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master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

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Promotor :
Prof. dr. Niels HELLINGS

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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ABBREVIATIONS

APC	antigen-presenting cell
AS	ankylosing spondylitis
BBB	blood-brain barrier
BMSC	bone marrow stromal cell
BSA	bovine serum albumin
CCR	C-C motif receptor
CD	cluster of differentiation
CD(…)L	CD(…) ligand
CFSE	carboxyfluorescein succinimidyl ester
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CX_3CL1	fractalkine
CX_3CR1	fractalkine receptor
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr Virus
EDSS	expanded disability status scale
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
γ_c	common cytokine-receptor γ-chain
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gr	granzyme
HC	healthy control
HLA	human leukocyte antigen
HP	homeostatic proliferation
HRP	horseradish peroxidase
Iba1	ionized calcium binding adaptor molecule 1
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
IL-(…)R	interleukin-(…) receptor
KIR	killer immunoglobulin-like receptor
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
LSP	long signal peptide
MFI	mean fluorescence intensity
MIC	MHC class I chain-related molecule
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NAWM	normal appearing white matter
NDC	non-demented control
NK	natural killer
PBMC	peripheral blood mononuclear cell

PBS	phosphate buffered saline
PE	phycoerythrine
PerCP	peridinin chlorophyll
PP	primary progressive
RA	rheumatoid arthritis
RR	relapsing-remitting
SEM	standard error of the mean
SNP	single-nucleotide polymorphism
SP	secondary progressive
SSP	short signal peptide
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tregs	regulatory T cells
TRITC	tetramethylrhodamine
ULBP	UL16-binding protein
VLA-4	integrin $\alpha_4\beta_1$
WG	Wegener's granulomatosis

PREFACE

My thesis is the result of doing 30-weeks intensive research at the Biomedical Research Institute (BIOMED) in Diepenbeek.

During this period, I participated in the multiple sclerosis (MS) and T cell research at the Biochemistry & Immunology department. I do not regret a minute my choice for this internship when looking back to this fantastic experience. Because science is teamwork, I would like to extend my sincere gratitude to everybody who has made it possible to bring my thesis to a good end. It is the team not the person that makes a good research!

First of all, I would like to thank Bieke Broux, my daily supervisor. - Thank you, Bieke, for your excellent guidance to realize this project. Thank you for giving me the chance to actively participate in the experiments. Thank you for sharing your scientific knowledge and tips with me, for the corrections and improvements you have provided me to lift my thesis to a higher level and, last but not least, for your pleasant company throughout my internship.

Furthermore, I would like to thank my promoter, Prof. dr. Niels Hellings, for his research insights and valuable advice during the meetings. - Niels, thank you for giving me the opportunity to perform my internship at BIOMED. Thank you for introducing me to the fascinating world of cytotoxic $CD4^{+}CD28^{null}$ T cells.

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I would like to thank MS patients and healthy controls for their blood donations, and Mrs Anne Bogaers for assistance in collecting the blood samples.

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Thanks to my fellow students Ilse, Jessica, Jo, Joke, Karen, Kenneth, Lauren, Mandy, Marjan, Nathalie, Sarah, Selien, Stefanie, Sylvia and Yanick for their direct and indirect help, and for the fun during the breaks and in the lab. Without them, the atmosphere wouldn't be the same.

Finally, a special word of thanks goes to my parents because they have given me the chance going to the university. They have supported me throughout my entire studies, especially these last few months. Thanks for always believing in me!

ABSTRACT

Introduction: Multiple sclerosis (MS) is a chronic degenerative disease of the central nervous system characterized by inflammation, demyelination, oligodendrocyte loss and neuroaxonal damage. The exact etiology of MS remains unclear, although it is regarded to be an autoimmune disease in which autoreactive T cells play a pivotal role. Interestingly, "premature immunosenescence" is shown to occur in patients with autoimmune disorders including MS. One of the main characteristics of these age-inappropriate immune system abnormalities is the expansion of $CD4^{+}CD28^{null}$ T cells in the peripheral circulation. These T cells have already been reported to be capable of migrating towards MS brain lesions. Moreover, they possess cytotoxic properties, indicating a potential role in MS disease pathology. Since IL-15 increases cytotoxicity of killer cells, and it is increased in serum and cerebrospinal fluid of MS patients, we aimed to investigate whether IL-15 is able to enhance cytotoxic functioning of $CD4^{+}CD28^{null}$ T cells.

Materials & Methods: Peripheral blood mononuclear cells of healthy controls and MS patients (both with expanded $CD4^{+}CD28^{null}$ T cells) were treated with IL-15 to analyze the proliferative capacity and cytotoxic profile of $CD4^{+}CD28^{null}$ T cells in response to this cytokine. We performed a CFSE-based proliferation assay, a flow cytometric staining for several cytotoxic markers (NKG2D, perforin, granzyme B, IFN-γ and IL-17A) and a CD107a-mobilization flow cytometry-based assay. Additionally, using immunohistochemistry, we identified $CD4^{+}$ T cells in close proximity to IL-15-expressing cells in MS brain.

Results: Our *in vitro* data showed that IL-15 preferentially enhances the proliferation rate of $CD4^{+}CD28^{null}$ T cells in both healthy controls and MS patients. Moreover, IL-15 significantly increases the NKG2D surface expression on, as well as the perforin, granzyme B and IFN-γ production of $CD4^{+}CD28^{null}$ T cells. Our immunohistochemical analyses showed that prominent IL-15 levels are present in MS brain and that IL-15 is expressed by microglia/macrophages and astrocytes. We identified in the brain of MS patients a $CD4^{+}$ T cell infiltrate in the vicinity of IL-15, and future experiments will assess their cytotoxicity.

Conclusion: Results of the present study demonstrated that (MS-related) IL-15 aggravates the pathogenic potential of $CD4^{+}CD28^{null}$ T cells. So, they may participate in the inflammatory and degenerative processes seen in MS.

Keywords: multiple sclerosis, immunosenescence, $CD4^{+}CD28^{null}$ T cells, interleukin-15

SAMENVATTING

Inleiding: Multiple sclerose (MS) is een chronische degeneratieve aandoening van het centraal zenuwstelsel gekenmerkt door inflammatie, demyelinisatie, verlies van oligodendrocyten en neuroaxonale schade. De precieze etiologie van MS is tot op heden onduidelijk, hoewel MS beschouwd wordt als een auto-immune ziekte waarin autoreactieve T-cellen een belangrijke rol spelen. Patiënten met auto-immune aandoeningen, inclusief MS, vertonen tekenen van "premature immuunveroudering". Een hoofdkenmerk van deze niet-leeftijdsgebonden immuunsysteem veroudering is de expansie van $CD4^+CD28^-$ T-cellen in de perifere circulatie. Deze T-cellen kunnen migreren naar MS-hersenlaesies. Ook bezitten ze cytotoxische eigenschappen, duidend op een potentiële rol in de ziektepathologie van MS. Aangezien IL-15 de cytotoxiciteit van killercellen doet toenemen, en IL-15 in serum en cerebrospinale vloeistof van MS-patiënten verhoogd is, willen wij met deze studie nagaan of IL-15 ook in staat is om de cytotoxische activiteit van $CD4^+CD28^-$ T-cellen te versterken.

Materialen & Methoden: Perifere bloed mononucleaire cellen van gezonde controles en MS-patiënten (beiden met $CD4^+CD28^-$ T-cellen) werden behandeld met IL-15 ter analyse van het IL-15 geïnduceerde effect op de proliferatieve capaciteit en het cytotoxisch profiel van $CD4^+CD28^-$ T-cellen. Hiervoor werd een CFSE-proliferatieanalyse, een flowcytometrische kleuring voor verschillende cytotoxische merkers (NKG2D, perforine, granzyme B, IFN-γ en IL-17A) en een CD107a-degranulatieanalyse uitgevoerd. Met immunohistochemie, identificeerden we $CD4^+$ T-cellen in de nabijheid gelegen van IL-15 tot expressie brengende cellen in MS-hersenen.

Resultaten: Onze *in vitro* data toonden aan dat IL-15 het aantal prolifererende $CD4^+CD28^-$ T-cellen doet toenemen, zowel in gezonde controles als in MS-patiënten. Ook verhoogt IL-15 de NKG2D oppervlakte-expressie van, alsook de productie van perforine, granzyme B en IFN-γ door $CD4^+CD28^-$ T-cellen. Met immunohistochemische kleuringen, toonden we aan dat in MS-laesies uitgesproken hoge IL-15 concentraties aanwezig zijn en dat IL-15 gepresenteerd wordt door microglia/macrofagen en astrocyten. We identificeerden in MS-hersenen een $CD4^+$ T-celinfiltraat in de buurt van IL-15, en verdere experimenten zullen hun cytotoxiciteit inschatten.

Conclusie: Resultaten van deze studie bewezen dat (MS-gerelateerd) IL-15 het pathogeen potentieel van $CD4^+CD28^-$ T-cellen verergert.

Sleutelwoorden: multiple sclerose, immuunveroudering, $CD4^+CD28^-$ T-cellen, interleukine-15

1 INTRODUCTION

1.1 Immunosenescence – Aging of the immune system

The efficacy and functionality of the immune system is influenced by increasing age, resulting in a raised susceptibility to infectious diseases and pathological conditions related to inflammation and autoreactivity. This age-associated impairment in immunity is termed "immunosenescence". Aging is characterized by a significantly decreased T cell receptor (TCR) diversity. A diminished TCR repertoire increases the risk of an insufficient or absent immune response (1). A subgroup of patients with autoimmune diseases may show age-inappropriate immune system abnormalities that resemble the typical features of immune dysfunction described in the elderly population, named "premature immunosenescence" (2).

1.1.1 $CD28^{null}$ T cells are characteristic for immunosenescence

An important aspect in the progression to immunosenescence is thymic involution, which causes a decline in the output of new T cells from the thymus. Thymic atrophy arises during adolescence and progresses at a rate of 3% per year. Reduced thymic output induces a space-filling autoproliferative mechanism, known as homeostatic proliferation (HP). HP maintains peripheral T cell numbers at a constant level throughout life, but can also lead to premature T cell senescence (1, 2). Another phenomenon that could contribute to immunosenescence is a persistent infection by pathogens, such as the cytomegalovirus (CMV). CMV is a β-herpesvirus that continuously activates the immune system. Approximately 50% of the adult population and 90% of the elderly is CMV-positive. Maintaining protective immunity against CMV is essential, but may affect overall adaptive immunity due to the repeated stimulation of T cells (3, 4). Both compensatory HP and CMV infection induce replicative stress on peripheral T cells. As a result, these $CD4^+$ and $CD8^+$ T cells undergo several phenotypic and functional changes. Replicatively stressed T cells progressively downregulate their expression of the major costimulatory molecule CD28, ultimately leading to the appearance of $CD28^{null}$ T cells. $CD28^{null}$ T cells display a decreased TCR diversity and defects in antigen-induced proliferation. In addition, $CD8^+CD28^{null}$ T cells have enhanced cytotoxic activities, whereas $CD4^+CD28^{null}$ T cells gain various proinflammatory and cytotoxic properties. Therefore, senescent $CD28^{null}$ T cells could participate in the pathogenesis of autoimmune disorders in genetically predisposed individuals in a susceptible environment (1-6).

1.1.2 Phenotype and functional properties of $CD4^+CD28^{null}$ T cells

Repeated antigenic stimulation, due to aging and in situations of chronic infections, is demonstrated to cause progressive loss of the CD28 surface expression on $CD4^+$ helper and $CD8^+$ cytotoxic T cells (7). CD28 is a costimulatory molecule constitutively expressed on the surface of virtually all T cells. CD28 is essential following TCR engagement and plays multiple roles during T cell activation, proliferation and survival. Therefore, CD28 is crucial for the induction and maintenance of T cell-mediated immune responses (5, 8). Vallejo and colleagues reported that loss of the CD28 molecule is caused by transcriptional silencing of its encoding gene (9, 10). In addition, tumor necrosis factor (TNF)-α can directly influence CD28 gene transcription. This suggests that $CD28^{null}$ T cells could be generated in a proinflammatory environment and that persistent infections might drive their expansion (5, 11). Remarkably, CD28 downregulation occurs faster in $CD8^+$ T cells in contrast to the $CD4^+$ T cell population (12). Only $CD4^+CD28^{null}$ T cells will be described further, as our study focuses solely on these cells.

Oligoclonally expanded $CD4^+CD28^{null}$ T cells are terminally differentiated cells characterized by shortened telomeres, a decreased interleukin (IL)-2 homeostasis and an increased resistance to apoptosis. They are functionally active, but distinct from classical helper $CD4^+$ T cells (Figure 1). $CD4^+CD28^{null}$ T cells have lost their expression of the CD40 ligand (CD40L) and consequently are unable to provide helper signals for B cells. They are also incapable of promoting B cell differentiation, because of a deficient IL-2 production (6, 13, 14). $CD4^+CD28^{null}$ T cells have been shown to be resistant to apoptotic cell death in spite of normal expression of death-inducing receptors, for example CD95 (Fas). This resistance to apoptosis is a consequence of the upregulation of the anti-apoptotic protein Bcl-2 and may explain their unusual longevity and persistence (15). Phenotypical characterization of this specific T cell population revealed that $CD4^+CD28^{null}$ T cells are inflammation-seeking due to their significantly high surface expression of various adhesion molecules including CD11a (LFA-1), CD54 (ICAM-1) and CD49d (VLA-4), which are involved in leukocyte recruitment to sites of infection and inflammation. CCR5, a C-C motif chemokine receptor mediating inflammatory responses to infection, is also present on these senescent T cells. In contrast, they have a low expression of homing receptors to lymph nodes, such as CD62L (L-selectin) and CCR7. Recently, Broux *et al.* demonstrated that the $CD4^+CD28^{null}$ T cell population expresses high amounts of the fractalkine receptor (CX_3CR1) on their cell surface in contrast to their $CD28^+$ counterparts, making them capable of migrating to

inflamed tissues (16). Moreover, $CD4^+CD28^{null}$ T cells gain natural killer (NK) cell-like cytotoxic features. For instance, they express the calcium-dependent (C-type) lectin receptor NKG2D, a (co)activating NK cell receptor that is constitutively expressed on NK cells and $CD8^+$ T cells (1, 4, 5, 13, 16-18). NKG2D is reported to be involved in oligodendrocyte killing by $CD8^+$ T cells via NKG2D receptor-ligand interactions (19). Furthermore, the majority of $CD4^+CD28^{null}$ T cells exhibit intracellular granules consisting of cytolytic enzymes; like perforin, granzyme (Gr)A and GrB. These enzymes are usually present in cytotoxic $CD8^+$ T cells and NK cells, whereas conventional $CD4^+$ T cells lack them. $CD4^+CD28^{null}$ T cells can rapidly produce significant levels of proinflammatory cytokines, such as interferon (IFN)-γ and TNF-α. They can exert their cytotoxic activities after antigen-specific and polyclonal TCR stimulation (1, 4, 5, 13, 16-18). In summary, $CD4^+CD28^{null}$ T cells are a proinflammatory cytotoxic T cell population with an effector-memory phenotype. They have features that are a hallmark of an aged immune system.

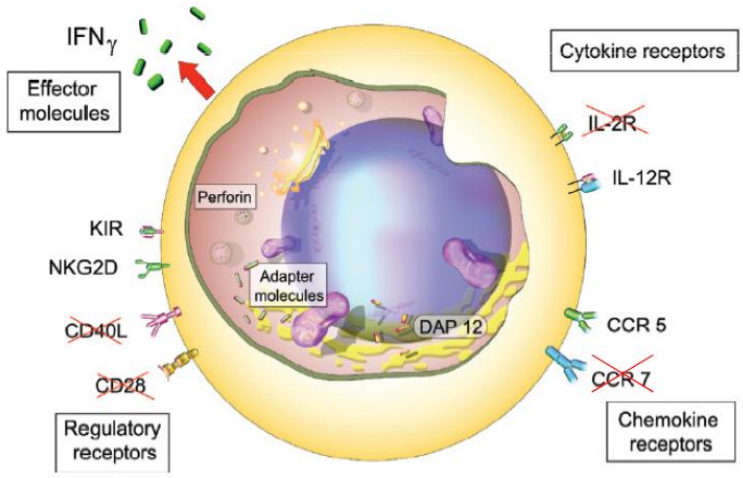


Figure 1: Characteristics of $CD4^+$ $CD28^{null}$ T cells. $CD4^+CD28^{null}$ T cells have an inflammation-seeking effector-memory T cell phenotype and gain several NK-like cytotoxic properties. CCR: C-C motif receptor; CD40L: cluster of differentiation 40 ligand; IFN: interferon; IL-(1)2R: interleukin-(1)2 receptor; KIR: killer immunoglobulin-like receptor.

1.1.3 $CD4^+CD28^{null}$ T cells in autoimmune diseases

Expansion of the $CD4^+CD28^{null}$ T cell population, the main characteristic of immunosenescence, is associated with several autoimmune and chronic inflammatory disorders including rheumatoid arthritis (RA) (20), Graves' disease (21), Wegener's granulomatosis (WG) (22), ankylosing spondylitis (AS) (23) and sporadic inclusion body myositis (24). Data from our group showed that in a subgroup of multiple sclerosis (MS) patients the frequency of $CD4^+CD28^{null}$ T cells has increased in the peripheral circulation (1). In addition, our group recently demonstrated that $CD4^+CD28^{null}$ T cells are present in MS brain lesions and exert cytotoxicity when stimulated with

MS-related autoantigens (16). Taken together, $CD4^{+}CD28^{null}$ T cells may contribute to MS disease pathology in a subgroup of patients. However, the underlying disease mechanisms of their killing activity to disease relevant cell types; like oligodendrocytes and neurons, have not been elucidated yet.

1.2 Multiple sclerosis

MS is a chronic inflammatory disease of the central nervous system (CNS), which is characterized by demyelination, oligodendrocyte loss and neurodegeneration. It is one of the most frequent causes of neurological impairment in young adults, between 20 and 40 years old. MS has a high prevalence rate that varies between 60-200 per 100 000 in Northern Europe and North America. Females are more commonly affected than males, in a ratio of approximately 2:1 (25-27).

MS can be categorized as being either relapsing-remitting (RR-) MS or primary progressive (PP-) MS in onset. The most frequent form is RR-MS (80-90%), which over time will enter a progressive form, known as secondary progressive (SP-) MS (25-27).

Although the exact etiology of MS remains unclear, researchers believe in a multifactorial cause with genetic susceptibility associated with environmental factors. Specific human leukocyte antigen (HLA) types; particularly DRB1*1501, DQA1*0102 and DQB1*0602, are associated with a predisposition for MS. Viruses e.g. Epstein-Barr Virus (EBV), and bacterial infections are proposed as environmental candidate triggers for MS, by either molecular mimicry or bystander activation (25-27).

1.2.1 Immunopathogenesis

MS is regarded to be an autoimmune disease in which self-reactive T cells play a pivotal role (25-27). $CD4^{+}$ T cells are traditionally considered the main effectors in the pathogenesis of MS, but $CD8^{+}$ T cells have been suggested to be responsible for the tissue damage (28, 29). Proinflammatory cytokines including IFN-γ, TNF-α, IL-2, IL-12, IL-17, IL-23 and recently IL-15 are believed to have a crucial contribution in MS disease pathology (30, 31).

Early pathogenesis of MS is mediated by the activation of autoreactive T cells in the periphery by self-antigens, giving rise to for example myelin-reactive T cells. It is still unknown how these antigen-reactive T cells become activated in the periphery (26, 27). However, several

mechanisms involving their peripheral activation have been hypothesized. Many of these are induced by microbial infection, such as microbial epitopes that share structural or sequential similarity with autoantigens. This mechanism is called molecular mimicry. Otherwise, viral and bacterial superantigens may activate myelin-specific T cells. Autoreactive T cells can also be triggered by bystander activation after an infection. Direct viral injury to oligodendrocytes is another mechanism that can lead to the activation of myelin-specific T cells (27, 32). Once activated, pathogenic T cells can migrate to the CNS and become reactivated when they encounter their specific antigen that is presented on CNS-resident antigen-presenting cells (APCs); like astrocytes, microglia, perivascular dendritic cells and macrophages. The blood-brain barrier (BBB) of MS patients is disrupted and more susceptible to cell infiltration, allowing autoreactive T cells to gain access to the CNS (26, 27). T cell entry is mediated by several adhesion molecules, chemokines and proinflammatory cytokines released by the activated T cells. Many of these mediators are upregulated in cerebrospinal fluid (CSF) and brain lesions of MS patients (33, 34). Recruitment of other immune cells including monocytes/macrophages, T cells and B cells, amplifies the inflammatory response. Ultimately, the inflammatory events lead to the formation of MS plaques in the CNS, characterized by demyelination, astrogliosis and oligodendrocytic and neuronal loss (26, 27).

1.2.2 $CD4^{+}CD28^{null}$ T cells in disease pathology – Cause or consequence ?

Increased amounts of $CD4^{+}CD28^{null}$ T cells were detected in one fourth of the MS patients. The enhanced frequencies of these T cells and their divergent characteristics make it likely that they are not just a marker of an aging immune system, but participate actively or passively to MS disease pathology. Several assumptions could link $CD4^{+}CD28^{null}$ T cells to MS, or to autoimmunity in general. The functionality of the immune system is highly dependent on the diversity of the naive T cell pool. Accumulation of $CD4^{+}CD28^{null}$ T cells leads to a diminished competence of the immune system because these T cells fill up immunological space which is otherwise available for functionally competent T cells. This alteration in T cell composition may cause an imbalanced immune system with a changed threshold for T cell activation. Moreover, $CD4^{+}CD28^{null}$ T cells have aberrant functional properties. Through the acquisition of NK-like features, they might sustain inflammation and promote self-perpetuating pathology. Thus, increased percentages in $CD4^{+}CD28^{null}$ T cells represent an important amplification mechanism

in MS and other immune disorders, rather than being involved in the initial breakdown of immunological self-tolerance (1, 13).

1.3 Interleukin-15

IL-15 is a proinflammatory cytokine that belongs to the 4-α-helix bundle cytokine family. Although its three-dimensional structure is similar to that of IL-2, both cytokines do not share a significant homology. Two IL-15 transcript isoforms exist: the 21 amino acid short signal peptide (SSP) and the 48 amino acid long signal peptide (LSP). The LSP IL-15 is the secreted form, while the SSP IL-15 is not secreted but located inside the cell in the cytoplasm and nucleus. IL-15 transcripts are expressed in different kinds of human tissue; for example placenta, skeletal muscle, heart, lung, liver and kidney. Activated peripheral blood monocytes, macrophages, dendritic cells and activated glial cells also have high IL-15 levels (31, 35-39).

IL-15 has biological functions which resemble those of IL-2. Induction of T cell proliferation and survival (of effector/memory T cells) are the most important functions. Besides acting as a T cell growth factor, IL-15 can promote the activation of cytolytic effector cells including NK cells and cytotoxic T cells. Moreover, it upregulates the production of proinflammatory cytokines (e.g. IFN-γ, TNF-α, IL-17) by NK cells and T cells, and induces B cell maturation and isotype switching. In addition, IL-15 is a selective chemoattractant for T cells and an anti-apoptotic cytokine. These functions are attributed to the binding of IL-15 to its receptor complex, which triggers different pathways in different cell types (37-42).

1.3.1 Interleukin-15 receptor complex

IL-15 binds to a specific heterotrimeric receptor complex consisting of three chains; namely IL-15Rα, IL-2/IL-15Rβ (CD122) and the common cytokine-receptor γ-chain (γ_c) (Figure 2A). The effects of IL-15 are mediated through the β- and γ_c-chain of the IL-2 receptor and a unique IL-15Rα-chain. IL-15Rα is expressed on NK cells, T cells, B cells, monocytes, macrophages, dendritic cells, vascular endothelial cells, as well as on thymic and bone marrow stromal cells (BMSCs). Transcripts also have a widespread tissue distribution including skeletal muscle, heart, lung, liver and spleen (38-40, 43).

IL-15Rα can be membrane-bound or soluble. Dubois and colleagues demonstrated that when IL-15 binds to membrane-bound IL-15Rα, this complex undergoes an endosomal recycling process

and subsequently can present IL-15 in *trans* to neighboring cells (Figure 2B). This reappearance can lead to the persistence of IL-15 on the plasma membrane even after its withdrawal from the environment. The IL-15/IL-15Rα complex has a wider biological activity than secreted IL-15 because the signal induced in *trans* can stimulate efficiently the proliferation of cells bearing β/γ_c and cells expressing the whole heterotrimeric IL-15R complex (44, 45). In contrast, soluble IL-15Rα has been reported to function as an IL-15 antagonist. It can also act as a carrier molecule or chaperon to prolong the half-life of secreted IL-15 by protecting it from proteolytic degradation (35, 45).

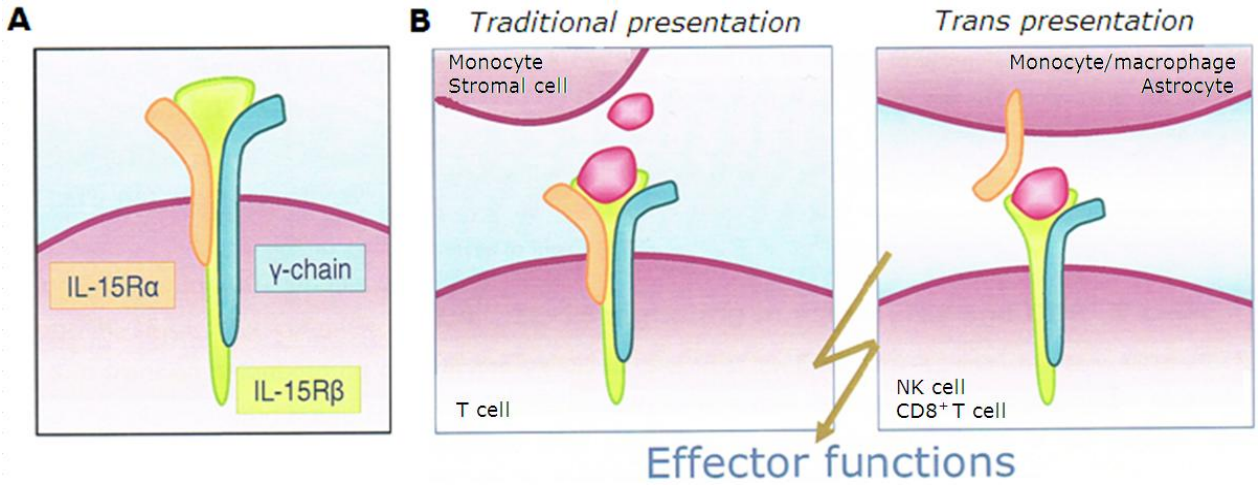


Figure 2: IL-15 and its receptor complex. A. The heterotrimeric IL-15R complex. **B.** Traditional and in *trans* presentation model of IL-15. IL-15 can be presented to cells in two ways. In the traditional model (left panel) IL-15 is shed from one cell (e.g. monocytes, thymic stromal cells and BMSCs) and captured by the heterotrimeric receptor complex on another cell (e.g. T cells). The *trans* presentation model (right panel) suggest that IL-15 bound to IL-15Rα presents the cytokine to opposing cells (e.g. NK cells and $CD8^+$ T cells) and signals via the IL-15Rβ/γ_c complex. Monocytes/macrophages and astrocytes can display the IL-15/IL-15Rα complex. IL-15R: interleukin-15 receptor; NK: natural killer.

1.3.2 Interleukin-15 in multiple sclerosis

The precise contribution of IL-15 to the pathogenesis of MS has not been elucidated yet, although IL-15 is found to be significantly elevated in the serum and CSF of MS patients compared with patients with inflammatory and non-inflammatory neurological disorders, and with healthy controls (HCs) (30, 37). Kivisakk *et al.* reported that the IL-15 mRNA expression is upregulated in blood and CSF mononuclear cells in MS (40). Moreover, increased IL-15 mRNA concentrations have been detected in patients with a relapse compared with patients in a stable

phase of the disease (46). Elevated IL-15 mRNA levels have also been found in active MS lesions (47, 48). These results suggest a role for IL-15 in the disease course of MS.

Monocytes/macrophages and dendritic cells have been reported as the main source of IL-15. They express biologically active IL-15 on their surface bound to IL-15Rα. Expression is upregulated by Toll-like receptor (TLR) ligands; such as lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF) and type I IFN. It has been suggested that IL-15 mRNA is present translationally in inactive pools because IL-15 is rapidly displayed on the cell surface upon activation (36). These cells can interact in *trans* with neighboring cells, like NK cells and $CD8^+$ T cells, and subsequently transduce the IL-15-mediated effects (44, 45).

Surface-bound IL-15 on astrocytes could also participate in MS disease pathology. Saikali and colleagues demonstrated that astrocytes in a proinflammatory milieu express sufficient IL-15 on their surface to enhance the effector functions of antigen-specific $CD8^+$ T cells. For example, astrocytic-bound IL-15 can significantly increase NKG2D expression and cytolytic enzymes (perforin and GrB) of $CD8^+$ T cells. In addition, IL-15 has been reported to be strongly expressed by astrocytes in MS lesions. Thus, astrocytes could contribute to perpetuating the activation of cytotoxic T cells via their IL-15 in *trans* presentation (48).

1.4 Outline of project

$CD4^+CD28^{null}$ T cells are expanded in a subgroup of MS patients. These T cells are suggested to contribute to the MS disease pathology because they gain several proinflammatory and cytotoxic properties. Moreover, they have been reported to be present in MS lesions.

The main goal of this study is to determine the role of membrane-bound IL-15 on the cytotoxic activity of the $CD4^+CD28^{null}$ T cell population. In addition, the effect of IL-15 on their proliferative capacity will be investigated. Several *in vitro* and *in vivo* experiments related to IL-15 were performed on $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells of HCs and MS subjects, in whom a $CD4^+CD28^{null}$ T cell population is present. These experiments will lead to a better understanding of the exact role of these detrimental T cells in the pathogenesis of MS, and more specific their response to (MS-related) IL-15. Long-term results may lead to a more personalized treatment of MS patients and patients with other autoimmune diseases in which $CD4^+CD28^{null}$ T cells are involved.

2 MATERIALS & METHODS

2.1 Study population and patient material

Peripheral blood samples were collected from 97 HCs and 42 clinically definite MS patients, established according to McDonald's criteria (49, 50). MS patients were recruited from the MS- & Revalidation Center in Overpelt, Belgium and the Orbis Medical Center in Sittard-Geleen, The Netherlands. Characteristics of patients and controls are listed in Table 1. For immunohistochemical analysis, frozen brain material from 12 MS patients and 2 non-demented controls (NDCs) was obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands). Clinical details are summarized in Table 2.

Table 1: Study population. Subjects used for analysis of $CD4^{+}CD28^{null}$ T cells in the peripheral blood.

	Total MS group (n = 42)	**RR-MS** (n = 13)	**CP-MS** (n = 9)	**Unknown type of MS** (n = 20)	**HCs** (n = 97)
Age (range)*	46 (21-74)	44 (28-67)	56 (39-72)	43 (21-74)	32 (23-59)
Female/male (ratio)	30/12 (2.5)	9/4 (2.3)	6/3 (2.0)	17/3 (5.7)	64/33 (1.9)
Disease duration (range)*	15.6 (1-35)	15.6 (1-35)	20.0 (14-23)	8.0 (1-12)	-
EDSS (range)	3.7 (0-7)	3.1 (2-4)	4.9 (3-7)	3.7 (0-7)	-
Treated **	19	8	6	5	-

* In years; ** Within three months before blood sampling.

RR: relapsing-remitting; CP: chronic progressive; HCs: healthy controls; EDSS: expanded disability status scale.

Table 2: Patient material. Subjects used to identify $CD4^+CD28^{null}$ T cells in brain tissue.

Patient ID	Age (years)	Gender	Specific	MS treatment
97/202	50	m	NAWM	Corticosteroids
01/058	48	v	NAWM	Corticosteroids, IVIG, IFN-β
01/058 (#6)	48	v	A	Corticosteroids, IVIG, IFN-β
01/298	53	v	A	Corticosteroids, Methotrexate
03/142	53	m	A	Methotrexate
07/314 (#6)	66	v	A	Chemotherapy
07/314 (#5)	66	v	CA	Chemotherapy
08/069	55	m	CA	Corticosteroids
00/043	52	v	CI	Symptomatic
01/058 (#4)	48	v	CI	Corticosteroids, IVIG, IFN-β
01/316	43	m	CI	Symptomatic
02/057	77	m	CI	Symptomatic
97/133	68	m	- (NDC)	-
97/185	61	v	- (NDC)	-

m: male; f: female.

NAWM: normal appearing white matter; A: active; CA: chronic active; CI: chronic inactive; NDC: non-demented control, IVIG: intravenous immunoglobulin.

2.2 Analysis of $CD4^+CD28^{null}$ T cell frequencies

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by means of a Ficoll-density gradient centrifugation (Cedarlane, Hornby, ON, Canada). Percentages of $CD4^+CD28^{null}$ T cells were determined by flow cytometric analysis. Cells were double stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 and phycoerythrin (PE)-labeled anti-CD28 antibodies (both from BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at room-temperature. Samples were analyzed by using a FACSCalibur flow cytometer (BD Biosciences). The $CD28^{null}$ T cell percentage within the $CD4^+$ T cell population was calculated by the BD CellQuest Pro Software (BD Biosciences). A minimal cut-off was set at 2% of the total $CD4^+$ T cell population.

2.3 PBMC culture preparation

PBMCs were cultured in 96-well plates with U-shaped bottom (Nunc, Roskilde, Denmark) at a density of 0.2×10^6 cells/well. Cells were maintained in culture medium that consisted of RPMI

1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids and 0.5% pen/strep (50 U/ml penicillin and 50 µg/ml streptomycin) (all from Invitrogen). Incubation took place at 37°C in a 5% CO_2 incubator.

2.4 CFSE-based proliferation assay

The effect of IL-15 on the proliferation of $CD4^+CD28^{null}$ T cells from HCs (n = 5) and MS patients (n = 5), with sufficient percentages of $CD4^+CD28^{null}$ T cells (> 2% of the total $CD4^+$ T cell population), was determined in a CFSE (carboxyfluorescein succinimidyl ester) assay. The PBMC population was labeled with 2 µM CFSE (Invitrogen) in phosphate buffered saline (PBS)/0.1% bovine serum albumin (BSA) (respectively from Lonza, Basel, Switzerland and from USBiological, Swampscott, MA, USA). After 7 min incubation at 37°C, cells were suspended in 300 µl culture medium. After a second incubation period of 15 min at 37°C, CFSE-labeled cells were dissolved in culture medium at 2 x 10^6 cells/ml and seeded in a 96-well plate (0.2 x 10^6 cells/well). Cells were left untreated or cultured in culture medium supplemented with 2 µg/ml anti-CD3 (clone 2G3; BIOMED, Diepenbeek, Belgium), 2 U/ml IL-2 (Roche, Basel, Switzerland), 50 ng/ml IL-15 (R&D Systems, Minneapolis, MN, USA) or 2 µg/ml anti-CD3 in combination with 2 U/ml IL-2 or 50 ng/ml IL-15. Flow cytometric analysis took place 5 days after incubation at 37°C. Peridinin chlorophyll (PerCP)-conjugated anti-CD4 (BD Biosciences), PE-conjugated anti-CD28 antibodies and dilution of the CFSE signal were used to evaluate the proliferative response of $CD4^+CD28^{null}$ T cells.

2.5 Characterization of cytolytic/proinflammatory molecules

The protein expression of the surface receptor NKG2D and of the intracellular deposits of the cytolytic/proinflammatory molecules (perforin, GrB, IFN-γ and IL-17A) of $CD4^+CD28^{null}$ T cells in the presence of IL-15 was determined. PBMCs of 4 HCs and 6 MS patients were evaluated. PBMCs were cultured in a 96-well plate (0.2 x 10^6 cells/well), treated with or without 50 ng/ml recombinant human IL-15 for 3 days. Cells were not stimulated prior to staining. $CD4^+CD28^{null}$ T cells surface markers were stained with anti-CD4 PerCP and anti-CD28 FITC (BD Biosciences) or anti-CD28 PE for 15 min at room-temperature. The surface receptor NKG2D was labeled with the PE-conjugated anti-NKG2D antibody (BD Biosciences; diluted 1/2 in FACS buffer). To

assess the intracellular protein level of perforin, GrB, IFN-γ and IL-17A; PBMCs were fixed and permeabilized in a cytofix/cytoperm solution (commercially available: BD Cytofix/Cytoperm Fixation and Permeabilization Solution; BD Biosciences) for 20 min at 4°C in the dark. After two washing steps in a perm/wash buffer, cells were 20 min incubated at 4°C in the dark with PE-labeled antibodies against perforin (BD Biosciences) or IFN-γ (ImmunoTools, Berlin, Germany) or with FITC-labeled anti-GrB or anti-IL-17A antibodies (both from BD Biosciences). The FACSCalibur flow cytometer was used to measure the mean fluorescence intensity (MFI).

2.6 Degranulation assay

To assess the percentage of $CD4^{+}CD28^{null}$ T cells that expel their intracellular deposits of cytolytic enzymes after IL-15 treatment, a CD107a-mobilization flow cytometry-based assay was performed. PBMCs of 7 HCs and 4 MS patients were seeded in a 96-well plate (0.2×10^6 cells/well). Cells were either treated with 50 ng/ml recombinant human IL-15 for 24, 48 or 72 h or left untreated. 4 h before flow cytometric analysis, PBMCs were stimulated with 2 µg/ml anti-CD3 (clone 2G3). As a control condition, cells were left unstimulated. In addition, 2 µl anti-CD107a PE and 1 µl GolgiPlug (diluted 1/3 in culture medium) (both from BD Biosciences) were added. After 4 h, $CD4^{+}CD28^{null}$ T cells were stained with PerCP- and FITC-conjugated antibodies specific for CD4 and CD28, respectively. Flow cytometry was performed to determine the amount of degranulation after different treatments. Samples were analyzed by using the FACSCalibur.

2.7 Immunohistochemistry

Non-perfused, snap-frozen brain material of NDCs (n = 2) and MS patients (n = 12) was cut with the Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany) to sections of 5-10 µm. Slides were fixed for 10 min in (cold) acetone (VWR International, Leuven, Belgium) and air-dried.

For 3,3'-diaminobenzidine (DAB) staining, slides were incubated for 10 min with 0.3% hydrogen peroxide (Merck, Whitehouse Station, NJ, USA) in methanol (VWR) to block the endogenous peroxidase activity. After washing in PBS, sections were blocked for 20 min in PBS containing 10% serum to prevent non-specific binding. Then, slides were incubated at room-temperature

with the primary antibody diluted in PBS containing 1% serum. Control staining was performed by omitting the primary antibody. After three rinses in PBS, slides were incubated at room-temperature with the complementary horseradish peroxidase (HRP)-labeled secondary antibody diluted in PBS containing 1% serum. If necessary, to amplify the staining reaction a biotinylated secondary antibody supplemented with avidin (commercially available: R.T.U. Vectastain Universal Quick Kit; Vector Laboratories, Burlingame, CA, USA) could be used. Antibodies and corresponding concentrations and incubation periods are listed in Table 3. After three wash steps in PBS, staining was performed with the DAB substrate (Sigma-Aldrich, St. Louis, MO, USA). Before the hematoxylin (Merck) nuclear staining (1 min), slides were briefly washed in PBS and Milli-Q and rinsed for 5 min with tap water. Finally, brain tissue sections were dehydrated in the alcohol series (70%, 80%, 95%, 2x 100% ethanol and 2x xylene; both from VWR) and covered with DPX neutral mounting medium (Prosan, Merelbeke, Belgium).

To assess the expression of membrane-bound IL-15 in brain tissue of NDCs and MS patients, slides were blocked in PBS with 10% rabbit serum (Merck Millipore, Billerica, MA, USA). Staining was performed overnight using a goat anti-human IL-15 primary antibody. Subsequently, sections were incubated for 1 h with a HRP-labeled rabbit anti-goat secondary antibody.

The presence of $CD4^+$ T cells in brain material of NDCs and MS patients was visualized by incubating slides with a mouse anti-human CD4 antibody and a HRP-labeled rabbit anti-mouse antibody, both for 1 h.

To stain the cytolytic enzyme GrB in brain tissues of NDCs and MS patients, slides were blocked in PBS/0.05% Tween 20 (Merck) containing 10% goat serum (Merck Millipore). GrB staining was performed by using a rabbit anti-human GrB antibody and a HRP-labeled goat anti-rabbit antibody, both 1 h incubated. Because $CD4^+CD28^{null}$ T cells store GrB in intracellular deposits, washing steps were done with PBS/0.05% Tween 20 to permeabilize tissues.

Table 3: Antibodies used for DAB staining on brain material. Dilution factors and incubation periods.

Antigen/Antibody	Commercial source	Concentration	Incubation period	Secondary/Tertiary antibody
IL-15	R&D Systems	5 µg/ml	ovn	rb anti-gt HRP
CD4	AbD Serotec	10 µg/ml	1 h	rb anti-ms HRP
GrB	Abcam	25 µg/ml	1 h	gt anti-rb HRP
rb anti-gt HRP	Dako	5 µg/ml	1 h	NA
rb anti-ms HRP	Dako	6.5 µg/ml	1 h	NA
gt anti-rb HRP	Dako	3 µg/ml	1 h	NA

NA: not applicable.

IL-15: interleukin-15; GrB: granzyme B; HRP: horseradish peroxidase, rb: rabbit; gt: goat; ms: mouse.

To investigate whether GrB^+ $CD4^+$ T cells (≈ $CD4^+CD28^{null}$ T cells) are located in the vicinity of IL-15-expressing cells, a triple fluorescent staining for IL-15, CD4 and GrB was performed on brain tissue of NDCs and MS patients. To stain IL-15, slides were blocked in PBS containing 10% donkey serum (Merck Millipore) for 20 min. Next, sections were incubated overnight with a goat anti-human IL-15 antibody diluted in PBS containing 1% donkey serum at room-temperature. Control staining was performed by omitting the primary antibody. After three rinses in PBS, slides were incubated for 1.5 h with a donkey anti-goat Alexa Fluor 555 (Cy3 or tetramethylrhodamine (TRITC)) antibody diluted in PBS containing 1% donkey serum, at room-temperature and protected from light. If necessary, to amplify the fluorescent intensity of the staining reaction an Alexa Fluor-conjugated streptavidin tertiary antibody could be used. Antibodies and corresponding concentrations and incubation periods are listed in Table 4. After three wash steps in PBS/0.05% Tween 20, GrB was stained by incubating sections 2 h with a rabbit anti-human GrB antibody and 1.5 h with a goat anti-rabbit Alexa Fluor 647 (Cy5) antibody, both diluted in PBS/0.05% Tween 20 containing 1% goat serum. To stain CD4, after three rinses in PBS/0.05% Tween 20, sections were incubated for 2 h with a mouse anti-human CD4 antibody and 1.5 h with a donkey anti-mouse Alexa Fluor 488 (Cy2 or FITC) antibody, both diluted in PBS containing 1% donkey serum.

To identify the IL-15-expressing cells in brain material of NDCs and MS patients, a double fluorescent staining for IL-15 in combination with Iba1 (ionized calcium binding adaptor molecule 1) or GFAP (glial fibrillary acidic protein) was performed. IL-15 was visualized as

mentioned above. To stain microglia/macrophages, slides were blocked with Protein Block (Dako). Next, sections were incubated overnight at 4°C with a rabbit anti-human Iba1 antibody and subsequently for 1.5 h with a biotinylated swine anti-rabbit antibody supplemented with an Alexa Fluor 488-conjugated streptavidin tertiary antibody, 1.5 h at room-temperature. To stain astrocytes, slides were blocked with rabbit serum. Then, a mouse anti-human GFAP antibody and a biotinylated rabbit anti-mouse antibody in combination with an Alexa Fluor 488-conjugated streptavidin tertiary antibody were used.

Table 4: Antibodies used for fluorescent staining on brain material. Dilution factors and incubation periods.

Antigen/Antibody	Commercial source	Concentration	Incubation period	Secondary/Tertiary antibody
IL-15	R&D Systems	5 µg/ml	ovn	dk anti-gt 555
CD4	AbD Serotec	10 µg/ml	2 h	dk anti-ms 488
GrB	Abcam	1.65 µg/ml	2 h	gt anti-rb 647
Iba1	Wako Chemicals	2 µg/ml	ovn	sw anti-rb biotin streptavidin 488
GFAP	Sigma-Aldrich	2 µg/ml	ovn	rb anti-ms biotin streptavidin 488
dk anti-gt 555	Invitrogen	5 µg/ml	1.5 h	NA
dk anti-ms 488	Invitrogen	5 µg/ml	1.5 h	NA
gt anti-rb 647	Abcam	5 µg/ml	1.5 h	NA
sw anti-rb biotin	Dako	2.5 µg/ml	1.5 h	NA
streptavidin 488	Invitrogen	1:2000	1.5 h	NA
rb anti-ms biotin	Dako	2 µg/ml	1.5 h	NA
streptavidin 488	Invitrogen	1:2000	1.5 h	NA

NA: not applicable.

IL-15: interleukin-15; GrB: granzyme B; Iba1: ionized calcium binding adaptor molecule 1; GFAP: glial fibrillary acidic protein; dk: donkey; gt: goat; rb: rabbit; ms: mouse; sw: swine.

Nuclear staining was performed using 4’,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Invitrogen) for 10 min. After three rinses in PBS, slides were treated for 10 min with 0.3% Sudan Black (Sigma-Aldrich) in 70% ethanol to minimize autofluorescence. After one dip in 70% ethanol, eight dips in PBS and one final wash step in Milli-Q (10 min), brain tissue slides were

covered using fluorescent mounting medium (Dako). Analyses were assessed using the Eclipse 80i Microscope and NIS-Elements BR 3.10 Software (both from Nikon, Tokyo, Japan).

2.8 Statistical analysis

Data were statistically analyzed using GraphPad Prism version 5.01. All data were reported as the mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using a two-tailed unpaired Student's t test (with Welch's correction if necessary). A paired t test was used for paired data. Probability values of less than 0.05 were considered to be significant: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. A probability value between 0.05 and 0.1 was considered to be a trend.

3 RESULTS

3.1 $CD4^+CD28^{null}$ T cells are expanded in a subgroup of multiple sclerosis patients

Our group and others have reported the age-inappropriate expansion of senescent $CD4^+CD28^{null}$ T cells in the peripheral circulation of patients suffering from various autoimmune and chronic inflammatory disorders (20-24) including MS (1). However, their potential participation in the pathogenesis of MS has not been elucidated yet.

3.1.1 Frequency of $CD4^+CD28^{null}$ T cells

The frequency of $CD4^+CD28^{null}$ T cells in the peripheral blood of 97 HCs and 42 MS patients was assessed by flow cytometric analyses. Our data indicate that 18% (17/97) of the HCs and 29% (12/42) of the MS patients have a $CD28^{null}$ T cell percentage within the $CD4^+$ T cell population above 2%. The fraction of this $CD4^+$ T cell subpopulation lacking the costimulatory molecule CD28 was higher in MS patients compared to HCs. However, no significant difference was reached (P = 0.3750) (Figure 1).

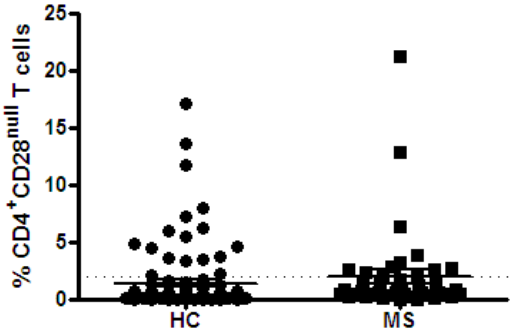


Figure 1: Frequency of $CD4^+CD28^{null}$ T cells in the peripheral circulation of HCs and MS subjects. Peripheral blood of HCs (n = 97) and MS patients (n = 42) were screened for $CD4^+CD28^{null}$ T cells. Results are obtained by flow cytometry and represent individual measurements. Data are expressed as mean ± SEM. Dotted line indicates the 2% cut-off value. HC: healthy control; MS: multiple sclerosis.

3.2 Effect of interleukin-15 on $CD4^+CD28^{null}$ T cells

In neurological disorders, including MS, increased levels of the proinflammatory cytokine IL-15 are reported. IL-15 is significantly upregulated in the serum, CSF and in active lesions; although the exact role in the pathogenesis of MS is still unclear (30, 37). The $CD4^+$ T cell population expresses the IL-15Rβ, which is necessary for IL-15 in *trans* signaling on T cells by e.g. monocytes/macrophages and astrocytes (51). Preliminary studies performed by Broux *et al.* demonstrated a threefold higher basal expression of IL-15Rβ on $CD4^+CD28^{null}$ T cells of HCs

and MS subjects compared to their $CD28^+$ counterparts (unpublished data). This indicates that $CD4^+CD28^{null}$ T cells are able to respond to membrane-bound IL-15.

3.2.1 Interleukin-15 increases proliferative capacity of $CD4^+CD28^{null}$ T cells

IL-15 is responsible for the induction of T cell proliferation and survival (37-42). However, there is little information regarding the effect of IL-15 on the expansion and maintenance of $CD4^+CD28^{null}$ T cells.

To investigate the IL-15-mediated effect on the proliferation of $CD4^+CD28^{null}$ T cells, PBMCs from HCs (n = 5) and MS patients (n = 5) were stained with CFSE and cultured in the presence or absence of recombinant IL-15 for 5 days. Dot plots show that both $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells proliferate in response to IL-15 pretreatment, with or without TCR stimulation (Figure 3A). Figure 3B shows that IL-15 significantly increases cell division and expansion of HC $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells. IL-15 in combination with anti-CD3 stimulation makes both $CD4^+$ T cell populations more susceptible to proliferate. The proliferating percentage of anti-CD3 activated $CD4^+$ T cells pretreated with IL-15 is significantly higher compared to those cultured in culture medium alone. IL-15 (supplemented with anti-CD3) has a more pronounced effect than IL-2 (supplemented with anti-CD3) (Figure 3B). $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells isolated from MS patients also display an enhanced proliferation rate in response to IL-15 alone or in combination with anti-CD3. Again, MS-related $CD4^+$ T cells are more susceptible for the IL-15-mediated proliferative effect than for IL-2 (Figure 3C). Remarkably, $CD4^+CD28^{null}$ T cells isolated from HCs and MS patients show a (slightly) overall higher proliferative capacity in response to IL-15 (supplemented with anti-CD3) with respect to their $CD28^+$ counterparts (Figure 3B and C). For HCs, a significant difference was observed for $CD4^+$ T cells treated with anti-CD3 alone or in combination with IL-2. A trend was observed when stimulated with anti-CD3 supplemented with IL-15 (Figure 3B). For MS subjects, only a significant effect was found for IL-15 (Figure 3C). Comparing the proliferating $CD4^+CD28^{null}$ T cell fraction from both study populations, no significant differences were reached (Figure 3B and C). Taken together, IL-15 stimulate the expansion of $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells by their proliferation inducing effect. This effect is more pronounced in the $CD4^+$ T cell population lacking the CD28 molecule. Overall, no significant differences were assessed between $CD4^+CD28^{null}$ T cells of HCs and MS subjects.

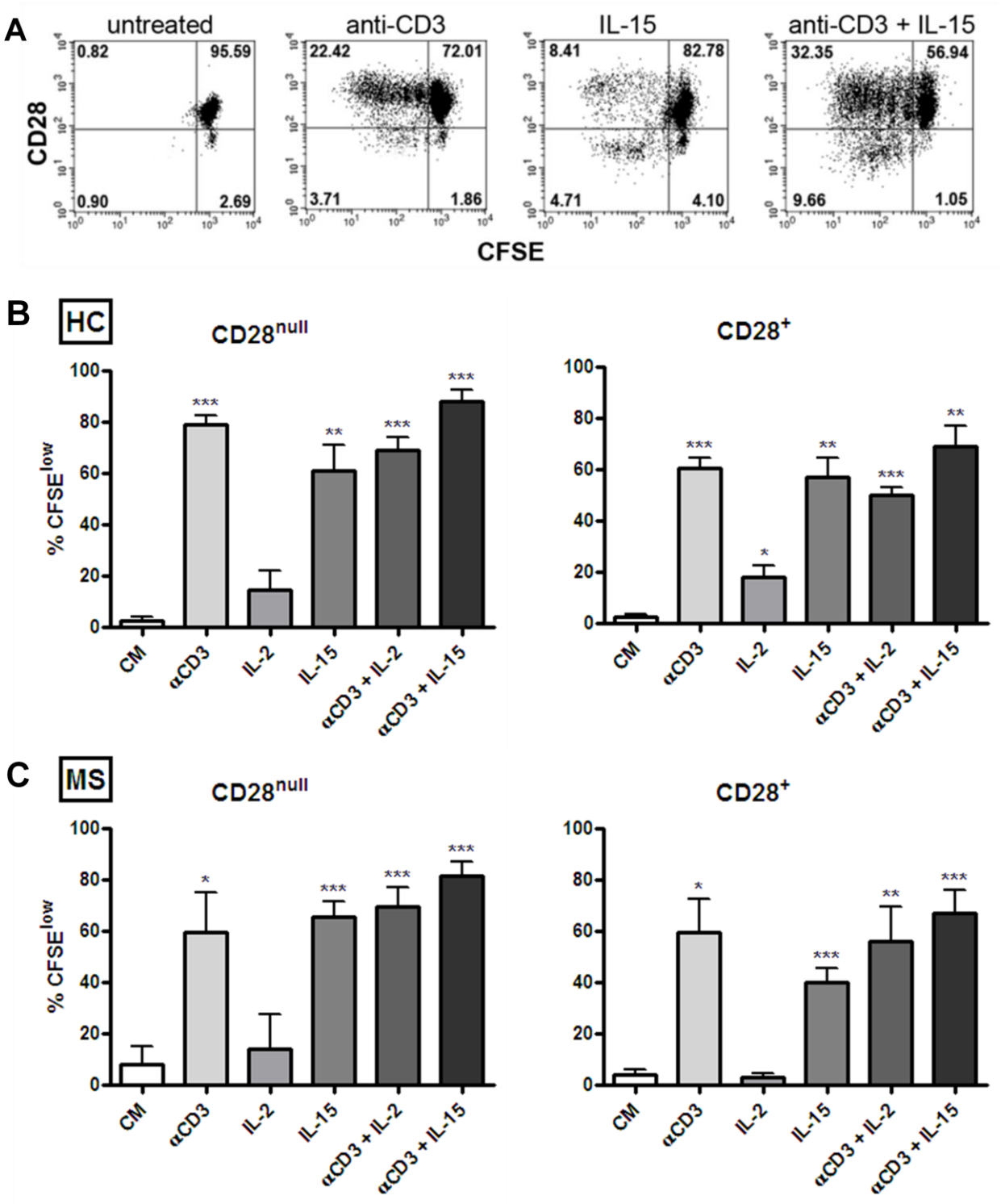


Figure 3: IL-15 preferentially enhances the proliferative capacity of $CD4^{+}CD28^{null}$ T cells in HCs and MS patients. Proliferation of $CD28^{null}$ and $CD28^{+}$ $CD4^{+}$ T cells obtained for 5 HCs and 5 MS patients. PBMCs were left untreated or treated for 5 days with various culture conditions: anti-CD3 (2 µg/ml), IL-2 (2 U/ml), IL-15 (50 ng/ml)

or anti-CD3 in combination with IL-2 or IL-15. **A.** Flow cytometric plots show the percentage of proliferating $CD4^+$ T cells in response to IL-15. **B** and **C.** Pooled data. Results represent independent experiments. Data are expressed as mean ± SEM. Significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. HC: healthy control; MS: multiple sclerosis.

3.2.2 Interleukin-15 enhances cytotoxic profile of $CD4^+CD28^{null}$ T cells

$CD4^+CD28^{null}$ T cells gain cytotoxic properties, similar to NK cells and $CD8^+$ T cells. For instance, they possess intracellular stores consisting of cytolytic enzymes (perforin and GrB) and IFN-γ, which are released after antigen-specific and polyclonal TCR stimulation. Moreover, they express the NKG2D receptor (1, 4, 5, 13, 16-18). Because it is documented for NK cells and $CD8^+$ T cells that the NKG2D expression and cytotoxicity are increased in response to IL-15 (48, 51-53), the influence of IL-15 on the protein expression of NKG2D, perforin, GrB, IFN-γ and IL-17A was analyzed in $CD4^+CD28^{null}$ T cells.

We evaluated the effect of IL-15 on the cytolytic and proinflammatory features of $CD4^+CD28^{null}$ T cells from 4 HCs and 6 MS patients via intracellular flow cytometry. Histogram plots show that IL-15 enhances the surface expression of NKG2D and increases the cytoplasmic stores of perforin, GrB and IFN-γ in the majority of $CD4^+CD28^{null}$ T cells (Figure 4A). Figure 4B and C represent the IL-15-mediated effect on the cytotoxic profile of $CD4^+CD28^{null}$ T cells of HCs and MS subjects. For HCs, a significant difference could be found for perforin, GrB and IFN-γ (Figure 4B). For MS patients, IL-15 significantly upregulates the protein expression of NKG2D, perforin, GrB and IFN-γ (Figure 4C). No significant differences were observed between $CD4^+CD28^{null}$ T cells of HCs and MS patients. These data indicate that (MS-related) IL-15 increases the cytotoxic profile of $CD4^+CD28^{null}$ T cells.

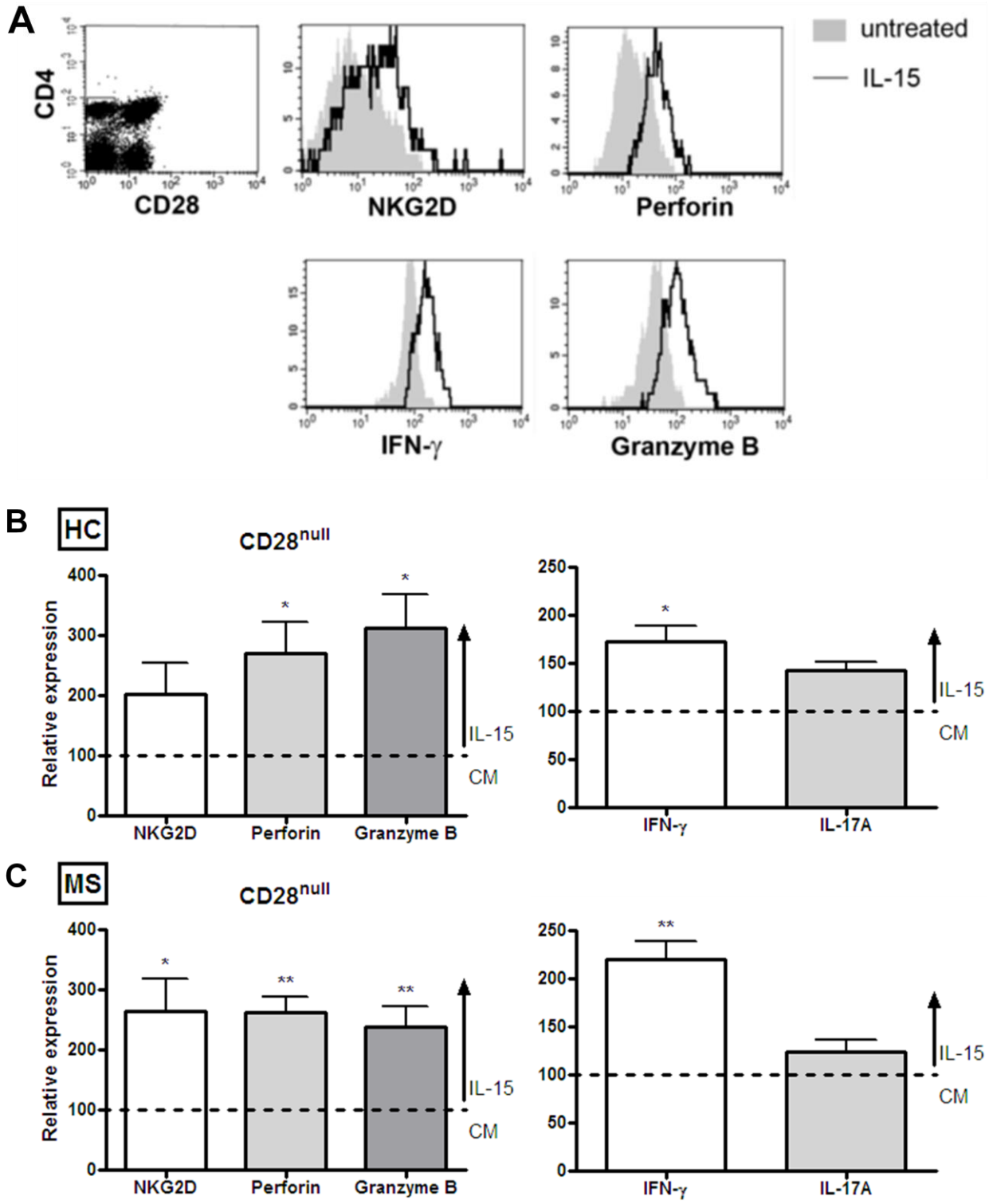


Figure 4: IL-15 upregulates cytolytic and proinflammatory molecules of HC and MS $CD4^{+}CD28^{null}$ T cells. PBMCs of 4 HCs and 6 MS patients were pretreated with IL-15 (50 ng/ml) for 3 days. **A.** Gating was performed on $CD4^{+}CD28^{null}$ T cells. Histogram plots are shown for NKG2D, perforin, granzyme B and IFN-γ. Grey peaks represent the untreated $CD4^{+}CD28^{null}$ T cells and black peaks correspond to the $CD4^{+}CD28^{null}$ T cells pretreated with

IL-15. **B** and **C.** Pooled data. Relative marker expression of NKG2D, perforin, granzyme B, IFN-γ and IL-17A of $CD4^+CD28^{null}$ T cells. Dashed lines signify the expression of untreated $CD4^+CD28^{null}$ T cells. Data are expressed as mean ± SEM. Significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. HC: healthy control; MS: multiple sclerosis.

To compare the amount of cytolytic and proinflammatory markers per cell between $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells, the MFI of each marker is analyzed in both $CD4^+$ T cell populations. Figure 5A shows the MFI of the cytolytic and proinflammatory markers of $CD4^+$ T cells obtained from HCs, while figure 5B represents the marker expression per $CD4^+$ T cell of MS subjects. The $CD4^+CD28^{null}$ T cell population of HCs contains more NKG2D, perforin and GrB per cell, under basal culture conditions and with IL-15 pretreatment, as compared to their $CD28^+$ counterparts. However, only a significant difference was found between both $CD4^+$ T cell populations for GrB without and with IL-15 stimulation. In addition, the MFI for GrB is higher in $CD4^+CD28^{null}$ T cells pretreated with IL-15. The amount of IFN-γ in HC $CD4^+CD28^{null}$ T cells is lower with respect to their $CD28^+$ counterparts. The IL-17A expression remains unchanged (Figure 5A). Like in HCs, $CD4^+CD28^{null}$ T cells of MS patients have a higher marker expression for NKG2D, perforin and GrB in respect with their $CD28^+$ counterparts. Significant differences were obtained for perforin and GrB. Again, the MFI of IFN-γ is lower in $CD4^+CD28^{null}$ T cells (Figure 5B). Comparing the expression of each marker for $CD4^+CD28^{null}$ T cells between HCs and MS subjects, data do not produce significant differences for the corresponding molecules (Figure 5A and B). These results illustrate that the $CD4^+CD28^{null}$ T cell population gain several intracellular stores containing cytolytic and proinflammatory molecules, which can be upregulated in the presence of IL-15. However, the cytotoxic properties of $CD4^+CD28^{null}$ T cells do not reach significant difference between HCs and MS patients.

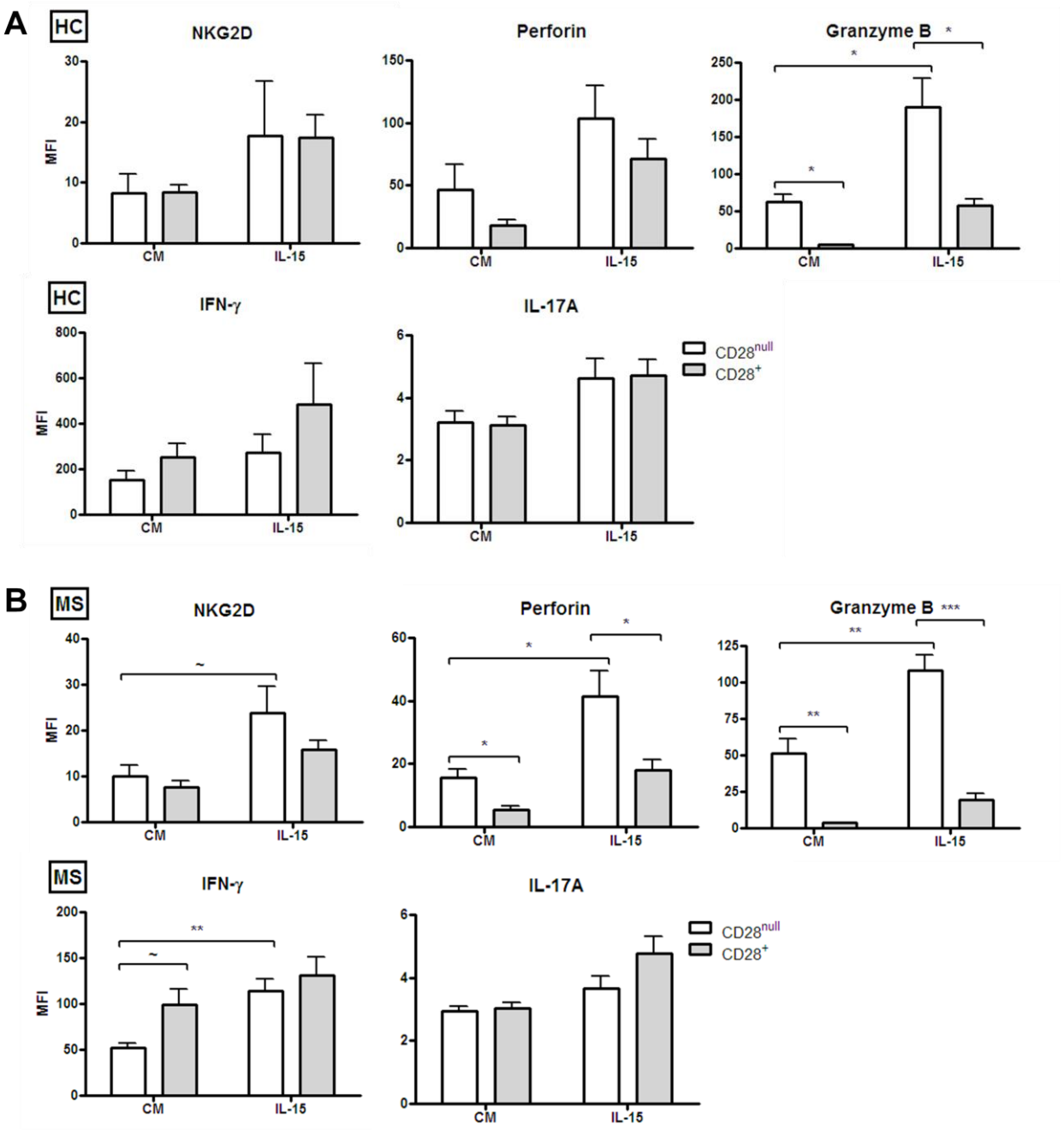
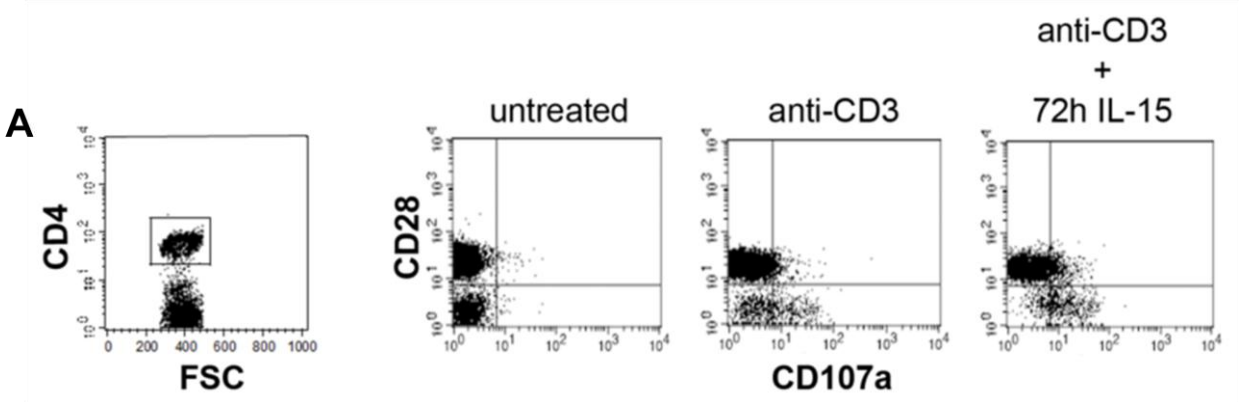


Figure 5: IL-15 is responsible for the additive raise in marker expression seen in CD4$^+$CD28^{null} T cells of HCs and MS subjects. A and **B.** MFI for NKG2D, perforin, granzyme B, IFN-γ and IL-17A of CD28^{null} and CD28$^+$ CD4$^+$ T cells isolated from 4 HCs and 6 MS patients. Data are expressed as mean ± SEM. Significant differences are indicated as * P < 0.05, ** P < 0.01, *** P < 0.001. A trend is represented by ~ (P value between 0.05 and 0.1). HC: healthy control; MS: multiple sclerosis; MFI: mean fluorescence intensity.

3.2.3 Interleukin-15 increases degranulation of activated $CD4^+CD28^{null}$ T cells

As established above, IL-15 can upregulate the protein expression of the cytolytic enzymes perforin and GrB in $CD4^+CD28^{null}$ T cells. Next, the IL-15-mediated effect on their degranulation is evaluated.

To determine the influence of IL-15 on the capacity of $CD4^+CD28^{null}$ T cells to expel their intracellular deposits after activation, the surface expression of CD107a on these T cells (HC: n = 7 and MS: n = 4) was assessed. Dot plots show a higher and more intense degranulating $CD4^+CD28^{null}$ T cell fraction with respect to their $CD28^+$ counterparts, when anti-CD3 stimulated and IL-15 pretreated (Figure 6A). $CD4^+CD28^{null}$ T cells of HCs and MS patients do not express CD107a immediately *ex vivo* (Figure 6B and C). Moreover, $CD4^+CD28^{null}$ T cells cultured in medium alone or in the presence of IL-15 without stimulation do not degranulate (data not shown). However, anti-CD3 stimulation induces a significantly higher percentage of CD107a-expressing $CD4^+CD28^{null}$ T cells in HCs (Figure 6B) and MS patients (Figure 6C). A 24, 48 or 72 h IL-15 pretreatment in combination with anti-CD3 remarkably increases the amount of degranulating $CD4^+CD28^{null}$ T cells in HCs (Figure 6B). For MS patients, the same conclusions could be made (Figure 6C). Activated $CD4^+CD28^{null}$ T cells of HCs degranulate more strongly compared to those obtained from MS patients, when 24 or 48 h pretreated with IL-15 (Figure 6B and C). As expected, the degranulating capacity of $CD4^+CD28^+$ T cells is significantly lower in HCs (Figure 6B) and MS subjects (Figure 6C). Thus, the classical helper $CD4^+CD28^+$ T cell population does not exhibit a meaningful CD107a surface expression under the different culture conditions. Taken these results together, IL-15 significantly increases the percentage of degranulating $CD4^+CD28^{null}$ T cells in HCs and MS patients upon activation.



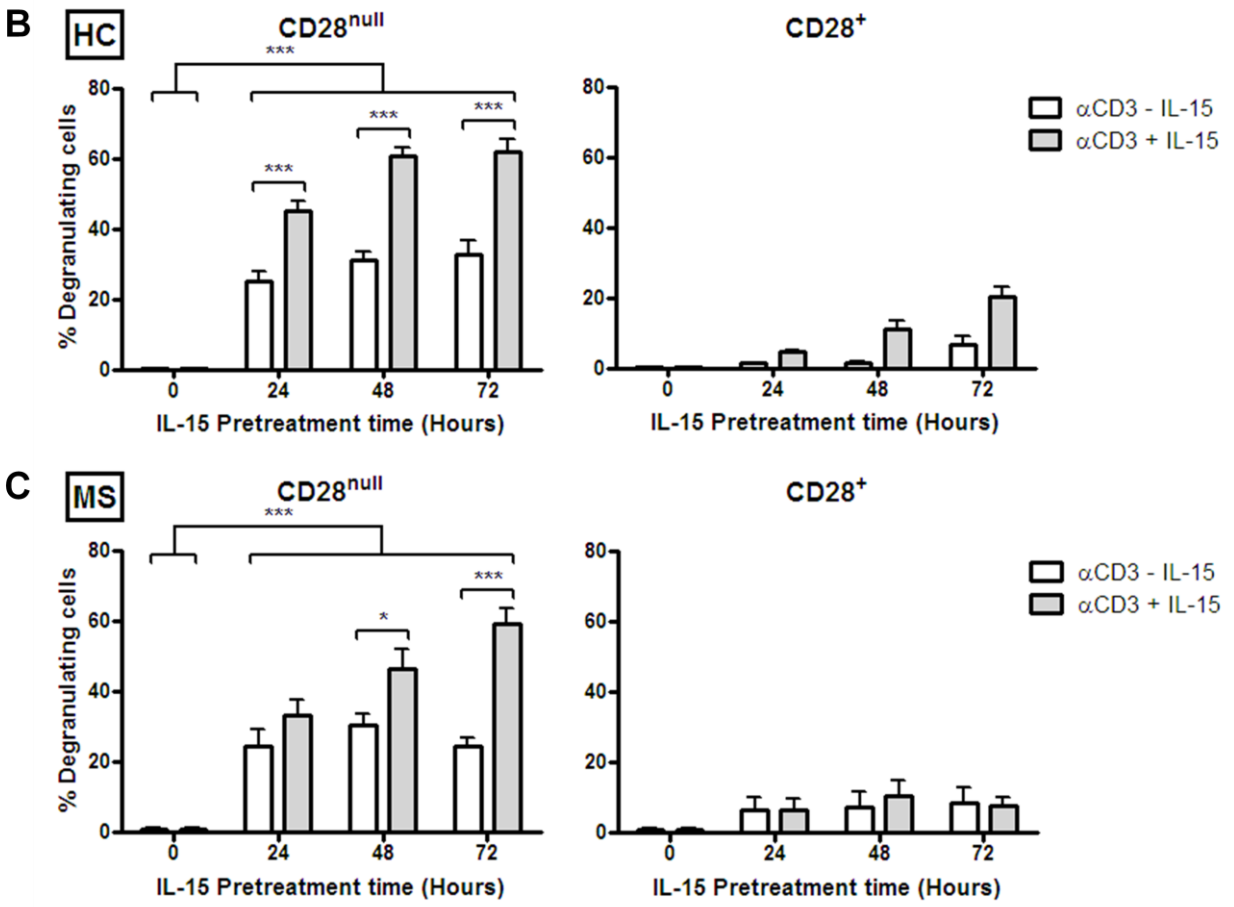


Figure 6: IL-15 increases the percentage of degranulating $CD4^+CD28^{null}$ T cells in HCs and MS patients. PBMCs of 7 HCs and 4 MS subjects were 4 h stimulated with anti-CD3 (2 µg/ml) alone or supplemented with IL-15 (50 ng/ml) for 24, 48 or 72 h. **A.** The fraction $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells expressing CD107a is represented by flow cytometric dot plots. **B** and **C.** Pooled data. Percentages degranulating $CD4^+$ T cells from HCs and MS patients. Results represent independent experiments. Data are expressed as mean ± SEM. Significant differences are indicated as * P < 0.05, ** P < 0.01, *** P < 0.001. HC: healthy control; MS: multiple sclerosis.

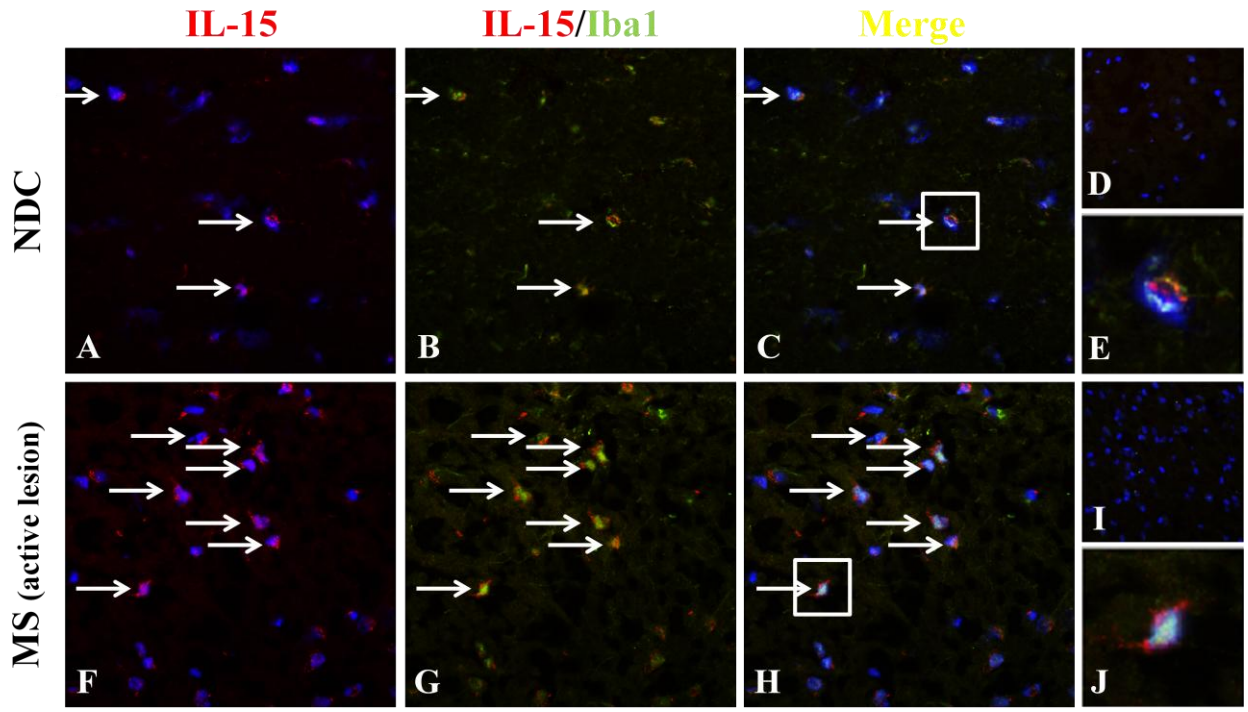
3.3 $CD4^+$ T cells in vicinity of interleukin-15-expressing cells in multiple sclerosis brain tissue

The presence of $CD4^+CD28^{null}$ T cells in brain tissue of MS patients has already been addressed, especially in active lesions (16). Moreover, high IL-15 levels are measured in acute and subacute/chronic MS lesions, appointing IL-15 as being a potential contributor to MS pathogenesis (30, 37, 48). We determined whether human CNS cells (e.g. microglia/macrophages and astrocytes) are a relevant source of providing functional IL-15 to $CD4^+CD28^{null}$ T cells in MS brain tissue.

3.3.1 Microglia/macrophages and astrocytes express interleukin-15 in the brain of multiple sclerosis patients

In vitro experiments have reported that myeloid cells (microglia, macrophages and monocytes) and astrocytes upregulate their IL-15 surface expression following a proinflammatory cytokine treatment (48).

To investigate whether microglia/macrophages and astrocytes express excessive membrane-bound IL-15 in MS brain, immunohistochemical analyses were performed. Postmortem brain tissue obtained from 1 NDC and 3 MS patients was stained for IL-15 and Iba1 or GFAP, markers for microglia/macrophages (Figure 7) and astrocytes (Figure 8) respectively. $Iba1^+$ myeloid cells are detected in MS brain tissue (Figure 7F-T). A high proportion expresses high levels of IL-15, especially within MS brain material characterized by a (chronic) active lesion (Figure 7F-O; white arrows). Few IL-15-expressing cells are $Iba1^-$. In contrast to MS brain tissue, less $Iba1^+$ microglia/macrophages are present in NDCs (Figure A-E). Only a proportion of them express detectable IL-15 surface levels (Figure A-E; white arrows). Thus, increased numbers of IL-15-expressing microglia/macrophages are shown in brain tissue of MS patients.



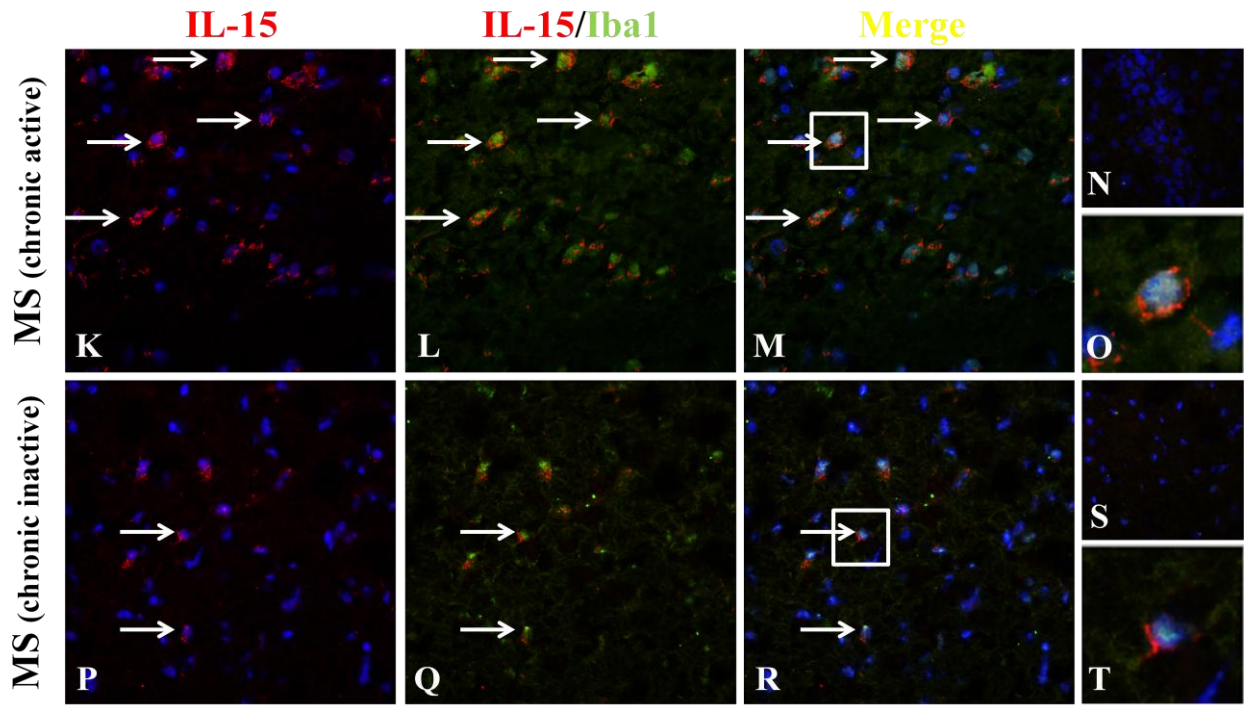


Figure 7: High numbers of microglia/macrophages expressing IL-15 in MS brain material. Brain material from 1 NDC (**A-E**) and 3 MS subjects (**F-T**) characterized by active (**F-J**), chronic active (**K-O**) and chronic inactive (**P-T**) lesions. Brain tissue was fluorescent stained for IL-15 (red), Iba1 (green) and DAPI (blue). White arrows indicate IL-15-expressing microglia/macrophages. **D, I, N and S.** Isotype controls. **E, J, O and T.** Magnified zones to show overlap between the three markers IL-15 (red), Iba1 (green) and DAPI (blue). NDC: non-demented control; MS: multiple sclerosis; IL-15: interleukin-15; Iba1: ionized calcium binding adaptor molecule 1.

Strong immunoreactivity for GFAP is shown in MS and NDC brain sections (Figure 8). In MS brain, the majority of $GFAP^+$ astrocytes display membrane-bound IL-15 (Figure 8F-T; white arrows). Like microglia/macrophages, astrocytes in acute and subacute/chronic MS lesions express IL-15. IL-15-expressing astrocytes are also detected near blood vessels with or without perivascular cuffs (data not shown). $GFAP^-$ cells are present, which express IL-15. $GFAP^+$ astrocytes are shown in NDC brain sections, although fewer of these cells have detectable IL-15 (Figure 8A-E; white arrows). To conclude, astrocytes express IL-15 in MS brain tissue.

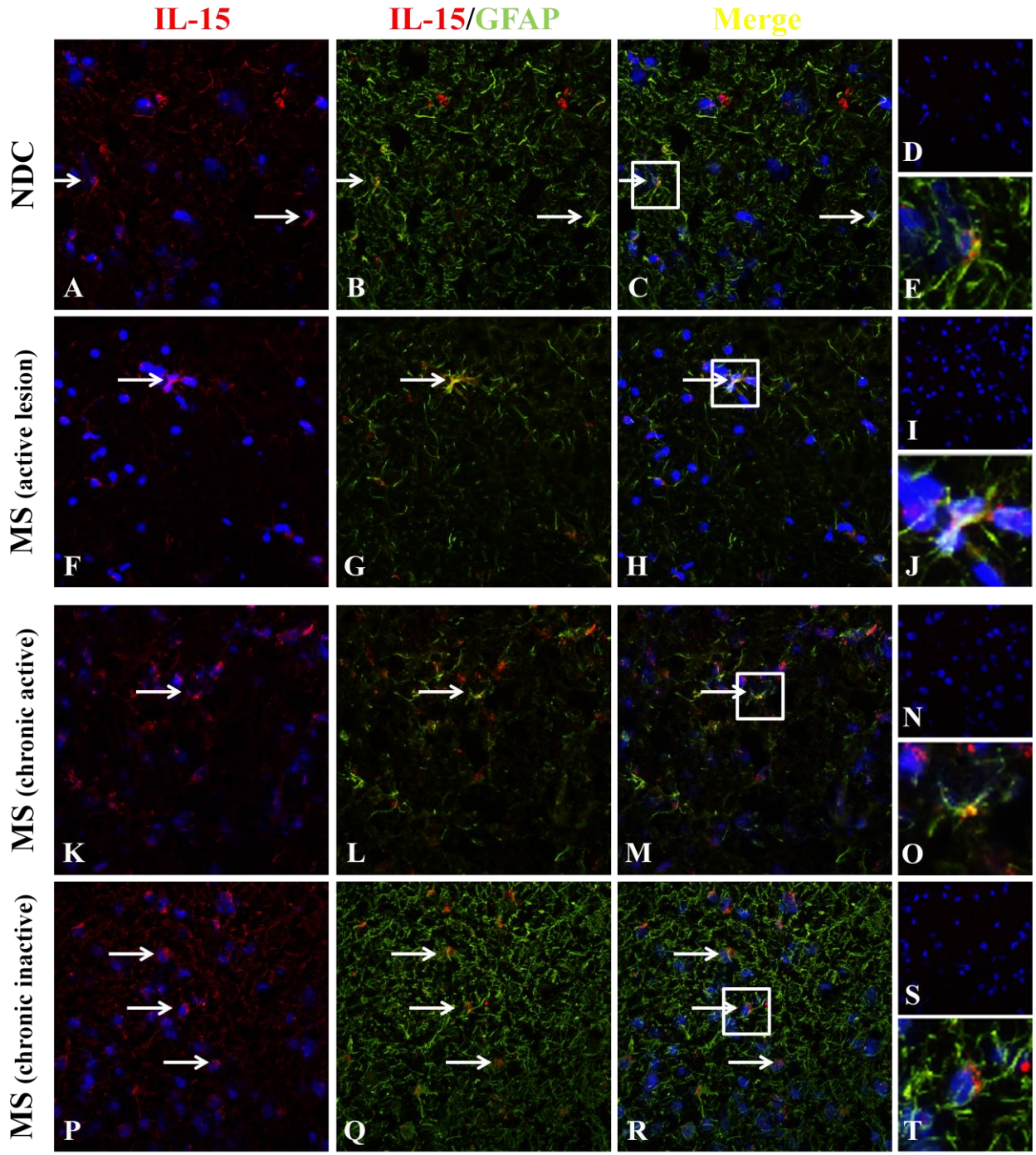


Figure 8: IL-15 expression on astrocytes in MS brain material. Brain material from 1 NDC (**A-E**) and 3 MS subjects (**F-T**) characterized by active (**F-J**), chronic active (**K-O**) and chronic inactive (**P-T**) lesions. Brain tissue was fluorescent stained for IL-15 (red), GFAP (green) and DAPI (blue). White arrows indicate IL-15-expressing astrocytes. **D, I, N and S.** Isotype controls. **E, J, O and T.** Magnified zones to show overlap between the three markers IL-15 (red), GFAP (green) and DAPI (blue). NDC: non-demented control; MS: multiple sclerosis; IL-15: interleukin-15; GFAP: glial fibrillary acidic protein.

3.3.2 $CD4^+$ T cells are found in close proximity to interleukin-15-expressing cells in the brain of multiple sclerosis patients

In this study, we demonstrated that membrane-bound IL-15 increases the proliferation rate of $CD4^+CD28^{null}$ T cells and enhances their cytotoxic functioning by upregulating the surface expression of the NKG2D receptor and by increasing the intracellular granules containing cytolytic enzymes (perforin and GrB) and proinflammatory cytokines (IFN-γ).

To assess whether microglia/macrophages and astrocytes could provide functional IL-15 *in vivo*, we investigated the presence of $CD4^+CD28^{null}$ T cells in the vicinity of IL-15-expressing cells in MS brain tissue. Postmortem brain tissue obtained from MS patients was stained for IL-15, CD4 and GrB (Figure 9). Active MS brain lesion is characterized by an immune cell infiltrate predominantly consisting of $CD4^+$ T cells (Figure 9A and B). Infiltrating $CD4^+$ T cells are localized in close proximity to IL-15-expressing cells. As identified previously, these IL-15-expressing cells could be myeloid cells and astrocytes. To see whether this $CD4^+$ T cell population also contains $CD4^+$ T cells that have lost their expression of the costimulatory molecule CD28, the infiltrate was intracellularly stained for GrB because, in contrast to the classical helper $CD4^+$ T cells, $CD4^+CD28^{null}$ T cells exhibit cytoplasmic stores of GrB. Unfortunately, due to technical issues, we could not identify $CD4^+$ T cells as being part of the $CD4^+CD28^{null}$ T cell population (Figure 9C and D).

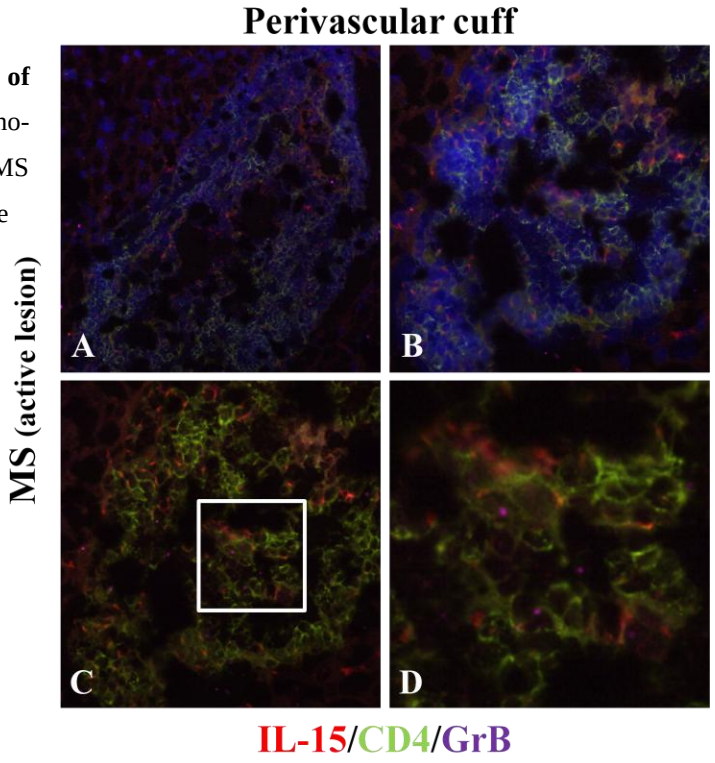


Figure 9: $CD4^+$ T cells are present in the vicinity of IL-15 in MS brain material. A. Immuno-histochemical staining in perivascular cuff of MS brain characterized by an active lesion. Brain tissue was fluorescent stained for IL-15 (red), CD4 (green), GrB (purple) and DAPI (blue). **B** and **C.** Immune cell infiltrate in perivascular cuff, respectively with and without DAPI. **D.** Magnified zone to assess GrB^+ $CD4^+$ T cells (≈ $CD4^+CD28^{null}$ T cells). MS: multiple sclerosis; IL-15: interleukin-15; GrB: granzyme B.

4 DISCUSSION

Understanding the precise role of autoreactive T cells in the pathogenesis of several chronic inflammatory and autoimmune diseases, including MS, is of vital importance to develop therapeutic strategies to overcome these disorders. Recently, Markovic-Plese and colleagues identified a $CD4^+$ T cell subpopulation that is characterized phenotypically by the persistent absence of the costimulatory molecule CD28 and functionally by CD28-independent activation (8). More specifically, this $CD4^+CD28^{null}$ T cell population has the potential to contribute actively or passively to disease pathogenesis.

In this study, we investigated several characteristics of $CD4^+CD28^{null}$ T cells to reveal their potential role in the pathogenesis of autoimmune disorders including MS. We determined the role of membrane-bound IL-15 on the proliferative capacity and cytotoxic activity of the $CD4^+CD28^{null}$ T cell population. We hypothesized that $CD4^+CD28^{null}$ T cells become more pathogenic in response to (MS-related) IL-15. We assessed increased frequencies of this T cell population in a subgroup of MS patients. We demonstrated that they show a (slightly) overall higher proliferative capacity with respect to their $CD28^+$ counterparts. IL-15 has an additive proliferative effect on activated and inactivated $CD4^+CD28^{null}$ T cells. Furthermore, we have shown that $CD4^+CD28^{null}$ T cells possess cytotoxic properties, similar to NK cells and $CD8^+$ T cells. They have increased surface expression of the NKG2D receptor and gain intracellular granules of the cytolytic enzymes perforin and GrB. Moreover, their cytotoxic profile and functioning could be enhanced by adding IL-15. Finally, we addressed the presence of the $CD4^+$ T cell population in the vicinity of IL-15-expressing cells (e.g. microglia/macrophages and astrocytes) in the CNS parenchyma and within perivascular cuffs of MS tissue. Unfortunately, we could not describe this $CD4^+$ T cell population as being $CD28^{null}$. Overall, our data suggest that (MS-related) IL-15 aggravates the pathogenic potential of $CD4^+CD28^{null}$ T cells, which make them a possible candidate to participate in the inflammatory and degenerative processes observed in MS.

$CD4^+CD28^{null}$ T cells are enriched in the peripheral circulation in a subgroup of MS patients (1). We confirmed the increased frequency of $CD4^+CD28^{null}$ T cells in a subset of MS patients. The proportion having an elevated percentage of these T cells is higher in respect to HCs. The reason

for their expansion remains elusive, although several studies regarding the antigen specificity of $CD4^+CD28^{null}$ T cells have reported that the majority of these T cells are CMV-reactive (54, 55) and that at least part of them proliferates in response to autoantigens (4, 8). In line with these results, Broux *et al.* addressed that MS-related autoantigens induce degranulation of $CD4^+CD28^{null}$ T cells after TCR stimulation in a donor dependent manner (16). Moreover, a variable fraction of $CD4^+CD28^{null}$ T cells of HCs and MS patients proliferate in response to CMV (4). It seems that CMV is the (main) driving force behind the generation of $CD4^+CD28^{null}$ T cells and age-associated changes in T cell phenotype and function are primarily related with CMV status and only secondary with age (HP) (56). Brok *et al.* described that the human CMV-UL86 peptide 981-1003 shares a cross-reactive T cell epitope with the encephalitogenic myelin oligodendrocyte glycoprotein (MOG) peptide 34-56 (57). Although the $CD4^+CD28^{null}$ T cell population do not appear to be directly autoreactive, their enrichment in patients suffering from autoimmune disorders supports their involvement in disease pathology.

As mentioned previously, accumulation of the aberrant $CD4^+CD28^{null}$ T cell population leads to a diminished competence of the immune system by limiting the peripheral space which is normally available for functional T cells; like naive T cells, competitor T cells or regulatory T cells (Tregs). This change in T cell composition may cause a breakdown in the immunological tolerance and therefore may contribute to autoaggressive immune manifestations. Moreover, Thewissen and colleagues demonstrated that $CD4^+CD28^{null}$ T cells are less susceptible for the suppressive function of Tregs. Tregs can inhibit the proinflammatory properties of $CD4^+CD28^{null}$ T cells, but cannot regulate their expansion (4). Treg suppression seems to be transcriptionally controlled via IL-2 in the effector cell (58). Because $CD4^+CD28^{null}$ T cells have a deficient IL-2 production (6, 13, 14), they could be resistant to the suppressive mechanisms of Tregs. The inability of Tregs to inhibit $CD4^+CD28^{null}$ T cell proliferation may contribute to their expansion. In addition, a decreased Treg function has been reported in MS (59, 60).

IL-15 is significantly overexpressed in MS compared with other inflammatory and non-inflammatory neurological disorders and with HCs (30, 37, 40, 46-48). It has been suggested that chronic exposure of IL-15 to $CD4^+CD28^{null}$ T cells is yet another mechanism responsible for their expansion (61). We speculate that IL-15 mediates its effects when presented in *trans* to neighboring cells, the biologically active form (44, 45). Preliminary studies performed by Broux

and colleagues demonstrated a threefold higher basal expression of IL-15Rβ on $CD4^+CD28^{null}$ T cells of HCs and MS subjects (unpublished data).

In the CFSE-based proliferation assay, we investigated whether membrane-bound IL-15 was responsible for the expansion of $CD4^+CD28^{null}$ T cells in MS patients. We found that IL-15 increases the percentage of both $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells in HCs and MS patients. Anti-CD3 activated $CD4^+$ T cells pretreated with IL-15 show even a higher proliferation rate. However, the amount of proliferating $CD4^+CD28^{null}$ T cells is higher than in the $CD28^+$ T cell subpopulation. Based on these results, it seems that IL-15 increases the frequency of $CD4^+CD28^{null}$ T cells by the preferential proliferation of these T cells, which could disturb the competence of the T cell composition and as a consequence leads to the development of autoimmune diseases. Remarkably, $CD4^+CD28^{null}$ T cells isolated from MS subjects do not show an enhanced proliferative capacity with respect to those from HCs. This means that $CD4^+CD28^{null}$ T cells of both study populations respond equally to IL-15, but the IL-15 mRNA expression is elevated in blood and CSF mononuclear cells in MS and in active MS lesions (40, 46-48). Therefore, it is more likely that $CD4^+CD28^{null}$ T cells in MS come in contact with IL-15-expressing cells, suggesting the potential relevance of IL-15 in their expansion. In our present study, we also demonstrated that IL-15 has a more pronounced effect than IL-2. This observation confirms our assumption that the IL-15/IL-15Rα complex is responsible for the increased proliferation seen in $CD4^+$ T cells. Namely, the biological effect of IL-2 is mainly achieved by its binding to the heterotrimeric high affinity IL-2R complex (IL-2Rα$\beta\gamma_c$ $K_d \approx 10^{-11}$ M). The IL-15Rα$\beta\gamma_c$ complex has the same high affinity. The β/γ_c heterodimer is characterized by an intermediate affinity for IL-2 and IL-15 ($K_d \approx 10^{-9}$ M). Importantly, the IL-2Rα-chain alone binds IL-2 with very low affinity, whereas IL-15Rα is capable of binding IL-15 with a high affinity similar to that of the heterotrimeric IL-15R (62). Because in our proliferation assay the proliferative effect of IL-2 is much lower than the IL-15-mediated effect on $CD4^+$ T cells, we may speculate that $CD4^+$ T cells respond via their IL-15Rβ/γ_c heterodimer to the IL-15/IL-15Rα complex expressed by e.g. monocytes/macrophages and astrocytes. Schneider *et al.* addressed that B cells also coexpress IL-15 bound to the IL-15Rα-chain. Like monocytes in MS, a significant higher proportion of B cells express the IL-15/IL-15Rα complex. However, IL-15Rα expression on monocytes and B cells is similar in HCs and MS patients (51).

$CD4^+CD28^{null}$ T cells have aberrant functional properties, resembling to those seen in NK cells and $CD8^+$ T cells. They contain the NKG2D receptor and gain large intracellular deposits consisting of various cytotoxic molecules; like perforin, GrB and IFN-γ (1, 4, 5, 13, 16-18), which are depleted upon TCR stimulation (4). Furthermore, it is known that NKG2D and IL-15 signaling pathways work in collaboration to each other (63).

We investigated the effect of membrane-bound IL-15 on the cytotoxic profile and functioning of $CD4^+CD28^{null}$ T cells. We found that IL-15 significantly increases the surface expression of NKG2D on $CD4^+CD28^{null}$ T cells of MS patients. However, it seems that this elevation is donor-dependent. NKG2D is a (co)activating receptor, which interacts with a family of ligands that respond to environmental triggers (e.g. stress, infection or inflammation), suggesting their role in alerting the immune system to the abnormal state of the ligand-expressing cells. The ligands include the MHC class I chain-related molecules (MICs: MICA/B) and the UL16-binding protein family (ULBP: ULBP1-4). NKG2D-NKG2D ligand interaction induces the secretion of cytokines and release of cytolytic enzymes perforin and GrB (19). Upregulation of NKG2D ligands have already been reported in the target organs of inflammatory disorders, such as RA (64) and celiac disease (65). In addition, Saikali *et al.* identified NKG2D ligand positive oligodendrocytes in the CNS, preferentially in MS lesions. Moreover, their NKG2D ligand expression is significantly upregulated by the cytokines TNF-α and IFN-γ (19), making them more vulnerable to NKG2D-mediated killing for example by $CD8^+$ T cells. The immune mediators TNF-α and IFN-γ are relevant in the context of multiple CNS inflammatory pathologies including MS (30, 31). In relation with our data, the IL-15 induced increase in NKG2D receptor expression on $CD4^+CD28^{null}$ T cells of MS patients may enhance $CD4^+CD28^{null}$ T cell effector functions and consequently exacerbate their killing activity against NKG2D ligand positive oligodendrocytes. We speculate that $CD4^+CD28^{null}$ T cells, in addition to $CD8^+$ T cells, take part in oligodendrocyte apoptosis induction. Identification of the IL-15-expressing cells in the CNS, which provide functional IL-15 to $CD4^+CD28^{null}$ T cells, will be discussed further on in the discussion (ref. immunohistochemistry).

Our *in vitro* experiments demonstrated that IL-15 has the capacity to increase $CD4^+CD28^{null}$ T cells effector functions. IL-15 presented in *trans* to $CD4^+CD28^{null}$ T cells increases the intracellular expression of perforin and GrB in HCs and MS patients, in the absence of antigenic or TCR stimulation. Against all expectations, $CD4^+CD28^{null}$ T cells of HCs show a higher

expression of both cytolytic enzymes under basal conditions and when pretreated with IL-15. In line with our results, Thewissen *et al.* addressed that $CD4^+CD28^{null}$ T cells of MS subjects contain significantly more perforin and GrB per cell as compared to their $CD28^+$ counterparts (1). To evaluate whether $CD4^+CD28^{null}$ T cells expel their intracellular deposits of perforin and GrB in response to IL-15, we performed a CD107a-based degranulation assay. As expected, we found a significantly higher degranulating capacity of TCR stimulated $CD4^+CD28^{null}$ T cells with respect to $CD4^+CD28^+$ T cells, both in HCs and MS patients. IL-15 pretreatment makes the $CD4^+CD28^{null}$ T cells more sensitive to degranulate. However, the fraction of degranulating $CD4^+CD28^{null}$ T cells is higher in HCs. $CD4^+CD28^{null}$ T cells of MS patients do not appear more pathogenic than those of HCs. This may indicate that the mere presence of the cytotoxic $CD4^+CD28^{null}$ T cells is not sufficient to initiate disease pathology. It seems more likely that these T cells participate in an indirect manner to the disease. Again, the overexpression of IL-15 in MS compared with other inflammatory and non-inflammatory neurological disorders and with HCs is of vital importance to exert their detrimental effects (30, 37, 40, 46-48).

Pretreatment with IL-15 also enhances the IFN-γ production of both inactivated $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells isolated from HCs and MS patients. Other studies demonstrated that $CD4^+CD28^{null}$ T cells produce massive amounts of IFN-γ upon stimulation. According to this study, IFN-γ has to be produced after stimulation, since $CD4^+CD28^{null}$ T cells do not have intracellular stores for IFN-γ (66). Another report addressed a higher IFN-γ production by both $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells in MS patients compared with those in HCs (67). This observation is in contrast with our data in which a twofold higher IFN-γ expression in $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells of HCs is observed. According to a study performed by Alonso-Arias and colleagues, IFN-γ production from $CD4^+CD28^{null}$ T cells is induced at much lower doses of anti-CD3 than in their $CD28^+$ counterparts (low activation threshold) and is independent of costimulation (68). These observations assign a potential pathogenic role to $CD4^+CD28^{null}$ T cells. Via the production of proinflammatory cytokines, like IFN-γ; $CD4^+CD28^{null}$ T cells could exert bystander activation of potential autoaggressive T cells (69).

IL-15 induces IL-17 production from synovial T cells in RA patients (70). We investigated whether IL-15 was also able to increase IL-17 expression in $CD4^+CD28^{null}$ T cells. $CD28^{null}$ and $CD28^+$ $CD4^+$ T cells of HCs and MS subjects show very low basal expression levels of IL-17 prior to no stimulation. Adding IL-15 do not cause an increase in expression.

Based on our *in vitro* experiments, we could summarize that the $CD4^+CD28^{null}$ T cell population displays a pathogenic profile that become aggravated by (MS-related) IL-15. In addition, IL-15 could give chemoattractive and prosurvival properties to for example $CD4^+CD28^{null}$ T cells (37-42, 71, 72). Therefore, $CD4^+CD28^{null}$ T cells may contribute to cell and tissue damage, and consequently to the release of self-antigens e.g. from immunoprivileged sites including the CNS. In addition, activation of these T cells could create a proinflammatory and cytotoxic environment in which autoreactive T cells may be activated in a non-specific manner, for example via bystander activation.

Broux *et al.* described that cytotoxic $CD4^+CD28^{null}$ T cells have the capability to migrate to brain lesions of MS patients via the fractalkine (CX_3CL1)-CX_3CR1 system (16). We investigated whether sources of functional IL-15 are present in the vicinity of $CD4^+CD28^{null}$ T cells in MS brain tissue. Our immunohistochemical analyses showed that IL-15 levels are higher in MS lesions compared with NDC brain material. Moreover, we identified the IL-15-expressing cells as being microglia/macrophages and astrocytes. Prominent membrane-bound IL-15 expression has been shown in acute and subacute/chronic MS lesions. These observations could indicate that microglia/macrophages and astrocytes in MS lesions have the capacity to provide functional IL-15 (in *trans*) to T cells, probably including $CD4^+CD28^{null}$ T cells. Moreover, astrocytes; the most abundant glial cell type within the CNS; are strategically positioned at the BBB, making them excellent cells to interact with CNS infiltrating immune cells. In line with our results, astrocytes and monocytes/macrophages have been shown to be the main source of IL-15, while microglia serve as an additional source (36). Dendritic cells; which are mainly found in the perivascular spaces in MS lesions; could also function as an additional source of IL-15 (73). As established in our *in vitro* experiments, IL-15 presented in *trans* to $CD4^+CD28^{null}$ T cells can enhance their proliferative capacity and cytotoxic activity, appointing myeloid cells and astrocytes as being potential candidates to participate in MS disease pathology.

Finally, we tried to demonstrate the presence of $CD4^+CD28^{null}$ T cells nearby IL-15-expressing cells, like microglia/macrophages and astrocytes. In MS brain tissue characterized by an active lesion, we found an immune cell infiltrate in perivascular cuffs, which mainly consists of $CD4^+$ T cells. Moreover, IL-15-expressing cells are also present. $CD4^+$ T cell infiltrates are only found in MS brain material because the BBB of MS patients becomes more susceptible to cell infiltration

during a systemic inflammatory response, allowing autoreactive T cells to migrate into the brain parenchyma (74). To indicate whether this infiltrating $CD4^+$ T cell population contains $CD4^+CD28^{null}$ T cells, we performed an intracellular fluorescent staining for GrB. We chose to stain for GrB because $CD4^+CD28^{null}$ T cells exhibit cytoplasmic stores of GrB and their $CD28^+$ counterparts do not. However, we did not succeed in identifying these $CD4^+$ T cells as being $CD4^+CD28^{null}$ T cells. Maybe because the fluorescent labeled antibody directed against GrB was not suitable. Another explanation could be that the MS brain material was not permeabilized enough to stain for intracellular GrB. Namely, we expected GrB^+ cells in the brain of MS patients, and more specifically GrB^+ $CD8^+$ T cells. First of all, $CD8^+$ T cells are characterized to exhibit large intracellular stores of GrB (75, 76). Secondly, $CD8^+$ T cells have been reported to be present in the parenchyma and in perivascular cuffs of MS brain (48). Thus, because we could not find any GrB^+ cells, we conclude that our immunohistochemical procedure for staining intracellular GrB was not optimal.

Taken our immunohistochemical analyses together, we have shown that the IL-15 expression is higher in the brain of MS patients. Besides, we identified in MS brain microglia/macrophages and astrocytes as being important sources of providing functional IL-15 to $CD4^+$ T cells, and probably to the cytotoxic $CD4^+CD28^{null}$ T cell population.

5 CONCLUSION & SYNTHESIS

We investigated several characteristics of $CD4^+CD28^{null}$ T cells to reveal their potential role in the pathogenesis of autoimmune disorders including MS. Our hypothesis stated that $CD4^+CD28^{null}$ T cells display a more pathogenic profile in response to (MS-related) IL-15. Indeed, we demonstrated that $CD4^+CD28^{null}$ T cells show a (slightly) overall higher proliferative capacity in response to this cytokine with respect to their $CD28^+$ counterparts. Furthermore, we have shown that $CD4^+CD28^{null}$ T cells possess cytotoxic properties, similar to NK cells and $CD8^+$ T cells. They have increased surface expression of the NKG2D receptor and gain intracellular granules of the cytolytic enzymes perforin and GrB. But more importantly, we assessed that IL-15 could enhance the cytotoxic profile and functioning of $CD4^+CD28^{null}$ T cells. Finally, we identified $CD4^+$ T cells in close proximity to IL-15-expressing cells (e.g. microglia/macrophages and astrocytes) in the CNS parenchyma and within perivascular cuffs of MS tissue, suggesting that $CD4^+CD28^{null}$ T cells can also interact with cells presenting IL-15. Overall, our data support our hypothesis that (MS-related) IL-15 aggravates the pathogenic potential of $CD4^+CD28^{null}$ T cells, which make them detrimental contributors in the inflammatory and degenerative processes observed in MS.

Future experiments need to be developed to answer the remaining questions regarding $CD4^+CD28^{null}$ T cells. For example, what is the origin of $CD4^+CD28^{null}$ T cells? Therefore, MS patients with and without expanded $CD4^+CD28^{null}$ T cells could be screened for single-nucleotide polymorphisms (SNPs) to see whether genetic background differences are associated with the development of these T cells. Furthermore, the influence of CMV on the presence of $CD4^+CD28^{null}$ T cells could be investigated. Clearly, a lot of research is needed to identify the causes that lead to the origin of expanded $CD4^+CD28^{null}$ T cells. To elucidate whether these T cells directly or indirectly contribute to MS disease pathology, killing assays could be performed on cocultures consisting of $CD4^+CD28^{null}$ T cells and MS-relevant cells; like astrocytes, oligodendrocytes and neurons. For example, to investigate whether $CD4^+CD28^{null}$ T cells could kill MS-relevant cells via a NKG2D-mediated mechanism, cocultured $CD4^+CD28^{null}$ T cells will be preincubated with either an isotype control or anti-NKG2D blocking antibody. Moreover, because our immunohistochemical procedure for GrB was not optimal, we could try to visualize

$CD4^+CD28^{null}$ T cells by using anti-CD4 antibodies in combination with anti-CX_3CR1 or anti-perforin antibodies.

Future experiments might increase our knowledge in targeting these T cells through therapeutic interventions. One way to manipulate these T cells is to induce re-expression of CD28. Warrington *et al.* discovered that *in vitro* activation of $CD4^+CD28^{null}$ T cells restore CD28 expression when adding IL-12 (77). Bryl *et al.* targeted the putative cause of CD28 loss, namely TNF-α (78). RA patients that receive an anti-TNF-α treatment show increased CD28 expression on $CD4^+$ T cells. However, TNF-α inhibition could have harmful consequences for MS patients (79). Blockade of systemic IL-15 in animal models characterized by an inflammatory autoimmune response (e.g. RA, diabetes and psoriasis) has been shown to decrease disease progression and severity (80-82). However, blocking IL-15; for example in order to prevent $CD4^+CD28^{null}$ T cells to become more pathogenic; will not be recommended as a therapeutic option in autoimmune disorders including MS. Recently, aggravated experimental autoimmune encephalomyelitis (EAE); an animal model for MS; has been reported in IL-15 knockout mice (83). It is also possible to manipulate the $CD4^+CD28^{null}$ T cell population by targeting CX_3CR1, a cell-specific receptor. Blocking CX_3CR1 prevents $CD4^+CD28^{null}$ T cells from entering their target tissue, such as the CNS in MS. However, this approach will also suppress the CX_3CL1-CX_3CR1 system in other cells; like NK cells, neurons and microglia. Induction of EAE in CX_3CR1 knockout mice leads to exacerbation of the disease because NK cell recruitment to the CNS is impaired (84). Therefore, other $CD4^+CD28^{null}$ T cell-specific target molecules need to be studied in detail before using them as targets in therapies.

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Interleukin-15 enhances cytotoxic activity of senescent CD4+CD28null T cells in multiple sclerosis patients

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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Datum: **11/06/2012**