From the Clinic for General Pediatrics, Neonatology and Pediatric Cardiology,

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Regulation of IL-7 receptor expression and the role of IL-7:
Impact on monocyte phenotypes and antimycobacterial effector functions.

Dissertation

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Zusammenfassung

Der Interleukin-7-Rezeptor-α (IL-7Rα) und Interleukin 7(IL-7) sind Schlüsselfaktoren für die Entwicklung und Aufrechterhaltung des menschlichen Immunsystems. Diese Moleküle wurden bei T-Zellen eingehend untersucht, ihre biologischen Funktionen bei Monozyten sind jedoch nach wie vor nicht klar. Eigene frühere Befunde zeigten eine geringere IL-7R-Expression und beeinträchtigte IL-7-vermittelte Funktionen in Monozyten von Tuberkulose-Patienten, was auf die Rolle der IL-7Rα-Regulierung im Zusammenhang mit Krankheiten hindeutet. In dieser Studie zielten wir darauf ab, in vitro Monozyten zelllinien-basierte Modelle der induzierten IL-7Rα-Expression und IL-7-Reaktion zu etablieren. In diesem Zusammenhang wollten wir die Mechanismen charakterisieren, die der IL-7Rα-Regulierung in Monozyten zugrunde liegen. Wir verwendeten zellkulturbasierte Systeme, In-vitro-Infektionstests und lentivirale Vektorsysteme. Für die Analysen wurden Durchflusszytometrie und molekulare Ansätze verwendet. Unsere Ergebnisse zeigten, dass die bakteriellen Komponenten die Zytokine, hauptsächlich TNF- α, IL-1-β und GM CSF, welche induzieren können autolog die IL-7Rα Expression in Monozyten fördern. Bei der weiteren Untersuchung dieser Ergebnisse konnten wir zeigen, dass die Expression von IL-7Rα in Monozyten und Monozyten-Zelllinien (MonoMac-Zellen) vorübergehend war. Der Transkriptionsfaktor FoxO1 war für die Expression von IL-7Rα unerlässlich. Anschließend wurde die IL-7-Signalübertragung in Monozyten untersucht, dabei stellten wir eine geringere IL-7-Empfindlichkeit in IL-7Rα+ Monozyten fest. Außerdem beeinflusste IL-7 den Phänotyp der Monozyten nur geringfügig. Interessanterweise aktivierten mit IL-7 gepulste T-Zellen die Monozyten erheblich. Darüber hinaus zeigten unsere Ergebnisse IL-7-abhängige antimykobakterielle Wirkungen in LD BCG Mykobakterien infizierten MDMs. Darüber hinaus wurden die Moleküle SOCS3 und CISH gleichzeitig mit IL-7Rα in Monozyten und MonoMac 6-Zellen induziert. Wir vermuteten, dass die Hochregulierung von SOCS3 und CISH-Molekülen für die IL-7-Hyposensitivität in Monozyten verantwortlich sein könnte. Allerdings unterstützen SOCS3/CISH-Knockdown-Zellen diese Hypothese nicht. Schließlich wurde der Zusammenhang zwischen SOCS3/CISH und IL-7Rα untersucht. Bei SOCS3/CISH über exprimierenden MonoMac 6 Zellen wurde die IL-7Rα Expression hochreguliert. Andererseits stellten wir eine geringere IL-7Rα Expression in SOCS3/CISH-Knockdown-Zellen fest, was darauf schließen lässt, dass diese Moleküle die IL-7Rα Expression in Monozyten regulieren. Weitere Analysen deuten darauf hin, dass SOCS3 die IL-7Rα Expression in Monozyten Post-transkriptionell regulieren könnte. Zusammenfassend lässt sich sagen, dass unsere Ergebnisse bisher unbekannte Mechanismen zur Regulierung von IL-7Rα in Monozyten aufzeigen. Weitere Studien können die Auswirkungen von SOCS3/CISH auf die IL-7Rα-Regulierung bei Antimykobakterielle Krankheiten umfassend aufschlüsseln.

Summary

Interleukin 7 receptor-α(IL-7Rα) and interleukin 7(IL-7) are key factors for the development and maintenance of the human immune system. These molecules have been extensively studied in T cells, but their biological functions remain elusive in monocytes. Own previous findings revealed lower IL-7Rα expression and impaired IL-7 mediated functions in monocytes of tuberculosis patients, implying the role of IL-7Rα regulation in diseases context. In this study, we aimed to establish *in vitro* monocyte cell line-based models of induced IL-7Rα expression and IL-7 response. Relatedly, we wanted to characterize the mechanisms underlying IL-7Rα regulation in monocytes. We used cell culture-based systems, in *vitro* infection assays and lentiviral vector systems. Flow cytometry and molecular approaches were used for the analyses. Our findings showed that the bacterial components can induce the cytokines, mainly TNF-α, IL-1β and GM CSF, which autologously promote IL-7Rα expression in monocytes. Interrogating these findings further, we showed that IL-7Rα expression was transient in monocytes and monocyte cell lines (MonoMac cells). FoxO1 transcription factor was essential for IL-7Rα expression. Next, IL-7 signaling in monocytes was investigated and we noted a lower IL-7 sensitivity in induced IL-7Rα expressing monocytes and IL-7 minorly affected monocyte phenotypes. Intriguingly, IL-7 pulsed T cells significantly activated monocytes. Moreover, our findings revealed IL-7 dependent antimycobacterial effects in LD BCG mycobacteria infected MDMs. Furthermore, monocytes or MonoMac 6 cells activated by bacterial components/cytokines concomitantly induced SOCS3/CISH molecules and IL-7Rα expression. We reasoned that the upregulation of SOCS3 and CISH could be responsible for IL-7 hyposensitivity in monocytes, but SOCS3/CISH knockdown cells did support this hypothesis. Finally, the association of SOCS3/CISH and IL-7Rα was investigated. SOCS3/CISH overexpressing MonoMac 6 cells upregulated IL-7Rα expression. On the other hand, we noted lower IL-7Rα expression in SOCS3/CISH knockdown cells, suggesting these molecules as regulators of IL-7Rα expression in monocytes. Further analyses point out that SOCS3 may post-transcriptionally regulate IL-7Rα expression in monocytes. In a nutshell, our findings revealed unprecedented IL-7Rα regulation mechanisms in monocytes. Further studies can comprehensively dissect SOCS3/CISH effects on IL-7Rα regulation in mycobacterial diseases.

List of Abbreviations

BCG: Bacillus Calmette-Guérin

BSA: Bovine serum Albumin

CD: Cluster of differentiation

CFU: Colony forming Unit.

CISH: Cytokine inducible SH2 containing protein.

CW_{Mtb}: Mycobacterial Cell wall

Fig: Figure

GM-CSF: Granulocyte macrophage colony stimulating factor.

IFN-γ: Interferon gamma

IL-7: Interleukin 7

IL-7R-α : Interleukin 7 receptor-α

JAK: Janus Kinase

Kd/oe: Knockdown/overexpression

LAM: Lipoarabinomannan

LM: Lipomannan

MDM: Monocyte derived macrophages

MM1/6: MonoMac 1/6

Pam3: Pam3CSK4

PAMPs: Pathogen associated molecular pattern.

PBMC: Peripheral blood mononuclear cells

qPCR: Quantitative polymerase chain reaction

SOCS: Suppressor of cytokine signalling.

TB: Tuberculosis

TNF-α : Tumor Necrosis Factor-α

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1.0 Introduction

1.1 Structure and function of IL-7Rα

Interleukin 7 receptor (IL-7R) is a heterodimer, consisting of two subunits such as IL-7Rα (also termed CD127) and common γ_c chain receptor (also termed CD132)[1]. The common γ_c receptor chain subunit is shared among common γ_c cytokines [2]. IL-7Rα is also part of the co-receptor of thymic stromal lymphopoietin receptor (TSLPR)[3].

Interleukin 7 receptor-α is encoded by IL-7RA gene. It consists of 8 exons and IL-7Rα transcripts display two main variants such as IL-7Rα with all exons (membrane IL-7Rα) and soluble IL-7Rα, lacking exon 6 coding for IL-7Rα transmembrane domain. Other variants were reported in T cells, IL-7Rα lacking exon 5,6 and exon 5,6 or 7 variants [4]. Structurally IL-7Rα comprises 3 domains including extracellular chains (EC), transmembrane domains (TM), cytoplasmic tails (CT). IL-7Rα is distant from γ_c receptor chain(110Å) but this space reduces during the signaling. [1].

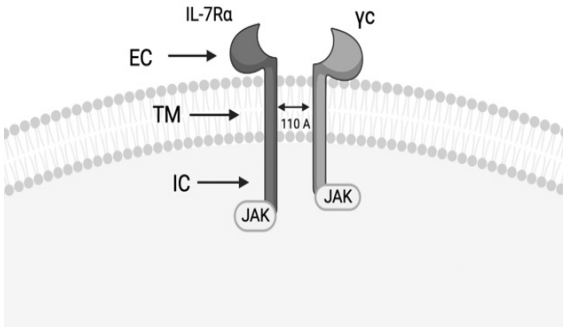

Fig. 1: Architecture of IL-7R on the cell membrane.
IL-7R consists of IL-7Rα and γ_c chain receptor. Both subunits are distant (110Å).IL-7Rα is composed of three domains including extracellular chain for ligand binding (EC), Transmembrane domain (TC) and intracellular tail (IC) for intrinsic signaling.

IL-7Rα is mainly expressed on T cells, B cells precursors and innate lymphoid cells. [3]. Monocytes express marginal IL-7Rα but activated monocytes can highly upregulate IL-7Rα [5]. IL-7Rα/IL-7 binding leads to different biological functions in T cells including proliferation, differentiation, and memory generation [3].

1.2 Structure and function of IL-7 cytokine

IL-7 is a soluble globular protein of 152aa, encoded by the IL-7 gene. It is pleiotropic, non-redundant cytokine, made up of a four-helix bundle with an up-up-down-down topology [6,7]. IL-7 is among the common γ_c chain cytokines, and it was initially recognized as a promotor of B cell development. [8,9]. Later, the role of IL-7 was expanded to other cells, and it is now evident that this cytokine is a key factor for the proliferation, survival, and memory generation of T cells [3,10,11]. IL-7 gene knocked out mice were featured by impaired immune system due to lymphopenia [12]. IL-7 is secreted by stromal cells of bone marrow and thymus [13]. Other tissues have also been reported to produce IL-7 such as epidermis, adult liver and intestines [3]. Hitherto 9 variants of IL-7 have been reported but only two isoforms were fully functional (IL-7 with all exons, and IL-7 lacking 5 and 7 exons) [14].

IL-7 initiates signaling by binding to the IL-7Rα and γ_c chain receptor is recruited. Structurally, the distance between IL-7Rα. and γ_c chain receptor is reduced from 110Å to 27Å. Jak1 and Jak3 proteins which bind to IL-7Rα and γ_c chain receptor get mutually phosphorylated. IL-7 mainly signals via Jak-STAT pathway. Following the phosphorylation of Jak molecules, Tyr449 is activated and serves as a docking site for STAT-5 proteins. Next, STAT-5 is phosphorylated and subsequently STAT-dimers will translocate into nucleus and promote gene transcription(i.e., BCL-2 and MCL-1) [1,6].

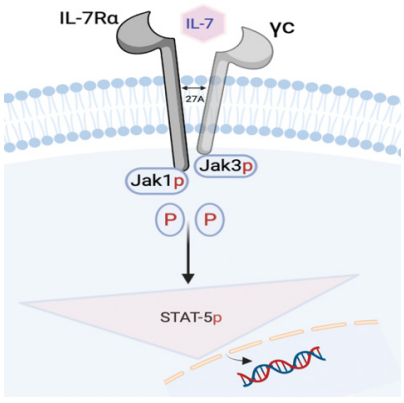

Fig. 2: Schematic depiction of IL-7 signaling.
Binding of IL-7 to IL-7Rα leads to the recruitment of γ_c chain receptor and both subunits get close. Next, Jak1 and Jak3 molecules are phosphorylated, and later STATs can be phosphorylated. Mainly STAT-5 dimers will translocate into nucleus and promote protein expression.

It is well described that IL-7 signaling can be repressed by Suppressor of cytokine signaling (SOCS) family proteins in T cells [15]. As previously reported, SOCS molecules act as negative feedback for JAK STAT signaling. SOCS family consist of 8 members such as SOCS1-7 and the cytokine inducible Src homology 2 protein (CISH) [16]. Besides the inhibitory roles of SOCS molecules, there is growing body of evidence that associate SOCS3/CISH with infectious diseases control including mycobacterial diseases [17,18]. Regulation of IL-7 signaling is well studied in T cells, but the information in monocytes is scarce.

1.3 Role of monocytes in mycobacterial diseases

Monocytes are part of the innate immune system. These cells are highly plastic, and they can differentiate into macrophages or dendritic cells. These cells are the first line of immune defence, and they play a pivotal role as effector cells. Importantly, monocyte-derived cells express chemokines which direct migrating immune cells towards inflamed or infected tissues. Monocytes/monocytes derived cells exert different functions including phagocytosis, antigen-presentation, and secretion of effector cytokines, which are crucial for initiation of adaptive immune responses.

Broadly, monocytes are classified according to CD14 and CD16 surface protein expression. Three subsets are known such as classical (CD14+ CD16-), these cells made up 80-90% of monocytes and are mainly involved in phagocytosis. Intermediate monocytes (CD14+CD16+) are approximately 2-10% and these subsets are mainly involved in antigen presentation and inflammatory responses. Alternative monocytes (CD14-CD16+) comprise 2-11% and they play pivotal roles in the maintenance of endothelium, antigen presentation and secretion of infection induced pro-inflammatory cytokines.

Monocytes and monocytes derived cells are the key components for the immune response against *Mycobacterium tuberculosis* infection [19]. Upon infection, the monocytes are mobilized and recruited to the lungs where they phagocytize and restrict mycobacteria to establish tuberculosis infection [20]. *Mycobacterium tuberculosis* use different mechanisms to evade the cellular innate immunity (i.e

inhibition of phagolysosomal maturation). Further, specialized monocytes derived macrophages together with the IFNγ producing T cells and B cells form tuberculoma structures to contain the mycobacteria in the lungs. At this stage the bacterial replication is controlled, leading to latent tuberculosis (TB). This condition can result to different clinical outcomes including elimination of the bacilli or persistent subclinical infection. Only 5-10% of infected individuals are reported to progress to active TB diseases due to primary infection or reactivation [21].

There is a growing understanding of considering monocytes as TB diagnostic marker. Monocytes shifts were suggested as TB development biomarker [22]. Recent studies also highlighted enrichment of CD16 expressing monocytes as a pathognomonic feature of acute tuberculosis [23]. Own study reported the increase of classical and intermediate monocytes proportions in tuberculosis and crucially, the findings demonstrated aberrant IL-7Rα expression[24], implying not only the changes of monocyte subsets but also expression of receptors in mycobacterial diseases.

1.4 Role of IL-7Rα /IL-7 in mycobacterial diseases

IL-7 and its receptor in monocytes or derived macrophages may be involved in the surveillance of mycobacteria and coordinated immune responses against TB diseases but the information is limited. Different studies highlighted the potential roles of IL-7Rα/IL-7 molecules in human tuberculosis. Own study revealed lower IL-7Rα expression and impaired IL-7 mediated monocytes/T cell functions in TB patients[24,25]. In addition, IL-7 was high and reverted to normal levels after TB treatment [25]. In mouse studies, IL-7 was shown to promote the production of IFNγ in T cells[26]. Persistent TB infection was observed in IL-7Rα_{low} expressing T cells of mouse [27]. In the same vein, IL-7 treated mouse survived mycobacterium disease and the splenocytes primed with IL-7 showed improved survival towards TB disease [28]. Prior studies also observed that administration of BCG vaccine synergized with IL-7 and IL-15 decreased the bacterial burden[29] and TB protein subunits vaccine, fused together with IL-7 and IL-15 induced long term immune responses in mouse [30].

IL-7Rα chain polymorphism was previous revealed in mycobacterial diseases. Own previous study assessed IL-7RA variants in purified CD4 T cells of TB patients and healthy individuals. IL-7RA full length(mIL-7Rα), IL-7R lacking exon 6(sIL-7Rα) and 5-6 isoforms were quantified and compared between rs11567764 allele carriers. Individuals carrying rs11567764 SNP displayed reduction of Δ6 IL-7Rα mRNA and Δ5-6 IL-7Rα mRNA expression. This study suggested the protective effects of IL-7RA SNPs (rs11567764/ rs1494558T) against tuberculosis infection [31]. All these above-described effects postulate that, IL-7Rα/IL-7 may be capable to promote immune responses against mycobacterial diseases.

1.5 Regulation of IL-7Rα expression

IL-7Rα is tightly regulated in T cells to avoid dysfunction of the immune system [7]. Mechanisms underlying IL-7Rα regulation are well described in T cells but less explored in monocytes. FoxO1 was reported to regulate IL-7Rα expression via a conserved non-coding sequence 1(CNS-1) region, located at 3.6kb upstream of the IL-7Rα promotor [32]. CNS-1 knockout mice displayed impaired survival and development of T cells and IL-7Rα was significantly low. FoxO1 is counter-regulated by phosphorylated AKT, which binds and sequestrates FoxO1 in cytoplasm [32,33]. Methylation of IL-7Rα promotor was postulated to silence IL-7Rα gene in CD8 T cells [34].

IL-7Rα regulation in monocytes is still in its infancy. Own findings and others have showed that IL-7Rα expression on monocytes is basal but it can be boosted by cytokines (TNF-α, IFNγ and IL-1β) [5,35] and bacterial components(i.e CW_{Mtb}, LPS and pam3csk4, termed Pam3 throughout) [5]. However, the mechanisms underlying these stimuli are still obscure.

1.6 Research objectives

Previous own findings indicated lower IL-7Rα expression and impaired IL-7 mediated monocyte functions. In contrast, the serum milieu of TB patients induced IL-7Rα expression in monocytes of healthy individuals, implying a possible complex mechanism of IL-7Rα regulation in diseases context. Whether the IL-7Rα regulation mechanisms and functions in monocytes are unique, the scientific literature is lacking. In the light with these above findings, we sought to:

1) Dissect the mechanisms underlying IL-7Rα regulation in monocytes and monocytes cell lines.
2) To establish an *in vitro* monocyte cell line-based model of induced IL-7Rα expression and IL-7 response.
3) Investigate IL-7 dependent effects in mycobacterial diseases using *in vitro* models.
4) Characterize the effects of IL-7 on monocyte phenotypes.
5) To investigate the role of T cells in IL-7 mediated effects on monocytes.

2.0 Materials and methods

The study was approved by the ethics committee of the University Hospital Düsseldorf- Heinrich-Heine-University Düsseldorf (ID: 5445).

2.1 Materials

2.1.1 Reagents

Item	Company
Distilled water Ampuwa®	Fresenius Kabi
AODC Medium	BD Biosciences
ATC	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
CelLytic™	Sigma-Aldrich
CS&T Research Beads	BD
Dimethyl-Sulfoxide (DMSO)	Sigma-Aldrich
DPBS – Dulbecco's Phosphate-Buffered Saline	Gibco
Ethylenediaminetetraacetic acid disodium salt solution (EDTA) (0,5M)	Sigma-Aldrich
FACS Clean Solution	BD
FACS Flow™	BD
FACS Rinse Solution	BD
Fetal Bovine Serum (FBS)	Gibco
Fixation buffer	BioLegend
HEPES (1M)	Invitrogen
Histopaque - 1077 Hybri Max (Ficoll)	Sigma-Aldrich
Human GM-CSF Recombinant Protein (GM-CSF)	ThermoFisher Scientific
Hygromycin	Gentour
IL-7 human	Sigma-Aldrich
Insulin Solution from Bovine Pancreas	Sigma Aldrich
L-Glutamine	Sigma-Aldrich
Lipopolysaccharide from Escherichia coli O127:B8	Sigma-Aldrich
Medium, 7H11	BD Biosciences
Medium, ADC	BD Biosciences
Medium,7H9	BD Biosciences
Methanol	VWR
Milk Powder	Sigma-Aldrich
NuPAGE™ Antioxidant	Invitrogen
NuPAGE™ LDS Sample Buffer (4X)	Invitrogen
NuPAGE™ MOPS SDS Running Buffer (20X)	Invitrogen

NuPAGE™ Sample Reducing Agent (10X)	Invitrogen
NuPAGE™ Transfer Buffer (20X)	Invitrogen
Pam3-Cys-OH (Pam3)	Sigma-Aldrich
Penicillin-Streptomycin	Sigma-Aldrich
Phosphatasehemmer-Cocktail 3	Sigma-Aldrich
PMA	Sigma-Aldrich
Polybrene Infection / Transfection Reagent	Sigma-Aldrich
Precision Plus Protein™ All Blue, Protein Standards	BioRad
Precision Plus Protein™ Dual Color Standards	BioRad
Proteasehemmer-Cocktail	Sigma-Aldrich
ReBlot Plus, stripping solution0x	Sigma-Aldrich
RPMI 1640 Medium	Gibco
SuperSignal West Femto Maximum Sensitive	Sigma-Aldrich
Tris Buffered Saline (TBS) 10x	Cell signaling
Trypan Blue Solution, 0.4%	Gibco
Tween® 20	Sigma-Aldrich
X-vivo 15	Lonza

2.1.2 Consumables

Item	Company
Beveled Tips, refill 1000µl	StarLab
Beveled Tips, refill 200µl	StarLab
Beveled Tips, refill 10µl	StarLab
Biobanking and Cell Culture Cryogenic Tubes 1ml Nunc™	ThermoFisher Scientific
Cell culture Microplate 96 Well U-Bottom plates	Greiner Bio-One
Cell culture Microplate 96 Well F-Bottom plates	Greiner Bio-One
24 Well CytoOne® Platte, TC-behandelt	StarLab
Multiwell plate for suspension culture, 24 well, PS, transparent, cover plate with condensation rings, sterile, individually packed	Greiner Bio-One
Nunc MaxiSorp ™Flat Bottom (ELISA)	ThermoFischer
Corning® Costar® Stripette® serological pipettes 25ml	Corning
Corning® Costar® Stripette® serological pipettes 10ml	Corning
Corning® Costar® Stripette® serological pipettes 5ml	Corning
Falcon™ 50ml Conical Centrifuge Tubes	ThermoFisher Scientific
Falcon™ 15ml Conical Centrifuge Tubes	ThermoFisher Scientific

Graduated Filter Tips 1000µl	StarLab
Graduated Filter Tips 200µl	StarLab
Graduated Filter Tips 20µl	StarLab
Graduated Filter Tips 10µl	StarLab
Lid, 9mm, sterile	Greiner Bio-One
Nitrile Micro-Touch® Gloves	Ansell
Microdilution Tube 1,2ml	StarLab

2.1.3 Culture medium components

Medium type	Preparation
Monocyte complete medium	RPMI 1640 Medium, no glutamine, 10% FBS, 1% Penicillin/Streptomycin, 1% HEPES, 1% L-Glutamine
X-vivo medium	X-vivo 15, 1% Penicillin/Streptomycin
Freezing medium for MonoMac 6 cells	90% FBS, 10% DMSO
Freezing medium- Rest of the cells	70% complete medium, 20% FBS, 10% DMSO

2.1.4 Buffers

Item	Preparations
1x TBS	899ml Ampuwa, 100ml 10x TBS, 1ml Tween20
10x MACS	37,5ml DPBS, 10ml EDTA, 2,5ml BSA
1x NuPage MOPS SDS Running Buffer	950ml Ampuwa, 50ml 20x NuPage MOPS SDS Running Buffer
1x NuPage Transfer Buffer	1550ml Ampuwa, 400ml Methanol, 50ml 20x NuPage Transfer Buffer
Lysis Buffer	1ml CelLytic™, 10µl Protease inhibitor Cocktail, 10µl Phosphatase inhibitor Cocktail 3
Blocking Buffer	100ml 1x TBS, 5g milk Powder
Cell sorting Buffer	DPBS + 5% FBS

2.1.5 Kits

Item	Company
Monocytes isolation kits w/o CD16 depletion	Stem cell
Monocytes isolation kit	Stem cell
CD4 T cells isolation kit	Stem cell

Pierce™ BCA Protein Assay Kit	ThermoFischer Scientific
One step qMethyl Kit	Zymo Research

2.1.6 Conjugated antibodies for flow cytometry

Antibodies	Clone	Company
PE/Dazzle™ 594 anti-human CD70 Antibody	113-16	BioLegend
PE/Dazzle™ 594 anti-human CD11c Antibody	3.9	BioLegend
PE/Cyanine7 anti-human CD11b Antibody	ICRF44	BioLegend
PE Mouse IgG1, κ Isotype Ctrl (FC) Antibody	Mouse IgG1, K	BioLegend
PE anti-human CD252 (OX40L) Antibody	11C3.1	BioLegend
PE anti-human CD127 (IL-7Rα) Antibody	A019D5	BioLegend
eBioscience™ Fixable Viability Dye eFluor™ 780		Invitrogen
CD33 Monoclonal Antibody (WM-53 (WM53)), Alexa Fluor 700, eBioscience™	WM53	Invitrogen
Brilliant Violet 785™ anti-human HLA-DR Antibody	L243	BioLegend
Brilliant Violet 785™ anti-human CD25 Antibody	BC96	BioLegend
Brilliant Violet 750™ anti-human HLA-DR Antibody	L243	BioLegend
Brilliant Violet 650™ anti-human CD274 (B7-H1, PD-L1) Antibody	29E.2A3	BioLegend
Brilliant Violet 650™ anti-human CD14 Antibody	M5E2	BioLegend
Brilliant Violet 605™ anti-human CD14 Antibody	M5E2	BioLegend
Brilliant Violet 510™ anti-human CD80 Antibody	2D10	BioLegend
Brilliant Violet 510™ anti-human CD64 Antibody	10.1	BioLegend
Brilliant Violet 510™ anti-human CD4 Antibody	OKT4	BioLegend
Brilliant Violet 421™ anti-human CD40 Antibody	5C3	BioLegend
Brilliant Violet 421™ anti-human CD274 (B7-H1, PD-L1) Antibody	MIH3	BioLegend
APC anti-human CD16 Antibody	3G8	BioLegend
APC anti-human CD14 Antibody	M5E2	BioLegend
Alexa Fluor® 700 anti-human CD127 (IL-7Rα) Antibody	A019D5	BioLegend
Alexa Fluor® 647 Rat Anti-Human CD132	TUGh4	BD
Alexa Fluor® 488 anti-human CD40 Antibody	5C3	BioLegend
Alexa Fluor® 488 anti-human CD11c Antibody	Bu15	BioLegend
PerCP/Cyanine5.5 anti-human HLA-DR Antibody	L243	BioLegend

PerCP/Cyanine5.5 anti-human CD8a Antibody	HIT8a	BioLegend
PerCP/Cyanine5.5 anti-human CD86 Antibody	IT2.2	BioLegend
PerCP/Cyanine5.5 anti-human CD70 Antibody	113-16	BioLegend

2.1.7 Primary and secondary antibodies for western blot

Antibodies	Clone	Company
IL-7R	AF-306-PB	R&D Systems
Phospho-Stat5	D47E7	Cell signaling
Stat5	D2O6Y	Cell signaling
Phospho-Stat1	D4A7	Cell signaling
Stat1 Antibody	Y701	Cell signaling
FoxO1	C29H4	Cell signaling
Phospho-FoxO1	Ser256	Cell signaling
SOCS3	C204	IBL
CISH	D4D9	Cell signaling
Monoklonales Anti-β-Actin	AC-15	Sigma-Aldrich
ECL™ Anti-Mouse IgG, HRP-linked		Cytiva
ECL™ Anti-RabbitIgG, HRP-linked		Cytiva

2.1.8 Equipments

Item	Company
Centrifuge Rotina 420R	Hettich
Centrifuge 5415C	Eppendorf
Multifuge 3S-R	Heraeus
Centrifuge Fresco 17	Heraeus
Heracell™ VIOS 160i CO2-Incubator	Thermo Fisher Scientific
Fridge (4°C) and freezer (-20°C)	Liebherr
Freezer (-80°C) TSX Series V-drive	ThermoFisher
Flake Ice Marker SPR 80 W	NordCap

FACS™ LSR Fortessa	BD
Light microscope Olympus CK40-F200	Olympus
ChemiDoc Touch Imagine System	BioRad
Incubator- MAXQ4450 for growing bacteria	ThermoFischer Scientific
Trank transfer system unit TE22	Hoefer
Cooling system	Biometra
Power Supply Model 1000/500	BioRad
Cell Sorter machine, MoFlo XDP	Becker-Couler
Neubauer Counting chamber	Marienfeld
Multichannel Pipette ErgoOne® 30-300µl	StarLab
Finnpipette™ F1 Multichannel Pipette 5-50µl	Thermo Fisher Scientific
Pipette Controller Pipetboy	Integra
Research® Pipette 100-1000µl	Eppendorf
Research® Pipette 20-200µl	Eppendorf
Research® Pipette 10-100µl	Eppendorf
Research® Pipette 0,5-10µl	Eppendorf
qPCR machine CFX96	Biorad
Thermocyclers	Biometra
Nanodrop 2000	ThermoFischer
Tecan Infinite M200- Microplate reader	Tecan
Spectrophotometer-UV Mini 1240	SHIMADZU

2.1.9 Softwares

Software	**Company**
Zotero	Zotero- Corporation for digital scholarships
FACSDiva™	BD
FlowJo®	Treestar, Inc.
GraphPad PRISM, version 9	GraphPad
Biorender	Biorender
Geneious	Biomatters
ImageLab	Biorad
Office™ (Word, Excel, Powerpoint)	Microsoft
Magellan	Tecan
CFX-Maestro	R&D Systems

2.2 Methods

2.2.1 PBMC, monocytes and CD4 T cells isolation

Buffy coats (40-50 ml) of healthy individuals were retrieved from the transfusion medicine department at the Heinrich-Heine-University in Duesseldorf. Using density gradient centrifugation (Histopaque-1077; Sigma-Aldrich), Peripheral blood mononuclear cells (PBMC) were isolated from blood and buffy coats following manufactures instructions. In short, buffy coats were diluted in sterile PBS and carefully layered on Histopaque solution. The cells were centrifuged for 30 minutes at RT, followed by two washing steps (20 Min, 12 min) at 4^{o}C. For buffy coats, monocytes or CD4 T cells were purified from PMBC(5x10^{6}) by immunomagnetic non-contact methodology (Easy Sept ^{TM} Monocytes negative selection kit w/o CD16 depletion for monocytes, Easy Sept ^{TM} CD4 T cells negative selection for CD4 T cells; Stem cell technology). The purity of enriched monocytes ranged from 82 to 92% and CD4 T cells (95-99%).

2.2.2 Monocytes *in vitro* culture and generation of monocyte derived macrophages

Monocytes were cultured in monocyte medium containing RPMI1640 (Gibco), heat-inactivated FBS (10%, Gibco), Hepes (1 %, Gibco) and L-Glutamine (1%, Sigma 1%). (Termed; monocyte complete medium throughout). For short term culture, 5X10^{4} cells/well were cultivated in U-bottom plates (Cat: 650 180, Greiner Bio-One). To mature the monocytes derived macrophages (MDMs), enriched monocytes were incubated in monocyte complete medium for 4 days (37^{o}C, 5% CO_2) in flat-bottom 96-well plates (Cat: 655 980, Greiner Bio-One). Sterile PBS was added to the neighbouring wells. Duplicate or triplicates wells were considered for all experiments.

2.2.3 Induction of IL-7Rα expression on primary monocytes and monocytes derived macrophages.

To characterise IL-7Rα regulation mechanisms in monocytes or MDMs, we cultured $5x10^4$ cells per well for 24h (37°C, 5% CO_2) with different stimuli including cytokines; GM-CSF (5ng/ml; ThermoFischer Scientific), TNFα (10ng/ml; Sigma-Aldrich), IL-1β (10ng/ml; Biolegend), IFNγ (100ng/ml; Sigma-Aldrich, IL-10 (10ng/ml; Biolegend), IL-6 (10ng /ml; Biolegend) and mycobacteria or mycobacteria components; CW_{Mtb} proteins (1µg/mL), PAM3 (100ng/ml; Sigma-Aldrich), Bacillus Calmette Guerin (BCG) (MOI 0.5), H37Rv strain mycobacterial soluble cell wall proteins (CW; 1µg/mL), H37Rv strain Lipoarabinomannan (LAM1;µg/mL), H37Rv strain Lipomannan (LM;1µg/mL) and purified proteins derivatives (PPD; 1µg/mL). All H37Rv strain components were provided by BEI-Resources, USA). Lipopolysaccharide (LPS,1ng/mL) was also used for some stimulations. IL-7Rα expression was measured by LSR Fortessa flow cytometer (BD Bioscience).

2.2.4 Inhibition of cytokines induced by mycobacteria components.

To investigate the mechanisms underlying bacterial components induced- IL-7Rα expression, monocytes or MDMs were co-cultured with CW_{Mtb} (10 µg/mL) or PAM3 (100ng/mL) and single or cocktail of cytokine depleting antibodies such as anti-TNFα; Infliximab, Sigma-Aldrich), anti-IL-1β (InvivoGen), anti-GM-CSF (Biolegend) at different concentrations (1-10 µg/mL). After 24h of incubation, IL-7Rα expression was characterised by flow cytometry analyses.

2.2.5 FoxO1 inhibition experiments.

To associate IL-7Rα expression with FoxO1 transcription factor, we established inhibition assays. Human monocytic cell lines (MonoMac1 and MonoMac 6) and enriched primary monocytes($5X10^4$/well) were stimulated with PAM3(100ng/ml) for 6h and 24h respectively. Simultaneously the FoxO1 inhibitor (AS1842856, Sigma-Aldrich) was added at different concentrations (0.25, 2.5 and 10 µg/mL). DMSO controls were separately included and IL-7Rα expression was measured by Flowcytometry.

2.2.6 IL-7Rα mRNA transcripts export

Since FoxO1 expression was constitutive, next we analyzed differential IL-7Rα mRNA transcripts and methylation of IL-7R promotor. To perform IL-7Rα mRNA transcripts analysis, firstly polyadenylation sites prediction of IL-7Rα was performed by APARENT polyadenylation sites (PAS) prediction and the results revealed two sites, suggesting two potential IL-7Rα transcripts (Analyzed in collaboration with Research group of Prof. Dr. Heiner Schaal, Heinerich Heine University-Düsseldorf). Using Geneious software (Trial version, Biomatters), we designed the primers (termed; short or long) to reflect the primers which can amplify the short or the long transcript of IL-7Rα respectively. Forward 5′ GTTTTGTGTTGTCTGTGAATGG 3′ and reverse 3′ CCTGAAGAATAAGATGGTCTG 5′ for short transcript. Forward 5′CCAAAGTGCTCTCCTCTGC 3′ and reverse 3′ GGAGGTGGAAGGAATGC 5′ for long transcript. GAPDH gene was included as a housekeeping gene. Forward 5′ ATG GAAATCCCATCACCATCTT 3′ and reverse 3′ GGTTTTAGTTCACCCCGC 5′.
MonoMac 1 and MonoMac 6 cell lines were stimulated with Pam3(100ng/mL) for 6h and 24h. mRNA isolation was performed with Macherey Nagel RNA isolation kit per manufacturer guidelines. cDNA synthesis was performed for the samples using Maxima H Minus First Strand Kit (Thermo Fischer Scientific) by following manufactures instructions. We next performed qPCR with SYBR Green PCR Master mix (ThermoFischer) on CFX96 Real time machine (BioRad). The following program was used; Holding stage 2 min, 50°C; Holding stage 10 min, 95 °C; Cycling stage (15 sec ,95 °C; 30 sec, 53 °C; 30 sec for 40x cycles), Melt Curve Stage (30 sec, 53 °C; 60 sec, 60 °C, 30 sec; 30 sec, 95 °C; 15 sec, 60 °C).

2.2.7 IL-7Rα methylation experiment

To investigate the effects of methylation on IL-7Rα expression, we firstly analyzed IL-7Rα promotor (-1341-1bp) as previously described (GenBank accession number DQ821273) [34]. Using Geneious software (Trial version, Biomatters), we designed the primers which can amplify the potential methylation sites in IL-7Rα promotor as previously shown [36]. Forward: 5′CTACAAAGGGTCAGTAGTAAAG 3′, Reverse: 3′GATGACACCACACATCATATAC 5′. Restriction enzymes-based methylation assay was used per manufacturer guidelines (One step qMethyl Kit, Cat: D5310, Zymo

research). In brief, MonoMac 6 cells were stimulated with Pam3 for 6h and 24h. Subsequently genomic DNA was isolated using Macherey Nagel DNA isolation kit. DNA samples were split into DNA test reaction, containing the qPCR master mix with restriction enzymes and DNA reference with no restriction enzymes. The samples were incubated for 1h at 37^{o}C and qPCR analysis was performed under this program; Initial denaturation for 10 minutes at 95^{o}C, denaturation, annealing and extension (30 sec at 95^{o}C, 60 sec at 54^{o}C and 60 sec at 72^{o}C respectively for 40 cycles), final extension for 7 minutes at 72^{o}C. Finally, the percentage of delta values from reference and test reactions were calculated to determine the level of methylation.

2.2.8 STAT phosphorylation analysis

To characterize IL-7 signaling in activated monocytes and human monocytic cell lines (MonoMac cells, THP-1 cells), we used a concentration of 5x10^{4} cells per well. purified CD4 T cells were included separately as a control. cells were stimulated in monocyte complete medium. In short, the cells were incubated with pre-warmed medium containing IL-7(1-10 ng/mL, sigma Aldrich) cytokines for 15 minutes at 37°C. Next, the cells were fixed in 100 µl of true nuclear fixation buffer (Biolegend). The cells were then permeabilized in 130 µl of methanol on ice for 30 min. Methanol was eliminated by two washing steps in 5% FBS/PBS and the cells were stained with different antibodies; anti-pSTAT5 (PE, clone: SRBCZX; eBiosciences). Finally, the cells were measured using LSR-Fortessa flow cytometer (BD Bioscience) and analysis was performed with flowJo software.

2.2.9 IL-7 effects on monocyte phenotypes

Following IL-7 signaling assays, we investigated IL-7 mediated effects in monocytes. Enriched monocytes were stimulated with pam3(100ng/mL) and concomitantly cells were supplied with IL-7(1ng/mL) for 24h, 48h or 72h at 37°C, 5% CO_2. IL-7 untreated controls were considered in all conditions. To investigate possible IL-7 indirect effects via T cells, enriched CD4 T cells were shortly pulsed with IL-7 (0,1, 1 and 10ng/mL) and incubated for 30 minutes at 37°C, 5% CO_2. Subsequently, the supernatant was eliminated, and cells were washed twice to eliminate IL-7 cytokine residuals. The cells were incubated for 24h at 37°C, 5% CO_2. To assess if CD4 T cells could directly or indirectly affect the monocyte phenotypes, the transwell plates were used. As shown (Fig. 3), IL-7 supplied monocytes and CD4 T cells were cultured (ratio 1:10) separately and they could only communicate via soluble factors (a) and co-culture of monocytes and CD4 T cells in the presence of IL-7 (b). The cells were incubated for 24h at 37°C.

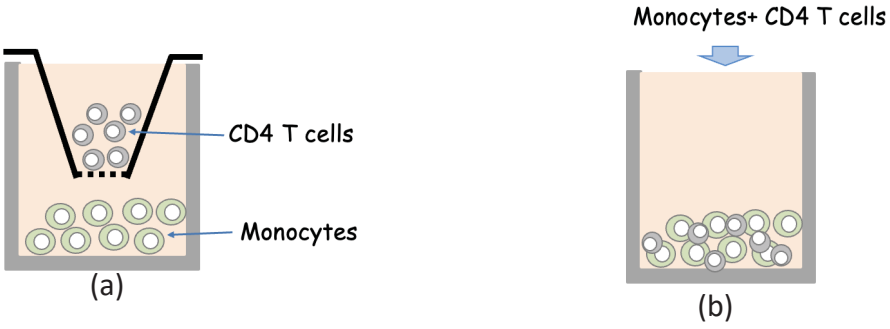

Fig. 3: Illustration of the transwell plates
CD4 T cells were cultured in upper chamber, while the monocytes were in the lower chamber. The cells could only communicate via membrane and only the soluble factors could pass through (**a**). Monocytes and CD4 T cells were co-cultured together (**b**). These two setups were supplied with IL-7(1ng/mL).

Different markers were stained and assessed on monocytes by multiparameter flow cytometry. We used different antibodies against CD40 (BV421, clone: 5C3; Biolegend), HLA-DR (PerCp-Cy5.5, Clone: L243; Biolegend), CD14 (BV605, clone: M5E2; BioLegend), CD11c (Alexafluor488, clone: Bu15; BioLegend), PD-L1 [BV650, clone:29EA3; BioLegend]), CD33 (Alexafluor700, clone: WM-53; Invitrogen), CD16 (APC, clone: 3G8; BioLegend), CD70 (PE-CF594, clone: 113-16; BioLegend), IL-7Rα (PE, clone: A019D5; BioLegend), CD11b [PE-Cy7, clone: ICRF44; BioLegend], CD64 [BV510, clone: 10.1; BioLegend], and viability dye (eFluor780; eBiosciences). We analysed the monocyte phenotypes using FlowJo

software (version 10; BD Biosciences, Franklin Lakes). MFI of monocytes activation markers (HLA DR and CD40) was computed. To characterize monocyte phenotypes, we visualized the cells with TriMap cell clustering algorithm as detailed below.

2.2.10 TriMap algorithm for visualization of IL-7 effects on monocytes

To characterize monocyte phenotypes, we used TriMap clustering algorithm following the guidelines of the author [37]. In brief, TriMap plugin was installed following the FlowJo instructions. The cells were imported in FlowJo software (version 10; BD Biosciences, Franklin Lakes) and the conditions were assigned the keywords. Subsequently, we gated on viable cells and HLA DR positive cells to exclude other cells other than monocytes. The cells were concatenated. IL-7 treated, and non-treated conditions were included. Thereafter, we used a TriMap clustering algorithm [37] for reduction of data complexity and visualization of monocyte phenotypes. This algorithm displays the cells in three regions and the global structure of the cells is maintained. Smoothed density plots were used to exhibit the monocyte phenotype changes. In addition, IL-7Rα+ monocytes were gated on HLA DR positive cells. Next, IL-7Rα+ monocytes were overlayed on the FACs plots. After localizing the monocytes regions, we manually gated IL-7Rα+ monocytes in dotted plots. Finally, multi-histograms overlaying IL-7 treated and non-treated conditions were also generated to confirm the changes suggested by TriMap plots.

2.2.11 Characterization of IL-7Rα expression on wild or modulated human monocytic cell lines (MonoMac and THP-1 cells).

Since primary monocytes are plastic and their life span is limited in culture. We used human monocytic cell lines for further characterization of IL-7Rα expression.
Human monocytic cell lines; MonoMac1 and MonoMac 6 cells (MonoMac 1: ACC 252, MonoMac 6: ACC 124) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH). THP1- cells were purchased from the Institute of Pharmaceutical Biology and Biotechnology, Düsseldorf. The cells were cultured in monocyte complete medium. For MonoMac 6 cells, medium was supplemented with insulin (10µg/ml; Sigma-Aldrich). MonoMac

cells were cultured in T-50 cell culture flask (Greiner bio-one) at a density of $0.3x10^6$ cells/ml for one week. THP-1 cells were cultured a density of $1\text{-}2x10^6$ cells/ml for a week. The cells were sub-cultured twice in 7 days. To determine IL-7Rα expression, MonoMac cells $5x10^4$ cells/well were taken from the T-50 flask and stimulated with different stimuli; Pam3(100ng/mL), GM CSF(5ng/mL) and IL-1β(10ng/mL) for 3h, 6h, 24h and 48h at 37°C 5% CO_2. Subsequently, cells were washed with 1x MACS buffer and stained for 30 minutes on ice in the dark with fluorescently labelled antibodies (anti-IL-7Rα (PE, clone: A019D5; Biolegend), $IgG1_K$ -PE isotype control (clone: MOPC-21, Biolegend) and viability dye (eFluor780; eBiosciences). Flow cytometry analyses of IL-7Rα expression was determined by LSR-Fortessa flow cytometer (BD Bioscience).

2.2.12 *In vitro* anti-mycobacterial assay

Following the investigation of IL-7 effects on monocytes. Next IL-7 mediated effects were assessed in mycobacterial infection using in *vitro* models.

2.2.12.1 Fluorescence based cytotoxicity assay.

BCG (Bacillus Calmette-Guérin) containing a live/dead reporter plasmid (known as LD BCG) were used to analyse mycobacterial infection of monocyte derived macrophages (MDMs). LD BCG were previously generated by our research group, and we used the same strain in this study [38]. We cultured the bacteria in 7H9 medium supplemented with 10% ADC (BD Biosciences) and hygromycin (50µg/mL, Gentaur) until the required log phase (OD_{600nm} between 0.8 and 0.9) was reached. The monocytes were matured for 4 days in monocyte complete medium supplemented with or without IL-7(10ng; Sigma-Aldrich). MDMs were infected with the log phase grown LD BCG treated with anhydrotetracycline (ATC,0.2 µg/mL, Sigma Aldrich) and non-ATC treated controls were considered. Rifampicin (0.5µM) was separately included as a control.

MOI of 1:1 was used and infected cells were incubated for 4 hours (37ºC, 5% CO_2). Subsequently, the extracellular bacteria were washed away using pre-warmed medium and the cells were incubated for 48h. The cells were characterised by flow cytometry using Mcherry and GFP proteins. LD BCG bacteria constitutively express Mcherry to mark their presence and GFP is a tetracycline inducible protein to indicate the viable bacteria. In short, the infected cells were placed on ice for 30

minutes in 1xMACS buffer to allow the detachment of the cells. Thereafter, the cells were stained with Anti-CD11b and the viability dye for 30 minutes on ice in the dark and then fixed (Perm/fix; Biolegend) according to manufacturers' instructions. Lastly, the cells were measured on LSR-Fortessa flow cytometer. During the samples acquisition, we added count beads ($1x10^4$; 123 count eBeads; Biosciences) to normalise MDMs counts. Data analysis was performed using FlowJo software. Absolute numbers and frequencies of live or dead BCG infected MDMs were characterised using stringent gating strategies. Uninfected and non-ATC treated MDMs controls were used.

2.2.12.2 Colony forming units.

To validate the IL-7 effects suggested by flow cytometry analyses, we performed colony forming units (CFUs), LD BCG infected MDMs were lysed in 120 µl/well PBS containing 0.5%Tween80 (Sigma Aldrich) for 30 minutes on ice. Following the lysis, the lysates were sequentially diluted in sterile PBS (10^{-1},10^{-2} and 10^{-3}). Using disposable loops, 50µl of lysates were inoculated on 7H11 agar plates supplemented with OADC (BD Biosciences). Hygromycin (50 µg/ml, Gentaur) was also added for the selection of LD BCG growth. The plates were incubated at 37°C for 3 weeks. The colonies on IL-7 treated MDMs and controls plates were recorded. CFUs were calculated by multiplying the counted colonies with dilution factors (i.e., 10,100,100) and divided with the inoculated volume(0.05mL).

2.2.13 IL-7Rα isoforms and SOCS family mRNA analysis

To analyze the transcripts of IL-7Rα and SOCS3 family, enriched monocytes and MonoMac 6 cells were stimulated for 3h, 6h and 24h. RNA extraction and cDNA synthesis were performed as described above. Finally, qPCR was performed under the following setup. Holding stage 2 min, 50°C; Holding stage 10 min, 95 °C; Cycling stage (15 sec ,95 °C; 30 sec, 53 °C; 30 sec for 40x cycles), Melt Curve Stage (30 sec, 53 °C; 60 sec, 60 °C, 30 sec; 30 sec, 95 °C; 15 sec, 60 °C).

Primers used for isoforms and SOCS family transcripts.

Gene	Forward (5´-3´)	Reverse (3´-5´)

Membrane IL-7Rα	AATAATAGCTCAGGGGAGATGG	GAGACAGCGAGACAACCAGTA
Soluble IL-7Rα	GATCAATAATAGCTCAGGATTAAGC	GTATTCTTCTGAGACCTTGTAGAA
SOCS1	GGCCCCTTCTGTAGGATGGT	CTGTTACGTCAGAGGTGTCGT
SOCS2	TGTTCACCTTTATCTGACCAAACC	AGAGACG TCGTAGAGACATCCG
SOCS3	GACCAGCGCCACTTCTTCAC	TTGTTGGACGCGTAGGTC
CISH	TGTGCATAGCCAAGACCTTCTC	ACCATAACCCCAAGGTAATGC

2.2.14 Lentiviral transduction of SOCS3, CISH or IL-7Rα proteins in monocytes cell lines (MonoMac6/THP-1).

IL-7Rα and SOCS3/CISH proteins were concomitantly expressed in activated monocytes and monocyte cell lines. We investigated if SOCS3/CISH molecules can be linked to lower IL-7 sensitivity or other functions.

Lentiviral vector systems for enforcing or knocking down biological molecules were previously established in our Laboratory [15,39]. In brief, shRNA candidates for SOCS3/CISH were purchased from sigma Aldrich and transferred into GFP containing LeGO plasmids. Potential candidates were tested in HEK293T cells. Finally, shRNA with high specificity were cloned into eBFP LeGO vector for further experiments. SOCS3/CISH cDNA containing plasmids were also transfected in HEK293T cells. Empty vector constructs were included as a control in all conditions. Next, to overexpress or knockdown SOCS3/CISH proteins in MonoMac6 cells, we used the viral vectors for SOCS3/CISH. The non-tissue treated plates (Cat: 351172, Corning) were coated overnight with RetroNectin (36µg/mL, Takara). Next, the RetroNectin was completely removed, and the plates were washed. We used 50% of the viral supernatant for each well and the viruses were centrifuged for one hour. Subsequently the cells were added and incubated for overnight in monocytes complete medium supplemented with insulin. The cells were transferred in T-50 flask cell culture. The cells were ready for further experiments after two weeks culture.

For overexpressing IL-7Rα in THP-1 cells, IL-7Rα consisting of plasmids(eGFP) and empty eGFP containing vector constructs were transfected in HEK293 T cells. Transfection protocol was previously optimized and the viral supernatant was generated [40]. In the presence of polybrene 8µg/mL, we incubated THP-1 cells ($1x10^{5,}$ per well), together with IL-7Rα containing viral vectors in the non-tissue treated plates for 3 days at 37°C, 5% $CO_{2.}$ Thereafter, the cells were grown in T-50

cell culture flasks (Greiner Bio-one) for two weeks. Finally, IL-7Rα transduced cells were sorted and used for further experiments.

2.2.15 Western blots analyses

To analyze total IL-7Rα expression (Soluble or Membrane), SOCS proteins, FoxO1 and phosphorylated proteins, we performed western blot. Human monocytic cell lines (MonoMac cells and enriched primary monocytes were stimulated with different stimuli; Pam3(100ng/mL), GM CSF(5ng/mL) and IL-1β(10ng/mL) for 3h, 6h, 24h at 37°C 5% CO_2. IL-7Rα$^+$ THP-1 cells were treated with IL-7(10ng/mL) or TSLP (10ng/mL). Following the incubation, the supernatant was discarded. The cells were washed in PBS and lysed in 80 µl of Cell Lytic solution supplemented with phosphatase inhibitors (P8340, P0044: Sigma Aldrich) according to manufactures guidelines. The lysates containing the proteins were harvested. To normalise our analyses, proteins were quantified using BSA protein Assay reagent (ThermoFisher Scientific). We added the protein reducing agents and NuPAGE and the proteins were denatured for 10 min at 80^{o}C and subsequently separated on NuPAGE 4-12% Novex Bis-Tri Gel (Invitrogen) in NuPAGE MOPS Buffer (Thermo Fischer Scientific) for 1h. The proteins were electrophoretically transferred to a 0.2µm nitrocellulose or PVDF membrane (Bio-Rad) in NuPAGE blotting Buffer (ThermoFisher Scientific) for 2h. Membranes were incubated in blocking buffer (1xTBS, 0.1% Tween-20, 5% skim milk) for 1h at room temperature. Thereafter, the membranes were probed overnight at 4^{o}C with the primary antibodies diluted per manufactures guidelines; polyclonal goat anti-human IL-7Ra (1:2000, cat: AF-306-PB, R&D systems),rabbit monoclonal antibodies anti-FoxO1 (1:1000, clone: S256, Cell signalling),rabbit polyclonal antibodies anti-pFoxO1 (1:1000, clone: C29H4, Cell signalling), rabbit monoclonal antibodies anti-SOCS3(1:1000, clone: C204, cat:18391-S, IBL), rabbit monoclonal antibodies anti-CISH(1:1000, clone: D4D9, cat: 8731, cell signalling), mouse monoclonal antibodies anti-β-actin (1:5000, clone: AC, Sigma-Aldrich). Matching secondary antibodies were applied for 1h at room temperature. We intermittently washed the membranes in (1xTBS, 0.1% Tween-20) between the proteins probing. Finally, we detected our target proteins on ChemiDoc Imaging Systems (Bio-Rad) using Super-Signal^{TM} West Femto Maximum Sensitivity

Substrate (Thermo Fischer Scientific) or BM chemiluminescence western blotting substrate (Sigma-Aldrich).

2.2.16 Analyses and statistics

LSR Fortessa (BD Bioscience) was used to measure the cells and FlowJo software (version 10; BD Biosciences, Franklin Lakes) was used for the analyses. GraphPad Prism v9 software (GraphPad Software, La Jolla CA, USA) was used to compute all statistical analyses. To compare different conditions, the non-parametric Mann-Whitney U test or Wilcoxon sign rank test were appropriately used. To statistically evaluate more than two groups, non-parametric Friedman test or Kruskal-wallis were applied. A p-value below 0.05 was considered statistically significant.

3.0 Results

3.1 Bacterial components induce IL-7Rα expression via autologous cytokines.

Regulation of IL-7Rα expression in monocytes is not well described. Bacterial components (i.e., LPS and PAM3) were reported to elicit IL-7Rα expression in monocytes [5]. Here, we aimed to investigate the regulation mechanisms underlying IL-7Rα expression on monocytes and monocytes derived macrophages. Firstly, we screened different potential IL-7Rα inducers including mycobacteria proteins (i.e., H37Rv LAM, LM, PPD and CW proteins), BCG mycobacteria and TLR agonists. As described, LPS strongly upregulated IL-7Rα on monocytes, ranging from 58% to 74% (Fig. 4a, left graph). Strikingly, Live BCG could induce approximately the same level of IL-7Rα expression as LPS (range 61% to 85%, left graph). CW_{Mtb}, dead BCG moderately upregulated IL-7Rα expression in monocytes. On the other hand, other H37Rv proteins; LAM, LM, PPD and CW proteins could not induce IL-7Rα expression on monocytes (Fig. 4a, left graph). We also investigated IL-7Rα expression in monocytes derived macrophages (MDMs), dead and viable BCG mycobacteria were the potent inducers of IL-7Rα expression on monocytes. CW_{Mtb} and LPS moderately induced IL-7Rα expression (Fig. 4a, left graph).

Cytokines(i.e TNFα, IL-1β) were reported to induce IL-7Rα expression on monocytes and macrophages [5,42]. Thus, we next evaluated the capacity of different recombinant cytokines for the induction of IL-7Rα expression. Bacterial components were also included as controls. As depicted by the proportions of IL-$7R\alpha_{high}$ monocytes, IL-1β ,TNFα and GM CSF cytokines significantly induced IL-7Rα expression but GM CSF was a potent inducer of IL-7Rα in both monocytes and MDMs (range 35 to 75%) (Fig. 4b). IFNγ, IL-6 and IL-10 did not induce detectable amount of IL-7Rα on monocytes (Fig. 4b, left graph). As expected, Pam3 and CW_{Mtb} significantly upregulated IL-7Rα in monocytes and MDMs (Fig. 4b). GM CSF and CW_{Mtb} strongly upregulated IL-7Rα on MDMs (Fig. 4b, right graph). IL-1β and TNFα induced approximately similar proportions of IL-7Rα expressing MDMs as monocytes. In contrast to IL-7Rα expression on monocytes, IFNγ induced IL-7Rα on MDMs (Fig. 4b, right graph).

We reasoned that, the bacterial components may induce cytokines, which autologously promote IL-7Rα expression in monocytes and MDMs. Hence, we investigated the effects of the cytokine depleting antibodies on IL-7Rα induction. We stimulated monocytes/MDMs with PAM3/CW_{Mtb} and simultaneously added cytokine blocking antibodies. TNFα and IL-1β depleting antibodies inhibited IL-7Rα expression in CW_{Mtb} activated monocytes in titration dependent manner. On the other hand, GM-CSF antibodies moderately repressed IL-7Rα expression (Fig. 5a).

As depicted by inhibition rates (Fig. 5b, upper graph), TNFα and IL-1β inhibitors strongly blocked IL-7Rα expression in CW_{Mtb} treated monocytes (70%) and anti-GM CSF moderately inhibited IL-7Rα expression in monocytes (20%). Contrastingly, TNFα and IL-1β depleting antibodies slightly blocked IL-7Rα expression in MDMs (20%). Notably, GM CSF inhibitor strongly blocked IL-7Rα induction in MDMs (80%). Inhibitory capacity of the mixture of all cytokines depleting antibodies were similar in both CW_{Mtb} stimulated monocytes and MDMs (80%). TNFα and IL-1β hardly affected IL-7Rα expression in MDMs (Fig. 5b, upper graph).

TNFα and IL-1β, GM-CSF depleting antibodies moderately inhibited IL-7Rα expression in PAM3 activated monocytes (40%,) (Fig. 5b, lower graph). Contrastingly, a strong inhibition of GM CSF cytokine was noted in PAM3 stimulated MDMs (80%) (Fig. 5b, lower graph). The cocktail of all blocking antibodies similarly inhibited IL-7Rα induction in both Pam3 activated monocytes and MDMs (81%) (Fig. 5b, lower graph). Taken together, these findings propose that cytokines induced by bacterial components are the central mediators of IL-7Rα expression in monocytes and MDMs.

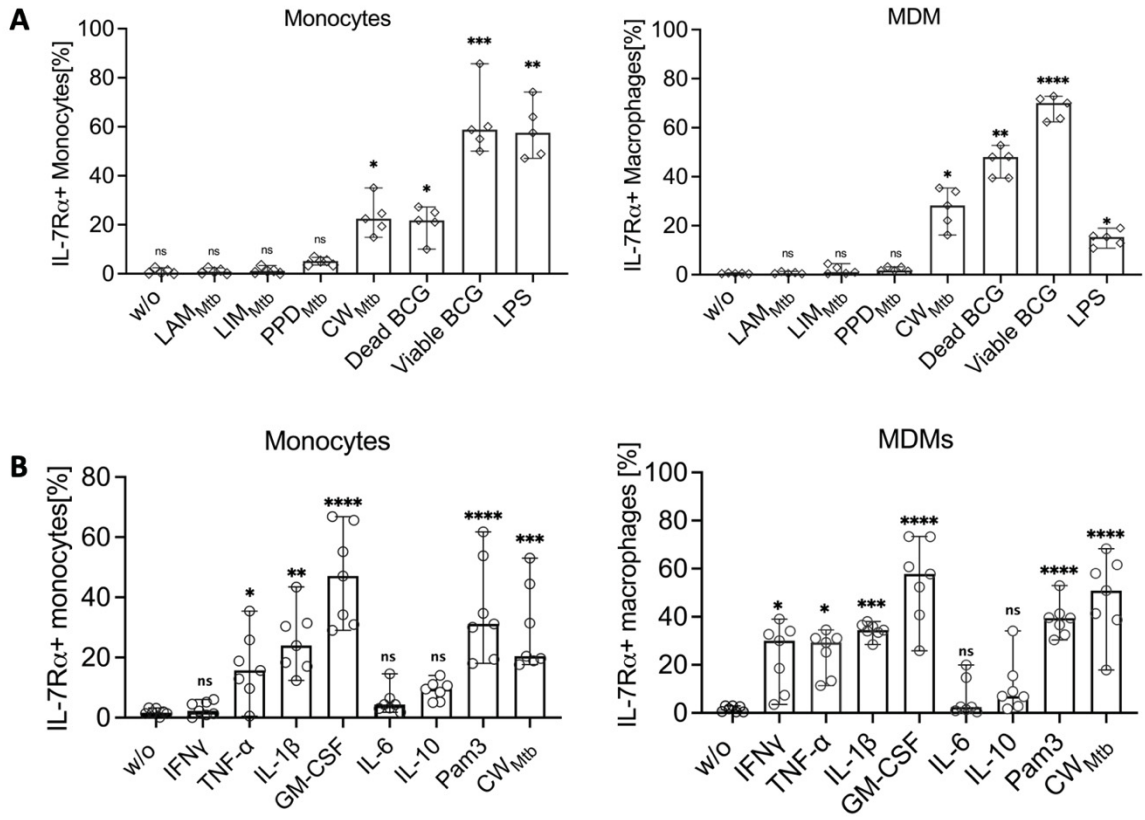

Fig. 4: Bacterial components and cytokines induce IL-7Rα in monocytes and MDMs.

The bar graphs depict the median values with ranges of IL-7Rα expressing monocytes or MDMs. **(A)** Different stimuli were used to induce IL-7α expression in monocytes (left graph) and MDM (right graph); H37Rv strain components: LAM_{Mtb}(1µg/mL), LIM_{Mtb} (1 µg/mL), PPD_{Mtb} (1 µg/mL), Viable/dead BCG(MOI:1) and toll like receptor 4 agonists (LPS). **(B)** In addition to H37Rv cell wall proteins, we used different cytokines to upregulate IL-7Rα in monocytes (left graph)and MDMs (right graph); IFN-γ (100 ng/mL); TNF-α (10 ng/mL); IL-1β (10 ng/mL); GM-CSF (5 ng/mL); IL-6 (10 ng/mL); IL-10 (10 ng/mL); PAM3 (100 ng/mL); CW_{Mtb} (1 µg/mL). Statistical differences were computed with Friedman test and p values were ****$p < 0.001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

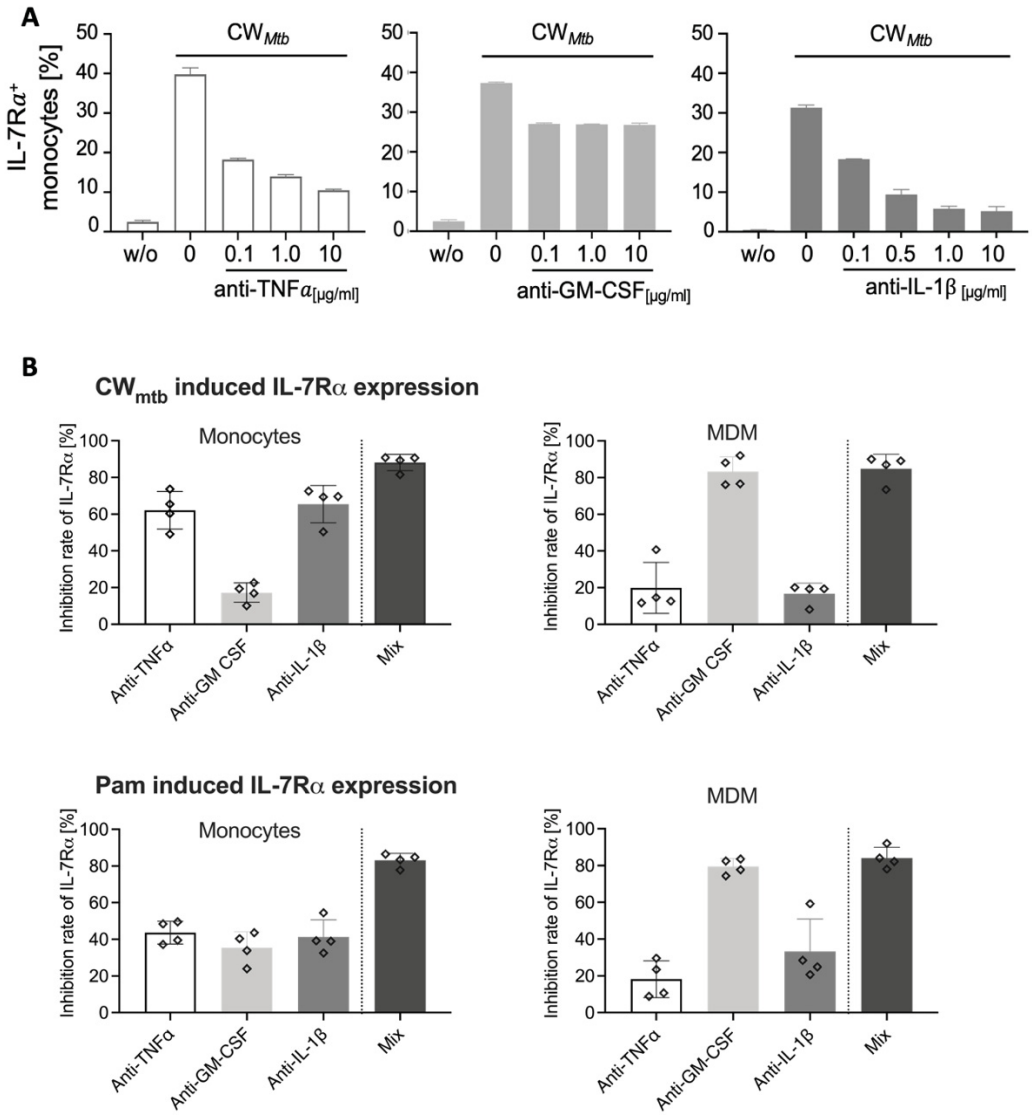

Fig. 5: Cytokines induced by bacterial components are the central mediators of IL-7Rα expression.
(**A**) The graphs depict the proportions of CW_{Mtb} induced IL-7Rα in monocytes in the presence or absence of cytokine depleting antibodies (Anti- TNF-α, Anti- GM-CSF or Anti- IL-1β) (0.1-10 µg). (**B**) Three independent experiments were performed. The findings show Inhibition rate of CW_{Mtb} or PAM3 induced IL-7Rα in monocytes (left graphs) or MDMs (right graphs) in the presence of the single or combination of inhibitory antibodies (10 µg each).

3.2 IL-7Rα expression is transient in monocytes and MonoMac cells.

Our *in vitro* findings suggested cytokines as key mediators of IL-7Rα expression. Consistently, serum milieu of TB patients promoted IL-7Rα expression on heterologous monocytes of healthy individuals. On the other hand, the cytokines of TB patients serum(i.e TNFα, GM-CSF) did not correlate with IL-7Rα induction [43]. These data prompted us to further characterize IL-7Rα expression on monocytes and monocytes cell lines.

In vitro time courses experiments were established (16h,24h and 48h). Monocytes were stimulated with GM-CSF, CW_{Mtb}, and LPS. After 16h of stimulation. One set of activated monocytes was pulsed after 16h. As shown by connecting line graph (IL-7Rα_{high} monocytes, mean proportions), IL-7Rα expression directly dropped after washing of GM- CSF stimulated monocytes (Fig. 6a, left graph). On the other hand, for washed LPS and Pam3 activated monocytes, IL-7Rα expression decreased progressively until 48h time point (Fig. 6a, middle and right graph). The findings posited that IL-7Rα expression is transient in primary monocytes. Gating strategies (Appendix.1).

Considering that monocytes are highly plastic, fragile and their life span is limited in culture, we considered to additionally include human monocytic cell lines(MonoMac cells), which were previously used as a model to investigate the effects of PAMPs [44–47]. MonoMac 1 and MonoMac 6 cells were stimulated with LPS and Pam3 for 3h, 6h and 24h. As depicted by the representative line graph (Mean proportions) both MonoMac 1(Fig. 6b, left graph) and MonoMac 6 (Fig. 6b, right graph) upregulated IL-7Rα in the presence of LPS or Pam3. IL-7Rα expression started at 3h, reached a plateau at 6h and drastically declined at 24h. Three independent experiments were performed. Collectively, these findings point out that IL-7Rα expression is transient in both primary monocytes and monocyte cell lines. Gating strategies for MM6 (Appendix. 2).

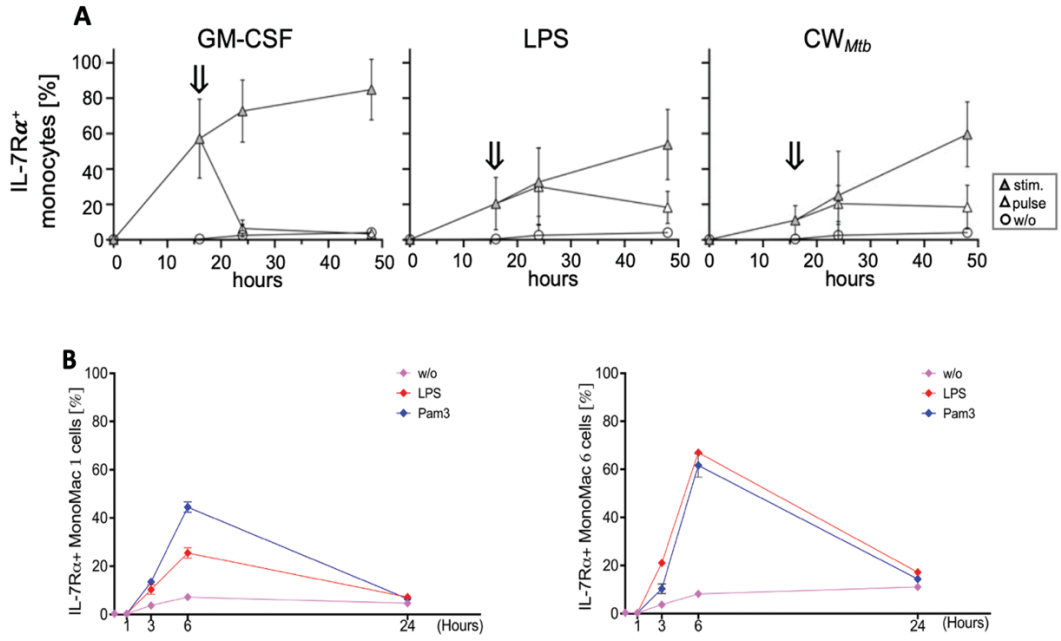

Fig. 6: IL-7Rα expression is transient in primary monocytes and Monocyte cell lines.
(**A**) Primary monocytes were activated with GM CSF(5ng/mL), LPS (1ng/mL) and CWmtb(1ug/mL) for 16, 24 and 48h. One set of cells were pulsed at 16h. (**B**) MonoMac 1(left graph) and MonoMac6 cells (right graph) were stimulated with Toll like receptor 2 agonists (Pam3, 100ng/mL) and toll like receptors 4 agonists (LPS, 1ng/mL) for 3h, 6h and 24h. The graphs depict the proportions of IL-7Rα expressing monocytes/MonoMac 6 cells.

3.3 FoxO1 is essential for IL-7Rα expression in primary monocytes and MonoMac cell lines.

Transient IL-7R expression in primary monocytes and monocyte cell lines prompted us to further dissect IL-7Rα regulation mechanisms at molecular level. Transcription factor FoxO1 is a key regulator of IL-7Rα expression in T cells [32]. It is likely that FoxO1 may play the same role in monocytes. Here, we characterized the association of FoxO1 and IL-7Rα induction in monocytes and MonoMac cells. *Ex vivo* PBMCs were used as a positive control and showed high IL-7Rα expression. As presented by western blot analyses (Fig. 7a), PAM3 induced IL-7Rα in both MonoMac1 and 6 cells and the expression reached a plateau at 6h and declined afterwards. The cells constitutively expressed FoxO1 over time course and there was no concomitant expression with IL-7Rα. Phosphorylated FoxO1 was constant for both PAM3 stimulated and unstimulated conditions (Fig. 7a). We next

investigated if blocking constitutive FoxO1 expression may affect IL-7Rα expression. Titrations of FoxO1 inhibitor (AS 1842856) were applied to PAM3 stimulated cells simultaneously. As indicated by both proportions of IL-7Rα positive cells and protein expression level on each cell (MFI), IL-7Rα induction was inhibited in titration dependent manner in both MonoMac 6 cells (Fig. 7b) and monocytes (Fig. 7c). These findings suggested that FoxO1 may be essential for IL-7Rα expression but additional cofactors may be involved.

Since FoxO1 inhibition decreased IL-7Rα induction in both monocytes and MonoMac 6 cells and we could not observe a concomitant IL-7Rα and FoxO1 expression, one of the underlying causes might be the methylation of IL-7Rα promotor or differential IL-7Rα mRNA transcripts expression. Therefore, we located the promotor of IL-7Rα(-1341-1bp) and performed restriction enzymes-based methylation assay. We expected an inverse correlational expression of IL-7Rα and methylation. As depicted by methylation rates generated from the qPCR analyses, we did not observe the differences in methylation rates of both MonoMac 1(Fig. 8a left graph) and MonoMac 6 cells (Fig. 8a right graph) (6h and 24h). As expected, methylation rate was 80% in positive control samples (Met DNA) and (23%) in negative controls (Fig. 8a). These data did not link IL-7Rα with epigenetic regulation mechanisms.

Recently, polyadenylation of the genes was associated with differential mRNA transcripts expression. We next assessed the possible alternative mRNA transcripts exports and their effects on IL-7Rα expression. We used two different primers which can depict two possible IL-7Rα transcripts which may result from differential nuclear exportation. As shown by fold changes, Pam3 upregulated both long and short IL-7Rα mRNA transcripts in both MM1(left graph) and MM6(right graph) cells (Fig. 8b). Expression reached a plateau at 6h and decreased dramatically at 24h (Fig. 8b). Taken together, these data did not suggest any association of IL-7Rα with IL-7R promotor methylation or differential mRNA transcripts exports. Altogether, these findings posit that IL-7Rα expression in primary monocytes and MonoMac 6 cells is dependent on FoxO1 but the underlying mechanisms remain obscure.

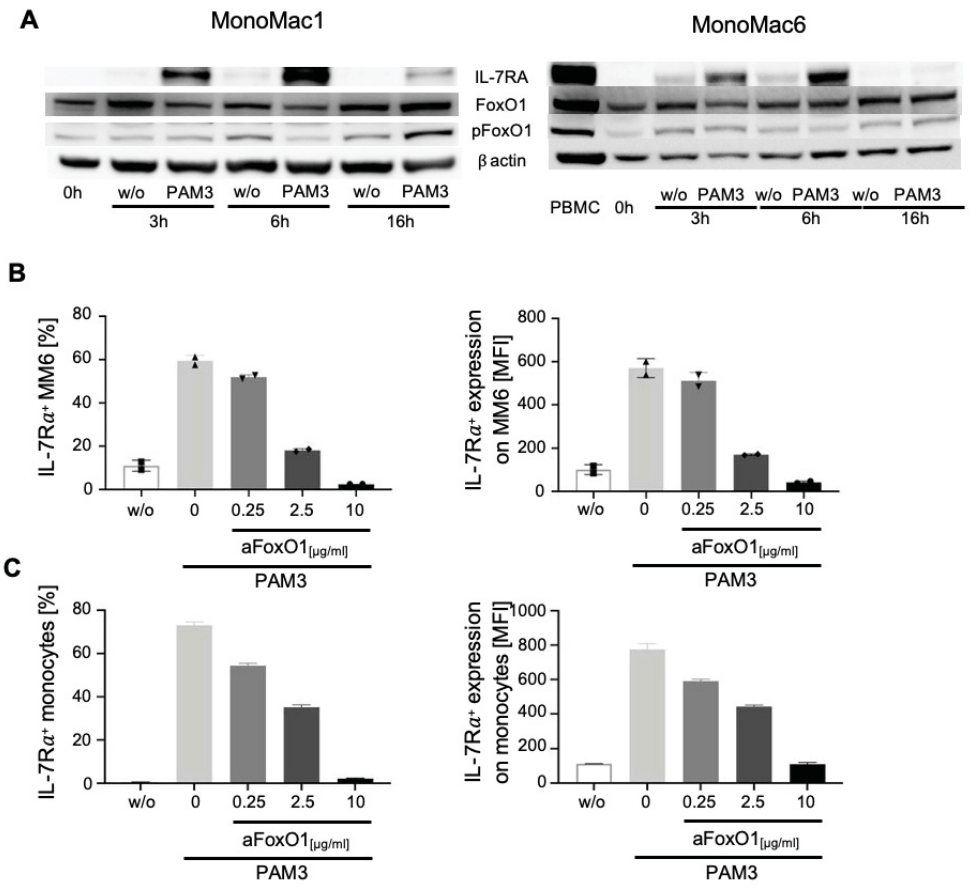

Fig. 7: FoxO1 is essential for IL-7Rα expression in monocytes and monocytes cell lines.

(A) MonoMac1 and MonoMac 6 cells were stimulated with/without pam3 for different time courses (0h, 3h,6h and 16h). PBMCs were used as a control as they constitutively express the target proteins. Induction of IL-7R, FoxO1 and pFoxO1 proteins are depicted by western blot bands. β-actin was included as a housekeeping gene. Data are a representative graph of three independent experiments. **(B, C)** the inhibitory effects of anti-FoxO1 on Pam3 induced IL-7Rα expression in MonoMac 6 cells **(B)** and monocytes **(C)** are shown. IL-7Rα proportions are depicted (left graph) and Mean fluorescence intensity (right graph). A representative graph of four independent experiments is illustrated.

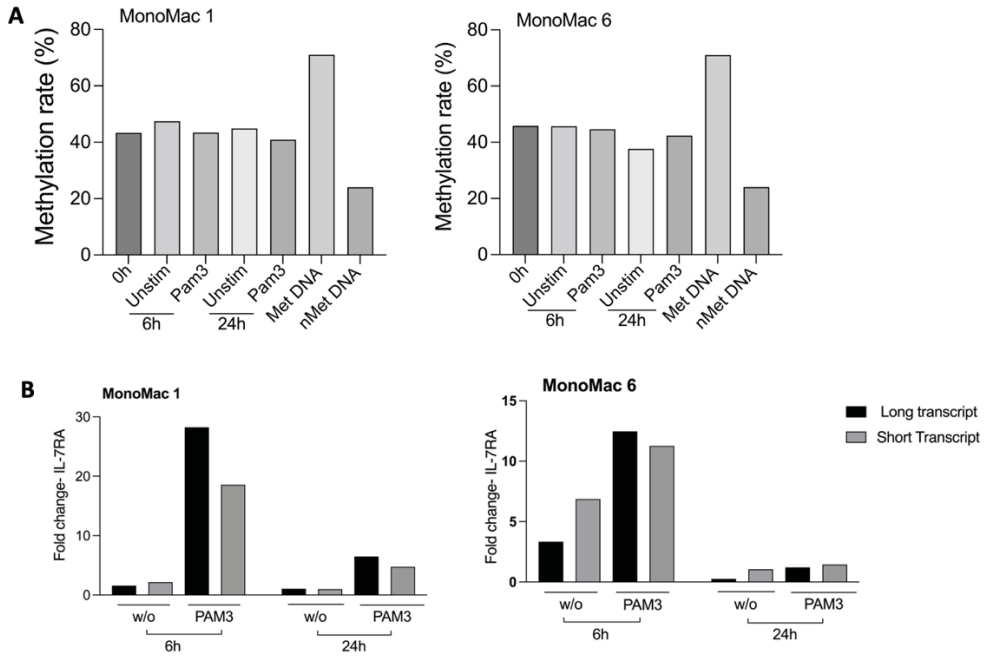

Fig. 8: Promotor methylation of IL-7Rα and IL-7Rα mRNA transcript export.

(A) Representative bar graphs depict the methylation rate of IL-7Rα promotor in MonoMac 1 cells(left) and MonoMac 6 cells (Right). Genomic DNA used for this experiment was extracted from 0h and 6h/24h pam3 activated MonoMac cells. Methylated DNA (Met DNA) and non-methylated DNA (nMet DNA) was included as controls. **(B)** Two types of IL-7Rα transcripts (termed long or short) were detected in 0h or 6h/24h stimulated MonoMac 1 cells (left graph) and MonoMac 6(Right graph). IL-7Rα transcripts expression was normalized with GAPDH as a constitutive gene. Two independent experiments were performed.

3.4 IL-7 moderately affects IL-7Rα expression on monocytes.

Previous studies and own findings revealed activation dependent IL-7Rα expression on monocytes but the function of this receptor is still elusive. It is well described that IL-7 induces rapid internalization of IL-7Rα expression in T cells [48]. Thus, we aimed to validate this effect in IL-7Rα expressing monocytes. Monocytes were stimulated with Pam3 (for 24h and 48h) and concomitantly IL-7 was supplied to the cells. Non-IL-7 treated conditions were considered. As presented by the paired graphs, IL-7 moderately decreased IL-7Rα expression on monocytes (Fig. 9). These data postulated that IL-7 signaling in monocytes is equivocal and needs more investigations.

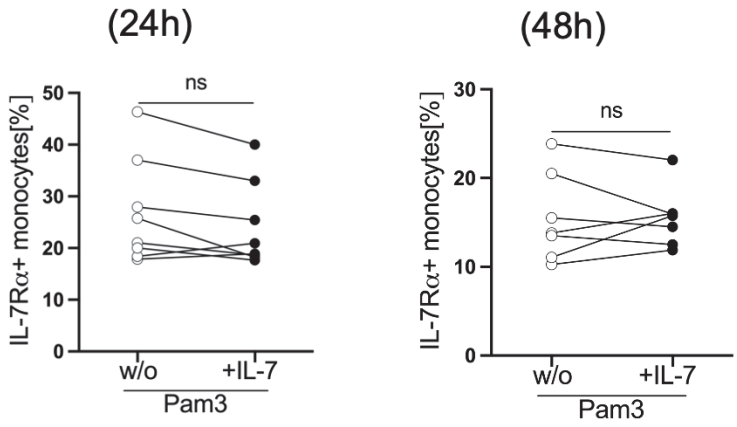

Fig. 9: IL-7 moderately affect IL-7Rα expression.
Paired analysis of the proportions of IL-7Rα+ monocytes**.** Monocytes were activated with Pam3(100ng/mL) in the presence or absence of IL-7(1ng/mL) for 24h and 48h.

3.5 IL-7 minorly affect the monocyte phenotypes and IL-7Rα expressing monocytes and monocyte cell lines are hyposensitive to IL-7.

In our experiments, sensitivity of IL-7 on monocytes was equivocal and we further investigated the possible IL-7 mediated phenotypic changes in monocytes. Pam3 activated monocytes were treated with or without IL-7(1ng/mL). We used different monocyte markers to depict the phenotypic changes over time courses (i.e., 24h, 48h and 72h). The FACs plots generated by TriMap cell clustering algorithm showed no monocytes phenotypic differences between IL-7 treated and non-treated cells. As displayed by TriMap plots, activated monocytes were phenotypically similar between IL-7 treated and IL-7 non-treated conditions (Fig. 10a, b). However, monocyte phenotypes were different between the time points (24h, 48h and 72h) (Fig. 10a, b), suggesting the culture environment effects on monocyte maturation. We have also overlayed IL-7 treated and non-treated conditions for all included monocyte markers but no difference of phenotypic markers expression was revealed (Fig. 10c).

IL-7 did not induce changes on monocytes. We reasoned that, IL-7Rα expressing monocytes may be less sensitive to IL-7. Thus, we next characterized IL-7 signaling in monocytes and monocytes cell lines. Monocytes and MonoMac 6 cells were pre-activated with Pam3 to induce higher IL-7Rα on monocytes. IL-7Rα expression on monocytes ranged between 14.5 and 41% (Proportions) (Fig. 11a). Afterwards, the

cells were shortly stimulated with different concentrations of IL-7(1-10^4 ng/mL). In titration dependent way, IL-7 induced pSTAT-5 in monocytes, (range 5 to 11%, IL-7Rα_{high} monocytes) for higher concentration, 10ng/mL (Fig. 11b). We further interrogated these data to compare pSTAT-5 induction between monocytes and T cells as a control. IL-7Rα expression on T cells was ranged between 97 to 99%. (Fig. 11c). Contrary to monocytes, the low concentration of IL-7(10pg/mL) could instigate the increase of pSTAT-5 (range 34 to 58%) in CD4 T cells, and the higher concentrations induced approximately between 93 and 97% of pSTAT-5 (pSTAT-5_{high} cells) (Fig. 11d). Finally, the proportions of pSTAT-5 inducing monocytes or CD4 T cells were normalized according to IL-7Rα expression and compared between monocytes and CD4 T cells. Among IL-7Rα expressing monocytes, only 20 to 50% monocytes (median range) were sensitive to IL-7(10^4 ng/mL) as opposed to 90-99% CD4 T cells (Fig. 11e). These data show that almost all IL-7Rα expressing CD4 T cells could respond to IL-7 but on the other hand, only lower proportions of IL-7Rα expressing monocytes were sensitive to IL-7.

To further explore IL-7 signaling in monocytes, we included human monocytic cell lines. MonoMac 6 cells were LPS pre-activated to upregulate IL-7α expression. The cells were supplied with IL-7(10ng/mL) and PBMCs were utilized as a control. As displayed by western blot analyses, pSTAT-5 induction was detected in all MonoMac 6 cells, regardless of IL-7 treated conditions (Fig. 11f) suggesting that other pSTAT-5 inducers were responsible for this effect as the cells had been in culture for 4 weeks. As expected, we noted an increase of the pSTAT-5 in IL-7 treated PBMCs (Fig. 11f).

We reasoned that, IL-7Rα expression on monocytes may be not sufficient to strongly respond to IL-7 cytokine. We overexpressed IL-7Rα on THP-1 and treated these cells with IL-7(10ng/mL). PBMCs were part of the experiment as a control. No pSTAT-5 induction was observed in these cells (Fig. 11g). Since TSLP cytokine uses IL-7Rα for signalizing, we included this cytokine to rule out if this receptor was functional after lentiviral transduction.

Interestingly, we detected pSTAT-5 increase in TSLP treated IL-7Rα overexpressing THP-1 cells but not in IL-7 stimulated cells (Fig. 11h). Together these results indicate that the monocytes may be less sensitive to IL-7.

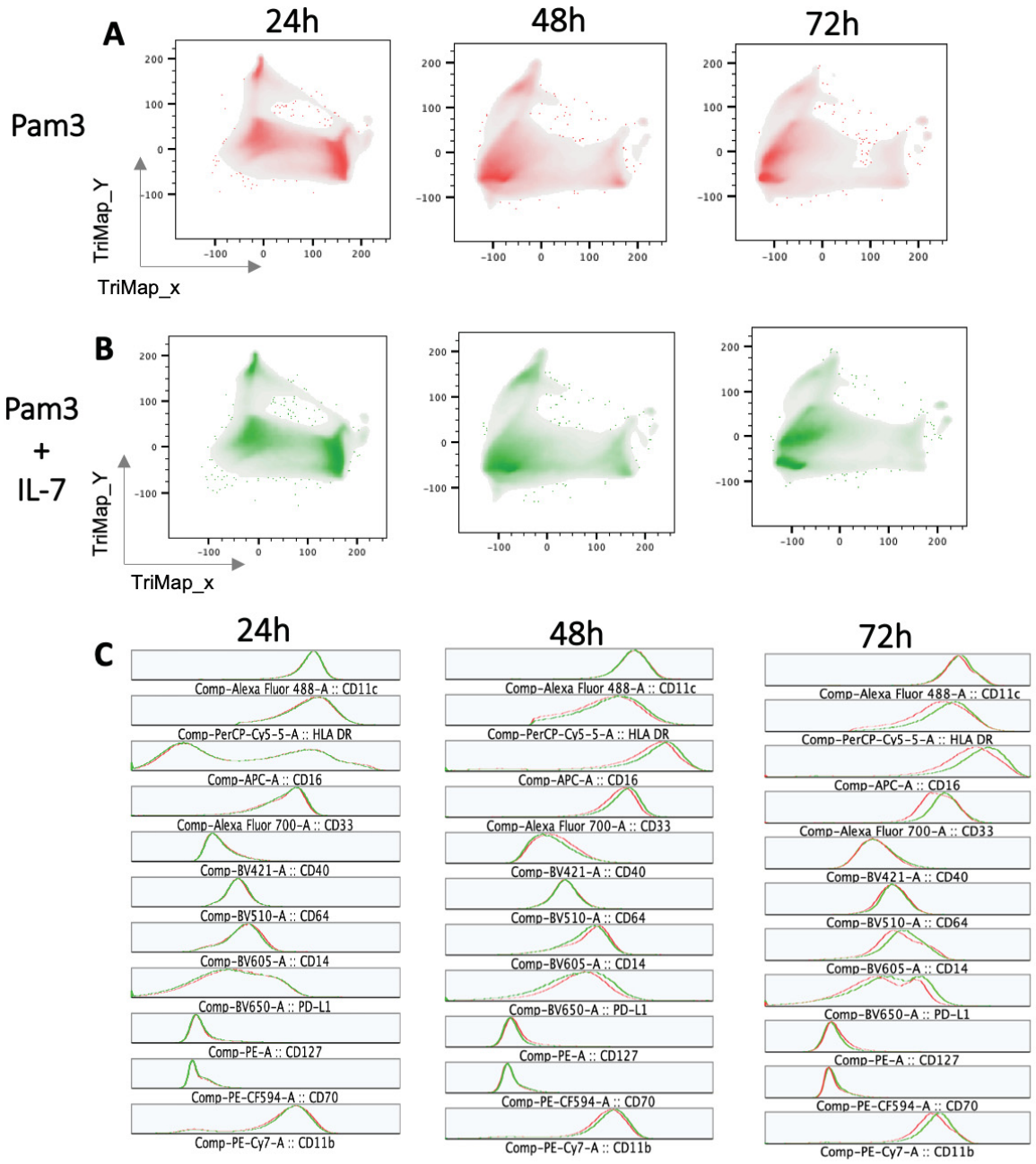

Fig. 10: IL-7 hardly affect the monocytes phenotypes.

The FACS plots display the distribution/similarities of monocyte phenotypes in three regions as computed by dimensionality reduction algorithm (TriMap). **(A)** Monocytes were stimulated with Pam3(100ng/mL) for 24h, 48h and 72h and the plots are visualized in red color. On the hand, **(B)** one set of monocytes were co-incubated with Pam3 and IL-7(1ng/mL) and the plots are presented in light green color. **(C)** Multi histograms visualize the comparison of different phenotypic markers between Pam3 activated (light green) and Pam3+IL-7 stimulated monocytes (Red color). FlowJo software was used to perform these analyses.

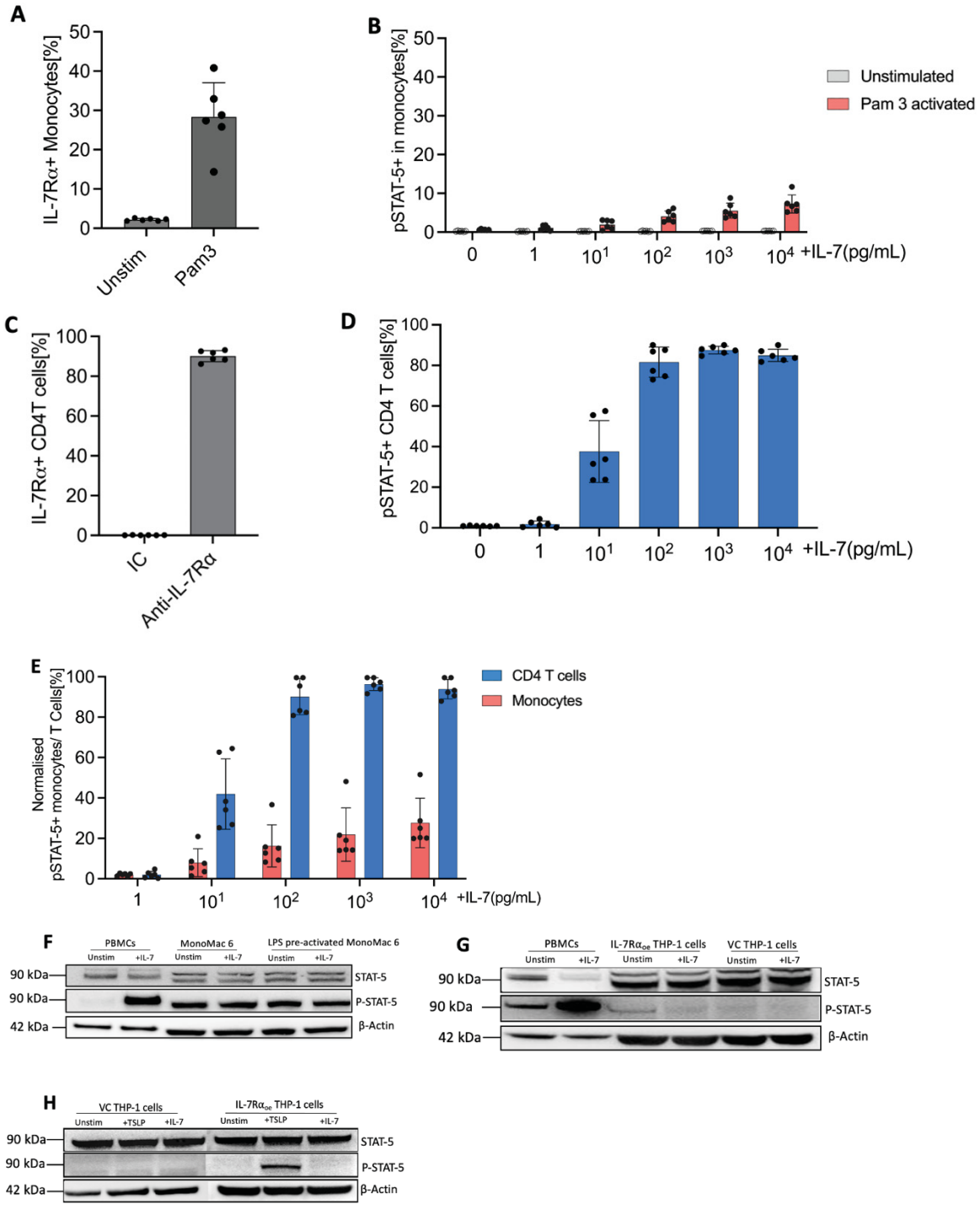

Fig. 11: IL-7 hyposensitivity in primary monocytes and MonoMac cells

(A) The bar graphs depict Pam3 induced IL-7Rα expression in monocytes as compared to isotype control (IC) and unstimulated cells. **(B)** IL-7 sensitivity is determined by the level of STAT-5 phosphorylated proteins(pSTAT-5) induction in both Pam3 pre-activated and unstimulated monocytes. The cells were shortly incubated with different concentrations of IL-7 (1-10^4 pg/mL). (**C**) IL-7Rα expression level in purified CD4 T cells is displayed. **(D)** pSTAT-5 level was determined in CD4 T cells. (**E**) Comparison of pSTAT-5$^+$ cells between monocytes and CD4 T cells. The proportions of pSTAT-5 inducing monocytes and CD4 T cells were normalized according to IL-7Rα expressing cells (Monocytes or CD4 T cells respectively). All analyses were performed by flow cytometry. **(F)** Western blot bands show the pSTAT-5/STAT-5 induction in LPS pre-stimulated MonoMac 6 cells and unstimulated conditions after the stimulation with IL-7(10ng/mL). **(G)** pSTAT-5/STAT-5 were probed in IL-7Rα overexpressing THP-1 cells and Vector controls after the incubation with IL-7(10ng/mL). PBMCs were used as controls. **(H)** In addition to IL-7, TSLP cytokine was involved as a control and pSTAT-5/STAT-5 were measured by western blots. All experiments included β-actin as a constitutive gene.

3.6 IL-7 affects the monocyte phenotypes indirectly via T cells *in vitro.*

Recently IL-7 was reported to activate the monocytes of PBMCs [41]. As we could not detect the effects of IL-7 on monocyte phenotypes, we thought that IL-7 may indirectly activate the monocytes via T cells. Therefore, we aimed to explore possible indirect effects of IL-7 on monocytes. As expected, the IL-7 treated monocytes used as a control did not upregulate the monocytes activation markers (Mean fluorescence intensity, MFI; CD40, HLA DR) as compared to non-treated conditions (Fig. 12a). However, IL-7 pulsed CD4 T cells significantly upregulated the monocytes activation markers (MFI, CD40 and HLA DR) (Fig. 12b).

Next, we evaluated if IL-7 stimulated T cells affect the monocytes via cell-cell contact or indirectly via soluble factors. We used the transwell plates separated by membranes. Monocytes and IL-7 treated CD4 T cells could only communicate via soluble molecules. As indicated by the MFI on the bar graphs, IL-7 treated CD4 T cells did not activate the monocytes in transwell plates. Interestingly, MFI analyses of monocytes co-cultured with IL-7 stimulated CD4 T cells revealed higher upregulation of CD40 and HLA DR expression (Fig. 12c).

We further characterized the phenotypes induced by IL-7 treated CD4 T cells in monocytes using TriMap cell clustering algorithm. As presented by the TriMap plots, IL-7 changed monocyte phenotypes (Fig. 13a). Further analyses show two potential distinct populations induced by IL-7. Among other markers, CD40 and HLA DR were highly expressed (Fig. 13b). Taken together, these data show that the IL-7 may indirectly activate monocyte via T cells.

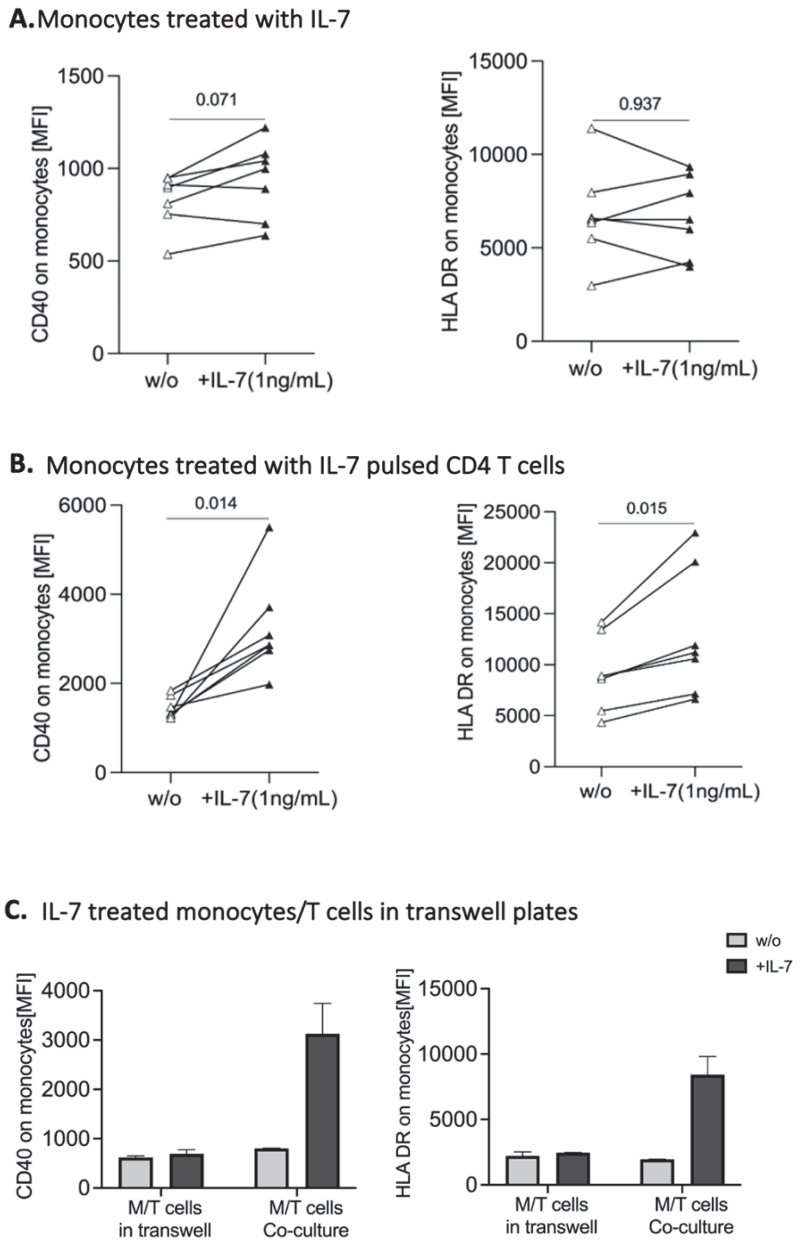

Fig. 12: IL-7 affect the monocyte phenotypes via CD4 T Cells

Paired analysis depicts the Mean fluorescence intensity (MFI) of monocyte activation markers (HLA DR, CD40). **(A)** Monocytes were stimulated in the presence or absence of IL-7(1ng/mL) for 24h. **(B)** Monocytes were treated with IL-7 pulsed T cells or co-incubated with CD4 T cells with no contact of IL-7. **(C)** The bar graphs show CD40 and HLA DR expression on monocytes from two different experiments. Monocytes and T cells were incubated in transwell plates (two different chambers separated by membrane). Second, monocytes and T cells were cocultured together. Every condition included IL-7 treated condition and controls.

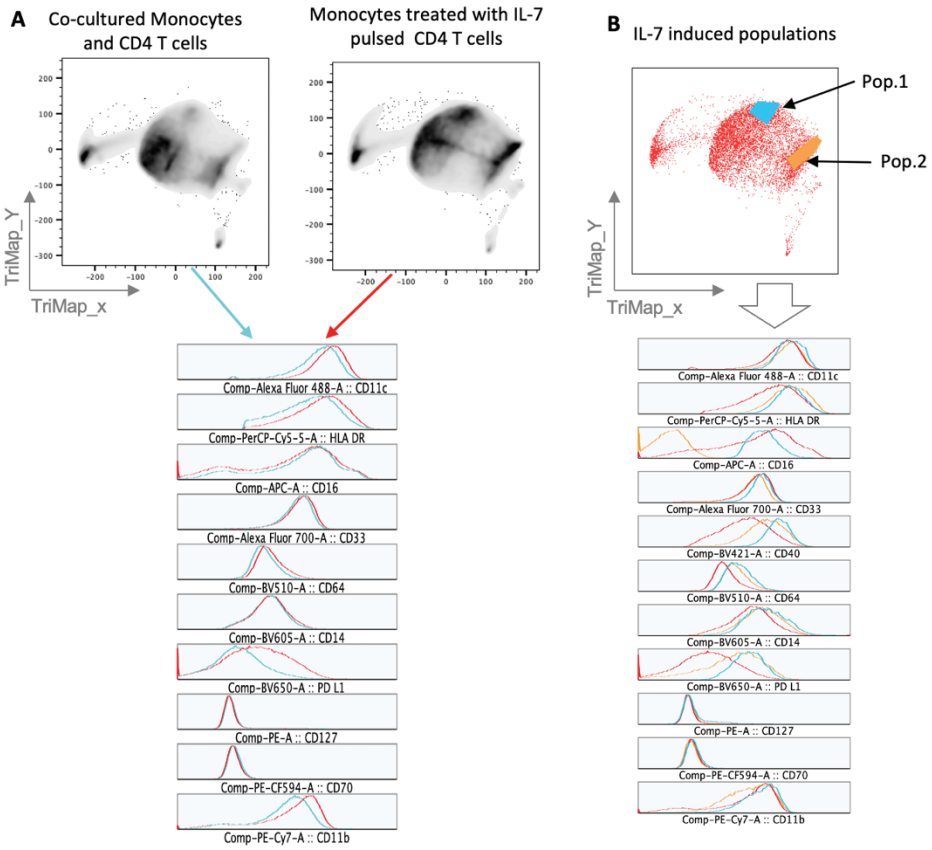

Fig. 13: IL-7 pulsed T cells induce distinct monocyte phenotypes.

The FACS plots illustrates the distribution/similarities of monocyte phenotypes in three regions as computed by dimensionality reduction algorithm (TriMap). (**A**) Overlay of monocytes co-incubated with T cells and monocytes treated with IL-7(1ng/mL) pulsed T cells. Multiparameter histograms display the comparison of different phenotypic markers between IL-7 pulsed T cells (Red line) and non-treated conditions (light blue line). (**B**) IL-7 induced phenotypes Population 1 (light blue), population 2(Pink) and other cells (Red) are overlayed with the rest of the cells. Histograms overlays compare different monocytes phenotypic markers.

3.7 IL-7 promotes anti-mycobacterial effector functions in live/dead LD BCG infected human monocyte-derived macrophages (MDMs)

In our previous experiments, mycobacterial components, and BCG induced IL-7Rα expression in both monocytes and MDMs. Here, we investigated the capacity of IL-7 polarized monocytes for inducing anti-mycobacterial effector functions.

To generate MDMs, monocytes were matured in the presence of IL-7 cytokine for 4 days. As shown by the overlay histograms, during the generation of MDMs, *in vitro* culture conditions upregulated IL-7Rα expression on the maturing cells at 24h and marked upregulation was observed (96h) (Fig. 14a). Therefore, we next investigated the effects of IL-7 on LD BCG infected MDMs. We used the previously established flow cytometry based assay for mycobacteria killing [38]. MDMs were infected with live/dead reporter strains which constitutively express mcherry proteins to mark all infected cells and viable bacteria can co-express GPF proteins under tetracycline (ATC) treatment. Hence, we can distinguish MDM containing dead or live BCG. We simultaneously measured the cells with count beads to derive both proportions and absolute numbers of infected LD BCG (Fig. 14b). As depicted by flow cytometry analyses, MDMs infected with LD BCG were 80% and 50% of the cells contained live bacteria. The proportions of MDMs consisting viable BCG decreased significantly in the presence of IL-7 (Mean 39%) (Fig. 14c). Next, we determined the effects of IL-7 on the viability of MDMs by measuring the absolute number of these cells. IL-7 decreased the numbers of viable BCG significantly (Fig. 14d). We also performed the colony forming units (CFUs) and consistently with the cytometry analyses, CFU were significantly lower in MDMs treated with IL-7 in comparison to IL-7 non treated conditions (Fig. 14e). Lastly, the BCG kill rate (Mean proportions) was computed between IL-7 treated and non-treated MDMs. IL-7 induced anti-mycobacterial MDM cytotoxicity about 27% (Fig. 14f). These findings point out that IL-7 may induce antimycobacterial effects in LD BCG infected MDMs.

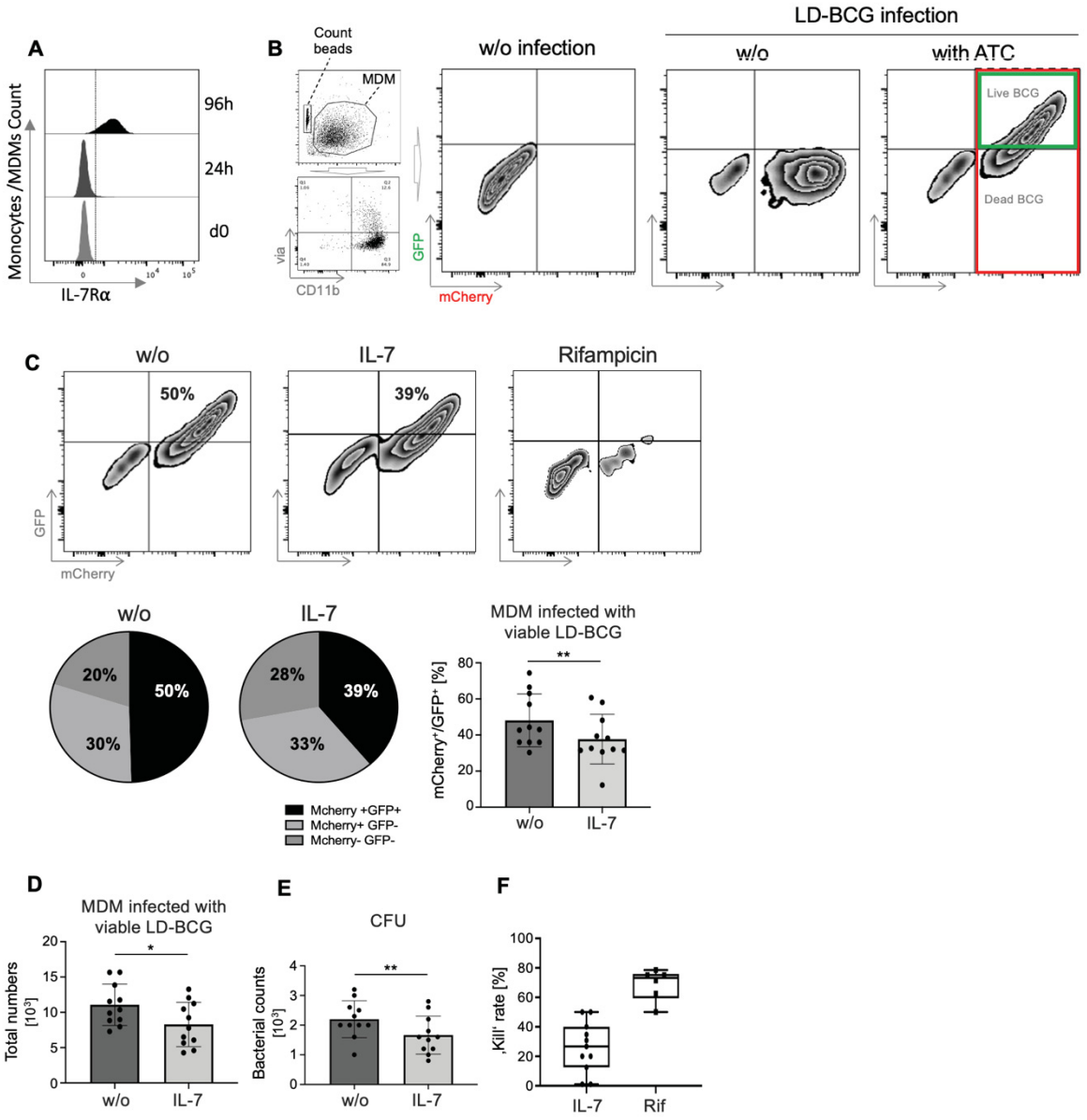

Fig. 14: IL-7 promotes anti-mycobacterial effects in LD BCG infected macrophages.

(A) Histogram displays the Mean Fluorescence intensity (MFI) of IL-7Rα expression on ex vivo(d0) monocytes and 24, 96 cultured monocytes. **(B)** the FACS plots depict the gating strategies of LD BCG infected MDM. Count beads were simultaneously measured to deduce the absolute number of MDM in culture. An ATC inducible protein (GFP, green) was used to mark the MDM containing viable BCG and Mcherry (red) for indicating all present BCG. **(C)** the plots show the proportions of MDM consisting viable BCG for untreated, IL-7 treated and Rifampicin as a control. The pie charts indicate the mean proportions of uninfected (dark grey), viable (dark) and dead (bright grey) LD BCG containing MDM. **(D)** the absolute number of MDM are shown. **(E)** the colony forming units of LD infected MDM are shown for IL-7 treated and untreated conditions. **(F)** LD BCG infected MDM kill rate are depicted between the samples with or without IL-7. The Wilcoxon signed rank test was performed for comparisons and p values were calculated. $^{**}p < 0.01$, $^{*}p < 0.05$.

3.8 SOCS family proteins are the potential inhibitors of IL-7 signaling in monocytes and monocytes cell lines.

Our findings suggested lower IL-7 sensitivity in monocytes. In light with these data, we reasoned that it is possible that IL-7 signaling may be decreased by some inhibitors. It is documented that SOCS3 family proteins dampen IL-7 signaling in T cells [49]. Thus, we sought to initially investigate SOCS molecules expression in monocytes and monocyte cell line. We stimulated the cells with Pam3 and cytokines to induce IL-7Rα expression. Using *in vitro* time courses (0h, 6h, 24h), SOCS proteins or mRNA transcripts were simultaneously analyzed. Expectedly, Pam3 and cytokines (IL-1β and GMCSF) upregulated both soluble and membrane IL-7Rα mRNA transcripts in monocytes at 6h and the induction was stronger at 24h. Pam3 was the potent inducer of IL-7Rα (Fig. 15a). Pam3 and cytokines concomitantly upregulated SOCS3/CISH and IL-7Rα mRNA transcripts in monocytes. We observed a moderate expression of the transcripts at 6h and higher induction at 24h. (Fig. 15b, c). SOCS1 and SOCS2 mRNA expression did not follow IL-7Rα expression pattern and no differences were noted between stimulated cells and non-activated conditions (Fig. 15d, e). Since we could reveal a concomitant expression of IL-7Rα and SOCS3/CISH transcripts, these proteins were considered as the candidates for IL-7 signaling inhibition.

We next analyzed the expression of SOCS3/CISH molecules at protein level. As shown by western blot analyses, Pam3 and cytokines (IL-1β and GMCSF) concomitantly induced both membrane and soluble IL-7Rα, SOCS3 and CISH proteins (6h and 24h). Strikingly, we detected a SOCS3 variant (termed; short) in monocytes which was mainly induced by the stimuli. β-actin was included as a housekeeping gene (Fig. 15f, g).

Lastly, we wanted to validate our findings in MonoMac6 cells. As depicted by fold changes, Pam3 transiently upregulated membrane and soluble IL-7Rα mRNA transcripts expression in MonoMac 6 cells (Fig. 16a). We could detect the expression of IL-7Rα transcripts at 3h and reached a plateau at 6h. Afterwards membrane and soluble IL-7Rα mRNA dramatically declined.

No mRNA expression could be detected in the presence of IL-1β and GMCSF cytokines (Fig. 16a). Differently to monocytes, only SOCS3 and IL-7Rα mRNA were parallelly induced in MonoMac 6 cells (Fig. 16b). No differences were deduced from CISH, SOCS1 and SOCS2 mRNA expression (Fig. 16c, d, e). Analysis of the proteins also revealed a concomitant expression of membrane and soluble IL-7Rα variants and SOCS3. Intriguingly, the long version of SOCS3 was constitutively expressed (3h,6h and 24) but we observed a downregulation of the short isoform at 24h (Pam activated conditions) (Fig. 16f, g, h). Collectively, these findings suggest that SOCS3/CISH molecules and IL-7Rα are concomitantly expressed in monocytes and these molecules may play some roles in IL-7Rα expression.

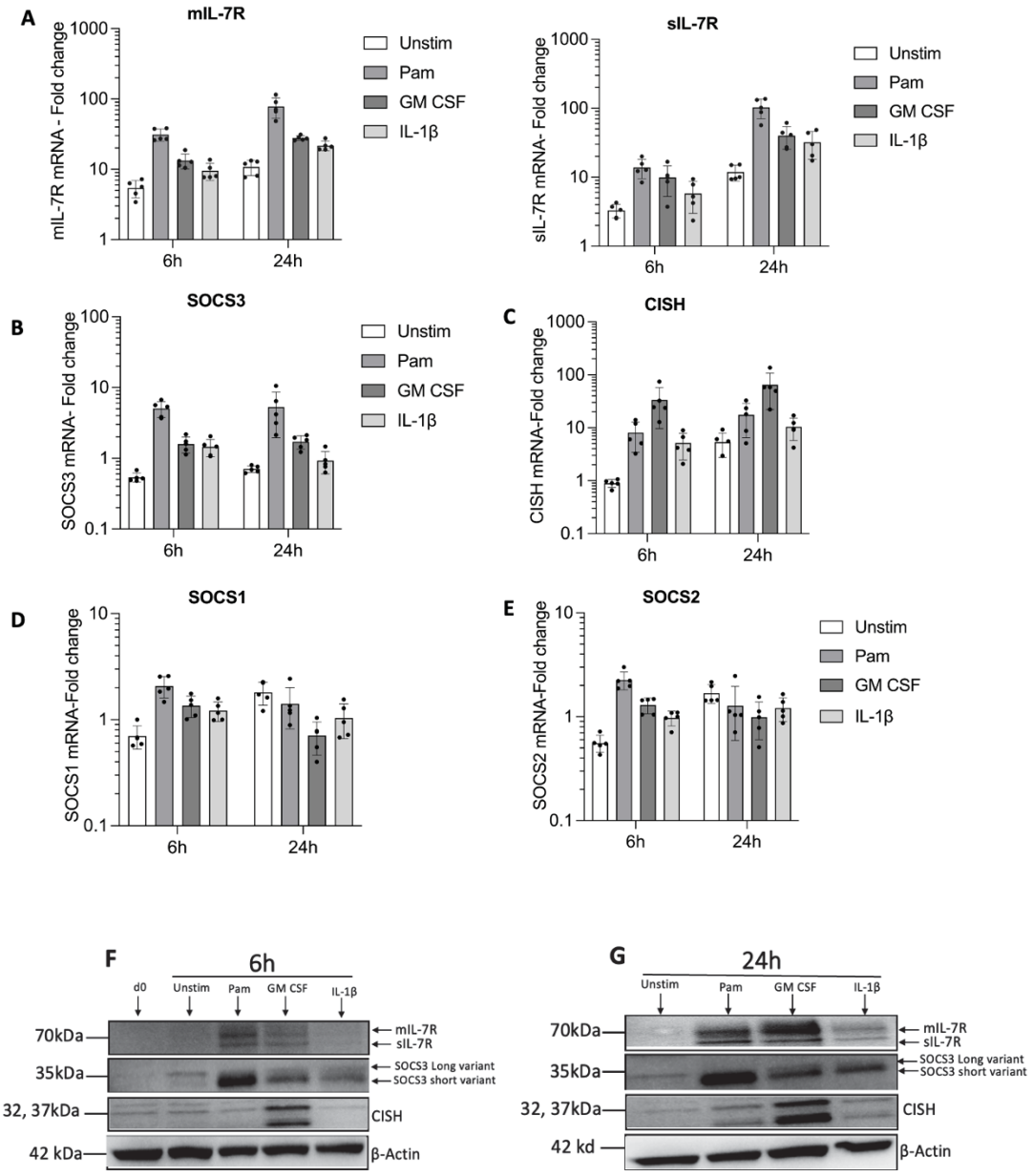

Fig. 15: SOCS family proteins are potential regulators of IL-7Rα expression in monocytes.
Bar graphs depict the logarithmic fold changes of time course analysis (d0, 6h, 24h) for mRNA expression in monocytes. Cells were stimulated with Pam(100ng/mL), GM CSF(5ng/mL) and IL-1β(10ng/mL). Five independent experiments were performed. **(A)** Membrane IL-7Rα(mIL-7Rα) mRNA (left graph and soluble IL-7Rα mRNA (right graph) are shown **(B)** SOCS3 mRNA expression **(C)** CISH mRNA expression **(D, E)** SOCS1 and SOCS2 mRNA expression respectively. **(F, G)** Western blots bands visualize membrane(m)and soluble(s)IL-7Rα, SOCS3 variants (Long and short isoforms) and CISH protein expression in monocytes for time courses (0h and 6h, left graph), (24h, right graph). β-actin mRNA expression was detected as a housekeeping gene. Images of the bands were analyzed with Image Lab software, Bio-Rad.

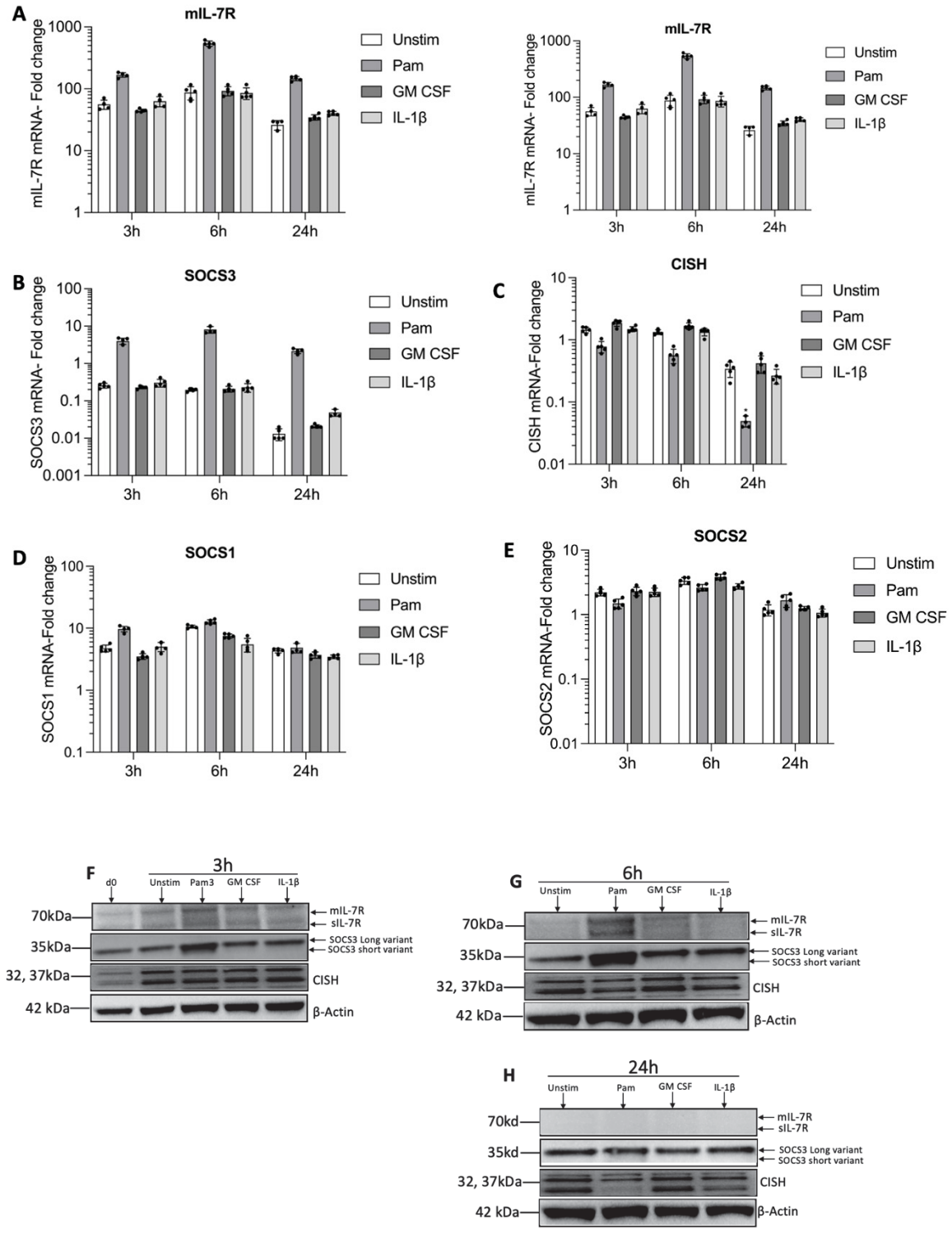

Fig. 16: SOCS family proteins are potential regulators of IL-7Rα expression in MonoMac 6 cells.
Bar graphs depict the logarithmic fold changes of time course analysis (d0, 3h, 6h, 24h) for mRNA expression in MonoMac 6 cells. Cells were stimulated with Pam(100ng/mL), GM CSF(5ng/mL) and IL-1β(10ng/mL). Five independent experiments were performed. **(A)** Membrane IL-7Rα(mIL-7Rα) mRNA (left graph and soluble IL-7Rα mRNA(right graph) are shown . **(B)** SOCS3 mRNA expression **(C)** CISH mRNA expression **(D,E)** SOCS1 and SOCS2 mRNA expression respectively. **(F,G)** Western blots bands visualize membrane(m) and soluble(s)IL-7Rα, SOCS3 variants (Long and short version) and CISH protein expression in monocytes for time courses (0h and 6h, left graph), (6h, right graph **(H)** Lastly, the findings for 24h stimulated cells are shown. β-actin mRNA expression was included as a housekeeping gene. The analysis of the images was performed in Image Lab software, Bio-Rad.

3.9 SOCS3/CISH proteins are not responsible for lower IL-7 sensitivity in monocytes cell lines.

Inducibility of SOCS3/CISH proteins in Pam3 activated monocytes and monocytes cell lines prompted us to further explore the functions of these molecules. Previous findings did not suggest any IL-7 dependent response on IL-7Rα overexpressing THP-1 cells (Monocyte cell lines). We reasoned that SOCS3/CISH molecules may inhibit IL-7 signaling in these cells and monocytes.

Using lentiviral vectors, we knocked down SOCS3/CISH proteins in IL-7Rα+THP-1 cells. Next, SOCS3/CISH$_{kd}$ IL-7Rα+ THP-1 cells were shortly stimulated with IL-7(1ng/mL) and STAT5 phosphorylated proteins were analyzed. As presented by the representative bar graphs, inhibition of SOCS3/CISH did not promote IL-7 signaling in IL-7Rα+ THP-1 cells (Fig.17a, b). Together these data indicate that, the lower sensitivity of IL-7 in monocyte cell lines is not caused by SOCS3/CISH proteins.

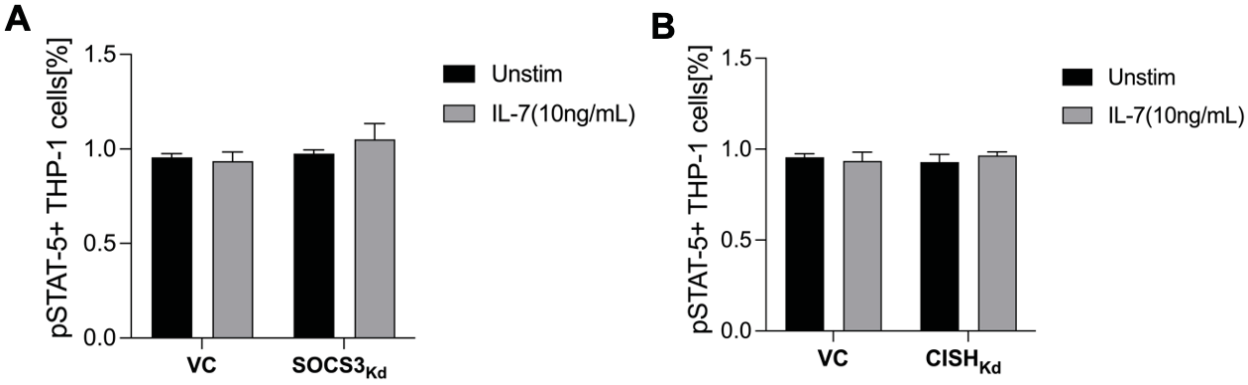

Fig. 17: SOCS3/CISH knockdown THP-1 cells did not promote IL-7 signaling.

Bar graphs depict the proportions of pSTAT-5 induction in modulated THP-1 cells. (**A**) SOCS3$_{kd}$ THP-1 cells (left graph) and (**B**)CISH$_{kd}$ THP-1 cells (right graph). The cells were stimulated for 15 minutes with IL-7(10ng/mL). kd: Knockdown, oe: overexpression

3.10 SOCS3/CISH proteins promote IL-7Rα expression in monocyte cell lines.

Previous findings did not associate SOCS3/CISH expression with IL-7 hyposensitivity in monocyte cell lines, but these molecules were concomitantly expressed with IL-7Rα in activated monocytes and monocyte cell lines. We reasoned that SOCS3/CISH expression may regulate IL-7Rα induction. We overexpressed and knocked down SOCS3/CISH proteins in MM6 cells.

SOCS3/CISH modulated MM6 cells were cultured for two weeks in non-tissue culture flasks. No concomitant SOCS3/ IL-7Rα was observed in MM6 cells and we thought that SOCS3 effects may need activation or cofactors. The cells were cultured in the presence or absence of Pam3 in TC treated places for 6h,24h and 48h. In general, IL-7Rα expression reached plateau at 6h and declined afterwards in MM6 cells. As provided by a representative line graph (IL-7Rα_{high} MM6, proportions) (Fig. 18a), unstimulated SOCS3$_{oe}$ MM6 cells moderately upregulated IL-7Rα as compared to VC. On the other hand, induction of IL-7Rα in SOCS3$_{kd}$ cells was lower. Activation of SOCS3 modulated MM6 cells with Pam3 did not show any differences of IL-7Rα expression between SOCS3$_{oe}$ and VC cells. In the same direction with unstimulated MM6 6 cells, IL-7Rα expression was lower in Pam3 activated SOCS3$_{kd}$ MM6 cells. Gating strategies (Appendix.3). These data indicated that IL-7Rα SOCS3 may promote IL-7Rα expression in monocytes and we next assessed if SOCS3 can affect IL-7Rα variants expression.

Membrane and soluble IL-7Rα mRNA transcripts and protein profile in SOCS3 modulated MonoMac 6 cells were investigated. As presented by bar graphs (fold changes), membrane and soluble IL-7Rα mRNA variants were comparable in all SOCS3 modulated cells as compared to VC controls (Fig. 18b, c). In contrast to mRNA transcripts analysis, protein analysis showed a higher soluble IL-7Rα expression in SOCS3$_{oe}$ MonoMac cells, followed by vector control and lastly SOCS3$_{kd}$ cells (Fig.18d).

Lastly, we investigated the effects of CISH protein on IL-7Rα induction. CISH molecule was enforced or knocked down in MonoMac 6 cells. After two weeks of

culture, cells were stimulated with Pam3 in TC treated plates for 6h, 24h and 48h. Unstimulated conditions were considered. As previously observed, IL-7Rα expression reached a plateau at 6h and declined afterwards in MM6 cells. As depicted by the connecting line graphs (IL-7Rα_{high} MM6, proportions) (Fig.19a), IL-7Rα expression was higher in unstimulated CISH$_{oe}$ MM6 cells as compared to VC cells. (30% at 6h, Mean proportions). Induction of IL-7Rα in VC cells and CISH$_{kd}$ MM6 was approximately similar (Fig.19a, b). Of note, we observed a moderate IL-7Rα expression on CISH$_{oe}$ MM6 cells directly after two weeks of CISH modulation (11% at 6h, Mean proportions) (Fig.19a). Pam3 activated CISH$_{oe}$ MM6 cells strongly upregulated IL-7Rα expression (Fig.19b) (80% at 6h, Mean proportions). Taken together, these findings demonstrate that SOCS3/CISH may promote IL-7Rα expression in monocytes and monocytes cell lines. Importantly SOCS3 may accumulate soluble IL-7Rα in the cells but underlying mechanisms remain elusive.

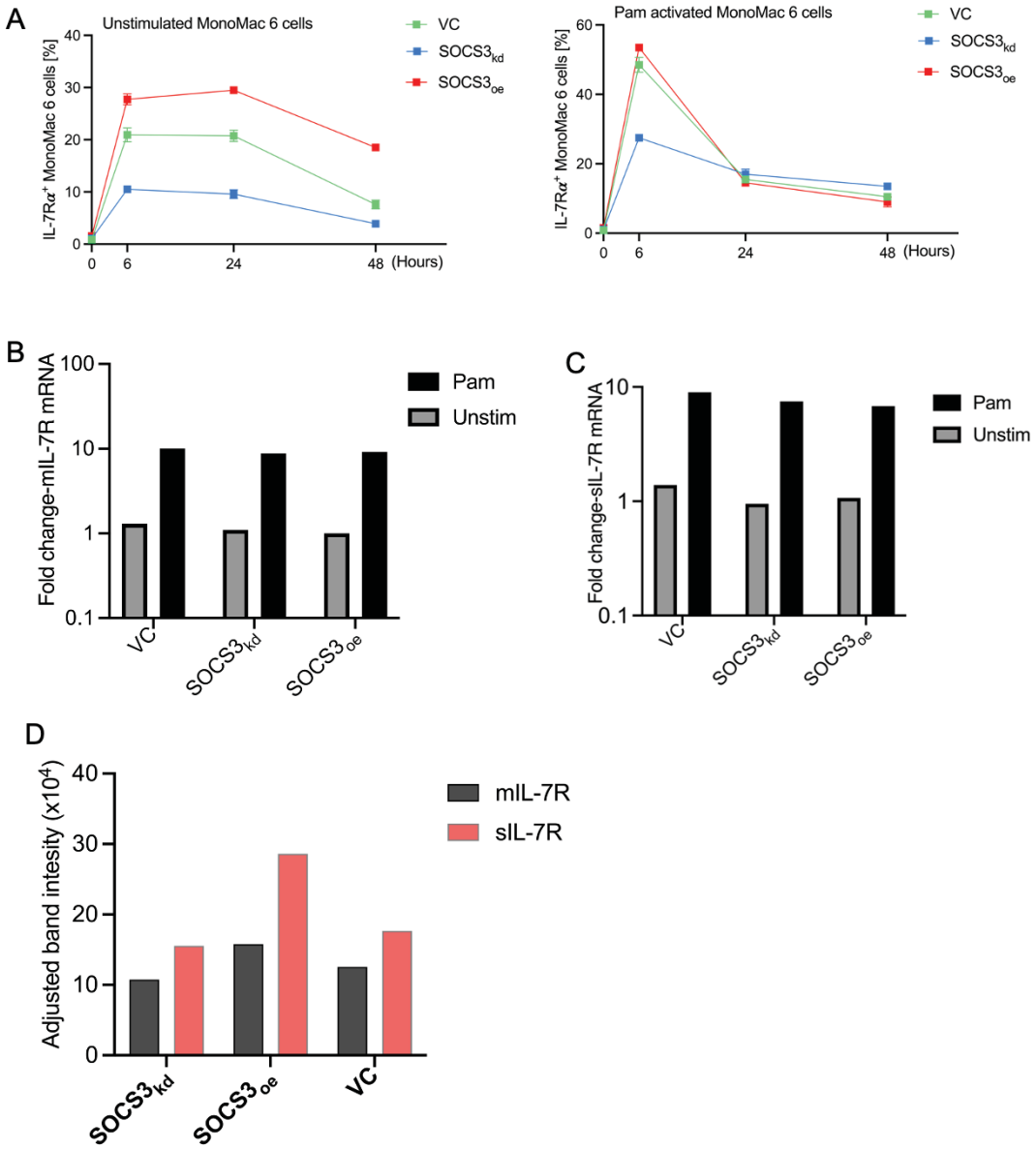

Fig. 18: SOCS3 proteins promote IL-7Rα expression in MonoMac 6 cells.
SOCS3 proteins were overexpressed or knocked down in MonoMac 6 cells using lentiviral vector systems. Vector control cells were included as well. The line graphs show the proportions of IL-7Rα expressing monocytes over time course; 6h, 24h and 48h incubated MonoMac cells. **(A)** IL-7Rα induction in unstimulated MonoMac 6 cells (left graph) and IL-7Rα expression in Pam3 activated MonoMac 6 cells (right graph). Red line depicts dynamic of IL-7α expression in SOCS3 overexpressing cells ($SOCS3_{oe}$), Blue line marks SOCS3 knockdown cells ($SOCS3_{kd}$) and green line represents vector controls (VC). **(B)** Bar histograms show the fold changes of membrane IL-7Rα mRNA (left graph), **(C)** soluble IL-7Rα mRNA (Middle graph) for 6h stimulated cells. **(D)** Histogram depicts the western blot analyses of band adjusted intensity for total IL-7Rα expression for 6h pam3 activated cells.

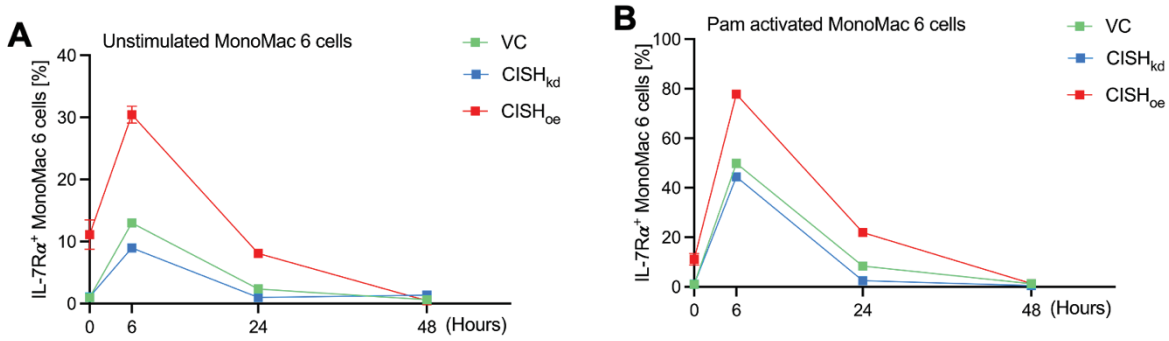

Fig. 19: CISH proteins promote IL-7Rα expression in MonoMac 6 cells.

CISH proteins were overexpressed or knocked down in MonoMac 6 cells using lentiviral vector systems. Vector control cells were considered. The line graphs show the proportions of IL-7Rα expressing monocytes over time course; 6h, 24h and 48h incubated MonoMac cells. (**A**) IL-7Rα induction in unstimulated MonoMac 6 cells and (**B**)IL-7Rα expression in Pam3 activated MonoMac 6 cells. Red line depicts dynamic expression in SOCS3 overexpressing cells ($SOCS3_{oe}$), Blue line marks SOCS3 knockdown cells ($SOCS3_{kd}$) and green line represents vector controls (VC).

4.0 Discussion

IL-7 mediated functions are well described in T cells. Own studies reported a lower IL-7Rα expression and impaired IL-7 mediated functions in monocytes and T cells of TB patients [24,43]. The findings of this study extend our understanding of IL-7Rα regulation in monocytes and IL-7 effects on monocyte phenotypes. Here, we display unprecedented IL-7Rα regulation mechanisms in monocytes and monocyte cell lines.

4.1 Cytokines induced by bacterial components promote IL-7Rα expression

IL-7Rα expression on monocytes is basal and it can be boosted by innate immune activators. Recent studies reported LPS and Pam3 as the inducers of IL-7Rα expression on monocytes [5,35,50]. Consistently, our findings showed significant LPS/Pam3 induced-IL-7Rα expression on monocytes and MDMs. In addition, own previous data revealed that serum milieu of TB patients can induce IL-7Rα expression on monocytes of healthy individuals [43]. Since the serum environment contains different soluble factors including the cytokines, we can suggest that serum containing cytokines were responsible for IL-7Rα expression in monocytes. On the other hand, own findings showed lower IL-7Rα expression in monocytes of TB patients [24]. Albeit these findings were controversial, but they posit some roles of IL-7Rα expression in mycobacterial diseases.

We next explored the capacity of mycobacteria and mycobacterial components to induce IL-7Rα *in vitro* as well as underlying mechanisms. Viable BCG robustly induced IL-7Rα in both monocytes and macrophages. Dead BCG and cell wall proteins of H37Rv mycobacteria moderately upregulated IL-7Rα. In contrast, other H37Rv strain components including LAM, LIM and PPD did not induce detectable IL-7Rα expression. Since the monocytes could respond to toll like receptor-2 agonist (Pam3), we may speculate that the molecules which are recognized by monocytes or macrophages via TRL-2 may induce IL-7Rα and this mechanism may play some roles in mycobacterial infection. However, LAM are well described to signal via TLR-2 and this component could not induce IL-7Rα upregulation on monocytes and

MDMs. While we may attribute IL-7Rα expression to TLR-2 independent mechanisms, there are also two different versions of LAMs such as AraLAM and ManLAM. It was demonstrated that these two variants of LAMs may possess different PRRs(pathogen recognition receptors) on the cells [51,52]. Therefore, it is likely that the mycobacterial components may induce IL-7Rα indirectly via soluble factors. Cytokines-induced IL-7Rα expression on monocytes and macrophages were reported to exacerbate some autoimmune diseases such as rheumatoid arthritis; mainly TNFα and IL-1β cytokines were identified [53,54]. Other cytokines were suggested to play roles in TB pathology (i.e. IL-6, IL-10, IFNγ, and GM CSF) [55,56]. In our experiments, except IL-6 and IL-10, the rest of cytokines significantly induced IL-7Rα on monocytes and MDMs. Our findings are in line with the previously published data. TNFα and IL-1β upregulated IL-7Rα on monocytes and MDMs [35,53]. Consistently with these studies, IFNγ only induced IL-7Rα expression on MDMs in our experiments. Our data may postulate that the various stages of monocytes maturation respond to the cytokines differently. Our findings also pointed out GM CSF as a strong inducer of IL-7Rα expression [43]. Crucially, inhibition of CW_{Mtb} -induced cytokines suggested GM CSF as the potent regulator of IL-7Rα expression in MDMs. Since macrophages play indispensable functions for the surveillance of mycobacteria, it is possible that, GM CSF induced-IL-7Rα expression may contribute to TB immune responses. Of note, recently GMCSF mediated antimycobacterial immune responses were reported and underlying mechanisms were not explored [56]. In addition, GM CSF and IL-7 synergetic effects were reported for the maturation and polarization of monocytes and dendritic cells [57,58]. Inhibition assays revealed TNFα and IL-1β as central mediators of IL-7Rα regulation in monocytes. Other studies observed TNFα-induced IL-7Rα expression in monocytes [5,50]. Whether the cytokines induced IL-7Rα on monocytes play roles in mycobacterial diseases, the information is scarce. We may suggest that, although the monocytes do not contact mycobacteria directly but TNFα and IL-1β induced-IL-7Rα may immunologically contribute to the control of TB diseases. Indeed, it well described that, TNFα and IL-1β soluble factors are enriched in tuberculosis. Dysregulation of these cytokines can be associated with TB progression [59]. Taken together, these findings suggest that the cytokines induced by mycobacterial components are key mediators of IL-7Rα regulation in both monocytes and MDMs.

Future studies may establish in *vitro* model to comprehensively analyze the mechanisms of IL-7Rα expression in monocytes/MDMs in mycobacterial diseases. There is also a need to translate our *in vitro* findings. Thus, next researchers may prospectively investigate the effects of cytokines on IL-7Rα expression in monocytes of TB patients. Broad analysis of serum proteomics of TB patients may exclude the serum confounding factors which contribute to IL-7Rα regulation in TB patients.

4.2 Regulation of IL-7Rα in monocytes and monocytes cell lines

IL-7Rα regulation mechanisms are well characterized in T cells, but the scientific literature is elusive in monocytes. IL-7Rα expression may generate different variants due to alternative splicing mechanism in T cells [14]. Similarly, both membrane and soluble IL-7R mRNA transcripts were induced by bacterial components or cytokines in monocytes and MM6 cells. Strikingly, our findings pointed out a higher soluble IL-7Rα expression as compared to membrane variant in monocytes. Consistently, similar effects were reported in LPS-elicited monocytes [5]. As previously reported, induction of soluble IL-7Rα may be attributed to SNPs rs6897932 located at exon 6 of IL-7R gene. Since the alternative splicing in T cells was associated with autoimmune diseases [4,5], it is likely that the monocytes may also display the same mechanisms but future studies may address this question. Of note, IL-7Rα expression was transient in monocytes and monocytes cell lines. These findings are discordant to IL-7Rα regulation in T cells, implying the unique features of IL-7Rα regulation in monocytes. Of note, MM6 cells were not sensitive to cytokines, probably due to biological changes of cell lines. In contrast, other studies showed that IFNγ, and GM CSF sensed MonoMac 6 cells could better respond to the bacteria and therefore increasing IL-6 and TNFα secretion [45,47].

Interrogating our findings further, we revealed the necessity of transcription factor FoxO1 for IL-7Rα regulation. Similarly, FoxO1 was reported to regulate IL-7Rα expression in T cells [60]. FoxO1 inhibition abrogated IL-7Rα expression in monocytes and monocytes cell lines. On the other hand, FoxO1 was constitutively

expressed, suggesting other cofactors involved in IL-7Rα regulation in monocytes and monocytes cell lines. One possibility can be the epigenetic mechanisms which can repress IL-7R promotor to prevent FoxO1 to bind to the promotor region. Previous studies associated lower IL-7Rα expression with hypermethylation in T cells [34,36] but our data did not suggest this mechanism in monocytes. It is also possible that, polyadenylation of IL-7R gene may regulate IL-7Rα mRNA expression and this mechanism has been well characterized recently [61]. Analysis of the possible IL-7Rα mRNA transcripts resulting from duo-polyadenylation of IL-7R gene did not suggest any association of this mechanism with IL-7Rα regulation in monocytes. In the light with these data, we may propose that, the regulation of IL-7Rα in monocytes may involve unique complex mechanisms which need more attention.

SOCS family proteins are linked with Jak-STAT pathway inhibition, but our findings did not support this fact. Unexpectedly, we observed a concomitant IL-7Rα expression and SOCS3/CISH in monocytes. Other SOCS proteins (SOCS1, SOCS2) did not indicate any association with IL-7Rα regulation. SOCS3 proteins were also simultaneously induced with IL-7Rα expression in activated monocyte cell lines (MM6 cells). In both monocytes and MM6 cells, SOCS3/CISH and IL-7Rα expression was comparable at mRNA and protein level. We reasoned that SOCS3/CISH may have regulation roles in monocytes and MM6 cells. Indeed, previously SOCS3 was associated with IL-7Rα regulation in T cells [15]. Of note, further analysis of SOCS3 expression revealed a SOCS3 short variant which was induced by bacterial components or cytokines, implying the possible role of this short version in regulation of IL-7Rα in monocytes. Other studies investigated the functions of SOCS3 variants and the half-life of this short isoform was longer as compared to longer version [62,63]. Contrastingly, our data suggested a transient expression of SOCS3 short variant and a constitutive expression of SOCS3 long isoform. We may speculate that, the changes of the normal half-life of SOCS3 short version may have some regulation roles in monocytes. To decipher the effects of SOCS3/CISH molecules, we modulated these proteins in MM6 cells.

We noted a marked decrease of membrane IL-7Rα proteins expression in $SOCS3_{kd}$ MM6 cells and a moderate upregulation of membrane IL-7Rα proteins was revealed in $SOCS3_{oe}$, implying the role SOCS3 in IL-7Rα regulation. Our data did not show any effects of SOCS3 at IL-7R mRNA expression, suggesting redundant regulation roles of SOCS3 at transcriptional level. Intriguingly, analysis of total IL-7Rα proteins (membrane and soluble) showed higher soluble IL-7Rα in $SOCS3_{oe}$ MM6 cells. As it is well documented, soluble IL-7Rα is continually released from the cells. We may speculate that, the accumulation of soluble IL-7Rα in MM6 cells may have some biological functions. These findings may suggest that SOCS3 proteins may post-transcriptionally regulate IL-7Rα expression. SOCS3 is well described for degradation of biological molecules including receptors via ubiquitination mechanism [64,65]. Recently, one study reported IL-7Rα as a key cargo of autophagy in CD8 T cells [66]. Considering our data, it is possible that SOCS3 may utilize autophagosomal pathway to regulate IL-7Rα in monocytes and monocyte cell lines. Therefore, reinforcing the increased soluble IL-7Rα observed in $SOCS3_{oe}$ MM6 cells. Since we noted a moderate increase of membrane IL-7Rα proteins in MonoMac 6 cells, one would also speculate that, SOCS3 may retain both membrane and soluble IL-7Rα in monocytes. Our data demonstrated a minor expression of IL-7Rα on the membrane of $SOCS3_{oe}$ MM6. It is unlikely that, SOCS3 can retain IL-7Rα in the cells and parallelly stabilize IL-7Rα on the membrane of the cells. Further studies can shed light on this question.

Since we could not detect IL-7Rα expression in SOCS3oe MM6 after two weeks of SOCS3 lentiviral transduction and the effects of SOCS3 on IL-7Rα expression was only observed after activation of the cells. We may postulate that, SOCS3 needs other cofactors such as CISH to fully engage in IL-7Rα regulation. Indeed, $CISH_{oe}$ MM6 cells induced moderate IL-7Rα after weeks of transduction. It is possible that CISH and SOCS3 synergically regulate IL-7Rα expression in monocytes and MM6 cells.

There is an increasing body of evidence postulating the role of SOCS3/CISH in infectious diseases [17,18,67]. Own study revealed that SOCS3 expression was

lower in T cells of TB patients and reverted to normal during the treatment, suggesting some key roles of SOCS3 in tuberculosis [25].In addition, IL-7Rα expression was lower in monocytes and T cells of TB patients [24,25]. We may speculate that, changes in SOCS3 proteins may affect IL-7Rα expression. To our knowledge, no study explored CISH effects on IL-7Rα expression. In our experiments, GM-CSF cytokine strongly upregulated CISH and IL-7Rα expression, implying possible biological crosstalk. Since GM CSF was reported to control TB disease, it is likely that CISH may indirectly contribute to TB immune responses via IL-7Rα regulation. Genetic polymorphism of CISH molecules was associated with TB susceptibility in different population [67,67–69]. CISH knockdown mice could not control early tuberculosis infection [17]. In the light with these data, future studies may clearly dissect how SOCS3/CISH proteins calibrate IL-7Rα expression in monocytes and MM6 cells. Importantly the role of these molecules in TB diseases. Ontology analyses of transcriptomic features at single cell level to single out the regulators of IL-7Rα in monocytes at transcriptional level may also be of interest. Investigating SOCS3/CISH and IL-7Rα interaction is of significance as well. Collectively, these findings posit that SOCS3/CISH molecules may tune IL-7Rα regulation in monocytes/MM6 cells and FoxO1 is crucial for IL-7Rα expression.

4.3 IL-7 sensitivity in monocytes and monocytes cell lines

IL-7 exerts different functions in T cells including differentiation, survival, and memory generation. On the other hand, IL-7 mediated functions in monocytes remains obscure. IL-7 mainly signals via Jak/Stat-5 axis. We observed a pSTAT-5 induction in IL-7 treated IL-7Rα+ monocytes but, a larger proportion of induced IL-7Rα expressing monocytes were not sensitive to IL-7. Our findings also suggested that only a lower proportion of IL-7Rα+ monocytes could be sensitive to IL-7. The question is whether all induced IL-7Rα expressing monocytes are less sensitive to IL-7 or if additional factors are required for signaling. Recent studies displayed four distinct phenotypic phenotypes and transcriptional profile of IL-7Rα expressing monocytes [50]. Therefore, it is possible that these identified monocyte subsets may respond to IL-7 differently. Further studies to characterize IL-7 dependent functions of these cells are warranted.

IL-7 signaling requires both IL-7Rα and chains. Thus, we reasoned that, the reduced responsiveness of IL-7 could be linked to the expression of γ_c chain receptor, plausibly IL-7Rα inducers may affect the expression of γ_c chain receptor. However, our data did not support this hypothesis as we observed a constitutive γ_c chain receptor expression in monocytes and monocyte cell lines. IL-7 may also signal via alternative pathways, but our data did not suggest this mechanism.

We reasoned that the cells may respond to IL-7 differently and we included T cells in our experiments as a control. Approximately all T cells were sensitive to IL-7. Thus, we posited that IL-7Rα expression on monocytes may not be sufficient to strongly bind to IL-7. However, IL-7Rα overexpressing THP-1 cells could not gain IL-7 responsiveness. Another possibility could be that the transduced IL-7Rα was not functional. Thus, we included TSLP cytokine which naturally needs IL-7Rα for signaling. Strikingly, TSLP induced pSTAT-5 in THP-1 and monocytes. Approximately all IL-7Rα /TSLPR expressing monocytes were sensitive to IL-7. Since IL-7 signaling needs the phosphorylation of Jak 1 and Jak3 and TSLP utilizes Jak 1 and Jak 2, it is likely that, the IL-7 hyposensitivity of monocytes and cell lines can be attributed to Jak 3 inhibitors. SOCS family proteins including SOCS3 and CISH were suggested to dampen IL-7 signaling [49,70]. SOCS3 and CISH molecules mainly affect Jak 1 and Jak3 phosphorylation respectively. Indeed, CISH/SOCS3 molecules were concomitantly induced with IL-7Rα in monocytes and monocytes cell lines. We reasoned that CISH/$SOCS3_{kd}$ MM6 may promote IL-7 signaling but our data did support this hypothesis, implying that other inhibitors may account for lower sensitivity of IL-7 in monocytes and monocyte cell lines.

Recently, the study of Zhang et al (2022) suggested IL-7 dependent functions in monocytes of Covid 19 and RA patients and they assumed that IL-7 cytokine milieu of the patients could activate IL-7Rα+ monocytes of the patients physiologically. In contrast to our findings, induced IL-7Rα expressing monocytes were not highly sensitive to nanogram concentrations *in vitro.* Considering the lower concentration of IL-7(picogram) *in vivo*, it is likely other confounding factors contributed to the effects attributed to IL-7. Whether *in vivo* monocytes need other cofactors to

respond to the physiological concentration of IL-7, future studies will shed light on this. In the same study, IL-7 sensed IL-7Rα expressing monocytes exhibited impaired secretion of inflammatory cytokines such as IL-6 and TNFα, implying unprecedented roles of IL-7Rα expression on monocytes/macrophages. These findings are fascinating as they contradict, the long-lasting dogma attributing IL-7Rα expression to exacerbation of autoimmune diseases(i.e. RA) [35].

To gain better understanding of IL-7 effects on monocytes, we evaluated the monocyte phenotypic features. Using TriMap clustering algorithm, IL-7 barely affected the phenotypes of monocytes. We may suggest that, plausibly IL-7 hardly induced any changes in monocytes, reinforcing the lower STAT-5 phosphorylation in monocytes. On the other hand, one would speculate that, IL-7 induced minor effects that could not be detected by TriMap algorithm. Thus, comprehensive analysis of molecular profile of IL-7 exposed IL-7Rα+ monocytes may display additional information. Indeed, a recent study depicted increase of c-Maf genes as a unique transcriptomic feature of IL-7 stimulated monocytes [50].

We reasoned that the observed IL-7 mediated functions in monocytes in the study of Zhang et al (2022) may have other contributing factors (i.e T cells). Indeed, IL-7 significantly activated monocytes of PBMCs *in vitro* [41]. Intriguingly, monocytes treated with IL-7 pulsed CD4 T cells, remarkably increased different surface proteins including the monocyte activation markers (HLA DR and CD40). These differences also were exhibited by TriMap clustering algorithm. We may speculate that the IL-7 signaling axis in CD4 T cells was able to activate monocytes. One would also suggest that the IL-7 may exert biological functions on monocytes via T cells. Since we assumed that, the monocytes could not be in contact with IL-7 and we used inactivated monocytes, we can also suggest that IL-7 sensed T cells may polarize monocytes in IL-7Rα-IL-7 independent pathways, but future studies may display further details. In line with our findings, IL-7 promoted the maturation of partially enriched monocytes towards dendritic cells in *vitro* [58]. These data solidify our observations, implying T cells as potential mediators for monocyte activation. Relatedly , IL-7 was reported to promote the functions of monocytes in lung cancer [71]. Our data also show that, these indirect effects of IL-7 on monocytes are not

supported by soluble factors but direct cell-to-cell interaction is required, suggesting the involvement of other co/receptors. The observed IL-7 mediated collaboration between T cells and monocytes postulate that, the IL-7 impaired responses of T cells may affect the monocyte functions. Own studies reported lower and impaired IL-7 mediated functions in both monocytes and T cells of TB patients [24,43]. Interaction of monocytes and T cells play indispensable roles in tuberculosis control [72]. Finally, the question remains obscure, the next studies may explore why PAMPs or cytokines-activated monocytes/monocyte cell lines upregulate IL-7Rα expression. It is possible that, the basal IL-7Rα expression may be sufficient for monocyte biology and induced IL-7Rα may not potentiate the functional effects of IL-7. In this regard, T cells may also indirectly potentiate IL-7 signaling on monocytes. Futures studies can comprehensively analyze the molecular futures of IL-7Rα expressing cells and rule out the controversial roles of these cells in both infectious and autoimmune diseases. Albeit we cannot exclude minor effects of IL-7 on monocytes, we concluded that IL-7 mediated functions in monocytes may be enhanced by T cells and cell-to cell contact is necessary. Recent studies suggested an assay system for monocytes-T cells communication in tuberculosis [72]. Next studies may deeply investigate the mechanisms underlying IL-7 mediated effects in monocytes/T cells interaction.

4.4 IL-7 mediated antimycobacterial effector functions in mycobacteria infected MDMs.

IL-7 effects on monocytes and MDMs are hardly defined. Although, induced IL-7Rα expressing monocytes were less sensitive to IL-7, it is possible that a minority of monocyte phenotypes may respond to IL-7 naturally and IL-7 polarized monocytes may exert different biological functions. Alternatively, as indicated by our data, IL-7 dependent functions can be accentuated by the presence of T cells.

Previous studies speculated possible functions of IL-7 in mycobacterial diseases. Using our *in vitro* model for mycobacterial infection, IL-7 showed increased killing of LD BCG mycobacteria as compared to non-treated conditions. These findings are consistent with the previously published data where IL-7 boosted the killing of

mycobacteria avium[73]. In the same direction, IL-7 exhibited antimycobacterial immune responses in mice studies [28]. Of note, IL-7Rα expression is moderate on monocytes and our findings showed an exponential increase of IL-7Rα expression on matured monocytes. Considering this effect, we may suggest that it is possible that a smaller subset of IL-7Rα+ monocytes may be polarized by IL-7. Therefore, we may postulate that, the observed IL-7 mediated effects may be attributed to IL-7/IL-7Rα signaling in a certain monocyte phenotype. Indeed, polarized monocytes towards dendritic cells were characterized by upregulation of CD40 [58]. In the light with our data, it is possible that, IL-7 matured monocytes may contribute to the killing of mycobacteria through antigen presentation mechanism. In this regard, the previous study has revealed the IL-7 induced CD40 and HLA DR expression in monocytes [41]. Considering immunological roles for the presentation of the antigens of CD40 and HLA DR molecules, it is likely the upregulation of these markers enhanced the mycobacteria antigen presentation. In the same vein our data noted IL-7 elicited- CD40 and HLA DR expression in monocytes. This effect was intensified by IL-7 treated T cells. Although we used enriched monocytes in our infection experiments, T cells contamination cannot be avoided. Thus, the killing of mycobacteria can be accelerated by the presence of T cells. Indeed, CD40-CD40L axis was associated with tuberculosis control [74]. IL-7 was shown to induce IFNγ in T cells and it is well documented that this cytokine can promote the control of mycobacteria [26]. We suggested that IL-7 may promote the killing of LD BCG mycobacteria in MDMs. Albeit IL-7Rα expression is low on monocytes but is functional but there is still a knowledge gap on how IL-7 signaling can establish antimycobacterial effector functions.

4.5 Conclusions and outlook

IL-7Rα expression and IL-7 mediated functions in monocytes and macrophages are poorly understood. Our *in vitro* findings have displayed unprecedented IL-7Rα regulation mechanisms in monocytes and monocyte cell lines. We have reported that the cytokines induced by bacterial components may be the key mediators for tuning IL-7Rα expression in monocytes and MDMs[43]. Importantly, our current data posit that SOCS3/CISH molecules may calibrate IL-7Rα expression in monocytes.

IL-7Rα+ monocytes are less sensitive to IL-7 but T cells exposed to IL-7 may potentiate monocytes activation. Interestingly, our findings also suggested that IL-7 matured MDMs enhanced the killing of LD BCG mycobacteria [24]. Albeit these data are immensely contributing to IL-7Rα regulation mechanisms and functions, other questions are still open. Our data pave the way to fully dissect the regulators of IL-7Rα in monocytes and monocyte cell lines. IL-7Rα regulation in monocytes may be different to the well described mechanisms in T cells. Further advanced analyses of SOCS3/CISH and IL-7Rα interaction may shed light on how IL-7Rα is calibrated in monocytes. Advanced analysis of IL-7Rα expressing monocytes clusters and how these subsets respond to IL-7 may add layer on the existing knowledge gap. Lastly, there is a need to understand the relevance of our data in disease context especially in tuberculosis pathology. Understanding biological crosstalk of SOCS3/CISH and IL-7Rα expression in tuberculosis disease may be of significance. In the same vein, a prospective study aiming to correlate IL-7Rα and TB prognostic markers would also be of interest. Investigating IL-7 effects on T cells/monocytes collaboration in disease context would be of relevance as well.

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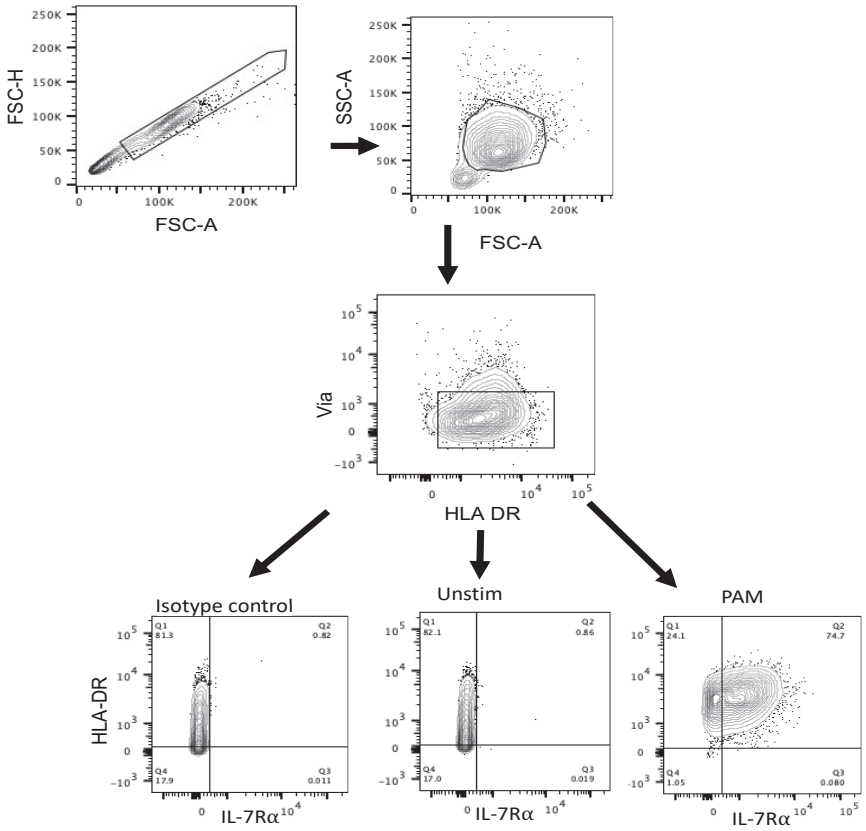
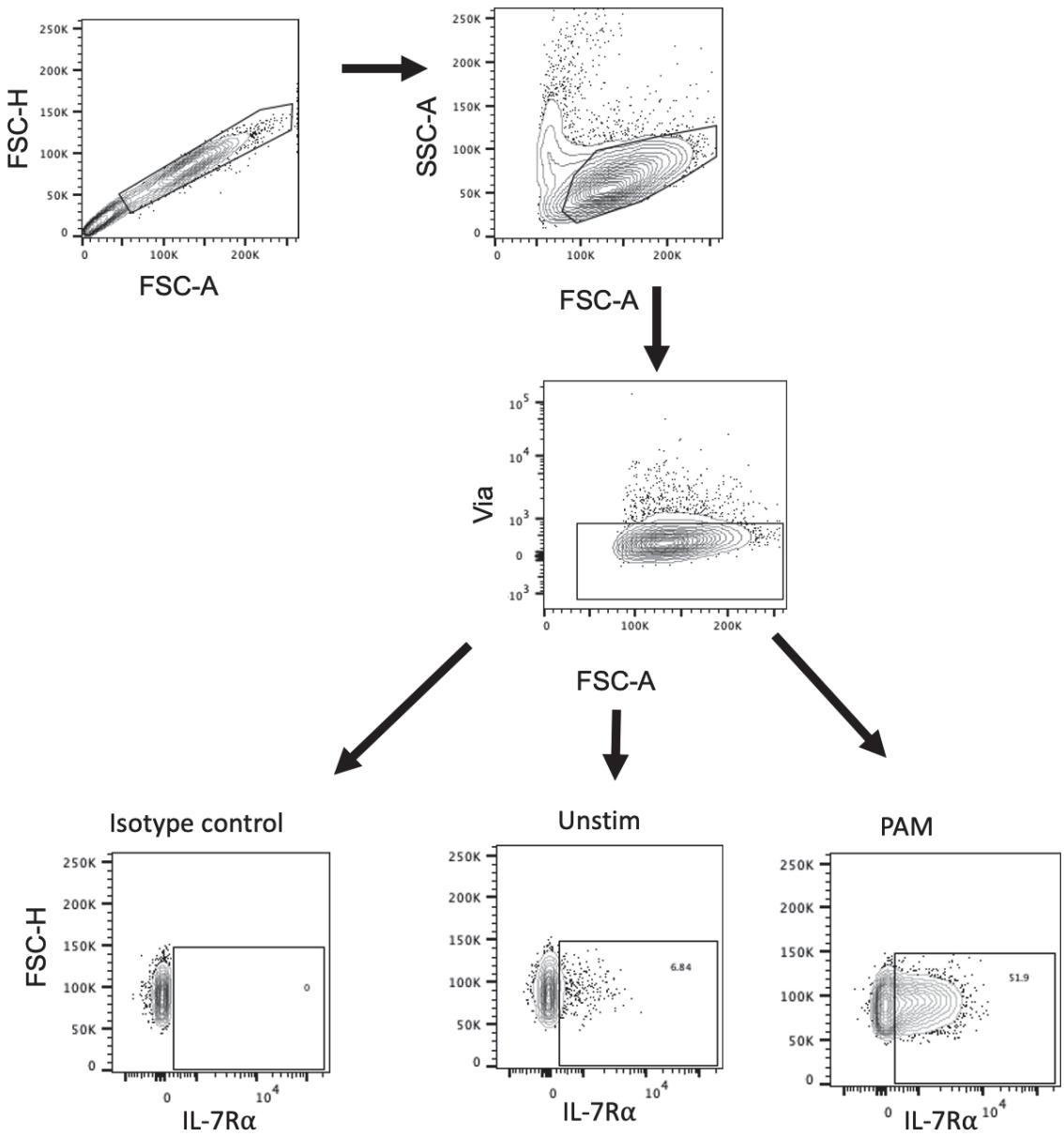
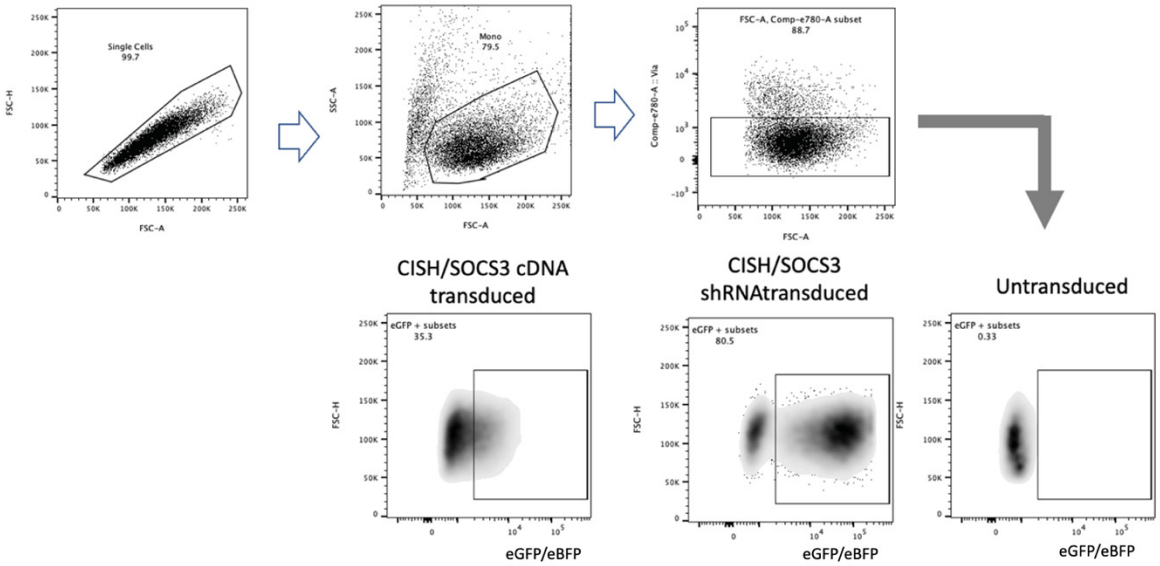
Appendix



Appendix 1: Flow cytometry gating procedure for Monocytes. Initially cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) contour plot with outliers. Next, the cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Subsequently the cells were gated on HLA DR, excluding viability dye (Via) positive (i.e., dead) monocytes. Finally, IL7Rα positive cells were gated basing on isotype control.



Appendix 2: Flow cytometry gating procedure for MonoMac6 cells. Initially cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) contour plot with outliers. Next, the cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A)). Subsequently the cells were gated on FSC-A, excluding viability dye (Via) positive (i.e., dead) MonoMac 6 cells. Finally, IL7Rα positive cells were gated basing on isotype control.



Appendix 3: Flow cytometry gating procedure for CISH/SOCS3 modulated MonoMac 6 cells. Initially cell duplets were excluded using a forward scatter height (FSC-H) vs. FSC area (FSCA-A) contour plot with outliers. Next, the cells were selected based on size (FSC-A) and granularity (Side scatter area, SSC-A). Subsequently the cells were gated on FSC-H, excluding viability dye (Via) positive (i.e., dead) cells. Finally, IL7Rα positive cells were gated transduced cells (eGPF+ cells for CISH and eBFP for SOCS3)