“The good thing about science is that it's true whether or not you believe in it.”

— Neil deGrasse Tyson

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List of Abbreviations

ADEM	acute disseminated encephalomyelitis
APC	Antigen presenting cell
BBB	blood-brain-barrier
Blimp-1	B lymphocyte-induced maturation protein-1
CD	cluster of differentiation
CD4+ CTL	CD4+ cytotoxic T lymphocyte
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CIS	clinically isolated syndrome
CM	Conditioned medium
CMV	cytomegalovirus
CNS	central nervous system
CPMS	Chronic progressive MS
CRTAM	MHC class I restricted T cell associated molecule
CSF	cerebrospinal fluid
CX3CR1	Fractalkine receptor
Cy	Cyanine
DNA	Deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
Eomes	Eomesodermin
EP	evoked potential
Exp	expansion
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FET	Fisher's exact test
FITC	Fluorescein isothiocyanate
Foxp3	forkhead box P3
GITR or GITRL	glucocorticoid-induced TNFR-related protein or ligand
GM-CSF	granulocyte macrophage colony-stimulating factor
GrB	Granzyme B
GWAS	genome-wide association studies
HC	Healthy control
HIV	human immunodeficiency virus
HOPX	Homeobox-only protein
IBD	Inflammatory bowel disease
IFN-γ	Interferon-γ
Ig	immunoglobulin
IL	interleukin
IL2RA	interleukin 2 receptor α

IMPC	intrinsic MS prognostic classification
MBP	myelin basic protein
MCMV	Murine cytomegalovirus
MHC	major histocompatibility complex
MICB	MHC class I polypeptide-related sequence B
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSSS	multiple sclerosis severity score
NK cell	Natural killer cell
OR	Odds ratio
PBMC	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD1	programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PPMS	Primary progressive MS
RA	Rheumatoid arthritis
RAF	risk allele frequencies
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RRMS	relapsing-remitting MS
Runx3	Runt-related transcription factor 3
SC	Spinal cord
SD	Standard deviation
SEM	Standard error of the mean
SNP	single nucleotide polymorphism
SPMS	Secondary progressive MS
T-bet	Tbx21
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR2	toll-like receptor 2
TMEV	Theiler’s murine encephalomyelitis virus
TNF-α	Tumor Necrosis Factor alpha
Tregs	Regulatory T cells
WT	Wild type

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INTRODUCTION AND AIMS

1.1 The origin, biology and role of cytotoxic CD4+ T cells in health and disease

1.1.1 Introduction

CD4+ T helper cells are central in the adaptive immune system, regulating both humoral and cellular immune responses (1). After positive and negative selection in the thymus, mature naive CD4+ T cells home to the peripheral lymphoid organs (2). Here, naive CD4+ T cells get activated and differentiate into subpopulations with distinct functions and properties. Depending on the cytokines present during activation, naive CD4+ T cells differentiate to one of the 3 main groups of effector cells; namely T helper cells, regulatory T cells or, as recent literature suggests, cytotoxic T cells (Figure 1.1).

As their name suggests, T helper cells help other immune cells (e.g. macrophages and B cells) in their response against pathogens. The best described pro-inflammatory Th subsets are Th1 and Th17 cells. Th1 cells express the transcription factor T-bet, produce IFN-γ and TNF-α and stimulate phagocyte-mediated functions (3, 4). Th17 cells express the transcription factor RORγt and produce IL-17A, IL-17F, IL-21, IL-22, TNF-α and GM-CSF (3, 5). In healthy individuals, Th17 cells provide protection against bacterial and fungal infection and are mostly found in the gut (4). Another widely known T helper subset, the Th2 cells, protects against extracellular parasites by activating the humoral immune response. They express the transcription factor GATA-3 and mainly produce IL-4 (6).

After antigen exposure and subsequent expansion and response, some T cells become memory cells that can respond faster after a second encounter with the same type of antigen (7). Two main types of memory T cells exist, central and effector memory T cells, which differ in function and phenotype. Central memory T cells express CCR7 (see also 1.1.3.4) and are able to home to the secondary lymph nodes, whereas effector memory T cells lack CCR7 expression and produce high levels of cytokines. Often researchers also refer to terminal effector memory T cells, which are effector memory T cells that re-express CD45RA and lose CD27 and CD28 (see 1.1.3.1) expression (T_{EMRA}) (8). For more in-depth information see (8).

Regulatory T cells control the immune response by suppressing effector T cells and antigen presenting cells. They express Foxp3 as transcription factor and produce IL-10 and TGF-β. Tregs are either derived from the thymus (natural Tregs) or induced in the periphery (induced Tregs) (3).

Classically, cytotoxic effector T cells originate from the CD8 lineage, while T helper cells are CD4 lineage-derived. It is now clear that CD4+ T cells can also acquire cytotoxic activity and damage tissues and cells, indicating that the T helper fate is not as fixed as previously thought (9). Different phenotypic markers are used to define CD4+ CTLs, however no consensus exists about their general phenotype. In disease, CD4+ CTLs can have direct protective functions, but can also become pathogenic under inflammatory conditions (10, 11).

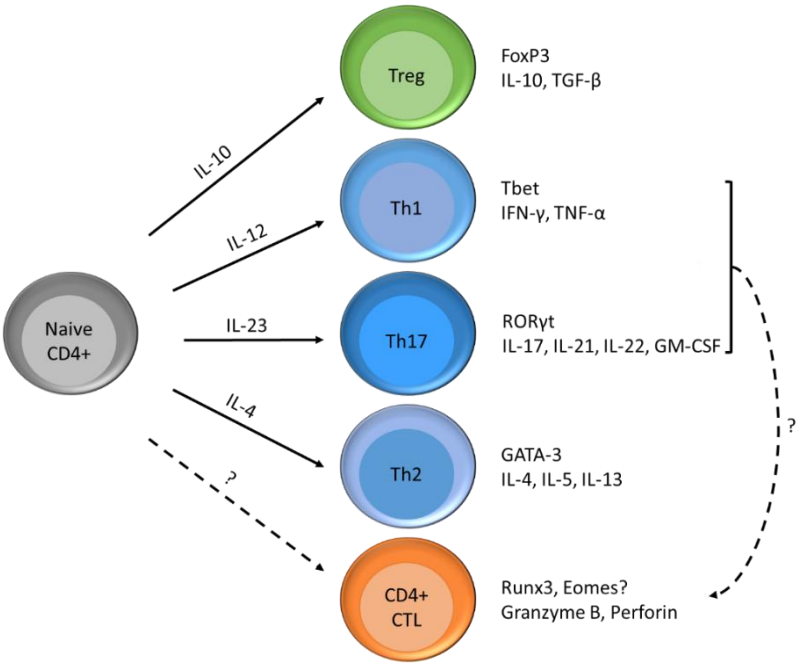


Figure 1.1: Differentiation possibilities of naive CD4+ T cells and formation of CD4+ CTLs. Naive CD4+ T cells can differentiate into T helper cells (e.g. Th1, Th2 and Th17), regulatory T cells and cytotoxic T cells according to the cytokines in their environment. Each subset has its specific transcription factor and produces its signature cytokines. Next to the differentiation of naive T cells into CD4+ CTLs, Th1 or Th17 cells are also thought to become cytotoxic.

In the next paragraphs, we discuss the characteristics of cytotoxic CD4+ T cells (CD4+ CTLs), the plasticity of helper T cells towards cytotoxic CD4+ T cells and the role of these CD4+ CTLs in the context of viral infections, cancer, cardiovascular and (auto)inflammatory disease.

1.1.2 The formation of cytotoxic CD4+ T cells

CD4+ CTLs are thought to originate either from naive CD4+ T cells or through plasticity of CD4+ effector Th cells (figure 1.1). The formation of CD4+ CTLs is controlled by a combined action of transcriptional regulation and extracellular cues. These extracellular cues could consist of cytokines, TCR stimulation and co-stimulatory signals, which lead to cytosolic signaling cascades (PI3K pathway and metabolic programs), ultimately altering gene transcriptional programs (9, 10). Indeed as shown for CD4+ effector T cells in mice, chronic or strong antigenic stimulation ensures the start of the cytolytic program (11). Cytotoxic CD4+ T cells express part of the CD8+ T cell lineage transcriptional program (12). Both CD4+ and CD8+ CTLs express e.g. transcription factors T-bet, Eomes, Runx3 and Hobit at higher levels compared to their naive counterparts (13-16). In this part, we discuss the most important transcription factors that are associated with the induction of cytotoxic properties in CD4+ T cells.

ThPOK. ThPOK is the master regulator of the T helper lineage. ThPOK is required for MHC-II restricted thymocytes to become CD4+ T cells, and inefficient upregulation of ThPOK in mice results in transdifferentiation into cytotoxic T cells. Furthermore, ThPOK blocks expression of Runx3, CD8 (a and b), Eomes, perforin and granzymes, which are implicated in the cytotoxic lineage (17, 18). In mice, ThPOK suppresses the cytolytic program in T helper cells. Loss of ThPOK expression coincides with the acquisition of cytolytic properties and differentiation to CD4+ CTL in the mouse gut (11). Whether the downregulation of ThPOK is required for the acquisition of cytotoxic functions of human CD4+ CTL remains to be determined.

T-bet. T-bet or Tbx21 is the master regulator for effector Th1 cells, as it induces the production of IFN-γ (19). T-bet expression is induced/maintained in CD8+ T cells by TCR signaling and IL-12, and is associated with upregulated perforin and granzyme B production in both humans and mice (15, 20). Based on this, T-bet is thought to be involved in the acquisition of cytotoxic activity by CD4+ Th1 cells. Indeed, T-bet has been described to be critical in the functionality of human effector and effector memory CD4+ T cells, as it controls lymphocyte trafficking via regulating chemokine expression and its downstream cytotoxic gene targets are perforin and granzyme B (15).

In vivo, T-bet deficiency during influenza virus infection leads to impaired expression of granzyme B in mouse CD4+ T cells (10).

Eomes. Eomesodermin (Eomes) has a similar activity as T-bet in both human and mouse cells, since it promotes IFN-γ production in CD8+ T cells and suppresses differentiation of Th cells other than Th1 (21). Its expression increases in human memory and effector cells (15). In mice, Eomes can induce both Th1 differentiation and expression of IFN-γ and perforin (15). In humans, Eomes+CD4+ T cells express IFN-γ and granzyme B and degranulate after activation (21). Eomes expression is reported to limit Foxp3 induction in mice, thereby blocking Treg formation (22). Transfection of Eomes in two murine CD4+ T cell lines resulted in the acquisition of IFN-γ expression, upregulation of FasL and expression of perforin and granzyme (23).

Runx3. Runt-related transcription factor 3 (Runx3) promotes the cytotoxic lineage. In CD8+ T cells, CD4 expression is terminated by binding of this transcription factor to the CD4 silencer element (24). Runx3 regulates granzyme B expression directly, but synergizes with T-bet and Eomes to promote IFN-γ and perforin expression (14). Indeed, in mouse CD8+ T cells, Runx3 induces the expression of Eomes and thereby promotes perforin gene expression (14). Runx3 is not present in naive CD4+ T cells, but it is upregulated during Th1 differentiation and it promotes IFN-γ expression (14). Upregulation of Runx3 expression in mouse intestinal CD4+ T cells leads to a parallel increase in CD8α, NK cell molecule CD244 and T-bet, and to a decrease in ThPOK expression (25).

Blimp-1. B lymphocyte-induced maturation protein-1 (Blimp-1) promotes the binding of T-bet to the promotors of cytolytic genes (granzyme B (*Gzmb*), perforin1 (*Prf1*)) in mouse CD4+ T cells and is required for cytolytic function (10). Blimp-1 deficiency impairs the expression of granzyme B and perforin in CD4+ T cells (via impaired binding of T-bet to their promotors) and reduces their cytotoxic function *in vivo* during influenza infection (10). CD4+ CTLs express high levels of Blimp-1 and low levels of Bcl-6 (26).

Hobit. Human homolog of Blimp-1 in T cells (Hobit) binds to Blimp-1 target sites, thereby regulating IFN-γ. Hobit is expressed in CD8+ effector T cells, especially long-lived effector T cells with strong effector functions (e.g. IFN-γ, granzyme B).

In mice, Hobit is predominantly expressed in CD4+ NKT cells, but also in tissue-resident memory CD4+ T cells and γδ T cells, making it a candidate regulator of cytotoxicity in CD4+ T cells (13, 27). Recently, Hobit expression was found in accumulating cytotoxic CD4+ T cells after primary hCMV infection. The Hobit+CD4+ T cells displayed highly overlapping characteristics with Hobit+CD8+ and Hobit+ γδ T cells, including the expression of cytotoxic molecules, T-bet and CX3CR1 (28).

Hopx. Homeobox only protein (Hopx) is induced by T-bet and increases after repeated antigenic stimulation of Th1 cells, conversely, its expression is high in memory and low in naive CD4+ T cells (human and mice). HOPX regulates genes involved in apoptosis and survival, and is thus a critical regulator for Th1 survival (29).

1.1.3 The biology of cytotoxic CD4+ T cells

CD4+ T cells are designated as CTLs when they acquire certain cytotoxic molecules, such as perforin and granzymes, and change the expression of transcription factors such as ThPOK and Eomes. Next to these cytotoxic properties, other phenotypic changes can occur. CD4+ CTLs have been described by various research groups, using a variable set of phenotypic markers. Here, we describe different molecules that identify CD4+ CTLs. It is not yet clear whether these phenotypes represent different subsets of CD4+ CTLs, or that all markers co-localize in one and the same subset. While TCRγδ CD4+ CTLs have also been described (30), we limit ourselves to TCRαβ CD4+ T cells.

1.1.3.1 CD28 loss

CD28 is a costimulatory molecule which binds to B7 on antigen presenting cells, providing the so-called second activation signal next to TCR triggering, needed for activation of naive T cells. During chronic antigenic stimulation, the CD28 molecule is downregulated on oligoclonal (derived from a few clones) T cells with restricted TCR diversity (31, 32). This loss of CD28 expression is attributed to two regulatory sequences sites α and β, in the gene promotor. Modulations at these sites, prevent the formation of α- and β-binding complexes, which renders the transcription initiator element inoperative (33). The CD4+CD28null T cells become CD28 signaling independent and not anergic as previously thought (34). CD4+CD28null T cells can be classified as Th1-derived cytotoxic T cells; Th1 because of their cytokine (e.g. IFN-γ, TNF-α) and chemokine receptor (e.g. CXCR3) expression profile and their similar DNA methylation patterns (35); cytotoxic, based on their production of perforin (cytolytic) and granzyme B (apoptosis induction) and expression of NK cell receptors (see 1.1.3.2) and CX3CR1, but not CCR7 (see 1.1.3.4)(36-38). Furthermore, they are resistant to apoptosis, since they upregulate anti-apoptotic molecule Bcl-2 and downmodulate pro-apoptotic molecules Bim and Bax (39), and can cause damage to e.g. endothelial cells (38, 39). Of note, CD27, another co-stimulatory molecule can also be downregulated in CD4+ CTLs (26, 40).

1.1.3.2 NK cell receptors

Natural killer (NK) cell receptors are, as their name suggests, classically only expressed on NK cells and can be divided into 2 main groups: activating and inhibiting receptors. The activating receptors induce cytotoxicity by NK cells upon binding to their ligands, whereas inhibitory receptors block cytotoxic activity (41). CD4+ CTLs express inhibitory NK receptors, namely killer cell lectin-like receptor subfamily B, member 1 (KLRB1/CD161) and Natural Killer Group 2A (NKG2A). Upon binding of KLRB1 or NKG2A with its ligand, respectively LLT-1 and HLA-E, cytotoxicity is inhibited (26, 42).

CD4+ CTLs also express activating NK receptors, such as Natural Killer Group 2D (NKG2D) and Natural Killer Group 2C (NKG2C). When CD4+ CTLs bind NKG2D (e.g. MICBs and ULBPs) or NKG2C (e.g. HLA-E) ligands, this leads to the release of cytokines and cytolytic molecules and augments molecules important for migration (e.g. adhesion molecules, chemokine receptors) (43-45). NK receptor-expressing CD4+ CTLs are mainly effector memory T cells, as demonstrated by their lack of CCR7 (45). They can kill stressed cells that overexpress the NKG2D/NKG2C ligands, e.g. infected and senescent T cells (45, 46). Other NK cell markers have also been described for CD4+ CTLs, such as neural cell adhesion molecule (NCAM/CD56) (45, 47, 48). CD56+ T cells display enhanced natural cytotoxicity compared to CD56- T cells (49). These CD4+ CTLs have a Th17-like phenotype since the majority produce IL-17 and have a high expression of CD161 (50). However, also IFN-γ production has been found, indicative of a Th1- or Th1/Th17-like phenotype (43, 48, 51).

1.1.3.3 CD8α

In CD4+ CTLs, CD8α expression was found either at protein or only at mRNA level (24, 52, 53). When CD8α is only expressed at the mRNA level, these cells are viewed as CD4+ CD8α+ CTL precursor cells. On these cells, expression of CRTAM (MHC class I restricted T cell associated molecule) is found (24).

CRTAM is a surface receptor predominantly expressed on activated CD8+ T cells, NK and NKT cells, where it promotes cytolytic function and IFN-γ production (24). CRTAM on CD4+ T cells was recently reported to lead to differentiation to pro-inflammatory and cytotoxic T cells, as evidenced by upregulated Eomes expression, IFN-γ, IL-17, granzyme B and perforin secretion (24).

CRTAM can be seen as an early marker of CD4+ CTLs, but its transient expression after stimulation, limits its usage as such (26).
CD4+ CTLs with CD8α expression at the protein level exhibit cytolytic functions (express Eomes, IFN-γ, CX3CR1, perforin and granzyme B) and have a highly differentiated effector memory phenotype, according to their reduced expression of CD28, CD45RO, CCR7 and CD127 (52, 53).

1.1.3.4 Chemokine receptors

Chemokine receptors and their ligands are involved in the migration of cells (chemotaxis) to sites of inflammation and homing to secondary lymphoid organs (54).
Ex vivo human perforin-expressing CD4+ T cells do not express C-C chemokine receptor type 7 (CCR7) (40). CCR7 is involved in homing of naive T cells to lymphoid organs, such as the lymph nodes and spleen, and its absence is indicative for an effector memory phenotype, since CCR7- T cells can migrate towards inflamed tissues and exert an immediate effector function (55).
The fractalkine receptor (CX3CR1) is expressed on CD4+ CTLs in various pathologies (36, 56). Fractalkine, the ligand of this receptor, can function as an adhesion molecule (bound) promoting adhesion to epithelial and endothelial cells, or as a chemoattractant (soluble), and accumulates in inflamed tissues (36, 54). Thus the presence of CX3CR1 on CD4+ CTLs induces migration towards inflamed tissues.

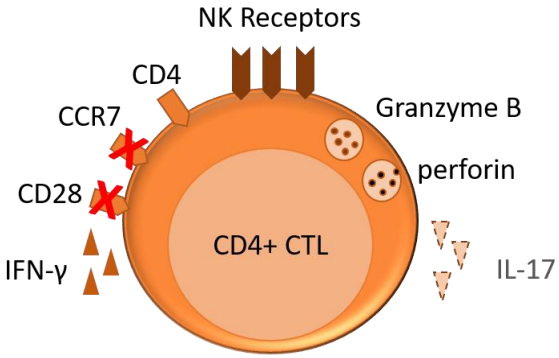


Figure 1.2: Proposed general phenotype of CD4+ CTLs. CD4+ CTLs are mostly effector memory T cells, as indicated by the absence of CCR7 and CD28. They produce IFN-γ, granzyme B and perforin, and express several NK cell receptors. Some of these subsets also produce IL-17.

Based on current findings (discussed in 1.1.3 and 1.1.4) we propose a common CD4+ CTL phenotype (Figure 1.2). CD4+ CTLs are mostly effector memory T cells, as indicated by the absence of CCR7 and CD28. They produce high levels of IFN-γ, granzyme B and perforin and gain the expression of several NK cell receptors. Some of the above mentioned CD4+ CTL subsets produce IL-17, whereas others clearly do not, this could indicate that they originate from different Th lineages. Another striking observation is that different phenotypes exist at various differentiation states, according to the loss of CD127 and the re-expression of CD45RA. Further research is needed to develop a consensus CD4+ CTL phenotyping panel, since e.g. CX3CR1 and CD8α could also be potential candidates, but have not been tested in all CD4+ CTL panels described here.

1.1.4 Cytotoxic CD4+ T cells in disease protection and exacerbation

Cytotoxic CD4+ T cells are associated with either beneficial or pathological immune responses, depending on the type of disease (Table 1.1).

They have protective functions in infections and cancer (10, 11). Indeed, CD4+ CTLs arise during both acute and chronic viral infections, and exert virus-specific cytotoxicity via alternative immune mechanisms that are not evaded by the virus. Evasion of immune surveillance by MHC I-restricted CD8+ CTLs is overcome by CD4+ CTLs lysis of infected MHC class II–expressing cells (11, 38, 57). The CD4+ CTLs in these infections are characterized by the transcription factors Blimp-1, T-bet and Eomes (10, 12, 24, 53, 58). They express NK receptors, but not CD28, and produce IFN-γ, perforin and granzyme B (24, 38, 47, 53, 59). In cancer, CD4+ CTLs have antitumor activities by e.g. lysing tumor cells and inducing tumor rejection/ regression via MHC class-II-restricted antigen recognition (60, 61). The phenotype of these killer T cells has not been studied in detail, but it has been shown that they produce IFN-γ and granzyme B (60, 61).

Cytotoxic CD4+ T cells can also display pathogenic activities under inflammatory conditions. Mechanisms include 1) production of pro-inflammatory cytokines, thereby augmenting inflammation, 2) infiltration of tissues, leading to destruction of target tissue and cells via their cytotoxic and autoreactive activity, and 3) recruitment of other harmful immune cells (e.g. macrophages) that contribute to inflammation (35, 62). Since CD4+ CTLs are increased in inflammatory conditions, these three mechanisms contribute to progression of autoimmunity or cardiovascular disease (10, 11).

Inflammatory bowel disease (IBD) is a group of autoimmune inflammatory conditions of the gastrointestinal tract. The principal types include Crohn's disease and intestinal colitis (63). In IBD, CD4+ CTLs are characterized by Eomes, T-bet and Runx3 expression and a downregulation of ThPOK (22, 24, 25). CD4+ CTLs can be found in the lamina propria and in colonic inflammatory sites, where they amplify inflammation via CTL activity (e.g. perforin, granzyme B and NK cell receptors) and pro-inflammatory cytokine production (e.g. IL-17 and IFN-γ) (24, 50, 64).

Another autoimmune disease is multiple sclerosis (MS), where autoreactive immune cells attack components of the central nervous system, leading to demyelination and axonal loss, causing a wide variety of symptoms (e.g. loss of vision, disability) (65), for more information see chapter 2, box 2.1. Autoreactive CD4+ CTLs, expressing Eomes and T-bet, can migrate towards lesions and cerebrospinal fluid of MS patients via CX3CR1 (21, 36, 43). They associate with neuroinflammation, since they are pro-inflammatory (e.g. IFN-γ, IL-17), and they kill oligodendrocytes (e.g. granzyme B, perforin, NK cell receptors) (43, 45). Furthermore, loss of CD28 has been described as one of the important markers for these CD4+ CTLs (36).

In rheumatoid arthritis, the main targets of autoreactive immune cells are the joints. CD4+ CTLs, mostly CD28-, are enriched at sites of inflammation and correlate with disease severity and extra-articular manifestations. This is probably due to their pro-inflammatory (e.g. IL-17, IFN-γ) and cytotoxic (granzyme B, perforin and NK cell receptors) nature (39, 52, 62, 66, 67).

The main subtypes of autoimmune myopathies include dermatomyositis (DM), polymyositis (PM), necrotizing autoimmune myositis (NAM) and sporadic inclusion body myositis (sIBM). All forms have a myopathy characterized by muscle weakness, which when severe, is almost always associated with muscular wasting (68). In DM, PM and sIBM, CD4+ CTLs, lacking CD28, were shown to infiltrate the inflamed muscle fibers and exert their pro-inflammatory (e.g. IFNγ) and cytotoxic (perforin, NK cell receptors) effects (39, 62, 69, 70).

Finally, cardiovascular diseases, such as acute coronary syndrome, are characterized by the involvement of heart or blood vessels. Mostly, atherosclerosis is the major precursor, caused by e.g. high blood pressure, smoking, diabetes and obesity (71). In atherosclerosis, CD4+ CTLs accumulate in rupture-prone regions and contribute to apoptosis and necrosis via perforin and granzyme B production and the expression of NK cell receptors (37, 51). In acute coronary syndrome, and more precisely unstable angina, CD4+ CTLs were shown to lyse endothelial and vascular smooth muscle cells. They are pro-inflammatory (e.g. IFN-γ), autoreactive and correlate with recurrent events and poor outcome (37, 39, 62).

Table 1.1: Involvement of CD4+ CTLs in different immune diseases

Disease	CD4+ CTL markers	Evidence for CD4+ CTL disease association	Refs.
Infections			
Persistent infections (EBV, CMV, HIV, parvovirus B19)	↑IFN-γ, IL-17, CX3CR1, CD8, GrB, perforin, NK receptors, Eomes, T-bet ↓CD28	Control of infection: lyse infected cells via FasL and perforin mediated mechanisms	(12, 38, 47, 52, 57-59)
Acute infections (Influenza, Hantaan and dengue virus)	↑T-bet, Blimp-1, GrB, perforin, CRTAM, Eomes, IFN-γ, CX3CR1, CD8α, NK receptors ↓CD28	Virus-specific cytotoxicity: directly kill infected MHC class II–expressing cells in an antigen-specific manner	(10, 24, 53, 72)
Tumors	↑IFN-γ, GrB	Tumor cell lysis, tumor rejection/ regression via MHC class-II-restricted antigen recognition	(60, 61)
Autoimmunity			
Crohn's disease (IBD)	↑Eomes, Runx3, NK receptors, IL-17, IFN-γ, GrB, perforin	Increased in lamina propria, pro-inflammatory cytokine production by Th17- and Th1-like CTLs	(50, 64, 73)
Intestinal colitis (IBD)	↑Runx3, Eomes, CD8α, GrB, IFN-γ, T-bet, CRTAM ↓ThPOK	Enriched in colonic inflammatory sites, induce inflammation via CTL activity and cytokine production	(22, 24, 25, 73)
Multiple sclerosis	↑Eomes, GrB, perforin, IFN-γ, IL-17, T-bet, NK receptors, CD8, CX3CR1 ↓CD28	Present in MS lesions and CSF, associate with neuroinflammation, kill oligodendrocytes, autoreactive and pro-inflammatory	(4, 21, 36, 43, 45, 52, 62)
Rheumatoid arthritis	↑NK receptors, IL-17, IFN-γ, CD8, CX3CR1 ↓CD28	Enriched at site of inflammation, autoreactive, cytotoxic and pro-inflammatory, correlate with severity and extra-articular manifestations	(39, 52, 62, 66, 67)
Autoimmune myopathies	↑IFN-γ, perforin, NK receptors ↓CD28	Pro-inflammatory, cytotoxic and tissue-infiltrating	(39, 62, 69, 70)
Cardiovascular diseases			
Acute coronary syndrome (Unstable Angina)	↑IFN-γ, perforin, GrB, NK receptors ↓CD28	Pro-inflammatory, lyse endothelial and vascular smooth muscle cells, autoreactive, correlate with recurrent events and poor outcome	(37, 39, 62)
Atherosclerosis	↑IFN-γ, perforin, GrB, NK receptors ↓CD28	Accumulate in rupture-prone regions, cytotoxic, augment apoptosis and necrosis	(37, 51)

EBV: Epstein-Barr virus, CMV: cytomegalovirus, HIV: human immunodeficiency virus, IBD: inflammatory bowel disease, GrB: granzyme B.

1.1.5 Conclusion

CD4+ CTLs are characterized by the expression of NK cell receptors and the production of IFN-γ, granzyme B and perforin. Most are effector memory T cells (CCR7-), although CD4+ CTLs are found in various states of differentiation, based on the expression or absence of CD45RA and CD28. Many other markers (e.g. IL-17 production, CD8α and CX3CR1 expression) have been reported, but further research is needed to include them in a common phenotype or use them to define CTL subsets.

The formation of cytotoxic CD4+ T cells is a result of the complex interplay between many different transcription factors and environmental factors. Based on our own preliminary data and on the literature reviewed here, we propose that effector T cells, rather than naive CD4+ T cells, differentiate towards cytotoxic T cells, because of the CD4+ CTL's advanced differentiation status as effector memory T cells that produce Th-related cytokines IFN-γ and IL-17. These effector T cells become cytotoxic under the right environmental circumstances, such as chronic antigenic stimulation, which is underscored by their oligoclonality and restricted TCR diversity. These extracellular cues may then alter their transcriptional program, influencing genes involved in the cytotoxic programming of CD4+ T cells, ultimately leading to the production of IFN-γ, granzyme B and perforin.

The role of CD4+ CTLs in the context of disease is dual. Protective effects have been noted in tumors and viral infections, where they exert virus- or tumor-specific cytotoxicity via their MHC II-restricted antigen recognition and NK cell receptors. Detrimental effects were found in cardiovascular and autoimmune diseases, CD4+ CTLs exacerbate inflammation and tissue destruction via the production of pro-inflammatory cytokines, their cytotoxic activity, tissue infiltrating capacity and recruitment of other harmful immune cells. Because of this dual role for CD4+ CTLs, it could be important to tailor therapy by boosting (infections and cancer) or inhibiting (autoimmunity and cardiovascular disease) them. Specific targeting of harmful CD4+ CTLs could include blocking of activation (e.g. blockade of co-stimulatory pathways), migration (e.g. blockade of CX3CR1), inflammatory cytokine production (e.g. statins), and cytotoxicity (e.g. cytotoxicity-blocking antibodies).

Future studies should indicate how cell type specific these approaches are, since other cell types could express common markers, and whether they are successful in different disease indications.

1.2 Aims of the study

The main goal of this thesis is to determine to what extent cytotoxic CD4+ T cells contribute to the pathogenesis of MS. We used CD28null as hallmark to define CD4+ CTLs. As a critical note, by using CD28 loss as a marker for these cells, we only focus on cytotoxic CD4+ T cells in the end stage of differentiation and thus exclude other relevant cytotoxic CD4+ T cells that still express CD28. We hypothesize that genetically predisposed patients, exhibiting CD4+CD28null T cell expansions, demonstrate a worse disease course compared to other patients. First, the involvement of cytomegalovirus (CMV) infection and chronic neuroinflammation in the formation and expansion of CD4+CD28null T cells is determined using *in vitro* and *in vivo* models. A possible genetic influence is also examined, via analysis of MS and CMV associated risk SNPs.
Next, the involvement of CMV infection and CD4+CD28null T cells in neuroinflammation and demyelination is investigated using mouse models of MS and CMV. To find further human evidence, clinical disease parameters are correlated with the presence of CD4+CD28null T cells. Lastly, *in vitro* experiments shed a light on the pro-inflammatory phenotype of CD4+CD28null T cells and their interaction with other MS relevant T cell subsets. Overall, this study aims to contribute to unravelling two key mechanisms that may exacerbate MS disease.

1.2.1 Aim 1: To determine the cause of the formation and expansion of CD4+CD28null T cells

CD4+CD28null T cells arise after chronic activation of the immune system. More precisely, repeated antigenic stimulation leads to the loss of CD28. Since MS is a chronic inflammatory disease, and CMV is a persistent virus, both actors can continuously challenge the immune system, making them valid candidates for the formation and expansion of CD4+CD28null T cells. In **chapter 3**, we investigate the CMV serostatus as well as immunoglobulin titers in donors with or without CD4+CD28null T cell expansions, to determine a link between CMV and CD4+CD28null T cells.

To further pinpoint CMV as an actual trigger for CD4+CD28null T cell expansions, CMV stimulation assays are performed both *in vitro* and *in vivo*. In an experimental autoimmune encephalomyelitis (EAE) model, we determine whether chronic neuroinflammation leads to the formation and expansion of CD4+CD28null T cells. Next to chronic antigenic challenge, perhaps genetic factors, such as single nucleotide polymorphisms (SNPs) predispose people to the expansion of CD4+CD28null T cells. In **chapter 4**, we focus on SNPs associated with CMV (*MICB*, *TLR2*) that have been reported to make people more susceptible to CMV infections and may thus enhance the likelihood for CD4+CD28null T cell expansions to occur. In addition, the MS associated SNP in *IL2RA* is investigated as this receptor is crucially involved in CD4+ T cell lineage homeostasis and could thus influence CD4+CD28null expansions.

1.2.2 Aim 2: To examine the role of CMV in MS pathology

The role of CMV in MS is controversial, different reports suggest detrimental but also beneficial effects. In **chapter 2**, we discuss current literature with regard to this controversy and propose mechanisms by which CMV could possibly contribute to MS. In **chapter 3**, an animal model combining EAE and CMV (MCMV) was set up to examine the role of CMV in EAE pathology. The amounts of disability, inflammation and demyelination are evaluated in these animals and an association with expanded CD4+CD28null T cells was pursued.

1.2.3 Aim 3: To analyze whether CD4+CD28null T cells exacerbate multiple sclerosis

Up to now, only circumstantial evidence (via *in vitro* experiments) links CD4+CD28null T cells with MS disease. In **chapter 3**, we aim to clarify the involvement of CD4+CD28null T cells in the pathogenesis of MS by using an EAE model. Disease pathology and course are evaluated in these animals as well as the relative expansion of CD4+CD28null T cells. In **chapter 5**, clinical parameters (disability scores, number of relapses, …) of MS patients are used to determine if expanded CD4+CD28null T cells contribute to a worse disease burden and clinical progression.

1.2.4 Aim 4: To elucidate by which mechanisms CD4+CD28null T cells contribute to the pathogenesis of multiple sclerosis

From previous research, we gathered that CD4+CD28null T cells are pro-inflammatory, autoreactive, cytotoxic and can migrate towards MS lesions. This indicates that they can directly contribute to inflammation and damage in the CNS. In **chapter 6**, the pro-inflammatory phenotype of CD4+CD28null T cells is further unraveled. And *in vitro* assays are set up to identify indirect actions of CD4+CD28null T cells via interactions with Tregs and Th17 cells, both T cell subsets implicated in MS pathology.

2

CYTOMEGALOVIRUS: A CULPRIT OR PROTECTOR IN MULTIPLE SCLEROSIS?

This chapter is based on:
Vanheusden M, Stinissen P, 't Hart BA, Hellings N.
Cytomegalovirus: a culprit or protector in multiple sclerosis?
Trends Mol Med. 2015;21(1):16-23.

2.1 Abstract

Multiple sclerosis (MS) is a chronic disabling autoimmune disease of the central nervous system. Cytomegalovirus (CMV), a β-herpes virus, may have a detrimental or beneficial role in MS pathology. Accumulating evidence indicates that CMV contributes to MS disease via interplay of different mechanisms such as molecular mimicry, bystander activation and epitope spreading. The activation and expansion of a specific T cell subset, CD4+CD28null T cells, via CMV infection could also contribute to MS pathology. Various additional observations also indicate a protective effect of CMV on autoimmune diseases. CMV immune evasion may mitigate the autoimmune reactions and pro-inflammatory milieu contributing to MS.

2.2 Introduction

2.2.1 Hurdles in CMV and MS research

In this chapter, we focus on cytomegalovirus (CMV), a member of the β-herpes family that establishes lifelong latent infections in ≥70% of the human population (74). CMV infection was considered 'innocent' in immunocompetent persons, but evidence is now emerging about the large impact of CMV infection on the aging immune system. In addition, the possible involvement of CMV in a wide range of diseases is being recognized, including in autoimmune diseases such as multiple sclerosis (MS).

MS is a chronic disabling autoimmune disease of the central nervous system (CNS; Box 2.1). Autoreactive immune cells attack the CNS myelin, leading to demyelination, axonal injury and ultimately neural cell loss. A wide range of symptoms can occur, including fatigue, muscle weakness and visual difficulties. MS is often preceded by clinically isolated syndrome (CIS), where patients experience a first episode of neurologic symptoms, such as optic neuritis, without a second event. Another disease, namely acute disseminated encephalomyelitis (ADEM) is clinically and pathologically similar to MS, and often manifests after an infection (75).

The role of CMV in MS disease is disputed. Our own research, together with that of others, supports a detrimental role of CMV, where the virus contributes to MS pathology, whereas others believe that CMV is disease limiting. Here we discuss viral mechanisms that are suggestive for promotion of autoimmunity and we summarize evidence arguing in favor of and against CMV involvement in MS etiology and progression.

Box 2.1: Disease course and types of MS

Patients often present themselves to the clinic with a first episode of neurologic symptoms where they are diagnosed with clinically isolated syndrome (CIS) until a second event occurs. After this second event, the McDonald criteria are fulfilled and the diagnosis is changed to clinically definite MS. With this second event we imply either a second clinical attack or secondary lesions that are disseminated in time and space, established via MRI. The majority of MS patients (85%) develop a relapsing-remitting (RRMS) disease with a duration ranging from several years to decades. In most patients, the episodes of recovery (remissions) gradually become less frequent and finally disappear completely, whilst their symptoms become more pronounced and their disability worsens. At this stage the disease converts to the secondary progressive (SP) phase.
In a minority of patients (10%), those with primary progressive MS (PPMS), the disease is progressive from onset.
A relatively rare (5%) form of MS, progressive relapsing MS, consists of steadily worsening of the disease, yet also comprises relapses. In some cases, there is no recovery, although in other cases there is. Thus the periods between relapses involve continuing progression of the disease instead of remission as in relapsing-remitting MS.
The lesions in relapsing-remitting MS are usually located in the white matter around ventricles and blood vessels and are characterized by sharply-edged focal areas of inflammation with a variable degree of demyelination, remyelination and axonal injury. Lesions in progressive MS are also found in the grey matter and are characterized by intensive demyelination with little inflammation but pronounced degeneration of oligodendrocytes and neurons.

2.2.2 Possible mechanisms of viral contribution to autoimmune disease

There are a number of different mechanisms by which viruses such as CMV could drive autoreactive T cell activation and thus lead to autoimmune disease (Figure 2.1).

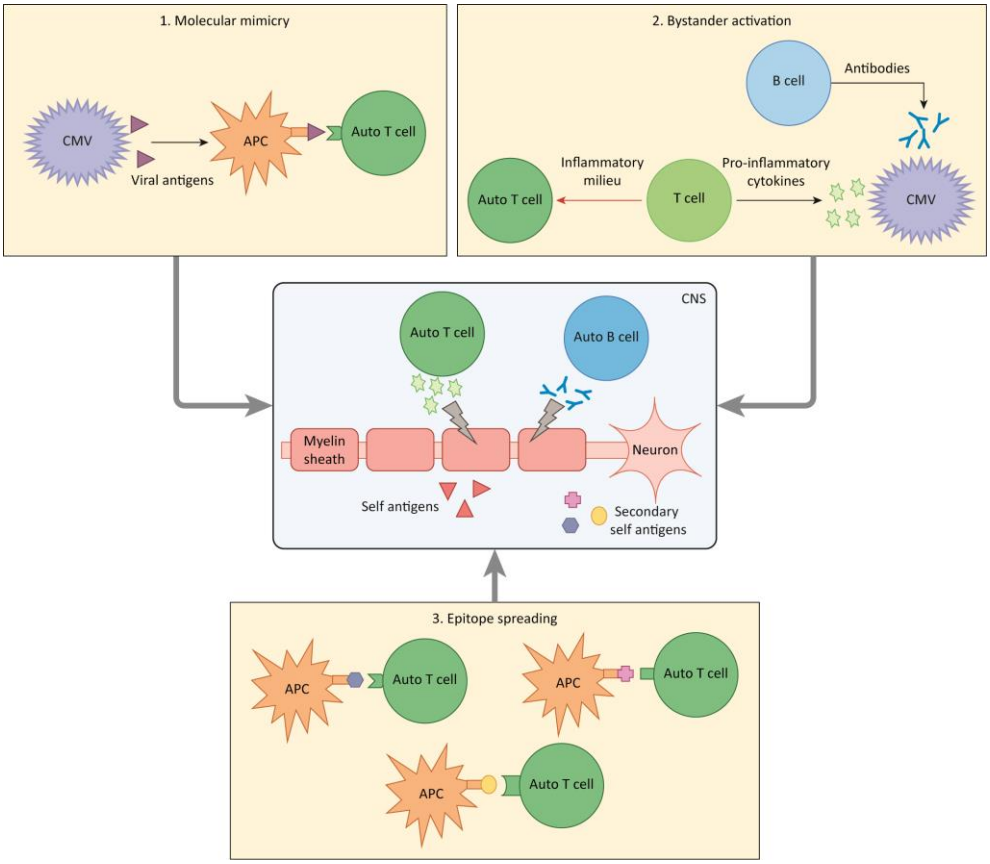


Figure 2.1: Hypothetical mechanisms by which CMV could contribute to MS disease. 1) Molecular mimicry: APC's present CMV-derived antigens, which resemble self-epitopes (MOG), to autoreactive T cells. Activated autoreactive T cells damage self-tissue (myelin) by the release of cytokines and toxic mediators, leading to release of secondary autoantigenic epitopes. 2) Bystander activation: CMV-specific T cells and B cells are activated to combat CMV; their response leads to strong inflammation and thus triggers the non-specific activation of autoreactive T cells. 3) Epitope spreading: persistence of CMV in the periphery causes damage to CNS myelin via the processes mentioned above (1+2). This results in the release of multiple self-antigens, activating an expanding repertoire of autoreactive T cells with different TCR specificities. All these processes combined (1+2+3) perpetuate MS disease.

Two hypotheses rely on the fact that potentially autoreactive T cells are already present in low numbers in each individual. These cells seem to escape negative selection in the thymus during normal T cell development (76, 77).

One hypothesis suggests the direct triggering of autoreactive T cells by infectious pathogens which express antigenic epitopes that structurally resemble epitopes of self-antigens (78). A well-known example of this molecular mimicry is the T cell cross-reaction between the MS-related autoantigen myelin basic protein and Epstein Barr Virus (EBV) (79). A second hypothesis, proposed by 't Hart *et al.*, suggests a variation on the molecular mimicry paradigm, namely a 'delayed molecular mimicry' model where latent chronic infections create a repertoire of long-living virus-specific memory T cells. These cells can be reactivated at any moment in time when they encounter molecular mimicry motifs present in self-antigens that are shed from injured tissues (80). Another hypothesis entails bystander activation, which comprises a variety of antigen-nonspecific theories. First, cytokines produced by virus-specific immune cells could lead to the accidental activation of autoreactive T cells. Second, host cell destruction by viral infection leads to the release of cryptic epitopes, including self-antigens that normally are not accessible to the immune system. Finally, a mitogen or superantigen, released from the infectious pathogen, could lead to polyclonal lymphocyte activation (76, 81). Thus, the inflammatory setting of a viral infection could elicit the activation and clonal expansion of autoreactive T cells resulting in autoimmune disease (82). McCoy *et al.* suggest a combination of both aforementioned hypotheses: viral epitopes that cross-react with self-antigens (molecular mimicry) prime genetically susceptible individuals. After this priming a non-specific immunologic challenge, leading to cytokine production (bystander effect), could provoke autoimmunity (83).

Another process closely linked to molecular mimicry and bystander activation is epitope spreading. After the initial reaction to a pathogen, antigens released from "primary lesions" in the target tissue will prime an expanding range of potentially autoreactive T cells due to T cell receptor (TCR) diversity (84, 85).
This cascade of self-recognition events provides a continuous inflammatory state that leads to chronic autoimmunity (86). Delogu *et al.* suggest that the three processes are linked, thus adding epitope spreading to the McCoy *et al.* hypothesis. Molecular mimicry would occur early in the development of autoimmunity, whereas bystander activation and epitope spreading occur later on, exacerbating the autoimmune responses (82).

The hypotheses of 't Hart *et al.* and McCoy *et al.* also comply with the so-called fertile field concept described by Fujinami *et al.* (87). The fertile field concept states that exposure to a potential immunogen is normally without consequence, but that under certain circumstances (e.g. viral infection) the immunological environment changes, leading to a dysregulated immune reaction. Thus the viral infection would create a fertile field in which immune responses to antigens could develop. Primed autoreactive T cells (by viral infections) also create a fertile field, since later events might trigger the expansion and activation of these cells leading to autoimmune disease.

2.3 Evidence in favor for the involvement of CMV in MS disease

In the etiology of MS, the interaction of environmental and genetic factors is thought to play a dominant role. It is envisaged that certain environmental factors (e.g. viruses) are potential triggers of the disease, while others (e.g. vitamin D or smoking) may also influence the disease course. There are several observations supporting a viral trigger for MS or ADEM. Many viruses are associated with encephalomyelitis, axonal damage and other demyelinating processes (88, 89).

2.3.1 Animal models

Most animal models used in translational MS research are based on inbred/SPF (specific pathogen free) laboratory strains of mice and rats. A minority of the research is based on non-human primates, man's closest kin in nature.

Rodents. In several mouse models, viral infection elicits an MS-like disease. Examples include Theiler's murine encephalomyelitis virus (TMEV), mouse hepatitis virus (MHV), Semliki Forest Virus (SFV) and canine distemper virus (CDV; Box 2.2). These models provide compelling evidence for a possible viral cause, or at least as part of the multifactorial and complex etiology of MS (80, 90).

Box 2.2: Viral animal models of MS

Many studies have examined the interaction between viruses and immune-mediated CNS disease *in vivo* by inoculating mice with different viral strains.

Theiler's murine encephalomyelitis virus (TMEV)
TMEV, a single-stranded virus of the Picornaviridae family, is a natural enteric mouse pathogen that can replicate and persist within the CNS. Intracerebral injection of mice causes paralysis, encephalomyelitis and demyelination, which is similar in pathology to MS (91, 92). Some strains cause a biphasic illness, starting with acute encephalomyelitis after which a relapse can occur (93). The chronic phase leads to slowly progressive disability and consists of demyelination, remyelination, inflammation and axonal damage (94).

Mouse hepatitis virus (MHV)
MHV is a natural mouse pathogen that infects all cell types within the CNS. Intracerebral or intranasal inoculation mounts a robust immune response against CNS myelin resulting in an influx of immune cells that largely clear the virus, although a low level viral infection persists. Infected mice develop a major symptomatic episode with inflammation and demyelination of brain and spinal cord leading to ataxia, hind limb paresis and paralysis (91, 94). Relapses could follow and lesion repair and remyelination may occur (91, 93).

Semliki forest virus (SFV)
SFV is a neurotropic alphavirus or togavirus that induces a demyelinating encephalomyelitis that is associated with the induction of T and B cell autoimmunity against CNS myelin. SFV is inoculated in the periphery (intraperitoneally), but can cross the blood-brain-barrier (BBB), infecting neurons and oligodendrocytes and inducing myelin damage (92). In mice, the virus is largely cleared from the CNS by 6 days post-infection, after which remyelination can take place (91).

Neurotropic strains of the canine distemper virus (CDV)
CDV is a single-stranded RNA morbillivirus, which can cause demyelinating leukoencephalitis in dogs and other carnivores. Infection in dogs occurs mainly via the oro-nasal route, after which infected immune cells could cross the BBB and cause CNS damage (95). Mice used in experiments are infected by intracranial injection. The virus persists and leads to multifocal, inflammatory demyelinating lesions similar to MS plaques (94).

Sindbis Virus (SV)
SV is a togavirus or alphavirus. Mice infected intracerebrally with a neuroadapted strain develop acute encephalomyelitis, experimental autoimmune encephalomyelitis- (EAE) like paralysis and demyelination (91, 96).

One of the most convincing mechanisms via which CMV could play a role in MS is molecular mimicry. Cross-reactivity between $hCMV_{981-1003}$ and myelin oligodendrocyte glycoprotein (MOG) residues 35-55 (MOG_{35-55}) in Lewis rats was found (97). Furthermore, sensitization of the rats against MOG_{35-55} triggered $CMV_{981-1003}$ specific lymphocytes, leading to clonal expansion and migration towards the spleen. This study provides further evidence of the "delayed molecular mimicry" theory. In another animal model, SJL/J mice were primed with vaccinia virus encoding proteolipid protein and subsequently challenged with murine CMV (MCMV) (87). These mice developed white matter lesions and had impaired righting reflex responses.
This experiment illustrates the fertile field concept, where the priming infection sets up the field by increasing the autoreactive T cell number, but still below the critical threshold, after which secondary infection leads to overt disease by bystander activation or heterologous immunity due to the activation and proliferation of the previously expanded autoreactive T cells.
This model indicates that one viral infection can prime for autoimmunity early in life while clinical disease is triggered by another infection later in life.

Non-human primates. A fundamental difference between SPF laboratory rodents and non-human primates is that the latter are exposed throughout their life to environmental pathogens that shape the immune repertoire. A cross-reactive epitope shared between human CMV ($UL86_{981-1003}$) and MOG_{34-56} was found (98). T cells specific for UL86 cross-reacted with MOG_{34-56}, eliciting mild inflammatory experimental autoimmune encephalomyelitis (EAE) in rhesus monkeys. This led to the hypothesis that T cells arising during primary infection may display autoreactive potential when they are reactivated by myelin antigen. This T cell repertoire may expand in response to CMV reactivation, and could be hyper responsive against MOG, thereby exacerbating autoimmunity. The pathogenic relevance of this repertoire in MS is illustrated by the observation that immunization of marmoset monkeys with MOG_{34-56} in incomplete Freund's adjuvant (IFA), activating only antigen-experienced effector memory T cells, triggers progressive MS-like disease (99).

2.3.2 Human studies

CMV causes a chronic latent infection that can be reactivated under conditions of inflammation or reduced immunity due to stress, medication or pregnancy. This pattern of latency and reactivation resembles, and may therefore underlie, the relapsing remitting form of MS (88).

MS relapses are often associated with common viral infections (100). Furthermore, long-acting viruses, such as CMV, could encourage modest immunological changes, eventually resulting in autoimmune demyelination (101).

The most prevalent exposure routes to CMV are sexual contact or contact with urine or saliva from infected persons. CMV mainly targets cells of the myeloid lineage (e.g. macrophages, dendritic cells) and replicates in the salivary glands and in brain tissue (88). The virus can cross the blood-brain-barrier (BBB) directly or indirectly as a viral load in cells (101). As CMV is present in the CNS, it could damage local cells and tissues (88). Indeed, CMV has been found in demyelinating plaques and the cerebrospinal fluid (CSF) of MS patients (89, 101), and causes demyelination in the CNS of mainly immunocompromised hosts (80, 90). Moreover, enrichment of EBV and CMV specific CD8+ T cell among T cells isolated from chronic inflammatory lesions of MS brain was reported (102).

Epidemiological studies: Epidemiological data supporting a role of CMV in MS pathology are scarce. It was shown that CMV DNA and CMV-specific IgG antibody titers are higher in MS patients compared to healthy controls, indicating a role of CMV in MS (100). Other researchers investigated a cohort of CIS patients, and found that CMV seropositivity was associated with reduced time to relapse and a higher number of relapses. CMV seropositivity was further associated with progression to clinically definite MS (103, 104). Another study performed on the same cohort of CIS patients found that Anti-CMV positivity was associated with greater total deep gray matter atrophy and whole brain atrophy (105). Newly diagnosed MS patients were examined via serological testing for a panel of viruses including CMV (101). They found that early CMV infection occurred in 86% of the MS patients, indicating that early CMV infections may affect the development of MS.

CMV infection and disease exacerbations. Many viral infections are associated with exacerbations of MS (83). Disease exacerbations in relapse remitting MS (RRMS) patients happen two to three times more frequently during or immediately after a viral infection (106, 107).

CMV infection has also been implicated in worsening of autoimmune diseases or in the progression of ADEM to clinically definite MS (89). CMV infection leads to cell death and could therefore enhance autoimmunity due to the release of self-antigen from degenerating tissue (81). Reactivation of CMV during ongoing MS could trigger the release of free virus in interstitial and cerebral fluids of the CNS. It is possible that autoreactive T cells (molecular mimicry) will migrate towards this region and induce demyelination. Our own research suggests that CMV exacerbation may indirectly lead to disease aggravation by the induction or expansion of cytotoxic CD4+CD28null T cells via repeated antigenic stimulation (108). These cells have an inflammation-seeking/pro-inflammatory effector-memory phenotype and are attracted to MS lesions via a fractalkine gradient (36, 109). Furthermore, at least a subpopulation of CD4+CD28null T cells proliferate and expel cytotoxic granules upon stimulation with MS-related autoantigens and may therefore contribute to MS pathology (62, 110). The fact that CD4+CD28null T cells are mostly found in CMV seropositive persons (31) hints at the involvement of CMV in the expansion of this oligoclonal subpopulation. Indeed, primary infection with CMV induces brisk expansion of CD4+CD28null T cells, but their appearance in the peripheral blood occurs only after cessation of viral replication (111).

2.4 Evidence contra CMV involvement in MS

Other studies are indicative for a protective effect of CMV on autoimmune diseases. CMV infection is associated with anti-inflammatory activities, which could lead to a less severe course of the disease (81). Furthermore, CMV encodes multiple factors that trigger immunomodulatory or evasion mechanisms (Box 2.3), which can decrease the immune response in MS patients (100, 112).

Box 2.3: Immune evasion mechanisms of CMV

Certain viruses have devised ways to evade the host immune system, allowing them to replicate, disseminate and persist throughout life, occasionally reactivating but mostly being latently present. CMV commits a large portion of its genome to modulating recognition by the immune system. It encodes multiple immune evasion proteins during the course of infection that target different arms of the immune system (113, 114).

Adaptive immunity
Expression of major histocompatibility complex (MHC) class I or II molecules on the surface of CMV-infected cells is downregulated, reducing antigen presentation (114). MHC class I molecules are degraded or retained in the endoplasmic reticulum by various viral proteins (74, 113). MHC class II molecules are also degraded and their upregulation is disrupted by CMV specific genes (113). Additionally, a viral homologue of the immunomodulatory cytokine interleukin 10 (IL-10), which is expressed during latency, has been shown to downregulate expression of MHC class I and II molecules, inhibit proliferation of peripheral blood mononuclear cells and inhibit the production of inflammatory cytokines (113).

Innate immunity
The lack of surface MHC class I molecules makes the infected cell prone to lysis by natural killer (NK) cells. However, CMV can evade NK cell lysis by expressing MHC class I homologs, or by upregulating expression of the host HLA-E, which bind an inhibitory NK receptor. Thus infected cells escape detection and subsequent lysis by NK cells. Furthermore, CMV glycoproteins can directly bind and sequester NK-activating ligands, minimizing their surface expression and preventing activating NK receptor signaling (113, 114). Additionally, selective, allele-specific degradation of MHC class I leads to protection against both CTL-mediated and NK-mediated cell lysis (114). Another strategy of CMV immune evasion may be to restrict the differentiation of CD34+ progenitor cells to dendritic cells (DCs), which are potent antigen presenting cells (APCs) (74, 114).

2.4.1 Animal models

In the TMEV mouse model of MS, researchers showed that MCMV infection two weeks before TMEV infection leads to a better disease outcome compared to TMEV alone. These mice showed better motor performance, and less T cell infiltration in the brain, suggesting an immunomodulatory effect of MCMV infection in the TMEV model (112). It should be noted that only the brain and not spinal cord tissue was examined, and TMEV is present in both compartments, which could have biased the results.

2.4.2 Human studies

An association between CMV-specific antibody titers and a better clinical outcome in a cohort of 140 MS patients was observed. CMV positive MS patients had a higher age at disease onset, and displayed less brain atrophy as measured by magnetic resonance imaging (MRI) (115). A case-control study comparing Swedish MS patients and healthy controls with reported data via meta-analysis indicated that CMV positivity reduces the risk of developing MS (116). It should be noted that this effect was only seen in the meta-analysis of the retrospective studies, and that only two out of 11 studies were significantly associated with MS, rendering the overall statistical value insignificant. The disadvantage of such retrospective studies is the time interval between onset of symptoms and collection of serum sample for virus analysis. In the Swedish study the mean time interval was 5 years, during which time patients could have seroconverted. This can obviously influence the results.

The possible involvement of CMV in pediatric MS in a cohort of early pediatric MS patients and age-matched control subjects was analyzed. They observed that CMV infection acquired during childhood reduced the risk of developing MS or CIS in subjects up to 18 years of age. However, the control group comprised, next to healthy individuals, ADEM patients and patients with other neurological conditions, which could have biased their findings (117).

2.5 The relationship between aging and CMV, and transition to progressive MS.

The biological factor that is most strongly associated with progressive MS is age. This notion has led to the hypothesis that age-associated changes in the immune system, a phenomenon known as immunosenescence, may underlie the conversion of relapsing-remitting to progressive disease (118). Of particular relevance are herpes viruses causing lifelong chronic latent infection, such as CMV and EBV. Observations in the aging immune system illustrate the substantial impact that these viruses, in particular CMV, have on the immune repertoire (119-121). For current knowledge on how CMV impacts immune aging see (122). As a conceptual basis, we propose the existence of two distinct compartments in the immune repertoire: an outward-directed compartment that deals with pathogens outside the body; and an inward-directed compartment that deals with pathogens inside the body. Studies in the extremely old (>90 yrs.), show decreased immunocompetence of the outward compartment, impairing the response to vaccination, while the inward compartment displays oligoclonal expansion of highly reactive T cell specificities (Figure 2.2) (123). This oligoclonal expansion combined with a decrease in age-dependent thymic output results in a dysregulation of the total T cell compartment, and thus may underlie the age-associated development of chronic inflammation in autoimmune disorders, such as MS and rheumatoid arthritis (RA) (118).

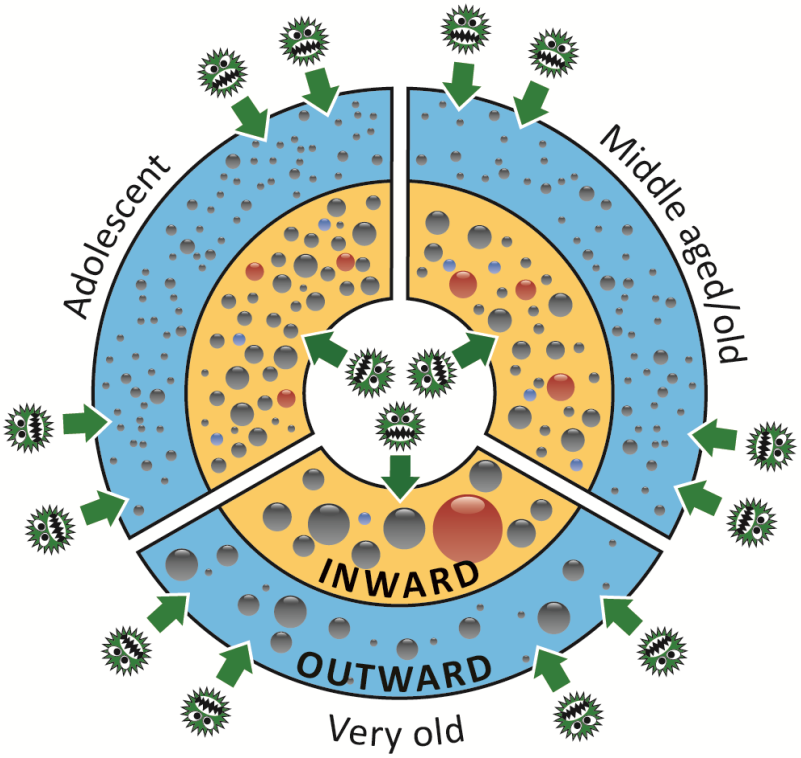


Figure 2.2: Changes in two distinct immune repertoire compartments throughout life. The pathogen-educated immune system deals with pathogens in the environment and those causing chronic latent infections (indicated by:). Studies in aging individuals show that distinct immune compartments may be involved in the defense against these outward and inward threats. Inflation of the outward compartment (blue ring) impairs the capacity of elderly to respond against vaccination. By contrast, the inward compartment (yellow ring) is characterized by oligoclonal expansion of immune specificities against chronic latent infections. The differently sized balls represent clones of different specificity. Based on studies in the non-human primate we hypothesize that T cells driving chronic inflammation (red color) are recruited from this inward compartment (123).

The main cause of progressive MS is thought to be the accumulating degeneration of neurons and oligodendrocytes, which may not have an immunological cause. Indeed, in contrast to lesions in the white matter, T cells and antibody deposition are rare in grey matter lesions. However, activation of microglia and the presence of immune cell infiltrates in adjacent meninges is prominent (124).

The presence of T cell and macrophage infiltrates is only evident in early but not chronic grey matter lesions (125), suggesting a role of the immune system during a restricted episode in progressive disease.

A new EAE model in marmosets has now been developed, which is induced by immunization with MOG_{34-56} in IFA (99). The model shares essential pathological characteristics with progressive MS that are not present in relapsing-remitting disease (126, 127), including prominent demyelination of the cortical grey matter (128, 129) and the expression of markers for mitochondrial dysfunction (own unpublished observations). Unraveling the immunopathogenic process that gives rise to MS-like pathology and disease in this model revealed a central role of CD3+CD56+CD28- cytotoxic T cells, which specifically recognize a mimicry motif shared between MOG and UL86 (CMV peptide) (98, 128). A similar type of T cell is present in the human anti-CMV repertoire (130) and in MS lesions (45). CTLs (cytotoxic T lymphocytes) depend on presentation of their specific antigen from B cells infected with lymphocryptovirus (LCV), a subgroup of primate-specific γ-herpes viruses of which EBV is the human representative. Thus, development of the primate-specific autoimmune pathway requires the involvement of at least two herpes viruses, both of which have been implicated in MS-like disease (118). Conceptually, chronic latent infection with CMV creates a progressively expanding repertoire of CTL, which display their encephalitogenic potency when they encounter MOG released by primary demyelination of CNS lesions. LCV infection presumably renders B cells capable of activating these strongly pathogenic T cells (131).

2.6 Concluding remarks

The role of CMV in MS pathology remains controversial, and may be detrimental or beneficial. A recent meta-analysis on 1341 MS patients and 2042 healthy controls did not yield a conclusive result on the effect of CMV infection and the occurrence of MS, since no significantly enhanced risk (expressed as an odds ratio) was found (132). Further studies are warranted to define a role of CMV as either culprit or protector. Future studies should take into account the different forms and stages of MS-like diseases, as well as the age of onset. The marmoset model suggests that CMV may have a stronger impact during late stage disease than in the early phase. Furthermore, the epidemiologic studies should be interpreted with care, since they have some limitations. Due to (possibly rapid) changes in the health and environmental status of some subjects (e.g. viral infections), the conclusions derived from previous analyses may not reflect the actual status (e.g. viral titers or status). Furthermore, case-control studies may prove an association, but they do not demonstrate causation. Retrospective studies should be examined critically since they could be influenced by confounding factors and bias due to missing or unknown information.

We strongly believe that CMV plays a role in MS pathology and disease progression, however CMV infection or reactivation by itself is not sufficient to induce MS disease. The combined effects of CMV infection with genetic and immune triggers should be investigated in further detail. It has already been shown that the synergy of viral (e.g. Herpes simplex virus 1) and immune triggers can elicit MS-like pathology, while the virus alone had no effect (133). Furthermore, EAE induction combined with viral infection accelerated or exacerbated disease, due to enhanced immune cell infiltration and polarization of the adaptive immune response (106, 134, 135).

Next to these triggers, another virus implicated in MS disease, such as EBV, could also play an added role. In marmoset EAE, EBV and CMV are both necessary for the development of the primate-specific autoimmune pathway, as already discussed. In humans, seropositivity for CMV and EBV is associated with significantly increased whole brain atrophy (103).

Since we reason that CMV plays a role in MS disease, we propose that vaccine or antiviral drug strategies to manage CMV responses should be taken into consideration as part of managing MS patients. These benefits may extend beyond MS, as CMV infection is also implicated in arteriosclerosis, vascular disease and immune aging (122, 136, 137).

Box 2.4: Outstanding questions

- Which exact mechanisms underlie the role of viruses in autoimmune diseases? How do viruses contribute to the development or exacerbation of autoimmune diseases?
- Is CMV involved in MS disease? If so, does it have a positive or negative influence on MS disease?
- Which *in vivo* model would be the best to study the role of CMV in MS?

3

CYTOMEGALOVIRUS INFECTION EXACERBATES AUTOIMMUNE MEDIATED NEUROINFLAMMATION

This chapter is based on:
Vanheusden M, Broux B, Welten S, Peeters L, Panagioti E, Van Wijmeersch B, Somers V, Stinissen P, Arens R, Hellings N
Cytomegalovirus infection exacerbates autoimmune mediated neuroinflammation
Scientific Reports 2017; 7(1):663

3.1 Abstract

Cytomegalovirus (CMV) is a latent virus which causes chronic activation of the immune system. Here, we demonstrate that cytotoxic and pro-inflammatory CD4+CD28null T cells are only present in CMV seropositive donors and that CMV-specific immunoglobulin (Ig) G titers correlate with the percentage of these cells. *In vitro* stimulation of peripheral blood mononuclear cells with CMVpp65 peptide resulted in the expansion of pre-existing CD4+CD28null T cells. *In vivo*, we observed de novo formation, as well as expansion of CD4+CD28null T cells in two different chronic inflammation models, namely the murine CMV (MCMV) model and the experimental autoimmune encephalomyelitis (EAE) model for multiple sclerosis (MS). In EAE, the percentage of peripheral CD4+CD28null T cells correlated with disease severity. Pre-exposure to MCMV further aggravated EAE symptoms, which was paralleled by peripheral expansion of CD4+CD28null T cells, increased splenocyte MOG reactivity and higher levels of spinal cord demyelination. Cytotoxic CD4+ T cells were identified in demyelinated spinal cord regions, suggesting that peripherally expanded CD4+CD28null T cells migrate towards the central nervous system to inflict damage.

Taken together, we demonstrate that CMV drives the expansion of CD4+CD28null T cells, thereby boosting the activation of disease-specific CD4+ T cells and aggravating autoimmune-mediated inflammation and demyelination.

3.2 Introduction

Multiple sclerosis (MS) is a disabling autoimmune disease of the central nervous system (CNS). Activated autoreactive immune cells infiltrate the brain and spinal cord leading to chronic inflammation, demyelination and ultimately axonal loss (65). Although the exact trigger for this activation has not been elucidated yet, a genetic predisposition in combination with environmental factors seems essential to develop MS (138). Worldwide, about 2.5 million people are affected, mostly young adults (20-40y) and females (3:1 ratio), although the disease progression in men can be more severe (139).

Naive T cells express CD28 on their cell surface, but due to repeated antigenic stimulation CD28 expression can be lost (140-142). CD4+CD28null memory T cells arise during chronic activation of the immune system, in a subset of healthy controls (HC) and patients with MS. These cells have a restricted T cell receptor (TCR) diversity (oligoclonal), are costimulation independent, more resistant to apoptosis, and less susceptible to suppression by regulatory T cells (Tregs) (31, 35, 37, 59, 110, 143). Relevant features suggesting their contribution to autoimmune-mediated CNS damage in MS include their autoreactive nature; their target tissue infiltration, via e.g. the fractalkine gradient; and their cytotoxic capacities, namely the expression of natural killer (NK) cell receptors and the production of perforin and granzymes (36, 110, 144).

So far, the trigger for the selective expansion of CD4+CD28null T cells and their contribution to MS disease pathology is poorly investigated. There is mounting evidence that CD4+CD28null T cell expansion occurs after infection with cytomegalovirus (CMV) (32, 35, 62, 111). CMV is a member of the β-herpesvirus family that establishes lifelong latent infections in ≥70% of the human population (74).

CMV commits a large portion of its genome to evade recognition and activation of the immune system: e.g. reduction of antigen presentation by interfering with the expression of MHC/HLA molecules, downmodulation of costimulatory molecules, and evasion of NK cell control (32, 113, 114, 145). However, as a result of cross-priming of CMV antigens, CMV-specific T cell responses develop.

Moreover, due to the persistent nature of CMV, substantial accumulation of CMV-specific memory T cells (on average 10% of the total memory T cell compartment) can occur (74, 120, 146, 147), albeit with varying degrees, which may be caused by differences in infectious dose (148). As a consequence of this large percentage of CMV-specific T cells, immune surveillance could become less effective over time, thereby compromising normal immunity (74, 149). Indeed, CMV seropositivity has been correlated with a worse MS disease course, although disease limiting effects have also been stated (Reviewed in chapter 2 (32)). The most important finding indicating a disease promoting role is the enrichment of CMV-specific antibodies in MS (100). When these antibodies were present in MS patients, this was correlated to a decreased time to relapse, an increase in the number of relapses and enhanced brain atrophy (103-105). In contrast, another study concluded that the presence of CMV-specific antibodies was associated with a better clinical outcome, an increased age of disease onset and decreased brain atrophy (115). A recent meta-analysis on 1341 MS patients and 2042 healthy controls did not yield a conclusive result on the relationship between CMV infection and the occurrence of MS (132).

In this study we investigated whether CMV by itself is able to trigger the expansion of CD4+CD28null T cells and aggravate MS disease, using a combination of human data and *in vivo* animal model systems.

3.3 Materials and methods

3.3.1 Study subjects

Human. Peripheral blood samples (Li-Heparin coated tubes) were collected from 63 healthy controls (HC) and 227 MS patients in collaboration with the University Biobank Limburg (UBiLim). CMV and Epstein-Barr virus (EBV) status and titers (CMV IgG and EBV EBNA IgG) were determined in serum samples via Vidas ELFA (bioMérieux, Marcy l'Etoile, France) and Architect immunoassay (Abbott, Illinois, USA). Clinical data are presented in table 3.1; there were no significant differences between CMV positive or negative donors, neither in MS patients nor in HC.

Mice. Female C57BL/6 mice were purchased from Harlan (Horst, the Netherlands). CD80/86$^{-/-}$ mice (150) were bred in LUMC to the C57BL/6 background.

Table 3.1: Study subjects for CD4+CD28null T cell analysis.

	MS patients		Healthy controls	
	CMV+	**CMV-**	**CMV+**	**CMV-**
Number	100	127	24	39
Age (y)	47 ± 13	44 ± 14	32 ± 9	32 ± 10
Male/Female (ratio)	26/74 (0.35)	35/92 (0.38)	7/17 (0.41)	14/25 (0.56)
EBV serostatus (- / border / +)	0/2/64	2/0/87	NA	
Disease duration range	1 mo - 40 y	0 mo – 37 y	NA	
EDSS range	0-7	0-7.5	NA	
Disease type			NA	
CIS	5	6		
RR-MS	63	78		
CP-MS	32	43		
Treatment[#]			NA	
No treatment	46	56		
IFNβ	25	47		
Glatiramer acetate	15	10		
Natalizumab	9	6		
Alemtuzumab	2	4		
Teriflunomide	/	3		
Dimethyl fumarate	2	/		
Methotrexate	1	1		

[#]within 3 months before blood collection. Alemtuzumab-treated patients just started treatment or received readminisration.MS, multiple sclerosis; EDSS, expanded disability status scale; CIS, clinically isolated syndrome; RR, relapsing remitting; CP, chronic progressive (=primary and secondary progressive MS); IFNβ, interferon beta; CMV, cytomegalovirus; NA, not applicable.

3.3.2 EAE induction

10 weeks old C57BL/6J mice were immunized subcutaneously with myelin oligodendrocyte glycoprotein 35–55 peptide ($MOG_{35–55}$) emulsified in complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis according to manufacturer's guidelines (Hooke Laboratories, Lawrence, USA).

Directly after immunization and 24 h later, mice were intraperitoneally injected with pertussis toxin. Mice were weighed and evaluated daily for neurological signs of disease using a standard 5-point scale; 0: no symptoms; 1: limp tail; 2: hind limp weakness; 3: complete hind limp paralysis; 4: complete hind limp paralysis and partial front leg paralysis; 5: moribund.

3.3.3 MCMV infection

MCMV-Smith was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and stocks were prepared from the salivary glands of infected BALB/c mice. C57BL/6J WT and CD80/86$^{-/-}$ mice were infected i.p. with 5×10^4 PFU. All mice were maintained under specific pathogen free conditions.

3.3.4 Flow cytometry

Human. All donors included in this study were analyzed for the percentage of CD4+CD28null T cells. This was done by isolating peripheral blood mononuclear cells (PBMCs) from whole blood by density gradient centrifugation (Cedarlane lympholyte, Sheffield, UK). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ). The gating strategy consists of a lymphocyte gate using the forward and side scatter signal, after which CD4$^+$ cells were gated and subsequently CD28 expression was monitored within this gate (Figure 3.1A). Cells were acquired using a FACSAria II cytometer, and data were analyzed using BD FACSDiva software. Significant expansion of CD4+CD28null T cells was arbitrary defined as a percentage ≥2% of the total CD4+ T cell population, as this was the minimal percentage of cells that allowed discrimination of a distinctive population (36).

Mice. Single cell suspensions were prepared from spleens by mincing the tissue through a 70-µm cell strainer (BD Bioscience). Erythrocytes were lysed in a hypotonic ammonium chloride buffer. The gating strategy consists of a lymphocyte gate using the forward and side scatter signal, after which CD3+CD4+ cells were gated and subsequently CD28 expression was monitored within this gate (Figure 3.1B). Surface and intracellular staining were used to identify and characterize CD4+CD28null T cells. MOG-specific CD4+ T cell responses were determined after *in vitro* stimulation with mouse MOG_{35-55} (10µg/ml, Hooke laboratories) peptides for 8 hours (6 hours in the presence of Brefeldin A).

Fluorochrome-conjugated antibodies specific for anti-mouse CD3, CD4, CD27, CD28, CD62L, CD127, IFN-γ and granzyme B were purchased from BD Biosciences, Biolegend or eBioscience. Cells were acquired using a BD LSR II flow or FACSAria II cytometer, and data were analyzed using FlowJo (TreeStar) or BD FACSDiva software.

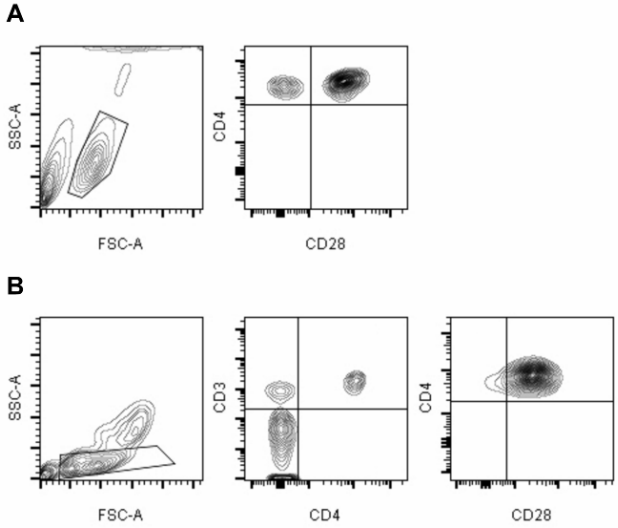


Figure 3.1. Gating strategy. (**A**) In human samples, the gating strategy consists of a lymphocyte gate using the forward and side scatter signal, after which $CD4^+$ cells were gated and subsequently CD28 expression was monitored within this gate. (**B**) In mouse samples, the lymphocytes were gated, after which $CD3^+CD4^+$ cells were targeted and CD28 expression was monitored within this gate.

3.3.5 Immunohistochemistry

Mice were perfused with Ringer's solution, spinal cords were dissected and, via a PFA/sucrose gradient, frozen in liquid nitrogen, 30 days after EAE induction. Ten micrometer cryosections were cut on the Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were fixed, blocked and incubated with anti-mouse antibodies against CD4 (1/100, BD Biosciences, 553043) and granzyme B (1/100, Abcam, Ab4059). Binding of these primary antibodies was visualized with the appropriate Alexa 488 or Alexa 555 (1/500, Life technologies, Merelbeke, Belgium) and nuclear staining was performed with DAPI (Life technologies). Autofluorescence was blocked using 0.1% Sudan Black in 70% ethanol. Demyelination and infiltration were visualized by 3, 30 diaminobenzidine (DAB) staining of myelin basic protein (MBP) with the envision kit according to the manufacturers protocol (dako Glostrup, Denmark) and subsequent hematoxylin counterstaining.

In short, peroxidase activity was inhibited with 0.3% H_20_2. Slides were blocked in PBS containing 10% protein block (dako Glostrup) and incubated with rat anti-mouse MBP (1/100, Millipore, MAB386) for 1h at room temperature. Following incubation with a peroxidase labelled polymer, staining was performed with DAB substrate and hematoxylin counterstain. Microscopical analysis was performed using a multiviewer DM 2000 LED microscope and DM 4000 LED microscope with Leica Application Suite software (Leica Microsystems).

3.3.6 Histological quantification

The extent of demyelination was evaluated in spinal cords of three mice per group (MCMV + EAE, EAE control and MCMV control group). Each mouse displayed a disease score close to the median of the respective group. Every 200 µm, an entire longitudinal spinal cord section was analyzed for immune infiltrates and demyelination, with a total of four sections for each animal.

Demyelinated area was assessed as loss of MBP staining within the white matter of these four sections covering the entire spinal cord. Microscopical analysis was performed using a multiviewer DM 2000 LED microscope (Leica Microsystems) and Fiji software (NIH ImageJ).

3.3.7 In vitro CMV stimulation assay

PBMCs from 12 HC and 8 MS patients were isolated from whole blood via density gradient centrifugation. These donors differed according to their CMV status and CD4+CD28null T cell expansions (Table 3.2). PBMCs were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Hyclone Europe, Erembodegem, Belgium), 1% nonessential amino acids, 1% sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin (all Life technologies). To mimic chronic CMV stimulation, cells were stimulated weekly with human CMVpp65 recombinant protein (10 µl/ml, Miltenyi Biotec, Bergisch Gladbach, Germany) or IL-2 (5 U/ml, Roche Diagnostics, Basel, Switzerland) for a maximum of 20 days. At different time points (d0, 1, 6, 9, 12, 15 and 20), the relative number of CD4+CD28null T cells was determined by flow cytometry as described above.

Table 3.2: Study subjects for *in vitro* CMV stimulation assay.

	MS patients (n=8)	Healthy controls (n=12)
CMV+ exp+	4	4
CMV+ exp-	1	4
CMV- exp-	3	4

MS, multiple sclerosis; CMV +/-, cytomegalovirus seropositive or negative; exp +/-, CD4+CD28null T cell expansions are present (≥2%) or not (<2%)

3.3.8 Generation of MBP reactive T cell clones

MBP-specific T cell clones were generated as described previously (151). Briefly, MBP-reactive T cell lines were generated from the blood of MS patients via limiting dilution analysis (LDA), cloned with phytohemagglutinin (PHA) in the presence of allogeneic accessory cells and further expanded by successive rounds of restimulation with human MBP or PHA and autologous antigen presenting cells (APCs).

3.3.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 and SAS 9.3. Parametric analyses include t-tests (2 groups), 1-way ANOVA and 2-way ANOVA (multiple groups). Nonparametric tests encompass Mann-Whitney tests (2 groups) and Kruskal-Wallis tests (multiple groups). Parametric data are shown as mean ± SD, nonparametric data as median ± interquartile range. A p-value <0.05 was considered significant.

3.3.10 Ethics approval and consent to participate

Experiments involving human samples and data were approved by the Medical Ethics Committee UZ KU Leuven and experiments were performed in accordance with its guidelines and regulations. Informed consents were obtained from all donors.

All animal studies were in accordance with the EU directive 2010/63/EU for animal experiments and were approved by the Ethical Committee Animal Experiments UHasselt.

3.4 Results

3.4.1 CMV expands CD4+CD28null T cells via repeated antigenic stimulation

To determine whether CMV infection is linked to expansion of CD4+CD28null T cells (>2% of CD4+ T cells), an association study between CMV serology and the percentage of CD4+CD28null T cells was performed. In our cohort, the percentage of CD4+CD28null T cells is significantly higher in CMV seropositive (CMV+) donors compared to CMV seronegative (CMV-) donors ($p<0.0001$, Figure 3.2A and B), with no differences between MS and HC, which is in line with other studies (35). Furthermore, CMV-specific IgG titers positively correlate with the percentage of CD4+CD28null T cells ($\rho_s=0.6$, $p<0.0001$, Figure 3.2C). To test whether this correlation is CMV specific, we examined the serology of EBV, another chronic and latent virus which has been implicated in MS (88). No significant correlation was found between the percentage of CD4+CD28null T cells and EBNA IgG titers (Figure 3.2D). Furthermore, EBV IgG levels did not differ between donors with versus without CD4+CD28null T cell expansion (respectively: 9 ± 4 vs 8 ± 4, $p>0.05$). In contrast, donors with CD4+CD28null T cell expansion have significantly higher CMV IgG titers compared to donors without these expansion (respectively: 219 ± 92.8 vs 5 ± 0, $p<0.0001$).

Since CD4+CD28null T cell expansion only occurred in CMV infected individuals and correlated with the level of CMV-specific antibody titers, we investigated whether CMV infection can drive expansion of CD4+CD28null T cells, using *in vitro* and *in vivo* models. Since there is no significant difference in the percentage of CD4+CD28null T cells between HC and MS patients, we did not discriminate between both populations in the following experiment. To mimic chronic TCR triggering by CMV, PBMCs from MS patients and HC, who were either CMV+ or CMV- and exhibited CD4+CD28null T cell expansion (exp+) or not (exp-), were repeatedly stimulated with a CMV peptide (CMVpp65) *in vitro*.

The percentage of CD4+CD28null T cells significantly increased over time in CMV+ exp+ donors, as opposed to CMV+ exp- and CMV-exp- donors (Figure 3.2E). IL-2 by itself did not induce expansion of CD4+CD28null T cells (Figure 3.2F). Repetitive CMV peptide stimulation *in vitro* did not induce the generation of CD4+CD28null T cells in exp- donors over the duration of the experiment (20 days).

To investigate the long term effect of CMV infection on formation and expansion of CD4+CD28null T cells, we used the *in vivo* MCMV mouse model, the most widely used and relevant model for human CMV infection (148). MCMV infected mice showed a significant increase of CD4+CD28null T cells in the spleen over time, with a 2-fold increase at day 8 ($p<0.05$) and 20-fold increase at day 250 post-infection compared to non-infected mice ($p<0.0001$, Figure 3.2G). In non-infected mice, the CD4+CD28null T cell levels were below the threshold for expansion (1±0.2%), indicating that CMV infection induces loss of CD28 in CD4+ T cells *in vivo*. In summary, repeated *in vitro* stimulation with CMV peptide expands pre-existing CD4+CD28null T cells, whereas *in vivo* CMV infection induces CD28 loss in CD4+ T cells and drives expansion of CD4+CD28null T cells.

To determine whether CMV induces the loss of CD28 on CD4+ T cells via repeated antigenic triggering or via interaction with its ligands CD80 and CD86, we infected CD80/86$^{-/-}$ mice with MCMV. MCMV infection induced the expansion of CD4+CD28null T cells to a similar extent in CD80/86$^{-/-}$ mice and WT mice (Figure 3.2H), indicating that the loss of CD28 is not caused by binding with their ligands CD80 and CD86. These findings further strengthen our notion that CD28 loss is caused by continues antigenic triggering via the TCR.

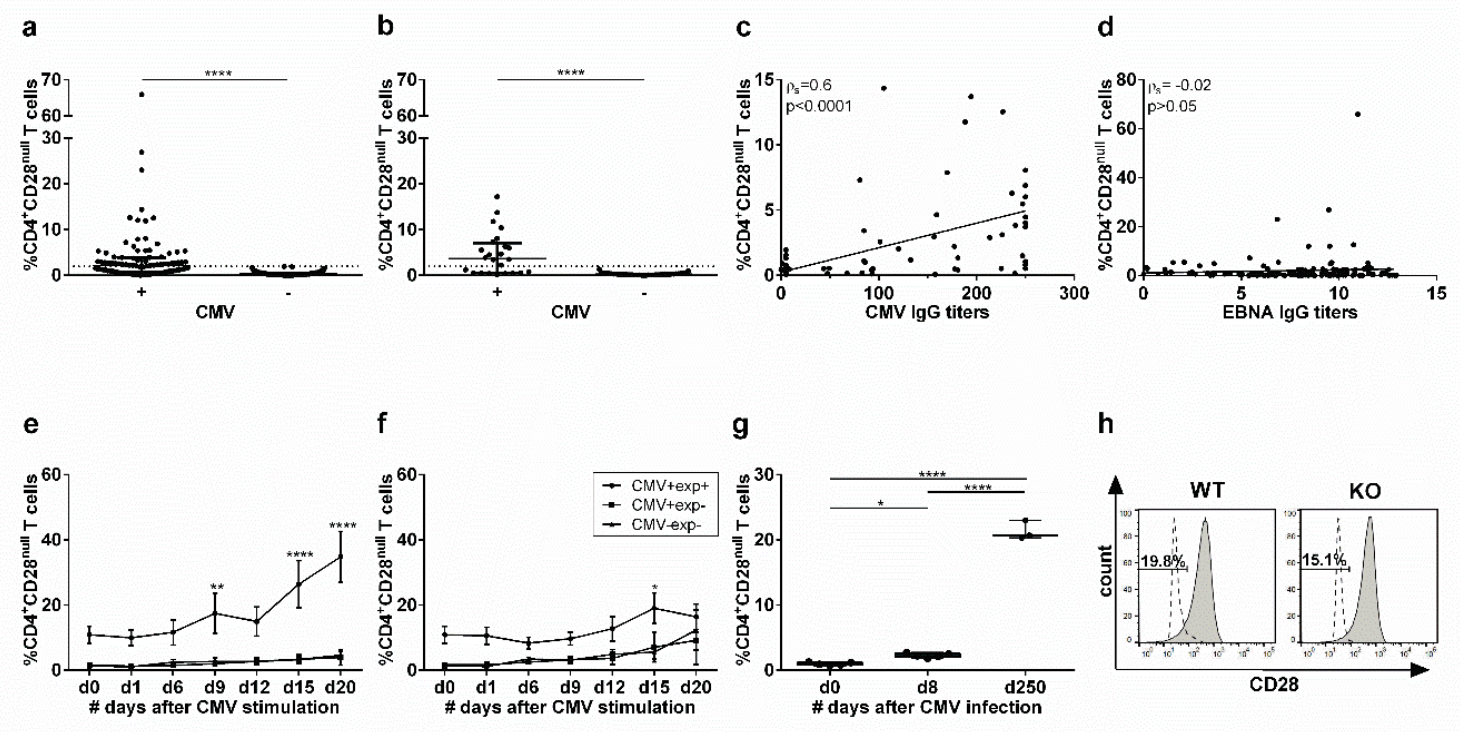


Figure 3.2: CMV infection expands CD4+CD28null T cells. Flow cytometry was performed to determine the percentage of CD4+CD28null T cells. CMV and EBV status and immunoglobulin titers were determined via ELFA. CD4+CD28null T cells in (**A**) CMV seropositive (n=100) compared to seronegative (n=127) MS patients and (**B**) CMV seropositive (n=24) versus seronegative healthy controls (n=39). (**C**) Correlation of CMV IgG levels with the percentage of CD4+CD28null T cells in MS patients and HC (n=140). (**D**) Correlation of EBV EBNA IgG titers in 155 MS patients. (**E**) Repeated stimulation of PBMCs from HC (n=12) and MS patients (n=8) with CMV pp65 (**E**) or IL-2 (**F**) *in vitro,* after which the number of CD4+CD28null T cells was determined at different time points. (**G**) Flow cytometry of splenocytes of MCMV infected mice on day 0, day 8 and day 250 post infection (n=5/ time point). (**H**) Splenocytes from MCMV-infected WT (n=5) and CD80/86$^{-/-}$ (n=4) mice were analyzed for the percentage of CD4+CD28null T cells at day 250 post infection. *p<0.05, **p<0.01, ****p<0.0001.

3.4.2 CD4+CD28null T cells are increased in EAE mice and correlate with severity

CD4+CD28null T cells are cytotoxic, accumulate in MS lesions and at least a subpopulation is autoreactive in nature (36). To test the hypothesis that CD4+CD28null T cells are associated with the severity of neuroinflammation, an EAE experiment was performed. Follow-up time (Figure 3.3A) was extended compared to the standard protocol (30 days p.i.), to test whether CD4+CD28null T cells expand during acute and chronic stages of EAE (Figure 3.3B). While limited numbers of CD4+CD28null T cells were found in CFA control mice, a significant increase above the 2% threshold for expansion was only found in the EAE mice (EAE: 3±0.7%, $p=0.004$ and control: 1.8±0.3%, $p>0.05$, Figure 3.3B).

From previous studies, it is known that human CD4+CD28null T cells produce IFN-γ and granzyme B, and that they show low expression of CD62L, CD127 and CD27 (35, 36, 45, 152, 153). To determine whether mouse CD4+CD28null T cells have a similar phenotype, we analysed these cells, which were present in the peripheral blood of EAE mice. We found that they indeed phenotypically resembled their human counterparts as evidenced by a low expression of CD62L, CD127 and CD27, and production of IFN-γ and granzyme B (Figure 3.3C), identifying them as pro-inflammatory and cytotoxic effector memory T cells. Furthermore, the percentage of CD4+CD28null T cells positively correlated with the EAE disease score ($\rho s=0.6$, $p=0.0002$, Figure 3.3D). The long-term follow-up indicated that there was no further expansion of CD4+CD28null T cells in the chronic phase of EAE (after d30), thus CD4+CD28null T cells especially expand in the developmental phase of EAE. The increase in CD4+CD28null T cells in EAE mice could result from repeated auto-antigenic stimulation. To test this hypothesis, human MBP-specific T cell clones, generated and sustained *in vitro* by stimulation rounds with MBP or PHA, were analyzed for the presence of CD4+CD28null T cells (Figure 3.3E). The number of CD4+CD28null T cells increased after each successive round of stimulation.

Thus, repeated MBP stimulation leads to the expansion of CD4+CD28null T cells *in vitro*, indicating that the expansion of CD4+CD28null T cells in MS patients may result from chronic auto-antigenic stimulation *in vivo*. Of note, *in vitro* stimulation with tetanus toxoid also induced expansion of CD4+CD28null T cells (Figure 3.4), indicating that the expansion is not antigen specific, but rather due to the chronicity of the antigen exposure.

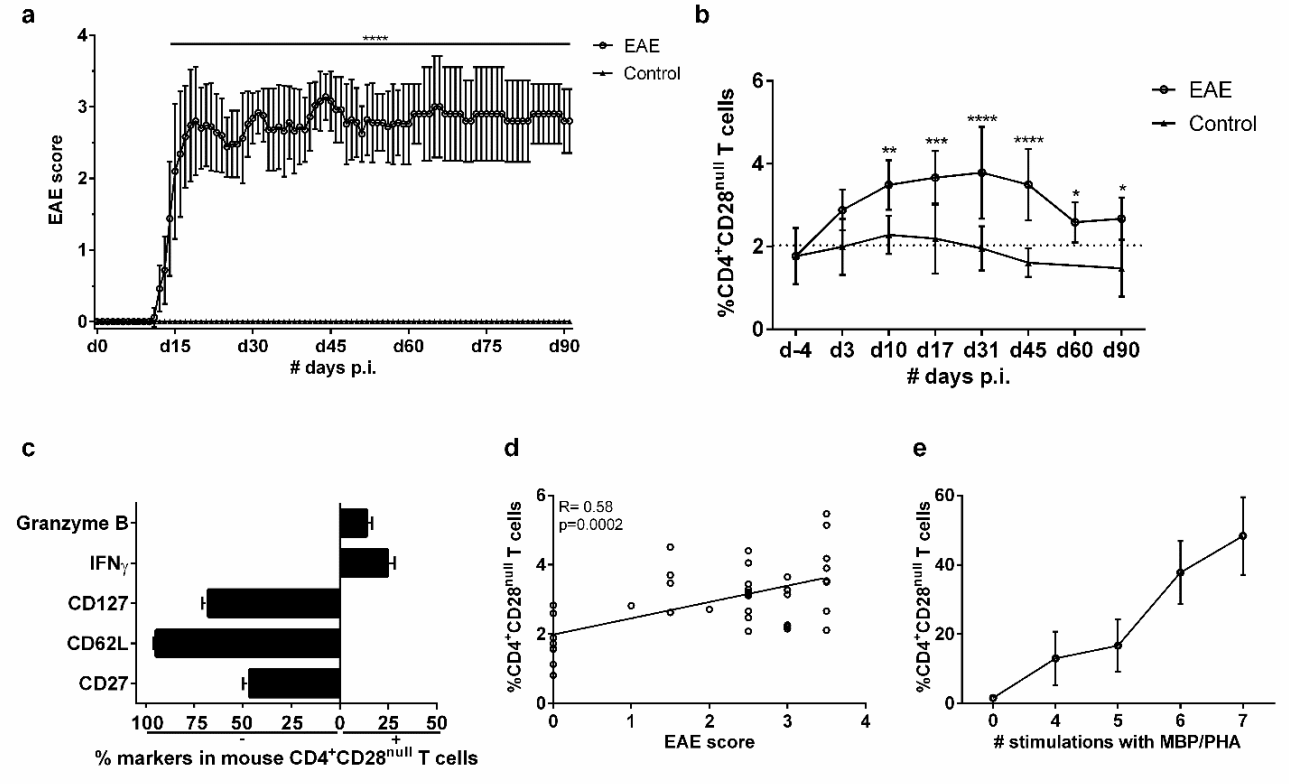


Figure 3.3: CD4+CD28null T cells are increased in EAE mice, as a result of auto-antigenic stimulation. (**A**) After induction, EAE mice (n=15) and CFA control mice (n=10) were scored for maximum 90 days according to their disability. (**B**) Blood was collected at different time point, to determine the number of CD4+CD28null T cells via flow cytometry. (**C**) The phenotype of blood-derived mouse CD4+CD28null T cells was measured via flow cytometry. (**D**) Correlation between CD4+CD28null T cells and EAE score. (**E**) Historical human MBP specific T cell clones repeatedly stimulated with MBP/PHA (n=8) were thawed and analyzed for the number of CD4+CD28null T cells via flow cytometry. **p<0.01, ***p<0.001, ****p<0.0001.

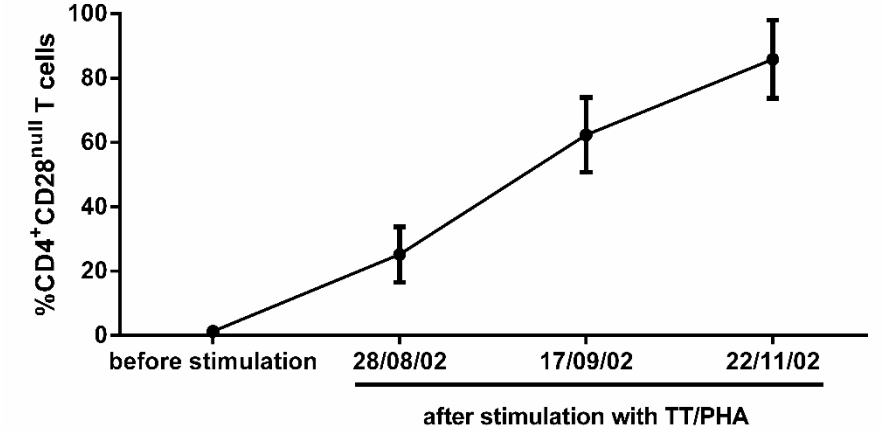


Figure 3.4: Tetanus toxoid induces expansion of CD4+CD28null T cells. Historical human tetanus toxoid (TT) specific T cell clones repeatedly stimulated with TT/PHA (n=5) were thawed and analyzed for the number of CD4+CD28null T cells via flow cytometry.

3.4.3 CMV infection exacerbates clinical symptoms of EAE

Our results indicate that CD4+CD28null T cells expand after repeated immune activation, either as a result of CMV infection or after the induction of autoimmunity. Here, we investigated whether CMV infection and subsequent expansion of CD4+CD28null T cells correlate with a worse EAE outcome. The interplay between these different factors was investigated by infecting mice with MCMV and subsequently inducing EAE 8 days later. The EAE disease score of mice that were pre-exposed to MCMV was significantly higher compared to the EAE control group (mean cumulative score: 56±4 vs 47±3, $p<0.01$; mean maximal score: 3.8±0.26 vs 3.5±0, $p<0.02$; mean end score: 3.1±0.35 vs 2.2±0.27 $p=0.002$). Furthermore, the MCMV group experienced a relapse between day 26 and day 30 after immunization, whereas EAE control mice did not (Figure 3.5A). The percentage of CD4+CD28null T cells in the spleen increased at least eight-fold in each group (CMV: 8±2%, $p<0.001$, EAE: 12±3%, $p<0.0001$ and CMV+EAE: 14±2%, $p<0.0001$) compared to baseline (1±0.2%) (Figure 3.5B). These results provide further evidence that both CMV infection and EAE induction lead to the expansion of CD4+CD28null T cells and that prior CMV infection aggravates EAE symptoms.

Since we showed that CMV exacerbates EAE disease, we asked whether this is due to increased autoimmune reactivity. To answer this question, CD4+ T cell reactivity to MOG peptide was measured in the spleen.

The MCMV infected EAE group displayed enhanced MOG-specific CD4+ T cell reactivity compared to the control groups (EAE: $p<0.004$, CMV: $p<0.002$). Furthermore, this MOG response correlated to the percentage of CD4+CD28null T cells in the spleen of these mice (Figure 3.5C). Also, we detected splenic CMV-specific CD4+ T cell reactivity in the MCMV infected groups, however they were not increased by EAE induction (data not shown). Viral load measured in the salivary glands at the end of the experiment indicate that the virus was still present in high amounts in both the MCMV and the MCMV infected EAE groups (data not shown).

These data indicate that CMV infection increases the percentage of MOG-specific CD4+ T cells, thereby increasing autoimmune-mediated neuroinflammation, and that CD4+CD28null T cells take part in this overall MOG response.

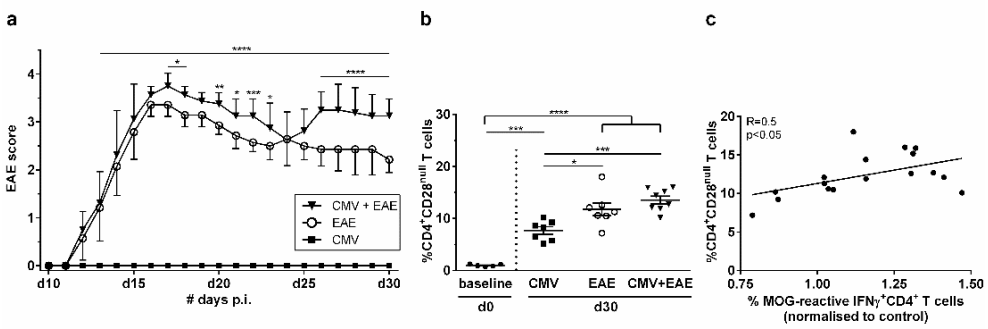


Figure 3.5: MCMV infected mice with EAE have a worse disease course. Mice were infected with MCMV and after 8 days EAE was induced. (**A**) Daily scoring of the CMV, EAE and MCMV infected EAE groups (n=8/group). (**B**) Splenocytes were isolated at day 0 (baseline) and day 30 and CD4+CD28null T cells were measured via flow cytometry. (**C**) After stimulation of splenocytes with a MOG_{35-55} peptide, the IFN-γ producing CD4+ T cells were measured via flow cytometry and normalized to the non-peptide control. The MOG response significantly correlated with the percentage of CD4+CD28null T cells in the spleen. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4.4 CMV infection increases demyelination in EAE

In MS patients, CD4+CD28null T cells accumulate in brain lesions and are in close contact with neural cells (36). Since, CMV infection leads to a worse EAE disease course, we next questioned whether demyelination of the spinal cord, the predominant location of lesions in this model, is also increased in these animals. No demyelination was found in the spinal cord of MCMV infected mice (Figure 3.6A and B). MCMV infected EAE animals exhibited enhanced demyelination compared to the EAE control group (Figure 3.6B), indicating that CMV infection accelerates autoimmune-mediated CNS damage.

Furthermore, the extent of demyelination is strongly correlated with the percentage of spleen-derived CD4+CD28null T cells (R=0.71, p<0.05, Figure 3.6C). We further identified CD4+Granzyme B+ T cells in the spinal cord (Figure 3.6D), suggesting that CD4+CD28null T cells, which are granzyme B+, are present in the spinal cord and possibly contribute to CNS damage.

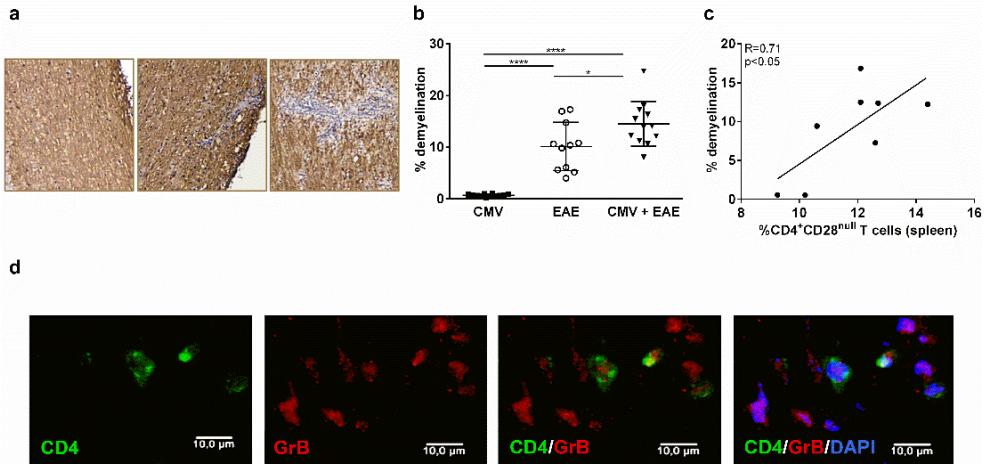


Figure 3.6: CMV infection increases EAE lesion size. (**A**) Representative staining of MBP in the CMV control group (left), the EAE control group (middle) and the CMV infected EAE animals (right). (**B**) The amount of demyelination within the CMV and EAE control groups and the CMV infected EAE group, calculated via dividing the demyelinated area (= loss of MBP) in the white matter of the spinal cord, over the total white matter area for each section. (**C**) Correlation between the amount of demyelination for each animal and the percentage of peripheral CD4+CD28null T cells. (**D**) Single and double staining of CD4 and Granzyme B, which points to the presence of CD4+CD28null T cells, in the spinal cord of EAE mice. *p<0.05, ****p<0.0001.

3.5 Discussion

Here, we demonstrate that CD4+CD28null T cells expand during EAE and positively correlate with disease severity. In addition, we show that CMV by itself is able to enhance activation of disease-specific CD4+ T cells, trigger the expansion of CD4+CD28null T cells and worsen EAE. Overall, our findings support a detrimental role for CMV in autoimmune neuroinflammation.

Our group, together with others have shown that CD4+CD28null T cells are associated with the pathogenesis of chronic inflammatory disorders (62, 154, 155). In MS, a direct link with disease severity has not been demonstrated so far. However, indirect evidence, such as their target tissue infiltrating capacity and cytotoxic activity towards oligodendrocytes, certainly alludes to this hypothesis (36, 45). In this study, we made use of the widely documented mouse model for MS, EAE. Although this model is certainly not fully equivalent to the human situation, it does recapitulate the inflammatory response that arises in patients with MS, which is the focal point of our study (156). Here, we demonstrate that peripheral CD4+CD28null T cells are increased in EAE animals and that the percentage of CD4+CD28null T cells strongly correlated with the amount of demyelination and disease severity. Mouse-derived CD4+CD28null T cells displayed an effector memory ($CD62L^{low}CD127^{low}CD27^{low}$IFN-γ+) and cytotoxic (granzyme B+) phenotype, indicating that they are similar to their human counterparts (35, 36, 45, 152, 153). Our findings are in line with evidence found in collagen-induced arthritis (CIA), the animal model for RA, where an increase in the number of CD4+CD28-NKG2D+ T cells was observed after immunization (157). The increase in peripheral CD4+CD28null T cells in EAE mice could be attributed to repeated autoantigenic stimulation caused by chronic autoimmune inflammation. Indeed, as evidenced by our *in vitro* data, repeated MBP stimulation of MBP-specific T cell clones leads to CD4+CD28null T cell expansions. *In vivo*, we found a direct correlation between the percentage of CD4+CD28null T cells and the anti-MOG response level in the spleen of EAE mice. Together, these findings confirm the autoreactive nature of CD4+CD28null T cells (110). After 30 days p.i., there was no further expansion of CD4+CD28null T cells in the blood of EAE mice. Instead, starting from day 60, the memory pool maintained a steady state.

This is as expected with regards to the homeostasis of the memory pool: expansion is followed by contraction and ultimately maintenance of the remaining memory T cell pool (158).

In contrast to EAE mice, not all MS patients have CD4+CD28null T cell expansions. Therefore, in humans additional components could be important in the generation of CD4+CD28null T cells. Potential triggers include: 1) chronic inflammation (33); and 2) viral infections (59), of which CMV, as a persistent virus, is a promising candidate. Our data demonstrate that repetitive *in vitro* CMV peptide stimulation of human PBMCs expands pre-existing CD4+CD28null T cells. IL-2, which enhances T cell proliferation and differentiation, does not lead to the expansion of CD4+CD28null T cells. EBV, another chronic and latent virus implicated in MS, is not associated with CD4+CD28null T cell expansion. These findings further support the hypothesis that CD4+CD28null T cells arise after CMV infection, which corresponds with previous reports by other groups (35, 111). Of note, we did not measure proliferation; therefore, the increase in CD4+CD28null T cells after CMV stimulation could be due to survival rather than proliferation. However, van Leeuwen et al. indicated that CD4+CD28null T cells proliferate after addition of CMV antigens, suggesting the latter is true (111). *In vivo,* CMV infection leads to continuous activation, enabling us to study chronic repeated antigenic challenge. Although human CMV and MCMV are different viruses, the MCMV mouse model is widely used and is the most relevant mouse model which mimics human CMV infection (148). MCMV virus in the salivary gland is thought to be important for spreading the virus from mouse to mouse. Whereas in all organs the virus is latent in less than a few weeks, in the salivary glands the virus replicates for months (159). Thus the amount of virus in the salivary gland is not influencing the titers in other organs, such as spleen and lymph nodes, but is instead set by the initial infection dose, and the local and pre-existing immunity conditions. Using this model, we clearly show formation and expansion of CD4+CD28null T cells in all MCMV infected animals over time. These findings are in line with those of other groups (59). Since CMV is unable to infect T cells, CMV cannot directly reduce CD28 expression on T cells, but rather exerts its effects due to its persistent nature.

In this study, we show that the loss of CD28 is caused by continued antigenic triggering and not by binding with their ligands CD80 and CD86, since the number of CD4+CD28null T cells did not differ between MCMV-infected CD80/86$^{-/-}$ mice and WT. Furthermore, studies in mice and humans have indicated that the number and phenotypes of CMV-specific T cells correlate with viral load (148, 160, 161); higher viral loads drive higher expansions, establishing the antigen-driven aspect of the response. In this study, we used a relatively high dose of MCMV leading to a higher amount of antigen-specific T cells, including CD4+CD28null T cells. In the human population, the dose of CMV is not evenly distributed, leading to variability in the number of antigen specific T cells between individuals. This heterogeneity explains the difference in the percentage of CD4+CD28null T cells among CMV seropositive donors. In this respect, it is of interest to note that the CMV Ig titers correlate with a higher percentage of CD4+CD28null T cells. This implies that individuals with a higher CMV exposure may develop more CD4+CD28null T cells and associated disease. Also, the proportion of CMV-seropositive individuals increases with age (162), as does the percentage of CD4+CD28null T cells (163).

In MS patients, CMV seropositivity and high IgG titers are correlated with increased percentages of CD4+CD28null T cells. This link between CMV and CD4+CD28null T cell expansion was previously also reported for RA, ankylosing spondylitis and cardiovascular diseases (111, 155, 164-168), indicating that CMV infection and CD28null T cell expansion form a common pathogenic background in these diseases (35).

The logical next step is to confirm the possible link between CMV, CD4+CD28null T cells and autoimmunity. Here, we demonstrate that CD4+CD28null T cells are increased in MCMV, EAE and MCMV infected EAE mice after 30 days p.i.. MCMV infected EAE animals had a higher disability score and experienced a relapse, compared to the EAE control mice. Furthermore, MCMV infection increased demyelination in EAE mice, which correlated with higher CD4+CD28null T cell percentages in the periphery.

Since we found CD4+GranzymeB+ T cells in the spinal cord of EAE and MCMV infected EAE mice, this suggests that CD4+CD28null T cells accumulate in the CNS to inflict damage in line with our previous observations in post-mortem MS brain material (36).

Thus, CMV infection exacerbates EAE disease course and does this by boosting the autoimmune response, as indicated by an increased MOG response. Indeed, T cell expansion preferentially occurred in MOG specific T cells, since the overall T cell responsiveness (no peptide control) in the spleen was comparable between all groups (data not shown). This is in accordance with others, where EAE induction combined with viral infection (γ-herpes virus, Semlike Forest virus or Sindbis virus) accelerated or exacerbated disease as a result of enhanced immune cell infiltration and polarization of the adaptive immune response (106, 134, 135). Furthermore, MCMV infection rendered EAE-resistant BALB/c mice susceptible for EAE induction (169). In another murine model of MS, namely Theiler's murine encephalitis virus (TMEV) model, opposite findings were demonstrated; CMV infection attenuated TMEV disease course (112). However, the immune response in TMEV is largely CD8-mediated, whereas in EAE and MS CD4+ T cells are the main players (170). We believe that the EAE model better represents what is going on in MS, namely a primary autoimmune-mediated attack of the CNS, in contrast to the TMEV model, where primary viral-induced neurotoxicity induces secondary autoimmunity.

An important question still remains to be answered: is the disease exacerbating effect and enhanced demyelination directly caused by CMV infection itself or attributable to the increased expansion of CD4+CD28null T cells? While technically challenging, an adoptive transfer study is needed to indisputably prove a direct cause-and-effect relationship of CD4+CD28null T cells and disease severity.

CMV was previously reported to be present in the CNS, where it could damage local cells and tissues directly (88). The ensuing cell death could then enhance autoimmunity as a result of the release and spreading of self-epitopes from degenerating tissue (81). However, since demyelination was not present in animals only infected with CMV, it is unlikely that CMV by itself leads to CNS damage as proposed by the epitope spreading hypothesis.

On the other hand, reactivation of CMV during ongoing MS could trigger the activation of autoreactive T cells (molecular mimicry) thereby enhancing subsequent demyelination. Of note, CMV-specific T cells were previously identified in MS lesions (102). Evidence for cross reactivity between a CMV antigen ($UL86_{981-1003}$) and the myelin oligodendrocyte glycoprotein epitope (MOG_{35-55}) has been found in rats and non-human primates (97, 98).

However, our data show that CMV infection alone did not mount a significant MOG response in the spleen, which would have been the case if molecular mimicry was involved. Another possible way by which CMV could directly contribute to autoimmunity is through bystander activation, where the immune response against CMV leads to robust inflammation, triggering the non-specific activation of autoreactive T cells (82). We postulate that these bystander-activated autoreactive T cells are mainly responsible for exacerbating EAE disease severity.

3.6 Conclusion

In summary, CMV infection and EAE induction lead to the expansion of CD4+CD28null T cells. Both CMV infection and CD4+CD28null T cells aggravate autoimmune-mediated inflammation, since EAE disease severity, measured by EAE score and the extent of neuroinflammation and demyelination, correlated with increasing amounts of CD4+CD28null T cells and the presence of a CMV infection. Overall, CMV infection drives the expansion of CD4+CD28null T cells, thereby amplifying the activation of disease-specific CD4+ T cells, and exacerbating EAE disease. Since these findings are based on an animal model, future studies will address whether this is also the case in MS patients. If so, CMV vaccination to prevent the formation of CD4+CD28null T cells and the adverse effects of the infection itself, could be beneficial for people at risk of developing MS.

4

ASSOCIATION OF CMV- AND MS-RELATED DNA POLYMORPHISMS WITH CD4+CD28NULL T CELL EXPANSION?

4.1 Abstract

CD4+CD28null T cells arise after chronic stimulation of the immune system in the context of persistent infections and autoimmunity. We previously showed that cytomegalovirus (CMV) is able to trigger the expansion of this highly cytotoxic subset. However, not all CMV infected people show these expansions in their peripheral blood. Therefore, other factors, such as genetic predisposition, may contribute to their appearance. In this pilot study, we investigated the possible contribution of two CMV-related single nucleotide polymorphisms (SNPs) in toll-like receptor 2 (TLR2) and flanking MHC class I polypeptide-related sequence B (MICB), to the expansion of CD4+CD28null T cells. Moreover, a SNP in the interleukin 2 receptor α (IL2RA) was taken along, as IL2R is decisive for T cell expansion and SNPs in this gene associate with the risk of developing MS. Both MS patients and controls were included in this study.

Although our cohort is a good representation of the general European population, our pilot study did not reveal an association between the investigated SNPs (*IL2RA, MICB, TLR2*) and the percentage or expansion of CD4+CD28null T cells. A larger sample size is needed to validate these conclusions in future studies.

4.2 Introduction

Terminally differentiated CD4+CD28null T cells arise after chronic activation of the immune system and were shown to be at least in part autoreactive, cytotoxic and have inflammation-seeking properties in the context of inflammatory diseases (36, 110, 144). We recently found that CD4+CD28null T cells are implicated in multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (110, 144). Specifically, CD4+CD28null T cells correlate with disease severity in a cohort of MS patients (chapter 5) and in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS (chapter 3). Functionally, we found that these pro-inflammatory effector memory T cells evade Treg suppression and stimulate the differentiation of Th17 cells (chapter 6). The precise etiology of MS is unknown, but it is clear that both genetic and environmental factors are essential for disease development (171). The most important risk alleles associated with MS are located in genes coding for the major histocompatibility complex (MHC). Since the appearance of large genome-wide association studies (GWAS), up to 110 MS-associated single nucleotide polymorphisms (SNPs) have been identified. The majority of these SNPs are related to the immune system, and more specifically to T cell processes (172-174). Furthermore, we recently found that cytomegalovirus (CMV) infection is able to induce the formation of CD4+CD28null T cells (chapter 3). However, not all CMV positive donors have CD4+CD28null T cells, indicating that other factors could be involved.

In this chapter, we investigate a possible genetic predisposition for CD4+CD28null T cell expansion. To this end, we selected 3 polymorphisms which are either linked with MS or CMV status.

Among the SNPs linked with MS susceptibility, interleukin-2 receptor α (*IL2RA* or CD25, rs2104286)(172) was chosen to study the possible association with expansion of CD4+CD28null T cells. IL-2 signaling promotes proliferation, survival and differentiation of effector T cells. On the other hand, the IL2RA also limits T cell responses via Treg-mediated suppression, based on IL-2 captivation by $CD25^{hi}$ Tregs (175, 176). The SNP leads to a reduced IL-2 responsiveness in Tregs (177), but increases IL2RA membrane expression on naive Th cells, promoting uncontrolled effector T cell function (178, 179). Recently, this SNP was shown to be associated with the level of GM-CSF production by effector T cells, a pathogenic cytokine in the setting of MS (180, 181). Together, these findings support a possible role for the *IL2RA* SNP in CD4+CD28null T cell expansions, since these cells show increased survival and expansion, high levels of GM-CSF production and evasion from Treg-suppression (31, 144).

Among the CMV-related genes, both toll-like receptor 2 (*TLR2*, rs5743708) and MHC class I polypeptide-related sequence B (*MICB*, rs2523651) were studied for association with CD4+CD28null T cell expansion. TLR2 is a receptor for pathogens expressed on many immune cells, including antigen presenting cells and T cells (182). During a CMV infection, recognition of envelope glycoprotein B and H by TLR2 leads to activation of these cells and the initiation of an immune response (183-185). However, when the CMV-associated risk allele is present, a functionally defective receptor is produced, leading to a reduced ability of TLR2 to respond to CMV (186). This in turn leads to higher levels of CMV replication, possibly contributing to the emergence of CD4+CD28null T cells. Second, MICB is a protein expressed on stressed cells (e.g. virally infected cells), which interacts with NKG2D on NK cells or T cells to induce cytotoxicity (187). However, during CMV infection, the CMV-specific glycoprotein UL16 retains MICB in the infected cells and prevents MICB-NKG2D interactions and thus NK or T cell-mediated cytotoxicity (188).

The phenotypical outcome of the CMV-associated SNP flanking the *MICB* gene is not known yet. We speculate that this SNP enhances the process of intracellular MICB detainment, further reducing the CMV-specific immune response. Similar to the *TLR2* SNP, the presence of the *MICB* SNP leads to a higher incidence of CMV infection (189), possibly contributing to the expansion of CD4+CD28null T cells.

In our study, we included a study population of healthy controls (HC) and MS patients, which represents the European population and is distributed according to the Hardy-Weinberg equilibrium. First, we explored the possible relation between the *IL2RA, TLR2* and *MICB* SNPs and the expansion of CD4+CD28null T cells. We could not reveal an association between the investigated SNPs and the percentage or expansion of CD4+CD28null T cells. A larger sample size is needed to validate these conclusions in future studies. In addition, we were not able to confirm the previously reported association of the *MICB* SNP with CMV seropositivity despite sufficient power, indicating that this published association may be coincidal or the investigated cohort is not a good representation of the total population.

4.3 Materials and methods

4.3.1 Study Subjects

Peripheral blood samples were collected from 68 healthy controls (HC) and 146 MS patients in collaboration with the University Biobank Limburg (UBiLim). CMV status was determined by measuring CMV-specific IgG titers in serum samples of MS patients via Vidas ELFA (bioMérieux, Marcy l'Etoile, France), IgG >6 AU/ml was classified as CMV positive, IgG <4 AU/ml as CMV negative. Clinical information is shown in table 4.1; there were no significant differences between HC and MS patients, concerning age and gender. This study was approved by the local ethical committee and written informed consents were obtained from all study subjects.

Table 4.1: Clinical data of healthy controls and MS patients

	TLR2 (n=142)	**MICB (n=167)**	**IL2RA (n=163)**
HC (n=)	29	44	36
M/F	10/19	18/26	14/22
Age (mean ± SD)	43 ± 17	39 ± 17	42 ± 17
CD28 exp: yes/no	6/23 (21%)	8/36 (18%)	8/28 (22%)
CMV status: pos/neg	ND	ND	ND
MS (n=)	113	123	127
M/F	34/79	38/85	38/89
Age (mean ± SD)	47 ± 13	47 ± 13	47 ± 13
CD28 exp: yes/no	18/95 (16%)	19/104 (15%)	19/108 (15%)
CMV status: pos/neg	39/56 (18 missing)	39/65 (19 missing)	41/67 (19 missing)
Type MS (n=)			
RRMS	69	77	77
CP-MS	40	40	44
Treatment			
No treatment	53	59	59
IFNβ	29	30	31
Glatiramer acetate	12	13	14
Natalizumab	12	12	12
Alemtuzumab*	3	5	5
Methotrexate	1	2	2
Teriflunomide	1	/	1
Tecfidera	1	1	1
EDSS (mean ± SD)	3 ± 2	3 ± 2	3 ± 2

*Alemtuzumab-treated patients either just started treatment or received readministration.
Toll-like receptor 2 (TLR2), MHC class I polypeptide-related sequence B (MICB), interleukin-2 receptor α (IL2RA), healthy control (HC), male (M), female (F), CD4+CD28null T cell expansion (CD28 exp): yes if >2%/no if <2%, cytomegalovirus (CMV), positive (pos), negative (neg), multiple sclerosis (MS), clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), chronic progressive MS (CP-MS; includes primary and secondary progressive MS patients), expanded disability severity scale (EDSS), not determined (ND).

4.3.2 Cell isolation and flow cytometry

All donors included in this study were analyzed for the percentage of CD4+CD28null T cells. This was done by isolating peripheral blood mononuclear cells (PBMCs) from whole blood by density gradient centrifugation (Cedarlane lympholyte, Sheffield, UK). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ). The gating strategy consists of a lymphocyte gate using the forward and side scatter signal, after which CD4+ cells were gated and subsequently CD28 expression was monitored within this gate. Cells were acquired using a FACSAria II cytometer, and data were analyzed using BD FACSDiva software. Significant expansion of CD4+CD28null T cells was arbitrarily defined as a percentage ≥2% of the total CD4+ T cell population, as this was the minimal percentage of cells that allowed discrimination of a distinctive population (36). A pellet of 3 million PBMCs per donor was stored at -80°C for genetic analysis.

4.3.3 Genomic DNA extraction

Genomic DNA extraction was performed using the ArchivePureTM DNA purification Blood Kit according to the manufacturers protocol (5PRIME, VWR International). The quantification, concentration and purity of the isolated DNA was carried out using NanoDrop 2000 UV-Vis Spectrophotometer and NanoDrop 2000/2000c software (Fisher Scientific Belgium, Belgium).

4.3.4 Taqman PCR

25 ng gDNA diluted in TE buffer (Tris, 10 mM, VWR international; EDTA, 1 mM, VWR international, Belgium; MiliQ) was added to a MicroAmp® Optical 96-well reaction plate (Applied Biosystems, USA). After drying of the DNA, Genotyping Master Mix (Applied Biosystems), TaqMan Assay (Applied Biosystems) and MilliQ was added. Negative controls consisted of TE-buffer only. After preparation, the reaction plate was covered with an optical adhesive film (Applied Biosystems).
PCR reaction and detection was performed by a 7300 Real-Time PCR system (Applied Biosystems). Detectors "FAM" and "VIC" were used to perform Allelic Discrimination. Background fluorescence was measured prior to the launch of PCR reaction. Data-analysis was carried out with 7300 System Software (Applied Biosystems).

4.3.5 Statistical analysis

The data set was analyzed using SAS JMP (JMP Pro12, SAS institute, USA) and Graphpad prism 6 (Graphpad software Ing, USA). To identify multicollinearity, all pairwise associations were computed. Normality was checked with d'Agostino-Pearson. Parametric analyses include t-tests (2 groups) and 2-way ANOVA (multiple groups). Nonparametric tests encompass Mann-Whitney tests (2 groups) and Kruskal-Wallis tests (multiple groups). A chi-square or fisher's exact test was used to investigate the significance of association between factors and risk allele frequency. A p-value <0.05 was considered significant.

Logistic or generalized linear regression models were used to test for the presence of effect of some factors of interest. Different models were constructed for the three genes (*TLR2*, *MICB* and *IL2RA*). A generalized linear model with exponential distribution was used when the distribution of the response variable was skewed. Logistic models were used for binary response variables. The analysis started with a full model with the main effects of genotype, CMV status, gender, age, medication, as well as the interaction effects of CMV status with the 4 other main effects (genotype, gender, age and medication). Backward model selection was run until all terms in the model or interactions involving them were significant ($p<0.05$).

Power and sample size analysis for the 3 selected SNPs was performed by using G*Power 3.0 (190). We used a Fisher's exact test for proportions of 2 independent groups with inequality, or 1-way ANOVA with fixed effects. Both tests were executed with type I error (α) = 0.05 and power (β) = 0.8.

4.4 Results

4.4.1 Characterization of the study cohort

4.4.1.1 Genotypic frequencies of the candidate genes in Belgian MS patients and HC

We compared the risk allele frequencies (RAF) and genotype frequencies from our study population (HC+MS patients) with the frequencies found in the European population (Ensemble) and with the estimated frequencies calculated from the Hardy-Weinberg equilibrium based on the European population (table 4.2). All genes of interest demonstrated a highly similar RAF and distribution of genotype frequencies as seen in the European population or compared to the theoretical estimation ($p>0.05$), indicating that our study cohort is a good representation of the overall European population.

Separate analysis of HC and MS patients also showed similar RAF and genotype frequencies compared to the European population and calculated Hardy-Weinberg equilibrium (data not shown).

Table 4.2: The risk allele frequencies (RAF) and genotype frequencies of *IL2RA*, *TLR2* and *MICB* genes in the European and our study population.

	IL2RA	***TLR2***	***MICB***
Chromosome	10	4	6
SNP rs number	rs2104286	rs5743708	rs2523651
Alleles (risk allele)	C/T	A/G	A/G
RAF (%)			
European population	78	2	34
Study population	**80.7**	**3.5**	**37.4**
Genotype frequencies (%)			
European population	CC: 6.4	AG: 4.8	AA: 12.1
	CT: 31.2	GG: 95.2	AG: 44.5
	TT: 62.4		GG: 43.3
Hardy-Weinberg Equilibrium	CC: 4.8	AG:4.7	AA:11.8
	CT: 34.3	GG:95.3	AG:45.1
	TT: 60.8		GG:43
Study population	**CC: 2.5**	**AG: 7**	**AA: 15**
	CT: 33.7	**GG: 93**	**AG: 44.9**
	TT:63.8		**GG: 40.1**

SNP: single nucleotide polymorphism, rs: reference SNP, RAF: risk allele frequency

4.4.1.2 Association of IL2RA with MS

Next, we sought to confirm the published association of the *IL2RA* SNP with MS (172, 174, 191). The SNP was distributed according to the Hardy-Weinberg equilibrium in both HC and MS patients. However, we could not detect a difference in the genotype or allele frequencies between HC and MS patients in our study cohort (table 4.3).

For *IL2RA*, we performed an a priori sample size calculation based on the Madrid cohort of Cavanillas et al. (175), which indicates that a sample size of 566 donors (246 MS patients and 320 HC) is needed. Our study only consisted of 163 donors (MS: 127, HC: 36), which is insufficient, as indicated by a power of 0.25 (Fisher's exact test).

Table 4.3: Genotype and allele frequencies of *IL2RA* SNP in HC and MS patients.

	HC	MS
IL2RA		
CC	0 (0%)	4 (3.1%)
CT	16 (44.4%)	39 (30.7%)
TT	20 (55.6%)	84 (66.1%)
C	16 (22.2%)	47 (18.5%)
T	56 (77.8%)	207 (81.5%)
Hardy-Weinberg equilibrium p-value	0.09	0.84
Allelic p-value	0.5	
OR (95% CI)	1.26 (0.66-2.39)	

Risk allele is underlined
HC: healthy control, MS: multiple sclerosis, OR: odds ratio, CI: confidence interval

4.4.1.3 Association of candidate genes TLR2 and MICB with CMV

The described associations between *TLR2* or *MICB* and CMV status were checked by investigating possible differences in risk allele frequencies and genotype distributions between CMV seropositive and seronegative MS patients (table 4.4). Both SNPs were distributed according to the Hardy-Weinberg equilibrium in both CMV+ and CMV- donors. However, we could not detect a difference in the genotype or allele frequencies between CMV+ and CMV- patients.

Table 4.4: Genotype and allele frequencies of *TLR2* and *MICB* SNPs in CMV+ and CMV- MS patients.

	CMV-	CMV+
TLR2		
AG	5 (8.9%)	1 (2.6%)
GG	51 (91.1%)	38 (97.4%)
A	5 (4.5%)	1 (1.3%)
G	107 (95.5%)	77 (98.7%)
Hardy-Weinberg equilibrium p-value	0.73	0.94
Allelic p-value	0.4	
OR (95% CI)	0.28 (0.03-2.43)	
MICB		
AA	10 (15.4%)	5 (12.8%)
AG	25 (38.5%)	20 (51.3%)
GG	30 (46.2%)	14 (35.9%)
A	45 (34.6%)	30 (38.5%)
G	85 (65.4%)	48 (61.5%)
Hardy-Weinberg equilibrium p-value	0.23	0.6
Allelic p-value	0.65	
OR (95% CI)	1.18 (0.66-2.11)	

Risk allele is underlined
CMV: cytomegalovirus, OR: odds ratio, CI: confidence interval

Based on the *MICB* results of Shirts et al. (189), our a priori sample size calculations (Fisher's exact test), indicate that 72 donors are needed, consisting of 13 CMV+ and 59 CMV- donors. Our study consisted of 104 donors (39 CMV+ and 65 CMV- persons) and yielded a power of $\beta = 0.99$. The population described by Shirts at al. only consisted of HC and was distributed according to the Hardy-Weinberg equilibrium, albeit borderline ($p<0.06$) according to our calculations.
For *TLR2*, a survival analysis has previously been performed (186). Based on this experiment, our population of 95 samples should yield sufficient power. However, since we do not perform a survival analysis, we recalculated the sample size and power for both our study as well as for the study of Kijpittayarit et al. Here, we demonstrate that neither studies reached sufficient power (Kijpittayarit: $\beta = 0.23$, $\alpha = 0.03$; own study: $n = 95$, $\beta = 0.28$, $\alpha = 0.04$, Fisher's exact test). To reach a power of 0.8, 447 donors, of which 117 CMV+ donors, should be included. Of note, according to our calculations, the CMV- population of Kijpittayarit et al. was not distributed according to the Hardy-Weinberg equilibrium ($p<0.05$).

4.4.2 The effect of genetic predisposition on CD4+CD28null T cell expansion

We analyzed a possible association between the SNPs in *IL2RA*, *TLR2* or *MICB* and CD4+CD28null T cell expansion. First, we validated the previously demonstrated association between CMV status and the percentage of CD4+CD28null T cells in both HC and MS patients (Chapter 3). In this study cohort, we confirm the significantly higher percentage of CD4+CD28null T cells in CMV+ MS patients. Furthermore, the expansion of CD4+CD28null T cells occurred in 40.5% CMV+ donors, but in none of the CMV- controls based on a threshold of 2% (figure 4.1).

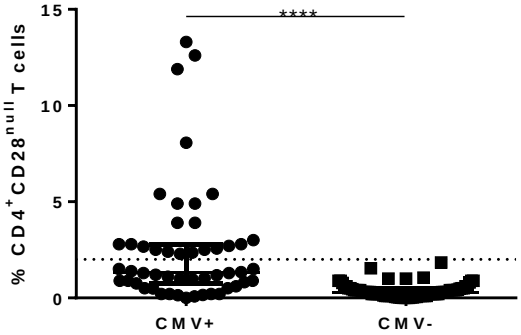


Figure 4.1: Association between CMV serostatus and CD4+CD28null T cell percentage in MS patients. Serostatus was determined via ELFA, CD4+CD28null T cell percentages were measured via flow cytometry. Median ± interquartile range, **** p<0.0001

Since CMV status and CD4+CD28null T cells are related, as shown in figure 4.1 and indicated by our backward selection ($p<0.05$), we corrected for CMV status. Both the CD4+CD28null T cell percentages and the presence or absence of CD4+CD28null T cell expansions were measured in our cohort of MS patients. Statistical analysis for each SNP did not reveal significant associations with CD4+CD28null T cells, as indicated in table 4.5.

Table 4.5: Statistical summary of *IL2RA*, *TLR2* and *MICB* SNPs in donors according to CD4+CD28null T cell percentages and expansions.

	% CD4+CD28null T cells		CD4+CD28null expansion	
	p-value	n	p-value	N
IL2RA	0.71	107	0.62	107
TLR2	0.39	96	0.34	96
MICB	0.4	107	0.25	108

Post hoc power and sample size calculations for the analysis of CD4+CD28null T cell percentages and expansions were based on our own preliminary data. We chose to perform a pilot study to determine the effect size and thus appropriate sample size for future studies. In table 4.6, sample sizes corresponding to $\beta = 0.8$ and $\alpha = 0.05$ are depicted for CD4+CD28null T cell expansions (Fisher's exact test for proportions) and CD4+CD28null T cell percentages (1-way ANOVA, fixed effects) for all 3 SNPs.

Table 4.6: Calculated sample size ($\beta = 0.8$, $\alpha = 0.05$) for CD4+CD28null T cell expansions and CD4+CD28null T cell percentages

	CD4+CD28null expansions		% CD4+CD28null T cells
	+	-	
IL2RA	4427	22310	41145
TLR2	471	2318	1718
MICB	43913	227909	4521

4.5 Discussion

Both environmental and genetic risk factors contribute to the susceptibility for disease development (192). For both CMV infection and MS disease, genetic factors have been identified which confer risk for increased susceptibility. In MS, 110 SNPs have been identified via GWAS, of which most are linked with T cell processes (172-174). Genetic factors involved in CMV infection have mostly been studied in the context of schizophrenia (189, 193-196) or transplantation (184, 186). Since we previously demonstrated that CD4+CD28null T cells are implicated in MS pathology and expand during CMV infection, SNPs involved in both MS and CMV were analyzed for their association with CD4+CD28null T cell expansion.
In this pilot study, we investigated whether an MS-related SNP in *IL2RA* and CMV-associated SNPs in *TLR2* and flanking *MICB* contribute to CD4+CD28null T cell expansion.

First, we analyzed our study population. The RAF and genotype frequencies of the three SNPs showed a highly similar distribution compared to the European population and calculated Hardy-Weinberg equilibrium. Further, we reproduced the finding from our large cohort (Chapter 3) that the CD4+CD28null T cell expansion could only be found in CMV seropositive donors. Taken together, these results indicate that our study cohort is a good representation of the general European population.
Next, we sought to confirm the published associations of the studied SNPs with either MS (for *IL2RA*) or CMV seropositivity (*TLR2* and *MICB*). We could not find a difference in *IL2RA* RAF and genotype frequencies between MS patients and HC. This is in contrast to the GWAS studies (172-174), which show that the risk allele and the TT and TT + TC genotypes of the *IL2RA* polymorphism are associated with MS. Furthermore, the minor allele frequency (allele C) of *IL2RA*, is significantly lower in MS patients compared to controls (172). We observe a similar downwards trend, although not significant. This is probably due to our low sample size and power. In contrast, the size of our cohort should not be a problem for biological association studies, as evidenced by Dendrou et al. (178).

For *MICB*, allele A is associated with CMV antibody seropositivity (189). We obtained sufficient power, but could not reproduce these findings. The main difference lies in the fact that Shirts et al. studied healthy controls and we focused on MS patients. It could be possible that during autoimmune inflammation genetic effects on immune biology are masked by non-genetic disease factors. Moreover, it should be noted that according to our calculations, the CMV+ and – populations of Shirts et al. were only borderline ($p<0.06$) distributed according to the Hardy-Weinberg equilibrium, which could indicate that their cohort is not a good representation of the general population. Further research is needed to determine the role of a SNP flanking *MICB* in CMV incidence. For *TLR2*, homozygosity for this SNP (AA) is associated with a significantly higher incidence of CMV infection compared with heterozygosity (AG) and wild type (GG). Furthermore, the *TLR2* SNP is associated with increased CMV antibody titers and CMV replication (184, 186). We could not reproduce these results, likely because of the lack of AA homozygotes, although our study had a similar sample size. Calculations of sample size and power indicate that for both the study of Kijpitayarit as well as our study, sample size was too small, yielding insufficient power to make any statements. Furthermore, The CMV- group of Kijpittayarit et al. was not distributed according to the Hardy-Weinberg equilibrium. The small sample size, low power and Hardy-Weinberg disequilibrium therefore undermine their published findings.

Other candidate-based gene studies identified genetic risk factors, such as *HLA-DRB1, IL-10, IL-12B, IL-18* pathway genes and *TNF* (194, 196-198), but a GWAS in 2012 could not reveal major genetic determinants involved in either susceptibility to or the strength of antibody response to CMV infection (199). Thus up until now, no confirmed CMV susceptibility genes have been identified.

Finally, a possible link between CD4+CD28null T cell expansion and SNPs in *IL2RA*, *TLR2* and *MICB* was explored. CD4+CD28null T cells do not express the IL-2R complex directly *ex vivo*, but upregulate CD25 expression after stimulation (31). Homozygosity for the *IL2RA* SNP could possibly lead to increased surface expression of this receptor, enhancing their expansion and survival as seen for naive T cells (178, 179).

In addition, it could also increase their granulocyte macrophage colony-stimulating factor (GM-CSF) production, as seen in T helper cells (180, 181). However, due to the small effect size, our study did not reach sufficient power to identify the *IL2RA* SNP as a genetic factor predisposing for the presence of CD4+CD28null T cell expansion.

With regard to the CMV-associated SNPs, a higher susceptibility to CMV infection could increase the formation and expansion of CD4+CD28null T cells. The *TLR2* SNP leads to a defective receptor, dampening the immune response against CMV (186). CD4+CD28null T cells express little or no TLR2 (200), suggesting that the SNP will not directly influence their function. However, a general reduction of the immune response against CMV could lead to higher CMV replication rates and predispose people for CD4+CD28null T cell expansion through indirect mechanisms. The functional outcome of the SNP flanking *MICB* is less clear. We speculate that this SNP prevents MICB-NKG2D interactions, thereby decreasing viral control by NK cells and cytotoxic T cells. Interestingly, CD4+CD28null T cells express NKG2D (35), thus a SNP in *MICB* might influence their cytotoxicity, but also their activation, since NKG2D can serve as a co-stimulatory signal (201).

However, for both CMV-associated SNPs, our pilot study did not reach sufficient power to make any statements about a possible association between SNPs in *TLR2* or *MICB* and CD4+CD28null T cell expansion.

In conclusion, our pilot study investigating the possible association between these SNPs and CD4+CD28null T cell expansion indicate that a larger sample size is required due to a small effect size, which should be considered for future studies.

5

EXPANSION OF CD4+CD28NULL T CELLS: ASSOCIATED WITH A WORSE PROGNOSIS IN MS?

5.1 Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), characterized by inflammation, demyelination and axonal loss. We recently identified CD4+CD28null T cells as autoreactive and cytotoxic effector memory T cells which have the ability to migrate to sites of inflammation, possibly contributing to local tissue damage. Expansion of CD4+CD28null T cells is driven by chronic antigenic stimulation and these cells are present in a subgroup of MS patients. We hypothesize that the presence of CD4+CD28null T cells in MS patients confers a worse clinical progression compared to patients without these cells. To correlate CD4+CD28null expansion with clinical progression, CD4+CD28null T cell percentages were measured in 269 MS patients and we introduced an intuitive classification provided by the attending neurologist called the "intrinsic MS prognostic classification" (IMPC), combining magnetic resonance data, expanded disability status scale (EDSS), disease type, medication, disease duration, evoked potentials (EP) and relapse frequency. The IMPC was correlated with clinical markers of disease severity and disability progression, validating its use in our study. Furthermore, we investigated EP, a useful prognostic tool for disability progression. Patients were scored for motoric (MEP), visual (VEP) and somatosensoric (SEP) evoked potentials, after which a global EP score was calculated. We found that the global EP score correlated with the IMPC, EDSS and MSSS. Within the relapsing remitting MS (RRMS) population, we found that patients with CD4+CD28null T cell expansion had a worse prognosis compared to patients without expansion. Specifically, RRMS patients with expansions were more frequently classified with a severe IMPC as compared to RRMS patients without expansions. In addition, when looking at the total MS population, patients with CD4+CD28null T cell expansion had a higher global EP score compared to patients without expansions, indicating a worse prognosis.

In conclusion, we validated the use of an MS outcome prediction model combining multiple parameters to analyze progression in MS patients. In addition, we found that CD4+CD28null T cells induce a worse disease progression in RRMS patients and predict a worse prognosis in both RRMS and CPMS patients.

5.2 Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) characterized by inflammation, demyelination and axonal loss (65). The leading hypothesis states that peripheral autoreactive T cells are activated after encountering self-antigens, after which they migrate across the blood brain barrier into the CNS (202). Here, these T cells are re-activated, leading to a perpetuation of the local immune response, causing migration of other immune cells to the CNS and ultimately triggering tissue damage (202). Typical MS symptoms include sensation deficits and motor, autonomic and neurocognitive dysfunction, but the clinical disease course of MS varies greatly between individuals making prognosis almost impossible (202). Nevertheless, it is clear from retrospective studies that some patients progress much more rapidly than others (203-206). Besides magnetic resonance imaging (MRI) scans, which visualize new CNS lesions, others clinical parameters are used in the assessment of MS progression. These include: age of onset, time from onset to the development of secondary progressive (SP) MS, relapse rate, Kurtzke expanded disability status scale (EDSS, ranging from 0 to 10 and quantifying the level of disability in MS patients), and multiple sclerosis severity score (MSSS, which combines disease duration and EDSS to compare progression rate within the population or calculate the disease severity of a given population). However, a comprehensive MS outcome prediction model combining multiple parameters is lacking.

CD4+CD28null T cells are effector memory T cells which have lost the expression of CD28. Normally, CD28 co-stimulation is necessary for the activation, proliferation and differentiation of T cells, but CD4+CD28null T cells are still able to induce an immune response (33). Chronic antigen presentation leads to the gradual loss of CD28 on CD4+ T cells and to the expansion of the CD4+CD28null T cells, in a subset of healthy controls (HC) and patients with MS (36).

Next to the loss of CD28, several phenotypic and functional changes occur compared to CD4+CD28+ T cells. CD4+CD28null T cells have shorter telomeres, limited TCR diversity, increased resistance to apoptosis and expression of natural killer (NK) and chemokine receptors. Furthermore, they produce large amounts of IFN-γ and cytotoxic molecules such as granzyme B and perforin (31, 35, 62).

Our group and others have shown that CD4+CD28null T cells are associated with chronic inflammatory disorders (31, 36, 39, 207-209). In atherosclerosis, high frequencies of human CD4+CD28null T cells correlated with disease severity and poor prognosis (154). In rheumatoid arthritis, the expansion of CD4+CD28null T cells positively correlated with the presence of extra-articular manifestations, and the progression of joint destruction occurred significantly faster in patients with CD4+CD28null T cells (62, 155). In MS, a direct link with disease severity has not been demonstrated so far. However, indirect evidence, such as their target tissue infiltrating capacity and cytotoxic activity towards oligodendrocytes, certainly alludes to this hypothesis (36, 45). In addition, we have recently shown that CD4+CD28null T cells expand in an animal model for MS (experimental autoimmune encephalomyelitis, EAE), in which they correlate with disease severity, demyelination and autoinflammatory responses (Chapter 3).

In this study, we aim to investigate whether MS patients with an expansion of CD4+CD28null T cells have a worse clinical disease course compared to MS patients without these expansions. For this, we introduce an intuitive classification provided by the attending neurologist called the "intrinsic MS prognostic classification" (IMPC) combining MRI data, EDSS, disease type, medication, disease duration, evoked potentials (EP) and relapses, thereby creating a powerful MS outcome prediction model.

5.3 Materials and methods

5.3.1 Study subjects

Peripheral blood samples (Li-Heparin coated tubes) were collected from 269 MS patients in collaboration with the University Biobank Limburg (UBiLim). Cytomegalovirus (CMV) status and IgG titers were determined in serum samples via Vidas ELFA (bioMérieux, Marcy l'Etoile, France). This study was approved by the local ethical committee and informed consents were obtained from all donors. Clinical data are presented in table 5.1. Of note, sampling of alemtuzumab-treated patients was done at start of treatment (n=8) or at readministration (n=2). Per definition they are treated, but effects of treatment are probably not present.

Table 5.1: Clinical data of MS patients

	All	**Non-expanded**	**CD28null expanded**
% CD4+CD28null T cells Mean ± SD Min-Max	N = 269 1.83 ± 4.14 0 – 38.5	N = 206 0.47 ± 0.44 0 – 1.94	N = 63 6.26 ± 6.28 2.03 – 38.5
CMV status	N = 202 (67 missing)	N = 156 (50 missing)	N = 46 (17 missing)
Positive	85 (43%)	39 (25%)	46 (100%)
Negative	117 (57%)	117 (75%)	0 (0%)
Gender	N = 268 (1 missing)	N = 205 (1 missing)	N = 63
Female	194 (73%)	145 (70%)	49 (77%)
Male	74 (27%)	60 (30%)	14 (23%)
Age (years) Mean ± SD Min-Max	N = 268 (1 missing) 45.5 ± 12,9 17 – 74	N = 205 (1 missing) 44.8 ± 12.8 17 – 74	N = 63 47.6 ± 12.9 17 – 71
Medication	N= 268 (1 missing)	N = 205 (1 missing)	N = 63
No treatment	119 (44%)	89 (43%)	30 (48%)
Treated	149 (56%)	116 (57%)	33 (52%)
Interferon-β	80	69	11
Glatiramer acetate	34	23	11
Natalizumab	18	12	6
Alemtuzumab	10	7	3
MTX	3	1	2
Teriflunomide	3	3	0
Cyclophosphamide	1	1	0
Disease duration (Years) Mean ± SD Min – Max	N = 230 (39 missing) 10,.53 ± 9.53 0 - 40	N = 176 (30 missing) 10.09 ± 9.6 0 – 40	N = 54 (9 missing) 11.9 ± 9.14 0 – 32
Type MS	N = 269	N = 206	N = 63
Chronic progressive	92 (34%)	68 (33%)	24 (38%)
Relapsing-remitting	177 (66%)	138 (67%)	39 (62%)

N = number of observations; SD = standard deviation; Min – Max = minimum and maximum value; CMV = cytomegalovirus; Disease duration = number of years between onset and sampling, chronic progressive MS = primary and secondary progressive MS.

5.3.2 Flow cytometry

All donors included in this study were analyzed for the percentage of CD4+CD28null T cells. This was done by isolating peripheral blood mononuclear cells from whole blood by density gradient centrifugation (Cedarlane lympholyte, Sheffield, UK). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ). The gating strategy consisted of a lymphocyte gate using the forward and side scatter signal, after which CD4+ T cells were gated and subsequently CD28 expression was monitored within this gate. Cells were acquired using a FACSAria II cytometer, and data were analyzed using BD FACSDiva software. Significant expansion of CD4+CD28null T cells was arbitrarily defined as a percentage ≥2% of the total CD4+ T cell population, as this was the minimal percentage of cells that allowed discrimination of a distinctive population (36).

5.3.3 Clinical markers of disability progression

The total number of relapses is defined as the number of relapses from the date of onset of the disease (=date of first relapse) until the date of sampling (= date of measuring the percentage of CD4+CD28null T cells). Relapses were defined as a worsening of neurological impairment or an appearance of a new symptom/abnormality attributable to MS, lasting at least 24 h and preceded by stability of at least one month. The EDSS, a scale from 0 to 10 measuring impairment or activity limitation, was used to measure disability in MS (210). The MSSS is an algorithm used to assess disease severity based on the EDSS and disease duration and was calculated using MSSStest, a program for implementing the method described by Roxburgh et al (2004) (211).

5.3.4 Evoked potentials

The EP measures include the performance of motoric evoked potential (MEPs), somatosensoric evoked potential (SSEPs) and monocular visual evoked potential (VEPs) to assess the function of sensory pathways. MEPs evaluate the corticospinal excitability via electromyographic (EMG) electrodes positioned at the musculus (m.) abductor pollicis brevis (APB) of the thumb or the m. abductor hallucis (AH) of the hallux (212). The motor cortex is stimulated by a magnetic impulse at the skull which causes contraction of the target muscle (213).

SSEPs assess the function of the dorsal column-lemnical system via electrical stimulation of the median nerve (MED) in the arm, which sends impulses to the brain. These impulses are intercepted via electrodes attached to the head (214). The VEPs measure the functional integrity of the visual pathway from retina, via the optic nerve, to the visual cortex, via electrodes placed at the mid-occipital location (Oz) and mid-frontal location (Fz). VEPs were elicited with a pattern-reversal check board screen. The size of the squares was adjusted to subtend a 15-min visual arc with an individual square (215). The MEP, SSEP and VEP parameters measured for each limb or eye were latency (µs) and amplitude difference (µV).

A global EP score was given to each patient, based on the VEPs, MEPs and SSEPs separately and for each limb of the hemisphere. A divergent parameter was scored 1, normal values were scored 0. The reference values for MEP and SSEP latency were 20 (upper limb) and 40 µs (lower limb), for VEP this was 106 µs. Higher values were deemed divergent. For the amplitudes, no more than 50% difference between the 2 hemispheres may occur to be within the normal range.

The higher the global EP score, the worse the neurophysiological result of the patient. An overview of the scores can be found in table 5.2.

Table 5.2: Overview EP scores

				Cut-off
Global EP N = 68 5.6 ± 2.6 Max score = 12	MEP N = 71 Max score = 6	Latency	**Right upper limb** (APB) 22.5 ± 5.4 (N = 82) Normal (0) 24 (30%) Elevated (1) 58 (70%)	20 µs
			Left upper limb (APB) 22.7 ± 6 (N = 83) Normal (0) 27 (33%) Elevated (1) 56 (67%)	20 µs
			Right lower limb (AH) 45.3 ± 13.3 (N = 80) Normal (0) 27 (33%) Elevated (1) 53 (66%)	40 µs
			Left lower limb (AH) 48.1 ± 13.8 (N = 77) Normal (0) 22 (28%) Elevated (1) 55 (72%)	40 µs
		Amplitude	**Upper limb (APB)** R: 2.5 ± 1.3 (N = 82), L: 2.2 ± 1.2 (N = 83) Normal (0) 68 (83%) Elevated (1) 14 (17%)	Diff. > 50%
			Lower limb (AH) R: 1.4 ± 0.9 (N = 75), L: 1.5 ± 1.1 (N = 74) Normal (0) 54 (75%) Elevated (1) 18 (25%)	Diff. > 50%
	VEP N = 79 Max score = 3	Latency	**Right eye** 118.5 ± 21.4 (N = 81) Normal (0) 26 (32%) Elevated (1) 55 (68%)	106 µs
			Left eye 118.7 ± 20 (N = 81) Normal (0) 26 (32%) Elevated (1) 55 (68%)	106 µs
		Amplitude	R : 8.9 ± 4.7 (N = 81), L : 8.6 ± 4.4 (N = 81) Normal (0) 73 (92%) Elevated (1) 6 (8%)	Diff. > 50%
	SEP N = 82 Max score = 3	Latency	**Right upper limb (MED)** 20.5 ± 2.2 (N = 82) Normal (0) 40 (49%) Elevated (1) 42 (51%)	20 µs
			Left upper limb (MED) 20.9 ± 3.6 (N = 83) Normal (0) 42 (51%) Elevated (1) 41 (49%)	20 µs
		Amplitude	R : 3.9 ± 2.5 (N= 82), L : 4.3 ± 2.8 (N = 83) Normal (0) 66 (80%) Elevated (1) 16 (20%)	Diff. > 50%

Motoric (MEP), visual (VEP) and somatosensoric (SEP) evoked potentials, abductor pollicic brevis (APB), abductor halluces (AH), median nerve (MED), right (R), left (L), difference (diff.). Mean ± SD

5.3.5 Definition of the intrinsic MS prognostic classification

We introduced an intuitive classification provided by the treating neurologist called IMPC. This classification is based on MRI, clinical (disease type, disease duration, EDSS, frequency of relapses) and electrophysiological data (multimodal EP) collected at the time of blood sampling or during the initial disease stage (<5 years). Patients were classified as having a mild, medium or severe expected disease course, based on the combination of the given data, but without using a fixed algorithm.

5.3.6 Statistical analysis

The data set was analyzed using SAS JMP (JMP Pro 12, SAS Institute, USA). A p-value < 0.05 was considered significant. Figures were plotted with Graphpad Prism 6.0 (Graphpad software Ing, USA, version 6). To identify multicollinearity, all pairwise associations were computed. After normality checks (d'Agostino-Pearson), a student's t-test was used to compare two populations when normality assumptions were met. Otherwise, non-parametric tests for differences between levels of a factor were conducted using a Kruskal-Wallis test, based on Wilcoxon scores. A chi-square or fisher's exact test was used to investigate the significance of association between factors. Five response variables were studies (IMPC, number of relapses, EDSS, MSSS and global EP score). Linear or generalized linear regression models were used to test for the presence of effect of the factors of interest. Linear models were used for continuous variables for which normality of the error terms could be assumed (EDSS, MSSS, global EP score). Logistic models were used for the nominal response variables (=IMPC). Generalized linear models with Poisson distribution were used when count data were analysed (=number of relapses).

The analysis started with a full model with the main effects of CD28nullexp-level (CD4+CD28null T cell expanded versus not-expanded), type MS-level (CP versus RR), CMV status-level (seropositive versus seronegative), gender (female versus male), age-level (17- 74 years), medication (treated versus not-treated), disease duration (0- 40 years), as well as the interaction effect of CD28nullexp-level and type MS-level. Backward model selection was run until all terms in the model or interactions involving them were significant.

5.4 Results

5.4.1 The IMPC is correlated with clinical markers of severity and disability progression

We hypothesize that MS patients with CD4+CD28null T cell expansion have a worse disease course compared to patients without expansion. However, a clinical prediction model combining multiple parameters is lacking. Therefore, we introduced the intuitive IMPC ranging from mild and medium to severe. Before this scoring system can be implemented, validation is needed. This is accomplished by studying the IMPC in relation to several established clinical markers of severity and progression. MS patients with a severe or medium IMPC have more relapses compared to MS patients with a mild IMPC ($p<0.05$, figure 5.1A). MS patients with a higher IMPC also have a significantly higher EDSS ($p<0.001$, figure 5.1B) and MSSS ($p<0.001$ and $p<0.0001$, figure 5.1C).

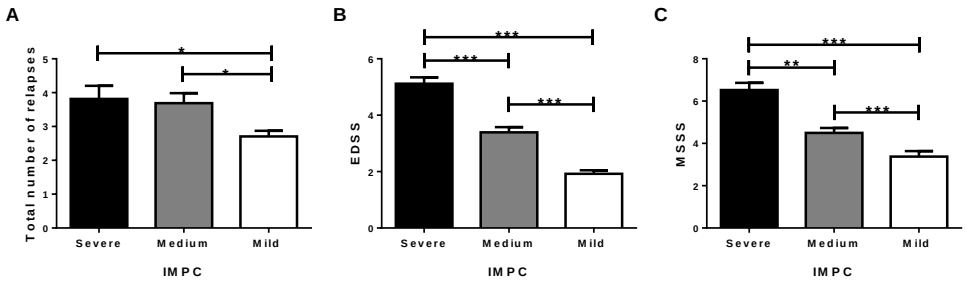


Figure 5.1: The IMPC is correlated with clinical markers of severity and disability progression: (**A**) Total number of relapses for patients with severe (N = 48), medium (N = 100) and mild (N = 65) IMPC; (**B**) Expanded Disability Status Scale (EDSS) for patients with severe (N = 60), medium (N = 112) and mild (N= 82) IMPC; (**C**) Multiple Sclerosis Severity Score (MSSS) for patients with severe (N = 44), medium (N = 96) and mild (N = 77) IMPC. Data are shown as mean ± SD, * $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

5.4.2 The IMPC is correlated with the global EP score, a prognostic marker for disability progression

We also investigated EP, which reveal early infra-clinical lesions on the long sensory-motor pathways and constitute a potentially useful prognostic tool for disability progression (216). Patients were scored for MEP, VEP and SEP, after which a global EP score was constructed. The separate MEP, VEP and SEP scores strongly correlated with EDSS, MSSS and more importantly with the IMPC (table 5.3).

Therefore, it is not surprising that the global EP score also correlated with EDSS ($p<0.001$), MSSS ($p=0.0001$) and IMPC ($p=0.0003$). We could not find a correlation with the total number of relapses for either separate or global EP scores.

Table 5.3: Correlation of evoked potential scores with clinical parameters and IMPC.

EP score	parameter	Statistical test	p-value
MEP	Total Nb of relapses	Log.regression	0.39
	EDSS	Log.regression	<0.0001
	MSSS		<0.0001
	IMPC	FET	0.0004
VEP	Total Nb of relapses	Log.regression	0.18
	EDSS	Log.regression	0.0007
	MSSS	Log.regression	0.0037
	IMPC	FET	0.04
SEP	Total Nb of relapses	Log.regression	0.18
	EDSS	Log.regression	<0.0001
	MSSS	Log.regression	<0.0001
	IMPC	FET	0.0003
Global EP	Total Nb of relapses	Log.regression	0.09
	EDSS	Log.regression	<0.0001
	MSSS	Log.regression	0.0001
	IMPC	FET	0.0003

motoric (MEP), visual (VEP) and somatosensoric (SEP) evoked potentials; Number (Nb), Expanded disability status scale (EDSS), Multiple Sclerosis Severity Score (MSSS), Intrinsic MS prognostic classification (IMPC), Logistic (log.), Fischer's exact test (FET)

5.4.3 Construction of the statistical model: association between independent variables

To investigate the impact of CD4+CD28null T cell expansion on MS disease progression, we constructed a statistical model including CD4+CD28null T cell expansion and following confounding variables: type MS, CMV status, gender, age, medication and disease duration. To test for multicollinearity, the associations between the independent variables in the statistical model were checked (table 5.4).

Table 5.4: Association between independent variables.

		Test	p-value
CD28nullexp	CMV status	FET	**p<0.0001**
	Gender	FET	p=0.33
	Age	Log. regression	p=0.14
	Medicated_cat	FET	p=0.56
	Disease duration	Log. regression	p=0.19
	Type_MS	FET	p=0.45
CMV status	Gender	FET	p=0.87
	Age	Log. regression	p=0.12
	Medicated_cat	FET	p=0.77
	Disease duration	Log. regression	p=0.10
	Type_MS	FET	p=0.99
Gender	Age	Log. regression	p=0.27
	Medicated_cat	FET	p=0.78
	Disease duration	Log. regression	p=0.93
	Type_MS	FET	p=0.88
Age	Medicated_cat	Wilcoxon	**p=0.002**
	Disease duration	Lin. regression	**p<0.0001**
	Type_MS	Wilcoxon	**p<0.0001**
Medicated_cat	Disease duration	Wilcoxon	p=0.32
	Type_MS	FET	**p=0.0001**
Disease duration	Type_MS	Wilcoxon	**p<0.0001**

Cd28nullexp: CD4+CD28null T cell expanded versus non-expanded; CMV: cytomegalovirus; medicated_cat: treated versus not treated; disease duration: number of years between onset of the disease and sampling; Type_MS = classification of MS disease type in chronic progressive MS (primary progressive and secondary progressive MS) and relapsing remitting MS; FET = Fisher's exact test; log. regression= logistic regression; lin. regresion = linear regression.

We found that CD4+CD28null T cell expansion occurred only in CMV seropositive MS patients in line with our earlier reports (chapter 3). Indeed, 54% of seropositive individuals showed CD4+CD28null T cell expansion versus 0% of seronegative individuals ($p<0.0001$, figure 5.2A). Treated MS patients were older compared to untreated MS patients ($p<0.05$, figure 5.2B) and CPMS patients were older compared to RRMS patients ($p<0.0001$, figure 5.2C). Evidently, disease duration increased with age ($p<0.0001$, figure 5.2D), explaining the longer disease duration in CPMS patients compared to RRMS patients ($p<0.0001$, figure 5.2E). Furthermore, RRMS patients were treated more often compared to CPMS patients ($p=0.0001$, figure 5.2F). There was no significant effect of gender, age, treatment, disease duration and MS type on CD4+CD28null T cell expansion or CMV status. In addition, there was no association between gender and age, treatment, disease duration or MS type, nor between treatment and disease duration.

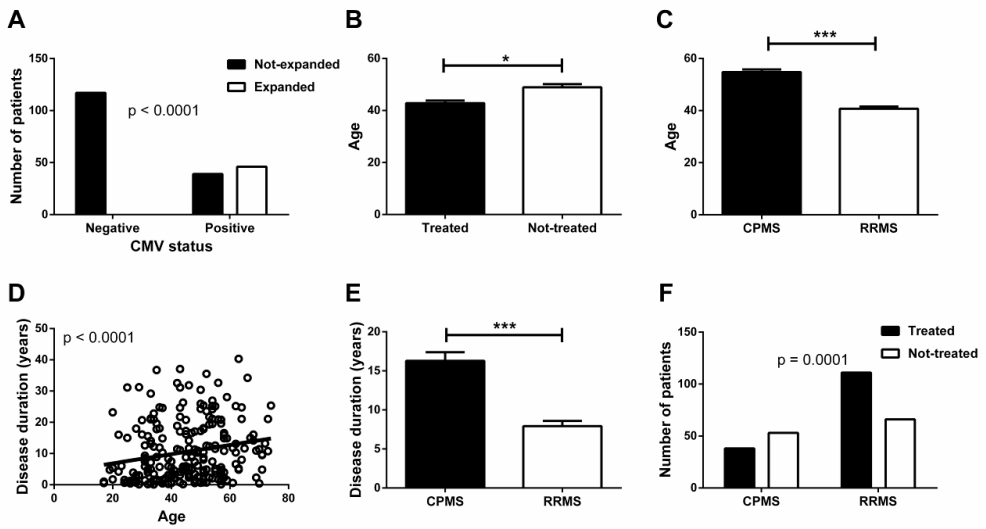


Figure 5.2: Association between independent variables: (**A**) Number of cytomegalovirus (CMV) seronegative (N = 117) and seropositive (N = 85) donors with versus without CD4+CD28null T cell expansion. (**B**) Mean age of treated (N = 149) versus not-treated (N = 118) MS patients. (**C**) Age of CPMS (N = 92) versus RRMS (N = 176) patients. (**D**) Age versus disease duration (N = 229). (**E**): disease duration in CPMS (N = 72) versus RRMS (N = 148) patients. (**F**): Treatment versus type of MS (CP: N = 92 and RR: N = 176). * p <0.05; *** p < 0.001.

5.4.4 RRMS patients with CD4+CD28null T cell expansion have a worse prognosis compared to patients without expansion

To investigate whether MS patients with CD4+CD28null T cell expansion have a worse disease course compared to patients without this expansion, we investigate the effect of CD4+CD28null expansion on 5 parameters measuring MS disease severity, disability progression and prognosis: 1) IMPC, 2) total number of relapses, 3) EDSS, 4) MSSS and 5) global EP score (table 5.5).

Table 5.5: Overview of response variables

	All	Non-expanded	CD28null expanded
IMPC	N = 269	N = 206 (75%)	N= 63 (25%)
Mild	86 (32%)	66 (32%)	20 (32%)
Average	120 (45%)	95 (46%)	25 (40%)
Severe	63 (23%)	45 (22%)	18 (28%)
Total relapses	N = 213 (56 missing)	N = 162 (44 missing)	N = 51 (12 missing)
Mean ± SD	3.4 ± 2.5	3.4 ± 2.4	3.5 ± 3.0
Min-Max	1 - 19	1 – 19	1 - 15
EDSS	N = 254 (15 missing)	N = 196 (10 missing)	N = 58 (5 missing)
Mean ± SD	3.3 ± 2.1	3.2 ± 2.1	3.7 ± 1.8
Min-Max	0 - 8	0 – 8	0 - 7
MSSS	N = 217 (52 missing)	N = 167 (39 missing)	N = 50 (13 missing)
Mean ± SD	4.5 ± 2.5	4.5 ± 2.5	6.3 ± 6.9
Min-Max	0.05 – 9.74	0.04 – 9.74	2.03 – 38.5
Global EP score	N = 68	N = 47	N = 21
Mean ± SD	5.6 ± 2.6	5.1 ± 2.4	6.7 ± 2.8
Min-Max	1 – 11	1 - 9	2 - 11

N = number of observations; IMPC = intrinsic MS prognostic classification; EDSS = expanded disability status scale; MSSS = multiple sclerosis severity score; EP = evoked potential

The analysis started with a full model including all independent variables, as well as the interaction effect of CD4+CD28null T cell expansion and MS type. Backward model selection was run until all terms in the model or interactions involving them were significant (table 5.6).

Table 5.6: Significance of factors in initial and final multivariable models.

	Total relapses		**EDSS**		**MSSS**		**Global EP**		**IMPC**	
test	*Gen. linear model*		*Linear regression*		*Linear regression*		*Linear regression*		*Nom.log. regression*	
model	*Full*	*Red.*	*Full*	*Red.*	*Full*	*Red.*	*Full*	*Red.*	*Full*	*Red.*
CD28nullexp*Type_MS	0,36	-	0,40	-	0,48	-	0,12	-	0,12	*
CD28nullexp	0,83	-	0,58	-	0,48	0,84	0,61	*	*	-
Type_MS	0,08	-	****	****	****	****	0,11	-	****	****
CMV status	0,78	-	0,33	-	0,81	-	0,67	-	0,24	-
Gender	0,17	**	0,26	-	0,11	*	0,33	-	0,72	-
Age	**	***	*	*	0,01	**	0,40	-	0,18	-
Medication	*	-	0,52	-	0,81	-	0,41	-	0,29	-
Disease duration	****	****	**	**	****	****	0,63	-	0,21	-
Observations	145	190	165	216	165	216	51	68	176	269

Expanded Disability Status Scale (EDSS), Multiple Sclerosis Severity Score (MSSS), global EP (evoked potential), Intrinsic MS prognostic classification (IMPC), nominal logistic regression (Nom. Log. Regression); generalized linear model (gen. linear model). The full model included all factors, the reduced model (Red.) included the factors following backward selection for IMPC. Cd28nullexp = CD4+CD28null T cell expanded versus non-expanded; Type_MS = classification of disease type in chronic progressive and relapsing remitting MS; CMV status = cytomegalovirus seropositive versus seronegative; Medication = treated versus untreated; Disease duration = number of years between disease onset and sampling. * p<0.05, **p<0.01, ***p<0.001**** p<0.0001

As expected, analyses showed that CPMS patients were more often classified with a severe IMPC as compared to RRMS patients ($p<0.0001$, figure 5.3A). There was no effect on number of relapses (figure 5.3B), but they had higher EDSS ($p<0.001$, figure 5.3C), MSSS ($p<0.001$, figure 5.3D) and global EP scores ($p<0.01$, figure 5.3E) compared to RRMS patients. This validates the model implemented here.

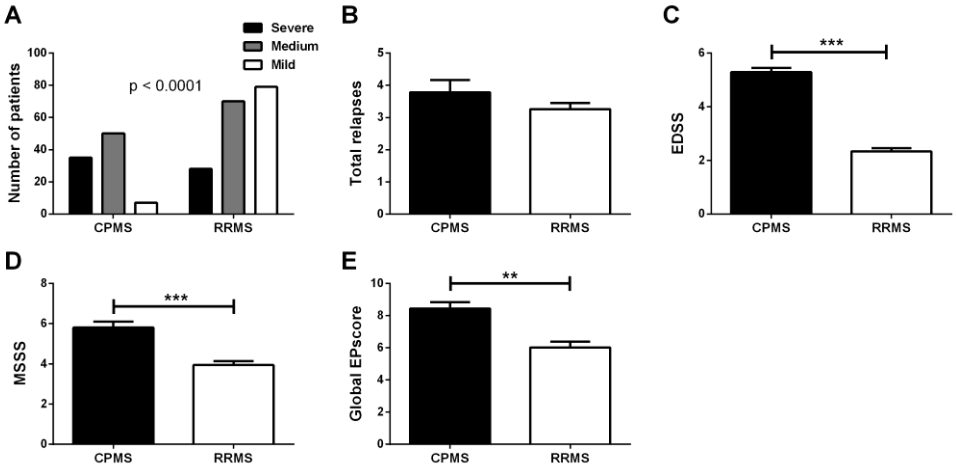


Figure 5.3: Effect of type of MS on progression parameters. Five different progression parameters were investigated: Intrinsic MS progression classification (IMPC, **A**); total number of relapses (**B**); Expanded Disability Status Scale (EDSS, **C**); Mean Multiple Sclerosis Severity Score (MSSS, **D**) and global evoked potential scores (global EP scores, **E**). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

For the IMPC, the effect of the interaction of CD4+CD28null T cell expansion and MS type (CD28nullexp*typeMS) was significant ($p<0.05$, table 5.6). This indicates that the effect of CD4+CD28null T cell expansion on the IMPC is different for RRMS patients compared to CPMS patients. Indeed, a hypothesis-driven approach showed that RRMS patients with CD4+CD28null T cell expansion were more often classified with a severe IMPC as compared to RRMS patients without expansion ($p<0.05$, figure 5.4, row 1). In contrast, CPMS patients with CD4+CD28null T cell expansion were less often classified with a severe IMPC compared to CPMS patients without expansion.

When combining all patients, no effect can be found ($p<0.05$, figure 5.4, row 1). With regard to other progression parameters, RRMS patients with versus without CD4+CD28null T cell expansion had a higher EDSS ($p<0.05$), which was not the case for CPMS or total MS patients (figure 5.4, row 3).

No significant effect of CD4+CD28null T cell expansion on number of relapses and MSSS was found (figure 5.4, rows 2 and 4). However, a significantly higher global EP score was found in patients with versus without expansion ($p<0.05$, table 5.6 and figure 5.4, row 5).

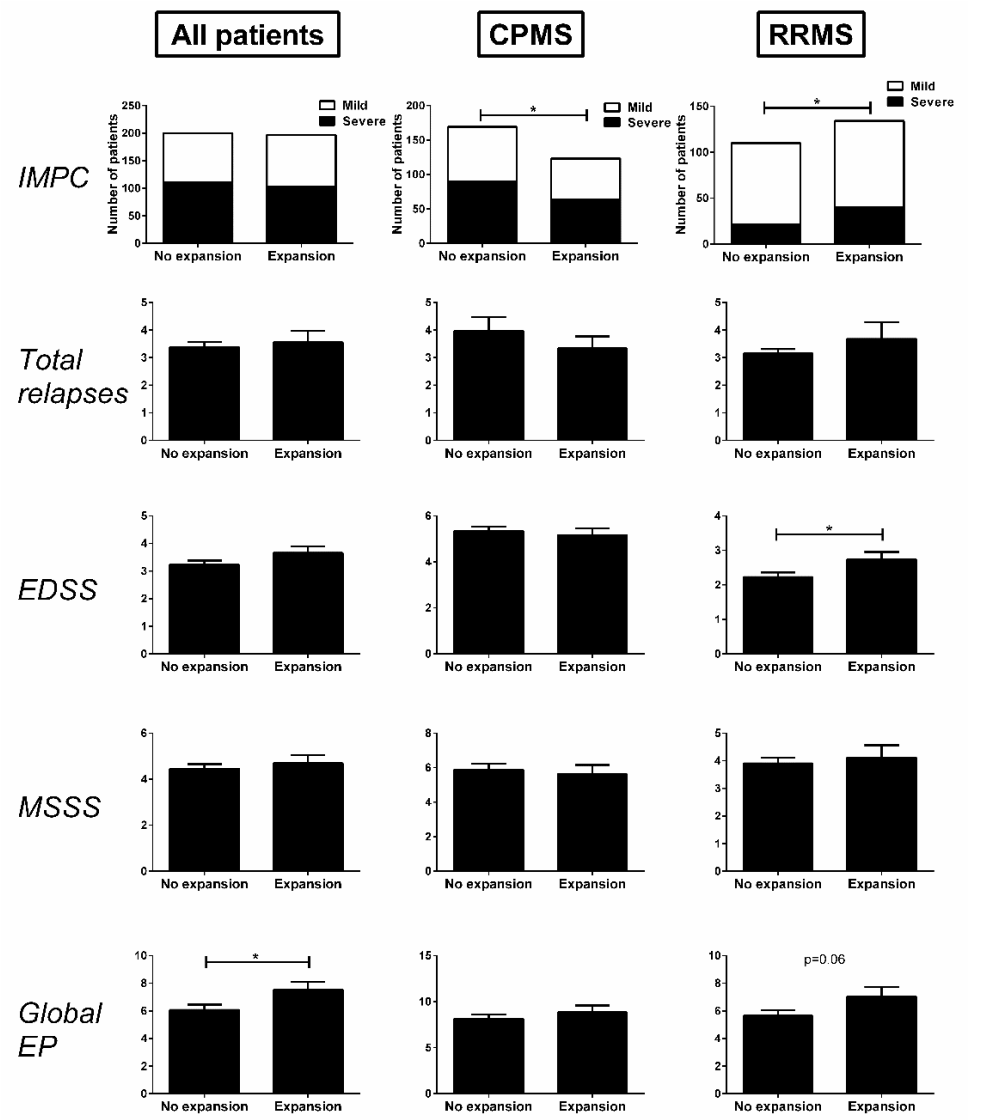


Figure 5.4: Effect of CD4+CD28null T cell expansion on progression parameters. Expansion is defined as a percentage of CD4+CD28null T cells above the threshold (>2% of $CD4^+$ T cells). Different progression parameters were investigated: Intrinsic MS progression classification (IMPC, row 1); total number of relapses (row 2); Expanded Disability Status Scale (EDSS, row 3); Multiple Sclerosis Severity Score (MSSS, row 4); and global evoked potential scores (global EP scores, row 5). The effect of CD4+CD28null T cell expansion differs for total (left panel), chronic progressive (CP, middle panel) and relapsing remitting (RR, right panel) MS patients. * $p<0.05$, without correction for multiple testing (hypothesis-driven approach).

5.5 Discussion

Although the etiology of MS is not fully understood yet, autoreactive T cells are considered to be one of the main players in the pathogenesis. Absence of CD28 expression on CD4+ T cells alters their phenotypic and functional characteristics, making them cytotoxic (36). Furthermore, we found that CD4+CD28null T cells migrate to sites of inflammation, where they potentially contribute to the inflammatory processes and local tissue damage. Since these T cells are present in a subgroup of MS patients, we investigated whether these patients showed a worse clinical progression compared to patients without expansion of CD4+CD28null T cells.

Expansion of CD4+CD28null T cells is found in several autoimmune and chronic inflammatory diseases, including atherosclerosis (154), RA (62, 155) Graves' disease (217), Wegener's granulomatosis (218) and sporadic inclusion body myositis (70). Importantly, the presence of these cells has been shown to be associated with disease severity and poor prognosis in atherosclerosis and RA (62, 154, 155). In MS, a direct link with disease severity and/or prognosis has not been demonstrated so far. This is possibly due to the clinical heterogeneity of MS between and within subjects, making prediction of the clinical course at the individual level challenging. MRI and CSF biomarkers are promising sources of prognostic information with a good potential of quantitative measure, sensitivity, and reliability. However, despite the large numbers of candidate molecular biomarkers proposed, very few biomarkers have been rigorously validated and used in clinical practice (219).

A comprehensive MS outcome prediction model combining multiple parameters is still lacking (220). To measure disability in MS, the standard assessment tool used is the EDSS (scale from 0 to 10 measuring impairment or activity limitation)(210). The MSSS is an algorithm used to assess disease severity based on the EDSS and disease duration (221). Others clinical parameters include: age of onset, time from onset to the development of SPMS, relapse rate and total number of relapses. However, some of these parameters are temporary and change with age and/or disease duration. Indeed, a significant effect of gender, age and/or disease duration was found when investigating number of relapses, EDSS and MSSS.

The reduced models for these parameters were more complex, since they needed to include these independent variables, thereby reducing the power of the analyses.

In this study, we introduced an intuitive classification provided by the treating neurologist called the IMPC. Patients were classified as having a mild, medium or severe expected disease course, based on the combination of information about MRI, clinical and electrophysiological data collected at the time of blood sampling or during the initial disease stage (<5 years). The use of a combination of prognostic parameters to determine the severity and prognosis for the individual patient was recently shown to be very useful to investigate prognosis (222). Furthermore, the IMPC takes a lot of confounding variables like gender, age, medication and disease duration into account, since the treating neurologist uses these parameters when classifying the patients, thereby increasing the power of the analyses. In our study, the IMPC correlated with clinical markers of disease severity and disability progression (total number of relapses, EDSS and MSSS) and with the global EP score, a prognostic marker for disability progression (216), validating its use as a predictive model. We realize that additional research is needed to the test the validity and reproducibility of the IMPC. One way forward could be a validation study through the MSBase Registry in which mathematical modelling is performed using the intuitive classification of many different neurologists combined with the already available clinical data from all the patient in this database.

We found that MS patients with CD4+CD28null T cells expansion had a worse prognosis compared to patients without expansion based on the IMPC (only RRMS) and global EP score (both RR and CPMS).

RRMS is characterized by self-limited attacks of neurologic dysfunction. These attacks develop acutely, evolving over days to weeks. Over the next several weeks to months, most patients experience a recovery of function that is often (but not always) complete. Between attacks the patient is neurologically and symptomatically stable. CD4+ T cells are considered to be the most critical in the RRMS disease pathology as they contribute to CNS inflammation directly through the secretion of pro-inflammatory cytokines or indirectly through attraction of other immune cells (223).

Since CD4+CD28null T cells are cytotoxic and pro-inflammatory, we hypothesize that they contribute to these inflammatory processes in a subgroup of RRMS patients. The effect of CD4+CD28null T cells in CPMS seems to be different. In CPMS patients, expansion of CD4+CD28null T cells appeared to be beneficial, since these patients were less often classified with a severe IMPC. This might be explained by the fact that in CPMS, the clinical course becomes characterized by a steady deterioration in function, due to ongoing demyelination as a result of intrathecal inflammation and less prominent involvement of peripheral autoreactivity (205, 224). Therefore, the role of CD4+CD28null T cells in CPMS patients might by comparable to their role in healthy individuals, where CD4+CD28null T cells have anti-viral and anti-tumor effects (10, 11).

In the total MS population, patients with CD4+CD28null T cell expansion had higher global EP scores compared to MS patients without expansion. Since this scoring system is already an established prognostic marker for progression, the presence of CD4+CD28null T cells could predict a worse outcome in MS patients. No difference was found in RRMS versus CPMS patients, possibly because the sample size was too low. Forthcoming research should investigate this in more detail.

5.6 Conclusion

In conclusion, we have found that an MS outcome prediction model combining multiple parameters can increase the power of analyses involving prognosis of MS patients. In this study, we introduced an intuitive classification provided by the treating neurologist, called the IMPC. This classification combined information about MRI, clinical and electrophysiological data collected at the time of blood sampling. Because multiple parameters were combined, variables like disease duration, age, gender, CMV status and medication had no effect. The IMPC correlated with clinical markers of disease severity and disability progression (total number of relapses, EDSS and MSSS) as well as with a prognostic marker (global EP), but is still an intuitive classification provided by one treating neurologist. To use this classification globally, standardisation is required, which we want to accomplish in the near future. We will determine and implement cut-off values for each individual parameter (EDSS, MSSS, …) in order to uniformly divide patients into the IMPC categories ranging from mild to severe. Additionally, MS patients could benefit from screening for CD4+CD28null T cells, since expansion of these cells predict a worse outcome. Therapies targeting CD4+CD28null T cells could be beneficial in the subgroup of MS patients exhibiting a CD4+CD28null T cell expansion, thereby preventing disease exacerbations.

6

CD4+CD28NULL T CELLS EVADE TREG-MEDIATED SUPPRESSION AND BOOST TH17 RESPONSES

6.1 Abstract

Cytotoxic CD4+CD28null T cells arise during chronic activation of the immune system. They are thought to contribute to many inflammatory diseases, including multiple sclerosis (MS). In this study, we found that CD4+CD28null T cells have an increased expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-22, IFN-γ and GM-CSF compared to CD4+CD28+ T cells, and display higher levels of HOPX, a survival-related transcription factor. Surface receptors IL-10R and GITR are decreased, whereas PD1 expression is increased on CD4+CD28null T cells. Furthermore, we demonstrate that the secretome of CD4+CD28null T cells induces *in vitro* differentiation of memory T cells towards Th17 cells and affects the phenotype of Tregs. Previous research has shown that CD4+CD28null T cells are resistant to Treg-mediated suppression *in vitro.* Here we demonstrate that addition of granzyme B and IFN-γ inhibitors rescues Treg-mediated suppression of CD4+CD28null T cells, suggesting that CD4+CD28null T cells evade Treg suppression via the production of both molecules.

Taken together, our *in vitro* experiments indicate that CD4+CD28null T cells may contribute to inflammation in three distinct ways: production of pro-inflammatory cytokines, evasion of Treg-mediated suppression and induction of pathogenic Th17 cells, all factors known to promote chronic autoimmune disease processes.

6.2 Introduction

Cytotoxic CD4+CD28null T cells arise during chronic activation of the immune system. Repeated antigenic stimulation causes the loss of CD28 expression, the emergence of their oligoclonal nature, coinciding with a restricted TCR diversity (35, 37, 140-142). Furthermore, CD4+CD28null T cells are co-stimulation independent, resistant to apoptosis and less susceptible to suppression by regulatory T cells (Tregs) (31, 35, 59, 110, 143). They are thought to contribute to many inflammatory diseases, because of their cytotoxic capacities via the expression of natural killer (NK) cell receptors and the production of perforin and granzymes; their capability of infiltrating target tissues via e.g. the fractalkine gradient; and their autoreactive nature (36, 110, 144)(chapter 1).

CD4+CD28null T cells were shown to play a role in the pathogenesis of several autoimmune diseases (chapter 1), including multiple sclerosis (MS), a demyelinating autoimmune disease of the central nervous system (CNS). T cells play a common role in the pathogenesis of autoimmune diseases, more specifically Tregs are shown to be dysfunctional and Th1 and Th17 cells are pathogenic (36, 65, 225, 226). Recently, we showed that CD4+CD28null T cells correlate with disability in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and migrate towards the inflamed CNS (chapter 3). We also demonstrated a correlation between CD4+CD28null T cell expansion and a worse prognosis in MS patients (chapter 5).

In this article, we aim to elucidate by which mechanism(s) CD4+CD28null T cells contribute to the inflammatory response in autoimmune diseases such as MS. Therefore, we investigated how these cells affect Treg phenotype and functionality, and whether they contribute to the differentiation of pathogenic T cell subsets (Th1, Th17) using *in vitro* assays.

6.3 Materials and methods

6.3.1 Study subjects

Peripheral blood samples were collected from healthy controls (HC) in collaboration with the University Biobank Limburg (UBiLim). This study was approved by the local ethical committee and informed consents were obtained from all donors.

6.3.2 Flow cytometry

All donors included in this study, were analyzed for the percentage of CD4+CD28null T cells. This was established by isolating PBMCs from whole blood by density gradient centrifugation (Cedarlane lympholyte, Sheffield, UK). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ). Expansion of this subset is defined as a percentage >2% of the CD4+ T cell population. The gating strategy consists of a lymphocyte gate, using forward and side scatter parameters, followed by a CD4 gate in which CD28 expression was monitored. Additional antibodies that were used for phenotypic characterization by flow cytometry are anti-human IL-10R PE (Biolegend), GITR APC and PD1 PE-Cy7 (eBioscience Buckinghamshire, UK). Cells were acquired using a FACSAria II cytometer, and data were analyzed using BD FACSDiva software.

6.3.3 Stimulation assays

Positive selection of CD4+ T cells from PBMCs was performed according to the manufacturer's protocol (Easysep, Stemcell technologies, Vancouver, Canada). Next, CD4+ T cells were stained with anti-human CD4 v4 FITC, CD28 APC (BD), CD25 PerCP Cy5.5 and CD127 PE (eBioscience), and CD4+CD28null T cells, CD4+CD28+ T cells and CD4+CD25^{hi}CD127^{low} Tregs were sorted using a FACSAria II cytometer and FACSDiva software (Figure 6.1).

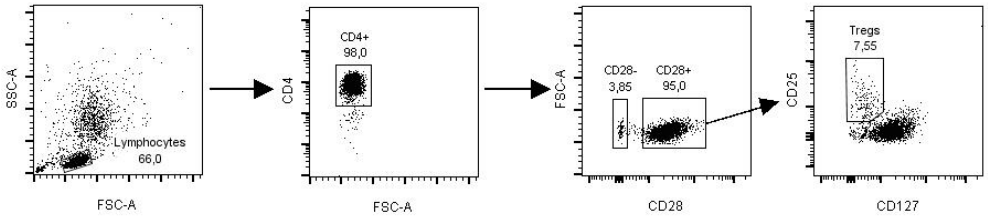


Figure 6.1: Sort strategy. First lymphocytes were gated, using the forward and side scatter (FSC, SSC). Next, CD4+ T cells were selected, followed by a discrimination of the CD28 signal (CD28- versus CD28+). Within the CD28+ gate, Tregs were gated according to CD25 and CD127.

CD4+CD28null and CD28+ T cells were stimulated with anti-human anti-CD3 (2 µg/ml, Biomed clone) and IL-2 (0.1 U/ml, Roche Diagnostics, Basel, Switzerland) in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Hyclone Europe, Erembodegem, Belgium), 1% nonessential amino acids, 1% sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin (all Life technologies). After 5 days, cells and supernatants (CM) were collected (centrifuged and frozen at -20°C (supernatant) and -80°C (cells)).

Tregs were stimulated with plate-bound anti-human anti-CD3 (1 µg/ml, OKT3, eBioscience), anti-CD28 (1 µg/ml, BD) and IL-2 (25 U/ml) in presence or absence of 50% (v/v) CM of activated CD4+CD28null T cells or CD4+CD28+ T cells in RPMI-1640. After 0 and 72h, cells were collected for subsequent qPCR.

6.3.4 qPCR

RNA was isolated using the RNeasy kit (Qiagen, Venlo, the Netherlands) and converted to cDNA via Qscript according to the manufacturer's protocol (Quanta bio, Beverly, MA). Human primers were purchased from IDT (table 6.1). All reactions, containing SYBR green (Applied Biosystems, Waltham, MA), were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Values are represented as the difference in CT values normalized to appropriate household genes (*ActB*, *CYCA* and *RPL13A*) for each sample.

Table 6.1: Human qPCR primers

gene	Full name	Forward primer	Reverse primer
A2AR	Adenosine A2A receptor	CGCTCCGGTACAATGGCTT	TTGTTCCAACCTAGCATGGGA
ActB	Actin B	GATCATTGCTCCTCCTGAGC	AAAGCCATGCCAATCTCATC
CD39	Ectonucleoside triphosphate diphosphohydrolase-1	ACTATCGAGTCCCCAGATAATGC	CCTGATCCTTCCCATAGCACAA
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	TGGATTTCAGCGGCACAAGGCT	CTGGGCCACGTGCATTGCTTTG
CYCA	Cyclin A	AGACTGAGTGGTTGGATGGC	TCGAGTTGTCCACAGTCAGC
FasL	Fas ligand	AAAGTGGCCCATTTAACAGGC	AAAGCAGGACAATTCCATAGGTG
GARP	Glycoprotein A Repetitions Predominant	AGACCCTTGATCTATCTGGGAAC	GAAGCTGATCTCATTGGTGCT
GITR	glucocorticoid-induced TNFR-related protein	CGAGTGGGACTGCATGTGTG	GGCAGGTCGTGCAGCAA
GM-CSF	Granulocyte-macrophage colony-stimulating factor	CCAGGAGCCGACCTGCCTACA	GAAGTTTCCGGGGTTGGAGGGC
GrB	Granzyme B	GCGAATCTGACTTACGCCATTA	CCAGAGTCCCCCTTAAAGGAA
HOPX	Homeodomain-only protein	TCAACAAGGTCGACAAGCAC	TCTGTGACGGATCTGCACTC
IFN-γ	Interferon-γ	GGGGCCAACTAGGCAGCCAAC	AAGCACTGGCTCAGATTGCAGGC
IL-10	Interleukin-10	GCTGTCATCGATTTCTTCCC	ATAGAGTCGCCACCCTGATG
IL-10Rα	Interleukin-10 Receptor α	CCTCCGTCTGTGTGGTTTGAA	CACTGCGGTAAGGTCATAGGA
IL-1β	Interleukin-1β	GATGAAGTGCTCCTTCCAGG	GCATCTTCCTCAGCTTGTCC
IL-22	Interleukin-22	AACCGCACCTTCATGCTGGCT	CGCTCACTCATACTGACTCCGTGG
IL-6	Interleukin-6	GAGGAGACTTGCCTGGTGAA	GCTCTGGCTTGTTCCTCACT
LAG3	Lymphocyte-activation gene 3	GCCTCCGACTGGGTCATTTT	CTTTCCGCTAAGTGGTGATGG
PD1	Programmed cell death protein 1	CTCAGGGTGACAGAGAGAAG	GACACCAACCACCAGGGTTT
RPL13A	Ribosomal Protein L13a	AAGTTGAAGTACCTGGCTTTCC	GCCGTCAAACACCTTGAGAC
TGF-β	Transforming growth factor β	GTGGAAACCCACAACGAAAT	CACGTGCTGCTCCACTTTTA

6.3.5 Treg suppression assay

CD4+ T responder cells ($CD28^-$ or $CD28^+$; $2x10^4$ cells/well) were stained with CFSE (2 µM, Invitrogen) and co-cultured with Tregs (1/1: $2x10^4$ or 1/0.5: $1x10^4$ cells/well) and irradiated feeders ($1x10^5$ cells/well) in 96-well round-bottom plates in culture medium as previously described (31, 227). Co-cultures were stimulated with human anti-CD3 (2 µg/ml, Biomed clone) and IL-2 (0.1 U/ml) for 5 days after which CFSE dilution was determined using FACS analysis. Supernatants was collected for IFN-γ ELISA which was executed according to the manufacturers' protocol (Ready-set-go kit, eBioscience). In parallel conditions, culture medium was supplemented with the following neutralizing anti-human antibodies or inhibitors: anti-IFN-γ (10 µg/ml; R&D systems), anti-GITRL (7µg/ml; R&D systems), granzyme B inhibitor (2µg/ml; Merck Chemicals) and anti-IL-10R (40µg/ml; Biolegend) or appropriate isotype controls. The antibodies/inhibitors were added as pretreatment of CD4+ T responder cells or Tregs 1 h before co-culture or added directly to the co-culture. Relative proliferation was calculated based on the 1/0 ratio for each condition (isotype or specific inhibitors).

6.3.6 Th differentiation assay

Memory CD4+ T cells were isolated from human PBMCs according to the manufacturer's protocol (>90%purity, Memory CD4+ T cell Isolation Kit, Miltenyi Biotec) and subsequently stimulated ($5x10^5$ cells/well) with plate-bound 2.5 µg/ml anti-CD3 (OKT3) and 2 µg/ml anti-CD28 for 5 days. The effect of CD4+CD28null T cells on Th differentiation was investigated by adding 50% (v/v) CM of activated CD4+CD28null T cells or irradiated feeders at day 0. Th1 and Th17 cocktails were used as positive controls. Th1 differentiation cocktail consisted of anti-human IL-4 neutralizing antibody (5 µg/ml, R&D systems), and recombinant human IL-12 (rIL-12; 10 ng/ml, R&D systems).

For Th17 differentiation, cells were cultured in the presence of anti- human IL-4 and anti-human IFN-γ neutralizing antibodies (5 µg/ml, R&D systems), and recombinant human IL-23 (rIL-23; 25 ng/ml; R&D systems). At the end of the culture period, cells were stimulated for 4h with calcium ionomycin (1 µg/ml, Sigma-Aldrich), Golgiplug (1/10, BD) and PMA (25 ng/ml, Sigma-Aldrich), and analyzed via flow cytometry using anti-human CD4 PE-Cy7, IL-4 APC, IL-17A PE and IFN-γ PercP-Cy5.5 (eBioscience).

6.3.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6. Parametric analyses include t-tests (2 groups) and 1-way or 2-way ANOVA (multiple groups). Data are shown as mean ± SD, unless indicated otherwise. A p-value <0.05 was considered significant.

6.4 Results

6.4.1 CD4+CD28null T cells evade Treg-mediated suppression via IFN-γ and granzyme B production

In physiological conditions, Tregs control the proliferation of pro-inflammatory lymphocytes and their immune effector function, thereby maintaining immunological tolerance (228). In a previous study, we showed that CD4+CD28null T cells evade Treg-mediated suppression of proliferation *in vitro* (31). Here, we confirm that Tregs are unable to suppress the proliferation of CD4+CD28null T cells (Figure 6.4), and in addition show that IFN-γ production by CD4+CD28null T cells is not inhibited by Tregs (CD4+CD28null T cells with Tregs: 710 ± 383 pg/ml, CD4+CD28null T cells alone: 902 ± 365 pg/ml, $p>0.05$). To unravel underlying mechanisms for this reduced suppression, we first determined differences between the respective responder cells: CD4+CD28null vs CD4+CD28+ T cells. We analyzed the expression of molecules that were previously reported to influence Treg functionality. More specifically, molecules that favor Th17 over Treg differentiation (IL-6, IL-22, GM-CSF) (229-232), that promote or reduce T cell escape and proliferation (IFN-γ, IL-1β, GITR, IL-10R) (229, 233-237), or that influence T cell survival (HOPX, PD1) (29, 233). CD4+CD28null T cells display a significantly higher mRNA expression of the pro-inflammatory cytokines interleukin- (IL-)1β, IL-6, IL-22, interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) compared to CD4+CD28+ T cells (Figure 6.2A). Homeobox-only protein (HOPX), a transcription factor important for survival, was also higher, while IL-10 receptor (IL-10R) was lower in CD4+CD28null T cells. Expression of the surface molecules IL-10R, GITR and PD1 were analyzed on the protein level. We confirmed the lower expression of IL-10R and further demonstrated a lower expression of glucocorticoid-induced TNFR-related protein (GITR), a co-stimulatory molecule, and a higher expression of programmed cell death protein 1 (PD1), which normally inhibits the T cell response (Figure 6.2B).

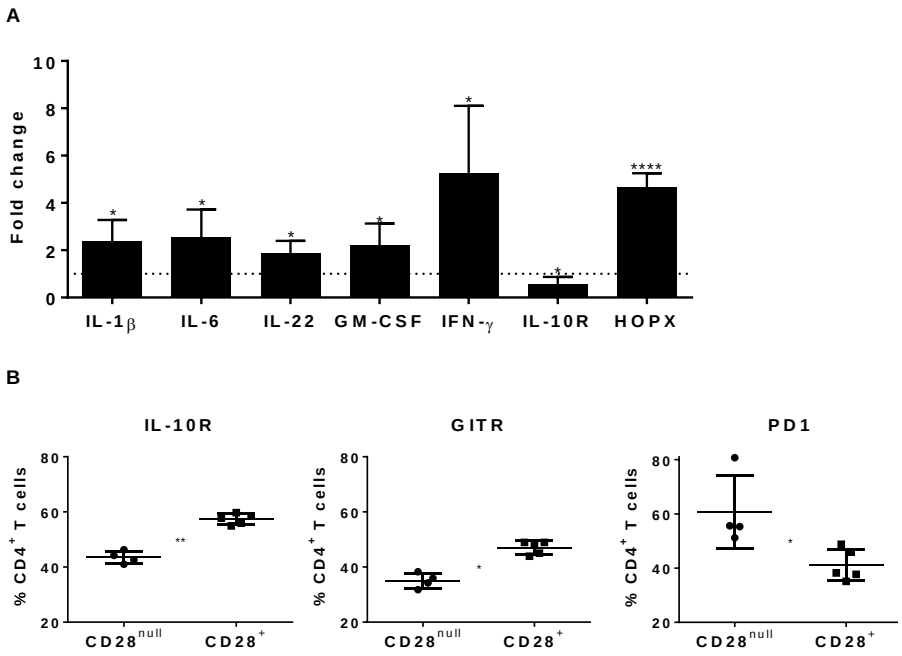


Figure 6.2: Expression of molecules influencing Treg interaction in CD4+CD28null T cells compared to CD4+CD28+ T cells. (**A**) Molecules influencing Treg interaction were measured on mRNA level (n=5-8 donors per group). Fold change is depicted and was calculated relative to the gene expression in CD4+CD28+ T cells (dotted line). (**B**) Surface molecules important in Treg interaction were studied via flow cytometry (n=4-5 donors per group). Percentage of CD4+ T cells positive for the surface molecule is depicted. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Next, we determined whether the secretome of CD4+CD28null T cell alters the expression of regulatory molecules on Tregs. Therefore, CM of activated CD4+CD28null or CD4+CD28+ T cells were added to Treg cultures, after which mRNA expression of the following molecules was investigated: co-inhibitory molecules (CTLA4, PD1, LAG3), immunosuppressive cytokines and their enhancers (TGF-β, IL-10, GARP), apoptosis-related molecules (granzyme B, FasL), molecules implicated in metabolic disruption (CD39, A_2AR receptor) and IFN-γ (233, 234, 237-240). A significant increase in cytotoxic T-lymphocyte-associated protein 4 (CTLA4), IL-10 and IFN-γ was found in Tregs treated with CM of CD4+CD28null T cells, compared to CM of CD4+CD28+ T cells.

An increasing trend for granzyme B and PD1 was observed ($p=0.09$). Furthermore, the increase in CTLA4, IL-10 and IFN-γ was significantly different from baseline in CD4+CD28null T cell CM conditions (only IL-10 was significant in the presence of CD4+CD28+ T cell CM).

The CM of CD4+CD28null T cells had no effect on Transforming growth factor β (TGF-β), GITR, glycoprotein A repetitions predominant (GARP), Fas ligand (FasL), adenosine A2A receptor (A2AR), Ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and lymphocyte-activation gene 3 (LAG3) expression (Figure 6.3).

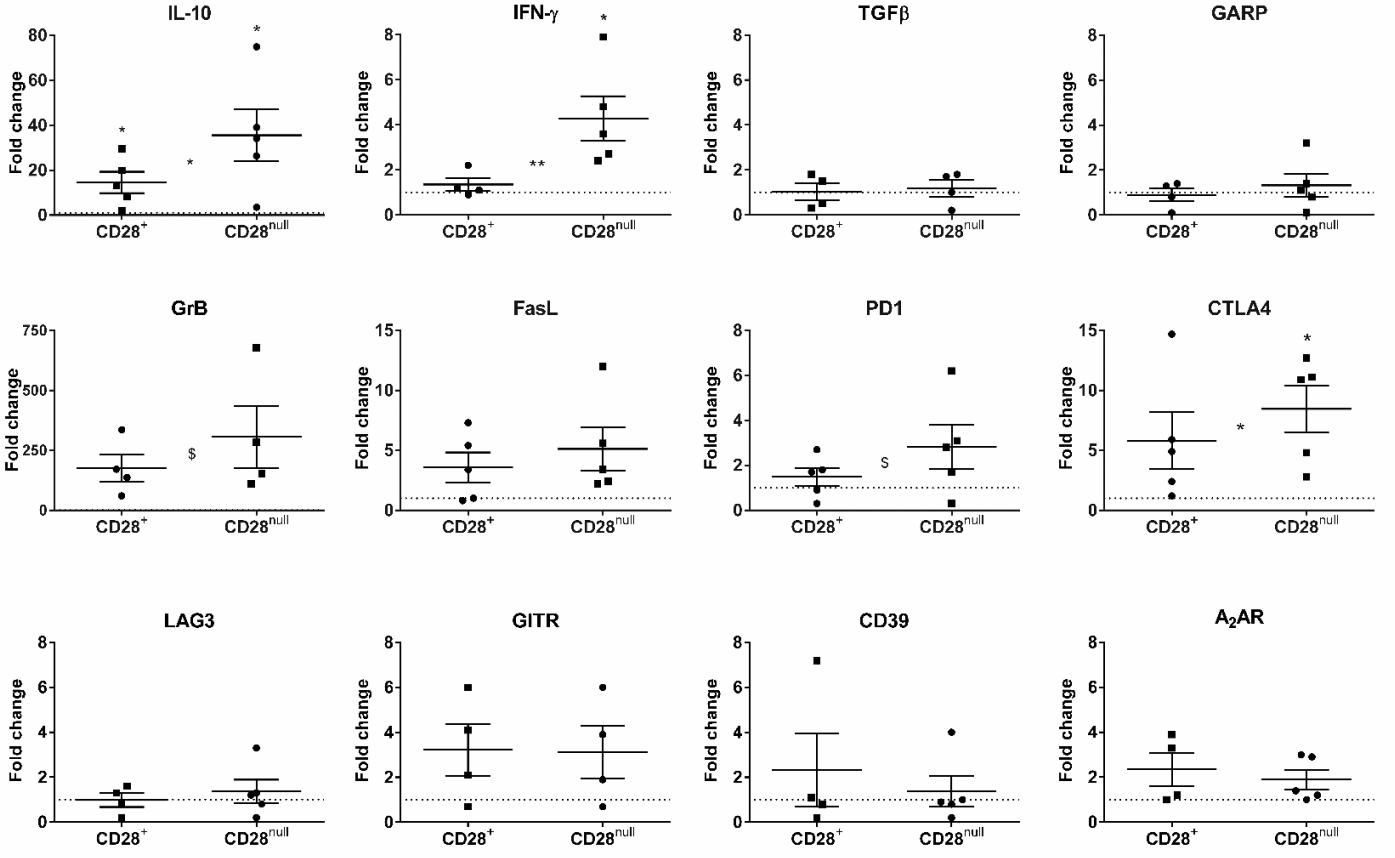


Figure 6.3: Influence of soluble factors produced by CD4+CD28null T cells on Treg phenotype. CM from activated CD4+CD28null T cells or CD4+CD28+ T cells was added to Tregs (n=5). After 72 h, molecules implicated in Treg suppression mechanisms were measured on mRNA level and depicted as the fold change from the baseline (0 h). Asterisks in between the groups signify differences between CD4+CD28null and CD4+CD28+ T cell CM conditions, asterisk above the groups indicate a difference between 72 vs 0 h. $ p=0.09, * p<0.05, ** p<0.01.

Lastly, to identify the mechanism of Treg evasion by CD4+CD28null T cells, we performed a suppression assay where the most promising molecules, found in the above mentioned experiments, were inhibited. IFN-γ (diminishes suppressive function of Tregs (234)), granzyme B (intracellular: leads to apoptosis; extracellular: implicated in Treg resistance (241)), IL-10R (Tregs use IL-10 to suppress proliferation of effector T cells (237)) and GITRL (triggering of GITR on Tregs abrogates their activation; GITR stimulation on T responders promotes survival (235)) were blocked in a Treg suppression assay (figure 6.4). Pretreatment of Tregs or CD4+CD28null T cells with anti-IFN-γ partly restored the suppression of CD4+CD28null T cells (respectively $p<0.09$ and $p<0.05$). Pretreatment of Tregs with a granzyme B inhibitor did not have any effect, while pre-incubation of CD4+CD28null T cells partly restored their suppression ($p=0.06$). Blocking GITRL or IL-10R in the co-culture system did not have any effect.

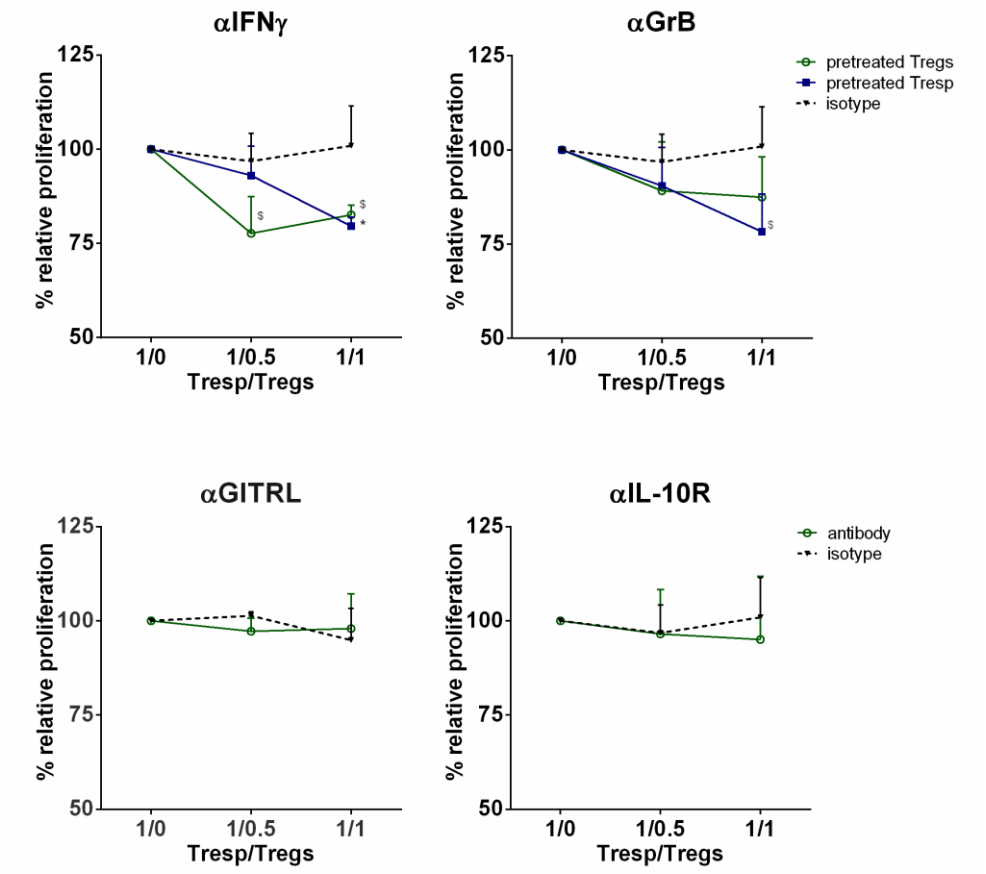


Figure 6.4: Co-culture of Tregs with CD4+CD28null T cells, with inhibitors of IFN-γ, granzyme B, GITRL and IL-10R (n=4). In the top panel, cells were treated with the inhibitor before the co-culture, in the lower panel the blocking antibody was added directly to the culture. The relative proliferation was calculated based on the 1/0 condition for each treatment (specific isotype or inhibitor). Statistical analysis was done compared to the isotype control. Data are depicted as mean ± SEM. * $p<0.05$, $^{\$}$ $p<0.09$. α: anti; GrB: granzyme B; Tresp: responder T cells.

In Treg suppression assays with CD4+CD28+ T cells as responder cells, we confirm that blocking of IFN-γ production but not of granzyme B, GITRL or IL10R enhances the suppression by Tregs ($p<0.05$, figure 6.5).

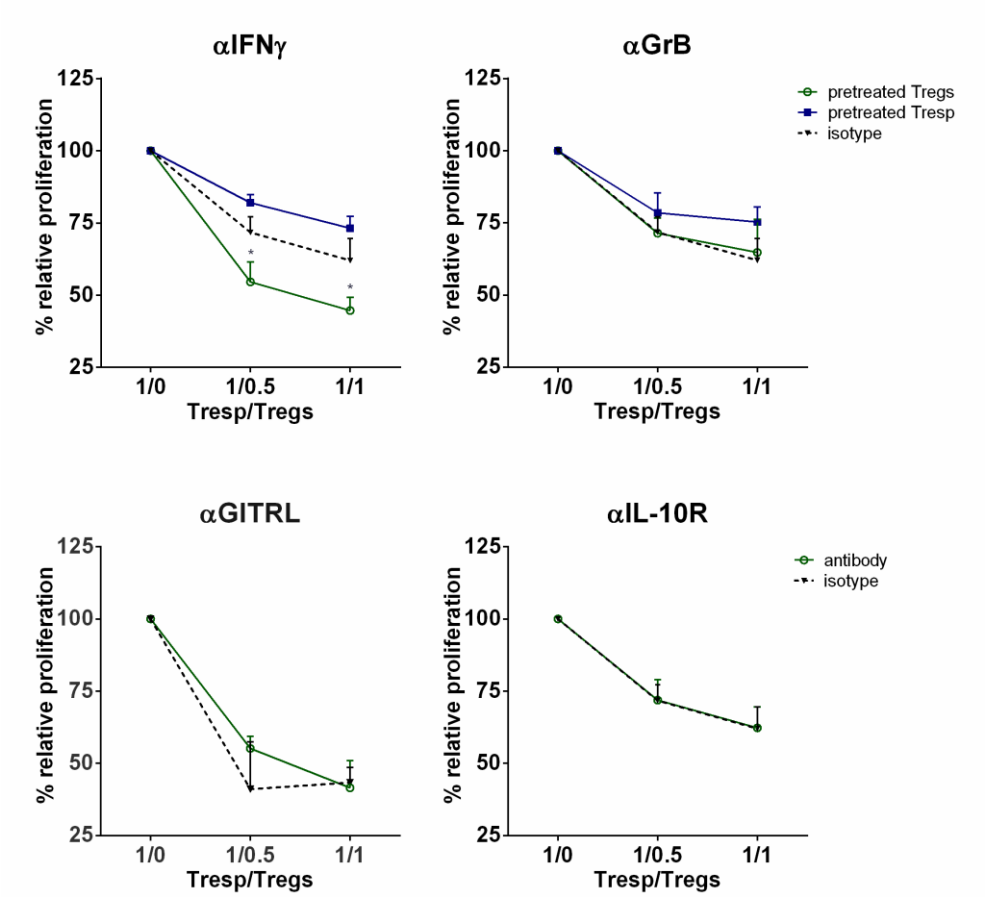


Figure 6.5: Co-culture of Tregs with CD4+CD28+ T cells, with inhibitors of IFN-γ, granzyme B, GITRL and IL-10R (n=4). In the top panel, cells were treated with the inhibitor before the co-culture, in the lower panel the blocking antibody was added directly to the culture. The relative proliferation was calculated based on the 1/0 condition for each treatment (specific isotype or inhibitor). Statistical analysis was done compared to the isotype control. Data are depicted as mean ± SEM. * $p<0.05$

6.4.2 CD4+CD28null T cells induce Th17, but not Th1 differentiation

To test the hypothesis that the CD4+CD28null T cell secretome by itself induces polarization towards pro-inflammatory Th1 or Th17 cells, memory CD4+ T cells were cultured in the presence of CD4+CD28null T cell CM and compared to standard differentiation cocktails.

We found that CM polarized CD4+ memory T cells towards a Th17 phenotype to a comparable level of the standard Th17 polarizing condition but had no influence on Th1 (figure 6.6).

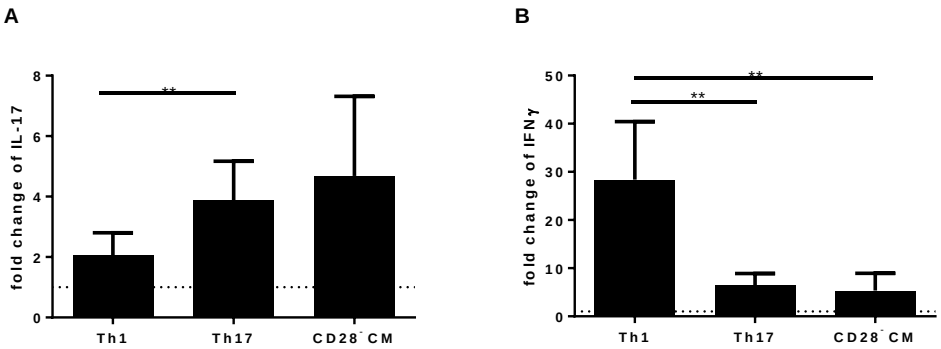


Figure 6.6: Th17 differentiation is favored by CD4+CD28null T cells. Differentiation towards Th17 (**A**) and Th1 cells (**B**) is measured by respectively IL-17 and IFN-γ production by CD4+ T cells. Th1 and Th17 conditions were acquired by adding appropriate differentiation cocktails. The $CD28^-$ CM condition comprised of supernatants of activated CD4+CD28null T cells. The fold change was calculated based on the control condition, which consisted of supernatants of activated feeders. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.5 Discussion

In this study, we show that CD4+CD28null T cells have a highly inflammatory phenotype based on its 1) increased expression of pro-inflammatory cytokines; 2) ability to polarize memory CD4+ T cells towards Th17 cells and 3) resistance to Treg-mediated suppression. These findings combined with the previously reported homing to inflamed tissue and highly cytotoxic nature (35, 36) underlies the overall disease promoting nature of CD4+CD28null T cells.

CD4+CD28null T cells are defined as Th1 cells, since they produce high levels of IFN-γ and TNF-α, but little or no IL-4 or IL-17 (109, 242). We now show that they also express IL-22 and GM-CSF, which are usually attributed to Th17 cells. In line with our findings, it has been shown that Th1 cells can produce these cytokines in the absence of IL-17 production (243, 244). CD4+CD28null T cells have a more pronounced pro-inflammatory phenotype compared to their CD28+ counterparts. Here, we demonstrated that they produce significantly higher amounts of IL-1β, IL-6, IL-22, GM-CSF and IFN-γ. This phenotype is in accordance with literature, where higher IFN-γ, TNF-α and GM-CSF production by CD4+CD28null T cells compared to CD4+CD28+ T cells was shown, underscoring their pro-inflammatory status (109, 144). With regard to the function of these cytokines in inflammatory diseases such as MS, IL-1β and IL-6 have been shown to promote migration of inflammatory immune cells (230, 245). Furthermore, IL-1β augments the production of IL-6 and IFN-γ and promotes apoptosis and tissue damage (245, 246). GM-CSF and IL-6 have been shown to be essential for EAE induction and to instigate CNS inflammation (247-249) and IL-22 is linked with MS disability (250). Thus the production of these cytokines by CD4+CD28null T cells supports their role in inflammatory diseases, including MS. In line with this, we show that conditioned medium of CD4+CD28null T cells promotes Th17 polarization. The enhanced expression of IL-1β, IL-6 and GM-CSF, support this observation. Indeed, these cytokines promote the generation and maintenance of Th17 differentiation and induce the production of Th17-related molecules (231, 246, 251).

We further confirmed the previous reported resistance of CD4+CD28null T cells towards Treg-mediated suppression. In this study, we investigated underlying mechanisms that may explain this resistance. First of all, the resistance could result from changes at the level of CD4+CD28null T cells themselves, reducing their susceptibility to Treg-mediated suppression. The observed downregulation of IL-10R on CD4+CD28null T cells could be an evasion mechanism, even though Tregs increase their IL-10 production in response to CD4+CD28null T cells. Blocking the IL-10/IL-10R axis in the co-culture system indicated that IL-10 signaling does not play a role in Treg suppression nor evasion in this context. Indeed, in a number of studies, neutralization of IL-10 did not abrogate suppression at least *in vitro* (252, 253). Further, we show a downregulation of GITR on CD4+CD28null T cells. Normally, triggering of GITR on Tregs abrogates their activation, whereas GITR stimulation on T responders promotes survival (235). In the context of Treg evasion, a lower expression of GITR on CD4+CD28null T cells could be beneficial, since GITRL, presented by APCs, will now have a greater impact on Tregs, leading to diminished suppression. However, adding anti-GITRL to the co-culture, thereby preventing GITR binding, did not influence suppression. This is in accordance with Nocentini et al., who claim that GITR-mediated suppression is only present in mice and not in humans (235). The absence of CD28 itself can provide another possible explanation why these cells are insensitive to Treg-mediated suppression. Normally, CTLA4 downregulates B7 expression on antigen presenting cells, which in turn hampers stable interactions with CD28 on effector T cells, diminishing activation and proliferation (237). Since CD4+CD28null T cells do not express CD28, downregulation of B7 should not affect them. Inducing re-expression of CD28 on CD4+CD28null T cells could answer this question in the future.

Treg evasion by CD4+CD28null T cells could also indicate resistance to apoptosis. Indeed, we found an increased expression of HOPX in CD4+CD28null T cells compared to CD4+CD28+ T cells, which according to Albrecht et al. conveys resistance to apoptosis, via the evasion of Fas-mediated apoptosis and respectively up- and downregulation of anti- and pro-apoptotic molecules (29). CD4+CD28null T cells are already known to express the anti-apoptotic molecules BCL-2 and cFLIP (254). The upregulation of anti-apoptotic molecules potentially drives the resistance to Treg suppression.

Alternatively, CD4+CD28null T cells could evade Treg-mediated suppression by directly neutralizing Treg activity. Indeed, increased expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF and IFN-γ have been demonstrated to abrogate Treg suppression, block Treg differentiation and promote effector T cell proliferation (230, 232, 234, 236, 255). Since CD4+CD28null T cells produce high amounts of IFN-γ, and Tregs increase their IFN-γ production in response to soluble factors released by CD4+CD28null T cells, we chose to inhibit this cytokine in the co-culture system. Blocking IFN-γ produced by either CD4+CD28null T cells or Tregs enhanced Treg-mediated suppression. This is in accordance with literature, where IFN-γ producing Tregs had a reduced suppressive capacity and blocking this cytokine recovered their suppressive capacity to some extent (234, 256). In addition to IFN-γ, we showed that blocking granzyme B of CD4+CD28null T cells reinstalled suppression by Tregs. Granzyme B production could induce apoptosis of Tregs, alternatively GrB has been reported to modulate Treg suppression by decreasing PD-L1 and CD39 expression in Tregs (241). Although we did not measure this, a decrease in PD-L1 could possibly influence suppression, since PD1 is increased on CD4+CD28null T cells versus CD4+CD28+ T cells.
Finally, we could not find a difference in the expression of GARP, TGF-β, GITR, LAG3, PD1 and A_2AR in Tregs stimulated with CD4+CD28null T cell CM. While this indicates that these molecules do not play a role in our *in vitro* suppression assays, we cannot rule out their potential involvement *in vivo*.

In summary, we identified CD4+CD28null T cells as IL-1β, IL-6, IL-22 and GM-CSF producing cells which show increased HOPX and PD1 expression, whereas IL-10R and GITR are decreased. We demonstrate that the CD4+CD28null T cell secretome induces Th17 differentiation, probably via the production of IL-1β, IL-6 and GM-CSF. Lastly, we show that CD4+CD28null T cells can evade Treg suppression via multiple mechanisms, including granzyme B and IFN-γ production. In the context of inflammatory diseases, the identified characteristics of CD4+CD28null T cells reported here, suggest direct contribution to the pathogenic immune responses (36, 39).

7

SUMMARY, GENERAL DISCUSSION & FUTURE PERSPECTIVES

7.1 Summary

The immune system, and more precisely the imbalance between protective and detrimental responses, is a major player in MS pathology (257, 258). This imbalance can be influenced by both environmental and genetic factors (257, 259). In this thesis, we aimed to identify the trigger(s) of CD4+CD28null T cell formation and expansion and investigated to which extent CMV and cytotoxic CD4+CD28null T cells contribute to MS disease. In the following paragraphs and figure 7.1, our main results are summarized.

7.1.1: CMV infection and chronic neuroinflammation, but not genetic variations in *TLR2*, *MICB* and *IL2RA*, contribute to the formation and expansion of CD4+CD28null T cells

CD4+ T cells lose CD28 via repeated antigenic stimulation, which occurs during chronic activation of the immune system (260). Since MS is a chronic inflammatory disease, and CMV is a persistent virus, both are valid candidates for triggering the formation and expansion of CD4+CD28null T cells.

In **chapter 3**, we investigated the CMV serostatus and IgG titers in donors with or without CD4+CD28null T cell expansion, revealing an association between CMV seropositivity and CD4+CD28null T cell expansion. In addition, CMV-specific IgG titers positively correlated with the percentage of these cells. In the future, longitudinal monitoring of IgG fluctuations combined with CMV DNA measurements could indicate whether the correlation of CD4+CD28null T cell frequency with CMV-specific IgG titers merely reflects the activity of CMV replication. To further pinpoint CMV as an actual trigger for CD4+CD28null T cell expansion, CMV stimulation assays were performed both *in vitro* and *in vivo*. Repeated stimulation of human PBMCs with a CMV peptide only led to expansion of pre-existing CD4+CD28null T cells, but did not induce the loss of CD28. However, the MCMV mouse model indicated that a long-lasting CMV infection can induce the formation and expansion of CD4+CD28null T cells *in vivo*. Thus, a full-blown CMV infection is necessary for CD4+CD28null T cell formation. In the EAE model, we determined whether chronic neuroinflammation leads to the formation and expansion of CD4+CD28null T cells.

Our results indicated that CD4+CD28null T cells arise during the acute phase of EAE, when neuroinflammation is most prominent, and correlate with the amount of demyelination. This increase in CD4+CD28null T cells can be attributed to repeated autoantigenic stimulation, since repeated MBP stimulation of MBP-specific T cell clones *in vitro* led to CD4+CD28null T cell expansions.

Next to chronic antigenic challenge, genetic factors, such as single nucleotide polymorphisms (SNPs) could predispose people to the expansion of CD4+CD28null T cells. In **chapter 4**, we confirmed the increased percentage of CD4+CD28null T cells in CMV seropositive donors and genotyped this population for SNPs associated with CMV (*MICB, TLR2*) or MS (*IL2RA*). While our pilot study may be underpowered, we could not find an evident correlation between any of the SNPs with either the percentage of CD4+CD28null T cells or the presence/absence of their expansion. In addition, we could not reproduce the previously published association with CMV for MICB, although we did reach sufficient power, indicating that the earlier study of Shirts et al. might not be accurate. The main difference between both studies is the use of healthy controls versus MS patients. Possibly, genetic effects on immune responses might be masked by non-genetic disease factors in MS.

Taken together, these results show that CD4+CD28null T cells arise during CMV infection and neuroinflammation, but are not influenced by the SNPs studied here.

7.1.2: CMV exacerbates clinical and pathological indices of EAE

The role of CMV in MS is controversial, different reports suggest detrimental but also beneficial effects. In **chapter 2**, we discussed current literature with regard to this controversy and propose mechanisms by which CMV could possibly contribute to MS. These mechanisms include molecular mimicry, bystander activation and epitope spreading. The activation and expansion of CD4+CD28null T cells, via CMV infection could also contribute to MS pathology. On the other hand, CMV immune evasion may mitigate the autoimmune reactions and pro-inflammatory milieu contributing to MS.

Literature indicating a disease promoting role show molecular mimicry between a CMV and MOG peptide in 2 different animal models (97, 98). Furthermore, CMV-specific antibodies are enriched in MS patients compared to healthy controls (100).

The presence of these antibodies correlated to a decreased time to relapse, an increase in the number of relapses and enhanced brain atrophy (103-105). In contrast, another study concluded that presence of CMV-specific antibodies led to better clinical outcome, an increased age of disease onset and decreased brain atrophy (115). In addition, in TMEV, a MS mouse model, MCMV infection 2 weeks before TMEV infection lead to a better disease outcome (112). However, a recent meta-analysis on 1341 MS patients and 2042 healthy controls did not yield a conclusive result on the relationship between CMV infection and the occurrence of MS (132). Further research is thus needed to establish the role of CMV in MS.

In **chapter 3**, we set up an animal model combining EAE and CMV (MCMV) to examine the role of CMV in EAE pathology. We demonstrated increased disability compared to non-infected EAE mice, as determined via daily EAE scoring. In addition, the MOG reactivity of CD4+ T cells was increased, leading to more pronounced inflammation. Lastly, the amount of demyelination in the spinal cord of CMV infected EAE mice was also higher. Our data support that the mechanisms by which CMV contributes to EAE exacerbation are not epitope spreading or molecular mimicry, but rather bystander activation of autoreactive T cells, including CD4+CD28null T cells. Indeed, increased percentages of CD4+CD28null T cells are present in these mice, which correlated with the observed exacerbations in CMV infected EAE mice.

In conclusion, although there is a lot of controversy about the role of CMV in MS disease, we demonstrate that CMV aggravates autoimmune-mediated inflammation and demyelination in an animal model of MS.

7.1.3: CD4+CD28null T cells contribute to worse MS disease

Up to now, only circumstantial evidence (via *in vitro* and *ex vivo* experiments) links CD4+CD28null T cells with MS disease (36, 109, 110, 144). In **chapter 3**, we showed that CD4+CD28null T cell expand after EAE induction and correlated with disease severity, inflammation and demyelination. We further suggested that CD4+CD28null T cells migrate towards the CNS to exert their cytotoxic and pro-inflammatory functions, since CD4+granzyme B+ T cells are present in the spinal cord of EAE mice.

The effector memory phenotype of these cells resembled that of their human counterparts; they had low or no expression of CD62L, CD27, and CD127 and produced IFN-γ and Granzyme B. In addition, we confirmed their autoreactive capacity, since they responded to MBP.

In **chapter 5**, clinical parameters (disability scores, number of relapses, …) of MS patients were monitored to determine if expanded CD4+CD28null T cells contribute to a worse disease burden and clinical progression. For these analyses, a new intuitive classification was constructed, namely the intrinsic MS prognostic classification (IMPC). This classification is based on MRI, clinical (disease type, duration, EDSS, MSSS and relapse frequency) and electrophysiological data (Multimodal Evoked Potentials). A severe IMPC is associated with an increased number of relapses, higher EDSS and MSSS, worse global electrophysiological score and occurs more often in chronic progressive MS (CPMS) patients. Within the RRMS population, patients with CD4+CD28null T cells expansion had a more severe IMPC compared to patients without expansion. This indicates that the presence of these cells are linked with a worse prognosis in RRMS patients.

In summary, we provide evidence that CD4+CD28null T cells contribute to MS pathology, both in an animal model as well as in RRMS patients.

7.1.4: CD4+CD28null T cells are pro-inflammatory, evade Treg suppression and induce Th17 cell differentiation

From previous research, we gathered that CD4+CD28null T cells are pro-inflammatory, autoreactive, cytotoxic and can migrate towards MS lesions (36, 109, 144). This indicates that they can directly contribute to inflammation and damage in the CNS. In **chapter 6**, the phenotype of CD4+CD28null T cells was further unraveled. Comparison between CD4+CD28null T cells and CD4+CD28+ T cells showed increased expression of pro-inflammatory cytokines IL-1β, IL-6, IL-22 and GM-CSF, and increased HOPX expression, a survival-related transcription factor. Surface receptors IL-10R and GITR were decreased, whereas PD1 expression was increased on CD4+CD28null T cells. Furthermore, we demonstrated that the secretome of CD4+CD28null T cells induces differentiation of memory T cells towards Th17 cells and that it changes the phenotype of Tregs towards the pro-inflammatory side. Previous research has shown that CD4+CD28null T cells can evade Treg-mediated suppression (31).

To further pinpoint the exact mechanisms involved, we inhibited granzyme B, IFN-γ, IL-10R and GITR in a Treg co-culture suppression assay. We found that addition of granzyme B and IFN-γ inhibitors restored suppression of CD4+CD28null T cells, suggesting that CD4+CD28null T cells evade Treg suppression via the production of both molecules.

Taken together, these findings indicate that CD4+CD28null T cells can directly contribute to inflammatory disease by producing pro-inflammatory cytokines, but also by evading Treg-mediated suppression and inducing differentiation of pathogenic Th17 cells, which could further amplify an ongoing immune response.

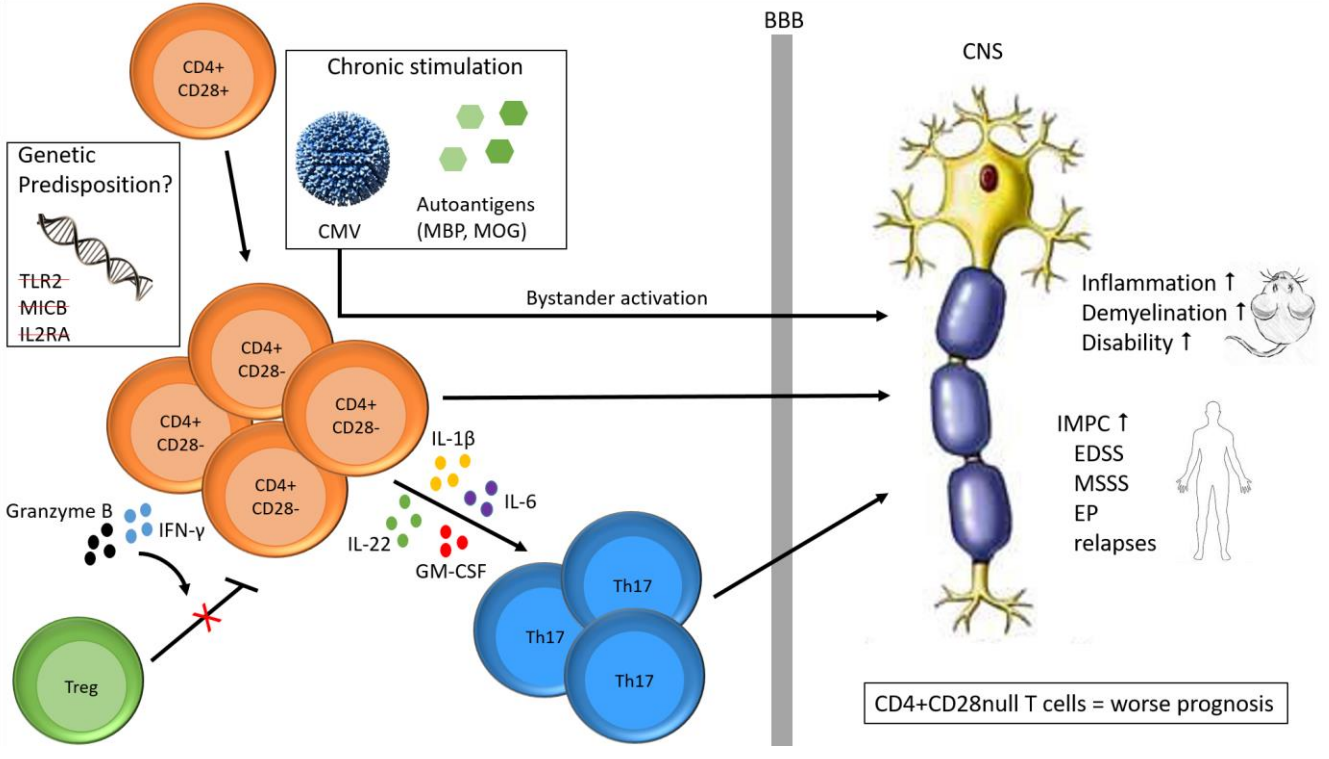


Figure 7.1: Mechanisms of CD4+CD28null T cell formation, expansion and function. CD4+CD28+ T cells lose CD28 after chronic antigenic stimulation, either by cytomegalovirus (CMV) infection or multiple sclerosis itself. Some individuals might have a genetic predisposition for the development of these cells, but this predisposition does not include mutations in toll-like receptor 2 (TLR2), interleukin 2 receptor α (IL2RA) or MHC class I polypeptide-related sequence B (MICB). CD4+CD28null T cells contribute to MS disease directly since they are autoreactive, produce pro-inflammatory cytokines, are cytotoxic and can migrate towards the central nervous system (CNS). Furthermore, CD4+CD28null T cells can evade Treg suppression via the production of granzyme B and interferon-γ (IFN-γ), making them resistant to regulation. In addition, CD4+CD28null T cells can promote the differentiation of Th17 cells, which cross the blood brain barrier (BBB) to inflict damage. In EAE, the mouse model of MS, CMV and CD4+CD28null T cells increased inflammation, demyelination and disability. In MS patients, CD4+CD28null T cells correlated with a severe intuitive MS prognostic classification (IMPC), indicating that the presence of these cells might lead to a worse prognosis.

7.2 General discussion

7.2.1 What is the origin of CD4+ CTLs?

We propose that CD4+ CTLs originate either from naive CD4+ T cells or through plasticity of CD4+ effector T cells (Chapter 1). In these 2 alternative pathways, both extracellular cues as well as transcription factors play an important role. Further research is warranted in this area, yet we propose 2 hypothetical models.

7.2.1.1 Hypothetical model 1: Transcriptional regulation - from naive CD4+ T cells to CD4+ CTLs

Based on current literature, we suggest the following model for the order of events in the differentiation process from naive CD4+ pre-CTL (before they acquire cytotoxicity) to effector CD4+ CTL: co-stimulation or TCR stimulation quickly induces T-bet in presence of Th1 polarizing cytokines (1). BLIMP-1 (and possibly Hobit) further promotes stable T-bet expression (10, 261). T-bet on its own can promote IFN-γ, granzyme B and perforin expression (262), but also induces Runx3 expression, which by itself promotes IFN-γ, granzyme B and perforin, but also upregulates Eomes and downregulates ThPOK, thereby completing the cytotoxic programming and subsequent production of IFN-γ, granzyme B and perforin (1, 14, 25, 263). It is noteworthy that CD4+ CTLs may be regulated by both T-bet and Eomes, depending on their maturation stage. In some cases, Eomes rather than T-bet contributes to CD4+ CTL function (26). As this is just a theoretical model (figure 7.2), further research is needed to validate it, certainly since most findings were established in mouse models and care should be taken to extrapolate this to the human situation and to generalize these specific models. Furthermore, the model depicted here is simplified. In the future the complex transcriptional network that exists between certain transcription factors such as Runx3, Eomes and T-bet, and Runx3 and ThPOK should be clarified (11, 14, 25, 264, 265).

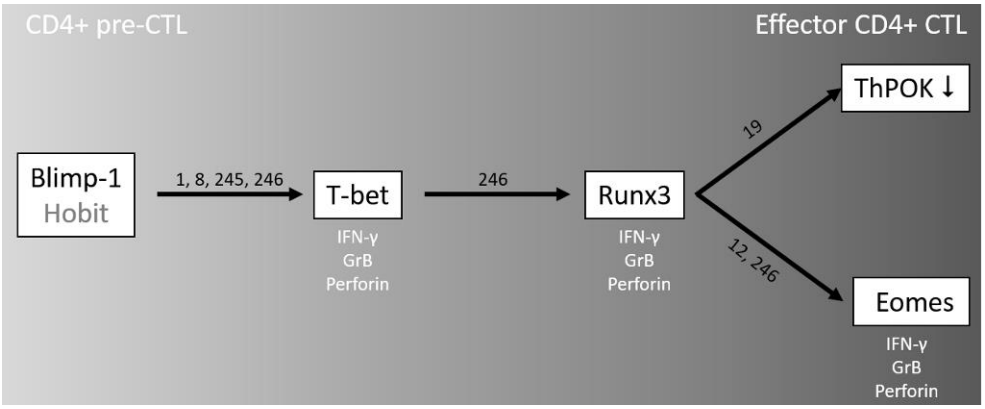


Figure 7.2: Hypothetical expression model of transcription factors involved in the formation of CD4+ CTL from naive CD4+ pre CTLs. TCR stimulation quickly induces T-bet, aided by BLIMP-1 (and possibly Hobit). T-bet will then induce Runx3 expression, which will in turn upregulate Eomes and downregulate ThPOK. GrB: Granzyme B.

In the future, it would be interesting to investigate the differentiation stages from CD4+ pre-CTLs to CD4+CD28null T cells, the probable end stage of CD4+ CTLs. This would certainly benefit a limitation of this study, namely the fact that only CD4+CD28null T cells are investigated and not the total population of cytotoxic CD4+ T cells that could be equally harmful. Perhaps precursor cells can be identified by the expression of a certain transcription factor or phenotypic marker (Chapter 1), and these pre-CTLs can be used as an indicator for future expansion of CD4+CD28null T cells, making it more feasible to identify donors at risk of developing aberrant CD4+ CTLs (since CD4+CD28null T cells are only present in 20% of the general population). Furthermore, if these CD4+ pre-CTLs exist, screening for these cells could lead to an earlier identification of a worse prognosis in MS patients (Chapter 5). Still, it should be kept in mind that these CD4+ pre-CTLs still need to become cytotoxic, indicating that not all pre-CTLs lead to worse prognosis. Future studies are therefore warranted to provide clarity.

7.2.1.2 Hypothetical model 2: Plasticity of effector CD4+ T cells towards cytotoxic CD4+ T cells

Next to the differentiation of naive CD4+ T cells into CD4+ CTLs, effector T helper cells may also become CD4+ CTLs. The ability of one differentiated effector CD4+ T cell to take on characteristics of a different effector T cell subset is called T cell plasticity (9). CD4+ CTLs could originate from Th0, Th1, Th2, Th17 or Treg cells (26), but we focus on Th1 and Th17 cells since these are more widely studied.

Evidence in favor for Th1 plasticity indicates that human CD4+ CTLs produce Th1-type cytokines (e.g. IFN-γ) and display a terminally differentiated effector memory phenotype, suggesting that they are induced by prolonged antigenic stimulation of Th1 cells (10, 40, 58, 266) (chapter 3). On the other hand, Th17 and Th1/17 cells can become cytotoxic as well. Pariente et al. demonstrated that CD4+ CTLs cells are a mixed population of Th1, Th17, Th1/17 and even Th22 and Th17/Th22 cells, since they produce IFN-γ, IL-17 and IL-22, either alone or in combination (IL-17 + IL-22, IFN-γ + IL-17) (50).

In chapter 6, we showed that CD4+CD28null T cells, classically defined as Th1 cells (109, 242), produce Th17-related cytokines IL-22 and GM-CSF, marking them as Th1/Th17 cells. However, it has been shown that Th1 cells can produce IL-22 and GM-CSF in the absence of IL-17 production, which is also the case for CD4+CD28null T cells (243, 244). Future studies should be set up to clarify if Th1 or Th17 cells can indeed differentiate towards CD4+ CTLs, e.g. by repeated *in vitro* activation or by *in vivo* single cell tracking in animal models of chronic infection or autoimmunity.

In summary, we suggest that there are two sources of which CD4+ CTLs can originate, namely naive CD4+ T cells and differentiated effector CD4+ T cells (probably Th1 or Th17). We believe that the plasticity of effector CD4+ T cells towards cytotoxic T cells is a more likely source than the differentiation of naive CD4+ T cells towards CD4+ CTLs, because of their advanced differentiation status as effector memory T cells that produce Th-related cytokines IFN-γ and IL-17.

7.2.2 Which triggers induce CD4+CD28null T cell formation?

After chronic antigenic stimulation, CD4+ T cells lose the expression of CD28. This repeated T cell activation process results in oligoclonality and a limited TCR diversity of CD4+CD28null T cells (39). The chronicity of the immune response leading to a complete loss of CD28 is underscored by the underlying molecular mechanisms of CD28 downregulation. Within the CD28 minimal promoter, 2 motifs (site α and β) are important for the expression of CD28. During normal T cell activation, a parallel decline in both site binding activities occurs, leading to a decrease in CD28 expression. *In vivo* expanded CD4+CD28null T cells uniformly lack both α- and β-bound complexes, resembling the pattern seen in chronically activated cells (34).

7.2.2.1 Environmental factors

Not just any inflammatory condition will lead to the formation of CD4+CD28null T cells; the chronic and repeated aspects are essential. However, even if a chronic immune response is ongoing, this does not automatically lead to the formation of CD4+CD28null T cells, as we and others have shown for EBV infection (164). This virus leads to a chronic and persistent infection, just like CMV. However, only CMV and not EBV infection is associated with the formation of CD4+CD28null T cells (Chapter 3). The underlying mechanism for this difference is unknown, but we speculate that two different CD4+ T cell populations are triggered by both viruses, just as in CD8+ T cells. EBV leads to a relatively low frequency of mostly central memory CD8+CD28+ T cells. By contrast, the majority of CMV-specific CD8+ T cells have an effector memory phenotype, do not express CD28 and are expanded (267, 268). In addition, the different targets of both viruses could also be involved in the differential effect on CD4+CD28null T cell expansion. EBV mainly targets B cells, whereas CMV infection occurs more broadly, in mononuclear cells, white blood cells, and epithelial cells (269). It would be worthwhile to check for CD4+CD28null T cell expansion in other chronic infections, since these cells have also been found in e.g. human immunodeficiency virus (HIV)+ persons (270). Also, our research should be broadened to other chronic inflammatory conditions, since EAE itself also triggered the expansion (Chapter 3).

7.2.2.2 Genetic predisposition

In Chapter 4, we investigated whether a specific genetic background could influence the formation and expansion of CD4+CD28null T cells. We could not find an association between CD4+CD28null T cells and TLR2, MICB (CMV-related) and IL2RA (MS-related). In the future, we should increase our sample size for analysis of these SNPs. However, the feasibility should first be investigated for each SNP.

Since we could not confirm the findings of MICB and TLR2 even though we had the same sample size as described, one could question their involvement in CMV susceptibility or chronicity, and thus their possible role in CD4+CD28null T cell expansion. Therefore, other SNPs should also be investigated and their effect on functional changes in CD4+CD28null T cells should be the main focus, instead of their expansion.

For example, *in vitro* experiments of a SNP in the EOMES gene, which is a transcription factor that drives perforin production, or of the already studied SNP in the IL2RA gene, which was reported to associate with the level of GM-CSF production (180, 181), could clarify if these SNPs are also associated with the function of CD4+CD28null T cells. Since both SNPs are involved in MS susceptibility, these experiments could be performed in MS patients and healthy donors.

In conclusion, we showed that CMV, but not EBV, seems to be the main mechanism for CD4+CD28null T cell expansion and that SNPs in *IL2RA*, *TLR2* and *MICB* are not involved in this process. However, a lot of questions still remain regarding the triggers for CD4+CD28null T cell expansions. Is there a genetic predisposition? Are there other environmental factors involved? Why do certain chronic viruses or inflammatory diseases cause expansion of CD4+CD28null T cells while others do not?

7.2.3 Which effector functions do CD4+CD28null T cells have?

CD4+CD28null T cells are often described as immunosenescent or even exhausted T cells (40, 271), implying that these cells do not function properly anymore. This is the case when you look at their classic function as helper cells, since they lose their ability to provide B-cell help (37). However, many reports have indicated that they have an effector memory phenotype, are cytotoxic and can rapidly produce pro-inflammatory cytokines upon stimulation, thereby disproving their exhausted state (62, 70, 260, 272).

In Chapter 3, we identified a similar population of CD4+CD28null T cells in mice, with an equivalent phenotype compared to their human counterparts. Next to the loss of CD28, CD4+CD28null T cells also acquire some typical NK cell or CD8+ T cell functions. CD4+CD28null T cells are cytotoxic, since they produce granzyme B and perforin and acquire NK cell receptors, such as NKG2D and KIRs.

Further phenotypic markers include the upregulated expression of adhesion molecules ICAM-1 and VLA-4, and chemokine receptors CCR5 and CX3CR1, which results in the migration towards MS lesions (35). They also produce high amounts of pro-inflammatory cytokines, such as IFN-γ, TNF-α, GM-CSF, IL-1β, IL-6 and IL-22, underscoring their contributing role to inflammation (Chapter 6).

So if they are not anergic, how do they become activated without CD28 binding? As a compensatory mechanism, CD4+CD28null T cells upregulate other co-stimulatory molecules, such as OX40 and 4-1BB (273). However, GITR, another molecule from the TNFR superfamily, was not upregulated as we illustrated in chapter 6. CD4+CD28null T cells express toll-like receptors (TLR2 and 4) and NK cell receptors (NKG2D, KIRs, CD161, CD11b), and can become activated via these routes as well (35).
In summary, CD4+CD28null T cells are not functionally exhausted as originally thought, but acquire alternative stimulation pathways. They have an effector memory phenotype and can rapidly produce pro-inflammatory cytokines and cytotoxic molecules upon stimulation.

7.2.4 CD4+CD28null T cells: friends or foes?

In **healthy** individuals, CD4+CD28null T cells can be considered as 'friends', since they have been shown to have anti-viral and anti-tumor effects. During infection, the presence of these cells could be beneficial to clear the pathogen, since the distinct mechanisms used by CD4+CD28null T cells are generally not blocked by the virus. For example, viral mimics could block MHC receptor activation, thereby circumventing the immune response of conventional T cells. However, CD4+CD8null T cells can become activated by TLR of NK cell receptor binding, in a non-MHC restricted way.
The same holds true for cancer: because of the different mechanisms used by CD4+CD28null T cells as compared to other immune cells, the CD4+CD28null T cells are still able to exert anti-tumor functions. For example, cancer cells have immune suppressive effects, by promoting Treg activity (274). As shown in chapter 6, CD4+CD28null T cells can evade Treg suppression, suggesting they are still able to recognize and respond to the tumor cells. Tumors can also reduce tumor antigen expression, by down-modulating MHC I (274). As already mentioned, CD4+CD28null T cells express TLRs and NK cell receptors, possibly circumventing this mechanism. Other evasion strategies of tumors include induction of anergy, by TCR stimulation in the absence of costimulation, or apoptosis (274). Yet again CD4+CD28null T cells are equipped to avoid these mechanisms, since they are apoptosis resistant and costimulatory independent (110, 254).

In chapter 6, we showed upregulation of HOPX (in line with (29) and Yasmina Serroukh, personal communication), a transcription factor important for survival, underscoring the resistance to apoptosis of CD4+CD28null T cells.

During **aging**, CD4+CD28null T cells are seen as 'foes', since they are a byproduct of immunosenescence and contribute to inflammaging. Indeed, in aging individuals, thymic output of naive T cells is decreased, which is compensated by homeostatic proliferation of memory T cells (=immunosenescence), leading to the formation and expansion of CD4+CD28null T cells. However, these CD4+CD28null T cells have only downmodulated site β binding activities of the CD28 minimal promoter, making them senescent rather than the above mentioned chronically activated CD4+CD28null T cells (34).
Because of the expansion of specific memory T cells, the T cell pool diversity decreases, reducing the potential to react to a wide variety of pathogens. Thus in the elderly, infections and inflammation are more common (=inflammaging), leading to an increased frequency of morbidity and mortality.

In **disease**, especially chronic inflammation, CD4+CD28null T cells can play a detrimental role. It is thought that in autoimmunity, premature immune aging occurs (275), diminishing the diversity of the immune system. In addition, CD4+CD28null T cells promote inflammation, migrate towards inflammatory sites and induce damage to cells and tissues in many different diseases, such as multiple sclerosis, rheumatoid arthritis and cardiovascular diseases (39, 62). Indeed, in Chapter 3, we showed that CD4+CD28null T cells correlate with disease severity, inflammation and demyelination in EAE, a mouse model of MS. Furthermore, CD4+granzyme B+ T cells were present in the spinal cord of EAE mice, indicating that CD4+CD28null T cells can migrate towards the CNS to inflict damage.
Other researchers have shown similar results for CD4+CTLs in MS/EAE: CD4+NKG2D+ T cells, which is also expressed on CD4+CD28null T cells (144), were shown to have elevated markers for migration, activation and cytolytic capacity in MS patients and EAE mice. Moreover, these cells were present in the cerebrospinal fluid and MS lesions, which indicates that these cells are involved in inflammatory CNS lesion development (43).

Furthermore, CD4+ CTLs expressing Eomes and T-bet, can also migrate towards lesions and cerebrospinal fluid of MS patients via CX3CR1 (21, 36). NKG2C+ or NKG2D+ CD4+ T cells associate with neuroinflammation, since they are pro-inflammatory and kill oligodendrocytes (44, 45). Neurotoxicity, neural damage as well as astrocyte lysis by CD4+CTLs have also been observed in the context of MS and EAE (276-279). In mice, the development of late/chronic EAE is promoted by cytotoxic Eomes+CD4+ T cells infiltrating the CNS, since knockout of Eomes in the CD4 lineage reduced the severity of this phase (21).

One could argue that we did not really prove the involvement of CD4+CD28null T cells in EAE. Therefore, in the future, an adoptive transfer of CD4+CD28null T cells should be done in EAE animals, to see whether the disease course is worse when these cells are present. Raveney et al. already performed an adoptive transfer with CD4+EOMES+ T cells, which resulted in EAE exacerbation thereby illustrating that CD4+ CTLs indeed worsen disease (21). Furthermore, labeling these cells will allow us to study their migration towards the CNS directly. Now we can only assume migration, because they are present in the CSF and/or lesions of MS patients and EAE animals. However, undertaking this experiment will be challenging, since a high number of cells is required, while on average less than 2% of the CD4+ population is CD28- in WT mice. One solution could be to perform an MCMV experiment and to wait until day 250, when a 20-fold increase of CD4+CD28null T cells will have taken place. Linked to the migration issue, is the question whether and how CD4+CD28null T cells can migrate across the BBB. It is known that CD4+CD28null T cells can damage endothelial cells via IFN-γ and TNF-α production (38). Therefore, it is reasonable to assume that they will attack the BBB as well. Another option could be that these cells only take part in the disease at a later stage, when the BBB is already leaky. Via the fractalkine gradient, they will then easily migrate towards the CNS. By using an *in vitro* BBB model, we could assess these questions.

In chapter 5, expansion of CD4+CD28null T cells was found to correlate with a worse prognosis, as indicated by a higher intrinsic MS prognostic classification (based on number of relapses, EDSS, MSSS and global electrophysiological score).

Other researchers have found indications for this correlation: CD4+NKG2D+ T cells were suggested to be associated with disease activity, since they are increased in the CSF of RRMS patients, with even higher frequencies in the active phases of MS (43). Furthermore, NKG2D+CD4+ T cells are associated with tissue destruction (45). Eomes+CD4+ T cells were also increased in the peripheral blood and CSF of MS patients in the progressive phase of the disease (21). Perforin-producing CD4+ T cells are activated and increased in active MS disease, again suggesting that cytotoxic CD4+ T cells may play a role in MS pathogenesis (280). Other CD4+ CTLs, namely cytolytic CD4+ T lymphocyte precursors and NKT cells, were also increased in MS patients (281, 282).
In rheumatoid arthritis, a correlation between pro-inflammatory CD4+ CTLs (mostly CD28-), which are enriched in inflamed joints, and disease severity has already been established (39, 52, 62, 66, 67). This is also the case for acute coronary syndrome, where higher numbers of CD4+CD28null T cells lead to an increased risk for recurrent severe acute coronary events and unfavorable prognosis (283). Furthermore, in atherosclerosis, CD4+CD28- T cells are involved in plaque destabilization and a correlation between these cells and the severity and extent of coronary artery disease has been found (284).

It should be noted that in this study, CD4+CD28null T cell expansions only occurred in CMV seropositive MS patients. As discussed in chapter 2 and 3, CMV infection can influence the disease course of MS, or its animal model EAE. In MS, conflicting literature exists (chapter 2), but in EAE, we showed that CMV itself can contribute to disease (chapter 3). MCMV infection leads to increased disability, inflammation and demyelination in EAE mice, probably via bystander activation of autoreactive T cells, including, CD4+CD28null T cells.

In summary, depending on the situation, the presence of CD4+CD28null T cells can be beneficial or detrimental.

7.2.5 How can the pathogenic role of CD4+CD28null T cells be blocked for therapeutic use?

First, current MS therapies might have an effect on CD4+CD28null T cell expansions. These drugs can be divided into first, second and third line treatments and reflect the treatment strategy.

First line drugs include injectable interferon-β (Avonex®, Rebif®, Betaferon®) and glatiramer acetate (Copaxone®), but also oral drugs such as teriflunomide (Aubagio®) and dimethylfumerate (Tecfidera®). Second line therapies consist of monoclonal antibodies, such as natalizumab (Tysabri®) and alemtuzumab (Lemtrada®), and fingolimod (Gilenya®). The third line treatment comprises mitoxantrone (MTX) (285).

In our study cohort, we have included interferon-β (IFN β), glatiramer acetate (GA), alemtuzumab (at starting or re-infusion time-point), natalizumab, MTX and fingolimod, and compared them to 'no treatment'. Recent drugs, such as teriflunomide and dimethylfumerate, were excluded because of small sample size.

IFN β is a recombinant form of a naturally occurring polypeptide produced by fibroblasts. It has anti-inflammatory effects, due to inhibition of T cell proliferation, induction of a shift from pro- to anti-inflammatory cytokine production, and reduction of leukocyte migration across the blood-brain barrier (286). In RRMS patients, the relapse rate, progression of disability and disease activity (MRI) is reduced after IFN β treatment (287). GA consists of synthetic peptides resembling MBP sequences. It is thought that its anti-inflammatory action is based on promoting GA-reactive Th2 cells, which lead to bystander suppression by anti-inflammatory cytokine production (288). In GA-treated RRMS patients, relapse rate and MRI-activity are reduced (287). Alemtuzumab is a recombinant monoclonal CD52 antibody. This CD52 glycoprotein is expressed on the surface of T and B cells, and binding of alemtuzumab on these cells will induce lysis and thus depletion (289). In RRMS patients treated with alemtuzumab, relapse rate, disability progression and MRI lesions were reduced (287). Natalizumab is another monoclonal antibody, directed against α4-integrin (VLA-4). Leukocytes expressing VLA-4 cannot bind their ligand and therefore cannot adhere to inflamed endothelium, for migration towards the CNS. RRMS patients treated with this antibody, had reduced relapse rate, disability progression and MRI lesions compared to placebo (290).

MTX is a synthetic drug that interacts with nuclear DNA and targets proliferating immune cells, inhibiting proliferation and inducing apoptosis (287). RR and SPMS patients experienced reduction in relapse rate, disability progression and MRI disease activity (291, 292). Fingolimod is a sphingosine 1-phosphate receptor antagonist. Upon binding this receptor on lymphocytes, it induces receptor degradation, thereby prohibiting autoreactive lymphocytes to migrate out of the lymph nodes towards the CNS (293). RRMS patients treated with fingolimod had a reduction in relapse rate, disability progression and MRI lesions (287). For in-depth information regarding MS treatments and underlying modes of action see Torkildsen et al. (287).

We could not find any difference in CD4+CD28null T cell percentages between treated and untreated MS patients, except for fingolimod (figure 7.3).

During fingolimod treatment, there is a relative increase in the proportion of CD4+CD28null T cells within the CD4+ T cell population. This is probably attributable to the fact that memory T cells remain in the circulation, whereas other T cells are retained in the lymph nodes (294), and not due to absolute expansion of the CD4+CD28null T cells. Indeed, during fingolimod treatment, the peripheral CD4+ T cell compartment changes drastically, enlarging the relative number of CD4+CD28null T cells within this compartment, whereas the absolute number most likely does not increase.

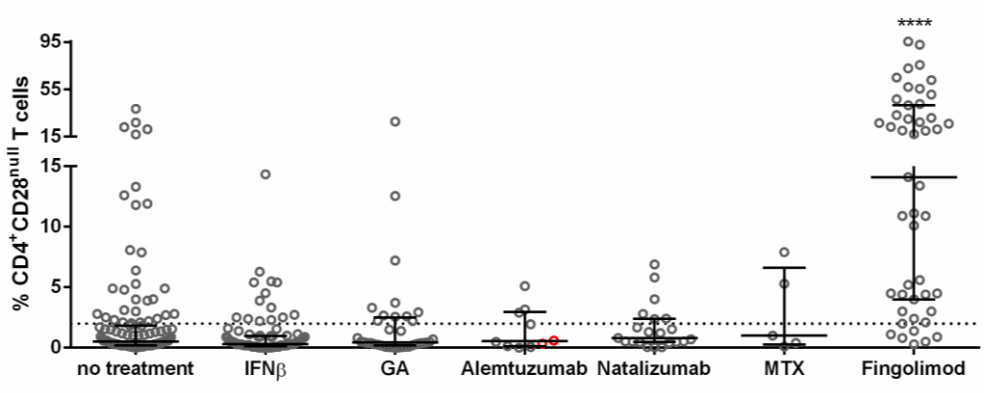


Figure 7.3: The influence of treatment on CD4+CD28null T cell percentages. Blood of MS patients treated with different established MS therapies was analyzed for CD4+CD28null T cell percentages via flow cytometry (dotted line represents threshold for expansion (≥2%). Median ± interquartile range, ****p<0.0001 compared to no treatment. Alemtuzumab-treated patient at re-infusion time-point are depicted in red (n=2), others are patients at start of treatment (n=8).

The lack of effect of Alemtuzumab may relate to the time of sampling. Most patients were sampled at start of treatment or just before re-infusion. Since induction of T cell lysis is the mode of action, sampling during treatment will be necessary to define an effect of treatment. Other MS treatment options, discussed above, do not decrease the percentage of CD4+CD28null T cells. Further studies are needed to investigate possible changes in function.

Are there other possibilities to block these cells? One option is to prevent them from forming and expanding, by targeting their most known trigger; CMV infection. Multiple vaccine candidates have been developed and the two main candidates are the gB/MF59 vaccine and the DNA vaccine TransVax, but still need to pass phase III trials (295). Another option consists of antiviral drugs, which are currently used for treatment and prophylaxis of CMV infection and disease. Ganciclovir (or its prodrug valganciclovir), foscarnet and cidofovir act by being a competitive inhibitor of viral DNA polymerase. Fomivirsen inhibits translation of the CMV major immediate early proteins. However, development of viral resistance, toxicities and inhibition of the host's immune response to CMV may limit the usefulness of these drugs. Therefore, new drugs are being synthesized and tested in clinical trials (296). Of course the benefits should always outweigh the costs, thus research is needed to see whether it is fruitful to prophylactically treat the entire population. Perhaps, in light of CMV infection itself, one could specifically target immunosuppressed adults or mothers-to-be (to prevent congenital HCMV infection). Based on the economic costs saved and the potential improvement in quality of life, the Institute of Medicine has identified CMV vaccine development as a major public health priority (295, 297, 298).

Direct targeting of CD4+CD28null T cells and their functions could also be an option. First, blocking the loss or re-establishing expression of CD28 on these cells could be investigated. Two cytokines, TNF-α and IL-12, have been described to influence CD28 expression. The addition of anti-TNF-α or IL-12 induces re-expression of CD28 (299-301). However, whether blockade or addition of these cytokines also alters the cytotoxic or pro-inflammatory function of CD4+CD28null T cells is not known and needs further study. Furthermore, TNF-α blockade induced severe side-effects in MS patients (302).

Therefore, a better option would be to block their activation and effector functions. Theoretically, targeting co-stimulatory pathways should hamper their activation. In this regard, OX40 and 4-1BB are interesting targets, since they are upregulated on CD4+CD28null T cells and seem to compensate for the loss of CD28, whereas they are absent on naive or inactive T cells (273).

When blocking effector functions of CD4+CD28null T cells, one should focus on reducing migration, production of pro-inflammatory cytokines and cytotoxic molecules. CD4+CD28null T cells express CX3CR1, whereas their CD28+ counterparts do not (36). CD4+CD28null T cells use this chemokine receptor to migrate towards MS lesions (36), thus blocking this receptor is recommended. However, CX3CR1 is also widely expressed on monocytes and microglia, making specific targeting of T cells difficult.

Production of pro-inflammatory cytokines could be stopped by blocking glycogen synthase kinase 3 (GSK3), which normally activates NFκB. This inhibition would also increase the production of anti-inflammatory cytokines, since GSK3 normally blocks IL-10 and TGF-β. Moreover, GSK3 inhibition has been shown to ameliorate EAE (303). Thus GSK3 inhibition could be beneficial in controlling CD4+CD28null T cell function, but certainly also in neuroinflammation in general. Since CD4+CD28null T cells also promote Th17 differentiation, probably via the production of pro-inflammatory cytokines (chapter 6), GSK3 inhibition would also influence this process, further reducing inflammation.

Blocking cytotoxicity could be accomplished by inhibiting Kv1.3 channels, which reduces the production of IFN-γ and perforin (304). Re-sensitizing CD4+CD28null T cells to apoptosis or Treg suppression, would mean that these cells could be controlled again. Dumitriu et al. demonstrated that addition of a proteasome inhibitor (MG-132) restored apoptosis sensitivity in CD4+CD28null T cells, but did not affect conventional T cells (39). Blocking anti-apoptotic molecules or HOPX, which are upregulated in CD4+CD28null T cells (chapter 6, (29) and Serroukh personal communication), might also reinstall apoptosis sensitivity. Evasion of Treg-mediated suppression could be blocked by adding specific inhibitors or antibodies against e.g. IFN-γ and granzyme B, as shown in chapter 6.

Next to Treg and Th17 interactions with CD4+CD28null T cells, many other immune cells could possibly interact with these cells, including macrophages and B cells.

In the future, experiments should be performed to investigate whether CD4+CD28null T cells also promote a pro-inflammatory phenotype in these cells. Co-cultures and subsequent phenotypic characterization could shed light on this hypothesis. Besides immune cells, neural cells are also an interesting target.
CD4+ CTLs are known to attack oligodendrocytes (45), is this also the case for neurons and microglia? To investigate this, *in vitro* cytotoxicity assays could be set up, e.g. chromium-release assay. If CD4+CD28null T cells indeed interact with other immune cells and neural cells, targeting these cells will be an even more important therapeutic strategy.

7.2.6 CD4+CD28null T cells as biomarker?

CD4+CD28null T cells are seen as a pathological hallmark for immune aging. Thus in the context of immunosenescence, the presence of these cells could be a biomarker. Indeed, they are included in the immune risk profile, which confers a higher all-cause mortality in the elderly. The immune risk profile consists of a relative deficit in the numbers and proportions of B cells, an accumulation of late-stage differentiated T cells (CD27-CD28-) and CMV seropositivity (305).
As already mentioned, CD4+CD28null T cells could predict a good, but also bad prognosis, depending on the circumstances. CD4+CD28null T cells are protective in healthy individuals, since they have anti-cancer and anti-viral effects, persons with expansion probably have a better prognosis compared to individuals without expansion. For chronic inflammatory diseases, the opposite is true.
The presence of CD4+CD28null T cells indicates a worse prognosis (Chapter 5), and thus more rigorous therapies should be started to prevent rapid progression, or therapies that affect CD4+CD28null T cell function. Indeed in chapter 5, we showed that CD4+CD28null T cell expansion correlates with a worse prognostic score in MS patients. However, only clinical parameters (EDSS, MSSS, number of relapses and electrophysiological scores) were investigated in MS patients with or without CD4+CD28null T cell expansions. In the future, it would be wise to screen for known biomarkers as well. Intrathecal oligoclonal IgM or neurofilament light chain are both related to disease progression and therefore are good candidates to study in correlation with CD4+CD28null T cells. If indeed a correlation exists, perhaps CD4+CD28null T cells can become part of the biomarker panel themselves.

In summary, CD4+CD28null T cells could be used as a prognostic biomarker in both aging and disease. Screening for this cell type might be beneficial for patients, since tailored therapies could be started, depending on the prognosis connected to the presence of CD4+CD28null T cells (friends vs. foes).

8

NEDERLANDSE SAMENVATTING

Een verstoord evenwicht tussen beschermende en schadelijke immuunresponsen is een belangrijk gegeven in het ontstaan en het verloop van de ziekte multiple sclerose (MS), een chronische aandoening van de hersenen en het ruggenmerg. Deze wanverhouding wordt beïnvloed door zowel omgevingsfactoren (bacteriën en virussen, dieet, klimaat, …) als genetische invloeden. In sommige MS-patiënten komen CD4+CD28null T-cellen voor. Deze cellen hebben schadelijke eigenschappen, die de ziekte zouden kunnen verergeren. In deze thesis proberen we de oorzaken van de vorming en vermenigvuldiging van CD4+CD28null T-cellen te achterhalen, alsook te onderzoeken of cytomegalovirus (CMV) en CD4+CD28null T-cellen bijdragen aan MS. In de volgende paragrafen worden de belangrijkste resultaten samengevat.

8.1 CMV-infectie en chronische ontsteking, maar niet genetische defecten in TLR2, MICB en ILRA, leiden tot de vorming en expansie van CD4+CD28null T-cellen.

CD4+ T-cellen verliezen CD28-expressie door herhaaldelijke stimulatie met hetzelfde antigen[1], dit komt voor tijdens chronische activatie van het immuunsysteem. Omdat MS een chronische ontstekingsziekte is en CMV een persistente infectie[2], zijn beide goede kandidaten voor het veroorzaken van CD4+CD28null T-celvorming en -expansie.

In **hoofdstuk 3** onderzochten we de CMV-status en CMV-specifieke antistoffen in het bloed van donoren met en zonder CD4+CD28null T-celexpansie. Hier zagen we een associatie tussen CMV-seropositieve donoren en de aanwezigheid van CD4+CD28null T-cellen. Bovendien vonden we een correlatie tussen de hoeveelheid CMV-antistoffen en het aantal CD4+CD28null T-cellen. In toekomstige experimenten, kan een opvolging van CMV-antistoffen gecombineerd met CMV-DNA metingen weergeven of deze correlatie enkel een weerspiegeling van de CMV replicatie activiteit is of niet. Om nu CMV echt als oorzaak voor CD4+CD28null T cell expansie aan te duiden, hebben we CMV-stimulatie testen gedaan, zowel *in vitro*[3] als *in vivo*[4].

[1] Soort eiwit waartegen een immuunrespons kan ontstaan
[2] Het virus blijft voor altijd aanwezig
[3] Buiten het lichaam, in schaaltjes in het lab
[4] In een levend wezen, in dit geval muizen

Herhaalde *in vitro* stimulatie van humane immuuncellen met een CMV-eiwit zorgde enkel voor expansie van bestaande CD4+CD28null T-cellen. Maar het CMV-muismodel toonde aan dat een langdurige CMV-infectie kan zorgen voor de vorming en expansie van CD4+CD28null T-cellen *in vivo*. Er is dus een daadwerkelijke CMV-infectie nodig voor de vorming van deze cellen.

In een muismodel van MS (experimentele autoimmune encefalomyelitis, EAE), onderzochten we of chronische neuroinflammatie[5] ook kan leiden tot de vorming en expansie van CD4+CD28null T-cellen. Onze resultaten toonden aan dat CD4+CD28null T-cellen ontstaan tijdens de acute fase van EAE, wanneer er veel ontsteking aanwezig is. Bovendien was de hoeveelheid van deze cellen evenredig met de hoeveelheid demyelinisatie[6]. De stijging in het aantal CD4+CD28null T-cellen kan toegeschreven worden aan herhaalde stimulatie met een autoantigen[7], aangezien herhaalde *in vitro* stimulatie van autoreactieve T-cellen met een myeline-eiwit zorgde voor de expansie van CD4+CD28null T-cellen.

Naast chronische stimulatie kunnen genetische factoren, zoals mutaties[8], mensen meer vatbaar maken voor CD4+CD28null T-celvorming. In **hoofdstuk 4** bevestigden we het verhoogde percentage CD4+CD28null T-cellen in CMV-seropositieve donoren en onderzochten we de aan- of afwezigheid van bepaalde mutaties die gelinkt zijn met CMV (*MICB*, *TLR2*) of MS (*IL2RA*). We konden geen correlatie vinden tussen deze mutaties en het percentage CD4+CD28null T-cellen of de aan- of afwezigheid van hun expansie. Dit is mogelijk te wijten aan de beperkte hoeveelheid stalen, aangezien het ging om een pilootstudie.

Bovendien konden we ook de reeds gepubliceerde associatie van MICB met CMV niet reproduceren, hoewel we hier wel genoeg stalen voor hadden. Dit kan er op wijzen dat de eerdere studie van Shirts et al. niet accuraat is. Het grote verschil tussen beide studies is dat zij gezonde controles en wij MS-patiënten onderzocht hebben. Het zou dan ook kunnen dat genetische effecten op de immuunrespons gemaskeerd werden door MS zelf.

Samenvattend tonen deze resultaten dat CD4+CD28null T-cellen ontstaan tijdens CMV-infectie en neuroinflammatie, maar dat ze niet beïnvloed worden door de hier onderzochte mutaties.

[5] Ontsteking in het centraal zenuw stelsel
[6] Verlies van isolerende laag (=myeline) rond zenuwbanen
[7] Lichaamseigen antigen
[8] wijzigingen in het DNA

8.2 CMV verergert klinische en pathologische symptomen van EAE

De rol van CMV in MS is controversieel; schadelijke maar ook beschermende effecten werden reeds aangetoond. In **hoofdstuk 2** bespraken we de huidige literatuur in verband met deze kwestie en stelden we mechanismen voor waarmee CMV mogelijk MS kan verergeren. Deze mechanismen zijn 'molecular mimicry'[9], 'bystander activation'[10] en 'epitope spreading'[11]. De activatie en expansie van CD4+CD28null T-cellen, via CMV-infectie, kan ook bijdragen aan het MS-ziektebeeld.

Aan de andere kant zou CMV via het ontwijken van het immuunsysteem, de autoimmune respons en het pro-inflammatoire milieu kunnen verminderen. De literatuur die CMV als schadelijk ziet, toonde aan dat er moleculaire mimicrie is tussen een CMV en een myeline-eiwit in 2 verschillende diermodellen. Bovendien zijn CMV-specifieke antistoffen verhoogd aanwezig in MS-patiënten in vergelijking met gezonde controles. De aanwezigheid van deze antistoffen correleerde met een verminderde tijdsspanne tussen relapsen[12], een verhoogd aantal relapsen en meer hersenatrofie[13]. Er is echter een andere studie die concludeerde dat de aanwezigheid van deze CMV-specifieke antistoffen leidde tot een beter klinisch verloop, een hogere leeftijd bij ziekteontwikkeling en minder hersenatrofie. Bovendien zorgde een MCMV-infectie 2 weken voor TMEV infectie (MS muismodel) voor een beter ziektebeeld. Toch vond een recente meta-analyse op 1341 MS-patiënten en 2042 gezonde controles geen sluitende bewijzen voor de relatie tussen CMV-infectie en MS. Verder onderzoek is dus nodig om de rol van CMV te achterhalen.

In **hoofdstuk 3**, hebben we de rol van CMV in EAE onderzocht aan de hand van een diermodel dat CMV en EAE combineerde. We toonden via een dagelijks scoresysteem aan dat deze dieren een erger ziektebeeld hadden dan EAE-dieren zonder infectie.

[9] Een eiwit van het virus bootst een lichaamseigen eiwit na
[10] Owv de immuunrespons tegen het virus, kunnen autoreactieve T-cellen geactiveerd worden
[11] Door de aangerichte schade komen er steeds meer autoantigenen vrij, die andere autoreactieve T-cellen kunnen activeren
[12] Het terugvallen na een periode zonder symptomen
[13] Het afsterven van hersenweefsel

Bovendien was de reactiviteit van CD4+ T-cellen t.o.v. een myeline-eiwit verhoogd, zodat er meer inflammatie kan ontstaan. Tot slot was er meer demyelinisatie in het ruggenmerg van CMV-geïnfecteerde EAE-muizen in vergelijking met controledieren. Onze resultaten ondersteunen de suggestie dat CMV bijdraagt aan EAE-verergering via 'bystander activation' van autoreactieve T-cellen, waaronder CD4+CD28null T-cellen, in plaats van 'molecular mimicry' of 'epitope spreading'. We vonden inderdaad een verhoogd percentage CD4+CD28null T-cellen in deze muizen, wat correleerde met de geobserveerde ziekteverergering in CMV-geïnfecteerde EAE-muizen.
In conclusie toonden we aan dat CMV autoimmuun-gemedieerde inflammatie en demyelinisatie verergert in een diermodel van MS.

8.3 CD4+CD28null T-cellen dragen bij tot een ernstiger MS ziektebeeld

Tot nu is er enkel indirect bewijs (via *in vitro* en *ex vivo* experimenten) dat CD4+CD28null T-cellen linkt met MS. In **hoofdstuk 3** toonden we aan dat CD4+CD28null T-cellen vermenigvuldigen na EAE inductie en dat ze correleren met de ziektegraad, inflammatie en demyelinisatie. We suggereerden verder dat CD4+CD28null T-cellen migreren naar het centraal zenuw stelsel om daar hun cytotoxische[14] en pro-inflammatoire functies uit te oefenen, aangezien CD4+granzyme B+ T-cellen aanwezig zijn in het ruggenmerg van EAE dieren. Het effector geheugen fenotype[15] van deze cellen is sterk gelijkend op dat van humane CD4+CD28null T-cellen; ze hebben een lage tot zelfs geen expressie van CD62L, CD27 en CD127 en ze produceren IFN-γ en granzyme B. Bovendien bevestigden we hun autoreactieve capaciteit, aangezien ze reageren op stimulatie met een myeline-eiwit.
In **hoofdstuk 5** werden klinische parameters (ziektegraad scores, relapsfrequentie,…) van MS-patiënten gebruikt om te bepalen of CD4+CD28null T-celexpansies bijdragen aan een erger ziektebeeld en ernstigere progressie. Voor deze analyses werd een nieuwe intuïtieve classificatie gemaakt, de intrinsieke MS prognostische classificatie (IMPC).

[14] De mogelijkheid om andere cellen te doden
[15] Eigenschappen van de cel

Deze IMPC is gebaseerd op MRI, klinische (type MS, ziekteduur, ziektegraad scores en relapsfrequentie) en elektrofysiologische ('evoked potentials') data. Een ernstige IMPC associeerde met een verhoogd aantal relapsen, hogere ziektegraad scores, een ernstigere globale elektrofysiologische score en kwam vaker voor bij chronisch progressieve MS-patiënten. Binnen de relapsing-remitting MS-patiëntpopulatie, hadden patiënten met CD4+CD28null T-cellen een ernstigere IMPC in vergelijking met patiënten zonder expansie. Dit wijst er op dat de aanwezigheid van deze cellen gelinkt is met een ernstigere prognose in RRMS-patiënten.
Samenvattend bewijzen we dat CD4+CD28null T-cellen bijdragen aan MS-ziekte, zowel in een diermodel als in RRMS-patiënten.

8.4 CD4+CD28null T-cellen zijn pro-inflammatoir, ontwijken Treg-suppressie en induceren Th17-differentiatie

Van voorgaande onderzoeken weten we dat CD4+CD28null T-cellen pro-inflammatoir, autoreactief en cytotoxisch zijn en dat ze kunnen migreren naar MS laesies[16]. Dit wijst er op dat ze rechtstreeks kunnen bijdragen aan inflammatie en ook schade kunnen berokkenen aan het centrale zenuwstelsel. In **hoofdstuk 6** werd het fenotype van CD4+CD28null T-cellen verder onderzocht. Een vergelijking tussen CD4+CD28null T-cellen en CD4+CD28+ T-cellen toonde aan dat er een verhoogde expressie is van pro-inflammatoire cytokines[17] IL-1β, IL-6, IL-22 en GM-CSF, en een verhoogde expressie van HOPX, een transcriptiefactor[18] dat overleving reguleert. Oppervlakte receptoren[19] IL-10R en GITR waren verlaagd, terwijl PD1 verhoogd tot expressie kwam op CD4+CD28null T-cellen.

[16] Plekken in het centrale zenuwstelsel waar geen myeline meer aanwezig is, maar wel vaak veel inflammatie
[17] Molecule dat een rol speelt in de immuunrespons, wordt geproduceerd door immuuncellen
[18] Eiwit dat de omzetting van DNA naar mRNA controleert, in dit geval DNA dat met overleving te maken heeft
[19] Hier kunnen cytokines op binden om hun functie uit te voeren

Bovendien toonden we aan dat het secretoom[20] van CD4+CD28null T-cellen de differentiatie van memory T-cellen naar Th17-cellen[21] induceerde en dat het het fenotype van Tregs[22] veranderde naar een pro-inflammatoir celsubset. Voorgaand onderzoek toonde aan dat CD4+CD28null T-cellen Treg-gemedieerde onderdrukking kunnen omzeilen.

Om het onderliggende mechanisme te achterhalen, onderdrukten we granzyme B, IFN-γ, IL-10R en GITR in een Treg suppressie assay *in vitro*.

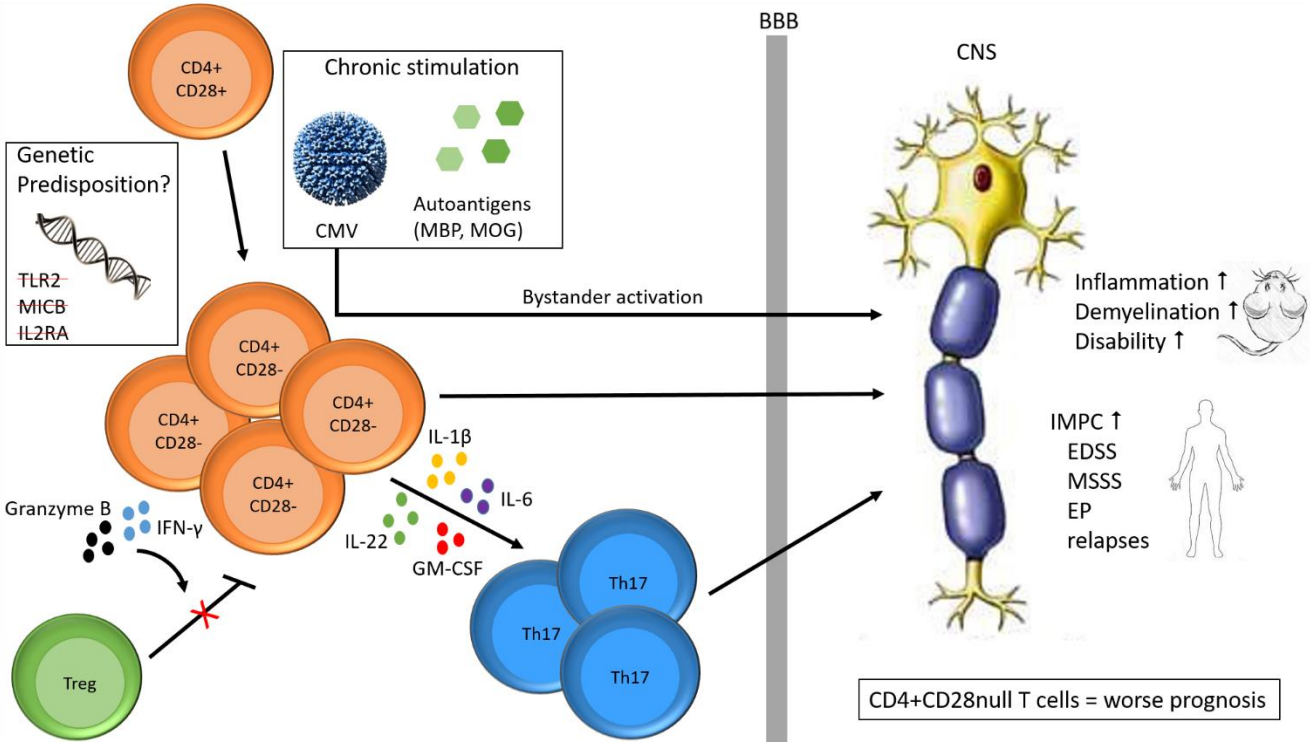
We vonden dat het blokkeren van granzyme B en IFN-γ de suppressie van CD4+CD28null T-cellen kon herstellen, wat suggereert dat CD4+CD28null T-cellen Treg suppressie omzeilen via de productie van deze molecules.

In conclusie tonen deze data aan dat CD4+CD28null T-cellen rechtstreeks kunnen bijdragen aan inflammatoire ziektes via de productie van pro-inflammatoire cytokines, maar ook via het omzeilen van Treg-gemedieerde onderdrukking en inductie van pathogene Th17-cellen, die de immuunrespons nog verder kunnen doen toenemen.

[20] Alles wat deze cel produceert (cytokines, cytotoxische stoffen,…)

[21] Pathogene pro-inflammatoire T-cellen, dragen bij aan MS ziekte

[22] Cellen die de immuunrespons reguleren, ze kunnen andere cellen stoppen (onderdrukken)



Figuur 8.1: Mechanismen van CD4+CD28null T-celvorming, -expansie en functie. CD4+CD28+ T-cellen verliezen CD28 na chronische antigen stimulatie, hetzij door cytomegalovirus (CMV) infectie of door multiple sclerose zelf. Sommige individuen kunnen een genetische predispositie hebben voor de ontwikkeling van deze cellen, maar deze predispositie bestaat niet uit mutaties in toll-like receptor 2 (TLR2), interleukin 2 receptor α (IL2RA) of MHC class I polypeptide-related sequence B (MICB). CD4+CD28null T-cellen dragen direct bij tot MS, aangezien ze autoreactief en cytotoxisch zijn, pro-inflammatoire cytokines produceren en kunnen migreren naar het centrale zenuwstelsel (CNS). Bovendien kunnen CD4+CD28null T-cellen onderdrukking door Tregs omzeilen via de productie van granzyme B en interferon-γ (IFN-γ), waardoor ze resistent zijn voor regulatie. Daarenboven promoten CD4+CD28null T-cellen de differentiatie van Th17 cellen, die door de bloed-hersen-barrière (BBB) kunnen om schade aan te richten. In EAE, het muismodel van MS, verhogen CMV en CD4+CD28null T-cellen de inflammatie, demyelinisatie en ziektegraad. In MS-patiënten, correleren de CD4+CD28null T-cellen met een ernstige intuïtieve MS prognostische classificatie (IMPC), wat er op wijst dat de aanwezigheid van deze cellen kan leiden tot een ernstigere prognose.

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Curriculum Vitae

Marjan Vanheusden werd geboren op 21 mei 1989 te Hasselt. In 2007 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Latijn-Wetenschappen aan het Heilig-Graf instituut te Bilzen. Vervolgens startte ze aan de opleiding Biomedische Wetenschappen aan de Universiteit Hasselt/transnationale Universiteit Limburg, waar ze in 2010 haar diploma bachelor in de Biomedische Wetenschappen met onderscheiding behaalde. In 2012 behaalde ze haar diploma master in de Biomedische wetenschappen, afstudeerrichting Klinische Moleculaire Wetenschappen, met grote onderscheiding samen met de certificaten voor proefdierkunde (FELASA C) en stralingsbescherming. Haar eindwerk, getiteld "Defining the neurotrophic and immunomodulatory properties of Wharton's jelly stem cells" werd uitgevoerd aan het Biomedisch Onderzoeksinstituut van de Universiteit Hasselt in de groep van Prof. dr. Niels Hellings en werd bekroond met een European Master in Neuroscience (EMiN) grant op de EURON PhD days (2012, Maastricht). In 2012 startte ze haar doctoraat onder het promotorschap van prof. dr. Piet Stinissen, prof.dr. Niels Hellings en dr. Bieke Broux. Gedurende deze periode deed ze onderzoek naar de rol van cytomegalovirus en cytotoxische CD4+ T-cellen in multiple sclerosis. Tijdens haar doctoraat bracht ze een studiebezoek van een maand aan het lab van dr. Ramon Arens in Leiden, Nederland. Ze schreef aanvragen voor onderzoeksmandaten (IWT, FWO) en -projecten (FWO). Daarnaast volgde ze verschillende cursussen (e.g. project and time management, good scientific conduct and lab book taking, biosafety) en was aanwezig op internationale congressen (e.g. ESNI, FOCIS, ABC5 summer school). Bovendien ontving ze reisbeurzen om congressen bij te wonen (ESNI, EFIS, international mobility, FWO) en won ze de prijs voor beste presentatie op de MS-onderzoeksdagen in 2015 (Oegstgeest, Nederland) en op het symposium 'The immune brain axis: from molecules to behavior' in 2015 (Hasselt, België).

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Publications from this work

1. Cytomegalovirus: a culprit or protector in multiple sclerosis?
Vanheusden M, Stinissen P, 't Hart BA, Hellings N.
Trends in Molecular Medicine. 2015 Jan;21(1):16-23.

2. Cytomegalovirus infection exacerbates autoimmune mediated neuroinflammation.
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3. Cytotoxic CD4+CD28null T cell expansions are associated with worse disease progression in patients with relapsing remitting multiple sclerosis.
Peeters L.M. & **Vanheusden M.**, Somers V., Van Wijmeersch B., Stinissen P., Broux B., Hellings N.
In preparation

4. The origin, biology and role of cytotoxic CD4+ T cells in health and disease.
Marjan Vanheusden, Bieke Broux, Piet Stinissen, Arnaud Marchant, Niels Hellings
Review, in preparation

5. CD4+CD28null T cells evade Treg-mediated suppression and promote Th17 responses.
Marjan Vanheusden, Bieke Broux, Liesbet M. Peeters, Veerle Somers, Piet Stinissen, Niels Hellings
In preparation

Publications in collaboration

1. IL-15 amplifies the pathogenic properties of CD4+CD28- T cells in multiple sclerosis.
   Broux B, Mizee MR, **Vanheusden M**, van der Pol S, van Horssen J, Van Wijmeersch B, Somers V, de Vries HE, Stinissen P, Hellings N.
   Journal of Immunology. 2015 Mar 1;194(5):2099-109.

2. Human Wharton's Jelly-Derived Stem Cells Display Immunomodulatory Properties and Transiently Improve Rat Experimental Autoimmune Encephalomyelitis.
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3. Age-associated B cells with proinflammatory characteristics are expanded in a proportion of multiple sclerosis patients
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4. Comparative gene expression analysis of mesenchymal-like stem cells derived from human umbilical cord and bone marrow.
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   Under review, Stem cells and development

Published abstracts

Does CMV Trigger CD4+CD28null T Cell Expansion in the Context of MS?
Marjan Vanheusden, Suzanne Welten, Bieke Broux, Liesbet M. Peeters, Ramon Arens, An Goris, Piet Stinissen, Niels Hellings
BIOMEDICA 2015, 2-3 June 2015, Genk, Belgium

Cytomegalovirus Infection Exacerbates Autoimmune Mediated Neuroinflammation
Marjan Vanheusden, Bieke Broux, Suzanne P.M. Welten, Liesbet M. Peeters, Bart Van Wijmeersch, Veerle Somers, Piet Stinissen, Ramon Arens, Niels Hellings
FOCIS 2016, 22-25 June 2016, Boston, USA

Oral Presentations

The cause and role for $CD4^{+}CD28^{null}$ T cells in patients with multiple sclerosis?
Marjan Vanheusden, Bieke Broux, Liesbet M. Peeters, An Goris, Piet Stinissen, Niels Hellings
IUAP meeting, 3 June 2014, Brussel Belgium

Does CMV trigger CD4+CD28null T cell expansion in the context of MS?
Marjan Vanheusden, Suzanne Welten, Bieke Broux, Liesbet M. Peeters, Ramon Arens, An Goris, Piet Stinissen, Niels Hellings

- 5th International Workshop on CMV and Immunosenescence, 20-21 November 2014, Amsterdam, the Netherlands
- The immune brain axis symposium: from molecules to behavior, 12-13 March 2015, Hasselt, Belgium
- MS Research Days, 28-29 May 2015, Oegstgeest, the Netherlands
- IUAP meeting, 6 July 2015, Diepenbeek, Belgium

CMV infection drives the expansion of cytotoxic CD4 T cells that aggravate autoimmune mediated neuroinflammation
Marjan Vanheusden, Bieke Broux, Suzanne P.M. Welten, Liesbet M. Peeters, Piet Stinissen, Ramon Arens, Niels Hellings
IUAP Meeting, 16 October 2015, Charleroi, Belgium

Cytomegalovirus Infection Exacerbates Autoimmune Mediated Neuroinflammation
Marjan Vanheusden, Bieke Broux, Suzanne P.M. Welten, Liesbet M. Peeters, Bart Van Wijmeersch, Veerle Somers, Piet Stinissen, Ramon Arens, Niels Hellings
EFIS-EIAS Summer School ABC5 - Autoimmunity: basic and clinical, 6-8 June 2016, Tartu, Estonia

Poster Presentations

Does CMV infection cause expansion of cytotoxic CD4+CD28null T cells?
Marjan Vanheusden, Bieke Broux, Liesbet M. Peeters, Ine Pauwels, An Goris, Piet Stinissen, Niels Hellings

- BIS Annual meeting, 8 November 2013, Liege, Belgium
- MS Research days, 27-29 November 2013, Hasselt, Belgium
- FWO-WOG-MS symposium, 16 May 2014, Leuven, Belgium
- Summer frontiers symposium: Age & Immunity, 12-13 June 2014, Nijmegen, the Netherlands

Does CMV Trigger CD4+CD28null T Cell Expansion in the Context of MS?
Marjan Vanheusden, Suzanne Welten, Bieke Broux, Liesbet M. Peeters, Ramon Arens, An Goris, Piet Stinissen, Niels Hellings

- BIOMEDICA 2015, 2-3 June 2015, Genk, Belgium
- FWO-WOG-MS symposium, 25 March 2016, Gent, Belgium

Cytomegalovirus Infection Exacerbates Autoimmune Mediated Neuroinflammation
Marjan Vanheusden, Bieke Broux, Suzanne P.M. Welten, Liesbet M. Peeters, Bart Van Wijmeersch, Veerle Somers, Piet Stinissen, Ramon Arens, Niels Hellings

- EFIS-EIAS Summer School ABC5 - Autoimmunity: basic and clinical, 6-8 June 2016, Tartu, Estonia
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Awards and grants

ESNI travel grant
ESNI congress, 2013, Porto (Portugal)

Travel grant for international mobility
- ESNI summer school, 2013, Porto (Portugal)
- Short stay abroad, 2014, LUMC, Leiden (the Netherlands)

EFIS travel grant
5th International Workshop on CMV and Immunosenesence, 2014, Amsterdam (the Netherlands)

Best oral presentation
The immune brain axis symposium: from molecules to behavior, 2015, Hasselt (Belgium)

Best oral presentation
MS research days, 2015, Leiden (the Netherlands)

FWO travel grant
FOCIS, 2016, Boston (USA)

Dankwoord

Vroeger beantwoordde ik de vraag: 'Wat wil je later graag worden?' steevast met: 'Archeoloog! Dokter! Onderzoeker!'. Niet echt de standaard antwoorden die je verwacht van een kind. Maar kijk, toen al had ik de microbe van de wetenschappen te pakken. Tijdens mijn studies is deze fascinatie alleen maar gegroeid, vooral voor levenswetenschappen. Misschien lag dat ook aan het feit dat ik doorhad dat archeologen helemaal niet 'Indiana Jones-gewijs' door het leven gingen ☺. Dan bleef er nog dokter of onderzoeker over; wat moest ik kiezen? Eigenlijk was de keuze snel gemaakt, ik wou ziektes helpen genezen, maar dan door de ziekte te ontrafelen en niet door patiënten te behandelen. En dan kwam de grootste uitdaging tot nu toe: doctoreren! Wat een avontuur waren de afgelopen vier jaar, maar eindelijk is het dan zo ver; ik sta aan de eindmeet. En dit had ik zeker niet gekund zonder de steun van heel wat mensen, die ik dan nu ook uitgebreid wil bedanken.

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"Want for nothing but patience - or give it a more fascinating name: Call it hope."

—Jane Austin, Sense and Sensibility (1811)