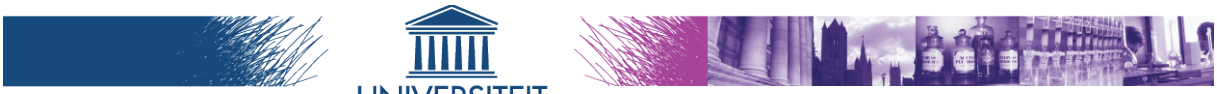

Teaching old drugs new tricks: selective serotonin reuptake inhibitors as a novel class of immunosuppressants

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Academic year 2014-2015

Thesis submitted in the fulfillment of the requirements for the degree of Doctor in Pharmaceutical Sciences

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List of abbreviations

5HT	5-hydroxytryptamine; serotonin
5HTR	5-hydroxytryptamine receptor
5HTT	5-hydroxytryptamine transporter
ACTH	adrenocorticotropic hormone
AP-1	activator protein 1
APAF-1	apoptotic peptidase activating factor 1
APC	antigen presenting cell
ATG	anti-thymocyte globulin
ATP	adenosine trisphosphate
AU	arbitrary unit
BAPTA-AM	1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester
BM	bone marrow
BMI	body mass index
BMT	bone marrow transplantation
CAD	caspase activated DNase
cAMP	cyclic adenosine monophosphate
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CIA	collagen-induced arthritis
CICR	Ca^{2+}-induced Ca^{2+} release
CKS1	cyclin-dependent kinase regulatory subunit 1
ConA	concanavalin A
Cp	plasma concentration
CRAC	calcium release-activated calcium channel
CRH	corticotropin releasing hormone
CRP	C-reactive protein
CS	contact hypersensitivity reaction
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	chemokine (C-X-C motif) ligand
CYP450	cytochrome P450
Cyt C	cytochrome C
DAG	diacyl glycerol
DC	dendritic cell
DDD	defined daily dose
ddPCR	droplet digital polymerase chain reaction
DISC	death inducing signaling complex
DMEM	dulbecco's modified eagle medium

DNA	deoxyribonucleic acid
DSS	dextran sulphate sodium
DTR	dextran texas red
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FYN	proto-oncogene tyrosine-protein kinase
GI	gastro-intestinal
GR	glucocorticoid receptor
GvHD	graft-versus-host disease
HEK	human embryonic kidney cell
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal axis
hSCT	hematopoietic stem cell transplantation
hSERT	human serotonin transporter
IC_{50}	half maximal inhibitory concentration
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
ITAM	immunoreceptor tyrosine-based activation motif
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
Kd	dissociation constant
LAT	linker for activation of T cells
LCK	lymphocyte-specific protein tyrosine kinase
LPS	lipopolysaccharide
MAO	monoamine oxidase
MAOi	monoamine oxidase inhibitor
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
miHA	minor histocompatibility antigen
MLC	mixed lymphocyte culture
Mls	minor lymphocyte stimulating antigen
MMTV	mouse mammary tumor virus
mRNA	messenger ribonucleic acid
MS	multiple sclerosis

NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB
NK	natural killer cell
NPE	1-(2-nitrophenyl)ethyl
OCD	obsessive compulsive disorder
ORAI	calcium release-activated calcium channel protein
PIP_2	posphatidyl inositol diphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidyl serine
PTK	protein tyrosine kinase
RA	rheumatoid arthritis
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RPMI	roswell park memorial institute medium
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RyR	ryanodine receptor
SERCA	sarco-endoplasmic reticulum calcium channel
SERT	serotonin transporter
SLC6A4	solute carrier family 6 member 4
SLP76	SRC-homology 2 -domain-containing leukocyte protein of 76 kD
SNRI	serotonin and noradrenaline reuptake inhibitor
SPL	spleen
SSRI	selective serotonin reuptake inhibitor
STIM1	stromal interaction molecule 1
$T_{1/2}$	half life
Tc	cytotoxic T cell
TCA	tricyclic antidepressant
TCR	T cell receptor
TG	thapsigargin
Th	T helper cell
TM	transmembrane domain
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
TRADD	tumor necrosis factor receptor type 1-associated DEATH domain protein
Vd	distribution volume
ZAP70	zeta-chain-associated protein kinase 70

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CHAPTER 1

INTRODUCTION

Parts of this chapter are based on "*Selective serotonin reuptake inhibitors as a novel class of immunosuppressants*." Veerle Gobin, Katleen Van Steendam, Damiaan Denys and Dieter Deforce. Int Immunopharmacol. 2014. 20: p. 148-156.

CHAPTER 1. INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are among the most prescribed drugs worldwide. Belonging to the overall group of antidepressants, SSRIs are primarily used for the treatment of major depressive disorder. In addition, they have proven to be effective in a number of psychiatric and neurological conditions such as obsessive-compulsive disorder, panic disorder and generalized anxiety disorder [1, 2]. Usually, SSRIs are well tolerated and have a beneficial side effect profile. However, recent evidence suggests that SSRIs might interfere with certain aspects of the immune system. Although confirmed by several research groups, this previously unnoticed adverse effect remains poorly characterized and the molecular mechanism underlying the immunological effects of SSRIs is not yet fully understood. Therefore, the first aim of this thesis was to characterize the immunological effects of SSRIs and to reveal the molecular mechanism behind them.

In drug discovery, compounds developed for the treatment of a specific medical condition often turn out to possess secondary effects that sometimes lead to marketing of the drug for a completely different indication. One well-known example of this principle, also called drug repositioning, is sildenafil: although this compound was originally investigated for its application in angina pectoris and hypertension, clinical studies demonstrated it could induce penile erection, leading to the marketing of this compound as Viagra®, the first approved drug for treatment of erectile dysfunction. Drug repositioning may also be applied to SSRIs: whereas these drugs have been used in the clinic for decades as antidepressants, they now turn out to exert interesting immunomodulatory effects that potentially could be used in the treatment of immune-mediated disorders. We thus investigated whether SSRIs could be ‘reinvented’ as a novel class of immunosuppressants. More particularly, we investigated the effect of SSRIs in acute graft-versus-host disease, an important complication of stem cell transplantation that limits the broad application of this life-saving treatment.

In order to provide the reader with the necessary background to interpret the results described in this thesis, this introductory chapter first describes the pharmacological background of SSRIs. Next, a general introduction on the immune system is given and the role of serotonin in immunity is discussed. In the third part, a brief background is given on the relation between depression and inflammation. The fourth part of the introduction consists of an overview of existing evidence on the impact of SSRIs on the immune system within and outside the context of depression. Finally, the last part provides an introduction on graft-versus-host disease as this condition was chosen to explore the *in vivo* immunosuppressive effects of SSRIs.

1. Selective serotonin reuptake inhibitors

1.1. History

Whereas many pharmacological compounds developed in the previous century were discovered by chance, the development of SSRIs is one of the first examples of 'rational drug design', a process in which the chemical structure of a drug is designed based on knowledge of its biological target. However, before one can design a pharmacological compound to treat a specific disease, the biological basis of that disease needs to be elucidated. As the biological basis of depression remained unknown until the second half of the twentieth century, the first antidepressants were discovered by chance. In the 1930s, a search for anti-malarial agents rendered a series of derivatives of phenothiazine which, although not effective against malaria, gained interest because of their antihistaminic, sedative properties. Further derivatization aiming on more effective compounds rendered chlorpromazine, which was marketed under the name Thorazine and became widely applied for the treatment of schizophrenia [3]. The finding that a pharmacological agent could alleviate psychosis, led to the recognition of schizophrenia having a biological basis. However, it was not until the 1960s that the biological basis of depression was unraveled. In the 1950s, in the course of a search for compounds effective against tuberculosis, isoniazid and iproniazid were synthesized and soon were found to be capable of enhancing mood in depressed patients [3]. At the time, the biological basis for this improvement in mental state was not known. Although effective in the treatment of depression, this class of drugs, now known as monoamine oxidase inhibitors (MAOi), was abandoned because of suspected induction of jaundice. In 1958, imipramine, a derivative of chlorpromazine and belonging to the class of tricyclic antidepressants, was equally found to alleviate depressive symptoms [4]. However, both types of antidepressants exhibited multiple side effects due to lack of selectivity. Not only did these drugs affect the availability of several neurotransmitters including serotonin, noradrenaline and dopamine, they also exerted antagonistic effects on histamine and acetylcholine receptors.

In 1961, Axelrod et al. discovered that neurotransmitters were not only broken down by monoamine oxidase (MAO) but were taken up back into the presynaptic neuron where they were stored (figure 1.1.)[5]. Two years later, it was established that antidepressant drugs available at the time worked through different mechanisms: whereas drugs like isoniazid and iproniazid inhibited MAO-mediated breakdown of monoamines, tricyclic compounds such as imipramine and amitriptyline blocked reuptake of neurotransmitters in the presynaptic nerves. Both resulted in increased levels of neurotransmitters, thus increasing their action on postsynaptic neurons. Understanding the way

monoamines were taken back up into nerve endings, would provide a clue on how to synthesize new compounds with higher selectivity [3].

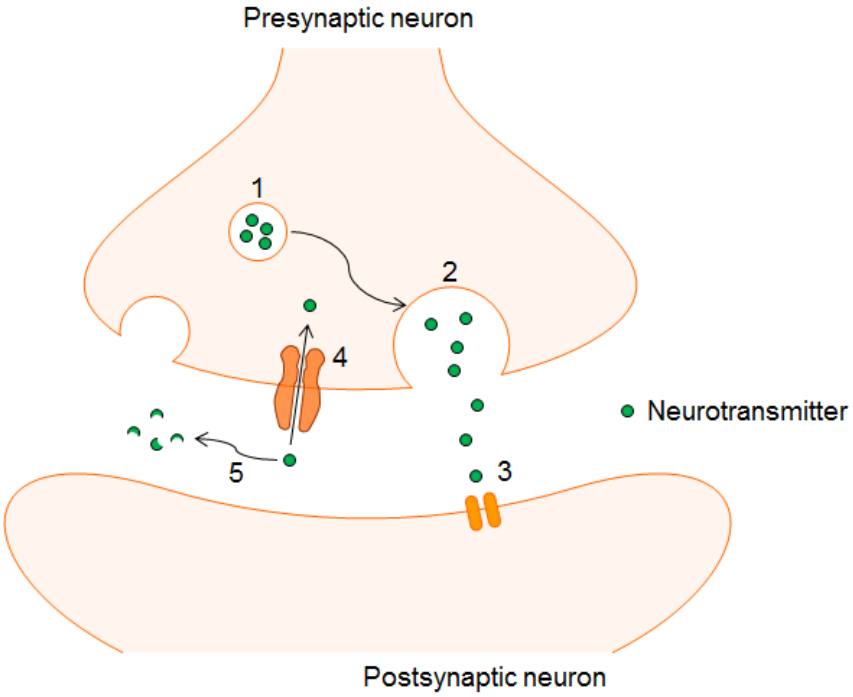


Figure 1.1. Mechanism of neurotransmission. (1) Neurotransmitters, such as noradrenaline, serotonin and dopamine are stored in vesicles in the presynaptic neuron. (2) Neurotransmitters are released into the synaptic cleft and (3) bind their specific receptors on the postsynaptic neuron. To terminate the signal, neurotransmitters are either taken back up into the presynaptic neuron (4), or degraded by monoamine oxidase (MAO)(5).

At about the same time, it became clear that serotonin, one of the newly discovered neurotransmitters, was involved in the pathology of depression. Inhibiting the reuptake of serotonin would thus be the target of choice for development of new antidepressants. A search began to synthesize compounds with high affinity for serotonergic neurons, but lower affinity for noradrenergic neurons. Zimelidine, a derivate of brompheniramine, was synthesized by Astra and was marketed in 1982 as the first SSRI [6]. Although clinical testing demonstrated equal effectiveness as compared to tricyclic antidepressants for the treatment of depression and a far more beneficial side effect profile [7], zimelidine was withdrawn from the market in 1983 because of a rare but serious side effect (Guillain-Barré syndrome, a condition involving degeneration of the peripheral neural system leading to paralysis) [6]. In 1971, a new method to study the uptake of different neurotransmitters, including serotonin, dopamine and noradrenaline by the nerves became available using synaptosomal preparations of rat brain homogenates [8]. By means of this method several

compounds could be screened on their relative capacity to inhibit uptake of different neurotransmitters. In 1972, Eli Lilly performed a screening of derivatives of diphenhydramine, an antihistaminic, and retained fluoxetine [8]. This compound was found to inhibit serotonin reuptake 200 times more potent than noradrenaline reuptake, and had no effect on acetylcholine nor histamine receptors. Although it would last until 1987 before fluoxetine, trademarked as Prozac, was approved by the FDA for treatment of depression, the most famous SSRI was born [3]. Soon, others would follow including paroxetine, sertraline, fluvoxamine and citalopram (figure 1.2). More recently, the active enantiomer of citalopram, escitalopram, was also marketed. Today, SSRIs are the most prescribed type of antidepressants worldwide. In 2012, Belgian physicians (both specialists and general practitioners) prescribed 165.59×10^6 defined daily doses (DDD) of SSRIs. The prescription of SSRIs (in DDD) accounted for 3.3% of all reimbursable drugs prescribed by Belgian physicians in the ambulatory practice. This places SSRIs at the 7^{th} place of most prescribed drugs in Belgium. Within the class of SSRIs, escitalopram is the most prescribed with 63.27×10^6 DDD. Paroxetine and sertraline follow with 34.21×10^6 and 33.13×10^6 DDD, respectively. Citalopram comes at the fourth place with 20.23×10^6 DDD. Fluoxetine and fluvoxamine close the list with 13.59×10^6 and 1.16×10^6 DDD, respectively [9].

1.2. Indications, adverse effects and interactions

Indications for SSRIs are broad and comprise major depression, panic disorder, obsessive-compulsive disorder and other less well-established indications such as obesity, eating disorders, post-traumatic stress disorder, social phobia and premenstrual disorder [6]. Within the group of SSRIs, all compounds appear to have equal efficacy in the treatment of depression. With respect to time to onset of action, limited evidence exists suggesting that fluoxetine, the SSRI with the longest half-life, takes slightly longer to reach its effect than do the other SSRIs [6].

In comparison to tricyclic antidepressants, SSRIs have a far more beneficial side effect profile. Frequent adverse effects of SSRIs include gastro-intestinal dysfunction (nausea, diarrhea) and central effects such as headache, dizziness, agitation, sedation and insomnia [2]. A third frequently reported side effect is sexual dysfunction [2]. Infrequent adverse effects include bleeding, serotonin syndrome and extrapyramidal symptoms such as tremor [10]. When side effects are compared between SSRIs, the majority of studies found no differences in overall adverse effects [6]. When however comparing specific side effects between two SSRIs, several studies did report differences.

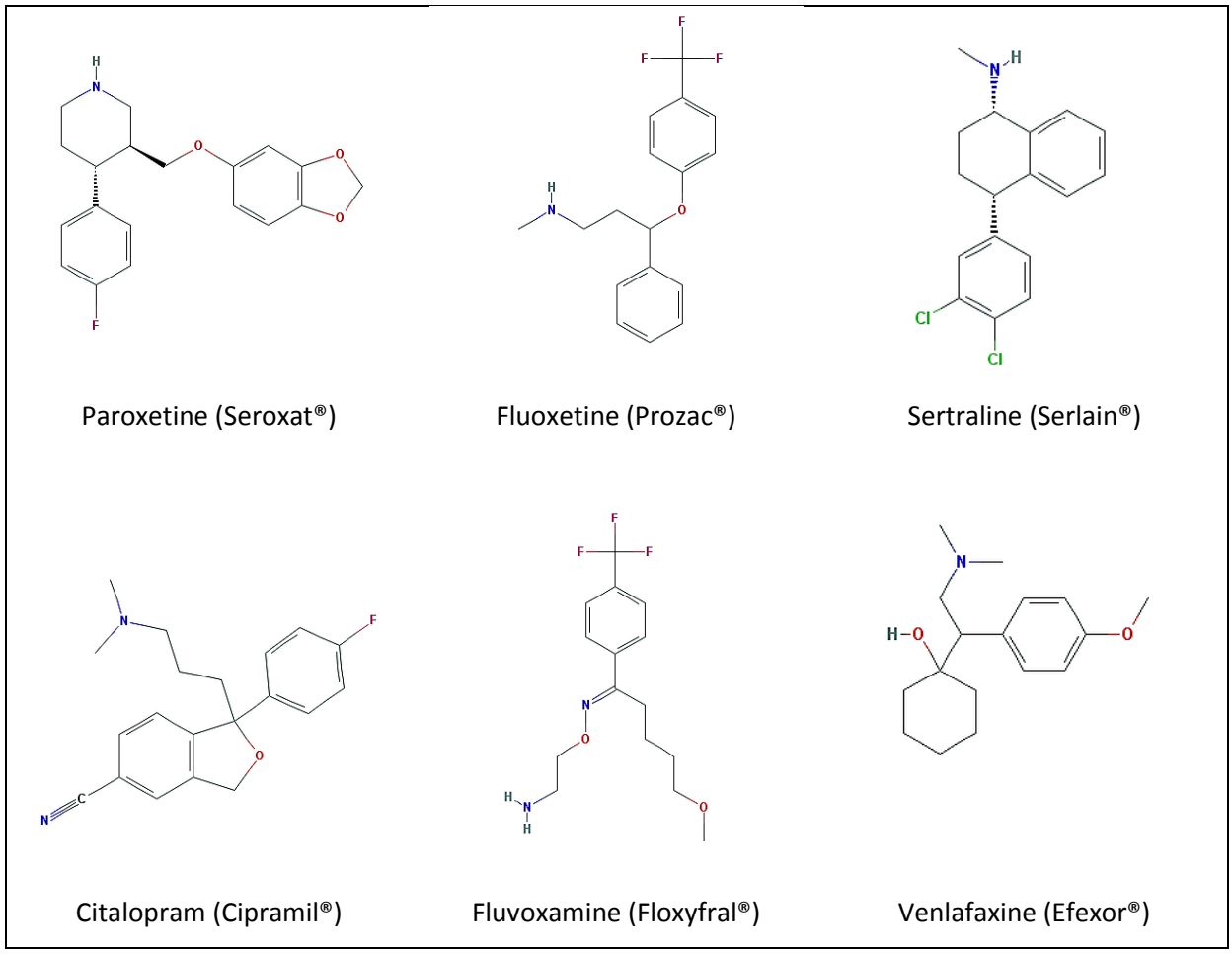


Figure 1.2. Chemical structures of the (S)SRIs investigated in this thesis. Whereas paroxetine, fluoxetine, sertraline, citalopram and fluvoxamine are SSRIs, venlafaxine is a serotonin and noradrenaline reuptake inhibitor. Belgian trade names are displayed between brackets. Structures obtained from http://pubchem.ncbi.nlm.nih.gov/.

From these studies it can be concluded that fluoxetine exerts more gastrointestinal effects than paroxetine or fluvoxamine, which in turn cause more nausea and diarrhea than citalopram [6]. With respect to central effects, fluoxetine was found to induce more agitation, insomnia and anxiety than sertraline [6]. Sexual dysfunction has been reported with all SSRIs and no clear evidence exists that one SSRI causes less sexual dysfunction than another [6]. Furthermore, SSRIs have been associated with an increased risk of suicide, especially in children and adolescents. However, suicide is also associated with depression and it is very difficult to distinguish between the possibility that a completed suicide was caused either by the SSRI or by the underlying disease it was prescribed for. From a comprehensive review of pediatric trials, it was concluded that the benefits of antidepressant treatment likely outweigh the risks in children and adolescents with depression or anxiety disorders

[11]. In addition to the adverse effects that are associated with the use of all SSRIs, some side effects are limited to one specific compound. For paroxetine, anticholinergic effects have been reported. Citalopram and escitalopram are associated with a lengthened QT interval (torsade de pointes) [10].

SSRIs have various inhibiting effects on CYP enzymes depending on the specific compound. Fluoxetine inhibits CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Fluvoxamine inhibits CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Citalopram, escitalopram, paroxetine and sertraline inhibit CYP2D6. Paroxetine is substrate to CYP2D6. Escitalopram is substrate to CYP2C19 [10]. Combination of SSRIs with a substrate of these enzymes can result in an interaction, although it is not always clear to what extent these interactions are clinically relevant. One important interaction can occur when SSRIs are combined with other serotonergic drugs such as dextromethorphan, narcotic analgesics (hydromorphone, pethidine, tramadol), certain antipsychotics, antidepressants (MAOi, tricyclic antidepressants, lithium, St-John's wort), triptans, ergot derivatives and linezolide. As these drugs all increase serotonin levels, the combination with SSRIs can lead to excessively high serotonin concentrations and result in a condition called 'serotonin syndrome'. This syndrome is characterized by hyperthermia, hyperreflexia, agitation and myoclonus [10]. In rare cases, convulsions and ventricular tachyarrhythmia occur, sometimes with fatal outcome. Serotonin syndrome rarely occurs after taking only a SSRI, except in overdose. It has been reported, however, when the wash-out period between a switch from one antidepressant to another was not sufficiently long.

Especially important in the context of this research project is the consideration of interactions of SSRIs with other drugs frequently used in patients receiving a hematopoietic stem cell transplantation (hSCT). Due to inhibition of CYP3A4, fluoxetine and fluvoxamine might increase plasma levels of cyclosporine, tacrolimus and sirolimus. Although no clinically significant pharmacokinetic interactions of SSRIs with immunosuppressive agents such as cyclosporine or tacrolimus have been reported thus far [12], it is recommended to monitor plasma levels of these immunosuppressive agents when combined with SSRIs and if necessary decrease their respective doses. No interactions are expected to occur between SSRIs and mycophenolate mofetil (MMF) [12, 13].

In addition to immunosuppressive therapy, hSCT patients commonly receive antifungal, antiviral and antibacterial agents. When combined with azole antifungals such as fluconazole, posaconazole or voriconazole, SSRIs might increase the QTc interval [13]. Close monitoring is therefore indispensable when this combination is administered. In addition, voriconazole is metabolized primarily by CYP2C19, and plasma levels might be increased by fluoxetine or fluvoxamine. QTc prolongation might

also occur when the antibiotic co-trimoxazol (sulfamethoxazole + trimethoprim) is combined with SSRIs [13]. In addition, inhibition of CYP2C9 by fluoxetine or fluvoxamine might result in increased plasma levels of sulfamethoxazole. No known interactions exist between SSRIs and the antifungal caspofungin, the antiviral agent acyclovir, and the commonly used antibiotics piperacillin + tazobactam, meropenem, or vancomycin [13]. Other supportive care commonly administered to hSCT patients includes ranitidine, a proton pump inhibitor. The combination of SSRIs with ranitidine is not expected to result in any interactions [13].

1.3. Pharmacokinetics

In table 1.1. an overview is given of pharmacokinetic properties of the five clinically available SSRIs and one serotonin noradrenaline reuptake inhibitor (SNRI). The SNRI, venlafaxine, has been added because it will also be investigated in this thesis (see also figure 1.2). For none of the SSRIs, a relationship between plasma concentration and clinical effectiveness has been established [6]. As SSRIs are lipophilic molecules, extensive accumulation in tissue occurs and brain-to-plasma ratios in patients range from 2,6:1 for fluoxetine to 24:1 for fluvoxamine [14]. In addition, antidepressants have been demonstrated to accumulate in liver, kidney and spleen [15]. The lipophilic nature of SSRIs is also visible in the large apparent distribution volumes. As some of the SSRIs display non-linear kinetics, a small change in the administered dose does not necessarily relate to a proportional change in plasma concentration. This is particularly true for paroxetine, fluoxetine and fluvoxamine. However, as the therapeutic-toxic window for SSRIs is relatively large, this does not usually pose any problems with respect to toxicity.

Table 1.1. **Pharmacokinetic properties of five SSRIs and one SNRI** [16].

(S)SRI	MW (g/mol)	Daily dose (mg)	$T_{1/2}$ (h)	Vd (L/kg)	Linear kinetics	Cp (µM)	Cp (ng/ml)
Paroxetine	329	20-50	20	3-12	No	0,03-0,20	10-75
Fluoxetine	309	20-80	96-144	20-42	No	0,43-1,45	150-500
Sertraline	306	50-150	26	20	Yes	0,15-0,73	50-250
Fluvoxamine	318	50-300	8-28	25	No	0,12-0,58	50-250
Citalopram	324	10-60	25-40	12-16	Yes	0,05-0,49	20-200
Venlafaxine (SNRI)	277	75-150	4	4-12	Yes	0,13-0,64	40-200

MW = molecular weight; $T_{1/2}$ = half-life; Vd = distribution volume; Cp = plasma concentration.

1.4. Mechanism of action

The three main classes of molecules which are used to treat depression (MAOi, TCAs, SSRIs) have different mechanisms of action. All of them increase neurotransmitter levels, but do so in a different way. MAOi inhibit the breakdown of monoamine neurotransmitters such as noradrenaline, serotonin and dopamine through inhibition of the enzyme monoamine oxidase (MAO). Antidepressant properties of TCAs are based on the inhibition of the reuptake of dopamine, noradrenaline and serotonin [17]. SSRIs were designed to selectively inhibit the reuptake of serotonin into the presynaptic neuron, thereby increasing the availability of serotonin for interaction with post-synaptic receptors (figure 1.1). In comparison to SSRIs, TCAs have a higher affinity for noradrenaline and dopamine transporters and also show affinity for acetylcholine and histamine receptors.

1.4.1. Serotonin (5HT)

A key factor in the mechanism of action of SSRIs is serotonin. Serotonin or 5-hydroxytryptamine (5HT) is synthesized from the essential amino acid tryptophan through hydroxylation and decarboxylation (figure 1.3). After synthesis, 5HT is stored in vesicles in presynaptic neurons and released into the synapse when the neuron is stimulated. Once its mission to trigger postsynaptic receptors has been accomplished, 5HT is either catabolized through MAO or taken up into the presynaptic nerve for reuse.

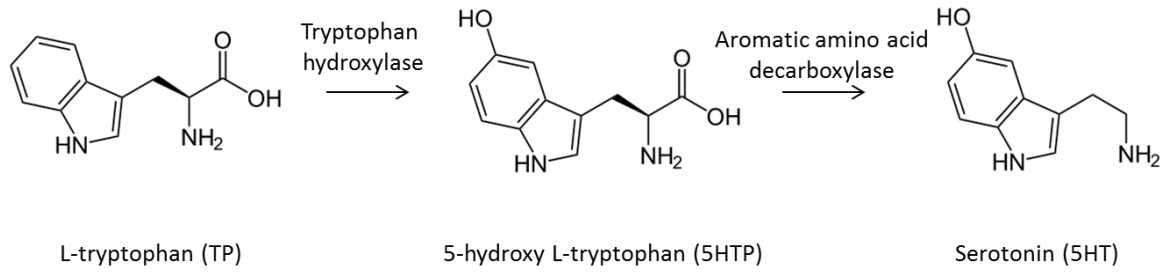


Figure 1.3. Synthesis of serotonin from tryptophan. L-tryptophan is hydroxylated into 5-hydroxy L-tryptophan by tryptophan hydroxylase. Subsequent decarboxylation by aromatic amino acid decarboxylase results in the formation of serotonin.

Within the brain, serotonin is almost exclusively produced in neurons originating in the raphe nuclei located in the midline of the brainstem. From these neurons, different regions of the brain are innervated forming a complex efferent system. Within the central nervous system (CNS) 5HT regulates behavioral effects such as mood, perception, reward, anger, aggression, appetite, memory,

sexuality and attention [18]. In addition, 5HT controls other centrally regulated processes such as motor control, sleep and circadian rhythms, emesis, respiratory drive and body temperature (figure 1.4).

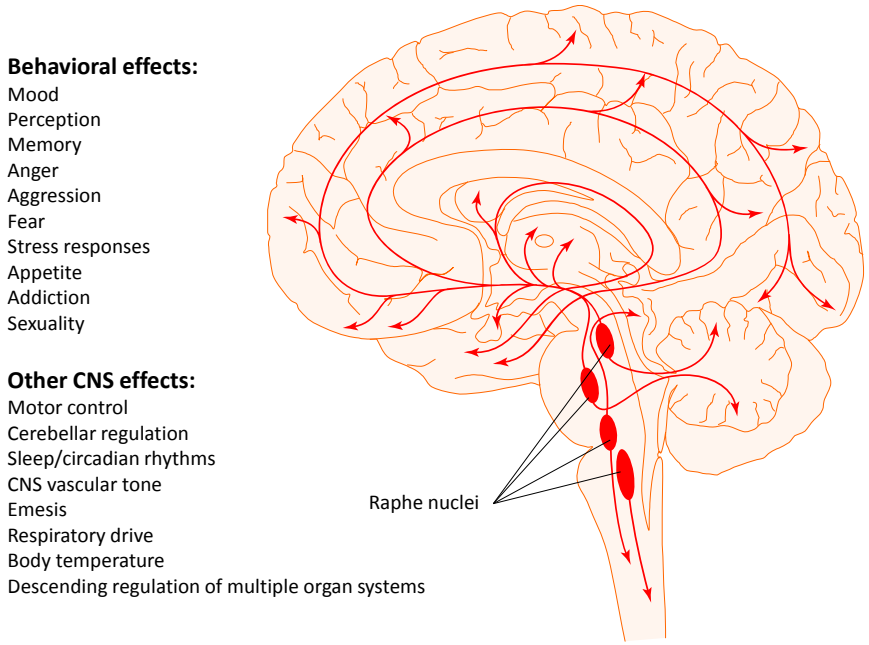


Figure 1.4. Central serotonergic pathways and effects. Within the brain, serotonin is almost exclusively produced in neurons originating in the raphe nuclei located in the midline of the brainstem. From these neurons, different regions of the brain are innervated forming a complex efferent system. Accordingly, serotonergic neurons control a wide variety of behavioral and neuropsychological functions, as well as many other CNS effects. Figure adapted from [18].

Although 5HT is mainly known for its role as a neurotransmitter in the brain, it is predominantly found in the periphery (>90%) where it is mainly released by enterochromaffin cells of the gut. In the gastro-intestinal tract, 5HT is involved in control of digestion by regulating both motility and secretion of digestive fluids [18]. In addition, 5HT is involved in vascular biology[1] through regulation of vasodilatation and vasoconstriction, depending on which receptors are present in the vessel wall and surrounding smooth muscles. Platelets take up 5HT from the plasma and secrete it during platelet activation, resulting in enhanced aggregation and local vasoconstriction and facilitating hemostasis [18]. Furthermore, 5HT plays a role in cardiac function, breathing and respiratory drive, endocrine system and metabolism, pain control, genitourinary function and reproduction [18]. As

[1] hence the name sero-tonin, which denoted the ability of a serum-derived factor to increase the tone of vascular smooth muscle [17].

demonstrated by these examples, 5HT exerts a wide variety of effects and plays a role in almost every function of the body. This also clarifies why SSRIs not only influence mood, but exert several other effects including nausea and sexual dysfunction.

1.4.2. The serotonin transporter

As described in 1.1., SSRIs were designed to block reuptake of serotonin in the presynaptic neuron. The protein responsible for (re)uptake of serotonin into a cell is the serotonin transporter (SERT or 5HTT). This is a 630 amino acid long protein composed of twelve membrane-spanning segments, with both N- and C-termini embedded in the cytosol. SERT contains two sites for N-glycosylation and multiple sites for phosphorylation, enabling post-translational regulation of its function (figure 1.5) [19].

The transporter is a member of the solute carrier family 6, which also includes transporters for e.g. dopamine, noradrenaline, and several amino acids. SERT is encoded by the solute carrier family 6 member 4 (SLC6A4) gene, which is localized on human chromosome 17. A 44-bp insertion or deletion polymorphism within the promotor region of the SLC6A4 gene has been described. The short allele of this polymorphism, the 5HTT-linked polymorphic region (HTTLPR), is associated with a reduced transcriptional activity of the SLC6A4 gene, resulting in decreased SERT expression and 5HT uptake [20].

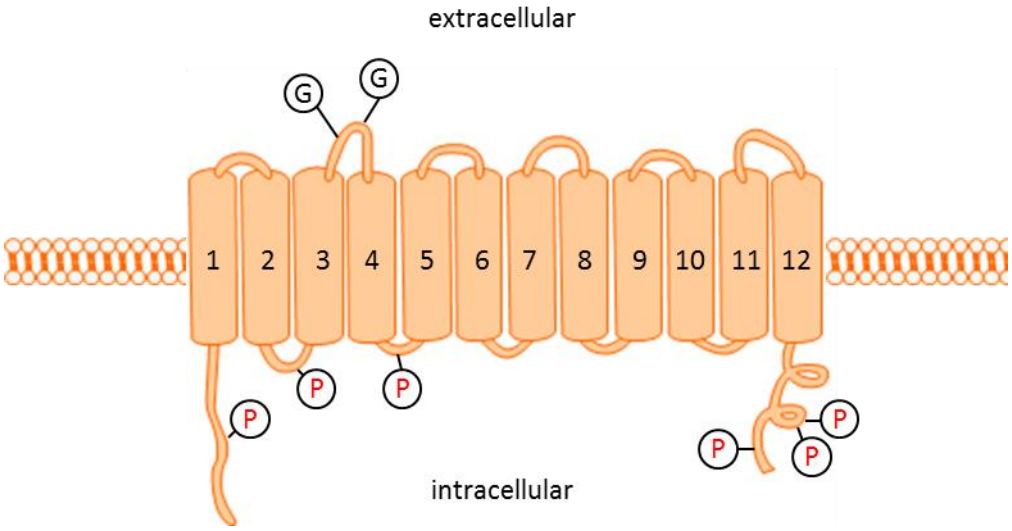


Figure 1.5. Structure of the serotonin transporter. The serotonin transporter is a twelve-membrane domain containing protein with cytoplasmic tails on both N- and C-termini. N-glycosylation sites are depicted with 'G', phosphorylation sites with 'P'.

The driving force for transport of serotonin into the cell is the energetically favorable co-transport of Na^+ and Cl^- ions. In the first step, a Na^+ ion binds the SERT, followed by the protonated form of 5HT ($5HT^+$). Then, a Cl^- ion binds and the complex of $5HT^+$, Na^+ and Cl^- induces a conformational change in the protein. From an outward open conformation, SERT changes to an inward open conformation thereby enabling release of $5HT^+$, Na^+ and Cl^- to the cytosol. Subsequently, a K^+ ion is bound and the transporter flips back to the original conformation, releasing the K^+ ion into the extracellular milieu (figure 1.6) [19].

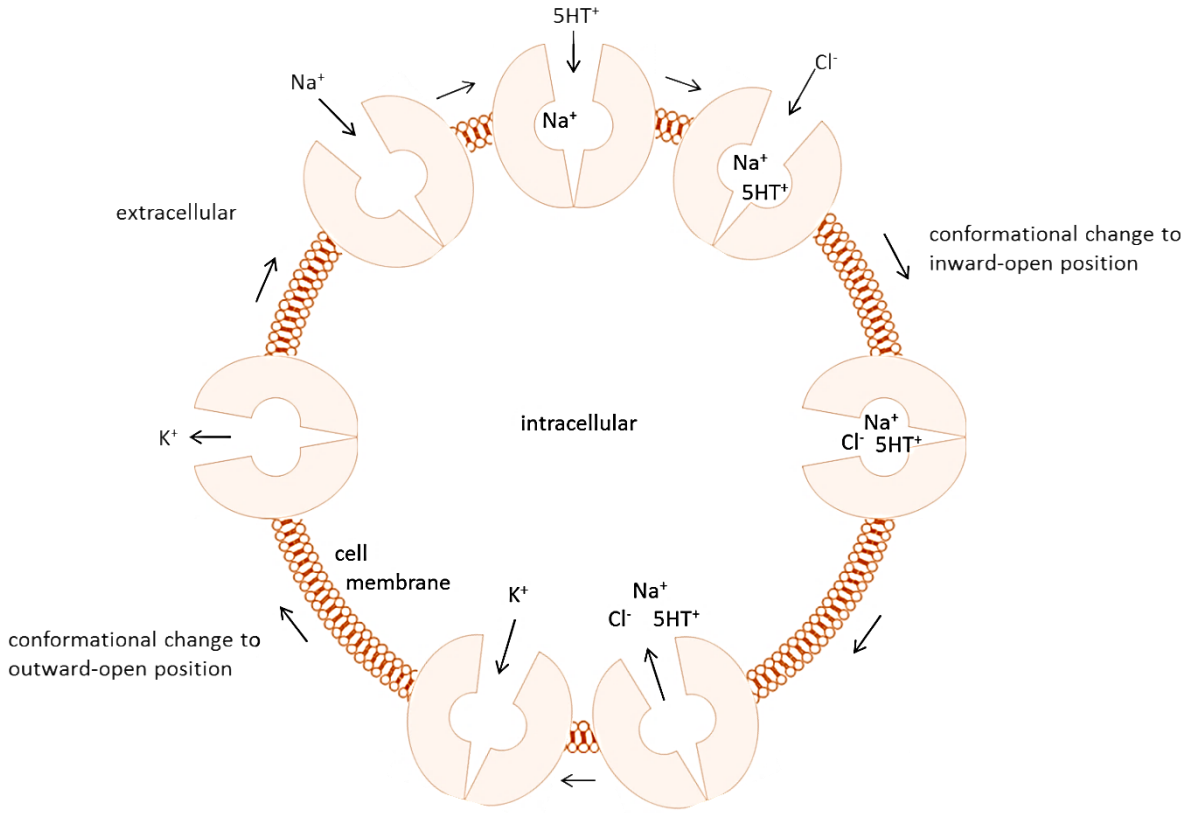


Figure 1.6. Schematic representation of 5HT transport through SERT. 5HT is transported into the cell simultaneously with Na^+ and Cl^-. A conformational change upon binding of all elements enables the release of 5HT, Na^+ and Cl^- into the cytosol. Subsequently, a K^+ ion is bound and the transporter flips back into the outward open position, thereby releasing the K^+ ion into the extracellular space.

1.4.3. Binding of SSRIs to SERT

As mentioned above, SSRIs block reuptake of serotonin in the presynaptic nerves, resulting in increased levels of serotonin in the synapse and enhanced serotonergic signaling. The binding characteristics of SSRIs to SERT have been subject of extensive research. Recently, a bacterial homologue of the eukaryotic monoamine transporter was mutated to produce a model that

approximates SERT pharmacology (Δ13 LeuBAT) [21]. According to this model, SSRIs bind to the primary binding pocket, which is also the binding site for serotonin [21]. SSRIs with different chemical structures (sertraline, paroxetine, fluoxetine and fluvoxamine) bind to SERT in a surprisingly similar way.

They all bind to the transmembrane helix 3 (TM3), and form a wedge between TM3/T8 and TM1/TM6, locking the transporter in the sodium and chloride-bound outward-open conformation (figure 1.7)[21]. Binding affinities (Kd) for human SERT range from 0,13 ± 0,01 nM for paroxetine to 2,2 ± 0,2 nM for fluvoxamine [22].

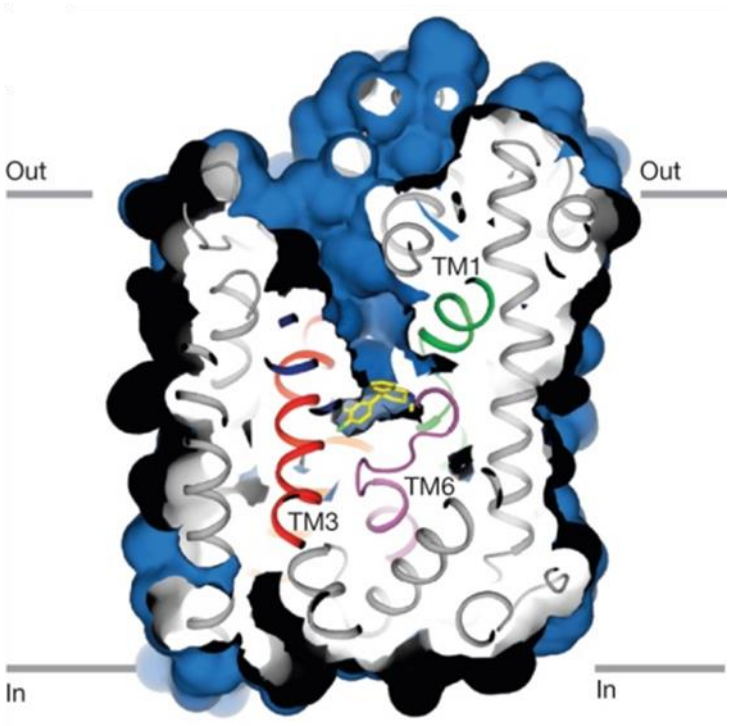


Figure 1.7. Binding of SSRIs to SERT. Cross-section of the crystal structure of the Δ13 LeuBAT-sertraline complex. Sertraline (shown in yellow sticks) binds to the primary binding pocket of the transporter, thereby locking it in the outward-open conformation. Figure obtained from [19].

However, in the case of citalopram it should be noted that both enantiomers act in a very different way. Whereas S-citalopram binds to SERT in a similar way as do the other SSRIs, R-citalopram is thought to alter the primary binding site trough allosteric modulation, which results in a decreased binding efficiency of S-citalopram [23]. This is the main reason why the purified S-enantiomer escitalopram was marketed (Belgian tradename Sipralexa®).

Whereas the blocking of 5HT transport through SERT occurs immediately after binding of a SSRI, the therapeutic effects of SSRIs usually take 2-3 weeks to fully develop. This discrepancy is explained by additional, adaptive mechanisms induced by SSRI-binding to SERT. Initially, SSRI-binding to SERT inhibits removal of 5HT from the synapse, thus increasing the available concentration of 5HT. However, 5HT not only binds post-synaptic receptors triggering signal transduction, but also pre-synaptic receptors of the 5HT1A-type. Stimulation of this receptor activates a negative feedback mechanism that results in a decreased release of 5HT from the presynaptic nerve. As a consequence, the 5HT concentration in the synapse returns to normal. Upon prolonged exposure to SSRIs, presynaptic 5HT1A receptors desensitize and internalize whereby the negative feedback mechanism is cancelled. Presynaptic neurons start to release 5HT and the synaptic concentrations are augmented again [24]. In addition, prolonged treatment with SSRIs has been shown to cause downregulation of SERT [25]. This downregulation is controlled on the posttranscriptional level, as SERT mRNA expression is not altered [26]. Thus, SERT function is not only abrogated by pharmacological inhibition, but the number of SERT molecules is also decreased resulting in a further impairment of serotonin uptake into the presynaptic neuron.

2. Immunology

The human immune system is divided into two parts, determined by the speed and selectivity of the reaction. These parts are the innate and adaptive branches of immunity. Whereas innate immunity delivers a fast, but non-selective immune response with no generation of memory, adaptive immunity generates a slower, but highly specific response that results in the generation of immunological memory. With respect to cellular distribution, innate immunity is carried out by neutrophils, monocytes, and macrophages whereas effector cells from adaptive immune responses are T and B lymphocytes [27]. This thesis focuses on the effect of SSRIs on T lymphocytes. Therefore in the next section an overview of T cells, their working mechanism and the effect of serotonin on T cells is given.

2.1. T lymphocytes

Lymphocytes are differentiated in the bone marrow out of lymphoid progenitor cells. In the case of T lymphocytes the maturation takes place in the thymus, where the T cell undergoes positive and negative selection generating mature, naive T lymphocytes that carry a T cell receptor (TCR) capable of recognizing non-self peptides presented on self major histocompatibility complex (MHC) molecules. After successful completion of the selection process, mature naive T cells leave the thymus and enter the periphery, where they circulate from one lymphoid tissue to the next through

lymph and blood [27]. The encounter between a naive T cell and its specific antigen occurs in peripheral lymphoid organs and is mediated by antigen presenting cells (APC). T helper cells, which are CD4+, recognize peptides presented on MHC-II by an APC. Once activated, they differentiate either to Th1 cells that aid macrophages to kill intracellular organisms or Th2 cells that facilitate B cell activation and antibody production. Communication between helper T cells and macrophages or B lymphocytes is accomplished by cytokine secretion. Th1 cells produce interleukin 2 (IL2), which induces T cell proliferation and interferon γ, which activates macrophages. Th2 cells produce IL4, IL5, IL6 and IL10 that stimulate B cells to produce antibodies. IL4 also induces class switching to IgE in B cells, and IL5 enhances eosinophil growth [27]. Other types of CD4+ cells include Th17 cells and regulatory T cells. Th17 cells secrete IL17, which recruits inflammatory cells such as neutrophils to sites of infection. Regulatory T cells suppress T cell responses. They have an important function in preventing autoimmunity and restrain the immune response in order to protect surrounding tissue from damage [28]. Cytotoxic T cells, CD8+, recognize peptides presented on MHC-I, which is present on all nucleated cells of the body. MHC-I typically presents peptides derived from intracellular viruses or tumor-specific proteins. If a cytotoxic T cell becomes activated, it inserts perforins in the target cells' plasma membrane and releases granzymes into the cytoplasm, resulting in the killing of that target cell (figure 1.8)[27].

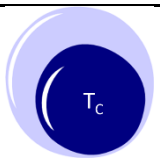
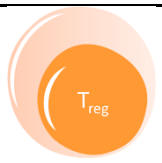
Type of effector T cell	**CD8+ Tc cells**	**CD4+ Th1 cells**	**CD4+ Th2 cells**	**CD4+ Th17 cells**	**CD4+ Treg cells**
					
Main function	kill virus-infected and tumor cells	activate infected macrophages	stimulate B cell antibody production	enhance neutrophil response	suppress T cell response
MHC interaction	class I	class II	class II	class II	class II
Effector molecules	perforins, granzymes	IL2, IFNγ	IL4, IL5, IL6, IL10	IL17, IL6	IL10, TGFβ

Figure 1.8. Different types of effector T cells and their function. Cellular immunity against intracellular pathogens is mainly regulated by T cells. CD8+ cytotoxic T cells kill virus-infected cells and tumor cells through release of perforins and granzymes. Macrophages containing intracellular pathogens are activated by CD4+ Th1 cells. CD4+ Th2 cells stimulate B cell antibody production and induce class switching. CD4+ Th17 cells recruit neutrophils to sites of infection through release of IL17. Finally, CD4+ Treg cells control T cell responses in order to prevent autoimmunity and protect surrounding tissue. Figure adapted from [28].

2.2. T cell activation

In order to induce the process of T cell activation, a naive T cell needs to obtain several signals: the first signal is the binding of the TCR with a peptide presented on a MHC-molecule by an APC. The TCR is formed by an α and β chain (or γ and δ in case of γδ T cells), associated with the CD3 complex that enables signal transduction (figure 1.9). CD4 and CD8 co-receptors bind to the MHC-molecule as well, but without binding to the antigen. The second signal is provided by CD80 (B7-1), CD86 (B7-2) and CD40 on the APC that bind with CD28, CTLA4 and CD40 ligand on the T cell [27]. Without the co-stimulatory signal, TCR triggering results in a non-responsive state (anergy) in which T cells do not longer respond to restimulation [29]. The encounter between a naive T cell and an APC takes place in lymphoid tissue, after an APC has endocytosed an antigen locally and brought it to the nearest lymph node. Naive T cells travel through the lymphatic system continuously (around the body in 1-2 days), so that an antigen-bearing APC is quickly detected by the right T cell. Once the T cell has found its antigen, activation occurs within 2-3 days [27].

So how does T cell activation work? If an antigen-MHC complex is bound by the TCR, aggregation of the receptor with tyrosine kinases like LCK and FYN takes place leading to phosphorylation of ITAM motifs in the cytoplasmic tail of the CD3 complex. Once phosphorylated, ZAP70 is recruited that in turn phosphorylates linker for activation of T cells (LAT) and SRC-homology 2 (SH2)-domain-containing leukocyte protein of 76 kD (SLP76). Phosphorylated LAT then recruits SLP76 to the membrane, and activates phospholipase Cγ (PLCγ). This enzyme converts phosphatidyl-inositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Whereas DAG activates protein kinase C and ras-dependent pathways resulting in the activation of gene transcription mediated by the transcription factors NFκB and AP-1, IP_3 migrates to the endoplasmic reticulum (ER) where it binds the IP_3 receptor (IP_3R) [28]. This ion channel is subsequently opened and ER Ca^{2+} stores are released into the cytoplasm. The release of Ca^{2+} from intracellular stores is further regulated by the ryanodine receptor (RyR)[30]. The initial release of ER Ca^{2+} into the cytoplasm in turn causes influx of extracellular Ca^{2+}. STIM1 molecules present in the ER wall sense the decrease in ER Ca^{2+} content and make contact with ORAI in the plasma membrane [31]. In turn, store-operated Ca^{2+} channels (Ca^{2+}-release activated Ca^{2+}-channel (CRAC)) are opened and extracellular Ca^{2+} enters the cell, thereby causing a sustained increase in cytosolic Ca^{2+} concentration [32]. Ca^{2+} influx is further regulated by voltage-gated K^+ channels, such as the Kv1.3 channel and Ca^{2+}-activated K^+ channels such as $K_{Ca}3.1$ [32]. The cytosolic Ca^{2+} binds calmodulin, which then activates calcineurin. Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), that subsequently migrates to the nucleus and stimulates transcription of a pleiotropic set of genes,

including IL2. As mentioned above, IL2 promotes long term T cell proliferation. The entire signaling pathway is illustrated in figure 1.9.

Once activated, T cells differentiate in either helper T cells or cytotoxic T cells. They start to proliferate, thereby producing large amounts of cells with the same antigen-specificity. Depending on the type of effector cell, different cytokines are secreted. Part of these cells will become long-lasting memory T cells, providing a strong and efficient mounting of an immune response in case of a second challenge with the same antigen.

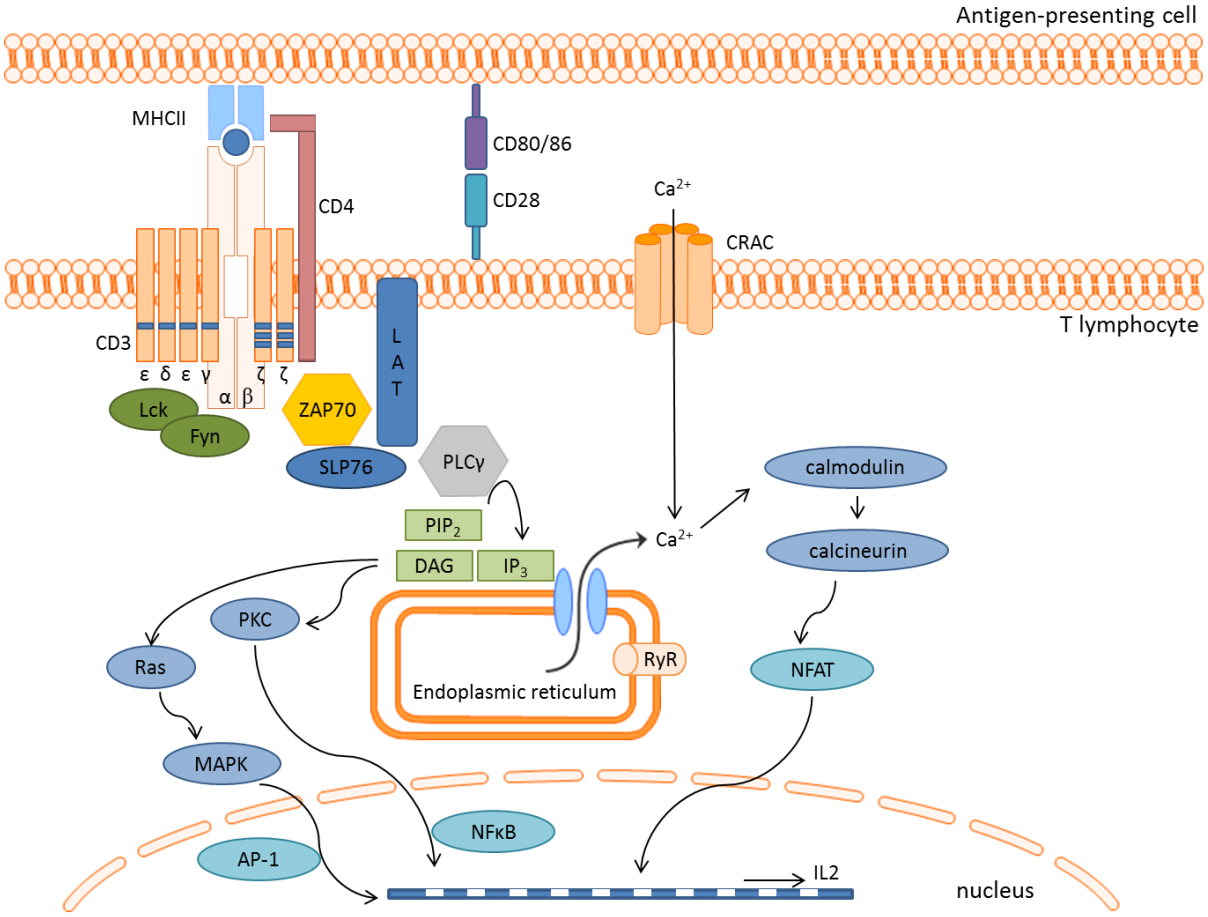


Figure 1.9. Signaling pathways involved in T cell activation. Interaction of the TCR with a peptide-MHC on an APC induces a complex signal transduction cascade eventually leading to activation, proliferation and differentiation of the T cell. Upon binding to the peptide-MHC complex, ITAM motifs in the CD3 tails are phosphorylated by PTKs LCK and FYN. ZAP70 is recruited and activates LAT and SLP76, that in turn activate PLCγ. PLCγ converts PIP_2 into DAG and IP_3. Whereas DAG activates PKC and Ras-dependent pathways leading to gene transcription mediated by NFκB and AP-1, IP_3 induces release of intracellular Ca^{2+} from the endoplasmic reticulum. This in turn causes influx of extracellular Ca^{2+} that binds to calmodulin and activates calcineurin. Calcineurin then dephosphorylates NFAT, a transcription factor that relocates to the nucleus and induces transcription of a pleiotropic set of genes, including IL2. Release of intracellular calcium is further regulated by RyR. Efficient activation only occurs when a costimulatory signal is present, provided by interaction of CD80/86 with CD28.

2.3. T cell apoptosis

In some situations, e.g. when the activation process is not optimal (co-stimulatory signal is absent) or when a T cell fails the negative or positive selection process in the thymus, T lymphocytes commit 'suicide', in a tightly controlled process called apoptosis. Equally, apoptosis has an important role in terminating an immune response by eliminating cells that are no longer needed after the infection has been cleared [28]. The apoptotic process can be induced either by the extrinsic or intrinsic signaling transduction pathway. The extrinsic pathway starts with the binding of a death factor (TNF, FasL) to its receptor on the plasma membrane. Activation of the receptor results in the formation of a death-inducing signaling complex (DISC), composed of the death receptor, FADD or TRADD and pro-caspase-8 that induces autocleavage of procaspase-8 or -10. These caspases, called initiator caspases, cleave caspase-3, -6 and -7, thereby activating them. The latter are also called effector caspases, and these carry out apoptosis through cleavage of intracellular proteins (figure 1.10, left). In addition, caspase-3 activates the caspase activated DNase (CAD) that degrades chromosomal DNA and induces chromatin condensation. Reorganization of the cytoskeleton occurs, and apoptotic bodies are formed [33]. As early apoptosis is accompanied by the flipping of phosphatidyl serine (PS) from the inside to the outside of the plasma membrane, apoptosis can be detected through binding of this exposed phospholipid with annexin V.

The intrinsic or mitochondrial pathway is initiated when a pore in the outer mitochondrial membrane causes cytochrome C (cyt C) to be released into the cytosol. This can be induced by e.g. ultraviolet irradiation, chemotherapeutic drugs, starvation or a lack of growth factors needed for survival [28]. Recent evidence indicates that the initial release of a small amount of cyt C by mitochondria activates IP_3R in the ER, thereby inducing the release of Ca^{2+} from ER stores. The rise in cytoplasmic Ca^{2+} in turn causes an orchestrated release of cyt C from all mitochondria [34]. Cyt C assembles, together with APAF-1 and pro-caspase-9, the apoptosome. Pro-caspase-9, an initiator caspase, is activated and in turn activates effector caspases [33] (figure 1.10, right).

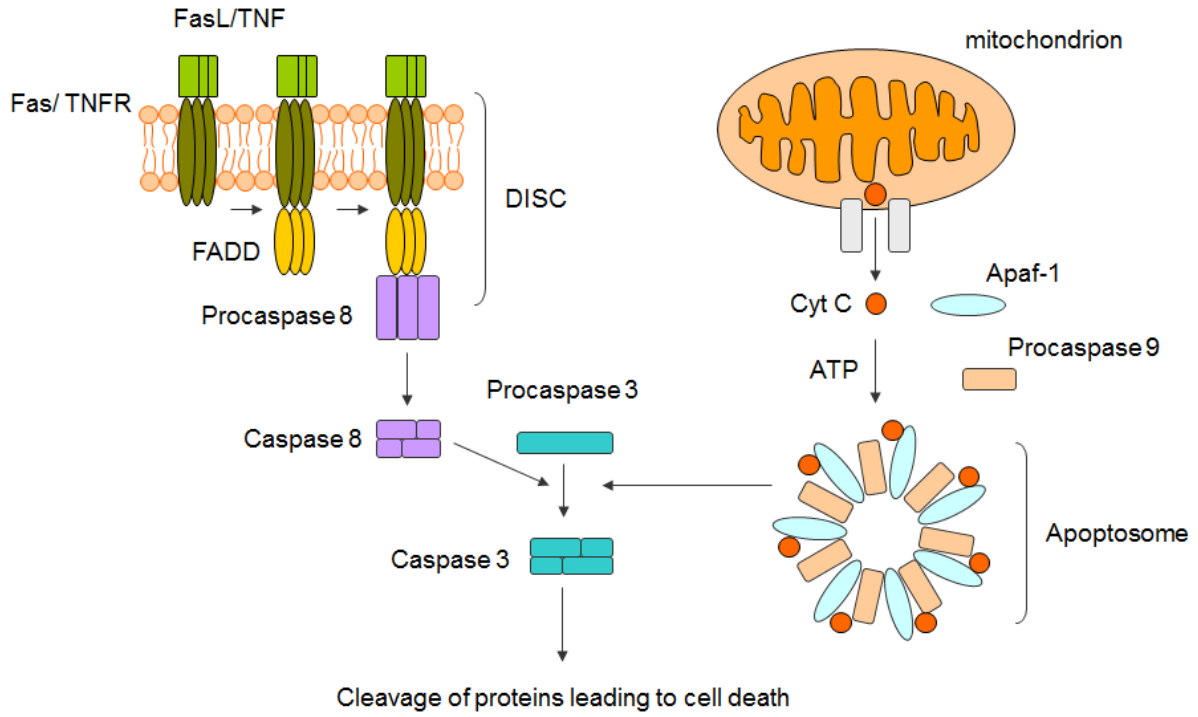


Figure 1.10. Pathways leading to T cell apoptosis. (Left) The extrinsic pathway starts with binding of a death factor (TNF, Fas) on its plasma membrane receptor. A death-inducing signaling complex (DISC) is formed that induces cleavage of pro-caspase-8 into active caspase-8. This in turn activates caspase-3. (Right) The intrinsic pathway starts with leakage of Cyt C into the cytoplasm. An apoptosome is formed through association of Cyt C with APAF-1 and pro-caspase-9. Finally, caspase-3 is activated and cleavage of intracellular proteins occurs.

2.4. Role of serotonin in immunity

Although cytokines are the best known molecules used by immune cells to communicate with each other, they are not the only messengers within the immune system. Small molecules such as neurotransmitters are used both centrally and in the periphery to convey a message between immune cells reciprocally and in their communication with other tissue cell types. As this thesis focuses on the effect of SSRIs on the immune system, the following overview is limited to the role of serotonin in immunity. The role of other neurotransmitters in the immune system is beyond the scope of this thesis and will not be discussed here.

As mentioned above, 5HT plays a role in almost every function of the body and the immune system is no exception. Enterochromaffin cells from the gut form the main source of 5HT in the periphery. Amongst other cell types, platelets take up 5HT and transport it through the blood stream, thereby providing a major source of 5HT in the circulation. 5HT is released in response to platelet activation by e.g. thrombin, IgE-containing immune complexes, platelet activating factor or certain complement factors [35]. Consequently, local 5HT concentrations can rise considerably in comparison with the

relatively low levels of free 5HT found in plasma. In addition, both primary and secondary lymphoid organs are innervated with nerves which release a variety of neurotransmitters, including 5HT. Lymphocytes can thus be exposed to 5HT both in the circulation and lymphoid organs. Furthermore, lymphocytes – especially when activated – can pass the blood brain barrier, thus exposure of lymphocytes to 5HT can occur both in the periphery and the central nervous system [35].

5HT receptors and SERT have been found in various types of immune cells. In human T cells, 5HT1, 5HT1A, 5HT1B, 5HT2A, 5HT3, and 5HT7 are expressed [36]. The expression of 5HT receptors is dependent on the subtype and appears very dynamic, changing in response to various stimuli. Some important factors that alter the expression of 5HT receptors in T lymphocytes are TCR activation, the cytokine environment and the neurotransmitter itself [36]. In naive murine splenic T cells, mRNA for both 5HT1B and 5HT7 was detected [37]. On the protein level however, only 5HT7 could be reliably detected [37]. A functional role for 5HT7 was confirmed using a 5HT7 receptor antagonist, which inhibited 5HT induced ERK activation and phosphorylation of IκBα [37]. Upon T cell activation, 5HT7 receptors, as well as 5HT1B and 5HT2A are upregulated [37]. Yin et al. demonstrated that antagonism of 5HT1B receptors in activated CD4+ T helper cells results in impaired proliferation [38]. Further, antagonists of 5HT2A receptors inhibit IL2 and IFNγ production in response to T cell receptor stimulation [39, 40]. Another study demonstrated 5HT to promote mitogen-activated T and B cell survival and proliferation via 5HT1A receptors [41]. Through activation of 5HT3 receptors, 5HT inhibits primary CD4+ T cell migration towards endothelial-bound CXCL12, thereby facilitating extravasation and migration of CD4+ T lymphocytes into inflamed tissue [42]. From these studies, it is clear that 5HT exerts a variety of effects through activation of several types of 5HT receptors.

In addition, tryptophan hydroxylase 1 (TPH1), the rate limiting enzyme in the synthesis of 5HT, is expressed in T lymphocytes and expression is upregulated during T cell activation [37, 43]. Thus, it appears that T cells are capable of synthesizing 5HT. Possibly, activated T cells secrete 5HT as a means of communication with other lymphocytes or even other cell types. Interestingly, 5HT has been shown to be involved in early T cell activation [37]. Dendritic cells (DC's), which have been shown to express SERT, are likely to take up 5HT at sites of inflammation and from activated T cells, and release it when encountering naive T cells. 5HT then acts as a cofactor that interacts synergistically with the TCR signaling to promote T cell activation and proliferation [37]. Furthermore, SERT expression has been shown in jurkat T lymphocytes and is upregulated in response to IFNα [44-46]. Thus, T lymphocytes also appear to be capable of taking up 5HT, and the uptake is regulated by inflammatory stimuli.

5HT not only plays a role in adaptive immunity, but has also been shown to exert several effects on innate immune cells. First, 5HT has been demonstrated to induce adhesion and chemotaxis in mast cells [40, 47]. A chemotactic role has also been described for 5HT in the recruitment of eosinophils [48]. Whereas in mast cells, 5HT1A receptors are believed to be responsible for the effect, eosinophil recruitment appears to be mediated through 5HT2A receptors. Furthermore, a chemotactic role for 5HT has been described in the attraction of neutrophils to inflammatory sites [49]. Activated platelets were shown to release 5HT, and this 5HT recruits neutrophils to sites of acute inflammation. In the absence of platelet 5HT, less neutrophil rolling on unstimulated endothelium was observed, and the neutrophils that did roll moved faster suggesting that less interaction with adhesion molecules occurred [49]. In LPS-stimulated endothelium, the absence of platelet 5HT resulted in less adhesion of leukocytes to the endothelium [49]. Finally, the migration, cytokine and chemokine secretion and T-cell priming capacity of dendritic cells is modulated by 5HT [50]. In accordance with data on mast cells and eosinophils, 5HT induced chemotaxis in immature DC's through interaction with 5HT1 and 5HT2 receptors [50]. In LPS-stimulated DC's, 5HT stimulated the production of CCL22 and IL6 and inhibited the production of CXCL10. As CCL22 is a typical chemoattractant for Th2 cells, 5HT appears to shift the immune response towards the Th2 response. This conclusion was further strengthened by the observation that 5HT-pretreated DC's induced a Th2 polarization in naive T cells [50].

In conclusion, 5HT influences several aspects of adaptive and innate immunity and is involved in the communication between both branches of immunity. In adaptive immunity, a stimulatory effect on T cell activation predominates, whereas in innate immunity the major role of 5HT is chemotaxis.

3. Immunological changes in depression

In order to be able to interpret immunological effects executed by SSRIs in depressive patients, understanding of the immunological changes at the basis of depression is required. For a long time, depression has been considered the consequence of a disturbed neurotransmission with changes in monoamine neurotransmitters such as noradrenaline, dopamine and serotonin lying at the basis of the disorder. However, more recent evidence suggests that monoamine deficiency is to be considered a secondary change owing to primary, more upstream abnormalities [17].

A hypothesis on the pathophysiology of depression receiving more and more attention is the role of glucocorticoids and cytokines. Hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis occurs in up to 80% of severely depressed patients [51]. The HPA axis is activated by environmental,

psychological and biological stressors and results in the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH stimulates receptors in the pituitary gland, that responds by release of corticotropin (ACTH) into the plasma. Once bound on its receptors in the adrenal cortex, ACTH induces secretion of cortisol, a glucocorticoid, into the blood [51]. Glucocorticoids exert their effects through binding on the glucocorticoid receptor, which is expressed by many different body tissues. A negative feedback mechanism involving glucocorticoid receptors in the hypothalamus ensures the maintenance of homeostasis. The main effects of glucocorticoids are the regulation of energy metabolism, anti-inflammatory action and adaption of behavior in stressful situations. In addition to different types of stress, biological stimuli can also induce the HPA axis. Pro-inflammatory cytokines such as IL1 and IL6 have been shown to stimulate the HPA axis, thereby increasing cortisol secretion into the blood. Cortisol in turn exerts an immunosuppressive effect on immune cells through binding on the glucocorticoid receptor, preventing further secretion of pro-inflammatory cytokines. Elevated levels of cortisol and CRH have repeatedly been found in the blood and cerebrospinal fluid of depressed patients [52]. Further, impairment of the negative feedback regulation of the HPA axis has been reported in depressed patients, as well as hypertrophy of the adrenal and pituitary glands [51].

Despite elevated levels of cortisol, two recent meta-analyses demonstrated that depression is associated with an elevation of pro-inflammatory cytokines such as CRP, IL6 and TNFα [53, 54]. Evidence for the consideration of depression being an inflammatory state comes from the observation that ‘sickness behavior’ associated with activation of an inflammatory response shares many symptoms with depression, including weakness, malaise, listlessness, inability to concentrate, lethargy, decreasing interest in the surroundings and reduced food intake [55]. Additionally, cytokine treatment (e.g. with IFNα) induces a depressive state in around 30% of patients [56]. Moreover, IFNα-induced depression is responsive to SSRI treatment [57, 58]. Finally, anti-TNFα therapy (etanercept) has been shown to exert antidepressive effects in psoriasis-associated depression, independent of improvement in psoriasis symptoms [59]. In treatment-resistant depression, anti-TNFα therapy (infliximab) improved depressive symptoms only in patients with high baseline TNFα levels [60]. Another clue to the link between inflammation and depression comes from the observation that there is a higher prevalence of depression in patients suffering from autoimmune disorders (e.g. rheumatoid arthritis) as compared to the general population [61].

Interestingly, pro-inflammatory cytokines such as IL6, TNFα and IFNγ have been demonstrated to induce indoleamine 2,3-dioxygenase (IDO) expression, which converts tryptophan into kynurenines

and thereby reduces available tryptophan for serotonin synthesis. Additionally, IDO has also been shown to directly metabolize serotonin [55]. Lowered tryptophan and serotonin availability, as well as the formation of neurotoxic tryptophan catabolites are thought to lie at the biological basis of depression. In addition, cytokines such as IL1 and TNFα have been shown to increase serotonin and noradrenaline transporter expression and activity through stimulation of p38 MAPK, which in combination with the increased catabolism of tryptophan results in lowered serotonin availability [62].

In conclusion, alterations in immune, endocrine and neurotransmitter systems are involved in the pathophysiology of depression. A considerable interplay between these three systems occurs, and abnormalities in any one of them can result in changes in both other systems. Accordingly, different causes might explain the occurrence of depression, including exposure to psychological stress or chronic low grade inflammation.

4. Immunological effects of SSRIs

Although antidepressant therapy has been developed for its restoring properties on neurotransmitter deficiency, immunomodulatory effects have also been described in patients with depression as well as healthy subjects. These immunomodulatory effects are described below in more detail. First, within the context of depression, SSRIs have been shown to alter cytokine plasma levels. Additionally, SSRIs have been shown to affect the *in vitro* proliferation and viability of lymphocytes from healthy human subjects. Whereas plasma concentrations in patients are typically below 1 µM, the majority of *in vitro* studies reports immunosuppressive effects at tenfold higher concentrations. Not only lymphocytes, but also cancer cells seem to undergo changes when they are incubated with SSRIs [63]. Finally, recent evidence showed an effect of fluoxetine on neutrophil adhesion and recruitment to inflammatory sites, demonstrating that not only cellular but also innate immunity is impacted by SSRIs [49].

4.1. Effect on cytokine levels and secretion

4.1.1. within depression

As described above, depression has been associated with an elevation of pro-inflammatory cytokines. Antidepressants, which have previously been shown to correct neurotransmitter levels in depression, also affect the altered cytokine levels in depression. Several antidepressants, including TCAs and SSRIs, have been shown to induce a shift from a Th1 type (pro-inflammatory) towards a Th2 type (anti-inflammatory) immune response [55, 64]. A reduced IFNγ/IL10 ratio was found in diluted

whole blood of both healthy subjects and fluoxetine-treated depressive patients [65, 66]. Cytokine measurements in serum or plasma of depressed patients before and after treatment with antidepressants have revealed *in vivo* suppression of cytokine secretion. From a comprehensive review, Miller et al. concluded that 11 out of 20 studies examining the impact of antidepressant treatment on the inflammatory response showed a decrease of inflammatory markers in serum/plasma [67]. Especially for IL6, multiple studies have demonstrated elevation in depressed patients compared to control, and reduction of IL6 serum levels in response to SSRI treatment [68, 69]. TNFα and CRP have also been demonstrated to decrease in response to SSRI treatment [70-72]. However, other studies have reported no changes or even increases in cytokine levels in response to antidepressant therapy [67]. Some of these studies reported an increase in body mass index (BMI) during antidepressant therapy, which itself has been shown to correlate with increases in inflammatory markers [67]. Thus, changes in BMI might complicate the establishment of a relationship between inflammation and antidepressant treatment [67]. Recently, an extensive study was performed analyzing the antidepressant effect on CRP, IL6 and TNFα in currently depressed or remitted patients taking lifestyle characteristics including BMI into account as covariate factors [73]. From this study, it was concluded that inflammation was present in depressed men, but not women. Different classes of antidepressants exerted different effects, with increased CRP and IL6 levels in response to SNRI treatment, increased CRP levels with tri- or tetracyclic antidepressant treatment and decreased IL6 in response to SSRI treatment [73].

In conclusion, these data suggest that antidepressants, especially SSRIs, reduce pro-inflammatory cytokine secretion in depression. The exact mechanism behind these alterations is currently unknown. However, it is intriguing to find that a condition which was previously thought to be solely the result of a disturbed neurotransmission now appears to be the result of a low grade, chronic inflammatory response. Even more surprising is the observation that pharmacological compounds designed for their capacity to correct a monoamine deficiency, now turn out to correct the inflammatory component of depression as well. At this moment, it is not clear to what extent the anti-inflammatory effects contribute to the therapeutic efficacy of SSRIs in depression. Do SSRIs suppress the inflammation through enhancement of serotonin neurotransmission? Or should the anti-inflammatory effect of SSRIs be considered a primary effect that contributes to the antidepressive effect? It is likely to assume that both effects are dependent upon each other, and further research will hopefully detangle the interplay between both major systems of the human body – and the effect SSRIs exert on them.

4.1.2. outside the context of depression

In addition to the effects of SSRIs on cytokine plasma levels in depression, these compounds have also been demonstrated to directly affect lymphocyte cytokine secretion from healthy subjects. For example, 20 μM citalopram decreased IL-2 and IFNγ secretion by mitogen-activated T cells [74]. Furthermore, paroxetine and sertraline (0-30 μM) have been demonstrated to reduce TNFα secretion by human anti-CD3 stimulated T lymphocytes [75]. Others showed that sertraline (0.01 and 1 μM) significantly decreases the IFNγ/IL-10 ratio in the supernatant of mitogen-stimulated whole blood [65, 66]. Although these studies point in the same direction, showing a suppressive effect of SSRIs on the production of pro-inflammatory cytokines, it should be noted that these studies are not equal in terms of experimental setup. Whereas the first two studies used purified lymphocytes, Maes et al. used whole blood assays [65]. In the latter model interactions between different types of blood cells are preserved, and this model is therefore believed to be more representative for the *in vivo* situation. Recently, Shenoy et al. demonstrated that not only peripheral blood lymphocytes but also thymocyte cytokine production is suppressed by citalopram [76]. Concentrations ranging from 25 to 250 μM citalopram completely suppressed anti-CD3 triggered IL2 production, severely reduced IL4 and partially suppressed IL17 production [76]. Overall, SSRIs appear capable of suppressing cytokine secretion in a concentration-dependent manner.

4.2. Anti-proliferative effect

Not only have SSRIs been shown to exert an anti-inflammatory effect under the form of cytokine suppression, they also directly interfere with lymphocyte proliferation. In the last decades, several research groups have demonstrated that micromolar concentrations of SSRIs are capable of altering lymphocyte proliferation. *In vitro* exposure to paroxetine, sertraline and fluoxetine has been shown to decrease the proliferation of mitogen-stimulated lymphocytes in a concentration-dependent manner [75, 77-81]. An anti-proliferative effect has also been observed in Jurkat T cells [82]. Pellegrino *et al.* found that *in vivo* administration of fluoxetine to rats similarly decreased lymphocyte proliferation when induced by mitogens *ex vivo* [83, 84]. The effect, however, seems to be dependent on the activation status of the cells. At suboptimal mitogenic Concanavalin A (ConA) concentrations, relatively low concentrations (0.1 – 1 μM) of fluoxetine have been found to stimulate T cell proliferation [80, 85]. In contrast, at optimal ConA concentrations, 1 μM fluoxetine inhibited T cell proliferation and a maximal suppressive effect was reached at 10 μM [80]. Although in some situations low levels of fluoxetine seem to stimulate lymphocyte proliferation, the majority of research in general points to a negative immunoregulatory effect of SSRIs on lymphocytes. Our own data support the observation that SSRIs reduce T cell proliferation in a concentration-dependent

manner at concentrations equal to or higher than 1 µM, when stimulated with anti-CD3/CD28 beads [86]. In addition to fluoxetine, other clinically available SSRIs (paroxetine, sertraline, citalopram, fluvoxamine) also appear to induce this anti-proliferative effect [86]. Like suppression of cytokine secretion, the anti-proliferative effect of SSRIs is concentration-dependent.

4.3. Pro-apoptotic effect

Finally, SSRIs have been shown to induce apoptosis in lymphocytes. Whereas paroxetine and sertraline were found to decrease activated T cell viability with an IC_{50} around 10 µM [75], other SSRIs exerted this effect only at tenfold higher concentrations. For citalopram, an IC_{50} of 180 µM was reported for pro-apoptotic action on naive T cells [87]. According to our own research, this apoptotic effect is induced by all SSRIs used in clinical practice (paroxetine, fluoxetine, sertraline, fluvoxamine and citalopram), albeit in different concentration ranges [86].

Not only do SSRIs induce apoptosis in healthy lymphocytes, they also seem capable of reducing the viability of several cancerous immune cells. Amit *et al.* showed that paroxetine (IC_{50}=18 µM) and sertraline (IC_{50}=9.5 µM) reduced the viability of Jurkat T cells [82]. Fluoxetine did not affect the viability of the leukemic T cells [82]. Another group demonstrated an inhibitory effect of fluoxetine on (T cell) lymphoma growth in mice [85]. However, the tumor suppressive effects were attributed to an enhancement of anti-tumor immunity instead of a direct effect on the tumor cells [85, 88]. These data appear contradictory with the observed immunosuppressive effects in *in vitro* experiments with lymphocytes and *in vivo* models of autoimmune disorders. However, anti-tumor immunity involves different T cell subsets and different cytokines than autoimmune disorders and thus the immunosuppressive or immunostimulatory outcome of SSRI treatment might be determined by the underlying pathology.

In Burkitt lymphoma cells, SSRIs (fluoxetine IC_{50}=9.3 µM, paroxetine IC_{50}=6.9 µM and citalopram IC_{50}=20.9 µM) were also found to induce apoptosis through cessation of DNA synthesis [89]. These findings raised the question whether SSRIs could be reinvented as a novel class of chemotherapeutic agents. Although some discussion was raised on whether SSRIs were specific enough to solely target the malignant cells [90, 91] and whether the effects were mediated through SERT [90-92], it was concluded that SSRIs are interesting candidates for further testing in B cell malignancies. The subsequent foundation of Celentyx by Nicholas Barnes and John Gordon warrants the further development of new SSRI derivatives for the treatment of B cell cancers. Although this group specifically focuses on B cell malignancies, further investigation into the usefulness of SSRIs in T cell cancers is equally promising.

Interestingly, in comparison with cancer cells, resting peripheral lymphocytes are much less sensitive to the effects of SSRIs [89]. In contrast, actively proliferating lymphocytes respond to SSRIs in a comparable way as cancerous immune cells [90]. Our own data support that there is a difference in sensitivity to the pro-apoptotic action of SSRIs between activated and resting T cells, and that activated T cells undergo apoptosis at significantly lower SSRI concentrations [93]. According to Schuster et al., this discrepancy between resting and activated lymphocytes is due to the intrinsic higher sensitivity of proliferating cells to undergo apoptosis [90]. However, since the exact mechanism by which SSRIs induce their effects is to be established, other possibilities explaining the different response, such as a possible role for the serotonin transporter (SERT) cannot be excluded. This SERT protein was undetectable or expressed in only small amounts in resting tonsilar B cells, while upon activation with mitogens, B cells upregulated SERT [94]. The observation that proliferating B cells were more sensitive to SSRI-induced effects than resting B cells [94] leads to the assumption that SERT expression might be an important factor in the execution of the immunological effect of SSRIs. Therefore, SERT expression in T lymphocytes will be further investigated in this thesis.

4.4. Potential mechanisms of action

Although the immunological effects of SSRIs have been described by several research groups, little is known about the mechanism underlying these effects. Initially, the inhibition of SERT and consequent rise in extracellular 5HT concentration were thought to be responsible for the anti-proliferative and pro-apoptotic action of SSRIs on lymphocytes. More recent research, however, provides several arguments against this assumption. Other research has focused on the participation of direct triggering or inhibition of signal transduction pathways in the immunological effects of SSRIs and on the pathways underlying the apoptotic action of SSRIs. Finally, some of the current views on antidepressant action in depression, such as modulation of membrane-associated lipid rafts or activation of the glucocorticoid receptor, may also be of importance in the immunomodulatory effects of SSRIs.

4.4.1. Involvement of 5HT and its transporter

Early work concerning the immunological effects of SSRIs assumed 5HT to be involved in the mechanism underlying the effects of SSRIs on lymphocytes. A role of 5HT was demonstrated by several research groups. Pellegrino and Bayer demonstrated that elevation of extracellular 5HT levels through administration of the 5HT precursor 5-hydroxytryptophan results in a decreased lymphocyte proliferation [83]. Also, when 5HT synthesis was inhibited *in vivo*, SSRIs were no longer capable of suppressing lymphocyte proliferation [83]. Lesioning of serotonergic neurons *in vivo* resulted in the

same inability of SSRIs to decrease lymphocyte proliferation [83]. Thus, if no 5HT was present, SSRIs were not able to increase the extracellular 5HT concentration and no effect on proliferation was observed. Also, fluoxetine and sertraline, two SSRIs with distinct chemical structures but with the same capacity to block SERT, were found to exert similar anti-proliferative effects on lymphocytes whereas dopamine and noradrenaline reuptake inhibitors did not [83]. These findings suggest an important role of 5HT in the anti-proliferative effect of SSRIs. In addition, 5HT itself has been shown to induce apoptosis in Burkitt lymphoma cells [95], and pro-apoptotic action of SSRIs thus might as well be explained through elevation of extracellular 5HT levels.

Several research groups have shown that antagonism of 5HT receptors, as well as inhibition of 5HT synthesis, results in impaired T cell activation and proliferation. Both 5HT-1A [96], -1B [37, 38] and 5HT-7 [37] receptors have been suggested to be involved in this process. Alternatively, it has been suggested that not the 5HT receptors, but the uptake of 5HT through SERT accounts for the mitogenic effect of 5HT [97]. Internalization of 5HT through SERT would lead to proliferation of the cells. Consequently, the anti-proliferative effect of SSRIs could be explained by the inhibition of 5HT uptake. These observations point to a stimulatory effect of 5HT on activation and proliferation of lymphocytes. The optimal activation of lymphocytes seems to require certain levels of 5HT, and both too low and too high concentrations result in sub-optimal lymphocyte activation, proliferation and viability. Taken together, these reports led to the postulation that SSRIs increased the extracellular 5HT concentration by blockage of 5HT uptake through SERT, which has been shown to be present on the cell surface of lymphocytes (figure 1.12A) [79, 98].

On the contrary, several arguments have come up recently that refute the involvement of 5HT and SERT in the immunosuppressive effect of SSRIs. First, acetylation of fluvoxamine suppressed the capability of the compound to inhibit 5HT uptake, but did not impair the anti-proliferative effect [90]. Nevertheless, acetylation of paroxetine resulted in an increase of the IC_{50} from 6.5 µM to 93.3 µM [90] and thus decreased the ability of paroxetine to suppress proliferation. Whereas the anti-proliferative effect of paroxetine shifted 15-fold by acetylation, the affinity for SERT decreased over 1000-fold demonstrating that both effects are not entirely dependent on each other [90]. However, It should also be noted that isomerization of fluvoxamine from the *trans* to the *cis* form cancelled its capability to suppress *in vitro* neural cell proliferation, as well as its ability to block 5HT uptake [99].

Second, the concentrations needed for inhibition of 5HT uptake are in the nanomolar range, while those exerting an anti-proliferative effect are in the micromolar range [77, 90]. Although Ferriere *et al.* found specific binding of ^{3}H-paroxetine in fish lymphocytes to be in the nanomolar range (0-10

nM), micromolar concentrations were needed to substantially inhibit 5HT uptake in these cells [100]. Thus, anti-proliferative action of SSRIs in the micromolar range might be explained by the substantial inhibition of 5HT uptake in this concentration range, notwithstanding specific binding of SSRIs to SERT already occurs in the nanomolar range.

Third, it was put forward that HEK293 cells, which were assumed not to express SERT, were still sensitive to the effects of SSRIs and thus these effects could not be mediated by SERT inhibition [90]. To this end, it should be noted that Chamba *et al.* found SERT expression in wild-type HEK293 cells both on mRNA and protein level [101], suggesting that these cells might yet encounter SSRI-induced effects through SERT inhibition.

Cloonan *et al.* pointed out that not all SSRIs induced a pro-apoptotic effect (citalopram did not induce apoptosis in any of the tested cell lines), whereas they all do inhibit 5HT uptake through SERT. Further, the same group also showed that 5HT was not able to prevent the induction of cell death by SSRIs, and that 5HT itself, amongst other SERT ligands, could not induce apoptosis in the tested malignant cell lines [92]. In addition, SSRIs did not induce more extensive cell death in cells expressing higher levels of SERT [92]. Whereas Pellegrino *et al.* reported that *in vivo* administration of noradrenaline and dopamine reuptake inhibitors in rats did not affect lymphocyte proliferation, Diamond *et al.* found that antidepressants, inhibiting the reuptake of noradrenalin (reboxetine, desipramine) or not inhibiting the reuptake of any monoamine (trimipramine), were still capable of inhibiting *in vitro* T cell proliferation, as well as IFNγ secretion [102].

Interestingly, it has been hypothesized that binding of monoamines on SERT can itself induce signal transduction pathways [95]. Possibly, binding of SSRIs on SERT induces the same changes in signal transduction pathways. Furthermore, 5HT uptake has been demonstrated to influence signal transduction directly through ‘serotonylation’ of small GTPases [103]. Thus, SSRIs might affect signal transduction through restriction of available 5HT for serotonylation.

As abovementioned arguments do not entirely in- nor exclude the SERT as a target through which SSRIs exert their immunosuppressive effects, other approaches to study the involvement of SERT are required. Convincing evidence for a role of SERT in the immunosuppressive effects of SSRIs might come from studies in SERT knockout mice. Limited evidence in SERT knockout mice has been gathered showing that SERT plays a role in experimental autoimmune encephalomyelitis (EAE)[104] and inflammatory bowel disease (IBD)[105], indicating that impaired serotonin transport indeed affects immunological mechanisms of these diseases. Further research comparing the immunological

effects of SSRIs in SERT knockout versus wild-type mice both in healthy conditions and in models for autoimmune disorders might reveal the role of SERT in the immunosuppressive effects of SSRIs.

Furthermore, a modified SERT knock-in mouse strain (SERT I172M) was recently developed that expresses a modified SERT protein with normal 5HT recognition and transport, but with a decreased sensitivity for antidepressants, including fluoxetine and citalopram [106]. The pranging question whether or not SERT is involved in the immunomodulating effects of SSRIs might be answered using this SERT I172M mouse model [107].

4.4.2. Effects on signaling transduction pathways

Regardless the blockage of SERT, further downstream events leading to SSRI-induced suppression of proliferation have been investigated by studying the interference of SSRIs with signal transduction pathways, such as the cAMP and phosphoinositol system. SSRIs have been demonstrated to interfere with the activation of the cAMP-dependent protein kinase A (PKA) pathway and the activation of protein kinase C (PKC), as well as with the influx of Ca^{2+} (figure 1.12B).

cAMP has been shown to be an important regulator of immune responses by inhibition of T cell proliferation [108]. Consequently, an increase in cAMP in response to SSRIs could explain the anti-proliferative action of SSRIs on lymphocytes. At optimal concentrations of ConA, fluoxetine induced a rise in intracellular cAMP concentration [77, 80]. Citalopram similarly elevated cAMP levels in T cells stimulated with phytohaemagglutinin [74]. However, Kenis *et al.* did not find any increase in cAMP in peripheral blood mononuclear cells exposed to 0.01 – 1 µM paroxetine [109]. The same group further examined the involvement of cAMP and PKA activation in the immunoregulatory effect of fluoxetine and concluded that the cAMP-dependent PKA pathway was probably not involved in the fluoxetine-induced suppression of the IFNγ/IL-10 ratio, but activation of PKA might contribute to the reduction in TNFα secretion [110].

On the other hand, PKC activation stimulates lymphocyte proliferation [77] and SSRI-mediated suppression of PKC translocation to the cell surface may account for the anti-proliferative effect. Translocation of PKC was inhibited by fluoxetine at optimal mitogenic concentrations [77], which might contribute to the observed anti-proliferative effect.

Further, cytosolic Ca^{2+} influx is an important factor in lymphocyte activation and subsequent proliferation [111]. Thus, SSRIs might interfere with lymphocyte proliferation through interference with Ca^{2+} influx. Edgar *et al.* demonstrated that fluoxetine exerted similar effects on mitogen-induced T cell proliferation as calcium ionophores [80]. At sub-optimal mitogen concentrations, both

fluoxetine and calcium ionophores stimulated T cell proliferation, whereas at optimal mitogen concentrations, both compounds inhibited T cell proliferation [80]. Thus, when suboptimal mitogen concentrations were used, fluoxetine possibly induced an influx of extracellular Ca^{2+} that enhanced T cell proliferation. In T cells exposed to optimal mitogen concentrations, however, fluoxetine caused an excessively high Ca^{2+} concentration resulting in impaired proliferation [80]. Further, fluoxetine, paroxetine and citalopram directly increased intracellular Ca^{2+} in malignant B cells [89].

In conclusion, interference with cAMP and phosphoinositol systems can explain some of the effects of SSRIs on lymphocytes, but the exact mechanism behind the immunomodulating effects of SSRIs remains unresolved and therefore requires further investigation. The direct effects of fluoxetine on Ca^{2+} signaling in T lymphocytes will be further investigated in this thesis.

4.4.3. Induction of the apoptotic cascade

Besides an anti-proliferative effect, SSRIs have been found to induce apoptosis in lymphocytes and cancer cells. The pathways involved in this apoptotic effect of SSRIs have been investigated extensively. Xia *et al.* showed that the decrease in cellular viability was due to the induction of apoptosis, and was accompanied by extensive DNA fragmentation [87]. In lymphocytes exposed to citalopram, the anti-apoptotic genes c-myc and bcl-2 were downregulated and Fas membrane expression was increased [112]. In cancer cells, the process involves caspase-3 activation, as was demonstrated in both Jurkat T cells [82] and acute myeloid leukemia HL-60 cells [113]. Early in the apoptotic cascade triggered by SSRIs in HL-60 cells, reactive oxygen species are formed, and this precedes the change in mitochondrial trans-membrane potential [114]. Further, Taler *et al.* showed an activation of the MAPK death signaling pathway and suppression of the anti-apoptotic protein bcl-2 in mitogen-activated rat splenocytes [81]. As an extensive cross-talk exists between bcl-2 and Ca^{2+}, and bcl-2 has been shown to exert its anti-apoptotic effects through regulation of ER Ca^{2+} stores [115], the observed effects of SSRIs on Ca^{2+} signaling might also relate to the observed decrease in bcl-2 and resulting apoptosis.

Finally, in human cervical cancer (SiHa) and breast cancer cells (MDA MB 231), fluoxetine was shown to induce an anti-proliferative and apoptotic effect which was mediated through cell cycle arrest at the G0/G1 phase [116]. By the use of bioinformatics tools, it was predicted that fluoxetine might interfere with CKS1, a protein involved in cell division. Further *in vitro* experiments confirmed functional inhibition of CKS1 by fluoxetine [116]. As the outcome of G1 arrest has been shown to be either differentiation or apoptosis, it was concluded that both the observed anti-proliferative and apoptotic effect of fluoxetine were mediated by inhibition of CKS1 [116]. Whether the same

mechanism accounts for the anti-proliferative and apoptotic effect of SSRIs in other types of cancer cells and non-malignant cells such as lymphocytes is not clear. In conclusion, several well-known mechanisms leading to apoptosis are involved in the process by which SSRIs reduce cellular viability of lymphocytes (figure 1.12C).

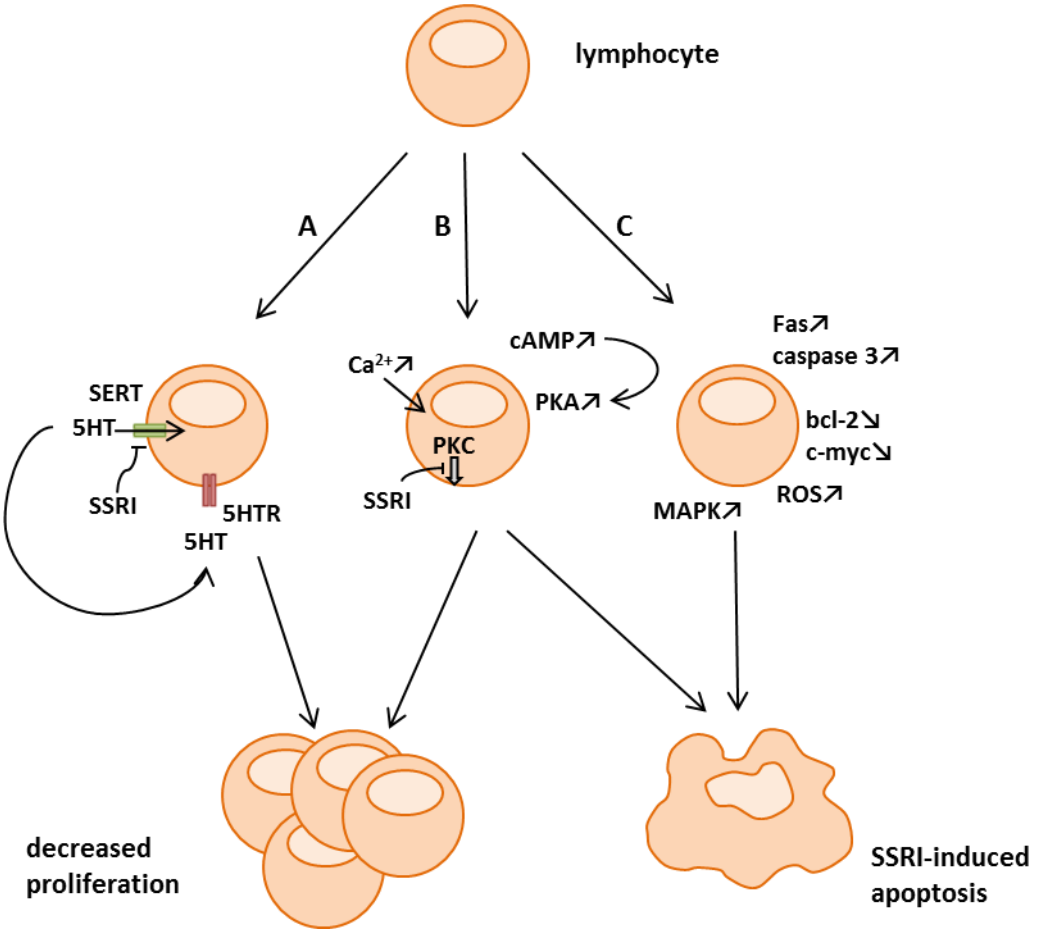


Figure 1.12. Possible mechanisms underlying the anti-proliferative and apoptotic effect of SSRIs on lymphocytes. A) Inhibition of 5HT uptake through SERT results in increased binding of 5HT on 5HT receptors, thereby reducing lymphocyte proliferation. The inability to take up 5HT itself might as well cause decreased lymphocyte proliferation. B) SSRIs increase cAMP levels, thereby activating the PKA pathway; SSRIs inhibit translocation of PKC, ultimately resulting in reduced lymphocyte proliferation and/or SSRIs increase Ca^{2+} influx, causing reduced T cell proliferation in response to optimal mitogen concentrations. Alterations in these signaling pathways, especially Ca^{2+} signaling, might elicit apoptosis. C) SSRIs induce activation of the apoptotic cascade, with activation of caspase 3 and MAPK, generation of reactive oxygen species (ROS), upregulation of Fas and downregulation of bcl-2 and c-myc.

4.4.4. Unexplored mechanisms

In addition to abovementioned targets that have been investigated in lymphocytes to a greater or lesser extent, other mechanisms explaining the antidepressive action of SSRIs might as well account for their immunological effects. Amongst others, it was suggested that SSRIs might directly influence mitochondrial pathways, as was demonstrated for clomipramine in human glioma cells [117, 118].

Furthermore, it was proposed that SSRIs could affect cell dynamics through e.g. phospholipid binding and lysosomal trapping, given their lipophilic and amphiphilic nature [117]. In this respect, SSRIs have been found to accumulate in membrane-associated lipid rafts in HEK293 and N1E-115 neuroblastoma cells [119]. Moreover, antidepressants have been shown to enhance G protein Sα migration from lipid rafts and thereby facilitate adenylyl cyclase activity and cAMP formation in C6 glioma cells [120]. As a result, signal transduction post G protein-coupled receptor activation is enhanced. The observed rise in cAMP after SSRI treatment of T lymphocytes activated with mitogens as described by Edgar et al. [77, 80] and Xia et al. [74] (see 4.4.2) might relate to the effects of SSRIs on lipid rafts. Given the presumed importance of lipid rafts in TCR clustering during T cell activation [121], SSRIs might disturb T cell function either directly via disturbance of lipid raft integrity or indirectly via enhanced G protein signaling.

Another possible mechanism is upregulation of the glucocorticoid receptor (GR). Antidepressants have been shown to increase GR expression, promote GR nuclear translocation and enhance GR function in mouse fibroblasts [51, 122]. As glucocorticoids have strong immunosuppressive effects, it is possible that SSRIs exert their immunosuppressive effects on T lymphocytes through GR modulation. However, these suggestions have not been investigated in lymphocytes and further research will be necessary to clarify whether the immunomodulating effects of SSRIs are mediated through any of the abovementioned mechanisms.

4.5. SSRI-mediated immunomodulation in animal models of autoimmune disorders

As it became more and more clear that SSRIs induced significant changes in immune cells, the possibility to use SSRIs in immune related pathologies was investigated. The potentially beneficial effects of SSRIs in autoimmune diseases have been tested in animal models of multiple sclerosis, rheumatoid arthritis, contact hypersensitivity reaction, inflammatory bowel disease, septic shock and allergic asthma. An overview is given in table 1.2.

In experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS), venlafaxine, paroxetine and sertraline were tested and both venlafaxine and sertraline were able to

ameliorate clinical symptoms of disease (tail limpness, paraparesis, hindlimb and forelimb paralysis) [123, 124]. Paroxetine did not affect the clinical progression of EAE [123]. However, animals were treated with only 5 mg/kg paroxetine, which may have failed to induce high enough plasma concentrations to reach an immunomodulatory effect. Cytokine secretion was also investigated and sertraline decreased the secretion of IFNγ, TNFα and IL-2 as well as the viability and *in vitro* proliferation of EAE splenocytes [123]. Histological examination of venlafaxine-treated animals revealed decreased central nerve system inflammation and infiltration of inflammatory cells in the brain and spinal cord [124]. Venlafaxine also reduced pro-inflammatory cytokine secretion (IL12 p40, IFNγ, TNFα) and diminished mRNA expression of inflammatory genes [124]. In a similar multiple sclerosis model in rats, fluoxetine has recently been shown to promote remission of EAE [125]. Fluoxetine-pretreated rats recovered faster and clinical scores during remission were lower than those found in vehicle-treated animals [125]. Spinal cord demyelination and inflammatory foci were reduced and IFNγ production was suppressed [125].

In a murine model for rheumatoid arthritis (RA), fluoxetine and citalopram were tested and both SSRIs were able to reduce clinical scores (based on the occurrence of erythema, swelling and joint deformity with ankylosis) [126]. Fluoxetine additionally improved paw thickness and significantly reduced IL12 secretion, whereas citalopram did not [126]. Histological examination of the affected joints revealed reduced inflammation, cartilage and bone erosion in fluoxetine-treated animals and a tendency towards reduced inflammatory cell infiltration, pannus formation and joint deformation in citalopram-treated mice [126]. Further, a beneficial effect of sertraline has been demonstrated in a rat model of RA [127]. The decrease in clinical symptoms was accompanied by an increase in IL10 secretion, and a decrease in TNFα and cox2 levels [127].

Recently, the effect of fluoxetine on murine contact hypersensitivity (CS) reaction of the skin has been studied [128]. CS is a T cell mediated immune reaction that was successfully suppressed by fluoxetine as determined by the reduction in swelling of the ear to which the contact allergen was applied. The weight of axillary lymph nodes was decreased and the production of IL10, an anti-inflammatory cytokine, was increased by fluoxetine [128]. A similar effect was observed for fluoxetine by the same group when using a different CS model [129].

Table 1.2: **Animal studies of SSRIs in autoimmune diseases.**

SSRI	pathology	animal model/species	beneficial effect	reference(s)
paroxetine	multiple sclerosis	EAE, murine	no	Taler et al, 2011
fluoxetine	allergic asthma	ovalbumin-sensitization, rat	yes	Roumestan et al, 2007
	septic shock	LPS-induced, murine	yes	Roumestan et al, 2007
	inflammatory bowel disease	acetic acid, rat	yes	Guemei et al, 2008
	rheumatoid arthritis	CIA, murine	yes	Sacre et al, 2010
	inflammatory bowel disease	DSS, murine	yes	Koh et al, 2011
	multiple sclerosis	EAE, rat	yes	Yuan et al, 2012
	contact hypersensitivity	picryl chloride, murine	yes	Kubera et al, 2012
	contact hypersensitivity	2,4-dinitrofluorobenzene, murine	yes	Curzytek et al, 2013
sertraline	multiple sclerosis	EAE, murine	yes, moderately	Taler et al, 2011
	rheumatoid arthritis	CIA, rat	yes	Baharav et al, 2012
citalopram	rheumatoid arthritis	CIA, murine	yes, partial	Sacre et al, 2010
venlafaxine	multiple sclerosis	EAE, murine	yes	Vollmar et al, 2008

EAE: experimental autoimmune encephalomyelitis, LPS: lipopolysaccharide, CIA: collagen-induced arthritis, DSS: dextran-sulphate sodium.

Inflammatory bowel disease is another example of an immunological disorder that might benefit from SSRI treatment. This disease is caused by a dysregulation of the gastro-intestinal immune system and is considered to be the result of an altered immune response to luminal antigens. In a dextran sulphate sodium (DSS)-induced murine model for colitis, fluoxetine showed to improve the disease activity index, consisting of a composite score for weight loss, stool consistency and gross rectal bleeding [130]. Histological examination of the proximal and distal colon showed less infiltration of inflammatory cells and reduced impairment of the glandular architecture in fluoxetine-treated animals versus controls. Another study demonstrated that fluoxetine and desipramine attenuate acetic acid-induced experimental colitis in rats [131]. In addition to a reduction of colonic damage, fluoxetine and desipramine suppressed serum cytokine levels (TNFα, IL1β) that were induced by experimental colitis [131].

Finally, in a lipopolysaccharide (LPS)-induced murine model of septic shock, preventive administration of fluoxetine diminished the expression of TNFα and the mortality rate [132]. In a rat model of allergic asthma, fluoxetine reduced lung inflammation and infiltration of inflammatory cell types [132]. Fluoxetine not only reduced the number of lymphocytes, but also macrophages, neutrophils and eosinophils [132]. *In vitro*, fluoxetine dose-dependently inhibited the release of TNF-α from LPS-treated monocytes [132].

4.6. SSRI-mediated immunomodulation in human autoimmune disorders

Although clinical evidence for SSRI use in autoimmune diseases is scarce, three studies have been conducted that demonstrate the usefulness of SSRIs in MS and RA. In undepressed patients with relapsing MS, fluoxetine (20 mg/d) reduced the occurrence of new enhancing lesions, as measured by MRI scan [133]. The beneficial effect was attributed to an anti-inflammatory effect of fluoxetine on astrocytes, rather than a suppressive effect on peripheral lymphocytes. The peripheral effects on immune cells, however, were not investigated.

In RA patients, a clinical trial was performed to evaluate the efficacy of paroxetine and amitriptyline for concurrent depression [134]. In addition to an improvement in depressive symptomatology, an improvement of RA associated pain and disability has also been detected with both paroxetine (20-40 mg/d) and amitriptyline (75-150 mg/d). Although this study did not measure direct immunological parameters, the improvement in RA symptoms seems to indicate a beneficial effect of paroxetine and amitriptyline in this pathology. It is not clear, however, whether this is a direct effect of the antidepressants on immune parameters, or an indirect effect through resolving the depressive

symptomatology which is known to exacerbate the arthritic symptoms [134]. In order to differentiate between both possibilities, studies in non-depressed RA patients should be conducted.

Furthermore, a patient suffering from RA was found to be in remission when treated with citalopram for concurrent depression and discontinuation of citalopram treatment resulted in reoccurrence of the rheumatic symptoms [135]. Although it can be argued that the mental state of a patient influences his perception of rheumatic symptoms, this case report mentions a significant improvement of DAS28 score, which is an objective measure of RA disease activity [136]. Therefore, it seems to indicate that there is a direct link between SSRI treatment and severity of RA symptoms in this patient.

Recently, a clinical study was performed evaluating the combination of a low dose prednisolone (3 mg) with 10 mg paroxetine (CRx-139^{low})(n=71) or 20 mg paroxetine (CRx-139^{high}) (n=69) in undepressed RA patients [137]. Comparison was made against treatment with prednisolone alone (n=69). The primary endpoint of the study was a 20% improvement of ACR score (ACR20) from baseline to the end of the study period (day 70). The ACR criteria were developed by the American college of Rheumatology to assess RA disease improvement, and are thus well suited to compare different treatment regimens [138]. Secondary endpoints were the difference in ACR50, ACR70, EULAR good response, remission, DAS28 score, CRP levels, and inflammatory cytokine levels. Although no significant effect was found for CRx-139^{low} and CRx-139^{high} on the primary endpoint (ACR20 at day 70), multiple secondary endpoints showed significant improvement in the group of patients receiving CRx-139^{high}. Whereas the ACR20 and ACR50 responses were significantly higher at day 42, no significant effect could be observed at day 70. Thus, the beneficial effect of CRx-139^{high} on ACR response appears to be transient. With respect to the EULAR good response and remission, a significant improvement could be detected for CRx-139^{high} at day 42 and day 70, demonstrating that the beneficial effect on these endpoints was maintained until the end of the study period [137]. From these data, it can be concluded that addition of 20 mg paroxetine to a low dose prednisolone yields a limited, but clinically meaningful decrease in disease activity. The lower dose of paroxetine, 10 mg, did not yield an added value to the standard therapy of 3 mg prednisolone with respect to RA disease control. Possibly, increasing the dose of paroxetine to 40 or 60 mg would augment the beneficial effect of this combination therapy on RA disease activity.

Abovementioned studies in humans and animals demonstrate the potential use of SSRIs in a wide variety of autoimmune diseases. Nonetheless, the data are limited and further research is needed to evaluate which SSRI, which dose and dosage regimen are optimal for each individual pathology. To

date, most preclinical evidence of immunosuppression exists for fluoxetine (table 1.2). Whereas fluoxetine is definitely a suitable candidate to proceed to clinical testing, it is worthwhile to screen the effect of other SSRIs in autoimmune disorders as well, as these might show to be equally or even more effective.

Other autoimmune disorders, such as diabetes mellitus type 1, lupus erythematosus, autoimmune thyroid diseases and others might as well benefit from SSRI treatment and studies exploring the potential use of SSRIs in these disorders should be encouraged. In addition, other conditions where unwanted immune activation occurs can potentially be controlled with SSRIs. Such conditions include several types of transplantation, where the host's immune system mounts an immune response against the transplanted organ (host-versus-graft response). Another unwanted immune reaction that might benefit from SSRI treatment occurs in patients receiving allogeneic stem cell transplantation. In these patients, immunocompetent cells in the stem cell graft can mount an immune response to the host's tissues and cells (graft-versus-host response).

5. Graft-versus-host disease

In this thesis, the potential application of SSRIs as immunosuppressants in graft-versus-host disease is further investigated. Therefore, an introduction on this major complication of stem cell transplantation is provided in the following section.

5.1. Definition

Graft-versus-host disease (GvHD) is a common complication after transplantation of haematopoietic stem cells (the 'graft') from one individual (the 'donor') to another (the 'host'). This type of transplantation, termed allogeneic hematopoietic stem cell transplantation (hSCT) is an increasingly applied treatment for inherited disorders of blood cells, immunodeficiencies or hematological malignancies such as acute and chronic leukemia. The transplantation is preceded by a conditioning regimen, which can be chemotherapy and/or irradiation and is intended to induce immunosuppression to prevent graft rejection, suppress the bone marrow of the patient to create a niche for stem cell engraftment and to eradicate the malignant cells (= myeloablative conditioning). Unfortunately, the immunosuppression not only aids to prevent graft rejection, but also compromises the patient's immune function and therefore increases the susceptibility to infection. In order to restore the blood forming compartment and immune function, a hematopoietic stem cell transplantation is provided. In addition, the graft provides an anti-tumor effect known as the graft-versus-leukemia (GvL) effect, that eliminates remaining cancer cells and prevents relapse [139].

Whereas the patient or host is pretreated with radio- and chemotherapy, the donor receives granulocyte-colony stimulating factor (G-CSF) to mobilize hematopoietic stem cells into the blood. There, they are collected through leukapheresis, a process that separates white blood cells from the rest of the blood. The resulting cell preparation, containing hematopoietic stem cells, is administered to the patient intravenously (figure 1.13). Neutrophils and platelets engraft within 10-20 days after transplantation. Other cell types, including T and B lymphocytes, macrophages, dendritic cells and erythrocytes may take longer to engraft [139]. The goal of hSCT is to replace the patient's blood cells entirely with donor cells, in which case full donor chimerism is obtained.

Whether a stem cell transplantation is effective largely depends on the genetic relationship between the donor and the host. Each individual has a specific tissue type that is defined by a set of histocompatibility genes. These genes encode histocompatibility antigens, which determine whether two tissues are either compatible or incompatible.

In men, major histocompatibility antigens are known as the human leukocyte antigens or HLA. In addition to HLA, minor histocompatibility antigens (miHA), that are encoded outside of the HLA loci, equally contribute to the (in)compatibility of a tissue [140]. In case of autologous stem cell transplantation, in which donor and host are one and the same individual, tissue types are identical and no immune response occurs. However, in case of allogeneic stem cell transplantation, in which donor and host are genetically disparate individuals, an immune response can be raised. The immune response against histocompatibility antigens, also called the allograft reaction, can result in a host-versus-graft reaction in which case the graft is rejected by the host. Alternatively, the immunocompetent cells present in the graft can mount an immune response against the host tissue. The latter event is called a graft-versus-host reaction. The consequences of a graft-versus-host reaction are immunological damage to the skin, liver and gastro-intestinal (GI) tract and are generally referred to as GvHD.

A distinction can be made between acute and chronic forms of GvHD, as well as an overlap syndrome which bears features of both. Whereas classic acute GvHD occurs within 100 days after transplantation, no time limits exist for chronic GvHD. Furthermore, a late-onset form of acute GvHD can occur more than 100 days post-hSCT [141]. Besides the time of onset, acute and chronic GvHD differ in many other aspects, including pathophysiological mechanism, target organs, clinical symptoms and treatment. Since the research in this thesis is only focusing on acute GvHD, the pathophysiology and treatment of chronic GvHD are not further discussed.

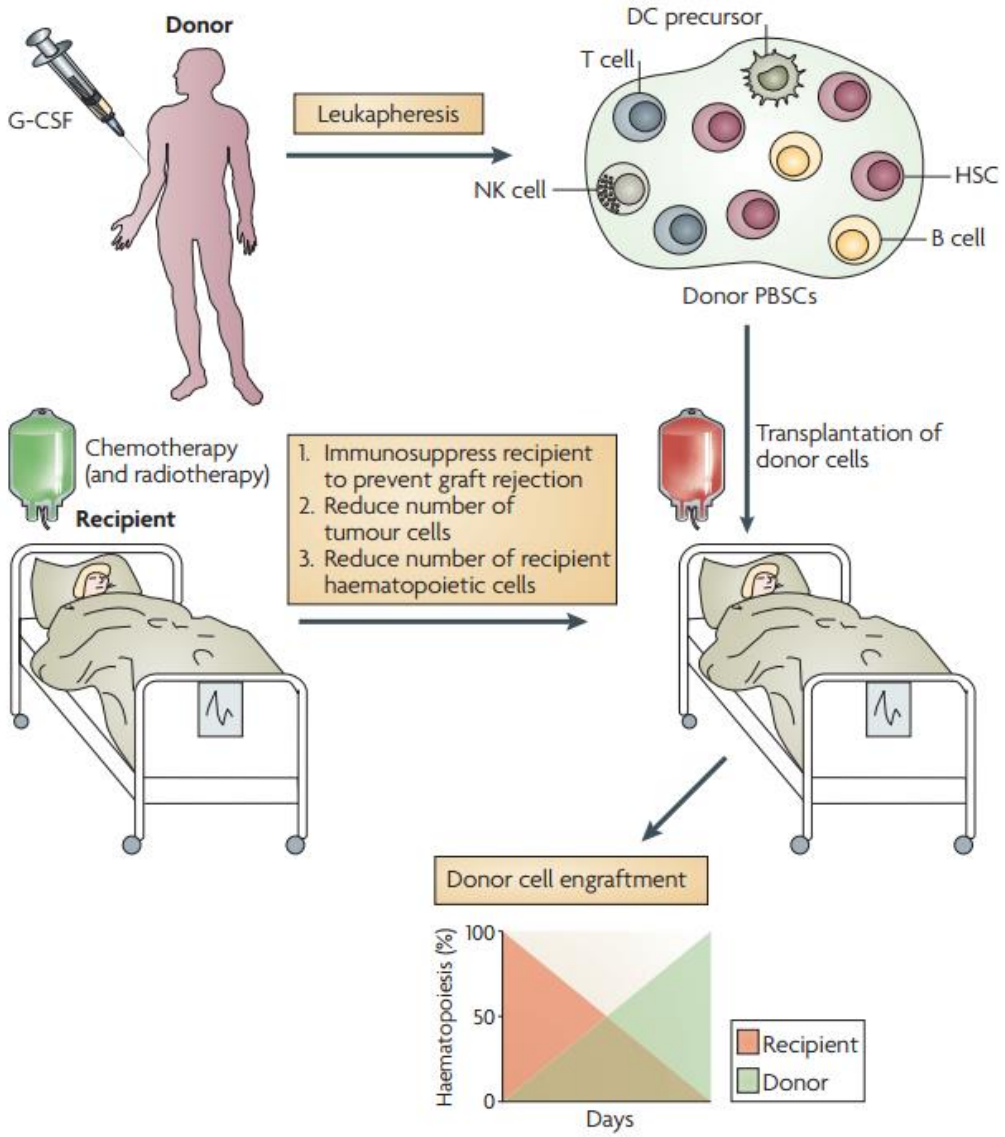


Figure 1.13. Principle of hematopoietic stem cell transplantation. The donor receives granulocyte-colony stimulating factor (G-CSF) to stimulate migration of hematopoietic stem cells into the blood, which are collected through leukapheresis. Meanwhile, the patient or recipient is treated with chemotherapy and/or radiotherapy to induce immunosuppression, create a niche for the hematopoietic stem cells and eradicate cancer cells. The cell preparation is administered to the patient intravenously. In the next 10-20 days, neutrophils and platelets engraft. Other cell types including T and B lymphocytes, macrophages, dendritic cells and erythrocytes may take longer to engraft. The goal of hSCT is to replace the patient's blood cells entirely with donor cells, in which case full donor chimerism is obtained. Figure obtained from [139].

5.2. Pathophysiology of acute GvHD

The development of acute GvHD can be divided in three phases: (1) activation of host antigen presenting cells (APC); (2) donor T cell activation, proliferation, differentiation and migration, and (3)

target tissue destruction (figure 1.14) [141]. The first step comprises activation of host APC as a result of the underlying disease and conditioning regimen. Damaged host tissue produces 'danger signals' such as pro-inflammatory cytokines, chemokines, MHC antigens and co-stimulatory molecules on host APC. Especially important is the damage to the GI tract following the conditioning regimen. Due to increased permeability of the GI mucosa, inflammatory molecules such as bacterial lipopolysaccharides (LPS) enter the body and additionally stimulate the activation of host APC [141, 142]. Activated host APC present self-antigens in association with MHC molecules.

The second phase is initiated when activated host APC, presenting self-antigens, encounter alloreactive donor T cells, which are efficiently activated as they recognize these self-antigens as non-self. The 'danger signals' produced in phase 1 further enhance this process. Activated T cells start to proliferate and differentiate into effector cells. Activation of donor T cells results in the production of large amounts of pro-inflammatory cytokines such as IL2, TNFα and IFNγ [141, 142].

In the third and last phase, both cellular and soluble inflammatory effectors work together to further promote inflammation and damage host tissues. Cellular effectors are cytotoxic T lymphocytes and natural killer (NK) cells that are attracted to target organs through chemokines. At the target organs such as skin, liver and GI tract, effectors use Fas/FasL or perforin/granzyme pathways to lyse target cells. Meanwhile, inflammatory effectors such as TNFα are produced in response to microbial products such as LPS leaking through the GI wall. TNFα is produced by both donor and host cells and causes activation of host APC, attracts effector cells to target organs and directly causes tissue necrosis [141, 142].

5.3. Prevention and treatment of acute GvHD

Standard pharmacological prevention of acute GvHD after myeloablative hSCT is a combination of a calcineurin inhibitor (cyclosporine or tacrolimus) and methotrexate or sirolimus. In case of non-myeloablative hSCT (see below), the golden standard is tacrolimus + mycophenolate mofetil (MMF). Cyclosporine and tacrolimus have similar mechanisms of action – inhibition of the enzyme calcineurin which plays an important role in T cell activation, see 2.2.–, clinical effectiveness and toxicity profiles, with transplant-associated thrombotic microangiopathy, neurotoxicity, nephrotoxicity, hypertension, hypomagnesaemia and hyperkalemia [141].

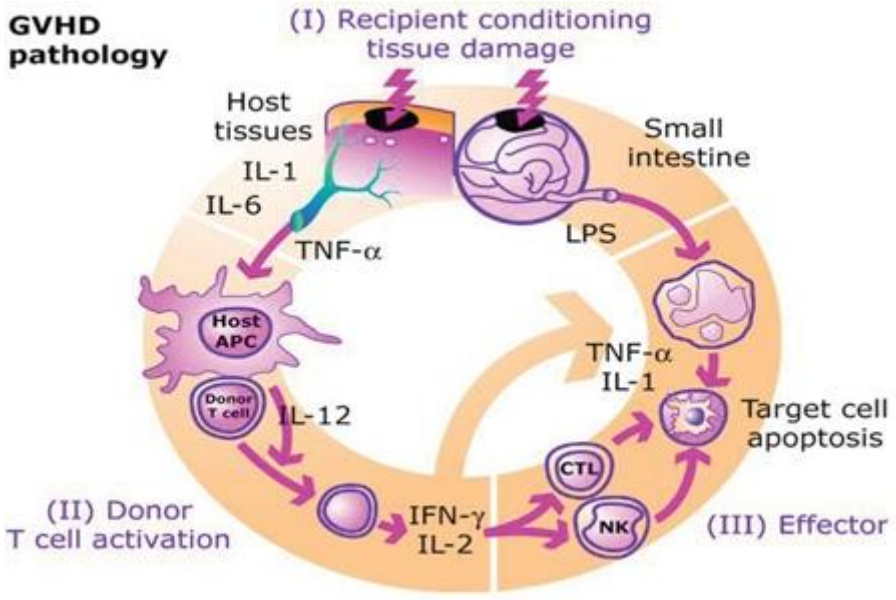


Figure 1.14. Pathophysiology of acute GvHD. In the initial phase, recipient conditioning through irradiation or chemotherapy causes tissue damage that induces leaking of bacterial products (e.g. LPS) through the intestinal wall and cytokine production in host tissues. Subsequently, donor T cells are activated by host antigens presented on host APCs, which is enhanced by circulating cytokines. In the effector phase, both cellular (cytotoxic T cells and NK cells) and soluble (TNFα, IL1) effectors induce target cell apoptosis, thereby causing damage to target organs such as liver, gastro-intestinal system and skin. Figure obtained from [142].

The immunosuppressive effect of methotrexate relies on its interference with the enzyme dihydrofolic acid reductase, thereby inhibiting the conversion of folic acid to tetrahydrofolate and preventing DNA synthesis, repair and cellular replication [143]. Adverse effects of methotrexate include mucositis, delayed neutrophil engraftment and liver toxicity [143]. Although the combination of a calcineurin inhibitor with methotrexate is successful in up to ¾ of patients, further progress is needed. Not only protection from developing acute GvHD is incomplete, as many patients still develop a degree of GvHD despite prophylaxis, but also problems with toxicity of methotrexate stimulate researchers to search for better alternatives [144]. Pharmacological alternatives include MMF and sirolimus, which mainly provide a benefit in terms of side effects. MMF is an antimetabolite that, like methotrexate, inhibits purine synthesis. In comparison to methotrexate, MMF induces significantly less severe mucositis and allows for more rapid neutrophil engraftment [141]. Sirolimus, although structurally related to tacrolimus, is not a calcineurin inhibitor. Instead, it binds to the FK binding protein 12 and forms a complex with the mammalian target of rapamycin (mTOR), resulting in decreased DNA transcription, translation and protein synthesis, as well as cell cycle arrest in activated T cells [145]. Sirolimus also inhibits the response of T cells to pro-

inflammatory cytokines, B cell proliferation and antibody production [145]. In contrast to cyclosporine and tacrolimus, sirolimus does not cause renal toxicity and hypertension, but it does induce microangiopathic hemolytic anemia, thrombocytopenia, hypercholesterolemia and hypertriglyceridemia [143].

Besides pharmacological alternatives, alterations to the conditioning regimen and stem cell transplantation itself have been considered. As mentioned in 5.2, the myeloablative conditioning regimen causes damage to the GI tract that results in the leakage of microbial products through the GI wall, thereby contributing greatly to the initiation of acute GvHD. As clinical experience with hSCT showed that myeloablative conditioning did not usually succeed in fully eradicating the malignant cells and the true therapeutic potential of hSCT actually relied on the anti-leukemia effect, reduced intensity conditioning (RIC) or non-myeloablative conditioning was developed. The primary goal of this approach was to induce sufficient immunosuppression to allow engraftment, and no longer to fully eradicate the cancer cells. RIC therefore greatly decreased the risk of developing acute GvHD, while maintaining the efficacy of the hSCT [141].

Further, *ex vivo* depletion of donor T cells from the graft has been proposed as a means of effectively preventing acute GvHD. However, this approach is associated with impaired immune reconstitution, infectious complications and an increased risk of primary disease relapse [144]. Another approach is the *in vivo* use of anti-lymphocyte antibodies, such as anti-thymocyte globulin (ATG). This approach has a double goal: it suppresses the host immune response thereby facilitating engraftment, and suppresses donor T cells after transplantation thus preventing GvHD. Although successful in the prevention of GvHD, this approach has the same disadvantages as *ex vivo* depletion of donor T-cells from the graft.

When prophylaxis has been unsuccessful and acute GvHD develops, the golden standard for treatment are corticosteroids. However, systemic glucocorticoid treatment results in complete remission in only 30 to 50% of patients [144], emphasizing the need for better treatment options. In case of steroid-refractory GvHD, other pharmacological approaches have been applied, including ATG, monoclonal antibodies against CD3 or CD25 (both specific T lymphocyte antigens), MMF and sirolimus, TNF-targeting agents such as infliximab and extracorporal photopheresis. The latter uses ultraviolet A light to damage the DNA of peripheral blood cells after exposing the cells to 8-methoxypsoralen. Unfortunately, these alternative treatments have shown to be effective in only a minority of cases, and are an additional source of toxicity [144]. Accordingly, there is a need for novel

preventive and curative treatment options for acute GvHD that can efficiently prevent and treat this major complication of hematopoietic stem cell transplantation.

5.4. Animal models of acute GvHD

In order to study the pathophysiology of acute GvHD, as well as to develop new preventive or curative treatment strategies, animal models for acute GvHD have been developed. The majority of these use mice as these animals are inexpensive and easy to maintain in a laboratory environment. Whereas at the present time, stem cells for transplantation in humans are increasingly obtained from peripheral blood, murine models use bone marrow as a source for transplantable stem cells. Comparable with the human HLA system, mice have the H-2 system defining their histocompatibility antigens. There are generally two types of animal models for acute GvHD: whereas the first type is based on a mismatch between one or more major histocompatibility antigens, the second type uses MHC-matched mouse strains. In the latter case, the graft-versus-host reaction is induced by disparities in minor histocompatibility antigens (miHA) [140]. As over 50% of human hSCT are MHC-matched [146], mouse models using MHC-matched, miHA-mismatched mouse strains resemble the human hSCT more closely.

In a primary immune response, less than 0.01% of T cells recognize a conventional antigen presented by MHC. However, minor lymphocyte stimulating (Mls) antigens are able to induce a response in up to 25% of T cells [147]. In contrast with conventional antigens, Mls antigens do not interact with the hypervariable regions of the TCR but rather bind to invariable parts of specific Vβ-chains. Therefore, a specific Mls antigen is recognized by all T cells of a given Vβ-type. This type of antigens does not (or minimally) show MHC restriction. However, the presence of MHC-II is required. This mechanism of T cell stimulation is also seen with bacterial superantigens (figure 1.15).

In mice, several genes encode Mls antigens. These genes are all encoded in mouse mammary tumor virus (MMTV) sequences, which have incorporated in the murine genome over time [147]. For each Mls antigen, a stimulatory (e.g. Mls-1a) and a null allele (e.g. Mls-1b) exists. When a specific Mls antigen is expressed at birth, the developing immune system deletes the T cells bearing the specific Vβ-chain that recognizes this Mls antigen. One example is the AKR mouse strain, that bears the Mtv-7 genome in which Mls-1a is encoded. In these mice, TCR-Vβ6+ T cells are deleted through negative selection in the thymus. When using donor mice that do not contain the Mtv-7 genome (e.g. C3H), these mice do not express the Mls-1a antigen and accordingly possess functional TCR-Vβ6+ T cells.

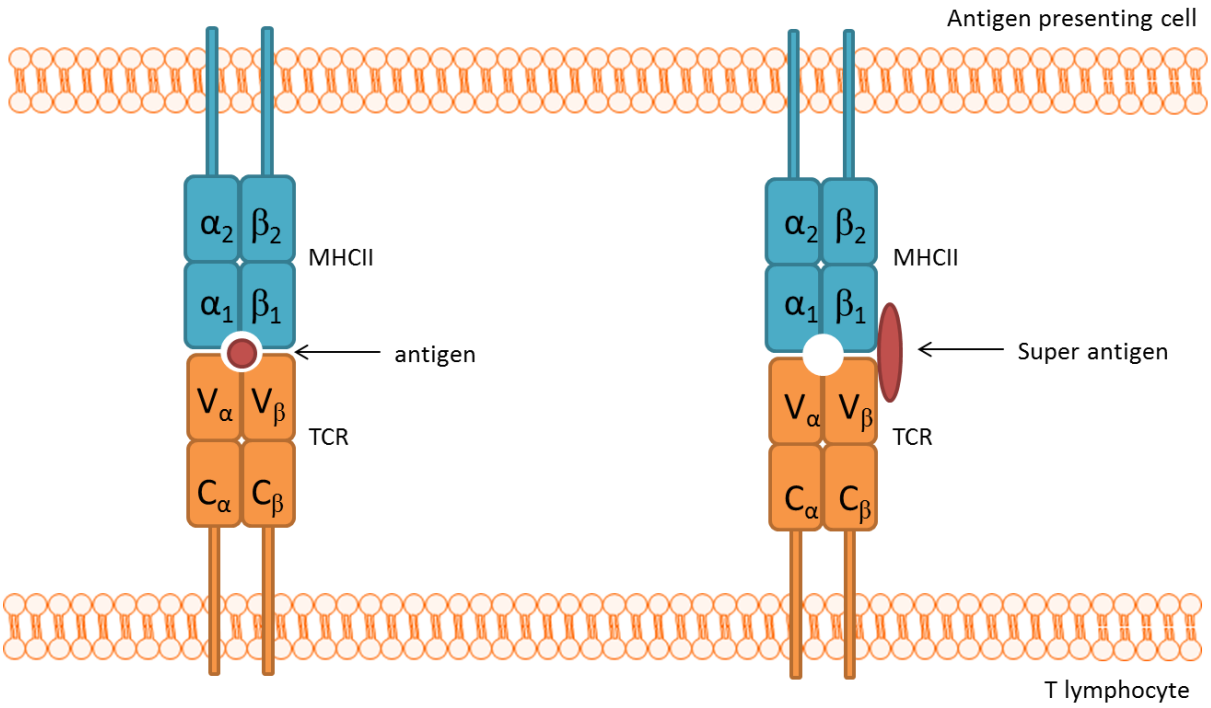


Figure 1.15. Mechanism of T cell stimulation by conventional antigens (left) and superantigens (right). Conventional antigens are presented by MHC and recognized by T cells through binding on both the antigen and the MHC molecule. Superantigens are bound on MHC molecules outside of the specific peptide-presenting region. T cells bind to the superantigens with invariable parts of their Vβ-chains. The mechanism through which Mls antigens stimulate T cells resembles the one used by superantigens.

The mouse model used in this thesis is based on the combination of AKR recipients, deficient of TCR-Vβ6+ T cells and C3H donor mice, that do have TCR-Vβ6+ T cells [148, 149]. Both mouse strains are MHC-matched, bearing H-2K^{k}. When TCR-Vβ6+ T cells from C3H donor mice are transplanted into AKR recipients, which do contain the Mtv-7 genome and thus express the Mls-1a antigen, donor TCR-Vβ6+ T cells recognize recipient Mls-1a antigens (figure 1.16). In this model, donor and recipient mice differ in their expression of the Mtv7-genome, which has been shown to be associated with a highly increased rate and severity of GvHD [150]. The result of a transplantation of C3H bone marrow cells (usually enriched with C3H spleen cells to ensure a high enough percentage of mature T lymphocytes to induce GvHD) into lethally irradiated AKR recipients, is the occurrence of acute GvHD. In mice, symptoms of acute GvHD include ruffled fur, hunched back, lethargy, diarrhea, inflammation of the eyes and weight loss [148, 149].

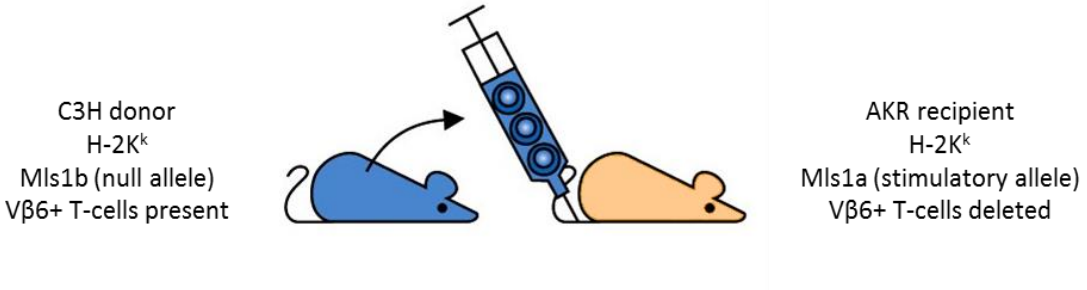


Figure 1.16. Schematic representation of the murine bone marrow transplantation model using C3H donor mice and AKR recipient mice. The mouse strains are MHC-matched (H-2K^k) but differ in the expression of Mls1. C3H mice express Mls1b, a null allele. Vβ6+ T cells are therefore retained in C3H mice. AKR mice express Mls1a, the stimulatory allele. In the latter mice, Vβ6+ T cells are deleted through negative selection in the thymus. When transferring C3H T cells into AKR recipients, Vβ6+ T cells from the C3H donors respond to Mls1a expressed in the recipient and mount an immune response.

References

1. Hughes, J., B. Lunn, and J. O'Brien, *SSRIs and patient groups with specific treatment problems.*, in *Selective Serotonin Reuptake Inhibitors (SSRIs). Past, Present and Future.*, S.C. Stanford, Editor. 1999, RG Landes: Austin, TX, USA. p. 47 - 64.
2. Vaswani, M., F.K. Linda, and S. Ramesh, *Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(1): p. 85-102.
3. Wrobel, S., *Science, serotonin, and sadness: the biology of antidepressants - A series for the public.* Faseb Journal, 2007. **21**(13): p. 3404-3417.
4. Kuhn, R., *The Treatment of Depressive States with G-22355 (Imipramine Hydrochloride).* American Journal of Psychiatry, 1958. **115**(5): p. 459-464.
5. Axelrod, J., L.G. Whitby, and G. Hertting, *Effect of Psychotropic Drugs on Uptake of H3-Norepinephrine by Tissues.* Science, 1961. **133**(345): p. 383-384.
6. Stanford, S.C., ed. *Selective Serotonin Reuptake Inhibitors (SSRIs). Past, Present and Future.* 1999, R.G. Landes Company: Austin, Texas, U.S.A. 221.
7. Siwers, B., et al., *Initial Clinical-Trial Based on Biochemical Methodology of Zimelidine (a Serotonin Uptake Inhibitor) in Depressed-Patients.* Clinical Pharmacology & Therapeutics, 1977. **21**(2): p. 194-200.
8. Wong, D.T., F.P. Bymaster, and E.A. Engleman, *Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication.* Life Sci, 1995. **57**(5): p. 411-41.
9. *http://www.riziv.be/drug/nl/statistics-scientific-information/pharmanet/Statistics-group/2012/pdf/all.pdf*.
10. *Belgisch centrum voor farmacotherapeutische informatie. http://www.bcfi.be.*
11. Bridge, J.A., et al., *Clinical response and risk for reported suicidal ideation and suicide attempts in pediatric antidepressant treatment: a meta-analysis of randomized controlled trials.* JAMA, 2007. **297**(15): p. 1683-96.
12. Leather, H.L., *Drug interactions in the hematopoietic stem cell transplant (HSCT) recipient: what every transplanter needs to know.* Bone Marrow Transplant, 2004. **33**(2): p. 137-52.
13. de Gier, J.J., *Commentaren medicatiebewaking*. 23 ed. 2008-2009: Health Base.
14. Hiemke, C. and S. Hartter, *Pharmacokinetics of selective serotonin reuptake inhibitors.* Pharmacol Ther, 2000. **85**(1): p. 11-28.
15. Uhr, M., M.T. Grauer, and F. Holsboer, *Differential enhancement of antidepressant penetration into the brain in mice with abcb1ab (mdr1ab) P-glycoprotein gene disruption.* Biol Psychiatry, 2003. **54**(8): p. 840-6.
16. Wille, S.M., et al., *Relevant issues in the monitoring and the toxicology of antidepressants.* Crit Rev Clin Lab Sci, 2008. **45**(1): p. 25-89.
17. Hasler, G., *Pathophysiology of depression: do we have any solid evidence of interest to clinicians?* World Psychiatry, 2010. **9**(3): p. 155-61.
18. Berger, M., J.A. Gray, and B.L. Roth, *The expanded biology of serotonin.* Annu Rev Med, 2009. **60**: p. 355-66.
19. Rudnick, G., et al., *The SLC6 transporters: perspectives on structure, functions, regulation, and models for transporter dysfunction.* Pflugers Arch, 2014. **466**(1): p. 25-42.
20. Heils, A., et al., *Allelic variation of human serotonin transporter gene expression.* Journal of Neurochemistry, 1996. **66**(6): p. 2621-2624.
21. Wang, H., et al., *Structural basis for action by diverse antidepressants on biogenic amine transporters.* Nature, 2013. **503**(7474): p. 141-5.

22. Tatsumi, M., et al., *Pharmacological profile of antidepressants and related compounds at human monoamine transporters.* Eur J Pharmacol, 1997. **340**(2-3): p. 249-58.
23. Sanchez, C., *The pharmacology of citalopram enantiomers: the antagonism by R-citalopram on the effect of S-citalopram.* Basic Clin Pharmacol Toxicol, 2006. **99**(2): p. 91-5.
24. Stahl, S.M., *Mechanism of action of serotonin selective reuptake inhibitors. Serotonin receptors and pathways mediate therapeutic effects and side effects.* J Affect Disord, 1998. **51**(3): p. 215-35.
25. Frazer, A. and S. Benmansour, *Delayed pharmacological effects of antidepressants.* Mol Psychiatry, 2002. **7 Suppl 1**: p. S23-8.
26. Lau, T., et al., *Antidepressant-induced internalization of the serotonin transporter in serotonergic neurons.* FASEB J, 2008. **22**(6): p. 1702-14.
27. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777-89.
28. Murphy, K., P. Travers, and M. Walport, *Janeway's immunobiology*. 7th ed. 2008.
29. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.
30. Thakur, P., S. Dadsetan, and A.F. Fomina, *Bidirectional coupling between ryanodine receptors and Ca2+ release-activated Ca2+ (CRAC) channel machinery sustains store-operated Ca2+ entry in human T lymphocytes.* J Biol Chem, 2012. **287**(44): p. 37233-44.
31. Cahalan, M.D., *Cell biology. How to STIMulate calcium channels.* Science, 2010. **330**(6000): p. 43-4.
32. Lewis, R.S., *Calcium signaling mechanisms in T lymphocytes.* Annu Rev Immunol, 2001. **19**: p. 497-521.
33. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
34. Boehning, D., et al., *Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis.* Nat Cell Biol, 2003. **5**(12): p. 1051-61.
35. Meredith, E.J., et al., *Close encounters of the monoamine kind: immune cells betray their nervous disposition.* Immunology, 2005. **115**(3): p. 289-95.
36. Levite, M., *Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors.* Curr Opin Pharmacol, 2008. **8**(4): p. 460-71.
37. Leon-Ponte, M., G.P. Ahern, and P.J. O'Connell, *Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor.* Blood, 2007. **109**(8): p. 3139-46.
38. Yin, J., et al., *5-HT(1B) receptors play a prominent role in the proliferation of T-lymphocytes.* J Neuroimmunol, 2006. **181**(1-2): p. 68-81.
39. Inoue, M., et al., *Regulation of antigen-specific CTL and Th1 cell activation through 5-Hydroxytryptamine 2A receptor.* Int Immunopharmacol, 2011. **11**(1): p. 67-73.
40. Ahern, G.P., *5-HT and the immune system.* Curr Opin Pharmacol, 2011. **11**(1): p. 29-33.
41. Abdouh, M., et al., *5-HT1A-mediated promotion of mitogen-activated T and B cell survival and proliferation is associated with increased translocation of NF-kappa B to the nucleus.* Brain Behavior and Immunity, 2004. **18**(1): p. 24-34.
42. Magrini, E., et al., *Serotonin-mediated tuning of human helper T cell responsiveness to the chemokine CXCL12.* PLoS One, 2011. **6**(8): p. e22482.
43. O'Connell, P.J., et al., *A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells.* Blood, 2006. **107**(3): p. 1010-7.
44. Tsao, C.W., et al., *Glycogen synthase kinase-3beta is critical for interferon-alpha-induced serotonin uptake in human Jurkat T cells.* J Cell Physiol, 2011. **227**(6): p. 2556-66.

45. Tsao, C.W., et al., *Serotonin transporter mRNA expression is decreased by lamivudine and ribavirin and increased by interferon in immune cells.* Scand J Immunol, 2006. **63**(2): p. 106-15.
46. Tsao, C.W., et al., *Interferon-alpha-induced serotonin uptake in Jurkat T cells via mitogen-activated protein kinase and transcriptional regulation of the serotonin transporter.* J Psychopharmacol, 2008. **22**(7): p. 753-60.
47. Kushnir-Sukhov, N.M., et al., *5-hydroxytryptamine induces mast cell adhesion and migration.* J Immunol, 2006. **177**(9): p. 6422-32.
48. Boehme, S.A., et al., *Cutting edge: serotonin is a chemotactic factor for eosinophils and functions additively with eotaxin.* J Immunol, 2004. **173**(6): p. 3599-603.
49. Duerschmied, D., et al., *Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice.* Blood, 2012. **121**(6): p. 1008-15.
50. Muller, T., et al., *5-hydroxytryptamine modulates migration, cytokine and chemokine release and T-cell priming capacity of dendritic cells in vitro and in vivo.* PLoS One, 2009. **4**(7): p. e6453.
51. Antonioli, M., J. Rybka, and L.A. Carvalho, *Neuroimmune endocrine effects of antidepressants.* Neuropsychiatr Dis Treat, 2012. **8**: p. 65-83.
52. Belmaker, R.H. and G. Agam, *Major depressive disorder.* N Engl J Med, 2008. **358**(1): p. 55-68.
53. Howren, M.B., D.M. Lamkin, and J. Suls, *Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis.* Psychosom Med, 2009. **71**(2): p. 171-86.
54. Dowlati, Y., et al., *A Meta-Analysis of Cytokines in Major Depression.* Biological Psychiatry, 2010. **67**(5): p. 446-457.
55. Muller, N. and M.J. Schwarz, *The immune-mediated alteration of serotonin and glutamate: towards an integrated view of depression.* Mol Psychiatry, 2007. **12**(11): p. 988-1000.
56. Loftis, J.M. and P. Hauser, *The phenomenology and treatment of interferon-induced depression.* J Affect Disord, 2004. **82**(2): p. 175-90.
57. Capuron, L., et al., *Treatment of cytokine-induced depression.* Brain Behav Immun, 2002. **16**(5): p. 575-80.
58. Musselman, D.L., et al., *Paroxetine for the prevention of depression induced by high-dose interferon alfa.* N Engl J Med, 2001. **344**(13): p. 961-6.
59. Tyring, S., et al., *Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial.* Lancet, 2006. **367**(9504): p. 29-35.
60. Raison, C.L., et al., *A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers.* JAMA Psychiatry, 2013. **70**(1): p. 31-41.
61. Dickens, C., et al., *Depression in rheumatoid arthritis: a systematic review of the literature with meta-analysis.* Psychosom Med, 2002. **64**(1): p. 52-60.
62. Capuron, L. and A.H. Miller, *Immune system to brain signaling: neuropsychopharmacological implications.* Pharmacol Ther, 2011. **130**(2): p. 226-38.
63. Abdul, M., C.J. Logothetis, and N.M. Hoosein, *Growth-inhibitory effects of serotonin uptake inhibitors on human prostate carcinoma cell lines.* J Urol, 1995. **154**(1): p. 247-50.
64. Martino, M., et al., *Immunomodulation Mechanism of Antidepressants: Interactions between Serotonin/Norepinephrine Balance and Th1/Th2 Balance.* Current Neuropharmacology, 2012. **10**(2): p. 97-123.
65. Maes, M., et al., *Negative immunoregulatory effects of antidepressants: inhibition of interferon-gamma and stimulation of interleukin-10 secretion.* Neuropsychopharmacology, 1999. **20**(4): p. 370-9.
66. Kubera, M., et al., *Anti-Inflammatory effects of antidepressants through suppression of the interferon-gamma/interleukin-10 production ratio.* J Clin Psychopharmacol, 2001. **21**(2): p. 199-206.

67. Miller, A.H., V. Maletic, and C.L. Raison, *Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression.* Biol Psychiatry, 2009. **65**(9): p. 732-41.
68. Basterzi, A.D., et al., *IL-6 levels decrease with SSRI treatment in patients with major depression.* Hum Psychopharmacol, 2005. **20**(7): p. 473-6.
69. Sluzewska, A., et al., *Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine.* Ann N Y Acad Sci, 1995. **762**: p. 474-6.
70. O'Brien, S.M., L.V. Scott, and T.G. Dinan, *Antidepressant therapy and C-reactive protein levels.* Br J Psychiatry, 2006. **188**: p. 449-52.
71. Sutcigil, L., et al., *Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy.* Clin Dev Immunol, 2007. **2007**: p. 76396.
72. Tuglu, C., et al., *Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder.* Psychopharmacology (Berl), 2003. **170**(4): p. 429-33.
73. Vogelzangs, N., et al., *Association of depressive disorders, depression characteristics and antidepressant medication with inflammation.* Transl Psychiatry, 2012. **2**: p. e79.
74. Xia, Z., J.W. DePierre, and L. Nassberger, *Tricyclic antidepressants inhibit IL-6, IL-1 beta and TNF-alpha release in human blood monocytes and IL-2 and interferon-gamma in T cells.* Immunopharmacology, 1996. **34**(1): p. 27-37.
75. Taler, M., et al., *Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression.* Eur Neuropsychopharmacol, 2007. **17**(12): p. 774-80.
76. Shenoy, A.R., et al., *Citalopram suppresses thymocyte cytokine production.* J Neuroimmunol, 2013. **262**(1-2): p. 46-52.
77. Edgar, V.A., et al., *Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation.* Eur J Pharmacol, 1999. **372**(1): p. 65-73.
78. Berkeley, M.B., et al., *In vitro effects of cocaine, lidocaine and monoamine uptake inhibitors on lymphocyte proliferative responses.* Immunopharmacol Immunotoxicol, 1994. **16**(2): p. 165-78.
79. Fazzino, F., et al., *Serotonin transporter is differentially localized in subpopulations of lymphocytes of major depression patients. Effect of fluoxetine on proliferation.* J Neuroimmunol, 2008. **196**(1-2): p. 173-80.
80. Edgar, V.A., et al., *Fluoxetine action on murine T-lymphocyte proliferation: participation of PKC activation and calcium mobilisation.* Cell Signal, 1998. **10**(10): p. 721-6.
81. Taler, M., et al., *Evidence for an inhibitory immunomodulatory effect of selected antidepressants on rat splenocytes: possible relevance to depression and hyperactive-immune disorders.* Int Immunopharmacol, 2008. **8**(4): p. 526-33.
82. Amit, B.H., et al., *Proapoptotic and chemosensitizing effects of selective serotonin reuptake inhibitors on T cell lymphoma/leukemia (Jurkat) in vitro.* Eur Neuropsychopharmacol, 2009. **19**(10): p. 726-34.
83. Pellegrino, T.C. and B.M. Bayer, *Specific serotonin reuptake inhibitor-induced decreases in lymphocyte activity require endogenous serotonin release.* Neuroimmunomodulation, 2000. **8**(4): p. 179-87.
84. Pellegrino, T.C. and B.M. Bayer, *Modulation of immune cell function following fluoxetine administration in rats.* Pharmacol Biochem Behav, 1998. **59**(1): p. 151-7.
85. Frick, L.R., et al., *Inhibitory effect of fluoxetine on lymphoma growth through the modulation of antitumor T-cell response by serotonin-dependent and independent mechanisms.* Biochem Pharmacol, 2008. **75**(9): p. 1817-26.
86. Gobin, V., et al., *Fluoxetine reduces murine graft-versus-host disease by induction of T cell immunosuppression.* J Neuroimmune Pharmacol, 2013. **8**(4): p. 934-43.
87. Xia, Z., et al., *Tricyclic antidepressants induce apoptosis in human T lymphocytes.* Int J Immunopharmacol, 1997. **19**(11-12): p. 645-54.

88. Frick, L.R., et al., *Oral administration of fluoxetine alters the proliferation/apoptosis balance of lymphoma cells and up-regulates T cell immunity in tumor-bearing mice.* Eur J Pharmacol, 2011. **659**(2-3): p. 265-72.
89. Serafeim, A., et al., *Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells.* Blood, 2003. **101**(8): p. 3212-9.
90. Schuster, C., et al., *Selective serotonin reuptake inhibitors--a new modality for the treatment of lymphoma/leukaemia?* Biochem Pharmacol, 2007. **74**(9): p. 1424-35.
91. Gordon, J., et al., *In response to "Selective serotonin reuptake inhibitors--a new modality for the treatment of lymphoma/leukaemia?" by C. Schuster, et al. [Biochem. Pharmacol. 74 (9) 2007 1424-1435].* Biochem Pharmacol, 2008. **75**(6): p. e1-3; author reply e4-5.
92. Cloonan, S.M., et al., *The antidepressants maprotiline and fluoxetine have potent selective antiproliferative effects against Burkitt lymphoma independently of the norepinephrine and serotonin transporters.* Leuk Lymphoma, 2010. **51**(3): p. 523-39.
93. Gobin, V., et al., *Fluoxetine Reduces Murine Graft-Versus-Host Disease by Induction of T cell Immunosuppression.* J Neuroimmune Pharmacol, 2013.
94. Chamba, A., et al., *SLC6A4 expression and anti-proliferative responses to serotonin transporter ligands chlomipramine and fluoxetine in primary B-cell malignancies.* Leuk Res, 2010. **34**(8): p. 1103-6.
95. Serafeim, A., et al., *5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors.* Blood, 2002. **99**(7): p. 2545-53.
96. Aune, T.M., H.W. Golden, and K.M. McGrath, *Inhibitors of serotonin synthesis and antagonists of serotonin 1A receptors inhibit T lymphocyte function in vitro and cell-mediated immunity in vivo.* J Immunol, 1994. **153**(2): p. 489-98.
97. Fanburg, B.L. and S.L. Lee, *A new role for an old molecule: serotonin as a mitogen.* Am J Physiol, 1997. **272**(5 Pt 1): p. L795-806.
98. Barkan, T., et al., *Biochemical and pharmacological characterization of the serotonin transporter in human peripheral blood lymphocytes.* Eur Neuropsychopharmacol, 2004. **14**(3): p. 237-43.
99. Miolo, G., et al., *Photoisomerization of fluvoxamine generates an isomer that has reduced activity on the 5-hydroxytryptamine transporter and does not affect cell proliferation.* European Journal of Pharmacology, 2002. **450**(3): p. 223-229.
100. Ferriere, F., et al., *Characterisation of serotonin transport mechanisms in rainbow trout peripheral blood lymphocytes: role in PHA-induced lymphoproliferation.* Developmental and Comparative Immunology, 1999. **23**(1): p. 37-50.
101. Chamba, A., et al., *Characterisation of the endogenous human peripheral serotonin transporter SLC6A4 reveals surface expression without N-glycosylation.* J Neuroimmunol, 2008. **204**(1-2): p. 75-84.
102. Diamond, M., J.P. Kelly, and T.J. Connor, *Antidepressants suppress production of the Th1 cytokine interferon-gamma, independent of monoamine transporter blockade.* Eur Neuropsychopharmacol, 2006. **16**(7): p. 481-90.
103. Walther, D.J., et al., *Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release.* Cell, 2003. **115**(7): p. 851-62.
104. Hofstetter, H.H., et al., *Absence of reuptake of serotonin influences susceptibility to clinical autoimmune disease and neuroantigen-specific interferon-gamma production in mouse EAE.* Clin Exp Immunol, 2005. **142**(1): p. 39-44.
105. Bischoff, S.C., et al., *Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(3): p. G685-95.

106. Thompson, B.J., et al., *Transgenic elimination of high-affinity antidepressant and cocaine sensitivity in the presynaptic serotonin transporter.* Proc Natl Acad Sci U S A, 2011. **108**(9): p. 3785-90.
107. Baganz, N.L. and R.D. Blakely, *A dialogue between the immune system and brain, spoken in the language of serotonin.* ACS Chem Neurosci, 2012. **4**(1): p. 48-63.
108. Heijink, I.H., et al., *Sensitivity of IL-5 production to the cAMP-dependent pathway in human T cells is reduced by exogenous IL-2 in a phosphoinositide 3-kinase-dependent way.* Eur J Immunol, 2003. **33**(8): p. 2206-15.
109. Kenis, G., et al., *Influence of antidepressants on intracellular levels of cyclic adenosine monophosphate in human peripheral blood mononuclear cells.* Eur Neuropsychopharmacol, 2003. **13**(1): p. 53-6.
110. Maes, M., et al., *The negative immunoregulatory effects of fluoxetine in relation to the cAMP-dependent PKA pathway.* Int Immunopharmacol, 2005. **5**(3): p. 609-18.
111. Feske, S., E.Y. Skolnik, and M. Prakriya, *Ion channels and transporters in lymphocyte function and immunity.* Nat Rev Immunol, 2012. **12**(7): p. 532-47.
112. Xia, Z., J.W. DePierre, and L. Nassberger, *Dysregulation of bcl-2, c-myc, and Fas expression during tricyclic antidepressant-induced apoptosis in human peripheral lymphocytes.* J Biochem Toxicol, 1996. **11**(4): p. 203-4.
113. Xia, Z., et al., *The antidepressants imipramine, clomipramine, and citalopram induce apoptosis in human acute myeloid leukemia HL-60 cells via caspase-3 activation.* J Biochem Mol Toxicol, 1999. **13**(6): p. 338-47.
114. Xia, Z., et al., *Changes in the generation of reactive oxygen species and in mitochondrial membrane potential during apoptosis induced by the antidepressants imipramine, clomipramine, and citalopram and the effects on these changes by Bcl-2 and Bcl-X(L).* Biochem Pharmacol, 1999. **57**(10): p. 1199-208.
115. Orrenius, S., B. Zhivotovsky, and P. Nicotera, *Regulation of cell death: the calcium-apoptosis link.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 552-65.
116. Krishnan, A., et al., *Fluoxetine mediates G0/G1 arrest by inducing functional inhibition of cyclin dependent kinase subunit (CKS)1.* Biochem Pharmacol, 2008. **75**(10): p. 1924-34.
117. Meredith, E.J., et al., *The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential anti-tumor target for psychotropics.* FASEB J, 2005. **19**(9): p. 1187-9.
118. Daley, E., et al., *Chlorimipramine: a novel anticancer agent with a mitochondrial target.* Biochem Biophys Res Commun, 2005. **328**(2): p. 623-32.
119. Eisensamer, B., et al., *Antidepressants and antipsychotic drugs colocalize with 5-HT3 receptors in raft-like domains.* Journal of Neuroscience, 2005. **25**(44): p. 10198-10206.
120. Donati, R.J. and M.M. Rasenick, *Chronic antidepressant treatment prevents accumulation of gsalpha in cholesterol-rich, cytoskeletal-associated, plasma membrane domains (lipid rafts).* Neuropsychopharmacology, 2005. **30**(7): p. 1238-45.
121. van der Merwe, P.A. and O. Dushek, *Mechanisms for T cell receptor triggering.* Nature Reviews Immunology, 2011. **11**(1): p. 47-55.
122. Pariante, C.M., et al., *Antidepressants enhance glucocorticoid receptor function in vitro by modulating the membrane steroid transporters.* British Journal of Pharmacology, 2001. **134**(6): p. 1335-1343.
123. Taler, M., et al., *The Immunomodulatory Effect of the Antidepressant Sertraline in an Experimental Autoimmune Encephalomyelitis Mouse Model of Multiple Sclerosis.* Neuroimmunomodulation, 2011. **18**(2): p. 117-122.
124. Vollmar, P., et al., *The antidepressant venlafaxine ameliorates murine experimental autoimmune encephalomyelitis by suppression of pro-inflammatory cytokines.* Int J Neuropsychopharmacol, 2009. **12**(4): p. 525-36.

125. Yuan, X.Q., et al., *Fluoxetine Promotes Remission in Acute Experimental Autoimmune Encephalomyelitis in Rats.* Neuroimmunomodulation, 2012. **19**(4): p. 201-208.
126. Sacre, S., et al., *Fluoxetine and citalopram exhibit potent antiinflammatory activity in human and murine models of rheumatoid arthritis and inhibit toll-like receptors.* Arthritis Rheum, 2010. **62**(3): p. 683-93.
127. Baharav, E., et al., *Immunomodulatory Effect of Sertraline in a Rat Model of Rheumatoid Arthritis.* Neuroimmunomodulation, 2012. **19**(5): p. 309-318.
128. Kubera, M., et al., *Inhibitory effect of antidepressant drugs on contact hypersensitivity reaction.* Pharmacol Rep, 2012. **64**(3): p. 714-22.
129. Curzytek, K., et al., *Inhibition of 2,4-dinitrofluorobenzene-induced contact hypersensitivity reaction by antidepressant drugs.* Pharmacological Reports, 2013. **65**(5): p. 1237-1246.
130. Koh, S.J., et al., *Fluoxetine inhibits NF-kappaB signaling in intestinal epithelial cells and ameliorates experimental colitis and colitis-associated colon cancer in mice.* Am J Physiol Gastrointest Liver Physiol, 2011. **301**(1): p. G9-19.
131. Guemei, A.A., et al., *Do desipramine [10,11-dihydro-5-[3-(methylamino) propyl]-5H-dibenz[b,f]azepine monohydrochloride] and fluoxetine [N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-propan-1-amine] ameliorate the extent of colonic damage induced by acetic acid in rats?* J Pharmacol Exp Ther, 2008. **327**(3): p. 846-50.
132. Roumestan, C., et al., *Anti-inflammatory properties of desipramine and fluoxetine.* Respir Res, 2007. **8**: p. 35.
133. Mostert, J.P., et al., *Effects of fluoxetine on disease activity in relapsing multiple sclerosis: a double-blind, placebo-controlled, exploratory study.* J Neurol Neurosurg Psychiatry, 2008. **79**(9): p. 1027-31.
134. Bird, H. and M. Broggini, *Paroxetine versus amitriptyline for treatment of depression associated with rheumatoid arthritis: a randomized, double blind, parallel group study.* J Rheumatol, 2000. **27**(12): p. 2791-7.
135. Krishnadas, R. and J. Cavanagh, *Sustained remission of rheumatoid arthritis with a specific serotonin reuptake inhibitor antidepressant: a case report and review of the literature.* J Med Case Reports, 2011. **5**: p. 112.
136. Prevoo, M.L., et al., *Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis.* Arthritis Rheum, 1995. **38**(1): p. 44-8.
137. Keystone, E., et al., *A clinical study of very low dose prednisolone and paroxetine for active rheumatoid arthritis.* Arthritis and Rheumatism, 2007. **56**(9): p. S395-396.
138. Arnett, F.C., et al., *The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.* Arthritis Rheum, 1988. **31**(3): p. 315-24.
139. Shlomchik, W.D., *Graft-versus-host disease.* Nat Rev Immunol, 2007. **7**(5): p. 340-52.
140. Schroeder, M.A. and J.F. DiPersio, *Mouse models of graft-versus-host disease: advances and limitations.* Dis Model Mech, 2011. **4**(3): p. 318-33.
141. Ferrara, J.L., et al., *Graft-versus-host disease.* Lancet, 2009. **373**(9674): p. 1550-61.
142. Reddy, P. and J.L.M. Ferrara, *Mouse models of graft-versus-host disease.* 2008.
143. Abo-Zena, R.A. and M.E. Horwitz, *Immunomodulation in stem-cell transplantation.* Curr Opin Pharmacol, 2002. **2**(4): p. 452-7.
144. Pidala, J., *Graft-vs-host disease following allogeneic hematopoietic cell transplantation.* Cancer Control, 2011. **18**(4): p. 268-76.
145. Glotzbecker, B., et al., *Important Drug Interactions in Hematopoietic Stem Cell Transplantation: What Every Physician Should Know.* Biology of Blood and Marrow Transplantation, 2012. **18**(7): p. 989-1006.

146. Hurley, C.K., M. Fernandez Vina, and M. Setterholm, *Maximizing optimal hematopoietic stem cell donor selection from registries of unrelated adult volunteers.* Tissue Antigens, 2003. **61**(6): p. 415-24.
147. Llewelyn, M. and J. Cohen, *Superantigens: microbial agents that corrupt immunity.* Lancet Infect Dis, 2002. **2**(3): p. 156-62.
148. Billiau, A.D., et al., *Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-leukemia responses in mice receiving allogeneic bone marrow transplants.* Blood, 2002. **100**(5): p. 1894-1902.
149. Sefrioui, H., A.D. Billiau, and M. Waer, *Graft-versus-leukemia effect in minor antigen mismatched chimeras given delayed donor leucocyte infusion: immunoregulatory aspects and role of donor T and ASGM1-positive cells.* Transplantation, 2000. **70**(2): p. 348-53.
150. Miconnet, I., et al., *Critical role of endogenous Mtv in acute lethal graft-versus-host disease.* Eur J Immunol, 1995. **25**(2): p. 364-8.

CHAPTER 2

AIMS AND OVERVIEW

CHAPTER 2. AIMS AND OVERVIEW

Pharmacological treatment of depression and mood disorders is most commonly based on antidepressants. Within the group of antidepressants, selective serotonin reuptake inhibitors (SSRIs) are most frequently prescribed, due to their efficacy, tolerability and safety [1]. Since their marketing, SSRIs have been shown not only to induce changes in the central nervous system, but also in the immune system. This project was initiated upon a study conducted by Denys *et al.* investigating the immunological changes in patients with obsessive-compulsive disorder (OCD) and the impact SSRIs and SNRIs (together SRIs) exert on them [2, 3]. In this study, it was observed that some of the patients treated with SRIs showed a complete inhibition of peripheral blood T lymphocyte proliferation. In one patient, the absence of an *in vitro* proliferative response was accompanied by the clinical manifestation of sinusitis, which persisted despite 3 antibiotic regimens [4]. Upon cessation of the antidepressant treatment, the sinusitis ceased. These findings raised the question whether SRIs were responsible for the observed immunosuppression. Accordingly, this project aimed at resolving this question. In the study conducted by Denys *et al.* paroxetine and venlafaxine were analyzed as these were prescribed most frequently to OCD patients at the time. However, immunosuppressive effects had been described for several SSRIs and therefore, we decided to investigate the immunological effects of the entire class of SSRIs. Venlafaxine, although a SNRI, was also retained in the analyses.

The first aim of this PhD thesis was to characterize the effects of SSRIs on the immune system. More specifically, we analyzed the effects of SSRIs on T cell proliferation and viability. These experiments were carried out *in vitro* using freshly isolated human lymphocytes. The results of these experiments are described in **chapter 3**. In the next phase of this research, we attempted to unravel the mechanism behind the observed immunosuppressive effects. Two hypotheses were investigated, namely the impact on [1] serotonin transporter expression and [2] calcium signaling in T lymphocytes. Equally, these experiments were performed *in vitro* using freshly isolated human lymphocytes or an immortalized human T lymphocyte cell line. The results of these experiments are described in **chapters 4 and 5**, respectively.

As the first phase of this research demonstrated that SSRIs possess interesting immunosuppressive characteristics, we and others hypothesized that SSRIs might be reinvented as a novel class of immunosuppressants. The application of SSRIs in several autoimmune disorders such as multiple sclerosis and rheumatoid arthritis has been investigated by others and the majority of research indeed demonstrated a beneficial effect of SSRIs. We decided to investigate whether SSRIs, and

fluoxetine in particular, could be used as a preventive and/or therapeutic strategy for acute graft-versus-host disease. To this end, we used a murine bone marrow transplantation model that closely resembles the human situation. The results of these experiments are described in **chapter 6**.

Throughout this thesis, the immunosuppressive properties of SSRIs became clear and the possible reinvention of this class of drugs as immunosuppressants was investigated. Some considerations with respect to the feasibility of using this class of drugs as immunosuppressants in the clinic are enumerated in **chapter 7**. Further, the consequences of the immunosuppressive effects for the use of SSRIs as antidepressants in the clinical practice are discussed. Finally, a perspective on future steps in the development of SSRIs as immunosuppressants is given.

References

1. Stafford, R.S., E.A. MacDonald, and S.N. Finkelstein, *National Patterns of Medication Treatment for Depression, 1987 to 2001.* Prim Care Companion J Clin Psychiatry, 2001. **3**(6): p. 232-235.
2. Denys, D., et al., *Decreased TNF-alpha and NK activity in obsessive-compulsive disorder.* Psychoneuroendocrinology, 2004. **29**(7): p. 945-952.
3. Denys, D., et al., *Effects of paroxetine and venlafaxine on immune parameters in patients with obsessive compulsive disorder.* Psychoneuroendocrinology, 2006. **31**(3): p. 355-60.
4. Denys, D., et al., *A case of venlafaxine-induced inhibition of T-lymphocyte proliferation.* Psychopharmacology (Berl), 2002. **164**(4): p. 432.

CHAPTER 3

CHARACTERIZATION OF THE IN VITRO EFFECTS OF SRI'S ON THE PROLIFERATION AND VIABILITY OF HUMAN T LYMPHOCYTES

Based on "*Fluoxetine reduces murine graft-versus-host disease by induction of T cell immunosuppression*." Veerle Gobin, Katleen Van Steendam, Sabine Fevery, Kelly Tilleman, An Billiau, Damiaan Denys* and Dieter Deforce*. J Neuroimmune Pharmacol, 2013; 8: 934-43.

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CHAPTER 3. CHARACTERIZATION OF THE IN VITRO EFFECTS OF SRI'S ON THE PROLIFERATION AND VIABILITY OF HUMAN T LYMPHOCYTES

Abstract

Serotonin reuptake inhibitors (SRIs) are widely used drugs in the treatment of depression and anxiety disorders. Although SRIs are generally regarded as safe drugs with relatively few side effects, literature suggests that high concentrations of SRIs may alter immune function. In this study, we analyzed the direct *in vitro* effect of six SRIs on the viability and proliferation of human peripheral T lymphocytes and found an anti-proliferative and pro-apoptotic effect that was significantly larger in activated than in resting T cells. We discuss these results in the light of potential future exploration of SRIs as a novel class of T cell immunosuppressive drugs.

1. Introduction

Serotonin reuptake inhibitors (SRIs) belong to the most frequently prescribed drugs worldwide. While originally introduced to treat major depressive disorder, they have proven to be effective in a number of psychiatric and neurological conditions such as obsessive-compulsive disorder, panic disorder and generalized anxiety disorder [1, 2]. In the past decades, it has become clear that SRIs not only affect biological mechanisms within the central nervous system, but also have an influence on immunity. Several *in vivo* and *in vitro* reports have demonstrated a negative effect of SRIs on mitogen-induced lymphocyte proliferation [3-9], pro-inflammatory cytokine secretion [8, 10-13] and lymphocyte viability [8, 10, 14]. Although it is clear that several research groups have investigated the anti-proliferative and pro-apoptotic effects of SRIs, variation in the SRIs studied, the concentrations used and the experimental read-out hampers comparison between studies and interpretation of results. Therefore, a comprehensive study comparing the anti-proliferative and pro-apoptotic effects of all available SRIs in both activated and resting human T lymphocytes would contribute to our understanding of the potential immunomodulatory effects of SRI treatment. Thus, the aim of this study was to determine and compare the direct *in vitro* effects of six different SRIs used in clinical practice (paroxetine, fluoxetine, sertraline, fluvoxamine, citalopram and venlafaxine) on the viability and proliferation of T lymphocytes from healthy human subjects. Whereas the first five compounds listed are SSRIs, venlafaxine is a mixed serotonin and noradrenalin reuptake inhibitor (SNRI), and was included in the study since it has also been shown to induce immunosuppression [15-17]. We found clear *in vivo* and *in vitro* evidence that SRIs may alter T cell responsiveness.

2. Materials and methods

2.1. Reagents

Citalopram, sertraline, fluvoxamine and venlafaxine were purchased from Sigma Aldrich (St-Louis, MO, USA). Paroxetine was purchased from Fagron (Nieuwerkerk a/d IJssel, The Netherlands), and fluoxetine from ABC chemicals (Wouters-Brakel, Belgium). The drugs were diluted in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin (100 U/ml penicillin G; 100 µg/ml streptomycin). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Apoptosis assay

Human peripheral blood mononuclear cells (PBMCs) from six healthy volunteers were obtained by Ficoll density centrifugation. T cells were isolated from PBMCs using a human T cell enrichment kit containing tetrameric antibody complexes recognizing CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123 and glycophorin A (STEMCELL technologies, Vancouver, Canada) according to the manufacturer's instructions. T cell purity was determined by staining with anti-CD3 PECy5 and flow cytometric analysis and was in each experiment greater than 97%. Anti-CD3/CD28 beads (Dynabeads® Human T-activator CD3/CD28, Life Technologies) were added in a 1:1 ratio and $2x10^5$ cells were seeded per well in a total volume of 200 µl. After a 24h-incubation in the presence of SRIs, cells were stained with 0.5 µl annexin V-FITC and 10 µl propidium iodide (PI) (BD Pharmingen, San Diego, CA, USA). Activation status was determined by staining with anti-CD69 PECy7 (eBiosciences, San Diego, CA, USA).

2.3. Proliferation assay

Isolated PBMCs from six healthy volunteers were stained with 10 µM carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, cells were activated with $4x10^5$ anti-CD3/CD28 beads per 10^6 PBMCs. $25x10^4$ PBMCs were seeded per well in a total volume of 250 µl and incubated for 6 days in the presence of SRIs. Thereafter, cells were stained with anti-CD3 PECy5 (eBiosciences, San Diego, CA, USA). All analyses were performed on a Cytomics FC500 flow cytometer (Beckman Coulter, Miami Florida). Dead cells were excluded based on FSC-SSC properties. For CD3+ T cells, a proliferation index (PI) was calculated according to the following formula [18]:

$$\text{Proliferation index} = \frac{\sum_0^i N_i}{\sum_0^i N_i / 2^i}$$

With i = generation number (0 is the undivided population) and Ni = the number of events in generation i. Using control samples without SRIs, the maximal proliferative response was determined. Relative to these samples, the percentage inhibition by SRIs was calculated. All *in vitro* experiments were approved by the Ethical Committee of the Ghent University Hospital.

2.4. Statistics

Wilcoxon signed ranks tests were used to identify statistically significant differences between treatment and control, and between activated and resting T cells. Results were considered statistically significant if one-tailed *p*-values were <0.05.

3. Results

3.1. Activated human T cells are more sensitive to SRI-induced apoptosis than resting T cells

We investigated the viability of both resting and activated T cells, obtained from 6 healthy human volunteers, when exposed to SRIs *in vitro*. In this study, we incorporated the most frequently used SRIs (paroxetine, fluoxetine, sertraline, citalopram, fluvoxamine and venlafaxine). T cells were stimulated using anti-CD3/CD28 beads and the activation status of the cells was confirmed by the expression of CD69. More than 80% of the T cells were CD69 positive after a 24h-incubation with anti-CD3/CD28 beads ('activated T cells'). In contrast, less than 2% of the T cells that were not stimulated expressed CD69 ('resting T cells').

To detect apoptotic cells, annexin V and PI staining was performed. PI staining correlated well with annexin V data (correlation coefficients were typically >0.99) and therefore, only annexin V data were further used for data analysis. The mean percentage annexin V+ cells in control samples (n=6) was 4.05 ± 1.72% for resting T cells and 5.99 ± 4.65% for activated T cells. In order to compare apoptosis rates in resting and activated T cells, annexin V+ percentages obtained in control samples were subtracted from the individual percentages determined in the test samples. Thus, differences in SRI-induced apoptosis found between resting and activated T cells cannot be ascribed to differences in basal apoptosis.

Detailed analysis of the annexin V+ T cell percentages revealed that paroxetine (p= 0.016) and sertraline (p=0.031) significantly induced apoptosis in activated T cells at 5 µM, while for the other SRIs, no apoptosis could be detected at this concentration. For fluoxetine on the other hand, apoptosis could be detected at concentrations of 10 µM or higher in activated T cells (p=0.016). Fluvoxamine showed a similar effect, but at a higher concentration range: apoptosis started to appear at concentrations of 50 µM (p=0.016). Citalopram only induced a slight increase in apoptotic cells, and statistical significance was reached at 100 µM only (p=0.016). No apoptosis could be detected after treatment with venlafaxine at concentrations up to 100 µM.

Interestingly, activated T cells were more sensitive to the apoptotic effect compared to resting T cells (figure 3.1). Paroxetine, fluoxetine, sertraline and fluvoxamine induced significantly more apoptosis in activated T cells than in resting T cells. For paroxetine, already the lowest tested concentration (5 µM) induced significantly more apoptosis in the activated cells (p=0.031). This difference was maintained with higher concentrations (10 µM, p=0.016 and 20 µM, p=0.016). For fluoxetine (p=0.016) and sertraline (p=0.031), a significantly higher effect could be detected in the activated T

cells at 10 µM. Fluoxetine maintained this significantly higher induction of apoptosis at 20 µM (p=0.031). In contrast, sertraline did not exert a differential effect on activated and resting T cells at the highest concentration tested (20 µM). The absence of a significant difference at this concentration might be due to an increased cytotoxicity of this high concentration of sertraline. Fluvoxamine induced significantly more apoptosis in the activated T cells at the 2 highest concentrations tested (50 µM, p=0.016 and 100 µM, p=0.016). Citalopram did not exert a differential effect on activated versus resting T cells, however this may be due to the fact that citalopram only induced a very low percentage of apoptosis in both cell populations. No apoptosis could be detected after treatment with venlafaxine in concentrations up to 100 µM.

3.2. SRIs can inhibit T cell proliferation at concentrations that do not affect T cell viability

In order to evaluate the effect of SRIs on T cell proliferation, PBMCs from healthy volunteers were labelled with CFSE, activated with anti-CD3/CD28 beads and incubated for 6 days in the presence of SRIs. The amount of T cells in each cell cycle was determined by flow cytometry and the results were expressed as a proliferation index.

All SRIs tested decreased the proliferation index in a concentration-dependent manner (figure 3.2). Paroxetine exerted an anti-proliferative effect at 10 µM (p=0.016). Fluoxetine and sertraline significantly decreased the proliferation index at concentrations as low as 1 µM (p=0.018 and p=0.047 respectively). Fluvoxamine and citalopram significantly decreased T cell proliferation at 2 µM (lowest dose tested, p=0.029 and p=0.016 respectively). For venlafaxine, higher doses were needed in order to reduce T cell proliferation: a significant decrease for venlafaxine was detected only at 20 µM (p=0.047). Importantly, the SRI concentrations needed to reduce T cell proliferation were, except for paroxetine, below those inducing apoptosis in activated and/or resting T cells (gray background in figure 3.2).

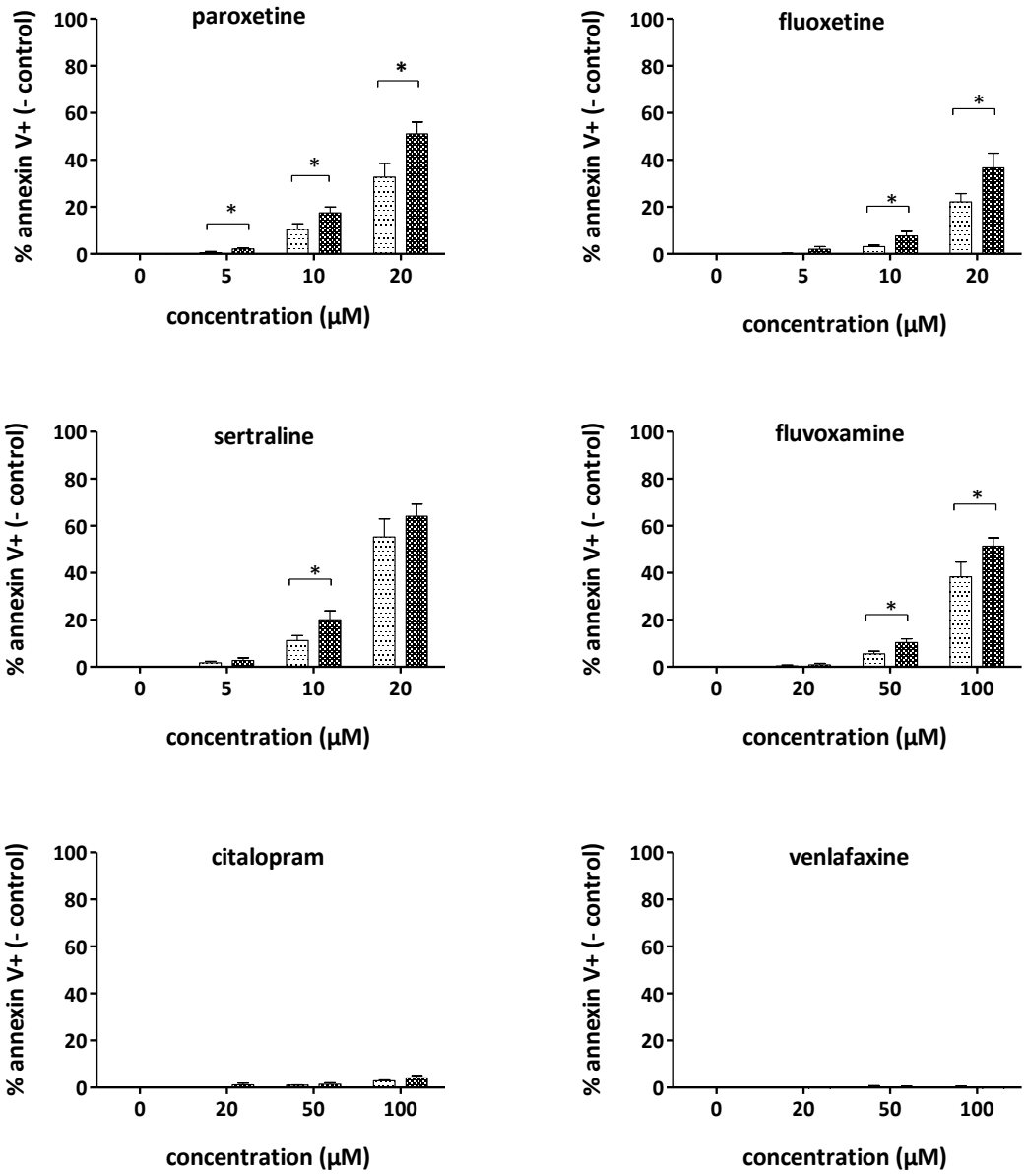

Figure 3.1. Apoptotic effect of SRIs on activated and resting T cells. *In vitro* activated and resting T cells were incubated for 24h with 0-20 µM paroxetine, 0-20 µM fluoxetine, 0-20 µM sertraline, 0-100 µM fluvoxamine, 0-100 µM citalopram, 0-100 µM venlafaxine and subsequently analyzed by annexin V staining. Mean ± SEM percentages of annexin V+ cells (- control) in activated and resting T cells are shown (n=6). ( ) = T cells activated with anti-CD3/CD28 beads in a 1:1 bead:cell ratio; ( ) = resting T cells. Statistically significant differences between activated and resting T cells are depicted with * (one-tailed $p<0.05$).

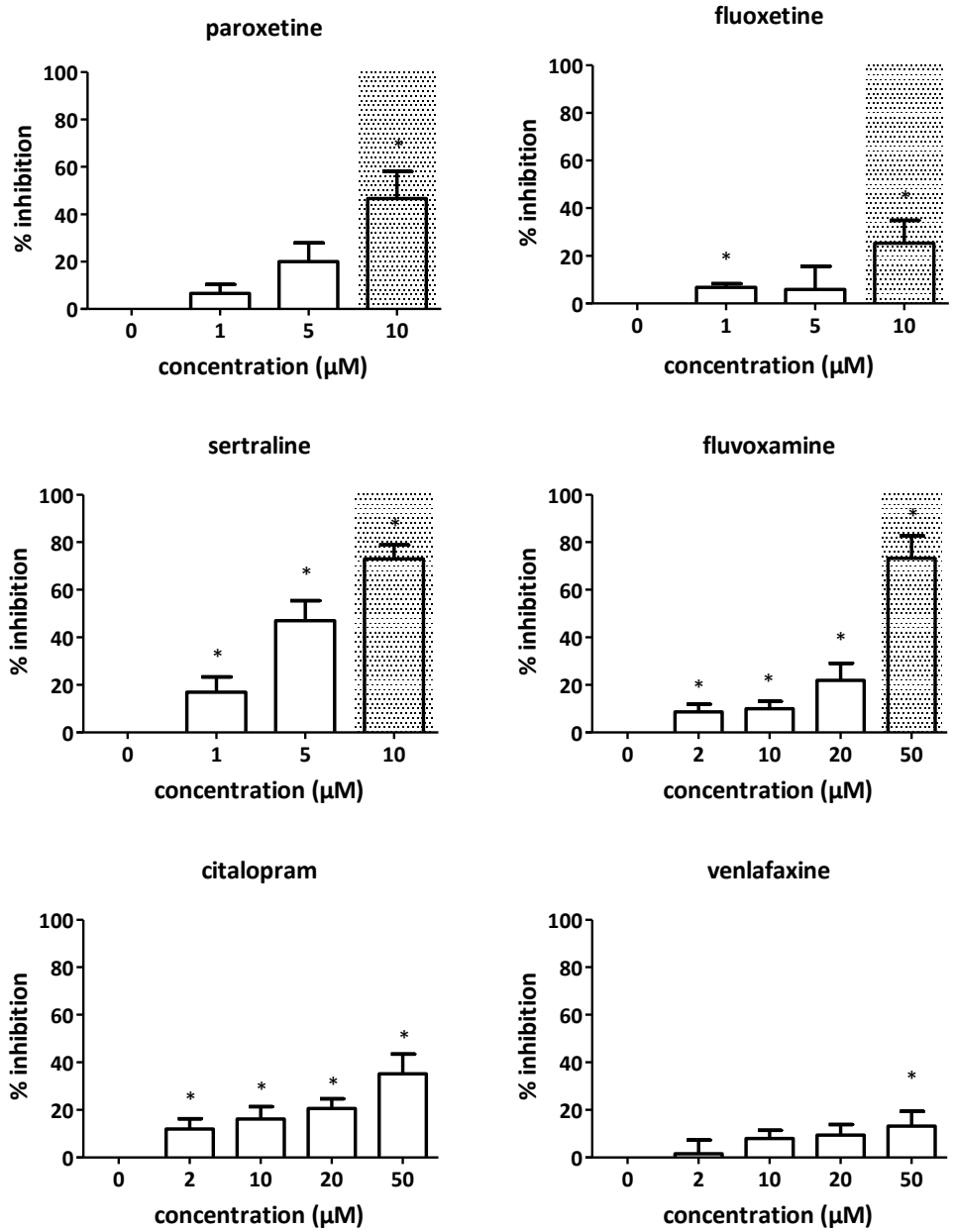

Figure 3.2. Inhibition of T cell proliferation by SRIs. PBMCs were labelled with CFSE, activated with $4x10^5$ anti-CD3/CD28 beads per 10^6 cells and incubated in the presence of 0-10 µM paroxetine, 0-10 µM fluoxetine, 0-10 µM sertraline, 0-50 µM fluvoxamine, 0-50 µM citalopram, 0-50 µM venlafaxine for 6 days. Viable CD3+ cells were gated and proliferation indices were calculated based on the number of cells in each division peak. Values are expressed as % inhibition in comparison to control cells not exposed to SRIs. Mean ± SEM % inhibition of 6 individual experiments are shown. Concentrations that induce more than 5% apoptosis in activated and/or resting T cells are displayed with gray background. Statistically significant differences in proliferation indices compared to control cells not exposed to SRIs are depicted with * (one-tailed $p<0.05$).

The strongest decrease in T cell proliferation was induced by sertraline. Whereas a 5 µM concentration only slightly affected the viability of resting T cells (an increase of 1.78 ± 1.38% annexin V+ cells was observed as compared to controls), this concentration also dramatically reduced the proliferation of activated T cells (a mean reduction of 39.4% was found) (figure 3.3). At even higher concentrations, proliferation was almost completely inhibited, but in this concentration range, also the viability of both resting and activated T cells was affected.

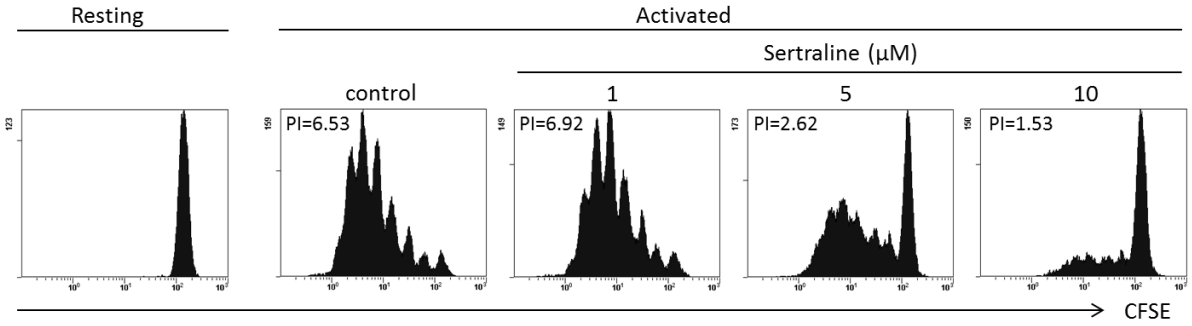

Fig. 3.3. Histogram plots of CFSE labelled PBMCs, gated on viable CD3+ T cells. Cells were loaded with 10 µM CFSE, activated with 10 µl anti-CD3/CD28 beads per 10^6 cells and cultured for 6 days with 0-10 µM sertraline. Thereafter, cells were stained with anti-CD3 antibody and analysed by flow cytometry. Dead cells were excluded based on FSC-SSC properties and gate was set on CD3+ cells. Proliferation indices (PI) were calculated based on the number of cells in each generation.

We provided a comprehensive study comparing the anti-proliferative and pro-apoptotic effects of all available SRIs in both activated and resting human T lymphocytes. We clearly demonstrated that *in vitro* exposure of human T cells to SRIs affects their responsiveness and viability.

4. Discussion

Several papers reported on the effect of SRIs on the immune response, but large differences are seen in the type of SRI, the experimental setup, the test species and the detection methods. Therefore, the need emerged to correlate all these previous findings and extend them in order to obtain one standardized study that gives an overview of the effect of all clinically available SRIs on both activated and resting T cells. This study compared the *in vitro* immunomodulatory effects of five selective SRIs (paroxetine, fluoxetine, sertraline, fluvoxamine, citalopram) and one serotonin and noradrenalin reuptake inhibitor (venlafaxine). We incubated human PBMCs or purified T cells with SRIs *in vitro* and determined the effect on viability and proliferation. SRIs were shown to exert direct *in vitro* effects on the viability and proliferation of T cells. Paroxetine, fluoxetine, sertraline,

fluvoxamine and citalopram were found to induce apoptosis in activated T cells, and this pro-apoptotic effect was significantly lower in resting T cells. For citalopram, only a slight increase in apoptosis could be detected and no differential effect on activated versus resting T cells was found. However, it should be noted that others showed a substantial pro-apoptotic effect in resting T cells for citalopram at 180 µM [14]. Therefore, it cannot be ruled out that citalopram exerts a differential effect at concentrations higher than 100 µM. Venlafaxine did not induce apoptosis in both activated and resting T cells at concentrations up to 100 µM.

Further, all SRIs were found to reduce T cell proliferation in a concentration-dependent manner, at concentrations below those inducing apoptosis (except for paroxetine, which inhibited T cell proliferation and viability at the same concentration). Since the concentrations needed to significantly reduce T cell proliferation are substantially lower than those affecting resting T cell viability, SRIs could be used to suppress proliferation of pathologically activated T cells while at the same time the repertoire of resting T cells remains unaffected and preserves the capability of reacting to pathogens and cancer cells at later stages.

In the central nervous system, SRIs inhibit reuptake of serotonin through the serotonin transporter (SERT) in the presynaptic neuron, resulting in increased synaptic serotonin concentrations [19]. Although SERT expression has also been shown in lymphocytes [9, 20], it is doubted that the immunosuppressive effects of SRIs are mediated through the serotonergic system [21, 22]. On the other hand, it has been suggested that the immunological effects of SRIs are due to induced changes in several signaling pathways. SRIs have been demonstrated to interfere with the activation of the cAMP-dependent protein kinase A (PKA) pathway and the activation of protein kinase C (PKC), as well as with the influx of Ca^{2+} [4, 5, 23, 24]. Furthermore, SRI-mediated induction of apoptosis was accompanied by activation of the MAPK signaling pathway and downregulation of the anti-apoptotic factor bcl-2 [8]. Finally, it has been suggested that triggering of SERT itself can induce changes in downstream signaling pathways [25], thus linking the known affinity of SRIs for SERT with the observed changes in signaling pathways. However, the exact mechanism through which SRIs induce immunosuppression requires further investigation.

Our *in vitro* data indicate that not all SRIs have the same magnitude of effect in lymphocytes. Whereas paroxetine, fluoxetine and sertraline exert immunosuppressive effects at concentrations below 10 µM, fluvoxamine, citalopram and venlafaxine only exert immunosuppressive effects at 50 µM or higher. Since the mechanism through which SRIs induce immunosuppression is not fully understood, it is difficult to interpret differences in SRIs that might explain the observed differences

in immunological effects. However, assuming that triggering of SERT is important for the observed effects, the difference in immunological effects might be explained by the different affinity of SRIs for SERT. The three SRIs that exert the strongest immunosuppressive effects (paroxetine, fluoxetine and sertraline), also have the highest affinity for SERT (Kd < 1 nM). The SRIs that show less or no immunosuppressive action, fluvoxamine, citalopram and venlafaxine, have a lower affinity for SERT (1 nM < Kd < 10 nM) [26]. Possibly, the lack of effect for venlafaxine might as well be explained by the fact that venlafaxine is a mixed serotonin and noradrenaline reuptake inhibitor, whereas the other tested SRIs are selective for serotonin. In the case of citalopram, it is known that the R-enantiomer induces an allosteric modification in SERT, thereby reducing the binding capacity of the active S-enantiomer escitalopram [27]. Possibly, the lack of immunosuppressive effect seen with the racemic mixture citalopram is due to the presence of the R-enantiomer.

References

1. Hughes, J., B. Lunn, and J. O'Brien, *SSRIs and patient groups with specific treatment problems.*, in *Selective Serotonin Reuptake Inhibitors (SSRIs). Past, Present and Future.*, S.C. Stanford, Editor. 1999, RG Landes: Austin, TX, USA. p. 47 - 64.
2. Vaswani, M., F.K. Linda, and S. Ramesh, *Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(1): p. 85-102.
3. Berkeley, M.B., et al., *In vitro effects of cocaine, lidocaine and monoamine uptake inhibitors on lymphocyte proliferative responses.* Immunopharmacol Immunotoxicol, 1994. **16**(2): p. 165-78.
4. Edgar, V.A., et al., *Fluoxetine action on murine T-lymphocyte proliferation: participation of PKC activation and calcium mobilisation.* Cell Signal, 1998. **10**(10): p. 721-6.
5. Edgar, V.A., et al., *Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation.* Eur J Pharmacol, 1999. **372**(1): p. 65-73.
6. Pellegrino, T.C. and B.M. Bayer, *Modulation of immune cell function following fluoxetine administration in rats.* Pharmacol Biochem Behav, 1998. **59**(1): p. 151-7.
7. Pellegrino, T.C. and B.M. Bayer, *Specific serotonin reuptake inhibitor-induced decreases in lymphocyte activity require endogenous serotonin release.* Neuroimmunomodulation, 2000. **8**(4): p. 179-87.
8. Taler, M., et al., *Evidence for an inhibitory immunomodulatory effect of selected antidepressants on rat splenocytes: possible relevance to depression and hyperactive-immune disorders.* Int Immunopharmacol, 2008. **8**(4): p. 526-33.
9. Fazzino, F., et al., *Serotonin transporter is differentially localized in subpopulations of lymphocytes of major depression patients. Effect of fluoxetine on proliferation.* J Neuroimmunol, 2008. **196**(1-2): p. 173-80.
10. Taler, M., et al., *Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression.* Eur Neuropsychopharmacol, 2007. **17**(12): p. 774-80.
11. Xia, Z., J.W. DePierre, and L. Nassberger, *Tricyclic antidepressants inhibit IL-6, IL-1 beta and TNF-alpha release in human blood monocytes and IL-2 and interferon-gamma in T cells.* Immunopharmacology, 1996. **34**(1): p. 27-37.
12. Maes, M., et al., *Negative immunoregulatory effects of antidepressants: inhibition of interferon-gamma and stimulation of interleukin-10 secretion.* Neuropsychopharmacology, 1999. **20**(4): p. 370-9.
13. Kubera, M., et al., *Anti-Inflammatory effects of antidepressants through suppression of the interferon-gamma/interleukin-10 production ratio.* J Clin Psychopharmacol, 2001. **21**(2): p. 199-206.
14. Xia, Z., et al., *Tricyclic antidepressants induce apoptosis in human T lymphocytes.* Int J Immunopharmacol, 1997. **19**(11-12): p. 645-54.
15. Basterzi, A.D., et al., *Effects of venlafaxine and fluoxetine on lymphocyte subsets in patients with major depressive disorder: A flow cytometric analysis.* Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2010. **34**(1): p. 70-75.
16. Denys, D., et al., *A case of venlafaxine-induced inhibition of T-lymphocyte proliferation.* Psychopharmacology (Berl), 2002. **164**(4): p. 432.
17. Vollmar, P., et al., *The antidepressant venlafaxine ameliorates murine experimental autoimmune encephalomyelitis by suppression of pro-inflammatory cytokines.* Int J Neuropsychopharmacol, 2009. **12**(4): p. 525-36.

18. Roederer, M., *Interpretation of Cellular Proliferation Data: Avoid the Panglossian.* Cytometry Part A, 2011. **79A**(2): p. 95-101.
19. Anderson, G.M., et al., *Serotonin in cisternal cerebrospinal fluid of rhesus monkeys: basal levels and effects of sertraline administration.* Psychopharmacology (Berl), 2002. **161**(1): p. 95-9.
20. Faraj, B.A., Z.L. Olkowski, and R.T. Jackson, *Expression of a high-affinity serotonin transporter in human lymphocytes.* Int J Immunopharmacol, 1994. **16**(7): p. 561-7.
21. Cloonan, S.M., et al., *The antidepressants maprotiline and fluoxetine have potent selective antiproliferative effects against Burkitt lymphoma independently of the norepinephrine and serotonin transporters.* Leuk Lymphoma, 2010. **51**(3): p. 523-39.
22. Diamond, M., J.P. Kelly, and T.J. Connor, *Antidepressants suppress production of the Th1 cytokine interferon-gamma, independent of monoamine transporter blockade.* Eur Neuropsychopharmacol, 2006. **16**(7): p. 481-90.
23. Maes, M., et al., *The negative immunoregulatory effects of fluoxetine in relation to the cAMP-dependent PKA pathway.* Int Immunopharmacol, 2005. **5**(3): p. 609-18.
24. Kenis, G., et al., *Influence of antidepressants on intracellular levels of cyclic adenosine monophosphate in human peripheral blood mononuclear cells.* Eur Neuropsychopharmacol, 2003. **13**(1): p. 53-6.
25. Serafeim, A., et al., *5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors.* Blood, 2002. **99**(7): p. 2545-53.
26. Tatsumi, M., et al., *Pharmacological profile of antidepressants and related compounds at human monoamine transporters.* Eur J Pharmacol, 1997. **340**(2-3): p. 249-58.
27. Sanchez, C., *The pharmacology of citalopram enantiomers: the antagonism by R-citalopram on the effect of S-citalopram.* Basic Clin Pharmacol Toxicol, 2006. **99**(2): p. 91-5.

CHAPTER 4

CHARACTERIZATION OF SEROTONIN TRANSPORTER EXPRESSION IN HUMAN T LYMPHOCYTES

Unpublished data

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CHAPTER 4. CHARACTERIZATION OF SEROTONIN TRANSPORTER EXPRESSION IN HUMAN T LYMPHOCYTES

Abstract

Serotonin transporter (SERT) expression has been demonstrated in human lymphocytes, including B lymphocytes, NK cells and other immune cells. However, discussion remains on whether human T lymphocytes express SERT. Given the potentially important role of serotonin (5HT) in lymphocyte activation and proliferation, we investigated SERT expression in purified human T lymphocytes both in resting and activated state. Blood samples were collected from 9 healthy volunteers. PBMCs were isolated using Ficoll density centrifugation and T lymphocytes were further purified with magnetic activated cell sorting. T cells were either processed for mRNA and protein isolation immediately, or after activation using anti-CD3/CD28 coated magnetic beads and proliferation for 72h at 37°C and 5% CO_2. SERT mRNA expression was measured using qRT-PCR and droplet digital PCR while SERT protein was detected on western blot. SERT expression was detected both on mRNA and protein level, although expression levels were low. On mRNA level, SERT was expressed in both resting and activated cells. On the protein level however, only activated cells displayed SERT expression. This observation might point to a 'translational readiness' where resting T lymphocytes already produce SERT mRNA, but translation is only induced after activation of the cells.

1. Introduction

In order to understand how SSRIs alter certain aspects of T lymphocyte functioning, we first investigated the most obvious possibility: as SSRIs are known to selectively block the uptake of serotonin (5HT) through the serotonin transporter (SERT) in the central nervous system, it was hypothesized that the anti-proliferative and pro-apoptotic action of SSRIs on T lymphocytes was related to their capacity to block 5HT uptake in this cell type. However, as described in more detail below, the presence of SERT in T lymphocytes had not been demonstrated with certainty and thus, we decided to investigate whether T cells express SERT as a first step in the search of the molecular mechanism behind the SSRI-induced immunomodulatory effects. The monoamine neurotransmitter 5HT is well known to act as a signaling molecule in the central nervous system [1]. In the past decades, 5HT has also been shown to exert several functions outside the brain, such as platelet coagulation and gastrointestinal function [1]. It has now been well established that 5HT also plays a role in several immune mechanisms, such as regulation of NK cell activity, chemotaxis, MHC expression by macrophages and delayed-type hypersensitivity [2-4]. More recently, a role for 5HT has been described in the communication between dendritic cells and T cells [5]. In general, the major role of 5HT appears to be stimulation of T cell activation within the adaptive immune system and chemotaxis within the innate immune system.

Several cells of the immune system have been shown to express 5HT receptors and SERT [3]. Whereas a consensus has been reached about SERT expression in B cells, macrophages, dendritic cells and mast cells [4], discussion remains on whether T lymphocytes express this membrane protein. The majority of research regarding SERT expression was conducted on peripheral blood lymphocytes, where no further discrimination was made between B, T and NK cells [6-12]. When using Ficoll density centrifugation, blood platelets can contaminate the peripheral lymphocyte fraction and concern has been raised on the impact hereof on the analysis of 5-HT uptake [13]. Although SERT expression has been described in Jurkat T cells [14, 15], evidence for SERT expression in primary T lymphocytes is scarce [16]. Others failed to detect SERT in T lymphocytes [17] or concluded that 5HT transport in T cells was mediated by other transporters than SERT [13].

Considering these contradicting research findings, our first aim was to determine SERT expression in purified human T lymphocytes. As different reports used techniques that either detected SERT on gene expression level or on protein level, we decided to compare both SERT mRNA and protein expression. Furthermore, the difference in SERT expression could be influenced by the activation status of the cells [18]. Therefore, we analyzed both resting and *in vitro* activated T cells. Further,

previous research indicated that activated T cells were more susceptible to SSRI-induced apoptosis than resting T cells. Therefore, differences between SERT expression in activated and resting T lymphocytes might explain the different response to SSRIs.

2. Methods

2.1. T lymphocyte isolation and culture

Venous blood was drawn from healthy human volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density centrifugation. T cells were further isolated by negative selection using the EasySep human T lymphocyte enrichment kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. Purity of the resulting T lymphocyte population was analyzed by staining with anti-CD3 PECy5 (eBioscience, San Diego, USA) and flow cytometric detection on a FC500 (Beckman Coulter, Miami, Florida, USA). Purified T cells were either used directly, or cultured at one million cells per ml in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (100 U/ml penicillin G; 100 µg/ml streptomycin). All cell culture reagents were purchased from Life Technologies (Carlsbad, CA, USA). Activation of the cells was obtained by stimulation with magnetic particles coated with anti-CD3 and anti-CD28 antibodies (Dynabeads® Human T-activator CD3/CD28, Life Technologies) in a 1:1 bead:cell ratio (25 µl per 10^6 cells) for 72h.

2.2. Cell lines

HEK293 cells stably transfected with human SERT were used as a positive control [19]. These cells, which were a kind gift of Randy Blakely (Vanderbilt university, Nashville, TN), were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% glutamine and 1% penicillin/streptomycin at 37°C and 5% CO_2. Jurkat and RAJI cells were cultured in RPMI supplemented with 10% heat-inactivated FBS, 1% glutamine and 1% penicillin/streptomycin at 37°C and 5% $C0_2$.

2.3. mRNA isolation and cDNA synthesis

0.5-4x10^6 cells were resuspended in 1 ml Trizol (Invitrogen) and stored at -80°C until further analysis. After thawing, samples were left at room temperature for 5 minutes to obtain total dissociation of nucleoproteins. 200 µl chloroform was added and samples were vortexed thoroughly for 15 seconds. After additional incubation for 2-3 minutes at room temperature, samples were centrifuged for 15 minutes at 15000g and 4°C. The watery phase containing RNA was aspirated and purified using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. On column digestion of genomic DNA was performed using the RNase-free DNase set (QIAGEN). RNA was finally

eluted in 40 µl DNase-free water and concentrated to 12.5 µl using Vivacon® 500-50.000 MWCO columns (Sartorius, Göttingen, Germany). RNA concentrations were determined with the Quant-it™ Ribogreen® RNA assay kit (Life Technologies). RNA quality assessment using microfluidic capillary electrophoresis (Experion RNA HighSens Chip, Bio-Rad) showed good quality RNA samples as determined by 18S/28S rRNA ratios (RNA quality index [RQI] 8.5 for positive control, 9.3-9.4 for representative T lymphocyte samples; a RQI >7 indicates good quality). mRNA was transcribed to cDNA using the iScript advanced cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and cDNA concentration was estimated using the Quant-it™ oligreen® ssDNA kit (Life Technologies). All kits were used according to the manufacturer's instructions.

2.4. Quantitative PCR and digital droplet PCR

A commercially available Taqman® assay (Hs00984349_m1; Life Technologies) was used for amplification of serotonin transporter cDNA. For RT-qPCR experiments, iTaq supermix with ROX (Bio-Rad) was used. RT-qPCR reactions were performed in a total volume of 25 µl, consisting of 5 µl cDNA (25-100 ng input material), 12.5 µl 2x iTaq supermix, 1.25 µl 20x Taqman® assay and 6.25 µl water. All analyses were performed in triplicate on an ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA, USA). For ddPCR experiments, ddPCR supermix for probes (Bio-Rad) was used. 20 µl reactions were performed containing 5 µl of cDNA (500 ng input material), 10 µl 2x ddPCR supermix, 1 µl Taqman® assay and 4 µl water. Briefly, droplets were generated in 8-channel cartridges containing the 20 µl samples plus 50 µl droplet generating oil using the QX100™ droplet generator (Bio-Rad). Subsequently, droplet-in-oil suspensions were transferred to 96 well plates and placed into a T100™ Thermal Cycler (Bio-Rad). Cycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Subsequently, the droplets were automatically read by the QX100™ droplet reader (Bio-Rad) and the data were analyzed with the QuantaSoft™ analysis software 1.2.10.0 (Bio-Rad). All samples were tested in duplicate. No-template controls (NTCs) were included in every ddPCR run. HEK 293 cells stably transfected with hSERT (kind gift from Randy Blakely, Vanderbilt University) were used as a positive control.

For normalization purposes, seven reference loci were screened with RT-qPCR and analyzed using the Genorm application in qBase$^+$ software version 2.6. Tested references were: ribosomal protein L13A (RPL13A), importin 8 (IPO8), beta-2-microglobulin (B2M), peptidylprolyl isomerase A (PPIA), glyceraldehyde 3-phosphate dehydrogenase (GAPHD), beta-actin (ACTB) and Alu repeats (AluR). Primer sequences and concentrations are listed in table 4.1. For SYBR green detection, the iTaq universal SYBR green supermix was used (Bio-Rad).

Table 4.1. Primer sequences, concentrations and detection methods used for RT-qPCR and ddPCR analysis.

Name	Primers	Conc. (nM)	Detection method
IPO8	FW: GTGTACACACTGGCAGAGC	300	SYBR green
	RE: GCCTCCCTGTTGTTCAATCT		
PPIA	FW: CAAATGCTGGACCCAATACAAA	300	6-FAM Probe:
	RE: GCCATCCAACCCCTCAGTCT		TGTTCCCAGTGTTTCATCTGCACTGCC
GAPDH	FW: AGCCTCAAGATCAGCAATG	300	6-FAM Probe:
	RE: ATGGACTGTGGTCATGAGTCCTT		CCAACTGCTTAGCACCCCTGGCC
ACTB	FW: AGAAAATCTGGCACCACACC	300	SYBR green
	RE: TAGCACAGCCTGGATAGCAA		
AluR	FW: CATGGTGAAACCCCGTCTCTA	250	SYBR green
	RE: GCCTCAGCCTCCCGAGTAG		
B2M	RTPrimerDB ID #2	250	SYBR green
RPL13A	RTPrimerDB ID #6	250	SYBR green
SERT	Taqman assay Hs00984349_m1		6-FAM probe

2.5. Western blot

10^7 cells were lysed in 1 ml radioimmunoprecipitation (RIPA) buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 supplemented with 5 mg/ml Complete Mini protease inhibitor cocktail (Roche, Basel, Switserland), 10 µl/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St-Louis, MO, USA) and 1 µl/ml benzonase® nuclease (Sigma-Aldrich). Protein concentrations were estimated using the Bradford assay. 50 µg of total protein was dissolved in laemlli buffer and incubated at 37°C for 30 minutes. Subsequently, the proteins were subjected to 10% sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to PVDF membrane using Tris-glycine buffer (25 mM Tris base, 190 mM glycine, 0.05% SDS) as described elsewhere [14]. Serotonin transporter protein was detected with 1:5000 dilution of ST51-1 (aa51-66) mouse monoclonal anti-human serotonin transporter antibody (Santa Cruz Biotechnology, CA, USA) overnight at room temperature in PBS + 0.3% Tween-20 and 10% nonfat dry milk. Blots were incubated with secondary goat anti-mouse poly-HRP antibody (1:1000 dilution) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) during 1h at room temperature in PBS + 0.3% Tween-20 + 5% nonfat dry milk. Protein bands were detected with enhanced chemiluminescence (Supersignal West Dura Extended Duration Substrate, Pierce,

Rockford, IL, USA). Specificity of the primary antibody was confirmed with a blocking peptide (PSPGAGDDTRHSIPAT) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The ST51 antibody was incubated for 2h at room temperature with the blocking peptide at 5- fold excess in PBS as described elsewhere [14]. Then, the antibody-peptide mixture was diluted in PBS containing 0.3% Tween-20 and 5% nonfat dry milk to obtain a final antibody concentration of 1:5000 and added to the membrane.

2.6. Statistical analysis

Statistical analysis was performed in Graphpad Prism 5. The difference in expression levels between resting and activated cells was analyzed with a paired t test. A two-tailed P-value < 0.05 was considered statistically significant.

3. Results

3.1. Serotonin transporter expression on the mRNA level

3.1.1. SERT expression in purified T lymphocytes

T lymphocytes from 9 healthy volunteers were isolated through negative selection from PBMCs. Purity of the obtained samples was checked by flow cytometric detection of CD3 expression and was in all samples greater than 98.7% (mean 99.4 ± 0.37%). From all samples, activated T lymphocytes were generated through stimulation with anti-CD3/CD28 beads and T cell activation was detected with flow cytometry using CD69 as an activation marker. A mean percentage of 86.76 ± 9.01% of CD69 positive T cells were found in the activated T cell samples. Resting T lymphocytes were not activated with anti-CD3/CD28 beads.

In order to select the most suitable reference loci, we screened seven references (RPL13A, IPO8, B2M, PPIA, GAPHD, ACTB and AluR) with RT-qPCR and performed a Genorm analysis to select the most stably expressed ones among them. In the stability ranking, IPO8, B2M and AluR were repeatedly found to be among the four most stable reference loci for this type of samples, indicating that the expression of these loci was not affected by T cell activation. The stability measure M-values for these loci were consistently below 1, which is considered acceptably stable for heterogeneous samples [20]. Therefore the geometric mean of the relative quantities of these three loci was used as a normalization factor.

Using reverse transcription quantitative PCR (RT-qPCR), serotonin transporter mRNA could be detected in all tested samples containing resting T cells. However, the obtained Cq-values were high

(~36 with 100 ng cDNA input) indicating a low abundancy of the transcript. In comparison, equal amounts of cDNA of the positive control, hSERT HEK293 cells, yielded Cq-values of ~22. The amount of SERT mRNA in T lymphocyte samples was found to be at the edge of the detection limit and we therefore concluded that the RT-qPCR assay could not be used to reliably estimate the amount of SERT mRNA present in the samples. Recently, digital droplet PCR (ddPCR) became available as an alternative to RT-qPCR promising increased sensitivity and precision for detection of low abundant transcripts [21]. We therefore analyzed the same set of samples with ddPCR using 500 ng of input cDNA per reaction. Since the sample is divided in a large amount of individual droplets, the ddPCR reaction is less subject to PCR inhibition than conventional PCR, making it possible to use larger amounts of input cDNA [22]. After optimization of the assay, SERT mRNA could be detected in all the samples, which contained either resting or activated T lymphocytes. An example of the data output is shown in figure 4.1.

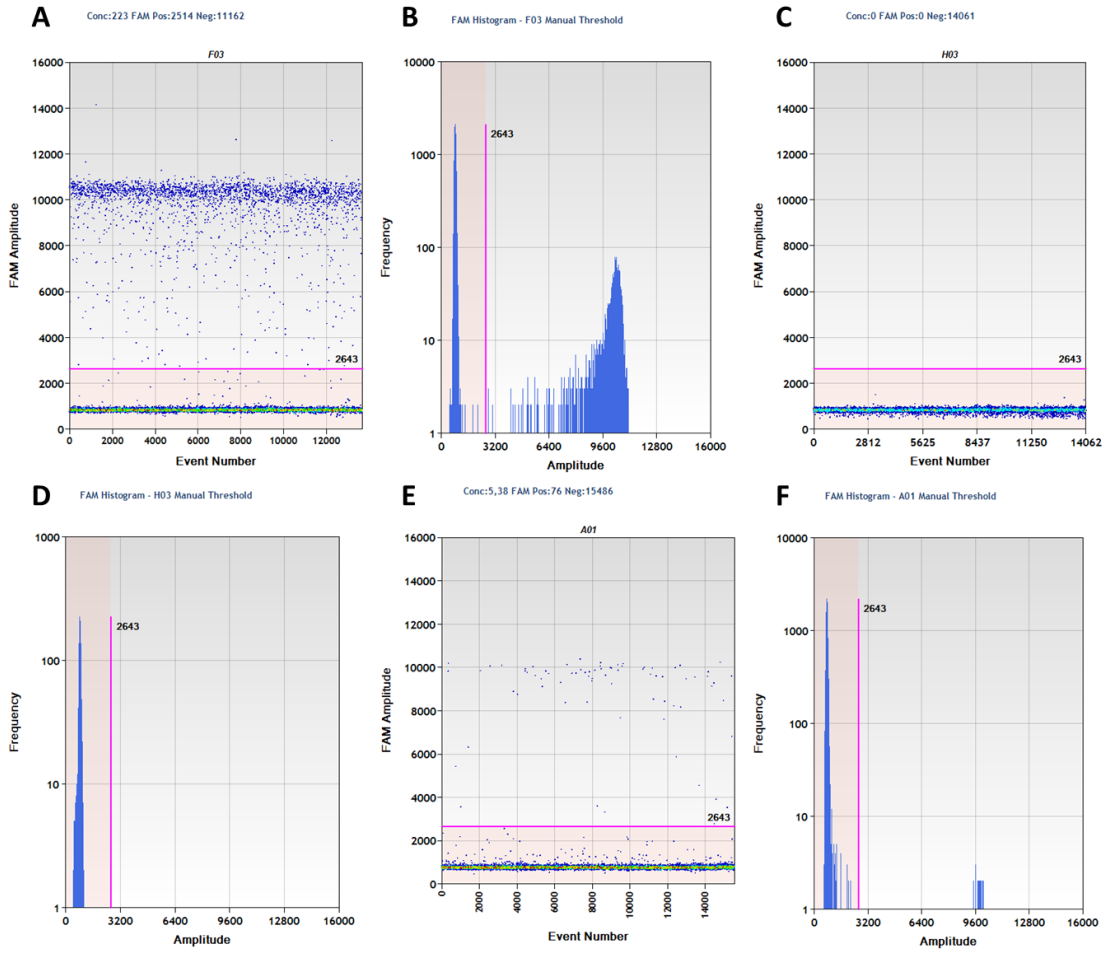


Figure 4.1. Examples of data output generated with ddPCR. One-dimensional scatter plots and histogram plots of a positive control (hSERT transfected HEK cells) showing clear separation of positive and negative droplets (A and B); no template control reactions (C and D) and a representative resting T lymphocyte sample (E and F).

No clear difference between resting and activated T cells was detected (figure 4.2), except for one sample (person 4 in figure 4.2) a remarkably higher SERT mRNA expression in resting T cells was detected. Upon activation, the SERT mRNA expression of these T cells decreased to a level comparable to the other samples, as can be seen in figure 4.2. In an attempt to confirm the high SERT expression of this blood donor, we analyzed a new blood sample and found that the SERT expression in resting T cells was decreased to normal values as compared to the other donor samples (data not shown). Thus, there appears to be high intra-individual variation in SERT mRNA expression in resting T lymphocytes.

Samples of T cells that had been activated for 72h *in vitro* all contained detectable amounts of SERT mRNA. Whereas 5 samples showed a decrease in SERT expression compared to the respective resting T cells, 2 samples had an increased SERT expression and 2 showed equal expression in both resting and stimulated T cells (figure 4.2A). Statistical analysis of resting vs. activated T cells did not shown a significant difference in SERT expression (p=0.304)(figure 4.2B). Thus, it appears that SERT mRNA expression is not consistently altered by T cell activation.

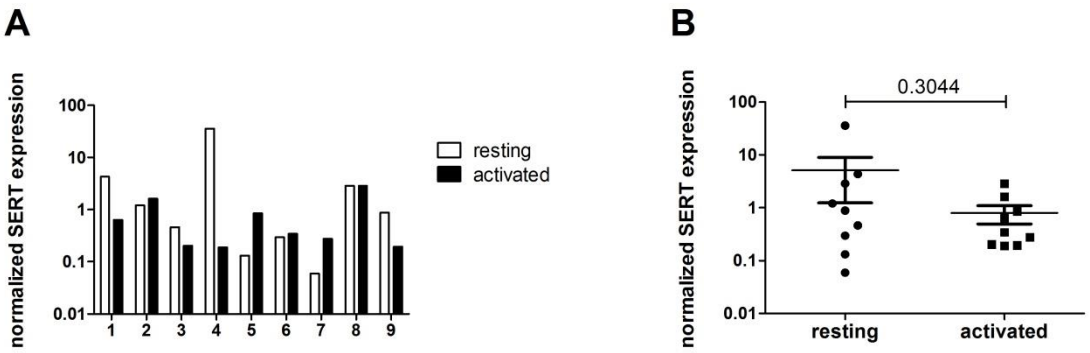


Figure 4.2. Serotonin transporter expression in resting and activated T lymphocytes. T lymphocytes were either analyzed directly (resting) or allowed to proliferate for 72h in culture in contact with anti-CD3/CD28 beads (activated). SERT expression was analyzed in duplo with ddPCR and results (copies per µl sample) were normalized against the geometric mean of IPO8, B2M and AluR expression. Blood samples from 9 donors were analyzed. A) normalized SERT expression in resting and activated T lymphocytes per blood donor. B) Comparison between normalized SERT expression levels in resting and activated T lymphocytes.

3.1.2. SERT expression in PBMCs different from T cells

In order to confirm that SERT mRNA found in isolated T cell samples originated from the T cells and not from the small percentage of other cell types present in the samples, we performed a double isolation using first a negative selection strategy as described in materials and methods and

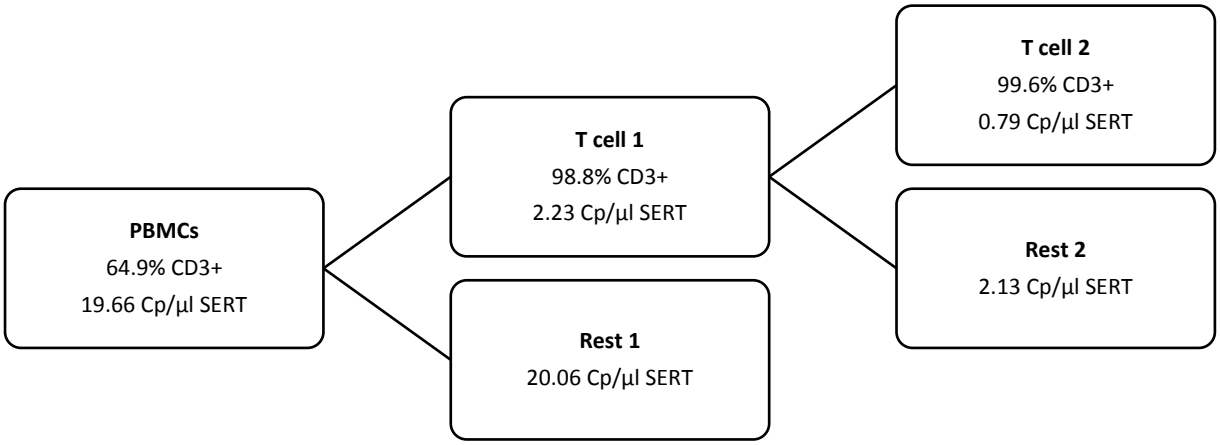
subsequently a positive selection strategy based on CD3 expression (Dynabeads FlowComp Human CD3, Life Technologies) according to the manufacturer's instructions. This double T lymphocyte isolation technique provided a T lymphocyte sample with 99.6 % purity (based on CD3 expression as measured with flow cytometry)(figure 4.3, experiment 1).

A genorm analysis was again performed to select the best reference loci among the seven available candidates. In this set of samples, IPO8, AluR and ActB were found to be the most stably expressed (M-values ranged from 0.513 to 0.607) and therefore, the geometric mean of the relative quantities of these three references was used as normalization factor.

Whereas the unpurified peripheral blood mononuclear cell (PBMC) sample contained 19.66 normalized copies SERT mRNA per µl sample, the T cell sample after the first negative selection (98.8% CD3+) expressed only 2.23 normalized copies/µl. The rest fraction generated by the first negative selection contained 20.06 normalized copies/µl showing that other blood cells, such as B lymphocytes, NK cells, monocytes, dendritic cells or contaminating blood platelets express higher levels of SERT. A second round of T lymphocyte isolation using a positive selection strategy generated a sample with 99.6% CD3+ cells. This sample contained 0.79 normalized copies/µl. The rest fraction generated after the second T lymphocyte isolation expressed SERT at 2.13 copies/µl. Thus, further elimination of contaminating cells from the sample did not abolish SERT expression, although SERT expression further decreased (figure 4.3, experiment 1). From this experiment it can be concluded that T lymphocytes most likely do express SERT mRNA at low levels, but that at least one other cell type present in PBMC preparations such as B lymphocytes, NK cells, dendritic cells or contaminating blood platelets expresses SERT at higher levels.

In order to confirm and extend these results, the experiment was repeated and the percentage of B lymphocytes was additionally determined in all fractions, as B cells are the second most abundant cell type in PBMC preparations and have been shown to express SERT [23](figure 4.3, experiment 2). Similar results were obtained with respect to T cell percentages and SERT expression levels as compared to the first experiment. It must be noted that the second rest fraction ("rest 2") also contained a large number of T cells, as the positive selection strategy did not capture all T cells present in the sample. SERT expression was also detected in RAJI cells, a malignant B cell line, with 0.80 copies/µl sample (data not shown). Thus, the observation from the first experiment that T lymphocytes express much lower but yet existing levels of SERT in comparison to at least one other PBMC cell type was confirmed. Surprisingly, these data also suggest that malignant B lymphocytes express SERT at comparable levels as T lymphocytes.

Experiment 1



Experiment 2

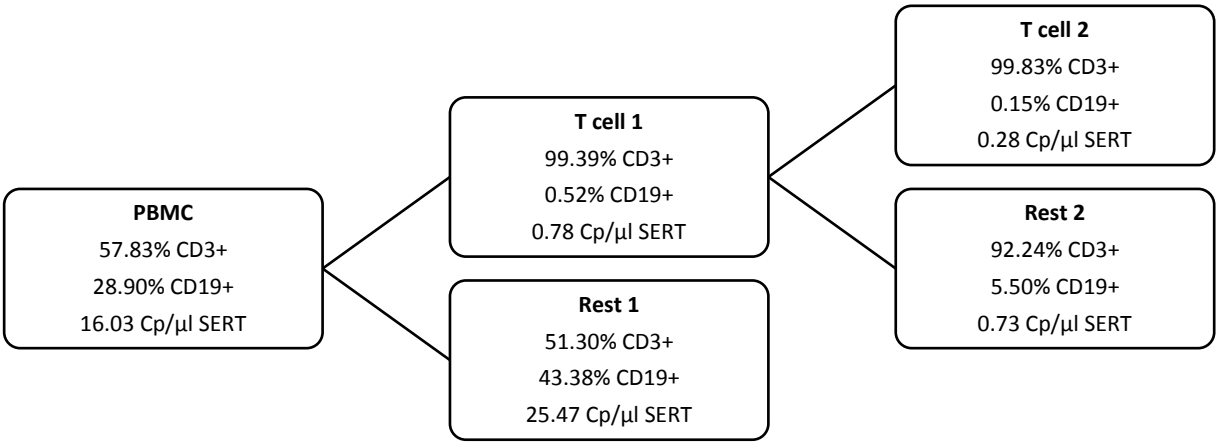


Figure 4.3. SERT mRNA expression in highly purified T cells. In the first isolation round, a negative selection strategy was used to purify T cells from PBMCs. The second isolation was based on a positive selection strategy, using antibodies against CD3. SERT expression was determined with ddPCR and the results (copies/µl) were normalized against the geometric mean of IPO8, AluR and ActB. Percentages of CD3+ cells (experiment 1) or CD3+ and CD19+ cells (experiment 2) were determined by flow cytometry. Cp/µl = normalized copies per µl sample.

3.1.3. SERT expression in Jurkat T cells

In order to confirm the finding that human T lymphocytes express low levels of SERT, the expression was also determined in a Jurkat T cell line, which can be considered 100% pure. In this sample, we found 0.51 copies SERT per µl sample (data not shown). Although it is not certain that SERT expression is not altered by the malignant nature of Jurkat cells, the finding that Jurkat T cells express similar amounts of SERT mRNA as freshly isolated T lymphocytes contributes to the conclusion that T lymphocytes express low levels of SERT.

3.2. Serotonin transporter expression on the protein level

Total cell lysates were prepared from both resting and activated T cell samples from 2 healthy volunteers. Protein extracts of hSERT HEK293 cells were used as a positive control. Using 50 µg of total protein, an intense protein band could be detected at 83.5 kDa for the positive control (figure 4.4). Although the theoretical molecular weight of SERT is 70.3 kDa, protein bands of higher molecular weight have been described using this transfected cell line, presumably due to glycosylation [14]. A second, less intensive band could be detected at 62.5 kDa. The latter presumably represents the original non-glycosylated SERT protein. For T cell samples, only the 62.5 kDa band could be detected (figure 4.4), which is in agreement with Chamba et al. who found that native cells expressed only the non-glycosylated protein [14].

To control for non-specific binding of the antibody to proteins other than SERT, a blocking peptide (PSPGAGDDTRHSIPAT) was used to which the antibody was raised. The peptide corresponds with the 51-66 amino acid sequence of SERT, which is situated at the cytoplasmic N-terminal region. With this approach a distinction can be made between specific binding of the antibody through its antigen-binding site and non-specific binding through another part of the antibody (e.g. its Fc domain). Both protein bands at 83.5 kDa (positive control) and at 62.5 kDa disappeared when the ST51 antibody was pre-incubated with the blocking peptide (figure 4.4). Hence, it can be concluded that both the 83.5 and 62.5 kDa bands are the result of specific binding of the primary antibody through its variable domain. Equal loading of the samples was checked through detection of beta-actin on the same blot (figure 4.4).

On the protein level, SERT expression was detected in activated T cells of two out of eight tested blood donors as well as in jurkat T cells (figure 4.4). These data show that activated human peripheral T lymphocytes can express SERT. Analysis of activated T cell lysates from the 6 other blood donors did not reveal SERT protein expression (data not shown). Thus, SERT protein expression appears subject to high inter-individual variation. In contrast, no SERT protein could be detected in resting T cells from eight analyzed blood donors.

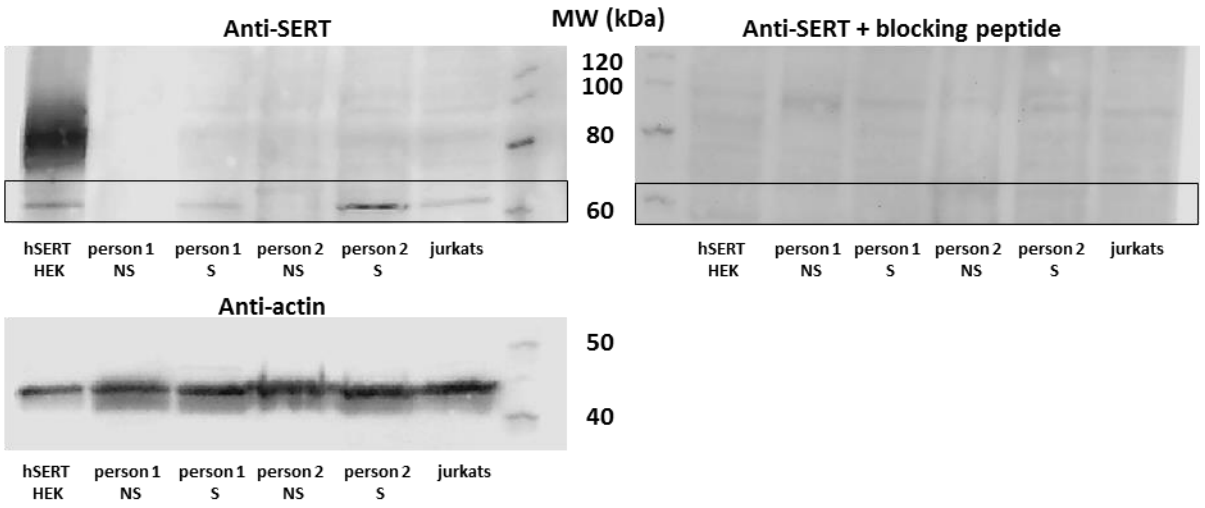


Figure 4.4. SERT protein detection on western blot. The upper left panel shows detection of SERT protein with ST51-1 antibody. Molecular weight (MW) is depicted on the right side (kDa). The upper right panel shows detection with ST51-1 antibody pre-incubated with 5-fold excess of a blocking peptide to demonstrate specificity of the antibody. The lower panel shows detection with anti-actin to ensure equal loading of the samples. hSERT transfected HEK cells were used as a positive control.

4. Discussion

Previous research has demonstrated that SSRIs induce immunosuppression in human T lymphocytes. As SSRIs are known to inhibit 5HT uptake through SERT in the central nervous system and a role for 5HT has been described in immunity, we investigated whether the immunosuppressive effects of SSRIs could be related to their ability to inhibit 5HT uptake through SERT in T lymphocytes. SERT expression has been shown in several types of immune cells including B cells, dendritic cells, macrophages, mast cells and platelets [4]. In dendritic cells, the role of 5HT uptake has been described by O'Connell et al. who found that dendritic cells take up 5HT on inflammatory sites and shuttle it to naive T cells thereby influencing their activation and proliferation [5]. In peripheral T lymphocytes however, the presence of SERT has not been demonstrated with certainty. Whereas some research points to the presence of SERT expression [8, 16], others concluded no SERT expression was present in T lymphocytes [5, 13]. Nevertheless, it is clear that 5HT uptake and release are involved in the functioning of the immune system. Therefore, we analyzed SERT expression in human peripheral T lymphocytes on both mRNA and protein level. As the activation status of the T cells might affect SERT expression, we investigated both resting and activated T cells. Furthermore, activated T lymphocytes have been shown to be more susceptible to SSRI-induced apoptosis than resting T cells. In this study, we investigated whether differences in SERT expression between both populations can explain this discrepancy.

The results of this study show that human peripheral T cells express SERT both on mRNA and protein level. However, expression levels are low and therefore highly sensitive techniques are necessary to study SERT in this cell type. Here, we demonstrate that digital droplet PCR can provide the extra sensitivity over quantitative PCR to achieve this goal. Possibly, others that have failed to detect SERT in human T cells have used techniques that did not provide the required sensitivity. Moreover, we observed large differences in SERT expression levels between different blood donors. Since standard lab techniques like RT-qPCR and western blotting are only just sensitive enough to detect SERT mRNA or protein in T lymphocytes, the inter-individual variability might as well explain the contradictory conclusions of different research groups regarding the presence of SERT in this cell type. Differences in SERT expression levels between individuals might be related to a polymorphism in the promotor region of the SLC6A4 gene (5HTT-linked polymorphic region, 5HTTLPR), which encodes SERT. A short and long allele for 5HTTLPR have been described, possibly influencing the transcription of SLC6A4 with a lower transcriptional activity in individuals carrying the short allele [24, 25]. Furthermore, whether or not the studied T lymphocytes were activated might also influence the detectability of SERT. Which factors influence the expression level of SERT in T lymphocytes is largely unknown. However, Tsao et al. demonstrated increased SERT mRNA expression and 5HT uptake in Jurkat T cells after exposure to IFNα [15], showing that inflammatory cytokines induce upregulation of SERT in these cells.

On mRNA level, we found SERT expression in both resting and activated T cells. On the protein level, we could detect SERT only in activated T lymphocytes. It is not clear why resting T cells do not express detectable SERT protein. Possibly, SERT protein is not detected in resting T cells by the antibody used because of different post-translational modifications. It is also possible that resting T cells express only mRNA for SERT, and that translation is induced when T cells become activated. Interestingly, the same observation has been made by Chamba et al. in normal resting B lymphocytes [23]. Equally, they detected SERT mRNA expression in resting B lymphocytes, but could only find SERT protein upon activation and proliferation of the B cells. These data suggest a ‘translational readiness’, where SERT gene expression is already present in resting T lymphocytes, but translation is only induced after activation of the cells. Further, we detected SERT protein at two different molecular weights in hSERT transfected HEK cells. Whereas the 83.5 kDa SERT presumably represents a highly glycosylated form of SERT, the 62.5 kDa protein most likely represents the original full length SERT protein [14]. In activated T lymphocytes, we only detected the 62.5 kDa form of SERT. As glycosylation of SERT has been shown to be important for its trafficking to the plasma membrane [26], it can be questioned whether the ~60 kDa SERT is a functional form and is available at the

plasma membrane for 5HT transport. Although it has been reported that the ~60 kDa SERT protein remained in the endoplasmic reticulum in SERT-transfected COS-7 cells [26], others have shown that this non-glycosylated form of SERT was present at the plasma membrane of Burkitt lymphoma cells, although the majority of the ~60 kDa SERT was situated in the cytoplasm [14]. As the same group previously found that Burkitt lymphoma cells actively transport 5HT, it was concluded that the non-glycosylated SERT protein was indeed a functional form [27].

In order to confirm specificity of the ST51 antibody, we used a blocking peptide. As both detected protein bands at ~90 kDa and ~60 kDa disappeared when the antibody was pre-incubated with the blocking peptide, it was concluded that the detected bands were the result of specific binding of the antibody through its antigen-binding site. However, these data do not exclude the possibility that the antibody might bind to similar epitopes on other proteins through its antigen-binding site. The latter possibility is unlikely though, as detection of SERT with ST51 and another anti-SERT antibody (C20, a goat polyclonal antibody directed to SERT C-terminus aa611–630 from Santa Cruz Biotechnology, CA, USA) gave rise to bands of equal molecular size in both hSERT HEK cells (~90 and ~60 kDa) and L3/bcl-2 cells (only ~60 kDa) [14]. In addition, enzymatic removal of N-linked glycans by PNGase treatment of the hSERT HEK samples resulted in a shift of the ~90 kDa band to 60 and 70 kDa bands [14].

In order to exclude the possibility that other cell types (e.g. B lymphocytes or platelets) contaminated the samples, we used purification techniques that generated T lymphocyte samples of very high purity. In addition, we determined percentages of T- and B lymphocytes in the isolated cell fractions, as well as in the rest fractions after negative and positive selection of T lymphocytes. In all these fractions, SERT mRNA expression was detected. From these data, we concluded that SERT mRNA was present in all isolated cell fractions, even those with approximately 100% T lymphocytes. However, we did find higher expression of SERT mRNA in total PBMC samples and rest fractions after the first (negative) selection round. Thus, one or more other blood cell types express higher levels of SERT than T lymphocytes. In addition to freshly isolated lymphocytes from peripheral blood, we analyzed SERT mRNA expression in two malignant cell lines: Jurkat leukemic T lymphocytes and RAJI Burkitt lymphoma B lymphocytes. Comparable SERT mRNA expression levels were found in both cell lines. As these cell lines can be considered 100% pure, the observation that Jurkat T lymphocytes express SERT mRNA contributes to the conclusion that human T lymphocytes express SERT. Furthermore, as Jurkat and RAJI cells expressed comparable levels of SERT mRNA, the high SERT levels found in PBMC samples and rest fractions are unlikely to originate from contaminating B lymphocytes. Instead, other cell types such as platelets might account for the high SERT levels. In order to confirm that platelets

are the source of high SERT expression in PBMC samples, the experiment should be repeated while monitoring the presence and abundance of platelets in the samples. Importantly, these data underscore the need for appropriate isolation kits and monitoring the purity of the generated samples when studying the expression of any type of gene or protein, especially when expression levels are low.

In this study, only pan T cells were studied and no further distinction was made between different subsets of T lymphocytes. However, as pointed out by Levite M., the expression of specific neurotransmitter receptors and transporters is dependent on the subset of T lymphocytes studied, and is dynamically regulated in response to TCR activation, cytokines and the neurotransmitter itself [18]. Thus, although we demonstrated that SERT expression is generally present in T lymphocytes, it might be absent or more expressed in certain subtypes, e.g. CD4+ T helper cells or CD8+ cytotoxic T cells. Also within the group of CD4+ T cells, differences might be detected depending on the type of T helper cell studied, e.g. Th1 cells, Th2 cells, Th17 cells or regulatory T cells. As cytokines and the neurotransmitter itself have an impact on the expression of neurotransmitter receptors and transporters, it is important to control for these parameters when designing experiments. Indeed, standard cell culture medium contains 10% fetal bovine serum (FBS) which is a source of 5HT as well as different cytokines. Thus, batch-to-batch variations in the levels of these molecules in the FBS used might affect the detected SERT expression levels.

In conclusion, this study demonstrates that human peripheral T lymphocytes express SERT both on mRNA and protein level. SERT mRNA expression is present in both resting and activated T cells, whereas SERT protein is only present in activated T lymphocytes. To the present, the role of SERT in T lymphocytes has not fully been established and therefore further research in this area should be encouraged. Considering that SERT is expressed in T lymphocytes, it can be expected that 5HT is actively taken up in these cells, especially in activated T cells since these have been shown to express SERT protein. It is thus likely to assume that SSRIs indeed inhibit 5HT uptake in T lymphocytes. The observation that SERT protein is upregulated in activated T cells as compared to resting T cells leads to the assumption that inhibition of 5HT uptake through SERT might be an important factor in the differential pro-apoptotic effect of SSRIs on activated versus resting T cells.

References

1. Berger, M., J.A. Gray, and B.L. Roth, *The expanded biology of serotonin.* Annu Rev Med, 2009. **60**: p. 355-66.
2. Mossner, R. and K.P. Lesch, *Role of serotonin in the immune system and in neuroimmune interactions.* Brain Behav Immun, 1998. **12**(4): p. 249-71.
3. Ahern, G.P., *5-HT and the immune system.* Curr Opin Pharmacol, 2011. **11**(1): p. 29-33.
4. Baganz, N.L. and R.D. Blakely, *A dialogue between the immune system and brain, spoken in the language of serotonin.* ACS Chem Neurosci, 2012. **4**(1): p. 48-63.
5. O'Connell, P.J., et al., *A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells.* Blood, 2006. **107**(3): p. 1010-7.
6. Faraj, B.A., Z.L. Olkowski, and R.T. Jackson, *Expression of a high-affinity serotonin transporter in human lymphocytes.* Int J Immunopharmacol, 1994. **16**(7): p. 561-7.
7. Marazziti, D., et al., *Presence and characterization of the serotonin transporter in human resting lymphocytes.* Neuropsychopharmacology, 1998. **19**(2): p. 154-9.
8. Barkan, T., et al., *Biochemical and pharmacological characterization of the serotonin transporter in human peripheral blood lymphocytes.* Eur Neuropsychopharmacol, 2004. **14**(3): p. 237-43.
9. Cedeno, N., et al., *Characterization of serotonin transporter in blood lymphocytes of rats. Modulation by in vivo administration of mitogens.* J Neuroimmunol, 2005. **159**(1-2): p. 31-40.
10. Lima, L., S. Mata, and M. Urbina, *Allelic isoforms and decrease in serotonin transporter mRNA in lymphocytes of patients with major depression.* Neuroimmunomodulation, 2005. **12**(5): p. 299-306.
11. Yang, G.B., et al., *Expression of serotonin transporters by peripheral blood mononuclear cells of rhesus monkeys (Macaca mulatta).* Cell Immunol, 2007. **248**(2): p. 69-76.
12. Singh, Y.S., et al., *Differential serotonin transport is linked to the rh5-HTTLPR in peripheral blood cells.* Transl Psychiatry, 2012. **2**: p. e77.
13. Beikmann, B.S., et al., *Serotonin uptake is largely mediated by platelets versus lymphocytes in peripheral blood cells.* ACS Chem Neurosci, 2013. **4**(1): p. 161-70.
14. Chamba, A., et al., *Characterisation of the endogenous human peripheral serotonin transporter SLC6A4 reveals surface expression without N-glycosylation.* J Neuroimmunol, 2008. **204**(1-2): p. 75-84.
15. Tsao, C.W., et al., *Interferon-alpha-induced serotonin uptake in Jurkat T cells via mitogen-activated protein kinase and transcriptional regulation of the serotonin transporter.* J Psychopharmacol, 2008. **22**(7): p. 753-60.
16. Fazzino, F., et al., *Serotonin transporter is differentially localized in subpopulations of lymphocytes of major depression patients. Effect of fluoxetine on proliferation.* J Neuroimmunol, 2008. **196**(1-2): p. 173-80.
17. Leon-Ponte, M., G.P. Ahern, and P.J. O'Connell, *Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor.* Blood, 2007. **109**(8): p. 3139-46.
18. Levite, M., *Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors.* Curr Opin Pharmacol, 2008. **8**(4): p. 460-71.
19. Qian, Y., et al., *Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression.* J Neurosci, 1997. **17**(1): p. 45-57.
20. Hellemans, J., et al., *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome Biol, 2007. **8**(2): p. R19.
21. Baker, M., *Digital PCR hits its stride.* Nature Methods, 2012. **9**(6): p. 541-544.

22. Huggett, J.F., et al., *The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments.* Clinical Chemistry, 2013. **59**(6): p. 892-902.
23. Chamba, A., et al., *SLC6A4 expression and anti-proliferative responses to serotonin transporter ligands chlomipramine and fluoxetine in primary B-cell malignancies.* Leuk Res, 2010. **34**(8): p. 1103-6.
24. Lesch, K.P., et al., *Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region.* Science, 1996. **274**(5292): p. 1527-1531.
25. Heils, A., et al., *Allelic variation of human serotonin transporter gene expression.* Journal of Neurochemistry, 1996. **66**(6): p. 2621-2624.
26. Nobukuni, M., et al., *The C-terminal region of serotonin transporter is important for its trafficking and glycosylation.* J Pharmacol Sci, 2009. **111**(4): p. 392-404.
27. Serafeim, A., et al., *5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors.* Blood, 2002. **99**(7): p. 2545-53.

CHAPTER 5

EFFECTS OF FLUOXETINE ON CALCIUM SIGNALING IN HUMAN T LYMPHOCYTES

Based on '*Fluoxetine suppresses Ca^{2+} signaling in human T lymphocytes through inhibition of Ca^{2+} release from intracellular stores*'. Veerle Gobin, Marijke De Bock, Bart Broeckx, Maja Kiselinova, Ward De Spiegelaere, Linos Vandekerckhove , Katleen Van Steendam, Luc Leybaert*, and Dieter Deforce*. Submitted to Cell Calcium.

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CHAPTER 5. EFFECTS OF FLUOXETINE ON CALCIUM SIGNALING IN HUMAN T LYMPHOCYTES

Abstract

Selective serotonin reuptake inhibitors, such as fluoxetine, have recently been shown to exert anti-inflammatory and immunosuppressive effects. Although the effects on cytokine secretion, proliferation and viability of T lymphocytes have been extensively characterized, little is known about the mechanism behind these effects. It is well known that Ca^{2+} signaling is an important step in the signaling transduction pathway following T cell receptor activation. Therefore, we investigated if fluoxetine interferes with Ca^{2+} signaling in jurkat T lymphocytes. Fluoxetine was found to suppress Ca^{2+} signaling in response to T cell receptor activation. Moreover, fluoxetine inhibited IP_3- and ryanodine-receptor mediated Ca^{2+} release from intracellular stores in a concentration-dependent manner. The Ca^{2+}-modifying effects of fluoxetine are not related to its capability to block the serotonin transporter, as even a large excess of 5HT did not abolish the effects. In conclusion, these data show that fluoxetine inhibits IP_3- and ryanodine-receptor mediated Ca^{2+} release in jurkat T lymphocytes, an effect likely to be at the basis of the observed immunosuppression.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) have been shown to exert anti-inflammatory and direct immunosuppressive effects such as suppression of T cell activation, cytokine secretion and proliferation and induction of apoptosis *in vitro* and *in vivo* [1-3]. Although it has been shown that these compounds have a high affinity for the serotonin transporter (SERT) in the central nervous system, it is not clear whether the immunological effects of SSRIs are mediated by inhibition of SERT-mediated serotonin (5HT) uptake in lymphocytes. On the contrary, several arguments oppose to the involvement of 5HT and SERT in the immunosuppressive effects of SSRIs, especially the discrepancy between the concentration needed for blockage of 5HT uptake on the one hand (nM range) and for *in vitro* immunosuppression on the other hand (µM range) [4, 5]. The actual mechanism underlying the immunosuppressive effects of SSRIs has not been elucidated yet.

Elevation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the key triggering signals for T-cell activation. The $[Ca^{2+}]_i$ is regulated through an intimate interplay between Ca^{2+} in the extracellular space and intracellular storage sites such as the endoplasmic reticulum (ER). Ca^{2+} signaling mechanisms mostly rely on Ca^{2+} release from the ER through inositol 1,4,5 trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) following the activation of G-protein coupled receptors on the plasma membrane. Subsequent depletion of the ER triggers store-operated, capacitative Ca^{2+} entry to replenish the ER [6]. SSRIs have been shown to affect Ca^{2+} signaling in several cell types. Fluoxetine inhibited ATP-induced Ca^{2+} increases in PC12 cells through inhibition of both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores [7]. Whereas fluoxetine has also been shown to suppress Ca^{2+} spikes in cultured rat hippocampal neurons, two other SSRIs, namely paroxetine and citalopram, did not [8]. Furthermore, chronic exposure of astrocytes to fluoxetine diminished RyR- and IP_3R-mediated Ca^{2+} release as well as the subsequent capacitative Ca^{2+} entry [9]. In microglia, pretreatment with paroxetine or sertraline reduced the amplitude of the Ca^{2+} increase induced by interferon-gamma (IFNγ) [10]. Oppositely, sertraline induced a Ca^{2+} rise in MG63 osteosarcoma cells [11]. Fluoxetine, paroxetine and citalopram induced a rise in $[Ca^{2+}]_i$ in Burkitt lymphoma cells [12]. In platelets, SSRIs (sertraline, paroxetine, fluoxetine) potentiated thrombin-mediated increases in intracellular Ca^{2+} [13]. Clearly, SSRIs are capable of interfering with Ca^{2+} signaling in a wide variety of cell types. Furthermore, it has been suggested that fluoxetine interferes with mitogen-induced Ca^{2+} influx in murine and human T lymphocytes as fluoxetine exerted similar effects as the Ca^{2+} ionophore A23187 on T cell proliferation, protein kinase C (PKC) degradation and cAMP levels [14, 15]. As ionophores promote Ca^{2+} entry, this suggests fluoxetine might increase $[Ca^{2+}]_i$ in lymphocytes.

Given the importance of Ca^{2+} signaling in T cell activation [6], we investigated whether interference with Ca^{2+} signaling might be at the basis of the immunosuppressive effects of fluoxetine in jurkat T lymphocytes. In addition, we investigated whether the observed effects on Ca^{2+} signaling are related to the inhibition of 5HT uptake.

2. Methods

2.1. Cell culture

Jurkat T cells, clone E6-1, were cultured at 37°C and 5% CO_2 in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin (100 U/ml penicillin G; 100 µg/ml streptomycin). All cell culture reagents were purchased from Life technologies (Carlsbad, CA, USA).

2.2. Buffers and chemicals

Krebs HEPES buffer contained 133.5 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM HEPES, 11.5 mM glucose and 1.5 mM $CaCl_2$, pH 7,4. In Ca^{2+}-free Krebs buffer, $CaCl_2$ was replaced by 4.47 mM EGTA, pH 7.4. 1,2-Bis(2-aminophenoxy) ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM), D-*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester ("NPE-caged IP_3"), dextran Texas Red 10000 MW (DTR), fluo3-AM and thapsigargin were purchased from Molecular Probes, Life technologies. Caffeine was from Sigma-Aldrich (St. Louis, MO, USA), ryanodine from Abcam (Cambridge, UK) and fluoxetine from ABC chemicals (Woutersbrakel, Belgium).

2.3. Visualization of intracellular Ca^{2+}

Dynamic changes in $[Ca^{2+}]_i$ were monitored using fluo3-AM. Cells in suspension were loaded with 5 µM fluo3-AM at $2x10^6$/ml in Krebs buffer for 1h at room temperature and subsequently washed 3x in Krebs buffer. Thereafter, cells ($0.5\text{-}1x10^6$) were allowed to adhere on poly-L-lysine (0.1%) coated 18mm diameter glass coverslips and left for 30 min at room temperature for de-esterification and settling on the dish. Cells were washed once in Krebs buffer to remove any unbound cells before imaging.

Intracellular Ca^{2+} imaging was performed in Krebs buffer at room temperature and was carried out using a Nikon Eclipse TE300 inverted epifluorescence microscope (Nikon Belux, Brussels, Belgium), equipped with a 40x oil-immersion objective (Plan Fluor, NA 1.30; Nikon) and an EM-CCD camera (QuantEM™ 512SC CCD camera, Photometrics, Tucson, AZ). We used a Lambda DG-4 filterswitch (Sutter Instrument Company, Novato, CA) to deliver excitation at 482 nm and captured emitted light via a 505-nm long-pass dichroic mirror and a 535 nm bandpass filter (35 nm bandwidth). Images (1/s)

were generated with custom-generated QuantEMframes software written in Microsoft Visual C^{++} 6.0. Fluo3 fluorescence-intensity changes were analyzed with custom-developed FluoFrames software (generated by L.L., Ghent University, Belgium). Background fluorescence was subtracted from all images. Traces of individual cells were obtained by point analysis in Fluoframes software.

2.4. Electroporation loading with NPE-caged IP_3 and photoliberation

In order to study IP_3-mediated Ca^{2+} release from the ER, T cells were loaded with NPE-caged IP_3 through electroporation, as described elsewhere [16]. Briefly, cells seeded on coverslips were washed 3x with a low conductivity electroporation buffer (4.02 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1.0 mM $MgCl_2$, 300 mM sorbitol, 2.0 mM HEPES, pH 7.4). The coverslips were then placed on the microscopic stage, 400 µm underneath a parallel wire Pt-Ir electrode and electroporated in the presence of 10 µl electroporation buffer containing 100 µM NPE-caged IP_3 and 100 µM DTR to visualize the electroporation zone. Electroporation was done with 50 kHz bipolar pulses applied as trains of 10 pulses of 2 ms duration each and repeated 15 times. The field strength was 100V peak-to-peak applied over a 500 µm electrode separation distance. After electroporation, cells were thoroughly washed with Krebs buffer. Electroporation was performed after fluo3 loading and did not result in loss of fluo3 from the cells [17].

After loading with NPE-caged IP_3, coverslips were transferred to the microscope stage for Ca^{2+} imaging. Photoliberation of IP_3 was done by spot (20 µm diameter) illumination with 1-kHz pulsed UV light (349 nm UV laser Explorer, Spectra-Physics, Newport, Utrecht, The Netherlands) applied during 50 ms (50 pulses of 90 µJ energy measured at the entrance of the microscope epifluorescence tube).

2.5. Activation with anti-CD3/CD28 beads

T cell receptor activation was achieved by adding magnetic particles coated with antibodies against CD3 and CD28 (Dynabeads® Human T-Activator CD3/CD28, Life technologies) at a concentration of 25 µl per 10^6 cells (1:1 bead:cell ratio). Cells were visually inspected under the microscope at the end of each experiment to determine which cells were making contact with at least one magnetic bead.

2.6. Data analysis

The statistical analysis was conducted in R [18]. Homoscedasticity and normality of residuals were visually checked using residuals vs fitted plots and QQ plots. If necessary, power transformations were applied, using a Box-Cox plot for guidance [19]. The datasets in section 3.1, 3.3. and 3.6 were analyzed using a one-way ANOVA or two-way ANOVA (to correct for the possible influence of time if the experiment was conducted for > 1 day). The other datasets (section 3.2, 3.4 and 3.5) were

analyzed using the non-parametric Kruskal-Wallis test. Student's T tests (parametric) or Wilcoxon rank sum tests (non-parametric) were used for post-hoc testing with a Holm Bonferroni correction for multiple testing being applied. Data are presented as mean ± SD or median and range, for parametric and non-parametric data, respectively. Significance was set at $p < 0.05$, all tests were two-tailed.

Data are visually presented as boxplots showing median, first and third quartile. Whiskers represent lowest and highest data within 1,5 interquartile range (IQR). Data exceeding the 1.5 IQR were omitted from the graphs for clarity.

2.7. Digital droplet (dd)PCR

mRNA was isolated using the RNeasy® mini kit (QIAGEN, Hilden, Germany) and on column digestion of genomic DNA was performed using the RNase-free DNase set (QIAGEN). RNA concentrations were determined with the Quant-it™ Ribogreen® RNA assay kit (Bio-Rad, Hercules, CA, USA). RNA quality assessment using microfluidic capillary electrophoresis (Experion RNA HighSens Chip, Bio-Rad) showed good quality RNA samples as determined by 18S/28S rRNA ratios (RNA quality index [RQI] 8.5 for positive control, 9.3-9.4 for representative T lymphocyte samples; a RQI >7 indicates good quality). mRNA was transcribed to cDNA using the iScript™ advanced cDNA synthesis kit (Bio-Rad). cDNA concentrations were subsequently estimated using the Quant-it™ oligreen® ssDNA kit (Life technologies). All kits were used according to the manufacturer's instructions. A commercially available Taqman® assay (Hs00984349_m1; Life technologies) was used for amplification of serotonin transporter cDNA. 20 µl reactions were prepared containing 5 µl of cDNA (500 ng input material), 10 µl 2x ddPCR™ super mix for probes (Bio-Rad), 1 µl Taqman® assay and 4 µl water. ddPCR assays were performed as described previously [20]. Briefly, droplets were generated in 8-channel cartridges containing the 20 µl samples plus 50 µl droplet generating oil using the QX100™ droplet generator (Bio-Rad). Subsequently, droplet-in-oil suspensions were transferred to 96 well plates and placed into a T100™ Thermal Cycler (Bio-Rad). Cycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Subsequently, the droplets were automatically read by the QX100™ droplet reader (Bio-Rad) and the data were analyzed with the QuantaSoft™ analysis software 1.2.10.0 (Bio-Rad). All samples were tested in duplicate. No-template controls (NTCs) were included in every ddPCR run. HEK 293 cells stably transfected with hSERT (kind gift from Randy Blakely, Vanderbilt University) were used as a positive control.

2.8. Western blotting

10^7 cells were lysed in 1 ml radioimmunoprecipitation (RIPA) buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and supplemented with 5 mg/ml Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland), 10 µl/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) and 1 µl/ml benzonase® nuclease (Sigma-Aldrich). 50 µg of total protein was subjected to 10% sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane using Tris-glycine buffer (25 mM Tris base, 190 mM glycine, 0.05% SDS), as described elsewhere [21]. Serotonin transporter protein was detected with a 1:5000 dilution of ST51-1 (aa51-66) mouse monoclonal anti-human serotonin transporter antibody (Santa Cruz Biotechnology, CA, USA) in PBS + 0.3% Tween-20 + 10% nonfat dry milk overnight at room temperature. Incubation with secondary goat anti-mouse poly-HRP antibody (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (1:1000 dilution) was performed during 1h at room temperature in PBS + 0.3% Tween-20 + 5% nonfat dry milk. Protein bands were detected with enhanced chemiluminescence. HEK 293 cells, stably transfected with hSERT were used as a positive control. Specificity of the primary antibody was confirmed with a blocking peptide (PSPGAGDDTRHSIPAT; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The ST51 antibody was incubated for 2h at room temperature with the blocking peptide at 5- fold excess in PBS, as described elsewhere [21]. Then, the antibody-peptide mixture was further diluted in PBS containing 0.3% Tween-20 and 5% nonfat dry milk and added to the membrane.

2.9. Detection of T cell activation

In order to detect T cell activation, T cells were stimulated with anti-CD3/CD28 beads in a 1:1 bead:cell ratio for 5h at 37°C and 5% CO_2. Fluoxetine or BAPTA-AM were added 30 minutes before addition of the T cell stimulus and were maintained in the culture medium throughout the experiment. After 5h incubation, cells were stained with anti-human CD69 PECy7 and anti-human CD3 PECy5 (eBioscience, San Diego, CA, USA) for 30 minutes in PBS + 1% bovine serum albumin and 0.1% NaN_3, washed once and analyzed on a FC500 (Beckman coulter, Fullerton, CA, USA).

3. Results

3.1. Fluoxetine suppresses Ca^{2+} signaling in response to T cell receptor activation

In order to analyze the effect of fluoxetine on Ca^{2+} signaling when T cells are activated through the T cell receptor (TCR), we activated jurkat T cells with magnetic particles coated with antibodies against CD3 and CD28 and analyzed the resulting changes in $[Ca^{2+}]_i$ through labeling with the fluorescent Ca^{2+}

dye fluo3-AM. The fluorescent images obtained and the response of T cells to the anti-CD3/CD28 beads are illustrated in figure 5.1A. After each experiment, T cells making contact with at least one magnetic bead were visualized and selected for analysis. The changes in fluorescent signal over time (arbitrary units, A.U.), which relate to changes in $[Ca^{2+}]_i$, were plotted and result in a Ca^{2+} trace. The majority of cells in the control samples (83%) responded to contact with a bead with a short period of Ca^{2+} oscillations followed by a sustained increase in $[Ca^{2+}]_i$. A small percentage of cells showed an oscillatory pattern (11%) or transient response (6%) after contact with a bead (figure 5.1B). The same types of responses were found in T cells pre-incubated with 10 µM fluoxetine. A slight shift from sustained responses (75%) towards oscillatory (13%) and transient (12%) responses was observed, but no significant changes were detected as compared to control. However, fluoxetine did affect the magnitude of the response to TCR activation. T cells that were pre-incubated for 30 min with 10 µM fluoxetine (F10) responded with oscillations with smaller amplitude and a weaker sustained increase in $[Ca^{2+}]_i$. At 100 µM fluoxetine (F100), the response to TCR activation was almost completely absent (figure 5.1C). Viability of the cells was assessed by trypan blue staining at the end of the experiment and no increased cell death was observed in samples pre-incubated with fluoxetine compared to control samples (data not shown). The absence of a response in samples incubated with 100 µM fluoxetine was thus not due to loss of viability. In order to quantify the different responses, we calculated the difference between the maximum of the Ca^{2+} peak and the baseline (mean of 0-120s). Whereas the mean ± SD peak height of control T cells was 59.18 ± 30.38 (A.U.), the mean peak height of T cells pre-incubated with F10 was 50.97 ± 28.16 ($p=0.041$) (figure 5.1E). Cells pre-incubated with F100 showed a dramatically reduced response to TCR activation, with a mean peak height of only 7.45 ± 7.05 ($p<0.0001$). Similar results were obtained when the area under the $[Ca^{2+}]_i$ trace was analyzed instead of the peak $[Ca^{2+}]_i$ change (data not shown). In resting T lymphocytes (no TCR activation), fluoxetine did not affect the $[Ca^{2+}]_i$ (figure 5.1D). These results show that fluoxetine inhibits the Ca^{2+} signaling pathway following TCR activation in a concentration-dependent manner in T lymphocytes.

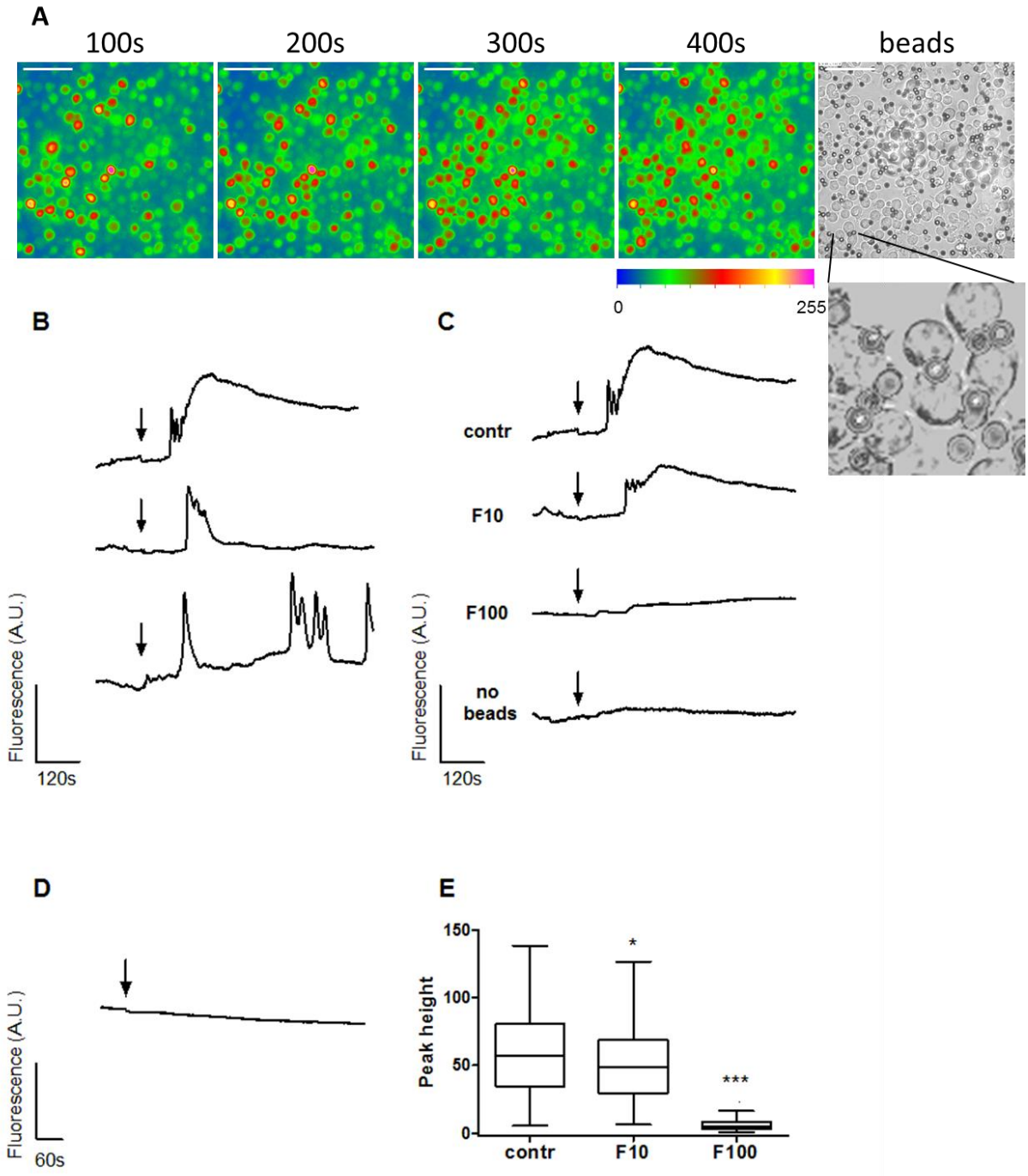

Figure 5.1: Effect of fluoxetine on Ca^{2+} signaling in response to TCR activation. Figure legend see opposite page.

Figure 5.1: Effect of fluoxetine on Ca^{2+} signaling in response to TCR activation. T cells were stimulated with magnetic beads coated with anti-CD3 and anti-CD28 antibodies. Figure 5.1A shows a series of images taken after addition of anti-CD3/CD28 beads (beads were added at 120s). The right image is a bright field image taken at the end of the experiment, in which the location of the beads can be seen. Scale bars are 50 µm. B) Representative Ca^{2+} responses induced by anti-CD3/CD28 beads in individual cells. Contact with a bead triggered sustained (top), transient (middle) or oscillatory (bottom) responses. The arrow indicates the addition of the beads. C) representative traces of T cells activated with anti-CD3/CD28 beads in Krebs buffer (contr), 10 µM fluoxetine (F10), 100 µM fluoxetine (F100) or without beads (negative control). For the negative control, the arrow indicates addition of an equal amount of buffer without beads. D) Effect of fluoxetine on $[Ca^{2+}]_i$ in resting T lymphocytes (no TCR activation). 100 µM fluoxetine was added after 60s (indicated by an arrow). Graph shows the mean trace of 89 cells. E) Peak height of the Ca^{2+} response of T cells when stimulated with anti-CD3/CD28 beads in Krebs buffer (contr) or fluoxetine (10 µM, F10 and 100 µM, F100). Peak height was calculated as the difference between the maximum and the baseline. Each condition was repeated at least three times and data were pooled for analysis. In total, 89-146 bead-bound cells per condition were analyzed. * = $p<0.05$. *** = $p<0.0001$.

3.2. Interference of fluoxetine with endoplasmic reticulum Ca^{2+} stores

We next questioned whether the observed suppression was due to either inhibition of capacitative Ca^{2+} entry or interference with the release of Ca^{2+} from intracellular stores. To this end we added thapsigargin (TG), a selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that prevents reuptake of Ca^{2+} into the ER, to the cells in Ca^{2+}-free buffer containing EGTA. Ca^{2+} exits the ER through a yet unidentified basal leak system, and blockage of SERCA consequently results in depletion of the ER. After a five-minute incubation period with TG that allowed the ER to be completely emptied, Ca^{2+}-containing buffer (1.5 mM Ca^{2+}) was added, thus allowing the cells to refill their ER with Ca^{2+} through capacitative Ca^{2+} entry (figure 5.2A). The impact of fluoxetine on both steps was analyzed. Interestingly, fluoxetine reduced the magnitude of the peak after TG addition in a concentration-dependent manner (control median 7.90, range [-75.54 – 192.90]; F10 3.35, [-58.45 – 180.27], $p<0.0001$; F100 1.12, [-22.14 – 99.43], $p<0.0001$; figure 5.2B). No significant differences could be detected with respect to the magnitude of the peak after addition of Ca^{2+}-containing buffer (control median 38.00, range [7.0-217.3]; F10 44.55, [5.2-196.1]; F100 41.00, [3.3-208.7]; $p=0.423$; figure 5.2C). These data suggest that fluoxetine might inhibit Ca^{2+} release from intracellular stores. In contrast, fluoxetine does not affect capacitative Ca^{2+} entry in T lymphocytes.

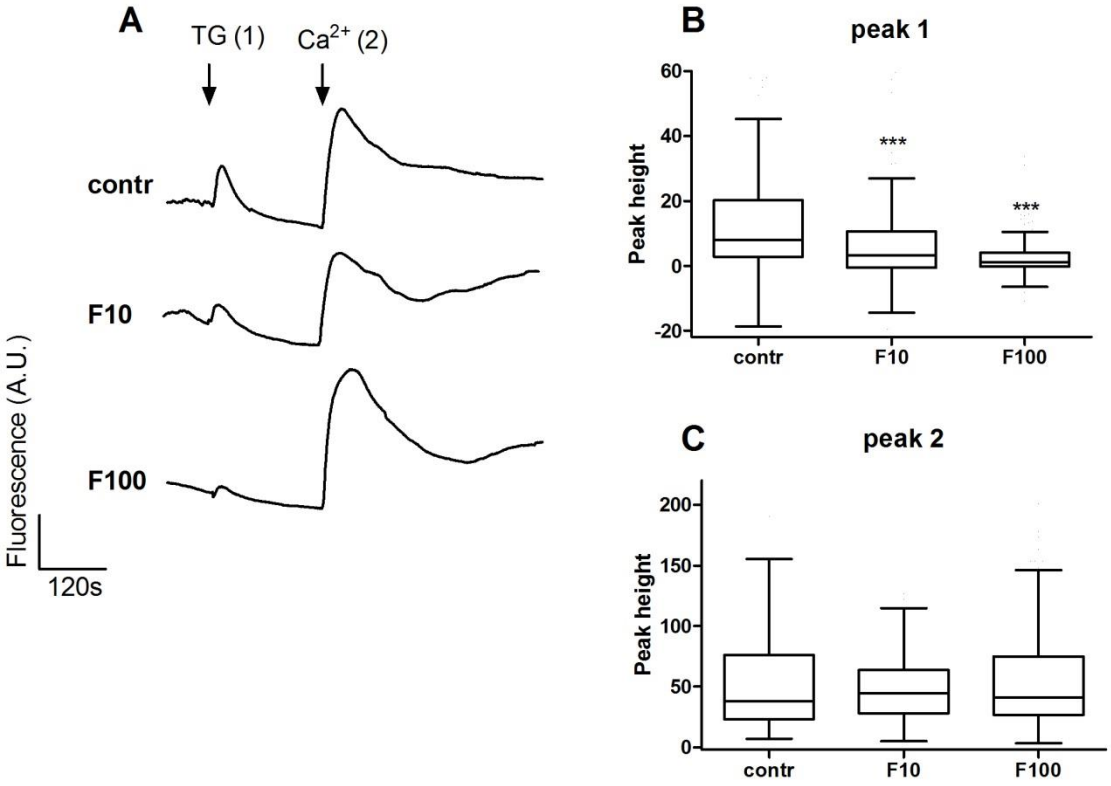

Figure 5.2. Effect of fluoxetine on thapsigargin-induced rise in $[Ca^{2+}]_i$ and capacitative Ca^{2+} entry. T cells were incubated in Ca^{2+}-free buffer and 10 µM thapsigargin was added at 120s. At 420s, when the ER was emptied, Ca^{2+}-free buffer was replaced for Ca^{2+}-containing buffer (1.5 mM Ca^{2+}). Cells were pre-incubated with fluoxetine (10 µM, F10 and 100 µM, F100) for 30 minutes, and fluoxetine was maintained in all added solutions. All conditions were repeated at least three times. Data were pooled for analysis. A) Mean traces of cells in Krebs buffer (contr), 10 µM fluoxetine (F10) and 100 µM fluoxetine (F100). Arrows indicate addition of thapsigargin (TG, peak1) and Ca^{2+}-containing buffer (Ca^{2+}, peak 2). B) Peak height of the Ca^{2+} change induced by thapsigargin (peak 1). Peak height was calculated as the difference between the maximum and the baseline. C) Peak height of Ca^{2+} change induced by re-introduction of Ca^{2+}-containing buffer (peak 2). *** = p<0.0001.

3.3. Interference of fluoxetine with IP_3-induced Ca^{2+} release

In order to study in more detail the effect of fluoxetine on the ER, T cells were loaded with NPE-caged IP_3 through electroporation, and IP_3 was released during imaging through flash photolysis. The height of the resulting $[Ca^{2+}]_i$ peak was measured. In accordance with the results of the TG experiment, fluoxetine reduced the height of the Ca^{2+} peak after release of IP_3, although statistical significance was only reached at 100 µM fluoxetine (mean control 11.43 ± 11.81; F10 9.98 ± 12.94, p=0.11, F100 2.13 ± 2.65, p<0.0001 (figure 5.3). Thus, fluoxetine suppresses IP_3-mediated Ca^{2+} release from the ER.

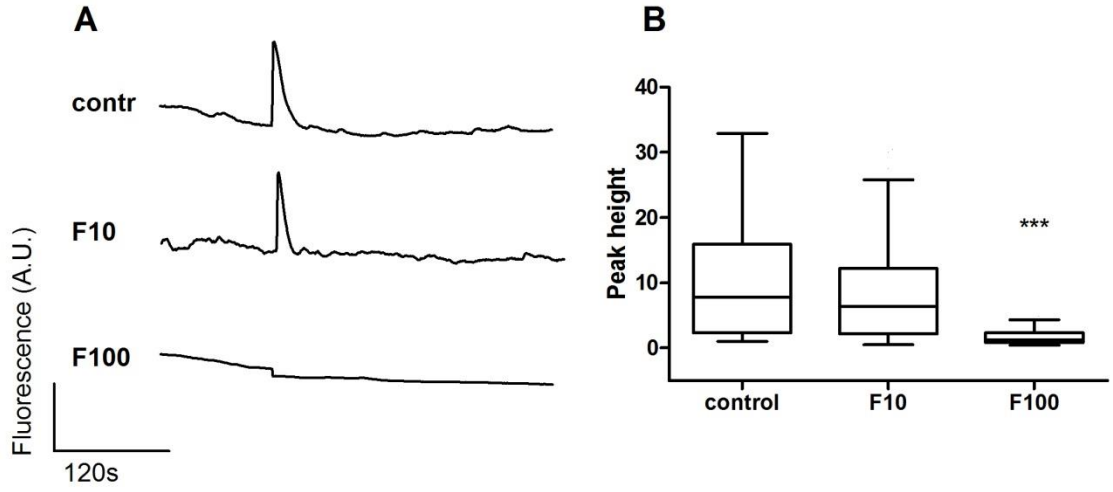

Figure 5.3. Effect of fluoxetine on IP_3-mediated Ca^{2+} release. T cells were electroporated with NPE-caged IP_3 and IP_3 was released by flash photolysis after 120s of imaging. Imaging was continued for 5 minutes. Fluoxetine (10 µM, F10 and 100 µM, F100) was added 30 minutes before the start of the experiment. The peak height of the Ca^{2+} change after photolytic release of IP_3 was calculated as the difference between the maximum and the baseline. A total of 61 – 111 cells per group were analyzed. A) mean traces of electroporated cells within the flash zone in Krebs buffer (contr), 10 µM fluoxetine (F10) and 100 µM fluoxetine (F100). B) Calculated peak heights of the recorded Ca^{2+} changes after photolytic release of IP_3. *** = p<0.0001.

3.4. Interference of fluoxetine with ryanodine receptor-mediated Ca^{2+} release

In addition to IP_3R, ryanodine receptors (RyR) are equally known to regulate Ca^{2+} release from intracellular stores. Ca^{2+} released by IP_3R in turn activates RyR, resulting in Ca^{2+}-induced Ca^{2+} release (CICR). The effect of fluoxetine on RyR-mediated Ca^{2+} release was analyzed by addition of caffeine, which is known to activate RyR [22]. Preliminary experiments to select the most suitable concentration of caffeine showed a concentration-dependent increase in $[Ca^{2+}]_i$ upon exposure to caffeine in a range from 10 to 50 mM (data not shown). As 50 mM caffeine induced the strongest effect, we selected this concentration to study the impact of fluoxetine hereon (figure 5.4A). Similar responses were obtained when the experiment was repeated in Ca^{2+}-free buffer, indicating that caffeine indeed released intracellular Ca^{2+} and did not induce influx of Ca^{2+} through the plasma membrane (data not shown). In order to confirm that the rise in $[Ca^{2+}]_i$ induced by caffeine was due to ryanodine receptor stimulation, cells were pre-incubated with an antagonistic concentration of ryanodine (200 µM). As shown in figure 5.4, ryanodine completely suppressed the rise in $[Ca^{2+}]_i$ induced by caffeine. Fluoxetine suppressed the rise in $[Ca^{2+}]_i$ in a concentration-dependent manner (figure 5.4A). In order to quantify the effect of fluoxetine on the RyR-mediated Ca^{2+} release, the

difference between the maximal and minimal $[Ca^{2+}]_i$ was calculated. Control cells showed a median peak height of 17.5, range [2.8 - 74.9], F10 12.3, [0.4 - 50.8], p<0.0001 and F100 4.1, [0.0 - 57.8], p<0.0001 (figure 5.4B). Thus, fluoxetine inhibits ryanodine receptor-induced Ca^{2+}-release in a concentration-dependent manner. The inhibitory effect of fluoxetine on caffeine-induced Ca^{2+} release is time-dependent, as longer incubation times resulted in a stronger inhibitory effect (figure 5.4C). However, as addition of fluoxetine at the same time as caffeine (T=0) already produced a significant inhibition of Ca^{2+} release, the effect of fluoxetine is manifested immediately.

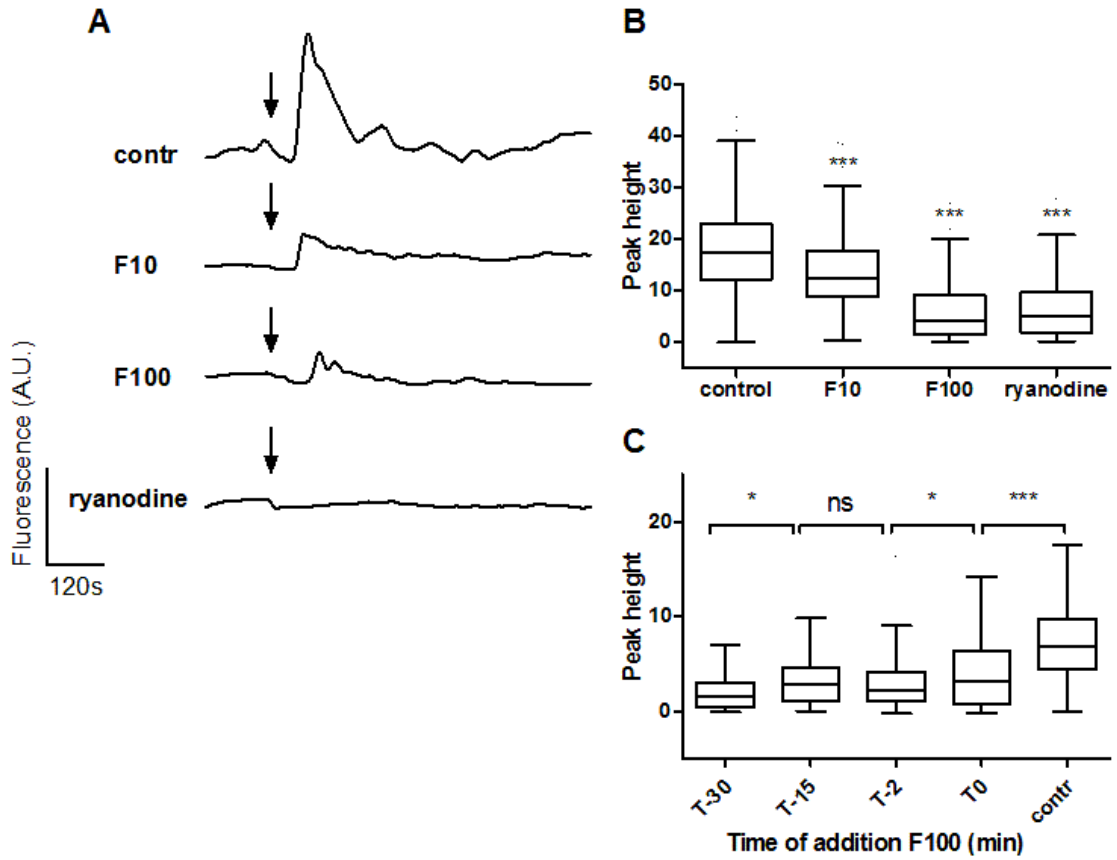

Figure 5.4. Effect of fluoxetine on RyR-mediated Ca^{2+} release. T cells were stimulated with 50 mM caffeine in the presence of different concentrations of fluoxetine. A) Individual traces of cells in Krebs buffer (contr), 10 µM (F10), 100 µM (F100) fluoxetine or 200 µM ryanodine and stimulated with 50 mM caffeine at 120s (arrow on the graph). Fluoxetine was added 30 minutes before the start of the experiment and maintained in all added solutions. B) Calculated peak heights of the recorded Ca^{2+} changes. Per sample, 100 arbitrary cells were analyzed. Each condition was performed in duplicate and results were pooled for analysis. C) Time-dependent effect of fluoxetine on caffeine-induced Ca^{2+} release. Caffeine was added at 120s. T-30 = addition of fluoxetine 30 min before the start of the experiment, T-15 = 15 minutes before the start, T-2 = at the start of the experiment; T0 = at the same time as addition of caffeine (120s). Contr = no fluoxetine added. * = p<0.05. *** = p<0.0001. ns = not significant.

3.5. Involvement of 5HT and SERT in the fluoxetine-induced effects on Ca^{2+} signaling

As fluoxetine is known to inhibit serotonin uptake through the SERT, we next questioned whether the observed effects on Ca^{2+} signaling could be initiated by SERT inhibition. Therefore, we first analyzed whether jurkat T lymphocytes express SERT. As shown in figure 5.5, T lymphocytes express SERT both at the mRNA level and the protein level. Whereas the positive control (hSERT transfected HEK293 cells) showed a protein band at ~80 and 60 kDa, only the 60 kDa band was found in T cells. As described previously, the ~80 kDa band presumably represents a highly glycosylated form of SERT, whereas the 60 kDa band is most likely the unmodified SERT protein [21]. Further, it should be noted that the large difference in expression levels between the positive control and the T cells is due to overexpression of SERT in the hSERT transfected cell line [21]. Specific binding of the primary antibody through its antigen-binding site was confirmed by incubation with a blocking peptide. The results of these experiments indicate that T lymphocytes do express SERT.

To investigate if the effects of fluoxetine on Ca^{2+} signaling are mediated by SERT inhibition, we analyzed the influence of a large excess (1 mM) 5HT on the fluoxetine-induced suppression of RyR-mediated Ca^{2+} release (100 µM fluoxetine). If inhibition of SERT by fluoxetine causes the observed decrease in Ca^{2+} signaling, it can be expected that 5HT reverses this effect by competing with fluoxetine for binding to SERT. As shown in figure 5.5C, 5HT did not inhibit the suppression of RyR-mediated Ca^{2+} release by 10 or 100 µM fluoxetine (control median 21.70, [0.0-70.7] vs 5HT 20.70, [3.1-71.1], p=0.61; F10 18.60, [0.9-60.3] vs F10 + 5HT 20.20, [-0.1-100], p=0.35; F100 7.30, [-0.9-62.2] vs F100 + 5HT 5.25, [-0.6-93.6], p=0.18). Thus, it is not likely that fluoxetine inhibits Ca^{2+} release from the ER through blockage of 5HT uptake by SERT.

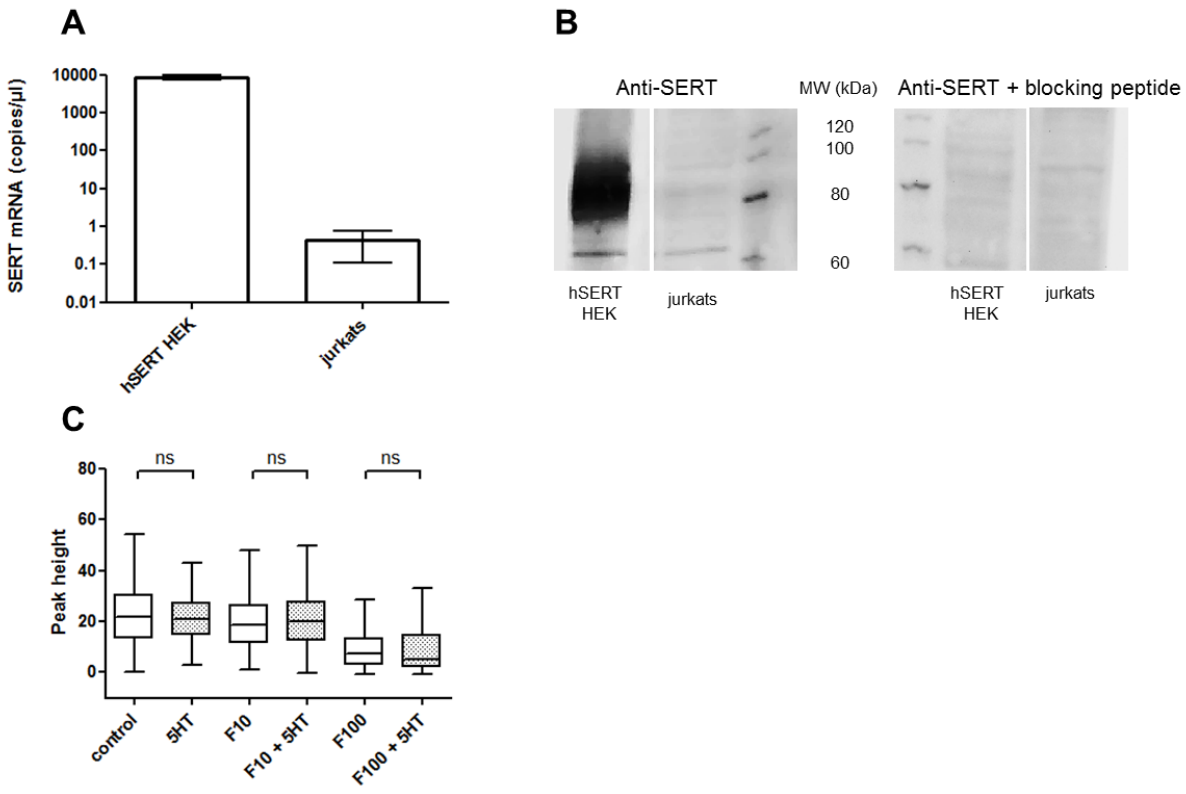

Figure 5.5. Analysis of SERT expression in jurkat T lymphocytes and involvement of 5HT in the fluoxetine-induced effects. A) SERT mRNA expression was detected with ddPCR. Samples were analyzed in duplo and compared to a positive control (hSERT transfected HEK293 cells); B) SERT protein expression in hSERT HEK cells (positive control) and T cells. Left: detection with anti-SERT; Right: detection with anti-SERT and a blocking peptide; C) Results of competition experiments with 5HT (1 mM) on fluoxetine (10 and 100 µM) inhibition of caffeine-induced Ca^{2+} release. Cells were incubated with fluoxetine and/or 5HT 30 minutes before the start of the experiment. All conditions were analyzed in triplicate and data were pooled for analysis. Peak height expresses the caffeine-induced change in Ca^{2+}. ns = not significant.

3.6. The effect of fluoxetine on T cell activation is mimicked by buffering of intracellular Ca^{2+}

In order to investigate whether the observed effect of fluoxetine on Ca^{2+} release from intracellular stores is at the basis of its immunosuppressive effect, we analyzed the effect of fluoxetine on CD69 expression, an early activation marker. Incubation of T cells with anti-CD3/CD28 beads during 5h in the absence of fluoxetine induced a strong upregulation of CD69 expression (figure 5.6). Non stimulated ('NS') cells showed a mean fluorescence intensity (MFI) of 10.29 ± 0.97, whereas stimulated cells ('S') had a MFI of 107.0 ± 3.0. Fluoxetine (100 µM) decreased the MFI to 9.77 ± 1.97 ($p<0.0001$ compared to 'S'). The same suppressive effect was found when cells were incubated with BAPTA-AM (50 µM), an intracellular Ca^{2+} chelator added to silence cytoplasmic Ca^{2+} changes, demonstrating that interference with intracellular Ca^{2+} signals after TCR stimulation indeed impairs T cell activation (MFI 18.27 ± 0.21, $p<0.0001$ compared to 'S'). Thus, the inhibitory effect of fluoxetine

on CD69 expression can be mimicked by buffering the intracellular Ca^{2+} of the cells with BAPTA-AM. These data show that interference with Ca^{2+} signaling in T lymphocytes results in impaired T cell activation, as estimated from CD69 expression, and that the effect of fluoxetine is comparable in magnitude to the effect of buffering $[Ca^{2+}]_i$ with BAPTA-AM.

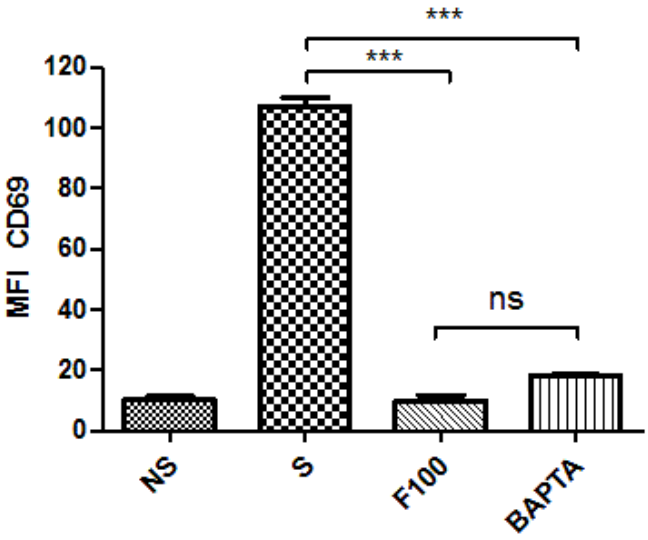

Figure 5.6. Effect of fluoxetine and BAPTA-AM on CD69 expression in activated T cells. T cells were activated with anti-CD3/CD28 beads. BAPTA-AM (50 µM) or fluoxetine (100 µM, F100) were added 30 minutes before addition of the beads and the cells were incubated for 5h at 37°C and 5% CO_2. Cells were stained with anti-human CD3 PECy5 and CD69 PECy7 and analyzed by flow cytometry. NS = non-stimulated cells. S = cells stimulated with anti-CD3/CD28 beads. Mean ± SD of mean fluorescent intensities (MFI) are shown. Each condition was analyzed in triplicate. *** = $p<0.0001$. ns = not significant.

4. Discussion

In this report, we investigated the impact of fluoxetine on Ca^{2+} signaling in jurkat T lymphocytes. Previous research has demonstrated that fluoxetine and other SSRIs exert anti-inflammatory and immunosuppressive effects on T lymphocytes [23, 24]. Similar suppressive effects have been described in jurkat T lymphocytes [25]. Although several hypotheses on the mechanism behind the observed effects were investigated (reviewed in [2]), the exact mechanism by which fluoxetine suppresses T cell activation and proliferation was not clarified. SSRIs have been shown to affect Ca^{2+} signaling in several cell types including neurons [8], astrocytes [9], microglia [10], osteosarcoma cells [11], platelets [13] and adrenal medulla PC12 cells [7, 26]. Since elevation of intracellular Ca^{2+} plays a major role in the pathway leading to T cell activation in response to antigens [6], we investigated if SSRIs, in particular fluoxetine, interfere with this signaling pathway in T cells.

In the case of T lymphocytes, Ca^{2+} is stored in the ER and release from the ER is mediated predominantly by binding of IP_3 to IP_3R, and is further regulated by RyR [27]. The majority of research conducted on the effect of antidepressants, including SSRIs, on Ca^{2+} signaling in other cell types suggests interference with intracellular Ca^{2+} stores [7, 9, 11, 13]. In accordance with these data, we demonstrated that fluoxetine interferes with the ER Ca^{2+} stores in T lymphocytes. As opposed to tricyclic antidepressants, we found that fluoxetine inhibits IP_3-induced Ca^{2+} release [28]. More specifically, we demonstrated that fluoxetine suppresses the rise in $[Ca^{2+}]_i$ in response to TCR activation. Additionally, we showed that the decreased Ca^{2+} signaling is due to the inhibition of IP_3- and RyR-mediated Ca^{2+} release from ER stores, rather than the blockage of capacitative Ca^{2+} entry. The observed inhibition of Ca^{2+} release from intracellular stores could be explained in two ways: either fluoxetine causes a depletion of stored Ca^{2+} thus leaving less Ca^{2+} available for release after IP_3R or RyR activation, or fluoxetine directly interferes with the Ca^{2+} channels blocking the Ca^{2+} release in response to IP_3R or RyR activation. In contrast to Serafeim et al., who found that fluoxetine and other SSRIs induced a rise in $[Ca^{2+}]_i$ in malignant B cells [12], the addition of fluoxetine to resting T cells did not result in any increase of the cytoplasmic Ca^{2+} concentration. Therefore, it is unlikely that fluoxetine would deplete the ER stores in this cell type. In addition, the inhibitory effect of fluoxetine on caffeine-induced Ca^{2+} release occurs almost immediately, indicating that a slow and therefore unnoticed depletion of the ER Ca^{2+} stores is unlikely. Instead, these results indicate that fluoxetine directly interferes with the ER Ca^{2+} channels and thereby inhibits release of Ca^{2+} in response to IP_3 or ryanodine receptor activation (figure 5.7).

As to how fluoxetine interacts with Ca^{2+} channels, we demonstrated that the effect is not mediated through blockage of 5HT transport by SERT since addition of even a large excess of 5HT did not abrogate the effect of fluoxetine on Ca^{2+} signaling. Instead, it has been proposed that fluoxetine, being a highly lipophilic molecule, interacts with the membrane lipid bilayer and thereby influences the ion channel structure and function [7]. Future research will be needed to elucidate how fluoxetine interacts with Ca^{2+} channels at the molecular level.

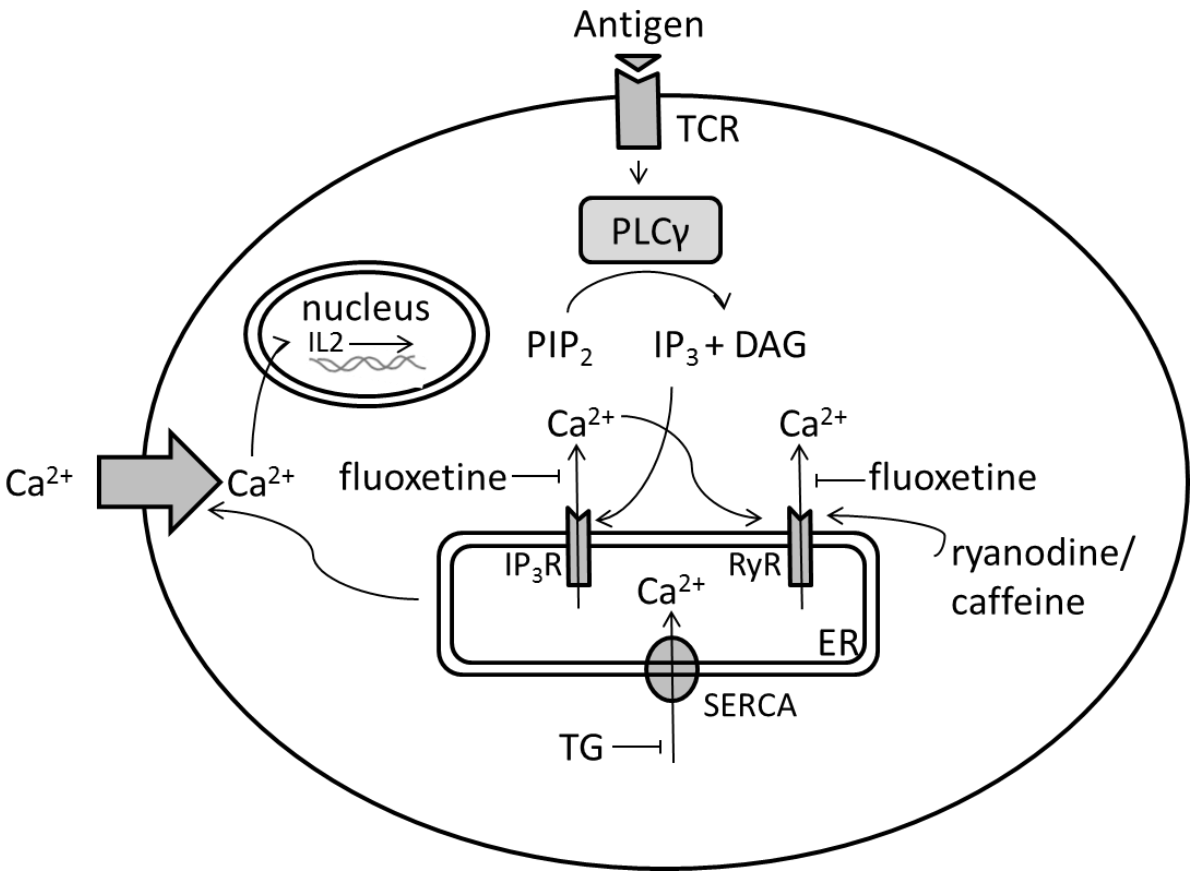

Figure 5.7. Schematic representation of fluoxetine-induced effects on Ca^{2+} signaling in T lymphocytes. In T cell activation, binding of an antigen to the TCR results in activation of PLCγ, which converts PIP_2 to IP_3 and DAG. IP_3 induces Ca^{2+} release from the ER through activation of IP_3R. Ca^{2+} in turn activates RyR thereby further stimulating Ca^{2+} release. RyR agonists such as ryanodine and caffeine also induce Ca^{2+} release by RyR. Secondary to the release of Ca^{2+} from the ER, influx of Ca^{2+} through the plasma membrane is induced. The rise in cytoplasmic Ca^{2+} eventually leads to transcription of a pleotropic set of genes, including IL2, resulting in T cell activation and proliferation. Fluoxetine inhibits IP_3- and RyR-mediated release of Ca^{2+} from the ER. As Ca^{2+} release from intracellular stores is an indispensable step in the pathway leading to T cell activation, inhibition of Ca^{2+} signaling by fluoxetine results in impaired T cell activation and proliferation. Intermediate steps in the signaling transduction pathway were omitted for clarity. TCR = T cell receptor, PLCγ = phospholipase Cγ; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = phosphatidylinositol 3,4,5-trisphosphate; DAG = diacylglycerol; ER = endoplasmic reticulum; TG = thapsigargin.

Finally, we demonstrated that the immunosuppressive effects of fluoxetine - under the form of decreased CD69 expression in response to TCR activation – can be mimicked by buffering of intracellular Ca^{2+} with BAPTA-AM. Others have shown that inhibition of IP_3- or RyR- mediated Ca^{2+} release downregulates jurkat T cell proliferation and IL2 production [27]. In primary human T cells, inhibition of RyR equally inhibited T cell proliferation [29]. These data suggest that inhibition of IP_3- and RyR-mediated Ca^{2+} release from ER stores plays an important role in the immunosuppressive

effects of fluoxetine, although it cannot be excluded that other mechanisms contribute to the immunosuppressive outcome.

It should be noted that the concentrations of fluoxetine used in this report are considerably higher than the plasma concentrations found in depressive patients. Whereas plasma concentrations of fluoxetine are usually below 1 µM, we applied concentrations of 10-100 µM to study the effects of fluoxetine on Ca^{2+} signaling. The applied concentrations are based on previous reports on *in vitro* T cell immunosuppression by SSRIs [24]. However, since SSRIs are lipophilic compounds that accumulate in tissues, significantly higher concentrations in organs than in plasma can occur. In that respect, it has been demonstrated that SSRIs can reach 10-fold higher concentrations in spleen than in plasma [30]. As the meeting of a naive T cell and its antigen occurs in lymphoid tissue such as the spleen or lymph nodes, it can be expected that T lymphocytes going through the activation process in lymphoid tissue are actually exposed to fluoxetine concentrations up to 10 µM, a concentration which we have demonstrated to exert inhibitory effects on Ca^{2+} signaling *in vitro*.

Finally, we selected fluoxetine to study the effects on Ca^{2+} signaling in T lymphocytes. As other SSRIs also induce immunosuppressive effects in T lymphocytes [24], it would be interesting to investigate whether these compounds also affect Ca^{2+} signaling in T lymphocytes.

In conclusion, these data show that fluoxetine suppresses intracellular Ca^{2+} signaling in jurkat T lymphocytes through inhibition of Ca^{2+} release from IP_3- and caffeine sensitive intracellular stores, an effect likely to be at the basis of the observed immunosuppression.

References

1. Janssen, D.G., et al., *A psychoneuroimmunological review on cytokines involved in antidepressant treatment response.* Hum Psychopharmacol, 2010. **25**(3): p. 201-15.
2. Gobin, V., et al., *Selective serotonin reuptake inhibitors as a novel class of immunosuppressants.* Int Immunopharmacol, 2014. **20**: p. 148-156.
3. Gobin, V., et al., *Fluoxetine Reduces Murine Graft-Versus-Host Disease by Induction of T cell Immunosuppression.* J Neuroimmune Pharmacol, 2013.
4. Schuster, C., et al., *Selective serotonin reuptake inhibitors--a new modality for the treatment of lymphoma/leukaemia?* Biochem Pharmacol, 2007. **74**(9): p. 1424-35.
5. Cloonan, S.M., et al., *The antidepressants maprotiline and fluoxetine have potent selective antiproliferative effects against Burkitt lymphoma independently of the norepinephrine and serotonin transporters.* Leuk Lymphoma, 2010. **51**(3): p. 523-39.
6. Lewis, R.S., *Calcium signaling mechanisms in T lymphocytes.* Annu Rev Immunol, 2001. **19**: p. 497-521.
7. Kim, H.J., et al., *Fluoxetine inhibits ATP-induced [Ca(2+)](i) increase in PC12 cells by inhibiting both extracellular Ca(2+) influx and Ca(2+) release from intracellular stores.* Neuropharmacology, 2005. **49**(2): p. 265-74.
8. Kim, H.J., et al., *Fluoxetine suppresses synaptically induced [Ca(2)(+)]i spikes and excitotoxicity in cultured rat hippocampal neurons.* Brain Res, 2013. **1490**: p. 23-34.
9. Li, B., et al., *Effects of chronic treatment with fluoxetine on receptor-stimulated increase of [Ca2+]i in astrocytes mimic those of acute inhibition of TRPC1 channel activity.* Cell Calcium, 2010. **50**(1): p. 42-53.
10. Horikawa, H., et al., *Inhibitory effects of SSRIs on IFN-gamma induced microglial activation through the regulation of intracellular calcium.* Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2010. **34**(7): p. 1306-1316.
11. Lin, K.L., et al., *Effect of sertraline on [Ca2+](i) and viability of human MG63 osteosarcoma cells.* Drug Chem Toxicol, 2013. **36**(2): p. 231-40.
12. Serafeim, A., et al., *Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells.* Blood, 2003. **101**(8): p. 3212-9.
13. Helmeste, D.M., et al., *Serotonin uptake inhibitors modulate intracellular Ca2+ mobilization in platelets.* Eur J Pharmacol, 1995. **288**(3): p. 373-7.
14. Edgar, V.A., et al., *Fluoxetine action on murine T-lymphocyte proliferation: participation of PKC activation and calcium mobilisation.* Cell Signal, 1998. **10**(10): p. 721-6.
15. Edgar, V.A., et al., *Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation.* Eur J Pharmacol, 1999. **372**(1): p. 65-73.
16. Monaco, G., et al., *Alpha-Helical Destabilization of the Bcl-2-BH4-Domain Peptide Abolishes Its Ability to Inhibit the IP3 Receptor.* PLoS One, 2013. **8**(8): p. e73386.
17. De Bock, M., et al., *Connexin 43 hemichannels contribute to cytoplasmic Ca2+ oscillations by providing a bimodal Ca2+-dependent Ca2+ entry pathway.* J Biol Chem, 2012. **287**(15): p. 12250-66.
18. R core team, *R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing*. 2014; Available from: http://www.R-project.org.
19. Venables, W.N., Ripley, B.D., *Modern Applied Statistics with S*. Fourth ed. 2002, New York: Springer.
20. Kiselinova, M., et al., *Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA.* PLoS One. **9**(1): p. e85999.

21. Chamba, A., et al., *Characterisation of the endogenous human peripheral serotonin transporter SLC6A4 reveals surface expression without N-glycosylation.* J Neuroimmunol, 2008. **204**(1-2): p. 75-84.
22. Zucchi, R. and S. Ronca-Testoni, *The sarcoplasmic reticulum Ca2+ channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states.* Pharmacol Rev, 1997. **49**(1): p. 1-51.
23. Taler, M., et al., *Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression.* Eur Neuropsychopharmacol, 2007. **17**(12): p. 774-80.
24. Gobin, V., et al., *Fluoxetine reduces murine graft-versus-host disease by induction of T cell immunosuppression.* J Neuroimmune Pharmacol, 2013. **8**(4): p. 934-43.
25. Amit, B.H., et al., *Proapoptotic and chemosensitizing effects of selective serotonin reuptake inhibitors on T cell lymphoma/leukemia (Jurkat) in vitro.* Eur Neuropsychopharmacol, 2009. **19**(10): p. 726-34.
26. Hahn, S.J., et al., *Inhibition by fluoxetine of voltage-activated ion channels in rat PC12 cells.* Eur J Pharmacol, 1999. **367**(1): p. 113-8.
27. Dadsetan, S., et al., *Store-operated Ca2+ influx causes Ca2+ release from the intracellular Ca2+ channels that is required for T cell activation.* J Biol Chem, 2008. **283**(18): p. 12512-9.
28. Shimizu, M., et al., *Ca2+ release from inositol 1,4,5-trisphosphate-sensitive Ca2+ store by antidepressant drugs in cultured neurons of rat frontal cortex.* J Neurochem, 1993. **60**(2): p. 595-601.
29. Thakur, P., S. Dadsetan, and A.F. Fomina, *Bidirectional coupling between ryanodine receptors and Ca2+ release-activated Ca2+ (CRAC) channel machinery sustains store-operated Ca2+ entry in human T lymphocytes.* J Biol Chem, 2012. **287**(44): p. 37233-44.
30. Uhr, M., M.T. Grauer, and F. Holsboer, *Differential enhancement of antidepressant penetration into the brain in mice with abcb1ab (mdr1ab) P-glycoprotein gene disruption.* Biol Psychiatry, 2003. **54**(8): p. 840-6.

CHAPTER 6

FLUOXETINE REDUCES MURINE GRAFT-VERSUS-HOST DISEASE BY INDUCTION OF T CELL IMMUNOSUPPRESSION

Based on "*Fluoxetine reduces murine graft-versus-host disease by induction of T cell immunosuppression.*" Veerle Gobin, Katleen Van Steendam, Sabine Fevery, Kelly Tilleman, An Billiau, Damiaan Denys* and Dieter Deforce*. J Neuroimmune Pharmacol, 2013; 8: 934-43.

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CHAPTER 6. FLUOXETINE REDUCES MURINE GRAFT-VERSUS-HOST DISEASE BY INDUCTION OF T CELL IMMUNOSUPPRESSION

Abstract

Serotonin reuptake inhibitors (SRIs) have been shown to possess immunomodulatory effects, which potentially could be used to treat immune-mediated disorders. After hematopoietic stem cell transplantation, immunocompetent cells present in the graft can mount an immune response to host antigens, resulting in graft-versus-host disease. We investigated whether high-dose treatment with fluoxetine was able to suppress acute graft-versus-host disease (GvHD) in a MHC-matched, minor histocompatibility antigen mismatched murine bone marrow transplantation model. We found that fluoxetine induces a significant reduction of clinical symptoms and increases survival of these animals. The amelioration of clinical GvHD was accompanied by a reduced expansion of alloreactive T cells. We discuss these results in the light of potential future exploration of SRIs as a novel class of T cell immunosuppressive drugs.

1. Introduction

Serotonin reuptake inhibitors (SRIs) have been shown to possess immunomodulatory effects, which potentially could be used to treat immune-mediated disorders. Evidence exists that SRIs may attenuate autoimmune responses in experimental autoimmune encephalomyelitis, collagen-induced arthritis, murine allergic asthma and contact hypersensitivity reaction [3-8]. A different type of immune-mediated disorders occurs in the transplantation setting. Whereas organ transplantation is often complicated by immunological reactions of host immune cells against the transplanted donor organ (host-versus-graft reaction), the opposite occurs in stem cell transplantation. After transplantation of donor hematopoietic stem cells into a patient or host, immunocompetent cells present in the graft can mount an immune response to allogeneic antigens expressed by the host (graft-versus-host reaction). When this immunological reaction causes damage to target organs such as skin, liver and gastro-intestinal tract, it is called graft-versus-host disease (GvHD). Hypothesizing that SRIs may hold potential as a novel class of immunosuppressive drugs, the aim of this study was to determine whether SRIs could suppress alloreactive T cell responses in murine GvHD. The host antigens responsible for allogeneic reactions can be divided into major and minor histocompatibility antigens (MHC and miHA, respectively). Since over 50% of hematopoietic stem cell transplantations in the clinical practice are MHC-matched, we used a MHC-matched, minor histocompatibility antigen (miHA)-mismatched model of allogeneic bone marrow transplantation (BMT). In this study, we deliver proof-of-concept evidence that SRIs may attenuate murine GvHD.

2. Materials and methods

2.1. Animals

Ten- to 12-week old female AKR (H-2^k, Thy1.1, Mls1a/2b) mice were used as recipients and 6- to 8-week old C3H (H-2^k, Thy1.2, Mls1b/2a) mice as donors. Mice were purchased from Harlan BV, The Netherlands. Recipients were housed in groups of four or five in individually ventilated cages. Animals were fed standardised pellet chow and water, decontaminated by UV irradiation or by acidification. All experiments were approved by the Ethical Committee for Animal Science of the University of Leuven.

2.2. Mixed lymphocyte culture (MLC)

Single cell suspensions were prepared from spleens obtained from donor C3H and recipient AKR mice using a gentle MACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and 70 µm cell strainers. Responder (C3H) splenocytes suspended in RPMI + 5% fetal bovine serum (FBS) + 1%

penicillin/streptomycin (100 U/ml penicillin G; 100 µg/ml streptomycin) were loaded onto a nylon-wool column (Nylon Wool Fiber, Baseclear B.V., Leiden, The Netherlands) and incubated for 60 min at 37°C and 5% CO_2. Following incubation, the column was washed with RPMI + 5% FBS + 1% antibiotics and the T cell-enriched eluate was resuspended at $5x10^6$ cells/ml in RPMI + 10% FBS + 1% antibiotics + 0.5% 2-mercaptoethanol. AKR splenocytes were inactivated with 32 ng/ml mitomycin C (Kyowa Hakko Kogyo Co, Ltd, Tokyo, Japan) for 20 min at 37°C and washed 4x in RPMI + 10% FBS and 1% antibiotics. MLC were performed at a concentration of $5x10^6$ cells per ml in a 1:1 ratio and a final volume of 200 µl per well in flat-bottom 96-well microculture plates. Cells were cultured for 5 days in RPMI + 10% FBS + 1% antibiotics + 0.5% 2-mercaptoethanol. Reactions were performed in quadruplicate. DNA synthesis was assayed by adding 1 µCi methyl-^{3}H-thymidine (Radio chemical centre, Amersham, Buckinghamshire, UK) per well during the last 18 hours of culture. Thereafter, cells were harvested on glass filter paper and the counts per minute were determined with a liquid scintillation counter. Results were calculated as the percentage suppression compared to control wells not containing SRIs:

$$\% \text{ suppression} = 100 - \frac{\text{cpm SRI-treated cells x } 100}{\text{cpm control cells}}$$

2.3. GvHD model and SRI treatment

Bone marrow (BM) cells were obtained by flushing RPMI containing 1% heparin through the shafts of the femurs and tibia of C3H donor mice. T cell depletion was performed using cytotoxic complement-fixing anti-Thy1.2 antibody and low toxic rabbit complement (Serotec, Oxford, United Kingdom) as described previously [9]. AKR recipient mice received a single dose of 9.5 Gy total body irradiation on day -1. Within 24h after completion of irradiation, either $5x10^6$ T cell depleted BM (BM only) or $5x10^6$ T cell depleted BM in combination with $50x10^6$ spleen cells (BM + SPL) were injected into a tail vein in a total volume of 250 µl. Recipient mice were treated with 20 mg/kg fluoxetine IP 2x/day at the day of transplantation, 1x/day for the following 11 days, and 3x/week for the rest of the experiment. Control animals received vehicle (PBS) only.

2.4. GvHD scoring

Animals were inspected on a daily basis. Signs of GvHD typically observed in this model are ruffled fur and hunched posture, lethargy, inflammation of the eyes, weight loss and diarrhea [9, 10]. The mice were weighed and scored for GvHD once weekly, using a GvHD scoring system previously described in this model [11]. Scoring was always done by the same person. Each parameter received a score as followed: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. For body weight, the following scoring

system was used: 0 = 100-90%, 1 = 90-80%, 2 = 80-70%, 3 = <70% of initial body weight. The maximum score was 15. Mice that succumbed to GvHD received the maximum score of 15.

2.5. Donor T cell chimerism and host-reactive donor T cell frequency

The percentage of donor T lymphocytes in peripheral blood was determined by flow cytometry, based on the differential expression of Thy1.2 (donor) and Thy1.1 (recipient). Following red blood cell lysis with NH_4Cl, cells were labelled with FITC- or PE-conjugated anti-Thy1.1 and anti-Thy1.2 (Serotec, Oxford, UK). The frequency of host-reactive TCR-Vβ6+ T cells was determined as a parameter of *in vivo* alloreactive T cell expansion [9, 10]. Cells were labelled with APC-, PE- or PerCP-conjugated antibodies against CD3 and TCR-Vβ6 (BD Biosciences, Erembodegem, Belgium).

2.6. Statistics

The Gehan-Breslow-Wilcoxon test was used to estimate the level of significance of the difference in survival between treatment groups. The Wilcoxon signed ranks test was used to identify statistically significant differences for GvHD scores and flow cytometry data between treatment groups.

3. Results

3.1. Selection of the most potent SRI

In order to select the most potent SRI for suppression of murine acute GvHD, a preliminary *in vitro* screening was performed by means of mixed lymphocyte culture (MLC). Paroxetine, fluoxetine and sertraline, the three SRIs that showed strongest anti-proliferative and pro-apoptotic effects in human T lymphocytes, were tested in concentrations ranging from 1 to 20 µM. The proliferative response of T cell-enriched C3H splenocytes was analyzed when stimulated by mitomycin C-inactivated AKR splenocytes, an *in vitro* situation mimicking the alloresponse that occurs in the *in vivo* mouse model. All tested SRIs induced a strong anti-proliferative effect that reached approximately 100% suppression at 10 µM (figure 6.1). At the lowest concentration tested, 1 µM, the strongest effect was observed for fluoxetine. These data correlated well with the human *in vitro* experiments (chapter 3) in which fluoxetine equally exerted an anti-proliferative effect at 1 µM. As expected plasma concentrations were in the lower micromolar range, fluoxetine was chosen to explore the *in vivo* immunosuppressive effect on murine acute GvHD.

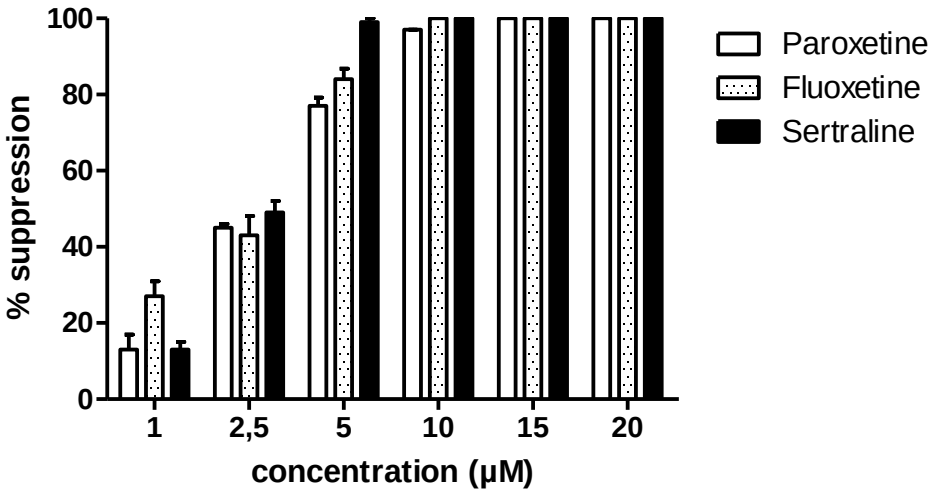

Figure 6.1. Preliminary screening of the anti-proliferative effect of three SRIs in a mixed lymphocyte culture with C3H responder cells and AKR stimulator cells. T cell enriched C3H splenocytes (responders) and mitomycin C-inactivated AKR splenocytes (stimulators) were co-cultured for 5 days at a concentration of $5x10^6$ cells per ml in a 1:1 ratio and total volume of 200 µl. DNA synthesis was assayed through ^{3}H-thymidine incorporation during the last 18h of culture. Data are presented as mean + SEM.

3.2. Fluoxetine delays the onset and attenuates the severity of GvHD

An established model of acute GvHD in a MHC-matched miHA-mismatched mouse strain combination was used [9, 10]. AKR recipient mice carry the Mtv-7 retrovirus which encodes the Mls-1 antigen, leading to deletion of TCR-Vβ6+ T cells. C3H donor mice do not carry the Mtv-7 virus and therefore TCR-Vβ6+ T cells are retained in these mice. In this model, donor and recipient mice differ in their expression of the Mtv7-genome, which has been shown to be associated with a highly increased rate and severity of GvHD [12].

An IP dose of 20 mg/kg fluoxetine was administered twice at the day of transplantation in order to achieve high enough plasma levels to prevent alloreactive T cells to initiate an immune reaction. To prevent alloreactivity during the immediate posttransplant period (day 2-12), fluoxetine was administered 1x/day. In this period, the frequency and severity of GvHD is higher, possibly because of overstimulation of host-reactive T cells by the remnants of the cytokine storm elicited by the conditioning regimen [13]. In our murine model, cytokine mRNA expression is diminished by day ten after irradiation [14]. During the rest of the experiment, a maintenance dose was given 3x/week. A 20 mg/kg IP dose was chosen as this dose gives rise to a plasma concentration of 4 µM [15], a

concentration that showed optimal anti-proliferative effect in preliminary murine mixed lymphocyte reactions without inducing cytotoxicity (figure 6.1).

Throughout the course of the experiment, mice were observed daily for clinical symptoms of GvHD and the GvHD score was recorded weekly. Mice that were treated with vehicle developed typical symptoms of GvHD (score > 2) after 4 weeks, whereas fluoxetine-treated mice only showed clinical signs of illness after 8 weeks (figure 6.2A). Although fluoxetine-treated mice did develop clinical symptoms in the course of the experiment, GvHD was less severe in this group compared to vehicle-treated mice (p<0.0001). Control mice receiving either BM+SRI (n=6), BM+vehicle (n=7) or BM only (n=5) did not develop clinical signs of GvHD (data not shown).

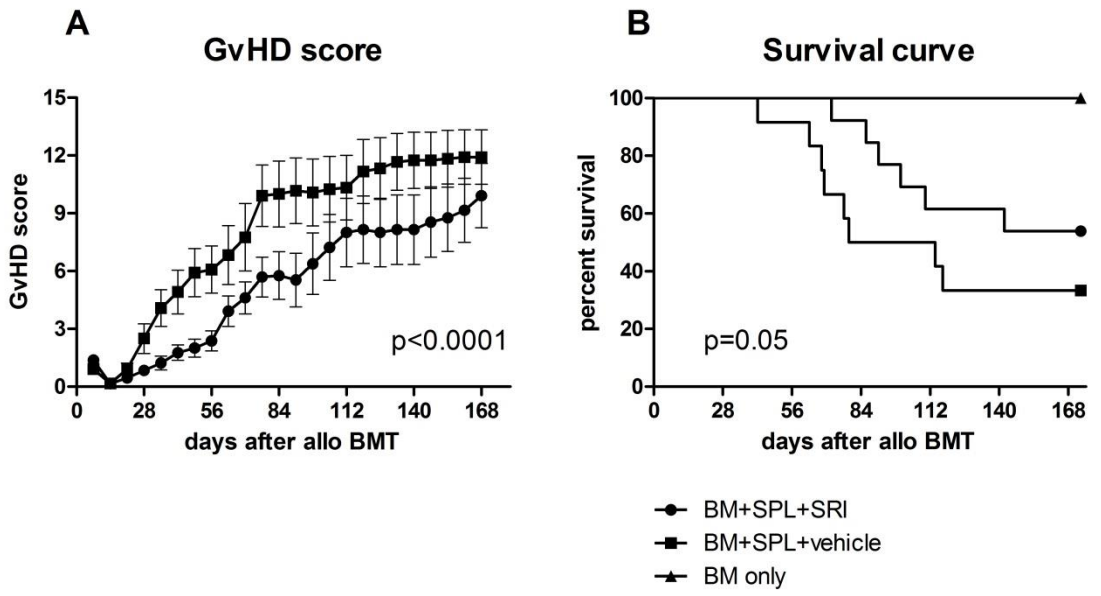

Figure 6.2. Effect of fluoxetine on GvHD score and survival. AKR mice were irradiated with 9.5 Gy on day -1 and transplanted with 5x10^6 C3H BM only or together with 50x10^6 SPL cells on day 0. Mice were treated with 20 mg/kg IP fluoxetine or vehicle 2x/day on the day of transplantation, 1x/day for the following 11 days and 3x/week for the rest of the experiment. (a) mean ± SEM GvHD scores from BM+SPL+SRI group (n=13) and BM+SPL+vehicle group (n=12). GvHD score was based on five parameters, each receiving a score of 0-3: ruffled fur and hunched back, inflammation of the eyes, weight, diarrhea and lethargy. (b) Survival curve. Results are the percentage survival from BM+SPL+SRI (n=13), BM+SPL+vehicle (n=12) and BM only (n=5) groups. Results are pooled data from two different experiments.

3.3. Fluoxetine reduces GvHD lethality

In figure 6.2B, the survival of AKR mice after transplantation of 5x10^6 C3H BM only or together with 50x10^6 SPL cells and treated with 20 mg/kg fluoxetine or vehicle is shown. Whereas only 4/12

(33.3%) animals from the vehicle-treated group survived 6 months after transplantation, 7/13 (53.8%) mice survived in the fluoxetine-treated group (p=0.05). Control mice receiving either BM+SRI (n=6) (data not shown), BM+vehicle (n=7) (data not shown) or BM only (n=5) all survived until the end of the experiment.

3.4. Fluoxetine does not interfere with engraftment of cells

In order to determine whether fluoxetine interferes with the engraftment of the allogeneic cells, peripheral blood donor T cell chimerism was determined at week 8 after transplantation, a time point at which donor T cell chimerism – in the absence of GvHD - can be expected to be near-complete [9]. Thy1.2 and Thy1.1 expression was used to discriminate between donor- and recipient-derived lymphocytes, respectively. Both fluoxetine-treated and vehicle-treated mice showed a donor T cell chimerism of more than 99%, indicating that the efficiency of the stem cell transplantation was equal in both groups and was not negatively influenced by fluoxetine. Consistent with previous work in this model, donor T cell chimerism of the 'BM only' group was around 90% (figure 6.3A).

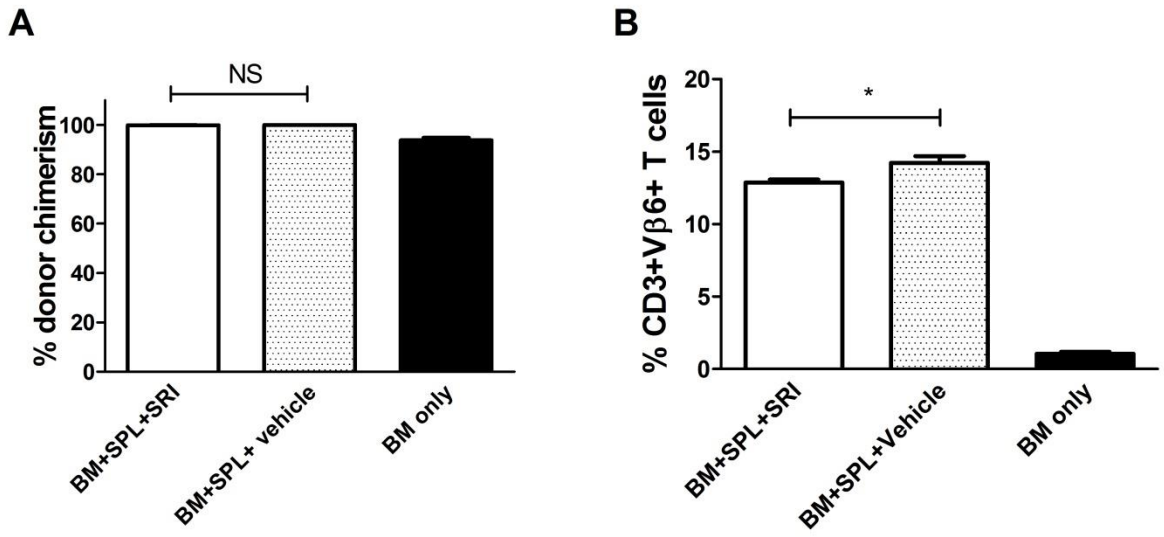

Figure 6.3. Donor chimerism and alloreactivity. (a) Percentage donor T cell chimerism in peripheral blood lymphocytes, determined by Thy1.1 (recipient) and Thy1.2 (donor) positivity. (b) percentage alloreactive CD3+Vβ6+ T cells. Results are mean ± SEM from 5 animals in BM+SPL+SRI and BM only group and from 4 animals in BM+SPL+vehicle group. Statistically significant differences are depicted with * (one-tailed p<0.05). NS = not significant.

3.5. Fluoxetine reduces the expansion of donor-type host-reactive T cells

In murine GvHD models involving Mls-disparate mouse strains, GvHD has been shown to be associated with expansion of donor-type T cells bearing TCR Vβ chains that are specific for host-type Mls antigens [9, 10, 16]. Accordingly, GvHD in C3H-AKR chimeras is associated with an expansion of TCR-Vβ6+ T cells [9, 10]. In order to determine whether fluoxetine suppressed the expansion of these host-reactive T cells, we determined the frequency of CD3+Vβ6+ T cells in peripheral blood of chimeras at week 8 after bone marrow transplantation, a time point at which control mice showed clear GvHD whereas SRI-treated mice were still free of GvHD clinical symptoms (score ≤ 2). The results are shown in figure 6.3B. Fluoxetine-treated mice showed a significantly lower percentage of peripheral blood CD3+Vβ6+ T cells than vehicle-treated mice (p=0.016), indicating that the beneficial effect of fluoxetine on GvHD is indeed associated with a reduced expansion of host-reactive T cells.

4. Discussion

In this study, we investigated whether high-dose treatment with fluoxetine was able to suppress acute graft-versus-host disease (GvHD) in a MHC-matched, minor histocompatibility antigen mismatched murine bone marrow transplantation model. Several lines of evidence exist that SRIs exert an influence on the immune system. First, *in vitro* studies have shown a suppressive effect of SRIs on both rat and human lymphocyte proliferation and viability [17, 18]. Second, animal studies have demonstrated that SRIs can attenuate symptoms and inflammatory activity in selected autoimmune disorders, such as experimental autoimmune encephalomyelitis [3-5], collagen-induced arthritis [6], septic shock and allergic asthma [7] and contact hypersensitivity reaction [8]. These studies suggest that SRIs might be beneficial in the treatment of autoimmune pathologies. Third, clinical case reports indicate that SRIs, when administered in high doses, may influence immune function. For instance, Reed and Glick report a reactivation of herpes simplex virus in patients receiving high doses of fluoxetine [19]. Also, a case of recurring sinusitis was associated with venlafaxine use [20].

The above mentioned papers indicate that SRIs might interfere with pathologically activated autoreactive T cells. Here, we investigated the role of SRIs in allo-antigen activated T cells in the course of acute GvHD. Acute GvHD after allogeneic haematopoietic stem cell transplantation (alloHSCT) is a cause of extensive morbidity and mortality. We used a murine MHC-matched, miHA-mismatched bone marrow transplantation model to investigate whether fluoxetine could reduce GvHD. The MHC-matched, miHA-mismatched model was chosen in analogy with the human situation where over 50% of alloHSCT patients receive an HLA-matched graft [21]. In our study, fluoxetine was

found to significantly delay and reduce clinical symptoms, without interfering with the reconstitution of the hematopoietic compartment. An improvement in survival rate was also noted. Furthermore, the percentage of CD3+Vβ6+ T cells was significantly reduced by fluoxetine, consistent with an inhibitory effect of this compound on the expansion of alloreactive T cells. This leads to the assumption that fluoxetine, and possibly also other SRIs can have a beneficial effect on the outcome of acute GvHD.

In our murine model, a 20 mg/kg fluoxetine dose was sufficient to delay and reduce clinical symptoms of GvHD. In comparison, fluoxetine doses used in mice to obtain an 'antidepressive' effect are around 10-18 mg/kg [22]. A single IP dose of 20 mg/kg fluoxetine in mice gives rise to a serum concentration around 4 µM, measured 30 min after administration [15]. Although plasma concentrations of SRIs in depressive patients are below 1 µM [15], SRIs are known to have a wide therapeutic-toxic range in humans and higher dosing may be achieved without serious adverse effects [23]. Therefore, plasma concentrations needed for immunomodulation are expected to be feasible.

In conclusion, this study shows that fluoxetine can delay and reduce clinical symptoms of experimental GvHD, along with an inhibition of the expansion of alloreactive T cells. Data on T cells from healthy human subjects (**chapter 3**) show that this effect may be attributed to a direct anti-proliferative and pro-apoptotic effect. Given the similar T cell immunosuppressive effects of other SRIs *in vitro*, the potential application of these compounds in GvHD should be investigated. Together with prior studies in EAE and CIA, our data from a GvHD mouse model support the exploration of the therapeutic value of SRI-induced T cell suppression in GvHD and other immune-mediated disorders. Moreover, the data underscore the need for further research into the potential immunomodulatory effects of the therapeutic use of SRIs in humans.

References

1. Hughes, J., B. Lunn, and J. O'Brien, *SSRIs and patient groups with specific treatment problems.*, in *Selective Serotonin Reuptake Inhibitors (SSRIs). Past, Present and Future.*, S.C. Stanford, Editor. 1999, RG Landes: Austin, TX, USA. p. 47 - 64.
2. Vaswani, M., F.K. Linda, and S. Ramesh, *Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(1): p. 85-102.
3. Vollmar, P., et al., *The antidepressant venlafaxine ameliorates murine experimental autoimmune encephalomyelitis by suppression of pro-inflammatory cytokines.* Int J Neuropsychopharmacol, 2009. **12**(4): p. 525-36.
4. Taler, M., et al., *The Immunomodulatory Effect of the Antidepressant Sertraline in an Experimental Autoimmune Encephalomyelitis Mouse Model of Multiple Sclerosis.* Neuroimmunomodulation, 2011. **18**(2): p. 117-122.
5. Yuan, X.Q., et al., *Fluoxetine Promotes Remission in Acute Experimental Autoimmune Encephalomyelitis in Rats.* Neuroimmunomodulation, 2012. **19**(4): p. 201-208.
6. Sacre, S., et al., *Fluoxetine and citalopram exhibit potent antiinflammatory activity in human and murine models of rheumatoid arthritis and inhibit toll-like receptors.* Arthritis Rheum, 2010. **62**(3): p. 683-93.
7. Roumestan, C., et al., *Anti-inflammatory properties of desipramine and fluoxetine.* Respir Res, 2007. **8**: p. 35.
8. Kubera, M., et al., *Inhibitory effect of antidepressant drugs on contact hypersensitivity reaction.* Pharmacol Rep, 2012. **64**(3): p. 714-22.
9. Billiau, A.D., et al., *Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-leukemia responses in mice receiving allogeneic bone marrow transplants.* Blood, 2002. **100**(5): p. 1894-1902.
10. Sefrioui, H., A.D. Billiau, and M. Waer, *Graft-versus-leukemia effect in minor antigen mismatched chimeras given delayed donor leucocyte infusion: immunoregulatory aspects and role of donor T and ASGM1-positive cells.* Transplantation, 2000. **70**(2): p. 348-53.
11. Fevery, S., et al., *CTLA-4 blockade in murine bone marrow chimeras induces a host-derived antileukemic effect without graft-versus-host disease.* Leukemia, 2007. **21**(7): p. 1451-1459.
12. Miconnet, I., et al., *Critical role of endogenous Mtv in acute lethal graft-versus-host disease.* Eur J Immunol, 1995. **25**(2): p. 364-8.
13. Antin, J.H. and J.L. Ferrara, *Cytokine dysregulation and acute graft-versus-host disease.* Blood, 1992. **80**(12): p. 2964-8.
14. Billiau, A.D., et al., *Transforming growth factor-beta inhibits lymphokine activated killer cytotoxicity of bone marrow cells: implications for the graft-versus-leukemia effect in irradiation allogeneic bone marrow chimeras.* Transplantation, 2001. **71**(2): p. 292-9.
15. Holladay, J.W., M.J. Dewey, and S.D. Yoo, *Quantification of fluoxetine and norfluoxetine serum levels by reversed-phase high-performance liquid chromatography with ultraviolet detection.* J Chromatogr B Biomed Sci Appl, 1997. **704**(1-2): p. 259-63.
16. Johnson, B.D., et al., *Use of anti-CD3 epsilon F(ab')2 fragments in vivo to modulate graft-versus-host disease without loss of graft-versus-leukemia reactivity after MHC-matched bone marrow transplantation.* J Immunol, 1995. **154**(10): p. 5542-54.
17. Pellegrino, T.C. and B.M. Bayer, *Specific serotonin reuptake inhibitor-induced decreases in lymphocyte activity require endogenous serotonin release.* Neuroimmunomodulation, 2000. **8**(4): p. 179-87.

18. Taler, M., et al., *Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression.* Eur Neuropsychopharmacol, 2007. **17**(12): p. 774-80.
19. Reed, S.M. and J.W. Glick, *Fluoxetine and reactivation of the herpes simplex virus.* Am J Psychiatry, 1991. **148**(7): p. 949-50.
20. Denys, D., et al., *A case of venlafaxine-induced inhibition of T-lymphocyte proliferation.* Psychopharmacology (Berl), 2002. **164**(4): p. 432.
21. Hurley, C.K., M. Fernandez Vina, and M. Setterholm, *Maximizing optimal hematopoietic stem cell donor selection from registries of unrelated adult volunteers.* Tissue Antigens, 2003. **61**(6): p. 415-24.
22. Dulawa, S.C., et al., *Effects of chronic fluoxetine in animal models of anxiety and depression.* Neuropsychopharmacology, 2004. **29**(7): p. 1321-30.
23. Barbey, J.T. and S.P. Roose, *SSRI safety in overdose.* J Clin Psychiatry, 1998. **59 Suppl 15**: p. 42-8.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Parts of this chapter are based on "*Selective serotonin reuptake inhibitors as a novel class of immunosuppressants*." Veerle Gobin, Katleen Van Steendam, Damiaan Denys and Dieter Deforce. Int Immunopharmacol. 2014. 20: p. 148-156.

CHAPTER 7. GENERAL DISCUSSION AND CONCLUSIONS

The research described in this thesis focused on the effects of SSRIs on the immune system. In the first part of this project, the effects of SSRIs on human peripheral T lymphocytes were characterized (**chapter 3**). Major functions of these cells within the body are 1) the ability to become activated when encountering an antigen, 2) the proliferation and differentiation into effector T cells and 3) the ability to commit 'suicide' through apoptosis when no longer needed or when responding to an inappropriate antigen (e.g. a self-antigen). In order to investigate the impact of SSRIs on these parameters, we isolated T lymphocytes from the blood of healthy volunteers. The lymphocytes were subsequently incubated with SSRIs and activated through triggering of the TCR, thereby mimicking the *in vivo* encountering of an antigen. We analyzed the effects of all clinically available SSRIs, namely paroxetine, fluoxetine, sertraline, fluvoxamine and citalopram. In addition, we also investigated the effect of venlafaxine, a mixed serotonin and noradrenaline reuptake inhibitor. All drugs were tested in different concentrations, thereby enabling us to identify possible concentration-dependent effects. T cell proliferation was analyzed with CFSE, a fluorescent dye that is distributed evenly over daughter cells upon mitosis and is thus decreasing in fluorescence with each cell division. Apoptosis was detected through annexin V and propidium iodide (PI) staining. With this method, a differentiation can be made between early apoptosis (annexin V positive and PI negative) and late apoptosis or necrosis (annexin V and PI double positive).

The results obtained in these experiments pointed out that all tested SSRIs exerted immunosuppressive effects on T lymphocytes. A concentration-dependent suppressive effect was observed on T cell proliferation for all compounds tested (both SSRIs and SNRI), albeit in different concentration ranges. With respect to apoptosis, all SSRIs induced this form of programmed cell death, but not venlafaxine. Further, we demonstrated that SSRIs induce apoptosis preferentially in activated T cells. After 24h incubation with SSRIs, activated T lymphocytes were in a late phase of apoptosis as they stained annexin V and PI positive. In contrast, resting T cells are less susceptible to SSRI-induced apoptosis. This is an interesting property of SSRIs, as the selective targeting of activated T cells opens possibilities for the treatment of autoimmune diseases and other immune-mediated disorders where unwanted T cell activation occurs. Indeed, selective targeting of activated T cells provides a means of eliminating unwanted active T cells while at the same time preserving the resting T cell pool. The latter are thus still capable of mounting an immune response in case of infection or cancer at later stages.

Abovementioned results are in accordance with other research conducted on the *in vitro* effects of SSRIs on lymphocytes [1]. The added value of this study is the comparability of different SSRIs and one SNRI with respect to the anti-proliferative and pro-apoptotic effects on T lymphocytes in one experimental setup. Additionally, prior reports only focused on a small number of SSRIs, each with their own methods, patients and setups giving rise to several small scale comparisons but lacking a total overview. In addition, we did not use polyclonal mitogens to activate the T cells, but instead used magnetic beads coated with anti-CD3 and anti-CD28 antibodies. Whereas polyclonal mitogens such as concanavalin A and phytohaemagglutinin are a non-physiological method to induce T cell activation, the coated beads simulate the *in vivo* situation where an antigen-presenting cell makes contact with a T lymphocyte through presentation of an antigen on a MHC-molecule. In the *in vivo* situation, binding of the TCR to this MHC-antigen complex results in clustering of TCRs and formation of an 'immunological synapse' between an APC and a T cell. The same clustering is induced by the use of antibody-coated beads. Therefore, this method of activation is considered superior to polyclonal mitogens.

The responsible mechanism for these observed *in vitro* effects of SSRIs on T lymphocytes is still unraveled. Two possible hypotheses were further investigated: 1) the expression of the serotonin transporter in T lymphocytes and 2) the interference of SSRIs with calcium signaling in response to T cell activation. The first hypothesis was based on the well-known inhibition of SERT activity by SSRIs in the central nervous system (**chapter 4**). SERT expression has been described in multiple cell types outside the central nervous system, including immune cells such as B lymphocytes, dendritic cells, macrophages and NK cells. Thus, we investigated whether T lymphocytes express SERT and whether upregulation or downregulation occurred in response to T cell activation. The underlying reasoning was that if the SERT expression level was different in activated versus resting T lymphocytes, this might explain the different susceptibility of both populations to SSRI-induced apoptosis. Although SERT expression in most cell types can be easily detected with classical methods such as RT-qPCR and western blotting, we experienced several technical difficulties to detect SERT in human T lymphocytes due to the low expression levels. On the mRNA level, we used ddPCR to allow for increased sensitivity and precision in comparison to RT-qPCR which was needed to detect the low levels of SERT mRNA present in the samples. mRNA was purified from resting and activated T cells from nine healthy volunteers and SERT mRNA could be detected in all of the samples. No significant differences in SERT expression were found between the resting and activated T lymphocytes. Thus, it can be concluded that transcription of the SLC6A4 gene is not affected by the process of T cell activation.

One aspect we turned our attention to is the possibility of other blood cells contaminating the samples. Previous research on SERT expression in lymphocytes was usually conducted on unpurified buffy coats after Ficoll density centrifugation. However, besides lymphocytes these buffy coats contain monocytes, dendritic cells and blood platelets and are thus not pure lymphocyte preparations. Therefore, detected SERT expression might be attributed to different cell types present in the samples and results should be interpreted with care. This problem has been notified by Beikmann et al. [2] who demonstrated that 5HT uptake was largely mediated by blood platelets present in the samples instead of the lymphocytes themselves. In order to exclude that SERT expression detected in our samples originated from other cell types than T lymphocytes, we performed a double T cell isolation thereby creating T cell samples with very high purity. In addition, we also analyzed the rest fractions containing all other cell types but T lymphocytes. Finally, we also analyzed SERT expression in a Jurkat human leukemic T cell line, which can be considered 100% pure. From these experiments, we concluded that SERT mRNA expression originated from the T lymphocytes and not from other cell types present in the blood, although one or more other cell types did express higher levels of SERT**.**

On the protein level, extensive optimization was conducted to achieve a highly sensitive western blotting technique. Despite the attempts to increase the sensitivity of this technique, protein SERT expression was only detected in two of the eight tested lymphocyte samples. Presumably, inter-individual differences in expression levels are at the basis of this discrepancy. Additionally, expression levels within individuals could change over time. Conclusions based on these experiments should therefore be interpreted with care, as they might not be representative for the entire population. Interestingly, we only detected SERT protein in activated T lymphocytes. Thus it appears that SERT protein is upregulated during T cell activation. These limited observations might point to a 'translational readiness' where resting T lymphocytes already produce SERT mRNA, but translation is only induced after activation of the cells. Similar conclusions were drawn by Chamba et al. with respect to B lymphocytes [3]**.** They found SERT mRNA in both resting and activated B cells, but protein was upregulated only upon activation of the cells. As SERT protein and not mRNA is the functional form of this transporter, conclusions with respect to the impact of SSRIs should be based on the presence or absence of SERT protein in T lymphocytes. As our limited data show that SERT protein is upregulated in activated T cells, it seems likely to assume that SSRIs have a more pronounced effect on activated T cells because of the higher expression of SERT protein. Solid evidence to either include or exclude the SERT as a target for SSRIs in their immunosuppressive effects might be obtained using genetically altered mice either completely lacking SERT (SERT

knockout mice) or expressing a mutated version of SERT. Recently, a modified SERT knock-in mouse strain (SERT I172M) was developed that expresses a modified SERT protein with normal 5HT recognition and transport, but with a decreased sensitivity for antidepressants, including fluoxetine and citalopram [4]. The question whether or not SERT is involved in the immunosuppressive effects of SSRIs might be answered using this SERT I172M mouse model [5].

The second investigated hypothesis proposed that the immunosuppressive properties of SSRIs might be due to their capability to interfere with Ca^{2+} signaling in T lymphocytes (**chapter 5**). This hypothesis was based on the work performed by Edgar et al. in which they compared the immunosuppressive effects of SSRIs with ionophores [6, 7]**.** They concluded that fluoxetine modulated calcium influx in a similar way as ionophores, thereby causing an anti-proliferative effect. Unfortunately, Edgar et al. did not directly investigate the intracellular Ca^{2+} content, but instead based their conclusions on indirect evidence through comparison with ionophores. However, SSRIs have also been shown to affect Ca^{2+} signaling in a variety of other cell types, including neurons [8], astrocytes [9], microglia [10], osteosarcoma cells [11], platelets [12] and adrenal medulla PC12 cells [13, 14]. As Ca^{2+}-mediated signaling transduction is an important step in T cell activation, we examined the impact of SSRIs on Ca^{2+} signaling in jurkat T lymphocytes. Jurkat T lymphocytes are a well-established model to study T cell activation [15] and were chosen instead of freshly isolated peripheral T lymphocytes because of practical reasons. From these experiments, we concluded that fluoxetine suppresses the increase in $[Ca^{2+}]_i$ following T cell receptor activation in jurkat T lymphocytes in a concentration-dependent manner. We next examined whether the observed suppression was due to inhibition of Ca^{2+} release from the endoplasmic reticulum, or influx of Ca^{2+} through the plasma membrane. These experiments pointed out that fluoxetine inhibits both inositol trisphosphate and ryanodine receptor mediated release of Ca^{2+} from intracellular stores. No effects were seen on Ca^{2+} influx through CRAC channels in the plasma membrane. Finally, we demonstrated that the immunosuppressive effects of fluoxetine - under the form of decreased CD69 expression in response to TCR activation – can be mimicked by buffering of intracellular Ca^{2+} with BAPTA-AM.

As we previously found that T lymphocytes express SERT and upregulate this transporter protein upon T cell activation, we next questioned whether the observed changes in Ca^{2+} signaling could be due to blockage of SERT. In order to provide an answer to this question, we performed competition experiments with a large excess 5HT. If inhibition of SERT accounts for the suppressive effect of fluoxetine on Ca^{2+} signaling, it could be expected that 5HT reverses this effect, as competition between fluoxetine and 5HT would occur for binding to SERT. However, the results of these

experiments showed no difference in Ca^{2+}-suppressing effects of fluoxetine in the presence or absence of 5HT. Thus, it is unlikely that inhibition of SERT accounts for the effects fluoxetine exerts on Ca^{2+} signaling.

In conclusion, we demonstrated that the immunosuppressive effects of SSRIs, at least for fluoxetine, can be explained by the inhibition of inositol trisphosphate and ryanodine receptor- mediated release of intracellular Ca^{2+}, thereby impairing the necessary signaling transduction step in response to T cell receptor triggering and leading to T cell activation, proliferation and differentiation. Although human peripheral T lymphocytes express SERT, it is unlikely that the mechanism through which SSRIs induce immunosuppression is related to the blockage of SERT. Not only interference with Ca^{2+} signaling can explain the anti-proliferative effect of fluoxetine, it might also be involved in the pro-apoptotic effect as interference with ER Ca^{2+} stores has been reported to activate apoptotic pathways [16]. As SSRIs inhibit release of Ca^{2+} from ER stores, they might cause ER Ca^{2+} overload which is associated with increased apoptosis [16].

Instead of directly interacting with the IP_3R and RyR, it has been proposed that fluoxetine, being a highly lipophilic molecule, interacts with the membrane lipid bilayer and thereby influences the ion channel structure and function [14]. Furthermore, SSRIs have been shown to disturb lipid rafts, cholesterol and sphingolipid-rich microdomains in the plasma membrane that function as signaling transduction platforms [17]. As lipid rafts have been found to be important in TCR clustering after T cell activation [18] and many of the regulatory proteins and ion channels involved in Ca^{2+} signaling are situated in lipid rafts [19], it is possible that the observed effects of fluoxetine on Ca^{2+} release from intracellular stores are due to disturbance of the lipid raft organization. Although highly speculative, it is possible that interference with lipid raft structure and function is at the basis of several results described by different research groups regarding the underlying mechanism of SSRI-induced immunosuppression. As described in chapter 1, others have found SSRIs to interfere with different signaling transduction pathways including cAMP, PKA and PKC pathways in lymphocytes [6, 7, 20]. Since the importance of lipid rafts in the regulation of signaling transduction has recently been acknowledged [19], these results might as well relate to the capacity of SSRIs to interfere with and accumulate in lipid rafts. Furthermore, extensive crosstalk exists between cAMP and Ca^{2+} signaling [21] and an effect of fluoxetine on either one of these pathways might induce secondary changes in the other pathway.

One other possibility worthy of further investigation is the effect SSRIs exert on glucocorticoid receptor (GR) expression and function. Several reports have described increased GR expression, translocation and function in response to antidepressants [22]. Therefore, it is possible that SSRIs increase the sensitivity of T lymphocytes for the immunosuppressive effects of glucocorticoids through enhanced GR expression and function.

From the abovementioned experiments, it is clear that SSRIs are capable of inducing T cell immunosuppression *in vitro*. Whether SSRIs are also successful in suppressing unwanted T cell activation *in vivo*, was tested in a murine bone marrow transplantation model for acute GvHD. This condition was chosen to explore the *in vivo* immunosuppressive effects of SSRIs because current treatment options for acute GvHD are inadequate. Corticosteroids form the golden standard therapy for acute GvHD, but these drugs can induce severe side effects such as increased infection risk, Cushing syndrome, diabetes, osteoporosis and myopathy [23]. Furthermore, steroid treatment results in complete remission in less than half of the patients [24], indicating that new treatment options are highly necessary. In comparison, SSRIs have a more beneficial side effect profile with nausea, diarrhea, sexual dysfunction, headache, dizziness, agitation and insomnia [25].

The model used in this thesis is based on transplantation of bone marrow and spleen cells from MHC-matched, minor HC-mismatched C3H donors into lethally irradiated AKR recipients. In this model, immunologically competent donor cells mount an immune response towards recipient antigens presented by antigen-presenting cells, thereby causing acute GvHD. We investigated whether treatment with 20 mg/kg fluoxetine could suppress acute GvHD in this model. The results of these experiments are described in **chapter 6**. Fluoxetine significantly suppressed clinical scores of acute GvHD and improved survival of the mice. Importantly, these beneficial effects were reached without a negative impact on the engraftment of donor cells. Thus, the concern raised by Foley et al. that antidepressants could negatively impact graft fate due to increased prolactin levels does not hold stand for fluoxetine in our model [26]. With respect to the underlying mechanism, we found that percentages alloreactive Vβ6+ T lymphocytes in peripheral blood were significantly lower in fluoxetine treated animals than control mice. These data indicate that the beneficial effect of fluoxetine on GvHD is indeed associated with a reduced expansion of host-reactive T cells.

In the past decades, it has become more and more clear that hematopoietic stem cell transplantation is a successful therapy for leukemia not only because of the replacement of the blood forming compartment, but also because of the anti-leukemia effect that is executed by the graft [27]. However, GvHD and graft-versus-leukemia effect often go hand-in-hand and are at least in part

mediated by the same effector cells and target antigens [27]. Whereas fluoxetine has been shown to suppress GvHD, the impact of this drug on the graft-versus-leukemia effect has not been investigated. Therefore, further research will be necessary to evaluate whether the anti-leukemia effect is maintained during SSRI therapy. Extension of the existing data in the murine BMT model to evaluate the effect of fluoxetine on the graft-versus-leukemia effect, as well as further characterization of the immunological changes induced by fluoxetine in this model is therefore desirable.

An important issue in the consideration of SSRIs as a novel class of immunosuppressants is the possibility to administer high enough doses to achieve an optimal immunosuppressive effect. There is a considerable difference between the SSRI concentrations that are reported to exert immunosuppressive effects *in vitro*, and the ones found in plasma of depressed patients. Concentrations used *in vitro* for immunosuppressive effects range from 1-20 µM for paroxetine, fluoxetine and sertraline and even higher for the other SSRIs. These concentrations are considerably higher than plasma concentrations found in depressed patients, which range from 10 - 600 ng/ml or 0.03 – 1.6 µM [28, 29]. However, various factors contribute to the reasoning that SSRIs might still be suitable for immunomodulation *in vivo*. First, SSRI concentrations might vary considerably between organs and lymphocytes may be exposed to high enough SSRI concentrations in peripheral compartments instead of in the blood. Uhr *et al.* determined plasma and organ concentrations of SSRIs after subcutaneous injection in mice and found 10-fold higher concentrations in spleen compared to plasma [30]. Thus, lymphocytes might be exposed to SSRI-concentrations high enough for immunomodulation in the spleen while plasma concentrations can be kept low.

Second, evidence exists that doses currently used in patients already exert immunomodulatory effects. For instance, Reed and Glick reported reactivation of herpes simplex virus in patients receiving high doses of SSRIs [31]. A case of recurring sinusitis was reported in a patient suffering from obsessive-compulsive disorder and treated with high doses of venlafaxine [32]. Thus, the concentrations needed to establish an immunosuppressive effect *in vitro* might not correlate with those exerting an immunosuppressive effect *in vivo*.

Third, the doses that exert immunomodulatory effects in some of the animal experiments give rise to plasma concentrations within the same range as concentrations found in patients. Chronic daily administration of 10 to 18 mg/kg fluoxetine orally given to mice gives rise to plasma concentrations within the same range as those found in patients (100 – 700 ng/ml) [33]. Several of the animal experiments analyzing the effect of fluoxetine on autoimmune diseases and cancer used doses below

20 mg/kg/day and reported significant changes in immune function and symptoms [34-36]. Others reported doses below 20 mg/kg/day to already exert small changes in immune function, but higher doses were needed in order to reach significance [36].

Finally, if higher dosing would be necessary, this may be achieved without severe adverse effects. SRIs are known to have a wide therapeutic-toxic range in humans and higher dosing may be achieved without serious adverse effects [37]. Doses two to three times higher than the ones used for treatment of depression are already being subscribed for other disorders, such as obsessive compulsive disorder without unacceptable side effects [38]. As most SSRIs have a non-linear kinetic profile, higher dosing might result in a disproportional increase in plasma concentration. This was confirmed for fluoxetine in mice, where a chronic dose of 25 mg/kg per day gave rise to a plasma concentration 3.15 times higher than the plasma concentration obtained after a chronic dose of 18 mg/kg (the latter dose gives rise to a plasma concentration within the same range as those found in patients) [33].

Nevertheless, one aspect that needs further attention is the potentially increased risk to commit suicide under treatment with SSRIs. There is limited evidence that antidepressant treatment might elevate the risk of suicide in depressed patients, especially at the start of treatment [39]. When using SSRIs as immunosuppressants in patients suffering from autoimmune disorders or GvHD, in particular when concomitant depression is present, the potentially increased risk of suicide should be considered. In undepressed patients, this seems less to be an issue, as the increased suicide risk with antidepressants is associated with the underlying depression [39]. Thus, although immunoregulatory application of SSRIs will probably require higher doses than the ones currently used for treatment of major depressive disorder, there are indications that achieving the needed plasma concentrations may be feasible without competing against unacceptable side effects.

The implications of the results described in this thesis are two-fold: on the one hand, SSRIs might be reinvented as a novel class of immunosuppressants and alleviate symptoms in a variety of (auto-) immune disorders. On the other hand, the observation that SSRIs induce immunosuppressive effects raises concern on the extensive clinical use of these drugs for the treatment of psychiatric disorders. As explained above, higher doses will probably be needed to obtain an optimal immunosuppressive effect in the context of (auto-)immune disorders, but immunosuppression has been described at doses already being prescribed in the clinic. Are we inducing immunosuppression in a considerable part of the population? What are the consequences for susceptibility to infection, efficacy of vaccination, spreading of diseases? No extensive research has been conducted to answer these

questions, and further investigation is highly desirable. To partially meet this need, we have set up a small scale clinical pilot study in which we will evaluate the efficacy of hepatitis B vaccination in obsessive compulsive disorder patients receiving 40-60 mg/day paroxetine. Whereas Denys et al. did not find any significant changes in immune parameters in these patients in the absence of an immunological challenge [40]**,** we attempt to analyze whether SSRI-treated patients are still capable of mounting a sufficient immune response to an immunological challenge, under the form of a hepatitis B vaccine.

The research conducted in this PhD thesis can be considered as an example of 'drug repositioning'. Whereas SSRIs have been used in clinical practice for decades in the treatment of psychiatric disorders, their potential in the treatment of immunological disorders is only now being discovered. The major advantage of drug repositioning is that phase I clinical trials, which are intended to characterize the drug's safety profile, have already been performed and an extensive experience in the clinical practice has been build up. Accordingly, development of a repositioned drug is cheaper and holds less risk of failure due to unexpected and unacceptable toxicity in comparison to a novel drug. As SSRIs have already proven to possess a beneficial side effect profile, it should be possible to conduct a small scale phase II pilot study to assess the effects of SSRIs in patients suffering from acute GvHD.

Overall, the research described in this thesis contributes to the growing evidence that SSRIs exert immunomodulatory effects which might be useful in the treatment of a wide variety of immune-mediated diseases. In autoimmune disorders such as rheumatoid arthritis and multiple sclerosis, the first clinical data have been gathered and results of these pilot studies are promising. With growing evidence in the future, SSRIs will hopefully find their way to the clinic as a novel class of immunosuppressants.

"The most fruitful basis of the discovery of a new drug is to start with an old drug." - James Black, Nobel laureate 1988.

References

1. Taler, M., et al., *Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression.* Eur Neuropsychopharmacol, 2007. **17**(12): p. 774-80.
2. Beikmann, B.S., et al., *Serotonin uptake is largely mediated by platelets versus lymphocytes in peripheral blood cells.* ACS Chem Neurosci, 2013. **4**(1): p. 161-70.
3. Chamba, A., et al., *SLC6A4 expression and anti-proliferative responses to serotonin transporter ligands chlomipramine and fluoxetine in primary B-cell malignancies.* Leuk Res, 2010. **34**(8): p. 1103-6.
4. Thompson, B.J., et al., *Transgenic elimination of high-affinity antidepressant and cocaine sensitivity in the presynaptic serotonin transporter.* Proc Natl Acad Sci U S A, 2011. **108**(9): p. 3785-90.
5. Baganz, N.L. and R.D. Blakely, *A dialogue between the immune system and brain, spoken in the language of serotonin.* ACS Chem Neurosci, 2012. **4**(1): p. 48-63.
6. Edgar, V.A., et al., *Fluoxetine action on murine T-lymphocyte proliferation: participation of PKC activation and calcium mobilisation.* Cell Signal, 1998. **10**(10): p. 721-6.
7. Edgar, V.A., et al., *Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation.* Eur J Pharmacol, 1999. **372**(1): p. 65-73.
8. Kim, H.J., et al., *Fluoxetine suppresses synaptically induced [Ca(2)(+)]i spikes and excitotoxicity in cultured rat hippocampal neurons.* Brain Res, 2013. **1490**: p. 23-34.
9. Li, B., et al., *Effects of chronic treatment with fluoxetine on receptor-stimulated increase of [Ca2+]i in astrocytes mimic those of acute inhibition of TRPC1 channel activity.* Cell Calcium, 2010. **50**(1): p. 42-53.
10. Horikawa, H., et al., *Inhibitory effects of SSRIs on IFN-gamma induced microglial activation through the regulation of intracellular calcium.* Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2010. **34**(7): p. 1306-1316.
11. Lin, K.L., et al., *Effect of sertraline on [Ca2+](i) and viability of human MG63 osteosarcoma cells.* Drug Chem Toxicol, 2013. **36**(2): p. 231-40.
12. Helmeste, D.M., et al., *Serotonin uptake inhibitors modulate intracellular Ca2+ mobilization in platelets.* Eur J Pharmacol, 1995. **288**(3): p. 373-7.
13. Hahn, S.J., et al., *Inhibition by fluoxetine of voltage-activated ion channels in rat PC12 cells.* Eur J Pharmacol, 1999. **367**(1): p. 113-8.
14. Kim, H.J., et al., *Fluoxetine inhibits ATP-induced [Ca(2+)](i) increase in PC12 cells by inhibiting both extracellular Ca(2+) influx and Ca(2+) release from intracellular stores.* Neuropharmacology, 2005. **49**(2): p. 265-74.
15. Abraham, R.T. and A. Weiss, *Jurkat T cells and development of the T-cell receptor signalling paradigm.* Nat Rev Immunol, 2004. **4**(4): p. 301-8.
16. Orrenius, S., B. Zhivotovsky, and P. Nicotera, *Regulation of cell death: the calcium-apoptosis link.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 552-65.
17. Eisensamer, B., et al., *Antidepressants and antipsychotic drugs colocalize with 5-HT3 receptors in raft-like domains.* Journal of Neuroscience, 2005. **25**(44): p. 10198-10206.
18. van der Merwe, P.A. and O. Dushek, *Mechanisms for T cell receptor triggering.* Nature Reviews Immunology, 2011. **11**(1): p. 47-55.
19. Pani, B. and B.B. Singh, *Lipid rafts/caveolae as microdomains of calcium signaling.* Cell Calcium, 2009. **45**(6): p. 625-633.
20. Kenis, G., et al., *Influence of antidepressants on intracellular levels of cyclic adenosine monophosphate in human peripheral blood mononuclear cells.* Eur Neuropsychopharmacol, 2003. **13**(1): p. 53-6.

21. Bruce, J.I., S.V. Straub, and D.I. Yule, *Crosstalk between cAMP and Ca2+ signaling in non-excitable cells.* Cell Calcium, 2003. **34**(6): p. 431-44.
22. Antonioli, M., J. Rybka, and L.A. Carvalho, *Neuroimmune endocrine effects of antidepressants.* Neuropsychiatr Dis Treat, 2012. **8**: p. 65-83.
23. Ferrara, J.L., et al., *Graft-versus-host disease.* Lancet, 2009. **373**(9674): p. 1550-61.
24. MacMillan, M.L., et al., *Response of 443 patients to steroids as primary therapy for acute graft-versus-host disease: comparison of grading systems.* Biol Blood Marrow Transplant, 2002. **8**(7): p. 387-94.
25. Vaswani, M., F.K. Linda, and S. Ramesh, *Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(1): p. 85-102.
26. Foley, K.F. and R.E. Kast, *Review of evidence that posttransplantation psychiatric treatment commonly affects prolactin levels and thereby influences graft fate.* Gen Hosp Psychiatry, 2006. **28**(3): p. 230-3.
27. Appelbaum, F.R., *Haematopoietic cell transplantation as immunotherapy.* Nature, 2001. **411**(6835): p. 385-9.
28. Wille, S.M., et al., *Relevant issues in the monitoring and the toxicology of antidepressants.* Crit Rev Clin Lab Sci, 2008. **45**(1): p. 25-89.
29. DeVane, C.L., *Metabolism and pharmacokinetics of selective serotonin reuptake inhibitors.* Cell Mol Neurobiol, 1999. **19**(4): p. 443-66.
30. Uhr, M., M.T. Grauer, and F. Holsboer, *Differential enhancement of antidepressant penetration into the brain in mice with abcb1ab (mdr1ab) P-glycoprotein gene disruption.* Biol Psychiatry, 2003. **54**(8): p. 840-6.
31. Reed, S.M. and J.W. Glick, *Fluoxetine and reactivation of the herpes simplex virus.* Am J Psychiatry, 1991. **148**(7): p. 949-50.
32. Denys, D., et al., *A case of venlafaxine-induced inhibition of T-lymphocyte proliferation.* Psychopharmacology (Berl), 2002. **164**(4): p. 432.
33. Dulawa, S.C., et al., *Effects of chronic fluoxetine in animal models of anxiety and depression.* Neuropsychopharmacology, 2004. **29**(7): p. 1321-30.
34. Frick, L.R., et al., *Inhibitory effect of fluoxetine on lymphoma growth through the modulation of antitumor T-cell response by serotonin-dependent and independent mechanisms.* Biochem Pharmacol, 2008. **75**(9): p. 1817-26.
35. Frick, L.R., et al., *Oral administration of fluoxetine alters the proliferation/apoptosis balance of lymphoma cells and up-regulates T cell immunity in tumor-bearing mice.* Eur J Pharmacol, 2011. **659**(2-3): p. 265-72.
36. Sacre, S., et al., *Fluoxetine and citalopram exhibit potent antiinflammatory activity in human and murine models of rheumatoid arthritis and inhibit toll-like receptors.* Arthritis Rheum, 2010. **62**(3): p. 683-93.
37. Barbey, J.T. and S.P. Roose, *SSRI safety in overdose.* J Clin Psychiatry, 1998. **59 Suppl 15**: p. 42-8.
38. Decloedt, E.H. and D.J. Stein, *Current trends in drug treatment of obsessive-compulsive disorder.* Neuropsychiatr Dis Treat, 2010. **6**: p. 233-42.
39. Stanford, S.C., ed. *Selective Serotonin Reuptake Inhibitors (SSRIs). Past, Present and Future.* 1999, R.G. Landes Company: Austin, Texas, U.S.A. 221.
40. Denys, D., et al., *Effects of paroxetine and venlafaxine on immune parameters in patients with obsessive compulsive disorder.* Psychoneuroendocrinology, 2006. **31**(3): p. 355-60.

SUMMARY

SAMENVATTING

SUMMARY

Selective serotonin reuptake inhibitors (SSRIs) are among the most prescribed drugs worldwide. They belong to the class of antidepressants and are used in the treatment of depression, as well as in anxiety disorders such as obsessive-compulsive disorder. Since their marketing, SSRIs have been shown not only to induce changes in the central nervous system, but also in the immune system. The effects of SSRIs on immunity are, however, not yet fully known. Therefore, the first objective of this thesis was to characterize the immunomodulatory effects of SSRIs. More specifically, we studied the influence of paroxetine, fluoxetine, sertraline, citalopram, fluvoxamine and venlafaxine on the proliferation and viability of human T lymphocytes. The results of these experiments are described in **Chapter 3**. Whereas the first five drugs belong to the class of SSRIs, venlafaxine is a mixed serotonin and noradrenaline reuptake inhibitor (SNRI). The latter was included in the study because it has equally been associated with immunological alterations. The experiments were performed *in vitro* using T lymphocytes isolated from the blood of healthy volunteers. All tested compounds were found to suppress the proliferation of T lymphocytes in a concentration-dependent manner. Furthermore, we found that all SSRIs reduce the viability of T lymphocytes by induction of apoptosis. Venlafaxine, the only tested SNRI, had no effect on T cell viability. Both the anti-proliferative and apoptotic effects were concentration-dependent. Furthermore, activated T lymphocytes were found to be more sensitive to SSRIs than resting T lymphocytes. In the context of immunological diseases, this finding implies that SSRIs might be able to suppress unwanted T cell activation without affecting resting T lymphocytes. Therefore, when SSRIs would be administered to patients with an immunological disorder, e.g. an autoimmune disease or in case of rejection after transplantation, it would in theory be possible to suppress only the activated T lymphocytes - which are responsible for the occurrence of the disease. At the same time resting T lymphocytes are left untouched thus remaining capable of responding to infection at a later stage.

In the second part of this work the underlying mechanism responsible for the immunosuppressive effects of SSRIs was studied. The first investigated hypothesis was related to the known mechanism of action of SSRIs in the brain. SSRIs block the uptake of serotonin (5HT), a neurotransmitter, in the presynaptic neuron by inhibition of the serotonin transporter (SERT), a protein responsible for the uptake of 5HT in the cell. Since conflicting evidence exists on whether human T lymphocytes express SERT, we decided to first determine whether this protein was present in human T lymphocytes. In order to get a complete picture on the expression of SERT in T lymphocytes, the experiments were performed at both messenger RNA (mRNA) and protein level. Because the activation state of a T

lymphocyte might be important for the expression of SERT, the experiments were carried out on both resting and activated T lymphocytes. In addition, differences in expression levels between activated and resting T cells might provide an answer on why especially activated T lymphocytes are suppressed by SSRIs. Again experiments were carried out on T lymphocytes isolated from the blood of healthy volunteers. The results of this research are described in **Chapter 4** and confirm that T lymphocytes express SERT both at the mRNA and protein level, although the expression levels were low. SERT mRNA was found in both resting and activated T lymphocytes, and no statistically significant differences were found between the expression levels in both groups. At the protein level, SERT could be detected only in activated T lymphocytes. These results might indicate that there exists a "translational readiness" for SERT: resting T lymphocytes already produce SERT mRNA, but translation of this mRNA into a protein is induced only when the T lymphocyte is activated. The observation that activated T cells express higher levels of SERT protein than resting T lymphocytes might explain why this population is more sensitive to the apoptotic effects of SSRIs.

A second hypothesis regarding the molecular mechanism at the basis of the immunosuppressive effects of SSRIs concerns an important signal transduction pathway, more specifically the calcium signaling pathway. This pathway is essential in T cell activation, and provides the intermediate step between the recognition of an antigen by the T cell receptor and the proliferation and differentiation of the T lymphocyte. As an effect of SSRIs on calcium signaling had already been reported in several other cell types, we hypothesized that SSRIs might disturb this pathway, and thereby impaired optimal T cell activation resulting in decreased proliferation. Further, interference with calcium signaling might also activate apoptotic pathways. Experiments to investigate this potential mechanism were performed using a human leukemic T cell line, a well-known model for T cell activation, which was chosen for practical reasons instead of peripheral blood T cells. Fluoxetine was used in these experiments as a model SSRI. The results of this study, as described in **Chapter 5**, indicate that fluoxetine suppresses the increase in the cytoplasmic Ca^{2+} concentration after T cell receptor activation. This increase is caused by both release of Ca^{2+} from the endoplasmic reticulum (ER), and influx of Ca^{2+} through the cell membrane (capacitative calcium entry). Further experiments showed that fluoxetine inhibits the release of Ca^{2+} from the ER when induced by inositol trisphosphate (IP_3) or ryanodine receptor activation. The capacitative calcium entry was not affected by fluoxetine. As we previously showed that T lymphocytes express SERT, we further investigated whether the observed inhibition of Ca^{2+} release from the ER is due to the blockade of 5HT uptake through SERT. From competition experiments with 5HT, it was concluded that the inhibition of Ca^{2+} release from the ER by fluoxetine is not related to inhibition of 5HT uptake through SERT. Finally, we

confirmed that buffering cytoplasmic Ca^{2+} changes with BAPTA-AM leads to the suppression of activation marker expression (CD69) in T cells, an effect that was also observed with fluoxetine. In conclusion, we demonstrated that the immunosuppressive effects of fluoxetine likely are related to the inhibition of IP_3 and ryanodine receptor- mediated release of Ca^{2+} from the ER during T cell activation.

In the last part of this work, we investigated whether SSRIs may be used as immunosuppressive drugs in the prevention and/or treatment of acute graft-versus-host disease (GvHD). Being a major complication of hematopoietic stem cell transplantation, GvHD is a major source of morbidity and mortality that limits the applications of this life-saving therapy. In order to study the effect of fluoxetine on acute GvHD, we made use of a murine bone marrow transplantation model for acute GvHD in which the donors and recipients were different in the expression of minor histocompatibility antigens (miHA). Donors and recipients were matched for major histocompatibility complex antigens (MHC), which is also in the clinic the most common situation. The choice for fluoxetine was based on two reasons: its immunosuppressive effects at relatively low doses which came to light in the *in vitro* experiments, and because there was the most evidence that this SSRI showed immunosuppressive activity in animal models of other (auto-) immune disorders. The results of this study, described in **Chapter 6**, demonstrate that fluoxetine suppresses the symptoms of acute GvHD and improves the survival after transplantation. Furthermore, no adverse effects were observed for fluoxetine on engraftment of the transplanted stem cells. The improvement of clinical symptoms as a result of fluoxetine treatment was associated with a suppression of allo-reactive T cells (the cells responsible for the development of acute GvHD) in the blood of the experimental animals. From this study, it was concluded that fluoxetine can exert a favorable effect on acute GvHD in mice, and that further investigation of the immunological alterations induced by fluoxetine in mice as well as the applicability of fluoxetine as an immunosuppressant in acute GvHD in the clinical context is desirable.

In **Chapter 7**, the findings of this work and the applicability of SSRIs as immunosuppressants in the clinical context are discussed. In particular, attention is paid to whether sufficiently high plasma concentrations can be achieved to induce the desired immunosuppression. Moreover, the impact of this research on the current application of SSRIs in the treatment of psychiatric disorders is also discussed, as immunosuppression may also be introduced unintentionally in these patients.

SAMENVATTING

Selectieve serotonine heropname inhibitoren (SSRIs) behoren wereldwijd tot de meest voorgeschreven geneesmiddelen. Ze maken deel uit van de klasse der antidepressiva en worden gebruikt in de behandeling van depressie, alsook bij angststoornissen zoals obsessief-compulsief gedrag. Hoewel het bijwerkingenprofiel van SSRIs over het algemeen als zeer gunstig wordt beschouwd, is de voorbije jaren uit onderzoek gebleken dat deze geneesmiddelen een invloed uitoefenen op het immuunsysteem. De effecten van SSRIs op de immuniteit zijn echter nog niet volledig gekend. Daarom was de eerste doelstelling van dit onderzoek om de immunologische effecten van SSRIs in kaart te brengen. Meer bepaald werd de invloed onderzocht van paroxetine, fluoxetine, sertraline, citalopram, fluvoxamine en venlafaxine op de proliferatie en leefbaarheid van humane T lymfocyten. De resultaten van deze experimenten worden beschreven in **hoofdstuk 3**. Daar waar de eerste vijf opgesomde geneesmiddelen behoren tot de SSRIs, is venlafaxine in feite een gemengde serotonine en noradrenaline heropname inhibitor (SNRI). Deze laatste werd eveneens getest, omdat uit onderzoek gebleken is dat ook dit geneesmiddel een invloed uitoefent op de immuniteit. Deze experimenten werden *in vitro* uitgevoerd op T lymfocyten die werden geïsoleerd uit het bloed van gezonde vrijwilligers. Uit de resultaten kon worden afgeleid dat alle geteste geneesmiddelen de proliferatie van T lymfocyten onderdrukken op een concentratie-afhankelijke manier. Bovendien werd vastgesteld dat alle SSRIs de leefbaarheid van T lymfocyten verlagen door geprogrammeerde celdood (apoptose) te induceren. Venlafaxine, de enige geteste SNRI, had geen invloed op de leefbaarheid van T lymfocyten. Ook deze effecten waren concentratie-afhankelijk. Bovendien bleek uit deze experimenten dat geactiveerde T lymfocyten gevoeliger waren voor de effecten van SSRIs dan rustende T lymfocyten. Deze laatste observatie is zeer interessant in het kader van immunologische aandoeningen omdat dit betekent dat SSRIs in staat zijn geactiveerde T lymfocyten te onderdrukken zonder de rustende T lymfocyten aan te tasten. Wanneer SSRIs dus zouden worden toegediend aan patiënten met een immunologische aandoening, bijvoorbeeld een auto-immuunziekte of bij afstoting na transplantatie, zou het in theorie mogelijk zijn om enkel de geactiveerde T lymfocyten – die verantwoordelijk zijn voor het optreden van de ziekte – te onderdrukken. Tegelijkertijd worden de rustende T lymfocyten ongemoeid gelaten, zodat deze in staat blijven om te reageren tegen infecties in een later stadium.

In het tweede gedeelte van dit onderzoek werd dieper ingegaan op het onderliggend mechanisme waardoor de immunosuppressieve effecten van SSRIs tot stand komen. Een eerste hypothese dewelke onderzocht werd, houdt verband met het gekende werkingsmechanisme van SSRIs in de

hersenen. Daar verhinderen SSRIs de opname van serotonine (5HT), een neurotransmitter, in het presynaptisch neuron. In het bijzonder blokkeren SSRIs de serotonine transporter (SERT), een eiwit dat instaat voor de opname van 5HT in de cel. Aangezien er in de literatuur nog geen consensus bereikt was of humane T lymfocyten de SERT tot expressie brengen, werd besloten eerst na te gaan of dit eiwit wel aanwezig was in humane T lymfocyten. Om een volledig beeld te krijgen van de aanwezigheid van de SERT in T lymfocyten, werd de bepaling uitgevoerd op twee niveaus: enerzijds werden boodschapper RNA (mRNA) gehalten bepaald, anderzijds werd het eiwit zelf gedetecteerd. Omdat de activatiestatus van een T lymfocyt bepalend kan zijn voor het tot expressie brengen van allerlei eiwitten, werden de experimenten uitgevoerd op zowel rustende als geactiveerde T lymfocyten. Bovendien zouden verschillen in SERT expressieniveaus tussen geactiveerde en rustende T cellen een verklaring kunnen bieden waarom vooral geactiveerde T lymfocyten door SSRIs worden onderdrukt. Opnieuw werd gewerkt met T lymfocyten die geïsoleerd werden uit het bloed van gezonde vrijwilligers. De resultaten van dit onderzoek worden beschreven in **hoofdstuk 4**. De resultaten van dit onderzoek bevestigen dat T lymfocyten de SERT tot expressie brengen, en dit zowel op mRNA als eiwit niveau. Er dient echter te worden opgemerkt dat de expressieniveaus laag waren. Er werd SERT mRNA teruggevonden in zowel rustende als geactiveerde T lymfocyten, en er werden geen statistisch significante verschillen gevonden tussen de expressieniveaus in beide groepen. Op eiwitniveau kon de SERT enkel gedetecteerd worden in geactiveerde T lymfocyten. Dit zou erop kunnen wijzen dat er een 'translationele paraatheid' bestaat voor dit eiwit: rustende T lymfocyten produceren reeds SERT mRNA, maar translatie van dit mRNA naar een eiwit wordt pas geïnduceerd wanneer de T lymfocyt geactiveerd wordt. De observatie dat geactiveerde T cellen meer SERT eiwit tot expressie brengen dan rustende T lymfocyten, zou kunnen verklaren waarom deze populatie gevoeliger is voor de apoptotische effecten van SSRIs.

Een tweede hypothese omtrent het moleculaire mechanisme aan de basis van de immunosuppressieve werking van SSRIs betreft de interferentie met een belangrijke signaaltransductie pathway, nl. de calcium signalisatie. Deze pathway is van zeer groot belang bij T cel activatie, en zorgt ervoor dat herkenning van een antigeen door de T cel receptor uiteindelijk leidt tot de proliferatie en differentiatie van de T lymfocyt. Bovendien zou het verstoren van de calcium signalisatie ook kunnen leiden tot activatie van apoptose pathways. Deze hypothese kwam tot stand doordat een invloed van SSRIs op calcium signalisatie reeds werd gerapporteerd in verscheidene andere celtypes. Deze experimenten werden uitgevoerd op een humane leukemie T cellijn, een gekend model voor T cel activatie dat omwille van praktische redenen werd gekozen in plaats van uit het bloed geïsoleerde T cellen. Fluoxetine werd in deze experimenten getest als model SSRI. De

resultaten van dit onderzoek, beschreven in **hoofdstuk 5**, wijzen erop dat fluoxetine de toename van de intracellulaire calcium concentratie na T cel receptor activatie onderdrukt. Deze stijging wordt veroorzaakt door enerzijds vrijstelling van calcium uit het endoplasmatisch reticulum (ER), en anderzijds opname van calcium doorheen de celmembraan. Verdere experimenten toonden aan dat fluoxetine de vrijstelling van calcium vanuit het ER blokkeert, wanneer dit geïnduceerd wordt door inositol trisfosfaat (IP_3) of ryanodine receptor activatie. De opname van calcium doorheen de celmembraan werd niet beïnvloed door fluoxetine. Verder werd onderzocht of de geobserveerde inhibitie van calcium vrijstelling vanuit het ER te maken had met de blokkade van 5HT opname doorheen de SERT. Uit competitie experimenten met 5HT kon besloten worden dat de inhibitie van calcium vrijstelling uit het ER door fluoxetine niet gerelateerd is aan de inhibitie van 5HT opname doorheen de SERT. Tenslotte werd bevestigd dat het bufferen van intracellulair calcium met BAPTA-AM leidt tot het onderdrukken van activatiemerker expressie (CD69) in T lymfocyten, een effect dat ook met fluoxetine werd geobserveerd. Hieruit kan dus besloten worden dat de immunosuppressieve effecten van fluoxetine waarschijnlijk gerelateerd zijn aan de inhibitie van IP_3- en ryanodine-receptor gemedieerde vrijstelling van calcium uit het ER bij T cel activatie.

In het laatste gedeelte van dit onderzoeksproject werd onderzocht of SSRIs kunnen worden aangewend als immunosuppressiva in de preventie en/of behandeling van acute graft-versus-host ziekte (GvHD). Deze aandoening kan optreden na hematopoietische stamceltransplantatie en is een belangrijke bron van morbiditeit en mortaliteit die de toepassingen van deze levensreddende therapie beperkt. Er werd gebruik gemaakt van een beenmergtransplantatie muismodel voor acute GvHD waarbij de donoren en ontvangers verschilden in de expressie van mineure histocompatibiliteitsantigenen (miHA). De majeure histocompatibiliteitscomplex antigenen (MHC) waren in donors en ontvangers dezelfde, hetgeen ook in de kliniek de meest voorkomende situatie is. Als vertegenwoordiger van de groep SSRIs werd opnieuw voor fluoxetine gekozen. Enerzijds was deze keuze gebaseerd op de immunosuppressieve effecten die bij de *in vitro* experimenten aan het licht kwamen en die als zeer gunstig werden beschouwd, anderzijds was voor fluoxetine het meeste bewijs voorhanden dat dit geneesmiddel immunosuppressieve werking vertoonde in diermodellen van andere (auto-)immuun aandoeningen. De resultaten van dit onderzoek, beschreven in **hoofdstuk 6**, tonen aan dat fluoxetine de symptomen van acute GvHD kan onderdrukken en de overleving na transplantatie verbetert. Bovendien werd geen nadelige invloed teruggevonden van fluoxetine op de innesteling van de getransplanteerde stamcellen. De verbetering van klinische symptomen onder invloed van fluoxetine ging gepaard met een onderdrukking van alloreactieve T cellen (de cellen verantwoordelijk voor het ontstaan van acute GvHD) in het bloed van de proefdieren. Uit dit

onderzoek werd besloten dat fluoxetine een gunstige invloed uitoefent op acute GvHD (althans in muizen) en dat verder onderzoek naar de immunologische wijzigingen die fluoxetine induceert in het muismodel, alsook de toepasbaarheid van fluoxetine als immunosuppressivum bij acute GvHD in de klinische context wenselijk is.

In **hoofdstuk 7** worden de bevindingen van dit onderzoek en de toepasbaarheid van SSRIs als immunosuppressiva in de klinische context besproken. In het bijzonder wordt aandacht besteed aan de vraag of voldoende hoge plasma concentraties kunnen bereikt worden om de gewenste immunosuppressie te induceren. Bovendien wordt hier ook besproken wat de impact is van dit onderzoek voor de huidige toepassing van SSRIs, nl. in de behandeling van psychiatrische aandoeningen. Immers, ook in deze patiënten wordt mogelijk een onderdrukking van het immuunsysteem geïntroduceerd.

CURRICULUM VITAE

CURRICULUM VITAE

1. GENERAL INFORMATION

Name	GOBIN Veerle
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Place of birth	Les Ulis (France)
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2. EDUCATION

2009-present PhD in Pharmaceutical Sciences, Ghent University

2004-2009 Master in Pharmaceutical Sciences, Ghent University, diploma obtained with great distinction

Dissertation: "Influence of TNFα on LTβ-receptor responsiveness in synovial fibroblasts"

1998-2004 Latin-Mathematics, Jan-van-Ruusbroeckollege, Brussel

3. SCIENTIFIC CURRICULUM

3.1. Articles in peer-reviewed journals (A1)

V. Gobin, M. De Bock, B. Broeckx, M. Kiselinova, W. De Spiegelaere, L. Vandekerckhove, K. Van Steendam, L. Leybaert, D. Deforce. Fluoxetine suppresses Ca^{2+} signaling in human T lymphocytes through inhibition of Ca^{2+} release from intracellular stores. Submitted to Cell Calcium. *IF 4.21; JCR rank 70/185 in category cell biology (2013).*

E. Scheerlinck, K. Van Steendam, M. Vandewoestyne, T. Lepez, V. Gobin, P. Meert, L. Vossaert, F. Van Nieuwerburgh, A. Van Soom, L. Peelman, B. Heindryckx, P. De Sutter, M. Dhaenens, D. Deforce. Detailed method description for non-invasive monitoring of differentiation status of human embryonic stem cells. Analytical Biochemistry, Anal Biochem. 2014 Sep 15;461:60-6. *IF 2.31; JCR rank 32/76 in category analytical chemistry (2013).*

V. Gobin, K. Van Steendam, D. Denys and D. Deforce. Selective serotonin reuptake inhibitors as a novel class of immunosuppressants. International immunopharmacology, 2014 May; 20(1): 148-56. *IF 2.71; JCR rank 95/256 in category pharmacology and pharmacy (2013).*

V. Gobin, K. Van Steendam, S. Fevery, K. ,Tilleman, A. Billiau, D. Denys and D. Deforce. Fluoxetine reduces murine graft-versus-host disease by induction of T cell immunosuppression. Journal of neuroimmune pharmacology, 2013 Sep; 8(4): 934-43. *IF 4.57; JCR rank 30/261 in category pharmacology and pharmacy (2011).*

3.2. Other publications

V. Gobin. Fluoxetine onderdrukt graft-versus-host ziekte bij muizen door T cel immunosuppressie. Tijdschrift voor Psychiatrie, 2013 Oct; 55 (10): 801-801.

3.3. Attended (inter)national conferences with active contribution

2nd International meeting on Nerve-driven Immunity, 20-21 Aug 2014, Stockholm, Sweden. Poster presentation: “Characterization of serotonin transporter expression in human T lymphocytes.” V. Gobin, K. Van Steendam, M. Kiselinova, W. De Spiegelaere, L. Vandekerckhove, D. Deforce.

Knowledge for growth, 8 May 2014, Ghent, Belgium. Poster presentation: “Characterization of serotonin transporter expression in human T lymphocytes.” V. Gobin, K. Van Steendam, M. Kiselinova, W. De Spiegelaere, L. Vandekerckhove and D. Deforce.

Knowledge for growth, 30 May 2013, Ghent, Belgium. Poster presentation: “Serotonin reuptake inhibitors as immunosuppressants in murine graft-versus-host disease.” V. Gobin, K. Van Steendam, S. Fevery, K. ,Tilleman, A. Billiau, D. Denys and D. Deforce.

11th International Congress of Neuroimmunology, 04-08 Nov 2012, Boston, USA. Poster presentation: “Serotonin reuptake inhibitors as immunomodulators: a proof-of-concept.” V. Gobin, K. Van Steendam, S. Fevery, K. ,Tilleman, A. Billiau, D. Denys and D. Deforce.

British Society for Immunology annual congress, 05-08 Dec 2011, Liverpool, UK. Poster presentation: “serotonin reuptake inhibitors selectively decrease proliferation and viability of activated T-cells.” V. Gobin, K. Van Steendam, D. Deforce.

3.4. Attended symposia and workshops

Bio-Rad droplet PCR road show, 13 June 2013, Grimbergen, Belgium.

Symposium Ghent Clinical Immunology, 7 June 2013, Ghent, Belgium.

Presentation skills, November – December 2012, Ghent, Belgium.

ANALIS – flow cytometry day, 25 Sept 2012, Brussels, Belgium.

Seminar on inflammation and vaccination, 19 Sept 2012, Ghent, Belgium.

Clinical studies: study design, implementation and reporting, August 2012, Ghent, Belgium.

Bio-Rad qPCR road show, 26 Oct 2011, Grimbergen, Belgium.

Introduction to mass spectrometry, 23-25 August 2011, Ghent, Belgium.

Academic English writing skills, February – May 2011, Ghent, Belgium.

Laboratory animal science I & II, October 2010, Ghent, Belgium.

7th Training course on 'Concepts and Methods in Programmed Cell Death', 1 Sept 2010, Ghent, Belgium.

Introduction course flow cytometry, 30 March 2010, Erembodegem, Belgium.

Course on qPCR experiment design and data-analysis, 10-11 September 2009, Ghent, Belgium.

4. EDUCATIONAL EXPERIENCE

4.1. Training

Practicumtraining, 18 February 2014, Ghent, Belgium.

Basisassistententraining, 24 June 2013, Ghent, Belgium.

Seminarie onderwijskunde: uitdagende practica, 26 March 2013, Ghent, Belgium.

Workshop het feedback gesprek, 22 May 2012, Ghent, Belgium.

4.2. Experience

Supervision of practical courses in Pharmaceutical Biotechnology 2009-2014.

Supervision of practical courses in Phytochemistry and Pharmacognosy 2009-2014.

Supervision of Integrated Bachelor Course 2010-2014.

Supervision of dissertations

- 2013-2014. Evi Standaert. Onderzoek naar serotonine transporter expressie in T-cellen. (master of Industrial Pharmacy)

- 2012-2013. Lisa D'Hondt. Onderzoek naar de serotonine transporter expressie van gestimuleerde en niet-gestimuleerde T-lymfocyten op mRNA- en eiwitniveau. (Master of Pharmaceutical care)
- 2011-2012. Celine Verhille. Onderzoek naar het verband tussen tryptofaan depletie en immunosuppressie door selectieve serotonine heropname inhibitoren. (Master of Pharmaceutical care)
- 2010-2011. Natascha Vandommele. Analyse van het immunomodulerend effect van selectieve serotonine heropname inhibitoren aan de hand van een human in vitro model. (Master of Pharmaceutical care)
- 2009-2010. Katrien Foubert. Het effect van selectieve serotonine heropname inhibitoren op het geactiveerde immuunsysteem. (Master of Pharmaceutical care)

5. OTHER WORKING EXPERIENCE

Administrative assistant at forensic DNA laboratory

DANKWOORD

Een doctoraat doe je niet alleen, en zonder de bijdrage van een heleboel anderen zou deze thesis dan ook niet tot stand gekomen zijn. Ik neem hieronder dan ook graag de gelegenheid om mijn dank te uiten aan allen die hun steentje hebben bijgedragen.

In eerste plaats een grote DANK U aan mijn promotor, Dieter, om me meer dan vijf jaar geleden op het idee te brengen om aan een doctoraat te beginnen. Had jij geen contact met me opgenomen, dan was ik wellicht nooit op dit labo terecht gekomen en had ik misschien wel nooit mijn weg gevonden naar de onderzoekswereld. Ook bedankt om me de voorbije vijf jaar de vrijheid te geven mijn eigen weg te zoeken, en tegelijkertijd beschikbaar te zijn om resultaten te bespreken en de weg uit te stippelen voor verder onderzoek.

Terwijl ik bij Dieter vooral terecht kon voor het bespreken van resultaten, kon ik bij Katleen eerder terecht voor dagelijkse raad bij kleine en grote problemen. Katleen, merci om me onder je vleugels te nemen en me tegelijkertijd, net zoals Dieter, de ruimte te geven mijn eigen ding te doen. Ook merci voor het veelvuldig en grondig nalezen van allerlei schrijfsels – inclusief deze thesis! Bovenal bedankt om me het gevoel te geven dat ik er niet alleen voor stond en om in een wereld vol mass specs en stamcellen de interesse in immunologie levend te houden. Ook aan Kelly ben ik een dankjewel verschuldigd, om me aan te stellen als opvolger voor dit project en me de basics bij te brengen in het begin van mijn tijd hier op het lab.

In de loop van dit doctoraat heb ik met heel wat andere labo's samengewerkt en zonder hen zou dit onderzoek nooit zo veelzijdig zijn geworden. Uit de vele samenwerkingen heb ik heel wat geleerd en zij hebben mij dan ook een veel ruimere blik op onderzoek bijgebracht. Prof. Damiaan Denys en Pelle, bedankt om me enthousiast te ontvangen op het AMC en met me in zee te gaan voor de studie rond hepatitis B vaccinatie. Er zijn dan nog wel geen resultaten, maar die komen er uiteindelijk wel.

Ook heel erg bedankt aan Sabien (met –ien!) om me met open armen te ontvangen in Leuven en enthousiast in de GvHD experimenten te duiken. Sabien, merci voor de toffe samenwerking! Als ervaren onderzoeker en mama van vier stond je ook naast het onderzoek altijd klaar voor goede raad. In Leuven kon ik ook rekenen op de hulp van Omer, die met zijn jarenlange ervaring als laborant heel wat tips en trics klaar had dewelke ik gretig mee terug naar Gent nam. Merci Omer voor je hulp en je gezelschap tijdens het bereiden van de stamcel grafts! Ook Leen en Li, bedankt voor jullie bijdrage in de GvHD experimenten (Li, thanks for your time-consuming help with the mice). Tenslotte ook An, bedankt voor het nalezen van de paper en het leveren van nuttige taalkundige en wetenschappelijke suggesties.

For the ddPCR experiments, I could count on the help of Maja to guide me from one building to the next (no need to do any physical exercise in the evenings ;-)) and teach me how to do ddPCR and what to conclude from the droplets. Thank you, Maja, to help me out at any time despite your sometimes very busy schedule! Ook merci Ward, voor de hulp bij statistische onduidelijkheden in de Quantasoft-ware.

Meer recent kwam ik terecht op het labo fysiologie voor de calcium experimenten waar ik opgevangen werd door Marijke. Marijke, merci voor de begeleiding en de veelvuldige uren samen

achter de microscoop. Tussen koetjes en kalfjes is gebleken dat we heel wat meer gemeen hebben dan de interesse voor onderzoek – ik denk aan toevalstreffers in De Stokerij en een voorliefde voor verre reizen! Ook merci om me last-minute doorheen de technische kantjes van de microscoop te loodsen, al had je gelijk dat ik het misschien toch wat ver aan het zoeken was ;-). Ook bedankt prof Leybaert, voor de brainstorm sessies in de keuken en voor het enthousiasme dat u toont voor dit project.

De vele experimenten op humaan bloed zouden niet kunnen doorgaan als er niet eerst een ader voor gesneuveld was. Ik ben dan ook een grote DANK U verschuldigd aan alle 'vampiers' die met veel plezier voor mij prikten: dankjewel voor jullie inspanningen! Weet dat zonder jullie de eerste 4 hoofdstukken van dit doctoraat niet tot stand hadden kunnen komen. Ook zonder bloeddonoren had dit natuurlijk niet gekund, dus bij deze een welgemeende merci aan iedereen die zijn bloed doneerde voor de wetenschap! Tenslotte ook een woordje van dank aan Yens voor het onderhouden van de jurkat cultuur. Je hebt me heel wat tijd bespaard (al hebben we die wellicht terug ingehaald met kletsen iedere keer ik langs het koffiekot passeerde –waarvoor minstens evenveel dank)!

Tijd uitsparen kon ook dankzij de thesisstudenten die in de loop der jaren op mijn project terecht kwamen. Katrien, Natascha, Celine, Lisa en Evi, merci voor jullie helpende handen en jullie interesse voor dit onderzoek. De ervaring was minstens even leerrijk voor mij als voor jullie!

Naast de vele mensen waar ik mee heb samengewerkt voor dit project, zijn er ook nog een heleboel collega's en ex-collega's die ik om andere redenen graag wil bedanken. Liesbeth en Pieter, wij zijn samen gestart en hebben samen de ups en downs van het 'PhD life' leren kennen, en dat schept een band! Meer dan op wie ook, kon ik op jullie steunen als de motivatie even zoek was. Van existentiële crisissen en het beantwoorden van grote levensvragen zoals 'wat-ben-ik-in-godsnaam-aan-het-doen', tot het delen van de kick bij het eerste aanvaarde artikel en de voldoening als 'het' eindelijk af was, bij jullie wist ik dat het gevoel van herkenning nooit veraf was. Van filosofische gesprekken op café die tot in de vroege uurtjes mochten duren, tot picknicks aan de coupure met bijbehorend droogje en (aangezoet ;-)) natje: Ik kan me niet inbeelden hoe het zonder jullie geweest zou zijn! Ook de andere collega's verspreid over den beneden, het tweede en ondertussen ook het derde: Maarten (master in 'out-of-the-box' denken), Marlies (een voorbeeld om naar op te kijken), Sandra (PhD in doorzettingsvermogen), Trees (de vriendelijkheid in eigen persoon en een waar organisatorisch talent), Paulien (down-to-earth, doelgericht en steeds constructief), Bart (extreem gemotiveerd en de King-of-Coffee), Ellen (steeds enthousiast en ondernemend), Elisabeth (mijn favoriete nieuwe linkerbuurvrouw met aanzienlijk overlappend interesse-profiel;-)), Lieselot (klein maar met véél inhoud), Yip (nog steeds trouw op post bij ieder doctoraat), Filip (avonturier en entertainer), Mado (merci voor ons appartement!), Christophe (vertaler-tolk van dienst bij Oekraïense meteorieten), Bert (10 km lopen – yes we can), Yannick en Dieter (IT-problem solvers ten allen tijde): ik ben blij dat ik deel mag uitmaken van zo'n vrolijke bende! Merci aan jullie allemaal om van labFBT te maken wat het is. De nieuwe garde, Laura en Senne, jullie eerste maanden op het lab zitten er ondertussen al op en in die korte tijd hebben jullie al bewezen volwaardige opvolgers te zullen worden!

Ook de collega's van het DNA lab wil ik natuurlijk bedanken: David (ik ben vereerd jouw favoriete Veerle te mogen zijn), Saskia (de vrolijkste persoon die ik ken), Petra, Sabine, Sylvie, Delphine,

Evelien en Evelyne. Fijn om met jullie samengewerkt te hebben! Ook Astrid, Nadine, Leen en Inge een welgemeende dankjewel om de administratieve taken uit onze handen te nemen.

Dat brengt me tenslotte bij een hoop mensen die de voorbije vijf jaar mijn mentale gezondheid op pijl hielden: merci aan 'de farma', de vriend(-innet)jes uit Brussel en uit Gent, om me met regelmaat te entertainen op week- en weekendavonden. We hebben samen al heel wat mijlpalen bereikt, sneeuwwitte bergen getrotseerd, kleine en grote feestjes gebouwd, en als het van mij afhangt blijven we dat doen tot we oud en versleten zijn! Merci om interesse te tonen voor wat ik doe, en om mijn wellicht soms ietwat onverstaanbaar antwoord aan te horen op de vraag: 'en wat onderzoek je nu weer precies?' ;-) Ik hoop dat ik jullie daar vanavond voor eens en voor altijd een verstaanbaar antwoord op zal geven! Ook een special thanks aan Sofie, om al zolang mijn grote steun en toeverlaat te zijn, om met me te komen vieren na mijn interne verdediging en om me meter te laten worden van jouw zoontje!

Dat brengt me tenslotte bij 'the inner circle': mama & papa, Peter, Jan, Marlies en kleine Jonas: simpelweg dankjewel om mijn familie te zijn. Jullie hebben me gemaakt tot wie ik ben, en zonder twijfel heb ik de wetenschappelijke 'neus' van jullie meegekregen. Een extra merci aan mama, om me de liefde voor het apothekersvak bij te brengen (hoe oud was ik toen ik mijn eerste gelulen mocht maken?) en me een extra duwtje in de rug te geven om aan dit doctoraat te beginnen. Je had gelijk, het was een kans die ik niet mocht laten schieten, en ook nu kan ik nog alle richtingen uit!

Last but not least, diegene die me het beste kent van allemaal – Goemé: merci voor de onvoorwaardelijke steun, de ontelbare duwtjes in de rug, de andere invalshoek, de afleiding, het ontkurken van 'de' fles champagne en de zoveel andere bijdragen die jij geleverd hebt aan dit doctoraat. Maar nog veel meer merci voor alles wat je voor mij betekent daarbuiten. Binnen enkele dagen zijn we tien jaar samen, en in tegendeel tot wat weleens beweerd wordt – alfabetische liefde in het practicum, geen zin om het ver te zoeken? – zou ik ook voor jou gekozen hebben en nog steeds kiezen als je aan de andere kant van de aardbol (Eeklo bijvoorbeeld) elektrieker geworden was. En liefje, helaas, jouw gloriedagen zijn voorbij: het wordt terug gelijkstand wat het aantal diploma's betreft! ;-)

Veerle