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Inflammation and mood: T cell phenotype in multiple sclerosis–associated depression

Dissertation

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1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS)[1]. MS frequently co-occurs with major depressive disorder (MDD)[2], which is characterised by low mood, loss of interest and a variety of somatic or further mood symptoms[3]. Although up to half of MS patients experience a depressive episode over the course of their life[4], little is known about how the two conditions are interconnected. Epidemiological studies have revealed that symptoms of mood disorders may be present years before a MS diagnosis is made[5], conveying that depression is not merely a psychologic reaction to a diagnosis of chronic disease. Accumulating evidence interrogating the immune, endocrine and central nervous systems suggest that MS and MDD may share overlapping pathogenic pathways that give rise to MS-associated depression.

In the following, a brief introduction will be given to the immune system and more specifically T cells as well as glucocorticoid (GC) signalling, before presenting background on MS, MDD and the combination thereof.

1.1 The immune system

Bacteria, viruses, fungi and parasites have accompanied mammalian evolution, posing as one of the greatest selectors. In response, all species have developed an immune system of varying complexity to detect and control pathogens, while limiting damage to affected tissues. For successful immune defence a distinction between foreign and self, dangerous and harmless is of the utmost importance. A failure of the immune system to correctly classify organisms, cells or surface molecules can lead to autoimmunity, allergy, severe infection, or cancer[6].

The human immune system is classically divided into two branches: the innate and the adaptive immune system, each comprising several functional groups of immune cells (leukocytes), as well as circulating proteins of the complement system. The innate immune system provides protection since birth, responds very quickly, and consists of dendritic cells (DCs), granulocytes, natural killer (NK) cells, macrophages and monocytes. DCs and macrophages are phagocytes that can engulf and eliminate pathogens, but also sense threat and initiate an immune response by secreting cytokines and chemokines and presenting antigen[6]. To detect pathogens, innate immune cells rely on pattern recognition receptors (PRR) to detect evolutionarily conserved pathogen-associated molecular patterns (PAMP)[7].

The sheer number of pathogens and their fast evolution exceeds the capacity of genetically encoded PRRs and is complemented by the adaptive immune system. The adaptive immune system develops throughout life and consists of lymphocytes that recognise specific chemical structures (so called antigens) due to their highly variable receptors. Antigen receptors of B and T cells are generated by somatic recombination, hypermutation and random nucleotide

insertion, yielding a copious number of possible receptors[6]. Importantly, after adaptive immune cells meet their antigen, they can generate immunological memory, which can be reactivated to create an immediate and stronger immune response in case of renewed infection. B cells produce and secrete soluble antibodies, which establishes humoral immunity. In contrast, T cells engage in so called cellular immunity.

1.1.1 T lymphocytes

T cells originate from bone marrow lymphoid progenitors and mature in the thymus. T cell receptor (TCR) recombination can mathematically produce between 10^{12} and 10^{15} possible TCRs, but after thymic selection conservative estimates have reported the human TCR repertoire to consist of about $2x10^7$ unique TCRs[8]. Unlike soluble and membrane-bound antibodies produced by B cells, TCRs can only bind pre-processed antigens in the context of a presenting molecule, the major histocompatibility complex (MHC). There are two major forms of MHC class I (encoded by the genes human lymphocyte antigen (HLA) -A, -B, -C) and class II (encoded by HLA-DR, -DQ, -DP). Equally there are two major T cell subtypes characterised by their TCR-co-receptors CD4, restricted to MHC-II, and CD8, restricted to MHC-I. The activation of naïve T cells requires three signals, making their activation highly specific: firstly, antigen recognition, secondly co-stimulation via CD80/CD86 – CD28 and thirdly a cytokine stimulus.[6]

$CD8^+$ T cells are also called cytotoxic T lymphocytes (CTL) that recognise viral or other non-self-epitopes presented by any nucleated cell via MHC-I. In response to antigen encounter, CTLs will kill the infected or transformed cell by secretion of cytotoxic molecules such as perforin, granzymes and granulysin or engagement of apoptosis-inducing surface receptors on the target cell (i.e. CD95 or TNFR1)[6]. $CD4^+$ T cells are also known as T helper (Th) cells. They are activated upon encountering their antigen in the context of MHC-II on antigen presenting cells (APC) of the innate immune system or B cells. Depending on the cause of immune stimulation and cytokines present, $CD4^+$ cells polarise into different subsets with different effector functions: interferon (IFN) -γ and interleukin (IL) -12 induce Th1 cells, characterised by activation of the transcription factor T-bet via STAT1 and -4 signalling, which produce IFN-γ. IL-4 induces Th2 cells, marked by activity of GATA3 and STAT6, which produce IL-4, IL-5 and IL-13. IL-6 and IL-23 polarise naïve $CD4^+$ T cells into Th17 cells, in which activation of STAT3 induces transcription factor RORγT and production of IL-17 and IL-22. IL-6 leads to the induction of Bcl-6 positive T-follicular helper (Tfh) cells, that produce IL-21 and provide help to B cell affinity maturation in germinal centres. Transforming growth factor-beta (TGF-β) and IL-2 induce FoxP3 transcription, creating regulatory T cells (T_{reg}), which produce TGF-β and IL-10 (**Figure 1.1**).[6,9]

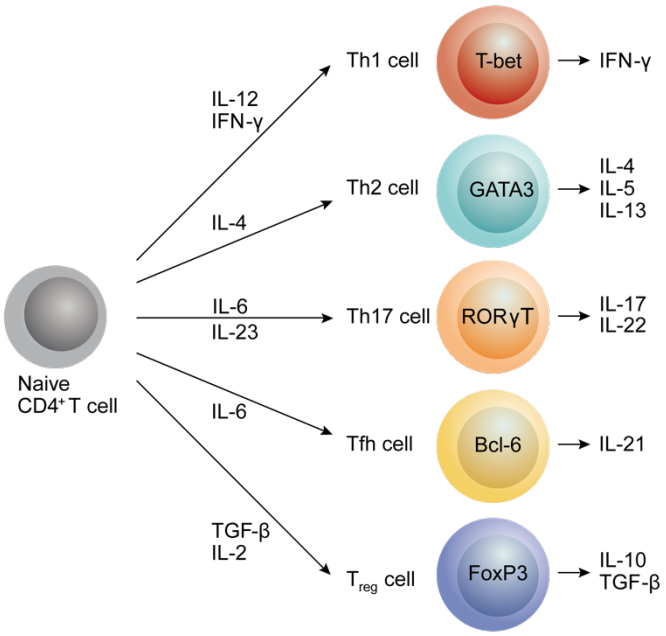

Figure 1.1: CD4⁺ T cell polarisation into Th subsets

Figure adapted from[10].

The Th cell fate varies according to immunological insult and shapes the immune response through the immune subsets recruited to the site of inflammation, i.e. Th1 cells are induced mainly in infections with intracellularly located viruses and microbes and enhance macrophage activity; Th17 cells are preferentially polarised in the case of extracellular microbial infection and activate neutrophils; Th2 cells are essential in parasitic infections, activating granulocytes.[6]

Next to the classical CTLs and Th cells, there are also unconventional T cell subsets, that can have properties typically attributed to innate immune cells, despite being derived from the T cell lineage. One example hereof are mucosal-associated invariant T (MAIT) cells, a population of T cells carrying a semi-invariant TCR that recognises a bacterial metabolite of the riboflavin pathway, presented by the MHC-I related (MR-1) molecule found on most cells[11]. MAIT cells have been implicated in various immune-mediated conditions, although whether their effect in asthma, ulcerative colitis and multiple sclerosis is protective or deleterious remains unclear[11].

Immune cell subsets are identified by combinations of surface molecules or intracellular cytokine production representing functions of the particular subset[12]. For example, within the human T cell population naïve cells can be distinguished from memory and effector cells by their expression of CD45RA or -RO and their expression of CCR7, marking their ability to home to secondary lymphoid organs[13].

1.1.2 Glucocorticoid signalling in T cells

GCs are involved in a variety of different bodily systems and exert a range of effects on different tissue and cell types, including immunosuppression. A clinical illustration hereof is Cushing syndrome, a condition caused by excess of GCs. Symptoms include cataracts, ulcers, skin

thinning, hypertension, masculine hair growth in women, immunosuppression and infections, osteoporosis, glucose elevation, impaired wound healing and mood changes or depression. The main endogenous GC is cortisol, which is systemically regulated by the hypothalamic-pituitary-adrenal (HPA) axis: the hypothalamus reacts to stress, inflammation and circadian cues by producing corticotropin-releasing-hormone (CRH) and arginine vasopressin (AVP); CRH and AVP cause the production of adrenocorticotropin hormone (ACTH) by the pituitary gland, which induces cortisol synthesis in the adrenal glands; cortisol enters the circulation and mediates its systemic effects including a direct negative feedback loop to the HPA axis via the hypothalamus and pituitary. Most circulating cortisol is bound by corticosteroid-binding globulin (CBG), enhancing its systemic distribution.[14]

Locally, GC signalling is regulated by receptor availability, posttranslational modifications to the receptor and the enzymes 11β-hydrosteroid dehydrogenase type 1 (HSD1) and type 2 (HSD2)[14]. HSD1 converts biologically inactive cortisone into cortisol, regulating intracellular cortisol availability, a mechanism reversed by HSD2[15]. Cortisol receptors are the high-affinity mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR)[14]. MR and HSD2 are highly expressed in heart, kidney, colon and hippocampus, and caused serious side effects of fluid retention and mineral imbalance when purified cortisone was first administered as an anti-inflammatory treatment in the late 1940s[14,16]. Since then, many synthetic glucocorticoids have been developed for clinical use, improving affinity to the GR, half-life and lipid permeability, while decreasing MR effects[14,16]. The main GC receptor in leukocytes is the GR, mediating the anti-inflammatory actions of GCs[14]. Transcription of GR from the *NR3C1* gene can yield isoforms GRα and GRβ[17]. GRβ is thought to exert a dominant negative effect on GRα without binding ligand and to promote GC insensitivity[17]. However, as GRα expression is considerably higher than that of GRβ in peripheral blood mononuclear cells (PBMC)[18], the present study reports GR signalling, neglecting the differentiation of isoforms.

Unbound GR is present in the cytoplasm in a complex with chaperone proteins, which increase ligand affinity, hinder degradation and are also involved in nuclear traslocation[19]. Upon ligand binding the GR is phosphorylated and translocates to the nucleus where it mediates its genomic effects[14]. Firstly, the GR binds to glucocorticoid response elements (GRE), acting as a transcription factor in a process called transactivation; negative GREs (nGRE) inhibiting transcription have also been reported[14]. Secondly, the GR can tether to other transcription factors, without direct contact to the DNA, thereby repressing their action[14]. Thirdly, the GR can bind to composite elements, by which both the GR and the composite transcription factor bind to the DNA, which leads to differential effects[14]. Furthermore, non-genomic effects of the GR are described as insertion into plasma membranes, altering cation transport or promoting proton leakage into mitochondria, interference with cytoplasmic signalling complexes, and translocation to mitochondria resulting in apoptosis[20]. Among the many read-outs of GR activity, glucocorticoid-induced leucine zipper (GILZ) is as prominent transcriptional GR target, expressed after GC stimulation and mediating anti-inflammatory downstream effects[21].

Many effects of GR activation in T cells have been reported, i.e. interference with TCR signalling via downregulation of AP-1, NFκB, NFAT and LCK, resulting in reduced proliferation and cytokine production[14,22,23]. Furthermore, GCs selectively affect some T cells subsets more than others, i.e. T_{reg} are less susceptible to GR-induced apoptosis and increase in numbers when GILZ is overexpressed[24,25]. Th1 and Th17 cells are more supressed after GC treatment through repression of IL-17α, IL-23R, RORγT, BATF, IL-12 and T-bet, possibly inducing a shift of Th1/Th17 towards Th2 and T_{reg} subsets[26–28]. GCs also decrease stimulation of T cells by downregulating expression of co-stimulatory molecules on DCs and cytokine production by DCs, i.e. CD1a, CD86, TNF-α, IL-12[29].

In summary, GCs affect a plethora of pathways. In T cells, signalling predominantly takes place via the GR. Gene expression of defined GC pathway elements *GR* and *MR*, activating enzyme (*HSD1*) and a downstream target (*GILZ*) provide a simplified read-out of T cells' potential to respond to GCs (**Figure 1.2**).

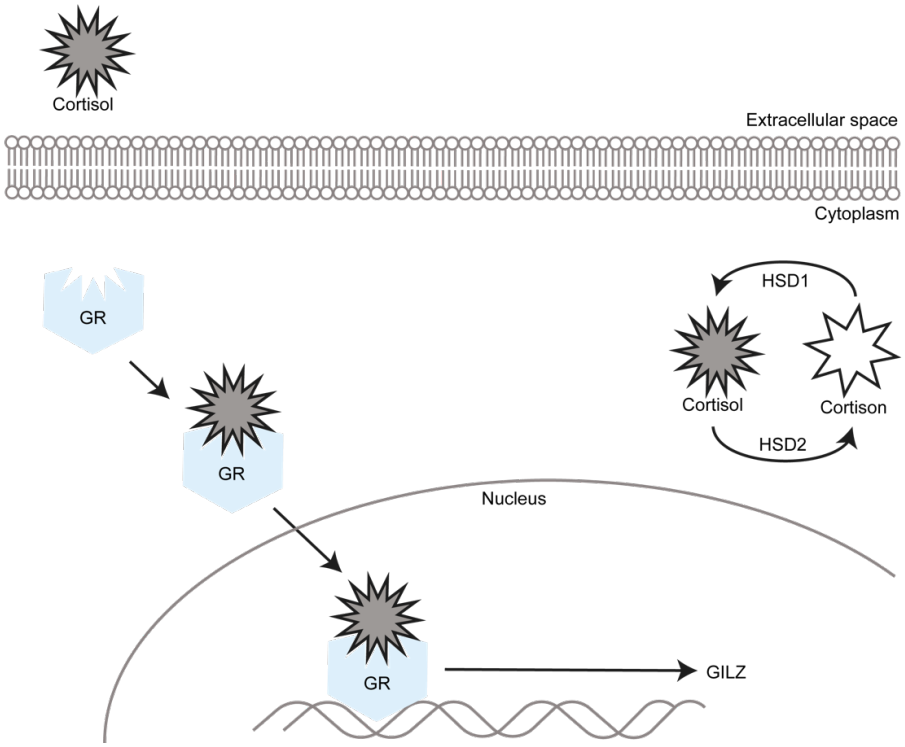

Figure 1.2: Simplified GR signalling and defined GC pathway elements examined in the present study

1.2 Multiple sclerosis

In 1838 Robert Carswell first recorded pathological lesions of multiple sclerosis (MS), which was described as a distinct disease entity in 1868 by Jean-Marin Charcot[30,31]. Today, MS affects around 2.3 million people worldwide and causing high socio-economic burden[1,32].

1.2.1 Clinical presentation

MS presents as a heterogeneous disease, with possible symptoms ranging from sensory symptoms, visual problems, autonomic dysfunction to motor impairment as well as cognitive impairment and fatigue, among others[33]. The diagnosis of MS is based on the detection of disease activity, disseminated in time and space. Indicators of disease activity are clinical symptoms, and more recently magnetic resonance imaging (MRI) with contrast enhancing agents or proof of intra-thecal antibody production[34].

Several disease subtypes of MS have been described: the most common form is relapsing-remitting multiple sclerosis (RRMS), affecting approximately 85% of patients, with disease onset in early adulthood[35]. RRMS is marked by inflammatory relapses of clinical symptoms, which initially subside within several weeks. Gradually however, remission tends to become incomplete, leading to an accumulation of disability. About 80% of RRMS patients progress to secondary progressive multiple sclerosis (SPMS) within 10 to 20 years of disease onset[35]. SPMS is characterised by slow progression of neurologic disability and atrophy as relapses become less dominant over time[35]. About 15% of MS patients are diagnosed with primary progressive multiple sclerosis (PPMS) at disease onset[32]. These patients, which typically experience disease onset a decade after RRMS patients, enter into the phase of progressive neurologic decline without evidence of inflammatory relapses[35].

1.2.2 Aetiology and epidemiology

To date, the aetiology of MS is unknown, although a multitude of environmental factors combined with genetic pre-disposition have been suggested[1]. While in the general Northern European population the risk of developing MS is estimated at 0.3%, this risk increases to 2-5% when having an affected first-degree relative, with familial re-occurrence rates of 20%[30]. Concordance of homozygotic twins is estimated between 30 and 50%[1], clearly indicating the importance of genetic factors in MS pathogenesis. Genome-wide association studies (GWAS) have found over 200 loci associated with MS risk, among them the IL-2Rα, IL-7Rα and HLA-DRB1 loci[35]. HLA-DRB1*15:01 confers an MS risk, described by an odds-ratio of over 7.0 in homozygous carriers[36]. Yet, interpreting the biological meaning of the multitude of associated loci has been challenging. Almost all of the non-MHC associations lie in non-coding regions, many are common variants and overlap with genetic risk loci found in other autoimmune diseases[36]. To date genetic studies have strengthened the notion of a complex multi-genic risk underlying MS, which requires further investigation of cell type-specific networks and epigenetics.

Environmental factors that have been proposed to impact risk of developing MS are smoking, obesity, late mononucleosis infection, cytomegalovirus infection, circadian disruption as well as geographic latitude, limited sun exposure and vitamin D deficiency[1,35]. These suggested

environmental factors are partly inspired by the global distribution of MS cases: MS prevalence increases moving north and south from the equator, with the highest prevalence in Western industrialised countries[32]. Interestingly, the environment or geographical latitude, which a person experiences before and during puberty, imposes the MS local risk; that is a child migrating from high to low risk countries or vice versa will adopt the MS risk of the destination, whereas migration during adolescence or later will not modify MS risk, acquired at the location of origin[30].

MS, as many autoimmune diseases, shows a female preponderance, affecting females approximately three times more often than males[1]. Intriguingly, this is true for RRMS, but in PPMS women and men are equally often affected[32]. Also, disease incidence of RRMS has been rising especially in females over the past decades, further increasing the sex bias in MS[37].

1.2.3 Pathology, pathogenesis and therapy

1.2.3.1 Neuropathology

The hallmark of MS pathology, first described in the 19th century, are lesions or plaques in the brain and spinal cord of patients[30]. Lesions are areas of demyelination and oligodendrocyte loss, infiltrated by mostly macrophages and CD8$^+$ T cells, fewer CD4$^+$ T cells, but also B- and plasma cells[35]. In older lesions or more progressed disease course, inflammatory infiltrates cease to be the prominent feature and glial scarring, axonal degeneration and neuronal loss are found[35]. Lesions can appear in white and grey matter of any part of the CNS and their location dictates the clinical presentation of symptoms, with spinal lesions usually causing most disability[1]. Remyelination of affected axons is possible[38], but chronic inflammation causes oxidative stress and excess glutamate stimulation, leading to mitochondrial dysfunction, intracellular calcium accumulation and compensatory redistribution of ion channels in affected axons[39]. The excitotoxicity related rise of sodium and calcium levels in neurons can at some point no longer be buffered by protective mechanisms and cell death ensues[39].

1.2.3.2 CNS extrinsic vs. intrinsic pathogenesis

The cause of MS has to date remained elusive. And while there are two opposing theories of CNS extrinsic and intrinsic MS pathogenesis, it is undisputed that multiple cell types contribute to pathology, making it difficult to pinpoint the origin of inflammatory activity.

The central hypothesis of MS puts forward that the trigger to neuroinflammation is CNS intrinsic and that infiltration of auto-reactive lymphocytes is a secondary phenomenon[35]. This hypothesis is fuelled by the fact that despite the ability of potent immunotherapy to suppress relapses, none of the licenced disease modifying therapies (DMT) seem to halt long-term neurodegeneration[39]. Further, neurodegeneration is correlated with clinical disability[39]. In addition, the phenomenon that PPMS develops without inflammatory relapse activity questions the peripheral hypothesis of MS. CNS resident cells as microglia and astrocytes can

themselves react in an inflammatory manner by producing cytokines, chemokines and reactive oxygen species (ROS)[35]. Thus, the initiation of an inflammatory cascade from within the CNS is possible, or as proposed by Dendrou and colleagues, peripheral inflammation may trigger CNS inflammation by soluble factors or nerve innervation, leading to subsequent inflammatory infiltration[35].

The peripheral hypothesis of MS suggests a CNS extrinsic cause of disease: peripheral activation and a break in tolerance to CNS self-antigens cause infiltration of immune cells into the CNS[35]. T lymphocytes mature in the thymus, where they undergo a two-step selection process, which ensures functionality of TCRs and deletion of self-reactive T cells[40]. In autoimmunity, self-reactive T cells may escape thymic selection and be activated by infection and molecular mimicry, bystander activation, co-expression of TCRs or novel auto-antigen presentation[35].

Molecular mimicry describes the cross-activation of T cells, specific to one antigen, that may also recognise another chemically similar antigen in a different context, i.e. in MS it has been proposed that T cells responsive to Epstein-Barr virus (EBV) may also respond to myelin basic protein (MBP)[41], implying that re-activation of EBV may cause demyelinating events. Bystander activation describes the phenomenon in which, during an antigen-specific immune response, T cells responsive to an unrelated antigen are activated by cytokines[42]. This antigen-independent activation has been shown for both $CD4^+$ and $CD8^+$ T cells[42]. Co-expression of more than one TCRα or TCRβ chain can occur through insufficient allelic exclusion during T cell maturation, meaning that such a T cell can theoretically respond to more than one antigen[43]. Cells with dual TCRs have been suggested to evade thymic selection and thus be potentially self-reactive[43]. Novel auto-antigens can be generated by epitope spreading, in which activated B cells ingest molecules initiating the autoimmune reaction, but then after internal processing present new, previously hidden epitopes of the self-molecule to T cells; hereby new T cell clones can be activated, hence accelerating autoimmune inflammation[6]. Likely causative cell populations in these scenarios are $CD4^+$T cells, $CD8^+$ T cells or B cells.

1.2.3.3 Immune pathology and therapy

Genetic studies (see. 1.2.2) have greatly favoured the peripheral hypothesis, as most genes implicated in MS risk are immune genes. So far, the most striking genetic associations have been found with MHC-II genes, implicating $CD4^+$ T cells as drivers of demyelinating disease. This is further supported by the fact that experimental autoimmune encephalomyelitis (EAE), the most common animal model of MS, is conferred via Th1 and Th17 cells, both in induced and genetic models[44]. Basic immunotherapies such as IFN-β, glatiramer acetate (Copaxone) and dimethyl fumarate (Tecfidera) are speculated to prevent relapses by shifting $CD4^+$ differentiation from a Th1/Th17 bias to Th2[45–47]. Further, myelin-reactive $CD4^+$ T cells have been found in the periphery of MS patients, but cells with this reactivity have also been detected in healthy donors[48]. Besides, upon examination of brain lesions in MS patients, $CD4^+$ cells are

not the major immune cell population present, but $CD8^+$ T cells are more frequent, clonally expanded and widely distributed in the parenchyma, suggesting their pivotal role in MS pathology[1,49].

Interestingly, of $CD8^+$ T cells found in MS brain lesions 5% on average are MAIT cells, but this fraction can reach up to 25%[50]. MAIT cells were reduced in frequency and produce more IL-17 in the blood of MS patients, compared to healthy controls[50,51]. Whether their homing to inflamed CNS tissue is antigen-driven and TCR-dependent or a generalised response to inflammatory cytokines is uncertain. Second line or escalation therapies are more effective in reducing relapses than the DMTs described above, but as these therapies affect both $CD4^+$ and $CD8^+$ T cells populations, it is difficult to disentangle the contributions of each cell population to MS pathology from these observations[35]. Alemtuzumab (Lemtrada) is a αCD52 monoclonal antibody, which targets mature B and T lymphocytes; fingolimod (Gilenya) is a sphingosine-1-phosphate receptor agonist, that traps lymphocytes in the lymph nodes; natalizumab (Tysabri) is a monoclonal α-very late antigen 4 (VLA-4) antigen, blocking migration of T cells across the endothelial barrier to the brain; teriflunomide (Aubagio) blocks T and B cell proliferation; cladribine (Mavenclad) is a synthetic deoxyadenosine analogue, causing depletion of T and B cells[33].

Auto-reactive B cells are also implicated in MS: clonally expanded B cells can be encountered in the meninges, parenchyma and cerebrospinal fluid (CSF), while oligoclonal bands, indicative of intrathecal antibody production, are used to diagnose MS[35]. Also, recently licenced B cell therapies rituximab and ocrelizumab (MabThea/Rituxan and Ocrevus, both αCD20) have proven their benefit in RRMS and possibly also PPMS, as ocrelizumab is the first anti-inflammatory drug licenced for PPMS[52].

Next to auto-reactive lymphocytes, a break of immunological tolerance may also be attributed to changes of their counterpart: regulatory lymphocytes. Insufficient suppression by T_{reg}, a shift in relative frequencies of naïve and memory T_{reg}, less regulatory $CD8^+$ cells or diminished production of IL-10 by regulatory B cells have been reported in MS[35].

Notably, the most potent therapy to diminish disease activity long term in highly active disease is hematopoietic stem cell transplantation, seeking to “re-boot” the immune system by eliminating all leukocytes and reconstituting with previously harvested autologous stem cells, from which then the complete immune system must again develop. This procedure seems to yield best results in young patients with high inflammatory activity but short disease duration[33]. For acute relapse treatment, high-dose corticosteroids are given orally or intravenously. Relapse treatment aims to shorten time to clinical remission, but it is not known to improve long term disability[33].

1.3 Major depressive disorder

MDD is a highly debilitating mood disorder affecting approximately 6% of the world's adult population each year[53]. The lifetime risk of experiencing at least one depressive episode is estimated between 11 and 20%, whereas female to male preponderance is 2:1[3,53].

1.3.1 Clinical presentation

Diagnostic criteria of MDD are specified in two taxonomies: the Diagnostic and Statistical Manual of Mental disorders, 5th edition (DSM-5) issued by the American Psychiatric Association[54] and the WHO's International Statistical Classification of Diseases and Related Health Problems 10th revision (ICD-10)[55]. While both classifications are widely used in clinical practice, DSM-5 is predominantly used in research[56].

DSM-5 characterises MDD as having depressed mood and/or strongly decreased interest and pleasure in most activities, present almost every day for at least two weeks. At minimum one of these key features must be accompanied by several of the following symptoms: considerable gain or loss of weight, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue, feelings of worthlessness, excessive or inappropriate guilt, indecisiveness or loss of the ability to concentrate, recurrent thoughts of death or suicidal ideation. A total of five depressive symptoms must be fulfilled for diagnosis and symptoms must be of clinical relevance, impairing daily life. Furthermore differential diagnosis is required rule out other psychiatric disorders or substances possibly causing these symptoms.[54]

The median onset of MDD is at 25 years of age[53]. Onset, disease symptoms and symptom severity do not differ considerably across countries or cultures, however access to appropriate treatment (pharmaco- or psychotherapy) and prevalence differs across countries. Notably however, prevalence of MDD across low- and high-income countries are on average comparable[3].

MDD disease course is heterogeneous with a high variation in remission, relapse and response to treatment: duration of depressive episodes has been estimated with a median around 12 weeks, but a considerably higher mean duration of 13-30 weeks[57]. Recurrence of depressive episodes is high: more than 80% of patients will go on to develop at least a second depressive episode[58]. In a population based sample, after one year, 76% of patients had recovered from a depressive episode[59]. However, amongst outpatients, recovery may be worse and in different surveys roughly 20% of patients did not remit[57,59].

Longitudinal studies have indicated, that being affected by MDD is associated with increased risk of developing various chronic medical disorders including diabetes mellitus, heart disease, stroke, hypertension, obesity, cancer, cognitive impairment or Alzheimer's disease[60].

Furthermore, MDD can provoke suicide; in fact estimates claim that half of all suicides committed take place during a depressive episode[3].

1.3.2 Aetiology

MDD, as MS, is thought to be a multifactorial disease, in which the combination of genetic susceptibility and environmental factors contribute to its manifestation. However, knowledge of the exact mechanisms of aetiopathogenesis and detectable pathobiological features remains incomplete.

Genetic contribution to risk of developing MDD is estimated at 35%[61]. Risk genes associated with MDD overlap with other psychiatric diseases as schizophrenia, bipolar disorder, obsessive compulsive disorder and anxiety disorders, but considerably less with MS and other neurologic disorders[61–63]. GWAS studies have yielded few replicable associations, however, a recent meta-analysis found 87 MDD-associated loci, many of which cluster in neuronal gene ontology (GO) terms, but also include MHC variants HLA-B, HLC-DQB1 and HLA-DQA1[64]. Environmental factors which are suggested to contribute to risk of developing MDD are stressful life events, trauma or physical abuse in childhood, but also severe infections and autoimmunity[3,65].

1.3.3 Pathology and pathogenesis

Pathological processes observed in MDD include disturbances in the central and autonomic nervous system, the immune system and neuro-endocrine systems; inflammation and hyperactivity of the HPA axis may converge in structural and functional alterations of specific brain regions and circuits, i.e. decreased hippocampal volumes, hyperactivity of brain regions conveying negative salience and a decrease of monoaminergic signalling[3,66,67]. Notably, none of these mechanisms by themselves have been able to explain MDD[3].

Classical antidepressants aim to enhance monoamine signalling in the CNS, assuming that increasing avidity of serotonin, noradrenalin or dopamine in the synaptic cleft counteracts depressive symptoms[68]. However, more recent monoamine modulating drugs such as selective-serotonin-reuptake inhibitors (SSRI) or serotonin-noradrenalin-reuptake inhibitors (SNRI) have a delayed onset of response, suggesting that they facilitate other effects than simply enhancing availability of monoamines during neurotransmission, possibly neuroplasticity, synaptic plasticity, neurotrophic support and neurogenesis[68,69]. Furthermore, the high number of non-responders to antidepressant therapy questions the monoamine-hypothesis – a third of patients do not respond to treatment after several therapeutic attempts[69].

The HPA axis has been a major focus of MDD research in the past decades. Elevated cortisol levels have been consistently reported, although variation of effect sizes between study

populations is very high and not all patients seem to be affected[70]. However, modulating HPA axis activity in clinical trials did not yield convincing results[3].

Genetic variants in the extended MHC region and cytokine encoding genes have been associated with MDD risk, pointing to an involvement of the immune system in MDD pathology[64,71–73]. Also, epidemiological findings have linked MDD risk to inflammation: a register-based Danish study found that hospitalisation for autoimmune conditions or infections increase the risk to develop subsequent mood disorders[65]. Interestingly, the number of hospitalisations for infections increased the risk of developing mood disorders in a dose-response relationship[65]. Furthermore, cytokines have been implied in depression and sickness behaviour, as treatment of chronic viral hepatitis C with IFN-α and antivirals induced depression in one out of four patients[74]. IFN-α treatment of melanoma patients also caused depression, which could be ameliorated by prophylactic antidepressant treatment[75]. Also, inflammatory challenge by endotoxin increased plasma levels of TNF-α and IL-6 in healthy controls and triggered depressed mood and feeling of social disconnect; in females cytokine levels correlated with affective symptoms[76]. In mice, depression-like sickness behaviour can be induced by elevating circulating cytokine levels, i.e. by injecting lipopolysaccharide (LPS)[77], and production of IL-6 by leukocytes is required to render animals susceptible to social stress[78].

Thus, circulating cytokines have received much attention in MDD research and meta-analyses showed increase of IL-6, TNF-α, IL-10, soluble IL-2 receptor, CC-chemokine ligand 2 (CCL2), IL-13, IL-18, IL-12, IL-1 receptor antagonist and C-reactive protein (CRP) level in MDD patients compared to healthy controls[79,80]. A recent study that assessed reproducibility among meta-analyses of different psychiatric disorders found the highest number of modulated immunological factors in MDD[81]. Next to the unspecific elevation of circulating cytokines, the cellular origin of these soluble inflammatory factors and the connection between proposed immune activation and suppression remains incompletely understood[82]. On the one hand, several studies reported inflammatory changes in innate immune cells as a shift towards non-classical monocytes, activation of monocytes, monocytic GC insensitivity and inflammasome induction in MDD patients[73,83]. On the other hand, increase of circulating T_{reg} and decreased chemokine receptor expression[84,85] suggest suppression of T cell responses. Evidence from animal studies implies that T cells may promote resilience to stress-induced depressive-like behaviour and that boosting T cell trafficking to the brain may alleviate depression and anxiety models[73,86]. Also, mildly decreased numbers of NK cells and reduced NK cell-mediated toxicity have been reported[87].

1.4 Multiple sclerosis-associated depression

1.4.1 Epidemiology

Next to motor symptoms, spasticity and visual impairment, MS patients frequently suffer from fatigue, cognitive decline and depression[33]. The 12-month prevalence of MDD in MS patients is estimated at 23.7%[88], while lifetime risk is approximated between 17 and 50%[4,89]. Clinically relevant depressive symptoms, short of a formal diagnosis, are even more prevalent at 35%[90]. These MS depression rates are higher than those in the general or populations matched for sex, age and geographic area[3,88,91]. Similarly, increased depression rates have also been reported in other chronic inflammatory conditions as rheumatoid arthritis and inflammatory bowel disease compared to matched healthy populations[92–94]. Interestingly, among the MS population, the female preponderance of MDD, which is seen in the general population, is less prominent or non-existent [91,95].

MS-associated depression is very relevant as it has been associated with poorer adherence to DMT for MS, negative effects on social life and decreased quality of life[96–98]. Anxiety co-occurs in almost half of depressed MS patients, which enhances somatic complaints and social dysfunction[89].

Depression may be associated with higher long term neurologic disability, as suggested by a Canadian register-based study, following MS patients longitudinally: presence of psychiatric comorbidity was associated with higher subsequent Extended Disability Status Scale (EDSS) scores and depression alone was also related to higher disability[99]. This finding was recently corroborated by a Swedish register-based study[100]. Yet, more than a dozen previous smaller studies have both confirmed and contradicted this finding[99,101–103]. An increase of depressive symptoms during MS relapse compared to remission has been reported[104]. Nonetheless, these findings do not exclude bidirectional impact of MS and MDD. Indeed, in the concept of a prodromal phase of MS, several register-based studies have found an increase of depression up to ten years prior to MS diagnosis, compared to matched controls[5]. Also reported were more encounters with psychiatrists[105] and in 43.5% of MS patients affected by mood or anxiety disorders evidence of these disorders was already present in the two years leading up to the first demyelinating event[99]. Notably, in contrast to other comorbidities including anxiety, depression prevalence does not decrease over time[92,106].

Taken together, these epidemiological observations indicate that depression in MS is not merely a psychological response following the diagnosis of a chronic debilitating disorder. The number of pathological processes proposed for both MS and MDD strongly suggests that both conditions are multifactorial. Most likely, the co-occurrence of MS and MDD also arises from multifactorial biological processes, but their possible interactions remain poorly understood.

1.4.2 Causal relationships linking MS and MDD

Given the presence of both MS and MDD, there are several possibilities of causal relationships between the two conditions (**Figure 1.3**).

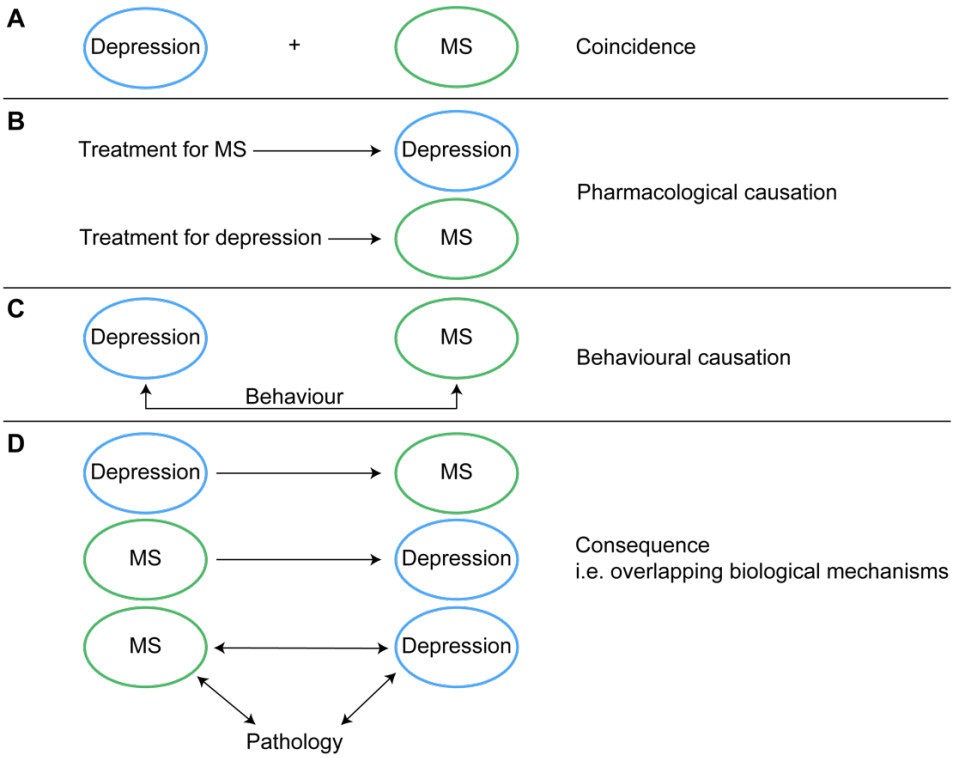

Figure 1.3: Possible causal relationships of MS and MDD

*(**A**) Coincidence, (**B**) pharmacological causation, (**C**) behavioural causation, (**D**) interconnection via overlapping pathological processes. Graph provided by Prof. Dr. S. M. Gold.*

A) The co-occurrence of MS and MDD may be a coincidence.

B) MS and MDD may be related by pharmacological causation, that is pharmacological treatment for either condition may be the cause of the other. It has been suggested that IFN-β may cause depression, although meta-analysis could not confirm this finding[107]. Also, depressed or hypomanic mood as side effects of high-dose corticosteroids in acute MS relapse treatment have been reported[108]. Antidepressant medication can have numerous side effects, but demyelinating or neurodegenerative events have not been reported.

C) MS and MDD might be linked by behavioural causation. Development of either MDD or MS may induce behavioural changes that in turn enhance risk for the other condition. Such a causation has been speculated for smoking, i.e. in depressed and anxious states people are more likely to smoke, which in turn enhances risk for developing MS or worsening the course of disease[99].

D) MS and MDD may be indirectly causal to one another by means of overlapping pathogenic pathways or processes, which render patients more susceptible to both conditions.

1.4.3 Pathogenic processes possibly overlapping between MS and MDD

In the search for possibly overlapping pathogenic processes, the literature presents several pathogenic findings that have been described both in MDD and MS independently. These Findings include hippocampal changes, inflammation and alterations of cortisol regulation. Decreased hippocampal volume has been described in MS and MDD patients[66,109]. Notably, in MS-associated depression a specific reduction in volume of the cornu ammonis (CA) 2-3 and dentate gyrus was measured compared to non-depressed MS patients and healthy controls[110]. Also, morphological changes of the right hippocampus, mainly in CA 2-3 and dentate gyrus have been found in depressed MS patients, correlating with affective, but not vegetative depressive symptoms[111]. As in MDD, increased microglia activation, indicative of neuroinflammation has been found in hippocampi of depressed MS patients[112,113]. Neuroinflammation was associated with severity of depressive symptoms and hippocampal volume in MS[113]. On top of that, a change in regional activity and functional connectivity in the limbic system during emotional processing was observed in MS-associated depression[114].

As in MDD, studies have sought to detect changes of circulating cytokines in MS-associated depression. Small studies examining all forms of MS found increased levels of circulating IL-6 and evidence of impaired redox balance of plasma proteins[115,116]. Also, elevation of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α, as well as elevated numbers of leukocytes in the CSF of MS patients was correlated with severity of depressive symptoms[117–119]. Aiming to pin-point the origin of altered circulating inflammatory mediators, increased production of IFN-γ of $CD4^+$ and $CD8^+$ T cells were found to be associated with depressive symptoms and fatigue in depressed MS patients[120,121], whereas both in MS and MDD aberrant T cell responses have been suggested (see **1.2.3.3** and **1.3.3**).

In MDD research, systemic control of cortisol by the HPA axis has been heavily studied confirming a modest elevation of cortisol in MDD patients[70,122]. Hyperactivity of the HPA axis has also been measured in MS patients without depression[123]. MS patients with depression showed elevated evening cortisol, resulting in a flattened cortisol slope compared to healthy controls and non-depressed MS patients[110,121]. Interestingly, impaired diurnal cortisol regulation was associated with elevation of leukocyte numbers in the CSF and numbers of gadolinium-enhancing lesions[110,119]. Also, cortisol slope was correlated with hippocampal volume of CA 2-3 and dentate gyrus, regions shown to express high amounts of GR and MR and to be vulnerable to damage by glucocorticoids[124,125]. On a functional level, in T cell proliferation assays, low concentrations of hydrocortisone inhibited proliferation significantly less in peripheral blood mononuclear cells (PBMC) of depressed MS patients, compared to cells of non-depressed MS patients[126], indicating possible loss of regulatory GC potential on T cells.

In summary there is accumulating evidence for hippocampal changes in MS-associated depression and alterations of the HPA axis. Despite elevated levels of cortisol, enhanced

inflammatory T cell responses have been suggested in MS patients with depression, possibly linking immunological, neuroendocrine and neurologic findings.

1.5 Aims

Epidemiological observations in MS, MDD and MS-associated depression suggest that depression is likely more than a psychological response to receiving a diagnosis of MS, but rather that MS and MDD might share pathogenic pathways explaining the high co-occurrence of the conditions. It can be hypothesised that putatively T cell-driven autoimmune inflammation in MS coincides with immune alterations in MDD, accompanied or facilitated by HPA axis hyperactivity. Endogenous GCs, regulated by the HPA axis, are among the most potent modulators of the immune system and the increased circulation thereof should result in a dampening of inflammatory responses. As this does not seem to be the case, these conflicting findings point towards a loss of regulatory potential of GCs on the immune system. A small study has shown that T cells of depressed MS patients are insensitive to lower concentrations of GCs[126]. To follow up this finding, pilot data were generated at the Institut für Neuroimmunologie und Multiple Sklerose (INIMS) which suggest that the GC insensitivity could be mediated by lower levels of *GR* and *HSD1* gene expression and significantly lower *GILZ* transcription in depressed MS patients - specifically in T cells (**Figure 1.4**).

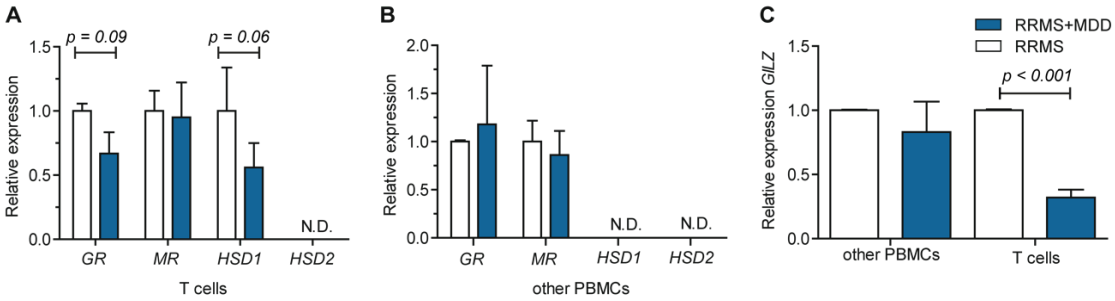

Figure 1.4: Pilot data suggest decreased expression of defined GC pathway elements

*Gene expression of defined GC pathway elements were measured by qPCR in T cells isolated by magnetic cell sorting in RRMS patients without depression (white) and with depression (blue). Relative expression GR, MR, HSD1 and HSD2 (**A**) in T cells and (**B**) in other PBMCs. (**C**) Relative gene expression of GILZ. Data are displayed relative to housekeeping gene TBP as mean and SEM of n = 7 patients per group matched for sex, age and EDSS. P values derive from one sample t-test. Data provided by Dr. Dr. K. Patas.*

Based on the evidence reviewed above, the present study aimed to elucidate T cell phenotype and GC signalling in T cells in a larger cohort of depressed and non-depressed MS patients as a putative biological substrate of MS-associated depression. Specifically, it was hypothesised that the decreased gene expression of GC pathway elements could mediate a loss of regulatory potential of GCs on T cells in MS-associated depression, as suggested by the pilot data.

Ratios of effector and memory T cell subsets vary greatly from one person to another, are influenced by environmental factors[127], and are likely to be differentially responsive to GC stimulation. Therefore, specific T cell subsets were chosen for analysis to exclude the possibility that changes in expression level of GC pathway elements might be mediated purely by

interindividual differences in ratios of memory to effector cells. Defined GC pathway elements were examined in four cell populations: $CD4^+$ memory T cells, $CD8^+$ memory T cells, T_{reg} and monocytes. Memory cells ($CD45RO^+$) were chosen as these antigen-experienced cells are most likely to react quickly to stimulation and cause inflammation. T_{reg} cells are the best known tolerogenic cells, interesting both in MS and MDD and thought to counteract activated memory T cells. Monocytes were also included in the analysis to represent the innate immune system. In idiopathic MDD patients, innate inflammation and loss of GC signalling has been suggested by decreased expression of *GR* and *GILZ* in monocytes, accompanied by a shift to pro-inflammatory monocytes. In the same cohort no changes of T cells were observed[83]. In contrast, in the present study it was expected that MS-associated depression would be mediated by changes in T cell populations, rather than innate cells.

To analyse possible GC disruption or phenotypic shifts in T cells in a well-controlled clinical setting the following aims were formulated:

1. Establishment of a well-characterised clinical cohort of 25 depressed RRMS patients, 25 RRMS patients free of depression and 25 healthy controls, matched closely for age and sex, and collection of detailed clinical and sociodemographic data as well as biological samples.

2. Analysis of the regulatory potential of GCs on T cell by measuring gene expression of defined GC pathway elements in $CD4^+$ and memory $CD8^+$ T cells, T_{reg} and monocytes, hypothesizing that memory T cells downregulate expression of GC pathway elements.

3. Measurement of circadian salivary cortisol levels as a read-out of HPA axis activity and availability of the endogenous ligand activating the GC pathway.

4. Screening for changes in immune subset composition of our cohort. To this end an extensive immunophenotyping panel was designed for flow cytometry including broad measurements of innate and adaptive immune cell populations and more detailed phenotyping of memory and naïve, regulatory and helper T cell populations as well as chemokine receptors.

To date, evidence alludes to the possibility that T cells might be the link between peripheral inflammation, neuroendocrine changes and neuronal damage of susceptible brain regions affecting mood and forming a biological substrate of MS-associated depression. Ultimately, this work sought to better understand the pathomechanisms involved in MS-associated depression.

2 Subjects, material and methods

2.1 Subjects

This study was approved by the responsible local ethics committee (Hamburger Ärztekammer, PV3792). All participants gave written informed consent prior to enrolment and received a complimentary breakfast. All patients were remunerated for their time and effort.

Patients were recruited through the Multiple Sklerose Tagesklinik of the Universitätsklinikum Hamburg-Eppendorf or through advertisement at local specialised neurologists, psychiatrists and psychologists or at the Deutsche Multiple Sklerose Gesellschaft Hamburg and their online presence. Healthy controls were also recruited through the Multiple Sklerose Tagesklinik and amongst colleagues and friends.

2.1.1 Inclusion criteria

For the MS groups, the following inclusion criteria were applied: A diagnosis of RRMS. Patients were assigned to the depressed MS group if one of two criteria was fulfilled: either the diagnosis of a current depressive episode in the mini international neuropsychiatric interview (MINI) or a depression score above the cut-off marking significant depressive symptomatology (Beck's depression inventory-II (BDI-II) score ≥ 14 and Montgomery-Åsberg Depression Rating Scale (MADRS) score ≥ 7)[128–130]. Non-depressed MS patients and healthy controls were free of depressive symptoms and had no history of depressive episodes.

2.1.2 Exclusion criteria

Exclusion criteria for all groups were:

- Other psychiatric disorders including personality disorder, schizophrenia, autism, bipolar disorder, alcohol- or substance abuse in the past 12 months
- Other autoimmune disorders including rheumatoid arthritis, asthma, psoriasis, diabetes, Hashimoto thyroiditis, lupus
- Other neurologic disorders including epilepsy, dementia, previous stroke, traumatic head injury (excluding concussions)
- Infection with hepatitis or HIV, coronary heart disease or previous myocardial infarct
- Change of antidepressant therapy or DMTs within the past three months
- Relapse or steroid treatment within the past month

- Fever in the past two months
- Vaccination in the past three months
- Pregnancy

2.1.3 Clinical assessment

Study appointments were conducted in the mornings between 8 and 10 am; the blood was sampled between 8.30 and 11 am (after a fasting period of at least 11 h). Body mass index (BMI), waist and hip circumference and blood pressure was measured for each participant. Socio-demographic information was collected in a questionnaire as well as family history of MS and psychiatric disorders.

Psychological status of all participants was assessed by a trained clinical rater using the structured MINI[131]. Depression severity was rated with the MADRS[130] by a trained rater. In case of a current depressive episode Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V)[54] specifiers of depression were assessed. All participants filled out the following self-reported questionnaires: BDI-II[129], Beck's anxiety inventory (BAI)[132], Fatigue Scale for Motor and Cognitive function (FSMC)[133] and Fatigue Severity Scale (FSS)[134]. MS patients also completed the Hamburg Quality of Life Questionnaire in Multiple Sclerosis version 10.0 (HAQUAMS 10.0)[135].

A trained neurologist recorded medical history, current medication and neurologic impairment of patients, using the EDSS[136]. Furthermore, patients completed the Multiple Sclerosis Functional Composite with the clinical observer (MSFC)[137]. The MSFC measures arm function in the nine-hole peg test (9-HPT); leg function and ambulation in the 25 foot walk; and cognition by means of the Single Digit Modality Test (SDMT), rather than using the PASAT as previously suggested due to its easier administration and better predictive validity[138]. Also, the timed tandem walk was assessed and vision was measured by the five meter visual acuity test.

Healthy controls were asked for any psychological or somatic disorders and current medication by the clinical rater and tested for cognitive function by the SDMT.

2.2 Material

2.2.1 Antibodies

Table 2.1: Antibodies for flow cytometry and fluorescence activated cell sorting

Antigen	Fluorochrome	Clone	Dilution	Company
CD3	Brilliant Violet 605	OKT3	1:300	Biolegend
CD3	Brilliant Violet 650	OKT3	1:100	Biolegend
CD4	Alexa Flour 700	RPA-T4	1:100	Biolegend
CD8a	Brilliant Violet 510	RPA-T8	1:100	Biolegend
CD8a	PE-Cy7	HIT8a	1:30	Biolegend
CD11c	PE-Dazzle	3.9	1:30	Biolegend
CD14	Brilliant Violet 711	M5E2	1:100	Biolegend
CD16	APC	B73.1	1:100	Biolegend
CD20	Alexa Flour 700	2H7	1:300	Biolegend
CD25	Brilliant Violet 421	M-A251	1:30	Biolegend
CD28	PE-Cy7	CD28.2	1:100	Biolegend
CD45	Brilliant Violet 510	HI30	1:100	Biolegend
CD45RO	Brilliant Violet 785	UCHL1	1:100	Biolegend
CD45RA	Brilliant Violet 711	HI100	1:100	Biolegend
CD56	Brilliant Violet 421	HCD56	1:100	Biolegend
CD123	PE-Dazzle	HNK-1	1:100	Biolegend
CD127	APC	A019D5	1:100 / 1:300	Biolegend
CD127	PE	A019D5	1:10	Biolegend
CD161	PE	HP-3G10	1:100	Biolegend
CXCR3 (CD183)	Brilliant Violet 711	GO25H7	1:100	Biolegend
CXCR5 (CD185)	PE-Dazzle	J252D4	1:100	Biolegend
CCR4 (CD194)	PerCP-Cy5.5	L291H4	1:100	Biolegend
CCR4 (CD194)	PE-Cy7	L291H4	1:100	Biolegend
CCR6 (CD196)	Brilliant Violet 421	GO34E3	1:100	Biolegend
CCR7 (CD197)	PE	G043H7	1:30	Biolegend
CCR10	APC	6588-5	1:100	Biolegend
PD-1 (CD279)	Brilliant Violet 605	EH12.2H7	1:100	Biolegend
Vα7.2	FITC	3C10	1:100	Biolegend
HLA-DR (MHC-II)	FITC	L243	1:100	Biolegend

2.2.2 Buffers

Table 2.2: Buffers

Buffer	Ingredients
dPBS	8 g/L NaCl
	200 mg/L KCl
	1.15 g/L Na_2HPO_4
	200 mg/L KH_2HPO_4
	in ddH_2O, pH 7.4

FACS buffer	0.01% BSA 0.02% NaN_3 in 1 x dPBS
Freezing solution 1 (sterile-filtered)	10% FCS in RPMI complete medium
Freezing solution 2 (sterile-filtered)	20% DMSO 40% FCS in RPMI complete medium
Sort buffer (sterile-filtered)	2 mM EDTA in 1 x dPBS
Sort collection medium (sterile-filtered)	25% FCS in RPMI complete medium

2.2.3 Consumables

Table 2.3: Consumables

Consumables	Company
30 µm Pre Seperation Filters	Miltenyi
5 mL polystyrene tubes non-sterile	Sarstedt
5 mL polystyrene tubes sterile	Falcon
Adhesive PCR seal	Sarstedt
Biosphere Filter Tips	Sarstedt
C-Chip	NanoEnTek
Cellstar tubes 15 mL	Greiner Bio-One
Cellstar tubes 50 mL	Greiner Bio-One
Combitips advanced	Eppendorf
Cryo.s 1.5 mL tubes	Greiner Bio-One
MicroAmp Optical 384 well reaction plate	Applied Biosystems/Thermo Fisher Scientific
Pipett tips	Sarstedt
Pipett tips with filter	Sarstedt
RNAse free tubes	Qiagen, Sarstedt
SafeSeal 1.5 mL tubes	Sarstedt
SafeSeal 1.5 mL tubes brown	Sarstedt
SafeSeal 2 mL tubes	Sarstedt
Serological pipettes	Sarstedt
Stericup 500 mL	Millipore
TC T75 flasks	Sarstedt

2.2.4 Equipment

Table 2.4: Equipment

Equipment	Company
ABI Prism 7900 HT 294 Fast Real-Time PCR system	Applied Biosystems/Thermo Fisher Scientific
BD Aria III cell sorter	BD Bioscience
BD LSR-II cell analyser	BD Bioscience
Centrifuge 5417P	Eppendorf
FlexCycler^2	Analytic Jena
Fridges (4 °C) and freezers (-20 °C, -80 °C)	Liebherr, Miele, Panasonic
Heraeus Fresco 21 Centrifuge	Thermo Fisher Scientific
Heraeus Multifuge 3SR+ Centrifuge	Thermo Fisher Scientific
Liquid Nitrogen Tank LABS-40K	Taylor Wharton
Nalgene® Mr Frosty Freezing Container	Merck
KS125 Orbital plate shaker	IKA Labortechnik
Quant Studio 6 Flex	Applied Biosystems / Thermo Fisher Scientific
µQuant Plate Reader	Bio-TEK

2.2.5 Primes and real-time PCR assays

Table 2.5: qPCR TaqMan assays

qPCR target	Gene name	TaqMan assay ID	Company
HSD1	HSD11B1	Hs01547870_m1	Thermo Fisher Scientific
IPO8	IPO8	Hs00183533_m1	Thermo Fisher Scientific
GR	NR3C1	Hs00353740_m1	Thermo Fisher Scientific
MR	NR3C2	Hs01031809_m1	Thermo Fisher Scientific
TBP	TBP	Hs00427620_m1	Thermo Fisher Scientific
GILZ	TSC22D3	Hs00608272_m1	Thermo Fisher Scientific

2.2.6 Reagents

Table 2.6: Reagents used for biological sample preparation

Reagent	Company
Biocoll	Biochrom
DMSO	AppliChem
dPBS	PAN Biotech
EDTA	AppliChem
FCS	Biochrom
Isopropanol	Roth
RPMI	PAN Biotech
S-Monovette EDTA (2.7 mL, 9 mL)	Sarstedt
S-Monovette 2.7 mL Li-Hep	Sarstedt
S-Monovette 7.5 mL Serum	Sarstedt
Salivette Cortisol, Code blau	Sarstedt
Trypan Blue	Sigma

Table 2.7: Reagents used for flow cytometry and cell sorting

Reagent	Company
Alexa-Flour 750 carboxylic acid succinimidyl ester (stock 0.8 mM in DMSO, live/dead stain)	Invitrogen / Thermo Fisher Scientific
Anti-Mouse Ig, κ/negative Compensation Particles Set	BD Bioscience
BD Cytofix	BD Bioscience
BD FACS Accu drop beads	BD Bioscience
dPBS	PAN Biotech
EDTA	AppliChem
Ethanol absolute	ChemSolute / T.H. Greyer
FACS Clean	BD Bioscience
FACS Flow	BD Bioscience
FACS Rinse	BD Bioscience
FACS Diva CS&T Research Beads	BD Bioscience
Fc block (human IgG)	Jackson's Immuno Research
FCS	Biochrom
Human serum	PAA
Lysis buffer	BD Bioscience
RPMI	PAN Biotech
Sodium azide	Roth
Sphero Rainbow Calibration Particles (8 peaks)	BD Bioscience

Table 2.8: Reagents used for gene expression analysis

Reagents	Company
DEPC-Treated H_2O	Ambion
DNase	Qiagen
QIAshredder	Qiagen
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Fisher Scientific
RNase-free DNase	Qiagen
RNAseZap	Invitrogen / Thermo Fisher Scientific
RNeasy Micro Kit	Qiagen
RNeasy Mini Kit	Qiagen
TaqMan Gene Expression Master Mix	Applied Biosystems / Thermo Fisher Scientific

Table 2.9: Reagents used for salivary cortisol analysis

Reagents	Company
Cortisol Saliva ELISA	IBL-International / Tecan

2.2.7 Software

Table 2.10: Software and algorithms

Software	Company
FACS Diva	BD Bioscience
FlowJo V10	Treestar
Thermo Fisher Connect™ RQ app	Thermo Fisher Scientific
Prism 5	GraphPad

R Studio	The R Project (non-commercial)
SDS 2.4	Applied Biosystems
SPADEVizR	Non-commercial
UMAP for FlowJo	Non-commercial

2.3 Methods

2.3.1 Preparation and storage of biological samples

2.3.1.1 PBMC isolation, cryopreservation and thawing

Blood samples were routinely processed by a laboratory technician according to the standard biobank operating procedure at the INIMS.

PBMCs were isolated from 40 – 64 mL whole blood, collected in EDTA-coated tubes by density gradient centrifugation, a method that allows isolation of large amounts of PBMCs without erythrolysis. Blood was diluted 1:1 with dPBS. 35 mL of diluted blood was layered on to 15 mL of Biocoll and centrifuged for 30 min at 860 xg. The majority of plasma was aspirated from the separated gradient, then PBMCs were carefully aspirated out of the gradient and washed twice in 50 mL cold dPBS (485 xg, 4 °C for 5 min).

Cells were suspended in 1 mL freezing medium 1 (see **Table 2.2**) for counting, then adjusted to a cell concentration of 10 million cells per mL in a 1:1 mixture of freezing medium 1 and 2. Cells were frozen in aliquots of 1 mL with approximately 10 million cells per aliquot. Cells were cooled slowly in isopropanol containing Mr Frosty freezing containers at -80 °C, decreasing temperature by 1 °C per minute. After 24 h cryovials were transferred into a bio-bank infrastructure at -195 °C and stored until further use.

For all following experiments, samples of a matched triplet were assayed together to avoid systematic bias across study groups.

When thawing PBMCs for further processing, cryovials were taken out of the liquid N_2 tank and set on ice. Cryovials were placed into a 37 °C water bath for about 1 min until only a small sphere remained frozen. Then under sterile conditions the cell suspension was transferred into a 15 mL tube, dPBS was added first drop by drop with a serological pipette, then with increasing speed up to a total volume of 10 mL, intermittently shaking the tube slightly. Cells were subsequently centrifuged (485 xg, 5 min, 4 °C), supernatant aspirated and further working steps followed.

2.3.1.2 Saliva collection and storage

Patients were instructed to sample their saliva at home in the morning at awakening and at 9 pm in the evening on the next two days following their study appointment, resulting in a total

of four samples and two biological replicates per participant. Participants received labelled sampling tubes and instructions of use during study visits.

Instructions according to the manufacturer's guidelines were to move the sampling swab around in one's mouth with one's tongue for one minute, to refrain from eating, smoking or physical activity 30 min prior to sampling and to note down the exact time of sampling. Also, participants were instructed to store all saliva samples at 4 °C until sampling had been completed and samples were returned to the INIMS by post. To avoid unnecessarily long amounts of time when temperature could not be controlled, participants were asked to avoid postage over the weekend and instead store the samples at 4 °C in the meantime.

Sample tubes were centrifuged to elute saliva out of the sampling swab (1000 xg, RT, 2 min) and aliquoted into two 1.5 mL tubes of at least 500 µL each, sample volume permitting, and then stored at -20 °C until further analysis.

2.3.2 Analysis of salivary cortisol

Salivary cortisol levels were measured with an enzyme-linked immunosorbent assay (ELISA). The assay was performed according to manufacturer's instructions. In brief, saliva samples were thawed, vortexed thoroughly and centrifuged (2000 xg, 10 min, RT). 50 µL samples, controls and standard dilutions were loaded onto a cortisol antibody-coated 96-well plate. 100 µL enzyme conjugate containing purified cortisol coupled to horseradish peroxidase (HRP) was added to the plate and incubated (2 h, RT, 500 rpm on plate shaker). The plate was washed (4 x 250 µL wash buffer) and 100 µL tetramethylbenzidine (TMB) substrate solution was added. After incubation (30 min, RT, 500 rpm on plate shaker) 100 µL stop solution containing sulphuric acid (H_2SO_4) was added to halt the reaction. Optical density, inversely proportional to cortisol amounts in the samples, were read immediately on a photometer at 450 nm wavelength. For analysis the standard curve was calculated by a four-parameter logistic curve fit using an online tool (https://www.mycurvefit.com).

Unfortunately, the manufacturer re-called the assay lot used on the complete cohort due to imprecisions in the supplied ELISA standards. Repetition of samples on a new assay lot verified a skewed standard curve and unreliable measurements at higher concentrations of cortisol in the re-called ELISA lot. Repeated analysis of the complete cohort was not possible in previously unused samples due to limited availability of aliquots. However, using samples that had been subjected to two freeze-thaw cycles, many samples could be re-measured with the new ELISA lot. Salivary cortisol levels have previously been reported to be unaffected by an additional freeze-thaw cycle[139]. Furthermore, all remaining samples received identical treatment and were thus considered comparable in terms of relative group shifts of cortisol levels.

2.3.3 Fluorescence activated cell sorting (FACS)

Cell populations were separated by FACS sorting, which disperses fluorescently labelled PBMCs into droplets containing single cells and sorts these into separate sample tubes via electric charge. This method allows for the use of a panel of surface markers to obtain well-defined cell subsets of high purity.

For cell sorting, two aliquots of each sample were thawed as described above (see 2.3.1.1.). All steps of preparation for sorting were performed under sterile conditions at a laminar flow work bench. Cells were cooled on ice or at 4 °C whenever possible and incubated in the dark. To avoid loss of cells by additional washing steps necessary for cell counting, each sample was assumed to contain roughly 15 million cells and staining concentrations were adapted accordingly. Cells were re-suspended in 96 µL of MACS buffer. The following antibodies were then added to the cell suspension in thrice the concentration used for standard flow cytometric analysis: BV605 αCD3, BV711 αCD14, BV786 αCD45RO, PE-Cy7 αCD8, BV421 αCD25, AF700 αCD4, PE αCD127 (see **Table 2.1**), as well as 7.5 µL human IgG making up a total staining volume of 150 µL. After 10 min of incubation at 4 °C, 10 µL live/dead staining (1:50 in dPBS) was added and incubated for further 20 min at 4 °C. Cells were then washed in 5 mL cold dPBS (485 xg, 5 min, 4 °C), re-suspended in 500 µL sorting medium and passed through a 30 µm cell strainer to avoid occlusion of the sort nozzle. The 15 mL tube was washed with 500 µL sorting medium, which was then also passed through the cell strainer and added to the sample, as well as 500 µL sort medium with which the cell strainer was rinsed to retrieve as many cells as possible for sorting. Total sort volume was 1.5 mL cell suspension.

Cells were sorted on a BD Aria III with a 70 µm nozzle and aerosol management. Compensation was transferred to all experiments and CS&T was routinely measured biweekly. Start-up and daily setup of the sorter were done in parallel to cell thawing and staining. Samples were processed in two batches: samples of triplets 1 - 13 were stained individually just prior to sorting. For the remaining triplets, samples of a triplet were stained simultaneously and kept on ice in the dark until being sorted. To avoid introducing systematic bias in the second batch, the order in which samples of the different groups were sorted was varied from triplet to triplet.

Populations that were sorted were monocytes ($CD14^+$, $CD3^-$), $CD8^+$ memory cells ($CD3^+$, $CD8^+$, $CD45RO^+$), T_{reg} ($CD3^+$, $CD4^+$, $CD25^+$, $CD127^{-/low}$), $CD4^+$ memory cells ($CD3^+$, $CD4^+$, not T_{reg}, $CD45RO^+$) (**Figure 2.1**). Before gating on the fluorescent surface markers, cellular debris, dead cells and doublets were excluded; monocytes and lymphocytes were separated by size and granularity. Sort gates were set conservatively to ensure purity of the sorted cell population. Gates were adjusted to each sample after having recorded a test measurement of 10000 events to ensure correct sorting despite variation between subjects.

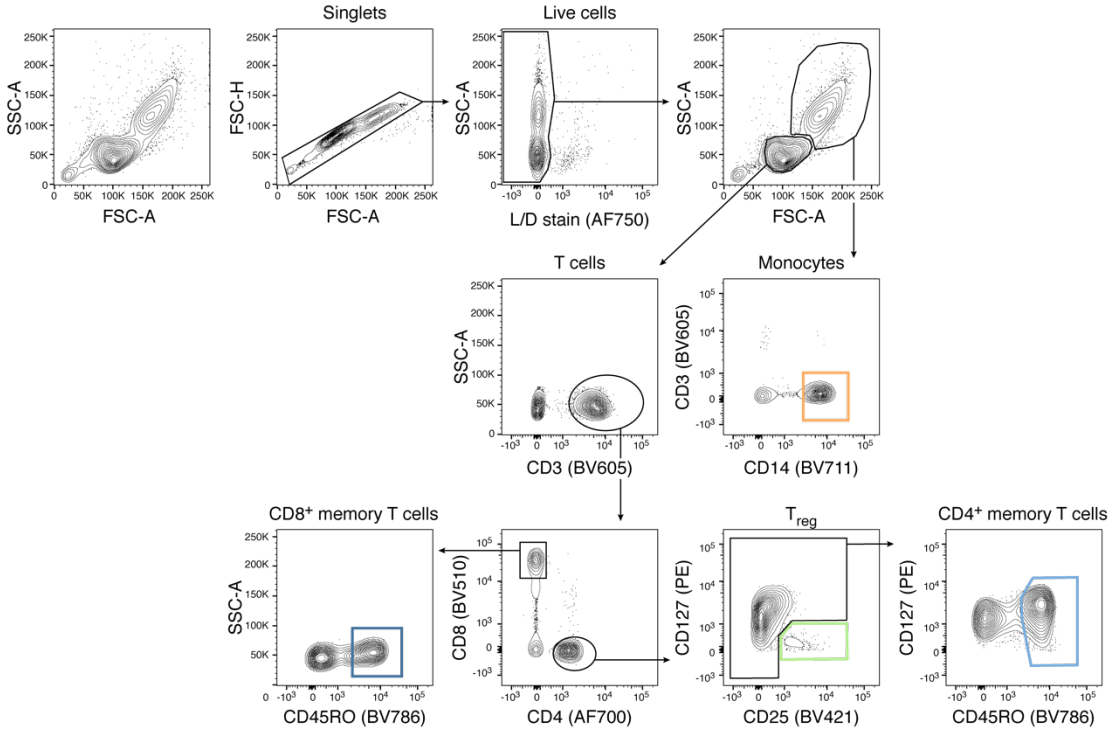

Figure 2.1: Sort strategy of cell populations for qPCR analysis

FACS gating strategy to sort for monocytes (yellow gate), $CD8^+$ memory T cells (dark blue gate), T_{reg} (light green gate), $CD4^+$ memory T cells (light blue gate).

Between 10 and 30 million cells per sample were sorted (**Figure 2.2A**). Sorting took about 60 to 75 min per sample, with an event rate of up to 5000 events/s. If the event rate reached more than 5000 events/s or the efficiency dropped below 90%, the volume of the cell suspension was increased to ensure sufficient sort efficiency at the expense of increased duration of the sort. Cells were collected in cooled uncoated 5 mL tubes containing 1 mL sorting collection medium. After sorting, cells were transferred into 2 mL tubes and pelleted at 3000 rpm, 4 °C for 5 min. Supernatant was carefully aspirated and dry pellets snap-frozen in liquid nitrogen. Pellets were stored at -80 °C until further processing. Purity checks showed that typically a sort purity of ≥ 90% in T_{reg} and ≥ 95% for the other subsets was achieved (**Figure 2.2B**). Sorting yield varied between samples and expectedly, between cell populations (**Figure 2.2C**).

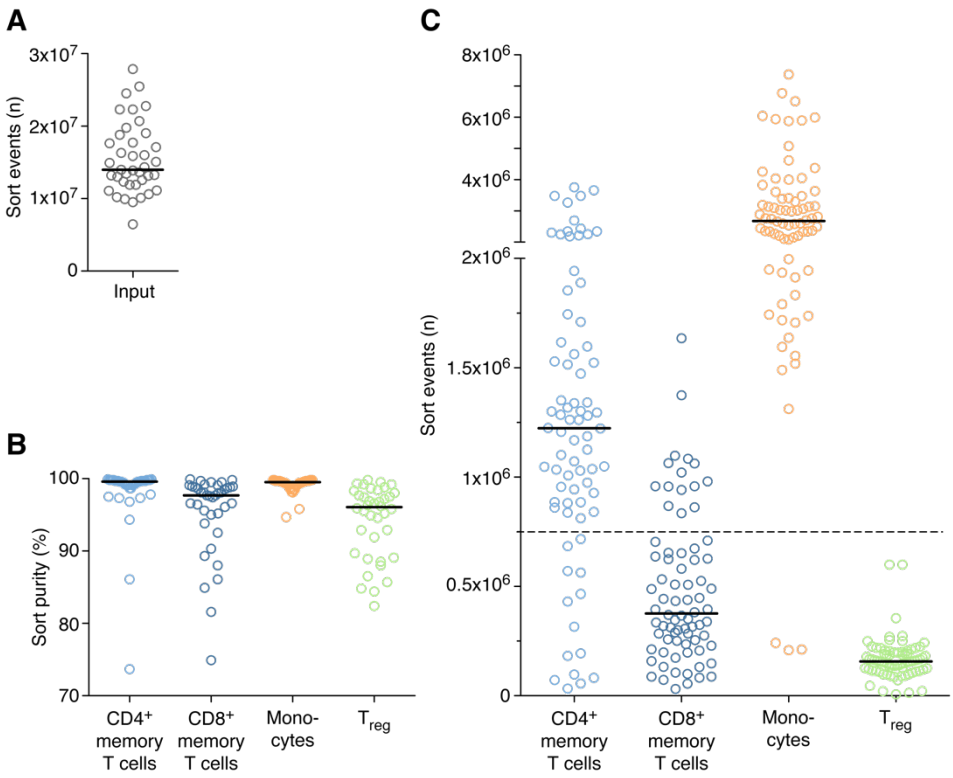

Figure 2.2: Sort yield, input and purity

*(**A**) Total event count during sort, representing the number of PBMCs as input to sorter. (**B**) Purity of each sorted cell population. (**C**) Sort yield as sorted events for each cell population. Data are displayed as individual samples and median (n = 75 per cell population): $CD4^+$ memory T cells (light blue), $CD8^+$ memory T cells (dark blue), monocytes (yellow) and T_{reg} (light green). The dashed line in **C** depicts the cut-off at which samples were processed with the different RNA kits.*

2.3.4 Analysis of gene expression

To analyse gene expression quantitative polymerase chain reaction (qPCR) was performed. For this method RNA is isolated, transcribed into cDNA and then quantified during amplification. Here we used TaqMan assays, which consist of target-specific non-fluorescent primers and a probe coupled to a fluorescent reporter and quencher.

2.3.5 RNA isolation

RNA was isolated with the Qiagen RNeasy Mini and Micro kits. All centrifugation and incubation steps were performed at room temperature.

Sorting yield and thus cell numbers in the dry pellets greatly differed between subjects and cell subsets. Therefore, two different RNA kits were used, in which protocols and reagents were identical and only the columns differed in RNA binding capacity and elution volume. Pellets with less than 750 000 cells were isolated on RNeasy Micro columns, larger pellets were isolated on RNeasy Mini columns (separation marked by dashed line in **Figure 2.2C**), while following the protocol of Qiagen's RNeasy Micro kit according to manufacturers' instruction. In brief, pellets were thawed on ice, resuspended in 350 µL RLT buffer, vortexed thoroughly and passed

through a QIAshredder (9000 xg, 15 s). 350 µL 70% ethanol was added and mixed well by pipetting. The cell lysate was transferred to a RNeasy spin column and RNA was bound to the silica membrane by centrifugation (9000 xg, 15 s). Remnants of cell lysate were washed off with 350 µL RW1 (centrifuged at 9000 xg, 15 s). Remaining DNA was digested by adding 80 µL DNase I working solution directly onto the membrane and incubating for 15 min, followed by several washing steps (350 µL RW1, 9000 xg 15 s; 500 µL RPE, 9000 xg 15 s; 500 µL 80% ethanol, 9000 xg 2 min). In between washing steps flow through was discarded, only after DNase digestion and the last washing step a new collection tube was used. Then the column was opened and dried (21000 xg, 5 min) before setting it into a new collection tube to elute the RNA: to RNeasy Micro columns 14 µL of RNase-free water was added, to RNeasy Mini columns 30 µL of RNase-free water was added and incubated for 3 min prior to centrifugation (21000 xg, 1 min).

To exclude contamination by genomic DNA (gDNA), DNase treatment was included in the RNA isolation protocol and exon-spanning primers were used. The absence of DNA was confirmed during establishment by use of controls missing reverse transcriptase during cDNA synthesis, which gave no signal during qPCR. After establishment this protocol was considered to routinely exclude gDNA.

2.3.5.1 cDNA synthesis

cDNA was synthesised using the RevertAid H Minus First Strand cDNA Synthesis Kit. Briefly, 1 µL random hexamer primers were annealed to 11 µL of RNA at 65 °C for 5 min. 4 µL 5x reaction buffer, 1 µL Ribolock RNAse inhibitor, 2 µL deoxyribose nucleoside triphosphate and 1 µL reverse transcriptase were added and carefully mixed. For primer annealing the mix was initially heated to 25 °C for 5 min, then cDNA synthesis took place at 42 °C for 60 min and was terminated at 70 °C for 5 min. cDNA was stored at -20 °C until quantification by qPCR.

2.3.5.2 qPCR

Analysis of the target genes *GR*, *GILZ*, *MR* and *HSD1* was performed on ABI Prism 7900 HT 294 Fast Real-Time PCR system (triplets 1 - 13) and Quant Studio 6 Flex (triplets 14 - 25), with *IPO-8* and *TBP* as housekeeping genes. Within cell subsets, analysis of matched triplets for both target and housekeeping genes were run on the same plate.

qPCR was run on 384 well plates with 10 µL total volume per well (pre-mixed: 5 µL TaqMan gene expression master mix, 2.5 µL H_2O, 0.5 µL TaqMan probe and 2 µL cDNA template (diluted 1:1 in water)). Each plate contained a non-template control per target. qPCR was started with 2 min 50 °C and 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s for denaturation and 60 °C for 1 min of primer alignment and polymerisation.

For analysis SDS 2.4, and the Thermo Fisher Connect™ RQ app were used: first triplets were inspected for technical errors or grave outliers, C_t values of over 35 were excluded. Then ΔC_t

values and relative expression were calculated in R using the mean of both housekeeping genes as reference.

2.3.6 Flowcytometric immunophenotyping

For immunophenotyping, fluorescent staining of surface proteins was analysed by flow cytometry. The panel used here consisted of three stainings of up to 12 antibodies, which were known markers of immune cell subsets, expanding on recommendations for human immunophenotyping[12].

Staining I: BV421 αCD56, BV510 αCD45, BV605 αCD3, BV711 αCD14, FITC αHLA-DR, PE-TexasRed αCD11c, PE-Cy7 αCD123, APC αCD16, AF700 αCD20, AF750 live/dead stain, Fc block

Staining II: BV421 αCD25, BV510 αCD8, BV605 αPD-1, BV650 αCD3, FITC αHLA-DR, PerCP-Cy5.5 αCCR4, PE αCCR7, PE-Cy7 αCD28, APC αCD127, AF700 αCD4, AF750 live/dead stain, Fc block

Staining III: BV421 αCCR6, BV510 αCD8, BV605 αCD3, BV711 αCXCR3, BV785 αCD45RO, FITC αVα7.2, PE αCCR7, PE-TexasRed αCXCR5, PE-Cy7 αCCR4, APC αCCR10, AF700 αCD4, AF750 Live/Dead stain, Fc block; (all antibodies see **Table 2.1**)

Antibodies were mixed on the day prior to the staining procedure as a cocktail thrice the final concentration. L/D stain pre-mix was prepared on the day of staining. For analysis one aliquot of cryopreserved PBMCs was thawed, as described above (see 2.3.1.1). Cells were resuspended in 1 mL dPBS, counted, and 1 million cells were transferred into one tube per staining and an unstained control. Volume was adjusted to 60µL, 30µL antibody cocktail was added and each tube was vortexed. After an incubation of 10 min (RT, in the dark) 10 µL L/D stain pre-mix (1:100 in dPBS) was added, vortexed and the cells were incubated (20 min, RT, in the dark), then washed with 1 mL dPBS, centrifuged (5 min, 485 xg, RT) and supernatant decanted. Cells were fixed by adding 100 µL BD Cytofix and incubating (20min, RT). After another washing step with 1 mL dPBS (485 xg, RT), cells were resuspended in 300 µL FACS buffer for analysis.

Flow cytometric measurements were always carried out on the same day or the day following staining on a BD LSRII cell analyser using BD FACS Diva software. The flow cytometer was calibrated by CS&T measurement on each day of analysis and application settings as well as standardised compensation where applied throughout measurements.

2.3.6.1 Analysis of immunophenotyping

Immunophenotypic flow cytometry data were analysed in an unsupervised manner by use of FlowJo, the FlowJo Uniform Manifold Approximation and Projection (UMAP) plugin[140] and the

SPADEVizR package in R[141]. Data were pre-processed and cleaned prior to automated data analysis to remove noise. For each staining this was achieved by selecting events in the time gate of constant data spread in the side scatter, excluding debris, doublets and dead cells and then gating on a target subpopulation. In staining I the target population included all leukocytes, marked by CD45 (**Figure 2.3**); in stainings II and III $CD3^+$ T cells were selected as the target population for automated analysis (**Figure 2.4** and **2.5**). Data were then checked for correct compensation and data spread between subjects for each staining. Compensation was manually adjusted where necessary. Random down-sampling to 10k events per sample was performed, then samples of the same staining were concatenated into one file, maintaining information on sample, triplet and group identity as keys. Concatenated data files were fed into two unbiased analyses: the UMAP algorithm and the SPADEVizR algorithm.

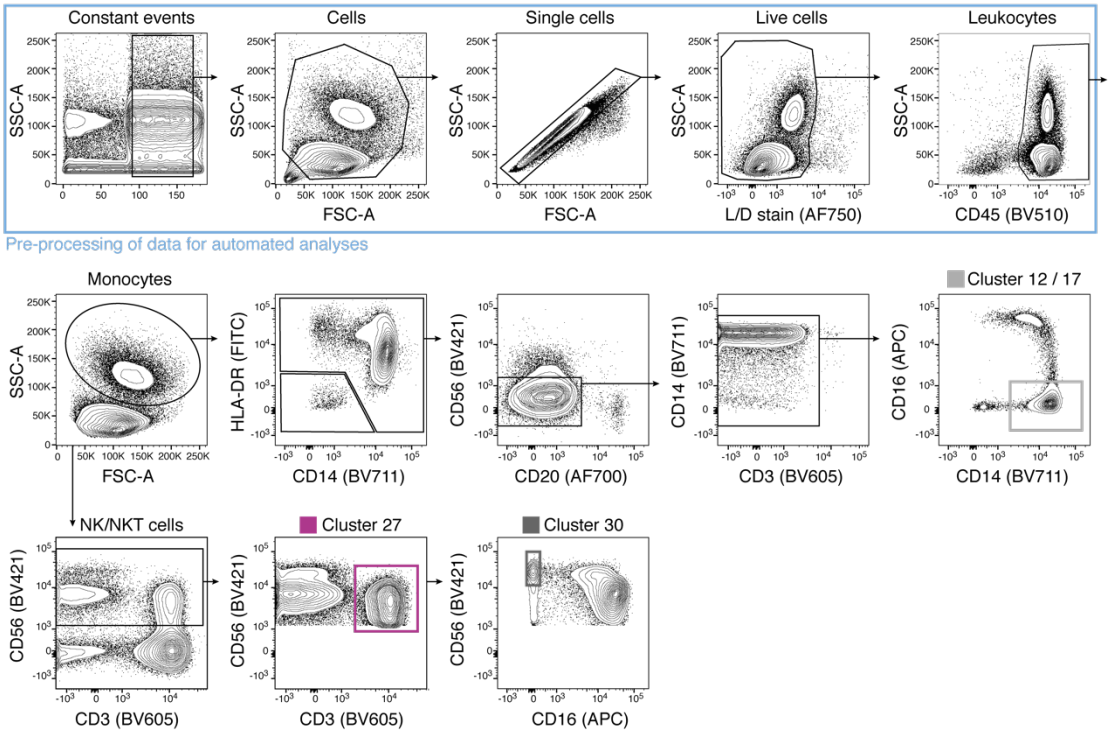

Figure 2.3: Manual gating strategy of staining I and pre-processing

The data files containing the pre-processed events from all donors were first clustered by SPADE into 30 clusters. Then each cluster was compared in abundance between groups using a students' t-test. Clusters that were significantly different in frequency between two of the three study groups ($p < .05$) were considered clusters of interest. As a quality control, equal contribution of each sample to the clusters of interest was verified. For each sample, clusters of interest were next manually gated in FlowJo (**Figure 2.3, 2.4, 2.5**) for each sample. Absolute numbers were calculated by referencing to blood counts: in staining I lymphocyte and monocyte numbers were equated to live, single, CD45+ cells; in staining II and III, live, single cells falling into the FSC/SSC lymphocyte gate were equated to lymphocyte number in the blood count. Frequencies of clusters of interest were analysed relative to the stainings' target population and a parent gate. Effects detected in automatic clustering were reported as verified if the manually

gated data showed significant differences in one omnibus test and at least one *post hoc* test with correction for multiple comparisons.

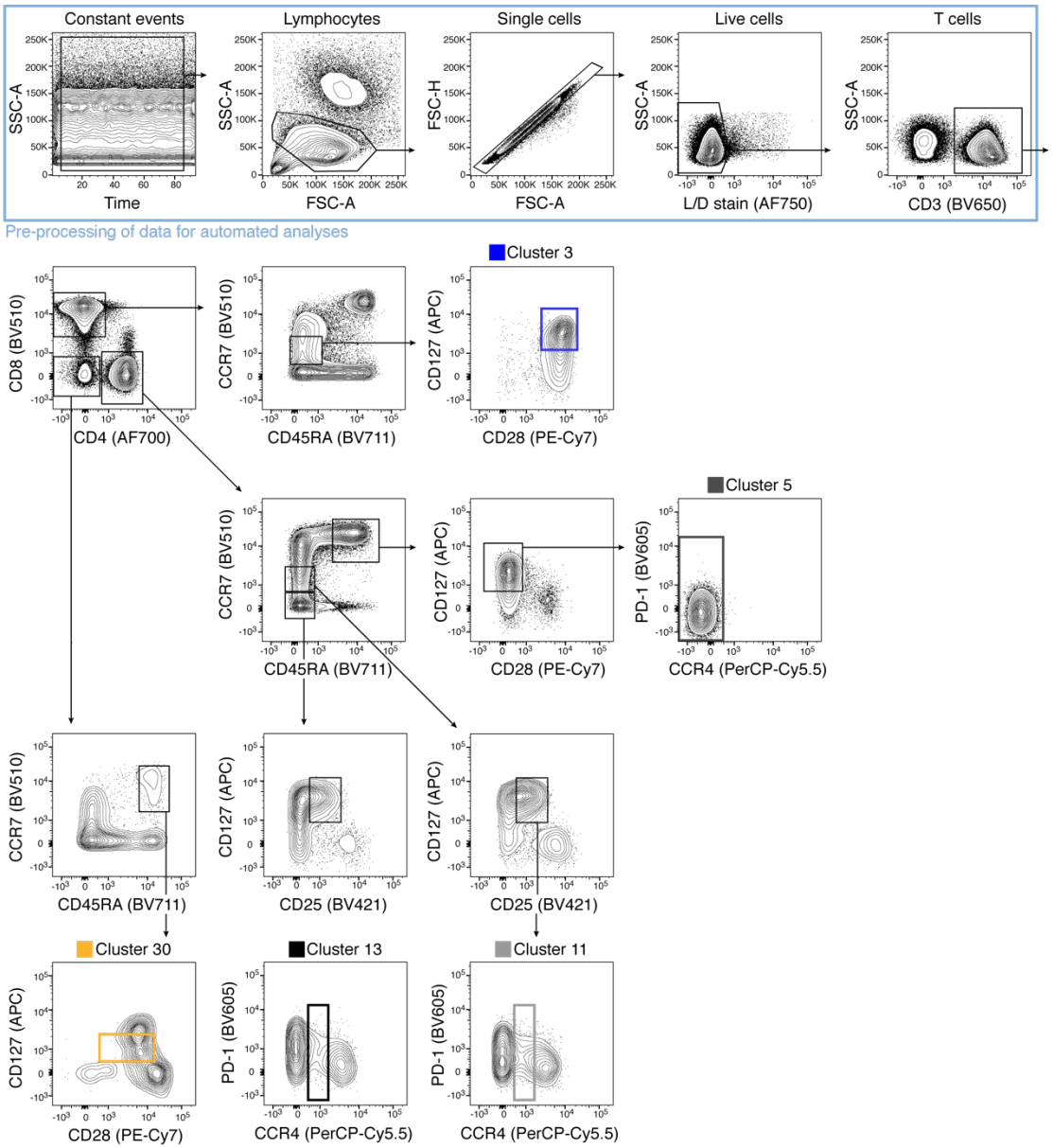

Figure 2.4: Manual gating strategy of staining II and pre-processing

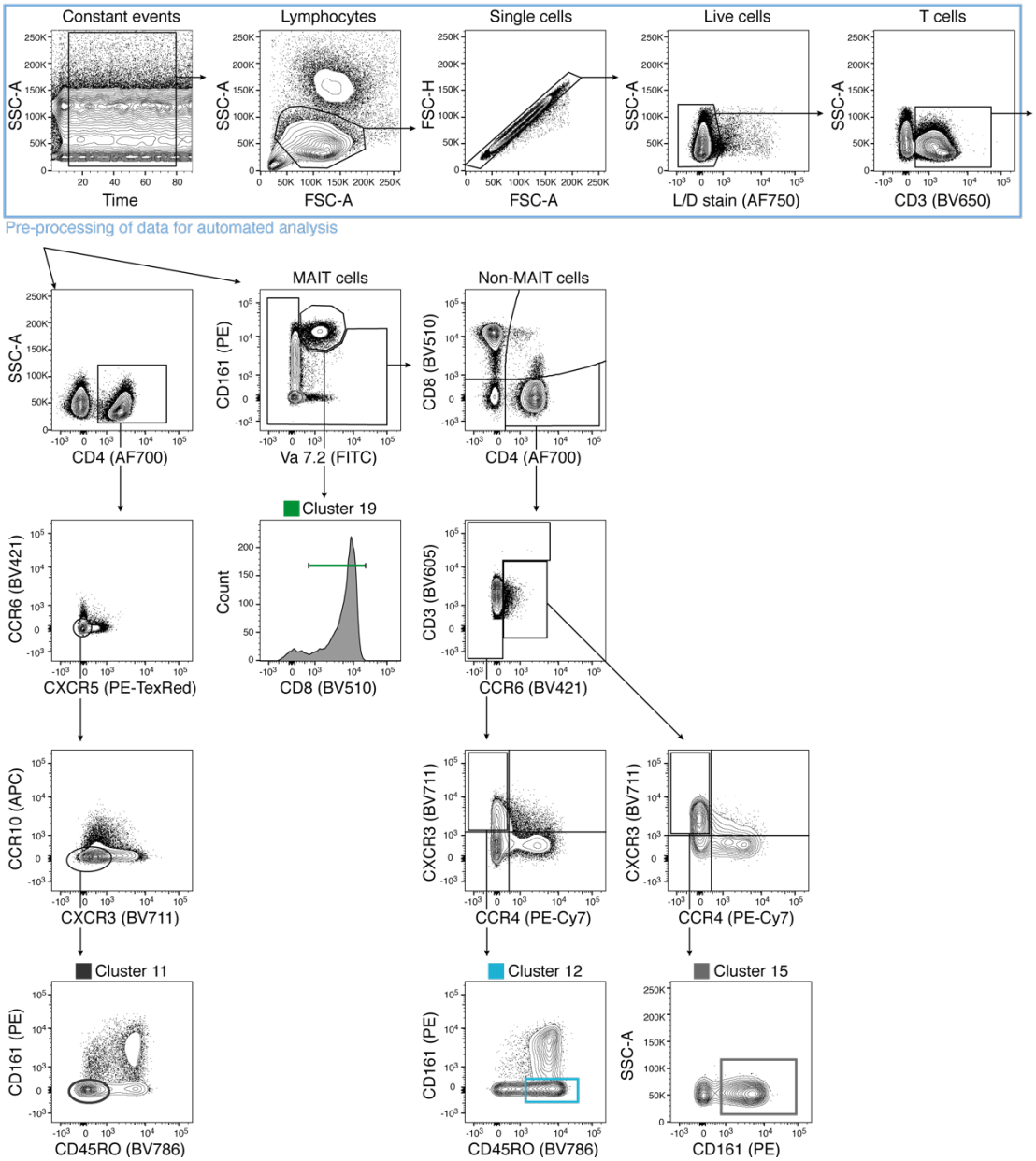

Figure 2.5: Manual gating strategy of staining III and pre-processing

The UMAP algorithm reduces dimensionality of multidimensional parameters (here all fluorescent channels per staining without live/dead, SSC, FSC and CD45 or CD3, respectively; Euclidean distance, nearest neighbours: 15, minimum distance 0.5) to two-dimensional space, allowing visualisation and exploratory analysis of multidimensional data. The UMAP map of each group (20k events per group) was displayed as a density plot representing cell frequencies and overlaid with manual gates of clusters of interest as identified above.

2.3.7 Statistics

Statistics were performed in R. *P* values < .05 were considered significant and *p* < .1 were considered a trend. As triplets of healthy controls, non-depressed MS patients and MS patients

were closely matched, data generated from a triplet were treated as paired observations where possible.

For socio-demographic data, metabolic data, blood counts and psychological descriptors variables of ordinal scale and above were analysed by Friedman's tests. If a significant difference was detected in this omnibus test, Wilcoxon paired tests were conducted as *post hoc* tests with Bonferroni correction for multiple comparisons (p values ≤ .017 were defined as significant). Categorical variables were tested using Pearson's Chi squared test. MS-descriptors were analysed by Wilcoxon paired tests. Correlation was assessed by Spearman's rank correlation.

For qPCR and ELISA data, missing data points did not allow for pairing, so Kruskal-Wallis tests were performed, followed by *post hoc* (non-paired) Wilcoxon tests with Bonferroni correction for multiple comparisons (p values ≤ .017 were defined as significant).

FACS data was analysed first in an exploratory and unsupervised manner by the SPADEVizR algorithm (for detailed information see **2.3.6.1.)**. This algorithm has built-in statistical testing that compares cluster abundance for each cluster between two groups by student's t-tests. Clusters that were significantly different in abundance between two of the three study groups (unadjusted p value < .05) were considered clusters of interest. Secondly, to confirm the exploratory analysis, all clusters of interest identified by SPADEVizR were manually gated and quantified in absolute numbers, relative to the stainings' target population (staining I: leukocytes; staining II and III: T cells) and to another relative parent gate. For the manually gated data differences across groups were tested by Friedman's tests. If in omnibus tests yielded p values < .05, a Wilcoxon paired tests was conducted as *post hoc* tests with Bonferroni correction for multiple comparisons (p ≤ .017 were regarded as significant). Group differences of a cluster of interest, were regarded as confirmed by manual analysis, if at least one of the comparisons (absolute numbers or a relative quantification) showed a significant difference across groups and at least one significant *post hoc* test after correction for multiple comparisons (p < .017) confirmed a difference in pair-wise group comparisons.

In boxplots, omnibus tests are depicted by a dashed line; post hoc tests are represented by solid lines and only depicted if the corresponding omnibus test was significant. Number of observations per group and tests used are specified in the figure legends, table legends or appendix (**Table 7.1 – 7.4**). Tables show mean (IQR) and respective statistic unless otherwise specified. Boxplots generally display median, 1st and 3rd quartile, as well as whiskers defined as 1.5 times the inter quartile range (IQR) subtracted from the 1st quartile and 1.5 times the IQR added to the 3rd quartile. Datapoints falling outside the whiskers are depicted as outliers but were included in all analyses. Figures were created in R or FlowJo and assembled in Adobe Illustrator.

3 Results

3.1 Cohort description

To address our hypothesis in a larger cohort of patients, we collected data from 25 healthy controls (HC), 25 MS patients (MS) and 25 MS patients suffering from depression (MS+MDD). Participants were matched in triplets by age and sex. Detailed socio-demographic, clinical and psychological data were recorded in order to provide a thorough cohort description and to identify possible confounders.

3.1.1 Socio-demographic descriptors

As **Table 3.1** indicates, age and sex did not differ between groups as a result of this rigorous matching. Also, hormonal contraception and somatic comorbidities were not overrepresented in any of the study groups. However, current employment status and years of education differed significantly across groups in omnibus tests, but no significant differences remained in adjusted *post hoc* tests. Furthermore, the most current smokers were amongst the depressed MS patients, but the difference across groups was not significant.

Table 3.1: Socio-demographic data

All data shown as median (IQR) unless otherwise specified. For categorical variables the Pearson's Chi-squared test for count data was applied, for variables of ordinal scale and above the Friedman's test was applied, using paired Wilcoxon tests with Bonferroni correction as post hoc tests. Bold digits indicate significance.

Variable	HC	MS	MS+MDD	Statistic	Post hoc test
Age in years	39 (16)	39 (18)	40 (12)	$X^2_{(df=2)} = 2.4$ $p = .307$	NA
Females / males, n (%)	22 (88) / 3 (12)	22 (88) / 3 (12)	22 (88) / 3 (12)	$X^2_{(df=2)} = 0$ $p > .999$	NA
Comorbidities (n): none / A / AH / HT / M / O	18 / 3 / 0 / 0 / 2 / 3	19 / 1 / 2 / 2 / 0 / 2	12 / 4 / 1 / 0 / 1 / 5	$X^2_{(df=10)} = 12.6$ $p = .249$	NA
Employment: at least part time / less than part time / not employed	18 / 3 / 4	17 / 4 / 4	11 / 1 / 13	$X^2_{(df=4)} = 11.3$ $\mathbf{p = .023}$	NA
Years of school / university education	13 (3)	16 (6)	10 (3)	$X^2_{(df=2)} = 7.4$ $\mathbf{p = .024}$	HC - MS: $p = .937$ HC - MS+MDD: $p = .024$ MS - MS+MDD: $p = .022$
Current smokers, n (%)	2 (8)	5 (20)	9 (36)	$X^2_{(df=2)} = 5.9$ $p = .053$	NA
Hormonal contraception among female participants, n (%)	7 (31.82)	8 (36.36)	4 (18.18)	$X^2_{(df=2)} = 2.4$ $p = .305$	NA

A: allergy, AH: arterial hypertonus, HT: hypothyroidism, M: migraine, O: other (among these: nail fungus, rosacea, thyroid nodules, anaemia, factor-V-Leiden mutation, hereditary polyneuropathy, neurodermatitis, renal artery stenosis, scoliosis)

3.1.2 MS descriptors

As displayed in **Table 3.2,** MS patients were matched pairwise for DMTs as closely as possible: 22 of 25 pairs could be successfully matched; healthy controls were all DMT and antidepressant free. A variety of disease and disability descriptors commonly used in MS research and clinical

care were recorded for all MS patients during the study visit. Depressed and non-depressed MS patients did not differ significantly in terms of DMTs, disease duration, disease progression and relapses in the past year. Of note, 11 matched triplets were free of DMTs or antidepressant therapy. Neurologic disability measured by the EDSS was slightly higher in the depressed MS group, yet the difference was not significant. Disability as measured by the MSFC did not reveal any group differences in fine motor hand function (9-HPT), walking ability (25-foot walk) or cognitive function (SDMT). Balance, gait and coordination measured by the timed tandem walk, also did not differ between groups. Only visual acuity of the right eye was significantly different between groups. As MS groups differed significantly in only one of many descriptors, it can be concluded that the level of disability between MS groups was not of relevant difference.

Table 3.2: Multiple sclerosis descriptors

All data shown as median (IQR), paired Wilcoxon tests were applied. Bold digits indicate significance.

Variable	MS	MS+MDD	Statistic
DMT (n): untreated / 1st line / 2nd line / monoclonal antibodies	12 / 5 / 6 / 2	11 / 5 / 5 / 4	$X^2_{(df=3)} = 0.8$ $p = .849$
Disease duration (years)	6 (7)	5 (7)	V = 172.5 $p = .798$
EDSS	1.5 (1)	2.0 (1.5)	V = 62 $p = .053$
Progression index	3.3 (5.2)	2.5 (3.5)	V = 221.5 $p = .115$
Participants with at least one MS relapse in last year, n (%)	8 (32)	11 (44)	$X^2_{(df=1)} = 0.3$ $p = .56$
Visual acuity left / right	1 (0.25) / 1 (0.25)	1 (0.49) / 1 (0.34)	V = 120, $p = .319$ / V = 117.5, **$p = .011$**
9-HPT dominant hand / non-dominant hand (s)	17.58 (2.81) / 18.65 (5.8)	17.97 (3.8) / 20.05 (3.5)	V = 128, $p = .546$ / V = 110, $p = .264$
25-foot walk (s)	3.87 (0.72)	4.14 (1.67)	V = 98 $p = .143$
SDMT (standard deviations from age/education appropriate controls)	0.5 (1.5)	-0.125 (1.25)	V = 176 $p = .11$
Timed tandem walk (s)	9.04 (5.64)	9.86 (5.32)	V = 113 $p = .303$

1st line: interferon, glatiramer acetate; 2nd line: fumarate, teriflunomid, fingolimod; monoclonal antibodies: alemtuzumab, daclizumab, natalizumab, rituximab

3.1.3 Psychological descriptors

As summarised in **Table 3.3**, participants were asked to complete several self-reported questionnaires as well as undergo observer-rated assessments (MINI, MADRS) to characterise depressive symptoms as well as to record common symptoms in MS which may overlap with depression, i.e. fatigue. Depression scores of the BDI-II and MADRS were factors contributing to the definition of the MS-associated depression group (see **2.1.1**) and were thus expected to be different between study groups. Despite having BDI-II scores below the cut-off of mild depression, significant group differences in the BDI-II were observed between healthy controls and non-depressed MS patients[129]. Significant differences between groups were also detected in measures of anxiety (BAI) and fatigue, both of the motor and cognitive domain (FSS, FSMC total, -motor, -cognitive). The *post hoc* analysis detected significant differences not only between depressed MS patients and healthy controls, but also between healthy controls and depression-free MS patients. Notably, the number of psychological comorbidities encountered in the MINI

interview among MS patients with depression was considerably higher compared to the other two groups. Amongst the MS patients with depression only five participants (20%) were currently experiencing their first episode of depression.

Table 3.3: Psychological descriptors

All data shown as median (IQR). For comorbidities detected by MINI the Pearson's Chi-squared test for count data was applied, for all other variables the Friedman's test was applied, using paired Wilcoxon tests with Bonferroni correction as post hoc tests. Bold digits indicate significance.

Variable	HC	MS	MS+MDD	Statistic	Post hoc test
BAI score	2 (3)	4 (6)	17 (16)	$X^2_{(df=2)} = 30.7$ ***p* < .001**	**HC - MS: *p* = .008** **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
BDI score	1 (2)	5 (4)	24 (6)	$X^2_{(df=2)} = 41.7$ ***p* < .001**	**HC - MS: *p* = .008** **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
MADRS score	1 (2)	2 (3)	15.5 (6.5)	$X^2_{(df=2)} = 39.2$ ***p* < .001**	HC - MS: *p* = .625 **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
FSS score	1.4 (0.7)	1.8 (2.7)	5.7 (2.03)	$X^2_{(df=2)} = 32$ ***p* < .001**	**HC - MS: p = .006** **HC - MS+MDD: p < .001** **MS - MS+MDD: p < .001**
FSMC total score	3 (5)	17 (21)	53 (26)	$X^2_{(df=2)} = 39.8$ ***p* < .001**	**HC - MS: *p* < .001** **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
FSMC motor score	1 (3)	8 (11)	25 (12)	$X^2_{(df=2)} = 35.3$ ***p* < .001**	**HC - MS: *p* < .001** **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
FSMC cognitive score	1 (3)	6 (15)	28 (14)	$X^2_{(df=2)} = 35.3$ ***p* < .001**	**HC - MS: *p* = .001** **HC - MS+MDD: *p* < .001** **MS - MS+MDD: *p* < .001**
Occurrence of MINI diagnoses: none / A / D / M / PTSD / S / other	25 / 0 / 0 / 0 / 0 / 0 / 0	21 / 2 / 0 / 2 / 0 / 0 / 0	1 / 20 / 18 / 4 / 2 / 10 / 3	$X^2_{(df=12)} = 94$ ***p* < .001**	NA
Patients experiencing their first episode of depression (n)	-	-	5	NA	NA

A: Panic disorder, agoraphobia, social phobia, generalised anxiety disorder; D: Major depressive disorder, dysthymia; M: hypomanic episode in past; PTSD: Post-traumatic stress disorder; S: Suicidal ideation; other: Bulimia, obsessive-compulsive disorder

3.1.4 Metabolic and inflammatory descriptors

Risk of MDD has been linked to metabolic syndrome and obesity[60]. Therefore, metabolic descriptors were documented as possible confounders. CRP was measured as an unspecific marker of inflammation.

As **Table 3.4** displays, the study groups did not differ significantly from one another in terms of BMI, but MS patients with depression had significantly higher waist-hip ratios than healthy controls, although the median values were well below the WHO recommended cut-off for abdominal obesity (> 0.85 for females and > 0.90 for males)[142]. Four participants had a BMI > 32. No differences across groups were seen for blood pressure, high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol, triglycerides or CRP levels.

Taken together, although study groups were not matched for BMI, there were no group differences of metabolic descriptors or CRP overall, apart from differences in waist-hip ratio.

Table 3.4: Metabolic descriptors

All data shown as median (IQR). Friedman's tests were applied, using paired Wilcoxon tests with Bonferroni correction as post hoc tests. Bold digits indicate significance.

Variable	HC	MS	MS+MDD	Statistic	Post hoc test
BMI (kg/m^2)	22.5 (3.3)	23.03 (4.8)	23.10 (4.2)	$X^2_{(df=2)} = 2.8$ $p = .246$	NA
Waist-hip ratio	0.74 (0.07)	0.76 (0.14)	0.79 (0.1)	$X^2_{(df=2)} = 7$ **$p = .03$**	HC - MS: $p = .121$ **HC - MS+MDD: $p = .009$** MS - MS+MDD: $p = .178$
Systolic blood pressure (mmHg)	120 (15)	119.5 (12.75)	120 (11.25)	$X^2_{(df=2)} = 1.4$ $p = .5$	NA
Diastolic blood pressure (mmHg)	70 (10)	75 (10)	70 (14.25)	$X^2_{(df=2)} = 5.5$ $p = .065$	NA
HDL (mg/dL)	68.5 (18)	68 (13.25)	67 (18)	$X^2_{(df=2)} = 1.9$ $p = .378$	NA
LDL (mg/dL)	94 (61.5)	100.5 (51.75)	95 (34)	$X^2_{(df=2)} = 0.3$ $p = .854$	NA
Total cholesterol (md/dL)	181.5 (60.75)	192 (48.5)	176 (32)	$X^2_{(df=2)} = 0.9$ $p = .646$	NA
Triglycerides (mg/dL)	76 (56.75)	90.5 (55)	74 (47.25)	$X^2_{(df=2)} = 0.4$ $p = .8$	NA
CRP (mg/L)	< 5 (0)	< 5 (0)	< 5 (0)	$X^2_{(df=2)} = 0.4$ $p = 0.819$	NA

3.2 Analysis of cell-specific gene expression within the GC signalling pathway

The present study had hypothesised a memory T cell-specific regulatory deficit of GCs, marked by decreased gene expression of *GR* and *HSD1*, but not *MR* in depressed MS patients compared to the other groups. As a consequence, lower expression levels of the downstream element *GILZ* were also expected in MS-associated depression, reflecting decreased GR signalling. This change of expression of defined GC pathway elements had been hypothesised to be especially pronounced in T cells, while in innate immune cells as monocytes no changes in gene expression were expected. To assess the present hypothesis, gene expression of *GR*, *GILZ*, *HSD1* and *MR* was analysed in $CD4^+$ memory T cells, $CD8^+$ memory T cells, T_{reg} and monocytes.

Neither *GR* (**Figure 3.1A**) nor *GILZ* (**Figure 3.1B**) were differentially expressed on mRNA level across groups in any of the examined cell subsets. Similarly, neither *HSD1* (**Figure 3.1C**) nor *MR* (**Figure 3.1D**) showed significant expression differences between groups in any of the immune cell subsets analysed. *HSD1* could only be reliably detected in $CD4^+$ memory T cells.

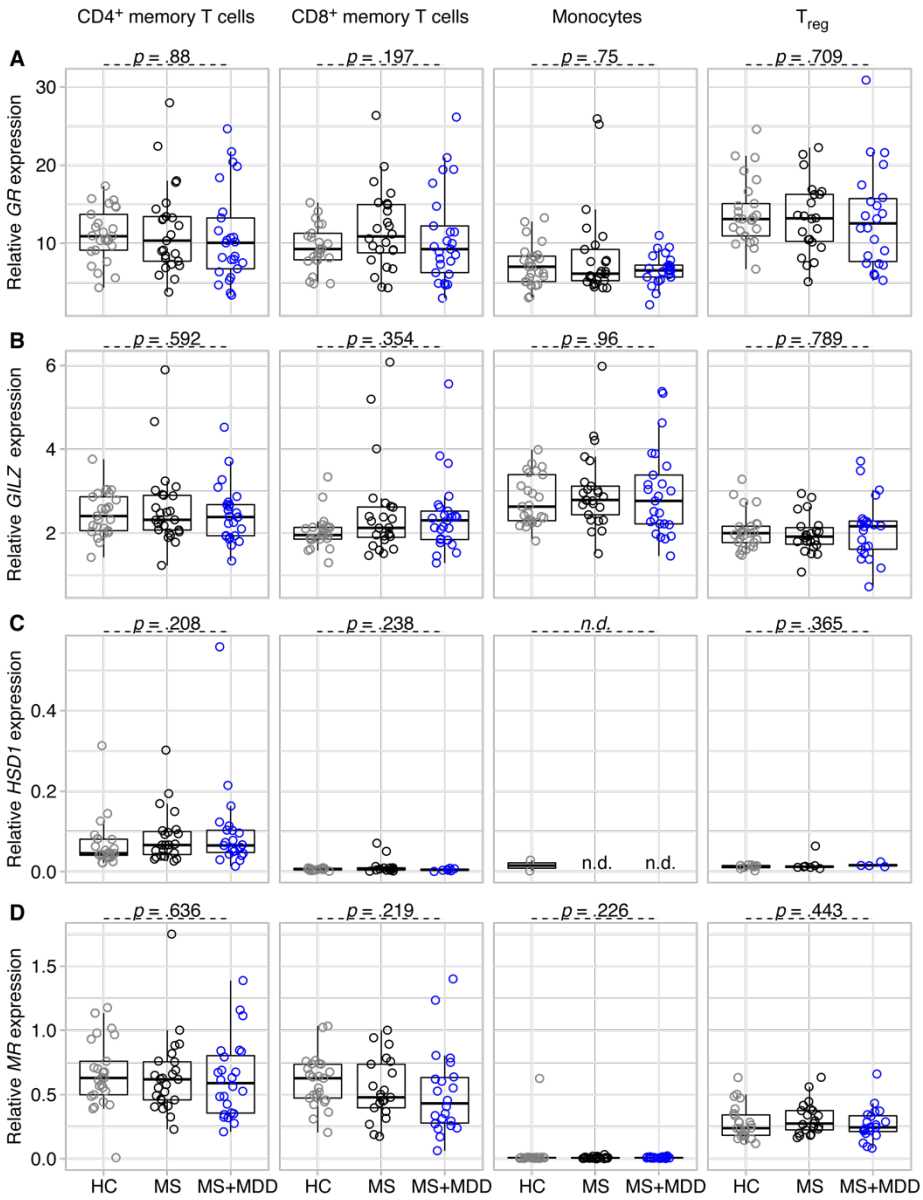

Figure 3.1: Expression of defined GC pathway elements was not altered in MS-associated depression

*mRNA expression of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes TBP and IPO8 in CD4⁺ memory T cells (first column), CD8⁺ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with individual data points, each representing an individual (n see **Table 7.1**): healthy controls (HC; grey), non-depressed MS patients (MS; black), depressed MS patients (MS+MDD; blue). P values derived from Kruskal-Wallis tests.*

As a quality control, sorting parameters were examined to exclude a systematic bias due to sample preparation. Sort purity and sort yield did not vary systematically across groups (**Figure 7.1**). When displaying sort yield (**Figure 7.2**) and purity (**Figure 7.3**), none of these parameters showed a systematic variation across the levels of relative gene expression, suggesting that sample preparation did not systematically affect the expression levels measured. Furthermore, the mean cycle threshold (C_t) values of the housekeeping genes did not vary systematically with BDI-II score, confirming that the chosen housekeeping genes *IPO8* and *TBP* were well suited to normalise gene expression in T cells and monocytes, and were not themselves modulated in depressed patients (**Figure 7.4**). Predictably, the C_t values measured for different cell populations varied considerably.

The housekeeping gene C_t value reflects the amount of cDNA loaded into the qPCR, when housekeeping genes are well chosen. As the different cell populations varied greatly in their sort yield (see **Figure 2.2A**) and RNA amounts transcribed into cDNA were not assimilated, this result was expected.

The study cohort was heterogeneous for DMTs and antidepressants, which could potentially confound effects of MS and depression on the defined GC pathway elements studied here. Also, sex differences could occlude differential regulation of genes. Therefore, as a next step subgroup analysis of the aforementioned data was performed on more homogeneous, but smaller cohorts. Subgroup analysis of DMT- and antidepressant free participants (**Figure 7.5**), female participants (**Figure 7.6**), and female participants free of DMTs or antidepressants (**Figure 7.7**) revealed no group differences in relative gene expression of *GR*, *GILZ*, *MR* or *HSD1* in any of the interrogated cell populations. Also, none of the mRNA targets analysed correlated with the BDI-II depression scores in all cell populations (**Figure 7.8**), nor did age seem to influence gene expression levels (**Figure 7.9**).

Taken together, these results do not support the hypothesis of a cell-specific decrease in gene expression of defined GC pathway elements in MS-associated depression in $CD4^+$ or $CD8^+$ memory T cells, T_{reg} or monocytes.

3.3 Endogenous cortisol levels

As hyperactivity of the HPA axis has been reported in both MDD and MS, this raised the question whether this phenomenon is also present in MS-associated depression, thus altering the circulating levels of the endogenous ligand to the GC pathway.

No differences were observed in levels of morning or evening cortisol between healthy controls, MS patients or MS patients with depression (**Figure 3.2A**). The delta between morning and evening measurements of cortisol was also not significantly different between the study groups (**Figure 3.2B**).

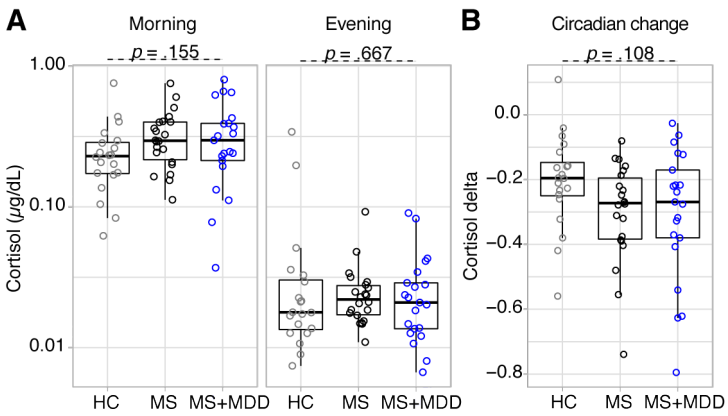

Figure 3.2: Circadian cortisol levels did not differ between healthy controls and MS patients

*(**A**) Morning and evening cortisol. (**B**) Change of circadian cortisol levels throughout the day as delta between morning and evening cortisol. Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 20; grey), MS patients (MS; n = 21; black), depressed MS patients (MS+MDD; n = 22; blue). P values derived from Kruskal-Wallis tests.*

3.4 Immunophenotyping and leukocyte shifts in MS-associated depression

The previous data did not reveal evidence of GC signalling alterations in memory T cells nor hypercortisolaemia in MS-associated depression. For the fourth aim of the present study, detailed immunophenotyping combined with blood cell counts were used to screen for possible phenotypic shifts of immune cell populations in MS-associated depression.

3.4.1 Higher numbers of neutrophils in depressed MS patients

Absolute numbers of lymphocytes (**Figure 3.3A**) and monocytes (**Figure 3.3B**) were not different between study groups. However, absolute and relative numbers of neutrophils were significantly increased in depressed MS patients compared to healthy controls (**Figure 3.3C, Table 3.5**). Depression-free MS patients also displayed a trend to higher numbers of neutrophils compared to healthy controls, but this comparison did not pass the adjusted threshold of significance ($p < .017$)Neutrophil number correlated with BDI-II score of depression (rho = .244; $p = .039$), additionally a correlation of similar degree could be noted between neutrophil number and the MADRS score, yet the correlation was not significant (rho = .222; $p = .062$). Further, the neutrophil-to-lymphocyte ratio (NLR) was significantly elevated in MS-associated depression compared to healthy controls (**Figure 3.3D**).

The relative frequency of lymphocytes was significantly lower in depressed MS patients compared to healthy controls, but not in absolute numbers. It is likely that the relative decrease of lymphocytes was caused by the increased absolute number of neutrophils, whereas absolute lymphocyte counts remained unchanged. Other parameters of the blood cell count did not differ across experimental groups (**Table 3.5**).

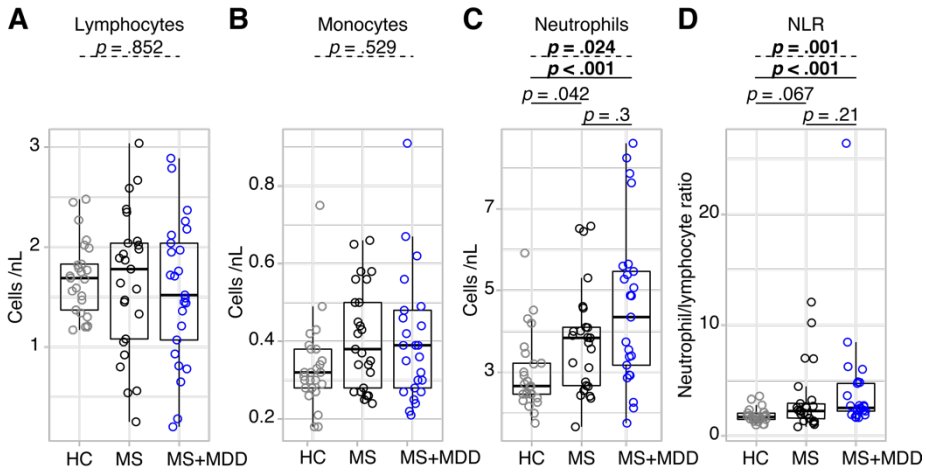

Figure 3.3: Higher numbers of neutrophils in depressed MS patients compared to healthy controls

*Absolute numbers of (**A**) lymphocytes (**B**) monocytes and (**C**) neutrophils. (**D**) Neutrophil-to-lymphocyte ratio of absolute cell counts. Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 25; grey), MS patients (MS; n = 25; black), depressed MS patients (MS+MDD, n = 25, blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

Table 3.5: Blood counts

All data shown as median (IQR). The Friedman's test was applied, using paired Wilcoxon tests with Bonferroni correction as post hoc tests. Bold digits indicate significance.

Variable	HC	MS	MS+MDD	Statistic	Post hoc test
Haemoglobin (g/dL)	13.2 (0.8)	13.6 (1)	13.6 (0.9)	$X^2_{(df=2)} = 4$ $p = .133$	NA
Haematocrit (L/L)	39.8 (2.6)	40.8 (2.6)	41.2 (3.8)	$X^2_{(df=2)} = 4.9$ $p = .087$	NA
Erythrocytes (cells/pL)	4.5 (0.24)	4.6 (0.38)	4.59 (0.66)	$X^2_{(df=2)} = 1.6$ $p = .441$	NA
Leukocytes (cells/nL)	5.2 (1.3)	6.2 (2.5)	6.5 (3.2)	$X^2_{(df=2)} = 5.5$ $p = .065$	NA
Thrombocytes (cells/nL)	261 (86)	239 (48)	222 (73)	$X^2_{(df=2)} = 4.9$ $p = .087$	NA
Neutrophils (cells/nL; %)	2.71 (0.42); 56.45 (6.9)	3.84 (1.43); 60.40 (14.2)	4.35 (2.3); 63.9 (10.2)	$X^2_{(df=2)} = 7.4$ **$p = .024$**; $X^2_{(df=2)} = 8.9$ **$p = .012$**	HC - MS: $p = .042$ **HC - MS+MDD: $p > .001$** MS - MS+MDD: $p = .3$; HC - MS: $p = .071$ **HC - MS+MDD: $p > .001$** MS - MS+MDD: $p = .085$
Lymphocytes (cells/nL; %)	1.71 (0.42); 32.95 (8.32)	1.78 (0.96); 26.3 (11.8)	1.52 (0.97); 24.8 (12.1)	$X^2_{(df=2)} = 0.3$ $p = .852$; $X^2_{(df=2)} = 12.6$ **$p = .002$**	NA; HC - MS: $p = .071$ **HC - MS+MDD: $p > .001$** MS - MS+MDD: $p = .055$
Monocytes (cells/nL; %)	0.32 (0.1); 6.85 (1.8)	0.38 (0.22); 6.3 (1.2)	0.39 (0.2); 6 (2.3)	$X^2_{(df=2)} = 1.3$ $p = .529$ $X^2_{(df=2)} = 1.8$ $p = .399$	NA; NA
Eosinophils (cells/nL; %)	0.11 (0.04); 1.75 (1.03)	0.14 (0.1); 2.1 (2.7)	0.13 (0.08); 2 (2.5)	$X^2_{(df=2)} = 2.8$ $p = .248$; $X^2_{(df=2)} = 0.02$ $p = .99$	NA; NA
Basophils (cells/nL; %)	<0.1 (0); 0.7 (0.3)	<0.1 (0); 0.6 (0.3)	<0.1 (0), 0.5 (0.3)	$X^2_{(df=2)} = 1.1$ $p = .584$; $X^2_{(df=2)} = 5.8$ $p = .056$	NA; NA

3.4.2 Immunophenotyping staining I: lymphoid and myeloid subsets

Staining I of the immunophenotyping panel included surface markers to classify T cells, B cells, NK cells, monocytes and some DC sub-populations. SPADEVizR analysis of staining I grouped

cells into 30 clusters (**Figure 3.4A**) and identified four clusters of interest: three clusters that were significantly enriched in depressed MS patients compared to healthy controls (clusters 12, 17 and 30) and one cluster that was significantly enriched in healthy controls compared to depressed MS patients (cluster 27) (**Figure 3.4B**). No cluster was significantly different in abundance between MS patients and healthy controls or MS patients and depressed MS patients.

Next, manual gating (**Figure 2.3**), informed by the expression of surface markers within each cluster (**Figure 3.4A**), was used to verify the differential abundance of the four clusters of interest. To compare manual gating and automatic clustering, histograms displaying the surface marker expression of each of the manual gates (**Figure 3.5A**) were compared visually to the surface marker expression of the automatic grouping (**Figure 3.4A**). It was concluded that the manually gated cell populations were reasonably similar to automatically identified clusters.

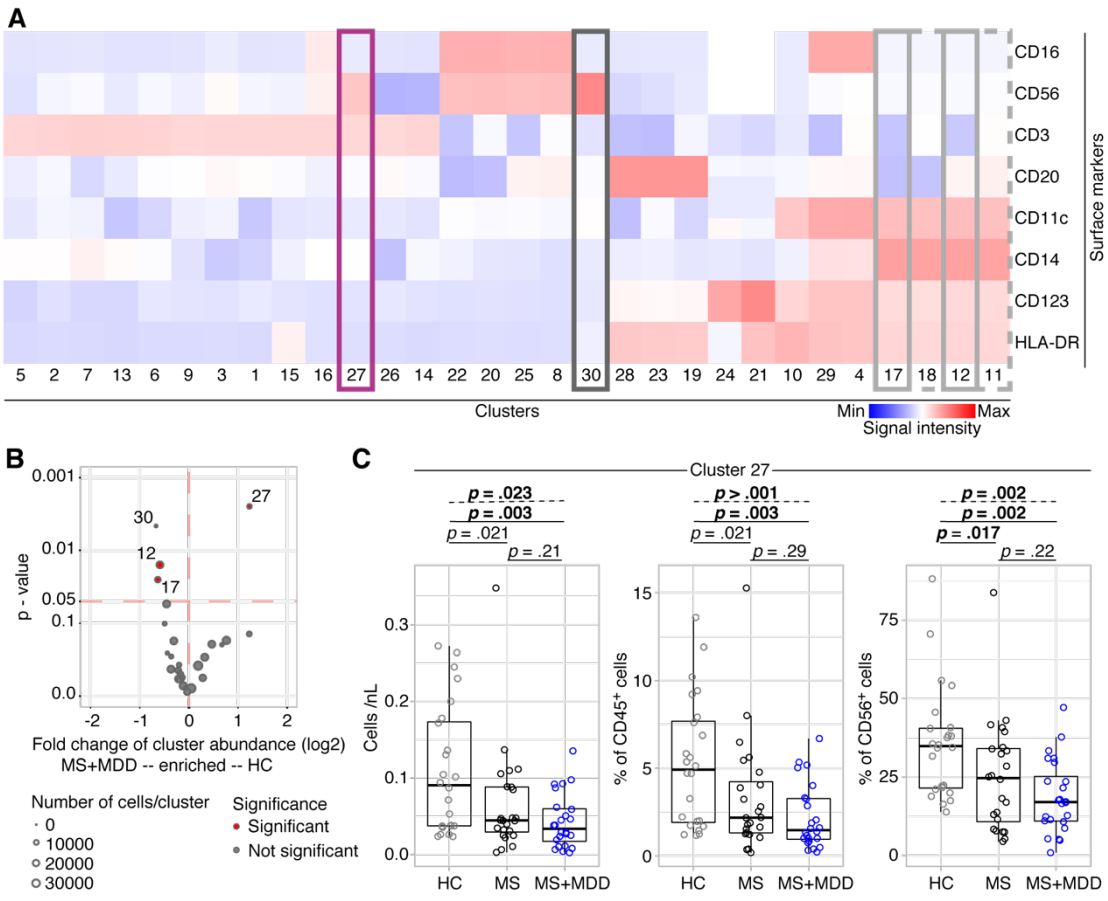

Figure 3.4: MS Patients showed decreased frequency of CD56⁺ T cells

(A) *Automated clustering of flow cytometry data of staining I into 30 clusters, revealed four clusters that were significantly enriched or de-enriched in depressed MS patients compared to healthy controls (**B**). (**C**) Manual quantification of cluster 27 (quantification of other clusters see **Figure 3.6**). In **A**, boxes highlight the clusters of interest identified by SPADEVizR. When manual gating expanded across several clusters, the additional clusters are marked by a dashed line. **B** shows clusters ordered according to their differential abundance between groups and the corresponding p value derived from student's T test. The size of each cluster is depicted by the diameter of plotted circles. In **C** data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

Clusters 12 and 17 were similar in terms of surface marker expression ($CD16^-$ $CD56^-$, $CD11c^+$, $CD14^{++}$ $CD123^+$ $HLA\text{-}DR^+$) and only differed from clusters 18 and 11 in the signal intensity of CD3 and CD20 antibodies within the "negative population" of these markers. This negative signal shift was likely due to data spread during measurement rather than being of biological relevance. Therefore, in the manual gating clusters 11, 12, 17 and 18 were gated together, resembling established gating strategies for classical monocytes[12] (**Figure 2.3**).

Cluster 27 was defined by high expression of CD56 and CD3 whereas all other surface markers of the staining gave negative signals; these markers could be present on a variety of T cells as discussed below. Cluster 30 was highly positive for CD56, but negative for all other markers and was most likely a $CD56^{bright}$ $CD16^-$ subset of NK cells.

As an overview of the flow cytometry data, dimensionality reduction by UMAP was performed in addition to automated clustering. Density plots for each group illustrate shifting densities of clusters, which depict changes in frequency of sub-populations. Clusters that vary in density across groups match the clusters of interest resulting from the automated clustering approach (**Figure 3.5B**).

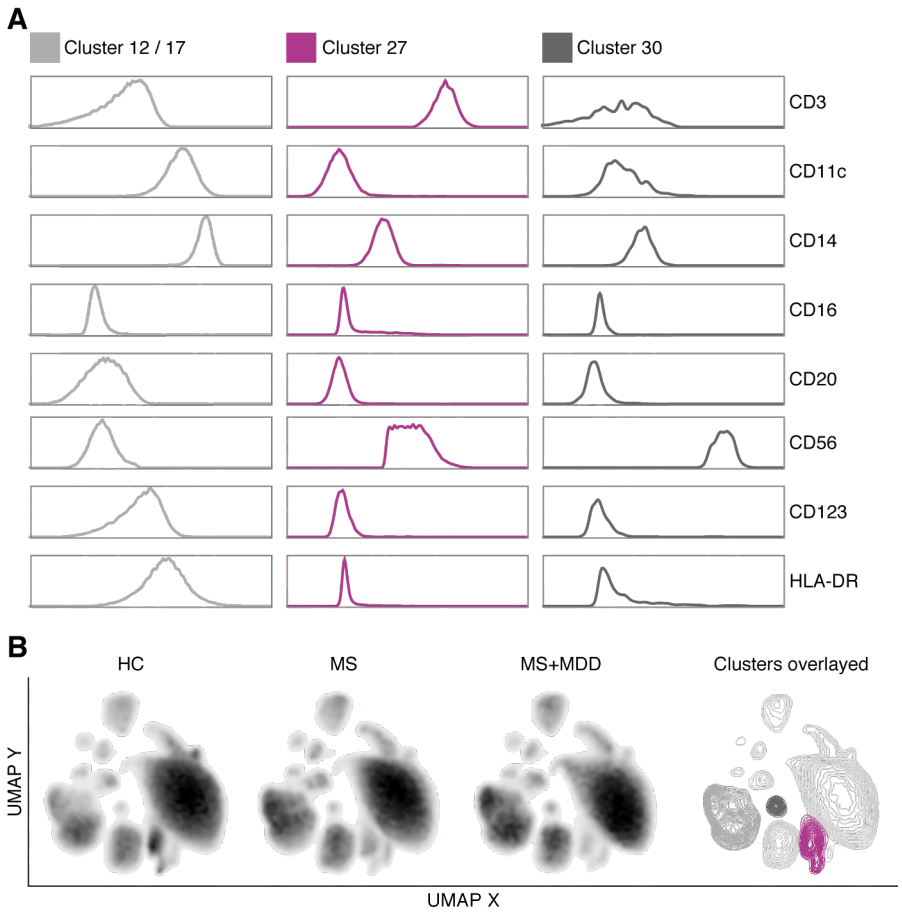

Figure 3.5: Manual gating of clusters of interest identified in staining I

*Manual gating of clusters 12/17, 27 and 30 informed by surface marker expression of each cluster (shown in **Figure 3.4A**) was performed (for quantification see **Figure 3.4C** and **Figure 3.6**). (**A**) Marker expression of each manually gated cluster is depicted as a histogram of a representative individual. (**B**) UMAP plots displaying dimensionally reduced, combined results of staining I for each group and overlaid with the clusters manually gated.*

3.4.2.1 Decreased frequency of CD56$^+$ T cells in MS patients

Manual quantification verified significantly lower frequencies of CD56$^+$ T cells (cluster 27) in MS patients with depression compared to healthy controls relative to all CD56$^+$ cells, all leukocytes and also in absolute numbers. Relative to CD56$^+$ cells, also MS patients displayed a significant reduction in frequency of cluster 27 cells compared to healthy controls (**Figure 3.4C**). Cluster 27 cell frequencies correlated negatively with depression scores of BDI-II and MADRS, further corroborating a relation between lower CD56$^+$ T cell frequency and MS-associated depression (**Table 3.6**).

Table 3.6: Correlation of cluster 27 frequency with depression scores

	BDI-II score	MADRS score
Cluster 27 relative to all CD56$^+$ cells	rho = -.342 ***p* = .003**	rho = -.281 ***p* = .018**
Cluster 27 relative to leukocytes	rho = -.314 ***p* = .007**	rho = -.255 ***p* = .032**
Absolute cluster 27 numbers	rho = -.308 ***p* = .009**	rho = -.224 *p* = .061

When examining only DMT and antidepressant free patients and their respective matched healthy controls (n = 11 per group), significant differences across groups were detected for relative frequencies of cluster 27, but *post hoc* tests with correction for multiple comparisons did not detect significant group differences. Nevertheless data of unmedicated patients showed trends towards decreased cluster 27 frequencies in MS patients with and without depression (cluster 27 as % of leukocytes: ***p* = .035**, *post hoc*: HC vs. MS+MDD *p* = .018, HC vs. MS *p* = .123, MS vs. MS+MDD *p* = .577; cluster 27 as % of CD56$^+$ cells: ***p* = .017**, *post hoc*: HC vs. MS+MDD *p* = .067, HC vs. MS *p* = .067, MS vs. MS+MDD *p* = .638). There was no significant difference across groups in absolute abundance of cluster 27 (*p* = .234).

Differential abundance of clusters 12/17 (**Figure 3.6A**) and cluster 30 (**Figure 3.6B**) were not confirmed by manual quantification.

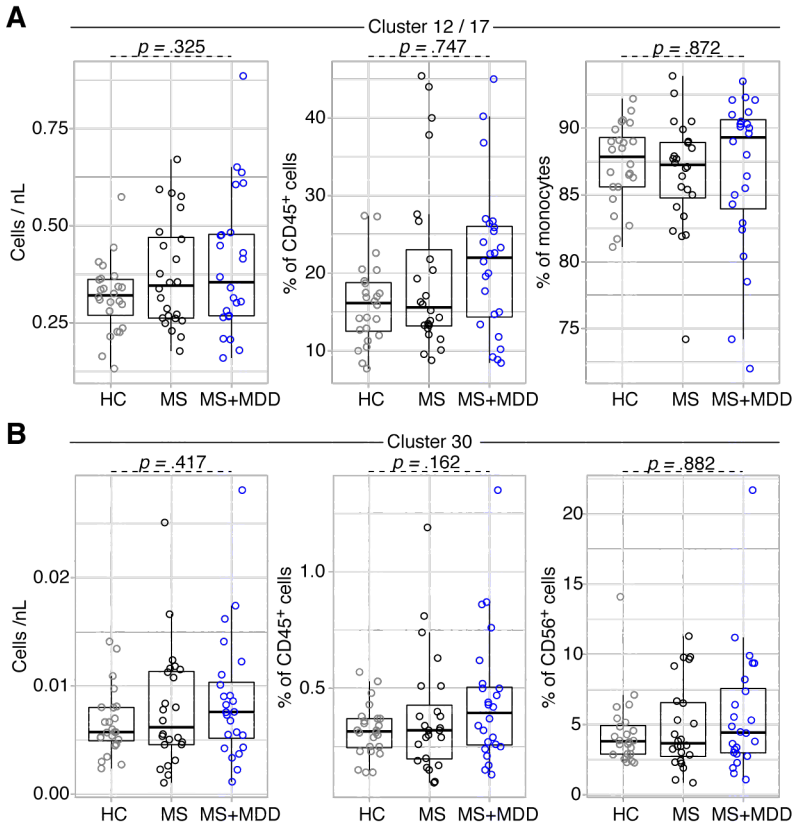

Figure 3.6: Verification of automatic cluster abundance analysis of staining I

*Quantification of manual gating of clusters of interest of staining I. (**A**) Cluster 12/17 and (**B**) cluster 30 (quantification of cluster 27 see **Figure 3.4**). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests.*

To summarise, $CD56^+$ T cells (cluster 27) were decreased in MS patients with depression compared to healthy controls and negatively correlated with depression scores. Relative to all $CD56^+$ cells, $CD56^+$ T cells were also significantly less frequent in MS patients than in healthy controls, pointing to a MS-specific effect, which may be enhanced in MS-associated depression as suggested by the negative correlation to depression scores.

3.4.3 Immunophenotyping staining II: effector and memory T cells

The immunophenotyping panel staining II focused on T cell subsets, including markers defining memory and naïve T cell subsets as well as T_{reg}. To investigate shifts in the frequencies of known T cell subsets in an unbiased way, data of staining II underwent the same automated data processing pipeline described above.

Again, SPADEVizR grouped cells into 30 clusters (**Figure 3.7A**), of which five were identified as clusters of interest: three were significantly de-enriched in depressed MS patients compared to healthy controls (clusters 3, 11, 13); two enriched (clusters 5, 30). Moreover, cluster 30 was also significantly more abundant in depressed MS patients compared to non-depressed MS patients (**Figure 3.7B**). Next, manual gating and quantification aimed to validate these group differences

as above (gating strategy see **Figure 2.4**, for control of manual gating success see **Figure 3.8A** and visualisation shifts across groups by UMAP see **Figure 3.8B**).

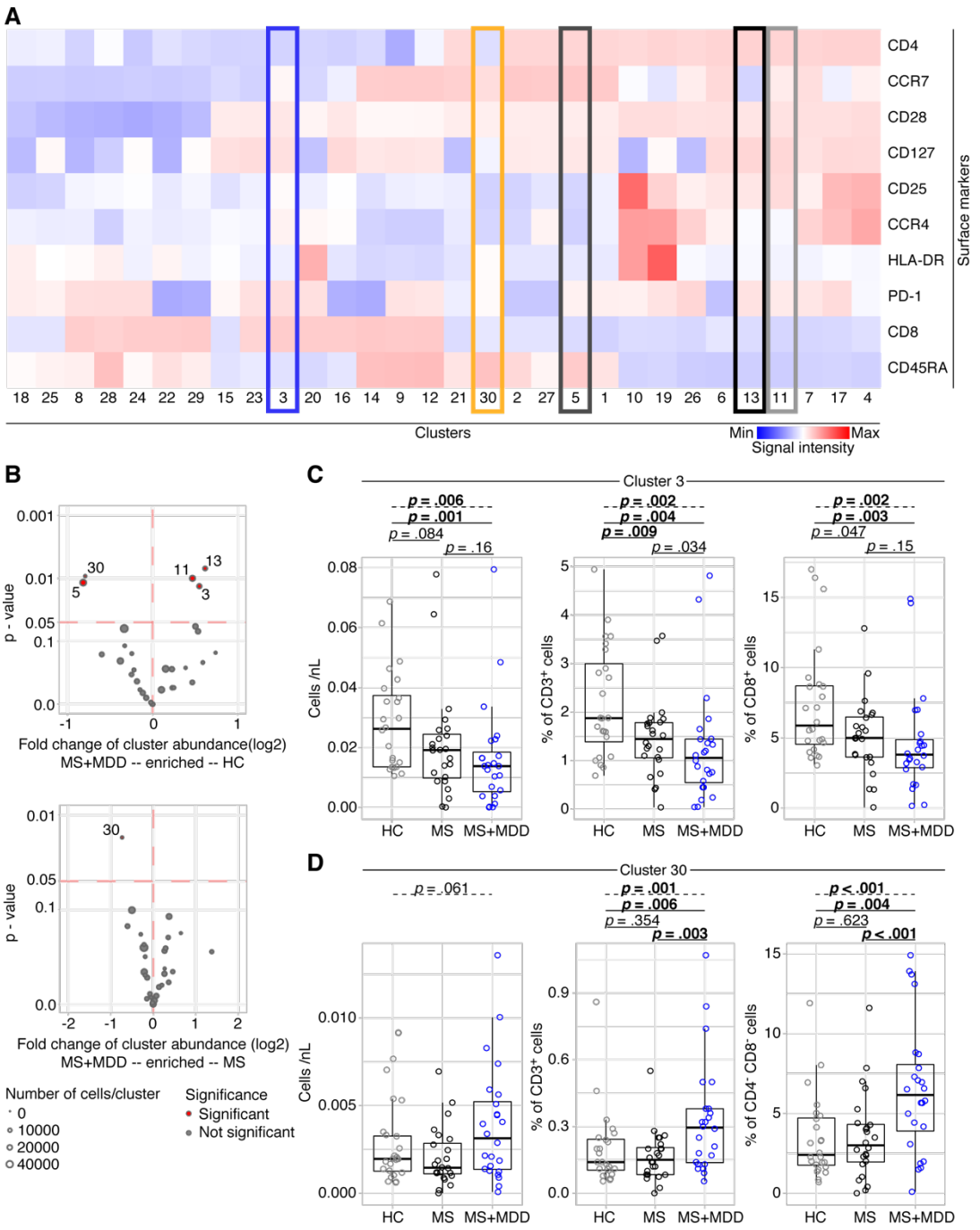

Figure 3.7: MS Patients showed decreased frequency of CD8⁺ T_{CM} and depressed MS patients showed increased frequency of DN naïve T cell subsets

*(**A**) Automated clustering of flow cytometry data of staining II into 30 clusters revealed five clusters, that were significantly enriched or de-enriched in depressed MS patients compared to healthy controls or MS patients (**B**). Manual quantification of cluster 3 (**C**) and cluster 30 (**D;** quantification of other clusters see **Figure 3.9**). In **A**, each cluster is described by the staining intensity of surface markers. Boxes highlight the clusters of interest identified by SPADEVizR. **B** shows clusters ordered according to their differential abundance between groups and their p value derived from student's T test. The size of each cluster is depicted by the diameter of plotted circles. In **C** and **D** data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

3.4.3.1 Reduction of $CD8^+$ central memory T cell subset in depressed and non-depressed MS patients

Cluster 3 was identified as a cluster of interest in the automated analysis of staining II. This cluster was classified as $CD4^-$ $CD8^+$ $CD45RA^-$ $CCR7^{low}$ and positive for CD28, CD127, PD-1 and $CD25^{low}$ $CCR4^{low}$ (**Figure 3.7A**). The marker combination of $CD4^-$ $CD8^+$ $CD45RA^-$ $CCR7^+$ describes $CD8^+$ central memory T cells (T_{CM})[12], of which cluster 3 was a subset with low CCR7 expression. T_{CM} are antigen-experienced cells, that can home to secondary lymphoid organs.

Decreased frequencies of cluster 3 cells in MS patients with depression compared to healthy controls were manually validated in absolute numbers and frequencies relative to $CD3^+$ T cells and $CD8^+$ T cells. Relative to all T cells, cluster 3 cells were also significantly decreased in MS patients compared to healthy controls and less frequent in depressed MS patients compared to non-depressed MS patients, although the latter comparison was not significant after correction for multiple comparisons (**Figure 3.7C**). In addition, cluster 3 frequencies correlated negatively with BDI-II scores, in line with the group analysis, but not with MADRS scores (**Table 3.7**). This again pointed to an MS-specific effect, possibly augmented by MS-associated depression.

Table 3.7: Correlation of cluster 3 frequency with depression scores

	BDI-II score	MADRS score
Cluster 3 as % of $CD8^+$	rho = -.336 ***p* = .004**	rho = -.148 *p* = .218
Cluster 3 as % of T cells	rho = -.324 ***p* = .005**	rho = -.143 *p* = .233
Cluster 3 absolute numbers	rho = -.318 ***p* = .006**	rho = -.135 *p* = .260

Subgroup analysis of untreated patients backed significant group differences of cluster 3 cells relative to $CD3^+$ T cells (***p* = .035**), with significant group difference in *post hoc* analysis only between healthy controls and non-depressed MS patients (HC vs MS: ***p* = .014**; HC vs. MS+MDD: *p* = .032; MS vs. MS+MDD: *p* = .365). Relative to $CD8^+$ T cells or in absolute numbers, cluster 3 was not significantly different in abundance across groups in untreated patients (cluster 3 as cells/nL: *p* = .078, cluster 3 as % of $CD8^+$ T cells: *p* = .234)

3.4.3.2 Increased frequencies of double negative $CD4^-$ $CD8^-$ T cells in MS patients with depression

Another cluster of interest resulting from automatic cluster analysis of staining II was cluster 30, which was enriched in depressed MS patients compared to healthy controls, but also non-depressed MS patients. Manual quantification confirmed the results of increased frequencies of cluster 30 relative to T cells and $CD4^-$ $CD8^-$ T cells in depressed MS patients compared to both healthy- and MS controls. Besides, no difference of cell abundance of cluster 30 was detected between healthy controls and non-depressed MS patients, making cluster 30 a very interesting cell population (**Figure 3.7D**).

Cluster 30 was classified as $CD4^-$ $CD8^-$ double negative (DN), but $CD45RA^+$ $CCR7^+$ CD28low (**Figure 3.7A, Figure 3.8A**). CD45RA/CCR7 positivity mark naïve T cells in the CD4+ and CD8+ compartment. Among DN T cells cluster 30 could belong to several unconventional T cell populations. Relative frequencies of cluster 30 correlated with BDI-II and MADRS depression scores, but absolute abundance of cluster 30 did not (**Table 3.8**).

Table 3.8: Correlation of cluster 30 frequency with depression scores

	BDI-II score	MADRS score
Cluster 30 as % of DN T cells	rho = .330 ***p* = .005**	rho = .269 ***p* = .023**
Cluster 30 as % of T cells	rho = .315 ***p* = .007**	rho = .328 ***p* = .005**
Cluster 30 absolute numbers	rho = .151 *p* = .206	rho = .194 *p* = .105

In the subgroup of unmedicated patients, significant differences in abundance of cluster 30 were not present, although relative to DN T cells there was a trend to differences across groups ($p = .078$).

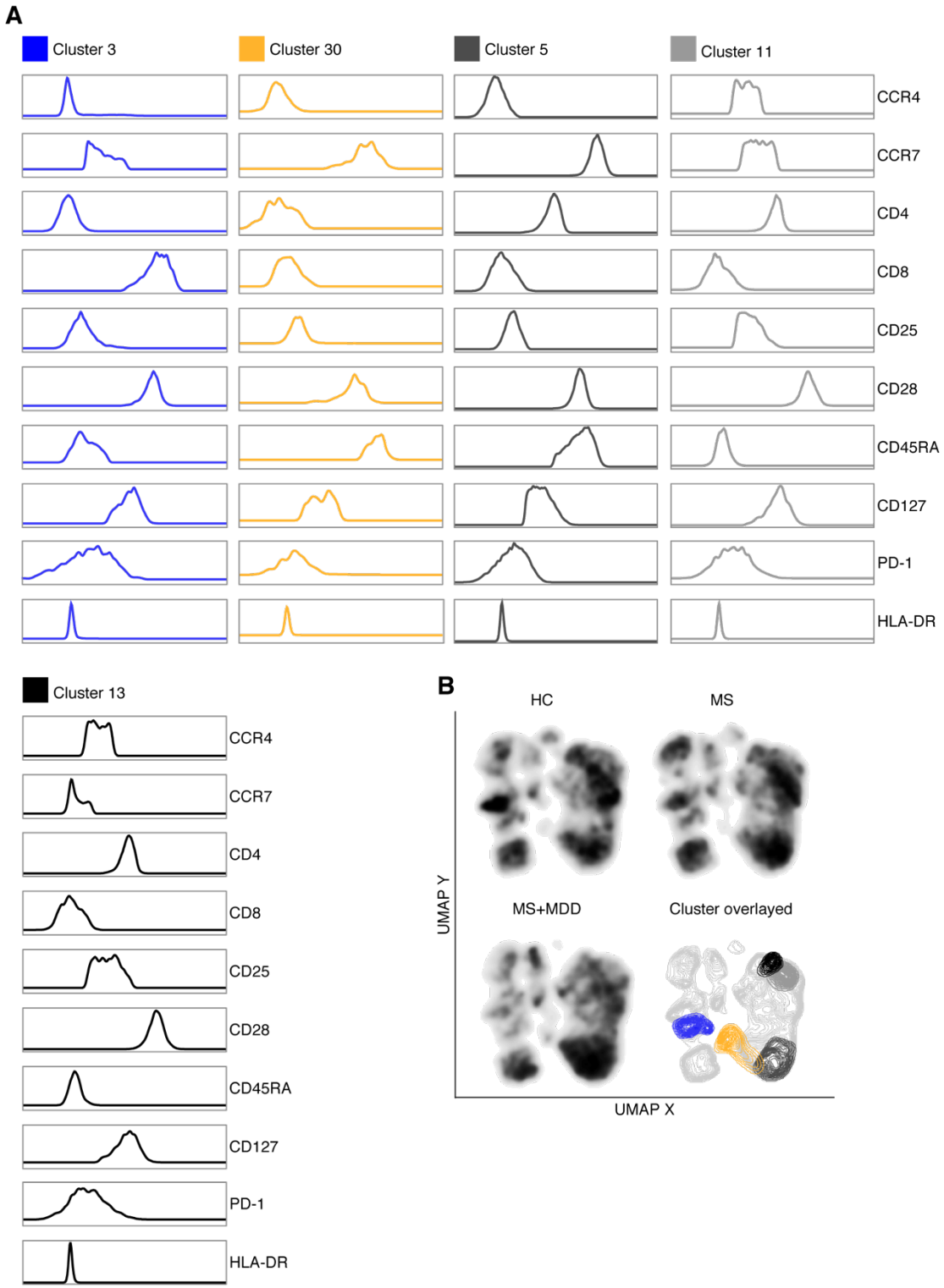

Figure 3.8: Manual gating of clusters of interest identified in staining II

*Manual gating of clusters 3, 5, 11, 13 and 30, informed by surface marker expression of each cluster (shown in **Figure 3.7A**) was performed (for quantification see **Figures 3.7C, D** and **3.9**). (**A**) Marker expression of each manually gated cluster is depicted in histograms of a representative individual. (**B**) UMAP plots displaying dimensionally reduced, combined results of staining II, for each group and overlaid with the clusters manually gated.*

Differential abundance of the other clusters of interest detected in staining II could not be validated by manual quantification: Cluster 5, a subset of naïve CD4⁺ T cells (**Figure 3.7A, Figure 3.8A**), which was found to be enriched in depressed MS patients by automatic analysis, displayed no

significant group differences in *post hoc* tests of manual quantification (**Figure 3.9A**). Clusters 11 and 13 of staining II, which were identified to decrease in abundance in MS patients with depression by the automatic analysis, did not convey differences between study groups when manually gated (**Figure 3.9B, C**). According to staining intensities of surface markers, cluster 11 matched a subset of CD4$^+$ T_{CM}, while cluster 13 was identified as a subset of CD4$^+$ effector memory T cells (**Figure 3.7A, Figure 3.8A**).

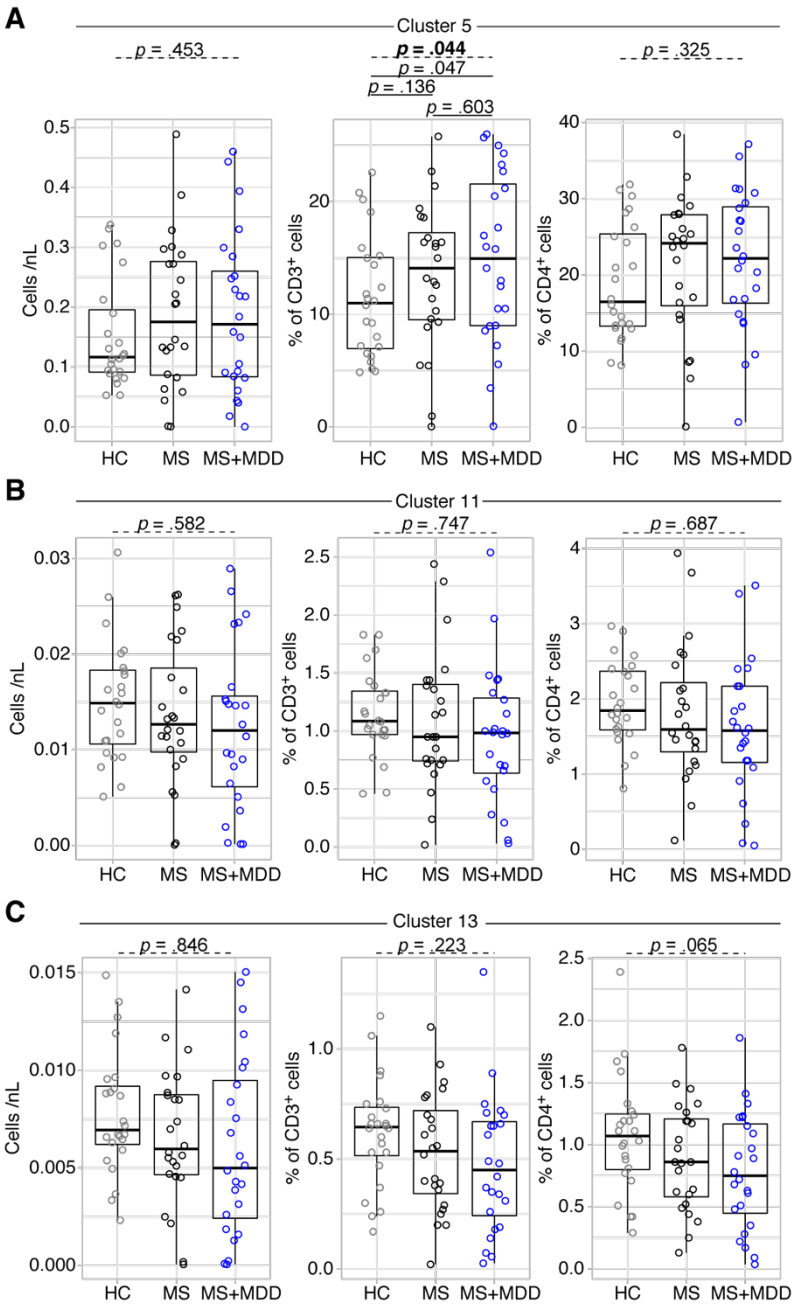

Figure 3.9: Verification of automatic cluster abundance analysis of staining II

*Quantification of manual gating of clusters of interest in staining II. (**A**) Cluster 5 and (**B**) cluster 11, (**C**) cluster 13 (quantification of clusters 3 and 30 see **Figure 3.7**). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

To conclude, firstly, a subset of $CD8^+$ T_{CM} was decreased in MS-patients with depression compared to healthy controls, but also in MS patients compared to controls (cluster 3). Cluster 3 negatively correlated in frequency with BDI-II depression scores, supporting an additive effect of MS and depression on the decrease of cluster 3 frequency. Secondly, a subset of DN T cells displaying "naïve" T cell surface markers (cluster 30) was significantly increased in relative frequencies compared to healthy controls and non-depressed MS patients. Relative frequencies of cluster 30 correlated with both BDI-II and MADRS scores of depression. The data suggest that the relative increase of these DN T cells may be associated with MS-associated depression.

3.4.4 Immunophenotyping staining III: MAIT cells and Th subsets

Staining III of the immunophenotyping panel contained many chemokine receptors designed to differentiate between different subsets of Th cells but also to detect MAIT cells.

Pre-processed flow cytometry data collected from staining III were analysed as described above. Of the 30 clusters grouped together by SPADEVizR (**Figure 3.10A**), four were distinguished as significantly different in abundance between healthy controls and MS patients with depression: clusters 12, 15 and 19 were de-enriched in depressed MS patients compared to healthy controls, whereas cluster 11 was enriched (**Figure 3.10B**). Again, manual gating (**Figure 2.5**, **Figure 3.11A**) and quantification aimed to corroborate the results of the automated analysis. UMAP dimensionality reduction was performed to visualise changes between groups across multiple parameters (**Figure 3.11B**).

Cluster 11 was not discernible in a biologically meaningful way from clusters 7, 13, 1, 14 and 10, as these clusters merely differed in the degree of negative signal for all surface markers apart from CD4 and CD3. Thus, these clusters (marked by dashed line in **Figure 3.10A**) were summarised together in the manual gating strategy (**Figure 2.5**).

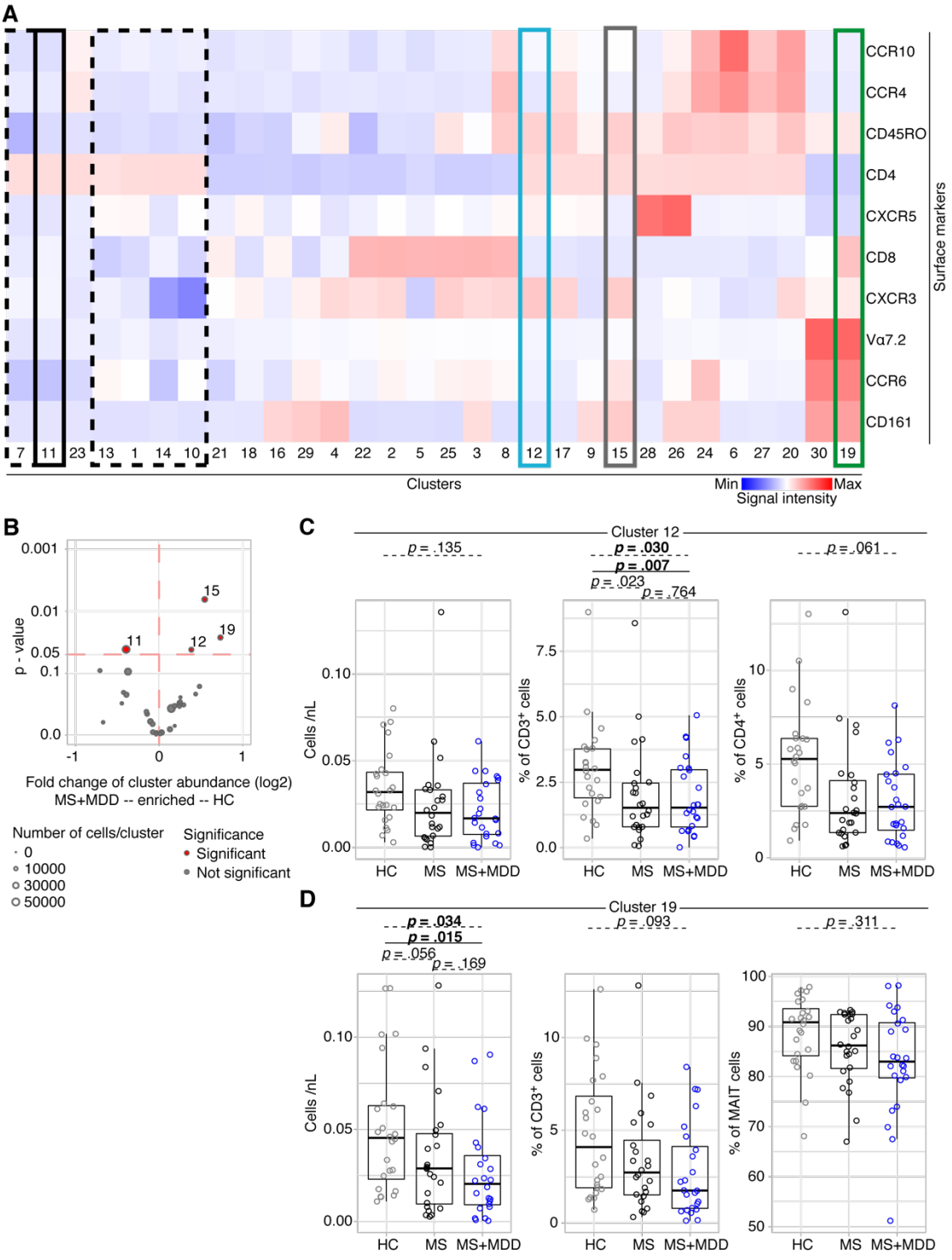

Figure 3.10: MS Patients show decreased frequency of Th1 cells and decreased frequency of CD8⁺ MAIT cells in MS-associated depression

*(**A**) Automated clustering of flow cytometry of staining III into 30 clusters revealed four clusters, that were significantly enriched or de-enriched in depressed MS patients compared to healthy controls (**B**). Manual quantification of cluster 12 (**C**) and cluster 19 (**D**; quantification of other clusters see **Figure 3.12**). In **A**, each cluster is described by the staining intensity of surface markers. Boxes highlight the clusters of significant group change as identified by SPADEVizR. When manual gating expanded across several clusters, the additional clusters are marked by a dashed line. **B** shows clusters ordered according to their differential abundance between groups and their p value derived from student's T test. The size of each cluster is depicted by the diameter of plotted circles. In **C** and **D** data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

3.4.4.1 Decreased frequency of Th1 cells in MS patients

Cluster 12 was detected as a cluster of interest in the automated analysis and characterised by the following combination of surface marker expressions: $CD4^+$ $CD8^-$ $CD45RO^+$ $CXCR3^+$ $CCR4^-$ $CCR6^-$ and negative signals for all other surface markers. This expression pattern conveyed that these cells belong to the Th1 population[12]. Manual quantification confirmed that cluster 12 frequency was significantly decreased in MS patients with depression compared to healthy controls relative to all T cells, but not relative to $CD4^+$ cells or in absolute numbers (**Figure 3.10C**). Relative to all T cells, cluster 12 was also substantially less frequent in MS patients compared to healthy controls, however this comparison was not significant after correction for multiple comparisons. No differences in cluster 12 abundance was seen between MS groups and none of the cluster 12 frequency measurements correlated to BDI-II depression scores. In the subgroup analysis looking only at unmedicated patients and their matched healthy controls, no difference across groups was detected. Taken together, these observations suggest a moderate MS-specific decrease of Th1 cells among T cells, independent of depression.

3.4.4.2 Fewer $CD8^+$ MAIT cells in MS and MS-associated depression

Another subgroup of cells identified as a cluster of interest was cluster 19, defined by an intense staining for CD161, Vα7.2 and CCR6 but also a positive signal for CD8. This identified cells of cluster 19 as a subset of $CD8^+$ MAIT cells[11]. Cluster 19 was detected as significantly less abundant in MS patients with depression compared to healthy controls by automated analysis (**Figure 3.10B**) and manual quantification confirmed this finding. Manual gating also detected a trend towards lower absolute abundance of cluster 19 in MS patients compared to healthy controls (**Figure 3.10D**). Relative to total T cells or all MAIT cells no significant group differences of cluster 19 frequency were observed, even though BDI-II depression score correlated negatively with all cluster 19 quantifications (**Table 3.9**). MADRS depression score, however, was not correlated to cluster 19 frequency.

Table 3.9: Correlation of cluster 19 frequency with depression scores

	BDI-II score	MADRS score
Cluster 19 as % of MAIT	rho = - .235 ***p* = .047**	rho = - .138 *p* = .251
Cluster 19 as % of T cells	rho = - 235 ***p* = .047**	rho = - .048 *p* = .689
Absolute numbers of cluster 19	rho = - .320 ***p* = .006**	rho = - .104 *p* = .388

Manual subgroup analysis of unmedicated patients did not reveal any significant modulation across groups for cluster 19 cells.

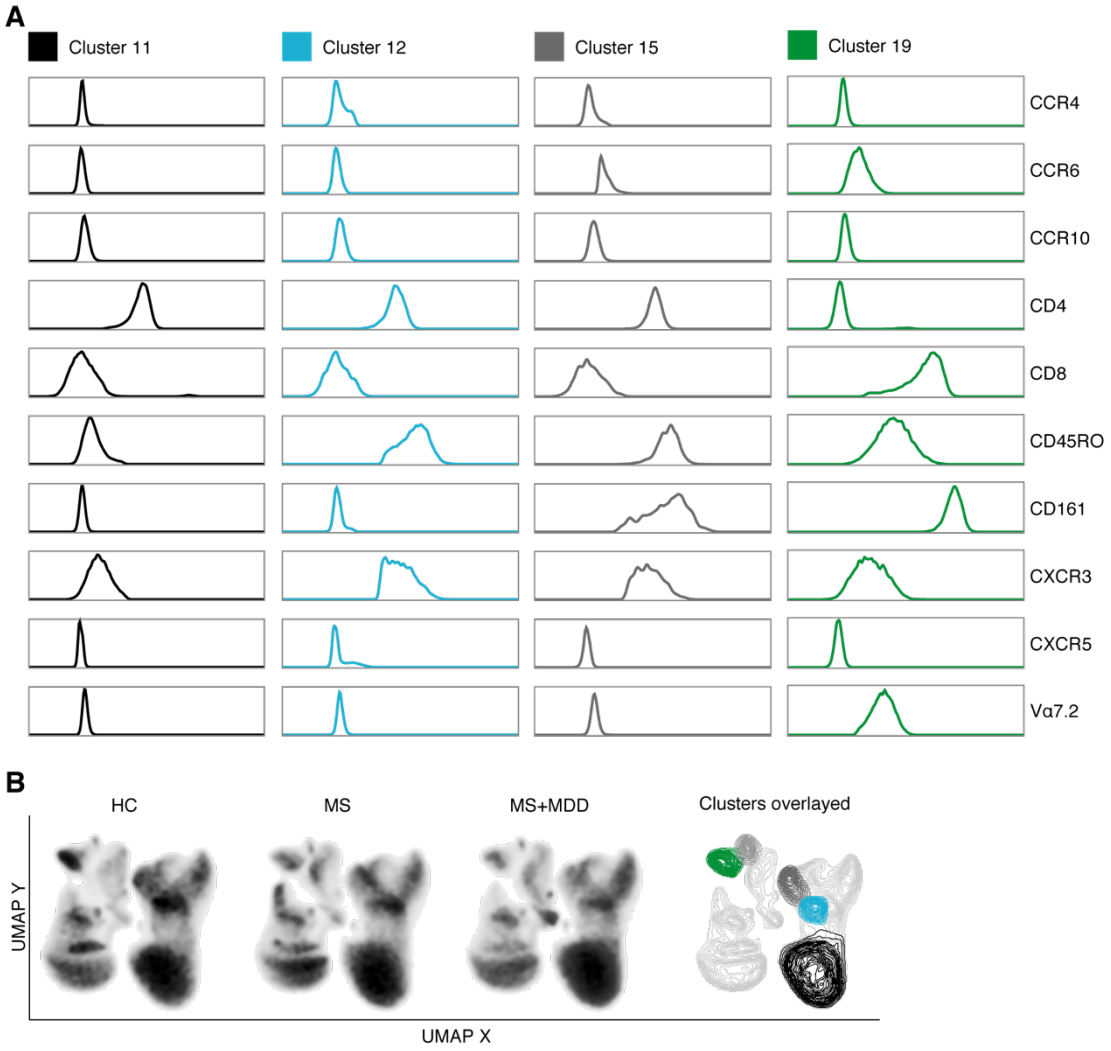

Figure 3.11: Manual gating of clusters of interest identified in staining III

*Manual gating of clusters 11, 12, 15, 19 informed by surface marker expression of each cluster (shown in **Figure 3.10A**) was performed (for quantification see **Figure 3.10C, D** and **Figure 3.12**). (**A**) Marker expression of each manually gated cluster is depicted in histograms of a representative individual. (**B**) UMAP plots displaying dimensionally reduced, combined results of staining III for each group and overlaid with the clusters manually gated.*

The remaining two clusters of interest (cluster 11 and 15) were not significantly different in abundance between groups after manual quantification (**Figure 3.12**). Cluster 11 consisted of $CD4^+$ $CD45RO^-$ T cells, negative for all other surface markers measured, marking them as naïve $CD4^+$ cells (**Figure 3.10A**, **Figure 3.11A**). Relative to $CD4^+$ T cells a significant difference between groups was detected across groups, however, *post hoc* tests adjusted for multiple comparisons did not yield significant group differences (**Figure 3.12A**). Cluster 15 was characterised as $CD4^+$ $CD8^-$ $CD45RO^+$ $CCR6^+$ $CXCR3^+$ $CCR4^-$, a marker combination with which cells fall in between the classical characterisation of Th1 and Th17 subsets[12]. Cluster 15 was also positive for CD161 (**Figure 3.10A**, **Figure 3.11A**). Manual quantification of cluster 15 did not recapitulate the effects detected by automatic analysis (**Figure 3.12B**).

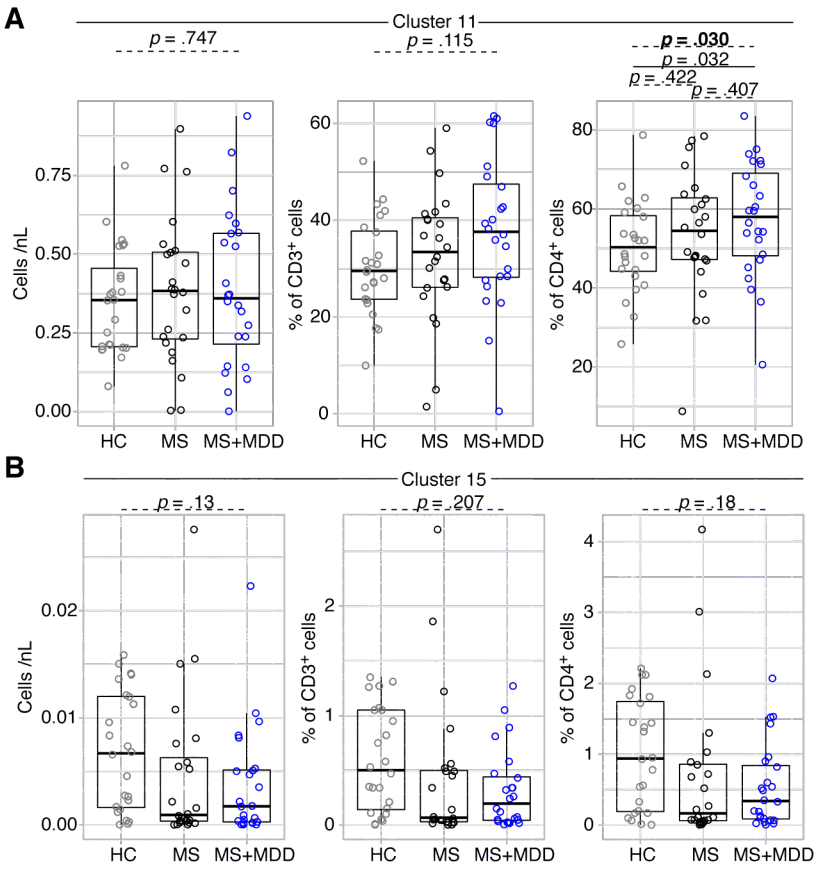

Figure 3.12: Verification of automatic cluster abundance analysis of staining III

*Quantification of manual gating of clusters of interest of staining III. (**A**) Cluster 11 and (**B**) cluster 15 (quantification of other clusters see **Figure 3.10C, D**). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points each representing an individual: healthy controls (HC; n = 24; grey), MS patients (MS; n = 24; black), depressed MS patients (MS+MDD; n = 24; blue). P values derived from Friedman's tests (dashed line) and post hoc Wilcoxon tests, with Bonferroni correction for multiple comparisons (solid line); bold digits indicate significance.*

To sum up, Th1 cells (cluster 12) were decreased in their frequency relative to T cells in MS-associated depression compared to healthy controls. Furthermore, there was a trend to decreased Th1 frequency in MS patients compared to healthy controls. However, depression scores did not correlate with Th1 frequency measurements. The fact that significant group differences could only be observed in one of three frequency measurements conveyed that the effect was moderate and absence of correlation to depression scores suggested an MS-specific effect. Furthermore, absolute $CD8^+$ MAIT cell numbers (cluster 19) were significantly decreased in MS patients with depression compared to healthy controls and $CD8^+$ MAIT cell numbers correlated negatively to depression scores. These findings propose that besides a possible MS effect, robust correlation of depression scores with all frequency measurements of $CD8^+$ MAIT cells suggests a possibly additive effect of depression.

3.4.5 Association of cortisol levels and cluster abundance

Of all the clusters of interest identified in this analysis, only absolute numbers of classical monocytes (staining I, cluster 12/17) correlated to evening cortisol (rho = .346, ***p* = .007**). Classical monocytes relative to leukocytes showed a trend in correlation to evening cortisol (rho = .238, $p = .067$).

3.4.6 Summary of immunophenotyping

In conclusion, broad immunophenotyping identified several clusters of interest that were either significantly enriched or de-enriched in MS-associated depression and could be confirmed by manual quantification. Taking into account the frequency changes and correlation of cluster frequency with depression scores three patterns of evidence emerged:

Firstly, Th1 cells (staining III, cluster 12) were found to be decreased in MS patients with depression compared to healthy controls, however only relative to T cells. As no difference between MS groups were observed, frequencies were not correlated to depression scores and in untreated patients this effect was absent, it can be concluded that the observed effect is possibly a moderate MS-specific effect, independent of depression.

Secondly, possibly additive effects of MS and depression were seen in $CD56^+$ T cell frequencies (staining I, cluster 27), which were consistently decreased in depressed MS patients, correlated to depression scores and the observed effects were largely recapitulated in unmedicated patients. A similar pattern was observed for a subset of $CD8^+$ T_{CM} (staining II, cluster 3), although subgroup analysis of unmedicated patients found a significant reduction of these cells in depressed MS patients only relative to all T cells. These observations point to a MS-specific effect that may further be enhanced by MS-associated depression. Also, $CD8^+$ MAIT cell numbers (staining III, cluster 19) were decreased in MS patients with depression compared to healthy controls. Interestingly, although none of the relative frequencies of this cluster conveyed any group differences, BDI-II scores were negatively correlated to every measure of $CD8^+$ MAIT cells, implying an interrelation with MS-associated depression. Increase of neutrophils also pointed to an additive effect of MS and depression.

Thirdly, a subset of DN T cells displaying a “naïve” phenotype (staining II, cluster 30) was significantly increased in relative frequencies in MS patients with depression, compared to both MS and healthy controls. Furthermore, depression scores correlated with relative frequencies of these cells, but in absolute numbers and among untreated patients this significant frequency increase was not seen. This was the only subpopulation in which a difference between depressed and non-depressed MS patients was observed in a direct comparison, suggesting a possible depression-specific effect in this MS cohort.

4 Discussion

MS patients frequently suffer from depression and evidence suggests that the high co-occurrence might be mediated by overlapping pathological processes. Observations in MS and MDD have indicated the involvement of the endocrine, immune and central nervous system. Here, the GC signalling pathway in T cells was investigated, which forms an important point of interaction between the immune and endocrine system.

4.1 Cohort establishment

To address the aims of this study a cohort of 25 MS patients with depression, 25 MS patients without depression and 25 healthy controls was recruited, well documented and matched for age and sex. Upon analysis of socio-demographic data, group differences were only detected in employment status and education, with fewest years of education and lowest employment in the MS group with depression. Lower socio-economic status, unemployment and low educational attainment have been associated with a higher risk of developing MDD[3,143]. However, a recent large cohort study of healthy individuals did not show an association of education or employment with variation of immune traits[127]. The same study found that the strongest non-genetic factors explaining variation within the healthy immune system were age, sex, smoking and latent CMV infection[127].

While the present study controlled for age and sex by matching, participants were not matched for smoking, which could potentially be a confounding factor. Furthermore, CMV status could confound frequencies of memory T cells, but was not assessed here. Metabolic descriptors did not differ between study groups, except for waist-hip ratio, however in the aforementioned resource, both BMI (between 18 and 32) and metabolic status had negligible influence on immune cell composition[127]. Thus, while smoking could be a confounder, it is unlikely that the socio-demographic or metabolic group differences in this cohort influenced the immunological parameters measured.

Measures of anxiety, depression and fatigue significantly differed between groups, as was expected in the case of the group with MS-associated depression. However, in all the self-reported questionnaires also non-depressed MS patients scored significantly higher than healthy controls. This observation emphasises the high overlap of non-affective MS symptoms with vegetative symptoms of anxiety and depression, that are included in the BAI and BDI, i.e. fatigue, numbness and tingling, wobbliness in legs, feeling unsteady, problems of concentration and decision making or changes in sleep. Despite the significant differences between healthy controls and non-depressed MS patients in scores of anxiety, depression and fatigue, the median values of the depression-free MS group of all these scales stayed below the cut-offs marking impairment, while the depressed MS group's median was above the respective cut-

offs[129,133,144,145], suggesting that the non-depressed MS group did not suffer from clinically meaningful fatigue, anxiety or depression. The potential of the BDI to yield false positives in MS patients has been discussed, but ultimately, the BDI-II is considered to be a valid tool to detect MS-associated depression[146–148], which has been described as clinically very similar to idiopathic MDD[149]. Notably, in the observer-rated depression score MADRS no differences were observed between healthy controls and non-depressed MS patients.

In summary, the strengths of the RRMS cohort recruited in the present study were the detailed description of subjects, the matching across groups, and the large proportion of MS patients matched pairwise for DMTs without differences in disability among groups. Also, the standardised sampling of blood samples in the morning after over-night fasting and paired experimental procedures add value to the present results. Possible limitations are sample size, unknown CMV infection status, lack of matching for smoking status as well as an unequal sex distribution. Measurement of CMV seropositivity should be considered in future studies.

4.2 No evidence for altered GC signalling in MS-associated depression

Based on pilot data and previous studies[110,121,126], the hypothesis of the present study was that in MS-associated depression T cells might have a deficit in GC signalling marked by downregulation of defined GC pathway elements. Contrary to this hypothesis, no deregulation of circadian cortisol was measured. Neither GC receptors nor enzyme expression was deregulated in memory T cells, T_{reg} or monocytes. Also, no evidence of decreased GR signalling was detected in levels of *GILZ* expression in MS-associated depression.

Changes of circadian cortisol have previously been described in MS and MS-associated depression and dysregulation of the HPA axis has been indicated, however the changes reported in MS were not consistent and the cohort sizes were small [110,123,150]. The present study did not show differences in circadian salivary cortisol in MS-associated depression, in contrast to previously published work. Yet, cortisol measurements in the present cohort had technical limitations. Firstly, unassisted sampling of saliva at home and return of samples by post made it impossible to control for compliance with instructions given to participants. To avoid this uncertainty, one would have to conduct such a study in an in-patient setting, track home sampling of saliva more reliably or change the sampling measures i.e. to newly developed wearable biosensors detecting cortisol in sweat[151]. Secondly, data on salivary cortisol were not available from all subjects of the cohort, due to technical complications. After having run all samples on assays with a technical fault, which were later recalled by the manufacturer, measurements could be repeated for most, but not all study participants.

In the present study, gene expression of GC receptors *GR* and *MR*, activating enzyme *HSD1* and *GILZ* downstream of GR activation were not differentially regulated in memory T cells, T_{reg} or monocytes in MS-associated depression. Nevertheless, a loss of regulatory potential of GCs

on T cells may still be present in MS-associated depression, but not detected by the present setup. Due to the complex nature of GR signalling many other mechanisms can modulate the regulatory potential of GCs in T cells.

Chaperone proteins form a complex with the GR in the cytoplasm to enhance the receptors' affinity to cortisol, by guiding its confirmation and to facilitate nuclear translocation after ligand binding[19]. The successful assembly of the multimeric chaperone complex is required to obtain a high affinity GR, and reduced availability of the contributing proteins, reduced availability of ATP or enhanced availability of negative modulators could influence GC signalling. For instance, during chaperone assembly of heat shock protein (Hsp) 40 and Hsp70 with the GR to the intermediate foldosome, Hsp70 interacting protein (Hip or ST13) facilitates foldosome assembly, while Bcl2-associated athanogene-1 (BAG-1) blocks it[19]. Thus, a shift of balance between Hip and BAG-1 availability could enhance or decrease GR activity. Of note, neuronal BAG-1 over-expression was reported to reduce manic and depressive phenotypes in mice and change expression of other GR chaperone proteins as Hsp70, possibly increasing neuronal resilience, but immune cells were not addressed in that study[152]. Completion of GR chaperone complex assembly to an active receptor complex is enabled upon binding of FKBP52, in contrast FKBP51 impairs complex activity and delays nuclear translocation *in vitro*[19,153]. Interestingly, in individuals carrying a functional polymorphism in the FKBP51 locus, early-life stress was associated with increased risk of developing post-traumatic stress disorder (PTSD) or MDD[154]. This gene environment interaction was associated with epigenetic changes within the FKBP51 locus in whole blood and long-term HPA alterations[155]. FKBP51 has been reported as an "ultra-short" negative feedback loop regulating GR signalling, as FKBP51 is also expressed in response to GR activation[156]. It has been put forward that the highly cell-specific effects of GCs are also mediated by chromatin accessibility of GREs and nGREs[14]. Therefore, comparing epigenetic marks and chromatin status of lymphocytes, as well as levels of competing chaperone regulators such as FKBP51/FKBP52 or BAG-1/Hip between depressed and non-depressed MS patients would be interesting.

Next, many post translational modifications to the GR have been described that modify its activity and stability[157]: phosphorylation and SUMOylation influence GR transcriptional activity and stability; ubiquitination regulates proteasomal degradation. Each of these modifications at different residues of the GR can regulate GC signalling and study thereof might provide clues of GR activity in lymphocytes of patients with MS-associated depression.

Further, additional downstream targets of GR activity beyond transactivation of GILZ could be analysed. Possible candidates are i.e. NFκB, AP-1 and NFAT, which are repressed in their transcriptional activity by GR tethering, or CREB, STAT3 and STAT5, the activity of which is enhanced by GR composite binding (CREB) or tethering (STAT3, -5)[14]. However, proving specificity of the GR effect *ex vivo* will be problematic as all these molecules are signal integrators receiving various inputs apart from the GR. Furthermore, fast non-genomic actions

of the GR that modulate T cells have not been addressed in the present study, such as interference with TCR signalling via transient phosphorylation of ZAP-70 and inhibition of LCK/FYN phosphorylation[20].

Finally, one could criticise that the immune cell populations chosen were not adequate to examine the presented hypothesis. Generally, analysis of pan T cells can bury or confound differential gene expression of T cell subsets. To avoid this issue, here $CD4^+$ and $CD8^+$ $CD45RO^+$ memory T cells, T_{reg} and $CD14^+$ monocytes were analysed separately, but still these populations might have been too heterogeneous to detect differential expression of GC pathway elements. Instead, one could assume that the examined subsets were irrelevant to the hypothesis, but that the hypothesis was actually true. In that case one would have to analyse other subsets for their GC pathway expression in future work, i.e. by FACS sorting other subsets to analyse gene expression or combining measurements of GC pathway expression with flow cytometry (i.e. intracellular protein staining, intracellular mRNA hybridisation, or intracellular phosphorylation staining). An alternative approach to the hypothesis driven setup conducted here would be to switch to unbiased expression analysis by single-cell RNA sequencing (RNA-Seq) or semi-hypothesis driven bulk RNA-Seq of chosen cell populations.

A limitation of the present study is sample size and heterogeneity of the cohort. It cannot be excluded that the effect size of the hypothesised changes in expression of GC signalling elements may be too small to detect in the given sample size, and even more so in the subgroup analysis.

In summary, a difference in expression of defined GC signalling elements in memory T cells, T_{reg} or monocytes was not detected. However, given the complexity of GC signalling, impaired GC signalling within the immune system cannot be ruled out as a biological substrate of MS-associated depression. A variety of different mechanisms that modulate GC signalling through epigenetic modifications, differential gene expression, protein availability, posttranslational modifications and protein interactions has been presented above. However, before setting out on further mechanistic studies, the functional relevance of GC insensitivity in MS-associated depression would be advisable to corroborate in defined immune subsets. Functional analysis of proliferation and cytokine production suggesting GC resistance of T cells have been published in small cohorts of depressed MS patients[121,126]. Similar experiments in a larger more controlled cohort, as the present cohort assembled here, could show to what extent potential defects in the presented mechanisms of GC signalling are functionally relevant. Also, the strength of GC signalling could be influenced by local cortisol concentrations depending on CBG levels and local steroid synthesis[158].

4.3 Shifting frequencies of unconventional T cell subsets and neutrophils in MS-associated depression

The hypothesis-driven approach of the present study was accompanied by the aim to screen the T cell compartment within the circulation in depth. In a detailed immunophenotyping approach with unbiased analysis, several shifts within the immune cell composition of patients with MS-associated depression were identified. The identified shifts can be classified into three patterns: firstly, MS-specific effects; secondly, potential additive effects of MS and depression, which were seen in MS patients as a tendency and amplified in MS-associated depression; and thirdly depression-specific effects.

Firstly, an MS-specific decrease of Th1 cells was observed, when comparing healthy controls to MS patients, independent of depression status or - severity. Th1 cells had first been suspected as causal to MS, before more recently Th17 cells have been proposed as the disease-mediating cell population[159]. Functional changes of Th1 cell have been reported in MS: when PBMCs were cultured *ex vivo* without external stimulus, cells from MS patients proliferated more than cells from healthy donors[160]. This phenomenon, which the authors named autoproliferation, was most pronounced in MS patients carrying the risk haplotype HLA-DR15 and was pinpointed to expansion of classical and non-classical Th1 cells. In MDD research, a T cell-specific decrease of surface CXCR3, a Th1-defining chemokine receptor, was found in MDD patients[161]. In contrast, the present study suggests that while Th1 cells are less abundant in MS patients, co-occurring depression does not influence Th1 abundance.

Secondly, a potential additive effect of MS and depression was detected in several T cell subsets. $CCR7^{low}$ $CD8^+$ T_{CM}, $CD56^+$ T cells and absolute numbers of $CD8^+$ MAIT cells were decreased in depressed MS patients compared to healthy controls and subset abundance was negatively correlated with depression scores. Descriptively, frequencies were highest in healthy controls, decreased in MS patients and further deminished in depressed MS patients. Given this descriptive pattern and the correlation with depression severity one can speculate that the immunophenotypic shifts observed might be present in MS patients, and augmented in MS patients with comorbid depression.

T_{CM} have been studied in MS, especially in light of the clinical benefit of the DMT fingolimod, which particularly affect T cells that home to lymph nodes, as T_{CM}. Fingolimod is a sphingosine-1-phosphate receptor agonist that blocks the egress of T cells from secondary lymphoid organs, thus lowering the numbers of circulating T_{CM}[33,162,163]. Patients relapsing while on fingolimod treatment had elevated levels of $CD4^+$ T_{CM} in peripheral blood[162]. Also most T cells in the CSF of MS patients are T_{CM}[164] , indicating that T_{CM} may play a detrimental when being reactivated in the brain parenchyma of MS patients.

MAIT cells are of great interest in autoimmunity research, but interestingly, whether they play a protective or detrimental role is debated in the literature[11]. The results of this work are in line

with two studies reporting a decreased MAIT cell frequency in the blood of MS patients during remission and relapse[50,165]. Also, peripheral blood from MS patients contained more IL-17-producing MAIT cells, and these cells expressed higher levels of RORγt and CCR6 than their non-MAIT counterparts[51]. Reduction of MAIT cells in peripheral blood and increase of MAIT cells in the affected tissues was reported in various other immune-mediated diseases and some cancers[11], assuming that MAIT cells contribute to inflammation in the tissue they home to. On the other hand, a protective role of MAIT cells in EAE has been reported, although the identification of murine MAIT cells at the time lacked tetramer staining, and should thus be interpreted with care[166]. To date, no studies investigating MAIT cells frequencies in MDD could be found.

$CD56^+$ T cells were consistently decreased in MS-associated depression. Formerly, T cells expressing NK markers as CD161 or CD56 were broadly named NKT cells[167,168]. With advances in knowledge and techniques the definition of NKT cells has been narrowed down to αβ T cells, restricted by CD1d, a MHC class Ib molecule, that presents lipids and is expressed on classical APCs but also on endothelial cells[169]. There are two classes of NKT cells presently described: NTK I cells are also called invariant NKT (iNKT) cells and express a semi-invariant TCR Vα24Jα18 paired with Vβ11; NKT II or variant NKT (vNKT) cells have a diverse TCR repertoire and have been suggested to possess regulatory functions[168]. In MS, a reduction of iNKT cells as well as decreased cytokine production has been reported, although inconsistent usage of markers for NKT cell classification limits the comparability of studies[168]. The findings that vNKT cells were enriched in the CNS in EAE and that most murine vNKT cells respond to sulphatide, a self-glycolipid which is abundant in the myelin sheath, sparked enthusiasm for this cell population as the driving force of demyelinating disease[170]. Yet, follow-up EAE studies have produced conflicting results[171] and only few NKT cells are found in MS lesions, questioning translatability[168]. In a cohort of female patients suffering from fibromyalgia syndrome, $CD56^+$ T cells were significantly decreased in patients with depressive symptoms[172], similar to the findings of the present study. In contrast, in a small cohort of geriatric depressed patients, $CD16^+$ and/or $CD56^+$ T cells were increased in unmedicated MDD patients but not in MDD patients on antidepressant therapy compared to population based controls[173]. Thus, $CD56^+$ T cells have been described in the context of MS and MDD, however what role they might play is unclear and requires more detailed studies in larger cohorts.

In the T cell compartment CD56 is expressed not only on NKT cells, but also γδ T cells, MAIT cells as well as $CD8^+$ T cells and expression levels are often increased in response to activation[11,174]. The $CD56^+$ T cells identified in cluster 27 could indeed include unconventional T cells, but they could also be T_{CM}[174], which might overlap with changes of $CD8^+$ T_{CM} frequency mentioned above. Furthermore, CD56 can also be expressed by some γδ T cells. γδ T cells are non-classical T lymphocytes which, instead of expressing an αβ TCR, express diverse TCRs made up of a γ and a δ chain and recognise a variety of antigens[169]. Unlike classical αβ TCRs, γδ TCRs have less possibilities of genetic recombination, but can recognise larger

surface molecules that must not necessarily present antigens but can signal stress, infection or transformation of a cell (i.e. MICA, ULBP4, EPCR, CD1c or CD1d-presented αGalCer)[169]. In mice many γδ T cell subsets have been described that home to mucosa or epithelium, where they become tissue-resident cells[175]. In humans two main γδ T cells subsets have been reported: Vγ9Vδ2 are most predominant in blood and Vδ2$^-$ cells are more predominant in tissues as skin and gut[175]. Ultimately, cluster 27 could be a heterogeneous pool of T cells expressing CD56, including subsets of the populations described above and characterisation would require further phenotypic markers.

Thirdly, CD4$^-$CD8$^-$ DN T cells carrying a "naïve" phenotype were increased in MS-associated depression, but not in depression free MS patients, thus indicating an association with depression. Commonly, naïve αβ T cells commit to either the CD4 or the CD8 lineage in the thymus, so DN naïve αβ T cell in the periphery are rare and poorly studied. Few reports of αβ DN T cells in the peripheral blood claim a regulatory function[176], but also pathogenicity[177,178]. However, no more reports of DN T cells have been published in the past years. Technical advances have allowed for more detailed classification of unconventional T cells (reviewed in[169]). Hence, one may question if the aforementioned reports on DN T cells might have described clusters of unconventional T cells, which are now defined by their TCR chain usage or restriction to MHC-Ib molecules. As the CD3 antibody used in the present study did not differentiate between an αβ or γδ TCR, cluster 30 could consist of γδ T cells expressing CCR7 and CD45RA[175]. As stated above for CD56$^+$ T cells, more phenotypic markers would be needed to clarify the identity of cells within these clusters of interest and to develop hypotheses as to their role in MS-associated depression.

Aside the shifts observed in the immunophenotypic analysis, blood counts revealed that neutrophils and NLR, were significantly elevated in MS-associated depression when compared to healthy controls and there was a trend to higher neutrophils also in depression-free MS patients. Neutrophils have been reported to be elevated in MS patients and exhibit a primed phenotype[179]. NLR, which is a metric adapted from intensive care as a measure of systemic inflammation[180], was elevated in MS patients compared to healthy controls and associated with EDSS, depression and stress scores[181–183], in line with the present results. Interestingly NLR has also been investigated in MDD and was shown to be elevated in a recent meta-analysis[184]. In an LPS-induced model, depression-like behaviour was accompanied by neutrophil infiltration into the brain and administration of anti-polymorphonuclear antibody rescued the depressive phenotype[77]. Further study of neutrophils would be highly interesting in MS-associated depression, however for future studies sample preparation would have to be adjusted, as classical PBMC isolation protocols lose granulocytes during gradient centrifugation.

To sum up, potentially additive effects of MS and MDD were observed in a decrease of CD56$^+$ T cells and CD8$^+$ MAIT cells, as well as CCR7^{low} CD8$^+$ T_{CM}, that correlated negatively with depression scores. T_{CM} and MAIT cells have previously been implicated in MS patients,

but not in MDD, and have been speculated to home to the inflamed CNS, thus decreasing their peripheral levels. One could speculate that further decreased numbers of these populations in depressed MS patients point to a larger cell infiltrate into the CNS, enhancing inflammation in the brain. A depression-specific shift of immune cell populations in MS patients was observed in a relative increase of DN T cells of naïve phenotype, possibly also representing an unconventional T cell population. Moreover, neutrophils were increased in MS-associated depression, which may convey higher systemic inflammation or be involved in migration to the CNS and in amplification of inflammatory brain damage, a speculation that would need to be substantiated by examining brain tissue.

The strength of this detailed immunophenotyping procedure are the technically standardised measurements in a well-described cohort, aiming to avoid systematic bias. The data were analysed in an objective and unbiased manner followed by a confirmation by manual gating and conservative statistical analysis. Despite the great effort taken to ensure technical consistency, a batch effect was discernible in the cytometry data, caused by a renewal of the cytometer baseline during the period of measurements. This batch effect added variance and data spread especially in the negative populations. The variance may have exaggerated differences of negative signals in the automated clustering, but this was accounted for in the manual analysis, where differences in negative signals were considered as biologically irrelevant, i.e. in staining III, manual gating of cluster 11 included clusters 7,13,1,14, and 10 as the only differences between these clusters were slight shifts in negative signals of surface markers.

A limitation of this immunophenotyping is that CMV status of subjects was not known. As mentioned above, CMV seropositivity can strongly shape the memory T cell compartment. Here, no changes were observed in effector memory T cells re-expressing CD45RA (T_{EMRA}) frequency between groups, but when examining relative numbers, shifts of T_{EMRA} frequency can shift relative frequencies of other T cell subsets. Also, some DMTs influence frequencies of circulating lymphocytes. To minimise this bias, efforts were made to match DMTs between the two MS groups. To exclude any effect of DMTs one would have to recruit an untreated cohort, which given the strict inclusion criteria and matching of the present study would not be realistic in a single centre. Simultaneously, the strict inclusion criteria add great value to the present results.

For convincing classification of the cluster 27 (staining I) and cluster 30 (staining II), additional markers as CD4, CD8, γδ TCR, CD161, Vα7.2, Vα24, Vβ11 and αGalCer-loaded CD1d tetramer staining would be necessary. Despite the need for more detailed classification the results of the present study hint to a dysregulation of unconventional T cells in MS-associated depression. This is an intriguing finding, as unconventional T cells can function in both innate and adaptive immune capacities and are thought to form an important connection between the innate and adaptive arms of the immune system[185]. In MS the adaptive immune response is

dysregulated, whereas in MDD, immunological aberrations observed are shown more robustly in innate cell populations and only recently changes of lymphocytes have been reported. Cells that can transverse between the two systems are therefore highly interesting when contemplating a co-occurrence of MDD and MS. Further, there is evidence that unconventional T cells do not necessarily require antigen-specific TCR stimulation to become activated, but activation can also be mediated by cytokines or cellular stress markers[11,169]. This might put forward unconventional T cells as potential supporters of a low-grade inflammatory milieu that, on the one hand, may kindle sickness behaviour and on the other hand, may facilitate a break of tolerance to CNS antigens.

4.4 Conclusion

The high co-occurrence of MS and MDD, combined with epidemiological and pathophysiological observations suggest that MS and MDD may share overlapping pathogenic pathways. The present study observed potentially additive effects of MS and depression as well as a depression-specific effect on the composition of the T cell compartment. Contrary to the hypothesis, in MS-associated depression there was no difference in circadian cortisol levels nor evidence suggesting a loss of regulatory potential of GCs on T cells, T_{reg} or monocytes, although much of the complex GC signalling remains to be investigated. This is the first study that examined frequencies of circulating immune cells in MS-associated depression in such detail and revealed clues pointing to a deregulation of unconventional T cell populations that should be explored and characterised further in future studies. Implication of unconventional T cells in MS-associated depression is an intriguing avenue for further research, as unconventional T cells encompass functions usually attributed to either innate or adaptive immune cells and are therefore promising candidates in the search of overlapping pathogenic processes between MS, an adaptive autoimmune condition and MDD, which seems most robustly described by innate immune activation.

5 Summary

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) and affects around 2.3 million people worldwide. MS patients frequently suffer from major depressive disorder (MDD). MDD is characterised by low mood or loss of interest, accompanied by a variety of somatic symptoms. Although up to half of all MS patients experience a depressive episode in the course of their life, little is known about how the two conditions are interconnected. Affective symptoms may present themselves years before MS is diagnosed, suggesting that depression is not merely a psychologic reaction to a diagnosis of chronic disease. Accumulating evidence suggests that MS and MDD may share overlapping pathogenic pathways involving the immune, endocrine, and central nervous system.

Increased levels of cortisol, enhanced T cell cytokine production and glucocorticoid (GC) insensitivity of T cells have been reported in MS-associated depression. This suggests a possible loss of regulatory potential of GCs on T cells. In the present study it was hypothesised that in MS-associated depression GCs lose their regulatory potential on T cells. Pilot data suggested that this loss may be mediated by lower expression levels of defined GC pathway elements *GR, HSD1* and *GILZ* in T cells.

A matched cohort of 25 healthy controls, 25 depression-free MS patients and 25 depressed MS patients were examined. But contrary to the hypothesis, no changes in circadian cortisol levels, nor any evidence suggesting loss of regulatory potential of GCs were observed. Instead, broad immunophenotyping of peripheral immune cells revealed frequency shifts of several T cell populations. Potentially additive effects of MS and depression were seen in frequencies of $CD8^+$ MAIT cells, $CD8^+$ central memory T cells and $CD56^+$ T cells. These cell populations were significantly decreased in MS patients with depression compared to healthy controls and slightly decreased compared to depression-free MS patients. Furthermore, the frequency of these populations correlated negatively with depression scores, implicating their association with MS-associated depression. Interestingly, a subset of $CD4^-CD8^-$ double negative T cells were elevated only in depressed MS patients. Also, neutrophils were increased in MS-associated depression compared to healthy controls.

This is the first time that circulating cell populations are examined in such detail in MS-associated depression. Notably, changes of the T cell compartment were identified mostly in populations of unconventional T cells, which may be an intriguing avenue for future research of MS-associated depression. MS is a condition marked by adaptive immune activation, whereas MDD is most robustly associated with innate immune activation. Therefore, unconventional T cells, which can encompass functions of both innate and adaptive immune cells, are promising candidates in the search of overlapping pathogenic processes between MS and MDD.

6 Zusammenfassung

Multiple Sklerose (MS) ist die häufigste chronisch-entzündliche Erkrankung des zentralen Nervensystems (ZNS). Weltweit sind davon etwa 2,3 Millionen Menschen betroffen. Viele MS-Patienten leiden an Depression. Die Anzeichen dafür können sich in einer niedergedrückten Stimmung, in Interessenverlust sowie in körperlichen Symptomen manifestieren. Obwohl rund die Hälfte aller MS-Patienten im Laufe ihres Lebens eine depressive Episode erleiden, ist über den Zusammenhang zwischen MS und Depression wenig bekannt. Da affektive Symptome schon Jahre vor Diagnosestellung auftreten können, ist anzunehmen, dass Depression nicht bloß die Folgeerscheinung einer MS-Diagnose ist. Der aktuelle Stand der Forschung legt nahe, dass der MS und der Depression überlappende pathogene Mechanismen im Hormonsystem, im Immunsystem und im ZNS zugrunde liegen.

In Studien mit depressiven MS-Patienten wurden erhöhte systemische Cortisolspiegel, vermehrte T-Zell-Zytokinproduktion und eine Desensibilisierung von T-Zellen gegenüber Glucocorticoiden (GC) gemessen. Diese Ergebnisse ließen vermuten, dass die GC bei depressiven MS-Patienten ihr regulatorisches Potential in T-Zellen verlieren. Pilotdaten stützten die Hypothese, dass dieser Verlust einer verringerten Genexpression von *GR, HSD1* und *GILZ* zuzuschreiben ist. Die gegenwärtige Studie konnte diese Hypothese nicht bestätigen. Untersucht wurden 25 gesunden Patienten, 25 nicht-depressive und 25 depressive MS-Patienten. Die Untersuchung ergab weder eine Veränderung des Cortisolspiegels noch Beweise für einen Verlust des regulatorischen Potentials von GC in T-Zellen. Stattdessen wurde im Blut eine Verschiebung von unkonventionellen T-Zellpopulationen festgestellt. Detektiert wurde ein möglicher additiver Effekt von MS und Depression bei den Frequenzen von $CD8^+$ MAIT-Zellen, $CD8^+$ zentralen T-Gedächtniszellen und $CD56^+$ T-Zellen. Diese Zellpopulationen waren bei depressiven MS-Patienten im Vergleich zu Gesunden signifikant verringert und im Vergleich zu nicht depressiven MS-Patienten leicht verringert. Die Häufigkeiten dieser Zellpopulationen korrelierten zudem negativ mit der Schwere der depressiven Symptome. Interessanterweise war eine doppelt negative $CD4^-CD8^-$ T-Zellpopulation gegenüber den anderen Vergleichsgruppen nur bei depressiven MS-Patienten erhöht. Weiterhin wurden im Blut depressiver MS-Patienten mehr Neutrophile gefunden, als bei gesunden Kontrollen.

Dies ist die erste Studie, die bei depressiven MS-Patienten auf detaillierte Weise zirkulierende Immunzellpopulationen untersucht. Bemerkenswert ist, dass die die beobachteten Veränderungen vor allem unkonventionelle T-Zellpopulationen betreffen, deren Eigenschaften sonst entweder dem angeborenen oder dem adaptiven Immunsystem zugeschrieben werden. MS ist gekennzeichnet durch adaptive Immunaktivierung, Depression dagegen vorwiegend durch angeborene Immunaktivierung. Unkonventionelle T-Zellen könnten das Verbindungsglied zwischen MS und Depression sein und sollten in weiteren Studien untersucht werden.

7 Abbreviation list

α-GalCer	α-Galactosylceramide
ACTH	Adrenocorticotropic hormone
AP-1	Activator protein 1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BAG-1	Bcl-2 associated athanogene 1
BAI	Beck's anxiety inventory
BATF	Basic leucine zipper ATF-like transcription factor
Bcl	B-cell lymphoma
BDI-II	Beck's depression inventory II
BMI	Body mass index
BSA	Bovine serum albumin
BV	Brilliant Violet
CBG	Cortisol binding globulin
CA	Cornu ammonis
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CNS	Central nervous system
CREB	Cyclic adenosine monophosphate response element-binding protein
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CS&T	Cytometer setup and tracking
C_t	Cycle threshold value
CTL	Cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DMT	Disease modifying therapy
DN	Double negative ($CD4^-CD8^-$)
dNTP	Deoxyribose nucleoside triphosphate
dPBS	Dulbecco's phosphate buffered saline
DSM-5	Diagnositic and Statistical Manual of Mental Disorders, 5^{th} Edition
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Extended disability status scale
EDTA	Ethylenediaminetetraacetic acid
EPCR	Endothelial protein C receptor
FACS	Fluorescence activated cell sorting

FCS	Foetal calf serum
FKBP51	FK506 binding protein 51
FKBP52	FK506 binding protein 52
FoxP3	Forkhead box protein 3
FSC	Forward scatter
FSMC	Fatigue scale for motor and cognition
FSS	Fatigue severity scale
FYN	Proto-oncogene protein-tyrosine kinase Fyn (*FYN*)
GATA3	GATA binding protein 3 (*GATA3*)
GC	Glucocorticoid
GILZ	Glucocorticoid-induced leucine zipper (*TSC22D3*)
GO	Gene ontology
GR	Glucocorticoid receptor (*NR3C1*)
GRE	Glucocorticoid response element
GWAS	Genome-wide association studies
HAQUMS	Hamburg Quality of Life Questionnaire in Multiple Sclerosis version 10.0
HC	Healthy controls
HDL	High-density lipoprotein
Hip	Hsp70 interacting protein or ST13
HLA	Human leukocyte antigen
HPA axis	Hypothalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase
HSD1	11β-Hydroxysteroid dehydrogenase type 1 (*HSD11B1*)
HSD2	11β-Hydroxysteroid dehydrogenase type 2 (*HSD11B2*)
Hsp	Heat shock protein
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th revision
IFN	Interferon
IL	Interleukin
IL-23R	Interleukin-23 receptor
IL-2Rα	Interleukin-2 receptor alpha chain
IL-7Rα	Interleukin-7 receptor alpha chain
INIMS	Institute für Neuroimmunology und Multiple Sklerose
iNKT cell	Invariant NKT cell (also NKT I)
IPO8	Importin 8 (*IPO8*)
IQR	Interquartile range
LCK	Lymphocyte-specific protein tyrosine kinase
L/D	Live/dead
LDL	Low-density lipoprotein
LPS	Lipopoly saccharide
MACS	Magnetic activated cell sorting
MADRS	Montgomery-Åsberg Depression Rating Scale
MAIT cell	Mucosal-associated invariant T cell
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A

MINI	Mini International Neuropsychiatric Interview
MSFC	Multiple Sclerosis Functional Composite
MR	Mineralocorticoid receptor (*NR3C2*)
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MDD	Major depressive disorder
MS	Multiple sclerosis (in graphs also group of non-depressed MS patients)
MS+MDD	Multiple sclerosis patients with depression
NA	Not applicable
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	Neutrophil-to-lymphocyte ratio
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PPMS	Primary progressive multiple sclerosis
PRR	Pattern recognition receptor
PTSD	Post-traumatic stress disorder
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RORγt	Retinoic acid-related orphan receptor γt
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SDMT	Symbol digit modalities test
SNRI	Serotonin noradrenalin reuptake inhibitor
SPMS	Secondary progressive multiple sclerosis
SSC	Side scatter
SSRI	Selective serotonin reuptake inhibitor
ST13	Suppression of tumorgenicity 13
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor TBX21
TBP	TATA-box binding protein (*TBP*)
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EMRA}	Effector memory T cells re-expressing CD45RA
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TMB	Tetramethylbenzidine
TNFR1	Tumour necrosis factor receptor 1
T_{reg}	Tegulatory T cells
ULBP4	UL16 binding protein 4

UMAP	Uniform manifold approximation and projection
VLA-4	Very late antigen 4
vNKT cell	Variable NKT cell (also NKT II)
ZAP-70	Zeta-chain-associated protein kinase 70
9-HPT	Nine-hole peg test

8 Appendix

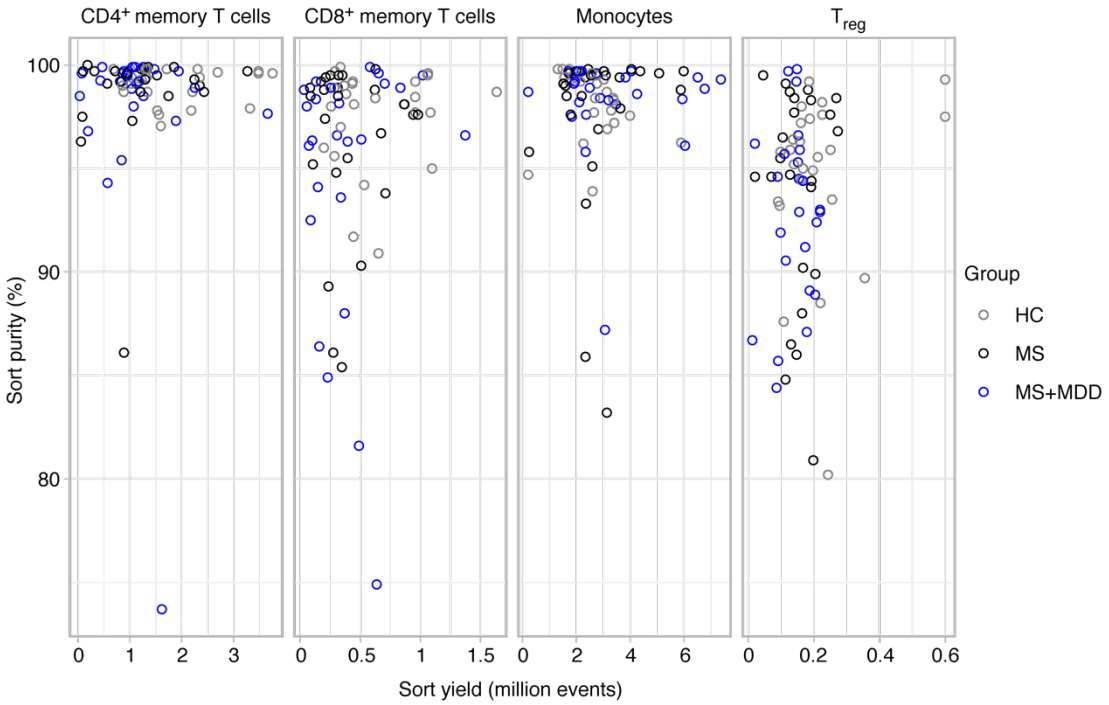

Figure 7.1: Sort purity and sort yield did not systematically vary across study groups

Sort purity over sort yield for $CD4^+$ memory T cells (first box), $CD8^+$ memory T cells (second box), monocytes (third box) and T_{reg} (fourth box). Data points display individual samples, colours depict groups: healthy controls (HC; n = 25; grey), MS patients (MS; n = 25; black); depressed MS patients (MS+MDD; n = 25, blue).

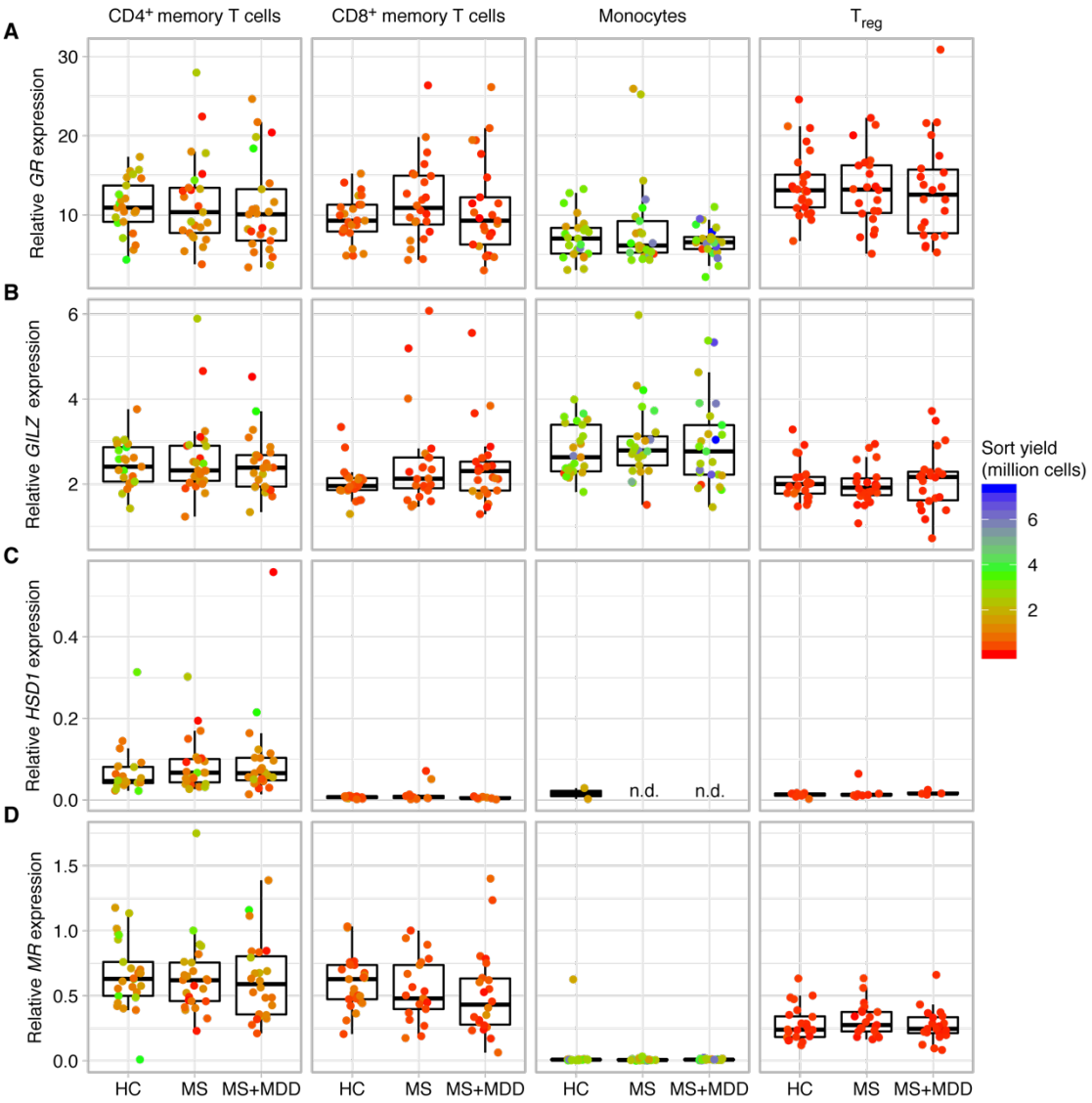

Figure 7.2: Sort yield did not affect expression of defined GC pathway elements

*mRNA expression of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4$^+$ memory T cells (first column), CD8$^+$ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with individual data points, each representing an individual (n see **Table 7.1**) coloured by number of events sorted for this sample: lower sort yield is depicted in red, higher sort yield in green to purple.*

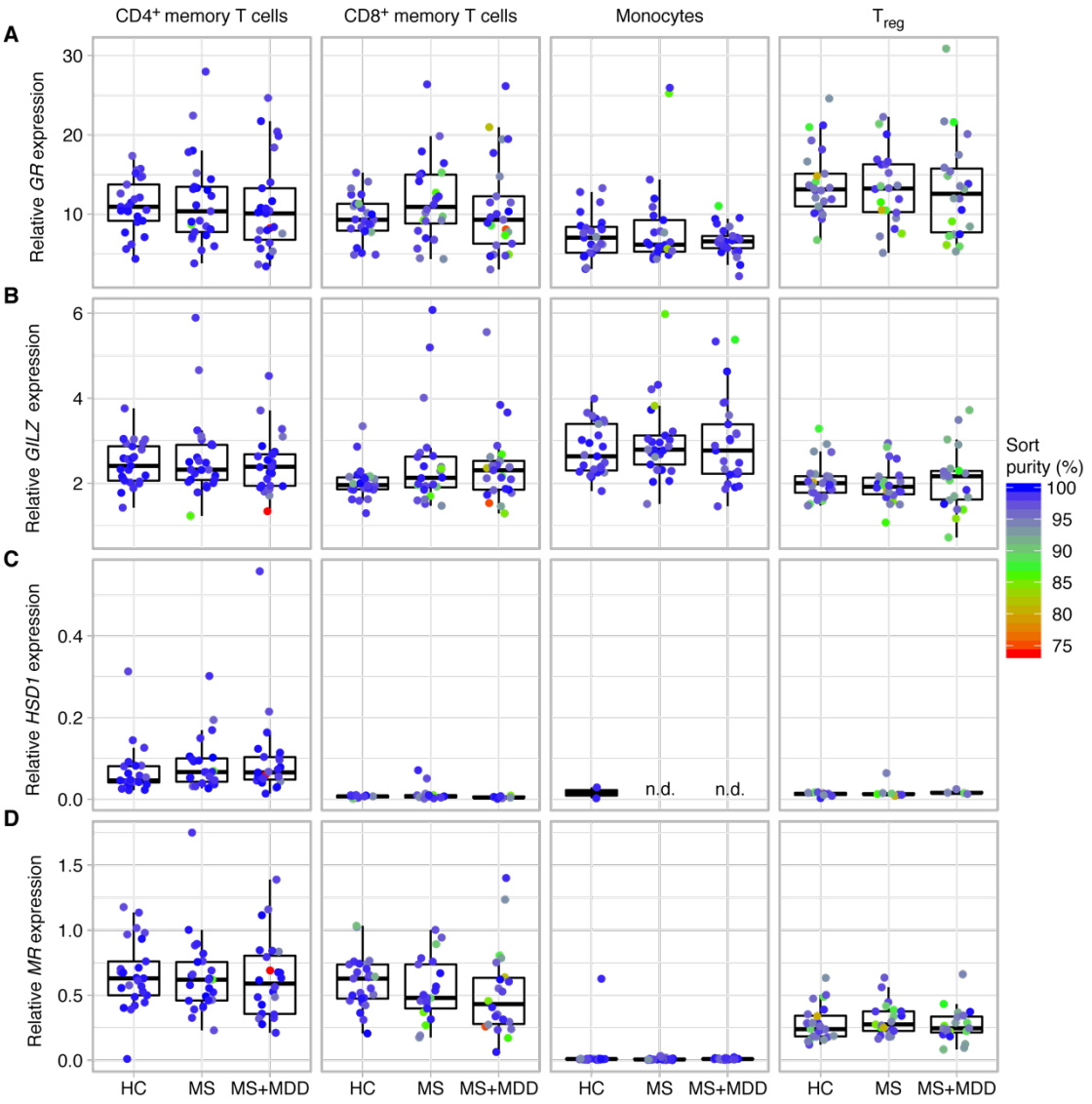

Figure 7.3: Sort purity did not affect expression of defined GC pathway elements

*mRNA expression of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4⁺ memory T cells (first column), CD8⁺ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with individual data points, each representing an individual (n see **Table 7.1**) coloured by sort purity measured for this sample: lower purities are depicted in red, higher purities in purple to blue.*

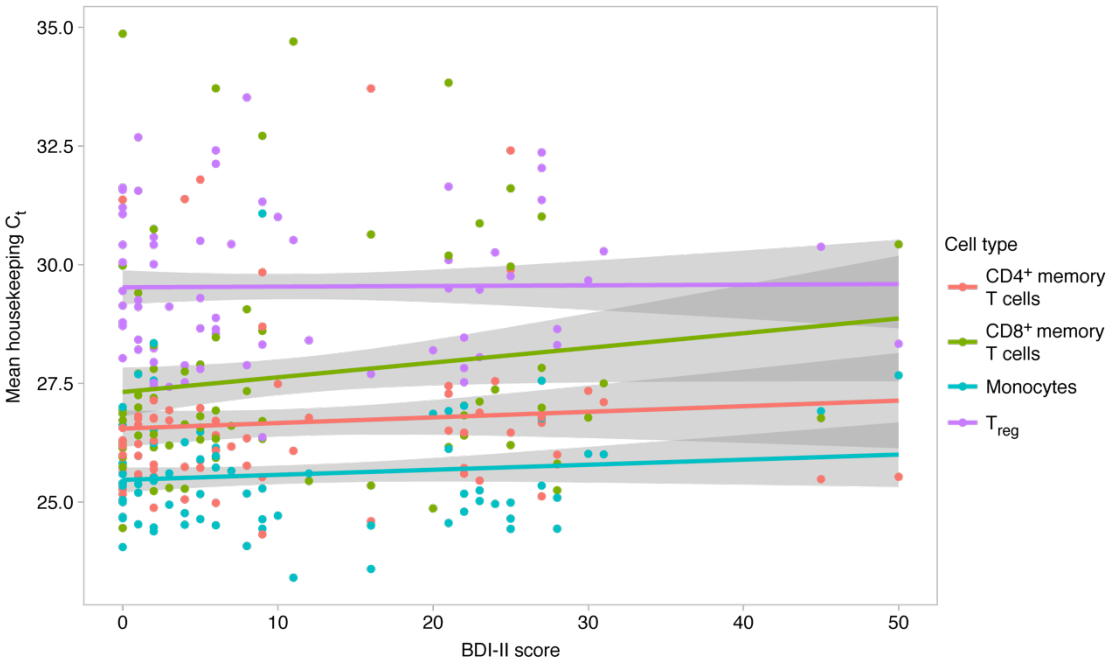

Figure 7.4: No variation of mean housekeeping C_t values across depression scores

Mean housekeeping C_t value, representing sample input to the qPCR over BDI-II depression score. Data are depicted as individual samples coloured for their cell type: $CD4^+$ memory T cells (pink), $CD8^+$ memory T cells (green), monocytes (turquoise), T_{reg} (purple), overlaid with a linear regression lines and 95% confidence intervals.

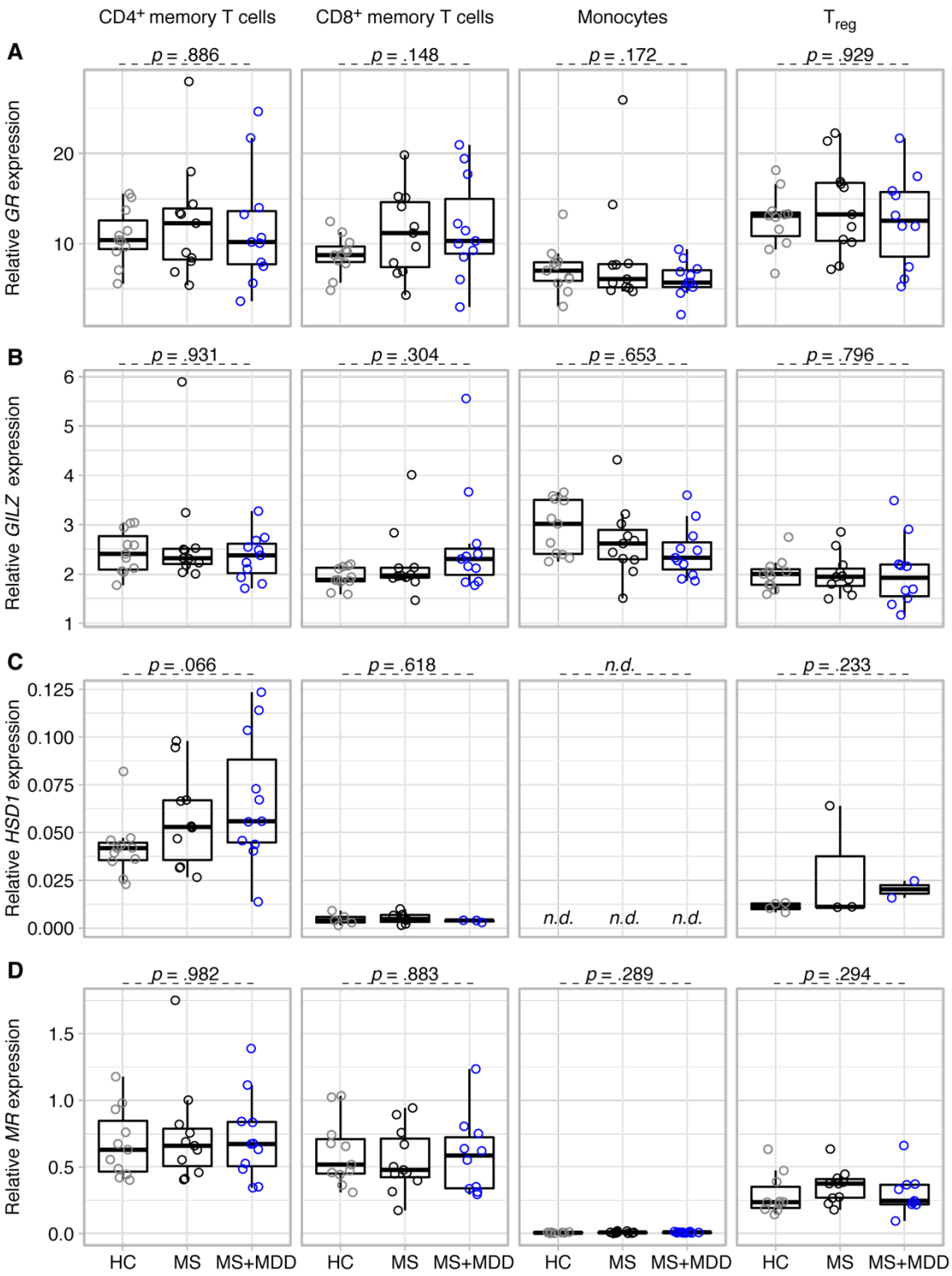

Figure 7.5: Expression of defined GC pathway elements was not altered in unmedicated depressed MS patients

*mRNA expression of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4⁺ memory T cells (first column), CD8⁺ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points, each representing an individual (n see **Table 7.2**): healthy controls (HC; grey), non-depressed MS patients (MS; black), depressed MS patients (MS+MDD; blue). P values derived from Kruskal-Wallis tests.*

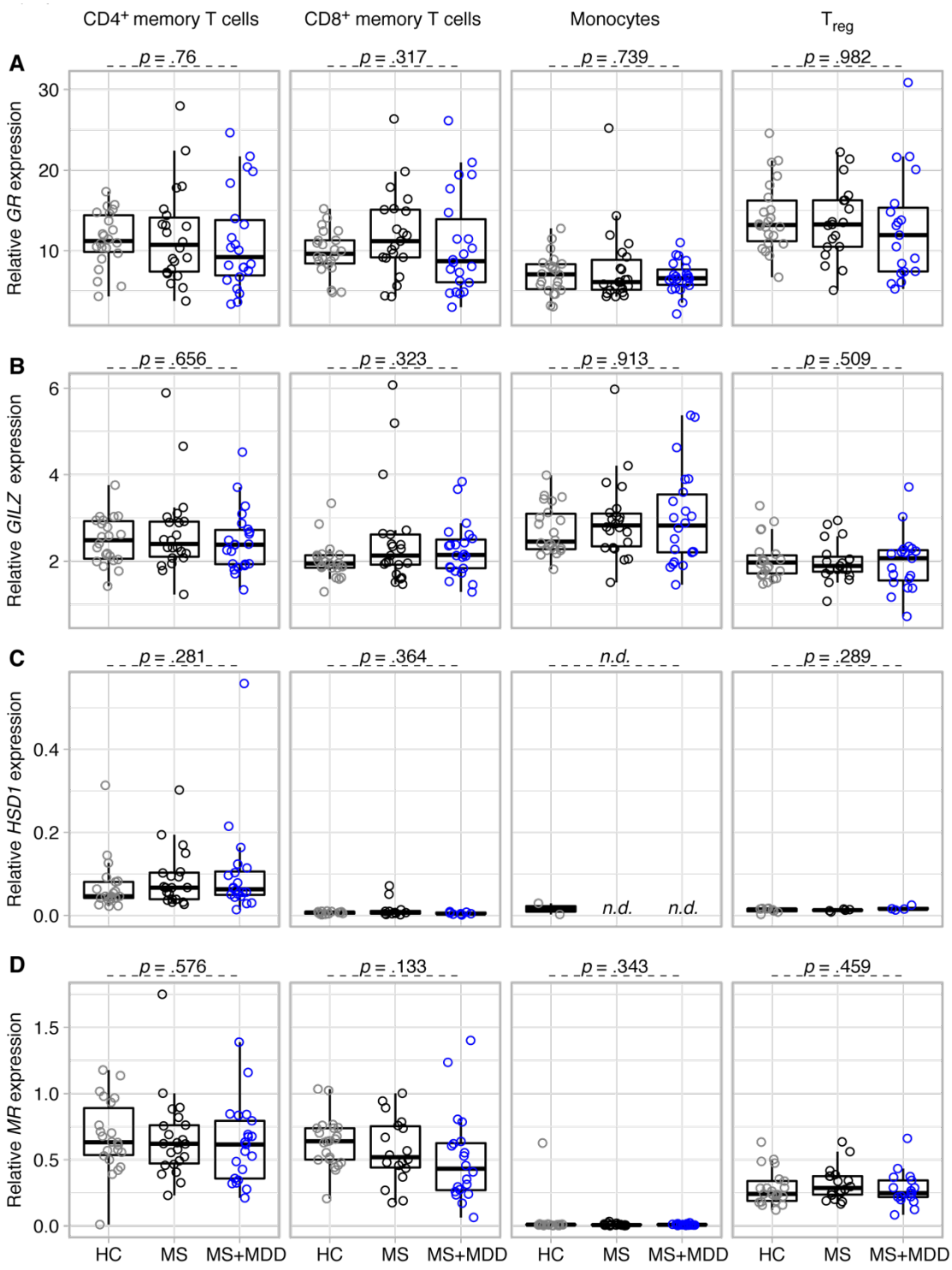

Figure 7.6: Expression of defined GC pathway elements was not altered in female depressed MS patients

*mRNA expression of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4⁺ memory T cells (first column), CD8⁺ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points, each representing an individual (n see **Table 7.3**): healthy controls (HC; grey), non-depressed MS patients (MS; black), depressed MS patients (MS+MDD; blue). P values derived from Kruskal-Wallis tests.*

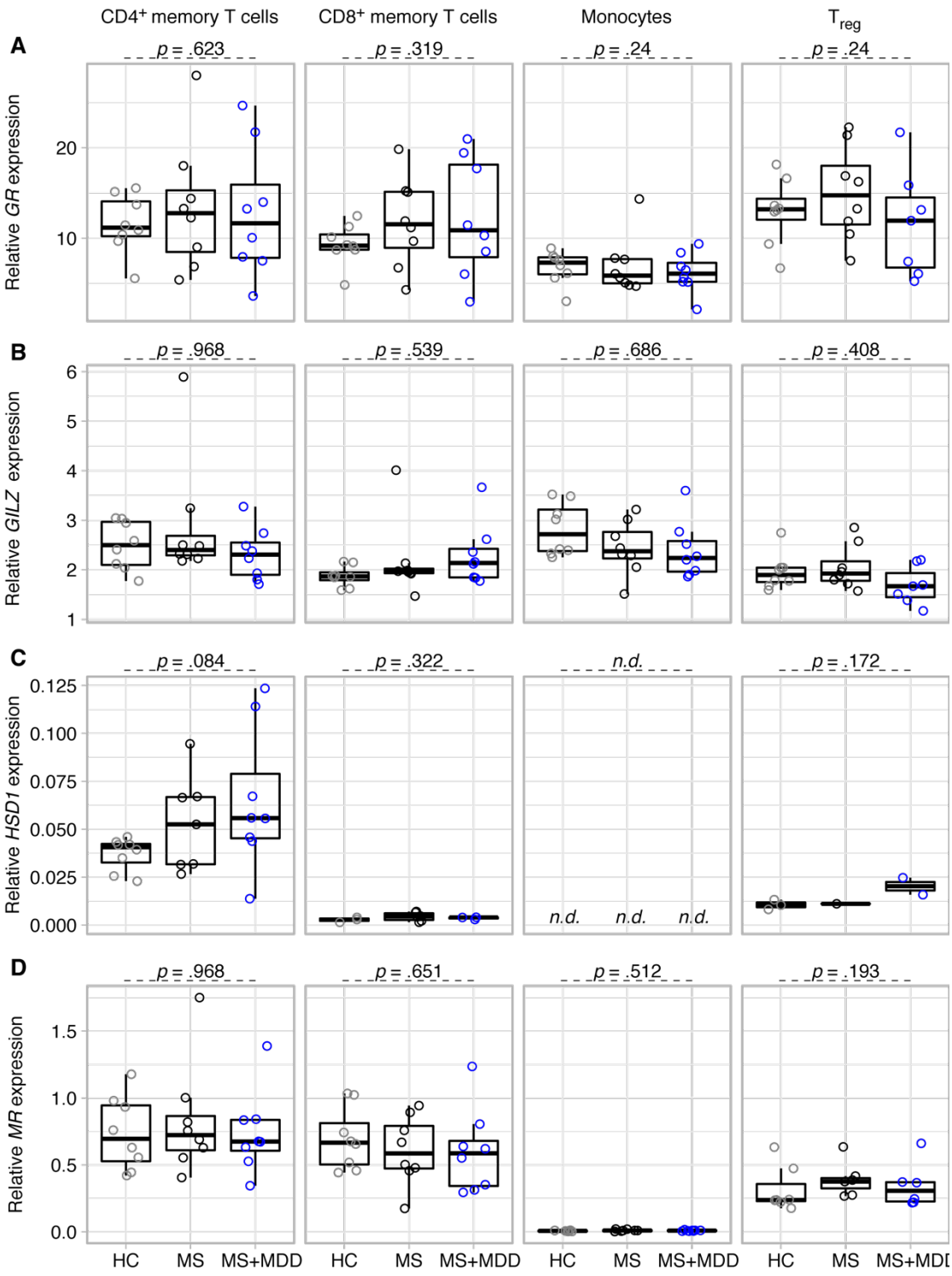

Figure 7.7: Expression of defined GC pathway elements was not altered in unmedicated female MS patients with depression

*mRNA expression, of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4$^+$ memory T cells (first column), CD8$^+$ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points, each representing an individual (n see **Table 7.4**): healthy controls (HC; grey), non-depressed MS patients (MS; black), depressed MS patients (MS+MDD; blue). P values derived from Kruskal-Wallis tests.*

Figure 7.8: Relative gene expression of defined GC pathway elements was not correlated with depression scores

*(**A**) Relative mRNA expression of GILZ (pink), GR (green), HSD1 (turquoise), MR (purple) over BDI-II depression scores of individual subjects are depicted for $CD4^+$ memory T cells (first box), $CD8^+$ memory T cells (second box), monocytes (third box) and T_{reg} (fourth box). (**B**) shows the same data with a smaller scale of the Y axis. Data are displayed as individual data points (n see **Table 7.1**) with superimposed linear regression lines and 95% confidence intervals.*

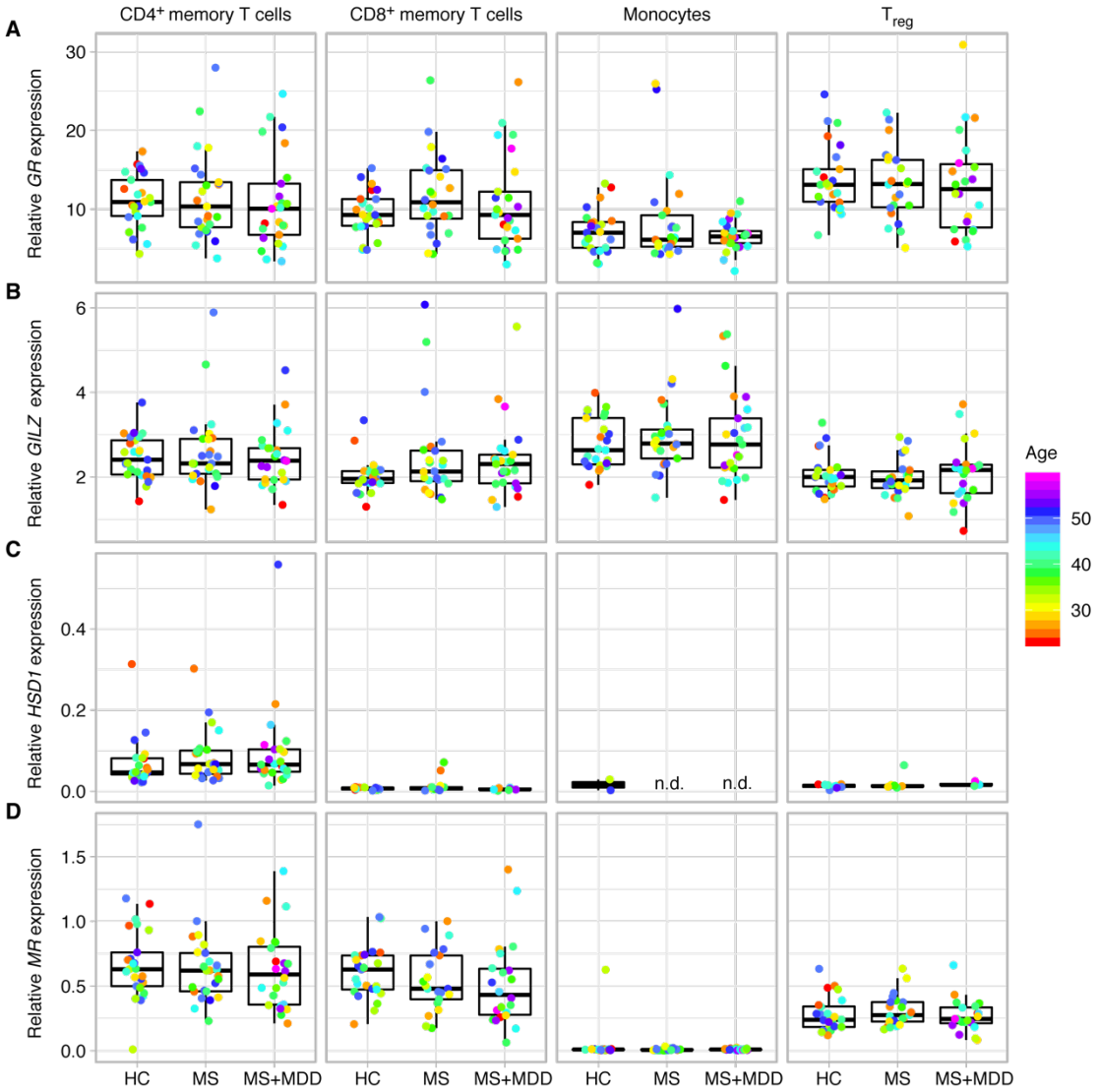

Figure 7.9: Age of participants did not influence gene expression of defined GC pathway elements

*mRNA expression, of GR (**A**), GILZ (**B**), HSD1 (**C**) or MR (**D**) relative to housekeeping genes in CD4⁺ memory T cells (first column), CD8⁺ memory T cells (second column), monocytes (third column) and T_{reg} (fourth column). Data are displayed as box plots showing median and 1st to 3rd percentile; whiskers depict range excluding outliers; overlaid with data points, each representing an individual (n see **Table 7.1**), coloured by the individuals age.*

Table 7.1: Sample number analysed in qPCR analysis of complete cohort

Cell populations	qPCR target	HC (n)	MS (n)	MS+MDD (n)
$CD4^+$ memory T cells	GR	25	25	25
	GILZ	25	25	25
	HSD1	24	23	23
	MR	25	25	24
$CD8^+$ memory T cells	GR	25	24	25
	GILZ	25	24	25
	HSD1	15	13	7
	MR	25	21	22
Monocytes	GR	25	25	25
	GILZ	25	25	25
	HSD1	2	0	0
	MR	21	23	21
T_{reg}	GR	25	25	25
	GILZ	25	25	25
	HSD1	9	7	4
	MR	21	23	21

Table 7.2: Sample number analysed in qPCR subgroup analysis of unmedicated patients

Cell populations	qPCR target	HC (n)	MS (n)	MS+MDD (n)
$CD4^+$ memory T cells	GR	11	11	11
	GILZ	11	11	11
	HSD1	11	10	11
	MR	11	11	11
$CD8^+$ memory T cells	GR	11	11	11
	GILZ	11	11	11
	HSD1	5	7	3
	MR	11	11	10
Monocytes	GR	11	11	11
	GILZ	11	11	11
	HSD1	0	0	0
	MR	8	10	11
T_{reg}	GR	11	11	10
	GILZ	11	11	10
	HSD1	4	3	2
	MR	10	10	9

Table 7.3: Sample number analysed in qPCR subgroup analysis of females

Cell populations	qPCR target	HC (n)	MS (n)	MS+MDD (n)
$CD4^+$ memory T cells	GR	22	22	22
	GILZ	22	22	22
	HSD1	21	20	20
	MR	22	22	21
$CD8^+$ memory T cells	GR	22	21	22
	GILZ	22	21	22
	HSD1	13	12	7
	MR	22	18	20
Monocytes	GR	22	22	22
	GILZ	22	22	22
	HSD1	2	0	0
	MR	20	21	18
T_{reg}	GR	22	19	19
	GILZ	22	19	19
	HSD1	8	5	4
	MR	21	18	17

Table 7.4: Sample number analysed in qPCR subgroup analysis of unmedicated females

Cell populations	qPCR target	HC (n)	MS (n)	MS+MDD (n)
$CD4^+$ memory T cells	GR	8	8	8
	GILZ	8	8	8
	HSD1	8	7	8
	MR	8	8	8
$CD8^+$ memory T cells	GR	8	8	8
	GILZ	8	8	8
	HSD1	3	6	3
	MR	8	8	8
Monocytes	GR	8	8	8
	GILZ	8	8	8
	HSD1	0	0	0
	MR	7	8	8
T_{reg}	GR	8	8	7
	GILZ	8	8	7
	HSD1	3	1	2
	MR	7	7	6

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11 Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

Publications

1. **Ramien, C.*,** Yusko, E.C. *, Engler, J.B., Gamradt, S., Patas, K., Schweingruber, N., Willing, A., Rosenkranz, S.C., Diemert, A., Harrison, A., Vignali, M., Sanders,C., Robins, H.S., Tolosa, E., Heesen,C., Arck, P.C., Scheffold, A., Chan, K., Emerson, R.O., Friese, M.A.*, and Gold, S.M.* (2019) T Cell Repertoire Dynamics during Pregnancy in Multiple Sclerosis. *Cell Reports*, 2019, 29. https://doi.org/10.1016/j.celrep.2019.09.025

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12 Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

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