Pharmacological targeting of acid sphingomyelinase increases CD4$^+$ Foxp3$^+$ regulatory T cell subsets in patients with major depression

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Pharmakologische Hemmung der sauren Sphingomyelinase verstärkt CD4$^+$ Foxp3$^+$ regulatorische T-Zell-Subpopulationen bei Patienten mit Depression

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1 ABSTRACT

Lack of acid sphingomyelinase (ASM) activity, either through genetic deficiency or through pharmacological inhibition, is linked with increased activity and frequency of Foxp3$^+$ regulatory T cells (T_{reg}) among cluster of differentiation (CD) 4$^+$ T cells in mice *in vivo* and *in vitro*[1]. Thus, pharmacological blockade of ASM activity, which catalyzes the cleavage of sphingomyelin to ceramide and phosphocholine, might be used as a new therapeutic mechanism to correct numeric and/ or functional T_{reg} deficiencies in diseases like multiple sclerosis or major depression.

In the present study, the effect of pharmacological inhibition of ASM in humans, *in vitro* and *in vivo*, was analyzed. In the *in vitro* experiments, peripheral blood mononuclear cells (PBMC) of healthy human blood donors were treated with two widely prescribed antidepressants with high (sertraline, Ser) or low (citalopram, Cit) capacity to inhibit ASM activity. Similar to the findings in mice an increase in the frequency of T_{reg} among human CD4$^+$ T cells upon inhibition of ASM activity was observed. For the analysis *in vivo*, a prospective study of the composition of the CD4$^+$ T cell compartment of patients treated for major depression was done. The data show that pharmacological inhibition of ASM activity was superior to antidepressants with little or no ASM-inhibitory activity in increasing CD45RA$^-$ CD25^{high} effector T_{reg} (efT_{reg}) frequencies among CD4$^+$ T cells to normal levels. Independently of ASM inhibition, correlating the data with the clinical response, i.e. improvement of the Hamilton rating scale for depression (HAMD) by at least 50 per cent (%) after four weeks of treatment, it was found that an increase in efT_{reg} frequencies among CD4$^+$ cells during the first week of treatment identified patients with a clinical response.

Regarding the underlying mechanism, it could be found that the positive effect of ASM inhibition on T_{reg} required CD28 co-stimulation suggesting that enhanced CD28 co-stimulation was the driver of the observed increase in the frequency of T_{reg} among human CD4$^+$ T cells. Inhibition of ASM activity was further associated with changes in the expression and shuttling of CTLA-4, a key inhibitory molecule expressed by T_{reg}, between cellular compartments but the suppressive activity of CTLA-4 through its transendocytosis activity was unaffected by the inhibition of ASM activity.

In summary, the frequency of (effector) T_{reg} among $CD4^+$ T cells in mice and in humans is increased after inhibition of ASM activity suggesting that ASM blockade might beneficially modulate autoimmune diseases and depression-promoting inflammation.

2 ZUSAMMENFASSUNG

Ein Mangel an Aktivität der sauren Sphingomyelinase (ASM), entweder durch genetisches Defizit oder durch pharmakologische Hemmung, ist mit einer erhöhten Aktivität und Häufigkeit von Foxp3$^+$ regulatorischen T-Zellen (T_{reg}) innerhalb der CD4$^+$ (cluster of differentation 4) T-Zellen in Mäusen *in vivo* und *in vitro* verbunden[1]. Daher könnte die pharmakologische Blockade der ASM-Aktivität, die die Spaltung von Sphingomyelin in Ceramid und Phosphocholin katalysiert, als neuer therapeutischer Mechanismus zur Korrektur von numerischen und/oder funktionellen T_{reg}-Defiziten bei Erkrankungen wie Multipler Sklerose oder schwerer Depression eingesetzt werden.

In der vorliegenden Studie wurde die Wirkung der pharmakologischen Hemmung von ASM beim Menschen, *in vitro* und *in vivo* analysiert. In den *In-vitro*-Experimenten wurden die peripheren mononukleären Blutzellen (PBMC) gesunder menschlicher Blutspender mit zwei weithin verschriebenen Antidepressiva mit hoher (Sertralin, Ser) oder niedriger (Citalopram, Cit) Fähigkeit zur Hemmung der ASM-Aktivität untersucht. Ähnlich wie bei Mäusen wurde bei Hemmung der ASM-Aktivität ein Anstieg der Häufigkeit von T_{reg} innerhalb der menschlichen CD4$^+$ T-Zellen festgestellt. Für die Analyse *in vivo* wurde eine prospektive Studie über die Zusammensetzung des CD4$^+$ T-Zellkomplexes bei Patienten, die wegen einer Depression im Krankenhaus behandelt wurden, durchgeführt. Die Daten zeigen, dass die pharmakologische Hemmung der ASM-Aktivität Antidepressiva mit geringer oder keiner ASM-hemmenden Aktivität überlegen war, was die Vermehrung der CD45RA$^-$ CD25^{hoch}-Effektor-T_{reg} (efT_{reg})-Frequenzen innerhalb der CD4$^+$ T-Zellen betraf. Unabhängig von der Untersuchung zur ASM-Aktivität beobachteten wir, dass die klinische Reaktion (d.h. der Verbesserung der Hamilton-Bewertungsskala für Depressionen (HAMD) um mindestens 50 Prozent (%) nach vierwöchiger Behandlung) mit einem frühen Anstieg der efT_{reg}-Frequenzen unter CD4$^+$-Zellen während der ersten Behandlungswoche positiv korrelierte.

Hinsichtlich des zugrunde liegenden Mechanismus konnte festgestellt werden, dass die positive Wirkung der ASM-Hemmung auf T_{reg} eine CD28-Kostimulation erforderte, was darauf hindeutet, dass eine verstärkte CD28-Kostimulation die Ursache für den beobachteten Anstieg der Frequenz von T_{reg} innerhalb menschlicher CD4$^+$ T-Zellen war. Die Hemmung der ASM-Aktivität war darüber hinaus mit Veränderungen in der

Expression und im zellulären Umsatz von CTLA-4, einem von T_{reg} exprimierten inhibitorischen Schlüsselmolekül, verbunden. Die suppressive Aktivität von CTLA-4 durch seine Transendozytose-Aktivität wurde jedoch durch die Hemmung der ASM-Aktivität nicht beeinflusst.

Zusammenfassend lässt sich sagen, dass die Häufigkeit von (Effektor-)T_{reg} unterhalb der $CD4^+$ T-Zellen in Mäusen und beim Menschen nach Hemmung der ASM-Aktivität erhöht ist, was darauf hindeutet, dass eine ASM-Blockade Autoimmunerkrankungen und depressionsfördernde Entzündungen vorteilhaft modulieren könnte.

3 INTRODUCTION

3.1 IMMUNE SYSTEM

A host defense system against all kinds of diseases is called immune system. The main function of the immune system is to detect a broad range of pathogens, from viruses to parasitic worms. But it is important that the immune system can distinguish them from the healthy tissue of its own organism, which is known as self-tolerance. To function properly, the immune system involves different organs and consists of numerous cell types and various humoral factors. In general, one differentiates between two subsystems, the innate and the adaptive immune system.

3.1.1 INNATE IMMUNE RESPONSE

The innate immune defenses provide a first and immediate, but not long-term defense. This immune response is non-specific, which means that it is responding to pathogens in a general way[2]. Hereby, it uses different mechanisms of protection from epithelial barriers to specialized cells to soluble factors.

Epithelial barriers are the first line of defense and prevent invasion of microbes. If the microbes or pathogens manage to bypass the epithelial barriers then the specialized cells of the innate immune system engulfs and digests microbes or kills the pathogens or infected cells[3]. These cells are phagocytes, like macrophages and neutrophils. The cells of the innate immune system recognize the invaders by their specific molecular structures, pathogen-associated molecular patterns, which bind to pattern recognition receptors (PRRs) of the cells. The PRRs recognize further damage-associated molecular patterns. They are found on damaged or dead host cells[2, 4, 5].

Other cell types like eosinophils and mast cells also play a role in the innate immune response by releasing pro-inflammatory mediators upon contact with pathogens or infected cells. Thereby, recruiting the natural killer (NK) cells and cells of the adaptive immune system [2, 3]. NK cells play a critical for the innate immune system. They have in contrast to the immune cells of the adaptive immune system the ability to recognize and kill virus-infected stressed cells in the absence of major histocompatibility complexes (MHC) and antibodies[6].

3.1.2 Adaptive immune response

In contrast to the innate immune system, the adaptive immune system provides a stronger and highly specific immune response. Furthermore, it allows for immunological memory, which means that each encountered pathogen is remembered by its signature antigen.

The adaptive immune response is mediated by lymphocytes and more specifically their main cell types, B and T cells. These cells derive from hematopoietic stem cells in the bone marrow[7]. T cells then develop and mature in the thymus whereas B cells stay in the bone marrow for development and maturation. During the maturation process both cell types acquire their antigen specificity through random somatic recombination of receptor-encoding gene segments. In the end of the recombination process and after passing the test for non-self recognition insuring central and peripheral tolerance[8, 9], each cell has unique receptors, which are called T cell receptors (TCR) for T cells and B cell receptors for B cells. Each cell type has its own specific task. The B cells are involved in the humoral immune response as they secrete antibodies (AB) upon activation and T cells in cell-mediated immune responses. Both cell types can develop memory cells, which allows for a faster immune response to recurring pathogens.

As mentioned, self-tolerance is important for the immune system but likewise an overshooting immune response has to be avoided as it causes immunopathology. Therefore, the immune system has regulatory mechanisms to avoid an overreaction of the immune response but still allow an effective immune response[10]. In the presented work, I focused on regulatory T cells – key mediators of immunosuppression.

3.2 T lymphocytes in the immune system

3.2.1 Development of T cells

As explained before, T cells do develop in the thymus and circulate between secondary lymphoid tissues like the lymph nodes and the spleen, and the blood[11]. Two T cell lineages can be distinguished according to their TCR type. The TCRs are heterodimers with two transmembrane polypeptide chains which are bound covalently with disulfide bridges. In 95 % of the T cells the TCR consists of an alpha (α) and a beta (β) chain whereas in 5 % of T cells the TCR consists of a gamma (γ) and a delta (δ) chain. This ratio might change during diseased states[9, 12]. Furthermore, the cells can be divided

by their co-receptor into $CD8^+$ and $CD4^+$ T cells. $CD8^+$ T cells interact with MHC class I molecules and defend the body against viruses and intracellular pathogens due to their cytolytic activity and interferon γ production. $CD4^+$ T cells interact with MHC class II molecules[3].

3.2.2 T CELL ACTIVATION AND DIFFERENTIATION

During an infection, antigen-presenting cells (APC), like dendritic cells, encounter microbes and then internalize them. Afterwards, they present antigenic peptides on MHC molecules. Additionally, B cells are another cell type, which can present antigenic peptides on MHC molecules. They take T cell dependent antigens up by receptor-mediated endocytosis and after degradation, present them on MHC molecules[7]. As mentioned above, there are two types of MHC molecules, class I and class II. MHC class I molecules present mainly cytosolic antigens but can also take up phagocytized proteins via cross presentation. Contrary to this MHC class II molecules mainly present phagocytized extracellular proteins, but if the cytosolic antigens are processed correctly, they can also be presented on MHC class II molecules[3]. Naïve T cells roam through the body via the blood stream and lymph vessels and scan the APC in the lymphoid organs. If they recognize their cognate antigen, T cells initiate an intracellular signaling cascade and get fully activated upon a co-stimulatory signal (Fig. 1). Without a co-stimulatory signal, the cells either die through apoptosis or get anergic, meaning they cannot react to their specific antigen anymore[13, 14]. For T cells to differentiate into different T helper cell subsets different cytokine signals are additionally necessary during the activation of the cells[15, 16].

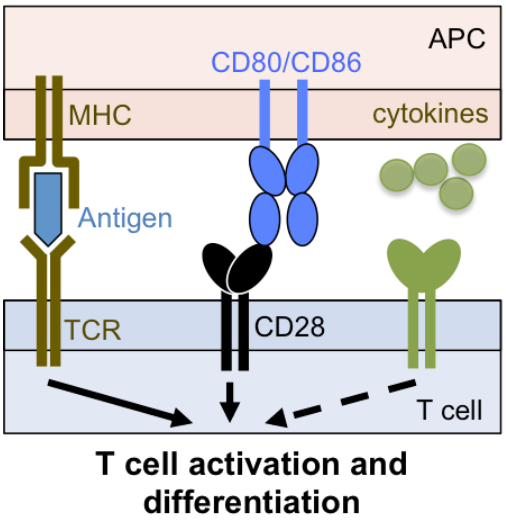
3.3 REGULATION OF THE IMMUNE SYSTEM

3.3.1 STIMULATORY IMMUNE CHECKPOINT: CD28 CO-STIMULATION

The main co-stimulatory receptor is CD28 (Fig. 1). It belongs like inducible T cell costimulator (ICOS), another co-stimulatory molecule, to the immunoglobulin superfamily and is constitutively expressed on naïve T cells. CD28 is involved in the initiation of the T cell signaling cascade and upon binding to its ligands prompts T cell expansion[17].

CD28 is a homodimer glycoprotein[18] and it binds monovalently[19] to CD80 and CD86 presented on activated APC[13]. CD86 is amply expressed in steady state and faster

induced upon activation in APC, indicating that CD86 might be a major ligand for initial T cell priming[20]. CD80, on the other side, is the more potent ligand during T cell and APC interaction[21]. CD28 signaling in conventional T (T_{conv}) cells is needed for interleukin (IL) 2 production of the cells. The secreted IL-2 and CD28 signaling is important for regulatory T (T_{reg}) cells[22].



T cell activation and differentiation

Figure 1 Three signals are needed for T cell activation and differentiation

For the T cell to get fully activated a TCR and a CD28 costimulatory signal is needed. For signaling they bind to an antigen presented on MHC and to CD80 or CD86, respectively. In addition, T cell differentiation needs a signal through cytokines, depending on which cytokines are in the periphery different T helper cell subsets develop[15, 16].

3.3.2 Inhibitory immune checkpoint: CTLA-4

Next to stimulatory checkpoints, the immune system has inhibitory immune checkpoints. One of them is the inhibition through cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), which shares its ligands with CD28. CTLA-4 binds bivalently to its ligands CD80 and CD86 and has therefore a 20 times higher binding affinity than CD28[19, 23].

CTLA-4 is constitutively expressed on T_{reg} and up-regulated upon activation in T_{conv}[24, 25]. This suggests that CTLA-4 is important for the suppressive function of T_{reg} and allows for effective control of T cell responses[19]. There are three ways for CTLA-4 to mediate inhibition of the immune response. Firstly, CTLA-4 binds to its ligands and induces a signaling cascade in the APC, which leads to the expression of immunosuppressive genes. Secondly, it inhibits the TCR/CD28 signaling in the T cell and lastly,

captures its ligands from the APC and in-cooperation them into the T cell[26-28] (Fig. 2). Thereby, CTLA-4 takes away ligands for the stimulatory immune pathway via CD28. For these suppressive functions of CTLA-4 and to get in contact with its ligands, it must be brought to the cell membrane from its intracellular storages. This is achieved through constant turnover of CTLA-4 at the cell membrane. When CTLA-4 is endocytosed, which occurs very efficiently, it is either degraded in lysosomes or recycled to the plasma membrane[27]. In previous work, it could be shown that CTLA-4 turnover is dependent on the activity of acid sphingomyelinase[1] (ASM, see 3.5.3 Sphingomyelinase and their activation). The process of transendocytosis of CTLA-4 is clathrin- and dynamin-dependent[29]. It is the same for the transendocytosis of transferrin. For the transferrin receptor this process is ceramide dependent and the receptor activates ASM upon binding, thus leading to the generation of the necessary ceramide at the cell membrane[30]. Therefore, it is reasoned that ASM activity might play a role in transendocytosis of CTLA-4.

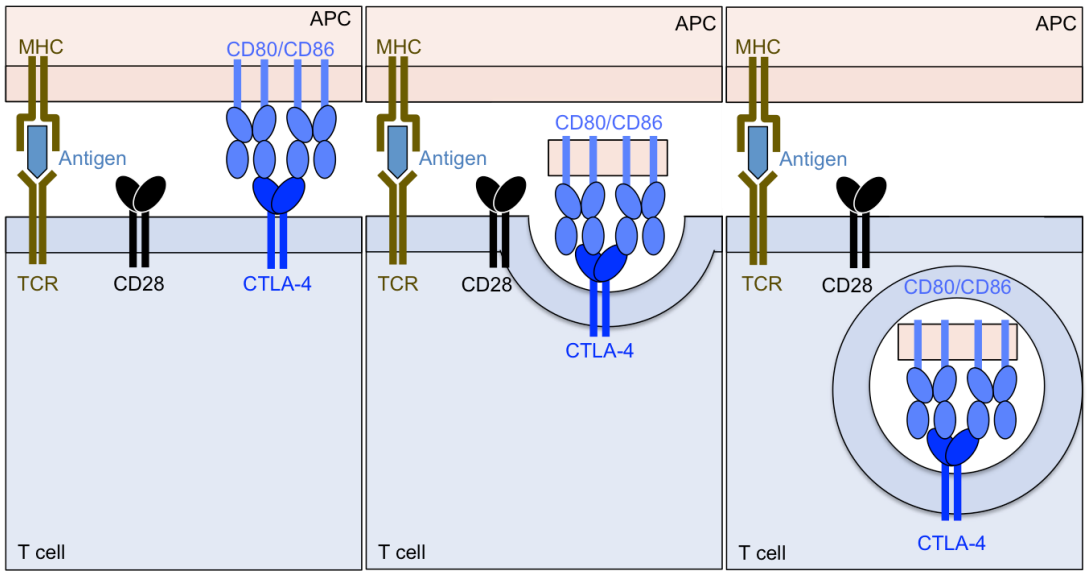


Figure 2 Transendocytosis of CTLA-4-CD80/CD86 complex

CTLA-4 cycles from its intracellular stores to the cell membrane and back. Therefore, it gets endocytosed and transported through the cell in vesicles. At the cell membrane CTLA-4 can bind to its ligands CD80 or CD86 which are present on APC. When CTLA-4 gets endocytosed while being bound to CD80/CD86, CTLA-4 takes CD80/CD86 and the surrounding membrane of the APC and transendocytoses it into the T cell.[26-28] Moreover, CTLA-4-mediated transendocytosis leads to a down regulation of CD80 and CD86 expression in the APC[26, 31].

3.3.3 *Inhibitory immune checkpoint: PD-1*

Programmed cell death-1 (PD-1) is another inhibitory immune checkpoint. As CTLA-4, PD-1 suppresses T cell activation and is an important cancer immunotherapy target. Binding of PD-1 to programmed cell death ligand 1 (PD-L1) protein transmits an inhibitory signal based on interaction with phosphatases, i.e. Src homology region 2 domain-containing phosphatases-1 (SHP-1) and SHP-2 via immunoreceptor tyrosine-based switch motif (ITSM)[32]. This reduces the proliferation of antigen-specific T-cells in lymph nodes, while simultaneously reducing apoptosis in regulatory T cells.

Engagement of PD-1 on T cells by its ligand PD-L1 delivers a signal that inhibits CD28- and TCR-mediated activation of T cells. The main target of PD-1 is CD28 which is signaling gets inhibited by dephosphorylation[33].

3.4 $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells

In 1995, $CD4^+$ T cells, constitutively expressing CD25, were described to have a suppressive function[34]. These cells inhibit other immune cells and thereby regulate or terminate immune responses[34, 35].

Later, forkhead box protein 3 (Foxp3) was found to be the master regulator transcription factor of $CD4^+$ $CD25^+$ T_{reg}[36-39]. This discovery was based on previous observations in Scurfy mice[40, 41] and immune-dysregulation, polyendocrinopathy and enteropathy X-linked syndrome (IPEX) patients who suffer from autoimmunity as a consequence of mutations in the Foxp3 gene[42].

The function of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} is to inhibit other immune cells and thereby regulating or terminating immune responses. The cells are important for self-tolerance[34]. As explained before, patients missing this cell type suffer from autoimmunity.

3.4.1 *Development of regulatory T cells*

T_{reg} can either develop in the thymus, natural T_{reg}, or differentiate in the periphery, induced T_{reg}, from naïve $CD4^+$ T cells[43]. For the development of T_{reg} in the thymus, their affinity for self-peptide MHC complexes is crucial. Hereby, one distinguishes between a strong, an intermediate and a weak affinity. Strong and intermediate affinity leads to cell death of T_{conv} (through apoptosis), but not T_{reg}[44]. Lastly, T_{reg} require CD28 signaling for their full development. It is needed for Foxp3 up-regulation[45]. In CD28

deficient mice a reduction by approximately three fourths of the T_{reg} population can be observed compared to wild type mice[46-50].

3.4.2 *Effector mechanism of regulatory T cells*

T_{reg} need to be activated to perform their suppressive function. Though once the T_{reg} is activated through antigen binding to its TCR and CD28 co-stimulation, it can suppress antigen independently. This effect is called Bystander-Suppression[51]. Dependent on the immune status, the type and the locus of the immune response, the activated T_{reg} use different mechanisms for their suppressive function[43, 52-54].

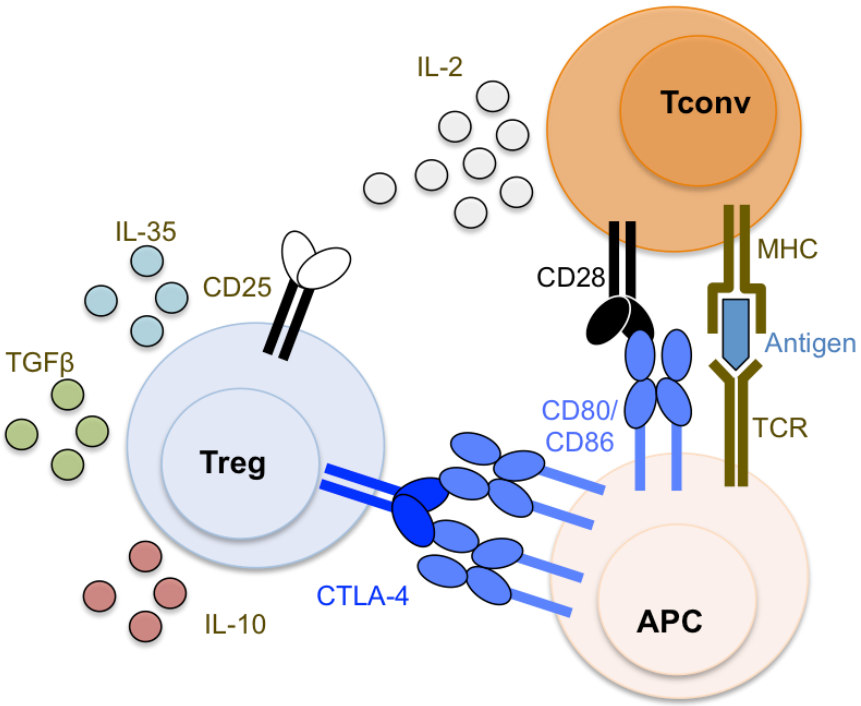


Figure 3 Effector mechanisms of T_{reg}

T_{reg} use different mechanisms to influence other immune cells. As mentioned in part 3.3.2, T_{reg} use CTLA-4 for transendocytosis of CD80/CD86 thereby inhibiting the costimulatory effect of CD28. Other mechanisms are through the release of inhibitory cytokines or cell-cell contact. Furthermore, T_{reg} have a higher affinity receptor for IL-2, which limits IL-2 accessibility for T_{conv}.

Firstly, T_{reg} suppress through the secretion of the anti-inflammatory cytokines IL-10, IL-35 and transforming growth factor β. Secondly, they reduce the availability of the cytokine IL-2 for T_{conv} as they constitutively express CD25 which forms the trimeric high affinity IL-2 receptor together with CD122 and CD132. Thirdly, T_{reg} express granzyme

B, a serine protease, and perforin, a pore inducing protein[55, 56]. They induce apoptosis in the target cells and thereby, T_{reg} can reduce or stop the immune response. Lastly, as previously explained, T_{reg} constitutively express CTLA-4 and can thereby remove CD80 and CD86 from the cell surface of activated APC through a process called transendocytosis. This process blocks the CD28 co-stimulation of T_{conv} and therefore the activation of T_{conv}[26].

3.4.3 *Subpopulations of regulatory and conventional CD4⁺ T cells*

For the presented work, the $CD4^+$ T cell population was divided either into $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} and $CD4^+$ $CD25^-$ $Foxp3^-$ T_{conv} or further classified into subpopulations according to Miyara et al.[57] Here, five subpopulations are defined by their expression of CD45RA, a marker for naïve cells, and CD25, a marker for activation. This results in $CD45RA^+$ $CD25^-$ naïve conventional T cells (nT_{conv}), $CD45RA^-$ $CD25^-$ memory conventional T cells (mT_{conv}), $CD45RA^-$ $CD25^{low}$ memory conventional T cells ($CD25^{low}$ mT_{conv}), $CD45RA^+$ $CD25^+$ resting regulatory T cells (rT_{reg}) and $CD45RA^-$ $CD25^{high}$ effector regulatory T cells (efT_{reg}) [57, 58].

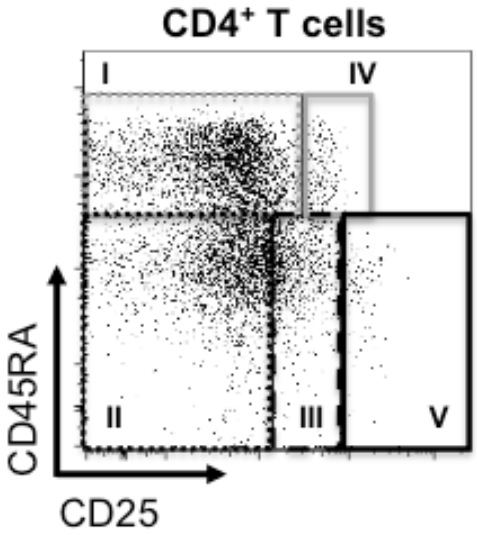


Figure 4 Exemplary gating strategy for the five subpopulations of $CD4^+$ T cells

For $CD4^+$ T cells, the two markers CD45RA and CD25 are plotted against each other: (I) Naïve conventional T cells, (II) memory conventional T cells, (III) $CD25^{low}$ memory conventional T cells, (IV) resting regulatory T cells and (V) effector regulatory T cells[57, 58].

3.5 SPHINGOLIPIDS

Eukaryotic cell membranes have three main structural components; cholesterol, glycerophospholipids and sphingolipids[59-61]. Sphingolipids form large and condense membrane domains upon cell activation[62] and therefore play a major role in signaling complexes near to the membrane and the sorting processes of membrane proteins[63].

Furthermore, active sphingolipids can modulate different important functions of the cell like cell proliferation, differentiation or apoptosis[64, 65]. They can do this by changing the fluidity and stiffness of cell membranes[63].

3.5.1 STRUCTURE OF SPHINGOLIPIDS

The defining structure of sphingolipids is the long-chained unsaturated aminoalcohol backbone, which is called sphingosine. The simplest sphingolipid is ceramide which forms the center of the sphingolipid metabolism. It consists of the sphingosine backbone, a fatty acid (of C-atom chain lengths of C16 to C24) and hydrogen. For more complex sphingolipids, ceramide is metabolized by acylation of the amino group, adding a once or twice unsaturated fatty acid, and esterification of the polar head group. Sphingolipids are located in the bilayers of cell membranes and can be found on organelles thanks to the fact that they can be compartmentalized. They have various physical properties and functions depending on their location[59, 66, 67].

3.5.2 SPHINGOLIPID METABOLISM

Sphingolipid metabolism is complex and highly regulated. Several bioactive molecules, like ceramide, sphingosine and sphingosine-1-phosphate, can be quickly converted and formed[68]. The metabolism centers on ceramide, which is synthesized in the endoplasmic reticulum (ER). From the ER, ceramide is further distributed to various cellular compartments, like the Golgi apparatus, for further synthesis.

There are three pathways, de novo pathway, salvage pathway and sphingomyelin hydrolysis, through which ceramide can be synthesized[69].

De novo pathway

The de novo synthesis of ceramide (Fig. 5, red) is a four-step pathway, which happens in the ER. The first step is a condensation of palmitate, a fatty acid, and serine, an

amino acid, to build 3-ketodihydrosphingosine. This process is catalyzed by the enzyme serine palmitoyl transferase. Then follows the reduction of the product to dihydrosphingosine. Afterwards, dihydrosphingosine is further acylated to dihydroceramide by the enzyme dihydroceramide synthase. Lastly, dihydroceramide desaturase catalyzes dihydroceramide to ceramide.

The produced ceramide is then transported from the ER to the Golgi apparatus. Therefore, either the ceramide transfer protein or vesicular trafficking are needed as ceramide, as a lipid, is highly hydrophobic and thus unable to leave the membrane and move through the cytosol on its own[70]. At the Golgi apparatus, other parts of the sphingolipid metabolism happen and ceramide can be further processed to other sphingolipids and glycosphingolipids[59, 69].

Salvage pathway

In this pathway, sphingolipids and glycosphingolipids get degraded to ceramide (Fig. 5, green and orange). This happens in the acidic compartments of the cells, like the lysosomes and the late endosomes. In these conditions, sphingolipids and glycosphingolipids get first degraded to ceramide but as it can't leave the compartments, it is further hydrolyzed to sphingosine and a fatty acid by acid ceramidase. These products are able to leave and to reenter the ceramide and sphingosine-1-phosphate synthesis pathway at the surface of the ER[69].

Sphingomyelin hydrolysis

The synthesis of ceramide from sphingomyelin is catalyzed by sphingomyelinase (Fig. 5, grey). The pathway is activated by exogenous stress signals[71] and happens at the cell membrane. The released ceramide can then be further converted in other bioactive molecules[68].

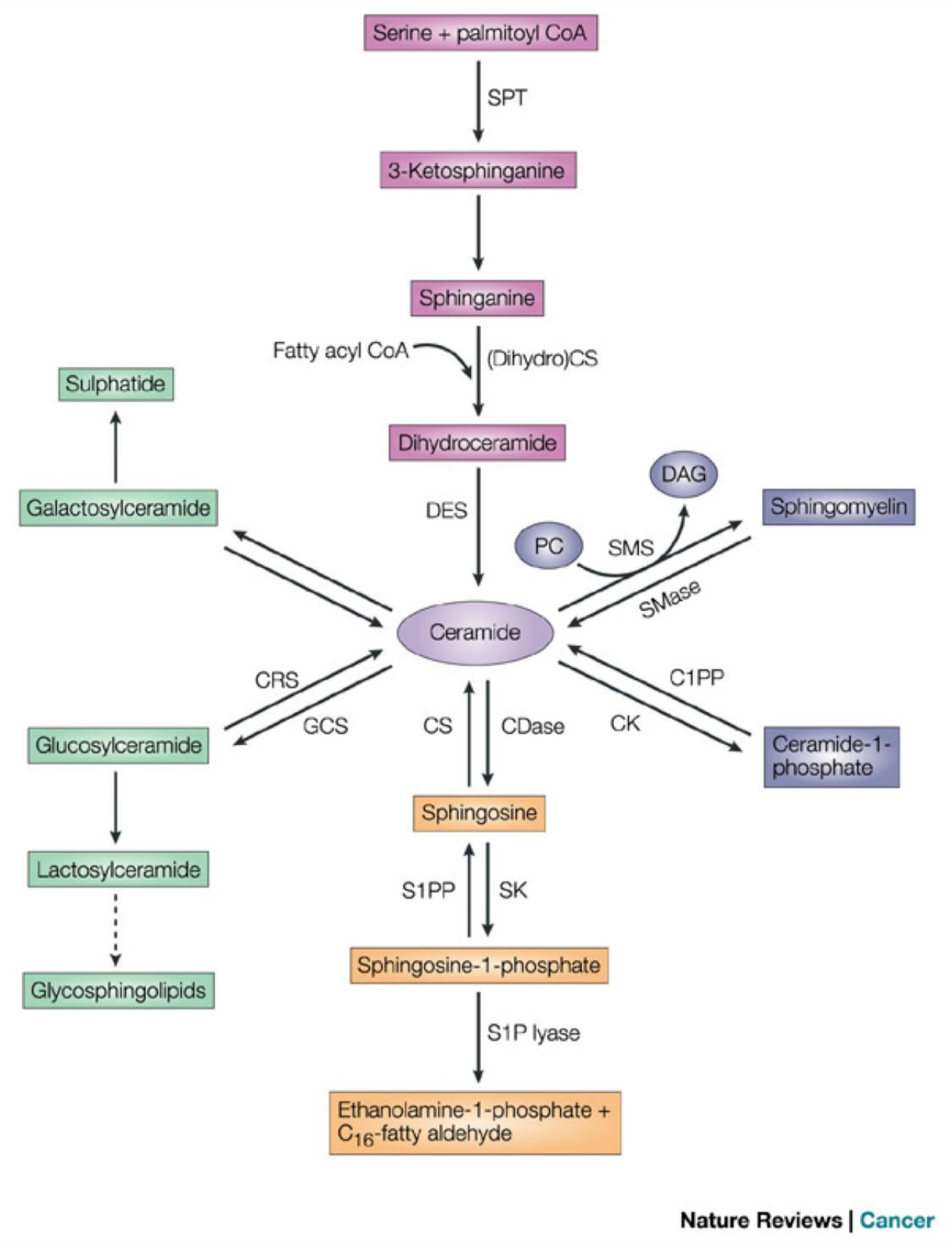


Figure 5 Sphingolipid metabolism.

B. Ogretmen & Y. A. Hannun, Nature Reviews Cancer, 2004[72]

3.5.3 SPHINGOMYELINASES AND THEIR ACTIVATION

Acid sphingomyelinase

The main interest of the presented work was the acid sphingomyelinase (human: ASM; mouse: Asm). Thus, it was focused on sphingomyelin hydrolysis. As mentioned above, the acid sphingomyelinase catalyzes the cleavage of sphingomyelin to ceramide and phosphocholine. Therefore, the ASM is a key modulator of the cellular signaling pathways mediated by bioactive sphingolipids. Sphingomyelinases are classified by two characteristics: their cation dependence and pH optima. The latter is which they are named after. Therefore, acid sphingomyelinase is active in the acid pH range with an optimum at a pH of 5. Such a pH level is found in the late endolysosomal vesicles, where ASM is compartmentalized. More precisely, the ASM can be found at the inner membrane leaflet of the lysosome. After activation by external stimuli, the ASM (within lysosomes) is transported to the cell membrane[66, 67]. Fusion of the vesicle with the plasma membrane relocates the ASM to the outer leaflet of the cell membrane[73-75]. Here, the ASM gets in contact with sphingomyelin, which the plasma membrane is rich of, and it can do its part of the sphingolipid metabolism[66, 67] (Fig. 6).

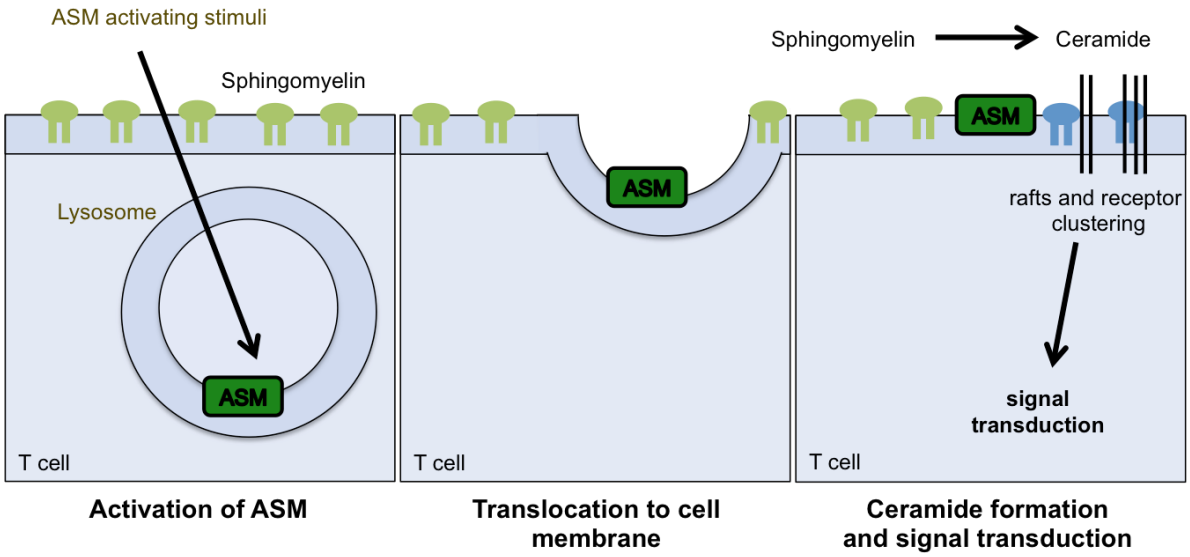


Figure 6 Translocation of activated ASM and its modulation of ceramide signaling clusters

ASM is activated through external stimuli (left) and upon activation is relocated from the inner leaflet of the lysosome to the outer leaflet of the cell membrane (middle). Here, ASM catalyzes sphingomyelin to ceramide. Ceramides accumulate at the cell membrane, which influences the distribution of proteins and receptors at the cell membrane and also clustering of certain receptors. Thus, ASM can modulate the formation of ceramide signaling clusters.

Secretory acid sphingomyelinase

Various forms of ASM are generated by alternative splicing. If the gene for ASM, sphingomyelin phosphodiesterase 1 (SMPD1), is spliced differentially from the main lysosomal form, the resulting ASM form gets secreted through the Golgi apparatus secretion way. It differs from the ASM residing in the lysosome in its degree of glycosylation and its dependency on zinc ions[76, 77].

Neutral sphingomyelinase

The neutral sphingomyelinase (NSM) is active around a pH of 8, i.e. in the neutral pH range[78]. In contrast to the acid sphingomyelinase, it can be found at the inner instead of the outer leaflet of the cell membrane. There are four forms of the neutral sphingomyelinase: NSM1, NSM2, NSM3 and mitochondria-associated NSM. Of these forms, only the NSM2 has the same function as the ASM and metabolizes sphingomyelin to ceramide and phosphocholine[59].

The activation of sphingomyelinases

For the activation of sphingomyelinases, an external stimulus is necessary. These stimuli can be the binding of antibodies (AB) to dendritic cell-specific ICAM-grapping non-integrin (DC-SIGN) or the ligation of pro-apoptotic receptor molecules like TNF, IL-1β or CD95 receptors[79-84]. Furthermore, a costimulatory signal through CD28 activates the ASM[85]. If the cells receive additionally to a signal through CD28, one through CD3 no activation of ASM activity is observed[73, 83, 86]. In contrast, the activation of the NSM requires CD3 or both CD3 and CD28 stimulation[85-87].

3.5.4 Function of sphingolipids and sphingomyelinases in T cells

The products of the sphingomyelin pathway, ceramide, sphingosine and sphingosine-1-phospate influence T cells through two ways. First, they play a role in membrane properties and composition and T cells are highly dependent on their cell membrane for their biological activity. These include T cell activation, clonal expansion and apoptosis. The generation of ceramide through sphingomyelin cleavage by sphingomyelinases leads to an accumulation of ceramide and the formation of ceramide-rich membrane domains. These domains are highly hydrophobic, leading to an exclusion of cho-

lesterol in these areas[79, 88, 89]. Furthermore, the lateral and vertical distribution of receptors, as well as the position and signaling of proteins, is influenced by ceramide-rich domains. The accumulation of these domains in the cell membrane leads to a reduction of actin cytoskeleton dynamics in T cells and thereby to a reduced spreading and polarization of T cells[86, 90, 91]. Moreover, CD95 clustering is especially dependent on ceramide-rich domains[74, 75, 92]. CD95 is part of the CD95/Fas apoptosis pathway, which is an important mechanism for the regulation of T cell homeostasis. Furthermore, it is part of the termination system for T cell responses by depletion of activated T cells[3].

Second, they have a bioactive effect. For ceramide, it is known that it is involved in the inhibition of cell growth and differentiation[93] and that it promotes cell death through apoptosis[73, 94-96], necrosis[97] and senescence[96, 98]. Sphingosine has a similar effect and acts pro-apoptotically and anti-mitogenicly. Sphingosine inhibits protein kinase C and thus is part of the actin-cytoskeleton regulation, thereby being involved in cell growth, proliferation and survival[67, 99]. Sphingosine-1-phospahte (S1P) has an enhancing role in chemokine and cytokine production, and vascular integrity and is of importance for the immune system[100]. Therefore, S1P has opposite effects compared to ceramide and sphingosine, which means that it has anti-apoptotic effects and promotes cell growth and proliferation[101].

3.6 Sphingolipids and sphingomyelinases in various diseases

A genetic deficiency in ASM activity in human leads to a disease called Niemann-Pick disease. It is a lysosomal storage disease, in which sphingomyelin cannot be catalyzed into ceramide and phosphocholine, leading to an accumulation of sphingomyelin in endo-lysosomal compartments[79, 91].

Furthermore, a targeted inhibition of ASM activity could be beneficial for multiple sclerosis. In an experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, it could be shown that genetic deficiency for the ASM inhibits EAE development[102]. In a study analyzing patients affected by systemic lupus erythematosus (SLE), it was shown that $CD4^+$ T cells have an increased glycosphingolipid transport and subsequently an accumulation in intracellular compartments. It is assumed that this disbalance is responsible for the abnormal, hyperactive T cell function

in SLE patients[103]. Moreover, the turnover rate of CTLA-4 proteins at the cell surface is increased in T cells from SLE patients[103, 104].

Furthermore, other studies with functional ASM inhibitors showed an improved disease progression in T cell mediated autoimmunity and graft-versus-host disease (GvHD)[105]. For a mouse model of allergic rhinitis, functional inhibition of Asm leads to a shift in T cell frequencies from pathogenic T helper cells expressing IL-17 (T_h17) cells to protective T_{reg}[106].

3.7 DEPRESSION

Major depressive disorder (MDD) is a complex disease and affects the person concerned in several domains, including cognition, emotion, motivation and physiology. Furthermore, MDD has an inflammatory component[68, 107] and thus cooperation between psychologists and immunologists is essential to further understand MDD.

In 1980, MDD was recognized as a disease and added to the Diagnostic and Statistical Manual of Mental Disorders (DSM-III). Today it is the leading cause of disability worldwide and in 2015, 216 million people were diagnosed with MDD accounting for 3 % of world's population. Overall, this disease will affect one out of every five people in their lifetime[108, 109] and in general, females are twice as often affected than men. The first onset of MDD happens usually between 20 and 40 years of age[109, 110]. It can be caused by major life changes or be a side effect of some medications. Furthermore, people with a chronic health problem, a family history of the disease or a problem with substance abuse have a higher predisposition for MDD[110].

Clinically, MDD is characterized by constantly low mood regardless of the situations over a time frame of at least two weeks[111] and mostly accompanied by aversion to activity. Common symptoms of MDD are sadness, difficulties with concentration and thinking and a significant change in sleeping time and in appetite. On the emotional side patients often state feelings of hopelessness, dejection and suicidal thoughts[109]. There are three different forms of depression. The first is a unipolar depression, people suffering from it have recurring episodes of depression interspaced with depression

free intervals. The second is a bipolar depression and differs from the first as the depressive episodes are interchanged with manic episodes. The last is an ongoing depressive mood for at least two years and is called dysthymia[111].

MDD is diagnosed by a mental status examination and a personal report of experiences by the person concerned. In our study, the 21-item Hamilton rating scale for depression (HAMD)[112] together with the 10-item Montgomery-Åsberg Depression Rating Scale (MADRS)[113] was used to analyze the severity of the patient's depression and to evaluate the progression of their recovery during treatment[114, 115]. A trained rater filled out the 21 or 10 questions, respectively, after interviewing the patients. For every question, the rater will evaluate the severity of a symptom of MDD and give it a value between 0 and 4 or 0 and 6, respectively. In the end, the overall score is calculated and the severity of MDD determined[116, 117] (Tab. 1).

Depression Rating Scales

HAMD	MADRS	Diagnosis
0-8	0-6	no depression
9-16	7-19	mild depression
17-24	20-34	moderate depression
25 and higher	34 and higher	major depression

Table 1 Depression Rating scales are used to diagnose the severity of depression

Trained raters answer 21 questions of the HAMD with 0 to 4 points for each patient and calculate the overall score through adding all points together. For MADRS scores 10 questions are answered by trained raters. In the present work, the patients that were recruited all had a HAMD score of higher than 14 at admission and were diagnosed with at least mild depression.

The psychiatrist decides then the best form of treatment. In general, treatment is a mix of counseling by a psychiatrist or psychologist and antidepressant medication. If no fitting antidepressant is found for a patient, it can be decided to use electroconvulsive therapy (ECT)[118].

3.7.1 ANTIDEPRESSANTS

The medications that are used to treat MDD are called antidepressants. Furthermore, they are often used to treat some anxiety disorders, some chronic pain conditions and to help with some addictions[119]. But on the other side, antidepressants can also have side effects. One critical side effect is the increase of suicidal thoughts in young patients diagnosed with depression. Common side effects in adults are dizziness, dry mouth, headaches, sexual dysfunction, and weight gain[120-122]. Additionally, it can come to a discontinuation syndrome after stopping antidepressant treatment, which might falsely be seen as recurrent depression[123, 124].

The pharmacological principle behind all currently used antidepressants is based on the monoamine hypothesis. It says that depression occurs due to an imbalance of the monoamine neurotransmitters, like serotonin, norepinephrine and dopamine. Therefore, the currently marketed antidepressants are classified into subgroups characterized by their mode of action. Antidepressants exert further anticholinergic, anti-histaminergic and anti-inflammatory actions[79]. For the purpose of this work, the antidepressants were further characterized by their ability to have an effect on the sphingolipid metabolism, more precisely an inhibitory effect on ASM activity[79, 125, 126]. For *in vitro* studies, I concentrated on the two antidepressants sertraline (Ser) and citalopram (Cit) (Fig. 7). Both are widely used antidepressants in clinical treatment of MDD[127] and are of the same class of antidepressants, called selective serotonin reuptake inhibitors (SSRI). SSRIs block the reuptake of serotonin into the presynaptic cell via the serotonin transporter, which is a membrane protein, thus increasing its extracellular levels. Therefore, more serotonin is present in the synaptic cleft, which can then bind to the postsynaptic receptor. Furthermore, sertraline has a high capacity to inhibit the ASM activity whereas citalopram has a low capacity, 88 % and 20 % respectively[125, 126]. Important for this is their level of lipophilicity and their protonation in an acidic environment like the one in the lysosome (intralysosomal). Both antidepressants are similarly protonated in an acidic environment, i.e. have similar acid dissociation constant (pK_a) values, but they differ in their level of lipophilicity (logP) with sertraline being more lipophilic than citalopram. The lipophilicity is important as the compound has to reach the ASM in the lysosome and therefore has to diffuse through several membranes. The protonated state in an acidic environment keeps the drug in the lysosome and prevents

the diffusion back through the membrane leading to an accumulation of the compound in lysosomes.

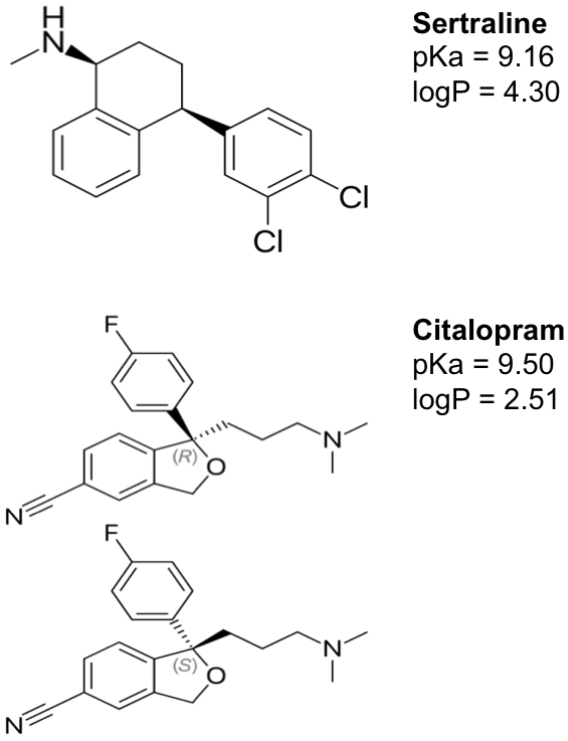


Figure 7 Chemical structures and physical properties of sertraline and citalopram

Sertraline and citalopram are both widely used antidepressants of the SSRI group. But they have different capacities to functionally inhibit ASM activity as their logP values differ. The logP value reflects the level of lipophilicity of the molecules. A positive value stands for lipophilic properties and the higher the more lipophilic is a molecule. The pK_a stands for the protonated state of molecules in an acidic environment. If the value is above 8, the molecules are mostly in a protonated state in such an environment.

The mode of inhibition of the ASM activity is that the protonated sertraline interacts with the inner leaflet of the lysosome membrane and accumulates there. ASM interacts also with the membrane via a proton and if the competition is too high, it gets displaced from the membrane and afterwards degraded in the lysosome[125, 126, 128]. Therefore, ASM is functionally inhibited as it cannot be transported to the outer leaflet of the cell membrane, where the catalyzation of the cleavage of sphingomyelin to ceramide and phosphocholine takes place (Fig. 8).

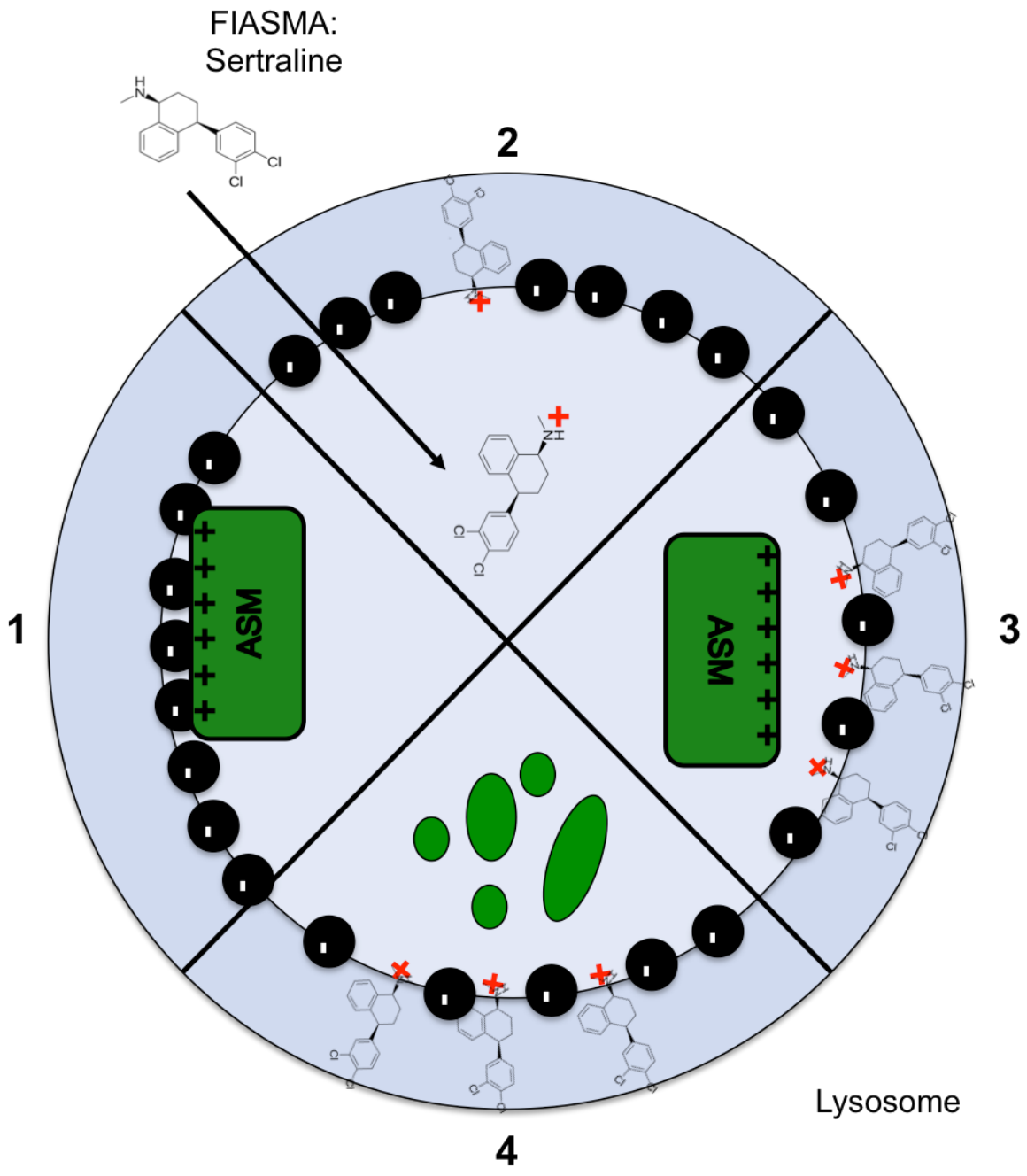


Figure 8 Sertraline's mode of action

(1) ASM is located at the inner leaflet of lysosomes and its positively charged amino acids (+) bind to the negatively charged polar headgroup of membrane lipids (black circle). (2) Functional inhibitors of acid sphingomyelinase (FIASMA), like sertraline, inhibit ASM by accumulating in lysosomes. Here, they get protonated (red +) and can therefore interfere with the binding of ASM to the membrane. (3) This causes the ASM to disassociate from the membrane and to get inactivated. (4) The disassociated ASM gets protolyzed and degraded.

3.8 OBJECTIVES OF THE DOCTORAL THESIS

T cell function is highly dependent on their cell membrane and its components like sphingolipids[129-131]. Sphingolipids influence the signaling of surface receptors and co-stimulatory molecules due to their importance in forming platforms for signal transduction. Furthermore, they play a role in vesicular transport and compartmentalization[132] and can act as a second messenger[66].

In previous work of the laboratory, it could be shown that both genetic ablation and pharmacologic inhibition of Asm in mice increases the activity and frequency of $CD4^+$ $Foxp3^+$ T_{reg} among $CD4^+$ T cells[1]. For the immune response, this leads to a dampened autoimmune or inflammatory reaction, but also to a reduced antiviral immune response.

A long-term goal of the work is to understand the effects of changes in sphingolipid metabolism on the immune response. The final goal would be to manipulate the immune response in a directed way by influencing sphingolipid metabolism. Either by increasing T_{reg} suppressive or T_{conv} effector activity this would be beneficial for autoimmune and inflammatory diseases or viral infections, respectively.

The goal of the presented work was to analyze the effect of pharmacological inhibition of ASM on human T cells, *in vitro* and *in vivo.* Moreover, the underlying mechanism, which leads to the observed effects, was studied and differences in sphingolipid content and functionality of affected and unaffected cells characterized.

4 MATERIALS

4.1 CELLS

Cell lines

Cell line	Cell type	Species	Origin
MEF	mouse embryonic fibroblast	mouse	University Freiburg, Dr. J. Rohr
MEF-CD80-mScarlet	mouse embryonic fibroblast	mouse	University Freiburg, Dr. J. Rohr

Primary cells

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from leukocyte reduction chambers supplied by the University Hospital Würzburg, Institute for Transfusion Medicine and Haemotherapy.

PBMC from patients were isolated from blood supplied by the University Hospital Würzburg, Department of Psychiatry, Psychosomatics and Psychotherapy, Center of Mental Health. Prior to inclusion into the prospective observatory study, all participants had to give written informed consent. The local Ethics Committee of the Faculty of Medicine at the University of Würzburg gave its approval for the clinical study protocol (vote no. 128/15). In total 70 participants, depressed patients, were recruited (mean age = 45.64±13.43 SD, 45.8 % female). This was done within the first 2-5 days after admission to the Department of Psychiatry, Psychosomatics and Psychotherapy of the University Hospital Würzburg.

4.2 MICE

To obtain primary mouse cells, C57BL/6 wild type mice of different age and gender were used, which were bred under specific pathogen-free conditions in the animal facility of the Institute for Virology and Immunobiology of the University of Würzburg in accordance with German Animal Protection Law.

4.3 BIOCHEMICAL AND CHEMICAL REAGENTS

Chemical	**Manufacturer**
2-mercaptoethanol	Invitrogen/Life technologies
4-OH Tamoxifen	Sigma-Aldrich/Merck KGaA
Amitriptyline	Sigma-Aldrich
Ammonium chloride (NH_4Cl)	Sigma-Aldrich/Merck KGaA
Bovine Serum Albumin (BSA)	AppliChem GmbH
Calcium chloride	AppliChem GmbH
Citalopram	Sigma-Aldrich/Merck KGaA
Citric acid	Merck KGaA
Disodium hydrogen phosphate (Na2HPO4)	Carl Roth GmbH & Co. KG
Desipramine	Sigma-Aldrich
Entellan	Merck KGaA
Ethanol	AppliChem GmbH
Ethylendiaminetetraacetic acid (EDTA)	AppliChem GmbH
Ficoll (Histopaque 1077)	Sigma-Aldrich/Merck KGaA
Formaldehyde (37 %)	AppliChem GmbH
Fetal Calf Serum (FCS)	Gibo/Life technologies
GW4869	Sigma Aldrich
L-Glutamine	Carl Roth GmbH & Co. KG
LPS	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	AppliChem GmbH
Magnesium Sulfate ($MgSO_4$)	Merck KGaA
Neomycin	Belapharm
Non-essential amino acids MEM	Gibco/Life Technologies
Normal rat serum	In-house
Penicillin	InfectoPharm Arzneimittel und Conilium GmbH
PMA	Sigma-Aldrich
Polymyxin B	Sigma-Aldrich/Merck KGaA
Potassium bicarbonate ($KHCO_3$)	AppliChem GmbH
Potassium chloride (KCl)	AppliChem GmbH
Potassium di hydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG
Proleukin (recombinant, human IL-2)	Novartis AG
Roti-Mount Fluor Care	Carl Roth GmbH & Co. KG
Sertraline	Sigma-Aldrich/Merck KGaA
Sodium azide (NaN_3)	AppliChem GmbH
Sodium Carbonate (Na_2CO_3)	AppliChem GmbH
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG
Sodium Citrate ($Na_3C_6H_5O_7$)	AppliChem GmbH

Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG
Sodium Pyruvate ($C_3H_3NaO_3$)	Sigma-Aldrich/Merck KGaA
Streptomycin	AppliChem GmbH
Tris ($C_4H_{11}NO_3$)	Merck KGaA
Tween 20	Sigma-Aldrich/Merck KGaA
Xylene	AppliChem GmbH

4.4 BUFFERS, SOLUTIONS AND CULTURE MEDIUM

Chemical	**Company or Recipe**
3, 6 µm beads	Polysciences
Agarose, ultrapure	BioFroxx
ATP (10mM)	Thermo Fischer
ATV	136.89 M NaCl 5.36 M KCl 3.22 M D(+)-Glucose 6.90 M $NaHCO_3$ 0.05 % Trypsine 0.54 M EDTA ad aqua dest., pH 7.4
BSS	50 g BSS I 9.25 g BSS II Mix 125 ml
CFSE labelling washing buffer	5 % FCS in PBS
Chloroform	AppliChem
Complete, EDTA-free, Protease Inhibitor Cocktail	Roche
DMSO	AppliChem
dNTP Mix, 10 mM each	Thermo Fisher Scientific Inc
EDTA	Sigma-Aldrich
Ethanol	AppliChem
FACS buffer	PBS (w/o Ca^{2+}/Mg^{2+}) 0.5 % (w/v) BSA 0.02 % (w/v) NaN_3
FCS	Biochrome AG
Heparin	Sigma-Aldrich
Histopaque-1077	Sigma-Aldrich
HMU-PC	Moscerdam Substrates
HMU-PC solution	1.35 mM HMU-PC 0.25 M Na-Acetate 30 µM Na-Taurochlorate ad aqua dest., pH 7.4 or 5.2
HPLC-grade water	AppliChem

Human T cell media	RPMI 1640 1 % Sodium pyruvate 100 mM (100x) 1 % MEM NEAA (100x) 1 % Hepes buffer solution 1 M 1 % Penicillin/Streptomycin 0.1 % β-Mercaptoethanol 10 % human AB serum
Isopropanol	AppliChem
Lysis buffer	50 mM Tris-HCl 100 mM NaCl 1 mM DTT ad aqua dest., pH 7.4
Minimal essential medium	Gibco
Methanol	AppliChem
Mouse T cell media	RPMI-1640 5 % FCS 2 mM Glutamine 10 mM HEPES 50µM 2-Mercaptoethanol 100 µg/ml Streptomycin 100 U/ml Penicillin
Na_2SO_4	AppliChem
NSM Assay resuspension buffer	20 mM HEPES pH 7.4 15 mM $MgCl_2$ 10 mM β-Glycerolphosphate protease inhibitor cocktail ad aqua dest.
NSM/ASM Assay lysis buffer	20 mM HEPES pH 7.4, 2 mM EDTA 5 mM EGTA 5 mM DTT 1 mM Na-Ortho-Vanadate 10 mM β -Glycerolphosphate protease inhibitor cocktail ad aqua dest.
NSM/ASM Assay stop buffer	0.2 M glycine 0.2 M NaOH 0.25 % Triton-X ad aqua dest., pH 11
NTE	0.1 M Tris 1 M NaCl 0.01 M EDTA ad aqua dest.
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 x H_2O 1.8 mM KH_2PO_4 1mM$CaCl_2$ x2H_2O 0.5 mM $MgCl_2$ x 6 H_2O ad aqua dest.
PBS (w/o Ca^{2+}, Mg^{2+})	137 mM NaCl

	2.7 mM KCl 10 mM Na_2HPO_4 x H_2O 1.8 mM KH_2PO_4 ad aqua dest.
Penicillin/Streptomycin (100 i.e./mL)	Sigma-Aldrich
Propidium Iodide	Immunotools
RPMI-1640	Gibco
TAE-buffer	25 mM Tris 0.57 % (v/v) acetic acid 0.6 M EDTA ad aqua dest., pH 8.0
TRIzol reagent	Invitrogen
Trypanblue	0.25 % (w/v) in PBS (w/o Ca^{2+} /Mg^{2+})

4.5 KITS

Kit	**Manufacturer**
Anti-Biotin MicroBeads	Miltenyi Biotec Gmbh
Foxp3 Transcription Factor Fixation kit	eBioscience, Inc./Thermo Fisher Scientific Inc.
MagniSort CD4 T cell isolation kit human	Affymetrix/Thermo Fisher Scientific Inc.
MagniSort CD4 T cell isolation kit mouse	Affymetrix/Thermo Fisher Scientific Inc.

4.6 ANTIBODIES, DYES AND DRUGS

Specificity	**Species**	**Conjugate**	**Clone**	**Manufacturer**
Phosphatidylserine (Annexin V)	-	APC		BD BioScience
CCR7	human	Alexa488	G043H7	Biolegend
CD11b	mouse	biotin	M1/70	BD BioScience
CD127 (IL-7Ra)	human	PE	A019D5	Biolegend
CD15s	human	BV510	Cslex1	BD BioScience
CD152 (CTLA-4)	human	PE	L3D10	Biolegend
CD152 (CTLA-4)	human	PE-Cy7	L3D11	Biolegend
CD25	human	APC	BC96	Biolegend
CD25	mouse	biotin	7D4	BD BioScience
CD3	human	PE-Cy7	SKX7	Biolegend
CD4	human	FITC	RPA-T4	Biolegend
CD4	human	PerCP	RPA-T4	Biolegend
CD4	human	PE-Cy7	RPA-T4	Biolegend
CD4	human	PB	RPA-T4	Biolegend
CD4	human	Alexa 700	Okt4	Biolegend

CD4	mouse	biotin	RM4-5	BD BioScience
CD45R/B220	mouse	biotin	RA3-6B2	BD BioScience
CD45RA	human	PerCP-Cy5.5	HI100	Biolegend
CD49b	mouse	biotin	(Dx5) NK pan	BD BioScience
CD80	mouse	FITC	16-10A1	Biolegend
CD86	mouse	biotin	B7-2 (GL1)	BD BioScience
CD86	mouse	FITC	BU63	Biolegend
Foxp3	human	PB	259D	Biolegend
IgG	mouse anti-rat	biotin	-	dianova
Ki67	human	Alexa700	Ki-67	Biolegend
KI67	human	PE	Ki-67	Biolegend
PD-1	human	PE-Cy7	EH12.1	BD BioScience
Streptavidin	-	PE-Cy5	-	BD BioScience
Viability Dye	-	eFluor780	-	Invitrogen, Life technologies
CD28	human	-	CD28.2	BD BioScience
CD28	human	-	CD28.3 Fab	University Nantes, Dr. B. Vanhove
αCD3	human	-	Hit3a	BD Biosciences
αCD3	human	-	UCHT-1	BD Biosciences
CFSE	-	-	-	eBioscience
eFluor670	-	-	-	Affymetrix
PD-1	human	-	Pembrolizumab	Creative Biolabs
CTLA-4	human	-	Tremelimumab	Creative Biolabs

4.7 CONSUMABLES

Consumables	**Manufacturer**
6-, 12-, 24-, 48 and 96- well cell culture plates	Greiner Bio One
12-well microscopy slide	ibidi
25, 75 and 125 cm^2 cell culture flasks	Greiner Bio One
70 µM cell strainer	SPL Life Sciences
Cell strainer	Corning, Inc.
Cover slips	Paul Marienfeld GmbH & Co. KG
Cryo-tubes	Greiner Bio One
Dispenser tips	BRAND GmbH & Co. KG
FACS bullets	A. Hartenstein GmbH
FACS-tubes (5 ml)	Sarstedt AG & Co
Glass pipettes	VIM (Würzburg)
MS, LS and LD Columns	Miltenyi Biotec GmbH
Petri tissue culture dishes	Greiner Bio One

Plastic tubes (15 and 50 ml)	Sarstedt AG & Co
Pipette tips (300 µl)	Brand GmbH & Co. KG
Pipette tips (10, 100 and 1000 µl)	A. Hartenstein GmbH
Polystyrene round-bottom tube with cell-strainer cap (5 ml)	Falcon
Reaction tubes (0.5, 1.5, 2.0 ml)	Eppendorf
Serological pipettes (5, 10, 25 ml)	Sarstedt AG & Co
Syringe plunger (1 ml)	B. Braun Melsungen
Syringe (60 ml)	Infuject

4.8 INSTRUMENTS

Instrument	**Manufacturer**
Analytical Balance	Mettler Toledo GmbH
Centrifuge (5415C)	Eppendorf AG
Centrifuge (Megafuge 1.0R)	Heraeus/Thermo Fisher Scientific Inc.
Confocal microscope LSM 780	Zeiss
Disscetion set	A. Hartenstein
FACS Aria III	Becton Dickinson
FACS Attune NxT	Thermo Fisher Scientific Inc.
FACS Celesta	Becton Dickinson
FACScan Calibur	Becton Dickinson
FACS LSR II	Becton Dickinson
Freezer (-80°C)	Thermo Fisher Scientific Inc.
Fridge	Candy Hoover Group S.r.l.
Haematocytometer (Neubauer)	A. Hartenstein GmbH
Heatblock	Eppendorf AG
Incubator 37 °C, 5 % CO_2 (HeraCell 240i)	Hearaeus/Thermo Fisher Scientific Inc.
Laminar flow hood (Heracell)	Thermo Fisher Scientific Inc.
Light-optical microscope	Leica Microsystems GmbH
Magnetic cell separators	Miltenyi Biotec GmbH and Affymetrix
Microliter pipettes	Eppendorf
Multichannel pipette	Eppendorf AG
PCR cycler	ML Research
Pipette Controller	BRAN GmbH & Co. KG
Safire2 – Fluorescence plate reader	Tecan
Vortex mixer (Genie 2)	Scientific Industries, Inc.
Water bath	A. Hartenstein

4.9 SOFTWARE

Program	**Application**
EndNote X7	Reference Management
FACS Diva	FACS acquisition and recording

FlowJo v9 and v10	FACS analysis
Graphpad Prism v6 and v7	Statistical analysis and graphs
ImageJ 1.50i	Fluorescence microscopy image analysis
Mass Hunter Agilent Technologies	Mass spectroscopy
Microsoft Office	Data management and manuscript preparation
SPSS	Statistical analysis

5 METHODS

5.1 CLINICAL SAMPLES

For the prospective clinical study, blood samples of patients treated for major depressions were obtained. The local Ethics Committee of the Faculty of Medicine at the University of Würzburg had approved the clinical study protocol (vote no. 128/15). A written informed consent was given by all participants before the inclusion into the study.

In total 70 depressed patients (mean age = 45.64±13.43 SD, 45.8 % female) were recruited within the first 2-5 days after admission to the Department of Psychiatry, Psychosomatics and Psychotherapy of the University Hospital Würzburg. For the patients to be considered as participants, they had to have as a minimum mild depressive episode, meaning a 21-item HAMD equal or higher than 14 and be above 18 years of age. The participants' treatment with antidepressants was decided upon by the doctor within a clinical routine setting. The HAMD was assessed weekly by trained raters and a positive response to clinical treatment was defined as a reduction by at least 50 % of the initial HAMD at admission after week 4 of treatment[133]. Participants were excluded when they had severe general or neurological medical conditions or acute infections. Therefore, different tests were done on their blood samples to control coagulation, C-reactive protein concentration, differential blood count, liver enzymes and renal function. Furthermore, vital signs, like pulse and blood pressure, were checked. Blood samples for the analysis of $CD4^+$ T cell frequencies were collected from the participants at 6 pm using ammonium-heparin (NH_4-heparin) tubes (Sarstedt) and stored at room temperature overnight and processed in the laboratory the next morning.

The participants were divided into two categories depending on the antidepressant's capacity to functionally inhibit the ASM [134, 135]:

- strong inhibitors (27 patients): amitriptyline, doxepin and sertraline.
- weak inhibitors (33 patients): citalopram/escitalopram, venlafaxine, bupropion, mirtazapine and agomelatine
- dropouts or insufficient blood quality for at least one timepoint (10 patients)

Other studies analyzing the T_{reg} compartment in patients with major depression showed that a patient group of 27 patients per group allowed for sufficient statistical power[136, 137].

5.2 CELL PREPARATION

5.2.1 CELL ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC were either obtained from healthy donors of the Department of Transfusion Medicine or from patients of the Department of Psychiatry, Psychosomatics and Psychotherapy, both of the University Hospital Würzburg.

For healthy donors, leucocyte reduction chambers were used to gain a high concentration of leucocytes in the 10 ml blood sample. First, the blood was diluted with 25 ml of Versene buffer and then layered onto 15 ml Histopaque (Sigma-Aldrich). To separate the cells according to their density, the tube, containing the diluted blood and the Histopaque, was centrifuged at 400 g for 15 min at room temperature (RT). Afterwards the layer of PBMC was extracted from the density gradient. The cells were washed twice with balanced salt solution / bovine serum albumin (BSS/BSA) and resuspended in 10 ml human cell culture medium and the cell concentration was determined.

For patients, heparin tubes were used to obtain up to 10 ml of blood. The blood was layered onto 3 ml Histopaque and then centrifuged (15 min, 400 g, RT). The layer of PBMC was extracted and washed twice with BSS/BSA. Afterwards, the cells were resuspended in 1 ml human cell culture medium and three times 50 µl (2 x 10^5 cells/50 µl) were distributed into wells of a 96-V bottom well plate to be used for FACS analysis. Cells not immediately used were frozen using 10 % dimethyl sulfoxide (DMSO) in FCS at -140°C.

5.2.2 CELL SORTING

Cell separation with magnetic beads to obtain $CD4^+$ T cells

The MagniSort Human CD4 T Cell Enrichment Kit (eBioscience, ThermoFischer) was used for the isolation of $CD4^+$ T cells. The kit consists of magnetic streptavidin-coated

particles and biotinylated antibodies against every cell type but $CD4^+$ T cells. Therefore, unlabeled $CD4^+$ T cells could be negatively selected using a magnet. The isolation was done according to the manufacturer's instruction, but only half of the amount of antibodies and of magnetic particles was used. The purity of the obtained $CD4^+$ T cells was confirmed to be above 90 % purity by flow cytometry. For the isolation, the single cell suspension was adjusted to a concentration of 1 x 10^8 cells/ml and then 10 µl per 100 µl of cells of the antibody mix was added. After an incubation time of 10 min at RT, the cells were washed and readjusted to 1 x 10^8 cells/ml. Afterwards, 10 µl per 100 µl of cells of the magnetic streptavidin-coated particles were added to the suspension and well mixed by 5 times pulse vortexing, followed by a 5 min incubation at RT. The cell suspension, maximum 4 ml, was then transferred to 5 ml tubes and the tube was inserted into the magnet. After waiting for further 5 min, the 5 ml tube, still inside the magnet, was inverted and non-adherent cells were collected. Finally, the number of obtained cells and their purity for $CD4^+$ T cells were determined.

Flow cytometric cell sorting to obtain $CD4^+$ T cell subpopulations

The $CD4^+$ T cell subpopulations were sorted by fluorescence-activated cell sorting (FACS) on a BD FACSAria III (BD Bioscience). The PBMC were enriched for $CD4^+$ T cells as described above. To prepare the $CD4^+$ T cells for sorting, they were stained with anti-CD4, anti-CD45RA and anti-CD25 antibodies and afterwards resuspended in PBS containing 20 % FCS at a concentration of 2 x 10^7 cells/ml. Then the $CD4^+$ T cells were sorted with the BD FACSAria III into either T_{reg} ($CD4^+$ $CD25^+$) and T_{conv} ($CD4^+$ $CD25^-$) or the five subpopulations of $CD4^+$ T cells, nT_{conv} ($CD45RA^+$ $CD25^-$), mT_{conv} ($CD45RA^-$ $CD25^-$), $CD25^{low}$ mT_{conv} ($CD45RA^-$ $CD25^{low}$), rT_{reg} ($CD45RA^+$ $CD25^+$) and efT_{reg} ($CD45RA^-$ $CD25^{high}$). The sorted cells were collected in fresh human cell culture medium and the number of obtained cells together with their purity was determined.

5.2.3 Isolation of mouse splenocytes

For our cross-species transendocytosis experiment, mouse splenocytes were isolated. To obtain the splenocytes, the mice were killed with CO_2 and the vital reflexes (lid closure reflex, tail pinch reflex and pedal withdrawal reflex) tested to confirm death. Afterwards the corpses were dissected to take out the spleen. The spleen is then kept in a small petri dish with BSS/BSA to avoid the drying out of the cells and transferred

to a sterile hood. Here, a nylon cell strainer (40 µm) and a plunger were used to mash the spleen. The splenocytes were small enough to be filtered through the cell strainer and be collected in a new petri dish containing fresh BSS/BSA.

The splenocytes were a mix of leucocytes and erythrocytes, but for the experiment only leucocytes were wanted. Therefore, an erythrocyte lysis was done. Hereby, the principle of osmosis is used, leading to the bursting of erythrocytes due to hypoosmotic shock. Thus, the cell pellet was gently vortexed, and 3 ml of double distillated H_2O shortly followed by 3 ml of 1.8 % NaCl solution was added. To prepare the cells for cell culture, they were washed twice with BSS/BSA and afterwards resuspended in 10 ml of mouse cell culture medium. Furthermore, the cell concentration was determined.

5.3 Cell culture

5.3.1 Culture conditions

For all experiments, the cells were grown in a humid atmosphere (37 °C, 5 % CO_2) in the presence of either mouse or human cell culture medium. For mixed cultures of mouse and human cells, the cells were cultured in human cell culture medium.

5.3.2 Culturing of cell lines

The mouse embryonic fibroblast (MEF) cell lines expressing CD80 or CD80-mScarlet were cultured in 75 cm^2 cell culture flasks (Greiner Bio One) and split every 2nd or 3rd day. After they reached 90 % confluence, the medium was aspired, and the cells were washed with PBS. Then they were treated with 2 ml ATV/trypsin for 3-5 min at 37 °C to detach the cells from the plastic. Adding 8 ml of mouse cell culture medium to the cells stopped the activity of ATV/trypsin. The cells could now be adjusted to the desired density. Then, 0.5 or 1 ml of this cell suspension was transferred into a new flask and diluted with 20 ml of new mouse cell culture media.

5.3.3 Determination of cell concentration

The cell concentration was determined with a Neubauer counting chamber. Therefore, an aliquot of 2-10 µl of the cell suspension was diluted 1:10 or for high concentrations 1:50 with 0.04 % trypan blue solution. Afterwards, 10 µl of the mix was transferred to the Neubauer counting chamber and the number of living cells was counted for each of the four quadrants (Q) of the counting chamber. The cell concentration (cells/ml)

was calculated with the following formula: (Sum of counted cells per quadrant/number of quadrants) * dilution factor * chamber factor (10^4)

5.3.4 *Freezing of cells*

For longtime storage, cells were stored in freezing medium at -140 °C. To survive the storage, they were first centrifuged for 5 min at 1000 rpm. Then the resulting cell pellet, up to 1 x 10^7 cells, was resuspended in 1 ml of cold freezing medium, which consists of 70 % human or mouse cell culture medium, 20 % FCS and 10 % DMSO. For the cells obtained from patients, the freezing medium was changed to 90 % FCS and 10 % DMSO as the viability of T_{reg} were low in previous thawed cell samples. The cell suspension was then transferred into a cryotube and frozen at -80 °C for one week before the tube was transferred to -140 °C. If the cell number was > 1 x 10^7 cells, a bigger volume of freezing medium was used, and several aliquots containing 1 x 10^7 cells each were frozen.

5.3.5 *Thawing of cells*

To thaw the cells, 10 ml of human or mouse cell culture medium was preheated to 37 °C. The cells were then taken out of storage and quickly thawed in a 37 °C water bath. After thawing, the cells were diluted in medium and cultured in the incubator. To reduce cellular stress the medium is changed after 24 h.

5.4 Assays

5.4.1 *ASM activity assay*

To measure the ASM activity in the absence or presence of antidepressants, 3 x 10^5 human T cells or 5 x 10^5 mouse T cells were suspended in ASM lysis buffer (250 mM of Na-acetate pH 5.2, 1.3 mM of EDTA, 0.2 % Na-taurocholate) and lysed by several rounds of freezing and thawing. After centrifugation at 244 g for 5 min (Biofuge pico), 10 µl of the supernatant were added to 20 µl of ASM activity assay master mix containing 1.35 mM of an artificial sphingomyelinase substrate, 6-hexadecanoylamino-4-methyumbelliferyl-phosphorylcholine (Moscerdam Substrates). The sphingomyelinase substrate is metabolized by the ASM upon incubation at 37 °C and after 17 h the reaction was stopped by a stop solution. One of the resulting products is fluorescent with

the excitation maximum at 404 nm and emission at 460 nm, which can then be measured in a fluorescence reader (Safire2; Tecan). This assay was used to either compare the ASM activity of different samples or to calculate the absolute ASM activity. For the latter, a standard series with different amounts of the fluorescent product (0, 15, 60, 150, 300, 450, 600, 750 ng) was measured in parallel. Through the measured standard row, it was possible to calculate the amount of cleaved sphingomyelinase substrate for each sample. Furthermore, this allowed for the comparison of samples measured on different days.

5.4.2 CTLA-4 CAPTURE ASSAY

CTLA-4 is a protein receptor and is constantly shuttled from its intracellular stores to the cell membrane. This assay was used to analyze, how many protein receptors are shuttled to the cell membrane in a specific time. Therefore, 2 x 10^5 PBMC (1 x 10^6 cells/ml) were cultured in a 96-well round bottom plate (Greiner Bio One) in the presence of 1 µg/ml anti-CD3 monoclonal antibody (mAB) (Clone HIT3a, Biolegend), 0.1 µM human IL-2 (Proleukin, Novartis) and anti-CTLA-4-PE or a matching PE-labeled isotype control (1:200, Biolegend, Fig. 10). The effect of the ASM on CTLA-4 shuttling was analyzed by adding the ASM activity inhibiting antidepressants sertraline (1 µM) and citalopram (1 µM) to the culture. The cells were cultured for 24 h at 37 °C and then washed and further stained with antibodies to CD4, CD25, Foxp3, and CD45RA. To determine the total CTLA-4 amount per cell, parallel samples were first fixed and permeabilized before staining of CTLA-4 using the same antibody as for CTLA-4 capturing.

5.4.3 CTLA-4 TRANSENDOCYTOSIS ASSAY

Analysis of CTLA-4 transendocytosis activity with MEF-CD80-mScarlet cell line

MEF expressing CD80-mScarlet were seeded at a concentration of 1 x 10^4 cells/well in a 48-well tissue culture plate (Greiner Bio One). After 24 h the cells were washed and 1 x 10^6 freshly isolated PBMC were added and the cells were further cultivated for 12 h. The cells were cultured in the presence of 0.1 µg/ml of an anti-CD3-antibody (LEAF purified anti-human CD3, Clone HIT3a, 0.1 µg/ml, Biolegend) and of 1 µM of the ASM inhibitor sertraline or 1 µg/ml of the CTLA-4 inhibiting mAb tremelimumab.

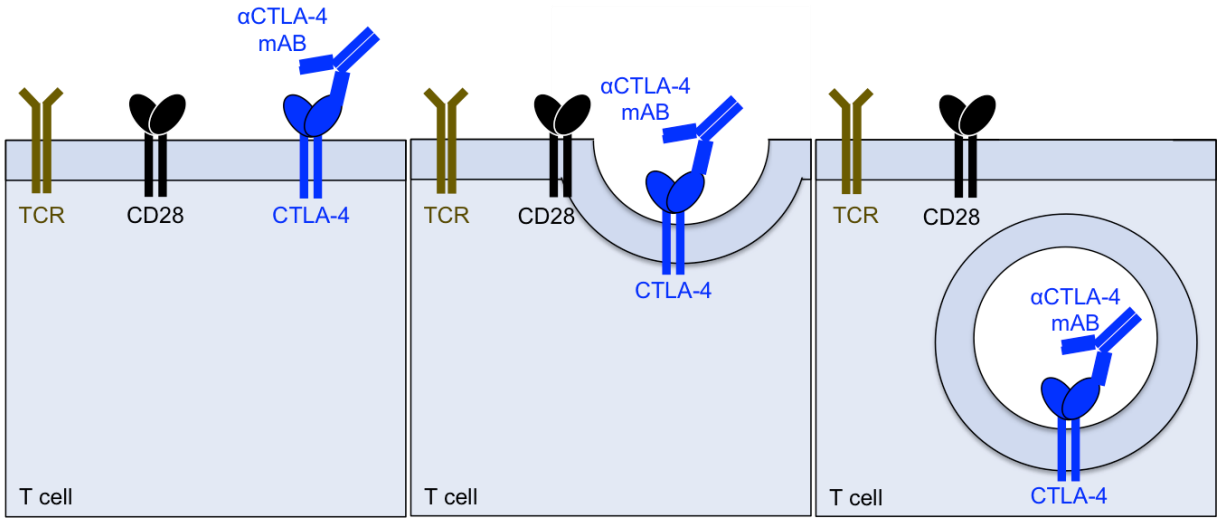


Figure 9 Schematic representation of CTLA-4 capturing assay

At the cell membrane CTLA-4 binds to the extracellular monoclonal antibody against CTLA-4 conjugated to a fluorochrome. After 24 h of culturing the amount of αCTLA-4 mAB in differently treated cells is measured.

Analysis of CTLA-4 transendocytosis activity with mouse splenocytes

In a variation of the CTLA-4 transendocytosis activity assay, mouse splenocytes were used as CD86 donor cells. Therefore, the cells had to be activated with LPS (1µg/ml) in an overnight (ON) culture. The cell concentration was 1 x 10^7 cells/ml and 24-well plates were used. Similarly, to the previous assay, the cells were washed the next day and cultivated together with 1 x 10^6 freshly isolated PBMC for 16 h. For this culturing step human cell culture medium was used. It contained 0.1 µg/ml of an anti-CD3-antibody (LEAF purified anti-human CD3, Clone HIT3a, 0.1 µg/ml, Biolegend) and of 1 µM of the ASM inhibitor sertraline or 1 µg/ml of the CTLA-4 inhibitor tremelimumab.

5.4.4 In vitro cultures in the presence of ASM inhibitors

PBMC from human blood samples were pre-cultivated in a high-density culture, 1 x 10^7 cells/ml, in human cell culture medium for two days at 37 °C. A 24-well plate (Greiner) was used for the culture. This step is necessary to restore tonic TCR signaling in T cells. In the high-density culture of cells, the PBMC have tissue-like conditions leading to a higher susceptibility to the influence of immunomodulating agents[138]. Thereafter, the cells are washed and resuspended to a density of 1 x 10^6 cells/ml and

further cultivated under different conditions for four days. If required, various concentrations of the ASM activity inhibitors sertraline or citalopram (both Sigma-Aldrich), of an anti-CD28-Fab (clone CD28.3; 1, 0.5, 0.25 µg/ml)[139, 140], of the PD-1 inhibiting mAb pembrolizumab (Pem) (BioVision), or 1 µg/ml of the CTLA-4 inhibiting mAb tremelimumab (Tre) (Creative Biolabs) were added to the culture medium. For analysis, the cells were washed, stained with ABs and then measured by FACS.

5.4.5 *Carboxyfluorescein succinimidyl ester (CFSE) labeling*

In order to determine the proliferation of cells, PBMC were washed thrice with BSS and then resuspended in BSS containing CFSE (1:2000) at a concentration of 1 x 10^7 cells/ml. The cell suspension was incubated for 5 min at RT and the labeling stopped by diluting the cells with 45 ml BSS/BSA. The cells were washed and resuspended in human cell culture medium and the number of cells was determined. Evaluation of the CFSE incorporation was done after 3 days by flow cytometry.

5.4.6 *Suppression Assay*

The suppressive activity of cells under different conditions was analyzed using the suppression assay. For the assay, one part of PBMC was sorted to obtain $CD4^+$ $CD25^{hi}$ T_{reg} and $CD4^+$ $CD25^-$ T_{conv} and another part of the PBMC was taken for CFSE labeling.

For the suppression assay, the CFSE labeled cells were co-cultured with either T_{reg} or T_{conv}. 1 x 10^4 cells labeled cells per well were seeded into a 96-well V-bottom plate (Greiner) and either T_{reg} or T_{conv} were added at a ratio of 1:1, 1:4 and 1:16. Furthermore, the cells were stimulated with 1 µg/ml anti-CD3 mAB and cultured in 200 µl human cell culture medium either containing 1 µM sertraline or without ASM activity inhibiting drug. The culture lasted for 4 days at 37 °C and then the cells were washed and stained for FACS analysis. The cell proliferation was analyzed by dilution of the cell marker CFSE and the suppressive function of T_{reg} and T_{conv} determined by the formula: 100 x (division index [T_{ind}] – division index [T_{ind} + T_{reg}])/ division index [T_{ind}]

5.5 Flow cytometry analysis

For flow cytometric analysis, up to 10^6 cells were placed per well of a 96-well V-bottom plate and unspecific binding of fluorochrome-labeled antibodies was blocked with an αCD16/αCD32 antibody (clone 2.4G2 supernatant, diluted 1:10 in FACS buffer) for

mouse cells and with normal mouse Immunoglobulin G (nmIgG, diluted 1:50 in FACS buffer) for human cells for 15 min at 4 °C. Cell surface markers were then stained with the respective fluorochrome-conjugated antibodies at a saturating concentration for 15 min at 4 °C and either directly used for flow cytometric analysis or washed with PBS and fixed with a fixation/permeabilization step (Fix/Perm buffer, eBioscience) for 30 min at RT for subsequent intracellular staining. Antibodies directed against intracellular markers were diluted in permeabilization buffer (eBioscience) at a saturating concentration and incubated with the cells for 45 min at RT. Fluorescently labeled cells were resuspended in 50 µl FACS buffer and analyzed on an LSR II flow cytometer (BD Bioscience) equipped with FACS Diva software (BD Bioscience). Exceptions were the transendocytosis and suppression assays which were measured on an Attune NxT flow cytometer (Invitrogen, ThermoFisher) and a FACSCelesta flow cytometer (BD Bioscience), respectively. For further analyses of the data, FlowJo software (BD Bioscience) was used.

5.6 MASS SPECTROSCOPIC ANALYSIS

The cells for the lipid analysis were suspended in 500 µl Methanol and were stored at -80 °C. Afterwards, they were sent on dry ice to Dr. Fabian Schumann in the laboratory of Prof. Dr. B. Kleuser, University of Potsdam.

The lipids were extracted by using 1.5 ml methanol/chloroform (2:1, v:v) as described in Gulbins et al. 2018[141]. The extraction solvent contained d_7-sphingosine (d_7-Sph), d_7-sphingosine 1-phosphate (d_7-S1P), C17-ceramide (C17 Cer) and C16-d_{31}-sphingomyelin (C16 d_{31}-SM) (all Avanti Polar Lipids, Alabaster, USA) as internal standards. Chromatographic separations were achieved on a 1260 Infinity HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Poroshell 120 EC-C8 column (3.0 × 150 mm, 2.7 µm; Agilent Technologies). MS/MS analyses were carried out using a 6490 triple-quadrupole mass spectrometer (Agilent Technologies) operating in the positive electrospray ionization mode (ESI+). The following mass transitions were recorded (collision energies (CE) in parentheses): *long-chain bases*: *m/z* 300.3 → 282.3 for Sph (8 eV), *m/z* 307.3 → 289.3 for d_7-Sph (8 eV), *m/z* 380.3 → 264.3 for S1P (16 eV) and *m/z* 387.3 → 271.3 for d_7-S1P (16 eV); *ceramides* (CE = 25 eV for all transitions): *m/z* 520.5 → 264.3 for C16 Cer, *m/z* 534.5 → 264.3 for C17 Cer, *m/z* 548.5 →

264.3 for C18 Cer, *m/z* 576.6 → 264.3 for C20 Cer, *m/z* 604.6 → 264.3 for C22 Cer, *m/z* 630.6 → 264.3 for C24:1 Cer and *m/z* 632.6 → 264.3 for C24 Cer; *sphingomyelins* (CE = 25 eV for all transitions): *m/z* 703.6 → 184.1 for C16 SM, *m/z* 731.6 → 184.1 for C18 SM, *m/z* 734.8 → 184.1 for C16 d_{31}-SM, *m/z* 759.6 → 184.1 for C20 SM, *m/z* 787.7 → 184.1 for C22 SM, *m/z* 813.7 → 184.1 for C24:1 SM and *m/z* 815.7 → 184.1 for C24 SM. Quantification was performed with MassHunter Software (Agilent Technologies).

5.7 Statistical analysis

The parametric data was analyzed through a two-way a repeated measure analysis of variance (ANOVA) followed by Tukey's post-hoc test or a two-tailed unpaired Student's t test depending on the number of groups. (OriginPro 2016G, OriginLab). If the analysis showed a $p<0.05$, the difference between two groups was considered significant and is depicted with one star (*). Two or three stars stand for a p-value of $p<0.01$ or $p<0.001$, respectively. Furthermore, the summarized data are shown as means and its corresponding standard deviation (SD) except if specifically written otherwise. For further analysis of the patient data, general linear models (GLM) with and without repeated measures were used to investigate the associations of $CD4^+$ T cells frequencies, the type of antidepressants (strong vs. weak ASM inhibition) and response after 4 weeks of treatment with the antidepressants. Furthermore, the clinical and sociodemographic variables between the two patient groups, with strong or weak ASM activity-inhibiting antidepressants, was compared and for quantitative data statistically analyzed with a Student's t test for independent samples and for qualitative data with Fisher exact or Pearson chi-square tests. These additional analyses of the patient data were done with the software program SPSS for Windows (Releases 25, SPSS Inc., Chicago, IL, USA).

6 RESULTS

6.1 ANALYSIS OF CD4$^+$ T CELLS FROM PATIENTS TREATED WITH ASM-INHIBITING ANTIDEPRESSANTS

6.1.1 ANTIDEPRESSANTS INCREASE EFFECTOR REGULATORY T CELL FREQUENCIES AMONG CD4$^+$ T CELLS IN PATIENTS

In biomedical research the ultimate goal is to bring findings obtained by the research to patients. Therefore, the obtained results in mouse studies and *in vitro* with human cells must be verified *in vivo* for patients. As several functional inhibitors of acid sphingomyelinase (ASM) activity are in clinical use as antidepressants, a collaborative study with the Department of Psychiatry, Psychosomatics and Psychotherapy of the University Hospital Würzburg was started in order to analyze their effects on T cell subpopulations. The 60 participating patients underwent treatment for major depression with antidepressants or electroconvulsive therapy.

The antidepressants have different capacities to inhibit the activity of ASM[135] (Fig. 7), which allowed for a control group (33 patients, Fig. 12, gray) receiving an antidepressant with weak or no inhibition of ASM activity. These were analyzed in comparison to the 27 patients (Fig. 12, black) receiving strongly ASM-inhibiting drugs. This study was undertaken to confirm the results obtained in mice[1] and with healthy human samples *in vitro*[142] (shown in 6.2.1.), which showed an increased frequency of regulatory T (T_{reg}) cells among CD4$^+$ T cells after inhibition of ASM activity. Therefore, blood samples from patients at day zero (start of therapy), after one week and four weeks were analyzed.

After receiving the blood samples, without knowing the kind of treatment that the patient had received, the PBMC were isolated and stained with different markers (among others CD3, CD4, CD25 and Foxp3) to analyze the distribution of CD4$^+$ subpopulations through FACS (Fig. 11, dot plot). Excess cells were frozen and kept at -140°C for further analysis.

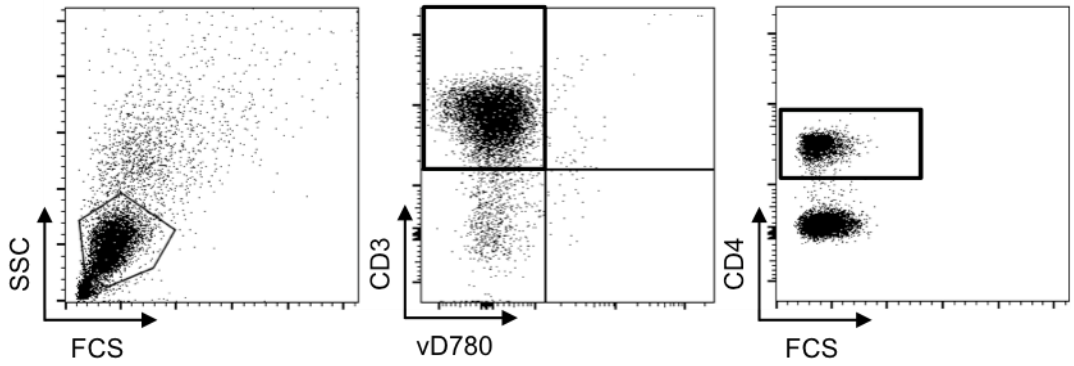


Figure 10 Gating strategy for CD4⁺ T cells

First lymphocytes were gated for their size and granularity. To obtain CD4⁺ T cells, the lymphocytes were gated for viability and expression of CD3 and CD4.

As human peripheral blood contains resting and effector T_{reg} another interest of this study was to further define the specific T_{reg} subset, which causes the change in frequencies among CD4⁺ T cells. Therefore, the memory and naïve cell subsets were further defined by their expression of CD45RA and CD25 leading to five different subsets of CD4⁺ T cells[57] (Fig. 11, dot plot left). The gating strategy was controlled by the expression of Foxp3 and CTLA-4 (Fig. 11, histograms middle and right).

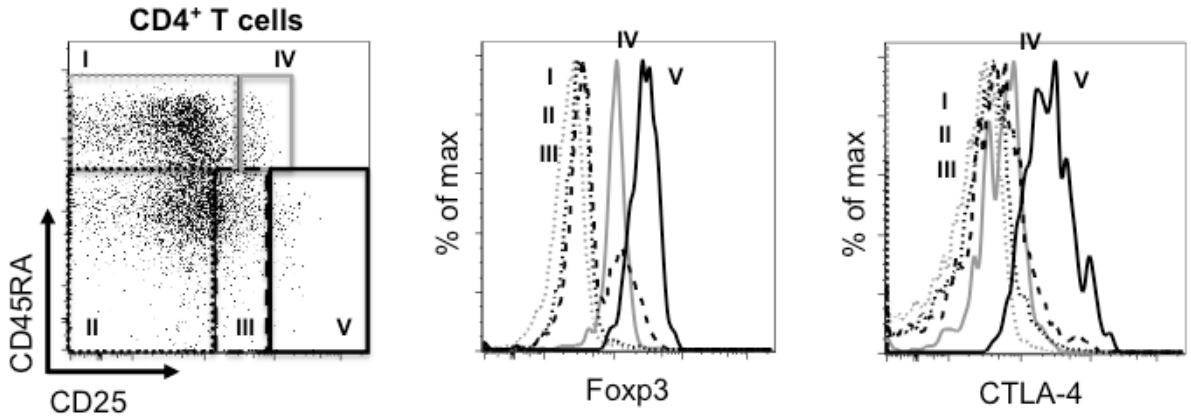


Figure 11 Gating strategy for cells of patients treated for major depression

CD4⁺ T subpopulations are gated for their expression of CD45RA and CD25 (dot plot, left) and 5 subpopulations are defined: (I) Naïve conventional T cells, (II) memory conventional T cells, (III) $CD25^{low}$ memory conventional T cells, (IV) resting regulatory T cells and (V) effector regulatory T cells. The correct position of the gates is controlled by the expression of Foxp3 (middle) and CTLA-4 (right).

The strongest change in frequencies was seen in the CD45RA$^-$ CD25^{high} effector regulatory T (efT$_{reg}$) cell subset (Fig. 12 (V)). Over the study period of four weeks the relative increase in frequency was about 78 % for the patient group treated with strongly ASM-inhibiting antidepressants. In the control group the increase is significantly lower with around 35 % (Fig. 12 (V)). Furthermore, in the group of patients treated with strong functional ASM inhibitors, there was a significant increase in the frequencies of efT$_{reg}$/CD4$^+$ T cells after one week of treatment.

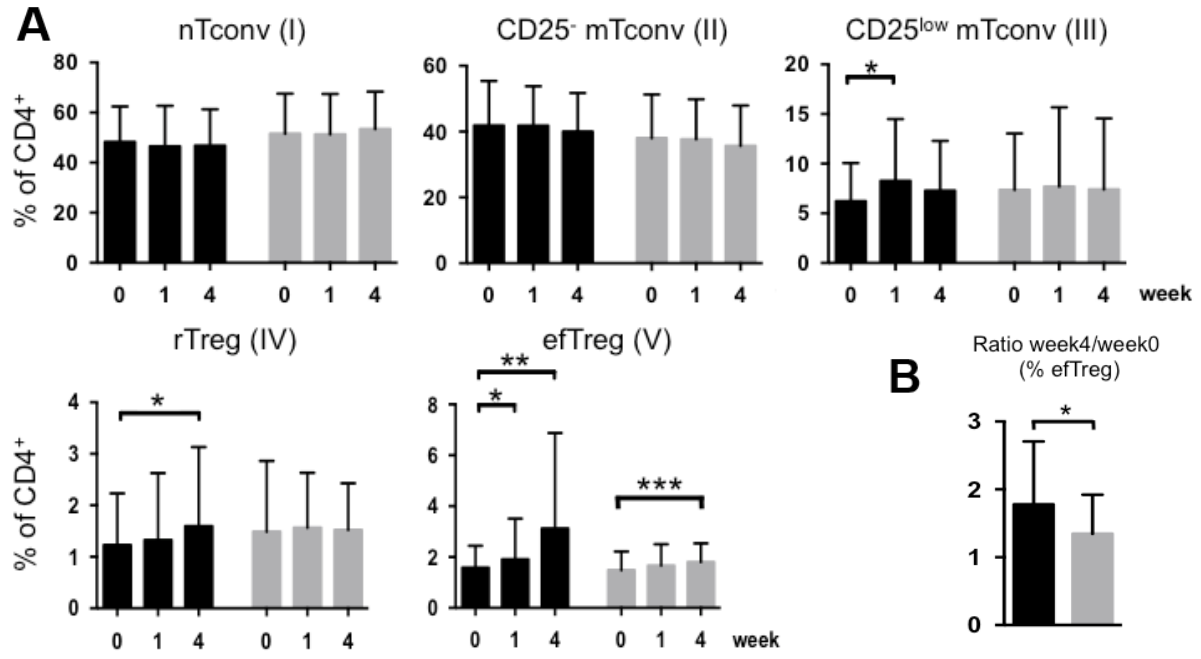


Figure 12 Increase in T$_{reg}$ frequencies among CD4$^+$ T cells in patients treated for major depression dependent on the capacity of the antidepressant to inhibit ASM activity.

(A) The graphs show a summary of the frequencies of the five CD4$^+$ T cell subpopulations over the time of treatment. Here, the two patient groups, treated with antidepressants with strong (black, n= 27) or weak (grey, n= 33) capacity to inhibit ASM activity, are shown separately. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. The statistical analysis revealed significant changes in three CD4$^+$ T subpopulations, CD25^{low} mT$_{conv}$ (III), rT$_{reg}$ (IV) and efT$_{reg}$ (V). (B) Furthermore, the comparison of the ratio of efT$_{reg}$ frequencies among CD4$^+$ T cells at week four over week zero for both groups shows a significant contrast between the patients treated with strongly (black, n= 27) or weakly (grey, n= 33) ASM-inhibiting antidepressants. *p<0.05, **p<0.01, ***p<0.001

Moreover, for the patients treated with drugs with strong inhibitory capacity of the ASM activity, other CD4$^+$ T cell subsets showed also a significant change in frequencies among CD4$^+$ T cells. The CD45RA$^+$ CD25^{lo} resting regulatory T (rT$_{reg}$) cell subset increased significantly after four weeks of treatment (Fig. 12 (IV)) and in the subset of

conventional $CD4^+$ T cells, the $CD45RA^-$ $CD25^{lo}$ memory conventional T cells ($CD25^{low}$ mT_{conv}) showed an increase in frequencies after one week of treatment (Fig. 12 (III)).

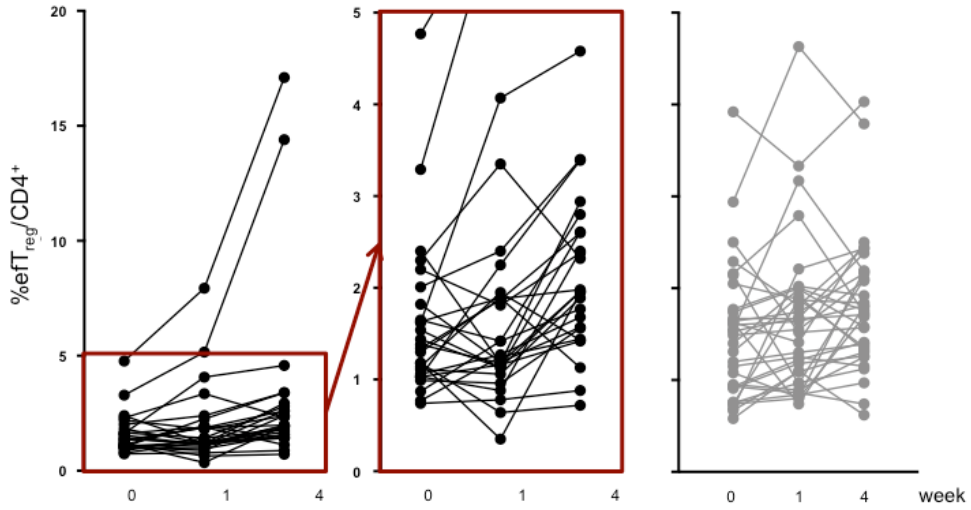


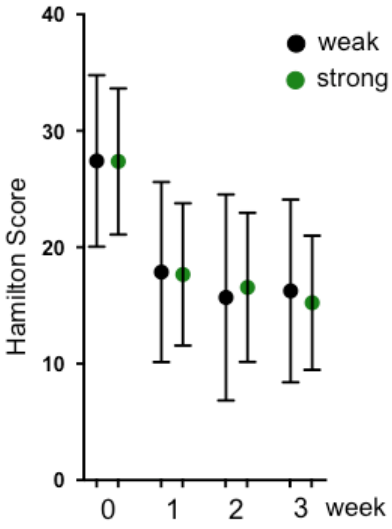
Figure 13 Presentation of efT_{reg} frequency development among $CD4^+$ T cells for each patient during the time of treatment

The same efT_{reg} (V) data as in Fig. 12 are depicted, but instead of presenting the mean frequencies and their standard deviation (SD), the efT_{reg} frequency development among $CD4^+$ T cells for each individual patient is presented. Again, the patients treated with strongly (black, n= 27) or weakly (grey, n= 33) ASM-inhibiting antidepressants are presented separately. This representation of the data shows the divers results that were obtained within the patient groups, which is normal for human samples. In the statistical analysis, the development of the efT_{reg} frequencies among $CD4^+$ T cells for each patient was analyzed. Overall, a clear trend towards an increase in efT_{reg} frequencies among $CD4^+$ T cells over the 4 weeks can be seen in the data set for the patients receiving a strong ASM-inhibiting antidepressant. In contrast, the data set for patients treated with a week ASM-inhibiting antidepressant shows a higher diversity and overall, for most patients no significant change in frequencies.

In the analysis of the data, the development of the $CD4^+$ T cell subset frequencies for each patient over the four-week period was analyzed. To gain a better understanding of the heterogenicity between the patients, the development of the efT_{reg} frequencies among the $CD4^+$ T cells for each patient is shown in Fig. 13. Here, one sees as in the plot showing the mean values (Fig. 12 (V)) a difference between the data obtained from patients receiving strongly and weakly ASM-inhibiting antidepressants. The treatment with strong ASM inhibitors led to an overall to a higher increase in efT_{reg} frequencies among the $CD4^+$ T cells over the four-week period than treatment with weak ASM inhibitors.

6.1.2 THERAPEUTIC IMPROVEMENT INDEPENDENT OF THE ASM-INHIBITORY ACTIVITY OF ANTIDEPRESSANTS

Low T_{reg} frequencies among $CD4^+$ T cells are associated with a depressed mood in patients with major depression[137] and to increase during antidepressive therapy[136]. Therefore, it was analyzed whether the significantly higher increase in T_{reg} frequencies among $CD4^+$ T cells in patients treated with strongly ASM-inhibiting antidepressants correlated with an improved clinical response of the patient. Thus, the patients were scored with the Hamilton Depression Rating Scale (HAMD, Fig. 14) and a positive response was defined by a decrease in the Hamilton score by at least 50 % compared to the initial score taken at the beginning of the treatment. To study the effect of ASM activity inhibitors on the health of the patient, the change in HAMD score was analyzed for both branches of the study and compared to each other (Fig. 14). The comparison of the HAMD score development for both patient groups over the period of four weeks showed no significant differences. The mean HAMD score decreased by about 42 % for both patient groups and the decrease was not dependent on the capacity of the antidepressant to inhibit ASM activity.



Hamilton Rating Scale for Depression

Points	Diagnosis
0-8	no depression
9-16	mild depression
17-24	moderate depression
25 and higher	major depression

Response: Reduction by 50%

mean ±SD (Hamilton Score)

day 0: 27.39 ±6.74

week 4: 15.94 ±7.00

Figure 14 Clinical response is independent of ASM activity inhibition

HAMD scores are plotted for each week of hospitalization. Means and standard derivation are shown for patients treated with strong (green, n= 27) and weak (black, n= 33) ASM-inhibiting antidepressants. The statistical analysis with an ANOVA showed no significant differences between the two groups. For both patient groups the mean HAMD score decreased by about 42 % over three weeks independent of the treatment with or without ASM activity inhibiting antidepressants.

6.1.3 *Early increase in efT$_{REG}$ frequencies among CD4$^+$ T cells correlated with better clinical outcome in patients treated for major depression*

As mentioned before, low T$_{reg}$ frequencies among CD4$^+$ T cells and a depressive mood in patients with major depression are positively correlated. Therefore, the data were further analyzed for a correlation between clinical response and an increase in efT$_{reg}$ frequencies. While analyzing the obtained data, a significant increase of 29 % in efT$_{reg}$ frequencies among CD4$^+$ T cells after one week of treatment was observed for the patients who had a positive clinical response after four weeks of treatment (Fig. 15 A, black). For patients, who did not show a positive clinical response after four weeks of treatment (Fig. 15 A, white), no early changes in efT$_{reg}$ frequencies among CD4$^+$ T cells could be seen. The ASM activity of the cells seemed not to correlate with the clinical response (data not shown). Analyzing the change in efT$_{reg}$ frequencies among CD4$^+$ T cells after four weeks of treatment no difference could be observed between the patients with a positive clinical response and no clinical response (Fig. 15 B). These results are independent of ASM-inhibiting treatment as the patients receiving a strongly and a weakly ASM-inhibiting antidepressant had a probability of 60 % and 55 %, respectively, to have a positive clinical response after four weeks of treatment.

Lastly, the ratio of week 1 over week 0 of percentage of efT$_{reg}$ among CD4$^+$ T cells were correlated with the percentual HAMD score of week 1 over week 0, which shows no clear correlation between both values. Therefore, the percentage of efT$_{reg}$ among CD4$^+$ T cells is most likely an independent biomarker.

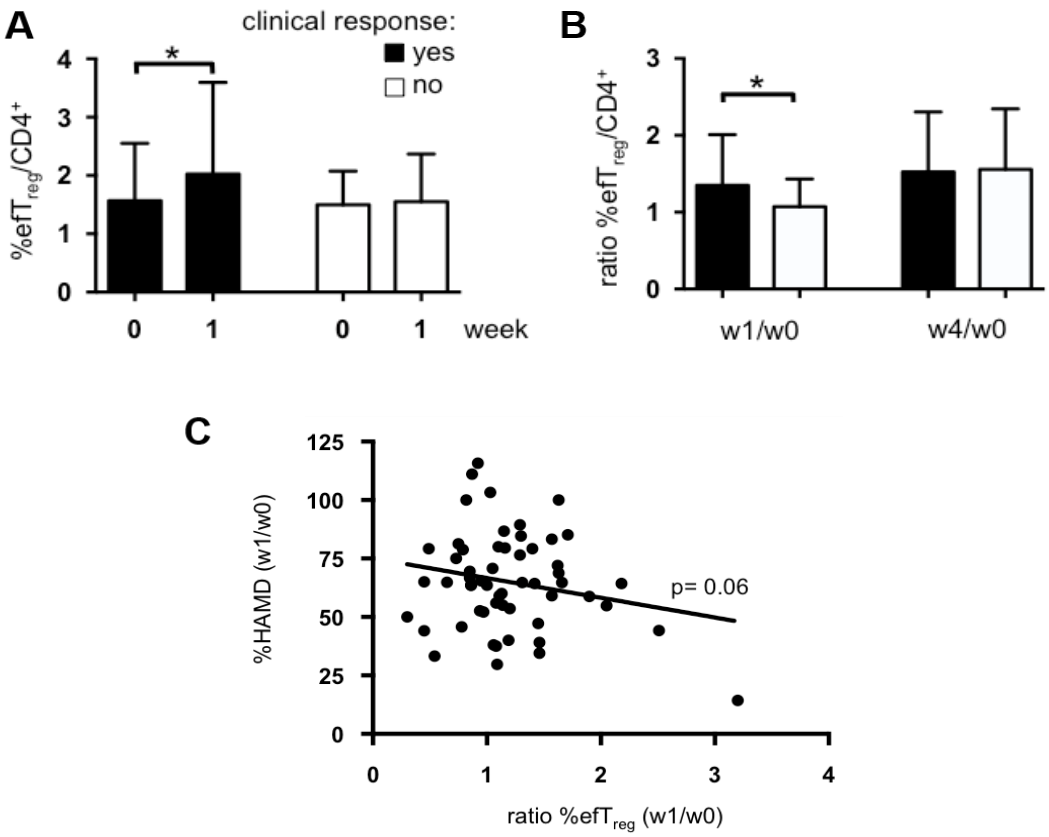


Figure 15 Early increase in efT$_{reg}$ frequencies among CD4$^+$ T cells correlates with clinical response to the treatment

(A) The mean efT$_{reg}$ frequencies among CD4$^+$ T cells for week zero and week one is shown for patients with a positive (black, n= 28) and no (white, n= 32) clinical response to treatment. A positive clinical response is defined as a decrease in HAMD score by more than 50 % after four weeks of treatment. A repeated measures ANOVA followed by a Tuckey post-hoc test showed a significant increase in efT$_{reg}$ among CD4$^+$ T cells for patients with a positive clinical response. (B) A comparison of the ratio of efT$_{reg}$ among CD4$^+$ T cells at week one over week zero showed a significant difference between the responding and non-responding patients. For the ratio at week four over week zero no differences could be seen. (C) Correlation of percentual HAMD scores at week 1 with ratio of %efTreg week1 to week 0. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05

6.2 T_{REG} AND T_{CONV} DIFFER IN SPHINGOLIPID CONTENT

The sphingolipid concentration, especially the ceramide concentration, in cellular membranes plays an important role in the homeostasis of $CD4^+$ T_{reg} and T_{conv}. This could be shown in the laboratory by adding C6-ceramide to cultures, which led to decrease in T_{reg} frequencies among $CD4^+$ T cells caused by a stronger reduction of T_{reg} numbers, 74 % and 49 % respectively (data not shown). This might indicate that T_{reg} tolerate ceramide less than T_{conv} in human. Therefore, it was investigated if differences in sphingolipid composition correlated with ceramide tolerance and also ASM activity, which has been described to be higher in T_{reg} than in T_{conv} as was previously observed in mice[1] and human[142], but which was not confirmed by our newest experiment which showed no significant differences of ASM activity between human efT_{reg} and human T_{conv} (data not shown).

The $CD4^+$ T cell subsets (Fig. 16 A) were isolated. Afterwards, their total membranes were prepared and sent to the laboratory of Prof. Burkhard Kleuser in Potsdam for mass spectroscopic analysis. Here, different ceramide (Fig. 16 B) and sphingomyelin (Fig. 16 C) species were quantified. The two T_{reg} subsets, rT_{reg} and efT_{reg}, had similar amounts and distributions of the different ceramides and sphingomyelins. The same was true for the different subsets of T_{conv}: nT_{conv}, $CD25^-$ mT_{conv} and $CD25^{low}$ mT_{conv}. Differences between efT_{reg} and T_{conv} could be observed in the total levels of ceramide. EfT_{reg} contain less total ceramide due to reduced amounts of C16 and C24:1 ceramide (Fig. 16 B). These results stand in contrast to our findings in mouse T_{reg}[1, 181] and suggests human T_{reg} might overcompensate increased ASM activity by even higher activity of cellular ceramidases or less *de novo* ceramide production in human T_{reg} compared to T_{conv}.

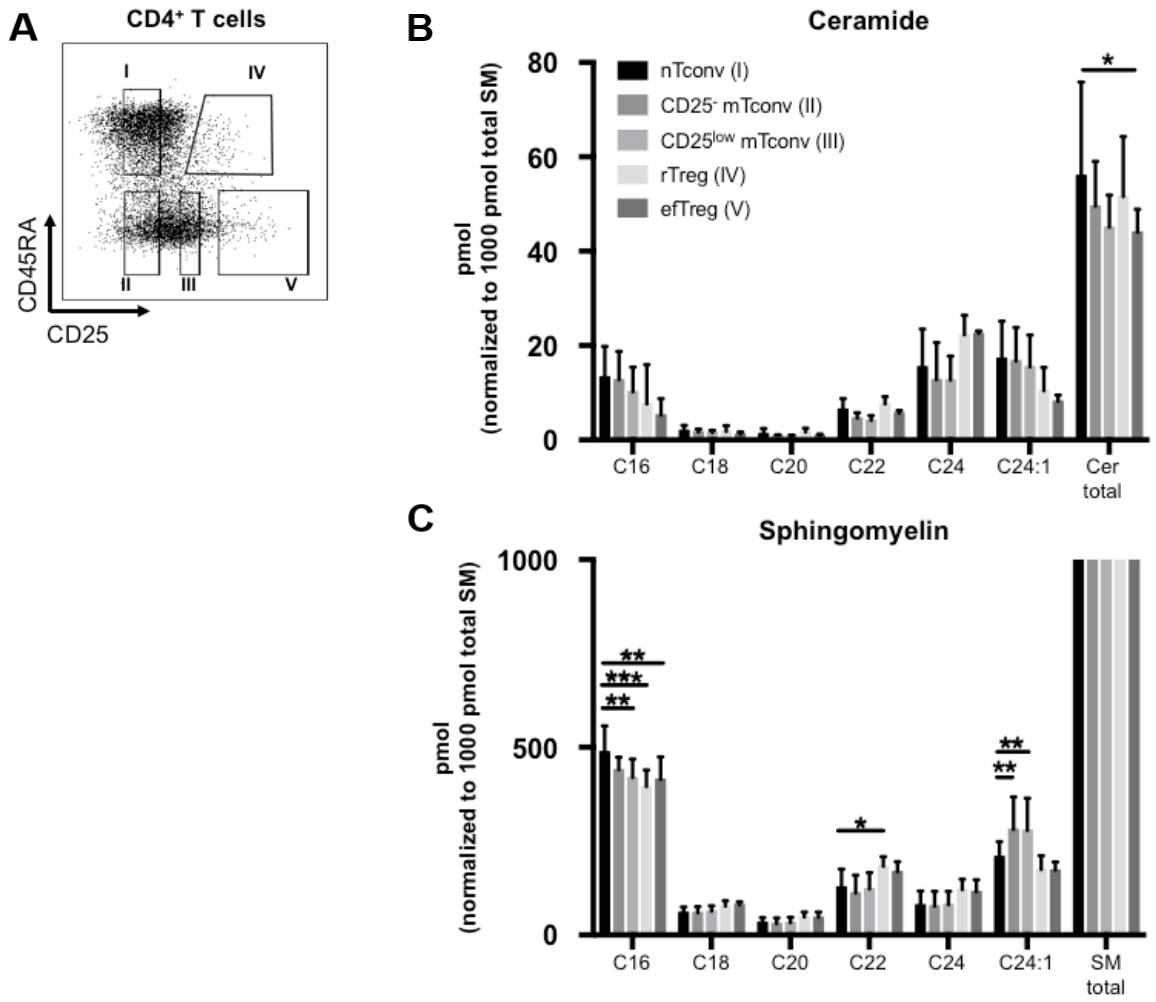


Figure 16 Human T_{reg} contain more ceramide and sphingomyelin than T_{conv} subsets

The $CD4^+$ T cell subsets were sorted as indicated (dot plot): (I) Naïve conventional T cells, (II) memory conventional T cells, (III) $CD25^{low}$ memory conventional T cells, (IV) resting regulatory T cells and (V) effector regulatory T cells. Their sphingolipid content was determined by mass spectrometry and the results normalized to their total sphingomyelin content. (n= 3, individual experiments/ donors) The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05, **p<0.01, ***p<0.001

6.3 Analysis of the effect of functional ASM inhibition and immune checkpoint inhibition on T_{REG} frequencies among $CD4^+$ T cells *in vitro*

6.3.1 Functional ASM inhibition increased efT_{REG} frequencies among $CD4^+$ T cells in vitro

Previously to and during the prospective clinical study of changes in the cellular compartments of patients treated for major depression, cells of healthy human donors were investigated in tissue culture to obtain mechanistic data. PBMC were cultured for two days in a high-density culture to obtain T cells with tissue-like responsiveness. This is necessary to analyze effects of immunomodulatory reagents *in vitro* [138]. As it became clear in the clinical study that especially efT_{reg} are affected, it was decided to focus on them in the analysis of the *in vitro* measurements. Therefore, the top 1 % of untreated $CD4^+$ T cells which is the 1 % with the highest expression of CD25 and Foxp3 ($CD25^{high}$ $Foxp3^{high}$), were gated (Fig. 17 A) and the increase in cell number after treatment with different concentrations of the ASM-inhibiting antidepressant sertraline in this gate was analyzed. For cells treated with 0.2 µM sertraline (Fig. 17 B, bright green) the frequencies of $CD25^{high}$ $Foxp3^{high}$ cells increased 1.80-fold and for the ones cultured with 1 µM sertraline (Fig. 17 B, dark green) the frequencies of $CD25^{high}$ $Foxp3^{high}$ cells increased 1.41-fold. Compared to controls, under sertraline treatment the proportion of efT_{reg} among $CD4^+$ T cells increased by 80 % and 41 %, respectively.

Additional work done by a medical doctoral student[142] in our laboratory showed that the increase in T_{reg} frequencies among human $CD4^+$ T cells caused by the treatment with 0.2 and 1 µM of the ASM-inhibiting antidepressant sertraline was due to a higher absolute number of T_{reg} cells compared to untreated cultures. In contrast, the absolute cell number of T_{conv} was unaffected by these concentrations of sertraline. It could be shown that T_{reg} treated with sertraline seem to be dose-dependently protected from cell death, whereas the cell death of T_{conv} is unaffected (data not shown). In higher concentrations (≥ 5 µM) sertraline becomes toxic for both T_{reg} and T_{conv} and cell numbers are reduced (data not shown). *In vivo*, the toxic effect of high sertraline concentrations is unimportant as these concentrations are not reached in humans treated with

sertraline. In patients a concentration of about 1 µM in the blood is common, which is non-toxic for cells.

6.3.2 *Inhibition of the immune checkpoint CTLA-4 increased efT$_{REG}$ frequencies among CD4$^+$ in vitro*

After observing similar effects of functional ASM inhibition on T_{reg} frequencies among CD4$^+$ T cells, *in vitro* and *in vivo*, the next step was to analyze the underlying mechanism.

In previous mouse studies, it was focused on CTLA-4, which is important for the suppressive activity of T_{reg}[1]. Cells from mice treated with functional ASM inhibitors showed an increased turnover of CTLA-4 from its intracellular stores to the cell membrane in mice. To test whether CTLA-4 was involved, the PBMC of healthy human donors were treated *in vitro* with the CTLA-4 checkpoint inhibitor tremelimumab[28, 145] (Tre) and analyze whether this led to an increase in T_{reg} frequencies among CD4$^+$ T cells. Furthermore, a combined treatment of tremelimumab and sertraline was done. This would allow to asses if both inhibitors affect the same pathway or if the effects were independent. As previous results showed that especially efT_{reg} are affected by the treatment with ASM-inhibiting antidepressants like sertraline, it was gated for the top 1 % highest Foxp3- and CD25-expressing CD4$^+$ T cells, which correspond with the efT_{reg} subpopulation (Fig. 17 A).

In cells treated with 1 µg/ml of the CTLA-4 inhibitor tremelimumab a significant increase by on average 64 % in efT_{reg} frequencies among CD4$^+$ T cells was observed (Fig. 17 B, blue). When the treatment was combined with 1 µM of the strong ASM activity inhibitor sertraline no additional increase could be seen (average increase of 34 %) (Fig. 17 B, green with blue border). The effect of the combinational treatment was similar to that of the separate treatments with the two drugs. Therefore, both inhibitions might target the same pathway and have no additive effect.

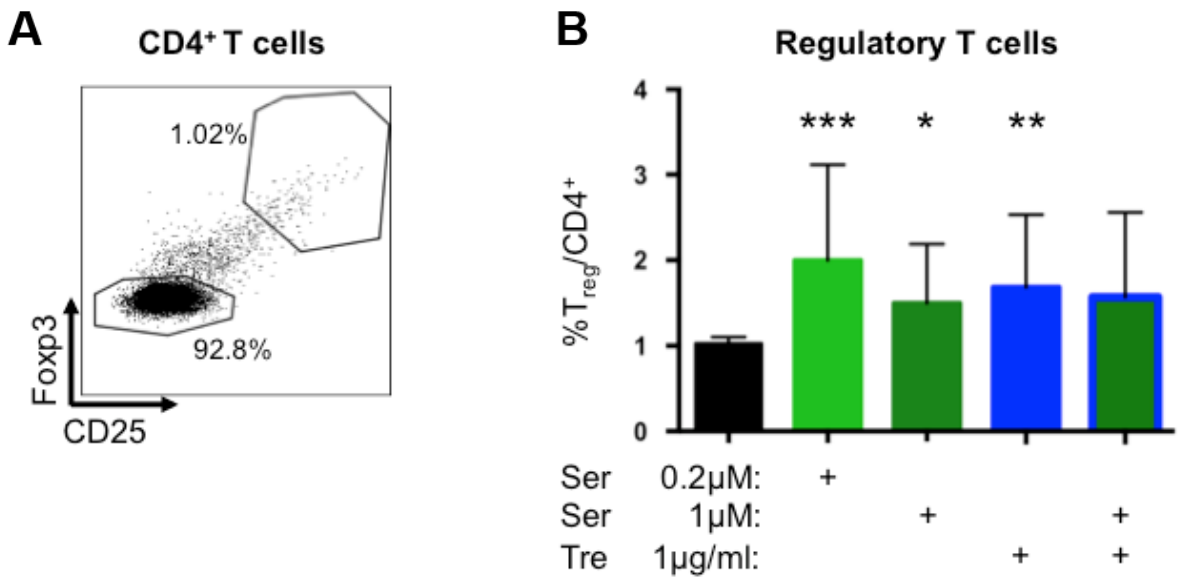


Figure 17 Increased T_{reg} frequencies/ $CD4^+$ T cells are induced by functional ASM inhibition and CTLA-4 blockade

(A) Gating strategy for efT_{reg}-like cells which were defined by their high expression of CD25 and Foxp3 (around 1 % of $CD4^+$ T cells for the untreated cells). (B) The cells were treated for 4 days with either 0.2 µM or 1 µM sertraline (Ser, green, n= 30), 1 µg/ml tremelimumab (Tre, blue, n= 24) or a combination of 1 µM sertraline and 1 µg/ml tremelimumab (green/blue, n= 18). All treatments show an increase of about 50 – 100 % in efT_{reg} frequencies among $CD4^+$ T cells and no additive effect can be seen for the combinational treatment. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05, **p<0.01, ***p<0.001

6.4 Impact of PD-1 immune checkpoint inhibition on T_{REG} frequencies among $CD4^+$ in vitro

A second important checkpoint molecule is PD-1 which like CTLA-4 negatively influences CD28 co-stimulation[27, 33]. After blocking PD-1 with the mAB pembrolizumab[146] it was checked if similar effects were observed as after treatment of cells with anti-CTLA-4 antibodies.

To analyze the effect of PD-1 blockade on the cells *in vitro* two concentrations of pembrolizumab were used. With concentrations of 0.1 and 1 µg/ml pembrolizumab a tendency towards an increase in the frequencies of efT_{reg} among the $CD4^+$ T cells could be seen (Fig. 18). However, these results were not statistically significant.

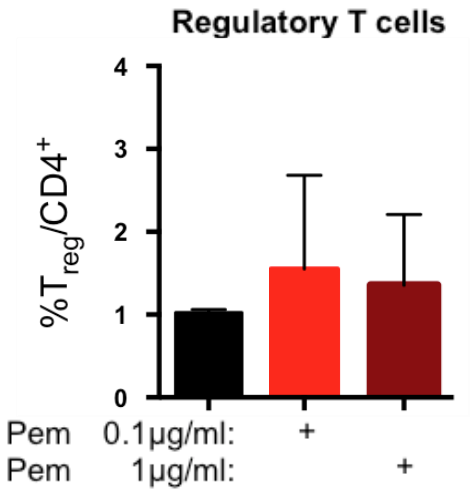


Figure 18 PD-1 inhibition induces an increase in efT$_{reg}$ frequencies among CD4$^+$ T cells

The PBMC were gated as in Fig. 17 for CD4$^+$ CD25^{high} Foxp3^{high} T$_{reg}$. Furthermore, the cells were cultured for 4 days with either 0.1 µg/ml or 1 µg/ml of the anti-PD-1 mAB pembrolizumab (Pem, red) (n= 10). The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test.

6.5 CD28 CO-STIMULATION IS REQUIRED FOR AN INCREASE IN EFT$_{REG}$ FREQUENCIES AMONG CD4$^+$ T CELLS UPON ASM INHIBITION

The co-stimulatory molecule CD28 and the co-inhibitory molecule CTLA-4 share its ligands, CD80 and CD86. In T cells, CD28 binding to a mAB has been shown to lead to highly activated ASM in T cells[85]. Furthermore, the balance between T$_{reg}$ and T$_{conv}$ in the CD4$^+$ T cell compartment is crucially affected by CD28 co-stimulation[46]. Moreover, as aforementioned both CTLA-4 and PD-1 signaling have an inhibitory effect on the CD28 signaling pathway[27, 33]. Therefore, the inhibition of CD28 with an anti-CD28-Fab fragment[139, 140] was tested in different concentrations. Additionally, combinations of the anti-CD28-Fab with sertraline, tremelimumab or pembrolizumab were analyzed to see whether CD28 co-stimulation is required for the increase in T$_{reg}$ frequencies among CD4$^+$ T cells induced by ASM or checkpoint inhibition. Treating the cells with different concentrations of the anti-CD28-Fab fragment led to a dose-dependent decrease in the T$_{reg}$ frequencies among the CD4$^+$ T cells (Fig. 19, black). The strongest effect was obtained with 1 µg/ml CD28-Fab (42 % decrease). Treatment with anti-CD28-Fab in combination with the checkpoint inhibitors anti-CTLA-4 (Fig. 19, blue) or anti-PD1 (Fig. 19, red) or the functional ASM inhibitor sertraline (Fig. 19, green)

showed that CD28 co-stimulation is required to reach peak efT_{reg} frequencies among $CD4^+$ T cell. Anti-CD28-Fab treatment totally blocked an increase in efT_{reg} frequencies among $CD4^+$ T cells. Only the combined treatment with the lowest dose of anti-CD28-Fab and sertraline led to a slight increase of about 46 % in efT_{reg} frequencies among $CD4^+$ T cells. Thus, the positive effect of CD28 co-stimulation on efT_{reg} is enhanced by ASM inhibition.

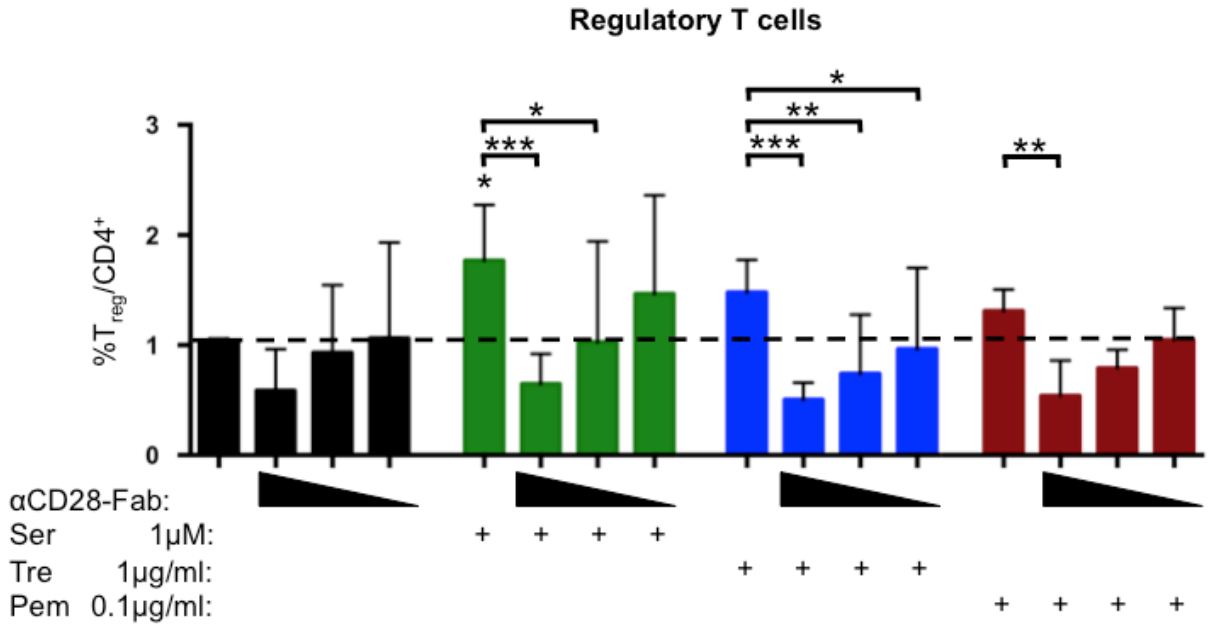


Figure 19 ASM inhibition-induced increase in efT_{reg} frequencies among $CD4^+$ T cells is CD28-dependent.

The PBMC were gated as in Fig. 17 for $CD4^+$ $CD25^{high}$ $Foxp3^{high}$ T_{reg}. PBMC were additionally co-cultured with different concentrations (1, 0.5, 0.25 µg/ml) of an anti-CD28 Fab (black, n= 12), thus, inhibiting CD28 co-stimulation. The ASM-inhibiting antidepressant sertraline was added at 1 µM (green, n= 9), the anti-CTLA-4 mAB tremelimumab was added at 1 µg/ml (blue, n= 3) and the anti-PD-1 mAB pembrolizumab was added at 0.1 µg/ml (red, n= 3). ASM inhibition enhances the positive impact of CD28 co-stimulation on efT_{reg}. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05, **p<0.01, ***p<0.001

6.6 Analysis of the effect of functional ASM inhibition on trans-endocytosis activity of CTLA-4

6.6.1 Functional ASM inhibition increased CTLA-4 turnover in T_{REG} in vitro

CTLA-4 is a endocytic receptor, 90 % of CTLA-4 is intracellular, that undergoes both recycling to the plasma membrane and degradation in the lysosomes[29, 147]. It is expressed in T_{reg} and activated T_{conv} and has in T_{reg} a direct impact on their functionality and homeostasis[148-150]. A dysfunction of CTLA-4 after treatment with strongly ASM activity inhibiting antidepressants could explain the changes in frequencies among $CD4^+$ T cells *in vivo* and *in vitro* (Fig. 12 and Fig. 17). In mice, it was shown that T_{reg} from Asm-deficient mice had a higher turnover of CTLA-4 in the cell membrane than T_{reg} from wild-type mice[1]. Therefore, I investigated if this is also the case in human cells.

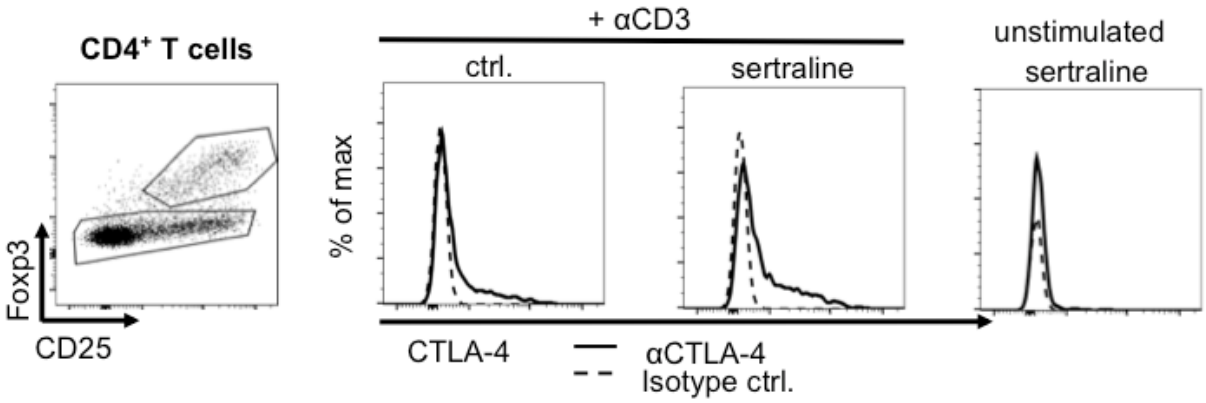


Figure 20 CTLA-4 turnover is modulated by functional ASM inhibition through sertraline

Human PBMC were cultured for 24 h with 1 µM sertraline or without sertraline (ctrl.). Furthermore, the cells were cultured in the presence or absence of an anti-CD3 mAB as indicated. T_{reg} were gated as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (left, dot plot). To investigate the number of CTLA-4 molecules at the cell surface during the time of culture, an anti-CTLA-4 mAB was added to bind to CTLA-4 molecules at the cell surface (middle and left, histograms). An isotype matched mAB with irrelevant specificity was added to parallel cultures and used as signal control.

In anti-CD3-activated cells significantly more CTLA-4 (34 % more) was shuttled to the cell surface of T_{reg} within 24 h in the presence of sertraline (Fig. 20 and Fig. 21 A,

green) than in untreated activated cells (Fig. 20 and Fig. 21 A, black). It was further analyzed if the treatment with sertraline leads to an increase in total CTLA-4 expression. However, no changes in total expression could be observed (Fig. 21 B).

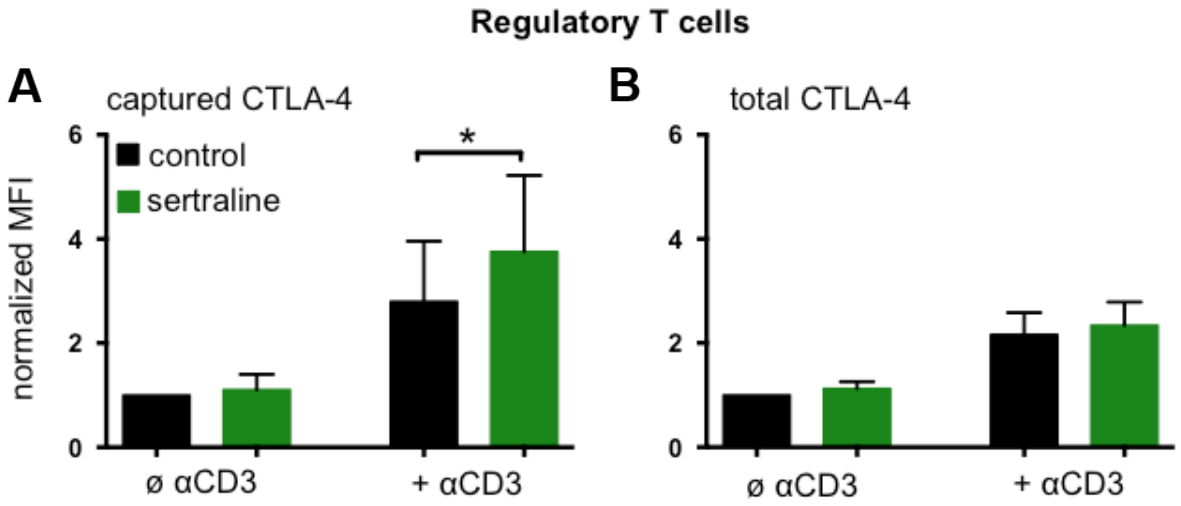


Figure 21 Summary graph of measured mean fluorescence intensity of anti-CTLA-4 mAB

(A) The cells were cultured for 24 h together with an anti-CTLA-4 mAB, which can bind to CTLA-4 molecules on the cell membrane. (B) After culturing, cells from parallel cultures were permeabilized and stained for the total amount of CTLA-4 molecules in the cells (right). The data show the mean fluorescence intensity (MFI) of the anti-CTLA-4 mAB normalized to the unstimulated control. (n=14) The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05

6.6.2 *Transendocytosis activity of CTLA-4 remains unchanged after ASM inhibition*

CTLA-4 plays an important role in maintaining immune homeostasis as a negative regulator of the immune system. It does this through its binding to the ligands CD80 and CD86, which are present on APC. It removes these molecules from the surface and internalizes them, which is called transendocytosis[26-28] Therefore, these ligands are not available for binding to CD28 and the subsequent co-stimulation of the cell. Moreover, CTLA-4 also inhibits TCR/CD28 intracellular signaling and induces immunosuppressive genes in APC. In this experiment, it was focused on the transendocytosis of the ligands CD80 and CD86.

To test if CD80/CD86 transendocytosis (Fig. 2) is changed by sertraline treatment, for which a higher turnover of CTLA-4 on the surface was observed (Fig. 20), MEF expressing mouse (m)CD80-mScarlet were used as donor cells and co-cultivated with human PBMC. After 12 h of co-cultivation and activation of the cells with anti-CD3 mAB transendocytosis of mCD80 by T_{reg} was measured (Fig. 22). Additional treatment of the cells with sertraline did not lead to a difference in transendocytosis activity.

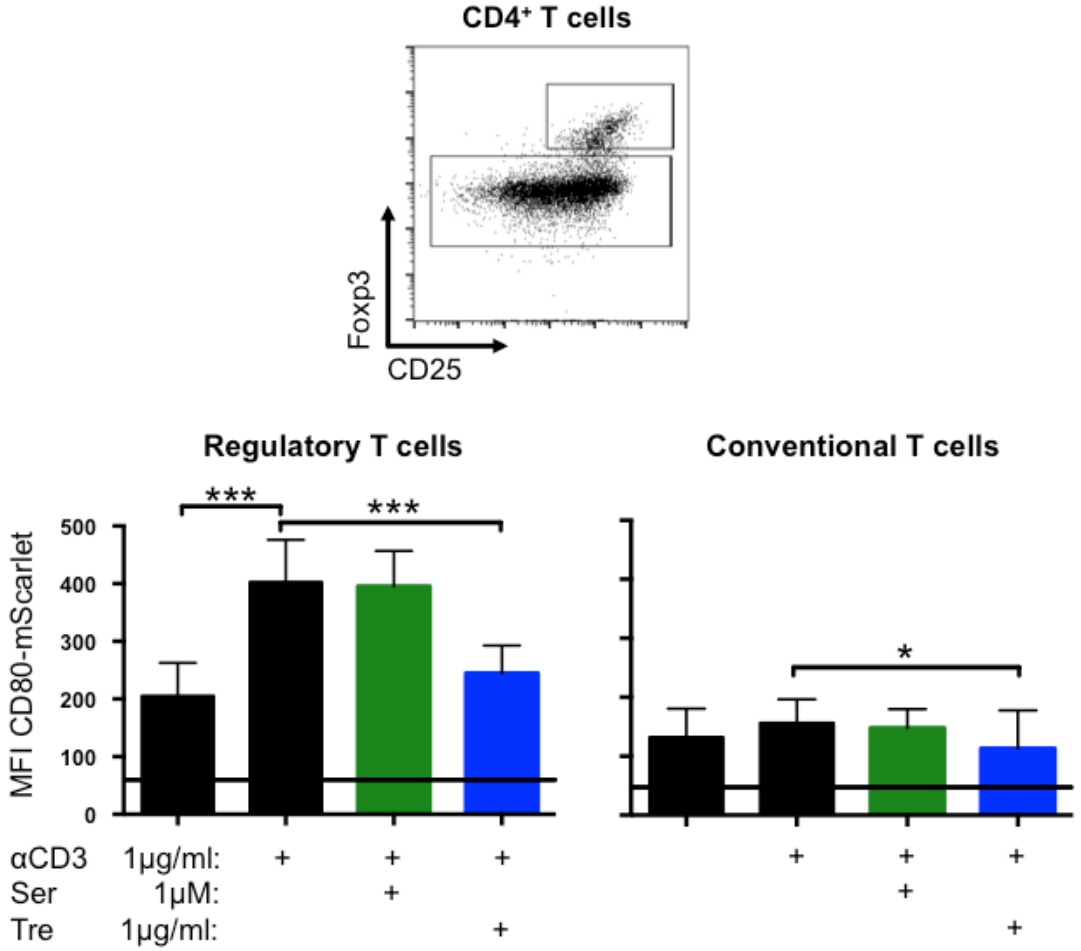


Figure 22 CTLA-4-mediated transendocytosis of ligands is independent of ASM activity

For this experiment $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} cells (dot plot, upper gate) and $CD4^+$ $Foxp3^-$ T_{conv} cells (dot plot, lower gate) were gated and their MFI for mCD80 molecules measured. PBMC were co-cultured for 12 h with mCD80-mScarlet expressing MEFs and activated with anti-CD3 mAB. Furthermore, the cells were treated either with 1 µM of ASM-inhibiting antidepressant sertraline (Ser, green) or 1 µg/ml of anti-CTLA-4 mAB tremelimumab (Tre, blue) to analyze the effect of functional ASM inhibition on transendocytosis activity of T_{reg} cells (n=8). The black line indicates the background signal obtained upon co-incubation of human PBMC with MEF lacking mCD80-mScarlet expression. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. *p<0.05, **p<0.01, ***p<0.001

In contrast, the anti-CTLA-4 mAB tremelimumab partially blocked the transendocytosis activity of the cells by about 80 % in comparison to the transendocytosis activity induced by anti-CD3 mAB alone (Fig. 22, blue). For T_{conv}, a significant decrease of transendocytosis activity under tremelimumab treatment was also observed. The signal of transferred mCD80-mScarlet measured in the presence of tremelimumab was even below the level of spontaneous transendocytosis activity of unstimulated cells.

Transendocytosis means that mCD80 is captured by CTLA-4 and internalized into the T_{reg}. Therefore, to check for the internalization of the CTLA-4/mCD80 complex, the cells were additionally stained for mCD80 on the surface and the results showed that the cells positive for mScarlet were all additionally positive for mCD80 on the surface (Fig. 23). As the cells were disrupted after culturing and the MEF could be clearly distinguished by their higher granularity and their bigger size, this means that mCD80 is removed from target cells, but not completely internalized by T_{reg}. As this might be due to ectopic expression of mCD80 by MEF rather than APC, we repeated the transendocytosis experiment using mouse APC.

The previous experiment showed that the used system was not optimal for analyzing the transendocytosis activity of T_{reg}. Therefore, the experiment was repeated using LPS-pre-activated mouse splenic lymphoblasts as CD86 donor cells. Here, the goal was to see the internalization of the CTLA-4/mCD86 complex and whether this process is influenced by sertraline treatment. It could be shown in the new experimental set-up that mCD86 was internalized by human T_{reg} cells. In the previous experiment, all T_{reg} were double positive for surface mCD80 and CD80-mScarlet (Fig. 23 B, lower dot plot). In comparison to this, in the new set up, T_{reg} were stained for surface CTLA-4 and mouse CD86 (Fig. 24). Here, it is seen that some T_{reg} are double positive for both CTLA-4 on the surface and mCD86 (Fig. 24, right dot plot, 19.6 %), but also another population of T_{reg} are only positive for mCD86 (Fig. 24, right dot plot, 27.7 %). For T_{reg} to be only positive for mCD86, it has to be internalized as the T_{reg} would have been double positive for surface CTLA-4 and mCD86 if the CTLA-4/mCD86 complex was not internalized into the T_{reg}. Therefore, this set up allows for transendocytosis and was used to further analyze the impact of the ASM-inhibiting antidepressant sertraline on transendocytosis and the degradation of CD86.

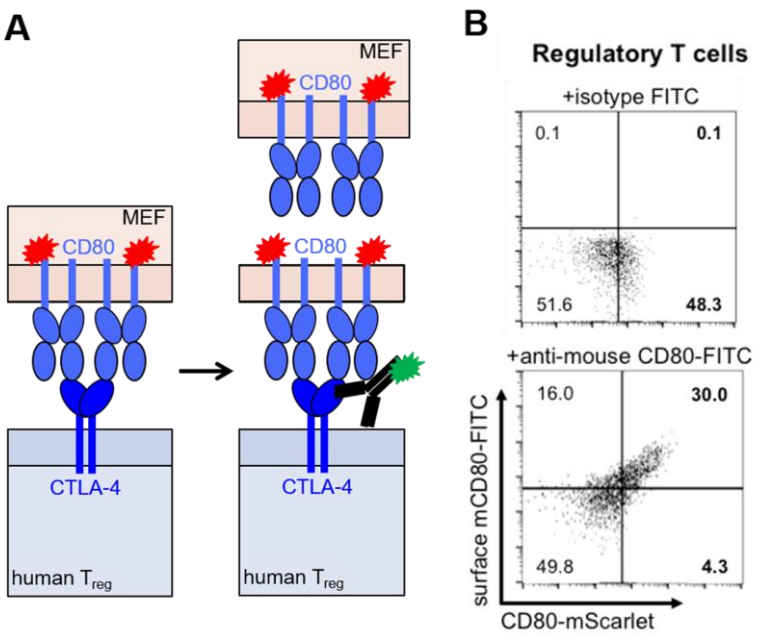


Figure 23 CTLA-4-mCD80-mScarlet complex is not internalized by human Treg

(A) Schematic representation of results, the MEF express CD80-mScarlet, which was measured in the FACS machine. CTLA-4 binds to CD80-mScarlet but does not internalize into the T_{reg}. (B) PBMC were co-cultured for 12 h with mCD80-mScarlet expressing MEFs and anti-CD3 mAB for stimulation. After culture the cells were additionally stained with an anti-mCD80 mAB on the surface to detect whether the CTLA-4-mCD80 complex is internalized or not. The results show that the cells are double positive for both mScarlet and FITC. FITC shows the CD80 bound to CTLA at the cell surface. Therefore, the CD80-mScarlet captured from the MEF is not internalized into the T_{reg}. An isotype mAB was used as signal control. Regulatory T cells were defined as $CD4^+$ $CD25^+$ $Foxp3^+$ and numbers indicate percent cells/quadrant.

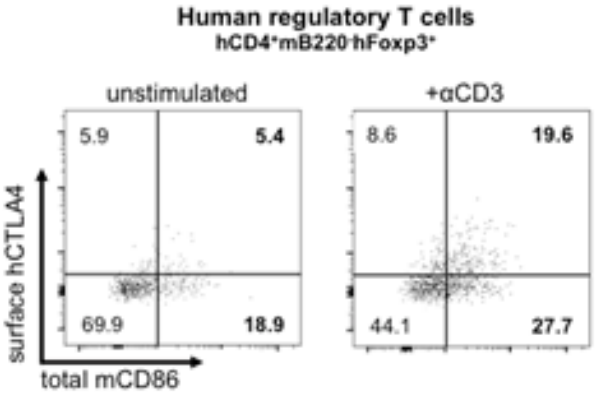


Figure 24 CTLA-4-mCD86 complex gets internalized by human Treg

PBMC were co-cultured for 16 h with LPS-pre-activated mouse splenic lymphoblasts and anti-CD3 mAB for stimulation. After culture the cells were stained for human (h)CTLA-4 on the surface and additionally permeabilized and stained with an anti-mCD86 mAB to detect whether the CTLA-4-mCD86 complex is internalized or not. Regulatory T cells are defined as $hCD4^+$ $mB220^-$ $hCD25^+$ $hFoxp3^+$ and numbers indicate percent cells/quadrant.

Next, it was checked if the treatment with sertraline led to changed endocytosis or degradation of the CTLA-4-mCD86 complex in this new experimental set-up. Therefore, mouse splenic lymphoblasts and PBMC were again co-cultivated and then human T_{reg} were analyzed at different time points (data shown for 16h) to optimize the assay. The treatment with ASM-inhibiting antidepressant sertraline did not lead to a change in acquisition of CD86 to the cell surface of T_{reg} (Fig. 25 A). Also, the internalization/degradation of the CTLA-4-mCD86 complex was unaltered (Fig. 25 B, green). Furthermore, the blocking of CTLA-4 by the anti-CTLA-4 mAB tremelimumab led to a partial decrease in transendocytosis activity of T_{reg} (Fig. 25, blue). Even though ASM inhibition increased the turnover rate of CTLA-4, it did not affect the CTLA-4-mediated transendocytosis of CD80 and CD86.

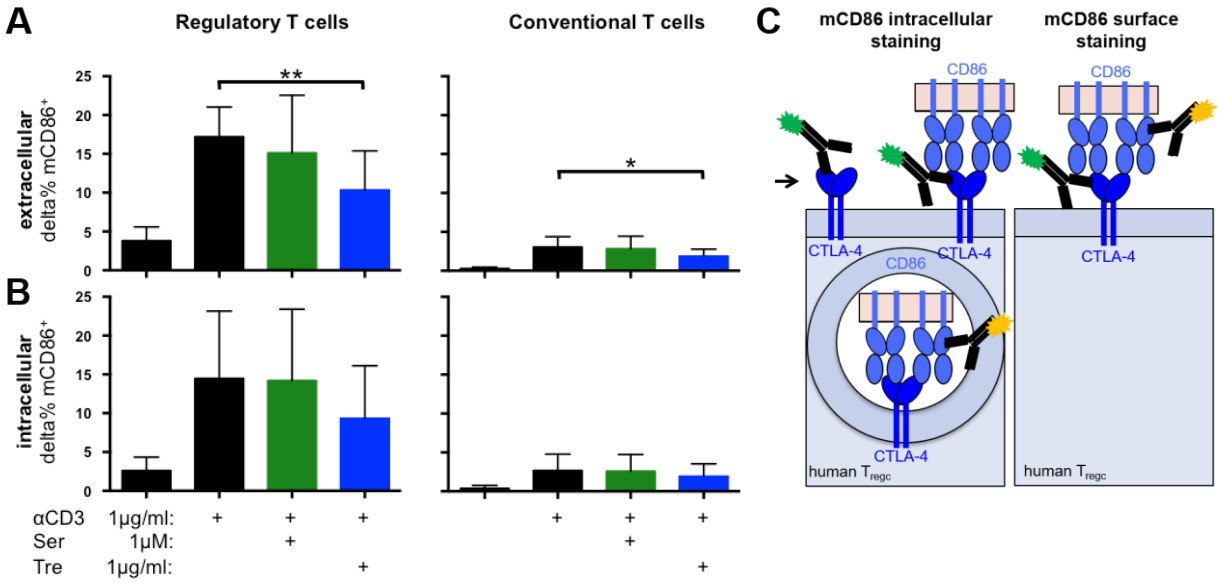


Figure 25 CTLA-4-mediated transendocytosis of mCD86 is unaffected by functional ASM inhibition by sertraline

PBMC were co-cultured for 16 h with LPS-pre-activated mouse splenic lymphoblasts and anti-CD3 mAB for stimulation. After the culture, cell-cell contact was prevented through dilution and different time points were analyzed. Here, the transendocytosis of mCD86 from mouse splenic LPS blasts to human T_{reg} (left) or T_{conv} (right) at the time point of 16 h is shown. The background noise was subtracted from the measured percentage of cells for (A) extracellular and for (B) intracellular mCD86 signal. Additionally, the cells were treated with 1 µM sertraline (green) or 1 µg/ml tremelimumab (blue). Cells treated with sertraline show no significant difference in their transendocytosis activity, whereas tremelimumab blocks the transendocytosis activity of the T cells. (C) Schematic representation of the staining done for obtaining the data. The results were statistically analyzed with ANOVA followed by a Tuckey post-hoc test. (n=8) *p<0.05, **p<0.01

6.7 SUPPRESSIVE ACTIVITY OF T_{REG} IS UNAFFECTED BY FUNCTIONAL ASM INHIBITION

The key parameter for T_{reg} is their suppressive function. The changes in proportion in the $CD4^+$ T cell compartment in human PBMC *in vitro* and in patients are important, but without analyzing the functionality of T_{reg} after treatment with the ASM-inhibiting antidepressant sertraline, it is not possible to hypothesize what the increase in frequencies could mean and how it could be exploited.

Therefore, indicator cells (T_{ind}) were labeled with CFSE (Fig. 26) and T_{reg} or T_{conv} cells to indicator cells were added to indicator cells at various ratios (1:1, 1:4 and 1:16) to measure the proliferation of indictor cells in the absence and presence of sertraline (Fig. 26, left, green). As T_{conv} (Fig. 26, right) do not have a suppressive activity, they were used as control. After 4 days of culture, no significant changes in the suppressive activity of T_{reg} was observed in the presence compared to the absence of sertraline.

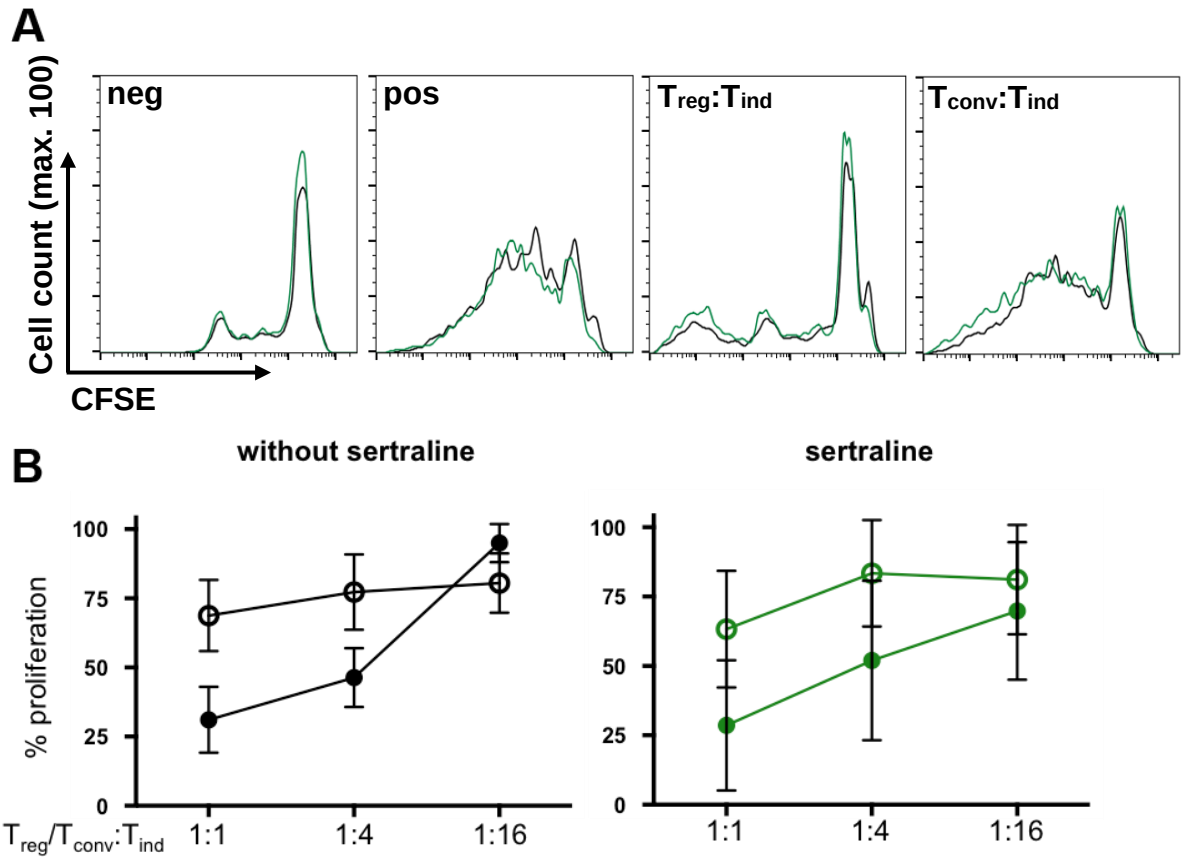


Figure 26 The suppressive activity of T_{reg} cells is not affected by sertraline

(A) PBMC were labeled with CFSE as a proliferation marker and gated $CD4^+$ T cells used as indicator cells (Ind). $CD25^{high}$ T_{reg} and $CD25^-$ T_{conv} were sorted by cell sorter. The two cell groups were co-cultivated for 4 days with (green) or without (black) the ASM-inhibiting antidepressant sertraline and with 1 µg/ml anti-CD3 mAB. The maximum number of cells cultured was 2 x 10^4 cells. An exemplary result is shown for the ratio 1:1 for $T_{reg}/T_{conv}:T_{ind}$. (B) The cells were cultivated with different ratios, 1:1, 1:4 and 1:16 for $T_{reg}/T_{conv}:T_{ind}$. The mean percentage of proliferation is shown for each ratio, cultures with T_{reg} are represented by a filled circle and cultures with T_{conv} are shown with an empty circle. Again, results for cells treated with sertraline are shown in green and controls are shown in black. T_{conv} have no significant effect on the proliferation of T_{ind}. T_{reg} had a dose-dependent effect on the proliferation of T_{ind}, but no effect of sertraline treatment can be seen. (n= 4, individual experiments/ donors)

7 DISCUSSION

T cells are major players in the adaptive immune response. Even though the T cells undergo a rigorous selection process in the thymus, some autoreactive T cells slip through[106, 151-153]. To defend the body against these cells and an “overshooting” or unwanted adaptive immune response, also T_{reg} are generated in the thymus[154]. In the absence of T_{reg} healthy tissue can be attacked which could lead to autoimmune diseases[155]. Furthermore, T cells are necessary for maintaining or restoring tissue homeostasis after muscle damage[156], myocardial infarction[157-159] or stroke[160]. T cells function mainly through extracellular signals induced by cell surface receptors, allowing them to achieve all these diverse tasks.

Here, lipids play a major role as they form the cell membranes. Nearly one third of the lipids are part of the sphingolipid group[161]. Depending on the differentiation state and function of the cells, they undergo metabolization and the needed sphingolipids are formed[162]. The sphingomyelins of various chain lengths can be cleaved into ceramides and phosphocholine by ASM and NSM2.

In previous work, the absence of Asm in mice, either by genetic ablation or pharmacologic inhibition, led to an increase in frequency and activity of $CD4^+$ $Foxp3^+$ T_{reg} among $CD4^+$ T cells[1]. Even though much work was done to advance the understanding of sphingolipids and their roles in different diseases, the underlying mechanistic function of ASM during the adaptive immune response in humans is still unclear. Therefore, in the present thesis the focus was to analyze the effect of pharmacological inhibition of ASM in human, *in vitro* and *in vivo.* Moreover, the underlying mechanism, which leads to the observed effects, was studied. In addition, it was aimed for a further characterization of the affected cells. Therefore, the impact of pharmacological ASM inhibition on the balance of the $CD4^+$ T cell subsets was analyzed in human samples and especially it was distinguished between conventional and regulatory T cells. Both cell populations differ in ASM activity, lipid order, and content of sphingolipids of various chain lengths in their membranes.

7.1 PHARMACOLOGICAL INHIBITION OF ASM IN HUMANS

Previous work in our laboratory showed promising results in mice[1] that if verified in human, could possibly be a therapeutic option for the treatment of autoimmune diseases. In the thesis by F. Dennstädt[142] and in the present work, it could be demonstrated that treating human peripheral blood mononuclear cells *in vitro* with ASM-inhibiting drugs leads to an increased frequency of effector regulatory T cells among $CD4^+$ T cells (Fig. 17). They are more resistant to pharmacological inhibition of ASM activity than conventional T cells.

Based on these findings with human PBMC *in vitro*, a co-operation was started with the Department of Psychiatry, Psychosomatics and Psychotherapy of the University Hospital Würzburg to obtain blood samples of patients treated for major depression. In the clinic, ASM-inhibiting antidepressants are the most broadly used drugs in patients treated for major depression (prevalence in depression 5,000/100,000)[111]. Here, several different antidepressants, which were categorized into strongly and weakly/ non-ASM-inhibiting, or electroconvulsive therapy, categorized as non-ASM-inhibiting, were used as therapy. The results showed that as found *in vitro*, *in vivo* treatment with ASM-inhibiting drugs led to an increased frequency of T_{reg}, especially of efT_{reg}, but also of rT_{reg} and of the subpopulation of $CD25^{low}$ mT_{conv} (Fig. 12). For the patients treated with weakly ASM-inhibiting drugs a significant increase in the frequency of efT_{reg} could be seen after four weeks of treatment (Fig. 12). When one compares the observed increases in efT_{reg}, it clearly is dependent on the ASM-inhibiting capacity of the used drug for treatment. Patients receiving strongly ASM-inhibiting drugs have a significantly higher increase of about 78 % over the time of four weeks, whereas the increase is about 35 % for patients treated with weakly ASM-inhibiting drugs (Fig. 12). Therefore, the data indicate that the increase in T_{reg} frequencies among human $CD4^+$ T cells *in vivo* is part of the mode of action of ASM-inhibiting drugs in routine clinical use.

T_{reg} cells have an important function in suppressing autoreactive T cells, their generation is also called the third function of the thymus[154]. Even though, the process of negative selection is highly efficient, it is not able to completely remove maturing autoreactive T cells. For healthy individuals with an intact immune system these cells are not problematic, but for the few individuals that have an impaired immune system, espe-

cially in the T_{reg} compartment, it can lead to autoimmune diseases like multiple sclerosis[106, 151-153]. The modulation of the balance in the $CD4^+$ T cell compartment towards T_{reg} by ASM-inhibiting antidepressant suggests an immunosuppressive function and therefore, a novel indication for the use of ASM inhibitors for inflammatory and autoimmune diseases, like multiple sclerosis and rheumatoid arthritis[163, 164], in which a defect in T_{reg} numbers and function has been shown[165, 166]. In a mouse model for experimental autoimmune encephalomyelitis a positive effect of Asm-deficiency was observed[102]. In a double-blind placebo-controlled clinical trial in patients with multiple sclerosis the ASM inhibitor fluoxetine led to a reduction in inflammation[167].

This is in accordance with the findings of the work presented here, which showed that ASM-inhibiting antidepressants can help to increase the T_{reg} frequencies among the $CD4^+$ T cells to normal levels.

The connection of the immune system and major depression has become clearer over the last decades[168] as several studies[169, 170] showed that anti-inflammatory treatments led to an improved depression in patients, but not all patients benefited from these treatments[171]. Therefore, responses to ASM-inhibiting drugs were analyzed as a possible decisive factor, but no significant role could be seen.

Furthermore, it was of interest if the increase in efT_{reg} frequencies among $CD4^+$ T cells in patients treated with ASM-inhibiting antidepressants reflected the clinical response of the patients. Recovering patients from severe depression show a similar increase in efT_{reg} frequencies among $CD4^+$ T cells as seen *in vitro* and *in vivo* upon ASM activity inhibition[136]. Therefore, the changes of the HAMD scores over the treatment of four weeks were compared between the two patient groups (Fig. 14 and Fig. 15). Here no significant differences between the patients being treated with strongly or weakly ASM-inhibiting drugs could be seen. Thus, clinical improvement of patients was ASM independent as was expected due to well documented clinical efficacy of all antidepressants used in the study.

Nevertheless, even though the clinical response is independent to the capacity of the used antidepressant to inhibit ASM activity, while analyzing the data set, a correlation between an early, after one week, increase in efT_{reg} frequencies among $CD4^+$ T cells and a positive clinical response after four weeks of treatment was found (Fig. 15). EfT_{reg}

among $CD4^+$ T cells could be used as a biological marker and predictor of a positive clinical response in patients.

Clinical improvement of patients is a process affected by multiple factors, for example on a molecular level the improvement is influenced by serotonin levels, which are increased by SSRIs[172] like sertraline and citalopram. Furthermore, it is still not known what causes the low T_{reg} frequencies among $CD4^+$ T cells in patients with major depression prior to treatment.

7.2 ASM INHIBITION PROVIDES FOR BETTER CD28 CO-STIMULATION OF T_{REG}

By analyzing the cell numbers of unstimulated cells *in vitro* before and after four days of treatment with sertraline, it could be seen that the total number of cells decreased over the time of culture and that the decrease is less pronounced in T_{reg} (data not shown) than in T_{conv}, which died consistently at low doses, independently of the dose (high doses are toxic for both cell types). Moreover, it could be shown that it was the subpopulation of efT_{reg} that was most affected by sertraline treatment and efT_{reg} have the lowest dose-dependent decrease in cell numbers. It was checked if the differences in cell numbers were caused by *de novo* development of T_{reg}, T_{conv} upregulating Foxp3 and developing into T_{reg}, or by enhanced survival of T_{reg}. *De novo* development of T_{reg} and T_{conv} upregulating Foxp3 could be ruled out by depleting T_{reg} from PBMC and treating them and the T_{reg}-sufficient PBMC separately with sertraline. Increases in T_{reg} frequencies were only seen in T_{reg}-sufficient cultures[142].

Next, the mechanism how inhibition of the ASM might enhance survival of T_{reg}, but not T_{conv}, was investigated. ASM activity leads to changes in cell membranes and could play an important role in associated cellular processes like exocytosis and vesicular transport of receptors involved in the regulation of survival. Therefore and because of its important role in suppressive activity of T_{reg}, it was decided to firstly focus on CTLA-4. It has been demonstrated before that CTLA-4 is involved in the regulation of proliferation of T_{reg}. An absence of CTLA-4 signaling leads to an increased cell proliferation of about 50 % in T_{reg}. CTLA-4 signaling regulates the proliferation of T_{reg}, but not T_{conv}[173], which could be explained by the higher expression of CTLA-4 in T_{reg} compared to T_{conv} (Fig. 11).

Therefore, the anti-CTLA-4 mAB tremelimumab was used to inhibit CTLA-4 *in vitro* in

human PBMC. The results showed that CTLA-4 blockade with and without inhibiting ASM activity, led to an increase in efT_{reg} frequencies among $CD4^+$ T cells (Fig. 17). These results are in accordance with the results of Kolar et. al[173] who showed *in vitro* in mice that blocking CTLA-4 with anti-CTLA-4 Fab fragments led to a duplication in proliferation. These results are consistent with the work of others[174, 175]. To test whether both inhibitions use the same mechanism to cause the increase, both treatments, inhibition of ASM and CTLA-4 activity, were combined. No additive effect was seen (Fig. 17). Therefore, it is reasonable to assume that both inhibitions use the same mechanism to cause the increase of T_{reg} frequencies among $CD4^+$ T cells.

A function of CTLA-4 is its transendocytosis activity. It was hypothesized that this function, which happens at the cell membrane, could be influenced by functionally inhibiting ASM. In previous work, it could be shown that the turnover of CTLA-4 from its intracellular storage to the cell membrane in mice is Asm dependent[1]. This result could be confirmed in humans *in vitro* (Fig. 20 and 21). In T_{reg}, CTLA-4 is constitutively expressed, whereas its expression is induced upon activation in T_{conv}. After treatment of the cells with sertraline the overall expression of CTLA-4 is not increased (Fig. 21) and the fraction of CTLA-4 molecules reaching the cell surface is significantly higher (Fig. 21).

Due to these results and the fact that inhibition of the ASM activity might lead to changes in the property of cell membranes, it was reasoned that the transendocytosis activity of CTLA-4 might also be affected. However, the experiments showed no significant difference in transendocytosis activity in cells treated with the strongly ASM-inhibiting drug sertraline and untreated cells despite the increased expression and circulation of CTLA-4 (Fig. 22). In the first experimental set-up, in which CD80-mScarlet MEFs were used as donor cells, it was noticed that the CTLA-4-mCD80-mScarlet complex was not internalized (Fig. 23), an important aspect of transendocytosis and something, which could affect the results. Therefore, a second set-up was chosen with splenic lipopolysaccharide blasts as mCD86 donors. Here, the complex was internalized (Fig. 24), but again no significant difference in transendocytosis activity could be observed. Afterwards the sertraline treated cells were checked if treatment leads to differences in CTLA-4 degradation. Here, the sertraline treated cells showed again no significant differences compared to the

untreated cells (Fig. 25). As more total CTLA-4 is expressed, the transendocytosis function per CTLA-4 molecule appears to be reduced. Thus, the hypothesis that the increase in T_{reg} frequencies among $CD4^+$ T cells is caused by an altered CTLA-4 transendocytosis activity (per cell) was shown to be false.

Next, we checked whether the observed increase in T_{reg} frequencies among $CD4^+$ T cells was exclusive to inhibition of CTLA-4 or if the inhibition of another suppressive receptor (PD-1) would cause the same results. The results showed that treatment with the anti-PD-1 mAB pembrolizumab also led to an increase in T_{reg} frequencies among $CD4^+$ T cells although the data did not reach statistical significance (Fig. 18).

Both suppressive receptors have in common that they inhibit the co-stimulatory signaling of CD28[27, 33] (Fig. 30). The obtained results and this fact led us to analyze the effect of CD28 on the frequencies of efT_{reg} among $CD4^+$ T cells. Therefore, different concentrations of an anti-CD28-Fab were used to inhibit CD28 co-stimulation. It could be shown that inhibition of CD28 co-stimulation led to a dose-dependent decrease in T_{reg} frequencies among $CD4^+$ T cells (Fig. 19). This corresponds with results obtained by others who induced deletion of CD28 in mice and saw a decline in T_{reg} cell numbers and impaired effector functions[46, 176], showing the importance of CD28 for T_{reg} homeostasis. Furthermore, several groups showed that T_{reg} cell numbers and function are sustained by signaling through CD28 and CD25 a subunit of the high affinity IL-2 receptor[46-48, 177-179]. CD28 is one of the cell surface receptors whose activation leads to increased ASM activity in the lysosome and lysosomal fusing with the cell membrane[85]. The latter exposes ASM to sphingomyelin at the cell surface and leads to its cleavage into ceramide and phosphocholine[180].

When in addition to anti-CD28-Fab inhibitors for ASM, CTLA-4 and PD-1 were added to the cultures then the increase in T_{reg} among $CD4^+$ T cells caused by these inhibitors was abolished (Fig. 19). Thus, these data suggest that the increase in the T_{reg} compartment *in vitro* was caused by enhancing the positive impact of CD28 co-stimulation on the T_{reg} compartment and that ASM activity might negatively modulate CD28 co-stimulatory signaling (Fig. 30). This might be caused by a lower amount of ceramide in T_{reg} after ASM inhibition. Ceramide has a negative effect on the mTOR/Akt pathway[181] and a decrease in ceramide levels might therefore lead to an increased CD28 signaling[14]. Therefore, the next steps would be to analyze CD28-induced

signaling pathways, like mTORC and pAkt, in cells treated with the ASM-inhibiting antidepressant sertraline[182].

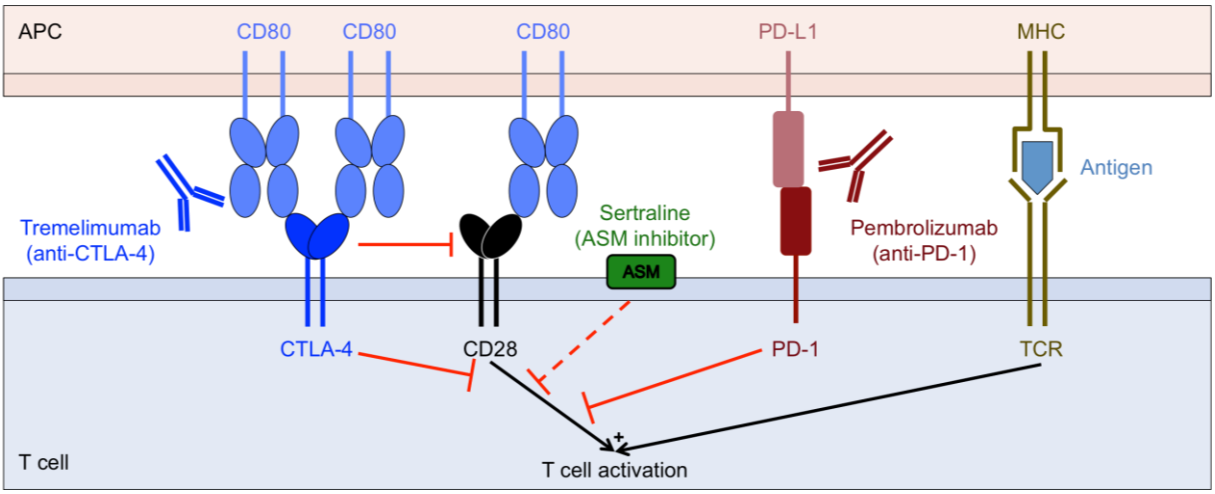


Figure 27 ASM might inhibit CD28 co-stimulatory signaling

Graphical summary of the obtained results. CTLA-4 (blue), PD-1 (red) and ASM (green) are known or might negatively influence CD28 (black) signaling.

Further analyses showed that the suppressive activity of T_{reg} is not influenced by functional ASM inhibition through the antidepressant sertraline *in vitro* (Fig. 26). This is important if one wants to use the findings of this study for treatment of autoimmune diseases, like multiple sclerosis or rheumatic arthritis, which are characterized by an overshooting immune reaction and fewer T_{reg} among $CD4^+$ T cells. These results could be explained by the described effects of ASM inhibition on both CD28 and CTLA-4 signaling. An enhanced CD28 signaling leads to a better suppressive activity of T_{reg}, whereas a decreased CTLA-4 signaling leads to less suppression by T_{reg}[14].

7.3 Differences in sphingolipid content in T_{REG} and T_{CONV}

T_{reg} and T_{conv} differ in their ASM activity, lipid order, and content of sphingolipids of various chain lengths in their membranes. Especially the ceramide concentration in cellular membranes plays a major role in the homeostasis of $CD4^+$ T_{reg} and T_{conv}. It could be shown that T_{reg} frequencies decrease in culture with additional C6-ceramide due to higher changes in cell numbers compared to T_{conv}, reduction of cell number was about 74 % and 49 % respectively (data not shown). Therefore, our newest working hypothesis is that human T_{reg} tolerate ceramide less than human T_{conv}.

The mass spectroscopic analysis showed for the T_{reg} subsets that the distribution of sphingolipids of various chain lengths was comparable for rT_{reg} and efT_{reg}, the same is true for the T_{conv} subsets. When the content of ceramide and sphingomyelin was normalized to the total amount of sphingomyelin, it could be seen that in relation to the total amount of sphingomyelin T_{conv} have a higher proportion of ceramide (Fig. 16 B). This is in contrast to findings in mouse T_{reg}[1, 181] and suggests human T_{reg} might overcompensate increased ASM activity by even higher activity of cellular ceramidases or less *de novo* ceramide production in human T_{reg} compared to T_{conv}.

It would be further interesting to measure the sphingolipid content for the five $CD4^+$ T cell subsets after sertraline treatment, which was impossible as the extracted cell number of especially efT_{reg} and rT_{reg} was just enough for mass spectroscopic analysis, but not for further treatment and a subsequent mass spectroscopic analysis. Therefore, one would have to establish a new protocol. Another possibility would be to use cells of patients with Niemann Pick disease, but also here it would be difficult to obtain enough PBMCs, which would lead to a high enough number of cells after sorting for each subset.

8 OUTLOOK

Sphingolipids play a role in many central cell processes like apoptosis, proliferation, secretion and signaling. Therefore, a whole understanding of sphingolipids in the underlying process would allow a target modulation of these processes through for example modulating ASM. In the presented work, it was possible to build a bigger picture of the influence of ASM on the $CD4^+$ T cell compartment. But it will be necessary to further analyze the sphingolipid metabolism and its role for the immune cells and therefore for diseases.

For a better analysis of the ASM a specific inhibitor would be needed as the analyzed FIASMAs also inhibit the acid ceramidase and as SSRIs influence serotonin levels, at least in vivo. Even though the used ASM inhibitors are good for analyzing the effect of ASM inhibition on cells, a specific inhibitor would allow a precise investigation of the ASM effect on cells and tissue. This knowledge could be used for targeted therapies and the specific inhibitors would lead to less side-effects.

Furthermore, the increase in CTLA-4 turnover in T_{reg} has to be further investigated. In the presented work, it was focused on the role of CTLA-4 in transendocytosis, which was unaffected by ASM-inhibiting treatment, but another suppressive mechanism of CTLA-4 could be affected and should be experimentally analyzed. Moreover, membrane bound CTLA-4 could have an inhibitory effect on ASM through internal signaling.

Lastly, the connection between CD28 signaling and ASM should be further analyzed to better understand the seen effects, which would be important for target therapeutic use as CD28 is an important co-stimulatory molecule.

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10 ABBREVIATIONS

%	percent
$^{+}$	expressed in cells
$^{-}$	not expressed in cells
α	anti (prefix), alpha
AB	antibody
Akt	proteinkinase B
ANOVA	Analysis of variance
APC	antigen-presenting cell
ASM/Asm	acid sphingomyelinase (human/mouse)
β	beta
BSS/BSA	balanced salt solution / bovine serum albumin
γ	gamma
°C	degree Celsius
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
Cit	citalopram
CO_2	carbon dioxide
CTLA-4	cytotoxic T lymphocyte antigen-4, CD152
δ	delta
DMS II	Diagnostic and Statistical Manual of Mental Disorders
DMSO	dimethyl sulfoxid
EAE	experimental autoimmune encephalomyelitis
ECT	electroconvulsive therapy
EDTA	ethylenediaminetetraacetic acid
efT_{reg}	effector regulatory T cell
ER	endoplasmic reticulum
et. al	and others (lat. et alii, et aliae)
FCS	fetal calf serum
FIASMA	functional acid sphingomyelinase inhibitor
Foxp3	forkhead box protein 3
g	gram
GLM	generalized linear model

GvHD	graft versus host disease
h	hours
HAMD	Hamilton rating scale for depression
^{high}	high-level expression in cells
ICOS	inducible T cell costimulator
IL	interleukin
T_{ind}	indicator cells
IPEX	immune-dysregulation, polyendokrinopathy and enteropathy X-linked syndrome
l	liter
logP	logarithmic partition coefficient
^{low}	low-level expression in cells
M	molar (mol/l)
m	milli (prefix, 10^{-3}); mouse (prefix, in front of CDX); meter (suffix)
µ	micro (prefix, 10^{-6})
mAB	monoclonal antibody
MADRS	Montgomery-Åsberg Depression Rating Scale
MDD	major depressive disorder
MEF	mouse embryonic fibroblast
MEF-CD80-mScarlet	transgenic mouse embryonic fibroblast expressing CD80-mScarlet
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
mT_{conv}	memory conventional T cell
mTOR	mammalian target of rapamycin
n	nano (prefix, 10^{-9})
n=	number of experiments carried out
NaCl	natrium chloride
NK	natural killer cell
NSM/Nsm	neutral sphingomyelinase (human/mouse)
nT_{conv}	naïve conventional T cell

ON	over night
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
Pem	pembrolizumab
pH	potential of hydrogen
pK_a	acid dissociation constant
PRRs	pattern recognition receptors
Q	quadrant
RT	room temperature
rT_{reg}	resting regulatory T cell
S1P	sphingosine-1-phosphate
Ser	sertraline
SLE	systemic lupus erythematodes
SMPD1	sphingomyelin phosphodiesterase 1
SSRI	selective serotonin reuptake inhibitor
T_{conv}	conventional T cell
TCR	T cell receptor
T_h17	T helper cells expressing IL-17
Tre	tremelimumab
T_{reg}	regulatory T cell
v:v	volume percent

11 TABLE OF FIGURES AND TABLES

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