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The role of IL-18 and IL-1 receptor signaling in T cell development and T cell exhaustion in a murine pancreatic cancer model

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I Abbreviations

%	Percent
°C	Degree Celsius
Akti	Akt inhibitor VIII
ANOVA	Analysis of variances
APC	Antigen-presenting cell
APC	Allophytocyanin
APC/Cy7	Allophytocyanin/Cyanine 7
B cells	Bone marrow-derived lymphocytes
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
Ct	cycle threshold
CTL	Cytotoxic T cell
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting
FCS	Fetal calve serum
FIR	FOXP3-IRES-mRFP
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box protein 3
fwd	Forward
g	gravity
g	gram
h	hour
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
IL	Interleukin
IL-1R	IL-1 receptor

IL-18R	IL-18 receptor
i.p.	intraperitoneally
ISQ	$OVA^{323-339}$
iTreg	in vitro-induced regulatory T cell
k	kilo
l	liter
LAG-3	Lymphocyte activation gene 3
M	Molar (mol/l)
m	milli
MACS	Magnetic Cell Separation
MDSC	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
min	minute
mTOR	Mammalian target of rapamycin
n	nano
n	Sample number
$NaHCO_3$	Sodium Bicarbonate
NaPyr	Sodium pyruvate
NFkB	Nuclear factor k-light-chain-enhancer of activated B cells
NH_4Cl	Ammonium chloride
NK	Natural killer
NLRP3	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing protein 3
ns	not significant
p	pico
p-	phospho-
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PD-L1	Programmed cell death 1 ligand 1
PE	Phycoerythrin
PE/Cy7	Phycoerythrin/cyanine7
Pen-Strep	Penicillin-streptomycin

PerCP	Peridinin-chlorophyll-protein-complex
PerCP/Cy5.5	Peridinin-chlorophyll-protein-complex/Cyanin5.5
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PMA	12-O-Tetradecanoylphorbol-13-acetate
pTreg	peripherally derived regulatory T cell
RFP	red fluorescent protein
rh	recombinant human
rm	recombinant mouse
RORγt	RAR-related orphan receptor gamma
RT	Room temperature
RT qPCR	Real time quantitative PCR
s	second
s.c.	subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
STAT5	Signal transducer and activator of transcription 5
STAT5i	STAT5 inhibitor
T cells	Thymus-derived lymphocytes
TAM	Tumor-associated macrophage
T-bet	T-box transcription factor expressed in T cells
Tcf1	T cell factor 1
TCR	T cell receptor
Th cell	helper T cell
TIL	Tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high mobility group box
Treg	regulatory T cells
tTreg	thymus-derived regulatory T cell
U	Unit
µ	micro

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1 Introduction

1.1 The Immune system and T cells

The term "immune system" describes a network of cells, processes and chemicals that protect the organism against pathogens and cancer. It consists of two lines of defense, which work cooperatively. The first line, innate immunity, works fast, but non-specific and consists of macrophages, neutrophils, dendritic cells (DCs), mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid cells. The second line, adaptive immunity, works slower but in an antigen-specific manner, resulting in immunologic memory. Cells of the adaptive immune system include thymus-derived lymphocytes (T cells), antigen-presenting cells (APCs, such as DCs, macrophages, fibroblasts, and epithelial cells) and bone marrow derived lymphocytes (B cells) (Marshall et al. 2018).

T cells conduct central functions in the adaptive immune system as they mediate the immune response against pathogens, allergens, and tumors. They express antigen-binding receptors on their surface, the T cell receptors (TCR) and a co-receptor (CD4/CD8). T cells originate from hematopoietic stem cells in the bone marrow and mature in the thymus and enter the periphery as $CD4^+$ or $CD8^+$ naïve T cells. Naïve T cells require antigen presentation on major histocompatibility complex (MHC) molecules by APCs which causes activation of the TCR and subsequent differentiation into effector T cells. Effector T cells are then able to fulfill their task, i.e. pathogen clearance by production of cytotoxic cytokines T cells that express the co-receptor CD4 recognize class II MHCs (MHC II) and differentiate into helper T cells (Th cells) upon activation, whereas T cells that express the co-receptor CD8 recognize class I MHCs (MHC I) and differentiate into cytotoxic T cells (CTLs) (Kumar et al. 2018; Marshall et al. 2018).

1.1.1 $CD4^+$ T cells

$CD4^+$ T cells, or Th cells, play a key role in the immune response, mainly by supporting or activating other immune or non-immune cells. They are able to enhance and maintain CTL responses and to recruit immune cells to the site of infection (Zhu et al. 2010; Zhu and Paul 2008; Luckheeram et al. 2012). In particular, $CD4^+$ T cells regulate the immune response by the production and secretion of cytokines, small proteins that are secreted by cells and influence interactions between cells (Zhu and Paul 2008; Dong 2021; Luckheeram et al. 2012; Zhang and An 2007).

$CD4^+$ T cells can be further subdivided into different subsets with distinct functions that are characterized by their induction, cytokine profile and key transcription factors (Dong 2021). After the initial discovery of the Th1 and the Th2 subset in 1986 (Mosmann et al. 1986), further subsets of Th cells were identified, amongst them Th17 cells (Harrington et al. 2005; Park et al. 2005).

Th1 cells are induced by interleukin- (IL-)12 and interferon (IFN)-γ and the transcription factor T-box transcription factor TBX21 (T-bet), produce IFN-γ and TNF and mediate cellular immunity. Th2 cells are induced by IL-25, IL-33, and IL-4, regulated by the transcription factor GATA3, and produce IL-4, IL-5, and IL-13 in order to mediate parasitic immunity. Th17 cells are induced by a combination of transforming growth factor- (TGF-)β, IL-6 and IL-23 and the expression of the transcription factor RAR-related orphan receptor gamma t (RORγt). They produce IL-17, IL-21 and IL-22 and are responsible for immunity against pathogens (Dong 2021; Raphael et al. 2015).

A special subset of $CD4^+$ T cells is represented by $CD25^+$ regulatory T cells (Tregs) which are characterized by the expression of the transcription factor Forkhead box protein 3 (FOXP3) (Hori et al. 2003; Sakaguchi 2000). Tregs can either be generated in the thymus and are therefore termed thymus-derived Tregs (tTreg), differentiated from $CD4^+$ T cells in the periphery (peripherally derived Tregs (pTregs)) or differentiated *in vitro* in the presence of TGFβ; *in vitro*-induced Tregs (iTregs) (Shevach and Thornton 2014). Tregs regulate the immune response in order to prevent excessive and long-lasting immune reactions by inhibiting proliferation, differentiation, and effector function of T cells as well as function of other cells of the immune system like NK cells and B cells. The suppressive activity of Tregs can be mediated by the induction of immunosuppressive cytokines (like IL-10 or TGFβ) or by cell-contact (Sakaguchi et al. 2008).

1.1.2 $CD8^+$ T cells/CTLs

CTLs are key mediators of the adaptive immunity because of their ability to kill infected or malignant target cells. CTLs derive from naïve $CD8^+$ T cells and undergo differentiation upon activation by APCs. CTLs migrate to the effector site and induce death of their target cells by producing effector cytokines and molecules like IFN-γ, tumor necrosis factor (TNF), perforin, granzymes and Fas-ligand (Zhang and Bevan 2011; Halle et al. 2017). IFN-γ has anti-viral and anti-tumoral functions and can inhibit proliferation and induce apoptosis (Castro et al. 2018). In particular, IFN-γ has been shown to increase the motility and cytotoxicity of the producing CTLs (Bhat et al. 2017). TNF has pro- and anti-inflammatory functions and therefore plays an important role in many functions of the adaptive immune system, in anti-tumor response (Mehta et al. 2018).

After antigen clearance, most CTLs die by apoptosis but 5-10 % differentiate into memory T cells and contribute to the immunological memory (Kaech and Cui 2012). When those memory cells are exposed to the same antigen again, they are able to expand quickly and regain higher effector functions compared to naïve cells being exposed to the same antigen(Schluns and Lefrançois 2003; Baitsch et al. 2012).

1.1.3 T cell exhaustion

The term "T cell exhaustion" describes a type of T cell dysfunction that is characterized by loss of effector function and arises when immune response fails (Saeidi et al. 2018; Wherry 2011). It was first observed in a model of chronic viral infection, where dysfunctional antigen-specific CTLs were found that failed to perform cytotoxic functions and were finally deleted (Zajac et al. 1998; Gallimore et al. 1998; Moskophidis et al. 1993). Subsequently, this phenomenon was observed in chronic infections and cancer in mice and humans (Virgin et al. 2009; Wherry 2011; Woroniecka et al. 2018; Baitsch et al. 2011).

T cell exhaustion generally arises when the immune response is persistent, accompanied by the lack of $CD4^+$ T cell support (Virgin et al. 2009; Wherry 2011; Wherry and Ahmed 2004) and is mediated by the quantity of antigen, rather than the specificity (Alfei et al. 2019; Utzschneider et al. 2016a). T cell exhaustion is thought to be an "adaptive state of hyporesponsiveness" in chronic infections, which prevents immunopathology (Philip and Schietinger 2022).

Exhausted T cells are typically characterized by the expression of multiple coinhibitory receptors, reduced proliferation, alterations in the transcriptional and epigenetic features and metabolic requirements (Saeidi et al. 2018). Additionally, exhausted T cells lose their effector function in a hierarchical manner. Typically, the *ex vivo* killing and IL-2 production capacity is lost first (Wherry 2011) followed by the ability to produce TNF and at the advanced stage IFN-γ and Granzyme B (GrzB) production is lost (Wherry et al. 2003). Finally, exhausted T cells undergo apoptosis and are physically deleted (Wherry 2011; Wherry et al. 2003; Zajac et al. 1998; Moskophidis et al. 1993).

Typical inhibitory receptors expressed by exhausted T cells are programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG3), T cell immunoglobin domain and mucin domain-containing protein 3 (TIM3) and 2B4. It has been shown that inhibitory receptors differ in their regulation of cellular functions and therefore seem to influence T cell function in different ways (Wherry 2011). PD-1, for example, has an impact on survival and proliferation (Petrovas et al. 2006; Petrovas et al. 2007; Blackburn et al. 2010; Blackburn et al. 2008). PD1 is expressed on tumor infiltrating lymphocytes (TILs) and binds to its ligands programmed cell death 1 ligand 1 (PD-L1) and programmed cell death 1 ligand 2 (PD-L2) causing dampened T cell response. PD-L1 and PD-L2 can inter alia be expressed on solid tumors (Saka et al. 2020).

Inhibitory receptors are also expressed by activated T cells (Wherry and Kurachi 2015) and therefore, the individual expression of one receptor cannot be used to determine the state of exhaustion. Nevertheless, co-expression of multiple inhibitory receptors is a feature of exhaustion and more exhausted T cells express more inhibitory receptors on their surface (Saeidi et al. 2018;

McLane et al. 2019; Wherry and Kurachi 2015; Blackburn et al. 2009). For example, PD-1 and LAG3 are typically expressed already at the early stages of exhaustion, whereas TIM3 is expressed at the late stage (Philip et al. 2017). Consequently, simultaneous expression of PD-1 and TIM3 often accounts for an advanced exhausted phenotype, characterized by the lost production of IL-2, TNF and IFN-γ. (Jones et al. 2008; Sakuishi et al. 2010).

Several transcription factors have shown to been involved in the process of T cell exhaustion, for example: T-bet and Eomesodermin (Eomes) (Saeidi et al. 2018). More recently, the transcription factor thymocyte selection-associated high mobility group box protein (TOX) has been shown to play a role in this phenomenon (Saka et al. 2020). In exhausted T cells T-bet is able to suppress the expression of inhibitory receptors (Kao et al. 2011) and high T-bet expression is therefore characteristic for a subset of progenitor exhausted T cells with intermediate PD-1 expression and maintained proliferate capacity during chronic infection (Paley et al. 2012). Eomes on the other hand has been shown to be highly expressed in exhausted CTLs, causing an increased Eomes:T-bet ratio that induces expression of genes involved in T cell exhaustion (Li et al. 2018; McLane et al. 2021). Additionally, Eomes is highly expressed by a terminally exhausted subset of exhausted T cells with high PD-1 expression and lost proliferative capacity. Therefore, T-bet and Eomes are expressed by exhausted T cells in a somewhat mutually exclusive pattern (Paley et al. 2012). It has also been stated that Eomes and T-bet might compete for the same binding sites and therefore antagonize each other (Li et al. 2018; McLane et al. 2021).

TOX expression is induced by TCR stimulation and has been shown to be involved in exhaustion (Scott et al. 2019; Khan et al. 2019; Seo et al. 2019; Alfei et al. 2019). Interestingly, TOX also plays a role in maintaining dysfunctional T cell populations during chronic infections (Alfei et al. 2019).

Several cell populations and cytokines are potentially involved in the induction and progression of exhaustion, for example IL-10 and TGFβ (Wherry 2011; Wherry and Kurachi 2015). Those cytokines are produced by Tregs (Veiga-Parga et al. 2013), but it is not clear whether Tregs have a direct role in the induction and progression of exhaustion in CTLs (Saeidi et al. 2018; Wherry and Kurachi 2015). However, PD-1 blockade in combination with Treg depletion caused increased viral control and reduced exhaustion in a murine chronic infection model (Penaloza-MacMaster et al. 2014). Other cell types that possibly contribute to T cell exhaustion are MDSCs, NK cells, DCs and TAMs (Wherry and Kurachi 2015; Jiang et al. 2015).

Exhausted T cell populations are typically heterogenic (Blackburn et al. 2008; Im et al. 2016; Paley et al. 2012; Utzschneider et al. 2016b; He et al. 2016; Miller et al. 2019). This feature is of particular importance as early, but not late exhausted T cells, are able to recover when continuous antigen

stimulation is removed (Philip and Schietinger 2022) and respond to checkpoint blockade (Daud et al. 2016; Kurtulus et al. 2019; Huang et al. 2019; Huang et al. 2017; Sade-Feldman et al. 2019). In particular, exhausted T cells with intermediate PD-1 expression respond to PD-1 blockade, while exhausted T cells with high PD-1 expression do not (Blackburn et al. 2008; Miller et al. 2019). The transcription factor T cell factor 1 (Tcf1) has been identified as an marker for progenitor exhausted T cells (Chen et al. 2019; Im et al. 2016; Utzschneider et al. 2016b; Wu et al. 2016). Based on the expression of Ly108 (which surrogates Tcf1) and CD69, Beltra et al. defined four subsets of exhausted T cells which differ in their transcriptional and epigenetic profile and showed distinct characteristics *in vivo* (Beltra et al. 2020).

The fact that PD-1 blockage is able to reverse features of exhausted T cells (Barber et al. 2006) shows that T cell exhaustion is not a terminal state (Wherry 2011) and is a promising target for immunotherapy (Nguyen and Ohashi 2015).

Although most studies regarding T cell exhaustion focus on $CD8^+$ T cells, $CD4^+$ T cells can also show features of exhaustion (such as expression of inhibitory receptors, loss of cytokine production and proliferation) and become functionally impaired during chronic infections (Wherry and Kurachi 2015; Miggelbrink et al. 2021). The functionality and potential exhaustion of $CD4^+$ T cells is of particular interest as $CD4^+$ T cell response directly influences $CD8^+$ T cells and $CD8^+$ T cell exhaustion (Wherry and Kurachi 2015; Kravtsov et al. 2022).

1.1.4 T cells and cancer

The immune system plays a key role in the prevention and rejection of cancer. CTLs for example are able to directly kill cancer cells, while Th cells mediate anti-tumor response of CTLs, B-cells and NK cells via cytokines and influence the composition of the tumor microenvironment (TME) (like IL-2, IFN-γ and TNF) (Gonzalez et al. 2018; Borst et al. 2018). Additionally, $CD4^+$ T cells can have independent anti-tumoral effects by directly lysing tumor cells and by activating the innate immune system and inhibiting angiogenesis (Miggelbrink et al. 2021; Kravtsov et al. 2022; Borst et al. 2018).

However, during tumor progression, different mechanisms arise that mediate cancer immune evasion. For example, antigen stimulation of the TCR causes more severe exhaustion and therefore lack of effector function of T cells (Philip and Schietinger 2022). The antigen presentation process is also often is impaired in TME, partly mediated by Th2 cells, causing insufficient activation of T cells (Baitsch et al. 2012; Miggelbrink et al. 2021). The expression of PD-L1 and PD-L2 also favors immune evasion and is associated with the survival of patients with several types of cancer (Jiang et al. 2015).

Tregs can also contribute to tumor immune evasion. This can be mediated by suppressing the effector function of T cells by secreting inhibitory cytokines (like IL-10, IL-35 and TGβ) and by scavenging IL-2 from the TME, which is than no longer available for effector T cells (Miggelbrink et al. 2021; McRitchie and Akkaya 2022). Of note, the altered nutrient, cytokine and chemokine composition caused by tumor cells during tumor progression, favors the infiltration of Tregs (McRitchie and Akkaya 2022). Therefore, the level of Treg infiltration can serve as an indicator for tumor progression and therapy response in cancer patients (Plitas et al. 2016; Verma et al. 2019).

1.2 The NLRP3 inflammasome and NLRP3-dependent cytokines

Inflammasomes are multiprotein complexes with important functions in the immune response (Kanneganti 2015). The NLRP3 inflammasome is a prominent member of this family of Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) inflammasomes (Schroder et al. 2010; Strowig et al. 2012; Yin et al. 2018). Inter alia, the NLRP3 inflammasome is responsible for the processing of the cytokines IL-1β and IL-18, by cleaving their pro-domains upon activation (Martinon et al. 2009). Active IL-1β and IL-18 have functions in innate and adaptive immunity and are also involved in diseases affecting the immune system (e.g. multiple sclerosis and asthma) (Sims and Smith 2010). Additionally, elevated levels of IL-1β and IL-18 have been found in various tumors (Wang et al. 2018).

1.2.1 IL-1

IL-1, the first cytokine that was described, exists in two forms, IL-1α and IL-1β, both having the same biological functions. However, some differences exist; IL-1β is a secreted cytokine that circulates systemically and IL-1α is bound to the plasma membrane and can therefore only act locally. Furthermore, while IL-1α is produced by many cell types, IL-1β is mainly produced by monocytes and macrophages. Finally, their genes are differently regulated, indicating different functional contributions (Sims and Smith 2010; Garlanda et al. 2013).

Binding of IL-1 to the IL-1 receptor (IL-1R) causes structural rearrangements inducing the recruitment of MyD88 and subsequent activation of pro-inflammatory signals. (Dinarello 2018).

IL-1 signaling promotes the activity of DCs and therefore facilitates T cell priming (van den Eeckhout et al. 2020). Additionally, IL-1 increases $CD4^+$ T cell-mediated immune responses, facilitates the differentiation of naïve $CD4^+$ T cells, and enhances survival and antigen-stimulated proliferation of Th1, Th2 and Th17 cells (Ben-Sasson et al. 2009; van den Eeckhout et al. 2020), although IL-1R is usually not detected on Th1 cells (Taylor-Robinson AW 1994). IL-1 blocks thymic development of Tregs and prevents Treg mediated inhibition of proliferation (O'Sullivan

et al. 2006; Nikolouli et al. 2021). Additionally, IL-1 contributes to the differentiation of T cells via DC regulation (Guo et al. 2003; Luft et al. 2002; Wesa and Galy 2002).

The role of IL-1 in tumor progression however is more complex. As an pro-inflammatory cytokine, IL-1 has been linked to tumor promotion caused by inflammation. (Menu and Vince 2011; Dinarello 2010; Elinav et al. 2013; Briukhovetska et al. 2021) but also to anti-tumor responses (Menu and Vince 2011; Briukhovetska et al. 2021). In any case the positive effects of IL-1β on T cells have been shown to be substantial for tumor rejection after chemotherapy (Ghiringhelli et al. 2009). Several studies detected increased levels of IL-1β in pancreatic cancer patients (Yako et al. 2016) and high levels of IL-1 in the TME was associated with poor prognosis (Apte and Voronov 2002). Additionally, it has been shown that IL-1β, modulates the stroma in a pro-tumorigenic way by inducing desmoplasia and immune suppression (Das et al. 2020).

1.2.2 IL-18

IL-18 is a pro-inflammatory, IFN-γ inducing cytokine that belongs to the IL-1 family of cytokines and has been shown to induce Th1 and Th2 immune response dependent on the context. It is released by inter alia macrophages and DCs and can regulate cell function, proliferation and survival (Sims and Smith 2010; Dinarello et al. 2013; Li et al. 2019; Nakanishi 2018).

IL-18 binds to the IL-18 receptor (IL-18R), which consists of the binding chain (IL-18Rα) and the co-receptor chain (IL-18Rβ). Binding of IL-18 to IL-18Rα causes coupling of the two receptor chains and downstream signaling of inflammatory processes (Rex et al. 2020). IL-18R is already expressed on naïve T cells and is upregulated by $CD4^+$ T cell differentiation under Th1 conditions, enabling IL-18 to induce IFN-γ production. $CD4^+$ T cell differentiation under Th2 conditions, however, causes downregulation of IL-18R (Smeltz et al. 2001). Additionally, IL-18R is expressed on *ex vivo* $CD4^+$ T cell subsets (Harrison et al. 2015). IL-18 signaling has been shown to inhibit Tregs in their accumulation and function (Carroll et al. 2008) and therefore possibly supports immune responses of effector T cells (Sims and Smith 2010).

The role of IL-18 in cancer is disputed. On the one hand, IL-18 increases IFN-γ and Th1-type response, which is an antitumoral function (Eberl et al. 2000; Chang et al. 2007; Coughlin et al. 1998; Park et al. 2007). On the other hand, IL-18 treatment had no therapeutic effects in melanoma patients (Tarhini et al. 2009). Furthermore, IL-18 was shown to have pro-tumorigenic effects via the activation of NFkB, which causes increased proliferation and prevented apoptosis of tumor cells (Deswaerte et al. 2018; Park et al. 2007; Terme et al. 2011; Guo et al. 2016). Additionally, IL-18 may be involved in PD-1 dependent immunosuppression of NK cells in breast cancer patients

(Terme et al. 2011). Likewise, NLRP3 activity and, as a result, increased IL-18 levels in lymphoma tissue correlated with PD-L1 expression in lymphoma tissue (Lu et al. 2021).

IL-18 is produced by cancer cells contributing to tumor progression (Yao et al. 2020) and is highly expressed in pancreatic cancer (Usul Afsar et al. 2017). In the TME of pancreatic cancer, IL-18 can be additionally secreted by activated duct cells and macrophages (Ahmed et al. 2022; Dinarello et al. 2013). High levels of IL-18 in the blood have shown to be correlated with decreased survival (Carbone et al. 2009) and IL-18 levels in the serum and plasma of pancreatic cancer patients were higher than in healthy subjects (Bellone et al. 2006; Carbone et al. 2009; Usul Afsar et al. 2017; Guo et al. 2016), The correlation between high IL-18 levels in the serum and overall survival however remains contradicting, probably caused by different treatments of PDAC patients prior to the respective study (Li et al. 2019). Nevertheless, higher IL-18 levels in cancer tissue have been associated with shorter survival, tumor progression and abundancy of $CD8^+$ $PD\text{-}1^+$ cells in the tumor (Guo et al. 2016; Li et al. 2019; Ahmed et al. 2022).

Recently, IL-18 has been shown to promote T cell exhaustion of antigen-specific CTLs in a murine pancreatic cancer model and to induce the activation of the IL-2/ Signal transducer and activator of transcription 5 (STAT5) and the Phosphoinositide 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) pathway (Lutz et al. 2023).

1.3 Pancreatic cancer

Pancreatic cancer is one of the worst types of cancer with a 5-year survival rate of 11 % (Siegel et al. 2022), over 466,000 deaths (4.7 % of all cancer related deaths) and over 495,000 new cases (2.6 % of all new cancer cases) in the developed world in 2020 (Sung et al. 2021). Pancreatic ductal adenocarcinoma (PDAC) is the most common tumor type in the pancreas and accounts for more than 90 % of pancreatic malignancies (Kleeff et al. 2016). It is characterized by low mutational burden, which results in a lack of neoantigens, and poor antigenicity (Knudsen et al. 2017). Additionally, PDAC tumors are characterized by a desmoplastic and immunosuppressive TME which impairs drug delivery and limits therapy response (Saka et al. 2020).

PDAC is often detected in a late stage, which is of particular importance as the efficacy of treatments depends on the time of diagnosis. Only 10-20 % of patients are diagnosed with still resectable stages of PDAC. For the remaining patients, PDAC is either present in a locally advanced state and therefore not resectable, or with distant metastases. For non-resectable tumors, patients are treated with chemotherapy alone or combined with radiotherapy (Orth et al. 2019). FOLFIRINOX (a poly-chemotherapeutic regimen that consists of folinic acid, 5-FU, irinotecan

and oxaliplatin) has been shown double the median survival of patients with metastasis compared to gemcitabine treatment alone (Conroy et al. 2011).

T cells have been shown to play an important role in the progression of PDAC as higher levels of intratumoral $CD4^+$ and $CD8^+$ T cells correlated with better survival (Ino et al. 2013; Tewari et al. 2013; Fukunaga et al. 2004; Carstens et al. 2017). Nevertheless, the role of T cells in PDAC seems to be strongly dependent on the respective subset, the spatial distribution and other cells in the TME (Huber et al. 2020).

PDAC has evolved mechanisms to escape the immune response (for example downregulation of the antigen presentation machinery) (Ben-Sasson et al. 2009; Agata et al. 1996) and the TME of PDAC is highly immunosuppressive with high abundancies of inter alia Tregs, TAMs and MDSCs. These cells are already present in pancreatic intraepithelial neoplasia and therefore may even contribute to tumor initiation and progression (Saka et al. 2020; Martinez-Bosch et al. 2018). The TME of PDAC restricts the infiltration of antitumoral immune cells, inhibits T cell activation and contributes to exhaustion (Balachandran et al. 2019; Bernstorff et al. 2001).

25 % of $CD4^+$ T cells in the TME of PDAC are Tregs that contribute to immune suppression (Tan et al. 2009) and the ratio of Tregs to $CD4^+$ T cells correlated with shorter survival (Ino et al. 2013), while high abundance of Tregs correlated with cancer progression and worse survival (Hiraoka et al. 2006). Tregs have not only been high in number in the tumor, but also show higher FOXP3 expression (Stromnes et al. 2017). In particular, Tregs can induce increased expression of inhibitory receptors and decreased effector function, promoting an exhausted phenotype of tumor-infiltrating CTLs (Bauer et al. 2014). In addition, the engagement of CD11c and DCs by Tregs limited the interaction of DCs with $CD8^+$ T cells. Likewise, depletion of Tregs caused increased CTL-mediated anti-tumor immunity in PDAC (Jang et al. 2017).

1.3.1 Immunotherapy

The cancer immunity cycle describes the interaction between tumors and immune cells, in particular T cells. It starts with the release of an antigen due to the death of a cancer cell and subsequent presentation of the antigen to T cells by APCs. This causes the priming and activation of the T cell and migration and infiltration to the tumor. Here, the CTL recognizes and kills the cancer cells, causing the cycle to start again at the release of cancer cell antigens. In most cases, the cancer immunity cycle works well but fails when the T cell is supposed to kill the tumor cell. Therefore, treating cancer by modulating the immune system, for example by improving the killing capacity of T cells is a promising approach (Chen and Mellman 2013).

Immune checkpoint therapy does not target the tumor directly but aims to remove inhibitory pathways in T cells in order to prevent exhaustion and improve their effector function. The inhibitory receptors that can be targeted by immune checkpoint therapy include inter alia PD-1, LAG-3 and TIM-3 (Sharma and Allison 2015). However, not all patients respond to monotherapy (e.g., with anti-PD-1). Therefore, combinational therapies with more than one inhibitory receptor blocked or blockade of inhibitory receptors combined with treatment that target a cytokine or an inhibitory cell type are promising approaches (Wherry and Kurachi 2015; Jiang et al. 2015). For example, in a murine chronic infection model, treatment with anti-PD-1 in combination with IL-2 increased the amount and function of $CD8^+$ T cells and enhanced viral control (Hashimoto et al. 2022) and combination of PD-1 blockade, neoantigen vaccination and Treg depletion increased tumor rejection in a murine model (D'Alise et al. 2021).

PD-L1 is frequently expressed in PDAC causing the inhibition of anti-tumoral T cell response. Therefore, blockade of the PD-1/PD-L1 pathway is an interesting approach for immunotherapy of pancreatic cancer (Macherla et al. 2018). However, the efficiency of immune checkpoint inhibition in PDAC is limited (Brahmer et al. 2012; Royal et al. 2010; Saka et al. 2020; Bauer et al. 2016). This may be caused by a low number of neoantigens (caused by low tumor burden) and the strongly immunosuppressive TME. Therefore, immune checkpoint inhibition is only approved as a treatment for PDAC tumors with high microsatellite instability, which only account for 1-2 % of all cases (Orth et al. 2019).

1.4 Aim of this study

Successful immune response against pathogens and cancer is highly dependent on effector T cells. Their function or dysfunction is mediated by cytokines like IL-1β and IL-18. Therefore, the aim of this study was to further characterize the effects of the NLRP3-dependent cytokines IL-1 and IL-18 on the phenotypical and function plasticity of intratumoral T cells. Hereby, the focus was laid on:

I. The effect of IL-18 on T cell exhaustion *in vitro*
II. The molecular mechanisms of IL-18-mediated T cell exhaustion
III. The effects of IL-1β and IL-18 on Th1 and iTreg differentiation *in vitro*
IV. The effect of IL-1R and IL-18R signaling in adoptively transferred $CD4^+$ T cells on endogenous T cell populations in a murine pancreatic cancer model
V. The effect of IL-1R and IL-18R signaling in adoptively transferred $CD4^+$ T cells on tumor rejection and on the function of adoptively transferred tumor specific CTLs in a murine pancreatic cancer model.

2 Material

2.1 Laboratory Equipment

Table 1: List of laboratory equipment.

Device	Type	Company
Autoclave	VW 150 DX 65 DV 65	Systec GmbH (Linden, GER)
Balance	Precision balance Scout Pro 4000 g	Denver Instrument GmbH (Göttingen, GER) OHAUS (Parippany, USA)
Caliper	16FN	Mahr (Göttingen, GER)
Centrifuge	Megafuge 1.0R Heraeus Megafuge 8 Heraeus Mikro200R Zentrifuge Minicentrifuge Z100M	Thermo Fisher Scientific Inc. (Waltham, USA) Thermo Fisher Scientific Inc. (Waltham, USA) Hettich GmbH & Co. KG (Tuttlingen, GER) Hermle Labortechnik GmbH (Wehingen, D)
Flow cytometer	BD FACS Canto II CytoFLEX	Becton Dickinson (Franklin Lakes, USA) Beckman Coulter (California, USA)
Gel documentation	GEL Jet Imager with UV-Transilluminator	Intas Science Imaging Instruments (Göttingen, GER)
Gel electrophoresis	Easyphor gel electrophoresis chamber EC250-90 Electrophoresis Power Supply	Biozym Scientific GmbH (Hessisch Oldendorf, GER) EC Apparatus Corporation (Maynard, USA)
Heating block	ThermoCell Mixing Block MB-102	BIOER (Hangzhou, CN)
Ice machine	AF80	Scotsman Ice Systems (Mailand, IT)
Incubator	HERAcell 240iCo_2 Incubator	Thermo Fisher Scientific Inc. (Waltham, USA)

Magnet	MojoSort™ Magnet	Biolegend (San Diego, USA)
Magnetic stirrer	MR2000	Heidolph Instruments (Schwabach, GER)
Microscope	Light microscope Olympus IMT-2 Axio Cam MRm fluorescence microscope	Olympus Corporation (Tokyo, JPN) Carl Zeiss Sports Optics GmbH (Wetzlar, GER)
Microwave	VIP20, 900 W	Whirlpool (Benton Harbor, USA)
Spectrophotometer	Nanodrop 1000	Peqlab Biotechnologies GmbH (Erlangen, GER)
Neubauer chamber	Neubauer improved	Plan Optik AG (Elsoff, GER)
PCR machine	7500 Fast Real Time PCR Systems Thermocycler T100	Applied Biosystems (Foster City, USA) BioRad Laboratories GmbH (München, GER)
pH-meter	EL20	Mettler-Toledo (Columbus, USA)
Photometer	Multiskan FC Photometer	Thermo Fisher Scientific Inc. (Waltham, USA)
Pipettes	Discovery comfort Multichannel pipette (12-channel, 20-200 µl) Multipette® plus	Kinesis (Langenfeld, GER) Witeg Labortechnik (Wertheim, GER) Eppendorf AG (Haburg, GER)
Pipetting aid	Pipetboy acu	Integra Biosciences Deutschland GmbH (Biebertal, GER)
Shaker	3 DP PMS-1000i Microplate Shaker Orbital Shaker-Incubator ES_20	Ditabis (Pforzheim, GER) Grant (Cambridgeshire, GB) Grant Bio (Shepreth, GB)
Sterile bench	MSC Advantage	Thermo Fisher Scientific Inc. (Waltham, USA)
Storage devices	Fridge Premium (4 °C) Freezer Premium (-20 °C) Herafreeze HFU T series (-80°C)	Liebherr (Bulle, CH) Liebherr (Bulle, CH) Thermo Fisher Scientific Inc. (Waltham, USA)
Tally counter	4 digits	Veeder-Root (Weatogue, USA)

Vortex mixer	REAX 2000	Heidolph Instruments (Schwabach, GER)
Water bath	1002 1083	GFL (Burgwedel, GER)

2.2 Consumable materials

Table 2: List of consumable materials.

Product	Company
Cryotubes Cryo.s™ 2 ml	Greiner Bio-One (Kresmüster, AT)
Combitips advanced (500 µl – 5 ml)	Eppendorf (Hamburg, GER)
Tube, 5 ml, PP	Sarstedt AG & Co (Nümbrecht, GER)
Microtestplate 96 well, R	Sarstedt AG & Co (Nümbrecht, GER)
Microtestplate 96 well, V	Sarstedt AG & Co (Nümbrecht, GER)
Parafilm „M“ Laboratory FIl	Pechiney Plastic Packaging (Chicago, USA)
TC-dish 60	Sarstedt AG & Co (Nümbrecht, GER)
Depilatory cream	Veet (Slough, GB)
Flasks and beakers	Duran Group GmbH (Wertheim/Main, GER)
Pipette tips 10, 200 and 1000 µl	Sarstedt AG & Co (Nümbrecht, GER)
Reactions vessels 0.5, 1.5 and 2 ml	Sarstedt AG & Co (Nümbrecht, GER)
Centrifuge tubes 15 and 50 ml	Sarstedt AG & Co (Nümbrecht, GER)
Cannula 27G Sterican®	B. Braun (Melsungen, GER)
Cell strainer 30 µm	Miltenyi Biotec (Bergisch Gladbach, GER)
Cell strainer 70 µm	Miltenyi Biotec (Bergisch Gladbach, GER)
Scalpel	B. Braun (Melsungen, GER)
Disposable syringe, 1 ml	B. Braun (Melsungen, GER)
Multiply Pro vessel 0.2 ml, PP	Sarstedt AG & Co (Nümbrecht, GER)
Cell culture plates, 6-,12-,24-,48- and 96-well	Greiner Bio-One (Kresmüster, AT)
TC flask T75	Sarstedt AG & Co (Nümbrecht, GER)
TC suspension flask T75	Sarstedt AG & Co (Nümbrecht, GER)
Serological pipettes, 2-50 ml	Sarstedt AG & Co (Nümbrecht, GER)
Microamp™ Fast Optical reaction microtiter plate, 96-well	Sarstedt AG & Co (Nümbrecht, GER)

2.3 Chemicals and Enzymes

Table 3: List of chemicals.

Chemical	Company
12-O-Tetradecanoylphorbol-13-acetate (PMA)	Sigma Aldrich (St. Louis, USA)
Agarose Standard	Merck KGaA (Darmstadt, GER)
Ammonium chloride (NH_4Cl)	Carl Roth GmbH (Karlsruhe, GER)
Aqua ad.	B. Braun (Melsungen, GER)
Bovine serum albumin (BSA)	Sigma Aldrich (St. Louis, USA)
Brefeldin A	Sigma Aldrich (St. Louis, USA)
Citric Acid	Carl Roth GmbH (Karlsruhe, GER)
Collagenase D	Roche (Basel, CH)
DMSO	Carl Roth GmbH (Karlsruhe, GER)
DNase I, recombinant	Roche (Basel, CH)
dNTP Mix (10 mM)	Thermo Fisher Scientific Inc. (Waltham, USA)
Dulbecco's Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific Inc. (Waltham, USA)
Ethanol absolute (100 %)	Merck KGaA (Darmstadt, GER)
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH (Karlsruhe, GER)
Paraformaldehyde (4 %) (PFA)	Thermo Fisher Scientific Inc. (Waltham, USA)
HDGreen Plus DNA stain	Intas (Göttingen, GER)
Heparin	Ratiopharm (Ulm, GER)
Ionomycin	Sigma Aldrich (St. Louis, USA)
Methanol ≥ 98 %	Merck KGaA (Darmstadt, GER)
Potassium bicarbonate ($KHCO_3$)	Carl Roth GmbH (Karlsruhe, GER)
Sodium chloride (NaCl)	Merck KGaA (Darmstadt, GER)
Sodium hydroxide (NaOH)	Carl Roth GmbH (Karlsruhe, GER)
TAE buffer (10x)	Carl Roth GmbH (Karlsruhe, GER)
Tris (alkaline)	Carl Roth GmbH (Karlsruhe, GER)
Tris hydrochloride (Tris HCl)	Carl Roth GmbH (Karlsruhe, GER)
Triton X-100	Carl Roth GmbH (Karlsruhe, GER)

Trypan blue	Thermo Fisher Scientific Inc. (Waltham, USA)
Trypsin-EDTA (0.5 %, 10x)	Thermo Fisher Scientific Inc. (Waltham, USA)
Tween20	Carl Roth GmbH (Karlsruhe, GER)

2.4 Media, buffers, and additives

2.4.1 Media and buffers

Table 4: List of media and buffers.

Medium/buffer	Composition
Tumor medium	DMEM 10 % FCS (v/v) 1 % Pen-Strep (v/v)
Red blood cell (RBC) lysis buffer	155 mM NH_4Cl 10 mM KCO_3 0.1 mM EDTA ad H_2O
T cell medium	RPMI 10 % FCS (v/v) 3 % HEPES (v/v) 2 % Pen-Strep (v/v) 2 % NaPyr (v/v) 0.1 % 2-mercaptoethanol (v/v)
1 x TAE buffer	10 x TAE buffer ad H_2O
TBS	10 mM Tris HCl 150 mM NaCl ad H_2O pH 7.4
FACS buffer	PBS 3 % FCS
MACS buffer	0.5 % BSA (w/v) 2 mM EDTA ad PBS

	pH 7.2
Lyse buffer	25 mM NaoH 0.2 mM EDTA pH 12.0
Neutralization buffer	40 mM Tris HCl ad H_2O pH 5
Wash buffer	2 % FCS 0.2 % Tween20 ad PBS
ELISA wash buffer	0.05 % Tween20 ad PBS
Tumor lyse buffer	3 mg/ml Collagenase D 100 U/ml DNase I ad HBSS

2.4.2 Additives, cytokines, and inhibitors

Table 5: List of additives, cytokines, and inhibitors.

Product	Company
2-mercaptoethanol	Merck KGaA (Darmstadt, GER)
Akt inhibitor VIII (Akti)	Sigma Aldrich (St. Louis, USA)
CyclosporinA (CSA)	Sigma Aldrich (St. Louis, USA)
Dubecco's modified eagle's medium (DMEM)	Thermo Fisher Scientific Inc. (Waltham, USA)
Fetal calve serum (FCS)	Thermo Fisher Scientific Inc. (Waltham, USA)
G418 disulfate solution	Sigma Aldrich (St. Louis, USA)
Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific Inc. (Waltham, USA)
HEPES (1 M)	Thermo Fisher Scientific Inc. (Waltham, USA)
$OVA^{257-264}$ (SIINFEKL) (OVA)	Invivogen (San Diego, USA)
$OVA^{323-339}$ (ISQAVHAAHAEINEAGR) (ISQ)	Invivogen (San Diego, USA)

Penicillin-Streptomycin (Pen-Strep)	Thermo Fisher Scientific Inc. (Waltham, USA)
Rapamycin	Sigma Aldrich (St. Louis, USA)
recombinant human (rh) TGFβ_1	Peprotech (London, GB)
recombinant murine (rm) IL-12	Peprotech (London, GB)
rm IL-18	BioLegend (San Diego, USA)
rm IL-1β	Peprotech (London, GB)
rm IL-2	Peprotech (London, GB)
Rosewell Park Memorial Institute (RPMI) 1640 + L-Glutamin	Thermo Fisher Scientific Inc. (Waltham, USA)
Sodium Pyruvate (NaPyr) (100 mM)	Thermo Fisher Scientific Inc. (Waltham, USA)
STAT5 inihibitor (STAT5i)	Sigma Aldrich (St. Louis, USA)
Tacrolimus	Selleckchem (Houston, USA)

2.5 Kits and dyes

Table 6: List of kits and dyes.

Kit	**Company**
1st Strand cDNA Synthesis Kit	Thermo Fisher Scientific Inc. (Whatman, USA)
BD Phosflow™ Lyse/Fix-Buffer & BD Phosflow™ Perm/Wash-Buffer I	BD (Franklin Lakes, USA)
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific Inc. (Whatman, USA)
Fixation/Permeabilization Kit	BD (Franklin Lakes, USA)
iTaq Universal SYBR Green Supermix	BioRad Laboratories GmbH (München, GER)
LEGENDplex™ Human Inflammation Panel 1 with V-bottom Plate	BioLegend (San Diego, USA)
MojoSort Mouse CD4 Naive T Cell Isolation Kit	BioLegend (San Diego, USA)
MojoSort Mouse CD8 T Cell Isolation Kit	BioLegend (San Diego, USA)

Mouse IL-18 Uncoated ELISA Kit	Thermo Fisher Scientific Inc. (Whatman, USA)
peqGOLD Total RNA Kit	VWR (Darmstadt, GER)
Zombie Aqua™ Fixable Viability Kit	BioLegend (San Diego, USA)
Zombie NIR Fixable Viability Kit	BioLegend (San Diego, USA)

2.6 Antibodies

2.6.1 Antibodies for cell culture

Table 7: List of antibodies for cell culture.

Antibody	Clone	Company
Ultra-LEAF™ α-mCD122	TM-β1	BioLegend (San Diego, USA)
Ultra-LEAF™ α-mCD25	PC61	BioLegend (San Diego, USA)
Ultra-LEAF™ α-mIL-2	JES6-1A12	BioLegend (San Diego, USA)
anti-mCD28	37.51	BioLegend (San Diego, USA)
anti-mCD3ε	145-2C11	BioLegend (San Diego, USA)
anti-mIFN-γ	XMG1.2	BioLegend (San Diego, USA)
anti-mIL-4	11B11	BioLegend (San Diego, USA)

2.6.2 Antibodies for flow cytometry

Table 8: List of antibodies for flow cytometry.

Antigen	Reactivity	Conjugate	Clone	Company
Armenian Hamster IgG (Isotype)	armenian hamster	PE	HTK888	BioLegend (San Diego, USA)
CD121a (IL-1R, Type I/p80)	mouse	PE	JAMA-147	BioLegend (San Diego, USA)
CD122 (IL-2Rβ)	mouse	PE	TM-β1	BioLegend (San Diego, USA)
CD16/32 (Fc block)	mouse	purified	93	BioLegend (San Diego, USA)
CD218a (IL18Rα)	mouse	APC	A17071D	BioLegend (San Diego, USA)
CD223 (LAG-3)	mouse	APC	C9B7W	BioLegend (San Diego, USA)

CD223 (LAG-3)	mouse	PerCP/Cy5.5	C9B7W	BioLegend (San Diego, USA)
CD244.2 (2B4 Alloantigen)	mouse	PE/Cy7	m2B4 (B6)458.1	BioLegend (San Diego, USA)
CD25 (IL-2Rα)	mouse	APC/Cy7	PC61	BioLegend (San Diego, USA)
CD25 (IL-2Rα)	mouse	APC	PC61	BioLegend (San Diego, USA)
CD279 (PD-1)	mouse	PD-1	RMP1-30	BioLegend (San Diego, USA)
CD279 (PD-1)	mouse	APC	RMP1-30	BioLegend (San Diego, USA)
CD366 (Tim-3)	mouse	BV421	B8.2C12	BioLegend (San Diego, USA)
CD4	mouse	FITC	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	Alexa Fluor® 700	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	PE/Cy7	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	BV510	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	APC	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	PE	RM4-5	BioLegend (San Diego, USA)
CD4	mouse	FITC	RM4-5	BioLegend (San Diego, USA)
CD44	mouse/human	FITC	IM7	BioLegend (San Diego, USA)
CD44	mouse/human	PE	IM7	BioLegend (San Diego, USA)
CD45.1	mouse	APC/Cy7	A20	BioLegend (San Diego, USA)

CD45.1	mouse	PE/Cy7	A20	BioLegend (San Diego, USA)
CD45.1	mouse	PE	A20	BioLegend (San Diego, USA)
CD45.2	mouse	PerCP/Cy5.5	104	BioLegend (San Diego, USA)
CD45.2	mouse	APC/Cy7	104	BioLegend (San Diego, USA)
CD62L	mouse	APC/Cy7	MEL-14	BioLegend (San Diego, USA)
CD69	mouse	PE	H1.2F3	BioLegend (San Diego, USA)
CD69	mouse	PE	H1.2F3	BioLegend (San Diego, USA)
CD8a	mouse	FITC	53-6.7	BioLegend (San Diego, USA)
CD8a	mouse	Pacific Blue	53-6.7	BD (Franklin Lakes, USA)
CD8a	mouse	APC/Cy7	53-6.7	BioLegend (San Diego, USA)
CD8a	mouse	PerCP	53-6.7	BioLegend (San Diego, USA)
EOMES	mouse	PerCP eFluor710	Dan11mag	Thermo Fisher Scientific Inc. (Waltham, USA)
EOMES	mouse	Alexa Fluor® 488	Dan11mag	Thermo Fisher Scientific Inc. (Waltham, USA)
FOXP3	mouse	PE	3G3	Thermo Fisher Scientific Inc. (Waltham, USA)
Granzyme B	mouse	Pacific Blue	GB11	BioLegend (San Diego, USA)

IFN-γ	mouse	APC	XMG1.2	BioLegend (San Diego, USA)
IFN-γ	mouse	Alexa Fluor® 488	XMG1.2	BioLegend (San Diego, USA)
IL-10	mouse	BV421	JES5-16E3	BioLegend (San Diego, USA)
IL-17	mouse	PE	TC11-18H10.1	BioLegend (San Diego, USA)
IL-2	mouse	PE	JES6-5H4	BioLegend (San Diego, USA)
ki67	mouse	APC	16A8	BioLegend (San Diego, USA)
LAP (TGF-β1)	mouse	FITC	TW7-16B4	BioLegend (San Diego, USA)
Ly108	mouse	Pacific Blue	330-AJ	BioLegend (San Diego, USA)
Mouse IgG1, κ (Isotype)	mouse	Alexa Fluor® 488	MOPC-21	BioLegend (San Diego, USA)
Mouse IgG1, κ (Isotype)	mouse	APC	MOPC-21	BioLegend (San Diego, USA)
Mouse IgG1, κ (Isotype)	mouse	PE	MOPC-21	BioLegend (San Diego, USA)
Mouse IgG1, κ (Isotype)	mouse	PE/Cy7	MOPC-21	BioLegend (San Diego, USA)
Mouse IgG1, κ (Isotype)	mouse	FITC	MOPC-21	BioLegend (San Diego, USA)
Mouse IgG2a, κ (Isotype)	mouse	PE	MOPC-173	BioLegend (San Diego, USA)
Phospho-Akt (Ser473)	mouse	purified		Cell Signaling (Davers, USA)
Phospho-mTOR (Ser2448)	human/mouse	PE	MRRBY	Thermo Fisher Scientific Inc. (Waltham, USA)

Phospho-S6 (Ser235, Ser236)	human/mouse	APC	cupk43k	Thermo Fisher Scientific Inc. (Waltham, USA)
Phospho-S6 (Ser235, Ser236)	human/mouse	FITC	cupk43k	Thermo Fisher Scientific Inc. (Waltham, USA)
Phospho-STAT5 (Tyr694)	human/mouse	PE	SRBCZX	Thermo Fisher Scientific Inc. (Waltham, USA)
rabbit IgG	rabbit	FITC	Donkey Polyclonal Ig	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	Alexa Fluor® 488	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	APC	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	APC/Cy7	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	FITC	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	PE	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	PerCP/Cy5.5	RTK2071	BioLegend (San Diego, USA)
Rat IgG1, κ (Isotype)	rat	Alexa Fluor® 488	eBR2a	Thermo Fisher Scientific Inc. (Waltham, USA)
Rat IgG1, λ (Isotype)	rat	APC/Cy7	G0114F7	BioLegend (San Diego, USA)
Rat IgG1, λ (Isotype)	rat	APC	G0114F7	BioLegend (San Diego, USA)
Rat IgG2a, κ (Isotype)	rat	APC	RTK2758	BioLegend (San Diego, USA)

Rat IgG2a, κ (Isotype)	rat	Pacific Blue	RTK2758	BioLegend (San Diego, USA)
Rat IgG2a, κ (Isotype)	rat	PerCP eFluor710	eBR2a	Thermo Fisher Scientific Inc. (Waltham, USA)
Rat IgG2b, κ (Isotype)	rat	APC	RTK4530	BioLegend (San Diego, USA)
Rat IgG2b, κ (Isotype)	rat	BV421	RTK4530	BioLegend (San Diego, USA)
Rat IgG2b, κ (Isotype)	rat	PE	RTK4530	BioLegend (San Diego, USA)
T-bet	mouse/human	Alexa Fluor® 488	4B10	BioLegend (San Diego, USA)
T-bet	mouse/human	PE/Cy7	4B10	BioLegend (San Diego, USA)
TCR Vα2	mouse	APC	B20.1	BioLegend (San Diego, USA)
TCR Vα2	mouse	FITC	B20.1	BioLegend (San Diego, USA)
TNF	mouse	APC/Cy7	MP6-XT22	BioLegend (San Diego, USA)
TNF	mouse	FITC	MP6-XT22	Thermo Fisher Scientific Inc. (Waltham, USA)
TNF	mouse	PE	MP6-XT22	BioLegend (San Diego, USA)
TOX	mouse	eFluor 660	TXRX10	Thermo Fisher Scientific Inc. (Waltham, USA)
TOX	mouse	PE	TXRX10	Thermo Fisher Scientific Inc. (Waltham, USA)

2.7 Primer

2.7.1 Genotyping

Table 9: List of primers for genotyping.

Name	Sequence	Company
IL-18R WT+KO fw	TCA GAT TTC CCC TGG AAC TG	Biomers (Ulm, GER)
IL-18R WT rev	ACA GAG GCG AGA ACA AGC AC	Biomers (Ulm, GER)
IL-18R KO rev	CTC GTG CTT TAC GGT ATC GC	Biomers (Ulm, GER)
IL-1R WT fw	GGT GCA ACT TCA TAG AGA GAT GA	Biomers (Ulm, GER)
IL-1R KO fw	CTC GTG CTT TAC GGT ATC GC	Biomers (Ulm, GER)
IL-1R WT+KO rev	TTC TGT GCA TGC TGG AAA AC	Biomers (Ulm, GER)
FIR WT/tg fw	CAA AAC CAA GAA AAG GTG GGC	Biomers (Ulm, GER)
FIR WT rev	CAG TGC TGT TGC TGT GTA AGG GTC	Biomers (Ulm, GER)
FIR tg rev	GGA ATG CTC GTC AAG AAG ACA GG	Biomers (Ulm, GER)
OTI-Va2 fwd	CAGCAGCAGGTGAGACAAAGT	Biomers (Ulm, GER)
OTI-Va2 rev	GGCTTTATAATTAGCTTGGTCC	Biomers (Ulm, GER)
OTI-Vb2 fwd	AAGGTGGAGAGAGACAAAGGATTC	Biomers (Ulm, GER)
OTI-Vb2 rev	TTGAGAGCTGTCTCC	Biomers (Ulm, GER)
OTII-Va2 fwd	AAAGGGAGAAAAAGCTCTCC	Biomers (Ulm, GER)
OTII-Va2 rev	ACACAGCAGGTTCTGGGTTC	Biomers (Ulm, GER)
OTII-Vb2 fwd	GCTGCTGCACAGACCTACT	Biomers (Ulm, GER)
OTII-Vb2 rev	CAGCTCACCTA CACGAGGA	Biomers (Ulm, GER)

2.7.2 Real time quantitative PCR (RT qPCR)

Table 10: List of primers for RT qPCR.

Name	Sequence	Company
HPRT fwd	CTGGTGAAAAGGACCTCTCG	Biomers (Ulm, GER)
HPRT rev	TGAAGTACTCATTATAGTCAAGGGCA	Biomers (Ulm, GER)
T-bet fwd	CAACAACCCCTTTGCCAAAG	Biomers (Ulm, GER)
T-bet rev	TCCCCCAAGCAGTTGACAGT	Biomers (Ulm, GER)
IFN-γ fwd	TTCTTCAGCAACAGCAAGGC	Biomers (Ulm, GER)
IFN-γ rev	AGCTCATTGAATGCTTGGCG	Biomers (Ulm, GER)
TNF fwd	CCCTCACACTCAGATCATCTTCT	Biomers (Ulm, GER)

TNF rev	GCTACGACGTGGGCTACAG	Biomers (Ulm, GER)
FOXP3 fwd	GGCCCTTCTCCAGGACAGA	Biomers (Ulm, GER)
FOXP3 rev	GCTGATCATGGCTGGGTTGT	Biomers (Ulm, GER)
IL10 fwd	ACAACATACTGCTAACCGACTCC	Biomers (Ulm, GER)
IL10 rev	CAAATGCTCCTTGATTTCTGGGC	Biomers (Ulm, GER)
TGF-β fwd	CTCCCGTGGCTTCTAGTGC	Biomers (Ulm, GER)
TGF-β rev	GCCTTAGTTTGGACAGGATCTG	Biomers (Ulm, GER)

2.8 Mice strains

Table 11: List of mice strains.

Strain	Description	Origin
C57BL/6 Ly5.1	congenic marker CD45.1	Institute for Medical Microbiology and Hygiene, Phillips University Marburg
OT-I	transgenic OVA specific TCR on $CD8^+$ T cells	Institute for Medical Microbiology and Hygiene, Phillips University Marburg
OT-II FIR	transgenic OVA specific TCR on $CD4^+$ T cells; RFP-tagged FOXP3	Institute for Medical Microbiology and Hygiene, Phillips University Marburg Department of Gastroenterology, Endocrinology, Metabolism, and Infectiology, Phillips University Marburg
OT-II *Il18r*$^{-/-}$	transgenic OVA specific TCR on $CD4^+$ T cells; deficiency in IL-18R1	Department of Gastroenterology, Endocrinology, Metabolism, and Infectiology, Phillips University Marburg
OT-II *Il1r*$^{-/-}$	transgenic OVA specific TCR on $CD4^+$ T cells; deficiency in IL-1R1	Department of Gastroenterology, Endocrinology, Metabolism, and Infectiology, Phillips University Marburg
OT-II *Il18r*$^{-/-}$ FIR	transgenic OVA specific TCR on $CD4^+$ T cells; deficiency in IL-18R1, RFP-tagged FOXP3	Department of Gastroenterology, Endocrinology, Metabolism, and Infectiology, Phillips University Marburg
OT-II *Il1r*$^{-/-}$ FIR	transgenic OVA specific TCR on $CD4^+$ T cells; deficiency in IL-1R1, RFP-tagged FOXP3	Department of Gastroenterology, Endocrinology, Metabolism, and Infectiology, Phillips University Marburg

All mice strain were kept in the Animal Facility of the Philipps-University of Marburg in specific pathogen free (SPF) conditions and in individually ventilated cages. Husbandry conditions were a light-dark cycle of 12 h, 22 ± 2 °C and *ad libitum* food and water supply. For animal experiments animals had a minimum age of 8 weeks. Animal experiments were approved by the Regierungspräsidium Gießen (G89/2020).

2.9 Cell lines

Table 12: List of cell lines.

Cell line	Origin
Panc02	murine, methylcholanthrene-induced pancreatic cancer (Corbett et al. 1984)
PancOVA	stably transfected Panc02 cells expressing ovalbumin (Jacobs et al. 2011)

2.10 Software

Table 13: List of software.

Software	Company
Adobe Illustrator 2022	Adobe Systems (San José, USA)
Citavi 6.3	Swiss Academic Software GmbH (Wädenswil, CH)
FlowJo (Version 10)	BD (Franklin Lakes, USA)
GraphPad Prism 8	GraphPad Software (La Jolla, USA)
Microsoft Office	Microsoft (Redmond, USA)
Microsoft Windows 10	Microsoft (Redmond, USA)
LEGENDplexXM data analysis software	BioLegend (San Diego, USA)
FACS Diva (8.0.1)	BD (Franklin Lakes, USA)
CytExpert Software	Beckman Coulter (California, USA)
7500 Fast System Software	Applied Biosystems by Life Technologies (Waltham, USA)
AxioVision Rel. 4.8	Carl Zeiss (Jena, GER)
Benchling platform	Benchling Inc. (San Francisco, USA)
Intas GDS	INTAS Science Imaging (Göttingen, GER)
NanoDrop Software	Thermo Fisher Scientific Inc. (Waltham, USA)

3 Methods

3.1 Cell culture

3.1.1 Cell cultivation

Panc02 and stably with $OVA^{257-264}$ transfected PancOVA cells were cultivated T75 flasks at 37 °C, 5 % CO_2 in tumor cell medium (DMEM containing 10 % FCS and 1 % Pen-Strep). For selection of PancOVA cells, 500 mg/ml G418 was added. To split the cells, medium was removed, cells were washed with PBS and incubated with Trypsin EDTA for 5 min at 37 °C. Medium was added, cells were centrifuged at 400 x g for 5 min and resuspended in fresh medium. According to the confluence and growth rate of the cells, they were seeded in to a new T75 flask.

3.1.2 Freezing and thawing of cells

Cells were cultivated and removed from the flask as described above. After centrifugation, cells were resuspended in FCS containing 10 % DMSO, counted and transferred to cryotubes. Cryotubes were placed in a freezing container at -80 °C and transferred to liquid nitrogen after 24 h.

Frozen Panc02 and PancOVA cells in cryotubes were removed from liquid nitrogen and immediately placed in a water bath at 37 °C until cells suspension was thawed. Cells were then transferred to pre-warmed tumor cell medium containing G418 for selection of PancOVA cells and cultivated at 37 °C, 5 % CO_2. After 24 h, the medium was removed and replaced with fresh tumor cell medium containing G418.

3.1.3 Counting of cells

For cell counting, cells were diluted with trypan blue in an appropriate ratio. 10 µl were placed in a Neubauer chamber. Cells were counted in the four quadrants and cell number per ml was calculated according to the following formula:

$$\frac{cell\ number\ in\ four\ quadrants}{4}\ x\ dilution\ factor\ x\ 10{,}000\ (chamber\ factor)$$

3.1.4 Mycoplasma testing

Mycoplasma testing was performed on a regular basis to ensure that Panc02 and PancOVA cells were free of mycoplasma contamination. Cells were seeded in a six well plate as described above and incubated for 24 h at 37 °C, 5 %. Medium was removed and cells were washed with PBS. 3.7 % formaldehyde solution was added to the cells, incubated for 10 min, and removed. Afterwards, cells were accordingly incubated with -20 °C cold methanol for 1 min, PBS for 5 min, TBS + 0.5 %

Triton X-100 for 10 min and 2 % BSA in TBS/Triton for 30 min. Afterwards, DAPI staining was added to the cells and incubated for 10 min followed by an incubation step with 2 % BSA in TBS/Triton for 5 min. Cells were than analyzed for mycoplasma contamination using a microscope.

3.2 *In vitro* T cell cultivation

3.2.1 Differentiation of CD4+ T cell subsets

For *in vitro* differentiation of OT-II $CD4^+$ T cells, OT-II mice were killed, spleen and lymph nodes were isolated and mashed through a 30 µm filter with HBSS. Cells were washed (centrifuged and 400 x g), resuspended in RBC lyse buffer and incubated at room temperature (RT) for 5 min. HBSS was added, cells were again washed and counted. $4x10^7$ cells per ml were incubated with 5 µM of the $OVA^{323-339}$ peptide ISQ at 37 °C for 1 h. Cells were washed and plated on a 96 well F-bottom plate ($4x10^5$ cells per well), 48 well F-bottom plate ($1x10^6$ cells/well) or 24 well F-bottom plate ($4x10^6$ cells per well) in T cell medium (RPMI containing 10 % FCS, 2 % Pen-Strep, 3 % HEPES, 2 % NaPyr and 0.1 % 2-Mercaptoethanol) for four days at 37 °C, 5 % CO_2. For Th1 and iTreg differentiation, the following components were added to the medium.

Table 14: Th1 cells/iTreg differentiation medium *in vitro*.

Th1 cells	iTregs
10 ng/ml IL-2	TGFβ
10 ng/ml IL-12	1 µg/ml αIL-4
1 µg/ml αIL-4	1 µg/ml αIFN-γ
	2.5 ng/ml TGFβ

Cells were additionally cultivated with or without 10 ng/ml IL-18 and 10 ng/ml IL-1β.

3.2.2 *In vitro* T cell exhaustion assay

For the *in vitro* T cell exhaustion essay, $CD8^+$ cells were isolated using the MojoSort™ Mouse CD8 T Cell Isolation Kit from Biolegend.

The day before $CD8^+$ T cell isolation, a 24 well plate was coated with 1 µg/ml anti-CD3 and 2 µg/ml anti-CD28 which was added to the respective wells in 1 ml PBS. The plate was incubated at 4 °C overnight.

OT-I mice were killed, spleen and lymph nodes were isolated, mashed through a 30 µm filter with HBSS and washed (centrifuged at 400 x g). Cells were resuspended in HBSS, passed through a 70 µm filter, counted, and adjusted to $1x10^8$ cells/ml in MACS buffer. 10 µl biotin-antibody cocktail per $1x10^7$ cells was added and after 15 min incubation at 4 °C 10 µl streptavidin nanobeads,

followed by 15 min incubation at 4 °C. 2.5 ml MACS buffer was added, and the suspension was transferred into a FACS tube which was placed in a MojoSort™ magnet for 5 min. The liquid was poured out into a fresh tube and another 2.5 ml of MACS buffer was added to the FACS tube, which was again placed at the magnet for 5 min and the liquid was poured out into the same tube as before. Cells were washed, counted and purity (% $CD8^+$ cells) was determined using flow cytometry.

$1x10^6$ cells per well were seeded in coated wells of a 24 well F-bottom plate in T cell medium containing 20 ng/ml IL-2 with or without 10 ng/ml IL-18 and incubated at 37 °C, 5 % CO_2 for 48 h.

After 48 h (day 2), cells were harvested, counted, and seeded in 96 well R-bottom plates ($1x10^5$ cells/well) in T cell medium containing 20 ng/ml IL-2 with or without 10 ng/ml IL-18. For repetitive stimulation, 10 ng/ml $OVA^{257-264}$ was added. A control group was left untreated and cultivated without IL-18 and without $OVA^{257-264}$ throughout the assay.

For inhibitor treatments, the following inhibitors in respective concentrations were added to the culture medium.

Table 15: Inhibitors/antibodies and concentrations used in the *in vitro* T cell exhaustion model.

Inhibitor/antibody	iTregs
Tacrolimus	1 nM
CSA	1 nM
anti-CD25	50 µg/ml
anti-IL-2	50 µg/ml
STAT5i	100 nM
Akti	2 nM
Rapamycin	0.05 nM

After another 48 h of incubation (day 4), cells were split 1:2 into the same conditions as before, incubated for 48 h (day 6) and split again. Cells were then cultivated in the same conditions as before but without $OVA^{257-264}$ treatment for another 48 h. After the total cultivation time of 192 h (8 days), cells were harvested, stained for viability, surface markers, transcription factors, cytokines and phosphorylation of molecules and analyzed using flow cytometry.

3.3 Animal experiments

For tumor inoculation, $1x10^6$ PancOVA cells in 100 µl PBS were injected subcutaneously (s.c.) into the left flank of Ly5.1 mice at day zero accompanied by intraperitoneal (i.p.) injection of $1x10^6$

naïve OT-II WT, *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ cells in 100 µl PBS. For experiments that focused on the *ex vivo* analysis of T cells, i.p. injection of naïve CD4^{+} T cells was repeated at day 5 and mice were killed at day 10. For growth curve experiments naïve CD4^{+} T cells were injected i.p. at day 0, 5, 10 and 15 and mice were killed at day 20.

For *ex vivo* analysis of cells in the blood, tumor draining lymph node and tumor, mice were killed by cervical dislocation and blood was immediately drained from the heart using a syringe. Spleen, tumor-draining lymph node and tumor was isolated afterwards and placed in HBSS.

Blood was placed in PBS containing 50 µl/ml heparin, centrifuged, and incubated with 5 ml red RBC lysis buffer for 5 min. Reaction was stopped by adding 5 ml HBSS, cells were washed (centrifuged at 400 x g for 5 min) and lysis was repeated if needed.

Spleens and lymph nodes were mashed through 30 µm filters, spleen cells were treated with RBC lysis buffer as described above and spleen and lymph node cells were washed.

Tumors were chopped into 1-2 mm pieces, placed in tumor lysis buffer, and digested at 37 °C for 45 min. Cells were mashed through 30 µm filters, RBC lysis was performed (if needed) and washed.

After preparation of organs, cells were resuspended in appropriate amounts of PBS and counted. Afterwards, cells were stained for surface markers, transcription factors and cytokines and analyzed via flow cytometry.

Mice in the animal experiments were monitored daily and weight and tumor growth were reported. For growth curve experiments, tumor sizes were used in order to track the growth and rejection of tumors. Hereby, the length and width of tumors were measured, and total tumor size was calculated using the following formula: $length\ x\ width^2\ x\ 0.5$

3.3.1 Purification of naïve OT-II CD4^{+} T cells

Naïve CD4^{+} T cells, which were injected into tumor bearing mice at day 0, 5, 10 and 15 were isolated from OT-II FIR, OT-II *Il18r*$^{-/-}$ and OT-II *Il1r*$^{-/-}$ mice using the MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit from Biolegend. Apart from components of the kit, the procedure was the same as described above for CD8^{+} T cells. After purification, CD4^{+} T cells were checked for purity (% of CD4^{+} CD62L^{+} cells) and 1x10^{6} cells in 100 µl PBS were injected into the mice.

3.3.2 Differentiation of OT-I CTLs

OT-I mice were killed at day 2 of the animal experiment, spleen and lymph nodes were isolated, mashed through a 30 µm filter and washed (centrifuged at 400 x g). Cells were resuspended in RBC lysis buffer, incubated for 5 min at RT, washed with HBSS and counted. 4x10^{7} cells/ml were

incubated in T cell medium containing 5 µM $OVA^{257-264}$ at 37 °C for 1 h. Cells were washed, resuspended in T cell medium and cultivated in a T75 suspension flask in T cell medium containing 10 ng/ml IL-12 at 37 °C, 5 % CO_2. After 48 h cells were washed and placed in fresh T cell medium containing 20 ng/ml IL-2, which was repeated after another 48 h. After 24 h (day 7 of the animal experiments) cells were harvested, counted, analyzed for CTL specific surface markers and cytokines using flow cytometry and injected into tumor bearing mice.

3.4 Flow cytometry

For flow cytometric analysis cells were stained for viability, surface molecules, transcription factors, intracellular cytokines and phosphorylated proteins using in staining protocols dependent on the molecule of interest. Staining was performed either in FACS tubes or in V-bottom plates. After staining, cells were analyzed on a BD FACSCanto™ or on a CytoFlex- Lx.

3.4.1 Phenotyping of OT-I and OT-II mice

Expression of the transgenic T cell receptor in OT-I and OT-II mice was determined by flow cytometric analysis of the blood. 20 µl blood were taken from the tail of the mice and 1 ml RBC lyse buffer was added. After 5 min, samples were centrifuged at 400 x g, washed with FACS buffer (centrifuged at 400 x g), and stained for CD4 (for OT-II mice) or CD8 (for OT-mice) and Vα2 expression for 30 min at RT. Cells were washed again and analyzed on a flow cytometer. Blood from mice that showed a clear population of $CD4^+/CD8^+$ and $V\alpha2^+$ cells were identified as $OT\text{-}II^+/OT\text{-}I^+$ respectively.

3.4.2 Live/Dead staining

Live/Dead staining was performed using Zombie Fixable Viability Kits from Biologend according to the manual. Cells were washed with PBS and incubated with Zombie dye (1:1000 in PBS) for 15 min at RT. For animal experiments, live/dead staining was accompanied by blocking of Fc receptors using anti-CD16/32 antibody. Cells were washed and further staining was performed if needed.

3.4.3 Surface staining

For surface staining, cells were washed with FACS buffer and stained with the respective antibodies (in FACS buffer) for 30 min at 4 °C. Cells were washed and further stained or analyzed on a flow cytometer.

3.4.4 Transcription factor staining

For transcription factor staining, Foxp3/Transcription Factor Staining Buffer Set from Thermo Fisher Scientific Inc. was used according to the manual. Cells were washed with PBS and fixed with

fixation/permeabilization concentrate (diluted 1:4 diluted in fixation/permeabilization dilutant) for 20 min at 4 °C. Cells were washed with FACS buffer and with permeabilization buffer (diluted 1:10 in water) and incubated for 30 min at 4 °C with antibodies against transcription factors in permeabilization buffer followed by washing with permeabilization buffer and FACS buffer. Staining of ki67 was performed accordingly, but with an additional incubation step with permeabilization buffer for 20 min at 4°C prior to staining.

3.4.5 Intracellular cytokine staining

For intracellular cytokine staining, cells were restimulated for 4 h at 37 °C either in an antigen-specific manner (in RPMI containing 1 µg/ml ISQ (for OT-II cells) or 100 ng/ml $OVA^{257-264}$ (for OT-I cells) and 1 µg/ml Brefeldin A) or in an antigen-unspecific manner (in RPMI containing 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 µg/ml ionomycin and 1 µg/ml Brefeldin A). *In vitro* differentiated Th1 cells were restimulated in an antigen-specific and in an antigen-unspecific manner. $CD8^+$ T cells from the *in vitro* exhaustion model were restimulated in an antigen-specific manner. In *ex vivo* experiments cells were restimulated in an antigen-unspecific manner.

After restimulation (and surface staining), cells were stained for intracellular cytokines using the Fixation/Permeabilization Kit from BD according to the manual. Cells were washed with PBS and incubated with fixation/permeabilization solution for 20 min at 4 °C. Cells were washed with FACS buffer and BD Perm/Wash™ Buffer (diluted 1:10 in water) and incubated with antibodies against cytokines in BD Perm/Wash™ Buffer for 30 min at 4 °C followed by washing steps with BD Perm/Wash™ Buffer and FACS buffer.

3.4.6 Phospho-protein staining

Phospho-protein staining was performed based on the molecules of interest by either using PFA fixation and methanol permeabilization or the BD Phosflow™ system.

For detection of phosphorylated STAT5 (p-STAT5) and phosphorylated Akt (p-Akt) cells were washed with PBS and fixed with 400 µl 2 % PFA (diluted in PBS) for 10 min at 37 °C. Afterwards, cells were permeabilized by slowly adding 3.6 ml ice-cold 98 % methanol, causing a total volume of 4 ml. Cells were incubated for 30 min on ice, centrifuged and washed with wash buffer. Cells were incubated with antibodies targeting the phosphorylated proteins (diluted in wash buffer) for 45-60 min on ice and washed with wash buffer. For detection of p-Akt, purified antibodies were used and therefore cells were incubated with fluorescence-labelled secondary antibodies targeting the primary antibodies (diluted in wash buffer) for another 30 min. Cells were washed with wash buffer and FACS buffer and analyzed.

For detection of phosphorylated mTOR (p-mTOR) and phosphorylated S6 (p-S6) cells were washed with PBS, fixed with BD Phosflow™ Lyse/Fix-Buffer (diluted 1:5 in water) for 12 min at 37 °C and washed with FACS buffer. BD Phosflow™ Perm/Wash Buffer I (diluted 1:10 in water) was added and cells were incubated at 4 °C for 30 min and washed twice with FACS buffer. Cells were incubated with antibodies targeting the phosphorylated proteins (diluted in FACS buffer) for 60 min on ice and washed with FACS buffer.

3.4.7 LEGENDplex™ assay

In order to determine the concentration of specific cytokines, IFN-γ, TNF, IL-10, IL-18 and IL-1β, in the serum of pancreatic cancer patients the LEGENDplex™ Human Inflammation Panel (13-plex) with V-bottom Plate from Biolegend was used according to the manual. Patient serum samples were provided by the Comprehensive Biomaterial Bank Marburg.

Serum samples were diluted with Assay Buffer and loaded on a V-bottom plate. Premixed beads were added to each well and the plate was incubated at RT for 2 h on a plate shaker (500 rpm). The plate was centrifuged, supernatant was removed, and detection antibody was added to each well followed by 1 h incubation at RT on a plate shaker (500 rpm). SA-PE was added to each well and the plate was incubated for 30 min at RT on a plate shaker (800 rpm). The plate was washed twice with wash buffer and 100 µl wash buffer was added to each well prior to measurement.

Samples were measured alongside standard samples with defined concentrations of cytokines on a BD FACSCanto™.

3.8 Polymerase chain reaction (PCR)

3.8.1 Genotyping

In order to obtain genomic DNA of transgenic mice strains, ear tags from respective mice were placed in a reaction tube and 75 µl alkaline lyse reagent was added. Tubes were placed on a heat block at 95 °C for 1 h, and subsequently on ice for 3 min. Lyse reaction was stopped by adding 75 µl neutralization reagent and samples were stored at -20 °C.

PCR was used in order to detect gene expression of FOXP3-IRES-mRFP (FIR), mice, which express a transgenic FOXP3 gene tagged with red fluorescent protein (RFP) and *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ mice, which are deficient in IL-18 and IL-1R expression, respectively. Additionally, expression of the transgenic T cell receptor in OT-I and OT-II mice was also determined using PCR.

The following reagents were mixed per sample in order to perform the PCR.

Table 16: Reagents for PCR per sample.

Reagent	Volume [µl]
H_2O	14.75
Buffer	5.0
Primer fwd	1.25
Primer rev	1.25
dNTPs	0.5
GoTaq polymerase	0.25
Sample	2
Total	25

For detection of the FIR, *Il18r*$^{-/-}$ and *Il1r*$^{-/-}$ allele PCR was performed using the following program.

Table 17: PCR program for FIR, *Il18r*$^{-/-}$ and *Il1r*$^{-/-}$ allele

Step	Temperature [°C]	Time [s]	comment
1	94	120	
2	94	20	
3	65	15	-0.5 °C per cycle decrease
4	68	10	
5			Repeat step 2-4 for 10 cycles (Touchdown)
6	94	15	
7	60	15	
8	72	19	
9			Repeat steps 6-8 for 28 cycles
10	72	120	
11	10		hold

For detection of the Vα2 and Vβ2 alleles, the PCR was performed using the following program.

Table 18: PCR program for Vα2 and Vβ2 alleles.

Step	Temperature [°C]	Time [s]	comment
1	95	30	
2	60	45	
3	72	120	
4			Repeat step 2-3 for 32 cycles
5	10		hold

In order to analyze the PCR products, a 1 % agarose solution was prepared and 1 µl HD Green-Plus was added. The agarose gel was poured and placed in a gel chamber containing 1 x TAE running buffer once it was solid. The samples from the PCR were placed in the pockets of the gel and bands were separated at 120 V for 30 min. In order to make the bands visible, the gel was irradiated with UV light.

3.8.2 RNA isolation, cDNA synthesis and RT qPCR

RT qPCR was performed in order to quantify the expression of genes of interest in *in vitro* differentiated OT-II Th1 cells and Tregs. For this purpose, OT-II splenocytes were cultivated on 24 well F-bottom plates ($4x10^6$ cells per well) under Th1 and Treg conditions with or without additional IL-18 and IL-1β treatment as described above. After 4 days of cultivation, cells were harvested, washed and supernatant was removed. The cell pellets were placed at -20 °C until RNA isolation was performed.

3.8.2.1 RNA isolation

RNA isolation was performed using the peqGOLD total RNA isolation kit by VWR according to the manual. Cells were resuspended in 350 µl lysis buffer and loaded on the peqGOLD RNA homogenizer column which was placed in a 2 ml collection tube. The sample was centrifuged at 1,200 x g for 2 min in order to collect the lysate. 350 µl 70 % ethanol was added, and the solution was mixed. The total volume (700 µl) was loaded on a peqGOLD RNA Mini Column placed in a 2 ml collection tube and centrifuged at 10,000 x g for 1 min. The flowthrough was discarded, 500 µl RNA wash buffer I was added to the column followed by a centrifugation step at 10,000 x g for 30 s. Flowthrough was again discarded, 500 µl 80 % ethanol was added to the column and the sample was centrifuged at 10,000 x g for 1 min. The washing step with ethanol was repeated and the empty column was centrifuged until it was dry. The column was placed into a fresh 1.5 ml collection tube and 50 µl nuclease free water was added to the column followed by a centrifugation step at maximum speed for 2 min in order to collect the RNA. The RNA concentration was determined using a NanoDop™ spectrophotometer and samples were stored at -80 °C until further processing.

3.8.2.2 cDNA synthesis

The RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher Scientific Inc. was used to subscribe RNA into cDNA. cDNA synthesis was performed according to the manual; 1 µg of total RNA was mixed with 1 µl Oligo$(dT)_{18}$ primer and nuclease water to a total volume of 12 µl. Subsequently, 4 µl 5X Reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl dNTP Mix and 1 µl RevertAid M-MuLV RT was added. The total volume of 20 µl was gently mixed and centrifuged

and samples were incubated at 42 °C for 60 min followed by a 5 min incubation step at 70 °C. The samples were diluted 1:50 to a final concentration of 1 ng/µl spectrophotometer and stored at -20 °C until further processing.

3.8.2.3 RT qPCR

RT qPCR was performed using the iTaq™ Universal SYBR® Green Supermix by Bio-Rad according to the manual. The following reaction mix was prepared for one sample.

Table 19: RT qPCR reaction mix per sample.

Reagent	Volume [µl]
iTaq™ Universal SYBR® Green Supermix (2X)	10
Primer fwd + rev	0.4 + 0.4
DNA template	5
GoTaq polymerase	0.25
H_2O	3.95
Total	20

RT qPCR was performed with Stratagene Mx3005P qPCR Sytem (Agilent) and the following program.

Table 20: RT qPCR cycling program.

Step	Temperature [°C]	Time
Denaturation	95	15 min
Amplification (40X)	94 55 72	30 s 30 s 30 s
Dissociation curve	95 55 55-95	1 min 30 s

The cycle threshold (Ct) values of a housekeeping gene and genes of interest obtained via RT qPCR were used to calculate the relative expressions of the genes of interest.

3.9 Enzyme-linked immunosorbent assay (ELISA)

For detection of IL-18 concentrations in the supernatant of *ex vivo* PancOVA tumors, tumors were isolated from mice seven days after tumor inoculation and cut into small pieces. 50 mg tumor was placed in 1 ml T cell medium and incubated at 37 °C, 5 % CO_2 for 24 h. Supernatant was collected and stored at -20 °C until further processing.

ELISA was performed using the Mouse IL-18 Uncoated ELISA Kit by Thermo Fisher Scientific Inc. according to the manual. 96 well plates were coated with 100 µl capture antibody per well (diluted 1:250 in coating buffer) overnight at 4 °C. Wells were washed twice with wash buffer and blocked with 200 µl blocking buffer for 2 h at RT. Samples and standard samples were placed in the wells diluted 1:2 with sample dilutant and 50 µl of detection antibody (diluted 1:250 in assay buffer) was added to each well. The plate was incubated at RT for 2 h on a plate shaker (400 rpm) and washed three times with wash buffer. 100 µl Streptavidin-HRP (diluted 1:100 in assay buffer) was added to each well followed by an incubation time of 1 h at RT on a plate shaker (400 rpm). The wells were washed three times with wash Buffer and 100 µl substrate solution was added per well. The plate was incubated for 15 min at RT and 100 µl stop solution were added per well. The plate was measured using a Multiskan™ FC Mikrotiterplate-photometer at 450 and 570 nm in order to calculate the standard curve and the respective concentrations of IL-18 in the samples.

3.10 Data analysis and statistics

Data obtained via flow cytometry was analyzed using the FlowJo™ (version 10) Software from BD apart from LEGENDplex^{XM} results which were analyzed using the LEGENDplex^{XM} data analysis software from Biolegend. Calculations of tumor size, IL-18 concentrations in tumor supernatant and relative gene expressions were performed using Microsoft Excel.

Data was further analyzed using GraphPad Prism (version 8). Outlier removal was performed using the ROUT method (Q =1 %) and normal distribution was evaluated by Shapiro-Wilk tests. If two groups were compared, unpaired, two-tailed t-test was performed. If samples were not normally distributed Mann-Whitney test was used. If variances differed significantly (evaluated by F test to compare variances) unpaired, two-tailed t-test with Welch's correction was performed. When more than two groups were compared Ordinary one-way ANOVA followed by Tukey's multiple comparisons (when each group was compared to every other group) or Dunnett's multiple comparisons (when groups were compared to a control group) was performed. For not normally distributed data Kruskal-Wallis test with Dunn's multiple comparisons was performed. When variances were significantly different between groups (evaluated by Brown-Forsythe test) Brown-Forsythe and Welch ANOVA test followed by Dunnett's T3 multiple comparisons was performed. When more than two groups, which were dependent on two factors were analyzed, two-way ANOVA was performed with Tukey's multiple comparison. Differences between tumor rejection in tumor growth curve experiments were evaluated using two-way ANOVA with subsequent Dunnet's multiple comparisons against a control group.

As a critical value for significance p=0.05 was used and marked with an asterisk (*). As further thresholds p=0.01 (**), p=0.01(***) and p=0.0001(****) were chosen and marked with the respective number of asterisks.

4 Results

4.1 Establishing OT-II *Il18r*$^{-/-}$ and OT-II *Il1R*$^{-/-}$ mice strains

OT-IIx*Il18r*$^{-/-}$ and OT-IIx*Il1r*$^{-/-}$ mice strains were established in order to investigate the influence of IL-18R and IL-1R signaling on differentiation of antigen-specific CD4^{+} T cells *in vitro* and *in vivo*. OT-II mice express a transgenic T cell receptor which specifically recognizes the ovalbumin peptide OVA$^{323-339}$ (ISQ). OT-II mice were crossed with mice deficient for IL-18R (*Il18r*$^{-/-}$) or IL-1R (*Il1r*$^{-/-}$). Expression of the transgenic T cell receptor was either assessed via staining for Vα2 and FACS analysis (Fig. 1A) or via PCR (Fig. 1B). Additionally, mice were evaluated for IL-18 or IL-1R deficiency via PCR (Fig. 1C, D). Homozygous mice were used for the experiments.

For future studies focusing on the influence of IL-18R and IL-1R signaling on differentiation of antigen-specific Tregs, OT-II *Il18r*$^{-/-}$ and OT-II *Il1r*$^{-/-}$ mice were crossed with OT-II FIR mice, which express RFP-tagged FOXP3. Offspring of those mice strains were assessed for OT-II expression and receptor deficiency as above. Additionally, those strains were evaluated for expression of FIR via PCR (Fig. 1E).

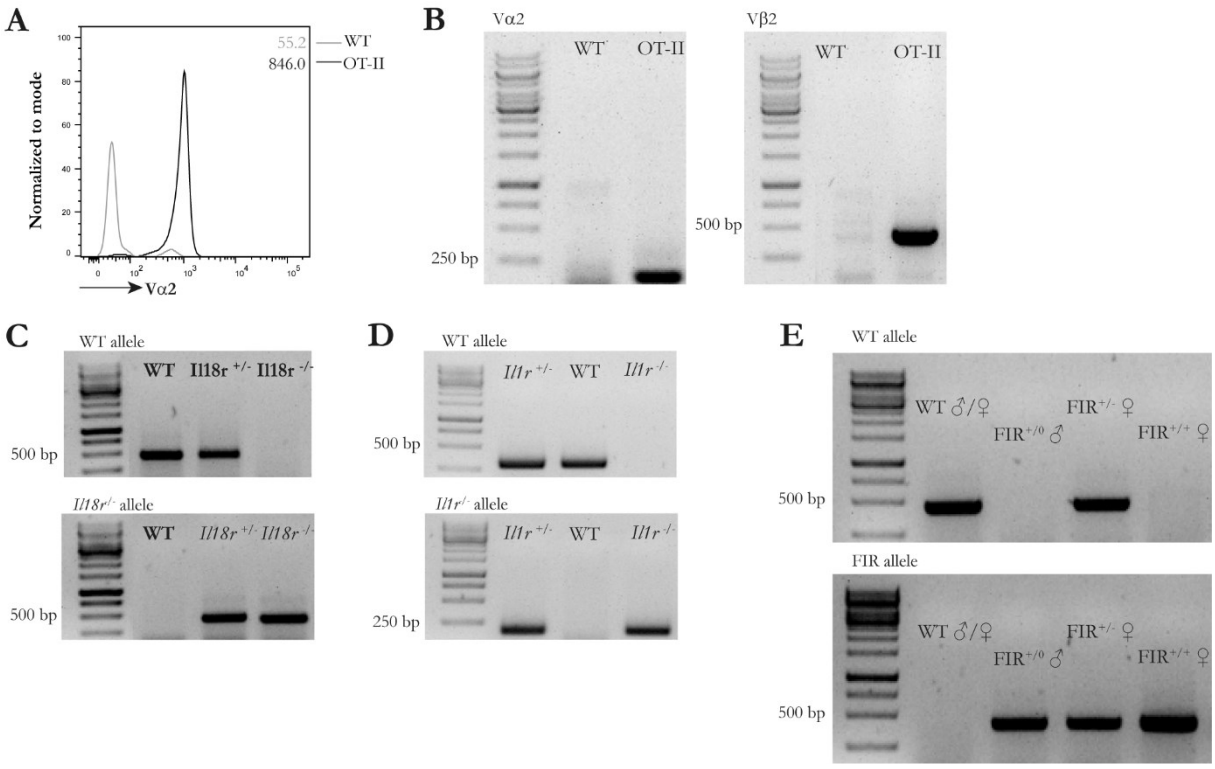


Figure 1: Pheno-/ Genotyping of newly established mice strains. A) Peripheral blood of mice was stained and evaluated for expression of the transgenic T cell receptor Vα2 via FACS. DNA was isolated from ear tags of mice and PCR was performed to test for B) transgenic T cell receptor Vα2 (160 bp) and Vβ2 (500bp), C) WT (538 bp) and *Il18r*$^{-/-}$ (550 bp) allele, D) WT (310 bp) and *Il1r*$^{-/-}$(150 bp) allele and E) WT (510 bp) and FIR (470) allele.

4.2 Cytokine profile in serum of PDAC patients

In this study, the NLRP3 dependent cytokines IL-18 and IL-1β and the Th1- and Treg-specific cytokines IFN-γ/TNF and IL10, respectively, play a leading role. Therefore, the concentration of those cytokines in serum of PDAC patients was analyzed via multiplex assay.

IL-18 levels ranged between 55.12 pg/ml and 1131 pg/ml around the average of 287.9 pg/ml. IL-1β levels were markedly lower with an average of 35.98 pg/ml and a range between 0 pg/ml and 563.8 pg/ml (Fig. 2A). IFN-γ and TNF, were found at low levels with an average of 5.131 pg/ml (IFN-γ; 0-32.99 pg/ml) and 33.23 pg/ml (TNF; 0-104.8 pg/ml). IL-10 was found at higher levels with an average of 41.4 pg/ml and a range between 0.7250 pg/ml and 352.9 pg/ml (Fig. 2B).

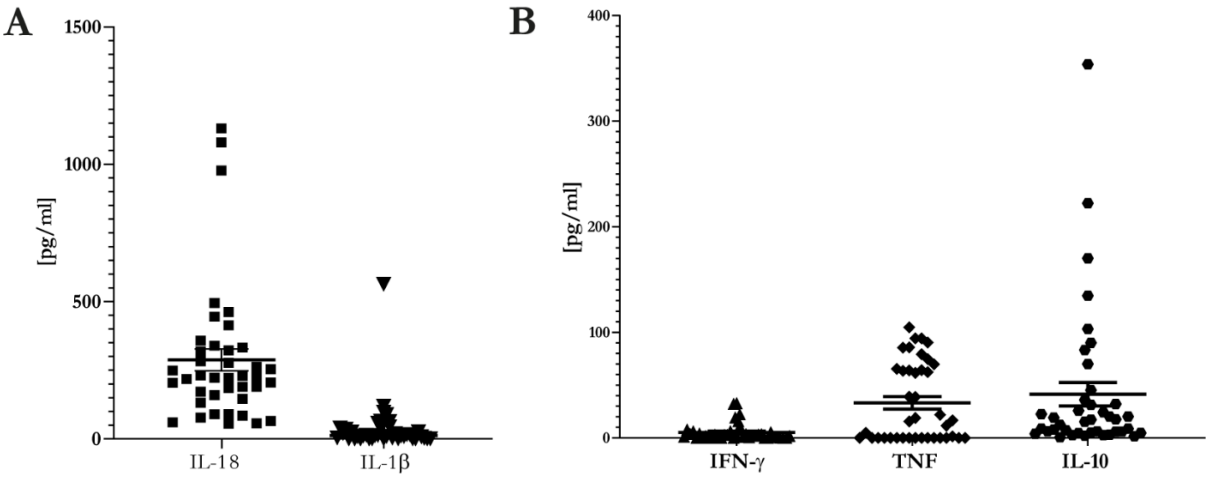


Figure 2: Cytokine levels in serum of PDAC patients. Multiplex analysis for serum of PDAC patients was performed to detect the levels of A) the NLRP3-dependent cytokines IL-18 and IL-1β and B) the Th1 or Treg specific cytokines IFN-γ, TNF and IL-10. Each data point represents one biologic replicate (n=37), graphs show mean ± SEM.

Interestingly, the average IL-18 level in serum of PDAC patients in the present study was higher than IL-18 levels in the serum of healthy subjects previously reported. These include for example an average IL-18 level of approximately 2.3 pg/ml (Ahmed et al. 2022), 64.17 pg/ml (Novick et al. 2001), 84.7 pg/ml (Bellone et al. 2006), approximately 230 pg/ml (Poch et al. 2007) and < 100 pg/ml (Kleiner et al. 2013). Furthermore, IL-18 levels reported in this study were higher than IL-18 level in serum of PDAC patients reported by two previous studies; approximately 2.0 pg/ml ($\log_{10}$) (Ahmed et al. 2022) and 192.8 pg/ml (Bellone et al. 2006), but lower than reported by Poch et al. (approximately 300 pg/ml) (Poch et al. 2007).

IL-1β levels in serum are in general very low, as shown by studies that were not able to detect IL-1β in the serum of healthy subjects or PDAC patients at all (Kleiner et al. 2013; Ebrahimi et al. 2004; Liu et al. 2021a) and two studies that detected relatively low levels of IL-1β in healthy subjects

with an average of 4.04 pg/ml (Bellone et al. 2006) and > 0.05 pg/ml (Poch et al. 2007) and significantly higher, but still low levels in PDAC patients with an average of 5.35 pg/ml (Bellone et al. 2006) and approximately 0.8 pg/ml (Poch et al. 2007). The levels of IL-1β detected in this study were therefore relatively high, but still lower than those detected by Basso et al., which were 57.8 ng/l in healthy subjects versus 62.8 ng/l in PDAC patients (Basso et al. 1995).

IFN-γ levels in this study were lower than reported for healthy subjects, for example 120-160 pg/ml (Liu et al. 2021a) and an average of 130 pg/ml (Kleiner et al. 2013). TNF levels were slightly higher and matched with TNF levels from healthy subjects from the literature, for example approximately 30 pg/ml (Kleiner et al. 2013) and 28-38 pg/ml (Liu et al. 2021a). However, TNF levels were generally found to be higher in PDAC patients than in healthy subjects (Yako et al. 2016) and the levels in the present study were relatively high compared to a study that reported significantly higher TNF levels in PDAC patients; <17 pg/ml in healthy subjects vs. 27 pg/ml in PDAC patients (Poch et al. 2007) and one study that did not detect a significant difference; 4.4-10.5 pg/ml in healthy subjects vs. 4.4-12.7 pg/ml in PDAC patients (Ebrahimi et al. 2004).

The levels of IL-10 in the present study were higher than levels reported for healthy subjects, with an average of 13.5 pg/ml (Bellone et al. 2006), approximately 5 pg/ml (Poch et al. 2007), 3.0-18 pg/ml (Ebrahimi et al. 2004), an average of 2.5 pg/ml (Bernstorff et al. 2001), ∅12.6 pg/ml (Kleiner et al. 2013) and 8.5-17 pg/ml (Liu et al. 2021a). Additionally, they matched with levels reported for PDAC patients, with an average of 15.5 pg/ml (Bellone et al. 2006), approximately 20 pg/ml (Poch et al. 2007) and 3.0-141 pg/ml (Ebrahimi et al. 2004), although there were markedly higher than IL-10 levels from PDAC patients reported by Bernstorff et al. 2001, with an average of 8.7 pg/ml.

In summary, this study revealed relatively high IL-18, IL-1β and IL-10 levels and relatively low IFN-γ levels in the serum of pancreatic cancer patients when compared to levels reported for healthy subjects in the literature. Reported TNF levels in the serum of healthy subjects have a broader range and therefore do not allow a conclusion about TNF levels found in the serum of pancreatic cancer patients during this study.

4.3 Molecular mechanisms of IL-18 mediated T cell exhaustion

4.3.1. Antigen-specific CD8$^+$ T cells obtain an exhausted phenotype upon repetitive simulation *in vitro*

Previous work reported by Lutz et al. confirmed a role of IL-18 signaling in T cell exhaustion in a murine pancreatic cancer model *in vivo* (Lutz et al. 2023). In order to further investigate the effects

of IL-18 on T cell exhaustion, an *in vitro* exhaustion model based on a protocol previously published (Zhao et al. 2020) was used. $CD8^+$ cells were purified from OT-I mice and activated for two days (primary stimulation) with anti-CD3 and anti-CD28. Subsequently, OT-I cells were cultivated with their cognate antigen $OVA^{257-264}$ for four days (= repetitive stimulation) followed by a two-day resting phase without $OVA^{257-264}$. A control group was cultivated without $OVA^{257-264}$ (Fig. 3A). After cultivation, typical features of exhausted T cells were examined to determine the phenotype of the T cells.

Repetitive stimulation *in vitro* increased the percentage of $PD\text{-}1^+$ cells in antigen-specific $CD8^+$ T cells. Additionally, the expression of TIM3 and LAG3 in repetitively stimulated cells was elevated (Fig. 3B). Additionally, the co-expression pattern of PD-1, TIM3 and LAG3 was evaluated. The number of $PD\text{-}1^+$ $TIM3^+$ double positive cells and $PD\text{-}1^+$ $TIM3^+$ $LAG3^+$ triple positive cells was increased from nearly zero in the control group to 40-50 % in the repetitively stimulated group (Fig. 3B; Lutz et al. 2023). Therefore, according to the surface markers, the repetitively stimulated cells show an exhausted phenotype. Nevertheless, surface markers cannot be solely used to infer an exhausted phenotype, as the expression of key transcription factors and production of cytokines also needs to be considered.

Typical transcription factors used to determine the developmental stage of T cells are T-bet, Eomes and Tox. Here, repetitively stimulated cells showed an increased expression of T-bet and TOX and a decreased expression of Eomes (Fig. 3C). Increased TOX expression corroborated the exhausted phenotype of repetitively stimulated T cells. T-bet and Eomes are known to regulate the function and dysfunction of T cells, T-bet is usually downregulated whereas Eomes is upregulated upon T cell exhaustion (Kao et al. 2011; Wherry et al. 2007; Paley et al. 2012). The observation of increased T-bet and decreased Eomes in our *in vitro* model might therefore arise from the fact that the expression of those transcription factors is dependent on the stage of exhaustion (Paley et al. 2012).

The capacity to produce effector cytokines is probably the most important feature to determine the condition of CTLs. Therefore, the percentage of IFN-γ, TNF, GrzB and IL-2 produced by *in vitro* cultivated $CD8^+$ T cells was examined. The percentage of IFN-γ-producing cells was not changed upon repetitive stimulation; however, the percentage of TNF-producing cells was significantly decreased. Likewise, the percentage of cells that produced both cytokines was significantly decreased (Fig. 3D, Lutz et al. 2023). Accordingly, the $PD\text{-}1^+$ $TIM3^+$ double positive cells were responsible for the loss of IFN-γ and TNF production, as those cells were producing both cytokines independently and simultaneously to a lesser amount (Fig. 3E, Lutz et al. 2023). The number of GrzB producing cells was significantly increased upon repetitive stimulation (Fig.

3D). Typically, TNF production is lost at the intermediate state of exhaustion, whereas IFN-γ and GrzB at advanced stage (Wherry et al. 2003) Therefore, the cytokine pattern observed would indicate an intermediate exhausted state of T cells. IL-2 production is typically lost at early stage of exhaustion (Wherry 2011) and was hardly detectable even in the control group (Fig. 3F). It is possible, that all cells in this model already lost the capacity to produce IL-2.

Repetitive stimulation neither affected T cell viability, as defined by the total number of living cells after cultivation (Fig. 3G), nor their proliferative capacity, determined by the expression of ki67 (Fig. 3H).

In summary, repetitive stimulation of $CD8^+$ T cells with their cognate antigen causes an increased expression of multiple coinhibitory receptors and the exhaustion specific transcription factor TOX. Additionally, the percentage of cells that produce both CTL-specific cytokines (IFN-γ and TNF) was reduced. Therefore, as repetitively stimulated $CD8^+$ T cells are characterized by phenotypical and functional traits specific for T cell exhaustion, those cells are termed as *in vitro* exhausted throughout this study.

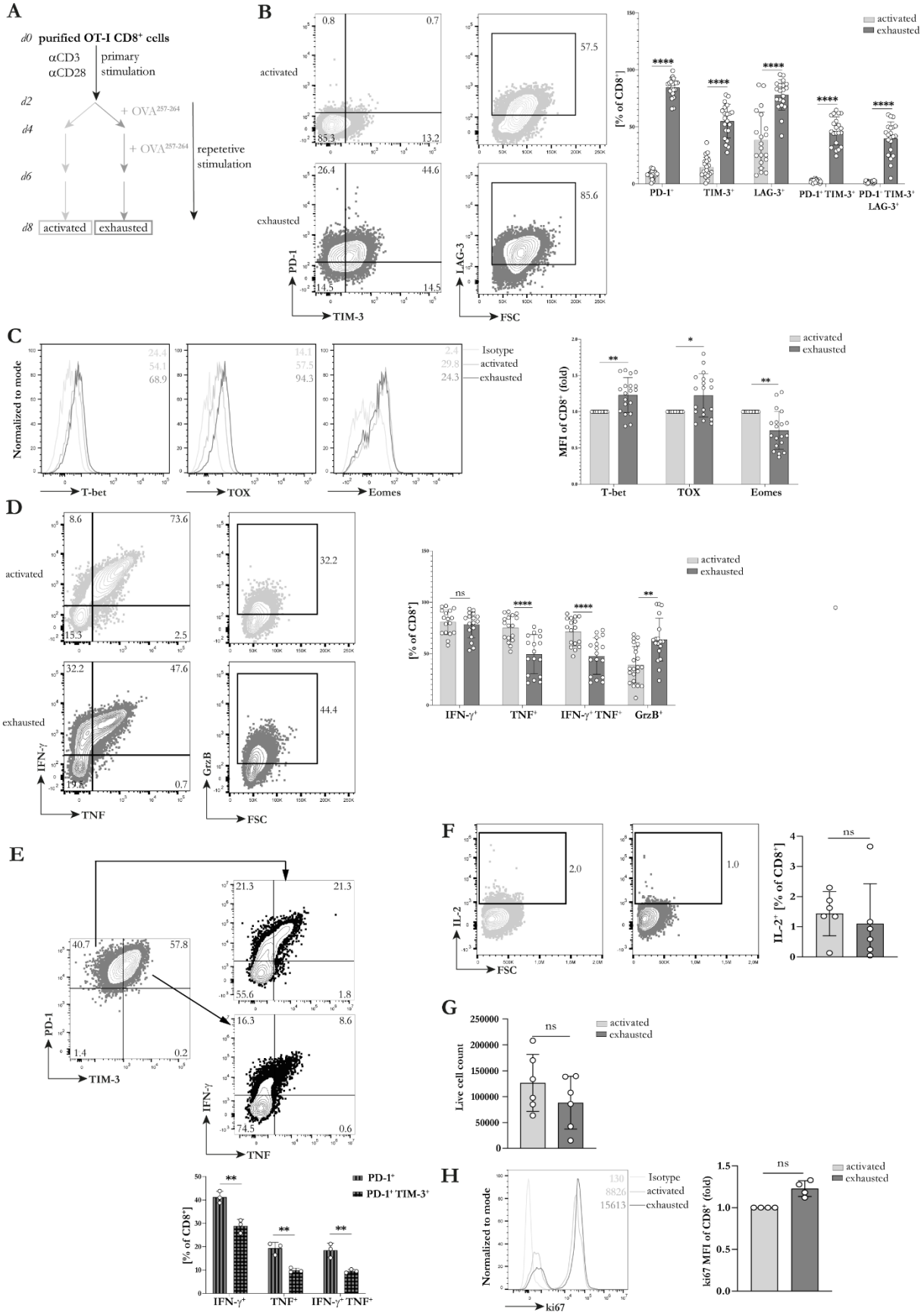


Figure 3: Repetitive *in vitro* stimulation of antigen-specific CD8+ T cells induces an exhausted phenotype. A) Schematic of the experimental setup of *in vitro* T cell exhaustion. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for two days (=primary stimulation) followed by cultivation with (=exhausted) or without (=activated) the model antigen OVA257-264 for four days and a

resting phase without $OVA^{257-264}$ peptide for two days. Cells were stained for typical exhaustion markers and analyzed by flow cytometry. Expression of B) surface markers (PD-1, TIM3 and LAG3; n=20-21), C) transcription factors (T-bet, TOX and Eomes; n=19) and D) production of cytokines (IFN-γ, TNF and GrzB; n=17) by activated and exhausted T cells. E) Production of cytokines of sorted PD-1$^+$ and PD-1$^+$ TIM3$^+$ exhausted T cells (n=3). F) Production of IL-2 by activated and exhausted T cells (n=6). G) Life cell count (n=6) and expression of ki67 (n=4) of activated and exhausted T cells. Fold change was normalized to activated T cells. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-tests with (B) or without (D, E, G) Welch correction, One sample Wilcoxon test (C, H) or Mann-Whitney test (F). *p<0.05, **p<0.01, ****p<0.0001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

4.3.1.1 Il18r$^{-/-}$ CD8$^+$ T cells obtain an exhausted phenotype upon repetitive stimulation in vitro

As Lutz et al. observed in a murine model that tumor specific *Il-18r$^{-/-}$* CTLs exhausted *in vivo* to a lesser extent (Lutz et al. 2023), their ability to obtain an exhausted phenotype *in vitro* was determined using the *in vitro* exhaustion model. Just as WT CD8$^+$ T cells, *Il18r$^{-/-}$* CD8$^+$ T cells showed elevated levels of PD-1$^+$, TIM3$^+$ and LAG3$^+$ cells in the *in vitro* exhaustion model. Additionally, the percentage of cells expressing PD-1 and TIM3 or even all three markers at the same time was significantly increased upon repetitive stimulation (Fig. 4A). Regarding the transcription factor pattern, *Il18r$^{-/-}$* CD8$^+$ T cells showed an increased T-bet and Tox expression upon repetitive stimulation. Interestingly, unlike in WT CD8 T cells, the expression of Eomes was unchanged (Fig. 4B). Repetitively stimulated *Il18r$^{-/-}$* CD8$^+$ T cells showed no difference in their capacity to produce IFN-γ compared to the control group, but the capacity to produce TNF was significantly decreased. Likewise, the percentage of cells producing both cytokines was reduced. The percentage of GrzB$^+$ cells was increased (Fig. 4C). Repetitively stimulated *Il18r$^{-/-}$* CD8$^+$ T cells therefore showed a cytokine pattern typical for intermediate exhausted T cells.

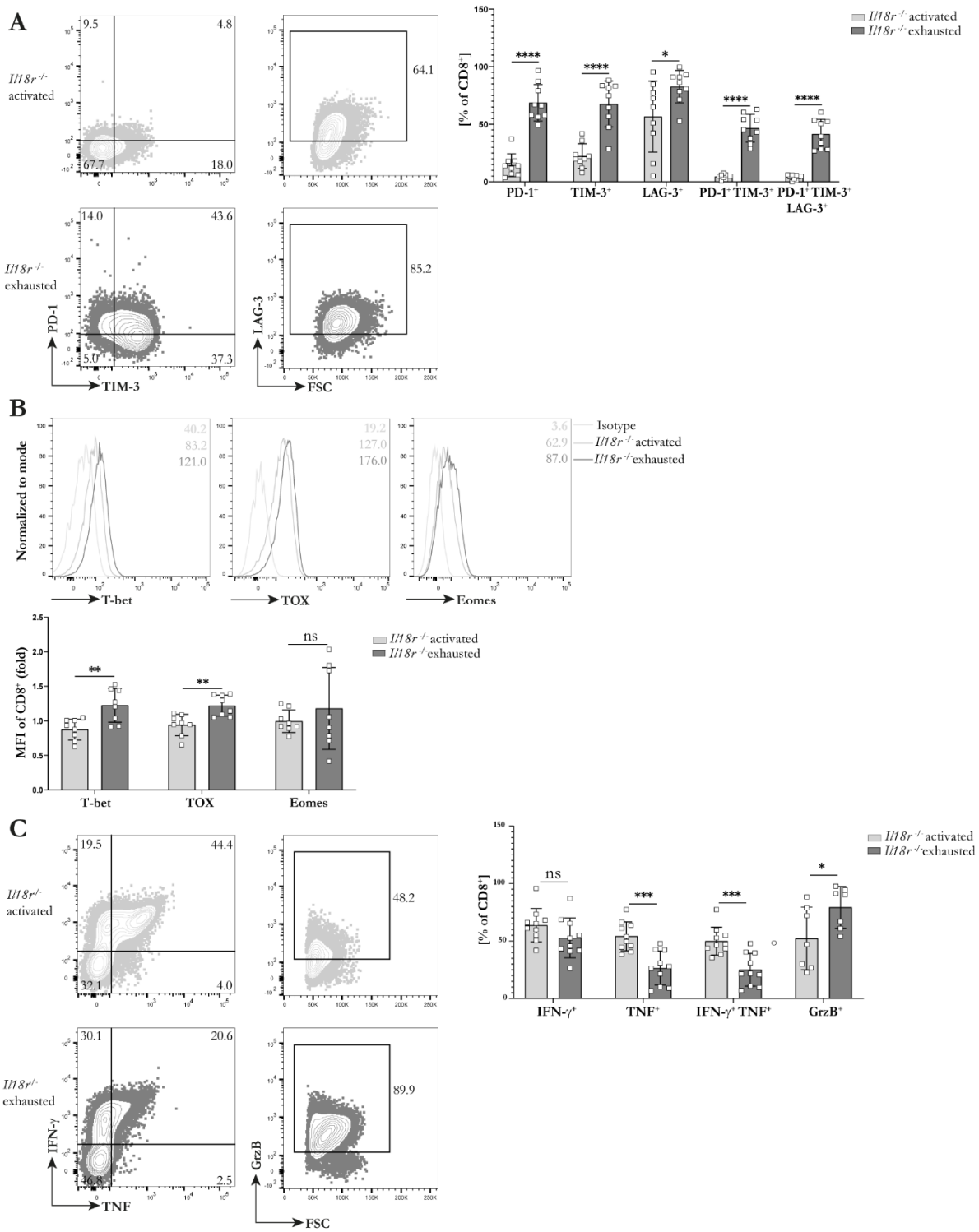


Figure 4: IL-18R deficient CD8+ T cells obtain an exhausted phenotype upon repetitive antigen stimulation *in vitro*. A-C) Antigen-specific IL-18R deficient CD8+ T cells were isolated from spleen and lymph nodes of OT-I *Il18r -/-* mice and activated with anti-CD3 and anti-CD28 for two days (=primary stimulation) followed by cultivation with (=exhausted) or without (=activated) the cognate antigen $OVA^{257-264}$ for four days and a resting phase without $OVA^{257-264}$ for two days. Cells were stained for typical exhaustion markers and analyzed via FACS. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=9), B) transcription factors (T-bet, TOX and Eomes; n=8) and C) production of cytokines (IFN-γ, TNF and GrzB; n=7-18) by activated and exhausted CD8+ T cells. Fold change was normalized to activated WT cells. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-tests with (A: PD-1+ TIM3+, LAG3+, PD-1+ TIM3+ LAG3+, B: Eomes) or without (A: PD-1+, TIM3+ B: T-bet, TOX, C) Welch correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.

Noteworthy, the percentage of PD-1$^+$ cells was significantly decreased in *Il18r*$^{-/-}$ CD8$^+$ T cells compared to WT CD8$^+$ T cells. Cells expressing TIM3, LAG3 or both markers in combination with PD-1 did not differ between WT and *Il18r*$^{-/-}$ CD8$^+$ T cells. Nevertheless, TIM3$^+$ cells were slightly, but not significantly, increased in *Il18r*$^{-/-}$ CD8$^+$ T cells compared to WT CD8$^+$ T cells (Fig. 5A). Therefore, there might be intrinsic differences between WT and *Il18r*$^{-/-}$ CD8$^+$ T cells regarding the expression of PD-1 and TIM3 in the *in vitro* exhaustion model. Nonetheless, the combinational expression of both markers, which is used to identify the percentage of *in vitro* exhausted cells during this study, does not differ between WT and *Il18r*$^{-/-}$ CD8$^+$ T cells. The expression of T-bet and Tox did not differ between WT and *Il18r*$^{-/-}$ CD8$^+$ T cells whereas the expression of Eomes was slightly, but not significantly, increased in *Il18r*$^{-/-}$ CD8$^+$ T cells (Fig. 5B). Speculatively, Eomes expression might be generally higher in *Il18r*$^{-/-}$ cells and therefore not further elevated upon repetitive stimulation. Compared to WT CD8$^+$ T cells, the percentage of IFN-γ, TNF and double producing cells was decreased. Additionally, the percentage of GrzB$^+$ cells was tendentially increased in *Il18r*$^{-/-}$ CD8$^+$ T cells compared to WT (Fig. 5C).

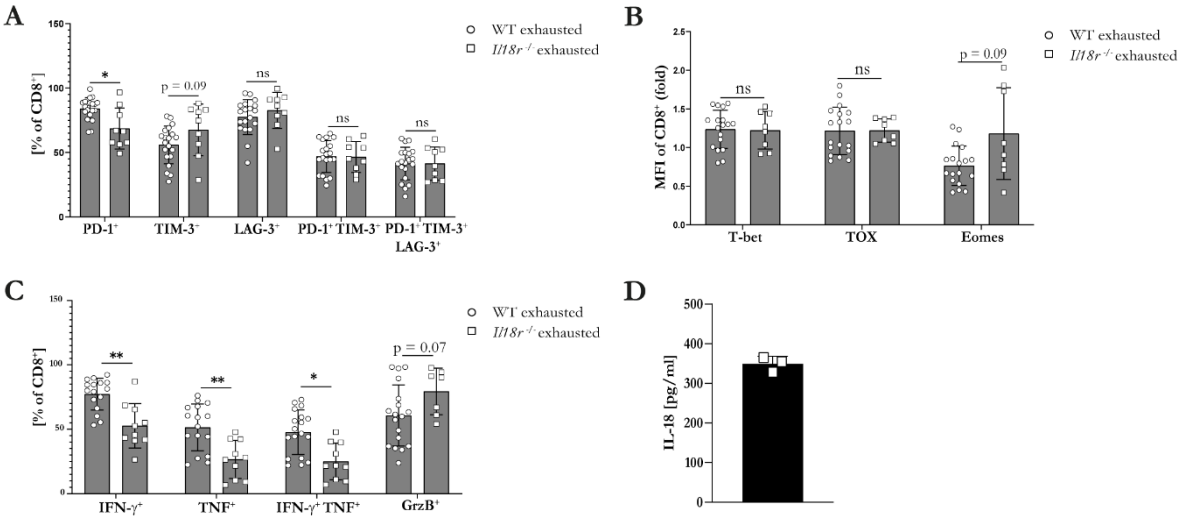


Figure 5: *In vitro* exhausted IL-18R deficient CD8$^+$ T cells show a similar phenotype compared to WT cells. A-C) Antigen-specific WT and IL-18R deficient CD8$^+$ T cells were isolated from spleen and lymph nodes of OT-I WT and OT-I *Il18r*$^{-/-}$ mice and activated with anti-CD3 and anti-CD28 for two days (=primary stimulation) followed by cultivation with (=exhausted) the cognate antigen OVA$^{257-264}$ for four days and a resting phase without OVA$^{257-264}$ for two days. Cells were stained for typical exhaustion markers and analyzed via FACS. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=9-20), B) transcription factors (T-bet, TOX and Eomes; n=8-18) and C) production of cytokines (IFN-γ, TNF and GrzB; n=7-18) by *in vitro* exhausted T cells. D) PancOVA tumors were isolated from mice and cultivated for 24 h in T cell medium. IL-18 levels in the supernatant were detected via ELISA (n=3). Fold change was normalized to activated WT cells. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-tests with (A: PD-1$^+$, B: Eomes) or without (A: TIM3$^+$, LAG3$^+$, PD-1$^+$ TIM3$^+$, PD-1$^+$ TIM3$^+$ LAG3$^+$, B: T-bet, TOX, C) Welch correction. *p<0.05, **p<0.01, ns: not significant.

These results indicate that deficiency of IL-18R does not change the level of exhaustion *per se*. Although there are differences detected between *in vitro* exhausted WT and *Il18r*$^{-/-}$ CD8$^+$ T cells, the overall pattern of exhaustion markers was the same. This indicated that differences in the exhaustion pattern of WT and *Il18r*$^{-/-}$ CD8$^+$ T cells *in vivo* must be mediated by extrinsic IL-18.

These results were supported by the fact that PancOVA tumors, which were used in the pancreatic cancer model revealing differences in the exhaustion patterns of WT and *Il18r* $^{-/-}$ antigen-specific CD8^{+} T cells (Lutz et al. 2023), produced detectable levels of IL-18 (Fig. 5D).

4.3.2 IL-18 increases effector T cell development of antigen-specific CD8^{+} T cells during primary stimulation *in vitro*

As IL-18 is typically known as an IFN-γ-inducing factor, the question arose how IL-18 supplementation would influence the phenotype of antigen-specific CD8^{+} T cells during the first two days of the *in vitro* exhaustion protocol, the phase of antigen-unspecific activation. Therefore, cells were stained for expression of coinhibitory receptors, transcription factors and cytokine production after that phase. The expression of coinhibitory receptors after non-specific activation of CD8^{+} T cells was not altered by IL-18 (Fig. 6A). Nevertheless, as coinhibitory receptors are also expressed by activated T cells (Wherry and Kurachi 2015), a notable number of cells expressed PD-1- and LAG3 were detectable (Fig. 6A). Further, IL-18 signaling did not change the expression of T-bet, TOX and Eomes during primary stimulation (Fig. 6B). Nevertheless, IL-18 significantly increased the percentage of the IFN-γ-producing cells and slightly, but not significantly, the percentage of TNF-producing cells. Consequently, the percentage of cells producing both IFN-γ and TNF (double positive) was increased upon IL-18 treatment, whereas the percentage of GrzB producing cells was unchanged (Fig. 6C).

Therefore, as expected, IL-18 increases the production of CTL-specific effector cytokines under T cell activating conditions.

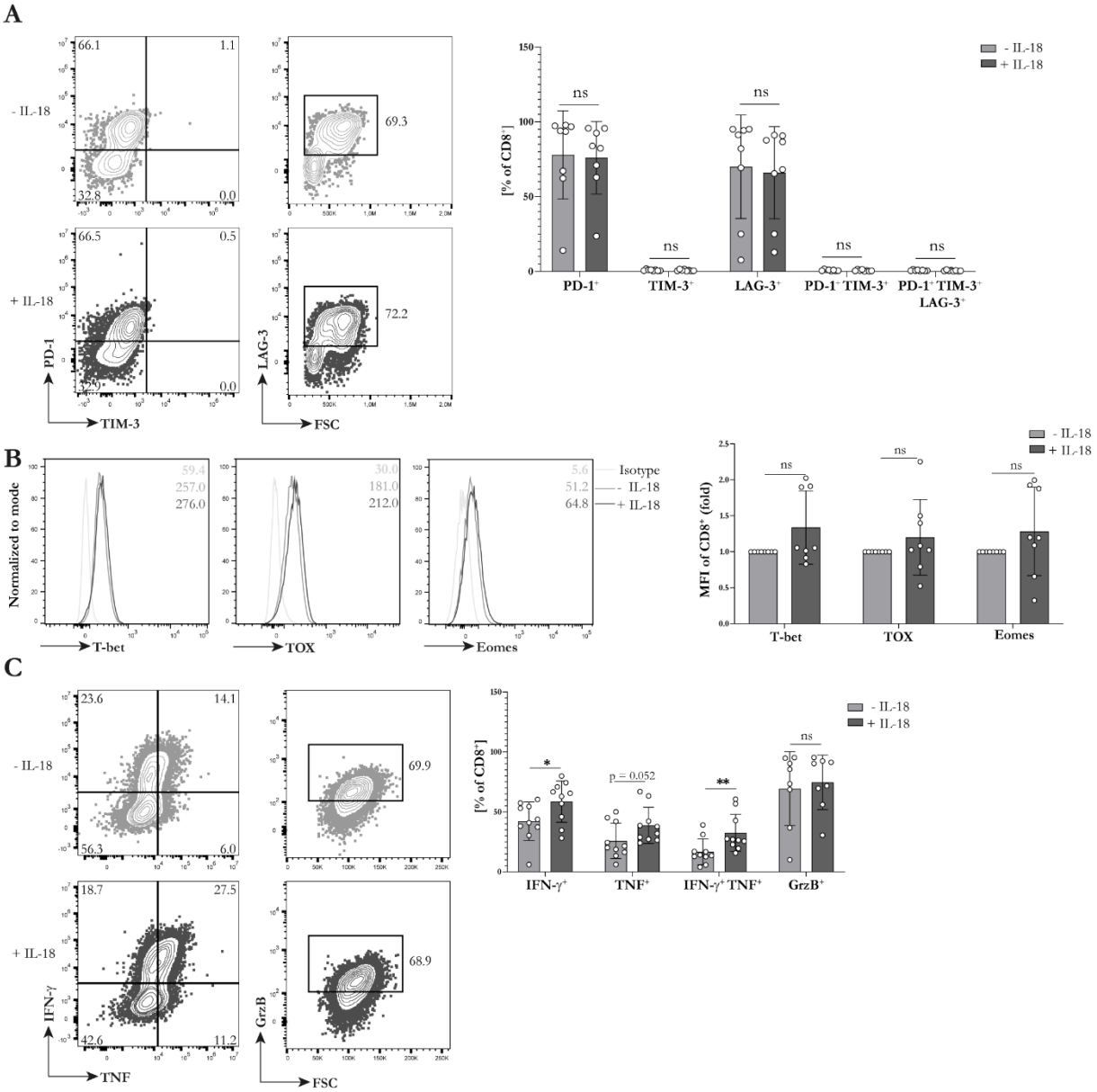


Figure 6: IL-18 enhances effector function of antigen-specific CD8+ T cells during primary stimulation of *in vitro* exhaustion. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) with or without IL-18. Cells were stained for typical exhaustion markers and analyzed via FACS. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=8), B) transcription factors (T-bet, TOX and Eomes; n=8) and C) production of cytokines (IFN-γ, TNF and GrzB; n=8-10) by T cells primarily stimulated with or without supplemented IL-18. Fold change was normalized to cells without IL-18 treatment. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-test (A: PD-1+ TIM3+, PD-1+ TIM3+ LAG3+, C: IFN-γ+, GrzB+), Mann-Whitney test (A: PD-1+, TIM3+, LAG3+, C: TNF+, IFN-γ+ TNF+) or One sample Wilcoxon test (B). *p<0.05, **p<0.01, ns: not significant.

4.3.3 IL-18 enhances *in vitro* T cell exhaustion

As IL-18 rather enhanced the effector phenotype of *in vitro* activated antigen-specific $CD8^+$ T cells but enhanced the exhausted phenotype of those cells in a murine pancreatic cancer model *in vivo* (Lutz et al. 2023) the question arose whether the influence of IL-18 on CTLs is dependent on their stage of differentiation. To gain further insight, $CD8^+$ T cells were repetitively stimulated *in vitro* with or without external IL-18 to examine the effect of IL-18 on *in vitro* exhausted $CD8^+$ T cells. PD-1 expression of exhausted T cells was not altered by IL-18 but TIM3 and LAG3 expression was significantly increased (Fig. 7A). Consequently, the percentage of cells expressing two (PD-1 and TIM3) or all three (PD-1, TIM3 and LAG3) coinhibitory receptors was significantly increased by IL-18 (Fig. 7A, Lutz et al. 2023). Furthermore, IL-18 caused an increased expression of the transcription factor TOX (Fig. 7B) indicating a more severe exhausted phenotype of those cells. Interestingly, the expression of T-bet and Eomes were both increased by IL-18 (Fig. 7B), although they are commonly believed to be alternatively regulated (Paley et al. 2012). The increased expression of Eomes might further indicate a more severe exhausted phenotype upon IL-18 treatment. Nevertheless, increased expression of T-bet rather indicated an activated phenotype of those cells. This might indicate a dual function, where prolonged IL-18 exposure increases exhaustion specific traits (like coinhibitory receptors and the expression of TOX and Eomes) but also increase the expression of effector specific traits (like T-bet expression). Consequently, IL-18 treatment did not increase the production of IFN-γ in the setting of *in vitro* exhausted T cells, although IL-18 usually increases IFN-γ production and did so in the primary stimulation phase of the *in vitro* exhaustion model, as shown above. Additionally, the percentage of TNF-producing cells was (not significantly) decreased and the percentage of IFN-γ and TNF double-producing cells was significantly decreased upon IL-18 treatment, while the percentage of GrzB-producing cells was increased (Fig. 7C, Lutz et al. 2023).

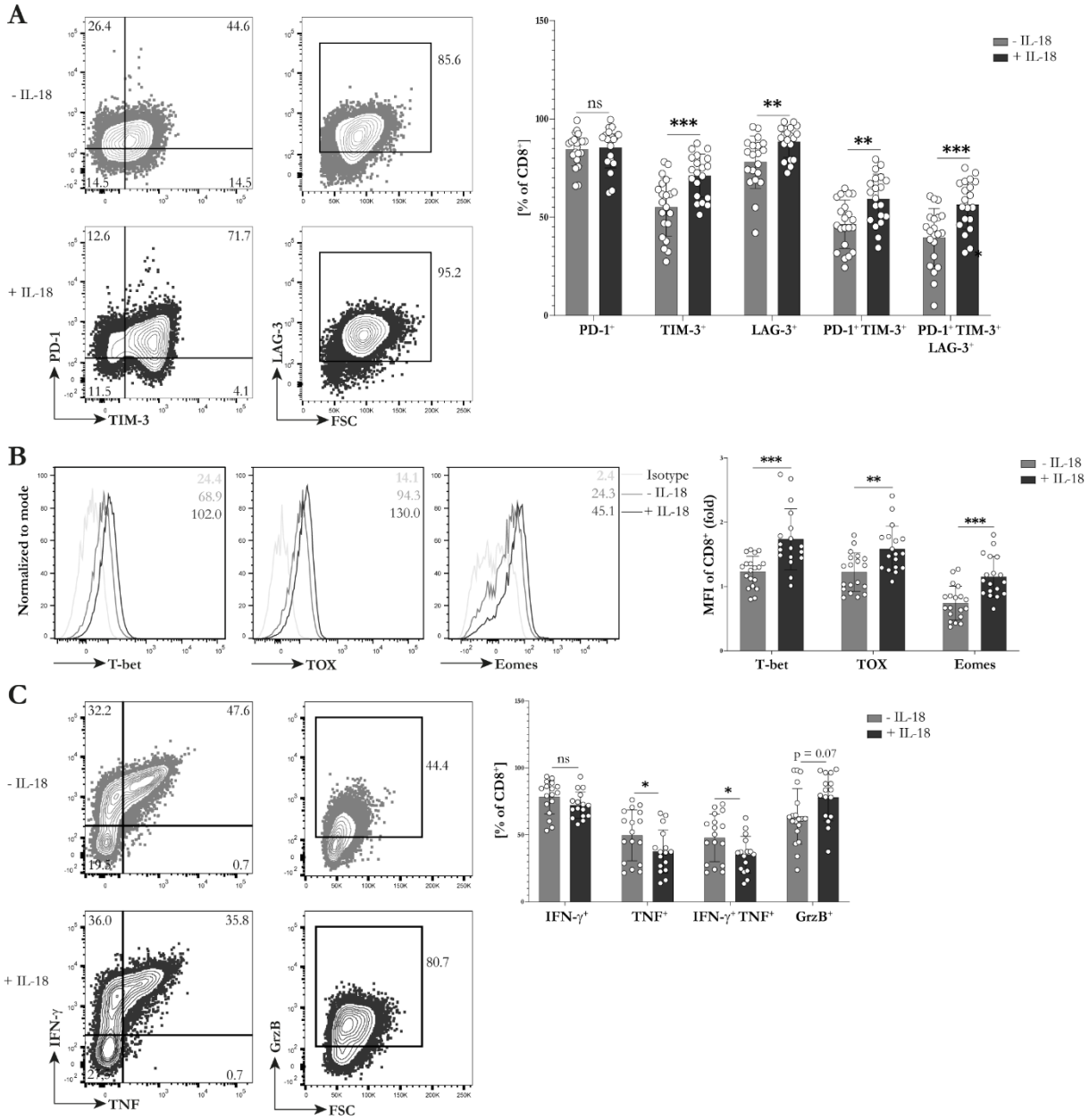


Figure 7: Treatment with IL-18 enhances the exhausted phenotype of repetitively stimulated antigen-specific CD8+ T cells *in vitro*. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen $OVA^{257-264}$ for 4 days and a resting phase without $OVA^{257-264}$ for 2 days. Cells were cultivated with or without IL-18 throughout the protocol, stained for typical exhaustion markers and analyzed via FACS. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=19-20), B) transcription factors (T-bet, TOX and Eomes; n=18) and C) production of cytokines (IFN-γ, TNF and GrzB; n=16) by *in vitro* exhausted T cells. Fold changed was normalized to activated cells without IL-18 treatment. Bars represent mean ± sd and statistics were evaluated by Mann-Whitney test (A: PD-1+) two-tailed, unpaired t-tests with (A: LAG3+, B: T-bet) or without (A: TIM3+, PD-1+ TIM3+, PD-1+ TIM3+ LAG3+, B: TOX, Eomes, C) Welch correction. *p<0.05, **p<0.01, ***p<0.001, ns: not significant**.** This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

Beltra et al. characterized four subsets of exhausted T cells based on their expression of CD69 and Ly108. The four subsets are progenitor exhausted T cells 1 (Tex^{prog1}; $Ly108^{+}$ $CD69^{+}$), progenitor exhausted T cells 2 (Tex^{prog2}; $Ly108^{+}$ $CD69^{-}$), intermediate exhausted T cells (Tex^{int}; $Ly108^{-}$ $CD69^{-}$), and terminally exhausted T cells (Tex^{term}; $Ly108^{-}$ $CD69^{+}$) (Beltra et al. 2020). IL-18 treatment caused an increased percentage of Tex^{prog1} cells and Tex^{term} cells while it (not significantly) decreased the percentage of Tex^{prog2} cells (Fig. 8A). This further supports the conclusion that IL-18 treatment enhances exhaustion.

As IL-18 affects exhaustion of *in vitro* stimulated $CD8^{+}$ T cells, the question arose whether it also influences the formation of memory T cells. Therefore, *in vitro* exhausted $CD8^{+}$ T cells were stained for the surface markers CD44 and CD62L and identified as effector T cells (T_{eff}; $CD44^{+}$ $CD62L^{-}$) and effector memory T cells (T_{EM}; $CD44^{+}$ $CD62L^{+}$). IL-18 treatment did not alter the abundance of one of those subsets (Fig. 8B, Lutz et al. 2023) and therefore does not influence the formation of memory T cells *in vitro*.

IL-18 significantly decreased the viability and proliferative capacity of *in vitro* exhausted $CD8^{+}$ T cells (Fig. 8C, D, Lutz et al 2023). This phenomenon is most likely a side effect of increased exhaustion, as exhausted T cells finally die by apoptosis (Wherry 2011).

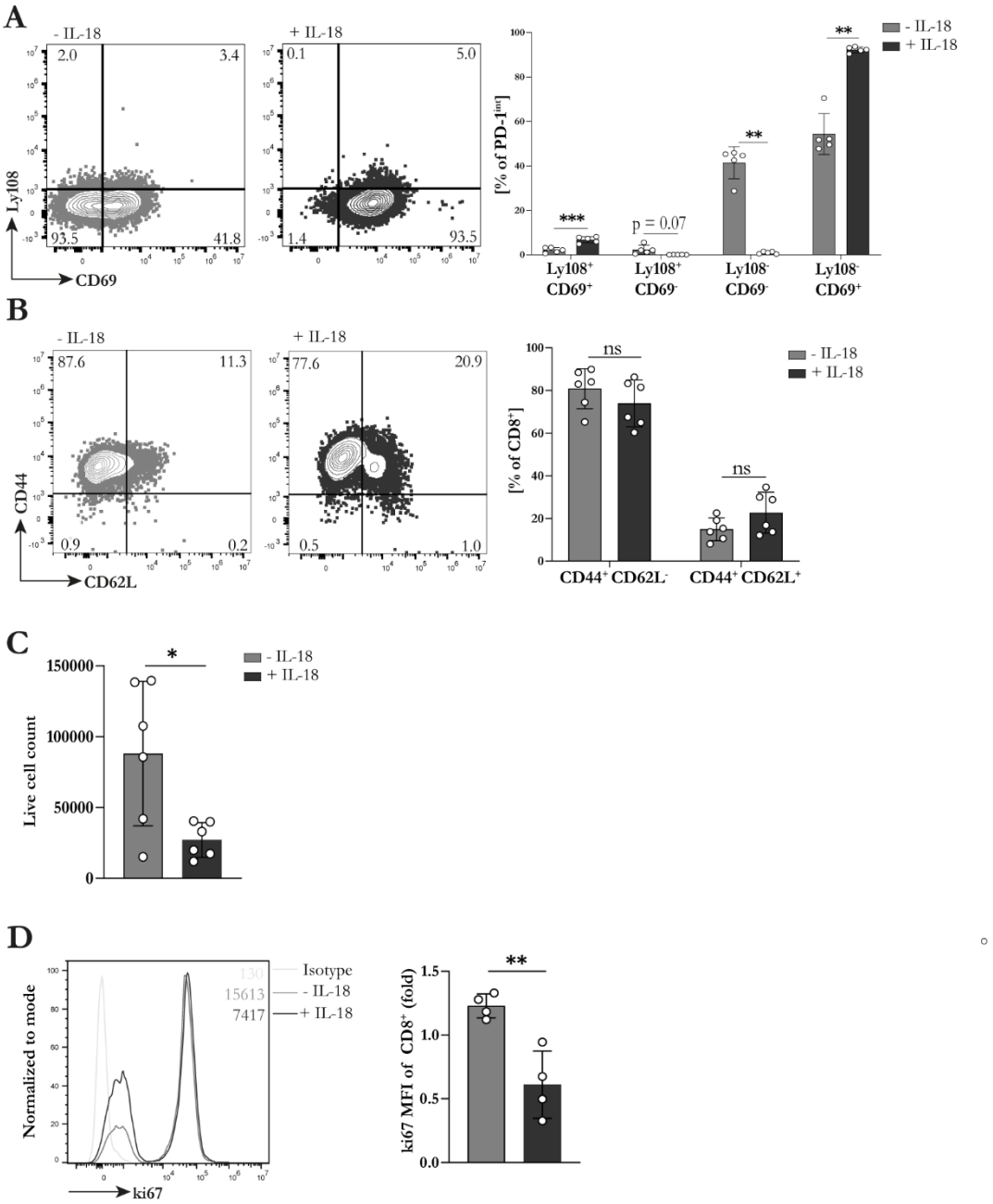


Figure 8: Treatment with IL-18 changes characteristics regarding development and viability of *in vitro* exhausted antigen-specific CD8⁺ T cells. Antigen-specific CD8⁺ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen $OVA^{257-264}$ for 4 days and a resting phase without $OVA^{257-264}$ for 2 days. Cells were cultivated with or without IL-18 throughout the protocol, stained surface markers, viability and ki67 and analyzed via FACS. Expression of A) CD69 and Ly108 (n=5) and B) CD62L and CD44 by *in vitro* exhausted T cells (n=3). C) Life cell count (n=6) and D) expression of ki67 (n=4) of *in vitro* exhausted T cells. Fold changed was normalized to activated cells without IL-18 treatment. Bars represent mean ± sd and statistics were evaluated by Mann-Whitney test (A: $Ly0108^{-}$ $CD69^{-}$, $Ly0108^{-}$ $CD69^{+}$) two-tailed, unpaired t-tests with (A: $Ly0108^{+}$ $CD69^{-}$, C) or without (A: $Ly0108^{+}$ $CD69^{+}$, C, D) Welch correction. *p<0.05, **p<0.01, ***p<0.001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al., 2023.

4.3.4 Inhibition of pathways involved in T cell development abrogates IL-18 mediated T cell exhaustion *in vitro*

RNAseq of tumor infiltrating CTLs revealed IL-18-mediated changes in the IL-2/STAT5 pathway and the Akt/mTOR pathway (Lutz et al. 2023). Therefore, the *in vitro* exhaustion model was used to gain further insight into the molecular mechanisms of IL-18-mediated T cell exhaustion,

4.3.4.1 Inhibition of IL-2 and STAT5 signaling partially abrogate IL-18 mediated effects on in vitro T cell exhaustion

RNAseq revealed changes in the IL-2/STAT5 pathway in IL-18R deficient CTLs compared to WT CTLs (Lutz et al. 2023). Liu et al. also confirmed a role of IL-2 signaling in T cell exhaustion (Liu et al. 2021b). Additionally, *in vitro* exhausted T cells showed increased expression of CD25, but not of CD122 and increased levels of phosphorylated STAT5 upon IL-18 treatment (Fig. 9A, Lutz et al. 2023) indicating an impact of IL-18 on the IL-2/STAT5 pathway during T cell exhaustion *in vitro*. Therefore, this pathway was inhibited at several points starting from day 2 of the *in vitro* exhaustion model in order to study its impact on IL-18-mediated T cell exhaustion *in vitro*.

Blockade of the IL-2 receptor CD25 decreased the percentage of PD-1$^+$ TIM3$^+$ cells but this effect was reversed when IL-18 was added (Fig. 9B). TOX expression was unchanged when CD25 was blocked but not further increased upon IL-18 treatment and therefore lower compared to the IL-18 treatment without CD25 blockade (Fig. 9C). IFN-γ and TNF double-producing cells nevertheless were less abundant when CD25 was blocked but did not differ dependent on the additional treatment with IL-18 (Fig. 9D). Therefore, blockade of CD25 was able to abrogate the IL-18-mediated increase of TOX expression but did not impact the IL-18-mediated increase in the expression of coinhibitory receptors and negatively impacted cytokine production independently of IL-18. Hence, CD25 was only partly involved in IL-18 mediated exhaustion *in vitro*.

IL-2 was blocked during the *in vitro* T cell exhaustion model starting from day 2, which caused a general decrease of the percentage of PD-1 and TIM3 double positive cells which was not increased by IL-18 treatment (Fig. 9E, Lutz et al. 2023). The expression of TOX nevertheless was not altered by IL-2 blockade and further increased by IL-18 treatment (Fig. 9F). Additionally, IL-2 blockade caused a reduction of cytotoxic cytokine-producing cells independently of IL-18, which was, however, not reinforced by IL-18 supplementation (Fig. 9G, Lutz et al. 2023). Furthermore, blockade of IL-2 had a negative impact on total live cell number and proliferative capacity, which was in turn rather increased by additional IL-18 treatment (Fig. 9H, I, Lutz et al. 2023). Hence, the negative impacts of IL-2 blockade seem to predominate in this model and therefore IL-2 blockade is not able to rule out IL-18-mediated exhaustion *in vitro*.

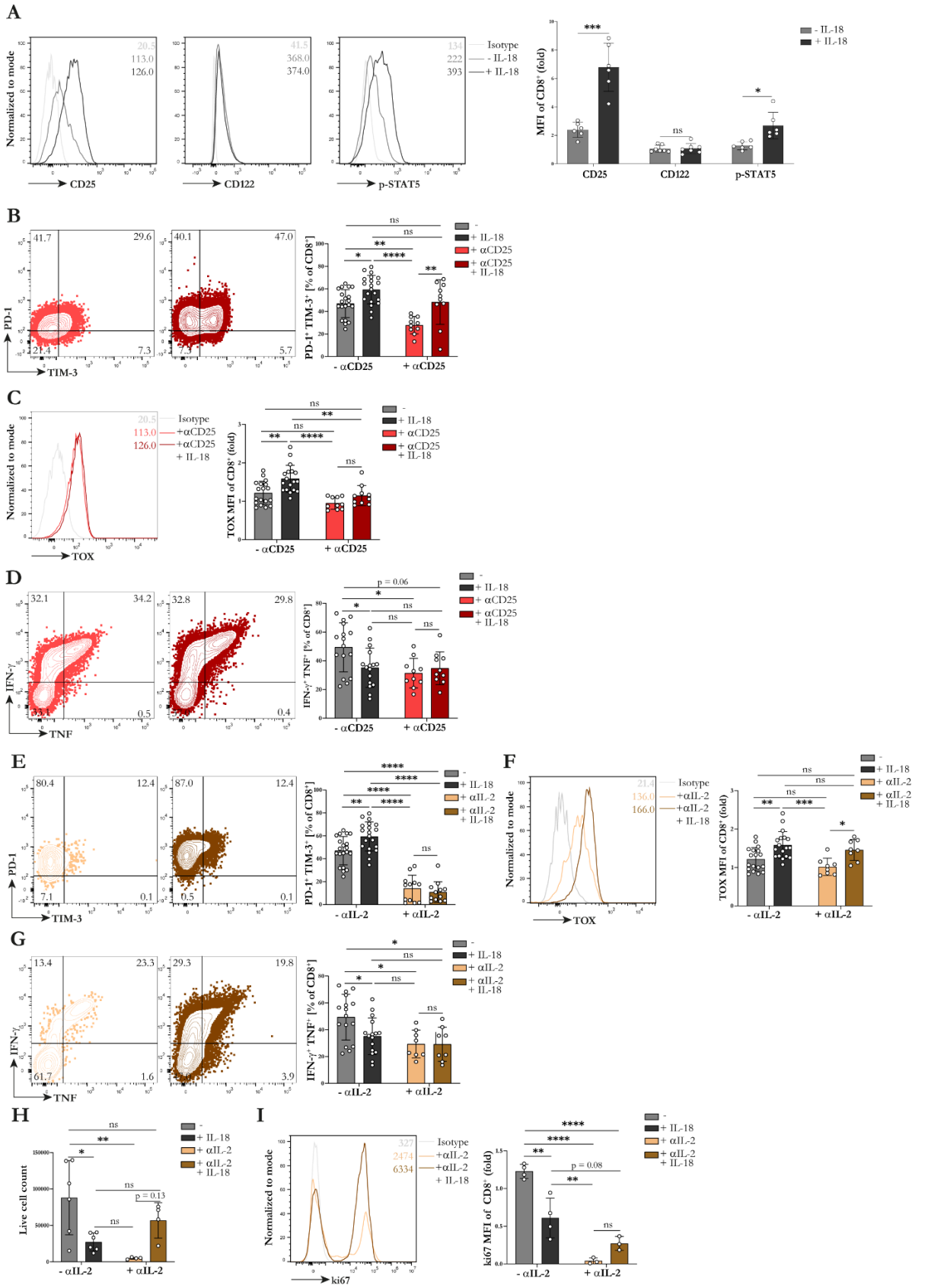


Figure 9: IL-2 signaling is partly involved in IL-18-mediated T cell exhaustion *in vitro*. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen OVA$^{257-264}$ for 4 days and a resting phase without OVA$^{257-264}$ for 2 days. A) Cells were cultivated with or without IL-18 throughout the study, stained for CD25, CD122 and p-STAT5 and analyzed via FACS. B-D) Cells were cultivated with or without IL-18 throughout the

protocol and with or without anti-CD25 starting from day 2. Expression of B) surface markers (PD-1, TIM3 and LAG3; n=10-20), C) transcription factors (T-bet, TOX and Eomes; n=10-18) and D) production of cytokines (IFN-γ, TNF and GrzB; n=10-16) by *in vitro* exhausted T cells. E-H) Cells were cultivated with or without IL-18 throughout the protocol and with or without anti-IL-2 starting from day 2. Expression of E) surface markers (PD-1, TIM3 and LAG3; n=12-20), F) transcription factors (T-bet, TOX and Eomes; n=12-18) and G) production of cytokines (IFN-γ, TNF and GrzB; n=12-16) by *in vitro* exhausted T cells. H) Life cell count (n=4-6) and I) expression of ki67 (n=3-4) of *in vitro* exhausted T cells. Fold change was normalized to activated cells without IL-18 or inhibitor treatment. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-tests with Welch correction (A: CD25, p-STAT5), Mann-Whitney test (A:CD122) or two-way-ANOVA followed by Tukey's multiple comparisons (B-I). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

As part of the IL-2/STAT5 pathway, STAT5 was inhibited during the *in vitro* T cell exhaustion model starting from day 2. STAT5 inhibition did not reduce the expression of PD-1, TIM3 and TOX in general but prevented the IL-18-mediated increase of these markers (Fig. 10A, B, Lutz et al. 2023). Additionally, STAT5 inhibition prevented the IL-18-mediated loss of effector cytokines (Fig. 10C, Lutz et al. 2023). Regarding the total live cell number and proliferative capacity, STAT5 inhibition prevented the IL-18-mediated reduction of both traits but negatively impacted total live cell number and proliferative capacity in general (Fig. 10D, Lutz et al. 2023).

In summary, inhibition of the IL-2/STAT5 pathway in the *in vitro* exhaustion model revealed that this pathway is partly involved in IL-18-mediated exhaustion as some effects of IL-18 were reversed by inhibition of this pathway. Nevertheless, the effects of IL-18 on T cell exhaustion *in vitro* cannot fully be explained by changes in the IL-2/STAT5 pathway, possibly because it also plays a key role in maintaining basic T cell development and function, irrespective of the induction of exhaustion.

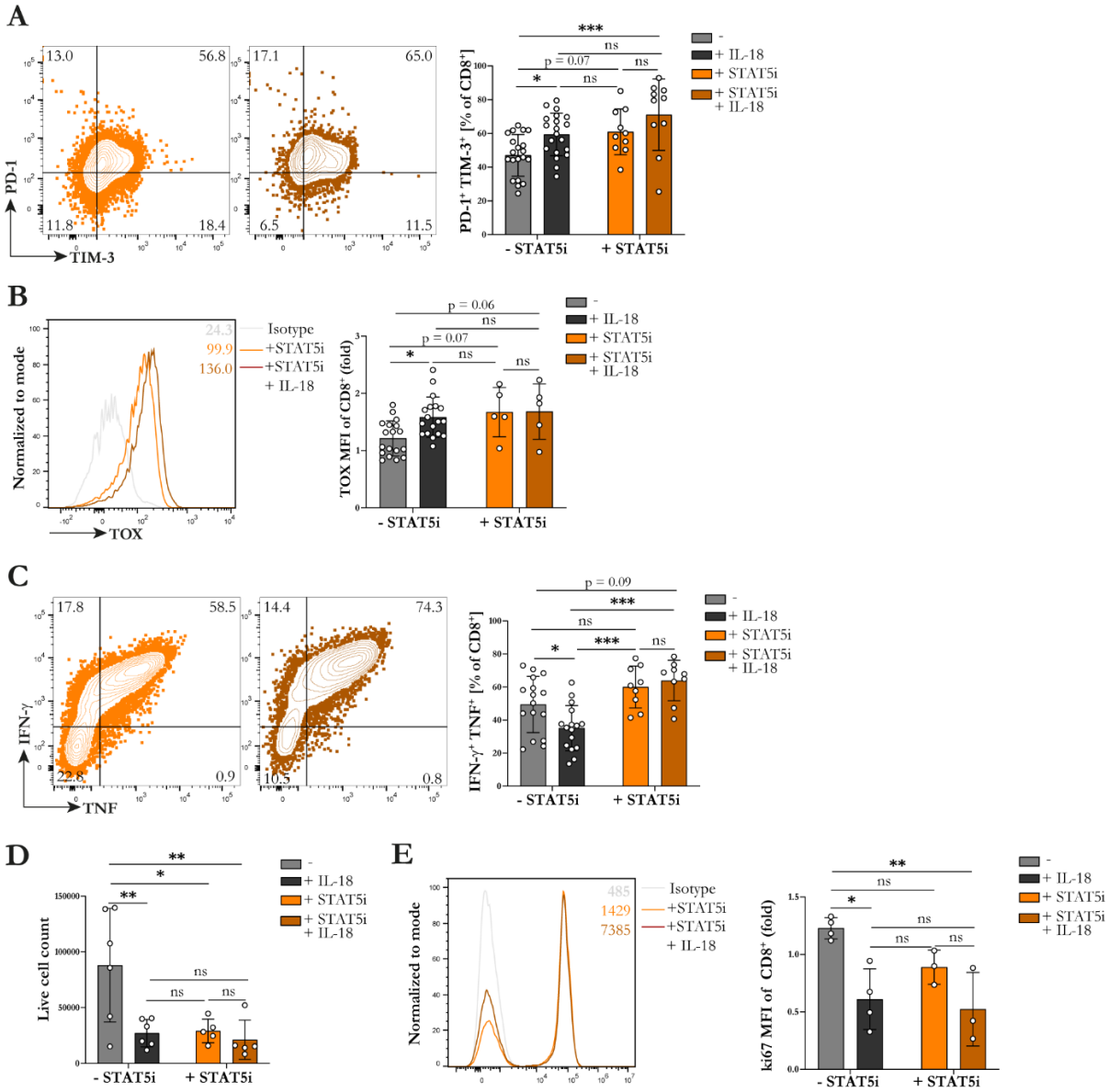


Figure 10: STAT5 signaling is partly involved in IL-18-mediated T cell exhaustion *in vitro*. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen $OVA^{257-264}$ for 4 days and a resting phase without $OVA^{257-264}$ for 2 days. Cells were cultivated with or without IL-18 throughout the protocol and with or without STAT5 inhibitor starting from day 2. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=9-20), B) transcription factors (T-bet, TOX and Eomes; n=9-18) and C) production of cytokines (IFN-γ, TNF and GrzB; n=9-16). D) by *in vitro* exhausted T cells. Life cell count (n=5-6) and E) expression of ki67 (n=3-4) of *in vitro* exhausted T cells. Fold change was normalized to activated cells without IL-18 or inhibitor treatment. Bars represent mean ± sd and statistics were evaluated two-way-ANOVA followed by Tukey's multiple comparisons (B-L). *p<0.05, **p<0.01, ***p<0.001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

4.3.4.2 The Akt/mTOR pathway engages in IL-18-mediated T cell exhaustion in vitro

IL-18R deficient CTLs showed differences in the Akt/mTOR pathway compared to WT CTLs *in vivo* revealed by RNA-Seq (Lutz et al. 2023). Furthermore, mTOR was shown to play a role in T cell exhaustion (Gabriel et al. 2021). The *in vitro* T cell exhaustion model revealed increased levels of phosphorylated Akt and mTOR as well as the mTOR substrate S6 caused by IL-18 treatment (Fig. 11A, Lutz et al. 2023). This indicated a role of the Akt/mTOR pathway in IL-18-mediated

T cell exhaustion *in vitro,* which was further studied by inhibiting Akt and mTOR starting from day 2 during the *in vitro* T cell exhaustion model.

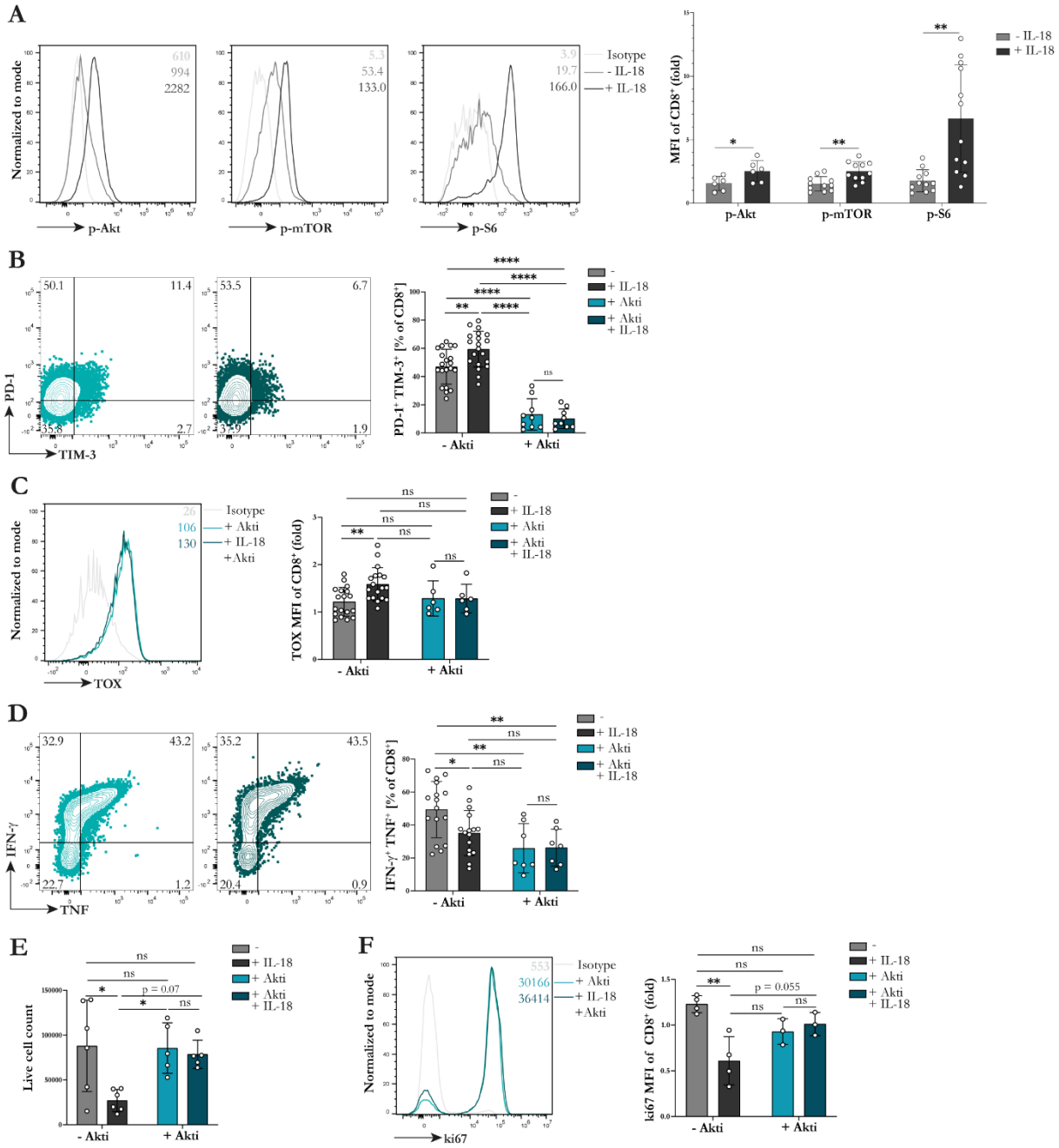


Figure 11: Akt signaling is partly involved in IL-18 mediated T cell exhaustion *in vitro*. Antigen-specific CD8⁺ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with anti-CD3 and anti-CD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen $OVA^{257-264}$ for 4 days and a resting phase without $OVA^{257-264}$ for 2 days. A) Cells were cultivated with or without IL-18 throughout the study, stained for p-Akt, p-mTOR and p-S6 and analyzed via FACS. B-D) Cells were cultivated with or without IL-18 throughout the protocol and with or without Akt inhibitor starting from day 2. Expression of B) surface markers (PD-1, TIM3 and LAG3; n=9-20), C) transcription factors (T-bet, TOX and Eomes; n=9-18) and D) production of cytokines (IFN-γ, TNF and GrzB; n=9-16) by *in vitro* exhausted T cells. E) Life cell count (n=5-6) and F) expression of ki67 (n=3-4) of *in vitro* exhausted T cells. Fold change was normalized to activated cells without IL-18 or inhibitor treatment. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-tests with (A: p-S6) or without (A: p-Akt, p-mTOR) Welch correction or two-way-ANOVA followed by Tukey's multiple comparisons (B-F). *p<0.05, **p<0.01, ****p<0.0001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

Inhibition of Akt decreased the percentage of PD-1 TIM3 double positive cells and prevented the IL-18-mediated increase of those cells (Fig. 11B, Lutz et al. 2023). Likewise, inhibition of Akt abolished the increase of TOX expression by IL-18 and did not influence TOX expression in general (Fig. 11C). The percentage of IFN-γ and TNF double producing cells nevertheless was decreased by inhibition of Akt but not further decreased by additional IL-18 treatment (Fig. 12D, Lutz et al.2023). The IL-18-mediated negative effects on T cell viability and proliferative capacity was abrogated by Akt (Fig. 11E, F, Lutz et al. 2023). Hence, inhibition of Akt was able to reverse some traits of IL-18-mediated exhaustion, indicating a role of Akt in this process. However, inhibition of Akt also negatively influenced cytokine production. Therefore, Akt activation did not fully explain the effects of IL-18 on T cell exhaustion *in vitro*.

mTOR inhibition by Rapamycin decreased the number of PD-1 TIM3 double positive cells and prevented the IL-18-mediated increase in PD-1 and TIM3 expression (Fig. 12A, Lutz et al. 2023). TOX expression was not generally affected by Rapamycin treatment, but IL-18 was not able to further increase TOX expression upon mTOR inhibition (Fig. 12B). Furthermore, mTOR inhibition prevented the IL-18-mediated loss of effector cytokines and did not negatively impact cytokine production itself (Fig. 12C, Lutz et al. 2023). Finally, mTOR inhibition caused a generally lower total live cell count and proliferative capacity but was able to abolish the IL-18-mediated decrease of those traits (Fig. 12D, E, Lutz et al. 2023). In summary, inhibition of mTOR prevented the IL-18-mediated increase of PD-1 TIM3 double positive cells and TOX expression while restoring the IL-18-mediated loss of effector cytokines. This indicated that mTOR activation is an important mediator of T cell exhaustion caused by IL-18R signaling *in vitro*.

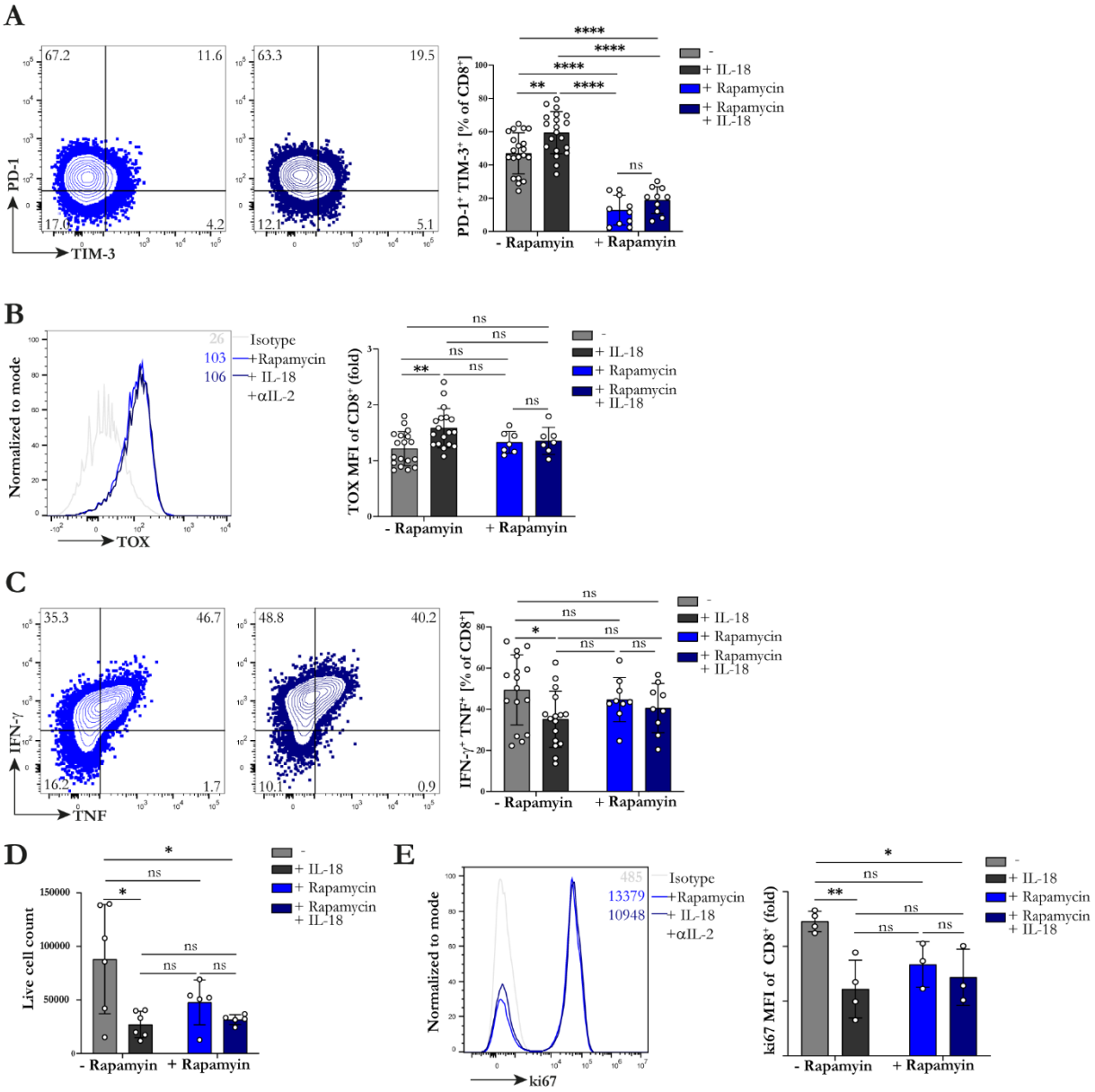


Figure 12: mTOR signaling engages in IL-18 mediated T cell exhaustion *in vitro*. Antigen-specific CD8+ T cells were isolated from spleen and lymph nodes of OT-I mice and activated with αCD3 and αCD28 for 2 days (=primary stimulation) followed by cultivation with the cognate antigen $OVA^{257-264}$ for 4 days and a resting phase without $OVA^{257-264}$ for 2 days. Cells were cultivated with or without IL-18 throughout the protocol and with or without the mTOR inhibitor Rapamycin starting from day 2. Expression of A) surface markers (PD-1, TIM3 and LAG3; n=10-20), B) transcription factors (T-bet, TOX and Eomes; n=10-18) and C) production of cytokines (IFN-γ, TNF and GrzB; n=10-16) by *in vitro* exhausted T cells. D) Life cell count (n=5-6) and E) expression of ki67 (n=3-4) of *in vitro* exhausted T cells. Fold change was normalized to activated cells without IL-18 or inhibitor treatment. Bars represent mean ± sd and statistics were evaluated two-way-ANOVA followed by Tukey's multiple comparisons (B-I). *p<0.05, **p<0.01, ****p<0.0001, ns: not significant. This Figure contains modified versions of graphs previously published in Lutz et al. 2023.

4.4 Influence of the NLRP3-dependent cytokines IL-18 and IL-1β on *in vitro* differentiated CD4+ T cell subsets

4.4.1 Expression of IL-18R and IL-1R on CD4+ subsets *in vitro*

In order to evaluate the upregulation of the receptors for the NLRP3-dependent cytokines IL-18 and IL-1β on $CD4^+$ subsets during differentiation, the expression of those receptors on $CD4^+$ T cells during Th1 and iTreg differentiation was measured every day. Both $CD4^+$ subsets markedly upregulated IL-18R after 48 h of differentiation. However, $CD4^+$ cells differentiated under iTreg conditions, upregulated IL-18R to a lesser extent than $CD4^+$ cells differentiated under Th1 conditions (Fig. 13A, B).

Contrary, the expression of IL-1R on $CD4^+$ cells differentiated under Th1 and iTreg conditions stayed at a marginal level, with a higher expression level on $CD4^+$ cells differentiated under iTreg conditions (Fig. 13 C, D). These results are in line with previous reports showing no IL-1R expression on Th1 cells but on Tregs (Carroll et al. 2008; Taylor-Robinson AW 1994).

Therefore, both receptors were expressed on *in vitro* differentiated $CD4^+$ cells at a detectable level, but IL-18R was markedly upregulated during differentiation while IL-1R was not. This raised the question if there might be a more prominent role for IL-18R signaling in $CD4^+$ T cell development *in vitro* and *in vivo*.

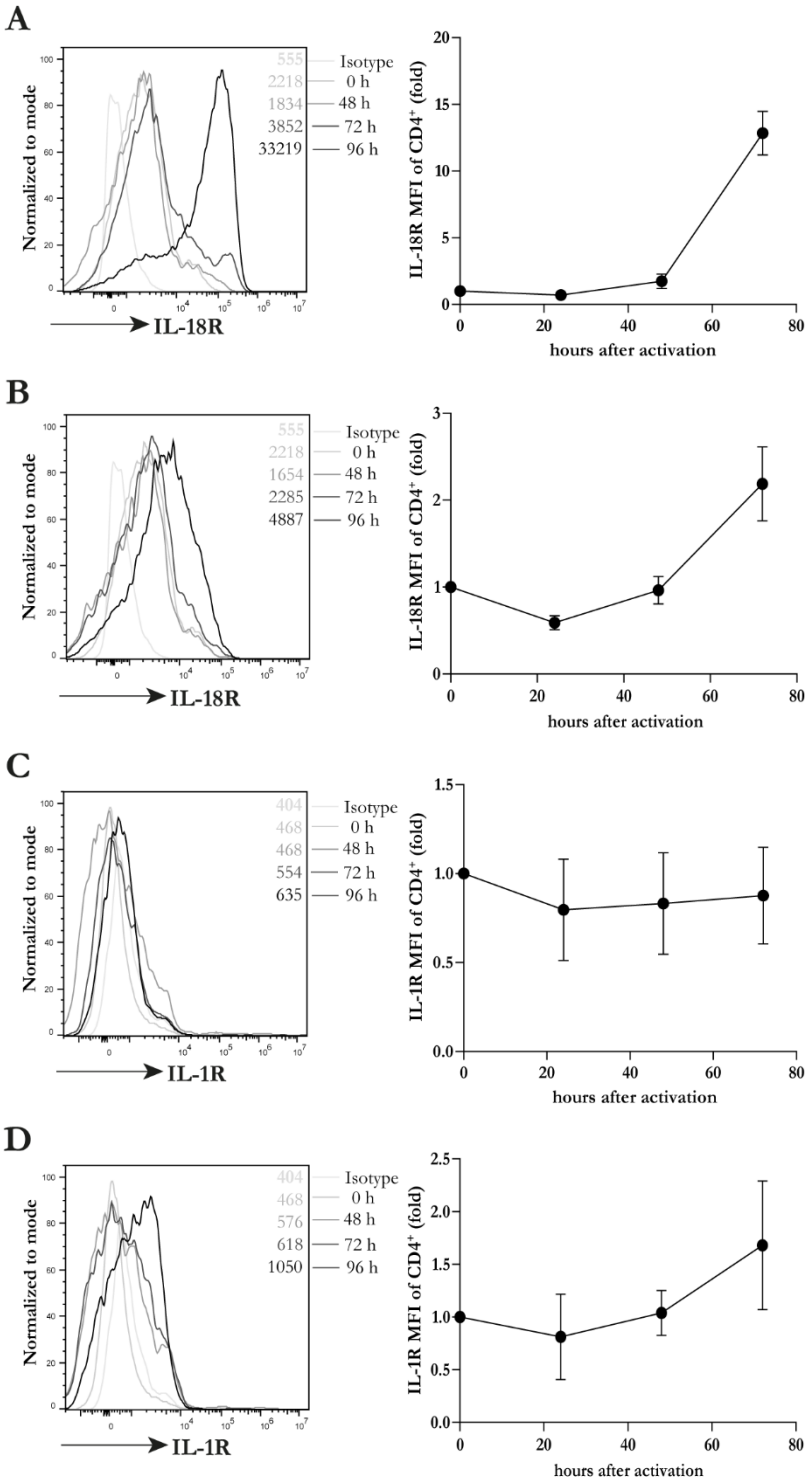


Figure 13: Expression of IL-18R and IL-1R on antigen-specific Th1 cells and iTregs *in vitro.* Splenocytes of OT-II mice were incubated with ISQ for 1 h and cultivated under A+C) Th1 conditions or B+D) iTreg conditions for 72 h. Cells were harvested after 24, 48 and 72 h, stained for A-B) IL-18R and C-D) IL-1R expression and analyzed via FACS. Data points represent mean ± sd (n=4).

4.4.2 IL-18 signaling affects CD4+ subset differentiation *in vitro*

In order to identify the effects of IL-18R signaling on CD4+ subsets *in vitro*, OT-II splenocytes were activated with ISQ and differentiated under Th1 and iTreg conditions with and without additional IL-18.

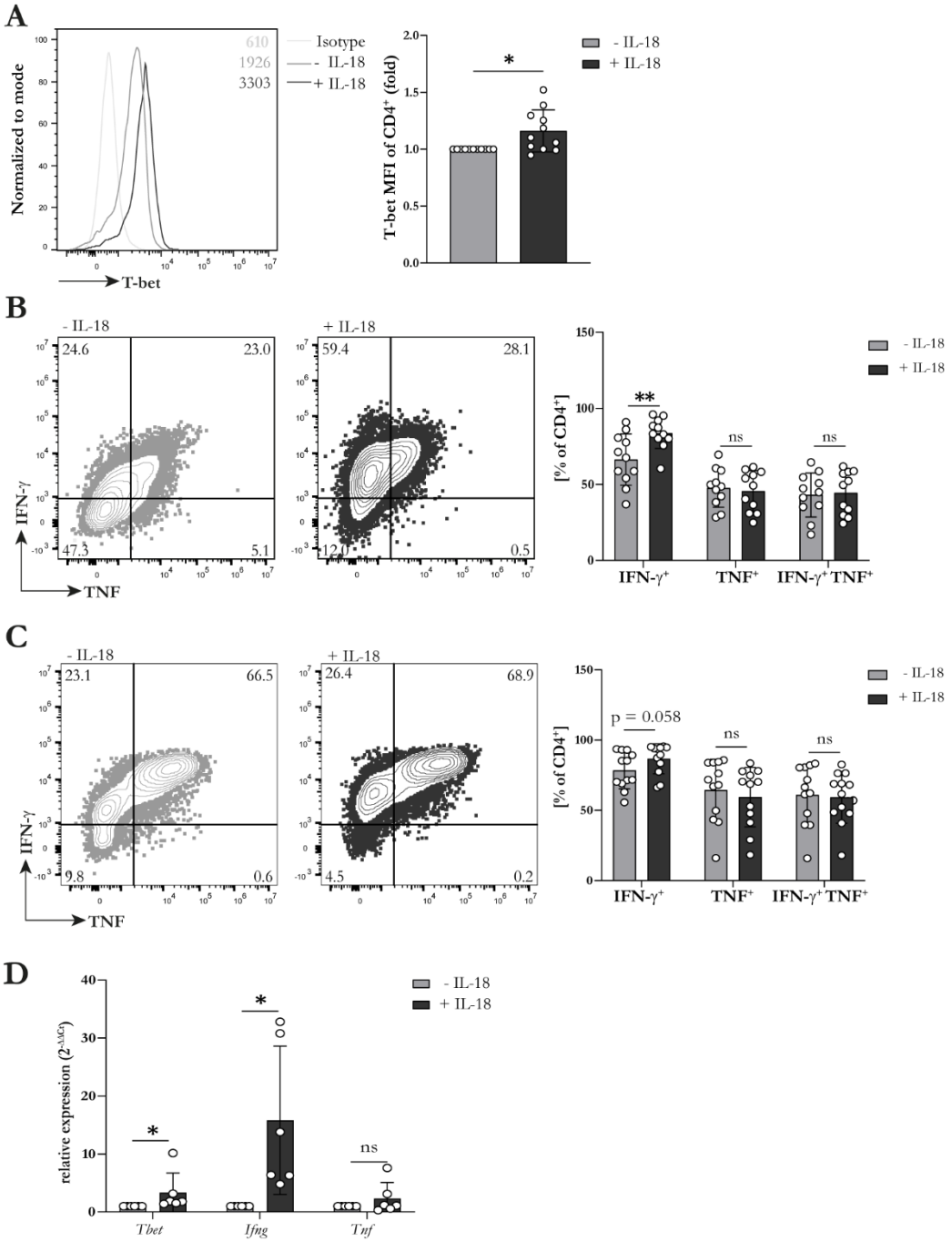


Figure 14: IL-18 impacts Th1 differentiation *in vitro*. Splenocytes of OT-II mice were incubated with ISQ for 1 h and cultivated under Th1 conditions with or without IL-18 for 72 h. Cells were harvested after 72 h, A-C) stained for Th1 markers and cytokines and D) used for RNA isolation and qPCR for respective genes. A) Expression of T-bet (n= 11) and production of cytokines after B) antigen-specific (n=11) and C) antigen-unspecific restimulation (n=12) of Th1 cells. D) Gene expression of *T-bet, Ifng* and *Tnf* of Th1 cells (n=6). Fold change was normalized to treatments without IL-18. Bars represent mean ± sd and statistics were evaluated by One sample Wilcoxon test (A), two-tailed, unpaired t-test (B, C: TNF+, IFN-γ+ TNF+, D) or Mann-Whitney test (C: IFN-γ+). *p<0.05, **p<0.01, ns: not significant.

Th1 cells differentiated with supplemented IL-18 showed increased T-bet expression compared to Th1 cells differentiated without additional IL-18 (Fig. 14A). Likewise, the percentage of IFN-γ-producing cells was increased upon IL-18 treatment when cells were restimulated in an antigen-specific manner. However, TNF production was not changed by IL-18 treatment and accordingly the number of cells producing both cytokines was unchanged (Fig. 14B). The IL-18-mediated increase of IFN-γ-producing cells was also (not significantly) present when cells were restimulated in an antigen-unspecific manner. The percentage of TNF and double-producing cells, however, remained unchanged (Fig. 14C). These results indicate an influence of IL-18 on T-bet expression and specifically on IFN-γ production of antigen-specific $CD4^+$ T cells differentiated under Th1 conditions. The influence of IL-18 on Th1 cells could also be validated via RT qPCR, which revealed an IL-18-mediated increase in *T-bet* and *Ifng* mRNA expression, while *Tnf* mRNA levels were unchanged (Fig. 14D).

$CD4^+$ T cells differentiated under iTreg conditions *in vitro* showed a lesser percentage of iTregs when supplemented with IL-18 compared to cells differentiated without IL-18 supplementation (Fig. 15A). Regarding the production of inhibitory cytokines, IL-18 had no effect on IL-10 production of *in vitro* differentiated iTregs. Of note, the IL-10 positive cells among *in vitro* differentiated iTregs were remarkably high. The number of TGF-β producing iTregs nevertheless was higher upon IL-18 treatment, which indicates an increased inhibitory potential of iTregs caused by IL-18 signaling (Fig. 15B). Therefore, IL-18 seems to generally impair iTreg differentiation *in vitro* but increased their inhibitory potential. This phenomenon was also supported by results from RT qPCR, where *Foxp3* mRNA expression was decreased, *Il10* expression was unchanged and *Tgfb* expression was (not significantly) increased (Fig. 15C).

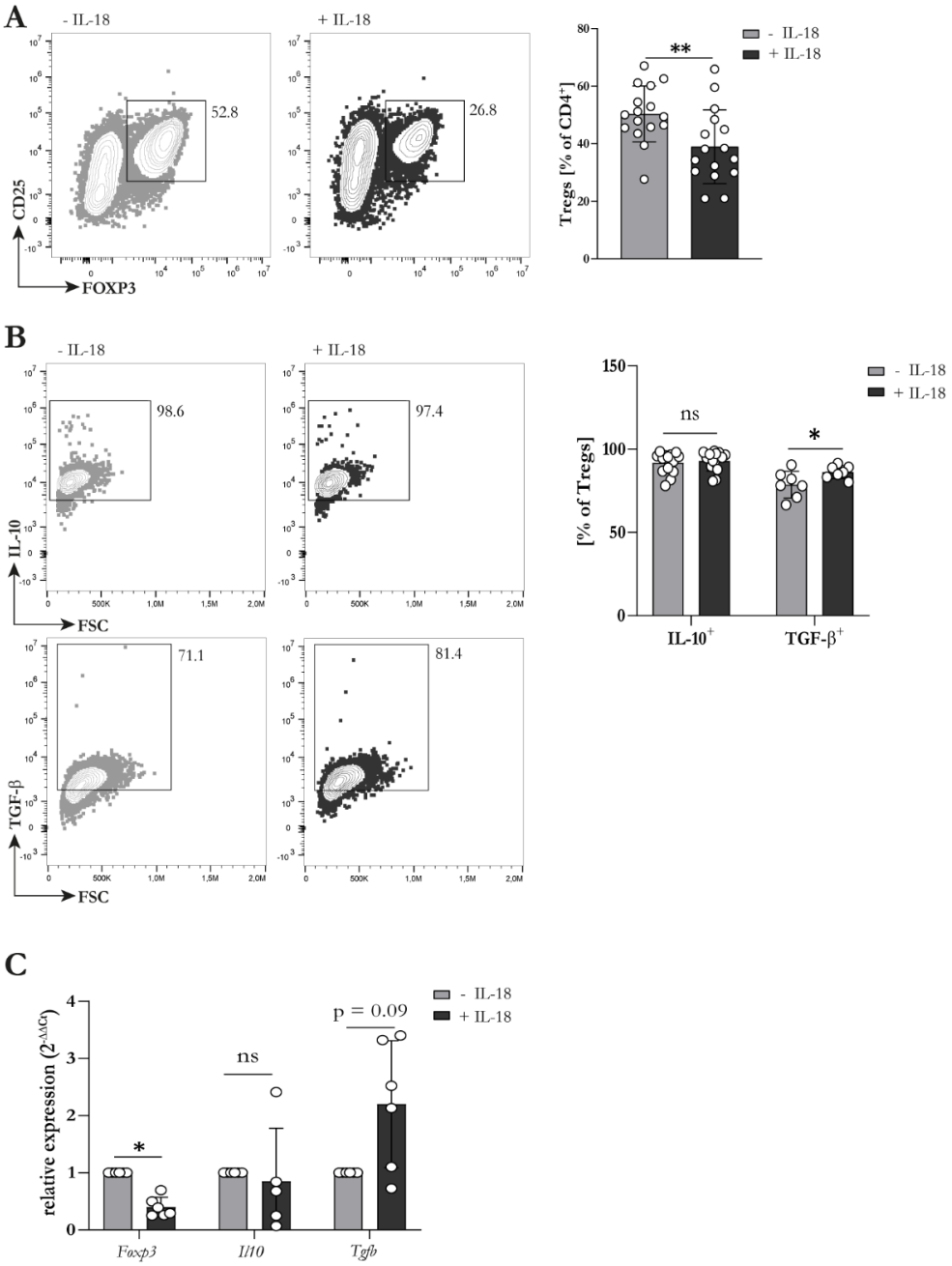


Figure 15: IL-18 impairs iTreg differentiation *in vitro.* Splenocytes of OT-II mice were incubated with ISQ for 1 h and cultivated under iTreg conditions with or without IL-18 for 72 h. Cells were harvested after 72 h, A-B) stained for Treg specific markers and cytokines and C) used for RNA isolation and qPCR for respective genes. A) Percentage of iTregs (n=16), B) percentage of IL-10^{+} and TGFβ-producing iTregs (n=7-14) and C) Gene expression of *Foxp3, Il19* and *Tgfb* of iTregs (n=6). Fold change was normalized to treatments without IL-18. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-test (A, B: TGF-β, C) or Mann-Whitney test (B: IL-10^{+}). *p<0.05, **p<0.01, ns: not significant.

In summary, during *in vitro* differentiation of antigen-specific CD4^{+} T cells the differentiation of Th1 cells was induced and the differentiation of iTregs was suppressed by IL-18.

4.4.3 IL-1β signaling affects iTreg, but not Th1 differentiation *in vitro*

In order to identify the effects of IL-1β signaling on CD4$^+$ subsets *in vitro,* OT-II splenocytes were activated and differentiated under Th1 and iTreg conditions *in vitro* with and without additional IL-1β.

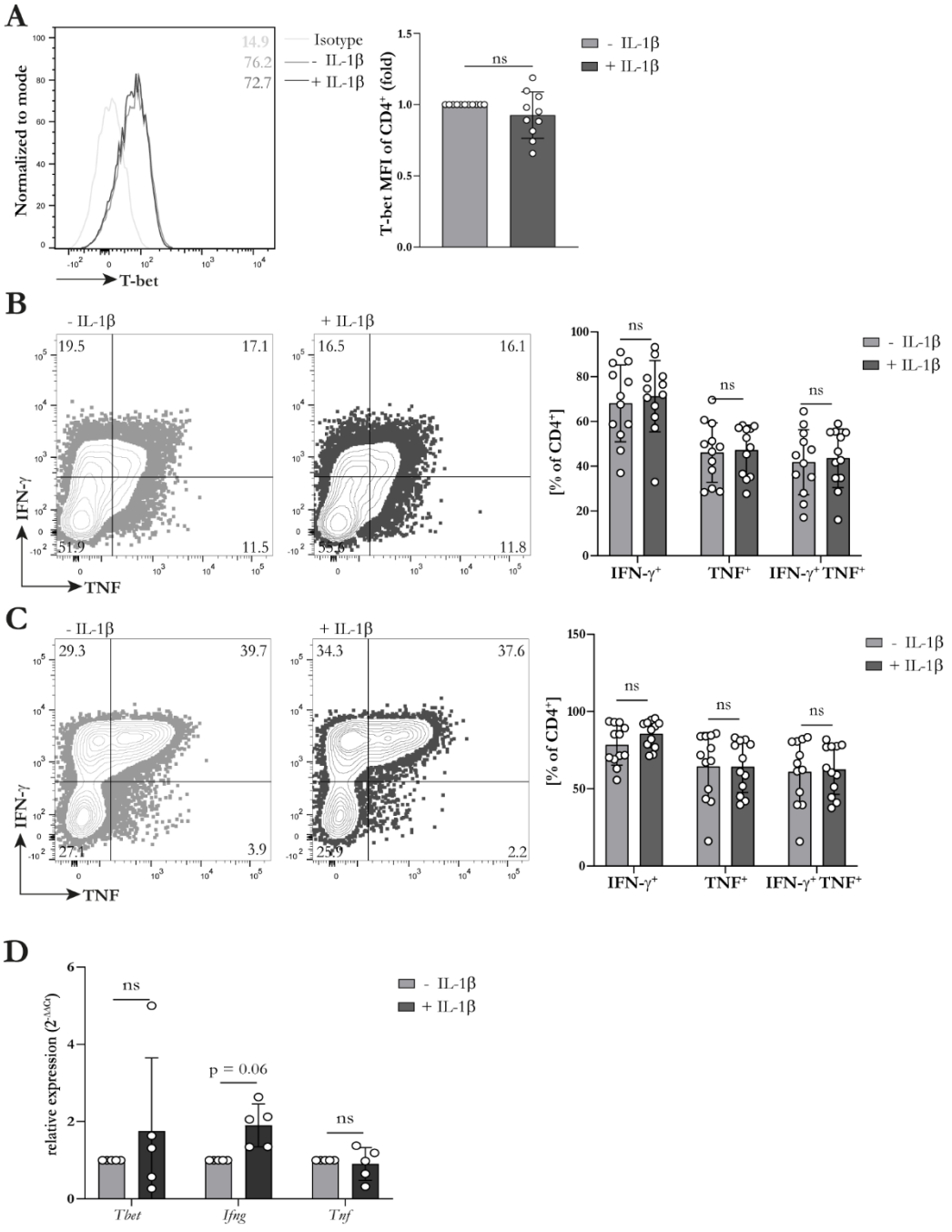


Figure 16: IL-1β does not have an impact on Th1 differentiation *in vitro*. Splenocytes of OT-II mice were incubated with ISQ for 1 h and cultivated under Th1 conditions with or without IL-1β for 72 h. Cells were harvested after 72 h, A-C) stained for Th1 markers and cytokines, and D) used for RNA isolation and qPCR for respective genes. A) Expression of T-bet (n=10) and production of cytokines after B) antigen-specific (n=12) and C) antigen-unspecific restimulation (n=12) of Th1 cells. D) Gene expression of *T-bet, Ifng* and *Tnf* of Th1 cells (n=5). Fold change was normalized to treatments without IL-1β. Bars represent mean ± sd and statistics were evaluated by One sample Wilcoxon test (A), two-tailed, unpaired t-test (B: IFN-γ$^+$, IFN-γ$^+$ TNF$^+$, C) or Mann-Whitney test (B: TNF$^+$). ns: not significant.

IL-1β treatment of Th1 cells differentiated *in vitro* neither changed T-bet expression (Fig. 16A) nor the percentage of IFN-γ- or TNF-producing cells, as well as double-producing cells, no matter if the cells were restimulated in an antigen-specific or unspecific manner (Fig. 16B, C). RT qPCR revealed a slight, but not significant, increase of *Ifng* mRNA expression upon IL-1β treatment, while *T-bet* and *tnf* mRNA expression was not altered (Fig. 16D).

Additionally, iTreg differentiation *in vitro* was not influenced by IL-1β treatment (Fig. 17A) and the percentage of IL-10-producing cells among iTregs was unchanged. Nevertheless, the percentage of TGFβ producing cells among *in vitro* differentiated iTregs was increased (Fig. 17B). RT qPCR however, showed that IL-1β supplementation did not alter the mRNA expression of *Foxp3, Il10* and *Tgfb* (Fig. 17C).

In summary, IL-1β, compared to IL-18, does not influence $CD4^+$ T cell development *in vitro* indicating a less prominent role of IL-1β *in vivo* as well.

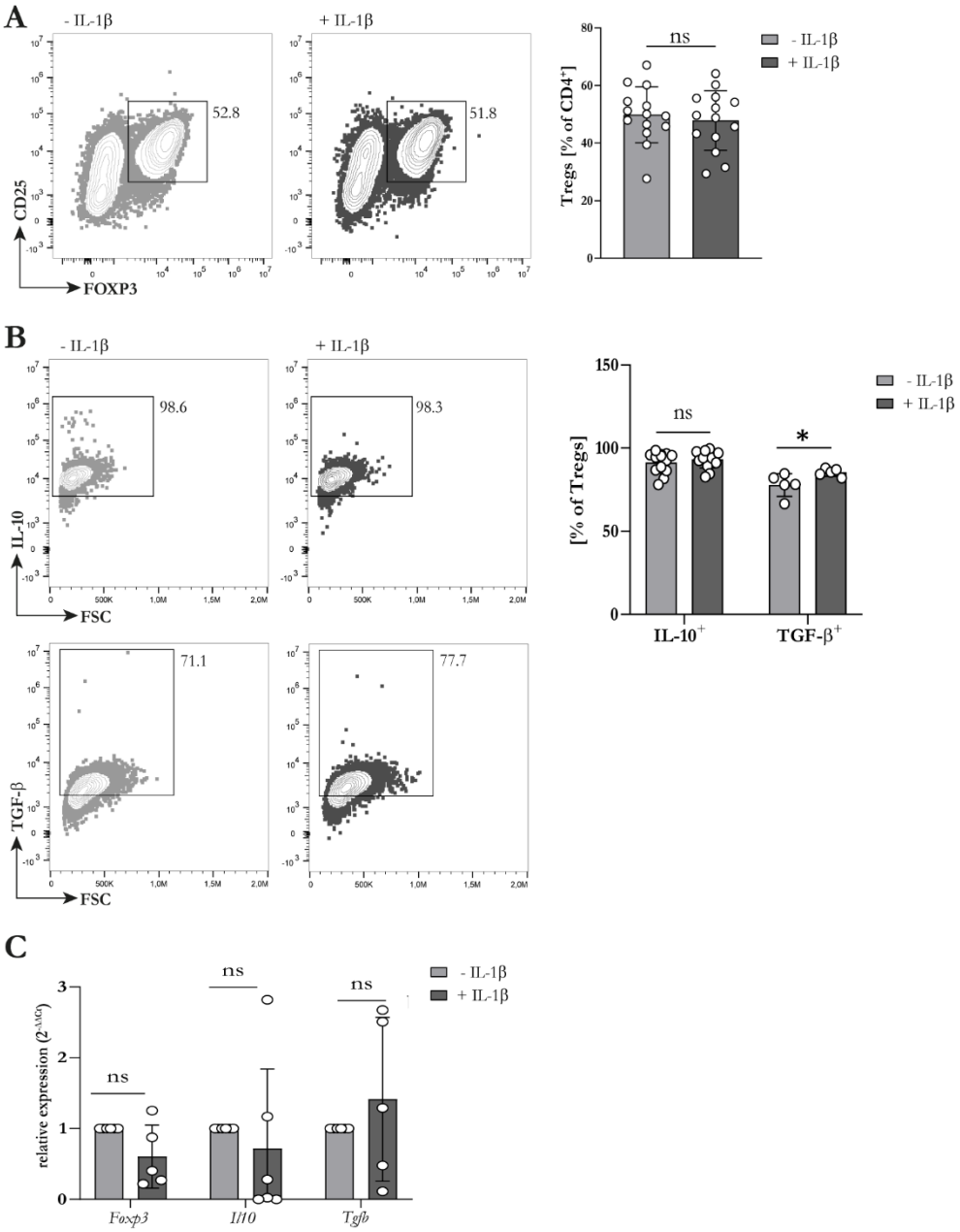


Figure 17: IL-1β influences cytokine production of iTregs. Splenocytes of OT-II mice were incubated with ISQ for 1 h and cultivated under iTreg conditions with or without IL-1β for 72 h. Cells were harvested after 72 h, A-B) stained for Treg specific markers and cytokines, and C) used for RNA isolation and qPCR for respective genes. A) Percentage of iTregs (n=14), B) percentage of IL-10$^+$ and TGFβ (n=5-13) producing iTregs and C) Gene expression of *Foxp3, Il10* and *Tgfb* of iTregs (n=5). Fold change was normalized to treatments without IL-1β. Bars represent mean ± sd and statistics were evaluated by two-tailed, unpaired t-test. *p<0.05, ns: not significant.

4.4.4 IL-18R and IL-1R deficiency does not impact the differentiation of CD4$^+$ T cell subsets *in vitro*

As treatment of CD4$^+$ subsets with the NRLP3-dependent cytokines can alter their differentiation *in vitro*, the question arose if CD4$^+$ T cells with deficiencies in the respective cytokine receptors exhibit plasticity of Th1 or iTreg differentiation that is different to the plasticity of WT cells. This question was of particular interest regarding planned *in vivo* studies focusing on the development and impact of those receptor deficiencies on T cell development *in vivo*.

Therefore, splenocytes of OT-II WT, *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ mice were incubated with ISQ and differentiated under Th1 and iTreg conditions. *In vitro* Th1 differentiation was not altered in CD4^{+} cells with IL-18 or IL-1R deficiency. T-bet expression was at similar levels as in WT cells (Fig. 18A) and IFN-γ, TNF or double-producing cells were unchanged regardless of whether the cells were restimulated in an antigen-specific (Fig. 18B) or unspecific manner (Fig. 18C).

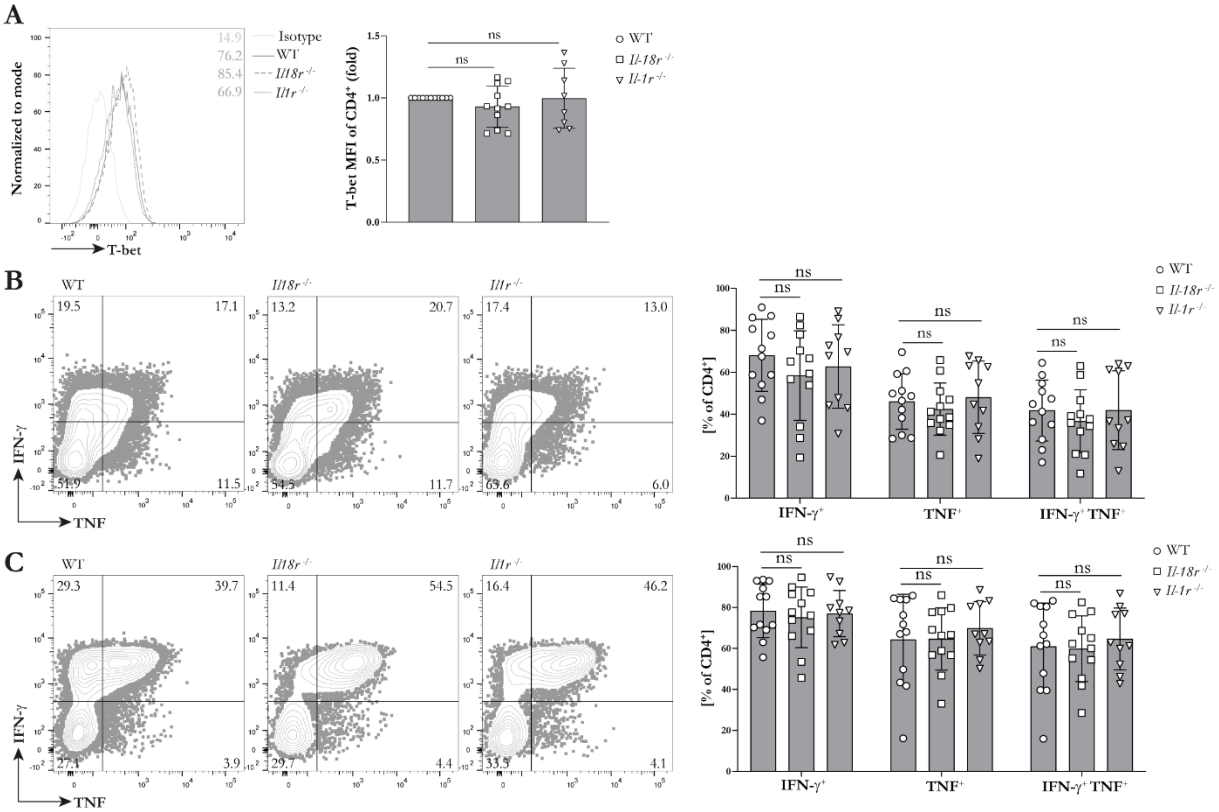


Figure 18: Th1 differentiation is not altered in antigen-specific *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ CD4^{+} T cells *in vitro*. Splenocytes of OT-II WT, *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ mice were incubated with ISQ for 1 h and cultivated under Th1 conditions or. Cells were harvested after 72 h and stained for Th1 specific markers and cytokines. A) Expression of T-bet (n=8-11) and production of cytokines after B) antigen-specific (n=10-12) and C) antigen-unspecific restimulation (n=10-12) of Th1 cells. Fold change was normalized to WT cells. Bars represent mean ± sd and statistics were evaluated by one-way ANOVA followed my Dunnett's multiple comparisons (compared to WT). ns: not significant.

Regarding iTreg differentiation, the percentage of FOXP3^{+} CD25^{+} iTregs did not differ between WT and *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4^{+} T cells (Fig. 19A). The percentage of IL-10- and TGFβ-producing cells among iTregs was also unaltered (Fig. 19B).

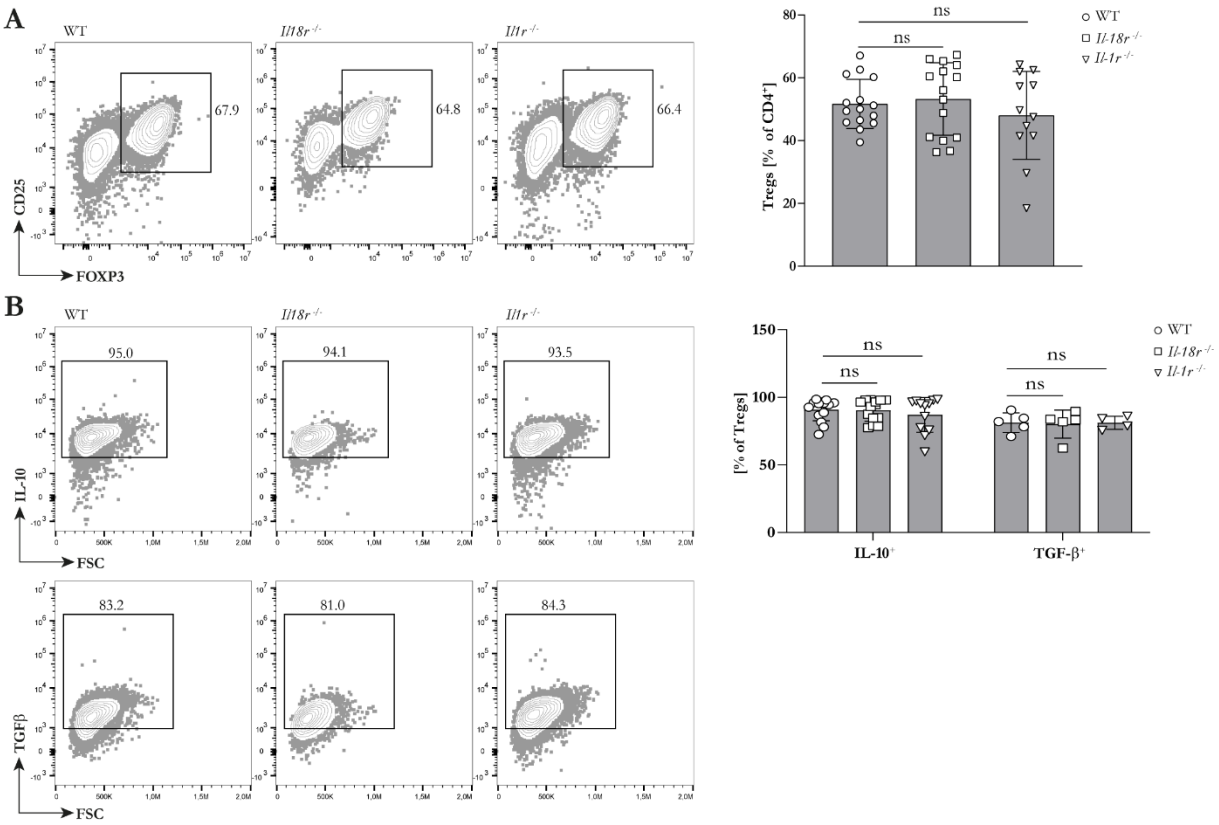


Figure 19: iTreg differentiation is not altered in antigen-specific *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ CD4^{+} T cells *in vitro*. Splenocytes of OT-II WT, *Il18r* $^{-/-}$ and *Il1r* $^{-/-}$ mice were incubated with ISQ for 1 h and cultivated under iTreg conditions. Cells were harvested after 72 h and stained for Treg specific markers and cytokines. Percentage of A) iTregs (n=10-15) and B) IL-10^{+} and TGFβ (n=4-15) producing iTregs. Fold change was normalized to WT cells. Bars represent mean ± sd and statistics were evaluated by one-way ANOVA followed my Dunnett's multiple comparisons (compared to WT). ns: not significant.

Therefore, the differentiation of Th1 cells and iTregs was not altered in antigen specific CD4^{+} T cells with an IL-18 or IL-1R deficiency, and potential differences to WT cells can only be mediated by external IL-18 and IL-1β, which can inter alia be supplied by cells in the tumor microenvironment.

4.5 Influence of IL-18R and IL-1R signaling in CD4^{+} cells on T cells in a murine pancreatic cancer model

4.5.1 Influence of IL-18 and IL-1R signaling in CD4^{+} T cells on abundancies of T cell populations in a murine pancreatic cancer model

In vitro experiments during this study revealed that IL-18 facilitated Th1 differentiation but suppressed iTreg differentiation. Therefore, there is a possibility that IL-18 strongly influences the Th1/Treg ratio *in vivo* and therefore shifts the immunological processes in the tumor microenvironment towards a tumor-rejecting type. Additionally, Lutz et al. confirmed a prominent role of IL-18 signaling on phenotype and function of adoptively transferred CTLs in a murine pancreatic cancer model (Lutz et al. 2023). Therefore, the influence of NLRP3-dependent cytokines on adoptively transferred CD4^{+} T cells in the same context was of particular interest.

In order to study these effects, PancOVA tumors were inoculated into the left flank of Ly5.1 mice and naïve OT-II CD4+ T cells were isolated from OT-II WT, *Il18r* -/- and *Il1r* -/- mice and intraperitoneally injected at day 0 and 5 of tumor inoculation. Mice were sacrificed at day 10 and cells from blood, tumor draining lymph node, spleen and tumor of those mice were stained for T cell populations (Fig. 20A).

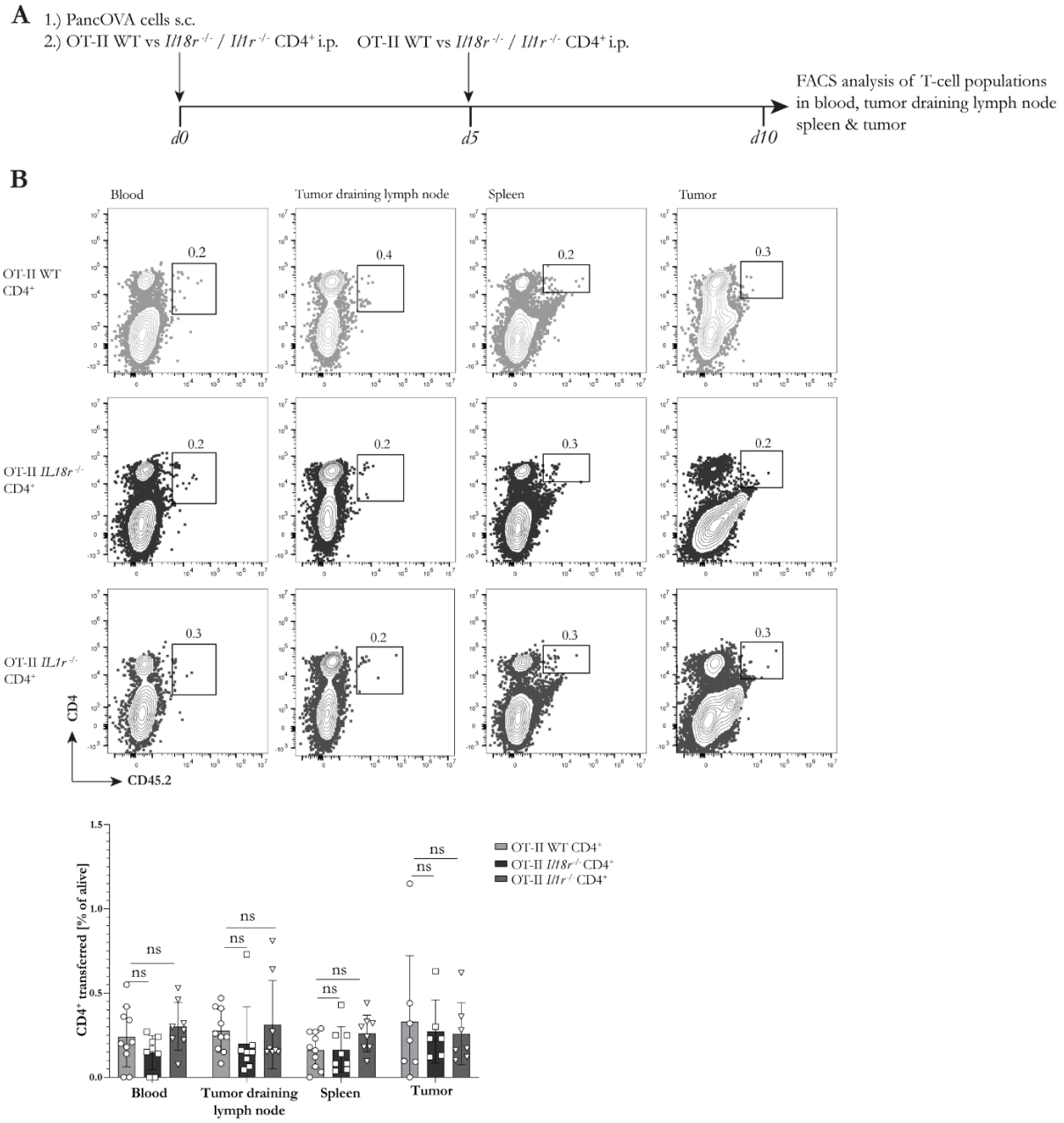


Figure 20: IL-18R or IL-1R deficiency of adoptively transferred OT-II CD4+ T cells does not influence their abundancy in lymphoid organs of tumor-bearing mice. A) Schematic of experimental procedure. PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* -/- or *Il1r* -/- naïve CD4+ T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. B) Amount of adoptively transferred OT-II WT, *Il18r* -/- or *Il1r* -/- CD4+ T cells found in blood, tumor-draining lymph node, spleen, and tumor (n=7-10). Bars represent mean ± sd and statistics were evaluated by one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT) (B: blood, spleen, tumor OT-II WT vs. OT-II *Il18r* -/- or *Il1r* -/- CD4+) or Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to OT-II WT CD4+) (B: tumor draining lymph node OT-II WT vs. OT-II *Il18r* -/- or *Il1r* -/- CD4+). ns: not significant.

Adoptively transferred OT-II $CD4^+$ T cells were found in the blood, tumor-draining lymph node, the spleen, and tumors of tumor-bearing mice with no differences between the WT and receptor deficient cells (Fig. 20B). Nevertheless, the number of adoptively transferred OT-II $CD4^+$ T cells was very low, which made further analysis of transcription factor and cytokine patterns of those cells difficult. For that reason, the focus of these studies was put on the effect of adoptively transferred OT-II $CD4^+$ T cells with or without IL-18 or IL-1R deficiency on endogenous T cell populations.

The adoptive transfer of tumor-specific OT-II WT $CD4^+$ T cells did not alter the amount of endogenous $CD4^+$ T cells in the blood, the tumor-draining lymph node, the spleen, or the tumor itself (Fig. 21A). Additionally, OT-II *Il18r*$^{-/-}$ and *Il1r*$^{-/-}$ $CD4^+$ T cells did not have an impact on this T cell population compared to OT-II WT $CD4^+$ T cells (Fig. 21B).

The amount of endogenous $CD8^+$ T cells, which migrated into the spleen, or the tumor was also neither changed by adoptive transfer of tumor-specific OT-II WT $CD4^+$ (Fig. 21C) nor by the adoptive transfer of OT-II *Il18r*$^{-/-}$ and *Il1r*$^{-/-}$ $CD4^+$ T cells (Fig. 21D).

In summary, the adoptive transfer of tumor-specific $CD4^+$ T cells caused low populations of these cells in the organs of tumor-bearing mice. Additionally, adoptive transfer of naïve $CD4^+$ T cells did not have an impact on endogenous T cell populations, no matter if the adoptively transferred $CD4^+$ T cells had an IL-18 or IL-1R deficiency or not.

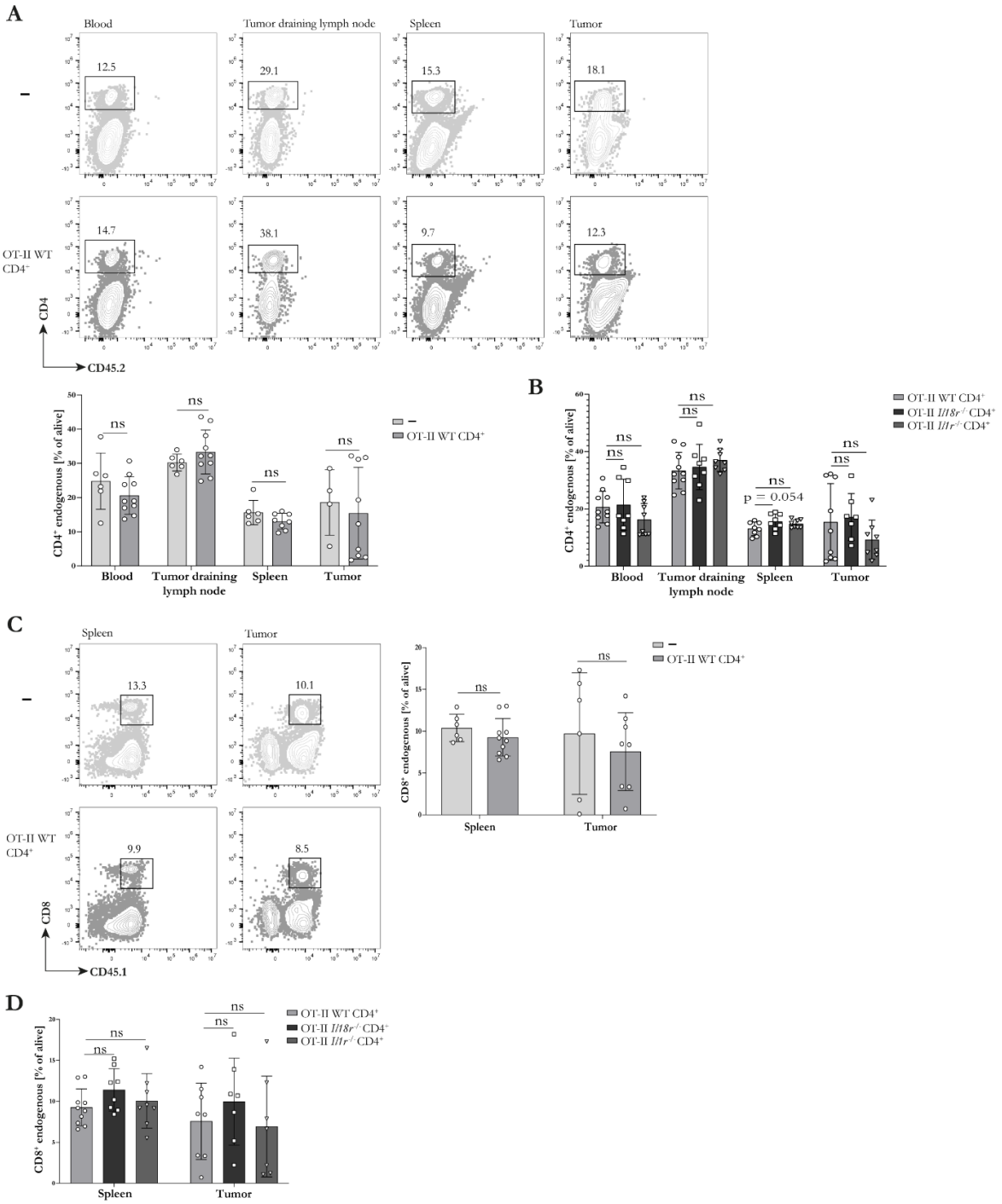


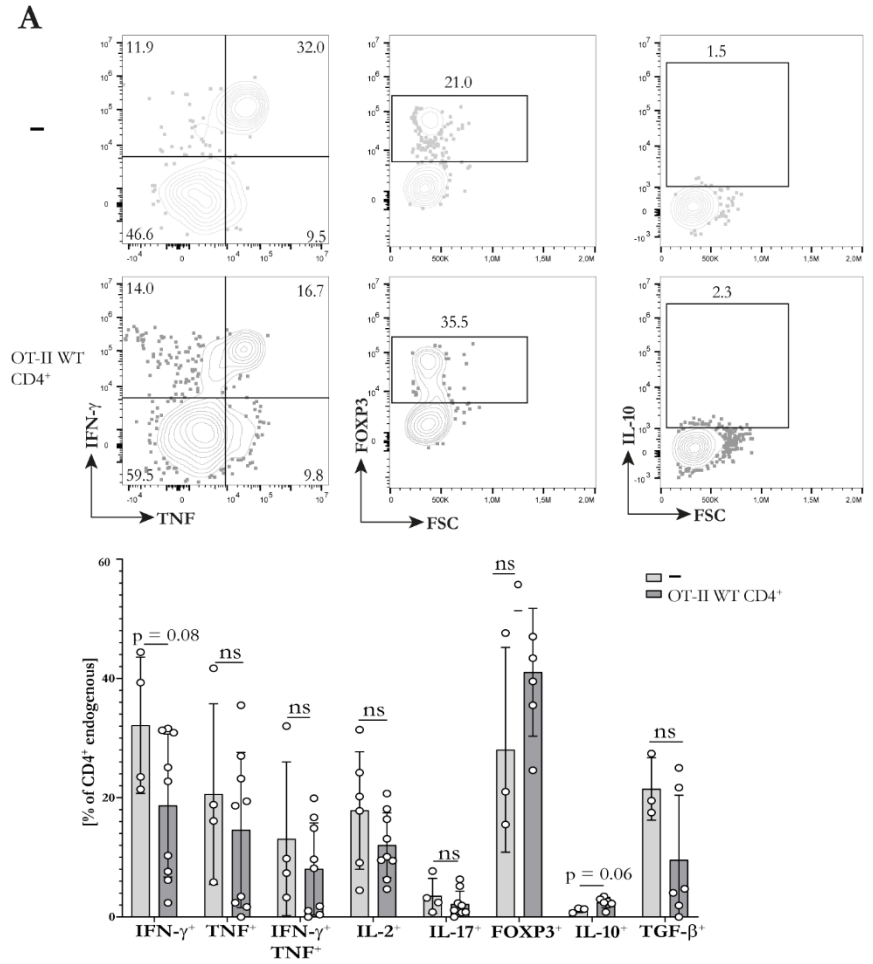
Figure 21: IL-18R or IL-1R deficiency of adoptively transferred OT-II CD4⁺ T cells does not influence the abundancy of endogenous T cell populations in lymphoid organs of tumor-bearing mice. . PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ naïve CD4⁺ T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. A+B) Amount of endogenous CD4⁺ T cells found in blood, tumor-draining lymph node, spleen, and tumor (n=6-10) and C+D) Amount of endogenous CD8⁺ T cells found in spleen and tumor of mice (n=6-10). Bars represent mean ± sd and statistics were evaluated by one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT) (B: blood, tumor draining lymph node, spleen, OT-II WT vs. OT-II *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$, D: OT-II WT vs. OT-II *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4⁺), Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to OT-II WT CD4⁺) (B: tumor OT-II WT vs. OT-II *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4⁺), two-tailed, unpaired t-test (A: blood, tumor draining lymph node, spleen – vs OT-II WT CD4⁺, D: – vs OT-II WT CD4⁺) or Mann-Whitney test (A: tumor – vs OT-II WT CD4⁺). ns: not significant.

4.5.2 Influence of IL-18R and IL-1R signaling in adoptively transferred CD4+ T cells on endogenous T cell populations

4.5.2.1 Influence of IL-18R and IL-1R signaling in adoptively transferred CD4+ T cells on endogenous CD4+ T cells

As the amount of endogenous CD4+ T cells that were found in the organs of tumor-bearing mice was not altered by adoptive transfer of OT-II WT and receptor deficient CD4+ T cells, the phenotype of endogenous CD4+ T cells in the tumor was investigated.

Endogenous CD4+ T cells showed a tending decrease in IFN-γ production when OT-II WT CD4+ T cells were adoptively transferred, while IL-10 production was slightly, but not significantly increased (Fig. 23A), indicating a shift from the Th1 to the Treg subtype upon adoptive transfer of tumor specific CD4+ T cells. Other markers used to determine the subset of endogenous CD4+ T cells (production of TNF, IL-2, IL-17A and TGFβ, expression of FOXP3) were not altered by adoptive transfer of OT-II WT CD4+ T cells (Fig. 22A). The adoptive transfer of OT-II *Il18r*$^{-/-}$ compared to OT-II WT CD4+ T cells did not alter the phenotype of endogenous CD4+ T cells in the tumor, except for a slight, but not significant, decrease in endogenous FOXP3 expressing cells and a significant decrease in IL-10 producing cells (Fig. 22B). As *in vitro* experiments confirmed a decreased FOXP3 expression upon IL-18 treatment, it might be the case that OT-II WT CD4+ T cells scavenge IL-18 in the TME while *Il18r*$^{-/-}$ do not. Intratumoral IL-18 would reduce the expression of FOXP3 in endogenous CD4+ T cells within this paradigm. The adoptive transfer of OT-II *Il1r*$^{-/-}$ CD4+ T cells did not change the tested markers of endogenous CD4+ T cells compared to OT-II WT CD4+ T cells (Fig. 22B).


A
–
OT-II WT CD4+
11.9
32.0
46.6
9.5
21.0
1.5
14.0
16.7
59.5
9.8
35.5
2.3
IFN-γ
TNF
FOXP3
FSC
IL-10
FSC
[% of CD4+ endogenous]
p = 0.08
ns
p = 0.06
IFN-γ+
TNF+
IFN-γ+ TNF+
IL-2+
IL-17+
FOXP3+
IL-10+
TGF-β+
OT-II WT CD4+

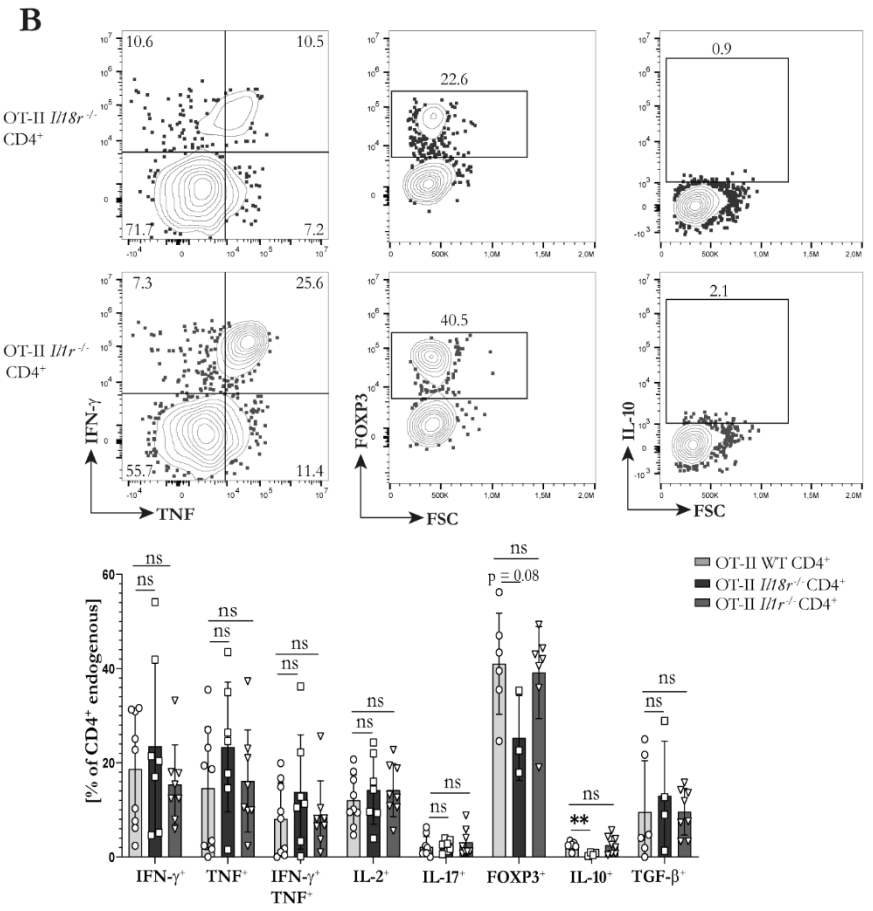

B
OT-II Il18r-/- CD4+
OT-II Il1r-/- CD4+
10.6
10.5
71.7
7.2
22.6
0.9
7.3
25.6
55.7
11.4
40.5
2.1
IFN-γ
TNF
FOXP3
FSC
IL-10
FSC
[% of CD4+ endogenous]
ns
p = 0.08
**
IFN-γ+
TNF+
IFN-γ+ TNF+
IL-2+
IL-17+
FOXP3+
IL-10+
TGF-β+
OT-II WT CD4+
OT-II Il18r-/- CD4+
OT-II Il1r-/- CD4+

Figure 22: Endogenous CD4$^+$ T cells in the tumor are marginally affected by adoptively transferred OT-II CD4$^+$ T cells. PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ naïve CD4$^+$ T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. Expression of FOXP3$^+$ and production of cytokines (n=3-9). Bars represent mean ± sd, and statistics were evaluated by tow-tailed, unpaired t-test (A: IFN-γ$^+$, TNF$^+$, IFN-γ$^+$, TNF$^+$, IL-2$^+$, FOXP3$^+$, IL-10$^+$, TGF-β$^+$), Mann-Whitney test (A: IL-17$^+$), one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT) (B: IFN-γ$^+$, TNF$^+$, IFN-γ$^+$, TNF$^+$, IL-2$^+$, FOXP3$^+$, TGF-β$^+$), Brown-Forsythe and Welch's ANOVA test followed by Dunnetts's T3 multiple comparisons (compared to OT-II WT CD4$^+$) (B: IL-10$^+$) or Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to WT CD4$^+$) (B: IL-17$^+$). **$p<0.01$, ns: not significant.

CD4$^+$ T cells have been described to show characteristics typical for exhausted CD8$^+$ CTLs (Miggelbrink et al. 2021). Hence, it was interesting to analyze endogenous CD4$^+$ T cells focusing on typical hallmarks of exhausted T cells. For that reason, the expression of typical surface markers (PD-1, TIM3, LAG3 and 2B4) by endogenous CD4$^+$ T cells was analyzed. Neither the adoptive transfer of OT-II CD4$^+$ T cells nor IL-18R or IL-1R deficiency altered the expression of the surface markers, or combinations of those, by endogenous CD4$^+$ T cells in the tumor (Fig. 23A, B). Likewise, the expression of the transcription factors T-bet, TOX and Eomes were unchanged by adoptive transfer of OT-II CD4$^+$ T cells (Fig. 23C, D).

Therefore, adoptive transfer of OT-II WT or receptor-deficient CD4$^+$ T cells does not have a strong impact on endogenous CD4$^+$ T cell populations regarding the expression of coinhibitory receptors, transcription factors involved in T cell development and function, or the production of subset-specific cytokines.

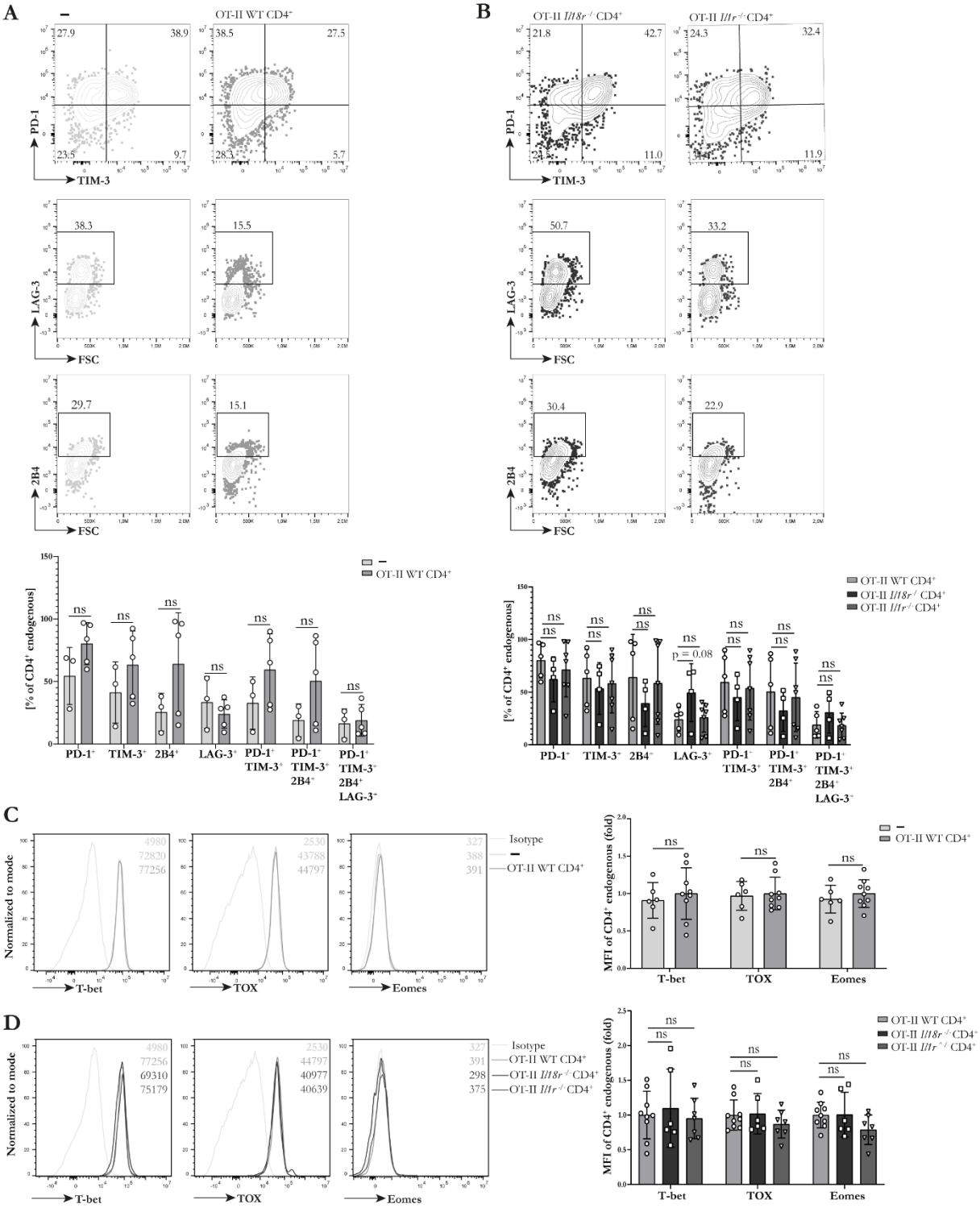


Figure 23: Expression of co-inhibitory receptors and transcription factors involved in T cell development and function by endogenous CD4+ T cells in the tumor is not affected by adoptively transferred OT-II CD4+ T cells. PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ naïve CD4+ T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. A+B) expression of coinhibitory receptors (n=3-7) and C+D) expression of transcription factors (n=6-9) by endogenous CD4+ T cells in the tumor upon adoptive transfer of OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4+ T cells. Bars represent mean ± sd, and statistics were evaluated by tow-tailed, unpaired t-test (A, C) or one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT CD4+) (B, D). ns: not significant.

4.5.2.2 Influence of IL-18R and IL-1R signaling in adoptively transferred CD4⁺ T cells on endogenous CD8⁺ T cells

CTLs are the most important mediator of T cell-mediated anti-tumor response (Raskov et al. 2021). Therefore, the effect of adoptively transferred OT-II CD4⁺ T cells with or without IL-18/IL-1R deficiency on endogenous CD8⁺ CTLs in the tumor was investigated. Adoptively transferred OT-II CD4⁺ T cells did not change the expression of coinhibitory receptors and combinations of those on CTLs in the tumor (Fig. 24A). Additionally, IL-18 or IL-1R deficiency of adoptively transferred OT-II CD4⁺ T cells did not change the expression of coinhibitory receptors by endogenous CTLs in the tumor (Fig. 24B).

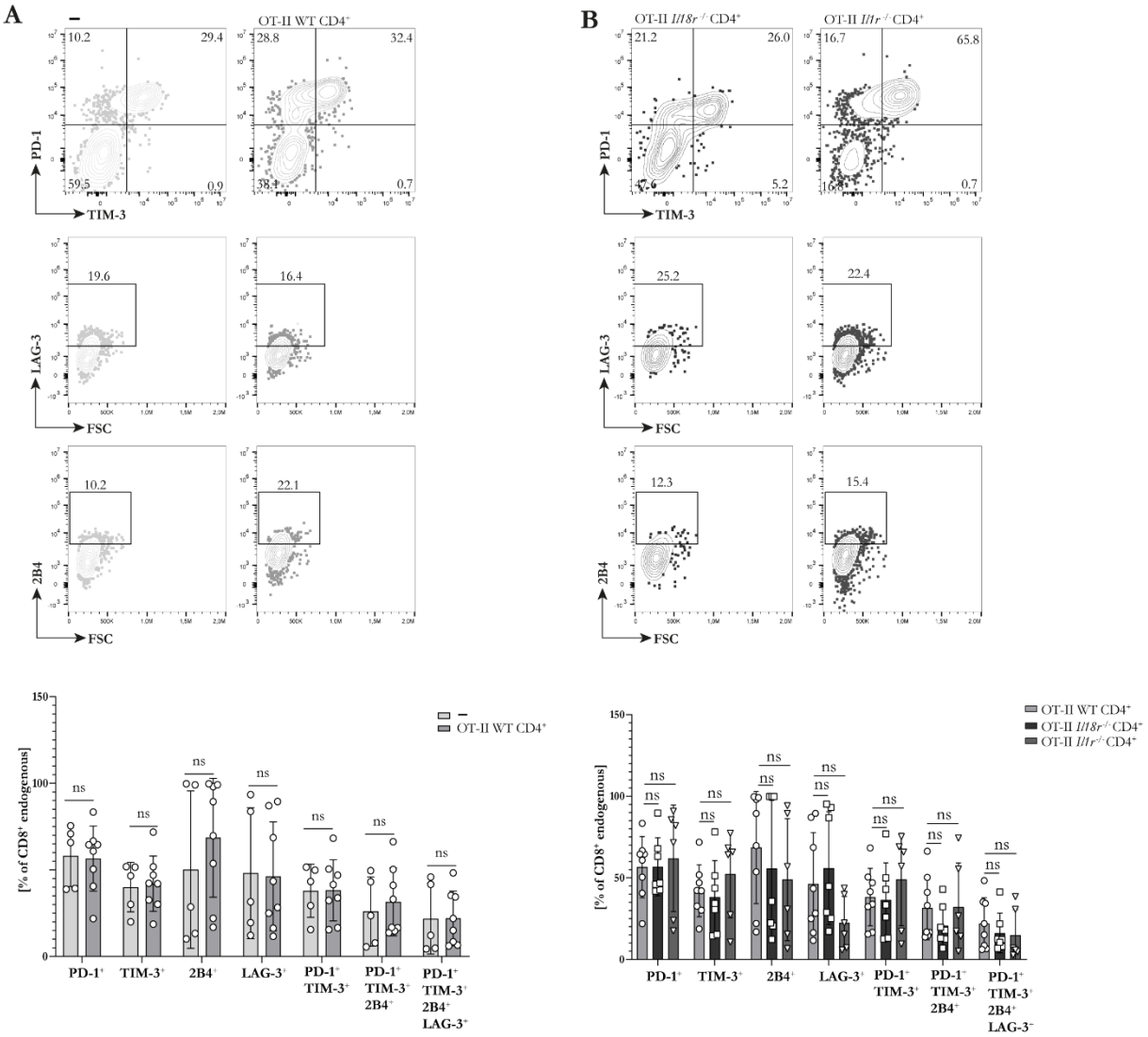


Figure 24: Adoptive transfer of OT-II WT, *Il18r* -/- or *Il1r* -/- CD4⁺ T cells does not impact exhaustion of endogenous CD8⁺ T cells in the tumor. PancOVA tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* -/- or *Il1r* -/- naïve CD4⁺ T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. A+B) Expression of coinhibitory receptors (n=5-8) by endogenous CD8⁺ T cells in the tumor upon adoptive transfer of OT-II WT, *Il18r* -/- or *Il1r* -/- CD4⁺ T cells. Bars represent mean ± sd, and statistics were evaluated by two-tailed, unpaired t-test (A), one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT CD4⁺)

(B: PD-1$^+$, TIM3$^+$ LAG3$^+$, PD-1$^+$ TIM3$^+$, PD-1$^+$ TIM3$^+$ 2B4) or Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to OT-II WT CD4$^+$) (B: 2B4$^+$, PD-1$^+$ TIM3$^+$ 2B4$^+$ LAG3$^+$). ns: not significant.

Likewise, the expression of the transcription factors T-bet, TOX and Eomes was not changed by adoptively transferred OT-II WT CD4$^+$ T cells although there was a tending increase in Eomes expression (Fig. 25A) indicating a more advanced stage of exhaustion (Paley et al. 2012). OT-II *Il18r*$^{-/-}$ or OT-II *Il1r*$^{-/-}$ CD4$^+$ T cells did not change the expression of these transcription factors compared to OT-II WT CD4$^+$ T cells (Fig. 25B).

The production of the CTL-specific cytokines IFN-γ, TNF and GrzB by CTLs was not altered by adoptive transfer of CD4$^+$ T cells (Fig. 25C). The production of IL-2 however was significantly reduced probably indicating an increase in early exhausted T cells upon adoptive transfer of OT-II CD4$^+$ T cells (Fig. 25C). The cytokine pattern of endogenous CTLs was not altered when OT-II *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ CD4$^+$ T cells were adoptively transferred although there was a tending increase of TNF production caused by OT-II *Il1r*$^{-/-}$ CD4$^+$ T cells (Fig. 25D).

In conclusion, there is no prominent effect of adoptively transferred CD4$^+$ cells on the functionality of endogenous CTLs in the tumor. Additionally, there was no difference in the effect of adoptively transferred CD4$^+$ T cells on endogenous CTLs with was dependent on IL-18R or IL-1R signaling in OT-II CD4$^+$ T cells.

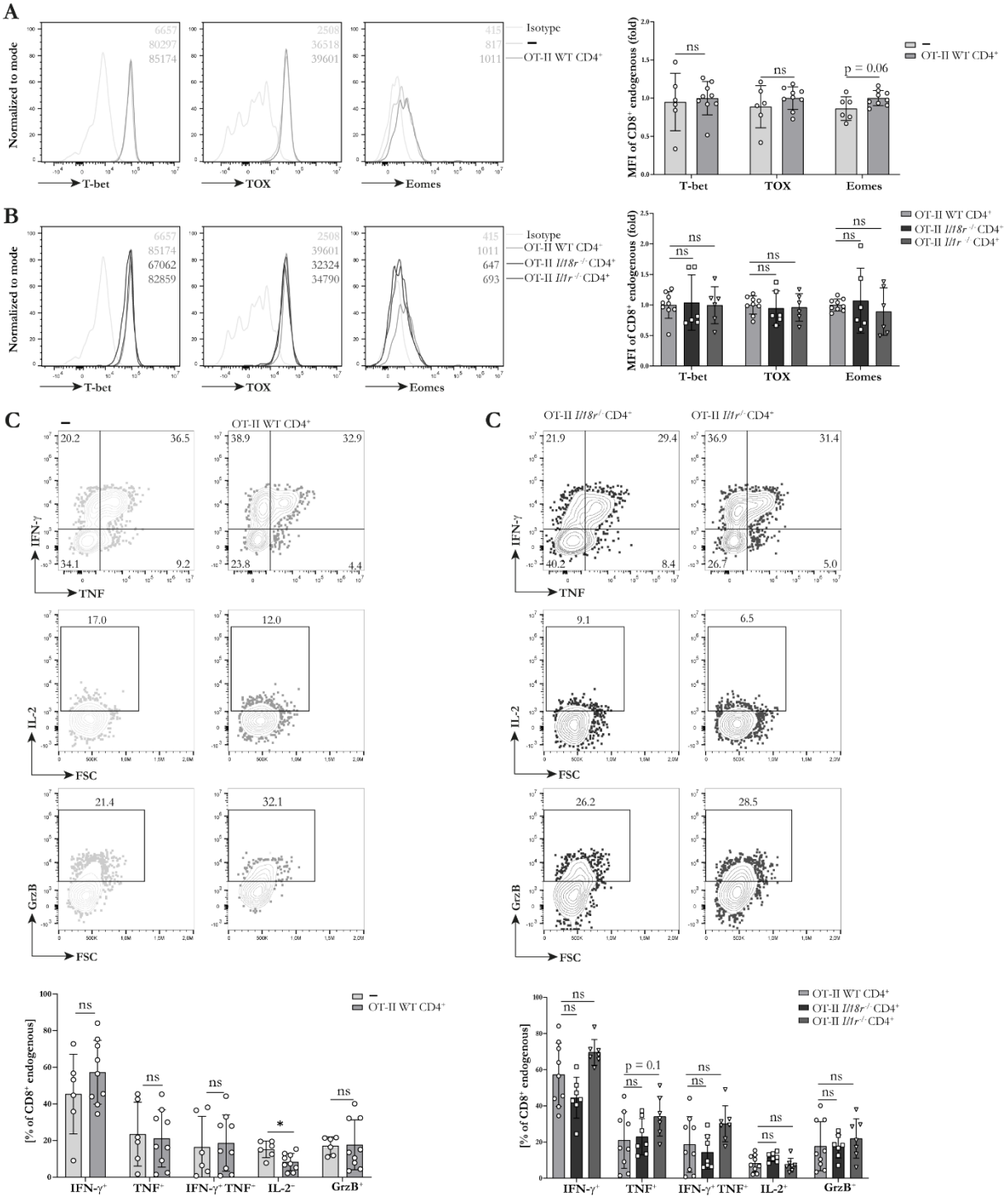


Figure 25: Adoptive transfer of OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4^{+} T cells does not impact expression of transcription factors and production of cytokines by endogenous CD8^{+} T cells in the tumor. PancOVA tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ naïve CD4^{+} T cells were injected i.p. at day 0 and day 5. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. A+B) expression of transcription factors (n=6-9) and C+D) production of cytotoxic cytokines (n=6-9) by endogenous CD8^{+} T cells in the tumor upon adoptive transfer of WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4^{+} T cells. Bars represent mean ± sd, and statistics were evaluated by two-tailed, unpaired t-test with (C: GrzB^{+}) and without (A, C: IFN-γ^{+}, TNF^{+}, IFN-γ^{+}, TNF^{+}, IL-2^{+}) Welch correction, one-way ANOVA followed by Dunnet's multiple comparisons (compared to OT-II WT CD4^{+}) (B: TOX, D: TNF^{+}, IFN-γ^{+}, TNF^{+}, IL-2^{+}, GrzB^{+}), Brown-Forsythe and Welch's ANOVA test followed by Dunnett's T3 multiple comparisons (compared to WT) (B: Eomes, D: IFN-γ^{+}) or Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to OT-II WT CD4^{+}) (B: T-bet). *p<0.05, ns: not significant.

4.5.3 Influence of IL-18R and IL-1R signaling in adoptively transferred CD4+ T cells on antigen-specific CTLs

4.5.3.1 Adoptive transfer of antigen-specific CD4+ T cells with or without IL-18 or IL-1R deficiency does not alter tumor growth or tumor rejection by antigen-specific CTLs

As adoptively transferred OT-II CD4+ T cells did not have an impact on endogenous T cell populations, their effect on tumor growth and antigen-dependent tumor rejection by CTLs was investigated. PancOVA tumor cells were inoculated into the left flank of Ly5.1. mice alongside with an intraperitoneal injection of naïve OT-II WT, *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ CD4+ T cells, which was repeated at day 5, day 10 and day 15. Tumor-specific OT-I CTLs were intraperitoneally injected at day 7. Tumor growth was tracked at day 5 and 7 after inoculation and at day 0, 3, 5, 7, 10 and 13 after CTL injection (Fig. 26A).

The tumor size at day 5 and 7 after tumor inoculation was not altered by adoptive transfer of WT or receptor deficient OT-II CD4+ T cells (Fig. 26B, C). Therefore, OT-II CD4+ T cells did not have an impact on tumor growth prior to CTL injection.

As expected, intraperitoneal injection of OT-I CTLs caused tumor rejection starting at day 3 after CTL injection (Fig. 26D). This phenomenon was not altered by additional intraperitoneal injection of OT-II WT CD4+ T cells (Fig. 26D). When OT-I CTLs were lacking, and only OT-II WT CD4+ T cells were adoptively transferred, tumors kept growing, causing significantly larger tumors compared to the OT-I CTL group starting from day 5 (Fig. 26D). The adoptive transfer of OT-II *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ CD4+ T cells does not alter tumor rejection compared to OT-II WT CD4+ T cells (Fig. 26E).

In conclusion, neither OT-II WT nor *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ CD4+ T cells influenced OT-I CTLs in a way that would alter their ability to reject PancOVA tumors in an antigen-specific manner.

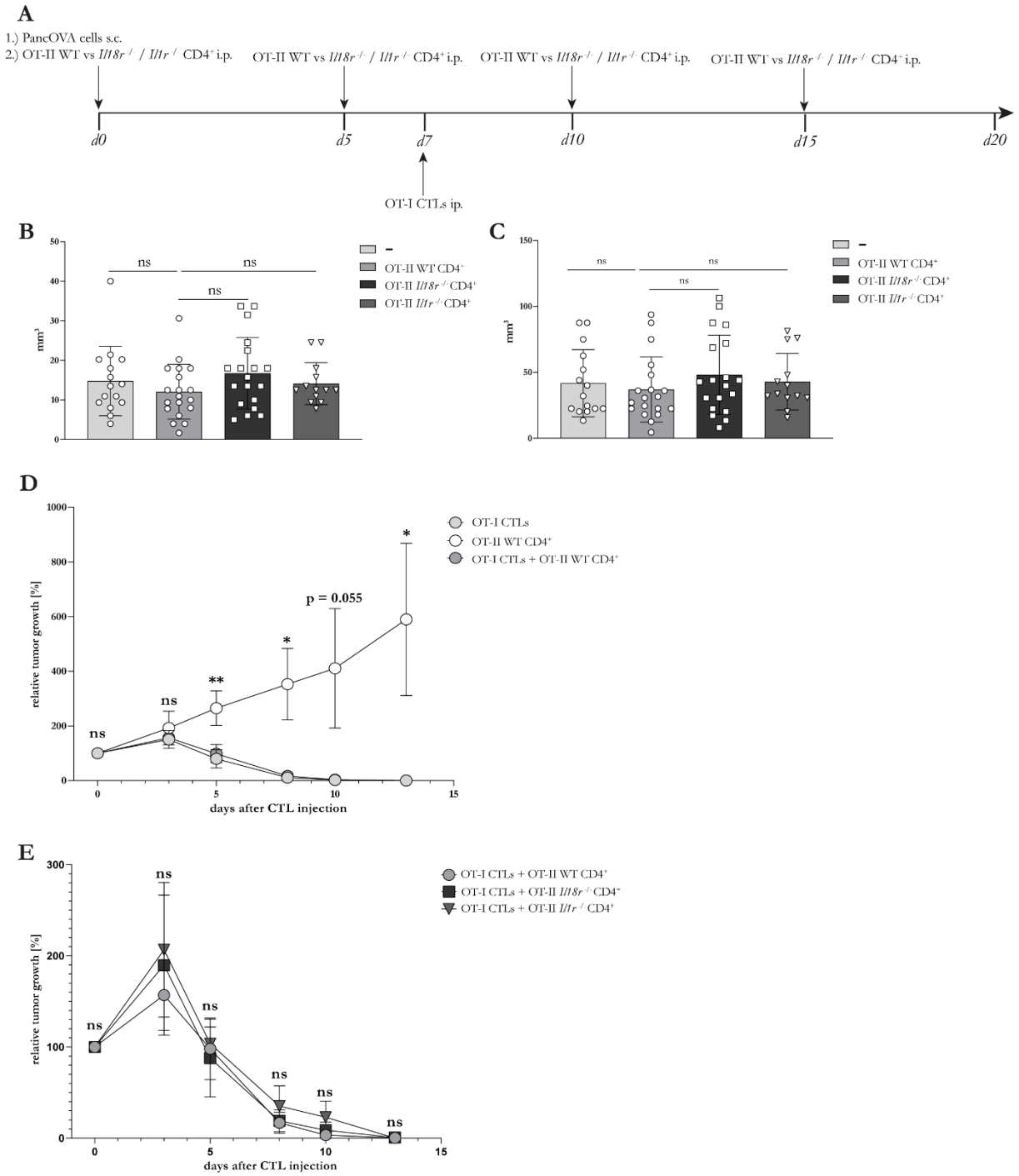


Figure 26: Adoptive transfer of OT-II WT or *Il18r* $^{-/-}$ / *Il1r* $^{-/-}$ CD4^{+} T cells does not impact tumor growth or rejection by OT-I CTLs. A) Schematic of the experimental setup. PancOVA tumor cells were s.c. injected into the left flank of Ly5.1 mice. OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ naïve CD4^{+} T cells were injected i.p. at day 0, 5, 10 and 15. OT-I CD8^{+} T cells were differentiated into CTLs with IL-12 and IL-2 and i.p. injected at day 7. Tumor growth was tracked, and mice were sacrificed at day 20. Tumor size in mm^{3} at B) day 5 (n=13-19) and C) day 7 (n=13-19) after tumor inoculation. Tumor rejection by D) OT-I CTLs with or without additional injection of OT-II WT CD4^{+} T cells (n=4-5) E) OT-I CTLs with additional injection of OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ CD4^{+} T cells (n=4-6). Bars/graphs represent mean ± sd, and statistics were evaluated by Kruskal-Wallis test followed by Dunn's multiple comparisons (compared to OT-II WT CD4^{+}) (B, C) or two-way ANOVA followed by Dunnett's T3 multiple comparisons (compared to OT-I CTLs) (D) or OT-I CTLs + OT-II WT CD4^{+} (E)). **p<0.01, ns: not significant.

4.5.3.2 Adoptive transfer of tumor antigen-specific CD4⁺ T cells with or without IL-18R deficiency does not alter the phenotype of adoptively transferred antigen-specific CTLs

In order to further study the effect of adoptively transferred OT-II WT or *Il18r*$^{-/-}$ CD4^{+} T cells on adoptively transferred OT-I CTLs, PancOVA tumor cells were inoculated into the left flank of Ly5.1. mice accompanied by an intraperitoneal injection of naïve OT-II WT or *Il18r*$^{-/-}$ CD4^{+} T cells, which was repeated at day 5. Tumor specific OT-I CTLs were intraperitoneally injected at day 7 and mice were sacrificed at day 10. Adoptively transferred OT-I CTLs in the tumor and the spleen of tumor bearing mice were analyzed with a focus on surface exhaustion markers (PD-1, TIM3, LAG3 and 2B4), exhaustion associated transcription factors (T-bet, TOX and Eomes) and production of effector cytokines (IFN-γ, TNF, IL-2 and GrzB) (Fig. 27A). Neither OT-II WT nor *Il18r*$^{-/-}$ CD4^{+} T cells influenced the invasion of OT-I CTLs in the tumor or the spleen (Fig. 27B).

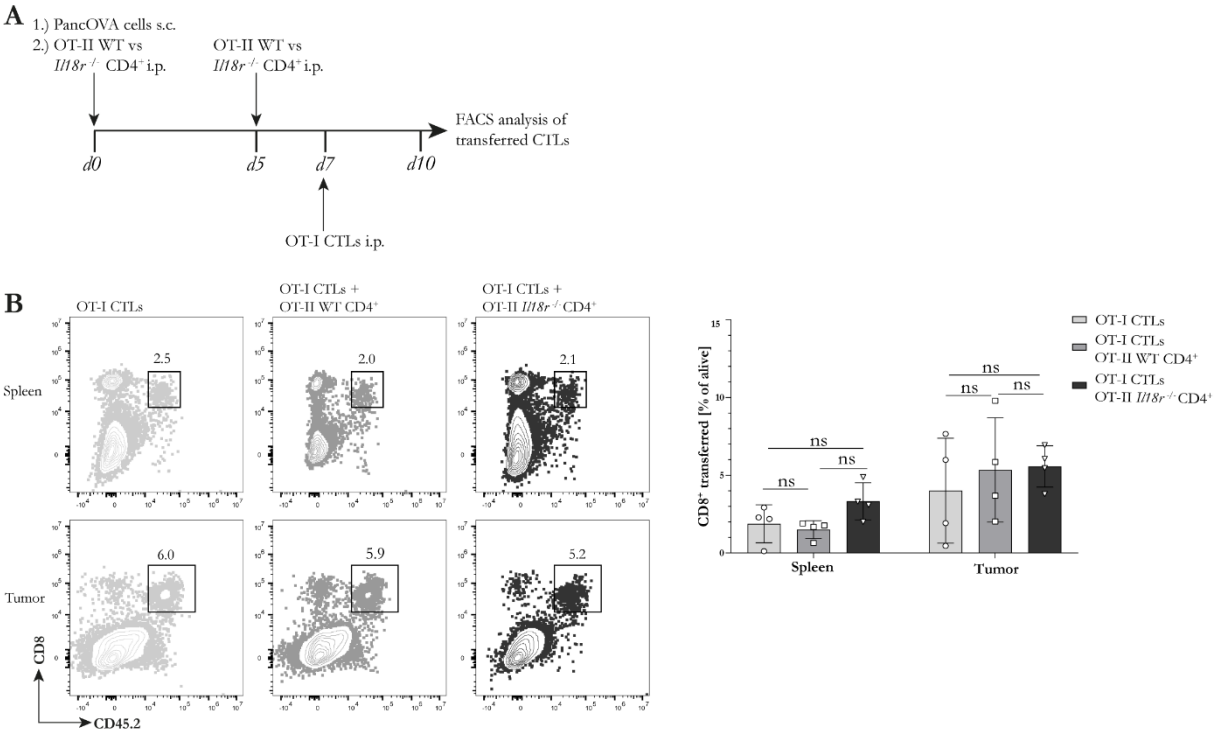


Figure 27. Adoptive transfer of OT-II WT or Il18r $^{-/-}$ CD4^{+} T cells does not impact abundancy of OT-I CTLs in organs of tumor bearing mice. A) Schematic of experimental procedure. PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT or *Il18r* $^{-/-}$ naïve CD4^{+} T cells were injected intraperitoneally at day 0 and day 5. OT-I CD8^{+} T cells were differentiated into CTLs with IL-12 and IL-2 and intraperitoneally injected at day 7. Mice were sacrificed at day 10 and T cell populations in blood, tumor draining lymph node and tumor of mice were analyzed via FACS. B) Amount of OT-I CTLs in spleen and tumor (n=4). Bars/graphs represent mean ± sd and statistics were evaluated by one-way ANOVA followed by Tukey's multiple comparisons (B). ns: not significant.

Additionally, OT-I CTLs showed no difference in the expression of coinhibitory receptors caused by OT-II WT or *Il18r*$^{-/-}$ CD4^{+} T cells (Fig. 28A). Likewise, there was no alteration in the expression of transcription factors and effector cytokines caused by OT-II WT or *Il18r*$^{-/-}$ CD4^{+} T cells (Fig. 28B, C).

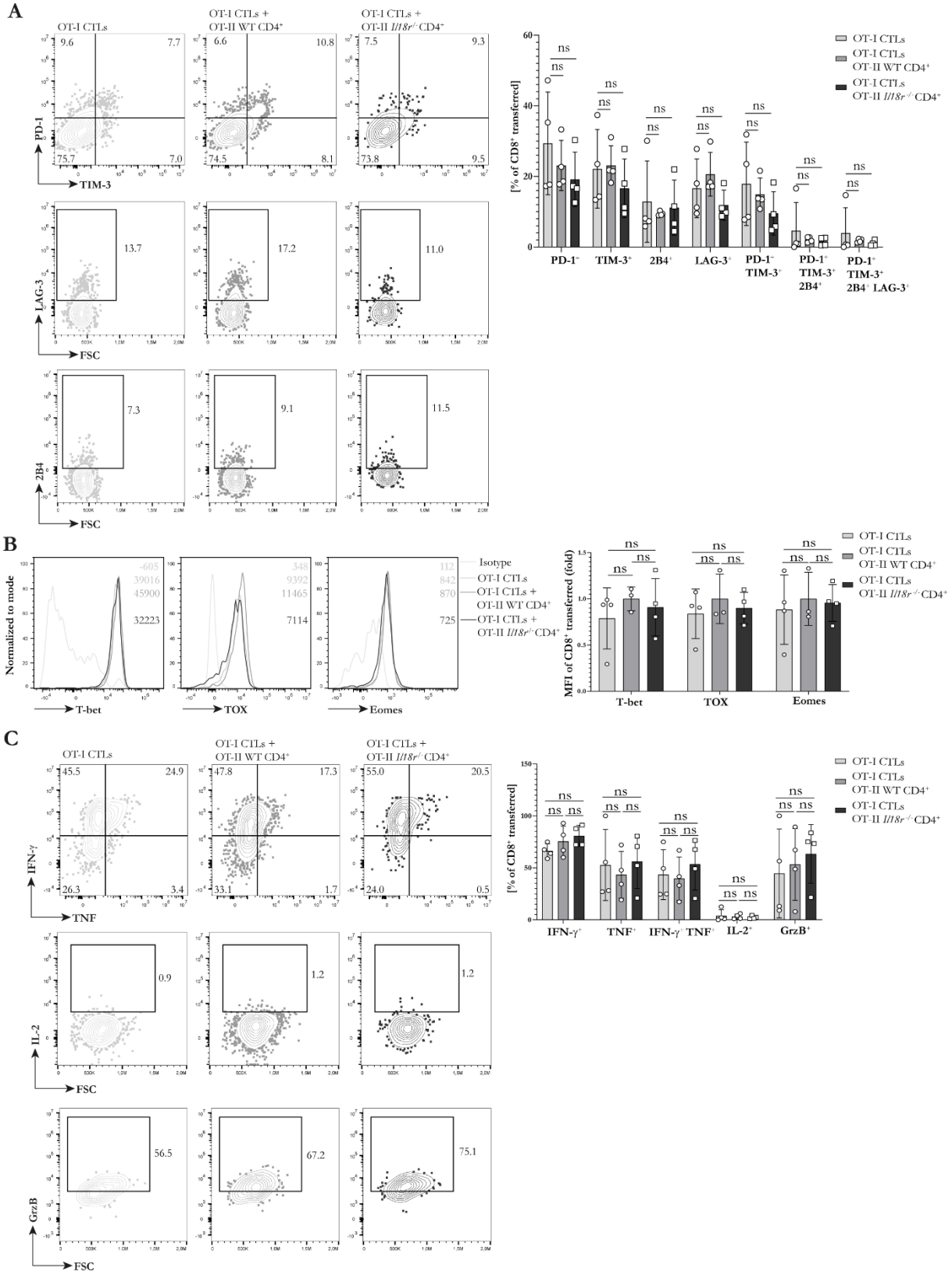


Figure 28: Adoptive transfer of OT-II WT or Il18r $^{-/-}$ CD4^{+} T cells does not impact exhaustion of OT-I CTLs. A) Schematic of experimental procedure. PancOVA Tumor cells were injected s.c. into the left flank of Ly5.1 mice. OT-II WT, *Il18r* $^{-/-}$ or *Il1r* $^{-/-}$ naïve CD4^{+} T cells were injected intraperitoneally at day 0 and day 5. OT-I CD8^{+} T cells were differentiated into CTLs with IL-12 and IL-2 and intraperitoneally injected at day 7. Adoptively transferred CTLs were stained for exhaustion markers and analyzed via FACS. Expression of A) coinhibitory receptors (n=4), B) transcription factors (n=4) and C) production of cytotoxic cytokines (n=4) by adoptively transferred CTLs. Bars/graphs represent mean ± sd and statistics were evaluated by one-way ANOVA followed by Tukey's multiple

comparisons (A: PD-1$^+$, TIM3$^+$, PD-1$^+$ TIM3$^+$, B: TOX, Eomes, C: IFN-γ$^+$, TNF$^+$, IFN-γ$^+$TNF$^+$, GrzB$^+$) or Kruskal-Wallis test followed by Dunn's multiple comparisons (A: 2B4$^+$, LAG3$^+$, PD-1$^+$ TIM3$^+$ 2B4$^+$, PD-1$^+$ TIM3$^+$ 2B4$^+$ LAG3$^+$, B: T-bet, C: IL-2$^+$). $^+$). ns. not significant.

Therefore, as indicated by no observed differences in tumor rejection by antigen-specific OT-I CTLs, adoptively transferred tumor-specific OT-II CD4$^+$ T cells did not influence the phenotype of adoptively transferred CTLs in the tumor.

5 Discussion

The effector function of T cells, especially CTLs, plays a major role in the immune response against cancer (Raskov et al. 2021). Nevertheless, dysfunction of the CTL response, caused by $CD8^+$ T cell exhaustion or interaction with other cell populations such as regulatory T cells, can impair this function (Zarour 2016). The role of cytokines in T cell exhaustion is not completely understood, although there is striking evidence that certain cytokines are able to modulate function or dysfunction of CTLs (Wherry 2011; Wherry and Kurachi 2015). The levels of NLRP3-dependent cytokines, Th1 specific cytokines and the immunosuppressive cytokine IL-10 in the serum of pancreatic cancer patients were therefore determined during this study. The effect of the NLRP3-dependent cytokine IL-18 is of particular interest, as data from our own lab indicated that IL-18R signaling mediates CTL exhaustion and decelerated tumor rejection in a murine pancreatic cancer model (Lutz et al. 2023). Therefore, the focus of this study was to uncover molecular mechanisms of this IL-18-mediated T cell exhaustion. Additionally, IL-18R and IL-1R signaling in CTLs influenced T cell populations in a murine pancreatic cancer model (Lutz et al. 2023). Hence, this study also aimed to investigate the role of IL-18 and IL-1R signaling in adoptively transferred $CD4^+$ T cells on T cell populations and tumor growth and rejection in a murine pancreatic cancer model.

5.1 The role of cytokines in pancreatic cancer patients

Cytokines play a key role in the field of tumor immunology because in the TME they mediate T cell interactions with other immune and non-immune cells (Briukhovetska et al. 2021). The levels of certain cytokines in the blood, serum, plasma, or tissue of pancreatic cancer patients have been widely studied. However, no conclusive picture on the role of cytokines as biomarkers in PDAC has emerged yet. Likewise, it has been stated that due to the differences between the data and insights gained from different studies, individual cytokines are not suitable for serving as a biomarker for diagnostics, prediction, or prognosis in PDAC (Yako et al. 2016).

In particular, the role of serum IL-18 levels in pancreatic cancer patients remains unclear. One study reported significantly lower IL-18 levels in the serum of PDAC patients than in healthy subjects , while other studies reported significantly higher levels of IL-18 in PDAC patients (Bellone et al. 2006; Carbone et al. 2009; Guo et al. 2016; Usul Afsar et al. 2017), and one study did not find any difference (Poch et al. 2007). Nevertheless, the fact that IL-18 is found at detectable levels in the serum of PDAC patients during this study implies a clinical relevance of IL-18.

IL-1β has been shown to be elevated in the serum of pancreatic cancer patients in several studies (Yako et al. 2016). Furthermore, higher levels of IL-1 in the TME of pancreatic cancer patients was correlated with poor prognosis (Apte and Voronov 2002). The IL-1β levels of the patient cohort

in this study support the conclusion that IL-1β levels in PDAC patients are elevated but comparing these levels with data from healthy subjects and PDAC patients from the literature (Kleiner et al. 2013; Ebrahimi et al. 2004; Liu et al. 2021a; Bellone et al. 2006; Poch et al. 2007; Basso et al. 1995) shows that general conclusions from IL-1β levels are not possible due to varying data between patient cohorts.

TNF levels in the serum of pancreatic cancer patients in this study either matched with values reported for healthy subjects or were higher (Kleiner et al. 2013; Liu et al. 2021a; Yako et al. 2016; Poch et al. 2007; Ebrahimi et al. 2004), further indicating that using cytokine levels as predictive markers is inappropriate.

As Tregs fulfill their regulatory role in the TME largely by the secretion of inhibitory cytokines like IL-10 (Sawant et al. 2019), IL-10 levels in the serum of PDAC patients are generally reported to be higher than in healthy subjects (Yako et al. 2016). The present patient cohort supports previous studies that found elevated levels of the immunosuppressive cytokine IL-10 in the serum of PDAC patients (Bellone et al. 2006; Poch et al. 2007; Ebrahimi et al. 2004; Bernstorff et al. 2001).

In summary, the cytokines that play a key role in this study were detected at distinct levels in the serum of pancreatic cancer patients. However, the role of these cytokines in the serum of those patients cannot be evaluated, inter alia because no data from healthy subjects was available and previously published data is inconsistent. Therefore, further studies focusing on the levels of certain cytokines and the respective impacts on pancreatic cancer progression are needed.

5.2 The role of IL-18R signaling in T cell exhaustion

It has been previously reported that stromal IL-18 levels correlated with the abundance of $CD8^+$ $PD\text{-}1^+$ T cells in the stroma of pancreatic cancer (Ahmed et al. 2022). Likewise, Lutz et al. were able to show that IL-18 signaling in a murine pancreatic cancer model caused a more severe exhausted phenotype of tumor-specific CTLs and reduced tumor rejection (Lutz et al. 2023). Both studies indicate a role of IL-18 signaling in the induction and/or enhancement of T cell exhaustion. Interestingly, Ahmed et al. concluded that the effect of IL-18 on T cell exhaustion is mediated by its effects on Th1 and Th2 cells, which in turn influence intratumoral CTLs (Ahmed et al. 2022). However, the present study confirms that there is a direct T cell-intrinsic effect of IL-18 on T cell exhaustion (Lutz et al. 2023).

In this study, an *in vitro* exhaustion model based on a report of Zhao et al. 2020 was established in order to gain further insights on IL-18-mediated T cell exhaustion. Repetitive stimulation of antigen-specific T cells with their cognate antigen induced an exhausted phenotype *in vitro*.

Interestingly, the expression of T-bet was also increased and the expression of Eomes was decreased, possibly indicating increased effector function (Paley et al. 2012). This pattern of T-bet and Eomes expression in *in vitro* exhausted T cells was also observed by Zhao et al. and might be explained by the dependency of T-bet and Eomes expression on the terminal versus preterminal state of exhaustion (Zhao et al. 2020).

The four subsets of exhausted T cells defined by Beltra et al. show dynamic changes of T-bet and Eomes expression. Hereby, Eomes is highest in Tex^{prog1}, declines in Tex^{prog2} and Tex^{int} and increases in Tex^{term}. T-bet, however, increased from Tex^{prog1} to Tex^{int} and decreased in Tex^{term} (Beltra et al. 2020). Therefore, cells that arise from the *in vitro* exhaustion model might be Tex^{prog2} or Tex^{int}. The capacity to produce cytokines by *in vitro* exhausted T cells, however, was markedly reduced, as the percentage of TNF-producing and IFN-γ and TNF double-producing cells was significantly lower (Lutz et al. 2023). In particular, the PD-1, TIM-3 double positive cells produced significantly less IFN-γ, TNF and both cytokines than PD-1 single positive cells, indicating that PD-1, TIM-3 double positive cells represent a more functionally impaired subset of T cells *in vitro* (Lutz et al. 2023). *In vitro* exhausted T cells were still able to produce GrzB. Alongside with the unchanged IFN-γ production, *in vitro* exhausted T cells are most likely intermediately exhausted (Wherry et al. 2003). Therefore, the *in vitro* exhaustion model gave rise to T cells with increased expression of inhibitory receptors and TOX and decreased effector function and can therefore be used to study effects of cytokines and inhibitors on T cell exhaustion *in vitro*.

IL-18R deficient CTLs showed a less exhausted phenotype in a murine pancreatic cancer model (Lutz et al. 2023). This phenomenon could have been explained by the fact that $CD8^+$ T cells with a deficiency in IL-18 signaling exhaust to a lesser extent in general. This possibility was ruled out by performing the *in vitro* exhaustion model with IL-18R deficient T cells, resulting in increased expression of inhibitory receptors, T-bet and TOX, increased production of $GrzB^+$ and decreased production of TNF, as well as IFN-γ and TNF. Additionally, the production of all cytokines was reduced in IL-18R deficient cells compared to WT cells, indicating an even more exhausted phenotype of IL-18R deficient cells *in vitro*. Therefore, the *in vitro* exhaustion model was used to demonstrate that reduced exhaustion and increased effector function of IL-18R deficient T cells compared to WT cells *in vivo*, were not mediated by an intrinsic mechanism in IL-18R deficient CTLs that causes a less exhausted phenotype, but a consequence of IL-18R signaling in the TME, inter alia supplied by the tumor himself. This conclusion is also supported by the fact that the expression of IL-18 is significantly increased in the tissue of pancreatic tumors and correlates with cancer progression and prognosis. Additionally, it has been shown the expression of IL-18R correlates with genes responsible for T cell exhaustion (Lutz et al. 2023).

Likewise, although IL-18 treatment increased the production of effector cytokines during primary activation, treatment of *in vitro* exhausted T cells with IL-18 caused a more severe exhausted phenotype (Lutz et al. 2023). It increased the expression of TIM-3, LAG-3 and the percentage of PD-1, TIM-3 positive cells (Lutz et al. 2023) as well as the percentage of PD-1, TIM-3, and LAG-3 positive cells. The expression of TOX was further increased, as well as the expression of T-bet and Eomes, which is unusual because T-bet and Eomes are usually regulated in a mutually exclusive manner in exhausted T cells (Paley et al. 2012). Therefore, the *in vitro* exhaustion model, especially under the influence of IL-18 might not fully resemble all features of exhausted T cells but can be used to study changes in certain hallmarks of T cell exhaustion. Most importantly, IL-18 treatment caused further reduced production of TNF as well as IFN-γ and TNF (Lutz et al. 2023), further indicating that IL-18 induces a more severe exhausted phenotype. However, retained ability to produce IFN-γ indicates that these are not yet terminally exhausted.

The definition of four subsets of exhausted T cells by Beltra et al. was used as a paradigm to further characterize the state of exhaustion *in vitro*. Interestingly, the expression pattern of Ly108 and CD69 revealed that most *in vitro* exhausted T cells without IL-18 were intermediate or terminally exhausted. IL-18 treatment, however, shifted this pattern even more towards the terminally exhausted phenotype. This would also match with the increased expression of Eomes upon IL-18 treatment. In addition, IL-18 treatment decreased the percentage of living cells and the proliferative capacity. As severely exhausted T cells die, resulting in a decline of antigen-specific T cells *in vivo* (Moskophidis et al. 1993; Wherry and Ahmed 2004; Williams and Bevan 2007), IL-18 seems to accelerate the process of T cell exhaustion. Therefore, IL-18 indeed seems to further promote T cells exhaustion, however, with the preservation to produce IFN-γ. It might be the case that the general IFN-γ-inducing capacity of IL-18 (Nakanishi 2018), which was also observed in activated T cells during this study, keeps IFN-γ production (and maybe T-bet expression) high, although it generally induces a more severely exhausted phenotype. Previous findings that $CD8^+$ T cells with high IL-18R expression mediate the immune response after immunotherapy (Kim et al. 2021) further highlights the dual role of IL-18 in the T cell life cycle. Nevertheless, the fact that the response to immune checkpoint therapy has been shown to be dependent on the stage of exhaustion (He et al. 2016; Im et al. 2016; Utzschneider et al. 2016b; Miller et al. 2019; Siddiqui et al. 2019), IL-18 could be considered as a therapeutic target in order to improve the efficiency of checkpoint therapy.

5.2.1 The role of signaling pathways during IL-18-mediated T cell exhaustion

RNAseq performed of intratumoral CTLs with or without IL-18R deficiency indicated an involvement of the IL-2/STAT5 and/or the PI3K/Akt/mTOR pathway in IL-18-mediated T cell dysfunction (Lutz et al. 2023). Accordingly, IL-18 treatment in the *in vitro* exhaustion model led to increased expression of CD25 and phosphorylation of STAT5, Akt, mTOR and S6 (Lutz et al. 2023). Therefore, CD25, STAT5, Akt and mTOR seem to be regulated by IL-18 signaling in the context of exhaustion and have therefore been identified as potential targets in order to reverse the induction of exhaustion mediated by IL-18.

IL-18 particularly increased the expression of TIM-3, and TIM-3 has been shown to increase activation of the PI3K/Akt/mTOR pathway. (Lee et al. 2011; Ferris et al. 2014; Avery et al. 2018). This indicates that IL-18R signaling possibly drives T cell exhaustion via those mediators and the more severe exhausted phenotype could therefore be mainly caused by increased TIM-3 expression and subsequent activation of the PI3K/Akt/mTOR pathway.

IL-2 and the IL-2/STAT5 pathway seems to have a contradictory role in T cell exhaustion. On the one hand, treatment with IL-2 in combination with PD-1 blockade has increased the effector function of $CD8^+$ T cells and improved viral control in a murine chronic infection model (Hashimoto et al. 2022; West et al. 2013). On the other hand, high IL-2 levels in cancer patients have been correlated to the expression of T cell exhaustion genes, and IL-2 has been shown to activate STAT5 and mediate exhaustion in a murine melanoma model (Liu et al. 2021b). It has been already shown that IL-18 is able to upregulate the expression of CD25 in NK cells (Lee et al. 2012; Stegmann et al. 2015). Interestingly, previous studies showed that IL-18 upregulated CD25 (and production of IFN-γ) only in effector and memory T cells and not in exhausted T cells (Ingram et al. 2011). In the present study, however, IL-18 has been shown to induce components of the IL-2/STAT5 pathway, namely the expression of CD25 and the phosphorylation of STAT5 (Lutz et al. 2023). Nevertheless, blocking CD25 or IL-2 did not revert IL-18-mediated T cell exhaustion. In fact, the production of cytokines was lower, no matter if IL-18 was supplemented or not. (Lutz et al. 2023). The negative effects of IL-2 and CD25 blockade were to be expected as IL-2 functions as a growth factor for T cells and can directly increase the production of cytokines (Ross and Cantrell 2018). Therefore, interfering with the IL-2/STAT5 pathway by blocking IL-2 or its receptor does not reverse the effects of IL-18 on T cell exhaustion *in vitro*, maybe because IL-2 serves as a growth factor for T cells (Gillis et al. 1978).

IL-2 is an activator of STAT5 (Gilmour et al. 1995) which in turn is mediator of T cell effector function (Tripathi et al. 2010). *In vitro* exhausted T cells treated with STAT5 inhibitor, however,

showed high expression of inhibitory receptors (Lutz et al. 2023) and TOX, independently of IL-18. The cytokine production upon STAT5 inhibition was at similar levels compared to the exhausted T cells without IL-18 treatment, and IL-18 treatment was not able to reduce cytokine production upon STAT5 inhibition (Lutz et al. 2023). Therefore, it seems to be the case that STAT5 is at least partially involved in IL-18-mediated T cell exhaustion, especially regarding cytokine production (Lutz et al. 2023).

The clear impact of IL-2 signaling on T cell exhaustion previously reported (Liu et al. 2021b) however, could not be fully confirmed by the *in vitro* exhaustion model in this study. This might be due to the fact that an *in vitro* model lacks other growth factors present *in vivo* that are able to compensate for lack of IL-2. Additionally, it might be the case that the impact of IL-2 on T cell exhaustion is mainly mediated by the Akt/mTOR pathway, which can be activated by IL-2 signaling (Ross and Cantrell 2018).

IL-18 has been shown to promote mTOR activation in NK cells via leucine transport (Almutairi et al. 2019) and this study also confirmed an impact of IL-18 on phosphorylation levels of Akt and mTOR in T cells in the context of exhaustion (Lutz et al. 2023). The Akt/mTOR pathway has also been shown to be involved in T cell development because it mediates changes in the metabolism of exhausted T cells (Buck et al. 2015). Akt inhibition results in increased memory T cell characteristics and improved anti-tumor response of exhausted T cells (Crompton et al. 2015). Furthermore, ectopic expression of Akt reduced memory T cell formation (Hand et al. 2010). mTOR is a downstream target of Akt (Laplante and Sabatini 2012) and activates several downstream targets involved in immune receptor signaling, metabolism and migration (Geng et al. 2008). mTOR inhibition has been shown to increase effector function and memory formation of CTLs (Rao et al. 2010; Araki et al. 2009), to preserve metabolic features and therefore pre-exhausted T cells and improve their effector function (Gabriel et al. 2021). Therefore, it has been suggested that the Akt/mTOR pathway influences T cell exhaustion via regulation of memory formation and metabolism (Gabriel et al. 2021). In the *in vitro* exhaustion model, Akt inhibition caused reduced expression of inhibitory receptors (Lutz et al. 2023), prevented the IL-18-mediated upregulation of TOX expression, was able to abolish IL-18-mediated decrease of living cells and proliferation capacity, but at the same time caused decreased cytokine production (Lutz et al. 2023). mTOR inhibition, however, was able to fully revert IL-18-mediated effects on T cell exhaustion *in vitro*. Inhibitory receptors were expressed to a significantly lower extent (Lutz et al. 2023), TOX expression was not increased by IL-18, cytokine production was at a level comparable to exhausted T cells without IL-18 and without rapamycin treatment, and IL-18-mediated loss of proliferative capacity was lost (Lutz et al. 2023). Therefore, mTOR inhibition and, to a lesser extent, Akt

inhibition was able to revert IL-18-mediated T cell exhaustion *in vitro* (Lutz et al. 2023). This is probably mediated by the already mentioned effect of the Akt/mTOR pathway on memory formation and metabolism of T cells. Via activation of the Akt/mTOR pathway, IL-18 might change the metabolism of exhausted T cells and promote more advanced exhaustion (Lutz et al. 2023). PD-1 has been shown to inhibit the PI3K/Akt/mTOR pathway and therefore negatively impacts T cell function (Parry et al. 2005). The fact that PD-1 expression is unaffected by IL-18 treatment and therefore most likely not involved in IL-18-mediated exhaustion further underlines the importance of TIM-3 in IL-18-mediated exhaustion, most likely via activation of the Akt/mTOR pathway.

In summary, this study revealed an effect of IL-18R signaling on T cell exhaustion *in vitro* which is mediated by the IL-2/Akt/mTOR pathway.

5.3 Impact of the NLRP3-dependent cytokines IL-1β and IL-18 on CD4$^+$ T cells

The NLRP3-dependent cytokines IL-1β and IL-18 have been shown to impact the effector function and tumor rejection capacity of adoptively transferred CTLs *in vivo* (Lutz et al. 2023). Because CD4$^+$ T cells are also impacted by IL-1 and IL-18 (Ben-Sasson et al. 2009; van den Eeckhout et al. 2020; O'Sullivan et al. 2006; Nikolouli et al. 2021; Smeltz et al. 2001; Carroll et al. 2008; Sims and Smith 2010) and can also become exhausted (Wherry and Kurachi 2015; Miggelbrink et al. 2021) this study aimed to investigate the effects of those cytokines on CD4$^+$ T cell differentiation and function *in vitro* and *in vivo*. Another question that was supposed to be answered was how antigen-specific CD4$^+$ T cells with or without IL-1R or IL-18R deficiency, would influence endogenous and adoptively transferred T cell populations as well as tumor growth and rejection in a murine pancreatic cancer model.

5.3.1 Impact of IL-1β and IL-18 signaling on *in vitro* differentiated CD4$^+$ T cells

IL-18 has been shown to increase IFN-γ production by Th1 cells activated non-specifically with anti-CD3 (Okamura et al. 1995; Okamura et al. 1998; Nakanishi et al. 2001; Nakanishi 2018). This mechanism is most likely mediated in concert with IL-12 (Smeltz et al. 2001). Additionally, it has been shown that OVA-specific Th1 cells, which are stimulated with OVA$^{257-264}$ and IL-18, produce Th1 and Th2 cytokines (Sugimoto et al. 2004). IL-18 has also been shown to increase T-bet expression in Th1 cells in Coeliac disease patients (Salvati et al. 2002). The present study confirms that IL-18 increases T-bet expression and has an IFN-γ-inducing effect on *in vitro* differentiated antigen-specific Th1 cells, which was interestingly stronger when the cells were restimulated in an antigen-specific manner. It might be the case that antigen-unspecific restimulation with PMA and ionomycin is that strong, that IL-18-mediated effects do not have an impact anymore. Although

IL-1β has been shown to promote CD4$^+$ T cell response (Ben-Sasson et al. 2009; van den Eeckhout et al. 2020), no effects on antigen-specific Th1 cells *in vitro* were detected during this study, indicating a less prominent role of IL-1β on Th1 cell effector function. Interestingly, the effects of IL-1 signaling on antigen-specific CTLs *in vivo* were also less prominent (Lutz et al. 2023).

Th1 cells have a rather tumor-suppressive function, whereas Tregs have immune-suppressive functions *in vivo* (Gonzalez et al. 2018; Miggelbrink et al. 2021; McRitchie and Akkaya 2022). Likewise, the ratio of Tregs to CD4$^+$ T cells was shown to play a prognostic role in pancreatic cancer patients (Ino et al. 2013). The effects of IL-18 and IL-1β on iTreg differentiation *in vitro* is therefore of particular interest. *In vivo* it was already shown that IL-18 is able to promote effector T cells by inhibiting accumulation and function of Tregs (Carroll et al. 2008). In the present study, IL-18 impaired iTreg differentiation *in vitro*, but slightly increased their capacity to produce the immunosuppressive cytokine TGFβ. The results of this study need to be carefully considered because IL-18 might inhibit iTreg differentiation, but *in vivo* Tregs are usually differentiated in the thymus and therefore less susceptible towards peripheral IL-18. Therefore, the effects of IL-18 on Treg differentiation and progression and especially its effects on FOXP3 expression and stability in already established Tregs *in vitro* and *in vivo* needs to be further studied, for example by using OT-II *Il18r*$^{-/-}$ FIR mice generated during this study. It is possible that the TGFβ-promoting effects of IL-1β and IL-18 are of more importance *in vivo* and could increase the suppressive activity of Tregs. *In vivo* it was already shown that IL-18R deficient Tregs did not express FOXP3 differently than WT cells but showed downregulation of Treg effector function genes and elevated IL-10 levels (Harrison et al. 2015). These results indicate that IL-18 signaling *in vivo* indeed does not change FOXP3 expression and even enhances Treg effector function but decreases IL-10 production. Interestingly, it was previously reported that IL-1 interferes with Treg development and function *in vivo* (O'Sullivan et al. 2006; Nikolouli et al. 2021), which was not observed *in vitro* during this study. Therefore, results gained from this study provide interesting findings on how IL-18 and IL-1 could influence the development and function of CD4$^+$ T cells. The special case of iTregs limits the ability to simply transfer those findings to the situation *in vivo*.

Of note, *in vitro* differentiation of Th1 cells and Tregs from IL-1 or IL-18R deficient CD4$^+$ T cells was unchanged compared to WT, indicating that possible differences *in vivo* are most likely mediated by external IL-1 and IL-18.

5.3.2 Adoptively transferred CD4$^+$ T cells in a murine pancreatic cancer model

This study aimed to determine effects of IL-1 and IL-18 signaling on T cell development in a murine pancreatic cancer model. However, upon adoptive transfer, only small populations of CD4$^+$ T cells were found in blood, lymphoid organs and the tumor of tumor bearing mice. It might be

possible that the amount of adoptively transferred CD4$^+$ T cells was low in the blood, tumor draining lymph node and spleen of tumor bearing mice because the T cells had already infiltrated the tumors, which expressed their cognate antigen. This phenomenon has already been shown when antigen-specific iTregs were adoptively transferred (Bauer et al. 2014). In the tumor, and most likely also in the other organs however, it is possible that most adoptively transferred cells died. Therefore, the number of adoptively transferred T cells that were found in FACS analysis were not sufficient to draw conclusions towards possible influences on the development of specific CD4$^+$ T cell subsets mediated by IL-1 or IL-18.

Adoptively transferred CD4$^+$ T cells have been shown to successfully contribute to the immune response against cancer (Perez-Diez et al. 2007). However, during this study this was not the case. Future studies focusing on the development of naïve CD4$^+$ T cells with or without receptor deficiency might consider to either harvest organs earlier after adoptive transfer, increase the number of adoptively transferred CD4$^+$ T cells or focus on defined subtypes of CD4$^+$ T cells, for example Th1 cells and/or iTregs.

5.3.3 Influence of adoptively transferred CD4$^+$ T cells on endogenous T cell populations in a murine pancreatic cancer model

Although this study did not manage to reveal the development of adoptively transferred naïve CD4$^+$ T cells *in vivo*, some effects of those cells on endogenous T cell populations were detected. Most likely, this is because adoptively transferred CD4$^+$ T cells impacted endogenous T cells in advance of their migration to the tumor (or their death).

Adoptively transferring WT CD4$^+$ T cells did not influence the abundance of endogenous T cell populations in the blood, tumor draining lymph node, spleen, and tumor. There was also no difference detected between the adoptive transfer of WT and *Il18r*$^{-/-}$ or *Il1r*$^{-/-}$ cells. However, there was a strong tendency of an increased endogenous CD4$^+$ T cell population in the spleen when *Il18r*$^{-/-}$ cells were transferred compared to WT cells. Therefore, antigen-specific IL-18R deficient CD4$^+$ T cells might somehow influence the migration of endogenous CD4$^+$ T cells. However, further studies are needed to confirm and further characterize this effect.

Regarding the cytokine production of endogenous CD4$^+$ T cells in the tumor upon adoptive transfer of antigen-specific CD4$^+$ T cells, there were some effects detected. WT CD4$^+$ T cells caused a tending shift from the antitumoral cytokine IFN-γ to the immunosuppressive cytokine IL-10, indicating a less effective anti-tumor response by endogenous CD4$^+$ T cells. While IL-1R deficiency in adoptively transferred CD4$^+$ T cells did not cause any significant differences in the cytokine profile of endogenous CD4$^+$ T cells in the tumor, IL-18R deficiency caused a decrease in

IL-10-producing cells. Likewise, there was a tending decrease in FOXP3$^+$ cells, indicating a general decrease in immunosuppressive Tregs upon adoptive transfer of *Il18r*$^{-/-}$ CD4$^+$ T cells. Although the *in vitro* experiments focusing on the effects of IL-18 on the differentiation of iTregs should be considered with caution, as discussed earlier, these *in vivo* observations might indicate that IL-18 possibly has a negative effect on Treg accumulation. It might the case that adoptively transferred WT CD4$^+$ T cells scavenge IL-18, causing less free IL-18 in the TME. IL-18R deficient CD4$^+$ T cells, however, are not able to bind IL-18, leading to more IL-18, which can influence endogenous CD4$^+$ T cells causing a decrease in the amount of Tregs. This hypothesis would also explain the tending increase of IL-10-producing cells upon adoptive transfer of WT CD4$^+$ T cells. Nevertheless, they do not cause significant changes in the FOXP3 expression of endogenous CD4$^+$ T cells.

Adoptively transferred CD4$^+$ T cells, with or without an IL-18 or IL-1R deficiency, did not change the state of exhaustion in endogenous CD4$^+$ T cells in terms of the expression of inhibitory receptors or transcription factors. *Il18r*$^{-/-}$ CD4$^+$ T cells, however, seemed to cause a slight increase of LAG-3$^+$ cells. IL-18 has been shown to increase LAG-3 expression in CD8$^+$ T cells during this study (Lutz et al. 2023). Therefore, *Il18r*$^{-/-}$ CD4$^+$ T cells not scavenging IL-18 from the TME might also explain this phenomenon. However, other findings contradict this conclusion: First, if this was true, WT CD4$^+$ T cells would scavenge IL-18 from the TME which should cause a decrease in LAG-3$^+$ cells amongst endogenous CD4$^+$ cells compared to when no CD4$^+$ T cells were transferred, which was not the case. Second, IL-18 has been shown to have a major impact on TIM-3 expression in CD8$^+$ T cells, but the number of TIM-3 positive cells among endogenous CD4$^+$ T cells was not changed by any kind of adoptively transferred CD4$^+$ T cells. Therefore, it is more likely that adoptively transferred *Il18r*$^{-/-}$ CD4$^+$ T cells influenced endogenous CD4$^+$ T cells in a yet unknown way that caused a tending increase in LAG-3 expression.

In summary, the adoptive transfer of CD4$^+$ T cells with or without IL-18 or IL-1R deficiency had only isolated, minimal effects on endogenous CD4$^+$ T cells in the tumor and did not markedly change their cytokine profile or their state of exhaustion. This indicates that naïve CD4$^+$ T cells were not able to influence endogenous CD4$^+$ T cells in a way that would drastically impact their contribution to the immune response in the TME.

Regarding endogenous CD8$^+$ T cells in the tumor, adoptively transferred CD4$^+$ T cells did not influence the expression of inhibitory receptors and transcription factors linked to T cell exhaustion. However, there was a tending increase of Eomes expression in endogenous CD8$^+$ T cells caused by adoptively transferred WT CD4$^+$ T cells. Additionally, the IL-2 production in the same scenario was significantly decreased. As loss of IL-2 production is a hallmark of early

exhausted T cells (Wherry 2011) it might be concluded that WT $CD4^+$ T cells induce early exhaustion in endogenous $CD8^+$ T cells. Nevertheless, the IL-2 production of endogenous $CD8^+$ T cells was also relatively low when no $CD4^+$ T cells were adoptively transferred, indicating that the decreased IL-2 production did not impact anti-tumor immunity to great extent, especially because the production of IFN-γ, TNF and GrzB was not affected. The tending increase of Eomes expression would also indicate an accelerated exhaustion upon adoptive transfer of WT $CD4^+$ T cells, but this phenomenon was most likely irrelevant as the expression of T-bet and TOX was unchanged.

In summary, the adoptive transfer of antigen-specific $CD4^+$ T cells did not change the effector function of endogenous $CD8^+$ T cells in a murine pancreatic cancer model. This is somewhat surprising as lack of CD4 help is a driver of $CD8^+$ T cell exhaustion (Wherry 2011). Therefore, antigen-specific $CD4^+$ T cells would have been expected to be able to positively influence $CD8^+$ T cells in the TME. It is possible that adoptively transferred $CD4^+$ T cells develop pro-tumor and anti-tumor features that rule each other out. Another explanation might be that only a small proportion of adoptively transferred $CD4^+$ T cells survives over the course of time, resulting in only marginal effects on endogenous CTLs.

The fact that adoptively transferred $CD4^+$ T cells do not have a clear and major impact on the immune response of endogenous T cell populations is further supported by the fact that tumor growth is not affected by transferred $CD4^+$ T cells, no matter if they have a receptor deficiency or not. Therefore, future studies should focus on the effect of certain $CD4^+$ T cell subpopulations with or without IL-18 or IL-1R deficiency.

5.3.4 Influence of adoptively transferred $CD4^+$ T cells on antigen-specific CTLs

Earlier studies revealed a prominent effect of IL-18 signaling on antigen-specific CTLs in a murine pancreatic cancer model in terms of effector function and their ability to reject the tumor (Lutz et al. 2023). As the interaction between $CD4^+$ and $CD8^+$ T cells plays a leading role in the immune response against cancer and can influence the state of exhaustion (Wherry 2011; Borst et al. 2018) this study aimed to determine the effects of adoptively transferred $CD4^+$ T cells and IL-18 signaling in those cells on adoptively transferred CTLs. Adoptively transferred WT $CD4^+$ T cells were not able to inhibit tumor growth like CTLs did, but also did not influence CTLs in their ability to reject the tumor. IL-18R deficient $CD4^+$ T cells did not accelerate or decelerate antigen-specific tumor rejection by CTLs either. This finding further supports the conclusion that antigen-specific $CD4^+$ T cells were not able to influence other T cell populations, no matter if endogenous or adoptively transferred, in a way that would significantly influence their anti-tumor response. Likewise, neither

WT CD4$^+$ nor *Il18r*$^{-/-}$ CD4$^+$ T cells changed the amount of adoptively transferred CD8$^+$ T cells in the tumor, their expression of inhibitory receptors and transcription factors or their capacity to produce effector cytokines.

Antigen-specific CD4$^+$ T cells have been reported to support antigen-specific CD8$^+$ T cells in murine cancer models. For example, antigen-specific CD4$^+$ T cells were able to restore the number and cytokine production of antigen-specific CD8$^+$ T cells and enabled them to reject previously escaped tumors (Arina et al. 2017). Additionally, antigen-specific Th1 cells enhanced the tumor rejection of antigen-specific CTLs in a murine melanoma model. Hereby, Th1 cells that were differentiated *in vitro* for a shorter time, proliferated better *in vivo* and were therefore more effective (Li et al. 2016; Li et al. 2017). Therefore, the effects of antigen-specific CD4$^+$ T cells are either marginal or simply not present in our model. Additionally, IL-18 signaling seems to have a far less prominent effect on antigen-specific CD4$^+$ T cells than on CD8 T cells (like in Lutz et al. 2023).

It might be the case that antigen-specific CD4$^+$ T cells did not have an effect on antigen-specific CD8$^+$ T cells in this study because naïve CD4$^+$ T cells were adoptively transferred and were probably not able to differentiate *in vivo*. Nevertheless, Arina et al. reported that naïve antigen-specific CD4$^+$ T cells had an impact on antigen-specific CD8$^+$ T cells. However, the adoptive transfer of antigen-specific subtypes of CD4$^+$ T cells (for example Th1 cells or iTregs) would probably have a higher impact on antigen-specific CD8$^+$ T cells because Th1 cells and Tregs are known to influence CD8$^+$ cells in the TME (Gonzalez et al. 2018; Miggelbrink et al. 2021; McRitchie and Akkaya 2022). Additionally, this study revealed differences in the Th1 and iTreg differentiation *in vitro* caused by IL-18 and IL-1β and those cytokines have been shown to be produced in the TME of pancreatic cancer and modulate its composition (Apte and Voronov 2002; Das et al. 2020; Ahmed et al. 2022; Dinarello et al. 2013). Likewise, how IL-18 and IL-1R signaling in this tumor specific Th1 cells and Tregs influences their impact on antigen-specific CD8$^+$ T cells would be of particular interest.

5.4 Conclusion and outlook

The present study revealed a direct effect of IL-18 on T cell exhaustion mediated by the PI3K/Akt/mTOR pathway and at least in parts by the IL-2/STAT5 pathway (Fig. 29). These results are novel and important, as they can help to understand the processes and mediators of intratumoral T cell exhaustion in cancer patients. In particular, IL-18, as a pro-inflammatory cytokine with IFN-γ enhancing capacities, was generally believed to increase anti-tumor immunity. This study shows that this assumption has severe limitations when T cells are already in an exhausted state or in an exhaustion-promoting context. Future studies are needed to clarify the

exact pathway of IL-18-mediated exhaustion and the roles of signaling molecules like STAT5, Akt and mTOR especially *in vivo*.

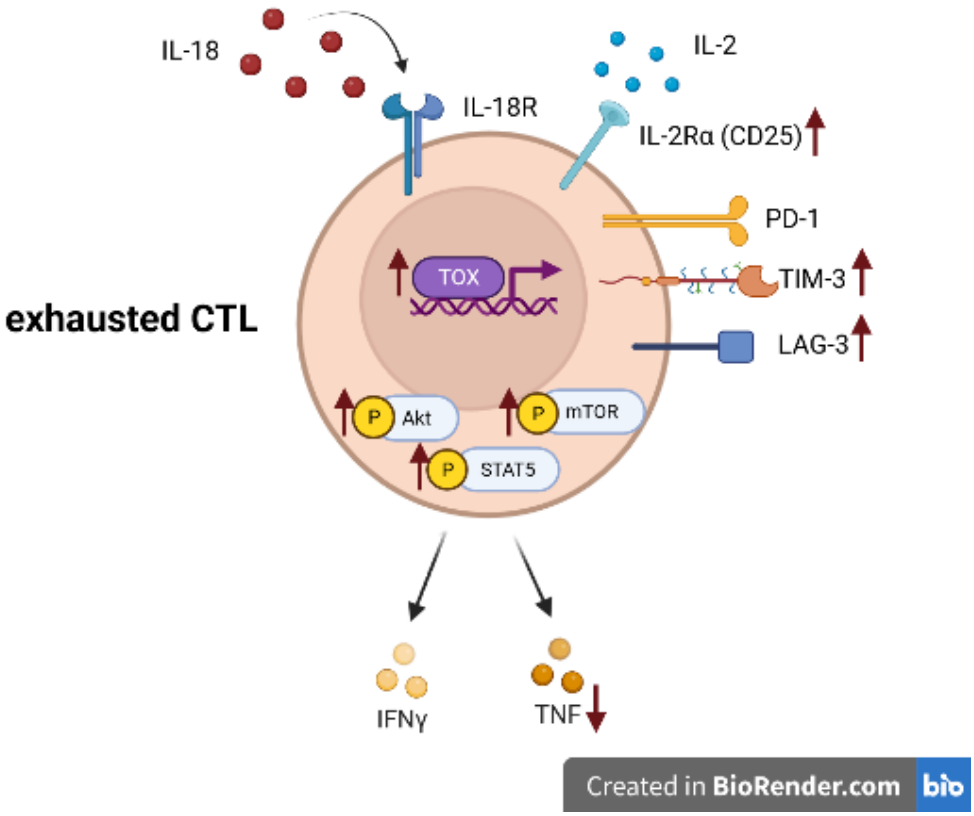


Figure 29: IL-18 mediated exhaustion in CTLs. IL-18 signaling increases TOX, inhibitory receptors, CD25 and phosphorylation of Akt mTOR and STAT5 in exhausted CTLs. Production of effector cytokines is reduced by IL-18 signaling. This Figure is based on data and information published in Lutz et al., 2023. Created in BioRender.com.

This study also aimed to detect effects of IL-1 and IL-18 signaling on $CD4^+$ T cells *in vitro* and *in vivo* and revealed Th1-promoting effects of antigen-specific $CD4^+$ T cells *in vitro* (Fig. 30A). Interestingly, effects of IL-18 on iTreg differentiation revealed decreased iTreg differentiation but increased TGFβ production, limiting the simple classification of IL-18 as a Treg suppressing cytokine (Fig. 30A). The impact of IL-1β on $CD4^+$ cells *in vitro* was marginal and only increased TGFβ production of iTregs (Fig. 30B). The impact of IL-1 and IL-18 on other T cell subsets, like Th2 and Th17 cells *in vitro* could be an interesting approach for future studies.

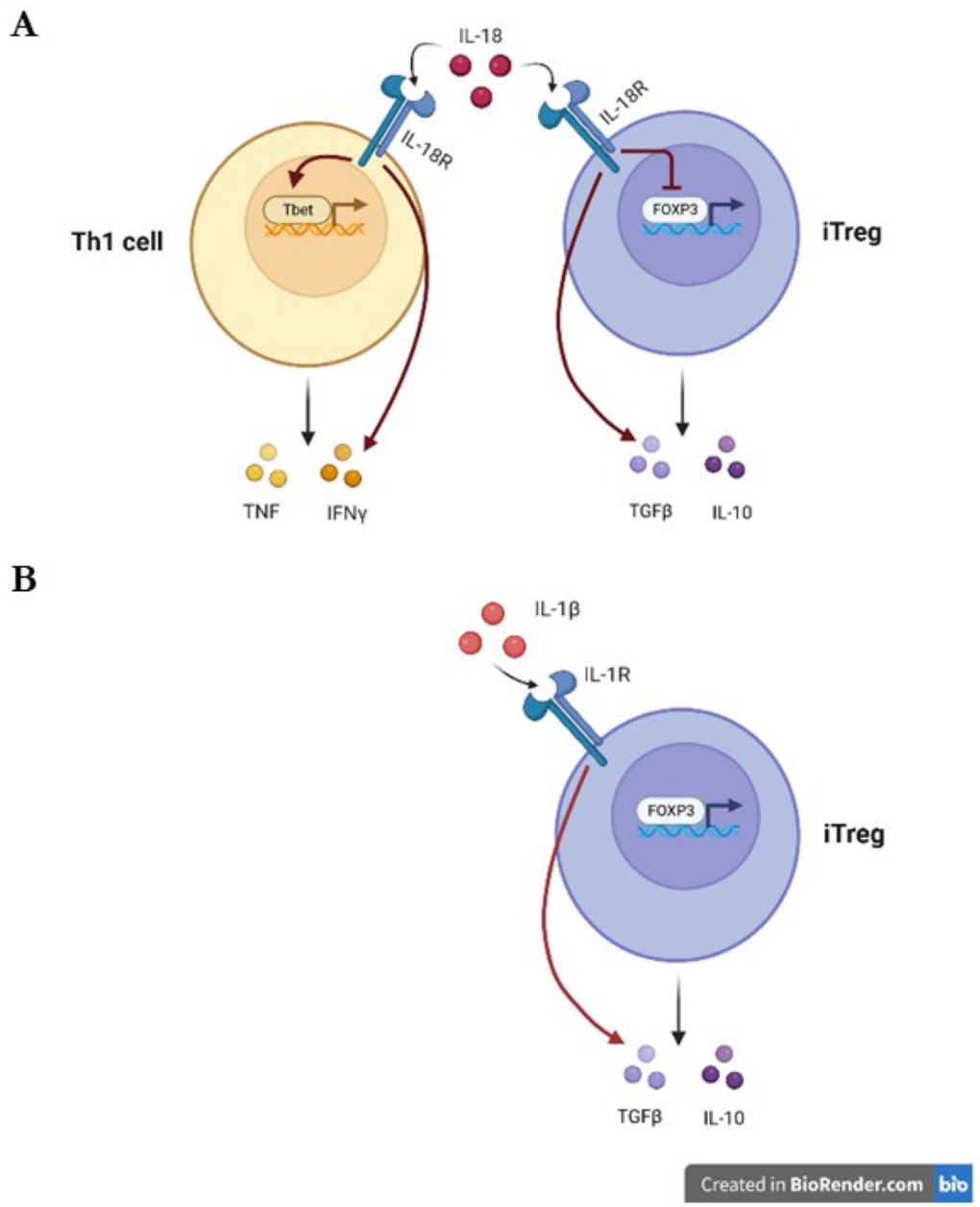


Figure 30: Impact of IL-18 and IL-1b on Th1 and iTreg differentiation *in vitro*. A) IL-18 signaling causes increased T-bet and production of effector cytokines in *in vitro* differentiated antigen-specific CD4 Th1 cells. *In vitro* differentiated iTregs show decreased FOXP3, but increased production of TGFβ upon IL-18 exposure. B) IL-1β signaling in *in vitro* differentiated iTregs causes increased production of TGFβ. Created in BioRender.com.

The adoptive transfer of antigen-specific $CD4^+$ T cells with and without IL-18 or IL-1R deficiency was performed in order to clarify the role of IL-1 and IL-18 in $CD4^+$ T cell development *in vivo*. Unfortunately, the number of living cells found at the end of the experiments was too low to identify possible effects of IL-1 or IL-18 on cytokine production and $CD4^+$ subset differentiation *in vivo*. Additionally, the present study revealed no considerable effects of antigen-specific $CD4^+$ T cells on endogenous T cell populations, adoptively transferred CTLs, tumor growth and tumor rejection. In this case, it was also irrelevant whether the adoptively transferred $CD4^+$ T cells had a receptor deficiency or not. Future studies should focus on the improvement of the murine model

in order to detect effects of IL-1 and IL-18 signaling on adoptively transferred $CD4^+$ T cells in a pancreatic cancer model. Increasing the number of injected $CD4^+$ T cells, choosing an earlier endpoint of the experiments, or injecting *in vitro*-activated/-differentiated $CD4^+$ T cells or subsets of those.

In summary, this study gained further insight into the effects of the NLRP3-dependent cytokines IL-18 and IL-1β on T cell development, characterizing the T cell exhaustion-promoting mechanisms of those cytokines in the context of antitumor immune responses. This study highlights the possibility to use anti-IL-18 strategies as an adjuvant therapy in cancer treatment.

6 Summary

Pancreatic cancer is one of the deadliest cancer types and is characterized by a highly immunosuppressive tumor microenvironment. The abundance of T cells in the tumor microenvironment has been shown to improve the prognosis for pancreatic cancer patients. Nevertheless, T cell exhaustion can cause a lack T cell effector function and therefore contribute to cancer immune evasion. Cytokines, like the NLRP3-dependent cytokines IL-18 and IL-1β, are highly involved in the regulation of the function and exhaustion of T cells. Additionally, elevated levels of IL-18 and IL-1β have been found in various cancer types, inter alia pancreatic cancer. Therefore, this study aimed to characterize the role of IL-18 and IL-1β in T cell exhaustion and dysfunction in a pancreatic cancer model.

It is highly important to understand the function and plasticity of T cells in pancreatic cancer in order to find new therapy approaches. Therefore, the goal of this study was to determine i) the effect of IL-18 on T cell exhaustion in vitro, ii) the molecular mechanisms of IL-18-mediated exhaustion of CTLs, iii) the effects of IL-1β and IL-18 on Th1 and Treg differentiation in vitro, iv) the effect of IL-1R and IL-18R signaling in adoptively transferred CD4+ T cells on endogenous

T cell populations in a murine pancreatic cancer model and v) the effect of IL-1R and IL-18R signaling in adoptively transferred CD4+ T cells on tumor rejection and on the function of adoptively transferred tumor specific CTLs in a murine pancreatic cancer model.

During this study, an in vitro exhaustion model was used which showed that IL-18 induces a more severe exhausted phenotype of antigen-specific CD8+ T cells in vitro. Additionally, it was used to determine that the IL-2/STAT5/Akt/mTOR pathway plays a key role in the IL-18 mediated induction of exhaustion. Therefore, this study identified IL-18 and the IL-2/STAT5/Akt/mTOR pathway as potential targets for immunotherapy in cancer.

This study also revealed a Th1 inducing and iTreg inhibiting function of IL-18 receptor signaling in vitro and therefore provides valuable information on IL-18 receptor signaling in CD4+ T cell subsets that are important for future in vitro and in vivo studies.

IL-18R and IL-1R signaling in adoptively transferred CD4+ T cells in a murine pancreatic cancer model neither had an impact on endogenous CD4+ or CD8+ T cells nor did they influence tumor growth or tumor rejection by antigen-specific CTLs. Nevertheless, the mice strains generated during this study are a helpful tool for future studies focusing on the role of IL-18 and IL-1 receptor signaling in antigen-specific CD4+ T cells and Tregs.

7 Zusammenfassung

Das Pankreaskarzinom ist eine der tödlichsten Krebsarten und zeichnet sich durch ein immunsuppressives Tumormilieu aus. Es konnte gezeigt werden, dass die Anzahl an T-Zellen in der Tumorumgebung die Prognose der Patienten verbessert. Die Dysfunktion von intra-tumoralen T-Zellen kann ihre Effektorfunktion jedoch verringern und daher zur Immunevasion beitragen. Zytokine, wie die NLRP3 abhängigen Zytokine IL-18 und IL-1β, sind stark an der Regulation der Funktion und Dysfunktion von T Zellen beteiligt. Zusätzlich wurden erhöhte Mengen von IL-18 und IL-1β bei verschiedenen Krebsarten gefunden, unter anderem in Pankreaskarzinomen. Die vorliegende Arbeit zielte daher darauf ab, die Rolle von IL-18 und IL-1β bei der T Zell Dysfunktion in einem murinen Pankreaskarzinommodell zu charakterisieren.

Es ist von großer Bedeutung, die Funktion und Plastizität von T-Zellen in Pankreaskarzinomen zu verstehen, um neue Therapieansätze zu finden. Daher war das Ziel dieser Arbeit i) die Wirkung von IL-18 auf die T-Zell Dysfunktion in vitro zu untersuchen, ii) die molekularen Mechanismen der IL-18-vermittelten Dysfunktion von CTLs zu identifizieren, iii) die Wirkungen von IL-1β und IL-18 auf Th1- und iTreg-Differenzierung in vitro zu zeigen, iv) den Effekt der IL-1- und IL-18-Rezeptor Signalgebung in transferierten CD4+-T-Zellen auf endogene T-Zellpopulationen und v) den Effekt von IL-1 und IL- 18-Rezeptor Signalisierung in transferierten CD4+ T-Zellen auf die Tumorabstoßung und die Funktion von transferierten tumorspezifischen CTLs in einem murinen Pankreaskarzinommodell zu untersuchen.

In dieser Arbeit wurde ein in vitro Modell für T Zell Dysfunktion verwendet in dem IL-18 eine erhöhte Dysfunktion in antigen-spezifischen CTLs induzierte. Es konnte gezeigt werden, dass der IL-2/STAT5/Akt/mTOR-Signalweg eine wichtige Rolle bei der IL-18-vermittelten Induktion von Dysfunktion spielt. Daher konnte diese Arbeit IL-18 und den IL-2/STAT5/Akt/mTOR-Signalweg als potenzielle Ziele für die Immuntherapie bei Krebs identifizieren.

Diese Arbeit konnte außerdem zeigen, dass IL-18 in vitro eine Th1-induzierende und iTreg-hemmende Funktion hat und liefert daher wichtige Informationen zur Rolle des IL-18-Signalweges in CD4+-T Zell Subtypen, die für zukünftige in vitro und in vivo Studien wichtig sind.

IL-18R und IL-1R Signaltransduktion in adoptiv transferierten CD4+ T Zellen in einem murinen Pankreaskarzinom Modell hatte weder einen Einfluss auf endogene CD4+ oder CD8+ T Zellen, noch beeinflussten sie das Tumorwachstum oder die Tumorabstoßung durch antigen-spezifische CTLs. Jedoch sind die während dieser Arbeit generierten Mäusestämme ein nützliches Werkzeug für zukünftige Studien, die sich auf die Rolle der IL-18- und IL-1-Rezeptorsignalisierung in antigen-spezifischen CD4+ T Zellen und Tregs fokussieren.

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9 Appendix

A Curriculum vitae

B Verzeichnis der Akademischen Lehrer/-innen

Meine akademischen Lehrenden an der Universität zu Köln waren:

Altenhain, Arndt, Baumann, Becker, Beyer, Blenau, Bonkowski, Borcherding, Bowagner

umer, Bucher, Büschges, de Meaux, Dohmen, Griesbeck, Häusler, Hafner, Hermann, Hess, Höcker, Hoef-Emden, Horstmann, Flügge, Frerigmann, Hofmann, Hoppe, Hülskamp, Kappes, Kisters-Woike, Klassmann, Klein, Kloppenburg, Kopriva, Korff, Kroiher, Krüger, Langer, Linne von Berg, Ludewig, Marin, Melkonian, Mörsdorf, Neuber, Neupert, Niefind, Nitsche, Nothnagel, Plickert, Predel, Roth, Sausen, Scherwaß, Schilke, Schnetz, Schwarz, Schössow, Schultz, Tresch, Schwarz, von Elert, Waffenschmidt, Waldkowiak, Wellmann, Wiehe, van der Meer, Venter, Zuccharo

Meine akademischen Lehrenden an der Universität Oulu waren:

Aspi, Haapakoski, Karppinen, Koivula, Markkola, Taulavuori, Vuoksiola

Meine akademischen Lehrenden an der Philipps-Universität Marburg waren:

Bange, Bauer, A. Becker, S. Becker Bökel, Bölker, Dolnik, Eickmann, Hassel, Huber, Kahmann, Kiontke, Krol, Maisner, Mösch, Sandrock, Schneider, Serrania, Sobetko, Strecker, Taxis, Waldminghaus, Visekruna, Yu

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D Ehrenwörtliche Erklärung

„Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „The role of IL-18 and IL-1 receptor signaling in T cell development and T cell exhaustion in a murine pancreatic cancer model“ in der der Klinik für Innere Medizin, Schwerpunkt Gastroenterologie, Endokrinologie, Infektiologie und Stoffwechsel, unter Leitung von Prof. Dr. Thomas Gress mit Unterstützung durch PD Dr. Christian Bauer und Prof. Dr. Malte Buchholz ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Ich versichere, dass ich sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate kenntlich gemacht habe.

Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Vorliegende Arbeit wurde in folgenden Publikationsorganen veröffentlicht:

Cancer Immunol Res., 2023 DOI: 10.1158/2326-6066.CIR-22-0398.
IL18 Receptor Signaling Regulates Tumor-Reactive CD8+ T-cell Exhaustion via Activation of the IL2/STAT5/mTOR Pathway in a Pancreatic Cancer Model.”

Ort, Datum, Unterschrift Doktorandin/Doktorand

„Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen.“

Ort, Datum, Unterschrift Referentin/Referent
