The role of SOCS3 in the regulation of T-cell activation and IL-7Rα expression

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Summary

Suppressor of cytokine signalling 3 (SOCS3) was initially described as a negative feedback inhibitor of cytokine signalling, but has also been associated with the regulation of T cell signalling and other pathways. Appropriate regulation of T-cell activation is crucial for both, pathogen clearance and for preventing excessive inflammatory responses. Interleukin-7 (IL-7) signalling is important for the homeostasis of naïve T helper cells and the development and maintenance of memory T helper cells, and mutations in the IL-7 signalling pathway result in severe immunological disorders. Impaired expression of the IL-7 receptor (IL-7R) has been associated with chronic infections and impaired development of memory T cells. IL-7R and SOCS3 are involved in the signalling of several mutual pathways and are dysregulated concomitantly in several diseases, among them tuberculosis. We showed previously elevated SOCS3 expression accompanied by decreased responsiveness against IL-7 in T cells of tuberculosis patients.

The aim of this study was to elucidate the role of SOCS3 during T-cell activation in primary human T helper cells.

In activated T helper cells, SOCS3 expression increased two days after T-cell activation and accompanied the re-expression of IL-7Rα. SOCS3 expression was modulated with lentiviral constructs to overexpress or knockdown SOCS3 in activated primary human T cells. The specificity of constructs was verified in 293T HEK cell line by comparing it to the closely related protein SOCS1. Lentiviral transduction of T helper cells affected SOCS3 expression starting on day two after T-cell activation, without affecting the activation of resting T cells. Knockdown of SOCS3 ($SOCS3_{kd}$) in T helper cells led to impaired re-expression of the IL-7Rα, and this was independent from signalling by IL-2 and IL-7.

No differences in the expression or phosphorylation of Akt and FoxO1 were detected, both being regulators of IL-7Rα expression and downstream targets of SOCS3. In accordance, IL-7Rα mRNA levels were similar between $SOCS3_{kd}$ and control transduced T helper cells. Therefore, regulation of IL-7Rα by SOCS3 might be independent of the Akt/FoxO1 pathway and regulated on a post-transcriptional level.

To address functional implications of differential IL-7Rα expression, T helper cells were co-transduced with $SOCS3_{kd}$ and control constructs thereby sharing the same culture conditions and competed for available growth factors and IL-7.

$SOCS3_{kd}$ T helper cells showed lower proliferation rates, leading to lower proportions and absolute numbers in co-culture with control cells only in the presence of IL-7. This competitive disadvantage of $SOCS3_{kd}$ T cells could be attributed to decreased IL-7-induced signal transducer and activator of transcription 5 (STAT5) activation over the course of IL-7 signalling.

Overall, these experiments provide evidence for a regulation of IL-7Rα expression during T-cell activation by SOCS3 and further implicate a role of SOCS3 in the development of memory T cells by promoting the re-expression of IL-7Rα after T-cell activation.

List of abbreviations

AD-HIES	autosomal-dominant hyper IgE syndrome
Akt	protein kinase B
ALL	acute lymphoblastic leukemia
CD	cluster of differentiation
CISH	cytokine-inducible SH-2-containing protein
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
FoxO	forkhead box O
G-CSF	granulocyte colony-stimulating factor
Gfi-1	growth factor independent-1
gp130	glycoprotein 130
IFN-γ	interferon-γ
IL	interleukin
IL-2Rβ	IL-2 receptor β-chain
IL-7R	interleukin-7 receptor
IL-7Rα	interleukin-7 receptor α-chain
JAK	Janus kinase
KIR	kinase inhibitory region
Lck	lymphocyte-specific protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
PI3K	phosphoinositide 3-kinase
Ras	Rat sarcoma
RasGAP	Ras GTPase activating protein
SCID	severe combined immunodeficiency
SH2	Src homology 2
SOCS	suppressor of cytokine signalling
$SOCS3_{kd}$	SOCS3 knockdown
$SOCS3_{oe}$	SOCS3 overexpression
STAT	signal transducer and activator of transcription
TB	tuberculosis
TCR	T cell receptor
TGF-β	transforming growth factor-β
T_h2 cell	type 2 T helper cell
TSLP	thymic stromal lymphopoietin
TYK2	tyrosine kinase 2

1 Introduction

T cells are lymphocytes of the adaptive immune system and therefore capable to exert immunological memory, which leads to long-lasting immunity against infections or after vaccination. Before encountering their specific antigen, T cells rest as naïve T cells. Upon antigen recognition these cells become activated, proliferate and differentiate into effector T cells. After pathogen clearance, some of these effector T cells survive in a resting state and become memory T cells whereas the majority is deleted to restore homeostasis, a process called clonal contraction. Following a second encounter with their specific antigen, these memory T cells are reactivated, proliferate and clear the pathogen much faster compared to the first encounter (Murphy and Weaver, 2016). In addition to antigen recognition, T cells need co-stimulatory signals to achieve full activation. The co-receptor CD28 is expressed by T cells and utilized by antigen-presenting cells for co-stimulation during antigen presentation. T cell receptor (TCR) signalling in the absence of co-stimulatory signals either leads to apoptosis or anergy (Kuklina, 2013).

Maintenance of T cells in the naïve state, the differentiation into a memory phenotype and persistence in a resting state are promoted by interleukin (IL)-7 signalling (Schluns et al., 2000, Tan et al., 2001, Li et al., 2003, Dooms et al., 2007). Furthermore, IL-7 can facilitate the activation and responsiveness of T cells to antigen presentation (Saini et al., 2009).

Suppressor of cytokine signalling 3 (SOCS3) has a well-described role in the feedback inhibition of cytokine signalling (Krebs and Hilton, 2001, Linossi et al., 2013). In addition, a role in the regulation of T cell signalling has been attributed to SOCS3 (Fletcher and Starr, 2005, Matsumoto et al., 2003, Li et al., 2017). Upon T-cell activation, CD28-mediated IL-2 expression and subsequent signalling leads to the expansion of T cells (Matsumoto et al., 2003, Yu et al., 2003), and SOCS3 inhibits both, CD28 and IL-2 signalling (Matsumoto et al., 2003, Cohney et al., 1999). An overlap of proteins that regulate or are regulated by SOCS3 and IL-7 signalling exists (Matsumoto et al., 2003, Fabre et al., 2005, Yu et al., 2003, Shi et al., 2017, Pellegrini et al., 2011, Ouyang et al., 2009), and SOCS3 and the IL-7 receptor α-chain (IL-7Rα) are dysregulated concomitantly in several diseases (Harling et al., 2018, Lundtoft et al., 2017, Siegel et al., 2011).

1.1 The JAK/STAT signalling pathway and its feedback regulation

When a cytokine receptor binds its cognate cytokine on the cell surface, intracellular signalling cascades are activated, which deliver the information to the nucleus and activate the transcription of target genes (Murray, 2007). Commonly, proteins are phosphorylated at intracellular receptor domains and then phosphorylate downstream proteins, which act as transcription factors (Murray, 2007). About 40 cytokines activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Murray, 2007). One of its targets is the family of SOCS proteins, which act as negative feedback regulators of cytokine signalling (Yoshimura et al., 2007).

The JAK/STAT-pathway comprises four JAK (JAK1-3, tyrosine kinase 2 (TYK2)) and seven STAT (STAT1-4, STAT5a, STAT5b, STAT6) family members (Murray, 2007). The SOCS family consists of eight proteins: SOCS1-7 and cytokine-inducible SH2-containing protein (CISH) (Yoshimura et al., 2007). Specificity in the distinct activation of a cell program upon JAK/STAT activation is achieved by differential expression of cytokine receptors on different cell types, co-expression of other cytokine receptors, the microenvironment of the cell and cell type dependent responses (Murray, 2007). In addition, receptors utilize different combinations of JAK proteins, and JAK, STAT and SOCS proteins have different affinities to receptors and binding partners (Murray, 2007).

Other mechanisms regulating receptor signalling involve tyrosine phosphatases and degradation of signalling molecules, receptor internalization and receptor antagonists, and protein inhibitors of activated STATs (PIAS) (Croker et al., 2008, Henriques et al., 2010, Greenhalgh and Hilton, 2001). Indeed, an overlap in binding sites has been reported for SOCS3 and the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-2 (SHP-2), which bind the same binding site on the glycoprotein 130 (gp130) receptor (Nicholson et al., 2000, Lehmann et al., 2003).

1.2 Inhibition of cytokine signalling by SOCS proteins

Several mechanisms can mediate or synergize in the inhibition of cytokine signalling by SOCS proteins. The main functional domains shared by all SOCS proteins are the Src homology 2 (SH2) domain and the SOCS box (Linossi et al., 2013). SOCS1

and SOCS3 are unique in the way that they carry an additional domain called kinase inhibitory region (KIR) (Linossi et al., 2013). The SH2 domain is a binding domain for phosphorylated tyrosine residues on target proteins and receptors (Linossi et al., 2013). The SOCS box is recruiting an E3 ubiquitin ligase complex for degradation of SH2-bound target proteins, which leads to their ubiquitination and subsequent proteasomal degradation (Zhang et al., 1999, Kershaw et al., 2014, Linossi et al., 2013). The KIR domain is an inhibitory region which blocks the activity of JAKs (Babon et al., 2012, Sasaki et al., 1999, Linossi et al., 2013).

Thus, SOCS proteins can inhibit JAK phosphorylation by competitive binding to the receptor (or by binding JAKs) in an SH2-dependent manner (Linossi et al., 2013). Furthermore, receptors or JAKs can be ubiquitinated for degradation after SH2 binding in a SOCS box-dependent manner (Zhang et al., 1999, Kershaw et al., 2014), and SOCS1 and SOCS3 are capable of inhibiting JAK kinase activity in a KIR-dependent manner after SH2-mediated binding (Linossi et al., 2013, Sasaki et al., 1999). Thereby, specificity is achieved by the individual SH2 domains bearing different affinities for phosphorylated tyrosine target motifs (Linossi et al., 2013, Babon et al., 2012).

1.3 Induction of SOCS3 expression and regulation of signalling pathways

1.3.1 Induction of SOCS3 expression by cytokine signalling

SOCS3 expression is induced upon signalling by many cytokines (Greenhalgh and Hilton, 2001), including IL-2 (Cohney et al., 1999). Binding of STAT1 and STAT3 homodimers, and STAT1/STAT3 heterodimers to the *Socs3* promoter and subsequent activation of gene expression is well characterized (Gatto et al., 2004, Auernhammer et al., 1999), and SOCS3 messenger RNA (mRNA) transcription in a STAT5-dependent manner has been assumed (Ghazawi et al., 2016). Moreover, it has been proposed that STAT proteins activate a generic set of genes regardless of the cytokine that induced their activation (Murray, 2007). Therefore, it is not surprising that SOCS3 expression is activated by many different cytokines.

Nevertheless, post-transcriptional regulation and other factors play an important role in the regulation of SOCS3 expression. For example, while the IL-7 receptor (IL-7R) can signal via STAT1, STAT3 and STAT5 (Spolski et al., 2017), and modest amounts of SOCS3 mRNA are being expressed in cytotoxic T cells upon IL-7

signalling in a JAK/STAT5-dependent manner, no significant increase of SOCS3 protein expression could be detected (Ghazawi et al., 2016). In contrast, the IL-2R utilizes the same set of STAT proteins (Spolski et al., 2017), and IL-2 signalling leads to the induction of SOCS3 protein (Cohney et al., 1999).

1.3.2 SOCS3 in the feedback regulation of cytokine signalling

In hematopoietic cells, SOCS3 is known to inhibit cytokine signalling of IL-2 receptor β-chain (IL-2Rβ) (Cohney et al., 1999), gp130 (Nicholson et al., 2000, Kershaw et al., 2013), erythropoietin (EPO) receptor (Hortner et al., 2002a, Sasaki et al., 2000), granulocyte colony-stimulating factor (G-CSF) receptor (Croker et al., 2004, Hortner et al., 2002b), IL-12Rβ2 (Yamamoto et al., 2003, Seki et al., 2003) and leptin receptor (Bjorbak et al., 2000), and to inhibit JAK1 (Babon et al., 2012), JAK2 (Masuhara et al., 1997, Sasaki et al., 1999, Babon et al., 2012) and TYK2 activation (Babon et al., 2012).

Preceding binding of SOCS3 to the receptor is likely necessary for subsequent inhibition of JAK activity (Babon et al., 2012, Kershaw et al., 2013, Linossi et al., 2013). Detailed structural analyses revealed that SOCS3 binds the tyrosine-phosphorylated IL-6R in an SH2-dependent manner and simultaneously JAK2 in a phospho-independent manner with the help of the SOCS3 KIR domain (Kershaw et al., 2013, Babon et al., 2012). A similar mechanism was shown for EPO-R/JAK2 signal inhibition by SOCS3 (Sasaki et al., 2000). Notably, the KIR domain alone is not sufficient to inhibit JAK2 activity, and specificity of inhibition of JAKs by SOCS3 is achieved by simultaneous binding of JAKs associated to receptors bearing phosphorylated tyrosine motifs, which are bound by the SH2 domain of SOCS3 (Kershaw et al., 2013). The main mode of inhibition thereby is mediated by the KIR domain blocking substrate binding of JAK2 (Kershaw et al., 2013). This model of inhibition is believed to be generally applicable for all cytokine receptors which are targeted by SOCS3 (Kershaw et al., 2013, Babon et al., 2012). Nevertheless, high concentrations of SOCS3 can overcome the necessary ternary high-affinity binding of SOCS3 consisting of receptor phospho-tyrosine motif and JAK, leading to an inhibition of JAK signal transduction in a non-receptor and therefore non-specific manner (Kershaw et al., 2013).

1.3.3 SOCS3 in the regulation of cytokine independent pathways

In addition, cytokine independent functions for SOCS3 have been described. A tyrosine phosphorylation site on the C terminus of SOCS3 leads to the inhibition of Ras GTPase activating protein (RasGAP) activity and therefore enhances the phosphorylation of extracellular signal-regulated kinase (ERK) (Cacalano et al., 2001). SOCS3 has also been shown to interact with downstream signalling molecules of the TCR (Masuhara et al., 1997, Banerjee et al., 2002), and with the T cell co-receptors CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Matsumoto et al., 2003, Yu et al., 2013).

1.4 SOCS3 mRNA and protein stability are regulated on different levels

Given its nature as a negative feedback regulator, signal attenuation by SOCS3 itself diminishes its expression (Auernhammer et al., 1999). Furthermore, SOCS3 has a relatively short half-life and succumbs to proteasomal degradation due to an N-terminal lysine ubiquitination site (Sasaki et al., 2003) and to proteasomal-independent degradation via a proline, glutamate, serine and threonine (PEST) rich sequence (Babon et al., 2006). Interestingly, the expression of a truncated form of SOCS3 is induced under stress conditions via an internal start-codon that lacks the N-terminal ubiquitination site and therefore exhibits stronger protein stability (Sasaki et al., 2003).

It has also been suggested that SOCS Box-mediated recruiting of the E3 ubiquitin ligase complex for proteasomal degradation of SOCS3 targets leads to degradation of SOCS3 protein itself (Zhang et al., 1999). Contrariwise, phosphorylation of SOCS3 upon cytokine signalling (Cacalano et al., 2001) disrupts association with elongin C, which is part of the E3 ubiquitin ligase complex, and increases proteasome-dependent degradation, while interaction with elongin C increases SOCS3 protein half-life (Haan et al., 2003).

Furthermore, SOCS2 mediates proteasome-dependent degradation of SOCS3 in experiments with cell lines ectopically overexpressing SOCS2 (Tannahill et al., 2005, Piessevaux et al., 2006). While SOCS2 and SOCS3 are reciprocally expressed on mRNA level during T-cell activation (Yu et al., 2003), and both being induced upon IL-2 signalling and therefore potentially relevant in activated T cells (Tannahill et al., 2005), physiological relevance of this mechanism is controversial

(Kiu et al., 2009). Kiu et al. demonstrate that SOCS3 expression, IL-2-induced STAT5 activation and proliferation of T cells are not affected in SOCS2 knockout mice compared to wildtype mice (Kiu et al., 2009).

SOCS3 expression can also be negatively regulated by cytokine signalling. SOCS3 mRNA and protein are downregulated upon IL-7 signalling in T cells (Li et al., 2017, Pellegrini et al., 2011), and participation of forkhead box O (FoxO) transcription factors were assumed, as mice deficient in these transcription factors showed diminished levels of SOCS3 expression (Pellegrini et al., 2011). Similarly, growth factor independent-1 (Gfi-1), which is also a negative regulator of FoxO1 (Shi et al., 2017), was shown to directly supress *Socs3* promoter activity (Jegalian and Wu, 2002). Gfi-1 itself is downregulated in a STAT5 dependent and mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) pathway independent manner (Jegalian and Wu, 2002), and therefore potentially relevant in the regulation of SOCS3 during T-cell activation. Likewise, IL-6-induced *Socs3* promoter activity is decreased upon transforming growth factor-β (TGF-β) signalling (Qin et al., 2009).

SOCS3 expression is a target of transcriptional and post-transcriptional modifications by promoter methylation and microRNAs (miRNAs) (Boosani and Agrawal, 2015). Post-transcriptional fine-tuning affecting SOCS3 mRNA stability has also been reported: zinc finger protein tristetraprolin (TTP) destabilizes whereas activation of the MAPK pathway stabilizes SOCS3 mRNA and prolongs SOCS3 expression (Ehlting et al., 2007). SOCS3 mRNA is a target of methylation, leading to an increase in degradation (Li et al., 2017). Evidence for post-transcriptional regulation of SOCS3 protein stability is furthermore underlined by a study with regulatory T cells, showing that both, T helper cells as well as regulatory T cells express SOCS3 mRNA, while SOCS3 protein is only being expressed in T helper cells (Pillemer et al., 2007).

1.5 The role of SOCS3 in the polarization of T helper cells

The regulation of cytokine signalling plays an important role in T helper cell differentiation, and dysregulated SOCS expression can lead to a shift in the polarization of different T helper cells (Yoshimura et al., 2007). In the case of SOCS3, maintenance of low levels of protein expression in regulatory T cells seems to be obligatory for suppressive functionality on T helper cells (Pillemer et al., 2007, Yu et al., 2013). In contrast, SOCS3 expression is important for the differentiation

of type 2 T helper (T_h2) cells by suppressing IL-12/STAT4-induced T_h1 cell differentiation (Seki et al., 2003, Egwuagu et al., 2002). For the differentiation of T_h17 cells, initial downregulation of SOCS3 is essential for polarization towards this cell subset (Qin et al., 2009, Taleb et al., 2009), whereas differentiated T_h17 cells benefit from higher SOCS3 expression levels (Kleinsteuber et al., 2012). Given the role of SOCS3 in T-cell polarization, its regulated expression is also of importance concerning prevention against autoimmune and allergic diseases (Tamiya et al., 2011).

1.6 Experimental modulation of SOCS3 expression

Most relevant information has been gained from knockout studies concerning the role of SOCS3 under physiological conditions in cytokine signalling and disease. SOCS3-deficient mice die in utero at mid-gestation (Roberts et al., 2001, Marine et al., 1999). Extensive erythropoiesis due to aberrant EPO receptor signalling was proposed to be the cause of embryonic lethality (Marine et al., 1999), while others hold placental defects caused by uncontrolled leukemia inhibitory factor (LIF) signalling responsible and could not find any differences in hematopoiesis and EPO responsiveness (Roberts et al., 2001, Robb et al., 2005).

Deletion of SOCS3 in the hematopoietic compartment leads to neutrophilia, neutrophil infiltration of tissues and consequent inflammatory pathologies due to enhanced G-CSF signalling in adult mice (Croker et al., 2004). SOCS3 deficiency in the myeloid compartment in mice leads to a severe form of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, by enhanced STAT signalling in macrophages and subsequent induction, activation and infiltration of inflammatory T_h1 and T_h17 cells in the brain (Qin et al., 2012).
Interestingly, SOCS3 deficiency in murine macrophages causes a STAT1-mediated interferon-like response. Although STAT3 phosphorylation is stronger in the absence of SOCS3, STAT1 phosphorylation in the absence of SOCS3 is thought to dominate the cell response. Consequently, SOCS3 not only mediates feedback inhibition and thus quantity of STAT signalling, but also qualitatively determines the type of STAT signalling being forwarded to the nucleus (Lang et al., 2003, Croker et al., 2003).
Mice with a conditional knockout of SOCS3 in T cells and natural killer T cells seem to develop normally up to at least nine months of age, but show enhanced T-cell

activation-induced proliferation and prolonged STAT activation upon IL-6 and IL-27 stimulation (Brender et al., 2007).

Conditional knockout of SOCS3 only in T cells does supposedly not alter T cell numbers, ratios of cytotoxic T cells and T helper cells, and T-cell activation measured by CD25 and CD69 (Kinjyo et al., 2006). Deletion of SOCS3 in T cells of mice also leads to prolonged STAT activation (Kinjyo et al., 2006, Yu et al., 2013), and an IL-10 mediated anti-inflammatory phenotype, and protects mice from developing chronic experimental autoimmune uveoretinitis by increasing the amount and functionality of suppressive regulatory T cells (Yu et al., 2013). Similarly, SOCS3-deficient T cells are associated with a reduced pathology mediated by TGF-β and IL-10 in a model of induced asthma in mice, but also with reduced immune responses against infection with an intracellular protozoan parasite (Kinjyo et al., 2006). Therefore, the authors proposed SOCS3 suppression as a potential target to ameliorate autoimmune and allergic pathologies (Kinjyo et al., 2006). Knockdown of SOCS3 in lymphocytic choriomeningitis virus (LCMV)-specific cytotoxic T cells leads to an impaired development from effector to memory T cells in mice, and this is possibly associated with a lack of SOCS3-mediated reduction of pro-inflammatory cytokine signalling (Cui et al., 2011). Interestingly, cytotoxic memory T cells with decreased SOCS3 expression show an impaired expression of the IL-7R (Cui et al., 2011).

1.7 The role of SOCS3 in the feedback regulation of T-cell activation

1.7.1 T-cell activation leads to a temporary decrease of SOCS3

SOCS3 is constitutively expressed in naïve T helper cells (Matsumoto et al., 2003, Yu et al., 2003, Owaki et al., 2006, Yu et al., 2013). TCR ligation leads to the activation of the MAPK/ERK pathway (Carey et al., 2000, Karsunky et al., 2002), while signalling of the T cell co-receptor CD28 activates the PI3K/protein kinase B (PI3K/Akt) pathway (Pages et al., 1994, Prasad et al., 1994, Truitt et al., 1994). CD28 signalling further increases the activation of ERK (Carey et al., 2000). SOCS3 has an inhibitory role in CD28/PI3K signalling (Matsumoto et al., 2003, Owaki et al., 2006). T-cell activation leads to the downregulation of SOCS3 (Matsumoto et al., 2003, Owaki et al., 2006) in an antigen-concentration dependent manner (Yu et al., 2003). This mechanism is, at least partly, mediated by the induction of Gfi-1 upon T-cell activation and its inhibitory capacity on the *Socs3* promoter (Yu et al., 2003,

Jegalian and Wu, 2002, Karsunky et al., 2002). Gfi-1 is a DNA binding transcriptional repressor protein and a transcription factor regulating S-phase entry in T helper cells upon antigenic T-cell activation (Karsunky et al., 2002, Pargmann et al., 2007, Moroy and Khandanpour, 2011). In addition, as described above, proteasomal-dependent and independent mechanisms lead to the degradation of SOCS3 (Sasaki et al., 2003, Babon et al., 2006).

Downregulation of SOCS3 enables IL-2 signalling and promotes clonal expansion after antigen encounter (Yu et al., 2003, Matsumoto et al., 2003). IL-2Rα is not expressed in naïve T cells (Tan et al., 2001, Dooms et al., 2007). Upregulation of IL-2Rα is mainly mediated by TCR activation, while co-stimulation of CD28 is needed for maximum production of IL-2 (Matsumoto et al., 2003, Owaki et al., 2006, Akaishi et al., 1998). IL-2 production is mediated by PI3K binding to phosphorylated CD28 (Pages et al., 1994, Stein et al., 1994), and IL-2 signalling further enhances IL-2Rα expression (Xue et al., 2002, Akaishi et al., 1998).

Taken together, SOCS3 is essential for maintaining T helper cells in a quiescent state (Yu et al., 2003), and transient downregulation of SOCS3 allows T cells to become activated and proliferate in an IL-2 dependent manner (Yu et al., 2003, Pillemer et al., 2007).

1.7.2 SOCS3 is re-expressed in the late T cell effector phase

IL-2 signalling leads to the activation of the transcription factors STAT3 (Lin et al., 1995) and STAT5, and to the re-expression of SOCS3 (Cohney et al., 1999). STAT5 has been shown to promote SOCS3 expression by counteracting the inhibition of *Socs3* by Gfi-1 upon EPO signalling (Jegalian and Wu, 2002). Whether this holds true for IL-2-induced STAT5 signalling remains to be investigated. Induction of SOCS3 during T-cell activation therefore requires CD28-mediated IL-2 signalling and leads to a decline of IL-2 secretion and IL-2 promoted proliferation (Yu et al., 2003, Matsumoto et al., 2003, Owaki et al., 2006). This effect is mediated by SOCS3 in two distinct ways. IL-2 secretion is inhibited by SOCS3 binding to the phosphorylated CD28 receptor and subsequent inhibition of PI3K association with CD28 (Matsumoto et al., 2003, Owaki et al., 2006), and by direct inhibition of STAT5 activation at the IL-2R (Cohney et al., 1999, Owaki et al., 2006). While inhibiting STAT5-mediated IL-2 signalling and therefore cytokine-mediated growth, SOCS3

expression preserves ERK activation by binding to the Rat sarcoma (Ras) inhibitor RasGAP, maintaining ERK dependent cell survival (Cacalano et al., 2001).

In summary, re-expression of SOCS3 may be important in prohibiting excessive CD28-mediated T-cell activation and IL-2 production (Matsumoto et al., 2003, Pillemer et al., 2007), while expression of SOCS3 prior to pathogen clearance contributes to chronic viral infection (Pellegrini et al., 2011).

1.8 Further implications for SOCS3 during T-cell activation

There is evidence for a role of SOCS3 in the direct regulation of TCR mediated signalling during T-cell activation (Masuhara et al., 1997, Banerjee et al., 2002). Lymphocyte-specific protein tyrosine kinase (Lck) is a Src family kinase which phosphorylates the TCR and initiates a cascade leading to Calcium mobilization and activation of nuclear factor of activated T cells (NFAT) via calcineurin (Huang and Wange, 2004, Crabtree, 1999). Activation of NFAT leads to the production of the cytokine IL-2 (Crabtree, 1999, Huang and Wange, 2004). SOCS3 is known to bind Lck (Masuhara et al., 1997). Furthermore, in an in vitro assay SOCS3 inhibits the activation of NFAT via interaction with calcineurin, which subsequently inhibits the production of IL-2 (Banerjee et al., 2002). Besides, SOCS3 has been shown to bind CTLA-4, a co-inhibitory receptor of the TCR, and regulate its expression in T helper cells (Yu et al., 2013). Knockout of SOCS3 in murine T cells leads to higher CTLA-4 expression, and these cells display a less activated phenotype (Yu et al., 2013). Whether these mechanisms play a role in vivo in establishing a threshold for T-cell activation in resting T cells or rather play a role in the inhibition of activated T cells needs to be investigated, although these two possibilities are not mutually exclusive.

Given the role of SOCS3 as a regulator of T-cell activation, the mechanisms regulating SOCS3 re-expression after antigen-encounter are important for understanding chronic infectious diseases. Untimely expression of SOCS3 before pathogen clearance contributes to the development of chronic infections and T cell exhaustion (Pellegrini et al., 2011, Harling et al., 2018). In addition, constant exposure to antigen as well as higher expression of SOCS3 are associated with impaired IL-7Rα expression and responses in chronic infections (Alves et al., 2008, Harling et al., 2018, Lundtoft et al., 2017, Lang et al., 2005).

1.9 IL-7R and the family of common γ-chain cytokines

The IL-7R belongs to the family of common γ-chain cytokine receptors, which comprises the cytokine receptors IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R. Besides having unique α-chain receptors, all these receptors share the common γ-chain receptor as their co-receptor (Ozaki and Leonard, 2002). IL-2R and IL-15R also share the IL-2Rβ as an additional receptor chain (Ozaki and Leonard, 2002). Common γ-chain cytokines generally act as growth factors. In T cells, IL-2 serves as the major growth factor promoting T cell expansion upon antigen encounter, whereas IL-15 is essential for the expansion of T effector memory cells. IL-4, IL-9 and IL-21 have more specific functions or target other lymphocytes than T cells (Ozaki and Leonard, 2002). IL-7 is essential for the development of T cells in the thymus (Jiang et al., 2005), and an important survival and maintenance factor for naïve and memory T cells. Homeostatic proliferation of naïve T cells depends on IL-7 signalling (Schluns et al., 2000, Dooms et al., 2007). Furthermore, IL-7 is necessary for the generation and survival of memory T cells (Schluns et al., 2000, Dooms et al., 2007, Li et al., 2003, Jiang et al., 2005).

Another receptor utilizing the IL-7Rα is the thymic stromal lymphopoietin (TSLP) receptor, which does not belong to the family of common γ-chain receptors (Ozaki and Leonard, 2002). TSLP exerts its function mainly by activating antigen-presenting cells and has promoting effects on B and T cells (Ozaki and Leonard, 2002).

1.10 IL-7R signalling

The IL-7R belongs to the type I cytokine receptor family and does not bear intrinsic kinase activity. For signal transduction, the receptor utilizes receptor associated kinases (Jiang et al., 2005). JAK1 and JAK3 are generally utilized by the family of common γ-chain receptors (Murray, 2007). The IL-7Rα is associated to JAK1, while the common γ-chain is associated to JAK3 (Carrette and Surh, 2012). Upon IL-7 signalling, both kinases phosphorylate each other, and activated JAK3 phosphorylates IL-7Rα (Carrette and Surh, 2012). The IL-7Rα phosphorylation site serves as a binding site and recruits STAT5, which is in turn phosphorylated by JAK1 and/or JAK3, and translocates into the nucleus (Carrette and Surh, 2012). IL-7/STAT5 signalling elicits proliferation signals and is indispensable in its pro-

survival function for peripheral cytotoxic T cells and T helper cells (Jiang et al., 2005).

Although other kinases and pathways, such as kinases from the Src family tyrosine kinases, other STAT molecules and the PI3K/Akt pathway, are being associated with IL-7 signalling, there is little evidence for an essential or non-redundant role of these pathways in mature T cells. Moreover, activation of the PI3K/Akt pathway seems to have a rather synergistic effect on T-cell activation (Jiang et al., 2005).

1.11 The source of IL-7 production

T cells do not express IL-7, but rely on its expression by stromal cells (Kroncke et al., 1996). Although some dendritic cells also produce IL-7 (Kroncke et al., 1996, de Saint-Vis et al., 1998), it has been suggested that the main IL-7 source for lymphocytes is of non-bone marrow derived origin (Schluns et al., 2000). However, other non-lymphoid cells and tissues are also capable of producing IL-7, and the essential source of IL-7 and whether IL-7 synthesis is regulated or constitutively expressed is not entirely dissolved (Jiang et al., 2005).

1.12 Cytokine-mediated regulation of IL-7R expression

1.12.1 IL-7Rα expression is regulated by pro-survival cytokines

Besides negative regulation of the IL-7Rα by IL-7, IL-7Rα is also downregulated by pro-survival cytokines IL-2, IL-4, IL-6 and IL-15 (Kerdiles et al., 2009, Swainson et al., 2006, Ghazawi et al., 2013, Park et al., 2004, Xue et al., 2002). Withdrawal of growth factors on the other hand leads to the upregulation of IL-7Rα in a FoxO1 dependent manner (Kerdiles et al., 2009). As a crucial cytokine for naïve and memory T-cell homeostasis (Schluns et al., 2000, Dooms et al., 2007, Li et al., 2003, Jiang et al., 2005), IL-7 availability is limiting the amount of T cells in the periphery (Bolotin et al., 1999, Fry et al., 2001, Napolitano et al., 2001, Seddon and Zamoyska, 2002a). Downregulation of IL-7Rα by other pro-survival cytokines is an important mechanism to maintain the peripheral T cell pool, preventing competition for remaining IL-7, maximizing the availability for naïve T cells and effectively removing activated T cells from the competition for IL-7 (Park et al., 2004). In addition, IL-7 levels in vivo are not sufficiently high to fully downregulate IL-7R on resting naïve and memory T cells (Li et al., 2003), and this might be an important mechanism to prevent cytokine deprivation-induced apoptosis in these cells (Chetoui et al., 2010).

1.12.2 Feedback regulation of the IL-7R upon IL-7 binding

In the absence of IL-7 signalling, expression levels of IL-7Rα on the cell surface are maintained relatively stable (Henriques et al., 2010, Ghazawi et al., 2013). Even though the receptor is being constantly endocytosed, only a minor part is degraded while the majority is recycled and transported back to the cell surface (Henriques et al., 2010). The recycling of the IL-7R depends on Vps34, which belongs to the family of Class III PI3Ks (Carrette and Surh, 2012). However, upon IL-7 signalling most of the internalized receptor is degraded, leading to a lower expression of the receptor at the cell surface (Henriques et al., 2010). Degradation of IL-7Rα is mediated by JAK3 signalling (Henriques et al., 2010). Therefore, while receptor signalling for most cytokines is at least partly feedback regulated by e.g. SOCS proteins, strength and duration of IL-7 signalling are primarily inhibited by IL-7R internalization upon IL-7 binding (Ghazawi et al., 2013, Henriques et al., 2010), although this might not fully explain the feedback regulation by IL-7 signalling. IL-7 mediated downregulation of the IL-7Rα occurs on mRNA and protein level and is dependent on new transcription and protein synthesis (Alves et al., 2008, Park et al., 2004), and re-expression after IL-7 withdrawal is dependent on new transcription and protein synthesis as well (Alves et al., 2008, Park et al., 2004). Nevertheless, IL-7 mediated receptor downregulation shows a faster kinetic compared to T-cell activation mediated receptor downregulation, which accounts for a primary signal inhibition by receptor internalization (Alves et al., 2008, Ghazawi et al., 2013). Transcriptional suppression by IL-7 is mediated in a JAK/STAT5-dependent and PI3K-independent manner, while removal from the cell surface is independent of JAK/STAT5 or PI3K signalling (Ghazawi et al., 2013). IL-7Rα internalization occurs before transcriptional suppression and might rather be mediated by receptor dimerization or conformational changes (Ghazawi et al., 2013, Henriques et al., 2010). Moreover, IL-7-induced downregulation seems to involve the transcriptional repressor protein Gfi-1 in cytotoxic T cells but not in T helper cells (Park et al., 2004, Alves et al., 2008).

IL-7-induced transcriptional suppression and downregulation at the cell surface are both dose- and time-dependent, and provide a sensitive mechanism in which cells can directly and sensitively react to IL-7, which further prevents competition and maintains the T cell pool (Ghazawi et al., 2013, Henriques et al., 2010).

1.12.3 Feedback regulation of IL-7R signalling by SOCS proteins

SOCS1 plays an additional role in the feedback regulation of IL-7 signalling (Cornish et al., 2003). SOCS1 is induced upon and inhibits IL-7 signalling by preventing STAT5 activation (Cornish et al., 2003, Fujimoto et al., 2000). Furthermore, CISH and, to a lesser extent, SOCS2 have been associated with targeting IL-7Rα upon stimulation for proteasomal degradation (Ghazawi et al., 2016).

1.13 The role of TCR/MHC stimulation and IL-7R signalling in the maintenance of naïve, effector and memory T cells

In addition to IL-7 signalling, homeostatic proliferation and survival of naïve T cells depend crucially on TCR/major histocompatibility complex (MHC) interaction (Tan et al., 2001, Murali-Krishna et al., 1999, Tanchot et al., 1997, Rooke et al., 1997, Takeda et al., 1996, Seddon and Zamoyska, 2002b, Seddon and Zamoyska, 2002a), while there are contrary reports on the importance of MHC interaction in memory T cells.

Some studies show that cytotoxic memory T cells and memory T helper cells survive without MHC-interaction (Lau et al., 1994, Murali-Krishna et al., 1999, Swain et al., 1999, Seddon and Zamoyska, 2002b), while others report that low affinity MHC stimulation is indispensable for memory T cell survival and functionality (Kassiotis et al., 2002, Tanchot et al., 1997, Markiewicz et al., 1998). However, the transition from an effector to a memory T cell phenotype is independent of MHC interaction (Li et al., 2003).

In the absence of TCR/MHC stimulation or cytokine signalling, T cells undergo cytokine deprivation-induced apoptosis (Chetoui et al., 2010). The protectory effect of IL-7 is executed by the upregulation of the pro-survival protein B cell lymphoma-2 (Bcl-2) (Chetoui et al., 2010, Li et al., 2003, Surh and Sprent, 2008). For memory T helper cells it has been shown that this happens in a STAT5-dependent, and PI3K/Akt and MAPK/ERK independent manner (Chetoui et al., 2010, Surh and Sprent, 2008).

1.14 Regulation of IL-7R signalling during T-cell activation

IL-7Rα expression is high in naïve and memory T cells, but low in effector T cells (Alves et al., 2008, Schluns et al., 2000, Dooms et al., 2007, Li et al., 2003, Xue et al., 2002), and T-cell activation has been shown to downregulate IL-7Rα expression

(Dooms et al., 2007, Schluns et al., 2000, Xue et al., 2002). Interestingly, on a transcriptional level IL-7Rα mRNA downregulation upon T-cell activation is prolonged compared with IL-7 stimulation (Swainson et al., 2006). The mechanism for IL-7Rα downregulation is fairly well studied, although not always in a comprehensive context of T-cell activation.

1.14.1 Maintenance of IL-7Rα expression in naïve T cells

Gfi-1 is downregulated during thymocyte development to allow the upregulation of proteins that are characteristic for mature thymocytes, e.g. FoxO1 and IL-7Rα (Shi et al., 2017). A loss of Gfi-1 leads to the upregulation, while retroviral ectopic expression leads to a decrease of FoxO1 and IL-7Rα expression (Shi et al., 2017). In mice, Gfi-1 mRNA expression is low whereas FoxO1 and IL-7Rα mRNA expression are high in naïve T cells (Shi et al., 2017). A regulatory role of FoxO1 in preventing T-cell activation in naïve T cells has been assumed, as knockout of FoxO1 in these cells leads to spontaneous activation (Ouyang et al., 2009). FoxO1 is a direct regulator of IL-7Rα, being able to bind to the *Il7r* gene and positively influence its expression (Ouyang et al., 2009, Kerdiles et al., 2009). Its expression is essential for the survival of naïve T cells (Ouyang et al., 2009, Kerdiles et al., 2009).

1.14.2 T-cell activation leads to a temporary decrease of IL-7Rα

Gfi-1 is upregulated upon T-cell activation (Karsunky et al., 2002), although a direct link in T cell signalling mediated regulation of the IL-7Rα has not been demonstrated. Nevertheless, regulated expression of Gfi-1 during effector stages seems to be important as a lack of Gfi-1 expression increases activation induced cell death in Gfi-1 deficient memory cells (Pargmann et al., 2007). Furthermore, TCR signalling leads to the sequestration of FoxO1 from the nucleus in a PI3K/Akt dependent manner and therefore inhibits its function as a transcription factor, which allows cells to grow after T-cell activation (Fabre et al., 2005). Activation of PI3K inhibits IL-7Rα expression (Kerdiles et al., 2009). Additionally, T-cell activation-induced IL-2 signalling leads to the downregulation of IL-7Rα in an Akt-dependent manner; and during the presence of IL-2, IL-7Rα expression is blocked (Xue et al., 2002).

Generally, TCR/CD28 mediated IL-7Rα downregulation occurs on mRNA and protein level, is dependent on new transcription and protein synthesis, and leads to

a chronical suppression of IL-7Rα re-expression on a transcriptional level (Alves et al., 2008).

1.14.3 IL-7Rα is re-expressed in the late T cell effector phase and essential for the formation of memory T cells

IL-2-mediated expansion is feedback regulated by SOCS3 (Cohney et al., 1999). T cells start to re-express IL-7Rα while IL-2Rα expression is diminished in a reciprocal manner (Dooms et al., 2007). Withdrawal of growth factors is known to lead to the upregulation of IL-7Rα expression in a FoxO1-dependent manner (Kerdiles et al., 2009), and particularly STAT5, which is activated by IL-2 signalling (Lin et al., 1995), is known to inhibit Gfi-1 expression (Jegalian and Wu, 2002). In addition, the repression of the negative regulator of IL-7Rα, Gfi-1, seems to be important for memory formation, as Gfi-1 deficiency leads to higher memory frequencies in cytotoxic T cells and T helper cells (Pargmann et al., 2007). While the initial downregulation of IL-7Rα during T-cell activation is IL-2 independent, re-expression of IL-7Rα after T-cell activation requires previous IL-2 signalling (Dooms et al., 2007). In the absence of IL-2 signalling, activated T cells fail to re-express IL-7Rα, which leads to diminished memory formation and survival (Dooms et al., 2007). Therefore, IL-7Rα re-expression as well as IL-7 signalling is indispensable for the generation of memory T cells (Dooms et al., 2007, Li et al., 2003).

1.15 SOCS3 and IL-7 signalling in infectious diseases and immunity

SOCS3 expression and IL-7 signalling are dysregulated alongside in several diseases (Harling et al., 2018, Lundtoft et al., 2017, Siegel et al., 2011). Proper IL-7 signalling itself is important for a functional immune system. The importance of accurately regulated IL-7 signalling in the lymphocyte compartment is evident from patients that lack proper IL-7 signalling or constitutively signal over the IL-7 pathway. Defective IL-7R expression or downstream signalling proteins result in severe combined immunodeficiency (SCID) (Puel et al., 1998, Macchi et al., 1995). In contrast, constitutive activation of the IL-7 pathway can be causative for T cell acute lymphoblastic leukemia (ALL) (Zenatti et al., 2011), and aberrant high levels of IL-7 are characteristic for diseases with genetic or acquired lymphopenia, e.g. SCID and ALL (Bolotin et al., 1999).

Similarly, SOCS3 itself is a hallmark of many diseases. Due to its role in the development of T_h2 cells, it is associated with allergic diseases like atopic dermatitis

and asthma, with SOCS3 expression correlating with disease severity (Seki et al., 2003). For SOCS3, a mutant that is impaired in regulating EPO signalling has been reported in humans, but its effects and whether it causes a pathology have not been studied extensively (Suessmuth et al., 2009).

1.15.1 Interplay of SOCS3 and IL-7 signalling in infectious diseases

High SOCS3 expression and impaired IL-7 signalling have been linked to various infectious diseases and T cell exhaustion. Many bacterial and viral pathogens, such as LCMV or mycobacteria, trigger the expression of SOCS3 in myeloid or lymphoid cells, which hampers pro-inflammatory responses and subsequently pathogen clearance (Pellegrini et al., 2011, Harling et al., 2018, Carow and Rottenberg, 2014). In addition, persistent antigen exposure leads to suppressed IL-7Rα expression in cytotoxic T cells and is associated with T cell exhaustion in chronically LCMV infected mice (Lang et al., 2005). While augmentation of immune responses after pathogen clearance is important to prevent tissue damage (Matsumoto et al., 2003), SOCS3 expression in chronic infections contributes to the failure of pathogen clearance and T cell effector functionality (Pellegrini et al., 2011).

In a mouse model of chronic viral infection with LCMV, in which SOCS3 is highly expressed, administration of IL-7 promoted antiviral responses, facilitating pathogen clearance while limiting cytotoxic tissue destruction (Pellegrini et al., 2011). Importantly, IL-7 leads to a decrease in SOCS3 levels, which accounts for the increase in antiviral effector functions and pathogen-specific T cell numbers (Pellegrini et al., 2011). Furthermore, decreased SOCS3 expression leads to higher T_h17 cell numbers, and IL-22 production of these cells is responsible for limiting tissue destruction despite the ongoing immunological boost by IL-7 (Pellegrini et al., 2011).

Similarly, SOCS3 is highly expressed in T cells of tuberculosis (TB) patients (Jacobsen et al., 2011, Mistry et al., 2007, Harling et al., 2018), and this is accompanied by impaired T cell functions and an IL-10-mediated immunosuppressive phenotype (Harling et al., 2018, Lundtoft et al., 2017). Constitutive active STAT3 signalling is thought to be causative for high SOCS3 expression in TB patients, and this correlates with impaired IL-2 signalling and elevated plasma IL-7 levels (Harling et al., 2018, Lundtoft et al., 2017). TB patients show impaired IL-7Rα expression, and together with elevated plasma IL-7 levels,

this accounts for a decreased consumption and responsiveness of T cells towards IL-7 (Lundtoft et al., 2017). Concomitantly, IL-7 induced cytokine release as well as STAT5 activation are impaired in TB patients (Lundtoft et al., 2017). Constant exposure to antigen in latently infecting viral diseases has been proposed to be responsible for a loss of IL-7Rα expression (Alves et al., 2008). Furthermore, patients are impaired in T_h17 cell responses while producing elevated levels of IL-6 and IL-10 cytokines (Harling et al., 2018).

1.15.2 Interplay of SOCS3 and IL-7 signalling in AD-HIES

In contrast, autosomal-dominant hyper IgE syndrome (AD-HIES) patients carry dominant-negative hypomorphic mutations in the STAT3 gene (Holland et al., 2007, Renner et al., 2008), and naïve and central memory cells of AD-HIES patients show a decreased expression of STAT3 targets, among them SOCS3 (Siegel et al., 2011). These patients show skeletal and connective tissue abnormalities and are susceptible to recurrent bacterial and fungal infections, partly due to aberrant T and B cell development (Holland et al., 2007, Minegishi et al., 2007, Renner et al., 2008). Defects in T_h17 cell differentiation have been reported (Renner et al., 2008), and previous studies in our group emphasized the importance of SOCS3 in T_h17 maintenance (Kleinsteuber et al., 2012). Furthermore, it has been shown that AD-HIES patients have a decrease in the frequency of central memory T cells and an increase in the number of naïve T cells (Siegel et al., 2011). While their naïve T cells seem to be phenotypically and functionally intact, central memory T cells of AD-HIES patients show lower levels of IL-7Rα and a decreased upregulation of the memory marker CD45RO upon IL-7 treatment in T helper cells (Siegel et al., 2011). The authors of this study hypothesize that differences in effector responses together with decreased expression of STAT3 target genes and aberrant IL-7Rα expression cause an abnormal memory phenotype in these patients, which increases the susceptibility to reoccurring viral diseases (Siegel et al., 2011).

As in AD-HIES patients lacking functional STAT3, mice deficient of STAT3 in T cells show perturbed development of cytotoxic memory T cells with a shortened lifespan and respond poorly upon a secondary infection (Cui et al., 2011). Induction of SOCS3 expression upon cytokine signalling is impaired, and knockdown of SOCS3 in cytotoxic T cells leads to deficiencies in the transformation of cytotoxic T cells from an effector to a memory phenotype (Cui et al., 2011). Moreover, these memory

T cells are characterized by decreased IL-7R expression (Cui et al., 2011). Consequently, it seems plausible that some of the defects in the memory compartment of AD-HIES patients are rather an effect of impaired SOCS3 induction than being directly mediated by the lack of proper STAT3 signalling.

2 Research objectives

Proper regulation of T-cell activation and IL-7 signalling are crucial for pathogen clearance and memory formation, and therefore important hallmarks of a functional immune system. From the activation of a naïve T cell to fulfil its effector functions to the inhibition of T cell signalling and the development towards a memory T cell, the regulation of IL-7 signalling and SOCS3 expression are important mechanisms which are tightly regulated.

The overall aim of the study is to better understand the mechanisms underlying impaired SOCS3 or IL-7Rα expression in chronic infectious diseases or autoimmunity, which is of utmost interest for therapeutic implications. Therefore, to determine the effects of SOCS3 expression on IL-7Rα during T-cell activation in primary human T helper cells, the specific objectives of this study are:

- To characterize the expression of SOCS3 and IL-7Rα during the course of T-cell activation.
- To generate vectors for lentiviral modulation of SOCS3 expression and evaluation of their efficacy and specificity in a novel in vitro assay.
- To evaluate the effects of modulated SOCS3 expression in activated T helper cells on IL-2Rα, IL-7Rα and the common γ-chain.
- To determine the cytokine dependency of decreased IL-7Rα expression in SOCS3 knockdown ($SOCS3_{kd}$) T helper cells.
- To establish a novel competitive in vitro assay with concomitantly transduced T helper cells.
- To evaluate the competitive proliferative capacity of $SOCS3_{kd}$ T helper cells upon IL-7 stimulation, and IL-7 signal transduction measured by STAT5 activation.

3 Paper: Suppressor of cytokine signalling 3 is crucial for interleukin-7 receptor re-expression after T-cell activation and interleukin-7 dependent proliferation

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- in vitro experiments with untransduced primary T cells
- generation of vectors for lentiviral transduction
- vector testing in HEK293T cells for efficacy and sensitivity
- virus propagation and transduction of primary T cells
- analysis of transduced primary T cells
- FACS analyses
- Western blot analyses
- qPCR analyses
- data analyses
- writing of the manuscript

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Suppressor of cytokine signalling 3 is crucial for interleukin-7 receptor re-expression after T-cell activation and interleukin-7 dependent proliferation

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SOCS3 is a crucial feedback inhibitor of several cytokine pathways with potential regulatory functions during T cell receptor activation. A role of SOCS3 in IL-7-dependent homeostatic mechanisms has been assumed but the underlying mechanisms remain unclear. We investigated the role of SOCS3 in IL-7 receptor α-chain (IL-7Rα) expression and IL-7 effects on activated human CD4$^+$ T cells. SOCS3 expression modulation by lentiviral transduction combined with T cell phenotyping, receptor signalling analysis, and a novel competitive in vitro assay were applied. Time course analyses following T-cell activation showed IL-7Rα re-expression after initial down-regulation that was accompanied by increased SOCS3 expression starting on day 2. T cells with low SOCS3 expression (SOCS3$_{kd}$) had decreased IL-7Rα levels due to impaired re-expression. SOCS3 mediated effects on IL-7Rα were not affected by recombinant IL-7 or blocking of IL-2. We found no evidence for SOCS3 effects on *IL7RA* transcriptional regulation. Functionally, SOCS3$_{kd}$ T cells showed decreased IL-7-dependent proliferation as compared to vector control T cells under competitive in vitro conditions. This impaired IL-7 response of SOCS3$_{kd}$ T cells was accompanied by decreased STAT5 phosphorylation late during IL-7 signalling. We identified a novel SOCS3 function in IL-7Rα regulation during T-cell activation with crucial implications for IL-7-dependent mechanisms.

Keywords: interleukin-7 · interleukin-7 receptor α-chain · proliferation · SOCS3 · T-cell activation

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

IL-7 is a central factor for homeostatic T-cell proliferation and survival. Especially naïve and memory T cells depend on IL-7 and these subpopulations are characterised by high membrane-associated IL-7 receptor (IL-7R) α expression [1]. IL-7Rα expression is essential for IL-7 sensitivity and is tightly regulated [2]. Both, IL-7Rα and T cell receptor signals cause down-regulation of IL-7Rα expression via internalization and inhibition of mRNA transcription [1]. Rapid re-expression of the IL-7Rα occurs in T cells when IL-7 is removed after stimulation [3, 4], whereas contrary effects of T cell receptor activation on IL-7Rα re-expression have been described [4, 5]. The mechanisms influencing IL-7Rα expression during T-cell activation are biologically relevant, since IL-7Rα expression is central for

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generation of long-term protective T cell memory [6]. In accordance, impaired generation of T cell memory in chronic viral infections is accompanied by reduced IL-7Rα expression of T cells [7]. Previously, we found lower IL-7Rα expression accompanied by dysfunctional IL-7 mediated T cell responses in patients with acute tuberculosis [8].

SOCS3 is a major regulator of cytokine signalling with crucial functions during chronic infection and inflammation [9]. STAT phosphorylation, including STAT1, STAT3 [10], and STAT5 [11] is inhibited by SOCS3, which has high-affinity binding sites for JAK molecules and target receptors [12]. Inhibition of signal transduction and also degradation of bound receptors via ubiquitination are general functions of SOCS molecules with multifaceted effects on T cells. Amongst others, SOCS3 was shown to polarize T cell immunity [13] and to suppress T-cell proliferation by blocking IL-2R signalling [14, 15]. IL-2 induces proliferation during T-cell activation in a pSTAT5 dependent manner and SOCS3 acts as a feedback inhibitor of IL-2 signalling [11, 16]. T cell mediated functions of SOCS3 may not solely be exerted via regulation of cytokine signals, since also SOCS3 interference with T-cell activation has been shown [14, 17]. SOCS3 binds the T cell co-receptor CD28 with inhibitory effects on activation as well as proliferation. Competitive inhibition of CD28-dependent PI3K/Akt activation likely accounts for SOCS3 function [18, 19]. PI3K/Akt activation inhibits the transcription factor FoxO1, an important regulator of T-cell homeostasis and memory generation [20]. FoxO1 induces IL-7Rα gene expression crucial for IL-7-dependent T cell function [20]. Previous studies suggested SOCS3-mediated effects on IL-7 function during T-cell homeostasis [21] and $CD8^+$ memory T-cell development [22], in this regard transcriptional regulation of IL-7Rα via the PI3K/Akt pathway is a possible explanation.

The basic assumption of the present study was that SOCS3 has a significant role in IL-7Rα regulation and IL-7 mediated T cell functions. To address this question, we characterized SOCS3 and IL-7Rα expression after T-cell activation and applied lentiviral modulation of primary human $CD4^+$ T cells to characterize SOCS3 functions in vitro. Especially effects of decreased SOCS3 expression (SOCS3 knock-down; termed $SOCS3_{kd}$) were considered relevant since affected functions likely indicate the role of SOCS3 at physiological expression levels during T-cell activation. An in vitro competitive model of T cell survival was established to assess functional implications of low SOCS3 expression and IL-7 dependency.

Results

IL-7Rα re-expression is accompanied by increased SOCS3 levels after T-cell activation

IL-7Rα expression is tightly regulated and differs between T cell subpopulations. T cell subpopulation differences were also described for SOCS3 but only sparse data for the expression of both molecules during T-cell activation exist. Therefore, we initially determined SOCS3 and IL-7Rα expression during the time course of T-cell activation (i.e. CD3/CD28 stimulation). IL-7Rα mean expression decreased rapidly after T-cell activation in all donors (Fig. 1A) and this was reflected by lower T cell proportions characterized by high IL-7Rα expression levels (Supporting Information Fig. 1A and B). Considerable variability was seen for different donors concerning the time course of IL-7Rα re-expression. T cells from all donors, however, started to re-express IL-7Rα between d3 and d4 of T-cell activation and reached a plateau level until d10 (Fig. 1A; Supporting Information Fig. 1B). For SOCS3, a basal expression level was detected prior to T-cell activation (d0) and at any time point of $CD4^+$ T cells cultured without activation (Fig. 1B). After T-cell activation, markedly enhanced SOCS3 protein expression was found on d2 remaining at high levels until d7 before decreasing on d10 (Fig. 1B). Therefore, IL-7Rα re-expression was accompanied by increased SOCS3 expression of $CD4^+$ T cells suggesting promoting SOCS3 effects on IL-7Rα re-expression.

$SOCS3_{kd}$ $CD4^+$ T cells express lower IL-7Rα levels

We characterised the role of SOCS3 during $CD4^+$ T-cell activation by using lentiviral transduction with SOCS3 cDNA ($SOCS3_{over\text{-}expression}$, $SOCS3_{oe}$) or SOCS3 shRNA ($SOCS3_{knock\text{-}down}$, $SOCS3_{kd}$). Since SOCS3 has structural and functional similarity to SOCS1, we initially tested specificity and efficacy of SOCS3 shRNAs in HEK293T cells. shRNA75 was the most potent inhibitor of SOCS3 (about 86% reduction) and had only marginal effects on SOCS1 (Supporting Information Fig. 2). Next, we transduced $CD4^+$ T cells (i.e. $SOCS3_{kd}$, $SOCS3_{oe}$, vector control; Fig. 1C) and compared IL-7Rα, IL-2Rα, and common-γ chain expression. $SOCS3_{oe}$ T cells had moderately increased IL-7Rα expression ($p = 0.06$; Fig. 1D). $SOCS3_{kd}$ T cells, however, showed significantly lower IL-7Rα expression as compared to vector control T cells ($p = 0.0002$). IL-2 signalling is a well-known target of SOCS3 [14, 15] and, in accordance, $SOCS3_{kd}$ T cells showed higher IL-2Rα expression ($p = 0.0001$). $SOCS3_{oe}$ T cells did not show differences in IL-2Rα expression. In addition, no differences in common-γ chain expression were found for $SOCS3_{kd}$ or $SOCS3_{oe}$ T cells (Fig. 1D). These results indicated specific SOCS3 effects on IL-7Rα expression which were impaired in $SOCS3_{kd}$ T cells. Against this background, we focused on $SOCS3_{kd}$ T cells in the following experiments.

Impaired IL-7Rα re-expression after T-cell activation in $SOCS3_{kd}$ T cells is independent of IL-7

To further characterize IL-7Rα expression in $SOCS3_{kd}$ T cells, we performed time course experiments. Lentiviral transduction requires T-cell activation for two days and additional three days for optimal lentiviral expression [23]. Consequently, IL-7Rα expression was measured on day 5, 8, 12, and 14 in $SOCS3_{kd}$ and vector control transduced T cells (Fig. 2A). IL-7Rα expression increased until day 8 and remained stable thereafter (Fig. 2B; upper graph). Notably, $SOCS3_{kd}$ T cells showed lower IL-7Rα

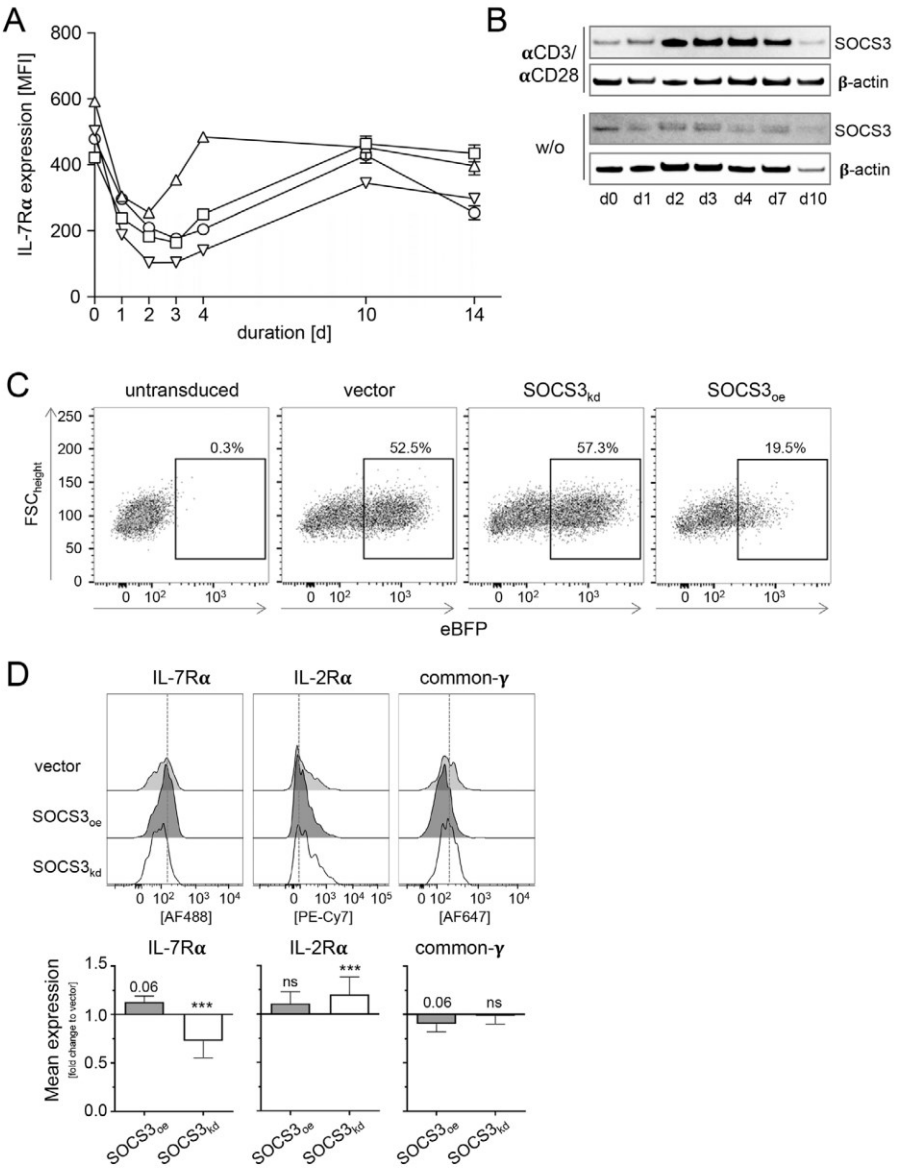

Figure 1. CD4$^+$ T cell IL-7Rα and SOCS3 expression are concomitantly regulated during T-cell activation and lentiviral SOCS3 modulation affects IL-7Rα expression. CD4$^+$ T cells activated with CD3/CD28 antibodies were analysed at different time points for IL-7Rα expression by flow cytometry (A) and for SOCS3 protein expression on western blots (B). (A) IL-7Rα membrane protein expression is indicated as MFI analysis. Different symbols indicate mean of duplicate measures from individual healthy donors. (B) For SOCS3 protein analyses also unstimulated (w/o) CD4$^+$ T cells are shown and β-actin bands were determined as loading controls for all samples. (C) Flow cytometry dot plots from a representative experiment show lentiviral marker (eBFP) expression of activated CD4$^+$ T cells transduced with a vector control (vector), SOCS3shRNA75 (SOCS3$_{kd}$), SOCS3cDNA (SOCS3$_{oe}$), or untransduced. (D) Representative flow cytometry histograms (upper graphs) and groups comparisons (lower graphs) of cytokine receptor chain (i.e. IL-7Rα, IL-2Rα, common-γ) mean expression on day 8 for SOCS3$_{kd}$, SOCS3$_{oe}$, and vector transduced T cells are shown. For group comparisons vector control MFI was set to 1. Wilcoxon signed-rank tests were performed and significant differences are indicated by asterisks (i.e. ***, $p < 0.001$). Repeated experiments have been done (SOCS3$_{kd}$: $n = 14$; SOCS3$_{oe}$: $n = 5$). ns: not significant; vector: empty vector control; d: day. n: number of experiments (equal to donor numbers).

mean expression during increase and at any time point as compared to vector controls (d5: $p = 0.004$; d8: $p = 0.0001$; d12: $p = 0.008$; d14: $p = 0.03$; Fig. 2B, lower graphs).

IL-7 is produced by nonimmune cells and largely absent from T cell in vitro culture. Since IL-7 is known to affect IL-7Rα expression, we next determined IL-7 effects on SOCS3 regulated IL-7Rα expression (Fig. 2C). IL-7Rα re-expression was even more pronounced in the presence of IL-7 and higher IL-7Rα plateau levels were reached (Fig. 2C; upper graph). As for non-IL-7 supplemented cultures, significant differences between SOCS3$_{kd}$ and

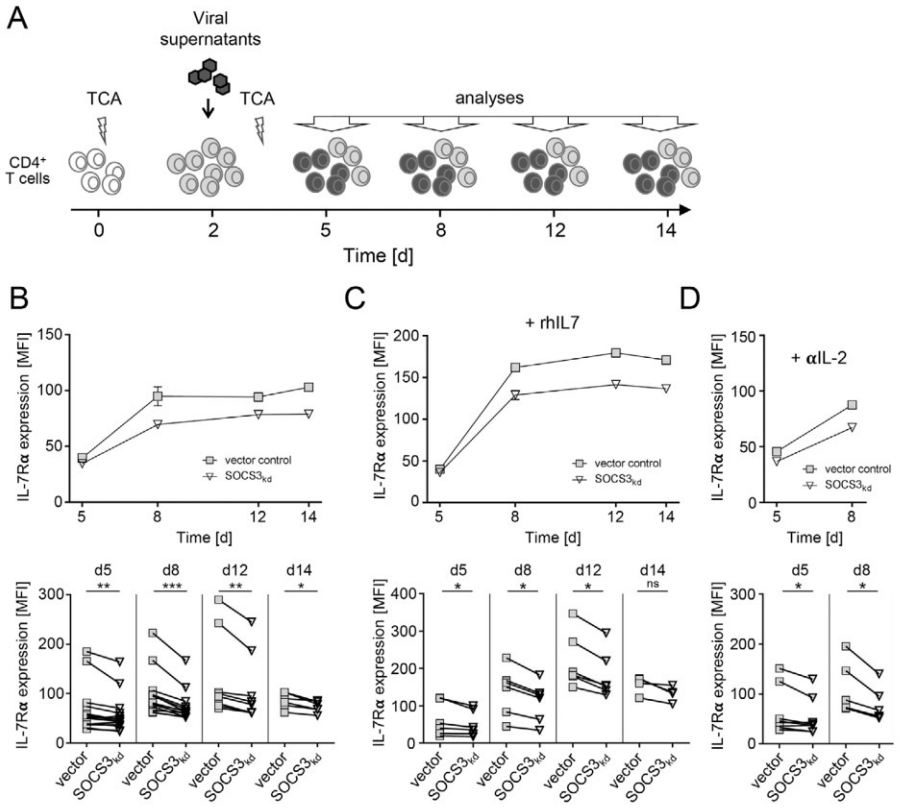

Figure 2. $SOCS3_{kd}$ effects on IL-7Rα expression at different time points after T-cell activation compared to vector control transduced T cells. (A) Schematic depiction of T-cell activation and lentiviral transduction procedure for time course experiments. (B, C, D) IL-7Rα mean expression of $SOCS3_{kd}$ and vector control transduced T cells in the absence of IL-7 (B), with IL-7 (C), and with IL-2 depleting antibodies (D). Upper graphs show representative experiments and lower graphs indicate comparisons between IL-7Rα mean expression of $SOCS3_{kd}$ and vector control for each time point. Wilcoxon signed-rank test analyses were performed and significant differences are indicated by asterisks (i.e. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Repeated experiments have been done (B: d5: $n = 14$, d8: $n = 14$, d12: $n = 8$, d14: $n = 6$; C: d5: $n = 6$, d8: $n = 6$, d12: $n = 6$, d14: $n = 4$; D: d5: $n = 8$, d8: $n = 6$). ns: not significant; vector: empty vector control; d: day. n: number of experiments (equal to donor numbers).

vector control T cells were detected also in the presence of IL-7 (d5: $p = 0.03$; d8: $p = 0.03$; d12: $p = 0.03$; Fig. 2C, lower graph).

IL-2 blocking does not affect IL-7Rα re-expression of $SOCS3_{kd}$ T cells

IL-2 has been shown to regulate IL-7Rα expression of T cells [24]. Since IL-2 is strongly produced by T cells early after activation, we next inhibited IL-2 signalling after T cell transduction by adding blocking aIL-2 antibodies to determine IL-2 effects on differential IL-7Rα regulation in $SOCS3_{kd}$ T cells. As expected, blocking of IL-2 markedly impaired proliferation of T cells (data not shown) and time course analyses could only be measured until d8 (Fig. 2D). However, IL-7Rα re-expression differences between $SOCS3_{kd}$ and vector control T cells were also found in the presence of IL-2 blocking antibodies (d5: $p = 0.04$; d8: $p = 0.03$; Fig. 2D). These results did not suggest IL-2-mediated mechanisms underlying $SOCS3_{kd}$ effects on IL-7Rα re-expression. Next, we investigated additional possible mechanisms focusing on described SOCS3 targets during T-cell activation.

No effects of $SOCS3_{kd}$ T cells on IL-7Rα transcription as well as Akt and FoxO1

T-cell activation requires T cell receptor and co-receptor signalling. SOCS3 has been shown to bind the co-stimulatory receptor CD28 causing inhibition of Akt phosphorylation [18]. Phosphorylated Akt interferes with IL-7Rα expression by inhibiting the IL-7Rα promoting transcription factor FoxO1 [25]. Therefore, we next determined Akt and FoxO1 activation of $SOCS3_{kd}$ and vector control T cells. Akt phosphorylation was similar between $SOCS3_{kd}$ and control T cells on day 5 and even lower in $SOCS3_{kd}$ T cells on day 8 ($p < 0.01$; Supporting Information Fig. 3A). No differences of Akt protein expression were seen in western blot analysis (Supporting Information Fig. 3B). In addition, FoxO1 and pFoxO1 levels were comparable between $SOCS3_{kd}$ T cells and vector controls (Supporting Information Fig. 3B). Since no explanatory differences of IL-7Rα transcriptional regulators, Akt and FoxO1, were identified in $SOCS3_{kd}$ T cells, we next performed qPCR analysis of IL-7Rα mRNA expression. No significant differences of IL-7Rα mRNA expression were found between $SOCS3_{kd}$ and vector control T cells

(Supporting Information Fig. 3C). $SOCS3_{kd}$ effects on transcription regulation could not be confirmed by these experiments.

$SOCS3_{kd}$ T cells had a competitive disadvantage in vitro exclusively in the presence of IL-7

Next, we addressed the question if decreased IL-7Rα expression of $SOCS3_{kd}$ $CD4^+$ T cells affected IL-7 sensitivity. We established an in vitro competitive assay based on concomitant transduction of $SOCS3_{kd}$ and vector control $CD4^+$ T cells (Fig. 3A). IL-7 was then added on day 5 or 8 to indicated samples (Fig. 3A). Results of a representative experiment are shown for T cell proportions (Fig. 3B) and absolute numbers (Supporting Information Fig. 4A). Without IL-7, $SOCS3_{kd}$ and vector control transduced T cells were similar in proportions at any time point (Fig. 3B), whereas numbers decreased at late time points (i.e. day 12/16; Supporting Information Fig. 4A). Addition of IL-7 increased T cell numbers on the following days as compared to non-IL-7-treated T cells (Supporting Information Fig. 4A). Notably, $SOCS3_{kd}$ transduced T cells responded less to IL-7 leading to decreased absolute numbers (Supporting Information Fig. 4A) and proportions (Fig. 3B). Normalized results from repeated experiment showed significantly reduced IL-7 responses for $SOCS3_{kd}$ T cells in absolute numbers (IL-7 on d5: $p = 0.002$; IL-7 on d8: $p = 0.008$; Supporting Information Fig. 4B) and proportions (IL-7 on d5: $p = 0.004$; IL-7 on d8: $p = 0.008$; Fig. 3C) as compared to vector control cells. These results indicated a competitive disadvantage of $SOCS3_{kd}$ T cells in the presence of IL-7 leading to lower T cell proportions as well as absolute numbers in culture.

Impaired IL-7 dependent proliferation of $CD4^+$ $SOCS3_{kd}$ T cells

IL-7 has well described effects on survival as well as proliferation of T cells. To investigate underlying mechanisms of $SOCS3_{kd}$ impaired T cell culture, we determined proliferation of $SOCS3_{kd}$ and vector-transduced T cells with or without IL-7. Since individual proliferation stages could not be analysed in transduced T cells, we compared populations of 'slow', 'medium', and 'fast' proliferating T cells between $SOCS3_{kd}$ and vector controls. IL-7 induced increased proportions of 'medium' and 'fast' proliferating T cells in all samples at both time points (Fig. 4A). However, $SOCS3_{kd}$ T cell showed generally impaired IL-7 effects on proliferation with lower 'medium' and 'fast' proliferating T cell proportions at both time points (i.e. day 8, day 12; Fig. 4A). These results indicated reduced IL-7 dependent proliferation as a consequence of $SOCS3_{kd}$ impaired IL-7Rα re-expression.

Decreased IL-7 induced STAT5 phosphorylation of $SOCS3_{kd}$ $CD4^+$ T cells

Finally, we investigated the mechanisms underlying IL-7-dependent impaired proliferation of $SOCS3_{kd}$ T cells. The JAK/STAT signalling pathway is central for IL-7 (as well as IL-2) signalling and mainly STAT5 phosphorylation is induced. $SOCS3_{oe}$ has been included since inhibitory effects on IL-2 signalling via STAT5 phosphorylation have been shown [11]. IL-7 induced highest pSTAT5 levels at 0.25 h and decreased after 2 h and 6 h (Fig. 4B). Similar results were obtained for IL-2 (data not shown). SOCS3 effects on cytokine-induced pSTAT5 were assessed by calculating the ratio of $SOCS3_{oe}$ or $SOCS_{kd}$ compared to the vector control from the respective experiment (Fig. 4C). $SOCS3_{oe}$ T cells showed significantly reduced pSTAT5 levels at any time point for IL-7 and IL-2 as compared to the vector control (Fig. 4C). Notably, we detected lower IL-7 induced pSTAT5 for $SOCS3_{kd}$ T cells after 2 h and 6 h ($p = 0.03$; Fig. 4C), whereas no differences were found for IL-2 in $SOCS3_{kd}$ and vector control T cells at any time point (Fig. 4C). These results suggested that $SOCS3_{kd}$ T cells showed impaired IL-7 response in functional assays due to lower IL-7-induced pSTAT5 at late time points.

Discussion

We report a novel role of SOCS3 in IL-7Rα regulation and functional implications for T-cell proliferation and survival. Reduced SOCS3 levels during T-cell activation impaired IL-7Rα re-expression after T-cell activation. Low IL-7Rα expression accompanied by low SOCS3 levels in $SOCS3_{kd}$ T cells resembled a phenotype found in T cells of patients with the autosomal dominant hyper IgE-Syndrome (AD-HIES) [26, 27]. AD-HIES patients have strongly impaired generation of $CD4^+$ and $CD8^+$ memory T cells leading to high susceptibility against recurrent infections [26, 27]. SOCS3 deficiency has also been shown previously to affect memory generation of $CD8^+$ T cells [22]. We assume that decreased IL-7Rα expression accompanied by decreased IL-7 induced STAT5 phosphorylation is causative for impaired $CD8^+$ T cell memory. These findings suggested that $SOCS3_{kd}$ effects on IL-7Rα regulation found in the present study are of central relevance for T cell memory development.

Fine balanced and strictly controlled regulation of IL-7Rα expression is crucial for both T-cell homeostasis and memory T-cell development [1]. IL-7Rα down-regulation after T cell receptor activation has been described by several studies [3, 4, 28, 29], and IL-7Rα re-expression is induced after pathogen clearance in memory T cell precursors of infected animals [28]. In accordance we detected initial down-regulation of IL-7Rα after T-cell activation followed by IL-7Rα re-expression of $CD4^+$ T cells at later time points. Previous in vitro studies also described initial down-regulation of IL-7Rα in $CD4^+$ and $CD8^+$ T cells on the protein and mRNA level [3, 4, 29]. These studies mainly focused on early hours and days of IL-7Rα regulation. Alves et al. detected higher IL-7Rα expression levels after 72 h but this was ignored because of increased isotype background [4]. We also found increasing background levels of IL-7Rα mainly after 7 and 10 days but subtraction of isotype control measures (i.e. mean, $IL\text{-}7R\alpha_{high}$ proportions) confirmed IL-7Rα re-expression after T cell receptor activation in vitro. We showed IL-7Rα re-expression was accompanied by increased SOCS3 expression starting on day 2. This finding was

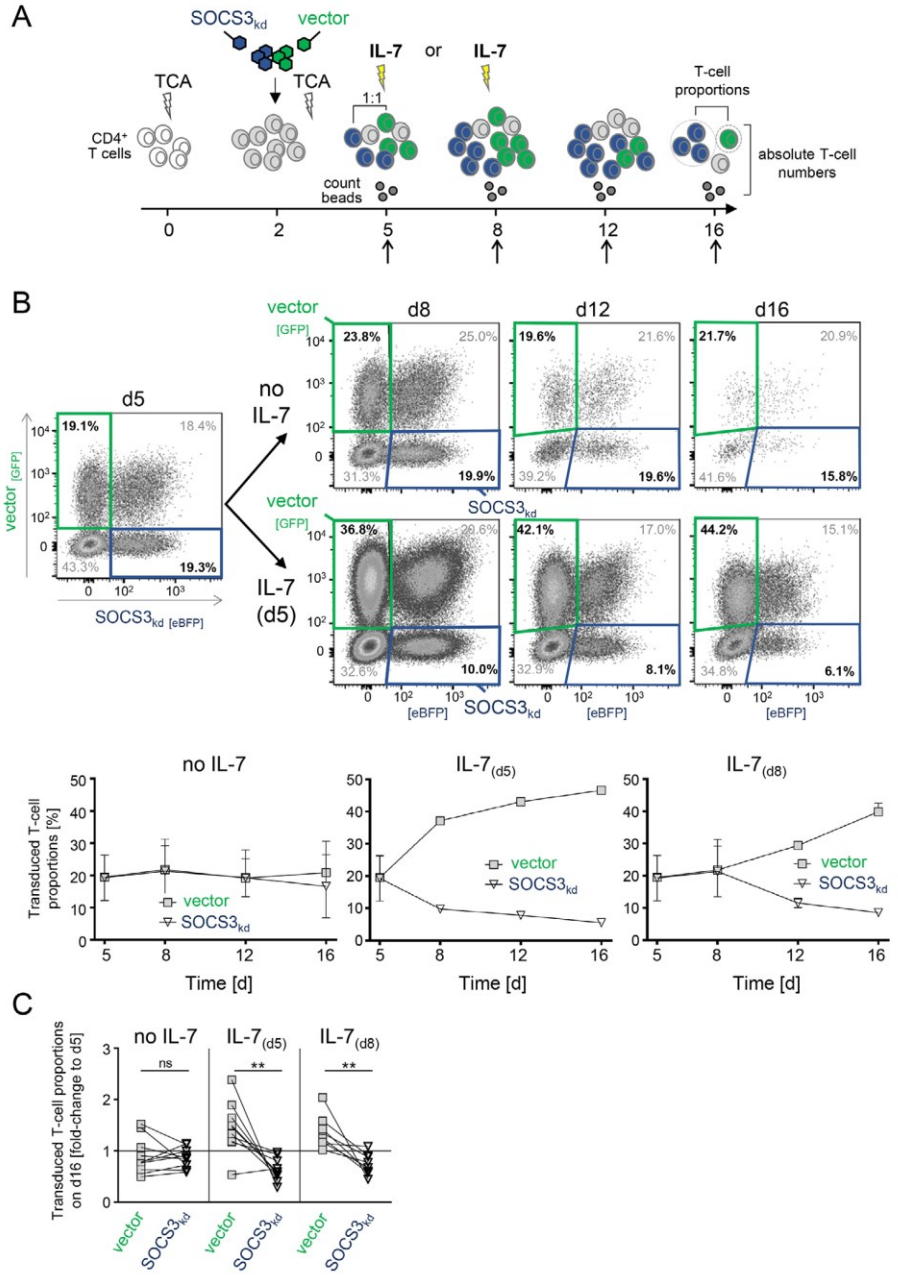

Figure 3. IL-7-dependent functional in vitro assay based on concomitant transduction of $SOCS3_{kd}$ and vector control positive $CD4^+$ T cells. (A) A schematic depiction of the assay procedure is shown. T cells were activated (i.e. aCD3/aCD28) on d0 and transduced with adjusted titres of vector control (green) and $SOCS3_{kd}$ (blue) on d2. After transduction (i.e. day 3) T cells received again CD3/CD28 stimulation. IL-7 was added to indicated samples either on day 5 or day 8. Arrows indicate time points of analyses. Normalization of absolute numbers was performed by adding counting beads to each sample at analysis. Differences in absolute numbers as well as proportions of marked $SOCS3_{kd}$ and vector control $CD4^+$ T cells were calculated for different time points. (B) Time course analyses of a representative experiment at indicated time points are shown as density dot plots (for IL-7 added on d5) and symbol graphs. T-cell proportions for $SOCS3_{kd}$ (lower right quadrants) and vector controls (upper left quadrants) are given for day 5, 8, 12, and 16 after T-cell activation. SOCS3kd (eBFP expression) is depicted on the x-axis, vector control (GFP expression) is depicted on the y-axis. Double transduced T cells (vector control and $SOCS3_{kd}$ positive; upper right quadrant) are not included in the graphs. Symbols indicate mean values of duplicates. (C) Proportions of transduced T cells on day 16 adjusted to respective vector control on d5 are shown for repeated experiments (no IL-7: n = 10; IL7 (d5): n = 10; IL-7 (d8): $n = 8$). Symbols indicate mean values of duplicates. Wilcoxon signed-rank tests were performed and significant differences are indicated by asterisks (i.e. **, $p < 0.01$). ns: not significant; vector: empty vector control; d: day; n: number of experiments (equal to donor numbers).

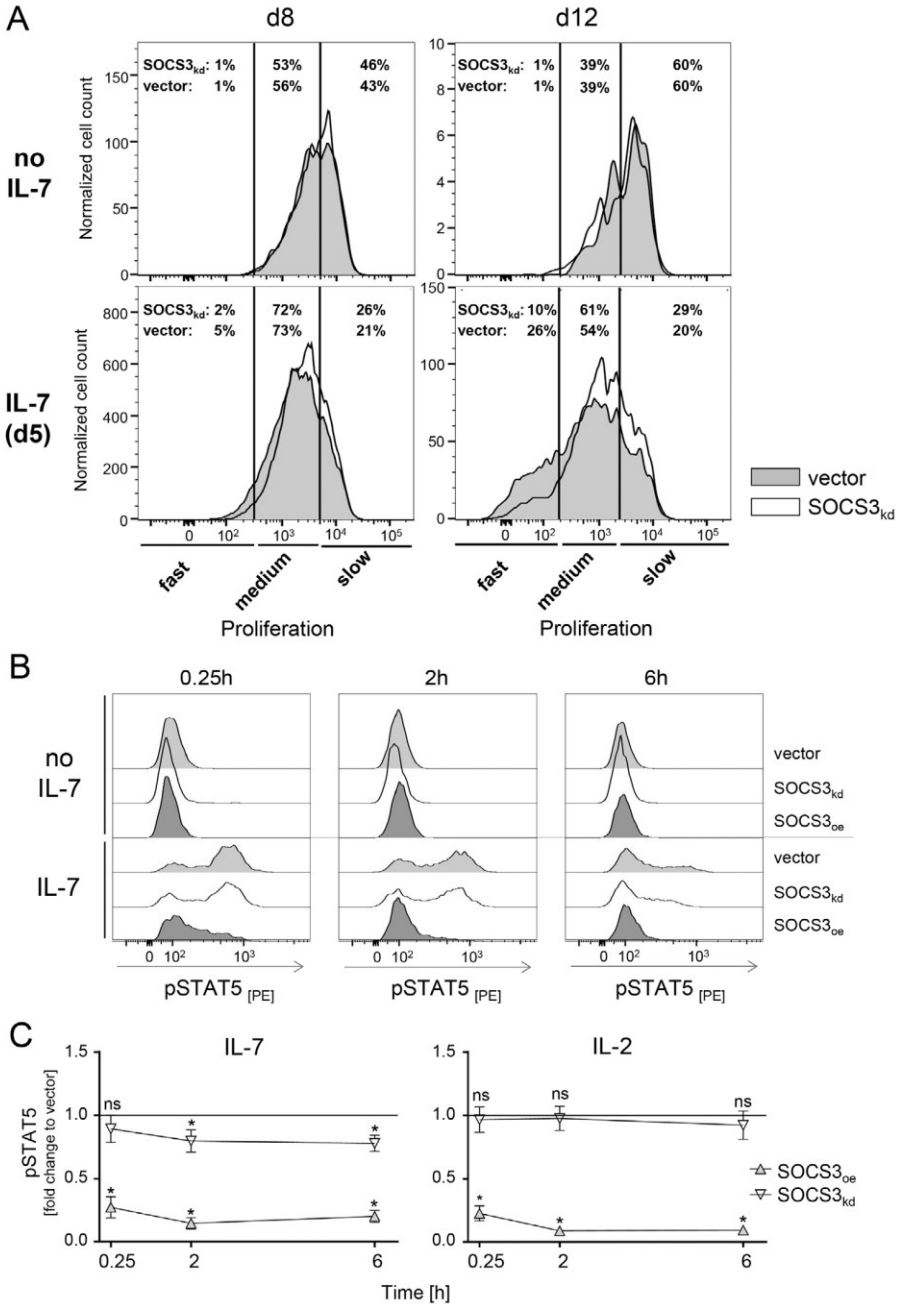

Figure 4. IL-7-dependent proliferation and STAT5 phosphorylation of transduced $SOCS3_{kd}$ or vector control expressing $CD4^+$ T cells. (A) Histogram plots show proliferation dye intensity for vector control (grey) and $SOCS3_{kd}$ (open) co-transduced T cells cultured with IL-7 (lower graphs) or without IL-7 (upper graphs). Percentages of proliferation dye high ('slow' proliferating), intermediate ('medium proliferating'), and low ('fast proliferating') T cells are given. (B, C) STAT5 phosphorylation induced by IL-7 or IL-2 in $CD4^+$ T cells transduced with $SOCS3_{kd}$, $SOCS3_{oe}$, or vector control for 15 min, 2 h, and 6 h are shown. MFI determined by flow cytometry for a representative experiment (B) as well as adjusted pSTAT5 expression values (relative to vector control MFI, set to 1) of repetitive experiments ($n = 6$) (C) are given. Wilcoxon signed-rank test analyses were performed and significant differences are indicated by asterisks (*, $p < 0.05$). ns: not significant; vector: empty vector control; d: day; PE: phycoerythrin; n: number of experiments (equal to donor numbers).

in accordance with a previous study [18] and strengthened our hypothesis that SOCS3 is functionally involved in the regulation of IL-7Rα re-expression.

Since T-cell activation is prerequisite for lentiviral transduction, we could not address the question if basal SOCS3 expression is also crucial for stable IL-7Rα levels in naïve and memory T cells in our model. However, the fact that $SOCS3_{kd}$ T cells have lower IL-7Rα plateau levels suggested a role of SOCS3 in promoting IL-7Rα expression beyond the stage of re-expression after T-cell activation.

IL-7 signalling affects IL-7Rα regulation on mRNA and protein levels [4]. We showed that SOCS3-mediated effects on IL-7Rα expression after T-cell activation are independent of IL-7. This is in accordance with a previous study, which showed that generation of IL-7Rα re-expressing memory $CD8^+$ T cells is independent from IL-7/IL-7Rα interaction [30]. T-cell activation effects on IL-7Rα expression have been characterised and underlying mechanisms centrally involve the transcription factor FoxO1 [20]. FoxO1 binds an *IL7RA* promotor enhancer and induces IL-7Rα transcription [20]. Several studies confirmed the important role of FoxO1 during establishment of memory and naïve T cell homing [20, 31]. Therefore, we determined FoxO1 expression and phosphorylation as well as its regulator Akt (target of SOCS3 and induced during T-cell activation) but did not find differences in $SOCS3_{kd}$ T cells. In accordance, only minor differences in IL-7Rα mRNA expression were seen in $SOCS3_{kd}$ T cells and this rendered an Akt/FoxO1 independent pathway or posttranscriptional SOCS3 effects on IL-7Rα expression likely.

The performed competitive T cell assays indicated that impaired response to IL-7 of $SOCS3_{kd}$ T cells caused decreased proliferation. Against the background of the IL-7 mediated central roles in homeostatic proliferation and memory T-cell generation, we assume that both functions may be affected. Evidence for low IL-7Rα expression effects on decreased homeostatic proliferation were found for cell-division cycle (cdc)42, a described regulator of IL-7Rα expression [32]. Guo et al. showed that cdc42 knock-out led to a partial decrease of IL-7Rα expression and a marked reduction of naïve T cells [32]. As for $SOCS3_{kd}$ T cells in the present study, decreased IL-7Rα expression was moderate in $CD4^+$ T cells demonstrating that already minor reduction of IL-7Rα expression has severe consequences for T-cell homeostasis [32]. As mentioned above, T-cell activation was a prerequisite for lentiviral transduction and, therefore, effects of low SOCS3 expression on naïve T-cell homeostasis cannot be investigated. Animal experiments with conditional SOCS3 knock-out T cells, however, may address the question if low SOCS3 levels impair homeostatic proliferation of naïve and/or memory T cells.

Evidence for a role of SOCS3 regulated cytokine signalling pathway during $CD8^+$ T cell memory generation was found in chronic infections [22]. Cui et al. demonstrated a critical role of the IL-10/IL-21/STAT3 pathway for the generation of memory $CD8^+$ T cells against acute LCMV infection [22]. $CD8^+$ T cells deficient for SOCS3 were impaired in memory generation and had decreased proportions of $IL\text{-}7R\alpha_{high}$ memory T cells [22]. Notably, also aberrant high SOCS3 expression—a feature of T cell exhaustion in chronic infections—impaired T cell functions and memory generation [33]. IL-7 was capable of reverting T cell exhaustion in chronic viral infection by inhibiting FoxO1, which is also essential for transcription of SOCS3 expression [34]. Altogether these studies indicate a complex interaction between SOCS3 and IL-7-dependent T cell functions. Aberrant high as well as low SOCS3 expression may impair IL-7 sensitivity by affecting IL-7Rα expression.

The mechanisms underlying $SOCS3_{kd}$ effects on IL-7Rα re-expression after T-cell activation could not be identified as part of this study. However, we provided evidence based on concomitant regulation of SOCS3/IL-7Rα expression during T-cell activation and impaired IL-7Rα re-expression in $SOCS3_{kd}$ T cells that SOCS3 plays a role in IL-7Rα regulation. Importantly, we found functional effects on IL-7 sensitivity leading to lower pSTAT5 levels of $SOCS3_{kd}$ T cells at late time points of IL-7 stimulation. Since no $SOCS3_{kd}$ effects were found for IL-2, SOCS3 specific effects on IL-7Rα expression were likely causative. $SOCS3_{oe}$ inhibited both IL-2 and IL-7 induced pSTAT5 but this may be partially explained by unspecific SOCS3 effects at artificial high levels (induced by lentiviral transduction) since no direct interaction of SOCS3 with the IL-7Rα chain has been described.

Initial experiments on IL-7Rα regulatory pathways did not suggest SOCS3 effects on transcriptional regulation and this narrows potential targets for future studies on relevant mechanisms.

Material and methods

Donor recruitment

Healthy adult blood donors were recruited from the staff of the University Children's Hospital at the Heinrich-Heine University, Duesseldorf. All donors gave written informed consent. The study was approved by the local ethics committee (ID5445).

$CD4^+$ T-cell purification and measure of IL-2Rα and IL-7Rα after T-cell activation in vitro

PBMCs were isolated from heparinized whole blood by density centrifugation (Biocoll Separating Solution, Biochrom) according to manufacturer's instructions. Enriched $CD4^+$ T cells were purified of PBMCs by magnetic cell sorting (anti-human CD4 particles, IMag, BD Biosciences) as described previously [35]. An enrichment purity >95% was confirmed by flow cytometry and 1.5×10^5 $CD4^+$ T cells/well were cultured in 96-well round bottom plates using X-vivo15 medium (200 µL/well, Lonza) supplemented with Penicillin (100 U/mL)/Streptomycin (100 µg/mL) (Sigma-Aldrich) at 37°C and 5% CO_2 for different in vitro assays. For T-cell activation, we added aCD3 (1 µg/mL, clone Okt-3, BioLegend) and aCD28 (1 µg/mL, clone CD28.2, BioLegend). Cytokine receptor measures were performed during the time course after T-cell activation. IL-2Rα and IL-7Rα expression was determined ex vivo and on day 1, 2, 3, 4, 10,

14 after activation using monoclonal antibodies aIL-2Rα (0.1 μL, clone 2A3, PE-Cy7, BD), aIL-7Rα (0.5 μL, clone A019D5, AF488, BioLegend), and viability dye eF780 (eBioscience). An IgG1 isotype control (Clone MOPC-21, AF488, BioLegend) has been used to determine nonspecific binding of the IL-7Rα antibody. IL-2Rα receptor measures were included to define activated T cells. IL-7Rα MFI as well as proportions of IL-7Rα_{high} CD4$^+$ T cells were determined. For the latter an arbitrary threshold was set according to isotype control stained CD4$^+$ T cells. Measurements were done using an LSR Fortessa (equipped with blue, red, violet, and yellow/green lasers, BD Biosciences). Data processing was performed using FlowJo software (FlowJo v10, LLC). We adhered to the 'Guidelines for the use of flow cytometry and cell sorting in immunological studies'. A representative experiment to indicate the gating procedure is shown as Supporting Information Fig. 1A.

SOCS3 shRNA/cDNA plasmids for lentiviral T cell transduction tested in HEK293T cells

For shRNA specificity and efficacy testing, five commercially available shRNA candidates for SOCS3 (SHCLNG-NM_003955; Sigma-Aldrich) were transferred into a GFP containing Lentiviral Gene Ontology plasmid (LeGO, kindly provided by B. Fehse, University Hospital Eppendorf, Hamburg, Germany) as described [23]. SOCS3 cDNA containing plasmids (eBFP) were co-transfected with shRNA plasmids or empty GFP-containing vector constructs into HEK293T cells. Transfections were performed as described [36]. In brief, 1.5×10^4 HEK293T cells were co-transfected with 100 ng of both plasmids using the Calcium Phosphate Transfection Kit (Sigma-Aldrich). DMEM medium was changed again 6 h after transfection. SOCS1 was used as a specificity control for SOCS3 shRNAs. After culture (i.e. 24h) HEK293T cells were measured by flow cytometry. shRNA candidate effects on SOCS3 cDNA were calculated as MFI differences compared to SOCS3 cDNA plus empty GFP vector. A representative example of flow cytometry histograms for SOCS3 cDNA transfected HEK293T cells with or without SOCS3 shRNA75 is shown as Supporting Information Fig. 2. For T-cell transduction, shRNA75 was cloned into an eBFP containing LeGO vector.

Lentiviral transduction of CD4$^+$ T cells and in vitro time course experiments

Transduction of primary CD4$^+$ T cells was performed as described [23] with minor modifications. In brief, T cell activating antibodies were added on day 0 (i.e. aCD3, 3 μg/mL; aCD28, 5 μg/mL) and lentiviral transduction was done on day 2. Results shown in Fig. 1C and D were determined on day 8 of culture (six days after transduction). For time course experiments including time points beyond day 8, a second stimulation with T cell activating antibodies was done (i.e. aCD3, 1 μg/mL; aCD28, 1 μg/mL) on day 3 (see Fig. 2A and 3A). Optimal expression of lentiviral plasmids in primary T cells is detected after 3 days [23], and, therefore, day 5 after T-cell activation was selected as the first time point for analysis. SOCS3 shRNA75 transduced T cells were termed SOCS3$_{kd}$, SOCS3 cDNA transduced T cells were termed SOCS3$_{oe}$ T cells throughout this manuscript. T cells transduced with an empty vector construct (termed vector control) have been included for comparison in all experiments.

Modulation of transduced T cell culture was performed by adding aIL-2 inhibitory antibodies (2 μg/mL, clone 5334, R&D Systems) on day 3. Human recombinant IL-7 (10 ng/mL, Sigma-Aldrich) was added together with aCD3/aCD28 or at the indicated time points.

Flow cytometric, quantitative PCR, and western blot analyses of transduced T cells

For flow cytometry, transduced T cells (i.e. SOCS3$_{oe}$, SOCS3$_{kd}$, vector control) have been analysed for mean expression of cytokine receptors. Akt phosphorylation was determined in SOCS3$_{kd}$ and vector control cells. Cytokine receptor staining was done using monoclonal antibodies against IL-2Rα (0.1 μL, clone 2A3, PE-Cy7, BD), IL-7Rα (0.5 μL, clone A019D5, AF488, BioLegend), common-γ chain (0.5 μL, clone TUGh4, AF647, BD), and viability dye eF780 (eBioscience). For Akt phosphorylation, T cells were harvested on day 5/8 and were measured without restimulation. Staining included antibodies against anti-human CD4 (0.5 μL, clone RPA-T4, PE-Cy7, BioLegend) and pAkt1 (0.5 μL, clone SDRNR, PE, eBioscience). Flow cytometry measures and analyses were performed as described above.

IL-7Rα qPCR analysis was done as described [8]. In brief, mRNA was isolated from SOCS3$_{kd}$ and vector control transduced CD4$^+$ T cells (NucleoSpin RNA, Macherey-Nagel) and was reverse transcribed to cDNA. The QuantiTect SYBR Green PCR kit (Qiagen) was used for qPCR quantification and GAPDH was determined as a housekeeping gene. Data from duplicate reactions was evaluated using the $2^{-\Delta\Delta Ct}$ method.

For western blots, SOCS3$_{kd}$ and vector control transduced T cells from the indicated time points were lysed in CelLytic Lysis/Extraction Reagent (Sigma-Aldrich) including phosphatase inhibitors P8340 and P0044 (Sigma-Aldrich) according to manufacturer's instructions. Proteins were denatured for 10 min at 80°C, separated using a 4–12% NuPAGE Novex Bis-Tris Gel (Life Technologies) and subsequently blotted on a nitrocellulose membrane. After blocking (1x TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature primary antibody incubation was performed over night at 4°C with rabbit monoclonal antibody against FoxO1 (1:1000, clone C29H4, Cell Signalling), rabbit polyclonal antibodies against pFoxO1 (1:1000, #9461, Cell Signalling), and mouse monoclonal antibodies against Akt (1:2000, clone 40D4, Cell Signalling) and β-actin (1:5000, clone AC-15, Sigma-Aldrich). Detection of proteins was carried out using BM Chemiluminescence Blotting Substrate (Roche Diagnostics) after incubation with secondary antibodies for 1 h at room temperature.

SOCS3 western blot analyses were done in untransduced T cells using rabbit polyclonal antibodies against SOCS3 (1:1000, ab16030, Abcam) following the above described procedure.

In vitro competitive T cell survival assay

To analyse functional effects of reduced SOCS3 and IL-7Rα expression, we established a novel in vitro assay based on concomitant transduction with lentiviruses containing SOCS3 shRNA ($SOCS3_{kd}$) and an empty construct (vector control) labelled with different fluorescent molecules, i.e. eBFP and GFP. Adjustment of added lentiviral supernatant volumes (according to single transduction experiments) led to comparable proportions of single-transduced $CD4^+$ T cells (Fig. 3; Supporting Information Fig. 4). The experimental scheme and representative FACS-plots are depicted in Fig. 3A and B, and Supporting Information Fig. 4A. Measures were performed on d5, d8, d12, and d16 after T-cell activation. IL-7 was added on d5 or d8 to a subset of indicated samples. Absolute numbers have been calculated by normalizing transduced T cell numbers to counting beads (10 μL; 123 count eBeads, Thermo Fischer Scientific) added before measurement. For comparison of day 16, T cell numbers between different experiments, we normalized the initial cell count of transduced T cells to 10^4. Equal proportions of single transduced T cells at start allow direct comparison of population changes in culture. For comparison of different experiments, proportions were set to 1 and relative changes are depicted.

T-cell proliferation assay of concomitantly transduced T cells

Concomitantly transduced $SOCS3_{kd}$ and vector control T cells were stained on day 5 with Cell Proliferation Dye eFluor 670 (eBioscience) at a concentration of 2 μM and re-seeded in 96-well plates afterwards. IL-7 was added on d5 to the subset of indicated samples. Flow cytometry measures were performed on d8 and d12 after T-cell activation as described before. For quantification, we gated Cell Proliferation Dye high ('slow proliferating'), intermediate ('medium proliferating'), low ('fast proliferating') T cell proportions. These subsets were classified based on the respective vector control samples without IL-7 for each time point. We used this strategy since identification of individual proliferation cycles was not possible for lentivirus transduced T cells. Cell numbers were adjusted for illustration using the FlowJo Plugin Tool 'DownSample'.

IL-7 and IL-2 induced STAT5 phosphorylation of transduced T cells

$CD4^+$ T cells transduced either with $SOCS3_{oe}$, $SOCS3_{kd}$, or vector control were analysed on day 5. STAT5 phosphorylation was done as described [33] with minor modifications. In brief, cells were incubated in X-vivo15 medium for 15 min, 2 h, and 6 h with human recombinant IL-7 (10 ng/mL, Sigma-Aldrich), IL-2 (25 U/mL, BioLegend) or without cytokines at 37°C and 5% CO_2. The cells were then permeabilised and fixed before staining with anti-human CD4 (0.5 μL, clone RPA-T4, PE-Cy7, BioLegend) and pSTAT5 (0.5 μL, clone SRBCZX, PE, eBioscience). Thereafter, flow cytometry analyses of $CD4^+$ T cell MFI was measured as described above.

Statistical analyses

GraphPad Prism (Version 7.04, GraphPad Software) was used for statistical analyses. Because of moderate numbers of experimental values, nonparametric distributions were assumed and the nonparametric Wilcoxon Rank Sum test was used. *p*-values below 0.05 were considered statistically significant.

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Author contributions: A.G. performed experiments, analysed data, and wrote the paper. M.L.V. and E.A. performed experiments and analysed the data. E.M. and N.N. contributed to the study design and wrote the paper. M.J. designed the study, analysed the data, and wrote the paper.

Conflict of interest: The authors declare no commercial or financial conflict of interests.

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Abbreviation: **IL-7Rα**: IL-7 receptor α-chain

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4 Discussion

The role of SOCS3 in the regulation of IL-7Rα re-expression during T-cell activation in primary human T helper cells was investigated in this study. SOCS3 was expressed concomitantly with the re-expression of the IL-7Rα during T-cell activation. Targeting SOCS3 expression with a small-hairpin RNA against SOCS3 by lentiviral transduction of activated primary human T cells revealed impaired re-expression of the IL-7Rα in $SOCS3_{kd}$ T helper cells, independently from signalling by IL-2 and IL-7. Gene modulation by lentiviral transduction was an appropriate method for this study, as lentiviral transduction needs prior T-cell activation, and knockdown of SOCS3 by this method inhibits SOCS3 expression around the time of SOCS3 and IL-7Rα re-expression. In concomitantly transduced T helper cell cultures, in which control transduced and $SOCS3_{kd}$ T helper cells compete for available factors, $SOCS3_{kd}$ T helper cells were impaired in proliferation in the presence of IL-7 due to decreased STAT5 activation over the course of time. Therefore, lower IL-7Rα expression impaired the responsiveness of T helper cells towards IL-7 stimulation. IL-7Rα mRNA expression, as well as its regulators Akt and FoxO1, were not affected by $SOCS3_{kd}$. Nevertheless, the effects of $SOCS3_{kd}$ on T helper cells provide evidence for a role of SOCS3 in the regulation of IL-7Rα expression during T-cell activation (Guler et al., 2019).

4.1 Interdependencies of SOCS3 and IL-7Rα expression during T-cell activation

4.1.1 Expression of SOCS3 and IL-7Rα in naïve T cells

IL-7Rα and SOCS3 were expressed in non-activated T cells (Guler et al., 2019), a finding which has been described by others previously (Dooms et al., 2007, Matsumoto et al., 2003). While the importance of IL-7Rα expression in naïve T cells is well established (Tan et al., 2001, Schluns et al., 2000, Kerdiles et al., 2009), the role of SOCS3 was not extensively studied. SOCS3 is known to regulate signalling by the TCR (Masuhara et al., 1997, Banerjee et al., 2002), its co-receptor CD28 (Matsumoto et al., 2003, Owaki et al., 2006), and IL-2 (Cohney et al., 1999). Consequently, a role for SOCS3 in maintaining T cells in a quiescent state has been proposed (Yu et al., 2003, Li et al., 2017).

SOCS3 might also play a role in maintaining IL-7Rα expression in these cells. T-cell activation as well as IL-2 signalling lead to the downregulation of IL-7Rα (Xue et al.,

2002, Dooms et al., 2007), and therefore, by controlling T-cell activation and the responsiveness towards IL-2, SOCS3 expression in naïve T cells could potentially be important in stabilizing IL-7Rα expression. Indeed, in our experiments ectopic overexpression of SOCS3 ($SOCS3_{oe}$) during T-cell activation led to a tendency towards higher IL-7Rα expression levels (Guler et al., 2019).

Similarly, the expression and maintenance of IL-7Rα is fairly well studied (Kerdiles et al., 2009), while it remains to be investigated how SOCS3 expression is induced and maintained in naïve T cells. Gfi-1 is a negative regulator of SOCS3 and FoxO1 (Shi et al., 2017, Jegalian and Wu, 2002), and FoxO1 is positively influencing the expression of IL-7Rα (Ouyang et al., 2009). Gfi-1 is downregulated in mature thymocytes, leading to the expression of FoxO1 and the IL-7Rα (Shi et al., 2017). The lack of Gfi-1 expression in resting T cells might be an important mechanism to prevent cell cycle progression while promoting IL-7Rα expression and regulating T cell signalling by allowing SOCS3 to be expressed.

4.1.2 SOCS3 and IL-7Rα are downregulated upon T-cell activation

SOCS3 expression did not show significant changes one day after T-cell activation (Guler et al., 2019). Nevertheless, downregulation of SOCS3 upon T-cell activation has been described by others (Yu et al., 2003, Matsumoto et al., 2003), and stronger T-cell activation with more rapid IL-2-mediated SOCS3 induction could potentially account for the differences in our in vitro model. Additional measurements within the first 2 days after T-cell activation are likely necessary to detect downregulated expression of SOCS3 early after T-cell activation. However, the mechanisms controlling the downregulation of SOCS3 remain vastly elusive. The antigen-concentration dependent manner of SOCS3 downregulation narrows down potential targets to pathways activated by the TCR (Yu et al., 2003). Similar to the downregulation of SOCS3, upregulation of the common regulator of SOCS3 and FoxO1 expression, Gfi-1 (Shi et al., 2017, Jegalian and Wu, 2002), is antigen-concentration dependent (Yu et al., 2003). Further studies are required to determine if SOCS3 is solely regulated by this mechanism, or if other pathways synergize in the downregulation of SOCS3 expression.

We showed that IL-7Rα expression was downregulated for two to three days after T-cell activation (Guler et al., 2019). IL-7Rα downregulation upon T-cell activation and T-cell activation-induced IL-2 signalling in a PI3K/Akt-dependent manner have

been well described (Fabre et al., 2005, Kerdiles et al., 2009, Xue et al., 2002), the latter being promoted by the downregulation of SOCS3 (Yu et al., 2003). In addition, Gfi-1 is upregulated upon T-cell activation (Karsunky et al., 2002), and IL-7Rα suppression is known to be dependent on new protein synthesis (Alves et al., 2008). Xue et. al further show that the initial downregulation of the IL-7Rα during T-cell activation is independent of IL-2 signalling (Xue et al., 2002). This is reasonable, as T-cell activation-induced PI3K/Akt signalling precedes the induction of IL-2 signalling in activated T cells and seems to be sufficient for its downregulation (Prasad et al., 1994, Truitt et al., 1994, Owaki et al., 2006, Xue et al., 2002).

4.1.3 SOCS3 and IL-7Rα are re-expressed after initial downregulation

We showed that SOCS3 was highly expressed two days after T-cell activation and preceded IL-7Rα re-expression, which was initiated between three to four days after T-cell activation (Guler et al., 2019). Similar expression patterns have been described for SOCS3 or IL-7Rα expression (Matsumoto et al., 2003, Dooms et al., 2007).

Expression of SOCS3 might be mainly driven by IL-2-induced STAT3 activation (Lin et al., 1995, Cohney et al., 1999), and further promoted by STAT5-mediated inhibition of Gfi-1 (Jegalian and Wu, 2002). The latter mechanism has been described for EPO signalling, but its relevance in T-cell activation-induced IL-2 signalling needs to be proven. Nonetheless, IL-2-mediated STAT5 activation and feedback regulation by SOCS3 expression have been described (Cohney et al., 1999).

Several T-cell activation-induced pathways are worth being considered in the regulation of IL-7Rα re-expression. Downregulation of Gfi-1 upon IL-2-induced STAT5 signalling is one possible mechanism for decreasing suppressive effects on the re-expression of IL-7Rα (Jegalian and Wu, 2002, Shi et al., 2017). In addition, as described above, the regulation of co-receptor CD28 and the associated inhibition of the PI3K/Akt signalling as well as inhibition of IL-2 signalling by SOCS3 are likely obligatory requirements for the upregulation of the IL-7Rα (Matsumoto et al., 2003, Cohney et al., 1999, Xue et al., 2002). Indeed, it has been shown that during the presence of IL-2 signalling, IL-7Rα expression is blocked in activated T cells (Xue et al., 2002). This mechanism is promoted by the fact that SOCS3 expression preceded IL-7Rα re-expression in our experiments, and ectopic

expression of SOCS3 in T cells after T-cell activation showed higher IL-7Rα expression by tendency (Guler et al., 2019). In addition, while ectopic expression of SOCS3 did not change IL-2Rα expression, probably due to abundant physiological SOCS3 expression after T-cell activation, knockdown of SOCS3 caused higher IL-2Rα expression in T cells eight days after T-cell activation, and this was accompanied by lower levels of IL-7Rα expression (Guler et al., 2019).
Therefore, although initial downregulation of the IL-7Rα does not depend on IL-2 signalling (Xue et al., 2002, Dooms et al., 2007), IL-2 signalling rather than T cell signalling might suppress IL-7Rα re-expression in the late stages of T-cell activation. Evidence for this comes from the fact that T-cell activation-induced PI3K/Akt signalling in cell lines starts to decline approximately 15 minutes after stimulation (Prasad et al., 1994, Truitt et al., 1994).

4.1.4 IL-7Rα re-expression is decreased in $SOCS3_{kd}$ T cells

$SOCS3_{kd}$ in T helper cells led to a decrease in the re-expression of IL-7Rα after initial downregulation by T-cell activation and to lower plateau expression levels throughout the course of measurement compared to control transduced T cells (Guler et al., 2019). Similarly, STAT3 deficiency has been described to cause decreased expression of SOCS3 and IL-7Rα in AD-HIES patients (Siegel et al., 2011), and knockout of SOCS3 in murine T cells was associated with impaired T cell memory development and IL-7Rα expression in these cells (Cui et al., 2011). Nevertheless, to our knowledge, a direct link between decreased SOCS3 and impaired IL-7Rα re-expression after T-cell activation has not been described previously.

We could not find higher levels of activated Akt and FoxO1 proteins in $SOCS3_{kd}$ T helper cells five and eight days after T-cell activation, with differences in IL-7Rα expression starting to become or being already evident, respectively (Guler et al., 2019). In accordance, we could not determine differences in IL-7Rα mRNA expression eight days after T-cell activation in these cells (Guler et al., 2019). Similarly, Alves et. al do not report significant differences in the expression of Gfi-1 upon T-cell activation (Alves et al., 2008). Nevertheless, Yu et. al show a narrow time window of two days with effects of IL-2 signalling being most prominent and preceding the re-expression of SOCS3 (Yu et al., 2003). It is possible that initial signalling by IL-2 and subsequent inhibition by SOCS3 at the early stages of T-cell

activation is sufficient to initiate mechanisms which lead to the re-expression of the IL-7Rα. In accordance with this, we did not see a comparable decrease in the re-expression of IL-7Rα in control cells when adding IL-2 blocking antibodies three days after T-cell activation (Guler et al., 2019), while IL-2 signalling deficient T helper cells completely fail to re-express IL-7Rα after T-cell activation (Dooms et al., 2007). In this regard, our experimental approach is limited in determining effects which occur during the early days of T-cell activation, and it is possible that mechanisms are initiated before lentiviral knockdown of SOCS3 reaches its full efficacy at around five days after T-cell activation.

Nevertheless, the lower re-expression and plateau levels of IL-7Rα in $SOCS3_{kd}$ T helper cells underlined stabilizing functions of SOCS3 in IL-7Rα expression, regardless of mechanisms initiating the re-expression itself (Guler et al., 2019). Additional IL-2-dependent pathways, as discussed above, could potentially synergize in the re-expression of the receptor. It has been shown that IL-2 provides signals necessary for the re-expression of IL-7Rα in activated T cells (Dooms et al., 2007), and it seems highly probable that this is an effect of IL-2/STAT3 mediated SOCS3 induction (Guler et al., 2019, Cui et al., 2011, Siegel et al., 2011), which synergizes with IL-2/STAT5 mediated suppression of Gfi-1 (Jegalian and Wu, 2002). Therefore, Gfi-1/SOCS3 double-knockout T cells would be an interesting approach in determining if additional IL-2-mediated effects synergize in the re-expression of IL-7Rα.

This study could not resolve the pathway leading to a decrease of IL-7Rα expression in $SOCS3_{kd}$ T helper cells. It remains to be investigated if SOCS3 is directly involved in pathways which regulate IL-7Rα expression, or rather exerts its promoting effect on IL-7Rα expression by inhibiting IL-2 and T-cell activation-mediated suppression of its expression, the latter being pronounced by the fact that T helper cells benefit from the absence of MHC signalling during memory transition (Li et al., 2003).

Whether other pathways synergize in the downregulation of the IL-7R or can even partly compensate for a loss of SOCS3 remains to be investigated. SOCS1 inhibits signalling by the common γ-chain, and signal inhibition has been shown for various common γ-chain cytokines, whereas SOCS1 deficiency leads to hypersensitivity towards these cytokines, including IL-2 and IL-7 (Fletcher and Starr, 2005). Furthermore, SOCS1 is upregulated early upon TCR activation (Yu et al., 2003),

and SOCS1 as well as SOCS3 have been shown to be involved in the regulation of TCR signalling (Fletcher and Starr, 2005). It is feasible that SOCS1 might synergize with SOCS3 in the feedback regulation of T-cell activation and IL-2 signalling. A double-knockdown of SOCS1 and SOCS3 in T cells could resolve the question of redundancies in the functionality of both SOCS proteins.

4.2 Comparable experiments targeting the expression of IL-7Rα

In a competitive in vitro assay, $SOCS3_{kd}$ T cells with decreased expression of IL-7Rα showed impaired proliferation only in the presence of IL-7 stimulation compared with control transduced T cells, and this could be attributed to lower STAT5 phosphorylation over the course of IL-7 signalling (Guler et al., 2019). The importance of IL-7/STAT5 signalling in promoting T-cell proliferation and survival is well established (Jiang et al., 2005). Comparable co-survival assays by co-transfer of FoxO1 deficient or IL-7Rα deficient and wildtype T cells into the same host with similar expansion kinetics to our experiments have been described in mouse models (Li et al., 2003, Ouyang et al., 2009, Buentke et al., 2006).

$SOCS3_{kd}$ T-cell proportions declined while control transduced T-cell proportions increased upon IL-7 stimulation under competitive conditions (Guler et al., 2019). Similarly, co-transfer of same ratios of IL-7Rα deficient and wildtype murine effector T_h2 cells into intact hosts leads to a decrease of the IL-7Rα deficient T cell population while wildtype T-cell proportions increase (Li et al., 2003). In addition, knockout of IL-7Rα leads to impaired memory development (Li et al., 2003), underlining the significance of SOCS3 as a mediator of perturbed memory development in AD-HIES patients (Siegel et al., 2011). Li et. al argue that IL-7 enhances the survival of T cells by upregulating anti-apoptotic proteins rather than promoting their proliferation (Li et al., 2003). Nevertheless, $SOCS3_{kd}$-mediated decrease in IL-7Rα expression led to proliferative deficiencies compared to control cells in our experiments (Guler et al., 2019).

Likewise, FoxO1 deficient murine T cells co-transferred with wildtype T cells into the same host were outcompeted due to proliferative defects (Ouyang et al., 2009). Similar to our experiments, IL-7-induced STAT5 phosphorylation was impaired in these cells (Ouyang et al., 2009). In addition, FoxO1 deficiency in murine T cells leads to a spontaneous activation of T cells and subsequently to decreased numbers of naïve T cells, and is associated with a T cell expansion-mediated

lymphadenopathy (Ouyang et al., 2009). Murine T cells deficient in FoxO transcription factors show drastically reduced SOCS3 protein expression (Pellegrini et al., 2011), and this emphasizes the role of SOCS3 in maintaining naïve T cells in a resting state. Interestingly, on a transcriptional level SOCS3 is expressed higher in FoxO1-deficient murine T cells (Ouyang et al., 2009). Nevertheless, SOCS3 mRNA transcription without SOCS3 protein being expressed has been described for T cells (Ghazawi et al., 2016, Pillemer et al., 2007).

In our experimental setup, SOCS3 expression is knocked down after initial T-cell activation around the time of SOCS3 re-expression. Therefore, SOCS3 and subsequently IL-7Rα expression are not disturbed in resting T cells and do not affect initial T-cell activation (Guler et al., 2019). The most similar in vivo experimental setup to our experiments was attained by tetracycline-inducible IL-7Rα expression in a mouse model (Buentke et al., 2006). Thymic developmental block of IL-7Rα deficient murine T cells was overcome by this method, and deprivation of doxycycline after activation of cytotoxic effector T cells and co-transfer with control cells into replete hosts allowed the analysis of IL-7Rα-mediated effects regarding the survival and development of cytotoxic memory T cells (Buentke et al., 2006). IL-7Rα deficient activated cytotoxic T cells are similarly competent in effector functions compared to control cells, while under competitive conditions these cells fail to survive and to develop into memory cells (Buentke et al., 2006). This is in accordance with our data and further underlines the in vivo significance of our results in a relevant mouse model. Nevertheless, in lymphopenic hosts survival of IL-7Rα deficient cytotoxic T cells is maintained by IL-15 (Buentke et al., 2006). The effects of defect IL-7 signalling in T helper cells might therefore be even more pronounced.

4.3 Unspecific effects of ectopic SOCS3 expression

4.3.1 IL-7 induced STAT5 phosphorylation is inhibited in $SOCS3_{oe}$ T cells

Overexpression of SOCS3 led to a strong inhibition of IL-7-mediated STAT5 phosphorylation over the course of time compared to control transduced T cells (Guler et al., 2019). However, overexpression of SOCS proteins has been shown to be impractical or misleading, as, for example, ectopic expression of SOCS3 inhibits interferon-γ (IFN-γ) signalling in mice, while SOCS3 deficiency seems to be dispensable for signalling regulation of IFN-γ (Croker et al., 2008). Similarly, while

SOCS3 deficiency in murine macrophages does not alter IL-10 signalling (Lang et al., 2003), ectopic overexpression has been shown to affect IL-10 signalling (Berlato et al., 2002), an observation we shared in regards to IL-7/STAT5 signalling (Guler et al., 2019).

Both, IL-10R and IL-7R are not targets of SOCS3, as binding sites for SOCS3 at the IL-7R and IL-10R have not been described. Moreover, it has been shown that SOCS3 achieves specific inhibition of cytokine signalling by binding simultaneously to its target receptors and their associated JAK proteins (Kershaw et al., 2013, Babon et al., 2012). However, high expression levels of SOCS3 might influence IL-7R signalling by inhibition and degradation of phosphorylated JAKs in a receptor-independent manner. Likewise, it has been proposed that the necessity for simultaneous binding of the receptor and its JAK protein can be overcome by high concentrations of SOCS3, leading to the binding and subsequent inhibition of JAK proteins independently from a receptor (Kershaw et al., 2013). The IL-7R signals via JAK1 and JAK3 (Carrette and Surh, 2012). JAK1, but not JAK3, is a target of SOCS3 (Babon et al., 2012). A possible mechanism leading to the inhibition of IL-7 signalling, when SOCS3 is ectopically expressed, is the inhibition of JAK1 activation by abundantly expressed SOCS3 protein in a receptor independent manner.

Whether high levels of SOCS3 expression can lead to receptor independent suppression of JAK activation in vivo remains to be investigated. Indications for a role of aberrant high SOCS3 expression influencing IL-7 signalling comes from TB patients, where high SOCS3 expression is accompanied with decreased responsiveness to IL-7 signalling (Harling et al., 2018, Lundtoft et al., 2017). Nevertheless, TB is a complex disease, and other mechanisms might contribute to or account for decreased IL-7 responsiveness in this regard. However, precise regulation of SOCS3 seems to be important for IL-7 signalling, as low levels of SOCS3 expression led to decreased IL-7 signalling as well (Guler et al., 2019).

4.3.2 IL-7Rα expression is not affected in SOCS3$_{oe}$ T cells in vitro

Despite decreased STAT5 phosphorylation, ectopic expression of SOCS3 did not lead to a decrease in IL-7Rα expression in our experiments (Guler et al., 2019). On the contrary, a tendency towards higher levels of IL-7Rα expression could be observed eight days after T-cell activation (Guler et al., 2019), which is probably caused by stronger suppression of T-cell activation and IL-2 signalling. This further

argues for a receptor independent regulation of IL-7 signalling, as SOCS3 is able to degrade target receptors (Kershaw et al., 2014, Irandoust et al., 2007). Ubiquitination of the G-CSF receptor by SOCS3 has been shown to be important for intracellular trafficking from early endosomes to lysosomes (Irandoust et al., 2007), and SOCS3 deficiency leads to an increase in gp130 mRNA and protein expression (Lang et al., 2003). In addition, degradation of IL-7Rα depends on JAK3 signalling (Henriques et al., 2010), which is not a target of SOCS3 (Babon et al., 2012). This further renders a role of SOCS3 in the degradation of the IL-7Rα unlikely.

4.4 SOCS3 as a regulator of T cell anergy and exhaustion

The role of SOCS3 in impairing functionality of T effector cells and promoting T cell exhaustion, a hallmark of TB infection (Henao-Tamayo et al., 2011), has already been pointed out. Concisely, expression of SOCS3 in effector T cells before pathogen clearance contributes to T cell exhaustion in chronic infections (Pellegrini et al., 2011, Harling et al., 2018).

In resting T cells, due to the antigen-concentration dependent nature of its downregulation (Yu et al., 2003), SOCS3 could potentially play an important role as a ‘gatekeeper’ by prohibiting T-cell activation upon self-antigen recognition by setting a threshold for strength and duration for the TCR to be activated. On the other hand, TCR signalling in the absence of CD28-mediated co-stimulatory signals leads to T cell anergy (Kuklina, 2013), and aberrant high levels of SOCS3 might cause anergy by blocking CD28 signalling.

Furthermore, besides inhibiting the co-stimulatory receptor CD28 (Matsumoto et al., 2003), SOCS3 regulates signalling by the T cell co-inhibitory receptor CTLA-4 (Yu et al., 2013). Low levels of SOCS3 expression in naïve and activated T cells promote CTLA-4 expression (Yu et al., 2013), and therefore could lead to anergy in a CTLA-4 dependent manner. Evidence for this comes from the fact that SOCS3 deficient T cells exhibit a less activated phenotype (Yu et al., 2013). In addition, IL-2 induces the expression of SOCS3 (Cohney et al., 1999), and in the absence of IL-2 signalling during T-cell activation, T cells fail to survive and to develop into memory cells (Dooms et al., 2007, Xue et al., 2002). Hence, the induction of CTLA-4 expression in the absence of SOCS3 expression in effector T cells could be a useful mechanism to prohibit extensive T-cell activation and subsequent inflammatory pathology.

Another characteristic of anergic T cells is dysregulated Ras expression and subsequently decreased signalling of the MAPK/ERK pathway (Kuklina, 2013). SOCS3 preserves Ras expression and therefore MAPK/ERK signalling (Cacalano et al., 2001).
The maintenance of T cells in a resting state, T-cell activation and subsequent inhibition of T cell effector functions, as well as promoting memory development are at least partly regulated by the appropriate expression of SOCS3 during these stages (Yu et al., 2003, Matsumoto et al., 2003, Cui et al., 2011). Therefore, accurate regulation of SOCS3 expression in naïve T cells, and downregulation during T-cell activation and re-expression in activated T cells is important for avoiding T cell anergy and exhaustion, although other mechanisms might participate in these pathways.

Pathways in T cell anergy share distinct similarities with regulatory T cells and both are associated with higher expression levels of CTLA-4 (Kuklina, 2013, Yu et al., 2013). Regulatory T cells depend on low levels of SOCS3 expression, which promotes the expression of CTLA-4 and regulatory T cell functionality (Pillemer et al., 2007, Yu et al., 2013). Furthermore, these cells are capable of inducing anergy in effector T cells (Kuklina, 2013).
Functionality of regulatory T cells depends on IL-2 signalling (Pillemer et al., 2007), and it has been shown that IL-2 only signals via STAT3 in activated T cells (Lin et al., 1995). Therefore, utilization of differential STAT proteins might be an important mechanism in maintaining low levels of SOCS3 in regulatory T cells by avoiding the induction of SOCS3 via IL-2 mediated STAT3 activation. Nevertheless, expression of SOCS3 mRNA has been shown for regulatory T cells, although SOCS3 expression could not be observed on protein level (Pillemer et al., 2007), and similar results have been obtained for IL-7/STAT5 mediated induction of SOCS3 mRNA (Ghazawi et al., 2016). Further experiments might determine the mechanisms leading to the suppression of IL-2 mediated SOCS3 protein expression in regulatory T cells. Concisely, low levels of SOCS3 expression promote the development of regulatory T cells (Pillemer et al., 2007, Yu et al., 2013), which could further contribute to anergy in effector T cells.

4.5 Therapeutic approaches for SOCS3

SOCS3 is dysregulated in many diseases due to its role in the feedback regulation of cytokine signalling, and studies have been performed to determine therapeutic effects of SOCS3 modulation.

In a mouse model of arthritis, adenoviral ectopic expression of SOCS3 or a dominant negative STAT3 in the joints of mice delays and reduces disease severity (Shouda et al., 2001). Similar results have been achieved for other types of inflammation (Yoshimura et al., 2007). Moreover, several types of cancer are characterized by hyperactivation of STAT3 (Yoshimura et al., 2007). For example, colorectal cancer is driven by continuous activation of STAT3 by IL-6, and ectopic expression of SOCS3 reduces STAT3 mediated inflammation (Linossi et al., 2013, Rigby et al., 2007).

Several viruses, among them human immunodeficiency virus-1 (HIV-1), induce SOCS3 and profit from its inhibitory functions on cytokine signalling to evade immune responses in hematopoietic cells (Akhtar and Benveniste, 2011). Therapeutic inhibition of SOCS3 could therefore be beneficial in restoring the integrity of the immune system in viral diseases. Besides, upon viral infection specific T helper cells mainly develop into T_h1 cells, which is mediated by a cytokine milieu containing IFN-γ among others (Swain et al., 2012). Previously, IL-6 signalling in SOCS3 deficient murine macrophages was shown to induce gene expression patterns mimicking an IFN-γ-like response (Croker et al., 2003, Lang et al., 2003), which could further support pathogen clearance in chronic viral diseases.

Perturbed SOCS3 expression in allergic diseases due to its role in T_h2 cell differentiation has been described (Seki et al., 2003). SOCS3 deficiency in a murine model of chronic experimental autoimmune uveoretinitis and in a model of induced asthma leads to a reduced pathology (Yu et al., 2013, Kinjyo et al., 2006), and beneficial effects of SOCS3 suppression in autoimmune and allergic diseases have been proposed (Kinjyo et al., 2006).

In contrast, therapeutic approaches targeting the role of SOCS3 in T-cell activation and exhaustion have not been undertaken extensively. SOCS3 is associated with T cell exhaustion in chronic infections, and SOCS3 deficiency or IL-7-induced SOCS3 suppression were shown to be beneficial for effector T cell functionality and pathogen clearance in a model system of chronic viral infection (Pellegrini et al., 2011). Pellegrini et. al show that IL-7 administration in chronic viremia decreases

SOCS3 expression. However, a significant protective effect of IL-7 administration is attributed to the mobilization of recent thymic emigrants and the increase in thymic egress, and increased T_h17 cell numbers and IL-22 secretion were protective against tissue damage (Pellegrini et al., 2011). Therefore, given the role of IL-7 in promoting T-cell proliferation and memory development, therapeutic administration of IL-7 to regulate SOCS3 expression rather than directly targeting SOCS3 might circumvent several potential side-effects of SOCS3 suppression, e.g. excessive inflammation due to uncontrolled T-cell activation (Matsumoto et al., 2003) or T cell anergy promoted by CTLA-4 expression (Yu et al., 2013). However, IL-7 has been proposed to render effector T cells unsusceptible towards inhibition by regulatory T cells in inflamed tissues (Ruprecht et al., 2005).

4.6 Outlook

The role of SOCS3 in T cell signalling, homeostasis and memory development is an interesting emerging function of SOCS3 other than in cytokine signalling. Furthermore, the importance of SOCS3 in T-cell homeostasis, especially in governing T cell fate between anergy and exhaustion, has been discussed, and future research will reveal its potential as a therapeutic target against T cell exhaustion in relevant diseases such as TB. Elucidating these pathways is of utmost interest for a better understanding of chronic infections and autoimmune disorders.

Given the role of SOCS3 in feedback regulating immune responses and its potential role in memory T-cell generation (Matsumoto et al., 2003, Cui et al., 2011), targeting SOCS3 expression in diseases might come with certain disadvantages. Low levels of SOCS3 could possibly cause aberrant immune responses due to a lack in the feedback inhibition of cytokine signalling and T-cell activation (Alexander, 2002, Matsumoto et al., 2003), or lead to anergy (Yu et al., 2013). It remains to be investigated if and under which circumstances SOCS3 deficient cells cause excessive inflammatory responses or undergo anergy. Furthermore, we showed promoting effects of SOCS3 in the re-expression of IL-7Rα during T-cell activation (Guler et al., 2019), which is a necessary feature for the development of memory T cells (Dooms et al., 2007, Li et al., 2003).

Well-dosed and time restricted suppression of SOCS3 expression to circumvent T cell exhaustion while allowing pathogen clearance in chronic infections might be necessary to circumvent excessive immune responses or anergy, and to allow

IL-7Rα re-expression and memory development, the latter being particularly important to achieve long-lasting immunity against the targeted disease.

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Statutory declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolgreichen oder erfolglosen Promotionsversuche unternommen.

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Vor- und Nachname

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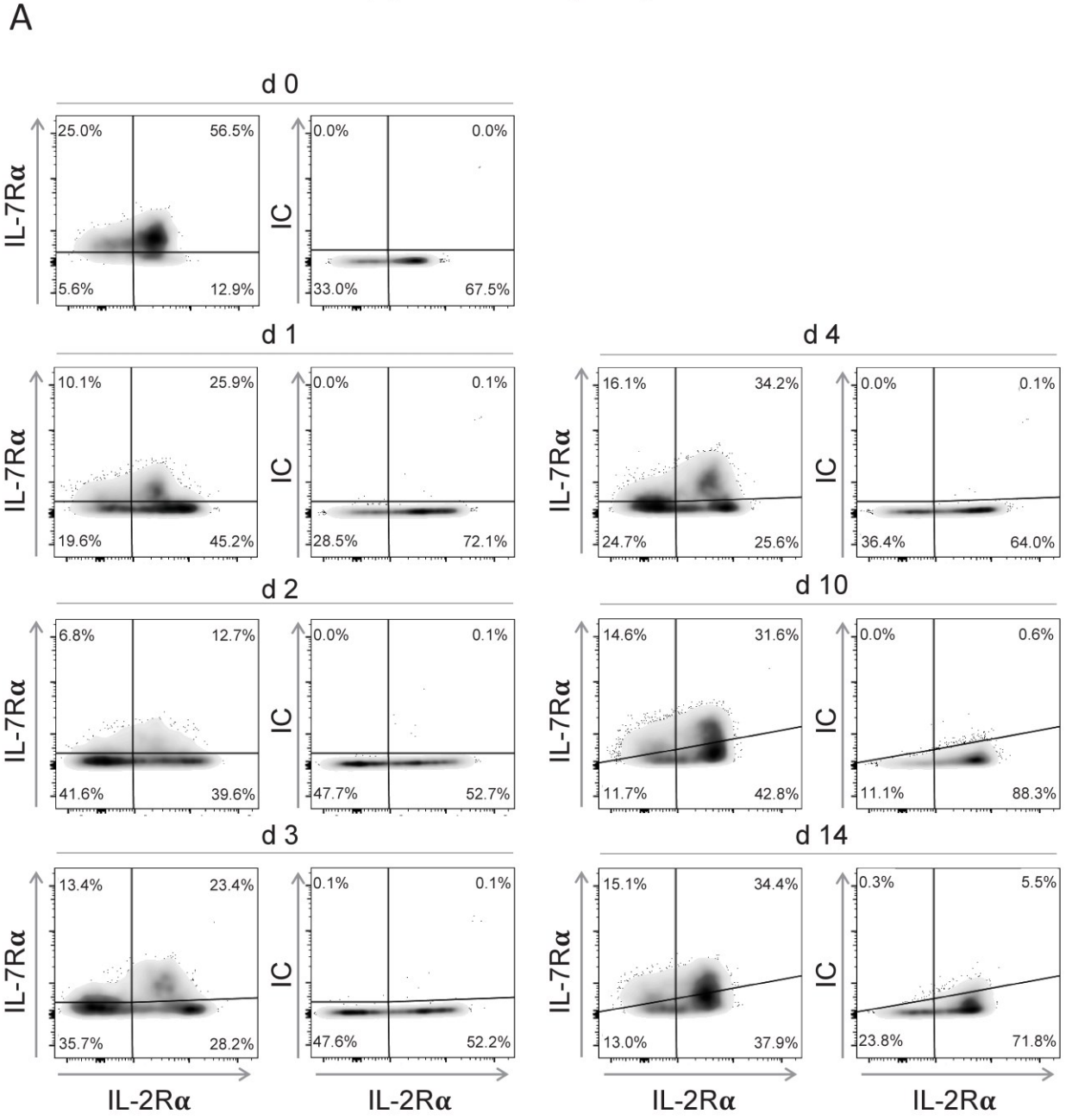
Datum und Unterschrift

Supplementary Figures of the Paper

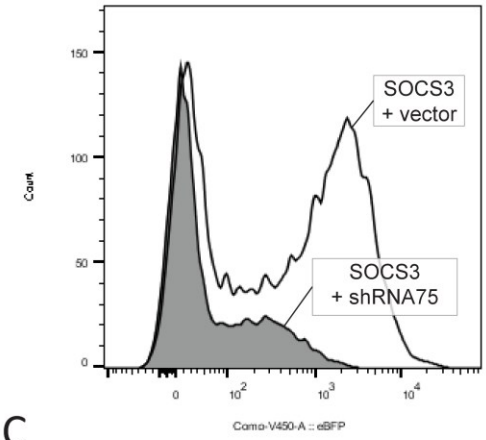
Supplementary Figure 1



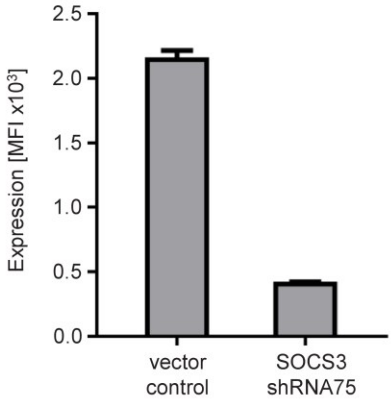
Time course of IL-7Rα expression for CD4⁺ T cells after T-cell activation. (**A**) Representative flow cytometry dot plots of IL-7Rα (left graphs) and isotype controls (right graphs) are given for d0, d1, d2, d3, d4, d10, d14. Quadrants are set according to the respective isotype control on each day. IL-2Rα expression has been included as a positive control for T-cell activation. Upper quadrants were summed for calculation of IL-7Rα_{high} T-cell proportions. (**B**) Proportions of IL-7Rα_{high} T cells during the time course after T-cell activation are shown for four healthy individuals indicated by different symbols. Isotype control: IC; d: day.

Supplementary Figure 2

A



B

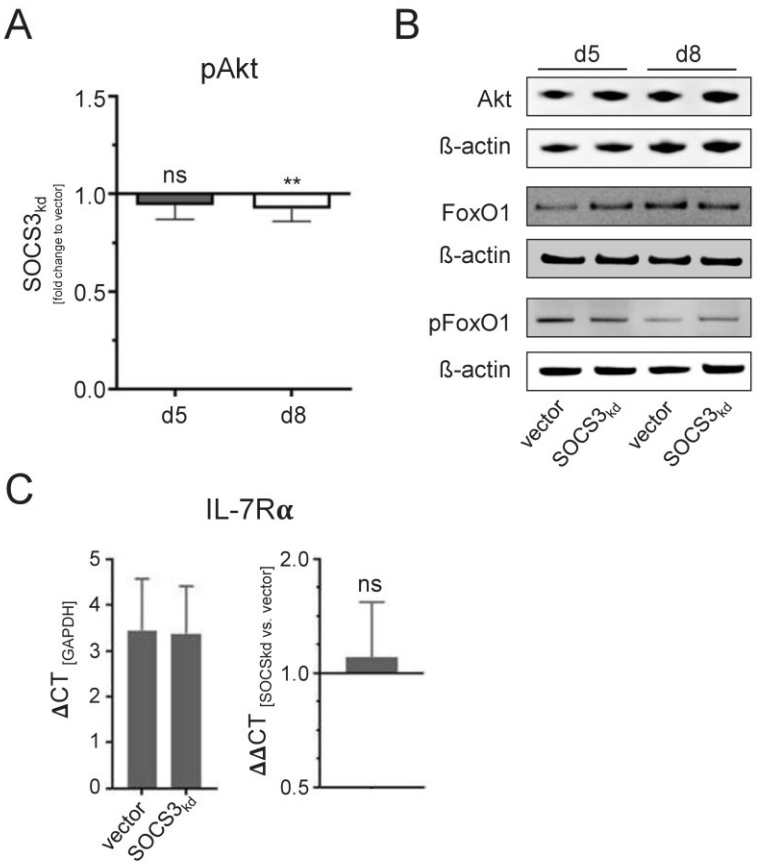


C

	SOCS1	SOCS3
Relative expression with shRNA75 [%]	99	14

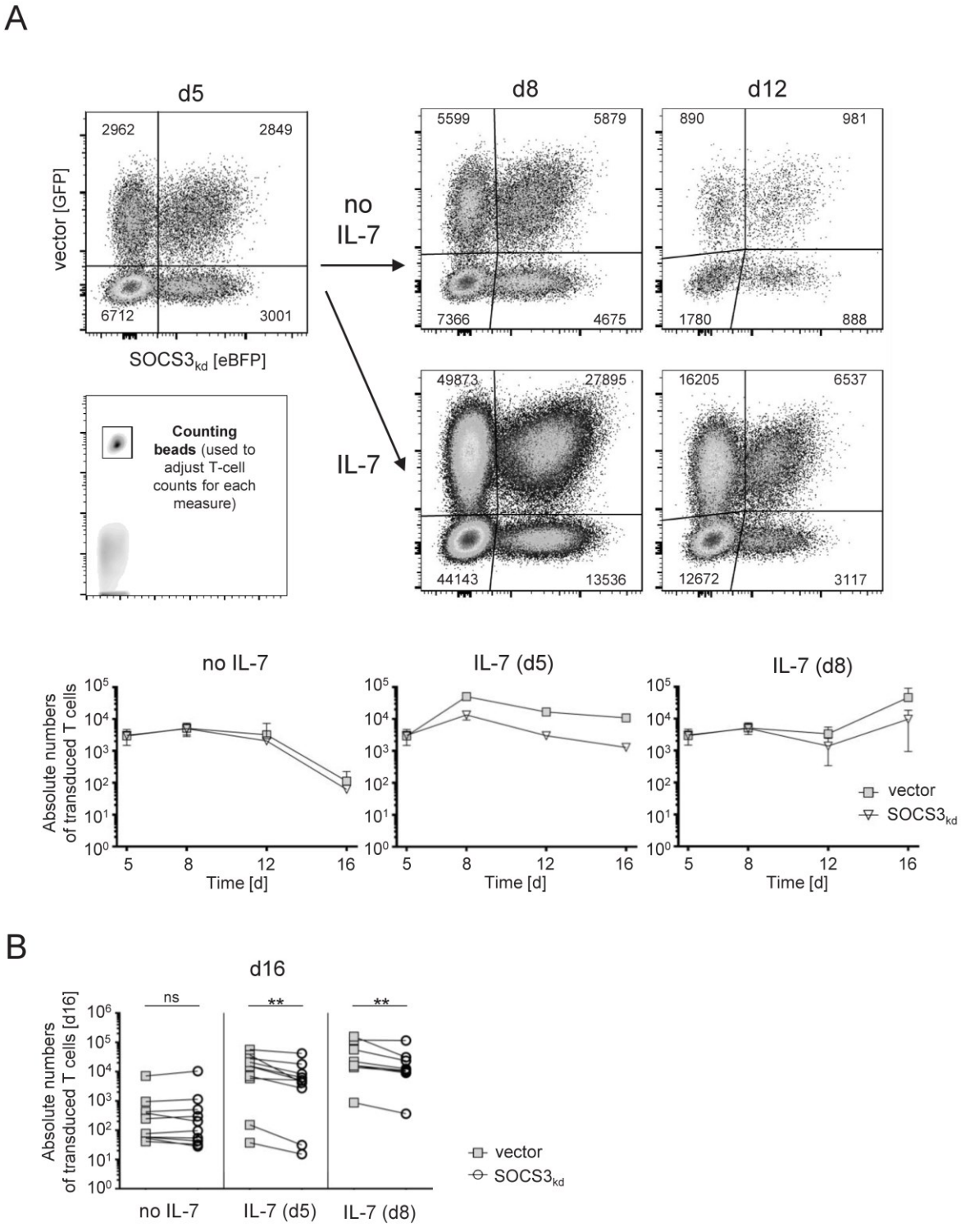
Efficacy and specificity of shRNA75 to inhibit SOCS3 expression. (**A, B**) HEK293T cells co-transfected with SOCS3 cDNA and shRNA75 or empty construct (vector control) are shown. A histogram of transfected HEK293T cells (**A**) and mean expression of the SOCS3 cDNA protein marker eBFP (**B**) are given. (**C**) shRNA75 mediated SOCS3 inhibition and cross-reactivity with closely related SOCS1 are indicated as SOCS1 or SOCS3 expression in the presence of shRNA75.

Supplementary Figure 3



Characterization of SOCS3 targets potentially involved in IL-7Rα regulation. (**A**) Akt phosphorylation comparison by flow cytometry for $SOCS3_{kd}$ and vector control transduced T-cells on d5 and d8 after activation. Vector control values are set to one and fold change differences of $SOCS3_{kd}$ are given. Analysis of repeated experiments are shown (d5: n=6, d8: n=10**)**. (**B**) Akt, FoxO1, and pFoxO1 protein analyses by western blot for $SOCS3_{kd}$ and vector control transduced T-cells on d5 and d8 are given. β-actin bands were determined as loading controls. (C) IL-7Rα mRNA expression on day 8 for $SOCS3_{kd}$ and vector control transduced T-cells determined by quantitative PCR. A total of 3 experiments have been performed (n=6). Wilcoxon signed-rank test analyses were performed and significant differences are indicated by asterisks (i.e. **, $p<0.01$). ns: not significant.

Supplementary Figure 4



A functional competitive in vitro *assay for comparison of SOCS3$_{kd}$ and empty vector control transduced T-cell proportions and absolute numbers.* (**A**) Gating strategy as dot plots of a representative experiment showing lentiviral marker expression in primary CD4$^+$ T cells co-transduced with SOCS3$_{kd}$ (eBFP) and vector control (eGFP) on d5, d8, and d12 after T-cell activation with or without IL-7 added on d5. Counting beads are used to adjust T-cell numbers for each individual measure. (**B**) For comparison of T-cell numbers on d16, numbers were adjusted to d5 values, where 10^4 transduced T cells were set as the mean initial population. Results from repeated experiments are provided (no IL-7: n=10; IL7 (d5): n=10; IL-7 (d8): n=8). Wilcoxon signed-rank test analyses were performed and significant differences are indicated by asterisks (i.e. **, p<0.01). d: day; ns: not significant.