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Role of Interleukin-33 in the Activation, Differentiation and Migration of CD8$^+$ T Lymphocytes

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Die Rolle von Interleukin-33 in der Aktivierung, Differenzierung und Migration von CD8$^+$ T Lymphozyten

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Für Dieter Hubert Dreis

1951- 2005

Wir Endlichen mit dem unendlichen
Geist sind nur zu Leiden und Freuden geboren, und beinahe
könnte man sagen, die Ausgezeichnetsten erhalten durch
Leiden Freude.

Ludwig van Beethoven (1770 – 1827)

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I. Summary

1.1. English

Interleukin- (IL-) 33 is an exceptional cytokine of the IL-1 family released upon tissue damage which exerts multiple functions in adaptive and innate immunity. While the cytokine has primarily been associated to atopic diseases, IL-33 has now been proven to induce cytotoxic and T_H1, T_H17 and regulatory T cell (T_{reg}) functions reaching far beyond T_H2 immunity. In the autoimmune disorder multiple sclerosis (MS), IL-33 is reportedly released from necrotic plaques of the central nervous system (CNS) caused by infiltrated autoreactive $CD8^+$ T cells. In this context, it was considered fundamental to first clarify if IL-33 is systemically or rather locally active, as local, but not systemic IL-33, might modulate the activity of the autoreactive $CD8^+$ T cells. Moreover, conditions and co-factors supporting the pro- and anti-inflammatory function of IL-33, as well as the contribution of IL-33 to the peripheral trafficking of $CD8^+$ T cells were investigated in this doctoral thesis.

In the first part of the present work, it was demonstrated that IL-33 in blood is biologically inactive. Naturally occurring IL-33 detected by enzyme-linked immunosorbent assays (ELISA) in healthy samples exerted no biological effects in a cell-based reporter assay. Concurrently, also bioactivities of recombinant bioactive IL-33 isoforms were significantly decreased in blood. Although the circulating decoy receptor of IL-33, soluble suppression of tumorigenicity 2 (sST2), was suspected to sequester exogenous IL-33, sST2 was revealed here to not efficiently decrease IL-33 bioactivity in the presence of the transmembrane signaling receptor ST2 long (ST2L) and co-receptor IL-1RAcP. Even though the precise mechanism leading to the observed decrease in IL-33 bioactivity remains to be elucidated, it was concluded that *in vivo*, IL-33 released from necrotic tissue like MS plaques would rather exert its effects on infiltrated and $CD8^+$ T lymphocytes. However, conditions promoting either IL-33-dependent activation of cytotoxic T cells or differentiation of T_{reg} are poorly described. The nutrient sensor

mammalian target of rapamycin (mTOR) is a central regulator of T cell differentiation, whose inhibition promotes immunosuppression. Since IL-33 activates mTOR complex 1 (mTORC1) in murine T_H2 cells, it was asked if $CD8^+$ T cells are IL-33-dependently differentiated through modulation of the mTOR activation status. Here was shown for the first time that inhibition of mTORC1 caused by serum withdrawal mediated expression of IL-33 receptor ST2L on human $CD8^+$ T cells. Starvation moreover triggered emergence of a subset with reduced CD8 expression, requiring distinction between $CD8^{high}$ and $CD8^{low}$ T cells. $CD8^{high}$ were in the following characterized as naive T cells expressing the T_H1 transcription factor T-box transcription factor TBX21 (T-bet). IL-33 potently enhanced the T cell receptor (TCR)- and IL-12-induced effector function of $CD8^{high}$, resulting in proliferation, secretion of Interferon-(IFN)γ and cytotoxic degranulation. In contrast, $CD8^{low}$ T cells did not proliferate after TCR activation but expressed the hallmark transcription factors for T_H2 and T_{reg} subsets, GATA3 and forkhead box P3 (FoxP3), respectively, upon stimulation with IL-33 and IL-12. Importantly, IL-33 stimulation promoted an anti-inflammatory function in IL-12-stimulated $CD8^{low}$ T cells, which were subsequently denominated T_{reg}-like $CD8^+$ T cells. Finally, it was investigated whether IL-33 re-activated mTOR but IL-33 failed to activate mTORC1 or to enhance mTORC2 activity.

Both IL-33 and the metabolic status are described to affect the migratory potential of T lymphocytes. Peripheral distribution of effector and regulatory T cells and invasion into tissue are however central aspects in the progression of autoimmune diseases. In the third part, starvation was shown to significantly alter the migratory potential of $CD8^+$ T cells and to enhance expression of lymph node homing CC-chemokine receptor 7 (CCR7), especially on T_{reg}-like $CD8^{low}$ T cells. Induction of the effector phenotype using TCR activation and IL-33 stimulation increased expression of tissue residency marker CD69 and reduced messenger ribonucleic acid (mRNA) expression of sphingosine-1-phosphate receptor 1 (S1P1), a receptor required for lymphocyte egress from secondary lymphoid organs (SLO). In contrast, IL-33 alone induced mRNA expression of *S1P1*, but a decreased transcriptional expression of both *S1P1* and the gene of its transcription factor Krüppel-like factor 2 (KLF2) in T_{reg}-like $CD8^+$ T cells. It was concluded that the ability of TCR activated or T_{reg}-like $CD8^+$ T cells to egress from lymph nodes and tissue would be

diminished, while IL-33 alone would promote re-circulation of $CD8^+$ T cells. Furthermore, IL-33 was found here to promote expression of sphingosine-1-phosphate receptor 4 (S1P4) in T_{reg}-like $CD8^+$ T cells, a receptor with a postulated but poorly described role in the differentiation of T_{reg} subsets. Here a new subpopulation of regulatory $CD8^+$ T cells differentiated by IL-33 has been identified. Therefore, it was finally examined in a pilot study if the T_{reg}-like $CD8^{low}$ T cells might be of clinical relevance. In the samples of patients suffering from relapsing-remitting (RRMS) it was observed that the ratios of the presumable effector $CD8^{high}$ to regulatory $CD8^{low}$ were altered compared to healthy controls. The percentage of $ST2L^+$ $CD8^{low}$ was significantly decreased, raising the question if this observation was a result of a reduced T_{reg} abundance described for the autoimmune disease.

In summary, it was revealed here that the systemically inactive, but locally active cytokine IL-33 induces a novel T_{reg}-like phenotype in $CD8^{low}$ T lymphocytes during nutrient withdrawal. The population was found to express a distinct $CCR7^+$ $S1P1^-$ $S1P4^+$ expression pattern presumably supporting migration of the T cell subset into SLO *in vivo*. A pilot study on relapsing-remitting MS further revealed a possible clinical significance of the T_{reg}-like $CD8^+$ T cells, which were shown to be less abundant. It is therefore recommended to perform a large cohort patient study to investigate the function of IL-33 in MS.

1.2. Deutsche Zusammenfassung

Interleukin-(IL-) 33 ist ein außergewöhnliches Zytokin der IL-1 Familie, welches nach Gewebsschädigung freigesetzt wird und verschiedene Funktionen in der adaptiven und angeborenen Immunität erfüllt. Während das Zytokin ursprünglich vor allem mit atopischen Erkrankungen in Verbindung gebracht wurde, wurde nun bewiesen, dass IL-33 ebenso zytotoxische und T_H1, T_H17 und regulatorische (T_{reg}) Aktivitäten induzieren kann, die weit über T_H2 Immunität hinausgehen. Bei der Autoimmunerkrankung der Multiplen Sklerose (MS), bei welcher autoreaktive $CD8^+$ T-Zellen in das zentrale Nervensystem transmigrieren und den Abbau axonaler Myelinscheiden bewirken, wird IL-33 beispielsweise aus nekrotischen Plaques freigesetzt. In Rahmen dessen wurde es hier als grundlegend erachtet, zunächst zu klären ob IL-33 systemisch oder lokal aktiv ist, da lokales, aber nicht systemisches IL-33 möglicherweise die Aktivität autoreaktiver $CD8^+$ T-Zellen moduliert. Darüber hinaus wurden die Bedingungen und Ko-Faktoren, unter welchen IL-33 entweder pro- oder anti-inflammatorische Immunantworten begünstigt, sowie die mögliche Beteiligung von IL-33 an der peripheren Migration und Gewebsinfiltration von $CD8^+$ T-Zellen in der vorliegenden Arbeit untersucht.

Im ersten Teil der vorliegenden Arbeit wurde vorgestellt, dass IL-33 in Blut inaktiv ist. In Blut gemessenes natürlich vorkommendes IL-33 erwies sich in einem Zell-basierten Reportergenassay als biologisch inaktiv. Entsprechend waren ebenso die Bioaktivitäten von rekombinanten bioaktiven IL-33 Isoformen signifikant in Blut reduziert. Obwohl vermutet wurde, der lösliche zirkulierende Rezeptor sST2 das exogene IL-33 abfängt, zeigte sich hier, dass sST2 nur ineffizient IL-33 Bioaktivität in Anwesenheit des membranständigen signalgebenden Rezeptors ST2L und des Korezeptors IL-1RAcP hemmt. Der genaue vorliegende Mechanismus, der zu der Reduktion der IL-33 Bioaktivität in Blut führte, konnte abschließend nicht vollständig aufgeklärt werden. Jedoch wurde gefolgert, dass IL-33, welches aus nekrotischem Gewebe wie MS Plaques freigesetzt wird, insbesondere die Aktivität infiltrierender $CD8^+$ T-Zellen modulieren würde. Dennoch sind die Bedingungen unter denen IL-33 entweder zytotoxische $CD8^+$ T-Zellen oder die

Differenzierung regulatorischer T-Zellen unterstützt, ungenügend beschrieben. Der Sensor für Nährstoffverfügbarkeit mTOR ist ein zentraler Regulator der T-Zell Differenzierung, dessen Inhibition zu Immunsuppression führt. Da IL-33 mTORC1 in T_H2 Zellen aktiviert, wurde nachfolgend gefragt, ob die Art der IL-33-abhängigen Differenzierung von $CD8^+$ T-Zellen von mTOR beeinflusst wird.

Im zweiten Teil dieser Arbeit wurde zum ersten Mal gezeigt, dass Serumentzug, der eine Inhibition der mTORC1 Aktivität zur Folge hat, ST2L Expression auf humanen $CD8^+$ T-Zellen induziert. Eine nährstoffarme Umgebung verursachte zudem ein Aufkommen einer CD8 Population mit einer reduzierten CD8 Expression, sodass im Folgenden zwischen $CD8^{high}$ und $CD8^{low}$ unterschieden wurde. $CD8^{high}$ wurden im Folgenden als naive T-Zellen charakterisiert, die den T_H1 Transkriptionsfaktor T-bet exprimieren. In dieser Subpopulation verstärkte IL-33 eine T-Zell Rezeptor- und IL-12-induzierte Effektoraktivität, was in Proliferation, Sekretion von Interferon-γ und zytotoxischer Degranulation resultierte. Im Gegensatz dazu proliferierten die $CD8^{low}$ nach T-Zell Rezeptor Aktivierung nicht und exprimierten die für T_H2 und T_{reg} Immunität charakteristischen Transkriptionsfaktoren GATA3 und FoxP3 nach Stimulation mit IL-33 und IL-12. Wichtig ist weiterhin, dass eine IL-33 Stimulation eine anti-inflammatorische Funktion auf IL-12-stimulierte $CD8^+$ T-Zellen ausübte. IL-33/ IL-12-stimulierte $CD8^{low}$ wurden daher in Folge als T_{reg}-ähnliche $CD8^+$ T-Zellen bezeichnet. Schließlich wurde untersucht, ob IL-33 mTOR unter Hunger reaktivieren kann. Jedoch stellte sich heraus, dass IL-33 weder mTORC1 aktivieren noch die Aktivität von mTORC2 verstärken kann.

Sowohl IL-33 als auch dem metabolischen Status wird ein Einfluss auf das Migrationspotential von T Lymphozyten zugesprochen. Die Verteilung von Effektor und regulatorischen T-Zellen in der Peripherie und in Gewebe ist dabei ein zentraler Aspekt für die Progression von Autoimmunerkrankungen. Im dritten Teil dieser Arbeit wurde beobachtet, dass eine geringe Nährstoffverfügbarkeit potentiell zu einem veränderten Migrationspotential von $CD8^+$ T-Zellen führt, indem die Expression des Chemokinrezeptors CCR7 insbesondere auf T_{reg}-like $CD8^{low}$ verstärkt wurde. Die Induktion des Effektorphänotypen mittels T-Zell Rezeptor Aktivierung und Stimulation mit IL-33 verstärkte zudem die Expression von CD69, einem Rezeptor, der für den Verbleib von Lymphozyten in Gewebe und sekundär lymphatischen Organen erforderlich

ist und eine reduzierte Expression von Sphingosin-1-Phosphat Rezeptor 1 (S1P1), welcher die Auswanderung von Lymphozyten aus Lymphknoten vermittelt. Im Gegensatz dazu induzierte IL-33 alleine die Expression des *S1P1* Gens und dessen Transkriptionsfaktor Krüppel-like Factor 2 (KLF2). Darauf wurde geschlossen, dass die Fähigkeit T-Zell Rezeptor aktivierter oder T_{reg}-ähnlicher $CD8^+$ T-Zellen aus dem Lymphknoten und Gewebe auszuwandern, reduziert wäre, während IL-33 alleine die Auswanderung der $CD8^+$ T-Zellen fördern würde. Weiterhin wurde hier beobachtet, dass IL-33 die Expression des Sphingosin-1-Phosphat Rezeptors 4 (S1P4) in T_{reg}-ähnlichen $CD8^+$ T-Zellen vermittelt. S1P4 ist allerdings ein schlecht beschriebener Rezeptor mit vermuteter Funktion in der Differenzierung regulatorischer T-Zell Subtypen.

In dieser Thesis wurde eine neue regulatorische $CD8^+$ T-Zell Population beschrieben, deren Differenzierung durch Stimulation mit IL-33 induziert wird. Es wurde schließlich im Rahmen einer Pilotstudie untersucht, ob T_{reg}-ähnliche $CD8^{low}$ T-Zellen eine klinische Relevanz innehaben könnten. In den Proben von Patienten mit schubförmig remittierender MS waren die prozentualen Verhältnisse der Effektor $CD8^{high}$ T-Zellen zu regulatorischen $CD8^{low}$ T-Zellen im Vergleich zu gesunden Kontrollen signifikant verändert. Zudem war der Anteil $ST2L^+$ $CD8^{low}$ signifikant reduziert, was die Frage aufwarf, ob dies mit einer insgesamt reduzierten Anzahl von T_{reg} einhergehen könnte, einem für Autoimmunerkrankungen beschriebenen Effekt.

Zusammenfassend wurde gezeigt, dass das systemisch inaktive, aber lokal bioaktive Zytokin IL-33 unter einer reduzierten Nährstoffverfügbarkeit einen hier erstmals beschriebenen T_{reg}-ähnlichen Phänotypen in $CD8^+$ T-Zellen induziert. Die Population exprimierte spezifisch ein $CCR7^+$ $S1P1^-$ $S1P4^+$ Expressionsmuster, welches *in vivo* vermutlich die Migration von T-Zellen zu sekundären lymphatischen Organen und die Transmigration in Gewebe unterstützt. Anhand einer Pilotstudie mit Patienten, die an schubförmig remittierender MS leiden, konnte gezeigt werden, dass die beschriebene Subpopulation von klinischer Signifikanz sein könnte. Daher wird abschließend die Durchführung einer umfassenden klinischen Studie empfohlen, um die Rolle von IL-33 in der MS zu untersuchen.

II. Introduction

2.1. Short Introduction to the Immune System

The mammalian immune system is composed of an innate and adaptive immunity. The innate immune system comprises antigen presenting cells (APC), NK cells, granulocytes and mast cells, whereas the adaptive immune system is constituted by T and B lymphocytes contributing to a lifelong, constantly evolving protection. Immune cells are generated in primary lymphoid organs and descend from a common hematopoietic stem cell progenitor. Immune cells have a surveillance function and circulate in the lymphatic and blood vascular system, through which they also immigrate into SLO. Specialized subpopulations of leukocytes however reside in organs, while memory T and B cells generated during infections patrol organ tissue to rapidly counteract re-infection. Endogenous soluble mediators and alarmins released e.g. upon tissue damage or secreted by immune cells orchestrate the primary response for acute inflammation after infection. As the first defense line, granulocytes and macrophages are activated and recruited to the site of infection. APC like dendritic cells (DC) are connectors of the innate and adaptive immunity and potently activate antigen-specific T cells in SLO. T cells are classified according to expression of cluster of differentiation (CD) 4 or CD8 on the cell surface, promoting either a T helper (T_H) or cytotoxic cell function of the subpopulation, respectively. A particularity of $CD8^+$ T cells is the ability to specifically lyse and destroy virally infected or transformed cells, a property that has come into focus for the research on the pathogenesis of autoimmune and cancer diseases.[1]

2.2. Immune Homeostasis and Immunometabolism

2.2.1. Differentiation of Effector and Regulatory T cell Subsets

T cells are classically activated antigen-specifically in draining lymph nodes (LN) or spleen through interaction of the T cell receptor (TCR) with a pathogen-derived peptide constituting the antigen that is presented in major histocompatibility (MHC) class I or II molecules expressed on professional APC. Efficient activation further requires interaction of the co-stimulatory molecules CD28 on the T cell with CD80/ CD86 expressed on the APC, as well as stimulation by pro-inflammatory cytokines activating clonal proliferation and determining the direction of polarization of T cell subsets (**Figure 1**).[2]

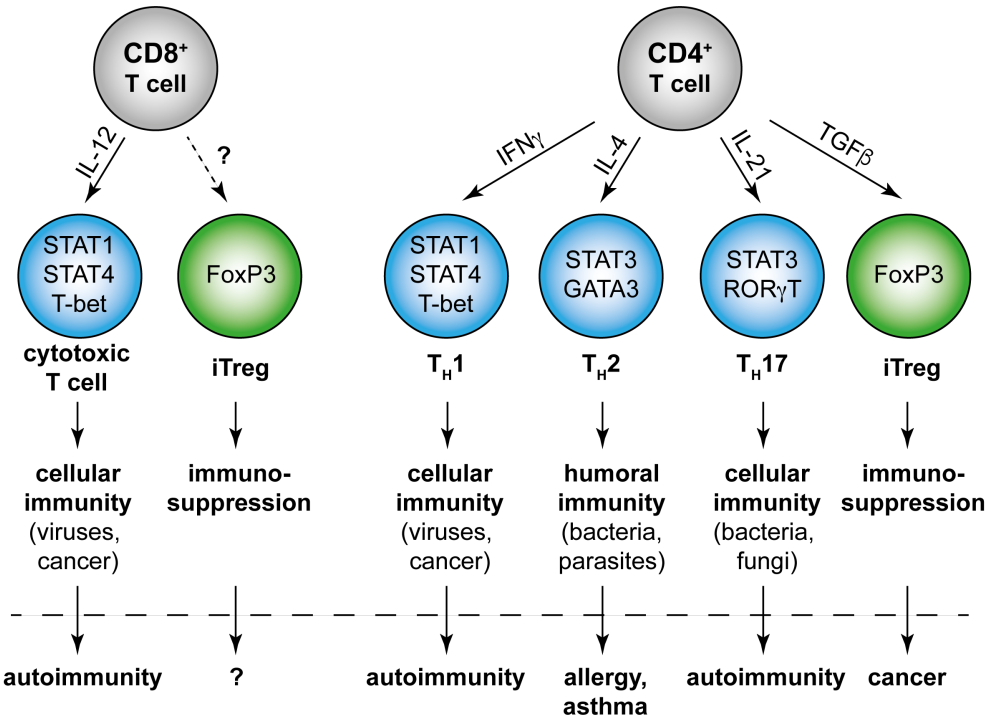


Figure 1: Differentiation of T cell subsets. $CD8^+$ T cells are differentiated to cytotoxic T cells or to T_{reg} cells (iT_{reg}), $CD4^+$ T cells to T_H1, T_H2, T_H17 or iT_{reg} cells. Differentiation to cytotoxic or T_H1 cells leads to expression of transcription factor T-bet, while T_{reg} differentiation requires FoxP3 expression. T_H2 and TH17 polarization is characterized by expression of transcription factors GATA3 and retinoic acid receptor related receptor gamma T (RORγT), respectively. Cytotoxic, T_H1 and T_H17 cells contribute to the pathogenesis of autoimmune diseases, T_H2 cells to allergy and asthma and iTreg to the progression of cancer (modified from Rink, Kruse and Haase[1]).

The type of cytokines secreted for T cell polarization depends on the origin of the antigen, the cytokine milieu and pattern recognition receptors activated on the APC to induce immune responses against bacterial, fungal, viral or parasitic infections. Respectively, stimulation of T cells with cytokines activates downstream signaling pathways and transcription factors initiating differentiation towards T_H1, T_H2, T_H17 or T_{reg} subsets. Herein, the interleukin IL-12 signaling pathway is required for differentiation of T_H1 polarized $CD4^+$ T cells and cytotoxic $CD8^+$ T cells. Secretion of IL-12 during TCR-dependent stimulation induces activation of Janus kinases and the signal transducer and activator of transcription (STAT) pathway, including STAT4 and STAT1. STAT1 signaling mediates expression of T-bet, a driver of tumor necrosis factor alpha (TNFα) and IFNγ secretion required for an enhanced cytotoxic activity of $CD8^+$ T cells during viral infection. $CD8^+$ T cells play an essential role in the identification of cells with an "altered self". Intracellular peptides, including "self-peptides" are continuously presented in MHC-class I to the TCR of $CD8^+$ T cells. Identification of viral or neo-peptides arising from malignant transformation triggers a cell killing program in $CD8^+$ T cells, inducing Fas ligand-mediated apoptosis or release of perforins and granzymes for caspase-dependent apoptosis in target cells. T_H2 differentiation is induced by expression of transcription factor GATA3 through the STAT3 pathway. In a positive feedback loop, GATA3-induced IL-4, IL-15 and IL-13 secretion activates immune cells, e.g. mast cells and eosinophils, to perform an anti-parasitic response during helminth infection. However, T_H2 cells are highly involved in the pathogenesis of atopic diseases like allergies and asthma, in which a contribution of IL-1 family member IL-33 has been extensively described[3–5]. T_H17 differentiated cells are co-workers of T_H1 and T_H2 cells and support clearance of pathogens at barrier sites. T_H17 cells are associated to chronic inflammation and to the pathogenesis of several autoimmune diseases. Problematically, transforming growth factor beta (TGFβ) contributes to both the generation of T_H17 cells, as well as to the differentiation of T_{reg}[1,2]. T_{reg} cells have formerly been named suppressor cells due to their distinctive function in the maintenance of homeostasis between pro- and anti-inflammatory immunity and are essential for resolution of inflammation and upholding self-tolerance. T_{reg} limit the activity of T_H1, cytotoxic, T_H2 and T_H17 cells through secretion of anti-inflammatory cytokines like IL-10 and TGFβ, or

expression of immune checkpoint receptors like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed-death 1 (PD-1). The T_{reg} population is highly heterogenous, as they are either generated in the thymus or are induced during inflammation in the periphery. The driver of T_{reg} differentiation and generation is transcription factor forkhead box P3 (FoxP3).

Although the T_{reg} population primarily consists of $CD4^+$ T cells, Gershon and Kondo identified in 1970 a small subpopulation of $CD8^+$ T_{reg}[6]. In spite of this early discovery, to date little is known about the function of this population for regulation of inflammation. Unfortunately, the lack of reliable surface markers to discriminate $CD8^+$ T_{reg} from ordinary $CD8^+$ T cells further complicates research. In experimental systems, $CD8^+$ T_{reg} cell surface expression patterns differed significantly[7]. In experimental autoimmune encephalomyelitis (EAE), $CD8^+$ $CD28^-$ T_{reg} inhibited secretion of IFNγ by pro-inflammatory $CD4^+$ T cells through direct cell-cell contact[8,9]. In cancer patients, $CD8^+$ T_{reg} induced by DC were described to express a C-C motif chemokine receptor 7 (CCR7) and memory $CD45RO^+$ expression pattern. Those cells seemingly dampened inflammation through secretion of IL-10, thus contributing to immune evasion of cancer cells. Interestingly, $FoxP3^+$ $CD8^+$ T_{reg} were described to be inducible *in vitro*[10].

2.2.2. Regulation of T Cell Metabolism by mTOR

mTOR is a serine/ threonine protein kinase in the phosphoinositide 3-kinase (PI3K) pathway and a key regulator of innate and adaptive immunity. Due to its prominent role in controlling T cell generation, differentiation and development, modulation of the mTOR signaling pathway for resolution of autoimmune diseases and cancer has gained great importance[11].

mTOR forms the catalytic subunit for two protein complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (**Figure 2**). A central function of mTORC1 is to drive cell growth by supporting anabolism through inhibition of catabolism, most importantly autophagy[12]. mTORC1 is further composed of mTOR itself, a regulatory protein associated with mTOR (Raptor), mammalian lethal with Sec18 protein 8 (GβL) and DEP domain containing mTOR interacting protein (DEPTOR)[13].

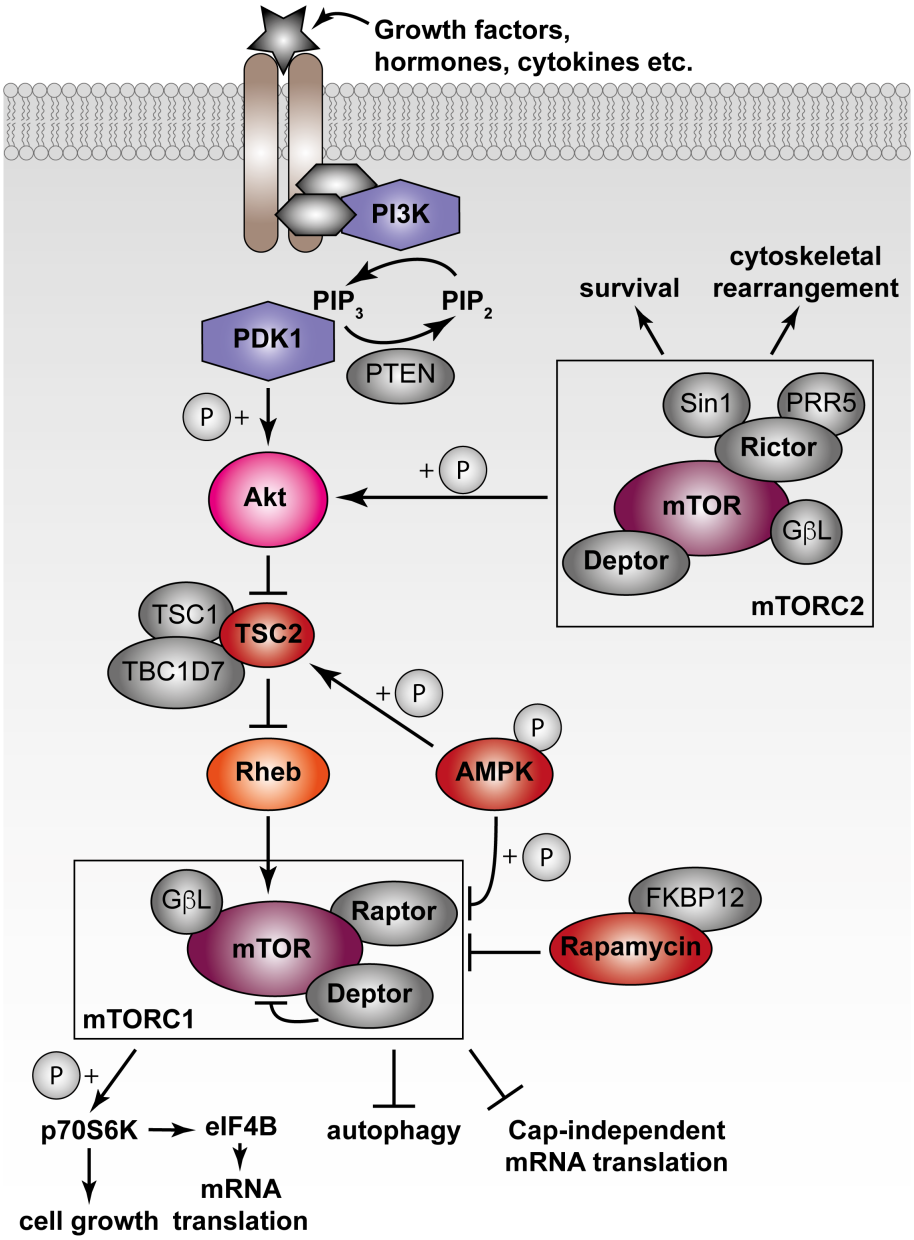


Figure 2: The mTOR signaling pathway. Growth factors, hormones and cytokines activate phosphoinositol 3-kinase (PI3K). Activation of protein kinase-1 (PDK1) enables activating phosphorylation of Akt, an inhibitor of tuberous sclerosis complex 2 (TSC2). TSC2 is a component of a heterotrimeric complex with TSC1 and TBC1D7 and inhibits the Rheb, an activator of mTORC1. mTORC1 promotes cell growth through activation of the p70 S6 kinase (p70S6K), regulates eukaryotic translation initiation factor 4B (eIFB) dependent mRNA translation and inhibits autophagy. Starvation promotes AMP-activated proteinkinase (AMPK)-dependent inhibition of mTORC1 by phosphorylation of TSC2 and Raptor. Rapamycin associates to FKBP12 to inhibit activity of mTORC1. mTORC2 contains the subunit rapamycin-insensitive companion of mTOR (Rictor) instead of regulatory protein associated with mTOR (Raptor) in mTORC1. Active mTORC2 activates Akt through phosphorylation and contributes to cell survival and cytoskeletal rearrangement (figure based on Saxton and Sabatini[13]).

During homeostasis, growth factors, hormones and cytokines of a nutrient rich environment induce activation of PI3K, a kinase activating protein kinase-1 (PDK1). PDK1 activates Protein Kinase B (Akt) through phosphorylation. Akt is an inhibitor of tuberous sclerosis complex 2 (TSC2), a component of the heterotrimeric TSC consisting of TSC1 and TBC1D7. TSC2 is a GTPase activating protein and inhibits the Rheb GTPase[14,15]. Rheb, in turn, is an activator of mTORC1. Importantly, Akt phosphorylates and inhibits TSC2, thus indirectly enabling Rheb to activate mTORC1[16]. Moreover, Akt is able to relieve mTORC1 from the inhibitory subunit proline-rich Akt substrate of 40 kDa (PRAS40) through phosphorylation[17]. The p70 subunit of the S6 kinase (p70S6K) is a well described substrate of mTORC1. Phosphorylated p70S6K promotes cell growth and activates eukaryotic translation initiation factor 4B (eIF4B), which positively regulates 5' cap-dependent mRNA translation.

Respectively, low energy and nutrient levels naturally lead to inhibition of mTORC1[18]. One reason is that during energy starvation, AMP-activated protein kinase (AMPK) supports inhibition of mTORC1 by phosphorylation of TSC2 and Raptor[14,19]. Rapamycin was early described to mediate immunosuppressive and anti-tumoral properties[20,21]. It was later revealed that rapamycin specifically inhibits activity of mTORC1 through inhibition of p70S6K-dependent phosphorylation of ribosomal protein S6[22–24]. mTORC2 is insensitive to acute treatment with rapamycin and consists besides of mTOR of the subunits GβL and DEPTOR like mTORC1, but instead of Raptor contains the rapamycin insensitive companion of mTOR (Rictor) and the regulatory subunits Sin1 and proline-rich 5 (PRR5)[13]. Unlike mTORC1, mTORC2 phosphorylates and activates multiple downstream proteins like Akt, and contributes to T cell survival, responses to osmotic stress and regulation of the cytoskeletal rearrangement[25,26].

2.2.3. Migration of $CD8^+$ T cells

Trafficking of effector and regulatory T cells is a highly complex system directing migration of immune cells to, emigration from SLO and transition into peripheral organs. Activation of T cells induces dramatic shifts in the expression patterns of receptors and integrins required for retention in SLO or extravasation into blood[27] (**Figure 3**).

T cell migration is a central aspect of an immune response, as immune cells initially primed in SLO by professional APC need to be released into the periphery to search for their specific target[28]. Besides TCR stimulation the activation status of nutrient sensing pathways such as mTOR is decisive for the modulation of T cell trafficking[29,30].

CCR7 is a G-protein coupled chemokine receptor expressed on several T cell subtypes. It guides chemokine-directed motility of T cells through activation of trimeric G-proteins or the JNK pathway[31,32]. CCR7 is considered as the principal chemokine receptor that controls and facilitates interactions between T cells and DC in LN[32]. Interestingly, ligands of CCR7 have previously been suggested to promote homeostatic expansion and survival of naïve T cells and seems to be required for T_{reg} homing and positioning in LN[33,34]. In this context, CCR7 deficiency in mice was associated to defective T_{reg} function[35,36].

The chemokines CC-chemokine ligand (CCL) 19 and CCL21 are constitutively released by high endothelial venules (HEV) and fibroblast reticular cells. They attract CCR7 expressing T cells to SLO, in particular to LN where the T cells encounter antigens presented by activated DC[28]. While CCR7 is mainly responsible for luring T cells to LN, retention requires expression of early activation marker and type II C-lectin receptor CD69[37]. Expression of CD69 on the cell surface of T cells is induced by type I IFN and other pro-inflammatory stimuli released during the interaction of APC with $CD8^+$ T cells[38]. CD69 is also expressed on γδ or resident memory T cell subsets residing in peripheral tissues[37]. Mechanistically, CD69 complexes sphingosine-1-phosphate receptor 1 (S1P1), a receptor mediating egress from LN, and induces its internalization to trap naïve lymphocytes in SLO and to prolong the time of T cells to survey for cognate antigens and interaction with APC[39,40]. S1P1 is the best explored receptor of a family comprising five known receptors (S1P1-S1P5). S1P receptors consist of seven transmembrane domains anchoring the protein within the cell membrane. Interestingly, expression of both *S1P1* and *CCR7* is transcriptionally regulated by Krüppel-like transcription factor 2 (KLF2)[41]. KLF2 itself has recently been described as a connector of mTOR to the regulation of T cell trafficking. Inhibition of mTORC1 triggers re-expression of KLF2 and confers T cells the ability to migrate to LN. Those changes in the migratory potential were attributed to a restored expression of CCR7, but not S1P1[29]. Consistent with this finding, another study showed that signaling through CCR7 counteracts the function of S1P1[42].

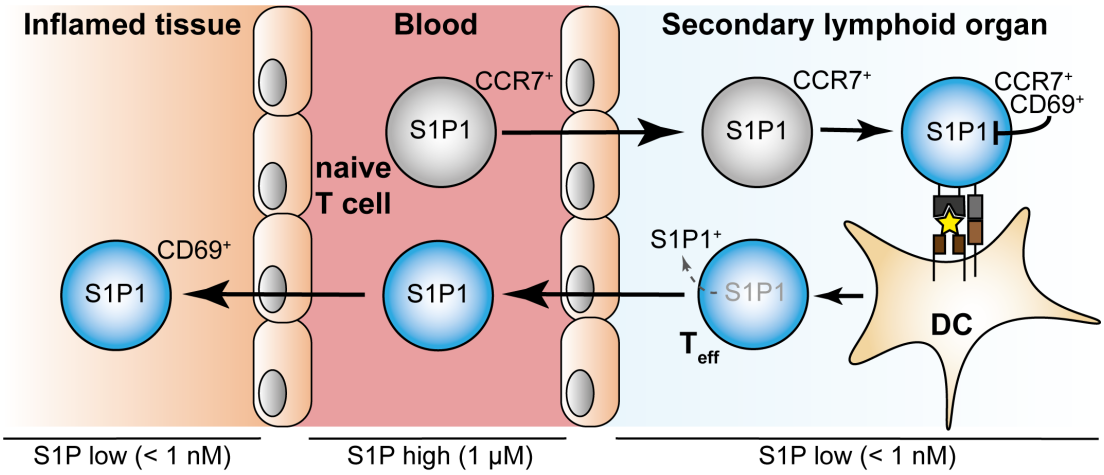


Figure 3: Expression of Sphingosine-1-Phosphate receptor 1 (S1P1), CCR7 and CD69 on migrating T cells. Naïve T cell circulating in blood highly express CCR7 on the cell surface and transmigrate through high endothelial venules to access SLO. TCR activation by dendritic cells (DC) stimulates additional expression of CD69, which complexes and blocks S1P1 on the cell surface. For egress from lymph nodes, CCR7 and CD69 expression is reduced, allowing S1P1 to translocate to the cell membrane of effector T cells (T_{eff}). S1P1 expressing T cells follow the gradient of sphingosine-1-phosphate (S1P) from the lymph node into blood. Loss of S1P1 expression allows migration into inflamed tissue, and T cell retention is provided by CD69 expression (modified from Aoki *et al.*[43]).

The precise mechanisms favoring either expression of CCR7 or S1P1 in spite of a common transcription factor however remain to be resolved. Baeyens *et al.* suggested that a central aspect of migration is how, in the end, T cells manage to balance signals triggering migration to LN and retention against S1P signals mediating egress into the blood stream[40]. For example, fibroblasts located in SLO reduce their secretion of CCL19 and CCL21 after antigen priming of T cells by APC, allowing the newly activated T cells to express S1P1 instead of CCR7[27,28]. S1P1 expressing cells follow a compartment-specific gradient of S1P, and binding of S1P leads to activation of intracellular G-proteins and pathways coupled to $G_{\alpha i}$, $G_{\alpha Q}$ or $G_{\alpha 12/13}$[44]. S1P is highly concentrated in blood (100 nM - 1 µM) and lymph (100 nM), but low concentrated in lymphoid organs (1 nM)[45]. Immune cells located in compartments with low S1P concentrations internalize and recycle S1P1. Erythrocytes are a major source of S1P, which they release into the blood stream where it is stabilized by the carrier proteins apolipoprotein M and high-density lipoprotein[40]. S1P4 is less studied than other S1P receptors, in spite of its abundant expression on immune cells. It has been postulated that S1P4 signaling plays a major role in the cytoskeletal rearrangement and indirectly contributes to the regulation of T cell differentiation[46,47].

2.2.4. Involvement of mTOR in Immune Homeostasis and Migration

The maintenance of immune homeostasis is a challenging task, which requires a continuous orchestration of pro- and anti-inflammatory signals by innate and adaptive immunity. Failure of or excessive T_{reg} function result in the pathogenesis of severe autoimmune or cancer diseases, respectively. Early patient studies on multiple sclerosis (MS)[48], systemic lupus erythematosus[49], rheumatoid arthritis[50] and type 1 diabetes[51] demonstrated that either T_{reg} numbers were decreased or the T_{reg} were dysfunctional, revealing an imbalance between effector and regulatory T cells.

Due to its central role in the regulation of immune cell metabolism, the PI3K-mTOR axis has gained attention as a molecular target for the treatment of autoimmune diseases. In this context, rapamycin, the inhibitor of mTORC1, was shown to be involved in the differentiation of T cell subsets. For example, the immunosuppressive effects mediated by rapamycin were related to an augmented production of $FoxP3^+$ T_{reg}[52,53]. Those findings were confirmed in an *in vivo* model, where inhibition of mTOR preserved the anti-inflammatory state of T cells, as it abrogated the reprogramming of $CD4^+$ $FoxP3^+$ T_{reg} into pathogenic T_H1/ T_H17 effector cells[54]. Importantly, similar observations were made for T_{reg} isolated from patients suffering from relapsing-remitting MS (RRMS), where inhibition of the PI3K/ Akt pathway re-established the suppressive function of the T_{reg}[55,56]. While in autoimmunity, restoration of T_{reg} function and numbers seems to be essential to limit disease progression, in cancer, T_{reg} dampening the activity of effector T cells support immune evasion and survival of malignant cells. Inside tumor tissue, nutrient deprivation results from irregular perfusion and uncontrolled growth of tumor mass. This effect naturally dampens activity of mTOR and is detrimental for the activity and fitness of infiltrating $CD8^+$ T cells[57,58]. mTORC1 inhibition occurring during antigen presentation was moreover shown to mediate T cell anergy and failure to activate in spite of antigen recognition[59]. Activity of mTORC1 facilitating the switch toward an anabolic metabolism, was described to be elementary for a potent T cell activation and expansion, a desirable effect for the induction of anti-tumor immunity[13]. Recent findings implicated that the balance between activated mTORC1 and mTORC2 is involved in the maturation of $CD8^+$

memory T cells. Pollizzi and colleagues found that during asymmetric division occurring during proliferation of activated $CD8^+$ T cells, mTORC1 activity was high in the daughter cells with an effector-like phenotype and low in the daughter cells resembling memory T cells[60]. The group further found that a shift in the activity of mTORC1 to mTORC2 was elementary for an enhanced generation of memory $CD8^+$ T cells[61].

Due to its function in promoting differentiation of cytotoxic, T_H1 and T_H17 cells while inhibiting the suppressive function and generation of T_{reg}, mTORC1 forms a central target, which can be differentially exploited to modulate T cell function[52,62,63]. Although both mTORC1 and mTORC2 have been attributed important functions for T cell responses, it has been suggested that particularly mTORC2 plays a central role in the regulation of survival and migration. mTORC2 actively engages in the regulation of cytoskeletal rearrangement, a fundamental mechanism for migration of immune cells. *In vivo* and *in vitro*, selective targeting of mTORC2, but not mTORC1, has been proven to efficiently inhibit migration of breast cancer cells and to induce apoptosis[64]. Active mTORC2 also stimulates migration of follicular $CD4^+$ T helper (T_H) cells during viral infection[65].

Sinclair and colleagues showed that the PI3K/ mTOR axis determines the repertoire of chemokine receptors and with this regulates peripheral trafficking[29]. Inhibition of mTORC1 with rapamycin in effector cells herein re-enabled T cells to migrate to LN through re-expression of CCR7. Importantly, S1P, which mediates T cell egress from SLO upon interaction with the S1P1, was also proven to cross-activate mTOR independent of Akt and PI3K[66]. Song and colleagues analyzed blood samples from RRMS patients treated with the S1P receptor modulator and sphingosine analogue Fingolimod. They revealed that the percentages of regulatory $CD4^+$ and $CD8^+$ T cells significantly increased in non-relapsing patients during Fingolimod therapy, which went along with a decelerated disease progression[67]. This finding implicated that the S1P signaling is presumably not only involved in migration of T cells, but also in generation of T cell subsets.

The gene of KLF2, the transcription factor for *S1P1* and *CCR7*, is a target of transcription factor forkhead box protein O1 (FoxO1). Interestingly, *in vitro* with IL-12 generated $CD4^+$ T_{reg} from T_H1-like cells exhibit an activated PI3K/ Akt pathway leading to inactivation of FoxO1, which reduces their suppressive capacity[68]. In line with this observation, Schröder *et al.* described that S1P1 signaling counteracts lipopolysaccharide (LPS)-

induced production of IL-12p17 by splenocytes[69] and Fingolimod was shown ameliorate T_H1-associated ulcerative colitis by supporting differentiation and function of $CD4^+$ T_{reg}[70].

The least explored S1P receptor 4 (S1P4) seemingly plays a less prominent role in the migration of immune cells than in the regulation of T cell fate. Interestingly, Weigert and colleagues have most recently identified the gene of phosphoinositide 3-kinase adaptor protein 1 (PIK3AP1) to be significantly and strongly induced in *S1P4* knockout cells (*unpublished results*). Deason *et al.* in turn found that PIK3AP1, which is also named BCAP, links the IL-1R and Toll-like receptor (TLR) signaling to the PI3K/ mTOR pathway. The group described this connection to be crucial for the induction of pathogenic T_H17 cell differentiation[71]. Although commonly expressed on immune cells, expression of S1P4 on myoblasts was reported to be inducible by TGFβ, which through inhibition of Akt signaling resulted in apoptosis[72]. In an *in vitro* model, priming of human DC in co-culture with autologous T cells with apoptotic debris of breast cancer cells prevented cytotoxicity towards live breast cancer cells. The immunosuppression was mediated by T_{reg}, which had been differentiated upon S1P/ S1P4-induced IL-27 secretion by DC[73]. Recent findings further implicated that binding of S1P to S1P4 might mediate production of cytokines fueling T_H17 and T_{reg} immunity[74].

Taken together, the findings on the contribution of the PI3K/ Akt pathway to the development of T_{reg} led to the view that the plasticity of T cell function, differentiation and migration must be strongly influenced by the environment and metabolism.

2.3. IL-33 – an Exceptional IL-1 Family Member

2.3.1. Characteristics of IL-33

IL-33 was identified 2005 as a novel member of the IL-1 family and ligand of hitherto orphan receptor Interleukin-1 receptor-like 1 (IL-1RL1, ST2L)[75]. Since then, the role of IL-33 in innate immunity, inflammation and atopic diseases has been widely proven[76]. IL-33 exerts a dual function as an intracellular chromatin-associated negative regulator of nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF-κB), and extracellularly as a cytokine[75,77,78]. Besides asthma and allergic diseases[79], high concentrations of IL-33 in blood have been suggested as a diagnostic tool for ischemic stroke[80], chronic heart failure[81,82] and relapsing-remitting MS[83]. In spite of its well described function as an extracellular cytokine, little is known about possible molecular mechanisms promoting active secretion of IL-33. In homeostasis, IL-33 is constitutively expressed in the nuclei of epithelial, endothelial and fibroblastic reticular cells of SLO, and is passively released from cells during necrosis and tissue damage[78,84,85].

Human IL-33 consists of 270 amino acids (aa) constituting an N-terminal non-classical nuclear localization domain (aa 1-65) with a chromatin-binding domain, a central (aa 66-111) and an IL-1 like cytokine domain for binding to ST2L (aa 112-270)[86]. During apoptosis, full length IL-33 is intracellularly cleaved by caspases-3 and -7, generating biologically inactive fragments due to cleavage of the IL-1 like cytokine domain[87,88]. Extracellular IL-33 is cleaved at the central domain by pro-inflammatory serine proteases released from neutrophils and mast cells, which results in maturation of the cytokine and a postulated increased biological activity[3,4]. For example, proteolytic maturation of IL-33 by chymases was described to produce the hyperactive isoforms IL-$33_{95\text{-}270}$ and IL-$33_{109\text{-}270}$, processing by tryptase generates the isoform IL-$33_{107\text{-}270}$[3] (**Figure 4**).

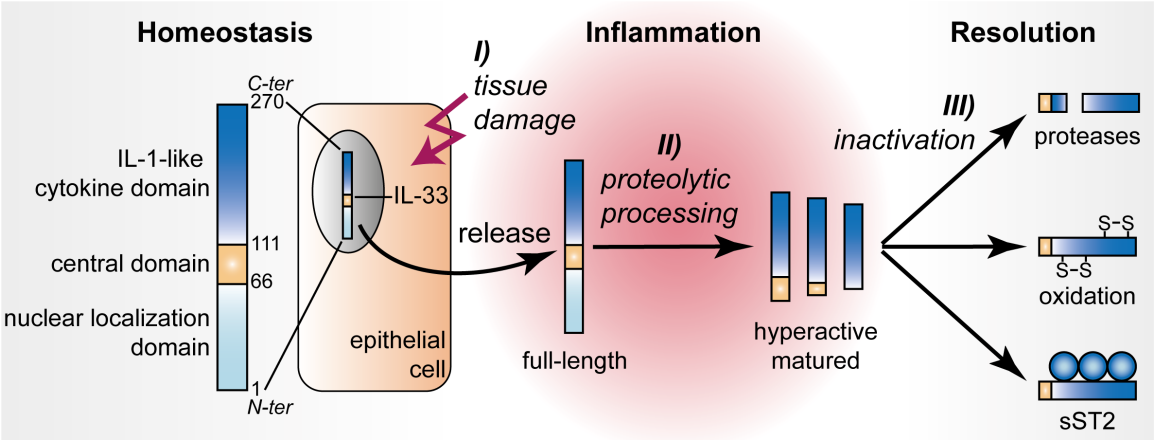


Figure 4: Regulation of IL-33 bioactivity. IL-33 consists of 270 amino acids and a C-terminal (C-ter) IL-1like cytokine, central and N-terminal (N-ter) nuclear localization domain. **(I)** During homeostasis, IL-33 is expressed in the nuclei of e.g. epithelial cells and negatively regulates activity of NF-κB. **(II)** Tissue damage resulting from inflammation or mechanical stress enables passive release of intracellular full-length IL-33, which is proteolytically processed and hyperactivated by pro-inflammatory proteases. **(III)** To limit range and duration of IL-33 bioactivity, the cytokine is inactivated by proteases, oxidation and presumably binding by decoy receptor soluble ST2 (sST2).

Extracellular, bioactive IL-33 binds to the membrane-bound receptor ST2L expressed on T_H2[89] and type 2 innate lymphoid cells (ILC2)[90,91], basophils and eosinophils[92]. ST2L was later discovered to also be expressed on natural killer (NK), NK T cells[93], activated T_H1, cytotoxic T cells[94,95] and T_{reg}[96,97]. The extracellular half-life of IL-33 is restricted by different mechanisms to limit range and duration of its biological activity. Neutrophil proteinase 3 enzyme is a protease digesting bioactive IL-33 and generates first a matured cytokine, but upon long-term exposure produces inactive IL-33 fragments[98]. Oxidation of IL-33 leads to formation of two disulfide bridges within the IL-1-like cytokine domain, promoting inaccessibility of the receptor binding site through conformational changes[99]. Recent findings implicate that allergen proteases further degrade processed isoforms of oxidized IL-33[100]. Soluble ST2 (sST2) respectively lacks a transmembrane and intracellular domain and is constitutively released by mast cells and different T_H2 cells. Decoy receptors have been identified for members of the IL-1 and TNF family, and for IL-6. IL-6 is similarly to IL-33 a mediator of acute inflammation. Interestingly, the soluble IL-6 receptor (sIL-6R) significantly prolongs the plasma half-life of IL-6 *in vivo*[101]. Although the accepted function of sST2 is to act as a decoy receptor for IL-33[102], it has been correspondingly to IL-6/ sIL-6R suggested that sST2 might serve as a reservoir for IL-33 by protecting the cytokine from proteolytic cleavage[82,103,104]

2.3.2. The IL-33/ ST2L Signaling

The ST2 gene is transcribed from two different promotors, resulting in expression of two isoforms[105]. The ST2L transcript is the longer isoform and consists of eleven exons. The transcript encodes a ligand-binding, a transmembrane and intracellular Toll-interleukin receptor (TIR) domain for signaling[86] (NM_016232.5). The sST2 transcript sequence respectively lacks the exons encoding the transmembrane and cytoplasmic domains and consists of eight exons (NM_003856.4). IL-1 receptor accessory protein (IL-1RAcP) is the transmembrane co-receptor of ST2L and both together with IL-33 form a high affinity signaling complex in the presence of IL-33[86].

Recruitment of IL-1RAcP has been proven to be absolutely required for IL-33 signaling and stabilizes binding of ST2L to its ligand[106]. Binding of the co-receptor promotes an intracellular dimerization of the TIR domains, although current findings implicate that the receptors might rather form oligomers in the plasma membrane[86,107]. Dimerization of the TIR domains consequently allows association of the adaptor protein myeloid differentiation primary response 88 (MyD88) to the scaffold, and subsequently, recruitment of the serine-threonine kinases interleukin-1 receptor-associated kinase (IRAK) 4, IRAK1 and IRAK2, which are recruited to the N-terminal death domain of MyD88 via their own death domains[108,109]. Primary activation of IRAK1 through phosphorylation by IRAK4 initiates a cascade of signals, which results in activation of tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 further activates transforming growth factor beta activated kinase 1 (TAK1), resulting in activation of the NF-κB pathway, the JNK and stress-activated protein kinase p38/ mitogen-activated protein kinases (MAPK)[110] (**Figure 5**).

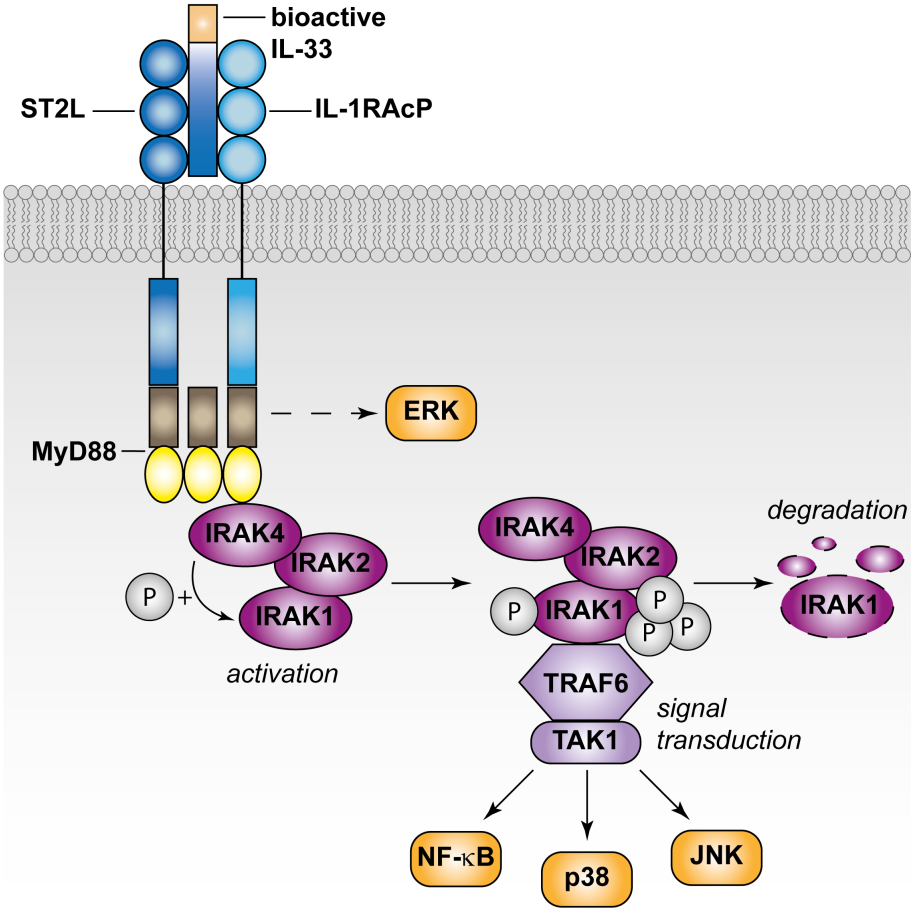


Figure 5: The IL-33/ ST2L/ IL-1RAcP signaling complex. Binding of IL-33 to ST2L mediates recruitment of IL-1 family co-receptor IL-1RAcP and subsequent dimerization of the intracellular TIR domains, a prerequisite for binding of the adaptor protein myeloid differentiation primary response 88 (MyD88). Interleukin 1-receptor associated-kinase (IRAK) 4, IRAK2 and IRAK1 bind to MyD88, and IRAK4 activates IRAK1 through initial phosphorylation. IRAK1 in the following hyperactivates itself by self-phosphorylation and allows recruitment of tumor necrosis factor receptor associated factor 6 (TRAF6) and transforming growth factor beta activated kinase 1 (TAK1). Signal transduction through TRAF6 and TAK1 downstream activates the signaling pathways for nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB), p38 and c-Jun-N-terminal kinase (JNK) activation. Degradation of IRAK1 terminates the signaling cascade (modified after Martin[86]).

2.3.3. Influence of IL-33 on T Cell Differentiation

Cytokines of the IL-1 family exert multiple functions in the regulation of immune and inflammatory responses. Among these, IL-33 seems to be the most promiscuous member of the IL-1 family. Different studies implicate that IL-33 exerts pathogenic effects in T_H2 mediated atopic diseases, but promotes wound healing, acts as a cardioprotective

cytokine and mediates neuronal protection in diseases of the central nervous system (CNS)[111]. The role of IL-33 in cancer immunity however remains to be elucidated, as IL-33 has been interpreted to either promote or counteract cancer progression. Peine, Marek and Löhning lately suggested that the promiscuity of IL-33 resulting in controversial interpretation on its function in disease is based on its property to fuel positive feedback loops enhancing different differentiation programs of T cell subsets[96]. For murine $CD8^+$ T cells, IL-33 was proven to synergize with the TCR signaling and IL-12 to promote enhanced cytotoxic effector functions and secretion of type-I cytokines[94,112]. This important property of IL-33 is beneficial for anti-viral immunity and might support anti-tumor responses[95,113]. In $CD4^+$ T_H1 cells, ST2L expression was reported to be dependent on transcription factor T-bet and activation of STAT4. Inversely, ST2 deficiency in T_H1 cells promoted reduced levels of T-bet and IFNγ[114]. These findings implicate a positive feedback loop, in which the IL-33 signaling enhances expression of T-bet and T-bet in turn supports expression of ST2L. Unsurprisingly, transcription factor binding studies revealed that T-bet and STAT4 bind to the *il1rl1*, the gene locus for ST2L[115,116].

The function of IL-33 as a promotor of GATA3-dependent T_H2 immunity was described early. IL-33 activates the p38/ MAPK pathway, which in consequence activates translocation of GATA3 through phosphorylation. Additionally, IL-33 promotes expression of STAT5, which together with GATA3 induces expression of ST2L[117]. GATA3 is a functional repressor of pro-inflammatory, T-bet dependent effector differentiation, and *in vivo* contributes to the dysfunction of tumor infiltrating $CD8^+$ T cells[118,119]. For this reason, it had been suggested that IL-33 is able to indirectly counteract T_H1 immunity by preferably inducing T_H2 immunity[120]. It has however been proven that IL-33 has a direct effect on $ST2L^+$ $Foxp3^+$ T_{reg} by enhancing their TGFβ-mediated differentiation, accumulation and maintenance in inflamed tissue[97]. In a murine model, the IL-33-induced phosphorylation of GATA3 led to recruitment of RNA polymerase II to the promotor of *Foxp3* and *Il1rl1*[97]. In consequence, expression of both T_{reg} hallmark FoxP3, as well as of ST2L was enhanced by IL-33.

This property would indeed be beneficial to counteract autoimmunity, but detrimental for anti-cancer immunity. Concurrently, the anti-inflammatory function of IL-33 was shown to counteract inflammation during cytomegaloviral infection, because IL-33 supported a

T_{reg} dependent suppression of liver damage[121]. IL-33 has further been linked to anti-inflammatory immunity in experimental colitis, where ST2L was preferentially expressed on colonic T_{reg}[97]. IL-33 is strongly expressed in white matter and plaque areas of patients suffering from RRMS, a disease mediated by exaggerated T_H1/ T_H17 immunity[83,122]. In a mouse model of MS, EAE, IL-33 administered after disease onset ameliorated disease progression. Concurrently, ST2 deficient mice developed enhanced symptoms[123]. Furthermore, Alvarez and colleagues confirmed that IL-33 conferred resistance of $CD4^+$ T_{reg} to acquiring an effector T_H17 phenotype. Remarkably, in the absence of ST2L, T_{reg} were prone to losing FoxP3 expression[124], supporting the hypothesis that the IL-33/ ST2L signaling might be involved in positive feedback loops to drive differentiation of T cells.

Interestingly, increasing evidence implicates that IL-33-mediated protection or inflammation is strongly dependent on the time point in the course of the disease. While administration of IL-33 at the onset of dextran sulfate sodium-induced colitis was proven to fuel inflammation, administration during remission contributed to recovery of homeostasis[125]. Based on those findings, it is tempting to speculate that IL-33 – in the context of homeostasis - in the first place strengthens FoxP3 dependent T_{reg} responses but is able to promote potent pro-inflammatory immunity in the case of inflammation. The function of IL-33 may be crucially driven by the presence of co-stimulatory cytokines determining the type of polarization, as well as the cell type on which ST2L is expressed.

III. Objectives

IL-33 is a promiscuous cytokine, whose role as a co-factor in inflammation and homeostasis is controversially discussed. IL-33 has been attributed anti-inflammatory properties in autoimmune diseases and chronic inflammation[97,121,124], while on the other hand, IL-33 was shown to drive the cytotoxic anti-viral activity of $CD8^+$ T cells [94,95,112]. Additionally, recent findings implicated that IL-33 is involved in the peripheral trafficking of immune cells – an essential process for the transmigration of autoreactive T cell subsets into tissue, as well as for the execution and termination of inflammatory immune responses by effector T cells and T_{reg}, respectively[41,126,127]. Still, conditions favoring either an anti- or pro-inflammatory function of IL-33 largely remain to be elucidated. Postulating a complex regulatory network polarizing the function of IL-33, the cytokine's diversity also offers opportunities for specific modification of the immune response. In this context, the autoimmune disorder MS represents an important clinical condition for the understanding of IL-33 function *in vivo*. IL-33 is released from necrotic MS plaques[83] and might play a central role in the activation of autoreactive $CD8^+$ T cells and their transmigration through the blood-brain-barrier. To understand the function of IL-33 in this disease, it was considered fundamental to first investigate if IL-33 is a locally or systemically active cytokine, secondly to determine the circumstances supporting IL-33-mediated $CD8^+$ T effector or T_{reg} functions and third, how IL-33 is involved in T cell migration. The thesis was divided in three major chapters and an additional chapter evaluating the role of IL-33 on T lymphocytes in the clinical setting of MS. The objectives of the chapters are described in detail below.

Part 1:

Circulating IL-33 detectable in blood has been suggested as an indicator for disease progression of MS[83] and is already used as a prognostic biomarker for chronic heart failure[81], although its systemic function is hardly described. Bioactivity of IL-33 is strictly

regulated in order to limit range and duration of IL-33 effects. It is thus doubtful that IL-33 exerts systemic functions, but likely rather locally regulates adaptive immunity prior its inactivation in blood. To analyze this hypothesis, bioactivity of IL-33 detected in blood was determined using a cell-based bioassay. Furthermore, the underlying mechanisms limiting bioactivity of IL-33 in serum were investigated.

Part 2:

It was postulated here that local IL-33 mediates activation and differentiation of T cell subsets by regulation of lineage-specifying transcription factors. mTOR, a central regulator of metabolism, T cell generation and differentiation, was described to be activated by IL-33[11,128] in T_H2 cells and is pharmacologically targeted for induction of immunosuppression[129]. The aim was to elucidate how the function of IL-33 in $CD8^+$ T cells is influenced by mTOR activity, TCR activation and the presence of T_H1 cytokine IL-12.

Part 3:

Transmigration of autoreactive $CD8^+$ T cells into e.g. the CNS in MS centrally contributes to disease progression. Arising evidence implicates that IL-33 is involved in processes regulating T cell homing to, retention in and egress from lymphoid organs and peripheral tissue[41,94,130,131]. It was here analyzed if IL-33 is involved in the CCR7-dependent migration of $CD8^+$ T cells and in which way IL-33 modulates transcriptional expression of *S1P1* and *S1P4* upon TCR-dependent and -independent stimulation.

Part 4:

Although highly controversially discussed, it is suggested that IL-33 ameliorates disease progression of MS by re-establishing T_{reg} function[123]. A pilot study here served to evaluate the potential of IL-33 as an object of interest for a large cohort patient study. It was asked whether ratios of suspected effector and regulatory $CD8^+$ T cell subsets expressing IL-33 receptor ST2L were disturbed. For this outlook, leukocytes from patients with relapsing-remitting MS treated with natalizumab, an inhibitor of CD8 transmigration, were analyzed.

IV. Material and Methods

4.1. Material

4.1.1. Cells

4.1.1.1.Reporter Cell Line HEK293-ST2L

HEK293-blue IL-33/ IL-1β (further named HEK293-ST2L) (Invivogen, Toulouse, France) is a stably transfected cell line expressing IL-1 receptor IL-1R1, IL-33 receptor ST2L and the shared co-receptor IL-1RAcP. The reporter gene for secreted alkaline phosphatase (SEAP) is located downstream of the IFN-β minimal promotor and was fused to five NF-κB and activator protein 1 (AP-1) binding sites, respectively. Treatment of the reporter cells with IL-33 or IL-1β results in the NF-κB or AP-1 dependent expression of SEAP. The cells were cultured with selective antibiotics Blasticidin, Hygromycin B Gold and Zeocin in order to maintain the transgenic expression of IL-1R1, ST2L and IL-1RAcP.

4.1.1.2. Primary Human Immune Cells

Peripheral blood mononuclear cells (PBMC) and serum samples from buffy coats were obtained from anonymous healthy donors of the DRK-Blutspendedienst Baden-Württemberg-Hessen (Institut für Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Frankfurt, Germany). All participants gave written informed consent prior blood sampling.

4.1.2. Laboratory Material

4.1.2.1. Interleukins and Stimuli

All Interleukins and stimuli (**Table 1**) were purchased at the highest purity and cell culture grade. Interleukins were delivered in a lyophilized state, reconstituted to a stock concentration of 10 µg/ml in PBS/ 0.1% BSA and stored at -80°C according to the manufacturer's recommendations.

Table 1: Interleukins and stimuli

Stimulus	Manufacturer
IL-1β	Peprotech (Hamburg, Germany)
IL-33	Peprotech (Hamburg, Germany)
IL-33 (C208S/C232S)	AdipoGen (San Diego, USA)
LPS	Sigma-Aldrich (St. Louis, USA)
Rapamycin	LC Laboratories (MA, USA)
sST2-His	Sino Biological (Beijing, China)

4.1.2.2. Antibodies

Antibodies were stored at 4°C and were used for treatment of primary immune cells, for flow cytometry analysis or for staining after western blotting (**Table 2**).

Table 2: Antibodies

Antigen	Clone	Fluorochrome	Manufacturer
CCR7	G043H7	APC	Biolegend (San Diego, USA)
CD107a	H4A3	PE	Biolegend (San Diego, USA)
CD2/3/28		-	Stemcell Technologies (Köln, Germany)
CD45RA	HI100	PE/Cy7	Biolegend (San Diego, USA)
CD45RO	UCHL1	APC	Biolegend (San Diego, USA)
CD69	FN60	PerCP	Biolegend (San Diego, USA)
CD8	RPA-T8	V450	BD Bioscience (Heidelberg, Germany)

Donkey anti-rabbit IgG		AlexaFluor488	Invitrogen (NY, USA)
Fc Block	-	-	BD Bioscience (Heidelberg, Germany)
Foxp3	259D	AlexaFluor647	Biolegend (San Diego, USA)
GATA-3	TWAJ	PE	Invitrogen (NY, USA)
Granzyme B	QA15A02	PE/Cy7	Biolegend (San Diego, USA)
hIL-33-IgG	19G8	-	Invivogen (Toulouse, France)
KLRG1	14C2A07	PE	Biolegend (San Diego, USA)
LC3B			Abcam (Cambridge, UK)
ST2L	B4E6	FITC	MD Biosciences (Zürich, Switzerland)
T-bet	eBio4B10	PE	Thermo Fisher (Waltham, USA)
P-Akt *(Ser473)*	D9E, rabbit mAb		Cell signaling technologies (MA, USA)
P-FoxO1 *(Ser256)*	rabbit pAb		Cell signaling technologies (MA, USA)
FoxO1	C29H4, rabbit mAb		Cell signaling technologies (MA, USA)
P-p70S6K *(Thr389)*	108D2, rabbit mAb		Cell signaling technologies (MA, USA)
β-Aktin	AC-15, mouse mAb		Sigma-Aldrich (St. Louis, USA)
hIL-33	Nessy-1, mouse mAb		Enzo life sciences
hIL-33	goat pAb		R&D Systems (Minneapolis, USA)

4.1.2.3.Kits

Kits were stored and used according to the manufacturer's protocols (**Table 3**).

Table 3: Kits

Kit	Manufacturer
DuoSet ELISA for human IL-33, sST2, IFNγ	R&D Systems (Minneapolis, USA)
EasySep^{TM} CD8^{+} T cell negative selection Kit	Stemcell Technologies (Köln, Germany)
FoxP3 Staining Buffer Set	Miltenyi Biotec (Bergisch Gladbach, Germany)
ISOLATE II RNA Micro Kit	Bioline (Memphis, USA)
Monolith NT Protein Labeling Kit RED-NHS	Nanotemper Technologies (München, Germany)
MTT assay	Roche Life Science (Basel, Switzerland)
peqGOLD Total RNA Kit	PEQLAB (Erlangen, Germany)
PureLink^{TM} HiPure Maxiprep Kit	Thermo Fisher (Waltham, USA)

4.1.2.4. TaqMan assays

5'FAM marked TaqMan probes specific for the target genes (**Table 4**) were stored at -20°C and used in a final concentration of 250 nM in a complete reaction mixture using iTaq Universal Probes Supermix (BioRad, Hercules, USA).

Table 4: Taqman assay probes for qRT-PCR

Gene	Catalog#	Manufacturer
BLIMP-1	Hs00153357_m1	Applied biosystems (Waltham, USA)
FOXP3	Hs00203958_m1	Applied biosystems (Waltham, USA)
GAPDH	Hs02758991_g1	Applied biosystems (Waltham, USA)
GATA3	Hs00231122_m1	Applied biosystems (Waltham, USA)
KLF2	Hs00360439_g1	Applied biosystems (Waltham, USA)
PIK3AP1	Hs00381030_m1	Applied biosystems (Waltham, USA)
RPL13A	Hs01578912_m1	Applied biosystems (Waltham, USA)
S1P1	Hs00173499_m1	Applied biosystems (Waltham, USA)

S1P3	Hs01019574_m1	Applied biosystems (Waltham, USA)
S1P4	Hs02330084_s1	Applied biosystems (Waltham, USA)
S1P5	Hs00924881_m1	Applied biosystems (Waltham, USA)
SOCS1	Hs00705164_s1	Applied biosystems (Waltham, USA)
SOCS3	Hs02330328_s1	Applied biosystems (Waltham, USA)
SST2	Hs01073297_m1	Applied biosystems (Waltham, USA)
ST2L	Hs00249389_m1	Applied biosystems (Waltham, USA)
TBX21	Hs00203436_m1	Applied biosystems (Waltham, USA)

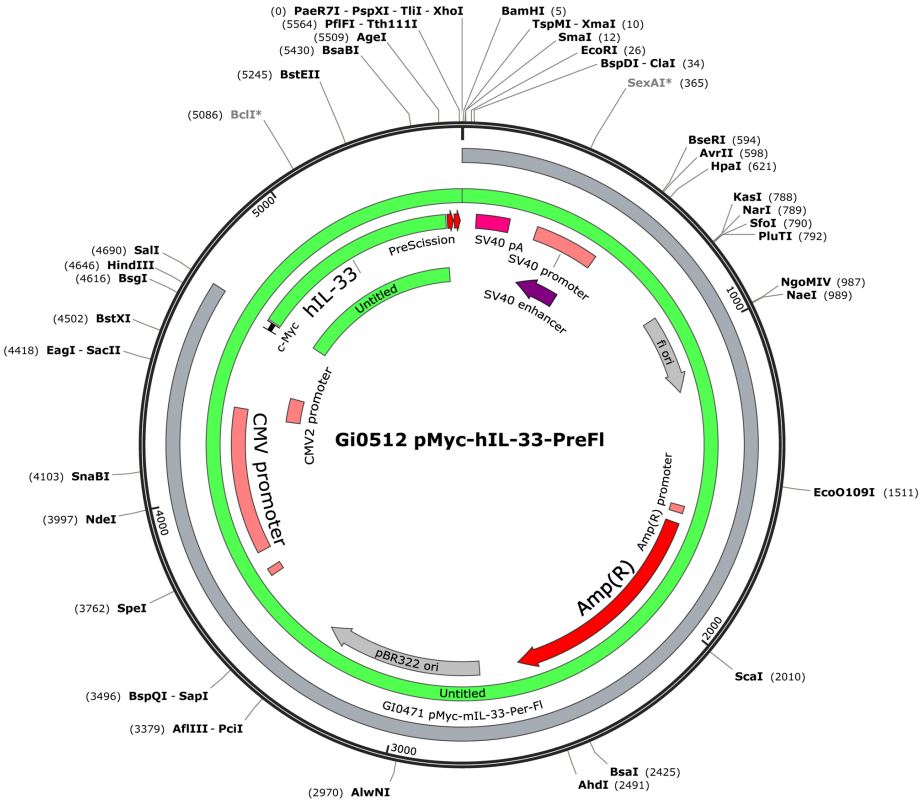
4.1.2.5. Plasmids

For the generation of recombinant human IL-33 isoforms, a plasmid encoding the full-length sequence for human IL-33 (amino acid 1-270) was kindly provided by Michael U. Martin (Justus-Liebig-University, Gießen, Germany) **(Figure 6A)**. The plasmid was stored at a concentration of 1 mg/ml at -20°C in *A. bidest*. Recombinant IL-33 proteins were generated according to full-length IL-33 consensus sequences (NM_033439). Sequences encoding truncated IL-33 isoforms (amino acid 95-270 or 179-270) were sub-cloned into bacterial expression plasmids before transformation of *Escherichia coli* **(Figure 6B, C)**. Expression and purification of the recombinant IL-33 isoforms in *E. coli* was performed by collaboration partners Andreas Ernst and Mateusz Putyrski (Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Frankfurt am Main) (**Table 5**).

Table 5: Properties of plasmids used for the generation of recombinant human IL-33 isoforms

Plasmid	**pMyc-hIL-33-PreFl**	**pET(+)-Ub-IL-33 aa95-270**	**pET(+)-Ub-IL-33 aa179-270**
Insert	Full length IL-33 (Met1-Thr270)	Truncated IL-33 (Ala95-Thr270)	Truncated IL-33 (Gly179-Thr270)
Total Vector size	5573 bp	6070 bp	5818 bp
Insert size	819 bp	531 bp	279 bp
Resistance	Amp	Km	Km
Promotor	Eukaryotic, CMV	Bacterial, T7	Bacterial, T7
Molecular weight	35 kDa	19.8 kDa	10.4 kDa

A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BamHI (5)
(5564) PflFI - Tth111I
TspMI - XmaI (10)
(5509) AgeI
SmaI (12)
(5430) BsaBI
EcoRI (26)
(5245) BstEII
BspDI - ClaI (34)
SexAI* (365)
(5086) BclI*
BseRI (594)
AvrII (598)
HpaI (621)
KasI (788)
NarI (789)
SfoI (790)
PluTI (792)
(4690) SalI
(4646) HindIII
(4616) BsgI
NgoMIV (987)
NaeI (989)
(4502) BstXI
(4418) EagI - SacII
EcoO109I (1511)
(4103) SnaBI
(3997) NdeI
(3762) SpeI
ScaI (2010)
(3496) BspQI - SapI
(3379) AflIII - PciI
BsaI (2425)
AhdI (2491)
(2970) AlwNI
PreScission
SV40 pA
SV40 promoter
SV40 enhancer
hIL-33
c-Myc
Untitled
f1 ori
CMV2 promoter
CMV promoter
Gi0512 pMyc-hIL-33-PreFl
Amp(R) promoter
Amp(R)
pBR322 ori
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GI0471 pMyc-mIL-33-Per-Fl
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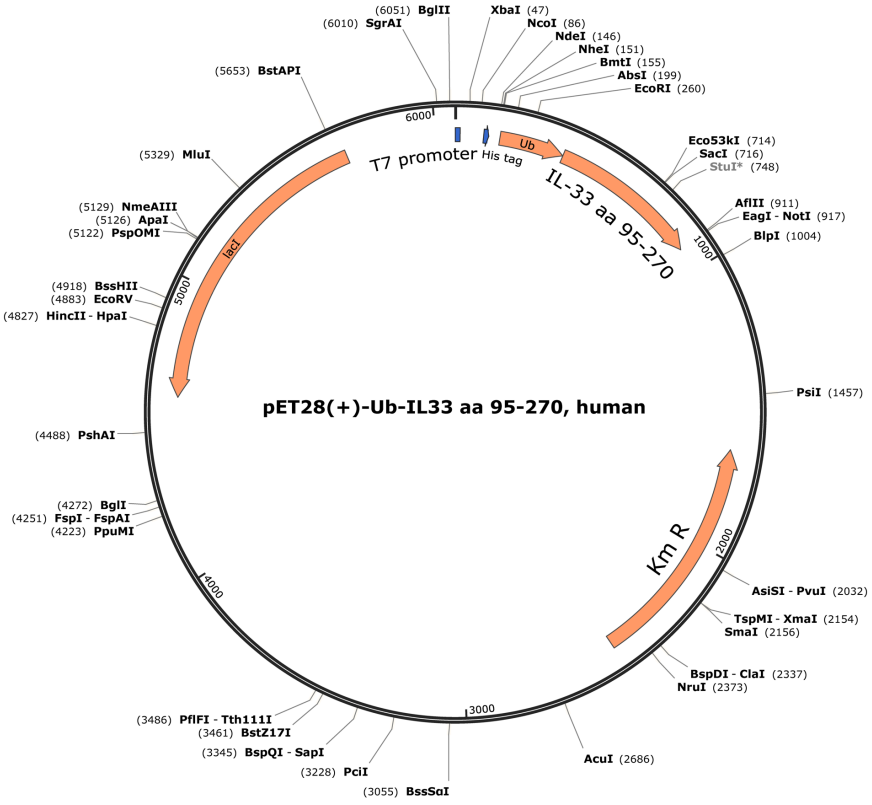
4000
2000
3000

B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XbaI (47)
(6010) SgrAI
NcoI (86)
NdeI (146)
NheI (151)
BmtI (155)
(5653) BstAPI
AbsI (199)
EcoRI (260)
Eco53kI (714)
SacI (716)
(5329) MluI
StuI* (748)
T7 promoter
His tag
Ub
IL-33 aa 95-270
AflII (911)
(5129) NmeAIII
EagI - NotI (917)
(5126) ApaI
BlpI (1004)
(5122) PspOMI
lacI
(4918) BssHII
(4883) EcoRV
(4827) HincII - HpaI
pET28(+)-Ub-IL33 aa 95-270, human
PsiI (1457)
(4488) PshAI
(4272) BglI
(4251) FspI - FspAI
(4223) PpuMI
Km R
AsiSI - PvuI (2032)
TspMI - XmaI (2154)
SmaI (2156)
BspDI - ClaI (2337)
NruI (2373)
(3486) PflFI - Tth111I
(3461) BstZ17I
(3345) BspQI - SapI
AcuI (2686)
(3228) PciI
(3055) BssSαI
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1000
5000
2000
4000
3000

C

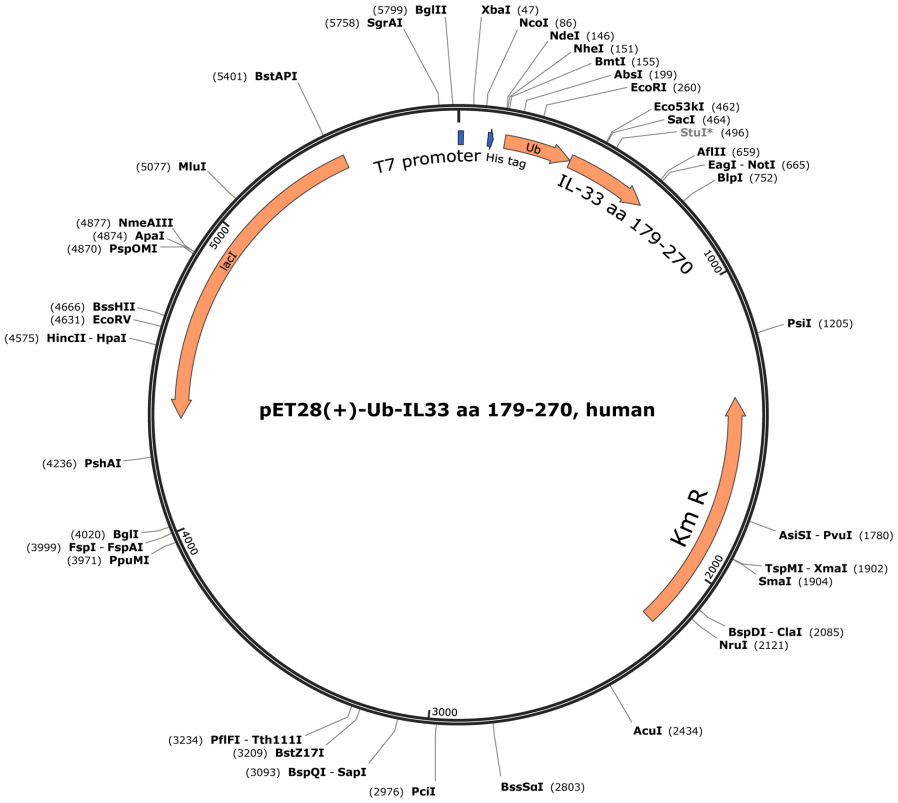

Figure 6: Plasmids for (A) eukaryotic expression of full-length human IL-33 and bacterial expression of (B) isoform IL-33$_{95\text{-}270}$ or (C) isoform IL-33$_{179\text{-}270}$.

4.1.2.6. Media and Buffer

Cell culture media were stored at 4°C and used for up to six weeks (**Table 6**). Buffers were stored at room temperature or at 4°C if required (**Table 7**).

Table 6: List of cell culture media used for cell lines and primary human immune cells.

Cells	Medium	Supplements	Manufacturer
HEK293	DMEM	10% FCS	Biochrom AG (Berlin, Germany)
HEK293-blue IL-33/ IL-1β (HEK293-ST2L)	DMEM	100 µg/ml streptomycin	Thermo Fisher (Waltham, USA)
		100 IU/ml penicillin	Thermo Fisher (Waltham, USA)
		30 µg/ml blasticidin	Invivogen (Toulouse, France)
		200 µg/ml hygromycin B gold	Invivogen (Toulouse, France)
		100 µg/ml zeocin	Invivogen (Toulouse, France)
		10% FCS	Biochrom AG (Berlin, Germany)
PBMC/ $CD8^+$ T cells	RPMI 1640 + Glutamax	50 mM β-ME	Thermo Fisher (Waltham, USA)
		1 mM sodium pyruvate	Thermo Fisher (Waltham, USA)
		100 µg/ml streptomycin	Thermo Fisher (Waltham, USA)
		100 IU/ml penicillin	Thermo Fisher (Waltham, USA)
		25 mM HEPES	Sigma-Aldrich (St. Louis, USA)
		1% human AB serum	Sigma-Aldrich (St. Louis, USA)

Table 7: List of buffers. Abbreviations: PBS = phosphate buffered saline, TBS = Tris-buffered saline.

Buffer	Supplements	Manufacturer
CD8 lysis buffer with supplements	50 mM Tris/ HCl pH 7.4	Applichem (Darmstadt, Germany)
	150 mM NaCl	Sigma-Aldrich (St. Louis, USA)
	10% Glycerol	Sigma-Aldrich (St. Louis, USA)
	2 mM EDTA	Applichem (Darmstadt, Germany)
	2 mM EGTA	Applichem (Darmstadt, Germany)
	2 mM DTT	Sigma-Aldrich (St. Louis, USA)

	1% Triton X 100	Carl Roth (Karlsruhe, Germany)
	10 mM β-glycerophosphate	Sigma-Aldrich (St. Louis, USA)
	1x Phosphatase-Inhibitor Cocktail 2	Sigma-Aldrich (St. Louis, USA)
EasySep™ buffer	1x PBS	Thermo Fisher (Waltham, USA)
	2% BSA	Sigma-Aldrich (St. Louis, USA)
	2 mM EDTA	Applichem (Darmstadt, Germany)
FACS buffer	1x PBS	Thermo Fisher (Waltham, USA)
	1% FCS	Biochrom AG (Berlin, Germany)
	0.1% NaN_3	Applichem (Darmstadt, Germany)
FACS fixation	1x PBS	Thermo Fisher (Waltham, USA)
	1% FCS	Biochrom AG (Berlin, Germany)
	0.1% NaN_3	Applichem (Darmstadt, Germany)
	1% paraformaldehyde	Applichem (Darmstadt, Germany)
4x Laemmli buffer	1.5 M Tris pH 6.8	Applichem (Darmstadt, Germany)
	50% Glycerol	Sigma-Aldrich (St. Louis, USA)
	20% SDS	Merck (Darmstadt, Germany)
	0.5% Bromophenol Blue	Applichem (Darmstadt, Germany)
	10% β-ME	Thermo Fisher (Waltham, USA)
10x PBS	4.3 mM Na_2HPO_4 x 7 H_2O	Sigma-Aldrich (St. Louis, USA)
	1.4 mM KH_2PO	Applichem (Darmstadt, Germany)
	137 mM NaCl	Sigma-Aldrich (St. Louis, USA)
	2.7 mM KCl, pH 7.3	Sigma-Aldrich (St. Louis, USA)
10x SDS buffer	35 mM SDS	Merck (Darmstadt, Germany)
	250 mM Tris	Applichem (Darmstadt, Germany)
	1.92 M Glycerol	Sigma-Aldrich (St. Louis, USA)
10x TBS	150 mM NaCl	Sigma-Aldrich (St. Louis, USA)
	10 mM Tris, pH 7.5	Applichem (Darmstadt, Germany)

TBST	1x TBS 0.05% Tween 20	 Applichem (Darmstadt, Germany)
Thermophoresis Buffer	1x PBS 0.1% Tween 20	Thermo Fisher (Waltham, USA) Applichem (Darmstadt, Germany)
Western Blot Buffer A	300 mM Tris 20% Methanol pH 10.4	Applichem (Darmstadt, Germany) Fisher Scientific (NH, USA)
Western Blot Buffer B	25 mM Tris 20% Methanol pH 10.4	Applichem (Darmstadt, Germany) Fisher Scientific (NH, USA)
Western Blot Buffer C	25 mM Tris 0.04 M ε-amino-n-caprone acid 20% Methanol pH 9.4	Applichem (Darmstadt, Germany) Sigma-Aldrich (St. Louis, USA) Fisher Scientific (NH, USA)

4.1.2.7. Chemicals

All chemicals were stored at room temperature or 4°C according to the manufacturer's instructions indicated in the respective safety data sheets **(Table 8)**.

Table 8: List of chemicals

Substance	Manufacturer
Acrylamide/ Bis-Acrylamide	Applichem (Darmstadt, Germany)
Dimethyl sulfoxide (DMSO)	Applichem (Darmstadt, Germany)
Ethanol	Sigma-Aldrich (St. Louis, USA)
Histopaque®-1077	Sigma-Aldrich (St. Louis, USA)
Monensin A	Sigma-Aldrich (St. Louis, USA)
Polyethylenimine (PEI)	Sigma-Aldrich (St. Louis, USA)
QUANTI-blue	Invivogen (Toulouse, France)
Sodium thiosulfate	Sigma-Aldrich (St. Louis, USA)
Silver nitrate	Riedel-de Haen (Seelze, Germany)
Trypan blue	Sigma-Aldrich (St. Louis, USA)
Trypsin/ EDTA	Thermo Fisher (Waltham, USA)

4.1.2.8. Equipment and Software

Equipment **(Table 9)** and software **(Table 10)** were provided by and used within the pharmazentrum Frankfurt.

Table 9: Laboratory equipment

Description	Equipment	Manufacturer
Centrifuges	Heraeus Rico 17	Heraeus Instruments (Hanau, Germany)
	Biofugefresco	Hermle Labortechnik (Wehingen, Germany)
	Z513K	Kern&Sohn GmbH (Balingen, Germany)
Cell counter	TC10	Bio-Rad (München, Germany)
Flow cytometer	FACS Canto II	BD Biosciences (Heidelberg, Germany
Microscope	Axio Vert 25	Zeiss (Jena, Germany)
Nanotemper	Monolith NT.115	Nanotemper Tech (München, Germany)
PCR thermocycler	Tpersonal	Biometra (Göttingen, Germany)

Photometer	SpektraMax M5e	Molecular Devices (Sunnyvale, USA)
qPCR system	7500 Fast System	Applied Biosystems (Darmstadt, Germany)
Spectrophotometer	ND-1000 UV/Vis	NanoDrop Technologies (Wilmington, USA)
Vortex mixer	Reax top	Heidolph (Schwabach, Germany)

Table 10: Software

Software	**Manufacturer**
7500 Fast System 2.0.6	Applied Biosystems (Darmstadt, Germany)
FACS Diva 6.1.3	BD Biosciences (Heidelberg, Germany)
FlowJo 7.6.5	Tree Star (Ashland, USA)
GraphPad Prism 6	GraphPad Software, Inc. (La Jolla, USA)
ImageJ	Wayne Rasband (Wisconsin, USA)
Mendeley 1.19.3	Elsevier (Amsterdam, Netherlands)
Microsoft Office 2016	Microsoft (Unterschleißheim, Germany)
MO.Affinity Analysis	NanoDrop Technologies (Wilmington, USA)
NanoDrop 3.1.0	NanoDrop Technologies (Wilmington, USA)
SnapGene Viewer	GSL Biotech, LLC (Chicago, USA)
Soft Max Pro 5	Molecular Devices (Sunnyvale, USA)

4.2. Methods

4.2.1. Cell culture

4.2.1.1. Cultivation of the HEK293-ST2L Reporter Cell Line

HEK293-blue IL-33/ IL-1β are referred to as HEK293-ST2L in the following. HEK293-ST2L were cultured in their respective media in a humidified incubator at 37°C and 5% CO_2. For subcultivation, HEK293-ST2L were passaged at 90% confluency. The monolayer was carefully washed with sterile PBS and harvested upon incubation with 5 ml of Trypsin/ EDTA for 5 minutes. The reaction was stopped by addition of 15 ml of cell culture medium containing FCS before centrifugation. The pelleted HEK293-ST2L cells were resuspended in full cell culture medium, seeded in a concentration of 2.5 x 10^5 cells/ ml and passaged 72h later by seeding out in a concentration of 1.0 x 10^5 cells/ml. The cells were then cultivated for 96h prior further subcultivation. In order to maintain expression of ST2L, IL-1R1 and IL-1RAcP, HEK293-ST2L were cultivated with the selection antibiotics blasticidin, hygromycin B gold and zeocin.

4.2.1.2. Determination of Unknown Concentrations of Bioactive IL-33

IL-33 bioactivity was assessed using HEK293-ST2L cells according to the manufacturer's protocol and as described in the doctoral thesis of Florian Ottenlinger[132]. 5x10^4 HEK293-ST2L cells were resuspended in 180 µl of medium and supplemented with 20 µl of sample containing IL-33. To minimize variations, the assay was performed in duplicates in a 96-well plate. The cells were stimulated for 22h at 37°C, 5% CO_2. The substrate for secreted alkaline phosphatase (SEAP) QUANTI-blue was prepared by reconstitution in *aqua bidest*. 40 µl of supernatant from treated HEK293-ST2L cells were transferred per well of a 96-well plate and 160 µl of QUANTI-blue were added bubble-free using a multichannel pipette. Activity of SEAP was indirectly determined by photometrical measurement of converted substrate at 635 nm every minute for 2h at 37°C.

Cell viability of the remaining cells was assessed by MTT assay in a remaining volume of 100 µl to detect cytotoxic effects occurring upon stimulation.

4.2.1.3. Long-term Storage of Cell Lines

For long-term storage in liquid nitrogen, cell lines were resuspended in 90% of FCS and 10% of DMSO. The cells were stored in a freezing container (Mr Frosty™, Thermo Fisher, Waltham, USA) for 24h and stored in the vapor phase above liquid nitrogen. For re-cultivation, the frozen cells were thawed using a 37°C water bath and rapidly transferred into FCS. The cells were resuspended in their respective cell culture medium after centrifugation (400 x g, 5 min.) and were cultivated for 7 days before experimentation. All cell lines were routinely tested for mycoplasma infection using VenorGeM Classic Kit (Minervera Biolabs, Berlin, Germany).

4.2.2. Cultivation of Primary Human Leukocytes

4.2.2.1. Ethical Approvement for the Use of Healthy PBMC

PBMC were isolated from buffy coats obtained from anonymous healthy donors (DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Frankfurt, Germany). All participants gave written informed consent prior blood sampling. According to the conventions of the institutional ethics committee of the Goethe University Hospital, Frankfurt, Germany and the local legislation, the need for a consent concerning the here presented experiments with samples obtained from healthy donors has been waived. In order to respect this convention, besides gender and age, no other data was collected.

4.2.2.2. Ethical Approvement for Patient Samples

Whole blood samples from RRMS patients receiving Natalizumab were obtained from the *Biomaterialbank* of the Center of Neurology and Neurosurgery (University Clinic Frankfurt/ Main, Germany). The *Biomaterialbank* received permission by the ethical board

with the ethical vote #110-11 granted on the 9th of May 2011. All patients received written and personal information before voluntarily signing a declaration of consent. The samples were pseudo-anonymized at the Center of Neurology and Neurosurgery according to the handbook of the *Biomaterialbank*. The use of the samples for further analysis was permitted by the ethical board with the ethic vote #429/14 on the 15th of January 2015 and an amendment on 7th of May 2016.

4.2.2.3. Inclusion and Exclusion Criteria for RRMS Samples

RRMS patient samples were chosen according to the inclusion and exclusion criteria listed in **Table 11**.

Table 11: Inclusion and exclusion criteria for the selection of RRMS patients.

Inclusion criteria	Exclusion criteria
• Diagnosed RRMS • Age: 18-60 years • Minimum of three months of natalizumab medication	• Acute infection • Atopic and allergic diseases • Tumor • Liver- or kidney insufficiency • Immunosuppressive treatment (mitoxantron, metotrexat, cyclophosphamide within the last year) • Other medical treatments • Pregnancy

4.2.2.4. Isolation of Human Primary Immune Cells from Buffy Coats

The steps for isolation of immune cells from buffy coats were performed at room temperature. 12 ml of blood were gently diluted with 20 ml of PBS. 12 ml of Histopaque were placed at the bottom of a 50 ml reaction tube and overlaid with the total volume of diluted blood without disturbing the surface plane. Density gradient cell separation was performed at 750 x g for 35 minutes with brakes off. The plasma phase was removed carefully, and the layer of mononuclear cells was collected avoiding aspiration of Histopaque. PBMC were washed at least twice at 400 x g for 10 minutes with PBS. After

the final wash, cells were resuspended in 20 ml of PBS to determine the cell number. PBMC were either cultivated in a cell density of $1x10^6$ cells/ml in a T75 cell culture flask or were directly used for isolation of $CD8^+$ T cells.

4.2.2.5. Isolation of $CD8^+$ T cells from PBMC

$CD8^+$ T cells were prepared from freshly isolated PBMC following the instructions for the usage of EasySep™ Human $CD8^+$ T Cell negative isolation kit. In brief, PBMC were resuspended in EasySep™ isolation buffer, transferred into a polystyrene tube (12 x 75 mm) and incubated with $CD8^+$ T cell isolation cocktail in order to eliminate all $CD3^-$ $CD8^+$ and $CD3^-CD8^-$ populations. The tube containing all cells was placed into the EasySep™ Magnet (Stemcell Technologies, Köln, Germany) for 10 minutes. The $CD8^+$ T cells were obtained by inverting the magnet and transfer of unbound cells into a new collection tube. The procedure was repeated in order to assure a high purity grade. The purity was determined by flow cytometry, in average representing at least 96%.

4.2.2.6. Stimulation of PBMC and $CD8^+$ T cells

$CD8^+$ T cells isolated from PBMC were seeded in a density of 0.5 x 10^6/ ml and left untreated or, if indicated, cultivated with human AB serum in T cell medium for 20h prior further analysis. For inhibition of mTOR, cells were treated with 100 nM of rapamycin in T cell medium containing human serum. In order to exclude the cytotoxic effects of DMSO used as a diluent for rapamycin, an appropriate control containing the final concentration of 0.1% DMSO during stimulation was included. $CD8^+$ T cells were cultivated under serum withdrawal (starvation) for 20h to induce expression of ST2L and were then stimulated with 20 ng/ml IL-33, 5 ng/ml IL-12 or both. For TCR activation, the cells were equally cultivated in serum-free medium for 20h before stimulation with IL-33 and/ or IL-12 and/ or 25 µl of T Cell Activator αCD3/CD28/CD2 (αCD). For Western Blot analysis, $CD8^+$ T cells were stimulated with αCD for 5h before lysis. PBMC were stimulated in serum-free T cell medium with 25 µl/ml of αCD beads or 1 µg/ml of LPS. All primary human immune cells were stimulated for a time period of 20h prior further analysis.

4.2.3. Molecular Biological Methods

4.2.3.1. Cell lysis and Protein Isolation

At least $2x10^6$ cells $CD8^+$ T cells per stimulation were used for generation of sufficient amounts of protein isolates. The cells were harvested through careful scraping, transferred to reaction tubes and centrifuged for 5 minutes at 600 x g. The pelleted $CD8^+$ T cells were then resuspended in 30 µl of complete CD8 lysis buffer. Nuclei were opened by six to eight freeze-thaw cycles in liquid nitrogen and on a heat-block preheated to 37°C. Nuclei and debris were pelleted by centrifugation at 13000 rpm for 20 min. at 4°C, the supernatants containing cytosolic proteins were stored at -20°C until further analyzes.

4.2.3.2. Polyacrylamide Gel Electrophoresis

Recombinantly generated proteins or proteins from cell lysates were separated according to their electrophoretic mobilities by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Acrylamide was used in a concentration of 7.5% or 12.5% for the separating gel and in a concentration of 5% for the stacking gel **(Table 12)**.

Table 12: Composition of a separating and stacking gel for SDS-PAGE.

Separating gel (12.5%)	
A. dest.	1.28 ml
1.5 M Tris/ HCl pH 8.8	1.38 ml
1% SDS	0.55 ml
30% Acrylamide/ 0.8% Bis-acrylamide	2.29 ml
TEMED	8 µl
40% Ammoniumperoxodisulfate (APS)	8 µl

Stacking gel	
A. dest	1.22 ml
1M Tris/ HCl pH 6.8	0.25 ml
1% SDS	0.2 ml
30% Acrylamide/ 0.8% Bis-acrylamide	0.33 ml
TEMED	8 µl
40% Ammoniumperoxodisulfate (APS)	8 µl

The samples were resuspended in Laemmli buffer supplemented with the reducing agent β-mercaptoethanol for cleavage of disulfide bridges. For disruption of secondary and tertiary structures, the samples were then heated to 95°C for 5 minutes. After cooling to room temperature, all samples were loaded onto the acrylamide gel. Migration of the proteins was achieved by application of voltage (120 V) for approximately 1.5h.

4.2.3.3. Silver Staining

The purity of recombinantly generated IL-33 proteins was assessed by silver staining upon protein separation by SDS-PAGE. The gel was transferred to a denaturing and precipitating fixative solution containing 10% acidic acid and 30% ethanol, in order to limit protein diffusion. After extensive washing in *A. dest.* (20-60 minutes), the gel was incubated in 0.02% sodium thiosulfate and washed twice for one minute in *A. dest*. The gel was then incubated in 0.1% silver nitrate solution for 30 minutes to allow silver ions to bind to negatively charged chains of the proteins. To gel was repeatedly washed in water and treated with development solution containing 0.04% formaldehyde and 2% Na_2CO_3. Finally, the gel was incubated in 1% acidic acid to stop the reduction of the silver ions to elemental silver by the development solution and was extensively washed in *A. dest*. For prolonged storage, the membrane was treated with Gel-Dry™ Drying Solution.

4.2.3.4. Western Blot

Proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane by electroblotting. The technique involves an electric current leading to the transfer of negatively charged proteins from the gel to a membrane. For semi-dry blotting, the membrane was placed between western blot filters humidified with buffers A, B and C **(Table 13)** and electroblotting was performed for 90 minutes and an electric current of 0.08-0.12 A.

Table 13: Organization of the apparatus for electroblotting of proteins

Top	**Cathode (-)**
	9 filters humidified in buffer C
	Acrylamide gel
	PVDF membrane
	3 filters humidified in buffer B
	6 filters humidified in buffer A
Bottom	**Anode (+)**

The membrane was then washed for 15 min. in 0.1% TBST and unspecific protein binding sites were blocked by incubation in 5% non-fat dry milk in TBST for PVDF or 5% BSA/ TBST for nitrocellulose membranes for 60 min. For specific identification of the target proteins, the washed membrane was incubated in the respective primary antibody diluted in 1% non-fat dry milk/ TBST or 5% BSA/ TBST at 4°C overnight. After the incubation period, the membrane was washed in TBST 3x 15 min. to wash off unbound primary antibodies. The membrane was then incubated with a secondary antibody linked to horseradish peroxidase diluted in 1% non-fat dry milk/ TBST or 5% BSA/ TBST at room temperature for 60 min. and washed 3x 15 min. in TBST. The ECL Western Blotting substrate of horseradish peroxidase, was prepared according to the manufacturer's instructions and added to the membrane for detection using a medical X-ray film.

4.2.3.5. Analysis of Cell Surface and Intracellular Markers by Flow Cytometry

Fluorescence activated cell sorting (FACS) was used to detect the physical characteristics of cell populations. As ideally one cell at a time flows through a capillary with a laser beam, information on the size (FSC, forward scatter) and granularity (SSC, sideward scatter) of the cell can be drawn. The constitutive or changes in the expression of intracellular or cell surface markers is analyzed using specific antibodies labeled with fluorochromes. The fluorochromes are excited by the laser beam, and light emitted in a band of wavelengths is detected. For flow cytometry analysis, the cells were first washed in FACS buffer. In order to avoid unspecific antibody binding, all cells were blocked for 15 min. at room temperature with FACS buffer containing human Fc Block (αCD16/ αCD32) in a dilution of 1:200. For detection of cell surface markers, the cells were stained in 100 µl of FACS buffer containing antibodies specific for the markers of interest for 30 minutes at 4°C in the dark. The cells were washed twice and either stored in fixation buffer until further analysis or prepared for intracellular staining. Intracellular staining was performed according to the manufacturer's recommendations for the FoxP3 Staining Buffer Set. In brief, the cell surface staining was fixed and the cells were permeabilized. Intracellular target proteins were then stained with specific antibodies for 30 min. at 4°C in the dark, were extensively washed in the following and stored in FACS fixation buffer at 4°C until measurement. CD107a also known as Lamp-1 (lysosomal-associated membrane protein 1) expressed on lymphocytes is a reliable indicator of degranulation, as it is translocated from the lysosome to the cell membrane during lysosomal fusion needed for release of cytotoxic granules. For the cytotoxicity assay involving measurement of cell surface CD107a, T cells were plated in 12-well plates and cultivated in serum-free medium for 20h. Following the cultivation period, the cells were left untreated or stimulated with 20 ng/ml IL-33 and/ or 5 ng/ml of IL-12 and/ or 25 µl/ml of αCD T cell activator. Antibodies directed against CD107a were added at the starting point of the treatment to detect CD107a translocation to the extracellular membrane. Re-internalization of CD107a by the Golgi network was blocked by addition of monensin A 60 minutes after beginning in a final concentration of 1 µM. The cells were stimulated for a total of 5h before additional staining of cell surface proteins for detection in flow cytometry.

4.2.3.6. RNA Isolation

Total RNA was isolated from PBMC using the peqGold Total RNA Kit, while RNA from $CD8^+$ T cells was extracted using the Isolate II RNA Isolation Kit for low amounts of RNA. RNA extraction was performed according to the manufacturers' instructions. The isolation is based on a reversible binding of RNA to silica columns and elution in 10-35 µl of RNAse-free water. To eliminate chromatin- or double- and single-stranded deoxyribonucleic acid (DNA), RNA extracted from $CD8^+$ T cells was additionally treated with DNAse.

4.2.3.7. Reverse Transcription of RNA for the Generation of cDNA

Concentrations of isolated RNA were determined using a spectrophotometer. For each sample, equal amounts of RNA (200 - 300 ng) were transcribed into cDNA using the High capacity cDNA RT kit for a reaction mixture (**Table 14**).

Table 14: Reaction mixture for the synthesis of cDNA

Component	**Volume per sample [µl]**
Aqua dest	4.2
10x Reverse Transcriptase Buffer	2
Random primers	2
dNTPs, 10 mM	0.8
Reverse Transcriptase	1
RNA	200 – 300 ng
Aqua dest	Ad to 20

The reverse transcriptase was performed with the following program in a thermocycler (**Table 15**).

Table 15: RT-program for the synthesis of cDNA.

Step	Temperature [°C]	Time [min]
1	25	10
2	37	120
3	85	5
4	4	On hold

4.2.3.8. Quantitative real-time PCR

In order to quantify the mRNA expression of different target genes, specific 5' carboxyfluorescein (FAM) marked probes were used. Quantitative real-time PCR (qRT-PCR) was performed in duplicates in 96-well plates (Biozym Scientific, Vienna, Austria) for every sample. The reaction was performed with 1 µl of cDNA and 9 µl of reaction mix. The composition of the reaction mixture is listed below **(Table 16)**. For detection, a 7500 Fast Realtime PCR System was used applying a defined program **(Table 17)**.

Table 16: Reaction mixture for qRT-PCR

Component	Volume per sample [µl]
Aqua dest	3.5
Precision FAST Mastermix	5
Taqman probe	0.5
cDNA	1

Table 17: qRT-PCR program

Step	Temperature [°C]	Time [min]	Cycles
1	94	2	1
2	94	1	27-33
3	60	1	
4	72	1	
5	72	10	1
6	4	On hold	1

The results were evaluated by the ΔCt method. The expression levels of the target genes were compared to the expression levels of the non-regulated housekeeping genes *GAPDH* and *RPL13A*. The relative gene expression was calculated as indicated in the following:

$$relative\ gene\ expression = 2^{-\Delta Ct}$$

4.2.4. Measurement of Protein-Protein Interactions by Microscale Thermophoresis

4.2.4.1. Microscale Thermophoresis

Microscale thermophoresis (MST) is a method for the analysis of biophysical interactions between proteins. The principle is based on the detection of temperature-induced changes in the fluorescence of a target protein, which is affected by the complexation by a binding partner. During the analysis, not only the change in fluorescence is detected, but also the directed movement of particles in a temperature gradient. The temperature gradient is generated by an infrared laser, which is directed on a capillary containing the probe. The binding affinity is obtained by a serial dilution of a ligand and a constant concentration of a fluorochrome-labeled binding molecule. Denoting the normalized fluorescence of the unbound molecule $F_{norm}(A)$, the normalized fluorescence of the bound complex $F_{norm}(AT)$ and the fraction of molecules bound to the target x, the changing fluorescence signal F_{norm} is calculated by:

$$F_{norm} = (1 - x)F_{norm}(A) + xF_{norm}(AT)$$

F_{norm} is plotted against the logarithm of the different concentrations of the serial dilutions, providing a sigmoidal curve. Fitting the sigmoidal curve to the nonlinear solution of the law of mass action, dissociation constant K_D is obtained, describing the strength of the binding affinity between the two partners[133,134].

4.2.4.2. Labeling and Purification of sST2 for MST

For interaction studies of IL-33 isoforms to sST2, recombinant sST2-His was labeled based on the instructions for the Monolith NT Protein Labeling Kit RED-NHS. The dye contains a reactive N-Hydroxy-Succinimidyl (NHS)-ester group that modifies primary amines, e.g. lysine residues, by covalent binding. sST2 was labeled in a concentration of 250 µg/ml, representing 6.85 µM, in a total volume of 100 µl.
The molarity was calculated by:

$$c(sST2)[Mol/l] = c(sST2)\ [mg/ml]\ /\ MW(sST2)\ [Da]$$

c = concentration
MW = molecular weight

For labeling of sST2, the dye was reconstituted in DMSO at a concentration three-fold of sST2 (20.55 µM) and a total volume of 100 µl. sST2 and the dye were co-incubated for 30 minutes at room temperature in the dark in a ratio of 1:1. Labeled sST2 protein was subsequently purified using purification columns and by elution of fractions by gravity. The quality control of the sST2 labeling was performed by measurement of the fractions' fluorescence in the thermophoresis instrument Monolith NT.115. The fractions used in the following experiments were selected depending on the fluorescence. The optimal fluorescence was between 200 and 1500 fluorescence counts measured at 20% of LED/ excitation power. Labeled sST2 was stored at -80°C.

4.2.4.3. Affinity Measurements of IL-33 Isoforms and sST2

The affinities of sST2 to IL-33 isoforms were determined by MST. Recombinant IL-$33_{112-270}$ was diluted in MST buffer in a serial dilution of 1.5:1, with a maximum concentration of 600 nM. IL-33_{95-270} and IL-$33_{179-270}$ were diluted 2:1 in a serial dilution, with a maximum concentration of 3037.5 nM. 10 µl of labeled sST2 fraction were added to 10 µl of ligand dilution. The samples were transferred into capillaries. Unspecific adsorption or aggregation were excluded by using optimized MST buffer. The samples were measured at an LED power of 20% to minimize the effects of bleaching. The K_D was calculated using the MO.Affinity Analysis software.

4.2.5. Statistics

All data are presented as mean ± SD (standard deviation). Appropriate statistical tests for multiple comparisons (RM one-way ANOVA and Friedman test for matched data, one-way ANOVA, Kruskal-Wallis for data not matched) or Wilcoxon matched-pairs signed rank test for matched t-test were used upon statistical testing of normal distribution with ns for $p > 0.05$, */# for $p \leq 0.05$, **/## for $p < 0.01$, ***/### for $p < 0.001$ and ****/#### for $p < 0.0001$. Asterisks (*) show comparisons as indicated, hashtags (#) to controls.

4.3. Copyright statement

Parts of the present work have been published in Frontiers in Immunology (Dreis *et al*., 2019. Front. Immunol. 10:1698. doi: 10.3389/fimmu.2019.01698). Based on a Creative Commons CC-BY license (Attribution 4.0 International), the copyright in the text of individual articles is not property of Frontiers, and its ownership is not affected by its submission to or publication in Frontiers (as of 25th May, 2018). All graphs and data of the publication are licensed to be shared and adapted for any purpose. The following figures are part of the publication and have been adapted for facilitated understanding: **Figure 10**, **Figure 11**, **Figure 14**, **Figure 15**, **Figure 16**, **Figure 17**, **Figure 18**, **Figure 19**, **Figure 20**, **Figure 21**.

V. Results

5.1. Bioactivity of IL-33 in Blood

Bioactivity of intra- and extracellular IL-33 is highly controlled in order to limit the range and duration of IL-33-induced inflammation. Although IL-33 has been suggested as a clinical prognostic marker detectable in blood samples and is secreted from necrotic plaques in MS[135,80,136, 83], so far little is known about bioactivity of IL-33 in blood or its systemic function. Based on the current knowledge, IL-33 bioactivity is controlled by proteolytic processing or oxidation resulting in conformational changes and disruption of the receptor binding sites within the IL-1 like cytokine domain[87,137,99]. In this chapter, bioactivity of IL-33 isoforms in blood, as well as the capacity of decoy receptor sST2 in blocking IL-33 bioactivity, have been analyzed to investigate if IL-33 exerts a systemic function.

5.1.1. Generation of Recombinant IL-33 Isoforms

Proteolytic processing of extracellular IL-33 *in vivo* produces isoforms with different biological activity[88,138]. Recombinant isoforms of IL-33 have been generated to characterize their biological activities in blood. Isoforms IL-33$_{95\text{-}270}$ and IL-33$_{179\text{-}270}$ were generated in a cooperative project with Mateusz Putyrski and Andreas Ernst (Department of Biochemistry II, University Clinic Frankfurt/ Main). For the generation of the isoforms, fragments encoding the aa sequences from positions 95 to 270 or from 179 to 270 within the full-length IL-33 molecule were sub-cloned from the eukaryotic plasmid pMyc-hIL-33-PreFl into bacterial vectors (**Figure 6**).

pMyc-hIL-33-PreFl was kindly provided by Prof. Michael U. Martin (Justus-Liebig University, Giessen, Germany).

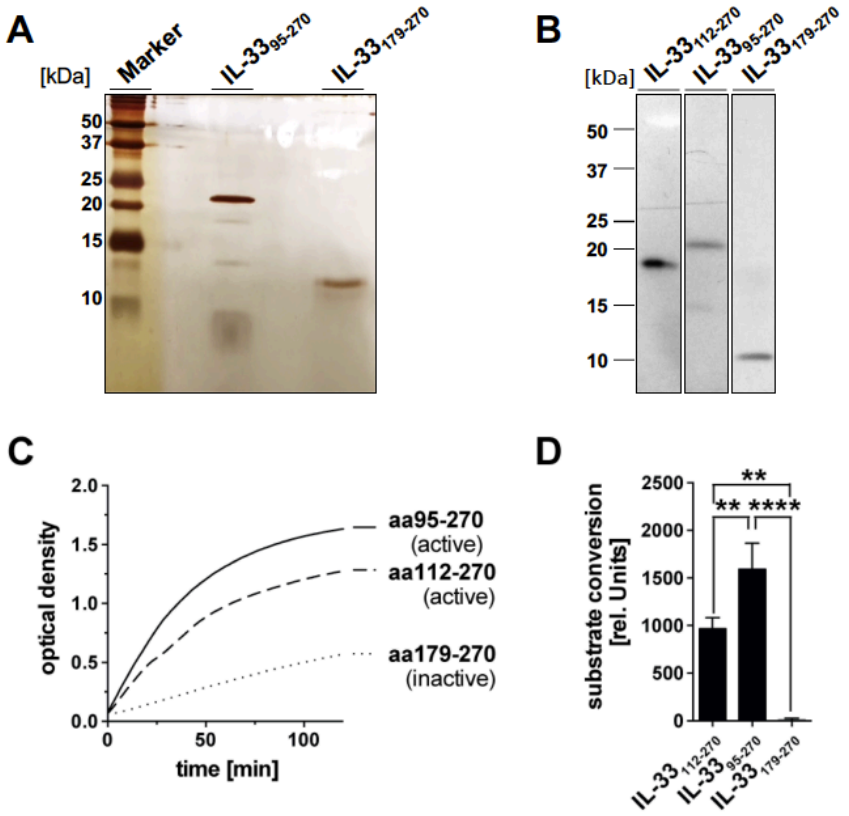

Figure 7: Quality control of recombinant IL-33$_{95\text{-}270}$ and IL-33$_{179\text{-}270}$. Purity of recombinant isoforms IL-33$_{95\text{-}270}$ (19.8 kDa) and IL-33$_{179\text{-}270}$ (10.4 kDa) was assessed by (A) silver staining of recombinant proteins loaded on an SDS-PAGE or (B) detection by a polyclonal, IL-33 specific antibody in Western Blot. Commercially available IL-33$_{112\text{-}270}$ (17.9 kDa) was used as a control. To assess the biological activity of the isoforms, HEK293-ST2L cells were treated with (C, D) 1 ng/ml of recombinant human IL-33$_{112\text{-}270}$ or self-generated IL-33$_{95\text{-}270}$ and IL-33$_{179\text{-}270}$ for 22h. (C) SEAP activity was assessed by photometrical measurement of the converted substrate QUANTI-Blue at 635 nm for 120 minutes. Representative data of n=3 independent experiments. (D) The substrate conversion in relative Units corresponds to the respective bioactivity of IL-33$_{112\text{-}270}$, IL-33$_{95\text{-}270}$ and IL-33$_{179\text{-}270}$. ** for $p < 0.01$, **** for $p < 0.0001$ using *one-way ANOVA with Tukey's posttest*. Data are shown as mean ± SD of n=3 independently performed experiments.

First, the generated isoforms IL-33$_{95\text{-}270}$ and IL-33$_{179\text{-}270}$ underwent quality controls involving assessment of purity, detection by specific anti-hIL-33 antibodies in western blot and bioactivity (**Figure 7**). Purity was determined by separation of the products containing either isoform IL-33$_{95\text{-}270}$ or IL-33$_{179\text{-}270}$ in an SDS-PAGE and silver staining (**Figure 7A**). In both cases, comparison of the molecular weight of the dominantly appearing bands to the protein standard corresponded to the expected molecular weights of 19.8 kDa for IL-33$_{95\text{-}270}$ or 10.2 kDa for IL-33$_{179\text{-}270}$.

The produced isoforms were analyzed by western blotting and compared to commercially available IL-$33_{112-270}$ to specifically identify the proteins as IL-33 isoforms (**Figure 7B**). Bioactivity of IL-33 was assessed using a commercially available reporter cell system as described in chapter 4.2.1.2. The positive control IL-$33_{112-270}$ and self-generated IL-33_{95-270}, but not IL-$33_{179-270}$, induced dose- and time-dependent increases in SEAP activity related to IL-33/ ST2L-stimulated NF-κB activity (**Figure 7C**). Calculation of the substrate conversion in relative units revealed significant differences between the bioactivities of all three isoforms, with IL-33_{95-270} most potently inducing a significantly higher SEAP activity compared to IL-$33_{112-270}$ ($p < 0.01$) and IL-$33_{179-270}$ ($p < 0.0001$). With this and as described in literature, IL-$33_{179-270}$ was proven to exhibit no biological activity (**Figure 7D**). After this initial experimental validation, the generated isoforms were used for the subsequent experiments on the detectability and regulation of IL-33 bioactivity in blood.

5.1.2. IL-33 Inactivated by Cleavage or Bound by sST2 is not Detectable by ELISA

Having previously confirmed that generated IL-33_{95-270} was bioactive and IL-$33_{179-270}$ was inactive, serial dilutions of both isoforms ranging from 1 ng/ml at the highest to 0.01 ng/ml at the lowest concentration were used for detection in an IL-33-specific commonly used ELISA (**Figure 8**). The aim of the experiment was to confirm the hypothesis that bioactive IL-33, but not IL-33 inactivated by proteases, is detectable by antibody-based techniques due to loss of epitopes occurring during proteolytic processing.

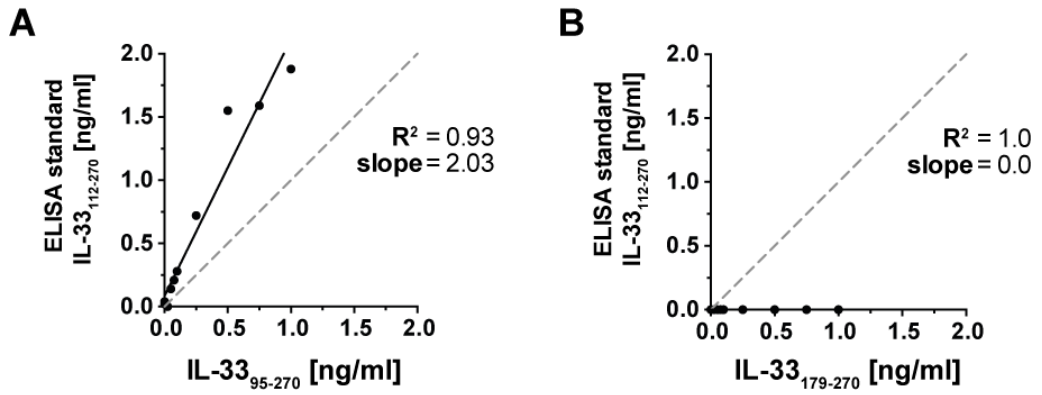

Figure 8: Bioactive IL-33$_{95-270}$, but not inactive IL-33$_{179-270}$, is detectable by ELISA. Total protein concentrations of self-generated, purified IL-33 isoforms were determined by Bradford protein assay. A serial dilution of either (A) IL-33$_{95-270}$ or (B) IL-33$_{179-270}$ ranging from 0.01 to 1 ng/ml was then applied in a commercially available ELISA and compared to the standard consisting of IL-33$_{112-270}$. The experiment has been performed n=1.

IL-33$_{95-270}$ was detectable by ELISA (**Figure 8A**). Although protein concentrations of the produced isoforms had been determined before using a Bradford assay, concentrations determined by Bradford and ELISA through comparison to IL-33$_{112-270}$ as a standard strongly differed. Accordingly, 1 ng/ml of IL-33$_{95-270}$ corresponded to approximately 2 ng/ml of IL-33$_{112-270}$. Moreover IL-33$_{179-270}$ was not detectable by ELISA (**Figure 8B**), disclosing possible limitations in the precise determination of total IL-33 concentrations in blood due to limited detectability of different isoforms. It was next asked if IL-33$_{112-270}$ complexed by sST2 was detectable by ELISA (**Figure 9**).

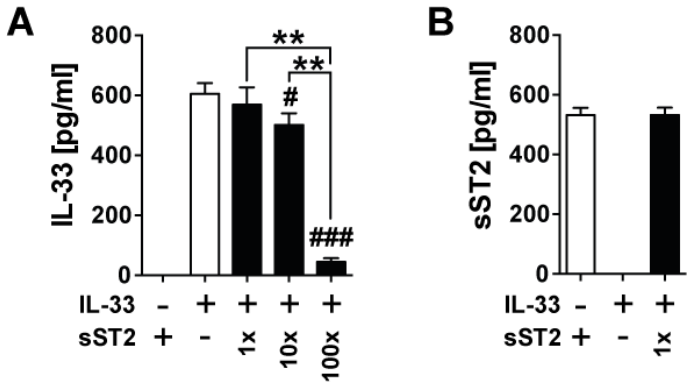

Figure 9: IL-33 complexed by sST2 is not detectable by ELISA. Recombinant IL-33$_{112-270}$ and 1x, 10x or 100x of sST2 were co-incubated for 30 minutes before application in a commercially available ELISA specific for either human (A) IL-33 or (B) sST2. Data are shown as mean ± SD of n=3 independently performed experiments. $^{\#}$ for $p \leq 0.05$, ** for $p < 0.01$, $^{\#\#\#}$ for $p < 0.001$ using *one-way ANOVA with Tukey's posttest*. Hashtags (#) indicate comparisons to IL-33 without sST2.

Addition of sST2 to IL-33 in equal molar concentrations failed to reduce the amount of IL-33 detected by ELISA compared to the control. However, addition of a tenfold molar excess of sST2 to 600 pg/ml of IL-33 resulted in a significant reduction ($p \leq 0.05$) and a hundredfold excess almost completely abrogated detection of IL-33 ($p < 0.0001$) (**Figure 9A**). In contrast, co-incubation of sST2 with IL-33 did not interfere in the detection of sST2 using an sST2-specific ELISA (**Figure 9B**). In summary, these findings implicate that IL-33 complexed by sST2 is not detectable, whereas sST2 bound to IL-33 remains detectable in ELISA.

5.1.3. IL-33 in Blood is Biologically Inactive

I*n vivo* bioactivity of IL-33 is highly limited. In contrast to ELISA, determination of the bioactivity of IL-33 in patient samples might represent a more conclusive tool for understanding the function of IL-33 in disease. Exemplary, IL-33 concentrations of 30 blood samples were first assessed by ELISA. Subsequently, HEK293-ST2L reporter cells were used for detection of bioactive IL-33 in the same samples (**Figure 10**).

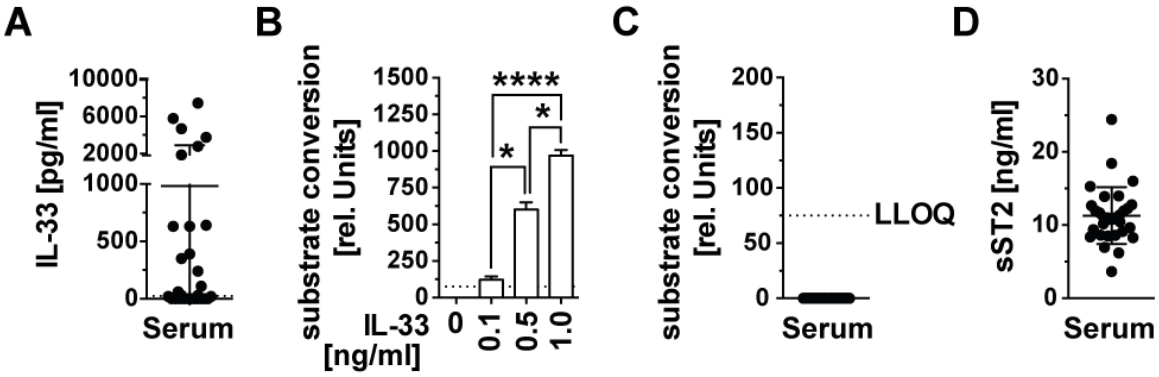

Figure 10: IL-33 detected in serum is not biologically active. (A) Determined IL-33 concentrations in serum samples of 30 healthy blood donors by ELISA. (B) Validation of the HEK293-ST2L reporter system with known concentrations of recombinant IL-$33_{112\text{-}270}$ before (C) measurement of bioactive IL-33 in serum. 1 relative (rel.) unit is equal to 1 pg/ml. (D) Soluble ST2 (sST2) concentrations were determined in the same serum samples by ELISA. Data are shown as mean ± SD of n = 30 serum samples for (A, C, D) or n=3 independently performed experiments with recombinant IL-33 for (B). * for $p \leq 0.05$, **** for $p < 0.0001$ using *RM one-way ANOVA with Tukey's posttest*. LLOQ = lowest level of quantification.

IL-33 concentrations of sera from 30 blood donors (mean age 56.4 years, range 28-72) were assessed by ELISA (**Figure 10A**). IL-33 was detectable in a total of 18 samples and concentrations ranged from 23 to 64 pg/ml in twelve and 1870 to 7440 pg/ml in six samples. In twelve samples, no IL-33 was detected (lowest limit of quantification 23 pg/ml). The HEK293-ST2L reporter cell system was validated using known concentrations of recombinant IL-$33_{112\text{-}270}$ (**Figure 10B**). IL-$33_{112\text{-}270}$ dose-dependently induced NF-κB activation indirectly determined by SEAP-dependent substrate conversion indicated in relative (rel.) Units. 100 pg/ml IL-33 induced 121.7 ± 23.2 rel. Units, 500 pg/ml 600.9 ± 49.1 and 1000 pg/ml 969.4 ± 37.86 rel. Units. After validation of the reporter system, the same serum samples used for ELISA were then applied to stimulate HEK293-ST2L reporter cells to investigate whether IL-33 detected by ELISA was bioactive (**Figure 10C**). IL-33 detected by ELISA was biologically inactive. Moreover, high concentrations of sST2 (11.27 ± 3.9 ng/ml) were detected in the samples (**Figure 10D**), but as described previously IL-33 complexed by sST2 is not detectable by ELISA. The experiment was performed in collaboration with Florian Ottenlinger and is fundamental for the ensuing experiments checking for the biological activity of IL-33 in blood[132].

Herein it was suggested that IL-33 is detectable by ELISA if the IL-1 receptor like domain is accessible, which was the case for isoforms that were not bound by sST2 or - at least in the above presented experiments – were not shorter than IL-$33_{95\text{-}270}$. However, high concentrations of IL-33 determined by ELISA in serum appeared to consist of biologically inactive IL-33 isoforms. For this reason, it was investigated if bioactivity of IL-33 in blood was reduced due to proteolytic processing or oxidation. As HEK293-ST2L also express membrane-bound receptor IL-1R1, IL-1β represented a suitable control to identify specific effects of serum on the regulation of IL-33 bioactivity (**Figure 11**).

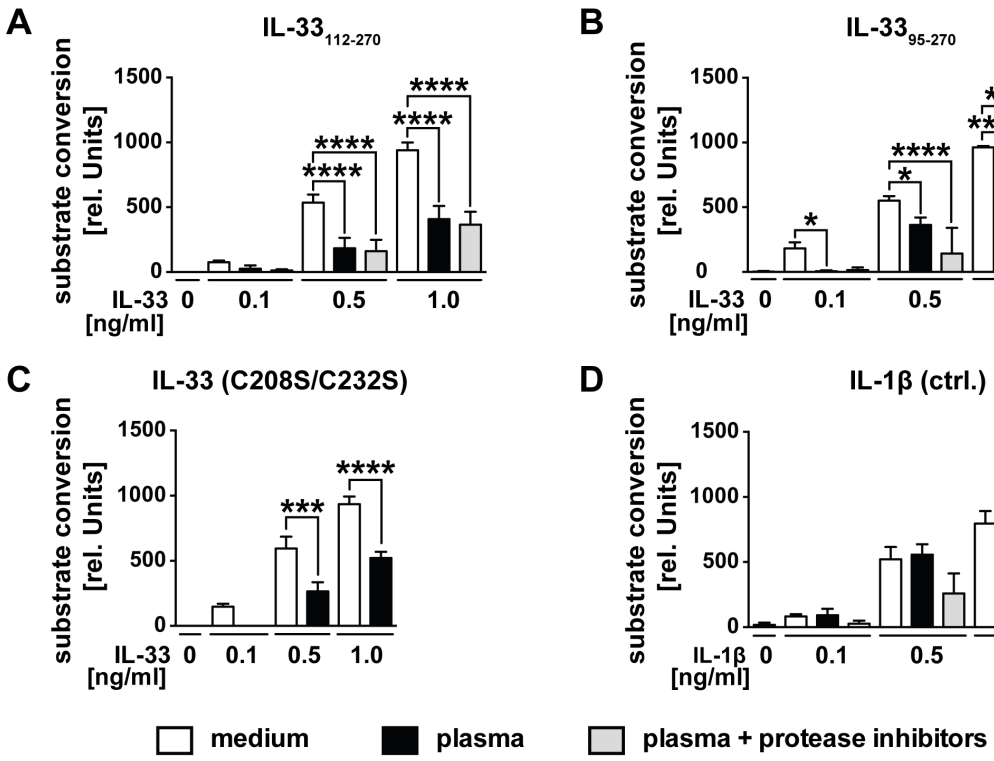

Figure 11: IL-33 bioactivity is reduced in blood. HEK293-ST2L reporter cells were either cultured in medium (control) or in human plasma obtained from healthy blood donors. The HEK293-ST2L cells were stimulated with 0.1, 0.5 or 1.0 ng/ml of (A) IL-$33_{112-270}$, (B) IL-33_{95-270}, (C) oxidation resistant mutant C208S/C232S or (D) IL-1β as control. Human plasma was added natively or supplemented with protease inhibitors. Viability of cells was examined by MTT viability assay, revealing no cytotoxic effects of plasma samples (data not shown). (A-D) Data are presented as mean ± SD of n = 3-10 different plasma samples with at least n=3 independently performed experiments. * for $p \leq 0.05$, *** for $p < 0.001$ and **** for $p < 0.0001$ using *one-way ANOVA with Tukey's posttest*.

HEK293-ST2L cells were stimulated with 0.1, 0.5 or 1 ng/ml of the bioactive isoforms IL-$33_{112-270}$ (**Figure 11A**) or IL-33_{95-270} (**Figure 11B**). To investigate if blood generally affects bioactivity of IL-33, stimulated HEK293-ST2L cells were co-incubated with human plasma. Compared to medium controls, SEAP-dependent substrate conversion was significantly reduced upon addition of plasma when treated with 0.5 ng/ml ($p < 0.0001$) and 1.0 ng/ml of IL-$33_{112-270}$ ($p < 0.0001$) or 0.1 ng/ml ($p \leq 0.05$), 0.5 ng/ml ($p \leq 0.05$) and 1 ng/ml of IL-33_{95-270} ($p < 0.0001$). Addition of inhibitors specific for serine-, amino-, cysteine-, acid- and aspartic proteases failed to reverse the observed downregulation of bioactivity. Oxidation abrogates IL-33 bioactivity through formation of disulfide bridges within the ST2L binding site[99]. Therefore, IL-33 mutant C208S/C232S resistant to oxidation was used to stimulate HEK293-ST2L cells (**Figure 11C**). Addition of plasma

led to a similar reduction in the bioactivity of mutated IL-33 ($p < 0.001$ for 0.5 ng/ml and $p < 0.0001$ for 1.0 ng/ml for comparisons to medium controls) observed for the non-mutated IL-33 isoforms, therefore excluding oxidation as a cause for reduced bioactivity. Importantly, plasma did not impede bioactivity of IL-1β, implicating a specific modulation of IL-33 bioactivity (**Figure 11D**). To exclude cytotoxic effects of plasma on the HEK293-ST2L cells, cell viability was determined through assessment of the cellular metabolic activity using an MTT assay. Viability of HEK293-ST2L cells was not affected by incubation with plasma and/ or stimulation with IL-33 or IL-1β compared to medium controls (*data not shown*).

5.1.4. Limited Blockade of IL-33 by Decoy Receptor sST2

sST2 is constitutively and abundantly expressed in sera of healthy individuals. The soluble isoform of membrane-bound IL-33 receptor ST2L, namely sST2, is a suggested decoy receptor of IL-33 circulating in blood[139,140], although an additional function of sST2 as a reservoir for IL-33 has been postulated[103]. It was questioned if high concentrations of circulating sST2 were able to efficiently block exogenously added IL-33, which would explain the above observed decrease in bioactivity. First, binding affinities of recombinant sST2 to bioactive IL-$33_{112\text{-}270}$ and IL-$33_{95\text{-}270}$, as well as to IL-$33_{179\text{-}270}$ were determined by MicroScale Thermophoresis (**Figure 12**).

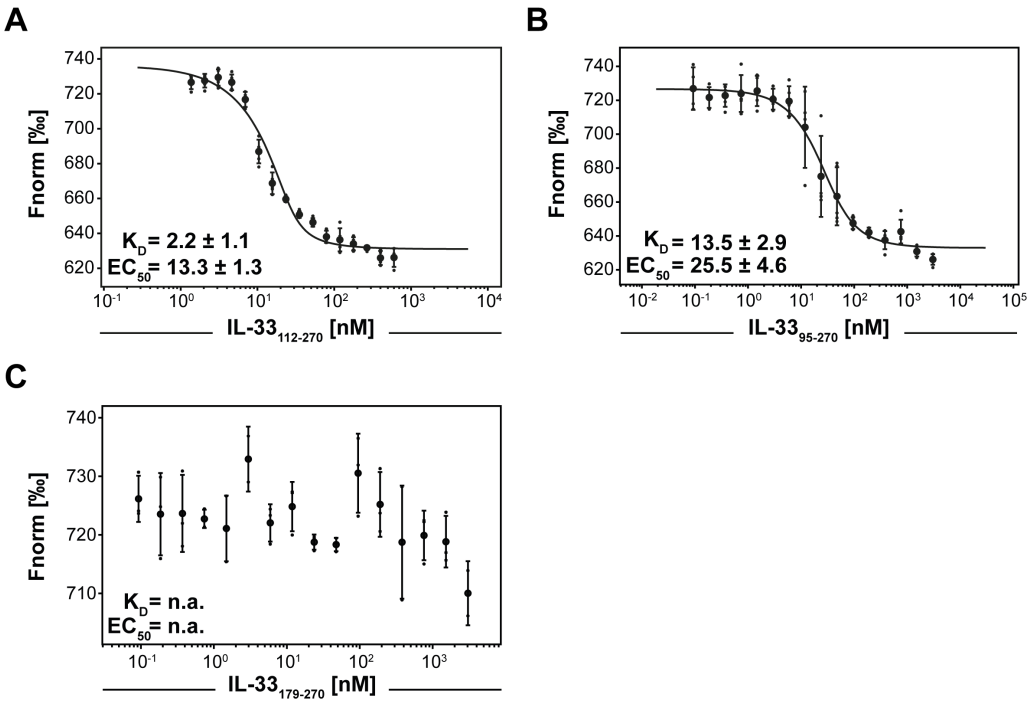

Figure 12: Affinity measurements of IL-33 isoforms with sST2. Affinities of (A) IL-$33_{112-270}$, (B) IL-33_{95-270} and (C) IL-$33_{179-270}$ (negative control) to sST2 were determined by MicroScale Thermophoresis to assess dissociation constant K_D and the half maximal effective concentration (EC_{50}). Data are shown as mean ± SD of n=3-5 independently performed experiments.

Affinity measurements disclosed high affinity interactions of sST2 with the bioactive isoforms of IL-33, namely IL-$33_{112-270}$ and IL-33_{95-270}. Calculations of the dissociation constant revealed a K_D of 2.2 ± 1.1 nM for the interaction of sST2 to IL-$33_{112-270}$ (**Figure 12A**) and a K_D of 13.5 ± 2.9 nM for the interaction of sST2 to IL-33_{95-270} (**Figure 12B**). In line with the expectations, sST2 did not bind to the N-terminally truncated isoform IL-$33_{179-270}$ (**Figure 12C**).

IL-1RAcP contributes to the stabilization of the complex leading to high affinity binding of IL-33 to ST2L[141]. As a naturally occurring soluble isoform of IL-1RAcP is to date not known, the following experiments asked to what extent IL-33 bioactivity is blocked by sST2 in the presence of ST2L and IL-1RAcP. Therefore, a competitive assay using the HEK293-ST2L reporter cells was conducted. Beforehand, molarities of recombinant IL-$33_{112-270}$, IL-33_{95-270}, sST2 and supposedly neutralizing anti-IL-33-immunoglobulin G (IgG) antibodies (nAb) were calculated based on their respective molecular weights **(Table 18)**.

Table 18: Molecular weights and molarities of IL-33 isoforms and antagonists.

Protein	Molecular weight [kDa]	pM at 1 ng/ml
IL-$33_{112\text{-}270}$	17.9	55.9
IL-$33_{95\text{-}270}$	19.8	50.4
sST2	36.5	27.4
Anti-IL-33-IgG	150	6.7

For co-incubation experiments with IL-$33_{112\text{-}270}$ and IL-$33_{95\text{-}270}$, binding partners sST2 and nAb were in molar ratios of 1:1, 1:10 and 1:100 (ratio of IL-33 : sST2/ nAb). For example, for a molar ratio of 1:10, sST2 was added in a concentration of 15.9 ng/ml to 0.5 ng/ml of IL-$33_{112\text{-}270}$ (**Table 19**).

Table 19: Concentrations sST2 and anti-IL-33-IgG (nAb) used for co-incubation experiments with IL-33 isoforms in molar ratios of 1:1, 1:10 and 1:100.

Antagonist	Ratio	Concentration [ng/ml] for 0.5 ng/ml of IL-$33_{112\text{-}270}$	Concentration [ng/ml] for 0.5 ng/ml of IL-$33_{95\text{-}270}$
sST2	1:1 (1x)	1.6	1.4
	1:10 (10x)	15.9	14.4
	1:100 (100x)	159.2	143.7
nAb	1:1 (1x)	4.2	3.8
	1:10 (10x)	41.9	37.8
	1:100 (100x)	419.0	378.2

HEK293-ST2L cells were treated for 22h with IL-$33_{95\text{-}270}$ or IL-$33_{112\text{-}270}$ together with either sST2 or nAb using concentrations for the molar ratios 1:1, 1:10 and 1:100 before measurement of IL-33 bioactivity (**Figure 13**).

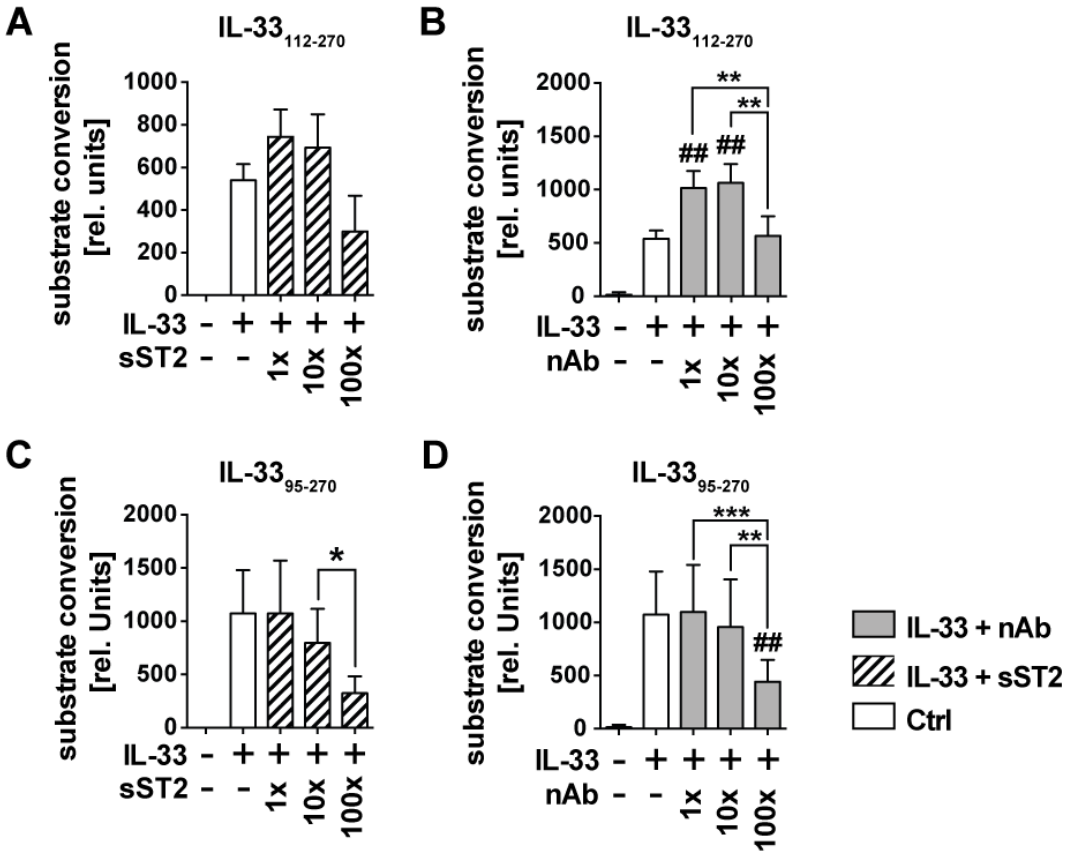

Figure 13: Limited blockade of IL-33 bioactivity by sST2 and neutralizing antibodies. The blocking capacity of 1x, 10x and 100x excess of sST2 (shaded bars) and neutralizing antibodies (nAb, grey bars) towards recombinant (A, B) IL-$33_{112\text{-}270}$ and (C, D) IL-$33_{95\text{-}270}$ was assessed within the HEK293-ST2L bioactivity assay. Bioactivity of IL-33 within the HEK293-ST2L reporter system was indirectly determined by a dose-dependent increase of a converted substrate by a secreted alkaline phosphatase (SEAP). Data are shown as mean ± SD of n = 5 independently performed experiments. * for $p \leq 0.05$ $^{\#\#}$/ ** for $p < 0.01$, *** for $p < 0.001$ using *RM one-way ANOVA with Tukey's posttest*. rel. = relative. Hashtags (#) indicate comparisons to the IL-33 treated control, asterisks (*) indicate comparisons as indicated.

In the competitive assay sST2 failed to significantly downregulate IL-$33_{112\text{-}270}$ bioactivity upon co-incubation in equal molar amounts, but also in tenfold or hundredfold molar excess of sST2 (**Figure 13A**). Addition of nAb in equal or tenfold excess significantly increased bioactivity of IL-33 ($p < 0.01$ each). This effect was not observed when the HEK293-ST2L cells were treated with IL-$33_{112\text{-}270}$ and a hundredfold surplus of nAb (**Figure 13B**). Bioactivity of IL-$33_{95\text{-}270}$ was weakly but significantly reduced if sST2 was added in a hundredfold excess ($p \leq 0.05$ compared to tenfold sST2) (**Figure 13C**). In contrast to IL-$33_{112\text{-}270}$, bioactivity of IL-$33_{95\text{-}270}$ was not increased by co-incubation with nAb, but nAb reduced bioactivity if added in a hundredfold of surplus ($p < 0.01$ compared

to the untreated control) (**Figure 13D**).

In these experiments, sST2 exerted a low blocking capacity in the presence of ST2L and IL-1RAcP on HEK293-ST2L reporter cells, but in the absence of the signaling receptor, sST2 selectively bound to bioactive IL-33 with high affinity. In consequence, high concentrations of sST2, as observed in the sera of healthy volunteers (**Figure 10D**), would be required to noteworthy block IL-33 bioactivity in the presence of $ST2L^+$ circulating immune cells in blood. It was further asked if sST2 expression is an inducible mechanism in response to pro-inflammatory stimulation, which would point to the mechanism of a negative or positive feedback loop, depending on the function of sST2 as only a decoy receptor or a reservoir for IL-33. For this purpose, transcription of *ST2L* and *SST2* mRNA in response to stimulation of the adaptive (αCD) or innate immunity (LPS) in total PBMC was assessed (**Figure 14**).

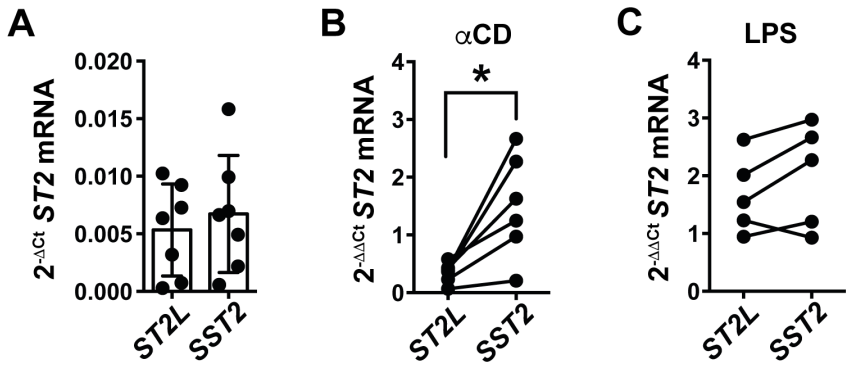

Figure 14: TCR activation of human PBMC induces transcriptional expression of *SST2*. qRT-PCR of human PBMC isolated and treated for 20h with αCD2/3/28 (αCD) or with LPS. Total RNA was isolated and (A) basal *ST2L* and *SST2* mRNA expression was determined in untreated cells by comparison to the housekeeping genes *GAPDH* and *RPL13A* ($2^{-\Delta Ct}$). Relative expression of *ST2L* mRNA and *SST2* mRNA compared to untreated control after stimulation with (B) αCD or (C) LPS was determined by comparison of $2^{-\Delta Ct}$ to the untreated control ($2^{-\Delta\Delta Ct}$). Data are shown as mean ± SD of (A) n=7, (B) n=6 and (C) n=5 different human RNA samples with at least n=3 independently performed experiments. * for $p \leq 0.05$ using *paired t-test.*

Untreated PBMC constitutively expressed basal, equal levels of both *ST2L* and *SST2* mRNA (**Figure 14A**). Stimulation with αCD for specific activation of T cells increased expression of *SST2* mRNA, which significantly exceeded the induction of *ST2L* expression ($p \leq 0.05$) (**Figure 14B**). In contrast, treatment of PBMC with LPS increased the overall expression of *ST2L* and *SST2* mRNA, but resulted in no significant differences in the

expression levels of *ST2L* and *SST2* mRNA (**Figure 14C**). This experiment showed that upon activation of the adaptive immune system, *SST2* mRNA expression was strongly induced and exceeding the mRNA expression levels of *ST2L*.

In the end, the cause for the downregulation of bioactivity of exogenous IL-33 was not clearly determined, but the experiments emphasized that IL-33 bioactivity in general is highly regulated in blood. The arising suggestion using circulating IL-33 as a prognostic biomarker has to be re-considered, as the data strongly implicate that IL-33 most likely exerts no systemic functions.

5.2. Differentiation of CD8$^+$ T cells during Nutrient Deprivation

Although the role of IL-33 in T_H2 immunity and atopic diseases has been widely proven, evidence arising in latest publications implicate a function of IL-33 in the modulation of T_H1 immunity and effector cell cytotoxicity. Controversially, IL-33 was described to induce differentiation of T_{reg}[97], a functionally important T cell subset required for limiting the activity of autoreactive cytotoxic T cells, for example. T cell programming, differentiation and memory generation is strictly dependent on T cell metabolism, which is in turn orchestrated by mTOR, a sensor for nutrient deprivation[52,11]. This chapter focuses on the complex interplay between nutrient deprivation and the IL-33-dependent differentiation of CD8$^+$ T cells.

5.2.1. Starvation Mediates Expression of ST2L and Emergence of CD8^{low} and CD8^{high} T cells

To date, the role of IL-33 in the modulation of the CD8-dependent cytotoxicity remains unclear and conflictive findings either report regulatory or inflammatory properties of IL-33[94,97,142,143]. Based on the findings presented in the previous chapter implicating that IL-33 is inactivated in blood, it was assumed that the cytokine mainly exerts its effects on immune cells nearby its source and most likely, directly in tissue. In a hypothetical setting, tumor infiltrating cytotoxic CD8$^+$ T cells would encounter immunosuppressive mediators, gradients of nutrients and oxygen, but also bioactive IL-33 released from necrotic tumor cells. In this context, nutrient deprivation leading to alterations in the regulation of the immunometabolism constitutes an important factor influencing fitness and differentiation of CD8$^+$ T cells. Furthermore, treatment with rapamycin is strongly associated to an enhanced generation of T_{reg}[53,144]. It was therefore asked whether the promiscuity of IL-33, which has been described to be involved in the generation of T_H1, T_H2 and opposing T_{reg} subsets, is rendered possible through mutual modulation between mTOR and IL-33. To begin with, it was investigated if nutrient deprivation affects the responsiveness of human CD8$^+$ T cells to IL-33 (**Figure 15**).

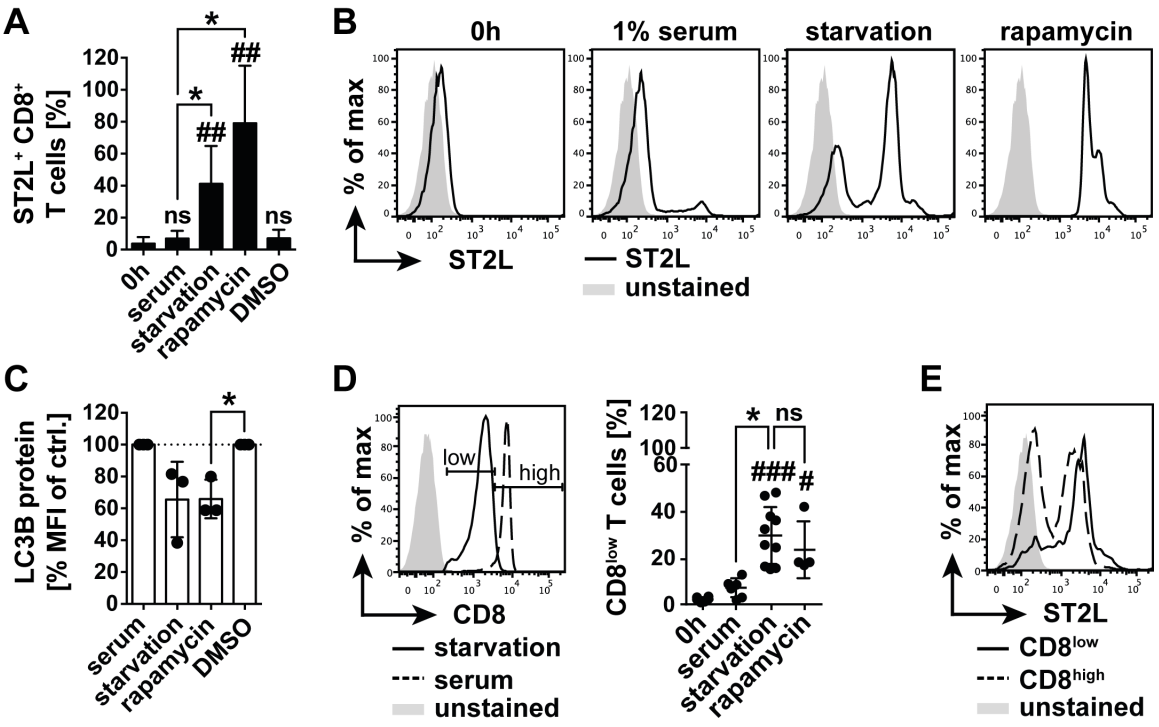

Figure 15: Serum withdrawal mediates emergence of ST2L$^+$ and CD8^{low} T cells. (A) Expression of ST2L on CD8$^+$ T cells determined directly after isolation, 20h of cultivation with 1% human serum or under serum withdrawal (starvation). mTOR inhibition by addition of rapamycin to serum with DMSO in serum as control (DMSO). (B) Exemplary histograms of CD8$^+$ T cells after isolation (0h) or cultured with different conditions for 20h. (C) Turnover of intracellular autophagy marker light chain 3 beta (LC3B) assessed by flow cytometry in treated CD8$^+$ T cells. (D) Exemplary histograms showing the gating strategy for discrimination of CD8^{high} and CD8^{low} T cells and percentage of CD8^{low} of total CD8$^+$ T cells. (E) Representative data for expression of ST2L on CD8^{low} and CD8^{high}. Data are shown as mean ± SD of (A, D) 0h n=6, starvation n=10, serum n=7, rapamycin n=4, DMSO n=4 or (C) n=3 per treatment from different donors and at least n=3 independently performed experiments. * for $p \leq 0.05$, $^{\#\#}$ for $p < 0.01$ and $^{\#\#\#}$ for $p < 0.001$ using *Kruskal-Wallis test with Dunn's posttest* for (A) and (D) or *one-sample t test* for (C). Hashtags (#) indicate comparisons to the 0h time point, asterisks (*) for comparisons as indicated.

Expression of ST2L and CD8 on the cell surface of isolated human CD8$^+$ T cells was either analyzed directly (0h) or after cultivation with human AB serum, under serum deprived conditions (starvation) or with serum supplemented with rapamycin to pharmacologically imitate starvation through inhibition of mTOR[18]. Detection by flow cytometry revealed that ST2L expression was significantly induced by starvation and rapamycin compared to the 0h control (both $p < 0.01$) or to cultivation in 1% serum (both $p < 0.01$) (**Figure 15A, B**). Degradation of microtubule-associated protein 1 light chain 3 beta (LC3B) indicates induction of autophagy resulting from inhibition of mTOR. Turnover of LC3B was significantly increased upon treatment with rapamycin compared to the respective DMSO

control ($p \leq 0.05$) (**Figure 15C**). Also, downregulation of CD8 expression was observed upon starvation (**Figure 15D**). Suspecting changes not only in the phenotype but also in their functions, $CD8^+$ T cells with diminished CD8 expression were named $CD8^{low}$, while T cells with CD8 expression levels comparable to freshly isolated cells were denominated $CD8^{high}$. The percentage of $CD8^{low}$ in the total $CD8^+$ T cell population significantly increased upon starvation compared to the 0h control ($p < 0.01$) or compared to cells cultivated in serum ($p \leq 0.05$), resulting from downregulation of CD8 expression. Correspondingly, $CD8^+$ T cells treated with rapamycin in serum exhibited a phenotype similar to starved T cells, showing that the changes in the expression of both ST2L and CD8 were associated to inhibition of mTOR. As $CD8^{low}$ were identified as the main population expressing ST2L (**Figure 15E**), both $CD8^{high}$ and $CD8^{low}$ were further phenotypically characterized (**Figure 16**).

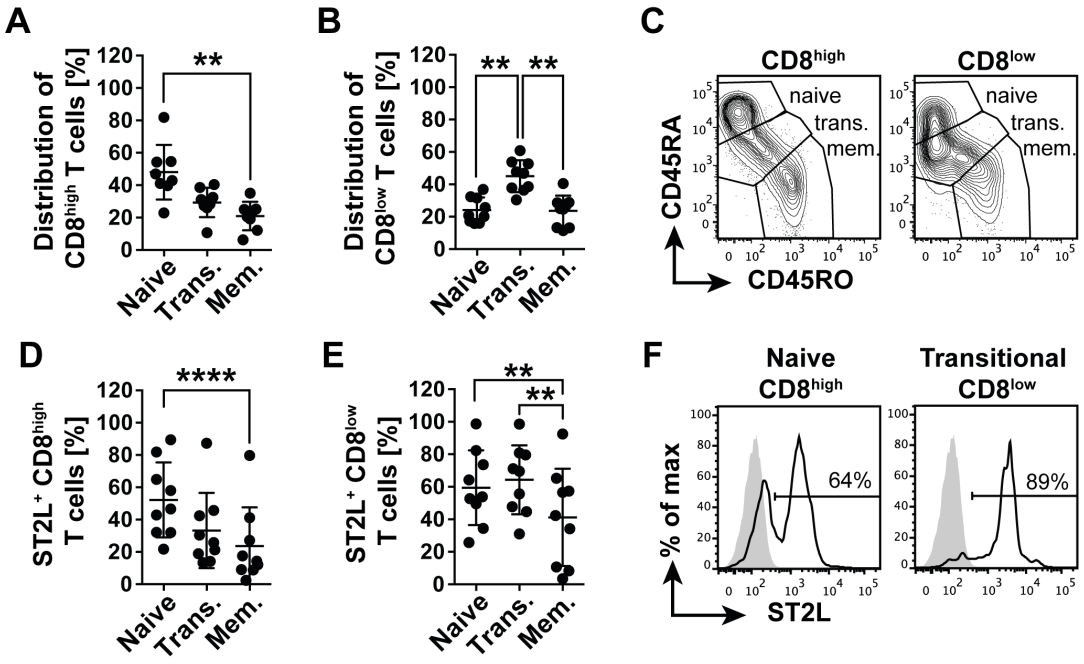

Figure 16: $ST2L^+$ $CD8^{low}$ are transitional $CD45RA^+RO^+$ and $ST2L^+$ $CD8^{high}$ are $CD45RA^+RO^-$ naïve T cells. Distribution of naïve ($CD45RA^+RO^-$), transitional (Trans., $CD45RA^+RO^+$) and memory type (Mem., $CD45RA^-RO^+$) subpopulations of (A) $CD8^{high}$ and (B) $CD8^{low}$ T cells determined by flow cytometry. (C) Representative data for the gating strategy of naïve, transitional and memory type $CD8^{high}$ and $CD8^{low}$. Percentage of ST2L expressing naïve, transitional or memory type (D) $CD8^{high}$ and (E) $CD8^{low}$. (F) Representative histograms of ST2L expression of transitional $CD8^{high}$ and naïve $CD8^{low}$. Data are shown as mean ± SD of (A-F) n=9 with at least n=3 independently performed experiments. ** for $p < 0.01$, **** for $p < 0.0001$ using (A, B) *Kruskal-Wallis test with Dunn's posttest* or (D, E) *Friedman test with Dunn's posttest*.

The characteristics of $CD8^{high}$ and $CD8^{low}$ T cells were further examined to understand why nutrient deprivation promotes responsiveness to IL-33 and which are the consequences for the pro- or anti-inflammatory activity of $CD8^+$ T cells. As starvation had been associated to inhibition of mTOR (**Figure 16**) and rapamycin has been reported to be involved in the generation of memory and regulatory T cells[145], expression of naïve T cell marker CD45RA and regulatory and memory T cell marker CD45RO were assessed by flow cytometry. Populations with the expression pattern $CD45RA^+RO^-$ were hence defined as naïve, $CD45RA^-RO^+$ as classical memory and $CD45RA^+RO^+$ were defined as T cells in a transitional differentiation phase. $CD8^{high}$ T cells were constituted mostly by naïve cells (48.0 ± 16.9%), as shown by comparison to the percentages of transitional (29.4 ± 9.1%) or memory type T cells (21.0 ± 8.8%, $p < 0.01$) (**Figure 16A, C**). In contrast, $CD8^{low}$ T cells were significantly dominated by the transitional type subpopulation (45.0 ± 9.8%) compared to the percentages of naïve (24.1 ± 7.9%, $p < 0.01$) or memory T cells (23.6 ± 9.5%, $p < 0.01$) (**Figure 16B, C**). Those results were proportional to the expression patterns of ST2L. The percentage of $ST2L^+$ naïve $CD8^{high}$ (52.2 ± 23.2%) significantly exceeded the percentage of $ST2L^+$ $CD8^{high}$ exhibiting a memory phenotype (23.6 ± 24.0, $p < 0.0001$) (**Figure 16D, F**). Furthermore, naïve (59.4 ± 23%) and transitional $CD8^{low}$ (64.3 ± 21.2%) were identified as the main populations expressing ST2L compared to the population characterized as memory T cells (41.2 ± 30.0%, both $p < 0.01$) (**Figure 16E, F**).

Since generation of memory T cells would classically require antigen encounter, it was suggested that the transitional $CD45RA^+RO^+$ phenotype of $CD8^{low}$ T cells might rather be associated to a reduced sensitivity towards TCR stimulation due to downregulation of CD8, rather than to generation of memory T cells. To assess possible differences between $CD8^{high}$ and $CD8^{low}$ concerning the TCR-dependent activation, the ratios of $CD8^{high}$ to $CD8^{low}$ upon treatment with IL-33 and αCD or IL-12 activation were determined (**Figure 17**).

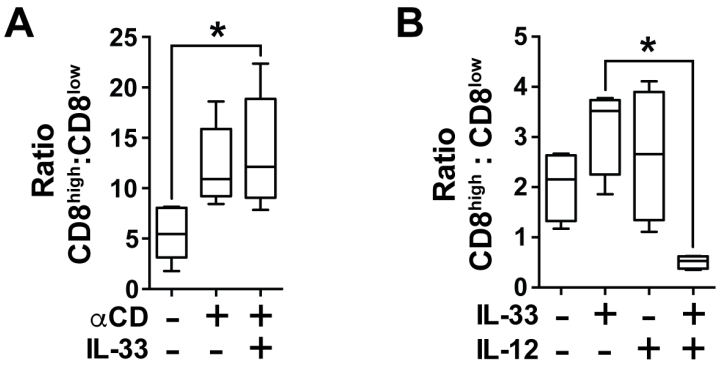

Figure 17: Stimulation dependent shift in the ratio of $CD8^{high}$ to $CD8^{low}$ T cells. Starved $CD8^+$ T cells were (A) activated TCR dependently (αCD) ± IL-33 or treated with (B) IL-33 ± IL-12 for 20h. Ratios were calculated from the percentages of $CD8^{high}$ and $CD8^{low}$ T cells determined by flow cytometry. Mean ± SD of n=5 different donors with at least n=3 independently performed experiments. * for $p \leq 0.05$ using *RM one-way ANOVA with Bonferroni's posttest.*

The ratio of $CD8^{high}$ to $CD8^{low}$ T cells was first assessed after treatment with TCR activators (αCD) with and without IL-33 stimulation (**Figure 17A**). The ratio of $CD8^{high}$ to $CD8^{low}$ was significantly increased by co-stimulation of the TCR and IL-33 compared to the untreated control ($p \leq 0.05$), implicating a predominance of $CD8^{high}$ T cells. However, TCR-independent co-stimulation of starved $CD8^+$ T cells with IL-33 and IL-12 led to a significant reduction in the ratio of $CD8^{high}$ to $CD8^{low}$ T cells ($p \leq 0.05$) (**Figure 17B**).

5.2.2. IL-33 TCR-dependently Induces T-bet-mediated Effector Functions

$CD8^{high}$ T cells resembling naïve "non-starved" T cells were expected to be able to exert effector functions. This assumption was based on the observation that $CD8^{high}$ predominated the population of $CD8^{low}$ after stimulation with IL-33 and activation of the TCR, implicating proliferation. In the following it was therefore investigated whether IL-33 also supports cytotoxic effector functions in human $CD8^+$ T cells during TCR activation and IL-12 stimulation (**Figure 18**).

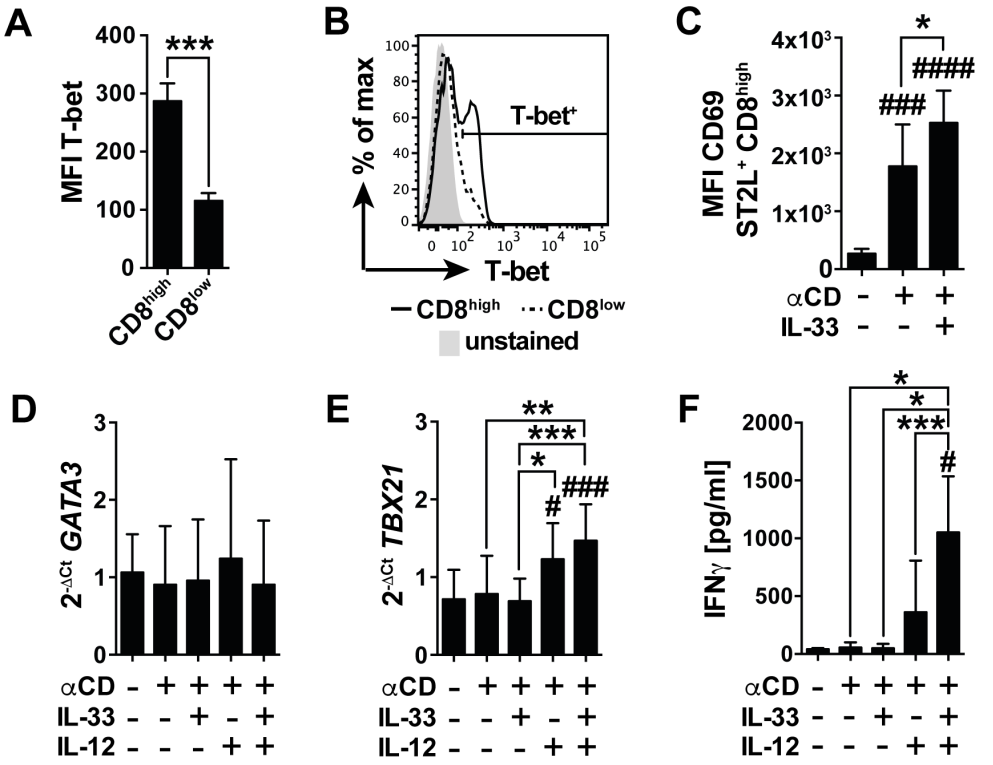

Figure 18: IL-33 supports TCR-dependent T_H1 effector functions. (A) Mean fluorescence intensity (MFI) of T-bet in $CD8^{high}$ and $CD8^{low}$ with representative histogram. (B) Ratio of $CD8^{high}$ to $CD8^{low}$ T cells and (C) MFI of CD69 on $ST2L^+$ $CD8^{high}$ during starvation without or with TCR-specific αCD2/CD3/CD28 (αCD) stimulation ± IL-33. qRT-PCR relative mRNA expression ($2^{-\Delta Ct}$) of (D) *GATA3* or (E) *TBX21* (T-bet). (F) Concentrations of secreted IFNγ by $CD8^+$ T cells treated with αCD ± IL-33 ± IL-12 for 20h. The expression levels were normalized to the housekeeping genes GAPDH and RPL13A. Mean ± SD of n=5 different donors with at least n=3 independently performed experiments. $^{\#}$/* for $p \leq 0.05$, ** for $p < 0.01$, $^{\#\#\#}$/*** for $p < 0.001$ or $^{\#\#\#\#}$ for $p < 0.0001$ using *RM one-way ANOVA with Bonferroni's posttest*. Hashtags (#) indicate significance compared to the untreated control, asterisks (*) indicate comparisons as indicated.

$CD8^{high}$ and $CD8^{low}$ T cells showed significant differences in the protein expression of T_H1-specific transcription factor T-bet ($p < 0.01$). $CD8^{high}$ expressed high levels (MFI 286.8 ± 30.8), $CD8^{low}$ expressed low levels of T-bet (MFI 115.6 ± 13.2) (**Figure 18A**). In line with these results, expression of activation marker CD69 on $ST2L^+$ $CD8^{high}$ was induced by TCR activation ($p < 0.001$) and in comparison further significantly increased by co-stimulation with IL-33 ($p \leq 0.05$) (**Figure 18B**). Expression of mRNA encoding T_H2 transcription factor GATA3 was not regulated by activation with the different stimuli (**Figure 18C**). However, T-bet mRNA *TBX21* was significantly increased by TCR/ IL-12 activation ($p \leq 0.05$ to untreated control) and by co-stimulation of the TCR with IL-12 and IL-33 ($p < 0.001$) (**Figure 18D**). T-bet is a key transcription factor driving IFNγ production

in T cells. Amounts of secreted IFNγ protein measured in the supernatants of the stimulated $CD8^+$ T cells were comparable to the expression levels of *TBX21* mRNA detected in the same cells (**Figure 18E**). The TCR/ IL-12-induced secretion of IFNγ (361.3 ± 199.3 pg/ml) was further significantly increased by addition of IL-33 (1051 ± 216.3 pg/ml, $p < 0.001$). To additionally confirm an effector function mediated by $CD8^{high}$, stimulation-dependent degranulation occurring during cell-mediated cytotoxicity was assessed (**Figure 19**).

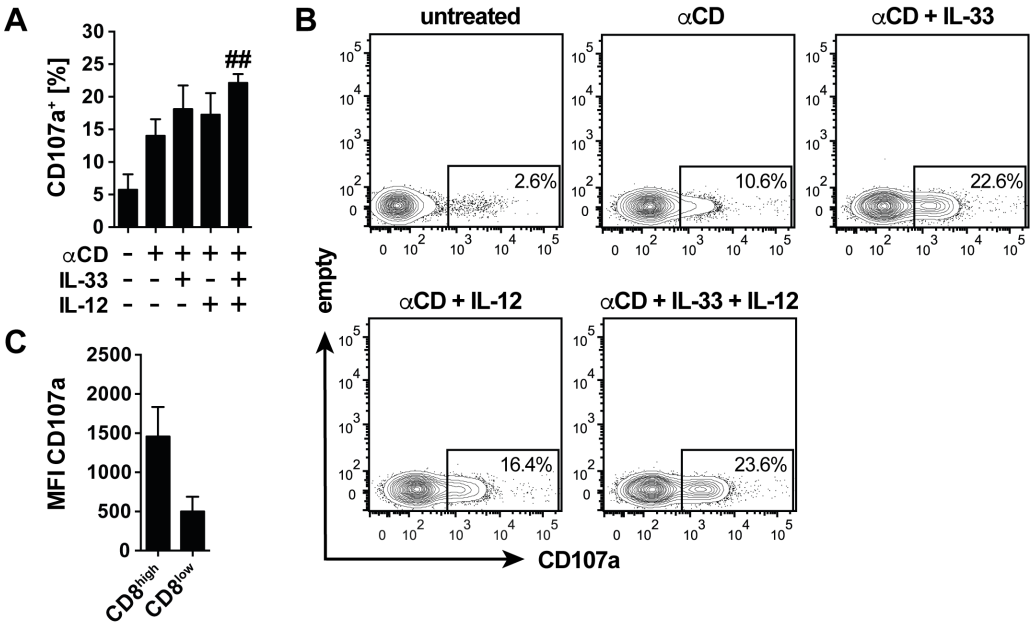

Figure 19: Co-stimulation of the TCR, IL-12 and IL-33 promotes degranulation of $CD8^+$ T cells. $CD8^+$ T cells were treated with αCD stimulus, IL-12 and IL-33 for 5h and co-incubated with an anti-CD107a antibody to detect (A) the percentage of $CD107a^+$ degranulating $CD8^+$ T cells. (B) Respective representative flow cytometry data and (C) assessment of the mean fluorescence intensity (MFI) of CD107a in $CD8^{high}$ and $CD8^{low}$. Mean ± SD of n=4 different donors. $^{\#\#}$ for $p < 0.01$ using *Friedman test with Dunn's posttest*. Hashtag (#) indicates significance compared to the untreated control.

It was found that expression of the degranulation marker CD107a on the cell surface was significantly increased compared to the untreated control ($p < 0.01$) if the $CD8^+$ T cells were activated TCR-dependently and co-stimulated with IL-33 and IL-12 (**Figure 19A, B**). In this context, $CD8^{high}$ expressed high levels, $CD8^{low}$ expressed low levels of CD107a (**Figure 19C**). Based on these findings, it was suggested that the pro-inflammatory effect of IL-33 was dependent on expression of transcription factor T-bet and TCR-dependent activation of $CD8^{high}$.

5.2.3. IL-33 and IL-12 Induce a T_{reg}-like Phenotype in $CD8^{low}$

The CD8α chain is required for stabilization of the MHC-class I/ TCR complex[146]. $CD8^{high}$ were characterized to exert effector functions after TCR activation, while $CD8^{low}$ did not perform cytotoxicity. It was postulated that downregulation of CD8 led to desensitization towards antigen-dependent stimulation. It was asked, which impact the TCR-independent IL-33 signaling on the activity or differentiation on $CD8^{low}$ might exert. To clarify this question, expression of granzyme B (GrzmB) was determined in starved $CD8^+$ T cells exhibiting the $CD8^{low}$ phenotype and stimulated with IL-12 without or with IL-33 (**Figure 20**).

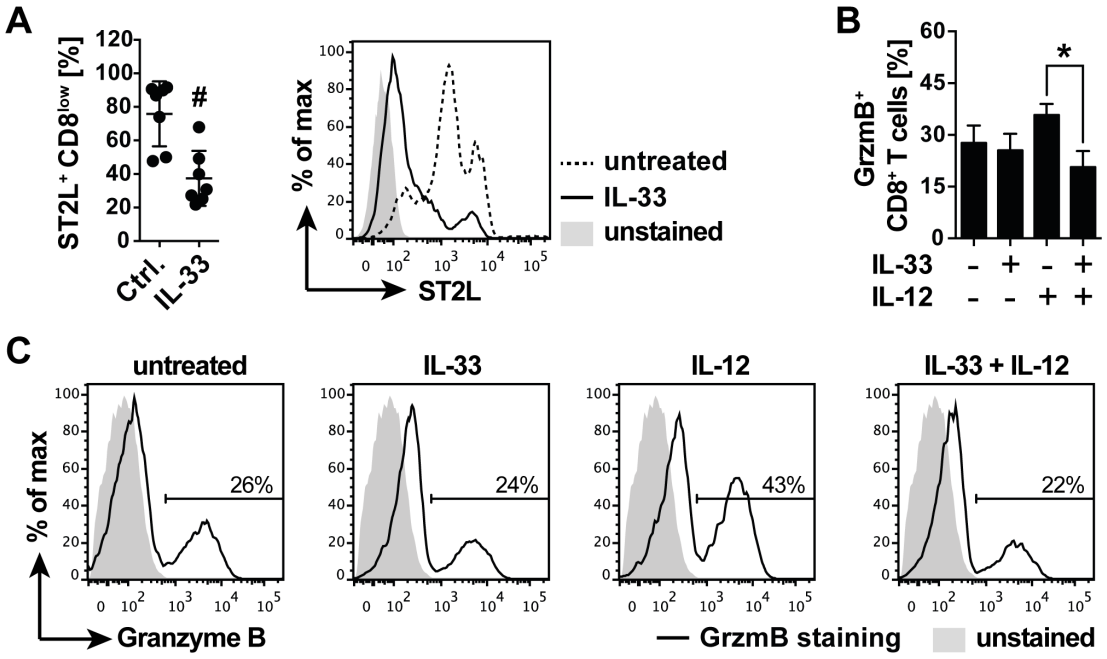

Figure 20: IL-33 inhibits the IL-12-induced expression of granzyme B during nutrient deprivation. (A) Starved $CD8^+$ T cells were treated with 20 ng/ml of IL-33 before determination of the percentage of $ST2L^+$ $CD8^{low}$ by flow cytometry. Representative histograms for ST2L expression of untreated and stimulated $CD8^{low}$. (B) Percentage of $CD8^+$ T cells expressing intracellular granzyme B (GrzmB) after stimulation with IL-33 ± IL-12. (C) Representative data showing the modulation of GrzmB. Mean ± SD of (A) n=7, (B) n=4 of different donors with at least n=4 independently performed experiments. */# for $p \leq 0.05$ using (A) *Wilcoxon matched pairs signed rank test*, (B) *Friedman test with Dunn's posttest*. Hashtags (#) indicate significance compared to the untreated control, asterisks (*) indicate significance as indicated.

To assess whether $CD8^{low}$ T cells were responsive to IL-33, expression of ST2L on $CD8^{low}$ T cells was examined upon stimulation with IL-33 for 20h by flow cytometry. Expression of ST2L was significantly reduced by stimulation (untreated 75.9 ± 19.4%, stimulated 37.5 ± 16.4%, $p \leq 0.05$), indicating receptor internalization (**Figure 20A**). Determination of intracellular GrzmB expression revealed a significant reduction of IL-12-induced GrzmB expression (35.8 ± 3.2%) by co-stimulation with IL-12 and IL-33 (20.7 ± 4.6%, $p \leq 0.05$) (**Figure 20B**, **C**). It was questioned if $CD8^{low}$ T cells IL-33-dependently acquire an anti-inflammatory phenotype. Therefore expression of hallmark transcription factors for T_H1 (*TBX21*), effector memory (*BLIMP-1*), T_H2 (*GATA3*) and T_{reg} (*FOXP3*) subsets were determined in IL-33 and/ or IL-12 stimulated $CD8^{low}$ T cells (**Figure 21**).

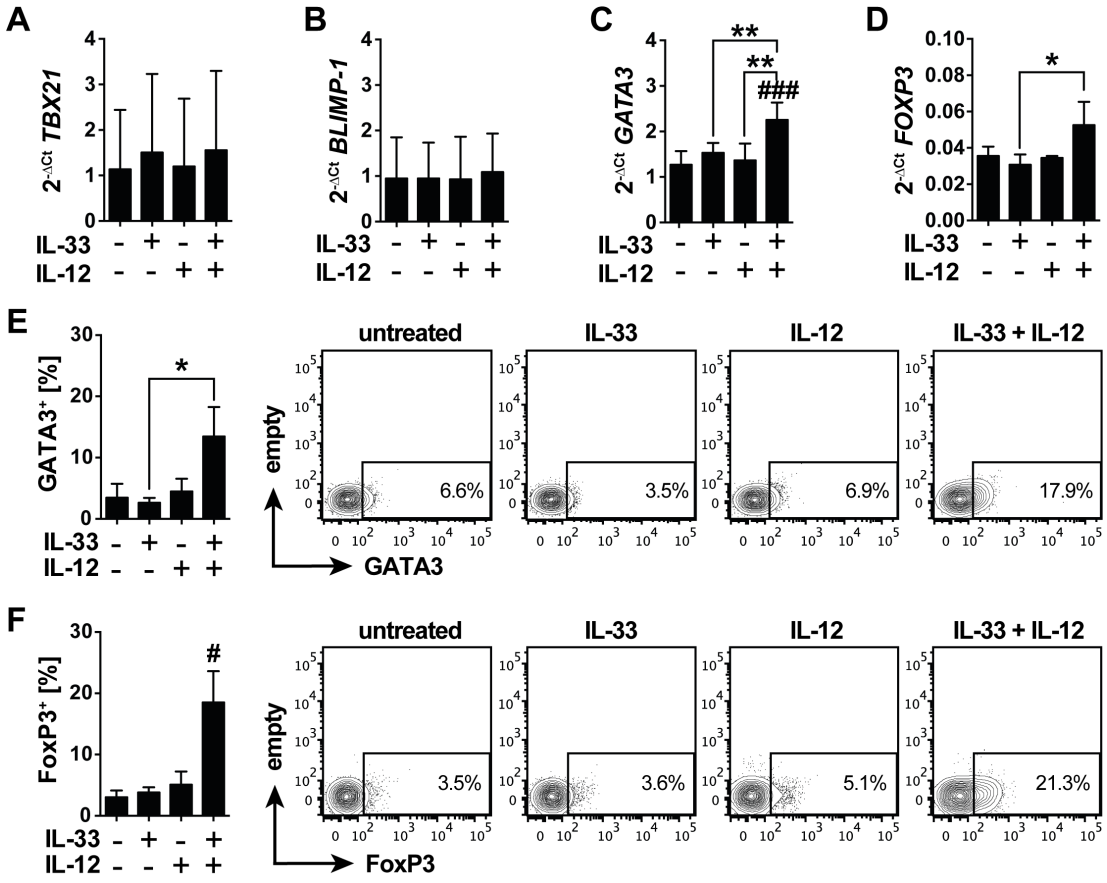

Figure 21: IL-33 and IL-12 co-induce an anti-inflammatory, regulatory differentiation profile in starved $CD8^+$ T cells. $CD8^+$ T cells cultured without serum were left untreated for 20h before stimulation with IL-33 and/ or IL-12 for 20h. qRT-PCR relative mRNA expression ($2^{-\Delta Ct}$) of (A) *TBX21* (T-bet), (B) *BLIMP-1*, (C) *GATA3* and (D) *FOXP3*. The expression levels were normalized

to the housekeeping genes *GAPDH* and *RPL13A*. Intracellular expression of transcription factors (E) GATA3 and (F) FoxP3 protein was additionally assessed by flow cytometry. Mean ± SD of (A, B, E) n=4 and (C, D) n=3 from different donors. $^{\#/*}$ for $p \leq 0.05$, ** for $p < 0.01$ and $^{\#\#\#}$ for $p < 0.001$ using (A-D) *RM one-way ANOVA with Bonferroni's posttest* or (E, F) *using Friedman test with Dunn's posttest.* Hashtags (#) indicate significance compared to the untreated control, asterisks (*) indicate significance as indicated.

Starved $CD8^+$ T cells were stimulated with IL-33 with or without IL-12 for 20h prior isolation of total RNA. mRNA Expression of transcription factors T-bet (*TBX21*), *BLIMP-1*, *GATA3* and *FoxP3* conducted by qRT-PCR analysis revealed no regulation of *TBX21* mRNA (**Figure 21A**) or *BLIMP-1* mRNA (**Figure 21B**), but significant induction of *GATA3* and *FOXP3* mRNA upon co-stimulation with IL-33 and IL-12 (**Figure 21C, D**). Those results were confirmed on protein levels by flow cytometry analysis, proving significant IL-33- and IL-12-dependent increases in the intracellular expression of both GATA3 ($p \leq 0.05$ compared to IL-33 stimulation alone; **Figure 21E**) and FoxP3 protein ($p \leq 0.05$ to untreated control; **Figure 21F**). These results supported the hypothesis, that in the absence of TCR activation, IL-33 and IL-12 induced an anti-inflammatory phenotype in $CD8^{low}$ T cells.

5.2.4. IL-33 does not re-activate mTORC1 or enhance mTORC2 Activity

Recently, Deason and colleagues revealed that the adaptor protein PIK3AP1 links the IL-1R signaling to the PI3K-mTOR pathway[71]. Although not yet described, it was suggested here that induction of IL-33 responsiveness on nutrient deprived $CD8^+$ T cells might enable IL-33 to modulate the PI3K-mTOR-Akt pathway through PIK3AP1. To begin with, it was investigated whether IL-33 with and without TCR stimulation influences expression of *PIK3AP1* mRNA in nutrient deprived $CD8^+$ T cells. (**Figure 22**).

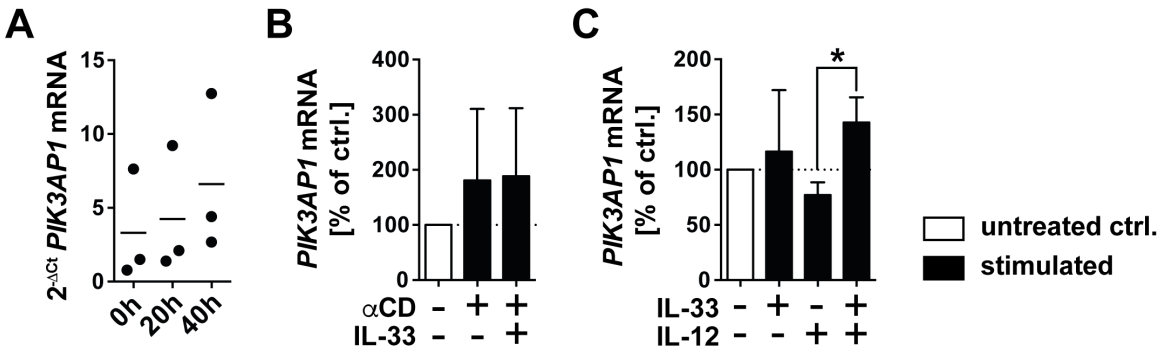

Figure 22: IL-33/ IL-12 co-stimulation induces expression of *PIK3AP1* mRNA. *PIK3AP1* mRNA expression was determined by qRT-PCR in $CD8^+$ T cells cultured for (A) 0h, 20h or 40h under serum-free conditions, (B) activated TCR-dependently with and without IL-33 or (C) were treated with IL-33 and/ or IL-12 after 20h of starvation and additional 20h of stimulation. Data are shown as mean ± SD of at least n=3 independently performed experiments. *$p \leq 0.05$ *using one-way ANOVA with Tukey's posttest*.

PIK3AP1 mRNA expression levels in $CD8^+$ T cells directly before, after 20h or 40h of starvation were not significantly different (**Figure 22A**). TCR activation without or with IL-33 did not mediate any differences in the transcriptional expression of *PIK3AP1* mRNA under nutrient withdrawal (**Figure 22B**). However, TCR-independent co-stimulation with IL-33 and IL-12 led to a significant increase in the expression of *PIK3AP1* mRNA compared to $CD8^+$ T cells stimulated with IL-12 alone ($p \leq 0.05$) (**Figure 22C**).

IL-33 has been described to induce mTOR activation through activation of the PI3K subunit p110δ in murine T_H2 cells[128]. It was examined whether similarly to the IL-1R signaling, expression of PIK3AP1 links the IL-33/ ST2L signaling to the mTOR pathway. To address this question, activation of mTORC1 was indirectly assessed through detection of the phosphorylated p70 subunit of S6K at the aa position threonine (Thr) 389. In contrast to mTORC1, activity of mTORC2 is usually not directly affected by nutrient deprivation but is able to indirectly activate mTORC1 through activating Akt, which blocks the activity of the mTORC1 by inhibiting the TSC complex. mTORC2 potently enhances activity of Akt through phosphorylation at the aa serine (Ser) on position 473 (**Figure 23**).

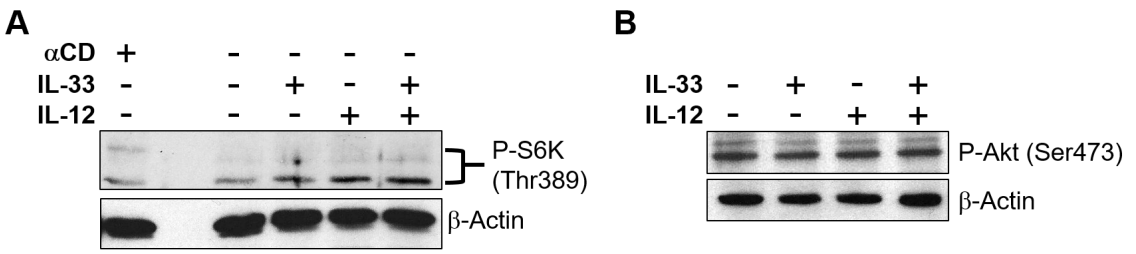

Figure 23: TCR-independent IL-33/ ST2L signaling does not alter phosphorylation of p70S6K or Akt. $CD8^+$ T cells were cultivated under nutrient deprivation and stimulated with IL-33 and IL-12 to induce a regulatory phenotype before western blot analysis of (A) phosphorylated p70S6K (P-S6K) at threonine 389 (Thr389) or (B) phosphorylated Akt (P-Akt) at serine 473. As a positive control for P-S6K, $CD8^+$ T cells were activated TCR-dependently using αCD2/3/28 antibodies (αCD). β-Actin as internal loading control. Representative data of n=3 independently performed experiments.

$CD8^+$ T cells were starved before differentiation with IL-33 and IL-12 to induce the previously observed regulatory phenotype. Subsequently, phosphorylation of p70S6K (P-S6K) at Thr389 and Akt (P-Akt) at Ser473 were determined. As a positive control for P-S6K, the $CD8^+$ T cells were additionally activated TCR dependently for five hours prior cell lysis. Western blot analysis revealed no phosphorylation of p70S6K at Thr389 associated to IL-33 and/ or IL-12 stimulation, implicating that mTORC1 was not reactivated (**Figure 23A**). Similarly, IL-33 and/ or IL-12 stimulation did not result in an enhanced phosphorylation of Akt (Ser473), which would be relatable to an amplified activity of mTORC2 (**Figure 23B**).

5.3. Contribution of IL-33 to CD8$^+$ T Cell Trafficking

Peripheral T lymphocyte trafficking is *in vivo* an essential process for the distribution of effector und regulatory T cells and the transmigration of lymphocytes into tissue. This process is centrally regulated by mTOR. In the previous chapter it has been discovered that during nutrient withdrawal, IL-33 was able to either induce a cytotoxic or T_{reg}-like phenotype in CD8$^+$ T cells. Lately arising evidence implicates that IL-33 might be involved in processes requiring strict coordination of signals promoting T cell homing, egress and retention in tissue[41,130,131]. It was therefore investigated in the following if IL-33 might contribute to alterations in the migratory behavior of CD8$^+$ T cells during starvation.

5.3.1. Starvation induces CCR7 but reduces S1P Receptor Expression

Receptors for chemokines and S1P receptors are essential regulators of migration and immune cell trafficking. While interaction of homing receptor CCR7 with its ligands mediates immigration into lymph nodes, tissue residency marker CD69 promotes retention through inhibition of S1P1, and activation of S1P1 itself triggers extravasation into blood[37,147,39]. To investigate if nutrient deprivation alters the migratory potential of CD8$^+$ T cells to migrate to and stay in LN, expression of CCR7 and CD69 was assessed on starved CD8^{high} and CD8^{low} T cells (**Figure 24**).

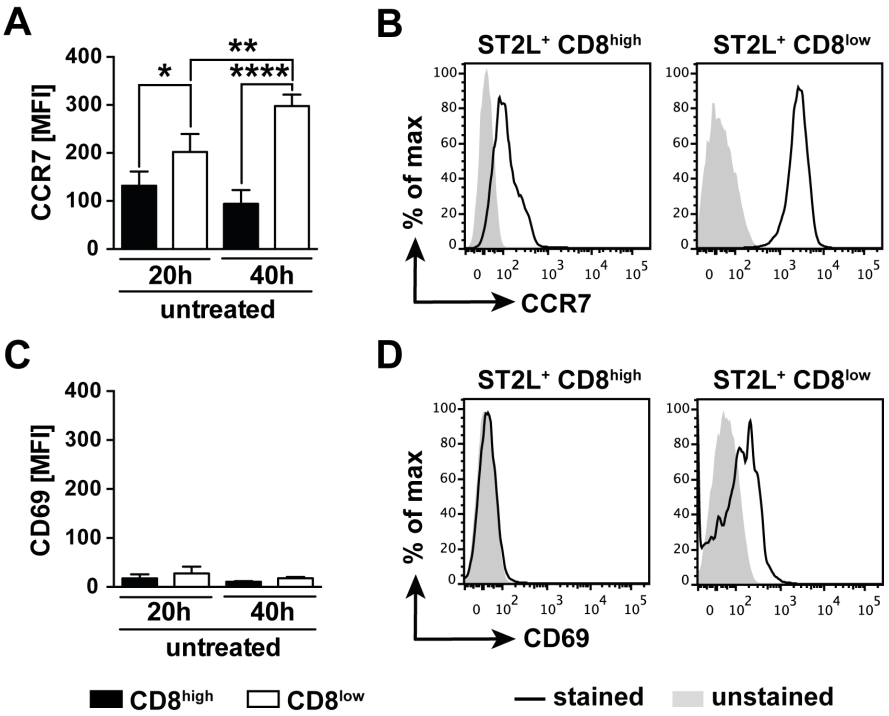

Figure 24: Starvation promotes expression of homing receptor CCR7 but not tissue residency marker CD69. Detection of (A, B) SLO homing receptor CCR7 and (C, D) tissue residency marker CD69 on the cell surface of ST2L$^+$ CD8^{high} and ST2L$^+$ CD8^{low} T cells. The mean fluorescence intensity (MFI) was determined by flow cytometry after 20h or 40h of cultivation under starvation without any stimulation (A, C). Representative flow cytometry histograms for (B) CCR7 and (D) CD69 on ST2L$^+$ CD8^{high} and CD8^{low} T cells. Data are shown as mean ± SD of at least n=3 independently performed experiments. * for $p \leq 0.05$, ** for $p < 0.01$, **** for $p < 0.0001$ using *one-way ANOVA with Tukey's posttest.*

CD8^{low} T cells expressed significantly higher levels of lymph node homing receptor CCR7 compared to CD8^{high} after 20h of starvation ($p \leq 0.05$) (**Figure 24A, B**). The expression levels were further increased until 40h of nutrient deprivation, suggesting starvation dependent regulation of CCR7 expression on CD8^{low}. However, expression of the retention marker CD69 was detected neither on CD8^{high}, nor on CD8^{low} T cells (**Figure 24C, D**). Activation of T cells reportedly induces dramatic shifts in the expression patterns of proteins involved in T cell trafficking[27]. Therefore, cell surface expression of CCR7 and CD69 was analyzed upon stimulation with IL-33 with and without TCR activation of CD8^{high} and CD8^{low} T cells. (**Figure 25**).

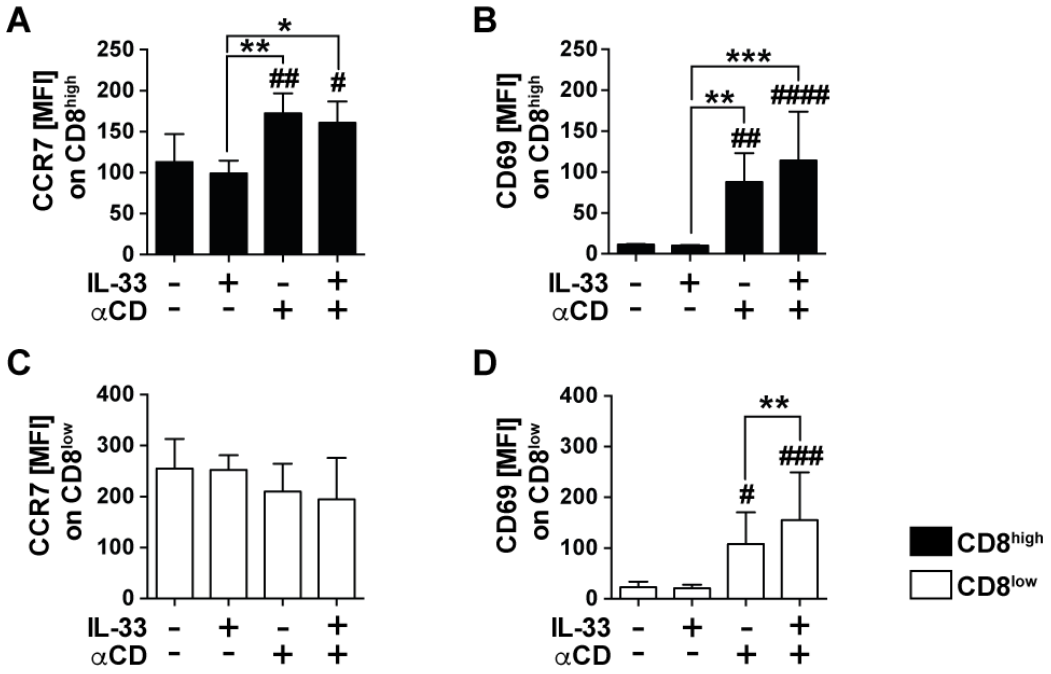

Figure 25: IL-33 enhances the TCR mediated expression of tissue residency marker CD69 on CD8^{low}, but not of lymph node homing receptor CCR7. Mean fluorescence intensities (MFI) of (A) CCR7 and (B) CD69 on CD8^{high} (black bars) and of (C) CCR7 and (D) CD69 on CD8^{low} (white bars) upon treatment with 20 ng/ml IL-33 or αCD2/3/28 (αCD) for 20h under starvation. Data are shown as mean ± SD of at least n=3 independently performed experiments. $^{\#}$/* for $p \leq 0.05$, $^{\#\#}$/** for $p < 0.01$, $^{\#\#\#}$/*** for $p < 0.001$ $^{\#\#\#\#}$/**** for $p < 0.0001$ using *one-way ANOVA with Tukey's posttest.* Hashtags (#) indicate comparisons to the untreated control, asterisks (*) indicate comparisons as indicated.

Starved CD8^{+} T cells were treated with IL-33 ± αCD TCR activators for additional 20h before analysis of CCR7 and CD69 cell surface expression on CD8^{high} and CD8^{low} T cells. TCR activation conferred a significantly higher expression of CCR7 ($p < 0.01$ to untreated) (**Figure 25A**) and induction of CD69 on CD8^{high} T cells ($p < 0.01$ to untreated) (**Figure 25B**). However, no synergistic effects of IL-33 and TCR activation were observed for expression of either cell surface marker on CD8^{high} T cells. In contrast to CD8^{high}, both TCR activation and IL-33 stimulation failed to further increase expression of CCR7 on CD8^{low} T cells (**Figure 25C**) but TCR activation mediated a significant induction of CD69 ($p \leq 0.05$ to untreated), an effect that was further enhanced by IL-33 ($p < 0.01$) (**Figure 25D**).

Observing that starvation promoted expression of CCR7 on $CD8^{low}$ T cells, it was expected that the ability of $CD8^+$ T cells to circulate was reduced during nutrient deprivation. As reliable antibodies for the detection of S1P receptors are currently not available, expression levels of *S1P1*, *S1P3*, *S1P4* and *S1P5* were determined on mRNA levels (**Figure 26**).

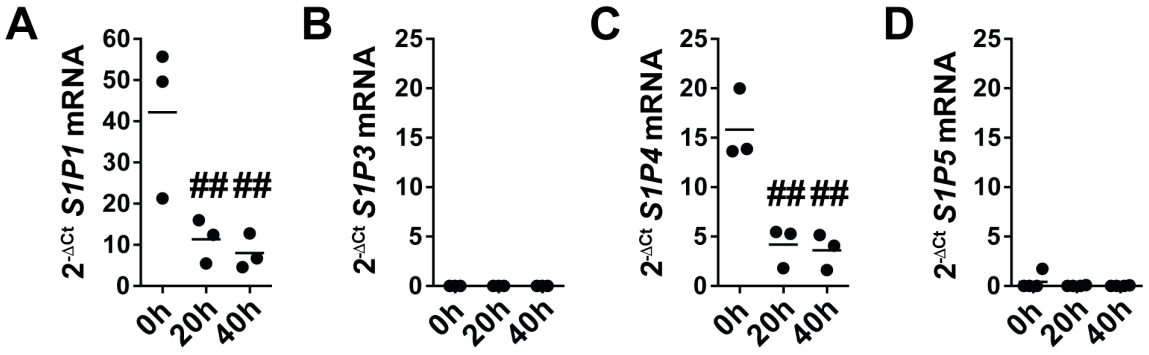

Figure 26: Expression of *S1P1* and *S1P4* mRNA is downregulated in starved $CD8^+$ T cells. Isolated $CD8^+$ T cells were cultivated under serum-free conditions for 0h, 20h or 40h prior cell lysis and isolation of RNA. qRT-PCR for analysis of mRNA expression of **(A)** *S1P1*, **(B)** *S1P3*, **(C)** *S1P4* and **(D)** *S1P5*. The expression levels were normalized to those of the housekeeping genes *GAPDH* and *RPL13A* (2^{-DCt}). Data are presented as mean ± SD representative of n=3 independent experiments. $^{\#\#}p < 0.01$ using *one-way ANOVA with Tukey's posttest*. Hashtags (#) indicate comparisons to the 0h control.

S1P1 mRNA was highly expressed in freshly isolated $CD8^+$ T cells and significantly declined during prolonged starvation ($p < 0.01$ for both 20h vs. 0h and 40h vs. 0h) (**Figure 26A**). Concurrently *S1P4* mRNA expression levels significantly decreased during nutrient deprivation ($p < 0.01$ for both 20h vs. 0h and 40h vs. 0h) (**Figure 26C**). Neither *S1P3* nor *S1P5* mRNA were expressed or induced by starvation (**Figure 26B, D**). As *S1P1* and *S1P4* mRNA expression levels appeared to be modulated during nutrient deprivation, it was asked whether IL-33, was directly involved in the modulation of S1P receptor expression upon TCR-dependent and -independent activation.

5.3.2. IL-33 Modulates *S1P1* mRNA and *KLF2* mRNA Expression

KLF2 is the transcription factor for both the *S1P1* and the *CCR7* gene[41,148], although conditions favoring KLF2-dependent expression of either gene remain to be resolved. As it had been observed that CCR7 protein expression was increased, while *S1P1* mRNA expression was decreased during starvation it was suggested that starvation influences the transcriptional activity of KLF2 on the *CCR7* gene. In the following, it was first investigated whether starvation influences *KLF2* mRNA expression levels (**Figure 27**).

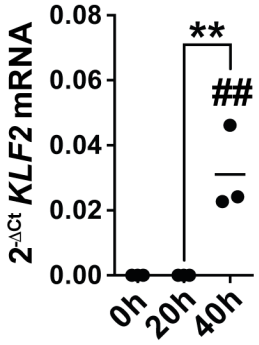

Figure 27: Starvation drives mRNA expression of S1P1 transcription factor *KLF2*. Isolated $CD8^+$ T cells were cultivated under serum-free conditions for 0h, 20h or 40h prior cell lysis and isolation of RNA. qRT-PCR for analysis of mRNA expression of *KLF2*. The expression levels were normalized to the housekeeping genes *GAPDH* and *RPL13A* (2^{-DCt}). Data are presented as mean ± SD representative of n=3 independent experiments. **/ $^{\#\#}p < 0.01$ using *one-way ANOVA with Tukey's posttest*. Hashtags (#) indicate comparisons to the 0h control, asterisks (*) as indicated.

KLF2 mRNA expression levels were determined for $CD8^+$ T cells cultivated for 20h or 40h under nutrient withdrawal. Determination of *KLF2* mRNA levels revealed a significant increase of expression after 40h of starvation compared to mRNA levels determined at 0h ($p < 0.01$) or 20h of cultivation ($p < 0.01$) (**Figure 27**).

In the following, $CD8^+$ T cells were stimulated with a combination of IL-33 and IL-12 and activated TCR-dependently in order to induce the previously described effector phenotype, Alternatively, they were stimulated TCR-independently to induce differentiation of the T_{reg}-like phenotype. The mRNA expression levels for *S1P1* and transcription factor *KLF2* were subsequently assessed (**Figure 28**).

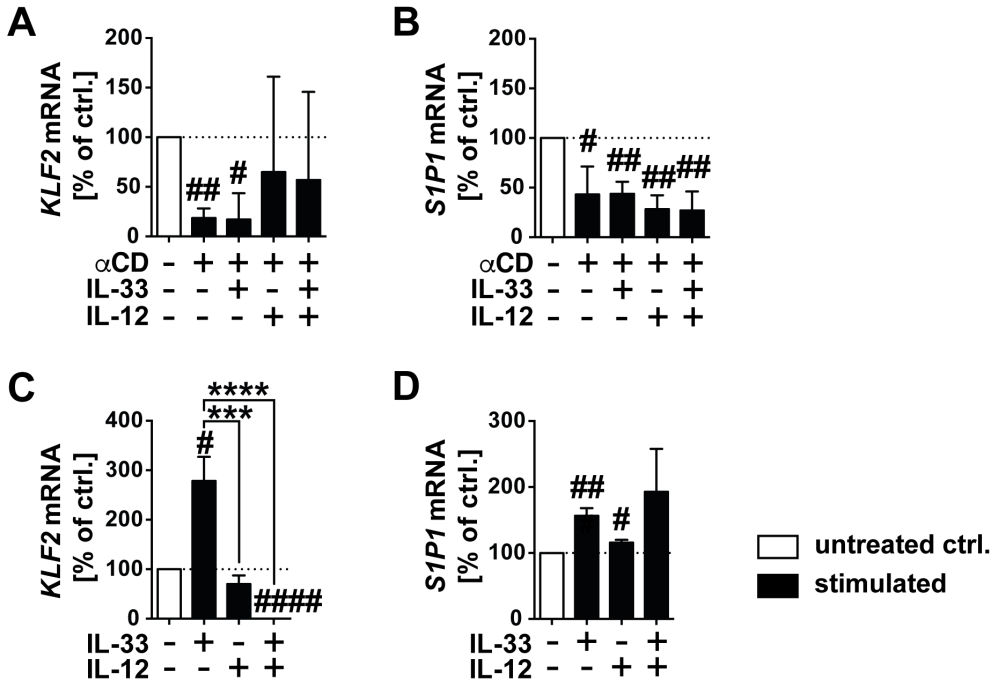

Figure 28: IL-33 regulates gene expression of *KLF2* and *S1P1* TCR-independently. Isolated $CD8^+$ T cells were cultivated under serum-free conditions for 20h before co-stimulation (A, B) with or (C, D) without TCR activation cocktail (αCD), IL-33, IL-12 or a combination of the stimuli for additional 20h. qRT-PCR for analysis of the mRNA expression of (A, C) *S1P1* and (B, D) *KLF2*. The expression levels were normalized to the untreated control set to 100% (white bars). Data are shown as mean ± SD of at least n=3 independently performed experiments. $^{\#}p \leq 0.05$, $^{\#\#}p < 0.01$, ***p < 0.001 and ****/$^{\#\#\#\#}p < 0.0001$ *using one-way ANOVA with Tukey's post-test*. Hashtags (#) indicate comparisons to the untreated control, asterisks (*) for comparisons as indicated.

TCR activation of $CD8^+$ T cells significantly reduced the relative expression of *KLF2* mRNA ($p < 0.01$) (**Figure 28A**) and *S1P1* mRNA ($p \leq 0.05$) (**Figure 28B**) compared to untreated cells. Addition of IL-33 and/ or IL-12 did not significantly further decrease *KLF2* or *S1P1* mRNA expression. Interestingly, in the absence of TCR activation, IL-33 alone significantly stimulated *KLF2* mRNA expression ($p \leq 0.05$) (**Figure 28C**), resulting in an increased gene expression of *S1P1* ($p < 0.01$ compared to untreated) (**Figure 28D)**. IL-33 and IL-12 co-stimulation abrogated *KLF2* mRNA expression, leading to an *S1P1* mRNA expression that remained unchanged compared to untreated cells. This finding raised the question how transcriptional expression of KLF2 is modulated. The *KLF2* gene is a target of transcription factor FoxO1, a member of the forkhead box proteins essentially required for the generation of $FoxP3^+$ T_{reg}[149]. Unphosphorylated FoxO1 is an active transcription factor located in the nucleus, while acetylation affects its DNA binding capacity and phosphorylation by Akt promotes its exclusion from the nucleus[150,151].

To assess whether IL-33 modulates FoxO1 and thus indirectly regulates *KLF2* mRNA expression, expression and phosphorylation of FoxO1 at aa Ser256 (P-FoxO1) was determined in starved $CD8^+$ T cells stimulated with IL-33 and IL-12 (**Figure 29**).

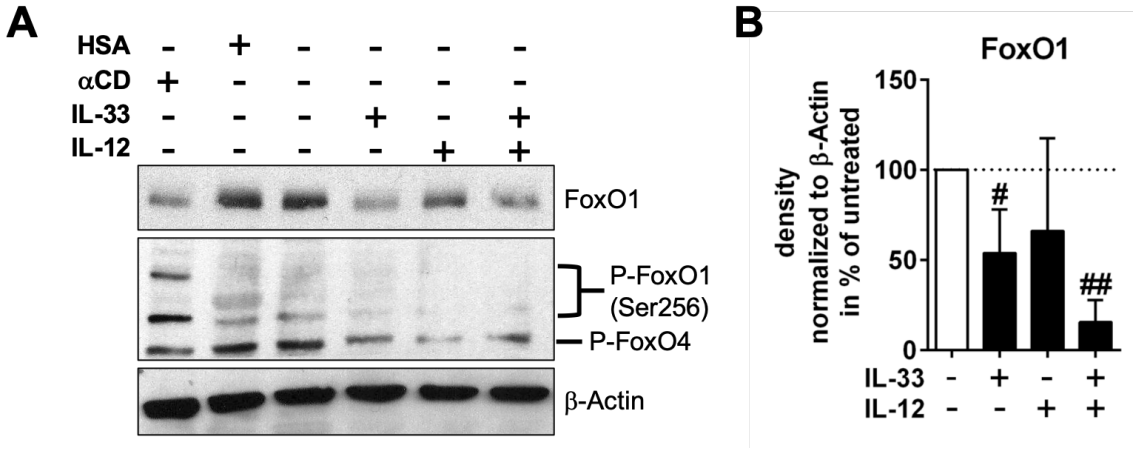

Figure 29: IL-33 modulates expression of FoxO1. (A) $CD8^+$ T cells were cultivated with human serum (HSA) or activated TCR-dependently (αCD) as positive controls. Expression of FoxO1 and phosphorylated FoxO1 (P-FoxO1) at serine 256 (Ser256) was assessed by western blotting. Besides P-FoxO1, phosphorylated FoxO3 (P-FoxO4) was detected by the polyclonal antibody. β-Actin was used as an internal loading control. (B) Densitometric measurement for quantification of FoxO1 expression. Densities of detected FoxO1 bands were normalized to the densities of β-Actin and indicated in percent (%) compared to the untreated control. Data are shown as mean ± SD of at n=3-4 independently performed experiments. $^{\#}p \leq 0.05$ *using one-sample t-test*. Hashtags (#) indicate comparisons to the untreated control.

$CD8^+$ T cells were starved for 20h and were differentiated for additional 20h with IL-33 in the absence of IL-12 in order to obtain the previously described T_{reg}-like phenotype. As FoxO1 is reportedly phosphorylated upon TCR stimulation (αCD) and is constitutively expressed under nutrient-rich conditions, $CD8^+$ T cells were activated TCR-dependently or cultivated in human serum for positive controls. Western blot analysis revealed a decrease in FoxO1 protein expression resulting from IL-33 stimulation, but no stimulation-dependent phosphorylation of Ser256 in the absence of TCR activation (**Figure 29A**). For quantification, the densities of FoxO1 bands were assessed and normalized to the respective β-Actin controls. The relative FoxO1 protein expression was significantly downregulated compared to untreated cells if the starved $CD8^+$ T cells were stimulated with IL-33 ($p \leq 0.05$) or co-stimulated with IL-33 and IL-12 ($p < 0.01$) (**Figure 29B**). The analysis revealed a discrepancy between expression of unphosphorylated FoxO1 protein

and expression of *KLF2* mRNA. Although *KLF2* mRNA expression was significantly induced by IL-33, expression of FoxO1 was significantly downregulated. However, a decrease in FoxO1 expression resulting from co-stimulation with IL-33 and IL-12 corresponded to the abrogated *KLF2* mRNA expression.

5.3.3. IL-33 maintains Expression of *S1P4* mRNA during T_{reg}-like Differentiation

S1P4 mRNA expression levels were found to be significantly reduced during starvation. S1P4 has been attributed important functions in the differentiation of effector T_H17 cells and T_{reg}[74]. It was therefore asked if IL-33 regulates *S1P4* mRNA expression during differentiation of T_{reg}-like $CD8^+$ T cells (**Figure 30**).

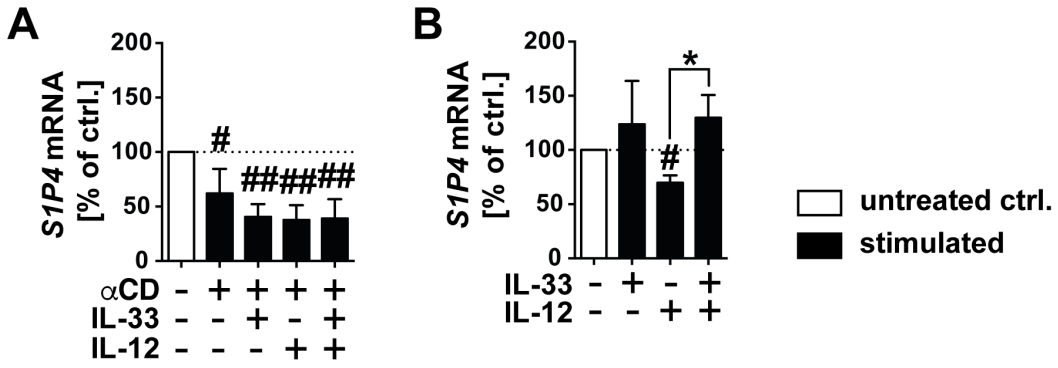

Figure 30: IL-33 re-induces *S1P4* gene expression. Starved $CD8^+$ T cells were stimulated with IL-33, IL-12 and (A) with or (B) without TCR activation (αCD) prior qRT-PCR analysis of *S1P4* mRNA expression. The expression levels were normalized to the untreated control (white bars). Data are shown as mean ± SD of at least n=3 independently performed experiments. $*/^{\#}p \leq 0.05$ and $^{\#\#}p < 0.01$ *using one-way ANOVA with Tukey's post-test*. Hashtags (#) indicate comparisons to the untreated control.

Compared to untreated $CD8^+$ T cells, TCR activation led to a significant decrease in the relative expression of *S1P4* mRNA ($p \leq 0.05$), as similarly observed for *S1P1* mRNA expression. Addition of IL-33 and/ or IL-12 did not further decrease the *S1P4* gene expression levels compared to TCR activated $CD8^+$ T cells (**Figure 30A**). In the absence of TCR activation, stimulation with IL-12 significantly reduced basal *S1P4* mRNA expression ($p \leq 0.05$). Addition of IL-33 reversed the IL-12 effect and significantly

increased *S1P4* mRNA expression ($p \leq 0.05$ for IL-12 vs. IL-33/ IL-12 co-stimulated T cells (**Figure 30B**). The regulation of S1P4 expression is to date poorly investigated but described to be impeded by pro-inflammatory signals. For the first time it was shown here that the signaling of IL-33 interferes in the IL-12-mediated downregulation of *S1P4* mRNA expression. Suppressor of cytokine signaling (SOCS) proteins 1 and 3 block IL-12-induced STAT4 activation[152]. It was therefore investigated whether IL-33 interferes in the IL-12 signaling through induction of SOCS1 or SOCS3 (**Figure 31**).

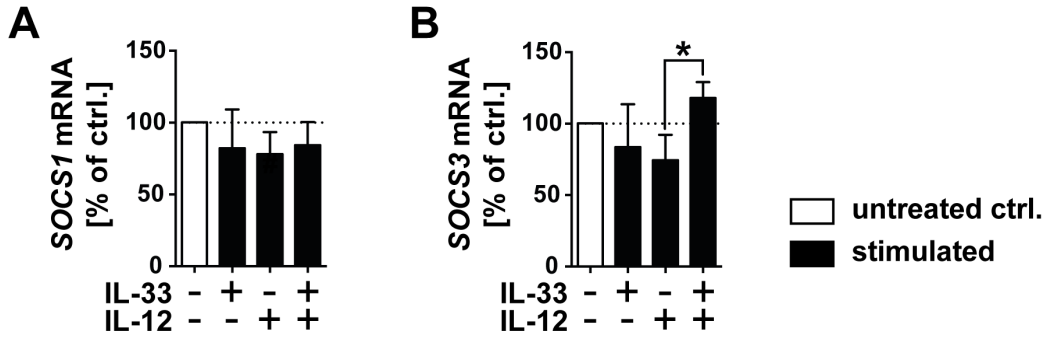

Figure 31: IL-33 induces *SOCS3* mRNA expression during co-stimulation with IL-12. Starved $CD8^+$ T cells co-stimulated with IL-33 and IL-12 prior qRT-PCR analysis of (A) *SOCS1* and (B) *SOCS3* mRNA expression. The expression levels were normalized to the untreated control set to 100% (white bars). Data are shown as mean ± SD of at least n=3 independently performed experiments. $*/^{\#}p \leq 0.05$ and $^{\#\#}p < 0.01$ *using one-way ANOVA with Tukey's post-test*. Hashtags (#) indicate comparisons to the untreated control.

Co-stimulation of starved $CD8^+$ T cells with IL-33 and IL-12 did not affect *SOCS1* mRNA expression levels (**Figure 31A)** but significantly induced expression of *SOCS3* mRNA compared to $CD8^+$ T cells treated with IL-12 ($p \leq 0.05$) (**Figure 31B**).

5.4. Investigations on the Role of IL-33 in Multiple Sclerosis

A central aspect of MS is the transmigration of autoreactive $CD8^+$ T cells through the blood brain barrier, which demyelinate the neuronal axons of the CNS[153]. Moreover, the disease is aggravated by a failure of the central and peripheral tolerance to re-induce the self-tolerance state, leading to persisting autoimmunity[153]. Although its role largely remains unexplored, elevated levels of IL-33 released from MS plaques have been detected in serum and cerebrospinal fluid of patients[154,155]. Based on the above presented findings, it was suggested that IL-33 affects the activity of autoreactive $CD8^+$ T cells or is involved in the differentiation of T_{reg}-like $CD8^+$ T cells in MS. IL-33-dependent differentiation of Treg-like CD8+ T cells required inhibition of mTORC1, and mTORC1 inhibitor rapamycin has been described to ameliorate disease progression[55,56]. It was thus tempting to investigate in a small group of RRMS patients treated with natalizumab, an inhibitor for transmigration of autoreactive T cells into the CNS, whether IL-33 might make a target of interest for research on the restoration of the effector T cell and T_{reg} balance in MS.

5.4.1. Demographic Details of Relapsing-Remitting MS Patients

Blood samples were collected at the Center for Neurology and Neurosurgery at the University Clinic Frankfurt/ Main, Germany, between 2017 and 2018 during the routine checkup (Dr. Robert Brunkhorst and others). RRMS was diagnosed by specialists from the department of neurology and patients were chosen respecting the exclusion and inclusion criteria (chapters 4.2.2.2 and 4.2.2.3). Blood samples from healthy controls were obtained from anonymous donors of the blood donation center (chapter 4.2.2.1). The demographic details of RRMS patients are listed in **Table 20**.

Table 20: Demographic details of RRMS patients.

	RRMS patients
n =	9
sex [m/f]	1 / 8
Age [mean]	37.7
Age [range]	28.8 – 47.2
Treatment	Natalizumab; integrin-α4 inhibitor
Disease duration [years, mean]	10.3
Disease duration [years, range]	2.3 – 19.6
EDSS [mean]	3.1
EDSS [range]	2 – 4
Disease progression [yes/ no; n]	4 / 5
Relapse free last 12 months [yes/ no; n]	3 / 6
Recent relapse [months, mean]	4.7
Recent relapse [months, range]	1 - 10

Abbreviation: EDSS; expanded disability status scale

5.4.2. Disbalances in the Frequencies of $CD8^{high}$ and $CD8^{low}$ in RRMS

PBMC of healthy donors or isolated from patients suffering from RRMS were examined by flow cytometry. The cells were cultured for 20h under serum deprived conditions before analysis of CD8 expression on the cell surface to determine the percentages of $CD8^{high}$ and $CD8^{low}$ of all $CD8^{+}$ cells (**Figure 32**).

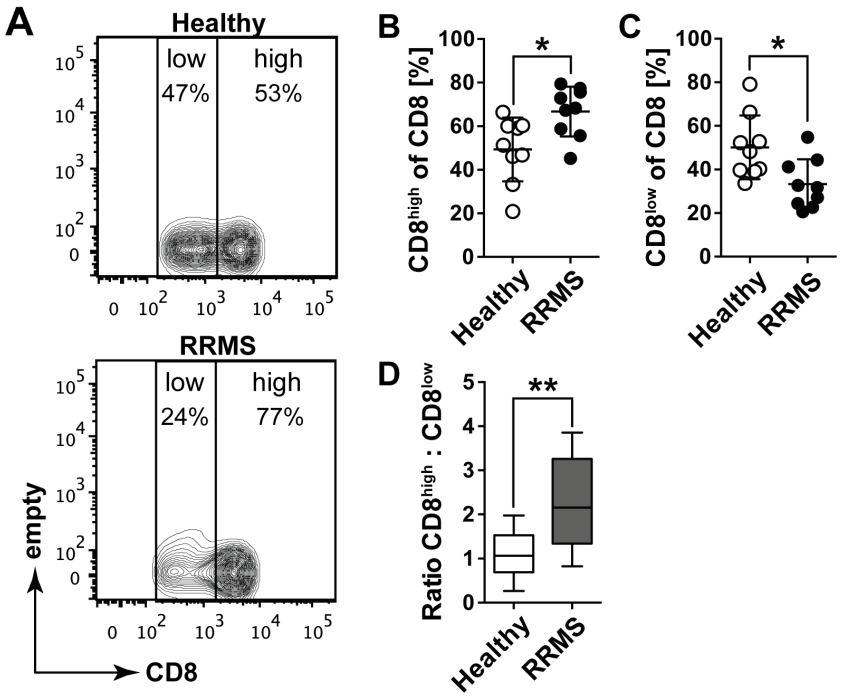

Figure 32: Differences in the frequencies of $CD8^{high}$ and $CD8^{low}$ in PBMC of healthy controls and RRMS patients. PBMC of healthy volunteers (white) or RRMS patients (black, gray) were cultivated for 24h under serum-free conditions before (A) analysis of CD8 expression by flow cytometry. Percentages of (B) $CD8^{high}$ and (C) $CD8^{low}$ of total $CD8^{+}$ cells were assessed for calculation of the (D) ratio of $CD8^{high}$ to $CD8^{low}$. Data are shown as mean ± SD of n=9 healthy and RRMS samples, respectively. *$p \leq 0.05$ and **$p < 0.01$ using *unpaired t-test*.

CD8 expression was examined on total PBMC of healthy controls and RRMS patients, discriminating between high and low expression (**Figure 32A**). Determination of the frequencies of $CD8^{high}$ and $CD8^{low}$ revealed a significantly altered distribution in comparison to the healthy control group. While in healthy controls, a mean percentage of 49.3 ± 14.6% of $CD8^{high}$ was assessed, a significantly higher percentage of $CD8^{high}$ was detected in samples of RRMS patients (66.7 ± 11.4%; $p \leq 0.05$) (**Figure 32B**). Consequently, the percentage of $CD8^{low}$ was significantly decreased in RRMS compared to the healthy control group (RRMS 33.3 ± 11.4%; healthy 50.2 ± 14.7%; $p \leq 0.05$) (**Figure 32C**). Healthy samples revealed an equal ratio of $CD8^{high}$ to $CD8^{low}$ (1.1 ± 0.5), while a significant shift in the ratio of $CD8^{high}$ to $CD8^{low}$ was observed for PBMC isolated from RRMS patients (2.3 ± 1.0) (**Figure 32D**).

Having observed that the balance of $CD8^{high}$ to $CD8^{low}$ cells was significantly altered in RRMS samples, it was next investigated if this disruption could be similarly observed for expression of ST2L on both CD8 subsets.

Therefore, expression of ST2L on CD8^{high} and CD8^{low} was assessed by flow cytometry (**Figure 33**).

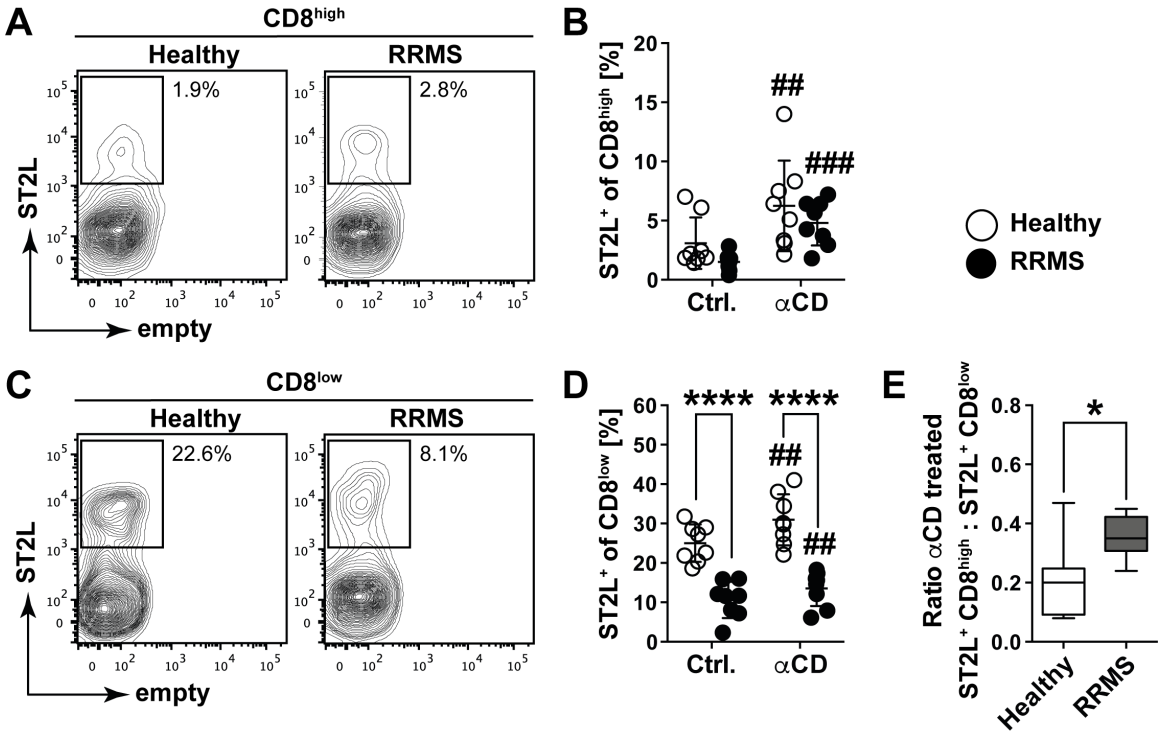

Figure 33: Expression of ST2L on CD8^{high} and CD8^{low} in PBMC of RRMS patients. PBMC of healthy volunteers (white) or RRMS patients (black, gray) were cultivated for 24h under serum-free conditions before flow cytometry analysis of ST2L expression on untreated (A) CD8^{high} and (B) percentages of ST2L$^+$ CD8^{high} upon TCR activation (αCD). (C) ST2L expression on untreated CD8^{low} and (D) percentages of ST2L$^+$ CD8^{low} after αCD stimulation. Data are shown as mean ± SD of n=9 healthy and RRMS samples, respectively. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and ****$p < 0.0001$ using *unpaired t-test* for comparisons between healthy controls and RRMS samples (*) and *paired t-test* for comparisons to the respective untreated control ($^{\#}$; ctrl.).

ST2L expression was examined by flow cytometry on CD8^{high} and CD8^{low} of healthy and RRMS PBMC. Herein, no significant differences in the percentages of ST2L$^+$ CD8^{high} from either untreated control subjects (3.1 ± 2.2%) or RRMS patients (1.5 ± 0.7%) were detected (**Figure 33A, B**). TCR activation conferred an equal increase in the amount of ST2L$^+$ CD8^{high}. On the other hand, a significantly reduced percentage of ST2L$^+$ CD8^{low} was determined in the untreated RRMS samples (10.6 ± 4.6%) compared to the healthy control (25.0 ± 4.8%; $p < 0.0001$) (**Figure 33C, D**). TCR stimulation significantly increased the percentages of ST2L$^+$ CD8^{low} in both healthy and RRMS samples, revealing no further differences related to TCR stimulation.

To determine possible imbalances in the proliferation of effector T cells, the ratio of $ST2L^+$ $CD8^{high}$ to $ST2L^+$ $CD8^{low}$ after TCR stimulation was determined (**Figure 33E**). Herein, the ratio of $ST2L^+$ $CD8^{high}$ to $ST2L^+$ $CD8^{low}$ was significantly higher in PBMC isolated from RRMS patients compared to samples of healthy controls ($p \leq 0.05$).

This first evidence from this pilot study with a small number of RRMS patients strongly suggests further investigations focusing on the role of IL-33 as a possible object of interest for further research on the restoration of anti-inflammatory immunity in the autoimmune disease of MS.

VI. Discussion

Since the discovery of IL-33 more than a decade ago, efforts have been made to understand its function in the modulation of immune responses. While the cytokine has been associated with adaptive type 2 immune responses in the first reports, more recent studies showed that IL-33 also contributes to the activity of cytotoxic $CD8^+$ T cells, the T_H1 activity of $CD4^+$ T cells and to the anti-inflammatory function of T_{reg}[156,157,96].

An important aspect for the function of IL-33 is whether the cytokine re-activates circulating T lymphocytes or acts as a local mediator. Despite numerous patient studies associating high serum levels of IL-33 to disease severity[79,126,158,159], little is known about the systemic function of IL-33. Therefore, the first part of this thesis aimed to clarify a potential role for systemic IL-33 in the activation, differentiation and effector function of T lymphocytes. However, to date, the precise conditions guiding the IL-33 mediated induction of differentiation pathways in different T cell subsets remain unknown. Hence in the second and third part of this thesis, it was investigated how inhibition of mTOR during nutrient withdrawal, TCR activation and stimulation with co-factor IL-12 affect the IL-33-dependent initiation of distinct differentiation programs in $CD8^+$ T cells. As a central aspect for the functionality of effector and regulatory T cells, it was furthermore asked if IL-33 alters expression patterns involved in the peripheral trafficking of $CD8^+$ T cells. For example, CCR7-mediated homing to SLO, CD69-dependent tissue retention and S1P receptor mediated recirculation.

MS is a condition in which autoreactive $CD8^+$ T cells are re-activated, transmigrate into the CNS and destroy the neuronal myelin sheath[153]. Reportedly, IL-33 is secreted in large amounts from cerebral MS plaques[83]. In the last chapter, the potential role of IL-33 in MS was addressed in a translational pilot study. Based on the findings concerning the local function of IL-33 in T cell differentiation and migration, the aim was to investigate whether IL-33 might be clinically relevant in MS and a possible object of interest for future investigations in a large cohort patient study.

6.1. IL-33 is a Local Regulator of Adaptive Immunity

Although high concentrations of IL-33 are detectable in the blood of patients with atopic, autoimmune or cancer diseases[79,126,158,159], little is known about the ability of systemic IL-33 to modulate inflammation. The reason why the systemic function of IL-33 is poorly described is that IL-33 concentrations are determined by ELISA, an antibody-based detection technique. Bioactivity of IL-33 is however controlled by proteolytic processing, by receptor binding or oxidation[99,102]. These might lead to disruption of the epitope, a specific amino acid sequence recognized by monoclonal antibodies. It was therefore first investigated in this chapter whether IL-33 is biologically active in blood and in the following if the soluble decoy receptor sST2 efficiently blocks IL-33 bioactivity.

6.1.1. IL-33 detected in Serum by ELISA is Inactive

To investigate the potential systemic function of IL-33, it was first investigated whether IL-33 detected by ELISA is bioactive. It was hypothesized that due to modifications of the IL-33 molecule after release, the binding sites for monoclonal antibodies, the epitopes, might be inaccessible. The disadvantage of detecting IL-33 in blood by ELISA might thus be that no conclusions on the systemic function of the cytokine can be drawn from the elaborate, published patient studies. To begin with, a recombinant bioactive and inactive isoform of IL-33 was generated.

While inflammation drives proteolytic maturation of extracellular IL-33, apoptotic signaling cascades result in activation of caspase-3 that cleaves human intracellular IL-33 at an aspartic acid on position 178, generating the inactive isoform IL-$33_{179\text{-}270}$ [87,88]. IL-$33_{179\text{-}270}$ and IL-$33_{95\text{-}270}$ produced *in vivo* by chymases[3] were recombinantly generated. IL-33-induced T_H1 inflammation is associated to NF-κB activation[110]. Therefore, a commercially available NF-κB-dependent reporter gene detection system for bioactive IL-33 was used to validate the bioactivities of the recombinant IL-33 isoforms generated

in vitro. Fulfilling the expectations, IL-33$_{95\text{-}270}$ induced a time- and dose-dependent substrate conversion by NF-κB-inducible SEAP **(Figure 7C)**, whereas recombinant IL-33$_{179\text{-}270}$ was not bioactive and subsequently served as a negative control for the following experiments **(Figure 7D)**.

Monoclonal capture antibodies are specific for unique epitopes within an antigen. Disruption of the epitope consequently results in loss of the antibody binding. It was suspected here that measurement of different IL-33 isoforms by antigen-based detection systems such as ELISA is technically limited, resulting in assessment of concentrations that most likely differ from the actual *in vivo* situation. This assumption was based on the knowledge that the IL-33 cytokine structure is modified by cleavage, oxidation or binding to its soluble receptor, eventually leading to conformational changes and inaccessibility or loss of epitopes for antibody binding. Therefore, not surprisingly, the bioactive isoform IL-33$_{95\text{-}270}$, but not inactive IL-33$_{179\text{-}270}$, was detectable by ELISA **(Figure 8)**. The soluble IL-33 receptor sST2 represents a proposed control mechanism for the bioactivity of extracellular IL-33 at the level of receptor binding[102]. IL-33 complexed by sST2 was not detectable, whereas sST2 bound to IL-33 was still recognized by specific ELISA **(Figure 9)**. The circumstance that IL-33 inactivated by cleavage or bound by sST2 remained invisible to the ELISA used here, probably causes a discrepancy in the determined concentrations of the cytokine and the actual amount of IL-33 in blood as the amount of IL-33 that is cleaved and free or bound by sST2 remains unknown. However it is clear that more investigations on the functions of sST2 for the bioactivity of IL-33 are to be done.

To investigate if IL-33 detected in blood samples by ELISA is biologically active, 30 samples from healthy volunteers were analyzed. Although declared as healthy, varying, high concentrations of IL-33 ranging from 23 to 7440 pg/ml were detected in the sera of 18 of 30 healthy volunteers **(Figure 10)**. A likely cause for these varying concentrations is the high prevalence of allergic diseases in the western world. Importantly, allergic persons are not excluded from blood donation. IL-33 is strongly involved in the activation of type-2 inflammation and in the pathogenesis of atopic diseases[157,79]. High concentrations of IL-33 correlating with disease severity have indeed been detected in the sera of patients suffering from asthma[160] and allergic rhinitis[157,161]. In 2016, almost 20% of the German population

aged between 18 and 79 years suffered from at least one type of allergy[162]. In the present experiment, 6 of 30 serum samples showed unusually high amounts of IL-33 in serum (1870 – 7440 pg/ml), representing 20% of the donors. As the anonymous donors were not tested for the allergy marker IgE, allergic and non-allergic persons had not been distinguished. Besides allergic diseases, high expression of IL-33 has also been linked to obesity, another widespread disease in western civilization[163,164]. Still, the high concentrations of IL-33 in blood were surprising, because IL-1 family cytokines are usually described to induce potent inflammatory signals already at low concentrations in circulation[102]. In contrast to the high concentrations of IL-33 detected in the serum samples, those same samples exerted no biological effects on the HEK293-ST2L reporter cells (LLOQ corresponding to 75 pg/ml), indicating total inactivity of circulating IL-33 at least in a healthy state **(Figure 10)**. Further investigations focusing on the mechanisms promoting inactivation of IL-33 in blood would involve immunoprecipitation of IL-33 in order to determine whether the cytokine is inactive due to proteolytic processing or oxidized. As IL-33 reportedly functions as an alarmin, it was asked whether its bioactivity is generally limited in blood to avoid systemic inflammation.

6.1.2. Bioactivity of Exogenous IL-33 is Reduced in Blood

Assuming that bioactivity of IL-33 is generally reduced in blood would consequently mean that IL-33 exerts its effects mainly in tissue during a short time frame after release. Interestingly and in support, bioactivities of both exogenous IL-$33_{112\text{-}270}$ and IL-$33_{95\text{-}270}$ were significantly reduced in human plasma **(Figure 11)**. Although oxidation of IL-33 has been reported to promote conformational changes and disruption of the ST2L binding sites[99], oxidation, but also proteolytic inactivation were excluded as reasons for the reduction. Measurement of the cellular metabolic activity of the HEK293 cells using an MTT assay revealed no cytotoxic effects of the plasma samples used in the experiment *(data not shown)*. sST2 is constitutively expressed and released by immune cells like mast cells, that are assigned to T_H2 immunity. Therefore, and because sST2 concentrations were shown to be high in the samples of the healthy individuals used here **(Figure 10)**, it was

asked if the decrease in bioactivity of exogenously added recombinant IL-33 resulted from binding to the naturally occurring sST2. Using microscale thermophoresis, binding affinities of sST2 to isoforms IL-$33_{112\text{-}270}$, IL-$33_{95\text{-}270}$ and IL-$33_{179\text{-}270}$ were determined **(Figure 12)**. In this experiment, IL-$33_{112\text{-}270}$ and sST2 bound with a dissociation constant (K_D) of 2.2 ± 1.1, and IL-$33_{95\text{-}270}$ and sST2 with a K_D of 13.5 ± 2.9 nM. Comparable to this observation, Lingel and colleagues determined a KD of 4 nM for the complex of IL-$33_{112\text{-}270}$ to sST2 and an affinity of 76 nM for the binding of the IL-33/ sST2 complex to extracellular region of IL-1RAcP in a 1:1:1 stochiometry[165]. Based on the high binding affinities of sST2 towards IL-$33_{95\text{-}270}$ and IL-$33_{112\text{-}270}$ it was expected that the decoy receptor traps IL-33 and prevents the cytokine from binding to ST2L. Recombinant sST2 however failed to inhibit IL-33 bioactivity completely. For a 50% inhibition, a 100-fold molar excess of sST2 was required. Interestingly, addition of supposedly neutralizing antibodies achieved similar results and surprisingly rather increased bioactivity of IL-$33_{112\text{-}270}$ at low concentrations **(Figure 13)**. Within this experiment, sST2 and ST2L with its co-receptor IL-1RAcP had been set in competition for binding to IL-33. Recruitment of the co-receptor is described not only to be essential for the IL-33 signaling, but moreover to stabilize the interaction between ST2L and its ligand[86]. Interestingly, Palmer and colleagues generated a recombinant soluble form of IL-1RAcP, which associated to the sST2/ IL-33 complex and increased the inhibitory capacity of sST2[141]. As to date no naturally occurring soluble isoforms of IL-1RAcP have been found, it is tempting to speculate that the co-receptor increases the binding affinity of IL-33 towards the membrane-bound ST2L, exceeding the high affinity of IL-33 towards sST2 resulting in preferential binding to the signaling receptor.

Previous studies addressed the hypothesis of a secondary function of sST2 as a reservoir for bioactive IL-33 like the sIL-6R for IL-6[101], protecting the cytokine from modifications decreasing its bioactivity. This function would however require proximity of the sST2/ IL-33 complex to $ST2L^+$ cells and a prompt delivery of the cytokine. In this context, IL-1RAcP as an "enhancer" of the IL-33-ST2 binding affinity might facilitate the transfer of IL-33 from sST2 to ST2L. Yang and colleagues developed small molecules that promote release of IL-33 from circulating sST2[103], a tool which might in the future gain importance to respond to the question whether sST2 prolongs the half-life of IL-33.

6.1.3. Activation of T cells Promotes Transcriptional Expression of *sST2*

It was next found that activation of the adaptive and innate immunity promoted a differential expression of *ST2L* and *SST2* mRNA. In spite of large sequence homologies, mRNA transcripts of *ST2L* and *SST2* can be specifically discriminated due to regions distinctive for the ST2 isoforms. Therefore, qRT-PCR probes spanning the ST2L specific exons 10 to 11, or the sST2 specific exons 1 to 2 were used for the discrimination of the receptor isoforms **(Figure 14)**. TCR stimulation activates T cells, the regulatory of adaptive immunity. On the other hand, the bacterial cell surface component LPS is recognized by TLR4, a pattern recognition receptor expressed e.g. on APC and B cells. Therefore LPS directly activates cells of the innate immune system[166]. Examining the *ST2* mRNA expression of PBMC, TCR activation, but not LPS stimulation, led to a significantly higher expression of *SST2* mRNA compared to *ST2L* mRNA. Those data led to the assumption that *SST2* mRNA expression is a direct answer to T cell activation. In line with these data, increased plasma sST2 levels were observed to positively correlate with $CD8^+$ T cell count and activation observed during early human immunodeficiency virus, dengue virus or hepatitis B infection[167–170]. On the other hand, epithelial cells have previously been identified as the source of secreted sST2 upon LPS stimulation. Mildner *et al.* found that *in vivo* LPS stimulation in humans triggered a short-term inflammatory response, followed by strong enhancement of sST2 secretion primarily by cardiac myocytes and lung alveolar epithelial cells[139]. According to that, activation of the T cells as a part of the adaptive immune system would thus induce immune cell derived expression of sST2, whereas activation of the innate immune system would promote sST2 secretion from epithelial and mast cells, for example. Although in the end, the reasons for the observed downregulation of IL-33 bioactivity in blood remain to be further analyzed, the findings presented here support the hypothesis that systemic IL-33 is not bioactive. IL-33 released into the extracellular space is most likely rapidly inactivated through proteolytic processing or oxidation to avoid long-term systemic inflammation. Consequently, IL-33 would exert local functions as a regulator of adaptive immunity, an important feature for the modulation of tumor infiltrating or autoreactive $CD8^+$ T cells in autoimmune diseases like MS.

6.2. IL-33 drives Activity of CD8$^+$ T Effector and Differentiation of CD8$^+$ T_{reg} Cells

Activation and proliferation of CD8$^+$ T cells is highly dependent on their metabolism and availability of nutrients in their environment. T cell generation, differentiation and development is centrally regulated by mTOR, a sensor for nutrient deprivation and a key regulator of innate and adaptive immunity. For this reason, modulation of the mTOR signaling pathway for resolution of immune mediated diseases and induction of anti-cancer immunity has recently gained importance[11]. The IL-33 signaling, which depending on the presence of co-factors in the microenvironment, promiscuously promotes expansion of functionally opposing T cell populations associated to T_H1 or cytotoxic, T_H2 and T_H17 immunity versus suppressive T_{reg}.

From the first part of this experimental thesis, it was concluded that IL-33 must be rapidly inactivated upon entry into blood. It was therefore reasoned that hypothetically, IL-33 would most likely exert its effects on CD8$^+$ T cells in close proximity of its source, e.g. necrotic cells and in defective barrier sites, in tissue. *In vivo*, mTOR is inhibited by nutrient deprivation, a phenomenon occurring by irregular perfusion and uncontrolled growth of tumor mass, an effect influencing the activity and fitness of both cancer cells and infiltrating CD8$^+$ T cells[57,58]. Pharmacological inhibition of mTORC1 proved to be beneficial for the course of autoimmune diseases by promoting expansion and accumulation of T_{reg}[171]. It was asked in the following whether the function of IL-33 on CD8$^+$ T cells is modulated or influenced by the mTOR pathway.

Starvation is a critical state in which only proteins essential for the survival of the cell are synthesized. Nutrient deprivation here resulted in responsiveness of human CD8$^+$ T cells to IL-33 by strong expression of ST2L (**Figure 15A**), suggesting that the IL-33 signaling pathway might play a role in the activity, differentiation or survival of CD8$^+$ T cells during starvation. Induction of ST2 expression during starvation has also been observed by Kunze and colleagues. They found that under translation-inhibitory conditions, translation of *SST2* mRNA was initiated cap-independently, as the *ST2* gene contains an internal ribosome

entry site (IRES)[172]. Although investigated not further, it is tempting to speculate that besides sST2, ST2L expression is also induced IRES-dependently in $CD8^+$ T cells. Besides induction of ST2L expression, $CD8^+$ T cells underwent further phenotypic changes under nutrient withdrawal. Next to $CD8^+$ T cells with CD8 expression levels comparable to naïve T cells ($CD8^{high}$), surprisingly a second population emerged with a significantly reduced expression of CD8 ($CD8^{low}$) (**Figure 15D, E**). Downregulation of CD8 has been controversially discussed to either increase effector activity during viral infection, or to reduce responsiveness towards TCR dependent activation[173–175]. An explanation arguing for low responsivity of $CD8^{low}$ towards TCR activation would be that the CD8α chain is required as a co-receptor for the TCR to stabilize interaction with MHC-class I[146]. A reduced expression of CD8 would therefore result in impairments in the TCR-MHC-class I interaction. Phenotypical characterization of both $CD8^{high}$ and $CD8^{low}$ by flow cytometry revealed that the population of $ST2L^+$ $CD8^{high}$ T cells mostly contained naïve $CD45RA^+RO^-$ T cells (**Figure 16A, D**). In contrast, $ST2L^+$ $CD8^{low}$ expressed reduced levels of CD45RA and co-expressed CD45RO (**Figure 16B, E**). CD45RO is a classical marker for memory T cells, but is also expressed on $CD25^{high}$ $FoxP3^{high}$ activated T_{reg}, a highly proliferative subset with potent suppressive activity[176,177]. Moreover, TCR activation led to a significant increase in the ratio of $CD8^{high}$ to $CD8^{low}$, an effect possibly relatable to proliferation of $CD8^{high}$ as effector cells (**Figure 17A**). Concurrently, TCR-independent stimulation significantly decreased the ratio of $CD8^{high}$ to $CD8^{low}$, implicating a predominance of $CD8^{low}$ (**Figure 17B**). Based on this finding, it was conceived that $CD8^{high}$ might be able to exert effector functions, whereas a regulatory function was postulated for $CD8^{low}$.

Supporting this hypothesis, $CD8^{high}$ expressed T-bet (**Figure 18A**), a transcription factor essentially driving T_H1 immunity. Interestingly, T-bet and STAT4 have previously been found to promote expression of ST2L on $CD4^+$ T_H1 effector cells, explicitly linking IL-33 to T_H1 immunity[114]. Additional studies revealed an IL-33-driven positive feedback loop, in which the IL-33/ ST2L signaling itself promoted T-bet and STAT4 to bind to the *il1rl1* gene locus resulting in increased expression of ST2L in T_H1 cells[115,116]. As a part of its function in T_H1 immunity, T-bet expression is enhanced by IL-12 and IFNγ in a positive feedback loop, further increasing the cytotoxic function of effector T cells [178,179].

Concurrently, TCR activation of $CD8^+$ T cells with IL-12 and IL-33 stimulation further enhanced transcriptional expression of the *TBX21* gene (**Figure 18E**). For murine $CD8^+$ T cells, Yang and Ottenlinger independently demonstrated that the TCR-, IL-33- and IL-12-signaling synergize and enhance IFNγ secretion[94,95,112]. The pro-inflammatory function of IL-33 observed before for murine $CD8^+$ T cells was proven here respectively for human $CD8^{high}$ T cells, as TCR activation and co-stimulation with IL-33 and IL-12 analogously promoted secretion of IFNγ protein (**Figure 18F**) and expression of cytotoxicity and marker CD107a (**Figure 19**) on the cell surface as a result of degranulation. With this, IL-33 was shown to enhance the TCR-mediated induction of effector immunity in $CD8^+$ T cells, and to overcome the immunosuppressive effect of starvation.

In contrast to $CD8^{high}$, $CD8^{low}$ expressed only low levels of T-bet protein, suggesting properties of $CD8^{low}$ independent from T-bet (**Figure 18A**). T-bet expression indirectly prevents induction of an endogenous T_H2 differentiation program during initial T_H1 cell responses[179], which might explain the differing functions of $CD8^{low}$ observed in the following compared to the above described features of $CD8^{high}$. Evidence supporting the hypothesis that $CD8^{low}$ do not exhibit T_H1-dependent functions was found when revealing that in the absence of TCR activation, IL-33 stimulation significantly inhibited the IL-12-induced increase in intracellular Granzyme B protein levels (**Figure 20B, C**). Kienzle *et al.* suggested that the $CD8^{low}$ phenotype is an alternative differentiation pathway for naïve $CD8^+$ T cells. Their assumption was based on the finding that T_H2 polarization generated high numbers of poorly cytolytic $CD8^{low}$ T cells expressing low amounts of perforin and GrzmB[180]. An important finding supporting the hypothesis of Kienzle and colleagues was that IL-33 and IL-12 co-induced expression of T_{reg} transcription factor FoxP3 and T_H2 transcription factor GATA3, on both transcriptional and protein levels in $CD8^{low}$ T cells (**Figure 21**). IL-33 was described to be essential for *in vivo* differentiation, accumulation and maintenance of $ST2L^+$ $FoxP3^+$ T_{reg} in inflamed tissue, acting as a co-factor of TGFβ[97]. Further data by Alvarez *et al.* described that expression of ST2L conferred resistance to losing FoxP3 expression, inhibiting transition of the T_{reg} to a T_H17 effector phenotype[124]. IL-33 here enhanced on the one hand the TCR/ IL-12-induced expression of T-bet and promoted cytotoxic effector functions. On the other hand, IL-33 stimulation mediated a $GATA3^+$ $FoxP3^+$ T_{reg}-like phenotype in $CD8^+$ T cells in the absence of TCR activation.

It was lately postulated that IL-33 induces GATA3 and STAT5, which leads to activation of the p38/ MAPK pathway and subsequently to activation of mTOR, the central regulator of T cell differentiation[96]. IL-33 has already been described to directly activate mTORC1 through activation of the PI3K subunit p110δ in murine T_H2 cells[128]. Further investigations revealed that pharmacological inhibition of the mTOR pathway in T_H2 and ILC2 cells in turn blocked an IL-33-induced expression of IL-5 and IL-13[156]. Interestingly, Deason and colleagues recently provided experimental proof that adaptor protein PIK3AP1 is directly recruited to TIR domain containing receptors and links the IL-1 signaling to the PI3K-mTOR pathway. This mechanism was held responsible for providing pathogenic T_H17 and T_H1 cell differentiation in EAE[71]. On the other hand, PIK3AP1 was described to brake potentially excessive TLR4-dependent immune responses[181]. Herein, *PIK3AP1* mRNA was basally expressed in $CD8^+$ T cells and was not significantly altered during starvation. While TCR-stimulation did not modulate *PIK3AP1* mRNA expression, co-stimulation with IL-33 and IL-12 led to a significant increase in the mRNA expression levels of *PIK3AP1* (**Figure 22**).

Based on this finding it was questioned whether ST2L expression was established during starvation to enable IL-33-dependent reactivation of the mTOR pathway. Activation of mTORC1 was indirectly assessed through phosphorylation of the S6K subunit p70, activity of mTORC2 through determination of the mTORC2-specific phosphorylation site Ser473 of Akt (**Figure 23**). IL-33 and/ or IL-12 stimulation neither re-activated mTORC1, nor enhanced the activation status of mTORC2. It is tempting to speculate that starvation induces IL-33 responsiveness to avoid antigen-unspecific inflammation under conditions limiting fitness and survival of immune cells. It seems likely that re-activation of mTORC1 would lead to an increased metabolic activity and accelerated cell death due to the absence of nutrients. In contrast, mTORC1 re-activation would be required to perform efficient effector functions upon antigen-dependent stimulation related to e.g. viral infection, representing an acute "life-threatening" situation. These findings are supported by an observation in a murine model, in which IL-33 ameliorated inflammation during experimental colitis by facilitating T_H2/ T_{reg} responses. Interestingly, this effect was related to inhibition of mTORC1, as autophagy was enhanced in macrophages[182]. In summary, IL-33 was shown to exert a dual function for $CD8^+$ T cells. As presented here, starvation

leading to inhibition of mTORC1resulted in responsiveness of $CD8^+$ T cells to IL-33. Although reported for T_H2 and ILC2 cells, IL-33 failed to re-activate mTORC1 or to enhance mTORC2 activity. IL-33 induced potent cytotoxic effector functions related to T-bet expression and helped to overcome the immunosuppressive effects of nutrient deprivation in order to respond to acute inflammation. Importantly, IL-33 counteracted induction of energy-consuming antigen-independent inflammation and induced expression of GATA3 and FoxP3 related to a T_{reg}-like $CD8^+$ T cell phenotype (**Figure 34**).

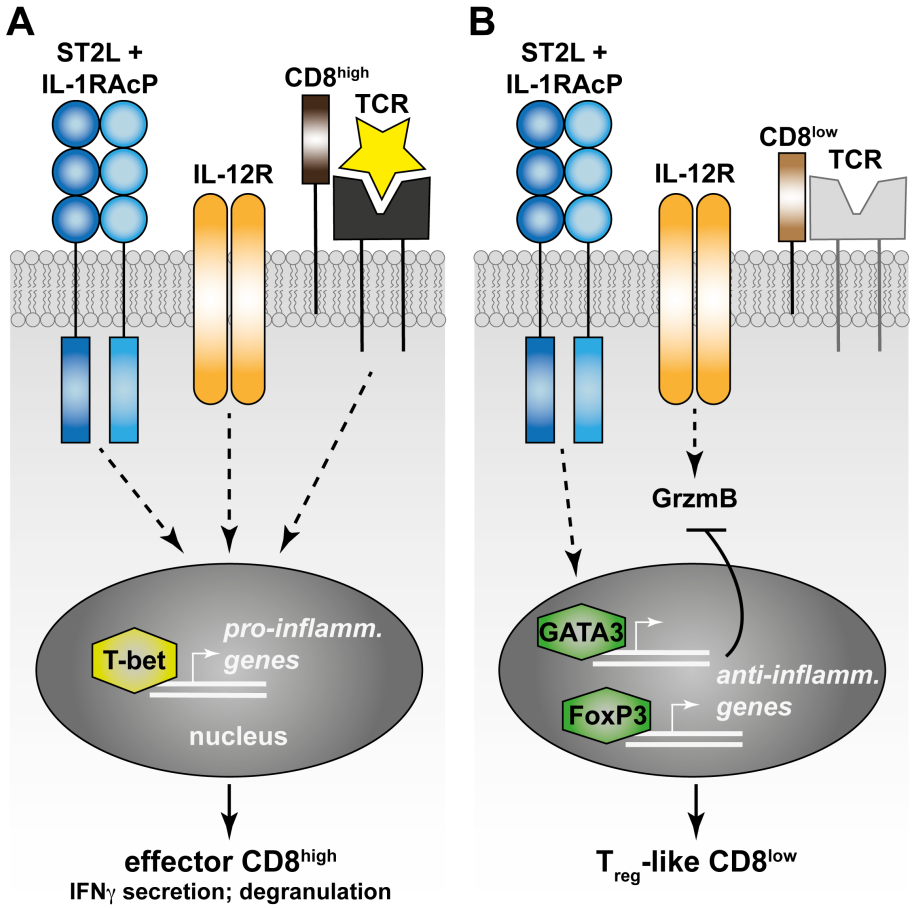

Figure 34: IL-33 promotes activation of cytotoxic effector and differentiation of T_{reg}-like $CD8^+$ T cells. $CD8^+$ T cells become responsive to IL-33 during starvation through expression of ST2L on the surface. (A) The IL-33 signaling synergizes with the IL-12R and TCR leading to expression of T-bet-dependent in $CD8^{high}$. Expression of T-bet is associated to the transcriptional expression and secretion of IFNγ and mediates degranulation. (B) In the absence of a TCR stimulus, IL-33 induces expression of GATA3 and FoxP3 in $CD8^{low}$ T cells. The IL-33 signaling furthermore counteracts an IL-12-mediated increased expression of granzyme B (GrzmB).

Taken together, the property of IL-33 to promote differentiation of T_{reg}-like $CD8^+$ T cells involved in homeostasis during limited nutrient availability might provide positive side-effects during the treatment of autoimmune diseases with the approved mTORC1 inhibitory drug rapamycin. In fact, mTORC1 inhibitors like rapamycin or sirolimus are currently undergoing clinical trials for the treatment of diabetes mellitus (112 studies) or MS, autoimmune diseases in which IL-33 has been suggested to ameliorate disease severity[123,183–186].

6.3. Starvation Results in CCR7 Expression on $CD8^+$ T cells

Effector and regulatory T cells *in vivo* need to be distributed in lymphoid and non-lymphoid tissues to efficiently accomplish their tasks. This requires a complex interaction between immune cells and chemokines promoting attraction to SLO, then prolonged retention to assure adequate T cell activation, followed by emigration and extravasation through the blood vessel wall to the site of inflammation[187]. This highly complex process is orchestrated by differential expression of chemokine receptors, adhesion molecules and S1P receptors on T cells. Interestingly, the activation status of mTOR has been proven to be decisive for the modulation of T cell trafficking[29,188]. IL-33 has been described to promote egress of ILC2 from bone marrow[131], to induce neutrophil migration[126] or to induce active migration of $ST2L^+$ $CD4^+$ $FOXP3^+$ T_{reg} to tumor tissue[127]. In this part of the project, it was investigated which impact IL-33 stimulation has on the migratory behavior of $CD8^+$ T cells expressing ST2L during starvation. To investigate this question, the dynamic expression of SLO homing receptor CCR7, of cell adhesion and LN retention molecule CD69 as well as of the S1P receptors mediating SLO egress, was analyzed. Mechanistically, binding of the ligands CCL19 and CCL21 to CCR7, which are constitutively expressed by HEV, induces migration of lymphocytes to SLO[32,147]. Activated effector T cells lose expression of CCR7 and with this their ability to access SLOs through HEV[27]. Starvation of untreated $CD8^+$ T cells promoted a significant increase in the expression of CCR7 on T_{reg}-like $ST2L^+$ $CD8^{low}$, and an evenly high CCR7 expression

on effector type ST2L$^+$ CD8^{high}. Expression of tissue retention marker CD69 was not detected on either population (**Figure 24**). *In vivo*, following CCR7-dependent migration of T cells to SLO, the cell adhesion molecule and early activation marker CD69 mediates retention within lymphoid organs and peripheral tissue[37]. Induction of CD69 expression requires TCR-dependent antigen recognition and stimulation with pro-inflammatory cytokines like IFNα, IFNβ or TNFα[189]. It was therefore not surprising to find that only TCR-dependent stimulation of the CD8$^+$ T cells mediated expression of CD69 on CD8^{high}, implicating induction of tissue retention (**Figure 25B**). IL-33 further significantly enhanced expression of CD69 on CD8^{low} (**Figure 25D**). Interestingly, CD69 has strongly been associated to secretion of TGFβ, a key cytokine involved in T_{reg} differentiation, and is expressed on T_{reg} precursors leaving the thymus[37,39]. As IL-33 was here associated to a T_{reg}-like differentiation of CD8^{low}, CD69 expression might be a component supporting the IL-33-dependent differentiation of the CD8$^+$ T_{reg} – a hypothesis requiring further investigation.

The CD69-dependent T cell retention is mediated by formation of a complex between CD69 and S1P1, in consequence negatively regulating the egress function of S1P1[37,39]. The S1P-dependent migration is driven by a compartment-specific distribution and gradients of S1P[40]. Concurrently, induction of S1P1 expression after T cell activation enables their egress from SLO into the periphery[45]. As during starvation, the ability of the CD8$^+$ T cells to migrate CCR7-dependently to LN might be enhanced, it was expected here that the S1P receptor expression would be reduced. Since reliable antibodies for detection of S1P receptors are currently not available, expression of *S1P1*, *S1P3, S1P4* and *S1P5* was determined on mRNA levels (**Figure 26**). Although highly expressed in CD8$^+$ T cells before cultivation, gene expression of *S1P1* and *S1P4* was significantly downregulated during nutrient deprivation. *S1P3* and *S1P5* mRNA concentrations were however below the limit of detection. *S1P1* gene expression is transcriptionally regulated by KLF2, a transcription factor connecting the nutrient sensing pathways and regulation of T cell trafficking[29,41]. Besides the *S1P1* gene, KLF2 transcribes the *CCR7* gene and represses expression of pro-inflammatory CXC chemokine receptor 3[29,148]. In spite of their common transcription factor, *S1P1* mRNA levels were negatively associated to high CCR7 protein expression. The *KLF2* mRNA expression levels were significantly induced upon 40h of

cultivation under nutrient deprivation (**Figure 27**), implicating to date unknown mechanisms modulating the transcriptional activity of KLF2 favoring either expression of CCR7 or S1P1. Indeed, it is an open question how S1P1 signaling interacts with signaling through CCR7, and it remains unknown if different T cell subsets are differently dependent on S1P signaling[40]. Sinclair and colleagues showed that pharmacological inhibition of mTORC1 with rapamycin concurrently led to re-expression of KLF2, and the cells regained the ability to migrate to LN. Those changes in the migratory behavior were attributed to a restored expression of CCR7 in rapamycin treated T cells. Liu and colleagues earlier suggested that mTOR is a downstream target of the CCR7/ CCL19 signaling pathway[190]. Others supported this hypothesis by showing that the CCR7 signaling pathway activated the mTOR signaling in DC, resulting in a prolonged survival of the cells[191]. The possible role of CCR7 in the modulation of the immunometabolism might be an explanation for the preferential transcription of CCR7 in starved $CD8^+$ T cells by KLF2, rather than of S1P1. The anti-inflammatory effects of rapamycin have in the past been partially explained by re-expression of KLF2 mediated by the drug[148]. Moreover, Pabbisetty and colleagues suggested KLF2 to be essential for the generation of antigen-induced or peripheral $CD4^+$ $FoxP3^+$ T_{reg}[192]. Therefore, KLF2-induced CCR7 expression might support induction of the T_{reg}-like $CD8^{low}$ phenotype and explain the higher expression of CCR7 on $CD8^{low}$.

6.4. IL-33 Modulates mRNA Expression of *S1P1* and *S1P4*

S1P1 and S1P4 promote differential functions in immune cells. While S1P1 has in the first line been described to regulate the egress of $CD8^+$ T cells from SLO, evidence arose that S1P4 contributes to the differentiation and maintenance of T_{reg}. This chapter focused on the question, if IL-33 exploits the S1P/ S1P1/ S1P4 signaling to support the above discussed induction of a T_{reg}-like phenotype in starved $CD8^+$ T cells.

6.4.1. *S1P1* mRNA Expression is suppressed during T_{reg}-like Differentiation

In line with previous reports[193], TCR activation of starved $CD8^+$ T cells resulted in downregulation of *KLF2* mRNA, which was concurrent with a reduction in the transcriptional expression of S1P1 (**Figure 28A, B**). IL-33 did not further reduce or restore mRNA expression of *KLF2* or *S1P1* in TCR-activated $CD8^+$ T cells. Of highest interest for the following experiments, IL-33 alone significantly induced expression of both *KLF2* and *S1P1* mRNA, but induction of a T_{reg}-like phenotype with IL-33 and IL-12 conferred a significant abrogation of *KLF2* mRNA expression in these cells and a non-significant *S1P1* mRNA expression (**Figure 28C, D**). Skon *et al.*, observed that IL-33 promoted establishment of tissue-resident lymphocytes by inhibition of KLF2 expression in T cells and through synergism to the signaling of the anti-inflammatory cytokine TGFβ[41]. This effect leading to a decrease in *S1P1* mRNA expression was related to activation of the PI3K/ Akt pathway, though. However, it was shown here before that IL-33 failed to re-activate mTORC1 or enhance Akt activation (**Figure 23**) in starved $CD8^+$ T cells. Skon and colleagues explained that *KLF2* gene expression is mechanistically dependent on the transcription factor FoxO1[41]. FoxO1 plays a central role in the differentiation of $FoxP3^+$ T_{reg}[149], in the regulation of the immunometabolism and also controls the development and functions of T_H9 cells[194]. Blom *et al.* discovered that IL-33 *in vitro* initiates IL-9 secretion by human $CD4^+$ T cells and basophils, with this indirectly connecting the IL-33/ ST2L signaling to FoxO1[195]. Unphosphorylated FoxO1 is an active transcription factor located in the nucleus. Acetylation of FoxO1 reduces its DNA binding capacity and promotes Akt-mediated phosphorylation, in turn resulting in exclusion of FoxO1 from the nucleus[151]. Based on the hypothesis that IL-33 might indirectly regulate *S1P1* expression through modulation of FoxO1, protein expression and phosphorylation of FoxO1 in starved $CD8^+$ T cells stimulated with IL-33 and IL-12 was investigated by western blot analysis. Surprisingly, IL-33 stimulation did not mediate inactivation of FoxO1 through phosphorylation but significantly decreased FoxO1 protein expression (**Figure 29**). This finding revealed a discrepancy to the *KLF2* mRNA expression pattern.

It had beforehand been postulated that the IL-33-enhanced *KLF2* mRNA expression might be caused by an increased expression of FoxO1, but FoxO1 protein expression was significantly downregulated by IL-33 stimulation. It should be emphasized that in this experiment, activity of FoxO1 was not directly determined, although it was shown that FoxO1 is at least not inactivated through phosphorylation. As specific antibodies for acetylated FoxO1 are currently not available, it remains unclear whether IL-33 contributes to inactivation of FoxO1 through acetylation. The attempt to link IL-33 to an increased expression of the deacetylase Sirtuin1 (SIRT1) failed, as SIRT1 was revealed to not be expressed on protein levels in $CD8^+$ T cells (*data not shown*). It is furthermore questionable whether the increase in *KLF2* mRNA expression is a direct cause of an increased transcriptional expression mediated by FoxO1. Nutrient deprivation highly influences mRNA stability to promote gene expression of proteins supporting survival, while inhibiting mRNA translation of proteins involved in energy-consuming processes or apoptosis[196]. An explanation for the increase in *KLF2* mRNA levels might also be a disbalance in the synthesis and degradation of *KLF2* mRNA, leading to accumulation of mRNA and not an increased expression. Furthermore, under certain conditions, accessory and transactivating proteins required for the FoxO1-mediated gene expression of *KLF2* might be recruited IL-33-dependently. Based on these speculations, it is suggested to perform further experiments including reporter gene assays and specific silencing of FoxO1 expression using small interfering (si)RNA.

Importantly, FoxO1 expression was even more significantly reduced if the $CD8^+$ T cells were treated with both IL-33 and IL-12 (**Figure 29**). This finding is in line with the observation that *KLF2* mRNA expression was abrogated by IL-33/ IL-12 co-stimulation. FoxO1 expression was here not expected to be completely suppressed in starved T_{reg}-like $CD8^+$ T cells as FoxO1 deficiency is described to severely curtail the development of T_{reg} cells, to diminish expression of immune checkpoint CTLA-4 and confer inability of TGFβ to suppress T-bet mediated effector functions[149]. Endogenous FoxO1 expression is also required for autophagy and promotes survival of naïve T cells during nutrient withdrawal[197,198]. Although *S1P1* mRNA expression appeared to be downregulated through a decrease in FoxO1 or KLF2 expression, the controversially discussed role of S1P1 in the differentiation and activation of $CD8^+$ T_{reg} remains to be elucidated in

consecutive investigations. Liu and colleagues stated that the S1P1 signaling inhibits T_{reg} development and functions through activation of Akt/ mTOR, leading to antagonization of TGFβ signaling by attenuating activity of Smad3[199,200]. In contrast, Radeke and colleagues demonstrated that S1P1 contributes to T_{reg} development and is induced via Smad3 and Smad4 upon TGFβ stimulation in DC[201]. Concurrently, the S1P/ S1P1 signaling was recently shown to directly activate the TGFβ signaling pathway, inducing TGFβ and IL-10 secretion promoting expansion of T_{reg}[202]. Importantly, deletion of S1P1 in T_H17 cells conferred resistance to development of EAE although permanent deletion in T_{reg} resulted in autoimmunity, an increased sensitivity to apoptosis and convergence to effector T_{reg}[203]. This finding raises the question for future experiments, whether the observed IL-33/ IL-12-dependent inhibition of *S1P1* mRNA expression was permanent or transient. The findings presented here implicate that in the absence of antigen-specific stimulation, the combination of IL-33 and IL-12 might support the ability of T_{reg}-like $CD8^+$ T cells *in vivo* to migrate into LN CCR7-dependently through inhibition of S1P1-dependent peripheral trafficking.

6.4.2. IL-33 Re-induces Expression of *S1P4* mRNA

Here, it was observed for the first time that a cytokine, in this case IL-33, stimulates re-expression of *S1P4* mRNA in IL-12-stimulated $CD8^+$ T cells. Although remaining mostly unexplored to date, several recent publications emphasized a possible role of S1P4 in the activation, differentiation of T_{reg} and trafficking of immune cells[204]. IL-33 alone did not alter *S1P4* mRNA expression levels, while IL-12 significantly reduced *S1P4* mRNA levels. Strikingly, the IL-12-dependent transcriptional downregulation of *S1P4* expression was reversed by addition of IL-33 (**Figure 30B**). Hence, it was questioned if S1P4 expression is associated with the maintenance or function of T_{reg}-like $CD8^+$ T cells. S1P4 was reported to be inducible by stimulation with TGFβ and to contribute to cytokine production promoting both T_H17 and T_{reg} immunity[72,74]. It was suggested here that IL-33 either directly or indirectly interferes with the pro-inflammatory IL-12 pathway. SOCS proteins regulate the responses of immune cells to cytokines in a negative feedback loop

to inhibit cytokine signal transduction[205]. It was observed here that IL-33 enhanced the expression of *SOCS3* mRNA, but not *SOCS1* mRNA upon co-stimulation with IL-12 (**Figure 31**). SOCS3 has been attributed a function as a mediator of anti-inflammatory responses, although pro-inflammatory factors such as LPS and IL-6 significantly induce expression of SOCS3[205]. Mechanistically, SOCS3 blocks the STAT4 binding site in the IL-12R β2 subunit and with this inhibits IL-12-induced STAT4 activation[206]. Interestingly, SOCS3 is also described to be involved in the negative regulation of pro-inflammatory immunity mediated by the TLR- and IL-1R/ TIR signaling[205]. However, the data presented here implicate an agonistic function of SOCS3 in the IL-33 signaling, reversing the IL-12-dependent inhibition of *S1P4* mRNA expression.

Besides the described inhibitory function of SOCS3 on the TLR and IL-1R signaling, it is tempting to speculate a possible mode of action in which SOCS3 supports the IL-33-dependent anti-inflammatory function (**Figure 35**). To inhibit the pro-inflammatory TLR or IL-1R signaling, SOCS3 inhibits activation of TRAF6 and TAK1 which are recruited to MyD88. Both are essential for activation of NK-κB and T_H1 immunity[207] (**Figure 35A**). According to unpublished data from the collaboration partners of this project, Weigert and Olesch, next generation sequencing revealed that expression of the gene of a central component of the PI3K/ mTOR pathway, *PIK3AP1*, was significantly and highly expressed in $S1P4^{(-/-)}$ knockout mice. Although IL-33/ IL-12 did not re-activate mTORC1 here, it is tempting to speculate that under certain conditions, the IL-33/ ST2L signaling, possibly with a contribution of the adaptor protein PIK3AP1, downstream leads to *SOCS3* gene expression (**Figure 35B**). The contribution of PIK3AP1 also seems likely, because Deason and colleagues associated the recruitment of PIK3AP1 to the IL-1R to induction of T_{reg} and T_H17 immunity[71]. The S1P4 signaling is in turn described to play a major role in the pathogenesis of T_H17-mediated diseases[204]. Mechanistically, activation of the PIK3AP1/ mTOR pathway might result in the expression of SOCS3 protein and blockade of the STAT4 binding sites on the IL-12R β2 subunit. As a consequence of the interruption in the IL-12 signaling pathway, mechanisms inhibiting the mRNA expression of S1P4 are not induced. This hypothesis might be additionally supported by findings from Yamamoto *et al*., showing that transgenic expression of SOCS3 in T cells resulted in strong T_H2 cell polarization, a process to which IL-33 has primarily been associated to after its

discovery[206]. The decisive elements which promote either an anti- or pro- "IL1R family function" of SOCS3 remain to be elucidated.

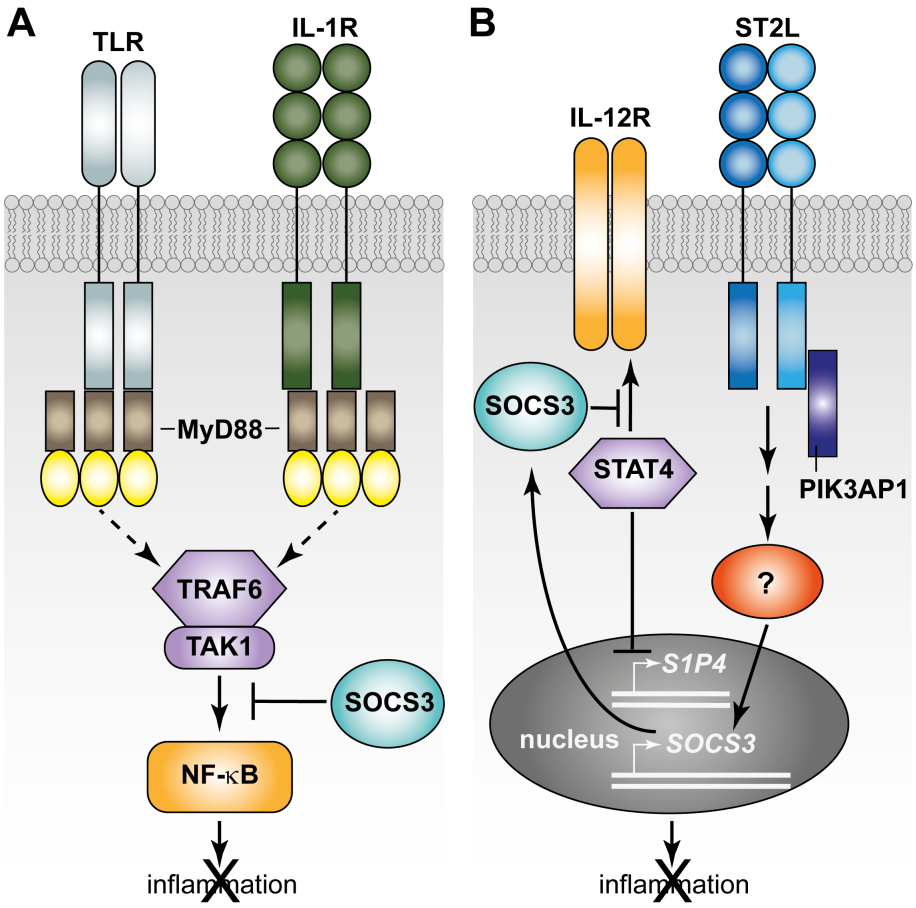

Figure 35: Suggested dual function of SOCS3 in the pro-inflammatory TLR/ IL-1R and anti-inflammatory IL-33/ ST2L pathways. (A) Suppression of the TLR and IL-1R pathways by SOCS3. Activation of TLR4 by LPS and or the IL-1R by IL-1 leads to recruitment of the adaptor protein MyD88, TRAF6 and TAK1. SOCS3 inhibits the subsequent NF-κB dependent transcription by inhibition of the complexation of TRAF6 and TAK1. (B) Hypothetical mode of action for the inhibition of the IL-12 signaling pathway by SOCS3 induced by IL-33/ ST2L signaling. IL-33/ ST2L signaling mediates expression and recruitment of PIK3AP1. Activation of the PI3K/ mTOR pathway and under contribution of unidentified downstream proteins (?) might lead expression of SOCS3. SOCS3 protein inhibits the IL-12-dependent activation of STAT4 by blockade of the STAT4 binding site at the IL-12R β2 chain. Figure B modified according to Yoshimura *et al.*[205].

Yasukawa *et al*. suggested that the function of SOCS3 is highly dependent on and controls duration of STAT3 activation. Sustained STAT3 activation was reported to suppress LPS-dependent inflammation, whereas transient expression promoted inflammation[208,209]. Interestingly, Lee and colleagues revealed that STAT3-induced S1P1 expression resulted

in a feed forward loop, in which the S1P1 signaling mediated persistent STAT3 activation in tumors[210]. Concurrently, the S1P1-dependent sustained STAT3 activation promoted metastasis and cancer cell proliferation. This project revealed evidence for future studies, implicating that IL-33 modulates the transcriptional expression of the *S1P1* and *S1P4* genes in different ways, possibly to maintain an anti-inflammatory function $CD8^+$ T cells during starvation. The here presented findings suggest that in the absence of antigen-specific stimulation, IL-33 upholds *S1P4* mRNA expression by counteracting IL-12-mediated *S1P4* mRNA reduction.

6.5. Potential of IL-33 as an Object of Interest in a Patient Study on Multiple Sclerosis

MS is a severe autoimmune disorder characterized by demyelination of neuronal axons and dendrites in the CNS due to an immune response including autoreactive antibody-dependent reactions and $CD8^+$ T cells. About 85% of all MS patients suffer from the most common disease course, the relapsing-remitting MS (RRMS). RRMS involves relapses followed by initially complete or later incomplete remission with periods of relative clinical stability[211,212]. One central aspect in the pathogenesis of MS is thought to be a functional T_{reg} deficiency resulting in failure of maintaining a self-tolerance state[153]. Pharmacological restoration of immune homeostasis is therefore a central aim for the treatment of autoimmune diseases like MS. The findings presented in the previous chapters of this thesis implicated that IL-33 contributes to the differentiation of a T_{reg}-like $CD8^{low}$ phenotype, a mechanism that could potentially be of interest to restore the imbalance of effector to regulatory T lymphocytes in MS. As recently published, IL-33 is released from MS plaques, raising the question whether the released cytokine promotes or dampens inflammation[83]. Also, in another investigation, the function of IL-33 for disease progression of MS remained highly disputed. Jiang and colleagues showed that administration of IL-33 ameliorated disease severity in the murine MS model, EAE[123]. Furthermore, as it was shown in this thesis, the bioactivity of IL-33 was

significantly reduced in the systemic blood compartment (chapter 5.1.3). Therefore, it might be postulated that IL-33 released within the CNS is meaningful for the modulation of infiltrating $CD8^+$ T cells.

To estimate whether IL-33 might be of interest in a future patient study with a large cohort of MS patients, samples of nine patients suffering from RRMS were analyzed in the small pilot study presented here. Importantly, all of the patients included in the study had been treated with natalizumab and were in remission at the time of blood withdrawal (**Table 20**). Natalizumab is a monoclonal antibody directed against integrin-α4 and blocks transmigration of T leukocytes across the blood-brain-barrier[213]. Consequently, the choice of patients treated with natalizumab increased the chances that possible effector or T_{reg} cells involved in RRMS were not located in the CNS but are more likely to be detectable in blood. Above, $CD8^{high}$ were described as effector cytotoxic T cells after TCR activation, while $CD8^{low}$ T lymphocytes seemed to differentiate to T_{reg}-like cells by IL-33/ IL-12 co-stimulation. Here, in blood samples of patients flow cytometric analysis revealed significant differences in the frequencies of $CD8^{high}$ and $CD8^{low}$ T cells compared to healthy controls. Interestingly, in RRMS, the percentage of $CD8^{high}$ T cells was significantly increased, and the percentage of $CD8^{low}$ T cells was decreased (**Figure 32**). Li *et al.* observed that the proportion of T_{reg} defined by $CD4^+$ $CD25^+$ $FoxP3^+$ was decreased in MS and suggested that this deficiency contributed to autoimmunity[214]. Although extensive studies concerning the functional suppressive activity of $CD8^{low}$ in RRMS will be required, based on the data shown in this thesis, it may be hypothesized that in addition to $CD4^+$ T_{reg}, the percentage of T_{reg}-like $CD8^{low}$ are diminished in RRMS as well.

The theory of "molecular mimicry" implies that presentation of viral peptides resembling components of the cerebral myelin sheath may trigger relapses by re-activation of effector cells[215], while at the same time a dysfunctional or a diminished T_{reg} population would fail to limit the cytotoxic activity of effector cells. IL-33 might exert a dual role and either promote pro-inflammatory responses through $CD8^{high}$ or anti-inflammatory immunity mediated by $CD8^{low}$. In order to mimic antigen-dependent activation by viral peptides, PBMC were activated TCR dependently (**Figure 33**). The frequencies of $ST2L^+$ $CD8^{high}$ and $ST2L^+$ $CD8^{low}$ were significantly and equally increased in healthy or RRMS PBMC upon TCR activation. Remarkably, the proportion of $ST2L^+$ $CD8^{low}$ remained

significantly lower in PBMC of RRMS patients with and without TCR activation (**Figure 33A-D**). Consequently, the ratio of the assumed effector ST2L$^+$ CD8^{high} to T_{reg}-like ST2L$^+$ CD8^{low} was consequently significantly higher in RRMS samples (**Figure 33E**).

In vivo, differentiation of ST2L$^+$ CD8^{low} might potentially be enhanced through pharmacological treatment with rapamycin. The reason is that a prerequisite for IL-33 to induce an anti-inflammatory phenotype in CD8$^+$ T cells was based on the inhibition of rapamycin target mTORC1 during nutrient deprivation. In EAE, rapamycin has been described to ameliorate clinical and histological signs for disease activity when administered during the ongoing disease[216,144,217,218]. Similar beneficial effects of rapamycin in human MS have been reported[171,219]. Indeed, the positive effects of the mTORC1 inhibitor were discussed to rely on its characteristics to suppress T effector functions, as well as to increase the percentage of T_{reg}[217]. Another aspect suggesting that clinical RRMS pathophysiology fits very well to the function of IL-33 described herein is supported by the cytokine's contribution to the modulation of S1P1 expression, although the role of S1P1 in T_{reg} development remains largely unclear. Fingolimod, an approved therapeutic drug for the treatment of RRMS, has been described to increase percentages of CD4$^+$ and CD8$^+$ T_{reg} in non-relapsing patients, which resulted in a delayed disease progression[67]. In summary, the pilot study revealed interesting clues suggesting disturbances in the proportions of ST2L$^+$ CD8^{high} and ST2L$^+$ CD8^{low}.

6.6. Concluding Remarks

IL-33 is a cytokine with great functional diversity. Although it has been described for the first time more than a decade ago, mechanisms promoting either a pro- or anti-inflammatory function of IL-33 in adaptive immunity remain mostly unclear. It is tempting to speculate that the potential of IL-33 to activate immune cells is limited in time and space, because the cytokine is most likely bioactive in tissue only. Therefore, the data presented above are strongly questioning whether IL-33 can be used as a prognostic biomarker when detected in blood. Interestingly, as revealed here, also nutrient withdrawal was a decisive factor promoting responsivity of CD8$^+$ T cells to IL-33. Whether ST2L expression during

starvation is IRES-dependently induced yet remains to be clarified yet. However, starved $CD8^+$ T cells could profit from the versatility of IL-33 to either promote effector activity or differentiation of T_{reg}, allowing efficient responses to infection or raising the threshold for antigen-independent inflammation, respectively. In the end both instances would promote long-term survival of the organism. Moreover *in vivo*, IL-33 might block S1P1-dependent recirculation of T_{reg}-like $CD8^+$ T cells located in LN but might support expression of largely unexplored S1P4 with largely unexplored consequences so far. Overall, the *in vitro* observations revealed interesting insights into mechanisms possibly influencing bioactivity and functions of IL-33 *in vivo*.

VII. References

1. Rink, L., Kruse, A. & Haase, H. *Immunologie für Einsteiger*. *Immunologie für Einsteiger* (2012). doi:10.1007/978-3-8274-2440-2
2. Murphy, K., Travers, P. & Walport, M. *Janeway Immunologie*. *Janeway Immunologie* (2009). doi:10.1007/978-3-8274-2219-4
3. Lefrancais, E. *et al.* Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc. Natl. Acad. Sci.* **111**, 15502–15507 (2014).
4. Cayrol, C. *et al.* Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat. Immunol.* **19**, 375–385 (2018).
5. Palmer, G. & Gabay, C. Interleukin-33 biology with potential insights into human diseases. *Nature Reviews Rheumatology* **7**, 321–329 (2011).
6. Gershon, R. K. & Kondo, K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* (1970).
7. Yu, Y. *et al.* Recent advances in CD8+ regulatory t cell research (Review). *Oncology Letters* (2018). doi:10.3892/ol.2018.8378
8. Yang, N. *et al.* A Trichosanthin-derived peptide suppresses type 1 immune responses by TLR2-dependent activation of CD8+CD28− Tregs. *Clin. Immunol.* **153**, 277–287 (2014).
9. Vuddamalay, Y. & van Meerwijk, J. P. M. CD28- and CD28lowCD8+ Regulatory T Cells: Of Mice and Men. *Front. Immunol.* **8**, 31 (2017).
10. Wei, S. *et al.* Plasmacytoid Dendritic Cells Induce CD8 $^{+}$ Regulatory T Cells In Human Ovarian Carcinoma. *Cancer Res.* **65**, 5020–5026 (2005).
11. Rostamzadeh, D. *et al.* mTOR Signaling pathway as a master regulator of memory CD8 + T-cells, Th17, and NK cells development and their functional properties. *Journal of Cellular Physiology* (2019). doi:10.1002/jcp.28042

12. Kim, J. & Guan, K.-L. mTOR as a central hub of nutrient signalling and cell growth. *Nat. Cell Biol.* **21**, 63–71 (2019).
13. Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* **168**, 960–976 (2017).
14. Inoki, K., Zhu, T. & Guan, K. L. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* (2003). doi:10.1016/S0092-8674(03)00929-2
15. Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C. & Blenis, J. Tuberous Sclerosis Complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* (2003). doi:10.1016/S0960-9822(03)00506-2
16. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. Rheb binds and regulates the mTOR kinase. *Curr. Biol.* (2005). doi:10.1016/j.cub.2005.02.053
17. Sancak, Y. *et al.* PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. *Mol. Cell* (2007). doi:10.1016/j.molcel.2007.03.003
18. Thomson, A. W., Turnquist, H. R. & Raimondi, G. Immunoregulatory functions of mTOR inhibition. *Nat. Rev. Immunol.* **9**, 324–37 (2009).
19. Gwinn, D. M. *et al.* AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Mol. Cell* (2008). doi:10.1016/j.molcel.2008.03.003
20. SEHGAL, S. N. & BANSBACH, C. C. Rapamycin: In Vitro Profile of a New Immunosuppressive Macrolide. *Ann. N. Y. Acad. Sci.* (1993). doi:10.1111/j.1749-6632.1993.tb35852.x
21. Douros, J. & Suffness, M. New antitumor substances of natural origin. *Cancer Treat. Rev.* (1981). doi:10.1016/S0305-7372(81)80006-0
22. Holz, M. K., Ballif, B. A., Gygi, S. P. & Blenis, J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* (2005). doi:10.1016/j.cell.2005.10.024

23. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N. & Sonenberg, N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* (1996). doi:10.1002/j.1460-2075.1996.tb00398.x
24. Kuo, C. J. *et al.* Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* (1992). doi:10.1038/358070a0
25. Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science (80-.).* (2005). doi:10.1126/science.1106148
26. Sengupta, S. *et al.* Regulation of OSR1 and the sodium, potassium, two chloride cotransporter by convergent signals. *Proc. Natl. Acad. Sci. U. S. A.* (2013). doi:10.1073/pnas.1318676110
27. Slaney, C. Y., Kershaw, M. H. & Darcy, P. K. Trafficking of T cells into tumors. *Cancer Res.* **74**, 7168–74 (2014).
28. Krummel, M. F., Bartumeus, F. & Gérard, A. T-cell Migration, Search Strategies and Mechanisms. *Nat. Rev. Immunol.* **16**, 193 (2016).
29. Sinclair, L. V *et al.* Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat. Immunol.* **9**, 513–521 (2008).
30. Odoardi, F. *et al.* T cells become licensed in the lung to enter the central nervous system. *Nature* **488**, 675–679 (2012).
31. Asperti-Boursin, F., Real, E., Bismuth, G., Trautmann, A. & Donnadieu, E. CCR7 ligands control basal T cell motility within lymph node slices in a phosphoinositide 3–kinase– independent manner. *J. Exp. Med.* **204**, 1167–1179 (2007).
32. Noor, S. & Wilson, E. H. Role of C-C chemokine receptor type 7 and its ligands during neuroinflammation. *J. Neuroinflammation* **9**, 604 (2012).
33. Ploix, C., Lo, D. & Carson, M. J. A Ligand for the Chemokine Receptor CCR7 Can Influence the Homeostatic Proliferation of CD4 T Cells and Progression of Autoimmunity. *J. Immunol.* **167**, 6724–6730 (2001).
34. Link, A. *et al.* Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat. Immunol.* **8**, 1255–1265 (2007).

35. Menning, A. *et al.* Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur. J. Immunol.* **37**, 1575–1583 (2007).
36. Schneider, M. A., Meingassner, J. G., Lipp, M., Moore, H. D. & Rot, A. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J. Exp. Med.* **204**, 735–45 (2007).
37. Cibrián, D. & Sánchez-Madrid, F. CD69: from activation marker to metabolic gatekeeper. *Eur. J. Immunol.* **47**, 946–953 (2017).
38. Bankovich, A. J., Shiow, L. R. & Cyster, J. G. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J. Biol. Chem.* **285**, 22328–37 (2010).
39. González-Amaro, R., Cortés, J. R., Sánchez-Madrid, F. & Martín, P. Is CD69 an effective brake to control inflammatory diseases? *Trends Mol. Med.* **19**, 625–632 (2013).
40. Baeyens, A., Fang, V., Chen, C. & Schwab, S. R. Exit Strategies: S1P Signaling and T Cell Migration. *Trends Immunol.* **36**, 778–787 (2015).
41. Skon, C. N. *et al.* Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat. Immunol.* **14**, 1285–1293 (2013).
42. Pham, T. H. M., Okada, T., Matloubian, M., Lo, C. G. & Cyster, J. G. S1P1 Receptor Signaling Overrides Retention Mediated by Gαi-Coupled Receptors to Promote T Cell Egress. *Immunity* **28**, 122–133 (2008).
43. Aoki, M., Aoki, H., Ramanathan, R., Hait, N. C. & Takabe, K. Sphingosine-1-Phosphate Signaling in Immune Cells and Inflammation: Roles and Therapeutic Potential. *Mediators Inflamm.* **2016**, 1–11 (2016).
44. Bryan, A. M. & Del Poeta, M. Sphingosine-1-phosphate receptors and innate immunity. *Cell. Microbiol.* **20**, e12836 (2018).

45. Lee, Y.-M., Venkataraman, K., Hwang, S.-I., Han, D. K. & Hla, T. A novel method to quantify sphingosine 1-phosphate by immobilized metal affinity chromatography (IMAC). *Prostaglandins Other Lipid Mediat.* **84**, 154–162 (2007).
46. Sit, S.-T. & Manser, E. Rho GTPases and their role in organizing the actin cytoskeleton. *J. Cell Sci.* **124**, 679–683 (2011).
47. Olesch, C., Ringel, C., Brüne, B. & Weigert, A. Beyond Immune Cell Migration: The Emerging Role of the Sphingosine-1-phosphate Receptor S1PR4 as a Modulator of Innate Immune Cell Activation. *Mediators Inflamm.* **2017**, 1–12 (2017).
48. Viglietta, V., Baecher-Allan, C., Weiner, H. L. & Hafler, D. A. Loss of Functional Suppression by CD4 $^{+}$ CD25 $^{+}$ Regulatory T Cells in Patients with Multiple Sclerosis. *J. Exp. Med.* **199**, 971–979 (2004).
49. Alvarado-Sánchez, B. *et al.* Regulatory T cells in patients with systemic lupus erythematosus. *J. Autoimmun.* (2006). doi:10.1016/j.jaut.2006.06.005
50. van Roon, J. A. G., Hartgring, S. A. Y., van der Wurff-Jacobs, K. M. G., Bijlsma, J. W. J. & Lafeber, F. P. J. G. Numbers of CD25+Foxp3+ T cells that lack the IL-7 receptor are increased intra-articularly and have impaired suppressive function in RA patients. *Rheumatology* (2010). doi:10.1093/rheumatology/keq237
51. Brusko, T. M., Wasserfall, C. H., Clare-Salzler, M. J., Schatz, D. A. & Atkinson, M. A. Functional defects and the influence of age on the frequency of CD4 +CD25+ T-cells in type 1 diabetes. *Diabetes* (2005). doi:10.2337/diabetes.54.5.1407
52. Delgoffe, G. M. *et al.* The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* **30**, 832–844 (2009).
53. Kopf, H., de la Rosa, G. M., Howard, O. M. Z. & Chen, X. Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells. *Int. Immunopharmacol.* **7**, 1819–1824 (2007).

54. Yurchenko, E. *et al.* Inflammation-driven reprogramming of CD4 +foxp3 + regulatory T cells into pathogenic Th1/Th17 t effectors is abrogated by mTOR inhibition in vivo. *PLoS One* **7**, e35572 (2012).
55. Huynh, A. *et al.* Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. *Nat. Immunol.* **16**, 188–96 (2015).
56. Shrestha, S. *et al.* Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat. Immunol.* **16**, 178–87 (2015).
57. Goel, S. *et al.* Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* **91**, 1071–121 (2011).
58. Jain, R. K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62 (2005).
59. Zheng, Y. *et al.* A Role for Mammalian Target of Rapamycin in Regulating T Cell Activation versus Anergy. *J. Immunol.* (2007). doi:10.4049/jimmunol.178.4.2163
60. Pollizzi, K. N. *et al.* Asymmetric inheritance of mTORC1 kinase activity during division dictates CD8+ T cell differentiation. *Nat. Immunol.* (2016). doi:10.1038/ni.3438
61. Pollizzi, K. N. *et al.* mTORC1 and mTORC2 selectively regulate $CD8^+$ T cell differentiation. *J. Clin. Invest.* **125**, 2090–2108 (2015).
62. Chi, H. Regulation and function of mTOR signalling in T cell fate decisions. *Nat. Rev. Immunol.* **12**, 325–338 (2012).
63. Battaglia, M., Stabilini, A. & Roncarolo, M.-G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* **105**, 4743–4748 (2005).
64. Li, H. *et al.* Targeting of mTORC2 prevents cell migration and promotes apoptosis in breast cancer. *Breast Cancer Res. Treat.* **134**, 1057–1066 (2012).
65. Hao, Y. *et al.* The Kinase Complex mTOR Complex 2 Promotes the Follicular Migration and Functional Maturation of Differentiated Follicular Helper CD4+ T Cells During Viral Infection. *Front. Immunol.* **9**, 1127 (2018).
66. Maeurer, C., Holland, S., Pierre, S., Potstada, W. & Scholich, K. Sphingosine-1-phosphate induced mTOR-activation is mediated by the E3-ubiquitin ligase PAM. *Cell. Signal.* **21**, 293–300 (2009).

67. Song, Z. Y. *et al.* Peripheral blood T cell dynamics predict relapse in multiple sclerosis patients on fingolimod. *PLoS One* **10**, e0124923 (2015).
68. Kitz, A. *et al.* AKT isoforms modulate Th1-like Treg generation and function in human autoimmune disease. *EMBO Rep.* **17**, 1169–1183 (2016).
69. Schröder, M. *et al.* The sphingosine kinase 1 and S1P1 axis specifically counteracts LPS-induced IL-12p70 production in immune cells of the spleen. *Mol. Immunol.* **48**, 1139–1148 (2011).
70. Daniel, C. *et al.* FTY720 Ameliorates Th1-Mediated Colitis in Mice by Directly Affecting the Functional Activity of $CD4^+$ $CD25^+$ Regulatory T Cells. *J. Immunol.* **178**, 2458–2468 (2007).
71. Deason, K. *et al.* BCAP links IL-1R to the PI3K-mTOR pathway and regulates pathogenic Th17 cell differentiation. *J. Exp. Med.* **215**, 2413–2428 (2018).
72. Cencetti, F. *et al.* TGFβ1 evokes myoblast apoptotic response *via* a novel signaling pathway involving $S1P_4$ transactivation upstream of Rho-kinase-2 activation. *FASEB J.* **27**, 4532–4546 (2013).
73. Sekar, D., Hahn, C., Brüne, B., Roberts, E. & Weigert, A. Apoptotic tumor cells induce IL-27 release from human DCs to activate Treg cells that express CD69 and attenuate cytotoxicity. *Eur. J. Immunol.* **42**, 1585–1598 (2012).
74. Schulze, T. *et al.* Sphingosine-1-phospate receptor 4 (S1P 4) deficiency profoundly affects dendritic cell function and T H17-cell differentiation in a murine model. *FASEB J.* **25**, 4024–4036 (2011).
75. Schmitz, J. *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**, 479–490 (2005).
76. Cayrol, C. & Girard, J.-P. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr. Opin. Immunol.* **31**, 31–7 (2014).
77. Ali, S. *et al.* The Dual Function Cytokine IL-33 Interacts with the Transcription Factor NF- B To Dampen NF- B-Stimulated Gene Transcription. *J. Immunol.* **187**, 1609–1616 (2011).

78. Carriere, V. *et al.* IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 282–7 (2007).
79. Bahrami Mahneh, S. *et al.* Serum IL-33 Is Elevated in Children with Asthma and Is Associated with Disease Severity. *Int. Arch. Allergy Immunol.* **168**, 193–196 (2015).
80. Qian, L. *et al.* Serum IL-33 Is a Novel Diagnostic and Prognostic Biomarker in Acute Ischemic Stroke. *Aging Dis.* **7**, 614–622 (2016).
81. Xie, S.-L. *et al.* Altered serum levels of IL-33 in patients with advanced systolic chronic heart failure: correlation with oxidative stress. *J. Transl. Med.* **10**, 120 (2012).
82. Sanada, S. *et al.* IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J. Clin. Invest.* **117**, 1538–1549 (2007).
83. Christophi, G. P. *et al.* Interleukin-33 upregulation in peripheral leukocytes and CNS of multiple sclerosis patients. *Clin. Immunol.* **142**, 308–19 (2012).
84. Moussion, C., Ortega, N. & Girard, J.-P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* **3**, e3331 (2008).
85. Pichery, M. *et al.* Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel Il-33-LacZ gene trap reporter strain. *J. Immunol.* **188**, 3488–95 (2012).
86. Martin, M. U. Special aspects of interleukin-33 and the IL-33 receptor complex. *Seminars in Immunology* **25**, 449–457 (2013).
87. Ali, S., Nguyen, D. Q., Falk, W. & Martin, M. U. Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation. *Biochem. Biophys. Res. Commun.* **391**, 1512–6 (2010).
88. Lüthi, A. U. *et al.* Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31**, 84–98 (2009).

89. Lohning, M. *et al.* T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl. Acad. Sci.* **95**, 6930–6935 (1998).
90. Monticelli, L. A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* **12**, 1045–54 (2011).
91. Mjösberg, J. M. *et al.* Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat. Immunol.* **12**, 1055–1062 (2011).
92. Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. & Dahinden, C. A. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* **113**, 1526–1534 (2009).
93. Smithgall, M. D. *et al.* IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK Cells. *Int. Immunol.* **20**, 1019–1030 (2008).
94. Ottenlinger, F. *et al.* Fingolimod targeting protein phosphatase 2A differently affects IL-33 induced IL-2 and IFN-γ production in CD8+ lymphocytes. *Eur. J. Immunol.* **46**, 941–951 (2016).
95. Bonilla, W. V *et al.* The alarmin interleukin-33 drives protective antiviral $CD8^+$ T cell responses. *Science* **335**, 984–9 (2012).
96. Peine, M., Marek, R. M. & Löhning, M. IL-33 in T Cell Differentiation, Function, and Immune Homeostasis. *Trends in Immunology* **37**, 321–333 (2016).
97. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
98. Bae, S. *et al.* Contradictory functions (activation/termination) of neutrophil proteinase 3 enzyme (PR3) in interleukin-33 biological activity. *J. Biol. Chem.* **287**, 8205–8213 (2012).
99. Popovic, B. *et al.* Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation. *Nat. Commun.* **6**, 8327 (2015).

100. Scott, I. C. *et al.* Interleukin-33 is activated by allergen- and necrosis-associated proteolytic activities to regulate its alarmin activity during epithelial damage. *Sci. Rep.* **8**, 3363 (2018).
101. Peters, M. *et al.* The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6. *J. Exp. Med.* **183**, 1399–406 (1996).
102. Palomo, J., Dietrich, D., Martin, P., Palmer, G. & Gabay, C. The interleukin (IL)-1 cytokine family – Balance between agonists and antagonists in inflammatory diseases. *Cytokine* **76**, 25–37 (2015).
103. Yang, C.-Y. *et al.* Small Molecule ST2 Inhibitors Cause Reduction of Soluble ST2 and Improve Gvhd and Survival In Vivo. *Blood* **128**, (2016).
104. Yin, X., Cao, H., Wei, Y. & Li, H.-H. Alteration of the IL-33-sST2 pathway in hypertensive patients and a mouse model. *Hypertens. Res.* 1–8 (2019). doi:10.1038/s41440-019-0291-x
105. Tominaga, S. *et al.* Nucleotide sequence of a complementary DNA for human ST2. *Biochim. Biophys. Acta* **1171**, 215–8 (1992).
106. Chackerian, A. A. *et al.* IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* **179**, 2551–5 (2007).
107. Motshwene, P. G. *et al.* An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. *J. Biol. Chem.* **284**, 25404–11 (2009).
108. Pinto, S. M. *et al.* A network map of IL-33 signaling pathway. *J. Cell Commun. Signal.* **12**, 615–624 (2018).
109. Lin, S.-C., Lo, Y.-C. & Wu, H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* **465**, 885–90 (2010).
110. Wasmer, M. H. & Krebs, P. The role of IL-33-dependent inflammation in the tumor microenvironment. *Frontiers in Immunology* **7**, 682 (2017).
111. Chen, W.-Y., Tsai, T.-H., Yang, J.-L. & Li, L.-C. Therapeutic Strategies for Targeting IL-33/ST2 Signalling for the Treatment of Inflammatory Diseases. *Cell. Physiol. Biochem.* **49**, 349–358 (2018).

112. Yang, Q. *et al.* IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8 + T cells. *Eur. J. Immunol.* **41**, 3351–3360 (2011).
113. Gao, X. *et al.* Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *J. Immunol.* **194**, 438–45 (2015).
114. Baumann, C. *et al.* T-bet- and STAT4-dependent IL-33 receptor expression directly promotes antiviral Th1 cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 4056–61 (2015).
115. Nakayamada, S. *et al.* Early Th1 Cell Differentiation Is Marked by a Tfh Cell-like Transition. *Immunity* **35**, 919–931 (2011).
116. Wei, L. *et al.* Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* **32**, 840–51 (2010).
117. Guo, L. *et al.* IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc. Natl. Acad. Sci.* **106**, 13463–13468 (2009).
118. Usui, T., Nishikomori, R., Kitani, A. & Strober, W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rβ2 chain or T-bet. *Immunity* **18**, 415–428 (2003).
119. Singer, M. *et al.* A Distinct Gene Module for Dysfunction Uncoupled from Activation in Tumor-Infiltrating T Cells. *Cell* **166**, 1500-1511.e9 (2016).
120. Pei, C. *et al.* Emerging role of interleukin-33 in autoimmune diseases. *Immunology* **141**, 9–17 (2014).
121. Popovic, B. *et al.* IL-33/ST2 pathway drives regulatory T cell dependent suppression of liver damage upon cytomegalovirus infection. *PLOS Pathog.* **13**, e1006345 (2017).
122. McFarland, H. F. & Martin, R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat. Immunol.* **8**, 913–919 (2007).

123. Jiang, H. R. *et al.* IL-33 attenuates EAE by suppressing IL-17 and IFN-γ production and inducing alternatively activated macrophages. *Eur. J. Immunol.* **42**, 1804–1814 (2012).
124. Alvarez, F. *et al.* The alarmins IL-1 and IL-33 differentially regulate the functional specialisation of Foxp3+ regulatory T cells during mucosal inflammation. *Mucosal Immunol.* **12**, 746–760 (2019).
125. Duan, L. *et al.* Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3$^+$ regulatory T-cell responses in mice. *Mol. Med.* **18**, 753–61 (2012).
126. Verri, W. A. *et al.* IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. *Ann. Rheum. Dis.* **69**, 1697–1703 (2010).
127. Pastille, E. *et al.* The IL-33/ST2 pathway shapes the regulatory T cell phenotype to promote intestinal cancer. *Mucosal Immunol.* **12**, 990–1003 (2019).
128. Akimoto, M., Hayashi, J.-I., Nakae, S., Saito, H. & Takenaga, K. Interleukin-33 enhances programmed oncosis of ST2L-positive low-metastatic cells in the tumour microenvironment of lung cancer. *Cell Death Dis.* **7**, e2057 (2016).
129. Geissler, E. K. The influence of mTOR inhibitors on immunity and the relationship to post-transplant malignancy. *Transplant. Res.* **2**, S2 (2013).
130. Mami-Chouaib, F. *et al.* Resident memory T cells, critical components in tumor immunology. *J. Immunother. cancer* **6**, 87 (2018).
131. Stier, M. T. *et al.* IL-33 promotes the egress of group 2 innate lymphoid cells from the bone marrow. *J. Exp. Med.* **215**, 263–281 (2018).
132. Ottenlinger, F. New Molecular Insights into the Treatment of Multiple Sclerosis. (Johann Wolfgang Goethe University Frankfurt/ Main, 2016).
133. Wienken, C. J., Baaske, P., Rothbauer, U., Braun, D. & Duhr, S. Protein-binding assays in biological liquids using microscale thermophoresis. *Nat. Commun.* **1**, 100 (2010).
134. Jerabek-Willemsen, M., Wienken, C. J., Braun, D., Baaske, P. & Duhr, S. Molecular interaction studies using microscale thermophoresis. *Assay Drug Dev. Technol.* **9**, 342–53 (2011).

135. Kieler, M. *et al.* Plasma levels of interleukin-33 and soluble suppression of tumorigenicity 2 in patients with advanced pancreatic ductal adenocarcinoma undergoing systemic chemotherapy. *Med. Oncol.* **36**, 1 (2019).
136. Hu, L.-A., Fu, Y., Zhang, D.-N. & Zhang, J. Serum IL-33 as a diagnostic and prognostic marker in non- small cell lung cancer. *Asian Pac. J. Cancer Prev.* **14**, 2563–6 (2013).
137. Cayrol, C. & Girard, J. P. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9021–9026 (2009).
138. Lefrançais, E. *et al.* IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 1673–1678 (2012).
139. Mildner, M. *et al.* Primary sources and immunological prerequisites for sST2 secretion in humans. *Cardiovasc. Res.* **87**, 769–777 (2010).
140. Wagner, A. *et al.* Increased Serum Levels of the IL-33 Neutralizing sST2 in Limited Cutaneous Systemic Sclerosis. *Scand. J. Immunol.* **82**, 269–274 (2015).
141. Palmer, G. *et al.* The IL-1 receptor accessory protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. *Cytokine* **42**, 358–364 (2008).
142. Brunner, S. M. *et al.* Tumor-infiltrating, interleukin-33-producing effector-memory CD8(+) T cells in resected hepatocellular carcinoma prolong patient survival. *Hepatology* **61**, 1957–1967 (2015).
143. Fournié, J. J. & Poupot, M. The pro-tumorigenic IL-33 involved in antitumor immunity: A yin and yang cytokine. *Frontiers in Immunology* **9**, 2506 (2018).
144. Donia, M. *et al.* Treatment with rapamycin ameliorates clinical and histological signs of protracted relapsing experimental allergic encephalomyelitis in Dark Agouti rats and induces expansion of peripheral CD4+CD25+Foxp3+ regulatory T cells. *J. Autoimmun.* **33**, 135–140 (2009).
145. Araki, K. *et al.* mTOR regulates memory CD8 T-cell differentiation. *Nature* **460**, 108–12 (2009).

146. Tanabe, M., Karaki, S., Takiguchi, M. & Nakauchi, H. Antigen recognition by the T cell receptor is enhanced by CD8 α-chain binding to the α 3 domain of MHC class I molecules, not by signaling via the cytoplasmic domain of CD8α. *Int. Immunol.* **4**, 147–152 (1992).
147. Förster, R., Davalos-Misslitz, A. C. & Rot, A. CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* **8**, 362–371 (2008).
148. Preston, G. C., Feijoo-Carnero, C., Schurch, N., Cowling, V. H. & Cantrell, D. A. The Impact of KLF2 Modulation on the Transcriptional Program and Function of CD8 T Cells. *PLoS One* **8**, e77537 (2013).
149. Kerdiles, Y. M. *et al.* Foxo Transcription Factors Control Regulatory T Cell Development and Function. *Immunity* **33**, 890–904 (2010).
150. Tzivion, G., Dobson, M. & Ramakrishnan, G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochimica et Biophysica Acta - Molecular Cell Research* **1813**, 1938–1945 (2011).
151. Daitoku, H., Sakamaki, J. ichi & Fukamizu, A. Regulation of FoxO transcription factors by acetylation and protein-protein interactions. *Biochimica et Biophysica Acta - Molecular Cell Research* **1813**, 1954–1960 (2011).
152. Yamamoto, K., Yamaguchi, M., Miyasaka, N. & Miura, O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor β2 subunit. *Biochem. Biophys. Res. Commun.* **310**, 1188–1193 (2003).
153. Gonsette, R. E. Self-tolerance in multiple sclerosis. *Acta Neurol. Belg.* **112**, 133 (2012).
154. Alsahebfosoul, F. *et al.* Interleukin-33 plasma levels in patients with relapsing-remitting multiple sclerosis. *Biomol. Concepts* **8**, (2017).
155. Jafarzadeh, A. *et al.* Increased Concentrations of Interleukin-33 in the Serum and Cerebrospinal Fluid of Patients with Multiple Sclerosis. *Oman Med. J.* **31**, 40–45 (2016).

156. Salmond, R. J. *et al.* IL-33 induces innate lymphoid cell-mediated airway inflammation by activating mammalian target of rapamycin. *J. Allergy Clin. Immunol.* **130**, 1159-1166.e6 (2012).
157. Glück, J., Rymarczyk, B. & Rogala, B. Serum IL-33 but not ST2 level is elevated in intermittent allergic rhinitis and is a marker of the disease severity. *Inflamm. Res.* **61**, 547–550 (2012).
158. Zhang, H. F. *et al.* Altered serum levels of IL-33 in patients with advanced systolic chronic heart failure: correlation with oxidative stress. *J. Transl. Med.* **10**, 120 (2012).
159. Zhou, Y., Ji, Y., Wang, H., Zhang, H. & Zhou, H. IL-33 Promotes the Development of Colorectal Cancer Through Inducing Tumor-Infiltrating ST2L+ Regulatory T Cells in Mice. *Technol. Cancer Res. Treat.* **17**, 1533033818780091 (2018).
160. Momen, T. *et al.* Comparison of Interleukin-33 Serum Levels in Asthmatic Patients with a Control Group and Relation with the Severity of the Disease. *Int. J. Prev. Med.* **8**, 65 (2017).
161. Asaka, D. *et al.* Elevated Levels of Interleukin-33 in the Nasal Secretions of Patients with Allergic Rhinitis. *Int. Arch. Allergy Immunol.* **158**, 47–50 (2012).
162. Bergmann, K.-C., Heinrich, J. & Niemann, H. Current status of allergy prevalence in Germany. *Allergo J. Int.* **25**, 6–10 (2016).
163. Tu, L. & Yang, L. IL-33 at the Crossroads of Metabolic Disorders and Immunity. *Front. Endocrinol. (Lausanne).* **10**, 26 (2019).
164. Hasan, A. *et al.* IL-33 is negatively associated with the BMI and confers a protective lipid/metabolic profile in non-diabetic but not diabetic subjects. *BMC Immunol.* **15**, 19 (2014).
165. Lingel, A. *et al.* Structure of IL-33 and Its Interaction with the ST2 and IL-1RAcP Receptors-Insight into Heterotrimeric IL-1 Signaling Complexes. *Structure* **17**, 1398–1410 (2009).
166. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**, 373–384 (2010).

167. Mehraj, V. *et al.* The plasma levels of soluble ST2 as a marker of gut mucosal damage in early HIV infection. *AIDS* **30**, 1617–1627 (2016).
168. Wu, X. *et al.* Increased Expression of sST2 in Early HIV Infected Patients Attenuated the IL-33 Induced T Cell Responses. *Front. Immunol.* **9**, 2850 (2018).
169. Becerra, A., Warke, R. V., de Bosch, N., Rothman, A. L. & Bosch, I. Elevated levels of soluble ST2 protein in dengue virus infected patients. *Cytokine* **41**, 114–120 (2008).
170. Wang, J. *et al.* Serum IL-33 levels are associated with liver damage in patients with chronic hepatitis B. *J. Interf. Cytokine Res.* **32**, 248–253 (2012).
171. Bagherpour, B. *et al.* Promising effect of rapamycin on multiple sclerosis. *Mult. Scler. Relat. Disord.* **26**, 40–45 (2018).
172. Kunze, M. M. *et al.* sST2 translation is regulated by FGF2 via an hnRNP A1-mediated IRES-dependent mechanism. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1859**, 848–859 (2016).
173. Liu, B. *et al.* CD8 low CD100 − T Cells Identify a Novel CD8 T Cell Subset Associated with Viral Control during Human Hantaan Virus Infection. *J. Virol.* **89**, 11834–11844 (2015).
174. Trautmann, A. *et al.* Human CD8 T cells of the peripheral blood contain a low CD8 expressing cytotoxic/effector subpopulation. *Immunology* **108**, 305–312 (2003).
175. Xiao, Z., Mescher, M. F. & Jameson, S. C. Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. *J. Exp. Med.* **204**, 2667–2677 (2007).
176. Chen, X. & Oppenheim, J. J. Resolving the identity myth: key markers of functional CD4+FoxP3+ regulatory T cells. *Int. Immunopharmacol.* **11**, 1489 (2011).
177. Miyara, M. *et al.* Functional Delineation and Differentiation Dynamics of Human CD4+ T Cells Expressing the FoxP3 Transcription Factor. *Immunity* **30**, 899–911 (2009).

178. Knox, J. J., Cosma, G. L., Betts, M. R. & McLane, L. M. Characterization of T-bet and eomes in peripheral human immune cells. *Front. Immunol.* **5**, 217 (2014).
179. Zhu, J. *et al.* The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* **37**, 660–73 (2012).
180. Kienzle, N., Baz, A. & Kelso, A. Profiling the CD8low phenotype, an alternative career choice for CD8 T cells during primary differentiation. *Immunol. Cell Biol.* **82**, 75–83 (2004).
181. Ni, M. *et al.* B-cell adaptor for PI3K (BCAP) negatively regulates Toll-like receptor signaling through activation of PI3K. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 267–72 (2012).
182. Wang, Z., Shi, L., Hua, S., Qi, C. & Fang, M. IL-33 ameliorates experimental colitis involving regulation of autophagy of macrophages in mice. *Cell Biosci.* **9**, 10 (2019).
183. Zdravkovic, N., Shahin, A., Arsenijevic, N., Lukic, M. L. & Mensah-Brown, E. P. K. Regulatory T cells and ST2 signaling control diabetes induction with multiple low doses of streptozotocin. *Mol. Immunol.* **47**, 28–36 (2009).
184. Rui, T. *et al.* Reduction in IL-33 expression exaggerates ischaemia/reperfusion-induced myocardial injury in mice with diabetes mellitus. *Cardiovasc. Res.* **94**, 370–378 (2012).
185. Milovanovic, M. *et al.* Deletion of IL-33R (ST2) Abrogates Resistance to EAE in BALB/C Mice by Enhancing Polarization of APC to Inflammatory Phenotype. *PLoS One* **7**, e45225 (2012).
186. U.S. National Library of Medicine. ClinicalTrials.gov. Available at: https://clinicaltrials.gov/ct2/home. (Accessed: 8th November 2019)
187. Masopust, D. & Schenkel, J. M. The integration of T cell migration, differentiation and function. *Nat. Rev. Immunol.* **13**, 309–320 (2013).
188. Ma, Q. *et al.* Rapamycin Regulates the Expression and Activity of Krüppel-Like Transcription Factor 2 in Human Umbilical Vein Endothelial Cells. *PLoS One* **7**, e43315 (2012).

189. Hunter, M. C., Teijeira, A. & Halin, C. T Cell Trafficking through Lymphatic Vessels. *Front. Immunol.* **7**, (2016).

190. Liu, F.-Y. *et al.* Mammalian target of rapamycin (mTOR) is involved in the survival of cells mediated by chemokine receptor 7 through PI3K/Akt in metastatic squamous cell carcinoma of the head and neck. *Br. J. Oral Maxillofac. Surg.* **48**, 291–296 (2010).

191. López-Cotarelo, P. *et al.* A Novel MEK-ERK-AMPK Signaling Axis Controls Chemokine Receptor CCR7-dependent Survival in Human Mature Dendritic Cells. *J. Biol. Chem.* **290**, 827–840 (2015).

192. Pabbisetty, S. K. *et al.* KLF2 is a rate-limiting transcription factor that can be targeted to enhance regulatory T-cell production. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 9579–9584 (2014).

193. Schober, S. L. *et al.* Expression of the transcription factor lung Kruppel-like factor is regulated by cytokines and correlates with survival of memory T cells in vitro and in vivo. *J. Immunol.* **163**, 3662–3667 (1999).

194. Malik, S. & Awasthi, A. Transcriptional control of Th9 cells: Role of Foxo1 in interleukin-9 induction. *Frontiers in Immunology* **9**, 995 (2018).

195. Blom, L., Poulsen, B. C., Jensen, B. M., Hansen, A. & Poulsen, L. K. IL-33 Induces IL-9 Production in Human CD4+ T Cells and Basophils. *PLoS One* **6**, e21695 (2011).

196. Yaman, I. *et al.* Nutritional control of mRNA stability is mediated by a conserved AU-rich element that binds the cytoplasmic shuttling protein HuR. *J. Biol. Chem.* **277**, 41539–46 (2002).

197. Kerdiles, Y. M. *et al.* Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat. Immunol.* **10**, 176–184 (2009).

198. Zhao, Y. *et al.* Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. *Nat. Cell Biol.* **12**, 665–675 (2010).

199. Liu, G. *et al.* The receptor S1P1 overrides regulatory T cell-mediated immune suppression through Akt-mTOR. *Nat. Immunol.* **10**, 769–777 (2009).

200. Liu, G., Yang, K., Burns, S., Shrestha, S. & Chi, H. The S1P 1-mTOR axis directs the reciprocal differentiation of T H 1 and Treg cells. *Nat. Immunol.* **11**, 1047–1056 (2010).

201. Radeke, H. H. *et al.* Overlapping Signaling Pathways of Sphingosine 1-Phosphate and TGF-β in the Murine Langerhans Cell Line XS52. *J. Immunol.* **174**, 2778–2786 (2005).

202. Liu, Y.-N. *et al.* Sphingosine 1 phosphate receptor-1 (S1P1) promotes tumor-associated regulatory T cell expansion: leading to poor survival in bladder cancer. *Cell Death Dis.* **10**, 50 (2019).

203. Eken, A. *et al.* S1P1 deletion differentially affects TH17 and Regulatory T cells. *Sci. Rep.* **7**, 12905 (2017).

204. Olesch, C., Ringel, C., Brüne, B. & Weigert, A. Beyond Immune Cell Migration: The Emerging Role of the Sphingosine-1-phosphate Receptor S1PR4 as a Modulator of Innate Immune Cell Activation. *Mediators Inflamm.* **2017**, 1–12 (2017).

205. Yoshimura, A., Naka, T. & Kubo, M. SOCS proteins, cytokine signalling and immune regulation. *Nature Reviews Immunology* **7**, 454–465 (2007).

206. Yamamoto, K., Yamaguchi, M., Miyasaka, N. & Miura, O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor beta2 subunit. *Biochem. Biophys. Res. Commun.* **310**, 1188–93 (2003).

207. Frobøse, H. *et al.* Suppressor of Cytokine Signaling-3 Inhibits Interleukin-1 Signaling by Targeting the TRAF-6/TAK1 Complex. *Mol. Endocrinol.* **20**, 1587–1596 (2006).

208. Yasukawa, H. *et al.* IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* **4**, 551–6 (2003).

209. Kortylewski, M. *et al.* Regulation of the IL-23 and IL-12 Balance by Stat3 Signaling in the Tumor Microenvironment. *Cancer Cell* **15**, 114–123 (2009).

210. Lee, H. *et al.* STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. *Nat. Med.* **16**, 1421–1428 (2010).

211. McKay, K. A., Kwan, V., Duggan, T. & Tremlett, H. Risk factors associated with the onset of relapsing-remitting and primary progressive multiple sclerosis: A systematic review. *BioMed Research International* **2015**, 817238 (2015).
212. Lublin, F. D. *et al.* Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology* **83**, 278–286 (2014).
213. Hutchinson, M. Natalizumab: A new treatment for relapsing remitting multiple sclerosis. *Ther. Clin. Risk Manag.* **3**, 259–68 (2007).
214. Li, Y.-F. *et al.* The proportion of peripheral regulatory T cells in patients with Multiple Sclerosis: A meta-analysis. *Mult. Scler. Relat. Disord.* **28**, 75–80 (2019).
215. Cusick, M. F., Libbey, J. E. & Fujinami, R. S. Molecular Mimicry as a Mechanism of Autoimmune Disease. *Clin. Rev. Allergy Immunol.* **42**, 102 (2012).
216. Schwarz, A. *et al.* Fine-tuning of regulatory T cell function: the role of calcium signals and naive regulatory T cells for regulatory T cell deficiency in multiple sclerosis. *J. Immunol.* **190**, 4965–70 (2013).
217. Lisi, L. *et al.* Rapamycin reduces clinical signs and neuropathic pain in a chronic model of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **243**, 43–51 (2012).
218. Esposito, M. *et al.* Rapamycin inhibits relapsing experimental autoimmune encephalomyelitis by both effector and regulatory T cells modulation. *J. Neuroimmunol.* **220**, 52–63 (2010).
219. Salehi, M., Bagherpour, B., Shayghannejad, V., Mohebi, F. & Jafari, R. Th1, Th2 and Th17 Cytokine Profile in Patients with Multiple Sclerosis Following Treatment with Rapamycin. *Iran. J. Immunol.* **13**, 141–7 (2016).

VIII. Abbreviations

aa	amino acid
Akt	Protein Kinase B
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APC	antigen presenting cells
CCL	CC-chemokine ligand
CCR7	CC-chemokine receptor 7
CD	Cluster of Differentiation
CD107a	Lamp-1, lysosomal-associated membrane protein 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Ctrl	control
DC	dendritic cell(s)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
eIF4B	eukaryotic translation initiation factor 4B
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FoxO1	forkhead box protein O1
FoxP3	forkhead box P3
FSC	forward scatter
GATA3	glutamyl amino transferase subunit A
GrzmB	granzyme B

HEV	high endothelial venules
IFNγ	interferon-γ
IgG/ IgE	immunoglobulin G/ immunoglobulin E
IL-	interleukin-
IL-1RAcP	interleukin-1 receptor accessory protein
ILC2	innate lymphoid cell group 2
IRES	Internal ribosome binding site
K_D	dissociation constant
KLF2	krüppel-like factor 2
LC3B	microtubule-associated protein 1 light chain 3 beta
LPS	lipopolysaccharide
mem	memory
MFI	mean fluorescence intensity
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
mTORC1/ -2	molecular target of rapamycin complex 1/ -2
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	myeloid differentiation primary-response gene 88
nAb	neutralizing antibodies
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
p70S6K	p70 subunit of S6 kinase
PBMC	peripheral blood mononuclear cells
PD-1	programmed death-1
PDK1	protein 3-phosphoinositide-dependent protein kinase 1

PI3K	Phosphoinositide 3-kinase
PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1
PRAS40	proline rich Akt substrate of 40 kDa
PRR5	proline rich 5
qRT-PCR	quantitative real-time PCR
Raptor	Regulatory-associated protein of mTOR
rel.	relative
Rictor	Rapamycin-insensitive companion of mTOR
RNA	ribonucleic acid
RORγT	retinoic acid receptor related receptor gamma
RRMS	Relapsing-remitting multiple sclerosis
S1P	sphingosine-1-phosphate
S1P1,-3,-4,-5	sphingosine-1-receptors 1, 3, 4 and 5
S6K	Ribosomal protein S6 kinase beta -1
SEAP	secreted alkaline phosphatase
sIL-6R	soluble IL-6 receptor
SLO	secondary lymphoid organ
SOCS1/ 3	suppressor of cytokine signaling 1/ 3
SSC	sideward scatter
sST2	soluble ST2
ST2L	suppression of tumorigenicity 2 long
STAT3/ 4	signal transducer and activator of transcription 3/ 4
T-bet	T-box transcription factor TBX21
TAK1	TGFβ-activated kinase 1
TCR	T cell receptor
TGFβ	tumor growth factor beta
T_H	T helper

TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	TNF-receptor-associated factor 6
trans	transitional
T_{reg}	regulatory T cells
TSC	tuberous sclerosis complex
αCD	anti-CD2/3/28

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

XI. Publications and Conference Contributions

11.1. Publications

Dreis C, Ottenlinger F, Putyrski M, Ernst A, Huhn M, Schmidt KG, Pfeilschifter JM, Radeke HH (2019). *Tissue cytokine IL-33 modulates the cytotoxic T lymphocyte activity during nutrient deprivation by regulation of lineage-specific differentiation programs.* Front. Immunol. 10:1698. doi: 10.3389/fimmu.2019.01698

Schmidt KG, Nordin A, Trautmann S, Wagner A, Ottenlinger F, Thomas D, Schwiebs A, Dreis C, Huhn M, Ackermann H, Larsson A, Pfeilschifter JM, Jakobsson PJ, Radeke HH (2019). *Activity-dependent stratification of SSc patients unravels new markers of disease progression at the endothelial–platelet interface.* Front. Immunol. (in revision)

Huhn M, Herrero San Juan M, Melchert B, Dreis C, Schmidt KG, Pfeilschifter JM, Vieth M, Radeke HH. *Identification of tryptophan metabolite 3-hydroxyanthranilic acid as a novel tool for the differentiation of Crohn's Disease phenotypes.* J Crohns Colitis (in revision).

11.2. Conference Contributions

Dreis C, Ottenlinger F, Schwiebs A, Schmidt KG, Herrero San Juan M, Pfeilschifter JM, Radeke HH. *Role of the IL-33/ ST2L-axis for the CD8-dependent anti-cancer cytotoxicity.* (workshop presentation). March 15-18, 2017. World Immune Regulation Meeting XI. Davos, Switzerland.

Dreis C, Ottenlinger F, Schwiebs A, Schmidt KG, Herrero San Juan M, Pfeilschifter JM, Radeke HH. *Role of the Interleukin-33/ ST2L-axis for the CD8-dependent anti-cancer cytotoxicity – Protease- versus receptor-dependent regulation of Interleukin-33 bioactivity.* (poster). May 15-17, 2018. 16th CIMT Annual Meeting – Pushing Frontiers in Cancer Immunotherapy. Mainz, Germany.

Dreis C, Ottenlinger F, Schwiebs A, Schmidt KG, Herrero San Juan M, Pfeilschifter JM, Radeke HH. *Role of the Interleukin-33/ ST2L-axis for the CD8-dependent anti-cancer cytotoxicity.* (poster) September 2-5, 2018. 5th European Congress of Immunology. Amsterdam, the Netherlands.

XII. Schriftliche Erklärung

Hiermit versichere ich, die vorgelegte Thesis selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt zu haben, die ich in der Thesis angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Thesis erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ‚Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis' niedergelegt sind, eingehalten. Gemäß § 25 Abs. 6 der Allgemeinen Bestimmungen für modularisierte Studiengänge dulde ich eine Überprüfung der Thesis mittels Anti-Plagiatssoftware.

Ort, Datum	Unterschrift