



The C-terminal region of IL-22R α : a novel potential
therapeutic target for psoriasis

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SUMMARY

Interleukin-22 is a cytokine that acts on various tissues including the skin, the gut, the lungs and the liver. In these organs, IL-22 is involved in tissue regeneration, homeostasis and defense against pathogens by inducing cell proliferation, recruitment of neutrophils and production of anti-microbial peptides. Although these functions are beneficial in various contexts such as wound healing, colitis or hepatitis, prolonged and exacerbated production of IL-22 in an inflammatory environment is associated with the development of pathologies such as psoriasis. Therefore, we think that it may be useful to develop methods to partially block IL-22 functions.

Unlike most cytokines, IL-22 is particular in that it induces STAT activation via two mechanisms. In addition to the classical activation of STAT1-3-5, STAT3 is phosphorylated in a receptor tyrosine-independent manner. This alternative mechanism relies on the coiled-coil domain of STAT3 and the C-terminal (C-ter) part of IL-22R α , which is devoid of tyrosines. This redundant activation of STAT3 could be a potential target to partially block IL-22 activities, particularly when IL-22 production is exacerbated.

In the first part of this thesis, we were able to confirm the existence of the alternative STAT3 activation pathway in cells endogenously expressing the IL-22R, and *in vivo*. Interestingly, the involvement of this alternative pathway in IL-22 signaling and functions seems to be organ-dependent. While the truncation of the C-ter IL-22R α strongly reduced IL-22 signaling in the skin, the effect was mild in the gut. In addition, we showed that this pathway is involved in the imiquimod-induced model of psoriasis because skin lesions are weaker in mice in which the IL-22R α is truncated. By contrast, the absence of the C-ter has limited impact on IL-22 protection observed during *Citrobacter rodentium* infection. In conclusion, this study suggests that targeting the alternative pathway could be of interest in the context of psoriasis.

In order to inhibit the alternative activation of STAT3 induced by IL-22, we took advantage of a monoclonal antibody targeting the coiled-coil domain of STAT3. *In vitro*, we could show that the

monobody strongly affects the alternative activation of STAT3 and not, or only slightly, the tyrosine-dependent activation. Strikingly, the monobody prevents the nuclear translocation of STAT3, which globally decreases its transcriptional activity. Hence, this monobody does not specifically inhibit the alternative pathway of IL-22 but, since it acts as a STAT3 inhibitor, it might still be useful for psoriasis treatment.

RÉSUMÉ

L'IL-22 est une cytokine qui agit sur différents organes dont la peau, l'intestin, les poumons et le foie. Dans ces organes, l'IL-22 est impliquée dans la régénération, l'homéostasie tissulaire et la défense contre les pathogènes en induisant la prolifération des cellules, le recrutement de neutrophiles et la production de peptides antimicrobiens. Bien que ces fonctions soient bénéfiques dans de nombreux contextes tels que la cicatrisation, la colite ou l'hépatite, une production prolongée et excessive d'IL-22 dans un environnement inflammatoire est associée au développement de maladies tel que le psoriasis. Dès lors, nous pensons qu'il pourrait être intéressant et bénéfique de bloquer partiellement les fonctions de l'IL-22.

Contrairement à la majorité des cytokines, l'IL-22 a la particularité d'induire l'activation des STATs via deux mécanismes. En effet, en plus de l'activation classique de STAT1-3-5, STAT3 est également phosphorylé de façon tyrosine-indépendante. Ce mécanisme alternatif requiert le domaine coiled-coil de STAT3 et la partie C-terminale (C-ter) de l'IL-22R α qui est dépourvue de tyrosines. Cette activation redondante de STAT3 pourrait être une cible potentielle pour bloquer partiellement les activités de l'IL-22, particulièrement dans un contexte où sa production est exagérée.

Dans la première partie de cette thèse, nous avons pu confirmer l'existence de la voie alternative dans des cellules exprimant l'IL-22R α de façon endogène et *in vivo*. Curieusement, l'implication de cette voie alternative dans la signalisation de l'IL-22 et ses fonctions semble être organe-dépendante. En effet, alors que le fait de tronquer le récepteur entraînait une forte diminution de la signalisation de l'IL-22 dans la peau, l'effet était modéré dans l'intestin. De plus, nous avons pu montrer que cette voie est impliquée dans le modèle de psoriasis induit par l'imiquimod car les lésions cutanées étaient réduites dans les souris exprimant un IL-22R α tronqué. Au contraire, l'absence de la queue C-ter a un impact limité sur la protection fournie par l'IL-22 dans le modèle d'infection au *Citrobacter rodentium*. En conclusion, cette étude suggère que cibler la voie alternative pourrait être une stratégie intéressante dans le contexte du psoriasis.

Afin d'inhiber la voie alternative d'activation de STAT3 induite par l'IL-22, nous avons utilisé un monobody ciblant le domaine coiled-coil de STAT3. *In vitro*, nous avons pu montrer que ce monobody impacte fortement l'activation alternative de STAT3 et pas, ou seulement légèrement, l'activation tyrosine-dépendante. Étonnamment, le monobody empêche la translocation nucléaire de STAT3 ce qui entraîne une diminution globale de son activité transcriptionnelle. Ce monobody ne permet donc pas de cibler spécifiquement la voie alternative induite par l'IL-22. Néanmoins, puisqu'il agit comme un inhibiteur de STAT3, il pourrait tout de même être intéressant comme traitement contre le psoriasis.

ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
AOM	2-azoxymethane
APC	Adenomatous polyposis coli
AuNP	Gold Nanoparticle
BBB	Blood brain barrier
CCD	Coiled-coil domain
CCL	(C-C) motif ligand
CD	Chron's disease
CD	Cluster of differentiation
CDR	Complementarity determining region
C _{H1}	First constant domain of Ig heavy chain
C _L	Constant domain of Ig light chain
CPP	Cell-penetrating peptide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCL	Chemokine (C-X-C) motif ligand
DBD	DNA binding domain
dDC	dermal dendritic cell
DETC	Dendritic epidermal T cell
DSS	Dextran sodium sulfate
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FERM	band 4.1, Ezrin, Radixin, Moesin
FN3	Fibronectin type III
Gb3/CD77	Globotriaosylceramide
GCSF	Granulocyte colony-stimulating factor
gRNA	guide RNA
GSK-3b	Glycogen synthase kinase 3 beta
HDR	Homology-directed repair
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IMQ	Imiquimod
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KIR	Kinase inhibitory region
KO	Knock-out
LPS	Lipopolysaccharide

LTi	Lymphoid tissue inducer (cell)
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MUC	Mucin
NHEJ	Non-homologous end-joining
NK	Natural killer (cell)
NKT	Natural killer T (cell)
NOD	Non-obese diabetic (mice)
NOD2	Nucleotide-binding oligomerization domain 2
NTD	N-terminal domain
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphatidylinositol 3-kinase
PSORS	Psoriasis susceptibility
PTP	Phosphotyrosine phosphatase
PTPRT	Receptor-type tyrosine-protein phosphatase T
Rag	Recombination-activating gene
RBX1	RING-Box Protein 1
ROR	RAR-related orphan receptor
scFv	single-chain fragment variable
SH2	Src homology 2
SHP1/2	Src homology region 2 domain-containing phosphatase-1
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TA	Transactivating
Tc	Cytolytic/cytotoxic T (cell)
Th	T helper (cell)
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor α
TPO	Trombopoietin
TYK	Tyrosine Kinase
UC	Ulcerative colitis
V _H	Variable domain of Ig heavy chain
VHL	von Hippel-Lindau
V _L	Variable domain of Ig light chain
WT	Wild-type

INTRODUCTION

Introduction

INTRODUCTION

This thesis focuses on IL-22 signaling, notably in the context of inflammatory diseases. Therefore, an overview of cytokine and cytokine signaling in general will be introduced, followed by a more detailed section on IL-22 production, signaling and functions. Binding proteins will be briefly described in the third part of this introduction.

1. CYTOKINES AND CYTOKINE SIGNALING

1. Cytokines and cytokine receptors

Cytokines are secreted glycosylated proteins that ensure communication between cells to control hematopoiesis, immune responses and inflammatory processes. There are different ways to classify cytokines, notably according to their production or function. However, these classifications are not optimal since some cytokines belong to multiple families. Instead, cytokines can be divided into families based on their structure: IL-1, TNF- α , TGF- β , chemokines and (at least) 4 α -helix bundle cytokines^{1, 2}. This last family can be further divided into class I and class II receptor cytokines, and includes interleukin-22, of interest in our laboratory.

Class I and II cytokine receptors are multimeric receptors composed of at least two chains, usually a high-affinity and a low-affinity chain although there are also homodimeric receptors. The extracellular part of the receptors, responsible for cytokine binding, is composed of two fibronectin type III domains but can also contain additional domains. Conserved cysteines are found in both class I and class II receptors while a WSXWS motif is located in the second fibronectin domain of class I receptors. The intracellular portion of cytokine receptor is unstructured but provide docking sites for JAKs (Janus Kinases) and STATs (Signal Transducers and Activators of Transcription)¹.

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2. JAK-STAT pathway

To exert their functions, class I and class II receptor cytokines induce the JAK-STAT pathway. This pathway is a multi-step process that involves several proteins (Figure 1). Since cytokine receptors do not possess an intrinsic tyrosine kinase activity, signaling relies on the JAK tyrosine kinases that are associated with the receptor and get activated following ligand binding. Once activated, JAKs phosphorylate tyrosines of the receptor itself, which recruits the STAT transcription factors that subsequently get phosphorylated. Phosphorylated STATs form homo- or hetero-dimers and migrate into the nucleus to modulate gene expression^{1, 3, 4}.

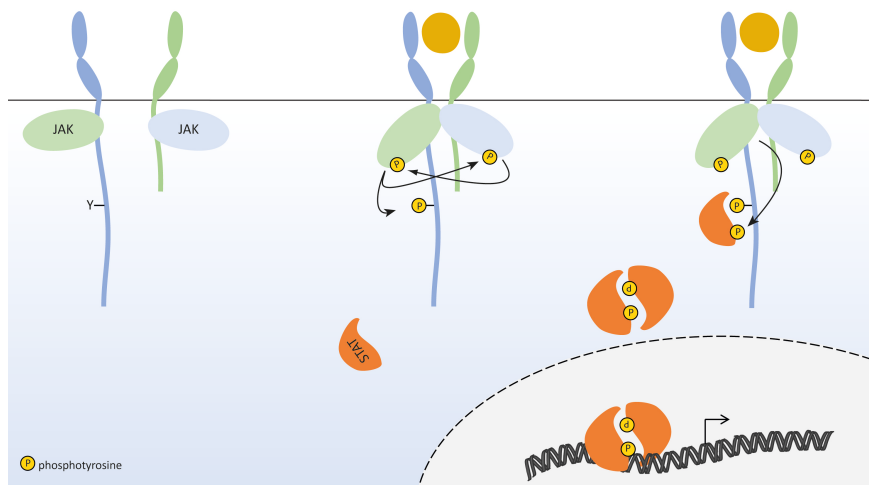


Figure 1: JAK-STAT pathway.

The JAK-STAT pathway is a cascade of signaling that involves a cytokine receptor, JAK kinases and STAT transcription factors. The binding of the ligand to its receptor induces a conformational change of the receptor and the activation of JAKs by trans-phosphorylation. Activated JAKs phosphorylate tyrosine residues of the receptor, which recruit STAT. Following phosphorylation by JAKs, STATs form dimers and migrate into the nucleus, resulting in the regulation of gene expression.

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2.1. Janus Kinases

JAKs are non-receptor tyrosine kinases responsible for signaling downstream of cytokine receptors. The JAK family is composed of four members: JAK1, JAK2, JAK3 and TYK2. While JAK1, JAK2 and TYK2 are ubiquitously expressed, JAK3 expression is restricted to the hematopoietic lineage. Structurally, JAKs are composed of 4 domains (Figure 2). The N-terminal FERM (4.1 band ezrin/radixin/moesin) and SH2 (Src homology 2) domains are required for the binding of JAK to the receptor, and the C-terminal pseudokinase and kinase domains are responsible for the kinase activity of JAK. Despite being catalytically defective, the pseudokinase domain modulates the activity of the kinase domain, required for the phosphorylation of receptors, STATs and other targets^{1, 3, 4, 5}.

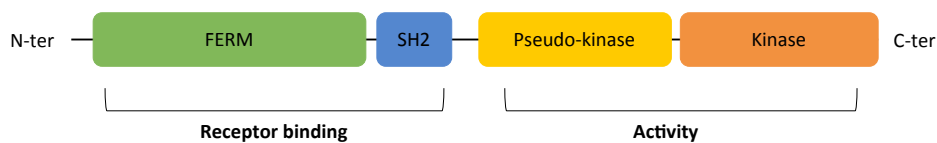


Figure 2: Janus Kinase structure.

JAKs are large proteins (>1000 amino acids) composed of 4 domains. The FERM and SH2 are involved in the binding to the receptor. The kinase domain, regulated by the pseudokinase domain, is responsible for the phosphorylation of targets.

JAKs are constitutively associated with the juxtamembrane domains of receptors. Several crystal structures have demonstrated that the FERM and SH2 domains are tightly associated to form a single module that binds two distinct motifs of the receptor: the leucin-rich “box 1” and the hydrophobic “box 2”. Of note, while SH2 domains usually bind phosphotyrosines, the SH2 domain of JAK binds a glutamate located in the box 2. Binding of the cytokine to its receptor induces a conformational change leading to the juxtaposition and the reciprocal interaction of the kinase domains of two JAKs. The subsequent trans-phosphorylation activates the JAKs which then transduce the signal via phosphorylation of targets^{1, 5, 6}.

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2.2. STATs factors

STATs are transcription factors activated downstream of JAKs. The family comprises 7 members: STAT1, 2, 3, 4, 5A, 5B and 6. All STATs share the same structure with an N-terminal domain (NTD) followed by a 4 antiparallel α -helix coiled-coil domain (CCD), a DNA binding domain (DBD), a linker region, an SH2 domain and a transactivation domain (Figure 3). Between the SH2 and the transactivation domains is located the conserved tyrosine that, when phosphorylated by JAK, activates STAT. Tyr⁷⁰¹, Tyr⁷⁰⁵ and Tyr⁶⁹⁴ of STAT1, STAT3 and STAT5, respectively, are phosphorylated by JAKs. Besides this phosphorylation site, other post-translational modifications are described. For example, lysine acetylation (Lys⁶⁹⁵), serine phosphorylation (Ser⁷²⁷) and tyrosine phosphorylation (Tyr⁶⁴⁰) were shown to regulate STAT3 activity^{4, 7, 8}.

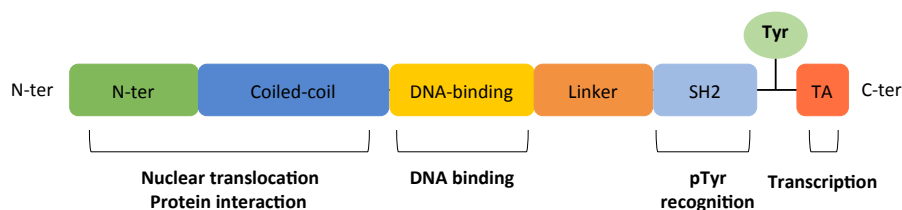


Figure 3: STAT structure.

STATs are transcription factors activated downstream of JAKs. The structure composed of 6 domains is conserved amongst STATs. Between the SH2 domain and the transactivation (TA) domain is located a conserved tyrosine that is phosphorylated by JAK, leading to active STAT dimer formation.

STATs form inactive dimers in the cytoplasm by interaction between the DNA binding and coiled-coil domains of two monomers. Once the receptor is phosphorylated, the SH2 domain of STAT uses the phosphotyrosines of the receptor as docking sites. Of note, each STAT has a preferential motif surrounding the phosphotyrosine. For example, STAT1, STAT3 and STAT5 have higher affinity for YxxP, YxxQ, YxxL, respectively. STAT, in turn, gets phosphorylated by JAK and forms an active dimer by the reciprocal interaction between the SH2 of one monomer and the phosphotyrosine of the other¹. Homo- or hetero-dimers are possible. The most well-known example of a hetero-dimer is the STAT1:STAT2 dimer whose

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formation is induced in response to type I IFN. For STAT3, besides the homo-dimer, STAT3:STAT1 and STAT3:STAT5 hetero-dimers are described. Hetero-dimers may have different target genes and recruit different partners than homo-dimers but this is still not fully elucidated⁹. Active dimers migrate into the nucleus. This process requires the formation of a complex comprising STAT, importins α/β and RanGDP. The asymmetric distribution of RanGDP between the cytoplasm (high concentration) and the nucleus (low concentration) drives the transport of the complex into the nucleus while importins are necessary to interact with specific motifs of nucleoporins in order to facilitate the translocation¹⁰. The N-terminal, coiled-coil and DNA binding domains of STATs are involved in the nuclear translocation. More precisely for STAT3, arginine residues within the coiled-coil and DNA binding domains were shown to be important for nuclear transport^{10, 11, 12}. Once in the nucleus, STAT dimers bind to DNA through its DNA binding domain by forming a scissor-like structure surrounding DNA. It interacts with other transcriptions factors via its coiled-coil domain and regulates the expression of various genes^{1, 13}. The palindromic nucleic sequence TTC(N₂₋₄)GAA is recognized by all STATs, with some preference within this motif¹. Finally, STATs are dephosphorylated by phosphatase, form a complex with exportin and RanGTP and shuttle back in the cytoplasm. Interestingly, unphosphorylated STAT (U-STAT) can also shuttle between the cytoplasm and the nucleus but this ligand-independent translocation is not well understood^{10, 14, 15}. In terms of signaling, U-STAT is also active. For example, it is known that U-STAT3 interacts with NF κ B and, thereby, promotes the expression of genes¹⁶.

2.3. Regulation

This cascade of signaling needs to be tightly regulated to avoid over-activation of signaling. Hence, several proteins regulate or switch off the system (Figure 4). The JAK-STAT pathway itself induces SOCS proteins while phosphatases are already present in the cells.

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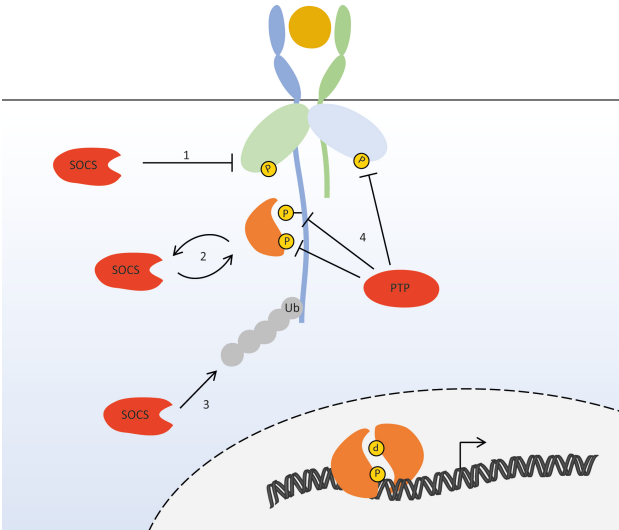


Figure 4: mechanisms of regulation of the JAK-STAT pathway.

SOCS proteins regulate the JAK-STAT pathway by (1) inhibiting JAK, (2) replacing STAT on receptor phosphotyrosines and (3) inducing the ubiquitination of JAK and cytokine receptor. As for phospho-tyrosine phosphatases (PTP), (4) they dephosphorylate JAK, receptor and STAT.

- SOCS

SOCS (Suppressor Of Cytokine Signaling) proteins are induced down-regulators of the JAK-STAT signaling. They are responsible for a negative feedback that limits the duration of the signaling pathway by switching it off. There are 8 SOCS: SOCS1-7 and CIS. All SOCS contain a SH2 domain and a SOCS box. In addition, SOCS1 and SOCS3 also contain a short motif called the Kinase Inhibitory Region (KIR). SOCS inhibit cytokine signaling through three distinct mechanisms (Figure 4). First, SOCS1 and SOCS3 can bind phosphorylated JAKs through their SH2 domain, leading to the direct inhibition of JAK via the KIR domain. Second, the SH2 domain of SOCS also binds phosphotyrosines of the receptor, competing with binding by STAT and therefore preventing signaling. The binding of SOCS to the receptor can also impair the interaction between JAK and the receptor. Finally, SOCS induces the proteasomal degradation of cytokine receptors and JAK through ubiquitination. This mechanism requires the SOCS box that recruits E3 ligases^{1, 4, 17, 18}.

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- Phosphatases

Several phosphotyrosine phosphatases (PTP) are involved in the regulation of the JAK-STAT pathway, including PTPRT, SHP1 and SHP2. These proteins are constitutively expressed in cells. They limit the amplitude of the signaling, rather than switching it off, by dephosphorylating JAK, receptor and STAT (Figure 4)^{1, 4}.

2.4. Alternative JAK-STAT pathways

Although receptor tyrosines phosphorylation is considered to be mandatory for JAK-STAT pathway activation, exceptions exist. Indeed, some receptors signal independently from phosphotyrosines of the receptor: activation that we call “alternative” or “unconventional”. Two such mechanisms have been identified, requiring either a tyrosine-free region of the receptor or a direct interaction between STAT and JAK. This was the subject of a review (manuscript in preparation), and certain alternative mechanisms are here.

- Receptor phosphotyrosine independent mechanisms

Regions that are devoid of tyrosines have been shown to be relevant for cytokine signaling. Some receptors utilize an alternative mode of activation of STAT3. This is notably the case for the G-CSF-R and IFN- λ R α . Indeed, both G-CSF and IFN- λ 1 induce the phosphorylation of STAT3 even in the absence of the receptor tyrosines. The tyrosine-less IFN- λ R α also transduces STAT1 and STAT4 signaling^{19, 20, 21}. Of note, in both receptors, STAT3 also binds receptor phosphotyrosines in addition to the tyrosine-less region.

Conversely, type I IFNs induce the alternative activation of STAT1 and STAT2 but not of STAT3, which is not the major signaling protein for these cytokines. Three docking sites are described for STAT2. Two require phosphotyrosine residues, either on the IFNAR1 or IFNAR2c chains, while the third, located on the IFNAR2c chain, is devoid of tyrosines^{22, 23, 24}.

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²⁵. A pre-association was observed between this chain and the N-terminal domain (including the coiled-coil domain) of STAT2. Amino acids 418-462 of the IFNAR2c chain, and particularly the acid block DDED, are required for this association^{22, 23, 24, 26, 27, 28}. While the binding sites of STAT2 on IFNAR1 and IFNAR2c are well described, there are conflicting data about the importance of these binding sites in STAT2 activation. The evidence would seem to suggest that the tyrosines of IFNAR1 play an accessory role²³. On the contrary, both sites on IFNAR2c seem to be equally involved in STAT2 activation, even though some studies claim that IFNAR2c tyrosines are required and the constitutive binding site is dispensable^{23, 24, 25, 28}. In addition, many studies show that STAT1 activation relies on STAT2 phosphorylation, suggesting that phosphorylated-STAT2 is used as a docking site by STAT1^{22, 27, 29}. Finally, concerning STAT3, two tyrosines on IFNAR2c are involved in the signaling³⁰. Hence, STAT2 can be activated by both classical and alternative means while STAT1 phosphorylation relies on STAT2 phosphorylation.

- JAK-direct mediated mechanisms

At least two studies have demonstrated that STAT5 and JAK1/2/3 constitutively interact, using yeast-two hybrid, co-immunoprecipitation and GST-pull down experiments^{31, 32}. This interaction requires the pseudo-kinase domain of JAK and the C-terminal part of STAT5. Interestingly, the JAK-phosphorylable tyrosine is not required for this interaction but rather the region 680-688. By contrast, STAT1 or STAT3 do not interact with JAKs³¹. Consistently with this, STAT5 phosphorylation following IL-6 stimulation can occur in the absence of gp130 phosphotyrosines. Instead, the membrane-proximal region, required for the binding of JAKs, is sufficient to transduce the signal. This suggests that STAT5 activation occurs through direct interaction with JAK³¹. On the contrary, STAT1 and STAT3 phosphorylation following IL-6 stimulation requires receptor phosphotyrosines³³. Similarly, STAT5 and STAT6 phosphorylation does not require IL-4R α tyrosines. In absence of IL-4R α tyrosines, STAT5 and STAT6 phosphorylation is dependent on the membrane-proximal region, suggesting the direct interaction of STAT with JAK^{32, 34, 35}.

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3. MAPK pathway

Another important signaling pathway induced by cytokines is the Mitogen-Activated Protein Kinase (MAPK) pathway. This pathway is characterized by 3 levels of kinases that are activated following ligand binding and activation of a small GTP-binding protein. Briefly, adaptors (Shc, Grb2) bind to phosphotyrosines of the receptor and bring a guanine nucleotide exchange factor (Sos) in close proximity to a small G-protein. This causes the activation of the G-protein (Ras, Rac) by swapping GDP for GTP. Activated small G-proteins activate the cascade of phosphorylation: MAPKKK (Raf, MEKK) phosphorylates and activates MAPKK (MEK1/2) which, in turn, phosphorylates and activates MAPK (ERK1/2, JNK, p38) that phosphorylates its targets (transcription factors, cytoskeleton...). In this manner, the MAPK pathway regulates cell survival, proliferation and metabolism³⁶.

2. INTERLEUKIN 22

Originally described as IL-TIF for IL-10 related T-cell derived inducible factor, IL-22 was discovered in our lab in 1998³⁷. Since then, many studies have focused on elucidating IL-22 production, functions and involvement in diseases.

1. Structure

IL-22 belongs to the class II receptor cytokines and, more precisely, to the IL-10 family, also composed of IL-19, -20, -24, -26, IFN- λ 1, - λ 2 and - λ 3. Based on their receptor subunits and biological activities, this family can be further divided into 3 subfamilies. The first contains IL-10 itself, whose properties are anti-inflammatory. The second one, named the IL-20 subfamily, includes IL-19, IL-20, IL-22, IL-24 and IL-26. These cytokines are implicated in host defense against pathogens as well as in tissue repair and homeostasis. Finally, the type III interferons (IFNs), IFN- λ 1, - λ 2 and - λ 3 (also called IL-29, -28A and -28B), are involved in antiviral responses^{38, 39}.

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Human IL22 is encoded by a gene located on chromosome 12q15 (chromosome 10 for the mouse), in a cluster with the *IL26* and *IFNG* loci. This gene is composed of 5 exons, coding for a 179 amino acids protein^{40,41}. Human and mouse IL-22 share 79% identity at the protein level, while hIL-22 shares 22% identity with hIL-10. IL-22 is composed of 6 α -helices (helices A to F) organized in an anti-parallel conformation (Figure 5). Helices are connected by loops and stabilized by two disulfide bonds, folding the structure into a compact bundle. The protein is also glycosylated. Unlike IL-10, the IL-22 and other IL-10 family cytokines act as monomers, although high IL-22 concentrations can lead to dimer or even tetramer formation^{37, 41, 42, 43, 44, 45}.

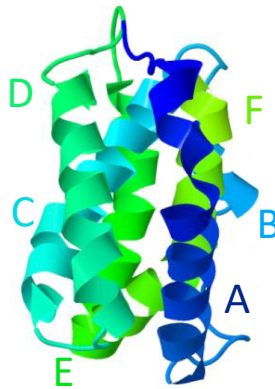


Figure 5: 3D structure of IL-22.

IL-22 is composed of 6 α -helices folded into a compact bundle. The structure follows a color gradient with the N-terminal part in dark blue.

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2. Production

Cells belonging to both the innate and adaptive immune systems produce IL-22, sometimes in combination with other cytokines (Figure 6). Mainly, IL-22 is co-produced with IL-17, with which it acts synergistically. IL-22 production, as well as IL-22-producing cell development and maintenance, is regulated by cytokines and transcription factors.

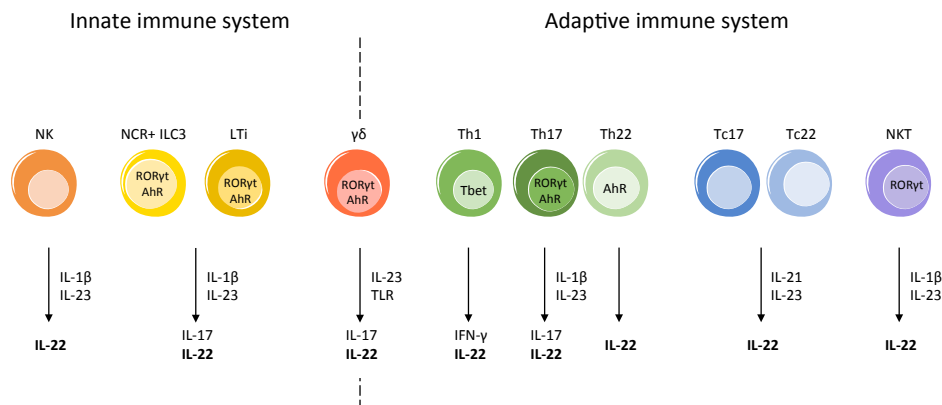


Figure 6: IL-22 producing cells and transcription factors associated with its production.

Cells from the innate and adaptive immunity produce IL-22. IL-23 and IL-1 β are inducers of IL-22 production. AhR and ROR γ T are transcription factors involved in IL-22 producing cell development and/or in IL-22 production.

2.1. $\alpha\beta$ T cells

Amongst $\alpha\beta$ T cells, CD4 $^{+}$ T cells, also called T helper cells, are important sources of IL-22. These cells are divided into subpopulations according to transcription factor expression and cytokines production profiles. Three types of T helpers are able to produce IL-22: Th1 (also producing IFN- γ), Th17 (co-expressing IL-17) and Th22. IL-6 is required for Th17 and Th22 differentiation and increases *IL23R* expression, which further promotes the expression of *IL22*. Indeed, IL-23 acts through STAT3 to induce IL-22 production in these cells. In addition, IL-1 β induces the expression of IL-22 via the NF κ B pathway^{41, 46, 47}. In contrast, TGF- β , although required for Th17 differentiation, inhibits IL-22 production by repressing *IL23R* and

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ROR γ T expression. *ROR γ T* is a transcription factor expressed by Th17 that is indispensable for differentiation. It can also directly induce *IL22* expression^{47, 48, 49}. Of note, IL-22 expression by Th17 seems to be more dependent on AhR (Aryl Hydrocarbon Receptor), another transcription factor expressed by Th17 and Th22, shown to be required for IL-22 production by Th17 *in vitro* and *in vivo*^{41, 50, 51}.

CD8⁺ T cells are also described to produce IL-22, but are less important than their CD4⁺ counterpart. They are called Tc17 (also producing IL-17) or Tc22. IL-23 and IL-21 promote IL-22 production in these cells^{52, 53}.

2.2. $\gamma\delta$ T cells

In addition to the conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells are also able to produce IL-22. These cells are at the interface between the innate and adaptive system. Indeed, they express a less-diversified TCR, and do not express the co-receptors CD4 or CD8. Moreover, antigens do not need to be presented by MHC molecules to activate $\gamma\delta$ T cells. They can produce IL-22 independently of TCR stimulation, in response to IL-23 and to innate ligands through Toll-like Receptors (TLRs). $\gamma\delta$ T cells also produce IL-17 and express IL-23R, AhR and *ROR γ T*^{41, 54, 55, 56}.

2.3. Innate lymphoid cells (ILC)

Innate lymphoid cells from group 3 are the initial sources of IL-22. They mirror Th17 in terms of cytokine production (IL-22 and IL-17) and transcription factor (*ROR γ T*). ILC3s can be divided into 2 subpopulations, both of which are potent IL-22 producers. The first subpopulation is called NCR⁺ ILC3s and expresses NKp46 (mice) or NKp44 (human) markers. LT α i (Lymphoid Tissue Inducer) constitutes the second population and plays an important role in the development of secondary lymphoid tissue. As with other cell types, IL-23 and IL-1 β enhance IL-22 production and AhR was shown to be important for IL-22 production as well as maintenance and/or recruitment of ILC3s^{41, 47, 51}.

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2.4. NK and NKT

IL-22 can be produced by natural killer (NK) cells, cytotoxic cells of the innate immune system. Another cell type that shares T cell and NK properties, called NKT, is also able to produce IL-22. Both produce IL-22 in an IL-23/IL-1 β -dependent manner⁴¹.

2.5. Non lymphoid cells

Some studies claim that macrophages of the lung, neutrophils in the gastrointestinal tract, keratinocytes and fibroblasts can produce IL-22. However, little data is available, making these non-lymphoid sources of IL-22 controversial⁴¹.

3. Receptor

3.1. Structure

The IL-22 receptor complex is a heterodimer formed of the subunits IL-22R α and IL-10R β ^{44, 57, 58}. The human IL-22R α is encoded by the *IL22RA1* gene, located on chromosome 1p36.11 (near *IFNLR1*) while *IL10R2* is located on 21q22.11 (near *IFNARA*, *IFNARB* and *IFNGRB*). Both genes share the same genomic structure composed of 7 exons (Figure 7). Exon 1 code for the signal peptide, exons 2-3 and 4-5 for the two fibronectin domains of the extracellular moiety, exon 6 for the transmembrane domain and exon 7 for the intracellular domain⁵⁶.

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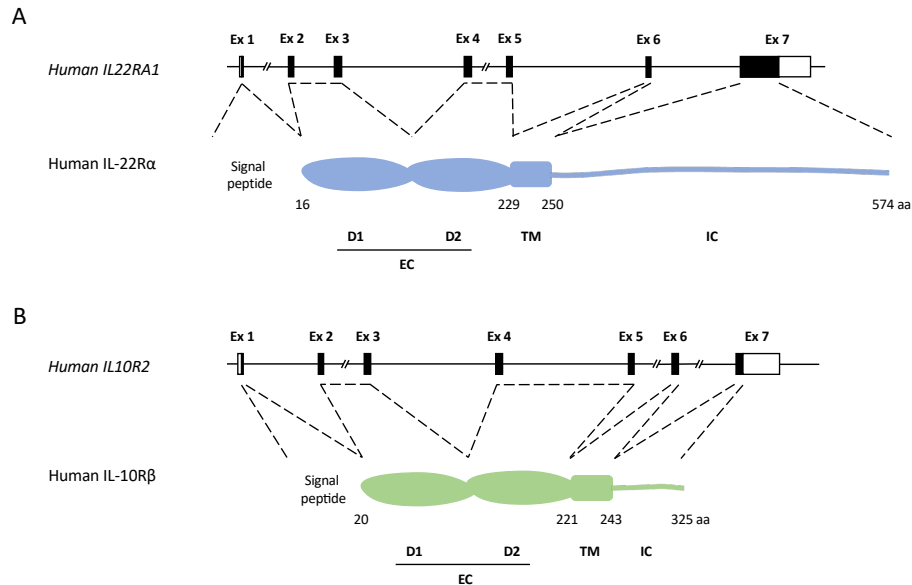


Figure 7: schematic representation of genomic and protein organization of IL-22Rα (A) and IL-10Rβ (B). (A) IL-22Rα and (B) IL-10Rβ share the same genomic and protein organization. Both genes are composed of 7 exons, each one coding for a (part of) domain of the protein. Each fibronectin domain of the extracellular part is about 100 amino acids long, followed by a short 20 amino acids transmembrane domain. The intracellular part of IL-22Rα is longer (324 aa) than that of IL-10Rβ (82 aa) and possesses tyrosines, which are important signaling residues. EC: extracellular, D1: domain 1, D2: domain 2, TM: transmembrane, IC: intracellular, Ex: exon

Both IL-22Rα and IL-10Rβ extracellular moieties are composed of two fibronectin type III domains, called D1 and D2 (Figure 7). Each domain is composed of two β -sheets in sandwich, containing 7 β -strands connected by loops. Interestingly, IL-22 has no affinity for IL-10Rβ but has instead a high affinity for IL-22Rα. IL-10Rβ has, however, a higher affinity for the IL-22/IL-22Rα complex. Hence, the binding of IL-22 to its receptor is a multi-step process: IL-22 first binds IL-22Rα, driving a conformational change, which in turn leads to the binding of this IL-22/IL-22Rα complex to IL-10Rβ. Multi-stage binding is similarly observed for IL-10 and its receptor^{59, 60}.

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The intracellular part of IL-22R α is longer than that of IL-10R β and possesses putative binding sites for STAT, which makes IL-22R α the signaling chain of the receptor^{58, 61}. While the extracellular structure of IL-22R α and IL-10R β is well described, the crystal structure of the intracellular part of the IL-22R complex has not been solved.

3.2. IL-22 binding protein

In addition to the membrane-anchored receptor, IL-22 also binds to a soluble form of the receptor. This soluble form, called IL-22 binding protein (IL-22BP), shares 34% identity with the extracellular part of the IL-22R α . It is encoded by the *IL22ra2* gene which is expressed in various tissues including breast, lungs, intestinal tract, skin, testis, brain, heart and thymus. IL-22BP can block the effects of IL-22 *in vitro*. Indeed, the affinity of IL-22 for IL-22BP is 20 to 1000 fold higher than for IL-22R α . Therefore, IL-22BP prevents the binding of IL-22 to its transmembrane receptor^{62, 63, 64}. The effects of IL-22BP have also been studied *in vivo*, in inflammatory models. By blocking the effects of IL-22 *in vivo*, IL-22BP impacts the course of diseases in which IL-22 is involved^{65, 66}.

3.3. Other IL-10 family receptor complexes

Both IL-22R α and IL-10R β subunits can associate with other chains to form receptor complexes (Figure 8). Indeed, IL-10R β pairs with IL-10R α to form the receptor of IL-10. When associated with IL-20R α or IFN- λ R α , it binds IL-26 or IFN- λ 1, IFN- λ 2 and IFN- λ 3, respectively^{41, 58}. IL-22R α combined with IL-20R β binds IL-20 and IL-24. Of note, this complex is not the only receptor for IL-20 and IL-24. Indeed, in addition to this type 2 IL-20R, IL-19, IL-20 and IL-24 signal via a type 1 receptor composed of the subunits IL-20R α and IL-20R β ^{67, 68}.

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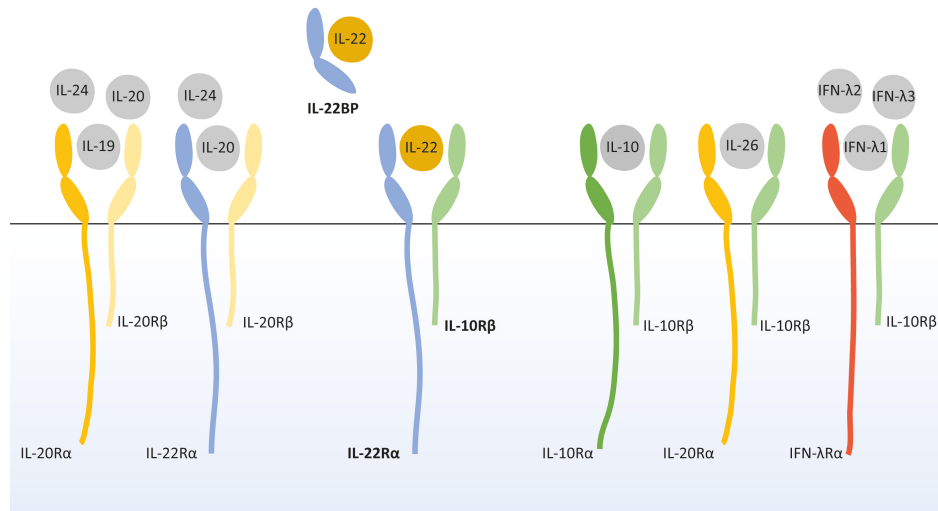


Figure 8: receptor complexes of the IL-10 family cytokines

The receptor of IL-22 is composed of IL-22Rα and IL-10Rβ. The IL-22Rα also associates with IL-20Rβ to bind IL-20 and IL-24. IL-10Rβ associates with IL-10Rα, IL-20Rα and IFN-λRα to bind IL-10, IL-26 and IFN-λ1/-λ2/-λ3 respectively. In addition, IL-22 binds to a soluble form of receptor - the IL-22 binding protein.

3.4. Expression

While IL-10Rβ is ubiquitously expressed, IL-22Rα expression is restricted to certain cell types, defining cells that respond to IL-22. Conversely to many other cytokines, IL-22 does not act on immune cells, neither resting nor activated⁶⁹. Rather, IL-22Rα expression is limited to stromal and epithelial cells, suggesting that IL-22 is a link between the immune system and tissues. IL-22Rα expression is detected in various organs, including the skin, gastro intestinal tract, liver, lungs, heart, pancreas and placenta. Interestingly, lots of IL-22Rα expressing organs have a barrier function^{61, 69}. In the skin, keratinocytes are the main target of IL-22 although dermal fibroblasts also express the IL-22Rα at a lower level⁷⁰. In the gut, both epithelial cells and subepithelial myofibroblasts express the receptor while, in the lungs, it is only expressed by epithelial cells. In the liver, hepatocytes are the IL-22Rα expressing cells^{41, 44}.

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Little is known about IL-22R α expression regulation. Nevertheless, the promoter of *IL22ra1* contains consensus binding-sites for STAT factors as well as for NF κ B, suggesting that both pathways are implicated in *IL22ra1* expression regulation⁶¹. One micro RNA (miR-197) was shown to regulate post-transcriptional *IL22RA1* expression in keratinocytes by repressing *IL22RA1* translation. Interestingly, IL-22 signaling enhances miR-197 expression in keratinocytes, leading to a negative feedback loop⁷¹. At the protein level, two mechanisms were demonstrated to regulate IL-22R α expression in murine lung epithelial cells: first, the ubiquitination of IL-22R α on Lys449, leading to the proteasome-mediated degradation, and second, its stabilization through phosphorylation of Ser410 and Ser414 by GSK-3 β (Glycogen Synthase Kinase-3 β). By inducing the inactivation of GSK-3 β by its phosphorylation and, therefore, impacting on IL-22R α stability, IL-22 again plays a negative feedback role⁷². While proteasomal degradation was demonstrated for both mouse and human IL-22R α , the effect of GSK-3 β on human IL-22R α has not been explored since the consensus site for this kinase is not present in the human receptor⁷³.

4. IL-22 signal transduction

4.1. Classical pathway

JAK1 and TYK2 are constitutively associated with the cytoplasmic part of IL-22R α and IL-10R β , respectively, and are rapidly activated after IL-22 stimulation. Downstream of the JAKs, STAT1, STAT3 and STAT5 are activated by IL-22. Nevertheless, while STAT3 is strongly phosphorylated in all IL-22R-expressing cells, this is not always the case for STAT1 and STAT5. Therefore, STAT3 is the main signal transducer activated by IL-22 while STAT1 and STAT5 activation seem to be cell-dependent^{44, 57, 58, 69, 74, 75}. Of note, STAT3 can be phosphorylated on its Tyr⁷⁰⁵ but also on Ser⁷²⁷, although this is less frequently observed^{74, 76}. Besides STAT factors, IL-22 can also activate the MAPK pathway. Phosphorylation of ERK, MEK1/2, JNK and P38 is observed in keratinocytes and other cell types after IL-22 stimulation^{74, 75, 76, 77}. Finally, activation of the PI3K/Akt/mTOR pathway was reported after

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IL-22 stimulation in normal human epidermal keratinocytes and fibroblast-like synoviocytes⁷⁸.

A study from the lab enabled the attribution of IL-22-induced pathways to specific residues of the IL-22R α . This information is summarized in Figure 9. Briefly, three independent regions of the human IL-22R α mediate Tyr⁷⁰⁵-STAT3 phosphorylation. First, Tyr³²⁶ of the IL-22R α seems to be important for STAT3 phosphorylation but not absolutely required. Second, one or several tyrosines between Tyr³⁵¹ and Tyr⁴⁰³ are implicated in STAT3 activation, as deletion of this region decreases STAT3 activation. Of note, three of these, as well as Tyr³²⁶, are comprised in a STAT3 binding motif (YxxQ). Third, STAT3 activation can occur in an IL-22R α phosphotyrosine independent manner that relies on the C-ter of the IL-22R α (see later). On the other hand, Ser⁷²⁷-STAT3 phosphorylation does not rely on IL-22R α tyrosines but rather on the juxtamembrane domain, which is sufficient to obtain activation. STAT1 and STAT5 phosphorylation seem to depend mostly on Tyr³²⁶. Finally, Tyr³⁰¹ and Tyr³²⁶ are important for ERK and p38 phosphorylation, respectively⁷⁹ (unpublished).

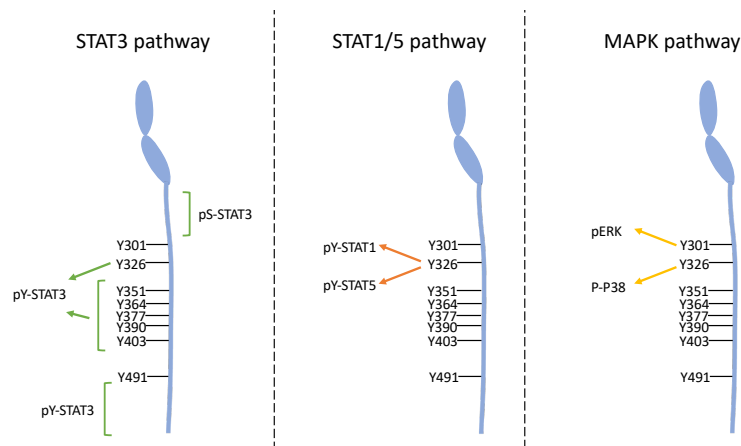


Figure 9: scheme of IL-22R α tyrosines involved in signaling pathways.

Phosphorylation of STAT3 on its tyrosine relies on 3 regions of the IL-22R α : Y³²⁶, tyrosines located between 351-403 and the C-ter tail. Phosphorylation on serine depends on the membrane-proximal region. STAT1, STAT5 and p38 phosphorylation requires Y³²⁶ while ERK phosphorylation depends on Y³⁰¹.

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4.2. Alternative activation of STAT3

Surprisingly, STAT3 activation can occur in an IL-22R α -phosphotyrosine independent manner. This so-called alternative/unconventional activation of STAT3 was demonstrated in the lab by the use of mutant forms of IL-22R α . Indeed, IL-22R α lacking all its tyrosines is still able to induce STAT3 phosphorylation, but not STAT1 and STAT5. By contrast, the C-ter part of IL-22R α (aa 492-574), devoid of tyrosines, is required and sufficient to mediate this alternative activation of STAT3⁸⁰. No specific residues of the IL-22R α C-ter could be determined to be responsible for this activation of STAT3 as progressive truncation of the IL-22R α C-ter leads to a progressive decrease in STAT3 phosphorylation (unpublished results). In summary, it was demonstrated that STAT3 is constitutively associated with the C-ter of IL-22R α through its coiled-coil domain (CCD), allowing for the tyrosine-independent activation of STAT3 upon IL-22 binding. This mechanism specifically requires the CCD of STAT3 since replacement of the NTD and CCD of STAT5 by those of STAT3 is sufficient to allow the phosphorylation of STAT5 in the absence of receptor tyrosines. Importantly, both classical and alternative pathways act synergistically to strongly induce STAT3 activation (Figure 10)⁸⁰.

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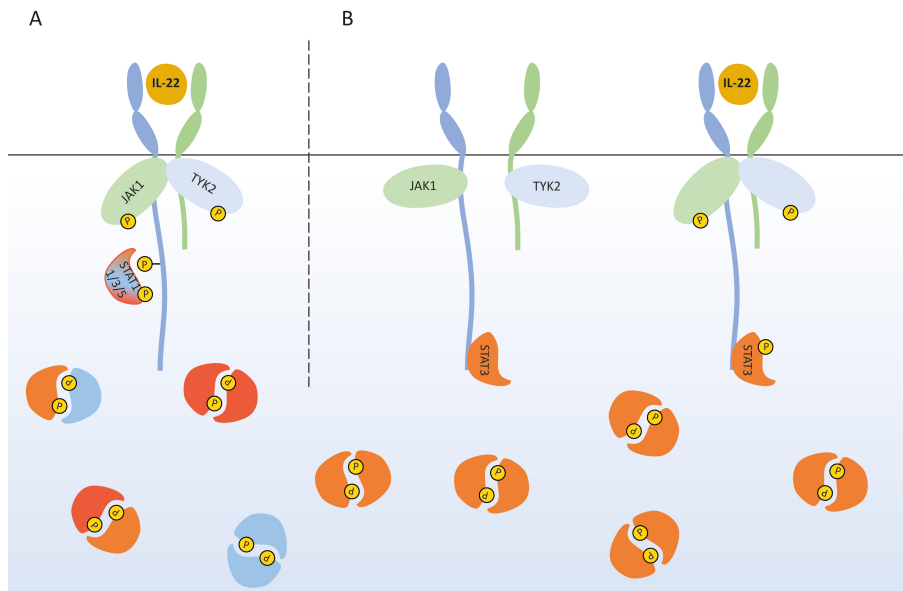


Figure 10: classical and alternative JAK-STAT pathways induced by IL-22.

(A) IL-22 induces, in a phosphotyrosine-dependent manner, STAT1, STAT3 and STAT5 phosphorylation. In this case, STAT binds a phosphotyrosine of the receptor via its SH2 domain. (B) Besides this classical signaling, STAT3 is constitutively associated with the C-ter part of the IL-22R α through its coiled-coil domain. Following IL-22 binding, STAT3 is activated independently of receptor tyrosines. The classical and alternative pathways synergize.
In orange: STAT3, in red: STAT1, in blue: STAT5.

5. IL-22 functions

Although cytokines can often be categorized as pro-inflammatory or anti-inflammatory, this is not the case for IL-22. Indeed, the inflammatory environment influences IL-22 production and thereby, the impact of this cytokine on the tissue. Limited amounts of IL-22 generally provide beneficial effects; by contrast, a prolonged/over-production of IL-22 leads to excessive effects that become deleterious. This part will review the roles of IL-22 in various tissues, both in physiological and pathological contexts (summarized in Figure 11), with a focus on the skin and gastro-intestinal tract.

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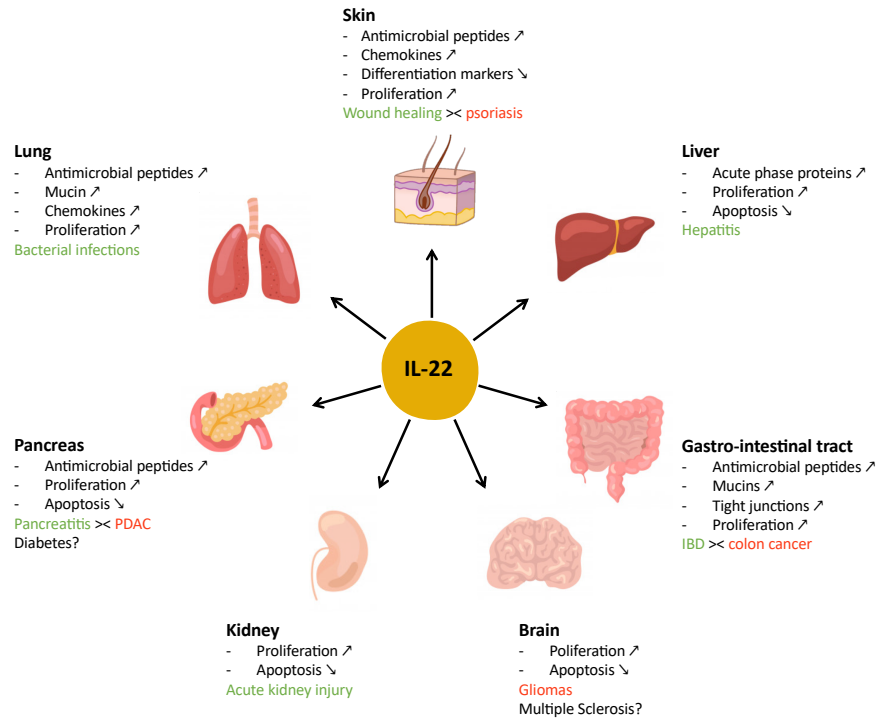


Figure 11: roles of IL-22 in different organs and implications in diseases.

In different organs, IL-22 promotes the production of antimicrobial peptides, chemokines, mucin and acute phase proteins, favors proliferation and prevents apoptosis. Therefore, IL-22 is beneficial in some diseases (in green) while deleterious in others (red).

5.1. Skin

• Global functions

IL-22 exerts multiple effects on keratinocytes linked with innate defense, cell proliferation, differentiation and migration. First, IL-22 induces the production of antimicrobial peptides (AMP), mainly S100A7, S100A8, S100A9, β -defensin 2 and 3 and lipocalin 2. These proteins work by directly targeting pathogens, interfering with microbial DNA and protein of membrane synthesis or depleting the environment in essential nutriment. Therefore, IL-22 contributes to the clearance of skin pathogens^{46, 69, 75, 81, 82}. By stimulating the production of CXCL1, CXCL3, CXCL5 and CXCL8, IL-22 promotes the recruitment of neutrophils to the skin.

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On the contrary, IL-22 repressed the production of CCL22, a Th17-recruiting chemokine^{70, 81, 82}. IL-22 also induces the proliferation of keratinocytes^{70, 81, 82}. Moreover, MMP-1 and -3 expression are increased by IL-22. These proteins are important for cell migratory capacity during epithelial regeneration, degrading the extracellular matrix^{75, 81, 82}. Finally, IL-22 inhibits keratinocytes differentiation and maturation. Indeed, the expression of several genes associated with keratinocytes differentiation is down regulated by IL-22. This is the case for keratin-1, -10, profilaggrin, involucrin and loricrin^{70, 75, 81}.

Because of these effects, IL-22 has a beneficial role in wound healing. IL-22, which is expressed following tissue injury, helps to regenerate the skin, mainly through its action on dermal fibroblasts⁸³. By contrast, an excess of IL-22 in the skin and the effects that follow are deleterious and lead to pathologies such as psoriasis.

- Pathological context: psoriasis

Description of the disease

Psoriasis is a chronic immune-mediated inflammatory disease of the skin that affects around 2 to 3 % of the population. Several comorbidities have been observed, including cardiovascular diseases, type 2 diabetes, Crohn's disease, obesity and dyslipidemia. Psoriasis can be associated with arthritis in up to 30 % of the patients⁸⁴.

Typical skin lesions of psoriasis are red raised plaques with silvery-white dry scales. These lesions appear primarily around the elbow, the knee, on the scalp and in the lumbosacral area^{84, 85}. The histological features of psoriatic skin explain the appearance of these lesions. Before going into a detailed description of psoriatic skin, I will describe the structure of healthy skin. The skin is composed of three layers from the innermost to the outermost: the hypodermis, the dermis and the epidermis (Figure 12A). The hypodermis is mainly constituted of adipocytes while the dermis is composed of connective tissue and fibroblasts. As for the epidermis, it is composed of several layers of keratinocytes but also of

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melanocytes. The deepest layer of the epidermis, called basal layer, is formed of keratinocytes that proliferate. These cells undergo a progressive program of differentiation that leads to the spinous, granular and cornified layers. In the granular layer, keratinocytes accumulate granules that contain profilaggrin, loricrin and antimicrobial peptides. During the last step of differentiation, keratinocytes become flat, lose their nucleus and die. They are called corneocytes and form the cornified layer. This last layer constitutes the physical barrier that is the skin. Indeed, cells are surrounded by an envelope composed of lipids and proteins that are secreted by keratinocytes and aggregate. This cornified envelope prevents the skin from water loss and makes it pathogen-resistant^{85, 86}.

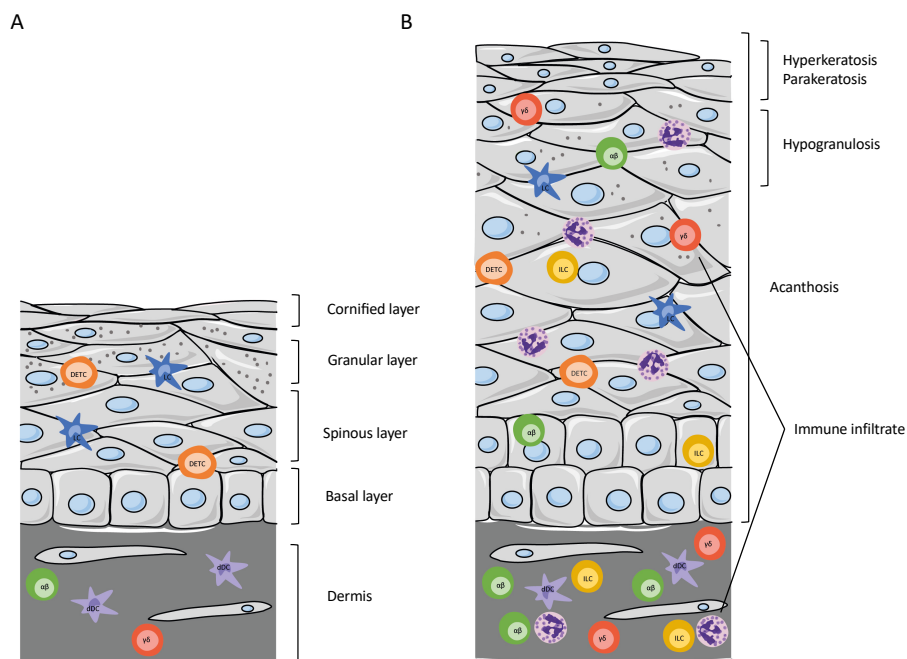


Figure 12: comparison of healthy (A) and psoriatic (B) skin.

(A) The skin is composed of the hypodermis, dermis and epidermis. The epidermis is divided into 4 layers: the basal, spinous, granular and cornified layers. Only few immune cells are present in the skin in physiological conditions.

(B) Psoriasis modifies the structure of the skin with immune cell infiltration, acanthosis, parakeratosis, hyperkeratosis and hypogranulosis. (DETC: dendritic epidermal $\gamma\delta$ T cells; dDC: dermal dendritic cells)

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Psoriasis alters the structure of the skin at multiple levels (Figure 12B). The most obvious characteristic is the thickening of the epidermis, called acanthosis. The basal layer of the epidermis undergoes excessive proliferation, leading to global thickening of the epidermis. The program of differentiation is also impaired, leading to a decrease of the granular layer, called hypogranulosis. By contrast, there is an increase of the cornified layer (hyperkeratosis). The defect in maturation also leads to the retention of nuclei in corneocytes (parakeratosis). An increase of vascularity is observed in the dermis^{84, 85}. Finally, while only few immune cells are present in the skin when healthy, there is a strong infiltrate of immune cells in psoriatic skin. In healthy skin, there are some $\alpha\beta$, $\gamma\delta$, dendritic cells, macrophages and mast cells in the dermis. The epidermis only contains Langerhans cells and dendritic epidermal $\gamma\delta$ T cells (DETC)⁸⁷. On the contrary, $\alpha\beta$, $\gamma\delta$, ILCs and neutrophils are recruited both in the dermis and the epidermis of psoriasis skin^{85, 88}.

Genetic and risk factors of psoriasis

Genetic, environmental and immunological factors can trigger the initiation of the disease. First, a genetic component is observed in psoriasis. Several studies and, more recently, GWAS could identify susceptibility loci and SNP associated with psoriasis. One particular locus is called PSORS1. It contains several genes but more particularly the allele HLA-Cw0602 that has been linked with the disease. This allele confers a 20-fold-increased risk of developing psoriasis and is carried by up to 60% of the patients. Besides this locus, other SNPs have been identified, including in genes involved in the Th17 pathway, namely the *IL23R*, *IL12B* and *IL23A*^{84, 85}. For IL-22, polymorphisms in both the *IL22* gene and promoter have been identified. A copy number variation of *IL22* was also associated with psoriasis severity^{89, 90, 91}. In addition to the genetic susceptibility, immunological events resulting from the actions of many cell types are involved in psoriasis pathogenesis. For example, molecules secreted by stressed keratinocytes are able to activate skin dendritic cells, which themselves trigger a cytokine cascade resulting in INF- γ , TNF- α , IL-17 and IL-22 production^{85, 92, 93}. Finally, streptococcal infection, physical trauma, medications, smoking and alcohol are also known to exacerbate psoriasis⁸⁴.

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IL-22 and psoriasis

IL-22 is upregulated, at the RNA and protein level, in the skin and serum from psoriasis patients as compared to healthy donor. On the contrary, neither *IL22RA1* nor *IL10R2* expressions are modified^{69,94}. *IL22* transgenic mice develop skin lesions that recapitulate the main features of psoriatic skin: skin is stiff, the epidermis is thicker and there is loss of the granular layer. Moreover, immune cells infiltrate the skin. In addition to these cutaneous lesions, *IL22* transgenic pups have 30 % less body weight compared to WT littermate and neonatal death is observed. Hence, this study highlights the deleterious effects of IL-22 when overexpressed in the skin⁷⁰. To study the role of IL-22 in depth *in vivo*, several mouse models have been developed, together with genetically modified mouse.

One of the models used to study psoriasis is based on repeated injections of IL-23. Daily intradermic injection of IL-23 promotes epidermal acanthosis, parakeratosis, neutrophils loci formation and inflammatory infiltrate⁹⁵. After IL-23 treatment, IL-22 is produced in the ear, mostly by T cells. As expected, IL-22 has a detrimental role in this model as ear thickness and epidermis acanthosis are reduced in *IL22*^{-/-} or neutralizing IL-22 antibody treated mice as compared to control mice. Importantly, mice are not completely protected, which implies that other actors besides IL-22 are involved in this model, TNF- α and other IL-20 subfamily cytokines for example^{95,96}.

The most commonly used psoriasis model, which we use in the lab, is the one induced by imiquimod, contained in Aldara® cream. After daily application on the shaved skin of the back or on the ears of mice, imiquimod causes psoriasis-like lesions⁹⁷. Interestingly, Aldara® is used in the treatment of genital and perianal warts and basal cell carcinoma and it has been observed that patients can develop psoriasis following treatment, making this model very relevant to the study of psoriasis *in vivo*⁹⁸. Imiquimod-treated mice display a similar phenotype to that of psoriasis patients, with skin thickening, acanthosis, hyperkeratosis, parakeratosis and immune cells infiltrate⁹⁷. The action of this compound relies on three distinct mechanisms: First, the imiquimod is an agonist of TLR-7 (and -8 in human), expressed

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on dendritic cells. Binding of imiquimod to its receptor induces the MyD88/NFκB pathway that promotes the production of pro-inflammatory cytokines, such as IFN-α, IL-23, TNF-α and IL-6. Second, by antagonizing the adenosine receptor, imiquimod also promotes pro-inflammatory cytokines production. Finally, imiquimod as well as isostearic acid, the vehicle in the cream, stimulate the inflammasome in keratinocytes, leading to the production of IL-1β and IL-18 as well as keratinocyte apoptosis⁹⁸. IL-22 is produced in this model, with a peak around day 3 to 5^{97, 99}. In WT mice, γδ T cells and αβ T cells are responsible for IL-22 production. Nevertheless, the imiquimod model also induces skin lesions and IL-22 production in *Rag2*^{-/-} mice. In the absence of B and T cells, IL-22 is produced by ILC3s^{51, 99}. *IL22* deficient mice are partially protected from the disease, at the macroscopic and microscopic levels. Moreover, the expression of several genes is less induced in these mice, as compared to WT treated mice, including the neutrophil-attracting chemokine *Cxcl3*. This correlates with a decrease of the percentage of neutrophils in the dermis⁹⁹. Same observations were made in *IL22ra1* deficient mice¹⁰⁰ (unpublished results). As for IL-22BP, it seems to be protective in the model because IL-22BP neutralizing antibody aggravates skin lesions in mice⁶⁶.

Finally, there are other models of psoriasis, more complicated to use routinely in laboratories. In the model of adoptive transfer, transferring naïve T cells (CD4⁺ CD45RB^{high}) depleted of CD25⁺ regulatory T cells into *scid/scid* recipient mice leads to the formation of skin lesions resembling psoriasis about two weeks after the transfer. The use of neutralizing IL-22 antibody is sufficient to prevent skin lesions, demonstrating, again, the deleterious effect of this cytokine. Interestingly, this model is also used to study inflammatory colitis in which, by contrast, IL-22 plays a beneficial role (see later)¹⁰¹. Finally, one study focused on IL-22 impact on two kinds of xenografts. Injection of IL-22 in normal human skin graft promotes the development of skin lesions. On the contrary, anti-IL-22 antibody injection in severe psoriatic skin graft is efficient to reduce skin inflammation¹⁰².

Altogether, these studies demonstrate that an excess of IL-22 in the skin is deleterious as it promotes inflammation and skin lesions.

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Current treatments for psoriasis

Various approaches are taken to treat psoriasis patients with. Topical treatments are the preferred first-line therapies. Corticosteroids, vitamin D and UV therapy (UVB or PUVA) can help mild to moderate psoriasis by slowing cell growth and inflammation. Unfortunately, in severe psoriasis, these approaches are not effective enough. Several immunosuppressive molecules can be used as second-line therapies. This is notably the case of cyclosporine A that acts by inhibiting T cells and cytokines production, methotrexate, tacrolimus, and phosphodiesterase inhibitor. Finally, various biologics have been developed, targeting TNF- α , IL-23 or IL-17. Nonetheless, despite their efficacy, their use should be limited in time because they are expensive, can cause side effects and lose efficiency after long-term use^{92, 103, 104}. This is why new approaches and/or molecules could still improve psoriasis treatment.

5.2. Gastro-intestinal tract

- Global functions

The global functions of IL-22 in the gut are similar to the ones observed in the skin: IL-22 is critical for the structural and functional aspects of the intestinal barrier. First, IL-22 increases cell proliferation and down-regulates apoptosis¹⁰⁵. IL-22 seems to induce claudin-1 expression, a protein participating in tight junctions between epithelial cells¹⁰⁶. These functions improve the barrier of the gut by enhancing the regeneration and the impermeability of the epithelium. It is facilitated by the production of proteases such as MMP⁴¹. Second, IL-22 is also engaged in the functional aspect of the barrier as it promotes the production of antimicrobial peptides from intestinal epithelial cells, such as β -defensin 2, S100A8, S100A9, Reg3 β and Reg3 γ ^{38, 107}. Production of mucus-associated proteins, MUC1, 3, 10 and 13, is also induced by IL-22 in colonic epithelial cells. These glycoproteins are necessary to create a protective layer of mucus covering the epithelium¹⁰⁸. Hence, IL-22 is important for ensuring the integrity of the intestinal barrier against pathogens. Because of these effects, the role of IL-22 has been studied in diseases where intestinal barrier integrity is impaired.

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- Pathological context: inflammatory bowel diseases

Description of the disease

Inflammatory bowel diseases (IBD) are a group of distinct diseases, the principal types being Crohn's disease (CD) and ulcerative colitis (UC). CD can affect the entire gastro-intestinal tract but preferentially the ileum and the proximal colon while UC is usually restricted to distal colon and rectum. Symptoms are abdominal pain, diarrhea, bloody stool and weight loss.

The gastro-intestinal tract is a tube composed of three layers named, from outside to inside, mucosal, submucosal and muscularis propria. The mucosa consists of the epithelium layer, the lamina propria (extracellular matrix, fibroblasts and immune cells) and a muscular layer (muscularis mucosa). The epithelium forms crypts all along the tract and also villi in the jejunum-ileum (Figure 13A). As already mentioned, the intestinal barrier plays an essential role in gut homeostasis and is the result of multiple cell types. Indeed, tight junctions between epithelial cells ensure impermeability; mucus protective layer is produced by goblet cells; Paneth cells, located at the base of intestinal crypts, and epithelial cells produce AMP; and the dendritic cells, macrophages, ILC and neutrophils located in the gut are the first line of defense. All these factors can be affected in IBD^{109, 110, 111, 112}.

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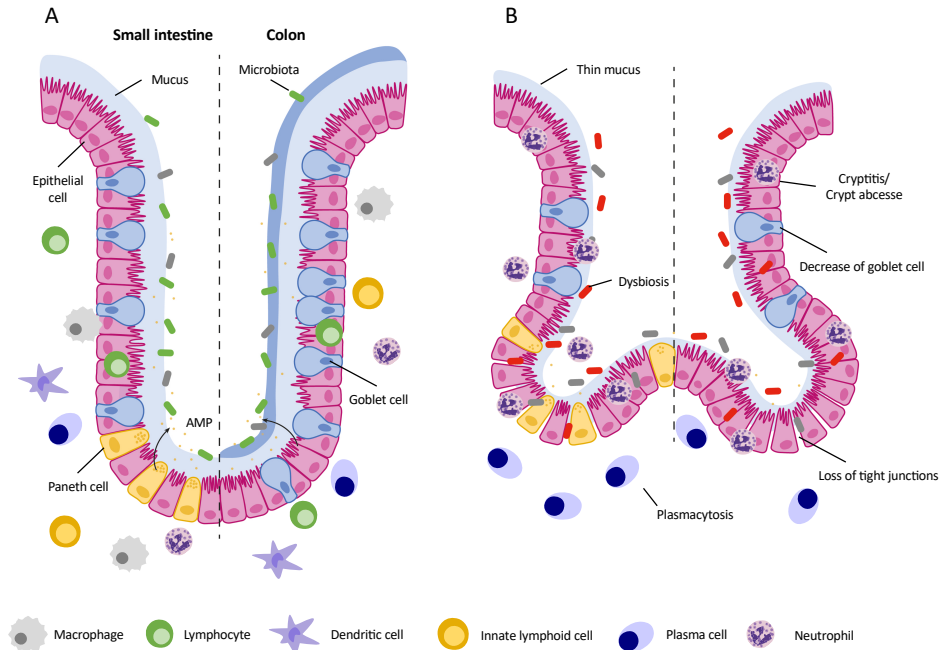


Figure 13: comparison of healthy (A) and colitis (B) intestinal tract.

(A) The epithelium of healthy intestine is constituted of epithelial, Paneth and goblet cells. Paneth cells are present in the small intestine and are responsible for AMP production whereas this production is carried out by epithelial cells in the colon. Goblet cells produce mucus and are more abundant in the colon than in the small intestine. In the colon, the mucus is composed of two layers: a thick inner and a thin outer layer. The intestinal tract is colonized by the bacterial microbiota. Some immune cells are present in the healthy ileum/colon. (B) In colitis, the epithelium is characterized by a decrease in goblet cells, a neutrophilic and plasma cells infiltrate, a thinner mucus layer and a loss of tight junctions. Moreover, crypts are shorter, form distortion and branching. Dysbiosis (microbial imbalance) is observed with bacteria infiltrating the epithelium. For clarity, immune cells are not represented in the colitis part, except plasma cells and neutrophils which are recruited.

At the histological level, 4 features are affected in UC and CD: mucosal architecture, lamina propria cellularity, neutrophil infiltrate and epithelial abnormalities (Figure 13B). Although both diseases resemble each other, histopathological analysis enables the discrimination of one from the other. Indeed, in UC, inflammation is diffuse in the colon but immune cells infiltration is confined to the lamina propria. It is characterized by the presence of plasma cells between the end of the crypts and the muscularis mucosae and in the muscularis mucosae (basal plasmacytosis) as well as inflammatory cells, notably neutrophils, in the

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crypts (cryptitis) or in the lumen of crypts (crypt abscesses). Mucosal architecture is affected, with crypt shortening, distortion and branching. Finally, there is a mucus depletion resulting from a decrease of goblet cells and mucin expression. The epithelium also displays some damage, with focal cell loss, erosion and ulcers^{109,113}. In contrast, inflammation affects all layers in CD. Mucosal alterations affecting crypts structures are also observed, although cryptitis and crypt abscesses are less common. One of the hallmarks of CD is the presence of granulomas, which are structures composed of a collection of epithelioid cells and macrophages formed during inflammation, in the mucosa. Finally, fissuring ulcerations are possible in CD¹⁰⁹.

IBD pathogenesis

CD and UC are multifactorial diseases whose pathogenesis is a result of the interplay between genetic susceptibility, microbiota deregulation, environmental factors and immunological abnormalities. The major factor is the microbiota as it plays a crucial role in gut homeostasis. Indeed, the commensal flora is composed of several strains that co-exist with the epithelium, with benefits for both players. AMP produced by epithelial and immune cells control microbiota while microbiota influences the type of immune responses promoted in the gut. Pathological colonization of the gut with specific bacteria completely deregulates the balance, leading to pathological pro-inflammatory immune response against both commensal and pathogenic bacteria. Dysbiosis (microbial imbalance) with a decrease of microbiota diversity has been described in IBD patients. Environment is another important factor in the regulation of microbiota. Indeed, food intake, smoking, medication, stress and so on influence the composition of the flora, favoring some types of bacteria over others. For example, diets rich in fatty acid and antibiotics are associated with IBD development. Finally, polymorphisms have been linked with IBD, notably in *NOD2*. *NOD2* is a receptor for muramyl dipeptide, a component of Gram+ and Gram- bacteria that induces immune responses following bacteria recognition. The loss of function mutation leads to an impairment of bacterial clearance^{110, 111, 114}.

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IL-22 and IBD

While IL-22 is not detected in colonic samples from healthy patients, its production is increased in UC and even more in CD patients^{108, 115, 116, 117}. Therefore, the role of IL-22 in these diseases was studied *in vivo* in mice. Several studies, using mouse models of IBD, could demonstrate that IL-22 has a protective effect in colitis by reinforcing the epithelial barrier. Three of them will be described in the following paragraphs.

The DSS (Dextran Sodium Sulfate) model is a chemically induced model of colitis that recapitulates IBD and particularly UC. Through a toxic effect on epithelial cells, DSS leads to epithelium injury, disruption of the intestinal barrier and resulting intestinal inflammation. DSS is provided to mice through drinking water (between 1 and 5 %) for various periods of time. Mice are characterized by weight loss, bloody stool and diarrhea^{118, 119}. At the histological level, several features observed in UC patients are present. Indeed, the colon is the most affected part, with a decrease in tight junctions, mucin and goblet cells, and a shortening of its length. A prominent infiltrate of neutrophils into the lamina propria is also observed^{119, 120}. IL-22 expression is induced in the colon following DSS treatment and deficiency in *Il22* leads to more pronounced weight loss and mortality, as compared to WT mice. This is notably explained by a defect in goblet cells and mucus production, affecting intestinal barrier integrity^{108, 121}. Interestingly, *Rag1*^{-/-} mice, lacking T and B cells, still produce IL-22 in the DSS model, suggesting that innate immunity plays a role in the disease. *Rag1*^{-/-} *Il22*^{-/-} mice are also more susceptible to DSS than *Rag1*^{-/-}. Altogether, it was shown that IL-22 is protective during both innate and adaptive phases of colitis¹²¹. Finally, another study supports the results obtained concerning the beneficial role of IL-22 in colitis. Indeed, *Il22ra2*^{-/-} rats, deficient for IL-22BP, are less sensitive to the DSS model than WT controls. As the absence of IL-22 antagonist is beneficial, it strengthens the protective role of IL-22 in DSS-induced colitis¹¹⁷.

The second model is the infectious colitis induced by *Citrobacter rodentium*, a mouse-specific pathogen. *Citrobacter rodentium* is a Gram⁻ bacterium that colonizes intestinal

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mucosa. Typically, *Citrobacter rodentium* is used to mimic and study infection with *Escherichia Coli* (enteropathogenic and enterohaemorrhagic), with which it shares 67% of genes. It is however also used to study colitis because infection with *Citrobacter rodentium* induces dysbiosis in the microbiota, promoting inflammation in the intestine. This is the model used in our laboratory to study colitis. In practice, mice are exposed to the bacteria via oral inoculation. *Citrobacter rodentium* then colonizes the caecum and colon, and causes, within 4 days, an immune cell infiltrate in the mucosa. Mucosa is also characterized by crypt hyperplasia (crypt elongation and mucosa thickening). WT C57BL/6 mice are able to clear the infection^{122, 123}. IL-22 is produced in this model with a peak around 4 days following infection. As in the DSS model, deficiency in *Il22* exacerbates disease. Indeed, there is significant weight loss and high mortality within two weeks in *Il22*^{-/-} or anti-IL-22 antibody treated mice. Histological features are also worsened with increase of mucosal hyperplasia, submucosal inflammation, ulceration and bacteria in colonic crypts. It was shown that the protection provided by IL-22 emanates, notably, from the induction of *Reg-3 β* expression. The protective effects of IL-22 seem to occur at the early stage of the model because mice who receive an anti-IL-22 antibody at D0 of the model lost weight and died rapidly. By contrast, the administration of the antibody at D8 did not interfere with weight or mortality¹⁰⁷. Therefore, several studies suggest that, although produced by both ILC3 and Th17/Th22 in this model, early IL-22 production by ILC3s plays the critical role in efficiently limiting disease^{107, 124, 125}.

As previously mentioned, the adoptive transfer of naïve T cell (CD45Rb^{high} CD4⁺) into *Rag*^{-/-} deficient mice induces, in addition to psoriatic lesions, colitis that recapitulates CD. IL-22 is expressed in this model, around two weeks post-transfer^{108, 121}. As observed in the other models, IL-22 production by ILCs plays a crucial role in protection. Indeed, expression of IL-22 by innate cells only is sufficient to limit weight loss and colon damage¹²¹.

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- Pathological context: colorectal cancer

Colorectal carcinogenesis

Colorectal cancer is the third most common cancer and its mortality rate is very high. Several genes are commonly mutated and have been associated with colorectal cancer initiation, amongst which *APC* (adenomatous polyposis coli) mutations occur in 80 % of cases. *APC* is a regulator of the Wnt/ β -catenin pathway. Inactivating mutations of *APC* lead to its truncation and consequently, to the constitutive translocation of β -catenin into the nucleus to regulate the expression of target genes involved in cell-cell adhesion, migration and apoptosis^{126, 127, 128}. Interestingly, polymorphisms in *IL22* was positively associated with colon cancer as well^{129, 130}. In addition, many environmental risk factors favor colorectal cancer development: sedentary lifestyle, high-unsaturated fat diet, alcohol and medication. Chronic inflammation also promotes tumor development. Finally, IBD is described to frequently evolve into colon cancer. Indeed, IBD patients are 4 times more likely to develop colon cancer as a result of colitis (colitis-associated cancer)^{126, 127, 128}.

IL-22 and colorectal cancer

IL-22 expression is increased in colon cancer samples. It is accompanied by an increase of the percentage of Th17 and Th22 among circulating lymphocytes of colon cancer patients compared to healthy donors and among tumor infiltrating lymphocytes compared to non-tumor infiltrating lymphocytes^{131, 132}. *In vitro*, IL-22 promotes the proliferation of colon-cancer derived cell lines and represses apoptosis¹³¹. Therefore, the role of IL-22 in colorectal carcinogenesis was studied *in vivo* in two mouse models of colon cancer. The first model is triggered by the dominant mutation of *Apc*^{Min} (multiple intestinal neoplasia), that causes protein truncation. It leads to spontaneous, non-inflammatory, carcinogenesis that recapitulates the genetic forms of colon cancer^{127, 133}. The second is a model of colitis-associated cancer. Mice are pre-treated with 2-azoxymethane (AOM), an alkylating agent that produces free radicals that bind to DNA and cause mutations. Then, a minimum of one cycle of DSS-induced inflammation is performed, as in the model of

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chemically-induced colitis. Together, AOM and DSS enable the recapitulation of the process in which colitis evolves into colon cancer^{127, 133}.

Apc^{Min/+} mice deficient in *IL22* develop fewer tumors in the colon as compared to control mice, suggesting that IL-22 is deleterious in carcinogenesis. Unexpectedly, *IL22*^{-/-} mice undergoing the AOM/DSS model develop more tumors as compared to WT mice. Nevertheless, IL-22 neutralization by antibody injection at a late stage of the DSS cycle (day 12) decreases tumor development. This is explained by the fact that since IL-22 is protective in the colitis, its total deficiency increases inflammation and tumor development, in contrast to the effect of late-stage IL-22 neutralization. These two models suggest that IL-22 plays a deleterious role in tumor development. This was further confirmed with *IL22ra2*^{-/-} mice. In both AOM/DSS and *Apc*^{Min} models, IL-22BP deficient mice display an increase in tumor number and size, demonstrating that the absence of IL-22 soluble receptor is deleterious and, thereby, that IL-22 itself is deleterious in colon carcinogenesis¹³⁴. In conclusion, IL-22 does not trigger carcinogenesis by itself, as *IL22* transgenic mice do not develop cancer. Nevertheless, by its pro-proliferative, anti-apoptotic and pro-angiogenesis effects, IL-22 can favor tumor development.

5.3. Lungs

IL-22 effects on lung epithelial cells are similar to those observed in the skin or in the gastro-intestinal tract. Indeed, IL-22 plays an important role in epithelial barrier integrity. It induces the expression of antimicrobial peptide (β -defensin 2, lipocalin 2), mucin (MUC1) and chemokines (CXCL5). IL-22 also promotes the proliferation of epithelial cells, favoring tissue repair after injury^{41, 135}.

IL-22 production is increased following lung bacterial infections (*Streptococcus Pneumoniae* and *Klebsiella pneumoniae* for example) and has a protective effect on bacterial clearance through AMP production^{135, 136}. Following influenza A virus infection, IL-22 is also produced in the lungs but does not participate in viral clearance. However, it is important for the

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regeneration of epithelial damage and cell death occurring during viral infection and is protective in case of secondary bacterial infection^{137, 138, 139, 140}.

5.4. Liver

In the liver, hepatocytes are targets of IL-22. In these cells, IL-22 promotes the expression of acute-phase proteins such as serum amyloid A, haptoglobin, fibrinogen and LPS-binding protein, demonstrating a role of IL-22 in liver inflammation^{44, 116, 141, 142}. In addition, IL-22 enhances hepatocytes proliferation by inducing the expression of mitotic and pro-proliferative genes. Finally, IL-22 mediates anti-apoptotic effects^{142, 143, 144, 145}.

In a pathological context, IL-22 is beneficial in various hepatitis models (LPS- or concanavalin A-induced hepatitis) by promoting regeneration of the liver. Indeed, exacerbation of hepatitis, monitored by liver damage and transaminase level, is observed in IL-22 neutralizing antibody treated mice and in *IL22*^{-/-} mice^{142, 143, 146}. It is also beneficial after partial hepatectomy¹⁴².

5.5. Pancreas

IL22ra1 is highly expressed in the pancreas, both in acinar and islet cells^{147, 148}. Within acinar cells, IL-22 induces the expression of Reg3 β , Reg3 γ and osteopontin, which are anti-microbial and pro-regenerative proteins. IL-22 also promotes proliferation and inhibits autophagy and apoptosis^{148, 149, 150}. This explains why acute pancreatitis severity is decreased upon injection of IL-22 or in *IL22* transgenic mice. IL-22 limits tissue damage, cell infiltrate and apoptosis of cells^{149, 150}. In contrast, IL-22 seems to play a deleterious role in the development of pancreatic tumors by promoting acinar to ductal metaplasia, tumor growth and the invasive capabilities of cells¹⁵¹. As in the islet cells, IL-22 induces Reg1 and Reg2 expression and proliferation, suggesting a possible role for IL-22 in type I diabetes¹⁵². However, injection of IL-22 in NOD mice (Non-Obese Diabetic mice that develop diabetes) does not prevent or delay the appearance of the disease¹⁵³.

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5.6. Kidney

Tubular epithelial cells of the kidney are target of IL-22 as they express the IL-22R α . IL-22 promotes the expression of pro-proliferative, survival and anti-apoptotic genes in these cells. Indeed, it was shown in acute kidney injury models that IL-22 favors the regeneration of the epithelium, with renal damage, serum urea and creatinin levels being reduced by IL-22 treatment. By contrast, IL-22 blockade results in impairment of regeneration^{154, 155}. More recently, *Il22ra1* expression was also shown in the glomerular compartment. However, IL-22 does not impact the course of glomerulonephritis positively nor negatively, as *Il22* deficient and WT mice display similar immune infiltrate, tissue damage and renal function impairment¹⁵⁶.

5.7. Brain

There is some evidence suggesting that IL-22 acts in the brain. First, *Il22* is constitutively expressed in the brain³⁷. Second, IL-22 acts on the rat neuronal cell line PC12, which IL-22 protects from cell death induced by serum deprivation¹⁵⁷. Also, IL-22 was shown to disrupt the blood-brain barrier (BBB) through action on BBB-endothelial cells that express *Il22ra1*. IL-22 enhances the permeability of the BBB for soluble molecules and immune cells¹⁵⁸. IL-22 is also increased in the serum from multiple sclerosis patients. Nevertheless, the role of IL-22 is still unclear as *Il22* deficient and WT mice are equally sensitive to experimental autoimmune encephalomyelitis^{158, 159}. Finally, glioblastoma cells express *Il22ra1*, with pro-proliferative and anti-apoptotic effects of IL-22 on these cells¹⁶⁰. *Il22*^{-/-} mice show increased survival and decreased tumors as compared to WT mice¹⁶¹.

6. Physiopathology of STAT3 : Link with IL-22 mediated diseases

STAT3 is an important transcription factor activated by several cytokines and growth factors, including IL-6, IL-10, IL-22, EGF, G-CSF, PDGF etc. It is involved in many cellular activities and promotes cell proliferation, survival, differentiation and migration. In fact, it is absolutely essential, notably in development since *Stat3* deficiency in mice leads to

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embryonic lethality¹⁶². Dysregulation of STAT3 activity, whether an increase or a decrease, causes disease (Figure 14). Indeed, as explained above, in normal conditions, STAT phosphorylation is a transient signal, the amplitude and duration of which are regulated and controlled. Constitutive and prolonged activation of STAT3 is observed in pathological conditions such as solid and blood tumors¹⁶³. Similarly, gain-of-function mutations of *STAT3* are associated with autoimmune diseases^{164, 165}. By contrast, loss-of-function mutations of *STAT3* were identified to be the cause of immunodeficiencies such as the autosomal dominant hyper IgE syndrome^{162, 166}.

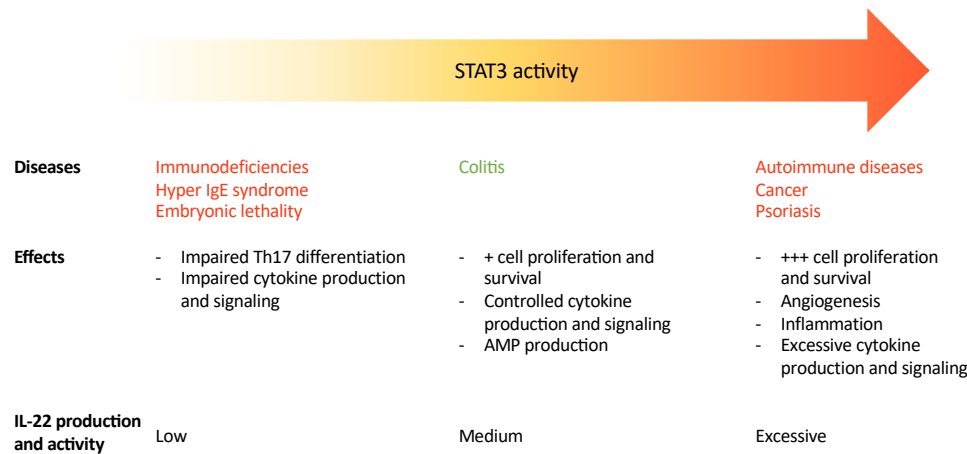


Figure 14: STAT3 activity and implication in diseases

While STAT3 activity is protective in contexts such as colitis (in green), an increase or an absence of STAT3 activity is linked with diseases (in red). The list of diseases and effects of STAT3 is not exhaustive and focuses mainly on diseases in which IL-22 was described to be involved. Indeed, IL-22 production and activity are also regulated by STAT3.

In cancers, STAT3 is over-activated, generally due to persistent stimulation by cytokines/growth factors or aberrant activation of kinases rather than mutations, although mutations in the SH2 domain of STAT3 are reported, in hepatocellular adenomas for example^{163, 167, 168, 169}. A study showed that injection of fibroblasts expressing constitutively active STAT3 (STAT3C) into nude mice induces the formation of tumors, demonstrating the

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oncogenic potential of STAT3¹⁷⁰. The activity of STAT3 in tumor cells but also in tumor environment, induced by IL-6 or VEGF for example, is crucial for tumor progression as it promotes inflammation and angiogenesis^{163, 171}. To draw a parallel with a type of cancer mentioned above, colon tumors are hardly present and smaller in mice with STAT3 deletion in intestinal epithelial cells (*Stat3^{lox/lox}* x *villin-Cre* mice) while hyperactivation of STAT3 (*gp130^{Y759F}* mice) favors tumor development^{172, 173}.

Phosphorylation of STAT3 is increased in inflammatory bowel disease but, in this case, has a protective effect, notably via the effects of IL-6 and IL-22^{174, 175}. Several mouse models were developed with STAT3 deficiency in a particular compartment. STAT3 deficiency in intestinal epithelial cells (*Stat3^{lox/lox}* x *villin-Cre* mice) increases the susceptibility of mice to DSS-induced colitis model and delayed intestinal wound healing^{105, 174}. IL-22 is thought to be the major phospho-STAT3 inducer in intestinal epithelial cells because *IL22^{-/-}* mice did not display a phospho-STAT3 signal in intestinal epithelium¹⁰⁵. Mice deficient in STAT3 in immune cells (CD4 and ROR γ T cells) are also more susceptible to the colitis model, suggesting that STAT3 activity in CD4 and ROR γ T+ cells protects from colitis. Interestingly, this correlates with a reduced level of IL-22^{124, 125}.

STAT3 was shown to be upregulated in psoriatic skin compared to healthy donors at the mRNA and protein level. STAT3 phosphorylation (Y705 and S727) is also increased in psoriatic skin^{70, 176, 177}. In fact, many cytokines involved in psoriasis pathogenesis signal via phosphorylated STAT3, making it a key player in various stages of psoriasis pathogenesis¹⁷⁸. As evidence of the deleterious effect of STAT3 in the skin and in psoriasis, the expression of constitutively active STAT3 in the basal layer of the epidermis (K5.*Stat3C* mice) is sufficient to promote spontaneous psoriatic lesions after two weeks¹⁷⁷. This mouse model demonstrates that STAT3 hyperactivation in keratinocytes is sufficient to trigger psoriasis pathogenesis. This can be explained by the fact that several cytokines act on keratinocytes to promote inflammation and proliferation via STAT3, notably IL-22^{70, 179}. In addition, STAT3 is involved in cytokine production. Indeed, STAT3 favors the production of IL-1 α by

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keratinocytes, leading to the activation of Langerhans cells and production of IL-23 by them¹⁸⁰. In turn, IL-23 drives the production of IL-17 and IL-22 by Th17 and $\gamma\delta$ T cells, via STAT3-dependent mechanisms, and ensures the amplification and maintenance of these cells¹⁸¹. Finally, IL-6-induced STAT3 is essential for Th17 differentiation as its deficiency prevents the development of Th17^{182, 183}.

In conclusion, STAT3 activity must be tightly balanced because its over-activation is associated with pathological effects. Therefore, targeting the STAT3 pathway could prove to be useful in cancer but also other diseases. One approach consists in blocking the kinase responsible for STAT3 phosphorylation. Several inhibitors of JAKs have been developed and some are currently used to treat cancer patients. This is notably the case for ruxolitinib, a selective and potent inhibitor of JAK1 and JAK2¹⁸⁴. JAK inhibitors have also been recently tested in clinical trials for psoriasis and show efficacy in decreasing disease score. The most well-studied in the context of psoriasis is tofacitinib, a JAK3/2/1 inhibitor^{184, 185}. Tremendous research efforts are being made to develop specific and potent inhibitors for STAT3. Several inhibitors are available: it includes peptides, peptidomimetics, non-peptide small molecules and oligodeoxynucleotide decoys. Most of these molecules target the SH2 domain of STAT3, critical for its activity. Nevertheless, none of these molecules is used in clinical practice¹⁸⁴. This is why there is still a need for new approaches and molecules to target STAT3.

3. BINDING PROTEINS

Targeting proteins with binding proteins is a powerful tool in biology. Various binding proteins have been developed, based on different scaffolds. Diversifying surface-exposed amino acids of the protein scaffold creates libraries of variants from which one protein able to bind a specific target can be selected. The first part of this chapter will briefly describe some binding proteins. Amongst binding proteins, monobodies are of great interest and are described in detail in the second part of this chapter.

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1. Protein scaffolds

Antibodies are the gold standard of binding protein. Nevertheless, their large size (150 kDa) and the presence of disulfide bonds are disadvantages in terms of tissue distribution and intracellular action. To overcome these problems, smaller variants of antibodies have been derived, such as Fab, scFv and nanobodies. Briefly, these variants are based on the part of antibody that recognizes the antigen and are devoid of the Fc portion. Fab comprises light chain ($V_L + C_L$) and heavy chain ($V_H + C_{H1}$) domains while scFv is composed of just two variable domains (V_L and V_H) fused with a flexible linker. As for nanobodies, they derive from camelid antibodies which are different from conventional antibodies as they do not contain light chains. Nanobodies correspond to the antigen-binding V_HH domain¹⁸⁶. In parallel, non-immunoglobulin scaffold-based binding proteins have also emerged, expanding the world of interacting proteins. Anticalin, affibody, DARPIn and monobody are based on different scaffolds, resulting in binding proteins with different structures (β -sandwich, β -barrel, α -helices...). These binders share several features such as small size, variable amino acids positions to enable the creation of libraries, stable scaffold, and low nM affinity, and they frequently lack disulfide bonds^{186, 187, 188, 189}. The characteristics of these proteins are summarized in table 1.

Name	Scaffold	Structure	Library	Disulfide bond	Size
Fab	Immunoglobulin	Ab light chain ($V_L + C_L$) and heavy chain ($V_H + C_{H1}$)	CDR loops	Yes	50 kDa
scFv	Immunoglobulin	Ab $V_L + V_H$ linked with a flexible peptide	CDR loops	Yes	25 kDa
Nanobody	Camelid Immunoglobulin	Single variable domain (V_HH)	CDR loops	Yes	12-15 kDa
Affibody	Z domain of protein A of <i>staphylococcus aureus</i>	Bundle of 3 α -helices	13 residues across two helices	No	6,5 kDa
Anticalin protein	Lipocalin	β -barrel with a long α -helix	Loops connecting β -strands	Yes	16-19 kDa
DARPIn	Ankyrin repeat proteins	2-3 $\alpha 2/ \beta 2$ repeats (2 antiparallel α -helices linked with a loop connected to another unit with a β -turn)	6 residues of the β -turn and the first α -helix of each repeat	No	18 kDa
Monobody	Human 10 th Fibronectin III	β -sandwich of 7 β -strands	a) Upper connecting loops (BC, DE, FG) b) Upper loop FG, lower loop CD and β -strands C and D	No	10 kDa

Table 1: Ig-derived and non Ig-derived scaffold protein, their structure and characteristics

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2. Monobody

2.1. Structure

Developed in 1998 by the team of S. Koide, monobodies are engineered binding proteins derived from a fibronectin III (FN3) domain of human fibronectin¹⁹⁰. FN3 are naturally present in about 2 % of animal proteins. Although monobodies are non-antibody scaffold molecules, their structure is similar to antibody variable domains. Indeed, the FN3 domain is formed of a β -sandwich composed of 7 β -strands (versus 9 for immunoglobulin); the two β -sheets are composed of β -strands A/B/E and C/D/F/G respectively (Figure 15). It contains loops joining β -strands, named according to the β -strands they connect, that are located above and below the β -sandwich. These loops are comparable to the Complementarity Determining Region (CDR) of variable regions of Ig. Contrary to Ig, FN3 domains do not contain a disulfide bond, facilitating their action in the cytoplasm and robust expression in bacteria. Monobodies are small proteins of about 10 kDa (95 amino acids), more than ten times smaller than antibodies, which enables interaction with smaller targets or less accessible sites. It is also believed that this improves their tissue distribution *in vivo*. The small size of monobodies reduces half-life within the organism, however, which could be limiting^{188, 190, 191, 192}.

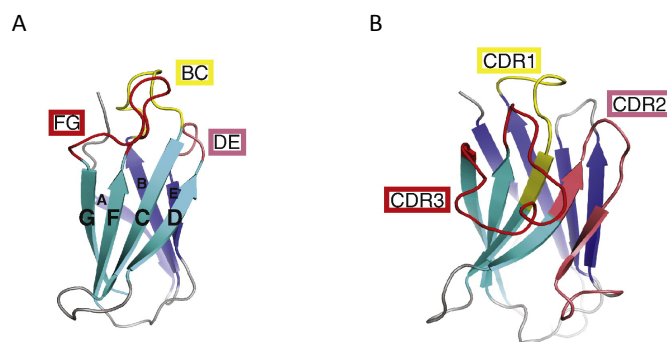


Figure 15: Comparison of FN3 and V_HH domain.

Both (A) FN3 and (B) V_HH domains are composed of two β -sheets forming a β -sandwich. The upper loops of the FN3 correspond to the CDR of V_HH. β -strands and loops of the FN3 are labelled¹⁹³.

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2.2. Diversification and selection

Two kinds of libraries have been developed by diversification of the monobody scaffold (between 10 and 20 residues). In the first, called “loops library”, only amino acids of the upper loops (BC, DE and FG) are diversified (Figure 16A). The second library, called “side and loops”, is diversified at the loops FG and CD and also at the β -strands C and D (Figure 16B)^{190, 193}. The loops library generates monobodies binding concave surfaces, while side and loops monobodies instead tend to bind convex surfaces¹⁹³. Libraries are screened by yeast- and phage-display to obtain potent and selective monobodies against one specific target. The affinity of monobodies is high with a K_d of low nM^{188, 191, 193}.

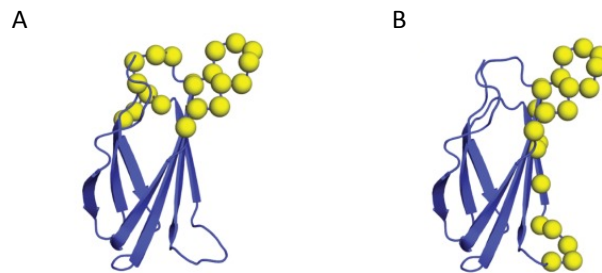


Figure 16: “loops” and “side and loops” libraries.

Three-dimensional structures of monobodies with, in yellow circles, amino acids that are diversified in the loops (A) and side and loops (B) libraries to generate binding proteins¹⁹².

2.3. *In vitro* and *in vivo* applications

The first application of monobody was in interfering selectively with a target function. Many examples of inhibitory monobodies can be found in the literature. To cite only a few, monobodies targeting BCR-ABL, Ras or Src have been developed, showing effective inhibition in *in vitro* experiments. The *in vivo* potential of monobodies has also been demonstrated. In a mouse model of leukemia, for example, engraftment of cells expressing WDR5-targeting monobody in lethally irradiated mice inhibits progression of the disease¹⁹⁴. Interestingly, the efficacy of monobody can be increased by its fusion to the von

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Hippel-Lindau (VHL) protein, leading to specific degradation of the target. The VHL protein is the substrate receptor for the Cullin2/RBX1 E3 ubiquitin ligase complex, which implies that, upon expression in cell, the monobody-VHL fusion will force the ubiquitination and the subsequent degradation of the target¹⁹⁵. There are other applications besides inhibition for monobodies: for example, when used as chaperones, monobodies improve and facilitate crystallization¹⁹⁶. Moreover, monobodies can be fused to a fluorescent protein to study localization of the target in cells¹⁹⁷.

The delivery of monobodies inside cells is still a challenge. Several strategies have been proposed, including fusion to cell-penetrating peptides (CPP) or bacterial toxin subunits as well as the use of nanocarriers. CPPs are short peptides (5 to 40 amino acids) that are capable of internalization. It is the amino acid composition of CPPs that enables them to cross the lipid membrane. Most depend on positively charged amino acids (arginines are the most effective) that can interact with negatively charged cell-surface glycoproteins. Another class is composed of highly amphipathic CPPs, and a third category shares the two properties. Two types of transport are described for CPPs: direct penetration and endocytosis (comprising micropinocytosis). Thereby, they can mediate the intracellular uptake of molecules¹⁹⁸. A second approach is to fuse the monobody to the catalytic and binding/translocation domains of a bacterial toxin, exotoxin A from *Pseudomonas aeruginosa* for example. The toxin binds its receptor, resulting in internalization of the monobody by endocytosis, followed by escape from the endosome-lysosome pathway or the retrograde trafficking¹⁹⁹. Finally, monobodies can be packed in liposomes or nanoparticles such as polymer-based or gold nanoparticles²⁰⁰. These various approaches can be combined to improve intracellular delivery efficacy.

AIM OF THE STUDY

AIM OF THE STUDY

This study aims to better characterize the alternative pathway induced by IL-22 to activate STAT3. On the one hand, we studied the presence of this pathway in an endogenous context *in vitro* and *in vivo*, as well as its role in inflammatory diseases. On the other hand, we used monoclonal antibodies to attempt to specifically block this alternative pathway.

First study

In the first study, we took advantage of CRISPR/Cas9 technology to truncate the C-terminal part of IL-22R α . The first objective was to define whether the alternative activation of STAT3 takes place in cells endogenously expressing the receptor, in contrast to the overexpression performed in the original study. Second, the alternative signaling of IL-22 was studied *in vivo* in mice expressing a truncated IL-22R α . Finally, the impact of the alternative pathway induced by IL-22 was evaluated in inflammatory diseases in mice. For this purpose, we used the imiquimod-induced psoriasis model and the *Citrobacter rodentium*-induced colitis model and compared the evolution of disease between WT, *Il22ra1* deficient and *Il22ra1* truncated mice.

Second study

In a second phase, we analyzed the effect of STAT3-targeting monoclonal antibodies on IL-22 signaling. The overall goal was to establish whether it was possible to specifically target the alternative activation of STAT3 induced by IL-22. First, we analyzed the effect of the monoclonal antibody on cells expressing mutant forms of IL-22R α and dissected the mechanism by which it impacts IL-22 alternative signaling. Second, we studied whether the monoclonal antibody affected cytokine-induced STAT3 phosphorylation in general. Finally, the effect of the monoclonal antibodies on STAT3 transcriptional activity was assessed using luciferase assays.

RESULTS – PART 1

Results – part 1

RESULTS

Preamble

The original study showing that IL-22 induces an alternative phosphotyrosine independent activation of STAT3 was performed in BW5147 cells stably transfected with mutant forms of IL-22R α ¹. These are not cells that endogenously express the IL-22R α so the first aim of my thesis was to study the alternative activation of STAT3 in a more physiological context. For this purpose, we used the CRISPR/Cas9 technology to mutate the C-terminal part of IL-22R α , endogenously expressed, and analyzed the impact on IL-22 signaling. Therefore, a guide RNA (gRNA) targeting the region downstream of the last tyrosine of IL-22R α (Tyr⁴⁹¹) was designed (Fig. 1). Briefly, the gRNA directs the nuclease Cas9 at the DNA site of interest and the Cas9 is responsible for DNA double-stranded break (DSB). Of note, the sequence recognized by the gRNA must be followed by a PAM sequence (NGG) to ensure the function of the Cas9. The DSB will be repaired by the NHEJ (Non-Homologous End-Joining) repair pathway, which leads to insertion or deletion of nucleotides, a shift in the open reading frame (ORF) and the early onset of a stop codon². The objective was to prevent the alternative activation of STAT3 by mutating the C-ter part of IL-22R α without affecting the classical pathway, depending on receptor tyrosines.



Figure 1: CRISPR/Cas9 design in mouse (A) and human (B) IL-22R α .

gRNA.A and gRNA.C were designed to target mouse (A) or human (B) *il22ra1*. Sequence targeted by the gRNA is marked in color with, in bold, the PAM sequence. Dotted line represents the theoretical DSB induced by the Cas9, 3 nucleotides upstream of the PAM. The last tyrosine (Tyr⁴⁹¹) is highlighted in bold.

Results – part 1

This CRISPR/Cas9 strategy was validated in various cell lines (MES13, A549, Colo205 and H4IIE). For this preamble, experiments performed in MES13, a murine mesangial cell line, and in A549, a human pulmonary cancer cell line, are presented. After transfection of the vector coding for the gRNA, the Cas9 and the GFP, GFP⁺ cells were sorted by FACS. Cells were then cloned and sequenced to define mutations induced in the *IL22ra1* by the CRISPR/Cas9. In both cell lines, the CRISPR/Cas9 enabled the introduction of mutations in the C-ter part of IL-22R α . These mutations lead either to a new C-terminal sequence due to a shift in the ORF or to an in-frame deletion of some amino acids (table 1). It is important to mention that, as planned, none of the cytoplasmic tyrosines of IL-22R α was mutated by the CRISPR/Cas9 and, thereby, it should not affect the classical pathway.

	Clone	Mutation (DNA)	Mutation (protein)	Protein size
Mouse receptor (581 aa)	MES 13 A+2	21 bp deletion	Deletion aa de 503 to 509	574 aa
		7 bp deletion	Frameshift from aa 504	572 aa
	MES13 A+4	1 bp insertion	Frameshift from aa 504	541 aa
	MES13 A+7	4 bp deletion	Mutation aa 505	580 aa
		+ 1 bp insertion	+ Deletion aa 504	
Human receptor (574 aa)	A549 C+5	1 bp insertion	Frameshift from aa 504	541 aa
		13 bp deletion	Frameshift from aa 503	561 aa
	A549 C+6	1 bp insertion	Frameshift from aa 507	541 aa
		14 bp deletion	Frameshift from aa 499	499 aa
		1 bp insertion	Frameshift from aa 505	541 aa

Table 1: summary table of mutations obtained in MES13 and A549 following CRISPR/Cas9.

Cells were transfected with the PX458 vector, coding for gRNA, Cas9 and GFP. Two days later, GFP⁺ cells were sorted by FACS. Cells were cloned after 7 days of culture. Genomic DNA was extracted and sequenced after cloning of the *IL22ra1* in pGEM-T easy vector to be able to discriminate mutations of the different alleles.

Once the various clones were obtained and sequenced, the signaling of IL-22 was assessed. As shown in figure 2, the mutation of the IL-22R α lead to a strong decrease of STAT3 phosphorylation as compared to the parental cells in both MES13 and A549 cell lines. Strikingly, STAT1 and STAT5 phosphorylation were also impacted by the mutation of the

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C-terminal tail of IL-22R α , contrary to what was obtained in BW5147 cells. Hence, these results suggest that most of IL-22 signaling requires the C-ter part of IL-22R α since mutation of the receptor in MES13 and A549 almost completely abrogated STAT1, STAT3 and STAT5 phosphorylation. Unfortunately, we were not able to analyze the expression of the receptor by flow cytometry in MES13 or A549 because of a lack of sensitivity. However, it was analyzed by western-blot in A549 and we observed that the protein level of the IL-22R α was similar between parental and mutant cells (Figure 2B).

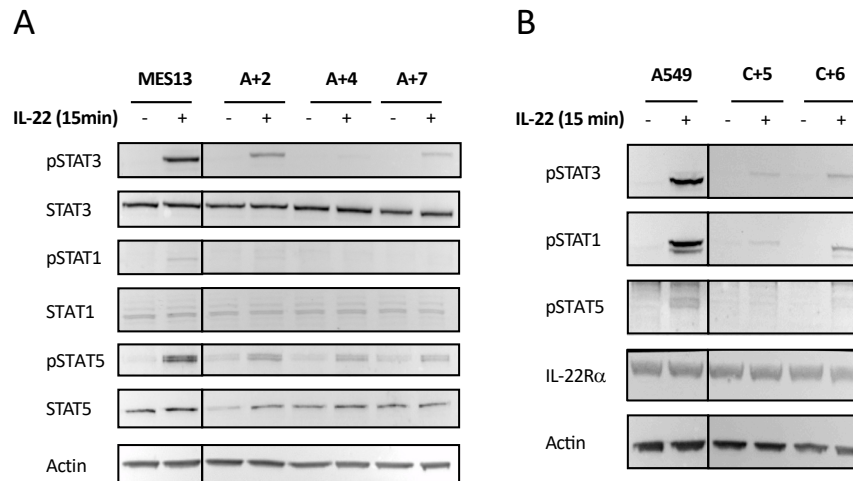


Figure 2: IL-22 signaling in MES13 and A549.

Parental MES13 or A549 and clones were stimulated with control medium or mouse (A) or human (B) IL-22 for 15 minutes (500 ng/ml). After stimulation, cells were lysed in O'Farrell buffer and western-blot against phospho- or total- STAT were performed. Blots are representative of 3 experiments.

We decided to pursue with the same strategy to create mice with an IL-22R α C-ter mutated. Amongst mutations we obtained, two mouse strains were kept. The first strain, called *GCC*, leads to a +2 frameshift and a long new sequence of 71 amino acids before introducing a STOP codon. The second strain, *Δ GA*, leads to a +1 frameshift and a shorter C-ter of 37 amino acids before the introduction of a STOP codon (Fig. 3). No tyrosines were added in

Results – part 1

the new C-ter tail in $GCC^{mut/mut}$ nor in $\Delta GA^{mut/mut}$. Both strains were used in the project since we postulated that, if adding a new C-ter tail would perturb the IL-22R α folding, interaction or function, it should not be the case for both mutants.



Figure 3: mouse strains obtained with CRISPR/Cas9.

Two mouse strains were obtained: GCC and ΔGA . Mutation of both strains begins at the amino acid 503 but leads to a different frameshift and, thereby, a different new C-terminal sequence (in green or blue). The red dotted line represents the theoretical DSB induced by the Cas9.

In a first time, IL-22 signaling was studied *ex vivo* in epidermal cells. After IL-22 stimulation, hardly any STAT3 phosphorylation was observed by western-blot in cells from $\Delta GA^{mut/mut}$ mice or $GCC^{mut/mut}$ as compared to WT mice (Fig. 4A and data not shown). As observed *in vitro*, STAT1 and STAT5 phosphorylation were also reduced in cells from mutated mice. Moreover, the expression of *Socs3*, a gene regulated by STAT3, was not induced after IL-22 stimulation in $\Delta GA^{mut/mut}$ or $GCC^{mut/mut}$ epidermis cells (Fig. 4B and data not shown). Therefore, mutation of the C-ter part of IL-22R α in mice prevents IL-22 signaling in epidermal cells. These results were also confirmed in other organs - ileum, liver and lungs - by analyzing the expression of *Socs3* after *i.p.* injection of IL-22 or PBS (Figure 4C and data not shown). Indeed, in the ileum, the induction of expression of *Socs3* was reduced, but present, in mutated mice. In the liver and the lungs, the induction of *Socs3* after IL-22 injection was totally lost.

Results – part 1

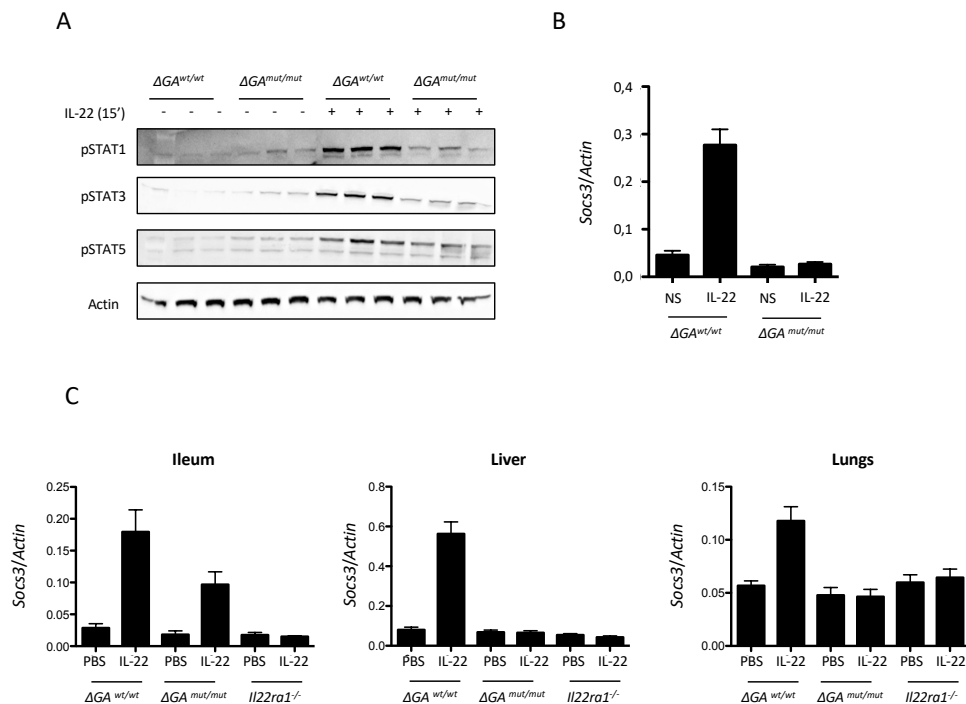


Figure 4: IL-22 signaling in epidermal cells.

(A) Western-blot analysis of IL-22 signaling in epidermal cells. After 24 hours of rest, cells were stimulated for 15 minutes with control medium or IL-22 (500 ng/ml) and lysed in O'Farrell lysis buffer. Blots representative of two independent experiments (n = 3 mice/group in each experiment). (B) *Socs3* expression was analyzed in epidermal cells after 60 minutes of IL-22 stimulation (500 ng/ml). *Socs3* gene expression is normalized against β -Actin. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = 3-5 mice/group in each experiment). (C) Ileum, liver and lungs were analyzed by RT-qPCR. 1 hour after PBS or IL-22 *i.p.* injection, organs were dissected and RNA was extracted. *Socs3* gene expression is normalized against β -Actin. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = 3-5 mice/group in each experiment).

As the impact on *Socs3* expression varies depending on the organ, we wondered if the expression of the receptor was similar between WT and mutated mice. We analyzed the expression of IL-22R α by RT-qPCR and by flow cytometry as, in the meantime, the IL-22R α staining was improved. In RT-qPCR analysis, *Il22ra1* seemed more expressed in the ileum as compared to the skin and this expression was similar between WT and $\Delta GA^{mut/mut}$ or $GCC^{mut/mut}$ mice (Figure 5A and C and data not shown). In contrast, in flow cytometry, the

Results – part 1

IL-22R α was detected at the surface of epidermal and intestinal epithelial cells of WT but not of mutated mice (Figure 5B and D and data not shown).

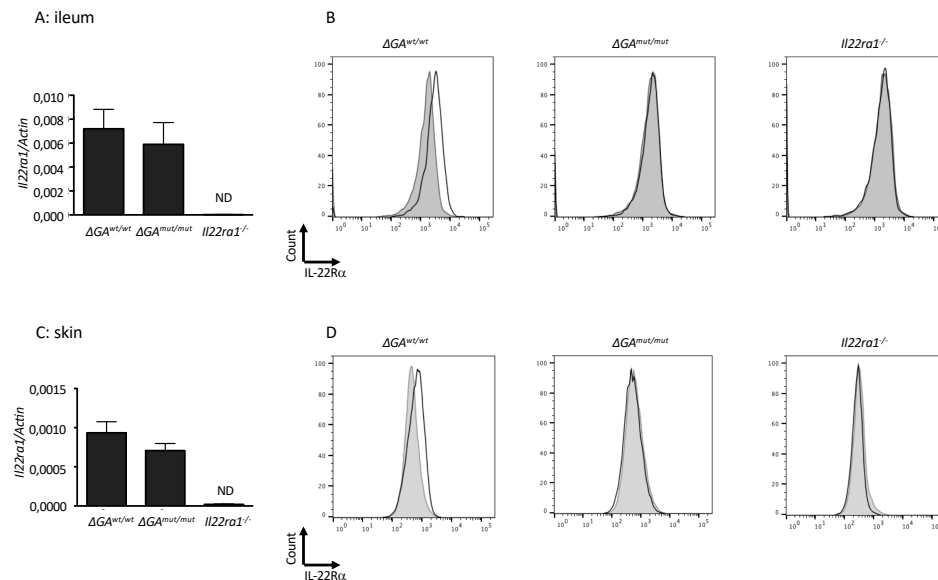


Figure 5: analysis of the expression of IL-22R α in the skin and the ileum.

(A and C) *Il22ra1* expression was analyzed by RT-qPCR in ileum and skin. *Il22ra1* gene expression is normalized against β -actin. Data are shown as mean $\pm$ SEM and are pooled from two independent experiments (n = 3 mice/group in each experiment). (B and D) Flow cytometry analysis of IL-22R α in intestinal epithelial (B) and epidermal cells (D). Cells were gated on living CD45⁻ cells and IL-22R α staining was analyzed in EpCAM⁺ (B) or CK10⁺ (D) cells. *Il22ra1*^{-/-} mice were used as negative control. Grey curve: no staining, empty curve: IL-22R α staining. Graph representative of three experiments performed in duplicates.

In conclusion, mutating the C-terminal part of IL-22R α affected the cell surface expression of IL-22R α . In contrast, the RNA expression was not affected. It suggests that the mutation impaired correct folding of the receptor, transport along the secretory pathway, half-life... Therefore, as the receptor is not correctly expressed at the surface, results about IL-22 signaling and impact on inflammatory models cannot be tackled with these mouse models. We generated a new receptor mutant, using CRISPR/Cas9 HDR (homology directed repair) approach to truncate the receptor instead of mutating it. The results obtained for this project are presented in the second part of this chapter.

Results – part 1

1. Dumoutier, L., de Meester, C., Tavernier, J. & Renauld, J.C. New activation modus of STAT3: a tyrosine-less region of the interleukin-22 receptor recruits STAT3 by interacting with its coiled-coil domain. *J Biol Chem* **284**, 26377-26384 (2009).
2. Sander, J.D. & Joung, J.K. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* **32**, 347-355 (2014).

RESULTS – PART 2

Results – part 2

Phosphotyrosine-independent STAT3 activation induced by IL-22 is involved in skin inflammation

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Abstract

Interleukin-22 is a cytokine with deleterious effects in psoriasis but beneficial in colitis. IL-22 signaling relies on two pathways: IL-22R α tyrosines-dependent and -independent. The C-ter region of IL-22R α , devoid of tyrosines, was shown to be required for this second signaling. In the present study, we investigated the role of the C-ter part of IL-22R α on IL-22 signaling *in vitro* and *in vivo*. We also dissected the implication of the IL-22R α and its C-ter part in skin and gut inflammatory diseases. Our results demonstrate that the tyrosine-independent activation of STAT3 is important for IL-22 signaling in the skin and in the imiquimod-induced model of psoriasis. In contrast, this alternative activation of STAT3 plays a secondary role in the gut and in the *Citrobacter rodentium* model of colitis. As for the IL-22R α , its deficiency partially protects mice from the imiquimod model and strongly affects mice in the *Citrobacter* model.

Results – part 2

Introduction

IL-22 is a member of the IL-10 related cytokines. This cytokine plays different functions during inflammatory processes, depending on the immune context. It exerts essential functions in barrier defense, tissue repair and homeostasis in various organs including the skin, gut, lungs and liver. Nevertheless, continuous and/or exacerbated action of IL-22 induces tissue inflammation, leading to immune disorders such as psoriasis. In inflammatory context, IL-22 is largely produced by T cells, including Th17, Th22 and $\gamma\delta$ T cells as well as by innate lymphoid cells type III (ILC3)¹. To signal, IL-22 acts on a receptor composed of two chains: IL-22R α and IL-10R β . While IL-10R β is ubiquitously expressed, IL-22R α is mainly expressed by epithelial cells, restricting cells responding to IL-22. Once bounded to its receptor, IL-22 induces the JAK-STAT and, to a lesser extent, the MAPK and AKT pathways².

JAK1 and TYK2, associated respectively with IL-22R α and IL-10R β , are activated upon IL-22 binding². In turn, these tyrosine kinases will phosphorylate other signaling proteins, including STAT-1, -3 and -5. Each STAT is composed of several conserved domains. The N-terminal domain of STAT proteins is involved in nuclear translocation and plays a role in cooperative DNA binding³. The coiled-coil domain, composed of 4 α helices, allows for interaction with other proteins and is involved in nuclear translocation^{3, 4, 5}. The DNA binding domain is essential for STAT to function as a transcription factor as it enables the binding to specific DNA sequences. The linker domain enables the correct structure of adjacent domains. The SH2 domain is required to bind to phosphotyrosines of the receptor as well as for the dimerization of two STATs. Finally, the tyrosine that is phosphorylated by JAK is located between the SH2 and the transactivation domains³. In the classical JAK-STAT pathway, the binding of the cytokine to its receptor induces the transactivation of the JAKs, pre-associated with the receptor. Then, JAK proteins phosphorylate tyrosine residues of the receptor that are recognized as docking sites by SH2 domain of STAT and phosphorylate STATs, inducing their dimerization and migration into the nucleus where they modulate gene expression. As mentioned, the classical JAK-STAT pathway requires the SH2 domain of STAT as well as tyrosines of the receptor³. Nevertheless, in some cases, STAT activation

Results – part 2

occurs in a phosphotyrosine-independent manner^{6, 7, 8, 9, 10}. This is true for the IL-22R α . Indeed, it was shown that STAT3 phosphorylation, but not STAT1 or STAT5, can arise in a receptor wherein all tyrosines are mutated to phenylalanine and this alternative activation requires the C-terminal part (C-ter, aa 492-574) of IL-22R α as the truncation of the Y->F mutant abolishes STAT3 activation. Our previous results demonstrated that STAT3, via its coiled-coil domain, is constitutively associated with the C-ter part of the IL-22R α , devoid of tyrosines¹¹.

Phosphotyrosines-dependent and -independent pathways lead to a massive activation of STAT3, which is involved in the vast majority of IL-22 functions. IL-22 displays beneficial effects as it promotes cell proliferation as well as antimicrobial peptides and chemokines production. In the case of colitis, IL-22 enables regeneration of the epithelium and the defense against pathogens. Studies have shown that, in the *Citrobacter rodentium*-induced model of colitis, *Il22* deficient mice exhibit increased epithelial damage, submucosal inflammation and colonic crypt bacteria penetration, leading to a more prominent weight loss and decreased survival rates as compared to WT mice. This phenotype is linked with a reduction of antimicrobial proteins expression (RegIII β , RegIII γ , S100A8 and S100A9), crucial for the protective effect of IL-22^{12, 13}. Nevertheless, in the case of an excess of IL-22, in an inflammatory environment, this cytokine becomes deleterious and was described being involved in diseases such as psoriasis^{14, 15, 16, 17, 18, 19}. Firstly, *Il22* transgenic mice present neonatal mortality and spontaneous typical psoriatic lesions (acanthosis and hyperkeratosis)¹⁴. Moreover, *Il22*^{-/-} or anti-IL-22 blocking antibody treated mice are partially protected in the imiquimod-induced model of psoriasis. Indeed, they exhibit reduced acanthosis, correlated with a decrease in neutrophils along with chemokines and differentiation genes expression¹⁵. The protection is also obtained in the IL-23 induced acanthosis and the adoptive transfer model in *Il22* deficient mice or with anti-IL-22 blocking antibody^{4, 18}.

Results – part 2

STAT3 was shown to play a considerable role in the development of psoriasis. Indeed, transgenic mice with expression of a constitutively active STAT3 in the basal layer of keratinocytes (K5.*STAT3C* mice) develop skin lesions that mimic psoriasis with hyperkeratosis, parakeratosis, defect in keratinocytes differentiation and hyperproliferation. STAT3 is also hyperactivated in psoriatic plaques from patients²⁰. Moreover, the deleterious effects of IL-22 observed in psoriasis are mediated by STAT3¹⁴. Therefore, preventing the alternative activation of IL-22 could be a targeted therapy in psoriasis. Such therapy might lead to a decreased activation of STAT3, blocking deleterious effects of IL-22 but leaving intact the beneficial ones. In this context, we investigated the implication of the C-ter part of IL-22R α in IL-22 signaling *in vivo* and in the development of IL-22 mediated inflammatory models. We could show that the alternative pathway plays a major role in IL-22 signaling in the skin and in the liver but only minor in the ileum. Mice deficient in *Il22ra1* or with a truncated receptor were partially protected from the imiquimod model. In contrast, only *Il22ra1* deficient mice were sensitive to the colitis model induced by *Citrobacter rodentium*.

Results

- Truncation of endogenously expressed IL-22R α decreases STAT3 phosphorylation

The C-ter tail of IL-22R α was shown to be essential in IL-22 signaling by the use of IL-22R α mutants expressed in BW5147 cells¹¹. Nevertheless, overexpression of the receptor could have impacted the results by forcing a signaling, even in the absence of tyrosines. To circumvent this issue, CRISPR/Cas9 approach was taken to truncate the receptor in a murine mesangial cell line (MES13) endogenously expressing IL-22R α . A STOP codon was inserted at position 500, 9 residues after the last tyrosine (Tyr491), creating the Δ Cter mutant cells. This enables the interruption, if it exists, of the alternative activation of STAT3. Of note, IL-22R α tyrosines were not mutated (Fig 1A). Truncation of the IL-22R α C-ter tail in MES13 lead to a decrease of STAT3 phosphorylation, demonstrating the role of the IL-22R α C-ter on STAT3 signaling (Fig 1B and Suppl. Fig. 1A). This difference was not due to a decrease of IL-22R α expression between MES13 and Δ Cter cells, as shown by flow cytometry (Suppl.

Results – part 2

Fig. 1B). Surprisingly, STAT5 phosphorylation was increased in these cells, which could be explained by a compensatory phenomenon, while STAT1 phosphorylation remained stable. As control, both WT and truncated cells were able to activate JAK1 to a similar extent (Fig 1B and Suppl. Fig. 1A).

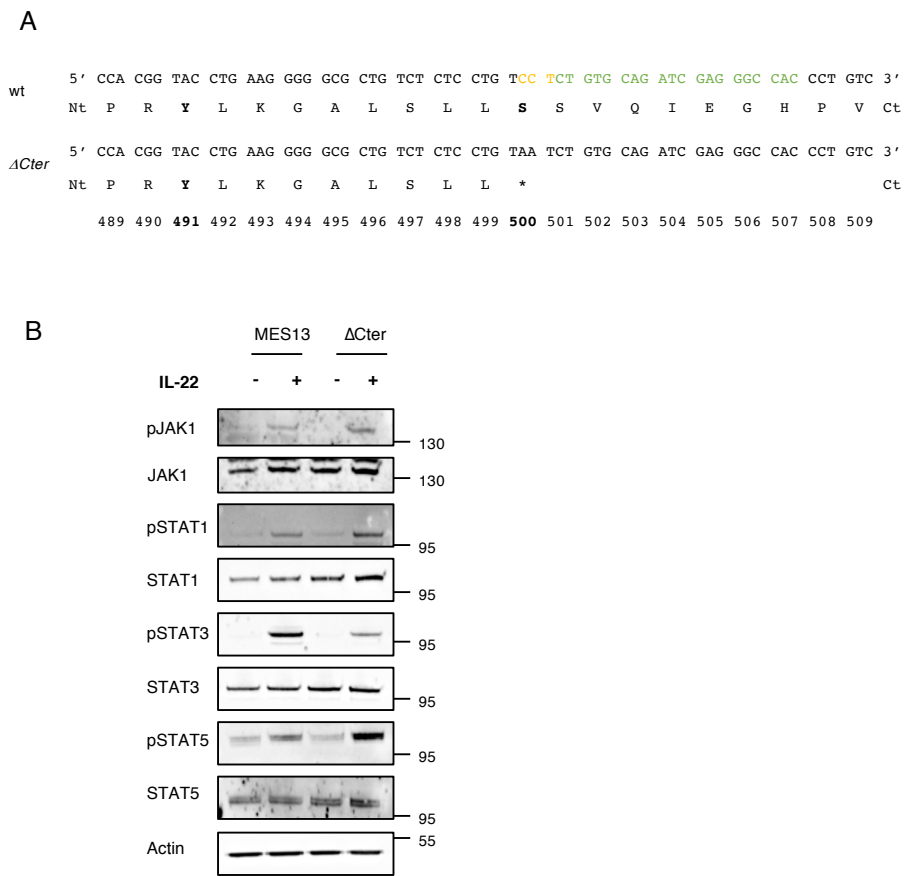


Figure 1: Truncation of endogenously expressed IL-22R α decreases STAT3 phosphorylation.
(A) Sequence of mouse IL-22R α (above: WT; below: Δ Cter) with in green the sequence targeted by the gRNA and in orange the PAM sequence. The last tyrosine (Tyr⁴⁹¹) and the serine mutated in stop are in bolt. (B) Western-blot analysis of IL-22 signaling in MES13 parental and Δ Cter. Cells were stimulated for 15 minutes with control medium or IL-22 (500 ng/ml) and lysed in a O'Farrel buffer. Blots representative of three independent experiments. Two clones Δ Cter were obtained with similar results in western-blot.

Results – part 2

- *The C-ter of IL-22R α plays a major role in IL-22 signaling in the skin but only minor in the gut*

In order to study the alternative activation of STAT3 *in vivo*, mice with a truncated receptor were generated using the same CRISPR design as for the MES13 cell line. Here again, the C-ter part of IL-22R α was truncated to prevent the pre-association of STAT3 with this region and to establish its relevance in IL-22 signaling *in vivo*. IL-22R α being expressed in several organs, IL-22 signaling was investigated in the skin, gut, lungs, liver and kidneys. First, after extraction of epidermal cells from ear skin, cells were stimulated *ex vivo* to analyze IL-22 signaling by western-blot. Reduced pSTAT3 levels were observed after truncation of the C-ter of IL-22R α ($\Delta Cter^{mut/mut}$ mice) as compared to littermate controls ($\Delta Cter^{wt/wt}$ mice) (Fig. 2A and Suppl. Fig. 2). As observed in MES13 cells, the phosphorylation of STAT5, but not STAT1, was slightly increased. JAK1 phosphorylation levels were similar between mutated and WT mice. In addition, the induction of *Socs3*, a STAT3-dependent gene, was weaker in epidermal cells from $\Delta Cter^{mut/mut}$ mice after IL-22 stimulation as compared to littermate controls (Fig 2B). Hence, in the epidermis, the truncation of IL-22R α partially represses STAT3 activation mediated by IL-22.

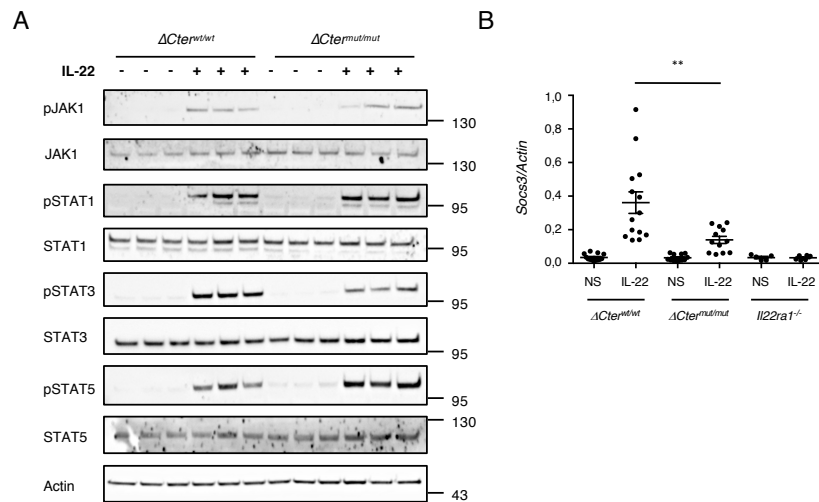


Figure 2: The C-ter of IL-22R α is involved in IL-22 signaling in the skin.

(A) Western-blot analysis of IL-22 signaling in epidermal cells. After 24 hours of rest, cells were stimulated for 15 minutes with control medium or IL-22 (500 ng/ml) and lysed in NP40 lysis buffer. 2

Results – part 2

µg or 10 µg of total proteins were charged on gels. Blots representative of three independent experiments (n = 3 mice / group in each experiment). (B) *Socs3* expression was analyzed in epidermal cells after 60 minutes of IL-22 stimulation (500 ng/ml). *Socs3* gene expression is normalized against β -Actin. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = 3-5 mice/group in each experiment). Statistical analyses: Mann Whitney (**p< 0,01).

Concerning other organs, IL-22 signaling was analyzed after *i.p.* injection of IL-22^{*1} by western-blot and RT-qPCR. Western-blot analysis on the distal ileum resulted in similar STAT3 phosphorylation level between $\Delta Cter^{mut/mut}$ and $\Delta Cter^{wt/wt}$ mice (Fig. 3A). Same results were obtained by immunohistofluorescence on ileum sections (Suppl. Fig. 3E). However, we observed that the expression of *Socs3* was reduced, but still induced, in $\Delta Cter^{mut/mut}$ mice as compared to WT mice (Fig. 3B). We also noted that the basal expression of *Reg3b* and *Fut2*, two antibacterial peptides whose expression is regulated by IL-22, was lower in $Cter^{mut/mut}$ mice as compared to $\Delta Cter^{wt/wt}$ mice. Interestingly, the level of induction of these three genes was similar between $Cter^{mut/mut}$ mice as compared to $\Delta Cter^{wt/wt}$ mice in the ileum as well and in the colon (Suppl. Fig. 3 A and D). These results suggest that the alternative pathway plays a role in the presence of physiological level of IL-22 in the gut, but not after the injection of exogenous IL-22. In other organs (liver, lungs, and kidneys), the induction of *Socs3* expression after IL-22 injection was reduced (Fig 3C, D, Suppl. Fig. 3 B and C + data not shown). The expression of *Saa1-2* in the liver was also impacted by the C-ter deletion (Fig. 3C). It correlated with a strong decrease of STAT3 phosphorylation in the liver of $\Delta Cter^{mut/mut}$ mice as compared to WT mice (Suppl. Fig. 3F). Of note, IL-22R α expression, at the gene and protein level, was similar, if not increased, in $\Delta Cter^{mut/mut}$ mice as compared to littermate controls (Suppl. Fig. 4A, B). Taking together, these data demonstrate that the C-ter of IL-22R α plays an important role in IL-22 signaling, and more particularly in STAT3 activation, *in vivo* as well. However, the level of involvement of the alternative pathway seems to depend on the organ. Hence, this region could turn out to be crucial in IL-22 dependent inflammatory models.

¹ Models used in this study are marked with * and are briefly described in the annex section.

Results – part 2

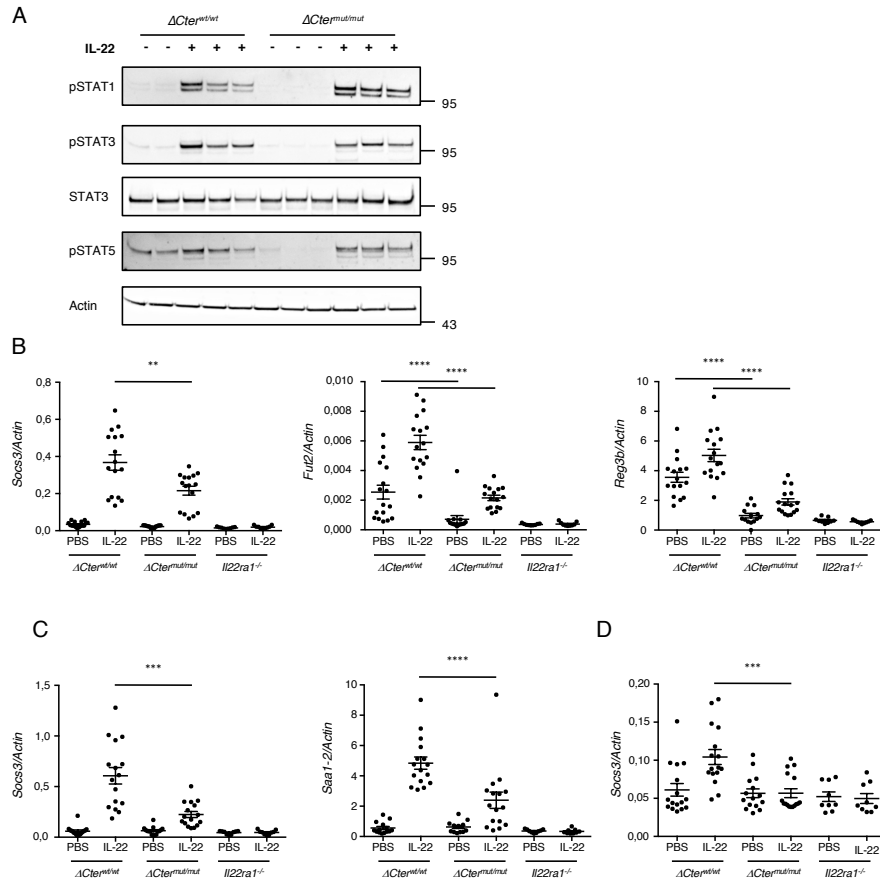


Figure 3: The C-ter of IL-22R α is involved in IL-22 activities.

(A) Western-blot analysis of IL-22 signaling in the ileum. 30 minutes after PBS or IL-22 injection, distal ileum was cut and freeze. Proteins were extracted and charged on gels. (B) Ileum, (C) liver and (D) lungs were analyzed by RT-qPCR. 1 hour after PBS or IL-22 injection, organs were dissected and RNA was extracted. *Socs3*, *Fut2*, *Reg3g* and *Saa1-2* gene expression are normalized against β -Actin. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = at least 5 mice/group in each experiment). Statistical analyses: Mann Whitney (**p< 0,01, ***<0,001 and ****<0.0001).

Results – part 2

- *Deficiency and truncation in IL-22R α protects in a psoriasis model*

First, we took advantage of the imiquimod-induced psoriasis model* to define the role of IL-22R α and its alternative activation of STAT3 in skin inflammation. Indeed, both IL-22 and STAT3 are known to be deleterious in this model. Mice were treated for 7 consecutive days with Aldara® and several parameters were analyzed and compared between B6 and *Il22ra1*^{-/-} or Δ Cter^{wt/wt} and Δ Cter^{mut/mut}. As shown in Fig. 4A, *Il22ra1*^{-/-} mice were protected from the ear thickening as compared to B6 mice. Δ Cter^{mut/mut} mice also displayed a significant reduced ear thickening as compared to WT littermate mice (Fig 4A). The protection of both *Il22ra1*^{-/-} and Δ Cter^{mut/mut} was also observed at the histological level by measuring the thickness of the epidermis and the number of crusts and pustules along the tissue (Fig 4B, C, D). As shown in a previous study, several genes were upregulated in this model in an IL-22 dependent manner, notably *Cxcl3* and *S100a8*¹⁵. Both genes were significantly less induced in *Il22ra1*^{-/-} and Δ Cter^{mut/mut} than in WT mice after imiquimod treatment (Fig 4E, F). Finally, the decrease of *Cxcl3* expression correlated with the decrease of neutrophils in the dermis and epidermis (Fig 4G, H). It is noteworthy that the *Il22ra1*^{-/-} mice were more protected than the Δ Cter^{mut/mut}, suggesting that the alternative activation of STAT3 is responsible for a part of IL-22 signaling and functions in this model. We conclude that the alternative pathway of STAT3 activation plays a role in IL-22 dependent skin inflammation.

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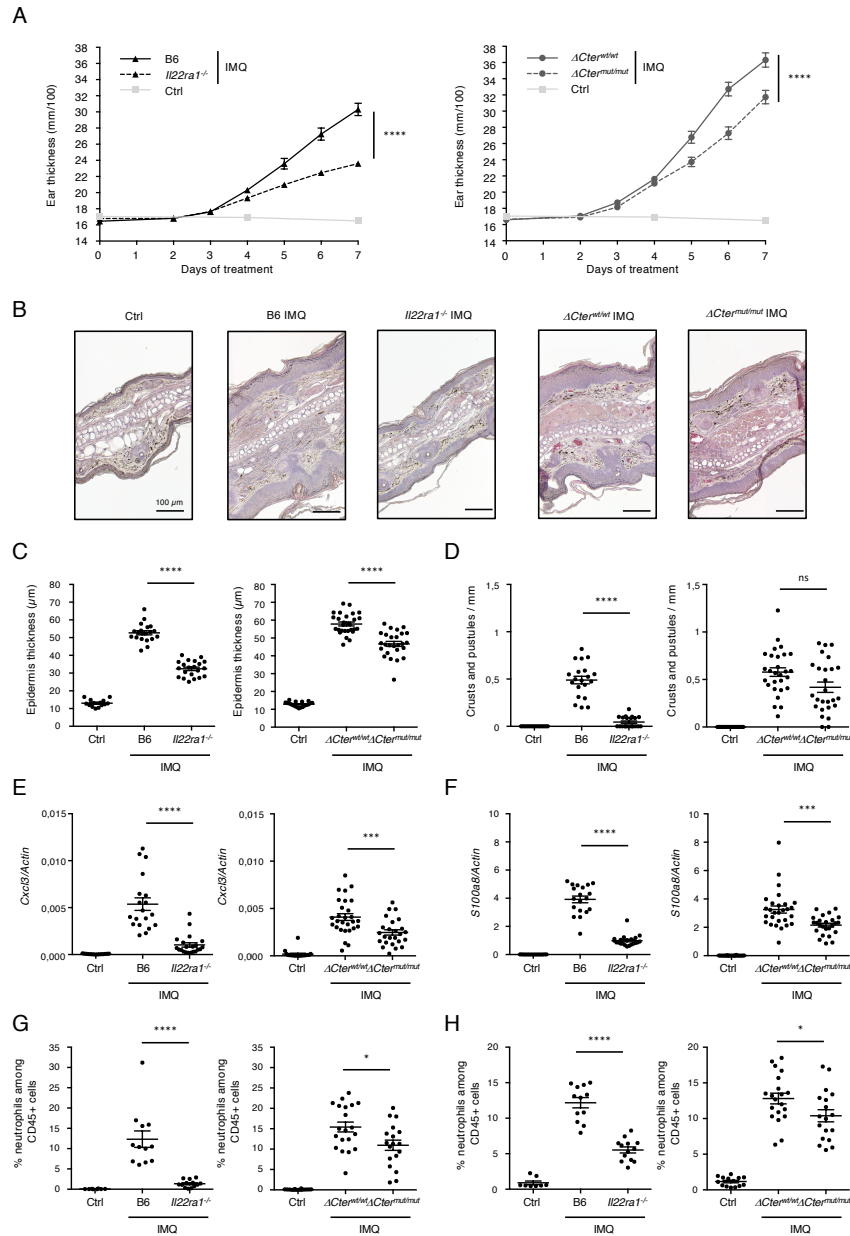


Figure 4: Deficiency and truncation in IL-22R α protects in the imiquimod psoriasis model.

(A-G) Mice were treated daily with imiquimod on the ears for 7 days. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments ($n = 8$ mice/group in each experiment). (A) Ear thickness was measured daily with a micrometer screw to evaluate the acanthosis. (B) H&E staining of ear sections (original magnification $\times 20$, scale bar = 100 μm). One representative picture is shown

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for each group. (C-D) Evaluation of acanthosis by measuring the epidermis thickness and counting the number of crusts and pustuls in the skin sections. (E-F) Ears were analyzed by RT-qPCR. *Cxcl3* and *S100a8* gene expression in ears were normalized against β -Actin. (G-H) Flow cytometry analysis of neutrophils in the epidermis (G) and dermis (H). Cells were gated on living CD45+ cells and neutrophils were analyzed using Ly6G and CD11b markers.

Statistical analyses: (A) Two-way ANOVA with Bonferroni post-test; (C-H) Mann Whitney (ns = not significant, * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$).

- *The tyrosine-dependent activation of STAT3 mediated by IL-22R α is sufficient to protect mice from colitis.*

In parallel to skin inflammation, the impact of the IL-22R α and its alternative pathway was also investigated in the gut using the *Citrobacter Rodentium*-induced colitis model*. After *Citrobacter rodentium* infection, mice weight was followed for two weeks. From day 7 after bacterial infection, *Il22ra1*^{-/-} and >IL-22R α treated B6 mice started losing weight in a similar manner, demonstrating the protective impact of IL-22 in this model (Fig. 5A). In contrast, neither Δ Cter^{mut/mut} nor Δ Cter^{wt/wt} mice presented significant weight loss, suggesting that the loss of the C-ter does not prevent IL-22 signaling, which is consistent with western-blot results obtained in the gut after IL-22 injection. However, the IL-22R α neutralizing antibody had a stronger effect on Δ Cter^{mut/mut} as compared to that on littermate controls (Fig. 5A). At the histological level, we could not detect major differences between groups (Fig. 5B and Suppl. Fig. 5A, B) regarding crypt length and the presence of goblet cells, even though *Il22ra1*^{-/-} and >IL-22R α treated mice tended to have less goblet cells. The expression of *Gob5*, a marker of goblet cells, in the colon was also decreased following >IL-22R α treatment in WT mice (Fig. 5C). Finally, *Reg3g*, *Reg3b* and *Fut2*, which are antimicrobial peptides, were less expressed in *Il22ra1*^{-/-} and Δ Cter^{mut/mut} as compared to WT mice. Similarly, the IL-22R α neutralizing treatment significantly reduced the expression of these genes in WT mice (Fig. 5D, E and Suppl. Fig. 5C). We conclude that *Il22ra1* is essential to protect mice against the *Citrobacter rodentium* model. In contrast, even though some parameters are reduced in Δ Cter^{mut/mut}, mice did not lose significant weight as compared to WT littermate mice. Hence, the absence of the alternative pathway does not prevent the

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beneficial impact of IL-22 in the colon, meaning that the tyrosine-dependent pathway is sufficient to mediate the protective STAT3 activation.

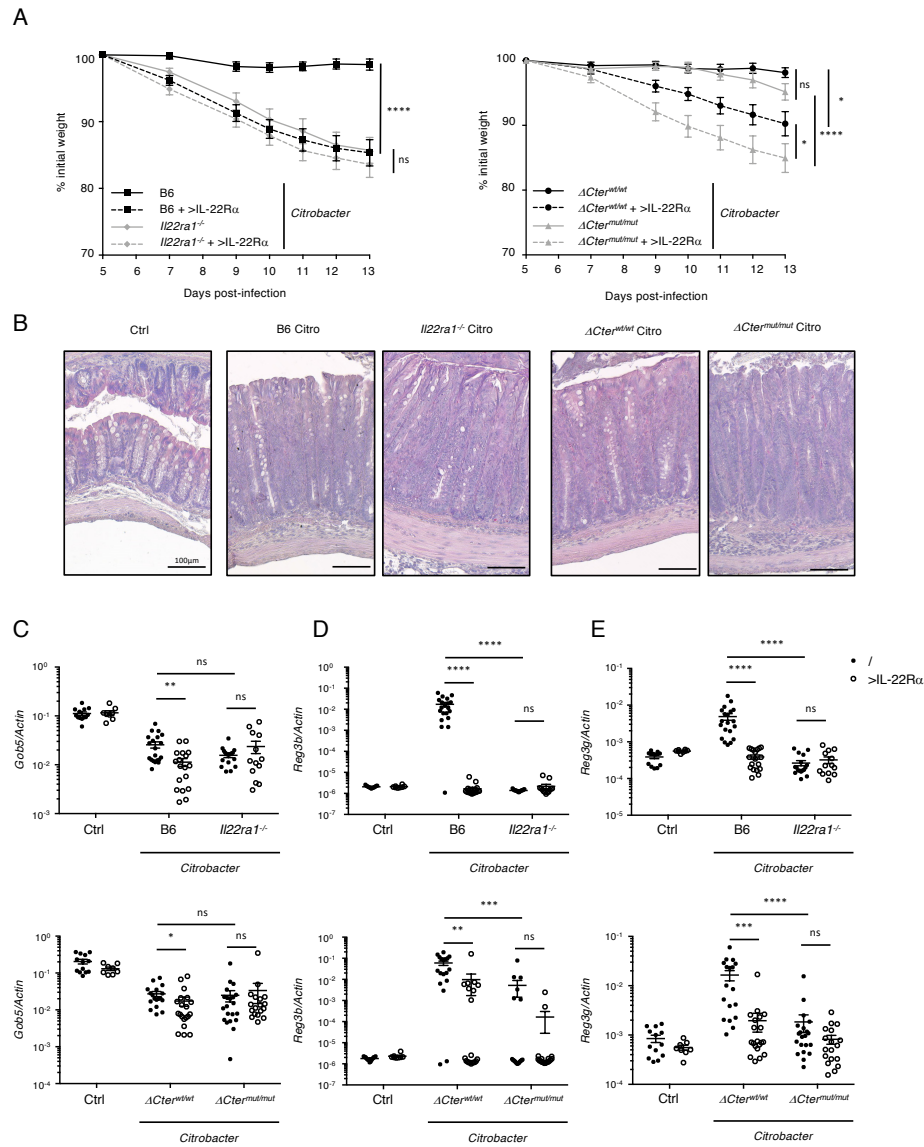


Figure 5: IL-22R α C-ter has only a minor role in *Citrobacter* rodentium-induced colitis.

(A-E) Mice were treated orally with *Citrobacter rodentium* (2.10^9 CFU/mice) and injected with antibodies (100 μ g/mice) one day prior gavage and two, five and nine days after. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = min 6 mice/group in each

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experiment). (A) Body weight was measured to evaluate the colitis. (B) H&E staining of colon sections (original magnification $\times 20$, scale bar = 100 μm). One representative picture is shown for each group. (C-E) Colons were analyzed by RT-qPCR. *Gob5*, *Reg3b* and *Reg3g* gene expression in colon were normalized against β -Actin.

Statistical analyses: (A) Two-way ANOVA with Bonferroni post-test; (C-E) Kruskal-Wallis (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Discussion

Our study confirms that IL-22 and its receptor are involved in skin and gut inflammation. Indeed, as previously described in *Il22^{-/-}* mice, *Il22ra1* deficient mice were partially protected in the imiquimod model and sensitive to the *Citrobacter rodentium* model. Our results also indicate that the C-ter part of IL-22R α is involved in the activation of STAT3, but not of STAT1 or STAT5, *in vitro* and *in vivo*. These results are consistent with our previous study showing this alternative activation of STAT3 in BW5147 engineered to express mutant forms of IL-22R α ¹¹. In addition, we show that this alternative activation of STAT3 is deleterious in the imiquimod-induced psoriasis model whereas its role in the *Citrobacter rodentium* induced colitis is limited.

Other tyrosine-independent activations of STAT have previously been described for cytokine receptors, including the IFN- λ R1, IFNAR, G-CSF-R and IL-23R^{6, 7, 8, 10, 21}. These alternative pathways enable a massive activation of STAT signaling and the pre-association of STAT might lead to faster response to cytokine stimulation. However, data about their biological relevance are still missing. This is the first time that such alternative pathway is demonstrated in physiological context, *in vitro* and *in vivo*, and not in cells transfected with mutant receptors.

In the imiquimod model, $\Delta\text{Cter}^{\text{mut/mut}}$ displayed a milder acanthosis than $\Delta\text{Cter}^{\text{wt/wt}}$ mice. It was accompanied by a reduced expression of *S100a8* and *Cxcl3*, correlating with a lower percentage of neutrophils in the dermis and the epidermis. The partial protection of $\Delta\text{Cter}^{\text{mut/mut}}$ mice from the imiquimod model was weaker than the one observed in *Il22ra1^{-/-}*

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mice, suggesting that the tyrosine-independent activation of STAT3 is partially involved in IL-22 signaling and effects in the skin. However, it seems that the IL-22R α was more expressed in the $\Delta Cter^{mut/mut}$ than in $\Delta Cter^{wt/wt}$ mice in epidermal cells. Hence, the effect of the IL-22R α Cter on STAT3 activation might be underestimated. Of note, the truncation of the IL-22R α might have deleted a ubiquitination site of the IL-22R α , increasing its expression, although the ubiquitination site described for the IL-22R α , Lys449, is not located in the C-ter²². Otherwise, the intracellular trafficking or circulation of the receptor could be increased if regulatory motifs are comprised in the C-ter sequence. For example, several cytokine receptors were shown to constitutively undergo internalization, not only following ligand binding, due to the presence of specific motifs^{23, 24}. Also, the expression of the receptor could be upregulated in a compensatory phenomenon to ensure minimal activity of IL-22 in the skin.

Strikingly, the effects of the truncation of the IL-22R α on IL-22 signaling in the gut is very limited as compared to the skin and liver, where STAT3 activation is strongly decreased in $\Delta Cter^{mut/mut}$ mice. This might be explained by the distribution of IL-22 in the different organs avec *i.p.* injection. Various amounts of IL-22 were injected in WT mice and we observed that 1 μ g of IL-22 was sufficient to induce the maximal expression of *Socs3* in the ileum whereas 10 μ g were required for the liver (data not shown). These results suggest that saturating effect of IL-22 is different in all the organs. This observation would not fit with those of the G-CSF-R, which also acts via a tyrosine-independent mechanism. In contrast to our results, it was shown that, at saturating G-CSF concentration, the activation of STAT3 is mediated by the C-ter of the receptor in a non-tyrosine dependent manner. By contrast, at low ligand concentration, tyrosine residues, and especially tyrosines 404 and 744, preferably support the activation of STAT3⁸. Analysis of IL-22 expression kinetics and level, in the skin and gut, during inflammatory models would help to demonstrate if our hypothesis is also applicable when IL-22 is endogenously produced.

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Since the alternative activation of STAT3 plays a deleterious role in the imiquimod model, it would be an interesting candidate to target as a therapeutic approach. Currently, treatments used in psoriasis patients include topical treatment and PUVA-therapy for the mild form of psoriasis and anti-inflammatory or immunosuppressive molecules such as corticosteroids, cyclosporin and methotrexate for more severe forms. Biologics targeting TNF- α , IL-23 or IL-17 are also used²⁵. However, these drugs cause side effects because they widely affect the immune system. Targeting IL-22 signaling might be advantageous because it would not affect the immune system since IL-22R α is not expressed in immune cells. On the other hand, IL-22 can also be beneficial in various contexts, in colitis, for example. This is why targeting the alternative activation of STAT3 would enable the partial decrease of IL-22 signaling to dampen its harmful effects in psoriasis while maintaining the beneficial ones. In addition, the C-ter dependent activation of STAT3 was also demonstrated in the human cell line A549 (data not shown), demonstrating that this mechanism also exists in human. Small molecules or binding proteins such as monobodies could be used to perturb the pre-association of STAT3 with the C-ter part of IL-22R α ²⁶.

Materials and methods

Mice and treatments

All mice used in this study were bred in our animal facility under specific pathogen-free conditions. Δ Cter mice were generated at the de Duve animal facility and kept in a littermate strain (B6D2xB6), Δ Cter^{wt/wt} mice were used as control of Δ Cter^{mut/mut}. *Il22ra1*^{-/-} and C57BL/6 (called B6 thereafter) were also used in this study. All animal experiments were performed in compliance with national and institutional guidelines and were approved by the local ethical committee.

Eight-week-old mice were shaved behind the ears and were treated with seven daily topical applications of imiquimod (0.8 mg per ear) from a commercially available cream (Aldara 5%; 3M Pharmaceuticals). Ear thickness was measured every day with a micrometer screw (Mitutoyo).

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The *Citrobacter Rodentium* inoculation was performed via oral gavage (2×10^9 CFU) on eight-week-old mice and body weight was measured frequently. Bacteria were prepared after overnight culture in LB at 37°C. Concentration was assessed by measuring absorbance at 600 nm. One day prior to gavage and on day 2-5-9 post-infection, anti-IL-22R α antibody (100 μ g/mice) was injected in half of the mice. The anti-IL-22R α antibody comes from ArgenX.

IL-22 (10 μ g) was injected *i.p.* and organs were dissected 1 hour after injection and incubated min 24 hours in RNA later (Thermofisher) before RNA extraction. For immunohistofluorescence and western-blot, mice were dissected 30 minutes after IL-22 or PBS injections.

CRISPR-Cas9 modification of cells

The single-stranded DNA used as template to achieve homology-directed repair was designed as described previously²⁷. gRNA was cloned into PX458 vector (Addgene), coding for Cas9 and GFP, in fusion with gRNA scaffold. MES13 ($5 \cdot 10^6$) were electroporated (250 V, 192 ohms, 1200 μ F) with the vector (5 μ g) and ssODN (5 μ g) and placed in culture with SCR7 (Sigma, 1 μ M) and RS1 (Sigma, 5 μ M), an inhibitor of NHEJ and an enhancer of HDR repair pathways, respectively. 48h after electroporation, cells were FACS sorted based on GFP staining (BD FACS Aria) and kept in culture 7 days before cell cloning. Clones were selected by PCR and sequenced (Macrogen). The sequence of gRNA and ssDNA (Eurogenec) is provided in Table 1.

	Sequence (5'-3')
<i>gRNA</i>	GTGGCCCTCGATCTGCACAG
<i>ssDNA</i>	ACAGACAGAGGACCTGACCTTCACACTGCGCAGTGAGGAACCAGAGACACCACGGTACCTG AAGGGGGCGCTGTCTCTCTGTAATCTGTGCAGATCGAGGGCCACCCTGTCTCCCTCCCTTTG

Table 1: sequence of gRNA and ssDNA designed for CRISPR/Cas9

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CRISPR-Cas9 modification of mouse zygotes

We used a cloning-free CRISPR/Cas9 system to target the mouse *il22ra1* gene²⁸. Guide RNA is split in two: crRNA (20 nucleotides), corresponding to the target sequence with a tail (16 nucleotides), and a tracrRNA (67 nucleotides), containing the hairpin recognized by Cas9 with an extremity complementary to the crRNA tail. Recombinant Cas9 protein, annealed crRNA/tracrRNA and single strand DNA donor (2.4 pmol/μl crRNA, 2.4 pmol/μl tracrRNA, 100 ng/μl Cas9 and 10 ng/μl single strand DNA donor), were mixed in IDTE buffer and injected into the pronucleus of B6D2 mouse zygotes. crRNA, tracrRNA, single strand DNA donor and recombinant Cas9 protein were purchased from Integrate DNA Technologies. Sequences of gRNA and ssDNA are the same as in the previous section. The presence of the mutation in pups was confirmed by PCR screening and sequencing. Mice carrying the mutation was crossed with B6 female and the strain was kept in a littermate background. Mice were selected based on the presence of the mutation in the *il22ra1* by PCR genotyping.

Epidermis and dermis single-cell suspension

Ears were dissected and incubated overnight at 4°C in dispase II at 1U/ml (Roche). After separation of epidermis and dermis with forceps, epidermal cells were isolated by digestion with DNase I (Roche) at 0.5 mg/ml during 20 min at room temperature while dermal cells were isolated by digestion with DNase I (Roche) at 0.1 mg/ml and collagenase D (Roche) at 2.6 mg/ml during 45 min at 37°C. After a mechanical disruption, cells were filtered on 40-μm nylon filters to obtain a single-cell suspension. FACS analysis were directly performed on cells. Western-blot and RT-qPCR were performed after 24 hours at 37°C in wells (48 wells plate).

Intestinal single-cell suspension

Intestines were dissected and cut longitudinally after removing the Peyer's patches and the fat. Small pieces of intestines were incubated with EDTA (2.5 mM) and dithiothreitol (72.5 mg/ml) during 20 min at 37°C with shaking (200 rpm.). After mechanical disruption, cells

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were passed through 70-mm cell strainer and cells were further centrifuged in a solution of 30% Percoll. FACS analyzes were directly performed on cells.

FACS staining

Cells were preincubated with 10 µg/mL of purified rat anti-mouse CD16-CD32 mAb (Fc Block; BD Pharmingen) before incubation with a viability marker (LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, Life) and 2 µg/mL of antibodies to extracellular staining for 30 min at 4°C. Then, for intracellular staining, cells were fixed, permeabilized, using the Intracellular Fixation & Permabilisation Buffer Set (eBiosciences) and finally stained 30 min at 4°C. Cells were analyzed with an FACS Fortessa (BD Biosciences). The following antibodies from Biolegend were used: PerCP or PE-Cy7-labeled anti-CD45 (30-F11), FITC-labeled anti-Ly6G/C (Gr1), PE-Cy7-labeled anti-CD4 (GK1.5), BV421-labeled anti-CD8α (53-6.7), APC-labeled anti-TCRb (H57-597), BV510-labeled anti-gd (GL3), PerCP-labeled anti-CD326/EpCAM (G8.8) and PE-labeled anti-mouse (Poly4053). The PerCP-labeled anti-cytokeratin-10 antibody (SPM 261) was purchased at Novus Bio and the PE-labeled anti-CD11b antibody (M1/70) at BD Biosciences. Anti-IL-22Rα (AM22R1.1) is unlabeled and produced in our laboratory. This IL-22Rα staining requires a bi-layer staining with, as second layer, the PE Goat anti-mouse IgG antibody (Biolegend, 405307).

Gating strategy included forward and side scatter, a viability marker and CD45 expression. Cytokeratin-10 and EpCAM markers allow discrimination of keratinocytes and intestinal epithelial cells, respectively. Ly6G and CD11b markers were used to analyze neutrophils. The specificity of the IL-22Rα staining was verified in *il22ra1^{-/-}* mice. Data were analyzed with the FlowJo software (Tree Star).

Western-blot

Cells were stimulated 15 min at 37°C with control medium or with murine IL-22 (500 ng/ml) before the lysis using buffer containing 50 mM tris(hydroxymethyl)aminomethane-HCl, 1% NP40, 150 mM NaCl, 1 mM EDTA, 15% glycerol, Halt Protease Inhibitor Cocktail 1/100 (ThermoFischer), phenylmethylsulfonyl fluoride 0.1 mM, and sodium orthovanadate 0.1

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mM. For mice, organs were frozen in liquid nitrogen before tissue disruption with Tissue Lyser LT (Qiagen) in the lysis buffer. Lysates were centrifuged and supernatants were kept. After protein quantification with Pierce BCA Protein Assay Kit (ThermoFischer), a total of 2 or 10 µg of proteins, diluted in LDS sample buffer and Sample Reducing Agent (ThermoFischer), were loaded on Bolt 4-12% Bis-tris gels (ThermoFischer) and transferred to nitrocellulose membrane (iBlot gel transfer stacks, ThermoFischer). The JAK-STAT signaling pathway was investigated with the following total or phospho-specific antibodies from Cell Signaling Technology: anti-pY701 STAT1 (#9167), anti-STAT1 (#9172), anti-pY705 STAT3 (#9131), anti-STAT3 (#12640), anti-pY694 STAT5 (#9351), anti-STAT5 (#9363), anti-pY1034/1035 JAK1 (#3331) and anti-JAK1 (#3332). The anti-β Actin antibody was also used as control (A5441, Sigma).

RT-qPCR

Total RNA was isolated from organs with the TriPure isolation reagent (Roche) after tissue disruption with Tissue Lyser LT (Qiagen). RNA extraction from cells was performed 1 hour after stimulation with control medium or with murine IL-22 (500 ng/ml), also with TriPure isolation reagent. Reverse transcription was performed on 1 µg of total RNA with oligo(dT) primer (Eurogentec) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) amplifications were performed on cDNA obtained from 25 ng of total RNA, using primer sets and TaqMan probes with qPCR Mastermix TaqMan or SYBS Green (Eurogentec). The sequences of primers and probe are provided in Table 2. Samples were first heated for 10 min at 95°C, before amplification as follows: 40 cycles of two-step PCR program at 95°C for 10 s and 60°C for 1 min. For SYBR Green qPCR, melting point analysis was carried out by heating the amplicon from 60 to 95°C. A standard curve with known concentrations of a cloned cDNA fragment was used for each gene.

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Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Probe (5'-3')
<i>Actin</i>	CTCTGGCTCCTAGCACCATGAAG	GCTGGAAGGTGGACAGTGAG	ATCGGTGGCTCCATCCTGGC
<i>Cxcl3</i>	CCATCCAGAGCTTGACGGTGAC	TGGACTTGCCGCTCTTCAGTATC	/
<i>Fut2</i>	AGTCTTCGTGGTTACAAGCAAC	TGGCTGGTGAGCCCTCAATA	/
<i>Gob5</i>	ACTAAGGTGGCCTACCTCAA	GGAGGTGACAGTCAAGGTGAG	/
<i>Il22ra1</i>	CACACCGGTCTCTCGGAAG	GGCACTTTCCTTGGACAATATCGG	CACCTACCAGATGCACCTTGAAG
<i>Reg3b</i>	CTACTGCCTTAGACCGTGCTTTC	GAGTCTTCACATTTGTCCCTTGTC	GTGAAGTTGCCCTATGTCTGC
<i>Reg3g</i>	GAGTGGAGCAATGCTGATGTGATG	GGGATCTTGCTTGCGTAGG	/
<i>S100a8</i>	CACCATGCCCTCTACAAGAATGA	CTCTGCTACTCCTTGCGCTGT	CCTTGGCGATGGTGATAAAAGTGGGTGTGGC
<i>Saa1-2</i>	TCTGCTCCCTGCTCTGGGA	TGGCTTCTGGTCAGCCATG	/
<i>Socs3</i>	GCCTCGGGGACCATAGGA	GCATCCCGGGGAGCTAGT	/

Table 2: RT-qPCR primer and probe sequences

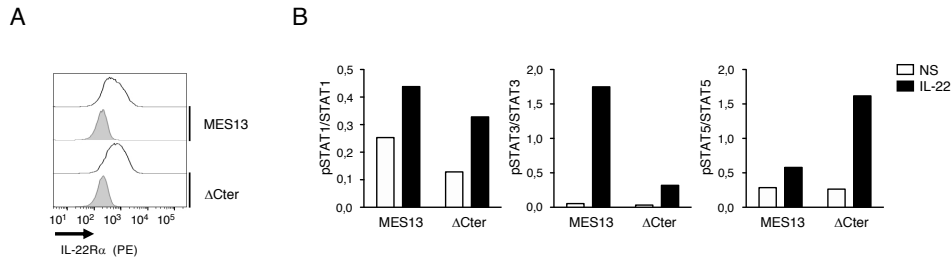
Histology and immunohistofluorescence

Paraffin embedding of ears were performed using routine methods. 6 µm sections were stained with H&E and scanned with Pannoramic 250 (3D Histech).

Intestines were dissected, cut longitudinally and washed briefly in PBS before fixation in PFA4% for 30 minutes at 4°C. Organs were then embedded and frozen in sucrose/gelatin. Immunofluorescence labeling was performed on 10 µm tissue sections after antigen unsmaking in a citrate buffer (0,01M pH 6.0). The following antibody allowed to study pY705 STAT3 (CST #9145, dil 1/200). Anti-E-Cadherin antibody was used as epithelial marker (BD biosciences #610181, dil 1/250). Secondary antibodies AF488 Donkey anti-Rabbit IgG (Thermo, A21206) and AF594 Donkey anti-Rabbit IgG (Thermo, A21207) as well as Hoeschst (Sigma) were applied at 1/1000. Slides were scanned with Pannoramic 250 (3D Histech).

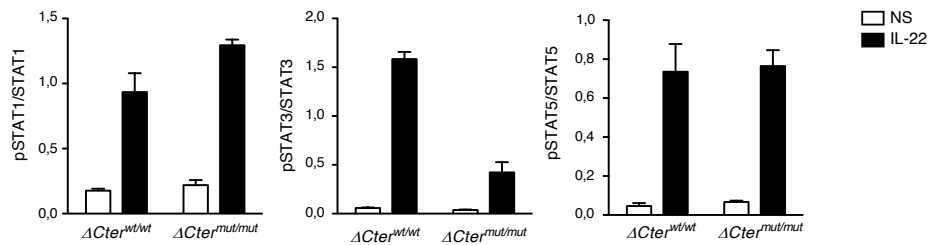
Results – part 2

Supplementary figures



Suppl. figure 1: flow cytometry analysis of IL-22Rα and IL-22 signaling in MES13 and ΔCter.

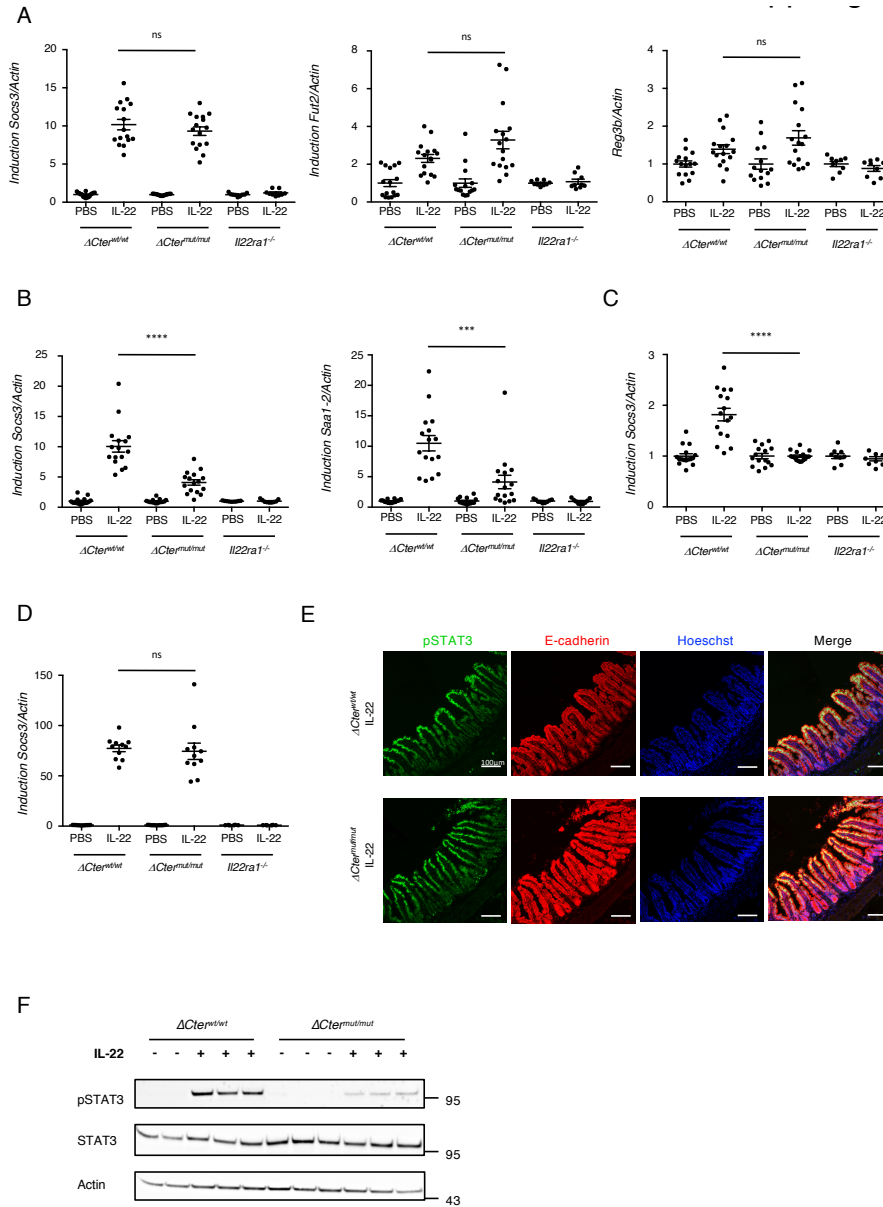
(A) Flow cytometry analysis of IL-22Rα in MES13 parental cells and ΔCter. Cells were gated on living cells. Grey curve: Goat anti-mouse Ab only, empty curve: IL-22Rα staining. Representative of 3 experiments. (B) Quantification of western-blot of Figure 1B on MES13 and ΔCter clone stimulated with IL-22 for 15 minutes.



Suppl. figure 2: quantification of western blot of Fig. 2A.

Quantification of western-blot of Figure 2A performed on epidermal cells stimulated 15 minutes with IL-22 (500 ng/ml). Data are shown as mean ± SEM of one representative experiment performed 3 times in triplicates.

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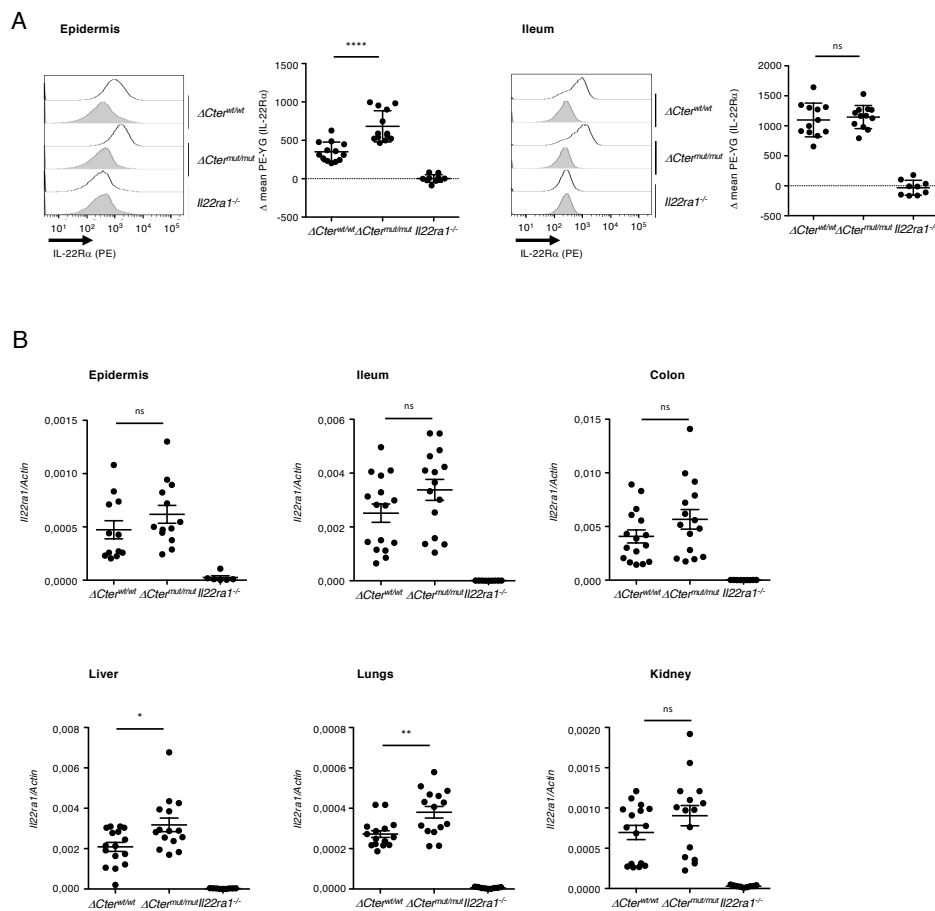


Suppl. figure 3: IL-22 signaling analysis in the ileum and liver after IL-22 injections.

(A-B-C-D) Ileum (A), liver (B), lung (C) and colon (D) were analyzed by RT-qPCR. 1 hour after PBS or IL-22 injection, organs were dissected and RNA was extracted. *Socs3*, *Fut2*, *Reg3g* and *Saa1-2* gene expressions are normalized against β -Actin and presented as induction compared to PBS injections. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = at least 5 mice/group in each experiment). Statistical analyses: Mann Whitney (**p<0,01, ***p<0,001 and

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****<0.0001). (E) Immunohistofluorescent staining on frozen sections of the ileum. 30 minutes after PBS or IL-22 *i.p.* injections, the ileum was cut and fixed in PFA 4%. After embedding in sucrose/gelatin and freezing, 10 μ m sections were performed. Slides were stained for pSTAT3, E-cadherin and Hoechst. Picture representative of two experiments. Original magnification $\times 20$, scale bar = 100 μ m. (F) Western-blot analysis of IL-22 signaling in the liver. 30 minutes after PBS or IL-22 *i.p.* injection, a piece of liver was cut and freeze. Proteins were extracted and 2 μ g of proteins was charged on gels. STAT3 and pSTAT3 were analyzed.

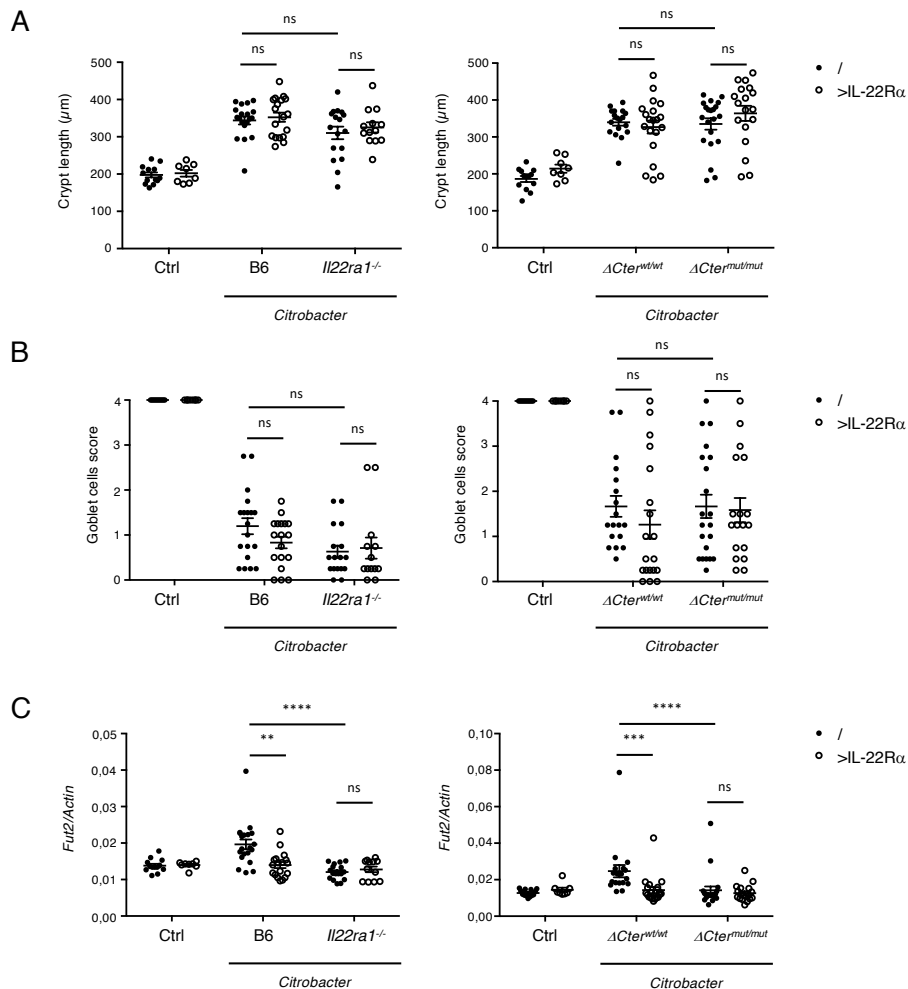


Suppl. figure 4: IL-22Rα expression analysis by flow cytometry and RT-qPCR.

(A) Flow cytometry analysis of IL-22Rα in epidermal and intestinal epithelial cells. Cells were gated on living CD45⁻ cells and IL-22Rα staining was analyzed in CK10⁺ or EpCAM⁺ cells. *Il22ra1*^{-/-} mice were used as negative control. Grey curve: Goat anti-mouse Ab only, empty curve: IL-22Rα staining. Staining was quantified using delta mean between stained and not stained cells. Data are shown as mean $\pm$

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SEM and are pooled from three independent experiments (n = 4-5 mice/group in each experiment). (B) *Il22ra1* expression was analyzed by RT-qPCR in epidermis cells, ileum, colon, liver, lung and kidney. *Il22ra1* gene expression is normalized against β -actin. Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = at least 5 mice/group in each experiment). Statistical analyses: Mann Whitney (ns = not significant, *p<0.05, **< 0.01 and ****<0.0001).



Suppl. figure 5: evaluation of colitis on H&E sections and by RT-qPCR.

(A-B) Evaluation of colitis on H&E sections of colon by (A) measuring crypt length and (B) scoring the number of goblet cells. (C) *Fut2* expression analysis in colon, normalized against β -Actin.

(A-C) Data are shown as mean $\pm$ SEM and are pooled from three independent experiments (n = min 6 mice/group in each experiment). Statistical analyses: Kruskal-Wallis (ns = not significant, *p<0.05, **< 0.01, ***<0.001 and ****<0.0001).

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References

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Selective inhibition of STAT3 alternative pathway induced by IL-22 using monobodies targeting the coiled-coil domain of STAT3

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Note

This work was done in close collaboration with the team of Pr. Hantschel and some of these results were recently accepted for publication in Nature Communications (La Sala *et al.*: Selective targeting and inhibition of STAT3 signaling using Coiled-coil and N-terminal domain specific monobodies. See annex). We decided to present this work in an IL-22 perspective and to add unpublished experiments to describe the entire study performed in the laboratory. Although, some experiments carried out in the laboratory of Pr. Hantschel by Grégory La Sala are also presented to explain the results and keep the story complete.

Abstract

Targeting IL-22 signaling could be a promising approach to treat psoriasis patients. Indeed, IL-22 promotes inflammation, keratinocytes proliferation and prevents differentiation. Nevertheless, in some cases, IL-22 can also be beneficial by reinforcing barrier defense, clearance of pathogens and regeneration of tissues. This is why we aim at specifically blocking the alternative activation of STAT3 induced by IL-22, which relies on the C-ter part of IL-22R α and the coiled-coil domain of STAT3, to partially interfere with IL-22 signaling. In this context, we analyzed the effect of a monobody targeting the coiled-coil domain of

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STAT3 on IL-22 signaling. Other STAT3-inducing cytokines were also analyzed, as well as the impact of the monobody on other STAT factors. We could show that this monobody is specific for STAT3. Moreover, it decreases the alternative activation of STAT3, but not the classical one induced by IL-22. Nevertheless, it decreases the transcriptional activity of STAT3 induced by all cytokines tested by reducing its nuclear translocation. This demonstrates that targeting the coiled-coil domain of STAT3 might be an alternative therapy in STAT3-driven diseases.

Introduction

IL-22 is a cytokine critical for tissue homeostasis that acts on various organs (skin, gut, lungs, liver...). It is involved in tissue regeneration, defense against pathogens and inflammation^{1, 2}. An overproduction of IL-22 has been linked with deleterious effects. Indeed, *IL22* transgenic mice develop spontaneous skin lesions that resemble psoriasis³. On the contrary, *IL22* deficient mice are partially protected from the imiquimod-induced psoriasis model⁴. Moreover, several studies showed that IL-22 favors the development of tumors, notably colon cancer, PDAC and liver cancer^{5, 6, 7, 8}. Targeting the IL-22 may be effective in the treatment of these diseases. Nevertheless, blocking completely IL-22 would induce side effects. Indeed, IL-22 can also be beneficial in wound healing, hepatitis or inflammatory bowel diseases^{9, 10, 11, 12}. In these contexts, IL-22 is important to regenerate tissues and for the clearance of pathogens. Hence, blocking IL-22 would increase the risk to develop pulmonary infection or colitis^{1, 2}. Another approach would be to partially block the effects of IL-22.

To exert its function, IL-22 induces the JAK-STAT pathway upon binding to its receptor composed of the IL-22R α and the IL-10R β ¹³. Upon IL-22 stimulation, STAT1, STAT3 and STAT5 are activated, STAT3 being the major signaling factor activated by IL-22^{13, 14}. As it is widely described, the binding of IL-22 to its receptor leads to the activation of the kinases associated with both chains of the receptor, JAK1 and TYK2, that will phosphorylate tyrosines of the receptor. Phosphotyrosines are used as docking sites by STAT factors,

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which, in turn, also get phosphorylated. Phosphorylated STATs dimerize and migrate into the nucleus to regulate gene expression¹⁵. In addition, STAT3, but not STAT1 and STAT5, can be activated in a mechanism that relies on the constitutive association between the C-ter part of IL-22R α and the coiled-coil domain of STAT3. In contrast to the classical pathway, this alternative activation of STAT3 is not dependent on receptor phosphotyrosines. Both pathways, phosphotyrosine dependent and independent, synergize to massively activate STAT3¹⁶. Hence, targeting one pathway would partially block IL-22 while maintaining some IL-22 activity.

In this perspective, we took advantage of monobodies targeting STAT3 developed in the lab of Pr. Hantschel. Monobodies are small binding proteins that derive from the tenth fibronectin type III (FN3) domain of human fibronectin as molecular scaffold. Contrary to antibodies or antibody derivatives, monobodies do not contain disulfide bonds, which enables its intracellular activity^{17,18}. We could show that monobody targeting the coiled-coil domain of STAT3 prevents the binding of STAT3 to the C-ter part of IL-22R α and, thereby, decreases STAT3 phosphorylation following IL-22 stimulation. Nevertheless, it also decreases the transcriptional activity of STAT3 induced by other cytokines by reducing its nuclear translocation.

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- *MS3-6 prevents the alternative activation of STAT3 induced by IL-22*

Monobodies against the N-terminal domain (aa 3-138) and the core fragment, which comprises the CCD, DBD and SH2 domains (aa 127-722), of STAT3 were identified from the combinatorial “side-and-loop” library. Two monobodies were used in this study: MS3-N3 (binding affinity of 40 ± 4 nM towards the NTD) and MS3-6 (binding affinity of 31 ± 6 nM towards the core fragment). To investigate the effect of monobodies on IL-22 signaling, we transiently expressed the monobodies in Ba/F3 cells expressing IL-22R α mutants. It includes a tyrosine-less mutant where all tyrosine residues located in the intracellular domain of IL-22R α were mutated to phenylalanine, and a C-terminal truncation (IL-22R α receptor

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lacking the last 84 amino acids: Δ C-ter). These IL-22R α mutants allowed us to dissect the impact of the monoclonal antibodies on conventional (Δ C-ter mutant) and alternative (tyrosine-less mutant) STAT3 activation. The Y705 phosphorylation status of STAT3 was assessed in cells expressing the different IL-22R α mutants by flow cytometry following IL-22 stimulation upon electroporation of the Myc tagged monoclonal antibody plasmids. This enabled the comparison of STAT3 phosphorylation between cells that expressed the monoclonal antibody (cMyc⁺) or not (cMyc⁻). The non-binding control Ha4-Y87A monoclonal antibody had no effect on STAT3 phosphorylation (Fig. 1A). In contrast, MS3-6, the monoclonal antibody generated against the core fragment of STAT3, strongly decreased STAT3 phosphorylation after IL-22 stimulation in cells expressing the wild-type and tyrosine-less receptor, but not the Δ C-ter receptor (Fig. 1A). Hence, it highlights the impact of the monoclonal antibody on the alternative IL-22 signaling pathway, but not on the tyrosine-dependent mechanism. Surprisingly, the monoclonal antibody generated against the N-ter domain of STAT3, the MS3-N3, increased the phosphorylation of STAT3 upon IL-22 stimulation in Ba/F3 expressing WT IL-22R α but not in the cells expressing the tyrosine-less mutant (Fig. 1B). Finally, the Y705-STAT3 phosphorylation was also analyzed in A549, a human lung cancer cell line that expresses the IL-22R α . A549 were engineered to stably express the monoclonal antibodies using a doxycycline inducible system. Here again, STAT3 phosphorylation following IL-22 stimulation was reduced upon expression of MS3-6 monoclonal antibody (Fig. 1C). This result confirms the impact of MS3-6 on IL-22-induced STAT3 phosphorylation in a context of endogenous expression of cytokine receptors.

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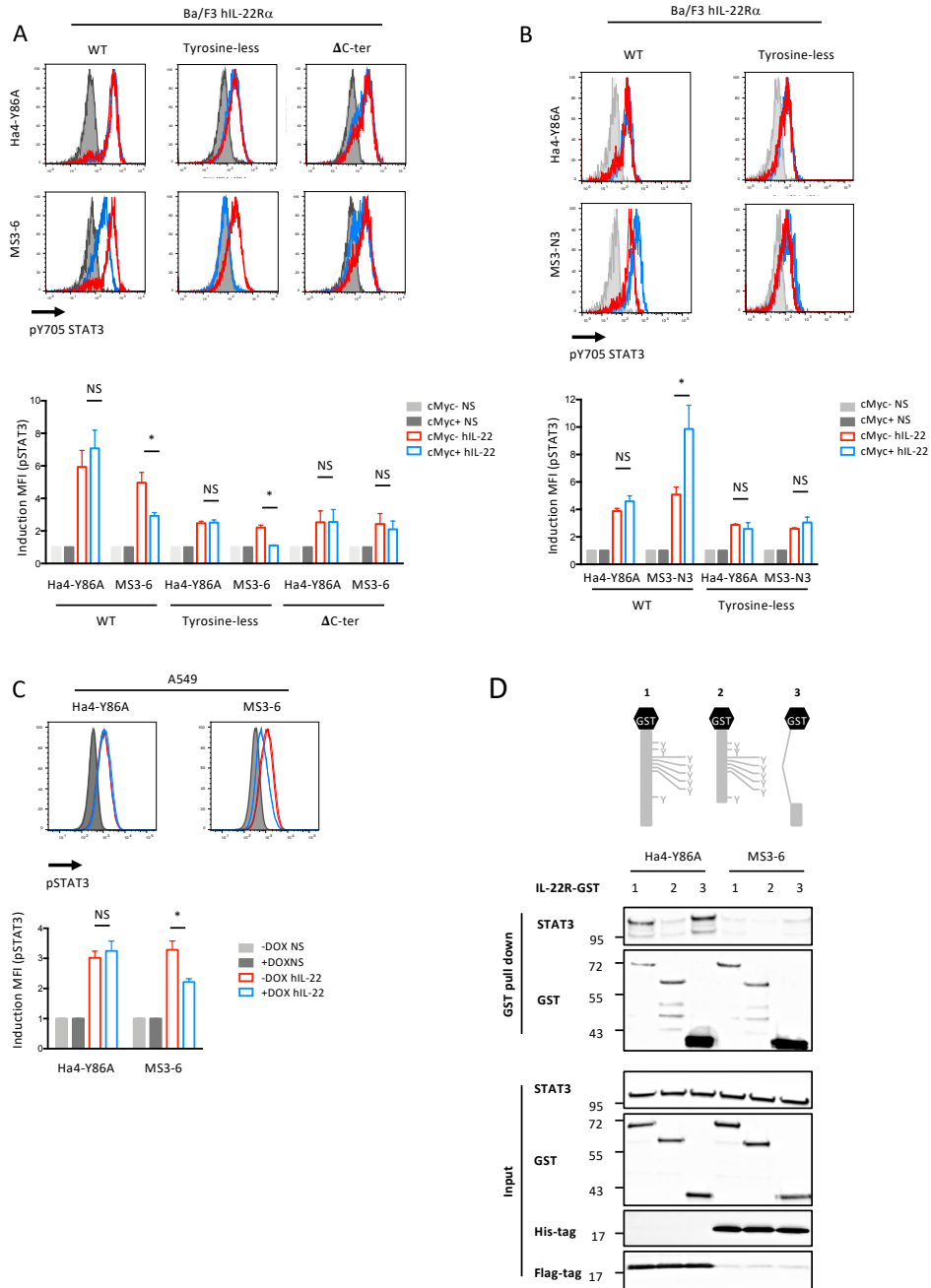


Figure 1: MS3-6 decreases IL-22 dependent STAT3 phosphorylation.

(A-B-C) Four hours after electroporation of the vector coding for monobody (Ha4YA, MS3-6 or MS3-N3), Ba/F3 cells expressing IL-22R α were stimulated with IL-22 (500 ng/ml) for 15 minutes and

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staining was performed. A549 with inducible monobody (Ha4YA or MS3-6) expression were treated with control medium or doxycycline (1 µg/ml). After 48 hours, cells were stimulated IL-22 (500 ng/ml) for 15 minutes and staining was performed. Cells were gated on living cells and phospho-STAT3 staining was analyzed and quantified in cMyc+ and cMyc- cells. Data are presented as mean ± SEM of two independent experiments performed in duplicates. Significance according to a Mann Whitney test analysis: *P ≤ 0.05. (D) Upper panel, schematic representation of GST fusion proteins with intracytoplasmic domain of IL-22Rα. Lower panel, COS-7 cells were transfected with a vector coding for the GST-fusion protein or STAT3. Cells were lysed and the recovered STAT3 protein was mixed with the recombinant monobody (10 µM) before incubation with IL-22R-GST overnight. Proteins eluted on GST SpinTrap columns as well as input samples were analyzed by western-blot with an anti-STAT3 antibody. Membrane was then re-probed with anti-GST and anti-tag antibodies.

- *MS3-6 binds the coiled-coil domain of STAT3 and thereby interferes with the unconventional binding of STAT3 on IL-22Rα*

To further corroborate the inhibition of the alternative activation of STAT3 by MS3-6, we performed GST-receptors pull-down experiments to detect the unconventional binding of STAT3 on the C-ter part of IL-22Rα. STAT3 was robustly co-purified with the recombinant GST-IL-22Rα intracellular domain mutants in cells that expressed the control HA4-Y87A monobody, as long as the IL-22Rα contained its C-terminal part (Fig. 1D). In contrast, the expression of MS3-6 resulted in the loss of STAT3 co-purification. This result reveals that MS3-6 prevents the binding of STAT3 to the C-terminus of IL-22Rα. This can be explained by the crystal structure of MS3-6 in complex with STAT3-Core Fragment obtained by Gregory La Sala (Fig. 2A and B). Indeed, the structure reveals that MS3-6 binds to the helices α1, α2, and to the loop between helices α3 and α4 of the CC domain of STAT3. Moreover, superposition of the MS3-6/STAT3-CF structure and several previously published structures of STAT3 showed that binding of MS3-6 results in the torsion of the 4-helix bundle (helices α1-4) of the coiled-coil and in the bending of the α1 and α2 helices by 4-12 degrees relative to the reference structures (Fig. 2C). Hence, MS3-6 binding provokes a conformational change of the coiled-coil domain orientation. This torsion might prevent the binding of STAT3 to the C-ter of IL-22Rα. Alternatively, MS3-6 binding could mask residues of the coiled-coil required for the interaction with IL-22Rα.

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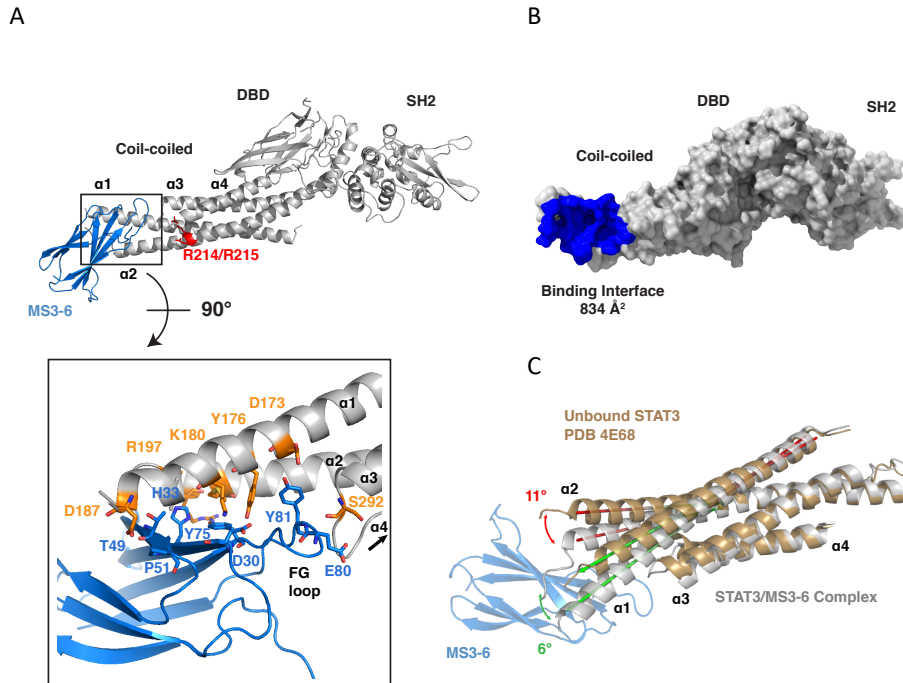


Figure 2: Co-crystal structure of MS3-6 bound to the STAT3 (Grégory La Sala's results).

(A) Cartoon representation of the structure of STAT3 (light grey) and monobody MS3-6 (blue). The upper panel shows an overview of MS3-6 binding to the coiled-coil domain of STAT3, with key residues of the nuclear localization sequence (NLS) in close proximity to the monobody binding site highlighted in red. The lower panel shows a magnification of the binding interface with epitope residues (threshold set at 4 Å) depicted as sticks. (B) Surface representation of STAT3 (light grey), with the area covered by the monobody colored in blue. (C) Structural alignment of the coiled-coil domains of STAT3 in complex with MS3-6 together with an unbound STAT3 structure previously published (PDB: 4E68). Monobody binding leads to a conformational distortion of the helices $\alpha1$ and $\alpha2$. The angles formed between helices are indicated in green and red, respectively.

- *MS3-6 does not affect STAT3 phosphorylation induced by all cytokines.*

To define whether the effect of MS3-6 on STAT3 phosphorylation was specific for IL-22 signaling, we analyzed STAT3 phosphorylation induced by various cytokines in multiple cellular contexts. On A549 cells for which monobody expression is Dox inducible, we observed that MS3-6 expression had no significant effect on pY705 STAT3 upon IL-6 stimulation (Fig. 3A). We also took advantage of BW5147 and Ba/F3 cells expressing a panel

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of cytokine receptors known to signal through STAT3. IL-9 stimulation on BW5147 and Ba/F3 stably expressing the hIL-9R was slightly affected after electroporation of the vector coding for MS3-6 compared to Ha4-Y87A (Fig. 3B and C). Strikingly, the electroporation of the MS3-6 plasmid strongly decreased STAT3 phosphorylation following IL-24 stimulation in Ba/F3 cells expressing the IL-20R α/β (Fig. 3D). Hence, while MS3-6 does not affect IL-6-induced STAT3 phosphorylation, it partially or strongly reduced STAT3 phosphorylation in response to IL-9 and IL-24, respectively.

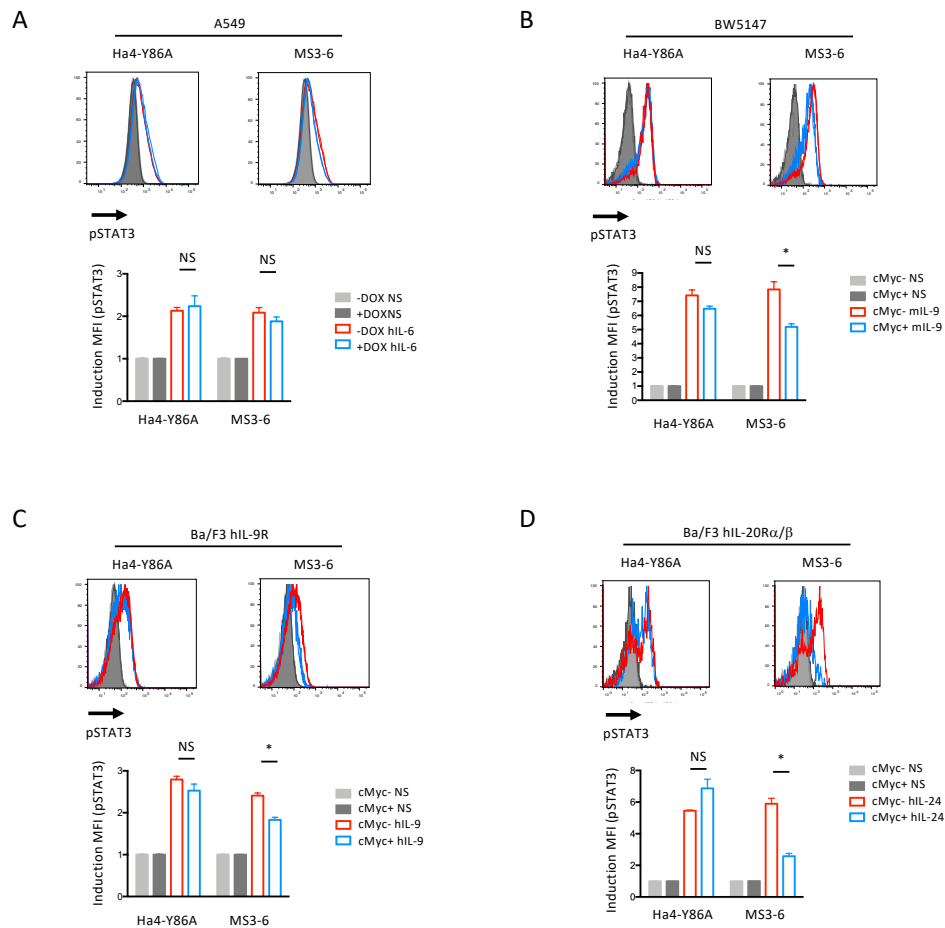


Figure 3: MS3-6 does not impact STAT3 phosphorylation.

A549 with inducible monobody (Ha4YA or MS3-6) expression were treated with control medium or doxycycline (1 μ g/ml). After 48 hours, cells were stimulated with IL-6 (100 U/ml, A) for 15 minutes and

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staining was performed (A). BW5147 and Ba/F3 cells were electroporated with the vector coding for monobody, stimulated with mIL-9 (100 U/ml, B), hIL-9 (1000 U/ml, C) or hIL-24 (HEK293 supernatant 2%, D) for 15 minutes and staining was performed. Cells were gated on living cells and phospho-STAT3 staining was analyzed and quantified in cMyc+ and cMyc- cells. Data are presented as mean $\pm$ SEM of two (A, D) or three (B, C) independent experiments in duplicates. Significance according to a Mann Whitney test analysis (ns: not significant, * $p \leq 0.05$)

- *MS3-6 is selective towards STAT3*

Next, we have determined whether MS3-6 monobody was specific for STAT3 or if it also impacted the function of other STATs. STAT1 and STAT5 phosphorylation were analyzed by flow cytometry in Ba/F3 cells expressing WT IL-22R α after electroporation with monobody plasmids. As shown in Fig. 4A and 4B, STAT1 and STAT5 phosphorylation were not significantly impacted by the expression of MS3-6 monobody. Nevertheless, IL-22 is not a strong inducer of STAT1 and STAT5 in these cells. Therefore, STAT1 and 5 phosphorylation were also studied upon other cytokines stimulation. First, A549 cells were used for their ability to respond to IFN γ , which is known to strongly induce STAT1 phosphorylation. In these cells, the expression of MS3-6 did not decrease STAT1 phosphorylation induced by IFN γ stimulation (Fig. 4C). Second, the phosphorylation of STAT5 after IL-3 stimulation of Ba/F3 cells was similar upon the expression of HA4-Y87A or MS3-6 (Fig. 4D). Altogether, these results show that the monobody MS3-6 is specific for STAT3. That was also confirmed by Grégory La Sala who performed monobody affinity-purification mass spectrometry experiments. After tandem affinity purification, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis identified that STAT3 was found 500-fold more abundant than STAT1 or STAT5B (data not shown).

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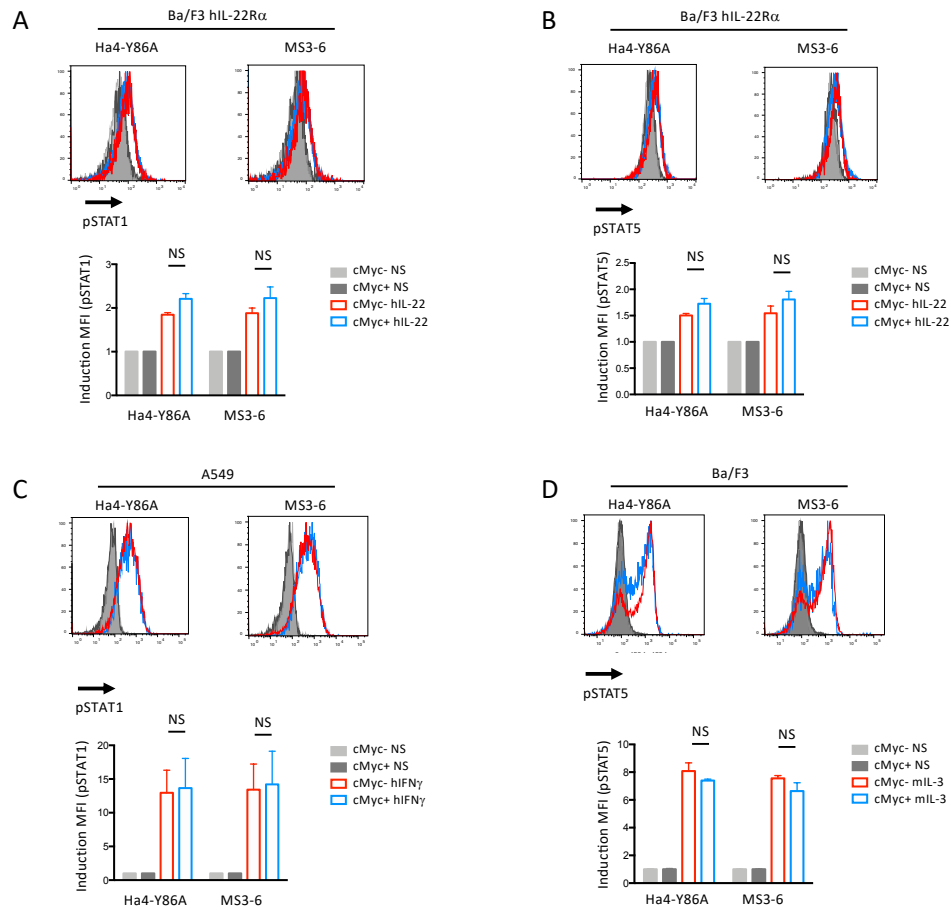


Figure 4: MS3-6 does not impact STAT1 and STAT5 phosphorylation.

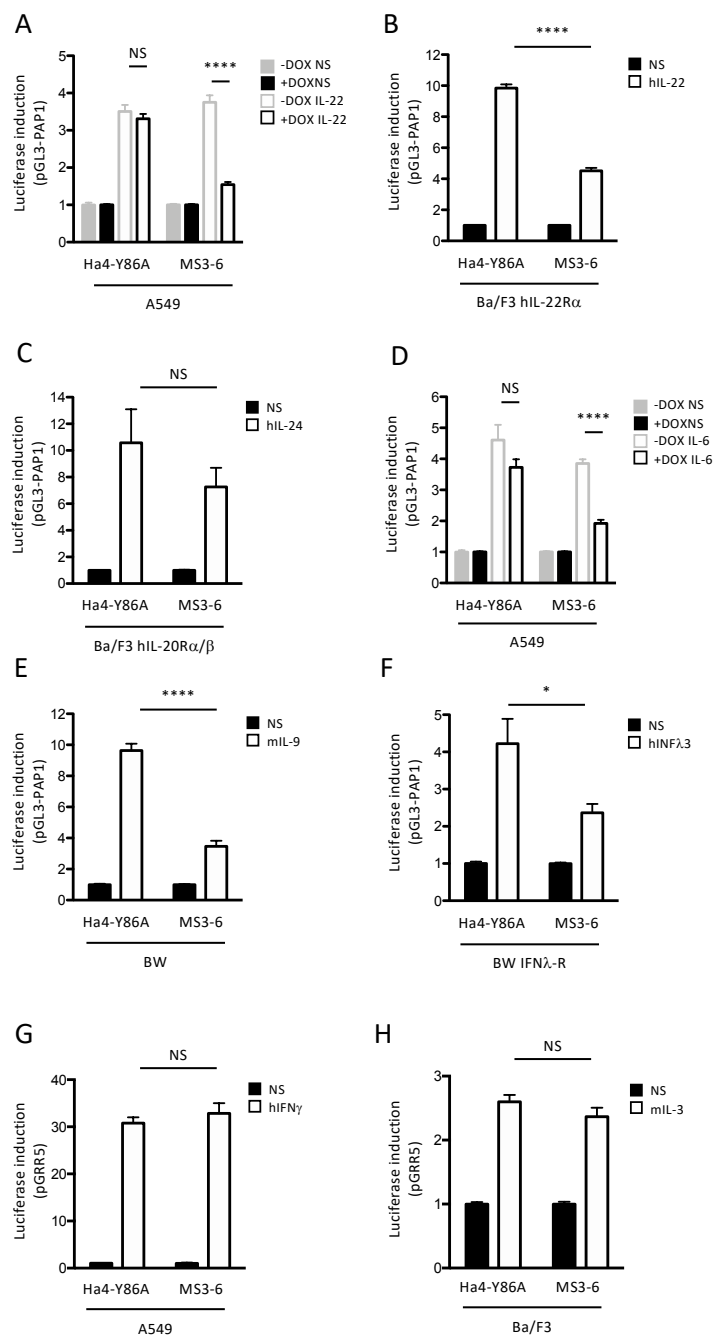
Four hours after electroporation of the vector coding for monobody (15 μ g), cells were stimulated with IL-22 (500 ng/ml, A-B), IFN γ (10 ng/ml, C) or IL-3 (150 U/ml, D) and staining was performed. Cells were gated on living cells and phospho-STAT1 or phospho-STAT5 staining was analyzed in cMyc+ and cMyc- cells. Data are presented as mean $\pm$ SEM of two (A, B, D) or three (C) independent experiments performed in duplicates. Significance according to a Mann Whitney test analysis (ns: not significant)

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- *MS3-6 decreases the transcriptional activity of STAT3*

We further investigated the effect of MS3-6 on STAT3 transcriptional activity in response to IL-22. Since MS3-6 decreased STAT3 phosphorylation in response to IL-22, the transcriptional activity of STAT3 should also be decreased. Two cell types were used: the A549 cells engineered to express monobodies using a doxycycline inducible system that we transiently transfected with a STAT3-responsive luciferase reporter and Ba/F3 cells stably expressing the IL-22R α that we transiently transfected with plasmid coding for monobody together with the STAT3-responsive luciferase reporter. In both cell lines, IL-22 induced Firefly/Renilla luciferase signals in the presence of the HA4-Y87A monobody control. In contrast, MS3-6 expression massively decreased STAT3 activity following IL-22 stimulation (Fig. 5A and B). Likewise, STAT3 transcriptional activity was also decreased, although not significantly, by MS3-6 in Ba/F3 hIL-20R α / β upon IL-24 stimulation, as observed for STAT3 phosphorylation (Fig. 5C). Surprisingly, STAT3 activity was also decreased following other cytokines stimulation where STAT3 phosphorylation was not or not much affected. Indeed, MS3-6 significantly reduced STAT3 activity in A549 upon IL-6 stimulation as compared to HA4-Y87A control monobody (Fig. 5D). Similarly, stimulation of BW with IL-9 and BW IFN- λ R, engineered to express IFN- λ R, with IFN- λ 3 induced STAT3-dependent luciferase signals in the presence of the HA4-Y87A control monobody. In contrast, the expression of MS3-6 significantly reduced STAT3 activity upon stimulation with both cytokines (Fig. 5E and F). Hence, contrary to what is observed for STAT3 phosphorylation, transcriptional activity was strongly affected by MS3-6 in all cytokine contexts that we studied. On the other hand, MS3-6 did not affect STAT1 or STAT5 transcriptional activity, measured with a STAT1/3/5-responsive luciferase reporter. Indeed, luciferase activity was not decreased by MS3-6 following IFN γ (STAT1) and IL-3 (STAT5) stimulation in A549 and Ba/F3 cells, respectively (Fig. 5G and H).

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Figure 5: MS3-6 impacts STAT3 transcriptional activity but not STAT1 and STAT5.

(A-D) Luciferase assay on A549 with inducible monobody (Ha4YA or MS3-6) expression. After 24 hours treatment with control medium or doxycycline (1 µg/ml), cells were transiently transfected with the pGL3-Pap1 luciferase reporter plasmid and the pRL-TK plasmid as internal control of transfection. 4 hours after transfection, cells were stimulated with control medium or human IL-22 (500 ng/ml, A) or human IL-6 (100 ng/ml, D) for 20 hours in the presence or not with doxycycline. Data are presented as mean ± SEM of three independent experiments in triplicates. (C-E-F-H) BW5147 or Ba/F3 cells were electroporated with the monobody, the pGL3-Pap1 luciferase reporter plasmid or pGRR5 luciferase reporter plasmid and the pRL-TK plasmid and stimulated with control medium or human IL-22 (500 ng/ml, B), human IL-24 (HEK293 supernatant 2%, C), murine IL-9 (100 U/ml, E), human IFN13 (HEK293 supernatant 2%, F) or mouse IL-3 (150 U/ml, H) for 4 hours. Data are presented as mean ± SEM of two or three independent experiments in triplicates. (G) Luciferase assay on A549. Cells were transiently transfected with the pGRR5 luciferase reporter plasmid and the pRL-TK plasmid as internal control of transfection. 4 hours after transfection, cells were stimulated with control medium or human IFN-γ (10 ng/ml) for 20 hours. Data are presented as mean ± SEM of two independent experiments in triplicates.

- MS3-6 prevents the nuclear translocation of STAT3.

In the crystal structure, it was observed that MS3-6 binds to helix α2 of the coiled-coil domain, which includes the nuclear localization signal (NLS) of STAT3 (Fig. 2A). Residues R214/215 are critical for the efficient nuclear import of active phosphorylated STAT3 dimers through binding to importins⁴⁹. Thus, MS3-6 binding may prevent interaction with importins and indirectly perturb efficient nuclear import of pY705 phosphorylated STAT3 dimers, which could explain diminished STAT3 transcriptional activity. To evaluate the impact of monobodies on nuclear translocation, Grégory La Sala took advantage of confocal immunofluorescence microscopy. He analyzed STAT3 localization after stimulation with IL-6 in HEK293 cells expressing GFP-monobody fusions upon doxycycline treatment. A reduced nuclear STAT3 level was observed in cells that expressed MS3-6 as compared to cells expressing HA4-Y87A (Fig. 6). Quantitative image analysis corroborated these qualitative results. These data determine that the inhibition of the transcriptional activity of STAT3 by MS3-6 is mediated by reduced STAT3 nuclear translocation.

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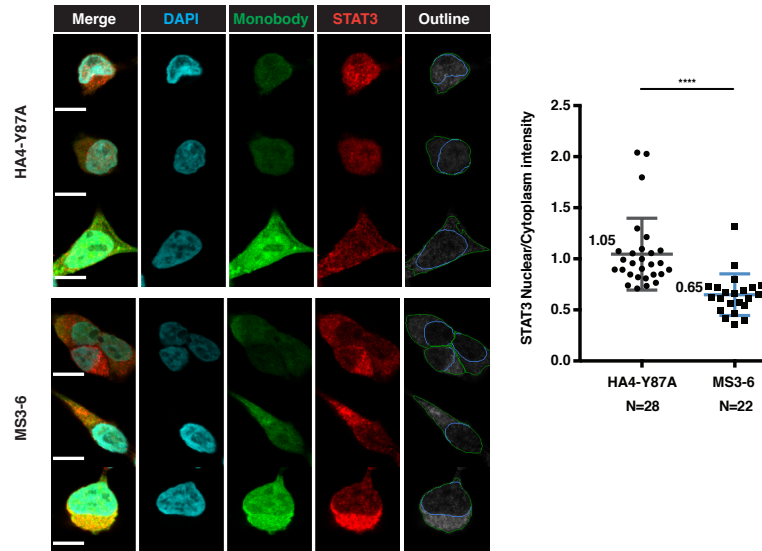


Figure 6: MS3-6 prevents the nuclear translocation of STAT3 (Grégory La Sala's results).

Confocal microscopy images of HEK293 cells expressing a monobody-GFP fusion and treated with IL-6 to assess nuclear translocation. Scale bar represents 10µm. The right column shows the outlines used to determine nuclear and cellular compartments indicated by blue and green lines, respectively, using the CellProfiler software. Quantification of STAT3 nuclear/cytoplasmic levels from two independent experiments is shown.

Significance according to an unpaired t-test analysis (**** $p \leq 0.0001$). The number of analyzed cells is indicated below the graph.

Discussion

In this study, we show that the binding of MS3-6 to the coiled-coil domain of STAT3 interferes with the signaling of IL-22. More precisely, the monobody completely blocks the alternative activation of STAT3. Indeed, STAT3 phosphorylation was absent in cells expressing a tyrosine-less IL-22R α and MS3-6 monobody. MS3-6 perturbs STAT3 phosphorylation in A549 endogenously expressing the IL-22R α as well. This specific inhibition is explained by the fact that MS3-6 prevents the interaction between the C-ter of IL-22R α and STAT3, indispensable for the alternative activation. In contrast, MS3-6 did not affect the classical pathway, tyrosine-dependent, induced by IL-22. Hence, the use of monobody strengthened the previous study that demonstrated the alternative pathway induced by IL-22. First, it confirms the importance of the coiled-coil domain of STAT3 in IL-22 signaling, and more particularly in the alternative activation of STAT3. Besides, while the alternative pathway was completely abrogated by MS3-6, the signaling of IL-22 with a WT receptor was only partially decreased by MS3-6, demonstrating the collaboration of the classical and alternative pathways to fully activate STAT3.

Interestingly, IL-24-induced phosphorylation of STAT3 was also strongly reduced by MS3-6 (around 50 % of inhibition). IL-24 is a cytokine that is related to IL-22: both belong to the IL-20 subfamily of cytokines²⁰. Therefore, we hypothesize that IL-24 also induces an alternative activation of STAT3 that relies on the coiled-coil domain of STAT3. Actually, we have evidences in the lab that an alternative pathway takes place for this receptor as an IL-20R α tyrosine-less receptor still leads to STAT3 activity (data not shown). Nevertheless, the exact mechanism underlying this pathway is not yet known and the requirement of the coiled-coil domain of STAT3 has to be evaluated. Hence, the MS3-6 monobody might be a useful tool to explore and discover new alternative mechanisms of activation of STAT3 that rely on the coiled-coil domain of STAT3.

Concerning other cytokines, a slight effect of the MS3-6 monobody was observed after IL-9 stimulation. Nevertheless, this effect is limited as compared to what was obtained for IL-22

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or IL-24, suggesting that only a small part of signaling was affected (20 to 30 %). Theoretically, IL-9 should not lead to an alternative activation of STAT3 since one tyrosine was shown to be responsible for STAT3 phosphorylation upon IL-9 stimulation²¹. Therefore, we hypothesize that, although MS3-6 does not target the SH2 domain of STAT3, the fact that it binds and induces a conformational change of the coiled-coil domain could indirectly perturb the docking of STAT3 on receptor phosphotyrosines and its subsequent phosphorylation. In addition, a study showed that the coiled-coil domain of STAT3 is implicated in the recruitment of STAT3 to the receptor, especially helices $\alpha 1$ and $\alpha 2$ that are bound by MS3-6²². It is therefore not unlikely that the monobody interferes with this process. In that case, why is this small impact not observed for IL-6? We noticed that the design of experiments was not the same for Ba/F3 or BW5147 and for A549. In Ba/F3 and BW5147 cells, the vector coding for the monobody was transiently transfected 4 hours before stimulation, which induces a high level of vector expression. In contrast, A549 cells stably expressed the monobody under the control of doxycycline, which leads to a smaller expression of the monobody as compared to electroporation. It suggests that only high expression of MS3-6 is able to provoke a slight decrease of STAT3 phosphorylation. In contrast, the smaller expression of MS3-6 obtained in A549 is not sufficient to significantly affect STAT3 phosphorylation induced by IL-6. To support this hypothesis, electroporation of the vector coding for MS3-6 in A549 also leads to a decrease in STAT3 phosphorylation, similarly to what is observed for IL-9 (data not shown). Therefore, MS3-6 slightly decreases STAT3 phosphorylation when highly expressed. Nevertheless, the impact of the monobody on STAT3 classical phosphorylation is minor as compared to the effect obtained on alternative pathways.

While MS3-6 does not equally affect the phosphorylation of STAT3 in response to all cytokines, we observed a prominent decrease of the transcriptional activity of STAT3 by MS3-6 following all stimulation tested. This phenomenon results from a decrease in nuclear translocation, as shown by the immunofluorescence experiment of Grégory La Sala. The structure revealed that MS3-6 binds the coiled-coil domain of STAT3, which includes an NLS composed of R214 and R215¹⁹. Therefore, MS3-6 could interfere with the binding of

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importin to this NLS. However, another NLS comprised in the DBD of STAT3 is proposed, which could explain that MS3-6 does not completely block the nuclear translocation of STAT3^{19, 23}. Therefore, the mechanism of action of the MS3-6 monoclonal antibody is a combination of multiple factors as it interferes with STAT3 signaling at various levels: it blocks phosphorylation (for IL-22 and IL-24), decreases nuclear translocation and partially inhibits DNA binding (data not shown, obtained by Grégory La Sala). Interestingly, despite the fact that the coiled-coil domain of STAT3 shares 42 % and 26 % identity with that of STAT1 and STAT5, respectively, MS3-6 specifically targets STAT3 over other STATs. Indeed, STAT1 and STAT5 phosphorylation were not affected, neither is the transcriptional activity of STAT1 and STAT5.

In conclusion, the monoclonal antibody MS3-6 is not the perfect tool to specifically block the alternative activation of STAT3 induced by IL-22 because it widely interferes with STAT3 function. Rather, a monoclonal antibody targeting the C-terminus of IL-22R α would be more specific. Still, this monoclonal antibody could be a potential therapeutic approach in the context of psoriasis, a STAT3-driven disease. Indeed, many cytokines involved in the pathogenesis signal via STAT3. Nevertheless, STAT3 blockade could cause deleterious side effects (increased sensitivity to infections or colitis), which implies that this monoclonal antibody should be used locally in the skin. In this perspective, the *in vivo* potential of the monoclonal antibody should be explored, as well as a skin delivery strategy.

Materials and methods

Antibodies and cytokines

Antibodies used to measure STAT phosphorylation by flow cytometry were purchased from BD Biosciences: APC-labeled anti-STAT3-pY705 (557815), APC-labeled anti-STAT1-pY701 (612597) and APC-labeled anti-STAT5-pY694 (612599). AF488 labeled anti-cMyc tag (2279) was purchased from Cell signaling technologies. For western-blot, anti-STAT3 (12640) and anti-His tag (12698) antibodies were purchased at Cell signaling technologies while anti-GST (RPN1236V) and anti-Flag tag (F1804) antibodies were purchased at Sigma.

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Various cytokine stimulations were used in the experiments: human IL-22 (500 ng/ml), human IL-6 (100 ng/ml), murine IL-9 (100 U/ml), human IL-9 (1000 U/ml), human IL-24 (HEK293 supernatant 2%), human IFN- λ 3 (HEK293 supernatant 2%) and murine IL-3 (100 U/ml). Cytokines are produced in the house.

Monobody expression and flow cytometry analysis

10⁷ BW5147 or Ba/F3 cells were electroporated with the plasmid coding for monobody (15 μ g) under specific conditions (74 Ω , 270 V, 1200 μ F) and used after 4 hours of incubation at 37°C. Ba/F3 cells were washed 3 times in PBS prior the electroporation. Parental A549 cells were transiently electroporated with vector coding for the monobody under specific conditions (74 Ω , 250 V, 1200 μ F) and used after 4 hours of incubation at 37°C. A549-MS3-6 and A549-HA4-Y87A cells, which stably integrated the doxycycline inducible monobody construct, were plated (10⁵ cells) in 6-well plates. The next day, cells were treated with control medium or Doxycycline (Sigma) at 1 μ g/ml for 48 hours. Cells were stimulated with control medium or with cytokine for 15 min at 37°C before FACS staining.

After incubation with a viability marker (LIVE/DEAD Fixation Near-IR Dead Cell Stain Kit, Live), cells were fixed in paraformaldehyde 2% for 10 min at 37°C and then permeabilized in 90% methanol for 30 min on ice. After two washes with PBS-EDTA 1% FCS, cells were stained with AF488 labeled anti-cMyc tag (2279, CST) and APC-labeled anti-STAT3-pY705 (557815, BD Biosciences) or APC-labeled anti-STAT1-pY701 (612597, BD Biosciences) or APC-labeled anti-STAT5-pY694 (612599, BD Biosciences) for 1 hour at room temperature. Cells were analyzed on BD FACSVerser flow cytometer.

GST pull down assay

To monitor the interaction between STAT3 and the different GST-IL-22R mutants generated previously¹⁶, the proteins were expressed independently in COS-7 cells. Briefly, 4x10⁵ COS-7 cells plated in a 6-well plate were transiently transfected using ViaFect (Promega), according to the manufacturer's recommendations. Two days later, cells were lysed in 500 μ l of lysis buffer (1% Triton X-100, 10% glycerol, 10 mM Tris (pH 8), 150 mM sodium vanadate, 1 mM

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sodium fluoride, 5 mM EDTA, 1 mM DTT and cOmplete Protease Inhibitor Cocktail (Sigma)) and cell debris were removed by centrifugation. STAT3 was mixed with recombinant monobody at 10 μ M for 8 hours at 4°C. Then, GST-proteins were added for an additional 16 hours. Purification of GST was performed using GST SpinTrap columns (GE Healthcare Life Sciences) according to the manufacturer's recommendations. Input and eluted samples were analyzed by western-blot with an anti-STAT3 antibody. The membranes were then reprobed with anti-GST, anti-Flag tag antibody and anti-His tag antibody.

Luciferase assay

Luciferase assays were performed with the pGL3-Pap1 and pGRR5 luciferase reporter plasmid as a specific promoter for STAT3 activation or STAT1/3/5 activation, respectively. pRL-TK plasmid was used as internal control of transfection.

10⁷ BW5147 or Ba/F3 cells were electroporated with vector coding for the monobody (7,5 μ g), pGL3-Pap1 or pGRR5 luciferase reporter plasmid (7,5 μ g) and pRL-TK plasmid (1 μ g) under specific conditions (74 Ω , 270 V, 1200 μ F). Ba/F3 cells were washed 3 times in PBS and rested for 3 hours prior the electroporation. Parental A549 cells were transiently electroporated (74 Ω , 250 V, 1200 μ F) with vector coding for the monobody (7,5 μ g), pGRR5 luciferase reporter plasmid (7,5 μ g) and pRL-TK plasmid (1 μ g). Cells were then stimulated with control medium or with cytokine for 4 hours. A549 with inducible monobody (HA4-Y87A or MS3-6) expression (5x10³ cells) were plated in a 96-well plate. After 24 hours of treatment with Doxycycline (Sigma) at 1 μ g/ml, cells were transiently transfected using ViaFect (Promega), according to the manufacturer's recommendations, with the pGL3-Pap1 luciferase reporter plasmid and pRL-TK plasmid. 4 hours after transfection, cells were stimulated with control medium or with cytokine for 20 hours at 37°C.

Luciferase activity was revealed with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's recommendation.

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Recombinant protein expression and X-ray crystallography (Grégory La Sala)

BL21 (DE3) *E. coli* cells were transformed with pHBT-monobody and pHBT-STAT3-CF constructs. An overnight pre culture was added to LB medium with an original OD at 0.05 to 0.1. Cells were grown at 37°, 200 rpm until OD reached 0.5-0.8 and the culture was next induced with 0.5 mM IPTG final concentration. Cells were further grown overnight at 18°C 200 rpm. The next day, cells were harvested at 6000g for 10 minutes at 4°C and resuspended in 40ml/L of culture in Buffer A [25 mM Tris (pH 7.5), 300 mM NaCl, 5% glycerol, and depending on sample, 1 mM DTT]. Cells were lysed using an Avestin Emulsiflex homogenizer and the soluble proteins were isolated from the cell debris by centrifugation at 41'000g for 30 min at 4°C. Proteins of interest were next purified by affinity using a Ni-NTA resin and size-exclusion chromatography using a Superdex 200 16/600 GL column. Complex formation was performed by mixing TEV cleaved-MS3-6 and STAT3-CF in a 1:1 molar ratio into gel filtration buffer (20mM HEPES pH 7, 200 mM NaCl, 10mM MgCl₂ and 2mM DTT). The complex was concentrated in Amicon vivaspin tubes to 11mg/ml and 0.6mM dsDNA (forward: TGCATTTCCTGAAATCT3' and reverse: 5'AAGATTACGGGAAATGC3') was added to the drop before crystallization trials in hanging drop plates by mixing 100 nL of protein with 100 nL of buffer conditions. Addition of DNA was necessary for crystallization, despite not appearing in the final structure. Crystals were optimized in a 24-well plate format (hanging drop) by mixing 1µL of protein with 1µL of buffer. The best diffracting crystals were obtained in 19% PEG300, 70mM Calcium acetate dihydrate, 100mM imidazole pH 7 conditions, frozen in 15 % (v:v) glycerol in liquid nitrogen. Data were collected at the beam-line PXIII of the Swiss Light Source (SLS), Villigen, Switzerland. Data were collected at 1Å and at 100K. Data was processed in XDS (Kabsch, 1993). The structure was determined by the molecular replacement method (molrep, PHENIX) using a previously determined STAT3 structure (PDB:4E68) and a homology model of the monobody MS3-6 as initial search models. Manual model building of the structure was performed using Coot and refined with REFMAC59. The final model geometry was with 93.2% in favored regions of the Ramachandran plot with 1.8% of Ramachandran outliers.

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Immunofluorescence (Grégory La Sala)

Immunofluorescence was performed using transiently transfected HEK293 cells (Polyfect Qiagen No. 301107) that expressed an inducible GFP-tagged monobody upon doxycycline treatment (1µg/ml) for 48 hours. Cells were stimulated with IL-6 (100ng/ml) for 20 minutes at 37°C and were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Slides were next washed with PBS containing 0.01% of Triton-X and stained for 1 hour at room temperature using a primary antibody (mouse anti-STAT3). Secondary antibody detection was performed using an anti-mouse Alexa Fluor 568 antibody. Hoechst staining was performed for 5 minutes during the last wash. Cells were imaged with a Zeiss LSM 700 confocal microscope. Image analysis and cellular compartments were defined according to a CellProfiler workflow: a primary object detection defined the nucleus according to the Hoechst staining. Secondary object detection was performed by expansion of the GFP signal around the nucleus, which defined the cell outlines. The tertiary object corresponded to the nucleus area removed from the cell outline, which defined the cytoplasm. Mean intensity of both GFP and the anti-mouse Alexa Fluor 568 secondary antibody signals (STAT3) were quantified in all objects. Data were presented as a ratio of nuclear STAT3:cytoplasm STAT3. A total of approximately 30 cells from two individual experiments were acquired and quantified. Cells whose automatic boundary detection failed were discarded from final analysis.

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DISCUSSION

DISCUSSION

In previous studies, IL-22 was shown to be deleterious in the skin disease psoriasis. However, all IL-22 activities are not detrimental. Contrariwise, it is beneficial in colitis or wound healing by promoting regeneration of tissues and defense against pathogens. Hence, partially blocking IL-22 activities might be useful in the treatment of psoriasis. In this perspective, the project of my thesis was based on the alternative activation of STAT3 induced by IL-22. We studied the role of this pathway *in vivo*, by generating mice with a truncated IL-22R α , characterized its involvement in the imiquimod-induced psoriasis and *Citrobacter rodentium*-induced colitis. In addition, we investigated a strategy to specifically inhibit this pathway using monobodies.

In the first part of this work, we showed that truncation of the C-ter of IL-22R α decreases STAT3 activation in epidermal cells following IL-22 stimulation. As a result, the absence of the alternative pathway partially protects mice from the imiquimod model of psoriasis, showing that the alternative activation of STAT3 is relevant in the skin. By contrast, our results suggest that the alternative pathway is less involved in IL-22 signaling in the gut than in the skin. First, STAT3 phosphorylation is still observed in the absence of C-ter following IL-22 stimulation. Second, the absence of IL-22R α C-ter did not impact the *Citrobacter rodentium* model, contrary to *Il22ra1* deficiency which strongly affects mice.

In the second study, the impact of a monobody targeting the coiled-coil domain of STAT3 on IL-22 signaling was studied *in vitro*. We showed that this monobody enables the decrease of the alternative activation of STAT3 but not the classical one, depending on tyrosines. However, it also perturbs the nuclear translocation of this factor, thus affecting the signaling of all STAT3-activating cytokines.

These results have already been discussed in the results section of this thesis. However, some questions, observations and perspectives can be deepened. The first point of this

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discussion is dedicated to the limitations of CRISPR/Cas9 model used *in vitro* and *in vivo*. Second, I will discuss the alternative activation of STAT3, its mechanism and role *in vivo*. Finally, the potential of monobodies as therapy will be the subject of the last part of this discussion.

1. GENOME EDITING WITH CRISPR/CAS9 TECHNOLOGY: LIMITATIONS AND INFLUENCE ON PROTEIN EXPRESSION

1. General limitations

CRISPR/Cas9 system is based on a simple DNA-gRNA binding with the gRNA directing the nuclease Cas9, which is responsible for the double-strand break. Hence, limitations of this system are the occurrence of off-target mutations if the gRNA binds unspecific sequences. Indeed, a limited number of mismatches is tolerated, especially in 5' of the gRNA^{201, 202}. This is therefore important to choose a gRNA for which no highly probable off-targets are predicted. In MES13, the three most likely off-targets were sequenced and no mutations were found in all the clones analyzed. As for the mice, we did not analyze off-targets in the Δ GA and GCC mice but the whole genome sequencing of the Δ Cter mice is ongoing, although no mutations were found in the five most likely off-target sites. Hence, this technology must be used carefully or knowing that other mutations can occur, alter the function of a gene and impact on the results. In addition, we used cellular clones in our *in vitro* experiments, which could also lead to the selection of clones that acquired a mutation. This is why, in our project, we worked with multiple cell lines and kept several clones, when possible, to compare the results with each other. In one set of experiments performed in HT-29, we encountered such problems. Indeed, cells possessing similar *IL22RA1* mutations did not show similar results in terms of IL-22 signaling. This suggests that, in these cells, another event occurred because of the CRISPR/Cas9 or the cellular cloning and influence IL-22 signaling.

Discussion

2. A case study: Δ GA and GCC mice

In the first years of my thesis, we took advantage of CRISPR/Cas9 technology to mutate the C-ter part of IL-22R α in several cell lines and in mice (called Δ GA and GCC). The strategy to use a gRNA to induce DNA double break and NHEJ repair pathway seemed appropriate as we wanted to modify the sequence of the C-ter. Keeping a small sequence instead of truncation of the receptor even seemed to be an advantage because it would less disturb the structure of protein. Moreover, in cells or in mice, we worked with at least two mutants to make sure that the results we obtained were not influenced by the new C-ter sequence that was changed. Unfortunately, the sequence of the IL-22R α implies that a frameshift does not cause the rapid introduction of a STOP codon: both frameshifts lead to a new sequence of 71 and 37 amino acids before the STOP codon. As a result, there were problems with the expression of these mutated receptors. While mRNA expression of the WT and mutant receptors were similar, we could not detect the IL-22R α at the cell surface. It is important to mention that it is unlikely that the receptor is completely absent. Indeed, following IL-22 stimulation, we observed a small response in Δ GA and GCC mice, suggesting that these mice are not full knockout for the *Il22ra1*. Flow cytometry is probably not sensitive enough to detect low amounts of receptors at the surface of cells. Unfortunately, detecting the IL-22R α is quite complicated and we did not succeed in analyzing it by western-blot on organ lysates.

Since we do not know if the total level of IL-22R α protein is similar between WT and mutated mice, we cannot exclude that translation of the mutated mRNA is decreased. Otherwise, we postulate that mutations of the C-ter perturbed the trafficking of the receptor within the cells. First, receptors constitutively undergo endocytosis, after which they are either recycled at the membrane or degraded in lysosome. This process regulates the number of receptors at the membrane and depends on the presence of internalization motif^{203, 204}. The mutant sequences of the IL-22R α C-ter could contain new internalization motifs, causing an acceleration and/or an increase of the internalization of the receptor, leading to a decrease of its surface expression. Second, to be exported to the membrane,

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receptors need to follow the secretory pathway involving their proper folding and maturation in the endoplasmic reticulum and Golgi followed by their transport to the membrane through vesicles. In case of misfolding or defect in the maturation, proteins are retained in the reticulum/Golgi and degraded^{205, 206}. If the new C-ter prevented the receptor folding or maturation, it could have affected the secretory pathway and, thereby, its surface expression. Another hypothesis is that changing the sequence of the C-ter tail of IL-22R α affected the interaction between the receptor and JAK1 because of a change in the receptor conformation. The binding of JAK to cytokine receptors was shown to be required for surface expression of various receptors. For example, EPO-R and JAK2 are already bound together in the endoplasmic reticulum and the absence of this interaction prevents the correct surface expression of the receptor²⁰⁷. A similar mechanism was also described for TPO-R, IFNAR1 and IL-10R β ^{208, 209}. These studies suggest that JAKs enhance the maturation of cytokine receptors in the Golgi and also stabilize receptors at the cell surface by preventing internalization and degradation. Hence, if Δ GA or GCC mutations prevented the binding of JAK, it could have impacted the surface expression of the receptor.

These expression problems were not observed when we truncated the receptor. In conclusion, CRISPR/Cas9 is a great tool to easily create genetically modified mice, but it has to be used carefully since modifying protein sequence using NHEJ can have side effects besides modifying the function of the protein.

2. THE ALTERNATIVE PATHWAY: MECHANISM AND ROLE *IN VIVO*

1. What is the mechanism of the alternative activation of STAT3?

Although the alternative activation of STAT3 was discovered in 2009, questions remain about the involvement of JAKs in the trigger of the activation or about the residues of IL-22R α that are important. To address the first question, IL-22 signaling was studied *in vitro* using luciferase assay in cells expressing mutants IL-22R α in the presence of CMP6, a pan-JAK inhibitor. As expected, JAK inhibitor blocked the classical pathway induced by IL-22.

Discussion

The alternative pathway was also affected by the inhibitor, as shown in cells expressing the tyrosine-less receptor. Therefore, this suggests that both classical and alternative pathways are JAK-dependent (Figure 1).

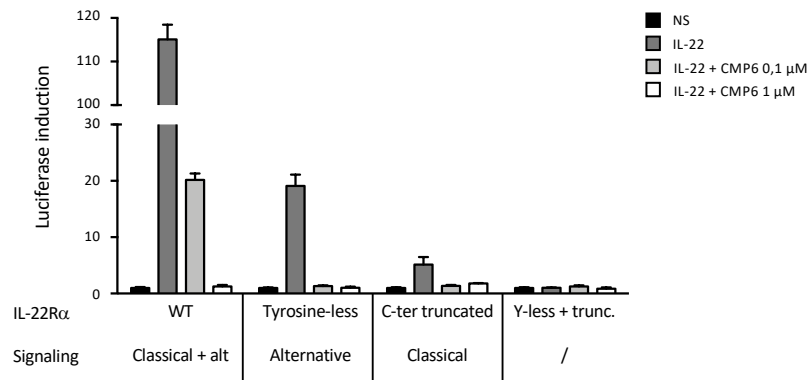


Figure 1: The classical and alternative pathways require JAK to be activated.

Luciferase assay on BW5147 cells stably expressing mutants IL-22Rα. Cells were electroporated with pGL3-PAP1, a STAT3-luciferase reporter, and pRL-TK, as internal control of transfection, and were left untreated or stimulated with IL-22 (500 ng/ml) in the presence or not of CMP6 (0,1 or 1 μM) for 4 hours before luciferase activity was analyzed. Data are presented as mean ± SEM.

The second important point is the fact that the alternative pathway is based on the constitutive interaction between the C-ter of IL-22Rα and the coiled-coil domain of STAT3. However, the exact residues required for this interaction are not known. The involvement of only some amino acids is unlikely because progressive truncations of the C-ter of IL-22Rα progressively causes a decrease in STAT3 activation (unpublished results). Coiled-coil domains are composed of at least 2 α-helices wrapped around each other. The structure of the helices follows a periodicity of seven-residue repeat (heptad repeat) noted *a-b-c-d-e-f-g*, in which amino acids *a* / *d* are typically hydrophobic and *e* / *g* are polar residues. Residues *a* / *d* interact with residues *a'* / *d'* from another helix to stabilize the dimerization and *e* / *g* form interhelical electrostatic interactions²¹⁰. The analysis of the IL-22Rα C-ter protein sequence on *Paircoil*²¹¹ or *Coils*²¹² does not predict it to form a

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coiled-coil. On the other hand, the secondary structure prediction of the C-ter on Phyre^{2 213}, despite the low confidence score, suggests that it might contain a small α -helix. Hence, amino acids of the C-ter part of IL-22R α might form an α -helix capable of interaction with the coiled-coil domain of STAT3. Interestingly, the alignment of human and mouse IL-22R α shows that residues possibly involved in the α -helix, in green, are conserved (Figure 2).

		*		20		*		40		*		60		*		80																																																																							
hIL-22Ra	L	K	G	Q	L	P	L	S	S	V	Q	I	E	G	H	P	M	S	L	P	L	Q	P	P	S	R	P	C	S	P	S	D	Q	G	P	S	P	W	G	L	L	E	S	L	V	C	P	-----	K	D	E	A	K	S	P	A	P	E	T	S	D	L	E	Q	P	T	E	L	D	S	L	F	R	G	L	A	L	T	V	Q	W	E	S				
	L	K	G		L		L	S	S	V	Q	I	E	G	H	P	+	S	L	P	L		S		C	S	P	S	D	+	G	P	S	P	W	G	L	+	S	L	V	C	P		+	E	A		P	+		S	+	L	E	Q		T	E	L	D	S	L	F	+	G	L	A	L	T	V	Q	W	E	S												
mIL-22Ra	L	K	G	A	L	S	L	S	S	V	Q	I	E	G	H	P	V	S	L	P	L	H	V	H	S	V	S	C	S	P	S	D	E	G	P	S	P	W	G	L	D	S	L	V	C	P	K	D	E	G	P	A	V	E	T	A	M	C	P	S	A	A	A	S	E	L	E	Q	S	T	E	L	D	S	L	F	K	G	L	A	L	T	V	Q	W	E	S

Figure 2: alignment of human and mouse IL-22R α C-ter parts.

Alignment of the 83/90 last amino acids of human and mouse IL-22R α using diagonals method in DNA strider software. In green are the residues of the human IL-22R α possibly involved in a α -helix.

2. What are the advantages of alternative pathways?

A tyrosine-independent activation of STAT was demonstrated for IL-22R α and other cytokine receptors, suggesting that it might provide some advantages to these cytokines. A first advantage would be quantitative. Indeed, classical and alternative mechanisms synergize to strongly induce STAT3 signaling in response to IL-22. Although the alternative pathway seems to be responsible for a bigger part of STAT3 activation *in vitro*, both are required to fully activate STAT3⁸⁰. On the contrary, micro-array data from the lab suggest that alternative and classical activation of STAT are not qualitatively different in terms of gene regulation since they both induce the same profile of expression with various levels of induction (unpublished results). Hence, the alternative pathway is added to the classical one to activate STAT3, the major actor of IL-22 signaling, rather than inducing different cellular responses. This strong activation of STAT3 by IL-22 is probably beneficial in hepatitis, colitis or pancreatitis in which the beneficial role of IL-22 was shown to be STAT3-dependent^{106, 143, 214}. However, this synergy also has a dark side since IL-22-mediated activation of STAT3 was related to several malignancies and psoriasis^{96, 127, 131, 215}.

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Another hypothesis is the kinetic advantage possibly provided by the pre-association of STAT3 with the IL-22R α . Indeed, it could enable a quick response following cytokine binding since STAT3 is directly accessible at the receptor⁸⁰. Interestingly, a pre-association was also demonstrated for STAT2 with the IFNAR^{22, 23}. Hence, the pre-association of STATs could facilitate effective and rapid defense against bacterial and viral pathogens in which IL-22 and IFNs are involved. In addition, SOCS-mediated downregulation implies binding to receptor phosphotyrosines to prevent the docking of STAT. Therefore, phosphotyrosine-independent mechanisms could partially escape SOCS regulation and, this way, enable a prolonged action of the cytokine. This hypothesis was tested previously in the lab for IL-22R α . Transfection of *Socs3* into cells expressing mutants IL-22R α showed that the classical pathway is more sensitive to SOCS3 than the alternative pathway (unpublished results). Here again, a prolonged action of IL-22 could provide a powerful defense against pathogens or better tissue regeneration. Whether for rapid or prolonged action, kinetics of classical and alternative signaling of IL-22 should be studied to confirm these hypotheses. As far as we know, no difference in kinetics were observed between tyrosine-dependent and -independent mechanisms for other cytokines than IL-22, notably G-CSF and EPO^{20, 216}.

Finally, the alternative activation of STAT3 could constitute a backup pathway in case of mutations in the IL-22R α . From the study previously performed in the lab, some tyrosines of the receptor were shown to be involved in STAT3 activation induced by IL-22, especially Tyr³²⁶ and tyrosines between residues 351 and 403⁷⁹. Therefore, if these tyrosines were absent, STAT3 phosphorylation would still be maintained by the alternative pathway. By analyzing the *IL22RA1* on GnomAD, the Genome Aggregation Database, no polymorphisms were found in Tyr³²⁶, but three missense polymorphisms are described in tyrosine residues (Tyr³⁵¹Cys, Tyr³⁷⁷Cys and Tyr⁴⁹¹Asn). However, these polymorphisms are extremely rare as they were found only once amongst the high number of alleles analyzed (<<0,1%). Hence, there is no evidence that the alternative pathway brings a significant advantage in terms of backup pathway.

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3. Are all IL-10 family cytokines able to induce an alternative activation of STAT?

An alternative activation of STAT that is independent of receptor tyrosines has already been described for IL-22R α and IFN- λ R, two receptors that bind cytokines belonging to IL-10 family. Therefore, we wondered if all cytokines of this family are able to activate an alternative pathway. Regarding IL-10, two tyrosines of the IL-10R α were shown to be required for STAT3 activation. An alternative activation of STAT3 for IL-10 is thus unlikely²¹⁷. During my thesis, we started a small project to attempt to answer this question concerning another subset of cytokines of the IL-10 family: IL-19, IL-20 and IL-24. As a reminder, IL-20 and IL-24 bind two complexes of receptors so the ability to induce a tyrosine-independent activation of STAT3 was evaluated for both.

Logically, the action of IL-24 on its type 2 receptor (IL-22R α + IL-20R β) leads to both classical and alternative activation of STAT3 (Figure 3A). Indeed, IL-24 still induced the phosphorylation of STAT3 in the absence of IL-22R α tyrosines. The tyrosine-independent activation of STAT3 is mediated by the C-ter part of IL-22R α because its truncation decreased STAT3 phosphorylation or abolished STAT3 phosphorylation in the absence of IL-22R α tyrosines. These results were expected since cytokines signaling is based on the α chain. Of note, the profile of phosphorylation of STAT3 was similar to that observed following IL-22 stimulation (Figure 3B).

Discussion

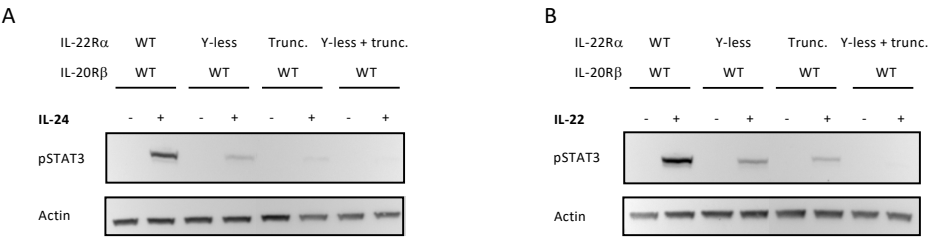


Figure 3: STAT3 is phosphorylated in the absence of IL-22Rα tyrosines following IL-24 stimulation. Western-blot analysis of BW5147 cells expressing mutants IL-22Rα and WT IL-20Rβ. Cells were stimulated with control medium or IL-24 (A) or IL-22 (B) at 500 ng/ml for 15 minutes before lysis in O'Farrell buffer. STAT3 phosphorylation was analyzed by western-blot. Y-less = all cytoplasmic tyrosines mutated in phenylalanine. Trunc. = C-ter part truncated

In parallel, we also studied IL-24 signaling on its type 1 receptor (IL-20Rα + IL-20Rβ). To investigate a tyrosine-independent mechanism in the IL-20Rα, the 11 tyrosine residues of the cytoplasmic part were mutated in phenylalanine (Figure 4A). This mutant receptor (F1-11) was transfected in COS-7 cells together with a STAT3-responsive luciferase reporter, and the luciferase signal was compared with that of the WT receptor. Interestingly, in the absence of IL-20Rα cytoplasmic tyrosines, one third of luciferase signal was still obtained compared to the signal with the WT receptor (Figure 4B). This result suggests that STAT3 can be activated independently of IL-20Rα tyrosines.

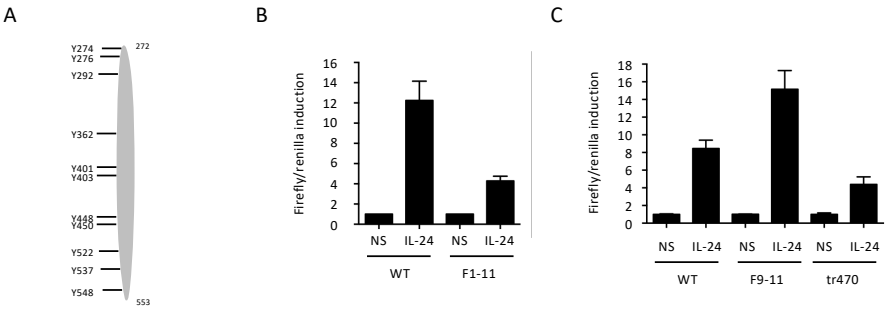


Figure 4: STAT3 activity is maintained in the absence of IL-20Rα tyrosines. (A) Scheme of the intracellular domain of IL-20Rα and its tyrosines. (B-C) Luciferase assay on COS-7 cells transfected with mutant IL-20Rα, WT IL-20Rβ, pGL3-PAP1 and pRL-TK. Four hours after transfection, cells were stimulated for 20 hours with control medium or IL-24 (100 ng/ml). Data are presented as mean ± SEM and are pooled from three independent experiments in triplicates. Results obtained with Ornella Calà

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To define whether this mechanism is similar to that observed in IL-22R α , the implication of IL-20R α C-ter tail in the alternative pathway was studied by truncating the receptor at amino acid 470. However, unlike the IL-22R α , this region contains tyrosines in the IL-20R α . To discriminate the role of C-ter tail and its tyrosines, another mutant was added in the study in which tyrosines 522, 537 and 548 comprised in the C-ter tail are mutated in phenylalanine (F9-11). Truncation of the IL-20R α lead to a decrease of luciferase signal while the mutation of tyrosines did not (Figure 4C). Hence, this suggests that the C-ter part of IL-20R α is implicated, regardless of its tyrosines, in an alternative activation of STAT3. It should be now interesting to define whether STAT3 is also pre-associated with this receptor and attempt to restrict, within this region, the amino acids that are implicated in the alternative pathway. By aligning the C-ter parts of IL-22R α and IL-20R α , some identity is found all along the C-ter tail (Figure 5). This suggests that, similarly to IL-22R α , it is unlikely that only one motif is responsible for this pathway. Performing progressive truncations could confirm this hypothesis or help to define the region required for the alternative pathway.

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hIL-22Ra LKGQLPLLSSVQIEGHMPSLPLQPPSRPCSPSD-QG--PS-PWGL-LESIVCPKDEAKSPAPETSDLEQPTELDLSLFRGLALTQWES
          G   S+  ++  P +  L  PS      D +G  PS   GL  E L+   E   PAP+   E  T  L       L  VQ  E+
hIL-20Ra --GPPEEPSTTLVDWDPTGRLCIPSLSSFDQDSEGCEPSEGDLGEEGLLSRLYE--EPAPDRPPGENETYLMQFMEEWGLYVQMEN
          *           20           *           40           *           60           *           80

% Identity = 28.0 (25/88)      % Homology = 9.0 (8/88)      % Total = 37.5 (33/88)

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Figure 5: alignment of IL-22R α and IL-20R α C-ter parts.

The 83/84 last amino acids of IL-22R α and IL-20R α were aligned using diagonals method in DNA strider.

Finally, the use of the monobody MS3-6 suggests that the coiled-coil domain of STAT3 plays an important role in IL-24 signaling and strengthens the observations of an alternative pathway. Indeed, the monobody strongly reduced STAT3 phosphorylation in response to IL-24. Therefore, the interaction between the IL-20R α and STAT3, in particular the coiled-coil domain, should be analyzed.

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4. How to explain the difference between the skin and gut?

From our study, we conclude that the alternative pathway induced by IL-22 is involved in IL-22 signaling *in vivo* in the skin and in the imiquimod model. Similar observations were made in the liver and lungs. By contrast, it has only a minor role in the gut because we did not notice a strong reduction of STAT3 activation nor an effect on the *Citrobacter* model in $\Delta Cter^{mut/mut}$. Therefore, an interesting question concerns the factors that could influence IL-22 signaling and explain the differences we observed between organs.

A first hypothesis could be related to the expression of STAT3. With a high expression of STAT3, the interaction between STAT3 and IL-22R α C-ter could be possible because STAT3 is in excess. On the contrary, if STAT3 concentration is lower, this interaction would not, or at least to a lesser extent, take place. It implies that the alternative pathway would not be induced, or only partially. The analysis of *Stat3* expression by RT-qPCR in the ileum and the skin supports this hypothesis (Figure 6A). Indeed, *Stat3* is less expressed in the ileum than in the skin. Similarly, the expression of *Stat3* is higher in MES13 cells, in which we showed that the alternative pathway is responsible for a part of STAT3 activation. However, even though there is a significant difference of expression between the skin and the ileum, *Stat3* is still highly expressed in the ileum, suggesting that STAT3 level might not be the explanation.

Second, the level of expression of the receptor could also influence the signaling. Indeed, surface expression of the IL-22R α measured by flow cytometry is higher in intestinal epithelial cells than in keratinocytes (Figure 6B). At the mRNA level, *il22ra1* is also more expressed in the ileum than in the skin (Figure 6C). Therefore, truncation of the IL-22R α and the subsequent loss of alternative pathway might have less impact on IL-22 signaling in the gut. Indeed, as the receptor is more expressed, the classical pathway might be sufficient to trigger a strong activation of STAT3.

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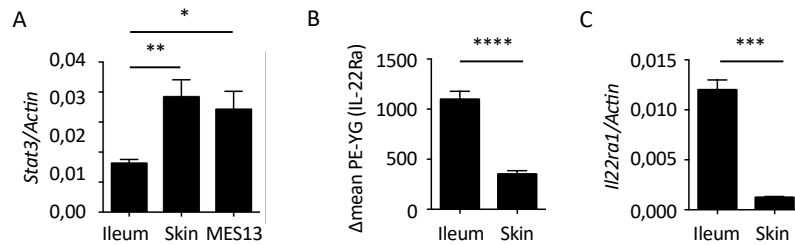


Figure 6: surface and mRNA expression of the IL-22Rα.

(A) RT-qPCR analysis of *Stat3* expression in the ileum, in the skin and in MES13 cells. Expression related to β -Actin. Data are shown as mean $\pm$ SEM of three independent experiments ($n = 3$ -5 samples/group in each experiment). (B) Flow cytometry experiment to measure IL-22R α expression at the surface of intestinal epithelial cells and keratinocytes. Staining was quantified using delta mean between stained and not stained cells. Data are shown as mean $\pm$ SEM of three independent experiments ($n = 4$ -5 mice/group in each experiment). (C) RT-qPCR analysis of *Il22ra1* expression in the ileum and in the skin. Expression related to β -Actin. Data are shown as mean $\pm$ SEM of two independent experiments ($n = 3$ -5 samples/group in each experiment). Statistical analysis: (A) Kruskal Wallis, (B, C) Mann Withney (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Finally, an intermediate protein might be required for the pre-association of STAT3 with the IL-22R α C-ter. If this protein is differentially expressed between organs, this could also explain the difference between the skin and the ileum. The interaction between STAT3 and IL-22R α C-ter has been demonstrated by immunoprecipitation and GST pull down assay, which cannot distinguish direct and indirect interactions. However, Grégory La Sala performed ITC (Isothermal titration calorimetry) experiments and confirmed the direct interaction between the IL-22R α C-ter and STAT3, which suggests that there is no intermediate protein required for this interaction. On the other hand, we cannot exclude that inhibitory proteins prevent this interaction. If expressed in the ileum and not in the skin, this could explain that the Δ Cter mutation did not strongly affect IL-22-mediated STAT3 activation in the gut. However, the pre-association of STAT3 with the IL-22R α was detected in HT-29 and Colo205 cell lines, two epithelial cell lines derived from colorectal cancer, suggesting that the alternative pathway can take place in the gut⁸⁰. Nonetheless, these are cells in culture and it does not reflect a physiological context where environmental factors might influence the expression of such inhibitory protein *in vivo*. The gut is an organ in which

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the microbiota plays a crucial role in immune regulation. This could be possible that the gut microbiota specifically induces the expression of an inhibitory protein to silence the alternative activation of STAT3 to limit the effects of IL-22 on intestinal cells and, consequently, on microbiota. However, data from the literature suggests that microbiota tends to increase IL-22 by favoring the development of IL-22-producing cells^{218, 219}.

5. Is the alternative pathway a good target for psoriasis treatment?

Since the absence of the C-ter of IL-22R α partially protects mice from the imiquimod model, we could consider targeting the alternative pathway as a therapeutic approach. This strategy presents some positive factors but also drawbacks.

Our results suggest that inhibiting the alternative pathway would partially block IL-22 signaling as well as a part of IL-20 and IL-24 activities because these cytokines can signal through IL-22R α . Although not much is known about IL-20 and IL-24, their role in psoriasis was studied and was shown to be similar to that of IL-22. Indeed, *Il20* and *Il24* transgenic mice display skin lesions with acanthosis and hyperkeratosis as well as neonatal mortality^{220, 221}. In addition, these cytokines are able to induce acanthosis on human epidermis models⁷⁰. Hence, compared to targeting IL-22, the approach to target the alternative pathway of IL-22R α has the advantage to partially block these three cytokines.

The fact that mice lacking the alternative pathway, the $\Delta Cter^{mut/mut}$, are not as sensitive to the *Citrobacter rodentium* model as the *Il22ra1*^{-/-} mice is also an encouraging result. Indeed, psoriasis and inflammatory bowel diseases are associated conditions sharing environmental risk factors, genetic predisposition and immunological mechanisms²²². The prevalence of inflammatory bowel disease, mostly Crohn's disease, is increased in psoriasis patients^{223, 224}. Since IL-22 is protective in the development of colitis, blocking this cytokine or its receptor could be deleterious and favor the development or worse IBD in psoriasis patients. In our study, blocking the alternative activation of STAT3 induced by IL-22 did not strongly affect

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the course of colitis. Therefore, an inhibitor of this alternative pathway might be an interesting option to treat psoriasis without inducing side effects in the gut, even if it was systemically administered. In contrast, STAT3 phosphorylation and/or STAT3-dependent gene expression were decreased in the liver and in the lungs, suggesting that side effects are possible for these organs. To evaluate this, the impact of the alternative pathway on hepatitis or lung infection should be investigated.

Nevertheless, we can wonder about the potential effectiveness of the inhibition of the alternative pathway. Indeed, following imiquimod treatment, *Il22ra1*^{-/-} mice are half protected from ears thickening. By contrast, the ear thickening is reduced by a quarter between $\Delta Cter^{mut/mut}$ and $\Delta Cter^{wt/wt}$. Regarding the other parameters that we analyzed, the protection was more prominent between *Il22ra1*^{-/-} mice and B6 than between $\Delta Cter^{wt/wt}$ and $\Delta Cter^{mut/mut}$, suggesting that targeting the C-ter of IL-22R α might probably modestly prevent skin lesions. However, we noticed that the receptor is more expressed in the skin of $\Delta Cter^{mut/mut}$ mice than in $\Delta Cter^{wt/wt}$. The protection provided by the inhibition of the alternative pathway is thus perhaps underestimated. Anyway, it might be interesting to combine IL-22R α C-ter strategy with another drug to improve the efficacy of the treatment.

3. ARE MS3-6 OR MONOBODIES GOOD TOOLS...

1. ... to target the alternative activation of STAT3 induced by IL-22?

As described in the results, the MS3-6 strongly impacted the alternative pathway induced by IL-22. However, it also slightly decreased tyrosine-dependent phosphorylation of STAT3 triggered by other cytokines. In terms of STAT3 transcriptional activity, MS3-6 strongly reduced it by preventing the nuclear translocation of STAT3. Hence, monobodies targeting the coiled-coil domain of STAT3 are not the optimal tool to specifically block the alternative activation of STAT3 induced by IL-22. Instead, developing monobodies targeting the C-ter of IL-22R α would be a more specific approach. C-ter-binding monobodies could also help to determine important residues within the IL-22R α that are required for its interaction with

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the coiled-coil of STAT3, either by comparing epitopes bound by monobodies and the effect on IL-22 signaling or by obtaining the crystal structure of the C-ter part of IL-22R α . Concerning MS3-6 monobody, it could still enable the discovery of new alternative pathways that require the coiled-coil domain of STAT3.

2. ... to use as treatment of psoriasis?

Many drugs used currently in psoriasis patients have the disadvantage to induce adverse effects after long-term use²²⁵. This is notably the case for Cyclosporin A that causes kidney and liver toxicity, methotrexate that is teratogenic or phosphodiesterase inhibitors that increase respiratory infections. Biologics, >TNF α , >IL-23 or >IL-17 antibodies, have also side effects such as increased risk of respiratory infections, worsening of IBD, hypertension... This is why there is still a need for new therapeutics. The use of JAK inhibitors is also studied in the context of psoriasis. As a reminder, Tofacitinib, an inhibitor of JAK3/JAK2/JAK1, has shown promising results in the treatment of psoriasis in mouse models and clinical trials. TYK2 inhibitors are also under studied^{185, 226}. However, these approaches might present some disadvantages. First, by inhibiting cytokines signaling in general, JAK inhibitors also prevent the action of anti-inflammatory cytokines. IL-10, for example, is an anti-inflammatory cytokine signaling through JAK1 and TYK2 that was shown to be protective in psoriasis^{227, 228}. Second, JAK inhibitors induce some side effects, an increased risk of infection for example, similar to biologics already used in clinic^{226, 229}.

Would the MS3-6 be efficient against psoriasis and overcome these problems? Since STAT3 was shown to be deleterious in this disease, it could be relevant to use MS3-6 as a STAT3 inhibitor. By specifically inhibiting STAT3 over other STATs, MS3-6 would have the advantage to reduce cytokines signaling but not completely, which could limit side effects. However, as it partially targets cytokines signaling, MS3-6 could be less efficient than JAK inhibitors or biologics.

Discussion

3. ... to use *in vivo*, and more particularly in the skin?

Several criteria suggest that monobodies are potentially good therapeutic tools. First, the absence of cysteine bonds facilitates their cytosolic action. Second, monobodies bind their target with a low nanomolar affinity. In addition, as FN3 domains are physiologically found in human proteins, monobodies present low immunogenicity. Finally, its small size likely increases the tissue distribution of monobodies but reduces the half-life, which is not optimal to reach therapeutic concentrations^{188, 191}. Unfortunately, delivery of monobodies into tissues remains the limiting factor. With the idea of using monobodies in therapy for psoriasis, would it be possible to deliver it into the skin?

The skin is an interesting organ for local delivery of therapeutics as it is large and easily accessible. Two kinds of deliveries are possible: topical (restricted to the skin) or transdermal (reaching the systemic circulation). Skin delivery has the advantage to limit side effects, contrary to drugs systemically delivered. However, the structure of the skin makes drug delivery complicated. Indeed, as described in the introduction, the upper part of the epidermis, the cornified layer, is composed of dead keratinocytes surrounded by a lipid envelope. Therefore, the skin is highly impermeable to the entry of external material. Furthermore, endocytosis is limited as cells in the upper layer are dead²³⁰. A study suggests that only molecules below 500 Da can be absorbed by the skin²³¹. This “500 Da rule” is a limiting factor for using larger molecules as new topical treatments. Methods were developed to enhance the entry of molecules in the skin by chemical or physical disruption of the skin barrier (electroporation, ultrasounds, microinjections) but these strategies are invasive and would not facilitate the treatment²³⁰. In addition, in psoriasis patients, lesions can appear in several places at the same time. Cream seems thus more appropriate than electroporation or microinjections. Hence, a better strategy would be to fuse the monobody with a small carrier to enhance drug delivery. These approaches were studied in the context of the skin, but not for monobodies. Yet, it highlights what the possibilities are. First, liposomes have been studied for a long time for their ability to enhance skin penetration of drugs because they have several advantages: they are notably biocompatible and enable

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the loading of hydrophobic and hydrophilic drugs. However, by fusing rapidly with cells, they are not believed to penetrate deeply in the skin, which could limit their efficacy. Modified liposomes were thus developed to improve their penetration²³⁰. In 1980, liposomes were used to increase the topical delivery of corticosteroids in psoriasis²³². More recently, topical applications of liposomal gel containing cyclosporin A on imiquimod-treated back skin of mice showed efficacy to reduce skin lesions²³³. Another class of nanodelivery is based on nanoparticles. By modifying their size, charge, shape or composition, it is possible to improve their skin delivery. Gold nanoparticles (AuNP), carrying VEGF for example, were shown to penetrate into the epidermis and dermis^{234, 235}. CPPs are also widely used to improve skin delivery of drugs and some studies show encouraging results. For example, while cyclosporin A cannot cross the skin, the conjugation of cyclosporin A to heptamer of arginines enables its penetration into the skin. It was observed in the epidermis and the dermis after topical applications on mice skin, but also on human skin grafted on mice²³⁶. Other studies used the GFP fused to different CPPs and showed a GFP signal in the epidermis 2 or 24 hours after topical application on the shaved skin of mice. It seems that GFP-CPPs accumulate in the hair follicles, suggesting that it uses the transappendageal pathway (along hair follicles and glands) to enter the skin^{237, 238}. Regarding the last strategy mentioned in the introduction, the fusion of monoclonal antibodies to toxin bacteria, nothing seems to be described in the literature about their use in the skin. Nevertheless, this strategy is studied in other contexts. One of the toxins, the nontoxic B-subunit of the bacterial Shiga toxin (StxB), is particularly interesting in the context of cancer because many cancer types were shown to upregulate its receptor, the Gb3/CD77²³⁹. The receptor is also highly expressed in the kidney and lungs and the enzyme responsible for its synthesis, the Gb3-synthase, was shown to be expressed moderately in the skin²⁴⁰, suggesting that it might be an interesting strategy in the skin.

To target IL-22 signaling in the skin, the best strategy would be to induce the delivery of monoclonal antibodies in keratinocytes. Hence, if CPPs preferentially lead to the transappendageal pathway, it might not be the best option. Instead, it would be better to favor the transcellular or intercellular pathways in which the carrier diffuses through the

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keratinocytes or the lipid envelope, respectively. Liposomes or nanoparticles might thus be more efficient to deliver the monobody in the epidermis²³⁰. Moreover, it could be interesting to add a molecule to the outer membrane of the vesicles in order to target specific cells. For example, targeting keratinocytes that highly express the IL-22R α could enhance the efficacy of the monobody. The profile of the expression of the IL-22R α within the epidermis is not well-known but, in our flow cytometry experiments, we noticed that cells expressing cytokeratin 10, which correspond to the spinous layer, co-expressed higher level of IL-22R α than other keratinocytes. Another strategy could be to target IL-22R α expressing keratinocytes in general. To do that, the Fab fragment of anti-IL-22R α antibodies could be fused to the liposome.

The monobody project presented in this thesis was entirely performed *in vitro*. We tried to study it in the skin by electroporating the vector coding for monobody but, unfortunately, we did not succeed in expressing the monobody in keratinocytes. Indeed, no expression of cMyc-tagged monobody in epidermis cells was detected by flow cytometry. In the future, it would be interesting to confirm the effects of the MS3-6 monobody *in vivo* in the skin. In this perspective, the electroporation method should be improved. Another solution would be to create a transgenic mouse by inserting the sequence coding for the monobody into the Rosa26 locus with a STOP cassette flanked by two loxP sites upstream of the transgene. Crossing these mice with K14-Cre mice will induce the specific expression of the monobody in the skin and help to determine its role in IL-22 signaling in keratinocytes and in the imiquimod-induced psoriasis model. This strategy, although not physiological, would be a proof of concept that the monobody is effective *in vivo*.

CONCLUSION

Conclusion

CONCLUSION

IL-22 is a cytokine involved in organ homeostasis, defense against pathogens and regeneration. However, prolonged and massive production of IL-22 is involved in psoriasis pathogenesis. Affecting around 2 % of the population, psoriasis is a skin disorder for which treatments are still limited. This is why we aimed at studying IL-22 signaling in the context of psoriasis. More particularly, since IL-22 induces a tyrosine-independent activation of STAT3, we used the imiquimod model of psoriasis in mice where the C-ter of IL-22R α , required for this alternative pathway, was truncated. We could show that the alternative pathway is partially involved in IL-22 effects in the skin because, following imiquimod treatment, C-ter truncation lead to less pronounced skin lesions. Contrariwise, the effect of the C-ter truncation on IL-22 signaling was limited in the gut and in *Citrobacter rodentium*-induced colitis. Hence, this study shows that the alternative pathway of IL-22 is a new candidate to target for psoriasis treatment. Of note, the role of the alternative activation of STAT3 induced by IL-22 should be studied in other organs such as the lungs or the liver to evaluate potential side effects of an IL-22R α C-ter targeting therapy.

In the second part of this work, a monobody targeting the coiled-coil domain of STAT3 was used to target the alternative pathway of IL-22. We studied its effects *in vitro* in the context of cytokines signaling. Although it decreased STAT3 phosphorylation mediated by the alternative pathway of IL-22R α , the monobody MS3-6 also affected STAT3 transcriptional activity in general. Hence, this monobody is not a suitable tool to inhibit specifically the alternative activation of STAT3 induced by IL-22. Nevertheless, it seems now important to investigate the effects of MS3-6 *in vivo*, notably to evaluate its protective effect on skin lesions induced by the imiquimod treatment. Indeed, STAT3 is known to be deleterious in this disease. For this purpose, the skin delivery of the monobody should be improved. Otherwise, it could be possible to generate a transgenic mouse expressing the monobody. Finally, deriving monobodies against the C-ter of IL-22R α would specifically target the alternative activation of STAT3 induced by IL-22 and not STAT3 induction mediated by all cytokines. In addition to analyze its effects on IL-22 signaling and functions in the imiquimod-induced psoriasis model, such monobodies could be used to better understand mechanisms of the alternative pathway.

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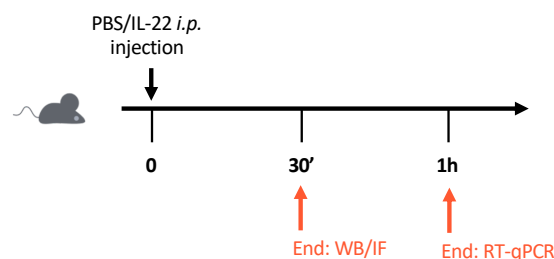
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ANNEX

1. MODELS USED IN OUR *IN VIVO* STUDY

1. Injection of PBS/IL-22 *i.p.*

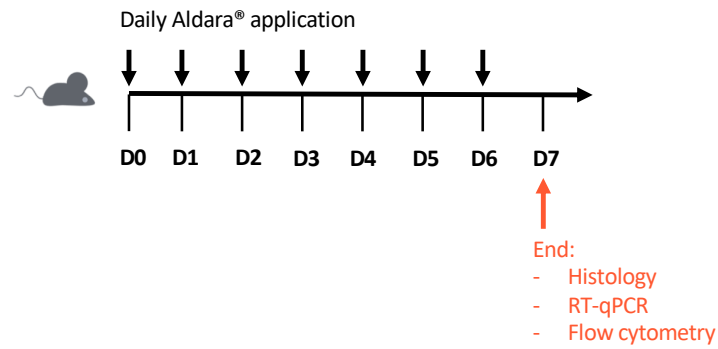
To analyze IL-22 signaling *in vivo*, PBS or IL-22 (10 µg) was injected *i.p.* in mice. After 30 minutes or 1 hour, organs were collected (ileum, liver, lung, colon and kidney). IL-22 signaling was analyzed by western-blot or immunohistofluorescence staining 30 minutes after injection and the expression of genes was analyzed by RT-qPCR after 1 hour.



2. Imiquimod model: model of psoriasis

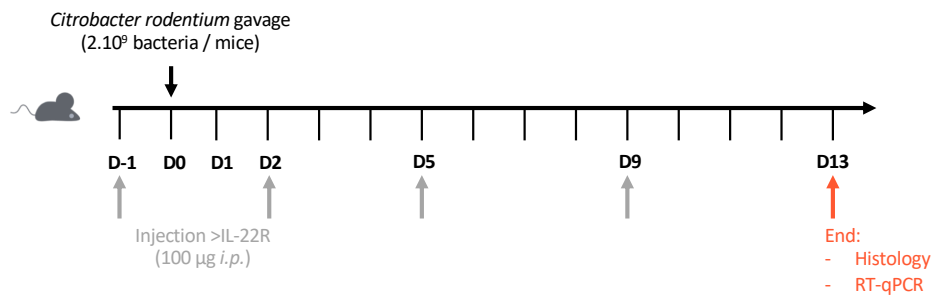
To define the impact of the IL-22R α and its C-ter part on the development of psoriasis, mice were treated daily with Imiquimod, contained in Aldara® cream, on both ear (0.8 mg per ear). Imiquimod induces the production of multiple pro-inflammatory cytokines, IFN- α , IL-23, IL-18, IL-1 β , TNF- α and IL-6. After daily application on the ears of mice, imiquimod causes psoriasis-like lesions. Ear thickness was measured every day with a micrometer screw to evaluate the disease. At the end of the model, ears were collected to perform histology, RT-qPCR and flow cytometry analysis.

Annex



3. *Citrobacter rodentium* model: model of colitis

To study the role of IL-22R α and its C-ter part on the development of colitis, mice were treated with *Citrobacter rodentium* via oral inoculation (2×10^9 CFU/mice) on day 0. Half of the mice were injected with an anti-IL-22R α antibody (100 μ g *i.p.*) on day -1, 2, 5 and 9. *Citrobacter rodentium* infection induces a dysbiosis which promotes inflammation in the colon. Mice were frequently weighted to monitor the evolution of the disease. At the end of the model, colons were collected and histology and RT-qPCR analysis were performed.



2. SELECTIVE INHIBITION OF STAT3 SIGNALING USING MONOBODIES TARGETING THE COILED-COIL DOMAIN AND N-TERMINAL DOMAINS



ARTICLE


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OPEN

Selective inhibition of STAT3 signaling using monobodies targeting the coiled-coil and N-terminal domains

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The transcription factor STAT3 is frequently activated in human solid and hematological malignancies and remains a challenging therapeutic target with no approved drugs to date. Here, we develop synthetic antibody mimetics, termed monobodies, to interfere with STAT3 signaling. These monobodies are highly selective for STAT3 and bind with nanomolar affinity to the N-terminal and coiled-coil domains. Interactome analysis detects no significant binding to other STATs or additional off-target proteins, confirming their exquisite specificity. Intracellular expression of monobodies fused to VHL, an E3 ubiquitin ligase substrate receptor, results in degradation of endogenous STAT3. The crystal structure of STAT3 in complex with monobody MS3-6 reveals bending of the coiled-coil domain, resulting in diminished DNA binding and nuclear translocation. MS3-6 expression strongly inhibits STAT3-dependent transcriptional activation and disrupts STAT3 interaction with the IL-22 receptor. Therefore, our study establishes innovative tools to interfere with STAT3 signaling by different molecular mechanisms.

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The signal transducer and activator of transcription (STAT) family is composed of STAT1-4, STAT5A, STAT5B, and STAT6 and is implicated in inflammation, proliferation, differentiation, cell survival and immune responses¹. These responses are driven by cytokines stimulating receptors upstream of the Janus kinases (JAKs) that are responsible for the phosphorylation of STAT proteins^{2,3}. This signal leads to the dimerization of STATs mediated by their SH2 domains and phosphotyrosine residues. STAT dimers then translocate into the nucleus and bind specific DNA sequences to regulate target gene expression^{4,5}.

STAT3 is ubiquitously expressed and activated by various interleukins, interferons, growth factors, as well as directly by oncogenic kinases such as Src, Abl or Met^{6–9}. Additionally, STAT3 is frequently activated in human cancers^{10–13} and is implicated in the regulation of cancer cell survival, proliferation, angiogenesis and metastasis^{4,14,15}. Gain-of-function mutations of STAT3 have recently been identified in patients suffering from various haematological malignancies and found to be associated with reduced overall survival^{16,17}. Hence, the direct therapeutic inhibition of STAT3 is highly desirable but remains challenging as evident from the lack of FDA approved drugs^{18,19}.

STAT3 shares sequence and structural homology with other STAT family members and is composed of six domains: N-terminal domain (NTD), coiled-coil domain (CC), DNA-binding domain (DBD), linker domain (LD), Src homology 2 domain (SH2) and transactivation domain (TAD, Fig. 1a)²⁰. Canonical STAT3 activation relies on the phosphorylation of a key tyrosine residue by JAKs, Y705, which results in SH2 domain-mediated STAT3 dimerization²¹. Most inhibitory approaches aim to preclude STAT3 dimerization, but they have shown only limited clinical efficacy so far³. By contrast, the inhibition of STAT3 by targeting other domains remains largely unexplored, although the CC and NTD are also implicated in mediating critical functions of STAT3. The NTD mediates the recognition of weaker DNA binding sites and preferentially mediates the formation of an anti-parallel dimer of un-phosphorylated STAT3^{20,22,23}, whereas the CC is important for nuclear translocation^{24–26}. Additionally, the CC domain of STAT3 has a pivotal role in IL-22 receptor (IL-22R) signaling, where it constitutively associates with the C-terminal region of IL-22R and leads to a non-conventional activation of STAT3 that is independent of phospho-tyrosines on the IL-22R²⁷. This mechanism complements the conventional phospho-tyrosine residue-dependent IL-22R/STAT3 activation mechanism²⁷. IL-22 signaling is a potent mediator of cellular inflammatory responses and is unique among the cytokines, as it only acts on non-hematopoietic stromal cells²⁸. The IL-22 signaling axis is a key player in chronic inflammatory disorders, such as psoriasis, but also promotes tumor growth, metastasis and inhibition of apoptosis²⁸. Therefore, achieving a specific blockade of alternative STAT3 activation in IL-22R signaling is of particular interest.

Monoclonal antibodies are synthetic binding proteins, which are built using the tenth fibronectin type III (FN3) domain of human fibronectin as the molecular scaffold²⁹. They are obtained from large combinatorial libraries by sequential phage and yeast display selection^{30,31}. Monoclonal antibodies can be selected to bind to proteins that lack deep catalytic grooves, which are difficult to target by small molecules³². Over the past decade, monoclonal antibodies targeting different oncoproteins, such as BCR-ABL^{33,34}, SRC³⁵ and Aurora A kinases³⁶, the SHP2 phosphatase³⁷ and the RAS GTPase³⁸ have been developed. Intracellular expression of these monoclonal antibodies resulted in inhibition of oncogenic signaling and cell proliferation. These studies established monoclonal antibodies as highly specific intracellular antagonists and their utility in advancing a mechanistic understanding of targeted proteins and in discovering new sites for interfering with their functions^{39,40}.

Nonetheless, no monoclonal antibodies antagonizing a transcription factor, which were deemed as untreatable proteins for decades, are yet available.

Here, we develop monoclonal antibodies to different STAT3 domains to interfere with critical cellular and pathophysiological processes. The monoclonal antibodies bind the N-terminal or coiled-coil domain with high affinity and selectivity, and inhibit the transcriptional activity of STAT3. We present a crystal structure of a lead monoclonal antibody MS3-6 bound to the CC domain of STAT3 and insights on its molecular mode of action in interfering with conventional and non-conventional STAT3 activation. Our results demonstrate the utility of blocking intracellular STAT3 signaling upon targeting of the therapeutically unexplored CC domain of STAT3 and pave a way towards inhibitory strategies in diverse diseases including cancer and autoimmune disorders.

Results

Generation of high affinity monoclonal antibodies targeting STAT3. To generate monoclonal binders against human STAT3, we first recombinantly expressed and purified the core fragment (CF) of STAT3, which encompasses the CC, DBD and SH2 domains (STAT3-CF, a.a. 127–722, Fig. 1a), as well as the N-terminal domain (NTD, a.a. 3–138; Fig. 1a). Both recombinant proteins were confirmed to be of high purity and monomeric in solution (Supplementary Fig. 1a, b). We identified monoclonal antibodies from the combinatorial side-and-loop library using phage and yeast display library sorting^{30,31,33}. Seven monoclonal antibody clones binding to STAT3-CF and five clones binding to STAT3-NTD were isolated and further characterized (Table 1). The recombinant monoclonal antibodies expressed in *E. coli* with high yield and solubility and size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) indicated the formation of a monodisperse 1:1 complex with STAT3-CF when the two components were mixed (Supplementary Fig. 1c, d). In order to measure the binding affinities to their respective targets, increasing concentrations of recombinant STAT3 proteins were titrated to yeast cells displaying the monoclonal antibodies. Each clone exhibited an apparent dissociation constant (K_D) in the mid-nanomolar range (Table 1 and Supplementary Fig. 1e). Monoclonal antibody MS3-6 had the highest apparent affinity toward STAT3-CF (31 ± 6 nM). For the STAT3-NTD, several clones showed comparably high affinities. Of those, monoclonal antibody MS3-N3 had the highest solubility with an apparent binding affinity of 40 ± 4 nM (Table 1 and Fig. 1b). Interestingly, competition assays for STAT3 binding in the presence of MS3-6 showed that all STAT3-CF monoclonal antibodies share an overlapping or possibly common epitope despite their different sequences (Supplementary Fig. 1f). We thus concentrated our efforts on the characterization of the MS3-6 monoclonal antibody. The thermodynamics of MS3-6 binding to STAT3-CF was investigated using isothermal titration calorimetry (ITC). We found a dominantly enthalpically driven binding ($\Delta H = -22.5 \pm 0.5$ kcal/mol) with a binding stoichiometry of 1:1 and an ensuing high binding affinity ($K_D = 7.6 \pm 4.5$ nM; Fig. 1c). Over the past decade, a number of STAT3 oncogenic mutants have been identified in patients suffering from solid and hematological malignancies⁴¹. MS3-6 bound recombinant STAT3-CF proteins harboring the S614R and D661V oncogenic mutations in their SH2 domain, as well as the Y705F dominant negative mutant, with apparent K_D values of 94.7 ± 16 nM, 18.5 ± 6 nM and 17.6 ± 7 nM, respectively, in yeast display binding assays, which was in a comparable range to the wildtype STAT3 protein (Supplementary Fig. 1g).

In summary, we have developed monoclonal antibodies that bind with high affinity either to the STAT3-CF or the STAT3-NTD, and that are capable of binding to oncogenic STAT3 mutants.

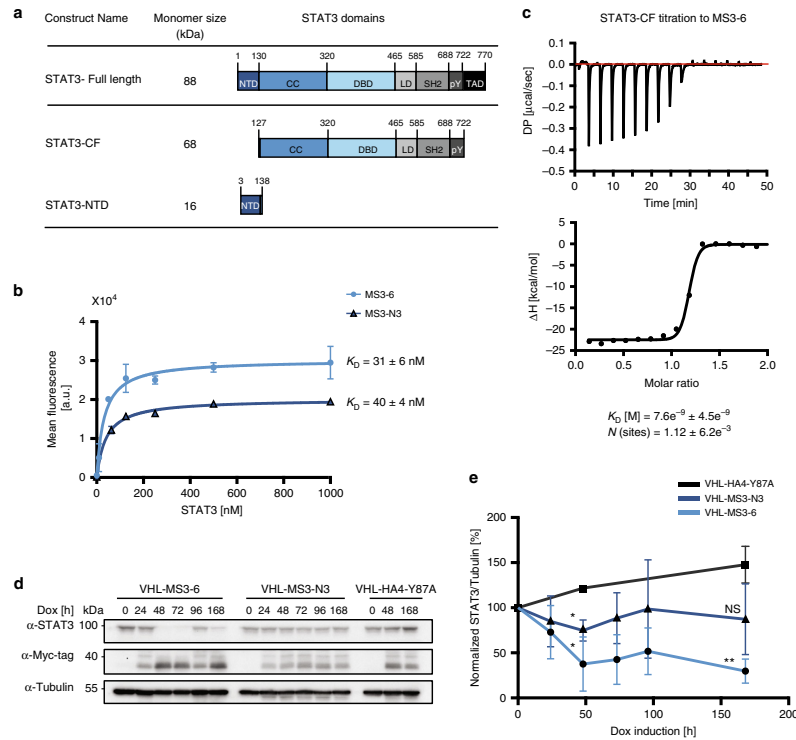


Fig. 1 Selection of high affinity, STAT3-selective monobody binders. **a** Schematic representation of the recombinant STAT3 constructs used for monobody selection. **b** Binding titration of yeast cells displaying monobodies (MS3-6; light blue line, MS3-N3; dark blue line) at their surface to recombinant STAT3 proteins. The mean fluorescence intensity of yeast cells bound to the target are plotted as a function of the protein concentration. Data from three individual experiments, mean $\pm$ SD are shown and a curve fitting 1:1 binding model was used. **c** Isothermal calorimetric titration (ITC) of STAT3 (100 μ M) to a MS3-6 solution (10 μ M) performed at 25 $^{\circ}$ C. The upper panel shows raw heat signal, while the lower panel shows the integrated calorimetric data of the area for each peak. A best fit 1:1 binding model was used and is illustrated by a black line (Microcal software). K_D and stoichiometry values (N) are indicated in the figure. ΔH (kcal mol $^{-1}$) = -22.5 ± 0.5 ; ΔG (kcal mol $^{-1}$) = -11.1 . **d** Representative immunoblot analysis from three independent experiments of monobody-VHL fusion expression overtime upon doxycycline treatment (1 μ g ml $^{-1}$) leading to STAT3 degradation. **e** STAT3 degradation levels were quantified and plotted from three independent experiments. Mean $\pm$ SD are shown and significance is indicated according to a two-sided unpaired t-test against HA4-Y87A control: MS3-6 * $P = 0.033$, ** $P = 0.0011$, MS3-N3 * $P = 0.0125$. Source data are provided as a source data file.

STAT3 monobodies are selective over other STATs in cells. Due to the high sequence and structural conservation among the STAT family members, the development of selective chemical probes remains challenging. To address selectivity of the developed STAT3 monobodies, we assayed binding of MS3-6 to the recombinant core fragment of STAT5B (a.a. 129–714), which encouragingly could not be detected in vitro (Supplementary Fig. 1g). To evaluate whether MS3-6 and MS3-N3 are able to bind to STAT3 in the complex cellular environment of mammalian cells, we transiently transfected HEK293 cells with 6xMyc-tagged monobodies and tested for binding to endogenous STAT3.

Enrichment of STAT3 in the Myc-tag pull down fraction was observed by immunoblot suggesting an effective binding between STAT3 and MS3-6 or MS3-N3 in cells (Supplementary Fig. 2a).

To assess functional engagement of STAT3 by the selected monobodies in cells, we engineered a targeted degradation system by fusing the monobodies to the Von Hippel–Lindau (VHL) protein^{42,43}, which is the substrate receptor of the Cullin2/RBX1 E3 ubiquitin ligase complex. Inducible expression of MS3-6-VHL fusions in NPM-ALK expressing mouse thymoma cells resulted in the degradation of endogenous STAT3 protein (Fig. 1d, e). To a lower extend, MS3-N3-VHL expression led to the degradation

Table 1 Original monobody library and selected STAT3 binders.

Library/Clone	K _d (nM)	Amino acid sequence
		10 20 30 40 50 60 70 80 90
Side-and-loop		VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(A ₁₀)-----SP(SINVRT
MS3-1	127 ± 6	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MGQVPSQIKA)-----SP(SINVRT
MS3-2	104 ± 10	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(VSVPETVFP)-----SP(SINVRT
MS3-4	18 ± 11	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MSDTFLWQSSP)-----SP(SINVRT
MS3-5	88 ± 14	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MATYEL)-----SP(SINVRT
MS3-6	8 ± 5	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(VSVPETVFP)-----SP(SINVRT
MS3-7	115 ± 23	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MGQVPSQIK)-----SP(SINVRT
MS3-8	235 ± 48	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MGQVPSQIK)-----SP(SINVRT
MS3-N1	65 ± 10	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MYTSQIENS)-----SP(SINVRT
MS3-N3	40 ± 4	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MYTSQIENS)-----SP(SINVRT
MS3-N5	39 ± 6	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MYTSQIENS)-----SP(SINVRT
MS3-N7	38 ± 5	VSSVPTKLEVAATPTELLISWDAPAVT-VQVYITVGTGQNSPV-QEPVVGSKSTATISGLKPGVDITTVVA(MYTSQIENS)-----SP(SINVRT

Amino acid sequences of the side-and-loop monobody library are shown on top, where "X" corresponds to a blend of 30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp and 2.5% each of all remaining amino acids with the exception of Cys. "B" denotes a mixture of Gly, Ser and Tyr. "J" corresponds to a mixture of Ser and Tyr. "O", a mixture of Asn, Asp, His, Ile, Leu, Phe, Tyr and Val. "U", a mixture of His, Phe and Tyr. "Z", Ala, Glu, Lys and Thr. Monobodies selected against STAT3-CF are indicated in the upper panel (MS3-1 to MS3-8), while monobodies selected against STAT3-NTD are shown in the lower panel (MS3-N1 to MS3-N7). Dissociation constants (K_d) are indicated according to yeast binding assays. Binding curves are shown in Supplementary Fig. 1c.

of STAT3 over time as compared to the non-binding HA4-Y87A-VHL control (Fig. 1d, e). STAT3 degradation by MS3-6-VHL was implied upon proteasome inhibition, indicating ubiquitin-dependent proteasomal degradation (Supplementary Fig. 2b). These results show that the monobodies mediated a targeted degradation of STAT3 in cells, thus highlighting successful target engagement.

To comprehensively assess the selectivity of MS3-6 and MS3-N3 in an unbiased way on a proteome-wide scale, we performed monobody affinity-purification mass spectrometry experiments^{37,44}. Tandem affinity purification (TAP)-tagged monobodies were stably expressed in two cell lines that endogenously express STAT3 and various other STAT family members at different levels: K562 chronic myeloid leukemia cells and Jurkat acute lymphoblastic leukemia T-cells. Immunoblot analysis of fractions from the TAPs of ~10⁹ cells each showed that both the monobody and its on-target STAT3 were efficiently recovered after the second affinity purification step (Supplementary Fig. 2c). The composition of the recovered fractions was assessed by SDS-PAGE analysis (Supplementary Fig. 2d) and identified two main bands at ~20 kDa and ~80 kDa corresponding to the molecular weight of the monobodies and STAT3, respectively. Subsequent liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis identified both the monobody itself and full-length STAT3 to be highly abundant among the identified proteins (Table 2). In contrast, only very low levels of STAT1 and STAT5B were identified in both cell lines. Other STATs were not detected despite expression in both K562 and Jurkat cells (Table 2 and Supplementary Fig. 2e). For MS3-N3, abundance of STAT3 was overall lower, but either no other STATs were detected (K562 cells) or much less abundant (STAT5B in Jurkat; Table 2). The modest abundance of proteins other than STAT3 isoforms, their inconsistent identification across the purifications and general high cellular abundance suggest that these are nonspecifically recovered in the TAP experiments, rather than being true off-targets of these monobodies.

Taken together, these results show that MS3-6 and MS3-N3 engage STAT3 as compared to other STAT family members in a highly selective manner in cells without apparent off-targets.

Monobodies potentially inhibit STAT3 transcriptional activity. We first tested whether the high-affinity and selective binding of MS3-6 and MS3-N3 interfered with STAT3 cellular functions in a reporter cell line expressing luciferase under the transcriptional control of STAT3 response elements⁴⁵. MS3-6, MS3-N3 and a non-binding monobody control (HA4-Y87A)⁴⁴ were expressed as eGFP fusion proteins, along with eGFP as further negative

control, in a doxycycline-inducible expression system. In all, 48 h after the induction of GFP-monobody expression, GFP-expressing cells were FACS-sorted and luciferase activity was measured to infer STAT3 activation upon oncostatin M (OSM) treatment (Fig. 2a). MS3-6 strongly reduced the transcriptional activity of STAT3 as compared to eGFP alone and to the HA4-Y87A monobody control (Fig. 2b). Strikingly, the perturbation of STAT3 activity by MS3-6 were comparable to the effects caused by expression of a transcriptionally inactive dominant negative mutant (STAT3-Y705F), siRNA knock-down of STAT3's upstream kinase JAK1 or treatment of cells with the potent JAK1/JAK2 kinase inhibitor Ruxolitinib (Fig. 2b). Expression of MS3-N3 inhibited STAT3 transcriptional activity to a lower extent as compared to the positive controls used (Fig. 2b).

As these results demonstrated potent STAT3 inhibition by a monobody, we investigated the activity of MS3-6 in different cellular and stimulatory contexts. We took advantage of BW5147 and Ba/F3 cells that we had engineered to express a panel of cytokine receptors including the IFNAR, the IL-22R and the IL-20Ra/b, known to signal through STAT3^{46,47}. Cells were electroporated with plasmids containing either MS3-6 or the negative control HA4-Y87A monobody together with a dual Firefly/Renilla luciferases system under the transcriptional control of STAT3. Stimulation of the respective engineered receptor-expressing cell lines with the mIL-9, hIL-22, hIL24 or hIFNAR3 cytokines strongly induced Firefly/Renilla luciferase signals in the presence of the non-binding monobody control HA4-Y87A (Fig. 2c). In contrast, expression of MS3-6 strongly reduced STAT3 activity upon stimulation by all four cytokines (Fig. 2c). We further tested if MS3-6 inhibits STAT3 transcriptional activity in A549 lung cancer cells, which endogenously express the hIL-6 and hIL-22 receptors. A549 cells were engineered to stably express the monobodies using a doxycycline inducible system and then transiently transfected with a STAT3-responsive Firefly/Renilla luciferase reporter. MS3-6 expression strongly decreased STAT3 activity as compared to the HA4-Y87A control following IL-6 and IL-22 stimulation (Fig. 2d), therefore showing that MS3-6 inhibits endogenous STAT3 activation. The inhibitory effect of MS3-6 in the luciferase reporter assays were paralleled by changes in STAT3 target gene expression as analyzed by RT-qPCR in A549 cells. IL-22 stimulation in the presence of MS3-6 severely decreased gene expression of the direct STAT3 target genes SOCS3, MMP9, IL-6 and BCL-3 as compared to the HA4-Y87A monobody control while the effects of MS3-N3 were more modest (Fig. 2e). Interestingly, expression of CLND2 was mildly increased in the presence of MS3-6, but remained unaffected by MS3-N3.

Table 2 Monobody MS3-6 and MS3-N3 interactome.						
Bait monobody	Cell line	STAT family proteins identified ^a	Spectrum counts of razor and unique peptides ^b	iBAQ (intensities) ^c	Rank out of all identified proteins ^d	
MS3-6	Jurkat	Monobody	498	6.74 × 10 ⁹	2/578	
		STAT3-FL	599	1.23 × 10 ⁹	6/578	
		STAT3 Del-701	7	1.22 × 10 ⁹	7/578	
		STAT3-β	3	1.21 × 10 ⁹	8/578	
		STAT1	2	4.52 × 10 ⁵	425/578	
	K562	Monobody	1967	1.38 × 10 ¹⁰	1/566	
		STAT3 [a.a. 3-84]	5	5.05 × 10 ⁹	3/566	
		STAT3-FL	3318	3.11 × 10 ⁹	4/566	
		STAT3-β	14	3.11 × 10 ⁹	5/566	
		STAT3 Del-701	29	2.99 × 10 ⁹	6/566	
		STAT1	7	2.56 × 10 ⁵	253/566	
		STAT5B	5	1.55 × 10 ⁵	321/566	
		STAT3-FL	173	4.63 × 10 ⁷	41/399	
		STAT3 Del-701	4	4.60 × 10 ⁷	42/399	
STAT5B	27	6.55 × 10 ⁶	119/399			
STAT3-β	1	5.98 × 10 ⁶	120/399			
K562	Monobody	517	1.91 × 10 ¹⁰	2/578		
	STAT3-FL	142	1.31 × 10 ⁷	39/578		
	STAT3-β	2	1.11 × 10 ⁷	41/578		
	STAT3 Del-701	1	1.64 × 10 ⁶	123/578		
	Exclusive spectrum counts of monobodies MS3-6 and MS3-N3 and STAT family members upon tandem affinity purification in Jurkat and K562 cells. The complete list of all identified proteins, including keratin contaminants and highly endogenously expressed proteins, is available as a PRIDE database submission: PXD018374.					
	^a Including STAT3 isoforms.					
	^b All shared peptides between STAT family members and isoforms are associated to STAT3-FL, in addition to its unique peptides. All other STAT family members and isoforms display their unique peptides counts only.					
	^c iBAQ values result from the sum of intensities of all tryptic peptides divided by the number of all theoretically observable peptides for each protein. These values provide an accurate determination of the relative abundance of all STATs identified in the samples.					
	^d All identified proteins, including common contaminants, such as keratin, were sorted by their iBAQ values from highest to lowest.					

In summary, these data indicate that expression of MS3-6 and, to a lower extent, MS3-N3 potentially diminishes the transcriptional activity of STAT3 and the expression of STAT3 downstream target genes.

MS3-6 is not an SH2-pY inhibitor and decreases DNA binding.

We next surveyed likely mechanisms underlying the molecular action of MS3-6. One way to inhibit STAT3 relies on the blockade of the interaction between its SH2 domain and pY ligands, which perturbs its dimerization and its interaction with upstream cytokines receptors⁴⁸. To investigate whether MS3-6 targets the SH2 domain, we established a fluorescence polarization binding assay with recombinant STAT3-CF and a fluorescent pY-peptide encompassing the pY905 docking site of gp130 (GMPKSpYLPQTVR), the co-receptor of the IL-6 cytokine receptor family. This peptide bound STAT3-CF with a *K_D* value of 930 nM (Supplementary Fig. 3a). The addition of increasing concentrations of recombinant MS3-6 did not affect binding of the gp130 pY-peptide to STAT3 (Fig. 3a), excluding the possibility that MS3-6 interferes with the SH2-pY ligand interaction.

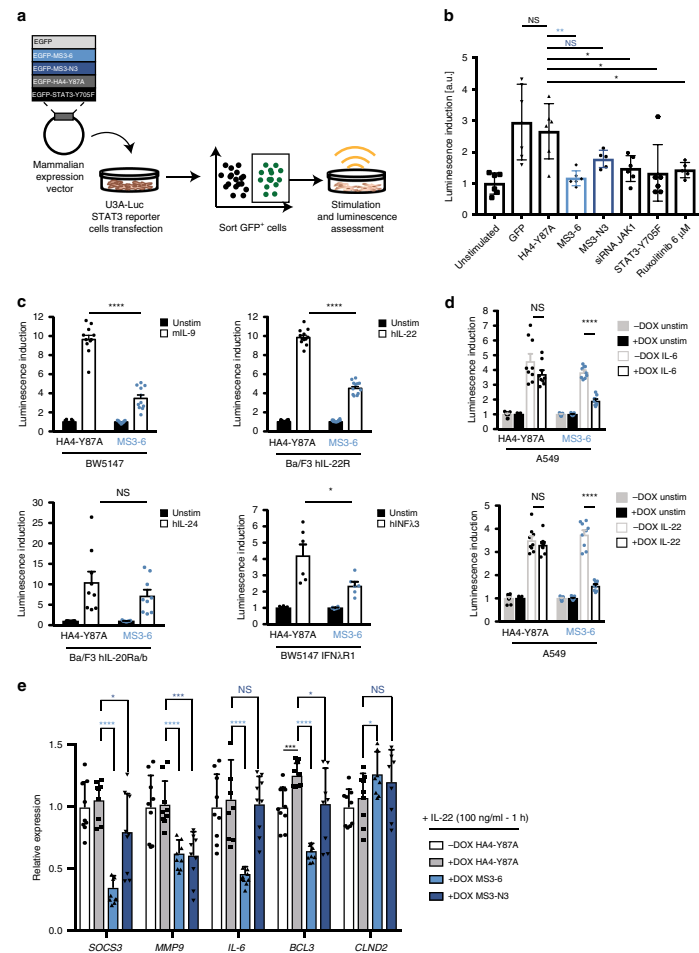
To assess whether the inhibitory activity of MS3-6 is due to interference with STAT3-DNA binding, we prepared a recombinant tyrosine phosphorylated STAT3 dimer by co-expression of STAT3-CF and the ABL1 tyrosine kinase domain in *E. coli*. Size exclusion chromatography and immunoblot analysis showed dimer formation and strong phosphorylation of Y705 (Supplementary Fig. 3b, c). The purified recombinant STAT3 dimer was assessed for in vitro binding to a fluorescently-labeled

double-stranded oligonucleotide corresponding to the STAT3 binding sites in the SOCS3 promoter region, as well as to a higher affinity STAT3 site (a2M)²⁵. *K_D* values of 1.21 μM and 0.21 μM to the SOCS3 and a2M probes, respectively, were derived from fluorescence polarization binding experiments, in line with previously reported values (Supplementary Fig. 3d)²³. The addition of recombinant MS3-6, but not of the HA4-Y87A control, decreased binding of the STAT3 dimer to both the SOCS3 and a2M DNA probes in a dose-dependent manner (Fig. 3b). Complete DNA binding inhibition could not be achieved even at high monobody concentration (>1000 fold above *K_D*). This result thus argues for a contribution of an indirect allosteric inhibition of DNA binding, but cannot explain the strong inhibition of STAT3 signaling by MS3-6 in cells on its own, suggesting the presence an additional mode of action.

Crystal structure of MS3-6 bound to STAT3. To understand the mode-of-action of MS3-6 on the molecular functions of STAT3, we solved the crystal structure of MS3-6 in complex with STAT3-CF at a resolution of 2.9 Å (Fig. 4a; Table 3; PDB: 6TLC). The asymmetric unit contains an anti-parallel (unphosphorylated) STAT3 dimer, as previously observed in STAT5A and STAT1 structures (PDB: 1Y1U and 1YVL, respectively). Two monobodies bind one STAT3 dimer, in line with the 1:1 binding stoichiometry determined by ITC (see Fig. 1c). The structure reveals that MS3-6 binds to the helices α1, α2, and to the loop between helices α3 and α4 of the CC domain. Interactions of the monobody with STAT3 are mediated by residues located

Annex

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in its diversified FG loop (a.a. 75–85), together with a diversified position (H33) located in strand BC of the monobody (Fig. 4a; Supplementary Fig. 4a). The overall buried surface area of the STAT3-M53-6 interface is 834 Å² (Fig. 4b). Protein sequence analysis of all STAT family members showed that most residues implicated in hydrogen bonds or salt bridges with M53-6 are unique to STAT3 and are not conserved in other STATs (Supplementary Fig. 4b). Additionally, the structure explains the high selectivity of M53-6 for STAT3 over STAT5, as helix α2

in STAT5 is more than two turns longer and superposition of the structures indicates incompatibility of MS3-6 binding to STAT5 (Supplementary Fig. 4c). Superposition of the MS3-6/STAT3-CF complex and an active STAT3-CF parallel dimer bound to DNA shows that monobody binding does not sterically interfere with DNA binding or pY705-dependent dimerization of the SH2 domains in line with the fluorescence polarization results described above (Supplementary Fig. 4d). Similarly, binding of MS3-6 is compatible with the formation of

Fig. 2 Monobody inhibition of STAT3 transcriptional activity. **a** Scheme depicting the initial screening strategy to identify monobodies with STAT3 inhibitory activity. **b** Luciferase reporter activity inductions normalized to that of unstimulated cells, which was arbitrarily set to 1, are reported for the expression of the indicated monobodies and control treatments. Results from two individual experiments performed in triplicate and/or duplicate are shown (data presented as mean $\pm$ SD, $n = 5$ or 6). Two-tailed unpaired *t*-test analysis was performed against the HA4-Y87A monobody control: MS3-6 $^{**}P = 0.0027$, Ruxolitinib $^{*}P = 0.0158$, siRNA JAK1 $^{*}P = 0.0147$, STAT3-Y705F $^{*}P = 0.028$. **c** BW5147 or Ba/F3 cells (10^7) were electroporated with the monobody plasmid, the pGL3-Pap1 luciferase reporter plasmid as a specific promoter for STAT3 activation and the pRL-TK plasmid. Cells were stimulated with control medium or murine IL-9 (100 ng ml^{-1} , left upper panel, $n = 11$), human IL-22 (500 ng ml^{-1} , right upper panel, $n = 12$), human IL-24 (HEK293 supernatant 2%, lower left panel, $n = 9$) or human IFN γ 3 (HEK293 supernatant 2%, lower right panel, $n = 6$) for 4 h. Data are presented as mean $\pm$ SEM from two to four independent experiments performed in triplicates. **d** Luciferase assay on A549 with inducible monobody (HA4-Y87A or MS3-6) expression. A549 cells (5×10^3) were plated in 96-well plate and treated for 24 h with control medium or doxycycline ($1 \mu\text{g ml}^{-1}$). Cells were transiently transfected with the pGL3-Pap1 luciferase reporter plasmid and the pRL-TK plasmid as internal control of transfection. In all, 4 h after transfection, cells were stimulated with control medium or human IL-6 (100 ng ml^{-1} , upper panel) or human IL-22 (500 ng ml^{-1} , lower panel) for 20 h. Data are presented as mean $\pm$ SEM of three independent experiments performed in triplicates. Significance in **c, d** is shown according to two-tailed Mann-Whitney test: $^{*}P = 0.026$, $^{***}P \leq 0.0001$. **e** RT-qPCR for expression of STAT3 downstream genes in A549 cells expressing monobodies upon IL-22 stimulation (100 ng ml^{-1} , 1 hr at 37°C). Gene expression was normalized to actin and is presented as fold change against that of untreated A549 cells (no monobody expression), which was set to 1. Data from three independent experiments performed in triplicates ($n = 9$) are presented as mean $\pm$ SD. Significance was calculated against mRNA levels in the HA4-Y87A monobody control conditions. Significance is shown according to two-tailed unpaired *t*-test analysis: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Source data are provided as a source data file.

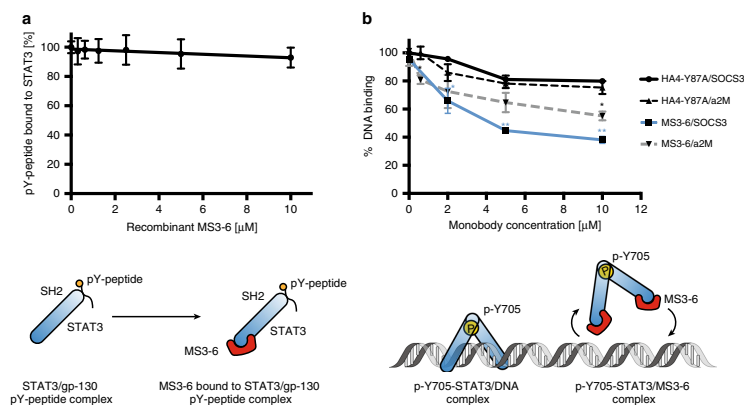


Fig. 3 MS3-6 inhibits STAT3 DNA binding, but does not bind the SH2 domain. **a** Addition of increasing recombinant MS3-6 concentrations to a STAT3/pY-peptide complex. Fluorescence polarization was measured at the indicated concentration and bound pY-peptide relative to samples in the absence of MS3-6 are plotted. Data from three independent experiments (mean $\pm$ SD). **b** Addition of increasing concentrations of recombinant MS3-6 (blue line and grey dashed line) to a pY705-STAT3 dimer bound to fluorescent DNA probes corresponding to downstream promoter sequences (SOCS3 and a2M). Fluorescence polarization was measured at the indicated concentrations of monobodies and bound DNA levels relative to samples in the absence of monobodies are plotted. All fluorescence polarization experiments were performed at 25°C . Data from two independent experiments (mean $\pm$ SD). Significance according to a two-tailed unpaired *t*-test analysis: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. Source data are provided as a source data file.

an anti-parallel unphosphorylated STAT3 dimer (Supplementary Fig. 4c).

Superposition of the MS3-6/STAT3-CF structure and several previously published structures of STAT3 as well as other STAT family members showed that binding of MS3-6 results in the torsion of the 4-helix bundle (helices $\alpha 1$ – $\alpha 4$) of the CC and in the bending of the $\alpha 1$ and $\alpha 2$ helices by 4–12 degrees relative to the reference (apo) structures (Fig. 4c and Supplementary Fig. 4f). Hence, MS3-6 binding provokes a conformational change of the CC domain orientation, which may allosterically perturb DNA binding. We furthermore realized that MS3-6 binds close to the nuclear localization signal (NLS) of STAT3, which includes residues R214/215 in helix $\alpha 2$ of the CC domain (Fig. 4a). These

two residues are critical for the efficient nuclear import of active phosphorylated STAT3 parallel dimers through binding to importins²⁶. Thus, MS3-6 binding may indirectly perturb efficient nuclear import of pY705 phosphorylated STAT3 dimers, leading to a diminished STAT3 transcriptional activity.

In summary, the structural data show that MS3-6 allosterically affects the conformation of STAT3 by altering the CC orientation, thus potentially impairing the nuclear translocation of STAT3.

MS3-6 reduces STAT3 nuclear translocation upon stimulation.

The close proximity between the monobody binding site and the NLS led us to hypothesize that MS3-6 impairs STAT3 nuclear

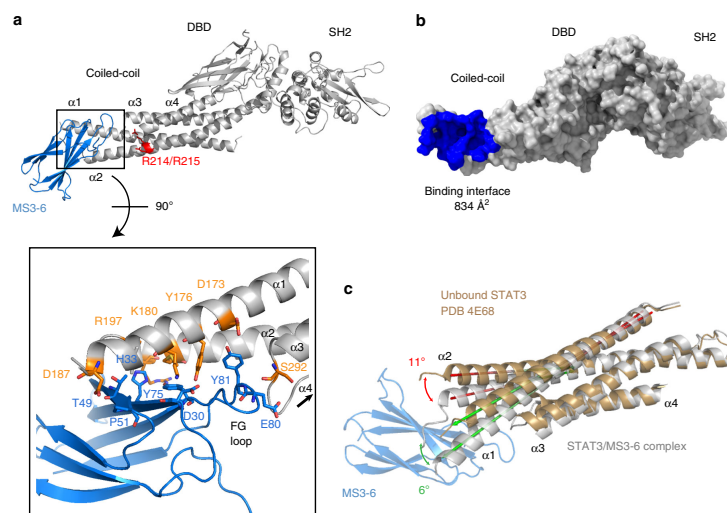


Fig. 4 Co-crystal structure of MS3-6 bound to the STAT3. **a** Cartoon representation of the structure of STAT3 (light gray) and monobody MS3-6 (blue). The upper panel shows an overview of MS3-6 binding to the coiled-coil domain of STAT3, with key residues of the nuclear localization sequence (NLS) in close proximity to the monobody binding site highlighted in red. The lower panel shows a magnification of the binding interface with epitope residues (threshold set at 4 Å) depicted as sticks. **b** Surface representation of STAT3 (light gray), with the area covered by the monobody colored in blue. **c** Structural alignment of the coiled-coil domains of STAT3 in complex with MS3-6 together with an unbound STAT3 structure previously published (PDB: 4E68, light brown). Monobody binding leads to a conformational distortion of the helices $\alpha 1$ and $\alpha 2$. The angles formed between helices are indicated in green and red, respectively.

translocation. To test this model, we performed subcellular fractionation and immunofluorescence experiments. The levels of STAT3 in the nuclear and cytoplasmic fractions were determined by immunoblot in A549 cells stimulated with IL-6 or IL-22 in the presence of MS3-6, MS3-N3 or the negative control HA4-Y87A monobody. MS3-6 led to a reduced level of nuclear STAT3 upon stimulation with either IL-6 or IL-22 (Fig. 5a). Similarly, MS3-6 decreased the nuclear:cytosolic-ratio of STAT3 and pY705-STAT3 (Fig. 5b and Supplementary Fig. 5a, b) as well as the STAT3:RCC1- and pY705-STAT3:RCC1-ratio used to correct for different fractionation efficiencies (Supplementary Fig. 5c). MS3-N3 failed to inhibit nuclear translocation of STAT3, in line with the distinct binding site of this monobody in the N-terminal domain, which is not in close proximity to the NLS. Therefore, in order to further evaluate the impact of monobodies on nuclear translocation, we additionally took advantage of confocal immunofluorescence microscopy using HEK293 cells inducibly expressing GFP-monobody fusions upon doxycycline treatment, which were stimulated with IL-6. Again, we observed reduced nuclear STAT3 levels in cells that expressed MS3-6 as compared to cells expressing HA4-Y87A (Fig. 5c and Supplementary Fig. 5d). Quantitative image analysis corroborated these qualitative results (Fig. 5d).

Altogether, these data determine that the inhibition of the transcriptional activity of STAT3 by MS3-6 is mediated by reduced STAT3 nuclear translocation.

MS3-6 reduces STAT3 Y705 phosphorylation in IL-22R signaling. To investigate the effects of the monobodies on endogenous STAT3 signaling, we inducibly expressed monobodies in A549 lung cancer cells. Expression of MS3-6, MS3-N3 or the HA4-Y87A control did neither strongly impact STAT1 tyrosine phosphorylation nor STAT3 serine 727 phosphorylation (Fig. 6a, b and Supplementary Fig. 6a). Interestingly, MS3-6 led to an increased STAT5 phosphorylation upon IL-22 stimulation (Supplementary Fig. 6a, b). While MS3-6 induction had no significant effect on pY705 STAT3 upon IL-6 stimulation, we observed a strong reduction of Y705 phosphorylation following IL-22 stimulation by immunoblotting (Fig. 6a, b). This unexpected finding was confirmed by FACS analysis. We again detected reduced pY705 STAT3 levels upon IL-22 stimulation, but not following IL-6 stimulation (Supplementary Fig. 6c–g). We reasoned that different molecular mechanisms of STAT3 activation by the two cytokine receptors may account for the observed difference, as IL-22 stimulation was reported to utilize a non-canonical constitutive association of STAT3 with the IL-22 receptor in the absence of cytokines²⁷. This alternative STAT3 stimulation is mediated by the constitutive interaction between the CC of STAT3 and the 84 C-terminal amino acids of the IL-22R, which lack tyrosine residues. IL-22-dependent STAT3 activation can be triggered by a phosphotyrosine independent mechanism in addition to the conventional (phospho-tyrosine dependent) recruitment of STAT3 to the IL-22R following stimulation.

Table 3 Crystal structure data collection and refinement statistics.

MS3-6/STAT3-CF	
Data collection	
Space group	P 41 21 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	111.31, 111.31, 483.47
α , β , γ (°)	90.00, 90.00, 90.00
Resolution (Å)	49.78–2.90 (3.1–2.9) ^a
<i>R</i> _{meas}	34.4 (447.9)
<i>CC</i> _{1/2}	99.8 (25.2)
<i>I</i> / σ <i>I</i>	8.5 (0.61)
Completeness (%)	99.9 (99.8)
Redundancy	14.27 (14.55)
Refinement	
Resolution (Å)	2.9
No. reflections	68566
<i>R</i> _{work} / <i>R</i> _{free}	0.246/0.280
No. atoms	
Protein	10063
Water	2
<i>B</i> -factors	
Protein	83.24
Water	35.64
R.m.s. deviations	
Bond lengths (Å)	0.015
Bond angles (°)	1.934
Molprobtity score	2.61

^aOne crystal per structure. PDB: 6TLC. Values in parenthesis are for the highest resolution shell.

To determine if the CC-binding monobody MS3-6 perturbs IL-22 signaling, we used Ba/F3 cell lines expressing IL-22R mutants, which include a tyrosine-less mutant where all tyrosine residues located in the intracellular domain of IL-22R were mutated to phenylalanine, and a C-terminal truncation (IL-22R receptor lacking the last 84 amino acids; Δ C-ter). These IL-22R mutants allowed us to dissect the impact of the MS3-6 monobody on conventional (Δ C-ter mutant) and alternative (tyrosine-less mutant) STAT3 activation. The Y705 phosphorylation status of STAT3 was assessed in cells expressing the different IL-22R mutants by flow cytometry following IL-22 stimulation upon electroporation of the Myc tagged monobody plasmids. MS3-6 strongly decreased STAT3 phosphorylation after IL-22 stimulation in cells expressing the wild-type and tyrosine-less receptor, but not the Δ C-ter receptor, highlighting the impact of the monobody on the non-canonical IL-22 signaling pathway (Fig. 6c, d). To further corroborate this result, we performed GST-receptors pull-down experiments. STAT3 was robustly co-purified with the recombinant GST-IL-22R intracellular domain mutants in cells that expressed the control HA4-Y87A monobody, as long as the IL-22R contained its C-terminal part (Fig. 6e). In contrast, expression of MS3-6 resulted in the loss of STAT3 co-purification.

These results reveal that MS3-6 prevents the binding of STAT3 to the C-terminus of IL-22R, which indicates selective perturbation of alternative non-canonical IL-22 mediated STAT3 activation.

Discussion

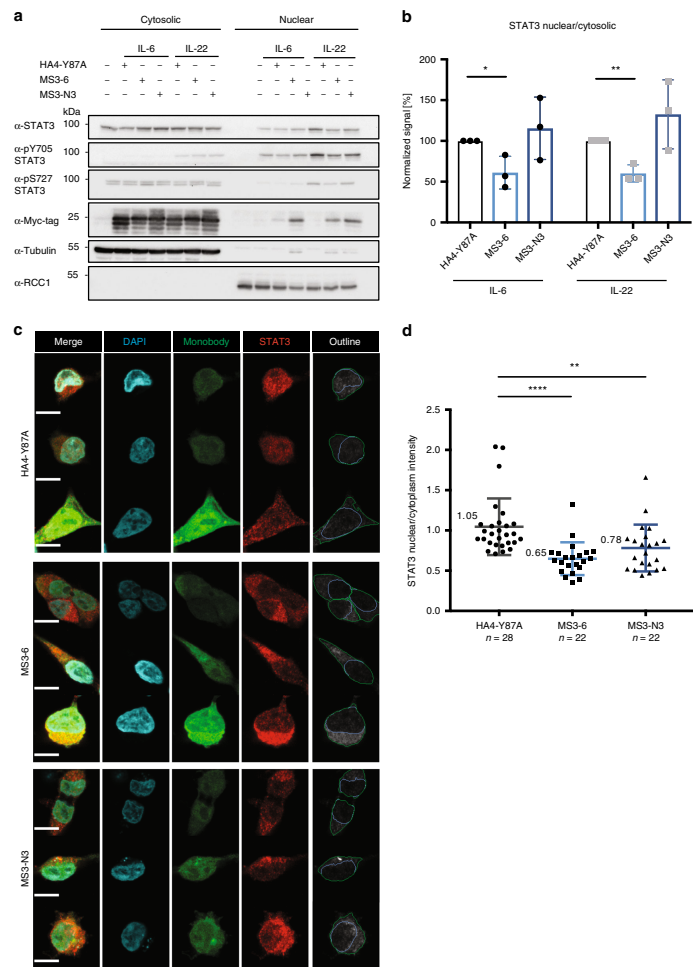
Transcription factors are essential players in the development of pathological conditions such as cancer⁴⁹. Nonetheless, targeting transcription factors is very challenging due to the lack of deep pockets able to fit an antagonistic small molecule inhibitor⁵⁰. The inhibition of STAT3 in cancer is highly desirable^{51,52} and a lot of

effort is invested towards the therapeutic inhibition of STAT3 in academia and the pharmaceutical industry. Still, there are no FDA-approved drugs targeting STAT3 directly yet. Current efforts focus on inhibiting STAT3-dimerization by developing antagonists of the SH2 domain¹⁹, whereas targeting additional domains that are important for pathological STAT3 signaling remain poorly studied. In this work, we establish monobodies as tools to antagonize the activity of a transcription factor. Our results indicate that both MS3-6 and MS3-N3 bind to STAT3 with low nanomolar affinities in both yeast binding and ITC assays, but the former assay consistently resulted in lower apparent binding affinities. Given that yeast display is not a true equilibrium binding assay, these differences are not surprising. Thus, while yeast display is less laborious, as no purified high-quality monobody protein is required, a true equilibrium binding assay, such as ITC, is indispensable to precisely determine *K_D* values of lead candidate monobodies.

In contrast to the monobodies previously developed against several other SH2 domain-containing oncogenes^{33,35,37}, none of the tested STAT3 monobodies target the SH2 domain, but instead bind the CC domain and inhibit transcriptional activity of STAT3. In a separate selection, monobodies targeting the NTD were developed. The NTD has been shown to fine tune transcriptional responses of STAT3 by favoring the formation of STAT3 tetramers, which allow the recognition of weaker DNA binding sites and regulation of chromatin organization^{13,22,53}. Similarly, the NTD of STAT3 was found to repress the expression of pro-apoptotic genes^{23,54}. The use of MS3-N3 to study NTD functions may prove as a very valuable tool to understand STAT3 biology in the future. Because the specific blockade of the NTD using MS3-N3 monobody was found to interfere to a lower extent with the transcriptional activity of STAT3, we concentrated our efforts on the characterization of MS3-6 in this study. Therefore, the mode of action of MS3-N3 remains to be studied in more detail, as part of an independent study in the future. In particular, the differential regulation of STAT3 target gene expression by MS3-6 and MS3-N3 may hint towards interesting underlying biology requiring a more in-depth study. Likewise, the observed trend of an inhibitory activity of MS3-N3 on IL-6 induced nuclear translocation of STAT3 should be followed-up.

We showed binding of MS3-6 in proximity to a proposed NLS including residues R214/R215²⁶ and inhibition of nuclear translocation. It is therefore not unlikely that MS3-6 interferes with importin- α 3 binding to this NLS. The lack of a complete inhibition of nuclear translocation can possibly be explained by the presence of a second NLS in the DBD. This NLS includes residues R414/R417, which are recognized by importin- α 5 and - α 7²⁴ and whose accessibility is predicted to be not affected by MS3-6 binding. Therefore, the relative contributions of the two NLSs to overall STAT3 nuclear translocation will determine the severity of MS3-6 perturbation. In summary, our data indicate that monobody MS3-6 interferes at various levels to inhibit STAT3 phosphorylation, reduce its nuclear translocation and diminish DNA binding. Hence, our results suggest that the inhibitory mode of action of MS3-6 relies on a cumulation of distinct mechanisms acting in synergy towards the overall reduction of STAT3 transcriptional activity (Fig. 7).

A strong limitation of many targeted therapies is off-target toxicity, in particular when protein classes with hundred and more homologous members, such as protein kinases or transcription factors are targeted. In contrast, our monobodies were found to be highly selective for STAT3 in different cellular proteomic contexts. While structural modeling indicated that the different lengths of helices in STAT1 and STAT5 would hinder MS3-6 binding, the residual amounts of STAT1 and STAT5 in



the TAP-MS experiments can be explained by the ability of STAT3 to form heterodimers with other STATs. The preferential binding of our monobodies to STAT3 may also prove useful for combining inhibition of STAT3 signaling with targeted degradation approaches, as exemplified by the degradation of STAT3 upon expression of monobody-VHL fusions. The distinct degradation efficiencies between MS3-6 and MS3-N3 may be explained by their differences in affinity for STAT3 or by the positioning of lysine residues for ubiquitylation in proximity of the monobody binding site. Altogether, our work highlights how the rapid development of high affinity protein binders, such as monobodies, appear particularly suited for a targeted degradation strategy to mediate the efficient suppression of undruggable target proteins for which we currently lack small molecule binders.

Future efforts in validating the use of these monobodies as protein-based therapeutics in physiological systems should aim at

Fig. 5 MS3-6 reduces STAT3 nuclear localization. **a** Representative immunoblot analysis of cellular fractionation experiments from three independent experiments. A549 expressing monobodies upon 48 h of $1\ \mu\text{g ml}^{-1}$ doxycycline treatment were stimulated with either IL-6 or IL-22 for 15 min. Nuclear (RCC1) and cytosolic (Tubulin) fractions were recovered and probed for total, p-Y705 and p-S727 STAT3. Quantification of three independent experiments normalized to the HA4-Y87A monobody control are shown in **b** and are plotted as mean $\pm$ SD. Significance according to a two-tailed unpaired t-test analysis: * $P = 0.0278$, ** $P = 0.0028$. **c** Confocal microscopy images from two independent experiments of HEK293 cells expressing a monobody-GFP fusion and treated with IL-6 to assess nuclear translocation. Scale bar represents $10\ \mu\text{m}$. The right column shows the outlines used to determine nuclear and cellular compartments indicated by blue and green lines, respectively, using the CellProfiler software. The number of individual cells analyzed (n) is indicated on the figure. Quantification of STAT3 nuclear/cytoplasmic levels from two independent experiments is shown in **d**. Significance according to a two-tailed unpaired t-test analysis: ** $P = 0.0065$, **** $P \leq 0.0001$. The number of analyzed cells is indicated below the graph. Source data are provided as a source data file.

exploring delivery strategies for therapeutic approaches and in vivo applicability. Importantly, our study shows that in addition to the inhibition of the transcriptional activity of STAT3, the specific blockade of the CC domain with MS3-6 provided additional insights in a poorly characterized cytokine signaling pathway, namely into alternative IL-22R signaling in which STAT3 is pre-associated via its CC domain to the IL-22R in the absence of cytokine. Therefore, our study highlights the role of monobodies as molecular tools to dissect poorly understood signaling pathways. Lastly, our study provides a proof of concept towards the inhibition of transcription factors implicated in human cancer and other human diseases using monobody inhibitors and lays the foundations for the targeting of additional transcription factors currently regarded as undruggable.

Methods

Antibodies, cell lines, and reagents. Antibodies against STAT1 (No. 9172), pY701 STAT1 (No. 9167), STAT2 (No. 4597), STAT3 (No. 9139), pY705 STAT3 (No. 9145), pS727 STAT3 (No. 9134), STAT4 (No. 2653) and pY694/pY699 STAT5 (No. 9359) were purchased from Cell Signaling Technology. The antibody against STAT5B (sc-1656) was obtained from Santa Cruz Biotechnology. The ubiquitin antibody (No. 04-263, clone FK2) was purchased from Merck. The anti-Myc tag antibody directly coupled to Dylight800 was purchased from ThermoFisher (No. MA1-21316-D800). The antibody against penta-His was ordered from Qiagen (No. 34610) and the anti-alpha Tubulin antibody (T9026) was ordered from Sigma-Aldrich. All primary antibodies were used at a 1:1000 dilution in 5% BSA TBS-T solution or 5% milk TBS-T according to manufacturer instructions. Secondary antibodies used in western blots were obtained from LiCOR: anti mouse IRDye680 (No. 926-68070), anti-mouse IRDye800 (No. 926-32210), and anti-rabbit IRDye680 (No. 925-68071). Similarly, the secondary anti-mouse or anti-rabbit HRP coupled antibodies were purchased from Cell Signaling Technology (No. 7076 and 7074 respectively). Streptavidin Magnosphere Paramagnetic Particles (Promega #Z5481) were used for the recombinant biotinylated protein pull-down and during the monobody selection. Ba/F3, K562 and Jurkat cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, Cat# ACC-300, ACC-10 and ACC-282, respectively). A549 cells were a kind gift from E. Meylan (EPFL). NPM-ALK cells were established from mouse thymoma in the lab of V. Sedl (Vet Med Uni Wien). HEK293 cells were obtained from ATCC (Cat# CRL-1573) and U3A-luc cells were a kind gift from David A. Frank (Harvard). The cytokines used in this work, human Oncostatin M (O9635) and IL-22 (SRP3089) as well as Human IL-6 (MAN0003501) were purchased from Sigma-Aldrich and Gibco, respectively.

Plasmids and cloning. Human STAT3 cDNA was purchased from the Gene Expression Core Facility at EPFL. The NTD [aa 3–138] and core fragment of STAT3 [aa 129–722] were cloned using the restriction enzymes BamHI and XhoI into a pHBT vector³⁵ comprising an N-terminal 6xHis tag, Avi-tag for biotinylation and tobacco etch virus (TEV) protease cleavage site. For lentiviral transduction, monobodies and VHL-monobodies fusions were cloned into an inducible pEM24 vector (modified pcW2239 obtained from E. Meylan, EPFL) using InFusion recombinase (Clontech). The lentiviral expression system vectors pCMV-R8_74 (encoding gag and pol proteins) and pMD2_G (encoding VSV-G envelope) were obtained from the Trono Lab at EPFL. Retroviral transduction was performed using a constitutive pRV-NTAP vector containing a 2xProteinG-TEV-cMyc tag. Monobodies were inserted in the pRV-NTAP vector using gateway cloning. Retroviral expression system encoding the VSV-G envelope (pCMV-VSV-G) was obtained from the SuperT-Fungo lab. Monobody-GFP fusions were introduced into a doxycycline inducible pEBietD vector using gateway cloning. Similarly, monobodies were inserted into a pCS2 N-term 6x-Myc tag vector using gateway cloning. All DNA constructs were confirmed by DNA sequencing (Microsynth). A complete

list of primers and description of vectors used in this work is provided in Supplementary Tables 1–4.

Monobody selection. Biotinylation of Avi-tagged proteins targets (STAT3) was performed in vivo by co-transforming *Escherichia coli* cells with a BirA plasmid and a pHBT plasmid containing the AviTag gene fusion. Cells were induced with 0.5 mM of IPTG once the OD600 of the expression culture medium between 0.5 and 0.8. A final Biotin concentration of $50\ \mu\text{M}$ from a $50\ \text{mM}$ stock in DMSO was added. Cells were further grown overnight before purification. Monobodies were selected according to methods previously described^{31,35}. Briefly, four rounds of phage display were followed by the amplification and transformation of yeast cells with the DNA sequences corresponding to binding monobodies. The yeast cells were next sorted using FACS based on a strict gating strategy comprising double positive cells for monobody display and binding to biotinylated target. Isolated clones were next sequenced and cloned into pHBT vectors for further characterization.

Yeast binding assay. Increasing concentrations of recombinant biotinylated protein targets resuspended in Tris-buffered saline (TBS) with 0.1% BSA were incubated with yeast cells displaying monobodies and a mouse anti-V5 antibody for 30 min at room temperature. Three washes with TBS with 0.1% BSA followed together with incubation for 30 min with a streptavidin-DyLight650 and a FITC-coupled anti-mouse IgG at room temperature in the dark. Samples were analyzed on a Gallios flow cytometer (Beckman Coulter). Data were fitted on a 1:1 binding model using the Prism software (Graphpad 7) to determine K_D values.

Isothermal titration calorimetry. Recombinant proteins were dialyzed overnight at 4°C in Isothermal titration calorimetry (ITC) buffer (25 mM Hepes (pH 7.5) and 150 mM NaCl) and were degassed. Total protein concentration was measured using the 280 nm protein absorbance. ITC measurement were acquired on a MicroCalPEAQ (Malvern analytical) instrument and consisted in the titration of the protein solution from the syringe in 16 steps with $0.49\ \mu\text{L}$ for the first injection followed by $2.49\ \mu\text{L}$ for the remaining steps. Protein concentrations used were $100\ \mu\text{M}$ (syringe) titrated to a $10\ \mu\text{M}$ solution (cell) as indicated on the figure legends. The MicroCal software was used to determine thermodynamic parameters.

Quantitative reverse transcription-polymerase chain reaction. Total RNA from A549 cells expressing the monobodies for 48 h followed by IL-22 ($100\ \text{ng ml}^{-1}$) stimulation (1 h at 37°C) was isolated using TRIzol reagent (Ambion, life technologies No. 15596-026). Primers for known STAT3 downstream target genes were obtained from the Etienne Meylan lab (EPFL) and were originally purchased from ThermoFisher (TaqMan Assays). Primer sequence references (cat# 4331182) are as follow: SOCS3 (Hs02330328_s1), BCL3 (Hs00180403_m1), MMP9 (Hs00234579_m1), IL-6 (Hs00985639_m1), Cldn2 (Hs00252666_s1), Actin (Hs01060665_g1) and 18S (Hs99999901_s1). Each PCR reaction contained $1\ \mu\text{L}$ of cDNA (from a RT-PCR reaction using $1000\ \text{ng}$ of total RNA) and $0.5\ \mu\text{L}$ of each $10\ \mu\text{M}$ primer mix. PCR conditions consisted in 95°C for 5 min followed by 35 cycles of $30\ \text{s}$ at 60°C . Actin housekeeping gene was used for normalization of signals. All Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analyses were performed in triplicates from three independent experiments.

Recombinant protein expression and X-ray crystallography. BL21 (DE3) E. coli cells were transformed with pHBT-monobody and pHBT-STAT3-CF constructs. An overnight pre culture was added to LB medium with an original OD at $0.05\text{--}0.1$. Cells were grown at 37°C , 200 rpm until OD reached $0.5\text{--}0.8$ and the culture was next induced with $0.5\ \text{mM}$ IPTG final concentration. Cells were further grown overnight at 18°C , 200 rpm. The next day, cells were harvested at 6000 g for 10 min at 4°C and resuspended in $40\ \text{mL}$ of culture in Buffer A [25 mM Tris (pH 7.5), 300 mM NaCl, 5% glycerol, and depending on sample, 1 mM DTT]. Cells were lysed using an Avestin Emulsiflex homogenizer and the soluble proteins were isolated from the cell debris by centrifugation at $41,000\ \text{g}$ for 30 min at 4°C . Proteins of interest were next purified by affinity using a Ni-NTA resin and

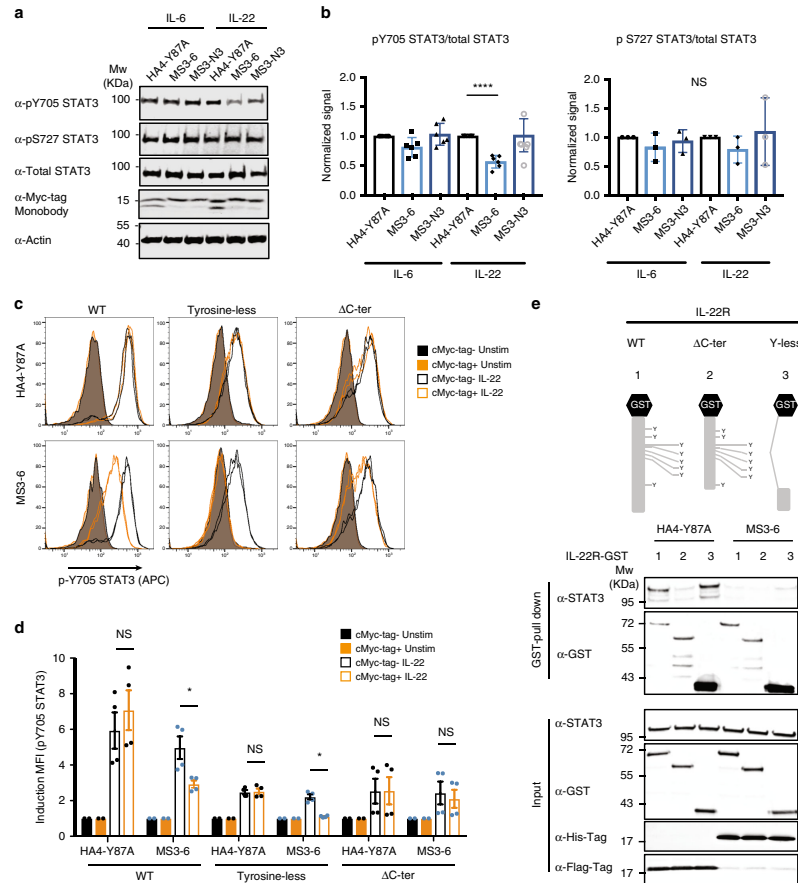


Fig. 6 MS3-6 reduces STAT3 Y705 phosphorylation levels upon IL-22 stimulation. **a** Representative immunoblot from five (pY705) and three (pS727) independent experiments of STAT3 phosphorylation levels following IL-6 and IL-22 stimulation for 15 min. All immunoblots are available as Source data Files. **b** Quantification (mean $\pm$ SD) of the results in **a** from all independent experiments for the indicated STAT3 phosphorylation sites. Significance according to an unpaired two-tailed t-test analysis: **** $P \leq 0.0001$. **c** Flow cytometry analysis on Ba/F3 cells expressing IL-22R. Four hours after electroporation of the monobody vectors (15 μ g), cells were stimulated with IL-22 (500 ng ml⁻¹) and staining was performed. A live-cell gating strategy was applied and phospho-STAT3 staining was analyzed in cMyc-tag⁺ and cMyc-tag⁻ cells. Unstimulated cells are reported as colored bar plot and stimulated cells are reported as empty bar plots. **d** Quantification of pY705-STAT3 staining from **c**. Data are presented as mean $\pm$ SEM of two independent experiments performed in duplicates ($n = 4$). Significance according to a two-tailed Mann-Whitney test analysis: * $P \leq 0.05$. **e** Upper panel, schematic representation of GST fusion proteins with intracytoplasmic domain of IL-22R. Lower panel, COS-7 cells were seeded in six-well plate and transfected with a vector coding for the GST-fusion protein or STAT3. Cells were lysed and the recovered STAT3 protein was mixed with the recombinant monobody (10 μ M) before incubation with IL-22R-GST overnight. Proteins eluted on GST SpinTrap columns as well as input samples were analyzed by western blot with an anti-STAT3 antibody. Membrane was then re-probed with anti-GST and anti-tag antibodies. Source data are provided as a source data file.

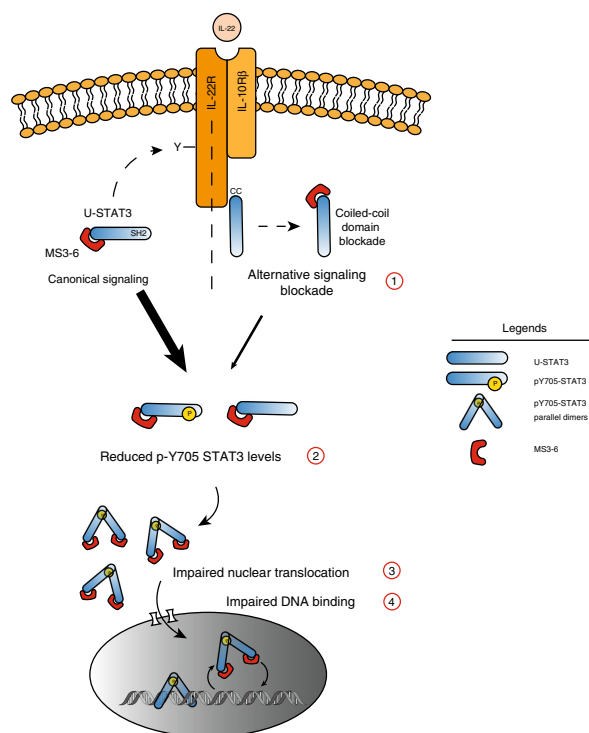


Fig. 7 Model of the mode-of-action of MS3-6. The cumulative influences (labeled 1–4) of MS3-6 binding on inhibition of STAT3 transcriptional activity is schematically illustrated based on the presented results. MS3-6 decreases STAT3 Y705 phosphorylation levels by impairing the binding of STAT3 to the IL-22R cytosolic tail, thus specifically blocking the alternative IL-22R/STAT3 signaling axis (1) and (2). Additionally, MS3-6 leads to the reduction of STAT3 translocation in the nucleus upon cytokine stimulation (3) and decreases STAT3 binding to DNA (4). Legend: U-STAT3: unphosphorylated STAT3.

size-exclusion chromatography using a Superdex 200 16/600 GL column. Complex formation was performed by mixing TEV cleaved-MS3-6 and STAT3-CF in a 1:1 molar ratio into gel filtration buffer (20 mM HEPES pH 7, 200 mM NaCl, 10 mM MgCl₂ and 2 mM DTT). The complex was concentrated in Amicon vivaspin tubes to 11 mg/ml and 0.6 mM dsDNA (forward: TGCATTTCGCGTAAATCT3' and reverse: 5'AAGATTTCAGGGAAATGCT3') was added to the drop before crystallization trials in hanging drop plates by mixing 100 nL of protein with 100 nL of buffer conditions. Addition of DNA was necessary for crystallization, despite not appearing in the final structure. Crystals were optimized in a 24-well plate format (hanging drop) by mixing 1 µL of protein with 1 µL of buffer. The best diffracting crystals were obtained in 19% PEG300, 70 mM Calcium acetate dihydrate, 100 mM imidazole pH 7 conditions, frozen in 15% (v/v) glycerol in liquid nitrogen. Data were collected at the beam-line PXIII of the Swiss Light Source (SLS), Villigen, Switzerland. Data were collected at 1 Å and at 100 K. Data was processed in XDS⁵⁶. The structure was determined by the molecular replacement method (molrep, PHENIX version 1.11.7) using a previously determined STAT3 structure (PDB:4E68) and a homology model of the monobody MS3-6 as initial search models. Manual model building of the structure was performed using Phaser and Coot and refined with REFMAC⁵⁷. The final model geometry was with 93.2% in favored regions of the Ramachandran plot with 1.8% of Ramachandran outliers.

Tandem affinity purification and mass spectrometry. Monobodies cloned into the retroviral TAP tagged vector were used to establish Jurkat and K562 stable cell lines. Cells expressing the IRES-GFP were selected and sorted using FACS. The TAP purification consisted in the lysis of used $2-3 \times 10^6$ cells as previously described⁵⁸. Briefly, the elution of the protein complexes following two steps of affinity purification was performed using 0.1 M hydrochloric acid immediately followed by the neutralization using 0.5 M Triethyl ammonium bicarbonate. Samples were then boiled and 10% of the eluates were resolved by SDS-PAGE (4–20% gel; Bio-rad) and silver stained to assess the efficiency of the pull-down. The rest of the eluates were then separated by SDS-PAGE and stained with R-250 Coomassie Blue Solution. For all single biological experiments, the entire SDS-PAGE gel lanes were first sliced into 4 fractions resulting in a total of 16 samples, which were washed twice in 50% ethanol and 50 mM ammonium bicarbonate (AB, Sigma-Aldrich) for 20 min and dried by vacuum centrifugation. Sample reduction was performed with 10 mM dithioerythritol (Merck-Millipore) for 1 h at 56 °C. A washing-drying step as described above was repeated before the alkylation step performed with 55 mM Iodoacetamide (Sigma-Aldrich) for 45 min at 37 °C in the dark. Samples were washed-dried again and digested overnight at 37 °C using modified mass spectrometry grade trypsin (Trypsin Gold, Promega) at a concentration of $12.5 \text{ ng } \mu\text{L}^{-1}$ in 50 mM AB and 10 mM CaCl₂. Resulting peptides were

extracted in 70% ethanol, 5% formic acid (FA, Merck-Millipore) twice for 20 min with permanent shaking. Samples were further dried by vacuum centrifugation and stored at -20°C . Peptides were desalted on StageTips⁴⁹ and dried under a vacuum concentrator prior to LC-MS/MS injection. Samples were resuspended in 2% acetonitrile (Biosolve), 0.1% FA and nano-flow separations were performed on a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo Fischer Scientific) on-line connected with a Q Exactive Orbitrap Mass Spectrometer (Thermo Fischer Scientific). Acquisitions were performed using Data-Dependent acquisition mode. First MS scans were acquired with a resolution of 70,000 (at 200 m/z) and the 12 most intense parent ions were selected and fragmented by High energy Collision Dissociation (HCD) with a Normalized Collision Energy (NCE) of 27% using an isolation window of 1.2 m/z. Fragmented ions were acquired with a resolution 17,500 (at 200 m/z) and then excluded for the following 30 s. A capillary precolumn (Acclaim Pepmap C18, 3 μm -100 Å, 2 cm $\times$ 75 μm ID) was used for sample trapping and cleaning. A 50 cm long capillary column (75 μm ID; in-house packed using ReproSil-Pur C18-AQ 1.9 μm silica beads; Dr. Maisch) was then used for analytical separations at 250 nl min⁻¹ over 150 min. biphasic gradients. Raw data were processed using SEQUEST (precursor and fragment ion mass tolerances were 10 ppm and 0.05 Da, respectively) in Proteome Discoverer v2.2 against a concatenated database consisting of the Uniprot human protein database (95796 entries) and monobodies sequences. Enzyme specificity was set to Trypsin and a minimum of six amino acids was required for peptide identification. Up to two missed cleavages were allowed. A cut-off was fixed at 1% FDR at the peptide and protein identification level. For the interactome database search carbamido-methylation was set as a fixed modification, whereas oxidation (M), acetylation (protein N-term), PyroGlu (N-term Q), were considered as variable modifications. Data was further processed and inspected in Scaffold 4.10 (Proteome Software, Portland, USA) and spectra of interest were manually validated. In order to analyze data in an unbiased way and filter for most unspecific protein contaminants, a selectivity score was calculated as a function of the number of TAP experiments similarly performed in Jurkat and K562 cells using unrelated monobodies (including the HA4-Y87A control)^{35,37,44,60} a given protein was detected in, as compared to the total number of TAP experiments we have performed and previously published. A threshold of >75% was applied to determine the likelihood of a protein being a specific monobody interactor. This scoring permits the identification of proteins found in only <25% of all the TAPs in our internal database, thus allowing most unspecific contaminating or highly expressed cytosolic proteins recovered from the pull down to be filtered out. Common contaminants and unspecific proteins identified were included in protein ranking shown in Table 2 for transparency. All mass spectrometry proteomics data from TAPs have been deposited to the ProteomeXchange Consortium via the PRIDE⁶¹ partner repository with the dataset identifier PXD018374.

Western blot analysis. STAT3 phosphorylation in A549 cells was assessed upon treatment with IL-6 or IL-22 (500 ng ml⁻¹) for 15 minutes at 37 °C. Total protein extraction was done in lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100) supplemented with 50 mM NaF, 1 mM Vanadate, 1 mM PMSF, 10 μg ml⁻¹ TPCK and protease and phosphatase inhibitors (Roche). Protein concentration was measured using a Bradford assay (Bio-Rad No. 500-006) or BCA (Thermo scientific No. 23225) and equal amounts of proteins were loaded on a SDS-polyacrylamide electrophoresis (PAGE) gel. Transfer to nitrocellulose membranes was performed using the semi-dry (Bio-Rad) blotting system or to PVDF membranes by overnight wet transfer. Membranes were next incubated overnight at 4 °C with primary antibodies followed by 1 h room temperature incubation with secondary antibodies. Fluorescent or chemiluminescent detection was performed using the LICOR and Bio-Rad ChemiDoc Imaging systems. Protein expression levels were quantified using the ImageStudio or Image Lab software and their relative amounts with respect to tubulin were calculated. All blots were performed in three independent experiments. All uncropped western blots are provided in the source data file.

Nuclear/cytoplasmic fractionation and immunofluorescence. Nuclear fractionation was performed upon stimulation of A549 cells for 15 minutes at 37 °C with 100 ng ml⁻¹ of IL-6 or IL-22. Cytosolic fractions were recovered by lysing cells in cytosolic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA supplemented with 2 mM DTT, 0.4 mM Na-Vanadate, 25 mM Na-Fluoride, 1 mM PMSF, 20 μg ml⁻¹ Leupeptin and 20 U ml⁻¹ Aprotinin). Extensive washing in cold PBS followed (resuspended five times in 1 ml, for 5 min each), and the nuclear pellet was recovered by centrifugation (13,000 g at 4 °C) and lysed in nuclear buffer (20 mM HEPES pH 7.9, 25% Glycerol, 400 mM NaCl, 1 mM EDTA and 1 mM EGTA supplemented with 2 mM DTT, 0.4 mM Na-Vanadate, 25 mM Na-Fluoride, 1 mM PMSF, 20 μg ml⁻¹ Leupeptin and 20 U ml⁻¹ Aprotinin). Western blot analysis was performed as described above.

Immunofluorescence was performed using transiently transfected HEK293 cells (Polyfect Qiagen No. 301107) that expressed an inducible GFP-tagged monobody upon doxycycline treatment (1 μg ml⁻¹) for 48 h. Cells were stimulated with IL-6 (100 ng ml⁻¹) for 20 minutes at 37 °C and were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Slides were next washed with PBS containing 0.01% of Triton-X and stained for 1 h at room temperature using a primary antibody (mouse anti-STAT3). Secondary antibody detection was

performed using an anti-mouse Alexa Fluor 568 antibody. Hoechst staining was performed for 5 min during the last wash. Cells were imaged with a Zeiss LSM 700 confocal microscope. Image analysis and cellular compartments were defined according to a CellProfiler workflow: a primary object detection defined the nucleus according to the Hoechst staining. Secondary object detection was performed by expansion of the GFP signal around the nucleus, which defined the cell outlines. The tertiary object corresponded to the nucleus area removed from the cell outline, which defined the cytoplasm. Mean intensity of both GFP and the anti-mouse Alexa Fluor 568 secondary antibody signals (STAT3) were quantified in all objects. Data were presented as a ratio of nuclear STAT3:cytoplasm STAT3. A total of approximately 30 cells from two individual experiments were acquired and quantified. Cells whose automatic boundary detection failed were discarded from final analysis.

Fluorescence polarization. A pY-peptide corresponding to the cytosolic tail of the gp130-Y905 co-receptor was used (5FLU-GMPKSp1LPQ1YR-NH₂). A 50 μl solution consisting in 250 nM of the peptide mixed with 1.25 μM of recombinant STAT3-CF followed by an addition of increasing concentrations (0–10 μM) of recombinant MS3-6 in TBS buffer (50 mM Tris pH 7.5 and 150 mM NaCl) was prepared. Measurements were performed in FP compatible dark bottom 96-well plates at room temperature. Wavelength set for data acquisition was 525 nm with a filter at 515 nm. Data were obtained using a M5 plate reader from Molecular Devices. Raw data were normalized to free peptide in TBS solution as background measurement.

Similarly, two DNA oligonucleotides sequences corresponding to STAT3 binding sites were used: SOCS3 (forward: 5'FAM-GCAGTTCCAGGAATCGG3'/reverse: 5'CGTCAAGGTCTTAGCC3') and a2M (forward: 5'FAM-AGCAGTA ACTGGAAAGTCCTTAATCTCTTGGGAATCT3'/reverse: 5'AGAATTCCTCA GAAGGATTAAGGACTTTCACGTTACTGCT5'). Oligos were initially mixed at equimolar concentration in annealing buffer (10 mM Tris pH 7.5, 50 mM NaCl and 1 mM EDTA) and annealed by PCR (95 °C for 2 min, then slowly decrease to RT). Once double-stranded DNA was obtained, a solution consisting of 25 μl of 10 nM d4DNA probes were added to 12.5 μl of 2 μM recombinant p-STAT3 dimer and 12.5 μl of increasing MS3-6 concentrations (final concentrations: 0, 0.5, 1, 2, 5, and 10 μM). The final 50 μl solution was seeded in FP compatible dark bottom 96-well plates. Fluorescence polarization measurements were performed at room temperature. Wavelength set for data acquisition was 485 nm with a filter at 528 nm. Data were obtained using a M5 plate reader from Molecular Devices. Raw data were normalized to free DNA in solution as background measurement.

GST pull down assay. To monitor the interaction between the different GST-IL-22R mutants generated previously²⁷ and STAT3, the proteins were expressed independently in COS-7 cells. Briefly, 4×10^7 COS-7 cells plated in a six-well plate were transiently transfected using ViaFect (Promega), according to the manufacturer's recommendations. Two days later, cells were lysed in 500 μl of lysis buffer (1% Triton X-100, 10% glycerol, 10 mM Tris (pH 8), 150 mM sodium vanadate, 1 mM sodium fluoride, 5 mM EDTA, 1 mM DTT and cComplete Protease Inhibitor Cocktail (Sigma)) and cell debris were removed by centrifugation. STAT3 was mixed with recombinant monobody at 10 μM for 8 h at 4 °C. Then, GST-proteins were added for an additional 16 h. Purification of GST was performed using GST SpinTrap columns (GE Healthcare Life Sciences) according to the manufacturer's recommendations. Input and eluted samples were analyzed by western blot with an anti-STAT3 antibody (12640, CST). The membranes were then reprobed with anti-GST (RPNI236V, Sigma), anti-Flag tag antibody (F1804, Sigma) and anti-His tag antibody (12698, CST).

Monobody expression and flow cytometry analysis. Ba/F3 cells were washed three times in PBS and 10^7 cells were electroporated with the plasmid coding for monobody (15 μg) under specific conditions (74 Ω , 280 V, 1500 μF) and used after 4 h of incubation at 37 °C. A549-MS3-6 and A549-HA4-Y87A cells, which stably integrated the doxycycline inducible monobody construct, were plated (10^5 cells) in six-well plates. The next day, cells were treated with control medium or Doxycycline (Sigma) at 1 μg ml⁻¹ for 48 h. Cells were stimulated with control medium or with human IL-22 (500 ng ml⁻¹) or human IL-6 (100 ng ml⁻¹) for 15 min at 37 °C. After incubation with a viability marker (LIVE/DEAD Fixation Near-IR Dead Cell Stain Kit, Live), cells were fixed in paraformaldehyde 2% for 10 min at 37 °C and then permeabilized in 90% methanol for 30 min on ice. After two washes with PBS-EDTA 1% FCS, cells were stained with APC-labeled anti-STAT3-pY705 (557815, BD Biosciences) and AF488 labeled anti-cMyc tag (2279, CST) for 1 h at room temperature. Cells were analyzed on BD FACSVers flow cytometer.

Luciferase assay. For initial screening of the monobodies inhibitory activity, U3A-Luc cells stably expressing a STAT3 luciferase reporter system obtained from the D. Frank lab (Harvard) were used⁶². In all, 5×10^5 cells were seeded in six-well plates and transfected upon reaching 60–80% confluency with a pEFTed-eGFP monobody vector using Polyfect (QIAGEN). In all, 1 μg ml⁻¹ doxycycline was added to induce monobody expression for 48 h. Cells were next trypsinized and washed in FACS buffer (PBS, 0.5% BSA) and 8000 cells were directly sorted into 96-well plates in duplicate or triplicate using a SONY sorter SH800. Cells were then

incubated overnight at 37 °C in 5% CO₂ in the presence of doxycycline to ensure continuous monobody expression. The next morning, cells were stimulated using OSM 10 ng ml⁻¹ for 6 h and luciferase activity was measured using the Bright Glo luciferase assay system (Promega) according to the manufacturer's instructions. Untransfected cells treated with 6 μM Ruxolitinib for 7 h at 37 °C were used as positive control.

Additional Luciferase assays were done with 10⁷ BW5147 or Ba/F3 cells, which were electroporated with vector coding for the monobody (7.5 μg), pGL3-Pap1 luciferase reporter plasmid (7.5 μg) and pRL-TK plasmid (1 μg), as internal control of transfection, under specific conditions (74 Ω, 270 V, 1200 μF). Cells were then stimulated with control medium or with murine IL-9 (100 ng ml⁻¹) or IL-24 (HEK293 supernatant 2%) or IFNα3 (HEK293 supernatant 2%) for 4 h.

A549 with inducible monobody (H4A-Y87A or MS3-6) expression (5 × 10⁵ cells) were plated in a 96-well plate. After 24 h of treatment with Doxycycline (Sigma) at 1 μg ml⁻¹, cells were transiently transfected using ViaFect (Promega), according to the manufacturer's recommendations, with the pGL3-Pap1 luciferase reporter plasmid and pRL-TK plasmid. In all, 4 h after transfection, cells were stimulated with control medium or with human IL-22 (500 ng ml⁻¹) or human IL-6 (100 ng ml⁻¹) for 20 h at 37 °C. Luciferase activity was revealed with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's recommendation.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The crystal structure of the MS3-6/STAT3-CF was deposited at Protein Data Bank under the accession code 6TLC. Proteomics data was deposited at ProteomeXchange with identifier PXD018374. Source data are provided with this paper. All other data are available from the corresponding author on reasonable request. Source data are provided with this paper.

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Author contributions

G.L.S. conducted and analyzed most experiments. C.M. conducted the IL22R experiments, T.K. performed monoclonal selection and contributed to data analysis, T.B. and B.M. provided vital tools for experiments and contributed to data analysis, A.K. and S.K. provided vital tools for experiments and contributed to study design, data interpretation and paper writing. K.L. and F.P. performed crystallography data acquisition and support to solve the structure. V.S. and L.D. contributed to study design, data interpretation and contributed to paper writing. G.L.S. and O.H. designed and coordinated the study, planned the experiments, interpreted the data and wrote the paper. All authors edited the paper.

Competing interests

A.K. and S.K. are listed as inventors on issued and pending patents on the monoclonal antibody technology filed by The University of Chicago (US Patent 9512199 B2 and related pending applications). The other authors declare no competing interests.

Additional information

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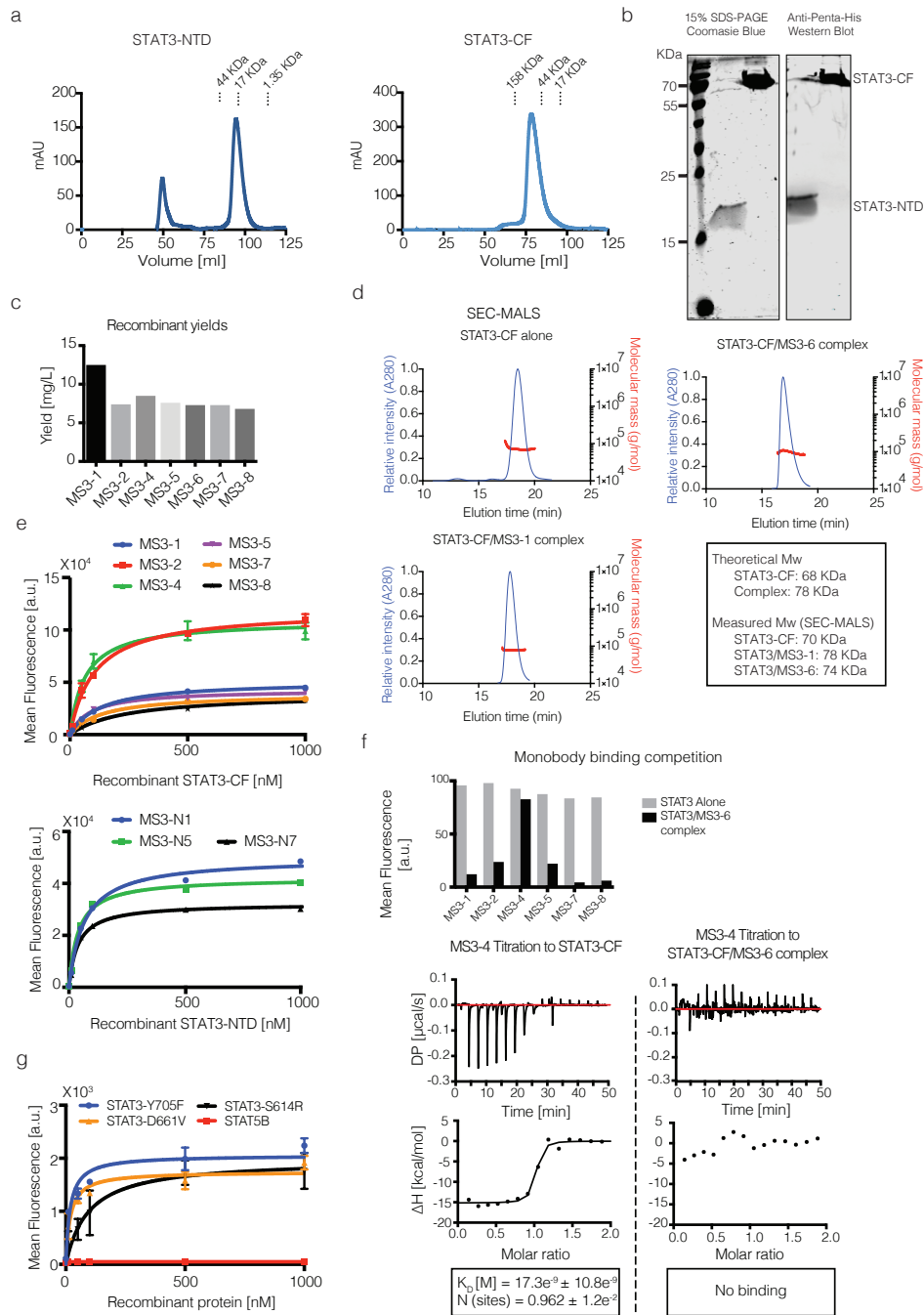


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Annex

Supplementary figures

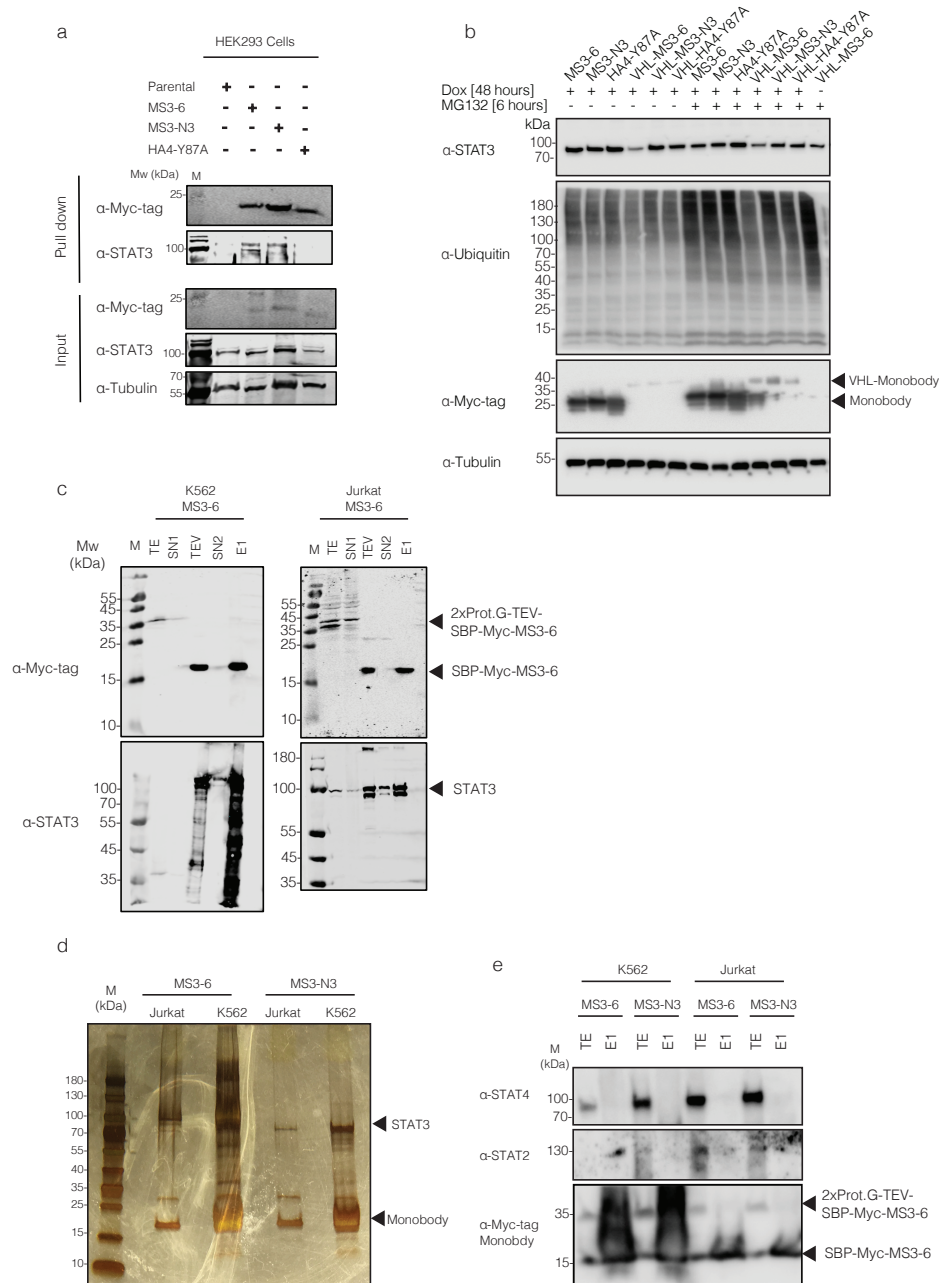


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Supplementary Fig. 1: STAT3 target protein constructs and monobodies characterization.

(a) Size exclusion chromatography traces of the purified STAT3 proteins. (b) Protein purity and identity was assessed by Coomassie staining and western blot analysis. (c) Monobodies recombinant yields expressed in *E. coli* reported in mg of protein per liter of lysogeny broth (LB) expression medium. (d) Gel filtration coupled with a multi-angle light-scattering analysis of STAT3-CF alone and in complex with monobodies. (e) Yeast binding assay of all additional monobody clones selected against the STAT3-CF (upper panel) and STAT3-NTD (lower panel). Affinities are reported in Table 1. Data presented as mean $\pm$ SD from one or two independent experiments. (f) Pre-complex formation of STAT3-CF/MS3-6 prevents other monobodies to bind to STAT3 as measured by yeast binding assay (upper panel) with the exception of MS3-4. This can be explained as the affinities of MS3-6 and MS3-4 are in a comparable range. Hence, to determine whether MS3-6 and MS3-4 shared a similar epitope, a higher sensitivity technique (ITC) was used to demonstrate that MS3-4 does not bind to the pre-formed MS3-6 complex as well (f - lower panel). MS3-4 binding to STAT3-CF alone: ΔH (Kcal/mol) = -15.2 ± 0.5 ; ΔG (Kcal/mol) = -10.6 . (g) Binding of MS3-6 to STAT3 point mutants located in its SH2 domain identified in patients suffering from various hematological malignancies. Data presented as mean $\pm$ SD from two or three independent experiments.

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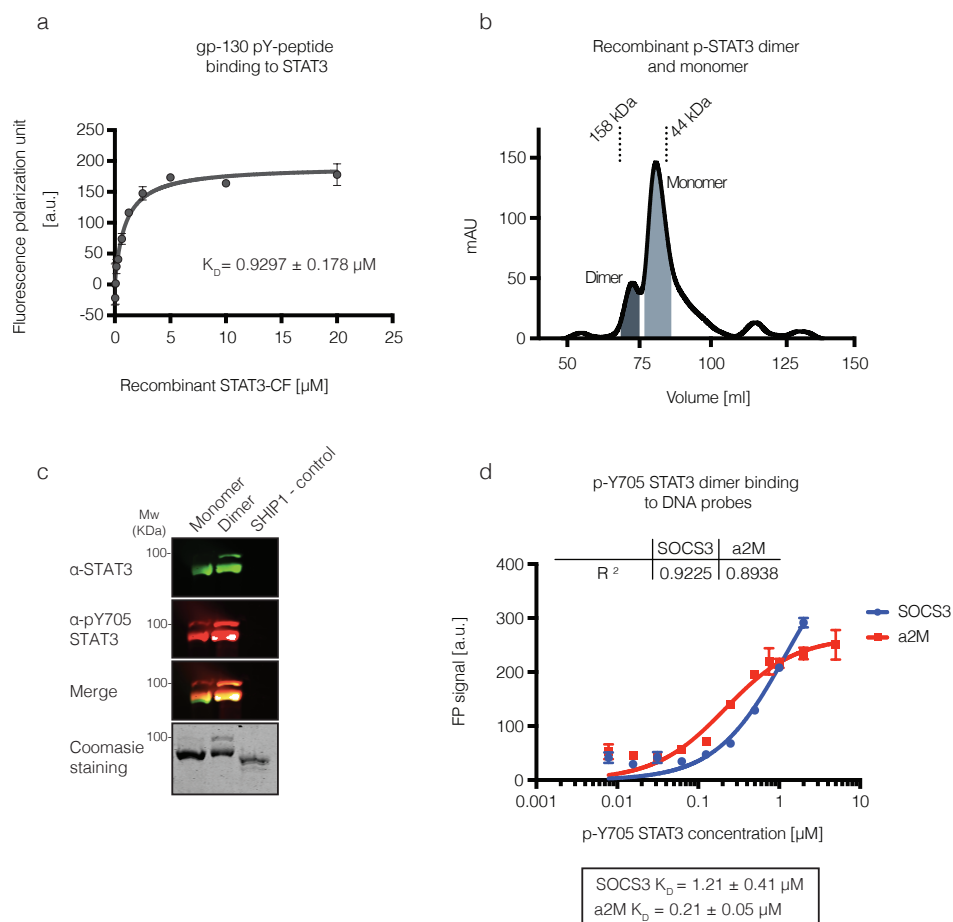
Supplementary Fig. 2: Monobodies MS3-6 and MS3-N3 binding to STAT3 in cellular contexts.

a) Myc-tagged monobody pull down upon transient transfections in HEK293 cells was performed to assess STAT3 binding in the complex reducing cellular environment. STAT3 Co-immunoprecipitation

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is detected with MS3-6 and MS3-N3, but not with HA4-Y87A control monobody.(b) STAT3 protein levels in presence or absence of a proteasome inhibitor MG132 upon monobody or VHL-monobody expression. The VHL-MS3-6 mediated STAT3 degradation is impaired in presence of MG132, highlighting the implication of the proteasome in the STAT3 targeted degradation. (c) Immunoblot analysis of Tandem-affinity purification (TAP) experiments from MS3-6 expression in Jurkat and K562 cells. Legends: M, Marker; TE, total extract; SN1 supernatant after protein G affinity capture; TEV, eluate after TEV cleavage; SN2, supernatant after streptavidin beads pull down; E1, eluate from streptavidin beads. Both monobody (bait) and STAT3 (target protein) were identified by immunoblotting using an anti-Myc tag and anti-STAT3 antibody detection. (d) 10% of the eluate (E1) fraction were resolved by SDS-PAGE and visualized by silver staining to assess the overall sample purity for MS3-6 and MS3-N3 in Jurkat and K562 cells. Major bands corresponding to the monobodies and STAT3 were identified in all fractions.(e) Total extracts (TE) and eluate fractions (E1) from Jurkat and K562 cells were probed for additional STAT family members. While both STAT2 and STAT4 are expressed at various levels in both cell lines, they could not be identified upon monobody pull down (E1 fractions). All data presented result from single experiments.

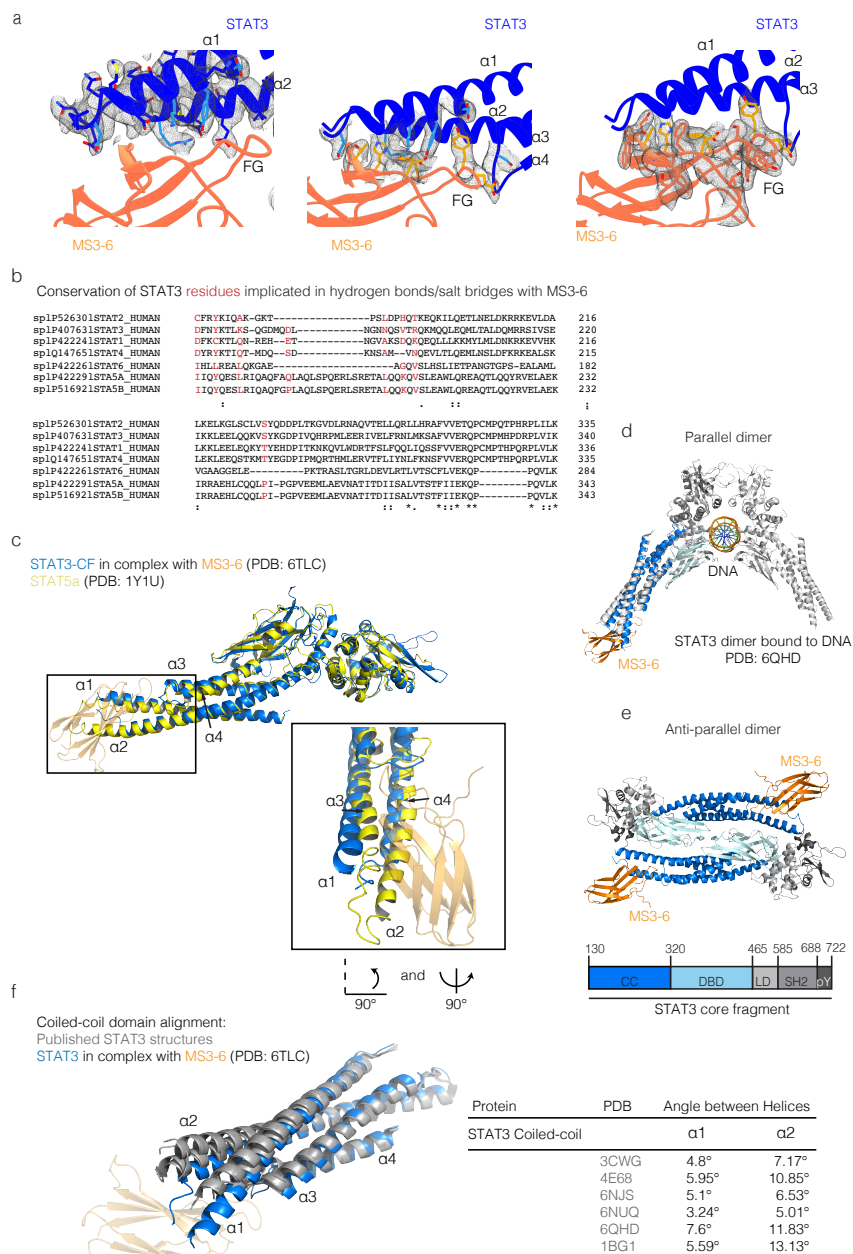
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Supplementary Fig. 3: Recombinant p-Y705 STAT3 dimer and In vitro Fluorescent polarization assays.

(a) Fluorescence polarization experiments were performed to monitor the pY-gp130 peptide binding to STAT3 SH2 domain. Increasing concentrations of recombinant STAT3-CF was added to a 250nM peptide solution at 25°C. Data from two technical replicates. (b) Size exclusion chromatography trace of a phospho-Y705 STAT3 expressed in *E. coli*. Monomeric and dimeric peaks are highlighted in light and dark blue respectively. (c) STAT3 Y705 phosphorylation was observed by immunostaining. Recombinant SHIP1 was used as a negative control. Data from a single experiment.(d) Fluorescence polarization experiments were performed to assess p-Y705 STAT3 dimer binding to two double stranded fluorescent DNA probes (full sequences in material and methods). Binding affinities are reported as K_D values. Data presented as mean $\pm$ SD from two independent experiments.

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Supplementary Fig. 4: Structural alignment of the STAT3/MS3-6 complex with previously published STATs structures.

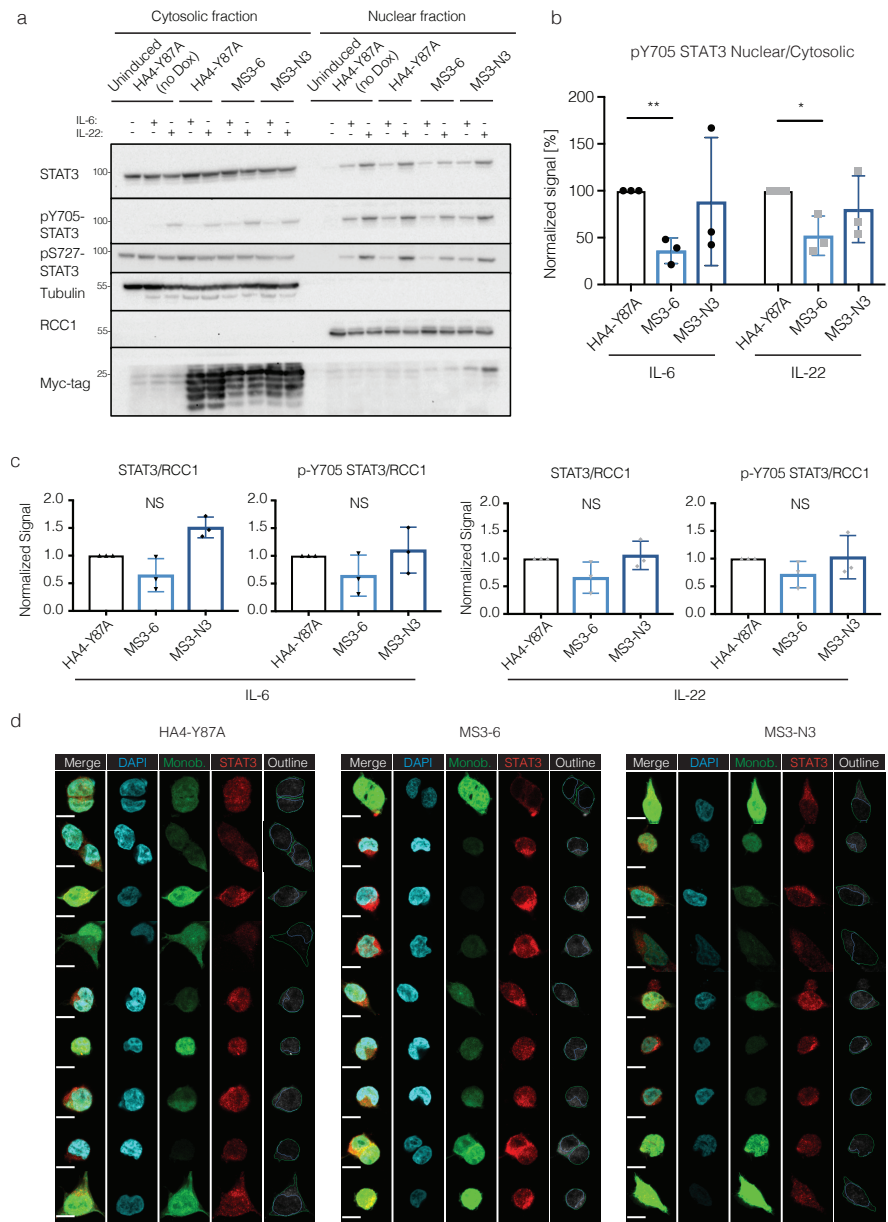
(a) Electron density map with solved model of STAT3 (blue) and MS3-6 (orange). Figure shows the 2mFo-DFc map at a 1 sigma contour level of the coiled-coil domain of STAT3 (left panel), of the binding

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interface (middle panel) and of MS3-6 (right panel). (b) Sequence alignment of all STAT family members. Residues implicated in MS3-6 binding are highlighted in red. (c) Alignment of STAT3 (blue)/MS3-6 (orange) complex (PDB: 6TLC) with a STAT5a (yellow) structure (PDB: 1Y1U). The magnification of the monobody binding interface highlights the torsion of the STAT3 coiled-coil domain (blue), as compared to the STAT5a coiled-coil domain (yellow). The longer helix $\alpha 2$ of STAT5a clashes with monobody binding (shown in transparency). (d) Structural alignment of the STAT3/MS3-6 complex with a STAT3 dimer bound to DNA (PDB: 6QHD) highlight the compatibility of the monobody binding with an efficient p-Y705 STAT3 parallel dimer formation. Individual domains are color coded as indicated.

(e) Formation of an anti-parallel STAT3 dimer, as observed in the crystal symmetry unit, is compatible with monobody binding. Individual domains are color coded as indicated. (f) MS3-6 binding to STAT3 (blue) leads to the torsion of the helices $\alpha 1$ and $\alpha 2$ of the coiled-coil domain as evidenced by the structural alignment with previously published STAT3 structures (in grey). Orientation vectors (Pymol) for each helices were used to measure the angle formed between the respective structures and are reported as a table.

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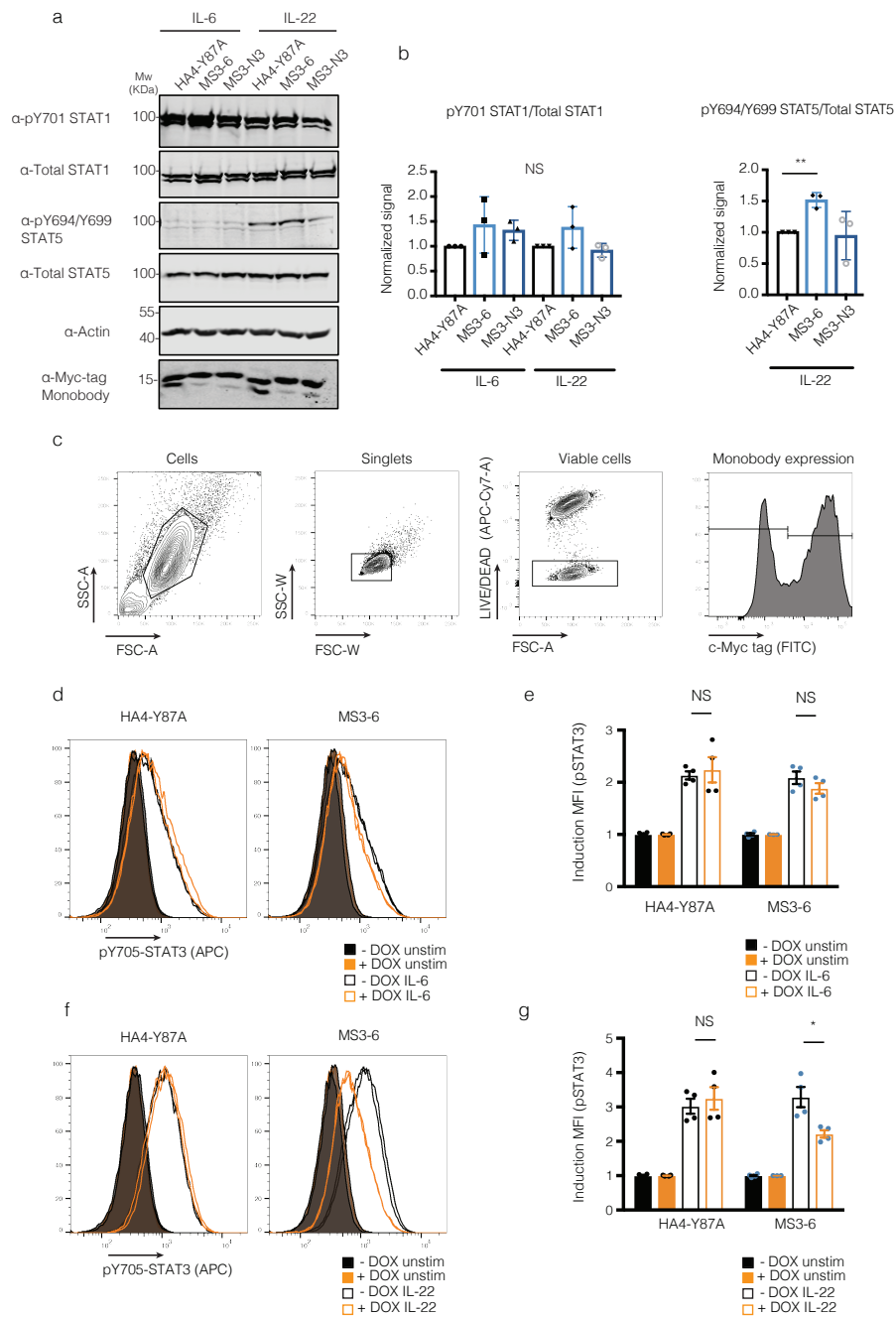


Supplementary Fig. 5: MS3-6 decreases the nuclear/cytosolic STAT3 ratio upon cytokines stimulation.

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(a) Representative immunoblot analysis of STAT3 nuclear translocation upon cytokine stimulation. MS3-6 reduces STAT3 nuclear levels as compared to the HA4-Y87A or no monobody (uninduced) control conditions. Experiment performed in three independent replicates. (b) Quantification of three independent immunoblot analysis of cellular fractionation experiments. A549 cells were treated with doxycycline to induce monobody expression for 48h, followed by IL-6 or IL-22 stimulation. Significance according to a two-tailed unpaired *t*-test analysis: **P* = 0.0168, ***P* = 0.0013. (c) Quantification of three independent immunoblot analysis of cellular fractionation experiments according to the protein levels loaded (RCC1 signal). (d) Additional representative images from confocal microscopy experiments performed in two independent experiments using HEK293 cells transiently transfected with a doxycycline inducible eGFP-monobody fusion and stimulated with IL-6 for 20 minutes at 37°C. Scale bar represents 10µm. The outline panel illustrates the threshold defined (CellProfiler) to determine the nucleus (blue line) and cytosolic compartments (green line).

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Supplementary Fig. 6: MS3-6 reduces STAT3 p-Y705 levels upon IL-22 stimulation.

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(a) Representative immunoblot analysis of STAT1 and STAT5 phosphorylation levels following stimulation with IL-6 and IL-22 in presence of monobodies. Data representative of three independent experiments. Actin, total STAT1/p-Y701 STAT1 and total STAT5/pY-694 -699 STAT5 blots were processed in parallel. (b) Quantification of STAT1 and STAT5 phosphorylation levels upon IL-6 or IL-22 stimulation in presence of monobodies. Quantifications were performed out of three independent experiments. Significance according to a two-tailed unpaired t-test analysis: $^{**}P = 0.0016$. (c) Flow cytometry gating strategy applied for all FACS data analysis. Viable cells were analyzed for monobody expression following doublets exclusion. (d) and (f) Flow cytometry on A549 cells with inducible monobody (HA4-Y87A or MS3-6) expression. Cells were treated with control or doxycycline (1 $\mu\text{g/ml}$) containing medium to induce monobody expression. Cells were stimulated with IL-6 (100 ng/ml) (e) or IL-22 (500 ng/ml) (g) and intracellular FACS staining was performed. (e) and (g) phospho-Y705 STAT3 levels were assessed, quantified and plotted as mean fluorescence intensities. Data are presented as mean $\pm$ SEM of two independent experiments performed in duplicates. Significance according to two-tailed unpaired t-test analysis: $^{*}P = 0.014$.

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Supplementary Tables 1-4.

Complete list of primers, including names and sequences

Supplementary Table 1: Primers used to clone the core fragment (CF) and NTD of STAT3.

hSTAT3-CF [aa 129-722]	Forward	CATGGGATCCGGCCAGGCCAACACC
	Reverse	CATGCTCGAGTCAAATGGTATTGCTGCAGGTCGTTG
hSTAT3-NTD [aa 3-138]	Forward	CATGGGATCCCAATGGAATCAGCTACAGCAGCTTG
	Reverse	CATGCTCGAGTCACGTCACACGCGCTGC
hSTAT5B [aa 129 - 714]	Forward	CATGGGATCC CCA GCT GGA AGC CTT GCT G
	Reverse	CTGCTCGAG TCA TGC GTT CAC AAA CTC AGG GAC

Supplementary Table 2: STAT3 oncogenic point mutants site directs mutagenesis primers.

STAT3-S614R	Forward	GATTCAGTGAAAGCAGGAAAGAAGGAGGCGTC
	Reverse	GACGCCTCCTTCTTCTGCTTTCACTGAATC
STAT3-D661V	Forward	GCTATAAGATCATGGTTGCTACCAATATCCTG
	Reverse	CAGGATATTGGTAGCAACCATGATCTTATAGC
STAT3 Y705F	Forward	GTAGCGCTGCCCCATTCTGAAGACCAAGTTATC
	Reverse	GATAAACTTGGTCTTCAGGAATGGGGCAGCGCTAC

Supplementary Table 3: Universal monobody gateway cloning primers.

For	GGGGACAAGTTTGTACAAAAAGCAGGCTCCATGGTTTCTTCTGTTCCGACCAAAC
Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGGTACGGTAGTTAATCGAG

Supplementary Table 4: List of all vectors used.

Vector name	Description
pHBT	Modified pET vector, N-term 6xHis-Avi-tag-TEV-BamHI-XhoI, used for bacterial expression of recombinant proteins inserted using BamHI/XhoI
pEM24	Modified pCW2239 obtained from E. Meylan, EPFL. Doxycycline inducible, N term 6xMyc-tagged, gateway vector, used for dox induced monobody expression in cells.
pCMV-R8_74	Lentiviral expression system encoding gag and pol proteins
pMD2_G	Lentiviral expression system encoding VSV-G envelope
pCMV-VSV-G	Retroviral expression system encoding VSV-G envelope. Obtained from the Superti-Furga lab
pRV-NTAP	2xProteinG-TEV-cMyc tag. Gateway vector. Used for tandem affinity purification pull downs.
pEBtetD	Doxycycline inducible, N-term eGFP-tag. Used for luciferase assays and immunofluorescence.
pCS2	N term 6x-Myc tag. Gateway vector. Used for transient transfections and luciferase assays.

