A SINGLE DOMAIN ANTIBODY DERIVED FROM ANTIBODY PHAGE DISPLAY LIBRARY FUSED WITH CELL PENETRATING PEPTIDE FOR THE CLEARANCE OF IL-23

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by

AHMAD ISMAIL KHALED ABDO

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The praise for the great Allah, as without his mercy, guidance and assistance, no success can be achieved.

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LIST OF ABBREVIATIONS

3′UTR	3′-untranslated region
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACR	American College of Rheumatology
ADA	Anti-drug antibodies
ADCC	Antibody-dependent cell cytotoxicity
AMPs	Antimicrobial peptides
APCs	Antigen presenting cells
APS	Ammonium persulfate
BME	ß-Mercaptoethanol
BSA	Bovine serum albumin
BSC	Biosafety cabinet
$CaCl_2$	Calcium chloride
Ca-D	Calcium dependent sdAB
CDRs	Complementary-determining regions
cfu	Colony forming unit
C_H	Constant heavy chain
CIA	Collagen-induced arthritis
C_L	Constant light chain
CMV	Cytomegalovirus
CPP	Cell-penetrating peptide
CV	Column volumes
DAB	3, 3'-diaminobenzidine
DCs	Dendritic cells

dH_2o	Distilled water
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's phosphate buffer saline
EBNA	Epstein-Barr nuclear antigen
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
FcRn	Neonatal Fc receptor
FLSs	Fibroblast-like synoviocytes
FRs	Framework regions
FRT	Flp recombination target
FSC	Forward scatter
Gam	Gamillus
gDNA	Genomic DNA
GFP	Green fluorescent protein
GST	Glutathione S-transferases
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
Histidine	His
HLA	Human leukocyte antigen
IBD	Inflammatory bowel diseases
IDT	Integrated DNA Technology
IEDB	Immune epitope database
IFN	Interferon-gamma

IL-23	Interleukin 23
IL-23R	Interleukin 23 receptor
ILCs	Innate lymphoid cells
JAK	Janus kinase
KCl	Potassium chloride
KCs	Keratinocytes
K_D	Dissociation constant
KH_2PO_4	Potassium dihydrogen phosphate
LPS	Lipopolysaccharides
MACE	Major adverse cardiac events
MBP	Mannose binding protein
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
Muc	Mucin
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NC	Nitrocellulose
NOD	Nucleotide-binding and oligomerization domain
PAGE	Poly acrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns

PASI	Psoriasis Area and Severity Index
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBS-T	PBS Tween 20
pDCs	Plasmacytoid dendritic cells
PEG	Polyethylene glycol
pfu	Plaque forming unit
pH-D	pH dependent sdAB
PMA	Phorbol 12-myristate-13-acetate
PMT	Photomultiplier tubes
PPs	Peyer's patches
PS	Psoriasis
RA	Rheumatoid Arthritis
RANK	Receptor activator of NF-κB
RANKL	RANK ligand
RegIII	Regenerating gene III
RORγt	Retinoic acid-related orphan receptor γ-T
RT-PCR	Real time PCR
ScFv	Single chain variable fragment
sdAB	Single domain antibody
SDS	Sodium dodecyl sulfate
SSC	Side scatter
ssDNA	Single strand DNA
STAT	Signal transducers and activators of transcription
TBE	Tri borate EDTA buffer

TCR	T cell receptor
TEMED	N,N,N′,N′ -TEtramethylethylenediamine
TLR	Toll-like receptor
TNF	Tissue necrosis factor
TNFR	Tissue necrosis factor receptor
TRX	Thioredoxin A
TTP	LPS-induced tristetraprolin
TYK	Tyrosine kinase
VEGFR	Vascular endothelial growth factor
V_H	Variable heavy chain
V_{HH}	Variable domain of the heavy chain
V_L	Variable light chain
VNAR	Variable domain of new antigen receptor

LIST OF SYMBOLS

A_{260}	Absorbance at 260 nm wavelength
bp	Base pair
Ct	Cycle threshold
Fw	Forward
g	Gram
KDa	Kilo Dalton
L	Liter
M	Molar
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimole
MW	Molecular weight
MWCO	Molecular weight cutoff
n	Sample size
NTC	No template control
OD_{600}	Optical density absorbance at 600 nm wavelength
pI	Isoelectric point
pKa	Acid dissociation constant
rpm	Round per minute
Rv	Reverse
s	Seconds
SD	Standard deviation
Tm	Primer melting temperature

UV	Ultraviolet
V	Voltage
xg	Times gravity
µg	Microgram
µl	Microliter

LIST OF APPENDICES

SATU DOMAIN ANTIBODI YANG DIPEROLEH DARIPADA PERPUSTAKAAN PAPARAN FAJ ANTIBODI DIGABUNGKAN DENGAN PEPTIDA PENEMBUS SEL UNTUK PENYINGKIRAN IL-23

ABSTRAK

IL-23 ialah sitokin proinflamasi dua subunit yang memainkan peranan penting dalam membentuk tindak balas imun. Ia dikaitkan dengan beberapa penyakit autoinflamasi dengan menjana gelung keradangan berterusan melalui laluan IL-17 dan IL-22 yang membawa kepada kerosakan tisu. Peneutralan antibodi terhadap IL-23 telah terbukti berkesan dalam memecahkan gelung ini dan memperbaiki penyakit. Walau bagaimanapun, antibodi mempunyai penembusan tisu yang terhad dan cenderung mencetuskan antibodi anti-ubat disebabkan saiznya yang besar. Selain itu, antibodi anti-IL-23 hanya menyasarkan satu subunit sitokin, sama ada p40 atau p19, meninggalkan subunit yang lain tanpa peneutralan. Kajian ini bertujuan untuk menghasilkan antibodi domain tunggal anti-IL-23 (sdAB) yang boleh membawa kepada penyingkiran antigen. Dua kaedah penyingkiran antigen diterokai, iaitu pengasingan sdAB kitar semula melalui pempaparan faga dan pengkonjugasian sdAB dengan peptida penembusan sel (CPPs). SdAB dihasilkan melalui pempaparan faga dengan biopanning terhadap salah satu subunit IL-23, p19, yang diekspreskan dalam *E. coli* dan diikat dengan protein Gamillus (Gam) sebagai penstabil. Biopanning gagal mengasingkan pengikat kitar semula yang spesifik dan berafiniti tinggi untuk p19, namun, pengikat standard yang spesifik dan berafiniti tinggi untuk p19 berjaya diasingkan. SdAB yang diasingkan, dinamakan H11, dikonjugasikan dengan dua CPPs, R9 dan TATk, masing-masing. Keputusan ELISA untuk pengikatan p19 kepada H11, H11-R9, dan H11-TATk yang telah disucikan, menunjukkan bahawa

pengkonjugatan CPP tidak mengubah afiniti pengikatan H11. Fungsi sdAB yang disucikan ini diperiksa lebih lanjut untuk menilai kesan penggabungan CPP. Analisis mikroskopi pendarfluor menunjukkan bahawa p19-Gam diinternalisasikan dalam kumpulan H11-R9 dan H11-TATk tetapi tidak dalam kumpulan H11, yang disahkan oleh sitometri aliran. Keputusan ELISA sandwich menggunakan IL-23R-IgG1 yang telah disucikan mendedahkan bahawa kira-kira 53% p19-Gam dibersihkan daripada medium dalam kumpulan H11-R9 dan H11-TATk berbanding hanya 4% dalam kumpulan H11. Akhir sekali, RT-PCR untuk PBMCs yang dirangsang IL-23 menunjukkan bahawa ketiga-tiga sdAB tersebut menghalang ekspresi IL-17 dan IL-22 dengan ketara, tetapi dengan variasi dalam keputusan yang mungkin disebabkan oleh interaksi CPP-PBMC. Kesimpulannya, sdAB IL-23 spesifik dengan keupayaan penyingkiran antigen yang berpotensi telah berjaya dihasilkan. Kompleks sdAB-CPP berkemungkinan mempunyai potensi terapeutik untuk penyakit autoimun berasaskan IL-23 yang mampu mengatasi batasan antibodi bersaiz penuh.

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ABSTRACT

IL-23 is a two-subunit proinflammatory cytokine that plays an important role in shaping the immune response. It is associated with several autoinflammatory diseases by generating sustained inflammatory loops through the IL-17 and IL-22 arms leading to tissue damage. Antibody neutralization of IL-23 was proven to be effective in breaking these loops and ameliorating disease. However, antibodies have limited tissue penetration and tend to elicit anti-drug antibodies due to their large size. Additionally, anti-IL-23 antibodies target only one subunit of the cytokine, either p40 or p19, leaving the other one unneutralized. This study aimed to generate an anti-IL-23 single domain antibody (sdAB) that leads to antigen-clearance. Two methods of antigen clearance were explored, the isolation of a recycling sdAB by phage display and conjugation of sdAB to cell penetrating peptides (CPPs). The sdAB was generated by phage display biopanning against one of the IL-23 subunits, p19, which was expressed in *E.coli* fused to Gamillus (Gam) protein as a stabilizer. Biopanning failed to isolate specific, high-affinity recycling binders for p19, however, a specific and high-affinity standard binder for p19 was successfully isolated. The isolated sdAB, designated as H11, was conjugated with two CPPs, R9 and TAT_k, respectively. ELISA results of p19 binding to purified H11, H11-R9, and H11-TAT_k , showed that CPPs conjugation did not alter the H11 binding affinity. The functionality of the purified sdABs was further examined to evaluate the CPP fusion effect. Fluorescent microscopy analysis revealed that p19-Gam was internalized in H11-R9 and H11-TAT_k

groups but not in H11 group which was confirmed by flow cytometry. Sandwich ELISA results using purified IL-23R-IgG1 revealed that around 53% of p19-Gam were cleared from the medium in H11-R9 and H11-TAT_k groups compared to only 4% in H11 group. Finally, RT-PCR for IL-23-stimulated PBMCs showed that the three sdABs significantly inhibited the IL-17 and IL-22 expression, but with a variation among the results probably due to the CPP-PBMC interaction. In conclusion, a specific IL-23 sdAB with potential antigen-clearance capability was successfully generated. SdAB-CPP complexes may have therapeutic potential for IL-23-based autoimmune diseases that can overcome full-length antibodies limitations.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Autoimmunity

The immune system's cells are activated upon receiving a danger signal or recognizing non-self antigens, which can be pathogens or diseases such as cancers. This activation initiates several inflammatory mechanisms including cytokines and antimicrobial peptides (AMPs) secretion, phagocytosis, antibody production and cell-mediated cytotoxicity (Medzhitov, 2007). Excessive effector inflammatory mechanisms, however, can injure the host. Hence, inflammation is tightly regulated by anti-inflammatory mediators. Both inflammatory and anti-inflammatory reactions shape the immune response. A balanced immune response can eliminate harmful and unwanted molecules or cells, while dysregulation and shifting the equilibrium towards one side can result in fatal diseases.

Immune-mediated disorders are conditions characterized by an uncontrolled immune response that leads to body damage. For example, overexpression of immune suppressive T regulatory cells (Treg), a subset of T cells which secrete anti-inflammatory cytokines such as TGF-β, IL-10 and IL-35, can attenuate cancer immune surveillance and contribute to tumor development (Ohue & Nishikawa, 2019). On the other hand, continuous inflammation leads to a deleterious impact on body tissues which can diminish or completely halt tissue function such as in rheumatism and type 1 diabetes. This can be due to B cell receptor (BCR) and T cell receptor (TCR) recognizing self-antigens, creating autoreactive lymphocytes; or by positive feedbacks inflammatory signaling through cytokine cross-talk among immune cells that keeps inflammation ongoing (Doria et al., 2012a). In both cases, the body own molecules are

the “fuels” for inflammation which create “auto” immune response activation leading to life-time chronic diseases.

In other words, autoimmune diseases are conditions where the body attacks and destroys itself. Several risk factors have been correlated with autoimmunity development. The genetic make-up of inflammatory mediators such as human leukocyte antigen (HLA), infections, tissue injuries and environmental factors such as UV and toxic chemicals exposure, are associated with several autoimmune diseases (Rosenblum et al., 2015). Even some vaccines were reported to cause autoimmune disorders as the contained antigens mimic host self-antigens, contributing to autoreactive lymphocytes generation (Vadalà et al., 2017). Nevertheless, the exact trigger and mechanism of initiating autoimmune response is not well defined, and thus, there isn’t any licensed vaccine or permanent treatment for these diseases. There are, however, some clinical trials that are aimed at protecting children of families of autoimmune history that are at high risk of developing autoimmune diseases (Doria et al., 2012b).

Current autoimmune diseases therapies are commonly immunosuppressive drugs, which are used for controlling rather than curing the condition. In autoimmune diseases where autoinflammation is characterized (instead of autoreactive lymphocytes), the treatments focus on targeting cytokines or their receptors. Several cytokines were well documented by their ability to create inflammatory loops by positive feedback mechanisms. Breaking these loops by cytokine neutralization was proven to attenuate inflammation and ameliorate the diseases. One of these cytokines is interleukin 23 (IL-23), which since its discovery two decades ago. It became an area of interest to many as it was to influence both phagocytes and lymphocytes, bridging innate and adaptive cellular immunity, and playing an essential role in body defense.

At the same time, if it's not controlled, it contributes to autoimmune diseases development (Croxford et al., 2012; Oppmann et al., 2000a).

1.2 IL-23 Biology

IL-23 is a heterodimer proinflammatory cytokine that belongs to the special IL-12 family which comprise of only heterodimeric cytokines. IL-23 is made up of two proteins subunits, the p40 subunit that is shared with IL-12 located on the 11q1.3 chromosome, and a unique p19 subunit on chromosome 12q13.2 that is composed of four α-helix strands. Both subunits are linked by a disulfide bond and they are attached only if they are synthesized in the same cell (Oppmann et al., 2000a).

1.2.1 IL-23 Regulation

IL-23 is mainly secreted by activated macrophages and dendritic cells (DCs). It can also be secreted by non-immune cells including keratinocytes (KCs) and synoviocytes. Stimulation of a few immune receptors can strikingly induce IL-23 expression. Peptidoglycan, which is abundant in *gram-positive* bacteria, and the *gram-negative* bacterial lipopolysaccharides (LPS) are potent IL-23 inducers via binding to toll-like receptor (TLR) 4 and 3 respectively (Re & Strominger, 2001; Smits et al., 2004). Zymosan, a β-glucan and a major fungus and yeast cell wall component, was also found to induce IL-23 secretion as a Dectin-1 receptor and TLR2 agonist (Lyakh et al., 2008). Other publications had additionally reported that antigen recognition by TLR9, TLR3 and TLR7 are involved in IL-23 induction either alone or cooperatively with other TLRs (Al-Salleeh & Petro, 2007; Bhan et al., 2010, 2013).

The captured antigens by professional antigen presenting cells (APCs), mainly macrophages and DCs, are presented to lymphocytes for adaptive immunity activation.

The process involves several receptor/ligand bindings including CD40/CD40L. This ligation induces IL-23 expression in APCs (Uhlig et al., 2006). Some receptors play a complex regulatory role in immune response, such as nucleotide-binding and oligomerization domain (NOD) 2, whereas it was found that NOD2 interacts synergistically with NOD1 and TLR8 to significantly increase IL-23 expression (Schwarz et al., 2013). On the other hand, NOD2 was reported to induce microRNA-29 which, is responsible for the downregulation of p40 and p19 (Brain et al., 2013).

Multiple cytokines play complex roles in IL-23 regulation. IL-23 p19 was shown to be upregulated in fibroblast-like synoviocytes (FLSs) in response to IL-1β (F.-L. Liu et al., 2007). Tissue necrosis factor-alpha (TNF-α) was discovered elevating IL-23 expression in KCs and FLSs (Goldberg et al., 2009a; H. Li et al., 2018). However, TNF-α has an opposite effect on macrophages and DCs as it was revealed that stimulation of tissue necrosis factor receptor (TNFR) 1 with TNF-α downregulate p40, leading to less IL-23 secretion (Zakharova & Ziegler, 2005). A similar effect goes for interferon-gamma (IFN-γ) with investigations indicating that IFN-γ has a negative impact on p19 mRNA stability. Moreover, IFN-γ is able to raise LPS-induced tristetraprolin (TTP), where TTP is an RNA binding protein and a suppressor of Adenylate-uridylate rich mRNA including p19 (Molle et al., 2013; Qian et al., 2011). Furthermore, IL-10 which, is a main anti-inflammatory cytokine, is capable downregulating IL-23 expression (W. Liu et al., 2009).

1.2.2 IL-23 Receptor and Influence

IL-23 is recognized by a heterodimer receptor protein complex that consists of IL-12β and IL-23R proteins that bind to p40 and p19 subunits respectively. Signaling through IL-23 receptor is mediated by two Janus kinase (JAK) family members; JAK2

which is attached to IL-23R, while tyrosine kinase 2 (TYK2) is linked to IL-12β. Upon ligation to IL-23, the two JAK members creates a docking site for signal transducers and activators of transcription 3 (STAT3) on IL-23R and STAT4 on IL-12β by phosphorylating the tyrosine residues on both protein subunits of the receptor complex. This in turn activates and phosphorylates both STATs followed by homodimerization of the STATs molecules then translocation into the nucleus for transcription (Parham et al., 2002; Vignali & Kuchroo, 2012) (**Figure 1.1**)

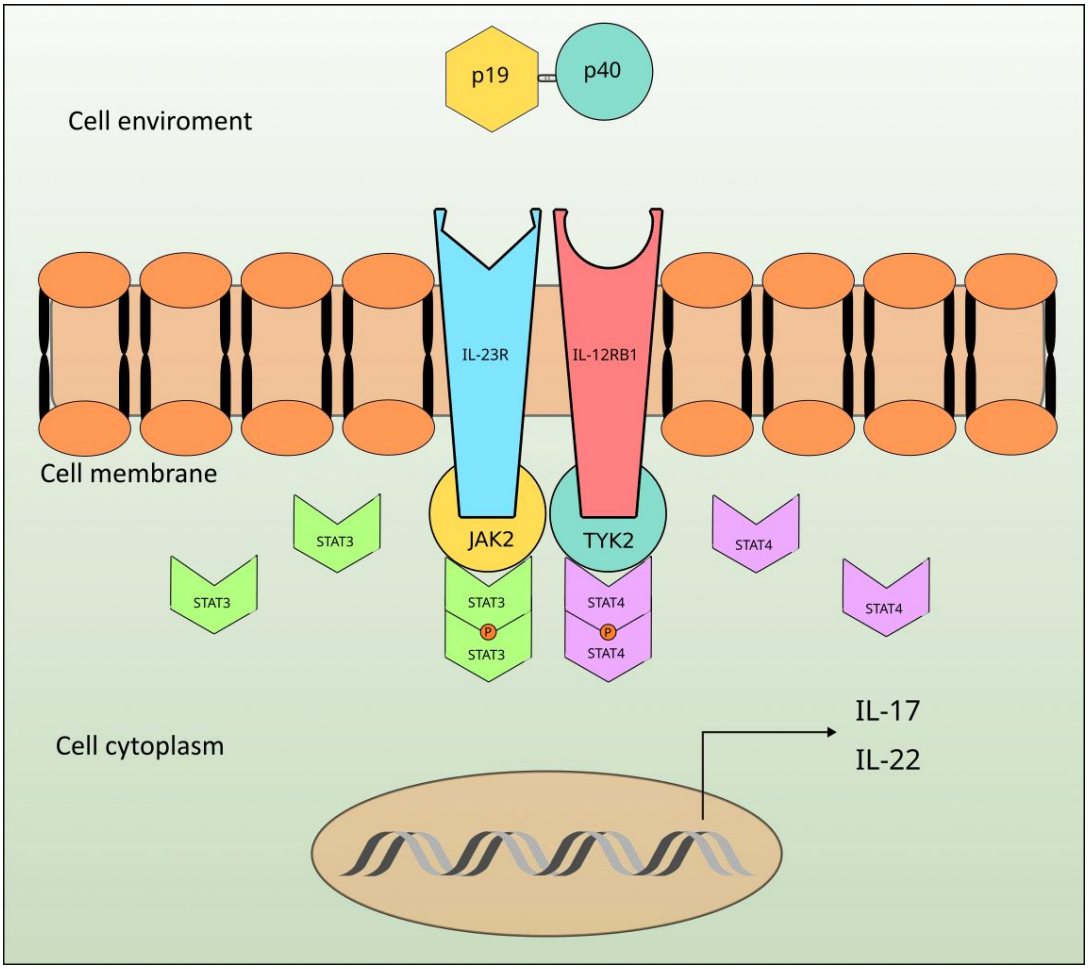

Figure 1.1 IL-23 protein, receptor and signaling pathway. IL-23 cytokine consists of a shared p40 subunit with IL-12 and a unique p19 subunit which are recognized by IL-12β and IL-23R proteins respectively. Both IL-23 receptor complex proteins are bonded to JAK signaling mediator proteins, TYK2 with IL-12β and JAK2 with IL-23R. JAK2 creates a docking station for STAT3 which phosphorylate, activate and homodimerize the STAT molecules. TYK2 also activates STAT4 by a similar JAK2-STAT3 mechanism. Both activated STAT3 and STAT4 enter the nucleus and start transcription of several cytokines such as IL-17 and IL-22.

A variety of cells bear IL-23R that endows IL-23 a wide range influence (Mezghiche et al., 2024). IL-23 exerts an autocrine effect on macrophages and DCs, enhancing CD40 expression with TNF and IL-1β secretion in macrophages (Cua et al., 2003), and induce antigen presentation in DCs (Belladonna et al., 2002). In addition to IL-23's ability in promoting inflammation, it can also induce tumorigenesis and improve cancer cell proliferation and renewal (Chan et al., 2014; D. Wang et al., 2016). Human plasma cells were found expressing IL-23R. The examination of IL-23 role on them revealed that, although they respond poorly to IL-23, the combination of IL-23 and IL-2 resulted in significant IgM elevation with remarkable IgG reduction. This may indicate the importance of IL-23 in the primary immune response (Cocco et al., 2011). Memory T cells were also found to express IL-23R on their surface. These memory cells are experienced cells located as resident tissue cells or circulate in the blood. IL-23 is capable of promoting memory T cells proliferation and induction of IL-17 production (Y. Li et al., 2016). IL-17 is a major proinflammatory cytokine which is responsible for initiating the secretion of several cytokines such as IL-1, IL-6, TNF, and GM-CSF, chemokines such as CXCL1, CXCL2, CCL2, CCL20 and IL-8, AMPs such as β-defensins and S100 proteins, and Matrix metalloproteinases (MMPs) such as MMP3 and MMP9. This points out the importance of IL-17 as a key mediator of both inflammation and autoimmune diseases (Amatya et al., 2017).

IL-23 plays a similar role in Th-17 cells activation in addition to its influence in Th-17 differentiation from naive T cells. $CD4^+$ naive T cells express little to none IL-23R, and thus respond poorly to IL-23 (Parham et al., 2002). However, there were findings that showed activation of human naive $CD4^+$ T cells enhancing the expression of IL-23R, making the cells sensitive to IL-23 (N. J. Wilson et al., 2007a). These $CD4^+IL\text{-}23R^+$ cells after stimulation with IL-23 were differentiated into the Th-17

phenotype, supported by the evidence of detection of IL-17. Th-17 differentiation and IL-17 expression require a specific transcriptional regulator, the retinoic acid-related orphan receptor γ-T (RORγt) It is a nuclear receptor regulated by STAT3 that can be activated by IL-23 (X. O. Yang et al., 2007a). Th-17 cells phenotype is not stable and can skew towards other T helper phenotypes according to cytokines milieu, where IL-23 was shown to maintain the Th-17 phenotype not only by providing the required signals but also by increasing glycolysis and avoiding of T cells anergy (Stritesky et al., 2008; Revu et al., 2018).

Upon stimulation of Th-17 with IL-23, Th-17 can also secrete IL-22. This cytokine is important for the mucosal barrier as IL-22 is able to stimulate mucus production by goblet cells in mucosal tissues and increase the secretion of AMPs and chemokines. IL-22 also, participate in tissue protection by enhancing anti-apoptotic proteins, mitogenic proteins and proliferation (Rutz et al., 2013). The effect of IL-23 on the production of IL-17 and IL-22 were discovered in other lymphoid cells as well. γδ T cells are a special subset of T cells consisting of γ and δ TCR chains instead of the common α and β chains in $CD4^+$ T cells. These cells upregulate the expression of IL-17 and IL-22 if stimulated with IL-23 (Malik et al., 2016). Additionally, group 3 of lymphoid cells especially characterized by the lack of antigen receptors defined as innate lymphoid cells (ILCs), has shown a similar IL-17-IL-22 response to IL-23 stimulation (Eberl et al., 2015). These IL-23-IL-17 and IL-23-IL-22 axes have been observed in neutrophils, macrophages and natural killer cells (NK) (Cella et al., 2009; F. Chen et al., 2016; Hou et al., 2018; Passos et al., 2010).

1.3 IL-23 pathology

The wide spectrum of pathogen-associated molecular patterns (PAMPs) and cytokines that can trigger IL-23-mediated inflammatory response added to IL-23 multiple effects make this cytokine the center of inflammation. Dysregulation of IL-23-mediated inflammatory response can cause chronic inflammation and tissue damage since both IL-17 and IL-22 axes can promote positive feedback loops, leading to inflammation permanence. This leads to extensive investigations into IL-23 and its involvement in a number of autoimmune diseases including psoriasis, rheumatoid arthritis and inflammatory bowel diseases.

1.3.1 Psoriasis

Psoriasis (PS) is an immune-mediated chronic skin-inflammation disease. It's classified under five main types, plaque PS (the most common PS), Pustular, guttate, inverse and erythroderma PS. Despite the difference in morphology in PS types, they all share aberrant KCs differentiation and proliferation. While the exact cause of PS is still unknown, several factors were found able to trigger the disease such as infection, skin injury, stress, immune response disorders and genetics factors (Di Meglio et al., 2014). The human endogenous AMP, LL-37, is considered a key mediator in psoriasis development. Studies by Lande and Ganguly, demonstrated that LL-37 which is secreted by stressed or injured KCs, is able to bind to self-DNA or RNA released from dying cells (Ganguly et al., 2009; Lande et al., 2007). The LL-37-DNA/RNA complex activates plasmacytoid dendritic cells (pDCs) through TLR7 and TLR9 producing IFN-α, whilst dermal DCs activates through TLR8 and secretes TNF-α, IL-6, IL-12 and IL-23. KCs are activated and releases cytokines including IL-1β, IL-6 and IL-23. This cytokines milieu creates an appropriate environment for Th-17 cells development.

Together, Th-17, γδ T cells, resident memory T cells and ILC3 start emanating IL-17 (Gallais Sérézal et al., 2018; Pantelyushin et al., 2012; Zaba et al., 2009), which exert a neutrophils recruitment effect by stimulating KCs to produce chemokines such as CXCL1 and CXCL8 alongside AMPs and other cytokines including IL-1β, IL-6, TNF-α and IL-23. The latter enhances and maintains IL-17 production, perpetuating inflammation and generating an inflammatory loop (Di Meglio et al., 2014) (**Figure 1.2**).

IL-22 is also secreted from IL-17 secreting cells as a result of IL-23 interaction, playing an important role in psoriasis pathogenesis. IL-22 causes abnormal differentiation and proliferation of KCs (hyperplasia) leading to nuclei retention in the stratum corneum layer defined as parakeratosis, and stratum spinosum layer thickening termed as acanthosis (Sa et al., 2007). Furthermore, IL-22 stimulates KCs to produce a group of AMPs, inflammatory cytokines and neutrophils recruitment chemokines (Ma et al., 2008). These recruited neutrophils accumulate and form Munro abscesses, while IL-22- induced cytokines provides positive feedback for IL-23 regulation.

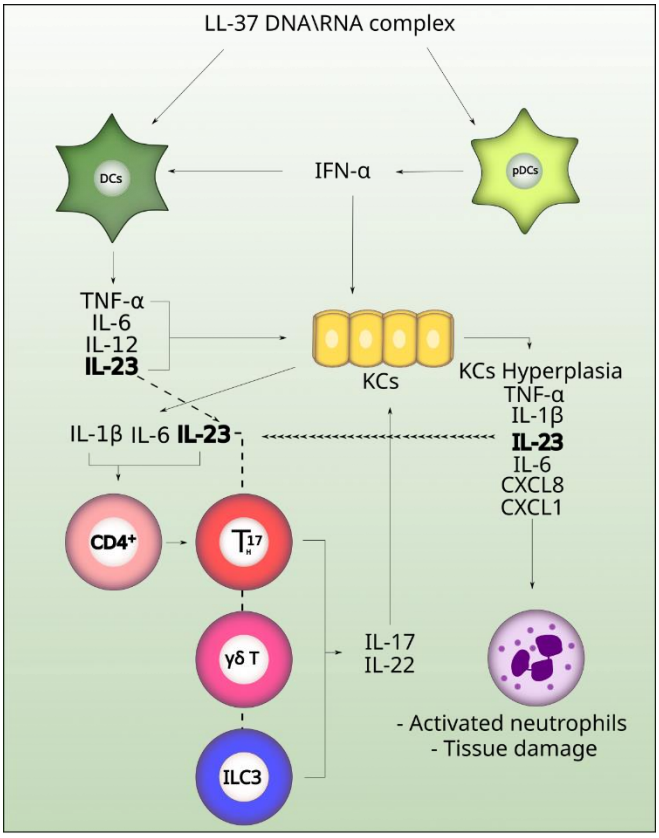

Figure 1.2 The proposed role of IL-23 in the development of PS. The released AMP LL-37 from injured cells binds to DNA\RNA forming a complex that able to activate pDCs to produce IFN-α, and stimulate dermal DCs to secrete TNF-α, IL-6, IL-12, and IL-23. The cytokines milieu stimulates KCs to produce IL-1β, IL-6, and IL-23. These cytokines push $CD4^+$ T cells differentiation forward to Th-17. IL-23 stimulates the production of IL-17 and IL-22 from Th-17, γδ T cells and ILC3. Both IL-17 and IL-22 activate KCs to secrete a wide array of cytokines including TNF-α, IL-1β, IL-6, CXCL1, CXCL8 and IL-23. KCs in the process begin abnormal differentiation and proliferation (hyperplasia). The secreted chemokines from KCs recruit activated neutrophils causing tissue damage while IL-23 form positive feedback (arrows line) which stimulate and maintain IL-17 and IL-22 production starting a loop of inflammation (dashed line). Adapted from (Abdo & Tye, 2020).

1.3.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease where the immune system attacks body joints causing inflammation, swelling and pain. This results in degradation of cartilages and bone destruction. Several risk factors have been identified to be related to RA including environmental factors such as smoking and infections; genetics factors such as human leukocyte antigen (HLA-DRB1); and sex where females are two to three times more prone for the disease compared to males (Smolen et al., 2018). IL-23 was found to be involved in the pathogenesis of RA by promoting inflammation and contributing to bone erosion (Yago et al., 2017). As the main cause of RA still unknown, its believed that activation of synovial tissue macrophages (type A synoviocytes) can lead to synovial membrane inflammation (synovitis), they are a major source of inflammatory cytokine including TNF-α, IL-1, IL-6, IL-12 and IL-23 (Firestein & McInnes, 2017; Kurowska-Stolarska & Alivernini, 2017), which creates a Th-17 differentiation environment.

IL-17 produced by Th-17 (and maintained by IL-23) is able to stimulate FLSs (type B synoviocytes) production of cytokines and chemokines including IL-8, IL-6, CCL20 and CXCL8 (Zrioual et al., 2008), thereby participates in inflammation and immune cells recruitment. IL-17 is also involved in bone erosion by disrupting bone remodeling and bone formation (osteogenesis) equilibrium. Osteogenesis is controlled by the balance between osteoblasts, cells that secrete the required components for bone synthesis, and osteoclasts, the multinucleated cells that responsible for bone resorption. Osteoclasts differentiation from osteoclast progenitors (monocyte/macrophage lineage) is significantly induced in the presence of IL-17 either directly (Yago et al., 2009), or by IL-17 influence through upregulation of receptor activator of NF-κB (RANK) ligand (RANKL). RANK/RANKL interaction

leads to osteoclast formation, where the RANK receptor is expressed on osteoclast progenitors. RANKL is found on synoviocytes and other immune cells such as $CD4^+$ T cells and Th-17 (Kotake et al., 1999; Sato et al., 2006). IL-17 aided by TNF-α stimulates IL-23 secretion from FLS (Goldberg et al., 2009b) , which in turn maintains IL-17 production leading to inflammation sustainability (**Figure 1.3**).

IL-23-induced IL-22 participate in RA as well. It showed a similar IL-17 effect on synoviocytes in regard to RANKL (K.-W. Kim et al., 2012). Additionally, IL-22 induction of synoviocytes proliferation might cause hyperplasia and thereby contribute to RA inflammation (Mitra et al., 2012). Moreover, challenging synoviocytes with IL-22 induced the matrix metalloproteinase, MMP1, which is associated in bone erosion (Cunnane et al., 2001).

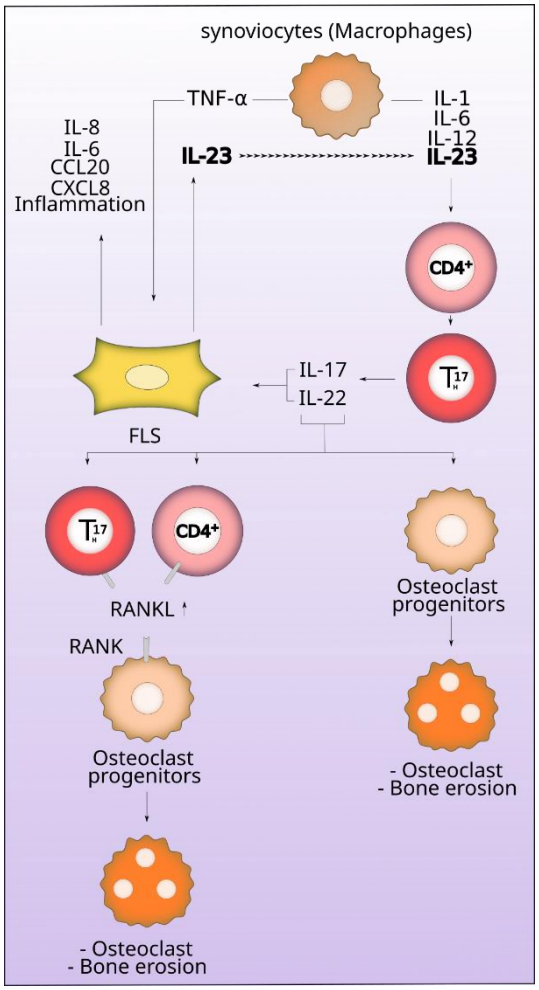

Figure 1.3 The proposed role of IL-23 in the development of RA. Activated macrophages in synovial tissues are sources of IL-1, IL-6, IL-12, TNF-α and IL-23. This cytokine environment stimulates the differentiation of Th-17 from $CD4^+$. Th-17 starts producing IL-17 and IL-22 which in turn stimulate FLS to secrete IL-8, IL-6, CCL20 and CXCL8 causing inflammation, while the macrophages-secreted TNF-α results in IL-23 production from FLS, forming a positive feedback loop (arrows line) that maintain the production of IL-17 and IL-22 from Th-17. Both IL-17 and IL-22 are able to induce osteoclast differentiation from osteoclast progenitor either directly, or by elevating RANKL on $CD4^+$ and Th-17 which upon ligation with the RANK receptor on osteoclast progenitor, stimulates osteoclast differentiation. Osteoclasts are a main cause of bone erosion. Adapted from (Abdo & Tye, 2020).

1.3.3 Inflammatory bowel diseases.

As the name implies, inflammatory bowel disease (IBD) is a chronic inflammation of the gut that can occur in several parts of the gastrointestinal tract. IBD commonly occurs at the small intestine, termed as Crohn's disease, or it can affect the colon and rectum named in this case ulcerative colitis. Similar to other autoinflammatory diseases, the initial trigger of IBD is still unknown, but several factors were found to be related to the disease including stress, drugs, genetic factors and infections. It's widely accepted that the disease is established by abnormal interaction between gut microbiota and immune system leading to uncontrolled inflammation which might occur if the gut mucosal barrier is breached (Y.-Z. Zhang & Li, 2014). Beneath the mucosal barrier, there is a layer of endothelial cells embedded with goblet cells and lymphoid follicles masses called Peyer's patches (PPs). The endothelial cells surrounding PPs contain microfold (M) cells that act as a terminal where they sample antigens from intestinal lumen and transport them to the lymphoid follicles which are loaded with wide array of immune cells arsenal (C. Jung et al., 2010). PAMPs of the captured antigens are presented by DCs and macrophages with the addition of CD40/CD40L ligation to T cells can stimulate IL-23 secretion (**Figure 1.4**).

This immune response also results in the production of a group of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12 (Hindryckx et al., 2016; Siakavellas & Bamias, 2012). Together, the cytokine environment drives Th-17 differentiation. Th-17 with γδ T cells and ILC3 begin IL-17 secretion (Geremia et al., 2011; Sun et al., 2017) which in turn induce a number of cytokines and chemokines that recruit neutrophils such as IL-6, CXCL1 and CXCL2 in the addition of MMP1 and MMP12 (Biancheri et al., 2013; J. W. Lee et al., 2008). The tissue damages caused

by pro-inflammatory cytokines, activated neutrophils and MMP, are well documented. Further, IL-17 is capable of promoting tumorigenesis and cause colorectal and colitis-associated cancer (Hyun et al., 2012; K. Wang et al., 2014).

IL-22, which is upregulated by IL-23, was found to be elevated in both Crohn's disease and ulcerative colitis. This pro-inflammatory cytokine has a dual role in IBD. IL-22 is able to induce differentiation and proliferation of epithelial cells and improve cells survival by upregulating anti-apoptotic proteins (K. Kim et al., 2014; L.-J. Li et al., 2014; Radaeva et al., 2004). On the other hand, IL-22 was found to be related to colitis-associated cancer via IL-22 receptor (IL-22RA1)-STAT3 signaling (L.-Z. Yu et al., 2013). Moreover, IL-22 induces the expression of AMPs molecules such as regenerating gene III (RegIII) γ and β, the latter (which also can be elevated by IL-23) showed the ability to recruit IL-22-secreting neutrophils (Aden et al., 2016). Calprotectin (e.g. S100A8 and S100A9) and β-defensin-2 AMPs are raised in response to IL-22 as well (Liang et al., 2006; Mizoguchi et al., 2018). However, while AMPs can restrain pathogens dissemination in the gut, some of them might also contribute to Crohn's disease and ulcerative colitis inflammation and colitis-associated cancer (X. Zhang et al., 2015, 2017).

Lastly, IL-22 can enforces the gut mucosal barrier and contribute to pathogen clearance. It induces the production of mucin (Muc) 1, 3, 10 and 13, crucial units of the mucosal barrier which prevent microbes from physical contact with endothelial cells limiting their attachment and colonization (Linden et al., 2008; Sugimoto et al., 2008). Additionally, IL-22 upregulates the expression of claudin-2, a protein that increase intestinal water efflux causing diarrhea and thus washing out microbes (Tsai et al., 2017).

However, IL-22 has no to negligible effect in some infection such as *Mycobacterium tuberculosis* and *Schistosoma mansoni* infections. On the contrary, IL-22 can exacerbate *Toxoplasma gondii* infection. It was shown that IL-22 expression do not aid in immunity development against *T. gondii* supported by the fact that IL-22 neutralization reduced infection-induced inflammation (M. S. Wilson et al., 2010). Furthermore, Behnsen et al. reported that AMPs induced by IL-22 expression are able to inhibit the growth of commensal *Escherichia coli* in gut. However, this allows *E. coli* competitor's, *Salmonella Typhimurium*, to grow, especially that *S. typhimurium* is able to overcome IL-22-induced AMPs (Visvanathan et al., 2019a).

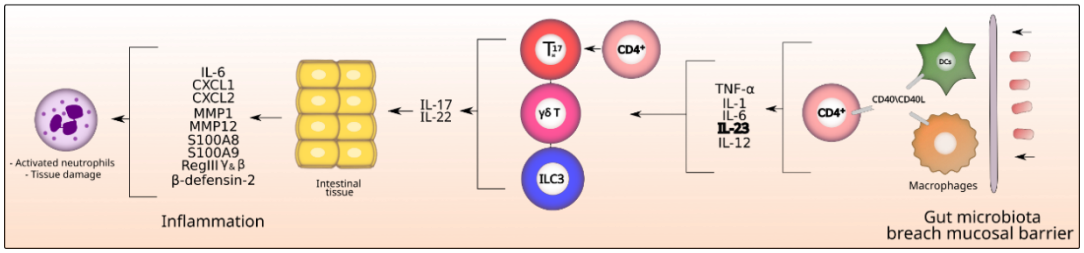

Figure 1.4 The proposed role of IL-23 in the development of IBD. Breaching of the mucosal barrier by gut microbiota activates phagocytosis and antigen presentation by macrophages and DCs, a process that includes CD40\CD40L interaction with CD4+, leading to TNF-α, IL-1, IL-6, IL-12 and IL-23 production. The cytokine milieu induces Th-17 formation. These cells beside γδ T cells and ILC3 are influenced by IL-23 and emanate IL-17 and IL-22. IL-17 and IL-22 effects result in the secretion of a wide array of cytokines, AMPs and chemokines from intestinal tissues including IL-6, CXCL1, CXCL2, MMP1, MMP12, S100A8 and others, leading to inflammation, activated neutrophils recruitment and eventually tissue damage. Adapted from (Abdo & Tye, 2020).

1.3.4 IL-23 as Diseases Initiator and Biomarker

Phagocytes play a complex role in PS, RA and IBD. These cells have ample receptors that respond to a plethora of immunogens by elevating IL-23 expression upon stimulation. The impact of IL-23 is summarized mainly by the differentiation and stimulation of IL-17 and IL-22 producing cells. IL-17 and IL-22 then induce KCs hyperplasia, parakeratosis and acanthosis in PS; osteoclast formations and bone erosion in RA; and elicit a large scale of inflammatory cytokines including neutrophils recruitment chemokines leading to tissue damage. This is exacerbated by IL-23-IL-17\IL-22 positive feedback loops that creates infinite inflammation. Based on these facts, IL-23 can be considered as a disease initiator as it is an inflammatory response key factor.

In IBD mouse model, the administration of anti-IL-23R antibodies affected the regulation of more than 50 protein such as S100A8, S100A9 and RegIII γ and β (Cayatte et al., 2012). Suppressing IL-23 by antibodies in PS patients showed genes expression downregulation of several inflammatory mediators including IL-8, CXCL1, β-defensins 2, S100A7, S100A8 and S100A9 (Sofen et al., 2014; Visvanathan et al., 2019b). Also, IL-23 serum levels were found to be positively correlated with inflammatory proteins such as C-reactive protein and rheumatoid factor in RA patients. The association of disease activity score (DAS) and rheumatoid arthritis severity scale (RASS) suggests that IL-23 can be used as a biomarker to reflect RA disease activity (Alsheikh et al., 2019). Similarly, Mirsattari and colleagues showed that serum levels of IL-23 were associated with ulcerative colitis severity and disease duration leading to it being a possible disease diagnostic marker (Mirsattari et al., 2012).

1.3.5 IL-23R Variants

Studies conducted on IL-23 signaling to elucidate its role in autoimmune diseases has intersected with IL-12 since both of them share the p40 subunit and both are implicated in inflammatory responses. Cua and colleagues investigated the effect of p19 in contrast to p35 (the second subunit of IL-12 heterodimer) to clarify IL-12 and IL-23 contribution in autoimmune diseases. They reported that p19 knockout mice but not p35 knockout mice were resistant to induced experimental autoimmune encephalomyelitis (EAE), a brain autoimmune disease (Cua et al., 2003). The same group also reported similar results in collagen-induced arthritis (CIA) (Murphy et al., 2003). The absence of IL-23 ($p19^{-/-}$) protected against CIA, while conversely the disease was exacerbated in $p35^{-/-}$ mice. Additionally, in IL-10 deficient mice that developed colitis by the third month, knocking out p35 resulted in colitis development at week 7. On other hands, $p19^{-/-}$ $IL\text{-}10^{-/-}$ double knock out mice remain colitis free after 12 months of birth (Yen et al., 2006). These data with many other reports emphasize that IL-23 rather than IL-12 is important for inflammation establishment, orienting researchers towards exploring p19 receptor (IL-23R). The receptor was investigated to better understand inflammatory signaling, including IL-23R variants to explicates autoimmune diseases susceptibility.

A single nucleotide polymorphism (SNP) in IL-23R can render ethnic groups prone or resistant to certain autoimmune diseases. For instance, SNP in mRNA of IL-23R at the 3′-untranslated region (3′UTR) is related to increased risk of developing IBD in some population (Peng et al., 2017; Zwiers et al., 2012). A single SNP at exon 9 of IL-23R leads to modification of amino acid glycine to arginine (Arg381Gln variant), called rs11209026 or R381Q, is considered a protective IL-23R variant against an array of diseases including IBD, RA, and PS (Abdollahi et al., 2016).

Examination of these variants revealed that disease-related variants such as rs10889677 exhibited an increased level of IL-23R mRNA and protein expression. The elevated level of receptor expression is ascribed to the lack of binding site of microRNA (miRNA) Let-7e and Let-7f. These miRNAs are implicated in IL-23R regulation by binding to the mRNA and degrading it (Zwiers et al., 2012). In contrast, protective variants such as R381Q and G149R had reduced IL-23R receptor expression that is attributed to reduced protein stability or impaired maturation due to endoplasmic reticulum retention. This weakens IL-23R signaling via decreased STAT3 and STAT4 activation and phosphorylation (Sivanesan et al., 2016).

1.4 IL-23 Therapy

The increased activity of IL-23 in autoimmune diseases due to excess secretion or signaling allowed its targeting as a therapy. This was indeed found to be effective and associated with diseases remission proven by clinical trials. IL-23-based therapy is either IL-23R antagonists which are mainly antibodies, or IL-23R downstream signaling suppressors such as JAK inhibitors. Both approaches have a few FDA-approved biologics that are available in the market and has been successfully used in reduction of disease severity (**Table 1.1**). Analysis of clinical trial results did not indicate benefits of using antibodies over JAK inhibitors or vice versa. Both IL-23 inhibitor types have similar results in some diseases and divergence in other areas. Even among antibodies or JAK inhibitors themselves, there are a varying degree of effectiveness. If a particular treatment is successful in reducing disease symptoms of a specific IL-23-related disease, it is not necessary to be able to treat another (Abdo & Tye, 2020).

For instance, Guselkumab is an FDA approved anti-IL-23 antibody that has one of the highest efficacy in regards of Psoriasis Area and Severity Index (PASI) in plaque PS, where 91.2% of patients have 75 % reduction of PASI score (Machado & Torres, 2018a), but it performs poorly in RA with an American College of Rheumatology (ACR20) score of 41.3% vs 40.0% placebo (Smolen et al., 2017). In contrast, the JAK inhibitor, Baricitinib, has been approved by FDA for RA therapy (ACR20 70.0% vs 40.0% placebo) (P. C. Taylor et al., 2017) but only achieved a subpar PASI 75 score of 54.1% in PS (Papp et al., 2016). Another example is Tofacitinib and Peficitinib JAK inhibitors. Clinical trial results in ulcerative colitis proves that Tofacitinib is superior (60.0% vs 19.2% placebo) (Motoya et al., 2018) compared to Peficitinib (54.5% vs 39.5% placebo) (Sands et al., 2018). Although both have similar targets (JAK 1,2,3 and TK2), the degree of inhibition for each JAK member is different (O'Shea et al., 2013a). This diversity in drug composition and its mechanism of action, dosage, time frame and disease nature results in variation of the outcome and makes it difficult to conclude a relation or preference between antibodies and JAK inhibitors.

Table 1.1 FDA-approved IL-23-assosiated medications

Therapeutics	Type/Target	Diseases
Ustekinumab (Stelara)	Human IgG1κ IL-12/IL-23 p40	PS, IBD
Risankizumab (SKYRIZI)	Humanized IgG1κ IL-23 p19	PS, Crohn's diseases
Guselkumab (Tremfya)	Human IgG1λ IL-23 p19	PS
Tildrakizumab (ILUMYA)	Humanized IgG1κ IL-23 p19	PS
Mirikizumab (Omvoh)	Humanized IgG4 IL-23 p19	PS and ulcerative colitis
Tofacitinib (XELJANZ)	JAK 1,2, 3 and TYK2 inhibitor	PS, RA, and ulcerative colitis
Baricitinib (Olumiant)	JAK 1 and 2 inhibitor	RA
Peficitinib (Smyraf)	JAK 1,2, 3 and TYK2 inhibitor	PS, RA, and ulcerative colitis

Adapted from (Abdo & Tye, 2020).

1.5 Current therapies limitations

Each of the aforementioned IL-23 treatments has its own side effects and limitations, either unique and specific depending on the composition and chemical formula, or generally based on the concept of targeting IL-23 itself. Experiments on p19 deficient mice showed decreased humoral immune response, particularly, T cell-dependent antigens-antibodies of all isotypes (Kljavin et al., 2014). This is in addition to other reduced pro-inflammatory cytokines such as IFN-γ, IL-6 and IL-17 (B.-J. Kim et al., 2013). Targeting IL-23 makes the patient immunocompromised, thus increasing their susceptibility to pathogens. It will be worsened if the drug inhibits other inflammatory mediators which might be fatal in antibiotic-resistant infections. This

unavoidable side effect of IL-23 based therapy is due to IL-23 remarkable regulation and influence over inflammatory response.

JAK inhibitors inhibit signaling transducers such as JAK2 and TYK2, but those signaling mediators are also essential for the upregulation of other cytokines; therefore, JAK inhibitors hinder several cytokine pathways and thus not considered to be IL-23 specific medications (**Figure 1.5**). Although, it's expected that JAK inhibitors render patients more prone to infections than IL-23 antibodies due to the wide array of restrained inflammatory cytokines, there are no head-to-head studies addressing this issue. However, patients' vulnerability to infections such as tuberculosis, pneumonia and hepatitis after JAK inhibitors administration is well defined (Alves et al., 2022).

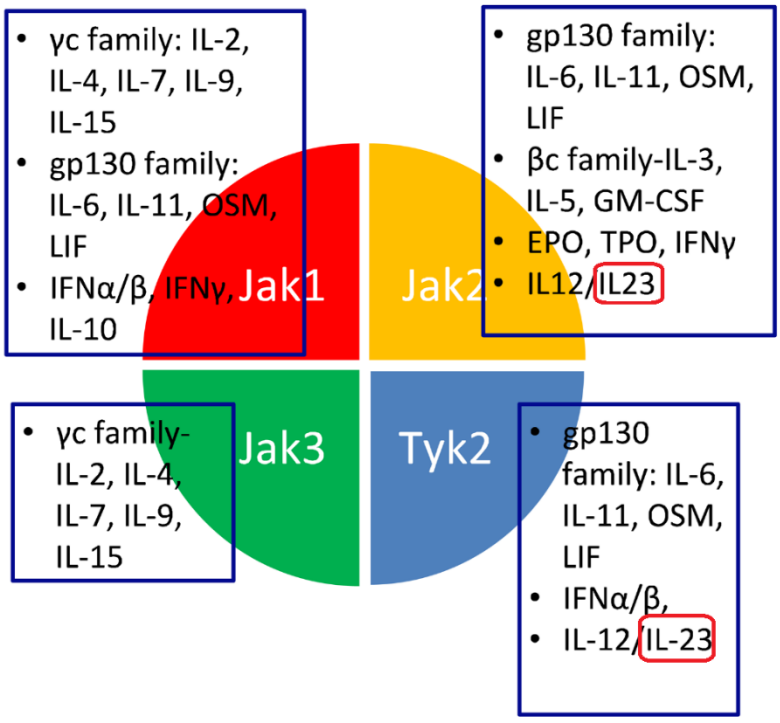

Figure 1.5 JAK signaling mediators' influence on several cytokines. Several cytokines share the same signaling mediator. Adapted from (O'Shea et al., 2013b).

While therapeutic antibodies are the best choice when it comes to target specificity compared to JAK inhibitors, but that's not without shortcomings. Producing full antibodies (with Fc region) is costly due to the glycosylation of the Fc region at Asparagine 297 (N297) that limit antibody production to mammalian expression systems (Dos Santos et al., 2018). Antibodies also are administered by subcutaneous and intravenous injections, which are considered invasive method compared to orally or topically administrated drugs. Immunogenicity of antibodies is another major concern in pharmaceutical industries. According to European Medicine Agency (EMA) committee for medical product for human use (CHMP) guideline, there is a variety of factors that can affect drug immunogenicity as classified under product, disease and patients related-determinants categories. These include similarity and foreignness of the drug, chemical structure and molecular weight (MW), genetics factor and MHC polymorphism, manufacturing process modifications and dose, duration and route of administration of the drug (CHMP, 2008).

Antibodies generally are immunogenic in terms of "foreignness" based on its amino acid structure and producing species. Therapeutic antibodies of murine origins were found to be immunogenic to a level which it can provoke anti-drug antibodies (ADA) production in almost 100% of patients. Several technologies were adopted to reduce therapeutic antibodies structure-based immunogenicity. Firstly, by replacing murine antibody Fc region with human sequence to develop chimeric antibody (**Figure 1.6**). Then utilizing humanoid antibodies which basically are human antibodies with murine complementary-determining regions (CDRs). Finally achieving full human antibody structure using transgenic mice. Indeed, these strategies significantly reduces antibodies immunogenicity but even a fully human antibody, can be immunogenic and able to provoke ADA production (Hwang & Foote, 2005; Santos et al., 2018).