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Immunological substrates of major depression and antidepressant treatment

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1. INTRODUCTION

Depression is a chronic and highly prevalent mood disorder that exacts a staggering toll on the affected individuals, their caregivers and society at large [1]. More than 15 years ago, it was predicted that by the year 2020 depression would represent the second leading cause of disability worldwide [2]. Strikingly, this position was already reached in 2013 [3]. To compound the predicament, at least 30% of patients suffering from depression are unable to respond to or tolerate existing antidepressant medications [4, 5], thereby emphasizing the need to expand our therapeutic arsenal.

Depression not only alters the brain of an individual but also affects bodily processes to a great extent. This is attested by the fact that depression alone produces decrements in health comparable to those produced by chronic physical diseases, i.e., angina, arthritis, asthma and diabetes [6]. Moreover, a comorbid state of depression is detrimental to overall health and worsens the prognosis of the above-mentioned primary diseases, including HIV infection and cancer [6-9]. Importantly, depression comes full circle, as it also functions as a risk factor for incident physical illness, e.g., coronary artery disease, stroke, type 2 diabetes, cancer and a wide range of infections [10-14].

This bidirectional relationship between depression and physical affliction is likely shaped by several pathophysiological processes, including immune system dysregulation [7, 15]. Indeed, ample evidence accrued over the past two decades demonstrates a mobilization of peripheral blood cytokines and activation of major pro-inflammatory signaling pathways in depressed individuals [16-20]. At the same time, vital immune cell populations (i.e., T cells and natural killer cells) have been long known to exhibit signs of functional suppression in the same individuals or a subset thereof [21, 22]. However, to date, much less attention has been paid to the characterization of these loss-of-function alterations [23]. The research objective of this thesis was to describe in greater detail the mechanisms underlying immune suppression, most notably T cell impairment, in depressed patients.

1.1. The immune system

The immune system consists of a complex and dynamic network of organs, cells and secreted molecules that have evolved to protect against a vast range of exogenous and endogenous threats. The fully functional immune system maintains the cellular integrity of the human body by mounting proper and efficient immune responses following recognition of specific molecules (called “antigens”) or molecular configurations, while at the same time safeguarding against collateral damage to healthy cells and tissues. The antigen repertoire available for immune recognition is immense, ranging from components of foreign pathogens (e.g. bacteria, viruses, fungi and other parasites) to antigens derived from foods, medications as well as damaged and healthy cells of the host itself. Hence, the immune system is responsible not only for responding to antigens with pathogenic potential but also tolerating those that pose no actual threat [24]. Failure to do so may result in

pathological conditions characterized by either insufficient responses (e.g., infectious and malignant diseases) or unwanted responses (e.g., allergic and autoimmune diseases).

1.1.1. Innate and adaptive immunity

Although reference is generally made to “the immune system”, immune responses are traditionally discriminated into innate (or natural) and adaptive (or acquired). Each component of the immune system has a distinctive function and role, but at the same time is integrated into highly interactive microenvironments [25].

Immune cells of the innate branch mount an initial rapid line of host defense against pathogenic microorganisms and tissue damage. Cells of the innate immune response include granulocytes (i.e., neutrophils, basophils, mast cells and eosinophils), natural killer (NK) cells as well as myeloid antigen-presenting cells (i.e., monocytes, macrophages and dendritic cells). Major functions of these types of leukocytes include phagocytosis (ingestion) of pathogens and associated particles, release of soluble antimicrobials, activation of the complement cascade, recruitment of other immune cells to sites of inflammation as well as engagement of the adaptive immune system through antigen presentation [26].

Unlike cells of the adaptive immune system, activity of the innate immune cells does not depend upon prior acquaintance with the antigenic stimulus. Instead, it is promptly generated by virtue of germline-encoded recognition molecules, collectively known as pattern recognition receptors (PRRs), which are naturally expressed by the majority of immune cells across species [27]. PRRs can detect a limited repertoire of common and highly conserved antigenic structures associated with pathogens or cell damage, thereby allowing an early, non-specific “profiling” of the threat under concern. Engagement of PRRs then triggers the activation of intracellular signaling cascades, leading to the induction of genes involved in antimicrobial host defense, such as pro-inflammatory cytokines and type I interferons [28].

By contrast, adaptive immune responses rely on B and T lymphocytes, which recognize much more subtle details of molecular organization called “epitopes”. Since the repertoire of epitope recognition is practically limitless, a vast number of lymphocytes is required to ensure that virtually every different epitope of foreign and self-antigens will be properly encountered. To this end, gene rearrangement events during lymphocyte development result in a highly diverse repertoire of membrane-bound B cell and T cell receptors (BCR and TCR, respectively) which subserve an equally diverse repertoire of epitope recognition specificities [29]. However, although the populations of B cells and T cells express a remarkably broad landscape of antigen-specific receptors, each individual cell will express only one type of receptors on its surface, as a result of a unique gene rearrangement in that cell. If a given lymphocyte divides, all progeny cells will bear receptors with antigen specificities identical to each other as well as to the progenitor lymphocyte, thereby constituting a “clone”.

Upon encountering their cognate antigen(s), naive B and T cells start to proliferate and differentiate into both short-lived effector cells – which carry out the primary immune response – and long-lived memory cells, which persist in the host after the primary response has resolved in order to mediate a faster and more efficacious secondary response upon re-challenge with the same antigen [30, 31]. Effector B cells, known as plasma cells, mediate humoral immune responses by virtue of secreting immunoglobulins (antibodies) which bear antigen-binding sites identical to those on the membrane-bound BCR. By integrally binding their corresponding epitopes on soluble antigens, antibodies form complexes that can be subject to various types of pro-inflammatory immune processes, including complement deposition, phagocytosis and enhancement of antigen presentation to T cells [32]. Unlike the BCR, the activated TCR is not released in a secreted form, but instead mobilizes cell-mediated immune responses involving antigen-specific effector T cells, release of various pro-inflammatory mediators and activation of phagocytes [33-35].

1.1.2. T cells

In contrast to B cells, which can exert effector functions from distant sites, T cells cannot directly recognize soluble antigens via their TCR. Instead, they make contact only with pieces of antigens that have been pre-processed by antigen-presenting cells (e.g., fragmented peptides) and which are bound to a genetically diverse group of glycoproteins known as major histocompatibility complex (MHC) molecules. In humans, these proteins are encoded by the human leukocyte antigen (HLA) system. Class I MHC molecules (HLA A, B and C) are expressed by nearly all nucleated cells and present intracellularly derived antigens, whereas class II MHC molecules (HLA DP, DM, DOA, DOB, DQ and DR) present extracellularly derived antigens and are normally expressed by professional antigen-presenting cells, i.e., dendritic cells, macrophages and B cells. During antigen presentation, MHC molecules interact with both the TCR and certain co-receptors on the T cell surface. In particular, T cells expressing the co-receptor CD4 recognize antigens in the context of class II MHC proteins, while T cells expressing the co-receptor CD8 recognize antigens coupled to class I MHC molecules.

1.1.2.1. Generation and maintenance of the TCR repertoire

T cell precursors originate from the bone marrow and reach the thymus via the blood. TCR gene rearrangement and strict precursor selection are the most decisive steps coordinating their subsequent maturational development. The vast majority of immature T cells, known as thymocytes, will die by apoptosis during an intensive screening process taking place in several distinct thymic microenvironments [36]. In particular, thymocytes expressing a TCR which binds ectopically expressed self-peptide-MHC complexes with high affinity are preferentially induced to die by negative selection [37], whereas those thymocytes that are not able to interact with the peptide-loaded self-MHC molecules will die by neglect. Only thymocytes with a TCR of intermediate affinity to self-peptide-MHC complexes will undergo positive selection [38], therewith resulting in their survival and further differentiation – largely

into either $CD4^+$ or $CD8^+$ mature naïve T cells that bear functional and adequately self-tolerant TCRs [36, 39].

The mature TCR is a heterodimeric protein which, in the majority of peripheral T cells, consists of an alpha (*α*) and a beta (*β*) chain. The extracellular domain of each chain is composed of germline-encoded subunits, including variable (V), diversity (D), joining (J) and constant regions. The by and large stochastic recombination of these germline gene segments during thymic development, along with the random insertion or deletion of nucleotides adjacent to the recombination sites, leads to the generation of numerous individual T cells with unique TCR amino acid sequences owing to a greatly diverse repertoire of rearranged VJ *α* chains and VDJ *β* chains [40]. This ensures the existence of a T cell repertoire that is many orders of magnitude more diverse than the singular genes encoding it, thereby covering a broad range of epitope recognition. Central to this diversity of antigen specificities is the particularly high sequence variation found in the complementarity determining regions (CDRs) which are contributed by both receptor chains and comprise the antigen-binding site of the TCR. Typically, TCR diversity is focused on the CDR3 region which is topologically oriented to allow for optimal interaction with the MHC-bound peptide antigen [41]. Characterization of CDR3 sequence variation therefore provides a measure of TCR diversity or bias within a polyclonal T cell repertoire [42].

Importantly, the TCR repertoire shaped by intrathymic selection can be further conditioned in the periphery. For instance, additional low-affinity interactions with self-peptide-MHC complexes take place in peripheral lymphoid organs in order to reaffirm the capacity of the mature naïve T cell pool to respond to higher affinity interactions with foreign antigens [43]. Substantial selection pressures owing to infections, self-interactions and aging processes will then greatly shape the clonal composition of the peripheral T cell pool [44, 45]. At the same time, multiple peripheral mechanisms of physical isolation or functional inactivation (e.g., anergy or active suppression) are at play to keep potentially harmful autoantigen-reactive clones sequestered from the functional repertoire [46]. On this account, the antigen-selected repertoire is destined to undertake the challenging task of keeping pace with trespassing pathogens and tissue demands while affording protection from pathological self-reactivity. Indeed, several preclinical studies have shown that reductions in TCR structural diversity can compromise resistance to certain pathogens (reviewed in [47]).

1.1.2.2. T cell activation, differentiation and migration

Upon recognition and binding to an MHC-peptide complex on the surface of their interacting cell, naïve $CD8^+$ T cells become activated and adopt an effector phenotype with substantial cytotoxic efficacy. Therefore, any kind of host nucleated cell that presents a foreign antigen coupled to a class I MHC molecule – namely, infected, allogeneic, neoplastic or otherwise aberrant somatic cell – will be subject to $CD8^+$ T cell-mediated apoptotic elimination by way of either secreted cytotoxins (i.e., perforin, granzymes and granulysin) or the Fas/FasL cytotoxic pathway [48].

However, to proliferate, differentiate and respond optimally, naïve $CD8^+$ T cells will usually need stimulatory signals from $CD4^+$ T cells as well [49].

When a naïve $CD4^+$ T cell becomes activated following interaction with a class II MHC-peptide complex on the surface of a professional antigen-presenting cell, it can differentiate into one of several effector T helper subsets, depending mainly upon the "instruction" received by the cytokine milieu of the microenvironment. Each T helper subset is characterized by a distinctive gene expression profile as well as the release of signature effector cytokines, all being under the control of lineage-determining master transcription factors (**Fig. 1.1.**). These transcription factors include TBX21 (or T-bet) which is expressed in T helper type 1 cells (associated with immune responses against intracellular pathogens [50, 51]), GATA3 in T helper type 2 cells (associated with responses against extracellular pathogens and allergens [52, 53]), RORγt in T helper type 17 cells (associated with responses against bacteria and fungi at mucosal surfaces [54]) and BCL-6 in T follicular helper cells (associated with B cell maturation and antiviral humoral immunity [55]).

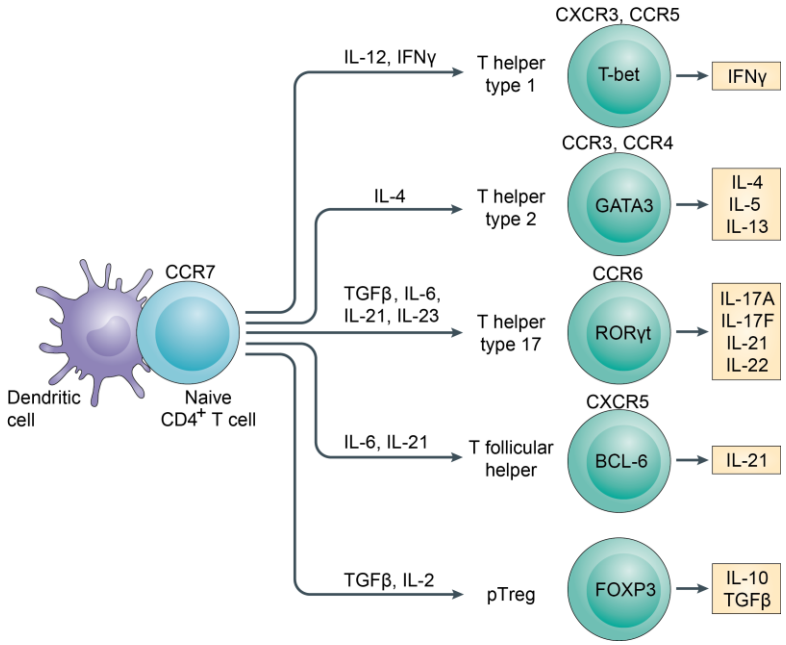


Figure 1.1. Effector $CD4^+$ T cell populations.
Subsets of effector $CD4^+$ T cells involved in immune protection and regulation. Polarizing cytokines and lineage-defining transcription factors as well as signature effector cytokines and homing receptors are depicted. IL: interleukin; IFN: interferon; TGF: transforming growth factor; pTreg: peripherally induced regulatory T cell; CCR: receptor for CC chemokine; CXCR: receptor for CXC chemokine. Figure modified from [51].

Another important subset of $CD4^+$ T cells, the regulatory T (Treg) cells, are characterized by the expression of the lineage-defining transcription factor FOXP3 and display the unique capacity to maintain immunologic self-tolerance as well as negatively control various immune responses via cell-cell contact and/or secretion of anti-inflammatory cytokines, e.g., IL-10, IL-35, TGFβ [56, 57]. In humans, these cells arise mainly from self-reactive T cells during thymic selection and maturation (natural Treg, [58]). In addition, they may develop extrathymically during a peripheral immune

response, usually following TCR stimulation and in a TGFβ-dependent manner [59-61]. However, phenotypic Treg induction may not always be accompanied by functional immunosuppression [62]. Interestingly, recent data suggest that Treg cells may also display tissue-protective functions independent of their typical immunosuppressive duties [63].

In addition to differences in cytokine repertoire, $CD4^+$ T cell subsets also exhibit a variety of homing patterns in order to maximize their opportunities for antigen detection. Targeted T cell migration is highly integrated with differentiation and function [64] and dependent upon the combinatorial expression of a broad set of adhesion and homing molecules (e.g., selectins, integrins, chemokine receptors and their ligands) that dictate the sequence of events with regard to T cell extravasation and positioning in various tissue microenvironments [65, 66]. For instance, the majority of T helper type 1 cells preferentially express the chemokine receptors CXCR3 and CCR5, whereas T helper type 2 and T follicular helper cells preferentially express the chemokine receptors CCR3/4 and CXCR5, respectively [55, 67-69]. At the same time, T helper type 17 cells seem to be characterized by the expression of the receptors CCR6 and CCR4 [70]. Finally, expression of homing receptors by Treg cells is dynamically defined by T helper polarizing signals in the immune microenvironment [71].

Of note, numerous preclinical and clinical studies have shown that certain adhesion molecules and chemokine receptors are implicated in the selective trafficking of patrolling T cells into central nervous system (CNS) compartments, i.e., cerebrospinal fluid, subarachnoid and brain perivascular spaces [72, 73]. In humans, the receptors CXCR3 and CCR5/6 noticeably belong to a narrow repertoire of chemokine receptors which are preferentially expressed on the surface of antigen-experienced T cells endowed with a constitutive (homeostatic) but also at times pathogenic CNS-homing capacity [74-81].

1.2. Major depressive disorder

1.2.1. Clinical presentation

Major depressive disorder (MDD) is a heterogeneous clinical syndrome characterized by a profoundly negative view of the world, oneself and the future [82]. It is currently diagnosed using criteria set forth by the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) and the International Statistical Classification of Diseases and Related Health Problems, 10th edition (ICD-10). Primary symptoms of MDD are a pervasive and persistent low mood and a loss of the ability to experience pleasure from activities that are normally enjoyable (anhedonia). The diagnosis also hinges on the copresence of an array of mental and physical features, including changes in weight or appetite, sleep and psychomotor disturbances, loss of energy, feelings of guilt or low self-worth and suicidal ideation. Inevitably, MDD heavily interferes with daily functioning as well as general physical and mental well-being [83].

1.2.2. Pathophysiology and pharmacotherapy

Despite its prevalence and socioeconomic impact, the cellular and molecular pathogenesis of MDD remains elusive [84]. This is testified by the high number of etiological theories that have been put forward over the years, which span a variety of neurobiological and somatic processes [85]. In this regard, prominent hypotheses propose that MDD arises from perturbations in monoamine metabolism, the hypothalamic-pituitary-cortisol system, glutamatergic neurotransmission, adult hippocampal neurogenesis and overall neurotrophic support [86-90]. Intriguingly, the "immune hypothesis" aspires to bring all these pieces together by suggesting that enhanced inflammatory responses may increase the risk for depression via incremental negative effects on the above-mentioned pathophysiological domains [19]. In addition, the "immune hypothesis" posits that impaired T cell responses may undermine resilience to depression owing to blunted anti-inflammatory and neuroprotective effects constitutively mediated by T cells [19, 91].

Despite the profusion of biological hypotheses, the monoamine-deficiency hypothesis has provided the only significant theoretical framework for antidepressant drug development, perhaps at the expense of comprehending the pathophysiology of depression [92]. As a result, the majority of currently used antidepressants (e.g., selective serotonin and/or norepinephrine reuptake inhibitors as well as newer "atypical" antidepressants) are simply refined versions of the original antidepressant medications (i.e., tricyclic antidepressants and monoamine oxidase inhibitors) [92]. Compounding the problem, currently available medications show only moderate response rates, while treatment resistance and relapse constitute significant clinical problems [93]. This highlights the need for approaches that target mechanisms beyond monoamines [94].

1.2.3. Immunology of MDD

Extensive psychoneuroimmunological research on the relationship between MDD and immunity has been conducted over the last 35 years. This huge effort included enumerative and functional measures of cellular immunity as well as *ex vivo* stimulated cytokine production and assessment of circulating levels of pro-inflammatory molecules. Overall, two classes of seemingly contradictory findings have reached meta-analytic confirmation: (1) MDD is characterized by innate immune activation and (2) MDD is characterized by functional, cell-specific immunosuppression. Astonishingly, these two sets of observations rarely overlapped such that only recently reviews in the field have started to hypothesize about the co-existence of both immunological alterations in MDD patients or a subset thereof [23, 91, 95]. Indeed, very recent peripheral blood gene expression [96] and flow cytometry studies [97] have shown that depression may be characterized by both immune activation and immune suppression.

1.2.3.1. Immune activation

A vast clinical literature has associated depressive symptoms and/or MDD with enhanced innate immune, pro-inflammatory as well as B cell-mediated autoimmune processes, as exemplified by increased peripheral blood concentrations of cytokines and associated soluble receptors (e.g., IL-6, TNF-α, IL-1β, sIL-2R), chemokines (e.g., CCL2), positive acute-phase reactants (e.g., CRP, haptoglobin), arachidonic acid derivatives (e.g., PGE_2) and a wide array of autoantibodies in depressed patients [22, 98-103]. In this context, a few clinical studies have suggested that lower frequency of Treg cells in MDD patients is permissive to chronic low-grade inflammation [97, 104, 105]. Furthermore, a plethora of preclinical studies in rodents as well as clinical studies in patients undergoing treatment with interferon-alpha have endorsed a depressogenic role for innate immune activation by virtue of diffuse autoinflammatory effects in mood-relevant neurocircuits [106-108]. This hypothesis is further corroborated, at least in part, by the anti-inflammatory effects of certain antidepressants, most prominently selective serotonin reuptake inhibitors [109-111].

Moreover, allelic variants of genes associated with innate immune responses (e.g., *IL1B*, *TNFA*, *CRP*), intracellular antigen processing (e.g., *PSMB4*, *PSMD13*) as well as T cell function and differentiation (e.g., *TBX21*, *CD3E*, *STAT3*) have been found to increase the risk for MDD and/or reduce patient responsiveness to antidepressant treatment [112-114]. Along the same line, evolution-based approaches proposed that allelic variants which confer risk for MDD have not been culled from the human genome because the physiological and behavioral processes they promote result in enhanced innate immune function and optimal host defense against pathogens [115, 116].

A recent randomized, placebo-controlled trial using the TNF antagonist infliximab in treatment-resistant MDD patients is the only study to date examining the antidepressant potential of cytokine antagonism [117]. In particular, TNF antagonism

showed therapeutic potential in patients exhibiting high levels of inflammation at baseline (CRP ≥ 5 mg/L). However, patients with lower levels of inflammation performed worse than placebo-treated patients, suggesting that low-grade inflammatory processes may also have an adaptive capacity in MDD. Indeed, evidence on this possibly beneficial aspect of inflammation is now emerging [118-122].

1.2.3.2. Immune suppression

Given the enthusiasm surrounding innate immune activation and depression, the role of adaptive immunity has been largely neglected [23, 91, 95]. Still, early meta-analytic work on this front indicated reduced mitogen-stimulated T cell proliferation in MDD patients [21]. This salient observation was confirmed by a second meta-analysis more than 15 years ago [22] and could be attributed, in part, to increased T cell apoptosis [123, 124].

In line with findings suggesting suboptimal T cell function, a recent systematic review and meta-analysis revealed significant cross-sectional associations between MDD and certain infectious agents, i.e., Borna disease virus, Herpes simplex virus 1, Varicella zoster virus, Epstein-Barr virus and *Chlamydophila trachomatis* [125]. Intriguingly, prospective population-based studies seem to suggest bi-directional causality behind these associations. That is, on one hand, depression may confer a general increased risk of various severe infections subsequent to the onset of the disorder [14], while on the other hand, a clinical history of severe infections may also increase the risk for a mood disorder diagnosis [126]. Therefore, a higher susceptibility to infections in MDD patients may not only be secondary to a depressive state but may, at least in some patients, precede the development of depression.

Equally important, it has been hypothesized that impaired T cell function may be central not only to increased infection susceptibility and somatic comorbidities in MDD but also to the cause as well as the treatment of the disorder [91] (**Fig.1.2.**). Indeed, extensive preclinical evidence now highlights the positive role of T cells – especially those that bear TCR specific for CNS-related antigens – in the development and maintenance of normal nervous system function, including mood, cognition and behavior [127-130]. In addition, a small but increasing number of independent studies demonstrate a specific role for the brain-localized $CD4^+$ T cell repertoire in sustaining neurotrophin production, promoting adult hippocampal neurogenesis and cognitive function as well as protecting from anxiety-like behaviors [131-136]. Although the role of TCR specificity has been questioned in regard to depressive disorders [137], this line of research is compatible with the emerging general notion that the self-reactive T cell pool can afford beneficial effects on tissue homeostasis [138]. It also coincides with a broader appreciation of the contribution of the immune system – most prominently T cells – to the maintenance of CNS integrity in the face of a wide range of neurological and neuropsychiatric conditions [139-141].

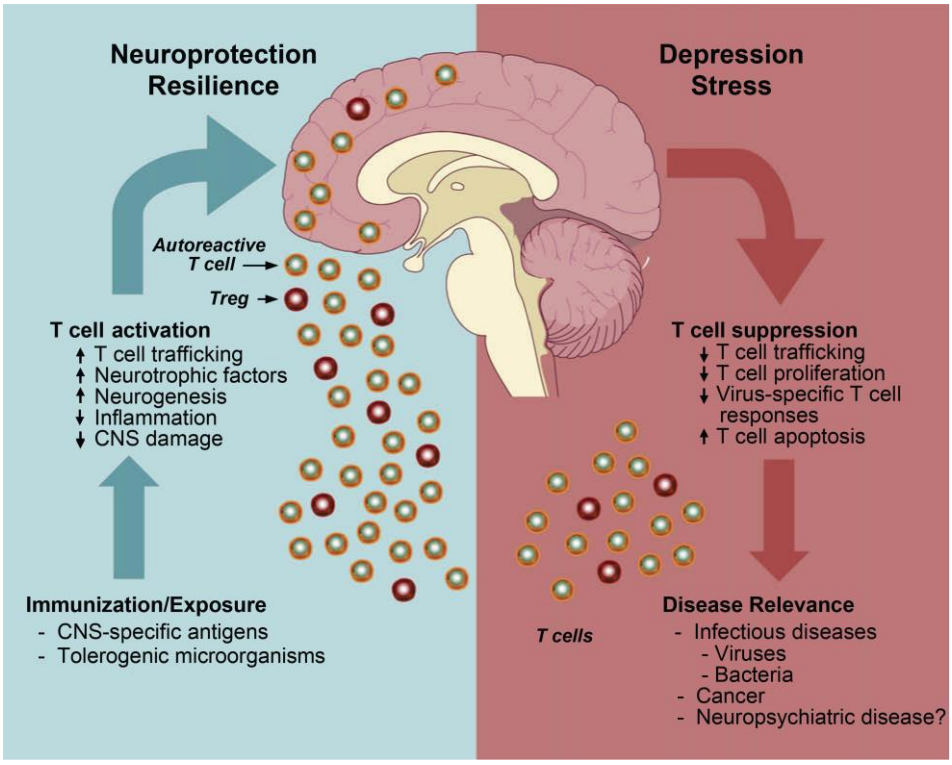


Figure 1.2. Brain-T cell interactions in health and depression.
Under physiological conditions, T cells – including those associated with CNS homeostasis – continuously receive tonic survival signals that depend on well-balanced self-reactivity. This provides subthreshold TCR stimulation that coordinates optimal T cell trafficking, homeostatic inflammation and neurotrophic/neurogenic support. By contrast, depression is characterized by T cell impairment as manifested by reduced T cell trafficking, decreased proliferative capacity and enhanced apoptosis. This in turn places a homeostatic challenge on the CNS and contributes to disease development, including infectious and malignant diseases. Figure modified from [91].

1.3. Aims

In view of the above-mentioned background, the current thesis paid attention to the role of T cells in the pathophysiology of MDD as well as in antidepressant treatment. In particular, the following aims were addressed:

1. Phenotypic identification of major lymphocyte subsets and in depth characterization of T cells in antidepressant-free MDD patients and matched non-depressed controls. To this end, recent guidelines for flow cytometric interrogation of human peripheral blood mononuclear cells were followed. Research endpoints included expression of T helper-associated chemokine receptors relevant to trafficking of T cells into both peripheral tissues and CNS compartments, frequency of circulating Treg cells and assessment of TCR repertoire diversity among $CD4^+$ and $CD8^+$ T cells.

2. Transcriptional characterization and analysis of the clonal composition of $CD4^+$ T cells derived from antidepressant-free MDD patients and matched non-depressed controls. For this purpose, magnetic-activated cell sorting, real time PCR and TCR β chain CDR3 sequencing were employed.

3. Examination of the possible association of the above-mentioned T cell parameters with antidepressant treatment as well as with the degree of clinical response to the treatment. To this end, depression symptom severity was assessed at baseline and after 5 weeks of antidepressant monopharmacotherapy through self-rated and clinician-rated questionnaires.

Ultimately, the goal of this work was to better elucidate the mechanisms underlying T cell dysregulation in depressed patients, as this could inform new research concepts towards novel, much needed antidepressant strategies.

2. SUBJECTS, MATERIALS AND METHODS

2.1. Subjects

Baseline characteristics of all study participants are shown in **Table 2.1**. Depressed patients (*n*=20) between 18 and 65 years of age were recruited in the depression outpatient clinic of the Department of Psychiatry and Psychotherapy, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Non-depressed controls (*n*=20), matched pairwise for age, sex, body mass index and smoking status, were recruited from the same geographical region by public advertisement and from the staff of the University Medical Center Hamburg-Eppendorf. All participants gave written informed consent for the study that was approved by the Ethics Review Committee of the Hamburg Medical Board (Ethikvotum PV4161 and PV4719).

Patient screening included a physical and neurological examination, blood laboratory tests, urine toxicology screen, electrocardiogram and a structured interview by a trained physician using the Structured Clinical Interview for the DSM-IV (SCID), axes I and II. Depression symptoms were assessed at baseline and after 5 weeks of antidepressant monopharmacotherapy through self-rated (Inventory of Depressive Symptomatology-Self Report, 30 item; IDS-SR_{30} [142, 143]) and clinician-rated questionnaires (Inventory of Depressive Symptomatology-Clinician rated, 30 item; IDS-C_{30} and Hamilton Rating Scale for Depression, 17 item; $HRSD_{17}$ [143, 144]). Summing of item responses was carried out by a rater blind to the immunological data. Patient total scores and other clinical characteristics are given in **Table 2.2**. Non-depressed controls filled in the Quick Inventory of Depressive Symptoms-Self Report, 16 item (QIDS-SR_{16}) [145].

Inclusion criteria for depressed patients were: (1) diagnosis of major depressive disorder, single or recurrent, according to *DSM-IV* criteria; (2) minimum baseline score of 18 points on the $HRSD_{17}$ and (3) at least 8 weeks free of any psychiatric medication, e.g., antidepressants, antipsychotics and mood stabilizers. Inclusion criteria for non-depressed controls were: (1) no current or lifetime mood disorder diagnosis and (2) a low QIDS-SR_{16} score (≤5).

Exclusion criteria for both groups were: (1) past or present self-reported diagnosis of a major medical condition, e.g., chronic or acute inflammatory, metabolic and neurological disorders; (2) frequent usage of either prescribed, over-the-counter or illicit drugs; (3) drinking of more than 100 g of alcohol per week; (4) current adverse life events; (5) pregnancy or nursing; (6) recent vaccination.

Additional exclusion criteria during patient screening were: (1) Axes I or II co-morbidities; (2) abnormal physical or neurological examinations; (3) basic blood laboratory test values deviating significantly from the normal range; (4) positive urine toxicology screen and (5) pathological initial electrocardiogram. Hypothyroidism in euthyroid state through hormonal substitution and hypertension in normotensive state through antihypertensive medication did not represent exclusion criteria.

Table 2.1. List of MDD patients at baseline and non-depressed controls, matched for age, sex, body mass index and smoking status (*n* = 40)

Case Control	Age	Sex	BMI	Currently smoking	QIDS SR_{16}
MDD01	29	M	25.95	No	15
CTR01	27	M	25.53	No	1
MDD02	41	F	17.93	Yes	18
CTR02	41	F	17.01	Yes	4
MDD03	32	M	24.17	No	17
CTR03	31	M	22.86	No	1
MDD04	49	M	23.99	No	21
CTR04	47	M	25.99	No	5
MDD05	24	M	24.11	No	19
CTR05	24	M	24.81	No	3
MDD06	20	M	19.75	Yes	9
CTR06	21	M	21.06	Yes	2
MDD07	52	F	20.70	Yes	17
CTR07	53	F	25.47	Yes	4
MDD08	53	F	20.83	Yes	21
CTR08	55	F	20.20	Yes	2
MDD09	42	F	32.87	No	15
CTR09	49	F	30.71	No	1
MDD10	34	F	30.85	No	14
CTR10	32	F	33.06	No	1
MDD11	27	F	26.26	No	23
CTR11	30	F	27.40	No	5
MDD12	30	F	23.33	No	16
CTR12	33	F	23.05	No	4
MDD13	41.5	F	22.49	No	19
CTR13	42	F	22.86	No	1
MDD14	37	M	20.67	Yes	17
CTR14	43	M	22.83	Yes	0
MDD15	61	M	28.07	No	20
CTR15	65	M	24.16	No	1
MDD16	36	F	26.23	No	23
CTR16	35	F	24.09	No	3
MDD17	32	F	17.58	Yes	19
CTR17	36	F	22.49	Yes	0
MDD18	35	F	32.79	No	18
CTR18	33	F	39.06	No	1
MDD19	46	M	28.03	Yes	26
CTR19	41	M	24.57	Yes	3
MDD20	38.5	M	27.16	No	24
CTR20	38	M	28.63	No	3

MDD: major depressive disorder; CTR: non-depressed control; BMI: body mass index; QIDS-SR_{16}: Quick Inventory of Depressive Symptoms-Self Report, 16 item

Longitudinal analyses were conducted within 15 patients receiving antidepressant monopharmacotherapy, namely escitalopram (*n* = 10), mirtazapine (*n* = 4) or venlafaxine (*n* = 1). A total of 5 patients were excluded due to dropouts and/or missing clinical data (see **Table 2.2**). Excluded patients were more likely to be non-smokers (*p* = .050) and to have received escitalopram (*p* = .085), but were not

different from included persons in terms of sex representation, age, body mass index and severity of depression (all $p > .40$).

Table 2.2. Clinical characteristics of MDD patients at baseline (n = 20) and after 5 weeks of antidepressant pharmacotherapy (n = 15)

Patient	Previous episodes	Antidepressant medication received	Post-treatment sample	IDS-SR_{30}		IDS-C_{30}		$HRSD_{17}$	
				pre	post	pre	post	pre	post
MDD01	0	Mirtazapine	Yes	37	43	28	30	20	9
MDD02	0	Mirtazapine	Yes	45	19	41	18	22	8
MDD03	1	Escitalopram	Yes	42	34	45	19	20	9
MDD04	0	Escitalopram	No (adverse event)	51	N/A	42	N/A	23	N/A
MDD05	1	Escitalopram	Yes	46	16	44	15	24	8
MDD06	1	Venlafaxine	No (lost to follow-up)	23	N/A	35	N/A	21	N/A
MDD07	1	Mirtazapine	Yes	41	19	46	5	30	4
MDD08	1	Escitalopram	Yes	49	38	43	37	18	13
MDD09	1	Venlafaxine	Yes	38	38	46	37	20	15
MDD10	3	Escitalopram	Yes	35	15	48	13	20	3
MDD11	0	Escitalopram	Yes	56	31	39	24	19	9
MDD12	1	Escitalopram	Yes	39	27	38	22	19	8
MDD13	0	Mirtazapine	Yes	46	5	44	4	18	1
MDD14	2	Escitalopram	Yes	42	14	37	8	23	6
MDD15	0	Mirtazapine	Yes (missing post-treatment data)	48	N/A	40	N/A	22	N/A
MDD16	1	Escitalopram	No (intercurrent illness)	58	N/A	57	N/A	28	N/A
MDD17	3	Escitalopram	No (adverse event)	46	N/A	45	N/A	24	N/A
MDD18	3	Escitalopram	Yes	44	19	37	17	19	8
MDD19	0	Escitalopram	Yes	65	42	44	27	24	10
MDD20	0	Escitalopram	Yes	61	28	50	21	26	6

MDD: major depressive disorder; IDS-SR_{30}: Inventory of Depressive Symptoms-Self Report, 30 item; IDS-C_{30}: Inventory of Depressive Symptoms-Clinician rated, 30 item; $HRSD_{17}$: Hamilton Rating Scale for Depression, 17 item; N/A: not applicable

2.2. Materials

2.2.1. Reagents

Table 2.3. Reagents for cell isolation and cell culture

Reagent	Company
BD IMag Human CD4 T Lymphocyte Enrichment Set	BD Biosciences
Dimethyl sulfoxid (DMSO) for cell culture	Applichem
Dulbecco's Phosphate Buffered Saline (PBS)	PAA Laboratories
Ethylenediaminetetraacetic acid (EDTA), 0.5 M	Sigma
Fetal calf serum (FCS)	Biochrom
Human serum	PAA Laboratories
Lymphocyte Separation Medium LSM 1077 (Ficoll)	PAA Laboratories
Penicillin/streptomycin, 10,000 units/ml	Invitrogen
RPMI 1640 with stable L-Glutamine	PAA Laboratories
Trypan blue staining solution, 0.4%	Sigma-Aldrich
Türk's staining solution	Merck Millipore

Table 2.4. Reagents for flow cytometry

Reagent	Company
BD Cytometer Setup and Tracking Beads	BD Biosciences
Bovine serum albumin (BSA)	PAA Laboratories
FACS Clean solution	BD Biosciences
FACS Flow, 20L	BD Biosciences
FACS Rinse solution	BD Biosciences
Fixation buffer	Biolegend
Human IgG, polyclonal	Jackson ImmunoResearch
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Life Technologies
Permeabilization Wash Buffer, 10X	Biolegend
Sodium azide (NaN_3)	Carl Roth

Table 2.5. Reagents for nucleic acid isolation, cDNA synthesis and real-time PCR

Reagent	Company
DNeasy Blood and Tissue Kit	Qiagen
Ethanol, absolute	Carl Roth
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Scientific
RNeasy Plus Universal Mini Kit	Qiagen
TaqMan gene expression Master Mix	Applied Biosystems
Trichlormethan/Chloroform	Carl Roth
Water for chromatography (LC-MS Grade)	Merck Millipore

Table 2.6. Reagents for bead-based serum immunoassays

Reagent	Company
LEGENDplex Kit, Mix and Match human subpanel	Biolegend

2.2.2. Antibodies for flow cytometry

Table 2.7. Antibodies for identification of major lymphocyte subsets and T cell characterization

Antigen	Clone	Dilution	Fluorochrome	Company
CD3	OKT3	1:100	BV605	Biolegend
CD4	RPA-T4	1:250	Alexa 700	Biolegend
CD8α	SK1	1:100	V500	BD Biosciences
CD196 (CCR6)	G034E3	1:100	PE/Cy7	Biolegend
CD183 (CXCR3)	G025H7	1:100	PE	Biolegend
CD127 (IL-7Rα)	A019D5	1:100	APC	Biolegend
CD25 (IL-2Rα)	M-A251	1:100	BV421	Biolegend
CD45RA	HI100	1:1,000	PE/Cy7	Biolegend
CD19	HIB19	1:100	V500	BD Biosciences
CD56	HCD56	1:100	PE/Cy7	Biolegend
CD20	2H7	1:10	PE	BD Biosciences
CD14	MΦP9	1:1,000	V450	BD Biosciences
CD16	3G8	1:500	FITC	BD Biosciences
HLA-A2	BB7.2	1:10	PE	Biolegend

For analysis of the T cell receptor (TCR) V*β* repertoire, the IOTest Beta Mark Kit from Beckman Coulter was used. The 24 antibodies are grouped into 8 tubes, as shown in **Table 2.8**, resulting in 8 reagent mixtures (10 µL/test).

Table 2.8. Antibodies for TCR V*β* repertoire assays

Tube	V*β* segment*	Clone	Fluorochrome
A	V*β* 5.3	3D11	PE
	V*β* 7.1	ZOE	PE+FITC
	V*β* 3	CH92	FITC
B	V*β* 9	FIN9	PE
	V*β* 17	E17.5F3	PE+FITC
	V*β* 16	TAMAYA1.2	FITC
C	V*β* 18	BA62.6	PE
	V*β* 5.1	IMMU157	PE+FITC
	V*β* 20	ELL1.4	FITC
D	V*β* 13.1	IMMU222	PE
	V*β* 13.6	JU74.3	PE+FITC
	V*β* 8	56C5.2	FITC
E	V*β* 5.2	36213	PE
	V*β* 2	MPB2D5	PE+FITC
	V*β* 12	VER2.32	FITC
F	V*β* 23	AF23	PE
	V*β* 1	BL37.2	PE+FITC
	V*β* 21.3	IG125	FITC
G	V*β* 11	C21	PE
	V*β* 22	IMMU546	PE+FITC
	V*β* 14	CAS1.1.3	FITC
H	V*β* 13.2	H132	PE
	V*β* 4	WJF24	PE+FITC
	V*β* 7.2	ZIZOU4	FITC

*Nomenclature from Wei et al. [146]

2.2.3. Gene expression assays

Table 2.9. Real-time PCR assays

Gene symbol	Gene name	TaqMan assay ID *
FOXP3	Forkhead box P3	Hs01085834_m1
GATA3	GATA binding protein 3	Hs00231122_m1
IPO8	Importin 8	Hs00183533_m1
RORC	RAR related orphan receptor C	Hs01076122_m1
RPL13A	Ribosomal protein L13a	Hs04194366_g1
TBP	TATA-box binding protein	Hs00427620_m1
TBX21	T-box 21 (T-bet)	Hs00203436_m1

*All purchased from Life Technologies

2.2.4. Buffers and media

Table 2.10. Buffers and media

Name	Content
FACS buffer	0.1% BSA 0.02% NaN_3 in PBS
Cell separation buffer	1% human serum 2 mM EDTA in PBS
Standard medium	5% human serum 100 units/ml penicillin/streptomycin in RPMI with stable L-Glutamine
Freezing medium 1	10% FCS in RPMI with stable L-Glutamine
Freezing medium 2	40% FCS 20% DMSO in RPMI with stable L-Glutamine

2.2.5. Consumables

Table 2.11. Laboratory consumables

Name	Company
C-Chip hemocytometers	NanoEntek
Cell culture plates	Greiner
Cryo tubes	Greiner
Eppendorf safe-lock tubes	Eppendorf
FACS tubes, 5 mL	Sarstedt
FACS tubes, sterile, 5 mL	BD Biosciences
Falcon tubes, 15 and 50 mL	Greiner
Sterican needles and syringes	B.Braun
Pasteur pipettes	Greiner
PCR 96 well plates and sealing tape	Sarstedt
Pipette tips	Sarstedt
Polypropylene bags	Sarstedt
Serological pipettes, 2 - 50 mL	Sarstedt
S-Monovette K3 EDTA tubes, 9 mL	Sarstedt
S-Monovette Serum-Gel tubes, 7.5 mL	Sarstedt
Tissue culture flasks	Sarstedt
Vacuum filter systems, sterile, 500 mL	Corning

2.2.6. Equipment

Table 2.12. Laboratory equipment

Name	Company
ABI Prism 7900 HT Fast Real-Time PCR system	Applied Biosystems
Accu-Jet pipette controllers	Brand
BD FACS LSR II flow cytometer	BD Biosciences
BD IMag cell separation magnets	BD Biosciences
Benchtop timers	Carl Roth
Centrifuges	Heraeus, Eppendorf
Coulter Ac·T Diff hematology analyzer	Beckman Coulter
CX21 Microscope	Olympus
FlexCycler for reverse transcription	AnalytikJena
Freezers, -20°C and -80°C	Liebherr, Sanyo
Freezing containers (Mr. Frosty)	Nalgene
Fridges, 4°C	Liebherr
Heracell 240 CO_2 incubator	Thermo Scientific
LABS-40K liquid nitrogen system	Tec-lab
NanoDrop ND-1000 spectrophotometer	PeqLab
Pipettes	Eppendorf, Gilson
Safe 2020 laminar flow cabinet	Thermo Scientific
Shakers and rotators	IKA, Heidolph, Sarstedt
Sonorex sonicator bath	Bandelin
Thermomixer	Eppendorf
Tube racks	Helma, Nalgene
Vortex mixers	Braun Biotech
Water bath	GFL

2.2.7. Software

Table 2.13. Software

Name	Developer
FACS Diva v8.0.1, acquisition	BD Biosciences
FlowJo v10.0.8, single cell analysis	Tree Star
Graphpad Prism v5.04, scientific graphing	GraphPad Software
LEGENDplex v7.0, immunoassay analysis	Biolegend
SDS v2.4, real-time PCR software	Applied Biosystems
RQ Manager v1.2.1, real-time PCR analysis	Applied Biosystems
SPSS Statistics v19	IBM
VDJtools v1.0.7, immune sequencing data analysis	Shugay et al. [147]

2.3. Methods

2.3.1. Sample collection

Peripheral blood samples from patients and controls were processed in the lab within 2 hours of collection. EDTA-treated samples were kept gently mixed at room temperature until processed for isolation of peripheral blood mononuclear cells (PBMCs). A small amount of whole blood (50 µL) was assayed for total and differential leukocyte counts using a Coulter Ac·T Diff hematology analyzer.

Samples for serum preparation were allowed to clot undisturbed for at least 1 hour at room temperature. The clot was then removed by centrifuging at 1,300 g for 10 min at 20°C. The resulting supernatant containing the serum was immediately aspirated, dispensed into 500 µL aliquots and stored at -80°C until the time of assay.

2.3.2. Cell isolation and cryopreservation

2.3.2.1. Isolation of PBMCs

Isolation of PBMCs from whole blood was accomplished through Ficoll density gradient centrifugation. Peripheral EDTA-anticoagulated blood was diluted 1:1 in room temperature PBS and stratified in 50 mL tubes by carefully layering over 15 mL Ficoll medium per tube. The samples were centrifuged at 863 g for 30 min at 20°C without brake and the supernatant containing diluted plasma was removed. PBMCs were then carefully recovered from the interphase of the gradient and transferred to a new 50 mL tube. After washing with cold PBS at 700 g for 10 min at 4°C, the cell pellet was loosened and washed again with cold PBS at 485 g for 5 min at 4°C. After removing all supernatant, the cells were resuspended in 1 mL of cold freezing medium 1 and leukocytes were counted excluding erythrocytes by incubation with Türk's solution 1:10.

2.3.2.2. Cryopreservation of PBMCs

For cryopreservation of the isolated PBMCs, a final concentration of 10% DMSO was used. To this end, 2×10^7 viable cells per mL in cold freezing medium 1 were resuspended with an equal volume of cold freezing medium 2 (see **Table 2.10** for media composition). The resulting cell suspension (1×10^7 cells per mL) was dispensed in 1 mL aliquots and placed at -80°C in a pre-cooled Mr. Frosty freezing container to achieve gradual cooling (-1°C per minute). Approximately 24 hours later, the cryovials were transferred to a liquid nitrogen tank for long term storage.

For thawing, the cryovials were retrieved from the liquid nitrogen tank, immediately placed on ice and transferred into a 37°C water bath until the medium was nearly liquified. The cryovial contents were slowly resuspended with additional 1 mL cold PBS until complete thawing and subsequently diluted with a 10X excess volume of cold PBS or standard medium. After washing at 485 g for 5 min at 4°C, the pellet was

loosened and resuspended in cold PBS or cell separation buffer. Cell viability was determined by staining 5 µL of thawed PBMCs with 1:10 trypan blue solution.

2.3.2.3. Magnetic separation of $CD4^+$ T cells

Untouched $CD4^+$ T cells were isolated from thawed PBMCs using the BD IMag Human CD4 T lymphocyte Enrichment Set. Cells resuspended in 1 mL cell separation buffer (see **Table 2.10** for buffer composition) were incubated with the biotinylated human CD4 T cell enrichment cocktail (7 µL per 1 × 10^6 PBMCs) for 15 min at room temperature. After washing with a 10X excess volume of cold cell separation buffer at 485 g for 5 min at 4°C, all supernatant was removed and biotin-labeled cells were mixed thoroughly with streptavidin-coated magnetic particles (5 µL per 1 × 10^6 cells). After an incubation period of 20 min at room temperature, cells were resuspended in 1.5 mL cell separation buffer and transferred into a 5 mL sterile tube, which was placed on the BD IMag magnet for 7 min. Using a sterile Pasteur pipette, the negative fraction was carefully aspirated and transferred into a new sterile tube which was placed once more on the magnet for 5 min to increase the purity of the enriched fraction (routinely above 90%; **Fig. 2.1**). For subsequent analyses, twice-enriched fractions were pelleted, snap frozen in liquid nitrogen and stored at -80°C until assayed.

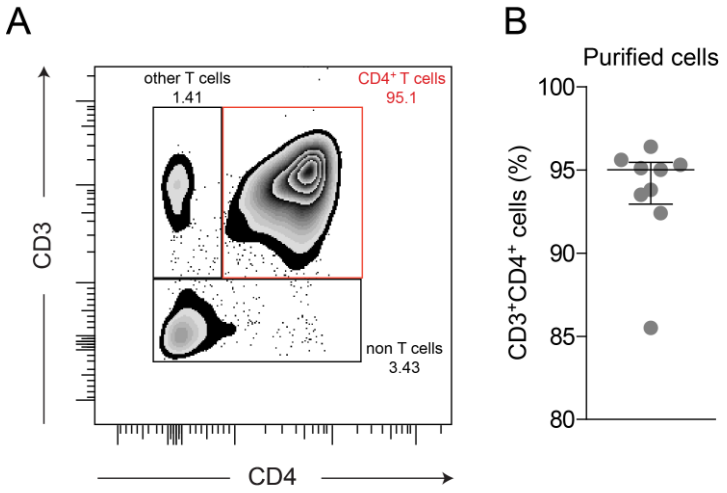


Figure 2.1. Magnetic separation of $CD4^+$ T cells.
(**A**) Single cell suspension was prepared from thawed PBMCs and untouched $CD4^+$ T cells (gate in red) were magnetically separated. Purity of the resulting enriched fraction was determined by flow cytometric analysis of CD3 and CD4 co-expression. Displayed values are percentages of total acquired events following debris exclusion. (**B**) Similar experiments yielded a median purity of 95% $CD4^+$ T cells.

2.3.3. Flow cytometry

In order to control for systematic variation in the reagents used, all procedures described in this section were applied concomitantly to PBMCs from both pre- and post-treatment patients as well as their corresponding controls.

2.3.3.1. Live/dead cell discrimination

To exclude dead cells from further analyses, the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit was used before applying any surface and intracellular stainings. Up to 1×10^6 thawed PBMCs were washed with protein-free cold PBS at 485 g for 5 min at 4°C and then resuspended in 100 µL cold PBS containing the amine-reactive dye in 1:1,000 dilution. After light-protected incubation for 30 min at 4°C, the cells were washed with cold PBS and subsequently stained for surface antigens.

2.3.3.2. Surface staining

Staining of cell surface molecules was achieved by using anti-human fluorescent-labeled monoclonal antibodies pre-tested with PBMCs from healthy donors and at final concentrations optimized by titration (see **Table 2.7**). Up to 1×10^6 PBMCs were transferred to 5 mL FACS tubes, resuspended in 90 µL FACS buffer and incubated with 0.1 µg/µL human IgG for 5-10 min at room temperature to prevent non-specific binding of antibodies to Fc receptors. Surface staining reactions were performed by light-protected incubation with 10 µL of V*β*-specific reagent mixture and/or 10 µL of 10X surface antibody cocktails for 20 min at 4°C. After washing with FACS buffer at 485 g for 5 min at 4°C, PBMCs were either resuspended in 250 µL FACS buffer for acquisition or fixed for intracellular staining.

2.3.3.3. Intracellular staining

In order to stain intracellular CXCR3 protein, fixation and permeabilization of surface-stained PBMCs was required (**Fig. 2.2**). To this end, cells were resuspended in 100 µL fixation buffer and incubated for 20 min at room temperature. Cells were then washed twice with 1X permeabilization wash buffer at 485 g for 5 min at 4°C and serially incubated with 0.1 µg/µL human IgG (5 min, at room temperature) and anti-CXCR3 antibodies for 30 min at room temperature. Cells were again washed twice with 1 mL permeabilization wash buffer and resuspended in 250 µL FACS buffer for acquisition.

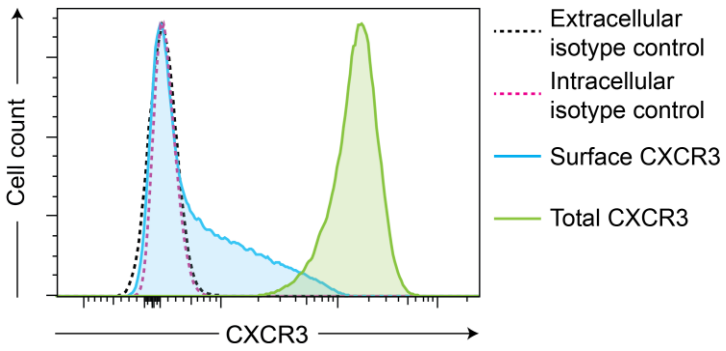


Figure 2.2. T cell surface and intracellular staining of CXCR3.
Cryopreserved PBMCs were thawed and stained as described in sections 2.3.3.1 - 2.3.3.3. Stained PBMCs were gated on live $CD3^+$ lymphocytes. Representative plot shows fluorescence intensity of CXCR3 expression in intact (surface CXCR3; blue-shaded curve) relative to fixed and permeabilized T cells (total cellular CXCR3; green-shaded curve). Isotype-matched negative controls were used at the same concentration before fixation (black-dashed curve) and after fixation-permeabilization (purple-dashed curve) and showed no positive staining for CXCR3.

2.3.3.4. Data acquisition and analysis

Data acquisition was carried out using a BD FACS LSR II flow cytometer and the FACS Diva v8.0.1 operating software. For immunophenotyping and V*β* repertoire assays, at least 100,000 live lymphocytes were acquired from case-control samples during the same session and using the same acquisition settings. For purity control after magnetic $CD4^+$ T cell separation, 25,000 events were usually acquired in the lymphocyte gate. Data analysis and plotting were always performed using FlowJo v10.0.8.

2.3.4. Gating strategy

Identification of major lymphocyte subsets (T cells, B cells and NK cells) and characterization of T cells via surface markers were carried out following the gating strategy shown in **Fig. 2.3.**

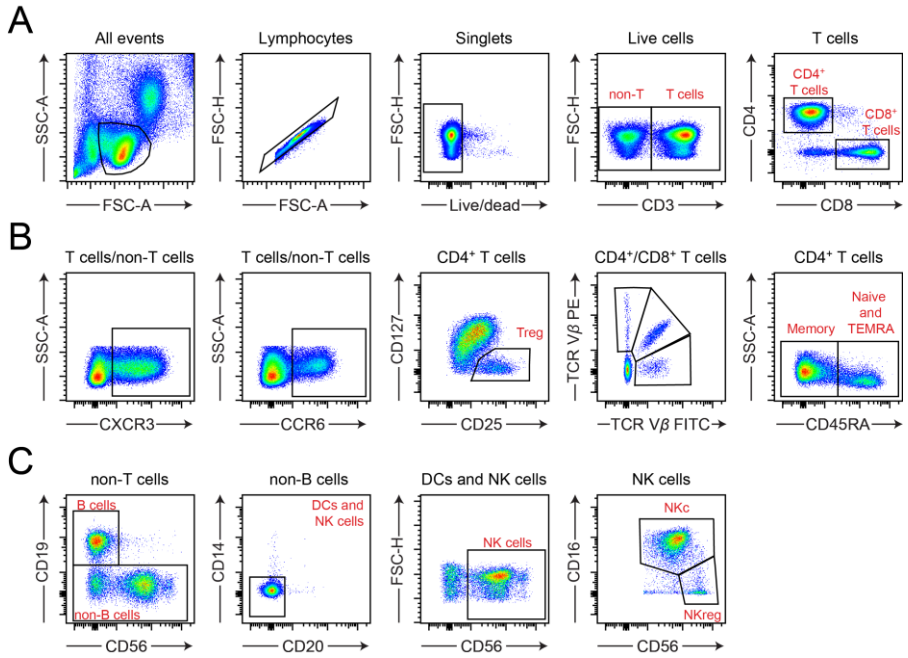


Figure 2.3. Example staining and gating strategy for identification of major lymphocyte subsets and T cell characterization.
Cryopreserved PBMCs from patients and matched controls were thawed and stained as described in sections 2.3.3.1 and 2.3.3.2. (**A**) A common gating was applied across panels allowing for identification of lymphocytes by forward (FSC) and side scatter (SSC), followed by exclusion of doublets (by FSC-height and FSC-area) and dead cells. Singlet live lymphocytes were selected on either CD3 positivity (T cells) or negativity (non-T cells). T cells were discriminated into $CD4^+CD8^-$ and $CD8^+CD4^-$ subsets. (**B**) T cells and non-T cells were further distinguished based on surface expression of CXCR3 and CCR6. Within $CD4^+$ T cells, regulatory T cells (Treg) were identified as $CD127^{low/-}$ while expressing high levels of CD25. The distribution of 24 V*β* families (3 V*β* specificities per tube) was assessed among $CD8^+$ and $CD4^+$ T cells, followed by an exploratory analysis in $CD45RA^-$ (memory) and $CD45RA^+$ (naïve and terminally differentiated effector) $CD4^+$ T cells. (**C**) Among non-T cells, B cells were defined as $CD56^-CD19^+$ and natural killer (NK) cells as $CD19^-CD20^-CD14^-CD56^+$, as suggested by Maecker et al. [148]. Within NK cells, $CD56^{low/-}CD16^+$ cytotoxic (NKc) and $CD56^{high}CD16^-$ regulatory (NKreg) subsets were identified as well. CXCR3: CXC-chemokine receptor type 3; CCR6: CC-chemokine receptor type 6; TCR: T cell receptor; TEMRA: T effector memory cells with reacquired RA; DCs: dendritic cells

2.3.5. RNA isolation, cDNA synthesis and real-time PCR

2.3.5.1. RNA isolation

Total RNA was isolated using the RNeasy Plus Universal Mini Kit according to manufacturer's instructions. Frozen cell pellets from patients and matched controls (approximately 5×10^5 purified $CD4^+$ T cells per pellet) were thoroughly resuspended and homogenized with 900 µL QIAzol lysis reagent. After an incubation of 5 min at room temperature, 100 µL gDNA eliminator solution was added and vigorously mixed with the homogenate to minimize genomic DNA contamination of the aqueous phase. After mixing thoroughly with 180 µL chloroform, the homogenate was left on the benchtop for another 3 min at room temperature and then centrifuged at 12,000 g for 15 min at 4°C. The upper, aqueous phase containing RNA was transferred to a new RNase-free tube and mixed thoroughly with 1 volume of 70% ethanol. Up to 700 µL of the lysate were immediately transferred to an RNeasy Mini spin column and centrifuged at > 8,000 g for 15 sec at 20°C. Column-bound RNA was then serially washed with 700 µL buffer RW1 (> 8,000 g, 15 sec, 20°C), 500 µL buffer RPE (> 8,000 g, 15 sec, 20°C) and once more with 500 µL buffer RPE (> 8,000 g, 2 min, 20°C) and finally eluted (> 8,000 g, 1 min, 20°C) with 30 µL RNase-free water into a new tube. The RNA concentration was photometrically determined using a NanoDrop ND-1000 spectrophotometer. All samples were kept on ice and directly used for cDNA synthesis without freezing.

2.3.5.2. cDNA synthesis

For reverse transcription, isolated RNA templates (250 - 500 ng) were added to 1 µL random hexamer primers in an initial reaction volume of 12 µL. After thermal denaturation at 65°C for 5 min, reaction components were added in the following order: 4 µL 5X reaction buffer, 1 µL RiboLock RNase inhibitor, 2 µL 10 mM dNTP mix and 1 µL RevertAid H minus M-MuLV reverse transcriptase. Samples were mixed gently and centrifuged before primer annealing at 25°C for 5 min. Synthesis of cDNA was then achieved at 42°C for 60 min. The reverse transcription reaction was terminated by heating at 70°C for 5 min and the products were stored at -20°C for less than a week.

2.3.5.3. Real-time PCR

Quantitative gene expression analyses were performed using predesigned 20X TaqMan gene expression assays (listed in **Table 2.9**) and an ABI Prism 7900 HT Fast Real-Time PCR system. Reactions were performed in triplicates in a total volume of 20 µL containing 1 µL cDNA template, 10 µL TaqMan gene expression mastermix, 8 µL HPLC-grade water and 1 µL TaqMan assay. Thermal cycling conditions included two initial incubation steps at 50°C for 2 min and 95°C for 10 min, respectively, followed by 40 PCR cycles of 15 sec denaturation at 95°C and 1 min primer annealing and extension at 60°C. Data analysis was performed using the SDS v2.4 and the RQ Manager v1.2.1 software. The expression levels of the genes of interest were calculated as $2^{-\Delta Ct}$ relative to the geometric mean expression of three

housekeeping genes (*IPO8*, *TBP*, *RPL13A*) which have been previously shown to be stably expressed in primary human T cells [149].

2.3.6. T cell receptor CDR3 sequencing

Total genomic DNA was isolated from negatively purified $CD4^+$ T cells of five matched HLA-$A2^+$ case-control pairs (n = 10) using the DNeasy Blood and Tissue Kit according to manufacturer's instructions. Anonymized DNA samples were outsourced to Adaptive Biotechnologies and sequencing of the CDR3 region of the TCR β chain was performed using the TCRB survey level assay [150]. Raw sequence data were pre-processed by Adaptive Biotechnologies for PCR and sequencing error correction [150] and uploaded into the Immuno-SEQ Analyzer. Post-analysis was conducted using VDJtools [147].

2.3.7. Immunoassays for serum CXCR3L

Quantification of the CXCR3 ligands CXCL10 and CXCL11 in sera of patients and controls was accomplished with the multiplexed bead-based immunoassay LEGENDplex according to manufacturer's instructions. Briefly, capture beads specific for CXCL10 and CXCL11, differentiated by size and internal APC fluorescence intensity, were incubated with 1:1 diluted sera from pre- and post-treatment patients as well as their matched controls. After washing, biotinylated detection antibodies specific for CXCL10 and CXCL11 were added, thus forming capture bead-chemokine-detection antibody sandwiches. PE-conjugated streptavidin was then added, providing signal intensities proportional to the amount of bound CXCL10 and CXCL11. The concentration of these chemokines was estimated based on a standard curve generated in the same assay. Values for CXCL10 and CXCL11 that were below the detection limit (5.7% and 17.3% of all resulting values, respectively) were set equal to the detection limit. For data acquisition and analysis, a BD FACS LSR II flow cytometer and the LEGENDplex v7.0 data analysis software were used, respectively.

2.3.8. Statistical analyses

All continuous variables are presented as median values with interquartile range, unless otherwise specified. Differences between patients at baseline and matched controls as well as between repeated (pre- and post-treatment) measurements were tested for statistical significance using the paired Wilcoxon signed-rank test. Due to sample size limitations, repeated measurement analyses stratified by type of medication were restricted mainly to patients treated with escitalopram. For dichotomous variables, the McNemar's test was used. Bivariate correlation analyses were conducted using Spearman's rank correlation test. Response to treatment was defined *a priori* as a ≥ 50% reduction in baseline total score derived from each rating scale (IDS-SR_{30}, IDS-C_{30} and $HRSD_{17}$). Cohen's kappa was used to evaluate the degree of agreement among the three instruments in identifying treatment responders [151]. All statistical analyses were performed using SPSS version 19. A two-tailed $p < .05$ was considered significant and $p < .10$ was considered a trend.

3. RESULTS

3.1. Baseline and clinical characteristics

3.1.1. Case-control component

Baseline demographics, lifestyle factors and clinical characteristics for the case-control component of the study are given in **Table 3.1**. On average, there were no differences in terms of sex representation, age, body mass index and current smoking status (yes/no) between unmedicated MDD patients and matched non-depressed controls. As expected, the two groups differed significantly only in severity of depressive symptoms, with a median total QIDS-SR_{16} score of 18.5 in the patient group indicating severe depression and a median score of 2 in the control group indicating no depression [145].

Table 3.1. Baseline characteristics (*n* = 40)

Characteristic	MDD (*n* = 20)	CTR (*n* = 20)	*p*-value*
% Females (*n*)	55 (11)	55 (11)	> .99
Age (years), median (IQR)	36.5 (30.5-44)	37 (31-46)	.28
BMI, median (IQR)	24.1 (20.7-27.8)	24.4 (22.8-27.1)	.37
% currently smoking (*n*)	35 (7)	35 (7)	> .99
QIDS-SR_{16}, median (IQR)	18.5 (16.5-21)	2 (1-3.5)	< .001

MDD: major depressive disorder; CTR: non-depressed controls; BMI: body mass index; IQR: interquartile range; QIDS-SR_{16}: Quick Inventory of Depressive Symptoms-Self Report, 16 item

* Based on the McNemar's test for dichotomous variables and the Wilcoxon signed-rank test for continuous variables

3.1.2. Longitudinal component

Overall, there was a significant reduction in depression severity over 5 weeks of antidepressant treatment, regardless of the rating scale used (**Table 3.2**). Pre- and post-treatment total scores indicated a median reduction from severe to moderate or mild depression. Similar results were obtained in subgroup analyses restricted to patients treated with escitalopram (**Table 3.2**).

Response to treatment was defined as a pre-treatment to post-treatment improvement of at least 50% on any of the depression severity scales shown in Table 3.2. Overall, thirteen patients (86.7% of the post-treatment sample; escitalopram-treated, *n* = 9; mirtazapine-treated, *n* = 4) responded to treatment according to the $HRSD_{17}$, whereas nine (60%; escitalopram-treated, *n* = 6; mirtazapine-treated, *n* = 3) and eight patients (53.3%; escitalopram-treated, *n* = 5; mirtazapine-treated, *n* = 3) were identified as treatment responders using the IDS-C_{30} and the IDS-SR_{30}, respectively. Kappa statistics showed only a fair agreement between the $HRSD_{17}$ and the IDS scales (IDS-C_{30}: kappa = .375, *p* = .063; IDS-SR_{30}: kappa = .299, *p* = .104), while there was a substantial to excellent agreement between the clinician-rated and

the self-rated IDS in identifying treatment responders (kappa = .865, p = .001). Similar results were obtained in subgroup analyses restricted to patients treated with escitalopram.

Table 3.2. Total scores of depression severity prior to and following 5 weeks of antidepressant pharmacotherapy (n = 15)

Rating scale	Pre-treatment	Post-treatment	p-value*
IDS-SR$_{30}$, median (IQR)			
Pooled treated	44 (39-49)	27 (16-38)	.001
Escitalopram-treated	45 (41.25-57.25)	27.5 (15.75-35)	.005
IDS-C$_{30}$, median (IQR)			
Pooled treated	44 (38-46)	19 (13-27)	.001
Escitalopram-treated	43.5 (37.75-45.75)	20 (14.5-24.75)	.005
HRSD$_{17}$, median (IQR)			
Pooled treated	20 (19-24)	8 (6-9)	.001
Escitalopram-treated	20 (19-24)	8 (6-9.25)	.005

IDS-SR$_{30}$: Inventory of Depressive Symptoms-Self Report, 30 item; IDS-C$_{30}$: Inventory of Depressive Symptoms-Clinician rated, 30 item; HRSD$_{17}$: Hamilton Rating Scale for Depression, 17 item; IQR: interquartile range

* Based on the Wilcoxon signed-rank test. Due to sample size limitations, subgroup analyses stratified by type of medication were restricted to patients treated with escitalopram (n = 10).

3.2. Circulating leukocyte subsets

Absolute leukocyte counts in whole blood were obtained using a Coulter cell counter. There was no difference in the number of total leukocytes between MDD patients at baseline (median [IQR]; 6.15 [5.02-8.35] × 10^3/μL) and non-depressed controls (6.0 [4.52-7.45] × 10^3/μL; p = .71). Similarly, no differences emerged between the two groups when comparing absolute counts of granulocytes, monocytes and lymphocytes in whole blood (all p > .35; **Fig. 3.1A**). Further analyses within lymphocytes using flow cytometry revealed no significant differences in the frequency of T cells (p = .147) and B cells (p = .50; **Fig. 3.1B**). In addition, no differences were observed between the two groups either for the $CD4^+$ (p = .17) or the $CD8^+$ T cell subset (p = .95; **Fig. 3.1C**). However, Treg cells displayed significantly higher frequency in MDD patients (p = .023; **Fig. 3.1C**)**.** By contrast, and in agreement with previous meta-analytic data [21, 22], natural killer (NK) cells showed a trend towards lower frequency in MDD patients (p = .062; **Fig. 3.1B**). Following up this finding, a significantly lower percentage of NK cells with putative immunoregulatory properties was observed in MDD (p = .018). Differences in the frequency of cytotoxic NK cells did not reach statistical significance (p = .100; **Fig. 3.1D**).

None of the abovementioned leukocyte populations varied significantly following antidepressant treatment, either within pooled treated (n = 15; all p > .15), escitalopram-treated (n = 10; all p > .24) or mirtazapine-treated patients (n = 4; all p > .27).

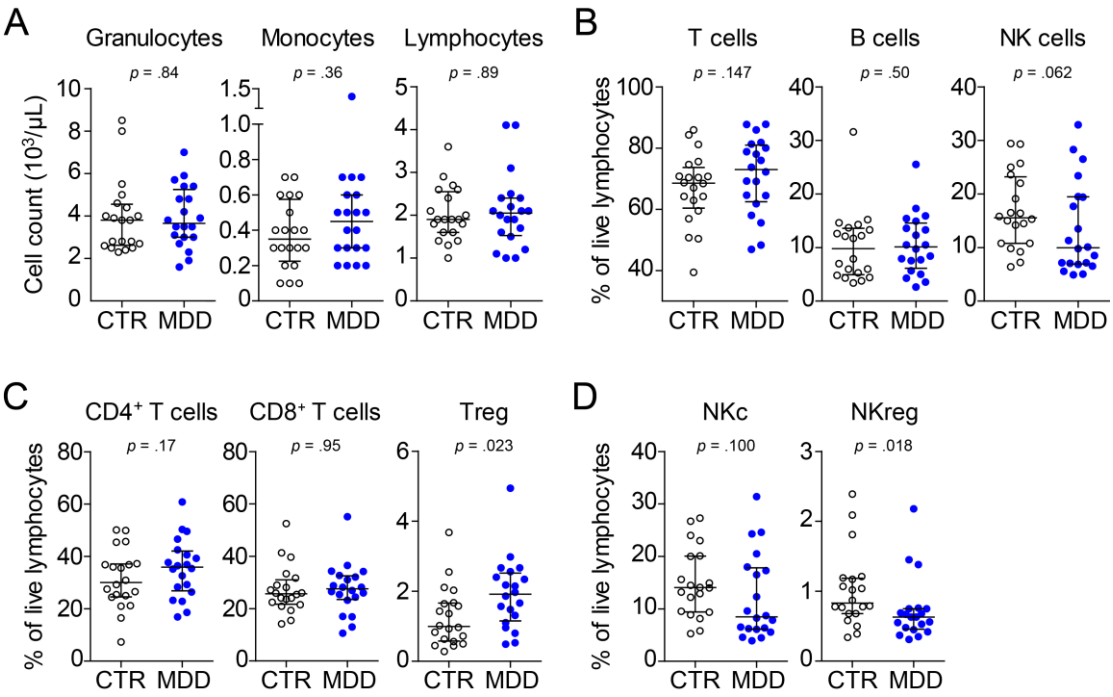


Figure 3.1. Peripheral blood counts and frequencies of major leukocyte subsets.
(**A**) Absolute peripheral blood granulocyte, monocyte and lymphocyte counts were obtained from major depressive disorder patients (MDD; *n* = 20; blue) and matched non-depressed controls (CTR; *n* = 20; white) using a Coulter Ac·T Diff hematology analyzer. (**B**) Frequencies of total $CD3^+$ T cells, $CD3^-CD56^-CD19^+$ B cells and $CD3^-CD19^-$ $CD20^-CD14^-CD56^+$ natural killer (NK) cells were obtained by flow cytometric analysis of thawed PBMCs. (**C**) T cells were discriminated into $CD4^+CD8^-$ and $CD8^+CD4^-$ subsets. Within $CD4^+$ T cells, $CD25^{high}CD127^{low/-}$ regulatory T cells (Treg) were quantified. (**D**) Within NK cells, $CD56^{low/-}CD16^+$ cytotoxic (NKc) cells and $CD56^{high}CD16^-$ regulatory (NKreg) cells were also identified. Graphs depict medians with interquartile ranges. For all comparisons, the Wilcoxon signed-rank test was used.

3.3. Chemokine receptor expression in T cells

3.3.1. Surface expression of CXCR3 and CCR6

3.3.1.1. Case-control analyses

To identify possible trafficking defects in peripheral blood T cells of MDD patients, the surface expression of the T helper-associated chemokine receptors CXCR3 and CCR6 was analyzed in PBMCs from unmedicated MDD patients and matched non-depressed controls [148, 152]. As shown in **Fig. 3.2A**, the percentage of CXCR3-expressing T cells was found to be substantially lower in MDD patients (21.5% of live $CD3^+$ lymphocytes) compared to their non-depressed counterparts (44.75%; p = .001). In particular, seventeen patients (85% of the patient sample) displayed lower frequency of $CXCR3^+$ T cells compared to their individually matched controls. This finding was equally striking in both $CD4^+$ (p = .001) and $CD8^+$ T cells (p = .002; **Fig. 3.2B**). However, it was not T cell-specific, as the percentage of CXCR3-expressing $CD3^-$ lymphocytes was also found to be significantly lower in MDD patients (p = .001; data not shown).

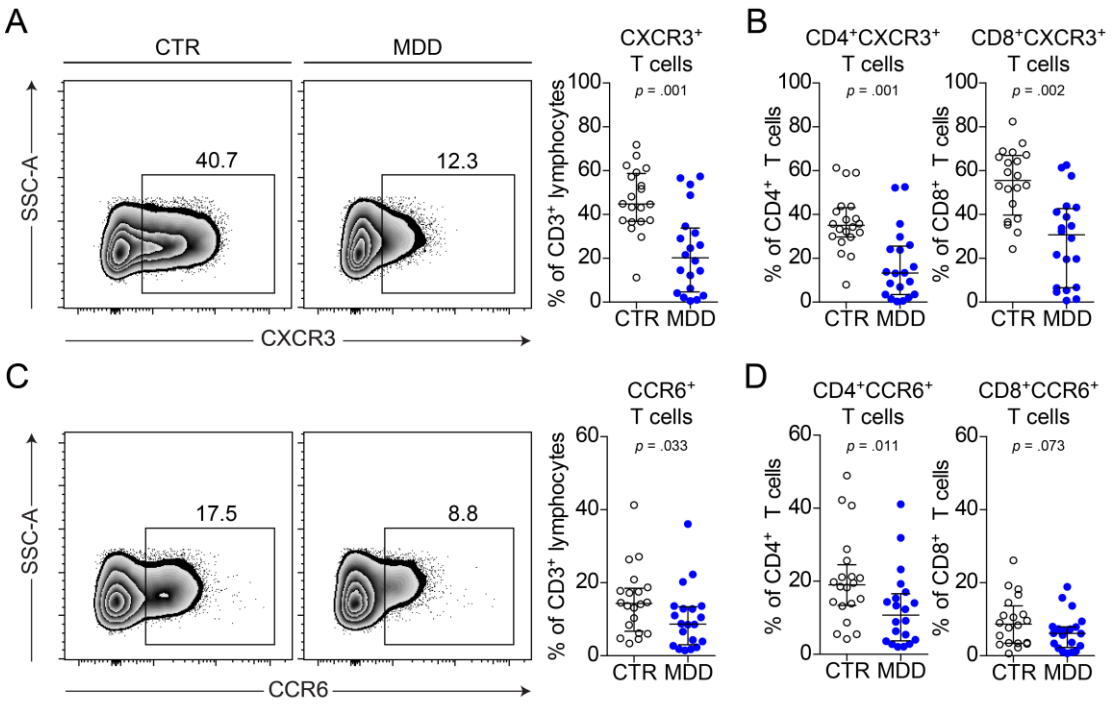


Figure 3.2. Lower surface expression of CXCR3 and CCR6 on T cells from MDD patients.
(**A**) CXCR3-expressing T cells were identified by flow cytometric analysis of PBMCs from major depressive disorder patients (MDD; *n* = 20; blue) and matched non-depressed controls (CTR; *n* = 20; white). Displayed values (*plots on left*) are frequencies of $CXCR3^+$ T cells expressed as a percentage of live $CD3^+$ lymphocytes from a representative case-control pair. (**B**) Percentages of CXCR3-expressing $CD4^+$ and $CD8^+$ T cells were also quantified. (**C**) Similar analyses were conducted for the surface expression of CCR6 on total T cells as well as on the $CD4^+$ and $CD8^+$ T cell subsets (**D**). Graphs depict medians with interquartile ranges. For all comparisons, the Wilcoxon signed-rank test was used. SSC-A: Side scatter-area

When T cell surface expression of CCR6 was comparatively analyzed in patients and controls, a significantly lower percentage of CCR6-expressing T cells was also observed in MDD patients (p = .033; **Fig. 3.2C**). Further analyses within T cells revealed a significantly lower percentage of CCR6-expressing $CD4^+$ T cells (p = .011) and a statistical trend towards a lower percentage of CCR6-expressing $CD8^+$ T cells in MDD patients (p = .073; **Fig. 3.2D**). Unlike CXCR3, lower surface expression of CCR6 was T cell-specific, as there was no difference in the frequency of CCR6-expressing $CD3^-$ lymphocytes between patients and controls (p = .60; data not shown).

3.3.1.2. Longitudinal analyses

To answer the question whether lower T cell surface expression of CXCR3 and/or CCR6 indicates a trait- or state-dependent immunological characteristic of MDD, the expression levels of these chemokine receptors were compared longitudinally, namely prior to and following 5 weeks of antidepressant treatment.

Regarding surface expression of CCR6, there was no significant change in the percentage of CCR6-expressing T cells with antidepressant treatment, as shown by either a pooled treatment analysis (n = 15, p = .92) or an exploratory subgroup analysis in escitalopram-treated patients (n = 10, p = .31). A statistical trend towards

a decrease in the percentage of CCR6-expressing T cells was however observed in the very small subgroup of mirtazapine-treated patients (n = 4, p = .068; data not shown).

Similarly, a pooled treatment analysis showed that use of antidepressants was not associated with variation in T cell surface expression of CXCR3 (p = .91; **Fig. 3.3A**). However, exploratory subgroup analyses stratified by type of medication suggested opposite effects of treatment on surface CXCR3 expression between patients treated with escitalopram and those treated with mirtazapine. Specifically, the percentage of CXCR3-expressing T cells tended towards an increase in escitalopram-treated patients (p = .114; **Fig. 3.3B**), a finding that reached a statistical trend in both the $CD4^+$ and $CD8^+$ T cell subsets (both p = .093; data not shown). By contrast, T cell surface expression of CXCR3 showed a statistical trend towards a decrease in mirtazapine-treated patients (p = .068; **Fig. 3.3C**), an effect that was equally evident in $CD4^+$ and $CD8^+$ T cells (both p = .068; data not shown).

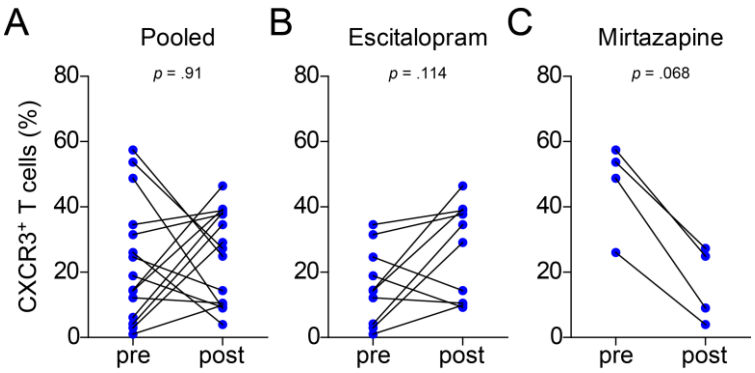


Figure 3.3. Opposite effects of treatment with escitalopram and mirtazapine on T cell surface expression of CXCR3.
CXCR3-expressing T cells, expressed as a percentage of $CD3^+$ live lymphocytes, were identified by flow cytometric analysis of PBMCs from major depressive disorder patients prior to (*pre*) and following (*post*) monopharmacotherapy with either escitalopram (n = 10), mirtazapine (n = 4) or venlafaxine (n = 1) over 5 weeks. Results of a pooled analysis (**A**) as well as of exploratory subgroup analyses stratified by type of medication are shown (**B**, **C**). For all comparisons, the Wilcoxon signed-rank test was used.

In view of the high pre- to post-treatment variation in surface CXCR3 expression within the escitalopram-treated subgroup (**Fig. 3.3B** and **3.4A**), it was next sought to explore whether this variation could be explained by the degree of clinical response to escitalopram. Indeed, on one extreme, a representative treatment non-responder (a patient who had a pre- to post-treatment improvement < 50% on the $IDS\text{-}SR_{30}$ total score) showed a decrease of 32.8% in surface CXCR3 expression over 5 weeks of escitalopram treatment (**Fig. 3.4A**, left histogram). On the other extreme, a representative treatment responder (a patient who had a pre- to post-treatment improvement > 50% on the $IDS\text{-}SR_{30}$ total score) showed a substantial increase (83.9%) of surface CXCR3 expression over 5 weeks of escitalopram treatment **(Fig. 3.4A**, right histogram). Overall, a significant negative correlation between the percent change on the $IDS\text{-}SR_{30}$ score and the percent change in CXCR3 median fluorescence intensity levels was observed in T cells of escitalopram-treated MDD patients (Spearman's rho = −.721, p = .019; **Fig. 3.4B**). A statistical trend towards a

similar but somewhat weaker correlation was observed for the clinician-rated IDS (rho = −.600, p = .067). However, no significance was reached for the $HRSD_{17}$ (rho = −.462, p = .18).

Taken together, these preliminary results suggested that lower T cell surface expression of CXCR3 may be a state-dependent characteristic of MDD.

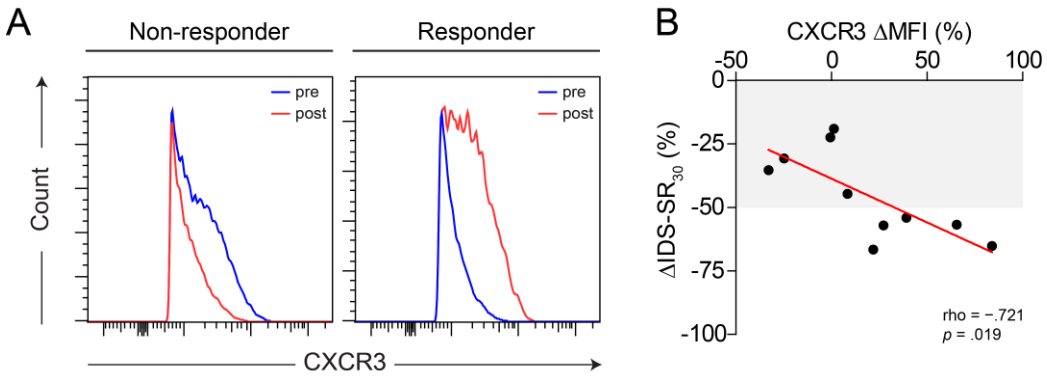


Figure 3.4. T cell surface expression of CXCR3 co-varies with the degree of clinical response to escitalopram treatment as assessed by the IDS.
(**A**) Surface CXCR3 MFI levels were measured by flow cytometric analysis of T cells from major depressive disorder patients prior to (*pre*) and following (*post*) monopharmacotherapy with escitalopram. Changes in T cell surface expression of CXCR3 in one representative treatment non-responder (*left histogram*) and one treatment responder (*right histogram*) are depicted. (**B**) The correlation between the percent change in surface expression of CXCR3 on T cells of escitalopram-treated patients and the concomitant percent change in baseline scores on the $IDS\text{-}SR_{30}$ is plotted (n = 10). The gray-shaded area represents the range of clinical non-response to 5 weeks of escitalopram treatment as assessed by the $IDS\text{-}SR_{30}$. Percent changes were calculated based on post-treatment minus pre-treatment differences divided by the respective pre-treatment value. MFI: median fluorescence intensity; $IDS\text{-}SR_{30}$: Inventory of Depressive Symptoms-Self Report, 30 item.

3.3.2. Intracellular staining of CXCR3

Given the significantly lower pan-lymphocyte surface expression of CXCR3 in antidepressant-free MDD patients as well as the association between T cell surface CXCR3 recovery and concomitant response to escitalopram treatment, it was next asked whether the phenomenon of lower surface expression of CXCR3 is associated with impaired CXCR3 protein output in lymphocytes of untreated MDD patients. To this end, the fluorescence intensity of total CXCR3 expression was comparatively analyzed in permeabilized PBMCs of MDD patients and matched controls (case-control analyses, n = 36; longitudinal analyses, n = 13).

Despite lower expression at the cell surface, CXCR3 protein was abundantly present intracellularly in all examined PBMC populations derived from MDD patients. In particular, median fluorescence intensities of total cellular CXCR3 expression were not significantly different between patients and matched controls, either in total $CD3^+$ lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells or $CD3^-$ lymphocytes (all p > .15; **Fig. 3.5**). In addition, total CXCR3 expression neither varied significantly following treatment with escitalopram (p = .21 for all tested populations) nor co-varied with the degree of clinical response over the same period (all p > .60; data not shown).

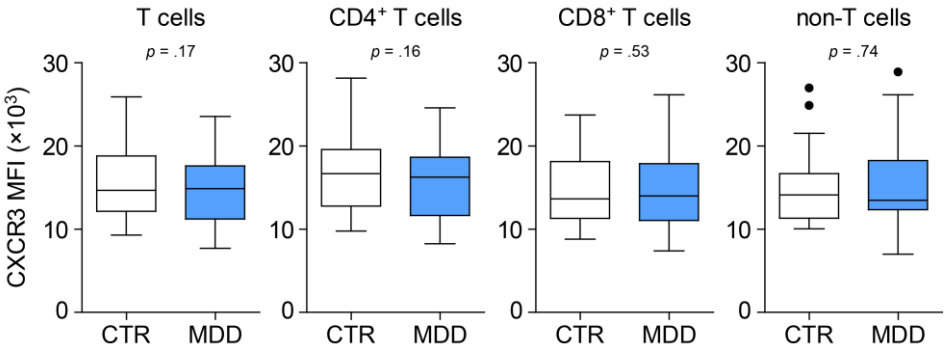


Figure 3.5. Intracellular CXCR3 protein levels.
Total cellular CXCR3 MFI levels were measured by flow cytometric analysis of fixed and permeabilized PBMCs from major depressive disorder patients (MDD; *n* = 18; blue) and matched non-depressed controls (CTR; *n* = 18; white). Stained PBMCs were gated on live $CD3^+$ lymphocytes (T cells), $CD4^+$ and $CD8^+$ T cell subsets as well as $CD3^-$ lymphocytes (non-T cells). Graphs depict Tukey boxplots. For all comparisons, the Wilcoxon signed-rank test was used. MFI: median fluorescence intensity

Taken together, these results suggested that lower intracellular amounts of CXCR3 protein cannot account for lower cell surface expression of the receptor in untreated MDD patients. Attention was therefore shifted towards potential mechanisms of increased receptor internalization [153].

3.3.3. Quantification of serum CXCR3L

To address the hypothesis that enhanced receptor endocytosis is responsible for lower T cell surface expression of CXCR3, the abundance of the CXCR3 ligands CXCL10 and CXCL11 was assessed in sera of patients and controls (case-control analyses, *n* = 38; longitudinal analyses, *n* = 14). These two chemokines were previously shown to be the most efficacious in inducing down-regulation of cell surface CXCR3 in human PBMCs [154, 155].

As shown in **Fig. 3.6** and in agreement with a previous study [112], a statistical trend towards higher serum CXCL10 protein levels was observed in antidepressant-free MDD patients (median levels: 89.23 pg/mL) compared to matched non-depressed controls (median levels: 49.78 pg/mL; $p = .091$). Differences regarding serum CXCL11 levels did not reach statistical significance ($p = .136$).

There was no correlation found between serum CXCR3L levels and median fluorescence intensity of surface CXCR3 expression in MDD patients at baseline (all $p > .55$). However, changes in serum CXCL10 levels co-varied negatively with changes in CXCR3 median fluorescence intensity levels in T cells of MDD patients during treatment with escitalopram (Spearman's rho = −.683, $p = .042$ for CXCL10; rho = −.533, $p = .139$ for CXCL11).

Thus, down-regulation of cell surface CXCR3 in T cells of untreated MDD patients could be accounted for, at least in part, by elevated levels of CXCL10 in the extracellular space.

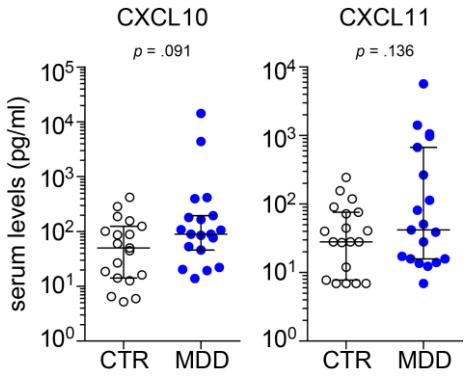


Figure 3.6. Higher serum CXCL10 protein levels in MDD patients.
The CXCR3 ligands CXCL10 and CXCL11 were quantified in sera of unmedicated major depressive disorder patients (MDD; *n* = 19; blue) and matched non-depressed controls (CTR; *n* = 19; white) using a cytometric bead array. Graphs depict medians with interquartile ranges. For both comparisons, the Wilcoxon signed-rank test was used.

3.4. Regulatory T cells

3.4.1. Case-control analyses

In the current study, regulatory T cells (Treg) were identified based on the expression patterns of certain surface molecules on $CD4^+$ T cells, namely high expression of CD25 (IL-2Rα) and low expression or negativity for CD127 (IL-7Rα) (**Fig. 3.7A**), as previously suggested [156-158].

A significantly higher frequency of circulating $CD4^+CD25^{high}CD127^{low/-}$ Treg was detected in untreated MDD patients compared to their non-depressed counterparts (*p*=.023; **Fig. 3.1C** and *p*=.048; **Fig. 3.7B**). In particular, fifteen patients (75% of the patient sample) displayed higher Treg frequency compared to their individually matched controls. To confirm this finding, the mRNA expression levels of the canonical Treg marker *FOXP3* were next assessed in negatively purified $CD4^+$ T cells, along with three signature transcription factors (*TBX21*, *GATA3*, *RORC*) associated with effector T helper subsets. These analyses were carried out in a subsample of MDD patients and controls where higher frequency of Treg occurred in nine out of 10 matched case-control pairs (*n* = 20, *p* = .007).

In accordance with the findings of flow cytometry, significantly higher mRNA expression of *FOXP3* was observed in $CD4^+$ T cells of MDD patients compared to controls (*p* = .007; **Fig. 3.7C**). In particular, overall expression levels of the gene *FOXP3* in purified $CD4^+$ T cells correlated positively with the frequency of Treg as assessed by flow cytometry in both patients and controls (**Fig. 3.7D**). By contrast, there were no significant differences in the expression levels of the T helper type 1-associated gene *TBX21* (*p* = .20), the T helper type 2-associated gene *GATA3* (*p* = .21) and the T helper type 17-associated gene *RORC* (*p* = .96) (**Fig. 3.7C**).

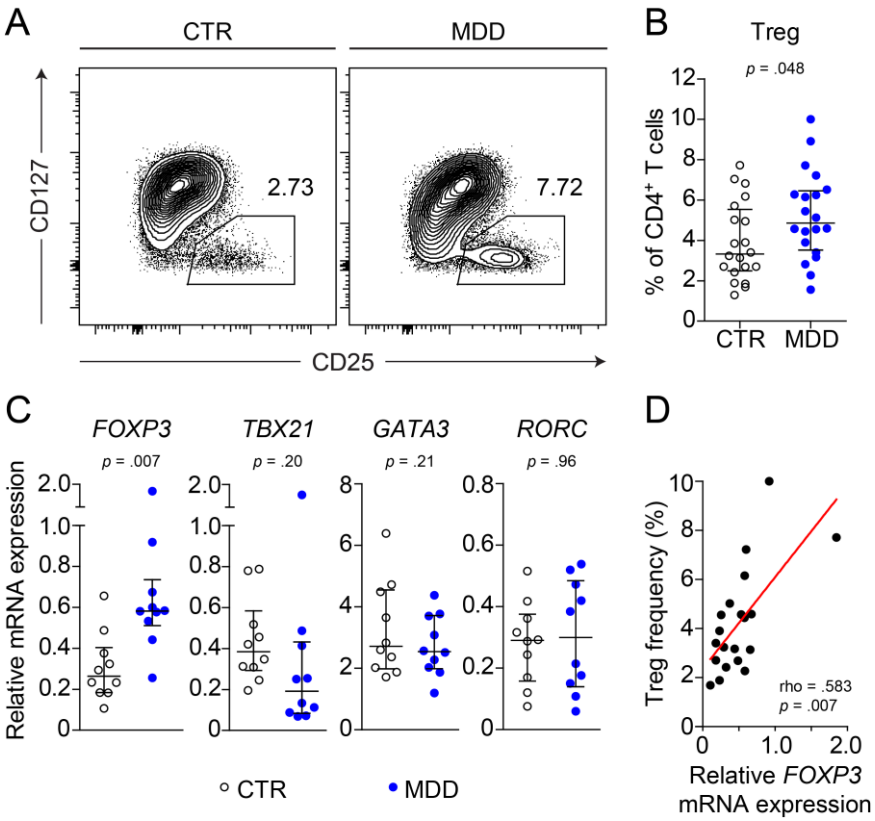


Figure 3.7. Higher Treg frequency and *FOXP3* expression in CD4$^+$ T cells from MDD patients. (**A**) Regulatory T cells (Treg) were identified by flow cytometric analysis of PBMCs from major depressive disorder patients (MDD) and matched non-depressed controls (CTR). Displayed values are frequencies of Treg expressed as a percentage of live CD4$^+$ T cells from a representative case-control pair. (**B**) Differences in frequency of Treg between MDD patients (*n* = 20; blue) and controls (*n* = 20; white) are depicted. (**C**) Untouched CD4$^+$ T cells were magnetically isolated from a subsample of patients and matched controls (*n* = 20) and analyzed for mRNA expression of the T helper-associated transcription factors Forkhead box P3 (*FOXP3*), T-box 21 (*TBX21* or *T-bet*), GATA binding protein 3 (*GATA3*) and RAR related orphan receptor C (*RORC*), respectively. Expression was normalized to the geometric mean expression of three housekeeping genes (*IPO8*, *TBP*, *RPL13A*). (**D**) The correlation between the expression levels of the gene *FOXP3* in purified CD4$^+$ T cells and the frequency of Treg expressed as a percentage of CD4$^+$ T cells is plotted (*n* = 20). Graphs depict medians with interquartile ranges. For all comparisons, the Wilcoxon signed-rank test was used.

3.4.2. Longitudinal analyses

Longitudinal data on the frequency of Treg were available only for the subgroup of patients treated with escitalopram (*n* = 10). No significant change in Treg frequency was observed (*p* = .51; **Fig. 3.8A**).

It was next sought to investigate whether changes in Treg frequencies would correlate with the degree of clinical response to escitalopram. Indeed, a significant positive correlation was observed between the percent change on the $HRSD_{17}$ score over 5 weeks of escitalopram treatment and the percent change in Treg frequency over the same period (Spearman's rho = .699, *p* = .024; **Fig. 3.8B**). However, no significant correlations were detected for the treatment response as assessed by the IDS-C_{30} (rho = .321, *p* = .36) and the IDS-SR_{30} (rho = .358, *p* = .31).

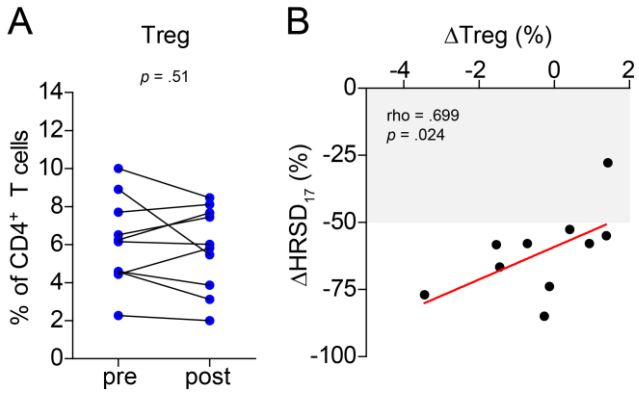


Figure 3.8. Treg frequency co-varies with the degree of clinical response to escitalopram treatment as assessed by the $HRSD_{17}$.
(**A**) $CD4^{+}CD25^{high}CD127^{low/-}$ Treg were identified by flow cytometric analysis of PBMCs from major depressive disorder patients prior to (*pre*) and following (*post*) monopharmacotherapy with escitalopram over 5 weeks (*n* = 10). (**B**) The correlation between the percent change in Treg frequency during treatment with escitalopram and the percent change in baseline scores on the $HRSD_{17}$ over the same period is plotted. The gray-shaded area represents the range of clinical non-response to 5 weeks of escitalopram treatment which was defined as a pre- to post-treatment improvement < 50% on the $HRSD_{17}$ score. $HRSD_{17}$: Hamilton Rating Scale for Depression, 17 item

3.5. T cell receptor V*β* repertoire

In the current study, the proportional TCR V*β* usage was measured in the $CD4^{+}$ and $CD8^{+}$ T cell subsets of MDD patients and matched controls (*n* = 40) by means of monoclonal antibodies specific for 24 human TCR V*β* segments (listed in **Table 2.8**). In order to have a direct measure of TCR V*β* distribution among $CD4^{+}$ and $CD8^{+}$ T cells, the Gini-TCR skewing index was next applied to the flow cytometric V*β* repertoire analysis [159].

Despite between-group variation in the usage of specific V*β* families, no significant skewing of the TCR V*β* repertoire was observed among $CD8^{+}$ T cells of untreated MDD patients compared to their matched controls (*p* = .36; **Fig. 3.9A**). On the other hand, a statistical trend was observed towards higher Gini-TCR skewing index values in the $CD4^{+}$ T cell subset of MDD patients (*p* = .057; **Fig. 3.9B**). In particular, fifteen patients (75% of the patient sample) displayed higher Gini-TCR skewing index values compared to their individually matched controls. Post hoc exploratory pairwise comparisons revealed higher frequency of usage of the families V*β*5.1 (*p* = .003) and V*β*22 (*p* = .040) as well as lower frequency of usage of the family V*β*11 (*p* = .015) among $CD4^{+}$ T cells of MDD patients. A statistical trend towards expanded usage of V*β*5.1 in MDD remained following Bonferroni correction for multiple comparisons (*p*=.072; **Fig. 3.9B**).

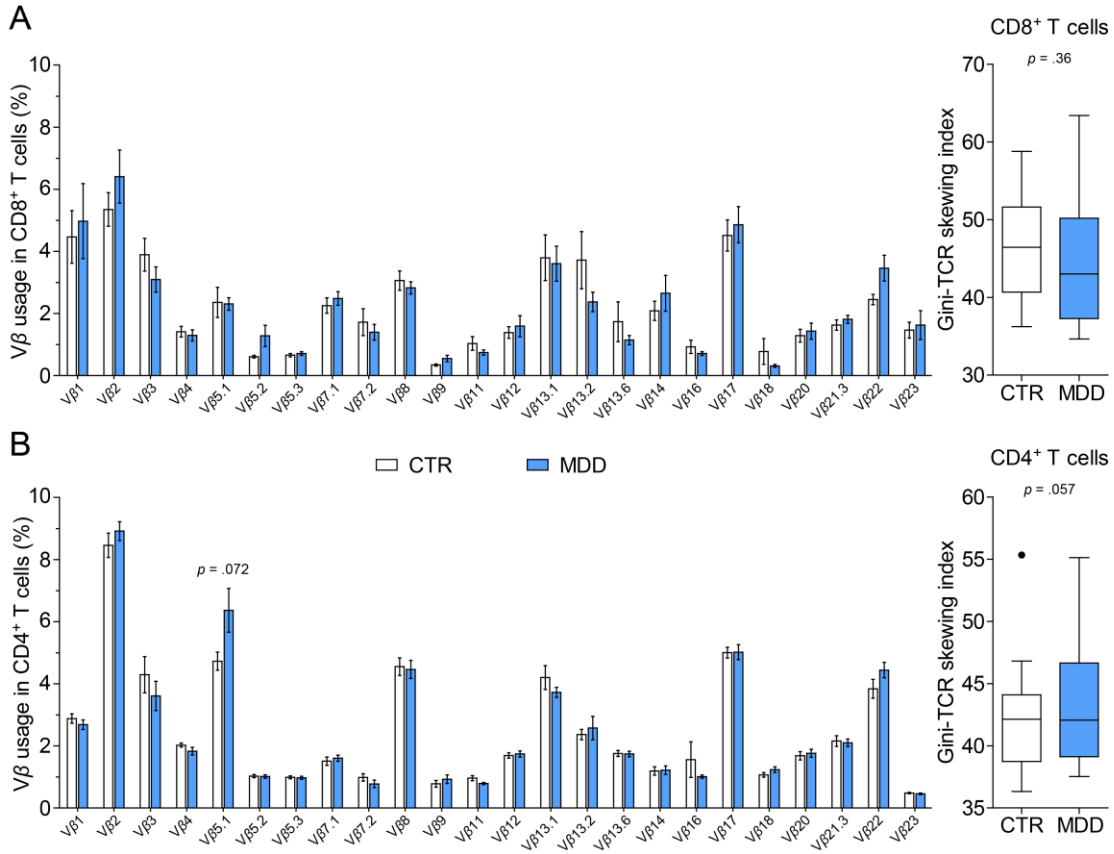


Figure 3.9. Skewing of the TCR V*β* repertoire among CD4$^+$ T cells from MDD patients.
(**A**) T cell receptor (TCR) variable *β* chain (V*β*) family distribution analysis was performed by means of flow cytometric interrogation of CD8$^+$ T cells from major depressive disorder patients (MDD; *n* = 20; blue bars) and matched non-depressed controls (CTR; *n* = 20; white bars). The resulting clonogram represents mean percentages ± s.e.m. of expression of 24 TCR V*β* families. The Gini-TCR skewing index was next applied to the flow cytometric V*β* repertoire analysis (*Tukey boxplots on right*). (**B**) Similar analysis was conducted in CD4$^+$ T cells from MDD patients and controls. Post hoc exploratory comparisons revealed proportional dominance of the family V*β*5.1 (the Bonferroni-corrected *p*-value is displayed). For all comparisons, the Wilcoxon signed-rank test was used.

To answer the question which subsets might be responsible for the observed TCR V*β* repertoire skewing in CD4$^+$ T cells of MDD patients, the Gini-TCR skewing index values were next calculated in the CD4$^+$CD45RA$^-$ (memory) and CD4$^+$CD45RA$^+$ (naïve and/or terminally differentiated effector) subsets of patients and controls. A statistical trend was noticed towards higher Gini-TCR skewing index values in the CD4$^+$CD45RA$^-$ T cell subset of MDD patients (p = .089), whereas no significant skewing of the TCR V*β* repertoire was found among CD4$^+$CD45RA$^+$ T cells (p = .41; **Fig. 3.10**).

Taken together, these results suggested a less evenly distributed TCR V*β* repertoire among CD4$^+$ T cells of untreated MDD patients, which could be accounted for, at least in part, by a skewing of the TCR V*β* repertoire among memory CD4$^+$ T cells.

Longitudinal data on TCR V*β* distribution following 5 weeks of antidepressant treatment were available only for the subgroup of patients treated with escitalopram

(n = 10). No significant changes were observed in Gini-TCR skewing index values either among $CD4^+$ T cells (p = .38) or $CD8^+$ T cells (p = .96) from this subgroup of patients (data not shown).

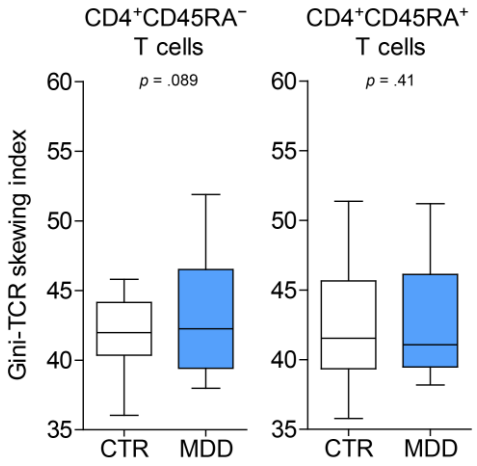


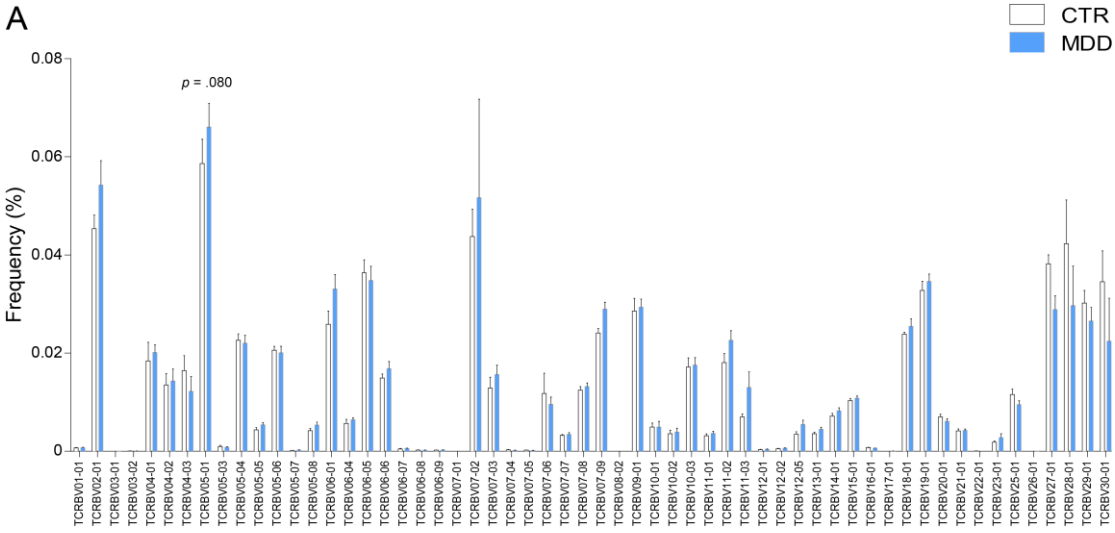
Figure 3.10. Skewing of the TCR Vβ repertoire among memory $CD4^+$ T cells from MDD patients. T cell receptor (TCR) variable β chain family distribution analysis was performed by means of flow cytometric interrogation of $CD45RA^-$ (memory) and $CD45RA^+$ (naïve and terminally differentiated effector) $CD4^+$ T cells from major depressive disorder patients (MDD; n = 20; blue bars) and matched non-depressed controls (CTR; n = 20; white bars). The Gini-TCR skewing index was next applied to the flow cytometric Vβ repertoire analyses. Graphs depict Tukey boxplots. For both comparisons, the Wilcoxon signed-rank test was used.

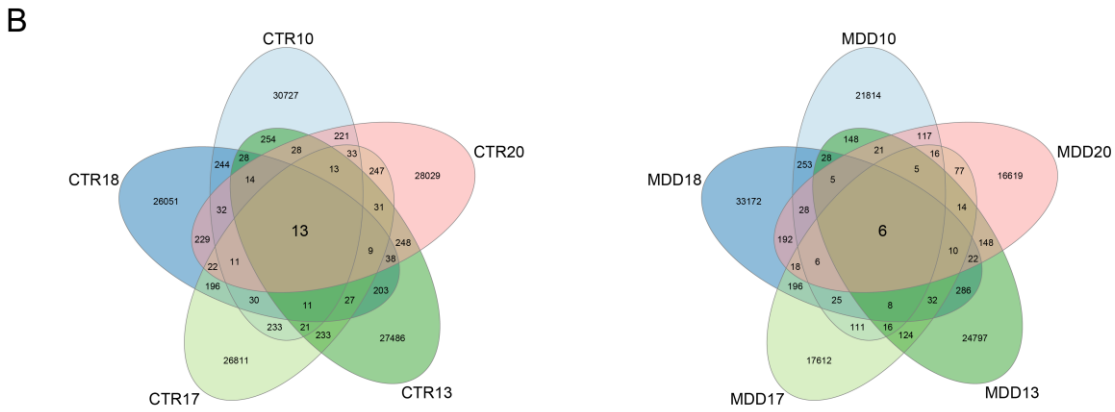
3.6. T cell receptor CDR3 sequencing

The $CD4^+$ T cell repertoire composition was next explored on a clonal basis by means of TCR β chain CDR3 sequencing. Given that CDR3 is the main complementarity determining region responsible for recognition of processed antigens and that numerous public T cell clones (i.e., T cells with common CDR3 amino acid sequences that dominate the response to the same antigen in multiple individuals) have been described in the HLA-A2 context [160], only $HLA\text{-}A2^+$ patients and matched controls were chosen to be included in this part of the study (n = 10).

First, expanded usage of the Vβ5.1 family (TCRBV05-01 according to the IMGT nomenclature [161]) was confirmed among $CD4^+$ T cells from MDD patients, reaching a statistical trend despite the very small sample size (p = .080; **Fig. 3.11A**). Second, sequencing data were used to screen for $CD4^+$ T cell clonotypes bearing the same CDR3 amino acid sequence within as well as between the two tested groups (**Fig. 3.11B**).

Thirteen clonotypes were thereby identified that were jointly present in all non-depressed subjects but not all MDD patients (**Fig. 3.11B**, left table). Conversely, 5 clonotypes were found to be jointly present in all MDD patients but not all non-depressed controls and, finally, 1 clonotype was found to be shared by all MDD patients but none of the controls (**Fig. 3.11B**, right table). Interestingly, the joint clonotype with the highest abundance among all MDD patients belonged to the Vβ5.1 family.





CDR3 (aa)	V name	D name	J name
CASSLGYEQYF	TCRBV07-08	unresolved	TCRBJ02-07
CASSLQETQYF	TCRBV07-09	TCRBD01-01	TCRBJ02-05
CASSQGETQYF	unresolved	unresolved	TCRBJ02-05
CASSLSYEQYF	TCRBV05-06	unresolved	TCRBJ02-07
CASSSSSYEQYF	TCRBV07-09	TCRBD02-01	TCRBJ02-07
CASSQGYEQYF	TCRBV07-02	TCRBD01-01	TCRBJ02-07
CASSLEETQYF	unresolved	unresolved	TCRBJ02-05
CASSQETQYF	TCRBV18-01	unresolved	TCRBJ02-05
CASSLGQQETQYF	unresolved	TCRBD01-01	TCRBJ02-05
CASSPNTGELFF	TCRBV18-01	unresolved	TCRBJ02-02
CASSLGGNQPQHF	TCRBV11-02	unresolved	TCRBJ01-05
CASSLAGGTEAFF	TCRBV05-04	TCRBD02-01	TCRBJ01-01
CASSLGGGTEAFF	TCRBV11-02	TCRBD02-01	TCRBJ01-01

CDR3 (aa)	V name	D name	J name
CASSLGETQYF	TCRBV05-01	unresolved	TCRBJ02-05
CASSLTENTEAFF	unresolved	TCRBD01-01	TCRBJ01-01
CASSLGQGYEQYF	TCRBV07-02	TCRBD01-01	TCRBJ02-07
CASSLGSNQPQHF	TCRBV11-02	unresolved	TCRBJ01-05
CASSLDSSGNTIYF	TCRBV05-04	TCRBD01-01	TCRBJ01-03
CASSLGGSYEQYF	TCRBV07-03	TCRBD02-01	TCRBJ02-07

Figure 3.11. T cell receptor *β* chain CDR3 sequencing of CD4$^+$ T cells from matched HLA-A2$^+$ MDD patients and non-depressed controls.

(**A**) The clonogram displays the complementarity determining region 3 (CDR3) sequencing data from matched HLA-A2$^+$ major depressive disorder patients (MDD; *n* = 5; blue bars) and non-depressed controls (CTR; *n* = 5; white bars). Usage of the family TCRBV05-01 was expanded among CD4$^+$ T cells from MDD patients (planned comparison, the uncorrected *p*-value is displayed). (**B**) Five-set Venn diagrams were used to visualize the overlap of clonotypes among non-depressed controls (*diagram and table on left*) and among MDD patients (*diagram and table on right*). Thirteen and six within-group shared clonotypes were thereby identified, respectively. The highlighted in blue CDR3 amino acid (aa) sequence was found to be shared by all tested MDD patients but none of their matched non-depressed controls.

4. DISCUSSION

In this thesis, converging evidence is provided from several cellular and molecular approaches in favor of impaired adaptive immunity in untreated patients suffering from MDD compared to closely matched non-depressed controls. The results indicate two potential mechanisms underlying T cell dysfunction in depressed patients: (1) suboptimal CXCR3- and CCR6-dependent T cell navigation and (2) suppression of beneficial T cell responses by Treg cells. As discussed in the following sections, these mechanisms might conspire in favor of both reduced immune surveillance and insufficient CNS homeostasis in MDD patients, thereby shaping the peripheral TCR repertoire in a different manner in these individuals.

4.1. Lower T cell surface CXCR3 expression in MDD

Chemokine function is pivotal for leukocyte motility which is required for a vast array of physiological and pathophysiological processes, ranging from immune cell development and homeostasis to immune responses to pathogens, allergens or autoantigens [162]. Hence, chemotaxis of T cells is central to their constant quest for detection of cognate antigens, interaction with other tissues as well as cell fate decisions [65, 162].

To identify possible defects in migration and homing capacities of circulating T cells from MDD patients, the surface expression of two major T helper-associated chemokine receptors, namely CXCR3 and CCR6, was analyzed. These receptors are members of a broad inflammatory chemokine receptor repertoire that is variably expressed on effector T cells following activation and mediates their trafficking to peripheral tissues and sites of inflammation [162-164]. A significantly lower percentage of CXCR3-expressing T cells was observed in the peripheral blood of untreated MDD patients compared to their matched non-depressed counterparts. Lower surface expression of CXCR3 could possibly extent to multiple lineages beyond T cells in MDD, as similar findings were reached in non-T cells as well (i.e., B cells, NK cells and dendritic cells). To the knowledge of the author, there is no previously reported work on the expression levels of the chemokine receptor CXCR3 in MDD patients, on either a transcriptional or translational level. However, it has been reported that an acute stressor (public speaking) selectively mobilizes CXCR2/3- as well as CCR5-expressing T cells in the peripheral blood of healthy volunteers [165]. Acute stressors have generally an immunostimulatory effect, however chronic stressors have been reliably associated with suppressive effects on the immune system [166]. Therefore, it could be hypothesized that chronic stress-induced immune mobilization in the context of MDD could predispose specific T cell populations, including CXCR3-expressing T cells, to exhaustion.

Despite lower expression of CXCR3 on the cell surface, total cellular amounts of the receptor as measured following intracellular staining were not found to be lower in either $CD4^+$ or $CD8^+$ T cells from untreated MDD patients. This finding is in agreement with data showing that human $CD4^+$ T cells store pre-formed CXCR3

protein in a distinct, regulated intracellular compartment [167]. Within a different disease context, $CD8^+$ T cells from cutaneous T cell lymphoma patients have been found to display a CXCR3-specific downregulation from the cell surface owing to increased receptor internalization and accumulation in endolysosomal compartments [168]. On account of these observations, it was hypothesized that an analogous mechanism of enhanced receptor internalization rather than an impairment in cellular protein expression may be at play in T cells of MDD patients. Indeed, extracellular ligand binding can enhance to a great extent the internalization rates of several chemokine receptors [153]. This was particularly evident in the case of the CXCR3 chemokine ligands in previously published *in vitro* studies, where cell surface levels of CXCR3 were shown to be rapidly reduced in a concentration- and time-dependent manner following incubation with the three natural ligands CXCL9, CXCL10 and CXCL11 [154, 155]. The concentrations of the most potent endocytosis-inducing ligands, CXCL10 and CXCL11, were therefore measured in serum samples of patients and matched controls.

In line with previously published data [112], circulating levels of CXCL10 protein tended to be higher in MDD patients, although group differences fell just short of statistical significance. Transcripts of this chemokine were also found by whole blood transcriptomics to be among the genes upregulated in chronic hepatitis C virus patients who developed IFN-α-induced depression [169]. Differences in serum CXCL11 protein levels did not reach statistical significance in the current study, yet previous preliminary data showed that this chemokine has higher plasma concentration in MDD patients, especially those with a history of childhood trauma [170]. Therefore, downregulation of T cell surface expression of CXCR3 in antidepressant-free MDD patients could be in part accounted for by relatively higher extracellular abundance of the chemokine CXCL10.

For proper interpretation, it is worth mentioning that downregulation of the receptor *in vivo* is likely to be the result of varying temporal and spatial expression patterns leading to a broader functional synergism among the three cognate ligands of CXCR3 (reviewed in [171, 172]). As a result, such a synergism may not be adequately captured by cross-sectional assessments of individual ligands. In addition, peripheral blood may not be the most informative biological specimen in case of distant, organ-specific inflammatory responses. Indeed, a very recent preclinical study demonstrated that brain endothelia-derived CXCL10 modulates sickness behavior (an inflammatory model of MDD) in a CXCR3-dependent manner [173], suggesting that a CXCL10 gradient in the peripheral blood may be accentuated towards inflamed CNS sites in MDD patients. This hypothesis is corroborated by clinical and postmortem data indicating blood-brain barrier dysfunction in MDD [174] as well as active neuroinflammatory processes within perivascular and disease-relevant brain regions [175-178].

Interestingly, mice lacking CXCR3 or CXCL10 have been found to be protected from IFN-β-induced depressive-like behavior [173]. Similarly, CXCR3 deficiency rescued cognitive deficits and was associated with significantly reduced brain *CXCL10* and enhanced *BDNF* mRNA expression in an Alzheimer's disease model [179]. These

preclinical findings suggest that downmodulation of CXCR3 in response to potentially depressogenic levels and/or action of CXCL10 may serve an adaptive role in depressed patients. Nevertheless, although conceivably adaptive in the short term, CXCR3 downregulation in T cells might impose an allostatic cost on the immunocompetence of these patients. Namely, numerous preclinical and clinical studies highlight the paramount importance of CXCR3 in effective T cell trafficking, differentiation and generation of timely effector and memory responses [163]. In this regard, preclinical research has shown that CXCR3 expression enhances the ability of tissue-localized $CD8^+$ T cells to locate and eliminate neoplastic, virus- or bacteria-infected cells [180-182] as well as the ability of parenchymal and tissue-resident $CD4^+$ T cells to safeguard against bacterial and parasitic infections [183, 184]. Strikingly, the CXCR3/CXCL10 axis plays also a pivotal role in controlling the migration of effector T cells to CNS sites of viral and parasitic replication [185-187]. Emphasizing their contribution to CNS immune surveillance, CXCR3-expressing $CD4^+$ and $CD8^+$ T cells have been found in clinical studies to be enriched in the cerebrospinal fluid as well as brain perivascular spaces, even in the absence of overt neuroinflammation [75, 77].

Loss of CXCR3-mediated T cell migration in MDD patients could thus compromise the ability of circulating T cells to extravasate into infected or otherwise endangered tissues, including the CNS, in response to CXC3L gradients. The resulting reduction of the capacity to detect pathogen-derived or other danger-associated antigens has possibly great relevance for explaining the accentuated association between the incidence of infections and MDD diagnosis [14, 125, 126]. In addition, CXCR3 may provide a molecular basis for the long-documented blunted lymphocyte responses to mitogen-induced stimulation in MDD patients [21, 22]. Indeed, the percentage of CXCR3-expressing T cells derived from healthy donors is rapidly increased upon polyclonal stimulation with the mitogen phytohaemagglutinin [167, 188].

Finally, the current findings might also have broader relevance for the study of immune surveillance mechanisms in the face of comorbid depression. For instance, it has been demonstrated that CXCR3 expression by $CD8^+$ T cells is significantly associated with enhanced survival in metastatic melanoma patients [189] owing to increased intratumoral localization of CXCR3-expressing antitumor T cells [180]. Given that both a history of depressive symptoms and the presence of comorbid MDD have been reliably associated with a poorer prognosis in cancer patients [9, 190-192], the results of this thesis may hold promise for identifying a possible link between impaired CXCR3-dependent T cell trafficking and depressive symptomatology in the context of chronic physical illness.

4.2. Lower T cell surface CCR6 expression in MDD

A recent whole blood transcriptomic analysis identified lower *CCR6* transcripts in both an antidepressant-treated discovery cohort and an antidepressant-free validation cohort [193]. Likewise, in the current study, T cell surface expression of CCR6 protein was found significantly lower in untreated MDD patients and was not modified by treatment with the selective serotonin reuptake inhibitor escitalopram. In

addition, lower surface expression of CCR6 was T cell-specific and it was mainly driven by $CD4^+$ T cells. Similar to $CXCR3^+$ T cells, reductions in CCR6-expressing $CD4^+$ T cells might further hinder immune surveillance in MDD patients or may reflect a homeostatic redistribution of this population to certain tissues in need.

Several preclinical studies highlight the role of CCR6 in the homing of $CD4^+$ T cells to gut-associated lymphoid tissues and mucosal defense [164]. In humans, CCR6-expressing T cells compartmentalize in diverse tissues and anatomical locations (e.g., nasal mucosa, lung, skin, cerebrospinal fluid), even in the absence of inflammation [75, 194]. CCR6-expressing $CD4^+$ T cells can however initiate harmful autoimmune responses in the CNS through the choroid plexus [76], suggesting that downregulation of CCR6 in MDD patients may have an adaptive aspect, e.g., to avoid unnecessary and potentially pathogenic recruitment of T cells into the CNS.

Furthermore, human memory $CD4^+$ T cells specific for the fungus *Candida albicans* are mainly $CCR6^+$, while those specific for *Mycobacterium tuberculosis* are enriched in the subpopulation of $CD4^+$ T cells expressing both CCR6 and CXCR3 [70, 195]. Intriguingly, tuberculosis was a prominent cause of mortality among severely depressed patients before the advent of antidepressants [196] and the first antidepressant drug that was introduced in the 1950s for use in clinical psychiatry (iproniazid) was at the time an established antitubercular agent [197, 198].

It is currently unclear what are the mechanisms responsible for reduced T cell surface CCR6 expression in MDD patients. However, it has been previously shown that TCR stimulation by plate-bound anti-CD3 for 24 hours leads to a downregulation of both CXCR3 and CCR6 in a human T cell line [199], thereby leaving open the possibility that recent antigenic stimulation *in vivo* may account for downregulation of both chemokine receptors in $CD4^+$ T cells of MDD patients. The possible nature of $CD4^+$ TCR engagement in MDD is discussed in section 4.4.

4.3. Elevated frequency of regulatory T cells in MDD

Regulatory T cells (Treg) are indispensable for the maintenance of immune self-tolerance and tissue homeostasis via negative regulation of adaptive immune responses (reviewed in [57, 58]). In the current study, Treg were identified among $CD4^+$ T cells using exclusively cell surface markers (CD25, CD127), as suggested by published guidelines for the identification of human Treg following PBMC cryopreservation [158]. Of note, the identification strategy hereby adopted (i.e., without intracellular staining for the canonical Treg marker FOXP3) is compatible with live Treg cell sorting needed for downstream *ex vivo* expansion and use in clinical trials [200-202].

Using flow cytometry, a significantly higher frequency of peripheral blood $CD4^+CD25^{high}CD127^{low/-}$ Treg was observed in untreated MDD patients compared to their non-depressed counterparts. Accordingly, significantly higher mRNA expression of the transcription factor *FOXP3* (but not *TBX21, GATA3* and *RORC*) was detected in negatively purified $CD4^+$ T cells from MDD patients, thereby confirming a

transcriptional shift towards a “tolerogenic” $CD4^+$ T cell phenotype. Of note, given that FOXP3 expression in human $CD4^+$ T cells is activation-dependent [62, 203], a negative selection strategy was opted for in order to avoid unwanted activation induced by positive selection. In keeping with previous studies showing enriched FOXP3 expression in human $CD4^+$ T cells that express low levels of CD127 [156, 157], mRNA expression of *FOXP3* in untouched $CD4^+$ T cells correlated positively with the frequency of $CD4^+CD25^{high}CD127^{low/-}$ Treg in both MDD patients and non-depressed controls.

A recent study showed higher Treg frequency using the same cell surface markers in older adults following acute psychological stress testing [204]. In line with the current findings, higher $CD4^+CD25^{high}CD127^{low}$ Treg percentages were associated with higher depressive symptoms and lower mental health status after adjustment for multiple covariates, including age, sex, BMI and smoking status [204]. Therefore, in addition to an impairment in CXCR3- and CCR6-dependent chemotactic capacity, T cell responses and effector proliferation may further be hampered in MDD patients by a relative increase in Treg cells. Intriguingly, preclinical studies with a primary focus on the neuroprotective potential of endogenous T cell responses seem to endorse this viewpoint by demonstrating that abrogation of Treg-mediated suppression is beneficial for withstanding CNS injury and stress-related pathology in laboratory animals [205-207]. Notably, a potent inhibition of T cell function owing to an increased proportion of myeloid-derived suppressor cells in the peripheral blood has been also documented in depressed patients [208], suggesting that an active suppression of T cell immunity in MDD may extend beyond the Treg compartment.

However, the current results are in apparent contradiction with other studies (except for one abstract [209]) which have suggested lower frequency of circulating Treg cells in MDD patients compared to controls [97, 104, 105]. It is difficult to discern the reasons behind this discrepancy, certain methodological and clinical considerations are nevertheless worth mentioning. First, different gating strategies have been used in these studies for Treg identification by flow cytometry. Second, none of these studies employed the surface marker CD127, which is an essential marker for the identification of Treg in clinical samples [210]. In particular, use of neither surface CD127 nor intracellular FOXP3 stainings minimizes the likelihood of discriminating human Treg cells from activated conventional T cells (discussed in [58]). Third, inclusion of antidepressant-treated patients at baseline and/or lack of adjustment for important covariates (e.g., BMI, smoking) might have confounded some results. Therefore, for proper interpretation, future studies on Treg cells in MDD need to harmonize their staining panels and research designs.

Of note, the current findings are restricted to the adaptive immune compartment and do not contradict the notion that MDD is characterized by insufficient innate immune regulation [19, 211, 212]. For instance, and in agreement with a previous report [209], MDD patients in the current study showed significantly lower frequency of peripheral blood $CD56^{high}CD16^-$ NKreg cells, a population with considerable cytokine-mediated regulatory properties in the innate immune system [213]. Therefore, insofar as MDD is characterized by both immune suppression and

immune activation, this “immunological conundrum” may as well be reflected in a commensurate variability of adaptive and innate immunoregulatory populations.

4.4. Skewed $CD4^+$ T cell receptor repertoire in MDD

The flow cytometry data indicated the presence of a skewing in the $CD4^+$ but not the $CD8^+$ TCR repertoire in untreated MDD patients. A proportional dominance in the usage of certain germline TCR Vβ genes was seen, perhaps most notably those belonging to the Vβ5.1 family. Preferential usage of this TCR Vβ family among $CD4^+$ T cells from MDD patients was confirmed by TCR sequencing data. In addition, post hoc analyses suggested that the detected repertoire skewing in MDD patients might primarily be driven by a less diverse TCR utilization profile among memory $CD4^+$ T cells.

These findings suggest that certain antigen-experienced T cells are dominating the $CD4^+$ repertoire in MDD patients. This could have arisen from an oligoclonal expansion in response to an antigen exposure, presumably in the context of a past infection. Insofar as pathogen-associated “public” clonotypes have been described in the HLA-A2 context [160], “footprints” of past infections on the T cell repertoire would be consistent with the identification of shared $CD4^+$ T cell clonotypes among HLA-$A2^+$ MDD patients and non-depressed controls in the current study. Thus, and in view of the strengthened association of MDD with certain infectious agents [125], the sequencing data could be further used to identify $CD4^+$ clonotypes of anti-pathogen specificity.

However, it cannot be precluded at this point that a skewed $CD4^+$ TCR repertoire is secondary to an oligoclonal expansion or contraction following an autoantigenic challenge. This scenario could be seen in concert with amplified autoimmune processes extensively described in MDD patients (reviewed in [101]). An informative example in this regard could be the prevalence of the TCR family Vβ5.1 among GAD65-specific $CD4^+$ T cells in type 1 diabetes patients [214].

Furthermore, a significant preclinical literature advocates a neuroprotective function of self-recognizing T cells in response to neural and behavioral distress [127-129] and the observed skewing of the memory $CD4^+$ TCR repertoire in MDD patients could be also discussed from this perspective. Namely, the human CNS is normally and specifically populated by antigen-experienced memory $CD4^+$ T cells in proportion to their abundance in the peripheral blood [215, 216] and elegant preclinical research has shown an enrichment of this memory $CD4^+$ TCR repertoire with CNS-specific clonotypes under physiological conditions [217]. Consequently, mice with clonally restricted T cell repertoire exhibited impaired cognitive performance, with CNS-directed $CD4^+$ (but not $CD8^+$) T cells being able to partially rescue this phenotype [135]. Along the same line, immunization with a CNS-related antigen was sufficient to counteract depression-like behavior and neurogenic deficits induced by chronic mild stress in rats [218].

Interestingly, a recent clinical study showed that effector memory $CD4^+$ T cells in the peripheral blood are inversely associated with cognitive performance in a healthy older cohort [219]. In addition, the majority of human T cells trafficking into CNS-associated compartments are CXCR3-expressing memory $CD4^+$ T cells [74, 75, 79]. In view of a spatial and functional association between CXCR3 and TCR activation upon CXCL10-induced T cell migration [220, 221], it is tempting to hypothesize that a skewing of the memory $CD4^+$ TCR repertoire in MDD patients might reflect a clonal contraction or redistribution of antigen-specific $CD4^+$ T cells equipped with a capacity for homing into the "depressed" CNS. Such a scenario warrants further translational exploration in view also of an emerging literature of independent preclinical studies demonstrating a specific role for the brain-localized $CD4^+$ (but not $CD8^+$) T cell repertoire in support of hippocampal neurogenesis, neurotrophin production, cognition and anxiolytic-like behavior [131-134, 136].

4.5. Antidepressant treatment effects on CXCR3 expression

In the current study, treatment with the selective serotonin reuptake inhibitor (SSRI) escitalopram was associated with increases in T cell surface expression of CXCR3 proportional to the degree of clinical response, as assessed by the self-rated, and to a lesser extent the clinician-rated, IDS. Conversely, and despite the very small sample size, treatment with the noradrenergic and specific serotonergic antidepressant (NaSSA) mirtazapine was associated with marked reductions in CXCR3 surface expression. It is not clear whether these medication-related findings are accounted for by disparate pharmacological actions on the T cell level or are secondary to more remote effects of medication, e.g., alterations in other cell types or humoral factors regulating chemokine receptor expression.

It is known that several diversely acting antidepressant medications display both direct and indirect immunomodulatory properties, at least when cytokine measures are used as a read-out (reviewed in [109]). For instance, escitalopram and mirtazapine were found *in vitro* to exert differential effects on the production of several pro-inflammatory cytokines (i.e., IL-1β, IL-17, IL-22 and TNF-α) by anti-CD3/CD40-stimulated whole blood cultures derived from antidepressant-free MDD patients [222]. In view of the well-described flexibility of chemokine receptor expression in human T cells in response to polarizing cytokine signals [68, 223, 224], it is conceivable that escitalopram and mirtazapine may be associated with differential modes of cytokine- and/or chemokine-mediated regulation of chemokine receptor expression. Of relevance to this hypothesis are *in vitro* findings on the capacity of the SSRI fluoxetine (but not the NaSSA mirtazapine) to suppress the LPS-induced expression of CXCL10 in a human monocytic cell line [225], thereby providing a plausible mechanistic insight into how suppression of innate inflammation could lead to increase of T cell surface expression of CXCR3 during SSRI treatment. Physiological doses of the SSRI citalopram have been also shown to suppress the expression of the HIV-associated chemokine receptors CCR5 and CXCR4 in human PBMCs [226], suggesting that related types of chemokine receptors may be also subject to direct regulation by antidepressant agents.

Future comparative pharmacological studies will be needed to dissect the underlying cellular and molecular aspects of antidepressant-induced T cell chemokine receptor modulation, as this may be relevant not only to the therapeutic properties but potentially also to the side effect profiles of these agents. One should however keep in mind that the *in vitro* effects of antidepressants on immune function may differ significantly from their *in vivo* effects (discussed in [110]).

4.6. Limitations and strengths

The results hereby presented are possibly limited by the small sample size and thus replication in larger, independent samples is required. The sample size may have also precluded a better agreement among the depression rating scales used in the current study, as they have been found to be highly correlated in adequately powered studies [142, 143]. Alternatively, disagreement among the rating scales might be accounted for by varying emphasis on symptom profiles. For instance, it is known that the IDS scales contain items relevant to symptoms of atypical depression (i.e., increased sleep and appetite, leaden paralysis and interpersonal rejection sensitivity) which are not assessed by the $HRSD_{17}$ [227]. Therefore, additional analyses of the immunological data on the basis of specific depression symptoms may be warranted [228].

However, particular care was taken to ensure the clinical homogeneity of the MDD group. To this end, only unmedicated patients were included (many of them being antidepressant-naive) with a minimum level of depression severity at baseline (at least 18 points on the $HRSD_{17}$). In addition, the MDD patients were individually and closely matched to non-depressed controls for important immune-related variables (age, sex, BMI and smoking status) in order to minimize confounding of the results. Thus, despite the small sample size, previous findings in MDD were replicated, such as higher serum levels of CXCL10 within the same concentration range as previously reported [112] and lower frequency of NK cells [96, 97], including the $CD56^{high}CD16^{-}$ NK subset [209]. It can therefore be claimed that the patient cohort of this study is representative of the MDD patient population.

Another strength of the current study was the employment of different methodological approaches. In particular, multi-parametric flow cytometry, magnetic cell separation and TCR sequencing allowed for interrogation of immunologically distinct PBMC subsets on a single-cell level. On this account, a more refined estimate of cell frequency and function was obtained as opposed to readouts from whole blood or bulk leukocytes.

4.7. Proposed model

Overall, the data support a model in which T cells of MDD patients display decreased potential to detect pathogen-associated antigens and/or CNS autoantigens owing to compromised chemotactic function and impaired tissue homing capacity (**Fig. 4.1.**). Endogenous T cell responses in these patients might further be hampered by excessive regulation imparted by elevated levels of Treg cells. Taken together, this could lead to impaired pathogen clearance and suboptimal memory formation, as reflected by a skewed T cell receptor repertoire among antigen-experienced $CD4^+$ T cells. Poor control over infection would further predispose to infection-associated inflammation, thereby reinforcing a "depressogenic feedback loop". In this context, memory $CD4^+$ T cells normally associated with CNS homeostasis would diverge from their usual endogenous function by acquiring a phenotype more appropriate for fighting recurrent infections and/or controlling the ensuing inflammation.

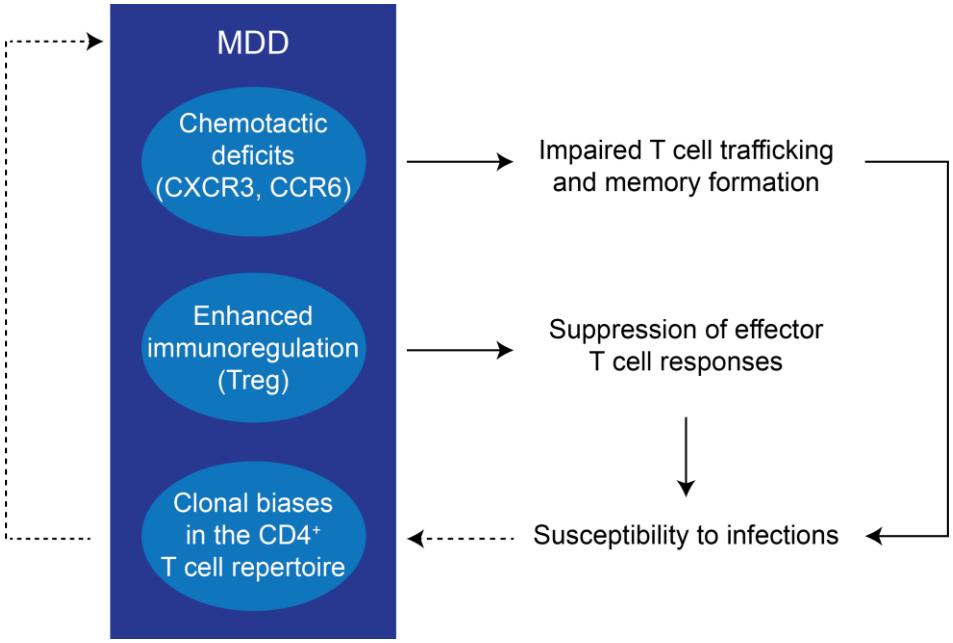


Figure 4.1. Conceptual framework on the link between impaired T cell function, infection susceptibility and TCR repertoire skewing in MDD patients.

In conclusion, this thesis extends earlier research on the role of adaptive immunity in MDD and reinforces the notion that T cells are a biologically and clinically relevant cell population in this disorder. Future clinical and translationally relevant animal studies are expected to examine pathogenetic implications of the observed T cell aberrations, to verify their potential utility for clinical outcome prediction and to apply similar research concepts to other fields of psychiatry and medicine.

5. SUMMARY

Epidemiological studies have provided evidence for a bidirectional relationship between major depressive disorder (MDD) and physical illness, including severe infections. This evidence is compatible with immunological findings on the presence of both low-grade inflammation and functional T cell suppression in MDD patients. In addition, an independent line of research suggests that a state of functional impairment in T cells, most prominently $CD4^+$ T cells, might be directly relevant to poor central nervous system (CNS) homeostasis in depressed patients. The goal of the thesis was to explore in greater detail the mechanisms underlying T cell dysregulation in patients suffering from MDD.

Antidepressant-free MDD patients (*n* = 20) with a minimum Hamilton Rating Scale for Depression score of 18 were recruited. Each patient was individually matched for sex, age, body mass index and smoking status to a non-depressed control (*n* = 20). Blood samples were obtained and peripheral blood mononuclear cells were isolated at baseline. A second sample was obtained from patients after 5 weeks of antidepressant monotherapy. T cell phenotype and repertoire were interrogated using a combination of multi-parametric flow cytometry, gene expression analyses and T cell receptor sequencing.

T cells from MDD patients showed significantly lower surface expression of the chemokine receptors CXCR3 and CCR6, which have been associated with trafficking of T cells to various tissues, including the human CNS. In addition, regulatory T cell frequency was increased in depressed patients as shown by phenotype and gene expression in purified $CD4^+$ T cells. Repertoire analyses and sequencing data further indicated a skewed $CD4^+$ T cell receptor repertoire in MDD patients. Finally, preliminary results suggested that lower T cell surface expression of CXCR3 may be a state-dependent characteristic of MDD.

These results identify impaired CXCR3- and CCR6-dependent immune surveillance and suppression of beneficial T cell responses as possible mechanisms underlying T cell dysfunction in MDD. Further elucidation of T cell pathology in this disorder could inform new research concepts towards novel, T cell-based antidepressant strategies.

Zusammenfassung

Epidemiologische Studien deuten auf eine gegenseitige Beeinflussung von Depression und somatischen Erkrankungen, wie zum Beispiel schweren Infektionen, hin. Dies ist im Einklang mit immunologischen Befunden einer niedriggradige Entzündungsreaktion sowie eines funktionellen Defizits von T-Zellen bei depressiven Patienten. Außerdem weisen unabhängige Studien darauf hin, dass ein funktionelles Defizit von T-Zellen, im Besonderen der $CD4^+$ T-Zellen, direkte Relevanz für eine verschlechterte Homöostase des zentralen Nervensystems (ZNS) bei depressiven Patienten hat. Ziel der vorliegenden Arbeit war es, die Mechanismen zu untersuchen, die der Dysregulation von T-Zellen bei depressiven Patienten zugrunde liegen.

Es wurden unbehandelte Patienten mit Depression (*n* = 20) und einem Hamilton Score von mindestens 18 rekrutiert. Zu jedem Patienten wurde außerdem eine bezüglich des Geschlechts, Alters, Body-Mass-Index, und Raucherstatus passende nicht-depressive gesunde Kontrollperson (*n* = 20) untersucht. Mononukleäre Zellen des peripheren Blutes wurden zum Zeitpunkt des Einschlusses in die Studie und im Falle der Patienten noch einmal nach 5-wöchiger Therapie mit einem Antidepressivum isoliert. Der Phänotyp der T-Zellen und das T-Zell-Rezeptor-Repertoire wurden mittels Durchflusszytometrie, Genexpressionsanalysen und T-Zell-Rezeptor-Sequenzierung untersucht.

T-Zellen von Patienten mit Depressionen wiesen eine signifikant verminderte Oberflächenexpression der Chemokinrezeptoren CXCR3 und CCR6 auf, die die Einwanderung von T-Zellen in verschiedene Gewebe, wie zum Beispiel das ZNS, vermitteln. Zusätzlich wurde anhand der Phänotypisierung und Genexpressionsanalysen isolierter $CD4^+$ T-Zellen eine erhöhte Frequenz regulatorischer T-Zellen bei depressiven Patienten gefunden. Die Analyse des Repertoires sowie die Sequenzierungsdaten ergab darüber hinaus eine Verschiebung des T-Zell-Rezeptor-Repertoires von $CD4^+$ T-Zellen bei Patienten mit Depression. Vorläufige Daten weisen zudem darauf hin, dass die verminderte Oberflächenexpression von CXCR3 auf T-Zellen ein erkrankungsabhängiges Charakteristikum der Depression darstellt.

Diese Ergebnisse identifizieren verminderte CXCR3- und CCR6-abhängige Immunreaktionen sowie eine Unterdrückung nützlicher T-Zell-Antworten als mögliche Mechanismen der T-Zell Dysfunktion bei Depressionen. Weitere Untersuchungen der T-Zell-assoziierten Pathologie bei Depressionen könnten zur Entwicklung neuer T-Zell-basierter Therapiestrategien führen.

6. ABBREVIATIONS

AA	Amino acid sequence
APC	Allophycocyanin
BCL-6	B-cell lymphoma 6
BCR	B cell receptor
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BSA	Bovine serum albumin
BV	Brilliant violet
CCL	Ligand for CCR
CCR	Receptor for CC chemokine
CD	Cluster of differentiation
CD3E	CD3e molecule, epsilon (CD3-TCR Complex)
cDNA	Complementary DNA
CDR	Complementarity determining region
CNS	Central nervous system
CRP	C-reactive protein
Ct	Cycle threshold
CTR	Control
CXCL	Ligand for CXCR
CXCR	Receptor for CXC chemokine
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DSM	Diagnostic and statistical manual of mental disorders
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GAD65	Glutamic acid decarboxylase, 65kD
GATA3	GATA binding protein 3
gDNA	Genomic DNA
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HRSD	Hamilton Rating Scale for Depression
ICD	International classification of diseases
IDS-C	Inventory of depressive symptomatology-clinician rated
IDS-SR	Inventory of depressive symptomatology-self report
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPO8	Importin 8
IR	Infrared
LPS	Lipopolysaccharides
MDD	Major depressive disorder
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NaSSA	Noradrenergic and specific serotonergic antidepressant
NK	Natural killer
NKc	Cytotoxic NK
NKreg	Regulatory NK

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptor
PSMB4	Proteasome subunit beta 4
PSMD13	Proteasome 26S subunit, non-ATPase 13
QIDS-SR	Quick inventory of depressive symptomatology-self report
RORγ	RAR-related orphan receptor gamma
RPL13A	Ribosomal protein L13a
RPMI	Roswell Park Memorial Institute medium
SCID	Structured clinical interview for the DSM
sIL-2R	Soluble IL-2 receptor
SNRI	Serotonin-norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
STAT3	Signal transducer and activator of transcription 3
TBP	TATA-box binding protein
TBX21	T-box 21
TCR	T cell receptor
TEMRA	T effector memory cells with reacquired RA
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
Vβ	T cell receptor beta chain, variable region

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CURRICULUM VITAE

Entfällt aus datenschutzrechtlichen Gründen.

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1. Ramien C, Taenzer A, Lupu A, Heckmann N, Engler JB, **Patas K**, Friese MA, Gold SM. Sex effects on inflammatory and neurodegenerative processes in multiple sclerosis. *Neurosci Biobehav Rev.* 2016; S0149-7634(15)30087-7.
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