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FLOW CYTOMETRY ANALYSIS OF T CELL SUBSETS IN CEREBROSPINAL FLUID AND PHERIPERAL BLOOD OF NARCOLEPSY TYPE 1 PATIENTS WITH LONG LASTING DISEASE

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ABSTRACT

Background: Type 1 Narcolepsy (NT1) is a central hypersomnia linked to the destruction of hypocretin-producing neurons. A great body of genetic and epidemiological data points to a likely autoimmune disease aetiology. Recent reports have characterized peripheral blood T-cell subsets in NT1, whereas data regarding the cerebrospinal fluid (CSF) immune cell composition are lacking. In this study, we aim to identify the T- and NK- cell subsets in NT1 patients with long-term disease course.

Methods: Immune cell subsets from CSF and peripheral blood mononuclear cell (PBMC) samples were analysed by flow cytometry in two age-, sex and BMI-balanced groups of 14 NT1 patients versus 14 healthy controls. The frequency of CSF cell groups were compared with PBMCs. Non-parametric tests were used for statistical analyses.

Results: NT1 patients did not show significant differences of CSF immune cell subsets compared to controls, despite a trend towards higher $CD4^+$ terminally differentiated effector memory T cells. T cells preferentially displayed a memory phenotype in the CSF compared to PBMCs. Furthermore, a reduced frequency of $CD4^+$ terminally differentiated effector memory T cells and an increased frequency of Natural Killer $CD56^{bright}$ cells were observed in PBMCs from patients compared to controls. Finally, the ratio between CSF and peripheral $CD4^+$ terminally differentiated effector memory T cells showed a two-fold increase in NT1 patients versus controls.

Conclusions: Significant differences in PBMCs and CSF immune cell profile were found between NT1 patients versus controls. These differences may reflect differences in HLA status, or be primary or secondary to hypocretin deficiency in narcolepsy.

CHAPTER 1

Introduction

1.1 NORMAL SLEEP STRUCTURE

Normal sleep is divided into two phases: non–rapid eye movement (NREM) and rapid eye movement (REM) sleep. NREM sleep is further distributed into progressively deeper stages of sleep: stage N1, stage N2, and stage N3. As NREM stages progress, stronger stimuli are required to result in an awakening. Stage REM sleep is characterized by tonic and phasic components. The phasic component is a sympathetically driven state of rapid eye movements, muscle twitches, and respiratory variability. Tonic REM is a parasympathetically driven state with no eye movements. Moreover, the REM period length and density of eye movements increases throughout the sleep cycle (Berry et al. 2012).

Waking usually transitions into light NREM sleep. NREM sleep typically begins in the lighter stages N1 and N2, and progressively deepens to slow wave sleep as evidenced by higher-voltage delta waves. N3 (slow wave sleep) is present when delta waves account for more than 20% of the sleep electroencephalogram (EEG). REM sleep follows NREM sleep and occurs 4-5 times during a normal 8-hour sleep period. The first REM period of the night may be less than 10 minutes in duration, while the last may exceed 60 minutes. The NREM-REM cycles vary in length from 70-100 minutes initially to 90-120 minutes later in the night.

Usually, N3 sleep is present more in the first third of the night, whereas REM sleep predominates in the last third of the night. This can be helpful clinically as NREM parasomnias such as sleep walking typically occur in the first third of the night with the presence of N3 sleep. This contrasts with REM sleep behaviour disorder (RBD), which typically occurs in the last half of the night (Berry et al. 2012).

1.2 NARCOLESPY TYPE 1 DISORDER

Narcolepsy is a life-long, underrecognized sleep disorder that was firstly described by Westphal in 1877 and Gelineau in 1880 (Schenck et al. 2007). It has a complex symptomatology that reflects the partial or global intrusion of REM sleep into wakefulness, causing excessive daytime sleepiness (EDS), cataplexy (i.e. sudden loss of muscle tone during wakefulness triggered by strong emotions), hypnagogic hallucinations, sleep paralysis, and disrupted nocturnal sleep (Dauvilliers et al. 2007; Abad & Guilleminault, 2009; Roth et al. 2013). While EDS with recurrent sleep attacks is the most common feature, cataplexy is pathognomonic, allowing us to clinically distinguish narcolepsy with cataplexy from narcolepsy without cataplexy. Along with the discovery of hypocretin deficiency as causal for narcolepsy, the disorder has been recently divided in narcolepsy type 1 (NT1), characterized by low cerebrospinal fluid (CSF) hypocretin-1 levels and cataplexy in most of these cases, and in narcolepsy type 2 (NT2), characterized by normal CSF hypocretin-1 levels and absence of cataplexy. The hypocretin neurons main function is to sustain wakefulness and suppress rapid eye

movement sleep (Bonnavion & de Lecea 2010), explaining why a loss of these neurons causes the narcoleptic phenotype. In addition to the lack of signal from preprohypocretin in the brains of NT1 patients, signals from the colocalizing proteins dynorphin and neuronal activity-regulated pentraxin have also been found lacking (Blouin et al. 2005; Crocker et al. 2012). Interestingly, nearby and intermingled melanin concentrating hormone-producing neurons were found to be intact (Peyron et al. 2000). The specific loss of hypocretin neurons in NT1 is now thought to result from an autoimmune attack on these neurons.

1.3 EPIDEMIOLOGY

The prevalence of NT1 is 0.02% to 0.05% (Silber et al. 2002). Globally, the prevalence varies from highest in Japan (0.16%) to lowest in Israel (0.0002%) (Longstreth et al. 2007). NT1 is prevalent in 0.02% to 0.18% in the United States and Western European populations (Darien 2014).

Narcolepsy onset usually arises in adolescence or young adulthood with a bimodal distribution: two peaks have been described, at the age of around 15 and 35 years, respectively (Dauvilliers et al. 2001; Dauvilliers et al. 2007).

The exact prevalence of NT2 is uncertain (Westchester, 2014). Cases of narcolepsy without cataplexy represent 15% to 25% of the clinic narcoleptic population. The age of onset mirrors that of NT1.

1.4 SYMPTOMS

The detrimental effect of narcolepsy on daily functioning and activities, psychosocial functioning, quality of life, work performances, and familial life are well described in the literature on adult patients (Ingravallo et al. 2012). Failure to recognize symptoms may delay diagnosis from 8.7 years up to 22.1 years (Thorpy & Krieger et al. 2014; Luca et al. 2013). Historically, narcolepsy was considered a disorder of adulthood, but accumulating evidence indicates that the disease most often starts during childhood / adolescence (Thorpy & Krieger et al. 2014), ages characterized by specific peculiarities in the clinical phenotype (Dahmen et al. 2001; Ohayon et al. 2005).

Extended sleep time and weight gain with rapid increase in body mass index (BMI) at disease onset are often noted in adult and even more in children patients (Rocca et al. 2015; Pizza et al. 2013; Dahmen et al. 2011), while the association with precocious puberty can obviously occur only in children especially with very early disease onset (Plazzi et al. 2006; Poli et al. 2013). Cataplexy in adults is characterized by paroxysmal episodes of loss of muscle tone triggered by strong, mainly positive, emotions. Typical cataplectic triggers are indeed laughing, sharp remarks, telling or listening to jokes, being tickled or tickling others, surprise, chuckling, feeling angry, stressed, or

embarrassed/ashamed (Overeem et al. 2011). The cataplexy childhood phenotype, especially close to the disease onset, often consists in a complex movement disorder with persistent hypotonia and prominent orofacial involvement (jaw opening, eyelid drooping, head rolling, or tongue thrusting movements), also called "cataplectic facies" even in the absence of emotional triggers (Rocca et al. 2015, Pizza et al. 2013).

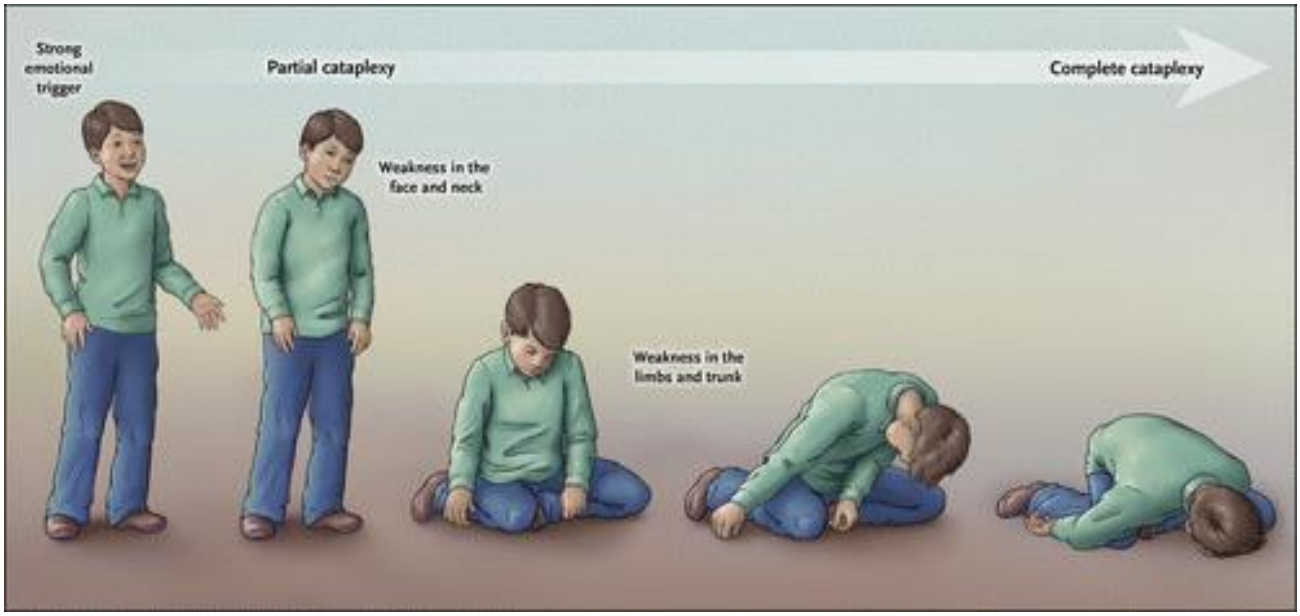

Figure 1: cataplexy features in NT1 patient (Scammel 2015).

Cognitive dysfunction includes difficulty focusing, thinking, and concentrating along with automatic behaviors (U.S. Food and Drug Administration 2013; Maski et al. 2017). Hyperactivity, aggressive behavior, problems interacting with peers, and psychopathological features may be additional clinical features very relevant in children affected by NT1 (Filardi et al. 2017). Moreover, nocturnal behavioral and movement disorders have been recently described as additional features in pediatric NT1 patients (Antelmi et al. 2017).

Narcolepsy in children represents a diagnostic challenge because of its variability in the presenting signs, and for the scarce awareness of the disease among physicians (Wise & Lynch, 2001). Associated symptoms such as cataplectic attacks may be confused with atonic seizures, especially in children under 5 years old (Guilleminault & Pelayo, 2000), or even with neuromuscular diseases (Plazzi et al. 2011). However, the preservation of consciousness despite EDS and the link to emotions remain the distinctive features of cataplexy. Sleep paralysis can be accompanied by hypnagogic hallucinations that consist in vivid visual or auditory experiences usually of benign content. Nevertheless, both these signs can be unrecognized or misinterpreted by young children or their parents (Yoss & Dali 1960).

Narcolepsy diagnosis is based on the combination of clinical assessment and objective confirmation by means of neurophysiological and biological examinations. The neurophysiological disease hallmark is the early appearance of REM sleep at sleep onset, named sleep onset REM period (SOREMP), a finding described in 1960 by Vogel (1960), that rapidly became the distinctive disease fingerprint along with the standardization of the Multiple Sleep Latency Test as in-laboratory diagnostic procedure to characterize EDS disorders in sleep laboratories worldwide (Littner et al. 2005). However, the discovery of the pathogenic relevance of hypocretin-1 as unique biological disease marker coupled with the increasing availability of cerebrospinal hypocretin-1 assay was recently integrated in the latest diagnostic requirements in the International Classification of Sleep Disorders, third edition (ICSD-3) (Darien 2014). Indeed, diagnostic criteria for narcolepsy now require the combination of different symptoms and objective neurophysiological or biological evidence; the ICSD-3 (Darien 2014) provides the diagnostic criteria that must be met for NT1 disease, i.e. the patient has EDS occurring for at least three months, and the presence of one or both of the following:

1. Cataplexy, and a mean sleep latency of ≤ 8 minutes and two or more SOREMPs on a multiple sleep latency test (MSLT). A SOREMP (within 15 minutes of sleep onset) on the preceding nocturnal polysomnogram may replace one of the SOREMPs on the MSLT.

2. CSF hypocretin-1 concentration, measured by immunoreactivity (RIA assay), is either ≤ 110 pg/mL or <1/3 of mean values obtained in normal subjects with the same standardized assay.

1.5 THE HYPOCRETIN SYSTEM IN NT1

The hypocretins are two peptides, hypocretin 1 (also named orexin-A) and hypocretin 2 (or orexin-B), generated from a single preprohypocretin peptide synthesized by a small number of neurons restricted to the lateral hypothalamus and perifornical brain area (de Lecea, 2012; Sakurai et al. 1998). In contrast, hypocretin axons are found throughout the central nervous system (CNS), with innervation of the hypothalamus, locus coeruleus, raphe, midline thalamus, all levels of spinal cord, sympathetic and parasympathetic centres, and many other brain regions (Peyron et al. 1998; van den Pol 1999). Two G protein–coupled receptors that respond to the hypocretins have been identified (Sakurai et al. 1998). In parallel to the wide distribution of axons, the two-hypocretin receptors show a widespread and heterogeneous pattern of expression throughout the CNS (Trivedi et al. 1998). Hypocretin raises synaptic activity in neurons of the hypothalamus (de Lecea et al. 1998) and locus coeruleus (Hagan et al. 1999; Horvath et al. 1999) and can act on postsynaptic receptors to increase cytosolic calcium or at presynaptic receptors on axon terminals to enhance release of glutamate and

GABA (van den Pol et al. 1998). The fact that hypocretin can enhance activity of either excitatory or inhibitory neurons suggests that the peptide could ultimately increase or decrease the activity of innervated brain circuits (van den Pol 2000).

The potential importance of hypocretin neurons in preventing NT1 was first suggested by the finding that narcoleptic dogs have mutations on the hypocretin receptors 2 (Lin et al. 1999). Even though different mutations were found in each of two narcoleptic breeds of dogs, in each breed the mutation was localized to the hypocretin receptor 2, rendering it non-functional. Parallel studies in hypocretin knockout mice revealed a similar narcoleptic phenotype (Chemelli et al. 1999), indicating that loss of either the peptide or one of the two peptide receptors results in narcolepsy in animal model (van den Pol 2000).

Following studies based on immunocytochemistry and in situ hybridization indicate that there is a substantially decreased number of neurons producing hypocretin in the hypothalamus of human NT1 patients (Peyron et al. 2000; Thannickal et al. 2000). Thannickal et al. show that the number of hypocretin neurons is decreased by about 90% in four narcoleptic human brains. In parallel, Peyron et al. show an absence of hypocretin mRNA from the hypothalamus, and the loss of hypocretin 1 and 2 peptides from extrahypothalamic hypocretin target regions of six narcoleptic brains. Both studies of narcoleptic brains report no decreased number of neurons containing melanin-concentrating hormone, a neuroactive peptide found in cells in the same hypothalamic region of hypocretin-producing neurons. This finding suggests a very selective absence of hypocretin neurons, rather than a general loss of neurons from the hypothalamus (van den Pol 2000) led to the hypothesis that narcolepsy is an autoimmune driven process within the hypothalamus.

1.6 ENVIRONMENTAL FACTORS AND GENETIC EVIDENCES SUPPORTING THE AUTOIMMUNE HYPOTESIS OF NT1

Autoimmune disease result from the interaction between environmental factors and a susceptible genetic background. It has become increasingly clear that environmental factors play an important role in the development and progression of a variety of autoimmune conditions (Molina & Shoenfeld, 2005). Currently the most important environmental factor for NT1 development is the influenza A H1N1 virus. Following the 2009-2010 campaign of pandemic H1N1 flu vaccination using Pandemrix, an AS03 (squalene, alpha-tocopherol) H1N1 vaccine developed in response to the 2009 H1N1 influenza pandemic, a significant six to nine fold increase in the risk of developing NT1 was reported in northern of Europe (Partinen et al. 2012), and a peak in the incidence of H1N1 infection in late 2009 was followed by a threefold increase in NT1 incidence in China (Han et al. 2011). A

phenomenon that was followed by a decrease in cases in the later years (Han et al. 2013). Intriguingly, the vast majority (>95%) of patients diagnosed with NT1 following the 2009 pandemic were not vaccinate for H1N1, indicating that naturally occurring influenza-A infections may increase the susceptibility of developing this disorder. Further reports discovered an association in Sweden, Finland, Ireland and in the United Kingdom subsequent the AS03-adjuvanted vaccine and an increased risk of developing NT1 especially in individuals between 5 and 19 years of age (Nohynek et al. 2012; Mahlios et al. 2013). On the other hand, there have been no reported increases in the incidence of NT1 in Italy, or the Netherlands, though the reason behind this phenomenon remains unclear (Wijnans et al. 2012; Mahlios et al. 2013). There is also evidence of an association with other common upper airway infections such as beta haemolytic streptococcal infections (Aran et al. 2009; Longstreth 2007), and lastly, the incidence rate of NT1 onset significant increase in the months following winter-related infections with a seasonal patterns (Han et al. 2011). Streptococcal infections have also been associated with several neurological autoimmune disorders, suggesting that certain immunological responses to infection may result in specific neurological damage (Mahlios et al. 2013; Snider & Swedo, 2003). Moreover, further epidemiological studies have revealed that individuals infected with streptococcus are more than five times as likely to develop NT1 (Koepsell et al. 2010).

The first suggestion, before any epidemiological data were reported, that NT1 might be an autoimmune disorder, came from HLA typing studies. In humans, more than 20 polymorphic HLA genes encode multiple subtypes of Major Histocompatibility Complex (MHC) class I and II proteins that present foreign peptides to T-cell during infections, triggering immune responses (Kornum et al. 2011). NT1 has the strongest HLA associations known, with more than 99% of patients carrying HLA *DQB1*06:02*. (Mignot et al. 2001). Among HLA class II genes, the *DRB1*15:01-DQA1*01:02-DQB1*06:02* is the disease-associated haplotype in NT1. In European populations, the three alleles of this haplotype are in complete linkage disequilibrium, so that any single allele is representative of the haplotype (Mignot et al. 2001; Hor et al. 2010; Tafti et al. 2014). Studies including molecular genotyping of HLA class I genes (*A*, *B*, and *C*) in large populations with NT1 are lacking. However, a recent study used molecular HLA class I typing in 304 patients and 304 controls and imputed class I genotypes from previous genome-wide (GW) association studies in White and Chinese populations. This study found, in addition to *DPB1*, significant associations between narcolepsy and HLA-*A*11:01*, *B*35:03*, and *B*51:01* (Ollila et al. 2015). HLA class II molecules are constitutively expressed only by antigen-presenting cells such as dendritic cells, macrophages, or B cells, while HLA class I molecules are ubiquitously expressed by nearly all nucleated cells.

Unique to NT1 is the genetic association with a polymorphism in the T cell receptor alpha (*TCRα*) gene, which encodes the alpha chain of the αβ-heterodimer of the T cell receptor on both CD4+ and cytotoxic $CD8^+$ T cells, which can bind to both MHC-I and MHC-II molecules (Hallmayer et al. 2009). The TCR consist of α and β chain and similarly to the immunoglobulin loci, DNA recombination occurs within the *TCR* loci to create an enormous diversity in the receptors expressed and leads to generation of a unique repertoire of TCR bearing T-cells maybe with autoreactive potential (Krangel 2009; Kornum et al. 2011).

Furthermore, a genetic association of NT1 with purinergic receptor subtype 2Y11 (*P2RY11*) was discovered, and since this receptor is highly expressed on cytotoxic lymphocytes suggests an involvement of immune responses in aetiology of the disease (Kornum et al. 2011). Another different mutation in the DNA Methyltransferase 1 (*DNMT1*) gene led to narcolepsy with or without cataplexy in the context of two different autosomal dominant progressive neurodegenerative disorders, named hereditary sensory and autonomic neuropathy with dementia and hearing loss type IE (HSAN-IE), and autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN) (Winkelmann et al. 2012; Moghadam et al. 2014).

As *DNMT1* is located in extremely close proximity to *P2RY11* (within 30 kb), it is possible that the genetic association in sporadic cases and the rare autosomal dominant ADCA-NC/HSAN-IE disease could be linked somehow (Singh et al. 2013).

Complementing this observation DNMT1 is highly expressed in immune system cells, and hypo methylation has been shown to be involved in other autoimmune diseases such as lupus (Singh et al. 2013). DNMT1 also plays an important role in differentiation of $CD4^+$ T cells into T regulatory cells by relieving repression of Foxp3 expression following TCR stimulation (Josefowicz et al. 2009). A possibility would be that an absence of T regulatory cells with specificity toward hypocretin neurons could result in autoimmunity.

In one more study, Faraco et al. (2013) used the Immunochip, an SNP chip designed to further fine map various autoimmune disease loci, and found that variants in two additional loci, Cathepsin H and Tumor necrosis factor super family member 4 (*TNFSF4*, also called *OX40L*), attained genome-wide significance (Faraco et al. 2013). As these genes are important for antigen processing and downstream T cell stimulatory effects, these findings further implicate the process of antigen presentation by HLA Class II APCs to T cells in the context of NT1 (Singh et al. 2013).

1.7 HUMORAL IMMUNITY AND ROLE OF AUTOANTIBODY IN NT1

The ultimate proof for autoimmunity would be the demonstration of T cell reactivity or autoantibodies directed towards hypocretin-producing neurons. Despite wide research, findings have been either controversial or negative. Post-mortem studies have only been conducted using brains from the late stages of NT1. While one study found an upregulation of glial fibrillary acidic protein (GFAP), a marker of astrogliosis, another study did not find any signs of inflammation (Peyron et al. 2000; Thannickal et al. 2003). Older studies have typically searched for a serum or CSF autoantibodies, but these studies have only found the examined autoantibodies in a minority of patients (Overeem et al. 2008). Moreover, reports of CSF and serum samples from patients sampled closer to the time of diagnosis have also shown varying results, possibly because of the latency between symptoms onset and clinical diagnosis (Degn and Kornum 2015; Kornum et al. 2015). For example, two studies have reported changed levels of the main subclasses of total IgG in NT1 and idiopathic hypersomnia (HI) (Tanaka & Honda 2010) and increased serum levels of total but not free IgG and IgM antibodies against hypocretin-1 in NT1, NT2 and HI diseases (Deloumeau et al. 2010). In 2010, the presence of autoantibodies against tribble homologues 2 (TRIB2) was detected in 14% of European patients versus 5% of controls (Cvetkovic-Lopes et al. 2010), a finding replicated in other patient populations (Kawashima et al. 2010; Toyoda et al. 2010). However, TRIB2 is expressed not only by hypocretin-producing neurons, but also present in many other cell populations, both in CNS and in the periphery, including immune cells (Kornum et al. 2011; Sung et al. 2006; Eder et al. 2008). Thus, TRIB2 autoantibodies are unlikely to be causative of the specific neuronal destruction; they could be reflective of a downstream effect that develops secondary to the neuronal destruction or they could be present before the disease in some individuals, causing higher risk of developing NT1 (Degn & Kornum 2015; Kornum et al. 2011). Another study discovered brain-specific autoantibodies in serum from NT1 patients (Bergman et al. 2014) do not target hypocretin neurons at all. Curiously, these autoantibodies were also detected in the serum of patients with other sleep disorders, and even in some healthy controls (Bergman et al. 2014). Finally, recent report investigated the presence of antibodies to the hypocretin receptor-2 in NT1 and NT2 patients, the results showed only 5% of patients positive for hypocretin receptor-2 autoantibodies with negative associations concluding that antibodies are not common in NT1 (Giannoccaro et al. 2017).

This development in NT1 research be like that of Rasmussen's encephalitis, an MHC class I-restricted $CD8^+$ mediated epilepsy syndrome. Initial research on Rasmussen's encephalitis was dominated by the detection of autoantibodies, with the identification of glutamate receptor 3 antibodies in a few patients (Wiendl et al. 2001) and later of α7 nicotinic acetylcholine receptors and Munc18-1 autoantibodies (Degn & Kornum 2015; Watson et al. 2011; Alvarez-Barón et al. 2008). Nevertheless, plasmapheresis was only effective in a few patients, and, similarly, the autoantibodies were only

identified in serum from a low fraction of patients (Varadkar et al. 2014). In neuromyelitis optica, antibodies against aquaporin 4 are identified in 60-80% of patients, and these respond well to intravenous immunoglobulin (IvIg) therapy (Jarius et al. 2014), suggesting a pathogenic role of aquaporin 4 autoantibodies. Because none of the NT1 related autoantibodies have been found in more than a small percentage of the patients, and since the clinical response to IvIg treatment is unpredictable at best (Knudsen et al. 2012), the role of CNS autoantibodies in NT1 disease remain unclear. Autoantibodies might be produced as part of secondary immune response to the initial neuronal damage in NT1, or they might increase the risk of developing NT1 by a yet unknown mechanism. As in the case of Rasmussen's encephalitis, NT1 is more likely caused by T cell, which is supported by the fact that other disorders involving a loss of neurons have also been shown to involve autoimmune T cells (Degn & Kornum 2015).

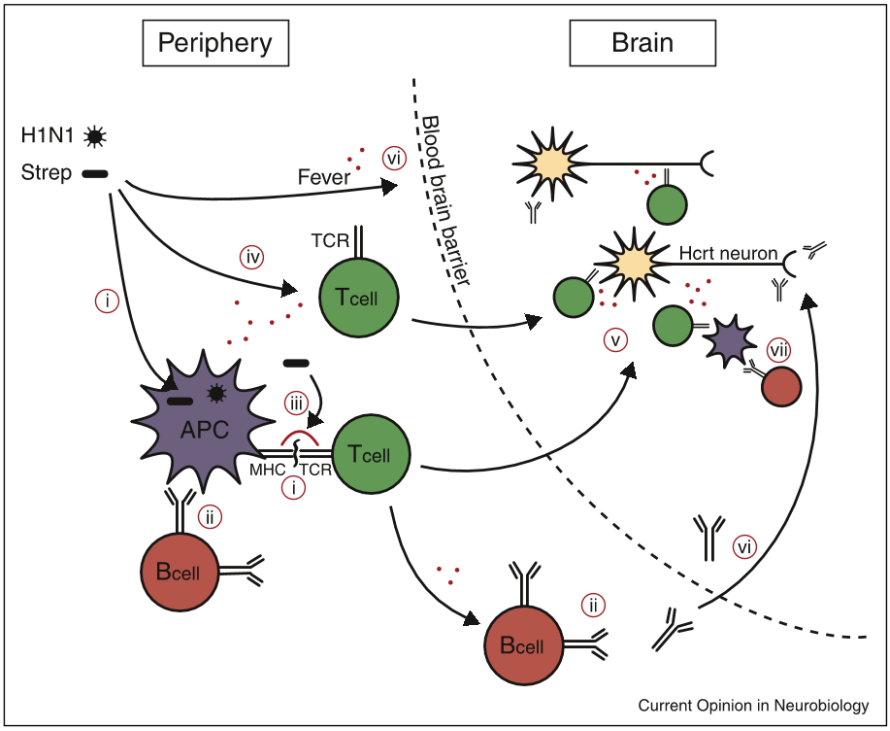

Figure 2: possible pathway for a role of influenza or streptococcus infections in the development of NT1. A H1N1 influenza or streptococcus pyogenes infection could stimulate autoreactive T cells or B cells via several possible mechanisms. Selected resting autoreactive T cells and B cells may have reactivity towards hypocretin-producing cells, having escaped negative selection. These could be activated in the following ways: (i) Molecular mimicry, T cells. Antigens from the virus or bacteria are presented by, for example, MHC *DQA1*01:02-DQB1*06:02* on an antigen presenting cell (APC). A T cells recognized the antigen and is activated. The same T cells migrates into the brain were it

recognized a hypocretin-cell specific antigen (cross-reactivity) inducing autoimmune process. (ii) Molecular mimicry, B cells. An autoreactive B cell can be triggered if it also recognizes an antigen from the microorganism. This process requires signals from activated T cell (T helper). (iii) Superantigens from streptococcus cross-link the MHC and TCR molecules independent of specific antigen specificity activating the autoreactive T cells. (iv) Bystander activation. Resting autoreactive cells are activated because of general immune activation independent of specific antigen. (v) Lymphocyte migration to CNS. Once activated, the T cells can migrate to the brain, depending on the type of T cell a variety of mechanisms could account for the autoimmune attack. (vi) Opening of the blood brain barrier. The immune response to infection also normally includes fever and other factors that can do the blood brain barrier more penetrant to lymphocytes and will allow antibodies to access to CNS. (vii) Production of autoantibodies can also occur as a secondary response to hypocretin producing-cell death via antigen presenting cells from the brain that have phagocytosed the dead neurons (Kornum et al. 2011).

1.8 T CELL MEDIATED IMMUNITY

T cell mediated immunity is an adaptive process that eliminates viral, bacterial, parasitic, or malignant cells by producing antigen-specific T lymphocytes. This type of immunity can also lead to the development of autoimmune disorders when the wrong recognition of a self-antigen occurs. The specificity of T lymphocytes is based on the recognition of antigenic peptides via the T cell receptor (TCR) receptor. Each T cell expresses on its surface a unique TCR because of the recombination processes that sort these highly variable receptors and the selection that occurs during the maturation of the lymphocytes in thymus. The pool of mature T cells leaving the thymus therefore has a vast repertoire of specific antigen receptors (Broere et al., 2011). The thymus selection process leads to the appearance of T cells with two types of TCRs. Most of them express a TCR formed by α and β chains. Lymphocytes carrying this TCR can be subdivided into several subgroups based on surface markers and different functional properties. Only a small fraction of T cells leaving thymus and expressing a TCR formed by the γ and δ chains, these lymphocytes appear much less heterogeneous than those with a TCR αβ and reside in the skin and in certain mucous surfaces, appear to have a role in the initial response to microbial invasions.

The mature T cells that leave the thymus, also known as naïve T cell (which have not yet encountered the antigen), circulate through the blood and the lymphatic system in order to concentrate in the secondary lymphoid organs. In these secondary lymphoid organs, namely: spleen, lymph nodes, and lymphoid tissues associated with mucous membranes (such as tonsils) that initiate the immune response mediated by T cells. These naïve cells reside in the spleen for a few hours and in the lymph

nodes for about a day, before leaving the latter through the splenic vein or efferent lymph vessels to reach the bloodstream again. Through the circulation, naïve T cells reach a new lymphoid organ, repeating this cycle until they will be activated by the encounter of the antigen or will die. Inside secondary lymphoid organs, antigenic peptides are presented to naïve T cells by antigen presenting cells (APCs). In particular, DCs (Dendritic Cells) are the most efficient APC cells as they provide T lymphocytes also costimulatory signals necessary for their activation. DCs capture antigens in the non-lymphoid tissues of the body, and then migrate into the secondary lymphoid organs. These cells processed the antigen to provide antigenic peptides exposed on the cell surface in the context of the MHC complex (Broere et al. 2011). The MHC complex is a set of surface proteins essential for the immune system, whose main function is to bind antigens derived from pathogenic agents and expose them to the cell surface to allow recognition by appropriate T cells. There are two main classes of MHC molecules: the MHC class I, which is present on all the nucleated cells; and MHC class II that is expressed only by "professional" APC cells, such as B cells, macrophages, and dendritic cells.

Naïve T cells recognize the antigen presented by MHC through TCR binding. $CD4^+$ T cells recognize the antigen in the context of class II MHC molecules, while T $CD8^+$ lymphocytes bind antigenic peptides presented by class I MHC molecules. Recruitment of TCR triggers an intracellular signalling cascade leading to naïve T cell activation. Activated T cell proliferates rapidly (i.e. clonal expansion) and migrates through the tissues to the inflamed site for exert their functions. In response to antigen stimulation, $CD4^+$ T cells differentiate into T helper (Th)-1 or Th2 cells. Th1 cells produce signature cytokine INF-γ, whereas Th2 cells produce IL-4, IL-5, IL-13, and IL-10. $CD4^+$ T helper lymphocytes produce cytokines that can stimulate the production of antibodies by B cells and the actions of other T lymphocytes. They are essential in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages.

Cytotoxic $CD8^+$ T cells are very effective in destroying directly infected or malignant cells that present the antigen. Virtually, every cell in the body expresses the class I MHC and is therefore able to present antigenic peptides thus becoming a potential target of cytotoxic $CD8^+$ T cells. These $CD8^+$ T cells are able to destroy more than one target cell, while saving the adjacent healthy cells. Two are the major cytotoxicity mechanisms activated by $CD8^+$ T cells: the release of lithic granules, and apoptosis induced by the Fas receptor, both triggered by antigen recognition via the TCR. Lithic granules are secreted lysosomes containing granzyme, and perforin. Granzymes are inducing apoptosis molecules on target cells. The Fas receptor (also known as CD95) is part of Tumor Necrosis Factor (TFN) superfamily; it is expressed on target cells and has an intracellular death domain capable of inducing caspase-dependent apoptosis when the receptor binds its ligand. Recruiting TCR

increases expression of Fas ligand (also called CD95L) on the surface of cytotoxic cells, and the binding between CD95 and CD95L induces the death of target cells. The cytotoxic T $CD8^+$ lymphocytes do not require costimulatory signals in addition to antigen recognition to perform their effector function, and consequently they can destroy a wide variety of target cells that exhibit exogenous antigens (Broere et al. 2011). Possible mechanisms of $CD8^+$ T cell mediated cytotoxicity are shown in Figure 3.

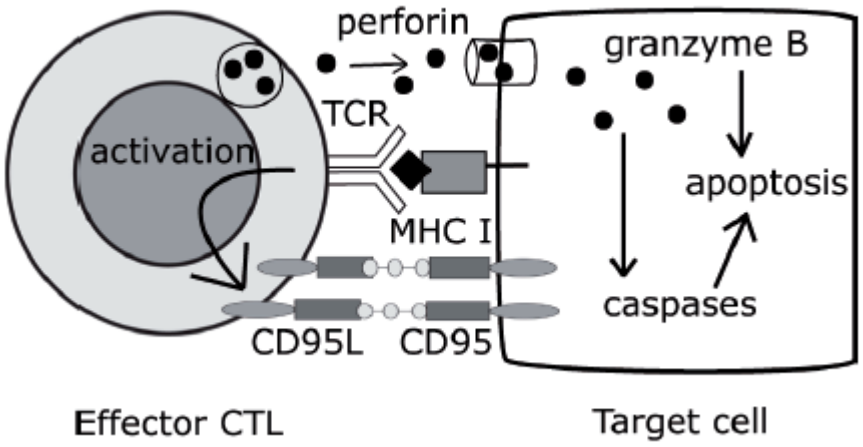

Figure 3. Mechanisms that mediate the cytotoxicity of T $CD8^+$ lymphocytes: 1) Secrete of lithic granules containing perforin and granzyme B. Perforin creates pores on the target cell membrane allowing granzyme B to activate caspases inducing cell apoptosis. 2) Interaction between CD95 (Fas) and CD95L (Fas ligand). Activation of T cell through TCR induces expression of CD95L binding CD95 to target cells causing death (Broere et al. 2011).

Most effector T cells disappear once the antigenic agent is eliminated, but a small portion of them remain and form the pool of memory T cells. Contrasting the naïve cells that live for a few months, and the effector cells that disappear after the pathogen's elimination, memory T cells can survive for years in the lymphoid organs and peripheral tissues. These lymphocytes can be easily activated and immediately acquire effective functions in peripheral tissues, while encountering clonal expansion in the lymphoid organs. This provides a rapid immune response (secondary response) when an antigen to which the organism has already been exposed appears again. Memory cells respond much more rapidly to the antigen than T naïve cells, so in the case of infection they help to eliminate the pathogen at early stages by preventing the spread of the disease (Broere et al. 2011).

1.9 THE CELLULAR BASIS OF IMMUNOLOGICAL MEMORY - CENTRAL MEMORY AND EFFECTOR MEMORY T CELL SUBSETS

Following antigen encounter and subsequent resolution of the immune response, a single naïve (N) T cell is able to generate multiple subsets of memory T cells with different phenotypic and functional properties and gene expression profiles. Single-cell technologies, first flow cytometry, have revealed the complex heterogeneity of the memory T cell compartment and its organization into subsets. However, a consensus has yet to be reached, regard nomenclature and phenotypic level, on the identification of these T cell subsets. Recent studies indicate that memory T lymphocytes contain distinct populations of central memory (CM) and effector memory (EM) cells characterized by distinct homing capacity and effector functions. After positive and negative selection T cells are released from the thymus as mature, N T cells harbouring a given epitope specificity. In response to antigen (Ag) encounter, N T cells proliferate and differentiate into effector cells, the vast majority of which migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets (Zhang & Bevan, 2011). Following Ag clearance, for example that in smallpox vaccination, more than 95% of the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells (Hammarlund et al. 2003; Mahnke et al. 2013). Single cell technologies, like flow cytometry; have revealed the complex heterogeneity of the T cell compartment and its organization into subsets. Together, monoclonal antibodies and flow cytometry have allowed the phenotypic examination of single cells level within heterogeneous cellular population, and the identification and isolation of certain cell types from biological fluid or tissue. CM- and EM-T cells were initially defined in the human system based on two criteria: 1) the absence or presence of immediate effector function and, 2) the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues (Sallusto et al. 1999; Sallusto et al. 2004). Human T CM cells are CD45R0+ memory cells that constitutively express CCR7 and CD62L, two receptors also found of N T cells, required for cells extravasation through endothelial tissue and migration to T cell areas of secondary lymphoid organs (Campbell et al. 1998; Förster et al. 1999). CD45R0 and CD45RA correspond to the high and low molecular weight protein products of splice variants of *CD45* gene, respectively (Terry et al. 1988; Mahnke et al. 2013). When comparing to N T cells, CM cells have higher sensitivity to antigenic simulation, are less dependent to co-stimulation and upregulate CD40L (a protein primarily expressed on activated T cells) providing a more effective stimulatory feedback to dendritic cells (DC) and B cells. Following TCR triggering, CM T cells produce mainly Interleukin (IL)-2, but after proliferation, they quickly differentiate to effector cells and produce large amounts of Interferon-γ (INF-γ) or IL-4 (Sallusto et al. 2004). Human EM T cells are memory cells that have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression, and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues (see Table 1). EM T cells are characterized by rapid effector functions (Lanzavecchia

& Sallusto, 2000; Sallusto et al. 2004). $CD8^+$ T EM cells carry the larger amount of perforin, and both $CD4^+$ and $CD8^+$ produce INF-γ, IL-4, and IL-5 within hours following antigenic stimulation. Some $CD8^+$ and with less frequency, $CD4^+$ EM cells express CD45RA and are defined as terminally differentiate EM (EMRA) cells. EMRA T cells have mostly been studied in the $CD8^+$ T cell compartment, where they are found at appreciable frequencies in most individuals (Tian et al. 2017). By contrast, the frequency of $CD4^+$ EMRA T cells varies drastically between individuals ranging from <0.3% to nearly 18% of total $CD4^+$ T cells in an apparently healthy population, and their functional role is less clear (Tian et al. 2017). In addition to exhibiting CD45RA+ and CCR7- phenotype, $CD4^+$ EMRA T cells have also been characterized by decreased expression of CD27 and CD28, as well as increased expression of CD57 and effector molecules such as perforin and granzyme B that resemble more terminally differentiated state (Tian et al. 2017). In addition, the $CD8^+$ EMRA T cells carry the largest amount of perforin.

Therefore, the EM T cell pool contains T helper 1, T helper 2, and cytotoxic T lymphocytes (CTL). The relative proportions of CM T cells and EM T cells in peripheral blood vary in the $CD4^+$ and $CD8^+$ compartments; CM cells are more prevalent in CD4 T, whereas EM cells are more frequent in $CD8^+$ lymphocytes. Within the tissues however the different T cell subsets shown characteristic distribution patterns (Sallusto et al. 2004). CM T cells are enriched in lymph nodes and tonsils, while lung, liver, and gut contain greater proportion of EM T cells (Campbell et al. 2001). In antigen-primed individuals, tetanus toxoid specific $CD4^+$ T cells can be detected in circulating CM and EM T cells up to 10 years after anti-immunization (Sallusto et al. 1999). The development of T cell memory subsets is shown in Figure 4.

T cell subsets	**Surface Markers**
Naïve	CCR7+, CD62L+, CD45RA+
CM	CCR7+, CD62L+, CD45RA-, CD45RO+
EM	CCR7-, CD45RA-, CD45RO+
EMRA	CCR7-, CD45RA+

Table 1. T cell subsets and corresponding surface markers

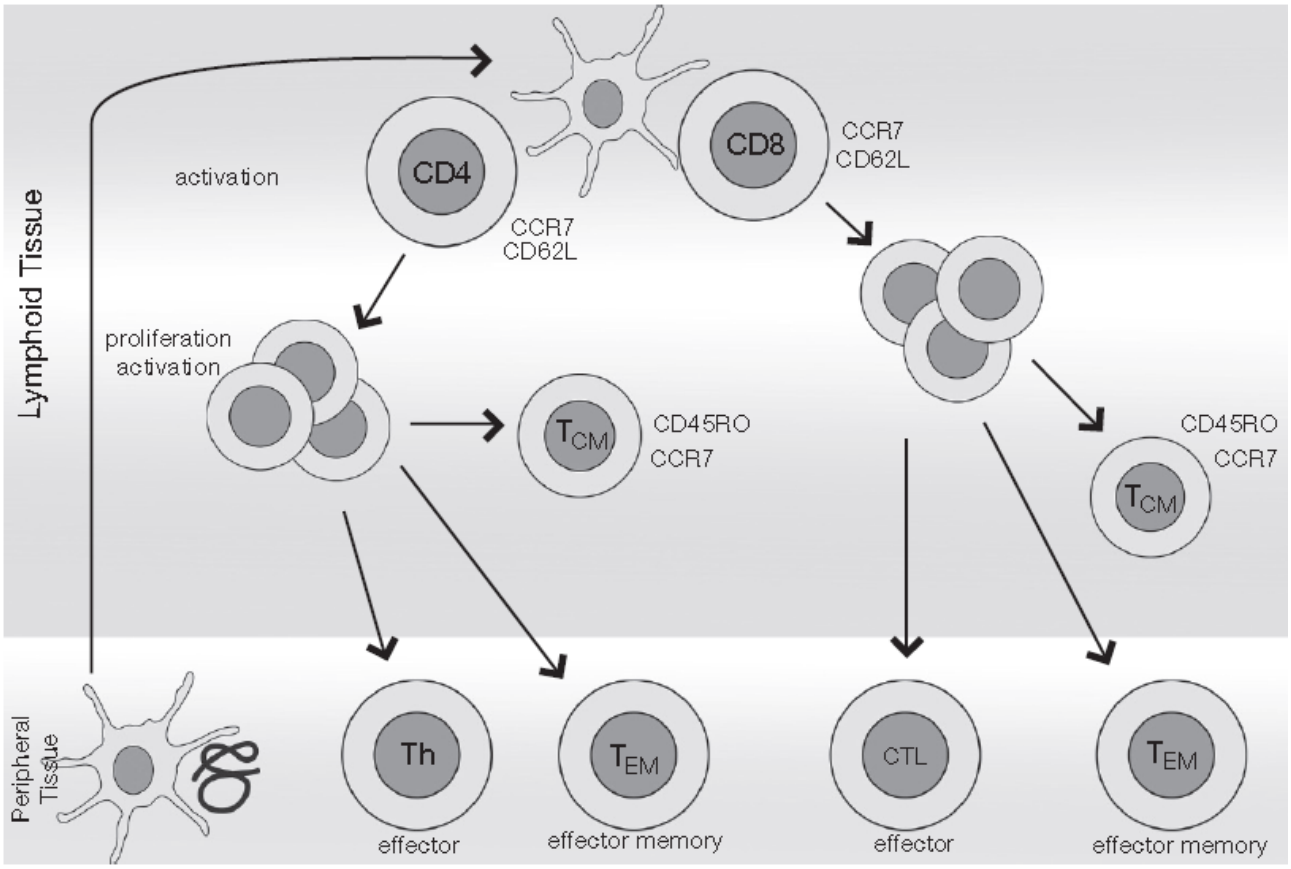

Figure 4. Development of T cell subsets. APC cells capture antigens in peripheral tissues and migrate to secondary lymphoid organs, where N T cells are activated by the recognition of the MHC-peptide complex. They proliferate and differentiate themselves into effective cells or memory cells. Both effective $CD4^+$ cells (Th, T helper) and $CD8^+$ efficacious (CTL, cytotoxic) migrate to peripheral tissues to perform their function. The memory cells themselves are subdivided into CM T cells expressing CCR7 and recirculating between lymph nodes, or in EM T cells that do not express CCR7 and migrate to peripheral tissues (Broere et al. 2011).

1.10 NATURAL KILLER CELL MEDIATED IMMUNITY

Natural Killer (NK) cells are effector lymphocytes of the innate immune system that control several types of tumours and microbial infections by limiting their spread and subsequent tissue damage. Recent studies highlights the fact that NK cells are also regulatory cells engaged in reciprocal interactions with DCs, macrophages, T cells, and endothelial cells. NK cells were recognized as a separate lymphocyte lineage, with both cytotoxicity and cytokine-producing effector functions (Trinchieri, 1989). In fact, NK cells can limit or exacerbate immune responses; they can regulate the immune response by killing APCs or over-activated T cells or by producing anti-inflammatory cytokines (such as IL-10) to prevent too strong inflammatory response (Vivier et al. 2011; Zhang et al. 2006). Nevertheless, the role of NK cells is not helpful in all situations. Over activation or

dysfunction of NK cells may be associated with pathogenesis of some diseases. For example, NK cells are found to act as a two edged weapon and play opposite roles with both regulatory and inducer activity in autoimmune diseases (Perricone et al. 2008; Zhang & Tian, 2017). NK cells are heterogeneous cell population and divided into different subsets based on their surface phenotype or cytokine secretion pattern. So far, various human NK subsets with distinct phenotypic and functional properties have been identified and they usually represent distinct stages of a linear development process. Furthermore, different NK cells are found to locate in several immune organs and anatomy sites, demonstrating that the microenvironment influences the differentiation and function of NK subsets (Zhang & Tian, 2017).

It is widely accepted that human NK cells can be divided into two subsets (showed in Figure 5) based on their cell-surface density of CD56: $CD56^{dim}$ NK and $CD56^{bright}$ NK cells (Cooper 2001; Cooper et al. 2001). In peripheral blood lymphocyte populations, approximately 10% of NK cells express high levels of CD56 ($CD56^{bright}$ $CD16^{dim/-}$), while the more abundant NK cell subset expressed CD56 at low density ($CD56^{dim}$ $CD16^{+}$). $CD56^{dim}$ $CD16^{+}$ NK cells exert higher cytotoxicity and express higher levels of Ig-like NK receptors than $CD56^{bright}$ NK cell subset, yet $CD56^{bright}$ NK cells exert relatively lower cytotoxic capacity but can produce abundant amounts of cytokines such as INF-γ, TNF-α, IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The $CD56^{dim}$ $CD16^{+}$ NK subset is predominant in peripheral blood, whereas $CD56^{bright}$ $CD16^{dim/-}$ NK cells are more abundant in secondary lymphoid tissues, particularly rich in immune tolerance organs, such as the liver and uterus (Cooper et al. 2001; Zhang & Tian, 2017). The distribution of NK cell subpopulations in the various tissues is determined by chemokine receptors on NK cells and chemokines secreted in sites of tissues. $CD56^{bright}$ NK cells express CCR7, which promotes entry into secondary lymphoid organs and contributes to migration toward sites of infection or inflammation. $CD56^{bright}$ NK cells also express high levels of CD62L a receptor required for lymphocytes homing to secondary lymphoid organs.

$CD56^{bright}$ NK cells are a regulatory NK subset and play major roles in maintaining immune homeostasis in both physiological and pathological conditions by secreting immune-regulatory cytokines. However, they can also become cytotoxic upon appropriate activation through inflammatory cytokines or triggering of co-activating receptors, thus can mediate killing of target immune cells such as autologous activated T cells or immature DCs for regulation of immune response. $CD56^{dim}$ NK cells are cellular cytotoxic killer cells and exert major early immune-surveillance against infected or malignant cells. They are also potent producers of cytokines (INF-γ and TNF-α) upon recognition of susceptible target cells (Zhang & Tian, 2017).

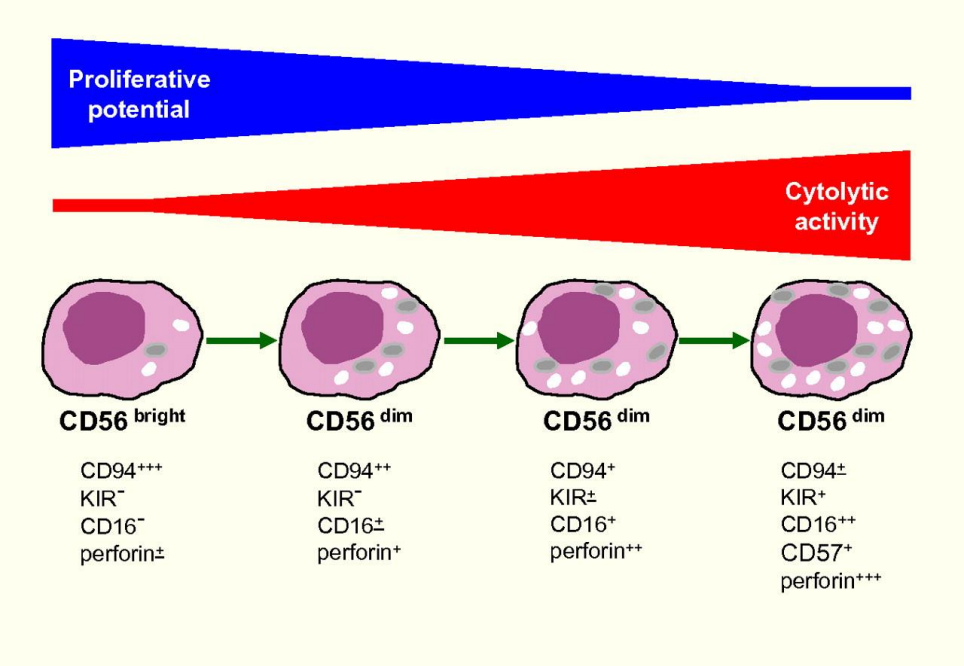

Figure 5. Model of human NK-cell differentiation (Moretta 2010)**.**

NK-T cells are another subset of NK cells and they are a population of autoreactive cells that mediate both protective and regulatory immune functions. NK-T cells express an invariant TCRα chain (Vα24-JαQ in humans) and recognize glycolipid antigens in association with the MHC class I-like molecules CD1d.

NK-T cells are different from functionally differentiated conventional αβ T cells in that they are autoreactive and produce both Th1 and Th2 cytokines, including IL-4, IL-10 and INF-γ, upon stimulation with their ligands (Taniguchi et al. 2003; Seino & Taniguchi, 2005). NK-T cells appear to have distinct functional capabilities; they have been shown to mediate both protective and regulatory immune functions. These includes tumour rejection, protection against infectious microbes, maintenance of transplant tolerance, and inhibition of autoimmune disease development (Taniguchi et al. 2003). NK-T cell functions and or cytokine profiles can be altered by modulation of TCR signalling. Research reported that the frequency of NK-T cell stimulation through the TCR affects their function (Kojo et al. 2005). Suggesting that the intensity of TCR signals influences the action of NK-T cells in a manner similar to the effect of TCR avidity on positive and negative selection of developing T cells in the thymus (Starr et al. 2003; Seino & Taniguchi, 2005).

1.11 IMMUNITY AND AUTOIMMUNITY IN CENTRAL NERVOUS SYSTEM

The CNS was considered an immunologically privileged compartment and protected from autoimmune process by both the blood-brain barrier (BBB) and by the lack of MHC class I expression

on neurons. Actually, this is known to not be the case. Research has shown that memory T cells cross the BBB and monitor the CNS by interacting with antigen-presenting DCs in the perivascular and subarachnoid space where they can be reactivated (Ransohoff & Engelhardt, 2012; Degn & Kornum, 2015). The fifth lumbar of the spinal cord has been suggested as a site of entrance; T cells can also enter the CNS through the choroid plexus (Ransohoff & Engelhardt, 2012).

The surveillance of the CNS by T cells is crucial for protecting the brain from infections; inhibition of T cells entry in humans can lead to progressive multifocal leukoencephalopathy caused by an uncontrolled infection by the Cunningham (JC) virus, which is asymptomatic in immunocompetent individuals. In general, virus specific T cells play a central role in the control of many neurotropic viruses, such as West Nile virus and herpes simplex virus type 1. For example, in mice and humans, herpes simplex virus enters in a lifelong latent state within infected sensory neurons. Activated $CD8^+$ T cells found in direct contact with infected neurons keep the virus in a latent state without causing the cells lysis (Degn & Kornum, 2015).

Another CNS disease, the paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections, also known as "PANDAS," is associated with obsessive-compulsive and tic disorders. The streptococcal infections may trigger an autoimmune reaction that exacerbates these conditions. Recurrent streptococcal tonsillitis is one of the frequent infections associated with PANDAS condition (Heubi & Shott, 2003).In the past, other neurological disorders including narcolepsy-like symptoms have been associated with H1N1 infections, most notably in the contest of the 1918 Spanish flu pandemic. Coincident with this epidemic a smaller one, called Encephalitis Lethargica (EL), a disease characterized by hypersomnolence and posterior hypothalamic lesions, occurred (von Economo 1931; Kornum et al. 2011). Although EL has largely disappeared, cases are still occasionally reported, and interestingly were found to be associated with higher ASO titers (Lopez-Alberola, 2009; Dale et al. 2001). Furthermore, autoimmune T cell responses directed against antigens that are derived from the CNS are thought to trigger several diseases, including multiple sclerosis, neuromyelitis optica and acute disseminated encephalomyelitis. Multiple sclerosis is thought to occur in genetically predisposed individuals following exposure to an environmental trigger that activates myelin- specific T cells, which allows the T cells to cross the BBB (Goverman, 2009). Reactivation of the T cells by CNS-resident APCs that present myelin antigens triggers the recruitment of innate immune cells, which have important roles in mediating demyelination and axonal damage (Goverman, 2009). Finally, relative recent studies suggest that psychiatric disorders (such as major depressive disorder, bipolar disorder and schizophrenia) could be considered as inflammatory conditions of CNS via microglial activation (Réus et al. 2015).

1.12 AIMS

Researches have investigated immune cell phenotype subsets in various CNS autoimmune diseases. In multiple sclerosis, patients have decreased frequency of EM and EMRA in $CD4^+$ and $CD8^+$ T cells, a finding present at onset and that persists throughout the clinical course (Pender et al. 2014; Teniente-Serra et al. 2016). Another larger study found a higher percentage of EM T cells in various CNS inflammatory syndromes in comparison to controls (Mullen et al. 2012).

In this study, we used flow cytometry to analyze the distribution of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells in the peripheral blood and, for the first time, in the CSF of NT1 patients compared to age and gender balanced healthy controls, differing for the presence of *HLA DQB1*06:02* allele. Finally, we analyzed T cell subsets differences between CSF and peripheral blood in NT1 patients versus controls.

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CHAPTER 2

Materials and Methods

2.1 PATIENTS AND CONTROLS

In our study of CSF immune subset characterization, we included 14 *HLA DQB1*06:02* positive NT1 patients, and 14 *HLA DQB1*06:02* negative healthy controls (HC).

The reason for only including *HLA DQB1*06:02* negative subjects was the fact that only approximately 13% of healthy Italian controls carry *DQB1*06:02* (http://www.allelefrequencies.net/). For PBMC immune cells phenotyping, we included 13 *HLA DQB1*06:02* negative HC. NT1 patients were diagnosed according to the ICSD 3rd edition criteria, and all had low (<110 pg/ml) or undetectable CSF hypocretin-1 levels. *HLA DQB1*06:02* negative HC were subjects who had been hospitalized for suspected hypersomnia of central origin not confirmed by normal neurophysiological (actigraphy, 48 hours polysomnography, and MSLT) evaluation (24). All HC had normal (> 200 pg/ml) CSF hypocretin-1 levels. All NT1 and HC were drug-naïve at time of evaluation.

Exclusion criteria were presence of cardiovascular diseases, other neurological conditions, psychiatric disorders, acute allergies, recent infections or immunologic diseases, and use of steroids, anti-inflammatory or immunosuppressive drugs. The local ethics committee approved the study (CE BO-IM protocol number: 17019), and all subjects signed a written informed consent.

2.2 COLLECTION OF CSF CELLS AND PBMCs

CSF samples were collected between 7 and 9 a.m. in NT1 patients and *HLA DQB1*06:02* negative HC for hypocretin-1 measurement under fasting condition. Lumbar puncture was performed using an atraumatic needle and the CSF volume withdrawn was 6-7 mL. The first aliquot (1 mL) of CSF was analyzed to exclude blood contaminations using routine laboratory testing procedures. The remaining CSF was centrifuged (1900 rpm for 10 minutes) and the supernatant frozen for hypocretin-1 measurement. Pellets (20 µL) were collected and used to manually count the number of CSF cells in a Neubauer chamber with erythrosine 1X (at the volume of 20 µL). Cellular number range was 5000-12000 cells and vitality >95%. Pellet-containing cells were then cryopreserved in freezing medium named *cryofluid* containing 90% Fetal Bovine Serum (FBS; Gibco®, Thermo Fisher Scientific) and 10% Dimethyl Sulfoxide (DMSO; Sigma Aldrich, http://www.sigmaaldrich.com) and then frozen into special container (Mr. Frosty, Nalgene®) filled with 250 mL of isopropanol to avoid membrane damage on the cells during freezing. The day after, the frozen cells were transferred in liquid nitrogen until further use.

Participants also provided a venous blood sample (20 mL) between 7 and 9 a.m. under fasting condition into two EDTA (EthyleneDiamine Tetra-acetic Acid) tubes to avoid blood clotting. PBMCs were extracted by density centrifugation; the blood was first diluted 1:1 with IMDM (Iscove's

Modified Dulbecco's Medium; Gibco®, Thermo Fisher Scientific) medium, placed over 10 mL of Ficoll-Paque (Histopaque 1.077, Sigma Aldrich, Dorset, UK) and then centrifuged at 2100 rpm for 30 minutes.

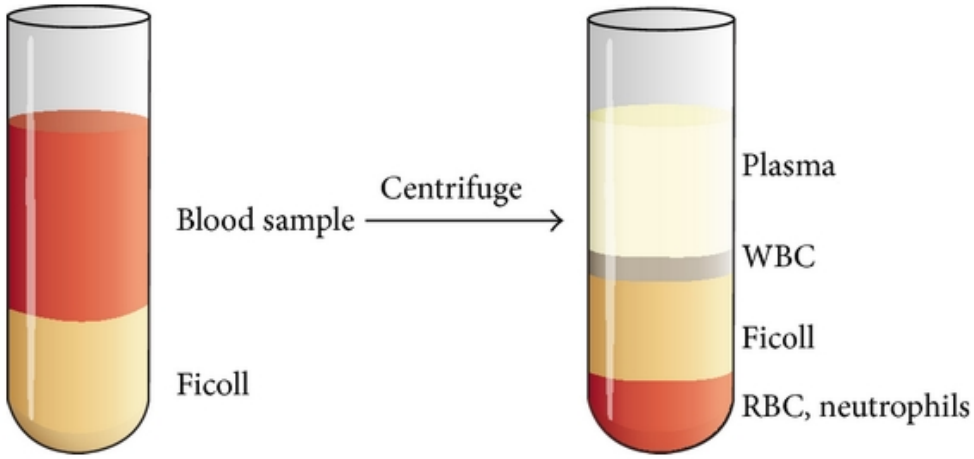

Figure 1. Principle of density centrifugation separation method. Sample is layered on top of a density gradient. Under centrifugal force, particles move through the medium and density gradient and be suspended at a point in which the density of the particles equals the surrounding medium. RBC: red blood cell, WBC: white blood cells.

After the centrifugation step showed in Figure 1, the PBMCs were withdrawn from the Ficoll-Paque layer and placed in another tube (approximately 8 mL). The white cells were then washed with IMDM medium at the final volume of 15 mL and centrifuged at 2100 rpm for 10 minutes. The cell pellet was re-suspended in 5 mL of medium for a second washing step at 2100 rpm for 10 minutes. Finally, the pellet containing cells was re-suspended in 1 mL of FBS and, in order to manually count the PBMCs number, we diluted 10 µL of cell solution into 190 µL of FBS in a separate tube.
At this point the PBMCs solution was enriched with *cryofluid* containing 80% FBS and 20% DMSO and placed in special freezing container as described above until further processing.

2.3 *HLA-DQB1*06:02* ALLELE TYPING

The presence of *HLA DQB1*06:02* allele was analyzed in all participants using Sequence Specific Primers, as previously described (Mignot et al. 1994). DNA was extracted from blood samples using a standard method with phenol-chloroform, and 1 µL of genomic DNA was quantified using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).
*DQB1*06:02* allele was determined by polymerase chain reaction (PCR) carried out in a Termocycler 2720 (Life Technologies/Applied Biosystems®, Foster City, CA, USA) using the following specific primers: DRBEX3F (5'-TGCCAAGTGGAGCACCCAA-3') and DRBEX4R (5'-

GCATCTTGCTCTGTGCAGAT-3') for amplifying the internal control region, 611F (5'-CCCGCAGAGGATTTCGTGTT-3') and 611R (5'-AACTCCGCCCGGGTCCC-3') for amplifying the specific *DQB1*06:02* genetic region.

The genetic amplification was carried out in a final volume of 25 µl of reaction mix showed in Table 1.

Reagent	**Volume**
Water	14.4 µl
10X PCR Buffer (Qiagen, Hilden, Germany)	2.5 µl
Dideoxynucleotide-triphosphates (ddNTPs) 2 mM	0.5 µl
611F (20 pg/µl)	1.25 µl
611R (20 pg/µl)	1.25 µl
DRBEX3F (20 pg/µl)	2 µl
DRBEX4R (20 pg/µl)	2 µl
HotStarTaq (Qiagen, Hilden, Germany)	0.1 µl
DNA	1 µl
Final volume	25 µl

Table 1. Composition of PCR reaction mix.

The cycling protocol was as follows: DNA denaturation at 94 °C for 15 minutes, 35 cycles of 94 °C for 1 minute, 63 °C for 1 minute, 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes.

To verify the samples' DNA amplification a horizontal electrophoretic run was performed on agarose gel. This method exploits the charges present in the DNA molecules, to migrate them through the presence of an electric field by a gel that acts as a molecular silicon. The agarose gel is made up of a network of pores that allow the molecules to separate according to their size and then their molecular weight. Agarose is a linear and neutral polysaccharide, soluble in water at boiling temperature.

When loading the sample, a glycerol-containing loading solution (bromophenol blue) is usually added to facilitate precipitation at the bottom of the well.

In particular, 1% agarose gel was obtained by dissolving 0.4 g of agarose in 40 mL of tris borate EDTA (TBE). Each well was loaded with 5 µL of DNA amplified plus 1 µL of bromophenol blue. *GelRed* (Biotium, Inc. Hayward, California, USA) was used as interlayer agent in an amount of 0.5 µL per single gel. The bands thus obtained were observed by placing the gel, after run, on an ultraviolet light trans-illuminator (Figure 2).

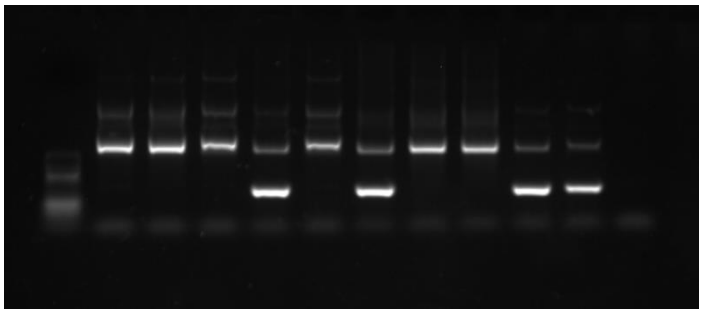

Figure 2. Representative gel in which both positive samples (where the lower band corresponding to 217 bp) and negative (where there is only one band over 700-730 bp) are observed for the *HLA DQB1 * 06: 02* allele

2.4 FLOW CYTOMETRY ANALYSIS

Flow cytometry allows automatic analysis of cell suspensions by measuring their physical and/or biochemical characteristics. These monodisperse cellular suspensions (such as blood peripheral cells) are conveyed by a fluid system that carries the cells in a separate and orderly manner, up to a point where a focused light band arrives from an excitation source. When the light beam intercepts the cells, signals are generated related to the physical characteristics of the cell itself (diameter, nucleus/cytoplasm ratio, internal granularity, membrane roughness) and the presence of molecular fluorochrome located at different sites. Once emitted, the signals are collected by a lens system, dichroic mirrors and optical filters, and sent to the respective sensors (photodiodes and photomultipliers), transforming them into electrical signals. These electric signals, appropriately amplified and digitized, are sent to a data software analyzer (Adan et al. 2017). A schematic flow cytometry operations diagram is shown in Figure 3.

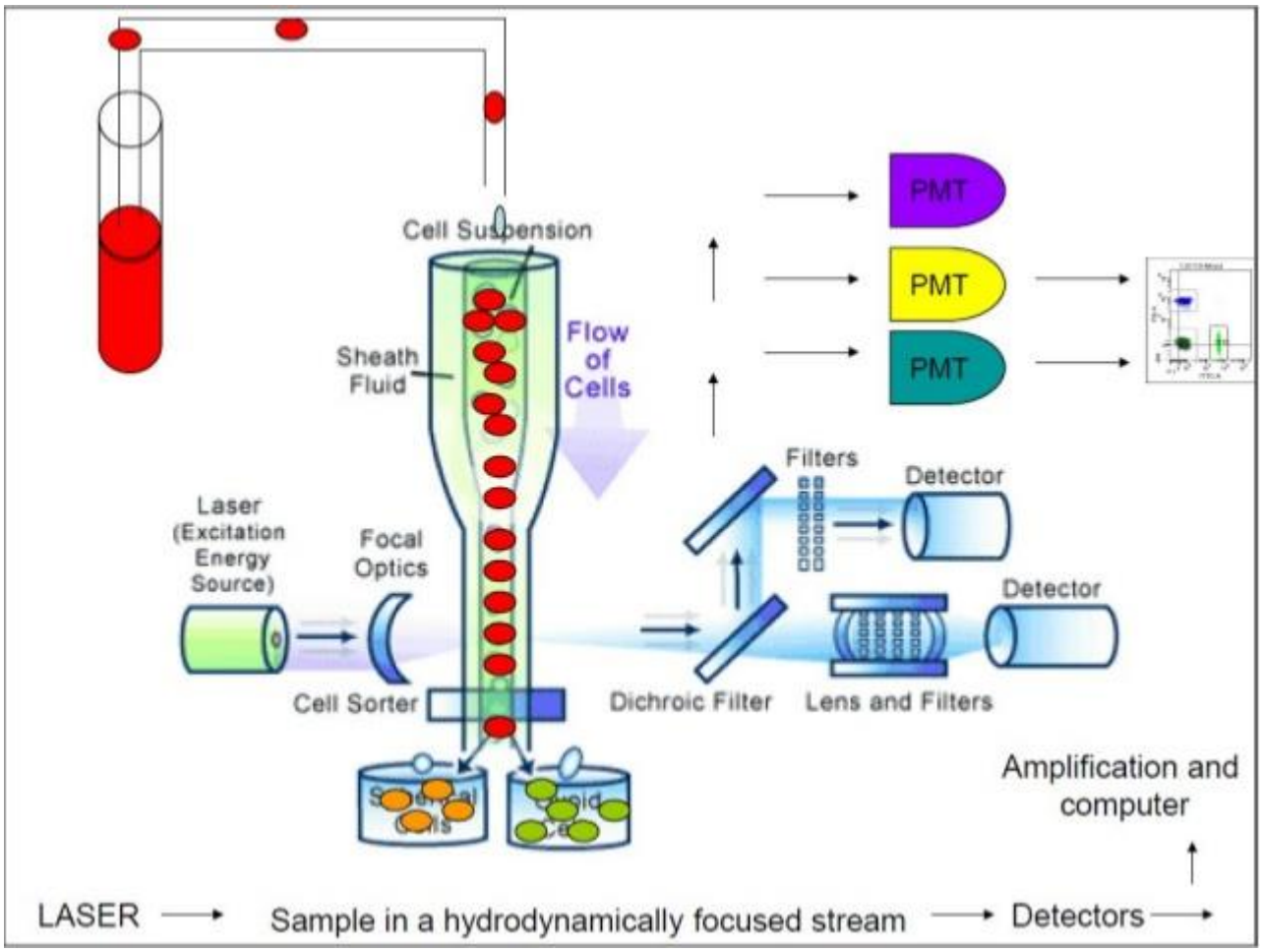

Figure 3. Flow cytometry operation diagram with cell sorter. Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier (PMT) tubes and digitized for computer analysis (Brown & Wittwer, 2000; Adan et al. 2017).

2.4.1 THE FLUIDIC SYSTEM

The liquid sample dispensing system provides an efficient means of presenting the cells individually to the measuring station where they intersect the light beam from the excitation system. The cell suspension is transported from the distribution system to the flow chamber where it is injected under pressure through a small needle (Valtriani & Hurle, 1997). Here, through the difference in pressure between the sample and the transport fluid, a concentric laminar flow of liquids is achieved in which the outer (transport liquid) introduces a thin fluid sample vein (hydrodynamic focusing process,

showed in Figure 4). In this way, the cells are forced to align and pass individually a measuring point interacting with the light beam of the excitation system. Push pressure and dilution of the sample allow analyzing up to several thousand cells per second. By acting on the pneumatic transport system that controls the pressure difference between core flow (sample) and the sheath fluid, the flow rate of the cells is normally determined by the number of events per second, which is the number of particles that met the bright spot in the unit of time. A high flow rate is generally used for methods such as immunophenotyping, where data is less resolved but is acquired more quickly. A lower flow rate is used instead for applications where optimal resolution and sensitivity are crucial (Brown & Wittwer, 2000; Valtriani & Hurle, 1997).

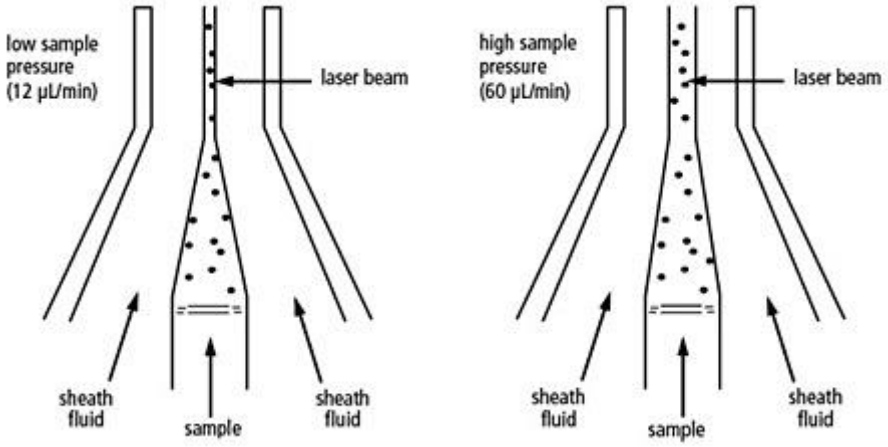

Figure 4. Hydrodynamic focusing process (picture from: https://www.bdbiosciences.com).

Cytometers are distinguished as "closed circuits" where the sample is lost, and "open circuit" instruments in which the sample meets the beam of laser light in the air, and then recovered in full or selectively after selecting the cells (cell sorters). These cytometers are able to physically separate homogeneous cells from heterogeneous populations: for this purpose, selection gating will be created that will match the population to be separated. Cells whose parameters fall into these gating will be separated and collected in isolated tubes. This is done by providing a suitable frequency vibration applied to the nozzle ("flow chamber terminal orifice"), which causes crushing of the fluid thread into tiny droplets that should contain a single cell. The droplet, containing the separating cell, will have a positive or negative charge, so that it can be diverted to the corresponding test tube from an electrostatic field (Valtriani & Hurle, 1997).

2.4.2 LIGHT SOURCES

The light source can be a laser, an arc lamp or even an LED. Currently, the majority of instruments use a laser. Lasers are chosen because they produce a high intensity beam of monochromatic light. They also have a small ‘spot’ size, which is important since the light needs to be focused into a small

volume to obtain maximum excitation of a single cell and to minimise the probability of there being more than one cell in the laser beam (Ormerod, 2008). Arc lamps need optical filters to select the appropriate wavelength. They do not give the sensitivity needed to observe weak fluorescence but offer a low-cost alternative for observing strong fluorescence, for example, in DNA analysis.

There is a large variety of air-cooled and solid state lasers available. The most common primary laser is an air-cooled argon-ion laser producing blue light at 488 nm. This wavelength is convenient for the excitation of fluorescein, the first immunofluorescent label to be used (Ormerod, 2008). Solid state lasers producing light from 355 nm, to 780 nm.

While a higher power output improves sensitivity, there is a consequence to pay in terms of increased cost, maintenance and size. A few specialized applications need higher laser powers, such as chromosome analysis and sorting (Ormerod, 2008).

2.4.3 THE PHYSICAL PARAMETERS

The single cell, affected by the focal light beam, first emits diffused light signals related to its physical and morphological characteristics (refraction, reflection and diffraction). The laser beam, intercepting the flow, causes most of the incident light to be dispersed. Obscuration bars block this radiation, which would represent a disturbance to the measurements. The forward scattered light (FSC) essentially depends on the cellular size and is collected by a sensor called photodiode. The 90 ° side-scattered (SSC) light is due to reflection and refraction phenomena, which are attributed to cellular morphology parameters such as cytoplasmic composition, nucleus-cytoplasm ratio, surface roughness and diameter. This light is subjected to spectral analysis (separation of the various components at different wavelengths with the help of dichroic mirrors and optical filters), thus collected from photomultipliers (PMT), since they are much less intense than the FSC signal. From the combination of FSC and SSC, we obtain a particular two-dimensional diagram, displayed in Figure 5, in which it is possible to detect different cellular populations based only on their physical characteristics (Valtriani & Hurle, 1997).

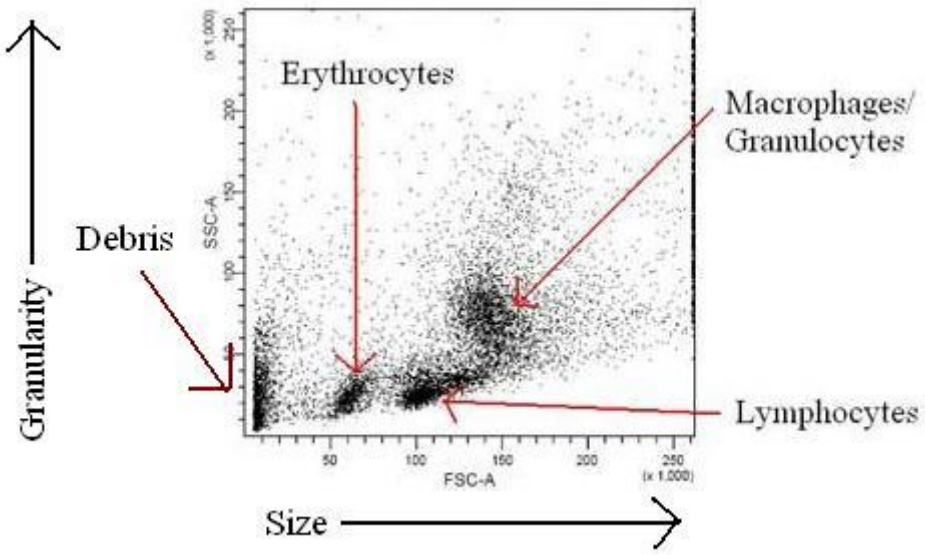

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Figure 5. Example of two-dimensional diagram that shows the different peripheral blood cells subtypes (Valtriani & Hurle, 1997).

2.4.4 FLUORESCENCE AND COMPENSATION

By detecting fluorescence, it is possible to highlight several cellular structures, in fact many membrane antigens or receptors, cytoplasm and nucleus can be highlighted with fluorescent ligands. These ligands may be polyclonal or monoclonal antibodies marked with fluorochromes, such as FITC (fluorescein isothiocyanate, green channel), PE (phycoerythrin, red channel), APC (allophycocyanin, blue channel) etc. Each fluorochrome has a characteristic wavelength band for excitation and emission. The wavelength of the excitation source therefore constitutes a limit to the choice of fluorochromes to be used individually or in combination.

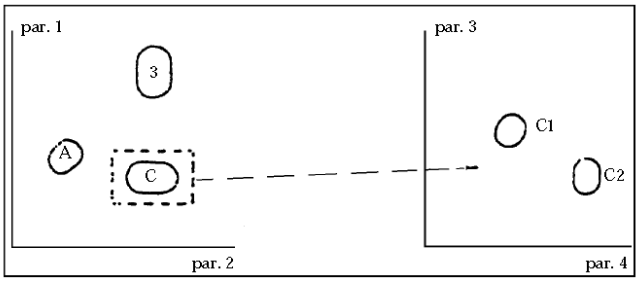
Despite the sophisticated wavelength selection system, it can happen that certain PMT can achieve a non-negligible light intensity of undesirable colour due to the excessive fluctuation of fluorochrome emission band and optical filter characteristics. This problem is surpassed by an electronic correction system of the defined compensation signals, which aims to subtract from a certain channel a fixed amount of signal related to the emission of another fluorochrome. A typical example is the detraction from the PE channel (red) of a fixed signal part due to the interference of the FITC (green). The compensation remains fixed for a given combination of fluorochromes, for a given set of filters, and for a given setting of PMT. Any change in these parameters requires a compensation adjustment. Finally, it is to be noted that there are cells or other elements not marked with any fluorochrome, which have a weak but measurable background fluorescence. In the case of staining that result in low colour intensity, autofluorescence is the real factor limiting the resolution power of the instrument (Valtriani & Hurle, 1997).

2.4.5 DATA COLLECTION AND DISPLAY

Once a data file has been saved, cell populations can be displayed in several different formats. The simplest representation of a cytometric data is a single parameter histogram that distributes cells (events) within a certain emitted fluorescence intensity.

Another possible representation of the cytometric data is the two-dimensional cytogram named dot-plot where one parameter is displayed on the x-axis and the other parameter is displayed on the y-axis simultaneously. However, the dot-plot representation has its limits since occupying all available points in a certain area; the arrival of a new event will not be able to produce any visual variation. To overcome this disadvantage, other diagrams have been developed to provide more information: contour-plot or contour diagrams, representing a dot-plot enhancement, as they display areas with the same probability density using concentric lines (Ibrahim & van den Engh, 2007).

Multi-parametric analysis is one of the most powerful aspects of this method, needed to address the biological problems of cell heterogeneity through two fundamental operations: gating and sorting. The gating is used to fully benefit from the analysis in several dimensions: a cell population can be isolated with the help of an electronic "window" (gate) based on the parameters 1 and 2, and then be divided into further sub-populations thanks to parameters 3 and 4 (Figure 6). Cell sorting, which is a physical gating, allows to physically collecting the cell population of interest in a separate tube.



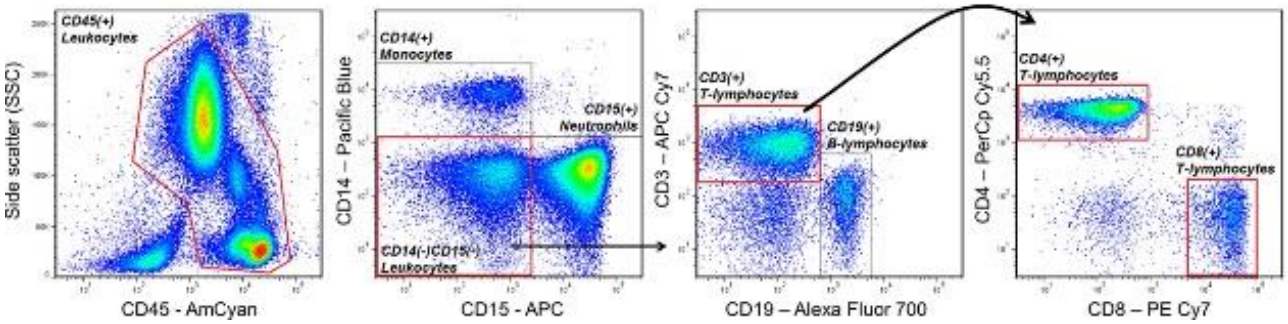


Figure 6. On left: example of multiparameters analysis, on the bottom: gating strategy example of peripheral blood analysis.

2.4.6 IMMUNOPHENOTYPING

The most common use of flow cytometry is in the identification of markers on cells, particularly in the immune system named immunophenotyping. Immunophenotyping can be used to simply identify

a cell by a single marker or more complex identification of cells, using homing profile, activation states and cytokine release all in one panel. Consequently, experimental protocols are often a combination of surface and intracellular staining. In addition to basic research, immunophenotyping is routinely used in clinical applications to diagnose diseases or to monitor and evaluate residual diseases. In fact, immunophenotyping can be performed on cellular samples from different body regions (peripheral, bones, lymphoid organ, etc.) or biological fluids such as CSF. This method requires negative and positive cell discrimination for markers of interest. The percentage of the latter provides the measure of the cells that have produced fluorescence due to specific antibodies that are related to the antigens. The criteria for setting the limit between negative and positive elements for a given marker must take into account some preliminary conditions, including the obligation to control for self-fluorescence and the control isotype (an antibody of identical isotype class and of identical conjugation, but not directed against the antigen of interest). Only comparing these controls signals can be determined whether the binding background is attributable to self-fluorescence or occurs through the Fc receptors (which bind the constant portion of the immunoglobulins) or other molecules. Based on the controls, therefore, a gating is placed in which the positive elements fall (Valtriani & Hurle, 1997).

2.4.7 FLOW CYTOMETRY ANALYSIS IN NT1

Using flow cytometry, we first assessed the following T cell markers: CD3, CD4, and CD8. To further define subsets of CD4 and CD8 T cells, we used a combination of CCR7 (a chemokine receptor that controls homing to secondary lymphoid organs), and CD45RA (the human leukocyte common antigen). This made it possible to distinguish four major differentiation states for CD4 and CD8 T cells: naïve (N) T cells ($CCR7^{+}$, $CD45RA^{+}$), central memory (CM) T cells ($CCR7^{+}$, $CD45RA^{-}$), effector memory (EM) T cells ($CCR7^{-}$, $CD45RA^{-}$), and "terminally differentiated" effector memory cells, expressing RA receptor (EMRA; $CCR7^{-}$, $CD45RA^{+}$) (Sallusto et al. 1999). NK cells were measured only in PBMCs (given low cell number and availability of CSF samples), using CD3, CD56, and CD16 markers distinguishing cytotoxic NK cell ($CD3^{-}$, $CD56^{dim}$, $CD16^{+}$), immune-regulatory cells ($CD3^{-}$, $CD56^{bright}$, $CD16^{+/-}$), and NK-T cells ($CD3^{+}$, $CD56^{+}$). Moreover, we computed the *fold-change* as the ratio between cells frequencies on CSF (central nervous system) and blood (periphery) compartments (CSF/PBMC ratio).

Previously extracted PBMCs and CSF cells were gently thawed at 37 ˚C in a distilled water bath and then washed with 20 ml of RPMI (Roswell Park Memorial Institute; Gibco®, Thermo Fisher Scientific) complete medium (enriched with: FBS, glutamine, HEPES, penicillin-streptomycin) at 2000 rpm for 5 minutes. About PBMC, the cell pellet was re-suspended in a volume of 1 mL of RPMI

medium and a manual cell count was performed in order to obtain 200,000 cells for cytometry analysis. To obtain the volume of PBMCs solution containing the desired cell number, the following formula was used:

(0.2 X Volume of Cell Solution) / Cell Number

The volume thus calculated was loaded into each labelling tube, together with a volume of Phosphate Buffered Saline (PBS; Gibco®, Thermo Fisher Scientific) to bring the final volume to 80 μL.

The staining protocol was performed in the dark for 20 minutes at room temperature using 2 μL of the following anti-human monoclonal antibodies (mAbs): allophycocyanin (APC)-conjugated CD56 (MEM188;), Pe-Cy7-conjugated CD8 (SK-1) and, phycoerythrin (PE)-conjugated CD4 (OKT4), CD8a (HIT8a) and CD16 (B73.1) from eBioscience (San Diego, CA, USA); Alexa Fluor®647-conjugated CD197 (CCR7; G043H) from Biolegend (San Diego, CA, USA); fluorescein isothiocyanate (FITC)-conjugated CD45RA (L48), and APC-Cy7 conjugated CD3 (SK7) from BD Biosciences (San Jose, CA, USA).

Negative controls were isotype-matched irrelevant MoAbs. For each sample and fluorescence parameter appropriate isotype-matched control mAbs were included: mouse IgG1 FITC (P3.6.2.8.1), mouse IgG1 PE (P3.6.2.8.1), mouse IgG1 Pe-cy7 (P3.6.2.8.1), mouse IgG2a APC (eBM2a), and mouse IgG2b PE (eBMG2b) mAbs from eBioscience, mouse IgG2a Alexa Fluor® 647 (MOPC-173), mAb from Biolegend, and mouse IgG1 APC-Cy7 (MOPC-21) from BD Biosciences.

Samples were washed in PBS at 2000 rpm for 5 minutes before running at FACS instrument. FACSCanto™ II (Becton Dickinson, Franklin Lakes, NJ, USA) was used for both PBMCs and CSF cells analysis. At least 20,000 events were collected for each PBMCs sample, while for CSF all cells extracted were analyzed on flow cytometry not performing cell counts to minimize cell losses. We only included CSF sample results containing a minimum of 1,000 gating events (Subirá et al. 2002). Data analyses were performed by using FCS Express 4 Flow Research Edition Software.

Representative figures displaying our gating strategy for PBMCs and CSF cells samples are shown in Figures 7 and 8, respectively.

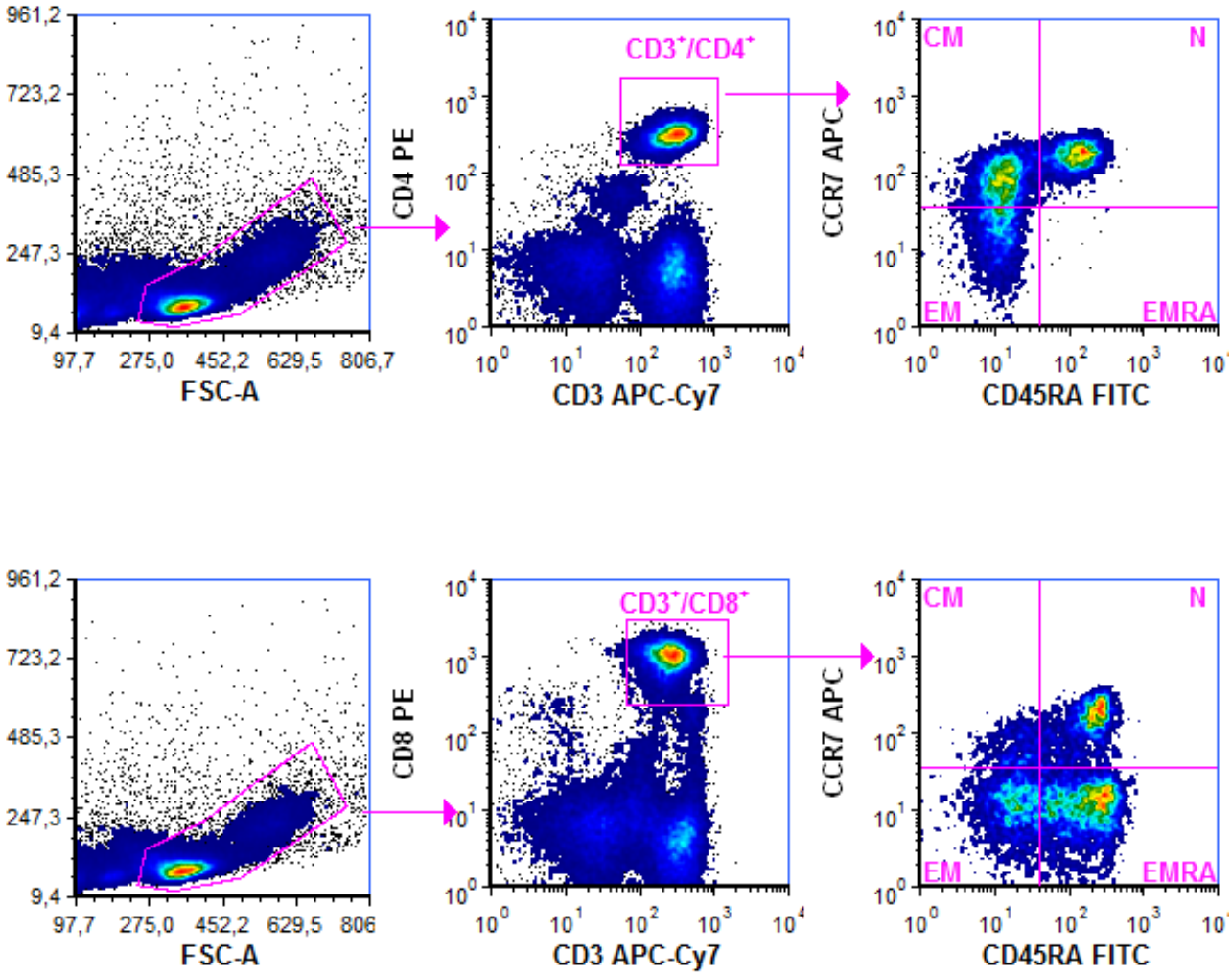


Figure 7. Representative gating strategy of flow cytometry analysis of PBMCs-derived $CD4^+$ and $CD8^+$ T cell subsets based on CCR7 and CD45RA cell-surface expression (N, CM, EM and EMRA).

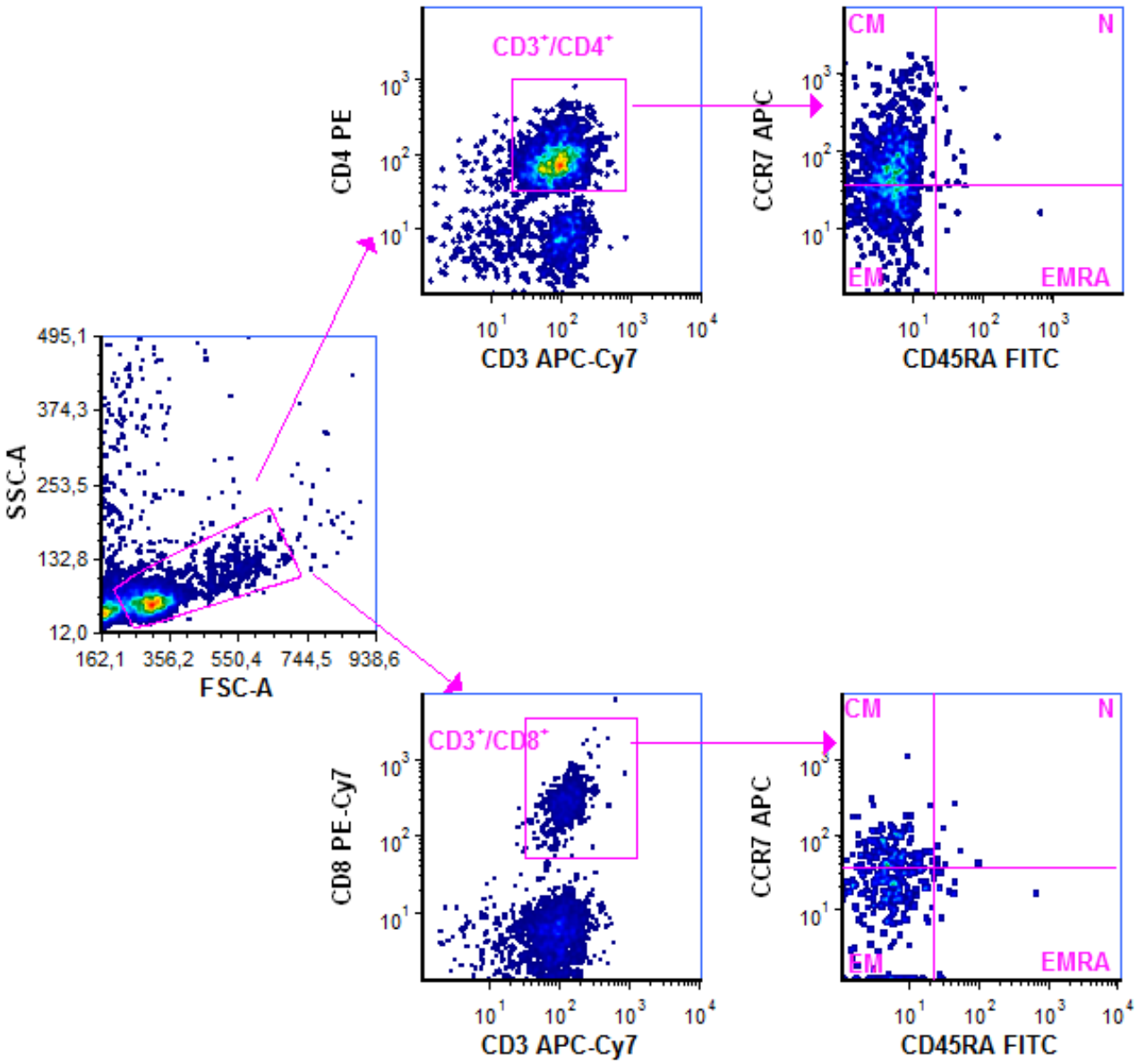


Figure 8. Representative gating strategy of flow cytometry analysis of CSF-derived $CD4^+$ and $CD8^+$ T cell subsets based on CCR7 and CD45RA cell-surface expression (N, CM, EM and EMRA).

2.5 STATISICAL ANALYSIS

Percentages of immune cells populations and demographic data are reported as mean ± standard deviation (SD). Group differences in sex distribution, age and body mass index (BMI) were explored with chi-square and independent sample t-tests, respectively. Differences between patients and controls in T cell subset distribution were analyzed using non-parametric Mann-Whitney U tests. Dissimilarities between T cell subset distribution in CSF and PBMC were analyzed, separately for NT1 and HC, using a Wilcoxon signed-rank test. Statistical analyses were conducted using SPSS 19.0 (SPSS Inc. Chicago, IL). A p value <0.05 was considered statistically significant. Given the explorative character of the analyses, alpha adjustment was not performed.

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CHAPTER 3

Results

3.1 DEMOGRAPHIC CHARACTERISTIC IN NT1 AND HC GROUP

No group differences were observed for age (mean age NT1 = 32 ± 11.15 years old, mean age HC = 38.8 ± 13.02 years old), sex (9 and 6 males in NT1 and HC, respectively), and body mass index (BMI) (mean NT1 = 27.31 ± 5.11, mean HC 24.21 ± 4.48).

NT1 patients had lower CSF hypocretin-1 levels (27.62 ± 30.79 pg/ml) compared to HC (321.20 ± 50.34 pg/ml; $p<0.0001$). Mean disease duration in NT1 patients was 12.50 ± 10.65 years.

3.2 CSF T CELLS AND PBMCs IN NT1 VERSUS *HLA DQB1*06:02* NEGATIVE HC

CSF cell distribution of $CD3^+$, $CD4^+$, $CD8^+$ T cells, and CD4/CD8 ratio did not differ between groups. NT1 patients however displayed a trend towards higher CSF $CD4^+$ T cells compared to HC (64.59 ± 4.77% in NT1, and 59.65 ± 9.05% in HC). Within $CD4^+$ cell subsets, we also observed 2-fold increase in EMRA T cells in patients versus controls that did not reach statistical significance (1.29 ± 1.27% in NT1, and 0.62 ± 0.87% in HC). Similar EM, N, and CM frequencies were observed in the two groups.

Regarding $CD8^+$ T cell subpopulations, we did not find any differences between NT1 patients and HC (see Table 1).

Concerning PBMCs, total frequencies of $CD3^+$, $CD4^+$, $CD8^+$ T cells, and CD4/CD8 ratio did not differ between groups. Within $CD4^+$ T cell subsets, EMRA had lower frequencies in NT1 versus HC (2-fold decreased); moreover, $CD4^+$ and $CD8^+$ memory subsets showed similar trends in NT1 versus HC, with decreased EM and EMRA and increased in N and CM T cells.

Regarding peripheral NK cells, their total frequency and cytotoxic phenotype were comparable in patients and controls. We observed a trend towards lower numbers of NK-T cells, and a significant difference in immune-regulatory $CD56^{bright}$ cells in NT1 versus HC groups with NT1 patients showing a 3-fold increase in NK immune-regulatory cell frequency compared to controls (see Table 1).

	NT1 (n=14) *Mean ± SD*	*HC (n=14)* *Mean ± SD*	*p*
CSF			
CD4 Naïve	3.19 ± 2.30	4.31 ± 2.93	0.26
CD4 CM	44.74 ± 14.84	41.16 ± 12.41	0.29
CD4 EM	50.74 ± 16.36	54.84 ± 13.04	0.36
CD4 EMRA	1.29 ± 1.27	0.62 ± 0.87	0.10
CD8 Naïve	2.74 ± 2.49	3.68 ± 2.42	0.32
CD8 CM	18.19 ± 8.10	15.20 ± 11.89	0.41
CD8 EM	72.32 ± 7.80	73.60 ± 13.62	0.53
CD8 EMRA	6.74 ± 4.79	7.51 ± 6.30	0.68
CD3	87.71 ± 3.72	86.38 ± 6.37	0.63
CD4	64.59 ± 4.77	59.65 ± 9.05	0.16
CD8	18.74 ± 5.39	21.26 ± 7.94	0.71
CD4|CD8 *ratio*	3.79 ± 1.37	3.24 ± 1.33	0.42
PBMC	*NT1 (n=14)*	*HC (n=13)*	
CD4 Naïve	42.92 ± 9.80	39.88 ± 15.56	0.73
CD4 CM	35.27 ± 8.55	32.92 ± 7.39	0.33
CD4 EM	21 ± 6.35	25.63 ± 13.64	0.38
CD4 EMRA	0.80 ± 0.88	1.52 ± 1.10	<0.01
CD8 Naïve	41.62 ± 19.87	35.75 ± 16.82	0.54
CD8 CM	10.98 ± 9.25	6.94 ± 4.18	0.13
CD8 EM	24.55 ± 7.88	27.27 ± 10.73	0.51
CD8 EMRA	22.85 ± 18.04	30.02 ± 20.33	0.31
CD3	60.72 ± 9.46	61.70 ± 10.44	0.96
CD4	35.49 ± 9.38	33.25 ± 7.85	0.48
CD8	20.04 ± 6.90	21.95 ± 10.42	0.79
CD4|CD8 *ratio*	1.98 ± 0.79	2.06 ± 1.59	0.47
NK Total	9.77 ± 5.48	7.86 ± 3.23	0.47
NK-T	2.42 ± 1.66	5.15 ± 3.70	0.06
Cytotoxic NK ($CD56^{dim}$)	24.18 ± 15.11	29.01 ± 9.75	0.29
Immuno-regulatory NK($CD56^{bright}$)	0.61 ± 0.52	0.20 ± 0.31	<0.01

Table 1. $CD4^+$, $CD8^+$ T cell subsets, and NK, frequencies (%) represented with mean ± standard deviation (SD) in CSF and PBMC from NT1 and HC.

3.3 CSF/PBMC FOLD-CHANGE IN NT1 VERSUS *HLA DQB1*06:02* NEGATIVE HC

CSF/PBMC fold-change scores for total $CD3^+$, $CD4^+$, $CD8^+$ cells, CD4/CD8 ratio and, $CD8^+$ T cell subsets did not differ between groups. However, NT1 patients showed a significant increase in $CD4^+$ EMRA cells (5-fold increase) compared to the HC group (2.52 ± 3.20 and 0.44 ± 0.68, respectively) without further differences in other $CD4^+$ T cell subsets (see Figure 1).

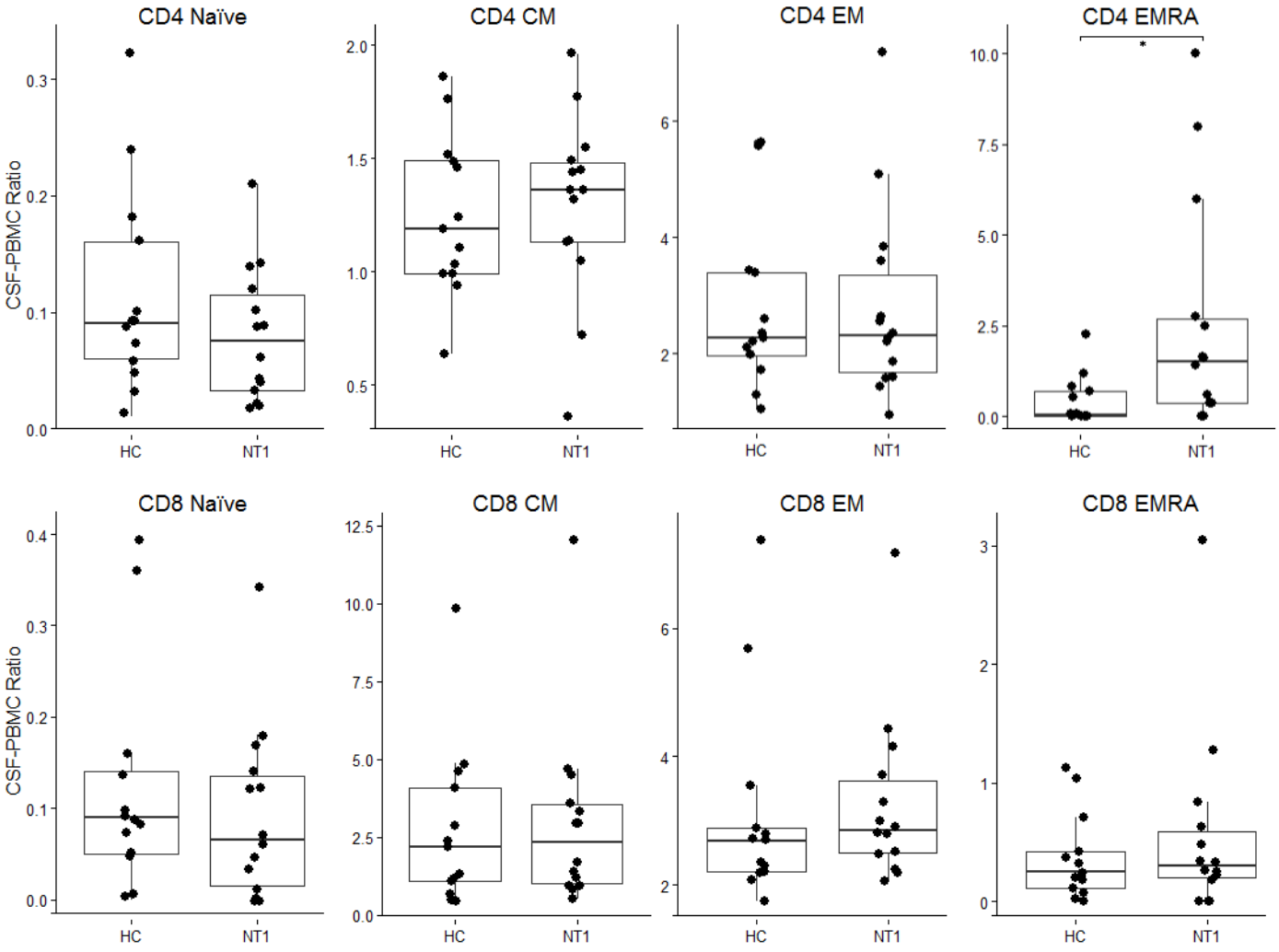
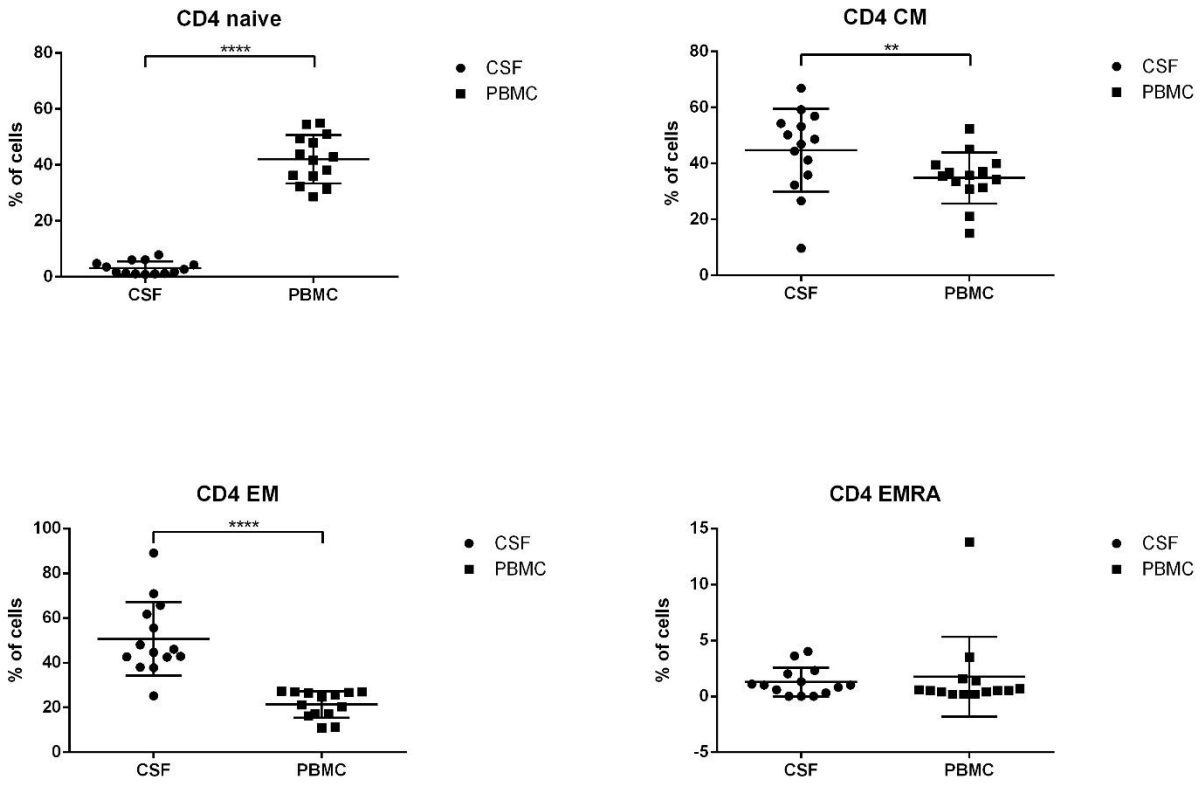


Figure 1. Box-plots of CSF/PBMC ratios for $CD4^+$ and $CD8^+$ T cell subsets in HC contrasted with NT1 subjects. Mann-Whitney U test *p=0.04.

3.4 CSF VERSUS PBMC T CELL SUBSETS IN NT1 AND *HLA DQB1*06:02* NEGATIVE HC

The composition of T cell populations in the CSF and PBMC of NT1 patients is shown in Figure 2. We found significant differences in all T cell subsets in CSF versus PBMCs except for $CD4^+$ EMRA cells. NT1 CSF composition was characterized by a higher prevalence of $CD4^+$ and $CD8^+$ EM and CM T cell phenotypes. In contrast, very low frequencies of $CD4^+$ and $CD8^+$ N T cells were observed in the CSF versus PBMC of NT1 patients. Regarding $CD4^+$ EMRA subsets, CSF frequency were similar to that of PBMC, whereas $CD8^+$ EMRA T cells showed elevated frequency in PBMCs versus CSF.

The composition of T cell populations in the CSF and PBMC of HC is depicted in Figure 3. HC showed a profile of different distributions in CSF vs PBMC comparable to NT1 in all T cell subsets, but $CD4^+$ EMRA. Indeed, $CD4^+$ EMRA were similarly distributed in CSF and PBMC of NT1.



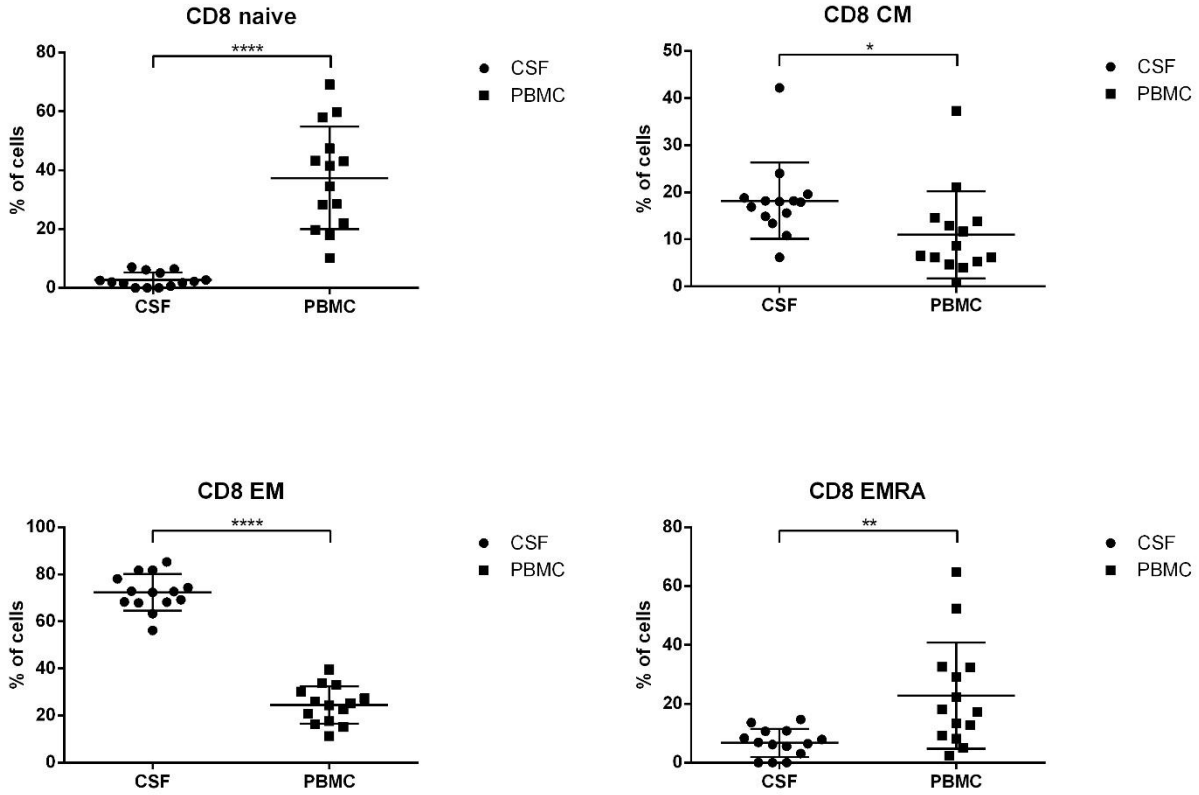
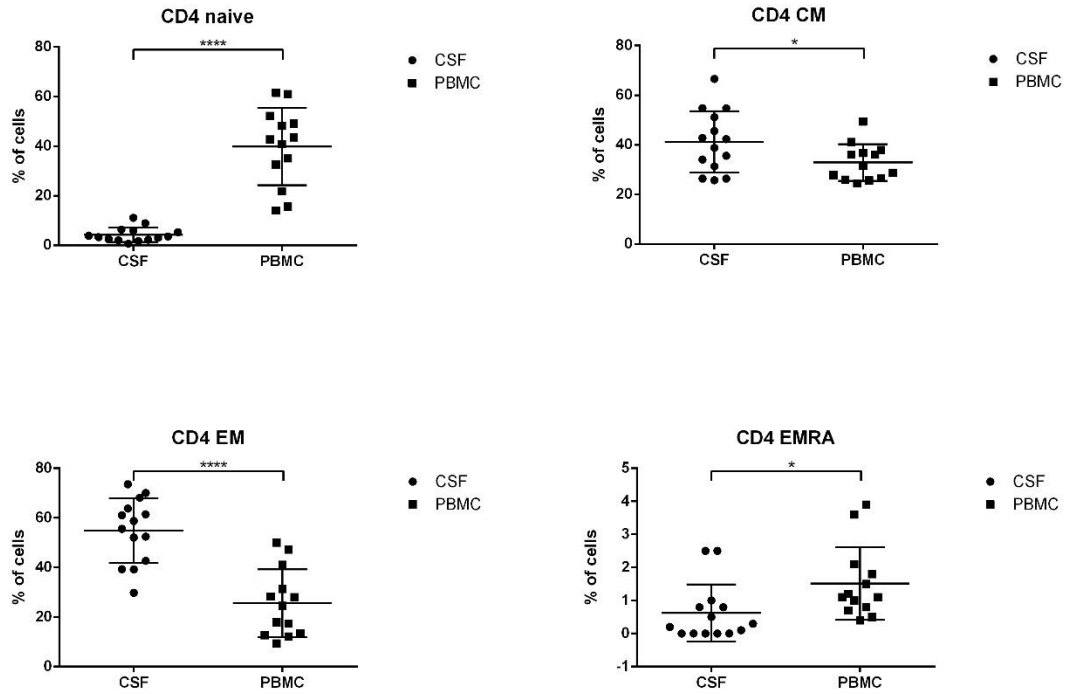


Figure 2. Dot-plot representation of CSF and PBMC $CD4^+$ and $CD8^+$ T cell subset frequencies in NT1 patients. Circle dots refer to CSF while square dots refer to PBMCs. **** p<0.0001; ** p<0.01; * p<0.05.



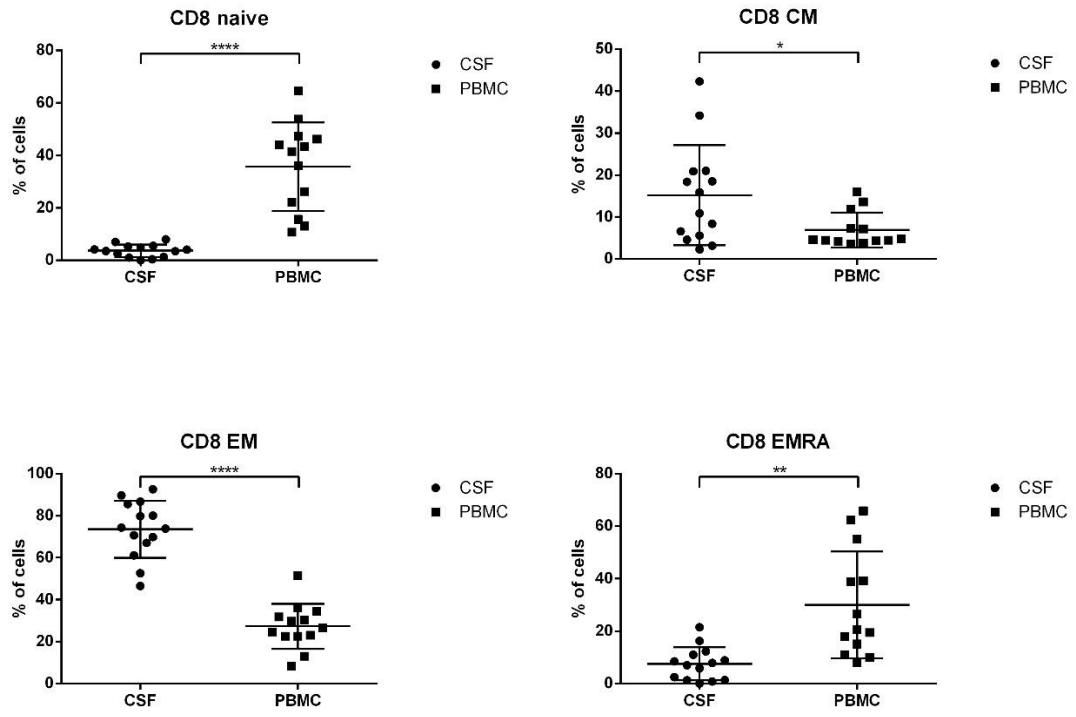


Figure 3. Dot-plot representation of CSF and PBMC $CD4^+$ and $CD8^+$T cell subset frequencies in HC. Circle dots refer to CSF while square dots refer to PBMCs. **** $p<0.0001$; ** $p<0.01$; * $p<0.05$.

CHAPTER 4

Discussion

In this study, we characterized immune cell subsets in the CSF cells and peripheral blood of NT1 patients in comparison to HC matched for sex, age, BMI, but differing for the presence of the *HLA DQB1*06:02* susceptibility allele. Moreover, we analysed the ratio between the CSF/PBMC cells of NT1 patients and HC group and differences in cell frequencies in CNS versus peripheral blood of NT1 patients. Although total frequency of $CD4^+$, $CD8^+$ T cells, and NK cells did not differ between groups in either CSF or PBMC compartments, we observed a trend towards higher frequency of $CD4^+$ EMRA T cells in the CSF and a 2-fold reduction in PBMCs of NT1 vs HC. EMRA T cells are a subpopulation of terminally differentiated effector memory (CCR7-) cells that express CD45RA marker and display the characteristic expression of chemokine receptors and adhesion molecules required for homing cells to inflammatory sites. Indeed, the EMRA T cells are characterized by rapid effector functions, the production of perforin, INF-γ, IL-4 and IL-5 following antigenic stimulation. EMRA T cells are at an advanced stage of differentiation, and thus have a low proliferation potential after engagement of TCR receptor. They however express granzyme B and have cytotoxic characteristics (Sallusto et al. 2004). Accumulating studies have indicated that infections with human cytomegalovirus (CMV) and Dengue virus are associated with an expansion of $CD4^+$ EMRA T cells (Libri et al. 2011; Tian et al. 2017). In fact, $CD4^+$ T cells with cytotoxic functions have been reported during a wide range of infections, including HIV, CMV, Epstein-Barr virus, mouse CMV, acute lymphocytes choriomeningitis virus, and influenza virus (Tian et al. 2016). The ability of $CD4^+$ T cells to acquire cytotoxic functions have been mostly attributed to Th1 cells after viral infections; however, it is now clear that other $CD4^+$ T cell subsets including regulatory T cells can also secrete effector molecules and exert cytotoxic effects (Grossman et al. 2004; Tian et al. 2016). Moreover, recent studies further suggest that cytotoxic $CD4^+$ T cells may represent a separate lineage independent of other $CD4^+$ T cell subsets and are induced by distinct environmental cues and transcriptional regulators, highlighting the versatility of $CD4^+$ T cell responses (Mucida et al. 2013; Cheroutre & Husain et al. 2013; Tian et al. 2016). Furthermore, as in our study, an elevated frequency of $CD4^+$ EMRA T cells was observed in patients affected with type 1 diabetes (Matteucci et al. 2011). The considerable accumulation of $CD4^+$ EMRA T cells in NT1 suggests life-long stimulation by protracted antigen exposure (H1N1 flu, streptococcal infections, or residual self-antigens), or a homeostatic defect in the regulation/contraction of immune responses.

Human NK cells can be divided into functionally distinct subsets based on the levels of CD56. Several studies indicate that the $CD56^{bright}$ NK subpopulation plays a critical role in the early innate immune response. These cells have a higher proliferative capacity than $CD56^{dim}$ NK cells (Cooper et al. 2001) and are the primary source of NK cell-derived cytokines including TNF-α, IFN-γ, IL-10, IL-13 (Cooper et al. 2001-b). Laroni *et al*. (2011) demonstrated that $CD56^{bright}$ NK cells are a regulatory

subset controlling proliferation of $CD4^+$ T cells through cytotoxic mechanisms. Another study reported that $CD56^{bright}$ NK cells could kill pre-activated T cells and markedly decrease autologous $CD4^+$ T cell proliferation in healthy subjects (Nielsen et al. 2012). This task was dependent on $CD56^{bright}$ NK-mediated cytotoxicity against autologous T cells undergoing activation, and may indicate that the role of $CD56^{bright}$ NK cells is to prevent excessive activation of $CD4^+$ T cells (Laroni et al. 2016). In multiple sclerosis the accumulation of $CD56^{bright}$ NK cells in the CSF, and the correlation of the expansion of this cell subset with decreased flares during effective immunotherapy support the hypothesis that NK cells play an immune-regulatory role in the disease (Fogel et al. 2013). Therefore, we may speculate that the expansion of $CD56^{bright}$ NK cells in NT1 patients can result from an excessive activation of $CD4^+$ T cells during disease course. Furthermore, $CD56^{bright}$ NK cells can also become cytotoxic in response to appropriate activation, particularly through inflammatory cytokines. In rheumatoid arthritis, it seems that $CD56^{bright}$ NK cells are pathogenic by playing an active role in sustained inflammation. NK cells in the synovial fluid are almost exclusively $CD56^{bright}$ and express high levels of activation molecules and secrets INF-γ after cytokines stimulation (Zhang & Tian, 2017). Our study evidenced also a trend towards reduction frequency of NK-T cell subset in NT1 patients. NK-T cells appear to have distinct functional capabilities; they have been shown to mediate both protective and regulatory immune functions especially in the context of autoimmune disease development (Taniguchi et al. 2003).

Recent studies have investigated the role of T cells in NT1 and reported associations with an increase and activation of regulatory T cells, a systemic activation of global T cells and increased production of the pro-inflammatory cytokines IL-12 and TNF (Hartmann et al. 2016; Lecendreux et al. 2017). In particular, the study of Lecendreux et al. (2017) found that 31 NT1 patients, 1.5 years after disease onset, displayed blood phenotypic changes characterized by an increased frequency of $CD4^+$ T regulatory cells associated with increased levels of activated and memory effector $CD4^+$ T cells in peripheral blood contrasted with healthy donors. Whereas, the study of Hartamann et al. (2016) showed complex patterns of immune activation in 39 NT1 patients characterized by a disease duration near 5 years and associated with $CD4^+$ and $CD8^+$ T cells-producing elevated levels of several cytokines involved in B cells differentiation (IL-4, IL-13, IL-21), supporting previous indications of autoantibodies in NT1.

In addition, our work showed that the overall frequencies of T cell subsets and NK cells in PBMC are in line with these recent studies on NT1 immune-phenotyping (Hartmann et al. 2016), although we adopted a different method and analysis.

Finally, we reported an expansion of the $CD4^+$ and $CD8^+$ subsets with EM phenotype in the CSF of NT1 patients with long disease course when compared with the corresponding peripheral blood compartment. This was especially true for cells expressing memory markers such as CD45RA, and CCR7, thus showing that CSF T cells compositions are dissimilar to PBMCs in both NT1 and HC. Furthermore, the different $CD4^+$ EMRA T cells distribution in CSF vs PBMC of NT1 compared to HC highlighted the increased presence of this cells subset in the CNS compartment of NT1 patients.

The main study limitations are the lack of a *HLA DQB1*06:02* positive HC group and the relatively small sample size, although sufficient to highlight significant differences between groups. Moreover, both circadian time and sleep deprivation affect immune cell subsets (Spitzer et al. 2015) especially for NK cells (De Lorenzo et al. 2015; Fondell et al. 2011) however we did not observe any alteration in overall frequency of T- and NK- cells between patients and controls.

4.1 CONCLUSION

Our results although not conclusive, point to an involvement of $CD4^+$ EMRA T cells and $CD56^{bright}$ immune-regulatory NK cells in NT1 with long disease course. The increased frequency of $CD4^+$ EMRA T cells in the CSF/PBMC ratio and the comparable percentages of CSF cells and PBMCs in NT1 group may equally support the two hypothesis of foreign- or self- antigen. The chronic immune stimulation with low antigen levels seemed necessary for the appearance of memory $CD4^+$ cells with the EMRA phenotype (Harari et al. 2004). Interestingly, Kornum *et al*. (2011) showed that NT1 patients frequently show mutations in the *P2RY11* genotype, a receptor mostly expressed in T lymphocytes and NK cells. These findings suggest that P2RY11 activity may be important for the selection of specific immune cells acting therefore as an important regulator of immune-cells survival, with possible implications in NT1 pathophysiology.

In conclusion, this study shows for the first time the CSF T cell subsets composition in NT1 patients with long-term disease courses. Similar studies in recent onset patients and functional *in vitro* studies on cytokines secretion and expression are warranted to gain further insight into NT1 pathophysiology.

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