs, the agglutinated cells do not settle to the bottom, whereas non-agglutinated cells appear as a halo at the bottom of the well. The halo of the partially agglutinated cells is less intense and has a larger diameter (**Figure 3**).
    3. Determination of 4 HA units.
    NOTE: The HA titration end point is the last well where complete hemagglutination occurs. This well contains 1 HA unit of virus. Because of the 2-fold dilutions of the antigen, two wells ahead of the HA titration endpoint is the well that contains 4 HA units of virus (**Figure 4**).

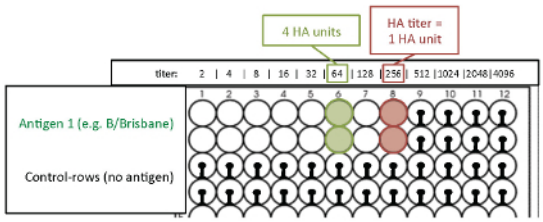

Figure 4: Readout of the HA titration with avian RBCs to determine the titer of 4 HA units. The optimal antigen amount required for hemagglutination is measured by the hemagglutination assay (antigen titration assay). The last well where complete hemagglutination occurs is the HA titration endpoint and contains 1 HA unit. Because of the 2-fold dilutions of the antigen, two wells ahead of the HA titration endpoint, the titer corresponds to 4 HA units. Please click here to view a larger version of this figure.

5. HI Assay

NOTE: The work-flow of the protocol has been optimized to allow a more efficient handling of multiple samples at the same time, by using PCR tube stripes and a thermo cycler (see below).

1. **Preparation of the Serum Samples**
   NOTE: Prepare serum samples in a BSL-2 laboratory.
   1. Thaw the frozen serum samples of each time point of every person (see step 1.2) at room temperature.
   2. Add an aliquot of 10 µL of each thawed serum sample to a tube of a PCR tube strip (10-tubes in one strip).
      NOTE: The big advantage of using PCR tube strips is that a multichannel pipette can be used for the following steps in the HI assay; this saves a lot of time when testing a large amount of serum samples and when performing repeated measures of the same samples for antibody titers against different virus strains.
   3. Store the aliquoted serum samples in the PCR tube strips at -80 °C until use.
   4. One day before the HI assay is performed, thaw the serum sample aliquots of interest at room temperature.
   5. Add 10 µL of the appropriate anti-serum to an empty PCR tube.
      NOTE: To serve as a positive control, the anti-serum against a specific virus must match the used virus. The positive control allows for standardization of plate performance over multiple plates.
   6. Add 30 µL of cholera filtrate solution to each serum aliquot and to the anti-serum (3 volumes of cholera filtrate to 1 volume of serum) by using a multichannel pipette.
   7. Keep the PCR tubes in a PCR 96-well rack or an empty tip-box and vortex for 5 s.
   8. Incubate the samples overnight at 37 °C using a thermo cycler.
   9. Incubate the samples at 56 °C for 30 min to inactivate the cholera filtrate using a thermo cycler.
      NOTE: Depending on the thermo cycler, this step can be programmed to further automate the process.
   10. Keep the PCR tubes in a PCR 96-well rack or an empty tip-box and vortex for 5 s.
   11. Store the samples at 4 °C in the fridge until use for the HI assay.

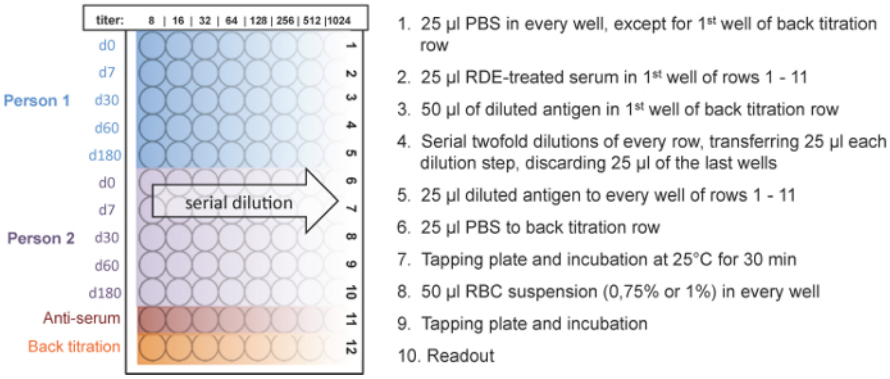

Figure 5: Plate design and workflow of the HI assay. Five time points of two people can be measured on one plate. The HI titer ranges from 8 to 1,024. An anti-serum of the used antigen served as a positive control and a back titration was performed to check if the antigen dilution equals 4 HA units. The serial dilution of the serum sample is shown for 2 individual vaccine recipients. Please click here to view a larger version of this figure.

2. **HI assay**
   NOTE: For an illustration of the principle of the HI assay see **Figure 1**. Depending on the virus, different species of RBCs are used for the assay (**Table 1**). The different species of RBCs are used in various types of 96-well plates, and the incubation time as well as the appearance of the non-agglutinated cells differs (**Table 2**). For the HI assay, 4 HA units of virus or antigen are added to the 2-fold dilution series of the samples.
   1. **Preparation of the antigen solution**
      1. Calculate the volume of antigen solution needed according to the number of 96-well plates used (25 µL antigen per well × 96 = 2,400 µL antigen per 96-well plate; add 100 µL per plate extra due to the usage of a reservoir for the multichannel pipette; a total 2.5 mL of antigen per plate).
         NOTE: For example, if measuring 100 serum samples then 10 plates are needed (10 samples per plate): 2.5 mL x 10 = 25 mL of antigen solution needed in total.
      2. Prepare the proper dilution of 4 HA units for the calculated volume using PBS.
         NOTE: 4 HA units are determined for the HA assay. For the appropriate amount of antigen, divide the calculated volume by the titer corresponding to 4 HA units. For example, 4 HA units correspond to a dilution of 1/64, and we needed 15,000 µL of antigen solution are needed: 15,000/64 = 234.4 µL of the dissolved lyophilized influenza antigen are added.
   2. **Preparation of the RBC suspension**
      1. Calculate the volume of RBC suspension needed according to the number of 96-well micro titer plates used (50 µL RBC suspension per well × 96 = 4,800 µL RBC suspension per 96-well plate; add 200 µL per plate extra due to the usage of a reservoir for the multichannel pipette).
      2. Dilute the RBC stock suspension (normally 10%, v/v; except human type O) with PBS to make the proper concentrations for avian and mammalian RBCs of 0.75% and 1%, respectively.

3. **Preparation of the 96-well micro titer plate**
    1. Label the 96-well micro titer plates (sample ID, positive control, and back titration). Please check the plate orientation in **Figure 5** carefully.
    2. Add 25 µL of PBS to every well except to the first well of the "back titration" row (**Figure 5**, 12th row) using the multichannel pipette.
       NOTE: A back titration was performed to check if the used antigen dilution equals 4 HA units. An antigen titer of 4 HA units is indicated if hemagglutination occurs in the first three wells of the "back titration" row, but not in the fourth well.
    3. Add 50 µL of the prepared antigen solution (described in 5.2.1) to the first well of the "back titration" row (12th row).
    4. Add 25 µL of the RDE-treated serum samples to the first wells of rows 1 to 10 on each plate, using the multichannel pipette.
    5. Add 25 µL of the appropriate anti-serum to the first well of the 11th row as a positive control.
    6. Perform serial 2-fold dilutions by transferring 25 µL from the first well of each row (1 - 12) to successive wells by using a multichannel pipette. Mix by pipetting up and down 10 - 15 times for each dilution step. The same tips can be used for each dilution step per sample.
    7. Discard the final 25 µL of the last wells.
    8. Add 25 µL of the antigen solution by using a multichannel pipette to each well of rows 1 to 11 (serum samples and anti-serum). The same tips can be used if they do not touch the wells.
    9. Add 25 µL of PBS instead of antigen to each well of the "back titration" row (12th row).
    10. Tap the plate carefully 10 times on all four sides to mix.
    11. Cover the plate with a lid and incubate at room temperature for 30 min. Do not move the plate while incubating.
    12. Add 50 µL of the RBC suspension to every well.
    13. Tap the plate carefully 10 times on all 4 sides to mix.
    14. Cover the plate with a lid and incubate at room temperature for the appropriate amount of time depending on the RBC species used (see **Table 2**). Do not move the plate while incubating.

4. **Reading the plate**
   NOTE: The HI titer is the reciprocal of the last dilution of (anti-) serum that completely inhibits hemagglutination. It is important to consider that the RDE-treated sera were already diluted 1:4 and after the serial dilution step, the sera in the first wells are diluted 1:8, which corresponds to a HI titer of 8.
    1. Readout of avian RBCs
        1. Tilt the plate 90° for 25 s.
           NOTE: Tilting the plate is crucial for the differentiation of avian patterns, because all three different types of agglutination patterns (completely agglutinated, partially agglutinated, and non-agglutinated) appear as a button when not tilted.
        2. Mark the results immediately, while the plate is still in a tilted position, on a printed scheme of the 96-well plate. The agglutination patterns of avian RBCs are shown in **Figure 3**.

    2. Readout of mammalian RBCs
        1. Mark the results on a printed scheme of the 96-well plate, without tilting the plate.
           NOTE: When hemagglutination occurs, the agglutinated cells do not settle down whereas non-agglutinated cells appear as a halo at the bottom of the well. The halo of the partially agglutinated cells is less intense and has a larger diameter (**Figure 3**).
        2. Determine the HI of each sample and transfer it to a computer-based table (**Figure 6**)
        3. NOTE: Partially agglutinated wells were determined as a lower titer. For example, if a serum sample completely inhibits hemagglutination up to the 4th well (1:64 dilution) and the 5th well (1:128 dilution) is partially agglutinated, then the HI titer is set to the lower titer 64 for the final analysis (**Figure 6**, 4th row).

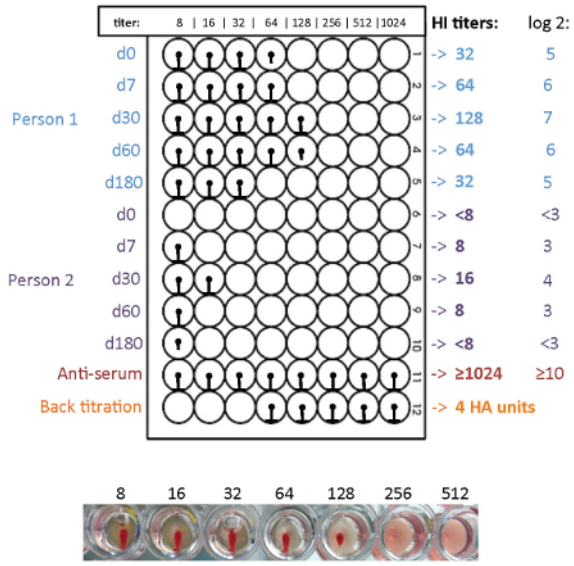

Figure 6: Readout of the HI assay with avian RBCs. The pre- and post-vaccination induced influenza specific antibody response is determined by HI assay. In this example, person one has higher HI titers than person two. Both persons show an antibody response after vaccination; 180 days after the vaccination the antibody titers of both persons are decreased again. Please click here to view a larger version of this figure.

Representative Results

Pre- and post-vaccination induced antibody response against Influenza A H3N2
The vaccine-induced antibody response was assessed in 26 healthy volunteers who received an inactivated trivalent subunit influenza vaccine containing Influenza A/H1N1/California/2009, A/H3N2/Texas/2012, and B/Massachusetts/02/2012 prior to the 2014/2015 influenza season. **Figure 6** shows a representative example of 2 vaccine recipients. Interestingly, during that particular influenza season, A/H3N2/Texas/2012 was not circulating, and instead the season included the somewhat different viral strain: A/H3N2/Switzerland/2013. The viral hemagglutinin of A/H3N2/Texas/2012 and A/H3N2/Switzerland/2013 show 97% sequence identity and differ in only eleven amino acids (see **Table 4**), whose positions are highlighted in **Figure 7**.

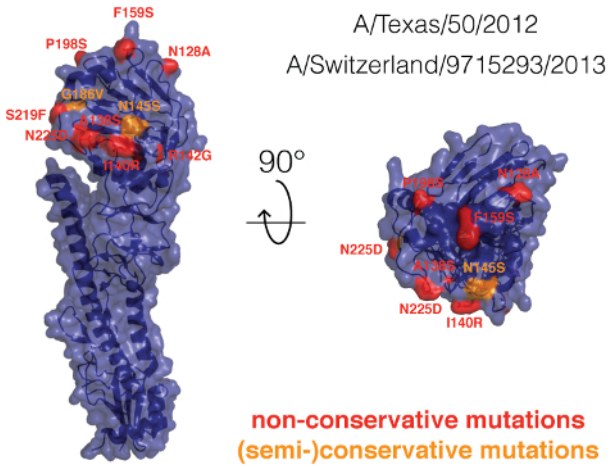

Figure 7: Hemagglutinin comparison of A/H3N2 influenza strains. We compared the hemagglutinin of the viral strains A/Texas/50/2012 and A/Switzerland/9715293/2013. Since there are no hemagglutinin crystal structures of these strains, we used the crystal structure of the highly similar hemagglutinin of the influenza strain A/Victoria/361/2011[18], which shows 98% sequence identity with the Texas strain and 95% sequence identity with the Switzerland strain. The amino acid positions in which the Texas and Switzerland strains differ are highlighted. Please click here to view a larger version of this figure.

We observed a cross-reactive immune response for the viral strains A/H3N2/Switzerland/2013 and A/H3N2/Texas/2012. HI titers against Influenza A/H3N2/Switzerland/2013 were significantly lower in terms of geometric mean titers and induced seroprotection (**Figure 8A**) in comparison to Influenza A/H3N2/Texas/2012 (**Figure 8B**).

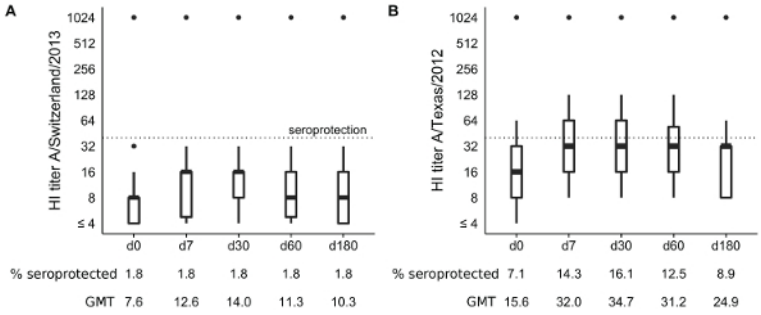

Figure 8: Geometric mean antibody-titers of healthy donors. The geometric mean antibody-titers (GMTs) of 25 healthy donors pre- and post-vaccination are determined using two different antigens. The mean titers of A/H3N2/Switzerland/2013 (**A**) and A/H3N2/Texas/2012 (**B**) are shown. An immune response due to the vaccination can be observed as increasing titers after the vaccination (d7-d60), compared to the GMTs before the vaccination (d0). 180 days after the vaccination, the GMTs decrease again. Of note, only A/H3N2/Texas/2012 (which was in the vaccine) reaches protective titers. Bars indicate geometric mean titers, and whiskers indicate the 95% confidence intervals. The dashed line indicates the seroprotection threshold. The % of seroprotected people (titer >1:40) is shown in the graph. Please click here to view a larger version of this figure.

After vaccination, the antibody titers against A/H3N2/Texas/2012 increased in most subjects; although the A/H3N2/Switzerland/2013 strain was not present in the vaccine, the titer against A/H3N2/Switzerland/2013 increased in some subjects as well. **Figure 9** shows the correlation between both titers over all time points with an R^2 of 0.745 for a linear regression model. As one would expect, the induction of the antibody response against A/H3N2/Switzerland/2013 was less potent.

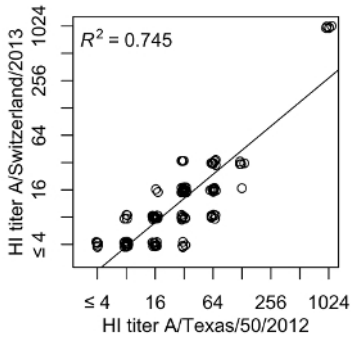

Figure 9: Cross-reaction between A/H3N2 influenza strains. The A/H3N2/Texas titers of every individual and time point are plotted against the corresponding A/H3N2/Switzerland titers. A linear regression model shows an R^2 of 0.745. Please click here to view a larger version of this figure.

Hemagglutination potential is based on the type of blood used

The viral hemagglutinin shows different species-dependent potential to hemagglutinate erythrocytes. This species-dependent effect also impacts the hemagglutination inhibition assay. To improve the specificity of measured anti-viral titers, we evaluated the best suited type of erythrocytes for five viral antigens (Influenza B/Brisbane/60/2008 and B/Massachusetts/02/2012, Influenza A/H1N1/California/2009, A/H3N2/Texas/2012, and A/H3N2/Switzerland/2013) to achieve the maximum hemagglutination but also the lowest cross-reactivity. We used positive control sera from the National Institute for Biological Standards and Control (NIBSC) against each antigen to perform these assays.

For Influenza B, we could observe that the B/Massachusetts/02/2012 induced antibody response does not provide protection against B/Brisbane/60/2008. In contrast, antibodies against B/Brisbane/60/2008 showed cross-reactivity against B/Massachusetts/02/2012 at a 4-fold lower titer across different erythrocytes (see **Table 3**). Of interest, guinea pig blood did not properly hemagglutinate with Influenza B. Turkey blood did best in showing the potential to hemagglutinate and the highest titers with relative low cross-reactivity apart from the previously mentioned A/H3N2/Texas and /Switzerland strains.

	Turkey	Guinea Pig	Chicken	Human type O
B/Brisbane	1024	-	1024	1024
B/Massachusetts	1024	384	768	1024
A/H3N2/Switzerland	1024	1024	-	1024
A/H3N2/Texas	1024	1024	512	1024
A/H1N1/California	1024	1024	768	768

Table 3: Positive control titers against the respective influenza HA antigen across different species.

No	A/H3N2/Texas/2012 strain	A/H3N2/Switzerland/2013 strain	Position
1	Asparagine (N)	Alanine (A)	128
2	Alanine (A)	Serine (S)	138
3	Isoleucine (I)	Arginine (R)	140
4	Arginine (R)	Glycine (G)	142
5	Asparagine (N)	Serine (S)	145
6	Phenylalanine (F)	Serine (S)	159
7	Glycine (G)	Valine (V)	186
8	Proline (P)	Serine (S)	198
9	Serine (S)	Phenylalanine (F)	219
10	Asparagine (N)	Aspartate (D)	225
11*	Lysine (K)	Arginine (R)	326
	*not shown in the crystal structure in Figure 8, because the hemagglutinin was cutted at residue 325.		

Table 4: List of different amino acids of hemagglutinin between A/H3N2/Texas/2012 and A/H3N2/Switzerland/2013 strains

Discussion

Quantification of pre- and post-vaccination influenza virus specific antibody titers is an important tool necessary for vaccine studies. Based on the surrogate measures of protection against virus infection, such as seroprotection (>1:40) or seroconversion (4-fold titer increase), vaccination strategies can be optimized[9]. Using the provided protocols can determine: (i) the hemagglutination potential of a particular virus, and (ii) the antibody titers for a virus of interest.

Modification and Troubleshooting:

This protocol is based on the WHO standard[12]. We modified the protocol by using PCR tube strips for serum preparations (see step 5). This modification helped to significantly reduce the workload and to increase the throughput of the assay. Further, we reduced the antigen amount by one fourth in the antigen titration step, which is cost effective overtime. A lower amount of serum (10 µL) can be used for the RDE treatment, which helps especially when the sample amount is limited (*e.g.*, mouse sera). The back titration and positive control are included in the antibody measurement plate to serve as a proper internal control and to monitor the aging of erythrocytes.

In addition, we used different erythrocyte concentrations than those in the WHO standard[12] to set the optimal size of the erythrocyte clot for a good visual readout. To guarantee this we suggest checking the erythrocyte concentration before the assay. Although, we have not optimized this part in our protocol, methods such as absorbance measurement with OD or cell counting could be used.

If the RDE is not completely inactivated, RBCs can be desialylated and reverse HA-positive wells when hemagglutination is measured at room temperature. Although we never observed this problem, in this case, we suggest performing the HI at 4 °C, since RDE activity is significantly lower at 4 °C. However, performing the HI assay at 4 °C is slower.

Limitations of the Technique:

A few critical aspects of the HI assay include the following points: Interestingly, the hemagglutination is strongly dependent on the particular type of erythrocyte (*e.g.*, turkey or guinea pig RBC). The optimal type of blood should be tested before a particular virus strain is evaluated and the same type of blood should be used throughout the assay. Although the induction of cross-reactivity between viruses may generate an immunological advantage in the case of a slightly new virus[19,20], this may cause some problems from a diagnostic point of view due to low specificity. Therefore, cross reactivity between similar viruses should be carefully addressed and discussed in studies. By choosing erythrocytes from a specific species, the amount of cross-reactivity can be somewhat lowered.

Significance with Respect to Existing Methods:

The HI is a well-established gold standard method providing highly reproducible and reliable results. Other techniques such as ELISA may detect non-neutralizing antibodies, whereas the HI only detects antibodies which bind to the HA stem loop and thereby correlate with neutralization.

Critical Steps Within the Protocol:

The most critical steps include the serum treatment with RDE to inactivate unspecific inhibitors and binding to the HA of the virus. Another critical step is to control for hemolysis of the erythrocytes as they age over time.

Future Applications:

The protocol may be used for other viruses with hemagglutination potential. Although we have only shown results on human sera samples here, the assay can also be used to measure antibody titers in mouse sera or in cell culture supernatant with stimulated B-cells (data not shown). In summary, the HI allows a rapid and reproducible assessment of vaccine-induced antibody titers.

Disclosures

A.E. was supported by a research grants from the "SNSF Ambizione Score" program (PZ00P3_154709), "Forschungsfond, Förderung strategischer Projekte" University of Basel, Stiftungsinfektionskrankheiten Basel, and Bangeter Rhyner Stiftung. L.K. was supported by a grant of the Technical University of Graz, Austria. J.L. acknowledges support by an iPhD fellowship of the SystemsX.ch initiative in systems biology program (9th call).

Acknowledgements

none.

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Materials List for:

An Optimized Hemagglutination Inhibition (HI) Assay to Quantify Influenza-specific Antibody Titers

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URL: https://www.jove.com/video/55833
DOI: doi:10.3791/55833

Materials

Name	Company	Catalog Number	Comments
25 ml Disposable Multichannel Pipette Reservoirs	Integra	4312	
8-well PCR tubes	Brand GMBH	781332	For serum aliquots
96-well microtiter plate, U-shaped	TPP	92097	For HI assay when using mammalian RBCs
96-well microtiter plate, V-shaped	Corning Costar	3897	For HI assay when using avian RBCs
Aqua ad iniect. Steril	Bichsel AG	1000004	For preparing influenza antigen and cholera filtrate solutions
Chicken RBC (10%)	Cedarlane	CLC8800	10% suspension of chicken red blood cells in Alsever's solution
Cholera filtrate	Sigma-Aldrich	C8772	Used as receptor destroying enzyme (RDE)
Dulbecco's PBS	Sigma-Aldrich	D8537	For diluting the serum samples, RBCs and antigens
Eppendorf Multichannel pipette, 12-channel, 10-100 µl	Sigma-Aldrich	Z683949	
Eppendorf Multichannel pipette, 8-channel, 10-100 µl	Sigma-Aldrich	Z683930	
Guinea Pig RBC (10%)	Cedarlane	CLC1800	10% suspension of guinea pig red blood cells in Alsever's solution
Influenza Anti-A/California/7/09 HA serum	NIBSC	14/134	Used as positive control at the HI assay
Influenza Anti-A/ Switzerland/9715293/2013-like HA serum	NIBSC	14/272	Used as positive control at the HI assay
Influenza Anti-A/Texas/50/2012-Like HA Serum	NIBSC	13/178	Used as positive control at the HI assay
Influenza Anti-B/Brisbane/60/2008-HA serum	NIBSC	13/254	Used as positive control at the HI assay
Influenza Anti-B/ Massachusetts/02/2012 HA serum	NIBSC	13/182	Used as positive control at the HI assay
Influenza antigen A/California/7/09 (H1N1)(NYMC-X181)	NIBSC	12/168	Inactivated, partially purified A/ California/7/09 (H1N1)(NYMC-X181) virus (ca. 46µgHA/ml)

Influenza antigen A/ Switzerland/9715293/2013 (NIB88)	NIBSC	14/254	Inactivated, partially purified A/ Switzerland/9715293/2013 (NIB88) virus (ca. 55µgHA/ml)
Influenza antigen A/Texas/50/2012 (H3N2)(NYMCX-223)	NIBSC	13/112	Inactivated, partially purified A/Texas/50/2012 (H3N2) (NYMCX-223) virus (ca. 74µgHA/ ml)
Influenza antigen B/ Brisbane/60/2008	NIBSC	13/234	Inactivated, partially purified B/Brisbane/60/2008 virus (ca. 42µgHA/ml)
Influenza antigen B/ Massachusetts/02/2012	NIBSC	13/134	Inactivated, partially purified B/ Massachusetts/02/2012 virus (ca. 35µgHA/ml)
Serum-Tubes	S-Monovette, Sardstedt	01.1601.100	For serum extraction with clotting activator
Single Donor Human RBC, Type 0	Innovative Research	IPLA-WB3	Suspension of single donor human red blood cells in Alsever's solution (ca. 26%)
Turkey RBC (10%)	Cedarlane	CLC1180	10% suspension of turkey red blood cells in Alsever's solution
Phosphate Buffered Saline (PBS)	Gibco		

5.5 IL-28B is a key regulator of B- and T-cell vaccine responses against influenza

Adrian Egli[1,2]*, Deanna M. Santer[2], Daire O'Shea[2,3], Khaled Barakat[2,4], **Mohammedyaseen Syedbasha**[1], Madeleine Vollmer[1], Aliyah Baluch[5], Rakesh Bhat[2], Jody Groenendyk[6], Michael A. Joyce[2], Luiz F. Lisboa[2], Brad S. Thomas[2], Manuel Battegay[1,7], Nina Khanna[1,7], Thomas Mueller[8], D. Lorne J. Tyrrell[2], Michael Houghton[2], Atul Humar[9¶], Deepali Kumar[9¶]

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¶ These authors are joint senior authors on this work.

Manuscript has been published in PLOS Pathogens, 2014; 11;10(12)
doi: 10.1371/journal.ppat.1004556.

Contribution of my work:
Design and performance of *in vitro* ELISA experiments to screen antagonistic peptides to block the binding of IFN-λs to IL28RA.
Figure 5C; Supplementary figure 5C and 5D

Note: The following part contains the paper abstract/ summary

5.5.1 Abstract/Summary

Influenza is a major cause of morbidity and mortality in immunosuppressed persons, and vaccination often confers insufficient protection. IL-28B, a member of the interferon (IFN)-l family, has variable expression due to single nucleotide polymorphisms (SNPs). While type-I IFNs are well known to modulate adaptive immunity, the impact of IL-28B on B- and T-cell vaccine responses is unclear. Here we demonstrate that the presence of the IL-28B TG/GG genotype (rs8099917, minor allele) was associated with increased seroconversion following influenza vaccination (OR 1.99 p = 0.038). Also, influenza A (H1N1)-stimulated T- and B-cells from minor-allele carriers showed increased IL-4 production (4-fold) and HLA-DR expression, respectively. In vitro, recombinant IL-28B increased Th1-cytokines (e.g. IFN-γ), and suppressed Th2-cytokines (e.g. IL-4, IL-5, and IL-13), H1N1-stimulated B-cell proliferation (reduced 70%), and IgG-production (reduced.70%). Since IL28B inhibited B-cell responses, we designed antagonistic peptides to block the IL-28 receptor a-subunit (IL28RA). In vitro, these peptides significantly suppressed binding of IFN-λs to IL28RA, increased H1N1-stimulated B-cell activation and IgG production in samples from healthy volunteers (2-fold) and from transplant patients previously unresponsive to vaccination (1.4-fold). Together, these findings identify IL-28B as a key regulator of the Th1/Th2 balance during influenza vaccination. Blockade of IL28RA offers a novel strategy to augment vaccine responses.

5.6 Sequential models in regression of vaccine-induced antibody titers in stem cell transplant recipients

Janina Linnik[1,2], Hans-Michael Kaltenbach[1,2], Yvonne Hollenstein[3], **Mohammedyaseen Syedbasha**[3], Dominik Vogt[3], Lukas Kaufmann[3], Kathrin Ullrich[3], Nathan Cantoni[4], Sabine Ruosch[5], Urs Schanz[6], Thomas Pabst[7], Georg Stüssi[8], Jörg Halter[9], Maya Weisser[10], Jörg Stelling[1,2] & Adrian Egli[3,11]

[1] Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland, [2] Swiss Institute for Bioinformatics, Basel, Switzerland, [3] Department of Biomedicine, University of Basel, Basel, Switzerland, [4] Division of Hematology, Cantonal Hospital of Aarau, Aarau, Switzerland, [5] Division of Hematology, Cantonal Hospital of Lucerne, Lucerne, Switzerland, [6] Division of Hematology, University Hospital Zurich, Zurich, Switzerland, [7] Division of Medical Oncology, University Hospital Bern, Bern, Switzerland, [8] Division of Hematology, Ospedale San Giovanni, Bellinzona, Switzerland, [9] Division of Hematology, University Hospital of Basel, Basel, Switzerland, [10] Division of Infectious Diseases, University Hospital of Basel, Basel, Switzerland, [11] Division of Clinical Microbiology, University Hospital of Basel, Basel Switzerland

Manuscript is close to submission

Contribution of my work:
Partly involved in the sample (PBMCs, EDTA Blood, Serum, Swabs) collection from HSCT patients and healthy controls after influenza vaccination (from season 2014-15 and 2015-16), maintaining of electronic database, establish HIA and ELISA methods to measure the antibody titers. Partly measured the antibody titers of the collected samples for the computational modeling to predict the vaccine responders.
Partly contributed to Figure 1A, 1B, 1C; Figure 2A, 2B, 2C

Note: The following part contains the paper abstract/ summary

5.6.1 Abstract/ Summary

Sequential models have been introduced by Tutz (1991) in categorical regression where response categories are reached successively step by step. Since then, sequential models have been successfully applied in social sciences, e.g. to model educational levels or career development. In biology and medicine, however, these models remain largely unknown. We propose that sequential models are a powerful tool for the regression of antibody titers – a commonly used outcome variable in vaccine trials. Antibody titers reflect a coarse-grained measure of antibody abundance in serum samples. They are obtained from serial dilution experiments and correspond to the minimal dilution of antibodies that is still able to perform certain functions against viruses, e.g. virus neutralization or hemagglutination inhibition. However, the standard approach to link patient characteristics to antibody titer response is based on dichotomization: patients are classified in responders and non-responders according to conventional thresholds on the titer response. This loss of information is particularly problematic if the sample size is small. Motivated by our vaccination trial in an immunosuppressed patient population consisting of stem cell transplant recipients (n = 144), we performed a simulation study to investigate how the size of effects (e.g. immunosuppressive drugs, time after transplantation), sample size, and unexplained variability in antibody response affect the power of sequential models in comparison with conventional classification methods. We show that the conventional methods identify only large effects for a sample size like ours, while sequential models are also able to detect moderate effects and require much smaller sample sizes to identify small effects. To facilitate the evaluation and interpretation of sequential models, we provide the *R* package *titer* for performing simulation studies for balanced and unbalanced designs, varying effect size and varying unexplained variability modelled by random effects. In addition, it allows for sample size and power calculation to guide the design of vaccination trials. We hope to encourage the application of sequential models in regression of antibody titers, and their extension to data obtained from similar serial dilution experiments.

5.7 Influenza vaccination of cancer patients during PD-1 blockade induces serological protection but may raise the risk for immune-related adverse events

Heinz Läubli[1,2], Catharina Balmelli[1], Lukas Kaufmann[3], Michal Stanczak[2], **Mohammedyaseen Syedbasha**[3], Dominik Vogt[3], Astrid Hertig[1], Beat Müller[4], Oliver Gautschi[4], Frank Stenner[1,2], Alfred Zippelius[1,2], Adrian Egli[3,5] and Sacha I. Rothschild[1,2] *

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Heinz Läubli and Catharina Balmelli are contributed equally to this work.
Adrian Egli and Sacha I. Rothschild are equally supervised this work.

Manuscript has been published in Journal for ImmunoTherapy of Cancer, 2018; 22;6(1):40. doi: 10.1186/s40425-018-0353-7.

Contribution of my work:
Design and performance of hemagglutination inhibition (HIA) assay to measure the antibody titers from cancer patients undergoing PD-1 blockade and age-matched healthy controls after influenza vaccination.
Partly contributed to Figure 1A, 1B, 1C, 1D

Note: The following part contains the paper abstract/ summary

5.7.1 Abstract/ Summary

Background: Immune checkpoint inhibiting antibodies were introduced into routine clinical practice for cancer patients. Checkpoint blockade has led to durable remissions in some patients, but may also induce immune-related adverse events (irAEs). Lung cancer patients show an increased risk for complications, when infected with influenza viruses. Therefore, vaccination is recommended. However, the efficacy and safety of influenza vaccination during checkpoint blockade and its influence on irAEs is unclear. Similarly, the influence of vaccinations on T cell-mediated immune reactions in patients during PD-1 blockade remains poorly defined.

Methods: We vaccinated 23 lung cancer patients and 11 age-matched healthy controls using a trivalent inactivated influenza vaccine to investigate vaccine-induced immunity and safety during checkpoint blockade.

Results: We did not observe significant differences between patients and healthy controls in vaccine-induced antibody titers against all three viral antigens. Influenza vaccination resulted in protective titers in more than 60% of patients/participants. In cancer patients, the post-vaccine frequency of irAEs was 52.2% with a median time to occurrence of 3.2 months after vaccination. Six of 23 patients (26.1%) showed severe grade 3/4 irAEs. This frequency of irAEs might be higher than the rate previously published in the literature and the rate observed in a non-study population at our institution (all grades 25.5%, grade 3/4 9.8%).

Conclusions: Although this is a non-randomized trial with a limited number of patients, the increased rate of immunological toxicity is concerning. This finding should be studied in a larger patient population.

5.8 Additional papers during my PhD time (not directly related to the thesis work)

1. Zimmermann M, Rose N, Lindner JM**,** Kim H, Gonçalves AR, Callegari I, **Syedbasha M,** Kaufmann L**,** Egli A, Lindberg RLP, Kappos L, Traggiai E, Sanderson NSR and Derfuss T **(2019)**. Antigen extraction from cell membranes identifies rare membrane antigen specific B-cells. **Front Immuno**l 10: 829. PMID: 31040853

2. Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, Gunten SV, Tzankov A, Tietze L, Lardinois D, Heinzelmann-Schwarz V, Bergwelt-Baildon MV, Zhang W, Lenz HJ, Han Y, Amos CI, **Syedbasha M**, Egli A, Stenner F, Speiser DE, Varki A, Zippelius A and Laubli H **(2018)**; Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T-cells. **J Clin Invest.** PMID: 30130255

3. Egli A, Saalfrank C, Goldman N, Brunner M, Hollenstein Y, Vogel T, Augustin N, Daniel Wüthrich D, Seth-Smith HMB, Roth E, **Syedbasha M**, Mueller NF, Vogt D, Bauer J, Amar-Sliwa N, Meinel DM, Dubuis O, Naegele M, Tschudin-Sutter S, Buser A, Nicke CH, Zeller A, Ritz N, Battegay M, Stadler T and Schneider-Sliwa R **(2019)**; Identification of influenza urban transmission patterns by geographical, epidemiological and whole genome sequencing data: Protocol for an observational study. **BMJ Open**. 9(8) PMID: 31434783

4. Egli A, Goldman N, Müller NF, Brunner M, Wüthrich D, Tschudin-Sutter S, Saalfrank C, Neher R, Hadfield J, Bedford T, **Syedbasha M**, Lang D, Roth E, Seth-Smith HMB, Hollenstein Y, Dubuis O, Naegele M, Buser A, Nickel CH, Ritz N, Zeller A, Stadler T, Battegay M and Schneider-Sliwa R; Socioeconomic impact on influenza transmission within and between urban quarters of a city (Manuscript is close to submission)

5. Müller NF, Wüthrich D, Seth-Smith HMB, Hollenstein Y, **Syedbasha M**, Lang D, Neher R, Dubuis O, Naegele M, Buser A, Nickel CH, Ritz N, Zeller A, Lang BM, Hadfield J, Bedford T, Battegay M, Schneider-Sliwa R, Egli A and Stadler T; Characterising the epidemic spread of Influenza A/H3N2 within a city through phylogenetics (Manuscript is close to submission)

6. Bienias M, **Syedbasha M**, Welzel T, Samba S, Wolf C, Kind B, Egli A, Kümmerle-Deschner J and Lee-Kirsch M; Autoinflammatory syndrome due to Interferon Lambda Receptor (IFNLR1) deficiency. (Manuscript in preparation)

6 Discussion

In this thesis, first, we developed sensitive *in vitro* ELISA assays to study the IFN-λ ligands and receptor interaction. Second, there are discrepancies in published data about the expression of the IFN-λ receptor in immune cells. The study of IFN-λ signaling in immune cells is very challenging due to low level of receptor expression in immune cells compared to epithelial cells, and the lack of tools such as: specific antibodies to detect functional IFN-λ receptor, and sensitive assays techniques. We established a highly sensitive phospho-flow cytometry and immunoblot assays to screen the specific responsiveness of various immune cells populations to IFN-λ. Next, we used the combination of *in vitro* assays and advanced transcriptomics techniques to investigate the immune modulatory role of IFN-λ in B-cells.

6.1 *In vitro* ELISA assays to study IFN-λ1, -λ2, -λ3 and the IFNLR1 interactions

We have established two ELISA based assays: a direct ligand-receptor interaction assay and a competition ligand-receptor interaction assay to study IFN-λs and the receptor (IFNLR1) interactions

First, we evaluated the binding affinities or disassociation constants (K_D values) of IFN-λ1-3 to IFNLR1 by direct ligand-receptor interaction assay. Our data indicates that IFN-λ1 has a higher binding affinity (K_D = 15.7 nM) for IFNLR1 compared to IFN-λ2 (K_D = 19.3 nM) and IFN-λ3 (K_D = 64.7 nM). This is mainly due to the differences in IFN-λs amino acid sequences. IFN-λ1 and IFN-λ2 have 33 amino acids difference while IFN-λ2 and IFN-λ3 have only 7 amino acid difference [40]. The helix A and the AB-loop of IFN-λ is involved in the interaction between IFN-λ and IFNLR1 [195]. The sequence alignment showed the significant differences in helix A and the AB-loop between IFN-λ1 and IFN-λ3. A strong salt bridge forms in the IFN-λ1-IFNLR1 complex between Arg54 and Glu119 (**Fig. 6-A**), whereas in IFN-λ3-IFNLR1 complex arginine is replaced by lysine 57, forming a less stable salt bridge between Lys-Glu (**Fig. 6-B**). This might be a reason why IFN-λ1 shows higher affinity compared to IFN-λ3 [196-198]. Since the amino acid sequence of helix A is identical in IFN-λ2 and IFN-λ3, the differences in helix A do not adequately explain the lower affinity of IFN-λ3 compared to IFN-λ2 for IFNLR1. It is thought that the main difference comes from changes in the

AB-loop, where Arg74 and His76 in IFN-λ2 are replaced by Lys70 and Arg72 in IFN-λ3 [40].

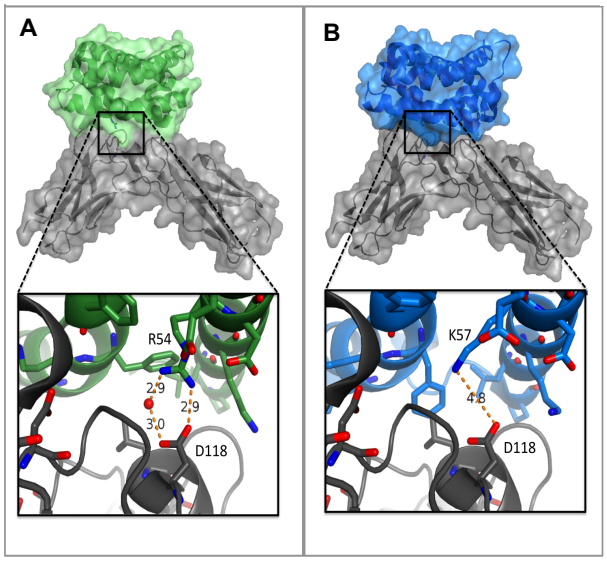


Fig. 6: Structural comparison of IFN-λ1 (green) and IFN-λ3 (blue). A) Salt bridge between Arg54 and D118. B) IFN-λ3 aligned to IFNLR1-IFN-λ1 complex (IFNLR1 is shown in grey). The alignment shows that the Arg-Glu salt bridge is replaced by a less stable Lys-Glu, a salt-bridge between Lys57 and D118. Syedbasha M., *et al,* 2017, J Vis Exp, 1 (130).

Next, we studied the competition of antagonistic peptides against the ligand-receptor interaction assay at the receptor (IFNLR1) binding sites. Short 14-20 amino acid (aa) length peptides were generated to inhibit IFN-λs and the potential receptor interaction sites. The inhibitory activity of antagonistic peptides was described in Egli A *et al,* PLoS Pathog, 2014. The interaction specificity was examined with peptide 15, which mimics the AB-loop of IFN-λ3. As mentioned before, the AB-loop plays an important role in the interaction of ligand and receptor, particularly in IFN-λ2 and IFN-λ3 [195]. The peptide more effectively blocks IFN-λ3 interactions to IFNLR1 than IFN-λ1 or IFN-λ2, occupying the binding pocket of the AB-loop of IFN-λ3 (peptide occupied region indicated in purple colour, **Fig. 7**). The amino acid alignment of peptide 15 and the AB-loop of IFN-λs shows the differences between each IFN-λ (see below table): 12 aa differ in IFN-λ1 while only 2 aa differ in IFN-λ2 (indicated in red colour).

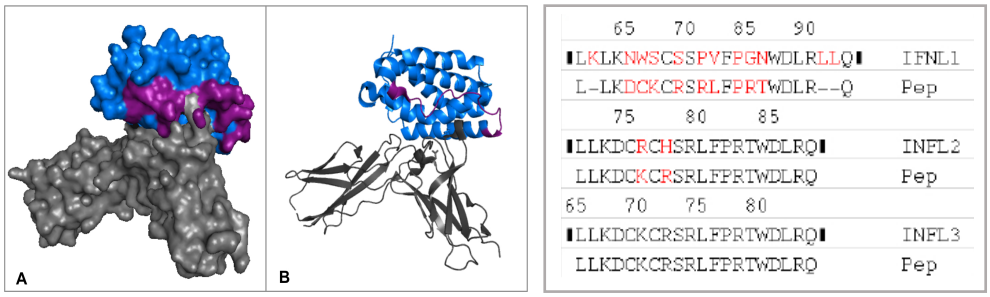
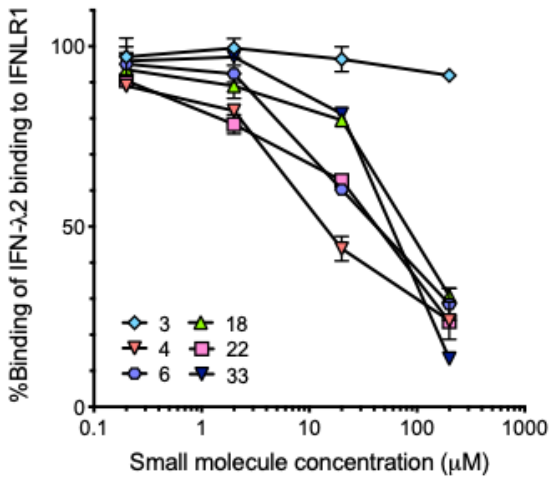


Fig. 7: The alignment of IFN-λ3 and the IFN-λ1-IFNLR1 complex (IFN-λ1 is not shown). IFN-λ3 in blue and IFNLR1 in grey colour. The regions corresponding to the blocking peptide are highlighted in purple. Sequence alignment of IFN-λ1-3 and inhibitory peptide with differing amino acids highlighted in red (in the right-side table). Syedbasha M., *et al,* 2017, J Vis Exp, 1 (130).

The blockade of IFN-λ signaling might be an interesting drug target for broad range of applications based on the immune modulatory functions. So, we have further performed the small molecules screen with an *in vitro* ELISA assay to identify the potential drug candidates to block the IFN-λ ligand-receptor interaction. These small molecules were *in silico* predicted, which are specific for IFNLR1 binding pocket. A series of small molecules were blocked the binding of IFN-λ ligand to IFNLR1 (**Fig. 8**). The small molecules compounds were further evaluated with cytokine release functional assay and MTT viability assay (data not shown in the thesis).



SM	IC50 (μM)
4	18.0
6	44.4
22	33.1
23	49.1
33	54.9

Fig. 8: Screening of small molecules with competition ligand-receptor interaction: The selected small molecules are shown the blocking of IFN-λ2 binding to IFNLR1. The IC50 inhibitory concentration of those selected small molecules are shown in the right-side table (data not published yet).

6.2 Responsiveness of immune cell populations to IFN-λ

In human immune cells, many conflicting data have been reported on the expression of IFNLR1. To resolve the discrepancies in the reported data, we performed extensive analysis to identify which immune cells directly respond to IFN-λ. We optimized a sensitive phospho-flow cytometry and immunoblot assays to measure IFN-α or IFN-λ induced STAT1 phosphorylation.

Our results showed that NK-cells, monocytes, CD3 (including CD4 and CD8) T-cells, and B-cells (including naïve, class switched and non-class switched memory B-cells) all respond to IFN-α stimulation and expressed pSTAT1, indicating that the receptor of IFN-α is expressed nearly by every cell type [75]. In contrast, IFN-λ did not induce any STAT1 phosphorylation in NK-cells, monocytes and T-cells including CD4 and CD8+ T-cells. Previously, NK-cells were shown not to be directly affected by IFN-λ, rather being activated by IFN-λ stimulated alveolar macrophages during influenza infection [83,91]. In addition, we observed pDCs strongly respond to IFN-λ as previously reported [88,89].

Next, the expression of IFNLR1 mRNA was described on B-cells [85,88]. We clearly showed that IFN-λ directly induces STAT1 phosphorylation in total B-cells and its subtypes (naïve, non-class switched and class switched memory B-cells). Based on JAK inhibitor assay and MX1 measurement assay, we confirmed that IFN-λ activates the classical JAK-STAT pathway to induce ISG expression in B-cells. Furthermore, IFN-λ induced ISG expression increased over 72h, which suggests that IFN-λ signaling is steady and prolonged compared to IFN-α signaling. The kinetic differences of both signaling pathways may be explained through specific feedback mechanisms [73,108,199].

6.3 IFN-λ increases mTORC1 activity in B-cells

B-cell transcriptomics in response to IFN-λ have been performed for the first time, to explore the immune modulatory role of IFN-λ in B-cells. The first evidence from gene set enrichment analysis indicates that the genes involved in mTORC1 signaling are significantly upregulated by IFN-λ in BCR-activated cells. This specific

IFN-λ effect was previously unknown. The activated mTORC1 phosphorylates its two downstream targets S6K and 4E-BP1, by which mTORC1 elicits numerous biological functions [143,155,200]. Our *in vitro* experiments showed that IFN-λ increases the BCR induced phosphorylation of mTORC1, S6 and 4E-BP1. In addition, IFN-λ prolongs S6 phosphorylation over 16h; blocking IFN-λ signaling using a JAK inhibitor (with ruxolitinib) specifically reduces the IFN-λ induced enhancement of mTORC1 activity. Further blocking of PI3K (with wortmannin) confirms the engagement of mTORC1 by BCR and IFN-λ receptor takes place via PI3K upstream. Similarly, IFN-α or IFN-γ can engage the mTOR/p70 kinase axis to generate of IFN responses through mRNA translation of interferon-stimulated genes [201-203]. However, it requires further evaluation to understand if IFN-λ increase the mTORC1 indirectly via its ISGs.

6.4 IFN-λ enhances cell cycle/proliferation of the BCR-activated B-cells

mTORC1 regulates cell proliferation and cell growth by modulating mRNA translation through the phosphorylation of its downstream targets 4E-BP1,2,3 and S6 kinases 1, 2 [159,204,205]. As IFN-λ increased mTORC1 activity, we investigated the effect of IFN-λ on B-cell proliferation or cell cycle progress. IFN-λ increased the expression of Ki-67 up to 2-fold in the BCR-activated state, the expression of Ki-67 actively increases during the S phase of cell cycle progression [204,206]. Similarly, IFN-λ increased the proliferation of BCR-activated B-cells. However, IFN-λ alone did not induce any proliferation. Additionally, hallmark gene set enrichment and Gene Ontology (GO) analysis signified that IFN-λ amplifies the expression of gene sets involved in G2M, E2F and other cell cycle related biological process in BCR-activated cells. Overall, our data suggest that IFN-λ enhances cell cycle progress by increasing mTORC1 activation. Because mTORC1/S6 axis plays an important role in cell cycle progression, it is shown that IFN-γ can mediate vesicular smooth cell proliferation in association with mTORC1/S6 phosphorylation; further blocking of mTORC1 with rapamycin was able to suppress the IFN-γ induced outcome [201,203].

6.5 IFN-λ boosts naïve B-cells into plasmablasts differentiation through mTORC1

Activation of mTORC1 signaling and cell cycle progression can promote cellular differentiation. The mTORC1 pathway is known to be involved in immune cell differentiation [207,208]. Our data showed that BCR-activation of naïve B-cells with IFN-λ greatly upregulates the expression of transcription factors such as IRF4 and PRDM1, which are essential for plasmablast differentiation. In addition, we observed changes in phenotypic markers (increase of CD38, CD71) and gaining effector functions like cytokine (IL-6, IL-10) and antibody (IgM) release. Overall, observations suggest that IFN-λ enhances the process of plasmablast differentiation. Further inhibition of PI3K or mTORC1 completely blocked IgM release, whereas inhibition of IFN-λ signaling blocked only IFN-λ induced boost of IgM release, independently of BCR response. This indicates that IFN-λ boosts the differentiation of naïve B-cells into IgM releasing plasmablasts by enhancing the mTORC1 pathway.

6.6 Overall conclusion

Taken together, in my thesis, the different binding affinities of IFN-λ1-3 to IFNLR1 and screening of blocking peptides to compete the binding of IFN-λ1-3 to IFNLR1 have been described. We have shown that B-cells and subtypes (naïve and non-class switch and class switch memory B-cells) directly respond to IFN-λ and activate the JAK-STAT pathway to induce ISG expression. Next, our work demonstrated how IFN-λ boosts naïve B-cells into plasmablast differentiation by enhancing the mTORC1 pathway and cell cycle progression in activated B-cells, which is a previously unknown immune modulatory role of IFN-λ.

The following schematic figure describes our working model (**Fig. 9**). These findings are particularly relevant for understanding the molecular mechanisms behind the immune modulatory function of IFN-λ signaling in B-cells. It may allow the optimization of strategies to target the immune cells involved in autoimmunity and infectious disease treatment and improve vaccine efficacy.

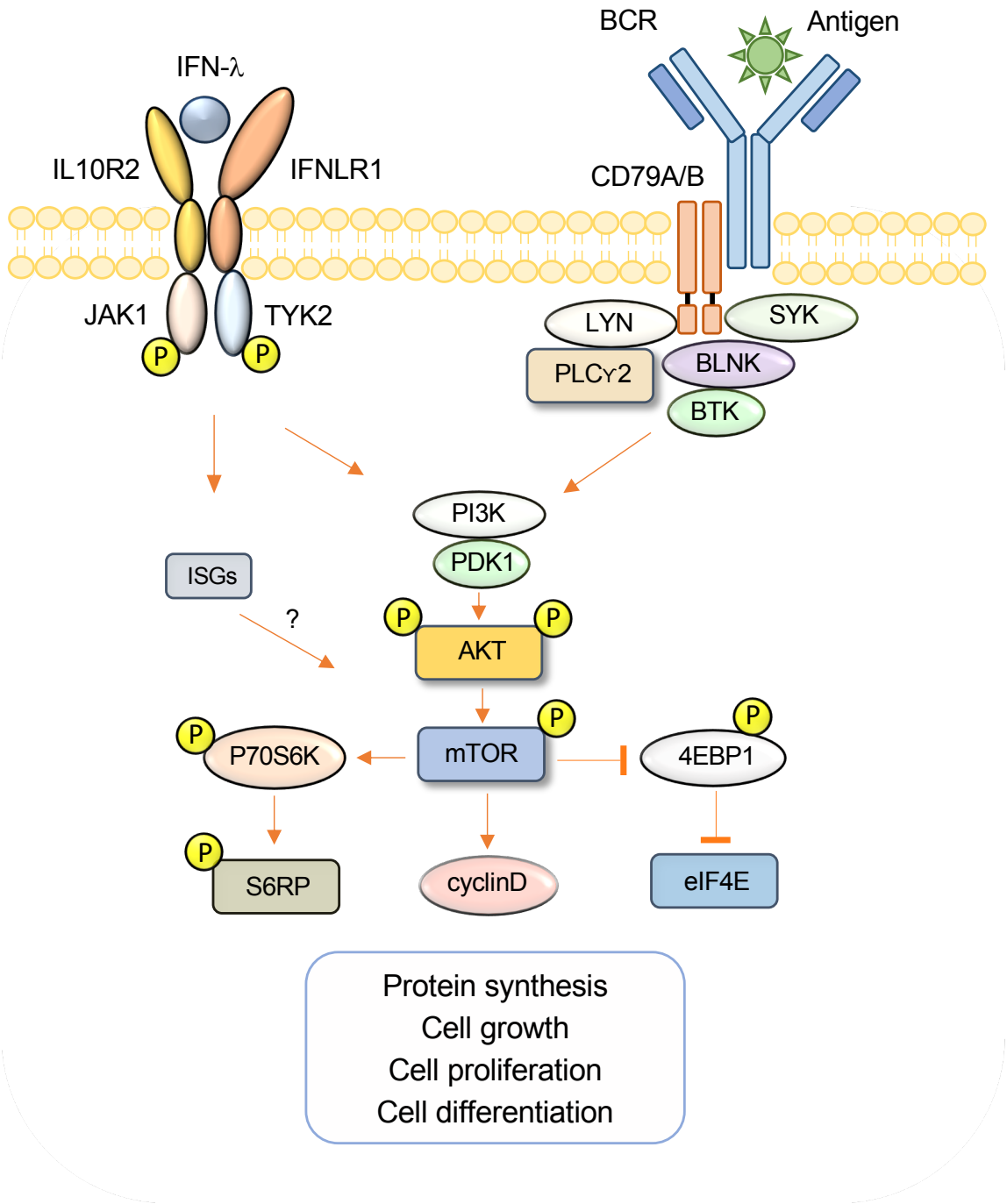


Fig. 9: IFN-λ synergizes with BCR signaling through the mTORC1 pathway

Schematic diagram demonstrates how IFN-λ and BCR signaling engage the mTORC1 axis to boost cell proliferation and differentiation of naïve B-cells into plasmablasts.

7 Outlook

To understand the role of IFN-λ in adaptive immunity

Future studies show focus on the mechanistic role of IFN-λ in adaptive immunity. In this thesis, we have shown the direct immune modulatory role of IFN-λ in human B-cells *in vitro*. In the mouse system, B-cells do not directly respond to IFN-λ, though IFN-λ indirectly triggers a GC reaction and enhances the antibody production through a thymic stromal lymphopoietin (TSLP) dependent mechanism [139]. Another mouse study described how an IFN-λ adjuvanted HIV vaccination lowers the number of regulatory T-cells and reduces Th2 cytokine IL-4 production; however, IFN-λ adjuvanted HIV vaccination increases IgG2a response compared to IL-12 adjuvanted vaccine [140]. In humans, IFN-λ augments the TLR mediated B-cell functions *in vitro* [85]. On the other hand, IFN-λ modulates Th1/Th2 balance and shifts the balance towards Th1 response [93,136,138]. In a human influenza vaccination cohort study, IFN-λ3 SNP rs8099917 TT allele correlated with high IFN-λ3 expression and a lower seroconversion rate. Also *in vitro* recombinant IFN-λ3 was found to increase Th1 cytokines (IFN-γ) and suppress Th2 cytokines (IL-4, IL-5 and IL-13) from H1N1 primed PBMCs, further it reduces the B-cell antibody production [93]. Overall, these observations from mouse and human suggest that the role of IFN-λ in adaptive immunity seems to be substantially different and context dependent. In such a complex setting, systematic studies of the human IFN-λ system is very a challenge, as controlled experiments cannot be transferred to animal models. Nevertheless, it will be tremendously important to further study the mechanistic role of IFN-λ in adaptive immunity. Especially how IFN-λ affects B-cells mechanistically during the complex interaction with migratory dendritic cells and T-cells in humans. To continue this evaluation, humanized mouse models may lead the path to even more profound insights.

To understand the extended role of IFN-λ in infectious diseases

In a series of infectious diseases IFN-λ plays an important role. One more recent discovery focuses on *Plasmodium* spp. The important role of IFN-α/β in *Plasmodium spp* infection has been explored [209-213]. In this thesis, we showed that IFN-λ enhances the differentiation of naïve or memory B-cells into plasmablasts and increases IgM release *in vitro*. In mice, *Plasmodium* specific IgM+ memory B-cells rapidly react and release somatically hyper mutated immunoglobulins IgM+ (IgM+) and provide early response during secondary *plasmodium* re-challenge [214]. It may be noteworthy to examine the role of IFN-λ in the host's IgM response to *Plasmodium* infection (and to other pathogens).

To understand the impact of IFN-λ signaling in SLE disease

B-cells play a major role in autoimmunity. The role IFN-λ signaling in autoimmune diseases is not clearly understood and should be examined, especially in hyperactive B-cell in systemic lupus erythematosus (SLE) patients. IFN-λ signaling might be a potential target for controlling mTOR activity. As the level of p-mTORC1 in CD19+ B-cells positively correlated with the amount of peripheral plasmablasts and SLE disease activity score index [215]. Therefore, modulation of the IFN-λ signaling pathway may be an interesting target for selected auto-immune diseases such as SLE.

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9 Acknowledgement

I would like to express my sincere gratitude to all the wonderful people I have come across during my PhD project. I start with my supervisor Dr. Adrian Egli, who provided me the opportunity to start my PhD in his Applied Microbiology Research group and guided me through many interesting translational research projects with invaluable support, encouragement and scientific discussions. In addition, I would like to thank Dr. Adrian Egli and Dr. Helena Seth-Smith for helping me with the development of my scientific writing skills.

I am very thankful to my follow-up PhD committee members Prof. Christoph Hess, Prof. Peter Stäheli and Prof. Daniel Pinschewer, who have provided stimulating scientific discussion and given me their constructive critical comments and useful advices for my thesis work.

I would like to express my gratitude to all the former and present members of Dr. Adrian Egli and Dr. Nina Khanna group members. I have been privileged to work with: Aline, Anne, Christian, Christina, Claudia, Daniel, Daniela, Darya, David, Dominik M, Dominik V, Ferdinando, Helena, Jana, Josiane, Julia, Kathrin, Lisandra, Lukas, Mathias, Olivia, Pascal, Richard, and Yvonne for your friendship and great support. In addition, I like thank Claudia, Pascal, Jana, Dominik V, Mathias K, Mathias S for all the inspiring scientific discussion during these years. I thank all my colleagues and friends in the DBM.

Many thanks to University of Basel for providing the excellent education and Department of Biomedicine for their excellent core facilities. I would like to especially thank DBM FACS facility former and current members Emmanuel, Lorenzo, Telma and Danny for their great support.

Finally, my special thanks go to all the members of my wonderful family and friends, who are always supporting me and helping in moving forward.

Mohammedyaseen Syedbasha
Basel, 2019