Tampere University

MAARIA PALMROTH

JAK-STAT Signaling and Inhibition in Immunological Diseases

Tampere University Dissertations 597

Tampere University Dissertations 597

MAARIA PALMROTH

JAK-STAT Signaling and Inhibition in Immunological Diseases

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the auditorium F114
of the Arvo building, Arvo Ylpön katu 34, Tampere,
on 3 June 2022, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

Responsible supervisor and Custos	Professor Olli Silvennoinen Tampere University Finland	
Supervisor	Docent Pia Isomäki Tampere University Finland	
Pre-examiners	Docent Heikki Valleala University of Helsinki Finland	Docent Tiina Kelkka University of Helsinki Finland
Opponent	Professor Mathias Müller University of Veterinary Medicine Vienna Austria	

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

Cover design: Roihu Inc.

ISBN 978-952-03-2396-7 (print)
ISBN 978-952-03-2397-4 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-2397-4

PunaMusta Oy – Yliopistopaino
Joensuu 2022

To my son.

ACKNOWLEDGEMENTS

This thesis work was carried out at Molecular Immunology research group at Faculty of Medicine and Health Technology of Tampere University, under the expert supervision of Professor Olli Silvennoinen, MD, PhD, and Docent Pia Isomäki, MD, PhD.

I wish to express my true gratitude to you Olli, for providing me with an excellent working environment and the opportunity to work on interesting and challenging research projects. I am thankful for all the guidance and trust you have given me during these years. You have let me work independently, which has taught me a lot. My deep gratitude also goes to you Pia, who introduced me to the interesting world of clinical research. Thank you for your guidance, support, and trust throughout the process. Thank you both for enabling me to gather many valuable experiences and learn how to make science.

I want to thank the pre-examiners of this thesis, Docent Heikki Valleala, MD, PhD, and Docent Tiina Kelkka, PhD, for their valuable comments and encouraging words. I would also like to thank the members of my thesis supervision committee, Professor Jukka Westermarck, MD, PhD, and Professor Vesa Hytönen, PhD, for their advice and constructive comments regarding my projects. Professor Mathias Müller, DVM, is acknowledged for doing me the great honor of being my opponent.

I am grateful to all my co-authors; only teamwork made these publications possible. Dr. Anniina Virtanen, DSc (Tech.), you have been mentoring me from the day 1. Thank you for everything, your friendship is truly valued. Dr. Teemu Haikarainen, PhD, thank you for your expert and friendly guidance and warm support. Antti Kurttila and Sanna Liukkonen, MSc (Tech.), thank you for your meticulous work in the lab as well as in result analysis. I wish to express my gratitude also to the collaborators in Helsinki and Basel: Dr. Krista Kuuliala, PhD and Antti Kuuliala, thank you for all the input, help and discussions about lab techniques, data analysis and manuscript writing; Dr. Markku Varjosalo, PhD, and Dr. Salla Keskitalo, PhD, thank you for your expert NanoString work and Professor Radek Skoda, MD, PhD, and Dr. Jacub Zmajkovic, PhD, for your great input in JAKinib selectivity manuscript. I wish to thank also all the clinical collaborators: Dr. Hanna Viskari, MD, PhD; Dr. Ritva Peltomaa, MD, PhD; Dr. Anna Kinnunen, MD; Docent

Marjatta Leirisalo-Repo, MD, PhD, and Docent Mikko Seppänen, MD, PhD for your valued contribution to this research.

I want to thank all the current and former members of our research group with whom I have had the privilege of working with. Dr. Juuli Raivola, PhD, we started our PhD journey on the same day and your peer support has meant the world for me. Thank you for all the fun moments in and outside the lab. Dr. Bobin George Abraham, DSc (Tech.), thank you for always being so kind and ready to help whatever the problem was. Thank you also Dr. Juha Saarikettu, PhD, Dr. Guillermo Martinez Nieto, PhD, Dr. Saara Lehmusvaara, PhD, Dr. Henrik Hammarén, PhD, and Dr. Atte Valli, MD, for all the help and support as well as for all the interesting discussions. All lab technicians over the years: Merja Peltomaa, Marika Vähä-Jaakkola, Merja Jokela, Krista Lehtinen, and Heidi Peussa, MSc (Tech.), thank you for all the excellent work and positive energy in the lab.

I also want to thank all the other colleagues at Arvo that have helped somewhere along the way. Especially, I want to thank members of Cancer Signaling group for being helpful lab neighbors, Dr. Laura Kummola, PhD, for the help with flow cytometry, Dr. Alejandra Rodriques Martinez, PhD, and Annika Kohvakka, MSc, for the help in gene expression studies; and Laura Oksa, MSc, for the help with sequencing data.

The Doctoral Programme of Medicine and Life Sciences is acknowledged for offering me a graduate school position, personal working grant and financial support for congress travel. The city of Tampere is thanked for supporting the publication of this thesis with a printing grant. I also wish to express my gratitude to all the patients and healthy blood donors for participating in the studies presented in this thesis.

I have been fortunate to have many friends that have supported me throughout this thesis project (and in life in general). Thank you, friends from the school years, for the friendship of over 20 years and counting. I am grateful for all the friendships made during my university studies, in Finland and abroad. Thank you for all the great discussions on science and other things. I also wish to thank those friends that I have made through parenthood. Peer support is needed also in that kind of project.

I wish to express my sincere gratitude to my family for their constant support and help during my studies. My parents, Anne and Jouko, have provided me with an excellent starting point for life and always encouraged me in my numerous endeavors. Your help for me in juggling the finishing PhD/starting new work/having family life combination has been invaluable. Thank you also my mother-in-law Heli for your great support.

Finally, I wish to express my greatest gratitude to my husband Aleksi for his constant support for my personal and professional goals. Many thanks also to my energetic son Konsta, who meritoriously keeps reminding me that the end of the day, a thesis is just a thesis.

11th of April 2022, Tampere

Maaria Palmroth

ABSTRACT

Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathway is one of the key signaling pathways in our bodies. JAK-STAT pathway regulates numerous biological functions including the immune response, metabolism, hematopoiesis, and development. Mutations in JAKs, STATs, or their regulatory proteins can cause defective JAK-STAT signaling responses resulting in human diseases. In addition, the cytokine environment typically present in inflammatory diseases can cause overactivation of the JAK-STAT pathway. Consequently, Janus kinases were identified as potential drug targets shortly after their discovery. To date, several JAK inhibitors are in clinical use for the treatment of both inflammatory diseases and hematological malignancies.

In this thesis project, a rare, previously uncharacterized, immunological disease was investigated focusing on the activation of JAK-STAT pathways, which was studied using flow cytometry. In the second study, we analyzed the *in vivo* effects of a JAK inhibitor, tofacitinib, on different JAK-STAT pathways in patients with rheumatoid arthritis using similar methodology. In addition, the selectivity of 20 clinical JAK inhibitors was analyzed and compared by using *in vitro* methods that measure enzymatic activity and cytokine inhibition.

The results presented in this thesis describe a new mutation in the *IRF2BP2* gene found in two patients having both inflammatory symptoms and lymphopenia. We showed that IRF2BP2 protein is particularly important in the regulation of the JAK-STAT-mediated interferon response. Studies done with JAK inhibitors showed that tofacitinib inhibits the activation of several JAK-STAT pathways, but the extent of inhibition depends on both the cytokine pathway and the cell population studied. On the other hand, the cytokine inhibition profile of tofacitinib also depends to some extent on the methodology, as differences were found between the *in vivo* and *in vitro* measurements. Comparison of 20 JAK inhibitors revealed inhibitors to possess distinct selectivity profiles and demonstrated that selectivity determination requires use of multiple measurement methods combined with pharmacokinetic variables.

The results described above add to our understanding of the role of JAK-STAT pathway and its inhibition in immunological diseases. By characterizing

immunological diseases and JAK inhibitors, more targeted and thus more effective and safer treatments can be achieved in the future for patients with various diseases of the immune system.

TIIVISTELMÄ

Janus kinaasi (JAK) – signaalinvälittäjä ja transkriptioaktivaattori (STAT) reitti on yksi keskeisimmistä signaalinvälitysreiteistä kehoissamme. JAK-STAT reitti säätelee lukuisia biologisia toimintoja, kuten immuunivastetta, aineenvaihduntaa, hematopoieesia sekä kehitystä. Mutaatiot JAKeissa, STATeissa tai niiden toimintaa säätelevissä proteiineissa voivat aiheuttaa virheellisen JAK-STAT signalointivasteen, joka voi johtaa vakaviin sairauksiin. Myös tulehduksellisissa sairauksissa tyypillisesti esiintyvä sytokiiniympäristö voi aiheuttaa JAK-STAT reitin yliaktivaation. Janus kinaasit nähtiinkin potentiaalisina lääkekehityskohteina jo pian niiden tunnistamisen jälkeen. Tällä hetkellä kliinisessä käytössä on jo useita hyväksyttyjä JAK-estäjiä tulehduksellisten sairauksien ja hematologisten syöpien hoitoon.

Tässä väitöskirjassa tutkittiin JAK-STAT reittien aktivaatiota harvinaisessa ja aiemmin määrittelemättömässä, immunologisessa sairaudessa virtaussytometria-menetelmää hyödyntäen. Toisessa osatyössä tutkittiin JAK-estäjä tofasitinibin *in vivo* vaikutusta eri JAK-STAT reitteihin lääkettä käyttävillä nivelreumapotilailla samankaltaisella metodologialla. Lisäksi analysoimme ja vertailimme keskenään 20 kliinisissä tutkimuksissa arvioidun JAK-estäjän selektiivisyyttä *in vitro* käyttäen sekä entsyymiaktiivisuutta että sytokiini-inhibitiota mittaavia menetelmiä.

Työssä kuvaillaan uusi mutaatio *IRF2BP2* geenissä, joka löydettiin kahdelta potilaalta, joilla oli sekä tulehduksellisia oireita että lymfopeniaa. Osoitimme, että IRF2BP2 proteiini on tärkeä erityisesti JAK-STAT välitteisen interferonivasteen säätelyssä. JAK-estäjillä tehdyissä tutkimuksissa osoitettiin, että tofasitinibi estää useiden JAK-STAT reittien aktivaation, mutta estämisen voimakkuus riippuu sekä tutkittavasta sytokiinireitistä että solupopulaatiosta. Toisaalta tofasitinibin sytokiinien estoprofiili myös jossain määrin riippuu tutkimusmenetelmästä, sillä eroavaisuuksia löydettiin *in vivo* ja *in vitro* mittausten välillä. JAK-estäjien vertailututkimus osoitti, että kliinisten JAK-estäjien selektiivisyydessä on eroja. Selektiivisyyden määrittäminen vaatii kuitenkin useiden mittausmenetelmien käyttämistä yhdistettynä farmakokineettisiin muuttujiin.

Edellä kuvatut tulokset lisäävät ymmärrystä JAK-STAT reitin ja sen estämisen merkityksestä immunologisissa sairauksissa. Immunologisia sairauksia ja JAK-estäjiä karakterisoimalla voidaan tulevaisuudessa saavuttaa entistä kohdennetumpia ja siten

tehokkaampia ja turvallisempia hoitoja erilaisista immuunijärjestelmän sairauksista kärsiville potilaille.

CONTENTS

List of Figures

List of Tables

ABBREVIATIONS

AA	alopecia areata
ACR	American College of Rheumatology
AD	atopic dermatitis
AS	ankylosing spondylitis
BCR	B cell receptor
CASP	caspase
CD	cluster of differentiation
CI	confidence interval
CIITA	class II major histocompatibility complex transactivator
CIS1	cytokine-inducible SH2-containing protein
CRP	c-reactive protein
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
csDMARD	conventional synthetic disease-modifying antirheumatic drug
CXCL	C-X-C motif chemokine ligand
DAS28	composite Disease Activity Score for 28 joints based on the c-reactive protein level (DAS28-4[CRP])
DUSP2	dual specificity protein phosphatase 2
EDTA	ethylenediamine tetra acetic acid
EPO	erythropoietin
ETO2	LIM domain-only 2 and eight-twenty-one 2
EULAR	European League Against Rheumatism
FDA	U.S. Food and Drug Administration
GAS	gamma-activated sequence
GBP2	guanylate binding protein 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
$gp91^{phox}$	cytochrome B-245, beta polypeptide
GR	glucocorticoid receptor
HAQ	health assessment questionnaire
IFN	interferon

IDO1	indoleamine 2,3-dioxygenase 1
IFI	interferon alpha inducible protein
IFI44L	interferon induced protein 44 like
IFIH1	interferon induced with helicase C domain 1
IFIT	interferon induced protein with tetracopeptide repeats
IL	interleukin
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
IQR	interquartile ranges
IRF	interferon regulatory factor
IRF2BP2	IRF2 binding protein 2
IRF2BPL	IRF2 binding protein like
JAK	Janus kinase
JAKinib	JAK inhibitor
KLF2	Krüppel-like factor 2
JIA	juvenile idiopathic arthritis
MACE	major cardiovascular event
MHC	major histocompatibility complex
MPN	myeloproliferative neoplasm
mRNA	messenger-RNA
MX1	MX dynamin like GTPase 1
NCOR1	nuclear receptor corepressor 1
NFAT1	nuclear factor of activated T cells 1
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRIF3	nuclear receptor interacting factor 3
NLRP3	NLR family pyrin domain containing 3
OAS1	2'-5'-oligoadenylate synthetase 1
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	pulmonary embolism
PRR	pattern recognition receptor
PTPN1B	protein tyrosine phosphatase 1B
PTPRD	protein tyrosine phosphatase receptor type D
PTPRT	protein tyrosine phosphatase receptor type T

REL	REL proto-oncogene, NFKB subunit
RING	really interesting new gene
RSAD2	radical s-adenosyl methionine domain containing 2
pSTAT	phosphorylated STAT
PIAS	protein inhibitor of activated STAT
PsA	psoriatic arthritis
RA	rheumatoid arthritis
SH2	Src homology 2
SHP	Src homology region 2 domain-containing phosphatase
SIGLEC1	SIGLEC family like 1
STAT	signal transducer and activator of transcription
SOCS	suppressor of cytokine signaling
SUMO	small ubiquitin-like modifier
TC-PTP	T cell protein tyrosine phophatase
TCR	T cell receptor
TNF	tumor necrosis factor
TPO	thrombopoietin
TYK2	tyrosine kinase 2
qPCR	quantitative PCR
UC	ulcerative colitis
USP18	uqiquitin specific peptidase 18
VAS	visual analogue scale
VGLL4	vestigial like family member 4
VTE	venous and arterial thrombosis

ORIGINAL PUBLICATIONS

I Palmroth M, Viskari H, Seppänen MRJ, Keskitalo S, Virtanen A, Varjosalo M, Silvennoinen O, Isomäki P. (2021) *IRF2BP2* mutation is associated with increased STAT1 and STAT5 activation in two family members with inflammatory conditions and lymphopenia. *Pharmaceuticals (Basel),* 14(8):797.

II Palmroth M, Kuuliala K, Peltomaa R, Virtanen AT, Kuuliala A, Kurttila A, Kinnunen A, Leirisalo-Repo M, Silvennoinen O, Isomäki P. (2021) Tofacitinib suppresses several JAK-STAT pathways in rheumatoid arthritis *in vivo* and baseline signaling profile associates with treatment response. *Frontiers in Immunology,* 12, 738481.

III Virtanen A, Palmroth M, Liukkonen S, Kurttila A, Haikarainen T, Zmajkovic J, Skoda R, Silvennoinen O. *In vitro* JAK-selectivity profiling and head-to-head comparison of 20 clinical JAKinibs. *Submitted manuscript.*

AUTHOR'S CONTRIBUTION

Article I: Maaria Palmroth wrote the manuscript as the main author and together with co-authors, planned the study. She collected and analyzed the flow cytometry samples, designed, and performed the cell model experiments and analyzed the results.

Article II: Maaria Palmroth wrote the manuscript as the main author and together with co-authors, planned the study. She set-up and optimized the methods (flow cytometry and qPCR) and participated in the collection and analysis of the data.

Article III: Maaria Palmroth planned the study together with co-authors, helped setting-up the barcoded flow-cytometric assay, performed partly the flow-cytometric assays and participated in data interpretation and manuscript writing.

1 INTRODUCTION

The immune system is essential for our survival, as it is responsible for defending the body against external pathogens, such as viruses, bacteria, and parasites. The immune system also protects us from dangers inside our bodies, as it can recognize and destroy transformed cells before they grow into tumors. Additionally, the immune system plays a significant role in tissue healing and regeneration. Thus, a malfunction of the immune system can have serious consequences, such as autoimmune diseases or cancer.

Giant leaps in our understanding of the immune system and exploitation of this knowledge for therapeutic or preventive approaches have improved the lives of patients with immunological diseases and eradicated several infectious diseases. Additionally, treatments that reactivate immune responses against tumors are available treatment options for certain cancer patients. Still, the pathogenesis of many immunological diseases remains poorly understood and many immunological diseases and cancers are incurable. Also, infectious diseases are still serious threats to human health, as the world-wide Covid-19 pandemic has demonstrated.

Molecular immunology investigates specific details of the immune system. A small, but rather significant component of our immune system is Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, which plays an indispensable role in development and function of immune cells as well as maintaining immune system homeostasis. Understanding the essential nature of JAK-STAT signaling in the immune system led soon to the idea that JAK inhibition could offer a therapeutic alternative in treating autoimmune diseases.

This thesis examines the function and inhibition of JAK-STAT pathway in a clinical perspective. The aim was to investigate JAK-STAT signaling in patients having immunological diseases, including a rare immunological disease caused by a novel mutation in *IRF2BP2* gene and rheumatoid arthritis. This study also investigates the specific mechanisms behind JAK inhibitors. Better understanding of disease and drug mechanisms would allow more targeted therapies for patients being affected by immunological disorders.

2 REVIEW OF THE LITERATURE

2.1 The immune response

The immune response is typically divided into two branches: the innate and adaptive immune response. The innate immune response is the first line of defense against invading microbes and is essential for the first hours or days of infection. The receptors of innate immune system can recognize structures common to related microbe groups, but cannot distinguish fine differences, so they are not antigen specific (Abbas et al., 2018). The adaptive immune response occurs later during infection manifesting refined specificity toward its target antigens (Paul, 2013). Importantly, cells of the adaptive immune system can remember the microbes encountered before. Even though the innate and adaptive immune responses are addressed separately below, they act together and only their synergy results as fully effective immune response (Chaplin, 2010).

2.1.1 Innate immune response

The main components of innate immunity are physical (skin and epithelium) and chemical (mucus, antimicrobial peptides and proteins, lactoferrin and microbiota) barriers, innate immunity cells (phagocytic neutrophils and macrophages, mast cells, eosinophils, basophils, dendritic cells, natural killer) and blood proteins, such as components of the complement system, which mediate inflammation (Abbas et al., 2018; Paul, 2013).

The two main types of protective reactions of the innate immune response are inflammation and antiviral response (Abbas et al., 2018). Tissue damage, which can be either caused by physical agents or by pathogens, induces inflammation (Male, 2013). Cells of the damaged tissue secrete cytokines, which act as drivers of inflammation. Key pro-inflammatory cytokines include tumor necrosis factors (TNFs), interleukin (IL)-1 and IL-6 (Abbas et al., 2018). The secretion of pro-inflammatory cytokines results in increased 1) blood supply to the damaged area, 2) capillary permeability (permitting larger molecules, such as complement system

proteins, to enter the tissue) and 3) migration of leukocytes from venules into the damaged area (Abbas et al., 2018; Male, 2013). Neutrophils constitute the majority of blood leukocytes and are the first leukocytes recruited to the site of inflammation, followed by blood monocytes that become macrophages once they enter the tissue (Abbas et al., 2018).

Inflammation can be divided into acute and chronic inflammation. Above-described inflammation process refers to acute inflammation, which typically resolves within days. Chronic inflammation takes over from acute inflammation when the infection is not eliminated or the tissue injury is prolonged and can persist for weeks, months or even years (Abbas et al., 2018; Lawrence & Gilroy, 2007). Chronic inflammation is mediated partly by different cytokines than acute inflammation and involves also participation of the adaptive immune system cells (Abbas et al., 2018; Feghali & Wright, 1997).

Recognition of viral molecular patterns that use host pattern recognition receptors (PRRs) triggers antiviral defense. Several different cell types express PRRs and their activation by viral molecular patterns induces the production of type I interferons (IFNs), which are the key mediators of antiviral defense (Male, 2013). Type I interferons (IFN-α and IFN-β) act by inducing interferon stimulated genes (ISGs) that enable the cells to enter antiviral state (Abbas et al., 2018; Male, 2013). Type I IFNs also activate natural killer cells and macrophages, which can then kill virus-infected cells (Male, 2013). Macrophages also phagocyte viruses and virus-infected cells and produce IFN-α and other key cytokines for fighting virus infection, such as TNF-α and nitric oxide (Male, 2013). The molecules produced during the antiviral defense reaction of innate immunity also act as activators of adaptive immune response, which take over during later stage of viral infection (Abbas et al., 2018).

2.1.2 Adaptive immune response

A specialized group of leukocytes called lymphocytes mediate the adaptive immune response. The two major types of lymphocytes are B and T cells. B cells are responsible for production of antibodies and mediate the humoral immune response, whereas T cells manage the cell-mediated immune response that eliminates intracellular pathogens. T cells also regulate responses of B cells and the overall immune response (Male, 2013). Both B and T cells represent enormous repertoire

of antigen-recognition receptors, which is achieved by rearrangement of antigen-receptor genes during maturation (Kondo et al., 2001).

The activation of adaptive immune response requires capture of antigens and their display to specific lymphocytes, which usually occurs in peripheral lymphoid organs, such as in the lymph nodes. One of the most specialized cell types for antigen presentation is dendritic cells. Before encounter with antigen, lymphocytes are called naïve. Upon activation by an antigen, lymphocytes go through proliferation, which increases the number of the antigen-specific clones and is referred as clonal expansion (Abbas et al., 2018; Male, 2013). Clonal expansion leads to lymphocyte differentiation into effector cells that can eliminate the antigen. Also, innate immune response cells, such as macrophages and neutrophils participate in antigen elimination. After the antigen has been eliminated, most of the effector cells die declining the immune response, but memory cells remain ready to elicit a response if the same pathogen is re-encountered (Abbas et al., 2018; Male, 2013).

Naïve B cells can recognize intact microbes, proteins, and other molecular structures through B cell receptors (BCR). Antibodies are soluble forms of BCRs, which can be secreted into extracellular space. Antibodies are multifunctional proteins that can fight pathogens through various mechanisms, including opsonization and toxin neutralization (Casadevall, 1998). During clonal expansion, BCRs are further modified through somatic hypermutation and antibody class switch recombination, to achieve maximum potential toward the antigen (Pieper et al., 2013).

T cells can be divided into several subgroups based on their T cell receptor (TCR) expression. Two major groups constitute cluster of differentiation (CD)4-positive T cells, called T helper cells, and CD8-positive T cells, called cytotoxic T cells. T cell receptors recognize infected cells, when antigen is bound to major histocompatibility complex (MHC) and antigen-presenting cells display the antigen-MHC complex (Alcover et al., 2018). Cytotoxic T cells can lyse infected cells by releasing contents of cytotoxic granules toward target cells and by producing cytokines, such as IFN-γ, which contribute to the damage (de Saint Basile et al., 2010). T helper cells in turn 'help' other immune cell types (e.g., activate macrophages to phagocyte infected cells) mainly by secreting different cytokines. Depending on the antigen-type T helper cell encounters for the first time (called priming), T helper cell differentiates into a subtype that produces IFN-γ and lymphotoxin (T_{H1} cells); IL-4, IL-13, IL-5, and IL-6 (T_{H2} cells) or IL-17 and related cytokines (T_{H17}) (Paul, 2013). T_{H1} cells are important components of antiviral and antibacterial immunity, T_{H2} cells are needed for fighting against extracellular pathogens, such as worms, and T_{H17} cells

play a critical role in anti-fungal responses and in host defense against bacterial infection (Saravia et al., 2019). Naïve T helper cells can also differentiate into regulatory T cells (T_{reg}) that have immunosuppressive functions (Saravia et al., 2019). In addition, CD4 cells can differentiate into T follicular helper cells (T_{FH}) that have the ability to enter B-cell follicles, where they help B cell develop into antibody-producing cells and undergo immunoglobulin class switch and affinity maturation (Paul, 2013). The main immune cell types and their functions are summarized in Table 1.

Table 1. Summary of cell types in innate and adaptive immune responses and their main functions.

	Immune cell type	Main functions
Innate immune response	Monocytes/macrophages	Cell recruitment to site of infection by cytokine secretion, phagocytosis (Abbas et al., 2018)
	Neutrophils	Principal cell type at site of acute inflammation; phagocytosis, production of antimicrobial substances and extracellular traps (Mortaz et al., 2018)
	Dendritic cells	Recognition of microbial molecules, cell recruitment and activation to the site of infection by cytokine secretion, antigen presentation to T cells, production of type I interferons (Abbas et al., 2018; Chaplin, 2010)
	Mast cells	Release of cytokines and other inflammatory mediators in response to parasite infections (Abbas et al., 2018; Chaplin, 2010)
	Basophils	Release of cytokines and other inflammatory mediators (Abbas et al., 2018; Chaplin, 2010)
	Eosinophils	Release of enzymes harmful for cell walls of parasites (Abbas et al., 2018; Chaplin, 2010)
	Natural Killer cells	Immunoregulation by release of cytokines, killing virus-infected cells and tumor cells (Vivier et al., 2008)
Adaptive immune response	Helper T cells ($CD4^+$)	B cell activation, macrophage activation, stimulation of inflammation by cytokine production (Abbas et al., 2018)
	Cytotoxic T cells ($CD8^+$)	Killing infected and tumor cells by release of cytotoxic granules and IFN-γ (de Saint Basile et al., 2010)
	B cells	Antibody production, immunoregulation by production of cytokines (Shen & Fillatreau, 2015)

2.1.3 Immunopathologies

Defects or malfunction of innate or adaptive immune responses can lead to diseases. In general, these diseases are caused by overactive immune system (hypersensitivity reactions), inappropriate self-reaction (autoimmunity) and ineffective immune responses (immunodeficiency) (J. S. Marshall et al., 2018).

The most classic examples of hypersensitivity reactions are allergic reactions. Re-exposure to an allergen – such as pollen or pet epithelium – leads to secretion of histamine, leukotrienes and prostaglandins by mast cells and basophiles, which have been sensitized by IgE type antibodies before. The production of histamine and other active mediators result in typical allergic airway symptoms (Rajan, 2003). Allergic reactions represent type I hypersensitivity reactions, which are also referred to as immediate hypersensitivity. Further hypersensitivity reaction classes are cytotoxic or antibody-dependent hypersensitivity (type II), immune complex disease (type III) and delayed-type hypersensitivity (type IV) (Rajan, 2003).

Autoimmunity develops, when the immune system recognizes self-antigens, despite several mechanisms maintaining immunotolerance. Main characteristics of autoimmune diseases include presence of autoantibodies, self-reactive T cells and inflammation (Castro & Gourley, 2010). Autoimmune diseases can be either systemic (such as systemic lupus erythematosus, SLE) or tissue-specific (such as type I diabetes mellitus, where immune cells attack the insulin-producing pancreatic cells). The development of an autoimmune disease is usually a result of complex mixture of genetic, hormonal and environmental factors (Khan & Wang, 2020). The region encoding MHC proteins seems to be the most important genetic factor in autoimmune susceptibility (Male, 2013). Autoimmune diseases typically affect more likely women than men with the exception of type I diabetes and small vessel vasculitis (Mouzaki et al., 2005). Even though the mechanisms how environmental factors contribute to development of autoimmune diseases are largely unknown, there is strong evidence that at least tobacco smoke, crystalline silica, solvents, pristine, mercury and pesticides are linked to pathogenesis of autoimmune diseases (Khan & Wang, 2020). Also, microbes seem to play a role in the development of autoimmune diseases. For example, infectious agents, such as Epstein-Barr virus, cytomegalovirus, proteus species, *Escherichia coli* and their products have been associated with the development of rheumatoid arthritis (McInnes & Schett, 2011).

In addition, dysbiosis of skin, gut and oral microbiome has been associated with auto-inflammation and tissue destruction in autoimmune diseases (Dehner et al., 2019).

Immunodeficiency is a state, where the immune system cannot fight infectious agents either with its full potency or at all. Immunodeficiency may arise from primary genetic defects or it may be acquired, and result e.g. from malnutrition, viral or bacterial infections, autoimmunity or use of immunosuppressants (J. S. Marshall et al., 2018). One example of primary immunodeficiency is severe combined immunodeficiency (SCID). The majority of the SCID cases are caused by mutations in common γ chain gene, which encodes a receptor component needed for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 mediated signaling (Notarangelo, 2010). Also, loss-of-function mutations in *JAK3* gene result in SCID, which is clinically inseparable from SCID caused by mutations in common-γ-chain gene (Notarangelo, 2010). The most known example of secondary immunodeficiency would probably be acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV). HIV infects T helper cells and induces T cell lymphopenia through multiple mechanisms (Chinen & Shearer, 2010).

2.1.3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease, where the immune system attacks synovial tissues as its primary targets. RA is a common autoimmune disease, affecting about 1% of adults world-wide, and is characterized by synovial inflammation and hyperplasia, autoantibody formation (rheumatoid factor and anti-citrullinated protein antibody), cartilage and bone destruction as well as systemic features, which include pulmonary, cardiovascular, skeletal and psychological disorders (McInnes & Schett, 2011; Mouzaki et al., 2005).

In synovial inflammation, leukocytes have infiltrated into the normally relatively acellular synovium. In synovium, $CD4^+$ T cells secrete proinflammatory cytokines, such as IFN-γ, which recruit and activate monocytes and macrophages (Mouzaki et al., 2005). Activated monocytes and macrophages as well as fibroblasts, produce more proinflammatory cytokines, such as TNF-α and IL-1, -6, -12, -15, -18, and -23, which e.g. induce the production of matrix metalloproteinases and osteoclasts, resulting in soft tissue and bone damage (McInnes & Schett, 2011; Mouzaki et al., 2005). As the presence of autoantibodies suggests, also B cells play a central role in RA pathogenesis. However, B cells remarkably add to pathogenesis of RA also by autoantigen presentation and cytokine secretion. This has been proven by the clinical

efficacy of rituximab, which targets $CD20^+$ B cells but not the antibody-producing plasma cells (McInnes & Schett, 2011).

Currently, there is no cure for RA. The first line pharmacological treatment option in Finland includes conventional systemic anti-rheumatic drugs (csDMARDs); methotrexate (MTX), hydroxychloroquine and sulfasalazine in combination with prednisolone (Rheumatoid arthritis: Current Care Guidelines Abstract, 2015). However, not all patients respond to csDMARD treatment. Other treatment options include biological treatments and JAK inhibitors. Biologics target a single cytokine or receptor and are administered subcutaneously or intravenously. To date, biologics for treating RA in Finland include suppressors of TNF-α, IL-1, IL-6, CD20, and CTLA4) (Rheumatoid arthritis: Current Care Guidelines Abstract, 2015). JAK inhibitors on the other hand are small molecules, which are taken orally and function by inhibiting kinase activity of Janus kinases, suppressing multiple cytokine-signaling pathways simultaneously. JAK inhibitors are addressed more in detail in later chapters of this literature review.

2.2 JAK-STAT pathway

Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway is one of the major signaling nodes of the immune system. The discovery of JAK-STAT pathway was preceded by groundbreaking work with a cytokine – namely IFN-α, which role in antiviral defense was described first in 1957 (Isaacs & Lindenmann, 1957).

JAKs are intracellular non-receptor tyrosine kinases that transmit signaling of over 50 different molecules, including cytokines, hormones, and growth factors (Hammarén et al., 2019). The signaling cascade is initiated, when a signaling molecule binds to its receptor, which activates JAKs and allows them to trans-phosphorylate. Then, JAKs phosphorylate the receptor enabling recruitment and phosphorylation of STATs. STATs dimerize and enter the nucleus, where they act as transcription factors (O'Shea et al., 2015). Schematic figure of JAK-STAT signaling pathway is presented in Figure 1.

The clinical significance of JAK-STAT pathway has become evident by the dramatic immunological phenotypes of animals and humans carrying loss- or gain-of-function mutations in genes encoding JAK-STAT pathway components (Alexander et al., 2021; Villarino et al., 2017). In addition, JAK-STAT signaling route can be hyperactive also without gene mutations, namely through excess of stimuli.

For instance, several cytokines that are implicated in the pathogenesis of rheumatological diseases employ JAK-STAT pathway (Banerjee et al., 2017).

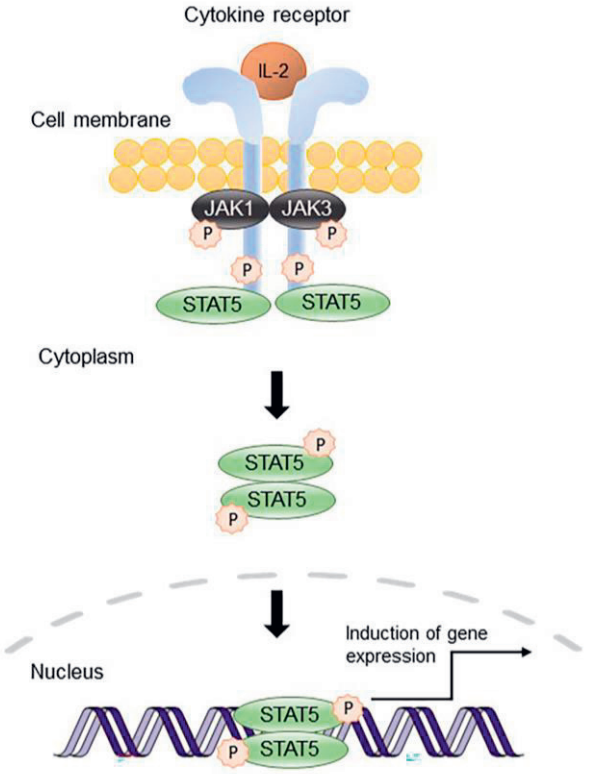


Figure 1. Schematic figure of JAK-STAT pathway. Cytokine binding to its receptor induces conformational changes in the receptor and brings JAKs close to each other, which enable trans-phosphorylation of the kinase domains. Then, receptor is phosphorylated, which enables STAT binding and phosphorylation. Phosphorylated STAT molecules dimerize and translocate to nucleus, where they act as transcription factors. Figure is based on following reviews (Dodington et al., 2018; Gadina et al., 2001; Spolski et al., 2018).

2.2.1 Cytokines and receptors

Cytokines are secreted proteins or peptides, which act as intercellular messengers. They mediate a plethora of functions, such as immune response, hematopoiesis and neural development (Deverman & Patterson, 2009; Kany et al., 2019; Metcalf, 2008). To forward the message, they bind to their specific receptors. Cytokine receptors are

usually produced by cells as transmembrane proteins that operate as oligomeric complexes, in most cases as dimers (Lokau & Garbers, 2020; R. Morris et al., 2018). However, most cytokine receptors exist also as soluble receptors that can either inactivate or activate the function of the cytokine (Lokau & Garbers, 2020).

JAK-STAT cytokines can be divided into two main groups based on sequence motifs in cytokine-binding modules found in their receptors, namely class I and class II (Mohr et al., 2012). Class I and II receptors are structurally related, but class II receptors lack the so called WSXWS motif, that is present in the extracellular part of type I receptors (R. Morris et al., 2018). Cytokine receptors can be further divided into cytokine families based on receptor subunit sharing. JAK-STAT cytokine families, their members and the main functions are summarized in Table 2.

Table 2. Summary of JAK-STAT cytokine receptor families, cytokines, and their main biological functions.

Receptor family	Cytokines	Main functions
Class I		
IL-2 family/common-γ -chain family	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21	T-, B- and NK-cell stimulation and homeostasis, T-cell differentiation, T and B cell growth factor (Lokau & Garbers, 2020)
IL-3 family/common-β-chain family	IL-3, IL-5, GM-CSF	Hematopoietic growth factor, B-cell development, inflammation (Dougan et al., 2019)
IL-6 family/gp130 family	IL-6, IL-11, IL-27, IL-31 LIF, CNTF, CT1, CLC, OSM, NP	Hematopoiesis, acute phase response, cardiac myocyte and neuronal growth factor, inflammation, T cell differentiation (Lokau & Garbers, 2020; R. Morris et al., 2018)
Homodimeric	G-CSF, EPO, TPO, GH, PRL, LEP	Blood cell differentiation, growth, mammary gland development, appetite regulation (Dwivedi & Greis, 2017; Mullen & Gonzalez-Perez, 2016; Nairz et al., 2012; Naylor et al., 2003; Smith & Murphy, 2014; Strous et al., 2020)
IL-12 family	IL-12, IL-23, IL-35	T-, B- and NK-cell activation, inflammation, anti-inflammatory (IL-35) (Jefremow & Neurath, 2020)
Others	IL-13, TSLP	Allergic response, inflammation (R. Morris et al., 2018)
Class II		
Type I interferon	IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω	Anti-viral defense (Lin & Young, 2014)
Type II interferon	IFN-γ	Inflammation, anti-microbial (De Benedetti et al., 2021)
Type III interferon	IFN-λ1, IFN-λ2, IFNλ3	Anti-viral defense (Lin & Young, 2014)
IL-10 family	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26	Anti-inflammatory (IL-10), inflammation, anti-microbial (R. Morris et al., 2018)

Abbreviations : CLC, cardiotrophin-like cytokine; CNTF, ciliary neurotrophic growth factor; CT1, cardiotrophin-1; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NK, natural killer; NP, neuropoietin; LEP, leptin; OSM, onkostatin M; PRL, prolactin; TPO, thrombopoietin; TSLP, thymic stromal lymphopoietin.

2.2.2 Janus kinases (JAKs)

The JAK family consists of four members, namely JAK1, JAK2, JAK3, and TYK2, which were discovered in the early 1990s. They were named after Janus, two-faced

roman god of gates, passages, and duality, because of their unusual structure of sequential kinase-like domains (Wilks, 2008). JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas the expression of JAK3 is restricted mainly to cells of hematopoietic origin (Salas et al., 2020). JAKs couple with their designed receptor and work in pairs. JAK1 can form heterodimers with other JAKs and JAK2 forms both homodimers and heterodimers with JAK1 and TYK2 (Winthrop, 2017). A summary of cytokine receptor families, JAK pairs and STATs that are activated by each receptor family is represented in Figure 2.

All JAKs share a similar structure consisting of four distinct domains: An N-terminal FERM (band 4.1 protein, ezrin, radixin and moesin) domain, followed by an SH2 (Src homology 2) domain, a pseudokinase domain (also known as JH2 domain) and a C-terminal kinase domain (also known as JH1 domain). FERM and SH2 domains are responsible for JAK association with cytokine receptors (Ferrao & Lupardus, 2017). Pseudokinase domain is catalytically inactive but plays a critical role in the regulation of the kinase domain, which in turn is required for phosphorylation of its target proteins (R. Morris et al., 2018). Pseudokinase domain seems to act as both positive and negative regulator, but the exact mechanisms are not fully understood (Raivola et al., 2021). The positive regulatory role was originally identified by the discovery of loss-of-function mutation in JAK3 JH2 resulting in SCID (Candotti et al., 1997; M. Chen et al., 2000). Also, domain deletions and mutations in pseudokinase domain of JAK2 and TYK2 have demonstrated impaired cytokine signaling (Saharinen & Silvennoinen, 2002; Yeh et al., 2000). The importance of the negative regulator role of the pseudokinase domain has been highlighted by the discovery of V617F point mutation, located in pseudokinase domain, in human JAK2. V617F mutation results in hyperactivation of JAK2 and is the driver mutation in most polycythemia vera patients as well as in some other myeloproliferative neoplasms (James et al., 2005).

JAK1 and JAK2 are indispensable during development. Homozygous JAK1 knock-out mice die perinatally due to ill-defined neurological defects and mice lacking JAK2 during embryogenesis, because of the failure of definitive erythropoiesis in the fetal liver (Gadina et al., 2001). Unsurprisingly, JAK1 or JAK2 deficient humans have not been reported. As mentioned earlier, the lack of JAK3 function causes SCID, which leads to recurrent infections and death in infancy, unless patients receive stem cell transplantation or enzyme replacement therapy (Aluri et al., 2019). As JAK3 is needed for the signaling of IL-2 family cytokines, patients are missing nearly all T and NK cells, and B cell functions are impaired (Luo et al., 2021). Identification of JAK3-SCID and immunodeficient phenotypes of

various knock-out mice eventually led to the idea that JAK inhibitors could be used as immunomodulatory treatment (Changelian et al., 2003; Liu et al., 2021). TYK2 deficiency has also been reported in humans, but with less serious and more heterogeneous symptoms, including susceptibility to intracellular pathogens and hyper-IgE syndrome (Karjalainen et al., 2020). The diversity of TYK2 deficiency symptoms has been speculated to relate to the type of loss-of-function mutation, as some mutations are probable to suppress all the signaling that goes through TYK2, whereas some mutations seem to suppress only IL-23 signaling (Luo et al., 2021).

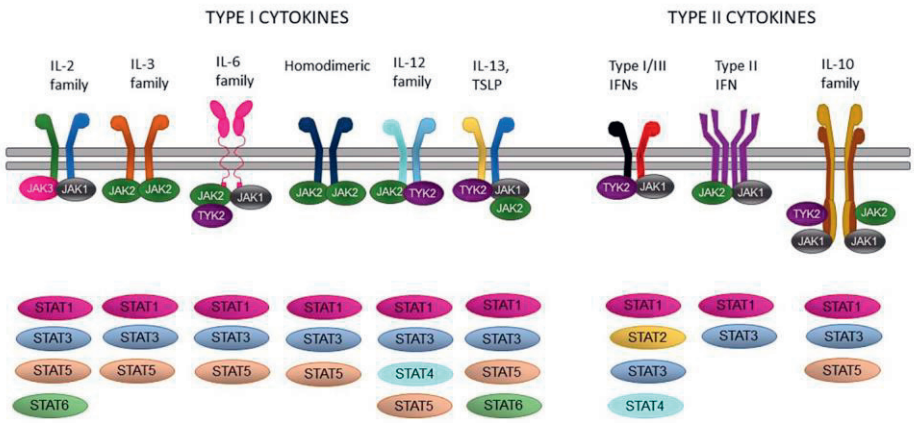


Figure 2. Schematic figure of cytokine receptor families, JAK pairs they use and STATs they activate. STATs can form homo- or heterodimers, depending on the stimulus. Specific cytokines typically have one major STAT they activate, but activation of additional STATs can fine-tune the response. Data presented in the Figure is composed from (Guschin et al., 1995; Kiu & Nicholson, 2012; Leonard et al., 2019; Luo et al., 2021; Nguyen et al., 2002; Pope & Shahrara, 2012; Salas et al., 2020; I. Wang et al., 2004; Wehinger et al., 1996; Zhong et al., 2014).

2.2.3 Signal transducers and activators of transcription (STATs)

Like JAKs, also STAT protein family was discovered somewhat thirty years ago. STAT family consists of seven proteins, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Cytokine signaling and JAK-STAT pathway represent the classic mode of STAT activation, but STATs can also be activated by other proteins, such as pyruvate kinase and fms-related receptor tyrosine kinase 3 (Banerjee et al., 2017).

STAT structure consists of several functional domains, including N-terminal STAT protein interaction domain, coiled-coil domain, DNA binding domain, linker domain, SH2 domain and transactivation domain (Kisseleva et al., 2002). STATs are recruited to the receptor via their SH2 domain. Then, JAKs phosphorylate a specific tyrosine residue on their cytoplasmic tail, which leads to dimerization of STATs (or in some cases, formation of higher-order complexes) through their phosphorylated tyrosine residues and SH2 domain and finally translocation to the nucleus, where STAT-dimers bind to their specific DNA binding elements and activate cytokine-responsive genes (Lim & Cao, 2006). Phosphorylated STAT complexes are retained in the nucleus, until their dephosphorylation by nuclear phosphatases (Mitchell & John, 2005).

STATs can regulate thousands of genes by directly engaging their DNA regulatory elements. The known STAT consensus motifs include gamma-activated sequence, GAS, which was first described in IFN-γ signaling, by the formation of STAT1 dimers (so called gamma-activated factors, GAFs). Later, GAS was noted to mediate also other responses. The other known consensus motif is called interferon-stimulated response element, ISRE, which seems to be involved only in type I/III interferon signaling (Lim & Cao, 2006). However, STATs can also bind DNA sequences that do not contain GAS or ISRE motifs, which can result from degeneration of the recognition code or tethering of other transcription factors (Villarino et al., 2017).

GAS element, $TTCN_{2\text{-}4}GAA$ (canonical GAS element having three nucleotides between palindromes), includes multiple GAS variants, such as prolactin response element (PRE), sis-inducible element (SIE) and acute phase response element (APRE) (Lim & Cao, 2006). Certain STATs bind certain GAS variants better than others, but all STATs are still capable of engaging GAS and can bind to each other's preferred sites (Villarino et al., 2017). However, STAT2 and STAT6 have other preferences over conventional GAS sites: STAT2 participates to type I and III interferon signaling by forming a complex called interferon-stimulated gene factor 3 (ISGF3) with STAT1 and IRF9 and by binding to ISRE, whereas STAT6 prefers to bind GAS element having four spacer nucleotides between palindromes (Platanitis & Decker, 2018; Villarino et al., 2017).

Pictures of cytokine-induced JAK-STAT pathways in textbooks usually describe canonical pathways: certain cytokine activates designated JAK pair, which leads to the activation of specific STAT pair. However, many cytokines, if not all, can activate multiple STATs. For example, type I interferons are the typical activators of STAT1 and STAT2, but can also activate STAT4 (Nguyen et al., 2002). The use of different

"assistant" STATs might partly explain the specificity of cytokine responses (Villarino et al., 2017). For instance, both pro-inflammatory IL-6 and anti-inflammatory IL-10 use STAT3 as their principal signal transducer, having still clearly different downstream effects. In addition to STAT3 activation, IL-6 stimulation leads to STAT1 activation and IL-10 stimulation to STAT1 and STAT5 activation (Guschin et al., 1995; Wehinger et al., 1996).

2.2.4 Interferon regulatory factors (IRFs)

Interferons were the first cytokines that were molecularly characterized and have been thus extensively studied in the context of host defense against viral infection (Taniguchi et al., 2001). Along with STATs, interferon regulatory factors (IRFs) represent important group of transcription factors in the regulation of interferon responses (Platanitis & Decker, 2018).

The IRF family consists of nine members (IRF1-9), which share similar domain structure of N-terminal DNA binding domain along with IRF-association domains (IAD) 1 and 2 in C-terminal region (Mogensen, 2019). IAD domains mediate homodimeric and heterodimeric interactions with other IRF family members, co-regulators and transcription factors (Mogensen, 2019). In the IAD domain of IRF9, but not in other IRFs, there is a STAT-binding site, which allows the ISGF3 formation upon type I/III IFN stimulation (Platanitis & Decker, 2018). The DNA binding domains recognize specific motifs called IRF elements (IRE), which are present within the promoters of *IFN-a* and *IFN-β* as well as in interferon-stimulated genes (ISGs). IREs are shorter versions of ISRE, but not recognized by ISGF3 (Michalska et al., 2018).

IRF1 and IRF2 were the first IRF family members to be recognized and along with IRF9, play a significant role in STAT mediated gene expression in response to interferon stimulation (Taniguchi et al., 2001). *IRF1* is a target gene of STAT1, and its expression is mainly induced by IFN-γ stimulation. Then, IRF1 participates in secondary type I and type II IFN responses by activating the transcription of ISRE-containing genes (Michalska et al., 2018). IRF1 target genes include known antiviral ISGs but also *STAT1*, *STAT2,* and *IRF9* (Michalska et al., 2018). Besides inducing the expression of ISGs and ISGF3 components through binding to ISRE, IRF1 seems to support also IFN-γ-induced function of STAT1, where ISRE is not employed. Even though no direct interaction has been detected between IRF1 and STAT1, IRF1 has been suggested to promote DNA binding of STAT1 dimers to

GAS (Zenke et al., 2018). Also, IFN-γ-induced gene expression of *CIITA*, *GBP2* and *gp91^{phox}* has been shown to be both STAT1 and IRF1 dependent (Kumatori et al., 2002; A. C. Morris et al., 2002; Ramsauer et al., 2007). Taken together, STAT1-IRF1-axis represents a positive feedback loop of interferon response.

IRF2 too is an important transcriptional regulator of interferon response, but with an opposite function of IRF1. IRF2 was first described to be able to bind *IFN-β* promoter region, and thereby outcompete IRF1 and suppress IRF1-mediated gene expression in multiple cell lines (Harada et al., 1989). IRF2 knock-out mice, which develop inflammatory skin disease and exhibit hyper-responsiveness to antigen stimulation and upregulation of type I induced genes, has further supported the suppressive role of IRF2 in interferon signaling (Hida et al., 2000).

2.2.5 IRF2 binding protein 2 (IRF2BP2)

The view of how IRF2 functions as repressor of interferon signaling has been further expanded by the discovery of IRF2 binding proteins, IRF2BP1 and IRF2BP2, which act as co-repressors of IRF2 (Childs & Goodbourn, 2003). However, IRF2BPs were also noted to be expressed in *Caenorhabditis Elegans* and *Drosophila Melanogaster*, which lack the IRF homologs, suggesting a wider role than just IRF2 co-repression (Childs & Goodbourn, 2003). Indeed, to date, especially IRF2BP2 has been shown to act as co-regulator with various other transcription factors as well, including VGLL4, NCOR1, ETO2, IRF2BPL, NFAT1, NRIF3, GR, and KLF2 (Carneiro et al., 2011; Jiang & Shen, 2021; Kim et al., 2019; Lempiäinen et al., 2017; Stadhouders et al., 2015; Teng et al., 2010; Tinnikov et al., 2009; Yeung et al., 2011). In addition to the immune response, IRF2BP2 plays a role in regulating apoptosis, angiogenesis, cell cycle and cell differentiation (Kim et al., 2019; Koeppel et al., 2009; Stadhouders et al., 2015; Teng et al., 2010; Tinnikov et al., 2009; Yeung et al., 2011). The observation that IRF2BP2 knock-out mice are rarely obtained and die before four weeks of age due to severe growth retardation emphasizes the importance of IRF2BP2 (Stadhouders et al., 2015).

IRF2BP2 consist of two conserved regions, the N-terminal zinc finger, which typically bind to specific DNA sequences, and C-terminal RING-domain, which is needed for protein-protein interactions between IRF2BP2 and its co-regulated transcription factors (Childs & Goodbourn, 2003; Laity et al., 2001; Stadhouders et al., 2015; Yeung et al., 2011). The clinical significance of IRF2BP2 in the regulation of immune response has become evident as heterozygous *IRF2BP2* germline

mutations causing inborn errors of immunity have been reported (Article I, Baxter et al., 2021; Keller et al., 2016). In addition, the sequencing of a cohort of 103 patients with common variable immunodeficiency (CVID), found 17 patients to have *IRF2BP2* mutations. However, most patients of these had also other rare mutations in CVID-related genes, so the role of these *IRF2BP2* variants in CVID development remained elusive (Bisgin et al., 2021).

2.2.6 Regulators of JAK-STAT pathway

The activation of JAK-STAT signaling pathway induces rapid signal transduction from the cell membrane to the nucleus followed by highly organized response and then, controlled attenuation and downregulation of the initial signal (R. Morris et al., 2018). Multiple proteins regulate the JAK-STAT pathway in every step of the signaling process. Central regulators include suppressors of cytokine signaling (SOCS) family, protein inhibitor of activated STAT (PIAS) family and protein phosphatases (Shuai & Liu, 2003).

Expression of SOCSs is induced upon cytokine activation of JAK-STAT pathway, creating thus a classic negative feedback system. SOCS family consists of eight members: SOCS1-7 and cytokine-inducible SH2-containing protein (CIS1). Of these, SOCS1-3 and CIS1 are inhibitors of cytokine receptors that use JAK-STAT pathway, whereas SOCS4-7 predominantly inhibit growth factor receptor dependent signaling (Trengove & Ward, 2013). SOCS share a domain structure of a low-conserved N-terminal domain, with varying lengths, a conserved central SH2 domain and a C-terminal SOCS box. SH2 domain is needed for association with target phosphotyrosine residues, which are typically in receptors (R. Morris et al., 2018). Once bound to their targets, SOCSs mediate protein degradation via SOCS box, which recruits elonging B and C along with components of E3 ligase ubiquitinoylation complex, leading eventually target ubiquitinoylation and proteasomal degradation (O'Sullivan et al., 2007). SOCSs can also use other methods to inhibit JAK-STAT signaling. SOCS1 and SOCS3 possess a unique motif in their N-terminal domain, called the kinase inhibitory region, which can directly bind to JAK kinase domain and inhibit its catalytic activity (R. Morris et al., 2018). In addition, SOCS1, SOCS2, and CIS1 can inhibit signaling by binding to phosphorylated tyrosine residues, typically on the receptor, and thereby block the association of STATs (Trengove & Ward, 2013).

PIAS family members are regulators of several different transcription factors, of which STATs happened to be the first to be discovered. PIAS can inhibit the function of transcription factors by multiple mechanisms, which include the induction of SUMOylation (SUMO: small ubiquitin-related modifiers), blocking the DNA-binding ability of transcription factors by binding them, recruiting histone deacetylase and sequestering of transcription factors to distinct subnuclear structures (Shuai & Liu, 2005). Protein phosphatases limit the signaling of JAK-STAT pathway by hydrolyzing phosphorylated tyrosine residues of JAKs, STATs and cytokine receptors. Protein phosphatases involved in JAK-STAT pathway regulation include e.g., two related cytoplasmic phosphatases PTP1b and TC-PTP, the receptor tyrosine phosphatases CD45, PTP-RT, and PTP-RD, the SH2 domain containing phosphatases SHP1 and SHP2 and dual specificity protein phosphatase DUSP2 (R. Morris et al., 2018; Villarino et al., 2017).

2.2.7 JAK-STAT pathway activity in rheumatoid arthritis

Cytokines are major drivers of rheumatoid arthritis and many of the key cytokines in RA, such as IL-6, interferons, and common-γ-chain cytokine family, signal through JAK-STAT pathway (Schwartz et al., 2016; Virtanen et al., 2019). Thus, not surprisingly, certain JAK-STAT pathways have been found to be constitutively active in immune cells isolated from patients with RA.

IL-6 is the prototypic proinflammatory cytokine and several studies suggest that serum IL-6 levels of patients with RA are higher than those of healthy controls (Helal et al., 2012; Hirano et al., 1988; Isomäki et al., 2015; Madhok et al., 1993). However, serum IL-6 levels might depend on the characteristics of the patient cohort, as also comparable IL-6 serum levels to those of healthy controls have been reported (Anderson et al., 2016), and different cytokines are likely to drive different phases of the disease process (McInnes et al., 2016a). Interestingly, in both kinds of situation, patients with RA have been shown to have increased constitutive phosphorylated (p)STAT3 levels in circulating $CD4^+$ T cells, which correlated with serum IL-6 levels, suggesting the hyperactivation of IL-6-STAT3 -axis (Anderson et al., 2016; Isomäki et al., 2015). The significance of constitutive STAT3 activation in RA has also been highlighted by the observation that constitutive pSTAT3 in $CD4^+$ T cells associates with disease activity and good response to csDMARD treatment in recent onset RA (Kuuliala et al., 2015).

Other STATs also seem to be overactive in RA and associated with treatment response. Increased constitutive pSTAT1 and pSTAT5 levels compared to healthy subjects have been measured in circulating T cells of patients with active RA (Isomäki et al., 2015; Pertovaara et al., 2015). Moreover, high IL-4-stimulated STAT6 phosphorylation in monocytes has been found to associate with good treatment response to csDMARDs in early RA, and decrease during successful treatment (Kuuliala et al., 2016). In the same paper, IFN-γ-stimulated pSTAT1 in lymphocytes was found to correlate positively with good treatment response to biological treatment in chronic RA (Kuuliala et al., 2016).

2.3 JAK inhibitors

Years of research that led to understanding of the significance of cytokine signaling in the pathogenesis of autoimmune diseases has revolutionized the treatment of many diseases. At first, cytokine signaling was targeted by biological treatments. Current biological treatments for autoimmune diseases include monoclonal antibodies, receptor antagonists and fusion receptor proteins (Moroncini et al., 2017). Etanercept, soluble TNF receptor fusion protein, was the first biologic treatment approved for rheumatoid arthritis in 1998 (Curtis & Singh, 2011). Since then, several biologics have entered the clinics for various indications, such as for juvenile idiopathic arthritis, ulcerative colitis, psoriatic arthritis, and atopic dermatitis (Curtis & Singh, 2011; Fabbrocini et al., 2018; Moroncini et al., 2017; Puthenpurail et al., 2021).

The potential of JAKs as drug development targets were recognized soon after their discovery. Conversely to biologics, JAK inhibition offers the possibility of targeting multiple cytokines simultaneously, inside the cell and by small molecules. First JAK inhibitor (also known as JAKinib), tofacitinib, which was originally screened against JAK3, but was found to inhibit also other JAKs with varying strenghts (Flanagan et al., 2010), was shown to prevent organ allograft rejection in animal models already about 10 years after JAK family discovery (Changelian et al., 2003; Kudlacz et al., 2004). The fast development of JAK inhibitors is also impressive in that viewpoint that structures of JAK kinase domains, which are targets for most of the current JAK inhibitors, were solved afterward, in the 2000s (Boggon et al., 2005; Chrencik et al., 2010; Lucet et al., 2006; Williams et al., 2009). The discovery of JAK2 V617F mutation as a driver of myeloproliferative conditions in 2005 (James et al., 2005) widened the applications for JAK inhibitors from

immunomodulators to cancer medication. Even though tofacitinib was the first JAK inhibitor entering clinical trials, ruxolitinib, was the first approved JAK inhibitor (O'Shea et al., 2015). Ruxolitinib, JAK1/2 inhibitors, was approved for treatment of myelofibrosis in 2011, tofacitinib receiving approval for treatment of RA in 2012 (Liu et al., 2021).

2.3.1 Clinical JAK inhibitors and their selectivity

Tofacitinib, ruxolitinib, baricitinib and peficitinib inhibit several JAKs simultaneously (Alexander et al., 2021) and are referred here as non-selective JAK inhibitors. Tofacitinib, baricitinib and peficitinib are approved for treatment of RA, tofacitinib having also approved indications for psoriatic arthritis (PsA), juvenile idiopathic arthritis (JIA), ankylosing spondylitis (AS) and ulcerative colitis (UC) and baricitinib for atopic dermatitis (AD) and emergency approval for treatment of Covid-19 (Liu et al., 2021). Ruxolitinib is approved for myelofibrosis, polycythemia vera, steroid refractory-graft-versus-host-disease and AD as topical cream (Alexander et al., 2021). They bind the adenosine triphosphate (ATP)-binding pocket of kinase domain of JAKs. ATP binding pocket of kinase domain between JAK family members is highly conserved and thus finding selective JAK inhibitors is challenging, however, JAK inhibitors in general show still reasonable kinome selectivity (Schwartz et al., 2016).

Non-selective JAK inhibitors have proven to be efficient and to have acceptable safety profile in clinical trials, but adverse events (which will be discussed more in detail later) have fueled the development of more selective JAK inhibitors. Anemia, neutropenia and thrombocytopenia are adverse events that are likely to arise from inhibition of erythropoietin and thrombopoietin, that signal through JAK2-JAK2 pairs (Virtanen et al., 2019). Thus, JAK1 targeted inhibitors have been developed for different inflammatory diseases in the hope of better safety profile. Filgotinib and upadacitinib are approved for treatment of RA, in addition upadacitinib has been approved for PsA, AS, and AD treatment and filgotinib for UC treatment (Alexander et al., 2021). Abrocitinib has been approved for the treatment of AD and itacitinib has been trialed for graft-versus-host disease (GHVD), asthma, bronchiolitis obliterans syndrome and MDS/MPN overlap syndrome (Alexander et al., 2021). Even though these inhibitors show relative selectivity toward JAK1 (Liu et al., 2021), like non-selective JAKinibs, these too target the ATP binding pocket of kinase domain, which means that they are not JAK1 specific.

As JAK3 functions are associated only with common-γ-chain cytokine family receptors and JAK3 is expressed mostly in cells of hematopoietic origin, inhibiting JAK3 offers significantly narrower cytokine inhibition spectrum compared to JAK1 inhibition. In addition to sparing JAK2-JAK2 mediated cytokine signaling, selective JAK3 inhibition could allow also signaling of anti-inflammatory cytokines that use JAK1, such as IL-10 (Verma et al., 2016). Decernotinib and ritlecitinib represent JAK3 targeted inhibitors, both targeting the ATP binding pocket of kinase domain (Farmer et al., 2015; Telliez et al., 2016). Ritlecitinib shows a unique binding mode among current JAKinibs, as it is the first inhibitor that binds covalently to a cysteine residue in position 909, which is specific for JAK3 among JAKs, as other JAKs have a serine residue in this position (Telliez et al., 2016). However, this cysteine residue is not unique structure among the whole kinome and ritlecitinib inhibits kinase activity of five members of the tyrosine kinase expressed in hepatocellular carcinoma (TEC) kinase family, with varying degrees (Telliez et al., 2016). Both decernotinib and ritlecitinib have been tested in clinical trials for the treatment of RA (Genovese et al., 2016; Robinson et al., 2020), but ritlecitinib is currently in phase III development for only alopecia areata (AA) (National library of Medicine (U.S.), https://clinicaltrials.gov). The development of decernotinib for the treatment of RA has been discontinued (Westhovens, 2019).

TYK2 is used by a relatively small number of cytokines, including IL-12, IL-23, and type I and II interferons, which play a central role in development of several autoimmune diseases (Virtanen et al., 2019). In addition, genome-wide association studies have indicated that some loss-of-function TYK2 mutations protect against autoimmunity, making TYK2 a tempting drug target (Dendrou et al., 2016; Enerbäck et al., 2018). Brepocitinib binds both JAK1 and TYK2 and has shown efficacy in treating AA in phase II trials, but trials for treating other autoimmune conditions are ongoing as well (King et al., 2021; Nogueira et al., 2020). Cerdulatinib is a dual kinase inhibitor, targeting JAK family members, (showing some selectivity toward TYK2) and spleen tyrosine kinase SYK (Coffey et al., 2014). Cerdulatinib has shown efficacy in treating lymphomas in phase II trials (Horwitz et al., 2019). Deucravacitinib is a TYK2 targeted inhibitor having a distinct binding mode, as it binds the pseudokinase domain (Wrobleski et al., 2019). Deucravacitinib binds also pseudokinase domain of JAK1, but shows markedly lower inhibition of IL-2- and IL-6-induced signaling than IFN-α-induced signaling (Wrobleski et al., 2019). Deucravacitinib has shown efficacy in treating psoriasis in phase II trials (Papp et al., 2018) and is currently evaluated in clinical trials for treatment of other inflammatory diseases as well (National library of Medicine (U.S.), https://clinicaltrials.gov).

JAK2 targeted inhibitors have been developed mainly for the treatment of MPN and other hematological malignancies. These inhibitors include fedratinib, pacritinib, momelotinib, lestaurtinib, ilginatinib, AT9283 and gandotinib, of which fedratinib has been approved for the treatment of myelofibrosis (Alexander et al., 2021; Berdeja et al., 2018; Bose & Verstovsek, 2017; Dawson et al., 2010; Mascarenhas et al., 2019). Of JAK2 targeted inhibitors, gandotinib has dose-dependent selectivity for the JAK2 V617F mutation (Berdeja et al., 2018). Clinical JAK inhibitors that have been approved or proceeded to Phase III clinical development along with their targeted conditions are summarized in Table 3.

2.3.2 Safety of JAK inhibitors

Approved JAKinibs have acceptable safety profiles in their designated indications. However, given that knocking out *JAK1* and *JAK2* is lethal, *JAK3* deficiency causes SCID and *TYK2* deficiency serious infections, it was not at all obvious at first. Infections do belong to common side effects of JAK inhibitors, and also serious infections including tuberculosis and opportunistic infections occur (Hu et al., 2021). However, in general, the incidence rate of infections seems similar to patients using TNF blockers or other biologics (Gadina et al., 2020). Herpes zoster is an exception, as it is more common in patients using JAKinibs (Nash et al., 2021; Olivera et al., 2020).

Cytopenias, such as neutropenia and anemia belong to common adverse effects of JAKinibs, probably due to JAK2 inhibition (Virtanen et al., 2019). Interestingly, in patients with RA, upadacitinib, JAK1 selective inhibitor, shows a similar effect on anemia as do non-selective and JAK2 selective inhibitors (Liu et al., 2021; Patel & Odenike, 2020). Another JAK1 selective inhibitor for treatment of RA, filgotinib, seems to produce less anemia (Liu et al., 2021). Other changes include decreased numbers of neutrophils, lymphocytes, NK cells and platelets (Hu et al., 2021; Liu et al., 2021).

JAK inhibitors alter cholesterol metabolism and increase both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol. The mechanism has been studied in patients with RA using tofacitinib and suggest that JAK inhibition decreases cholesterol ester catabolism (Charles-Schoeman et al., 2015). Cholesterol increment might even be beneficial, as despite the increased cardiovascular mortality and morbidity patients with RA have, decreased LDL and total cholesterol levels have been associated with active RA (Boers et al., 2003;

Maradit-Kremers et al., 2005). The use of tofacitinib increased cholesterol levels of patients with RA toward those of healthy controls and improved the markers of antiatherogenic HDL function (Charles-Schoeman et al., 2015).

Cardiovascular safety and malignancies have been major subjects of interest concerning the safety of JAKinibs. The United States Food and Drug Administration (FDA) requested a long-term safety study post-approval of tofacitinib in 2012, evaluating the safety of two doses of tofacitinib (5 and 10 mg) twice daily versus TNF inhibitors on patients with RA using methotrexate as a background medication, non-inferiority to TNF inhibitors regarding major adverse cardiovascular events (MACE) and malignancies being the co-primary endpoints (known as ORAL surveillance study). The study population consisted of patients of 50 years of age and older, who had at least one additional cardiovascular risk factor. Low incidence rates for MACE and malignancies were seen with both medications (Ytterberg et al., 2022). However, the incidences for MACE and malignancies were higher with the combined tofacitinib doses than with a TNF inhibitor and non-inferiority was not met in either endpoint (Ytterberg et al., 2022). Whether TNF inhibitors have similar effect over other JAK inhibitors in these events remains to be elucidated. Increased risk for MACE or malignancies has not been found for tofacitinib, baricitinib, upadacitinib or filgotinib compared to placebo or active comparator in meta-analysis of 36 studies including RA, UC, Chron's diseases, AS and psoriasis patients (Olivera et al., 2020).

Another safety concern of special interest includes pulmonary embolism (PE) and venous and arterial thrombosis (VTE). These concerns were raised during the phase III development program of baricitinib for treatment of RA, as VTEs occurred in 4 mg group, but not in the 2 mg or placebo groups (Liu et al., 2021). In addition, preliminary results of ORAL surveillance showed 5-fold increased risk in patients in tofacitinib 10 mg group for developing PE compared to TNF inhibitor arm, resulting in a dose reduction to 5 mg (European Medicines Agency. Increased risk of blood clots in lungs and death with higher dose of Xeljanz (tofacitinib) for rheumatoid arthritis. 2019, Ytterberg et al., 2022). However, later long-term studies with baricitinib have shown that the incidence rate of PE and VTE is similar among dose groups (Taylor et al., 2019). Other reports have suggested that there would not be evidence of short-term risk of VTEs in patients with RA starting JAKinibs (Liu et al., 2021). The mechanism that would explain increased risk of VTEs in patients using JAKinibs is currently unclear. One theory is, that JAK2 is needed for suppressing the actions of circulating TPO. JAK2 inhibition would result in the

expansion of megakaryocytes, which are responsible for platelet production, leading to thrombocytosis (Skoda, 2014).

Table 3. Summary of clinical JAK inhibitors that have been approved or proceeded to Phase III clinical development along with their targeted conditions. Letter A in brackets, (A), refers that inhibitor has been approved for specified indication. Data in the Table has been compiled from National library of Medicine (U.S.) (https://clinicaltrials.gov, accessed 1.4.2022), Food and Drug Administration (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&ApplNo=213871, accessed 1.4.2022), European Medicines Agency (https://www.ema.europa.eu/en/medicines/human/EPAR/cibinqo, accessed 1.4.2022) and (Alexander et al., 2021).

Condition	Non-selective	JAK1 targeted	JAK2 targeted	JAK3 targeted	TYK2 targeted
Inflammatory diseases					
Rheumatoid arthritis	Tofacitinib (A), Baricitinib (A[1]), Peficitinib (A[2]), Ruxolitinib,	Upadacitinib (A), Filgotinib (A)		Decernotinib	
Psoriatic arthritis	Tofacitinib (A)	Upadacitinib (A), Filgotinib			Deucravacitinib
Juvenile idiopathic arthritis	Tofacitinib (A), Baricitinib				
Uveitis	Baricitinib				
Systemic lupus erythematosus	Tofacitinib, Baricitinib	Upadacitinib			
Ankylosing spondylitis	Tofacitinib (A)	Upadacitinib (A[6]), Filgotinib			
Axial Spondylarthritis	Tofacitinib	Upadacitinib			
Giant cell arteritis		Upadacitinib			
Psoriasis	Tofacitinib, Baricitinib				Deucravacitinib
Atopic dermatitis	Baricitinib (A[3]), Ruxolitinib (A[4])	Upadacitinib (A), Abrocitinib (A)			

Alopecia areata	Baricitinib			Ritlecitinib	
Vitiligo	Ruxolitinib				Cerdulatinib
Hidradenitis suppurativa		Upadacitinib			
Dermatomyositis	Baricitinib				
Hand Eczema	Ruxolitinib				
Ulcerative colitis	Tofacitinib (A)	Filgotinib (A), Upadacitinib			
Crohn's disease	Tofacitinib	Filgotinib, Upadacitinib			
Takayasu's arteritis	Tofacitinib	Upadacitinib			
Nakajo-Nishimura Syndrome, Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome, STING-associated vasculopathy with onset in infancy, Aicardi Goutiers syndrome	Baricitinib				
Graft-versus-host disease	Ruxolitinib (A), Baricitinib	Itacitinib			
Macrophage activation syndrome	Ruxolitinib				
Hemophagocytic lymphohistiocytosis	Ruxolitinib				
Cancers					
Myelofibrosis	Ruxolitinib (A)		Fedratinib (A), Pacritinib, Momelotinib		
Polycythemia vera	Ruxolitinib (A)				
Acute lymphoblastic leukemia	Ruxolitinib		Lestaurtinib		

Metastatic pancreatic ductal adenocarcinoma			Momelotinib		
Peripheral T-cell lymphoma					Cerdulatinib
Pancreatic cancer	Ruxolitinib				
Other diseases					
Covid-19	Baricitinib (A[5]), Ruxolitinib, Tofacitinib		Pacritinib		

[1]2 mg and 4 mg doses approved by the European Medicines Agency (EMA) and 4 mg dose approved by the Food and Drug Administration (FDA). [2]Approved in South Korea and Japan only. [3]Approved by EMA, not FDA. [4]Topical treatment approved by FDA. [5]FDA emergency use authorization. [6]Approved by EMA, not FDA.

3 AIMS OF THE STUDY

Abnormalities in JAK-STAT signaling can arise from the immune system failing in self-recognition leading to autoimmune diseases or germline mutations in JAKs, STATs or their regulatory proteins. Recognition of novel patient mutations can reveal unexamined regulatory mechanisms and perhaps offer clues of effective therapies for patients with rare conditions.

JAKs have proven to be valid drug targets and clinical trials have demonstrated that JAK inhibition improves the symptoms of patients suffering from variety of inflammatory diseases and hematological cancers. JAK inhibitor research is fast-developing and competitive field, but the *in vivo* mechanisms in patients are not well-defined, even though they could add to our understanding about mechanisms behind efficacy and side-effects. Selectivity is one of the major subjects of interest in JAK inhibitor development. To understand the differences between ever-growing number of JAK inhibitors, they need to be examined together.

Specific aims of the current study were

1. Elucidation of the role of IRF2BP2 in JAK-STAT pathway regulation
    - Description of a novel *IRF2BP2* variant found in two family members with inflammatory conditions and lymphopenia
    - Studying the regulatory role of IRF2BP2 in interferon-induced response
2. Studying the *in vivo* mechanisms of JAK inhibitor tofacitinib in rheumatoid arthritis patients
    - Characterization of the effects of tofacitinib to various JAK-STAT signaling pathways in immune cell subtypes
3. Comparison of the selectivity of 20 clinically tested JAK inhibitors
    - Head-to-head comparison of JAK inhibitor selectivity by using several assays in combination with pharmacological parameters

4 PATIENTS AND METHODS

More detailed methodological information can be found in the online versions of the original publications, which are referred to by Roman numerals (I-III).

4.1 Human subjects

4.1.1 Patients carrying *IRF2BP2* mutation (I)

A family of two individuals (57 years old index male and his 72 years old sister) with inflammatory diseases and lymphopenia carrying a novel heterozygous mutation in *IRF2BP2* gene were recruited to the study. In addition, three healthy male subjects (54, 56 and 58 years old) and one healthy female subject (72 years old) were recruited as controls. Blood samples were drawn from the patients and healthy controls.

4.1.2 Rheumatoid arthritis patients starting tofacitinib treatment (II)

Patients with active rheumatoid arthritis (according to the 2010 ACR/EULAR classification criteria), were recruited to the study. Patient recruitment was performed at two rheumatology outpatient clinics, Tampere, and Helsinki University Hospitals. Suitable patients had active disease at screening/baseline visit: Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]) was >3.2 despite treatment with methotrexate and/or other csDMARDs. Key exclusion criteria were current infection, severe hepatic impairment, malignancy, pregnancy or lactation, lymphocyte count <0.75 x 10^9/l, neutrophil count <1.0 × 10^9/l, hemoglobin <90 mg/dl, or former treatment with biologics or JAK inhibitor.

The study included 2-3 visits: screening visit (0-3 months before baseline visit; could be combined with baseline visit), baseline visit (0 month) and follow-up visit (3 months). Tofacitinib 5 mg twice a day, was started at the baseline visit and

continued throughout the study. Patients continued their background prednisolone and csDMARD therapy.

The following clinical assessments and patient-reported outcomes were recorded at study visits: DAS28-4[CRP], physician's assessment VAS (0-100 mm), the number of swollen and tender joints (46 joint count), disability index (0-3), health assessment questionnaire (HAQ), patient general health visual analogue scale (VAS) (0-100 mm) and pain VAS (0-100 mm).

Blood samples were drawn at baseline and follow-up visits. At the follow-up visit, blood samples were taken 1-2 hours after the morning tofacitinib dose. Schematic overview of the study is presented in Figure 3.

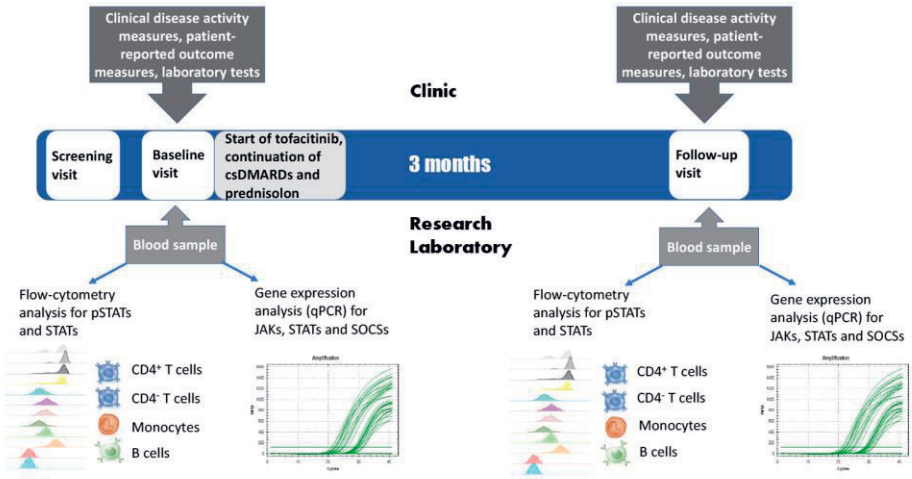


Figure 3. Schematic overview of the study. Blood samples were collected and clinical assessments and patient-reported outcomes were recorded at baseline visit and follow-up visit. Modified from Article II.

4.1.3 Healthy volunteers (III)

Blood samples for *in vitro* JAK inhibitor experiments were collected from three healthy volunteers.

4.1.4 Ethical considerations

Informed consent was obtained from all subjects involved in this study and the study was conducted according to the guidelines of Good Clinical Practice and the Declaration of Helsinki. The studies done involving human participants in Article I were approved by the Coordinating Ethics Committee of Hospital District of Helsinki and Uusimaa. The studies involving human participants in Article II were reviewed and approved by National Committee on Medical Research Ethics (TUKIJA) and Finnish Medicines Agency Fimea.

4.2 Whole blood cytokine signaling assay

4.2.1 Cytokine stimulation and flow cytometry for patient samples (I, II)

The activation of various JAK-STAT signaling pathways in circulating leukocytes of the patients was studied by measuring STAT phosphorylation in five-color flow cytometry. Also, the amount of total STAT1 and STAT3 was measured.

First, 50 µl (I) or 100 µl (II) aliquots of fresh blood samples were either left unstimulated or stimulated by 100 ng/ml recombinant cytokines, namely IL-2, IL-4, IL-6, IL-7, IL-15, IL-21, IFN-β, IFN-γ (all from Peprotech Rocky Hill, NJ, USA), IL-10 (R&D Systems, Minneapolis, MN, USA) or IFN-α (Cell Signaling Technology, Danver, MA, USA) for 15 minutes at 37°C. The stimulations were ended by transferring the samples on ice. Then, leukocytes were fixed, and erythrocytes were lysed with BD Phosflow Lyse/Fix buffer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), according to manufacturer's instructions. Cells were then washed with PBS and permeabilized in ice-cold methanol for 10 min on ice followed by 2-28 days preservation in methanol at -80°C. In preliminary experiments, STAT phosphorylation levels were determined to remain unchanged for at least four weeks at -80°C.

After methanol permeabilization and preservation, cells were washed twice with FACS buffer (PBS supplemented with 0,1% bovine serum albumin and 0,01% sodium azide) and stained with fluorochrome-conjugated antibodies, namely phycoerythrin-cyanin 7 (PE-Cy7)-conjugated anti-CD20 (clone H1(FB1)), allophycocyanin (APC)-conjugated anti-CD33, (FITC)-conjugated anti-CD3 (clone SK7) (BD), (APC-eFluor 780-conjugated anti-CD4) (clone SK3) (eBioscience, Santa

Clara, CA, USA) and phycoerythrin (PE)-conjugated STAT1 (clone 1/Stat1) (BD), phospho-STAT1 (clone 4a), STAT3 (clone M59-50) (BD), phospho-STAT3 (clone 4/P-STAT3) (BD), phospho-STAT4 (clone 38/p-Stat4) (BD), phospho-STAT5 (clone 47) (BD) or phospho-STAT6 (18/P-Stat6) (BD), at room temperature for 30 minutes, protected from light. After antibody staining, cells were washed twice with FACS buffer before fluorescence measurement.

Data collection was performed using FACS Canto II (BD) and the analysis of flow cytometer data using FlowJo Single cell analysis software (BD). Monocytes were gated based on light scattering properties (SSC-A and FSC-A) and $CD33^+$ positivity. Lymphocytes were gated first on light scattering properties (SSC-A and FSC-A) and among lymphocytes, $CD20^+$ B cells were gated from the $CD3^-$ population and $CD4^+$ and $CD4^-$ T cells from the $CD3^+$ population. PE fluorescence histograms were created for each cell population, and the median fluorescence intensity (MFI, arithmetic median) was calculated.

4.2.2 Cytokine stimulations, JAK inhibition and flow cytometry for *in vitro* selectivity experiments (III)

JAK inhibitor potency *in vitro* on selected JAK-STAT pathways was studied using barcoded flow cytometry assay (Krutzik et al., 2011).

20 μl of blood aliquots (from healthy volunteers) were incubated with JAK inhibitors (six concentrations ranging 0.1 nM-10 μM) in 96 deep well plates at 37° C for 60 min. Then, blood aliquots were stimulated with recombinant cytokines, namely IL-2 (100 ng/ml), IL-6 (100 ng/ml), GM-CSF (2 ng/ml), IFN-α (100 ng/ml) or IFN-γ (100 ng/ml) at 37° C for 15 min. Cytokine concentrations that elicit maximal pSTAT stimulation were used.

Leukocytes were fixed and erythrocytes were lysed using BD Biosciences lyse/fix buffer, according to manufacturer's instructions. After two washed with PBS, cells were permeabilized with cold methanol and stored at -80° C.

Samples were fluorescent barcoded in set of 18 samples by using different combinations of amounts 0/0.15/0.45/1.35/3/7.5 μg/ml of Pacific Blue and Pacific Orange NHS esters. After barcoding, samples that were intended to be stained with the same surface and intracellular fluorochrome-conjugated antibodies, could be combined. Cells were stained with CD33 (PE-Cy7; BD 333952), CD4 (APC-eFluor780 conjugate; eBioscience 47-0047-42), CD3 (PerCP-Cy5.5 conjugate; BD Cat# 332771), STAT1 pY701 (AlexaFluor488 conjugate, BD 612596), STAT3

pY705 (PE conjugate, BD 612569), and STAT5 pY694 (AlexaFluor647 conjugate, BD 612599) at room temperature for 30 min, protected from light. Next, cells were washed twice with FACS buffer and data were collected using FACS Aria Fusion flow cytometer (BD).

PE or AlexaFluor488 (depending on pSTAT) fluorescence histograms were created for cell population studied in each signaling pathway, and the median fluorescence intensity (MFI, arithmetic median) was calculated. The inhibition was calculated if cytokines could induce 50% or higher signal increase in pSTAT. IC50 values were calculated by fitting pSTAT-channel median fluorescence intensity against log inhibitor concentration. Plasma protein binding of the inhibitors were considered by calculating unbound IC50 values (IC50u), if unbound fraction (f_u) data were available:

$$IC50_u = IC50 * f_u$$

Unbound fraction values were as follows: ruxolitinib 0.03 (EMA, 2011), tofacitinib 0.61(EMA, 2017c), baricitinib 0.5 (EMA, 2017b), peficitinib 0.23 (Cao et al., 2016), filgotinib 0.45 (EMA, 2020a), upadacitinib 0.54 (EMA, 2020b), abrocitinib 0.36 (E. Q. Wang et al., 2021), itacitinib 0.26 (Barbour et al., 2021), fedratinib 0.09 (EMA, 2021), pacritinib 0.03 (EMA, 2017a), momelotinib 0.19 (Zheng et al., 2018).

The percent level of inhibition (ICxx) for JAKinibs at clinically relevant dose(s) in rheumatoid arthritis patients were calculated as follows:

$$ICxx = 100 * Cav/(IC50 + Cav)$$

where C_{av} is average plasma concentration of drug at selected dose in a steady state. Either the previously reported value for C_{av} was used or C_{av} was calculated by dividing reported area under curve (AUC) at steady state by dosing interval (Banfield et al., 2018; Center for drug evaluation and research, 2009; X. Chen et al., 2013; Chimalakonda et al., 2021; EMA, 2017c; Gerds et al., 2020; J. L. Marshall et al., 2005; Mohamed et al., 2019; Pardanani et al., 2013; Peeva et al., 2018; Robinson et al., 2020; Schroeder et al., 2020; Toyoshima et al., 2021; Vanhoutte et al., 2017; Verstovsek et al., 2017). All data presented are average of three individual experiments.

4.3 Genetic studies (I, II)

4.3.1 Whole exome testing (I)

Both family members in Article I were subjected to whole exome test targeting all protein coding exons, exon-intron boundaries (±20 base pairs) and selected clinically relevant non-coding variants. Blueprint Genetics executed the sequencing and data analysis using the Illumina sequencing system. Briefly, quality-controlled sequence reads of each sample were mapped to the human reference genome (GRCh37/hg19), following reads alignment using Burrows-Wheeler Aligner (BWA-MEM). Alignment post-processing and variant calling was conducted using GATK algorithms (Sentieon).

The pathogenic potential of variants was estimated by considering the biochemical properties of the codon change, predicted consequence, the degree of evolutionary conservation and allelic frequencies from large population studies (1000 Genomes project, gnomAD) and mutation databases (HGMD, ClinVar) as well as an in-house variant database.

4.3.2 mRNA expression analysis (I, II)

4.3.2.1 Peripheral blood mononuclear cell isolation and RNA extraction (I, II)

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and healthy controls in Article I and rheumatoid arthritis patients in Article II. Isolation was performed either by using BD Vacutainer® CPT™ Mononuclear cell preparation tubes (I) or by using Histopaque 1077 medium (II) (Sigma Aldrich, St. Louis, MO, USA) and density gradient centrifugation. Then, cells were washed two times with PBS supplemented with 2 mM EDTA. Cell pellets were snap frozen and stored at -80°C until RNA was extracted. Total RNAs were isolated from PBMCs using the Rneasy Mini-Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions.

4.3.2.2 Direct digital detection of mRNA (I)

Direct digital detection of mRNA by using NanoString technology was performed at the Molecular Systems Biology group, led by Professor Markku Varjosalo, Institute of Biotechnology, University of Helsinki. A custom gene panel (50 genes) including genes coding components of JAK-STAT and NFκB pathway, cytokines, interferon-inducible proteins, and inflammasome activation was studied.

Shortly, 100 ng of total RNA per sample was incubated with 5' reporter probes, tagged with fluorescent barcodes of targeted genes, and 3' biotinylated capture probes. The samples were hybridized overnight at 65 °C according to the manufacturer's protocol. Next, the reactions were purified and immobilized on the sample cartridge surface. The cartridge was scanned in triplicate using the nCounter Digital Analyzer (NanoString Technologies). Gene expression data were analyzed using nSolver™ 4.0 analysis software (Nanostring Technologies) and normalized gene counts from three replicates were used to calculate the fold changes between the patients and controls. Index patient data was compared to data from a 56-year-old healthy male subject and sister data to data from a 72-year-old healthy female subject. Genes, which fold changes exceeded the limit of detection, are presented in Results.

4.3.2.3 Quantitative PCR (II)

Total RNA (0.5 μg) was reverse-transcribed into cDNA by using M-MLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. 10 μl quantitative PCR (qPCR) reactions were done using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), according to the manufacturer's protocol. Primer sequences JAK-STAT pathway genes being studied (STAT1, STAT3, STAT4, STAT5A, STAT5B, STAT6, JAK1, JAK2, JAK3, TYK2, SOCS1, SOCS2, SOCS3, CIS1) and housekeeping gene (β-actin) are listed in Table 4. Quantitative PCR analyses were conducted with CFX384 (Bio-Rad Laboratories, Hercules, CA, USA) and gene expression was quantified using the delta C(T) method by normalizing to the expression of β-actin.

Table 4. Primer sequences for JAK-STAT pathway genes and housekeeping gene studied.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
STAT1	TCACATTCACATGGGTGGAG	CAAAGGCATGGTCTTTGTCA
STAT3	TCACATGCCACTTTGGTGTT	GCAATCTCCATTGGCTTCTC
STAT4	GGCAATTGGAGAAACTAGAGG	AGGGTGGGTTGGCATACAT
STAT5A	GCCAGATGCAGGTGCTGTA	GGGATTGTCCAAGTCAATGG
STAT5B	GCGTTATATGGCCAGCATTT	CTGGTGCTCTGCCTTCTTCT
STAT6	GGAAGGGCACTGAGTCTGTC	GGCTTTGGCATTGTTGTCTT
JAK1	CATGGTGGAAGAGTTTGTGGA	CAGCTGTTTGGCAACTTTGAATT
JAK2	CCGCCGGGTTTCAGAAG	GAAGAGGTGGATGTTCCCTCC
JAK3	AGTGGGACTTTCCTCTCGC	CTCTTCACTTGGAGGTGCCAT
TYK2	CCCATGGCTTGGAAGATGGT	ACTCAGCTTGATGAAGGGGC
SOCS1	CTGGGATGCCGTGTTATTTT	TAGGAGGTGCGAGTTCAGGT
SOCS2	CAGGGAATGGCAGAGACACT	TGGCAGAGAGAGAAGGGATG
SOCS3	GCCACCTACTGAACCCTCCT	ACGGTCTTCCGACAGAGATG
CIS1	AGCCCAGACAGAGAGTGAGC	TGACAGCGTGAACAGGTAGC
β-actin	TGGGACGACATGGAGAAAAT	AGAGGCGTACAGGGATAGCA

Abbreviations: CIS1, cytokine-inducible SH2-containing protein; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling.

4.4 Transcriptional activity measurements (I)

4.4.1 Plasmid constructs and mutagenesis

IRF2BP2 expression plasmid (IRF2BP2-pDEST-C1-FLAG-GFP11-GW), was a gift from Professor Jorma Palvimo from University of Eastern Finland (Manjur et al., 2019). IRF2BP2 patient mutation plasmid (IRF2BP2 Δ625-665) was created using QuikChange (AgilentTechnologies, Santa Clara, CA, USA) site-directed mutagenesis, following the manufacturer's protocol, and verified by Sanger sequencing. The backbone expression vector was used as a control in both reporter assay and immunoblotting experiments.

For a reporter assay (for studying transcriptional activation of STAT1), interferon-stimulated response element-Luc plasmid (ISRE-Luc) (Bluyssen & Levy, 1997) was used together with a constitutively expressing renilla luciferase plasmid.

4.4.2 Mammalian cell culture

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco modified Eagle medium (Lonza, Basel, Switzerland), which was supplemented with 10% FBS (Sigma, St Louis, MO, USA), 2 mmol/L L-glutamine (Lonza), and 0.5% penicillin/streptomycin (Lonza). For dual luciferase assay 120 000 cells/well and for immunoblotting experiments 100 000 cells/well, were seeded on 12-well plates. Transient transfections (24 to 48 hours) were done using FuGENE HD (Promega) following the manufacturer's instructions.

4.4.3 Dual luciferase assay

Ten nanograms of IRF2BP2 wild-type, IRF2BP2 Δ625-665 or empty backbone plasmid was co-transfected with 220 ng of ISRE-Luc and 60 ng of renilla luciferase plasmids. After 24 hours of transfection, cells were detached using trypsin-EDTA mix and re-plated to a 96-well plate. After 3-4 hours, when the cells had attached to bottom, medium was changed to either starvation medium (culture medium without FBS) or stimulation medium (starvation medium supplemented either with 100 ng/ml IFNα or 10 ng/ml IFNβ). Luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega) after 20 hours of starvation/stimulation using PerkinElmer Envision plate reader. Three independent experiments were performed, and each experiment included three technical replicates.

4.4.4 Immunoblotting

100 ng of IRF2BP2 wild-type, IRF2BP2 Δ625-665 or empty backbone plasmid was transfected to HEK293T cells. Following 48 hours of transfection, cell lysates were prepared for immunoblotting. First, cells were transferred on ice and washed with cold phosphate buffered saline (PBS). Triton X-100 lysis buffer supplemented with protease and phosphatase inhibitors (1 mM phenylmethanesulfonyl fluoride, 8.3 μg/mL aprotinin, 2 mM vanadate, and 4.2 μg/mL pepstatin) was used to lyse the cells. Then, whole cell lysates were centrifuged for 20 min at 16,000 g and total protein amount was determined from the supernatants by using Bradford assay (Biorad). Equal amount of total protein (30 μg) of each sample was loaded on 4–15% Mini-PROTEAN® TGX™ Precast Gels (BioRad, Irvine, CA, USA). Bovin serum albumin (BSA) was used for blocking of the immunoblots. Then,

immunoblots were incubated with primary antibodies for FLAG Tag (1:1000, F1804, clone M2, Merck, Kenilworth, NJ, USA) or actin (1:2000, MAB1501, Merc), which was used as a loading control, and with a mixture of goat anti-rabbit and goat anti-mouse DyLight secondary antibodies (both from Thermo Fisher Scientific, Waltham, MA, USA). Blots were then scanned with an Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA).

4.5 JAK inhibitors (III)

20 JAK inhibitors that have a clinical approval or have been studied in clinical trials Phase 2 or further were selected for the study. Inhibitors investigated in this study, their target JAKs and other targets are listed in Table 5.

Table 5. Clinical JAK inhibitors, their target JAKs and their other target proteins investigated in this study.

Inhibitor	Target JAKs	Other targets
Non-selective		
Ruxolitinib	JAK1, JAK2	
Tofacitinib	JAK1, JAK3, JAK2	
Baricitinib	JAK1, JAK2	
Peficitinib	pan-JAK	
JAK1 selective		
Filgotinib	JAK1	
Upadacitinib	JAK1	
Abrocitinib	JAK1	
Itacitinib	JAK1	
JAK2 selective		
Fedratinib	JAK2	BRD4, FLT3
Pacritinib	JAK2	IRAK1
Momelotinib	JAK2	
Lestauritinib	JAK2	ACVR1
Ilgatinib	JAK2	
AT9283	JAK2	AurA, AurB, FLT3, Abl
Gandotinib	JAK2 V617F	
JAK3 selective		
Ritlecitinib	JAK3	TXK, TEC, BMX, BTK
Decernotib	JAK3	
TYK2 selective		
Cerdulatinib	TYK2	SYK
Deucravacitinib	TYK2	
Brepocitinib	TYK2, JAK1	

Abbreviations: Abl, tyrosine-protein kinase ABL1; ACVR1, activin A receptor type 1; Aur, aurora kinase; BMX, Cytoplasmic tyrosine-protein kinase BMX; BTK, Tyrosine-protein kinase BTK; BRD4, Bromodomain-containing protein 4; FLT3, Fms-like tyrosine kinase 3; IRAK1, interleukin-1 receptor associated kinase 1; SYK, Tyrosine-protein kinase SYK; TEC, Tyrosine-protein kinase Tec; TXK, Tyrosine-protein kinase TXK.

4.6 Analysis of recombinant proteins (III)

4.6.1 Protein production and purification

Recombinant human proteins JAK1 JH1 (864-1155-His), JAK1 JH2 (553-836-His), JAK1 JH2-JH1 (553-1155-His), JAK2 JH1 (836-1132-His), JAK2 JH2 (503-827-

His), JAK2 JH2-JH1 (513-1132-His), JAK3 JH1 (811-1124-His), JAK3 JH2 (511-790-His), JAK3 JH2-JH1 (511-1124-His), TYK2 JH1 (886-1188-His), TYK2 JH2 (564-876-His), and TYK2 JH2-JH1 (564-1188-His) were first cloned into pFASTBAC1 vector (Invitrogen) and then expressed in *Spodoptera frugiperda* (Sf9) or High Five™ insect cells. Cells were infected with 5-10% P3 virus for 48-72 h and collected by centrifugation.

For protein purification, cell pellets were first resuspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% v/v glycerol and 20 mM imidazole). The lysis buffer was supplemented with protease inhibitors; sodium vanadate (10 μl/ml), phenylmethanesulfonyl fluoride (10 μl/ml), pepstatin A (1 μl/ml) and leupeptin (1 μl/ml). Cell lysis was executed by freeze-thawing, cell lysates clarified by centrifugation and recombinant proteins purified using Ni-NTA agarose (Qiagen). Purification was continued using size exclusion chromatography in a HiLoad 16/600 Superdex 75 pg column (GE Healthcare).

4.6.2 Kinase activity assay

JAK inhibitor potency to inhibit kinase activity of recombinant JAK JH2-JH1 proteins was assessed using LANCE Ultra kinase assay (PerkinElmer) in 384-plate format at reaction volume of 10 μl. Reaction conditions were as recommended by the manufacturer and consisted of 50 nM ULight-JAK-1 tyrosine kinase substrate, 2 nM Europium-labeled anti-phospho antibody, 1 mM ATP, JAK JH2-JH1 protein, and inhibitors at concentration range of 0.01 nM-100 μM in kinase buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl2, 0.5 mM TCEP, 0.01% Brij-35, 1 mM EGTA and 0.05% BSA). JAK protein concentration was selected based on protein titration to yield linear signal over 30 min and consistent signal to background ratio (S/B=1.5 at 30 min): 5 nM JAK1, 0.5 nM JAK2, 1 nM JAK3, or 5 nM TYK2. Phosphorylation of tyrosine kinase substrate was detected by measuring fluorescent resonance energy transfer (FRET; 320 nM excitation and 665 nM emission filters) in 5-minute interval for 30 minutes using EnVision Multilabel Plate Reader (PerkinElmer). IC50 values were obtained by fitting the slope of signal increase in function of time against the inhibitor concentration using GraphPad Prism. Fold-IC50 values were calculated for each inhibitor by dividing IC50 for each JAK by IC50 of the strongest inhibited JAK activity. Reactions were conducted in triplicate and data presented is a representative of 3 individual experiments.

4.7 Statistical analysis

In Article I, Student's two-tailed t-test was used to calculate significant differences between two groups in transcriptional activity experiments. Statistical analyses were performed using the measured values for unstimulated control plasmid as a normalizer for each independent experiment. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

In Article II, Wilcoxon signed rank test was used to study the statistical difference between matched samples, namely values measured at study entry and after 3 months. The correlation between variables was determined by Spearman rank correlation. No adjustment was made for multiple testing and p-values equal or less than 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS version 25 (IBM, Armonk, NY, USA), Stata version 15 (StataCorp LLC, College Station, TX, USA) and GraphPad Prism version 5 (GraphPad Software).

In Article III, one-way analysis of variance (ANOVA) with Tukey post hoc test was used for the assessment of statistically significant differences between multiple groups. Statistically significant differences in IC50 between 20 JAKinibs and in IC50u between 11 JAKinibs was investigated. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

5 SUMMARY OF THE RESULTS

5.1 Patients carrying *IRF2BP2* mutation show increased STAT1 and STAT5 activation (I)

5.1.1 Clinical description of patients

The index patient was a 57-year-old male, who had been suffering from various inflammatory conditions throughout his life. During adolescence and adulthood, he had experienced severe acne and hidradenitis suppurativa, which required surgical revisions. At the age of 51, he had uncomplicated cholecystectomy and umbilical hernia repair, and shortly after started to have subfebrile fever, fatigue, extensive weight loss, mild lymphadenopathy, moderate splenomegaly and marked lymphopenia (650 cells/μl). Healing erosion in duodenum was found by gastroscopy. The index was subjected to profound examinations including colonoscopy, capsule endoscopy, high resolution computed tomography (CT), positron emission tomography-CT, and bone marrow biopsy, but no other findings were made. After the examinations, the symptoms and weight loss eventually ceased. Symptoms returned after three years and new symptom of extensive painful oral and genital ulcers appeared. The index developed a deep neck abscess that was surgically drained with extraction of a molar tooth and he continued to have ulcer problems and occasional stomachaches. A strictured small intestine, probably leading to partial occlusion of the bowel was revealed in re-endoscopy. A biopsy was taken from tongue and genital lesions and showed unspecific ulceration with neutrophilic inflammation. No antibody deficiency was found in immunological analyses. Oral ulcers partially responded to repeated corticosteroid injections into oral mucosa, but new lesions still appeared regularly.

The index had 71-year-old sister, but no other close living relatives. The sister had also experienced inflammatory conditions for several years. She had had hidradenitis suppurativa for over ten years and she had significant lymphopenia (470 cells/μl). However, she showed no ulcer problems or marked infection history. She also suffered from diabetes mellitus type 2, hyperthyroidism and hypertension. At

the age of 71 years, she started to have arm cramps and difficulties in keeping her head position. She was diagnosed with dystrofia myotonica type 2 with typical CCTG expansion of *CNBP* gene.

As the index had unusual clinical features combined with lymphopenia, whole exome sequencing was performed. A novel heterozygous 41 base pair deletion in *IRF2BP2* gene (c.625-665del, p. (Ala209Glnfs*31)) was found. No previously established disease variants were detected. The novel variant generates a frameshift leading to a premature stop codon at position 31 in the new reading frame. Due to a novel finding, also sister was subjected to whole exome sequencing, which revealed that she also carried the same heterozygous 41-bp deletion in *IRF2BP2*.

5.1.2 Phosphorylation of STAT1 and STAT5 is altered in circulating leukocytes

To study the possible role of IRF2BP2 in JAK-STAT signaling, constitutive and *in vitro* cytokine-induced STAT phosphorylation was studied in circulating leukocytes of patients carrying *IRF2BP2* mutation as well as healthy controls, by using multi-color flow cytometry.

The most prominent findings concerned STAT1 and interferon signaling. Constitutive phosphorylation of STAT1 was slightly higher in monocytes and $CD4^+$ T cells of patients than in healthy controls (Figure 4). IFN-α-, IFN-β- and IFN-γ-induced STAT1 phosphorylation was markedly higher in $CD4^+$ T cells, monocytes, and B cells, when compared with healthy controls (Figure 4). The increased interferon-induced STAT1 phosphorylation was higher in index patient than in sister in all cell populations being studied and most notable in monocytes. IL-6-induced phosphorylation of STAT1 was higher in patients than in healthy controls in monocytes and $CD4^+$ T cells. Also, total STAT1 protein level was measured. Total STAT1 level was clearly elevated in the index and slightly increased in the sister in all cell populations studied.

In addition to STAT1, STAT5 phosphorylation differed between patients and healthy controls, namely constitutive STAT5 phosphorylation was increased in $CD4^+$ T cells (Figure 4). However, constitutive STAT5 phosphorylation in monocytes and B cells (Figure 4) and cytokine-induced STAT5 phosphorylation in all cell-types studied (data not shown), was at the same level for patients and healthy controls. Also, constitutive and cytokine-induced STAT3, STAT4, and STAT6

phosphorylation was studied, but there were no differences between patients and healthy controls (data not shown).

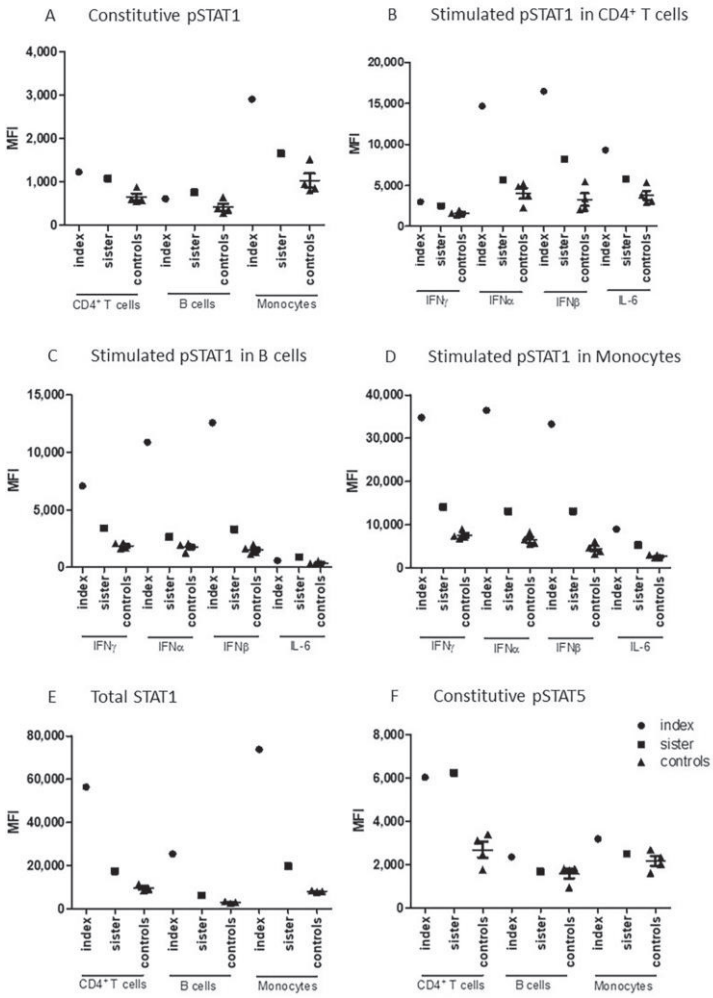


Figure 4. Median fluorescence intensities (MFI) measured in leukocytes from patients and controls. A) Constitutive pSTAT1 in CD4+ T cells, B cells and monocytes, B) Cytokine-induced pSTAT1 in CD4+ T cells, C) Cytokine-induced pSTAT1 in B cells, D) Cytokine-induced pSTAT1 in monocytes, E) Total STAT1 in CD4+ T cells, B cells and Monocytes, F) Constitutive pSTAT5 in CD4+ T cells, B cells and Monocytes. Modified from article I.

5.1.3 Expression of STAT1 and interferon-inducible genes is upregulated in patients

The expression of inflammation-related genes in peripheral blood mononuclear cells (PBMCs) of patients and controls was studied by NanoString analysis (Figure 5). *STAT1* was the only JAK-STAT pathway gene, which was upregulated (fold change ≥ 1.5) both in index and sister. However, there were several interferon-inducible genes, namely *IFIT1*, *IFIT3*, *IFI6*, *IFI27*, *IDO1*, *RSAD2,* and *ISG15*, that were overexpressed in both index and sister. In addition, common overexpressed genes included cytokines *CXCL9* and *CXCL10* as well as inflammasome gene *CASP5*. Index and sister did not have common down-regulated genes (fold change ≤ 0.5).

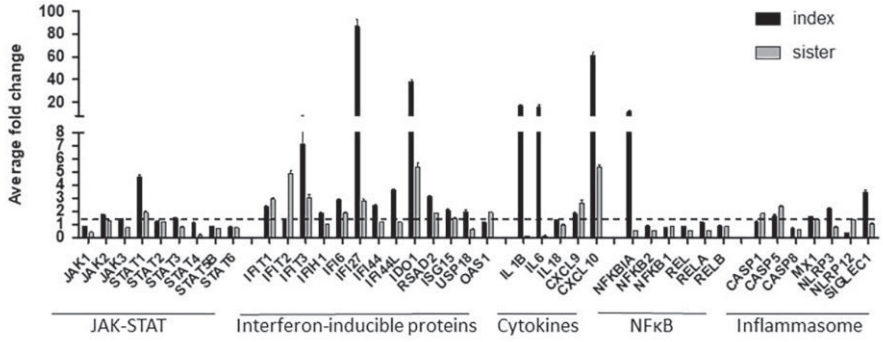


Figure 5. Fold changes of inflammation-related genes in peripheral blood mononuclear cells of index and sister. Average fold changes of three technical replicates are shown. Dashed line represents a fold change of 1.5, which was considered as a threshold for increased gene expression. Modified from Article I.

5.1.4 IRF2BP2 protein attenuates transcriptional activation of STAT1

As experiments performed with patient samples suggested IRF2BP2 to regulate interferon signaling, the role of IRF2BP2 in the type I IFN pathways was studied more directly using dual-luciferase assay in a cell model. Wild-type IRF2BP2 and Δ625-665 IRF2BP2 were co-transfected with reporter plasmid for STAT1 transcriptional activation (ISRE-Luc).

The reporter assay demonstrated that wild-type IRF2BP2 strongly attenuates unstimulated and IFN-α and IFN-β-induced STAT1 transcriptional activity (Figure

6). Interestingly, Δ625-665 IRF2BP2 failed to suppress both constitutive and IFN-induced STAT1 transcriptional activity. The expression of wild-type and Δ625-665 IRF2BP2 constructs was verified by immunoblotting.

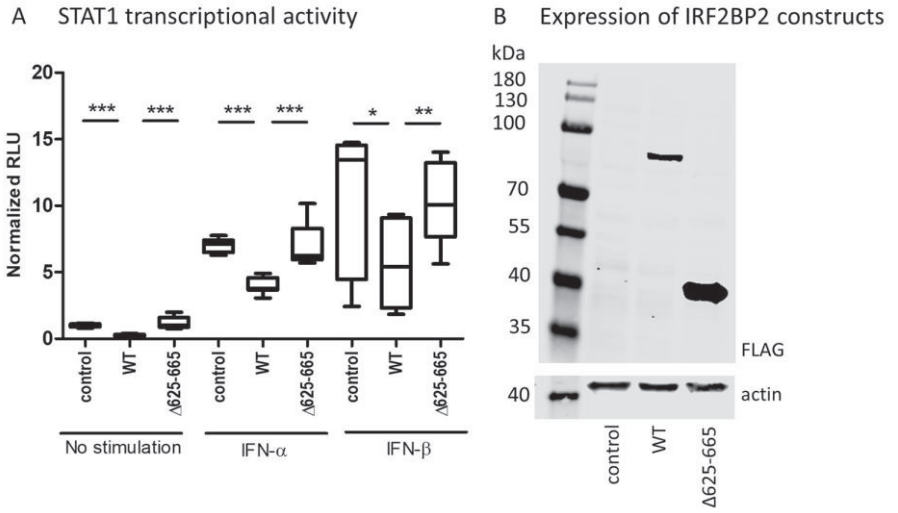


Figure 6. Transcriptional activity of STAT1 and expression of IRF2BP2 constructs. A) The effect of wild-type (WT) and Δ625-665 IRF2BP2 constructs on STAT1 transcriptional activity was assessed using ISRE-Luc reporter in HEK293T cells and presented as relative luciferase units. B) The expression of FLAG-tagged wild-type and Δ625-665 IRF2BP2 constructs was verified by immunoblotting. Actin was used as a loading control. p-values between control, wild-type IRF2BP2 and Δ625-665 IRF2BP2 are calculated by Student´s two-tailed T-test. Significant differences are marked with an asterisk: *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. Adapted from Article II.

5.2 Tofacitinib suppresses several JAK-STAT pathways in rheumatoid arthritis patients (II)

5.2.1 Clinical characteristics of patients and their response to tofacitinib treatment

Altogether 18 patients, nine from each outpatient clinic, were recruited. However, the final study population, who continued tofacitinib treatment until follow-up visit, constituted 16 patients. One patient did not start tofacitinib treatment after all and for another patient, flow cytometry results from the baseline sample were not obtained due to a technical issue. Characteristics of the patients at baseline visit are described in Table 6.

Table 6. Characteristics of patients with rheumatoid arthritis and their csDMARD regimen at baseline visit. Table adapted from Article II.

Patient characteristics and csDMARD regimen	
Female sex, n (%)	11 (69%)
Age, years, mean (range)	58.4 (36.6-72.9)
Disease duration, years, mean (range)	9.6 (0.5-48.0)
Rheumatoid factor positive, n (%)	11 (69%)
CCP-antibody positive, n (%)	12 (75%)
Erosive disease, n (%)	7 (44%)
Disease activity (DAS28), n (%)	
Moderate	13 (81%)
High	3 (19%)
csDMARD regimen, n (%)	
Triple	6 (37%)
Double	7 (44%)
Single	3 (19%)
Low-dose prednisolone, n (%)	8 (50%)

Abbreviations: CCP, cyclic citrullinated peptide; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; n, number of patients.

The disease activity of patients with RA decreased significantly during tofacitinib and background csDMARD treatment, according to both clinical measures of activity and patient-reported outcomes. The comparison of clinical and laboratory parameters at baseline and follow-up visits is presented in Table 7. Median DAS28-

4[CRP] decreased from 4.4 to 2.6. Nine patients (56%) achieved DAS28 remission at the follow-up visit. The disease activity was low in four patients, moderate in two patients and high in one patient at the follow-up visit, as defined by DAS28. Patients did not experience any serious adverse events during the study and safety laboratory tests remained within acceptable range (Table 7).

Table 7. Comparison of clinical and laboratory parameters pre-treatment (baseline visit) and post-treatment (follow-up visit). Table adapted from Article II.

	Pre-treatment, median (IQR)	Post-treatment, median (IQR)	p
Swollen joint count, 0-46	7 (6-9)	2 (0-3)	<0.001
Tender joint count, 0-46	11 (5-17)	1 (0-8)	<0.001
Swollen joint count, 0-28	5 (4-7)	1 (0-2)	<0.001
Tender joint count, 0-28	4 (2-10)	1 (0-3)	<0.001
General health, VAS, 0-100 mm	51 (37-65)	16 (7-29)	0.001
Pain, VAS, 0-100 mm	43 (22-64)	12 (5-38)	0.002
Physician's assessment, VAS, 0-100 mm	35 (31-46)	13 (11-18)	<0.001
DAS28	4.4 (3.6-4.9)	2.6 (1.9-2.9)	<0.001
Plasma C-reactive protein, mg/l	5 (3-17)	3 (3-4)	0.042
HAQ disability index, 0-3	0.813 (0.625-1.253)	0.130 (0-0.813)	0.011
Blood haemoglobin, g/l	129 (126-140)	132 (126-145)	0.775
Blood leukocyte count, $\times 10^9$/l	8.3 (6.1-9.1)	5.3 (4.3-6.9)	0.003
Blood neutrophil count, $\times 10^9$/l	5.32 (4.02-6.34)	2.88 (2.32-3.91)	0.003
Blood lymphocyte count, $\times 10^9$/l	1.40 (1.07-1.94)	1.41 (1.20-1.83)	0.959
Blood platelet count, $\times 10^9$/l	297 (274-334)	281 (225-302)	<0.001
Plasma alanine aminotransferase, U/l	20 (15-26)	21 (19-28)	0.224
Plasma creatinine, µmol/l	63 (56-83)	68 (56-86)	0.615

Abbreviations: DAS28, Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); HAQ, health assessment questionnaire; VAS, visual analogue score.

5.2.2 Tofacitinib suppresses constitutive and cytokine-induced STAT phosphorylation in cytokine and cell type specific manner

To investigate the *in vivo* effects of tofacitinib on JAK-STAT signaling pathways in patients with RA, STAT phosphorylation in monocytes, T cells and B cells was measured from samples collected at baseline and follow-up visits using multi-color flow cytometry.

Tofacitinib suppressed both constitutive and cytokine-induced STAT phosphorylation (Figure 7), but differences were seen between STAT molecules, cell types and cytokines.

When considering constitutive STAT phosphorylation, tofacitinib had the most consistent inhibitory effect on STAT3 phosphorylation. Constitutive STAT3 phosphorylation was significantly reduced after 3-month tofacitinib use in all four cell populations studied. pSTAT1 was downregulated in three cell types; monocytes and T cells, pSTAT4 in two cell types; $CD4^+$ T and B cells, pSTAT5 only in $CD4^+$ T cells and pSTAT6 was not significantly reduced in any cell type studied. Thus, these results suggest that $CD4^+$ T cells are the most sensitive for the inhibitory effect of tofacitinib *in vivo*.

Tofacitinib significantly suppressed cytokine-induced STAT phosphorylation, but the magnitude of inhibition varied depending on the cytokine and cell population (Figure 8). At least 50% inhibition was seen in IFN-γ-induced pSTAT1 in B cells, IFN-α-induced pSTAT1 in $CD4^+$ T cells, IL-2-induced pSTAT5, IL-4-induced pSTAT6, and IL-21-induced pSTAT3 in both T cell types studied and IL-15-induced pSTAT5 in $CD4^+$ T cells. Interestingly, 50% or higher inhibition was not achieved with any cytokine in monocytes. The lowest inhibition percentage was seen in IL-10-induced pSTAT3 in monocytes, being 10%.

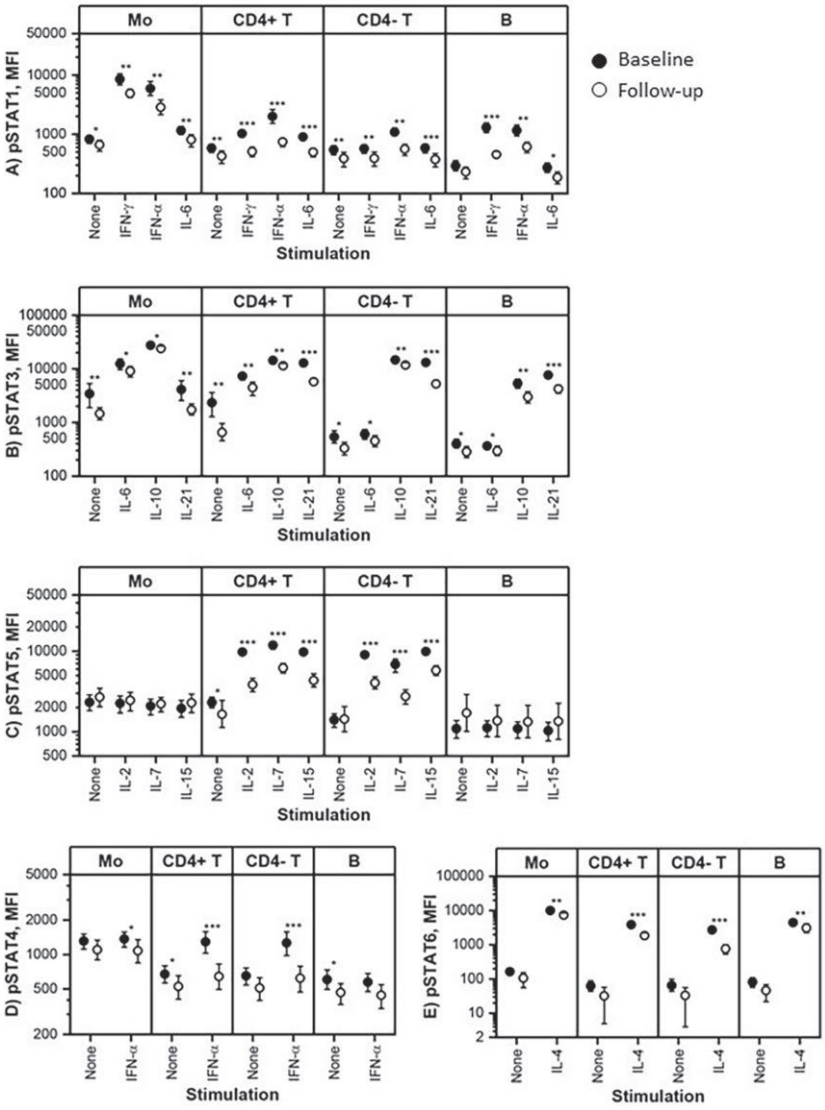


Figure 7. Means and 95% confidence intervals at baseline (filled symbols) and follow-up (open symbols) visits. A) Phosphorylated STAT1 (pSTAT1), B) pSTAT3, C) pSTAT5, D) pSTAT4 and E) pSTAT6 median fluorescence intensities (MFI) in monocytes (Mo), $CD4^+$ T cells ($CD4^+$ T), $CD4^-$ T cells ($CD4^-$ T) and $CD20^+$ B cells (B). p-values comparing baseline and follow-up values are calculated using Wilcoxon test. Significant differences are marked with an asterisk: *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. IFN, interferon; IL, interleukin. Modified from Article II.

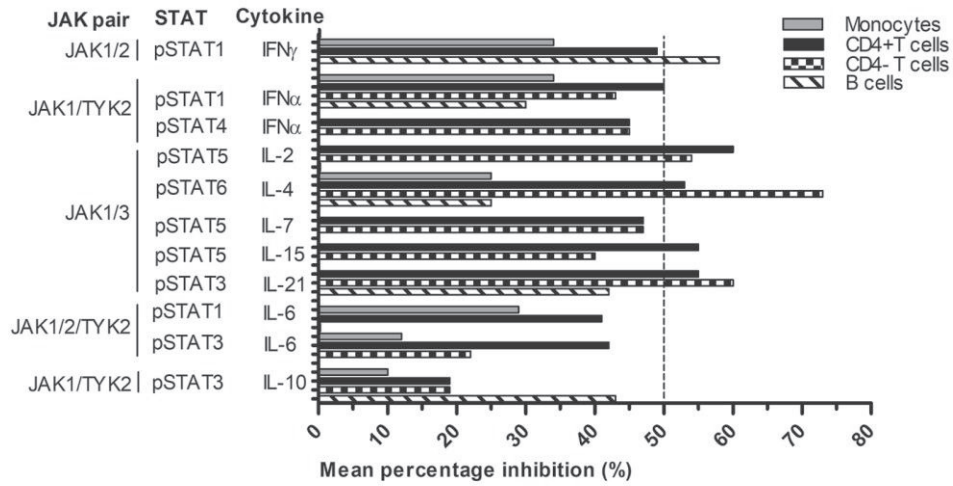


Figure 8. The mean percentage inhibition in different cell types. Inhibition percentages are shown for cytokine-stimulated pSTATs in cell populations, where the cytokine-induced phosphorylation level differs from the constitutive level. IFN, interferon; IL, interleukin.

5.2.2.1 Baseline pSTAT levels correlate with treatment response

Correlation coefficients were calculated between baseline pSTATs and the changes in DAS28 after 3 months of tofacitinib use, to study whether baseline pSTAT levels associate with treatment response.

Our data indicate that constitutive pSTAT1 and pSTAT3 levels positively correlated with treatment response in monocytes (pSTAT1: r=0.591, p=0.016; pSTAT3: r=0.635, p=0.008) and in $CD4^+$ T cells (pSTAT1: r=0.603, p=0.013; pSTAT3: r=0.732, p=0.001). Also, constitutive pSTAT5 levels correlated with treatment response in $CD4^+$ T cells (r=0.568, p=0.022). Interestingly, constitutive pSTAT4 correlated negatively with treatment response in monocytes (r=-0.506, p=0.046) and in $CD4^-$ T cells (r=-0.518, p=0.040).

In addition, some of the cytokine-induced pSTATs correlated with treatment response. IL-6-induced pSTAT1 and pSTAT3 positively correlated with treatment response in monocytes and in $CD4^+$ T cells, respectively (pSTAT1: r=0.556, p=0.025; pSTAT3: r=0.621, p=0.010). Also, IFN-γ-induced pSTAT1 in $CD4^+$ T cells correlated positively with treatment response (r=0.532, p=0.034). IFN-α-

stimulated pSTAT4 correlated negatively with treatment response in $CD4^+$ T cells (r=-0.568, p=0.022) and in $CD4^-$ T cells (r=-0.638, p=0.008).

5.2.3 Tofacitinib suppresses the expression of JAK-STAT pathway components and inhibitors

The effect of tofacitinib in PBMCs to JAK-STAT pathway gene expression was studied by qPCR (Figure 8). *JAK2, TYK2, STAT1, STAT5B,* and *STAT6* expression remained unchanged during tofacitinib treatment. However, *JAK1, JAK3, STAT3, STAT4, STAT5A* and *SOCS2* expression slightly decreased during tofacitinib treatment. The most prominent decrease in gene expression was seen with *SOCS1*, *SOCS3,* and *CIS1*.

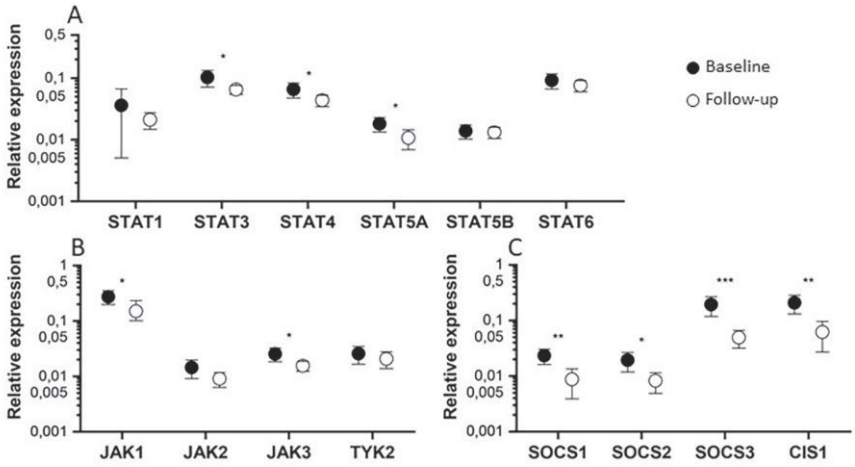


Figure 9. Means and 95% confidence intervals of mRNA expression levels. A) STAT, B) JAK and C) SOCS genes in peripheral blood mononuclear cells of rheumatoid arthritis patients pretreatment (baseline) and after 3-month tofacitinib use (follow-up). p-values comparing pretreatment and 3 months are calculated using Wilcoxon test. Significant differences are marked with an asterisk: *p ≤0.05, ** p ≤0.01, p***≤0.001. Modified from Article II.

5.2.3.1 Baseline *SOCS3* expression correlates with treatment response

Correlation coefficients were calculated also between baseline mRNA levels and treatment response. Baseline *SOCS3* level was the only variable that correlated with treatment response (r=0.532, p=0.034).

5.3 *In vitro* selectivity of JAK inhibitors (III)

5.3.1 JAK selectivity

JAK inhibitor selectivity between JAK isoforms was studied using enzymatic assay that measures the ability of kinase domain (JH1) to phosphorylate a tyrosine kinase substrate. Table 8 describes the obtained IC50 values for each inhibitor (left-hand side) and fold-IC50 values (right-hand side), where the IC50 values have been normalized to JAK, which activity is the most efficiently inhibited.

The non-selective JAK inhibitors showed strong inhibition of JAK1 and JAK2 activity. JAK3 was also inhibited effectively by tofacitinib and peficitinib, whereas inhibition of TYK2 was at low level for all non-selective JAKinibs. Ruxolitinib and baricitinib had selectivity for JAK1/2 over JAK3 (467- and 282-fold, respectively). Tofacitinib was observed to inhibit non-selectively JAK1/2/3 activity, whereas peficitinib possess slight selectivity for JAK1/3 over JAK2 (3.2-fold). All the non-selective JAKinibs possessed higher-degree of selectivity over TYK2 (92-, 148-, 79- and 7-fold for ruxolitinib, baricitinib, tofacitinib and peficitinib, respectively).

The JAK1-targeted JAKinibs filgotinib, upadacitinib, abrocitinib and itacitinib inhibited JAK1 activity most effectively, as expected, followed by inhibition of JAK2 to varying degrees, and minor inhibition of JAK3 (IC50 $\geq$ 500 nM) and TYK2 (IC50>650 nM) activity. The selectivity for JAK1 over JAK2 was highest for itacitinib (13-fold) and abrocitinib (12-fold), whereas filgotinib and upadacitinib possessed lower selectivity over JAK2 (2.4- and 1.8-fold, respectively).

The JAK2-targeted inhibitors were low nanomolar inhibitors of JAK2 activity. JAK1 activity inhibition was strongest after JAK2 inhibition for the group of targeted JAKinibs with exception of pacritinib, which inhibited JAK3 activity stronger than JAK1, and lestaurtinib, which inhibited JAK1 activity to the same extent as JAK2. Selectivity of the JAK2-targeted JAKinibs for JAK2 over JAK1 was high for pacritinib (41-fold), ilginatinib (22-fold), AT-9283 (19-fold) and momelotinib (13-fold), and moderate for fedratinib (9-fold) and gandotinib (2.6-

fold). All the JAK2-targeted inhibitors were > 10-fold selective for JAK2 inhibition over JAK3 and TYK2.

The JAK3-targeted JAKinibs ritlecitinib and decernotinib inhibited JAK3 at low nanomolar IC50 and with selectivity over other JAKs. Ritlecitinib shows specific inhibition of JAK3, fold-IC50 values for other JAKs being over 900. Decernotinib shows JAK3-selectivity, fold-IC50 values being 8 over JAK1, 20 over JAK2, and 1500 over TYK2. In the whole set of clinical JAKinibs, JAK3 activity inhibition was also potently inhibited by non-selective JAKinibs tofacitinib and peficitinib, JAK2-targeted lestaurtinib and AT-9283, and TYK2-targeted cerdulatinib.

TYK2-targeted inhibitor cerdulatinib and TYK2/JAK1-targeted brepocitinib are low nanomolar inhibitors of TYK2 recombinant protein activity but do not show selectivity for TYK2 over other JAKs. Cerdulatinib inhibited JAK2 and brepocitinib JAK1 more potently than TYK2. Inhibition of JAK3 and JAK1 was equal with TYK2 inhibition for cerdulatinib, and brepocitinib inhibits JAK2 and TYK2 with no statistically significant difference. Deucravacitinib, that targets the pseudokinase domain, was not observed to inhibit TYK2, JAK1, or JAK3 enzymatic activity, but showed low (micromolar) inhibition of JAK2 enzymatic activity. TYK2 activity was also potently inhibited by JAK2-targeted AT-928 and lestaurtinib and non-selective inhibitors peficitinib, ruxolitinib and baricitinib.

Table 8. IC50 concentrations (nM) and fold-IC50 values for JAK isoform inhibition. Fold IC50 values are normalized to JAK, which activity is the most efficiently inhibited. Statistical significance of pIC50 value compared to target JAK (bolded; non-selective JAKinibs were compared to JAK1) is indicated with stars (*). *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. ND, not detected or IC50 > 50 µM. Adapted from Article III.

		JAK-activity IC50				JAK-selectivity (fold-IC50)			
	Inhibitor	JAK1	JAK2	JAK3	TYK2	JAK1	JAK2	JAK3	TYK2
Non-sel.	Ruxolitinib	**5.0**	2.8	467***	92**	1.8	1.0	170	33
	Tofacitinib	**8.0**	5.0	6.3	391***	1.6	1.0	1.3	79
	Baricitinib	**3.1**	1.0	282***	148***	3.1	1.0	278	146
	Peficitinib	**5.7**	18*	6.6	42***	1.0	3.2	1.1	7
JAK1	Filgotinib	**36**	87	ND	1210***	1.0	2.4	399	34
	Upadacitinib	**3.7**	6.8*	541***	685***	1.0	1.8	145	184
	Abrocitinib	**21**	253***	12329***	3075***	1.0	12	581	145
	Itacitinib	**2.2**	29***	6385***	ND	1.0	13	2922	2885
JAK2	Fedratinib	75***	**8.6**	1009***	1591***	9	1.0	118	186
	Pacritinib	597***	**15**	296***	1781***	41	1.0	20	122
	Momelotinib	147***	**11**	1265***	940***	13	1.0	114	84
	Lestaurtinib	1.3	**2.0**	14***	31***	1.0	1.5	11	25
	Ilginatinib	28***	**1.2**	283***	1041***	22	1.0	227	835
	AT-9283	24***	**1.3**	41***	26***	19	1.0	32	21
	Gandotinib	11*	**4.3**	279***	157***	2.6	1.0	64	36
JAK3	Ritlecitinib	11447***	6261***	**6.9**	18185***	1671	914	1.0	2654
	Decernotinib	110***	295***	**13**	21472***	8.4	22	1.0	1634
TYK2	Cerdulatinib	71	7.6***	41	**42**	9.3	1.0	5.4	5.6
	Deucravacitinib	ND	24 417	ND	**ND**	2	1	3	ND
	Brepocitinib	5.1***	14	2566***	**32**	1.0	2.7	505	6.3

1	10	100	1000

5.3.2 Cytokine selectivity

Barcoded whole blood flow cytometry assay was used to investigate cytokine inhibition of JAKinibs. Cytokine inhibition IC50 values are presented in Figure 10. Several inhibitors, e.g. ruxolitinib, filgotinib, itacitinib, fedratinib, pacritinib and momelotinib demonstrate comparably high IC50 values throughout the cytokines tested. A major effector in the whole blood assays is the tendency of inhibitors to bind to plasma proteins, which effects on the obtained IC50 values. Parameter IC50u (unbound IC50) takes the reported unbound fraction of JAKinib into account. IC50u values are presented in Figure 11. Statistical tests presented in Figures 10 and

11 compare inhibitors to the strongest inhibitor of each pathway (ergo, column-wise).

JAK1/JAK3-mediated IL-2 signaling was inhibited most potently (in terms of IC50u or IC50 when unbound data not available) by tofacitinib, baricitinib, peficitinib, upadacitinib, ruxolitinib, AT9283, and brepocitinib with no statistically significant differences in IC50u (or IC50). When considering canonical JAK1/JAK2/TYK2 mediated IL-6-pSTAT3 signaling in $CD4^+$ T cells and in monocytes, non-selective ruxolitinib, tofacitinib, baricitinib and peficitinib along with JAK1 targeted upadacitinib and itacitinib, JAK2-targeted fedratinib and AT9283, as well as JAK1/TYK2-targeted brepocitinib were the most effective inhibitors. In addition, momelotinib inhibited IL-6 with no statistically significant differences in monocytes (IC50u). Deucravacitinib and baricitinib inhibited effectively JAK1/TYK2-mediated IFN-α signaling, followed by non-selective inhibitors ruxolitinib, tofacitinib and peficitinib as well as by AT9283 and brepocitinib without statistically significant differences in IC50u (or IC50). No remarkable differences in IC50u of IL-2, IL-6, IFN-α, or IFN-γ signaling were seen between the non-selective JAK inhibitors. Inhibition of JAK2-mediated GM-CSF signaling was more effective for baricitinib (IC50u 41 nM) and ruxolitinib (IC50u 85 nM) compared to tofacitinib (IC50u 279 nM) and peficitinib (IC50u 424 nM). In addition, AT9283 showed efficient inhibition of GM-CSF and there was no statistical difference compared to baricitinib.

Table 9. The heatmap of IC50 values (nM) for the cytokine receptor inhibition. Stars indicate the statistical difference in IC50 of JAKinibs compared to lowest IC50 among the JAKinibs (marked with bold and letter "a" in superscript) for each cytokine, pSTAT, and cell-type combination. Modified from Article III. Abbreviations: CD4+, $CD4^+$ T cells; GM-CSF, granulocyte-macrophage colony-stimulating factor, IFN, interferon; IL, interleukin; Mo, monocytes.

	IL-2 	IL-6 			IFN-α 	IFN-γ 				GM-CSF 
readout:	pSTAT5	pSTAT1	pSTAT3		pSTAT5	pSTAT1	pSTAT1	pSTAT3	pSTAT5	pSTAT5
cells:	CD4+	CD4+	CD4+	Mo	CD4+	Mo	Mo	Mo	Mo	Mo
Ruxolitinib	383***	231**	2848	1275***	190***	171*	1398***	92**	92**	2561***
Tofacitinib	9[a]	13[a]	372	74	12	13	62	7	5	457
Baricitinib	11	14	291[a]	47[a]	5[a]	5	17	3[a]	2[a]	83[a]
Peficitinib	35	75	526	141	23	35	241*	23	13	1842***
Filgotinib	206***	602***	3633	865***	177***	207**	2675***	187***	87***	10546***
Upadacitinib	17	85	361	136	75	55	59	13	12	508*
Abrocitinib	87***	182	3182	423**	38	273*	1125***	46*	31	7030***
Itacitinib	93***	136	1611	315	36	264**	205	22	4	4420***
Fedratinib	664***	3983***	4251*	1008***	885***	821***	4212***	597***	368***	4006***
Pacritinib	12572***	3539***	155460***	23817***	3726***	1982***	39727***	6036***	3524***	194210***
Momelotinib	411***	139*	13664**	457**	518	412**	379**	110**	125***	22850***
Lestaurtinib	1171***	3915***	11215**	3625***	1916***	2802***	5835***	2557***	2416***	27666***
Ilginatinib	1964***	1361***	32667***	3182***	533***	1128***	696**	253***	259***	6536***
AT9283	18	73	447	104	13	13	11[a]	21	12	316
Gandotinib	1281***	10913***	194643***	4715***	1558***	1175***	2744***	818***	826***	8842***
Ritlecitinib	124***	24447***	ND	18092***	400***	7479***	ND	10286***	6139***	ND
Decernotinib	318***	1875***	33587***	3646***	400***	317**	7994***	1879***	456***	21857***
Cerdulatinib	237***	351***	1912	377*	90***	127*	2146***	98**	212***	21047***
Deucravacitinib	1539***	801***	1629	367*	269***	4[a]	2726***	328***	377***	22303***
Brepocitinib	24	57	597	133	22	13	109	26	13	550*

Scale [nM]	<10	11-100	101-1000	1001-10 000	>10 000

Table 10. The heatmap of IC50 unbound values (nM) for the cytokine receptor inhibition. Stars indicate the statistical difference in IC50 compared to lowest IC50 among the JAKinibs (marked with bold and letter "a" in superscript) for each cytokine, pSTAT, and cell-type combination. Modified from Article III. Abbreviations: CD4+, $CD4^+$ T cells; GM-CSF, granulocyte-macrophage colony-stimulating factor, IFN, interferon; IL, interleukin; Mo, monocytes

Cytokine:	IL-2	IL-6			IFN-α	IFN-γ			GM-CSF
readout:	pSTAT5	pSTAT1	pSTAT3	pSTAT5	pSTAT1	pSTAT1	pSTAT3	pSTAT5	pSTAT5
cells:	CD4+	CD4+	CD4+ Mo	CD4+	Mo	Mo	Mo	Mo	Mo
Ruxolitinib	13	8	**94**[a]	42	6	6	46	3	3	85
Tofacitinib	**5**[a]	8	227	45	7	8	38	4	3	279**
Baricitinib	5	**7**[a]	145	**24**[a]	**3**[a]	**2**[a]	**9**[a]	**1**[a]	**1**[a]	**41**[a]
Peficitinib	8	17	121	32	5	8	55	5	3	424***
Filgotinib	92***	271***	1635*	389***	80***	93***	1204***	84***	39***	4746***
Upadacitinib	8	41	173	65	36	26**	28	6	6	244**
Abrocitinib	31**	66	1146*	152*	14	98***	405***	16***	11*	2531***
Itacitinib	137*	35	412	81	9	68***	52	6	1	1131***
Fedratinib	60***	358***	383	91	80**	74***	379***	54***	33**	361***
Pacritinib	377***	106**	4664**	715***	112***	59***	1192***	181***	106***	5826***
Momelotinib	79***	32	2623*	88	99**	79***	73*	21***	24**	4387***

Scale [nM]	<10	11-100	101-1000	1001-10 000	>10 000

5.3.3 Daily average inhibition percentages of JAKinibs intended for the treatment of rheumatoid arthritis

Dosing of the JAKinibs directly affects the clinical selectivity for the JAK-isoform and cytokine responses *in vivo*. Therefore, percent level of inhibition based on measured cytokine IC50 for recommended doses in rheumatoid arthritis patients was calculated. The average inhibition percentages for JAKinibs intended for the treatment of rheumatoid arthritis (tofacitinib, baricitinib, peficitinib, upadacitinib and filgotinib) are presented as spider diagrams in Figure 10. All the inhibitors showed in general similar inhibition profiles, upadacitinib and filgotinib representing scarcer inhibition profiles, having broadly speaking lower efficacy toward IL-6- and IFN-induced pathways. The spider diagrams also illustrate the cytokine inhibition characteristics of individual JAKinibs. Upadacitinib, for example inhibits IL-2-

pSTAT5 stronger than IL-6-pSTAT5, which for other JAKinibs for the treatment of RA was equal.

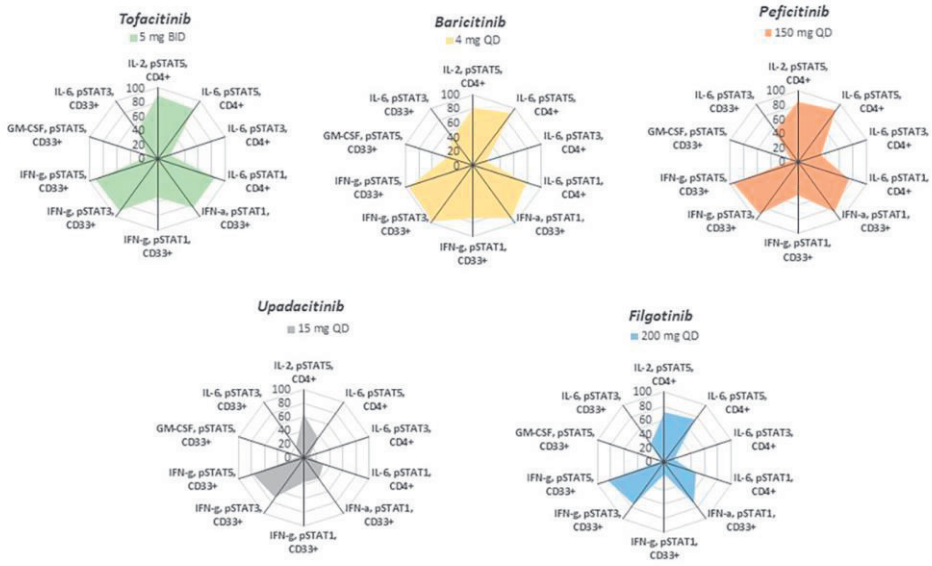


Figure 10. Percent level of daily inhibition of tofacitinib, baricitinib, peficitinib, upadacitinib and filgotinib at recommended doses in patients with RA. Modified from Article III. BID, twice a day; QD, once a day.

6 DISCUSSION

6.1 Characterization of mutations causing inborn errors of immunity could pave the way for targeted therapies

During the last 20 years, availability of whole exome and whole genome sequencing analysis has enabled discovery of increasing number of genetic disorders of the immune system (Delmonte & Notarangelo, 2020). The characterization of the molecular details behind genetic immunological disorders could ultimately lead to use of targeted therapies, including biologic treatments and JAK inhibitors. In article I, we found a novel *IRF2BP2* c.625_665del p.(Ala209Glnfs*31) variant from two family members with inflammatory conditions by whole exome sequencing and found out that IRF2BP2 protein is an important regulator of JAK-STAT pathway.

6.1.1 IRF2BP2 is needed to attenuate interferon responses

Even though IRF2BP2 was first discovered as transcriptional co-repressor of IRF2 (Childs & Goodbourn, 2003), which has a well-defined role as a negative regulator in type I interferon response, the role of IRF2BP2 as a regulator of JAK-STAT signaling has not been studied extensively before. We found that patients carrying *IRF2BP2* c.625_665del variant demonstrated increased constitutive STAT1 phosphorylation in monocytes and T cells and increased STAT1 phosphorylation in response to interferon stimulation in all leukocyte subtypes studied. In addition, several interferon-induced genes, *IFIT1, IFIT3, IFI6, IFI27, IDO1, CXCL9*, and *CXCL10* were overexpressed in PBMCs from patients. In addition, by using a cell model, we could show that overexpression of wild-type IRF2BP2 represses transcriptional activation of STAT1, whereas overexpression of Δ625-665 IRF2BP2 failed in repression, supporting the observations done with clinical samples.

Our results support the formerly proposed role of IRF2BP2 being a co-repressor of IRF2. Suggested role of wild-type IRF2BP2 and Δ625-665 IRF2BP2 in type I IFN response is represented in Figure 11. However, our results also raise questions of novel mechanisms in the regulation of interferon signaling. IRF2 is a transcription

factor that can bind ISREs in DNA – but not GAS elements, which are employed in type II interferon response.

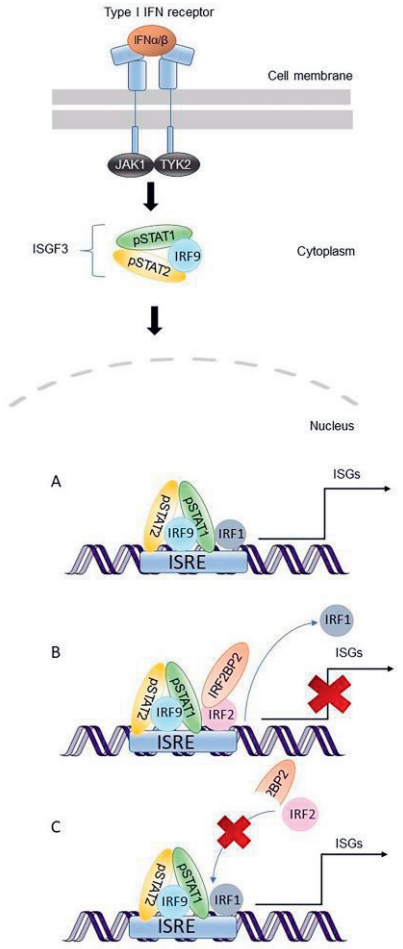


Figure 11. A schematic figure illustrating the suggested role of IRF2BP2 in type I interferon (IFN) signaling. IFN binding to their receptors activates JAKs, which phosphorylate STATs. Upon IFN-α or IFN-β stimulation, transcription factor complexes called interferon-stimulated gene factor 3 (ISGF3) that consist of STAT1, STAT2, and interferon regulatory factor 9 (IRF9) are formed. These transcription factor complexes translocate to the nucleus and bind to interferon-stimulated response elements (ISRE). (A) IRF1 is a transcription factor that co-operates with ISGF3 to induce interferon-stimulated genes (ISGs). (B) IRF2 restricts the induction of ISGs by competing with IRF1 for the same binding site. IRF2BP2 acts as a co-repressor of IRF2 by binding to it through the C-terminal RING domain and is essential for IRF2 ability to limit the expression of ISGs. (C)

Patient mutation IRF2BP2 c.625_665del generates a truncated IRF2BP2 protein (238 amino acids of 587) that entirely lacks the RING domain. Truncated IRF2BP2 cannot bind to IRF2, which in turn is unable to bind DNA and suppress IRF1 function. IFN; interferon, IRF; interferon regulatory factor, IRF2BP2; interferon regulatory factor 2 binding protein 2, ISGF3; interferon-stimulated gene factor 3, ISRE; interferon-stimulated response element, JAK; Janus kinase, STAT; signal transducer and activator of transcription. Figure adapted from Article I.

In this study, the patients showed increased pSTAT1 levels in response to both IFN-α/β and IFN-γ stimulus and there were both type I and type II interferon response genes among those that were overexpressed. IRF1 has been associated to positive regulation of IFN-γ-induced response before, even though direct interactions between GAS elements or STAT1 dimers have not been observed (Michalska et al., 2018; Zenke et al., 2018). More mechanistic data would be needed to determine if the regulation of STAT1 by IRF2BP2 occurs via IRF1/2.

In addition to altered STAT1 phosphorylation, we found that constitutive STAT5 phosphorylation was clearly increased in $CD4^+$ T cells but was approximately at the same level in other leukocyte subtypes studied. Also, STAT5 phosphorylation upon IL-2, IL-7, and IL-15 stimulation was similar to that of healthy controls in $CD4^+$ T cells. Interestingly, one previous study has found an association between IRF2BP2 and STAT5. Sécca et al showed that IRF2BP2 transduced mouse $CD4^+$ T cells had diminished STAT5 phosphorylation compared with empty vector transduced cells, which suggests a repressive role for IRF2BP2 in STAT5 regulation and is in accordance with our observations (Sécca et al., 2016). The mechanism how IRF2BP2 regulates STAT5 activity requires further studies. Noteworthy is that IRF1/IRF2 has not been reported to regulate STAT5 activity. Interestingly, a recent study showed that STAT5 and NFAT1 (nuclear factor of activated T cell 1) can compete from the same binding sites on DNA in T cells (G. Wang et al., 2021). As IRF2BP2 has been demonstrated to act as a co-repressor of NFAT1 (Carneiro et al., 2011), the functions of IRF2BP2 and STAT5 might relate to each other through NFAT1.

6.1.2 Could JAK inhibitors offer an effective treatment option for patients carrying *IRF2BP2* mutations?

As the family described in this study consisted of only two individuals, larger patient numbers would be needed to shed more light on the clinical phenotype and to confirm the statistical significance of the findings done from clinical samples. However, the current results still reveal novel insights on IRF2BP2 action and make one to consider, which would be suitable targeted therapy alternatives for these patients. Patients carrying IRF2BP2 mutation that were described in this study had

inflammatory conditions with no recurrent infections and had systematic hyperactivation of IFN-STAT1 – axis. Thus, they could benefit from treatment targeting interferon signaling. Also patients with SLE show increased circulating type I interferon levels as well as overexpression of IFN-induced genes (Paredes & Niewold, 2020). Anifrolumab, an antibody targeting IFNAR1, which is IFNα/β receptor, is the first approved IFN antagonist with approved indication to SLE (Mullard, 2021). Anifrolumab could be effective treatment option for patients described in this study.

Also JAK inhibitors baricitinib, tofacitinib and ruxolitinib have been shown to decrease clinical manifestations of other diseases that are characterized by enhanced type I interferon signaling, such as CANDLE syndrome (chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature) and STING-associated vasculopathy (Delmonte & Notarangelo, 2020). As the patients in this study had also increased STAT1 phosphorylation due to type II interferon signaling and increased STAT5 phosphorylation, JAK inhibitors that offer a wider inhibitory spectrum could indeed be more effective alternative than targeting specifically IFNAR1. In Article III, we compared the selectivity of different JAK inhibitors *in vitro*, which could offer clues, which JAK inhibitor would be the most effective in this case. Our cytokine inhibition data show that non-selective inhibitors tofacitinib, baricitinib, ruxolitinib and peficitinib inhibit potently IL-2-induced pSTAT5 as well as IFN-γ- and IFN-α induced pSTAT1. Also, JAK2-targeted AT-9283 and TYK2/JAK1 targeted brepocitinib potently inhibit these pathways.

Of tofacitinib, we have also *in vivo* data (Article II). These results confirm that tofacitinib efficiently inhibits cytokine-induced pSTAT5 and pSTAT1 also *in vivo*. In addition, in Article II we could study the effects of tofacitinib on constitutive STAT phosphorylation. Tofacitinib significantly decreased constitutive pSTAT1 levels in $CD4^+$ T cells and monocytes of patients with RA, which could be beneficial for patients carrying *IRF2BP2* mutations, as their pSTAT1 levels in these cell populations were slightly increased compared to healthy controls. Interestingly, tofacitinib decreased significantly constitutive pSTAT5 levels in $CD4^+$ T cells, which was the only cell population where pSTAT5 was notably increased in patients carrying *IRF2BP2* mutation, but did not alter pSTAT5 levels in other leukocyte populations studied. However, tofacitinib decreased significantly also constitutive pSTAT3 in the leukocyte populations being studied and constitutive pSTAT4 in $CD4^+$ T and B cells, which were not increased in *IRF2BP2* mutation carrying patients, which might result in adverse effects. In addition, results obtained in Article II describe the effects of tofacitinib in combination with csDMARDs in patient with

chronic rheumatoid arthritis and the *in vivo* effects of tofacitinib might of course differ in other patient groups.

Functional characterization of mutations causing inborn errors of immunity along with the clinical phenotype is of great importance, when thinking of targeted therapies. *IRF2BP2* variant described in Article I represents the third disease-causing *IRF2BP2* variant reported. Keller et al describe heterozygous point mutation in *IRF2BP2* (c.1652G > A: p. (S551N)) to be responsible for a familial form of CVID (Keller et al., 2016). The authors also showed *in vitro* that this variant decreased B cell maturation into plasmablasts. The second *IRF2BP2* variant was found by sequencing 123 patients referred with childhood-onset immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disease (Baxter et al., 2021). One patient was found to have a heterozygous mutation in *IRF2BP2* (c.1606insTTT, p.Q536delinsX), producing a truncated protein at position 536 of 587 (Baxter et al., 2021). All the three disease-causing IRF2BP2 variants effect on RING domain. Variant described in this study generates a truncated protein of only 238 amino acids of 587 and entirely lacks the C-terminal RING domain.

Interestingly, both previously reported *IRF2BP2* variants seem to cause immunodeficiency, as patients were described to have recurrent infections and hypogammaglobulinemia, which were not present in patients described in the current study (Baxter et al., 2021; Keller et al., 2016). However, patients in the current study still had decreased lymphocyte count. The patients described in the two previous studies also manifested inflammatory conditions, such as psoriasis, chronic diarrhea and/or severe eczema, suggesting wide defects in regulation of immune responses (Baxter et al., 2021; Keller et al., 2016). Given that IRF2BP2 seems to be a regulator of multiple transcription factors in various biological contexts, the polymorphous phenotype of the patients is not surprising. Even though JAK inhibitors could relief inflammatory conditions of these patients, infections belong to common side effects of these medications. Thus, risks might outweigh the possible benefits. However, except for increased risk of herpes varicella virus re-activation, studies done with RA cohorts indicate the risk of other infections of JAK inhibitors to be comparable to many other immunosuppressants, including csDMARDs and TNF inhibitor adalimumab (Nash et al., 2021; Virtanen et al., 2019).

6.2 Selectivity of JAK inhibitors for the treatment of rheumatoid arthritis

Five JAKinibs have obtained global approval for the treatment of RA, namely tofacitinib, baricitinib, peficitinib, filgotinib and upadacitinib (Alexander et al., 2021). As multiple cytokines contribute to RA pathogenesis (Schwartz et al., 2016), it is not surprising that these JAKinibs are not highly specific to any JAK isoform or cytokine. Tofacitinib, baricitinib and peficitinib are non-selective inhibitors, whereas filgotinib and upadacitinib show relative selectivity toward JAK1.

6.2.1 Assays for selectivity measurements

The selectivity of JAK inhibitors is usually determined by using recombinant protein and cell-based assays. Typically used recombinant protein assays include binding and enzymatic assays measuring kinase activity. Binding assays can measure for example the binding affinity of a compound, when it outcompetes ATP or some other binder, or compound's effect on thermal stability of the target protein or protein domain. However, binding assays do not describe the actual inhibitory potency to kinase activity of compounds. Enzymatic assays that measure tyrosine phosphorylation allow the determination of IC50 values, which describe the concentration of the inhibitor, where half of the activity is inhibited. These kinds of assays have been widely used for JAKinib selectivity determination (Fensome et al., 2018; Flanagan et al., 2010; Parmentier et al., 2018; Quintás-Cardama et al., 2010; Van Rompaey et al., 2013; Vazquez et al., 2018). However, the emergence of JAK inhibitors with distinct modes of action, such as deucravacitinib in our panel (Article III), highlights that selectivity assessment only with recombinant protein assays are not adequate.

Deucravacitinib is referred as TYK2 selective inhibitor, as it has been shown to bind specifically to JH2 domains of TYK2 and JAK1 and, in cell-based assays, to effectively inhibit JAK2/TYK2-mediated IL-23 and JAK1/TYK2-mediated IFN-α signaling, whereas the inhibition for JAK1/JAK3-mediated IL-2 or JAK2-mediated thrombopoietin signaling is low (Wrobleski et al., 2019). However, despite the specific binding to JH2 domains of TYK2 and JAK1, we showed that deucravacitinib does not inhibit enzymatic activity of TYK2 or JAK1 (Article III). The inhibitory mechanism of deucravacitinib thus obviously differs that of other current clinical JAK inhibitors (Article III). Deucravacitinib seems to effect on other interactions that are essential for signaling that could be e.g. inhibition of

transphosphorylation or receptor dimerization. However, the exact inhibitory mechanism remains to be elucidated.

Thus, assessment of cytokine inhibition profile of JAKinibs in patients is more relevant selectivity measure, especially in the case of JAKinibs developed for the treatment of inflammatory diseases. However, the availability of patient blood is often limited due to ethical and economic reasons. Thus, JAK inhibitor selectivity in respect to cytokine signaling inhibition has been in most cases determined using whole blood from healthy individuals (Fensome et al., 2018; Flanagan et al., 2010; Parmentier et al., 2018; Quintás-Cardama et al., 2010; Van Rompaey et al., 2013; Vazquez et al., 2018).

Due to different experimental conditions, the comparison of JAK inhibitor selectivity between different studies is often challenging. The results obtained from recombinant protein assays might vary depending on e.g. the protein construct (only kinase domain, JH1-JH2 construct or full-length protein), ATP concentration and substrates/tracers used (Virtanen et al., 2019). In human whole blood assays, the selection of cytokine concentration and cell preparation for flow cytometry and selection of which cell population the results are presented complicate the comparison of different studies.

As selectivity (or lack of it) is thought to stem many safety issues related to JAK inhibitors and more JAK inhibitors have been accepted to markets, papers comparing 3-4 JAK inhibitors for treatment of rheumatoid arthritis in respect to selectivity have been published in recent years by pharma companies (Dowty et al., 2019; McInnes et al., 2019; Traves et al., 2021). The comparison of selectivity in these articles has been assessed mainly by determining cytokine inhibition in whole blood or PBMCs.

6.2.2 Inhibition characteristics of tofacitinib *in vivo* versus *in vitro*

Selectivity measurement assays described in the previous chapter are of great importance in pre-clinical development of JAK inhibitors and are probably the only way how the selectivity can be compared between inhibitors in the same study. Studying JAK inhibitor selectivity by adding the inhibitors on top of blood samples derived from healthy individuals (referred as *in vitro* in this thesis) obviously differs from setting where selectivity is measured from blood samples derived from patients using JAK inhibitors orally (referred as *in vivo*). Indeed, the results we obtained on the inhibitory potency of tofacitinib toward different cytokine pathways in Article II

suggest that the effects *in vivo* differ from results obtained *in vitro* (Article III, Dowty et al., 2019; McInnes et al., 2019; Traves et al., 2021). As blood samples were drawn from patients using tofacitinib approximately one hour after morning medication, the results in Article II reflect the maximal inhibitory potency of tofacitinib. *In vitro* articles describe the inhibitory potential of JAKinibs by daily average inhibitory percentages.

Results obtained in Article II showed some inhibitory characteristics of tofacitinib action *in vivo* that differed from the results obtained *in vitro*. First, *in vitro* results might overestimate the inhibitory potential of tofacitinib. This was seen both with monocytes and $CD4^+$ T cells. The average inhibition percentage of IL-6-induced pSTAT3 in monocytes in Article III was approximately 50 %, 46% reported by McInnes et al and 10 % reported by Traves et al, whereas *in vivo* results presented in Article II suggest the maximal inhibitory percentage to be only 12 % (McInnes et al., 2019; Traves et al., 2021). A similar tendency was seen also with IL-4-induced pSTAT6 inhibition (Article II: 25% versus Traves et al 34% and McInnes et al 51%) and IL-10-induced pSTAT3 inhibition (Article II: 10% versus McInnes et al 25%) (McInnes et al., 2019; Traves et al., 2021). The maximal inhibitory percentage in Article II was the lowest for IL-10-induced pSTAT3 in monocytes throughout the cytokine panel in Article II. These results indicate that tofacitinib did not efficiently inhibit the anti-inflammatory effects IL-10 has on macrophages and monocytes (Sabat et al., 2010). In $CD4^+$ T cells, possible overestimation of inhibition capability *in vitro* was seen especially with common-γ-chain cytokines. For instance, maximal inhibitory percentage for IL-2 induced pSTAT5 in $CD4^+$ T cells in Article II was 60%, whereas *in vitro* studies report the daily average inhibition percentage in this cell type or lymphocytes between 53 and 90 % (Article III, Dowty et al., 2019; McInnes et al., 2019; Traves et al., 2021). Another interesting difference between *in vitro* and *in vivo* studies was that *in vitro* studies suggest systematically lower inhibitory potency of tofacitinib to IL-6 induced pSTAT3 than to pSTAT1 in T cells (Article III, 18% vs 85%; Traves et al, 53% vs 33%; Dowty et al approximately 74% vs 20%), whereas in Article II the maximal inhibition percentages of these two pSTATs were comparable (41% vs 42%) (Dowty et al., 2019; Traves et al., 2021). STAT3 represents the canonical downstream effector of IL-6/JAK1-JAK2/TYK2 pathway, however activation of STAT1 has been shown to be weaker in response to IL-6 stimulation than that of STAT3 (Haan et al., 2005). Thus, the obtained *in vitro* results would seem logical – lower phosphorylation level of IL-6-induced pSTAT1 could be inhibited with lower inhibitor concentration than higher phosphorylation level of IL-6-induced pSTAT3. However, it is important to note that patients with RA have been

shown to have altered STAT phosphorylation status compared to healthy controls, constitutive pSTAT1 showing increased levels in T cells (Isomäki et al., 2015; Pertovaara et al., 2015). In addition, *in vivo* setting obviously include more variables than *in vitro* settings. For example, in Article II, in addition to tofacitinib, the patients received also csDMARDs.

6.2.3 Several data points need to be assessed to determine selectivity

Studying JAK inhibitors in the same study and combining different selectivity measures to pharmacological data is essential for understanding the selectivity of one JAKinib in respect to another and for thinking the significance of the results from *in vivo* aspect. For instance, upadacitinib and filgotinib are both largely referred as JAK1 selective inhibitors in literature. Measured by kinase assay, both did show the strongest inhibition toward JAK1, followed by JAK2 with 1.8 (upadacitinib) and 2.4 (filgotinib) fold values in respect to JAK1 inhibition (Article III). Based on these results, they both are JAK1 selective inhibitors. However, when the results are examined together, upadacitinib inhibited JAK1 activity 10-times more potently than filgotinib and upadacitinib also inhibited JAK2 activity ~5-times more potently than filgotinib inhibited JAK1 (Article III). Thus, in respect to filgotinib, upadacitinib could also be referred to as JAK1/2 selective inhibitor.

IC50 values measuring the inhibition potency of RA JAKinibs to cytokine signaling showed that upadacitinib has similar inhibition profile as non-selective inhibitors, except for IFN-α- and IFN-γ-induced pSTATs in monocytes (Article III). Filgotinib on the other hand showed overall weaker inhibition potency toward cytokine signaling compared with other RA JAKinibs (Article III). However, the dosing between inhibitors varies. The recommended dose for patients with RA of filgotinib is about 13 times higher than that of upadacitib (recommended doses of JAKinibs intended for treatment of RA: tofacitinib, 5 mg twice daily; baricitinib 4 mg once a day; peficitinib 150 mg once a day; filgotinib 200 mg once a day and upadacitinib 15 mg once a day) (EMA Xeljanz product information, EMA Rinvoq product information, EMA Olumiant product information, EMA Jyseleca product information, Markham & Keam, 2019). Thus, dosing needs to be considered in selectivity estimations as well. Spider diagrams that estimate the daily average inhibition percentages in this study have been calculated using cytokine inhibition IC50 values and average plasma concentration of JAKinibs at selected dose in a steady state. These results indicated that tofacitinib, baricitinib and peficitinib show

similar cytokine inhibition profiles, whereas filgotinib and upadacitinib seem to have in general less inhibitory effect on interferon signaling than non-selective inhibitors (Article III). The inhibitory effect of upadacitinib seems also be lower toward IL-6 signaling than that of filgotinib and non-selective inhibitors.

As was discussed in the previous section, studying JAK inhibitors *in vivo* can reveal novel aspects of selectivity. It would be interesting to see, if the other RA JAKinibs would present similar *in vivo* actions and differences to *in vitro* data as did tofacitinib and if there would be analogous correlations between pSTATs and treatment response as was seen with tofacitinib. For instance, the lower inhibitory potency of upadacitinib and filgotinib toward interferon signaling that was seen from IC50 values/daily average inhibition percentages in Article III is interesting. Tofacitinib did inhibit quite efficiently interferon-induced pSTATs also *in vivo* (Article II), but the maximum inhibitory percentages calculated remained lower than those presented as average inhibitory percentages *in vitro* (Article III). Thus, it is possible that upadacitinib and filgotinib would show low interferon inhibition *in vivo*. Given the central role of interferon signaling in inhibiting varicella zoster replication (Ku et al., 2016; Shakya et al., 2019), lower inhibition of interferons might be beneficial in safety perspective. However, only filgotinib seems to cause less herpes zoster, whereas safety profile of upadacitinib resembles those of tofacitinib and baricitinib (Liu et al., 2021). On the other hand, interferons do belong to pathogenesis mediating cytokines in RA (Schwartz et al., 2016), so lower inhibitory potential toward interferons could in theory result in lower efficacy.

Even though selectivity of JAKinibs for treatment of RA (as well as of JAKinibs for treatment of other indications) is a subject of major interest, paradoxically, it is not known yet which selectivity profile would offer the best efficacy and safety combination for patients. In rheumatoid arthritis, distinct cytokine profiles dominate the different phases of the disease, such as early and established RA (McInnes et al., 2016b). Currently, biomarker profiles that would predict the optimal JAKinib or other treatment for each patient group have not been established. Thus, more research is needed that selectivity data of JAKinibs could be effectively used also in clinical contexts.

7 SUMMARY AND CONCLUSIONS

Genetic diagnoses of complex immunological conditions and functional characterization of novel disease-causing mutations can help us to understand more about immunoregulation and disease pathogenesis as well as guide clinical management decisions. Perhaps, targeted therapies for the treatment of immunological diseases will become reality in the future, as has happened in oncology during the past 20 years (Bedard et al., 2020). In this study, we found a novel mutation in *IRF2BP2* gene from two family members with inflammatory conditions and lymphopenia. *IRF2BP2* variant described in this study represents the third disease-causing mutation in this gene reported, emphasizing the central role of IRF2BP2 in regulation of immune responses. Our results indicate that IRF2BP2 is an essential regulator of JAK-STAT pathways.

The future of targeted therapies in immunology would require not only a better understanding of the diseases but also a better understanding of the medicines themselves. The selectivity of JAK inhibitors is currently a subject of great interest, as it impacts the efficacy and safety characteristics of this class of medicines. The work shown in this thesis presents the first comprehensive analysis and direct comparison of 20 clinical stage JAKinibs in terms of *in vitro* and cellular effects on JAK kinases and signaling. Additionally, this thesis provides novel information on the *in vivo* selectivity profile of one JAK inhibitor, tofacitinib, in patients with rheumatoid arthritis using tofacitinib orally. Our results indicated that certain immunological markers at baseline are associated with treatment response to tofacitinib. This preliminary result suggests that measuring certain signaling molecules pre-treatment could be used to predict the treatment response to advanced therapies, which would be of great value for clinics.

The main limitations of the study relate to low patient numbers. Novel *IRF2BP2* variant was found from only two family members, so the findings done should be considered preliminary. More patients would have been needed to demonstrate statistical significance of the difference in phosphorylated STATs and gene expression between patients and healthy controls. The *in vivo* effects of tofacitinib were studied with 16 patients with RA, which enabled only predictive conclusions on the clinical significance of the observed association between baseline

immunological parameters and treatment response. Article III was designed to provide a comprehensive analysis and comparison of current clinical JAK inhibitors. However, the analysis focused only to JAK kinases and JAK-STAT signaling, so the off-target effects of JAK inhibitors were not analyzed.

The optimal use of JAK inhibitor selectivity profiles in clinics still needs basic research as well as clinical data. For instance, different JAK isoforms seem to have dominance over another in certain receptor complexes (Raivola et al., 2019). Understanding the exact role of JAKs in each cytokine receptor would allow the development of even more selective inhibitors. On the other hand, more information on what kind of cytokine inhibition profile would be the most efficient and safest option in each disease and disease state is also required. In addition, patients who would benefit certain inhibition profile need to be recognized. This requires finding biomarkers that describe treatment responses for JAK inhibitors of different selectivity profiles.

The opportunity for attenuating undesired immune responses by JAK inhibition was recognized soon after the discovery of JAK-STAT pathway. To date, several JAK inhibitors have entered the clinics for treating inflammatory diseases, such as rheumatoid arthritis, ulcerative colitis, and dermatological conditions, and many are on their way. In addition, the characterization of rare immunological diseases can reveal new conditions that could be effectively treated with JAK inhibitors. However, better overall understanding of immunological diseases is still needed so that the full potential of JAK inhibitors can be unleashed.

8 REFERENCES

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2018). *Cellular and molecular immunology* (Ninth edit). Elsevier.

Alcover, A., Alarcón, B., & Bartolo, V. Di. (2018). Cell Biology of T Cell Receptor Expression and Regulation. *Https://Doi.Org/10.1146/Annurev-Immunol-042617-053429*, *36*, 103–125. https://doi.org/10.1146/ANNUREV-IMMUNOL-042617-053429

Alexander, M., Luo, Y., Raimondi, G., O'Shea, J. J., & Gadina, M. (2021). Jakinibs of All Trades: Inhibiting Cytokine Signaling in Immune-Mediated Pathologies. *Pharmaceuticals (Basel, Switzerland)*, *15*(1), 48. https://doi.org/10.3390/PH15010048

Aluri, J., Desai, M., Gupta, M., Dalvi, A., Terance, A., Rosenzweig, S. D., Stoddard, J. L., Niemela, J. E., Tamankar, V., Mhatre, S., Bargir, U., Kulkarni, M., Shah, N., Aggarwal, A., Lashkari, H. P., Krishna, V., Govindaraj, G., Kalra, M., & Madkaikar, M. (2019). Clinical, immunological, and molecular findings in 57 patients with severe combined immunodeficiency (SCID) from India. *Frontiers in Immunology*, *10*(FEB), 23. https://doi.org/10.3389/FIMMU.2019.00023/BIBTEX

Anderson, A. E., Pratt, A. G., Sedhom, M. A. K., Doran, J. P., Routledge, C., Hargreaves, B., Brown, P. M., Lê Cao, K. A., Isaacs, J. D., & Thomas, R. (2016). IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *75*(2), 466–473. https://doi.org/10.1136/annrheumdis-2014-205850

Banerjee, S., Biehl, A., Gadina, M., Hasni, S., & Schwartz, D. M. (2017). JAK–STAT Signaling as a Target for Inflammatory and Autoimmune Diseases: Current and Future Prospects. *Drugs*, *77*(5), 521. https://doi.org/10.1007/S40265-017-0701-9

Banfield, C., Scaramozza, M., Zhang, W., Kieras, E., Page, K. M., Fensome, A., Vincent, M., Dowty, M. E., Goteti, K., Winkle, P. J., & Peeva, E. (2018). The Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of a TYK2/JAK1 Inhibitor (PF-06700841) in Healthy Subjects and Patients With Plaque Psoriasis. *Journal of Clinical Pharmacology*, *58*(4), 434–447. https://doi.org/10.1002/jcph.1046

Barbour, A. M., Rockich, K., Cimino, E., Zhou, G., Leonetti-Whalen, C., Chen, X., Yeleswaram, S., Epstein, N., & Punwani, N. (2021). Effect of Hepatic Impairment on the Pharmacokinetics of Itacitinib. *Journal of Clinical Pharmacology*, *61*(7), 954–960. https://doi.org/10.1002/jcph.1814

Baxter, S. K., Walsh, T., Casadei, S., Eckert, M. M., Allenspach, E. J., Hagin, D., Segundo, G., Lee, M. K., Gulsuner, S., Shirts, B. H., Sullivan, K. E., Keller, M. D., Torgerson, T. R., & King, M.-C. (2021). Molecular diagnosis of childhood immune dysregulation, polyendocrinopathy and enteropathy, and implications for clinical management. *Journal of Allergy and Clinical Immunology*. https://doi.org/10.1016/j.jaci.2021.04.005

Bedard, P. L., Hyman, D. M., Davids, M. S., & Siu, L. L. (2020). Small molecules, big impact: 20 years of targeted therapy in oncology. *The Lancet*, *395*(10229), 1078–1088. https://doi.org/10.1016/S0140-6736(20)30164-1

Berdeja, J., Palandri, F., Baer, M., Quick, D., Kiladjian, J., Martinelli, G., Verma, A., Hamid, O., Walgren, R., Pitou, C., Li, P., & Gerds, A. (2018). Phase 2 study of gandotinib (LY2784544) in patients with myeloproliferative neoplasms. *Leukemia Research*, *71*, 82–88.

https://doi.org/10.1016/J.LEUKRES.2018.06.014
Bisgin, A., Sonmezler, O., Boga, I., & Yilmaz, M. (2021). The impact of rare and low-frequency genetic variants in common variable immunodeficiency (CVID). *Scientific Reports*, *11*(1). https://doi.org/10.1038/S41598-021-87898-1
Bluyssen, H. A. R., & Levy, D. E. (1997). Stat2 is a transcriptional activator that requires sequence-specific contacts provided by Stat1 and p48 for stable interaction with DNA. *Journal of Biological Chemistry*, *272*(7), 4600–4605. https://doi.org/10.1074/jbc.272.7.4600
Boers, M., Nurmohamed, M., Doelman, C., Lard, L., Verhoeven, A., Voskuyl, A., Huizinga, T., van de Stadt, R., Dijkmans, B., & van der Linden, S. (2003). Influence of glucocorticoids and disease activity on total and high density lipoprotein cholesterol in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *62*(9), 842–845. https://doi.org/10.1136/ARD.62.9.842
Boggon, T., Li, Y., Manley, P., & Eck, M. (2005). Crystal structure of the Jak3 kinase domain in complex with a staurosporine analog. *Blood*, *106*(3), 996–1002. https://doi.org/10.1182/BLOOD-2005-02-0707
Bose, P., & Verstovsek, S. (2017). JAK2 inhibitors for myeloproliferative neoplasms: what is next? *Blood*, *130*(2), 115–125. https://doi.org/10.1182/BLOOD-2017-04-742288
Candotti, F., Oakes, S. A., Johnston, J. A., Giliani, S., Schumacher, R. F., Mella, P., Fiorini, M., Ugazio, A. G., Badolato, R., Notarangelo, L. D., Bozzi, F., Macchi, P., Strina, D., Vezzoni, P., Blaese, R. M., O'Shea, J. J., & Villa, A. (1997). Structural and Functional Basis for JAK3-Deficient Severe Combined Immunodeficiency. *Blood*, *90*(10), 3996–4003. https://doi.org/10.1182/BLOOD.V90.10.3996
Cao, Y. J., Sawamoto, T., Valluri, U., Cho, K., Lewand, M., Swan, S., Lasseter, K., Matson, M., Holman, J. J., Keirns, J., & Zhu, T. (2016). Pharmacokinetics, Pharmacodynamics, and Safety of ASP015K (Peficitinib), a New Janus Kinase Inhibitor, in Healthy Subjects. *Clinical Pharmacology in Drug Development*, *5*(6), 435–449. https://doi.org/10.1002/cpdd.273
Carneiro, F. R. G., Ramalho-Oliveira, R., Mognol, G. P., & Viola, J. P. B. (2011). Interferon Regulatory Factor 2 Binding Protein 2 Is a New NFAT1 Partner and Represses Its Transcriptional Activity. *Molecular and Cellular Biology*, *31*(14), 2889–2901. https://doi.org/10.1128/mcb.00974-10
Casadevall, A. (1998). Antibody-mediated protection against intracellular pathogens. *Trends in Microbiology*, *6*(3), 102–107. https://doi.org/10.1016/S0966-842X(98)01208-6
Castro, C., & Gourley, M. (2010). Diagnostic testing and interpretation of tests for autoimmunity. *Journal of Allergy and Clinical Immunology*, *125*(2), S238–S247. https://doi.org/10.1016/J.JACI.2009.09.041
Center for drug evaluation and research. (2009). Clinical Pharmacology and Biopharmaceutics Review(s), application number 203214Orig1s000. *Food and Drug Administration*, 1–5.
Changelian, P. S., Flanagan, M. E., Ball, D. J., Kent, C. R., Magnuson, K. S., Martin, W. H., Rizzuti, B. J., Sawyer, P. S., Perry, B. D., Brissette, W. H., McCurdy, S. P., Kudlacz, E. M., Conklyn, M. J., Elliott, E. A., Koslov, E. R., Fisher, M. B., Strelevitz, T. J., Yoon, K., Whipple, D. A., … Borie, D. C. (2003). Prevention of Organ Allograft Rejection by a Specific Janus Kinase 3 Inhibitor. *Science*, *302*(5646), 875–878. https://doi.org/10.1126/SCIENCE.1087061
Chaplin, D. D. (2010). Overview of the Immune Response. *The Journal of Allergy and Clinical Immunology*, *125*(2 Suppl 2), S3. https://doi.org/10.1016/J.JACI.2009.12.980
Charles-Schoeman, C., Fleischmann, R., Davignon, J., Schwartz, H., Turner, S. M., Beysen, C., Milad, M., Hellerstein, M. K., Luo, Z., Kaplan, I. V., Riese, R., Zuckerman, A., & McInnes, I. B. (2015). Potential Mechanisms Leading to the Abnormal Lipid Profile in Patients With Rheumatoid Arthritis Versus Healthy Volunteers and Reversal by Tofacitinib,. *Arthritis & Rheumatology (Hoboken, N.J.)*, *67*(3), 616. https://doi.org/10.1002/ART.38974

Chen, M., Cheng, A., Candotti, F., Zhou, Y.-J., Hymel, A., Fasth, A., Notarangelo, L. D., & O'Shea, J. J. (2000). Complex Effects of Naturally Occurring Mutations in the JAK3 Pseudokinase Domain: Evidence for Interactions between the Kinase and Pseudokinase Domains. *Molecular and Cellular Biology*, *20*(3), 947. https://doi.org/10.1128/MCB.20.3.947-956.2000

Chen, X., Williams, W. V, Sandor, V., & Yeleswaram, S. (2013). Population pharmacokinetic analysis of orally-administered ruxolitinib (INCB018424 Phosphate) in patients with primary myelofibrosis (PMF), post-polycythemia vera myelofibrosis (PPV-MF) or post-essential thrombocythemia myelofibrosis (PET MF). *Journal of Clinical Pharmacology*, *53*(7), 721–730. https://doi.org/10.1002/jcph.102

Childs, K. S., & Goodbourn, S. (2003). Identification of novel co-repressor molecules for Interferon Regulatory Factor-2. *Nucleic Acids Research*, *31*(12), 3016–3026. https://doi.org/10.1093/nar/gkg431

Chimalakonda, A., Burke, J., Cheng, L., Catlett, I., Tagen, M., Zhao, Q., Patel, A., Shen, J., Girgis, I. G., Banerjee, S., & Throup, J. (2021). Selectivity Profile of the Tyrosine Kinase 2 Inhibitor Deucravacitinib Compared with Janus Kinase 1/2/3 Inhibitors. *Dermatology and Therapy*, *11*(5), 1763–1776. https://doi.org/10.1007/s13555-021-00596-8

Chinen, J., & Shearer, W. T. (2010). Secondary immunodeficiencies, including HIV infection. *The Journal of Allergy and Clinical Immunology*, *125*(2 Suppl 2), S195. https://doi.org/10.1016/J.JACI.2009.08.040

Chrencik, J., Patny, A., Leung, I., Korniski, B., Emmons, T., Hall, T., Weinberg, R., Gormley, J., Williams, J., Day, J., Hirsch, J., Kiefer, J., Leone, J., Fischer, H., Sommers, C., Huang, H., EJ, J., RE, T., AG, T., & TE, B. (2010). Structural and thermodynamic characterization of the TYK2 and JAK3 kinase domains in complex with CP-690550 and CMP-6. *Journal of Molecular Biology*, *400*(3), 413–433. https://doi.org/10.1016/J.JMB.2010.05.020

Coffey, G., Betz, A., DeGuzman, F., Pak, Y., Inagaki, M., Baker, D. C., Hollenbach, S. J., Pandey, A., & Sinha, U. (2014). The Novel Kinase Inhibitor PRT062070 (Cerdulatinib) Demonstrates Efficacy in Models of Autoimmunity and B-Cell Cancer. *Journal of Pharmacology and Experimental Therapeutics*, *351*(3), 538–548. https://doi.org/10.1124/JPET.114.218164

Curtis, J. R., & Singh, J. A. (2011). The Use of Biologics in Rheumatoid Arthritis: Current and Emerging Paradigms of Care. *Clinical Therapeutics*, *33*(6), 679. https://doi.org/10.1016/J.CLINTHERA.2011.05.044

Dawson, M. A., Curry, J. E., Barber, K., Beer, P. A., Graham, B., Lyons, J. F., Richardson, C. J., Scott, M. A., Smyth, T., Squires, M. S., Thompson, N. T., Green, A. R., & Wallis, N. G. (2010). AT9283, a potent inhibitor of the Aurora kinases and Jak2, has therapeutic potential in myeloproliferative disorders. *British Journal of Haematology*, *150*(1), 46–57. https://doi.org/10.1111/J.1365-2141.2010.08175.X

De Benedetti, F., Prencipe, G., Bracaglia, C., Marasco, E., & Grom, A. A. (2021). Targeting interferon-γ in hyperinflammation: opportunities and challenges. *Nature Reviews Rheumatology 2021*, 1–14. https://doi.org/10.1038/s41584-021-00694-z

de Saint Basile, G., Ménasché, G., & Fischer, A. (2010). Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nature Reviews Immunology 2010 10:8*, *10*(8), 568–579. https://doi.org/10.1038/nri2803

Dehner, C., Fine, R., & Kriegel, M. A. (2019). The Microbiome in Systemic Autoimmune Disease – Mechanistic Insights from Recent Studies. *Current Opinion in Rheumatology*, *31*(2), 201. https://doi.org/10.1097/BOR.0000000000000574

Delmonte, O. M., & Notarangelo, L. D. (2020). Targeted Therapy with Biologicals and Small Molecules in Primary Immunodeficiencies. *Medical Principles and Practice*, *29*(2), 101. https://doi.org/10.1159/000503997

Dendrou, C. A., Cortes, A., Shipman, L., Evans, H. G., Attfield, K. E., Jostins, L., Barber, T., Kaur, G., Kuttikkatte, S. B., Leach, O. A., Desel, C., Faergeman, S. L., Cheeseman, J., Neville, M. J., Sawcer, S., Compston, A., Johnson, A. R., Everett, C., Bell, J. I., … Fugger, L. (2016). Resolving TYK2 locus genotype-to-phenotype differences in autoimmunity. *Science Translational Medicine*, *8*(363), 363ra149. https://doi.org/10.1126/SCITRANSLMED.AAG1974

Deverman, B. E., & Patterson, P. H. (2009). Cytokines and CNS Development. *Neuron*, *64*(1), 61–78. https://doi.org/10.1016/J.NEURON.2009.09.002

Dodington, D. W., Desai, H. R., & Woo, M. (2018). JAK/STAT – Emerging Players in Metabolism. *Trends in Endocrinology & Metabolism*, *29*(1), 55–65. https://doi.org/10.1016/J.TEM.2017.11.001

Dougan, M., Dranoff, G., & Dougan, S. K. (2019). GM-CSF, IL-3, and IL-5 Family of Cytokines: Regulators of Inflammation. *Immunity*, *50*(4), 796–811. https://doi.org/10.1016/J.IMMUNI.2019.03.022

Dowty, M. E., Lin, T. H., Jesson, M. I., Hegen, M., Martin, D. A., Katkade, V., Menon, S., & Telliez, J. B. (2019). Janus kinase inhibitors for the treatment of rheumatoid arthritis demonstrate similar profiles of in vitro cytokine receptor inhibition. *Pharmacology Research and Perspectives*, *7*(6). https://doi.org/10.1002/prp2.537

Dwivedi, P., & Greis, K. D. (2017). Granulocyte Colony Stimulating Factor Receptor (G-CSFR) signaling in severe congenital neutropenia, chronic neutrophilic leukemia and related malignancies. *Experimental Hematology*, *46*, 9. https://doi.org/10.1016/J.EXPHEM.2016.10.008

EMA. (2011). *Jakavi assessment report*. *Ema*, *44*(September), 0–88.

EMA. (2017a). *Enpaxiq withdrawal assessment report*. *44*(September 2016).

EMA. (2017b). *Olumiant assessment report*. *44*(December 2016).

EMA. (2017c). *Xeljanz assessment report*. *44*(January).

EMA. (2020a). *Jyseleca assessment report*. *31*(July).

EMA. (2020b). *Rinvoq assessment report*. *31*(October 2019).

EMA. (2021). *Inrebic assessment report*. *31*(December 2020).

Enerbäck, C., Sandin, C., Lambert, S., Zawistowski, M., Stuart, P., Verma, D., Tsoi, L., Nair, R., Johnston, A., & Elder, J. (2018). The psoriasis-protective TYK2 I684S variant impairs IL-12 stimulated pSTAT4 response in skin-homing CD4+ and CD8+ memory T-cells. *Scientific Reports*, *8*(1). https://doi.org/10.1038/S41598-018-25282-2

Fabbrocini, G., Napolitano, M., Megna, M., Balato, N., & Patruno, C. (2018). Treatment of Atopic Dermatitis with Biologic Drugs. *Dermatology and Therapy*, *8*(4), 527. https://doi.org/10.1007/S13555-018-0258-X

Farmer, L. J., Ledeboer, M. W., Hoock, T., Arnost, M. J., Bethiel, R. S., Bennani, Y. L., Black, J. J., Brummel, C. L., Chakilam, A., Dorsch, W. A., Fan, B., Cochran, J. E., Halas, S., Harrington, E. M., Hogan, J. K., Howe, D., Huang, H., Jacobs, D. H., Laitinen, L. M., … Zuccola, H. J. (2015). Discovery of VX-509 (Decernotinib): A Potent and Selective Janus Kinase 3 Inhibitor for the Treatment of Autoimmune Diseases. *Journal of Medicinal Chemistry*, *58*(18), 7195–7216. https://doi.org/10.1021/ACS.JMEDCHEM.5B00301/SUPPL_FILE/JM5B00301_SI_002.CSV

Feghali, C. A., & Wright, T. M. (1997). Cytokines in acute and chronic inflammation. *Frontiers in Bioscience : A Journal and Virtual Library*, *2*. https://doi.org/10.2741/A171

Fensome, A., Ambler, C. M., Arnold, E., Banker, M. E., Brown, M. F., Chrencik, J., Clark, J. D., Dowty, M. E., Efremov, I. V., Flick, A., Gerstenberger, B. S., Gopalsamy, A., Hayward, M. M., Hegen, M., Hollingshead, B. D., Jussif, J., Knafels, J. D., Limburg, D. C., Lin, D., … Zhang, L. (2018). Dual Inhibition of TYK2 and JAK1 for the Treatment of Autoimmune

Diseases: Discovery of ((S)-2,2-Difluorocyclopropyl)((1R,5S)-3-(2-((1-methyl-1H-pyrazol-4-yl)amino)pyrimidin-4-yl)-3,8-diazabicyclo[3.2.1]octan-8-yl)methanone (PF-06700841). *Journal of Medicinal Chemistry*, *61*(19), 8597–8612. https://doi.org/10.1021/ACS.JMEDCHEM.8B00917

Ferrao, R., & Lupardus, P. J. (2017). The Janus Kinase (JAK) FERM and SH2 Domains: Bringing Specificity to JAK–Receptor Interactions. *Frontiers in Endocrinology*, *0*(APR), 71. https://doi.org/10.3389/FENDO.2017.00071

Flanagan, M. E., Blumenkopf, T. A., Brissette, W. H., Brown, M. F., Casavant, J. M., Shang-Poa, C., Doty, J. L., Elliott, E. A., Fisher, M. B., Hines, M., Kent, C., Kudlacz, E. M., Lillie, B. M., Magnuson, K. S., McCurdy, S. P., Munchhof, M. J., Perry, B. D., Sawyer, P. S., Strelevitz, T. J., … Changelian, P. S. (2010). Discovery of CP-690,550: A potent and selective janus kinase (JAK) inhibitor for the treatment of autoimmune diseases and organ transplant rejection. *Journal of Medicinal Chemistry*, *53*(24), 8468–8484. https://doi.org/10.1021/jm1004286

Gadina, M., Chisolm, D. A., Philips, R. L., McInness, I. B., Changelian, P. S., & O'Shea, J. J. (2020). Translating JAKs to Jakinibs. *The Journal of Immunology*, *204*(8), 2011–2020. https://doi.org/10.4049/JIMMUNOL.1901477

Gadina, M., Hilton, D., Johnston, J. A., Morinobu, A., Lighvani, A., Zhou, Y. J., Visconti, R., & O'Shea, J. J. (2001). Signaling by Type I and II cytokine receptors: ten years after. *Current Opinion in Immunology*, *13*(3), 363–373. https://doi.org/10.1016/S0952-7915(00)00228-4

Genovese, M. C., Van Vollenhoven, R. F., Pacheco-Tena, C., Zhang, Y., & Kinnman, N. (2016). VX-509 (Decernotinib), an Oral Selective JAK-3 Inhibitor, in Combination With Methotrexate in Patients With Rheumatoid Arthritis. *Arthritis & Rheumatology (Hoboken, N.J.)*, *68*(1), 46–55. https://doi.org/10.1002/ART.39473

Gerds, A. T., Savona, M. R., Scott, B. L., Talpaz, M., Egyed, M., Harrison, C. N., Yacoub, A., Vannucchi, A., Mead, A. J., Kiladjian, J.-J., O'Sullivan, J., García-Gutiérrez, V., Bose, P., Rampal, R. K., Miller, C. B., Palmer, J., Oh, S. T., Buckley, S. A., Mould, D. R., … Mascarenhas, J. O. (2020). Determining the recommended dose of pacritinib: results from the PAC203 dose-finding trial in advanced myelofibrosis. *Blood Advances*, *4*(22), 5825–5835. https://doi.org/10.1182/bloodadvances.2020003314

Guschin, D., Rogers, N., Briscoe, J., Witthuhn, B., Watling, D., Horn, F., Pellegrini, S., Yasukawa, K., Heinrich, P., Stark, G. R., & al., et. (1995). A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *The EMBO Journal*, *14*(7), 1421. /pmc/articles/PMC398228/?report=abstract

Haan, S., Keller, J. F., Behrmann, I., Heinrich, P. C., & Haan, C. (2005). Multiple reasons for an inefficient STAT1 response upon IL-6-type cytokine stimulation. *Cellular Signalling*, *17*(12), 1542–1550. https://doi.org/10.1016/J.CELLSIG.2005.03.010

Hammarén, H. M., Virtanen, A. T., Raivola, J., & Silvennoinen, O. (2019). The regulation of JAKs in cytokine signaling and its breakdown in disease. *Cytokine*, *118*, 48–63. https://doi.org/10.1016/j.cyto.2018.03.041

Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., & Taniguchi, T. (1989). Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell*, *58*(4), 729–739. https://doi.org/10.1016/0092-8674(89)90107-4

Helal, A. M. H., Shahine, E. M., Hassan, M. M., Hashad, D. I., & Moneim, R. A. (2012). Fatigue in rheumatoid arthritis and its relation to interleukin-6 serum level. *The Egyptian Rheumatologist*, *34*(4), 153–157. https://doi.org/10.1016/J.EJR.2012.08.004

Hida, S., Ogasawara, K., Sato, K., Abe, M., Takayanagi, H., Yokochi, T., Sato, T., Hirose, S., Shirai, T., Taki, S., & Taniguchi, T. (2000). CD8+ T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-α/β signaling. *Immunity*, *13*(5),

643–655. https://doi.org/10.1016/S1074-7613(00)00064-9
Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimi, M., Maid, R., Feldmann, M., & Kishimoto, T. (1988). Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *European Journal of Immunology*, *18*(11), 1797–1802. https://doi.org/10.1002/EJI.1830181122
Horwitz, S. M., Feldman, T. A., Hess, B. T., Khodadoust, M. S., Kim, Y. H., Munoz, J., Patel, M. R., Phillips, T. J., Smith, S. D., Smith, S. M., Wilcox, R. A., Birrell, M. R., Leeds, J. M., Conley, P. B., Michelson, G. C., Coffey, G. P., Curnutte, J. T., & Hamlin, P. A. (2019). A Phase 2 Study of the Dual SYK/JAK Inhibitor Cerdulatinib Demonstrates Good Tolerability and Clinical Response in Relapsed/Refractory Peripheral T-Cell Lymphoma and Cutaneous T-Cell Lymphoma. *Blood*, *134*(Supplement_1), 466–466. https://doi.org/10.1182/BLOOD-2019-123986
Hu, X., li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduction and Targeted Therapy 2021 6:1*, *6*(1), 1–33. https://doi.org/10.1038/s41392-021-00791-1
Isaacs, A., & Lindenmann, J. (1957). Virus interference. I. The interferon. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *147*(927), 258–267. https://doi.org/10.1098/RSPB.1957.0048
Isomäki, P., Junttila, I., Vidqvist, K. L., Korpela, M., & Silvennoinen, O. (2015). The activity of JAK-STAT pathways in rheumatoid arthritis: Constitutive activation of STAT3 correlates with interleukin 6 levels. *Rheumatology (United Kingdom)*, *54*(6), 1103–1113. https://doi.org/10.1093/rheumatology/keu430
James, C., Ugo, V., Le Couédic, J.-P., Staerk, J., Delhommeau, F., Lacout, C., Garçon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., & Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature 2005 434:7037*, *434*(7037), 1144–1148. https://doi.org/10.1038/nature03546
Jefremow, A., & Neurath, M. F. (2020). All are Equal, Some are More Equal: Targeting IL 12 and 23 in IBD – A Clinical Perspective. *ImmunoTargets and Therapy*, *9*, 289. https://doi.org/10.2147/ITT.S282466
Jiang, Y., & Shen, Q. (2021). IRF2BP2 prevents ox-LDL-induced inflammation and EMT in endothelial cells via regulation of KLF2. *Experimental and Therapeutic Medicine*, *21*(5). https://doi.org/10.3892/etm.2021.9912
Kany, S., Vollrath, J. T., & Relja, B. (2019). Cytokines in Inflammatory Disease. *International Journal of Molecular Sciences*, *20*(23). https://doi.org/10.3390/IJMS20236008
Karjalainen, A., Shoebridge, S., Krunic, M., Simonović, N., Tebb, G., Macho-maschler, S., Strobl, B., & Müller, M. (2020). TYK2 in Tumor Immunosurveillance. *Cancers*, *12*(1), 150. https://doi.org/10.3390/CANCERS12010150
Keller, M. D., Pandey, R., Li, D., Glessner, J., Tian, L., Henrickson, S. E., Chinn, I. K., Monaco-Shawver, L., Heimall, J., Hou, C., Otieno, F. G., Jyonouchi, S., Calabrese, L., van Montfrans, J., Orange, J. S., & Hakonarson, H. (2016). Mutation in IRF2BP2 is responsible for a familial form of common variable immunodeficiency disorder. *Journal of Allergy and Clinical Immunology*, *138*(2), 544-550.e4. https://doi.org/10.1016/j.jaci.2016.01.018
Khan, M. F., & Wang, H. (2020). Environmental Exposures and Autoimmune Diseases: Contribution of Gut Microbiome. *Frontiers in Immunology*, *0*, 3094. https://doi.org/10.3389/FIMMU.2019.03094
Kim, I., Kim, J. H., Kim, K., Seong, S., & Kim, N. (2019). The IRF2BP2-KLF2 axis regulates osteoclast and osteoblast differentiation. *BMB Reports*. http://www.ncbi.nlm.nih.gov/pubmed/31186082
King, B., Guttman-Yassky, E., Peeva, E., Banerjee, A., Sinclair, R., Pavel, A. B., Zhu, L., Cox, L.

A., Craiglow, B., Chen, L., Banfield, C., Page, K., Zhang, W., & Vincent, M. S. (2021). A phase 2a randomized, placebo-controlled study to evaluate the efficacy and safety of the oral Janus kinase inhibitors ritlecitinib and brepocitinib in alopecia areata: 24-week results. *Journal of the American Academy of Dermatology*, *85*(2), 379–387. https://doi.org/10.1016/J.JAAD.2021.03.050

Kisseleva, T., Bhattacharya, S., Braunstein, J., & Schindler, C. (2002). Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene*, *285*(1–2), 1–24. https://doi.org/10.1016/S0378-1119(02)00398-0

Kiu, H., & Nicholson, S. E. (2012). Biology and significance of the JAK/STAT signalling pathways. *Growth Factors (Chur, Switzerland)*, *30*(2), 88. https://doi.org/10.3109/08977194.2012.660936

Koeppel, M., van Heeringen, S. J., Smeenk, L., Navis, A. C., Janssen-Megens, E. M., & Lohrum, M. (2009). The novel p53 target gene IRF2BP2 participates in cell survival during the p53 stress response. *Nucleic Acids Research*, *37*(2), 322–335. https://doi.org/10.1093/nar/gkn940

Kondo, M., Scherer, D. C., King, A. G., Manz, M. G., & Weissman, I. L. (2001). Lymphocyte development from hematopoietic stem cells. *Current Opinion in Genetics & Development*, *11*(5), 520–526. https://doi.org/10.1016/S0959-437X(00)00227-6

Krutzik, P., Clutter, M., Trejo, A., & Nolan, G. (2011). Fluorescent cell barcoding for multiplex flow cytometry. *Current Protocols in Cytometry*, *Chapter 6*(SUPPL.55). https://doi.org/10.1002/0471142956.CY0631S55

Ku, C. C., Chang, Y. H., Chien, Y., & Lee, T. L. (2016). Type I interferon inhibits varicella-zoster virus replication by interfering with the dynamic interaction between mediator and IE62 within replication compartments. *Cell & Bioscience*, *6*(1), 21. https://doi.org/10.1186/S13578-016-0086-6

Kudlacz, E., Perry, B., Sawyer, P., Conklyn, M., McCurdy, S., Brissette, W., and, M. F., & Changelian, P. (2004). The Novel JAK-3 Inhibitor CP-690550 Is a Potent Immunosuppressive Agent in Various Murine Models. *American Journal of Transplantation*, *4*(1), 51–57. https://doi.org/10.1046/J.1600-6143.2003.00281.X

Kumatori, A., Yang, D., Suzuki, S., & Nakamura, M. (2002). Cooperation of STAT-1 and IRF-1 in interferon-gamma-induced transcription of the gp91(phox) gene. *The Journal of Biological Chemistry*, *277*(11), 9103–9111. https://doi.org/10.1074/JBC.M109803200

Kuuliala, K., Kuuliala, A., Koivuniemi, R., Kautiainen, H., Repo, H., & Leirisalo-Repo, M. (2016). Stat6 and stat1 pathway activation in circulating lymphocytes and monocytes as predictor of treatment response in rheumatoid arthritis. *PLoS ONE*, *11*(12). https://doi.org/10.1371/journal.pone.0167975

Kuuliala, K., Kuuliala, A., Koivuniemi, R., Oksanen, S., Hämäläinen, M., Moilanen, E., Kautiainen, H., Leirisalo-Repo, M., & Repo, H. (2015). Constitutive STAT3 Phosphorylation in Circulating CD4+ T Lymphocytes Associates with Disease Activity and Treatment Response in Recent-Onset Rheumatoid Arthritis. *PLOS ONE*, *10*(9), e0137385. https://doi.org/10.1371/journal.pone.0137385

Laity, J. H., Lee, B. M., & Wright, P. E. (2001). Zinc finger proteins: New insights into structural and functional diversity. In *Current Opinion in Structural Biology* (Vol. 11, Issue 1, pp. 39–46). Elsevier Ltd. https://doi.org/10.1016/S0959-440X(00)00167-6

Lawrence, T., & Gilroy, D. W. (2007). Chronic inflammation: a failure of resolution? *International Journal of Experimental Pathology*, *88*(2), 85–94. https://doi.org/10.1111/J.1365-2613.2006.00507.X

Lempiäinen, J. K., Niskanen, E. A., Vuoti, K. M., Lampinen, R. E., Göös, H., Varjosalo, M., & Palvimo, J. J. (2017). Agonist-specific protein interactomes of Glucocorticoid and androgen receptor as revealed by proximity mapping. *Molecular and Cellular Proteomics*, *16*(8),

1462–1474. https://doi.org/10.1074/mcp.M117.067488
Leonard, W. J., Lin, J. X., & O'Shea, J. J. (2019). The γc Family of Cytokines: Basic Biology to Therapeutic Ramifications. *Immunity*, *50*(4), 832–850. https://doi.org/10.1016/J.IMMUNI.2019.03.028
Lim, C. P., & Cao, X. (2006). Structure, function, and regulation of STAT proteins. *Molecular BioSystems*, *2*(11), 536–550. https://doi.org/10.1039/B606246F
Lin, F., & Young, H. A. (2014). Interferons: Success in anti-viral immunotherapy. *Cytokine & Growth Factor Reviews*, *25*(4), 369. https://doi.org/10.1016/J.CYTOGFR.2014.07.015
Liu, C., Kieltyka, J., Fleischmann, R., Gadina, M., & O'Shea, J. J. (2021). A Decade of Jakinibs: what have we learned and what may be the future. *Arthritis & Rheumatology*, art.41906. https://doi.org/10.1002/ART.41906
Lokau, J., & Garbers, C. (2020). Biological functions and therapeutic opportunities of soluble cytokine receptors. *Cytokine & Growth Factor Reviews*, *55*, 94–108. https://doi.org/10.1016/J.CYTOGFR.2020.04.003
Lucet, I., Fantino, E., Styles, M., Bamert, R., Patel, O., Broughton, S., Walter, M., Burns, C., Treutlein, H., Wilks, A., & Rossjohn, J. (2006). The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. *Blood*, *107*(1), 176–183. https://doi.org/10.1182/BLOOD-2005-06-2413
Luo, Y., Alexander, M., Gadina, M., O'Shea, J. J., Meylan, F., & Schwartz, D. M. (2021). JAK-STAT signaling in human disease: From genetic syndromes to clinical inhibition. *Journal of Allergy and Clinical Immunology*, *148*(4), 911–925. https://doi.org/10.1016/J.JACI.2021.08.004
Madhok, R., Crilly, A., Watson, J., Capell, H. A., & Madhok, R. (1993). Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity. *Annals of the Rheumatic Diseases*, *52*, 2021. https://doi.org/10.1136/ard.52.3.232
Male, D. K. (2013). *Immunology* (8th editio). Elsevier/Saunders.
Manjur, A. B. M. K., Lempiäinen, J. K., Malinen, M., Palvimo, J. J., & Niskanen, E. A. (2019). IRF2BP2 modulates the crosstalk between glucocorticoid and TNF signaling. *Journal of Steroid Biochemistry and Molecular Biology*, *192*, 105382. https://doi.org/10.1016/j.jsbmb.2019.105382
Maradit-Kremers, H., Crowson, C., Nicola, P., Ballman, K., Roger, V., Jacobsen, S., & Gabriel, S. (2005). Increased unrecognized coronary heart disease and sudden deaths in rheumatoid arthritis: a population-based cohort study. *Arthritis and Rheumatism*, *52*(2), 402–411. https://doi.org/10.1002/ART.20853
Markham, A., & Keam, S. J. (2019). Peficitinib: First Global Approval. *Drugs*, *79*(8), 887–891. https://doi.org/10.1007/S40265-019-01131-Y/TABLES/2
Marshall, J. L., Kindler, H., Deeken, J., Bhargava, P., Vogelzang, N. J., Rizvi, N., Luhtala, T., Boylan, S., Dordal, M., Robertson, P., Hawkins, M. J., & Ratain, M. J. (2005). Phase I trial of orally administered CEP-701, a novel neurotrophin receptor-linked tyrosine kinase inhibitor. *Investigational New Drugs*, *23*(1), 31–37. https://doi.org/10.1023/B:DRUG.0000047103.64335.b0
Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology 2018 14:2*, *14*(2), 1–10. https://doi.org/10.1186/S13223-018-0278-1
Mascarenhas, J., Baer, M. R., Kessler, C., Hexner, E., Tremblay, D., Price, L., Sandy, L., Weinberg, R., Pahl, H., Silverman, L. R., Goldberg, J. D., Kosiorek, H., Dueck, A. C., & Hoffman, R. (2019). Phase II trial of Lestaurtinib, a JAK2 inhibitor, in patients with myelofibrosis. *Leukemia & Lymphoma*, *60*(5), 1343–1345. https://doi.org/10.1080/10428194.2018.1532509
McInnes, I. B., Buckley, C. D., & Isaacs, J. D. (2016a). Cytokines in rheumatoid arthritis-shaping

the immunological landscape. In *Nature Reviews Rheumatology* (Vol. 12, Issue 1, pp. 63–68). Nature Publishing Group. https://doi.org/10.1038/nrrheum.2015.171

McInnes, I. B., Buckley, C. D., & Isaacs, J. D. (2016b). Cytokines in rheumatoid arthritis-shaping the immunological landscape. In *Nature Reviews Rheumatology* (Vol. 12, Issue 1, pp. 63–68). Nature Publishing Group. https://doi.org/10.1038/nrrheum.2015.171

McInnes, I. B., Byers, N. L., Higgs, R. E., Lee, J., Macias, W. L., Na, S., Ortmann, R. A., Rocha, G., Rooney, T. P., Wehrman, T., Zhang, X., Zuckerman, S. H., & Taylor, P. C. (2019). Comparison of baricitinib, upadacitinib, and tofacitinib mediated regulation of cytokine signaling in human leukocyte subpopulations. *Arthritis Research and Therapy*, *21*(1). https://doi.org/10.1186/s13075-019-1964-1

McInnes, I. B., & Schett, G. (2011). The Pathogenesis of Rheumatoid Arthritis. *Http://Dx.Doi.Org/10.1056/NEJMra1004965*, *365*(23), 2205–2219. https://doi.org/10.1056/NEJMRA1004965

Metcalf, D. (2008). Hematopoietic cytokines. *Blood*, *111*(2), 485–491. https://doi.org/10.1182/BLOOD-2007-03-079681

Michalska, A., Blaszczyk, K., Wesoly, J., & Bluyssen, H. A. R. (2018). A positive feedback amplifier circuit that regulates interferon (IFN)-stimulated gene expression and controls type I and type II IFN responses. *Frontiers in Immunology*, *9*(MAY), 28. https://doi.org/10.3389/fimmu.2018.01135

Mitchell, T. J., & John, S. (2005). Signal transducer and activator of transcription (STAT) signalling and T-cell lymphomas. *Immunology*, *114*(3), 301. https://doi.org/10.1111/J.1365-2567.2005.02091.X

Mogensen, T. H. (2019). IRF and STAT Transcription Factors - From Basic Biology to Roles in Infection, Protective Immunity, and Primary Immunodeficiencies. *Frontiers in Immunology*, *0*(JAN), 3047. https://doi.org/10.3389/FIMMU.2018.03047

Mohamed, M.-E. F., Zeng, J., Marroum, P. J., Song, I.-H., & Othman, A. A. (2019). Pharmacokinetics of Upadacitinib With the Clinical Regimens of the Extended-Release Formulation Utilized in Rheumatoid Arthritis Phase 3 Trials. *Clinical Pharmacology in Drug Development*, *8*(2), 208–216. https://doi.org/https://doi.org/10.1002/cpdd.462

Mohr, A., Chatain, N., Domoszlai, T., Rinis, N., Sommerauer, M., Vogt, M., & Müller-Newen, G. (2012). Dynamics and non-canonical aspects of JAK/STAT signalling. *European Journal of Cell Biology*, *91*(6–7), 524–532. https://doi.org/10.1016/J.EJCB.2011.09.005

Moroncini, G., Calogera, G., Benfaremo, D., & Gabrielli, A. (2017). Biologics in Inflammatory Immune-mediated Systemic Diseases. *Current Pharmaceutical Biotechnology*, *18*(12), 1008–1016. https://doi.org/10.2174/1389201019666171226152448

Morris, A. C., Beresford, G. W., Mooney, M. R., & Boss, J. M. (2002). Kinetics of a gamma interferon response: expression and assembly of CIITA promoter IV and inhibition by methylation. *Molecular and Cellular Biology*, *22*(13), 4781–4791. https://doi.org/10.1128/MCB.22.13.4781-4791.2002

Morris, R., Kershaw, N. J., & Babon, J. J. (2018). The molecular details of cytokine signaling via the JAK/STAT pathway. *Protein Science*, *27*(12), 1984–2009. https://doi.org/10.1002/PRO.3519

Mortaz, E., Alipoor, S. D., Adcock, I. M., Mumby, S., & Koenderman, L. (2018). Update on neutrophil function in severe inflammation. *Frontiers in Immunology*, *9*(OCT), 2171. https://doi.org/10.3389/FIMMU.2018.02171/BIBTEX

Mouzaki, A., Deraos, S., & Chatzantoni, K. (2005). Advances in the Treatment of Autoimmune Diseases; Cellular Activity, Type-1/Type-2 Cytokine Secretion Patterns and their Modulation by Therapeutic Peptides. *Current Medicinal Chemistry*, *12*(13), 1537–1550. https://doi.org/10.2174/0929867054039044

Mullard, A. (2021). FDA approves AstraZeneca's anifrolumab for lupus. *Nature Reviews. Drug*

Discovery, *20*(9), 658. https://doi.org/10.1038/D41573-021-00139-Y

Mullen, M., & Gonzalez-Perez, R. R. (2016). Leptin-Induced JAK/STAT Signaling and Cancer Growth. *Vaccines*, *4*(3). https://doi.org/10.3390/VACCINES4030026

Nairz, M., Sonnweber, T., Schroll, A., Theurl, I., & Weiss, G. (2012). The pleiotropic effects of erythropoietin in infection and inflammation. *Microbes and Infection / Institut Pasteur*, *14*(3), 238. https://doi.org/10.1016/J.MICINF.2011.10.005

Nash, P., Kerschbaumer, A., Dörner, T., Dougados, M., Fleischmann, R. M., Geissler, K., McInnes, I., Pope, J. E., Van Der Heijde, D., Stoffer-Marx, M., Takeuchi, T., Trauner, M., Winthrop, K. L., De Wit, M., Aletaha, D., Baraliakos, X., Boehncke, W. H., Emery, P., Isaacs, J. D., … Smolen, J. S. (2021). Points to consider for the treatment of immune-mediated inflammatory diseases with Janus kinase inhibitors: A consensus statement. In *Annals of the Rheumatic Diseases* (Vol. 80, Issue 1, pp. 71–87). BMJ Publishing Group. https://doi.org/10.1136/annrheumdis-2020-218398

Naylor, M. J., Lockefeer, J. A., Horseman, N. D., & Ormandy, C. J. (2003). Prolactin Regulates Mammary Epithelial Cell Proliferation Via Autocrine/Paracrine Mechanism. *Endocrine*, *20*(2), 111–114.

Nguyen, K., Watford, W., Salomon, R., Hofmann, S., Pien, G., Morinobu, A., Gadina, M., O'Shea, J., & Biron, C. (2002). Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science (New York, N.Y.)*, *297*(5589), 2063–2066. https://doi.org/10.1126/SCIENCE.1074900

Nogueira, M., Puig, L., & Torres, T. (2020). JAK Inhibitors for Treatment of Psoriasis: Focus on Selective TYK2 Inhibitors. *Drugs 2020 80:4*, *80*(4), 341–352. https://doi.org/10.1007/S40265-020-01261-8

Notarangelo, L. D. (2010). Primary immunodeficiencies. *Journal of Allergy and Clinical Immunology*, *125*(2), S182–S194. https://doi.org/10.1016/J.JACI.2009.07.053

O'Shea, J. J., Schwartz, D. M., Villarino, A. V., Gadina, M., McInnes, I. B., & Laurence, A. (2015). The JAK-STAT pathway: Impact on human disease and therapeutic intervention. *Annual Review of Medicine*, *66*, 311–328. https://doi.org/10.1146/annurev-med-051113-024537

O'Sullivan, L. A., Liongue, C., Lewis, R. S., Stephenson, S. E. M., & Ward, A. C. (2007). Cytokine receptor signaling through the Jak–Stat–Socs pathway in disease. *Molecular Immunology*, *44*(10), 2497–2506. https://doi.org/10.1016/J.MOLIMM.2006.11.025

Olivera, P., Lasa, J., Bonovas, S., Danese, S., & Peyrin-Biroulet, L. (2020). Safety of Janus Kinase Inhibitors in Patients With Inflammatory Bowel Diseases or Other Immune-mediated Diseases: A Systematic Review and Meta-Analysis. *Gastroenterology*, *158*(6), 1554-1573.e12. https://doi.org/10.1053/J.GASTRO.2020.01.001

Papp, K., Gordon, K., Thaçi, D., Morita, A., Gooderham, M., Foley, P., Girgis, I., Kundu, S., & Banerjee, S. (2018). Phase 2 Trial of Selective Tyrosine Kinase 2 Inhibition in Psoriasis. *The New England Journal of Medicine*, *379*(14), 1313–1321. https://doi.org/10.1056/NEJMOA1806382

Pardanani, A., Laborde, R. R., Lasho, T. L., Finke, C., Begna, K., Al-Kali, A., Hogan, W. J., Litzow, M. R., Leontovich, A., Kowalski, M., & Tefferi, A. (2013). Safety and efficacy of CYT387, a JAK1 and JAK2 inhibitor, in myelofibrosis. *Leukemia*, *27*(6), 1322–1327. https://doi.org/10.1038/leu.2013.71

Paredes, J. L., & Niewold, T. B. (2020). *Type I interferon antagonists in clinical development for lupus*. https://doi.org/10.1080/13543784.2020.1797677

Parmentier, J. M., Voss, J., Graff, C., Schwartz, A., Argiriadi, M., Friedman, M., Camp, H. S., Padley, R. J., George, J. S., Hyland, D., Rosebraugh, M., Wishart, N., Olson, L., & Long, A. J. (2018). In vitro and in vivo characterization of the JAK1 selectivity of upadacitinib (ABT-494). *BMC Rheumatology*, *2*(1), 1–11. https://doi.org/10.1186/S41927-018-0031-X/FIGURES/5

Patel, A. A., & Odenike, O. (2020). The Next Generation of JAK Inhibitors: an Update on Fedratinib, Momelotonib, and Pacritinib. *Current Hematologic Malignancy Reports 2020 15:6*, *15*(6), 409–418. https://doi.org/10.1007/S11899-020-00596-Z

Paul, W. E. (2013). *Fundamental immunology* (Seventh ed). Wolters Kluwer Health/Lippincott Williams & Wilkins.

Peeva, E., Hodge, M. R., Kieras, E., Vazquez, M. L., Goteti, K., Tarabar, S. G., Alvey, C. W., & Banfield, C. (2018). Evaluation of a Janus kinase 1 inhibitor, PF-04965842, in healthy subjects: A phase 1, randomized, placebo-controlled, dose-escalation study. *British Journal of Clinical Pharmacology*, *84*(8), 1776–1788. https://doi.org/https://doi.org/10.1111/bcp.13612

Pertovaara, M., Silvennoinen, O., & Isomäki, P. (2015). STAT-5 is activated constitutively in T cells, B cells and monocytes from patients with primary Sjögren's syndrome. *Clinical and Experimental Immunology*, *181*(1), 29–38. https://doi.org/10.1111/cei.12614

Pieper, K., Grimbacher, B., & Eibel, H. (2013). B-cell biology and development. *Journal of Allergy and Clinical Immunology*, *131*(4), 959–971. https://doi.org/10.1016/J.JACI.2013.01.046

Platanitis, E., & Decker, T. (2018). Regulatory Networks Involving STATs, IRFs, and NFκB in Inflammation. *Frontiers in Immunology*, *0*(NOV), 2542. https://doi.org/10.3389/FIMMU.2018.02542

Pope, R. M., & Shahrara, S. (2012). Possible roles of IL-12-family cytokines in rheumatoid arthritis. *Nature Reviews Rheumatology 2012 9:4*, *9*(4), 252–256. https://doi.org/10.1038/nrrheum.2012.170

Puthenpurail, A., Rathi, H., Nauli, S. M., & Ally, A. (2021). A BRIEF SYNOPSIS OF MONOCLONAL ANTIBODY FOR THE TREATMENT OF VARIOUS GROUPS OF DISEASES. *World Journal of Pharmacy and Pharmaceutical Sciences*, *10*(11), 14. /pmc/articles/PMC8775886/

Quintás-Cardama, A., Vaddi, K., Liu, P., Manshouri, T., Li, J., Scherle, P. A., Caulder, E., Wen, X., Li, Y., Waeltz, P., Rupar, M., Burn, T., Lo, Y., Kelley, J., Covington, M., Shepard, S., Rodgers, J. D., Haley, P., Kantarjian, H., … Verstovsek, S. (2010). Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood*, *115*(15), 3109. https://doi.org/10.1182/BLOOD-2009-04-214957

Raivola, J., Haikarainen, T., Abraham, B. G., & Silvennoinen, O. (2021). Janus Kinases in Leukemia. *Cancers*, *13*(4), 1–20. https://doi.org/10.3390/CANCERS13040800

Raivola, J., Haikarainen, T., & Silvennoinen, O. (2019). Characterization of JAK1 Pseudokinase Domain in Cytokine Signaling. *Cancers*, *12*(1). https://doi.org/10.3390/CANCERS12010078

Rajan, T. V. (2003). The Gell–Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends in Immunology*, *24*(7), 376–379. https://doi.org/10.1016/S1471-4906(03)00142-X

Ramsauer, K., Farlik, M., Zupkovitz, G., Seiser, C., Kröger, A., Hauser, H., & Decker, T. (2007). Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(8), 2849–2854. https://doi.org/10.1073/PNAS.0610944104

Robinson, M. F., Damjanov, N., Stamenkovic, B., Radunovic, G., Kivitz, A., Cox, L., Manukyan, Z., Banfield, C., Saunders, M., Chandra, D., Vincent, M. S., Mancuso, J., Peeva, E., & Beebe, J. S. (2020). Efficacy and Safety of PF-06651600 (Ritlecitinib), a Novel JAK3/TEC Inhibitor, in Patients With Moderate-to-Severe Rheumatoid Arthritis and an Inadequate Response to Methotrexate. *Arthritis & Rheumatology*, *72*(10), 1621–1631. https://doi.org/https://doi.org/10.1002/art.41316

Sabat, R., Grütz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., & Geginat, J. (2010). Biology of interleukin-10. *Cytokine and Growth Factor Reviews*, *21*(5), 331–344. https://doi.org/10.1016/j.cytogfr.2010.09.002

Saharinen, P., & Silvennoinen, O. (2002). The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *Journal of Biological Chemistry*, *277*(49), 47954–47963. https://doi.org/10.1074/jbc.M205156200

Salas, A., Hernandez-Rocha, C., Duijvestein, M., Faubion, W., McGovern, D., Vermeire, S., Vetrano, S., & Vande Casteele, N. (2020). JAK–STAT pathway targeting for the treatment of inflammatory bowel disease. *Nature Reviews Gastroenterology & Hepatology 2020 17:6*, *17*(6), 323–337. https://doi.org/10.1038/s41575-020-0273-0

Saravia, J., Chapman, N. M., & Chi, H. (2019). Helper T cell differentiation. *Cellular & Molecular Immunology 2019 16:7*, *16*(7), 634–643. https://doi.org/10.1038/s41423-019-0220-6

Schroeder, M. A., Khoury, H. J., Jagasia, M., Ali, H., Schiller, G. J., Staser, K., Choi, J., Gehrs, L., Arbushites, M. C., Yan, Y., Langmuir, P., Srinivas, N., Pratta, M., Perales, M.-A., Chen, Y.-B., Meyers, G., & DiPersio, J. F. (2020). A phase 1 trial of itacitinib, a selective JAK1 inhibitor, in patients with acute graft-versus-host disease. *Blood Advances*, *4*(8), 1656–1669. https://doi.org/10.1182/bloodadvances.2019001043

Schwartz, D. M., Bonelli, M., Gadina, M., & O'Shea, J. J. (2016). Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. *Nature Reviews Rheumatology*, *12*(1), 25–36. https://doi.org/10.1038/nrrheum.2015.167

Sécca, C., Faget, D. V., Hanschke, S. C., Carneiro, M. S., Bonamino, M. H., de-Araujo-Souza, P. S., & Viola, J. P. B. (2016). IRF2BP2 transcriptional repressor restrains naive CD4 T cell activation and clonal expansion induced by TCR triggering. *Journal of Leukocyte Biology*, *100*(5), 1081–1091. https://doi.org/10.1189/jlb.2A0815-368R

Shakya, A. K., O'Callaghan, D. J., & Kim, S. K. (2019). Interferon Gamma Inhibits Varicella-Zoster Virus Replication in a Cell Line-Dependent Manner. *Journal of Virology*, *93*(12), 257–276. https://doi.org/10.1128/JVI.00257-19

Shen, P., & Fillatreau, S. (2015). Antibody-independent functions of B cells: a focus on cytokines. *Nature Reviews Immunology 2015 15:7*, *15*(7), 441–451. https://doi.org/10.1038/nri3857

Shuai, K., & Liu, B. (2003). Regulation of JAK–STAT signalling in the immune system. *Nature Reviews Immunology 2003 3:11*, *3*(11), 900–911. https://doi.org/10.1038/nri1226

Shuai, K., & Liu, B. (2005). Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nature Reviews Immunology 2005 5:8*, *5*(8), 593–605. https://doi.org/10.1038/nri1667

Skoda, R. C. (2014). Less Jak2 makes more platelets. *Blood*, *124*(14), 2168–2169. https://doi.org/10.1182/BLOOD-2014-08-596361

Smith, B. W., & Murphy, G. J. (2014). Stem cells, megakaryocytes, and platelets. *Current Opinion in Hematology*, *21*(5), 430. https://doi.org/10.1097/MOH.0000000000000064

Spolski, R., Li, P., & Leonard, W. J. (2018). Biology and regulation of IL-2: from molecular mechanisms to human therapy. *Nature Reviews Immunology 2018 18:10*, *18*(10), 648–659. https://doi.org/10.1038/s41577-018-0046-y

Stadhouders, R., Cico, A., Stephen, T., Thongjuea, S., Kolovos, P., Baymaz, H. I., Yu, X., Demmers, J., Bezstarosti, K., Maas, A., Barroca, V., Kockx, C., Ozgur, Z., van Ijcken, W., Arcangeli, M.-L., Andrieu-Soler, C., Lenhard, B., Grosveld, F., & Soler, E. (2015). Control of developmentally primed erythroid genes by combinatorial co-repressor actions. *Nature Communications*, *6*, 8893. https://doi.org/10.1038/ncomms9893

Strous, G. J., Almeida, A. D. S., Putters, J., Schantl, J., Sedek, M., Slotman, J. A., Nespital, T., Hassink, G. C., & Mol, J. A. (2020). Growth Hormone Receptor Regulation in Cancer and Chronic Diseases. *Frontiers in Endocrinology*, *0*, 867.

https://doi.org/10.3389/FENDO.2020.597573

Taniguchi, T., Ogasawara, K., Takaoka, A., & Tanaka, N. (2001). IRF family of transcription factors as regulators of host defense. *Annual Review of Immunology*, *19*, 623–655. https://doi.org/10.1146/ANNUREV.IMMUNOL.19.1.623

Taylor, P., Weinblatt, M., Burmester, G., Rooney, T., Witt, S., Walls, C., Issa, M., Salinas, C., Saifan, C., Zhang, X., Cardoso, A., González-Gay, M., & Takeuchi, T. (2019). Cardiovascular Safety During Treatment With Baricitinib in Rheumatoid Arthritis. *Arthritis & Rheumatology (Hoboken, N.J.)*, *71*(7), 1042–1055. https://doi.org/10.1002/ART.40841

Telliez, J.-B., Dowty, M. E., Wang, L., Jussif, J., Lin, T., Li, L., Moy, E., Balbo, P., Li, W., Zhao, Y., Crouse, K., Dickinson, C., Symanowicz, P., Hegen, M., Banker, M. E., Vincent, F., Unwalla, R., Liang, S., Gilbert, A. M., … Thorarensen, A. (2016). Discovery of a JAK3-Selective Inhibitor: Functional Differentiation of JAK3-Selective Inhibition over pan-JAK or JAK1-Selective Inhibition. *ACS Chemical Biology*, *11*(12), 3442–3451. https://doi.org/10.1021/ACSCHEMBIO.6B00677

Teng, A. C. T., Kuraitis, D., Deeke, S. A., Ahmadi, A., Dugan, S. G., Cheng, B. L. M., Crowson, M. G., Burgon, P. G., Suuronen, E. J., Chen, H.-H., & Stewart, A. F. R. (2010). IRF2BP2 is a skeletal and cardiac muscle-enriched ischemia-inducible activator of VEGFA expression. *The FASEB Journal*, *24*(12), 4825–4834. https://doi.org/10.1096/fj.10-167049

Tinnikov, A. A., Yeung, K. T., Das, S., & Samuels, H. H. (2009). Identification of a Novel Pathway That Selectively Modulates Apoptosis of Breast Cancer Cells. *Cancer Research*, *69*(4), 1375–1382. https://doi.org/10.1158/0008-5472.CAN-08-2896

Toyoshima, J., Shibata, M., Kaibara, A., Kaneko, Y., Izutsu, H., & Nishimura, T. (2021). Population pharmacokinetic analysis of peficitinib in patients with rheumatoid arthritis. *British Journal of Clinical Pharmacology*, *87*(4), 2014–2022. https://doi.org/10.1111/bcp.14605

Traves, P. G., Murray, B., Campigotto, F., Galien, R., Meng, A., & Di Paolo, J. A. (2021). JAK selectivity and the implications for clinical inhibition of pharmacodynamic cytokine signalling by filgotinib, upadacitinib, tofacitinib and baricitinib. *Annals of the Rheumatic Diseases*. https://doi.org/10.1136/annrheumdis-2020-219012

Trengove, M. C., & Ward, A. C. (2013). Review Article SOCS proteins in development and disease. *American Journal of Clinical and Experimental Immunology*, *2*(1), 1–29. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3714205&tool=pmcentrez&rendertype=abstract

Van Rompaey, L., Galien, R., Van Der Aar, E. M. van der, Clement-Lacroix, P., Nelles, L., Smets, B., Lepescheux, L., Christophe, T., Conrath, K., Vandeghinste, N., Vayssiere, B., Vos, S. De, Fletcher, S., Brys, R., Klooster, G. van 't, Feyen, J. H. M., & Menet, C. (2013). Preclinical Characterization of GLPG0634, a Selective Inhibitor of JAK1, for the Treatment of Inflammatory Diseases. *The Journal of Immunology*, *191*(7), 3568–3577. https://doi.org/10.4049/JIMMUNOL.1201348

Vanhoutte, F., Mazur, M., Voloshyn, O., Stanislavchuk, M., Van der Aa, A., Namour, F., Galien, R., Meuleners, L., & van 't Klooster, G. (2017). Efficacy, Safety, Pharmacokinetics, and Pharmacodynamics of Filgotinib, a Selective JAK-1 Inhibitor, After Short-Term Treatment of Rheumatoid Arthritis: Results of Two Randomized Phase IIa Trials. *Arthritis & Rheumatology*, *69*(10), 1949–1959. https://doi.org/https://doi.org/10.1002/art.40186

Vazquez, M. L., Kaila, N., Strohbach, J. W., Trzupek, J. D., Brown, M. F., Flanagan, M. E., Mitton-Fry, M. J., Johnson, T. A., Tenbrink, R. E., Arnold, E. P., Basak, A., Heasley, S. E., Kwon, S., Langille, J., Parikh, M. D., Griffin, S. H., Casavant, J. M., Duclos, B. A., Fenwick, A. E., … Unwalla, R. (2018). Identification of N-{cis-3-[Methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}propane-1-sulfonamide (PF-04965842): A Selective JAK1 Clinical Candidate for the Treatment of Autoimmune Diseases. *Journal of Medicinal Chemistry*, *61*(3), 1130–1152.

https://doi.org/10.1021/ACS.JMEDCHEM.7B01598/SUPPL_FILE/JM7B01598_SI_002.CSV

Verma, R., Balakrishnan, L., Sharma, K., Khan, A. A., Advani, J., Gowda, H., Tripathy, S. P., Suar, M., Pandey, A., Gandotra, S., Prasad, T. S. K., & Shankar, S. (2016). A network map of Interleukin-10 signaling pathway. *Journal of Cell Communication and Signaling*, *10*(1), 61. https://doi.org/10.1007/S12079-015-0302-X

Verstovsek, S., Talpaz, M., Ritchie, E., Wadleigh, M., Odenike, O., Jamieson, C., Stein, B., Uno, T., & Mesa, R. A. (2017). A phase I, open-label, dose-escalation, multicenter study of the JAK2 inhibitor NS-018 in patients with myelofibrosis. *Leukemia*, *31*(2), 393–402. https://doi.org/10.1038/leu.2016.215

Villarino, A. V., Kanno, Y., & O'Shea, J. J. (2017). Mechanisms and consequences of Jak-STAT signaling in the immune system. In *Nature Immunology* (Vol. 18, Issue 4, pp. 374–384). Nature Publishing Group. https://doi.org/10.1038/ni.3691

Virtanen, A. T., Haikarainen, T., Raivola, J., & Silvennoinen, O. (2019). Selective JAKinibs: Prospects in Inflammatory and Autoimmune Diseases. In *BioDrugs* (Vol. 33, Issue 1, pp. 15–32). Springer International Publishing. https://doi.org/10.1007/s40259-019-00333-w

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology 2008 9:5*, *9*(5), 503–510. https://doi.org/10.1038/ni1582

Wang, E. Q., Le, V., O'Gorman, M., Tripathy, S., Dowty, M. E., Wang, L., & Malhotra, B. K. (2021). Effects of Hepatic Impairment on the Pharmacokinetics of Abrocitinib and Its Metabolites. *Journal of Clinical Pharmacology*, *61*(10), 1311–1323. https://doi.org/10.1002/jcph.1858

Wang, G., Tajima, M., Honjo, T., & Ohta, A. (2021). STAT5 interferes with PD-1 transcriptional activation and affects CD8+ T-cell sensitivity to PD-1-dependent immunoregulation. *International Immunology*, *33*(11), 563–572. https://doi.org/10.1093/INTIMM/DXAB059

Wang, I., Lin, H., Goldman, S., & Kobayashi, M. (2004). STAT-1 is activated by IL-4 and IL-13 in multiple cell types. *Molecular Immunology*, *41*(9), 873–884. https://doi.org/10.1016/J.MOLIMM.2004.04.027

Wehinger, J., Gouilleux, F., Groner, B., Finke, J., Mertelsmann, R., & Weber-Nordt, R. M. (1996). IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS Letters*, *394*(3), 365–370. https://doi.org/10.1016/0014-5793(96)00990-8

Westhovens, R. (2019). Clinical efficacy of new JAK inhibitors under development. Just more of the same? *Rheumatology (Oxford, England)*, *58*(Suppl 1), i27. https://doi.org/10.1093/RHEUMATOLOGY/KEY256

Wilks, A. F. (2008). The JAK kinases: Not just another kinase drug discovery target. *Seminars in Cell & Developmental Biology*, *19*(4), 319–328. https://doi.org/10.1016/J.SEMCDB.2008.07.020

Williams, N., Bamert, R., Patel, O., Wang, C., Walden, P., Wilks, A., Fantino, E., Rossjohn, J., & Lucet, I. (2009). Dissecting specificity in the Janus kinases: the structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 protein tyrosine kinase domains. *Journal of Molecular Biology*, *387*(1), 219–232. https://doi.org/10.1016/J.JMB.2009.01.041

Winthrop, K. L. (2017). The emerging safety profile of JAK inhibitors in rheumatic disease. *Nature Reviews Rheumatology 2017 13:4*, *13*(4), 234–243. https://doi.org/10.1038/nrrheum.2017.23

Wrobleski, S. T., Moslin, R., Lin, S., Zhang, Y., Spergel, S., Kempson, J., Tokarski, J. S., Strnad, J., Zupa-Fernandez, A., Cheng, L., Shuster, D., Gillooly, K., Yang, X., Heimrich, E., McIntyre, K. W., Chaudhry, C., Khan, J., Ruzanov, M., Tredup, J., … Weinstein, D. S. (2019). Highly Selective Inhibition of Tyrosine Kinase 2 (TYK2) for the Treatment of Autoimmune Diseases: Discovery of the Allosteric Wrobleski, S. T., Moslin, R., Lin, S.,

Zhang, Y., Spergel, S., Kempson, J., Tokarski, J. S., Strnad, J., Zupa-Fernandez, A., Chen. *Journal of Medicinal Chemistry*, *62*(20), 8973–8995. https://doi.org/10.1021/acs.jmedchem.9b00444

Yeh, T. C., Dondi, E., Uzé, G., & Pellegrini, S. (2000). A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-alpha signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(16), 8991–8996. https://doi.org/10.1073/PNAS.160130297

Yeung, K. T., Das, S., Zhang, J., Lomniczi, A., Ojeda, S. R., Xu, C.-F., Neubert, T. A., & Samuels, H. H. (2011). A novel transcription complex that selectively modulates apoptosis of breast cancer cells through regulation of FASTKD2. *Molecular and Cellular Biology*, *31*(11), 2287–2298. https://doi.org/10.1128/MCB.01381-10

Ytterberg, S. R., Bhatt, D. L., Mikuls, T. R., Koch, G. G., Fleischmann, R., Rivas, J. L., Germino, R., Menon, S., Sun, Y., Wang, C., Shapiro, A. B., Kanik, K. S., Connell, C. A., & ORAL Surveillance Investigators. (2022). Cardiovascular and Cancer Risk with Tofacitinib in Rheumatoid Arthritis. *The New England Journal of Medicine*, *386*(4), 316–326. https://doi.org/10.1056/NEJMOA2109927

Zenke, K., Muroi, M., & Tanamoto, K. ichi. (2018). IRF1 supports DNA binding of STAT1 by promoting its phosphorylation. *Immunology and Cell Biology*, *96*(10), 1095–1103. https://doi.org/10.1111/imcb.12185

Zheng, J., Xin, Y., Zhang, J., Subramanian, R., Murray, B. P., Whitney, J. A., Warr, M. R., Ling, J., Moorehead, L., Kwan, E., Hemenway, J., Smith, B. J., & Silverman, J. A. (2018). Pharmacokinetics and disposition of momelotinib revealed a disproportionate human metabolite - Resolution for clinical development. *Drug Metabolism and Disposition*, *46*(3), 237–247. https://doi.org/10.1124/dmd.117.078899

Zhong, J., Sharma, J., Raju, R., Palapetta, S. M., Prasad, T. S. K., Huang, T.-C., Yoda, A., Tyner, J. W., Bodegom, D. van, Weinstock, D. M., Ziegler, S. F., & Pandey, A. (2014). TSLP signaling pathway map: a platform for analysis of TSLP-mediated signaling. *Database: The Journal of Biological Databases and Curation*, *2014*. https://doi.org/10.1093/DATABASE/BAU007

9 PUBLICATIONS

PUBLICATION I

IRF2BP2 **mutation is associated with increased STAT1 and STAT5 activation in two family members with inflammatory conditions and lymphopenia.**

Palmroth M, Viskari H, Seppänen MRJ, Keskitalo S, Virtanen A, Varjosalo M, Silvennoinen O, Isomäki P.

Pharmaceuticals (Basel), 2021. 14(8):797.
doi: 10.3390/ph14080797

pharmaceuticals

MDPI

Case Report

IRF2BP2 Mutation Is Associated with Increased STAT1 and STAT5 Activation in Two Family Members with Inflammatory Conditions and Lymphopenia

Maaria Palmroth [1], Hanna Viskari [2,3], Mikko R. J. Seppänen [4], Salla Keskitalo [5], Anniina Virtanen [1], Markku Varjosalo [5], Olli Silvennoinen [1,6,7] and Pia Isomäki [1,8,*]

[1] Molecular Immunology Group, Faculty of Medicine and Health Technology, Tampere University, 33520 Tampere, Finland; maaria.palmroth@tuni.fi (M.P.); anniina.t.virtanen@tuni.fi (A.V.); olli.silvennoinen@tuni.fi (O.S.)
[2] Department of Internal Medicines, Tampere University Hospital, 33520 Tampere, Finland; hanna.viskari@pshp.fi
[3] Faculty of Medicine and Life Sciences, Tampere University, 33520 Tampere, Finland
[4] Rare Disease and Pediatric Research Centers, Children's Hospital, University of Helsinki and Helsinki University Hospital, 00290 Helsinki, Finland; mikko.seppanen@hus.fi
[5] Molecular Systems Biology Group, Institute of Biotechnology, University of Helsinki, 00790 Helsinki, Finland; salla.keskitalo@helsinki.fi (S.K.); markku.varjosalo@helsinki.fi (M.V.)
[6] Fimlab Laboratories, Pirkanmaa Hospital District, 33520 Tampere, Finland
[7] HiLIFE Helsinki Institute of Life Sciences, Institute of Biotechnology, University of Helsinki, 00790 Helsinki, Finland
[8] Centre for Rheumatic Diseases, Tampere University Hospital, 33520 Tampere, Finland
* Correspondence: pia.isomaki@tuni.fi

Citation: Palmroth, M.; Viskari, H.; Seppänen, M.R.J.; Keskitalo, S.; Virtanen, A.; Varjosalo, M.; Silvennoinen, O.; Isomäki, P. *IRF2BP2* Mutation Is Associated with Increased STAT1 and STAT5 Activation in Two Family Members with Inflammatory Conditions and Lymphopenia. *Pharmaceuticals* **2021**, *14*, 797. https://doi.org/10.3390/ph14080797

Academic Editor: Lawrence Marc Pfeffer

Received: 2 July 2021
Accepted: 11 August 2021
Published: 13 August 2021

Abstract: Interferon regulatory factor 2 binding protein 2 (IRF2BP2) is a transcriptional coregulator that has an important role in the regulation of the immune response. IRF2BP2 has been associated with the Janus kinase (JAK)—signal transducers and activators of transcription (STAT) pathway, but its exact role remains elusive. Here, we identified a novel clinical variant, *IRF2BP2* c.625_665del, from two members of a family with inflammatory conditions and investigated the function of IRF2BP2 and c.625_665del mutation in JAK–STAT pathway activation and inflammatory signaling. The levels of constitutive and cytokine-induced phosphorylation of STATs and total STAT1 in peripheral blood monocytes, T cells, and B cells from the patients and four healthy controls were measured by flow cytometry. Inflammation-related gene expression was studied in peripheral blood mononuclear cells using direct digital detection of mRNA (NanoString). Finally, we studied the relationship between IRF2BP2 and STAT1 activation using a luciferase reporter system in a cell model. Our results show that patients having the *IRF2BP2* c.625_665del mutation presented overexpression of STAT1 protein and increased constitutive activation of STAT1. In addition, interferon-induced JAK–STAT signaling was upregulated, and several interferon-inducible genes were overexpressed. Constitutive phosphorylation of STAT5 was also found to be upregulated in $CD4^+$ T cells from the patients. Using a cell model, we show that IRF2BP2 was needed to attenuate STAT1 transcriptional activity and that IRF2BP2 c.625_665del mutation failed in this. We conclude that IRF2BP2 has an important role in suppressing immune responses elicited by STAT1 and STAT5 and suggest that aberrations in IRF2BP2 can lead to abnormal function of intrinsic immunity.

Keywords: interferon regulatory factor-2 binding protein-2 (IRF2BP2); JAK–STAT pathway; interferons; inflammation

1. Introduction

Interferon regulatory factor 2 binding protein 2 (IRF2BP2) is a transcriptional coregulator having an important role in the regulation of immune response [1]. IRF2BP2 was originally identified as a co-repressor of IRF2 [2], which counteracts IRF1 and thereby

suppresses interferon signaling [3]. However, IRF2BP2 is also expressed in organisms lacking IRF2 [2], and subsequent studies have identified IRF2BP2 as a coregulator of various other transcription factors, such as VGLL4, NCOR1, ETO2, IRF2BPL, NFAT1, NRIF3, GR, and KLF2 [4–11]. In addition to the immune response, IRF2BP2 is involved in a variety of biological processes, such as apoptosis, angiogenesis, cell cycle, and cell differentiation [4–6,8,11,12]. The importance of IRF2BP2 is highlighted by the observation that IRF2BP2-deficient mice are rarely obtained and do not survive past 4 weeks due to severe growth retardation [5].

The Janus kinase (JAK)—signal transducers and activators of transcription (STAT) pathway constitutes a major signaling module in the immune system. JAKs are intracellular non-receptor tyrosine kinases that mediate signaling of about 60 different cytokines, hormones, and growth factors, including, but not limited to, interferons (IFN) and interleukins (IL) [13]. The JAK family consists of four members (JAK1–3 and TYK2), which transmit the cytokine-induced signal by phosphorylating the receptor and cellular signaling proteins such as STATs that act as transcription factors. Mammals encode seven STATs, namely, STAT1–4, STAT5a, STAT5b, and STAT6, which are major determinants of cytokine-specific gene responses [14]. The JAK–STAT signaling pathway is under tight regulation and is modulated by multiple regulatory proteins [15].

The clinical significance of IRF2BP2 has become evident in recent years, as familial *IRF2BP2* germline mutations causing inborn errors of immunity have been reported [16,17]. Defects in IRF2BP2 are also associated with cancer, as an *IRF2BP2–retinoic acid receptor alpha* (*RARA*) fusion gene and transcript has been detected in acute promyelocytic leukemia patients [18–23]. The association between IRF2BP2 and JAK–STAT signaling has been suggested previously. IRF2BP2 was shown to decrease IL-2-induced JAK–STAT5 signaling in mouse $CD4^+$ T cells [24]. In addition, since IRF2 has been shown to repress IFNα and IFNβ-induced gene expression in mice [3], IRF2BP2 as a co-repressor of IRF2, could be expected to have similar role in IFNα/β signaling.

In the current study, we identified the novel variant *IRF2BP2* c.625_665del from a family of two individuals with inflammatory conditions. We examined the connection between IRF2BP2 and JAK–STAT signaling in vivo using patient samples and studied the potential effects of the clinical variant on JAK–STAT signaling pathways in a cell model.

2. Results

2.1. Clinical History

Our index patient was a 57-year-old HIV-negative male who had had severe acne and hidradenitis suppurativa requiring surgical revisions during adolescence and adulthood. At the age of 51, he underwent uncomplicated cholecystectomy and umbilical hernia repair. Soon after, he started to have subfebrile fever and fatigue, and lost up to 24 kg of weight (starting weight, 103 kg). He presented with mild lymphadenopathy, moderate splenomegaly (15 cm), and marked lymphopenia (400–650 cells/uL). Gastroscopy revealed a healing erosion in the duodenum. After thorough examinations with no other finding (colonoscopy, capsule endoscopy, bone marrow biopsy, PET-CT, HRCT) the symptoms and weight loss ceased. Three years later, the symptoms returned, and a new symptom of extensive painful oral and genital ulcers presented. He developed a deep neck abscess that was surgically drained with the extraction of a molar tooth. Ulcer problems with occasional stomachache continued. A strictured small intestine, probably leading to partial occlusion of the bowel, was noted in re-endoscopy. Biopsies from tongue and genital lesions showed unspecific ulceration with neutrophilic inflammation. Immunological analyses did not reveal antibody deficiency (Supplementary Table S1). Oral ulcers have partially responded to repeated corticosteroid injections into oral mucosa, but new lesions appear regularly.

Besides his 71-year-old sister, the index had no close living relatives. His sister had also been suffering from hidradenitis suppurativa for over 10 years and showed significant lymphopenia but no ulcer problems or a marked infection history. In addition, she has diabetes mellitus type 2, hypertension, and hyperthyroidism. At the age of 71 years, she

developed difficulties keeping her head position and cramping of the arms. She was diagnosed with dystrofia myotonica type 2 with typical CCTG expansion in *CNBP* gene.

2.2. Family Members Possess a Heterozygous IRF2BP2 Variant

Due to unique clinical features combined with lymphopenia, the index patient was subjected to whole-exome sequencing. A novel heterozygous 41-bp deletion variant in *IRF2BP2*, c.625–665del, p. (Ala209Glnfs*31) was noted. This 41-bp deletion generates a frameshift leading to a premature stop codon at position 31 in the new reading frame. No previously established disease genes were detected for the index patient. The index patient's sister also carried the heterozygous 41-bp deletion in *IRF2BP2*.

2.3. Phosphorylation of STAT1 and STAT5 Are Altered in Circulating Leukocytes of the Family Members

Since IRF2BP2 is suggested to regulate cytokine-induced JAK–STAT pathways, we studied constitutive and cytokine-induced STAT phosphorylation in circulating immune cells from the index patient and his sister using a multicolor flow cytometric assay. As the staining procedures were performed without cell purification or manipulation using fresh blood, the results are likely to reflect the phosphorylation status of STAT proteins in vivo.

Constitutive phosphorylation of STAT1 in $CD4^+$ T cells and monocytes was slightly increased in the patients when compared with healthy controls (Figure 1A). Type I and type II interferon-induced phosphorylation of STAT1 was markedly higher in the patients than in the controls in all investigated cell populations (Figure 1B–D). This difference was more prominent in the index patient than in his sister in all cell populations, and the most notable differences were seen in monocytes. IL-6-induced STAT1 phosphorylation in $CD4^+$ T cells and monocytes was modestly increased in both patients.

Flow cytometric analysis showed also differences in the phosphorylation of STAT5. Constitutive STAT5 phosphorylation was significantly higher in $CD4^+$ T cells from the patients compared to the controls (Figure 1E). In contrast, cytokine-stimulated STAT5 phosphorylation in $CD4^+$ T cells did not generally differ between patients and controls, except for increased IL-7-induced STAT5 phosphorylation in the index patient (Figure 1F).

Constitutive as well as IFNα- and IFNβ-induced phosphorylation of STAT4 were also studied. STAT4 phosphorylation was induced by interferons only in $CD4^+$ T cells, and this induction was slightly higher in the index patient than in the controls. (Supplementary Figure S1). Constitutive or cytokine-induced phosphorylation levels of STAT3 and STAT6 did not differ between family members and controls in any leukocyte population studied (Supplementary Figure S1).

2.4. STAT1- and IFN-Regulated Genes Are Upregulated in the Family Members

Next, NanoString analysis was used for direct detection of mRNA levels of JAK–STAT pathway genes, genes involved in IFN response, and other inflammation-related genes in peripheral blood mononuclear cells (PBMCs) from patients and controls. Regarding the JAK–STAT pathway, increased mRNA levels of *STAT1*, but no other *JAK* or *STAT* genes, were demonstrated in both family members (Figure 2A). Upregulation of STAT1 was also shown at protein level, as STAT1 expression was strongly upregulated in the index patient and slightly increased in his sister in all studied cell populations, using flow cytometry (Figure 2B).

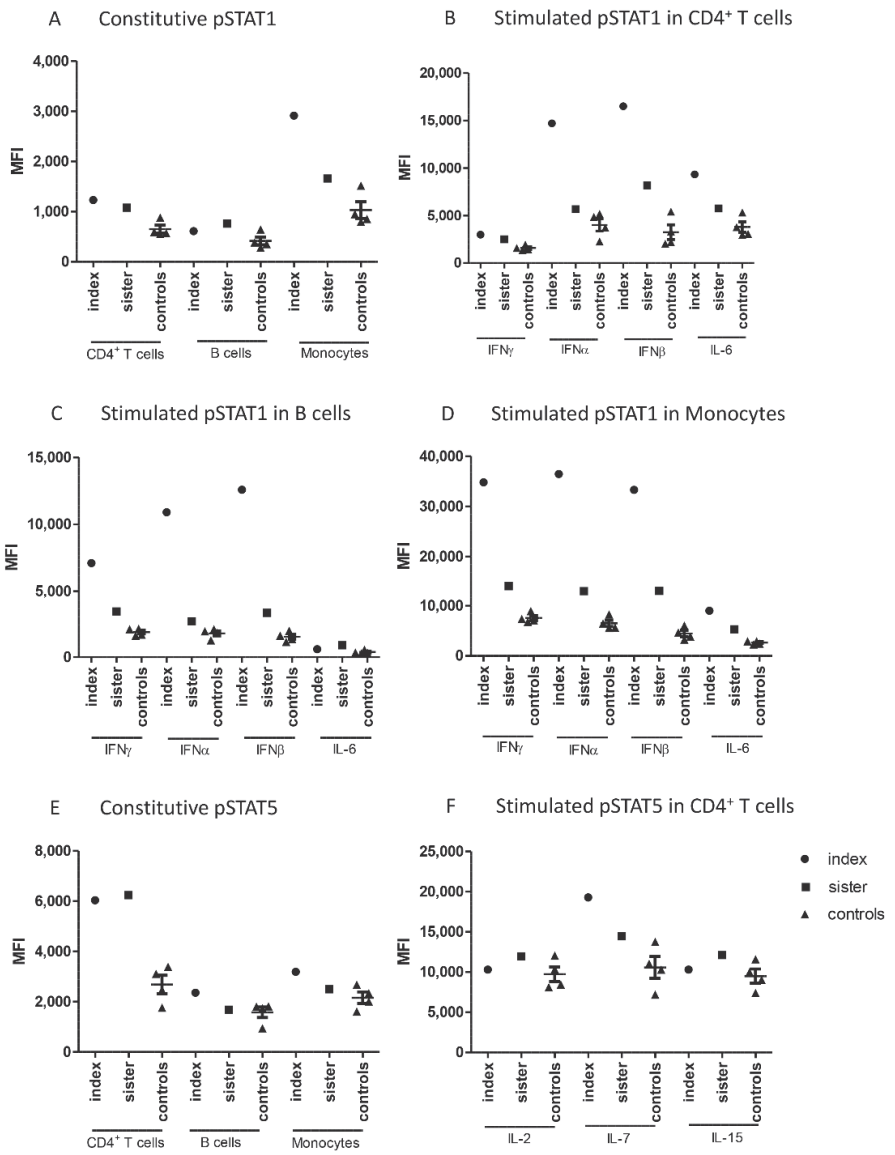


Figure 1. Median fluorescence intensities (MFI) measured in cells from patients and control subjects by flow cytometry for (**A**) Constitutive pSTAT1 in CD4$^+$ T cells, B cells, and monocytes, (**B**) Stimulated pSTAT1 in CD4$^+$ T cells, (**C**) Stimulated pSTAT1 in B cells, (**D**) Stimulated pSTAT1 in monocytes, (**E**) Constitutive pSTAT5 in CD4$^+$ T cells, B cells, and monocytes (**F**) Stimulated pSTAT5 in CD4$^+$ T cells. IFN; interferon, IL; interleukin, MFI; median fluorescence intensity, STAT; signal transducer and activator of transcription.

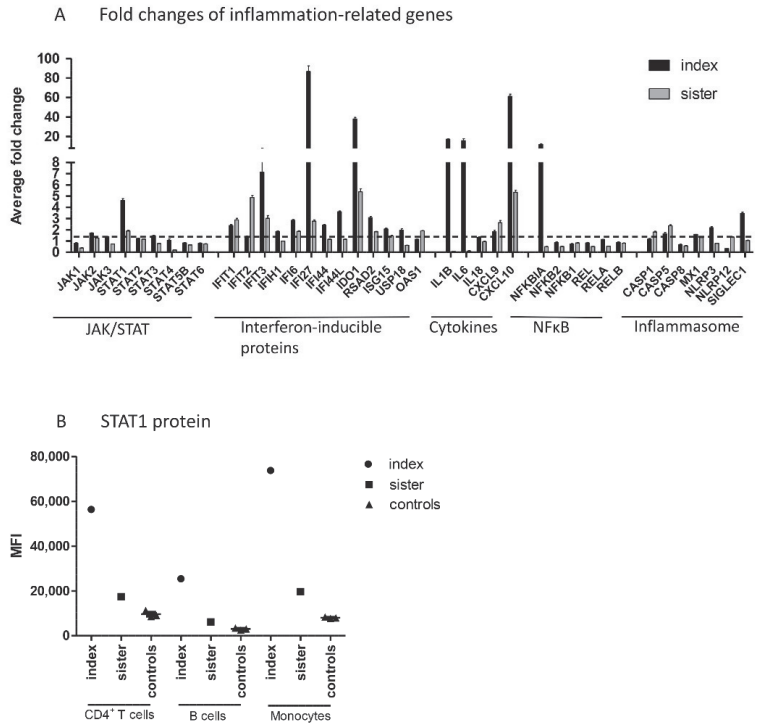


Figure 2. (**A**) Fold changes of inflammation-related genes in PBMCs from patients compared to those from control subjects. Inflammation-related custom gene set of the JAK–STAT pathway, interferon-inducible proteins, cytokines, NFκB pathway, and inflammasome activation were measured using the NanoString technology. Average fold changes of three technical replicates are presented. Dashed line represents a fold change of 1.5 that was considered as a threshold for increased gene expression, (**B**) STAT1 protein level in $CD4^+$ T cells, B cells, and monocytes measured in cells from patients and control subjects by flow cytometry. IFN; interferon, JAK; Janus kinase, MFI; median fluorescence intensity, NFκB; nuclear factor kappa-light-chain-enhancer of activated B cells, STAT; signal transducer and activator of transcription, PBMC; peripheral blood mononuclear cell.

In addition to *STAT1*, both family members had increased mRNA levels of interferon-inducible proteins *IFIT1*, *IFIT3*, *IFI6*, *IFI27*, *IDO1*, and *RSAD2*, cytokines *CXCL9* and *CXCL10*, and *CASP5* (10 common upregulated genes; fold change $\geq$1.5). In addition, the index patient, but not his sister, showed strong upregulation of *IL-1B*, *IL-6*, and *NFκBIA*. Altogether, the index patient had 12 upregulated genes that showed either normal or downregulated expression in the sister. The index patient and sister had no common downregulated genes (fold change $\leq$0.5).

2.5. IRF2BP2 Is Needed to Attenuate Constitutive and Cytokine-Induced STAT1 Transcriptional Activity

To study the relationship between IRF2BP2 and JAK–STAT pathway activation more directly, we used a dual-luciferase assay and analyzed the effects of wild-type IRF2BP2 and IRB2BP2 Δ625–665 on STAT1-mediated transcription. For this purpose, a reporter construct including an interferon-stimulated response element (ISRE-Luc) was used.

The reporter assay showed that wild-type IRF2BP2 significantly suppressed constitutive and cytokine-stimulated STAT1 transcriptional activity in HEK293T cells, when compared to control (Figure 3A). Importantly, IRF2BP2 Δ625–665 was not able to suppress

the transcriptional activity. The expression of wild-type and Δ625–665 IRF2BP2 in HEK293T cells was shown by immunoblotting (Figure 3B).

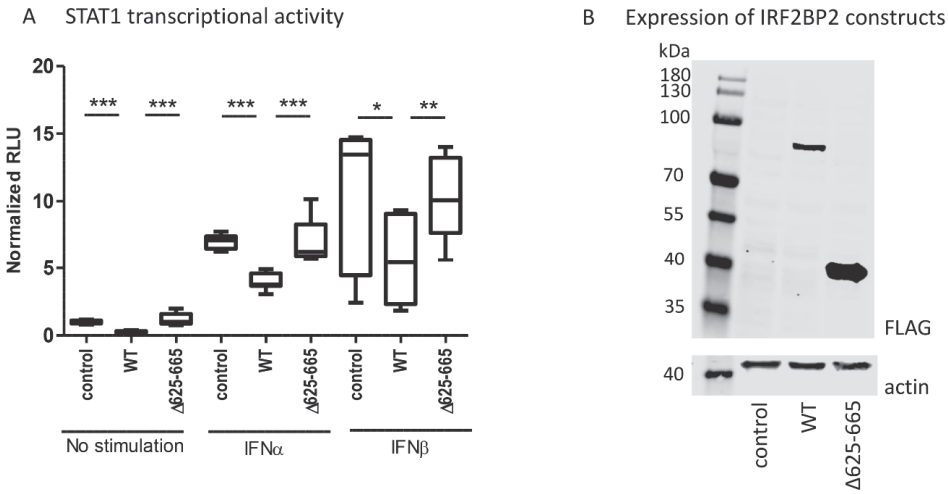


Figure 3. (**A**) The effect of wild-type IRF2BP2 and IRF2BP2 Δ625-665 on STAT1 transcriptional activity was studied using the ISRE-Luc reporter in HEK293T cells. (**B**) Detection of wild-type IRF2BP2 and IRF2BP2 Δ625-665 expression in HEK293T cells by immunoblotting. Statistical analyses were performed on three independent experiments, and Student's two-tailed T-test was used to calculate significant differences. Significant differences are marked with an asterisk: * $p \leq 0.05$, ** $p \leq 0.01$, p *** ≤ 0.001. IFN; interferon, IRF2BP2; interferon regulatory factor 2 binding protein 2, RLU; relative luciferase unit, STAT; signal transducer and activator of transcription.

3. Discussion

In this study, we describe a novel variant of *IRF2BP2*, c.625_665del p.(Ala209Glnfs*31), which was present in two members of a family with inflammatory conditions and lymphopenia. The patients demonstrated increased levels of STAT1 at both mRNA and protein levels, as well as increased constitutive and cytokine-induced STAT1 phosphorylation in circulating leukocytes. In addition, constitutive STAT5 phosphorylation was increased in $CD4^+$ T cells. We also demonstrate that wild-type IRF2BP2, but not IRF2BP2 Δ625–665, attenuated STAT1 transcriptional activity in a cell model.

The IRF2BP2 protein is an 87 kDa protein consisting of 587 amino acids. It contains two conserved regions: an N-terminal zinc finger and a C-terminal RING domain [2]. The zinc finger typically binds to specific DNA sequences [25], while the RING domain has been shown to be essential in protein–protein interactions formed by IRF2BP2 in transcriptional repressor complexes [2,5,6]. In addition, RING domain-containing proteins play a crucial role in transferring ubiquitin to substrate proteins or other RING domain-containing proteins [26].

Until now, germline *IRF2BP2* mutations have been reported in two families [16,17], this study reporting the third family and mutation described. Keller et al. identified a heterozygous point mutation in *IRF2BP2* (c.1652G > A: p. (S551N)) from three members of a family diagnosed with common variable immunodeficiency disorder (CVID) and showed in vitro that this variant decreased B cell maturation into plasmablasts [16]. Baxter et al., in turn, discovered a heterozygous *IRF2BP2* variant (c.1606insTTT, p.Q536delinsX) that produces a truncated protein at position 536 of 587. This variant was suggested as one of the candidate mutations in childhood-onset immune dysregulation, the patient having a similar phenotype as patients reported by Keller et al. [17]. The clinical variant described in the current study generates a truncated protein of only 238 amino acids of 587 and entirely lacks the C-terminal RING domain. Thus, all three described clinical *IRF2BP2*

variants affect the RING domain. Despite the low patient numbers, interestingly, the patient phenotype in the current study is more inflammatory than immunodeficient, as the patients did not present recurrent infections, in contrast to patients in earlier studies [16,17]. Mucosal ulcers in the index patient together with increased *CASP5* transcription resemble both the first described systemic non-canonical inflammasomopathy due to a neomorphic *CEBPE* mutation and Behçet disease [27] and require further study in a larger cohort.

IRF2BP2 was first described as a co-repressor of IRF2 [2]. IRF1 and IRF2 are transcription factors that have been shown to compete in binding to the same DNA-binding elements, interferon-stimulated response elements (ISRE), and are activated in response to type I interferons (IFNs). However, they have opposite functions: IRF1 activates IFN-induced gene expression, while IRF2 represses it [28,29]. IRF1 has also been shown to be as essential as STAT1 in activating IFNγ-induced gene expression [30], suggesting a role for IRF2 also in type II IFN signaling.

Our data showed a systematic hyperactivation of the IFN–JAK–STAT1 axis, as the patients had increased constitutive STAT1 phosphorylation in T cells and monocytes as well as increased IFN-induced STAT1 phosphorylation in all leukocyte subtypes studied. In addition, several type I IFN inducible genes, such as *IFIT1*, *IFIT3*, *IFI6*, and *IFI27*, as well as the type II IFN-induced genes *IDO1*, *CXCL9*, and *CXCL10*, were overexpressed in PBMC from the patients. Finally, a reporter assay including the ISRE binding site showed that IRF2BP2 has a repressive role in STAT1 transcriptional activity. Taken together, our data regarding type I IFN signaling are consistent with the prevailing hypothesis of IRF2BP2 being a co-repressor of IRF2 and suggest that the increased STAT1 activation observed in the patients could be explained by the lack of suppressive effects on IRF1 function. The suggested roles of wild-type and Δ625–665 variants of IRF2BP2 in the type I IFN signaling pathway are illustrated in Figure 4.

Since gamma-activated sequences (GAS) recognized by IFNγ-induced STAT1 dimers do not contain binding sites for IRF1 [31], the mechanism how IRF2BP2 regulates type II IFN signaling probably differs from that involved in type I IFN signaling. IRF1 has been shown to form heterodimers with STAT1 and regulate the expression of *LMP2*, whose promoter includes a GAS binding site [32], but also to promote STAT1 binding to GAS without direct interaction [33]. Thus, IRF2BP2 function in type II IFN signaling might also include IRF1/2. However, more mechanistic data are still needed to confirm whether the repressive function of IRF2BP2 on STAT1 activation and IFN-induced gene expression occurs via IRF1/2.

The patients in the current study had increased levels of STAT1, both at mRNA and at protein level. Interestingly, IRF1 has been shown to induce the expression of STAT1 and pSTAT1 during viral infection [34]. The expression of both STAT1 and IRF1 is induced upon IFN stimulation [30]. This suggests that the described IRF2BP2 variant indirectly upregulates pSTAT levels: the increased constitutive and IFN-stimulated pSTAT1 levels in the patients were likely to result from overexpression of STAT1, as the induction of IFN-stimulated genes is not limited by the IRF2–IRF2BP2 complex.

IRF2BP2 has been associated with STAT5 function in a previous study concerning the role of IRF2BP2 in $CD4^+$ T cell activation [24]. Sécca et al. demonstrated that overexpression of IRF2BP2 in mouse $CD4^+$ T cells impaired IL-2-induced STAT5 activation and thereby restricted IL-2 high-affinity receptor α-chain expression and $CD4^+$ T cell proliferation [24]. Interestingly, our data showed that both patients had increased constitutive STAT5 phosphorylation levels in $CD4^+$ T cells compared to healthy controls, but not in other cell types studied. This finding supports the hypothesis of IRF2BP2 being a repressor of $CD4^+$ T cell activation. However, the mechanism by which IRF2BP2 mutation affects STAT5 activation remains elusive and requires further studies.

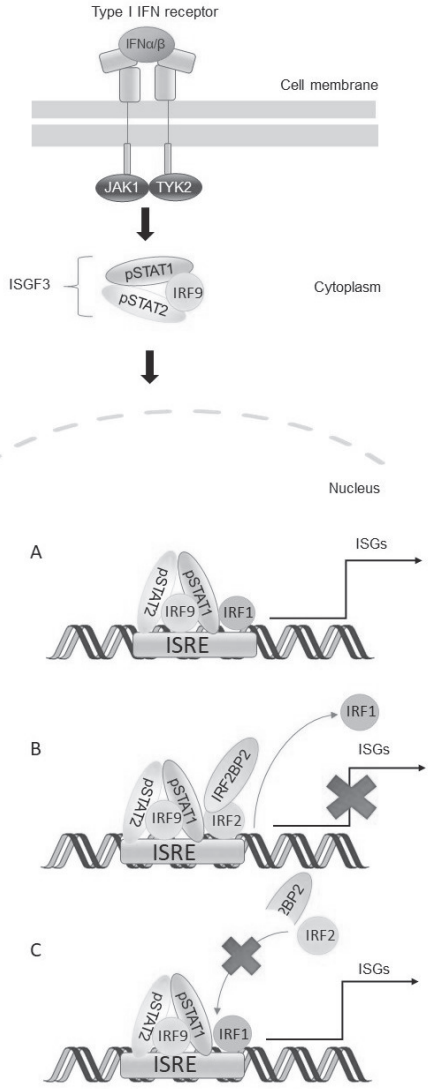


Figure 4. A schematic picture illustrating the suggested role of IRF2BP2 in type I interferon (IFN) signaling. IFN binding to their receptors activates JAKs, which phosphorylate STATs. Upon IFNα or IFNβ stimulation, the transcription factor complexes called interferon-stimulated gene factor 3 (ISGF3) that consist of STAT1, STAT2, and interferon regulatory factor 9 (IRF9) are formed. These transcription factor complexes translocate to the nucleus and bind to interferon-stimulated response elements (ISRE). (**A**) IRF1 is a transcription factor that co-operates with ISGF3 to induce interferon-stimulated genes (ISGs). (**B**) IRF2 restricts the induction of ISGs by competing with IRF1 for the same binding site. IRF2BP2 acts as a co-repressor of IRF2 by binding to it through the C-terminal RING domain and is essential for IRF2 ability to limit the expression of ISGs. (**C**) Patient mutation *IRF2BP2* c.625_665del generates a truncated IRF2BP2 protein (238 amino acids of 587) that entirely lacks the RING domain. Truncated IRF2BP2 cannot bind to IRF2, which in turn is unable to bind DNA and suppress IRF1 function. IFN; interferon, IRF; interferon regulatory factor, IRF2BP2; interferon regulatory factor 2 binding protein 2, ISGF3; interferon-stimulated gene factor 3, ISRE; interferon-stimulated response element, JAK; Janus kinase, STAT; signal transducer and activator of transcription.

Besides widening our knowledge on the mechanisms behind immunological diseases, studying immune responses in patients with novel genetic variants may provide clues to possible effective therapies. Our current results suggest that patients with loss-of-function IRF2BP2 mutations might benefit from treatments that inhibit interferon signaling. Finding an effective type I interferon antagonist therapy for systemic lupus erythmatosus (SLE) patients has been a major area of interest in recent years, as SLE patients show increased circulating type I interferon levels as well as overexpression of IFN-induced genes [35]. Anifrolumab, an antibody which blocks IFNAR1 receptor, is the first approved IFN antagonist therapy and received FDA approval in July 2021 [36]. Another alternative for inhibiting interferon signaling would be to block the signaling cascade one step further by using JAK inhibitor treatment. JAK inhibitors are small molecular kinase inhibitors that already include several approved inhibitors for rheumatologic, dermatologic, hematologic, and gastrointestinal indications, as well as emergency authentication for COVID-19 [37]. Given that also STAT5 was activated in T cells from patients in the current study, JAK inhibitors, that have the potential to inhibit multiple JAK–STAT pathways simultaneously, might provide a more potent therapeutic alternative than IFN antagonists for patients with loss-of-function *IRF2BP2* mutations.

The studied family had only two patients with *IRF2BP2*, c.625_665del, and the results should be considered preliminary. However, since similar effects of the *IRF2BP2* variant on STAT activation were demonstrated both with patient samples and using a cell model, the repressive role of IRF2BP2 on JAK–STAT signaling pathways seems credible. Therefore, the current study significantly adds to our knowledge of the regulatory function of IRF2BP2 in cytokine signaling.

4. Materials and Methods

4.1. Patient Samples

A family of two individuals (57-year-old index male and his 71-year-old sister) with inflammatory conditions were enrolled in the present study. Further, 3 healthy male subjects (54, 56, and 58 years of age) and 1 healthy female subject (72-year-old) were enrolled as controls. Blood samples were drawn at the same time for all the analyses, except for genetic studies that were performed separately.

4.2. Genetic Studies

Whole-exome test targeting all protein-coding exons, exon–intron boundaries (±20 bps), and selected clinically relevant non-coding variants was performed for both index patient and his sister. The analysis was performed using the Illumina sequencing system. Quality controlled sequence reads of each sample were mapped to the human reference genome (GRCh37/hg19), and reads were aligned using the Burrows–Wheeler Aligner (BWA-MEM). Alignment post-processing and variant calling were performed using GATK algorithms (Sentieon).

The pathogenic potential of variants was predicted by taking into account the predicted consequence, biochemical properties of the codon change, degree of evolutionary conservation, as well as allelic frequencies from large population studies (1000 Genomes project, gnomAD) and mutation databases (HGMD, ClinVar), as well as an in-house variant database.

4.3. Flow Cytometry

Constitutive and cytokine-induced STAT phosphorylation and level of total STAT1 were studied with multicolor flow cytometric analysis in circulating immune cells. First, 50 μL aliquots of fresh blood samples were either left unstimulated or stimulated with 100 ng/mL recombinant IFN-β, IFN-γ, IL-2, IL-4, IL-6, IL-7, IL-15, IL-21 (all from Peprotech, Rocky Hill, NJ, USA), IFN-α (Cell Signaling Technology, Danver, MA, USA), or IL-10 (R&D Systems, Minneapolis, MN, USA) for 15 min at 37 °C. The stimulation was terminated by transferring the samples on ice. Leukocytes were then fixed, and red blood cells lysed

using BD Phosflow Lyse/Fix buffer (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA) for 10 min at 37 °C, washed with PBS, and permeabilized in ice-cold methanol for 10 min on ice followed by 2–3 days at −80 °C. Following two washes with PBS, samples were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone SK7), allophycocyanin (APC)-conjugated anti-CD33, phycoerythrin-cyanin 7 (PE-Cy7)-conjugated anti-CD20 (clone H1(FB1)) (all from BD), APC-eFluor 780-conjugated anti-CD4 (clone SK3) (eBioscience, Santa Clara, CA, USA), phycoerythrin (PE)-conjugated STAT1 (clone 1/Stat1), phospho-STAT1 (clone 4a), phospho-STAT3 (clone 4/P-STAT3), phospho-STAT4 (clone 38/p-Stat4), phospho-STAT5 (clone 47), or phospho-STAT6 (18/P-Stat6) (all from BD) for 30 min at room temperature.

Data acquisition was performed using FACS Canto II (BD), and the analysis of flow cytometer data using Flowjo Single cell analysis software (BD). Monocytes were gated based on CD33 positivity and light scattering characteristics (SSC-A) (Figure 5A). Lymphocytes were gated based on light scattering characteristics (SSC-A and FSC-A) (Figure 5B). Among lymphocytes, $CD4^+$ T cells were gated from the $CD3^+$ lymphocyte population, and $CD20^+$ B cells from the $CD3^-$ lymphocyte population (Figure 5C,D). A PE fluorescence histogram was created for each cell population, and the median fluorescence intensity (MFI, arithmetic median) was calculated. Examples of cytokine-induced pSTATs in different cell populations are presented in Figure 5E–G.

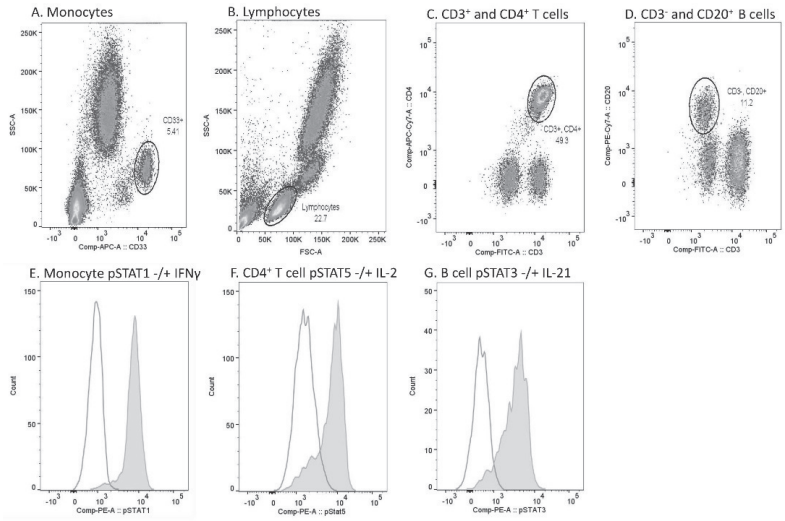


Figure 5. Flow cytometry gating strategy and examples of cytokine-induced phosphorylated STAT (pSTAT) in each cell population in one sample. (**A**) Monocyte gate was set based on CD33 positivity and light scattering characteristics (SSC-A). (**B**) Lymphocytes were gated based on light scattering characteristics (FSC-A and SSC-A). (**C**) Among lymphocytes, the $CD4^+$ T cell gate was set to comprise $CD3^+$ and $CD4^+$ populations. (**D**) Among lymphocytes, the B cell gate was set to comprise $CD3^-$ and $CD20^+$ populations. (**E**) pSTAT1 histograms in unstimulated (open peak) and IFN-γ-stimulated (grey peak) monocytes. (**F**) pSTAT5 histograms in unstimulated (open peak) and IL-2-stimulated (grey peak) $CD4^+$ T cells. (**G**) pSTAT3 histograms in unstimulated (open peak) and IL-21-stimulated (grey peak) B cells.

4.4. Peripheral Blood Mononuclear Cell Isolation and RNA Extraction

PBMCs were isolated by BD Vacutainer® CPT™ Mononuclear cell preparation tubes by density-gradient centrifugation and washed twice with PBS. Cells were snap-frozen without media and stored at −80 °C until RNA extraction. Total RNAs were isolated from PBMCs using the Rneasy Mini-Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

4.5. Nanostring Analysis

A custom gene panel (50 genes) containing genes involved in JAK–STAT pathway, interferon-inducible proteins, cytokines, NFκB pathway, and inflammasome activation was studied using NanoString analysis. In this analysis, patient data were compared to one gender- and age-matched control (for the index patient, a 56-year-old healthy male subject, and for his sister, a 72-year-old healthy female subject).

NanoString has been described in detail earlier [27]. Shortly, 100 ng of total RNA per sample were mixed with 5′ reporter probes, tagged with fluorescent barcodes of targeted genes, and 3′ biotinylated capture probes. The samples were hybridized overnight at 65 °C following the manufacturer's protocol. Then, the reactions were purified and immobilized on the sample cartridge surface. The cartridge was scanned in triplicate using the nCounter Digital Analyzer (NanoString Technologies, Seattle, WA, USA). Gene expression data were analyzed using nSolver™ 4.0 analysis software (NanoString Technologies). Normalized gene counts from three replicates were further used to calculate the fold changes between the patients and the controls. Genes, whose fold changes exceeded the limit of detection are presented in the manuscript.

4.6. Plasmid Constructs and Mutagenesis

The full-length IRF2BP2 expression plasmid (IRF2BP2-pDEST-C1-FLAG-GFP11-GW) was a kind gift from Professor Jorma Palvimo from the University of Eastern Finland [38]. The IRF2BP2 Δ625–665 plasmid construct possessing the patient's mutation was created by QuikChange (AgilentTechnologies, Santa Clara, CA, USA) site-directed mutagenesis, according to the manufacturer's instructions and verified by Sanger sequencing. An empty backbone expression vector was used as a control plasmid.

For luciferase reporter assays, firefly luciferase reporter constructs for STAT1 (interferon-stimulated response element-Luc (ISRE-Luc)) [39] were used together with a plasmid constitutively expressing renilla luciferase.

4.7. Mammalian Cell Culture

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco modified Eagle medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma, St Louis, MO, USA), 2 mmol/L L-glutamine (Lonza), and antibiotics (0.5% penicillin/streptomycin; Lonza). For dual luciferase assay and immunoblotting experiments, 120,000 or 100,000 cells were seeded on 12-well plates, respectively. Transient transfections were done using FuGENE HD (Promega, Madison, WI, USA) according to the manufacturer's instructions.

4.8. Dual Luciferase Assay

In total, 10 ng of control plasmid, IRF2BP2 wild-type plasmid, or IRF2BP2 Δ625–665 plasmid was co-transfected with 220 ng ISRE-Luc and 60 ng renilla luciferase plasmids. Then, after 24 h of transfection, cells were transferred to a 96-well plate. After 3–4 h, when the cells had attached to the bottom, the medium was changed to starvation medium (culture medium without FBS) or stimulation medium (starvation medium supplemented with 100 ng/mL IFNα or 10 ng/mL IFNβ). Luciferase activity was measured after 20 h of starvation/stimulation. Each experiment involved three technical replicates per sample, and three independent experiments were performed. Statistical analyses were performed using the measured values for unstimulated control plasmid as a normalizer for each independent experiment. Student's two-tailed T-test was used to calculate significant differences.

4.9. Immunoblotting

HEK293T cells were transfected with 100 ng of control plasmid, IRF2BP2 wild-type plasmid, or IRF2BP2 Δ625–665 plasmid. After 48 h of transfection, cell lysates were prepared for immunoblotting: cells were transferred on ice and washed with cold phosphate buffered saline (PBS). Triton X-100 lysis buffer with protease and phosphatase inhibitors (2 mM vanadate, 1 mM phenylmethanesulfonyl fluoride, 8.3 μg/mL aprotinin,

and 4.2 µg/mL pepstatin) was used to lyse the cells. Then, whole cell lysates were spun for 20 min at 16,000 g, and total protein amount in the cell lysates was determined from the supernatants by the Bradford assay (BioRad). Equal amounts of total protein (30 µg) were loaded on 4–15% Mini-PROTEAN® TGX™ Precast Gels (BioRad, Irvine, CA, USA).

Immunoblots were blocked with bovine serum albumin (BSA) and incubated with primary antibody for FLAG Tag (1:1,000, F1804, clone M2, Merck, Kenilworth, NJ, USA) or actin (1:2,000, MAB1501, Merc), which was used as a loading control, and with a mixture of goat anti-rabbit and goat anti-mouse DyLight secondary antibodies (both from Thermo Fisher Scientific, Waltham, MA, USA). Blots were scanned with an Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ph14080797/s1, Supplementary Table S1: Clinical parameters of index patient and his sister, Supplementary Figure S1: Constitutive and cytokine-induced STAT3, 4, and 6 phosphorylation in $CD4^+$ T cells, B cells, and monocytes.

Author Contributions: M.P., M.R.J.S., A.V., M.V., O.S. and P.I. planned the study. H.V. recruited the patients. M.P. and S.K. collected the data and analyzed the results. M.P., H.V. and P.I. wrote the manuscript, and all authors gave valuable comments on the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Molecular immunology laboratory at Tampere University is supported by Academy of Finland, Doctoral Programme of Medicine and Life Sciences at Tampere University, Sigrid Jusélius Foundation, Jane and Aatos Erkko Foundation, Tampere Tuberculosis Foundation and Pirkanmaa hospital district competitive research funding. PI has received funding from Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital (grant numbers 9U011 and 9X010). Molecular Systems Biology group is supported by Academy of Finland (nos. 288475 and 294173), Sigrid Jusélius Foundation, Finnish Cancer Foundation, University of Helsinki Three-year Research Grant, Biocentrum Finland, HiLIFE, Magnus Ehrnrooth Foundation and Instrumentarium Research Foundation. MS is funded by Hospital Research Funds, Pediatric Research Center, Helsinki University Hospital.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Coordinating Ethics Committee of Hospital District of Helsinki and Uusimaa (138/13/03/00/2013, 24 September 2013).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data underlying this article are available in the article and in its online Supplementary Material.

Acknowledgments: We thank Merja Peltomaa, Marika Vähä-Jaakkola, Sanna Liukkonen (Tampere University, Tampere, Finland), and Eira Leinonen (Folkhälsan Institute, Helsinki, Finland) for excellent technical assistance. Laura Oksa, MSc, is thanked for valuable comments on the manuscript.

Conflicts of Interest: M.P. has received a personal fee from Pfizer and is currently a part-time employee of MedEngine. H.V. has received travel grants from Merck and Gilead. O.S. has received personal fees from Pfizer and Abbvie and holds patents on JAK kinases, US Patent no. 5,728,536, US Patent no. 8,841,078, AU 2011214254, CAN 2789186, and EPO 11741946.5. P.I. has received a grant from Pfizer and personal fees from Pfizer, Eli Lilly and Company, Abbvie and Roche and non-financial support from Abbvie and Roche. M.R.J.S., S.K., A.V. and M.V. have nothing to declare.

References

1. Ramalho-Oliveira, R.; Oliveira-Vieira, B.; Viola, J.P.B. IRF2BP2: A new player in the regulation of cell homeostasis. *J. Leukoc. Biol.* **2019**, *106*, 717–723. [CrossRef]
2. Childs, K.S.; Goodbourn, S. Identification of novel co-repressor molecules for Interferon Regulatory Factor-2. *Nucleic Acids Res.* **2003**, *31*, 3016–3026. [CrossRef]
3. Hida, S.; Ogasawara, K.; Sato, K.; Abe, M.; Takayanagi, H.; Yokochi, T.; Sato, T.; Hirose, S.; Shirai, T.; Taki, S.; et al. CD8+ T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-α/β signaling. *Immunity* **2000**, *13*, 643–655. [CrossRef]

4. Teng, A.C.T.; Kuraitis, D.; Deeke, S.A.; Ahmadi, A.; Dugan, S.G.; Cheng, B.L.M.; Crowson, M.G.; Burgon, P.G.; Suuronen, E.J.; Chen, H.-H.; et al. IRF2BP2 is a skeletal and cardiac muscle-enriched ischemia-inducible activator of VEGFA expression. *FASEB J.* **2010**, *24*, 4825–4834. [CrossRef] [PubMed]
5. Stadhouders, R.; Cico, A.; Stephen, T.; Thongjuea, S.; Kolovos, P.; Baymaz, H.I.; Yu, X.; Demmers, J.; Bezstarosti, K.; Maas, A.; et al. Control of developmentally primed erythroid genes by combinatorial co-repressor actions. *Nat. Commun.* **2015**, *6*, 8893. [CrossRef] [PubMed]
6. Yeung, K.T.; Das, S.; Zhang, J.; Lomniczi, A.; Ojeda, S.R.; Xu, C.-F.; Neubert, T.A.; Samuels, H.H. A novel transcription complex that selectively modulates apoptosis of breast cancer cells through regulation of FASTKD2. *Mol. Cell. Biol.* **2011**, *31*, 2287–2298. [CrossRef]
7. Carneiro, F.R.G.; Ramalho-Oliveira, R.; Mognol, G.P.; Viola, J.P.B. Interferon Regulatory Factor 2 Binding Protein 2 Is a New NFAT1 Partner and Represses Its Transcriptional Activity. *Mol. Cell. Biol.* **2011**, *31*, 2889–2901. [CrossRef] [PubMed]
8. Tinnikov, A.A.; Yeung, K.T.; Das, S.; Samuels, H.H. Identification of a Novel Pathway That Selectively Modulates Apoptosis of Breast Cancer Cells. *Cancer Res.* **2009**, *69*, 1375–1382. [CrossRef] [PubMed]
9. Lempiäinen, J.K.; Niskanen, E.A.; Vuoti, K.M.; Lampinen, R.E.; Göös, H.; Varjosalo, M.; Palvimo, J.J. Agonist-specific protein interactomes of Glucocorticoid and androgen receptor as revealed by proximity mapping. *Mol. Cell. Proteom.* **2017**, *16*, 1462–1474. [CrossRef] [PubMed]
10. Jiang, Y.; Shen, Q. IRF2BP2 prevents ox-LDL-induced inflammation and EMT in endothelial cells via regulation of KLF2. *Exp. Ther. Med.* **2021**, *21*, 1–8. [CrossRef] [PubMed]
11. Kim, I.; Kim, J.H.; Kim, K.; Seong, S.; Kim, N. The IRF2BP2-KLF2 axis regulates osteoclast and osteoblast differentiation. *BMB Rep.* **2019**, *52*, 469–474. [CrossRef]
12. Koeppel, M.; van Heeringen, S.J.; Smeenk, L.; Navis, A.C.; Janssen-Megens, E.M.; Lohrum, M. The novel p53 target gene IRF2BP2 participates in cell survival during the p53 stress response. *Nucleic Acids Res.* **2009**, *37*, 322–335. [CrossRef]
13. Hammarén, H.M.; Virtanen, A.T.; Raivola, J.; Silvennoinen, O. The regulation of JAKs in cytokine signaling and its breakdown in disease. *Cytokine* **2019**, *118*, 48–63. [CrossRef] [PubMed]
14. O'Shea, J.J.; Schwartz, D.M.; Villarino, A.V.; Gadina, M.; McInnes, I.B.; Laurence, A. The JAK-STAT pathway: Impact on human disease and therapeutic intervention. *Annu. Rev. Med.* **2015**, *66*, 311–328. [CrossRef] [PubMed]
15. Villarino, A.V.; Kanno, Y.; O'Shea, J.J. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat. Immunol.* **2017**, *18*, 374–384. [CrossRef]
16. Keller, M.D.; Pandey, R.; Li, D.; Glessner, J.; Tian, L.; Henrickson, S.E.; Chinn, I.K.; Monaco-Shawver, L.; Heimall, J.; Hou, C.; et al. Mutation in IRF2BP2 is responsible for a familial form of common variable immunodeficiency disorder. *J. Allergy Clin. Immunol.* **2016**, *138*, 544–550. [CrossRef]
17. Baxter, S.K.; Walsh, T.; Casadei, S.; Eckert, M.M.; Allenspach, E.J.; Hagin, D.; Segundo, G.; Lee, M.K.; Gulsuner, S.; Shirts, B.H.; et al. Molecular diagnosis of childhood immune dysregulation, polyendocrinopathy and enteropathy, and implications for clinical management. *J. Allergy Clin. Immunol.* **2021**, in press. [CrossRef]
18. Yin, C.C.; Jain, N.; Mehrotra, M.; Zhang, J.; Protopopov, A.; Zuo, Z.; Pemmaraju, N.; DiNardo, C.; Hirsch-Ginsberg, C.; Wang, S.A.; et al. Identification of a novel fusion gene, IRF2BP2-RARA, in acute promyelocytic leukemia. *J. Natl. Compr. Cancer Netw.* **2015**, *13*, 19–22. [CrossRef]
19. Shimomura, Y.; Mitsui, H.; Yamashita, Y.; Kamae, T.; Kanai, A.; Matsui, H.; Ishibashi, T.; Tanimura, A.; Shibayama, H.; Oritani, K.; et al. New variant of acute promyelocytic leukemia with IRF2BP2–RARA fusion. *Cancer Sci.* **2016**, *107*, 1165–1168. [CrossRef]
20. Jovanovic, J.V.; Chillón, M.C.; Vincent-Fabert, C.; Dillon, R.; Voisset, E.; Gutiérrez, N.C.; Sanz, R.G.; Lopez, A.A.M.; Morgan, Y.G.; Lok, J.; et al. The cryptic IRF2BP2-RARA fusion transforms hematopoietic stem/progenitor cells and induces retinoid-sensitive acute promyelocytic leukemia. *Leukemia* **2017**, *31*, 747–751. [CrossRef] [PubMed]
21. Mazharuddin, S.; Chattopadhyay, A.; Levy, M.Y.; Redner, R.L. IRF2BP2-RARA t(1;17)(q42.3;q21.2) APL blasts differentiate in response to all-trans retinoic acid. *Leuk. Lymphoma* **2018**, *59*, 2246–2249. [CrossRef]
22. Liu, Y.; Xu, F.; Hu, H.; Wen, J.; Su, J.; Zhou, Q.; Qu, W. A rare case of acute promyelocytic leukemia with IRF2BP2-RARA fusion; and literature review. *Onco. Targets. Ther.* **2019**, *12*, 6157–6163. [CrossRef]
23. Alotaibi, A.S.; Abdulrazzaq, M.; Patel, K.P.; Ravandi, F.; Konoplev, S.; Bueso-Ramos, C.; Yin, C.C.; Muzzafar, T.; Tang, G.; Futreal, A.; et al. Acute promyelocytic leukemia (APL) with an *IRF2BP2-RARA* fusion transcript: An aggressive APL variant. *Leuk. Lymphoma* **2020**, *61*, 3018–3020. [CrossRef] [PubMed]
24. Sécca, C.; Faget, D.V.; Hanschke, S.C.; Carneiro, M.S.; Bonamino, M.H.; de-Araujo-Souza, P.S.; Viola, J.P.B. IRF2BP2 transcriptional repressor restrains naive CD4 T cell activation and clonal expansion induced by TCR triggering. *J. Leukoc. Biol.* **2016**, *100*, 1081–1091. [CrossRef]
25. Laity, J.H.; Lee, B.M.; Wright, P.E. Zinc finger proteins: New insights into structural and functional diversity. *Curr. Opin. Struct. Biol.* **2001**, *11*, 39–46. [CrossRef]
26. Joazeiro, C.A.; Weissman, A.M. RING Finger Proteins: Mediators of Ubiquitin Ligase Activity. *Cell* **2000**, *102*, 549–552. [CrossRef]
27. Göös, H.; Fogarty, C.L.; Sahu, B.; Plagnol, V.; Rajamäki, K.; Nurmi, K.; Liu, X.; Einarsdottir, E.; Jouppila, A.; Pettersson, T.; et al. Gain-of-function CEBPE mutation causes noncanonical autoinflammatory inflammasomopathy. *J. Allergy Clin. Immunol.* **2019**, *144*, 1364–1376. [CrossRef] [PubMed]

28. Harada, H.; Fujita, T.; Miyamoto, M.; Kimura, Y.; Maruyama, M.; Furia, A.; Miyata, T.; Taniguchi, T. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* **1989**, *58*, 729–739. [CrossRef]
29. Fujita, T.; Kimura, Y.; Miyamoto, M.; Barsoumian, E.L.; Taniguchi, T. Induction of endogenous IFN-α and IFN-β genes by a regulatory transcription factor, IRF-1. *Nature* **1989**, *337*, 270–272. [CrossRef]
30. Michalska, A.; Blaszczyk, K.; Wesoly, J.; Bluyssen, H.A.R. A positive feedback amplifier circuit that regulates interferon (IFN)-stimulated gene expression and controls type I and type II IFN responses. *Front. Immunol.* **2018**, *9*, 28. [CrossRef]
31. Feng, H.; Zhang, Y.B.; Gui, J.F.; Lemon, S.M.; Yamane, D. Interferon regulatory factor 1 (IRF1) and anti-pathogen innate immune responses. *PLoS Pathog.* **2021**, *17*. [CrossRef] [PubMed]
32. Chatterjee-Kishore, M.; Wright, K.L.; Ting, J.P.Y.; Stark, G.R. How Stat1 mediates constitutive gene expression: A complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J.* **2000**, *19*, 4111–4122. [CrossRef] [PubMed]
33. Zenke, K.; Muroi, M.; Tanamoto, K. ichi IRF1 supports DNA binding of STAT1 by promoting its phosphorylation. *Immunol. Cell Biol.* **2018**, *96*, 1095–1103. [CrossRef]
34. Xu, L.; Zhou, X.; Wang, W.; Wang, Y.; Yin, Y.; Van Der Laan, L.J.W.; Sprengers, D.; Metselaar, H.J.; Peppelenbosch, M.P.; Pan, Q. IFN regulatory factor 1 restricts hepatitis e virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *FASEB J.* **2016**, *30*, 3352–3367. [CrossRef] [PubMed]
35. Paredes, J.L.; Niewold, T.B. Type I interferon antagonists in clinical development for lupus. *Expert Opin. Investig. Drugs* **2020**, *29*, 1025–1041. [CrossRef]
36. Highlights of Prescribing Information. Available online: www.fda.gov/medwatch (accessed on 6 August 2021).
37. Liu, C.; Kieltyka, J.; Fleischmann, R.; Gadina, M.; O'Shea, J.J. A Decade of Jakinibs: What have we learned and what may be the future. *Arthritis Rheumatol.* **2021**. accepted author manuscript. [CrossRef] [PubMed]
38. Manjur, A.B.M.K.; Lempiäinen, J.K.; Malinen, M.; Palvimo, J.J.; Niskanen, E.A. IRF2BP2 modulates the crosstalk between glucocorticoid and TNF signaling. *J. Steroid Biochem. Mol. Biol.* **2019**, *192*, 105382. [CrossRef]
39. Bluyssen, H.A.R.; Levy, D.E. Stat2 is a transcriptional activator that requires sequence-specific contacts provided by Stat1 and p48 for stable interaction with DNA. *J. Biol. Chem.* **1997**, *272*, 4600–4605. [CrossRef]

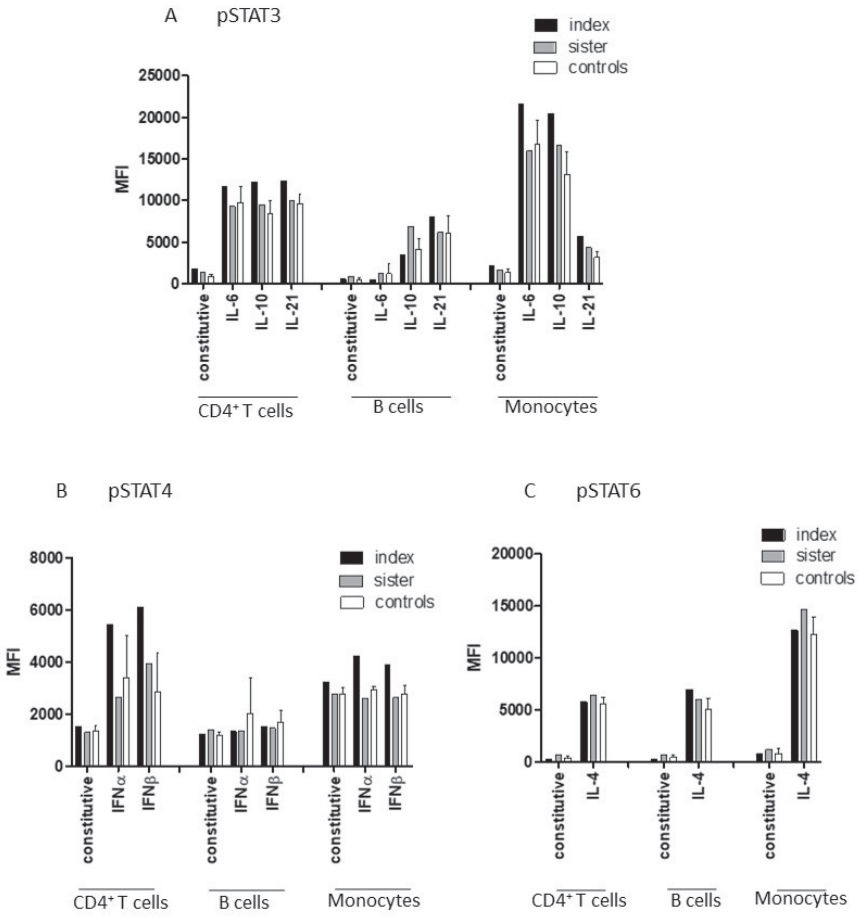
Supplementary material

Palmroth et al: IRF2BP2 mutation is associated with increased STAT1 and STAT5 activation in two family members with inflammatory conditions and lymphopenia

Supplementary Table S1: Clinical parameters of index patient and sister. Values differing from reference values are marked in bold.

	Index patient: Male		Sister: Female	
Clinical findings and symptoms	Oral and genital ulcers, Hidradenitis suppurativa, Acne, Periodic abdominal pain and fever, Weight loss		Hidradenitis suppurativa, Diabetes mellitus type 2, Hypertension, Hyperthyreosis, Dystrofia myotonica 2	
Age at sampling (years)	57		71	
		Normal range (males)		Normal range (females)
Leukocytes (cells/ul)	**2000**	3400-8200	5000	3400-8200
Lymphocytes (cells/ul)	**650**	1200-3500	**470**	1200-3500
Neutrophils (cells/ul)	**1290**	1600-6200	4080	1600-6200
Platelets (cells/l)	154	150 -360	272	150 -360
Hemoglobin (g/l)	**122**	134-167	140	117-155
CD4 (cells/ul)	**230**	520-1470	ND	
CD8 (cells/ul)	**190**	210-920	ND	
CD19 (cells/ul)	**40**	90-510	ND	
CD16/56 (cells/ul)	120	70-560	ND	
IgG g/l	12,6	6,8-15,0	**4,5**	6,8-15,0
IgA g/l	3,28	0,88-4,84	ND	
IgM g/l	0,79	0,36-2,59	0,59	0,47-2,84
IgE kU/l	12	0-100	ND	
Complement activation	normal		ND	
Polysaccaride vaccine response (pneumococcal)	normal		ND	

CD; cluster of differentiation, Ig; immunoglobulin, ND; no data



Supplementary Figure S1: Constitutive and cytokine-induced phosphorylation of A) STAT3, B) STAT4 and C) STAT6 in $CD4^+$ T cells, B cells and monocytes. CD; cluster of differentiation, IFN; interferon, IL; interleukin, MFI; median fluorescence intensity, STAT; signal transducer and activator of transcription.

PUBLICATION II

Tofacitinib suppresses several JAK-STAT pathways in rheumatoid arthritis *in vivo* and baseline signaling profile associates with treatment response.

Palmroth M, Kuuliala K, Peltomaa R, Virtanen AT, Kuuliala A, Kurttila A, Kinnunen A, Leirisalo-Repo M, Silvennoinen O, Isomäki P.

Frontiers in Immunology, 2021. 12, 738481
doi: 10.3389/fimmu.2021.738481

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 September 2021
doi: 10.3389/fimmu.2021.738481

Tofacitinib Suppresses Several JAK-STAT Pathways in Rheumatoid Arthritis *In Vivo* and Baseline Signaling Profile Associates With Treatment Response

*Maaria Palmroth[1], Krista Kuuliala[2], Ritva Peltomaa[3], Anniina Virtanen[1], Antti Kuuliala[2], Antti Kurttila[1], Anna Kinnunen[4], Marjatta Leirisalo-Repo[3], Olli Silvennoinen[1,5,6] and Pia Isomäki[1,4]**

[1] Molecular Immunology Group, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland, [2] Department of Bacteriology and Immunology, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [3] Department of Rheumatology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [4] Centre for Rheumatic Diseases, Tampere University Hospital, Tampere, Finland, [5] Fimlab Laboratories, Pirkanmaa Hospital District, Tampere, Tampere, Finland, [6] Institute of Biotechnology, HiLIFE Helsinki Institute of Life Sciences, University of Helsinki, Helsinki, Finland

OPEN ACCESS

Edited by:
Norma Maugeri,
San Raffaele Scientific Institute (IRCCS), Italy

Reviewed by:
Shingo Nakayamada,
University of Occupational and Environmental Health Japan, Japan
Yu-Jih Su,
Kaohsiung Chang Gung Memorial Hospital, Taiwan

***Correspondence:**
Pia Isomäki
pia.isomaki@tuni.fi

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: 08 July 2021
Accepted: 01 September 2021
Published: 24 September 2021

Citation:
Palmroth M, Kuuliala K, Peltomaa R, Virtanen A, Kuuliala A, Kurttila A, Kinnunen A, Leirisalo-Repo M, Silvennoinen O and Isomäki P (2021) Tofacitinib Suppresses Several JAK-STAT Pathways in Rheumatoid Arthritis In Vivo and Baseline Signaling Profile Associates With Treatment Response. Front. Immunol. 12:738481. doi: 10.3389/fimmu.2021.738481

Objective: Current knowledge on the actions of tofacitinib on cytokine signaling pathways in rheumatoid arthritis (RA) is based on *in vitro* studies. Our study is the first to examine the effects of tofacitinib treatment on Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathways *in vivo* in patients with RA.

Methods: Sixteen patients with active RA, despite treatment with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), received tofacitinib 5 mg twice daily for three months. Levels of constitutive and cytokine-induced phosphorylated STATs in peripheral blood monocytes, T cells and B cells were measured by flow cytometry at baseline and three-month visits. mRNA expression of JAKs, STATs and suppressors of cytokine signaling (SOCS) were measured from peripheral blood mononuclear cells (PBMCs) by quantitative PCR. Association of baseline signaling profile with treatment response was also investigated.

Results: Tofacitinib, in csDMARDs background, decreased median disease activity score (DAS28) from 4.4 to 2.6 ($p < 0.001$). Tofacitinib treatment significantly decreased cytokine-induced phosphorylation of all JAK-STAT pathways studied. However, the magnitude of the inhibitory effect depended on the cytokine and cell type studied, varying from 10% to 73% inhibition following 3-month treatment with tofacitinib. In general, strongest inhibition by tofacitinib was observed with STAT phosphorylations induced by cytokines signaling through the common-γ-chain cytokine receptor in T cells, while lowest inhibition was demonstrated for IL-10 -induced STAT3 phosphorylation in monocytes. Constitutive STAT1, STAT3, STAT4 and STAT5 phosphorylation in monocytes and/or T cells was also downregulated by tofacitinib. Tofacitinib treatment downregulated the expression of several JAK-STAT pathway components in PBMCs,

SOCSs showing the strongest downregulation. Baseline STAT phosphorylation levels in T cells and monocytes and SOCS3 expression in PBMCs correlated with treatment response.

Conclusions: Tofacitinib suppresses multiple JAK-STAT pathways in cytokine and cell population specific manner in RA patients *in vivo*. Besides directly inhibiting JAK activation, tofacitinib downregulates the expression of JAK-STAT pathway components. This may modulate the effects of tofacitinib on JAK-STAT pathway activation *in vivo* and explain some of the differential findings between the current study and previous *in vitro* studies. Finally, baseline immunological markers associate with the treatment response to tofacitinib.

Keywords: rheumatoid arthritis, cytokines, JAK inhibitor, monocytes, T cells, B cells

INTRODUCTION

Cytokines are important mediators of inflammation and tissue destruction in rheumatoid arthritis (RA) (1, 2). Several cytokines involved in the pathogenesis of RA, such as interleukin-6 (IL-6), interferons (IFNs), granulocyte-macrophage colony-stimulating factor (GM-CSF) and common gamma chain cytokine family, act through Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (3, 4). JAK-STAT pathways consist of four JAK kinases [JAK1-3 and tyrosine kinase 2 (TYK2)] and seven signal transducers and activators of transcription (STAT1-6, including the homologs STAT5A and STAT5B). The signaling cascade is initiated by a cytokine binding to its receptor, which enables JAK activation by trans-phosphorylation. Subsequently, JAKs phosphorylate the receptor and STATs that dimerize, and translocate to the nucleus to regulate the expression of their target genes. Each cytokine receptor employs a specific combination of JAK kinases, e.g. IL-6 signals through JAK1 and JAK2/TYK2 and common gamma chain cytokines through JAK1 and JAK3 (5). JAK-STAT signaling pathway is under tight regulation, which involves e.g. proteins of suppressors of cytokine signaling (SOCS) family SOCS1-3 and cytokine-inducible SH2-containing protein (CIS1) (6).

We and others have demonstrated that certain JAK-STAT pathways are constitutively active in rheumatic diseases (7–10). In RA, STAT3 is constitutively phosphorylated in circulating T cells and monocytes, and this correlates with serum IL-6 levels, suggesting hyperactivation of the IL-6 –STAT3 axis (7, 9). In addition to STAT3, constitutive phosphorylation of STAT1 and STAT5 is increased in peripheral blood T cells from patients with active RA (7, 10). Constitutive STAT3 phosphorylation in circulating T cells of patients with recent-onset RA associates with disease activity and good treatment response to conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) (8). In addition, we have demonstrated that cytokine-induced STAT1 and STAT6 phosphorylation in circulating leukocytes associates with treatment response to biological drugs in established RA and to csDMARDs in recent-onset RA, respectively (11).

Tofacitinib, which inhibits JAK1, JAK3, and to a slightly lesser extent JAK2, was the first JAK inhibitor developed for treatment of RA (12, 13) and its efficacy is comparable to that of TNF-inhibitor adalimumab (14). Tofacitinib has been shown to inhibit cytokine signaling and the effector functions of different immune cells and synovial fibroblasts *in vitro* (15–26) and in animal models of arthritis (15, 27, 28). However, information of the *in vivo* effects of tofacitinib on the activity of JAK-STAT pathways in RA patients is currently lacking and may differ from the results based on *in vitro* studies.

Gaining knowledge on how medicines, such as tofacitinib, function in patients with RA could help us to understand disease mechanisms, as well as both desired and undesired effects of JAK inhibitors. The current study is the first to investigate the JAK-STAT signaling profile by flow cytometry, and the expression of the signaling pathway components, in peripheral blood leukocytes of tofacitinib-treated RA patients. In addition, we examined associations between baseline immunological findings and the treatment response to tofacitinib.

MATERIALS AND METHODS

Patients

Patients fulfilling the 2010 ACR/EULAR classification criteria for RA were recruited for this clinical study in two rheumatology outpatient clinics (Tampere and Helsinki University Hospitals) between June 2018 and January 2020. Eligible patients had active disease at baseline visit: Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]) was >3.2 despite treatment with methotrexate and/or other csDMARDs. Key exclusion criteria were prior treatment with biologic therapies or JAK inhibitors, current infection, malignancy, severe hepatic impairment, pregnancy or lactation, hemoglobin <90 mg/dl, neutrophil count <1.0 × 109/l or lymphocyte count <0.75 x 109/l.

This study was approved by the National Committee on Medical Research Ethics (TUKIJA) and Finnish Medicines Agency Fimea and was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines. All patients gave their written informed consent.

Study Design

Graphical overview of the study is presented in **Figure 1A**. The study included a screening visit (0-3 months before baseline visit), a baseline visit (0 month) and a follow-up visit (3 months). Screening visit and baseline visit could be combined.

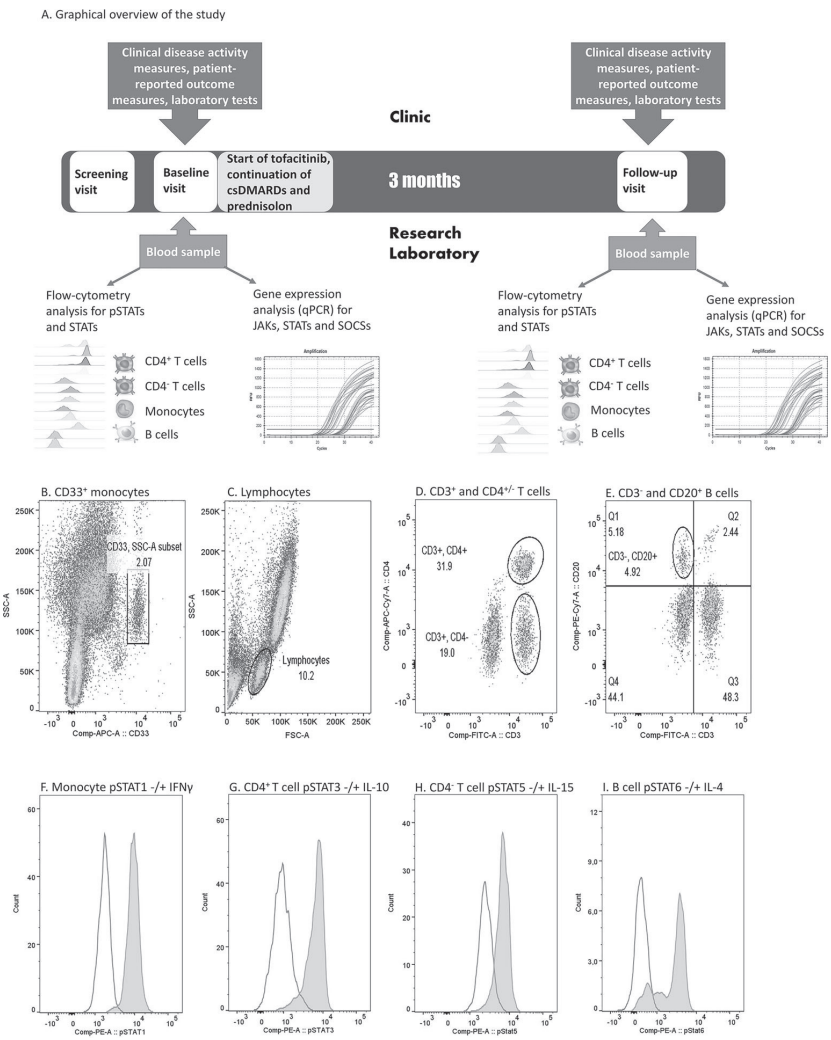


FIGURE 1 | **(A)** Graphical overview of the study and **(B–I)**. Flow cytometry gating strategy and examples of cytokine-induced phosphorylated STAT (pSTAT) in each cell population in one patient sample. **(B)** Monocyte gate was set based on CD33 positivity and light scattering characteristics (SSC-A). **(C)** Lymphocytes were gated based on light scattering characteristics (FSC-A and SSC-A). **(D)** Among lymphocytes, T cell gates were set to comprise $CD3^+CD4^+$ or $CD3^+CD4^-$ populations. **(E)** Among lymphocytes, B cell gate was set to comprise $CD3^-$ and $CD20^+$ population. **(F)** pSTAT1 histograms in unstimulated (open peak) and IFN-γ-stimulated (grey peak) monocytes. **(G)** pSTAT3 histograms in unstimulated (open peak) and IL-10-stimulated (grey peak) $CD4^+$ T cells. **(H)** pSTAT5 histograms in unstimulated (open peak) and IL-15-stimulated (grey peak) $CD4^-$ T cells. **(I)** pSTAT6 histograms in unstimulated (open peak) and IL-4 stimulated (grey peak) B cells. IFN, interferon; IL, interleukin, (p)STAT, (phosphorylated) signal transducer and activator of transcription.

Tofacitinib 5 mg twice daily was started at baseline visit and continued through the study. Patients continued their csDMARD therapy and prednisolone (0-10 mg/day) at a stable dose during the study.

The following patient-reported outcomes and clinical assessments were recorded at study visits: patient general health visual analogue scale (VAS) (0-100 mm), pain VAS (0-100 mm), health assessment questionnaire (HAQ) disability index (0-3), number of swollen and tender joints (46 joint count), physician's assessment VAS (0-100 mm) and DAS28. Tofacitinib treatment response was determined by the change from baseline DAS28-4[CRP].

Study-related blood samples were drawn at baseline and follow-up visits. Blood samples at the follow-up visit were taken 1-2 hours after the morning tofacitinib dose.

Cytokine-Stimulations and Flow Cytometry

Phosphorylation of STAT proteins, and levels of total STAT1 and STAT3 were studied using five-color flow cytometry. First, 100-µl aliquots of fresh blood samples were either left unstimulated or were stimulated by 100 ng/ml recombinant IL-2, IL-4, IL-6, IL-7, IL-10, IL-15, IL-21, IFN-α or IFN-γ (listed in detail in **Supplementary Table 1**) for 15 minutes at +37°C. The stimulation was terminated by transferring the samples on ice. Leukocytes were then immediately fixed and red blood cells lysed using BD Phosflow Lyse/Fix buffer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 10 min at +37°C, washed with PBS and permeabilized in ice-cold methanol for 10 min on ice followed by 1-4 weeks preservation in methanol at -80°C. In order to ensure the integrity of the results the samples that were collected in Helsinki were transferred in methanol on dry ice to Tampere, where sample preparation was continued and all stainings were performed. STAT phosphorylation levels were determined to be preserved for at least 4 weeks in methanol at -80°C in preliminary experiments (unpublished data).

Following two washes with FACS buffer (PBS supplemented with 0,1% bovine serum albumin and 0,01% sodium azide), samples were stained with fluorochrome-conjugated antibodies against CD33, CD3, CD4, CD20, STAT1, STAT3, phospho-STAT1 (pSTAT1), pSTAT3, pSTAT4, pSTAT5 and pSTAT6 (listed in detail in **Supplementary Table 2**) for 30 minutes at room temperature, protected from light. CD33 marker was selected to represent monocytes and CD20 to represent B cells, as the epitopes of more conventionally used markers CD14 and CD19, respectively, do not survive cold methanol permeabilization (29). Following antibody stainings the samples were washed twice with FACS buffer and fluorescence was measured with FACS Canto II (BD). To ensure consistent performance of the method throughout the study, 8-peak Rainbow calibration particles (BD) were used before every run.

Data acquisition was performed using FACSCanto II (BD) and the analysis of flow cytometer data using FlowJo Single cell analysis software (BD). $CD4^+$ and $CD4^-$ T cells were gated from the $CD3^+$ lymphocyte population, $CD20^+$ B cells from the $CD3^-$ lymphocyte population and $CD33^+$ cells represent monocytes (**Figures 1B–E**). A phycoerythrin (PE) fluorescence histogram was created for each cell population, and the median fluorescence intensity (MFI, arithmetic median) was calculated. Examples of cytokine-induced pSTATs are presented in **Figures 1F–I**. The change in STAT phosphorylation during the study was calculated by dividing the difference of fluorescence intensities at entry and after 3 months and dividing that by the fluorescence intensity at entry.

Peripheral Blood Mononuclear Cell Isolation, RNA Extraction, and qPCR Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077 medium (Sigma Aldrich, St. Louis, MO, USA), washed twice with PBS containing 2 mM EDTA and snap frozen. Samples were collected at both locations but transferred from Helsinki to Tampere on dry ice for further preparations and analysis. Total RNA was extracted from PBMCs using the RNeasy Mini-Kit (Qiagen, Valencia, CA, USA). Total RNA (0.5 µg) was reverse-transcribed using M-MLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were performed by using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Primer sequences for *STAT1, STAT3, STAT4, STAT5A, STAT5B, STAT6, JAK1, JAK2, JAK3, TYK2, SOCS1, SOCS2, SOCS3, CIS1* and *β-actin* are listed in **Supplementary Table 3**. The 10-µl real-time PCR reactions were performed with CFX384 (Bio-Rad Laboratories, Hercules, CA, USA) and gene expression was quantified by using the delta C(T) method by normalizing to the expression of *β-actin*.

Statistical Methods

Continuous variables are summarized as means with ranges or bootstrapped 95% confidence intervals (CI), or medians with interquartile ranges (IQR). Values measured at study entry and after 3 months were compared using Wilcoxon signed rank test. Correlation between variables was assessed by Spearman rank correlation. No adjustment was made for multiple testing and p-values equal or less than 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS version 25 (IBM, Armonk, NY, USA) and Stata version 15 (StataCorp LLC, College Station, TX, USA).

RESULTS

Patients

Eighteen patients, 9 from each outpatient clinic, were recruited to the study. Of these, one patient did not start tofacitinib treatment and for another patient flow cytometry results could not be obtained at baseline visit due to a technical problem with the flow cytometer. The final study population therefore consisted of 16 patients, who continued tofacitinib treatment until follow-up visit and had complete data sets from both visits.

Characteristics of the patient cohort at baseline visit are presented in **Table 1**. The background csDMARD therapy of each patient is described in detail in **Supplementary Table 4**. A total of 12 patients (75%) received methotrexate as part of their csDMARD regimen (6 with triple, 4 with double, and 2 with single csDMARD therapy).

Clinical Response to Tofacitinib

Tofacitinib with background csDMARD treatment significantly decreased the activity of RA during 3-month treatment, as defined both by clinical measures of activity, as well as by patient-reported outcomes (**Table 2**). Median DAS28 score decreased from 4.4 to 2.6. Nine patients (56%) were in DAS28 remission at the 3-month visit. Four patients had low, two patients moderate and one patient high

TABLE 1 | Characteristics of the patients (n=16).

Female sex, n (%)	11 (69%)
Age, years, mean (range)	58.4 (36.6-72.9)
Disease duration, years, mean (range)	9.6 (0.5-48.0)
Rheumatoid factor positive, n (%)	11 (69%)
CCP-antibody positive, n (%)	12 (75%)
Erosive disease, n (%)	7 (44%)
Disease activity (DAS28), n (%)	
Moderate	13 (81%)
High	3 (19%)
csDMARD regimen, n (%)	
Triple	6 (37%)
Double	7 (44%)
Single	3 (19%)
Low-dose prednisolone, n (%)	8 (50%)

CCP, cyclic citrullinated peptide; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; n, number of patients.

disease activity according to DAS28 following the treatment with tofacitinib and csDMARDs.

Safety laboratory tests remained within acceptable range (**Table 2**) and there were no serious adverse events during the study.

Tofacitinib Suppresses Both Constitutive and Cytokine-Induced STAT Phosphorylation *In Vivo* in Cytokine and Cell Type Specific Matter

Constitutive and *in vitro* cytokine-induced STAT phosphorylation in monocytes, T cells and B cells was measured using multi-color flow cytometry at baseline and three-month visits. Based on the pharmacokinetic profile of tofacitinib (29), our results describe the maximal *in vivo* effect of tofacitinib, as the blood samples at three-month visit were collected shortly after morning tofacitinib dose. The flow cytometric assay was performed using fresh blood without cell isolation, and thus is likely to reflect STAT phosphorylation *in vivo*. Cytokine-induced STAT phosphorylation was studied using selected cytokine or cytokines for each pSTAT (**Figure 2**).

Tofacitinib, in csDMARD background, decreased significantly constitutive phosphorylation of STATs (**Figure 2**). The decrease in constitutive phosphorylation was most consistent for STAT3 (shown in all cell types studied). In addition, constitutive phosphorylation of STAT1, STAT4 and STAT5 was also downregulated by tofacitinib in a cell type specific manner. Using similar methodology as in the current study, we have previously shown that constitutive pSTAT1, pSTAT3 and pSTAT5 are increased in circulating T cells and pSTAT3 in monocytes of RA patients compared to healthy volunteers (7, 10). In order to gain some insight into what extent these leukocyte signaling aberrations are normalized by tofacitinib treatment, we compared the differences in constitutive STAT phosphorylation between controls and RA patients (historical data) to those between tofacitinib-treated and untreated RA patients derived from the current study (**Supplementary Table 5**). This comparison suggests that the elevated pSTAT1, pSTAT3 and pSTAT5 levels in patients with RA are reversed to a significant degree by treatment with tofacitinib.

Cytokine-induced STAT phosphorylations were significantly decreased by tofacitinib in all studied leukocyte subtypes (**Figure 2**). In order to investigate the inhibitory potency of tofacitinib on STAT activation induced by different cytokines more closely, mean inhibition percentages between 3-month and baseline visits were calculated (**Table 3**). Depending on the cytokine and cell population, the mean percentage inhibition of STAT phosphorylation by tofacitinib treatment ranged from 10% to 73%. At least 50% inhibition was observed with STAT phosphorylations induced by IFN-α and common-γ-chain cytokines IL-2, IL-4, IL-15 and IL-21 in $CD4^+$ T cells, by IL-2, IL-4 and IL-21 in $CD4^-$ T cells and by IFN-γ in B cells. Lowest inhibition was demonstrated for IL-10 -induced STAT3 phosphorylation in monocytes. In general, monocyte responses were less sensitive to suppression by tofacitinib than those in $CD4^+$ T cells.

TABLE 2 | Comparison of clinical and laboratory parameters at baseline and after 3-month treatment with tofacitinib and csDMARDs.

	Before, median (IQR)	After 3 months, median (IQR)	p
Swollen joint count, 0-46	7 (6-9)	2 (0-3)	**<0.001**
Tender joint count, 0-46	11 (5-17)	1 (0-8)	**<0.001**
Swollen joint count, 0-28	5 (4-7)	1 (0-2)	**<0.001**
Tender joint count, 0-28	4 (2-10)	1 (0-3)	**<0.001**
General health, VAS, 0-100 mm	51 (37-65)	16 (7-29)	**0.001**
Pain, VAS, 0-100 mm	43 (22-64)	12 (5-38)	**0.002**
Physician's assessment, VAS, 0-100 mm	35 (31-46)	13 (11-18)	**<0.001**
HAQ disability index, 0-3	0.813 (0.625-1.253)	0.130 (0-0.813)	**0.011**
DAS28	4.4 (3.6-4.9)	2.6 (1.9-2.9)	**<0.001**
Plasma C-reactive protein, mg/l	5 (3-17)	3 (3-4)	**0.042**
Blood hemoglobin, g/l	129 (126-140)	132 (126-145)	0.775
Blood leukocyte count, $\times10^9$/l	8.3 (6.1-9.1)	5.3 (4.3-6.9)	**0.003**
Blood neutrophil count, $\times10^9$/l	5.32 (4.02-6.34)	2.88 (2.32-3.91)	**0.003**
Blood lymphocyte count, $\times10^9$/l	1.40 (1.07-1.94)	1.41 (1.20-1.83)	0.959
Blood platelet count, $\times10^9$/l	297 (274-334)	281 (225-302)	**<0.001**
Plasma alanine aminotransferase, U/l	20 (15-26)	21 (19-28)	0.224
Plasma creatinine, µmol/l	63 (56-83)	68 (56-86)	0.615

p-values are calculated using Wilcoxon test and shown in bold when $p \leq 0.05$.

DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; IQR, interquartile range; VAS, visual analogue scale.

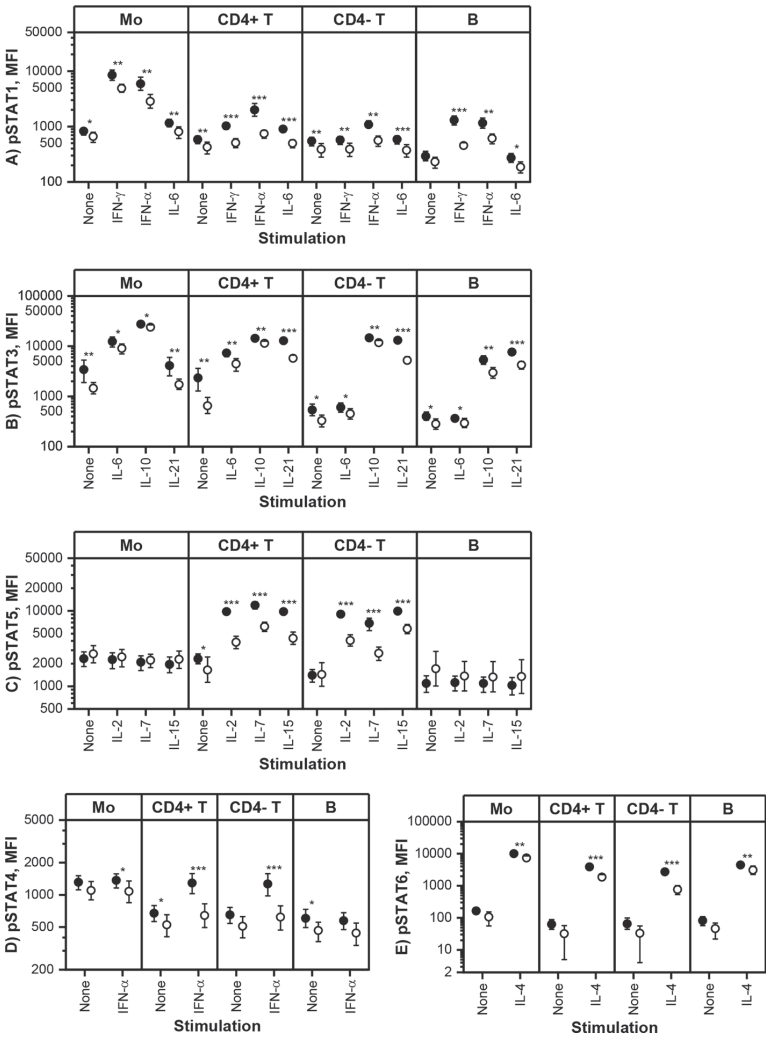


FIGURE 2 | Means and 95% confidence intervals of **(A)** phosphorylated STAT1 (pSTAT1), **(B)** pSTAT3, **(C)** pSTAT5, **(D)** pSTAT4 and **(E)** pSTAT6 median fluorescence intensities (MFI) in monocytes (Mo), $CD4^+$ T cells ($CD4^+$ T), $CD4^-$ T cells ($CD4^-$ T) and $CD20^+$ B cells (B) at baseline (filled symbols) and after 3-month treatment with tofacitinib and csDMARDs (open symbols). Note the logarithmic scale of the Y-axis. p-values comparing 0 and 3 months are calculated by Wilcoxon test. Significant differences are marked with an asterisk: *$p \leq 0.05$, ** $p \leq 0.01$, p***≤ 0.001. csDMARD, conventional systemic disease-modifying antirheumatic drug; (p)STAT, (phosphorylated) signal transducer and activator of transcription.

Tofacitinib Downregulates the mRNA Expression of JAK-STAT Signaling Pathway Components

We also measured the total amount of STAT1 and STAT3 proteins in monocytes, T cells and B cells by flow cytometry. Tofacitinib did not cause significant changes in total STAT1 and STAT3 protein levels (**Figures 3A, B**).

The effect of tofacitinib on the mRNA levels of JAK-STAT pathway components and inhibitors in PBMCs was studied using qPCR. Significant, but rather modest, decreases were observed in the expression of *STAT3*, *STAT4*, *STAT5A*, *JAK1*, *JAK3* by tofacitinib, whereas there was no effect on *STAT1*, *STAT5B*, *STAT6, JAK2* and *TYK2* expression (**Figures 3C, D**). In contrast, the expression of all *SOCS*s studied was significantly

TABLE 3 | Mean (95% confidence interval) percentage inhibition of cytokine-induced STAT phosphorylation by tofacitinib in different cell populations at three months compared to baseline.

Cytokine	JAKs	STAT	Percentage inhibition in different cell types			
			Monocytes	$CD4^+$ T cells	$CD4^-$ T cells	B cells
IFN-γ	JAK1/2	pSTAT1	34 (22;45)	49 (40;58)		**58** (45;68)
IFN-α	JAK1/TYK2	pSTAT1	34 (7;54)	**50** (35;63)	43 (31;56)	30 (0.4;52)
IFN-α	JAK1/TYK2	pSTAT4		45 (34;56)	45 (34;56)	
IL-2	JAK1/3	pSTAT5		**60** (50;69)	**54** (44;64)	
IL-4	JAK1/3	pSTAT6	25 (14;36)	**53** (44;61)	**73** (65;80)	25 (3;52)
IL-7	JAK1/3	pSTAT5		47 (37;57)	47 (25;63)	
IL-15	JAK1/3	pSTAT5		**55** (43;62)	40 (29;50)	
IL-21	JAK1/3	pSTAT3		**55** (47;62)	**60** (53;67)	42 (33;53)
IL-6	JAK1/2/TYK2	pSTAT1	29 (18;39)	41 (31;51)		
IL-6	JAK1/2/TYK2	pSTAT3	12 (-30;45)	42 (27;57)	22 (9;35)	
IL-10	JAK1/TYK2	pSTAT3	10 (-1;21)	19 (12;26)	19(10;27)	43 (28;56)

Results with percentages ≥ 50 are shown in bold. Spaces left empty denote the cases in which the stimulated phosphorylation level does not differ from the constitutive level. JAK proteins involved in each cytokine signaling pathway studied are presented in the Table.

IFN, interferon; IL, interleukin; JAK, Janus kinase; (p)STAT, (phosphorylated) signal transducer and activator of transcription; TYK2, tyrosine kinase 2.

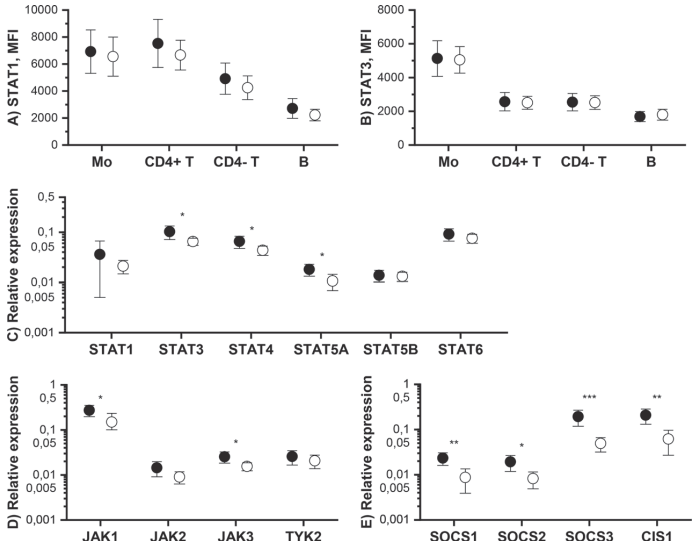


FIGURE 3 | Means and 95% confidence intervals of total **(A)** STAT1 and **(B)** STAT3 protein expression level in monocytes (Mo), $CD4^+$ T cells ($CD4^+$ T), $CD4^-$ T cells ($CD4^-$ T) and B cells and mRNA expression levels of **(C)** STAT, **(D)** JAK and **(E)** SOCS genes in PBMCs at baseline (filled symbols) and after 3-month treatment with tofacitinib and csDMARDs (open symbols). p-values comparing 0 and 3 months are calculated by Wilcoxon test. Significant differences are marked with an asterisk: *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. CIS1, cytokine-inducible SH2 domain-containing protein; csDMARD, conventional systemic disease-modifying antirheumatic drug; PBMC, peripheral blood mononuclear cell; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

downregulated during treatment with tofacitinib and csDMARDS (**Figure 3E**).

Baseline STAT Phosphorylation in Monocytes and T Cells and Expression of SOCS3 in PBMCs Correlate With Treatment Response

To study whether baseline signaling profile is associated with treatment response to tofacitinib, correlation coefficients were calculated between baseline pSTATs, total STAT1 and STAT3 and the change from baseline in DAS28 after 3 months of treatment with tofacitinib and csDMARDs (**Table 4**).

Constitutive pSTAT1 and pSTAT3 in monocytes and constitutive pSTAT1, pSTAT3 and pSTAT5 in $CD4^+$ T cells correlated positively with treatment response (**Supplementary Figures 1A–D, G**), while constitutive STAT4 phosphorylation in monocytes or in $CD4^-$ T cells correlated negatively with treatment response (**Supplementary Figures 1E, F**). In addition, IL-6-

TABLE 4 | Correlation of baseline pSTAT levels and total STAT1 and STAT3 expression levels with improvement in DAS28 during 3-month treatment with tofacitinib and csDMARDs.

Molecule	Stim.	Cell type							
		Monocytes		$CD4^+$ T cells		$CD4^-$ T cells		B cells	
		r	p	r	p	r	p	r	p
pSTAT1	None	**0.591**	**0.016**	**0.603**	**0.013**	0.391	0.134	0.280	0.294
pSTAT1	IFN-γ	0.379	0.147	**0.532**	**0.034**			0.062	0.820
pSTAT1	IFN-α	-0.079	0.770	-0.150	0.579	-0.088	0.745	0.038	0.888
pSTAT1	IL-6	**0.556**	**0.025**	0.282	0.289				
pSTAT3	None	**0.635**	**0.008**	**0.732**	**0.001**	0.424	0.102	0.426	0.099
pSTAT3	IL-6	0.385	0.141	**0.621**	**0.010**	0.126	0.641		
pSTAT3	IL-10	0.253	0.345	0.224	0.405	0.185	0.492	0.224	0.405
pSTAT3	IL-21			0.115	0.672	-0.009	0.974	0.085	0.753
pSTAT4	None	**-0.506**	**0.046**	-0.491	0.053	**-0.518**	**0.040**	-0.344	0.192
pSTAT4	IFN-α			**-0.568**	**0.022**	**-0.638**	**0.008**		
pSTAT5	None	0.350	0.184	**0.568**	**0.022**	0.271	0.311	0.276	0.300
pSTAT5	IL-2			0.147	0.587	0.159	0.557		
pSTAT5	IL-7			0.026	0.922	-0.147	0.587		
pSTAT5	IL-15			0.162	0.549	0.147	0.587		
pSTAT6	None	-0.175	0.517	-0.130	0.633	-0.219	0.414	0.218	0.418
pSTAT6	IL-4	-0.082	0.762	-0.300	0.259	-0.412	0.113	-0.279	0.295
STAT1	None	-0.291	0.274	-0.356	0.176	-0.488	0.055	-0.212	0.431
STAT3	None	-0.307	0.265	-0.354	0.196	-0.479	0.071	-0.432	0.108

Spearman correlation coefficients (r) are used. Results with p-values ≤0.05 are shown in bold. Spaces left empty denote the cases in which the stimulated phosphorylation level does not differ from the constitutive level.

DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; IFN, interferon; IL, interleukin; (p)STAT, (phosphorylated) signal transducer and activator of transcription; stim., stimulation.

stimulated pSTAT1 levels in monocytes, IFN-γ-stimulated pSTAT1 levels in $CD4^+$ T cells and IL-6-stimulated pSTAT3 in $CD4^+$ T cells correlated positively with treatment response. IFN-α-stimulated pSTAT4 in T cells correlated negatively with treatment response.

In addition, we studied the correlations between baseline demographic, clinical or laboratory variables and response to treatment with tofacitinib and csDMARDs. DAS28 (r=0.549; p=0.028) and CRP level (r=0.554; p=0.026) significantly associated with treatment response while the other parameters showed no correlation (**Supplementary Table 6**).

The association between the decrease in pSTAT or STAT levels during treatment and tofacitinib treatment response was also investigated. Decrease in constitutive STAT3 phosphorylation in monocytes (r =0.600, p=0.014) and in $CD4^+$ T cells (r=0.682, p=0.004) correlated positively with treatment response (**Supplementary Table 7**).

Finally, correlation coefficients were calculated between baseline *STAT*, *JAK* and *SOCS* mRNA levels in PBMC, or the change in mRNA expression levels, and the tofacitinib treatment response (**Supplementary Table 8**). Baseline *SOCS3* levels correlated positively with treatment response (r = 0.532; p = 0.034).

DISCUSSION

In this study we show that in patients with chronic csDMARD-unresponsive rheumatoid arthritis, tofacitinib suppresses multiple JAK-STAT pathways *in vivo* by decreasing both constitutive and cytokine-induced STAT phosphorylation in circulating leukocytes. However, the level of suppression by tofacitinib depends on the cytokine and cell type studied and is somewhat different from that reported in previous *in vitro* studies. Moreover, tofacitinib inhibits mRNA expression of several JAK-STAT pathway components and inhibitory proteins. We also show that baseline pSTAT levels in monocytes and T cells and *SOCS3* level in PBMCs correlate with treatment response.

The selectivity of JAK inhibitors is currently a major subject of interest. Recently, three *in vitro* articles comparing the potencies of several JAK inhibitors towards different cytokine signaling pathways in PBMC or blood of healthy donors have been published (17, 18, 26). Even though *in vitro* cellular modelling is a useful tool and allows direct comparison between different JAK inhibitors, the *in vitro* results do not necessarily demonstrate the actual effects of JAK inhibitors *in vivo*. Indeed, our current results reveal novel information on the effects of tofacitinib on different JAK-STAT pathways *in vivo*.

Regarding the cytokine pathways that are preferentially suppressed by tofacitinib, both *in vitro* studies (17, 18, 26) and our current study show that tofacitinib potently suppresses JAK1/JAK3 -mediated signaling induced by the common-γ-chain cytokines IL-2, IL-4, IL-15 and IL-21. Our results also indicate that both IFN-α and IFN-γ responses were suppressed by tofacitinib to almost the same extent as responses induced by the common-γ-chain cytokines, while *in vitro* studies have demonstrated variable effects of tofacitinib on IFN signaling. Regarding that interferons are crucial mediators of antiviral responses, and that the risk of herpes zoster infection is significantly increased upon JAK inhibitor use (30), the significant inhibitory effect of tofacitinib on interferon-

induced STAT1 signaling we revealed *in vivo* provides one possible mechanism for the herpes zoster infection susceptibility among tofacitinib users.

Our current *in vivo* results showed at least three inhibitory characteristics of tofacitinib that differed from the results obtained *in vitro*. First difference relates to the magnitude of inhibition of cytokine-induced STAT phosphorylation by tofacitinib. For example, the estimated daily average inhibition percentages of common-γ-chain cytokine (IL-2, IL-4, IL-15 and IL-21) responses in $CD4^+$ T cells (18, 26) or lymphocytes (17) were comparable to the maximal inhibition percentages that we demonstrate in $CD4^+$ T cells for these pathways *in vivo* (between 47 to 60%). Although the average and maximal inhibition percentages are not directly comparable, the current data nevertheless suggest that the *in vitro* studies may overestimate the inhibitory potential of tofacitinib on JAK-STAT pathway activation in RA patients *in vivo*. Our results also show that even after three months of tofacitinib treatment, cytokines can still induce STAT phosphorylation that exceeds the constitutive phosphorylation level. Therefore, the current results, although demonstrating a significant inhibitory effect of tofacitinib on constitutive and cytokine-induced STAT phosphorylation, further support the prevailing idea that oral dosing of JAK inhibitors, such as tofacitinib, allows only partial and reversible inhibition of JAK-STAT pathways (26, 31).

Second, the inhibitory effects of tofacitinib on IL-4, IL-6 and IL-10 -induced STAT phosphorylations in monocytes were lower *in vivo* than those described *in vitro* (17, 18, 26). IL-10 induced STAT3 phosphorylation demonstrated the lowest (10%) inhibition by tofacitinib in the current study. This indicates that the potent anti-inflammatory actions that IL-10 has on monocytes and macrophages (32) may not be efficiently suppressed by tofacitinib *in vivo*. In addition, regarding the potent anti-inflammatory and anti-arthritic effects of the IL-4/STAT6 pathway (33), the only modest *in vivo* potency of tofacitinib on IL-4-stimulated STAT6 phosphorylation, together with its weak effect on IL-10-stimulated STAT3 phosphorylation, may be advantageous features for the clinical efficacy of tofacitinib. It is also of note that in the present study, constitutive phosphorylation of STAT6, unlike that of the other STATs, was not decreased in any leukocyte subtype studied during 3-month tofacitinib treatment.

Third, *in vitro* data suggest that IL-6-induced STAT1 phosphorylation is more strongly inhibited by tofacitinib than IL-6-induced STAT3 phosphorylation in T cells (17, 18), whereas we demonstrate comparable inhibition of both IL-6-induced STAT pathways in $CD4^+$ T cells of RA patients *in vivo*. Actually, regarding our previous finding that constitutive STAT3 phosphorylation is common in circulating $CD4^+$ T cells in RA and associates with disease activity (8), one mechanism implementing the efficacy of tofacitinib in treating RA might be the relatively good inhibitory effect on $CD4^+$ T cell STAT3 phosphorylation *in vivo*.

There are several possible explanations for the above-mentioned differences. We used cytokine concentrations that are likely to induce maximal STAT phosphorylation, whereas lower cytokine concentrations were generally used in *in vitro* studies. *In vivo* conditions also include more variables than *in vitro* studies; for example, in our study RA patients were also treated with csDMARDs, which may influence the magnitude of the inhibitory responses seen with tofacitinib, and furthermore, the conditions in which circulating leukocytes sense tofacitinib after oral administration are surely different from those in *in vitro* incubations. Finally, during three-month treatment, tofacitinib is likely to cause long-term effects that influence the inhibitory potential of tofacitinib and its selectivity towards different cytokine pathways and thus, results obtained by studying samples of healthy volunteers in *in vitro* studies may in principle be somewhat different from ours. In fact, we demonstrate that tofacitinib treatment suppresses the mRNA expression of certain components of the JAK-STAT pathway. In particular, the expression of inhibitory molecules *SOCS*s were downregulated, which may affect the overall inhibitory state of JAK-STAT pathways and that way also influence tofacitinib's inhibitory potential *in vivo*.

The effect of tofacitinib on mRNA expression of *JAK*s, *STAT*s and *SOCS*s has not been extensively studied before. As cytokines are well-characterized inducers of *SOCS1-3* and *CIS1* expression (34), the observed decrease in their mRNA expression in the current study seems rational upon JAK inhibition. Decrease in *SOCS3* expression was the most prominent finding, and baseline *SOCS3* levels also correlated with the treatment response. This is an interesting observation regarding that STAT3-regulated *SOCS3* expression in $CD4^+$ T cells has been shown to be elevated in a cohort of 161 treatment-naïve early arthritis patients (35) and in PBMCs from patients with active RA (36). Our data suggests that tofacitinib treatment also slightly but significantly decreases *STAT3*, *STAT4*, *STAT5A*, *JAK1* and *JAK3* expression. However, as STAT3 protein levels were not repressed after the 3-month tofacitinib treatment, the mechanistic significance of the changes in STAT mRNA expression remains elusive. As tofacitinib has been shown to decrease expression of extracellular proteases, such as matrix metalloprotease 1 in rheumatoid arthritis synovial fibroblasts (37), it is possible that the inhibitory effect of tofacitinib extends also to intracellular protein degradation, such as the ubiquitin-proteasome system. This could explain the unchanged STAT3 protein level.

In order to estimate how tofacitinib-specific the observed decrease in constitutive and cytokine-induced phosphorylation of STATs is, we compared the current results to our previous results on csDMARD-unresponsive RA patients treated with biologic DMARD (11). During biological DMARD treatment, IL-4 -induced pSTAT6 was downregulated. Interestingly, the effect was clearly weaker than that shown with tofacitinib in the current study. Strikingly, neither constitutive nor IFN-γ-stimulated pSTAT1 showed significant changes during biological DMARD treatment, while the correspondent pSTAT1 levels fell significantly by tofacitinib use. For example, tofacitinib downregulated IFN-γ-stimulated pSTAT1 in monocytes by 34% and in $CD4^+$ T cells by 49%. Although results from different studies are not directly comparable, the data so far suggest that decrease in STAT phosphorylation is not a general effect achieved by any RA medication.

The association between baseline STAT phosphorylations and treatment response to tofacitinib was also examined. Baseline constitutive pSTAT1 and pSTAT3 levels in monocytes and $CD4^+$ T cells, and pSTAT5 levels in $CD4^+$ T cells correlated positively with the response. The strongest correlation was seen

with pSTAT3. The magnitude of decrease in pSTAT3 levels following tofacitinib treatment also correlated positively with treatment response. As we and others have previously shown that constitutive pSTAT1, pSTAT3 and pSTAT5 levels in T cells and pSTAT3 levels in monocytes are elevated in RA patients (7, 9, 10), tofacitinib obviously targets several RA-associated leukocyte signaling aberrations successfully. The comparison of STAT phosphorylation levels between controls and RA patients (historical data) to those between tofacitinib-treated and untreated RA patients derived from the current study suggests that the increased constitutive phosphorylation of STATs in patients with RA is reversed to a significant degree *in vivo* by tofacitinib. However, as healthy controls were not included in the current study, it remains unresolved whether the patients' STAT phosphorylation levels reached those of healthy individuals.

The only inverse correlation we found between baseline pSTAT levels and treatment response concerned STAT4. In this context it may be noteworthy that STAT4 is able to cause sustained expression of genes that increase sensitivity to IL-18 (38). IL-18 signaling does not take place *via* JAK-STAT pathways and hence, is not directly affected by tofacitinib. Both IL-18 receptor and STAT4 deficiency have suppressed the severity of arthritis in a murine model of RA (39, 40). Furthermore, STAT4 represses the genes of the Th2 cytokines IL-5 and IL-13 (41), both of which have been associated with arthritis-limiting capacity and decreased progression to fully established RA (33). Hence, STAT4 phosphorylation measured in the patients' leukocytes at baseline may be associated with immunological features that are not so easily amended by tofacitinib. It is also notable that genetic variation of STAT4 has been associated with the risk of autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis (42). It remains to be elucidated whether STAT4 variants are associated with STAT4 phosphorylation levels and RA patients' response to tofacitinib.

The effect of tofacitinib treatment on the pathobiology of rheumatoid synovium has been studied by Boyle et al. using immunohistochemistry (43). Their results showed a positive correlation between 4-month clinical improvement and reductions in STAT1 and STAT3 phosphorylation at day 28 (43). However, unlike ours, their results did not show associations between baseline STAT phosphorylation levels and the clinical response. This discrepancy might be due to methodological differences between the two studies, but it is also possible that constitutive and cytokine-stimulated pSTAT levels in circulating leukocytes, rather than in synovial cells, represent a more sensitive sensor of the overall immunological state. Nevertheless, further studies are needed to show whether STAT phosphorylation or *SOCS3* expression could be used as a biomarker to predict clinical response to tofacitinib treatment.

The strengths of the current study are: 1) prospective study on well-characterized RA patients in whom multiple JAK-STAT signaling pathways were analyzed close to *in vivo* conditions before and three months after starting tofacitinib therapy; 2) the use of whole blood flow cytometric assay, which minimizes inappropriate cell signaling pathway activation due to sample handling. The limitation of the study is that the relatively small patient cohort enables only predictive conclusions on the clinical significance of the observed association between baseline immunological parameters and treatment response. Even though it needs to be confirmed in further studies if the baseline JAK-STAT signaling profile has prognostic value for tofacitinib treatment responses, this study significantly adds to our understanding about the mechanisms of tofacitinib function in RA patients receiving this treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Committee on Medical Research Ethics (TUKIJA) and Finnish Medicines Agency Fimea. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MP, AV, KK, AKuu, ML-R, OS, and PI planned the study. RP, AKin, and PI recruited the patients. MP, KK, and AKur collected the data. MP, AV, AKuu, and AKur analyzed the results. MP, KK, AV, AKuu, and PI wrote the manuscript and all authors gave valuable comments on the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a grant from Pfizer. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank Merja Peltomaa for excellent technical assistance and Tuula Reini, Suvi Mäkinen and Arja Kaarto for their assistance in patient recruitment and follow-up. Molecular immunology laboratory at Tampere University is supported by Academy of Finland, Doctoral Programme of Medicine and Life Sciences at Tampere University, Sigrid Jusélius Foundation, Jane and Aatos Erkko Foundation, Tampere Tuberculosis Foundation and Pirkanmaa hospital district competitive research funding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.738481/full#supplementary-material

REFERENCES

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet* (2016) 388:2023–38. doi: 10.1016/S0140-6736(16)30173-8
2. McInnes IB, Buckley CD, Isaacs JD. Cytokines in Rheumatoid Arthritis-Shaping the Immunological Landscape. *Nat Rev Rheumatol* (2016) 12:63–8. doi: 10.1038/nrrheum.2015.171
3. Virtanen A T, Haikarainen T, Raivola J, Silvennoinen O. Selective JAKinibs: Prospects in Inflammatory and Autoimmune Diseases. *BioDrugs* (2019) 33:15–32. doi: 10.1007/s40259-019-00333-w
4. Schwartz DM, Bonelli M, Gadina M, O'Shea JJ. Type I/II Cytokines, JAKs, and New Strategies for Treating Autoimmune Diseases. *Nat Rev Rheumatol* (2016) 12(1):25–36. doi: 10.1038/nrrheum.2015.167
5. Gadina M, Le MT, Schwartz DM, Silvennoinen O, Nakayamada S, Yamaoka K, et al. Janus Kinases to Jakinibs: From Basic Insights to Clinical Practice. *Rheumatol (United Kingdom)* (2019) 58(Suppl 1):14–6. doi: 10.1093/rheumatology/key432
6. Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and Consequences of Jak–STAT Signaling in the Immune System. *Nat Immunol* (2017) 18(4):374–84. doi: 10.1038/ni.3691
7. Isomäki P, Junttila I, Vidqvist KL, Korpela M, Silvennoinen O. The Activity of JAK-STAT Pathways in Rheumatoid Arthritis: Constitutive Activation of STAT3 Correlates With Interleukin 6 Levels. *Rheumatol (United Kingdom)* (2015) 54(6):1103–13. doi: 10.1093/rheumatology/keu430
8. Kuuliala K, Kuuliala A, Koivuniemi R, Oksanen S, Hämäläinen M, Moilanen E, et al. Constitutive STAT3 Phosphorylation in Circulating CD4+ T Lymphocytes Associates With Disease Activity and Treatment Response in Recent-Onset Rheumatoid Arthritis. *PloS One* (2015) 10(9):e0137385. doi: 10.1371/journal.pone.0137385
9. Anderson AE, Pratt AG, Sedhom MAK, Doran JP, Routledge C, Hargreaves B, et al. IL-6-Driven STAT Signalling in Circulating CD4+ Lymphocytes Is a Marker for Early Anticitrullinated Peptide Antibody-Negative Rheumatoid Arthritis. *Ann Rheum Dis* (2016) 75(2):466–73. doi: 10.1136/annrheumdis-2014-205850
10. Pertovaara M, Silvennoinen O, Isomäki P. STAT-5 Is Activated Constitutively in T Cells, B Cells and Monocytes From Patients With Primary Sjögren's Syndrome. *Clin Exp Immunol* (2015) 181(1):29–38. doi: 10.1111/cei.12614
11. Kuuliala K, Kuuliala A, Koivuniemi R, Kautiainen H, Repo H, Leirisalo-Repo M. Stat6 and Stat1 Pathway Activation in Circulating Lymphocytes and Monocytes as Predictor of Treatment Response in Rheumatoid Arthritis. *PloS One* (2016) 11(12):e0167975. doi: 10.1371/journal.pone.0167975
12. Flanagan ME, Blumenkopf TA, Brissette WH, Brown MF, Casavant JM, Shang-Poa C, et al. Discovery of CP-690,550: A Potent and Selective Janus Kinase (JAK) Inhibitor for the Treatment of Autoimmune Diseases and Organ Transplant Rejection. *J Med Chem* (2010) 53(24):8468–84. doi: 10.1021/jm1004286
13. Haan C, Rolvering C, Raulf F, Kapp M, Drückes P, Thoma G, et al. Jak1 has a Dominant Role Over Jak3 in Signal Transduction Through γc-Containing Cytokine Receptors. *Chem Biol* (2011) 18(3):314–23. doi: 10.1016/j.chembiol.2011.01.012
14. Van Vollenhoven RF, Fleischmann R, Cohen S, Lee EB, Meijide JAG, Wagner S, et al. Tofacitinib or Adalimumab Versus Placebo in Rheumatoid Arthritis. *N Engl J Med* (2012) 367(6):508–19. doi: 10.1056/NEJMoa1112072
15. Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, et al. Modulation of Innate and Adaptive Immune Responses by Tofacitinib (CP-690,550). *J Immunol* (2011) 186(7):4234–43. doi: 10.4049/jimmunol.1003668
16. Migita K, Miyashita T, Izumi Y, Koga T, Komori A, Maeda Y, et al. Inhibitory Effects of the JAK Inhibitor CP690,550 on Human CD4+T Lymphocyte Cytokine Production. *BMC Immunol* (2011) 12:51. doi: 10.1186/1471-2172-12-51
17. Dowty ME, Lin TH, Jesson MI, Hegen M, Martin DA, Katkade V, et al. Janus Kinase Inhibitors for the Treatment of Rheumatoid Arthritis Demonstrate Similar Profiles of In Vitro Cytokine Receptor Inhibition. *Pharmacol Res Perspect* (2019) 7(6):e00537. doi: 10.1002/prp2.537
18. Traves PG, Murray B, Campigotto F, Galien R, Meng A, Di Paolo JA. JAK Selectivity and the Implications for Clinical Inhibition of Pharmacodynamic Cytokine Signalling by Filgotinib, Upadacitinib, Tofacitinib and Baricitinib. *Ann Rheum Dis* (2021) 80(7):865–875. doi: 10.1136/annrheumdis-2020-219012
19. Yarilina A, Xu K, Chan C, Ivashkiv LB. Regulation of Inflammatory Responses in Tumor Necrosis Factor-Activated and Rheumatoid Arthritis Synovial Macrophages by JAK Inhibitors. *Arthritis Rheum* (2012) 64(12):3856–66. doi: 10.1002/art.37691
20. Wang SP, Iwata S, Nakayamada S, Sakata K, Yamaoka K, Tanaka Y. Tofacitinib, A JAK Inhibitor, Inhibits Human B Cell Activation *In Vitro*. *Ann Rheumatic Dis* (2014) 73:2213–5. doi: 10.1136/annrheumdis-2014-205615
21. Kubo S, Yamaoka K, Kondo M, Yamagata K, Zhao J, Iwata S, et al. The JAK Inhibitor, Tofacitinib, Reduces the T Cell Stimulatory Capacity of Human Monocyte-Derived Dendritic Cells. *Ann Rheum Dis* (2014) 73(12):2192–8. doi: 10.1136/annrheumdis-2013-203756
22. Hodge JA, Kawabata TT, Krishnaswami S, Clark JD, Telliez JB, Dowty ME, et al. The Mechanism of Action of Tofacitinib - An Oral Janus Kinase Inhibitor for the Treatment of Rheumatoid Arthritis. *Clin Exp Rheumatol* (2016) 34:318–28.
23. Gao W, McGarry T, Orr C, McCormick J, Veale DJ, Fearon U. Tofacitinib Regulates Synovial Inflammation in Psoriatic Arthritis, Inhibiting STAT Activation and Induction of Negative Feedback Inhibitors. *Ann Rheum Dis* (2016) 75(1):311–5. doi: 10.1136/annrheumdis-2014-207201
24. Kawaguchi Y, Waguri-Nagaya Y, Tatematsu N, Oguri Y, Kobayashi M, Nozaki M, et al. The Janus Kinase Inhibitor Tofacitinib Inhibits TNF-α-Induced Gliostatin Expression in Rheumatoid Fibroblast-Like Synoviocytes. *Clin Exp Rheumatol* (2018) 36(4):559–67.
25. Furuya MY, Asano T, Sumichika Y, Sato S, Kobayashi H, Watanabe H, et al. Tofacitinib Inhibits Granulocyte-Macrophage Colony-Stimulating Factor-Induced NLRP3 Inflammasome Activation in Human Neutrophils. *Arthritis Res Ther* (2018) 20(1):196. doi: 10.1186/s13075-018-1685-x
26. McInnes IB, Byers NL, Higgs RE, Lee J, Macias WL, Na S, et al. Comparison of Baricitinib, Upadacitinib, and Tofacitinib Mediated Regulation of Cytokine Signaling in Human Leukocyte Subpopulations. *Arthritis Res Ther* (2019) 21(1):183. doi: 10.1186/s13075-019-1964-1
27. Pérez-Baos S, Gratal P, Barrasa JI, Lamuedra A, Sánchez-Pernaute O, Herrero-Beaumont G, et al. Inhibition of Pstat1 by Tofacitinib Accounts for the Early Improvement of Experimental Chronic Synovitis. *J Inflamm* (2019) 16(1):2. doi: 10.1186/s12950-019-0206-2
28. Gertel S, Mahagna H, Karmon G, Watad A, Amital H. Tofacitinib Attenuates Arthritis Manifestations and Reduces the Pathogenic CD4 T Cells in Adjuvant Arthritis Rats. *Clin Immunol* (2017) 184:77–81. doi: 10.1016/j.clim.2017.04.015
29. Cossarizza A, Chang H-D, Radbruch A, Akdis M, Andrä I, Annunziato F, et al. Guidelines for the Use of Flow Cytometry and Cell Sorting in Immunological Studies. *Eur J Immunol* (2017) 47(10):1584–797. doi: 10.1002/eji.201646632
30. Nash P, Kerschbaumer A, Dörner T, Dougados M, Fleischmann RM, Geissler K, et al. Points to Consider for the Treatment of Immune-Mediated Inflammatory Diseases With Janus Kinase Inhibitors: A Consensus Statement. *Ann Rheumatic Dis* (2021) 80:71–87. doi: 10.1136/annrheumdis-2020-218398
31. Clark JD, Flanagan ME, Telliez JB. Discovery and Development of Janus Kinase (JAK) Inhibitors for Inflammatory Diseases. *J Med Chem* (2014) 57:5023–38. doi: 10.1021/jm401490p
32. Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of Interleukin-10. *Cytokine Growth Factor Rev* (2010) 21(5):331–44. doi: 10.1016/j.cytogfr.2010.09.002
33. Chen Z, Bozec A, Ramming A, Schett G. Anti-Inflammatory and Immune-Regulatory Cytokines in Rheumatoid Arthritis. *Nat Rev Rheumatol* (2019) 15:9–17. doi: 10.1038/s41584-018-0109-2
34. Trengove MC, Ward AC. Review Article SOCS Proteins in Development and Disease. *Am J Clin Exp Immunol* (2013) 2(1):1–29.
35. Anderson AE, Maney NJ, Nair N, Lendrem DW, Skelton AJ, Diboll J, et al. Expression of STAT3-Regulated Genes in Circulating CD4+ T Cells Discriminates Rheumatoid Arthritis Independently of Clinical Parameters in Early Arthritis. *Rheumatol (United Kingdom)* (2019) 58(7):1250–8. doi: 10.1093/rheumatology/kez003
36. Isomäki P, Alanärä T, Isohanni P, Lagerstedt A, Korpela M, Moilanen T, et al. The Expression of SOCS Is Altered in Rheumatoid Arthritis. *Rheumatology* (2007) 46(10):1538–46. doi: 10.1093/rheumatology/kem198

37. Boor PPC, de Ruiter PE, Asmawidjaja PS, Lubberts E, van der Laan LJW, Kwekkeboom J. JAK-Inhibitor Tofacitinib Suppresses Interferon Alfa Production by Plasmacytoid Dendritic Cells and Inhibits Arthrogenic and Antiviral Effects of Interferon Alfa. *Transl Res* (2017) 188:67–79. doi: 10.1016/j.trsl.2016.11.006
38. SR G, VT T, AN M, Q Y, GL S, N Y, et al. Temporal Induction Pattern of STAT4 Target Genes Defines Potential for Th1 Lineage-Specific Programming. *J Immunol* (2009) 183(6):3839–47. doi: 10.4049/jimmunol.0901411
39. KM H, P S, I A, M D, B B, PR G, et al. Targeting of the Transcription Factor STAT4 by Antisense Phosphorothioate Oligonucleotides Suppresses Collagen-Induced Arthritis. *J Immunol* (2007) 178(6):3427–36. doi: 10.4049/jimmunol.178.6.3427
40. Y N, J R, K S, K N, K K, M F, et al. Inhibition of the IL-18 Receptor Signaling Pathway Ameliorates Disease in a Murine Model of Rheumatoid Arthritis. *Cells* (2019) 9(1):11. doi: 10.3390/cells9010011
41. SR GH, LD E, NS van O, JD F. STAT4-Mediated Transcriptional Repression of the IL5 Gene in Human Memory Th2 Cells. *Eur J Immunol* (2016) 46 (6):1504–10. doi: 10.1002/eji.201546050
42. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the Risk of Rheumatoid Arthritis and Systemic Lupus Erythematosus. *N Engl J Med* (2007) 357(10):977–86. doi: 10.1056/NEJMoa073003
43. Boyle DL, Soma K, Hodge J, Kavanaugh A, Mandel D, Mease P, et al. The JAK Inhibitor Tofacitinib Suppresses Synovial JAK1-STAT Signalling in Rheumatoid Arthritis. *Ann Rheum Dis* (2015) 74(6):1311–6. doi: 10.1136/annrheumdis-2014-206028

Conflict of Interest: MP has received a personal fee from Pfizer and is currently a part-time employee of MedEngine. KK has received a grant from Pfizer. RP has received personal fees from Pfizer, Eli Lilly and Company and Janssen. AKin has received non-financial support from Sandoz, Mylan, Celgene and Bristol-Myers Squibb. PI has received a grant from Pfizer and personal fees from Pfizer, Eli Lilly and Company, Abbvie and Roche and non-financial support from Abbvie and Roche. OS has received personal fees from Pfizer and Abbvie and holds patents on JAK kinases, US Patent no. 5,728,536, US patent no. 8,841,078, AU 2011214254, CAN 2789186, and EPO 11741946.5. OS is employed by Fimlab Laboratories Ltd.

The remaining 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers

Supplementary Material

Supplementary Table 1. Recombinant cytokines and their manufacturers.

Cytokine	Company
IL-2	Peprotech, Rocky Hill, NJ, USA
IL-4	Peprotech
IL-6	Peprotech
IL-7	Peprotech
IL-10	R&D Systems, Minneapolis, MN, USA
IL-15	Peprotech
IL-21	Peprotech
IFN-α	Cell Signaling Technology, Danvers, MA, USA
IFN-γ	Peprotech

Supplementary Table 2. Flow cytometry antibodies, conjugates, clones, and manufacturers.

Antibody	Conjugate	Clone	Company
CD33	APC	P67.7	Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA
CD3	FITC	SK7	BD
CD4	APC e-Fluor	SK3	eBioscience, Santa Clara, CA, USA
CD20	PE-Cy7	H1(FB1)	BD
STAT1	PE	1/Stat1	BD
STAT3	PE	M59-50	BD
pSTAT1	PE	4a	BD
pSTAT3	PE	4/P-STAT3	BD
pSTAT4	PE	38/p-Stat4	BD
pSTAT5	PE	47	BD
pSTAT6	PE	18/P-Stat6	BD

Supplementary Table 3. Primer sequences.

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
STAT1	TCACATTCACATGGGTGGAG	CAAAGGCATGGTCTTTGTCA
STAT3	TCACATGCCACTTTGGTGTT	GCAATCTCCATTGGCTTCTC
STAT4	GGCAATTGGAGAAACTAGAGG	AGGGTGGGTTGGCATACAT
STAT5A	GCCAGATGCAGGTGCTGTA	GGGATTGTCCAAGTCAATGG
STAT5B	GCGTTATATGGCCAGCATTT	CTGGTGCTCTGCCTTCTTCT
STAT6	GGAAGGGCACTGAGTCTGTC	GGCTTTGGCATTGTTGTCTT
JAK1	CATGGTGGAAGAGTTTGTGGA	CAGCTGTTTGGCAACTTTGAATT
JAK2	CCGCCGGGTTTCAGAAG	GAAGAGGTGGATGTTCCCTCC
JAK3	AGTGGGACTTTCCTCTCGC	CTCTTCACTTGGAGGTGCCAT
TYK2	CCCATGGCTTGGAAGATGGT	ACTCAGCTTGATGAAGGGGC
SOCS1	CTGGGATGCCGTGTTATTTT	TAGGAGGTGCGAGTTCAGGT
SOCS2	CAGGGAATGGCAGAGACACT	TGGCAGAGAGAGAAGGGATG
SOCS3	GCCACCTACTGAACCCTCCT	ACGGTCTTCCGACAGAGATG
CIS1	AGCCCAGACAGAGAGTGAGC	TGACAGCGTGAACAGGTAGC
β-actin	TGGGACGACATGGAGAAAAT	AGAGGCGTACAGGGATAGCA

Supplementary Table 4. csDMARD treatment of the patients at baseline.

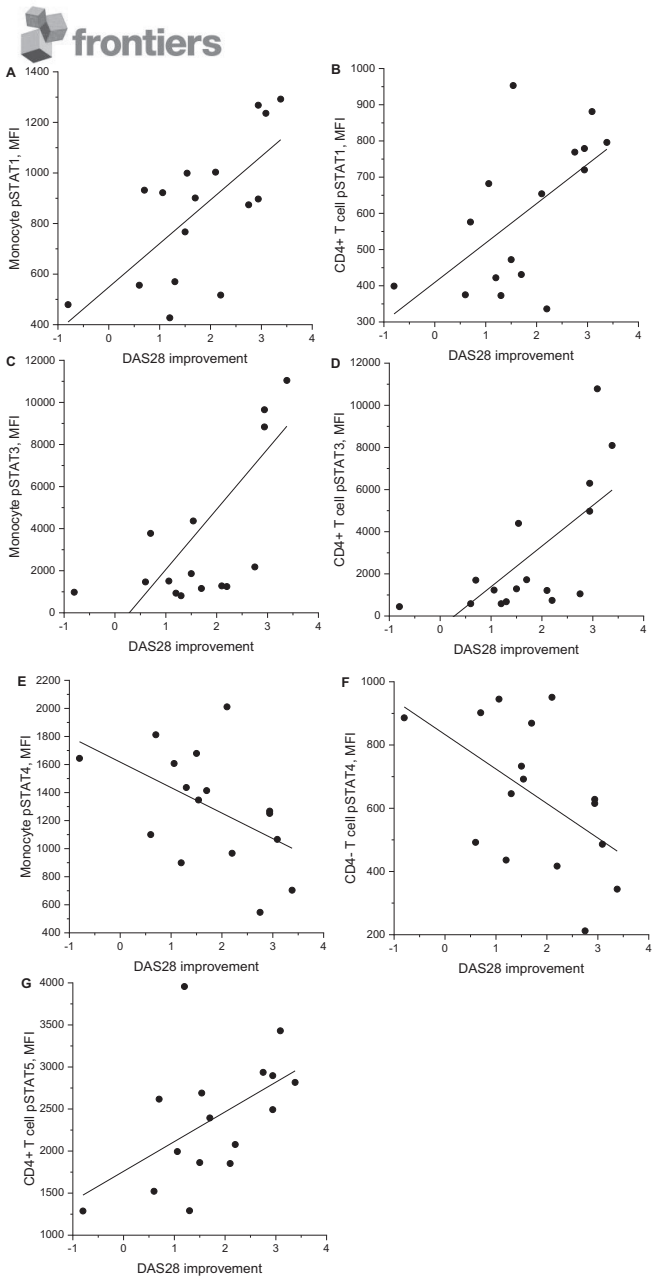
Patient	Methotrexate	Sulphasalazine	Hydroxychloroquine	Other csDMARD	Prednisolone
1	15 mg weekly po	2000 mg daily	300 mg daily		
2	20 mg weekly po				10 mg daily
3	25 mg weekly po	2000 mg daily			
4	15 mg weekly sc	2000 mg daily			
5		2000 mg daily	300 mg daily		
6	15 mg weekly po	2000 mg daily	300 mg daily		2.5 mg daily
7	25 mg weekly sc		300 mg 6 days/week		
8	20 mg weekly sc		300 mg 6 days/week		2.5 mg daily
9	25 mg weekly sc	2000 mg daily	300 mg 4-5 days/week		
10	25 mg weekly sc	1500 mg daily	300 mg 6 days/week		2.5 mg daily
11			300 mg daily		
12	20 mg weekly sc				5 mg daily
13		2000 mg daily		leflunomide 20 mg daily	5 mg daily
14			300 mg daily	azathioprine 50 mg daily	
15	25 mg weekly po	2000 mg daily	300 mg daily		5 mg daily
16	25 mg weekly sc	2000 mg daily	300 mg daily		7.5 mg daily

csDMARD, conventional synthetic disease-modifying antirheumatic drug; po, oral administration; sc, subcutaneous injection

Supplementary Table 5. The difference in constitutive STAT1, STAT3 and STAT5 phosphorylation in circulating monocytes and CD4-positive ($CD4^+$) and -negative ($CD4^-$) T lymphocytes between RA patients and healthy controls (left panels), and between RA patients before and after tofacitinib treatment (right panels). Comparison between healthy controls (n=11-13) and rheumatoid arthritis patients (n=12-14) was performed on data derived from previous studies (references 7 and 10), and comparison between untreated and tofacitinib-treated RA patients (n=16) on data from the current study. The experimental methods used in the previous and current studies were similar, but for better comparison of two separate studies, mean fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence intensity (MFI) value of each pSTAT with MFI value of mouse IgG isotype control. Both the absolute differences (ΔMFIR) and percentage differences in MFIR between the groups are presented. pSTAT levels of RA patients and healthy controls were compared using Mann-Whitney test, and pSTAT levels before and after tofacitinib treatment with Wilcoxon test and p-values are shown in the Table.

		RA patients vs healthy controls			**RA patients before vs after tofacitinib treatment**		
pSTAT	**Cell type**	**ΔMFIR (RA patients – healthy controls)**	**Percentage difference in MFIR**	**p**	**ΔMFIR (before tofacitinib – after tofacitinib)**	**Percentage difference in MFIR**	**p**
pSTAT1	Monocytes	0.00	0	0.846	0.68	26	0.002
	$CD4^+$ T cells	0.52	20	0.020	1.87	34	0.002
	$CD4^-$ T cells	0.22	11	0.139	1.00	30	0.002
pSTAT3	Monocytes	1.38	39	0.001	4.41	34	0.005
	$CD4^+$ T cells	2.43	55	<0.001	12.21	44	0.003
	$CD4^-$ T cells	0.71	31	0.024	0.95	23	0.019
pSTAT5	Monocytes	0.03	2	0.538	0.49	4	0.518
	$CD4^+$ T cells	1.54	38	<0.001	9.43	41	0.003
	$CD4^-$ T cells	0.49	19	0.006	1.31	10	0.187

*data derived from references 7 and 10. MFIR, mean fluorescence intensity ratio; (p)STAT, (phosphorylated) signal transducer and activator of transcription.



Supplementary Figure 1. Correlation of DAS28 improvement during tofacitinib and csDMARDs treatment and basal mean fluorescence intensity (MFI) levels at baseline of A) monocyte and B) $CD4^+$ T cell pSTAT1. C) monocyte and D) $CD4^+$ T cell pSTAT3. E) monocyte and F) $CD4^-$ T cell pSTAT4. and G) $CD4^+$ T cell pSTAT5. Lines of best fit are shown. csDMARD, conventional systemic disease-modifying antirheumatic drug; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); (p)STAT, (phosphorylated) signal transducer and activator of transcription.

Supplementary Table 6. Correlation of baseline demographic. clinical and laboratory variables with DAS28 improvement during 3-month treatment with tofacitinib and csDMARDs. Spearman correlation coefficients (r) are used. Results with p-values ≤0.05 are shown in bold.

	r	p
Swollen joint count. 0-46	0.167	0.537
Tender joint count. 0-46	0.124	0.647
Swollen joint count. 0-28	-0.051	0.852
Tender joint count. 0-28	0.297	0.264
General health. VAS. 0-100 mm	0.069	0.799
Pain. VAS. 0-100 mm	0.130	0.632
Physician's assessment. VAS. 0-100 mm	-0.155	0.566
HAQ disability index. 0-3	-0.235	0.381
DAS28	0.549	**0.028**
Plasma C-reactive protein. mg/l	0.554	**0.026**
Blood haemoglobin. g/l	-0.105	0.699
Blood leukocyte count. $\times 10^9$/l	-0.043	0.875
Blood neutrophil count. $\times 10^9$/l	0.103	0.704
Blood lymphocyte count. $\times 10^9$/l	-0.397	0.128
Blood platelet count. $\times 10^9$/l	0.227	0.399
Plasma alanine aminotransferase. U/l	-0.362	0.168
Plasma creatinine. µmol/l	0.121	0.656

DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); HAQ, Health Assessment Questionnaire; IQR, interquartile range; VAS, visual analogue scale

Supplementary Table 7. Correlation of change in pSTAT and total STAT1 and STAT3 levels with DAS28 improvement during 3-month treatment with tofacitinib and csDMARDs. Spearman correlation coefficients (r) are used. Results with p-values ≤0.05 are shown in bold. Spaces left empty denote the cases in which the stimulated phosphorylation level does not differ from the basal level

		Cell type							
		Monocytes		**CD4$^+$ T cells**		**CD4$^-$ T cells**		**B cells**	
Molecule	**Stim.**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
pSTAT1	None	0.313	0.256	0.407	0.132	0.375	0.168	0.000	1.000
pSTAT1	IFN-γ	-0.009	0.974	0.450	0.080			0.079	0.770
pSTAT1	IFN-α	-0.229	0.393	-0.285	0.284	-0.303	0.254	-0.256	0.339
pSTAT1	IL-6	-0.103	0.704	0.044	0.871				
pSTAT3	None	**0.600**	**0.014**	**0.682**	**0.004**	0.009	0.974	0.041	0.880
pSTAT3	IL-6	0.212	0.431	0.297	0.264	0.056	0.837	0.124	0.649
pSTAT3	IL-10	-0.050	0.854	0.074	0.787	0.112	0.680	0.482	0.058
pSTAT3	IL-21			0.174	0.520	0.168	0.535	-0.256	0.339
pSTAT4	None	-0.162	0.549	-0.135	0.617	-0.056	0.837	-0.132	0.625
pSTAT4	IFN-α			-0.368	0.161	-0.385	0.141		
pSTAT5	None	-0.374	0.154	0.071	0.795	-0.282	0.289	-0.200	0.458
pSTAT5	IL-2			0.109	0.688	0.021	0.940		
pSTAT5	IL-7			-0.026	0.922	-0.341	0.196		
pSTAT5	IL-15			0.021	0.940	-0.121	0.656		
pSTAT6	None	0.106	0.696	0.110	0.684	-0.077	0.778	0.232	0.387
pSTAT6	IL-4	-0.024	0.931	0.053	0.846	-0.226	0.399	-0.253	0.345
STAT1	None	-0.368	0.161	-0.224	0.405	-0.168	0.535	-0.124	0.649
STAT3	None	-0.164	0.558	-0.221	0.428	-0.268	0.334	-0.239	0.390

csDMARD, conventional systemic disease-modifying antirheumatic drug; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); IFN, interferon; IL, interleukin; (p)STAT, (phosphorylated) signal transducer and activator of transcription; stim., stimulation

Supplementary Table 8. Correlation of mRNA expression levels of JAK-STAT pathway genes in PBMCs with disease activity. A. expression at baseline vs the change from baseline in DAS28 during 3-month treatment with tofacitinib and csDMARDs; B. change in expression vs the change from baseline in DAS28 during 3-month treatment with tofacitinib and csDMARDs. Spearman correlation coefficients (r) are used. Results with p values ≤0.05 are shown in bold.

	A		B	
Transcript	**r**	**p**	**r**	**p**
STAT1	-0.065	0.812	0.094	0.729
STAT3	-0.021	0.940	-0.047	0.863
STAT4	-0.056	0.837	0.188	0.485
STAT5A	-0.297	0.264	0.309	0.244
STAT5B	-0.135	0.617	-0.147	0.587
STAT6	-0.015	0.957	-0.224	0.405
JAK1	-0.388	0.137	0.376	0.151
JAK2	-0.212	0.431	0.103	0.704
JAK3	0.229	0.393	-0.194	0.471
TYK2	0.153	0.572	-0.121	0.656
SOCS1	-0.044	0.871	0.462	0.072
SOCS2	-0.132	0.625	0.450	0.080
SOCS3	**0.532**	**0.034**	-0.229	0.393
CIS1	-0.174	0.520	0.471	0.066

CIS1, cytokine-inducible SH2 domain-containing protein; csDMARD, conventional systemic disease-modifying antirheumatic drug; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); JAK, Janus kinase; PBMC, peripheral blood mononuclear cell; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TYK2, tyrosine kinase 2

PUBLICATION III

***In vitro* JAK-selectivity profiling and head-to-head comparison of 20 clinical JAKinibs.**

Virtanen A, Palmroth M, Liukkonen S, Kurttila A, Haikarainen T, Zmajkovic J, Skoda R, Silvennoinen O.

Submitted manuscript.